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Solid-phase enzyme modification via affinity chromatography

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Abstract

In the present study antileukemic enzyme L-asparaginase (ASNase) and catalase (as a model enzyme) were modified in solid-phase with activated polyethylene glycol (PEG_2) by using ligand-immobilized affinity column systems L-asparagine-Sepharose CL-4B and Procion red-Sepharose CL-4B, respectively. Studies on change of specific activity with modification time showed negligible differences between batches of modified catalase. Modification of ASNase for 1 h resulted in 50.2% recovery of the specific activity and the attachment of 69 molecules of PEG_2 per molecule of ASNase forming 'PEGylated ASNase'. Sequential modification of ASNase by activated PEG and heparin resulted in coupling of about nine molecules of heparin per molecule of PEGylated ASNase. Intravenous (i.v.) administration of PEG_2-modified ASNase showed prolonged presence in the blood circulation and no adverse effects or symptoms of anaphylaxis were observed in presensitized mice.

Keywords: Enzymes; L-asparaginase

1. Introduction

Chemical modification of proteins became common in the late 1950s. The techniques were originally developed for use in the structural analysis of proteins. Since the late 1970s, many articles on the chemical modification of proteins with synthetic macromolecules have been published [1]. Modification of proteins with polyethylene glycol (PEG), a linear, non-toxic, non-immunogenic and amphipathic polymer has been studied as a potential means to add unique properties to proteins. The technique of modification with polyethylene glycol has opened a new avenue towards reduction of the immunoreactivity and prolonged the clearance time of foreign protein drugs used in biomedical applications [2–4].

The term 'site-specific modification' is used to describe the methods for chemical modification of bioactive proteins through their carbohydrate residues located on the outside of the active sites. Immobilization of glycoproteins on a support, using these techniques, through their carbohydrate residues resulted in high stability and activity. The binding

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sites of enzymes and antibodies are located in the protein moieties hence, the immobilization of the glycoproteins through their carbohydrate residues do not only stabilize the protein conformation but also provide better access of the substrate to the active sites [5,6].

In one such study, Petkov et al. [7] achieved a successful immobilization of glucose oxidase via activation of its carbohydrate residues by oxidation with galactose oxidase. The oxidized enzyme was coupled to hydrazine derivatives of *O*-α-D-galactosyl Sepharose-H1000 or Sepharose-4B. Glucose oxidase immobilized by this procedure showed higher activity than the glucose oxidases modified using other immobilization procedures.

Likewise, site-specific and site-directed modification of antibodies may be achieved by chemical or enzymatic oxidation of the carbohydrate residues located remote from the binding sites of the antibody, on the Fc fragment. Immobilization through this region does not usually impair the immunological activity of the antibody [8].

The protection of the activity of the enzyme during the immobilization procedure by the presence of competitive inhibitor has been reported by several researchers. In one such study, Blanco et al. [9] showed that multipoint covalent attachment of enzyme in the presence of competitive enzyme inhibitor to activated (aldehyde generated) support preserved the enzyme in fully active form. The inhibitor bound on the active centre of the enzyme protects the enzyme from distorting conformational changes by multipoint covalent attachment to aldehyde agarose gels.

In addition to protecting active sites, a different application of oriented immobilization of enzymes was demonstrated by Turkova et al. [10]. In this study, site specific crosslinking of antichymotrypsin with glutaraldehyde was achieved after biospesific adsorption of enzyme to natural inhibitor, antilysine immobilized on Sepharose 4B. This antilysine–chymotrypin crosslinked complex was further used as biospecific adsorbent for isolation of immunoglobulin G against antigenic sites outside of active site of chymotrypsin.

There are several approaches to oriented immobilization of enzymes using the power of molecular biology, including: (a) binding of site-specifically biotinylated enzymes to membrane immobilized streptavidin bridge; (b) fusion protein technology coupled with affinity tags on membranes; and (c) site-directed mutagenesis to introduce a cysteine into a protein with subsequent SH-specific coupling to the membrane support [11].

Increased circulatory half-life of some proteins upon modification with PEG or other high molecular mass polymers may be attributed to the decreased clearance by glomerular filtration by the kidney [12,13]. Conjugation of PEG to proteins caused a great increase in the size of the protein, presumably due to the hydration of PEG. Increasing the extent of modification increased the size of the protein, thereby decreasing the amount lost due to glomerular filtration.

Another possible mechanism for the prolonged half-life in the circulation includes the different actions of various proteases, such as trypsin and chymotrypsin, on the unconjugated form of ASNase. A third potential mechanism for PEG-asparaginase's prolonged half-life may be related to its decreased clearance by the reticuloendothelial system, which is responsible for the clearance of foreign proteins [14,15].

In order to accomplish effective modification of a bioactive agent with PEG, a balance must be maintained between the number of PEG chains (of a given molecular mass) which are required to prevent immunogenicity, and the decrease in enzyme activity resulting from the number of PEGs bound to or in proximity of active sites. In situations where standard PEG modification may block too many active sites on the target macromolecule, a higher molecular mass, branched PEG (U-PEG) would, in concept, be better because it would require only half the attachment sites to provide an equal coverage [16].

