Effect of natural PAL-enzyme on the quality of egg white and mushroom flour and study its impact on the expression of PKU related genes and phenylalanine reduction in mice fed on

Hesham A. Eissa a,b, Zeinab Y. Abdallah b, Wagdy K.B. Khalil c, Wafaa A. Ibrahim a, Hoda F. Booles c, Mahrous M. Hassanane c

a Food Technology Department, 33 El boohoos, National Research Centre, 12622 Cairo, Egypt
b Biochemical Genetics Department, 33 El boohoos, National Research Centre, 12622 Cairo, Egypt
c Cell Biology Department, 33 El boohoos, National Research Centre, 12622 Cairo, Egypt

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Abstract
PKU patients react to therapy with a low phenylalanine diet, but adherence to this diet is troublesome, subsequently the expansion of alternative ways is demand. Phenylalanine ammonia lyase (PAL) is one of this ways, which converts phenylalanine to harmless metabolites; trans-cinnamic acid and ammonia. In the current study, the extraction of PAL enzyme was used to investigate the efficiency for production of functional PKU egg white and mushroom flour with good quality by evaluation of colour characteristics, determination of phenylalanine concentrations and genetic materials expression of PKU related genes and DNA damage. Results indicated that the PAL enzyme treated of egg white and mushroom flour was stable colour and the calculated reduction per cent in phenylalanine concentration from female mice fed on untreated and PAL–treated samples was 22.77% in egg white and 31.37% in mushroom flour. Also, the results revealed that female mice fed on diet contained treated egg white exhibited low expression levels of PKU exons (3, 6, 7, 11, and 12) and low DNA damage which were similar to those in control mice.

1. Introduction
Phenylketonuria (PKU; OMIM 261600), the “poster child” for much of our understanding of metabolic disorders, affords a good model for effective management through dietary handling. PKU is an autosomal recessive genetic disorder, resulting from a defect in phenylalanine hydroxylase (PAH) or secondarily due to impairment in tetrahydrobiopterin metabolism, which is a cofactor for PAH [7]. All PKU patients are characterized by increasing and growing of phenylalanine (phe.) and its metabolites in blood and urine also, characterized by a severe mental retardation with reduced growth, microcephaly, neurological signs and behavioural problems also reduced hair skin, and iris pigmentations are observed [40]. Moreover, stress resulting from oxidation is coinciding with physiology and pathology of PKU which induce a neurodegeneration in this disease [37,29,31]. This neurodegeneration induces oxidative break in several kinds of cells such as protein, RNA, DNA and lipids [14]. In this regard, when Schulpis et al. [34] analysed the serum of PKU patients found correlation of Phe levels and DNA adducts (8-OhdG) which produced from exposure to oxidative stress. Furthermore, hyperphenylalaninemia was found in the DNA molecules extracted from animal models of PKU [36]. Dietary therapy with restriction of protein remains the cornerstone for treatment of PKU patients in order to keep blood phenylalanine level within safe limit that means no eggs, no cheese and no meat. Their food is depended on a special formula they eat at meal times, supplemented by fruits and vegetables. The diet therapy should be beginning early after birth; therefore, newborn screening (NBS) programmes are necessary to identify the PKU patients early [40].

Adherence to diet therapy is difficult especially in adolescence due to the off odour and bitterish taste of the amino acid recipes in addition to strong rival pressure to eat normal foods; therefore, new dietary routes are necessary to recover the palatability and variety of the low-Phe diet to promote compliance, metabolic monitoring and quality of life for PKU patient [20,42] such as, large neutral amino acids supplementation [23], a medical foods based upon glycomacropeptide, a naturally low-Phe protein, [25]
pharmacologic chaperones, cofactor. 5,6,7,8-tetrahydrobiopterin (BH4). Lastly, the subcutaneous injection of phenylalanine ammo-
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From literature effect of different traditional and nontraditional
processing methods on foods to reduce phenylalanine content was
rarely found. Also, there is no effective method can be suggested
for supplying phenylalanine hydroxylase to phenylketonuria
patients. The treatment for the food now in use can be producing
a free amino acid mixture from which phenylalanine has been
unnaturally removed. It is pointed out that the amino acid mixture
is commonly artificial as food for human [2].

