Iranian Journal of Fisheries Sciences 20(6) 1804-1821 2021
DOI: 10.22092/ijfs.2021.125502

Research Article

Comparison of glutaraldehyde cross linking versus direct Schiff base reaction for conjugation of L-asparaginase to nano-chitosan and improvement of enzyme physicochemical properties

Khounmirzaie N.[1]; Razijalali M.[1]; Shahriari A.[2*]; Tabandeh M.R.[2]

Received: July 2021 Accepted: September 2021

Abstract

The bacterial L-asparaginase (ASNase) has been used in the treatment of asparagine-associated tumors; however, the instability of the enzyme increases the number of injections as well as the side effects. In the present study, ASNase was conjugated to nanochitosan (ASNase -CSNPS) by direct (shiff-base) and indirect (glutaraldehyde linker) methods. In order to get the optimal conjugation, ASNase/CSNPS ratio was first investigated. The physicochemical properties (optimum pH, temperature, residual activity), enzyme kinetics (Michaelis constants; Km and maximal velocity; Vmax) and stability (against freezing, proteolysis, and chemical denaturation) were determined. The results showed that the highest residual enzyme activity (>85%) was obtained using a combination of ASNase and CSNPS at 1:5 mass ratio in both conjugation methods. ASNase -CSNPS prepared by glutaraldehyde linker had higher Km and Vmax values (69.7 μM, 20.6 mol/mL/minμ), wider range of optimum pH and higher temperature stability compared to ASNase-CSNPS produced by Schiff-base (Km: 105.8 μM, Vmax 14.5 mol/ml/minμ) method. ASNase-CSNPS produced by indirect method had more stability against freezing-thawing, and proteolysis when compared with ASNase-CSNPS prepared using direct method. The results showed that application of glutaraldehyde coupling was superior to Schiff base cross linking for conjugating of ASNase to CSNPS and for production of ASNase with better physicochemical properties for future cancer therapy.

Keywords: L-Asparaginase, Nanochitosan, Glutaraldehyde linker, Schiff base reaction, physicochemical properties

1-Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
2-Department of Basic Sciences, Division of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
*Corresponding author's Email: a.shahriari@scu.ac.ir

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

Introduction

In the past decades, the enzyme-therapy has been expanded and many enzymes have been approved as drugs and entered the pharmaceutical market, such as the enzymes with anticancer properties including arginine deaminase (Vellard *et al.*, 2003; Synakiewicz *et al.*, 2014), L-asparaginase (ASNase) (Tabandeh and Aminlari, 2009) and chondroitinase (Moon *et al.*, 2003). ASNase is an enzyme that breaks asparagine amino acid into the aspartic acid and ammonia. This enzyme which is obtained from *Erwinia carotovora* and *Escherichia coli* has been used in the treatment of asparagine-associated tumors, and in particular, acute lymphocytic leukemia (ALL) in children and adults (Aguayo *et al.*, 1999; Hawkins *et al.*, 2004). More recently, researchers also showed that treatment with ASNase can reduce metastasis of breast cancer (Knott *et al.*, 2018). Since the tumor cells do not have the ability to synthesize asparagine, ASNase significantly reduces the access of tumor cells to asparagine, thereby reducing the biosynthesis of the protein in these cells and stopping the cell division in the G1 stage. Natural cells are not affected due to their ability in biosynthesis of asparagine. On the other hand, the products of enzyme activity in the cell, especially ammonium ion, increase the pH and consequently lead to apoptosis in the treated cells (Aguayo *et al.*, 1999; Hawkins *et al.*, 2004).

The major clinical and dose-limiting toxicity of ASPase therapy is the development of acute hypersensitivity reactions shortly after administration of drug. ASNase has a short half-life in the serum and *frequent* need of *injections* increases the risk of hypersensitivity reactions. Conjugation of ASNase to natural or synthetic polymers has been extensively studied as the best available option to minimize hypersensitivity reactions of ASNase. The numerous conjugated forms of ASNase are produced for improving of immunogenicity and half-life of native ASNase. The PEGylated form of the ASNase (Pegaspargase) with longer half-life have been approved by FDA and the European Medicine Agency (EMA) for treatment of children and adults with newly diagnosed (Meneguetti *et al.*, 2019). Nanotechnology has made it possible to stabilize and integrate therapeutic proteins with a variety of nano-polymers. The stabilization of therapeutic proteins on nano-polymers generally intended to increase the stability of protein in the body and, stability to pH and temperature, as well as resistance to proteases and other denaturing compounds. The therapeutic efficacy and physichochemical properties of ASNase have been improved by application of various nanocompound such as poly(lactic-co-glycolic) nanoliposomes (Wolf *et al.*, 2003), hydrogel nanoparticles (Teodor *et al.*, 2009; Do *et al*., 2019) and hybrid nonoflowers (Noma *et al*., 2021).

Chitosan (poly [β-(1–4)-2-amino-2-deoxy-d-glucopyronose), is a natural polymer, resulting from full or partial

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

N-deacetylation of chitin under alkaline condition (Tikhonov *et al.*, 2006). The cuticle of crustaceans such as shrimp is reach in chitosan, thus, can be a cheap source of chitosan for the industry that are involved in the chemistry and biochemistry processes. Because of its excellent film forming ability, biocompatibility, nontoxicity, high mechanical strength, cheapness and a susceptibility to chemical modifications, chitosan has been extensively used for immobilization of various enzymes such as glucoamylase, lipase and trypsin. It had been observed that performance of enzyme immobilization onto chitosan nanoparticles (CNPs) is higher than those immobilized onto chitosan microparticles (Cheung *et al.*, 2015, Ribeiro *et al.*, 2021).

Several methods such as physical adsorption, entrapment (encapsulation) and cross linking by covalent bound are used for enzymes immobilization on nano materials. Covalent bonding is one of the most widely used methods for enzymes immobilization because the stability of the bonds formed between the enzyme and nano materials, which prevents enzyme release into the environment (Sheldon *et al*., 2021). The design of cross linking by covalent bound for enzyme immobilization has a great impact on the performance and physicochemical properties of the enzyme. To date, numerous methods of enzyme immobilization are available but the effectiveness of each one depends upon reaction conditions, process of product formation, and its cost evaluation. The use of glutaraldehyde cross linking and Shiff base reaction are the most frequently used techniques for covalent enzyme immobilization (Li *et al.*, 2013; Masarudin *et al.*, 2015).

