A PEGylation Technology of L-Asparaginase with Monomethoxy Polyethylene Glycol-Propionaldehyde

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Polyethylene glycol (PEG) conjugation technology has been successfully applied to improve the performance of protein drugs. In this study, L-asparaginase was N-terminal site-specifically modified by alkylating PEG with monomethoxy polyethylene glycol-propionaldehyde (mPEG-ALD_{20000}). The optimum reaction parameters were determined as pH 5.0, a molar ratio of mPEG-ALD_{20000} to L-asparaginase of 10:1, a reaction time of 16 h and temperature of 25 °C. PEG-L-asparaginase (PEG-L-ASNase) was isolated and purified with consecutive anion-exchange (XK, 16 × 20 cm, Q Sepharose FF) and gel-filtration (Tricorn, 10 × 600 cm, Sephacryl S-300 HR) chromatography, respectively. PEG-L-ASNase retained 43.5% of its activity and the N-terminal amino groups were modified to an extent of 3.67%.

Key words: PEGylation, L-Asparaginase, Purification

Introduction

L-Asparaginase (L-asparagine-amidohydrolase, E.C.3.5.1.1, L-ASNase), the enzyme that converts L-asparagine to L-aspartic acid and ammonia, has been a main component of treatment regiments for patients with acute lymphocytic, acute lymphoblastic leukaemia (ALL) and chronic myelogenous leukaemia (Narta *et al*., 2007). The clinical action of this enzyme is attributed to the reduction of L-asparagine since tumour cells, unable to synthesize this amino acid, are selectively killed by L-asparagine deprivation (Kwon *et al*., 2009). However, native L-ASNase is associated with a high incidence of allergic reactions, low stability, as well as short half-life due to rapid clearance in the body. Covalent attachment of polyethylene glycol (PEG) is one of the techniques currently employed to extend the circulation time of proteins in the blood (Kinstler *et al*., 1996; Park *et al*., 2010). A great deal of interest has been generated in PEGylation since it prolongs the circulation time, increases resistance against proteolytic digestion in serum, reduces the immunogenicity, as well as lowers its cytotoxicity (Lee *et al*., 2003; Narta *et al*., 2007; Roberts *et al*., 2002; Veronese, 2001; Veronese and Harris, 2008).

PEG-L-asparaginase (PEG-L-ASNase) has been developed since the early nineties and is used for the treatment of ALL in patients who are hypersensitive to native L-ASNase. PEG-L-ASNases, commercially available, are formed by non-specific random PEGylation of ε-amino groups on the lysine residues of enzymes (Narta *et al*., 2007). Although this type of conjugation has resulted in increased retention of the blood and hence circulation time, it can lead to steric hindrance of the specific binding of PEGylated proteins to their cellular receptors thus decreasing their bioactivity (Bailon and Berthold, 1998). PEGylation strategies have been improved in recent years so as to introduce a reactive amino acid at a desired site of a protein to achieve site-specific PEGylation (Lee *et al*., 2003; Veronese and Pasut, 2005). One such strategy available is alkylation, which maintains the positive charges of the starting amino groups required for retaining biological activity and solubility, rather than acylation (Veronese and Pasut, 2005). These strategies can also be utilized to achieve the mono-site-specific PEGylation by controlling the reaction conditions. It has been shown that alkylated PEGylation rhG-CSF could preserve the charges on the

N-terminal amino groups thus retaining the biological activity (Kinstler *et al*., 1996).

In the present study, we conjugated the N-terminus of L-ASNase with monomethoxy polyethylene glycol-propionaldehyde (mPEG-ALD_{20000}) using the alkylation strategy. The optimum reaction parameters and the purification strategies of bioconjugates have been assessed.

Material and Methods

Materials

L-ASNase (141 kDa) isolated from *E. coli* was obtained from Qianhong Bio-Pharma Co. Ltd. (Changzhou, China). mPEG-ALD_{20000} was purchased from JenKem Technology Co. Ltd. (Beijing, China). Sodium cyanoborohydrate was obtained from Acros Organics (Geel, Belgium). Fluorescamine and trypsin were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical reagent grade and were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Preparation of PEG-L-ASNase bioconjugates

Five mg of L-ASNase were dissolved in 1 mL phosphate-buffered saline (PBS, 0.1 M, pH 5.0) and mixed with mPEG-ALD_{20000} at a molar ratio of 1:10. Sodium cyanoborohydrate (20.4 μL, 1 M) was added, and the reaction mixture was diluted with PBS (0.1 M, pH 5.0) to provide a final enzyme concentration of 0.02 M. The conjugation reaction was allowed to proceed for 16 h at 4 °C.