Although the frequently question is to produce naturally occur-
ring proteins containing a very low quantity of phenylalanine, it
could be an effort to make up such a substance by making use of
new techniques. Yamashita et al. [43] explain how to apply a series
of enzymatic processes including the so-called plastein reaction for
prepare two kinds of peptide-type with low-phenylalanine sub-
stances. Not in animals or bacteria, but in some fungi and in all
higher plants analyzed to date has been contains PAL enzyme
activity. One of the best studied is PAL enzyme in higher plants
because of the important of PAL enzyme in the formation of lignin,
isoflavonoids, and other secondary metabolites [8]. PAL activity
has been extracted in many species of plants, such as monocots, dicots,
gymnosperms [24]. Moreover, PAL cDNAs were isolated, and
sequenced from different banana cultivars by Alvarez et al. [3].

Egg white and edible mushrooms are considered protein food
products [5,30]. The shelf life of oyster mushroom processed is
restricted to a few days, related to the enzymatic browning during
storage. It has been supposed that the PAL increases during the
minimum processing due to the damages caused in the cells
[32]. Many modern processes for food production employ enzymes
as important processing aids to obtain good food quality (espe-
cially colour characteristics) [10,38].

Too little of research papers were carried out in the field of PAL
enzyme extract to reduce of phenylalanine concentration in food
processing. In the present paper, we study the characterization
and quality of crude Phenylalanine Ammonia-lyase enzyme activ-
ity (PAL) from banana fruit (Musa cavendishii L., cv. Enana) to pro-
duce a functional PKU egg white and mushroom flour with good
quality by evaluation of colour characteristics, determination of
phenylalanine concentrations, genetic materials expression of
PKU related genes and DNA damage.

2. Material and methods

2.1. Fruit samples

Banana (Musa cavendishii, cv. Enana) fruits grown in Egypt were
obtained from a local supermarket in Cairo. Banana fruits were
placed in refrigerator at 3–4 °C for 4 h until used.

2.2. Enzyme preparation

Using blender, 10 g banana fruits were homogenized in 100 ml
of cold aceton and the soluble residue filtered and dried under
vacuum. It was extracted at 4 °C by gentle stirring with 50 ml of
extraction buffer which contained 100 mM sodium borate buffer
(pH 8.8), 2 mM EDTA (ethylenediaminetetraacetic acid) and PVPP
(polyvinylpolypyrrolidone) at 100 g kg⁻¹ of the fruit fresh weight.
After 1 h of extraction, the solution was filtered through two layers
of cheese clothes and centrifuged at 5000 rpm at 4 °C for 15 min
[41].

2.3. PAL enzyme activities assay

PAL enzyme activities in the buffer supernatant were deter-
mined by the production of trans-cinnamic and p-coumaric acids,
respectively, from 1-phenylalanine (PAL) during 1 h at 30 °C, as
measured by the absorbance change at 290 nm [41]. The assay
mixture contained 1.5 ml 30 mM sodium borate buffer (pH 8.8)
and 0.5 ml buffer supernatant, and 1 ml 0.015 M l-phenylalanine
for PAL assays. The substrates (l-phenylalanine for PAL) were
added after 10 min of pre incubation and the reactions stopped
with 0.1 ml 6 M HCl. Assays were performed in triplicate. l-
phenylalanine was obtained from Sigma (St. Louis, MO, USA).

2.4. Food processing

2.4.1. Egg white processing

Red chicken egg was obtained from a commercial super market,
Cairo, Egypt. Freshly egg white separated from the egg yolk. How-
ever, egg white was treated by 1% PAL enzyme and incubated at
room temperature for 2 h then added 0.05% SO₂ to stop of the
activity of PAL enzyme.

2.4.2. Mushroom floor production

Fresh mushroom (Pleurotus ostreatus) was obtained from a com-
mercial mushroom farm at Cairo, Egypt. The fresh mushroom
obtained was pre-treated with PAL enzyme (1%) and incubated at
room temperature for 2 h then added 0.05% SO₂ to stop the activity
of PAL enzyme and dried in an oven dried (60 °C) using hot air oven
(Shel, Lab 1370 FX, Shel Don manufacturing, Inc. and Germany)
for 8hr. to obtain a completely dried sample. Dehydration was contin-
ued until the moisture content of mushroom slices reached about
14%, using modified method of Parab et al. [28], it was then pulver-
ized, sieved and stored in polyethylene bag at room temperature at
20 °C for use afterwards.