PEG-conjugated proteins are expensive due to their difficult and long purification steps. Ultrafiltration is the major method to eliminate the unbound methoxy polyethylene glycol (mPEG) but it has a tendency to block the pores of the membrane filter because of its random coil structure that is well hydrated in aqueous solution. With mPEGs having an MW of 5000 Da or greater, it was noted that the ultrafiltration membrane was blocked rapidly owing

to clogging of the pores by free mPEG [17]. The major drawback of pressure-driven membrane processes, ultrafiltration in particular, is the build up of material at the membrane–solution interface [18].

In this study an alternative enzyme modification was tested; catalase and L-asparaginase were immobilized on an affinity matrix by adsorption on a biosorbent and to this structure activated polyethylene glycol chains were added. This novel method offers a means of protection of active sites during a modified process by orienting the enzyme active site towards the solid-phase and the presence of the solid support offers a convenient way of removing the unused reactive reagents without a need for excessive dialysis or ultrafiltration. Sequential modification of asparaginese first with PEG and then with heparin also served the purpose of investigating the suitability of solid-phase modification to controlled attachment of various polymers. In vivo studies were performed to study the pharmacodynamic behaviour and immunogenicity of solid-phase modified L-asparaginase preparations.

2. Experimental

2.1. Chemicals

Monomethoxy polyethylene glycol (PEG, MW 5000), bovine liver catalase (EC 1.11.1.6, 4000 units/mg solid), L-asparaginase (*Escherichia coli*, EC 3.5.1.1, 155 units/mg protein, in 50% glycerol), malic dehydrogenase (MDH, EC 1.1.1.37, porcine heart, 600 units/mg protein) and glutamic oxaloacetate dehydrogenase (GOT, EC 2.6.1.1, 309 units/mg protein), 1,4-butanediol diglycidyl ether (BDGE), carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (EDC), cyanuric chloride, Sepharose CL 4-B, heparin (porcine intestinal mucosa, Grade I-A) trinitrobenzene sulfonate (TNBS), and Procion Red H-E 3B were purchased from Sigma (St. Louis, MO, USA).

Nessler's reagent was purchased from Aldrich (Milwaukee, USA). Toluidine blue was from Merck (Darmstadt, Germany).

Balb/C mice were obtained from the Animal Care Facility of Gulhane Military Medical Academy (Ankara, Turkey).

All the other chemicals used were of analytical grade.

2.2. Activation of PEG

Activated PEG, 2-4-bis(*O*-methoxy polyethylene glycol)-6-chloro-*s*-triazine) (PEG_2), was prepared from monomethoxypolyethylene glycol according to the method of Ona et al. [19]. Monomethoxypolyethylene glycol (110 g) was dissolved in 500 ml of anhydrous benzene. The solution was refluxed in the presence of granular Molecular Sieves 4 A for 6 h to remove water. After cooling, zinc oxide (50 g) and cyanuric chloride (1.85 g) were added, and the mixture was refluxed for 53 h. The resulting mixture was diluted with 500 ml of benzene and filtered. The product (PEG_2) was precipitated by addition of petroleum ether twice the volume of the solution followed by drying under vacuum to obtain 108 g of activated PEG.

2.3. Activation of heparin

Heparin was activated according to method by Wissink et al. [20]. Heparin (15 mg) was dissolved in an aqueous solution of carbodiimide (0.8 m*M*) and *N*-hydroxysuccinimide (0.5 m*M*). The final pH was adjusted to 5.6 and the solution was stirred for 10 min at room temperature. The solution was dialyzed for 30 min against distilled water (500 ml, pH 5.6) and used immediately for enzyme conjugation.

2.4. Solid-phase oriented modification of catalase on Procion red-Sepharose

Procion red-Sepharose CL-4B conjugate was prepared according to the method of Stellwagen et al. [21]. Wet Sepharose CL-4B (20 g) in distilled water (70 ml) was mixed with Procion red HE-3B (200 mg) dissolved in distilled H_2O (20 ml). The mixture was stirred with a glass rod for 5 min. Into the slurry,

NaCl (10 ml, 20% (w/v)) was added and stirred for a further 30 min at room temperature. Finally, NaOH (0.5 ml, 5 *M*) was added to the mixture and incubated for 3 days while stirring on a magnetic stirrer. Procion red-Sepharose CL-4B was collected on a sintered glass filter and washed sequentially with an excess of deionized water, NaCl (1 *M*), urea (4–8 *M*) and distilled water.

2.4.1. Adsorption of catalase on Procion red-Sepharose

Catalase solution (5 ml, 1 mg/ml) in phosphate buffer (10 m*M*, pH 7.0) was circulated for 1 h using a peristaltic pump through a column (1×10 cm) packed with Procion red-Sepharose CL-4B. Enzyme not adsorbed onto the conjugate beads was washed by phosphate buffer (PB) (10 m*M*, pH 7.0).

