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ISSN: 2156-8456 (Print)   ISSN: 2156-8502 (Online)
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Advances in Bioscience and Biotechnology (ABB)

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Mitochondrial comparative proteomic analysis of sterile line and its maintain line of purple cytoplasmic rice (*Oryza sativa*)

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Received 23 May 2010; revised 7 June 2010; accepted 9 June 2010.

ABSTRACT

CMS/Rf systems in rice (*Oryza sativa*) have long been exploited for hybrid breeding to enhance productivity. Ying xiang CMS/Rf system is a new type. In this study, a mitochondrial comparative proteomic analysis of Ying xiang Sterile Line and its Maintain Line was started for a comprehensive investigation of the mitochondrial proteins’ functions in rice cytoplasmic male sterility. Mitochondria were prepared from rice shoots grown in the dark. Proteins were analyzed by two-dimensional electrophoresis and MALDI-TOF/MS. Using Mascot, it was found that 7 proteins were not described previously for plant mitochondria, indicating novel mitochondrial functions. 3 of them were characterized.

Keywords: ATP Synthase; Comparative Proteomic Analysis; Cytoplasmic Male Sterile (CMS); Ying xiang A; Ying xiang B

1. INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited phenotype characterized by the inability of a plant to produce functional pollen [1]. In rice (*Oryza sativa*), several CMS/Rf systems defined by the different CMS cytoplasm with distinct genetic features have been identified [2-4]. These systems have been widely used for hybrid rice breeding in China and other Asian countries as hybrid rice crops often produce higher yields than inbred varieties [5,6].

Cytoplasmic male sterility in plants is always associated with mitochondrial dysfunction. Molecular studies on CMS have revealed the existence of modifications in mitochondrial DNA and a correlation between the presence of chimaeric mitochondrial genes and the synthesis of new proteins [7-10], and some mitochondrial genes responsible for CMS are chimeric in structure [9,11]. Some of these genes encode a cytotoxic protein [12]. Proteomic analysis is a newly developed technique that has been demonstrated as a powerful approach in plant research [13-16].

Genetical resources play a key role in meeting global challenges in the fields of food security. For example, cytoplasmic diversity is very important for resistance to plant diseases and insect pests in agriculture. We constructed a new type of cytoplasmic male sterile (CMS) line ZidaoA. It can help us to reveal the mechanism of rice cytoplasmic male sterility because its microspores abort completely and earlier than the others, which is significant in hybrid breeding in rice [17,18]. Furthermore, it will enrich the diversity of the cytoplasmic genetic resource of rice, which can avoid the reduction of output caused by rice diseases and insect pests because of the genetic frangibility in rice. And Ying xiang CMS line Ying xiang A is a nucleus displacement product of ZidaoA of Purple rice CMS line and it has the identical cytoplasm and the same properties as the Purple-leaf rice CMS line, so research on Ying xiang CMS line and its maintenance can help us to elucidate the mechanism of purple-leaf rice cytoplasmic male sterility and be advantageous in hybrid breeding of rice. Our early study showed that RNA editing may play a role in the CMS. The translation of *atp9* transcript might interfere with the construction of F0F1-ATPase, resulting in the decrease of ATPase activity and the abortion of pollen in Ying xiang A. We also studied the total proteins of leaves and young panicle by two-dimensional electrophoresis [19,20]. However, little is known about the defects of mitochondrial proteome in the Ying xiang CMS line so far. In the present study, we demonstrate and characterize the mitochondrial proteomics difference of CMS line and its maintain line using 2D-PAGE and MALDI-TOF/MS (matrix-assisted laser desorption/ionisation-time of flight mass spectrometry).

2. MATERIALS AND METHODS

2.1. Materials

Cytoplasmic male sterile (CMS) line and its maintain
line of Zidao (Yunnan purple-leaf rice) type rice (Oryza sativa): Ying xiang A and Ying xiang B.

2.2. Rice Mitochondrial Isolation

The methods of rice mitochondrial isolation followed Mignouna et al. [21].

2.3. Two-Dimensional Gel Electrophoresis

Mitochondria protein samples (100 μg) were acetone extracted by addition of acetone to a final concentration of 80% (v/v) at −20°C. 1mg dried powder was used for two-dimensional electrophoresis. Proteins were visualized by Coomassie blue R350 staining for 10min at 100°C and destained overnight with 10% (v/v) acetic acid. Mw standards were used to identify apparent molecular masses on second dimension separation.

2.4. Mass Spectrometry Analysis and Protein Identification

Protein spots were manually excised from stained gels and characterized after in-gel trypsin digestion by Voyager-DE STR (Applied Biosystems, CA) according to Zhang et al. [22]. Then the peptide mixture was loaded onto a MALDI plate for MS analysis using a Reflex III type Maldi-Tof-MS (BRUKER) analyses. For protein identification purposes, mass spectrometric data were searched using Mascot software (www.matrixscience.com). The search parameters used with this software were as follows: one missed cleavage; 0.1 Da mass accuracy; six peptides allowed. For hits that did not fit with the tolerated mass accuracy, namely the occurrence of six peptides per protein and a coverage greater than 20%, cross-matching was carried out by comparing the molecular weight and pl of the predicted protein with the observed molecular mass and pl of the excision site on the two-dimensional gel. Database searches of MALDI-TOF/MS were performed using the NCBI database (O. sativa specified databases and the reversed databases as a control), and positive identifications were made when the scores were above the significance threshold value (P < 0.05). The proteomics tools at http://www.expasy.org/tools/ were used in computing the pl and Mw and functional analysis.

3. RESULTS AND ANALYSIS

3.1. Identification of Proteins via 2DE, Mass Spectrometry and Bioinformatics

In order to get the differentially accumulated mitochondrial proteins in sterile Ying xiang A and maintain lines Ying xiang B, the proteome maps visualized by Coomassie blue R350 of the mitochondria were compared, and protein spots with distinctly difference (Figure 1) were analysed using adobe Photoshop software. Only 13 protein spots were chosen (Figure 1). The 13 protein spots were excised manually and digested in-gel with trypsin, and analyzed using MALDI-TOF/MS and 12 protein spots were produced peptide mass fingerprint (PMF) data. The 13 spots were numbered, as indicated in Figure 1 and Table 1. Database searches using these PMF data at http://www.matrixscience.com/cgi/ using Mascot revealed the identities of 12 of the protein

![Figure 1](image-url)
spots and most of the 12 identified protein spots contained only one protein, while 4 spots (spots 5, 6, 12 and 13) contained two or more proteins with the sequences highly conserved to the best matched proteins. But only 3 spots (spots 3, 4 and 10) were significant after being compared with the observed molecular mass and pl of the excision site on the two-dimensional gel.

3.2. Functional Analysis of Identified Proteins

All 3 identified proteins were analyzed using ClustalX at home and BLAST at http://www.ncbi.nlm.nih.gov/. Furthermore, to get the function and other characters of the proteins proteomics, tools at http://www.expasy.org/tools/ were also used. To our knowledge, spot 3 is identifiable as r40c1 protein, whose function is not characterized and that belongs to family PD683597. Spot 4 is identifiable as hypothetical protein OsJ_002947, which has a domain of ATP synthase F1, beta subunit (Figure 2). Spots 10 is identifiable as OSJNBb0017I01.1 protein (GenBank: CAE05721.1), which function seem to be Phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (Table 2).

As for spot 4, this predicted molecular mass is slightly larger than the apparent molecular mass of the mature peptide as estimated by 2D-PAGE. We think it might be a defective protein. Marilyn and Robert [23] proposed that because the ATPase subunit 2 polypeptide was synthesized in the cytoplasm, it likely was synthesized as a larger precursor with a pre-sequence for mitochondrial targeting and localization. But it is not the case here. Because the protein spot is almost not detectable in the corresponding fertile line’s proteome map, if it is true, the fertile lines can not survive.

4. DISCUSSION

CMS was defined as maternally inherited male sterility resulting from a specific (mitochondrial) gene whose expression impairs the production of viable pollen without otherwise affecting the plant. That means that cytoplasmic male sterility in plants is associated with mitochondrial dysfunction. Up to now, 12 mitochondrion DNA regions associated with CMS have been identified, and most of them are involved in the genes encoding F0F1-ATPase subunits [11]. The N-terminal region of atp9 in petunia is present in the CMS-associated pcf gene. atp6 provides the 5’ regulatory sequences for the CMS associated urf13-T gene in maize [24]. The CMS-associated gene orf522 found in sunflowers, co-transcribes with atpA and a tissue-specific increase in the level of polyadenylated atpA-orf522 transcripts correlates with the tissue-specific instability of atpA-orf522 mRNA in male florets of the restored hybrid plants [25]. Rice (Oryza sativa L.) CMS-Boro II is associated with an abnormal copy of the mitochondrial gene atp6 [12,26] that transcribes aberrant mRNAs containing an additional orf named orf79 [27]. In Honglian (HL) rice orfH79 is a mitochondria chimeric gene being responsible for the CMS trait [28,29]. Furthermore, RNA editing plays a role in the CMS [10].

Mitochondrion is the site of both the tricarboxylic acid cycle and oxidative phosphorylation pathway and plays a crucial role in energy and carbon metabolism in eu-karyotic cells. The oxidative phosphorylation pathway-consists of the electron transfer chain (ETC), including complex I–IV, and F0F1-ATPase (complex V). The F1-ATP synthase beta subunit (β-subunit) is a highly con-

![Figure 2. Graphical view of domain structure at http://www.ebi.ac.uk/interpro/of spot 4.](image-url)
Table 1. Differentially accumulated proteins identified by mass spectrometry analysis and database searching.

| Spot No | Protein identity and Organism | Accession number | Score \( P < 0.05 \) | Matching peptides | Coverage (%) | \( M_w \) (KDa)/pI | Theoretical | Experimental |
|---------|--------------------------------|------------------|------------------------|------------------|--------------|------------------|-------------|--------------|
| 1       | D-galactose-binding periplasmic protein precursor *Salmonella enterica* | gi|56412897 | 58 | 6 | 24 | 35.689/6.24 | 90.0/5.78 |
| 2       | O-acetylserine lyase [Streptococcus suis] r40cl protein, *Oryza sativa* | gi|7489571 | 118 | 11 | 47 | 39.254/6.30 | 38.70/6.27 |
| 3       | Putative r40cl protein-rice, *Oryza sativa* | Q8H7M3 OR YSA | gi|108707932 | 96 | 9 | 44 | 42.237/6.25 | 31.136/6.36 |
| 4       | hypothetical protein *Oryza sativa* | gi|125571607 | 95 | 11 | 33 | 49.912/5.05 | 41.20/6.66 |
| 5       | Uncharacterized conserved protein, *Streptococcus suis* | gi|9656868 | 51 | 6 | 41 | 22.527/5.48 |
| 6       | Hypothetical protein, *Vibrio cholerae* | gi|146319136 | 53 | 5 | 62 | 14.271/5.28 | 51.4/6.66 |
| 7       | hypothetical protein *At2g22340* [imported], *Arabidopsis thaliana* | gi|25371335 | 79 | 6 | 28 | 42.644/7.48 | 94.1/9.58 |
| 8       | hypothetical protein *DDBDRAFT_0189008, Dictyostelium discoideum* | gi|66803747 | 84 | 8 | 11 | 140.692/8.95 | 78.3/9.16 |
| 9       | ∞ | ∞ | ∞ | ∞ | ∞ | ∞ | 81.4/9.83 |
| 10      | OSJNB001710 1.1 protein (GenBank: CAE05721.1), *Oryza sativa* | Q7XKF3 OR YSA | Q259D2 OR YSA | 81 | 8 | 18 | 41.386/9.62 | 41.20/5.37 |
Protein spots (1-13) from Figure 1 were excised, digested with trypsin, and analysed by MS and database searching. All the proteins were analysed by MALDI-TOF/MS. The GenBank accession number is shown. The Accession numbers correspond to possible proteins matching with the same peptides or part of them. The significance of matches is supported by the number of matching peptides, the combined MOWSE score (P < 0.05) of the matching peptides (Score) and the percentage of the predicted protein covered by the matched peptides (Coverage %).

Table 2. Functional analysis of identified proteins by ClustalX, BLAST and proteomics tools.

| Spot No | Proteins Functional analysis | Mw (KDa)/pI |
|---------|-------------------------------|-------------|
| 3       | Family PD683597               | 39.254/6.30 |
|         | This family was built using psi-blast, with Q8S756_ORYSA as query |             |
| 4       | ATP synthase F1, beta subunit | 49.912/5.05 |
|         | The sequences of ATP synthase F1 alpha and beta subunits are related and both contain a nucleotide-binding site for ATP and ADP. They have a common amino terminal domain but vary at the C-terminus. The beta chain has catalytic activity, while the alpha chain is a regulatory subunit. Proton translocating ATP synthase, F1 beta subunit is homologous to proton translocating ATP synthase archaeal/vacuolar(V1), A subunit. |             |
| 10      | Phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (By similarity). | 41.386/9.62 |

Protein spots (3, 4 and 10) from Table 1 were analysed and its function were characterised. The Mw (KDa) / pl were computed using Compute pl/Mw tool.

It plays an important role in the ATPase activity with the alpha subunit. If the β-subunit is defective, it will cause the dysfunction of F0F1-ATPase, which may impact the energy output of mitochondria, resulting in abnormal anther development with non-functional pollens. It has been reported that there are very highly energy demands.

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during pollen development in higher plants [31]. The dysfunction of F0F1-ATPase in CMS line Ying xiang A may impact the energy output of mitochondria, resulting in abnormal anther development with non-functional pollens.

A widely accepted hypothesis on the mechanism of CMS is that the increased demand for respiratory function and cellular energy in the form of ATP during anther development may be compromised by expression of the aberrant mitochondria genes. The mitochondrial enzyme F0F1-ATP synthase synthesizes adenosine triphosphate (ATP), the universal currency of chemical energy in the cell. The ATPase is installed in the inner membrane of the mitochondrion, with the F1-sector protruding into the matrix compartment and the F0-sector embedded in the inner membrane where it forms a proton-translocating channel. Using the pH-gradient between the cytosolic site and the matrix, the membrane embedded F0-part drives the synthesis of ATP in the F1-sector. The latter contains the three nucleotide binding pockets of the enzyme, which are formed mainly by the residues of the three β-subunits, which is thought to be the most conserved in its amino acid sequence [32].

In mitochondria, the majority of ATP is synthesized by F0F1-ATPase and driven by the electron transport on the inner membrane. If there are defects in the F0F1-ATPase, a further disturbance of the electron transport will magnify the disruptions of ATP synthesis, and consequently, the metabolism process in cells will be interfered with. For testing the link between F0F1-ATPase defects and CMS in HL (Hong-lian) rice, Zhang et al. [33] used inhibitor of ETC to interfere the respiration of seedlings and observed their growth. The results revealed that seedling growth delayed in the sterile line due to the disruption of phosphorylation, suggesting that F0F1-ATPase activity was suppressed in the CMS plants. Peng et al. [29] found that the content of reactive oxygen species (ROS) in the transformants that expressed ORFH79 was increased by 31%, and ATP was decreased by 41% compared with the control. Higher ROS content caused a more swift decrease of F0F1-ATPase activity and ATP contents in YTA than those in YTB [34]. Sun et al. [35] deduced that reduction of the proteins associated with energy production and lesser ATP equivalents detected in CMS anther indicated that the low level of energy production played an important role in inducing CMS-HL.

In conclusion, CMS of Ying xiang A in rice may have multiple causes. The abnormal protein complex of ATP synthase may cause the dysfunction of mitochondrial. We can propose that the cooperation of abnormal beta subunit and ATP9 protein may cause the cytoplasmic male sterility of Ying xiang A. Further research should be carried out to characterise the function of other different proteins.

5. ACKNOWLEDGEMENTS

We thank Dr. Josephine Richardson for her critical reading of the manuscript. This study was supported by the National Science Foundation of China (no. 30571143).

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In vitro demonstration of interactions among zinc-binding domains of cellulose synthases in Arabidopsis and aspen

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Received 16 April 2010; revised 25 May 2010; accepted 28 May 2010.

ABSTRACT

Plant cellulose synthases (CesAs) are the key enzymes necessary for cellulose biosynthesis. In Arabidopsis, two distinct groups of three CesAs each are necessary for cellulose synthesis during primary and secondary cell wall formation. It has also been suggested that such three CesAs interact with each other to form plasma-membrane bound rosette complexes that are functional during cellulose production. However, in vivo demonstration of such assemblies of three CesAs into rosettes has not been possible. We used yeast two-hybrid assays to demonstrate the possible interactions among several CesAs from Arabidopsis and aspen via their N-terminal zinc-binding domains (ZnBDs). While strong positive interactions were detected among ZnBDs from secondary wall associated CesAs of both Arabidopsis and aspen, the intergeneric interactions between Arabidopsis and aspen CesAs were weak. Moreover, in aspen, three primary wall associated CesA ZnBDs positively interacted with each other as well as with secondary CesAs. These results suggest that ZnBDs from either primary or secondary CesAs, and even from different plant species could interact but are perhaps insufficient for specificities of such interactions among CesAs. These observations suggest that some other more specific interacting regions might exist within CesAs. It is also possible that some hitherto unknown mechanism exists in plants for assembling the rosette complexes with different compositions of CesAs. Understanding how cellulose is synthesized will have a direct impact on utilization of lignocellulosic biomass for bioenergy production.

Keywords: Arabidopsis; Cellulose Synthase; Poplar; Protein-Protein Interaction; Yeast Two-Hybrid System; Zinc-Binding Domain

1. INTRODUCTION

Cellulose, the most abundant renewable biopolymer on earth, has a great potential to be used as a major raw material for bioenergy production. Cellulose synthases (CesA) play a pivotal role in the process of cellulose biosynthesis during the formation of primary and secondary cell walls of higher plants [1-2]. A better understanding of the process of cellulose biosynthesis will, therefore, have a far-reaching impact on agricultural and forest product industries that utilize cellulose as a main raw material. However, the precise molecular mechanism of cellulose biosynthesis has so far eluded scientists for a long time [3], and it is still unclear how plasma membrane bound CesA complexes regulate economically important characteristics of cellulose such as degree of polymerization, microfibril angle and crystallinity. It has been suggested that large enzyme complexes (> 500 kDa) consisting of 36 CesA subunits, arranged in the form of rosettes, synthesize about 36 glucan fibers-thick cellulose microfibrils at the plasma membrane [4]. Based on immunogold labeling experiments, CesAs appear to be a component of rosette complexes [5] although actual visualization of the 36 CesAs present in the rosettes has never been possible due to substantial technical limitations [6].

The Arabidopsis genome contains ten CesAs, at least six of which have been identified to be involved in cellulose biosynthesis based on mutant analysis: three (AtCesA1, 3 and 6) associated with primary cell wall formation and three (AtCesA4, 7, and 8) involved in secondary wall formation [1]. Such CesA mutant analysis in Arabidopsis has further delineated that the wild-type or unmutated status of these CesA proteins is essential for normal cellulose synthesis during primary or secondary wall formation, and that any such mutations in CesAs adversely affect the cellulose biosynthetic processes during the respective cell wall development [7-12].

Published Online August 2010 in SciRes. http://www.scirp.org/journal/abb
Three secondary wall associated CesAs (designated as secondary CesAs here) have been shown to be coordinately expressed during the secondary wall-enriched xylem formation and appear to be interacting with each other based on pull-down assays [10]. Moreover, mutation in any one of these three secondary CesAs resulted in failure of interactions with the other two CesAs [13]. Using co-immunoprecipitation and bimolecular fluorescence complementation it has been proved that in primary wall, AtCesA1 and AtCesA3 were required for composing the complex and AtCesA6 was partially needed but redundant with AtCesA2 and AtCesA5 [14]. Similar suggestions of possible involvement of CesA trios in secondary wall formation have been reported in rice [15], barley [16], aspen [17-18], and Eucalyptus [19] although such involvement has been suggested only on the basis of coordinate expression of three CesAs in respective cell wall forming plant cells.

The major question of how some of the three CesAs involved in secondary cell wall formation might be interacting with each other has been addressed in cotton using an in vitro approach with the help of yeast two-hybrid (Y2H) system [20]. Results of Y2H and co-immunoprecipitation assays showed that cotton GhCesA1 and GhCesA2 proteins, orthologous to Arabidopsis AtCesA8 and AtCesA4, respectively physically interact with each other to form homo- or heterodimers via their N-terminal zinc-binding domains (ZnBDs). Furthermore, CesA dimerization is important for the stability of the CesA complexes so reduced monomers were degraded rapidly while oxidized CesA dimers were resistant to degradation during in vivo and in vitro conditions [21]. It has been recently reported that the secondary CesA trios from Arabidopsis can interact with each other, but it seems that only AtCesA4 can form homodimers [22]. However, Atanassov et al. [23] successfully purified secondary wall specific CesA complex without other contaminating proteins and also showed that all three subunits are able to form homodimers independently. No complete interactions among primary and/or secondary CesA trios through Y2H experiments have been reported in Arabidopsis or any other plants.

In this study, we show that ZnBDs from AtCesA4, 7 and 8 from Arabidopsis positively interact with each other in the Y2H system and are capable of forming homodimers and heterodimers. We have previously cloned and characterized aspen orthologs of the same three secondary CesA genes (PtrCesA1, 2 and 3) that are coordinately expressed during xylem development [17-18]. We also demonstrate here that ZnBDs from PtrCesA1, 2 and 3 also strongly interact with each other. Furthermore, ZnBDs from 3 Arabidopsis secondary CesAs also interact with 3 aspen secondary CesAs, although much more weakly than the interactions among CesAs from the same species. Finally, we also demonstrate that ZnBDs from three aspen primary CesAs (PtrCesA4, 5 and 6), orthologous to three Arabidopsis primary CesAs (AtCesA1, 3 and 6), interact successfully with each other as well as with the three secondary CesAs from aspen, thus suggesting that ZnBDs are insufficient to confer specific interactions among CesAs.

2. MATERIALS AND METHODS

2.1. Plasmid Constructs

The cDNA regions encoding ZnBD regions of three secondary CesAs from Arabidopsis (GenBank accession nos. AtCesA4: AF458083, AtCesA7: AF088917, AtCesA8: AF267742) and six CesAs from aspen (Populus tremuloides) (GenBank accession nos. PtrCesA1: AFO72131, PtrCesA2: AV095297, PtrCesA3: AF527387, PtrCesA4: AV162181, PtrCesA5: AVO05724, PtrCesA6: AV196961) were PCR amplified and cloned into pGBKT7 DNA binding vector (BD) and pGADT7 activation vector (AD) from Matchmaker Two-Hybrid System 3 (Clontech, Palo Alto, CA). Similarly, the zinc binding domain-like region of one cellulose synthase-like D cDNA (PtrCSLD) from aspen (AV162184) was also cloned into these two AD and BD vectors. Since PtrCSLD does not contain a legitimate ZnBD, it was expected that inclusion of suchPtrCSLD constructs will provide an additional negative control for Y2H experiments. All vector constructs prepared and primer pairs used in this study are summarized in Tables 1 and 2.

2.2. Yeast Two-Hybrid Assays

The GAL4 fusion protein-based Matchmaker Two-hybrid System 3 from Clontech was used for yeast two-hybrid assays according to the manufacturer’s instructions. This system is a highly improved version of the original Y2H system that has been reengineered to significantly reduce the number of false positives. All BD-CesA plasmids were checked for the lack of autonomous activation of reporter genes in the yeast host strain AH109 provided by Clontech. Positive clones were selected by growth on a stringent medium (-Ade-His-Leu-Trp) and also by activation of α-galactosidase in the presence of X-α-Gal chromogenic substrate imparting blue color to yeast colonies.

Yeast transformations were performed by a small-scale lithium acetate method as recommended by the manufacturer. AH109 co-transformants containing empty GAL4AD and GAL4BD vectors as well as Lamin C and SV40 large T-antigen fusion constructs were used as negative controls, while vectors carrying p53 and the SV40 large T-antigen fusion constructs were used as positive controls.
Table 1. Vector constructs for yeast two-hybrid assays of ZnBDs from CesAs and CSLD.

| Construct Name | RE sites 5'/3' | AA | Construct Name | RE sites 5'/3' | AA |
|----------------|---------------|----|----------------|---------------|----|
| pGADT7(AD)     |               |    | pGBK7(BD)      |               |    |
| PtrCesA1       | EcoRI/XhoI    | 1-149 | PrtCesA1       | EcoRI/PstI    | 1-149 |
| PrtCesA2       | EcoRI/XhoI    | 1-131 | PrtCesA2       | EcoRI/PstI    | 1-131 |
| PrtCesA3       | EcoRI/XhoI    | 1-130 | PrtCesA3       | EcoRI/PstI    | 1-130 |
| PrtCesA4       | ClaI/SacI     | 1-245 | PrtCesA4       | NcoI/PstI     | 1-245 |
| PrtCesA5       | EcoRI/SacI    | 1-240 | PrtCesA5       | EcoRI/SalI    | 1-240 |
| PrtCesA6       | EcoRI/SacI    | 1-255 | PrtCesA6       | EcoRI/SalI    | 1-255 |
| PrtCslD        | EcoRI/XhoI    | 1-218 | PrtCslD        | EcoRI/PstI    | 1-218 |
| AtCesA4        | EcoRI/BamHI   | 1-109 | AtCesA4        | EcoRI/BamHI   | 1-109 |
| AtCesA7        | EcoRI/BamHI   | 1-100 | AtCesA7        | EcoRI/BamHI   | 1-100 |
| AtCesA8        | XmnI/BamHI    | 1-84  | AtCesA8        | XmnI/PstI     | 1-84  |

Table 2. Primers used for cloning ZnBDs of CesA cDNAs in AD and BD vectors in Y2H.

| CesA genes   | Primers name  | Sequence                      |
|--------------|---------------|------------------------------|
| PtrCesA1     | ABD_Ptr1Zn_F  | 5'-GGGAATTCCTGCTGATGGGAATCTGGG-3' |
|              | AD_Ptr1Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | BD_Ptr1Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| PtrCesA2     | ABD_Ptr2Zn_F  | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | AD_Ptr2Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | BD_Ptr2Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| PtrCesA3     | ABD_Ptr3Zn_F  | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | AD_Ptr3Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | BD_Ptr3Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| PtrCesA4     | AD_Ptr4Zn_F   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | AD_Ptr4Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | BD_Ptr4Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| PtrCesA5     | ABD_Ptr5Zn_F  | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | AD_Ptr5Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | BD_Ptr5Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| PtrCesA6     | ABD_Ptr6Zn_F  | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | AD_Ptr6Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | BD_Ptr6Zn_R   | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| PrtCslD      | ABD_PrtcslDZn_F | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | BD_PrtcslDZn_R | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| AtCesA4      | ABD_At4Zn_F   | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | AD_At4Zn_R    | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| AtCesA7      | ABD_At7Zn_F   | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | AD_At7Zn_R    | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
| AtCesA8      | ABD_At8Zn_F   | 5'-GGCAATTCGCAATCGATGGGAATCTGGG-3' |
|              | AD_At8Zn_R    | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |
|              | BD_At8Zn_R    | 5'-GGCTCGAGCTTCTCCTCCATCGGTTC-3' |

Note for the nomenclature system of primers: AD, vector for activation domain; BD, vector for DNA-binding domain; ABD, if the same primer could be used for making both AD and BD constructs; Ptr for aspen (P. tremuloides); At for Arabidopsis thaliana, gene number, 1-6 in the case of aspen or 4, 7, 8 in the case of Arabidopsis; Zn indicates it is a ZnBD primer; F or R indicates the forward or reverse primer.

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2.3. Analysis of interaction strengths between CesA pairs via α-Galactosidase activity assays

The relative strength of interactions among ZnBDs from CesAs was analyzed by a quantitative α-galactosidase (α-Gal) assay (Clontech). All combinations of CesAs used for measuring the strength of interactions were the same as described above. α-Gal activity [milliunits/ml × cell] was calculated according to Yeast Protocols Handbook from Clontech.

2.4. In Vitro Transcription/Translation

Two pairs of ZnBDs from primary and secondary CesA proteins (PtrCesA1 and PtrCesA4; PtrCesA3 and PtrCesA6) were synthesized by *in vitro* transcription/translation using the TNT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) and the fusion constructs obtained from the Y2H assays as described above. Synthesized proteins from pGBK7 and pGADT7 vectors carried c-Myc and HA tags, respectively.

2.5. Co-Immunoprecipitation (Co-IP) and Immunoblotting

Matchmaker CO-IP kit (Clontech, Palo Alto, CA) was used for the co-immunoprecipitation following the manufacturer’s protocol. Protein-protein interactions were confirmed based on the presence of BD constructs with c-Myc tags in the CO-IP reactions that were incubated with HA antibodies. Therefore, c-Myc antibody was used as the primary antibody for immunoblotting analysis. C-Myc-tagged proteins were visualized using secondary alkaline phosphatase (AP)-conjugated anti-mouse IgG (Sigma) antibody and BCIP/NBT as substrate.

3. RESULTS AND DISCUSSION

3.1. Interactions among Secondary Cell Wall-Specific CesAs

Previous genetic experiments with three cellulose synthesis-defective mutants from *Arabidopsis*, namely, *irx1*, *irx3*, and *irx5* exhibiting irregular xylem phenotypes have suggested that the presence of at least three secondary CesAs is essential for cellulose biosynthesis in secondary cell wall forming cells [9-11]. Two independent studies based on publicly available microarray data have further established that AtCesA4, 7 and 8 coordinately express during secondary cell wall development [24-25]. However, it is currently unknown whether these three *Arabidopsis* CesAs can actually form homo- as well as heterodimers *in vitro* via their ZnBDs as was suggested by Y2H results from using two cotton CesAs, GhCesA1 and GhCesA2 [20]. Such understanding has a direct impact on our concepts of rosette formation including the number and types of CesAs interacting during cellulose biosynthesis in higher plants. Moreover, no cotton ortholog of AtCesA7 has yet been reported and such interactions among three CesAs using the Y2H system have never been studied either in cotton or in *Arabidopsis* or any other plant. Therefore, we first set out to determine whether ZnBDs from AtCesA4, 7 and 8 can physically interact with each other and whether they are capable of forming homo- as well as heterodimers in the Y2H system. In order to examine the existence of such interactions, ZnBD regions from AtCesA4, AtCesA7 and AtCesA8 were cloned in both activation domain (AD) and binding domain (BD) vectors from Clontech’s Matchmaker system 3 (Tables 1 and 2). In all co-trans- formats Gal4-fusion proteins containing AtCesA ZnBD regions formed protein-protein interactions resulting in the activation of transcription of the α-galactosidase reporter gene and expression of histidine and adenine (Figure 1). These results show that all three secondary AtCesAs were able to interact with themselves to form homodimers and interacted with the remaining two CesAs to form heterodimers. Kurek et al. have previously confirmed the validity of such interactions through pull-down assays with recombinant GhCesA1 and GhCesA2 [20]. Therefore, rosettes in secondary wall forming *Arabidopsis* cells most likely contain at least up to 3 CesAs.

