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Effects of polyethylene glycol attachment on physicochemical and biological stability of *E. coli* L-asparaginase

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Abstract

L-asparaginase obtained from *E. coli* strains is an important enzyme widely used in leukemia treatment. However, hypersensitivity reactions must be considered a relevant adverse effect of asparaginase therapy. One approach to reduce the hypersensitivity reactions caused by this enzyme is to change its physicochemical and biological properties by means of polyethylene glycol (PEG) conjugation, resulting in a less immunogenic enzyme with much longer half-time of plasmatic life. This work investigated the factors that could interfere in PEG–enzyme's stability. The complexation did not affect the range of pH activity and stability was improved in acid medium remaining stable during 1 h at pH 3.5. The PEG–enzyme exhibited activity restoration capacity (32% after 60 min) when subjected to temperatures of 65 °C in physiological solution. The PEG–enzyme in vitro assays showed a very high stability in a human serum sample, keeping its activity practically unchanged during 40 min (strength to non-specific antibodies or proteases in serum). An increase of PEG–enzyme catalytic activity during the lyophilization was observed. The process of modification of L-asparaginase with PEG improved both physicochemical and biological stability. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: PEG–asparaginase; Leukemia; Stabilization; Storage

1. Introduction

Bacterial L-asparaginases (L-asparagine amidohydrolase, E.C. 3.5.1.1) are enzymes of high therapeutic value due to their use in leukemia treatment. *Escherichia coli* L-asparaginase, a high affinity periplasmic enzyme is particularly effective in certain kinds of cancer therapies (Marlborough et al., 1975). This enzyme, has been used as a therapeutic agent against lymphoblastic leukemia since Kidd (1953) discovered that L-asparaginase has the ability to avoid the increase of induced tumors in mice after a treatment with hamster serum. It was shown that hamster blood contains a high level of L-asparaginase which does

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not occur in other mammals. In a great number of patients with lymphoblastic leukemia, the malignant cells depend on an exogenous source of L-asparagine to be able to survive, meanwhile, the normal cells are able to synthesize L-asparagine (Broome, 1981; Ravindranath et al., 1992).

The most common therapeutic uses of L-asparaginase are: treatment of Hodgkin's disease, acute lymphocitic leukemia (mainly in children), acute mielomonocitic leukemia, lymphosarcoma and melanosarcoma (Ravindranath et al., 1992).

The main restrictions to the use of asparaginase in the therapeutic field include several types of side reactions, from mild allergies to dangerous anaphylactic shocks (Mashbum and Wriston, 1964; Marlborough et al., 1975). Several studies have shown that some complications can be bypassed replacing *E. coli* with *Erwinia* asparaginase, since the antigenic sites are different in both enzymes. The *Erwinia* enzyme can be used in the treatment of patients with allergic reactions from *E. coli* enzyme, and the reverse situation is also possible (Moola et al., 1994). Another approach to the problem is to deal with the hypersensitivity to this enzyme by changing the enzyme properties with polyethylene glycol (PEG) resulting in a less immunogenic enzyme. PEG has been used as a pharmaceutical aid since 1970, when it was approved by the FDA due to its suitable pharmaceutical properties: low toxicity as a vehicle in p.o. formulations as well as in i.v. injections (Burnham, 1994).

The reaction of proteins with PEG has certain requirements concerning the liability of proteins in many reaction media. The stable linkage between the proteins and PEG was made by using intermediate heterocyclic compounds such as cyanuric chloride, succinimide and any other intermediates which allow a reaction between PEG and proteins in mild conditions of pH and temperature, usually in a buffered medium. Activated PEG binds to the free amino group situated in the amino acid side chains of the proteins particularly lysine (Matsuyama et al., 1991).

The degree of substitution of protein amino groups and the length of the PEG chains are the main variables studied with the aim to obtain low immunogeniticity and good biological activity of the protein. The other advantage of the structural changes obtained with the enzyme PEGylation is the improvement of the enzyme's therapeutic properties, so that the modified enzyme has a plasmatic half-life longer than 24 h and better clearance (Harms et al., 1991; Wehner et al., 1992; Derst et al., 1992, 1994).

