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Tailoring structure-function properties of L-asparaginase: Engineering resistance to trypsin cleavage.

by

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RUNNING TITLE:

Engineering resistance to trypsin cleavage into *E. carotovora* L-asparaginase

KEY-WORDS:

L-asparaginase; hydrolase; enzyme engineering; leukaemia; PEG; PEGylation; proteolytic resistance.

ABBREVIATIONS USED:

ALL, acute lymphoblastic leukaemia; *Eca*L-ASNase, L-asparaginase from *Erwinia carotovora*; GDH, glutamate dehydrogenase; MALDI-TOF, Matrix Assisted Laser Desorption/Ionization-Time of Flight; mPEG-SNHS, methoxypolyethylene glycol succinate N-hydroxysuccinimide ester; ORF, open reading frame; R206H, Arg206His mutant enzyme; PEG, poly(ethylene glycol); PAGE, polyacryamide gel electrophoresis; PEG-*Eca*L-ASNase, PEGylated *Eca*L-ASNase; PEG-Arg206His, PEGylated Arg206His mutant enzyme; SDS, sodium dodecyl sulfate; Suc, succinic acid; FDA, Food and Drug Administration.

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ABSTRACT

Bacterial L-asparaginases (L-ASNases) catalyze the conversion of L-asparagine to L-aspartate and ammonia, and are widely used for the treatment of acute lymphoblastic leukemia (ALL). In the present study we describe an efficient approach, based on protein chemistry and protein engineering studies, for the construction of trypsin-resistant PEGylated L-ASNase from *Erwinia carotovora* (*Eca*L-ASNase). Limited proteolysis of *Eca*L-ASNase with trypsin was found to be associated with a first cleavage of the peptide bond between Lys53 and Gly54, and then a second cleavage at the Arg206-Ser207 position of the C-terminal fragment, peptide 54-327, showing that the initial recognition sites for trypsin are Lys53 and Arg206. Site-directed mutagenesis of Arg206 to His followed by covalent coupling of methoxypolyethylene glycol succinate N-hydroxysuccinimide ester (mPEG-SNHS) to the mutant enzyme resulted in an improved modified form of *Eca*L-ASNase that retains 82% of the original catalytic activity, exhibits enhanced resistance to trypsin degradation, and has higher thermal stability compared to the wild-type enzyme.

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INTRODUCTION

L-Asparaginase (L-ASNase) catalyzes the hydrolysis of the non-essential amino acid L-Asn to L-Asp and ammonia. L-ASNase is widely used for the treatment of haematopoetic diseases such as acute lymphoblastic leukemia (ALL). The anti-neoplastic activity results from depletion of the circulating pools of L-Asn by L-asparaginase [1,2]. Unlike normal cells, malignant cells can only synthesize L-Asn slowly and are dependent on an exogenous supply [1,2]. In contrast, normal cells are protected from Asn starvation by their ability to produce this amino acid [3]. Thus, unlike conventional cancer therapy, L-ASNase therapy is highly selective.

The main restrictions to the use of L-ASNase as a therapeutic agent include its premature inactivation and rapid clearance, thus necessitating frequent injections to maintain therapeutic levels, and several types of side effects ranging from mild allergies and development of immune responses to anaphylactic shock, which may be life-threatening [4-7]. To date, the most promising approach to extend the half-life and reduce the immunogenicity and antigenicity of therapeutic proteins is to covalently couple them to poly(ethylene glycol) (PEG) [8-10]. In this process (PEGylation), PEG is attached to the therapeutic protein by covalent linkage to residues located at the surface of the native protein [11]. PEGylation of proteins usually results in masking of some surface sites, increasing the molecular size and enhancing steric hindrance. Thus, attachment of PEG to proteins reduces their immunogenicity, improves pharmacokinetics [12,13], plasma half-life, and protects the proteins against proteolytic cleavage [14-17]. The *in vivo* activity of the modified protein can sometimes be enhanced due to longer half-life in the circulation after conjugation, even though there may be some reduction in *in vitro* activity.

PEGylation has been extensively used to modify various proteins such as adenosine deaminase [18], superoxide dismutase [19], asparaginase [20], urokinase [21], hirudin [22], hemoglobin [23], interferon [24], etc. In the USA, there are currently six polymer-modified proteins that have already been approved by the FDA and about a dozen more in various phases of clinical development [25].

The toxicity of L-ASNase is also partially attributable to the glutaminase activity of these enzymes [26]. Thus, L-ASNases with high specificity for L-Asn and low-to-negligible activity against L-Gln are reported to be less troublesome during the course of anti-cancer therapy [27]. The interest in L-asparaginase from *Erwinia*

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carotovora arose from the fact that it shows reduced glutaminase activity, and it is therefore believed to have fewer side effects when used in anti-cancer therapy [28].

All asparaginases are homotetramers with 222-symmetry and a molecular mass in the range of 140–150 kDa, with a highly conserved overall fold [29-31]. They are composed of four identical subunits denoted A, B, C, and D [32]. Each monomer consists of about 330 amino acid residues that form 14 β-strands and 8 α-helices, which are arranged in two easily identifiable domains: the larger N-terminal domain and the smaller C-terminal domain. These are connected by a linker consisting of ~20 residues. Each of the four active sites is located between the N- and C-terminal domains of two adjacent monomers. Thus, the asparaginase tetramer can be treated as a dimer of dimers, as the active site is derived from subunits A and C or from subunits B and D.