Reaction parameters

Optimum reaction conditions in terms of the molar ratio of the components of the mixture, pH value of the buffer, time, and temperature were investigated. L-ASNase and mPEG-ALD_{20000} were mixed at the molar ratios of 1:1, 1:3, 1:5, 1:7, 1:9, 1:11, 1:13, and 1:15, respectively, in 0.1 M PBS (pH 5.0) and the conjugation reaction was run for 16 h at 4 °C. In order to find the optimum pH, the reactions were carried out in buffers with different pH values (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0). We analysed the effect of time and temperature on the conjugation reaction by carrying it out for 8, 16, 24, 32, 40, 48, 56, 64, and 72 h at 4 °C, 25 °C, and 37 °C, respectively.

Purification of PEG-L-ASNase

For the best isolation and purification of the bioconjugates, a two-step procedure involving anion-exchange and gel-filtration chromatography was used. The samples were pooled and applied to a Q Sepharose FF column (XK, 16 × 20 cm) equilibrated with buffer A (20 mM PBS, pH 7.0) and eluted with buffer B (20 mM PBS, 1 M NaCl, pH 7.0). Afterwards, samples were subjected to Sephacryl S-300 HR (Tricorn, 10 × 600 cm) gel-filtration chromatography in 20 M PBS (15 mM NaCl, pH 7.2). The flow rate of buffers in both steps was 1 mL/min, and the samples were analysed at 214 nm with an ÄKTA Purifier 10 (GE Healthcare, Piscataway, NJ, USA).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed according to the method of Laemmli (1970) using 12.5% polyacrylamide gel. The SDS-PAGE gels were stained with silver nitrate (Pritsa and Kyriakidis, 2001) and scanned by Quantity One software (Bio-Rad, Hercules, CA, USA). The percentage of modified L-ASNase was analysed as follows: percentage of L-ASNase = (volume of modified L-ASNase bands/total volume of L-ASNase bands) · 100%.

Protein determination

Protein was determined by the method of Bradford as modified by Bearden (1978), using bovine serum albumin as a standard.

Determination of residual amino groups of the enzyme

The presence of unmodified amino acids was determined as described by Stocks *et al*. (1986). Briefly, proteins were dissolved in a small volume of PBS (0.1 M, pH 8.0). The solutions were diluted to different concentrations (0, 1, 2, 3, 4, 5, 6 μg/mL) with the same buffer. To 1.5 mL solution, 0.3 mL fluorescamine solution (0.3%, w/v, dissolved in acetone) was added. The fluorescence intensity was measured after 7 min at 475 nm with a Shimadzu UV-visible spectrophotometer (Kyoto, Japan).

Determination of enzyme activity

L-ASNase activities were assayed according to the method previously described by Matsuyama

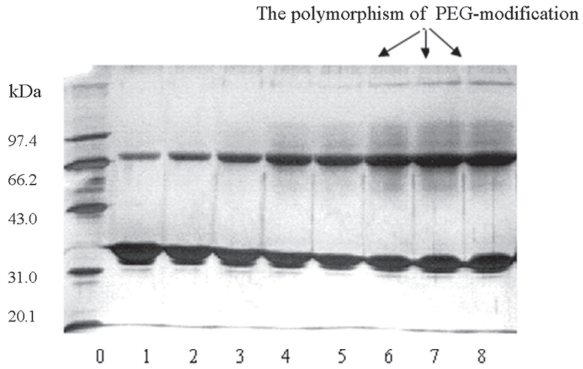

Fig. 1. SDS-PAGE analysis of the effect of the molar ratio of mPEG-ALD_{20000} to L-ASNase on PEGylation of the enzyme. Lane 0, protein marker; lanes 1–8, molar ratio of mPEG-ALD_{20000} to L-ASNase was 1:1, 3:1, 5:1, 7:1, 9:1, 11:1, 13:1, and 15:1, respectively.

et al. (1991). In brief, 1.9 mL L-asparagine (0.33%, w/v) were added to 0.1 mL enzyme solution (5 mg/mL in 0.1 M PBS, pH 8.0) and the mixture incubated at 37 °C for 3 min. The reaction was terminated with 0.5 mL 25% (w/w) trichloroacetic acid. The mixtures, 0.5 mL, were transferred into a new tube and treated with 1 mL Nessler's reagent and 7 mL double distilled water for 15 min at room temperature. The absorbance of the sample was then measured at 450 nm.