Similar to *Arabidopsis* secondary CesAs, ZnBDs of all three aspen homologous secondary CesAs were also able to interact with each other, and thus were able to form homo- and heterodimers (Table 3). This agrees with the coordinate expression of three secondary CesAs that we observed in aspen [17-18]. There is also a general opinion that Y2H leads to too many false positives. However, the Y2H system that we have currently used is a highly improved version of traditional Y2H system and this advanced system drastically reduces false positives. The current version 3 utilizes four strict nutritional selection markers and successful interactions can be monitored by expression of a visible marker gene. The general impression of high rate of false positives stems from the library screening of Y2H assays used to identify unknown members interacting with the bait proteins. In our case, however, both the bait and prey proteins are already well-characterized and we are confirming whether such CesA interactions occur via ZnBDs or not. We successfully used all the positive and negative controls that were provided with the Matchmaker Two-hybrid System 3 (Figure 1). In addition, the negative controls using empty AD/BD vectors showed that none of the interactions were caused by self-activation. Furthermore, failure of interactions between the PtrCslD and PtrCesAs demonstrated that the zinc binding-like domain in PtrCslD was unable to form dimers with the CesA ZnBDs (Table 3), thus serving as another biological negative control. Collectively, use of all these control experiments strongly support the notion that ZnBDs of...
Figure 1. Interactions among the ZnBDs of AtCesA4, AtCesA7 and AtCesA8. Schematic representation of the AtCesA4, AtCesA7 and AtCesA8 N-terminal regions containing ZnBDs is shown at the top. Yeast strains transformed with the plasmids containing the GAL-4 binding domain (GAL4BD: bait) and the GAL-4 activating domain (GAL4AD: prey), as indicated, were grown on synthetic dropout media lacking Leu and Trp (-LT), and lacking Ade, His, Leu, Trp and 5mM 3-amino1, 2, 4-triazole (-AHLT+3AT), respectively. α-Galactosidase activity (blue colonies) was assayed on –AHLT medium.

these three aspen secondary CesAs are able to physically interact with each other in vitro and these domains might be involved informing a functional terminal complex in vivo. Unlike in Arabidopsis, isolation of CesA mutations has not been reported in aspen and doing in vivo experiments of mutant complementation or protein-protein interactions are very difficult to perform using aspen system because of its long life cycle.

High similarity of ZnBDs of Arabidopsis and aspen secondary CesAs also prompted us to examine whether ZnBDs from Arabidopsis and aspen secondary CesAs could interact with each other in the Y2H system. As shown in Table 3, CesAs from aspen and Arabidopsis were also able to positively interact with each other. However, the time required to develop the blue color indicative of α-galactosidase activity was much longer in the case of intergeneric CesA interactions than the CesA interactions within the same species. Thus these qualitative estimates suggested that intergeneric CesA interactions may be weaker than the intraspecies CesA interactions.

3.2. Relative Strength of Interactions between Secondary CesAs

In order to quantitatively determine the relative strength of interactions between various secondary CesAs from Arabidopsis and aspen, the expression level of the MEL1 reporter gene was analyzed through measuring α-galactosidase (α-Gal) activity secreted in yeast cell supernatants (Figure 2). The α-Gal activity ranged from 229 to 354
Figure 2. Quantitative estimation of interaction strength between secondary CesA ZnBDs. Results shown are the average α-Gal activity of three cultures for each paired construct, assayed in triplicate. Error bars are standard errors (S.E.). B1-A1: BD-PtrCesA1 and AD-PtrCesA1; B1-A2: BD-PtrCesA1 and AD-PtrCesA2; B1-A3: BD-PtrCesA1 and AD-PtrCesA3; B2-A2: BD-PtrCesA2 and AD-PtrCesA2; B2-A3: BD-PtrCesA2 and AD-PtrCesA3; B3-A3: BD-PtrCesA3 and AD-PtrCesA3; At4A-4B: AD-AtCesA4 and BD-AtCesA4; At4A-7B: AD-AtCesA4 and BD-AtCesA7; At4A-8B: AD-AtCesA4 and BD-AtCesA8; At7A-7B: AD-AtCesA7 and BD-AtCesA7; At7A-8B: AD-AtCesA7 and BD-AtCesA8; At8A-8B: AD-AtCesA8 and BD-AtCesA8; At4A-B1: AD-AtCesA4 and BD-PtrCesA1; At4A-B2: AD-AtCesA4 and BD-PtrCesA2; At4A-B3: AD-AtCesA4 and BD-PtrCesA3; At7A-B1: AD-AtCesA7 and BD-PtrCesA1; At7A-B2: AD-AtCesA7 and BD-PtrCesA2; At7A-B3: AD-AtCesA7 and BD-PtrCesA3; At8A-B1: AD-AtCesA8 and BD-PtrCesA1; At8A-B2: AD-AtCesA8 and BD-PtrCesA2; At8A-B3: AD-AtCesA8 and BD-PtrCesA3; AD-BD (negative control): pGADT7 and pGBKT7; Lamin C-T (negative control): AD-Lamin C and BD-SV40 large T-antigen; p53-T (positive control): AD-p53 and BD-SV40 large T-antigen.

Table 3. Interactions among ZnBDs of PtrCesA1-6 and AtCesA4, 7, 8 with the Y2H system.

| Activation Domain | DNA-Binding Fusion Domain |
|-------------------|---------------------------|
|                   | PrtCesA | AtCesA |
|                   | 1 2 3 4 5 6 7 8 | BD-vector |
| PrtCesA1          | ++ ++ ++ ++ ++ ++ ++ |
| PrtCesA2          | ++ ++ ++ ++ ++ ++ ++ |
| PrtCesA3          | ++ ++ ++ ++ ++ ++ ++ |
| PrtCesA4          | ++ ++ ++ ++ ++ ++ ++ |
| PrtCesA5          | ++ ++ ++ ++ ++ ++ ++ |
| PrtCesA6          | ++ ++ ++ ++ ++ ++ ++ |
| AtCesA4           | + + N/A N/A N/A + + |
| AtCesA7           | + + N/A N/A N/A + + |
| AtCesA8           | + + N/A N/A N/A + + |
| PrtColD           | N/A N/A N/A N/A N/A |
| AD-vector         | N/A N/A N/A N/A N/A |

Note: ++ indicates interaction with fast color development; + stands for interaction with relatively slow color development; - represents absence of interaction without any blue color development of resultant yeast colonies; N/A means not available due to no primary wall AtCesAs involvement in this study.

milliunits/(ml × cell) for the yeast cultures containing CesA interaction partners from aspen, from 216 to 285...
milliunits /(ml × cell) for interacting Arabidopsis CesAs, and only from 59 to 148 milliunits /(ml × cell) for the yeast cultures containing CesAs from both Arabidopsis and aspen. These results suggested that the α-Gal activity was much stronger in the yeast cultures containing the interaction pairs from the same species as compared to the interaction pairs from two different plant species. Moreover, interaction strengths among Arabidopsis secondary CesAs were less than those for aspen CesAs. The negative controls, AD-BD and Lamin C-T co-transformants, showed very weak α-Gal background activity. The known positive control, p53-T combination, showed about 16-fold induction in this study as compared to empty negative controls. The CesA interaction partners for aspen members showed 9 to 14-fold inductions, 8 to 11-fold inductions were seen for Arabidopsis members, and only 2.5 to 5-fold inductions were seen in combinations of CesAs from both the species. These results suggested that stronger interactions may occur between the CesAs from the same plant species and weaker interactions may occur between CesAs from different genera. Additionally, the interaction strengths of ZnBDs from different genera could be different as observed here for the first time in Arabidopsis and aspen. It is well known that physical characteristics of natural cellulosy depend on the botanical origin of their source plants. Transfer of CesA genes from one species to another will possibly be attempted in the future to introduce novel cellulose characteristics in economically important plants [2]. An Y2H screen of compatibility of CesAs might be useful before beginning such cross-species CesA gene transfers.

It is currently unknown whether CesAs from one species can be functional in another species. We have attempted to complement three irx mutants (irx3, 1 and 5) with corresponding secondary CesAs from aspen to study functional conservation of these genes/proteins across the eudicots but such complementation experiments were not successful (data not shown). The weak interactions among Arabidopsis and aspen CesAs as reported here corroborate such observations. It is possible that such heterologous CesAs may not form the functional rosettes during genetic engineering experiments of expressing heterologous CesAs in transgenic plants resulting in either rapid turnover of the CesA transgenes [26] or ineffective impact of ectopic expression of CesAs in transgenic plants. Experimental proof for such hypothesis is currently missing but could be obtained in the future.

3.3. Interactions among Primary Cell Wall-Specific Cellulose Synthases

A phylogenetic tree developed on the basis of protein similarities among 52 CesAs indicated that higher plant CesAs could be divided into six groups, three primary and three secondary CesAs [27]. In Arabidopsis mutant studies, AtCesA1, AtCesA3, and AtCesA6 have been shown to be required for the cellulose synthesis in primary cell wall [1,7,12,28-31], indicating the potential of their interactions with each other. Earlier, we also isolated three cDNAs encoding aspen CesAs, PtrCesA4, 5 and 6, orthologs of primary CesAs from Arabidopsis, AtCesA1, 3 and 6 [27,32-33]. Y2H results using ZnBDs from these three aspen primary CesAs also showed strong positive interactions (Table 3). This demonstrates the in vitro interactions among primary poplar CesAs, suggesting that these three primary PtrCesAs may also be required during cellulose synthesis in the primary cell wall for aspen trees. Our results were consistent with the report by Desprez et al. [14] that AtCesA1, AtCesA3 and AtCesA6 or AtCesA6 partially redundant AtCesA2 and AtCesA5 are able to biophysically interact with each other based on the results of co-immunoprecipitation and bimolecular fluorescence complementation.

3.4. Interactions between Secondary and Primary Cell Wall-Specific Cellulose Synthases

It has been widely acknowledged that the secondary and primary cell wall-specific CesAs are differentially expressed in plants [24]. However, we do not know whether they are able to interact physically with each other during the transition from cessation of primary wall deposition and initiation of secondary wall formation. In order to explore such possible interactions between aspen secondary and primary cell wall-specific CesAs, yeast two-hybrid experiments were carried out to explore their interaction potentials via their Zn-binding domains. Our result shows that any two aspen CesA ZnBDs from these two categories were able to interact while the negative controls showed no interactions (Table 3). In order to validate these Y2H-mediated interactions among primary and secondary CesAs, we also utilized co-immunoprecipitation assays to examine interactions of some selected interacting partner pairs. By co-immunoprecipitating ZnBDs from secondary PrtCesA1 and primary PrtCesA4 with antibodies against HA, we observed that the c-Myc protein complex is present in an immunoblot hybridized with c-Myc antibodies (Figure 3). The positive and negative controls confirmed that the reaction conditions that we used were able to discriminate between a positive reaction and a negative reaction. The similar result was also obtained in a pair of secondary wall associated PrtCesA3 and primary wall associated PrtCesA6 ZnBDs, confirming that primary and secondary CesAs can interact with each other in vitro via...
ZnBDs (Figure 3). These results suggest that primary and secondary CesAs might also be able to physically interact with each other in vivo but experimental proof of such a claim is currently unavailable.

It is generally believed that secondary CesA genes involved in secondary wall formation begin their transcription only after cessation of primary cell wall deposition [34]. It is, however, not known whether primary CesAs continue to transcribe and translate during secondary wall formation or whether primary and secondary CesAs productions are temporally separated in a plant cell since primary CesAs do not appear to be coordinately expressed with secondary CesAs [24]. Although we have previously reported the presence of primary and secondary CesA transcripts in the developing xylem tissue [35], use of a mixture of primary and secondary wall forming tissues in the developing xylem (such as rays and developing vessels/fibers) precludes specific conclusions about temporal and spatial separation of primary and secondary CesA formation during aspen xylem development. Based on the data presented here it is possible that during the transition from primary to secondary cell wall formation, both primary and secondary CesAs might be co-expressed and might interact with each other in intact plants. This is further supported by two studies on the involvement of a secondary wall specific AtCesA7 in the cellulose synthesis during primary cell wall formation. Over-expression of one mutant form of AtCesA7, fra5, under the CaMV 35S promoter reduced cell wall thickness and cell elongation via affecting cellulose synthesis during primary wall formation as well as causes a dominant negative effect on cellulose synthesis during secondary wall formation [36]. This suggests that the fra5 mutant protein may also be incorporated into cellulose synthase terminal complex in the fra5-overexpressing plants, in elongating cells, so that affecting the normal function of CesA proteins involved in primary wall synthesis. Moreover, it is now still unclear whether and how two processes of primary and secondary wall formation are interrelated, and Bosca et al. [37] also reported that a widely recognized secondary wall-specific AtCesA7 (MUR10) is required for normal primary cell wall deposition and remodeling as well as plant growth, hypocotyl strength, and fertility. They further pointed out that there may exist a mechanism sensing secondary cell wall integrity and regulating primary cell wall structure and cellular differentiation [37]. It is, therefore, inferred that there may exist a stage in which primary wall-specific CesAs co-exist with some of secondary wall-specific CesAs if not all of them. Again more in vivo experimental evidence is needed for eventually proving such a possibility.

3.5. Lack of Specificity among CesA Interactions via Zinc-Binding Domains

The zinc-binding domains were thought to be the best candidate interaction regions between CesAs, as they were able to form both homodimers and heterodimers in the study of cotton fiber CesAs [20]. It was also shown that a mutation in the zinc-binding domain would inhibit the interaction between two CesA N-termini. In addition, Jacob-Wilk et al. [21] showed that the reduced CesA monomers were degraded rapidly while oxidized dimers were resistant to degradation during both in vivo and in vitro conditions, indicating the importance of CesA dimerization via zinc-binding domains for the stability of the CesA complexes. It seems that zinc-binding domains should play some specific roles in protein-protein interactions. However, our Y2H results showed that the interactions between CesA proteins via zinc-binding domains are insufficient to confer specificity of interactions among primary wall or secondary wall CesAs from aspen or Arabidopsis. CesAs are able to interact with each other either within primary and secondary wall specific groups or between these two groups. Moreover, the interactions even occurred between the inter-species CesAs, suggesting the structural capability of CesA zinc-binding domains for protein-protein interactions. Our results are consistent with the recent report [22], which showed that zinc-binding domains are not essential for the interactions between different CesAs. Using domain swapping techniques, Wang et al. [38] demonstrated that the catalytic or C-terminal domains were more important than other regions for the specific assembling in the CesA complex, which supports our results of lacking specificity of interactions between zinc-binding domains from different sources. While we
cannot exclude the role of zinc-binding domains in the protein-protein interactions between CesAs, the other more specific interaction regions or other regulatory mechanisms need to be further discovered.

4. CONCLUSIONS

Here, we have demonstrated that all interactions among secondary wall-specific CesAs in both Arabidopsis and aspen, and intergeneric interactions between these two plant species are possible. We identified both qualitatively and quantitatively that intraspecies CesA interactions are stronger than the intergeneric CesA interactions. We also showed the interactions among primary CesAs, and the ones between primary and secondary cell wall-specific CesAs in aspen. These results suggest that two possibilities exist about CesA interactions. First, dynamic interactions might exist between the CesAs from two distinct wall-specific groups in the same species at least during the transition stage from primary to secondary cell wall formation. However, currently there is no supporting evidence for such a possibility. Secondly, no strong specificity among N-terminal domains of these CesAs was found in this study, indicating the possible existence of other more specific regions in other domains of the CesAs that are required for the protein-protein interactions.

The genetic improvement of the cellulose biosynthetic process in crop plants or trees via overexpression of a single CesA has so far not been successful although such experiments are in progress in several laboratories including ours. We have hypothesized, therefore, that upregulation of CesA trios would improve cellulose production in cell walls [17-18]. ZnBD-mediated interactions of CesAs are difficult to demonstrate in vivo. Therefore, a systematic analysis of various combinations of CesA overexpressions in transgenic plants may be one of the methods available by which the CesA genes responsible for cellulose biosynthesis in economically important plants may be identified and manipulated for biofuel production.

5. ACKNOWLEDGEMENTS

This work was partially supported by the National Science Foundation’s CAREER award (IBN-0236492) to CPJ. This work was also partially supported by the World Class University project of the Ministry of Science and Technology of Korea (R31-2009-000-20025-0).

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Molecular differences in genotroph forms of common wheat (*Triticum aestivum* L.) and their initial cultivars

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Received 23 April 2010; revised 10 June 2010; accepted 17 June 2010.

ABSTRACT

By the modified ISSR-amplification method the comparison of the structural organization of enzyme loci in the initial common wheat cultivars and their genotrophs induced with epimutagens: plant niacin acid and niacin acid and its derivatives produced from β-picoline fraction of coal-tar pitch (niacin acid nitrile, isocinchomeronic and benzoic acids) was carried out. It is shown that niacin acid influence causes in genotrophs specific band appearance in PCR-profiles obtained on the DNA of enzyme loci. All these before mentioned epimutagens induced in limits of each genotroph differently directed changes in number and intensity of bands of PCR-profiles.

Keywords: Common Wheat; Genotrophs; Epimutagens; DNA

1. INTRODUCTION

In epigenetic investigations, a very important moment is induction of the alterations by means of epimutagens. The established epimutagen is 5-azacytidin causing cytosine demethylation in DNA and activation before inactive genes resulting in alteration of many morphological traits [1-5]. DNA status demethylated under 5-azacytidin action is able to be preserved during cell and sexual generations [1,2,4].

As it was shown earlier the epigenetic variability also could be induced by external factors such as nutrition. Leap changing in mineral nutrition of plantlets of different flax lines caused appearance of vigorous tall plants having augmented mass and size of seed and changed type of leaves pubescence [6-8]. Accrued alterations turned out hereditable and changed forms of flax plants had been called genotrophs [6-8]. Molecular investigations of flax genotrophs demonstrated that inheritable alterations of morphological traits were determined by means of alterations of quantity and structure of DNA in the cell nucleus [8,9].

Equally with 5-azacytidin in the capacity of mater which is able to induce inheritable alterations to row of sexual generations researchers also started to use niacin acid (NA) [10-12]. Treatment of spring common wheat (*Triticum aestivum* L.) plants of the cultivar Kazakhstanskaya-126 (K-126) with NA solutions resulted in appearance of the wheat form Genotroph-1 (G-1) having changed morphophysiological traits, enhanced viability and heightened resistance to extreme environmental conditions in comparison with the initial cultivar [10,11]. In crossing of these plants and the initial cultivar in hybrids F1 the traits of changed plants were dominant. The traits of these changed wheat plants are stable and there were no reversion to the initial type during 65 studied generations. Relying on it, niacin acid may be referred to epimutagens. Appearance of similar forms having changed morphological traits was revealed in the treatment of the winter common wheat cultivar Bezostaya-1 (B-1) with NA; induced alterations have been inherited during 44 investigated generations (Bogdanova et al., unpublished data). In crossing of changed plants and the initial cultivar B-1, reciprocal hybrids F1 inherited genotrophs' traits.

High prices of NA of natural origin and low biological activity of synthetic NA produced from the synthetic β-picoline led to necessity of producing as NA its analogues and derivatives from the β-picoline fraction of coal-tar pitch which are characterized with high biological activity. In the capacity of such analogues the following substances were synthesized and used in our investigations: niacin acid amid, niacin acid nitrile, isocinchomeronic acid, benzoic acid and others [10,11]. When using these analogues, appearance of tall, long-spiked genotrophs after the repeated (2nd) treatment of seed from generation in...
generation was also noted. The genotrophs differed from the initial cultivar B-1 on morphological traits and grain shape [10].

Using biochemical traits for comparison of the cultivar K-126 and the form G-1 allowed us to reveal differences between them on glutamate oxaloacetate transaminase (GOT2): the appearance of additional isozymes was noted in G-1 [11,12]. These isozymes were revealed constantly during several generations of G-1.

The stability of the changed traits makes the induced forms interesting both for practical activities and theoretical researches. For more particular studying of epigenetic variability, it was a great interest to compare expression of enzyme loci and peculiarities of DNA structure of the initial cultivars and their genotrophs induced by niacin acid, niacin acid nitrile, isocinchomeronic and benzoic acids. To study DNA structure, the modified ISSR-amplification method is successfully used. In this case in the pair with the specific to marker enzyme gene primer, the microsatellite primer is used [13-15]. Due to this method which allows researching the DNA-sites including the satellite primer mic2 the following specific primers were got: 1) got1 (direct orientation) and got2 (inverse orientation), which are specific to locus Got1; 2) malic1 (direct orientation) and malic 2 (inverse orientation), which are specific to locus Me1; 3) got1 (direct orientation) and got2 (inverse orientation), which are specific to locus Got1.

The primers having direct orientation allowed us to amplify a structural part of gene while the primers having inverse orientation allowed us to analyze its 5'-regulatory region of gene.

For PCR-amplification in the pair with the microsatellite primer mic2 the following specific primers were used: 1) adh1 (direct orientation) and adh2 (inverse orientation), which are specific to locus Adh1; 2) malic1 (direct orientation) and malic2 (inverse orientation), which are specific to locus Me1; 3) got1 (direct orientation) and got2 (inverse orientation), which are specific to locus Got1.

The electrophoregrams of the PCR-profiles were scanned using Biodoc2 device.

3. RESULTS AND DISCUSSION

Isozyme analysis of alcohol dehydrogenase (ADH) showed no differences between the initial cultivar K-126 and the line G-1. To determine differences between these forms at the genome level we used the modified ISSR-amplification method. Whereas the changes can take place as in the structural as in the regulatory part of the locus, in the pair with the microsatellite primer, differently directed specific primers were used. Primer adh1 (direct orientation) determines the amplification of the DNA extraction, PCR-amplification. Total plant DNA was extracted from plantlets by means of CTAB-method [16]. For PCR-amplification in the pair with the microsatellite primer mic2 the following specific primers were used: 1) adh1 (direct orientation) and adh2 (inverse orientation), which are specific to locus Adh1; 2) malic1 (direct orientation) and malic 2 (inverse orientation), which are specific to locus Me1; 3) got1 (direct orientation) and got2 (inverse orientation), which are specific to locus Got1.
structural part of the gene, while primer adh2 (reverse orientation) allows us to get the profiles of the 5'-regulatory area. The PCR-profiles obtained from the cultivar K-126 and from the line G-1 are presented in Figure 1. One can see that the pair of primers adh1 and mic2 reveals the specific band ~240 n.p. in G-1 (Figure 1, 7, 9, II).

Thus, having absence of differences in ADH1 isozyme spectra, we can observe the distinct difference between the initial cultivar K-126 and the line G-1 at the level of DNA structure.

The similar approach to use of the differently directed specific primers for the modified ISSR-amplification was applied to research locus Me1 controlling malic-enzyme. It was shown that the pair of primers malic1 and mic2 reveals the specific band ~100 n.p. in G-1 (Figure 2, 7, 9, II).

And also the PCR-profiles obtained with the use of the primer got1, specific to the locus Got1 controlling glutamate oxaloacetate transaminase-1, in the pair with mic2 turned out to be informative. These primers amplify the structural part of the gene Got1 and, on DNA of G-1 they produce the specific band ~720 n.p. (Figure 3, 4-6). The pair of primers got2 and mic2 determining the amplification of the regulatory 5'-area showed no differences between the initial cultivar K-126 and the line G-1.

It is significant that, among all investigated loci all the PCR-profiles of the genotroph plants are uniform and, in each investigated locus, the genotrophs have got the specific band.

The variability of enzyme loci was also investigated in the genotrophs obtained from the initial winter common wheat cultivar Bezostaya-1 (B-1) using other epimutagens: niacin acid nitrile (G-142/96), isocinchomeronic acid (G-143/96) and benzoic acid (G-145/96). In the genotrophs obtained from this cultivar by means of the before-mentioned epimutagens, an absolutely different picture of the enzyme loci variability was revealed.

The PCR-profiles of the locus Adh1 in the plants of the initial cultivar were identical while in the genotrophs one could see some variation of both quantity and intensity of the bands (Figure 4).

The locus Me1 has got slightly different reaction on
the epimutagen influence. The alterations were revealed not in all genotrophs. The PCR-profiles of G-142/96 (Figure 5, 7-12) and G-143/96 (Figure 5, 13-18) resemble the profiles of the initial cultivar, and the profiles of G-145/96 (Figure 5, 19-24) demonstrate some differences from the control variants.

The structure of locus Got1 also changed under the epimutagen influence. Profiles of G-142/96 are identical (Figure 6, 7-12) but they are different from the control variant. The PCR-profiles of G-143/96 (Figure 6, 13-18) and G-145/96 (Figure 6, 19-24) have got ranges of bands’ intensity, and that G-143/96 mostly differs from the control variant.

The information of all PCR-profiles described in our work is presented in the Table 1.

As you can see from the table the plant niacin acid acts in a specific way, causing the appearance of a definite band in each out of the investigated enzyme loci (Genotroph-1).

4. CONCLUSIONS

Thereby, the epimutagen influence results in hereditary variability in the plant genome. All the investigated genotrophs have got at least about 40 generations of the reproduction and carry induced morphological alterations. This investigation has shown that the base of the stability of morphological alterations is changes at the level of the DNA organization structure reproducing.
in the row of sexual generations. The character of the revealed DNA alterations depends on the used epimutagens and the researched enzyme locus. The influence of both niacin acid and its analogues in plants activates the appearance of changes in the structural part of the investigated enzyme loci. Though only the influence of niacin acid causes the appearance of the specific band in the PCR-profile. The before-mentioned abiotic epimutagens act different-directly causing the alterations of quantity and intensity of bands of the PCR-profile. Such diverse plants’ reaction to the influence of the abiotic epimutagens, synthesized from β-picoline fraction of coal-tar pitch, has testified that there are no specific acceptor zones for them. As it was shown before, at the morphological level, niacin acid nitrile induces the appearance of the homotypic tall and long-spiked genotrophs, while isocinchomeronic acid and benzoic acid induce the appearance of different types of morphological alterations at the level of each genotroph [10]. This statement well conforms to the data about significant variation of DNA changes in genotrophs obtained with the use of before-mentioned epimutagens. Thus, genome answer ambiguity of plants on the influence of the epimutagens, synthesized from β-picoline fraction of coal-tar pitch manifests itself at the level of morphological traits just as at the level of DNA genome. In contrast with it, the epimutagen of plant origin (plant niacin acid) acts in a specific way, causing the appearance of a definite band in each out of the investigated enzyme loci. This specificity points to that natural metabolites (in particular plant niacin acid) have their specific areas of the influence on the genome.

5. ACKNOWLEDGEMENTS

Investigations of Russian researchers were performed in frames of Integration Project #99 for 2009-2011, supported by Siberian Branch of Russian Academy of Sciences.

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A new form of Miscanthus (Chinese silver grass, *Miscanthus sinensis*—Andersson) as a promising source of cellulosic biomass

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Received 28 April 2010; revised 5 June 2010; accepted 7 June 2010.

**ABSTRACT**

The Far East population of *Miscanthus sinensis* (Andersson) was introduced into the West Siberia conditions. There was distinguished a form with a modified structure of the rootage which forms long shoots with leader buds and rapidly colonizes soil, thus forming a continuous and flat (without tussocks) plantation of miscanthus. It is shown that using usual agrotechnologies, it is possible to obtain 10-15 tons of dry biomass of high quality cellulose (about 40%) per ha/year.

**Keywords:** Cellulose; Lignin; Biomass; Cellulosic Biomass; *Miscanthus sinensis*; Vegetative Reproduction of Plants

**1. INTRODUCTION**

At present, only two sources of cellulose: cotton and wood biomass are of industrial importance. Cotton represents almost pure cellulose and does not require a complicated processing. Wood mass contains 40-44% of cellulose [1].

Plants with relatively a low content of lignin, in which the main mass of structural elements represents cellulose, can be used as an alternative cellulosic biomass. Introduction of plants species into the culture, yielding a high harvest of biomass with a high content of cellulose, cultivated by conventional agricultural methods can be quite a promising way to involve the new sources of high quality cellulose for the multi-purpose use. The research into evaluation of plants with high content of cellulose as promising raw sources for chemical industry and power production is carried out in the world [2].

In the Interactive European Network for Industrial Crops and their Application (IENICA) there is information about more than 90 kinds and species of promising plants that are studied as raw materials sources for chemical, fuel and power industries [3]. All these plants are herbs which are suitable for cultivation by generally accepted methods of agricultural production.

A special attention to such type of plants is quite justified, as the majority of them reproduces by seeds and can be cultivated in traditional agricultural regions and possess a high rate of accumulation cellulosic biomass in stalks. Among them there are well-known kinds: wheat, oats, corn, nettle, hemp, sunflower, flax etc. Currently the list of the kinds-candidates is actively supplemented.

One of the non-conventional species is *Miscanthus sinensis* (Andersson) to be called the Elephant grass. The plants are really giant of 3 meters high. Miscanthus is a sort of perennial herbs belonging to the bluegrass (Poa) family. More than twenty species belong to class of Miscanthus and they are widespread from the tropical and South Africa to the East and South East Asia. In Russia, in the Far East there are 3 kinds of miscanthus: *Miscanthus sacchariflorus, Miscanthus purpurascens, Miscanthus sinensis* [4].

Systematization of the genus in question is not steady and it is constantly exposed to revision.

Definition of chromosomal numbers of various kinds does not simplify the situation: from four from the fourteen kinds that were sufficiently studied, represent aneuploid populations (a plants mixture of various ploidy, probably, from diploids-38 chromosomes to hexaploids-114 chromosomes). There was fixed a various number of chromosomes in somatic cells at three varieties, including *Miscanthus sinensis* (Andersson): 35, 36, 38, 40, 41, 42, 57 [5].

Nowadays miscanthus is widespread as an ornamental plant. There was created a great number of forms and varieties of miscanthus, some of them are exotic
enough, for example, the species with striped leaves. In most cases miscanthus is attractive as a part of green hedges, and its panicle as an element of winter bouquets. The miscanthus is widely considered as a raw source of cellulose because of its high productivity of dry biomass, drought-tolerance and frost-resistance. Actually, according to the IENICA CROPS DATABASE (for example, for the giganteus species, used in Europe) its production is 11.7-25.3 tons of dry biomass per ha/year. In Denmark, the gathering of 44 tons of dry biomass per ha/year was fixed. The results of defining the chemical composition confirm the content of cellulose within 44%, lignin—17%, hemicellulose—24% [3,6].

2. RESULTS AND DISCUSSION

2.1. Reproduction and Cultivation of Miscanthus sinensis

By now, the general principles of reproduction and cultivation of miscanthus have been well developed for the conditions of Europe and the USA. Its existing species do not reproduce by seeds, their reproduction being possible only by rhizomes. Rhizomes are short enough (5-10 cm) and are formed during vegetation. They pass the winter and flush in spring. As a result there is a slow colonization of the soil space with formation of strongly rampant hummocks. Transplanting is carried out in spring with separate short rhizomes, usually placing them in rows with row-spacing of 60-75 cm. Miscanthus is unpretentious to a soil, but it is necessary to enrich it with a full norm of mineral fertilizers. The maximum productivity of planting is reached for the 3d or the 4th year, whereupon the annual biomass crop is saved up to 15-20 years [3].

2.2. A new form of Miscanthus sinensis (Anderson)

Availability of this species as a source of cellulose has stimulated the search for the new forms appropriate for agroindustrial technologies in the conditions of West Siberia.

More than 10 years ago an expedition from the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, supervised by academician V. K. Shumny (Dr. V. A. Godovikova’s team) worked in the Far East with participation of academician P. G. Gorovoy, a well-known botanist from the Far-East Branch of the Russian Academy of Sciences investigated populations of miscanthus plants on the Pacific ocean coast. The first samples of these populations were brought to Novosibirsk. They were reproduced and the population-genetic and selection research into this kind and replenishment of the collection of the Far East samples were begun at the Institute of Cytology and genetics of the Siberian Branch of the Russian Academy of Sciences.

As a result, there was obtained an unusual form of miscanthus with very long rhizomes which quickly colonized the soil and thus there was created a continuous and flat plantation of miscanthus.

In three years miscanthus plantation turned into 2-2.5 m tall thickets when planting such long rhizomes in furrows with row-spacings of 60 cm (Figure 1).