For many pharmaceutical proteins, enzymatic proteolysis makes maintaining appropriate serum levels problematic. The use of L-ASNase in therapy is limited by its premature inactivation. For example, it has been reported that serum trypsin levels increase significantly 10 and 20 days after the start of ASNase therapy [33,34]. Here we report an efficient approach for the construction of water soluble and trypsin-resistant PEGylated L-ASNase from *Erwinia carotovora*, based on protein chemistry and protein engineering.

MATERIALS AND METHODS

MATERIALS

L-Asn and L-Gln were obtained from Serva (Heidelberg, Germany). α-ketoglutaric acid, Sepharose CL6B, trypsin and methoxypolyethylene glycol succinate N-hydroxysuccinimide ester (mPEG-SNHS, mol wt ~5000) from Sigma Co. (St Louis, USA). NADH (disodium salt, grade II, ca. 98 %) and crystalline bovine serum albumin (fraction V), were purchased from Boehringer Mannheim (Germany). Nessler's reagent and glutamate dehydrogenase (GDH) were obtained from Fluka (Taufkirchen, Germany) and Sephadex G-25 from Pharmacia (Sweden). All primers were synthesized and purified by MWG-biotech AG (Ebersberg, Germany). TOPO cloning kit and all other molecular biology reagents were from Invitrogen (USA).

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METHODS

Cloning, expression, and purification of *Eca*L-ASNase

L-asparaginase from *Erwinia carotovora* NCYC 1526 (*Eca*L-ASNase) was cloned into a T7 expression vector (pCR®T7/CT-TOPO®), as described by Kotzia and Labrou [35]. The resulting expression construct pT7ASNase was used to transform competent BL21(DE3)pLysS *E. coli* cells, in which the synthesis of *Eca*L-ASNase was induced by 1 mM IPTG. The produced enzyme was purified from the other proteins using affinity chromatography on L-Asn-Sepharose CL6B column, according to Kotzia and Labrou [35].

Kinetic analysis of mutants and PEGylated enzymes

Enzyme assays were performed at 37 °C at a Hitachi U-2000 double beam uv-vis spectrophotometer carrying a thermostated cell holder (10 mm pathlength). Activities were measured by determining the rate of ammonia formation, by coupling with glutamate dehydrogenase (GDH), according to Balcao et al. [36]. The final assay volume of 1 ml contained 71 mM Tris-HCl buffer, pH 8.0; 1 mM Asn; 0.15 mM α-ketoglutaric acid; 0.15 mM NADH; 4 units GDH, and sample containing *Eca*L-ASNase activity. Alternatively, the rate of ammonia formation was measured at 37 ºC using the Nessler's reagent. One unit of Asparaginase activity is defined as the amount of enzyme that liberates 1 μmol of ammonia from Asn per minute at 37 °C. Protein concentration was determined at 25 °C by the method of Bradford [37] using bovine serum albumin (fraction V) as standard.

Steady-state kinetic measurements were performed in 0.1 M Tris-HCl buffer, pH 8.0 (or 8.2 for L-Gln), by varying the concentration of the substrate (L-Asn and L-Gln). The kinetic parameters were calculated by non-linear regression analysis of experimental steady-state data. Kinetic data were analyzed using the computer program GraFit (Erithacus Software Ltd., [38]). Kinetic experiments were repeated three times to verify the results.

Limited proteolysis of *Eca*L-ASNase by trypsin

*Eca*L-ASNase, in 10 mM Tris-HCl pH 7.4, was subjected to trypsin digestion. Proteolysis was initiated by adding trypsin (dissolved in 1 mM HCl, containing 1 mM

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$CaCl_2$) to *Eca*L-ASNase at a L-ASNase/trypsin ratio of 1:35 (w/w). Samples were withdrawn at selected incubation intervals and the trypsinolysis was stopped by freezing the samples with liquid nitrogen. All samples were lyophilized and then analyzed by SDS-PAGE.

Site-directed mutagenesis, expression and purification of mutants

Site-directed mutagenesis of Arg206 was carried out by overlap extension using the polymerase chain reaction (PCR) [39]. The pairs of oligonucleotide primers used in the PCR reactions for the Arg206His or Arg206Gln mutation were as follows: the first pair 5'-ATG TTT AAC GCA TTA TTC GTT GTT GTT TTT G -3' (P_1) and 5'-AAA CAC GGA *NTG* CGT GGT GTG AAC-3' (P_2), and the second pair 5'-GTT CAC ACC ACG *CAN* TCC GTG TTT-3' (P_3) and 5'-TTA AGC TTT TAA TAA GCG TGG AAG TAA TCC -3' (P_4). Sites of mutation are indicated in italics. The expression construct pT7ASNase [35] encoding the wild-type *Eca*L-ASNase was used as template DNA in all mutagenesis reactions. After completion of the PCR reactions (using primers P1, P2 and P3, P4), the PCR products were digested with DpnI to eliminate parental DNA, and purified by agarose-gel electrophoresis. Following gel extraction, the fragments containing either mutation (Arg206His or Arg206Gln) were used in another PCR reaction as templates using P_1 and P_4 primers to amplify the entire mutated gene. The latter was TOPO ligated into a T7 expression vector (pCR®T7/CT-TOPO®) and recombinant plasmids were isolated. Site-directed mutagenesis of Lys30 was carried out as described above. The pairs of oligonucleotide primers used in the PCR reactions for the Lys30Ala mutation were as follows: 5'-ACCACTGGGTAT*GCA*GCGGGTGCGC-3' and 5'-CGCACCCGC*TGC*ATACCCAGTGGT-3'. All mutations were verified by DNA sequencing.