Results

Effect of molar ratio on the rate of PEGylation

The molar concentration of modifying agents such as PEG has been shown to affect the extent of PEGylation (Harris *et al*., 2001). Fig. 1 shows the effects of different molar ratios of mixtures on the rate of PEG-L-ASNase modification. We observed that as the molar ratio of mPEG-ALD_{20000} to L-ASNase increased from 1:1 to 15:1, the PEG-L-ASNase protein modification rate increased by 43.1%, 46.7%, 48.5%, 51.7%, 54.3%, 55.3%, 54.6%, and 53.7%, respectively. The highest modification rate of 55.3% was obtained with an 11:1 molar ratio of mPEG-ALD_{20000} to L-ASNase. However, we also observed that a higher ratio resulted in increased polymorphism of PEGylation (Fig. 1, lanes 6 to 8). In order to avoid polymorphism and rather achieve mono-modification, a molar ratio of 10:1 was chosen in the subsequent experiments.

Effect of pH value on the rate of PEGylation

The N-terminal PEGylation of L-ASNase is obtained by conjugating methoxy-PEG derivatives at acidic pH conditions (Kinstler *et al*., 1996). We observed that the percentage of modified enzyme changed with different pH values (Fig. 2). We found that pH 5.0 gave the highest percentage of 59.1% before purification. L-ASNase, isolated from *E. coli*, consists of two types of subunits (Fig. 2, lane 9). As shown in Fig. 2, bands of subunit B of the enzyme were not present in lane 1 to lane 4 (pH 4.0 to 5.5). This indicates that PEGylation was preferentially on subunit B of the enzyme.

Effect of temperature on the rate of PEGylation

Time and temperature are two other parameters that affect the rate of the reaction. Comparing three different reaction temperatures, we observed that the reaction proceeded faster at 37 °C as compared to 25 °C and 4 °C (Fig. 3). However, the percentage of modified enzymes decreased rapidly at higher temperatures at longer reaction times (72 h).

Purification of PEG-L-ASNase

Mono-PEGylated enzyme produced in the PEGylation reactions was separated from higher PEGylated enzyme and unreacted enzyme as well as free PEG or low-molecular weight impurities by anion-exchange chromatography. As shown in Fig. 4, four peaks were obtained in the chromato-

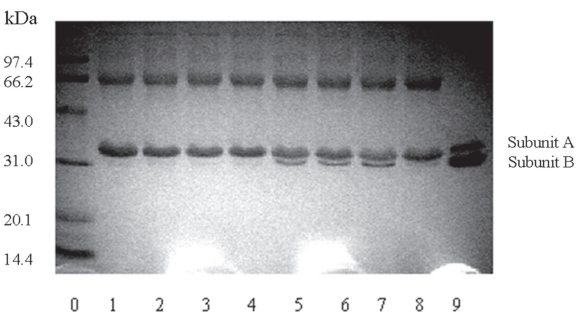

Fig. 2. SDS-PAGE analysis of the effect of pH value on the modification of L-ASNase by mPEG-ALD_{20000}. Lane 0, protein marker; lanes 1–8, pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5, respectively; lane 9, native L-ASNase.

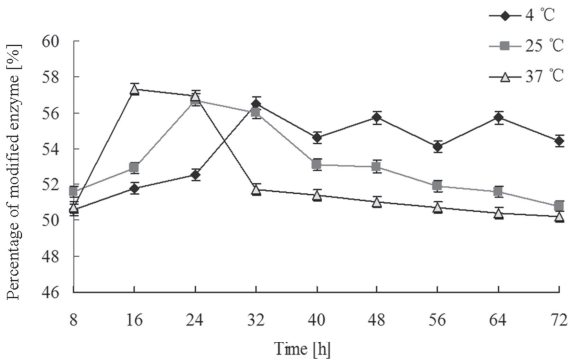

Fig. 3. Effect of the reaction time and temperature on the modification of $mPEG\text{-}ALD_{20000}$ to L-ASNase.