Next years the thickness of stalks was stabilized at a level of 200-220 per sq.m. In the soil rhizomes have formed a solid net at a depth of 5-20 cm and their length was 60-65 m/m2. Further study of the rhizomes formation has shown that they grow up to one meter and higher during the season, in addition, the buds, which provide a continuous regrowth of new shoots

Figure 1. On the top: The June shoots of miscanthus after landing in the second half of May; on the bottom: an eight-year plantation of miscanthus in August (Novosibirsk, 2008).
appear on them. Such a way of reproduction allows an essential simplification of plants transplanting: firstly, rhizomes are easy to separate from each other; secondly, they are easily placed in furrows by continuous tape.

To analyze the production of experimental plantations, the trial hay harvests were done. The straw exit was on the average 10-15 tons per hectare during years. A high quality straw with the content of cellulose, suitable for the multi-purpose use was about 40%. The characteristics obtained are comparable with the data from IENICA-CROPS DATABASE for the European species of miscanthus (see above), however they were obtained for conditions of the south of West Siberia in a shorter vegetative period and at rewinterings in a sharply continental climate.

Moreover, a plantation with a continuous application of plants allows one to carry out agrotechnical actions only during the early-spring period (May) and to introduce fertilizers with a subsequent soil loosening.

Studies on the biology of the new form of miscanthus have shown that it is possible to create an agroindustrial technology of cellulose biomass as a promising source of cellulose of the multi-purpose use. Clearly it is necessary to evaluate the efficiency of an agroindustrial way of manufacturing the cellulose biomass comparatively with a traditional cutting down of woods.

2.3. Efficiency of Agroindustrial Technology of Cellulose Production

We have shown that the new form of miscanthus and the proposed technology of its cultivation are capable of yielding a harvest of dry biomass (cellulosic biomass) at a level of 10-15 t/ha/year in the West Siberia conditions. For the formation of a long-term steadily functioning plantation (for 15-20 years) two-three years are needed. Forests accumulate a biomass during decades and 100-140 years is a normal age for a mature forest. It is easy to compare the efficiency of accumulation of dry biomass of woods of different breeds and of the miscanthus agroindustrial cultivation as an industrial crop [7,8].

Calculations of such a comparison are presented in Table 1 and on Figure 2. The production of wood breeds is taken for forests of the growth class Iа, i.e. the best ones [9]. The efficiency of accumulation of dry biomass by miscanthus plantation is calculated for a twenty years cycle, the mowing off beginning from the second year after landing and this is annually done.

After 20 years of vegetation a plantation is destroyed and a new one is made. Calculation is carried out based on a minimum efficiency of miscanthus in the conditions of West Siberia (10 t/ha/year), starting with the third year of its planting out. The production of the second year plantation is generally accepted as 5 t/ha/year. Thus, in 20 years, the production of Miscanthus plantation will make 185 tons per hectare. The period of hundred years, presented in Table 1, corresponds to five full agrotechnical cycles of miscanthus.

From Table 1 it follows that miscanthus plantations essentially surpass the best forests of a temperate Eurasian zone in efficiency of biomass accumulation.

It is possible to calculate a mid-annual increment of biomass according to the accepted plan of miscanthus cultivation for the comparison with the referenced data for the forests of the growth class Iа [7] (Table 2).

As a whole in Russia the average store of wood biomass per hectare in the ripe and overripe forests to be exploited fluctuates from 75 up to 175 tons, but it is necessary to take into account that more than one half of all the forests of Russia grows in permafrost soils of Siberia and Far East. This causes their low productivity. Only 55% of the areas of these forests are of interest

| Table 1. Biomass accumulation in miscanthus manufacturing in comparison with the major tree species of the growth class Iа. |
|---|---|---|---|
| Year | Miscanthus | Pine | Birch | Aspen |
| 5 | 5 | 25 | 25 | 25 |
| 10 | 85 | 56 | 68 | 54 |
| 20 | 185 | 112 | 112 | 84 |
| 40 | 370 | 170 | 152 | 116 |
| 50 | 455 | 224 | 187 | 148 |
| 60 | 555 | 269 | 215 | 175 |
| 70 | 640 | 308 | 239 | 196 |
| 80 | 740 | 340 | 259 | 212 |
| 90 | 825 | 368 | 275 | 223 |
| 100 | 925 | 392 | 287 | 229 |

Figure 2. Biomass accumulation in Miscanthus manufacturing in comparison with the major tree species of the growth class Iа.
for exploitation purposes. From these 55% many large forests are remote and unavailable [10].

The agroindustrial manufacturing technology of cellulosic biomass has undoubted social benefits.

Miscanthus is an industrial crop, and there is no necessity to use fertile arable lands for its plantations. Unpretentiousness to soils is its clear advantage. Only in the Novosibirsk region, more than one million of hectares were removed from a crop rotation for the last few years. These soils do not justify themselves when farming grains. In the presence of mineral fertilizers it appears possible to successfully develop miscanthus production. Thus, it is possible to make a conclusion that organization of agroindustrial manufacturing cellulosic biomass based on the technology of farming industrial crop—miscanthus as an additional source of high-quality cellulose seems quite promising.

Undoubtedly, a variety of similar industrial crops should be increased. It is necessary to search and to study non-conventional kinds for agriculture aimed at working out the technologies of their farming and processing.

The research into miscanthus population that was carried out in the Novosibirsk region conditions allows us to draw the following conclusions:

1) The population possesses a high frost-resistance in conditions of West Siberia, unlike the kinds of miscanthus used in the European countries.

2) Miscanthus is a long-term cereal and in three years of its cultivation, can annually (during 20 years) produce 10-15 t/ha of dry biomass on one field that corresponds to 4-6 t/ha of pure high-quality cellulose.

3. ACKNOWLEDGEMENTS

This work was done within the framework of the Interdisciplinary Integration Project of the Siberian Branch of the Russian Academy of Science № 73.

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Determining the specific status of the Iberian sturgeons by means genetic analyses of old specimens

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Received 5 April 2010; revised 22 April 2010; accepted 11 May 2010.

ABSTRACT

To clarify the species status of sturgeon from rivers of the Iberian Peninsula, eight molecular markers (4 nuclear and 4 mitochondrial) have been analysed in different specimens from historical museum samples and prehistoric samples from archaeological sites. These analyses indicate that one of these specimens (UGP captured in the Guadalquivir River in the 19th century) is A. sturio, based on all the eight molecular markers, four of them used from the first time in this study. In previous analyses based on 5 genetic markers, our group assigned two specimens captured in this river in the 1970-80s (EBD8173 and EBD8401) to the species A. naccarii, suggesting the presence of this species in the Iberian Peninsula. In this work, this conclusion is drawn after successfully obtaining a mitochondrial marker in a very old scute from a prehistoric site (Acinipo, about 1500 BC, from the Guadalquivir River basin). On the other hand, in the specimen EBD8174 captured in the Guadalquivir in 1975, we have obtained two new mitochondrial markers confirming that it can be considered A. sturio for all the mitochondrial markers, but nuclear ones identify it as A. naccarii. Finally, two very old samples (Nerja E-VI and Nerja N/62-63) were not successfully characterized by any molecular markers. Some aspects and consequences of our results are discussed, such as the origin of the “mosaic” specimen EBD8174 and, above all, the native status of A. naccarii in historic and prehistoric times in the southern Iberian Peninsula.

Keywords: Iberian Sturgeons; A. naccarii; A. sturio; Ancient DNA; Genetic Identification; Molecular Markers.

1. INTRODUCTION

The identification of sturgeon species inhabiting a certain geographical region has interest not only from the basic scientific standpoint but also for the conservation and recovery of this group of ancient fish so important from the evolutionary as well as the economic perspective [1]. Thus, the specific status of the Iberian Peninsula sturgeons is a debatable matter because, bearing in mind that they are currently almost extinct, it becomes necessary to analyse old museum specimens and even archaeological remains. In this sense, during the second half of the 20th century, it was traditionally considered that in the seas and southern rivers of Western Europe and, more concretely, in the southern Iberian Peninsula, there was only one sturgeon species, Acipenser sturio (Linnaeus 1758). However, from end of last century, the idea arose that until recently at least two species could have coexisted. In fact, based on morphologic and mainly genetic studies (including mitochondrial and nuclear markers) of old museum specimens of sturgeons from this region, it has been shown [2-4] that, in addition to specimens belonging to A. sturio, it is possible to find specimens belonging to another species, A. naccarii (Bonaparte 1836). This situation had been previously proposed by different authors who historically, although forgotten, cited A. naccarii in the Iberian Pen-
insula [5-13]. All these results would indicate that, in recent historical times, this latter species (*A. naccarii*), until now considered only endemic to the Adriatic region, would also have lived in rivers of the Iberian Peninsula.

However, these results have been questioned partly by other studies, which have not provided data to indicate the presence of the species *A. naccarii* in this region [14-16]. Finally, recently Ludwig *et al.* [17] studying the mitochondrial region control in five scutes of sturgeons from archaeological locations of historical times in the Iberian Peninsula, have recently found only mitochondrial haplotypes of *A. sturio*. Therefore, it becomes necessary to continue delving into the analysis of this issue.

In this work, our group, which has contributed to opening this new vision of the distribution of sturgeons in Southern Europe (*i.e.* the coexistence of *A. naccarii* with *A. sturio*), analyses and discusses the attempts to obtain eight molecular markers (mitochondrial and nuclear) in seven old specimens of historic and prehistoric times in southern Spain. These molecular markers are compared in several sturgeon species. Thus, the results previously reported by our group have been corroborated in four of these samples, in a scute of a very old specimen dating from 1500 BC, which again verifies the presence of the species *A. naccarii* in this region.

### 2. MATERIALS AND METHODS

#### 2.1. Samples

In this work, DNA was extracted from seven old sturgeon specimens from the southern Iberian Peninsula. Four of the specimens analysed had been captured in the Guadalquivir River, EBD8173, EBD8401, EBD8174 and UGP. Three of them (labelled EBD), captured in the 1970-80s, are conserved in the Biological Station of Doñana (Spain). The samples EBD8173 and EBD8401 are preserved in ethanol, whereas the EBD8174 is a dry skin. The fourth sample (labelled UGP) is a skin conserved in the Department of Biology Animal of the University of Granada and was also captured in the Guadalquivir River (19th century).

Additionally, three prehistoric samples are analysed for first time. One of them corresponds to a scute from 1500 BC which was found in the Acinipo archaeological deposit (Ronda, Malaga, Spain) (**Figure 1**). The archaeological deposit of Ronda la Vieja (called Acinipo, the name of the Roman city built on this site; [18]) is located in the depression of Ronda, 20 km from the city. The site is situated on a large limestone plateau, which provides a strategic view of the surrounding territory and provides communication with other areas, including the countryside of the Guadalquivir River. The bony sample of sturgeon analysed corresponds to the archaeological phase Acinipo III [19], prior to the Phoenician colonization around the second half of the II millennium B.C. Although it is difficult to assign its origin to the Guadalquivir River, the dates and the zone where it has been found would indicate its origin from this river. Finally, an attempt was made to extract DNA and amplify the different molecular markers from two very old scutes of sturgeons found at another prehistoric deposit (Cave of Nerja, Malaga, Spain). The Cave of Nerja has a long ichthyological record of the excavations made basically in the room of the Vestíbulo [20] on the stratum VII (about 12,000 years old). This level is correlated with Magdalenian occupation in the cave [21].

#### 2.2. DNA Extraction

The extraction and purification of DNA was carried out using ancient DNA techniques and according to the protocol described in Martínez-Espin *et al.* [22]. The first step consisted of cleaning the tissue samples in a polymethyl methacrylate (PMMA) box. A miniature Dremel drill was used to eliminate any polluting agents adhering to the surface. Then, the tissue samples were pulverized in liquid nitrogen using a Freezer Mill. After pulverization,
the powdered sample was transferred to a sterile 15 ml conical polypropylene tube.

To improve DNA recovery, in older samples (the scute from Acinipo and the two scutes from Nerja), we made some changes in the protocols. For these three samples, a protocol was adapted for demineralization of skeletal remains frequently used in mummies and historical identification [23,24]. To minimize the possibility of contamination by contemporary DNA of extraneous sources, these samples were extracted in the minimal-human-remains laboratory, where an animal sample had never before been extracted. Here, possible contamination was eliminated from the old samples. Only one specimen was cleaned and processed at the same time and a negative control was included with the analysis of each specimen. After adding demineralization buffer, the samples were incubated on an orbital shaker at 56ºC for 20-30h. The tubes were angled during agitation to ensure thorough mixing. At the beginning of the extraction, we first added 50 µl of proteinase K (20 mg/ml) and 25 µl again 18 h later. The extracts were purified using sterile water washes in Microcon YM-30 Millipore centrifugal filter units; in the other samples, Microcon YM-100 was used. As a final point, the concentrator was discarded, and 200 µl of the purified DNA were obtained. In this case, many inhibitors were also obtained owing to the fact that tissue is adsorbed into a mineral matrix, after the death of the animal. The following step was the purification of the GENECLEAN® (BIO 101) for Ancient DNA Kit (using the recommended protocol). To guarantee the absence of inhibitor, the Quantifiler® kit for 7500 Real-Time PCR (Applied Biosystems) was used. The Internal Positive Control detectors indicated the absence of PCR inhibitor in all samples.

2.3. Amplification, Cloning, and Sequencing of Molecular Markers

For each specimen an effort was made to characterize the following genetic markers: 1) four nuclear markers corresponding to two satellite-DNA families: the family HindIII [25] and the family PstI [26]; non-transcribed sequences of 5S ribosomal gene (NTS) [27] and 230 base pairs from nuclear DNA flanking the microsatellite Aox-23 [28]. 2) four mitochondrial markers corresponding to two fragments of the cytochrome b gene of 212 bp and 265 bp, respectively [29,30], one fragment of 210 bp corresponding to the mitochondrial region control, d-loop, [30], and one fragment of the 12S ribosomal gene of 139 bp [16]. In each case, the PCR reactions were carried out with the amplification conditions described in each of the references.

Each marker was cloned using the vector TOPO TA (TOPO TA Cloning® kit PCR® 2.1) and were used to transform the cells DH5α of E. coli, according to the supplier recommendations (Invitrogen Carlsbad, CA, USA).

Recombinant plasmids were sequenced on both strands using Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems) and T7 and M13 primers in an ABI Prims® 3100-Avant Genetic Analyzer DNA Sequencer (Applied Biosystems).

2.4. Sequence Analysis

Multiples alignments of sequences obtained from the samples and reference sequences from GenBank database were performed using ClustalX software [31]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [32]. Sequence divergences were calculated according to the Jukes-Cantor method and distance trees produced by UPGMA [33] and the neighbour-joining method [34].

3. RESULTS AND DISCUSSION

The (Table 1) presents a summary for all the seven sturgeon specimens from Iberian Peninsula analysed for different molecular markers. This table includes the data obtained in this new study, completed with the data from previous analyses made by us.

The specimen UGP (Table 1) had previously been analysed for four markers (HindIII and PstI satellite DNA family, 212-bp cytochrome b and 12S mitochondrial gene) and catalogued as A. sturio [4]. Considering nuclear markers, Garrido-Ramos et al. [4] analysed this specimen for the HindIII satellite DNA family, showing the lack of this repetitive sequence in its genome (its absence is characteristic of the species A. sturio; [35]) In the same study, these researchers showed that the sequences corresponding to PstI satellite DNA family analysed for this specimen UGP were grouped, in a phylogenetic tree, together with the sequences of A. sturio.

Now, nine clones have been sequenced for non-transcribed sequences of the 5S ribosomal nuclear genes (NTS), and their sequences were aligned with NTS ribosomal genes from other sturgeon species (Figure 2). Characteristic positions for A. sturio and A. oxyrinchus are present in the sequences isolated from UGP. Thus, in a phylogenetic tree based in genetics distances, all sequences belonging to this sample were grouped together with the NTS sequences of A. sturio (Figure 2).

Additionally, a new nuclear marker Aox23 locus [28] was amplified in this specimen. The sequence found, when compared with sequences of A. sturio and A. oxyrinchus taken from GenBank, proved similar to those of A. sturio (data not shown).
With respect to mitochondrial markers, Garrido-Ramos et al. [4] analysed in this specimen the fragments of the mitochondrial DNA 212-bp cytochrome b and 12S gene and considered UGP as A. sturio. In the present work, two new mitochondrial markers have been amplified for this sample (265-bp cytochrome b and d-loop). It was found that all diagnostic positions for these markers correspond to the species A. sturio (Figures 3(a) and (b)).

Therefore, the results of eight nuclear and mitochondrial markers confirm the classification of this sample (UGP) as A. sturio. This affirmation is not surprising if we bear in mind, as mentioned in the Introduction, that the species A. sturio has been broadly described in most of rivers of the Iberian Peninsula.

On the other hand, previous molecular analyses carried out by our group in three samples from the Biological Station of Doñana (Table 1), identified two of them, EBD8173 and EBD8401, as A. naccarii, based both on the mitochondrial and on the nuclear markers [2-4]. Thus, the samples EBD8173 and EBD8401 have the HindIII satellite DNA family in their genome. This satellite DNA, as commented above, is absent in the A. sturio genome [2].

The presence of this repetitive sequence means that these two samples cannot be assigned to A. sturio, the only species that had previously been considered to live in the rivers of the Iberian Peninsula. Additional results using the markers Psrl satellite DNA, non-transcribed sequences of 5S ribosomal gene (Figure 2), 212-bp cytochrome b and 12S mitochondrial gene (Figures 3(a) and 4) confirmed that EBD8173 and EBD8401 belong to A. naccarii [3,4].

However, the sample EBD8174 (Table 1) is a special specimen from the genetic perspective. For all the nuclear markers analysed to date, this sample EBD8174 cannot be assigned to A. sturio but to A. naccarii. The presence of HindIII satellite DNA family and the fact that all the sequences corresponding to the nuclear markers (the HindIII itself and satellite Psrl and NTS) are not grouped with A. sturio but with A. naccarii are indicative of this fact [3,4]. However, previous mitochondrial DNA studies using 212-bp cytochrome b and 12S mitochondrial gene DNA markers [3,15,16], conclude that, in this specimen, mitochondrial DNA markers are similar to A. sturio.

Thus, the results of nuclear and mitochondrial DNA are contradictory in this specimen because, the nuclear
Figure 2. UPGMA tree based on NTS sequences of 5S ribosomal nuclear genes.

DNA markers indicate its assignment to *A. naccarii* but mitochondrial DNA markers show identities to *A. sturio*. To confirm this situation, we analysed two new mitochondrial markers, 265-bp cytochrome b and d-loop (*Figures 3(a) and (b)*). And the results coincided with previous ones, demonstrating that this sample corresponds to *A. sturio* for all mitochondrial markers. In fact, in the mitochondrial sequences analysed in this study (265-bp cytochrome b and d-loop), we found positions fixed with those of the species *A. sturio*.

Thus, the specimen EBD8174 could be considered a “mosaic” sturgeon: having nuclear characteristics of *A. naccarii* but mitochondrial markers of *A. sturio*. Hybridization or introgression processes between *A. sturio* and *A. naccarii* could explain this phenomenon. In sturgeons, genetic evidence of hybridisation phenomena between sympatric sturgeon species has been shown for example in Arefjev [36], and more recently between *A. ruthenus* and *A. baerii* in the Danube River [37].

Also, similar introgression processes have been described previously in the Adriatic region (*A. gueldenstaedtii* introgressed into the *A. naccarii*) [38] and in the population of the Baltic Sea of *A. sturio* (*A. oxyrinchus* introgressed into the *A. sturio*; [39]).

Finally, we have tried to clarify the specific status of three samples from archaeological sites (*Table 1*). Two of these samples (about 12,000 years old found at an older prehistoric settlement, the Cave of Nerja) were not successfully analysed. Unfortunately, none of the markers used could be characterized for these samples. However, we succeeded in amplifying a fragment of the 12S mitochondrial gene from the prehistoric scute (Ronda, Malaga, of about 3500 years of antiquity) found at the archaeological site of Acinipo (*Table 1*). These results are tentative because the first samples were very old and it was difficult to extract enough quality DNA to amplify the molecular markers. However, in previous studies some samples with similar antiquity at Acinipo, have been used successfully in species identification [17,40].

The 12S mitochondrial gene obtained from the Acinipo sample was compared with other 12S sequences from different species of sturgeons in the GeneBank database. The diagnostic positions for this marker did not coincide with *A. sturio*, ruling out its assignment to this species (*Figure 4*). In fact, all diagnostic sites coincided with *A. naccarii*, although they are not exclusive of this species, sharing them with other sturgeon species such as *A. gueldenstaedtii*, *A. baerii*, *A. persicus* and *A. nudiventris* with a distribution far away from the Iberian Peninsula. Thus, in a phylogenetic tree, based on genetic distances, the sequence from the Acinipo scute is grouped with the sequences from *A. naccarii* (*Figure 5*).

**4. CONCLUSIONS**

The nuclear and mitochondrial markers show that the specimens EBD8173 and EBD8401 belong to the species *A. naccarii*, and the sample UGP to *A. sturio*. The specimen EBD8174, using mitochondrial markers can be catalogued as *A. sturio*, or as *A. naccarii* according to nuclear markers. Hybridization or introgression proc-
esses between *A. sturio* and *A. naccarii*, could explain this phenomenon, common in sturgeons in these species. On the other hand, we were able to analyse the 12S mitochondrial marker for the ACINIPO sample (3500 years old) demonstrating that it belongs to species *A. naccarii*. These analyses provide insights into the existence of specimens belonging to *A. naccarii* in the southern Iberian Peninsula in historic (EBDs samples) and prehistoric (ACINIPO) times. Thus, our analyses confirm old references mentioning the presence of *A. naccarii* in the Iberian Peninsula [5-13]. Therefore, although *A. naccarii* is currently considered endemic of the Adriatic Sea, in the past it could have had a broader distribution area, extending to the Iberian Peninsula, including the Guadarrameric peninsula.

(a) Multiple alignment of the sequences of a 265-bp cytochrome b fragment from UGP and EBD8174, respectively. They are compared with the same mitochondrial DNA region from *A. sturio* (STU AJ428389), *A. naccarii* (NAC AJ245834), *A. oxyrinchus* (OXY AJ245838), *A. persicus* (PER AJ245835), *A. gueldenstaedtii* (GUE AJ245827), *A. baeri* (BAE AJ245825), *A. sinensis* (SIN AJ252186), *A. stellatus* (STE AJ584668). The grey boxes show the diagnostic sites used in the analysis. The primer sequence is not used in the alignment; (b) Multiple alignment of the sequences of the d-loop from EBD8174 and UGP, respectively. These are compared with sequences of the same mitochondrial DNA region from 15 different species of sturgeon: *A. sturio* (STU AJ428389), *A. naccarii* (NAC AJ245834), *A. oxyrinchus* (OXY AJ245838), *A. persicus* (PER AJ245835), *A. gueldenstaedtii* (GUE AJ245827), *A. baeri* (BAE AJ245825), *A. sinensis* (SIN AJ252186), *A. stellatus* (STE AJ584668), *A. stellatus* (BAE AJ245825), *A. transmontanus* (TRA AJ249674), *A. fulvescens* (FUL AJ249661), *A. mediostris* (MED AJ27518), *A. mikadoi* (MIL AJ275189) and *S. platyrhynchos* (PLA AJ249676). The grey boxes show the diagnostic sites used in the analysis. The partial rRNA*" sequences are underlined. The alignment does not show the primer sequence.

Figure 3. (a) Alignment of sequences of a 265-bp cytochrome b fragment; (b) Alignment of partial d-loop sequences.
Multiple alignment of sequences of 12S mitochondrial gene from Acinipo. These are compared with the same mitochondrial-DNA region from the three EBD and UGP specimens, *A. sturio* (STU AJ549115), *A. naccarii* (NAC AJ549114) *A. oxyrinchus* (OXY AF402894), *A. guledenstaeidii* (GUE FJ392605), *A. baerii* (BAE AY544135), *A. persicus* (PER AY544139), *A. nudiventris* (NUD AY544148), *H. huso* (HUS AY544146), *A. stellatus* (STE AY544144), *A. ruthenus* (RUT AY544140), *A. fulvescens* (FUL AF402885), *A. brevirostrum* (BRE AF402886), *A. transmontanus* (TRA AF402893), *A. sinensis* (SIN AY544143), *A. schrenckii* (SCH AY544142), *H. dauricus* (DAU AY544147), *A. mikadoi* (MIK AY544141), *A. medirostris* (MED AF125598), *S. platyrinchus* (PLA AF402901), *S. albus* (ALB AY430247) and *S. tuttasi* (SUS AF402900). The grey boxes show the diagnostic sites used in the analysis. The alignment does not show the primer sequence.

**Figure 4.** Alignment of sequences of 12S mitochondrial gene from Acinipo.

![Alignment of sequences of 12S mitochondrial gene from Acinipo.](image)

Neighbour-joining tree based on 12S mitochondrial gene sequences and Jukes-Cantor distances calculated in MEGA 4. The tree shows the close relationships between sequences from Acinipo (*▼*), EBD8173 and EBD8104 with *A. naccarii* and between sequences from UGP and EBD8174 with *A. sturio*. Numbers indicate the bootstrap support for each node (10000 replicates). *Polypterus. ornatus* (Bichir NC001778) is used as outgroup.

**Figure 5.** Neighbour-joining tree based on 12S mitochondrial gene sequences.

dalquivir River. Similarly, *A. sturio* was distributed not so long ago throughout Europe whereas, at the present, only one population exists, in the Gironde-Garonne-Dordogne River, France [41-44]. Furthermore, to propose a broad distribution area for *A. naccarii* is consistent with the general observation that most sturgeon species inhabited vast areas of continents and river basins [45]. Thus, observations based on molecular analyses, as we present in this paper, or the finding of an “American” species in Europe (*i.e.* the movement of *A. oxyrinchus* into Europe during the Little Middle Ages [46,47]), require more studies in order to establish a more complete vision of the distribution of different sturgeon species in Western Europe.

5. ACKNOWLEDGEMENTS

This research has been financed by grants of the Junta de Andalucía, Consejería de Innovación, Ciencia y Empresa (Proyecto de Investigación de Excelencia P07-CVI-03296) (F. Robles is a postdoctoral grant holder in this Project). Nerja Cave was analysed in the Project “Revisión, estudio y contextualización cronoestratigráfica de los restos arqueológicos procedentes de las antiguas excavaciones del Patronato de la Cueva de Nerja”, authorized by Consejería de Cultura de la Junta.
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An *in vitro* infection model system to study proteins expressed during interaction of mycobacterium with murine macrophages

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Received 10 April 2010; revised 7 May 2010; accepted 30 June 2010.

**ABSTRACT**

Resurgence of mycobacterial diseases particularly tuberculosis has caused a renewed interest to unravel the strategies employed by mycobacteria for intracellular survival. In spite of advancement in mycobacterial research, our knowledge about genes and their corresponding functional proteins involved during the interaction of mycobacterium with host’s macrophages is fragmentary. This study pertains to development of a suitable *in vitro* model using murine macrophages and *Mycobacterium bovis* BCG to study proteins expressed during macrophage-mycobacterium interactions. Peritoneal macrophages from BALB/c mice were infected with *M. bovis* BCG and intracellular replication was assessed by [³H] thymidine uptake assay which was maximal when macrophage to mycobacterium ratio was 1:10. SDS-PAGE was employed to study the proteins expressed and selected proteins were subjected to mass spectrometry. Seven proteins found to be upregulated during macrophage-mycobacterium interaction were identified by MALDI-TOF. The results indicate that the present *in vitro* infection model was able to support the growth of *M. bovis* BCG in murine macrophages and is an ideal model to determine the pattern of functions of gene expression during the interaction of mycobacterium with macrophages. The differentially expressed proteins will help in understanding the mycobacterial molecular basis of adaptation to intracellular macrophage environment.

**Keywords:** Tuberculosis; Macrophages; Model System; Proteomics

1. INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculo-

sis* is a devastating health problem. World Health Organization (WHO) estimates that 2 billion people have latent TB while another 3 million people worldwide die of TB each year [1]. Resurgence of tuberculosis has intensified the necessity for new approaches to combat *M. tuberculosis*. *M. tuberculosis* is a facultative, intracellular pathogen which resides within the macrophages of the host. To survive intracellularly it must respond to the intracellular milieu of the macrophages. It has been well documented that intraphagosomal survival and growth of mycobacteria in macrophage is associated with changes in their gene expression and protein composition upon engulfment [2-4].

To date, in spite of advancement in mycobacterial research our knowledge about genes and their corresponding functional proteins involved during the interaction of mycobacterium with macrophages remains fragmentary. Though several research groups have done proteomic studies on *M. tuberculosis* and other members of the genus [5-7], only few researchers have utilized proteomics to determine changes in protein composition in response to the intraphagosomal environment [3-8]. Such studies have mostly been carried out using cell lines, which definitely have limitations to model *in vivo* situation. Several reports have demonstrated that the use of different cell lines and culture conditions can significantly influence the protein patterns and intracellular survival of mycobacteria [9,10].

In the present study we have worked to develop an *in vitro* model using murine peritoneal macrophages and *M. bovis* BCG. BCG, though an attenuated vaccine strain, retains its ability to survive within the macrophages hence expected to mimic some of its behavior with the pathogenic *M. tuberculosis* inside the macrophages.
Multiplication of the mycobacteria inside the macrophages was ascertained using thymidine uptake assay. SDS-PAGE was employed to study the proteins expressed and selected proteins were subjected to mass spectrometry.

2. MATERIALS AND METHODS

Mycobacteria and culture:
M. bovis BCG (Danish) procured from Mycobacterial Repository Centre at National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra was cultured in Sauton’s liquid medium at 37°C. Cells were harvested in late log phase (4 weeks) of growth.

Animals:
BALB/c mice (6-8 weeks old) of 20 ± 2 g of weight were obtained from Institute’s Animal House. The animals were given a standard pellet diet and water ad libitum. The animal studies were strictly performed following mandates approved by the Animal Ethical Committee of the Institute.

Macrophage culture and infection with M. bovis BCG:
BALB/c mice were sacrificed by cervical dislocation and murine peritoneal macrophages were collected as lavage in RPMI 1640 (Sigma). After washing, cells were adjusted to a density of 5 × 10⁶ cells/well (medium with 10% fetal calf serum, 2mM glutamine, 100 µg/ml antibiotic) of a 96-well culture plate which was kept for 48 hrs in a CO2 incubator. Prior to infection with mycobacteria, non-adherent cells were removed and macrophages were suspended in antibiotic free medium. M. bovis BCG cells, sonicated for 15sec to disperse clumps were added to the adhered macrophage monolayer at different infection ratios and left to phagocytose for 12 hrs. After 12 hrs extracellular mycobacteria were removed by extensive washing and the infected macrophages were replenished with medium containing gentamycin (10 µg/ml) to prevent extracellular replication of mycobacteria. Macrophage viability (never fell below 80%) was assessed by trypan blue exclusion. Incubations were carried out for 5 days in CO2 incubator and 18 hrs prior to termination the cultures were pulsed with 1µCi [³H] thymidine (methyl-T, specific activity approx. 17.2 Ci/ mmol from BRIT, India) per well. Cells were harvested on a cell harvester and the radioactivity was measured in a liquid scintillation counter.

