Biochimica et Biophysica Acta, 661 (1981) 136–141
Elsevier/North-Holland Biomedical Press

BBA 69343

THE SUBSTRATE SPECIFICITY OF PROTEINASE B FROM BAKER'S YEAST

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(Received March 23rd, 1981)

Key words: Proteinase B; Substrate specificity; (Baker's yeast)

The substrate specificity of proteinase B (EC 3.4.22.9) from Baker's yeast was studied. Experiments with unblocked synthetic peptides indicated that the enzyme has no aminopeptidase activity. The proteinase cleaves trypsin substrates like Bz-Arg-OEt, Bz-Arg-pNA and Bz-Ile-Glu-Gly-Arg-pNA and chymotrypsin substrates like Ac-Tyr-OEt and Bz-Tyr-pNA. The K_m value for Ac-Tyr-OEt is similar to that of chymotrypsin A, but the catalytic activity per mol proteinase B amounts to only 1/20 that of chymotrypsin A. K_m and k_{cat} for Bz-Arg-OEt are1/50 and 1/7 as high as the corresponding values determined for trypsin. Proteinase B cleaved the oxidized insulin B chain with an initial rapid cleavage step at Leu(15)–Tyr(16) and Phe(24)–Phe(25). Slower hydrolysis was observed at Gln(4)–His(5), Leu(11)–Val(12) Tyr(16)–Leu(17), Leu(17)–Val(18), Arg(22)–Gly(23) and Phe(25)–Tyr(26). These results suggest that the specificity of proteinase B is comparable to the specificity of porcine chymotrypsin C as well as of trypsin. When the hexapeptide Leu-Trp-Met-Arg-Phe-Ala was used as a substrate for proteinase B, the enzyme preferentially attacked at Arg-Phe and more slowly at Trp-Met.

Introduction

Yeast proteinase B is a serine proteinase (EC 3.4.22.9) [1–4] located in the vacuoles [5]. In a previous paper we reported a rapid purification method and some characteristics of the enzyme. Previous work indicated that proteinase B hydrolyzes synthetic substrates like Bz-Arg-OEt, a substrate for trypsin and Ac-Tyr-OEt, a substrate for chymotrypsin [1,3]. In the present study, kinetic constants of the action of proteinase B on these two synthetic substrates were compared with the parameters reported for cleavage of these substrates by bovine chymotrypsin A and by trypsin. Furthermore, the specificity of proteinase B was determined using the oxidized insulin B chain and the hexapeptide Leu-Trp-Met-Arg-Phe-Ala as proteinase substrates.

Materials and Methods

Materials. Bz-Arg-OEt, oxidized B chain of bovine insulin, hexapeptide Leu-Trp-Met-Arg-Phe-Ala, Leu-pNA, Dowex AG50 WX2 (200–400 mesh), trypsin and chymotrypsin were from Serva, (Heidelberg); Ac-Tyr-OEt, Bz-DL-Arg-pNA, Leu-pNA and Azocasein were from Sigma (München), Gbz-dipeptides, Ala-Leu-NH$_2$ were from Bachem (Bubendorf, Switzerland). Bz-Ile-Gly-Glu-Arg-pNA (S-2 222) was from Deutsche KABI GmbH (München); Azocoll was from Calibochem (La Jolla, CA). All other chemicals were obtained from Roth GmbH (Karlsruhe). Baker's yeast was obtained from Bäko Gesellschaft GmbH (Freiburg). Proteinase B was purified from baker's yeast (Pleser Hefe) as described by Kominami et al. [6].

Abbreviations: Ac, acetyl; Bz, benzoyl; Cbz, benzoyloxycarbonyl; pNA, *p*-nitroanilide; OEt, ethyl ester; Mops, 4-morpholinepropanesulfonic acid.

Hydrolysis of synthetic substrates. The hydrolysis of Bz-Arg-OEt and Ac-Tyr-OEt was measured at pH 7 by the method of Jusic et al. [7] and followed spectrophotometrically by continuous monitoring of the increase in absorbance at 340 nm. Activity on Bz-DL-Arg-pNA, Bz-Tyr-pNA, Lys-pNA, Arg-pNA, Leu-pNA and Bz-Ile-Gly-Glu-Arg-pNA was measured spectrophotometrically at 405 nm in 100 mM morpholinopropanesulfonic acid, pH 7.5 [8]. Cleavages of Cbz-dipeptides were measured by the method of Lewis and Harris [9]. Hydrolysis of Ala-Tyr-NH_2 was detected at pH by the release of ninhydrin-positive material.

