Production, characterization and immobilization of *Aspergillus versicolor* L-asparaginase onto multi-walled carbon nanotubes

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**ABSTRACT**

The aim of this work is to study the optimum production of L-ASNase and the influence of oxidized multi-walled carbon nanotubes (ox-MWCNTs) on the biological activity of the isolated L-ASNase. Isolation and production of L-ASNase from different screened fungi in the presence of L-asparaginase substrate were carried out. The isolated enzyme was identified by molecular18S rRNA analysis. The partial purified L-ASNase having 6.77 purification fold was immobilized onto ox-MWCNTs using physical adsorption technique with maximum yield about 54.4%. *In vitro* cytotoxicity of L-ASNase against normal fibroblast cell line (BHK-21) was examined relative to the immobilized one using SRB assay. Also, *in vivo* biological examination using liver and kidney dysfunction biomarkers in the treated mice (AST, ALT, LDH, lipase and α-amylase) was also investigated. Among the ten screened fungi, *Aspergillus versicolor* was the most potent one with maximum activity about 45.5 U/mL. The results showed that the most appropriate production condition was at asparagine concentration of about 0.04 M and pH 8. The immobilized L-ASNase retained a complete activity (100%) after 30 min at 45°C of incubation relative to that of the free one. The toxicity of L-ASNase was significantly decreased up to 50 μg/mL after immobilization onto MWCNTs. Besides, the immobilized L-ASNase exhibited good storage ability and stability at high temperature relative to that in case of the free one.

**Keywords:** Multi-walled carbon nanotubes; L-asparaginase; biochemical analysis; cytotoxicity; *Aspergillus versicolor*; normal fibroblast cell line.

1. INTRODUCTION

L-asparaginase (L-ASNase) is an effective chemical agent for acute lymphoblastic leukaemia (ALL) treatment and other hematopoietic malignancies. This is due to its hydrolysis capacity of L-asparagine, an essential amino acid in L-aspartic acid and ammonia. The enzyme inhibits the development of asparagine-dependent cancer cells, induces apoptosis and triggers tumor death [1, 2].

Current formulations of L-ASNase have been used for long-term control of ALL, but these formulations are not free of adverse reactions. The quest for alternative sources of this enzyme with reduced or no side effect is therefore an important objective [3]. The key limitations on the use of L-ASNase are its premature inactivation, faster plasma clearance and shorter drug effect length, thus requiring regular injections to maintain a therapeutic level [4, 5].

Nevertheless, in addition to its clinical applications, the market for L-ASNase will increase many times in the coming years due to its possible industrial applications as food processing aid [6]. L-ASNase was found in many tissues of animals and plants as well as in a large number of micro-organisms such as Enterobacter, Aerobacter, Bacillus megaterium, Erwinia, Streptomyces brotllosae, Halobacillus, Vibrio, Proteus and some marine sources [7-10]. The route of administration is the main factor as maximum depletion of amino acid and peak activity levels of enzymes are achieved within 5 days of intramuscular administration [11].

Enzyme immobilization is one of biotechnology’s major trends and goals. The use of immobilized enzymes reduces the cost of production as these can be quickly isolated from the mixture of reactions and can therefore be used repeatedly and continuously. Several methods of enzyme immobilization have been employed, including adsorption on insoluble products, encapsulation in polymeric gels, membrane encapsulation and crosslinking with bifunctional or multifunctional reagents [12-15].

As new research areas, enzyme immobilizations on CNTs for biocatalyst preparation are rapidly emerging. Due to its exceptional mechanical, electrical and chemical properties, CNTs were used in the field of nanotechnology to create new functional nanostructures. Consequently, the curiosity in knowing and managing their interiors is now growing [16-31]. Despite significant progress in the production of L-ASNase, existing formulations are not free of limitations. Major adverse events are associated with the protein asparaginase immune reaction. In addition, degradation of asparagine and subsequent inhibition of protein synthesis often tend to restrict the use of L-ASNase. It is important to need more useful protocols and to define the best method possible.