Mycobacterial cell lysate proteins:
Cell lysates were prepared according to the recommended protocol [11]. Cells were pelleted by centrifugation, washed and suspended (0.2 g/ml) in sonication buffer (50 mM Tris/HCl, pH 7.4 with 10 mM MgCl₂, 1 mM PMSF and 1mM EGTA) and sonicated for 10 min. Lysates were clarified by centrifugation and protein concentration was estimated using Bradford method [12]. For infected cell lysate, macrophages grown in 25 cm² culture flasks at density of 2-5 × 10⁶ cells/ml were infected with M. bovis BCG at ratio of 1:10. Finally cells were washed, collected in PBS and lysate prepared. Using similar protocol, non infected macrophage cell lysate was also prepared.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):
Mycobacteria, macrophage and infected cell lysates were subjected to SDS-PAGE. SDS-PAGE under reducing condition was done by the method of Laemmli [13] using 12% resolving gel. An exact amount (25 µg) of protein was loaded in each lane and experiment was repeated atleast three times. After electrophoresis the gels were stained for proteins with silver stain using Investigator Silver Stain Kit (Genomic Solutions).

In-gel digestion with trypsin and mass spectrometry (MS):
Method of Shevchenko et al was followed for in-gel digestion of proteins [14]. Protein spots of interest were excised from gel using Investigator ProPic (Genomic Solutions). Digestion of proteins and spotting of peptides on matrix assisted LASER desorption/ionization-time of flight (MALDI-TOF) target plate was carried out using Investigator ProPrep (Genomic Solutions). Gel plugs digested with K₃[Fe(CN)₆] and Na₂S₂O₃ solution were treated with 10 mM DTT followed with 55 mM iodoacetamide and incubated for 12 h at 37°C with trypsin (Promega). Samples were then desalted with C-18 ZipTips, which were eluted on target plate with 2 µl of α-cyano-4-hydroxycinnamic acid. Mass spectra were acquired with Autoflex II TOF/TOF 50 (Bruker Daltonics) in positive reflectron mode, in the detection range of 500-3000 m/z. Peak detection in MALDI spectra and submission of peak lists to the database were done using Mascot wizard (Matrix Science). Peptide mass tolerance was set to 50 ppm with carbamidomethyl-cystein set as fixed modification, oxidation of methionine as variable modification and 1 missed cleavage site allowed.

3. RESULTS

Intracellular replication of M. bovis BCG within murine peritoneal macrophages was assessed by measuring the [³H] thymidine uptake. Table 1 shows four-fold increase in thymidine uptake in test group which was maximal when macrophage to mycobacterium ratio was 1:10. It was observed that after this infection ratio there was decrease in thymidine uptake with increasing infection ratio, which could be due to clumping of M. bovis BCG. These observations suggested that this in vitro infection model was able to support the growth of M. bovis BCG.

The most striking difference observed by SDS-PAGE
was the induction of some proteins in infected cell lysate as compared to those of mycobacterial or macrophage cell lysates (Figure 1). Seven proteins were differentially expressed during macrophage-mycobacterium interaction and these proteins were further identified by MALDI-TOF (Table 2). Spot 1 (Mb2271) was identified as probable propionyl-CoA carboxylase beta chain 6 which is important for cell envelope lipid biosynthesis. Spot 2 (Mb3761c) encodes UPF0089 protein which is basically uncharacterized protein family. Spot 3 (Rv0046c) encodes a hypothetical protein and could not be assigned any function. Spot 4 was identified as riboflavin biosynthesis protein ribAB with accession number Mb1450. Spot 5 with accession number Mb2260c was identified as cobalamin biosynthesis protein cobD. Spot 6 (Mb2729) is a transcription initiation factor sigma B and is suggested to auto-amplify its own expression under certain stress conditions. Finally, spot 7 (Mb3581) was identified as putative CoA-transferase subunit alpha and has a role in lipid metabolism. Experiments were repeated three times. These proteins might play some role in intracellular survival of mycobacteria within the macrophages.

4. DISCUSSION

The present study deals with development of an in vitro infection model system to study protein profile during macrophage-mycobacterium interaction. Model systems have proved of central importance in determining the activities associated with mammalian cells. To explore the strategies employed by mycobacteria to survive within the macrophages, studies have mostly been carried out with cell lines [3,15]. Our study, however, varies with respect to host macrophage system as we have used natural macrophages from murine peritoneum. Mice are the most widely used vertebrates with short reproductive cycle, short life span, small size and low cost of maintenance making them suitable substitutes in biomedical research.

Our results with thymidine uptake assay clearly suggest that the present in vitro infection model was able to support the growth of M. bovis BCG in murine macrophages. The pattern of cell lysate proteins indicate that adjustments in the pattern of gene expression do occur during macrophage-mycobacterium interaction. The present study revealed seven proteins that were upregulated during macrophage-mycobacterium interaction. Four of the identified proteins (propionyl-CoA carboxylase beta chain 6, riboflavin biosynthesis protein ribAB, Cobalamin biosynthesis protein cobD and putative CoA-transferase subunit alpha) were found to belong to the lipid metabolism category. Propionyl-CoA carboxylase catalyses the carboxylation reaction of propionyl CoA and methylmalonyl CoA is formed. Propionyl CoA is a key precursor in several lipid biosynthetic pathways in M. tuberculosis [16]; however, accumulation of propionate is toxic to the cell and efficient mechanisms are required for its disposal [17]. This dual nature implies a central role for propionate metabolism in the growth and persistence of M. tuberculosis in vivo [18]. Savvi et al. [19] demonstrated the ability of M. tuberculosis to utilize propionate as the sole carbon source in absence of functional methylcitrate cycle provided cobalamin (vitamin B12) is supplied exogenously and thus reinforces the potential relevance of vitamin B12 to mycobacterial patho

Table 1. [3H] Thymidine Incorporation to Mycobacterium bovis BCG. The values are the mean cpm (counts per minute). The SEM of triplicate cultures was < 12% of the mean.

| Samples          | Infection Ratio (Macrophage: Mycobacterium) | cpm (Mean ± SEM) |
|------------------|---------------------------------------------|------------------|
| Control          | 1:0                                         | 377 ± 38         |
| (Only Macrophages)|                                             |                  |
|                  | 1:5                                         | 1081 ± 46        |
|                  | 1:10                                        | 1877 ± 200       |
| Test             | 1:15                                        | 1703 ± 80        |
|                  | 1:20                                        | 1520 ± 50        |

Lane 1: Molecular weight marker (Broad range, BioRad)  
Lane 2: Whole cell lysate of M. bovis BCG grown in macrophages  
Lane 3: Whole cell lysate of macrophages  
Lane 4: Whole cell lysate of M. bovis BCG  

Figure 1. SDS-PAGE profile showing proteins expressed during macrophage-mycobacterium interaction.
Table 2. Details of upregulated proteins identified by mass spectrometry.

| Spot No. | Protein identified | MASCOT Score | Nominal Mass (Da) | pI | Accession Number |
|----------|--------------------|--------------|------------------|----|-----------------|
| 1        | Probable propionyl-CoA carboxylase beta chain 6 | 48           | 50504            | 5.74 | Mb2271          |
| 2        | UPF0089            | 26           | 49501            | 6.36 | Mb3761c         |
| 3        | Hypothetical protein | 9            | 40183            | 5.02 | Rv0046/MT0052   |
| 4        | Riboflavin biosynthesis protein ribAB | 47           | 46331            | 5.46 | Mb1450          |
| 5        | Cobalamin biosynthesis protein cobD | 16           | 33212            | 10.85 | Mb2260c         |
| 6        | Transcription initiation factor sigmaB | 15           | 36347            | 6.19 | Mb2729          |
| 7        | Putative CoA-transferase subunit alpha | 87           | 31817            | 5.64 | Mb3581          |

genesis. Thus the differential expression of cobalamin biosynthesis protein in our experiment is significant. Riboflavin (vitamin B2) biosynthesis has been reported to be essential for in vivo survival of a number of bacterial species because of scarcity of riboflavin in mammalian cells [20]. Riboflavin is the precursor of the coenzymes flavin mononucleotide phosphate and flavin adenine dinucleotide phosphate that are essential compounds for basic metabolism. CoA transferases catalyze the reversible transfer of CoA from CoA-thioesters to free acids and are involved in lipid biosynthesis. Increased expression of these proteins directly or indirectly support their probable role during growth of mycobacteria within macrophages.

Transcription initiation factor sigma B is induced under various stress conditions like heat shock, cold shock, low aeration and limited or no substrate as seen in stationary phase [21,22]. Upregulation of Sigma factor B during macrophage-mycobacterium interaction suggests its role in intracellular survival. Earlier reports have revealed the upregulation of stress proteins under oxidative stress [23] and upon infection of macrophage [3]. No information however is available for the hypothetical protein and UPF0089 protein. Our findings indicate that M. bovis BCG mounts a specific response to the host intracellular environment and the expression of these preferred proteins plays an important role in its intracellular survival.

It is rather unlikely that only expression of seven proteins is sufficient for intracellular survival. Probable reasons for not finding more proteins could be due to technical reasons. Future studies would employ approaches like application of more sensitive and complementary proteome techniques or analyzing at a point where protein profile of mycobacteria is quite stable. Analysis of proteins by two-dimensional gel electrophoresis could not be carried out due to difficulty in recovery of sufficient amount of intracellular BCG proteins. MS identification of mycobacterial proteins from two-dimensional gels has mainly been applied to mycobacteria growing in broth grown cultures where abundant amounts of protein are available for analysis. Only a few studies have utilized the classical proteomic approach to study the mycobacterium-macrophage interactions due to the paucity in recovery of intracellular mycobacteria using infection models [8]. The present study is an attempt in the latter approach.

To summarize, our results clearly indicate that this in vitro infection system is an ideal model system to characterize the differentially expressed genes during the interaction of mycobacterium with macrophages. The employment of such model systems will help in understanding the early molecular events during mycobacterium-macrophage interactions, and thus mycobacterial virulence and pathogenicity. It is hoped that such proteomic studies may contribute significantly to understand mycobacterial pathogenesis and may help in the development of new drug targets and attenuated vaccines.

5. ACKNOWLEDGEMENTS

The authors are grateful to Dr. VM Katoch & Dr. UD Gupta, for their help. We also thank Mr. Ajeet Pratap Singh for technical assistance. This work was supported by grant from DST, New Delhi (No. SR/FTP/LS-A-80/ 2001). NS is SRF (CSIR-UGC). PS and BK are SRFs, ICMR.

6. DISCLOSURES

This paper is unique and is not under consideration by any other publication and has not been published elsewhere. This manuscript has been read and approved by all authors. The authors report no conflicts.
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Structure and properties of bone-like-nanohydroxyapatite/gelatin/polyvinyl alcohol composites

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Received 14 April 2010; revised 7 May 2010; accepted 8 May 2010.

ABSTRACT

Bone-like nanohydroxyapatite powders (b-nanoHA) were synthesized in simulated body fluid (SBF). The b-nanoHA, gelatin (Gel) and Polyvinyl Alcohol (PVA) were used to prepare bone-like composites (b-nanoHA/Gel/PVA) at room temperature. Characterizations of b-nanoHA powders and b-nanoHA/Gel/PVA composites were investigated by using X-ray diffraction (XRD), transmission electron microscopy (TEM), High-resolution transmission electron microscopy (HRTEM), scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR). Bending strength and compressive strength of the composite were tested. It was found that microstructure of the b-nanoHA powders was whisker shape and its crystalline degree was low similar to natural bone, bending strength and compressive strength of the b-nanoHA/Gel/PVA composite depended on the mixing ratio of HA, Gel and PVA, and also PVA could induce the network formation in the b-nanoHA/Gel/PVA composite.

Keywords: SBF; b-nanoHA/Gel Composites; Structure; Mechanical Strength; Cross-Linkage

1. INTRODUCTION

Inorganic bioceramic hydroxyapatite (HA), being the main inorganic composition of the hard tissues in natural bones, has been extensively studied for medical application due to its excellent bioactivity and biocompatibility [1-3]. However, pure HA is low fracture toughness and low degradation properties limiting its clinic application in hard tissue bones [4,5]. In order to solve these shortcomings, collagen, the main organic component of nature bone, is well used to prepare bone-like composites of collagen and HA. Gelatin, being a hydrolysate of collagen, recently is also studied to substitute the expensive collagen. Many kinds of HA/Gel composites were produced and their properties were investigated [6-9].

The dimensions and morphology of HA crystals in bone affects its mechanical properties [10]. It has been found that their length, width and thickness are almost in the range of nano-size [11]. Therefore, the studies of nano-HA/Gel composites have been received extensive attention.

To improve the mechanical properties, various kinds of aldehyde were used as cross-linkage in nano-HA/Gel composites [12-14]. It is known that the composites are limited to apply in bone reconstruction due to toxic aldehyde. In recent years, HA/PVA/Gel composite was prepared by some investigators [15-17].

In this paper, in order to repair the material similar to natural bone, the bone-like nanohydroxyapatite powders (b-nanoHA) was synthesized in simulated body fluid (SBF). The b-nanoHA, Gel and PVA were used to prepare bone-like composites (b-nanoHA/Gel/PVA) at room temperature. The characters of the b-nanoHA powders, the b-nanoHA/Gel and the b-nanoHA/Gel/PVA composites were studied.

2. MATERIAL AND METHODS

2.1. Production of Powders and Composites

SBF[18,19] was prepared by dissolving NaCl, NaHCO3, KCl, K2HPO4·3H2O, MgCl2·6H2O, CaCl2 and Na2SO4 in deionized water. Reagent was added (amount of each reagent given in Table 1), one by one after each reagent was completely dissolved in 2000 ml of deionized water, in the order given above. The concentration of prepared SBF was given in Table 2. A measured amount of CaCl2 (1.946 g) and K2HPO4·3H2O (1.596 g) was added to the prepared SBF solution, under continuous stirring, to produce suspension, according to the following reaction:

\[
14K_2HPO_4·3H_2O + 14CaCl_2 \rightarrow \\
HA + 4Ca(H_2PO_4)_2 + 28KCl + 50H_2O
\]

Then the suspension was transferred into a glass bottle.
and was tightly sealed. The bottle was kept at 37°C in a water bath for 72 hours. The resulting suspension was filtrated and washed several times with deionized water, then the HA slurry was obtained as a precursor to prepare the b-nanoHA/Gel and the b-nanoHA/Gel/PVA composites. On the other hand, HA slurry was dried at 50°C for 24 h, the b-nanoHA powders were obtained.

Commercial gelatin (molecular weight 50000-250000) was produced in Shanghai Chemical Reagent Company. The Gel was completely dissolved at 40°C in deionized water. The commercial PVA (molecular weight 75000-790000) was produced in Shanghai Chemical Reagent Company. The PVA was completely dissolved at 80°C in deionized water. The mixing ratio of HA, Gel and PVA was changed to prepared a series composite samples. Composites were shaped by using homemade cylindrical dies 8 mm in diameter and 25 mm in length. The shaped composites were placed at air for 24 h, then dried at 50°C in air drier for 72 h. Due to water loss, each dried sample was about 5 mm in diameter and 20 mm in length. The experiment schematic diagram for preparing the b-nanoHA/Gel/PVA composite is shown as Figure 1.

2.2. Testing Methods

The prepared samples were studied by XRD (Model: D-Max, Rigaku Co., Tokyo, Japan) at the step size of 0.02° (2θ) and the speed of 10° (2θ) per min. A Cu Kα tube operated at 40 Kv and 80 mA was used for the generation of X-rays. Morphological and sizes of the b-nanoHA powders were investigated by HRTEM (model: Philips Tecnai20U-TWIN). Microstructure characterization of cross-sections of the composites was observed by SEM (model: Quanta200) and by TEM (model: H800). Three point bending strength and compressive strength of composites were measured by universal testing machine (model: RG D-5), at a cross-head of 0.5 mm/min with a span of 15 mm for the samples of Φ 5 mm × 20 mm and at a head of 1 mm/min for the samples of Φ 5 mm × 10 mm, respectively. B-nanoHA/Gel/PVA powders were obtained by pulverizing and used in FT-IR (model: Nicolet Nexus470).

3. RESULTS AND DISCUSSION

3.1. XRD

Figure 2 is the X-ray diffraction spectra of PVA, Gel,
the X-ray diffraction peak shape of PVA was broad at \( \theta = 19.8^\circ \), as well as its peak shows that PVA was non-crystal structure. The X-ray diffraction peak shape of Gel in Figure 2(b) was similar to PVA, non-crystal structure. The X-ray diffraction peak of b-nanoHA powders in Figure 2(c) were good match with the JCPDS (09-0432) standard. In Figure 2(d) and Figure 2(e), the X-ray diffraction spectra of b-nanoHA/Gel composite and b-nanoHA/ Gel/PVA composite were very similar to that of b-nano HA powders, but their diffraction peak broadening was stronger than pure b-nanoHA powders, due to organic gelatin and organic PVA being introduced. Some study [20] has shown that human bone apatite is of diffraction peak broadening in XRD spectra.

3.2. Morphology analysis

The morphology and size of b-nanoHA powders under HRTEM was shown in Figure 3. It can be seen that b-nanoHA powders was composed of a large number of long and straight HA whiskers being of 3-6 nm in diameter and 50-120 nm in length. During high-magnification HRTEM observation (Figure 3(b)), it was found that the HA whiskers were very unstable under strong electron beam irradiation. After about 10 seconds of electron irradiation, the edges of the whiskers became irregular or even disappear.

SEM photographs of the cross-section of b-nanoHA/ Gel (HA:Gel = 6:4) composite and b-nanoHA/Gel/PVA (HA:Gel:PVA = 6:4:2) were shown in Figure 4(a) and Figure 4(b). It is apparent that distribution of b-nanoHA powders, gelatin and PVA in the composite was very uniform. The more high-magnification SEM photographs of the cross-sections of b-nanoHA/Gel composite and b-nanoHA/Gel/PVA composite were not obtained due to the composites unstable by electron irradiation under high-magnification SEM.

From the TEM photographs, the microstructures of b-nanoHA/Gel composite (HA:Gel = 6:4) and b-nano HA/Gel/ PVA (HA:Gel:PVA = 6:4:2) were observed, as shown in Figure 5(a) and Figure 5(b). Organic-inorganic interaction among gelatin, PVA and HA was very uniform. The size of HA whiskers in composites was similar to HA powders, about 50-150 nm in length. Figure 5 also shows the corresponding SAED pattern for the two composites. It was a typical micrograph for HA/ Gel nano-composite [21], being of the small spots and diffused ring pattern, indicating the existence of low degree crystalline nano-phase.

3.3. Mechanical Property Analysis

Bending strength and compressive strength of five samples were provided in Table 3, each datum was the average value of six samples with the same mass ratio (HA:Gel). It is seen that, with Gel content increasing, both bending strength and compressive strength of b-nanoHA/Gel composites increase initially, and decrease when the ratio of HA to Gel is more than 6:4. The values of bending strength and compressive strength of
Table 3. Bending strength and Compressive strength of b-nanoHA/gel composites.

| Order | Mass ratio  | Bending strength (MPa) | Compressive strength (MPa) |
|-------|-------------|------------------------|---------------------------|
| Sample 1 | HA:Gel = 8:2 | 16.83 | 10.79 |
| Sample 2 | HA:Gel = 7:3 | 19.06 | 19.82 |
| Sample 3 | HA:Gel = 6:4 | 35.97 | 39.20 |
| Sample 4 | HA:Gel = 5:5 | 22.38 | 29.77 |
| Sample 5 | HA:Gel = 4:6 | 18.35 | 17.06 |

Table 4. Bending strength and Compressive strength of b-nanoHA/gel/PVA composites.

| Order | Mass ratio  | Bending strength (MPa) | Compressive strength (MPa) |
|-------|-------------|------------------------|---------------------------|
| Sample 6 | HA:Gel = 6:4:1 | 36.22 | 39.73 |
| Sample 7 | HA:Gel = 6:4:2 | 58.11 | 60.21 |
| Sample 8 | HA:Gel = 6:4:3 | 49.79 | 52.17 |

sample 3 were the highest among the five samples, 35.97 MPa and 39.20 MPa, respectively.

Table 4 showed bending strength and compressive strength of three samples, each datum was the average value of five samples with the same mass ratio (HA:Gel:PVA). It is observed that bending strength and compressive strength of b-nanoHA/Gel/PVA composites are higher than those of b-nanoHA/Gel composites. Moreover, the values of bending strength and compressive strength of sample 7 being the highest, 58.11 MPa and 60.21 MPa, respectively, are higher than some reports [15,16]. It is thought that PVA has cross-linking effect in b-nanoHA/Gel/PVA composites.

3.4. FT-IR

Figure 6(a) showed chemical bond positions for the reference sample PVA, such as C-H stretching and bending at 2945 cm\(^{-1}\) and 1337 cm\(^{-1}\), C-C stretching at 854 cm\(^{-1}\), O-H stretching at 3328 cm\(^{-1}\), C-O stretching at 1098 cm\(^{-1}\) and CH-OH bending at 1427 cm\(^{-1}\). From Figure 6(b), the spectral intensity of O-H stretching at 3328 cm\(^{-1}\) was stronger than that of PVA in Figure 6(a) due to HA containing abundance of OH\(^{-}\) ions. It is suggested that new chemical bonds were not formed between HA and PVA in the HA/PVA composite because the bond frequencies (3328 cm\(^{-1}\), 1427 cm\(^{-1}\)) being relate to OH were not changed. Figure 6 (c) showed typical amide bond frequencies for the reference sample Gel, such as amide A at 3419 cm\(^{-1}\) for N-H stretching, amide I at 1659 cm\(^{-1}\) for C=O stretching, amide II at 1537 cm\(^{-1}\) for N-H bending and amide III at 1240 cm\(^{-1}\) for C-N stretching. From

Figure 6(d), the spectral feature of all amide bonds was obviously changed, such as the band frequencies of amide A and amide I were blue-shifted to 3406 cm\(^{-1}\) and 1649 cm\(^{-1}\), respectively, of amide II was red-shifted to 1548 cm\(^{-1}\) and of amide III disappeared. From the spectral change of the amide bonds between the reference Gel and the HA/Gel composite, it is indicated that the inorganic-organic (Ca\(^{2+}\)-COO\(^{-}\)) bonds were formed in the HA/Gel composite [9,21].

Figure 7 is FT-IR spectra of HA/Gel/PVA composites. It is observed that the band frequencies of amide A and amide I were blue-shifted, of amide II was red-shifted. From the spectral change of the amide bands, it is indicated that the hydrogen bond (OH…NH) was formed between the active group OH in PVA and the group NH in Gel. On the other hand, we can observe the intensity of C-H bond became stronger with the amount of PVA increasing. The optimal amount of PVA in HA/Gel/PVA composites was HA:Gel:PVA = 6:4:2 (mass ratio) in the range of experiments, corresponding to the mechanical
property analysis above.

From the above analysis for Figure 6 and Figure 7, it is considered that there was a complex network structure in HA/Gel/PVA composites, being existence of $\text{Ca}^{2+}$-COO$^{-}$ bond between HA and Gel, and being $\text{OH}$$\cdots$NH bond between Gel and PVA. Figure 8 is the sketch map of the complex network structure in HA/Gel/PVA composites. It is thought that PVA could be a good kind of cross-linkage in HA/Gel/PVA composites or other bone substitution materials.

4. CONCLUSIONS

Diameter and length of HA synthesized in SBF are almost in the range of nano-size and its crystalline is low similar to natural bone. The mechanical properties of b-nanoHA/Gel/PVA composites depended on the mass ratio of HA, Gel and PVA. The highest values of bending strength and compressive strength appeared when mass ratio was HA:Gel:PVA = 6:4:2 in the range of experiments. PVA might be a potential cross-linkage instead of toxic aldehyde in the field of preparing artificial bone.

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The lability of behavior as a marker of comorbid depression and anxiety

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Received 30 April 2010; revised 29 May 2010; accepted 7 June 2010.

ABSTRACT

This study examines nine dynamical and three emotional aspects of behavior in depression and anxiety, singly and comorbidly. The study employs the Structure of Temperament Questionnaire Compact (STQ 77), whose 12 scales assess the energetic, lability and sensitivity aspects of behavior in the physical, social, mental and emotional domains. The STQ 77 was administered to 86 patients with Major Depression, 85 patients with an anxiety disorder, 43 patients with comorbid depression and anxiety, and 71 subjects without depression or anxiety disorder all presenting to a private outpatient clinical practice. Results: 1) Depression was associated with self-reports of increased impulsivity and rigidity of behavior; 2) Depressed patients reported significantly lower physical energy, tempo of physical activity and plasticity of behavior. The presence of comorbid anxiety further worsened these effects; 3) The ability to sustain attention on a mental task and to learn new information was lower in depressed patients than in other groups. Conclusions: comorbid depression and anxiety might be associates, decreasing adaptivity and the self-regulatory balance of behavior, leading to the development of extremes in behavioral reactivity (impulsivity and rigidity).

Keywords: Comorbid Depression and Anxiety; Behavioural Reactivity; Lability; STQ-77

1. INTRODUCTION

Psychiatry is focused on the biological factors associated with mental disorders, hence it has been natural to emphasize symptoms that have a psychobiological character such as psychomotor retardation, fatigue, inattention and so on. These symptoms reflect the impact of mood upon the dynamics of behavior, in particular, its energetics. There are many additional dynamical aspects, however, but these have been relatively neglected in studies of depression and anxiety and in comorbidity in particular. Studies into psychobiological correlates in comorbidity have identified relationships between mood and factors such as neuroticism, harm avoidance, self directedness [1] but not with activity per se. This leaves a significant gap in our current understanding of the biological underpinnings of depression and anxiety and their comorbidity. Even basic questions related to whether depression and anxiety are separate or interrelated remain unsettled [2,3]. Since the regulation of activity is a basic psychobiological attribute, it is reasonable, therefore, to consider additional measures and modes of investigation that highlight correlations between mood and activity in an effort to achieve a greater understanding.

Correlations between mood and activity partly formed the basis of the earliest attempts to understand human character. About 2400 years ago Hippocrates and then Galen described four types of “temperaments”, or mixtures of bodily chemical components. According to their theory, in a healthy individual this mixture is balanced, and an imbalance in this mixture causes noticeable and consistent patterns of behavior: choleric (impulsive), melancholic (depressive), phlegmatic (socially detached) and sanguine (manic). It is probably safe to say that the Hippocrates-Galen theory of temperaments was the earliest theory of mood and personality disorders linked to human physiology, even while it described only four conditions. The first attempt to describe the basic factors underlying these behavioral patterns was made by Immanuel Kant [4]. Kant suggested that the Hippocrates-Galen four “temperaments” could be derived from two components, 1) “activity”, or energetic strength in behavior, and 2) “feelings” (emotionality), or the ability to control emotional reactions while focusing on an action. Choleric were described as emotional and energetic, Phlegmatics—as non-emotional and weak, Sanguines—as well-regulated and energetic, and Melancholics as emotional and weak.

These two factors, an “energetic” aspect and an
“emotionality” aspect were echoed in the writings of many psychiatrists and psychologists during the 20th century. In spite of a diversity of labels given to these traits, universally they were presented as traits of temperament. Eysenck designed his version of the pair “Activity and Emotionality” as “Extraversion and Neuroticism”, and the Eysenck Personality Questionnaire was used in several clinical investigations [5]. Thayer [6] proposed a similar model with dimensions of energetic arousal (energy vs. tiredness) and “tense arousal” (tension vs. calmness). Jeffrey Gray [7], separated two types of regulatory systems, calling them the Behavioural Activation System (BAS) and the Behavioural Inhibition System (BIS). An imbalance between these systems caused, according to Gray, clinically symptomatic behavior such as impulsivity (with an excess of BAS activation over BIS), anxiety-neuroticism (with an excess of BIS) and intermediate states of extraversion and withdrawal [8]. Other temperamental models tested in clinical practice include the Temperament and Character Inventory, TCI [9,10], Pleasure-Arousal-Dominance, PAD [11], Dimensions of Temperament Survey—Revised [12], Happiness-Unhappiness model [13], Big Five model [14], and Temperament & Personality Questionnaire [15].

Overall the list of temperament traits described in about 30 various temperament models and tests reported in the literature now exceeds 80 entries, and is not the subject of the present paper. The majority of these models have paid little attention to the multiple aspects of activity components, especially to the dynamical (energetic, tempo, plasticity) aspects, and the scales of multi-dimensional models have primarily considered aspects of emotionality, such as approach and withdrawal behavior. In contrast to these models, an experimental tradition involving the detailed study of aspects of activity, i.e. the dynamical properties of nervous systems (strength, mobility and balance) was started at the beginning of 20th century by Pavlov. During 30 years of experiments and extensive observations, Pavlov noticed that these properties might be linked to psychiatric disorders [16]. About the same time, observations from psychiatrists provided terminology which described consistent deficiencies in energetic and tempo-related features of behavior as contributing factors in psychiatric disorders. For example, concepts of “cyclothymia”, “psychoaesthesia” and “psychic tempo” suggested by the German psychiatrist Ernst Kretschmer [17].

Pavlov’s tradition was continued in the experiments of Teplov, Nebylitsyn, Strelau and Rusalov throughout the 20th century. In the 1980’s Rusalov studied consistent individual differences in psychophysiological and psychological data in a wide variety of settings, including: EEGs, evoked potentials, absolute thresholds in visual, auditory, and tactile modalities, strength of excitation and mobility in auditory and visual modalities, problem-solving in deterministic and probabilistic conditions, the speed of problem-solving using a variety of intellectual tests, the time spent in attempting unsolvable problems and the number of times a subject gave up while attempting to solve a task [18]. Based on these studies, Rusalov developed the Questionnaire of Formal-Dynamical Properties of Individuality later re-named as the Structure of Temperament Questionnaire (STQ). The STQ in its Extended version assesses 4 traits: 1) ergonicity (energetic component); 2) plasticity; 3) tempo of activity; and 4) emotionality, each according to three types of activity—physical (“object-related”), social-verbal and intellectual [19-22]. The Compact version of the STQ (STQ-77) was developed by a clinician for clinical usage as an abbreviated version of the Extended STQ, with additional scales following the neuropsychological models of Luria. The STQ-77 uses 6 out of 12 items from each original scale, rearranging and re-labeling the scales according to the traits of 1) arousal; 2) lability; and 3) sensitivity, each within the physical, social-verbal, and mental areas of activity, and emotionality, as shown in the Figure 1 [21,23]. A brief summary of the validation history of the Extended and Compact STQ is given in the Supplementary material.

In this paper, “temperament” refers to the consistent, biologically based dynamic aspects of behavior, and should not be conflated with “personality” which refers to those stable characteristics that arise through social developmental processes and social learning. Previous investigations of associations between temperament and depression and anxiety have mainly focused on issues of childhood temperament, and to a much lesser degree on adult temperament. The studies of the associations between adult temperament and depression showed that depression was associated with high so-called “harm avoidance” and low so-called “self-directedness” as measured by the TCI [9].

| Mental Activity | Physical Activity | Social-verbal Activity | Emotionality |
|-----------------|-------------------|-----------------------|--------------|
| Intellectual Ergonicity (ERI) | Motor Ergonicity (ERM) | Social/Ergonicity (ERS) | Self-confidence, SCF |
| Plasticity, PC | Motor Tempo, TM | Social Tempo, TMS | Impulsivity, IMP |
| .. to probabilities, PRO | .. to sensations, SS | to others/Empathy, EMP | Neuroticism, NEU |

Figure 1. The STQ-77 structure and its temperament scales.
The content of the harm-avoidance scale of the TCI includes the characteristics of anticipatory worry, fear of uncertainty, shyness and fatigability, and the content of the TCI self-directedness scale includes the characteristics of responsibility, purposefulness, resourcefulness, and self-acceptance. When analyzed against the STQ-77 scales, the TCI scale of Self-directedness includes features of the Plasticity scale of the STQ-77, and the fatigability descriptor in the TCI scale relates to the energetic components (Ergonicity) scales of the STQ-77.