2.4.2. Solid phase PEG_2 modification

Cyanuric chloride activated PEG (25 mg) in borate buffer (5 ml, 10 m*M*, pH 9.0) was circulated through the column at a flow-rate of 1.5 ml/min using a peristaltic pump (Master Flex®, Cole Parmer Instruments, USA). Unconjugated PEG_2 molecules were washed with PB (30 ml).

2.4.3. Desorption of modified catalase

In order to elute the solid-phase-modified catalase, 2 *M* NaCl solution in PB (10 m*M*, pH 7.0), was circulated through the system for 1 h. Finally, the eluted enzyme was dialyzed overnight against PB (10 m*M*, pH 7.0).

The number of PEG_2 molecules coupled to the ε-amino groups of catalase was calculated from the difference in the free amino contents of the unmodified enzyme (a native catalase molecule contains 112 amino groups) and that of the modified enzyme.

2.5. PEG_2 modification of ASNase on L-asparagine-Sepharose

Sepharose CL-4B was activated by reaction with 1, 4-butanediol diglycidyl ether (40 ml) and NaOH (4 l, 0.6 N) solution containing sodium borohydride (0.08 g) [22]. The activation was allowed to proceed at room temperature (RT) for 15 h in a 0.5 l flask in a rotary evaporator. The activated gel was recovered by vacuum filtration, washed seven times with water (1.5 l), and coupled to L-asparagine by the reaction of the gel with L-asparagine (20 g) in sodium carbonate buffer (0.5 *M*, 0.35 l, pH 8.5). The coupling reaction was allowed to proceed at room temperature for 15 h with slow rotation in the rotary evaporator. After the coupling step, the gel was washed seven times with water (1.5 l) and stored in sodium azide (0.02%).

2.5.1. Adsorption of L-asparaginase on activated matrix

Wet asparagine-Sepharose CL-4B (5 g) was packed into an affinity column ((1×10 cm, Amersham Pharmacia Biotech, Sweden) and equilibrated with phosphate buffer (10 m*M*, pH 7.0). L-Asparaginase solution (5 ml, 0.6 mg) was circulated by a peristaltic pump through the column for 1 h. Unadsorbed enzyme was washed away with phosphate buffer (10 m*M*, pH 8.5) until no protein appeared in the wash (about 30 ml).

2.5.2. Solid phase PEG_2 modification

Activated PEG (25 mg) was dissolved in borate buffer (5 ml, 10 m*M*, pH 9.5) and circulated through the affinity column for 1 h at 17 °C. Unconjugated PEG_2 molecules were removed by phosphate buffer (10 m*M*, pH 7.0) from the column.

2.5.3. Desorption of PEG_2-modified asparaginase

NaCl solution (0.5 *M*) containing L-asparagine (20 m*M*) was circulated through the affinity column to desorb the adsorbed enzyme until absorbance of the enzyme in eluent became steady (about 35 min).

The number of PEG_2 molecules coupled to ε-amino groups of asparaginase was calculated from the difference in the amino content of the unmodified enzyme (a native asparaginase molecule contains 92 amino groups) and that of the modified enzyme.

