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The Specificity of Prolyl Endopeptidase from *Flavobacterium meningoseptum*: Mapping the S′ Subsites by Positional Scanning via Acyl Transfer

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Abstract—The S_1'–S_3' subsite specificity of prolyl endopeptidase from *Flavobacterium meningoseptum* was studied by acyl transfer to libraries of amino acid amides and peptides. Whereas the S_1' and S_3' subsites influence the specificity for the amino component by approximately one order of magnitude, the S_2' subsite possesses a markedly higher specificity. Besides the high specificity for hydrophobic residues at P_1'–P_3', proline was efficiently bound by the S_2' and S_3' subsites of the enzyme. In contrast, no binding of P_1' proline-containing peptides was observed. It could be demonstrated that the specificity of the S′ subsite is not restricted to L-amino acids. Effective P′–S′ interactions were also found for β- and γ-amino acids indicating that the enzyme does not form close contacts to the backbone of P_1' and P_2' amino acid residues.

Introduction

Prolyl endopeptidase (PEP, EC 3.4.21.26), also known as post-proline cleaving enzyme, functions mainly as an intracellular serine peptidase which specifically hydrolyzes peptide bonds at the carboxyl side of proline and, more slowly, of alanine residues.[1,2] PEP is the only enzyme that cleaves prolyl residues within a peptide sequence. Such proline-specific enzymes are commonly found in small quantities in mammalian organs,[3–8] in plants[9–11] and in microorganisms.[12,13] In mammals the enzyme is considered to be involved in maturation and degradation of peptide hormones and neuropeptides.[14,15] Recent studies suggest that PEP is an enzyme involved in learning and memory processes[16] and possibly involved in the generation of the β-amyloid A4-peptide in Alzheimer's disease.[17] Sequencing of several proline-specific enzymes reveal that most of these enzymes belong to a subfamily of serine proteinases with a different primary sequence than the trypsin (type I) and subtilisin (type II) subfamilies of serine proteinases.[18] Kinetic studies show similar specificities for proline-specific enzymes from several sources.[7,10–13,19–22] By varying the chain length of peptide substrates a minimal chain length of three residues was discovered. Substrates containing only one or two residues on the amino side of the scissile bond are stable against PEP. However, further elongation to P_4 caused no further increase in activity, suggesting that the binding site of the enzyme consists of three subsites (S_3, S_2, S_1, binding site notation according to Schechter and Berger[23]). Furthermore, for the S′ subsite of PEP two additional binding sites, S_1' and S_2', have been postulated. The specificity of the S′ subsite was not systematically investigated. Only a few kinetic studies using small peptide libraries varying in the P_1' position have been described.[13,24] The specificity at the S_2' position is practically unknown. However, characterization of the S′ subsite specificity is essential for scanning native substrates, modeling of enzyme–substrate interactions and for designing specific and fine-tuned inhibitors which bind both to the S and S′ subsites of the enzyme.

Key words: Prolyl endopeptidase; enzyme specificity; acyl transfer; protease catalysis; S′ subsite mapping.
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In the present study the specificity of the S_1', S_2', and S_3' positions of recombinant proline-specific endopeptidase from *Flavobacterium meningoseptum* was investigated by S′ subsite mapping based on acyl transfer to added nucleophiles. In order to elucidate the influence of the chain length of the amino component on the P′–S′ interactions, amino acid amides and peptides up to five residues were used. Furthermore, the stereospecificity was investigated by using D-amino acids in P_1', P_2', and P_3' positions. The resulting data give a detailed picture of the S′ subsite specificity of this enzyme and suggest the presence of S_3'–P_3' interactions, in particular for basic amino acid residues.