Those few studies which investigated associations between adult temperament, depression or anxiety analyzed these disorders separately, without investigating the comorbidity condition. This is the first study to explore the impact of comorbidity between anxiety and depression and the resulting associations with patients’ reports of symptoms related to the formal dynamical aspects of their activity (such as energetic, lability, sensitivity and emotionality aspects). Clinically, both depressive and anxiety disorders have significant impact on the organization and tempo of activity and the motivation and endurance of the patient. These manifest in symptoms such as psychomotor retardation, agitation, lack of motivation and interest, inattention, impaired learning under stress, and the like. It is an interesting question whether these symptoms are wholly state determined reflecting the transient effect of the illness on psychophysiological functioning, or whether there might exist underlying dynamical properties of the nervous system, which either predispose an individual to depression or anxiety or which predispose an individual to expressing depression or anxiety through these particular symptoms complexes. These symptoms form an essential part of the diagnostic criteria for these disorders.

The fundamental role that these symptoms play provides justification for examining how the dynamical aspects of activity may be related to the depressive and anxiety disorders that distort such expression. Thus the goal of the study was to investigate the relationships between the indicated disorders and patients’ reports on their endurance, tempo, plasticity and emotionality of behavior.

### 2. METHOD

In this study we examined the intake records of 199 (86 males and 113 females) Caucasian Canadians, patients and associates of a private psychiatric and psychological practice located in Hamilton, Ontario, Canada and serving the Hamilton and Toronto areas. Eighty six subjects were diagnosed with Major Depressive Disorder on the basis of the structured DSM-IV clinical interview (conducted by licensed psychologist and psychiatrist), file review and the results of testing using the Beck Depression Inventory (scores of 20 or higher), Hamilton Depression Inventory (scores of 20 or higher on the Total scale) and Symptom CheckList-90 (scores of 40 or higher on the Depression scale). Eighty five subjects (31 males and 44 females) were diagnosed with a disorder within the DSM-IV Anxiety Disorders category (moderate to severe): GAD (78.6%), PTSD or specific phobia (16.7%), OCD (4.8%) on the basis of the structured DSM-IV clinical interview, file review and the results of testing using the Beck Anxiety Inventory (scores of 16 or higher), State Trait Anxiety Inventory (scores of 61 or higher), Detailed Assessment of Post-traumatic Stress inventory, Post-Traumatic Stress diagnostic scale and Symptom CheckList-90 (scores of 31 or higher on the Anxiety scale). Within the patient group, 43 patients had comorbid depression and anxiety. The patient groups were compared against 71 control subjects in whom the diagnoses of depression or anxiety disorder were ruled out. Control subjects were also clients and patients of the same private practice. Demographic information about the sample is presented in Table 1.

During intake testing each patient completed the Compact Structure of Temperament Questionnaire (STQ-77) [21] and gave consent to use the data from their file for research purposes. The STQ-77 consists of 77 statements, assigned to 12 temperament scales (6 statements each) and a validity scale (5 items, addressing social desirability bias), which are listed below. Subjects responded according to a 4-point Likert scale format: 1) “strongly disagree”, 2) “disagree”, 3) “agree”, 4) “strongly agree”. Protocols having scores of 15-20 on the validity scale were considered invalid as the respondents were likely to demonstrate a positive impression bias in their responses. The scales are:

1-3: Ergonicity group, scales of Motor, Social and Intellectual Ergonicity: the ability of an individual to sustain prolonged physical (ERM), social (ERS) or mental (ERI) activity.

4-5: Lability group, scales of Motor and Social Tempo: preferred speed of physical activity (TMM), speed of speech and reading and of other verbal activities (TMS) and Plasticity scale, assessing the ability to adapt quickly to changes in situations, to change the program of action, and to shift between different tasks (PL).

6-9: Sensitivity group: Sensitivity to Sensations scale (SS), assessing the sensitivity of an individual to basic physical sensations and pleasures, a tendency for sensation-seeking and risk-taking behaviour; Empathy scale (EMP) assessing sensitivity of an individual to another
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Table 1. Demographic characteristics of the sample.

|                | All     | Control group | Comorbid group | Depression only | Anxiety only | Combined Depression | Combined Anxious |
|----------------|---------|---------------|----------------|-----------------|--------------|---------------------|-----------------|
| Total          | 199     | 71            | 43             | 43              | 42           | 86                  | 85              |
| Males/Females  | 86/113  | 28/43         | 19/24          | 19/24           | 20/22        | 38/48               | 39/46           |
| Age range      | 18-87   | 18-87         | 18-82          | 19-81           | 19-72        | 18-87               | 19-81           |
| Mean           | 38.76   | 41.93         | 41.87          | 43.51           | 43.53        | 41.16               | 43.52           |
| SD             | 14.29   | 13.78         | 11.99          | 14.49           | 15.21        | 14.51               | 14.77           |
| Marital status (%) |        |               |                |                 |              |                     |                 |
| Married        | 59.30   | 64.79         | 48.84          | 53.49           | 66.67        | 51.16               | 57.65           |
| Divorced       | 14.57   | 9.86          | 25.58          | 16.28           | 9.52         | 20.93               | 17.65           |
| Single         | 26.13   | 25.35         | 25.58          | 30.23           | 23.81        | 27.91               | 24.71           |
| Education %    |         |               |                |                 |              |                     |                 |
| High school or less | 38.19   | 29.58        | 39.53          | 44.19           | 45.24        | 41.86               | 42.35           |
| College or higher | 61.81   | 70.42        | 60.47          | 55.81           | 54.76        | 58.14               | 57.65           |
| Work status %  |         |               |                |                 |              |                     |                 |
| Retired        | 6.53    | 4.23          | 11.63          | 6.98            | 4.76         | 9.30                | 8.24            |
| On disability  | 10.55   | 9.86          | 6.98           | 20.93           | 4.76         | 13.95               | 5.88            |
| Unemployed     | 22.11   | 19.72         | 18.60          | 27.91           | 23.81        | 23.26               | 21.18           |
| Employed       | 60.80   | 66.20         | 62.79          | 44.19           | 66.67        | 53.49               | 64.71           |

person’s emotional state, and Sensitivity to Probabilities (PRO) scale assessing ability of an individual for adequate understanding and expectations of probable events, the efficient extraction and processing of new knowledge.

10-12: Emotionality group: Self-confidence scale (SLF): the tendency to be optimistic and confident (sometimes overly optimistic) in own performance, to ignore other people’s warnings and criticism; Impulsivity scale (IMP): the lability of emotional reaction, a poor ability to control immediate impulses for actions; Neuroticism scale (NEU): low tolerance of uncertainty with expectations of a negative outcome.

3. RESULTS

ANOVA was carried out with post-hoc comparisons using the Tukey HSD method for unequal samples, for 3 separate analyses 1) between “Controls”, “Depression plus Comorbid” (Depression-C), and “Anxiety-only” groups; 2) between “Controls”, “Anxiety plus Comorbid” (Anxiety-C), and “Depression-only” groups; 3) between four groups: Control, Depression-only, Anxiety-only, Comorbid Depression-Anxiety. Table 2 shows the results on the scales which had significant effects.

Figures 2-4 show that the major effects involved the temperament scales of Motor Ergonicity, Motor Tempo, Intellectual Ergonicity, Sensitivity to Probabilities, Plasticity, and Impulsivity scales of STQ-77. The F and p values of the effects are given in the last three lines of Table 2. In the first analysis (Figure 2) the Depression-C group had significantly higher Impulsivity scores and significantly lower scores of Motor Ergonicity, Motor Tempo, Plasticity, Intellectual Ergonicity and Sensitivity to Probabilities as compared to the Control group.

The second analysis (Figure 3) showed that the group which combined both Anxiety-only and Comorbid-anxiety subgroups had higher Impulsivity and lower Motor Ergonicity and Motor Tempo scores than the Control group. The differences between the Depression-only and Control groups were similar to the first analysis, showing that depressed patients had significantly lower scores on Motor Ergonicity, Motor Tempo and Plasticity and Intellectual Ergonicity scales of STQ-77.

When the comorbid group was analyzed separately from the “depressed only” and “anxious only” groups in our third analysis there were no significant differences between the anxiety and control groups, nor between the depression and comorbid groups (Figure 4). The Comorbid group reported significantly lower physical ergonicity, plasticity, physical tempo and higher impulsivity than the Control group. The Depression group again showed lower scores of physical ergonicity, physical tempo, plasticity and intellectual ergonicity than the Control group.

4. DISCUSSION

There are three main findings.

1) The results show that depression had an impact on the lability of behavior. This is a new finding. When analyzed according to the dynamical aspects of activity, three out of four lability-related scales of
STQ-77 (the mobility of initiation or of changing an activity, the lability of emotional responses, *i.e.* impulsivity and tempo of physical activity) showed a significant effect in the Comorbid group, Depression

Figure 2. Means and conf. intervals (0.95) of the STQ-77 scores in scales with significant effects (p-level of post-hoc pairwise comparisons is indicated below each group). The “Depression” group consists of patients with and without comorbid anxiety.

Table 2. Means (M), confidence intervals (CI, 95%), standard deviations (SD) of the STQ scores, and ANOVA effects in three analyses 1) the Depression group including the Comorbid group (F3D), 2) the Anxiety group including the Comorbid group (F3A), 3) Comorbid group was an independent group (F4). Only the scales having significant effects are shown.

| Group: | Control, N = 71 | Depression-C, N = 86 | Anxiety (only), N = 42 |
|--------|-----------------|----------------------|------------------------|
| Control | ERM: 0.00, TMM: 0.00, IMP: 0.00 | ERM: 0.00, TMM: 0.00, IMP: 0.00 |
| Depression | PL: 0.00, ERI: 0.00, PRO: 0.01 |

| Group: | Motor Ergonicity | Motor Tempo | Intellectual Ergonicity | Plasticity | Sensitivity to Probabilities | Impulsivity |
|--------|------------------|-------------|-------------------------|------------|----------------------------|-------------|
| Control, M | 17.30 | 16.73 | 17.18 | 16.49 | 17.37 | 14.11 |
| CI | 16.6-18.0 | 16.0-17.4 | 16.5-17.9 | 15.9-17.1 | 16.7-18.1 | 13.5-14.7 |
| SD | 2.86 | 2.93 | 3.03 | 2.36 | 2.99 | 2.42 |
| Comorbid, M | 14.33 | 14.35 | 15.60 | 14.65 | 15.63 | 16.09 |
| CI | 13.0-15.7 | 12.9-15.8 | 14.2-17.0 | 13.4-15.9 | 14.5-16.8 | 15.0-17.2 |
| SD | 4.32 | 4.80 | 4.52 | 4.09 | 3.81 | 3.68 |
| Depression only, M | 14.40 | 14.26 | 15.12 | 14.88 | 15.98 | 16.09 |
| CI | 12.9-15.9 | 12.9-15.6 | 14.0-16.3 | 13.8-15.9 | 15.1-16.9 | 15.2-17.0 |
| SD | 4.81 | 4.49 | 3.70 | 3.42 | 2.94 | 3.05 |
| Anxiety only, M | 16.07 | 15.57 | 16.00 | 15.98 | 16.71 | 14.79 |
| CI | 14.8-17.3 | 14.3-16.8 | 14.9-17.1 | 15.1-16.9 | 15.7-17.8 | 13.7-15.9 |
| SD | 4.00 | 4.01 | 3.65 | 2.92 | 3.37 | 3.54 |
| Depression-C, M | 14.36 | 14.30 | 15.36 | 14.77 | 15.80 | 16.09 |
| CI | 13.4-15.3 | 13.3-15.2 | 14.5-16.2 | 14.0-15.6 | 15.1-16.5 | 15.4-16.8 |
| SD | 4.55 | 4.62 | 4.11 | 3.75 | 3.39 | 3.36 |
| Anxiety-C, M | 15.19 | 14.95 | 15.80 | 15.31 | 16.16 | 15.45 |
| CI | 14.3-16.1 | 14.0-15.9 | 14.9-16.7 | 14.5-16.1 | 15.4-16.9 | 14.7-16.2 |
| SD | 4.24 | 4.44 | 4.09 | 3.60 | 3.62 | 3.65 |
| F_{10}(2,196)/p | 11.15/0.00 | 7.35/0.00 | 4.86/0.01 | 6.18/0.00 | 4.58/0.01 | 8.22/0.00 |
| F_{1A}(2,196)/p | 8.84/0.00 | 6.28/0.00 | 4.93/0.01 | 4.26/0.02 | 3.47/0.03 | 6.21/0.00 |
| F_{1}(3,195)/p | 7.40/0.00 | 4.88/0.00 | 3.36/0.02 | 4.14/0.01 | 3.12/0.03 | 5.45/0.00 |
Means and confidence intervals (0.95) for the scales:

| Group                  | Control, N = 71 | Depression (only), N = 43 | Anxiety, N = 85 |
|------------------------|-----------------|--------------------------|-----------------|
| Control                | ERM: 0.00, TMM: 0.03 | ERM: 0.00, TMM: 0.01 | ERM: 0.00, TMM: 0.02 |
| Depression             | PL: 0.04, ERI: 0.02 | IMP: 0.03               | IMP: 0.01       |
| Anxiety                | IMP: 0.03       | ERI: 0.04, IMP: 0.02    |                 |

Figure 3. Means and confidence intervals (0.95) of the STQ-77 scores in scales with significant effects (p-level of pairwise comparisons is indicated below each group). The “Anxiety” group consists of patients with and without comorbid depression.

Means and confidence intervals (0.95) for the scales:

| Group                  | Control, N = 71 | Comorbid, N = 43 | Depression-only, N = 43 | Anxiety-only, N = 42 |
|------------------------|-----------------|-----------------|------------------------|----------------------|
| Control                | PL: 0.03, IMP: 0.01 | ERM: 0.00, TMM: 0.03 | ERM: 0.00, TMM: 0.02   |                       |
| Comorbid               | IMP: 0.01       | ERI: 0.04, IMP: 0.02 |                      |                      |

Figure 4. Means and confidence intervals (0.95) of the STQ-77 scores in scales with significant effects in post-hoc comparison between 4 groups (p-level of pairwise comparisons is indicated below each group).

The presence of comorbid depression and anxiety had a more significant positive impact on impulsivity than did either pure depression or anxiety.

While impulsive behavior, especially violent and self-destructive behavior is not uncommon in depression, it is not a fundamental defining characteristic. Its significant expression in the comorbid group could reflect the exhaustion of the energetic capacities, which in the STQ model are related to temperament. It implies that comorbid depression and anxiety have a more significant impact on impulsivity than pure depression or anxiety.
bidity of depression and anxiety lowers the self-regulatory capacity of an individual, as these two illnesses force an individual to live under conditions of lowered physical resources and elevated perceived situational demands. Lower scores on the Plasticity scale in depressed and comorbid patients indicate rigidity in adjusting to changing circumstances.

These results were consistent with higher rates of non-adaptive behaviour in depressed patients in comparison to non-depressed people: higher rates of suicidal attempts, shop-lifting, problems with initiation of activity perceived as apathy, problems in relationships and employment. Such behavior has components of impulsivity or rigidity in reaction to the circumstances. This was also consistent with the association between depression and scores on temperament measures from other reports. For example, Sellbom and colleagues [13] found association between depression, demoralization and the lower scores on the TCI scale of Self-directedness. These results were consistent with higher rates of suicide attempts, shop-lifting, problems with initiation of activity perceived as apathy, problems in relationships and employment. Such behavior has components of impulsivity or rigidity in reaction to the circumstances.

2) The most significant differences on the temperament scales of the STQ-77 reflected a lowered physical capacity in depression, i.e. patients with comorbid depression and depression-only had significantly lower scores than the control group on the scales of Motor Ergonicity, Motor Tempo and Plasticity. These scales describe (respectively) the ability of an individual to sustain intense and/or prolonged physical activity, to maintain a high speed in such activity or to change the course of activity in a timely manner. The lowered capacity described by these scales may reflect the effect of psychomotor retardation and lethargy induced by the depression.

3) The ability to sustain attention on a mental task (as measured by the Intellectual Ergonicity scale of STQ-77) and the ability to learn new information (as measured by the Sensitivity to Probabilities scale) were significantly lower in the combined Depression group than in the Depression-only, Anxiety and Control groups. This is consistent with such symptoms of depression as “inability to focus” and informational withdrawal. This was also consistent with the findings using other temperament models. Thus, Healy and Kulig [12] used the Dimensions of Temperament Survey and measures of anxiety and attentional control and found associations between anxiety and attentional control. Hundt and colleagues [8] used Gray’s 2-component model of Behavioral Activation/Behavioral Inhibition Systems and found that high inhibition predicted poor attention and ADHD symptoms.

From the standpoint of the STQ-77, the differences between the Anxiety groups and the normal controls were less significant than the differences between the Depression groups and controls. This could reflect a more debilitating effect of depression than anxiety on normal functioning and a stronger association of depression with the same chemical-biological factors that determine temperament. The differences in scores appear to show a graded response as a function of the disorder, depression having the greatest impact followed by the comorbid state, then anxiety and finally normality. This graded effect holds for both the positive as well as the negative effects and suggests that in their underlying effect on the neurophysiological underpinnings of temperament, depression, anxiety and normality lie along a continuum, in keeping with the current hypothesis that these disorders lie along a spectrum of psychopathology. That the comorbid group behaves more like the depressive group also suggests that depression may be the more dominant component and the comorbid group consists more of anxious depressives rather than de-
pressed worriers.

The main limitation of this study is associated with a use of a self-report measure, such as the STQ-77. While self-report measures are commonly used in psychiatric practice and research, one should always remember the limitations of their validity. The STQ-77 has a validity scale which allows one to screen for the high social desirability tendency and to select out invalid protocols. This improved the validity of our results but did not completely eliminate the measurement errors expected for self-report measures.

This study shows that depression has a significant impact on lability of activity (tempo, plasticity and impulse control), compromising the responsiveness of behavior through an increase in rigidity and impulsivity. Our results also show that far from simply providing an overall modulating effect, depression and anxiety appear to associate mostly with areas related to physical activity and not to intellectual or verbal activity.

5. ACKNOWLEDGEMENTS

The authors would like to acknowledge the hard work of Ms. Natalia Gregory in processing the data of this article.

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Supplementary Material: A Summary of the STQ Validation

During the experimental validation of the STQ in the 1980-90s, the performance of subjects on the following measures were compared with STQ scales in a series of studies: speed of writing, reading and generation of words, maximal and optimal tempo of performance in sensory-motor tasks and intellectual (including unsolvable) tasks, performance on non-verbal tasks with which subjects were unfamiliar, rigidity of perception in tactile and visual modalities, duration of the switch from one way of solving the task to another, mobility in attention, variability in line drawing (Rusalov, 1979, 1989, Rusalov and Trofimova, 2007). The first version of STQ had 8 scales: the four temperament scales (ergonicity, plasticity, tempo and emotionality) assessed in social and physical activity. Each scale had 12 items (Rusalov, 1989).

The Extended version included additional 4 scales related to intellectual activity. In the studies of the concurrent validity of the initial STQ it was compared to Eysenck’s EPQ (Brebner and Stough, 1993; Rusalov, 1989; Zinko, 2006), NEO-FFI (Bodunov et al., 1996, Dumenci, 1995), Strelau’s PTS (Bodunov et al., 1996; Ruch et al., 1991; Strelau, 1999, Trofimova, 2009), meaning attribution to neutral objects (Trofimova, 1999), the Motivation for Achievement scale (Vorobieva, 2004), adaptivity strategies in the Dembo-Hoppe Level of Aspiration experiment (Zin’ko, 2006), 25 measures of Mobility (Rathe and Singh, 2001), Dissociative Experiences Scale (Beere and Pica, 1995, Vasyura, 2008). References to STQ validation with the Rogers Adaptivity scale, the Torrance’s Nonverbal Tests of Creative Thinking, Rotter’s Locus of Control scale, a choice of profession, with other 8 measures of plasticity, STAI, MAS, Wechsler, Shepard and Gotschild Figure tests, Rosenzveig test, Cattell’s 16-PF inventory, and with the school grades of high-school students, can be found in the work of Rusalov and Trofimova (2007).

The administration of the English version of the STQ (STQ-E) to American, Australian and Canadian samples demonstrated that it has a factor structure similar to that of the Russian language version, and that it has good reliability and internal consistency (Bishop et al., 1993; Bishop, Hertenstein, 2004; Dumenci, 1995, 1996 (initial version of STQ); Rusalov, 1997, 2004; Stough et al., 1991; Rusalov and Trofimova, 2007). Chinese (STQ-C), Urdu (STQ-U) and Polish (STQ-P) Extended versions of the STQ, administered among corresponding populations, showed reliability coefficients in the range 0.70-0.86, item-total correlations in the range 0.42-0.73, and all versions demonstrated robust factor structures similar to those of the original version (Trofimova, 2010a).

The Confirmatory Factor Analysis of the Compact STQ (STQ-77) using data from a Canadian sample shows a satisfactory fit of the traditional 4-factor STQ activity-specific model, grouping the scales to the factors of Motor, Social, Intellectual activity and Emotionality and having 2 correlated residuals (from the new scale of Sensitivity to Sensations to Impulsivity and Neuroticism scales) with the CFI > 0.90, RMSEA < 0.07 and RMSR < 0.06 (Trofimova, 2010b).

The studies of reliability and content, concurrent and discriminant validity of STQ-77 scales showed that the reliability of these scales is in the range of 0.70-0.86. The time of testing correlated statistically significantly with the Social Tempo scale (r = –0.31) (Trofimova and Sulis, 2009), and the time of performance on a task involving the classification of 25 common words also had the most significant negative correlations with Social Tempo, as well as with Self-Confidence scale of STQ-77 (–0.36 and –0.29, respectively) (Trofimova, 2010c). High school grades show activity-specific correlations with the STQ-77 scales (Trofimova and Sulis, 2009): the grades in athletics correlate with the STQ-77 scales of Motor Ergonicity and Tempo (r = 0.53 and 0.45), the grades in verbal assignments have r = 0.28 with Social Ergonicity, and r = 0.27 with Social Tempo scale, and the grades in math and science correlate only with the scales of Intellectual Ergonicity and Plasticity (r = 0.26 and 0.22, respectively) (Trofimova and Sulis, 2009). Significant positive correlations are found between the new STQ-77 scales of Impulsivity, Sensitivity to Sensations, Empathy and the corresponding scales of Impulsiveness (r = 0.51), Venturesomeness (0.64) and Empathy (0.73) of Eysenck’s I-7 questionnaire (Trofimova and Sulis, 2009). STQ-77 Sensitivity to Sensation scale shows good agreement with Impulsivity scale (r = 0.68) and Zuckerman Sensation Seeking Scale (r = 0.37) (Trofimova, 2010b).

Extraversion, as measured by the Big Five (NEO-FFI), correlates with Social Ergonicity and Impulsivity with r = 0.46 and 0.52, respectively, and Neuroticism scales of NEO-FFI and STQ-77 correlate with r = 0.38, and all these values show large effect sizes (Trofimova, 2010b). Openness to Experience of Big Five correlate most significantly with the STQ-77 scales of Intellectual Ergonicity (r = 0.31), Sensitivity to Probabilities (r = 0.40), Impulsivity (r = 0.25) and Empathy (r = 0.52); the scale of Agreeableness of Big Five correlate most significantly with the Empathy scale of the STQ-77 (r = 0.46), and the scale of Conscientiousness of Big Five has the most significant correlation with the STQ-77 scales of Motor Ergonicity (r = 0.35) and Intellectual Ergonicity (r = 0.34).
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Silica matrix doped with calcium and phosphate by sol-gel

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Received 21 April 2010; revised 7 May 2010; accepted 10 May 2010.

ABSTRACT

Silica matrices doped with calcium and phosphate at various Ca/P molar ratios were prepared by the hydrolytic sol-gel methodology. Tetraethylorthosilicate (TEOS) was reacted with calcium ethoxide, in the presence of phosphoric acid as catalyst. Eu III ions were added to the resulting silica, in order to obtain structural information. The samples were dried at 50°C and characterized before and after contact with Simulated Body Fluid (SBF). The xerogels were analyzed by thermal analysis (TA), X-ray diffraction (XRD), photoluminescence (PL), and scanning electron microscopy (SEM). The PL spectra revealed Eu III lines characteristic of the $^5D_0 \rightarrow ^7F_J$ ($J = 0, 1, 2, 3, 4$) transition of this ion, and they indicated a nonhomogenous distribution of Eu III in the Ca-P-Si matrix. XRD and SEM confirmed the presence of an amorphous and crystalline system before and after contact of the samples with the SBF solution, and the crystalline phases were ascribed to hydroxyapatite and $\beta$-calcium triphosphate. The goal of the work is the preparation of a material can be used as biomaterials at low temperature.

Keywords: Luminescence; Biomaterials; Hydroxyapatite

1. INTRODUCTION

The bone tissue consists in the combination of a complex material containing an organic matrix (collagen) embedded with inorganic crystals of calcium phosphate (hydroxyapatite $\text{HA} = \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) [1]. HA is capable of stabilizing the structure of the bone, and it can occur as hydroxyapatite carbonate (HAC), which is present in the human body at 4-7%. HAC is known as biological hydroxyapatite (bio-HA), which also contains a small amount of sodium, potassium, magnesium, fluoride, and chloride, all of which play an important role in the osteoinductivity of bio-HA [2-5].

HA has been used as bioactive ceramics since the 1970s because of its good biocompatibility; however, its can be easily dissolved in the body fluid. Bioactive glasses are more satisfactory in this sense, since the formation of silica on their surface promotes crystallization of apatite containing calcium and phosphate [6,7]. The formation of hydroxyapatite nanocrystals makes these glasses potentially useful biomaterials, because they promote a favorable environment for osteoconduction, protein adhesion, and osteoblast proliferation [8].

The combination of HA and CaCO$_3$ with materials such as glasses, inorganic matrices, organic polymers, among others, results in biocompatible materials that facilitate biomineralization [9-13]. The sol-gel process, in turn, is an extremely interesting route for the synthesis of biomaterials due to the presence of hydroxyl groups in the silica matrix (Si-OH), which promote HA formation [14,15].

In this work, samples containing Si-Ca-P at different Ca/P molar ratios were prepared by the sol-gel route, by mixing tetraethylorthosilicate (TEOS), calcium ethoxide, and phosphoric acid. The synthesis consisted in hydrolysis and condensation of the alkoxide, using acid catalysis. Europium chloride was used as structural probe due to its luminescence properties and, because it can replace calcium ion in the phosphate, it furnishes information about the chemical surroundings of Ca II. The resulting xerogel structure, obtained at low temperature, was analyzed by thermal analysis (TG), X-ray diffraction (XRD), photoluminescence (PL), and scanning electron microscopy (SEM), before and after contact with Standard Body Fluid (SBF).

2. MATERIALS AND METHODS

2.1. Sample Preparation

The reagents were purchase at analytical grade (Sigma-Aldrich).

The samples were prepared with Ca/P molar ratios equal to or different from that encountered in natural hydroxyapatite. The sample denominated HA= had the same Ca/P molar ratio as HA, HA- sample had less Ca/P and the HA+ sample had more Ca/P than HA. The three samples, HA=, HA+ and HA-, were synthesized by the
sol-gel route. To this end, $9.60 \times 10^{-3}$ mols TEOS, $3.84 \times 10^{-3}$ mols calcium ethoxide, and $6.06 \times 10^{-4}$ (HA-), $6.73 \times 10^{-4}$ (HA=) or $7.40 \times 10^{-4}$ (HA+) mols phosphoric acid were added to 16.0 mL ethanol (solvent), under stirring. The resulting materials were dried at 50°C for 1 day. 1% Europium III chloride was added before gelation, in ethanolic solution, the Eu III ion can replace Ca II ion and used as structural probe.

The samples were obtained the powder form and analyzed by Thermal Analysis (TG), X-Ray Diffraction (XRD), Photoluminescence (PL), and Scanning Electron Microscopy (SEM). They were then immersed in SBF [16], pH = 7.40 at 37°C, 0.10 g of the powder was put in 10 mL of SBF solution for 19 days. The samples were characterized before and after contact with SBF.

2.2. Characterizations

2.2.1. X-ray Diffraction (XRD)

The XRD measurements were accomplished at room temperature, with 2θ values ranging from 4 to 80° at a resolution of 0.05°, using a Rigaku Geigerflex D/max-c diffractometer employing monochromated CuKα radiation ($\lambda = 1.54\text{Å}$).

2.2.2. Thermal Analysis (TG)

Thermal analysis (TG) was carried out in a thermal analyzer (TA – Instruments – SDT Q600 – Simultaneous DTA-TG) in nitrogen atmosphere, at a heating rate of 20°C/min, from 25 to 1100°C.

2.2.3. Photoluminescence (PL)

Photoluminescence data were obtained under continuous Xe lamp (450W) excitation in a spectrofluometer SPEX – Fluorolog II, at room temperature. The emission was collected at 90° from the excitation beam. The slits were placed at 2.0 and 0.5 mm for excitation and emission, respectively, giving a band width of 7.0 and 1.0 nm. Oriel 58916 (exc.) and Corning 97612 (em.) filters were employed. Decay curves were measured with a SPEX 1934 phosphorimeter, Xe lamp (5 J/pulse).

2.2.4. Scanning Electron Microscopy (SEM)

SEM analysis was performed with a Jeol JSM-T330A microscope, in order to investigate the morphology of the system.

3. RESULTS AND DISCUSSION

3.1. X-ray Diffraction (XRD)

Figure 1 shows the XRD patterns for the samples prepared with different Ca/P molar ratios, before contact with the SBF solution.

The three starting powders present crystalline and amorphous phases, with well-defined diffraction peaks. The crystalline phase displays peaks at 2θ = 26.5, 32.5, 33.0, 49.2, and 53.1, which can be ascribed to hydroxyapatite (HA), whereas the peaks corresponding to calcium triphosphate (TCP-β) appear at 2θ = 26.5, 30.2, and 53.1 [17]. Several other peaks due to other phosphate silicates, such as Ca$_5$(PO$_4$)$_2$SiO$_4$ and (Ca$_2$(SiO$_4$))$_6$(Ca$_3$(PO$_4$)$_2$), can also be observed.

In a previous work, we observed formation of crystalline phases only after sample contact with the SBF solution, but in that case the amount of phosphate in relation to calcium in the material was about 1% only [13]. The high percentages of phosphate ions (40%) in the present samples were crucial to the precipitation of crystalline phosphate nanoparticles in the silica matrix. However, according to literature reports, the double P=O bond favors phosphate phase formation in the silica network, thus increasing the tendency toward crystallization [18].

The peak broadening of the XRD reflection can be used to estimate crystallite size in a direction perpendicular to the crystallographic plane, based on the Scherrer equation:

$$L = \frac{K\lambda}{\beta\cos\theta}$$  \hspace{1cm} (1)

where $L$ denotes the average crystallite size, $\lambda$ represents the wavelength of the X-ray radiation ($\lambda = 0.154056 \text{nm}$), $K$ is a constant related to crystallite shape and is approximately equal to unity. $\beta$, which is experimentally measured, is the full width of the peak at half of the maximum intensity (rad) and is expressed in its squared form as a squared sum function of the two main contributions, according to Eq.2 depicted below. In the latter equation, $\beta_1$ represents the crystallite size contribution to the peak broadening, while $\beta_2$ is the instrumental broadening contribution [19].

$$\beta = \beta_1^2 + \beta_2^2$$  \hspace{1cm} (2)

On the basis of the XRD data, the average crystallite size was calculated as being approximately 2 nm, indicating the formation of calcium phosphate nanoparticles.

3.2. Scanning Electronic Microscopy (SEM)

The morphology of the system was investigated by SEM. Figure 2 depicts the micrographs obtained for the sam-
Figure 2. Micrographs of the samples. (a) = HA white field; (b) HA-white field; (c) HA+ dark field, before contact with SBF.

Sulfates prepared with different Ca/P molar ratios, before contact with the SBF solution.