Mutated expression constructs were used to transform competent BL21(DE3)pLysS *E. coli* cells. The *E. coli* cells were grown at 37 °C in 1 l LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The synthesis of the mutated enzymes was induced by the addition of 1 mM IPTG when the absorbance at 600 nm was 0.6-0.8. Five hours after induction, cells were harvested by centrifugation at 8000 r.p.m. and 4 ºC for 20 min, resuspended in 5 mM potassium phosphate buffer, pH 7.5,

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sonicated and left at 4 ºC under agitation. After 1h, cells were centrifuged at 10000g for 5 min and the supernatants were collected. Cell-free extracts of *E. coli* transformants showed high asparaginase activity. The collected supernatants were applied to L-Asn-Sepharose CL6B affinity column (2 ml, 1.5 x 1.5 cm I.D.) previously equilibrated with 5 mM potassium phosphate buffer, pH 7.5 [35]. Non-adsorbed protein was washed off with 20 ml equilibration buffer. Bound L-ASNase (Arg206His or Arg206Gln) was eluted with equilibration buffer containing 10 mM L-Asn (6 ml). Collected fractions (1 ml) were assayed for L-ASNase activity and protein (A_{280} or Bradford [37]). The results from a typical purification run showed for the Arg206Gln, 75.4-fold purification with 95.3% yield, for the Arg206His mutant showed 119.7-fold purification with 96.5% yield and for the Lys30Ala mutant 110-fold purification with 97% yield.

Modification of the *Eca*L-ASNase and mutant Arg206His with mPEG-SNHS and purification of the PEGylated enzymes

Purified L-ASNase (*Eca*L-ASNase and/or its mutant Arg206His, 25µg), in 50 mM potassium phosphate buffer pH 8.5, was mixed with different amounts (0.28mg, 0.7mg, 1.15mg, 2.14mg and 4.28mg) of mPEG-SNHS (methoxypolyethylene glycol succinate N-hydroxysuccinimide ester, MW 5000 Da) in order to determine the necessary amount for complete modification. Modification reactions were allowed to proceed for 22h at 4 ºC, before they were stopped by the addition of 12 mM glycine, pH 8. The effect of PEGylation on enzyme activity was evaluated by taking samples at various time intervals during the modification reaction and measuring the residual activity, using the coupled enzyme assay method described above. The purification of PEG-*Eca*L-ASNase and/or PEG-Arg206His was carried out using size exclusion chromatography on a Sephadex G-25 column (9.1 ml, 1.5 x 5 cm I.D.), equilibrated with 10 mM Tris-HCl buffer, pH 7.4 and eluted with the same buffer at a flow rate of 0.5 ml/min. The samples were analyzed by SDS-PAGE and MALDI-TOF MS as described below. Residues' accessibility was determined *in silico* using the WHAT IF software package [40].

Succinylation

*Eca*ASNase was succinylated with succinic anhydride as described in [41] with the following modifications: succinic anhydride crystals were added to 10 mM final

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concentration over a period of 1 h to a solution of 0.2 mg of the enzyme in 0.1 M potassium phosphate buffer (pH 8.5) at 4°C. The solution was constantly stirred and maintained at pH 8.5 by the addition of 0.01 M NaOH. Modification reaction was allowed to proceed for 6h at 4 ºC, before it was stopped by the addition of 12 mM glycine, pH 9. The extent of reaction with lysyl residues was determined from titration of free amino groups with trinitrobenzene sulfonic acid [42].

Limited proteolysis with trypsin of Arg206His and PEGylated enzymes

Agr206His, PEG-*Eca*L-ASNase and PEG-Arg206His, previously equilibrated in 10 mM Tris-HCl pH 7.4, were subjected to trypsin digestion. Proteolysis was carried out using L-ASNase/trypsin ratio of 1:35 (w/w). All samples were lyophilized and then analyzed by SDS-PAGE. Gels run with a Laemmli buffer system [43]. The intensities of bands were quantified with the 1D Image Analysis Software (Version 3.5, Kodak) analysis software. The intensity change versus time was fit to the first-order rate equation 1:

$$I = I_0 \, e^{-k_{obs}t} \qquad \text{equation 1}$$

where I is the intensity at time t, I_0 is the intensity at t=0, and k_{obs} is a first-order rate constant.

Thermal stability of *Eca*L-ASNase, Arg206His and PEGylated enzymes

Irreversible thermal inactivation of *Eca*L-ASNase, Arg206His and PEGylated enzymes was monitored by activity measurements. Samples of the enzymes, in 50 mM potassium phosphate buffer pH 8.5, were incubated at different temperatures (35 ºC-50 ºC) for 7.5 min. Subsequently the samples were assayed for residual activity, using the coupled enzyme assay method described above. The T_m values were determined from the plots of relative inactivation (%) *versus* temperature (°C). T_m value is the temperature at which 50% of the initial enzyme activity is lost after heat treatment.

The time course of thermal inactivation of *Eca*L-ASNase, Arg206His and PEGylated enzymes, in 50 mM potassium phosphate buffer pH 8.5, was studied at 37°C. The rate of inactivation was followed by periodically removing samples for assay of enzymatic

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activity. Observed rates of inactivation (k_{in}) were deduced from plots of log (% of remaining activity) versus time (h). Thermal inactivation experiments were repeated three times to verify the results.