Lyophilization has been used in pharmaceutical preparations in order to obtain an enzymatic drug in a dry and stable form prior to parenteral administration. The lyophilization process involves solidification of the proteinaceous solution by freezing, followed by sublimation of water from the solid state at reduced pressure. During this process, bulk water molecules are removed, leaving some residual moisture that remains immobilized in the protein structure. The conformational mobility of the proteins is related to their moisture content. Most protein preparations are at least partially denatured by the freezing and dehydration stresses encountered during lyophilization. A properly prepared lyophilized formulation should allow the protein to endure the lyophilization process and assure stability during long-term storage (Carpenter et al., 1997; Costantino et al., 1997).

The purpose of the present work is to investigate PEG–L-asparaginase stability and activity changes in different solutions and buffers with respect to the variations of pH, temperature and human serum reactivity. Furthermore, in trying to improve PEG–L-asparaginase shelf-life, we studied the stability of the modified enzyme during lyophilization.

2. Materials and methods

2.1. Materials

L-asparaginase was obtained in the form of the concentrate Elspar® with the lyophilized enzyme (Merck, Sharp and Dohme, Pennsylvania, USA) in 10 ml vials with 10 000 IU, through Prodome. Activated methoxypolyethylene glycol

5000 (succinimidyl succinate), L-asparagine, aspartate, molecular weight markers, Sephacryl S-300HR, Sephadex G-150 and bovine albumin serum were obtained from Sigma Chemical Co., St. Louis, USA. All the other chemicals were from Merck (Darmstadt, Germany) and were of analytical degree from Quimitra, Rio de Janeiro, Brazil.

2.2. Determination of enzymatic activity

L-asparaginase catalyzes the hydrolysis of L-asparagine producing L-aspartic acid and ammonia. After the adapted time of incubation, in buffer Tris–HCl 50 mM, pH 8.6 containing 10 mM L-asparagine, the reaction was interrupted with 1.5 M of trichloroacetic acid and the samples, after centrifugation, were treated with Nessler's reagent. The ammonia concentration produced in the reaction was determined on the basis of a standard curve previously obtained with ammonium sulfate as a standard.

The activity of L-asparaginase can also be measured by using the conductimetric method. The method is based on the increase of conductivity, which is due to the production of ammonia and/or aspartate. This conductivity is linear in relation to the time and to the enzymatic concentration and follows Michaelis kinetics (Drainas and Drainas, 1985). The addition of commercial L-asparaginase in the reaction mixture that contains the substrate L-asparagine causes a fast increase in conductivity. This increase is linear to the enzymatic concentration. This method can detect up to 0.001 IU of commercial L-asparaginase. Among the advantages of this method are: (a) it is faster because it does not require lengthy preparation and a long reaction time; (b) it is very sensitive; (c) it is reliable due its reproducibility and adapted to the enzyme kinetic study (Drainas and Drainas, 1985). An International Unit (IU) of L-asparaginase is the amount of enzyme that catalyzes the production of 1umol of ammonia liberated in 1 min under the conditions of the assay (Wriston, 1985; Wade and Phillips, 1971; Whelan and Wriston, 1969; Law and Wriston, 1971).

Results of enzymatic activity are the average of three experiments conducted either in parallel or independently.

2.3. Determination of protein concentration

The concentration of protein was determined by the Bradford method (Bradford, 1976) using bovine albumin serum as a standard. Where necessary, the Lowry method (Lowry et al., 1951) was used.

2.4. Modification and purification of L-asparaginase with activated PEG

The following was poured in a reaction vessel: 1.50 ml of phosphate buffer (PB) pH 7.0 (0.4 g NaCl, 0.01 g KCl, 0.0575 g Na_2HPO_4, 0.01 g KH_2PO_4 and water to obtain 50.0 ml). This solution was combined with 1 mL of L-asparaginase (4 mg/ml) and 80 mg activated PEG.