Results and Discussion

Deacylation kinetics

Serine and cysteine proteases are capable of catalyzing acyl transfer reactions. This reaction is the reverse of the acylation of the enzyme by a peptide substrate and therefore provides analogous specificity data.[25–27] The validity of this method has been demonstrated for various serine and cysteine proteases and is extensively discussed in the review of Schellenberger and Jakubke.[26] Since the acyl enzyme can be attacked by water or by the added nucleophile, two products are formed: (a) hydrolysis product; (b) aminolysis (peptide) product. The ratio between the formation of hydrolysis product and peptide product reflects the S′ subsite specificity of the enzyme. As an efficiency parameter for the deacylation of the acyl enzyme by added nucleophiles the partition value p was introduced, with a definition analogous to that of the Michaelis constant K_m (eq (1)).[26]

$$p = \frac{d[Ac-OH]}{d[Ac-N]}[HN] = \frac{k_3 K_n}{k_4} + \frac{k_5}{k_4}[HN] = p_0 + p_n[HN] \quad (1)$$

According to eq (1) the p value is defined as the nucleophile concentration at which the rate of the aminolysis reaction equals the rate of the hydrolysis reaction of the acyl enzyme intermediate. Consequently, a decrease in the p value can be directly correlated with the preference of the protease for a given nucleophile. Following Schellenberger et al.,[28] eq (1) can be integrated to give eq (2), which allows an uncomplicated determination of the nucleophile efficiency, since the value for p_0 can be calculated at any ratio between the acyl donor and the nucleophile by linear regression analysis from the slope of the plot of $[Ac-OH]/[Ac-N]$ versus $\ln([HN]_0/([HN]_0-[Ac-N]))/[Ac-N]$.

$$\frac{[Ac-OH]}{[Ac-N]} = p_n + p_0 \ln\left(\frac{[HN]_0}{[HN]_0-[Ac-N]}\right)/[Ac-N] \quad (2)$$

Reaction course of PEP-catalyzed acyl transfer reactions

An important side reaction in acyl transfer, which can lead to false p values, is secondary hydrolysis of the peptide bond formed. Usually, serine proteases possess a markedly higher catalytic activity towards ester substrates in comparison to amides. Therefore, the rate of secondary hydrolysis of the peptide product is normally low if esters are used as acyl donors. Secondary reactions of this type are sensitively detected by plotting the partition value p versus the ester consumption. Figure 1 shows plots of PEP-catalyzed acyl transfer from Bz-Gly-Pro-OMe (Bz, benzoyl-) to H-Phe-NH_2 and H-D-Phe-NH_2. In both cases a marked increase of p could be observed, independent of enzyme concentration, in particular after approximately 40% ester consumption, suggesting an unusually high amidase activity of PEP. The increase of p in the case of H-D-Phe-NH_2 also demonstrates that PEP is even capable of cleaving Pro-D-Xaa bonds. In contrast to carboxypeptidase substrates (Y-Gly-Pro-D-Xaa-OH; Y, amino protecting group), which seem to be stable against PEP,[19] Pro-D-Xaa bonds within peptide sequences could be hydrolyzed by the endopeptidase activity of the enzyme. As indicated by the slopes of the curves with both nucleophiles, the rate of secondary hydrolysis seems to be nearly independent of the conformation of the P_1' amino acid, suggesting similar specificities for both the corresponding peptide products.

Mapping the S_1'–S_3' subsites of PEP

For mapping the S_1'–S_3' subsites, amino acid amides (S_1'), dipeptides (S_2') and pentapeptides of the consensus sequence H-Ala-Ala-*Xaa*-Ala-Gly-OH (S_3') were used as nucleophiles. To avoid false estimates of p due to secondary reactions of the peptide products, the acyl transfer reactions were analyzed with an ester consumption of 30–40%. Figure 2 shows the p values determined for PEP-catalyzed acyl transfer using the acyl donor Bz-Gly-Pro-OMe. Except for P_1' Pro, all nucleophiles show productive binding at the S′ subsites of PEP resulting in peptide product formation. However, the generally high values of p illustrate less efficient S′ subsite interactions with this enzyme compared to type I and type II serine proteases.[29–31] Nevertheless, a marked influence of the amino acid side chain on the nucleophile efficiency can be observed. The highest differences in p were found for the S_2' subsite. For this position the p values cover a range of three orders of magnitude. Surprisingly, the best interactions were with P_2' Pro residues. However, the only slightly higher p values found for Leu, Met, Phe and Ala indicate a similar specificity for nonpolar amino acids. The specificity for polar residues (Ser and Asn) seems to be dependent on the particular side chain of these amino