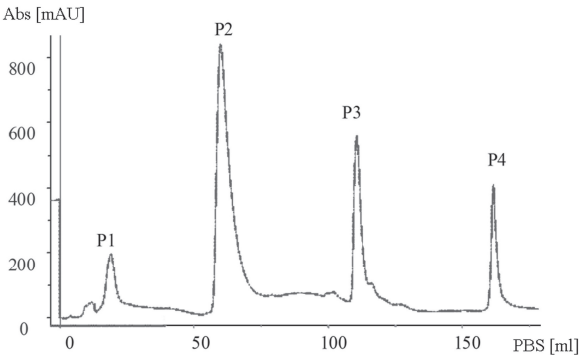

Fig. 4. Purification of PEG-L-ASNase by XK Q Sepharose™ FF column chromatography.

gram. These four peaks were collected and analysed as described in Materials and Methods. P1, the fraction of non-adsorbed material, contained no protein but only free PEG as detected by the iodine staining method (Nag *et al.*, 1996). P4 was also found to contain no protein but rather some low-molecular weight impurities. P2 and P3 contained both enzymes. SDS-PAGE analysis showed that P2 contained both PEGylated enzyme and unmodified L-ASNase (Fig. 5, lane 1 and lane 2), whereas P3 contained only unmodified L-ASNase (Fig. 5, lane 3). The tailing of P2 indicated that it had some unreacted L-ASNase. A sample of P2 was collected and further purified by gel-filtration chromatography.

As shown in Fig. 6, the retention time of P2 (PEG-L-ASNase) was 23.4 min (Fig. 6b), while that of native L-ASNase was 30.1 min (Fig. 6a). In size-exclusion chromatography (Fig. 6b), the peak had a narrow and symmetrical shape. This indicated that the target purified PEG-L-ASNase was of high purity and homogeneity. The purity of the sample was estimated to be in the range of 98% by comparison of the two peak areas. SDS-PAGE separated purified PEG-L-ASNase into two bands (Fig. 5, lane 4), likely indicating that some subunits of L-ASNase were not conjugated with PEG.

Determination of residual amino groups and enzyme activity of PEG-L-ASNase

From Fig. 7, in order to determine the percentage of modified enzymes, two equations were used:

linear equation of purified PEG-L-ASNase: $y = 1.9786x + 3.7429$, $R^2 = 0.9916$;

linear equation of native L-ASNase: $y = 2.0429x + 4.3429$, $R^2 = 0.9978$.

The percentage of modified enzyme can be calculated as follows:

percentage of PEG-L-ASNase = 1 – (linear slope of equation of PEG-L-ASNase/linear slope of equation of native L-ASNase) · 100% = 3.76%.

The activity retained in PEG-L-ASNase was determined according to the method of Matsuyama *et al.* (1991). PEG-L-ASNase had an activity of 89.659 IU/mg, while native L-ASNase had 205.901 IU/mg. Thus 43.545% of the enzyme activity were retained in the PEGylated enzyme.

Isoelectric focusing electrophoresis (IFE) of PEG-L-ASNase

IFE is used to isolate and characterize molecules by their different PI value. According to the

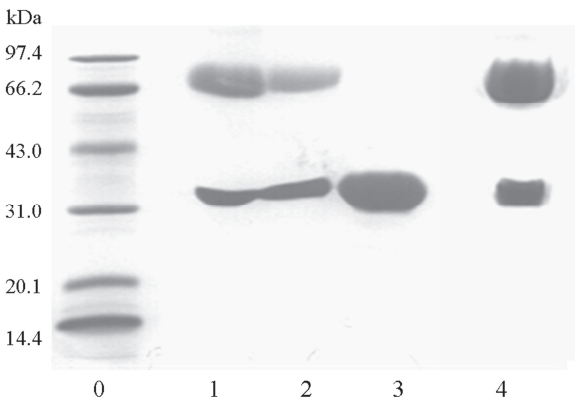

Fig. 5. SDS-PAGE of samples separated by XK Q Sepharose™ FF column chromatography. Lane 0, marker; lane 1, P2; lane 2, tailor of P2; lane 3, P3; lane 4, purified PEG-L-ASNase.