This study addresses the optimal production of L-ASNase from *Aspergillus versicolor* and the immobilization of the partially purified enzyme on MWCNTs through this trend. The effect of the assay temperature, pH, substrate concentration and storage time on the relative enzyme activity (%) was investigated compared to the immobilized one. In addition, the *in vitro* cytotoxicity of L-ASNase to normal fibroblast cell line (BHK) and biochemical parameters related to the immobilized one were examined using SRB assay and biochemical liver and kidney dysfunction.
2. MATERIALS AND METHODS

2.1. Materials.

Multi-walled carbon nanotubes (MWCNTs), carbon about 95%, O.D L 6-9 nm 5 μm obtained by Sigma-Aldrich. The used screened fungi were collected from the laboratory of Natural and Microbial Products Department, National Research Centre (NRC), Cairo, Egypt. They included the following cultures: Trichoderma viride, Aspergillus terrus, Aspergillus versicolor, Penicillium racinosum, Rhizopus sp., Paecilomyces lilacinus, Fusarium solani, Penicillium sp., Penicillium chrysogenum and Alternaria alternata. Anhydrous L-asparagine, trichloroacetic acid (TCA), Nessler reagent chemicals were purchased from Sigma Chemicals. Agar and sucrose were obtained by Fluka Company, Switzerland. HEPES sterile buffer 1M obtained by Bio-West Chemical Co. All sera biomarkers were determined using a commercial kit in accordance with manufacturer's instructions using a spectrophotometer (Shimadzu UV-VIS Recording 2401 PC, Japan). Lactate dehydrogenase (LDH: EC. 1.1.1.27), alpha amylase, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lipase kit received, were solved on 4°C, 3968 -2 μC, against 2°C, Paecilomyces lilacinus °C, 2 -e suspension of each culture was used to inoculate 50 ml of Czapek (PDA) and stored. The enzyme had the following composition of 0.5 mL of saline sterilizers and centrifuged for 10 min at 10,000 rpm (Sigma 3-16KL model).

2.2. Methods.

2.2.1. Purification and oxidation of MWCNTs.

In mixed concentrated sulphuric and nitric acids (3:1, v / v), pristine MWCNT (100 mg) was distributed at a ratio of 50 mL of acid mixture[32]. The resulting blend was then heated overnight at 110°C with continuous stirring to create oxidized carbon nanotubes (Ox-MWCNTs). The sample washed away with ultrapure water until neutral filtrate was obtained (pH 7.0). The collected solid was vacuum dried for 12 h at 70°C and kept for further investigation.

2.2.2. Maintenance of fungi and preparation of inoculum.

The fungi were cultured on potato-dextrose agar medium (PDA) and stored in a refrigerator with regular transfer every month and preserved at 8°C in 50% (v/v) glycerol. The spore suspension of each culture was used to inoculate 50 ml of Czapek peptone medium that contained the following: Glucose (30 g/L), peptone (10 g/L), yeast extract (2 g/L), NaNO3 (3 g/L), KH₂PO₄ (0.5 g/L), KCl (0.5 g/L) and MgSO₄•7H₂O (0.5 g/L). The medium was prepared in 250 mL flasks, incubated at 30°C for 3 days under shaking condition (200 rpm). On the other hand, the medium used for the production of the enzyme had the following composition: sucrose (2.0 g/L), L-asparagine (10.0 g/L), KH₂PO₄ (1.52 g/L), KCl (0.52 g/L) MgSO₄•7H₂O (0.52 g/L) and FeSO₄•7H₂O (0.002 g/L). The pH of the medium was initially adjusted at pH 6.2.

2.2.3. Partial purification of the produced L-ASNase.

The enzyme was produced on a large scale (2 L) and precipitated with ethanol at 4°C to obtain 80% saturation. The precipitated protein was obtained by centrifugation for 20 min, dissolved in Tris-buffer (0.2 M, pH8.0) and asparaginase activity was calculated.

2.2.4. 18SrRNA Analysis technique.