Separation and analysis of proteolytic fragments from polypeptides. Oxidized insulin B chain (10 mg in 0.1 M ammonium acetate, pH 7.2) was digested with 240 μg proteinase B for 30 min or 6 h at 25°C. The reaction was stopped by lowering the pH to 3.0 with acetic acid. The lyophilized sample was dissolved in a small volume of 0.2 M pyridine/acetic acid, pH 3.1. A modification of ion-exchange chromatography method of Schroeder [10] was used for the crude separation of peptides. The complete digest was applied to a column (1 X 50 cm) of Dowex AG 50 WX2 (200–400 mesh) equilibrated with 0.2 M pyridine/acetic acid, pH 3.1, with a flow rate of 9 ml/h at 30°C. The column was developed with a linear gradient composed of 300 ml 0.2 M pyridine/acetic acid, pH 3.1, and of 300 ml 2.0 M pyridine/acetic acid, pH 5.0. The effluent was collected in 1-ml fractions. Peptides were detected in the fractions with ninhydrin, after alkaline hydrolysis of aliquots of the fractions [11]. Tubes containing separated peptides were combined and dried under reduced pressure at 35°C. Further purification was achieved by thin-layer chromatography (Silica gel 60 from Merck, Darmstadt) in *n*-butanol/glacial acetic acid/H_2O (4 : 1 : 1). The separated bands were located by staining guide strips with 0.5% ninhydrin and eluted with 0.1 M acetic acid.

The hexapeptide Leu-Trp-Met-Arg-Phe-Ala was dissolved in 0.1 M ammonium acetate, pH 7.2, and adjusted to a final concentration of 10 mg/ml. Hydrolysis of 10 mg of the peptide was performed at 25°C by incubation with 0.6 mg proteinase B, 0.8 mg trypsin or 0.5 mg chymotrypsin. Hydrolytic products were separated with thin-layer chromatography on Silica gel 60 from Merck (Darmstadt) in *n*-propanol/formic acid/H_2O (75 : 12.5 : 12.5) and located by staining guide strips with ninhydrin. Proteolytic fragments were eluted from thin-layer sheets with 0.1 M acetic acid.

The peptides were hydrolyzed with 6 M HCl in evacuated sealed tubes, for 18 h at 105°C and analyzed on a Biotronik LC-6000 amino acid analyzer. The NH_2-terminal residue of the isolated peptides was determined by dansylation followed by thin-layer chromatography [12].

Results

Hydrolysis of synthetic substrates

At first the action of proteinase B on a series of N-substituted dipeptides was tested to determine

TABLE I

SPECIFICITY OF PROTEINASE B IN COMPARISON WITH TRYPSIN AND CHYMOTRYPSIN

n.d., not determined.

Substrate	Concentration	pH	Specific activity (units/mg protein)		
			Proteinase B	Trypsin	Chymotrypsin
Bz-Arg-OEt	1.0 mM	8.0	4.7	30	n.d.
Ac-Tyr-OEt	10.0 mM	8.0	17.0	2.0	120
Bz-DL-Arg-pNA	5.0 mM	7.5	0.0022	0.41	n.d.
Bz-Tyr-pNa	2.0 mM	7.5	0.12	n.d.	0.36
Bz-Ile-Glu-Gly-Arg-pNA	1.0 mM	7.5	0.8	33.7	0.48
Azocoll	2.4%	7.0	37.2	21.5	1.84
Azocasein	1.0%	7.0	4.1	4.8	1.23

whether proteinase B exhibits carboxypeptidase activity. No activity was found against the dipeptides Cbz-Ala-Tyr, Cbz-Glu-Tyr, Cbz-Gly-Leu, Cbz-Tyr-Leu, Cbz-Phe-Leu and Cbz-Gly-Phe, incubated at a concentration of 5 mM with 60 μg/ml proteinase B in 0.1 M potassium phosphate, pH 7.0, for 1 h at 25°C (data not shown). The action of the enzyme on aminopeptidase substrates, like Leu-pNA, Arg-pNA, Lys-pNA and Ala-Tyr-NH_2 was also examined. The esters were incubated at a concentration of 1 mM in 0.1 M Mops, pH 7.5, at 25°C with 50 μg/ml enzyme. No activity was observed, indicating that proteinase B has neither carboxypeptidase nor aminopeptidase activity.