The solution was maintained at 30 °C for 2 h and after this the reaction vessel was cooled to 8 °C and allowed to stand overnight. The purification of the PEG asparaginase was made trough a Sephadex G-150 column, equilibrated with 100 mM Tris buffer, pH 8.6 and eluted with the same buffer at a flow rate of 0.2 ml/min.

2.5. Electrophoresis (SDS-PAGE)

The experiments were carried out as described by Laemmli (1970) using 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

2.6. Freezing–thawing

Aliquots (1 ml) of PEG–L-asparaginase (0.03 IU/μl in a phosphate buffer pH 7.3, containing: 1.2 mg/ml NaH_2PO_4, 5.6 mg/ml Na_2HPO_4 and 8.5 mg/ml NaCl) solution were poured into freeze-drier vials. The samples were frozen at a cooling rate of 25 °C/min (rapid freezing in liquid nitrogen, measured with a thermoelement-IOP therm model 41, inserted into the vials). To measure the enzyme activity after freezing, the vials were immersed in a water bath at 37 °C and thawed for at least 10 min. Then aliquots were taken and the asparaginase activity was measured.

2.7. Freeze-drying

Aliquots (1 ml) of PEG L-asparaginase (0.03 IU/μl) solution in phosphate buffer pH 7.3, containing: 1.2 mg/ml NaH_2PO_4, 5.6 mg/ml Na_2HPO_4 and 8.5 mg/ml NaCl, were frozen as described above and transferred to an FTS Systems freeze-drier consisting of a control work station, sample extractor, isolation valve, moisture sensor and product thermocouples in vials. Condenser temperature (−70 °C), chamber pressure (0.1 mbar) and shelf temperature (−30 °C) were monitored for 8 h, and the samples were withdrawn at indicated times, reconstituted with the same amount of sublimated water and submitted to the analysis of enzymatic activity.

3. Results and discussion

At first, an electrophoresis in polyacrylamide gel (SDS-PAGE) was performed with pure enzyme solutions to get basic information about the purity of the L-asparaginase (Laemmli, 1970). The results revealed no detectable contamination and a single distinct band was observed with molecular weight of about 33 kDa, the same of the sub-unit of L-asparaginase (results not shown).

Native L-asparaginase was modified as described in Section 2.4 and the degree of modification of amino groups in the molecule was determined (Habeeb, 1966). Fifty amino groups had been substituted and the PEG–enzyme retained 30% of the original activity of non-modified asparaginase. PEG–asparaginase was subjected to chromatography with Sephacryl S-300HR and its elution corresponded to the molecular weight of approximately 400 000, which is far greater than of native L-asparaginase, 136 000.

Activity and stability experiments of both the native and the PEGylated enzymes are summarized in Fig. 1A and B, and represent the entire range of pH considered in this study. It is evident that the PEG–enzyme remained active in a wide range of pH (Fig. 1A). Furthermore, at a low pH (3.5) the stability was increased compared to unmodified enzyme (Fig. 1B) and this improvement was observed for 1 h. The possible mechanisms of protein stabilization causing an observed increase of enzymatic stability can be explained by the protection provided to the active sites by the PEG chains. Without the protecting effect, the structure in the active site of the native enzyme is more susceptible at low pH leading the active site to an irreversible change.

Previous studies have reported on the denaturation of L-asparaginase (Stecher et al., 1999). This denaturation, which can result in complete lose of