Despite of the studies conducted on the encapsulation or immobilization of ASNase on CNPs for improvement of its half-life and therapeutic efficiency, there is no report comparing the performance of ASNase-CNPs conjugates produced by glutaraldehyde cross linking versus Shiff base reaction. In the present study, ASNase-CNPs was produced by using glutaraldehyde cross linking and Shiff base strategies and their physicochemical properties, enzyme kinetics and stability were compared.

Materials and methods

Asparaginase assay

The activity of ASNase (Kidrolas^{R}, Jazz Pharmaceuticals, and France) was measured by modified method of Wriston (1970). The enzyme assay mixture consisted of 900 μL of freshly prepared L-asparagine (20mM) in 50mM Tris-HCl buffer (pH 8.0), 50 mM KCl, and 100 μL of ASNase (1mg/mL in PBS). The reaction mixture incubated at 37°C for 30 min and the reaction stopped by adding 100 μL of 15% trichloroacetic acid. The reaction mixture was centrifuged at 10,000 ×g for 5 min at 4°C to remove the precipitates. The ammonia released in the supernatant was determined using colorimetric technique by adding 100 μL Nessler's reagent into the sample

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

containing 100 μL supernatant and 800 μL distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min and OD was measured at 425 nm. The ammonia produced in the reaction was determined based on the standard curve obtained with ammonium sulfate. One unit of L-asparaginase activity is defined as the amount of the enzyme that liberates 1 μmol of ammonia per min at 37°C. The enzyme activity expressed as IU/mg protein. Protein concentration was measured using Bradford method.

Preparation of nano-chitosan

CNPs was prepared by using ionic gelation method as reported by Masarudin *et al*. (2015). Chitosan (30 kDa) with 85% deacetylation (Sigma, USA) was dissolved (5 mg/mL) in 1% acetic acid solution and pH of the sample was adjusted to 6.0 by adding 1 M aqueous sodium hydroxide solution. Tripolyphosphate (TPP) was dissolved in distilled water to make a concentration of 0.7 mg/mL and pH adjusted to 2.0 by using 1.0 M hydrochloric acid. CNPs was formed by adding 600 µL of CS to 250 µL of TPP solution. The sample was mixed on magnetic stirrer at room temperature for 15 min till obtaining clear solution. Then the mixture was centrifuged at 10000 rpm for 15 min to purify it. The sample containing CNPs was powdered using lyophilizer (Christ Alpha1-2 LD plus, Germany) for evaluation of physicochemical properties.

Preparation of activated CNPs

Glutaraldehyde activated CNPs (GA-CNPs) was synthesized by dissolving 1.0 g of chitosan into 25.0 mL of 1.0% acetic acid and then adding 15.0 mL 1% glutaraldehyde into the chitosan solution to form a water gel after 30 min of stirring at room temperature by using a magnetic stirrer. To remove the non cross-linked glutaraldehyde the samples were washed out for more than five times by double distilled water and desalted using NAP-5 column (NAP-5 Healthcare, USA) in sodium phosphate buffer (5 mM) at pH 5 for 1 h (Adriano *et al.*, 2005). The samples were powdered using the lyophilizer (Christ Alpha1-2 LD plus, Germany) and used for subsequent evaluation (Li *et al.*, 2013).

Immobilization of L-asparaginase on CNPs or GA- CNPs

ASNase (Kidrolas^{R}, Jazz Pharmaceuticals, France) enzyme solution (10 mg/mL) was prepared in 0.1 M phosphate buffer at pH 8.0. ASNase solution (1 mL) was mixed with 1 mL CNPs or GA-CNPs at different ASNase/CNPs weight ratio (1:2, 1:5, 1:10, 1:20 w/w). For conjugation of ASNase to GA-CNPs, the preparation kept under gentle stirring at 28°C for 22 h. For conjugation of ASNase to CNPs 10 µL, sodium cyanoborohydride (final concentration 50 mM) was added and stirred on the magnetic stirrer at 28˚ C for 4 h. To stop the reaction, 50 µL of Tris 1 M, pH 7.4 was added and maintained at room temperature for 30

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

min. The samples were then dialyzed in PBS and concentrated to 50-fold by using Vivaspin® ultrafiltration (Sartorious, USA) system and were used for further evaluation.

Characterization of CNPs

The size and morphologies of CNPs and ASNase-CNPs confirmed using a transmission electron microscopy (TEM) (Philips M20 Ultra Twin). For this purpose, the suspension of CNPs or ASNase-CNPs was prepared (100 ppm) and sonicated for 90 s. To prepare TEM images, the samples (10 µL) were placed on carbon-coated grids (300-mesh, Ted Pella, Inc., Redding, CA, USA) and air dried for 10 min. The plates placed on TEM with a voltage of 200 Kv. The average particle diameter was calculated by counting at least 100 particles and using the Paxit software.

Evaluation of the loading yield

The efficiency of conjugation methods was estimated by calculating the residual specific ASNase activities before (At_0) and after (At_t) conjugation using the following formula:

$$IY\% = \frac{At_0 - At_t}{At_0} \times 100$$

Evaluation of ASNase-CNPs

The SDS-PAGE was used to evaluate the accuracy of conjugation procedure. Briefly, 10 µg of ASNase-CNPs and ASNase were mixed with 10 µl of sample buffer and boiled for 5 min. Electrophoresis was performed on 10% SDSPAGE condition using electrophoresis instrument (Paya Pjohesh Pars, Iran) with 100 mA for 1 h. The gel stained with Coomassie Blue R-250 (Sigma, St. Louis, MO). The change in mobility shift of conjugated ASNase represented the efficiency of conjugation.

Determination of enzyme kinetics

Michaelis constants (Km) and maximum velocity (Vmax) of conjugated and non-conjugated enzymes were calculated from the Lineweaver–Burk plots of enzyme activity vs. L-asparagine concentrations 10–100 µM in 0.05 M phosphate buffer, and pH 8.0. All analyzes were performed with three replications.

Determination of optimum temperature and pH

The activity of conjugated and non-conjugated enzymes was evaluated in the presence of constant concentration of L-asparagine (0.01 M) at different temperatures 25, 35, 45, 55, 65 and 70°C. To determine the optimum pH, the activities of all enzyme preparations in the presence of a constant concentration of substrate (0.01 M) and in buffers with different pHs, including 0.05 M acetate buffer, pH 4-5, 0.05 M HEPS buffer, pH 6-8, and 0.05 M Tris buffer, pH 9-11 were measured. All experiments performed with three replications.