The action of proteinase B on derivatives of arginine and tyrosine was tested and compared with that of chymotrypsin A and trypsin. Each substrate was incubated with different amounts of the various proteolytic enzymes at 25°C for 10–200 min. As shown in Table I, proteinase B hydrolyzes both types of substrates. The rate of hydrolysis of the corresponding substrates with proteinase B is slower than with trypsin or chymotrypsin. The tetrapeptide Bz-Ile-Glu-Gly-Arg-pNA is also hydrolyzed rapidly. It was concluded that the enzyme released only pNA, because no ninhydrin-positive substances could be observed after the addition of mixtures of aminopeptidases to the reaction system. The proteolytic activities of proteinase B, trypsin and chymotrypsin against Azocoll and Azocasein were compared. Their relative specific activities were 100 : 58 : 5 for Azocoll and 100 : 117 : 30 for Azocasein at pH 7 and 25°C. The kinetics of the action of proteinase B on Ac-Tyr-OEt and Bz-Arg-OEt were measured and compared with those of chymotrypsin and trypsin. Lineweaver-Burk plots gave the K_m values listed in Table II. The values of k_{cat} were calculated from the V values and enzyme concentrations, taking 33 000 as the molecular weight for proteinase B [6]. The k_{cat} of proteinase B with Ac-Tyr-OEt is approx. 1/20 of the k_{cat} value with chymotrypsin A as determined by Bender et al. [13]. However, the data cannot be compared directly with those of Bender et al. [13] because the reaction conditions were slightly different.

Trypsin has a very low K_m value for Bz-Arg-OEt [14]. The corresponding K_m value with proteinase B was 25-fold higher. The k_{cat} for Bz-Arg-OEt with proteinase B was 7-fold lower than with trypsin.

Proteolysis of insulin B chain by proteinase B

The peptide bonds cleaved by proteinase B were determined using the oxidized insulin B chain as a model substrate. Insulin B chain was digested for 6 h at 25°C at a ratio of 500 : 1 (mol insulin B chain/mol proteinase B). Eight peptides and three amino acids were isolated from the digest by ion-exchange chromatography on a Dowex AG 50 WX2 column and by thin-layer chromatography. The peptides, shown in Table III, were identified with the data from amino acid composition and NH_2-terminal analysis. Free amino acids were identified as phenylalanine, leucine and tyrosine (Table III).

In order to identify the most susceptible peptide bonds, insulin B chain was digested for only 30 min (Table III). The digest, fractionated in the described

TABLE II

KINETIC CONSTANTS FOR THE HYDROLYSIS OF Ac-Tyr-OEt AND Bz-Arg-OEt

Substrate	Proteinase B			Trypsin			Chymotrypsin		
	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Ac-Tyr-OEt	$8 \cdot 10^{-4}$	9.6	$1.2 \cdot 10^4$	$4.2 \cdot 10^{-2}$	14.5	$3.45 \cdot 10^{2}$ [a]	$7 \cdot 10^{-4}$	192	$2.76 \cdot 10^{5}$ [b]
Bz-Arg-OEt	$1 \cdot 10^{-4}$	2.1	$2.1 \cdot 10^4$	$4.3 \cdot 10^{-6}$	14.6	$3.40 \cdot 10^{6}$ [c]	$1.5 \cdot 10^{-2}$	1.45	$9.6 \cdot 10^{1}$ [a]

[a] Data taken from Inagami et al. [15].

[b] Data taken from Bender et al. [13].

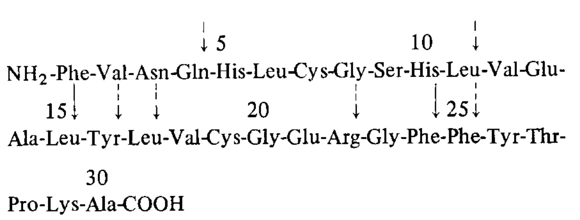
[c] Data taken from Bains et al. [14].