2.5. Nutrition experiment

Fifty Swiss albino female mice were obtained from the animal
house of the National Research Centre, Dokki, Giza, Egypt and
immediately after weaning, they were classified into 5 groups of
10 mice each as follows:

Group 1: used as a control group with normal feeding (22% pro-
tein, 3.48% fat and 3.71% dietary fibre as 60% yellow corn, 34% soya
bean, calcium phosphate, sodium chloride, calcium chloride, oil
soya, vitamins, antitoxins and molasses). Group 2: fed on raw
egg white without any treatment. Group 3: fed on PAL enzyme
treated egg white. Group 4: fed on dried mushroom floor without
any treatment. Group 5: fed on dried PAL enzyme treated mush-
room floor.

All groups were fed with 0.5 mg/kg m body weight for one
month. Animals were housed under temperature- and light-
controlled conditions with standard laboratory rodent chow and
water provided ad libitum. Animal procedures were performed in
accordance with the Ethics Committee of the National Research
Centre and followed the recommendations of the National Insti-
tutes of Health Guide for Care and Use of Laboratory Animals (pub-
lication no. 85-23, revised 1985). Diet and water were provided
ad libitum.
2.6. Determination of amino acids in egg white and mushroom flour

All amino acids were analyzed for untreated and PAL enzyme treated egg white and mushroom flour samples according to the method of A.O.A.C. [1].

\[
\% \text{ reduction} = \left(1 - \frac{C_{\text{phenylalanine treated}}} {C_{\text{phenylalanine untreated}}} \right) \times 100
\]

2.7. Colour characteristics determination of egg white and mushroom flour

Colour of untreated and PAL-enzyme treated egg white and mushroom flour was measured using a HunterLab colourimeter (Tristimulus Colour Machine) with the CIE lab colour scale (International Commission on Illumination) as mentioned by Hunter [16] and Sapers and Douglas, [33]. Colour of Fresh egg white and mushroom flour samples was measured using a HunterLab colourimeter Hunter a*, b* and L*. Parameters were measured with a colour difference meter using a spectro-colourimeter (Tristimulus Colour Machine) with the CIE lab colour scale (Hunter, Lab Scan XE - Reston VA, USA) in the reflection mode. The instrument was standardized each time with white tile of Hunter Lab Colour Standard (LX No. 16379): X = 72.26, Y = 81.94 and Z = 88.14 (L* = 92.46; a* = –0.86; b* = –0.16). The instrument (65°/0° geometry, D25 optical sensor, 10° observer) was calibrated using white and black reference tiles. The colour values were expressed as L* (lightness or brightness/darkness), a* (redness/greenness) and b* (yellowness/blueness). The Hue (H), Chroma (C) and Browning Index (BI) were calculated according to the method of Palou et al. [27] as follows:

\[
H^* = \tan^{-1}\left(\frac{1}{b^*/a^*}\right)
\]

\[
C^* = \sqrt{a^2 + b^2}
\]

\[
BI = \left[\frac{100(x - 0.31)}{10.72}\right]
\]

where \(X = (a^* + 1.75 L^*)/(5.645 L^* + a^* – 3.012b^*)\)

\[
\Delta E = (\Delta a^2 + \Delta b^2 + \Delta L^2)^{1/2}
\]

where all values were recorded as the mean of triplicate readings.

2.8. Non-enzymatic browning determination

Non-enzymatic browning was measured spectrophotometrically by 4054 - UV/Visible spectrophotometer, (LK Biochrom Comp., London, England), as absorbance at 420 nm using ethanol as a blank according to the method of Stamp and Labuza [39] and Birk et al. [6].

2.9. Determination of phenylalanine concentration in dried blood spot using Tandem mass spectrometry

Chemicals included MassChrom Reagent Kit for the LC-MS/MS analysis of amino acids and acylcarnitines from dried blood spot, non derivatized, which contained; mobile phase, lyophilized internal standard, rinsing solution, extraction buffer, 96 well plates V bottomed, protective sheets, piercable adhesive seal and amino acids, acylcarnitine dried blood spot control bi-level(1 + II)–GmbH, Guthrie filter paper cards (S&S 903; Schleicher and Schull, Dassel, Germany) were used to collect blood samples. The blood samples from female mice were spotted on Guthrie filter paper cards, left to dry at room temperature for at least 24 h. The procedures of extraction and mass analysis were applied according to instruction of manufacture chromsystems; briefly, 3 mm dried blood spot disk was punched out of the filter card from level 1 (target value of phenylalanine 167 µmol/l, range 107–227) level II (target value of phenylalanine 564 µmol/l, range 348–780) and sample into well of microtiter plate. For the extraction: 100 ul of the reconstituted internal standard with known concentration of phenylalanine-D5 (6.52 µmol/l) was added for each well. Sealed the microtiter plate and agitated at 600 rpm for 20 min. at ambient temperature. The supernatant was transferred into a new v-bottomed well plate. Sealed the plate with a piercable adhesive seal, 10 ul of the eluate was injected into the LC-MS/MS system, the concentration of the analyte in the sample was calculated according to: The concentration C Analyte, (µmol/l) = (signal intensity of analyte A in the sample spectrum/single intensity of the internal standard IS in the spectrum of sample) X sample related concentration C of the internal standard.