The temperature and pH with the highest enzyme activity considered as 100% activity and the enzyme activity in other conditions reported as a proportion of 100%.

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

Evaluation of half-life and stability of enzymes

In order to evaluate the enzyme half-life under environmental conditions, an enzyme solution with the activity of about 500 IU/mL was prepared in PBS and kept at ambient temperature. The residual enzyme activity was evaluated after 10 to 70 h. Results were expressed as % in relation to condition with the highest enzyme activity as 100%.

In order to evaluate the enzyme's stability against proteolysis, 500 IU/mL of the enzyme in PBS was mixed with 50 IU trypsin, and the residual enzyme activity was evaluated 5 to 30 min after proteolysis. All experiments performed with three replications. Results were expressed as % in relation to condition with the highest enzyme activity as 100%.

To determine the stability of all prepared enzymes during freezing and thawing, the enzyme solution containing 500 IU/mL of ASNase and 50 mg/mL mannitol in PBS was frozen at -20˚ C and then melted after 2-24 h. After the above-mentioned periods, the residual enzyme activity was evaluated. Results were expressed as % in relation to condition with the highest enzyme activity as 100%.

Conjugated and nonconjugated forms of ASNase (500 IU/mL in PBS) was exposed to guanidine hydrochloride (0.5 to 5 mM) as chemical denaturant for 0.5 to 5 h and then the residual enzyme activity was evaluated. Results were expressed as % in relation to condition with the highest enzyme activity as 100%.

Data analysis

All the experiments were performed in triplicate and data represent average ± standard deviation (SD). Statistical analysis was done with one-way ANOVA and p value < 0.05 was considered statistically significant.

Results

Characteristics of CNPs and GA-CNPs

Figure 1 shows the TEM images of CSNPs and ASNase-CNPs. It was observed that both CSNPs and ASNase-CNPs are spherical and exist as discrete spheres. The particle size distribution varied from 68.4 nm to 252.7 nm and the mean diameters of the CSNPs and GA-CNPs were 129.6 and 178.3 nm, respectively.

Efficacy of ASNase conjugation

To determine the best ratio of ASNase/CNPs for production of conjugated enzyme with the highest activity, the residual enzyme activities were determined at different ASNase/CNPs ratios. As shown in Fig 2A, the maximum residual activities of both conjugated forms of ASNase was archived at 1:5 ratio (Glutaraldehyde cross linking; 77.1±3.9%, Direct Schiff base; 60.2%±2.8 versus vs unconjugated ASNase), and the enzymes obtained from this ratio was selected as a suitable ratio and used for subsequent experiments (Fig. 2A). Loading yield for glutaraldehyde linker and Shiff base conjugation methods at ratio of ASNase/CNPs were 89.4 and 81.3, respectively (Fig. 2B). To determine the efficacy of conjugation,

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

electrophoresis was performed on 10% SDS-PAGE condition. The change in mobility shift of conjugated ASNase represented the efficiency of conjugation (Fig. 2C).

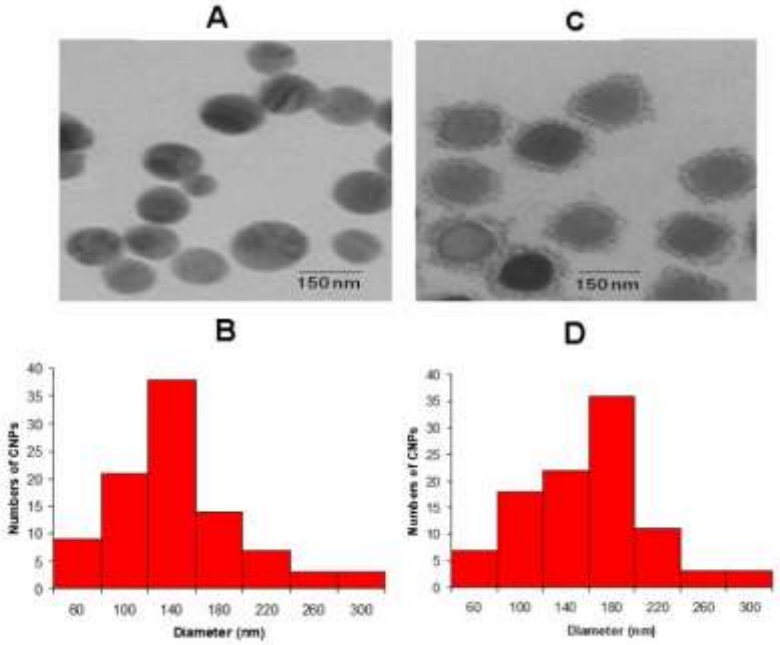

Figure 1: TEM images and particle size distribution histogram of chitosan nanoparticels (CNPs) (A, B) and glutaraldehyde linked ASNase-CNPs (C, D). The average particle diameter was calculated by counting at least 100 particles using the Paxit software.

Kinetic parameters of conjugated ASNase

In order to determine the Km and Vmax of the enzymes, Lineweaver–Burk plot was constructed using the inverse changes of the various concentrations of the substrate ($\frac{1}{[S]}$) (20-100 µM) against the changes of inverted initial velocity ($\frac{1}{[V]}$). The Km and Vmax values extracted for the native enzyme were 189.5 µM and 13.2 µmol/mL/min, respectively (Fig 3A-C). The Km and Vmax values of the enzyme conjugated by direct Schiff base method were 105.8 µM and 14.5 µmol/mL/min, and for the enzyme conjugated using glutaraldehyde crosslinking were 69.7 µM and 20.6 µmol/mL/min, respectively. Our results showed that the Km values of the enzymes conjugated to CNPs by both methods were lower than the native enzyme, indicating an increase in the affinity of the enzymes to substrate following its conjugation with CNPs. The enzyme conjugated to CNPs by glutaraldehyde linker showed lower Km compared to the enzyme conjugated by *direct Schiff base* method. Moreover, it was found that the Vmax of the enzyme conjugated by glutaraldehyde linker (Vmax=20.6) was more than that in native enzyme (Vmax=13.2) and in the enzyme conjugated by direct *Schiff base* method (Vmax=14.5) (Fig. 3A-C).