MALDI-TOF MS

The samples were desalted with ZipTip μ-C18 (Millipore, USA). A saturated solution of sinapinic acid in water/acetonitrile (50/50, v/v, 0.1 % trifluoroacetic acid) was used as the matrix solution. One μL of the sample and matrix mixture was spotted into a well of the sample plate and dried on the sample holder. Mass spectra were taken with a Voyager System 6322 (Applied Biosystems). Mass calibration was performed using bovine serum albumin, apomyoglobin and cytochrome c.

N-terminal amino acid sequence analysis

N-terminal amino acid sequence analysis of the proteolytic products resulting from the trypsin digestion of the *Eca*L-ASNase was carried out on a gas-phase Applied 542 Biosystems Protein sequencer, model 470A, equipped with an on-line phenylthiohydantoin analyzer.

Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli [43] on a slab gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Brilliant Blue R-250 or with silver nitrate.

RESULTS and DISCUSSION

Limited proteolysis of *Eca*L-ASNase

To obtain information about the protease-sensitive cleavage sites in *Eca*L-ASNase, limited proteolysis with trypsin was carried out. To follow the time course of formation and disappearance of proteolytic fragments, samples were withdrawn from the tryptic digestion mixture at intervals and subjected to SDS-PAGE for separation of the proteolytic products (Figure 1A). After 30 min of incubation with trypsin, one main fragment (~31.5 kDa) was easily identifiable by SDS-PAGE. As the digestion proceeded, the amount of this fragment decreased progressively with time, with concomitant formation of another smaller fragment with a molecular mass

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corresponding to ~25 kDa. N-terminal amino acid sequencing analysis of the two fragments gave two sequences: Gly-Glu-Gln-Val-Ala and Ser-Val-Phe-Asp-Val, corresponding to residues 54–58 and 207–211 of *Eca*L-ASNase for the 31.5 and 25 kDa fragments, respectively. Trypsin cleavage results in peptides with C-terminal Lys or Arg peptides. These results indicate that trypsin initially cleaves *Eca*L-ASNase at Lys53-Gly54, and then the C-terminal fragment, peptide 54–327, is cleaved again at the Arg206-Ser207 position. The loss of enzyme activity following trypsin treatment is a direct result of this cleavage, since the peptide fragment released contains the main part of the active site of the enzyme [35].

The locations of the two cleavage sites in *Eca*L-ASNase are illustrated in Figure 1B. Both cleavage sites are positioned at the external surface of *Eca*L-ASNase. In particular, Lys53 is located at the short β-sheet formed by residues Asn51-Val57. Arg206 is located at a flexible segment, formed by residues Asp201-Asp221, which connects the N-terminal and C-terminal domains (Figure 1B).

Our results thus showed that in order to engineer resistance to trypsin cleavage into *Eca*L-ASNase, two main amino acid targets should be considered: Lys53 and Arg206. Taking into account that Lys53 is located at a surface accessible to the solvent and that this residue can be efficiently protected through chemical modification with succinimide esters of poly(ethylene glycol), Arg206 was selected for site-directed mutagenesis.

Site-directed mutagenesis of *Eca*L-ASNase

On the basis of limited proteolysis and computational analysis studies, Arg206 was selected for site-directed mutagenesis. This residue was mutated to the conserved residues His and Gln, which are not recognized targets of trypsin. Kinetic analysis of the mutants was carried out to evaluate the effect of each mutation on substrate binding and catalysis, and the results are given in Table 1. Arg206His showed differences in K_m and specific activity values for L-Asn when compared to the wild-type enzyme. In particular, mutant Arg206His showed an increase in K_m value of about 6.4-fold for L-Asn and a slight decrease in specific activity (~9%) compared to the wild-type enzyme. Arg206Gln gave low expression levels and worse kinetic properties compared to Arg206His (data not shown); thus, it was not considered further.

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The mutant Arg206His was subjected to limited proteolysis with trypsin, under the same conditions as used for the wild-type enzyme, in order to verify the absence of the ~25 kDa fragment resulting from trypsin digestion. SDS-PAGE analysis of the tryptic digestion mixture showed that after 90 min of incubation, no band corresponding to ~25 kDa was formed (Figure 2).

PEGylation of *Eca*L-ASNase and the Arg206His mutant enzyme

PEGylation has become one of the most extensively used techniques, and has attracted increasing attention for enhancement of the therapeutic potential of protein-based pharmaceuticals [18-25]. Recent advances in recombinant technology and the development of functional group-selective reactive PEG derivatives have led to the creation of PEGylated proteins or mutants with markedly enhanced *in vitro* and *in vivo* characteristics and with minimal loss of bioactivity compared to their unmodified equivalents [13].

Various factors including inherent nucleophilicity, solvation, protein conformation, and also local electronic and pK_a effects determine the actual reactivity of the potential PEGylation sites [44]. The reaction chemistry and the length of PEG chains are the minimum set of variables that can determine the biological activity and stability of the modified protein [7,13]. In the present work, the N-hydroxysuccinamide derivative of PEG (mPEG-SNHS) was employed. mPEG-SNHS allows linkage between PEG and exposed Lys residues and/or the N-terminal amino group in proteins under mild conditions of pH and temperature [45]. This approach is suitable for *Eca*L-ASNase because the enzyme contains twenty lysine residues in addition to the N-terminal amino acid. None of these lysine residues are located in regions of the protein that are directly involved in substrate binding and catalysis [39].