2.10. Mass spectrometry (MS)

A waters Xevo triple quadrupole mass spectrometer (Milford, USA) equipped with electrospray ionization (ESI) source in positive ion mode was used for sample analysis, a multiple reaction monitoring (MRM) was used for analytes and internal standards (phenylalanine transition 165.98/120 and phenylalanine-D5 171.12/125.2). The automatic sampler and the pump of an LC apparatus were used to inject sample and mobile phase to MS, but no LC column was needed. The Masslynx V4.1 and Neolyx were used for data acquisition and processing of MS analysis. A designed MS/MS program for automatic profiling of acylcarnitines and amino acids was used.

2.11. Gene expression analysis

2.11.1. RNA extraction

The total RNA from brain tissues of female mice was extracted using TRizol® Reagent (Invitrogen, Germany) according to the manufacturer's instructions with minor modifications. Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at −80 °C [11].

2.11.2. Reverse transcription reaction

Complete Poly(A)+ RNA isolated from brain tissues was reverse transcribed (RT) into cDNA in a total volume of 20 µl using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). The reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative Real-time polymerase chain reaction (qPCR) [17,18].

2.11.3. Real-time PCR

One step's real-time PCR cycler (Applied Biosystem, USA) was used to determine the brain cDNA copy number. PCRs were set up in 25 µl reaction mixtures containing 12.5 µl 1× SYBR® Premix Ex Taq™ (TaKaRa, Biotech., Co. Ltd.), 0.5 µl 0.2 µM sense primer, 0.5 µl 0.2 µM antisense primer, 6.5 µl distilled water, and 5 µl of cDNA template. Each experiment included a distilled water control. The sequences of specific primer of the genes used are listed in Table 1. At the end of each qPCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers. The relative quantification of the target (PKU exons 3, 6, 7, 11 and 12) to the reference (β-actin) was determined using the 2−ΔACT method.

2.11.4. DNA fragmentation analysis

Apoptotic DNA fragmentation in liver tissues of female mice was qualitatively analysed by detecting the laddering pattern of nuclear DNA as described according to Lu et al. [21].
3. Results and discussions

3.1. Phenylalanine ammonia layase (PAL) enzyme activity in fresh egg white and mushroom slices

Fig. 1, showed that fresh egg white and mushroom flour had different activities of enzymes such as phenylalanine ammonia layase (PAL) being 0.41 “Unit/g/h” in fresh mushroom sample and 0.0062 “Unit/g/h” in fresh egg white sample. PAL enzymes are responsible for various changes in chemical ingredients that will affect the quality characteristics and deterioration of fresh or processed mushroom. However, enzyme activity of phenylalanine ammonia layase (PAL) in mushroom showed greater activity than in egg white (Fig. 1). As seen in Fig. 1, PAL enzyme activity increased noticeably in mushroom samples.

3.2. Effect of PAL enzyme treatment on phenylalanine concentration “Unit/g/h” in egg white and mushroom flour using amino acid analyser

Fig. 2 represents the content of phenylalanine concentration in the studied untreated and PAL – treated egg white and mushroom flour samples. The phenylalanine content was 0.8 and 0.14 “Unit/g/h” in fresh egg white and mushroom flour samples. But, the phenylalanine content was decreased as a result of PAL-enzyme activity to 0.71 and 0.09 in treated egg white and mushroom flour samples. The percentage reduction in phenylalanine concentration was 11.25% and 35.71% in Pal-enzyme treated egg white and mushroom flour, respectively.