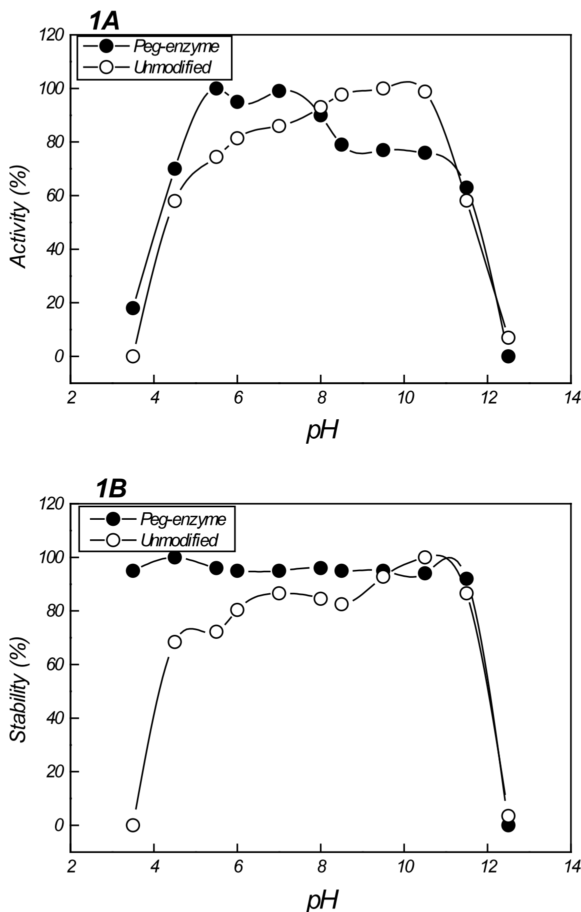

Fig. 1. (A) Enzyme activity; and (B) stability of L-asparaginase (unmodified -○- and PEGylated -●-). The activity was determined by incubation in different buffers varying the pH from 3.5 to 12.5, (3.5–5.5 acetate 0.05 M; 6.0–8.0 Hepes 0.05 M and 8.5–12.5 Tris–HCl 0.05 M), at 37 °C for 10 min. The stability was determined after incubation for 1 h in the same buffers at 37 °C. At the end of this time the enzyme activity was determined as described in Section 2.2 at pH 8.5.

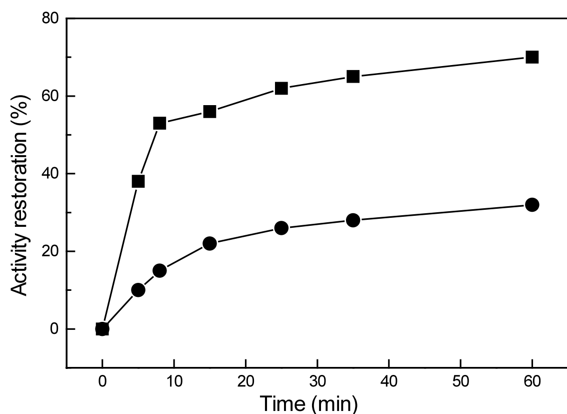

Fig. 2. L-asparaginase activity restoration in physiological solution. The enzymes were previously submitted to a thermal treatment in bathing (65 °C/30 min). Following this thermal treatment the samples were placed in ice-cold water until 40 °C and soon after placed in bathing to 37 °C. At the indicated times aliquots were withdrawn and assay of the activity was performed as described in Section 2.2. (-■- unmodified; -●- PEGylated).

enzyme activity, has been shown to be a consequence of dissociation of the enzyme sub-units. The sub-units can be re-associated, and the enzyme activity restored, depending on the experimental conditions. In our current investigation, we studied the restoration of PEG–L-asparaginase activity compared to the unmodified enzyme. Fig. 2 shows the activity recovery (restoration) after thermal treatment in order to verify a possible similarity between the effects observed in the treatment with urea (Marlborough et al., 1975) and the effects caused by the increase of temperature on native and modified L-asparaginases restoration activity. A total loss of PEG–L-asparaginase activity was observed after a few minutes at 65 °C, probably because the sub-units are dissociated. This sample, incubated at 65 °C for 30 min and then immediately incubated in physiologic solution at 37 °C, recovers 15% of its initial activity after 8 min and 32% after 60 min. Meanwhile, the native enzyme in the same conditions recovers 50 and 70%, respectively. These results show that there is much greater activity restoration, hence sub-unit re-association, for the native enzyme compared to the PEG–enzyme.

