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β-Aspartylpeptides as substrates of L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi*

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Abstract **L-Asparaginase is known to catalyze the hydrolysis of L-asparagine to L-aspartic and ammonia, but little is known about its action on peptides. When we incubated L-asparaginases purified either from *Escherichia coli* or *Erwinia chrysanthemi* – commonly used as chemotherapeutic agents because of their antitumour activity – with eight small β-aspartylpeptides such as β-aspartylserineamide, β-aspartylalanineamide, β-aspartylglycineamide and β-aspartylglycine, we found that both L-asparaginases could catalyze the hydrolysis of five of them yielding L-aspartic acid and amino acids or peptides. Our data show that L-asparaginases can hydrolyze β-aspartylpeptides and suggest that L-asparaginase therapy may affect the metabolism of β-aspartylpeptides present in human body.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Enzyme kinetics; Leukemia; Substrate specificity

1. Introduction

L-Asparaginase (L-asparagine aminohydrolase; EC 3.5.1.1) is widely used in the treatment of acute lymphoblastic leukemia (ALL) because of its ability to catalyze the deamidation of L-asparagine to L-aspartic acid. Its antitumour activity is based on the fact that certain tumour cells, especially ALL tumour cells, have a nutritional dependence on an external supply of Asn. In contrast, normal cells are protected from Asn-starvation during the L-asparaginase therapy due to their ability to produce this amino acid. In clinical use, the L-asparaginases, which are mainly purified from *Escherichia coli* or *Erwinia chrysanthemi*, are toxic drugs and their therapeutic response rarely occurs without some evidence of toxicity. The common toxic side effects of asparaginase therapy include hypersensitivity reactions, leukopenia, hyperglycemia, neurological seizures and pancreatitis [1–3]. The toxicity is partially attributable to the glutaminase activity of these enzymes [4,5]. Investigations of the substrate specificity of bacterial L-asparaginases have shown that, in addition to L-asparagine and L-glutamine, these enzymes are able to catalyze the hydrolysis of D-asparagine, succinamic acid [4], L-aspartate-β-hydroxamate, L-aspartate-β-methylester, DL-N^{α}-ethylasparagine [6] and certain N^4-substituted L-asparagine derivatives [7], and de-aminate small peptides with carboxyl-terminal asparagine residues [4].

β-Aspartylpeptides are present in normal mammalian body fluids [8,9] and tissues [10] and their formation in rat kidney has been demonstrated [10]. Some of these peptides have been suggested to represent end products of protein degradation [11]. We have recently shown that the lysosomal enzyme glycosylasparaginase (GA) is active toward β-aspartylpeptides [12]. In this study we have investigated whether two therapeutically used L-asparaginases, purified from *E. coli* and *E. chrysanthemi*, can catalyze the hydrolysis of β-aspartylpeptides. Our results show that both L-asparaginases catalyze the hydrolysis of certain β-aspartylpeptides, and particularly small size β-aspartyl amino acid amides such as H-Asp(Ser-NH_2)-OH (β-aspartylserineamide). The results suggest that at the doses used in ALL, L-asparaginase therapy may affect the metabolism of β-aspartylpeptides.

2. Materials and methods

EC-2-type L-asparaginase purified from *E. coli*, Elspar® (EcAII), was purchased from Merck, PA, USA. L-Asparaginase derived from *E. chrysanthemi*, Erwinase® (ErAII), was a product of Ipsen, Berkshire, UK. L-Asparagine monohydrate was purchased from Fluka, Buchs, Switzerland. GlcNAc-Asn (β-aspartylglucosamine), ASAT (aspartate aminotransferase) and MDH (malic dehydrogenase) were products of Sigma Chemical, MO, USA. NADH (nicotinamide adenine dinucleotide) was purchased from Boehringer, Mannheim, Germany. H-Asp(Phe-OMe)-OH (β-aspartame) and H-Asp(Gly)-OH (β-aspartylglycine) were products of Bachem Feinchemikalien, Bubendorf, Switzerland. H-Asp(Ser-NH_2)-OH and other amides were prepared as previously described [13,14]. Other peptides were prepared as reported [13].