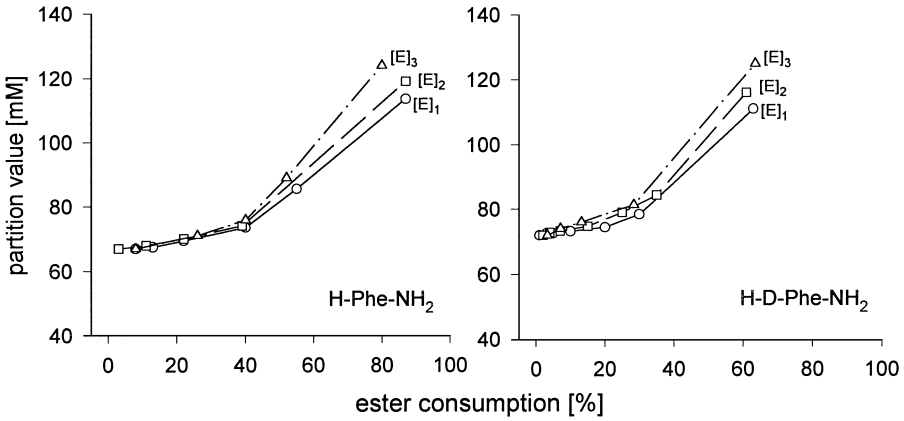

Figure 1. Dependence of p on the ester consumption and the enzyme concentration for the PEP-catalyzed acyl transfer from Bz-Gly-Pro-OMe to H-Phe-NH_2 and H-D-Phe-NH_2. Conditions: 0.025 M borate buffer, pH 8.5, 0.2 M NaCl, [acyl donor]: 2 mM, [acyl acceptor]: 50 mM, PEP: $[E]_1 = 3.6\times10^{-8}$ M, $[E]_2 = 6.8\times10^{-8}$ M, $[E]_3 = 1.8\times10^{-7}$ M.

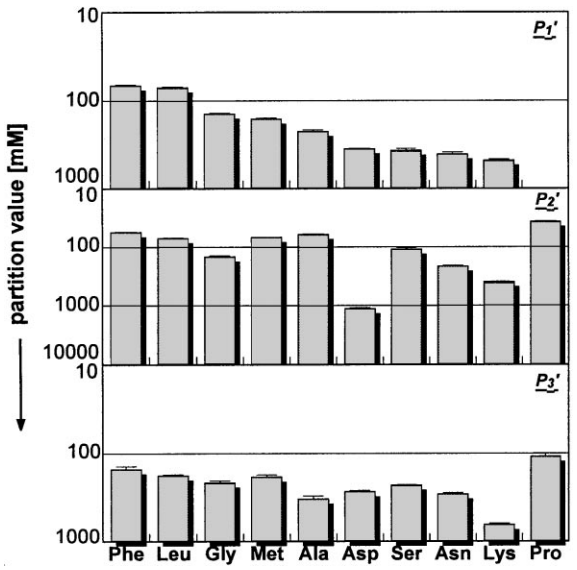

Figure 2. S′ subsite specificity of PEP determined by acyl transfer. Conditions: 0.025 M borate buffer, pH 8.5, 0.2 M NaCl, [acyl donor: Bz-Gly-Pro-OMe]: 2 mM, [acyl acceptor: P_1', H-*Xaa*-NH_2; P_2', H-Ala-*Xaa*-OH; P_3', H-$(Ala)_2$-*Xaa*-Ala-Gly-OH]: 1–100 mM, [PEP]: 6.8×10^{-8} M. The error bars give the ± standard deviation calculated from triplicate experiments.