3.3. Effect of PAL-enzyme on colour characteristics, parameters and non-enzymatic browning (A420 nm) in egg white and mushroom flour

Using the Hunter Lab colour scale to measure the surface colour of untreated and PAL-enzyme treated egg white and mushroom flour. The Hunter colour values of untreated and PAL-enzyme treated egg white and mushroom flour were determined immediately after processed. The results of the Hunter Lab colours showed that the changes in a’ values were inversely relationship to the changes in L’ values. Absorption at 420 nm, the L’, a’, b’, C’, H’ and BI values were found to be appropriate indicators for the brown pigment composition because of nonenzymatic browning after processing, as seen in Table 1. The a’ values and BI for fresh egg white were low (−1.55 and 1.91) in contrast to high values for untreated mushroom flour (6.05 and 94.12) sample. Whereas, the a’ values and BI for PAL enzyme egg white were low (−2.5 and 10.34) in contrast to high values for PAL enzyme mushroom flour (6.34 and 95.67) sample under all tested conditions, fresh egg white showed much lower efficient values based on L’, a’, b’, C’, H’ and A420 nm than ΔE values measurements, whereas the mushroom flour sample behaved an opposite trend. Such a trend is in agreement with previous studies of Ozoglu and Bayindirh [26]. These results explained that the increased browning was related to PPO enzyme activity in mushroom flour samples than in fresh egg white samples, as seen in Table 1. According to our results, the main colour changes by increasing of the BI and a’ value in mushroom flour, which were in high correlation to browning measurement. Other colour parameters, such as hue angle and chroma, also explained that egg white and mushroom flour caused a slight colour changes.

Table 1

| Treatments      | L’   | a’   | b’   | ΔE   | A400nm | A420nm | C’   | H’   | BI   |
|-----------------|------|------|------|------|--------|--------|------|------|------|
| Egg white       | 13.61| −1.55| 1.28 | 78.85| 1.86   | 1.56   | 2.01 | 39.56| 1.91 |
| STDEV           | 0.08 | 0.04 | 0.04 | 0.21 | 0.04   | 0.01   | 0.05 | 0.14 | 0.14 |
| Mush flour      | 73.02| 6.05 | 27.16| 74.22| 15.82  | 18.92  | 27.83| 77.45| 94.12|
| STDEV           | 0.15 | 0.04 | 0.09 | 0.06 | 0.1    | 0.08   | 0.08 | 0.08 | 0.21 |
| PAL-egg white   | 15.02| −2.5 | 2.63 | 76.3 | 1.96   | 2.05   | 3.62 | 47.01| 10.34|
| STDEV-EW        | 0.10 | 0.15 | 0.07 | 1.05 | 0.03   | 0.04   | 0.15 | 1.03 | 0.49 |
| PAL-Mush flour  | 73.05| 6.34 | 27.43| 77.37| 19.23  | 22.64  | 28.16| 77.00| 95.67|
| STDEV           | 0.18 | 0.066| 0.15 | 0.15 | 0.065  | 0.05   | 0.16 | 0.06 | 0.91 |

±Standard Deviation (SD)/SQR2 (n), where, n = 3.

Fig. 1. Phenylalanine ammonia layase activity (Unit/g)/h in fresh egg white and mushroom slices.
Our results are consistent with those of Palou et al. [27] and Ozoglu and Bayindirh [26]. In general, it was showed that mushroom flour samples had the higher increased in colour as optical density (A420 nm) of nonenzymatic browning compared with fresh egg white samples.

Hunter colour (L*, a* and b*) values were increased, while R420 (reflectance) were decreased in PAL treated egg white and mushroom flour samples compared with untreated samples. This is perhaps due to release of carotenoids and anthocyanins as a result of PAL enzyme addition. While, the addition of PAL enzyme resulted in an increase in the L* and b*-values but decrease in a*-value and R420 nm.

The increasing in colour browning values (as A420 nm) could be referred to the reaction occurred between amino groups and active carbonyl groups (Maillard reaction) after oven drying process. From our results, it could be concluded that pretreated oven drying process did not affect non enzymatic browning with optical density (A420 nm). However, the values of non enzymatic browning (A420 nm) confirm the data obtained for oven drying process showed higher values (18.92) in mushroom flour compared with (2.015) fresh egg white. This is perhaps due to release of carotenoids and anthocyanins as a result of PAL enzyme addition. While, the addition of PAL enzyme resulted in an increase in the L* and b*-values but decrease in a*-value and R420 nm.