In L-asparaginase while ionic forces and hydrogen bonds are the main factors responsible for the secondary and tertiary structure, the forces among the sub-units in the tetramer are predominantly hydrophobic (Cammack et al., 1972; Ryoyama, 1972; Shifrin et al., 1973; Stecher et al., 1999). The results (Fig. 2) indicate that the hydrophobic sites involved in the arrangement of the tetramer form are more susceptible to the heat treatment in the modified enzyme. Furthermore, it is probable that the PEG–enzyme recovers activity more slowly since it has a lower mobility (structural rigidity) as was discussed earlier.

The results of L-asparaginases stability towards human serum are summarized in Fig. 3. The native enzyme loaded in human serum without acute lymphoid leukemia at a final concentration of 1 IU/ml exhibited a reduction of 70% in enzymatic activity after 30 min. This fact can be explained by the presence of non-specific antibodies or proteases in serum. In other words, these results demonstrate that the human serum already contains natural antibodies and/or proteases capable of making L-asparaginase of *E. coli* inactive. In order to verify if the enzyme modified with PEG presented in this work really results in greater enzyme stability in the same conditions, the PEG–asparaginase obtained was submitted to

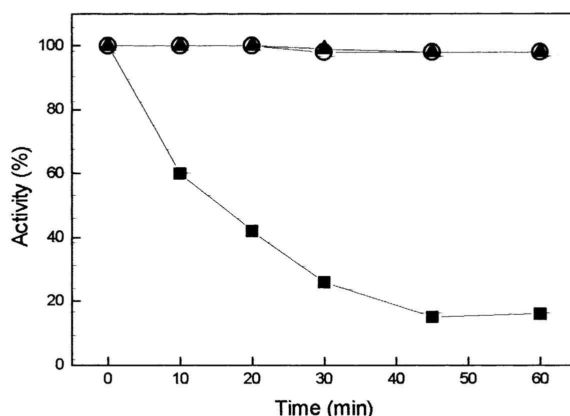

Fig. 3. Antigenic reactivity of L-asparaginases towards serum antibodies. Samples were incubated at 37 °C in human serum (-■- unmodified; -○- PEGylated) and physiological solution (-▲- PEGylated), at the indicated times aliquots were withdrawn and assay of the activity was performed as described in Section 2.2.

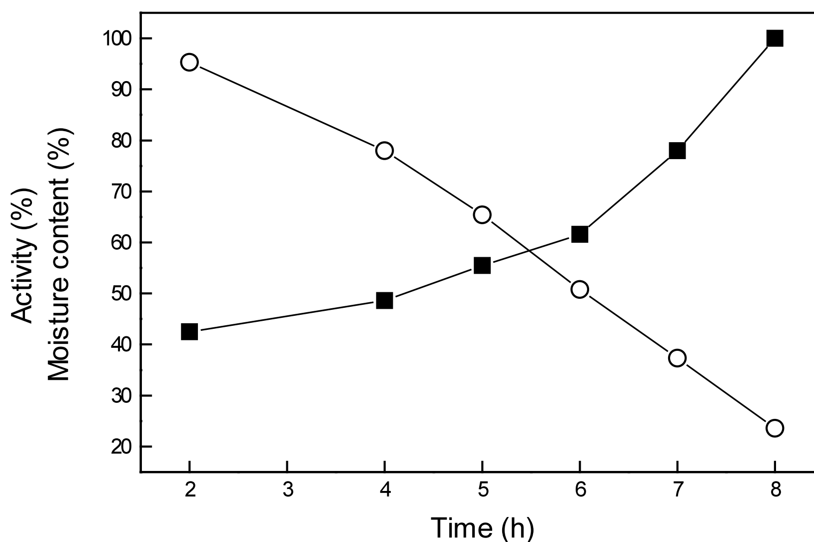

Fig. 4. Aliquots (1 ml) of PEG–L-asparaginase (0.03 IU/μl) solution in phosphate buffer of pH 7.3 containing: 1.2 mg/ml of NaH_2PO_4, 5.6 mg/ml of Na_2HPO_4, 8.5 mg/ml of NaCl, were frozen and lyophilized as described in Section 2.6. The samples were drawn at indicated times, reconstituted with the same amount of sublimated water (○) and assay of the activity (■) was performed as described in Section 2.2.