The kinetic parameters for the hydrolysis of β-aspartylpeptides were carried out in vessels thermostated at 23°C with a spectrophotometric assay [15]. Briefly, various concentrations of Asn, β-aspartylpeptides or GlcNAc-Asn were incubated for 15 min in microplate wells in the presence of stock solution containing NADH, MDH and ASAT. Then, either EcAII or ErAII was added to give a final volume of

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Abbreviations: ALL, acute lymphoblastic leukemia; H-Asp(Phe-OMe)-OH, β-aspartame; ASAT, aspartate aminotransferase, glutamic-oxaloacetic transaminase; H-Asp(Gly)-OH, β-aspartylglycine; H-Asp((Gly)$_4$-OH))-OH, β-aspartyltetraglycine; MDH, malic dehydrogenase; GlcNAc-Asn, β-aspartylglucosamine; H-Asp(Ser-NH_2)-OH, β-aspartylserineamide, 2-acetamido-1-N-(β-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; GA, glycosylasparaginase; NADH, nicotinamide adenine dinucleotide

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Table 1
Kinetic parameters of L-asparaginase from *E. coli* (EcAII) and *E. chrysanthemi* (ErAII) for the hydrolysis of L-Asn, β-aspartylpeptides and GlcNAc-Asn at 23°C

Compound	K_{cat}/K_m (M^{-1} s^{-1})		V_{max} (μM/min)		K_m (μM)		Relative rate (%)	
	EcAII	ErAII	EcAII	ErAII	EcAII	ErAII	EcAII	ErAII
H-Asn-OH[a]	973 000	3 180 000	22.7	16	130	90	100	100
H-Asp(Ser-NH_2)-OH	148 000	629 000	11.8	8.5	460	240	52	53
H-Asp(Ala-NH_2)-OH	76 400	833 000	1.9	1.4	140	30	8.2	8.6
H-Asp(Gly-NH_2)-OH	7 150	12 500	0.51	0.35	410	500	2.2	2.1
H-Asp(Gly-OH)-OH[b,c]	47	144	0.07	0.02	8 510	2 230	0.3	0.1
H-Asp(Phe-OMe)-OH	1 530	0	0.04	0	150	0	0.2	0
H-Asp((Gly)$_4$-OH))-OH	0	6 380	0	0.05	0	140	0	0.3
H-Asp(Gly-Gln-OH)-OH[b]	0	0	0	0	0	0	0	0
H-Asp(Gly-Val-OH)-OH[b]	0	0	0	0	0	0	0	0
H-Asp(GlcNAcNH)-OH[d]	0	0	0	0	0	0	0	0

Compounds marked with a–d are present in human body.
[a]Cooney et al. [28].
[b]Buchanan et al. [8].
[c]Pisano et al. [11].
[d]Rowley et al. [29].

250 μl and the final L-asparaginase activity of 91 U/l. Into blank wells, an aliquot of 50 mM Tris–HCl, pH 7.5, was added instead of enzyme to determine if there was any free Asp in the peptide solution. The blank values were subtracted from the actual measurements, because free Asp, as an impurity, could give false positive results. The rate of the absorbance decrease at 340 nm was followed with a Tecan SpectraFLUOR spectrophotometer, Tecan Austria, Grödig, Austria, and the change in the substrate concentration was calculated as described previously [16]. The kinetic parameters were calculated using the Enzpack for Windows, version 1.4, Biosoft software package.

3. Results

When L-asparaginases from *E. coli* (EcAII) and *E. chrysanthemi* (ErAII) were incubated in the presence of eight different β-aspartylpeptides at 23°C, both enzymes catalyzed the hydrolysis of five of these peptides to L-aspartic acid and the residual peptide or amino acid. The kinetic parameters of the hydrolysis of Asn, β-aspartylpeptides and GlcNAc-Asn are summarized in Table 1.

The relative hydrolysis rates of different β-aspartylpeptides varied markedly depending on the peptide. H-Asp(Ser-NH_2)-OH was hydrolyzed by the two L-asparaginases at half of the rate of Asn. The L-asparaginases also catalyzed the hydrolysis of other β-aspartylpeptide amides, but their relative rates were considerably lower: the hydrolysis rates of β-aspartylalanineamide and β-aspartylglycineamide were 1/10–1/50 of that of Asn. The hydrolysis rate of H-Asp(Gly)-OH was approximately 1/1000 of that of Asn. The two enzymes showed differences in their substrate specificity towards the β-aspartylpeptides that were hydrolyzed at low relative rates. ErAII catalyzed the hydrolysis of H-Asp((Gly)$_4$-OH))-OH, but this compound was not hydrolyzed by EcAII. EcAII could hydrolyze H-Asp(Phe-OMe)-OH, but this peptide remained unhydrolyzed by ErAII. The relative hydrolysis rate of β-aspartylglycineamide was approximately 10-fold higher than that of the corresponding unsubstituted peptide, H-Asp(Gly)-OH. Neither of the L-asparaginases was able to hydrolyze GlcNAc-Asn. Fig. 1 shows schematically the hydrolytic reactions catalyzed by L-asparaginases.