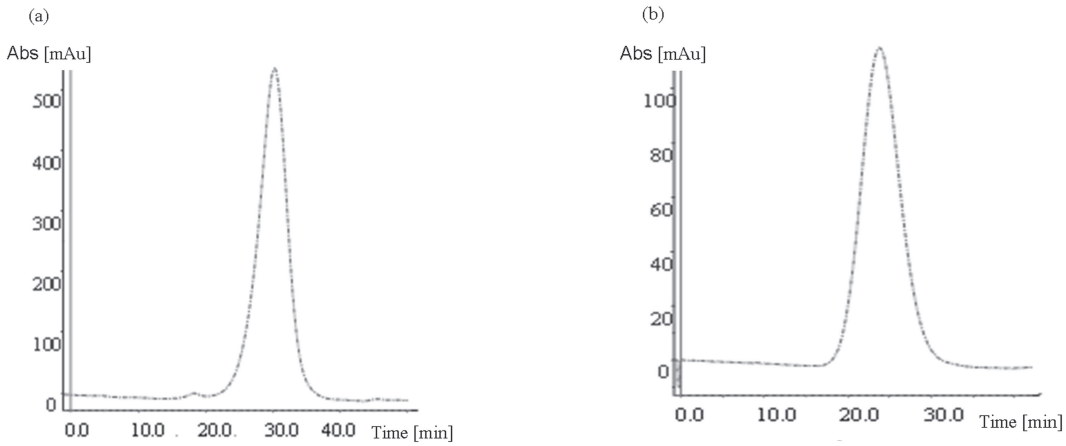

Fig. 6. (a) Chromatography of native L-ASNase on a Tricorn Sephacryl S-300 HR column. (b) Chormatography of P2 on a Tricorn Sephacryl S-300 HR column.

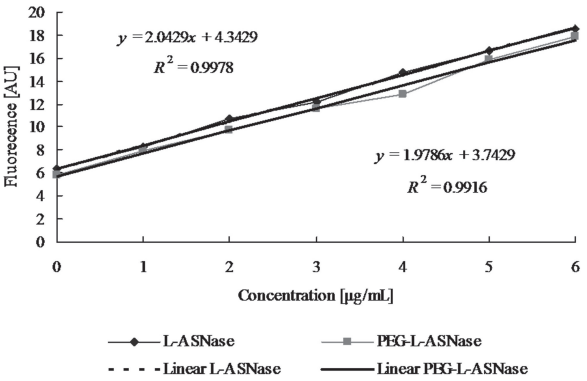

Fig. 7. Enzyme activity of PEG-L-ASNase and native L-ASNase as a function of enzyme concentration.

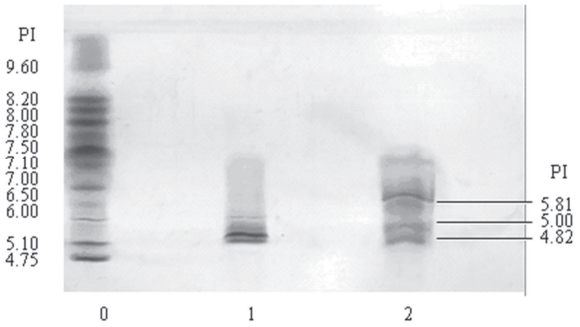

Fig. 8. Isoelectric focusing electrophoresis of L-ASNase and PEG-L-ASNase. Lane 0, PI marker; lane 1, native L-ASNase; lane 2, PEG-L-ASNase.

IFE (Fig. 8), the PI of native L-ASNase was 4.88. The three bands of PI, 5.81 (main), 5.00, and 4.82, thus are from PEGylated enzyme.