the same serum above and to a standard solution as described in Section 2. The modified enzyme retains its activity for 40 min when it was incubated in the same manner as the physiological solution at 37 °C (Fig. 3). These results suggest that the modification with activated PEG protects L-asparaginase by causing steric hindrance to the binding of non-specific antibodies or at enzymatic hydrolysis caused by proteases in serum. This results in a drug with longer plasmatic half-life and improved therapeutic properties (Harms et al., 1991; Wehner et al., 1992; Derst et al., 1992, 1994). It is important to observe that this data does not show that the PEG–enzyme is less immunogenic compared to the unmodified enzyme. The results obtained in Fig. 3 demonstrate that the PEG–enzyme is much more stable in human serum compared to unmodified enzyme.

Preliminary shelf-life studies showed that modified asparaginase was stable at 8 °C for a longer period than one month when maintained in phosphate buffer of pH 7.3 (results not shown).

The principal challenge associated with lyophilization of biological materials is loss of activity, during freezing, drying or storage. Fig. 4 shows the results of the lyophilization experiments. Aliquots of PEG–L-asparaginase solution in phosphate buffer were frozen and lyophilized as described in Section 2.6. Surprisingly, an increase in catalytic activity was observed after the samples had been drawn through the freeze-drier sample extractor, at indicated times, and reconstituted with the same amount of sublimated water.

Fig. 5 (PAGE) indicates that the loss of enzymatic activity (Fig. 4) occurs in the starting step due to freezing without any apparent structural

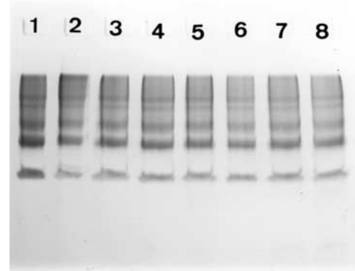

Fig. 5. Electrophoresis (SDS-PAGE) of PEG–L-asparaginase conjugate. Lane 1, sample after Sephadex G-150; lane 2, sample after freeze-thawing; lanes 3–8, time course of lyophilization (2–8 h).

alterations after freeze-thawing and during lyophilization once the banding pattern remains unchanged. In addition, the presence of various bands indicates that it is very likely that there is heterogeneity among the amines modified, probably due to the differential reactivities among these amines and to the heterogeneity of the activated PEG reagent which varies in polymer length by about 300 Da around the average molecular mass (5000 Da) (Veronese, 2001).

As the water was displaced by sublimation only the frozen water was removed (primary drying, Fig. 4). This results suggest that upon freezing, the sub-units become dissociated, and after thawing they re-associate poorly; hence the activity is lowered. This statement is in agreement to the observation regarding the thermal unfolding in Fig. 2. The noteworthy recovery of biological activity (Fig. 4) can be explained by the increase in molecular interactions between the enzyme and others medium components (including sodium) which occurs with water displacement. Also, upon drying the protein structure may be altered (reversibly) allowing for addition contacts and improved sub-unit re-association.

4. Conclusions

The results have demonstrated that the PEG–enzyme remains stable over a wide range of pHs (3.5–12.5). Furthermore, stability was greatly increased in the acidic medium, remaining stable for 1 h at pH 3.5. The PEG–enzyme also proved to have activity restoration capacity (32% after 60 min) when submitted to temperatures of 65 °C in physiological solution. The PEG–asparaginase showed high in vitro stability in human serum with activity remaining unchanged during 40 min. The lyophilization process conditions allowed the frozen PEG–enzyme a restoration of its enzymatic activity. The structural modifications performed by the reaction of activated PEG with L-asparaginase improved both physicochemical and biological properties which can lead to significant improvements in the therapeutic properties of L-asparaginase.

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