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### Table 2

| Treatments                     | Mice number | Phenylalanine (μmol/l) | ST. DEV | ST. Error |
|-------------------------------|-------------|------------------------|---------|-----------|
| Control                       | 10          | 70.32                  | ±11.90  | ±3.77     |
| Egg white untreated           | 10          | 84.46                  | ±14.13  | ±4.47     |
| Egg white treated             | 10          | 65.23                  | ±10.86  | ±3.44     |
| Mushroom flour untreated      | 10          | 79.16                  | ±5.15   | ±1.63     |
| Mushroom flour treated        | 10          | 54.33                  | ±14.13  | ±4.47     |

St. Error = SD/SQRT (n).
Concluded that the treated food with PAL extracted can reduce the phenylalanine level and open a new way to apply into different types of food for PKU patients to compensate the shortcomings in free phenylalanine formulas and decrease the compliances from PKU diets.
or treated egg white and mushroom flour. The results revealed that the expression levels of PKU exons 3, 6, 7, 11, and 12 were down-regulated in control mice fed on standard diet compared with female mice in other groups. In the same line, female mice fed on diet contained treated egg white exhibited relatively similar expression levels of PKU exons 3, 6, 7, 11, and 12 compared with those in control mice (Figs. 4–8). The expression levels of PKU exons (3, 6, 7, 11, and 12) in female mice fed on diet contained untreated egg white were higher but without significant differences compared with control mice (Figs. 4–8). On the other hand, the expression levels of PKU exons were up-regulated in mice fed on diet contained untreated mushroom flour compared those in female mice fed on standard diet or those fed on egg white; however, these levels of expression were only significant in exons 6 and
In the same line, female mice fed on a diet containing mushroom flour exhibited higher expression levels of PKU exons compared with those fed on a diet containing egg white, while these levels of expression were only significant in exon 6 (Fig. 5).

### 3.6. Effect of untreated or treated egg white and mushroom flour on the DNA fragmentation

The results of DNA fragmentation revealed that female mice fed on a diet containing untreated or treated egg white and mushroom flour showed different rates of DNA fragmentation (Fig. 9 and Table 3). The rate of DNA fragmentation in female mice fed on a diet containing treated egg white revealed similar rate of DNA fragmentation compared with control mice. However, the rate of DNA fragmentation in female mice fed on a diet containing untreated egg white increased slightly compared with those in control mice (Table 3). On the other hand, supplementation of female mice with a diet containing untreated mushroom flour increased significantly the rate of DNA fragmentation compared with those in control mice (Table 3). However, administration of female mice with a diet containing treated mushroom flour increased insignificantly the rate of DNA fragmentation compared with those in control mice (Table 3).

Several findings suggested that several oxidative stress resources are correlated with the development and acceleration of hyperphenylalaninemia in PKU patients. Based on these results, Rocha and Martins [31] and Ribas et al. [29] suggested that increasing the levels of Phe and/or its metabolites is coinciding with an increase in the reactive oxygen species and antioxidant status in the diets of PKU patients.

It has been reported that extreme generation of reactive oxygen species induce damage of the complete cell. The damage of the cell includes inhibition of the enzymes necessary for cellular processes, break of the DNA structure, and increase in the apoptosis [15]. This damage attributed to the generation of reactive oxygen species could be suggested by oxidation of the DNA bases and break the linkage between the structure of protein and DNA [14].

#### Table 3

| Treatments                          | % of DNA Fragmentation | Mean ± SEM |
|-------------------------------------|------------------------|------------|
| Control                             | 6–15                   | 7.8 ± 0.2b |
| Untreated egg white                 | 8–18                   | 10.3 ± 0.3b|
| PAL treated egg white               | 7–16                   | 8.2 ± 0.2b |
| Untreated mushroom flour            | 11–20                  | 15.9 ± 0.4a|
| PAL treated mushroom flour          | 7–19                   | 12.4 ± 0.3ab|

**Fig. 8.** The alteration of PKU exon 12-mRNA in brain tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. Data are presented as mean ± SEM. Mean values within tissue were insignificantly different ($P > 0.05$).