The SEM micrographs reveal the presence of crystalline nanoparticles with an average size of 2 nm, as evidenced by XRD. An amorphous phase is also detected. Crystal particles are also observed by electron diffraction, thus confirming the XRD results.

3.3. Thermal Analysis (TG/DTG)

Figures 3(a), (b) and (c) present the thermogravimetric (curve TG) and its derivative (DTG) for the starting samples, before contact with the SBF solution.

The thermogravimetric curve and its derivative reveal three distinct weight loss stages for all the samples. The first loss occurs at 100°C and is ascribed to water and solvent molecules adsorbed on the matrix. The other stages, which took place between 200 and 650°C, are...
possibly due to the decomposition of residual organic groups of the precursor. The HA= and HA+ samples have a total weight loss of 35%, while the HA- sample presents a total weight loss of 40%. Nevertheless, the initial weight loss for the samples HA- and HA+ was the same: 15%. The presence of phosphate ion at Ca/P molar ratios different from that encountered in natural HA may have contributed to the incomplete polycondensation of the silica matrix. This may have led to a larger quantity of residual –OH groups on the surface of the matrix and thus a larger number of water molecules adsorbed on the silica surface.

3.4. Photoluminescence (PL)

Figures 4 and 5 respectively show the excitation and emission spectra of the samples HA=, HA+, and HA- prepared by the sol-gel method, doped with Eu III ions before contact with the SBF solution. The maximum emission occurs at 590 nm (5D0 → 7F1), while the maximum excitation takes place at 394 nm (5L6 level). The intensities of the emission bands were normalized.

The emission spectra display transitions arising from 5D0 to 7FJ (J = 0, 1, 2, 3, and 4) manifolds. There are three main transitions, namely 5D0 → 7F0 (around 570 nm), 5D0 → 7F1 (around 595 nm), and 5D0 → 7F2 (around 610 nm). The first is a strongly forbidden transition not yet observed with appreciable intensity in some hosts. The 5D0 → 7F1 transition is forbidden as electric dipole, but allowed as magnetic dipole. This is the only transition that takes place when Eu III occupies a site coinciding with a symmetry centre. When the Eu III ion is situated at a site lacking an inversion centre, the transition corresponding to even J values (except 0) are electric dipole allowed, and red emission can be observed. The 5D0 → 7F1 transition can also be observed as a magnetic dipole allowed transition. Further on, all the lines corresponding to this transition split into a number of components depending on the local symmetry [20].

When Eu III is located at a low symmetry site (without an inversion center), the 5D0 → 7F2 emission transition often predominates in the emission spectrum, so the Eu III ions occupy a non-inversion symmetric site [21]. The 5D0 → 7F0 transition of Eu III is only allowed in the case of Cs, Cn and Cnv symmetries of the Eu III sites in the crystalline state [22]. The hexagonal unit-cell HA contains ten cations distributed between two crystallographic sites: four on type (1) sites and six on type (2) sites. Ca (1) ions present C3 symmetry and are surrounded by nine oxygen atoms. Ca (2) ions present Cs symmetry and are surrounded by six oxygen atoms [23-25]. There are literature reports stating that Ca (1) and Ca (2) ions (r = 0.99 Å) can be replaced with Pb II (r = 1.2 Å) [26], so the Eu III ion (r = 0.95Å) [27] can also occupy the Ca II sites in the crystalline hydroxyapatite and β-TCP, as well as in amorphous silica. The emission spectra of Eu3+ is not similar in glasses hosts, as discussed in literature [28-30].

The Eu III emission bands in the spectra of Figure 5 reveal a nonhomogenous distribution of the ion in the Si-Ca-P matrix [31-33].

Because of its electrical-dipole nature [34], the relative intensity of the 5D0 → 7F0 and 5D0 → 7F2 transitions is strongly dependent on the Eu III surroundings. In contrast, the band corresponding to the 5D0 → 7F1 transition has a magnetic-dipolar nature whose intensity is unaffected by the ion’s surroundings. Therefore, the latter transition can be considered a standard to measure the relative intensity of the other bands [35,36], and the 5D0 → 7F2 emission intensity can thus provide valuable information about environmental changes around the Eu III ions. Table 1 shows the intensity of the 5D0 → 7F0 and 5D0 → 7F2 transitions relative to the 5D0 → 7F1 transition.
Table 1. Relative intensity of the $^5D_0 \rightarrow ^7F_J$ transitions with respect to the $^5D_0 \rightarrow ^7F_1$ transition in the emission spectra of the samples HA$, HA^+$ and HA$^-$ doped with Eu III, before contact with SBF.

| Samples | $^5D_0 \rightarrow ^7F_0$ | $^5D_0 \rightarrow ^7F_1$ | $^5D_0 \rightarrow ^7F_2$ | $^5D_0 \rightarrow ^7F_3$ |
|---------|-----------------|-----------------|-----------------|-----------------|
| HA$=$   | 0.09            | 1.13            |                 |                 |
| HA$^-$  | 0.09            | 1.06            |                 |                 |
| HA$^+$  | 0.11            | 1.11            |                 |                 |

The similar relative intensities of the transitions indicate that the environment around the Eu III ion is similar in the three samples, and the ion can be located either in the silica matrix or in calcium phosphate.

The characterized samples were then placed in SBF solution for 19 days, followed by characterization using the same techniques mentioned above.

3.5. XRD and TEM

Figure 6 depicts the XRD patterns for the samples prepared with different Ca/P molar ratios, after contact with the SBF solution.

The XRD patterns depicted in Figure 6 display the same peaks observed for the samples not submitted to contact with SBF solution. Therefore, the crystalline phase formed during the sol-gel process remains intact in the silica matrix after sample immersion into SBF. TEM analysis also reveals that the crystalline phase does not disappear after contact with the SBF solution, and the presence of calcium phosphate nanoparticles in the samples is confirmed by this technique. Furthermore, the Sherrer formula corroborates the average size of the crystalline particles, which is 2 nm.

3.6. Thermal Analysis

Figures 7a, b and c present the thermogravimetric curve (TG) and its derivative (DTG) for the three samples, after contact with the SBF solution.

The thermogravimetric curve and its derivative give evidence of two weight losses for all the samples. The main loss occurs at about 100°C, ascribed to water molecules and solvent. As for the second weight loss stage, it took place between 200 and 650°C before the samples were immersed into SBF and the weight loss percentage was 20%. After contact with the SBF solution, this weight loss percentage reduced to 7% in the same temperature range. An exchange between the solid
sample and the SBF solution probably occurred, with residual product being removed from the silica matrix after contact.

### 3.7. Photoluminescence (PL)

Figures 8 and 9 respectively show the excitation and emission spectra of the samples HA=, HA+, and HA- prepared by the sol-gel method, doped with Eu III ions, after contact with the SBF solution. The maximum emission occurs at 590 nm (5D0 → 7F1), while the maximum excitation takes place at 394 nm (5L6 level). The intensities of the emission bands were normalized.

The emission spectra display transitions arising from 5D0 to 7FJ (J = 0, 1, 2, 3, and 4) manifolds. The Eu III emission bands in the spectra of Figure 9 are characteristic of a nonhomogenous distribution of this ion in the Si-Ca-P matrix, as observed for the samples before contact with the SBF solution.

The relative intensity of the 5D0 → 7F0 and 5D0 → 7F2 transitions was obtained by measuring the area under the emission band. Table 2 shows the intensity of the 5D0 → 7F0 and 5D0 → 7F2 transitions relative to the 5D0 → 7F1 transition of the samples HA=, HA+ and HA- doped with Eu III, after contact with SBF.

| Samples | 5D0 → 7F0/D0 → 7F1 | 5D0 → 7F2/D0 → 7F1 |
|---------|---------------------|---------------------|
| HA=     | 0.05                | 0.99                |
| HA-     | 0.09                | 1.11                |
| HA+     | 0.12                | 1.15                |

Table 2. Relative intensity of the 5D0 → 7F0 and 5D0 → 7F2 transitions with respect to the 5D0 → 7F1 transition in the emission spectra of the samples HA=, HA+ and HA- doped with Eu III, after contact with SBF.

There were only slight changes in the relative intensity of the emission bands, indicating that the environment around the Eu III ion is not affected upon contact of the samples with the SBF solution. This is because Eu III probably occupies a stable site in the matrix and in the phosphate nanoparticles. The change in the relative intensity of the 5D0 → 7F0 and 5D0 → 7F2 transitions was more significant for the sample HA=, which can be ascribed to possible dissolution of the matrix and phosphate into the SBF solution, as discussed in the literature [18].

### 4. CONCLUSIONS

The concentration of phosphate ions is crucial to the formation of crystalline calcium phosphate in the silica matrix, when compared with results from a previous work. The formation of nanocrystals is very important for the future application of these solids as biomaterials.

The Eu III ion can occupy different sites in these materials; that is, it can replace the calcium ion in the phosphate or in the matrix.

Thermal analysis revealed that residues from the synthesis can be removed after washing with water, but this does not affect nanocrystal formation.

The period during which the materials remain in contact with the SBF solution is not sufficient to promote their reaction with the solution. Literature reports mention that biomaterials need to be placed in SBF for a certain contact time before calcium phosphate starts to precipitate, but this process depends on sample composition. In our case, the sample HA=, which contains the same Ca/P molar ratio as natural HA, seems to undergo dissolution after a certain contact time with SBF, and we think that a longer contact time could lead to reprecipitation of the HA.

### 5. ACKNOWLEDGEMENTS

The authors acknowledge FAPESP, CNPq and CAPES (Brazilian
research funding agencies) for their financial support of this work.

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Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation

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Received 7 April 2010; revised 22 April 2010; accepted 25 April 2010.

ABSTRACT

A low-cost process for the production of laccases is necessary for a sustainable enzymatic wastewater treatment. Therefore, it is necessary to establish an easy and low-cost procedure for the production of laccase. In the present study the properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation is investigated. The application of the enzyme for dye decolorization is also studied. Crude laccase from the studied culture established maximal activity at 45°C. The enzyme retained over 90% of its activity in the temperature range 40-47°C and pH 4.5. The kinetic constants of the crude enzyme was also determined. In the presence of KCl, NaCl, CaCl₂, MnSO₄ and MgSO₄, laccase demonstrated high stability—over 50% of its initial activity was still retained after 4-month incubation. Complete loss of enzymatic activity was observed in the presence of CuCl₂, FeCl₃, FeCl₄ and NaN₃ after 30 min of incubation. 100% decolorization by investigated crude laccase was completed in the case of Indigo Carmine for 4 h, Remazol Brilliant Blue R—for 6 h, Orange II—for 48 h and Congo Red—for 13 d.

Keywords: Laccase; Solid-Substrate Fermentation; *Trametes versicolor*; Dye Decolorization

1. INTRODUCTION

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are extracellular, multicopper enzymes that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalysed reaction mechanism [1]. Laccases can convert o-and p-diphenols, aminophenols, methoxy-substituted phenols, benzenethiols, polyphenols, polyamines, hydroxyindols, some aryl diamines and a considerable range of other compounds but do not oxidize tyrosine (whereas the tyrosine ses do).

Laccases can be very strongly inhibited by various reagents. Small anions such as azide, halides, cyanide, thiocyanide, fluoride and hydroxide bind to the type 2 and type 3 Cu, resulting in the interruption of internal electron transfer and inhibition of activity. Other inhibitors include metal ions (e.g. Hg²⁺), fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, desferal and cationic quaternary ammonium detergents the reactions with which may involve amino acid residue modifications, conformational changes or Cu chelation [2,3]. Regarding conformational changes, it is known that these are highly dependent on the state of oxidation of the copper atoms. Laccases generally are more stable at alkaline pH than at acidic pH, probably due to the OH⁻ inhibition of auto-oxidation.

Laccases are increasingly being used in a wide variety of industrial oxidative processes such as delignification, dye or stain bleaching, bioremediation, plant fibre modification, ethanol production, biosensors, biofuel cells, etc. Industrial uses require overproduction of the enzyme, generally in a heterologous host, as an indispensable prerequisite. Indeed, most commercial laccases are produced in *Aspergillus* hosts. The functional expression of the *Myceliophthora thermophila* laccase in *S. cerevisiae* by directed molecular evolution has been reported, which enables this system to be tuned up for new and challenging applications [4]. Many white-rot fungi produce multiple laccase isoforms under the appropriate inductive conditions [5,6]. Most fungal laccases studied are extracellular proteins, but intracellular laccases have been detected in some fungi [7].

The genus *Trametes*, which belongs to the white-rot fungi, is assumed to be one of the main producers of laccases. *T. versicolor* produces laccase and MnP as major ligninolytic enzymes; however, the role of these enzymes in decolorization of azo dyes is not yet clear. Laccase and/or MnP activities in culture filtrate of *T. versicolor* were not able to decolorize azo dyes, thus indicating a role of other enzymes or cell-bound components in azo dye degradation [8].
In order to produce laccases, white-rot fungi have to be cultured under specific conditions. Two types of culture techniques are used: Solid-state fermentation (SSF) and Submerged fermentation (SmF). The SSF is defined as any fermentation process occurring in absence or near absence of free liquid, using an inert substrate or a natural substrate as a solid support [9]. The former works as an attachment place for the microorganism, whereas the latter also acts as a carbon source, which considerably reduces the production costs [10]. SSF is advantageous in obtaining concentrated metabolites and subsequent purification procedures are economical [11,12]. In SSF, the microorganisms grow under conditions close to their natural habitat. This may allow them to produce certain enzymes and metabolites, which usually would not be produced or would only be produced at a low yield in SmF [9]. Therefore, the selection of an adequate support is essential, since the success of the process depends on it.

Laccases have become important, industrially relevant enzymes that can be used for a number of diverse applications, including biocatalytic purposes such as delignification of lignocellulosics and crosslinking of polysaccharides, bioremediation applications such as waste detoxification and textile dye transformation [13], food technological uses, personal and medical care applications [14], and biosensor and analytical applications [15]. In view of the broad biotechnological applications of laccases [16-18], there is a scientific need to identify different sources of laccases having diverse properties so that suitable laccases for various applications could be identified.

The enzymatic treatment of wastewater requires the production of large amounts of enzymes, in this case laccases, at low cost. The current commercial price of laccases [16], there is a scientific need to identify different sources of laccases having diverse properties so that suitable laccases for various applications could be identified.

2. MATERIALS AND METHODS

2.1. Microorganisms and Inoculum

A fungal strain of *Trametes versicolor* 1A collected from hills in the city of Plovdiv, Bulgaria was used in this work. The culture belongs to the collection of the Department of “Biotechnology” at the University of Food Technologies in Plovdiv-Bulgaria. The culture is maintained on 2% lima bean agar plates and slants at 4°C. For enzyme production, a 7-day old plate culture grown on 2% potato dextrose agar (PDA) was used. Mycelial inoculum was prepared by inoculating 10⁷ spores of fungus from agar-slant culture to 300 ml shake flask containing 50 ml beer must 7.5°B. The pH of the media was adjusted with 1M NaOH to 6.5. The inoculated flasks were incubated at 30°C and 220 rpm for 72 h.

2.2. Solid-Substrate Fermentation (SSF)

The SSF was carried out using a medium consisted of 4.0 g wheat bran, 2.5 g oats straw and 2.5 g beetroot press in 300 ml flasks. The moist of the substrate was adjusted to 60% by salt solution containing (%): (NH₄)₂SO₄—0.14; KH₂PO₄—0.2; MgSO₄.7H₂O—0.03; CaCl₂—0.03; FeSO₄.7H₂O; ZnSO₄.7H₂O; MnSO₄.7H₂O and CoCl₂—0.002 (pH 4.5). After autoclaving (121°C for 30 min) and cooling, the substrate was inoculated with the appropriate mycelial inoculum prepared as mentioned above. SSF fermentation with monocultures was used as reference. All flasks were cultivated at 30°C for 7 days. Triplicate flasks were set up for each experimental variation.

2.3. Enzyme Extraction

The crude enzyme extract was obtained by adding 50 ml distilled water to the fermented matter. The flasks were mixed for 30 min at room temperature (25°C) using a shaker (220 rpm). Solids were removed first by filtering and then by centrifuging at 5000 g for 5 min. The supernatant obtained was used as crude enzyme extract.

2.4. Laccase Activity Measurement

Laccase activity was assayed according to Marbah *et al.* [20] using syringaldazine as a substrate. One unit of laccase activity was defined as 0.001 ΔA₅₃₀ for 1 min, pH 4.5 and 30°C

2.5. Determination of the Kinetic Parameters

*Kₘ* and *Vₘₐₓ* values were determined using 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine) and 2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as substrates [20] at different concentrations (0.01 ± 0.1 mM) in 0.2M citrate-phosphate buffer (pH 4.5). The computing procedures were performed by using a package of applied software programs of own development in the software media of Matlab and Eureka [21,22]. For analyzing the results two approaches were used:

\[
\text{Michaelis-Menten } V = \frac{V_{\max} S(t)}{K_m + S(t)} 
\]
Reversible substrate inhibition

\[ v = \frac{V_{\text{max}} S(t)}{k_m + S(t) + S^2(t)/k_i} \]  

(2)

The kinetic parameters in each of the models were calculated according to the optimization procedure which minimized a criterion of the type:

\[ J = \sum_{i=1}^{n} (v_{\text{calc},i} - v_{\text{mod},i})^2 \rightarrow \text{min} \]  

(3)

where \( v \) is the initial velocity of the enzyme reaction calculated by (1) and (2) and \( n \) is the number of analyzing points.

2.6. Effect of Ph on the Activity and Stability of the Enzyme

Laccase activity is a function of pH measured in 0.2M citrate-phosphate buffer over the range from 1.8 to 6.6. The highest activity was set as 100% of relative activity. For the stability experiments, the crude extract was incubated under initial conditions for 30 minutes at 30°C and residual laccase activity was determined at intervals according to the standard conditions cited above. The buffers used in that study were: citrate (pH 3.0, 3.5, 4.0), acetate (pH 4.0, 4.5, 5.0, 5.5), phosphate (pH 5.5, 6.0, 6.5, 7.0).

2.7. Effect of Temperature on the Activity and Stability of the Enzyme

Laccase activity was measured at pH 4.5 in 0.2M citrate-phosphate buffer at different temperatures over the range 10-70°C. The enzyme’s thermal stability was assayed by incubating the crude extract at different temperatures in the range of 30-70°C at different times using 0.2M citrate-phosphate buffer. After the incubation, the crude extract was cooled and laccase activity was determined at standard conditions.

2.8. Effect of Ions

The crude extract was incubated with different ionic solutions: KCl, ZnCl₂, CuCl, Pb(NO₃)₂, AgNO₃, ZnSO₄, MgSO₄, MnSO₄, NaCl and CaCl₂ at 10 mM; NaN₃ at 1.0 mM and residual laccase activity was determined at intervals according to the standard conditions cited above with syringaldazine as substrate. The activity of the enzyme immediately after ultrafiltration was set as 100% of relative activity.

2.9. Removing of Salts (Ultrafiltration)

Removing of salts in crude enzyme extract was carried out in ultrafiltration module by pressure of nitrogen of 0.3 MPa. Cellulose acetate membrane (500 Da) was used. The crude enzyme extract was rinsed and filtered until conductivity of 56 μS was achieved (Tungsram’s “Radelkis” conductometer).

2.10. Dye Decolorization Studies

Crude enzyme extract was collected at the maximum laccase activity (day 7), filtered, clarified by centrifugation at 8000 g for 15 min, frozen, defrosted and then filtered to remove the precipitated polysaccharides. The resulting clear filtrate was concentrated by ultrafiltration (as described above). In vitro decolorization experiments were performed with the concentrated clear filtrate. For the purpose of the investigation the crude enzyme extract was diluted twice, thus we used two samples—with 2000 and 1000 U/ml laccase activity. The dyes tested for the in vitro studies were: indigo carmine (indigoid) CI 73015, phenol red (sulfonaphthalein), Remazol Brilliant Blue R, and the azo-dyes Orange II and Congo Red. All were purchased from Aldrich (St. Louis, MO, USA) and were used in a concentration of 0.01%. The reaction mixture for dye decolorization consisted of equal volumes of an aqueous solution of dye and crude laccase (2000 or 1000 U/ml) in citrate phosphate buffer (pH 5.0). The residual dye concentration was measured spectrophotometrically at 608 nm for indigo carmine, 587 nm for Remazol Brilliant Blue R, 483 nm for Orange II, 497 nm for Congo Red and 475 nm for phenol red. A control test containing the same amount of a heatdenatured laccase was performed in parallel. The assays were done thrice, the experimental error being below 3%.

3. RESULTS AND DISCUSSIONS

3.1. Effect of pH and Temperature on Laccase Activity and Stability

Most of the fungal laccase have optimum pH in the range of 3.0-4.0 and temperature in the range of 40-60°C for oxidation of phenolic compounds. Crude laccase produced by Trametes versicolor 1A was found to express maximal activity at pH 4.5 (Figure 1(a)). The enzyme activity was also high in the pH range between 4.1 and 4.8, being respectively 99.3% and 95.2% of the maximum. Crude laccase was proved active in the range of pH modification from 3.3 to 6.0.

Crude laccase from the studied culture established maximal activity at 45°C (Figure 1(b)). The enzyme retained over 90% of its activity in the temperature range 40-47°C, the respective values being 94.2% and 93.3% of the maximum. Within the range 30-50°C the loss of enzymatic activity was below 30 %, and in particular—28.9% and 23.2%. At 60°C the loss of laccase activity was 83.6%.

In the range of pH values between 3.0 and 7.0 the enzyme was found to be most stable at pH 4.5 (Figure 2(a)). During the 48-hour follow-up of the investigation the enzyme activity was found to decrease only by 3.4%.
Minor losses of enzyme activity were registered at pH 5.0 and 5.5, as well, namely 7.1% and 9.2% for 48 h. Crude laccase was assessed as being most unstable at pH 7.0 – the reduction of enzymatic activity was by 57.2% for 48 h.

The thermal stability of crude laccase was followed within the temperature interval 30–60°C (Figure 2(b)). The greatest stability of the enzyme was observed at 30°C – the residual activity after 48 h incubation was 97.2%. The enzyme was still stable at 40°C – the loss of enzymatic activity for the same period of incubation was 9.8%. At 50°C the losses of enzymatic activity were 53.4% for 6 h and 90.0% for 48 h incubation. After 6 h incubation at 60°C the loss of activity reached 58.0%, while after 48 h incubation no enzymatic activity was registered. At 70°C the enzyme had lost 53.5% of its initial activity in 5 min, and had been completely inactivated in 20 min (data not shown).

### 3.2. Kinetic Analysis

Two compounds, ABTS and syringaldazine, were used as substrates for measuring the kinetic constants of crude laccase. The results from the investigation of process dynamics in the case of ABTS and syringaldazine as substrates are presented in Table 1.

The conditions for setting in the establishment of substrate inhibition are specified in (2): \( S_{\text{crit}} = \sqrt{k_s K_c} \). That correlation, as well as (3), was used as the grounds for an a priori choice of model type. The values of the kinetic parameters calculated by the two models, and the values of \( J \) and \( S_{\text{crit}} \) criteria for
Table 1. Dynamics of the process using model (1) and (2) for ABTS and Syringaldazine.

| S [mM] | $V_{opt}$ [nKat/cm$^3$] | $V_{mod}$ [nKat/cm$^3$] | $\epsilon = V_{opt} - V_{mod}$ [nKat/cm$^3$] | $V_{opt}$ [nKat/cm$^3$] | $V_{mod}$ [nKat/cm$^3$] | $\epsilon = V_{opt} - V_{mod}$ [nKat/cm$^3$] |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Substrate ABTS |
| 0.1 | 32.25 | 33.16 | -0.91 | 32.25 | 32.32 | -0.07 |
| 0.08 | 31.25 | 30.73 | +0.52 | 31.25 | 31.16 | +0.09 |
| 0.05 | 26.31 | 25.19 | +1.12 | 26.31 | 26.24 | +0.07 |
| 0.025 | 16.65 | 17.01 | -0.36 | 16.65 | 16.99 | -0.34 |
| 0.020 | 14.35 | 14.63 | -0.28 | 14.35 | 14.31 | +0.04 |
| 0.0125 | 9.93 | 10.37 | -0.43 | 9.93 | 9.65 | +0.28 |
| Substrate Syringaldazine |
| 0.1 | 2.325 | 2.315 | +0.010 | 2.325 | 2.294 | +0.031 |
| 0.05 | 2.040 | 2.050 | -0.010 | 2.040 | 2.075 | -0.035 |
| 0.03 | 1.770 | 1.780 | -0.010 | 1.770 | 1.796 | -0.026 |
| 0.02 | 1.530 | 1.526 | +0.004 | 1.530 | 1.528 | +0.002 |
| 0.01 | 1.079 | 1.071 | +0.008 | 1.079 | 1.049 | +0.03 |

Table 2. Kinetic constant using model (1) and (2) for ABTS and Syringaldazine.

| Substrate | ABTS | Syringaldazine |
|-----------|------|----------------|
| Kinetic constant | model (1) | model (2) | model (1) | model (2) |
| $V_{max}$ | 48.52 | 91.4100 | 2.6570 | 2.8820 |
| $k_m$ | 0.0463 | 0.1047 | 0.0148 | 0.0174 |
| $k_i$ | - | 0.1280 | - | 1.2130 |
| $J$ | 2.72 | 0.2097 | 3.67$e^4$ | 3.76$e^3$ |
| $S_{crit}$ | - | 0.116 | - | 0.145 |

Table 3. Effect of ions on enzyme activity.

| Salts (10 mM) | 1 day | 1 month | 2 months | 3 months | 4 months |
|---------------|-------|---------|----------|----------|----------|
| Control | 100 | 68.54 | 52.51 | 22.92 | 0 |
| KCl | 96.35 | 90.36 | 86.44 | 59.60 | 52.72 |
| NaCl | 112.38 | 91.26 | 87.40 | 75.70 | 74.53 |
| CaCl$_2$ | 111.69 | 94.01 | 94.01 | 73.36 | 68.82 |
| MnSO$_4$ | 111.69 | 82.50 | 66.96 | 59.87 | 51.24 |
| MgSO$_4$ | 109.63 | 87.81 | 40.12 | 35.99 | 28.42 |
| ZnSO$_4$ | 95.87 | 53.40 | 39.23 | 25.24 | 0 |
| ZnCl$_2$ | 68.82 | 46.11 | 22.29 | 17.41 | 9.84 |
| CuCl | 64.69 | 34.13 | 22.71 | 5.71 | 0 |
| Pb(NO$_3$)$_2$ | 66.75 | 66.07 | 29.59 | 24.50 | 2.75 |
| AgNO$_3$ | 85.57 | 45.83 | 386 | 26.56 | 3.16 |

Laccase to the first of the substrates.

3.3. Effect of Ions on Enzyme Activity

Laccase in crude enzyme extract (control) was found to lose its activity after an incubation period of 3 months (Table 4). In the presence of 5 of the investigated salts—KCl, NaCl, CaCl$_2$, MnSO$_4$ and MgSO$_4$, laccase demonstrated high stability—over 50% of its initial activity was still retained after 4-month incubation. It has been assumed that in the presence of various salts enzymes are more substantially influenced by cations. Data from our study revealed that the strongest positive impact on enzyme’s molecule was exercised by Na(I) ions, ensuring the molecule stabilization for a period of 4 months with the least loss of enzymatic activity—25%.

On the other extreme was the effect of Zn(II) and Cu(I) ions introduced into the medium as chlorides— their...
availability destabilized the enzyme molecule making it much more unstable than crude laccase; in the presence of the above cited salts a complete loss of enzymatic activity was registered after 25 and 15 d (in the respective case). In principle, heavy metals have been known to posses a negative impact on enzymes and this was confirmed by the results from our study. It is a clear fact, however, that their destabilizing influence does not go beyond the influence of ZnCl₂ and CuCl₂.

The suggestion that Na(I) added in the form of NaCl have a positive effect in terms of laccase stability was not confirmed by the experiment with NaN₃, in which after 30 min of treatment a complete loss of enzymatic activity was registered. It could be assumed that in the specific case dominated the influence of the respective anion. Azide is a very effective inhibitor of laccase II, and complete inhibition was observed with 1.0 mM NaN₃. Azide is thought to bridge both the type 2 and type 3 Cu in laccase. Our results are with correspondence with [25,26]. They reported for full or 98% inhibition of laccase activity of some basidiomycetes and ascomycetes by 1.0 mM sodium azide but for shorter period of time to compare to our crude enzyme.

Complete loss of enzymatic activity was observed in the presence of CuCl₂, FeCl₂ and FeCl₃ after 30 min of incubation, suggesting that Cu(I) and Cu(II) had a negative impact on the enzyme, regardless of being its co-factors. Probably that finding could be attributed to the high concentration of the introduced salts.

### 3.4. Dye Decolorization Studies

The ability of white-rot fungi to decolorize synthetic dyes has been widely studied, particularly with Phanerochaete chrysosporium and Trametes versicolor [27]. In the present study, we assessed the ability of the crude laccase (extracted fluid) from SSF culture of T. versicolor, to decolorize five structurally different synthetic dyes. The decoloration of type model dyes is a simple method to assess the aromatic degrading capability of ligninolytic enzymes [28]. Dye decolorization by laccase is a property of the crude enzyme that underlies one of its possible applications. The results concerning dye decolorization by laccase with enzymatic activities of 1000 and 2000 U/ml are presented on Figures 3(a) and (b). The application of enzyme solutions with higher activity proved better results for each of the five dyes studied. As it can be seen in Figure 3, the decoloration rate obtained was very different in each case. 100% decolorization by laccase (2000 U/ml activity) was completed in the case of Indigo Carmine for 4 h, Remazol Brilliant Blue R—for 6 h, Orange II—for 48 h and Congo Red—for 13 d. No complete decolorization of Phenol Red was achieved. Decolorization of Indigo Carmine by means of laccase with...
ent strains as well as to the difference in specificities to different dyes of diverse structures [31]. In addition, as commented above, it could also be due to the difference in the redox potential of laccases from different microorganisms.

Decolorization of the dyes covered by our study has been reported for laccase produced by *Trametes versicolor* ATCC 200801, as well, but authors do not specify dye concentrations [32]. Regarding *T. versicolor* CC BAS614, over 50% degradation of Remazol Brilliant Blue R and Orange 16 for 14 days has been reported [33]. Our data suggests that a more active form of the enzyme allows for the achievement of a complete or at least a higher degree decolorization, as well as for a successful reduction of decolorization time. The influence of laccase activity on dye decolorization is another topic not discussed in scientific literature.

4. CONCLUSIONS

The crude enzyme laccase from SSF culture of *Trametes versicolor* 1A was isolated and characterized. The unpurified enzyme was very stable with very good potential for application for industrial wastewater treatment.

On one hand, the results clearly showed the enormous potential of wheat bran, oats straw and beetroot press as a support-substrates for production of laccase at low cost by *T. versicolor* under solid-state conditions. In addition, the laccase produced presented a highly decolorizing ability, especially for indigo and anthracene dyes. This makes laccase from this fungus very attractive for further investigations as well as for its application to different biotechnology areas.

5. ACKNOWLEDGEMENTS

This work was supported by national Science Fund, Bulgaria.

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Molecular dynamics simulations of valinomycin interactions with potassium and sodium ions in water solvent

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Received 17 June 2010; revised 25 June 2010; accepted 26 June 2010.