A purely cultivated bacterium was used for classification. Colonies are collected in a 1.5 mL centrifugal tube with a sterilized toothpick suspended in 0.5 mL of saline sterilizers and centrifuged for 10 min at 10,000 rpm (Sigma 3-16KL model), supernatant was added, and Insta Gene Matrix (Bio-Rad, USA) suspended the pellet in 0.5 mL. Incubated for 30 min at 56°C, and then heated for 10 min at 100°C. Supernatant can then be used for PCR after heating. Template DNA 1μL was used for bacteria in 20 μL of PCR reaction solution 27F/1492R primers, then 35 amplification cycles were performed at 94°C for 45 sec, 55°C for 60 sec and 72°C for 60 sec. Approximately 1400 bp of purified PCR products were sequenced using 2 primers AGA GTT TGA TCM TGG CTC AGTAC GGY TAC CTT GTT ACG ACG T and CCA GCC GCC GTA ATG CGTAG CAG GTT ATC TAA TTC. Sequencing was performed using a large dye terminator cycle sequencing kit (Applied Biosystems, United States). The sequencing products were solved on an automated DNA sequencing system of Applied Biosystems model 3730XL, according to Baxevanis A.D. [33].

2.2.5. Immobilization of L-ASNase onto ox-MWCNTs.

L-asparaginase (3968 U/g carrier) was mixed with ox-MWCNTs aqueous solution at room temperature under stirring for 1 h, using physical adsorption technique. After that, the immobilized enzyme material was collected using centrifugation for 10 min at 10,000 rpm (Sigma 3-16KL model). The resulting sample washed with HEPES buffer and kept for further analysis.

2.2.6. Quantitative assay of L-ASNase activity.

L-ASNase activity was determined in the culture filtrates by quantifying the ammonia formation using Nessler’s reagent. The enzymatic reaction mixture was prepared including 0.5 mL of 0.04 M L-asparagine substrate, 0.5 mL of 0.05 M Tris-HCl buffer (pH 8.6) and 0.5 mL of the crude enzyme. The mixture was then incubated at 40°C for 30 min and the enzyme activity was stopped by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). The liberated ammonia was coupled using Nessler’s reagent for 15 min for development of colour. The optical density of the colour produced was measured at λ 450 nm using a double beam UV-visible spectrophotometer (ECIL model UV- 5704 SS). The liberated ammonia was determined using the standard curve of ammonium sulphate. The L-ASNase activity was expressed in terms of International unit (IU). One unit (IU) of enzyme was defined as the amount of enzyme which liberated one μmol of ammonia/mL/min (μmole/mL/min) at 40°C.

2.3. Characterization.

2.3.1. Influence of incubation parameters on L-ASNase activity.

2.3.1.1. Effect of pH.

The optimum pH of the free and immobilized L-ASNase activity was studied over a range of pH values 4.0-9.0 with asparaginase as a substrate dissolved in citrate-phosphate and 0.2 M Tris-buffer. The relative enzyme activities were plotted against different pH values.

2.3.1.2. Effect of substrate concentrations.

The initial velocity of the sample was estimated in a range of L-asparaginase concentrations (0.02 to 0.12 M). Using of Lineweaver-Burk plot [34], both Kₘ and Vₘₐₓ constants were determined.

2.3.1.3. Effect of storage time.

The free and immobilized L-ASNase (0.5mL) were mixed separately with 0.5 mL of substrate and stored at 4°C for different intervals of time (1, 2, 3 and 4 weeks), then the enzyme activity was calculated.
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2.3.1.4. Effect of assay temperature.

The optimum temperature for free and immobilized L-ASNase activity was determined by incubating the reaction mixture at different temperature from 30 to 60 °C. The values of the relative enzyme activities were plotted against temperature.

2.3.2. Thermal stability.

The filtrate medium containing enzyme (1 mL) was added to 0.5 mL of 0.05 M Tris-HCl buffer (pH 8.6) and pre-incubated for 15, 30 and 60 min at different temperature ranges from 30 to 60 °C. The relative activities were calculated.

2.3.3. Kinetic study.

The activation energy (Ea) was determined by plotting the log of the relative activity of the assayed temperature of free and immobilized L-ASNase against 1/T (Kelvin) cording to the following equation:

\[ E_a = \text{slope} \times 2.303 \text{ R (gas constant)} = 1.976. \] (1)

Also, half-life and the deactivation constant rate were determined by plotting the log of the relative activity against time according to the following equations:

- Half-life = 0.693/slope of the relative activity log against time.
- Deactivation energy = slope of the straight line.