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

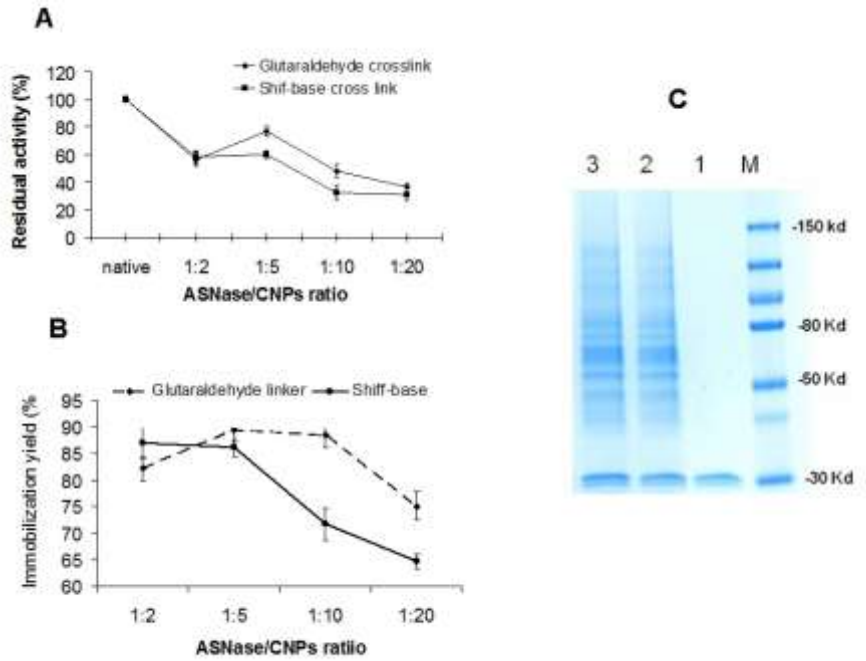

Figure 2: A: The residual enzyme activity of ASNase after conjugation to CNPs or GA-CNPs at different mass ratios in comparison to native enzyme (100% activity). The highest activity of the enzyme observed in the ratio of 1:5 and this ratio used for subsequent experiments. B: Loading yield for cross linking of ASNase to CNPS and GA-CNPs. C: SDS-PAGE (10%) of native (lanes 1), ASNase-CNPs (lane 2) and ASNase-GA-CNPs (lane 3). Lane1: protein marker. Each lane was loaded with 20 µg of the protein solution; the gel stained with Coomassie brilliant blue G-250. Band distribution indicates the conjugation of CNPs or GA-CNPs to the enzyme.

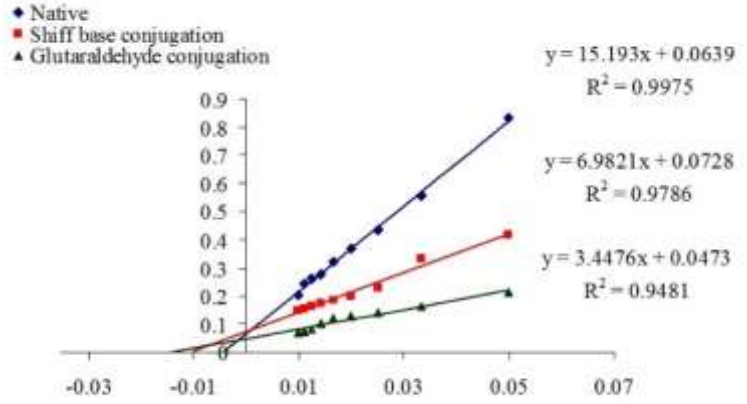

Figure 3: Double reciprocal Lineweaver-Burk plots for native and CNPs conjugated ASNase by Shiff-base or glutaraldehyde linker method. Reverse values of different concentrations of the asparagine as substrate (20, 30, 40, 50, 60, 70, 80, 90 and 100 mM) against the inverse velocity changes (µmol mL^{-1} min^{-1}) for the native and CNPs conjugated ASNase by Shif-base or glutaraldehyde linker methods.

Optimal pH and temperature of conjugated ASNase

The residual activities of native and conjugated ASNase at different pHs and temperatures are shown in Figure 4. It was found that the maximum activities of native and conjugated enzymes was at pH 8. ASNase that conjugated to

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

CNPs using glutaraldehyde crosslinking was active in a wider range of pH (6-9) than ASNase that conjugated using direct Schiff base method and native ASNase (Fig. 4A).

The temperature dependence of the ASNase activity ranged between 25 to 55°C. The maximum activities of conjugated and native enzymes observed at 45°C (Fig. 4B). A significant decrease in the activity was observed above these temperatures. However, the decrease of reaction rate at temperatures above the optimum was much slower than that of the native ASNase. Conjugated enzymes exhibited high activity at a wider temperature range (35-55°C), so that the conjugated enzymes maintained the highest enzyme activity (80%) at 55°C, whereas the highest activity of non-conjugated enzyme was 40% at this temperature. ASNase that conjugated to CNPs using glutaraldehyde crosslinking was more stable at temperature between 35-55° C than ASNase that conjugated using direct Schiff base method (Fig. 4B).

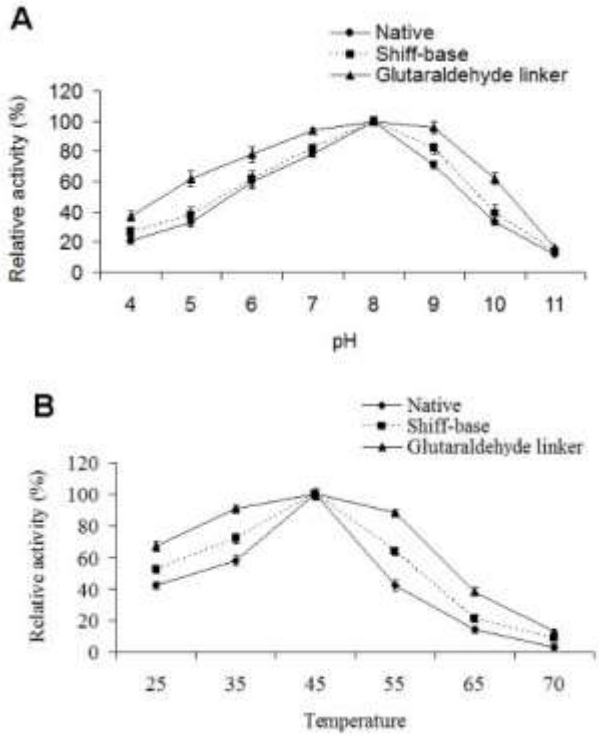

Figure 4: The activity of native and CNPs conjugated ASNase at different pHs (4-11) (A) and temperatures (25-70 °C) (B). Enzymes incubated for 1 h at the indicated temperatures. The enzyme activity was measured at 37 °C in different buffers with pH values ranging from 4 to 11. The highest activity considered as 100% and the residual activity at different conditions expressed as a ratio of 100%. The error bars represent the SD of the mean calculated for 3 replicates.