2.6. Sequential solid-phase modification of ASNase first with PEG_2 and then with heparin

Solid phase PEG_2 modification of ASNase was

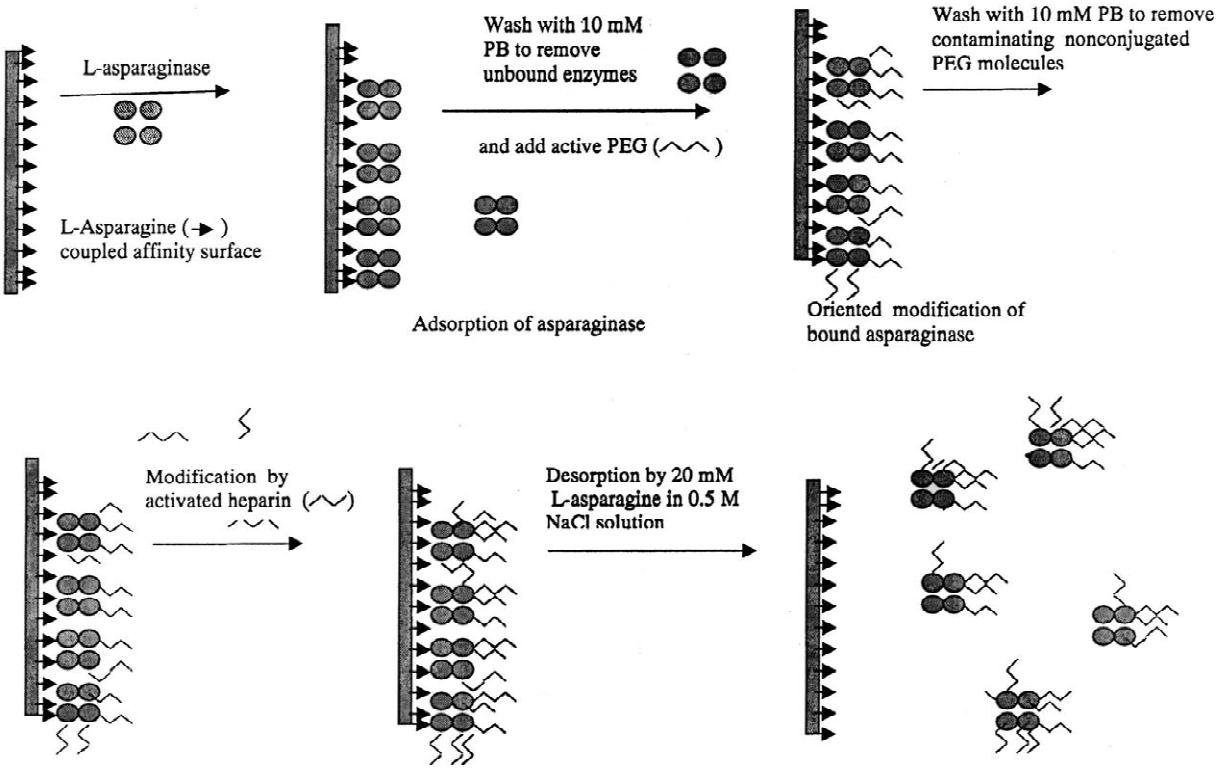

Fig. 1. Successive solid-phase modification of L-asparaginase.

done as described in Section 2.5. For sequential coupling of activated heparin (Fig. 1), column was washed with 30 ml distilled water (pH was adjusted to 5.6 with HCl) after PEG_2 modification. Then, 5 ml of dialyzed aqueous active heparin solution (20 mg, pH 5.6) was circulated through the column for 30 min. Desorption of PEG_2 and heparin conjugated ASNase was achieved by circulating 4 ml sodium chloride solution (0.5 *M*, containing 20 m*M* L-asparagine) for 30 min. The modified ASNase solution was dialyzed overnight in PBS at 4 °C.

2.7. Measurement of catalase and ASNase activity

2.7.1. Specific activity of catalase

The activity of catalase was determined spectrophotometrically by measuring the decrease in absorbance of H_2O_2 at 240 nm [23]. Enzyme solution (25 μl) was mixed with H_2O_2 (3 ml, 15 m*M* in 50 m*M* phosphate buffer, pH 7) and the decrease in absorbance in 2–3 min at 25 °C was recorded with a UV–Vis spectrophotometer (Shimadzu, Model 2100). The specific activity, U/mg protein, was calculated using the extinction coefficient for H_2O_2 of 0.0447 mM^{-1} cm^{-1} from the equation given below

$$\text{Specific Activity (U/mg protein)} = (\Delta A_{240}/\text{min})(3025\ \mu\text{l})(0.0447)^{-1}(25\ \text{ml})^{-1}(\text{mg prot. in } 25\ \mu\text{l})^{-1}$$

2.7.2. Specific activity of L-asparaginase in situ and in vivo

In situ L-asparaginase activities were measured by Nessler's method. The assay procedure is based on direct Nesslerization of ammonia [24]. Enzyme solution (10–100 μl) was added to Tris–HCl (pH

8.5, 50 m*M*) in a final volume of 1.5 ml. The reaction was started with addition of L-asparagine solution (0.5 ml, 10 m*M*, in 50 m*M* Tris–HCl, pH 8.5) and allowed to proceed for 5–60 min at 37 °C. The incubation was stopped with trichloroacetic acid (0.5 ml, 15%) and the volume was adjusted to 4.5 ml with distilled water. Nessler's reagent (0.5 ml) was added, and the mixture was allowed to stand at room temperature for 15 min, and then the absorbance at 500 nm was measured with UV–Vis spectrophotometer.

The extinction coefficient of NH_4Cl (used as a standard ammonia) was found to be 0.1383 μM^{-1} cm^{-1}.

L-asparaginase specific activity (Unit) was calculated as: $(\Delta A_{500}/\text{min})/(0.1383)/\text{mg}$.

To measure L-asparaginase activity in the serum of mice, coupled enzyme assay was used due to the higher sensitivity of the method compared with Nessler's method [25]. The enzyme mixture was prepared by mixing glycerol (100 ml, 87%), Tris–HCl buffer (pH 8.45, 50 ml, 0.5 *M*), NADH (50 mg), α-ketoglutaric acid (50 mg), GOT (180 U), MDH (110 U) and the volume was adjusted to 500 ml with distilled water. Asparaginase containing plasma (50 μl) was added into this enzyme-coupled activity assay mixture (2.25 ml) and incubated for 15 min at 37 °C. Then, 250 μl 10 m*M* L-asparagine in Tris–HCl buffer, pH 8.5, was added and the reduction in absorbance of NADH was recorded at 340 nm using UV–visible spectrophotometer.

The concentration of native and modified enzymes were calculated by the use of Lowry protein determination method [26].