Purified L-ASNases (wild-type and the Arg206His mutant) were subjected to PEGylation using different amounts (0.28–4.28 mg) of mPEG-SNHS in order to determine the optimum [enzyme]/[mPEG-SNHS] ratio for complete enzyme modification. The reactions were allowed to proceed for a long period of time (22 h, 4°C) in order to make sure that complete PEGylation would be occurred. SDS-PAGE analysis of the products of the modification reactions showed that the amount of mPEG-SNHS necessary for complete modification of the enzymes was 4.28 mg mPEG-SNHS/25 μg protein. This ratio promotes the formation of a single modified form of the enzyme with subunit molecular mass of approximately 70.5 kDa, as

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determined by SDS-PAGE analysis (Figure 3, see 'control' lanes). The other concentrations used gave a mixture of different forms with molecular masses between 36.5 to 70.5 kDa, due to incomplete modification (data not shown). The lowest amount used, 0.28 mg mPEG-SNHS, had no significant effect on the enzyme's molecular weight. MALDI-TOF was used to determine precisely the number of PEG molecules attached to the wild-type and mutant enzymes. Analysis of the mass spectra gave mass/charge ratios of 69,492.3 and 69,472.4 for the modified wild-type and mutant enzymes (subunit MW), respectively. Given the molecular weight of PEG and L-ASNase, the molecular weights of the modified enzymes corresponded to hepta-PEG-modified L-ASNases.

The effect of PEGylation (at 4.28 mg mPEG-SNHS/25 μg protein) on the enzyme activity of both the wild-type enzyme and the Arg206His mutant was evaluated by measuring the enzyme activity after the completion of the modification reaction. The PEG-*Eca*L-ASNase retained 78% of its initial activity and the PEG-Arg206His retained 82%. The tendency to lose activity can be attributed to the steric hindrance caused by the conjugation of PEG to the free amino groups on the protein surface, which prevents L-Asn from approaching the active site of the enzyme [46].

The modification of *Eca*L-ASNase and Arg206His with mPEG-SNHS led to a 3.6- to 5-fold increase in K_m value for L-Asn and L-Gln, respectively. The increased K_m value for L-Asn, although undesirable, is still within the range of acceptability for therapeutic applications [47]. On the other hand, the increase in K_m value by 5-fold (24.8 mM) for L-Gln is desirable, since the ability of therapeutic preparations of L-asparaginases to hydrolyze L-Gln has been implicated to cause several side effects [27].

To analyze whether the effect of PEGylation on K_m value (K_m =0.09 ±0.03 mM for L-Asn) is the result of modification of a specific Lys residue or from the structure/chemistry of PEG we employed protein chemistry and site-directed mutagenesis studies. Complete chemical modification of *Eca*L-ASNase by succinic anhydrite results to a succinylated enzyme (fifteen residues succinylated per subunit) which retains unaltered its K_m, in agreement with the results reported by Shifrin et al., [41]. It is apparent that extensive succinylation does not destroy enzyme function and therefore this finding indicates that none of the Lys residue is essential for catalytic activity or substrate binding.

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In silico analysis of residues' accessibility in *Eca*L-ASNase showed that the seven, out of twenty Lys residues, that exhibit high accessible surface area are: Lys30, Lys45, Lys53, Lys145, Lys216, Lys257, Lys269. The other Lys residues are either more buried or lie at the subunit interface. These seven residues are probable the targets of chemical modification by mPEG-SNHS. Analysis of the crystal structure of the enzyme showed that none of these lysine residues are located in regions of the protein implicated in substrate binding and catalysis with the only exception that of Lys30 which lies at the flexible loop (residues 10-40, Figure 1B) near to the enzyme active site [29,31,35]. Site-directed mutagenesis of Lys30 to Ala gave a mutant enzyme with kinetic parameters very close to the wild-type enzyme (K_m = 0.075 ± 0.03 mM for L-Asn). Therefore the results from site-directed mutagenesis and from extensive chemical modification by succinic anhydrite point to the conclusion that the influence of PEGylation on K_m value is an indirect effect and may be described as originating from the overall shielding effect of the PEG chains rather than from the modification of a specific Lys residue. This conclusion is also supported by the observation that Arg206His mutant enzyme, after PEGylation, exhibits K_m values or L-Asn and L-Gln comparable to that of the wild-type enzyme.

Trypsin digestion of PEGylated enzymes

To evaluate the effect of modification with PEG on the rate of trypsinolysis of PEG-*Eca*L-ASNase and the PEG-Arg206His mutant enzyme, limited proteolysis experiments were carried out under the same conditions as those reported for the unmodified enzymes. SDS-PAGE analysis of the products of the trypsinolysis reaction showed that after 90 min of reaction, trypsin had completely digested PEG-*Eca*L-ASNase, whereas PEG-Arg206His showed considerable resistance to proteolysis (Figure 3). The rate of breakdown of both enzymes was monitored by quantifying the amount of intact enzyme on SDS-PAGE gels as a function of incubation time. The decrease in intensity of the enzyme band was fitted to a first-order rate equation. The observed rates of digestion (k_{obs}) were found to be 1.54 ± 0.18 and 0.1 ± 0.12 for PEG-*Eca*L-ASNase and PEG-Arg206His, respectively. The protective effect of PEGylation has been described as originating from a shell of PEG chains surrounding the protein, with the shielding effect of the PEG chains contributing to the inaccessibility of the enzyme to trypsin [12,13].

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Thermal stability of *Eca*L-ASNase, Arg206His and PEGylated enzymes

To determine the structural stability of *Eca*L-ASNase, Arg206His, and the PEGylated enzymes, heat inactivation studies were carried out. The enzymes were incubated at different temperatures and subsequently assayed for residual activity (Figure 4). The corresponding T_m values are listed in Table 2.

