Specificity of prolyl endopeptidase

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Received 14 October 1986

A series of tetrapeptides, Cbz(Bz)-Gly-X-Leu-Gly, were synthesized and the kinetic parameters, k_{cat} and k_{cat}/K_m, determined for their hydrolyses by prolyl endopeptidase from *Flavobacterium*. The peptides with X = *N*-Me-Ala, Sar and Ala as well as the standard substrate (X = Pro) were found to be good substrates, while those with X = α-aminobutyryl, Hyp, Ser and Gly were poor substrates, and those with X = pipecolyl, α-aminoisobutyryl, *N*-Me-Val, *N*-Me-Leu, Hyp(O-Bzl) and Ser(O-Bzl) were not cleaved at all. These results suggest that the specificity-determining site or S1 subsite of the enzyme is designed to fit exactly the proline residue of the substrate with allowance for the residues carrying substituents at the N and/or C_α which must not exceed the size of the pyrrolidine ring of proline.

Prolyl endopeptidase *Primary specificity* *Synthetic substrate*

1. INTRODUCTION

Prolyl endopeptidase (EC 3.4.21.26, PEPase), originally designated post-proline cleaving enzyme [1] or proline-specific endopeptidase [2,3], is an endopeptidase which specifically cleaves the peptide bond at the carbonyl side of prolyl residues in polypeptides of relatively small M_r. Since its discovery in human uterus by Walter et al. [4] in 1971, it has been isolated from various mammalian tissues, especially brain, from the sperm of Ascidian [5] and from *Flavobacterium* [3]. A natural inhibitor was isolated from rat brain [6], and small inhibitors were also synthesized [7–9]. Extensive studies have been done on substrate specificity and subsite mapping by Yoshimoto and Walter [10,11]. They reported that the alanyl peptide bond was also cleaved by PEPase, although to a lesser extent than the prolyl bond. This raised the basic question as to the primary specificity of this enzyme, because apparently there is no structural relation between proline and alanine. In order to solve this question, a series of tetrapeptides, Cbz(Bz)-Gly-X-Leu-Gly, where X equals various usual and unusual amino acids, were synthesized and hydrolyzed by PEPase from *Flavobacterium* to determine the kinetic parameters, k_{cat} and k_{cat}/K_m.

Abbreviations: Hyp, hydroxyproline; Sar, sarcosyl or *N*-Me-Gly; Abu, α-aminobutyryl; Aib, α-aminoisobutyryl; Pip, pipecolyl; Cbz, *N*-α-benzyloxycarbonyl; Bz, *N*-α-benzoyl; Bzl, benzyl; MCA, 4-methylcoumaryl-7-amide; Suc, succinyl

2. MATERIALS AND METHODS

The standard substrate, Cbz-Gly-Pro-Leu-Gly, was purchased from Peptide Institute Inc., and other peptides were synthesized stepwise from the C-terminus by a liquid-phase method for various substituents (X) as shown in table 1.

The enzyme, proline-specific endopeptidase from *Flavobacterium*, was purchased from Seikagaku Kogyo (lot no.5220). This sample had some contaminants as judged by SDS gel electrophoresis and had Gly-Pro-MCA hydrolyzing activity about 1/4000 as large as that of Suc-Gly-Pro-MCA. However, it possessed no thermolysin-like activity since Cbz-Gly-Pip-Leu-Gly, a good substrate for thermolysin, was not cleaved at all.

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00145793/86/$3.50

The rate of peptide hydrolysis was determined at 30°C by quantitating the liberated Leu-Gly in the aliquots withdrawn at adequate time intervals, on a Hitachi 835 automatic amino acid analyzer by a shortened program of elution. The K_m and V_{max} values were determined from the usual Lineweaver-Burk plots for [S] = 0.25–10 mM and [E] = 100 or 500 nM.

3. RESULTS

3.1. *Synthesis of substrates*

The N-protected tetrapeptides were synthesized as crystalline or amorphous powder. A portion of each peptide was purified by HPLC on a reverse-phase column (Cosmosil 5C8, 8 × 250 mm) and lyophilized. Rechromatography on the same column showed a single and symmetrical peak. The purity was also confirmed by the amino acid composition.

3.2. *PEPase hydrolyses of the synthetic peptides*

The values of the rate constant (k_{cat}), Michaelis constant (K_m) and the specificity constant (k_{cat}/K_m) are listed in table 1. The peptides with X = *N*-Me-Ala, Sar and Ala are shown to belong to a group of good substrates with k_{cat} of 40–26% of that of the standard substrate Cbz-Gly-Pro-Leu-Gly, and k_{cat}/K_m being 43–16%. The second group composed of those with Abu, Hyp, Ser and Gly were poor substrates with k_{cat} of 4.1–0.14%, and k_{cat}/K_m of 2–0.06% compared to Pro-peptide. The third group of peptides with Pip, Aib, *N*-Me-Val, *N*-Me-Leu, Hyp(*O*-Bzl) and Ser(*O*-Bzl) were not cleaved at all.