2.8. Determination of the degree of modification of enzymes with PEG_2

The degree of modification with PEG_2 was determined through quantifying the amino groups in the modified enzyme by reaction with trinitrobenzene sulfonate (TNBS). An enzyme solution (250 μl, 0.5 mg protein/ml) was mixed with sodium bicarbonate buffer (250 μl, 0.5 *M*, pH 8.5) and then an aqueous solution of trinitrobenzene sulfonate (250 μl, 0.1%) was added. The mixture was incubated at 40 °C for 2 h to complete the reaction. After adding SDS (250 μl, 10%) and HCl (125 μl, 1 *M*), the absorbance of the solution at 335 nm was measured. A standard curve was obtained using glycine solutions (0–1 m*M*). The degree of modification is obtained by dividing the number of modified free amino groups by the total number of amino groups in the unmodified enzyme molecule.

2.9. Determination of heparin concentration

The amount of heparin conjugated to asparaginase was estimated by the Toluidine blue reaction as described by Smith et al. [27]. PEG_2-Heparin-ASNase sample (0.1 ml) was added into Toluidine blue solution (2.5 ml, 0.005%, 0.01 N HCl containing 0.2% NaCl) in a test tube. The mixture was vortexed vigorously for 30 s. It was diluted with NaCl (0.2%) to a total volume of 5 ml and agitated by a vortex mixer for 30 s. Hexane (5 ml) was then added to each tube and the tubes were shaken vigorously for another 30 s to separate the heparin–dye complex formed. Aqueous solution (0.5 ml) was mixed with ethanol (2.5 ml) and the absorbance was measured at 621 nm by using UV–visible spectrophotometer. To construct a standard curve with a concentration range of approximately 10–70 μg of heparin, varying amounts of heparin in saline solution (2.5 ml 0.2%) were prepared and mixed with the dye solution (2.5 ml). For the assessment of the number of heparin molecules bound on each asparaginase molecule, the average molecular masses for heparin and asparaginase were assumed to be 19 000 and 136 000 Da, respectively.

2.10. Determination of in vivo half-life of ASNase preparations

Studies to measure the residence of modified and unmodified asparaginase in the circulation were undertaken using female Balb-C mice. The animals were housed in the Animal Care Facility (METU), in an air-conditioned room with a 12 h light/12 h dark cycle. PEG_2 modified L-asparaginase (0.5 ml, 1.2 U) or unmodified free asparaginase (0.5 ml, 30 U) in

PBS was injected to the dilated lateral vein of mice. By using opposite lateral vein, blood samples were collected into heparin (10 μl, 5 mg/ml) containing plastic microtubes. After centrifugation, 50 μl of plasma was collected and added into enzyme assay mixture (2.25 ml) (for coupled enzyme activity assay). After incubating plasma in enzyme assay mixture 15 min at 37 °C, substrate solution (0.25 ml, 10 m*M*) was added and the reaction was started for asparaginase activity measurement.

2.11. Antigenicity of ASNase preparations

For toxicity studies, four Balb-C mice for solid-phase PEG-modified and three for unmodified ASNase, were sensitized with subcutaneous injections of 100 μg of unmodified, and PEG_2-modified ASNase on days 0, 10, and 20 [28]. On day 30, animals were challenged with the same dose of free and PEG-modified enzyme given by intravenous injection through their tail vein. Deaths and symptoms of hypersensitivity were recorded.

3. Results and discussion

3.1. PEG_2 modification of catalase on Procion red-Sepharose CL-4B

For solid-phase modification of catalase, a specific affinity column aimed to protect the active site of the enzyme was prepared and the enzyme was modified with activated polyethylene glycol. Procion Red H-E 3B dye molecule was reported to bind the empty NADPH cofactor space near the catalytic site of catalase (some subunits of catalase were detected to have no cofactor) [29]. As seen in Fig. 2, the number of attached PEG_2 increased almost linearly with the reaction time. At the end of a 1 h reaction time with PEG_2, 75 molecules of PEG_2 were conjugated per catalase molecule (75 per 112 amino groups occupied). In the same figure it is shown that as the conjugation of PEG_2 increases, the specific activity of catalase decreases in almost linear fashion. Some subunits in tetrameric catalase molecule may not be protected by the affinity matrix during PEG_2 modification due to the globular structure of enzyme.

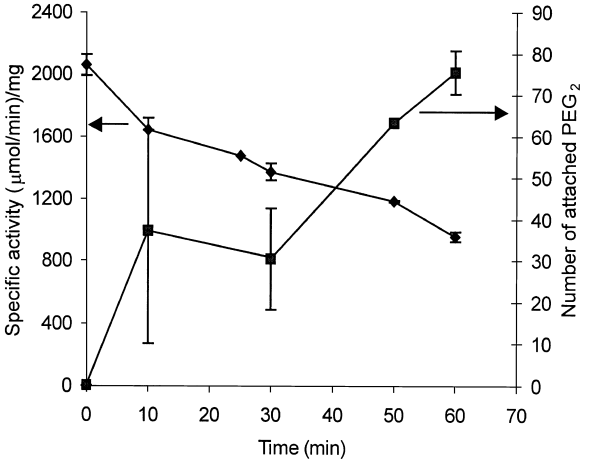

Fig. 2. Specific activity (-♦-) and number of coupled PEG_2 molecules (-■-) at specified times of solid-phase PEG_2 modification of catalase on Procion red-Sepharose. ($n=2$ for specific activity and $n=3$ for attached PEG determination. Modifications were performed at 17 °C with a PEG_2 flow-rate of 1.5 ml/min).