**Fig. 9.** DNA fragmentation in liver tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. M: DNA marker. Lane 1 represents PCR products of untreated control mice; lane 2 represents mice fed on egg white; lane 3 represents mice fed on treated egg white; lanes 4 represents mice fed on mushroom flour. Lane 5 represents mice fed on treated mushroom flour.
flour diets) increased the expression alterations of PKU exons 6 and 7 as well as increase the rate of DNA fragmentation compared with mice fed on standard diet. The miss-binding between the structure of protein and DNA may lead to induce mutations, alteration in the gene expression, chromosomal abnormalities, rearrangement of the DNA structure, cellular-toxicity [9]. On the other hand, the present study showed that feeding of female mice with diets contained egg white or mushroom flour treated with PAL enzyme decreased the expression alterations of PKU exons 6 and 7 as well as declined the rate of DNA fragmentation compared with mice fed on a standard diet. The protective effect of PAL against high levels of phenylalanine is that PAL is an autocatalytic protein responsible to catalyze the phenylalanine suppressing its genetic toxicity functions in female mice.

4. Conclusion

According to the application of recent treatment tool several points should be taken into consideration such as the scientific doubts, potential risks, overheads, and sociological outcomes for the kids and their families. Based on this study the addition of PAL enzyme in egg white and mushroom flour showed that improve colour characteristics, decrease phenylalanine concentrations and its genetic toxicity, which can be used and applied in different wide range of foods as a functional food for PKU patients.

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References

[1] A.O.A.C. (Official Method of Analysis). International no. 994.12. Chapter 4, p. 18–9. 19th ed., 2012 – Off J Eur Comm 19.9.98 - Biochrom 30 instruction manual (analyzer used). 2005. EZ Chrom manual (software used for data collection and processing); 2004.

[2] Altschul AM. In: New protein foods. New York: Academic Press; 1974. p. 1–9.

[3] Alvarez JC, Rodriguez HA, Rodriguez-Arango E, Monsalve ZI, Morales JG, Arango Palou E, Lopez-Malo A, Barbosa-Canovas G, Chanes-Welti J, Swanson W. Sreenath H, Nanjundaswamy A, Sreekantiah K. Effect of various cellulase and Strisciuglio P, Concolino D. Review new strategies for the treatment of Sitta A, Vanzin CS, Biancini GB, Manfredini V, de Oliveira AB, et al. Evidence Richard H, Forsythe H, Joseph F. Foster, egg white proteins I. Electrophoretic Saltveit MF. Physical and physiological changes in minimally processed fruits Gamez A, Wang L, Sarkissian CN, Wendt D, Fitzpatrick JF, et al. Structure-based epitope and PEGylation sites mapping of phenylalanine ammonia-lyase for enzyme substitution treatment of phenylketonuria. Mol Genet Metab 2007;91:325–34.

[4] Gámez A, Wang L, Straub M, Patch MG, Stevens RC. Toward PKU enzyme replacement therapy: PEGylation with activity retention for three forms of recombinant phenylalanine hydroxylase. Mol Ther 2004;9:124–5.

[5] Halliwell B, Gutteridge JC. Free radicals in biology and medicine, 14th ed. New York, Oxford; 2007.

[6] Höhn A, Jung T, Grune T. Pathophysiological importance of aggregated damaged proteins. In: Troullinou K, Ou P, editors. Metabolic Med 2004;1:253–60.

[7] Hunter RS. Scales for measurements of colour differences. In: Measurement for appearance. New York: Wiley Inter Science; 1975.

[8] Khalil WKB, Abd El-Kader HAM, Eshak MG, Farag IM, Ghanem KZ. Biological studies on the protective role of arbo1ch and green pepper against potential toxic effect of thermally oxidized oil in mung. Arab J Biotechnol 2005;12 (1):27–40.

[9] Khalil WKB, Mahmoud MA, Zahran MM, Mahrous KF. A sub acute study of metronidazole toxicity assessed in Egyptian Tilapia zillii. J Appl Toxicol 2007;27(4):380–90.

[10] Kim W, Erlandsen H, Surendran S, Stevens RC, Gamez A, Michols-Matalon K, et al. Trends in enzyme therapy for phenylketonuria. Mol Ther 2004;10:220–4.

[11] Koch R, Burton B, Hogsanog G. Phenylketonuria in adulthood: a collaborative study. J Inherit Metab Dis 2002;25:333–46.

[12] Lu T, Xu Y, Mericle MT, Mellgren RL. Participation of the conventional calpains in apoptosis. Bioch. et Bioph. Acta 2002;1590:16–26.

[13] MacDonald A, Rocha JC, van Rijn M, Feillet E. Nutrition in phenylketonuria. Genet Metab 2011:104.