Discussion

This work focuses on the preparation method and reaction parameters of N-terminal site-specific PEGylated L-ASNase retaining high enzymatic activity. The prolonged half-life of PEGylated proteins often compensates for the reduction of biologic activity *in vivo*, leading to an overall increase in their therapeutic effects (Yoshioka *et al*., 2004). Alkylation, which is a convenient way for conjugation when the positive charge of the amino group is critical for the retention of biological activity, was chosen here to obtain mono-N-terminal site-specific PEGylated L-ASNase. However, the reaction rate of the formation of the Schiff base is relatively low and the pH value of the reaction mixture is critical for selective α-amino terminal modification (Veronese, 2001). It is crucial to find optimum conditions for the modification. Therefore, the reaction parameters, *i.e.* the molar ratio of alkylated PEG to enzyme, pH value, reaction time, and temperature, have been investigated in this study.

The amount of PEG had significant effects on the degree of modification of the α-amino groups of the enzyme (Fig. 1). It was highest at a ratio of 11:1. However, a higher molar ratio of mPEG-ALD$_{20000}$ to enzyme resulted in higher

polymorphism (Fig. 1, lanes 5 to 8). Thus, mono-PEGylation was obtained at a molar ratio of 10:1. It is well-known that the pH value of the reaction plays a critical role in the extent and site of PEGylation, especially by alkylation. Different from acylating PEGs, PEG-aldehyde does not modify the charge of amino residues but forms a Schiff base which is then reduced with cyanoborohydride. We observed in this study that mono-N-terminal PEGylation of L-ASNase was obtained by conjugating methoxy-PEG derivatives at acidic pH conditions, *i.e.* pH 5.0 (Fig. 2, lane 4). Under acidic conditions, N-terminal α-amino groups are more reactive than the ε-amine groups in lysine residues (Lee *et al.*, 2003). The pH value not only affected the extent of enzyme modification but also reaction sites. As shown in Fig. 2, the B-subunit of L-ASNase was selectively modified. Thus, site-specific mono-PEGylation can be achieved by controlling the reaction conditions.

After PEGylation, the modified enzyme must be separated from PEG, unreacted enzyme, and multiple PEGylated enzymes. Due to the molecular weights of L-ASNase (141 kDa) and mPEG-ALD_{20000} (20 kDa), modified enzyme was difficult to get well separated from native enzyme by molecular-size chromatography only. Besides, some physicochemical properties of the parent molecules such as surface charge and molecular morphology in solution are also changed by PEGylation (Lee *et al.*, 2003). Therefore, a two-step strategy of anion-exchange chromatography and molecular size-exclusion chromatography was utilized in the present study. In the anion-exchange chromatography, we found that PEGylated enzymes are eluted in low-salt buffer. Mono-PEGylated enzyme was separated from higher PEGylated enzyme, unreacted enzyme, as well as free PEG or low-molecular weight impurities by anion-exchange chromatography (Fig. 4). Native L-ASNase is a tetramer of four dimers. However, it can form hexamers in elution buffer (Pritsa and Kyriakidis, 2001). Due to the strong hydrophilicity of mPEG-ALD_{20000}, the apparent molecular weight is higher than the actual molecular weight in the mobile phase buffer (Jackson *et al.*, 1987). L-ASNase eluted later than PEGylated L-ASNase (Fig. 6). Therefore, binding forces between enzymes conjugated with PEG and side chains of matrix were weakened in ion-exchange chromatography due to steric hindrance effects.

IFE separates proteins by their specific PI values. Due to different PEGylation sites, PEGylation proteins with the same molecular weights may be resolved into several bands in IFE. We found that purified PEG-L-ASNase consisted of three isomeric compounds in this study (Fig. 8). In a typical protein PEGylation via modification of lysine and N-terminal amino groups, respectively, PEG molecules attached to one of several potential sites on the protein, each attachment location defining a different isotype (Nag *et al.*, 1996). Thus, mono-PEGylated proteins may have more than one isomeric form and the different PEG isotypes have interesting implications in the drug development process.

Conclusions

The alkylating PEGylation of L-ASNase at the N-terminal α-amino groups was optimized. The reaction conditions were a pH value of 5.0, a molecular ratio of mPEG-ALD_{20000} to L-ASNase of 10:1, a reaction time of 16 h, and a temperature of 25 °C. The two-step purification procedure with anion-exchange (Q Sepharose FF) and gel-filtration (Sephacryl S-300 HR) chromatography can be utilized to purify PEG-L-ASNase. The retained enzyme activity was found to be 43.5%. This method of alkylating PEGylation and the purification strategy may be useful in the further application of this enzyme.

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