Evaluation of enzyme stability during reuse, proteolysis and freezing

To evaluate the environmental half-life, enzymes maintained at room temperature for 10 to 70 h and their residual activity was evaluated. The results showed that stability of ASNase during reuse increased after conjugation

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

to CNPs. The half-life of ASNase/CNPs conjugated by glutaraldehyde linker (60 h) was more than the native enzyme (20 h) and enzyme that conjugated by direct Schiff base method (50 h) (Fig. 5A). According to Fig 5B, evaluation of the enzyme's stability against proteolysis showed that conjugation of the ASNase to CNPs by glutaraldehyde linker led to higher stability of the enzyme compared to the other conjugated form of enzymes. ASNase/CNPs conjugated by glutaraldehyde linker retained >80% of initial activity 15 min after proteolysis, while ASNase/CNPs conjugated by direct Schiff base method and native enzyme retained 27% and 42% of initial activity 15 min after proteolysis, respectively (Fig. 5B). Our results indicated that the conjugated enzymes showed more resistance to freezing compared to the native enzyme. ASNase/CNPs conjugated by glutaraldehyde linker lost about nearly 20% of its activity 24 h after freezing; while ASNase/CNPs conjugated by direct Schiff base method and native enzyme lost about 30% and 42% of their activities 24 h after freezing (Fig. 5C).

The activity measurements of free and conjugated ASNase in aqueous solutions in the presence of guanidine hydrochloride are reported in Figure 5D. The results showed that IC_{50} of the enzyme conjugated by glutaraldehyde (3.5 mM) was more than that of conjugated by *Schiff base* method (3mM) and non-conjugated enzyme (2.5mM) (Fig. 5D). A second important observation from Figure 5D is that, in comparison with free lysozyme, the ASNase-CNPs showed a lower loss of activity for all guanidine hydrochloride concentrations.

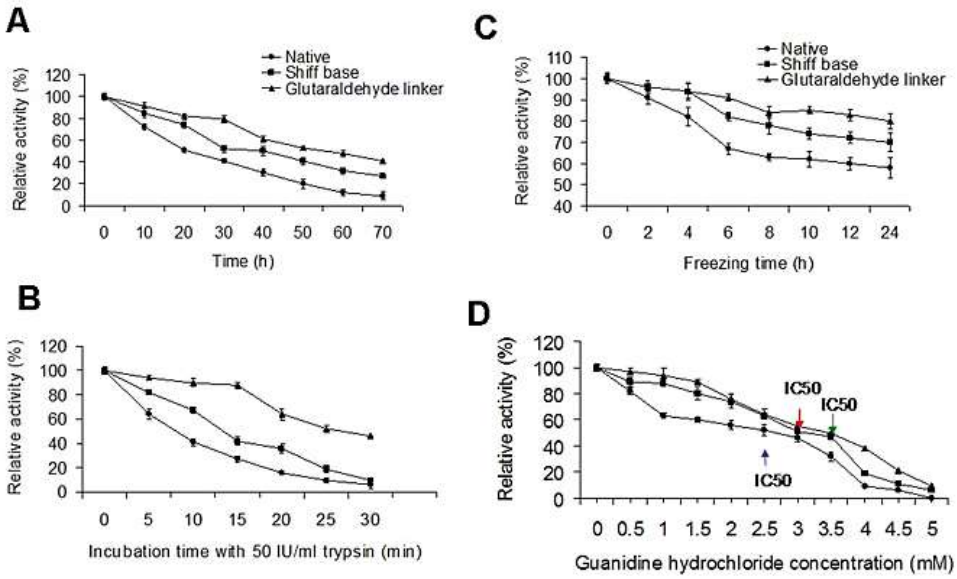

Figure 5: The stability of native and CNPs conjugated ASNase at room temperature from 10 to 70 h (half-life) (A), after digestion with 50 IU/ ml trypsin from 5 to 30 min (B), following freezing for 2 to 24 h and defrost at 37° C (C) and after exposure to guanidine hydrochloride (0.5 to 5 Mm) as denaturant IC50 (D). IC50 is the concentration of the denaturant that inhibits the activity of the enzyme by 50%. The highest activity considered as 100% and the residual activity at different conditions expressed as a ratio of 100%. The error bars represent the SD of the mean calculated for 3 replicates.

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

Discussion

Chitosan as a natural source of crustaceans such as shrimp is a biocompatible and biodegradable polymer, has been widely tested in a variety of fields for developing biocompatible protein drugs (Herdiana *et al.*, 2021). Its application in aquafeed as a part of additives can enhance the immune system of aquatic organisms including shrimp and fish. Additionally, substantial efforts have been devoted for the development and application of chitosan nanoparticles as vehicles for drug delivery (Cho *et al.*, 2010) as well as an immunostimulatory substance in aquaculture sector. Different methods have been developed for immobilization of enzymes on nanochitosan, but limited data are available about the comparative efficiency of various methods on stability and physicochemical properties of immobilized enzymes. In the present study, ASNase conjugated to CNPs by using Shiff base reaction and glutaraldehyde linker and stability, enzyme kinetic parameters and physicochemical properties of conjugated enzymes compared to each other.

In this study, different ratios of the enzyme conjugated with nano-chitosan used to achieve the best ratio that would maintain the activity of the enzyme. The results of this study showed that in the ratio of 1:5 (enzyme : nano-chitosan), more than 80% of enzyme activity was retained compared to free enzyme, while in ratios higher than 1:5, more than 60% of the enzyme activity was lost. Lower concentration of CNPs may be insufficient to give adequate rigidity and molecular crowding to enhance stability of conjugate, whereas higher concentrations may give more rigidity and block the active sites of enzyme causing decreased activity and stability.

Studies show that low concentrations of carbohydrate polymer, in spite of protein binding, have no effect on reducing the protein's flexibility and stability in the presence of unstable agents, while high concentrations of carbohydrate polymers by increasing non-invasive structures in the protein and cover the active site decrease the activity of the enzyme (Tabandeh and Aminlari, 2009; Sukhoverkov and Kudryashova, 2015).