The time course of thermal inactivation of *Eca*L-ASNase, Arg206His, and the PEGylated enzymes was also investigated at the temperature of therapeutic importance, i.e. 37ºC. The results indicated that the PEGylated enzymes are particularly stable at 37ºC whereas the *Eca*L-ASNase and Arg206His mutant enzyme have significantly lower thermostability. Kinetic analysis of the thermal inactivation at 37ºC gave linear plots (Figure 5), from which the first-order inactivation rate constants k_{in} could be calculated according to equation 3:

$$\log(\%\text{remaining activity}) = 2.303 \cdot k_{in} \cdot t \qquad \text{equation 3}$$

The results are summarized in Table 2. The PEGylated enzymes showed a dramatic improvement in thermal stability at 37ºC, which is about 30.7-fold and 55.6-fold higher (for PEG-*Eca*L-ASNase and PEG-Arg206His, respectively) than for non-PEGylated enzymes. It is believed that PEGylation usually enhances the thermal stability of proteins by sterically blocking degradation pathways induced by hydrophobic interactions [48], and by causing non-specific steric hindrance of the intermolecular interactions that are involved in thermal instabilities [49].

Conclusion

A short half-life in serum is a common trait of most proteins being used or developed for therapeutic purposes. Proteolysis is being increasingly acknowledged to play a significant role in the clearance of therapeutic proteins. Thus, the design of novel proteins that are resistant to digestion, represents important strategy for the creation of next-generation therapeutic products with improved pharmacological properties. In the present study, the *Eca*L-ASNase was genetically modified by site-directed mutagenesis and chemically modified with mPEG-SNHS. The modified form of the mutant enzyme (PEG-Arg206His) was found to have improved proteolytic stability, good catalytic activity for L-Asn, and high structural stability. This enzyme may be

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tested further on animals and/or humans in order to create a new drug for future therapeutic use.

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Table 1 Kinetic parameters of PEGylated and non-PEGylated enzymes

Steady-state kinetic measurements were performed at 37 ^{o}C. All initial velocities were determined in triplicate. The kinetic parameter K_m was calculated by non-linear regression analysis of experimental steady-state data using the GraFit (Erithacus Software Ltd.) program [38]. The specific activity was estimated as % of the wild-type *Eca*L-ASNase [35].

Substrates	Enzymes	Specific Activity (% of *Eca*L-ASNase)	K_m (mM)
L-Asn	*Eca*L-ASNase	100	0.085 ± 0.02*
	Arg206His	90.9 ± 1.9	0.55 ± 0.09
	PEG-*Eca*L-ASNase	78.0 ± 5.0	0.31 ± 0.05
	PEG-R206His	74.5 ± 2.7	0.36 ± 0.07
L-Gln	*Eca*L-ASNase	100	6.80 ± 1.32*
	Arg206His	137.5 ± 3.5	4.94 ± 0.58
	PEG-*Eca*L-ASNase	92.0 ± 2.6	24.69 ± 5.55
	PEG-R206His	83.6 ± 1.7	24.84 ± 4.90

* According to Kotzia and Labrou [35]

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Table 2 T_m (ºC) and k_{in} (h^{-1}, at 37 ºC) values of PEGylated and non-PEGylated enzymes.

Enzymes	T_m (ºC)	k_{in} (h^{-1}) x 10^{-3}
*Eca*L-ASNase	38.9±0.45	347.2±44.6
Arg206His	38.8±0.84	361.6±45.7
PEG-*Eca*L-ASNase	42.9±0.25	11.3±0.8
PEG- Arg206His	43.1±0.45	6.5±0.9

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FIGURE LEGENDS

Figure 1

A. Time course of tryptic digestion of *Eca*L-ASNase

Analysis was carried out by SDS-PAGE. Proteolysis was initiated by adding trypsin to the enzyme at an L-ASNase/trypsin weight ratio of 1:35 (w/w). Conditions are shown above lanes. A sample taken immediately after the addition of trypsin to *Eca*L-ASNase was used as control. The experiment was performed three times to verify the results.

B. Structural representations of limited proteolysis sites and critical PEGylation site

Lys30, Lys53 and Arg206 are shown in space-filling mode and are labelled. The figure was created based on the crystal structure of *E. carotovora* L-ASNase (PDB code 1ZCF) [29].

Figure 2 Time course of tryptic digestion of Arg206His mutant enzyme

Analysis was carried out by SDS-PAGE. Proteolysis was initiated by adding trypsin to the mutant enzyme at Arg206His/trypsin weight ratio of 1:35 (w/w). Conditions are shown above lanes. A sample taken immediately after the addition of trypsin to Arg206His mutant enzyme was used as control. The experiment was performed three times to verify the results.

Figure 3 Time course of tryptic digestion of PEGylated enzymes

Analysis was carried out by SDS-PAGE. Conditions are shown above lanes. A sample taken immediately after the addition of trypsin to purified PEGylated enzymes were used as control. The experiment was performed three times to verify the results.

Figure 4 Thermal inactivation curves of PEGylated and non PEGylated enzymes

The residual activities of the enzymes were measured after heat treatment at various temperatures for 7.5 min. *Eca*L-ASNase, (●); Arg206His mutant enzyme, (□); PEG-*Eca*L-ASNase, (■); and PEG-Arg206His mutant enzyme, (○). The data represent a mean of three independent experiments.