TABLE III

PEPTIDES AND AMINO ACIDS OBTAINED BY PROTEOLYSIS OF INSULIN B CHAIN BY PROTEINASE B

Residues (yield) from 30 min digestion		Residues (yield) from 360 min digestion	
16–24 (33%)	major peptide	5–11 (18.8%)	Phe (4.6%)
		26–30 (7.3%)	Leu (3.6%)
5–15 (11%)		18–24 (7.3%)	Tyr (3.6%)
25–30 (5%)	minor peptides	17–22 (4.9%)	
1–15 (2%)		1–11 (4.9%)	
		12–15 (3.6%)	
		1– 4 (2.4%)	
		23–24 (1.9%)	

manner, gave one major and three minor cleavage products. The major peptide contained the residues 16–24 of the insulin B chain (33%). The minor peptides could be assigned to the fragments 25–30 (5%), 5–15 (11%) and 1–15 (2%) of the insulin B chain. Since the major peptide was obtained in a higher yield after 30 min digestion than after 6 h, it is produced by a first cleavage at Leu(15)–Tyr(16) and Phe(24)–Phe(25) and then subjected to further attack. Tyrosine, leucine and the peptides 17–22, 18–24 and 23–24 may result from degradation of the major peptide. Peptide 5–11 was not detected in the 30 min digest, indicating that Leu(11)–Val(12) is a minor cleavage site. The peptide 25–30 obtained after 30 min digestion seems to be subjected to further degradation to give the fragment 26–30 and free phenylalanine. The time course of appearance of free amino acids during digestion indicates that the rapid release of phenylalanine is followed by tyrosine and then leucine. This may be consistent with the results described above. The major as well as the minor cleavage sites are summarized in Scheme I.



Scheme I. Sites of cleavage in oxidized insulin B chain.
The full-line arrows indicate major cleavage sites, while the dashed-line arrows indicate slowly cleaved peptide bonds.

Proteolysis of the hexapeptide Leu-Trp-Met-Arg-Phe-Ala

To obtain more information on the selectivity of proteinase B, proteolysis of a hexapeptide was examined. As shown in Fig. 1, the hexapeptide was cleaved very rapidly. The hydrolytic products were separated by thin-layer chromatography. After short-time incubation, two peptides were observed in addition to be hexapeptide uncleaved. The oligopeptides were separated by thin-layer chromatography and then isolated by elution with 0.1 M acetic

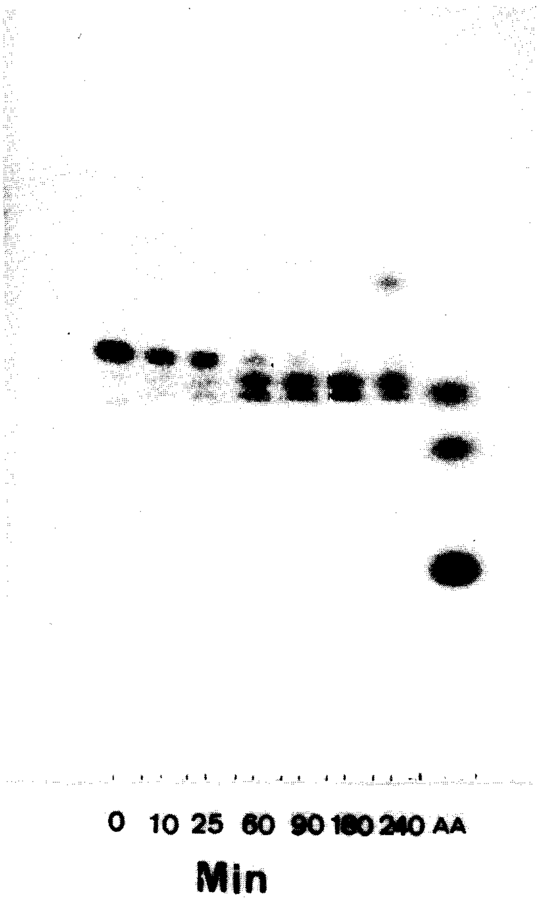


Fig. 1. Proteolysis of the hexapeptide Leu-Trp-Met-Arg-Phe-Ala by proteinase B. Hydrolysis of the hexapeptide was performed in a reaction mixture consisting of 0.6 mg proteinase B and 10.0 mg hexapeptide in 1.2 ml 0.1 M ammonium acetate, pH 7.2 at 25°C. 0.1 ml aliquots were removed at 0, 10, 25, 60, 90, 180 and 240 min, and the reaction was stopped by the addition of acetic acid to 10% (v/v). Analysis was done on silica plates. Reference amino acids (AA) from top to bottom are Phe, Met, and Ala.