Research on the conjugation of oxidized levan polymer to the asparaginase enzyme has shown that decreasing enzyme activity will not be noticeable if 20-15 amino acid groups of the enzyme react with the polymer (Výna *et al.*, 2001). In the study of (Tabandeh and Aminlari 2009), conjugation of inulin polysaccharide to asparaginase with ratio of 1:2 led to maintaining enzyme activity up to 67%, while residual enzyme activity was reported to be 30% when the ratio of 1:4 was used (Tabandeh and Aminlari,. 2009). Similar results had been obtained for the conjugation of various synthetic and natural polymers such as dextran sulfate, silk fibroin and sericin, and polyethylene glycol into the asparaginase enzyme (Karsakevich *et al.*, 1986; Zhang *et al.*, 2004; Zhang *et*

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

al., 2005). Moreover, in order to conjugate more polymeric units to the enzyme, it was necessary that the conjugation process performed at a higher pH to create the highest charged amino groups on the enzyme, which would increase the probability of degradation of the three-dimensional structure of the enzyme. According to the above, the production of asparaginase conjugated with nano-chitosan-glutaraldehyde is recommended with the ratio 1:5 at pH 8.

The SDS-PAGE results indicated the formation of protein bands in the range of 40 to 150 kD. This finding, along with the removal of the protein band of the natural enzyme, with an approximate molecular weight of 30 kD, indicates binding variable number of nano-chitosan groups to the enzyme and decreasing the electrophoretic mobility of the conjugated enzyme on the gel.

Kinetic parameters analysis showed that the Km of enzymes conjugated to nano-chitosan by *Schiff base* method (Km=105.8) and glutaraldehyde (Km=69. 7) at 37 °C was lower than the native enzyme (Km=189.5). This finding suggests an increase in the tendency of the enzyme to substrate following its conjugation into nano-chitosan. In addition, the enzyme conjugated to the nano-chitosan by glutaraldehyde linker showed an even lower Km compared to that by *Schiff base* method. The results of this study showed that the Vmax of the enzyme conjugated to nano-chitosan by glutaraldehyde linker was higher than that in native enzyme and the enzyme conjugated by *Schiff base* method. The Vmax of enzyme conjugated to nanocytosins by *Schiff base* method with ratio 1:5 was not different from the Vmax of native enzyme at 37 °C. It can be concluded that enzyme conjugation using the glutaraldehyde linker in comparison with *Schiff base* method could increase the tendency of the enzyme to substrate (reduction of Km) and enzymatic velocity, while in the *Schiff base* method, despite decreasing Km, the enzymatic reaction rate did not significantly change compared to native enzyme. The findings were consistent with those of the other studies, which used asparaginase enzymes conjugated to dextran sulfate, levan, and polyethylene glycol-chitosan (Karsakevich *et al.*, 1986; Tabandeh and Aminlari, 2009; Sukhoverkov and Kudryashova, 2015).

It is particularly important that enzymes conjugated to nanoparticles would be protected from denaturing, enzymatic degradation, or the general biochemical environment. Evaluation of enzyme activity at different temperatures and pH levels indicated that optimum enzyme activity was at 45°C and pH 8. However, the conjugated enzyme was active in a wider range of temperature and pH than non-conjugated enzymes. Conjugated enzyme was active more than 80% at pH 7 and 9, whereas the native enzyme activity in these pHs was less than 70%. Similar conditions were observed regarding the effect of temperature on

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

the conjugated enzyme, so that the conjugated enzyme had activity more than 70% at 55°C, while the non-conjugated enzyme activity was less than 40% at this temperature.

The results of this study showed that the half-life of the native enzyme was 20 h and the half-life of the conjugated enzyme was 50 h. Conjugated enzyme also showed high resistance to freezing, so that the conjugated enzyme retained 80% of the initial activity after 24 h of freezing and melting, whereas the non-conjugated enzyme showed up to 50% initial activity.

Research has shown that the binding of carbohydrate polymers such as dextran and polyethylene glycol to asparaginase with an appropriate ratio leads to resistance against degrading conditions such as high temperature and pH due to its high stability (Karsakevich *et al.*, 1986; Sukhoverkov and Kudryashova, 2015). In other words, the activation energy of protein folding decreases and the activation energy of unfolding increases in the conjugated enzyme. Another mechanism that could be considered for the greater stability of the conjugated enzyme under non-optimal conditions is the formation of enzymatic complexes mediated by the polysaccharides, which further protects the enzyme against structural changes induced by denaturation factors such as high temperature and pH (Výna *et al.*, 2001). To confirm this opinion, (Marlborough *et al.* (1975) showed that the asparaginase enzyme with 4 subunits has more activity and stability than the single subunit enzyme (Marlborough *et al.*, 1975; Vyna *et al.*, 2001). Furthermore, research by Miller *et al.* (1993) has shown that aspartic acid in the acidic pH with an inhibitory effect on the active site decreases the activity of the enzyme. It is hypothesized that the enzyme-linked carbohydrate chains reduce the inhibitory effect of aspartic acid in acidic conditions, and consequently, the enzyme is active in a wider range of pH (Miller *et al.*, 1993).

One of the main problems with the use of asparaginase in patients is low half-life of the enzyme due to the effect of serum proteases or reaction of enzyme with neutralizing antibodies (Soares *et al.*, 2002). The results of this study showed that the enzyme conjugated with nano-chitosan had more stability against trypsin digestion in vitro compared to non-conjugated enzyme. Our results showed that normal enzyme activity reached zero at the end of the 30-minute exposure to trypsin, while the conjugated enzyme still showed 40% of the initial activity under similar condition. Similar results had been reported to improve the enzyme's stability after the addition of other carbohydrate polymers. For instance, Qian *et al.* (1996) reported an increase in enzyme activity and resistance to trypsin digestion through the fixation of chitosan microspheres on the asparaginase enzyme. This finding can be due to the spatial inhibition of the carbohydrate chains from the access of proteolytic enzymes to the specific restriction sites or to the internal structure of the enzyme induced by the

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

intracellular interactions, resulting from the attachment of the nano-chitosan polymer to the protein in the enzyme structure (Qian *et al.*, 1996).