2.4. Toxicity studies.

2.4.1. In vitro cytotoxicity using SRB assay.

Potential cytotoxicity of prepared nanomaterials was tested using the Skehan et al. method [35] as follows: standard fibroblast cell line (BHk-21) was plated separately in 96-multwell plate (10 cells/well) for 24 hrs prior to treatment with prepared formulations (ox-MWCNTs, free and immobilized LASNase) to allow the cells to be attached to the plate surface. For each individual dose different concentrations (0, 12.5, 25, 50 and 100) μg/mL of the prepared formulations were prepared. Monolayer cells were incubated at 37°C in 5 % carbon dioxide atmosphere with the prepared materials for 48 h after restoring, cleaning and staining 48 h cells with sulpho-rhodamine-B stain (SRB). Excess stain sprayed with acetic acid and restored with buffer Tris-EDTA. In an ELISA reader, the strength of color was calculated. To obtain the survival curves of the used cell line, the relationship between the surviving fraction (number of viable cells) and the concentrations of the prepared formulation was plotted. In addition, the dead cells (%) were also measured.

3. RESULTS

3.1. Production and isolation of L-ASNase.

Different fungi were examined for the production of L-ASNase. The organisms were cultivated in L-asparagine medium to induce the production of the enzyme. The activity of the enzyme was assayed after 3, 4 and 6 days of incubation, as shown in Figure 1. It can be seen that *Aspergillus versicolor* was the superior to produce the enzyme where the maximum activity (45.53 U/mL) was achieved after 3 days of incubation at 28 °C and a great drop of the activity was obtained after 5 days of incubation followed by *Paecilomyces lilacinus* which exhibited a moderate enzyme activity (20.03 U/mL) after 3 days and lost 50% of the activity after 6 days of incubation. The fungus was identified on the basis of a molecular level by the use of 18 S rRNA (Ribonucleic acid) sequencing technique. The phylogenetic tree confirmed that the active strain was most closely related to *Aspergillus versicolor* strain KY908307.1, the similarity of the sequencing and multiple alignments confirmed that the fungus was in close relation to *Aspergillus versicolor* strain KY908307.1 (99% identity).

3.2. Immobilization and characterization of the produced L-ASNase.

The culture filtrate of *Aspergillus versicolor* (1500 mL) showed 30.78 U/mg specific activity. After further purification using 80% ethanol, the partially purified enzyme showed 208.52 U/mg specific activity. It had purification fold of 6.77 with enzyme recovery 3.68 %. The partial purified enzyme was immobilized onto ox-MWCNTs using physical adsorption technique with immobilization yield about 54.4 %.

As previously mentioned by Haroun et al. [38] the particle size of the immobilized L-ASNase onto ox-MWCNTs dependent on the used immobilization technique. The immobilized...
L-ASNase had particle size about 51 and 79 nm in case of the covalent and physical techniques, respectively. Free and immobilized L-ASNase were exposed to different pH values to cover the range from 3.0 to 9 using citrate buffer (pH 3-5), phosphate buffer (pH 6-7) and Tris-buffer (pH 8-9), as shown in Figure 1.

**Figure 1.** Screening of different strains of fungi for production of L-ASNase.

It was observed that both partially purified and immobilized L-ASNase exhibited maximum activity at pH 8. Moreover, the activity of the immobilized enzyme was less affected by the raise of pH than that of the free one. This clearly reflects the suitability of ox-MWCNTs as a carrier for this enzyme. Our results are in agreement with that of Tabandeh and Aminlari, [39] who reported that the optimum pH of modified L-ASNase and the native enzyme was at alkaline pH (pH 8) probably due to the produced L-aspartic acid which acts as a competitive inhibitor for enzyme in acidic condition. Both free and immobilized L-ASNase were exposed to a wide range of temperatures from 20–50°C, as shown in Figure 3. The maximum L-ASNase activity for both was obtained at 40°C. By increasing the temperature, the activity was gradually decreased. The immobilized enzyme retained 69% of its activity at 50°C while the free one retained 50.8%. Our results were in agreement with those obtained by Dutta et al. [40] who found that the optimum temperature of *Aspergillus fumigates* L-asparaginase was at 50°C.