acid. The results from amino acid analyses indicate that these fragments are generated by cleavage at Arg-Phe of the hexapeptide. After 240 min two additional peptides could be observed. From the amino acid analysis these fragments were identified as Leu-Trp and Met-Arg. Parallel experiments on the hydrolysis of the hexapeptide by trypsin and chymotrypsin revealed that trypsin cleaves the Arg-Phe bond. Chymotrypsin first hydrolyses the Trp-Met bond of the hexapeptide and later the Phe-Ala bond. These results show that proteinase B exhibits predominantly trypsin-like activity during hydrolysis of the hexapeptide Leu-Trp-Met-Arg-Phe-Ala.

Discussion

From these studies using polypeptide and synthetic substrates a uniqueness in the cleavage specificity of proteinase B appears: the enzyme shows both chymotrypsin-like and trypsin-like behavior. Synthetic substrates for chymotrypsin and for trypsin were hydrolyzed by proteinase B although the rates of hydrolysis were slower than those of the corresponding substrates by chymotrypsin and trypsin (Table I). The action of proteinase B on the oxidized B chain of insulin seems to be like porcine chymotrypsin C rather than like trypsin (Scheme I). Sites for rapid cleavage by proteinase B between Leu(15)–Tyr(16) and between (Phe(24)–Phe(25) are also major sites for porcine chymotrypsin C [16]. Minor sites for proteinase B between Tyr(16)–Leu(17) and between Phe(25)–Tyr(26) are also minor sites for porcine chymotrypsin C. The other minor sites for chymotrypsin C between Glu(4)–His(5), Leu(11)–Val(12) and Leu(17)–Val(18) were also minor sites for proteinase B. The sites between Leu(6)–Cys(7) and between Tyr(26)–Thr(27) were not attacked by proteinase B, but hydrolyzed by chymotrypsin A. The minor site for proteinase B between Arg(22)–Gly(23) is a major site for trypsin [17]. On the other hand, the action of proteinase B on the hexapeptide studied exhibits mainly trypsin-like rather than chymotrypsin-like activity (Fig. 1). The results suggest that purified proteinase B is a mixture of two types of proteinase, namely of a chymotrypsin-like and a trypsin-like proteinase. Actually, proteinase B appeared on polyacrylamide gel electrophoresis and electrofocusing in multiple forms, which exhibited Azocoll hydrolyzing activities [6]. Recent work by Wolf and Ehmann [17,18] and Zubenko et al. [19,20] with mutants of proteinase B suggests that the Azocoll-hydrolyzing activity in wild-type yeast extract is only derived from proteinase B. Thus, the purified proteinase B of high Azocoll-hydrolyzing activity should contain only very low amounts of other proteinases, if contaminated at all. Furthermore, preliminary experiments showed that either antipain, which preferentially inhibits trypsin [21], or chymostatin, a powerful inhibitor for chymotrypsin [21], depress both trypsin-like activities (hydrolysis of Azocoll and the hexapeptide) and chymotrypsin-like activities (hydrolysis of Ac-Tyr-OEt and insulin B chain) of proteinase B. This supports the idea that proteinase B is not a mixture of two types of proteinase but one enzyme, exhibiting both tryptic and chymotryptic activities.

The mixed specificity of yeast proteinase B may be explained by the model of the active site of proteinases as proposed by Schechter and Berger [22]. The authors found in the case of papain, that the active site extends over a range of about 25 Å which corresponds in size to at least seven amino acid residues (subsites). These subsites are located on both sides of the catalytic site of the proteinase (S_4-S_3-S_2-S_1-S_1'-S_2'-S_3'). Each of them accomodates one amino acid residue of the peptide substrates (P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'). The reactivity of a certain bond (P_1-P_1') in a peptide substrate depends not only on the two residues P_1 and P_1' (which occupy S_1 and S_1') but also on the nature of the residues in their neighborhood, occupying adjacent subsites [22]. Unexpected cleavages of the substrate may be favoured by the influence of adjacent subsites like S_2, S_3, S_4, S_2' and S_3' in the proteinase. We have, however, no experimental data to discuss extensively the broad substrate specificity of proteinase B on the basis of the Schechter and Berger model.

Acknowledgement

We thank Drs. John Phillips, Franz Meussdoerffer and Garfield P. Royer for reading and discussion of the manuscript.

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