Specific activity vs. modification time showed small differences in batches. As seen from the error bars of specific activity which correspond to two independent solid-phase preparations, the batch differences are quite small. This indicated that solid-phase modification yields quite homogenous PEG_2–protein conjugates at the end of the process. Other advantages of solid-phase enzyme modification are presented in Table 1.

3.2. PEG_2 modification of ASNase

In order to monitor the kinetics of enzyme adsorption, and possible changes during PEG_2 modification of solid-phase ASNase, a flow-through cell was connected to the outlet of an affinity column. Fig. 3 shows a rapid adsorption of the enzyme on the affinity matrix (removal from circulating solution); adsorption was accomplished within a few minutes and there was no change in the unadsorbed protein level after the 10th min indicating the efficiency of the system. It was seen that during the modification with PEG_2 the enzyme was not desorbed or did not leak from its affinity matrix. This indicates a strong adsorption by L-asparaginase on the matrix.

Table 1
Advantages and disadvantages of solid-phase oriented enzyme modification and free enzyme modification methods

Solid-phase oriented enzyme modification	Free enzyme modification
Active sites of enzymes can be protected from modification by active polymer	As the reaction proceeds randomly in nature, active sites can be harmed by the modification. Enzyme activity may be lost
By keeping column temperature and flow-rate constant, batch differences in enzyme modification can be avoided. Washing of active reagents is instantaneous, for that reason the level of modification is constant at specified conditions.	Modification level is adjusted by a ratio of enzyme functional group/active reagent. As the reaction can not be stopped instantaneously, batch differences by random modification is quite possible.
Modification control is easy. The modification can be stopped by simple column washing.	By changing reaction medium (sudden pH changes, neutralizing chemicals) modification can be stopped, but it is not instantaneous.
Modified enzyme can be desorbed from column in pure form.	Since there are active reagents and reaction by-products, the purification process requiring long and tedious purification and concentration steps.

Fig. 4 presents the desorption profile of L-asparaginase after modification with PEG_2 from the affinity matrix by 0.5 *M* NaCl containing 20 m*M* asparagine. Desorption was achieved in about 10 min. Since the desorption solution was circulated in a closed loop, the equlibration of the desorbed protein with the circulating solution required some time and within 30 min removal was completed. The number of PEG_2 molecules attached per molecule of asparaginase was determined using TNBS. A 1 h solid-phase modification resulted in the conjugation of 69.6 PEG_2 molecules per asparaginase molecule and 50.2% of the original specific activity was recovered in the desorbed asparaginase eluate (Table 2). The specific activities of the unmodified and modified asparaginase were 147.0 U/mg and 73.8 U/mg protein, respectively.

PEG_2 modification of asparaginase on asparagine-Sepharose-CL-4B was achieved with the attachment of about 69 PEG_2 molecules per asparaginase molecule with a retention of 50.2% specific activity for a 1 h of reaction time. Enzyme was partially protected from inactivation by PEG_2 modification process by binding it to affinity column. Inada et al. [15], reported 10% residual specific activity after 57% modification of asparaginase (52 of 92 amino groups on the enzyme were modified) with cyanuric chloride activated PEG. This result clearly shows that in

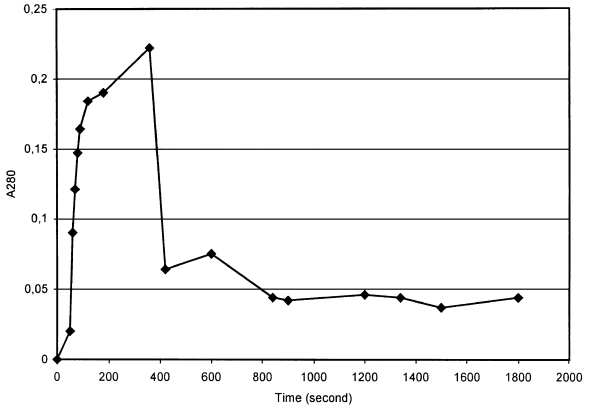

Fig. 3. Adsorption profile of ASNase (-♦-) on asparagine-Sepharose column (Test temperature: 17 °C, Enzyme Flow Rate: 1.5 ml/min).

