COMPARISON OF VARIOUS TYPES OF SUBTILISINS: SIZE AND PROPERTIES OF THE ACTIVE SITE

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SUMMARY

A comparative study was made on the size and properties of the active sites in five subtilisin types; Carlsberg, Novo, BPN', Fukumoto and SO4. The study was carried out using various synthetic peptides as substrates and their chloromethyl ketone derivatives as inhibitors. The results indicate that all five subtilisins have a large active site corresponding to at least six amino acid residues in peptide substrates, and that the properties of their active sites are qualitatively alike.

A DFP*-sensitive alkaline proteinase, called subtilisin, is produced by many strains of Bac. subtilis and related species. Representative types of this enzyme are known as Carlsberg, Novo, and BPN', though the latter two enzymes are believed to be identical (1). About one-third of the amino acid sequence in the Carlsberg enzyme differs from the sequence of BPN' (or Novo) enzyme (2), but the tertiary structures of both the enzymes are assumed to be identical or very similar (3). Enzymatic characteristics of the three subtilisins have been comparatively studied (4-8), and indicate that the Carlsberg enzyme is somewhat different from the BPN' or Novo enzyme, but that the latter two enzymes are remarkably similar.

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* Abbreviations: DFP, diisopropyl phosphofluoridate; ZPCK, benzyloxycarbonyl-L-phenylalanine chloromethyl ketone; ZAPCK, benzyloxycarbonyl-L-alanyl-L-phenylalanine chloromethyl ketone; ZAGPCK, benzyloxycarbonyl-L-alanyl-glycyl-L-phenylalanine chloromethyl ketone; Z, benzyloxycarbonyl; NH_2, amide. Abbreviated designations of amino acid derivatives, peptides, and their derivatives obey the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature. Except when specified, the constituent amino acids were all of L-configuration.

In the preceding papers (9, 10), we have shown that subtilisin BPN' possesses a large active site corresponding to at least six amino acid residues, four toward the amino-end and two toward the carboxyl-end from the catalytic point in peptide substrates. The present study was undertaken to compare the size and properties of the active sites in various types of subtilisin; Carlsberg, Novo, BPN' and two more enzymes obtained from Bac. subtilis var. amylosacchariticus Fukumoto and Bac. subtilis var. SO4. The comparative study was made using various synthetic peptides as substrates as well as some chloromethyl ketone derivatives of peptide substrates as inhibitors, as described in the previous papers (9-11).

MATERIALS AND METHODS

Subtilisins Carlsberg and Novo were kindly supplied by Novo Industri A/S, Copenhagen. Subtilisin BPN' and an alkaline proteinase of Bac. subtilis var amylosacchariticus Fukumoto (abb. Fukumoto) were obtained from Nagase & Co., Osaka, and Seikagaku Kogyo, Tokyo, respectively. An alkaline proteinase of Bac. subtilis var. SO4 (abb. SO4) was kindly donated by Prof. S. Murao of Osaka Prefectural University. The five samples were each crystalline and shown to be homogeneous. The chloromethyl ketone derivatives, ZPCK, ZAPCK and ZAGPCK, were synthesized as shown in the preceding paper (11). Z-Tyr-NH_2, Z-Gly-Tyr-NH_2, Z-Ala-Tyr-NH_2, Z-Tyr-Tyr-NH_2 and Z-Gly-Gly-Tyr-NH_2 were obtained from Cyclo Chemical Corporation, California. The other peptides were synthesized in this laboratory according to the method described previously (10).

The normality of each subtilisin was determined according to the method of Bender et al. (12) using N-trans-cinnamoylimidazole as titrant. The proteinase unit was determined using casein as substrate at pH 7.4 and 40°C (13), and was expressed as mg of tyrosine released per min per mg enzyme. Inactivation of enzyme by the chloromethyl ketone derivatives was carried out according to the method described previously (11). The reaction mixture contained 0.05 M Tris-buffer (pH

7), 10^{-3} M $CaCl_2$, 0.5 x 10^{-5} M of enzyme, 10^{-4} M of inhibitor, and 10% dioxane, and was kept at 40°C. The pseudo-first order reaction constant, k (sec^{-1}), was determined as usual.

Amidase and peptidase activity were determined by the ninhydrin method of Yemm and Cocking (14), in which calculations were made by using authentic compounds as standards. The reaction was carried out at pH 8.5 and 40°C in the presence or absence of 15% dimethylformamide (for low solubility of some peptides). Substrate concentration was 2 mM and enzyme concentration was suitably adjusted to determine the initial rate of hydrolysis. The sites of action of enzymes upon substrates were deduced from paper chromatograms of the hydrolysates, or by the usual DNP-method. Ammonia released was determined using Conway's apparatus.