acids. The lowest reactivity was found for P_2' charged amino acids, in particular for the acidic Asp residue, indicating a hydrophobic S_2' binding pocket in PEP. Regarding the S_1' subsite, the partition values cover a range of one order of magnitude. Therefore, compared to the S_2' position, a lower specificity can be postulated for S_1'. Nevertheless, an influence of P_1' amino acid residues on the nucleophile efficiency is recognizable. The most efficient contacts are formed by hydrophobic (Leu) and aromatic (Phe) amino acid residues, suggesting a hydrophobic S_1' subsite region. Correspondingly, polar and charged residues show the lowest reactivities. Except for P_3' Pro, the results indicate a specificity profile for the S_3' subsite the same as that found for S_1'. However, the p values observed for comparable nucleophiles are of the same order of magnitude and, therefore, the differences are lower. This finding indicates only a small contribution of S_3' to the S′ subsite specificity of PEP. However, the low reactivity of the peptide containing P_3' Lys is more marked than found for the P_1' Lys-containing nucleophile. Similar to P_2' Pro-containing nucleophiles, the best interactions were found for P_3' Pro residues. However, in this case the acyl transfer experiments were performed using the tripeptide H-Ala-Ala-Pro-OH, instead of the pentapeptide H-Ala-Ala-Pro-Ala-Gly-OH, to avoid proteolytic side reactions within the nucleophile. Investigations on the influence of the chain length of the amino component on the specificity show an increase of p on further elongation of the dipeptide up to the pentapeptide (Fig. 3). The efficiency optimum with dipeptides is in agreement with kinetic data given by Yoshimoto et al.[19] From Figure 3, the high specificity of the P_3' Pro-containing nucleophile may be partly the result of the shorter chain length of this nucleophile. Detailed studies on the proline specificity of the S′ subsite positions, using Pro-containing peptides of equal chain lengths, are included in Table 1. From the p values obtained for H-Ala-Pro-Ala-OH and H-Ala-Ala-Pro-OH, a higher proline specificity could be deduced for the S_2' compared with the S_3' subsite of PEP. Table 1 also shows that the presence of Pro residues at the P_2' or P_3' positions does not lead to productive binding of P_1' Pro residues. Since the Pro-analogue acetidine-2-carboxylic acid amide was also not accepted it can be concluded that P_1' imino acid derivatives are not substrates in PEP-catalyzed reactions.

S′ specificity of noncoded amino acid derivatives

As indicated by the acceptance of H-D-Phe-NH_2, PEP seems not to have a strict requirement for L-amino acids, at least in P_1' position. In order to confirm this suggestion, and to study the stereospecificity of the S_2' and S_3' subsites of the enzyme, further D-amino acid amides and peptides were used as deacylating amino components. The resulting *p* values for the acyl transfer reactions are summarized in Table 2. The values indicate productive binding of D-amino acid residues at all subsites. Except for H-D-Phe-NH_2, the replacement of L-amino acids at P_1' and P_2' position by the corresponding D-isomers causes an increase of *p* by a factor of only 3.1–3.7. When the P_3' residue was replaced by a D-amino acid, practically the same *p* value resulted. Consequently, stereospecificity is not evident within the S′ subsite of PEP. This finding suggests that the stereospecificity of PEP is determined exclusively by the S subsite of the enzyme, as was described for S_1 and S_2.[19] However, the stereospecificity of the S_1 subsite was not systematically investigated using corresponding substrate-isomers. Therefore, Bz-Gly-D-Pro-OMe was synthesized and used as a substrate for PEP. In accordance with the suggestion of Yoshimoto et al.,[19] no enzymatic hydrolysis of the P_1 D-Pro isomer could be found. Thus, the stereospecificity of PEP should be restricted to the S binding site of the enzyme.