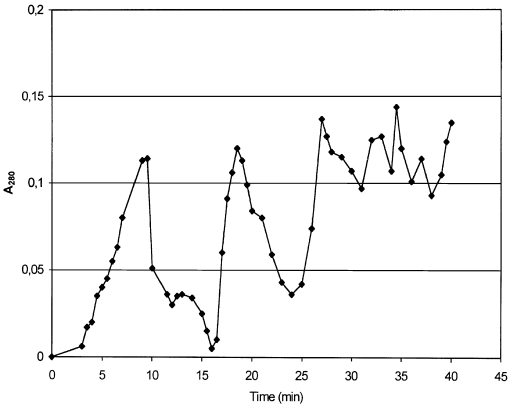

Fig. 4. Desorption profile of PEG_2-modified L-asparaginase (-♦-) from asparagine-Sepharose by 0.5 *M* NaCl containing 20 m*M* L-asparagine. (Flow rate: 1.5 ml/min).

Table 2
Changes in the activity and specific activity of asparaginase during solid-phase modification to PEG-ASNase

Steps in solid-phase modification	Total activity (Unit)	% Activity	Specific activity (Unit/mg)	% Specific activity
Before adsorption	78.8	100	147.0	100
Supernatant	6.0	7.6	–	–
Desorbed PEG-ASNase	18.6	23.6	73.8	50.25
Washing	15.6	19.8	–	–

Solid-phase modification conditions: PEG_2 (5 mg/ml, pH 9.5) at 17 °C and 1 h circulation.

the present study the asparagine-Sepharose matrix efficiently protected the active sites of the asparaginase against the detrimental effects of PEG_2 modification by orienting the active site of the subunit away from the mobile phase and towards the solid matrix. The active form of asparaginase is composed of four identical subunits, A, B, C, and D [30]. Since the enzyme is tetrameric and globular it is not possible to attach all the subunits to the affinity matrix. Consequently, the active sites of some subunits may not be protected against PEG_2 modification and the inactivation of those subunits is probably the cause of the decrease in the specific activity.

3.3. Determination of in vivo half life of ASNase preparations

One of the aims of this study was to increase the half-life and prevent the toxicity of L-asparaginase in the circulation by modifying with polyethylene glycol and heparin on a solid-phase affinity matrix, L-asparagine-Sepharose CL-4B. To assess the pharmacokinetic properties of PEG_2 modified ASNase and PEG_2-heparin conjugated ASNase these preparations were introduced to the blood circulation of Balb/C mice. Unmodified ASNase showed a very sharp clearance from the circulation as shown in Fig. 5. Slightly less than 50% of the activity remained after 10 h of circulation, a value very close to the half-life of 11 h reported in the literature [24].

Solid-phase PEG_2 modified ASNase, however, showed a prolonged clearance from the circulation (Fig. 5). Even after 2 days, more than 50% of the activity was still in circulation.

Although the half-life obtained with PEG_2 modified ASNase by Inada et al. [15] was similar to our result (56 vs. 50 h) their preparation had only 10% of the original specific activity whereas our preparation retained its specific activity to a level of 50.2% of the original. The high specific activity in our preparation must be the result of the differences in the PEGylation procedures. It seems that asparagine-Sepharose CL-4B affinity column significantly protects the active sites of ASNase.

Sequential PEG_2 and heparin conjugation was achieved on affinity matrix of L-asparaginase. Asparaginase modified for 1 h with PEG_2 and then conjugated with heparin had about nine molecules of heparin per asparaginase molecule. The residence half-lives ($t_{1/2}$) of unmodified, PEG_2-conjugated ASNase, and PEG_2-heparin conjugated ASNase in

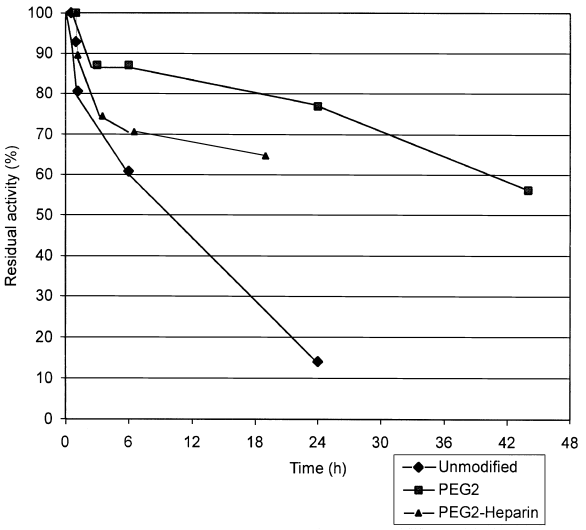

Fig. 5. Residual activity of unmodified (-♦-), solid-phase PEG_2 modified (-■-), and solid-phase PEG_2 and heparin conjugated (-▲-) ASNase in blood serum of mice. Injected doses: Native ASNase: 30 U, PEG_2-ASNase: 1.2 U, PEG_2-Hep-ASNase: 1.3 U.

the circulation were 10, 50 and 25 h, respectively. Compared with only-PEG_2 conjugate, however, the residence time of the ASNase modified by PEG_2 and heparin was decreased (half-lives 50 h with PEG-ASNase whereas 25 h with PEG-heparin-ASNase). Since, heparin is a negatively charged host-polymer and some cell types have heparin receptors, heparin attachment probably rendered asparaginase molecule more recognizable by endothelial cells and polymorphonuclear leukocytes. Nevertheless, the experiment showed the suitability of solid-phase modification to sequential conjugation of polymers to change the biological properties of enzyme molecules and target these pharmaceutical proteins to specific body sites.