Recently, Bahreini *et al.* (2014) have used the polytriphosphate interface to conjugate chitosan to the asparaginazase enzyme. This study showed that decrease in the enzyme activity was more than that of the present study (Bahreini *et al.*, 2014). The conjugation of chitosan to proteins is performed in the acid range (pH=7.5), with the most positive amino acid groups on the chitosan surface. Polytriphosphate is highly negative charged in this pH and most of the groups in chitosan that are highly positive at this temperature are covered. The neutralization of charges on chitosan reduces the solubility and deposition of chitosan in the environment, which is associated with decreasing chitosan solubility and decreasing the enzyme stabilization (Bahreini *et al.*, 2014). In the present study, the formation of nano-chitosan, nano-chitosan-glutaraldehyde and conjugation of nano-chitosan to the enzyme were carried out in acidic pH (pH=6), and the charge of remaining amino acids of the nano-chitosan caused the appropriate solubility of the chitosan-enzyme complex, due to the relative coverage of amino acids.

In this study, two conjugation strategies were compared for attachments of the ASNase to the nanochitosan. Based on the previous studies, the best conjugation results achieved when glutaraldehyde at concentration of less than 5% was used to activate the chitosan. Similar results were obtained by other authors (Li *et al.*, 2013), who studied enzyme immobilization on nanochitosan. The residual activity obtained by those authors was around 70%, which is close to the residual activity observed in the present work.

When environmental stability and kinetic parameters were concerned, it can be seen that application of glutaraldehyde linker strategy had an obvious different effect on the obtained results, since this strategy could produce more stable enzyme with higher tendency to substrate. These changes in the kinetic parameters indicate that the binding of ASNase onto GA-CNPs resulted in change of affinity for the substrate. This may be due to conformational changes in the protein and moderately increased substrate access to the active site of the conjugated enzyme. Catalytic activity of the enzyme depends on the ionizable groups in its active site. The charges on these groups depend on their accessibilities to the environment in different pH ranges. Variations in the pH lead to the changes in the ionic form of the active site and, consequently, changes in the activity of the enzyme. It seems that conjugation of ASNase with GA-CNPs had more effects on total net charge of the enzymes, distribution of charge on their exterior surfaces, and reactivity of the active groups compared with ASNase that conjugated to CNPs using Shiff base method.

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

The results of this study showed that the conjugation of glutaraldehyde-activated nano-chitosan to the ASNase enzyme at a ratio of 1: 5 (enzyme to nanocytosine) improved the activity, kinetic parameters and stability of the ASNase enzyme. The method in this study can be used to produce a stable form of asparaginase enzyme with improved physicochemical properties for future chemotherapy. Because in the indirect method, the activity of enzymatic residues, stability against heat, activity in the pressure range and the tendency to bond to the substrate are better than the direct method, this method is recommended. Also, evaluation of other features of the enzyme produced by this method, especially immunogenicity assessment, should be assayed in future studies.

Acknowledgement

This work was funded by a Grant from Shahid Chamran University of Ahvaz Research Council (Grant No: 96/3/02/16670).

References

Adriano, W.S., Costa Filho, E.H., Silva, J.A. and Gonçalves, L.R.B., 2005. Stabilization of penicillin G acylase by immobilization on glutaraldehyde-activated chitosan. *Braz. J. Chem. Eng*., 22, 529-538. DOI: 10.1590/S0104-66322005000400005

Aguayo, A., Cortes, J., Thomas, D., Pierce, S., Keating, M. and Kantarjian, H., 1999. Combination therapy with methotrexate, vincristine, polyethylene- glycol conjugated- asparaginase, and prednisone in the treatment of patients with refractory or recurrent acute lymphoblastic leukemia. *Cancer*, 86(**7**), 1203-1209.

Bahreini, E., Aghaiypour, K., Abbasalipourkabir, R., Mokarram, A.R., Goodarzi, M.T. and Saidijam, M., 2014. Preparation and nanoencapsulation of l-asparaginase II in chitosan-tripolyphosphate nanoparticles and in vitro release study. *Nanoscale research letters*, 9(**1**), 340. DOI:10.1186/1556-276X-9-340

Cheung, R.C.F., Ng, T.B., Wong, J.H. and Chan, W.Y., 2015. Chitosan: an update on potential biomedical and pharmaceutical applications. *Marine drugs*, 14(**8**), 5156-5186. DOI:10.3390/md13085156

Cho, Y., Shi, R. and Borgens R.B., 2010. Chitosan nanoparticle-based neuronal membrane sealing and neuroprotection following acrolein-induced cell injury. *Journal of Biological Engineering*, 4(**2**). DOI:10.1186/1754-1611-4-2

Do, T.T., Do, T.P., Nguyen, T.N., Nguyen, T.C., Vu, T.T. and Nguyen, T.G., 2019. Nanoliposomal L-asparaginase and its antitumor activities in Lewis lung carcinoma tumor-induced BALB/c mice. *Advances in Materials Science and Engineering*, Article ID 3534807. DOI:10.1155/2019/3534807

Hawkins, D.S., Park, J.R., Thomson, B.G., Felgenhauer, J.L., Holcenberg, J.S., Panosyan, E.H.

and Avramis, V.I., 2004. Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. *Clinical Cancer Research*, 10(**16**), 5335-5341. DOI:10.1158/1078-0432.CCR-04-0222

Herdiana, Y., Wathoni, N., Shamsuddin, S., Joni, I. and Muchtaridi, M., 2021. Chitosan-Based Nanoparticles of Targeted Drug Delivery System in Breast Cancer Treatment. *Polymers*, 13(**11**), 1717. DOI:10.3390/polym13111717

Karsakevich, A., Dauvarte, A., Zvirgzda, I., Lebedeva, L. and Vina, I., 1986. Effective complexes of the antileukemic enzyme L-asparaginase with dextran sulfate. *Voprosy Meditsinskoi Khimii*, 32(**4**), 47-51.

Knott, S.R.V., Wagenblast, E., Khan, S., Kim, S.Y., Soto, M., Wagner, M., Turgeon, M.O., Fish, L., Erard, N., Gable, A.L., Maceli, A.R., Dickopf, S., Papachristou, E.K., D'Santos, C.S., Carey, L.A., Wilkinson, J.E., Harrell, J.C., Perou, C.M., Goodarzi, H., Poulogiannis, G. and Hannon, G.J., 2018. Asparagine bioavailability governs metastasis in a model of breast cancer. *Nature*, 554, 378–381. DOI:10.1038/nature26162

Li, B., Shan, C.L., Zhou, Q., Fang, Y., Wang, Y.L., Xu, F., Han, L.R., Ibrahim, M., Guo, L.B., Xie, G.L. and Sun G.C., 2013. Synthesis, characterization, and antibacterial activity of cross-linked chitosan-glutaraldehyde. *Marine drugs*, 11(**5**), 1534-1552. DOI:10.3390/md11051534

Marlborough, D.I., Miller, D.S. and Cammack, K.A., 1975. Comparative study on conformational stability and subunit interactions of two bacterial asparaginases. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, 386(**2**), 576-589 .