The peptide, Bz-Gly-Pro-Leu-Gly, differing from the standard substrate only in the NH_2-protecting group, has both k_{cat} and K_m larger than those of the Cbz counterpart.

The Pip-peptide was a better substrate of thermolysin than Pro-peptide.

4. DISCUSSION

When using proteases to cleave peptides and proteins, it is essential to know their primary specificities. Trypsin is well known to cleave at the basic residues, Lys- and Arg-. However, it also cleaves Orn and *S*-aminoethyl–Cys bonds. *S. aureus* V8 protease cleaves Glu and Asp bonds, although in some cases it cleaves the Gly–Gly bond [12]. The present results indicate that PEPase cleaves the *N*-Me-Ala, Sar and Ala peptide bonds as well as the Pro bond at significant rates. The

Table 1

Kinetic parameters for the hydrolysis of synthetic substrates by PEPase

Substrate	V_{max} (nmol·min^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1}·mM^{-1})
Cbz-Gly-Pro-Leu-Gly	10.53	17.57	0.54	32.36
Cbz-Gly-Ala(*N*-Me)-Leu-Gly	4.24	7.07	0.51	13.94
Cbz-Gly-Sar-Leu-Gly	3.38	5.63	0.73	7.74
Cbz-Gly-Ala-Leu-Gly	2.74	4.57	0.86	5.31
Cbz-Gly-Abu-Leu-Gly	0.43	0.72	1.12	0.65
Cbz-Gly-Gly-Leu-Gly	0.015	0.025	1.33	0.019
Bz-Gly-Pro-Leu-Gly	17.54	29.23	1.37	21.24
Bz-Gly-Hyp-Leu-Gly	0.28	0.47	2.86	0.16
Bz-Gly-Ser-Leu-Gly	0.074	0.12	1.69	0.071
Cbz-Gly-Pip-Leu-Gly	no cleavage			
Cbz-Gly-Aib-Leu-Gly	no cleavage			
Cbz-Gly-Leu(*N*-Me)-Leu-Gly	no cleavage			
Cbz-Gly-Val(*N*-Me)-Leu-Gly	no cleavage			
Bz-Gly-Hyp(*O*-Bzl)-Leu-Gly	no cleavage			
Bz-Gly-Ser(*O*-Bzl)-Leu-Gly	no cleavage			

[S] = 0.25–10 mM, [E] = 1×10^{-7} M

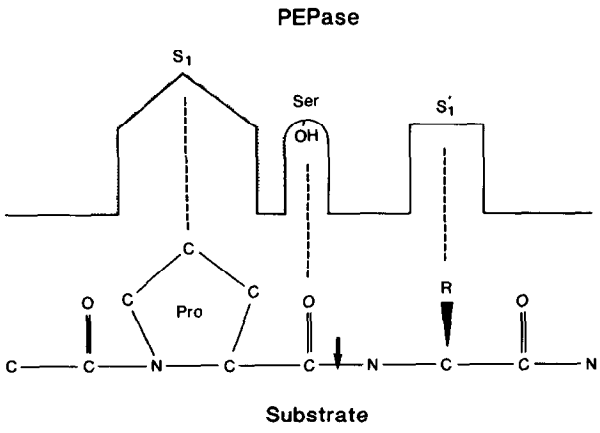

Fig.1. A schematic model of the active site of PEPase. The specificity-determining site or S1 subsite is presumed to accommodate those residues with size and shape similar to, and not larger than, proline. The S1' subsite can accommodate residues larger than proline.

above amino acid residues of the good substrates have a common structural feature, viz. an intact five-membered pyrrolidine ring (Pro) or part of it (Me group at N and/or C_α) and they have no group extending out of the ring. The residues in the poor or resistant substrates have a group extending out of the ring (Hyp) or a substituent not in the plane of the ring by rotation of a C_α–C_β bond. This leads to the conclusion that the specificity of PEPase is restricted to peptides with substituents at the N and/or C_α of the residue not exceeding the size and shape of the pyrrolidine ring. Among the peptides tested, only methyl substituents at the N and C_α are permissible. A schematic model for the specificity site of the enzyme is shown in fig.1.

Residues other than proline in the good substrates have freedom of rotation around the N–C_α bond. Only the conformation mimicking the plane of the pyrrolidine ring may be adopted at the S1 site, reminiscent of the requirement for the *trans* conformation of the Y–Pro imide bond in the Y-Pro-X sequence for the cleavage of the Pro–X bond by PEPase [13].

The possible cleavage at the Ala bond, which may proceed at a rate of 16% of that of the Pro bond cleavage, should be taken into account when estimating the results of PEPase digestion.

In this study only bacterial PEPase was used. Mammalian enzymes may have the same primary specificity, although S2 and S3 subsites have been postulated to have different requirements for substrates [14].

Similar specificity should also be studied for the post-proline dipeptidyl aminopeptidase using the above tetrapeptides after liberation of the NH_2-protecting groups, in analogy to the comparative studies by Yoshimoto et al. [10].

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