In conclusion, solid-phase PEG_2 modification of ASNase produced a very long circulatory-half life compared to unmodified ASNase preparations and heparin conjugation decreased this to some extent.

3.4. Antigenicity testing for ASNase preparations

In order to test the antigenicity, modified and native ASNase were introduced to mice by three subcutaneous (s.c) and one intravenous (i.v.) injections (challenge). Subcutaneous injections were for the sensitization of the animals before the i.v injection. When the native ASNase was injected, two of the three mice showed typical anaphylaxis and died within 30 min. Upon injection of modified ASNase, however, no adverse effects or symptoms of anaphylaxis were observed. These results indicate that solid-phase modification of enzymes on their affinity matrix prevented hypersensitivity reactions by covering antigenic sites of the enzyme by nonimmunogenic PEG_2 molecules and made them more suitable for pharmaceutical applications.

This study indicated that modification of enzymes by solid-phase oriented and non-random modification method presents important advantages both in practical applications (therapeutic) and basic enzymology: the accuracy of modification level (number of coupled polymers), protection of active sites from reactive polymers, desorption of pure modified enzyme from the affinity column thereby, not requiring extensive time consuming purification steps, and enabling a flexibility in subsequent modification of adsorbed enzyme by various polymers to change physical and biological properties of enzymes.

4. Conclusion

Solid-phase oriented enzyme modification employing catalase as a model enzyme was performed on the affinity medium Procion red-Sepharose. A significant amount of PEG was bound on the enzyme after 1 h reaction time (75 molecules of PEG_2 per 112 amino groups) and the results were reproducible. The retention of specific activity was at the level of 46.5%

The same approach was then tested on the antileukemic enzyme asparaginase using asparagine-Sepharose-CL-4B system as the solid support. A very significant level of occupation of the available amine groups was achieved with the attachment of about 69 PEG_2 molecules per asparaginase molecule with a retention of 50.2% specific activity within 1 h of reaction time.

Both these enzymes revealed that site-directed immobilization followed by PEG attachment retained a significantly higher fraction of the activity of the enzyme in question

The literature reveals that when L-asparagine was modified with PEG 5000 activated by succinimidyl succinate, the PEG-enzyme could retain only 30% of the original activity of nonmodified asparaginase and the extent of modification of amino groups in the molecule was determined to be 50 amino groups [31].

In another study L-asparaginase from *Escherichia coli* was modified with 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (activated PEG_2) [32]. The PEG_2-modified asparaginase retained approximately 30% (73 IU/mg of protein) of the enzymic activity of the native enzyme, while it had almost completely lost its immunoreactivity towards anti-asparaginase antibodies.

E. coli asparaginase was modified with poly(*N*-vinylpyrrolidone-co-maleic anhydride) approximately 69% of amino groups were modified but it retained only 8.3% of the native L-asparaginase activity [33].

All these studies had low yields of modification

and low retention of enzymatic activity revealing that site direction and modification on solid supports is a very effective way of enzyme modification.

ASNase could also be successfully and sequentially modified by first PEG_2 and then heparin. With this approach about nine molecules of heparin could be covalently attached per asparaginase molecule. This again is a proof of the effectiveness of the method.

Pharmacokinetic properties of solid-phase PEG modified ASNase preparations were studied in the blood circulation of mice. Unmodified ASNase showed a very sharp clearance from the blood. Less than 50% of the activity remained after 10 h in the circulation. PEG-ASNase, on the other hand, showed a prolonged circulation. Even after 2 days, more than 50% of the enzyme could be detected in the blood. The residence time of the PEG_2 and heparin conjugated ASNase was in between the PEG-ASNase and the untreated enzyme. After 10 h the residual enzyme content was around 70%. This could partially be explained by heparin interfering with the contribution of PEG in terms of hydrophilicity and surface coverage of the enzyme. In that sense this test showed that sequential modifications could be achieved but their effects may not always be synergistic.

No adverse effects or symptoms of anaphylaxis were observed upon injection of PEG_2-ASNase while the unmodified ASNase, resulted in hypersensitivity reactions in two out of the three mice tested. This revealed that surface masking of the enzyme through conjugation with PEG_2 is very important when its use as an anticancer agent is contemplated. All these results indicate that solid-phase oriented modification of enzyme is successful in preventing the detrimental effects of conjugation processes on the activity of the enzyme and it prolongs the circulation half-life and prevents the immunogenicity of the enzyme. In all this is a successful approach that has a potential for use in experimental cancer therapy studies.

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