Table 3 shows that in addition to D-amino acids, β- or γ-amino acids were also accepted by the S′ subsite of PEP. Surprisingly, H-β-Ala-NH_2 was bound more strongly than H-Ala-NH_2. A further elongation of the amino acid backbone in the case of H-γ-Abu-NH_2 causes an increase of *p* by a factor of approximately 2. The replacement of P_2' Ala by β-Ala is practically without any influence on the specificity for the nucleophile. These findings suggest that PEP does not form close

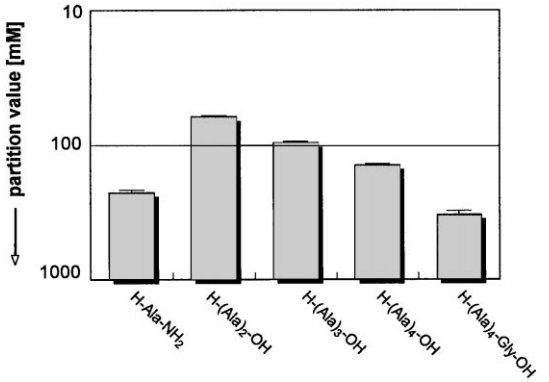

Figure 3. Partition values for the acyl transfer from Bz-Gly-Pro-OMe to amino components of varying lengths. Conditions: 0.025 M borate buffer, pH 8.5, 0.2 M NaCl, [acyl donor]: 2 mM, [acyl acceptor]: 1–100 mM, [PEP]: 6.8×10^{-8} M. The error bars give the ± standard deviation calculated from triplicate experiments.

Table 1. Partition values for PEP-catalyzed acyl-transfer from Bz-Gly-Pro-OMe to proline derivatives. Conditions: 0.025 M borate buffer, pH 8.5, 0.2 M NaCl, [acyl donor]: 2 mM, [acyl acceptor]: 1–100 mM, [PEP]: 6.8×10^{-8} M

Acyl acceptor	Product	*p*[mM]
H-Pro-NH_2	Bz-Gly-Pro-Pro-NH_2	n.s.
H-Aze-NH_2[a]	Bz-Gly-Pro-Aze-NH_2	n.s.
H-Pro-Pro-OH	Bz-Gly-Pro-Pro-Pro-OH	n.s.
H-Pro-Pro-Pro-OH	Bz-Gly-Pro-Pro-Pro-Pro-OH	n.s.
H-Ala-Pro-OH	Bz-Gly-Pro-Ala-Pro-OH	37.0 ± 0.5
H-Ala-Pro-Ala-OH	Bz-Gly-Pro-Ala-Pro-Ala-OH	68.4 ± 2.3
H-Ala-Ala-Pro-OH	Bz-Gly-Pro-Ala-Ala-Pro-OH	108 ± 9

n.s.: no synthesis.
[a]Aze; acetidin-2-carboxylic acid.

Table 2. S′ subsite stereospecificity of PEP determined by acyl transfer from Bz-Gly-Pro-OMe to various amino components. Conditions: 0.025 M borate buffer, pH 8.5, 0.2 M NaCl, [acyl donor]: 2 mM, [acyl acceptor]: 1–100 mM, [PEP]: 6.8×10^{-8} M

Acyl acceptor	Product	*p*[mM]
S_1' *stereospecificity*		
H-D-Ala-NH_2	Bz-Gly-Pro-D-Ala-NH_2	693 ± 20
H-Ala-NH_2	Bz-Gly-Pro-Ala-NH_2	223 ± 10
H-D-Phe-NH_2	Bz-Gly-Pro-D-Phe-NH_2	72.0 ± 1.4
H-Phe-NH_2	Bz-Gly-Pro-Phe-NH_2	67.0 ± 1.9
H-D-Leu-NH_2	Bz-Gly-Pro-D-Leu-NH_2	226 ± 4
H-Leu-NH_2	Bz-Gly-Pro-Leu-NH_2	70.5 ± 1.2
S_2' *stereospecificity*		
H-Ala-D-Ala-OH	Bz-Gly-Pro-Ala-D-Ala-OH	201 ± 5
H-Ala-Ala-OH	Bz-Gly-Pro-Ala-Ala-OH	61.1 ± 1.0
H-Ala-D-Phe-OH	Bz-Gly-Pro-Ala-D-Phe-OH	208 ± 3
H-Ala-Phe-OH	Bz-Gly-Pro-Ala-Phe-OH	55.9 ± 0.6
S_3' *stereospecificity*		
H-Ala-Ala-D-Ala-OH	Bz-Gly-Pro-Ala-Ala-D-Ala-OH	114 ± 2
H-Ala-Ala-Ala-OH	Bz-Gly-Pro-Ala-Ala-Ala-OH	95.7 ± 2.6