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Figure 5 Time course of inactivation of PEGylated and non PEGylated enzymes at 37 ºC

Time course of inactivation at 37 ºC of *Eca*L-ASNase, (□); Arg206His mutant enzyme (▲); PEG-*Eca*L-ASNase (■); and PEG-Arg206His mutant enzyme (●). The rate of inactivation was followed by periodically removing samples for assay of enzymatic activity. The data represent a mean of three independent experiments.

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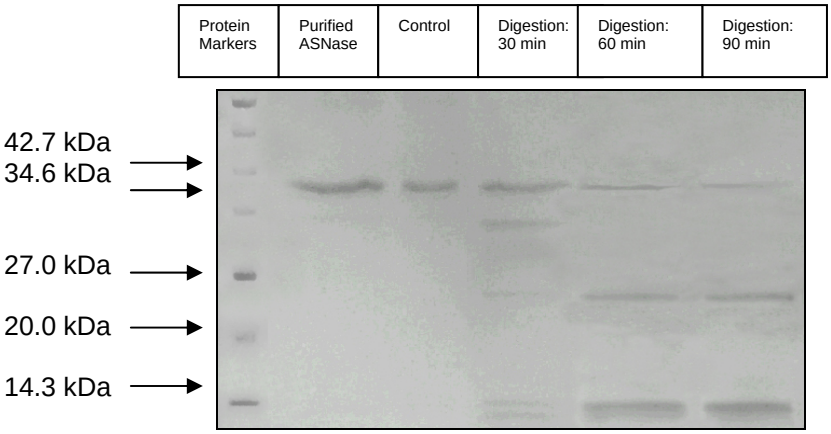

Figure 1A

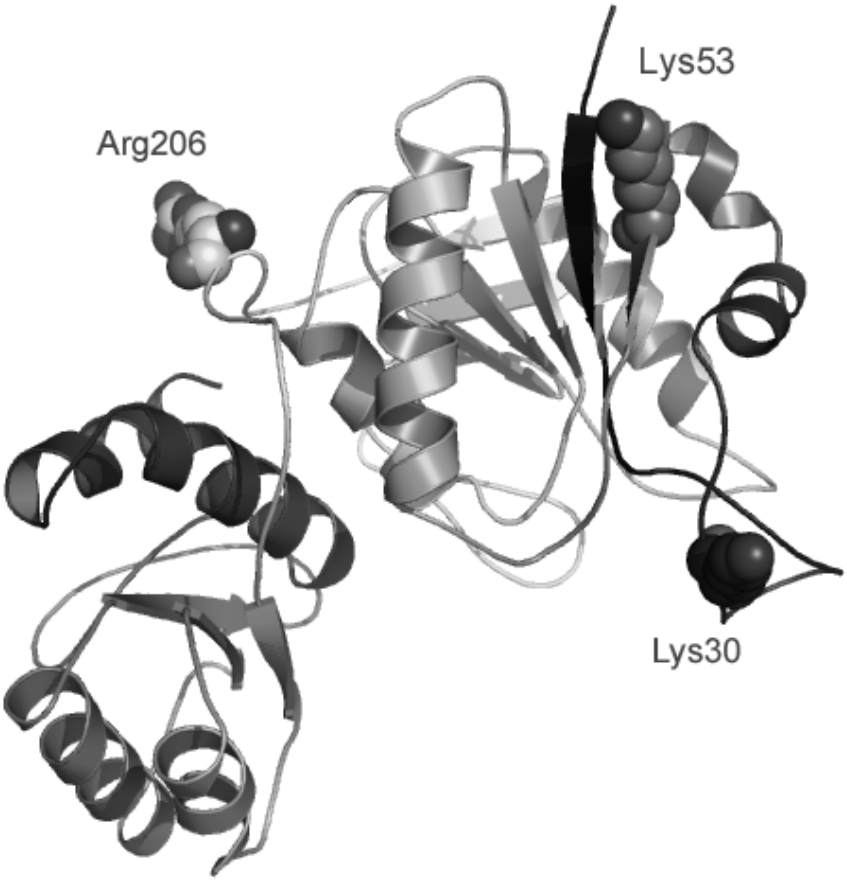

Figure 1B

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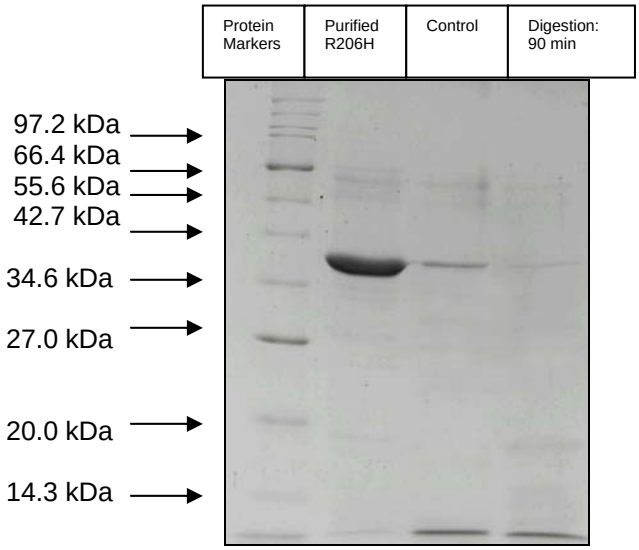