Masarudin, M.J., Cutts, S.M., Evison, B.J., Phillips, D.R. and Pigram, P.J., 2015. Factors determining the stability, size distribution, and cellular accumulation of small, monodisperse chitosan nanoparticles as candidate vectors for anticancer drug delivery: Application to the passive encapsulation of [14C]-doxorubicin. *Nanotechnology Science Applied*, 8, 67–80. DOI:10.2147/NSA.S91785

Meneguetti, G.P., Santos, J.H., Obreque, K.M., Barbosa, C.M., Monteiro, G., Farsky, S.H., Marim de Oliveira, A., Angeli, C.B., Palmisano, G., Ventura, S.P. and Pessoa-Junior, A., 2019. Novel site-specific PEGylated L-asparaginase. *PloS one*, 14(**2**), e0211951. DOI:10.1371/journal.pone.0211951

Miller, M., Rao, J., Wlodawer, A. and Gribskov, M. R., 1993. A left-handed crossover involved in amidohydrolase catalysis. *FEBS letters*, 328(**3**), 275-279.

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

Moon, L.D., Asher, R.A. and Fawcett, J.W., 2003. Limited growth of severed CNS axons after treatment of adult rat brain with hyaluronidase. *Journal of Neuroscience Research*, 71(**1**), 23-37 .

Noma, S.A., Yılmaz B.S., Ulu, A., Özdemir, N. and Ateş, B., 2021. Development of L-asparaginase@ hybrid Nanoflowers (ASNase@ HNFs) Reactor System with Enhanced Enzymatic Reusability and Stability. *Catalysis Letters*, 151(**4**), 1191-201. DOI:10.1007/s10562-020-03362-1

Qian, G., Zhou, J., Ma, J. and Wang, D., 1996. The chemical modification of E. coli L-asparaginase by N, O-carboxymethyl chitosan. *Artificial Cells, Blood Substitutes, and Biotechnology*, 24(**6**), 567-577.

Ribeiro, E.S., de Farias, B.S., Sant'Anna Cadaval Junior, T.R., de Almeida Pinto, L.A. and Diaz, P.S., 2021. Chitosan–based nanofibers for enzyme immobilization. *International Journal of Biological Macromolecules*, 183, 1959-1970.

Sheldon, R.A., Basso, A. and Brady, D., 2021. New frontiers in enzyme immobilisation: robust biocatalysts for a circular bio-based economy. *Chemical Society Reviews*, 50, 5850-5862. DOI:10.1039/D1CS00015B

Soares, A.L., Guimarães, G.M., Polakiewicz, B., de Moraes Pitombo, R.N. and Abrahão-Neto, J., 2002. Effects of polyethylene glycol attachment on physicochemical and biological stability of E. colil-asparaginase. *International Journal of Pharmaceutics*, 237(**1**), 163-170.

Sukhoverkov, K. and Kudryashova, E., 2015. PEG-chitosan and glycol-chitosan for improvement of biopharmaceutical properties of recombinant L-asparaginase from Erwinia carotovora. *Biochemistry (Moscow)*, 80(**1**), 113–119. DOI:10.1134/S0006297915010137

Synakiewicz, A., Stachowicz-Stencel, T. and Adamkiewicz-Drozynska, E., 2014. The role of arginine and the modified arginine deiminase enzyme ADI-PEG 20 in cancer therapy with special emphasis on Phase I/II clinical trials. *Expert Opinion on Investigational Drugs*, 23(**11**), 1517-1529. DOI:10.1517/13543784.2014.934808

Tabandeh, M. R. and Aminlari, M., 2009. Synthesis, physicochemical and immunological properties of oxidized inulin–l-asparaginase bioconjugate. *Journal of Biotechnology*, 141(**3**), 189-195. DOI:10.1016/j.jbiotec.2009.03.020

Teodor, E., Litescu, S.C., Lazar, V. and Somoghi, R., 2009. Hydrogel-magnetic nanoparticles with immobilized L-asparaginase for biomedical applications. *Journal of Materials Science: Materials in Medicine*, 20(**6**), 1307-1314. DOI:10.1007/s10856-008-3684-y

Tikhonov, V.E., Stepnova, E.A., Babak, V.G., Yamskov, I.A., Palma-Guerrero, J., Jansson, H.B. and Avdienko, I.D., 2006.

[DOR: 20.1001.1.15622916.2021.20.6.20.9]

Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2 (3)-(dodec-2-enyl) succinoyl/-derivatives .*Carbohydrate polymers*, 64(**1**), 66-72. DOI:10.1016/j.carbpol.2005.10.021

Vellard, M., 2003. The enzyme as drug: application of enzymes as pharmaceuticals. *Current Opinion in Biotechnology*, 14(**4**), 444-450.

Vyna, I., Karsakevich, A. and Bekers, M., 2001. Stabilization of anti-leukemic enzyme L-asparaginase by immobilization on polysaccharide levan. *Journal of Molecular Catalysis B: Enzymatic*, 11(**4**), 551-558.

Wolf, M., Wirth, M., Pittner, F. and Gabor, F., 2003. Stabilisation and determination of the biological activity of L-asparaginase in poly (D, L-lactide-co-glycolide) nanospheres. *International Journal of Pharmaceutics*, 256(**1**), 141-152.

Wriston, J. C., Jr., 1970. Asparaginase. *Methods Enzymol*, 98, 732–742 DOI:10.1016/0076-6879(71)17273-4

Zhang, Y.Q., Tao, M.L., Shen, W.D., Zhou, Y.Z., Ding, Y., Ma, Y. and Zhou, W.L., 2004. Immobilization of L-asparaginase on the microparticles of the natural silk sericin protein and its characters. *Biomaterials*, 25(**17**), 3751-3759 DOI:10.1016/j.biomaterials.2003.10.019.

Zhang, Y.Q., Zhou, W.L., Shen, W.D., Chen, Y.H., Zha, X.M., Shirai, K. and Kiguchi, K., 2005. Synthesis, characterization and immunogenicity of silk fibroin-L-asparaginase bioconjugates. *Journal of Biotechnology*, 120(**3**), 315-326. DOI:10.1016/j.jbiotec.2005.06.027

[DOR: 20.1001.1.15622916.2021.20.6.20.9]