[14] Matalon R, Michals-Matalon K, Bhatia G, Burlina AP, Braga C, et al. Double blind placebo control trial of large neutral amino acids in treatment of PKU: effect on blood phenylalanine. J Inherit Metab Dis. 2007;30:153–8.

[15] Min WH, Yeo HY, Jun YK, Seong HK. Fungal and plant phenylalanine-ammonia-lyase. Mycobiohistry 2011;39(4):257–65 [Rev. article].

[16] Ney DM, Gleason ST, van Calcar JC, MacLeod EL, Nelson KL, Ezzel MR, et al. Nutritional management of PKU: treatment with glycomacropeptide from cheese whey. J. Inherit. Metab. Dis. 2009;32:32–8.

[17] Oguzlu H, Bayyindirly A. Inhibition of enzymatic browning in cloudy apple juice with selected anti browning agents. Food Cont 2002;13:213–21.

[18] Palou E, Lopez-Malo A, Barrosa-Canoas G, Cha?es-Welti J, Swanson W. Polyphenoloxidase and colour of blanched and high hydrostatic pressure treated banana puree. J Food Sci 1999;64(1):42–5.

[19] Parab DN, Dahalaghe JR, Sahoo AK, Ranveer RC. Effect of incorporation of mushroom (Pleurotes suji-co) powder on quality characteristics of Papad (Indian snack food). Int J Food Sci Nutr 2012;63(7):866–70.

[20] Riba GS, Sitta A, Wajner M, Vargas CR. Oxidative stress in phenylketonuria: what is the evidence? Cell Mol Neurobiol 2011;31(5):553–62.

[21] Richard H, Forsythe H, Joseph F. Foster, egg white proteins I. Electrophoretic studies on whole white. J Biol Chem 1950:377–84.

[22] Rocha JC, Martins MJ. Oxidative stress in phenylketonuria: future directions. J Inherit Metab Dis 2012;35(3):381–98.

[23] Saltveit MF. Physical and physiological changes in minimally processed fruits and vegetable. In: Tomás-Barbáren FA, Robin RD, editors. Phytochemistry of fruits and vegetables. Oxford: Oxford University Press; 1997. p. 205–20.

[24] Sreenath H, Douglas F, Michals-Matalon K. Characterization of cut surfaces and in juice of raw apple and pear fruits. J Food Sci 1987;52:1258–62.

[25] Schulpis KH, Tsakiris S, Traeger-Synodinos J, Papassotiriou I. Low total antioxidant status is implicated with high L-hydroxy-2-deoxoguanosine serum concentrations in phenylketonuria. Clin Biochem 2005;38:239–42.

[26] Scriver CR, Kaufman S. Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill Inc; 2001. p. 1667–3301 (chapter 77).

[27] Simon KR, Dos Santos RM, Scaini G, Damiani AP, Furlanetto CB, et al. Phenylalanine hydroxylase and its genetic toxicity, which can be used and applied in different wide range of foods as a functional food for PKU patients.

[28] Sitta A, Vanzin CS, Biancini GB, Manfredini V, de Oliveira AB, et al. Evidence that l-carnitine and selenium supplementation reduces oxidative stress in phenylketonuric patients. Cell Mol Neurobiol 2011;31(5):429–36.

[29] Spreenath H, Nanjundaswamy A, Sreekantiah K. Effect of various cellular and pectinase on viscosity reduction of mango pulp. J Food Sci 1987;52:230–1.

[30] Stemp J, Labuza T. Kinetics of the Maillard reaction between aspartame and sucrose in solution at high temperatures. J Food Sci 1983;48:544–5. 547.

[31] Straccisiglio P, Concolino D. Review new strategies for the treatment of phenylketonuria (PKU). Metabolites 2014;4:1007–17.

[32] Teresa M, Molla E, Maria M, Francisco J. Effect of gibberellic acid (G3A) on strawberry PAL (phenylalanine ammonia-lyase) and TAL (tyrosine ammonia-lyase) enzyme activities. J Sci Agric Food 1995;77:720–4.

[33] Walter JH, White FJ. Blood phenylalanine control in adolescents with phenylketonuria. Int J Adolesc Med Health 2004;16:41–5.

[34] Yamashita M, Arai S, Fujimaki M. A low-phenylalanine, high-tyrosine plastein as an acceptable dietetic food. Method of preparation by use of enzymatic hydrolysis and resynthesis. J Food Sci 1976;41(5):1029–32.