**Figure 2.** Effect of pH on the relative activity of the immobilized L-ASNase relative to the free one.

Thermal stability of the immobilized L-ASNase was examined relative to the free one, as recorded in Table 1. It was observed that by elevating the temperature from 30°C to 60°C, the immobilized enzyme exhibited complete relative activity (100%) at different incubation times (15, 30 and 60 min). While heating the free enzyme at 45°C caused a loss of about 10, 13 and 19% of its activity at the same times, respectively. While, at 50°C, the activity of the immobilized enzyme started to decrease to about 7, 10 and 12% in comparison with the free one (24, 34 and 43 %) after the same incubation times, respectively. After 15 min of incubation at 60°C, the lost in the activity of the immobilized enzyme reached only the value (25.5 %) relative to that in case of the free one (84.5 %). After 1 hr, the activity of the immobilized enzyme was decreased to about 48% relative to that in case of the free one (4.04%). In other words, at the end of incubation, the relative activity of the immobilized enzyme was decreased to about 52% relative to that in case of free one (96%).

**Figure 3.** Effect of assay temperature on the immobilized L-ASNase activity relative to the free one.

It can be concluded that using ox-MWCNTs for enzyme immobilization for protection against deformation and denaturation processes by elevating temperature at different periods of time was significant. Our results were in accordance with that previously recorded by some researchers [41, 42] who mentioned that the maximum activity of the enzyme was at 30°C. The immobilized L-ASNase and free enzyme were stored with the substrate at 4°C for 4 weeks and the activity was calculated after each week. It can be concluded from the previous data that the storage process played a significant role in the activation of the immobilized enzyme by releasing the embedded enzyme from the carrier (ox-MWCNTs). The activity of the immobilized L-ASNase was increased 4 times (51.18 U/g) than that in case of the zero time.

**Table 1.** Relative L-ASNase activity (%) of the immobilized L-ASNase onto ox-MWCNTs at different temperatures relative to the free one.

| Temp °C | Free L-ASNase | Immobilized L-ASNase |
|---------|---------------|----------------------|
| 15 min  | 100           | 100                  |
| 30 min  | 100           | 100                  |
| 60 min  | 100           | 100                  |
| 15 min  | 100           | 100                  |
| 30 min  | 100           | 100                  |
| 60 min  | 100           | 100                  |

3.3. Kinetics study.

The data of temperature were recorded in the form of Arrhenius plots, as shown in Figure 4. Linear plots were drawn for the free and immobilized L-ASNase. The calculated activation energies (Ea) were about 4.363 and 3.015 Kcal/mol for immobilized and free L-ASNase, respectively. Similar results were previously obtained by El-Shora et al. [43] who found that the calculated activation energies were 43.2 and 30.8 KJ/mol for free and immobilized *Alternaria infectoria* chitinase, respectively. Log relative activity at the high temperatures (50, 55 and 60°C) was plotted against time, as shown in Figure 5 (A and B). The straight lines of the free and immobilized L-ASNase indicated that the thermal inactivation process for both free and immobilized enzyme is of the first order reaction. The values of the half-life were calculated as presented in Table 2.
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It was shown that the immobilized enzyme was more stable than the free one. The calculated half-life of the immobilized enzyme was 1155, 521.05 and 173.25 min, respectively at 50, 55 and 60°C, respectively, which were higher than those of the free one (173.25, 130.75 and 50.58 min, respectively). Moreover, the results of the deactivation constant rate which is the slope of the relative activity plot at high temperatures (50, 55 and 60°C). The immobilized enzyme was highly stable (6×10^{-4} and 4×10^{-3}) at 50 and 60°C, respectively, than the free one.