4. Discussion

The combined evidence indicates that in spite of the compact size of the catalytic cavity present in L-asparaginases [17,18] they are capable of accommodating small β-aspartylpeptides or amides leading to acylation of the L-asparagine β-carboxamido group. On the contrary, the lysosomal GA possesses a more spacious binding site, which is able to accept bulkier glucosyl residues, accounting for its activity towards larger size β-aspartylpeptides [12].

Earlier studies on the substrate specificity of bacterial L-asparaginases have characterized some special features for their potential substrates. A free α-carboxyl group of the L-asparagine moiety is essential for the hydrolysis of the substrate and a free α-amino group of L-asparagine participates in the binding of the amino acid to L-asparaginase [4]. Small peptides with a carboxyl-terminal L-asparagine residues are deaminated and β-aspartoalkylamides with straight chain alkyl substituents are hydrolyzed by *E. carotovora* L-asparaginase [4]. Derivatives of L-asparagine with substituents on N^4-position not greater than a methoxy group are hydrolyzed at a considerable rate by *E. coli* asparaginase [7]. In our study, H-Asp(Ser-NH_2)-OH, which is quite bulky when compared to N^4-methoxy-L-asparagine, is hydrolyzed half as fast as L-asparagine by both *Erwinia* and *E. coli* asparaginase. Furthermore, our finding that the L-asparaginase-catalyzed hydrolysis of β-aspartylpeptide amides is faster than that of the corresponding β-aspartylpeptides suggests that, because of its known hydrophobic character, the L-asparaginase substrate binding site does not tolerate a charged functional group on the C-terminal amino acid. This also applies to the GA-catalyzed hydrolysis of β-aspartylpeptides [12].

L-Asparaginases and GA have many biochemical properties

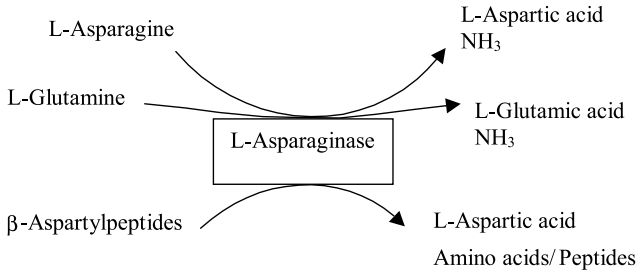

Fig. 1. Schematic illustration of the reactions catalyzed by L-asparaginase.

in common, including the polymeric subunit structure [19] and the ability to hydrolyze L-asparagine [20] to L-aspartic acid and ammonia. It has been suggested that the hydrolysis of β-aspartyl compounds catalyzed by GA is initiated by the binding of the aspartyl moiety to the active site of the enzyme through its free α-amino and α-carboxyl groups [19,21]. GA uses the γ-hydroxyl and α-amino group of its β-chain N-terminal threonine as an active site nucleophile and general base, respectively [22], in the formation of β-aspartylated enzyme, which is subsequently deacylated by water to L-aspartic acid. *E. coli* L-asparaginase [23], *Pseudomonas* 7A glutaminase-asparaginase [24] and *E. chrysanthemi* [25] use the γ-hydroxyl group of a threonine residue located in a flexible loop of the enzyme protein as an active site nucleophile.

In conclusion, our results suggest that high-dose L-asparaginase therapy, for example, used in the treatment of ALL may hydrolyze β-aspartylpeptides present in human body. The toxicity of L-asparaginases has partially been attributed to the glutaminase activity of these enzymes [4,5]. L-Asparaginases purified from *E. coli* (EC-2) or *Erwinia* have relative activities toward L-glutamine of 2–3% [26,27] or 9% [4] of that toward L-asparagine, respectively. We found that the relative rates of hydrolysis of H-Asp(Ser-NH_2)-OH by *E. coli* and *Erwinia* asparaginases were 52% and 53% of that of L-asparagine and thus significantly higher than those reported in literature for L-glutamine. The corresponding hydrolysis rates of β-aspartylalanineamide and β-aspartylglycineamide were 8–9% and ca 2% of that of Asn, respectively, and thus on the same range as the hydrolysis of L-glutamine by these enzymes. Whether the hydrolysis of β-aspartylpeptides is of significance in mediating the numerous side effects of L-asparaginase therapy in ALL patients remains to be elucidated.

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