ABSTRACT

The aim of this work is to estimate the value of the electric field (potentials) for the system of valinomycin + K⁺ and Na⁺ ions based on a molecular dynamics (MD) study. An analysis has been performed of the interaction processes for the system of valinomycin + K⁺(Na⁺) ion in water solvent. It is obtained that capturing a K⁺(Na⁺) ion in the valinomycin cavity is not possible for all values of the electric field strength. Each of the two kinds of ions (K⁺ or Na⁺) has its own critical electric field associated with ion binding to valinomycin, for which to exist, the ion has to remain localized inside the valinomycin cavity. The results obtained for the electrical potential reveal a stronger valinomycin binding—especially with the potassium ion. Valinomycin’s molecular structure efficiently surrounds the K⁺ ion, as this structure has to exactly correspond to the K⁺ ion in size. MD simulation results could be a prerequisite for studying a more complex scenario—for estimating ion transport in the cell membrane or physiological electric potential which is formed in the membrane or inside the cell relative to its surrounding medium.

Keywords: Molecular Dynamics Simulations; Valinomycin; Potassium and Sodium Ions

1. INTRODUCTION

Valinomycin was extracted for the first time from Streptomyces fulvissimus bacteria in 1955; in 1967, it was established that as a transporter, valinomycin catalyzes the exchange of K⁺ and H⁺ through a mitochondrial cell membrane, thereby causing no changes in the Na⁺ concentration [1-3]. In biological membranes, there are several kinds of ionic pumps, which work at the expense of the free energy of ATP hydrolysis—a special Na⁺/K⁺-ATPase system of integrated proteins known as the sodium-potassium pump. The ATPase mechanism of ion transport is realized as a transfer process conjugated with chemical reactions, which goes at the expense of the cell metabolism energies. During the functioning of the Na⁺/K⁺-ATPase at the expense of the chemical binding energy released in the hydrolysis of each ATP molecule, two sodium ions transfer into the cell with the simultaneous extraction of three potassium ions. Thus, an electric potential gradient is formed due to an increase in the concentration of potassium ions in comparison to that of the interstitial media and a decrease in the sodium concentration, which is physiologically important.

Specifically in neurons, the combination of the two mechanisms mentioned above corresponding to their state of rest is responsible for dynamical equilibrium stability. The internal sodium concentration in cells is ten times lower than in surrounding media; at the same time, the potassium concentration is ten times higher. Such a disbalance tends to equilibrate the streams going through narrow pores of the cell membrane. To control the necessary ion concentration in the cell, the membrane protein molecules (called “sodium pumps”) continuously pump potassium from, and sodium into, the cell. Each pump is able to transfer about 200 sodium ions and 130 potassium ions per second. A neuron, for example, does about a million of such pumps that transfer hundreds of millions of potassium and sodium ions through a cell membrane per second [2-4].

The potassium concentration in the cell is influenced by a large number of open potassium channels (i.e. protein molecules), which allow potassium ions to pass easily into the cell but suppress sodium ions passing through. For the transfer of potassium ions and other particles into the cell, special membrane transport proteins must be responsible. Valinomycin is an example of a transporter protein for potassium ions. Valinomycin has a macrocyclic (ring) structure as shown in Figures 1(a) and (b). The valinomycin molecule is highly selective to potassium ions as compared to sodium ions.
Figure 1. The valinomycin configuration. (a) a view from the molecule plane; (b) a side view. The color spheres represent nitrogen (blue), carbon (blue), hydrogen (white), and oxygen (red) atoms. The six oxygen atoms which are able to capture external solvent ions are denoted as Oe.

Within the cell membrane; it has 12 carbonyl groups for the binding of metal ions, and also for solvation in a polar solvent. The isopropyl groups and methyl groups are responsible for solvation in nonpolar solvents. Along with the molecule’s shape and size, this molecular duality is the main reason for its binding properties. In polar solvents, valinomycin will mainly expose the carbonyls to the solvent; in nonpolar solvents, the isopropyl groups are located predominantly on the exterior of the molecule. This conformation will change as valinomycin binds to a potassium ion. The molecule is “locked” into a conformation with the isopropyl groups on the exterior. Due to its chemical structure, valinomycin is able to form a complex with a potassium ion captured by the molecule—inside its ring; on the other hand, valinomycin is easily solvable in the membrane lipid phase—it is non-polar on the exterior part. Thus, the valinomycin molecules that are positioned on a membrane surface capture potassium ions from the surrounding solvent; then potassium ions are transferred by valinomycin by means of diffusion in the membrane, and, finally, ions are released in the solvent on the other side of the cell membrane. A gradient of the ion concentration in the cell membrane is thereby created; an electric potential relative to the cell surrounding varies from −70 mV to +50 mV. The potential stimulates a synaptic signal transmission necessary for biological functions [3-6].

In this work, based on the molecular dynamics (MD) simulation, we aimed to measure the electric field strength (potential gradients) for the model systems describing valinomycin with potassium (K⁺) and sodium (Na⁺) ions. To calculate electrostatics interactions, we used a reaction field algorithm [7]. We performed an MD analysis in comparison with the physiological data on the cell electric potential outlined above. It should be noted that MD simulation is one of the most applicable techniques used to study valinomycin’s dynamical and equilibrium properties in biological systems. For the valinomycin interaction with different cations and solutions, see, for example, references [8-10]. In [8], valinomycin selectivity for the transport of potassium ions (and not sodium ions) is studied by MD simulation. The process of potassium ion capture by a valinomycin molecule is illustrated in [9]. An estimation of the energy of the cation binding to valinomycin is performed in [10].

2. MATERIALS AND METHODS

The starting configuration of the molecular system consisting of valinomycin with potassium ions is shown in Figure 1. We have used periodic boundary conditions for all spatial axes; the geometry of the system configuration was a truncated octahedron with the side length of 42.86Å. Molecular dynamics (MD) simulations have been performed using the DL_POLY code, which was developed by the molecular simulation group at the Daresbury Laboratory (England) with the support of the...
Figure 2. A valinomycin molecule (a triangular shape chain is in the center) surrounded by potassium ions (green spheres) and water molecules (red and white are oxygens and hydrogens, respectively).

Research Council for Engineering and Physical Sciences (project CCP5 for simulating condensed phases). DL_POLY is a general-purpose MD simulation package developed by W. Smith, T.P. Forester, and I.T. Todorov [11]. We have employed version 2.19 of the DL_POLY; the initial geometry of the biphenyl molecule was chosen from the database of the program package at: http://www.cse.scitech.ac.uk/ccg/software/DL_POLY/

The configurational energy of the molecular model is represented as a sum of the energies of binding \(E_{\text{val}}\) and non-binding \(E_{\text{nb}}\) interactions:

\[
E = E_{\text{val}} + E_{\text{nb}}.
\]  

The energy of valence (binding) interactions \(E_{\text{val}}\) is given by the following formula:

\[
E_{\text{val}} = E_{\text{imb}} + E_{\text{ang}} + E_{\text{dih}} + E_{\text{inv}},
\]  

where \(E_{\text{imb}}\) is the energy of intermolecular bonds, \(E_{\text{ang}}\) is the energy of angular bonds, \(E_{\text{dih}}\) is the energy of dihedral bonds, and \(E_{\text{inv}}\) is inversion energy.

The energy of the non-valence (non-bound) interactions is a sum of the energies of the van der Waals (vdW), electrostatics (Coulomb), and hydrogen bonds:

\[
E_{\text{nb}} = E_{\text{vdW}} + E_{\text{coul}} + E_{\text{hb}}.
\]  

The valinomycin molecule consists of 168 atoms; the number of K'(Na') ions was 109. The water molecules were simulated as 3-site rigid bodies; the total number of water atoms was 3339 (1113 \(\times\) 3). Computer simulations were performed for a constant temperature of 300 K using the Nose – Hoover algorithm with the thermostat relaxation constant of 2 ps. For the van der Waals interactions, we have used the Lennard – Jones (LJ) potential. The interaction potential parameters and atomic masses and charges are shown in Tables 1 and 2. The integration of the equations of motion was performed using the Verlet integration scheme in quaternion. The integration step was 2 fs (femtoseconds). The intermolecular chemical bonds were estimated on the basis of the Shake algorithm to an accuracy of \(10^{-8}\).

The electrostatics forces were calculated using the so-called “reaction field” algorithm [7,11]. In this method, the molecule is surrounded by a spherical cavern of a limited radius where the electrostatics forces are calculated directly. Outside of the cavern, the system is represented as a dielectric continuum. In the reaction field algorithm, the Coulomb potential has the following form:

| Atomic pair | Potential | Functional form | Parameters | \(\varepsilon, \text{kcal/mol}\) | \(\sigma, \text{Å}\) |
|-------------|-----------|----------------|------------|----------------|----------------|
| C-C         | LJ        | \(U(r) = 4\varepsilon \left( \frac{\sigma}{r} \right)^{12} - \left( \frac{\sigma}{r} \right)^{6} \) | \(\varepsilon, \sigma\) | 0.12             | 3.30            |
| H-H         | …         | …              | …          | 0.02             | 1.78            |
| N-N         | …         | …              | …          | 0.16             | 3.12            |
| O-O         | …         | …              | …          | 0.20             | 2.85            |
| OS-OS       | …         | …              | …          | 0.15             | 2.94            |
| Oe-Oe       | …         | …              | …          | 0.20             | 2.85            |
| OW-OW       | …         | …              | …          | 0.16             | 3.17            |
| HW-HW       | …         | …              | …          | 0.02             | 1.78            |
| K-K         | …         | …              | …          | 0.32             | 3.13            |
| Na-Na       | …         | …              | …          | 0.08             | 2.73            |
Table 2. The mass and charge values in the system of valinomycin + K⁺(Na⁺) ion + water.

| Atom (md notation) | Mass m (mₐ, a.m.u.) | Charge q (e, proton charge) |
|--------------------|---------------------|-----------------------------|
| C                  | 12.01               | +0.47                       |
| H                  | 1.00                | +0.21                       |
| N                  | 14.01               | –0.40                       |
| O                  | 16.00               | –0.41                       |
| OS                 | 16.00               | –0.46                       |
| Oe                 | 16.00               | –0.41                       |
| OW                 | 15.99               | –0.82                       |
| HW                 | 1.00                | +0.41                       |
| K                  | 39.10               | +1.00                       |
| Na                 | 23.00               | +1.00                       |

\[ U(r_{ij}) = \frac{1}{4\pi\varepsilon_0} q_i q_j \left[ \frac{1}{r_{ij}} + \frac{B_0 r_{ij}^2}{2R_c^3} \right] , \]

where \( R_c \) is a cavern radius, the constant \( B_0 \) is the dielectric constant of the continuum media, and

\[ B_0 = \frac{2(\varepsilon_r - 1)}{(2\varepsilon_r + 1)} . \]

The non-bound vdW forces are calculated using the LJ potential of the standard form:

\[ U(r) = 4\varepsilon \left[ \left( \frac{\sigma}{r} \right)^{12} - \left( \frac{\sigma}{r} \right)^{6} \right] . \]

For different atoms, we applied the following averaged relations (the Lorentz–Berthelot combining rules):

\[ \varepsilon_i = (\varepsilon_i \varepsilon_j)^{\frac{1}{2}} \quad \text{and} \quad \sigma_i = \frac{1}{2}(\sigma_i + \sigma_j) . \]

In Table 2, the mass and charge values are presented for the system of valinomycin + K⁺(Na⁺) ion + water used in molecular dynamics simulations.

3. RESULTS AND DISCUSSIONS

We have fulfilled a series of MD calculations for the systems of valinomycin + K⁺ ions + water and valinomycin + Na⁺ + water with the same simulation parameters and temperature-pressure conditions as described above. In order to control the motion of K⁺(Na⁺) ions directed exactly to the valinomycin cavity (ring), an external electric field of different fixed strength values was applied. Without an external field, valinomycin’s interaction with K⁺(Na⁺) ions directed normally to the valinomycin cavity (ring) is efficient in the cavity itself. In Figures 3(a) and (b), we present the initial configuration of the valinomycin + K⁺ ions, where the electric field is directed normally to the molecule plane (water molecules are not shown). The orientation of valinomycin during the whole time of dynamical changes was fixed in space, so that the valinomycin molecule would be able only to vibrate (oscillate); the directional mobility of the valinomycin molecule was fixed in the initial position. In such conditions, valinomycin’s interaction with K⁺(Na⁺) ions and water molecules happen efficiently in the cavity (ring) region. Figures 4 (a) and (b) shows the equilibrium configuration of the valinomycin + Na⁺ ions surrounded by water molecules; six consequent snapshots show the valinomycin structure with a Na⁺ ion passing through the cavity.

It should be noted that K⁺(Na⁺) ion passing through valinomycin’s ring is not possible for all (arbitrarily) values of the electric field. Namely, for each ion type (K⁺ or Na⁺), a critical electric field value exists under which the ion remains captured (localized) in valinomycin’s ring cavity. The MD simulation results presented in Figures 5-8 illustrate K⁺ ion localization.

Figure 3. The valinomycin orientation (a) and the electric field direction (b) for potassium ions. The water molecules are not shown.
Figure 4. The valinomycin configuration (a) surrounded by sodium ions (blue spheres) in water. Six consequent configurations of valinomycin and a sodium ion penetrating into the cavity are shown (b). The snapshots correspond to the time moments of $t = 0, 1, 2, 3, 5$ and 10 ps (the electric field is directed from left to right).
(capture) by a valinomycin molecule inside the ring cavity. In Figure 5, the consequent dynamical configurations for valinomycin + K+ ion are shown. In Figures 6(a)-(c), trajectory diagrams are presented for three ions moving outside of valinomycin’s localization region. The diagrams in Figures 6(a)-(c) represent motion of ions in a periodic geometry. Figure 7(a) displays the trajectory diagram of a K+ ion captured by valinomycin’s localization ring. Figure 7(b) shows the consequent configurations of the system in the localization region.

Let us estimate the K+ (Na+) binding with a valinomycin molecule based on the critical values of the electric field. The simulation results show different critical values for K+ and Na+ ions. The critical values correlate with the difference in the ion masses (K/Na = 39.1/23.0). A summary of MD simulation results is presented in Table 3. It is seen that the critical electrical field, under which the ion remains localized inside valinomycin’s ring, is about 150 mV for K+ and about 90 mV for Na+. The critical values of the electrical field shown in Table 3 can be associated with the chemical binding strength between the ions and the valinomycin molecule. In our estimation of $U_{cr}$ in Table 3, we used the following simple relation: $U_{cr} = E_{cr}d$, where $d$=3Å was chosen as a length of valinomycin’s active region (the ring’s cross section length).

$U_{cr}(K^+) \sim 5 \times 10^8 \text{N/Q} \times 3 \times 10^{-10} \text{m} \sim 150\text{mV}$;
Table 3. The values of the critical electric fields for K$^+$ and Na$^+$ ions.

| Critical electric field | K$^+$ | Na$^+$ |
|-------------------------|-------|--------|
| $E_{cr} \times 10^8$ N/Q | 5     | 3      |
| $U_{cr} \times 10^{-3}$ V | 150   | 90     |

$U_{cr}(\text{Na}^+) \sim 3 \times 10^8$ N/Q $\times 3 \times 10^{-10}$ m $\sim 90$ mV.

In summary, the external electric field has been used to estimate the strength of two major (K$^+$ and Na$^+$) ion bindings with the valinomycin molecule in water solvent. A stronger valinomycin binding with the potassium ion is clearly observed. It is well known that the valinomycin molecular structure is folded in such a way that its chain conformation efficiently surrounds a metal cation [1-6,8-10,12]. Valinomycin is selective to the K$^+$ ion, because it folds in such a way that it forms an almost octahedral structure via its strong (non-polarizable) donors: carbonyl’s hydrogen atoms. This structure has to exactly correspond to the K$^+$ ion in size.

The ratio of the critical electrical potentials $U_{cr}(\text{K}^+)/U_{cr}(\text{Na}^+) \sim 1.7$ implies a stronger binding of valinomycin + K$^+$ compared to that of valinomycin + Na$^+$, which results in binding energy estimation that $W(\text{K}^+) > W(\text{Na}^+)$. The binding for valinomycin + K$^+$ is energetically stronger, which correlates well with a number of experimental observations. For example, in experimental salt extraction equilibrium measurements [13], the Na$^+$ $\to$ K$^+$ ion replacement revealed that valinomycin prefers binding K$^+$ to Na$^+$ by $\sim$5.4 kcal/mol. Other experimental studies of the permeability ratio in lipid membranes [14] show that valinomycin selects K$^+$ rather than Na$^+$ with a selectivity of about $\sim$6 kcal/mol. The correlation of the simulation results and experimental X-ray crystal structure measurements and related studies of a strongly selective K channel is straightforward [14-17].

The MD simulation results could be a prerequisite for
studi ng a more complex scenario—for example, protein-ion interactions involving valinomycin together with potassium and sodium ions. It should also be noted that some correlation can be found between the obtained values of the critical electric field strength and the electric potential which is formed in the cell membrane or inside the cell (~100 mV) relative to its surrounding medium. This aspect, however, is worth a more detailed study due to its complexity (in particular, the specifics of the cell membrane like its molecular formation, cross section size, etc. are more complex in terms of structure).

4. ACKNOWLEDGEMENTS

This work has been fulfilled under joint collaboration projects of JINR, Russia—Daresbury Laboratory, UK—Keio University, Japan. We would like to thank Prof. William Smith for the software support. This work was supported in part by Grant in Aid for the Global Center of Excellence Program for “Center for Education and Research of Symbiotic, Safe and Secure System Design” from the Ministry of Education, Culture, Sport, and Technology in Japan.

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Development of acidophilus milk via selected probiotics & prebiotics using artificial neural network

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Received 27 April 2010; revised 30 May 2010; accepted 7 June 2010.

ABSTRACT

Commercial interest in functional foods containing probiotic strains has consistently increased due to the awareness of gut health. Recent advancements are leading to development of synbiotic foods, containing prebiotics and probiotics bearing synergistic effects of the two. Thus, in present study, synbiotic acidophilus milk was developed satisfying functional dairy food properties. Different sets of milk were fermented with probiotic cultures (Lactobacillus acidophilus, Bifidobacterium bifidum, Lactobacillus casei, bioyoghurt culture) singly or in combination, and prebiotics namely inulin (I), oat fibre (O) and honey (H). Obtained 20 synbiotic samples were organoleptically tested, physico-chemically (titrable acidity percentage (TA) & pH) and microbiologically (total viable count (TVC), coliform count and yeast & mold count) analyzed. The incorporation of honey and inulin led to development of sweetened and low calorie sweetened synbiotic acidophilus milk, respectively. Incorporation of B. bifidum increased the flavour of synbiotic acidophilus milk when compared to L. acidophilus as control, where as L. casei culture showed thinner consistency in the product. Addition of prebiotic affected only the sensory scores, whereas the probiotics addition resulted in a marginal variation of pH and TA. TVC of all synbiotic acidophilus milk samples obtained were more than desirable limits for harvesting probiotic effects (>10^10 cfu/ml). Finally, a two layer feed-forward artificial neural network (ANN) was established to predict the sensory evaluation based on inputs of probiotic and prebiotic.

Keywords: Synbiotic Acidophilus Milk; Artificial Neural Network; Sensory Evaluation

1. INTRODUCTION

Notable fermented milks are buttermilk, acidophilus milk, bulgarian buttermilk, kumiss, kefir and yoghurt. Acidophilus milk is a sour product that has been allowed to ferment under conditions that favor the growth and development of thermophilic lactic acid bacteria. This type of fermented milk is produced by development in milk of Lactobacillus acidophilus. It is claimed that acidophilus milk has therapeutic and health-promoting properties. It is also claimed that the growth of Lactobacillus acidophilus under the condition existing in the intestinal tract can replace undesirable putrefactive fermentation with a beneficial lactic fermentation [1].

Probiotics are technically defined as live microbial food ingredients that have a beneficial effect on human health. Some of the important beneficial effects are antimicrobial activity, immune system modulation, antimutagenic activity, colonization resistance activity, maintenance of micro-ecology of bowel, stimulation of Bifidobacteria, deactivation of carcinogens etc. Commercially available probiotic strains belong to genera Lactobacilli, Bifidobacterium, Streptococcus, Bacillus, Bacteriodes, Pediococcus, Leuconostoc, Propionibacterium [2-4], Saccharomyces cerevisia and Aspergillus oryzae [5].

The other key component of functional dairy or other products is prebiotic ingredient. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in colon that can improve the host health [6].

When prebiotics are used in combination with probiotics or live bacteria, the resultant has synergistic effects, referred to as “synbiotic”. This is because in addition to the action of probiotics that promote the growth of existing strains of beneficial bacteria in the colon, prebiotics such as inulin and oligofructose also act to improve the survival, implantation and growth of newly added probiotics strains.

Inulin is a mixture of polymers consisting mainly of fructose unit; its partial enzymatic hydrolysis yields oligofructose. These are classified as prebiotic (dietary)
fibres, which help in stabilization of foams, assist in digestion, increase stool volume, stimulate *Bifidobacteria* and are used for formulation of low fat preparations as fat and sugar replacers, respectively [7-9].

As a prebiotic, honey contains carbohydrates called oligosaccharides, which may improve gastrointestinal health by stimulating the growth of good bacteria in the colon. Honey has been shown to enhance growth, activity of *Bifidobacteria* in fermented dairy food [10, 11]. Current market trends reveal that acidophilus milk could be a suitable vehicle to develop functional dairy foods. By incorporation of variations in terms of flavour, it could be easily used in wide range of nutritional and organoleptic qualities being demanded by consumers of all age, health status, and cultures.

In the food industry, end-products must achieve a compromise between several properties, including sensory, sanitary and technological properties. Among the latter, sensory property is essential because it influences consumer choice and performance. To produce a new product, if formulation of product is achieved automatically, production speed and efficiency can be improved in addition to the increased evaluation accuracy, with an accompanying reduction in production costs [12,13]. Artificial neural network (ANN) could be applied to evaluate its capability in predicting product composition involved in food formulation [14-19].

Scanty information is available on incorporation of prebiotic along with probiotic in acidophilus milk. Incorporation of prebiotic and probiotic for development of functional synbiotic acidophilus milk seem to be an effective area of research. In view of the above, present investigation was undertaken to develop quality of synbiotic acidophilus milk from toned cow’s milk, by use of ANN technique.

### 2. MATERIALS AND METHODS

#### 2.1. Materials and Chemicals

The freeze-dried cultures of *Lactobacillus acidophilus* NCDC No 015, *Bifidobacterium bifidum* NCDC No 255 and *Lactobacillus casei* NCDC No 063 were obtained from Culture Collection Centre, National Dairy Research Institute, Karnal (Haryana), India. Bioyoghurt culture (*L. bulgaricus* and *S. thermophilus*) was obtained from K.C. Das Pvt. Ltd. (Bangalore). Inulin was obtained from Nutraingredients, Netherlands. Pasteurized toned cow’s milk (3% fat and 8.5% Solid Non Fat), dietary fibre (oat), honey (Dabur India Ltd.), skim milk powder (Amul), polyethylene cups and aluminum foil were purchased from the local market. The chemicals and media used in the present investigation were of Analytical Reagent grade.

#### 2.2. Activation of Cultures

The freeze-dried cultures were activated according to the recommendations of suppliers and grown in sterile skim milk at 37 ± 1°C and then maintained by weekly transfers and stored at 4 ± 1°C between transfers. These cultures were activated by sub culturing 3-4 times before use.

#### 2.3. Preparation of Synbiotic Acidophilus Milk

Optimized level of skim milk powder (3% wt./v) based on sensory evaluation was added to pasteurized toned milk and reheated to at 40-45°C Prebiotics were added at optimized level based on sensory evaluation namely inulin (10% wt./v), oat fibre (0.2% wt./v) and honey (7% wt./v). The milk was inoculated with starter culture (*L. acidophilus*) as a control, and other probiotics namely *L. casei, B. bifidum, L. bulgaricus, S. thermophilus* singly or in combination with same ratio (1:1 and 1:1:1) at 7 percent v/v., and then incubated at 40°C for 4-4½ hours. Different type of synbiotic acidophilus milk thus prepared were cooled to 5°C and stored for about 2 hours for inducing cold gelation, then stirred for better homogenous consistency. Thus, obtained synbiotic acidophilus milk samples were tested for pH, TA, microbial analysis and sensory evaluation.

#### 2.4. Measurement of TA and pH of Samples

pH of the synbiotic acidophilus milk was measured directly using the digital pH meter (Digisun Electronics, Hyderabad). TA (as % lactic acid) of the samples was determined according to Ranganna [20].

#### 2.5. Total Viable Count

1 ml of sample was diluted in 9 ml distilled water and then serial dilutions were prepared. Different dilutions (10⁻⁴, 10⁻⁷ and 10⁻⁸) were used to check total viable count present per ml on DE Man, Rogosa, Sharpe and All purpose medium with tween so agar (Hi-Media Laboratories Pvt. Ltd., Mumbai). After incubation the average count of colonies present on Petri plates were multiplied by dilution factor and expressed as colony forming units (cfu) per ml.

#### 2.6. Yeast & Mold and Coliform Count

Potato dextrose agar and Acetamide media (Hi-Media Laboratories Pvt. Ltd., Mumbai) were used to enumerate the count of yeast & mold and coliform count, respectively. After incubation at 22 ± 1°C for about a week and 37°C for 24-48 hrs, respectively, the average count of colonies present on petri plates, if any, were multiplied by dilution factor and expressed as colony forming units (cfu) per ml.
2.7. Sensory Evaluation

Symbiotic acidophilus milk samples were evaluated for their sensory characteristics namely colour, flavour, body texture and overall acceptability (OA) using semi-trained sensory panel consisting of 10 judges drawn from faculty members of Brindavan college Banglor. The judges were requested to record their degree of liking/disliking on a scorer and using hedonic scale ranging from 1 to 9, where 1 represented dislike extremely and 9 represented like extremely [20]. The samples were served to panelist randomly.

2.8. Hybrid Analytical Approach

An artificial neural network is usually defined as a network composed of a large number of processors (neurons) that are massively interconnected, operate in parallel, and learn from experience (examples). The ANN used in this research is a feed forward network that can be used to estimate a vector X from a measured vector V. The ANN “learns” by adjusting the interconnection weights between layers. The answers produced by the network are repeatedly compared with the correct answers, and each time the connecting weights are adjusted slightly in the direction of the correct answers. Eventually, if the problem is learned, a stable set of weights adaptively evolves which will provide good answers for all of the sample predictions. When a new vector V is entered into the network, it is subtracted from the stored vectors representing cluster centers. The squares of the differences are summed and fed into a non-linear activation function to recognize the pattern which is most similar to the entered one [21].

Neural network (NN) approach is an effective method for generating new combination of input vector PP. Because of inherent parallel structure, a multi layer ANN can be trained for generating new combinations of prebiotics and probiotics in a very short running time. The Training set is \( \{ (PP, UG) \} \), \( PP \in R^r, UG \in R^t \), where PP is a sample random vector for the prebiotics and probiotics based on fractional factorial experiment. Generating new combinations by neural networks helps to approximate UG according to a given PP.

With a sigmoid activation function, a multi layer NN \( E[UG | PP] \) is trained and new interolation combination can be obtained.

The Model \( OA \approx f(PP) + \varepsilon \) is a nonlinear equation that has been approximated by quadratic equation as given in Formula (1).

\[
OA = \beta_0 + \sum_{i=1}^{p} \beta_i PP_i + \sum_{j=1}^{p} \sum_{i=1}^{p} \beta_{ij} PP_i PP_j + \varepsilon
\]  

where \( \beta_0 \) is equal to the mean of OA and \( \beta_i \) and \( \beta_{ij} \) are linear and quadratic coefficients, respect-ively. Moreover, the expected value of \( \varepsilon \) and OA to PP can be recognized by Eqs.2 and 3 respectively:

\[
E(\varepsilon | PP) = 0
\]

\[
E[OA | PP] = f(PP)
\]

For obtaining the parameters of Formula (1), we minimize squared error by the following equations.

\[
\varepsilon_j = OA_j - \left[ \beta_0 + \sum_{i=1}^{p} \beta_i PP_{ij} + \sum_{j=1}^{p} \sum_{i=1}^{p} \beta_{ij} PP_i PP_j \right]
\]

\[
S(\beta) = \sum_{j=1}^{r} \varepsilon_j^2
\]

\[
S(\beta) = \sum_{j=1}^{r} \left[ OA_j - \left[ \beta_0 + \sum_{i=1}^{p} \beta_i PP_{ij} + \sum_{j=1}^{p} \sum_{i=1}^{p} \beta_{ij} PP_i PP_j \right] \right]^2
\]

By using \( \frac{\partial S(\beta)}{\partial \beta} = 0 \), the corresponding parameters can be determined.

For higher polynomial degree of Eq.1, we used hypothesis test as follows:

\[
\{ \begin{align*}
H_0 & : \beta = 0 \\
H_1 & : \beta \neq 0
\end{align*} \]

The t-test has been used to test the degree of nonlinear equation model. For validity of approved model, we apply F-test for the result of actual and approximated TD as Formula (7) by F statistic in Formula (8).

\[
H_0 : F_0 = F \quad \quad H_1 : F_0 \neq F
\]

\[
F = \frac{SS_g / P_p}{SS_E / (n - P_p - 1)}
\]

where SS_g is sum of squared regression results and SS_E is the sum of squared error while \( SS_g \approx \chi^2 \) and \( SS_E \approx \chi^2(n - P_p - 1) \). Hence the F statistic has a Fisher distribution with safety interval of \( \alpha \), and \( P_p \) and \( n - P_p - 1 \) degree of freedom (\( F_{\alpha, P_p, n - P_p - 1} \)).

If \( F_0 \geq F_{\alpha, P_p, n - P_p - 1} \), then \( H_0 \) is rejected and the model would be valid, otherwise the model should be modified.

3. RESULTS AND DISCUSSIONS

3.1. Changes in TA and pH

The pH and TA for samples is shown in Table 1. They remained unaffected by the type of additive used but the type of culture combination altered them. Maximum acidity and lowest pH were observed in case of C1 culture (Lactobacillus acidophilus), followed by C2 cultures (Lactobacillus acidophilus + Bifidobacterium bifidum), C3 cultures (Lactobacillus acidophilus + Lactobacillus
casei) cultures, C4 cultures (Lactobacillus acidophilus + Bifidobacterium bifidum + Lactobacillus casei) and C5 cultures (Bioyoghurt culture). The range of pH and titratable acidity in synbiotic acidophilus milk was 4.70-4.84 and 1.23-0.88, respectively. Lowest TA and highest pH was obtained when C5 culture was used in preparation of synbiotic acidophilus milk, since it did not show acceptable quality. Similar observations were reported for different type of fermented milk by [20-24].

### 3.2. Changes in TVC, Coliform and Yeast & Mold Count

TVC of all synbiotic acidophilus milk samples obtained ranging from $10^6$ to $10^8$ cfu/ml, were higher than prescribed range of $10^6$ to $10^8$ cfu/ml at the end of storage period [25]. It can be inferred that the TVC obtained in synbiotic acidophilus milk samples in present investigation was equal to or higher than recommended range which were reported by [26,27]. No yeast & mold and coliform count appeared inferring that the product developed were of good quality.

### 3.3. Changes in Sensory Score

The sensory score for colour, flavour, texture and OA for samples is shown in Table 1. Synbiotic acidophilus milk prepared using starter culture (Lactobacillus acidophilus, B. bifidum and L. casei) and prebiotic additives (oat, inulin, honey) singly or in combination, significantly increased the colour, flavour, texture and OA of the synbiotic acidophilus milk. Addition of inulin (10%) or honey (7%) to C4 increased the sensory score for colour, flavour, texture and OA of the product developed. From the experimental data, it was found that C4 culture increased the sensory characteristic of the symbiotic acidophilus milk along with the honey or inulin; however addition of oat fibre did not affect the score of acidophilus milk sample much, and was not found to be as effective as inulin or honey. Thus, addition of inulin led to development of low calorie sweet acidophilus milk which is of value for recommendation to both diabetic and calorie conscious consumers.