Table 3. PEP-catalyzed acyl transfer from Bz-Gly-Pro-OMe to nucleophiles including noncoded amino acids. Conditions: 0.025 M borate buffer, pH 8.5, 0.2 M NaCl, [acyl donor]: 2 mM, [acyl acceptor]: 25 mM, [PEP]: 6.8×10^{-8} M

Acyl acceptor	Product	*p*[mM]
H-β-Ala-NH_2	Bz-Gly-Pro-β-Ala-NH_2	76.8 ± 6.2
H-γ-Abu-NH_2[a]	Bz-Gly-Pro-γ-Abu-NH_2	496 ± 21
H-Ala-NH_2	Bz-Gly-Pro-Ala-NH_2	223 ± 10
H-Leu-β-Ala-OH	Bz-Gly-Pro-Leu-β-Ala-OH	26.9 ± 0.3
H-Leu-Ala-OH	Bz-Gly-Pro-Leu-Ala-OH	19.2 ± 0.2

[a]Abu; γ-amino butyric acid.

contacts to the backbone of P_1' and P_2' amino acids, unlike those known for type I serine proteases.[32–34] Therefore, a high flexibility of these S′ subsite regions could be postulated, as was also found for type II serine proteases like subtilisin[31] and in particular for cysteine proteases.[35–37]

Experimental

Materials

PEP was obtained from Fluka Chemie AG, Buchs, Switzerland. Bz-Gly-Pro-OMe and the corresponding enantiomer were synthesized from Bz-Gly-OH and H-Pro-OMe or H-D-Pro-OMe, respectively, by standard procedures. H-β-Ala-NH_2, H-Aze-NH_2 and H-γ-Abu-NH_2 were synthesized by amidation of the corresponding methyl esters. The pentapeptides were synthesized as described by Ullmann et al.[35]

Acyl transfer experiments

The reactions were performed at 37 °C in a total volume of 100 μL 25 mM borate buffer, pH 8.5 containing 0.2 M NaCl. Stock solutions of the acyl donor (4 mM) were prepared in distilled water. Stock solutions of the nucleophiles were prepared in 50 mM borate buffer, pH 8.5 containing 0.4 M NaCl. Nucleophile concentrations were calculated as free, unprotonated species.[38] Before starting the acyl transfer by addition of 4 μL of the respective enzyme stock solution, the mixtures were thermostated for 10 min. For the HPLC analysis aliquots of 80 μL were withdrawn and diluted with a stop solution of 50% aqueous methanol containing 1% trifluoroacetic acid.

The partition values were determined from three independent experiments for each nucleophile concentration. Only p values based on correlation coefficients higher than 0.98 were used.

HPLC analyses

Samples were analyzed by analytical reversed phase HPLC using a SpectraSystem P 2000 system (Thermo-Separation Products, USA) on C18 polymer coated columns (Vydac 218TP54, 5 μm, 300 Å, [25×0.4 cm], The Separations Group, USA and Grom Capcell, 5 μm, 300 Å, [25×0.4 cm], Shiseido, Japan) which were thermostated at 25 °C and eluted with various mixtures of water/acetonitrile (11–18% v/v) containing 0.1% trifluoroacetic acid under isocratic and in several cases gradient conditions. All measurements were detected at 254 nm. The partition values were calculated from peak areas of ester substrates, hydrolysis products and aminolysis products using the software Hyperdata Chromsoft (Vers. 2.15, Autochrom Inc., USA).

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