Figure 2

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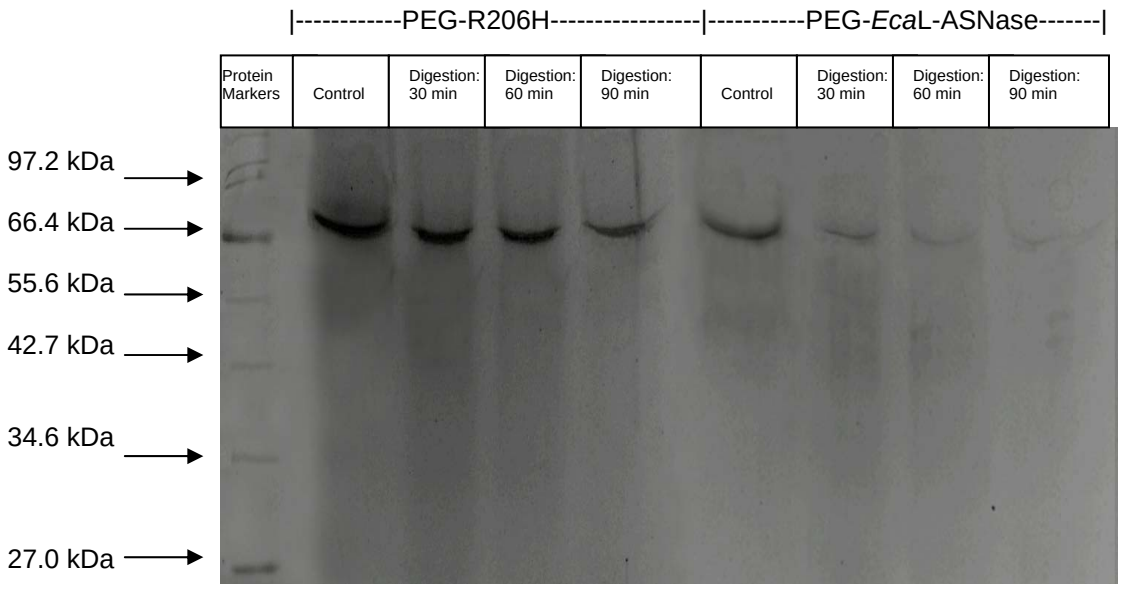

Figure 3

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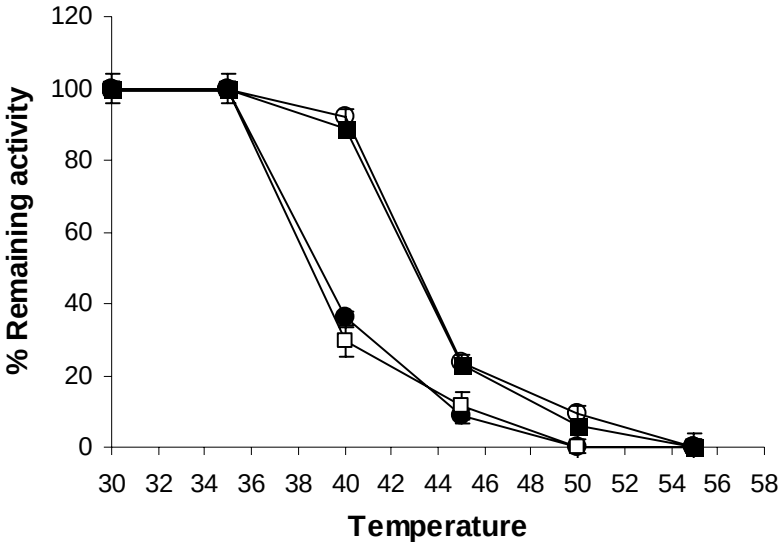

Figure 4

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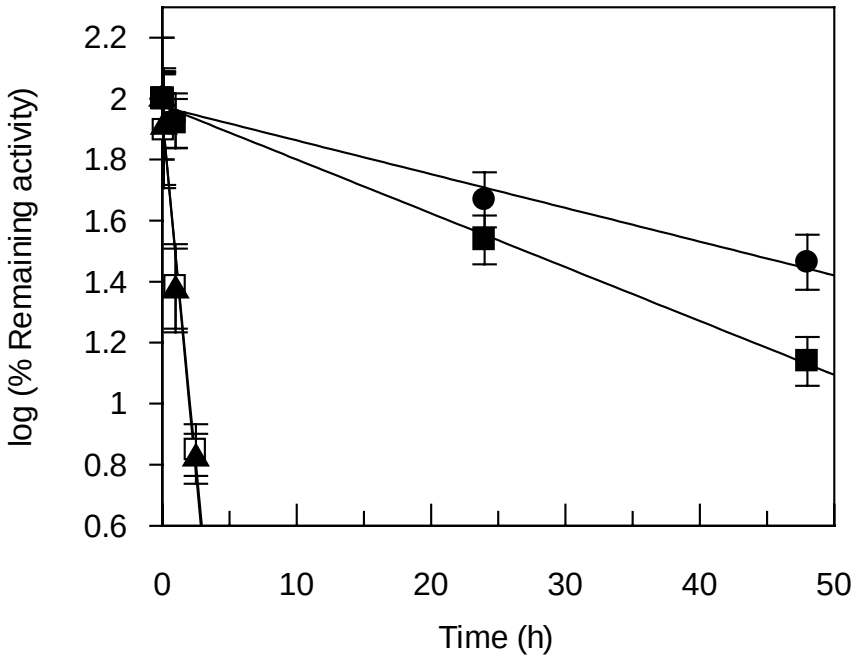

Figure 5

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