A fractional factorial experiment combination of each element by experiment is obtained for the problem as actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG) are put into a neural network and the output of ANN is obtained as UG actual UG. The training data (actual UG)...
Table 2. ANN Error (Δ), Actual and NN Output for different combinations.

| Input | Actual Output | NN Output | NN Error |
|-------|---------------|-----------|----------|
| [1;0;0;0;0;0;0;0] | [7;7;6;7] | [7.2743;7.422;7.0387;7.4512] | [-0.27433;–0.422;–1.0387;–0.4512] |
| [1;1;0;0;0;0;0;0] | [7;8;8;8] | [7.4576;7.7008;7.4737;7.6375] | [-0.45762;–0.7008;–0.4737;–0.6375] |
| [1;0;1;0;0;0;0;0] | [7;7;6;7] | [7.3409;7.3927;7.1037;7.3898] | [-0.34092;–0.3927;–1.1037;–0.38982] |
| [1;1;1;0;0;0;0;0] | [7;7.5;7.5;7.5] | [7.5242;7.6719;7.5386;7.5762] | [-0.5242;–0.6719;–0.5386;–0.5762] |
| [1;1,0;1;0;0;0;0;0.002] | [7;5;7;7.25] | [6.2442;6.2985;6.7955;6.4128] | [–0.7558;–0.2677;–0.7955;–0.5762] |
| [1;0;0;0;0;0;0;0.002] | [7;7.5;7.5;7.5] | [7.2742;7.422;7.0386;7.451] | [–0.27421;–0.422;–1.0386;–0.4512] |
| [1;1,0;0;0;0;0;0;0.002] | [7;7.5;7;7.25] | [6.2442;6.298;6.7955;6.4128] | [0.75584;1.021;2.045;0.83723] |

Table 4 indicates the t-test analysis results for the coefficients of the above-mentioned quadratic equation.

With the equation which approximates OA in terms of probiotic and prebiotic, as it is observed from the results reported in Table 4, the p-values of B.b, I, O, H, I^2, H^2, and some of other components are greater than 0.360. This means that with a confidence of %64, these factors are not important in approximating OA and can be safely removed from further consideration.

For optimization, the following quadratic mathematical model has been solved by LINGO 8 software package.

\[
\begin{align*}
\text{Max } \text{OA} &= 7.829(L.a) - 0.629(L.b) - 0.372(S.t) \\
&- 4428.58(O)^2 + 184.60(B.b)(O) + 4.80(L.c)(I) \\
&- 99.72(L.b)(O) - 5.14(S.t)(H) \\
&\text{subject to:} \\
&0 \leq I \leq 0.12 \\
&0 \leq O \leq 0.04 \\
&0 \leq H \leq 0.1 \\
&L.a, B.b, L.b, S.t, L.c \in \{0, 1\}
\end{align*}
\]

which lead to \{1,1,1,0,0,0.09,0.002,0.075\} as the optimum combination of prebiotic and probiotic.

4. CONCLUSIONS

From the results of the experiments it can be concluded that the addition of inulin or honey had synergistic effect on the physico-chemical and sensory quality of probiotic treated.
acidophilus milk. Also a satisfactorily good quality syn-
biotic acidophilus milk could be prepared by fermenting
milk with combinations of all cultures (C₄, Lactobacillus
acidophilus, Bifidobacterium bifidum and Lactobacillus
casei) along with 7 percent honey (sweetened acidophi-
lus milk) or 10 percent inulin (for low calorie sweet-
ened acidophilus milk). Moreover, in this research an
artificial neural network has been applied to create
more experimental data. Then by developing a quadratic
mathematical model, the optimum value of OA has been
approximated with respect to probiotics and prebiotics,
i.e. combination of Lactobacillus acidophilus, Bifido-
bacterium bifidum and Lactobacillus casei along with
7.5 percent honey, 9 percent inulin and 0.2 percent oat
fibre.

Figure 2. Block diagram of proposed NN.
Table 3. ANN output.

| L.a | B.b | L.c | L.b | S.t | I | O | H | OA | L.a | B.b | L.c | L.b | S.t | I | O | H | OA |
|-----|-----|-----|-----|-----|---|---|---|----|-----|-----|-----|-----|-----|---|---|---|----|
| 1   | 1   | 0   | 1   | 0   | 0.12| 0.003| 0.05| 6.451636804| 1   | 1   | 1   | 1   | 0   | 0.08| 0   | 0.05| 7.484155 |
| 1   | 1   | 0   | 1   | 0   | 0.12| 0.004| 0.07| 6.101418984| 1   | 1   | 1   | 0   | 0   | 0.12| 0.001| 0.08| 8.346724 |
| 1   | 0   | 0   | 1   | 1   | 0.06| 0.004| 0.01| 7.571022455| 1   | 1   | 0   | 0   | 0   | 0.02| 0.003| 0.02| 7.528115 |
| 1   | 0   | 0   | 1   | 0   | 0.02| 0.001| 0.06| 6.984832124| 1   | 1   | 0   | 0   | 1   | 0   | 0.06| 0.001| 0.03| 7.555261 |
| 1   | 0   | 0   | 1   | 0   | 0.06| 0.004| 0.05| 6.459366602| 1   | 0   | 0   | 1   | 0   | 0   | 0.02| 0.002| 0.09| 6.791768 |
| 1   | 1   | 0   | 0   | 1   | 0   | 1   | 0.004| 0.01| 6.149601739| 1   | 1   | 0   | 1   | 0   | 0.04| 0.003| 0.1| 7.339067 |
| 1   | 1   | 0   | 1   | 0   | 1   | 0.02| 0.002| 0.03| 7.552972025| 1   | 1   | 1   | 0   | 0   | 0.12| 0.002| 0.09| 8.346724 |
| 1   | 0   | 0   | 1   | 0   | 0.08| 0.001| 0.07| 6.854626756| 1   | 1   | 1   | 0   | 0   | 0.02| 0.001| 0.04| 8.154417 |
| 1   | 0   | 1   | 1   | 0   | 0.06| 0.004| 0.08| 4.657894747| 1   | 1   | 1   | 0   | 0   | 0.06| 0.002| 0.04| 7.423626 |
| 1   | 1   | 1   | 0   | 1   | 0   | 0.04| 0.001| 0.05| 7.48414076| 1   | 1   | 0   | 0   | 1   | 0.01| 0.003| 0.1| 7.536114 |

Table 4. The t-test and P-value analysis results for the coefficients. 

| Coefficients | B.b | L.b | S.t | I | O | H | F | O² | H² | IO | IH | OH |
|--------------|-----|-----|-----|---|---|---|---|----|----|----|----|----|
| T Stat        | -0.298 | -2.084 | -1.247 | -0.811 | 0.158 | 0.418 | 0.781 | -1.388 | 0.223 | -0.347 | -0.813 | -0.758 |
| P-value       | 0.76  | 0.04  | 0.21  | 0.42  | 0.87  | 0.67  | 0.43  | 0.16  | 0.82  | 0.72  | 0.41  | 0.45  |

| Coefficients | B.b | O.H | L.c | L.O | L.H | L.b | L.b.O | L.b.H | S.t | S.t.O | S.t.H |
|--------------|-----|-----|-----|-----|-----|-----|-------|-------|----|-------|-------|
| T Stat       | 0.477 | 2.045 | 0.217 | 1.974 | -0.963 | 0.442 | -0.669 | -1.285 | -0.661 | 0.187 | 0.235 | -1.59 |
| P-value      | 0.63  | 0.04  | 0.82  | 0.05  | 0.33  | 0.65  | 0.5   | 0.2   | 0.51  | 0.85  | 0.81  | 0.11  |
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Intra-renal and urinary RNA expression of podocyte-associated molecules in patients with IgA nephropathy

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Received 14 April 2010; revised 7 May 2010; accepted 12 May 2010.

ABSTRACT

Background Podocyte injury probably plays important roles in the pathogenesis of IgA nephropathy (IgAN). We studied intra-renal and urinary messenger RNA (mRNA) expression of podocyte-associated molecules in patients with IgAN.

Methods We studied 43 consecutive patients with biopsy-proven IgAN. Intra-renal and urinary expression of mRNAs was determined and compared to that of 20 patients with nephrectomy for kidney cancer and 12 normal subjects.

Results Intra-renal mRNA expression levels of nephrin, podocin and synaptopodin were significantly lower in patients with IgAN than that of controls. In contrast, their urinary mRNA expression levels were similar. Intra-renal gene expression of nephrin inversely correlated with proteinuria (r = –0.620, P < 0.001), GFR (r = 0.538, P < 0.001), and the degree of tubulointerstitial scarring (r = –0.423, P = 0.013). After followed for an average of 33.4 ± 12.6 months, intra-renal nephrin expression significantly correlated with the rate of GFR decline (r = 0.324, P = 0.041).

Conclusions Intra-renal mRNA expression of podocyte associated molecules was down-regulated in patients with IgAN, and the degree of down-regulation of nephrin correlated with disease severity and the rate of progression. Our result supports the hypothesis that podocyte injury is an important component in the pathophysiology of IgAN.

Keywords: Glomerulonephritis; Proteinuria; Chronic Kidney Diseases

1. INTRODUCTION

Immunoglobulin A nephropathy (IgAN) is the most common form of primary glomerulonephritis throughout the world, and a good portion of patients with biopsy-proven IgAN progresses to ESRD in 10 to 20 years [1-3]. The most prominent pathological changes of IgAN are deposition of pathogenic polymeric IgA1 in the mesangium, proliferation of mesangial cells, excessive synthesis of extracellular matrix (ECM), and infiltration of inflammatory cells. However, podocyte loss is a concomitant of increasing disease severity in IgAN [4]; podocyte loss may either cause or contribute to the progressive proteinuria, glomerular sclerosis and filtration failure in IgAN [4]. Podocyte injury might provide additional prognostic information in patients with IgAN [5]. More recently, it has been shown that Bcl-2 expression by podocytes exerts modulatory effects on cellular processes that contribute to progressive glomerular injury of IgAN [6], while humoral factors released from mesangial cells may alter glomerular permeability by reducing the expression of podocyte markers [7]. In the present study, we explore the intra-renal and urinary messenger RNA (mRNA) expression of podocyte-associated molecules in patients with IgA nephropathy.

2. PATIENTS AND METHODS

2.1. Subjects

We studied 43 consecutive patients with IgA nephropathy confirmed by kidney biopsy in the Prince of Wales Hospital, Hong Kong, between 2004 and 2007. Patients with other coexisting renal pathology, and recurrent IgA nephropathy after kidney transplantation, were excluded. The study was approved by the Clinical Research Ethical Committee of the Chinese University of Hong Kong, all patients provided informed consent. Clinical data including serum creatinine and 24 hours urine protein were recorded at the time of kidney biopsy. Glomerular filtration rate (GFR) was estimated by a standard equation [8]. We studied normal renal tissue from the nephrectomy...
specimen of 20 patients with renal cell carcinoma (all clear cell type) and urine sediment from 12 healthy subjects as controls for intra-renal and urinary mRNA expression study, respectively.

After renal biopsy, all patients were followed every 2 months for at least 12 months. Renal function and the degree of proteinuria were assessed at every visit. Disease progression was measured by the rate of GFR decline, which was calculated by the least-square regression method [9]. Treatment for individual patient was determined by the responsible physician and not affected by this study. All physicians were blinded from the results of mRNA expression.

2.2. Sample Preparation

Immediately after kidney biopsy, the renal tissue was fixed in 10% neutral buffered formaldehyde overnight and then dehydrated by alcohol and embedded in paraffin for intra-renal mRNA expression. Ten 10 μm sections were cut from the formalin-fixed and paraffin-embedded (FFPE) tissue blocks using a microtome and pooled in a 1.5 ml microcentrifuge tube. The sections were then treated by xylene for 3 minutes at 50°C and washed by 100% ethanol twice. Air dry the pellet for 30 minutes at room temperature.

Urine specimen was collected and processed immediately, or stored in 4°C overnight. Urine sample was centrifuged at 3000 g for 30 minutes and at 13000 g for 5 minutes at 4°C. Supernatant was discarded and the urinary cell pellet was lysed by RNA lysis buffer (Qiagen Inc, Ontario, Canada). Specimens were then stored at −80°C until use.

2.3. Measurement of MRNA Levels

RecoverALL™ total nucleic acid isolation kit and MirVana™ miRNA isolation kit (Ambion, Inc. Austin, TX, USA) were used for the extraction of total RNA from FFPE tissue and urinary sediment according to the manufacturer’s protocol.

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) were used for reverse transcription. 10 μl total RNA was mixed with 2 μl specific primers, 0.8 μl 100mM dNTPs (with dTTP), 2 μl 10x reverse transcription buffer, 1 μl (50U) MultiScribe™ Reverse Transcriptase, 1 μl RNase inhibitor (20 U/μl) and made up to 20 μl with H2O. Reverse transcription was performed at 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. The resulting cDNA was stored in −80°C until use.

Intra-renal and urinary mRNA expression of nephrin, podocin and synaptopodin were quantified by RT-QPCR using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Commercially available Taqman primers and probes, including 2 unlabeled PCR primers and 1 FAM™ dye-labeled TaqMan® MGB probe were used for all the targets (all from Applied Biosystems). The primer and probe set was deliberately designed across the intron-exon boundary so as not to detect probable genomic DNA. For RT-QPCR, 10 µl universal master mix, 1 µl primer and probe set, 1.33 µl cDNA and 7.67 µl H2O were mixed to make a 20 µl reaction volume. Each sample was run in triplicate. RT-QPCR were performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. β-Glucuronidase (GUSB, Applied Biosystems) was used as housekeeping genes to normalize the mRNA expression [10]. Results were analyzed with Sequence Detection Software version 2.0 (Applied Biosystems). In order to calculate the differences of expression level for each target among samples, the ΔΔCT method for relative quantitation was used. Average expression level of normal renal tissue from patients with kidney clear cell cancer and urinary sediment from healthy controls was used as calibrator for intra-renal expression and the expression level of targets was a ratio relative to that of the controls.

2.4. Assessment of Renal Scarring

Analysis of renal fibrosis was determined on 4 μm paraffin-embedded sections stained by Periodic Acid Schiff (PAS) or Jones silver stain. The severity of renal fibrosis was scored by an experienced pathologist who was blinded to the results of molecular studies. The severity of glomerulosclerosis was represented by the percentage of sclerotic glomeruli in total glomeruli obtained from biopsy. For tubulointerstitial scarring, ten microscopic fields were viewed at magnification of 200 × and scored subjectively from 0 to 100% for each patients. The severity of tubulointerstitial scarring was represented by the mean of ten scores.

2.5. Statistical Analysis

Statistical analysis was performed by SPSS for Windows software version 13.0 (SPSS Inc., Chicago, IL). All the results were presented in mean ± SD for data normally distributed and median (lower and upper quartiles) for the others. Since data of gene expression levels were highly skewed, either log transformation or nonparametric statistical methods were used. We used Mann-Whitney U test to compare gene expression levels between groups and Spearman’s rank-order correlations to test associations between gene expression levels and clinical parameters. When no detectable level of a transcript was found (defined as no detectable level after 40 cycles of RT-QPCR) and there was zero value, a value equal to half of the minimum observed gene expression
level was assigned. A P value of below 0.05 was considered statistically significant. All probabilities were two-tailed.

3. RESULTS

The demographic and baseline clinical data of the study subjects were summarized in Table 1. As compared to controls, patients with IgA nephropathy had significantly higher level of proteinuria and worse renal function. Histological studies showed that the percentage of glomerulosclerosis and tubulointerstitial scarring of patients were $28.33 \pm 26.97\%$ and $25.60 \pm 24.06\%$ respectively.

3.1. Levels of Gene Expression

The intra-renal and urinary expression of various mRNA species are compared and summarized in Figure 1. As compared to controls, patients with IgA nephropathy had lower intra-renal gene expression of nephrin ($0.16 \pm 0.07$ versus $1.02 \pm 0.81\%$, $P < 0.001$), podocin ($0.49 \pm 0.12\%$ versus $0.95 \pm 0.66\%$, $P = 0.019$) and synaptopodin ($0.60 \pm 0.31\%$ versus $0.92 \pm 0.72\%$, $P < 0.001$). Urinary mRNA expression of nephrin and synaptopodin, on the contrary, was marginally lower in patients with IgA nephropathy than controls ($0.16 \pm 0.06\%$ versus $0.80 \pm 0.27\%$, $P = 0.091$ for nephrin, and $0.31 \pm 0.14\%$ versus $0.94 \pm 0.30\%$, $P = 0.051$ for synaptopodin). In contrast, their urinary expression of podocin was similar ($0.21 \pm 0.06\%$ versus $0.40 \pm 0.00\%$, $P = 0.894$). There was no significant correlation between intra-renal and urinary mRNA expression of studied molecules (details not shown).

3.2. Relation with Clinical Data

Since intra-renal gene expression of nephrin, podocin and synaptopodin was significantly different between patients and controls, we further explored the relation between their level of intra-renal expression and clinical parameters. The result was summarized in Figure 2. In short, proteinuria inversely correlated with intra-renal gene expression of nephrin ($r = -0.620$, $P < 0.001$) and synaptopodin ($r = -0.389$, $P = 0.003$), but not podocin ($r = -0.237$, $P = 0.073$) (Figure 2(a)). GFR significantly correlated with intra-renal gene expression of nephrin ($r = 0.538$, $P < 0.001$), but not podocin ($r = 0.205$, $P = 0.123$) or synaptopodin ($r = 0.193$, $P = 0.147$) (Figure 2(b)).

3.3. Relation with Histological Damage

Relations between intra-renal gene expression of nephrin, podocin and synaptopodin were summarized in Figure 3. Briefly, tubulointerstitial scarring significantly correlated with intra-renal gene expression of nephrin ($r = -0.423$, $P = 0.013$), but not podocin ($r = -0.171$, $P = 0.333$) or synaptopodin ($r = -0.136$, $P = 0.445$). Glomerular scarring, however, did not significantly correlate with any of the targets studied (details not shown).

![Figure 1. Comparison of intra-renal and urinary expression of mRNAs between patients with IgA nephropathy and controls. Data are compared by Mann-Whitney U test. Levels are represented as ratio to the average of controls.](image-url)

![Table 1. Demographic and baseline clinical data of the subjects.](table-url)

|                | IgAN   | Biopsy control | Healthy subject |
|----------------|--------|----------------|-----------------|
| No. of case    | 43     | 20             | 12              |
| Sex (M:F)      | 27:16  | 13:7           | 7:5             |
| Age (year)     | $48.37 \pm 12.64$ | $52.20 \pm 8.30$ | $31.33 \pm 3.87$ |
| Proteinuria (g/day) | $1.13 \pm 1.13$ | $0.01 \pm 0.03$ | $0.00$ |
| Serum creatinine (μmol/l) | $175.07 \pm 123.03$ | $95.06 \pm 23.02$ | $--$ |
| GFR (ml/min/1.73m²) | $50.44 \pm 29.50$ | $76.09 \pm 26.02$ | $--$ |

IgAN, immunoglobulin A nephropathy; GFR, glomerular filtration rate.
3.4. Relation with Renal Function Decline

The average duration of follow up was 33.4 ± 12.6 months; the average rate of GFR decline was –0.24 ± 0.62 ml/min/month. The rate of GFR decline significantly correlated with intra-renal gene expression of nephrin \( (r = 0.324, P = 0.041) \) (Figure 4), indicating that the lower the intra-renal gene expression of nephrin, the faster the renal function decline. The rate of GFR decline did not significantly correlate with podocin \( (r = 0.177, P = 0.274) \) or synaptopodin \( (r = 0.224, P = 0.164) \) (Figure 4).

4. DISCUSSION

In the present study, we found intra-renal expression of
podocyte-associated molecules, nephrin, podocin and synaptopodin, was down-regulated in patients with IgAN while urinary expression of these molecules was unchanged. We also found intra-renal expression of these molecules, especially nephrin, significantly correlates with disease severity and progression of IgAN.

Podocyte injury in IgAN has been attributed to direct or indirect effects of serum IgA1 and mesangial-derived cytokines such as TNF-α or TGF-β [7,11]. Shedding of podocytes from glomerulus to urine and down-regulation of nephrin, a crucial component of the slit diaphragm, has been proved in patients with IgAN [12-15]. In line with these previous results, we found that besides nephrin, scaffolding and cytoskeleton related molecules podocin and synaptopodin were also down-regulated in patients with IgAN, as also proved by a recent in vitro study [7]. Though the exact mechanism by which these molecules are down-regulated has not been examined, these results suggest that there might be pervasive down-regulation of podocyte-associated molecules including slit diaphragm components, scaffolding and cytoskeleton related molecules in IgAN.

We have previously reported that urinary gene expression of podocyte-associated molecules in patients with other kidney diseases such as DN and HTN is significantly higher than normal controls [16,17], suggesting podocytes detach into the urine in these conditions. Indeed, more viable podocytes are proved to shed from the glomerulus in patients with glomerular diseases than healthy controls [18]. On the contrary, urinary gene expression of podocyte-associated molecules tends to be lower in patients with IgAN than that in normal subjects in the present study. This result suggests that podocyte loss in IgAN for a different mechanism (for example, apoptosis).

In the present study, the intra-renal expression of nephrin, but not podocin or synaptopodin, was found significantly correlated with disease severity and progression of IgAN. The explanation for this discrepancy among these podocyte-associated molecules remains unclear. Of note, nephrin has been proved to be an indispensable component of slit diaphragm and loss of nephrin is related to increased permeability of glomerular filtration barrier which may lead to proteinuria and disease progression [19,20]. Further, nephrin is a signaling adhesion molecule and link to the actin skeleton of podocyte [20]. Loss of nephrin may therefore facilitate unhinging adjacent foot processes through the dysfunction of the actin skeleton system. Taken together, our results suggest that nephrin loss may play a pivotal role in the impairment of slit diaphragm function.

There are a number of inadequacies of our study. First, we used normal renal tissue from the nephrectomy specimen of patients with renal cell carcinoma as control. However, the renal function of these patients was not normal (average GFR less than 80 ml/min), and intra-renal expression of podocyte-associated molecules might be affected by the renal insufficiency. In theory, renal tissue from subjects with normal renal function (for example, kidney donors) could be used as controls.

Secondly, we did not enumerate podocyte number in this study. It was likely that the change in intra-renal expression of podocyte-associated molecules is a result of decreasing podocyte number, but it was also possible that there was down-regulation of podocyte associated molecules per cell. Furthermore, this study is only cross-sectional, and it is possible that the mRNA expression of podocyte-associated molecules changes with disease progression and therapy. Future studies are needed to investigate the serial change in expression of these targets as the disease progresses or following various therapy strategies.

In summary, we show that intra-renal gene expression of podocyte-associated molecules, especially nephrin, is down-regulated in patients with IgAN, and the degree of down-regulation of nephrin correlates with disease se-

Figure 4. Relation between intra-renal expression of mRNAs and the rate of change in estimated glomerular filtration rate (GFR).

Data are compared by Spearman’s rank correlation coefficient. Negative change in GFR indicates worsening of renal function.
verity and rate of progression. Our result supports the hypothesis that podocyte injury is an important component in the pathophysiology of IgAN.

5. ACKNOWLEDGEMENTS

This study was supported in part by the CUHK research accounts 6901031 and 7101215. The results presented in this paper have not been published previously in whole or part, except in abstract format. All authors declare no conflict of interest.

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Photobiodegradation of halogenated aromatic pollutants

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Received 17 April 2010; revised 5 May 2010; accepted 7 May 2010.

ABSTRACT
Release of wide range of compounds as a consequence of industrial development is now a serious environmental problem. Numerous hazardous waste sites have been generated worldwide resulting from the accumulation of xenobiotics in soil and water. Aromatic compounds constitute a large and diverse group of chemicals that are responsible for causing widespread environmental pollution. Among them halogenated aromatic hydrocarbons are very stable to undergo degradation due to resonance energy and inertness of carbon-halogen, carbon-hydrogen and carbon-carbon covalent bonds. The physico-chemical remedial strategies to clean up sites contaminated by these compounds are inadequate and economically inefficient. Therefore, research is increasingly being focused on development of biological approaches for their remediation. The hunt for the microorganisms degrading halogenated aromatic pollutants has been successful in discovering a diverse range of aerobic, anaerobic and phototrophic bacteria. The bacteria mineralize the toxic halogenated pollutants into harmless products thereby contributing towards conservation of the environment quality.

Keywords: Haloaromatics; Photometabolism; Dehalogenation; Phototrophic Bacteria

1. INTRODUCTION
1.1. Halogenated Aromatic Pollutants Clorinated Aromatic Pollutants
Chlorinated aromatic compounds are major environmental pollutants because they are often released in substantial quantities, are toxic and resistant to degradation, and accumulate in sediment and biota. Although some compounds are degraded only slowly by soil and aquatic microorganisms, others are metabolized relatively quickly.

Some of the chlorinated aromatic compounds include chlorotoluene, chlorobenzenes chlorobenzoates, chlorophenols, 4-chlorophenylacetate and chlorophenoxyacetics [1]. Chlorobenzenes are used extensively as solvents, fumigants, and intermediates in the production of pesticides, dyes, disinfectants, room deodorants and moth control agents [2]. Chlorinated phenols are used as wood preservatives, herbicides, fungicides, and general biocides are a large group of toxic xenobiotics that are serious environmental pollutants [3-5].

Chlorinated derivatives of phenoxyacetics, such as Dichlorophenoxy acetic acid (2, 4-D) and 2,4,5-trichlorophenoxyacetic acid (2, 4, 5-T), have been released into the environment as herbicides over the past 40 years [6]. Unlike many of the recalcitrant synthetic compounds, 2, 4-D is rapidly degraded by soil microorganisms [7].

1.2. Brominated Aromatic Compounds
Brominated aromatic compounds have found use as flame retardants. Fluorinated and iodinated aromatic compounds are components of pharmaceutical agents.

The chemical inertness and hydrophobicity of many of these compounds has resulted in them becoming widely distributed in the environment; in particular accumulating in many terrestrial and aquatic organisms [8]. This coupled with their toxicity has given rise to concern about their fate in the environment. Some of the brominated persistent pollutants are 6-bromo-2,4,5-trichloro phenol (BrTriClP), pentabromophenol (PBP), 3,3′,5,5′ tetrabromobisphenol A (TBBPA), bromophenol (BP) and bromo benzoic acid (BBA). Brominated diphenyl ethers (BDEs), are environmentally persistent class of organic pollutants, in “biosolids” from four different regions of the United States. These compounds are widely used as flame retardants, and their presence suggests that the environmental consequences of land application of biosolids need further investigation. BDEs have been frequently detected in wild-caught fish, indicating another pathway for human exposure [4,9].

A diversity of natural aromatic brominated organic
compounds can be found in a variety of biota, mostly aquatic species such as algae and sponges [10]. The Aplysina aerophoba is an example of a marine sponge in which such compounds can be found [11]. Two examples of natural aromatic brominated compounds are shown in Figure 1. These brominated compounds may function as a chemical defence against predators and biofouling (unwanted accretion of biota on the outside of a-wet-organism).

1.3. Fluorinated Pollutants

Hazardous chemicals that have entered the environment because they have leaked out from products during use and waste treatment include chlorinated compounds and brominated flame-retardants (BFRs). Now the alarm is being sounded for a completely new group of problematic micropollutants—the fluorinated hazardous substances [12]. They are used in many types of products to achieve a smooth surface that is preferably stain- and water-repellent. The fluorinated chemicals can be found in certain cleaning agents, paint and varnish, wax, floor polishing agents, impregnation agents for textiles, carpets, paper, furniture and shoes, fire-extinguishing liquids and photo paper.

In the following section, the phototrophic bacterial degradation of some halogenated aromatic compounds is discussed.

2. PHOTOBIODEGRADATION

Utilization of light energy by organisms for growth and survival is called phototrophy. The function of the anoxygenic photosynthetic apparatus is the transformation of light energy into an electrochemical gradient of protons across the photosynthetic membrane, which can be used for ATP production, active transport, motility, and other energy-consuming processes.

Aromatic compounds, whether from natural or synthetic sources are ubiquitous in most ecosystems. Purple non sulphur photosynthetic bacteria are capable of degrading a wide variety of structurally diverse aromatic compounds anaerobically in the presence of light [13,14]. Among them Rhodopseudomonas palustris is perhaps the most nutritionally versatile. Members of Photosynthetic anoxygenic bacteria include purple sulfur bacteria and green and purple nonsulfur bacteria. Anaerobic soil environments would provide favorable conditions for the proliferation of these bacteria [15]. In addition to cyanobacteria, these may contribute to the productivity through carbon fixation [16].

2.1. Photobiodegradation of Halogenated Aromatic Compounds

Among all the halogenated aromatic pollutants the photobiodegradation of chlorobenzoates are well studied. However the reports on the photobiodegradation of other halogenated aromatic compounds are scanty.

The degradation of chlorobenzoates by soil microorganisms have been reported by many researchers [1] and also the photobiodegradation of chlorobenzoates have been extensively studied in the phototrophic bacteria like Rhodopseudomonas palustris. During the metabolic study of 3-chlorobenzoate by a mixed phototrophic culture in the presence of benzoate, Rhodopseudomonas palustris WS17 was the dominant phototroph [17]. The anoxygenic photoheterotroph Rhodopseudomonas palustris DCP3, isolated by Van der Woude et al. [18] is the first example of an R. palustris strain that can use 3-chlorobenzoate (3CBA) as sole source of carbon under anoxic conditions in the presence of light. A unique property of this bacterium is that it does not need a co-substrate for growth on the chlorinated compound, which is in contrast to previously described anaerobic phototrophic bacteria [17,19]. In an another degradation study, 3-chlorobenzoate was shown to be metabolized by Rhodopseudomonas palustris DCP3 under low-oxygen and phototropic conditions [20]. The phototrophic bacteria while metabolizing 3-chloro benzoate used the dechlorination pathway. In this the substrate 3-chlorobenzoate was dechlorinated to benzoate. If R. palustris grown on 3-chlorobenzoate, it can also use 2-chlorobenzoate, 4-chlorobenzoate or 3,5-dichlorobenzoate.

2.2. Dehalogenation in Phototrophic Bacteria

A few examples that phototrophic bacteria, including Rhodospirillum and Rhodopseudomonas sp., can grow phototrophically under anaerobic conditions using halo-carboxylic acids or 3-chlorobenzoate have been reported [3]. We are currently investigating the possible role of Pseudomonas stutzeri in metabolizing 2-chlorophenol and 4-chlorophenol under phototropic and denitrification conditions.

3. CONCLUSIONS

Purple non sulphur photosynthetic bacteria are capable of degrading a wide variety of structurally diverse aromatic compounds anaerobically in the presence of light.

Figure 1. Examples of natural aromatic brominated organic compounds. The left compound is isolated from the red alga Rypithelea tinctoria, the right one is isolated from the sponge Tedania ignis [10].

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Anaerobic soil environments would provide favorable conditions for the proliferation of these bacteria. Anaerobic processes are beneficial for eliminating pollutants from contaminated sites, in which oxygen is often unavailable due to its quick depletion with easily utilisable substrates, low solubility in water and low rate of transportation in saturated porous matrices such as soils and sediments. The metabolism of haloaromatics by anaerobic phototrophic bacteria is made possible by dislodging carbon-halogen bond by dechlorination or halorepiration, denitrifying conditions or under methanogenic environments. It is hoped that anaerobic and phototrophic microorganisms are better suited than aerobes for the remediation of natural halogenated aromatics and pollutants arising from industrial applications and effluents.

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