Different concentrations of L-Asparagine concentrations (0.02-0.12) M were prepared in the reaction mixture and incubated at 40°C for 30 min., the specific activity of the free and immobilized enzyme were calculated as shown in Figure 6. The potent relative activities of both free and immobilized enzyme were obtained at 0.04 M of asparagine and a slight decrease in activity in both formulations happened when 0.06 M asparagine was used. A gradual decrease in activity was shown by increasing the substrate concentrations from 0.08 to 0.12 M. The initial velocity was calculated as a function of substrate concentration in accordance with Lineweaver-Burk analysis (Figure 7 a and b). The calculated Michaelis constant Km and Vmax were about 0.02 M and 50 U/mg protein, respectively, for free enzyme. While, in case of the immobilized one, Km and Vmax were about 0.045 M and 90.90 U/mg, respectively suggesting that the enzyme diffusion resistance is strong.

**Figure 6.** Effect of L-asparagine substrate on the relative activity of the immobilized L-ASNase relative to the free one.

**Figure 7.** Lineweaver-Burk plot of (A) free L-ASNase and (B) immobilized L-ASNase.

3.4. Toxicity evaluation.

Figure 8 shows *in vitro* cytotoxicity of the immobilized L-ASNase onto ox-MWCNTs against BHK-21 normal cell line using SRB assay in comparison with ox-MWCNTs and the free enzyme. It can be seen that in case of the free L-ASNase and ox-MWCNTs, the cytotoxicity was gradually increased with an increase of the concentrations from 0 to 100 μg/mL. While, the immobilized L-ASNase exhibited steady state profile after 50 μg/mL concentration. In other words, the free enzyme had high cytotoxicity relative to ox-MWCNTs and immobilized one. It can be concluded that using ox-MWCNTs as a carrier for L-ASNase immobilization could be significantly retarded the cytotoxicity against BHK-21 normal cell line using SRB assay.

**Figure 8.** *In vitro* cytotoxicity of the immobilized L-ASNase onto ox-MWCNTs against BHK-21 normal cell line using SRB assay in comparison with ox-MWCNTs and free enzyme.

As previously mentioned that L-ASNase is commonly used for acute lymphoblastic leukemia (ALL), remission induction and intensification treatment in all pediatric regimens as well as in the majority of adult protocols [44]. In contrast, the adverse effects of
L-ASNase on the liver and pancreas of children and adult were reported in many patients. The biochemical biomarkers such as AST, ALT, LDH, lipase and alpha-amyrase were determined in the sera of the male mice, as shown in Figure 9. Treatment of mice with ox-MWCNTs had no to a low detectable significant effect on the activity of all the determined biochemical parameters relative to the control group.

Moreover, mice received the free L-ASNase showed highly significant increase in the activity of the aforementioned enzymes in comparison with the untreated ones. On the other hand, the immobilized L-ASNase onto ox-MWCNTs alleviated its harmful effect on the activities of the above measured biomarkers and maintained the activities or level close to near in comparison with the untreated ones.

Table 2. Kinetic parameters of the immobilized L-ASNase at different assay temperatures relative to the free one.

| Kinetic Parameters | Free L-ASNase | Immobilized L-ASNase |
|--------------------|---------------|----------------------|
| Activation Energy (Kcal/mol) | 3.015 | 4.36 |
| Assay temperatures (°C) | 50 | 55 | 60 |
| 173.25 | 130.75 | 50.58 | 115 | 521.05 | 173.25 |
| Deactivation rate constant | 4.0*10^{-3} | 5.3*10^{-3} | 1.37*10^{-2} | 6.0*10^{-4} | 1.33*10^{-1} | 4.0*10^{-3} |

4. CONCLUSION

L-ASNase can be isolated and produced from Aspergillus versicolor with max activity about 45.5 % U/mL. The partial purified L-ASNase was successfully immobilized onto ox-MWCNTs using physical adsorption technique with maximum yield about 54.4%. Using of ox-MWCNTs as a carrier could be protected the produced L-ASNase against deformation and denaturation effects by elevating temperature at different periods of time. Also, the storage process played a significant role in activation of the immobilized L-ASNase by releasing the adsorbed particles after time. On the other hand, ox-MWCNTs could also be reduced the adverse toxic effects of L-ASNase on both liver and pancreas of the treated male mice as well as against BHK-21 normal cell line using SRB assay.

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