RESULTS AND DISCUSSION

Some enzymatic characteristics of the five subtilisins were comparatively studied, and the results are summarized in Table I. The results show that Carlsberg enzyme has over three-times more proteinase activity than any of the other subtilisins. The behaviour of the five subtilisins against chloromethyl ketone derivatives are similar to each other; the degree of inactivation being as follows, ZAGPCK $\gg$ ZAPCK > ZPCK. The k value of Carlsberg enzyme against ZPCK or ZAPCK is highest among the five enzymes, but that against ZAGPCK is not. The sensitivities of the two subtilisins Fukumoto and SO4 against the chloromethyl ketone derivatives

Table I
Enzymatic characteristics of five subtilisins

Enzyme	Normality %	Proteinase unit, x10^3	Inactivation (k, x10^5, sec^{-1}) ZPCK	ZAPCK	ZAGPCK
BPN'	44.6	9.5	3.5	9.3	311
Novo	74.8	12.8	4.6	11.9	367
Carlsberg	57.7	34.0	9.2	26.8	284
Fukumoto	—*	9.5	0.4	4.2	167
SO4	63.8	8.8	1.3	4.5	184

* Not determined for low solubility of the crystalline enzyme.

appear to be somewhat smaller than those of the other subtilisins.

The size and properties of the active site in subtilisins were determined according to the method described previously (9, 10). For this purpose, Z-A-$(Gly)_n$-Tyr↑-NH_2, Z-A-$(Gly)_n$-Leu↑-NH_2 and Z-Gly-Leu↑-$(Gly)_n$-B (A or B = various amino acid residues; n = 0, 1 or 2) were used as substrates. These peptides were split by all the subtilisins at the linkage containing the carboxyl group of L-tyrosine or L-leucine (as shown by the arrow). Accordingly, by varying the value of n in these peptides, we expected to determine the influence on the hydrolysis of the distance of A and B from the catalytic point. To clarify the role of the terminal α-amino or α-carboxyl groups, further study was made using the above peptides in their Z-free form as substrates.

Table II

Hydrolysis of Z-A-$(Gly)_n$-Tyr-NH_2 and Z-A-$(Gly)_n$-Leu-NH_2

Reaction was carried out in the presence of 15% dimethyl formamide and the other conditions were described in MATERIALS AND METHODS. The positions of amino acid residues (P) in each peptide are numbered as P_1, P_2, etc. to the N-terminus, and as P_1', P_2', etc. to the C-terminus from the catalytic point. The arrow shows the bond split.

Peptide $P_5-P_4-P_3-P_2-P_1\downarrow P_1'$	Enzyme: BPN'	Novo	Carlsberg	Fukumoto	SO4
	Rate of hydrolysis, (μM substrate/mg enzyme/min)				
Z—Tyr–NH_2	0.03	0.04	0.07	<0.01	<0.01
H—Tyr–NH_2	<0.01	<0.01	<0.01	<0.01	<0.01
Z—Gly–Tyr–NH_2	0.10	0.10	0.09	0.07	0.04
Z—Ala–Tyr–NH_2	0.92	0.95	1.84	0.25	0.18
H—Ala–Tyr–NH_2	<0.01	<0.01	<0.01	<0.01	<0.01
Z—D-Ala–Tyr–NH_2	<0.01	<0.01	<0.01	<0.01	<0.01
Z—Tyr—Tyr–NH_2	0.02	0.01	0.04	<0.01	<0.01
Z — Gly–Gly–Tyr–NH_2	2.50	2.26	1.52	1.84	1.80
Z — Ala–Gly–Tyr–NH_2	7.05	7.22	4.78	4.67	2.70
H — Ala–Gly–Tyr–NH_2	0.02	0.04	<0.01	<0.01	<0.01
Z – D-Ala–Gly–Tyr–NH_2	0.03	0.04	0.05	0.02	0.02
Z— Ala — Gly–Gly–Leu– NH_2	5.0	7.70	14.68	6.09	4.90
H— Ala — Gly–Gly–Leu– NH_2	0.25	0.37	0.97	0.27	0.45
Z–D-Ala— Gly–Gly–Leu– NH_2	0.30	0.26	0.28	0.71	0.91

The results are summarized in Tables II and III, where the positions of amino acid residues (P) in each peptide are numbered as P_1, P_2, etc. to the N-terminus, and as P_1', P_2', etc. to the C-terminus from the catalytic point. Table II indicates that all the subtilisins show either stereo- or sidechain-specificity against amino acid residues at P_2, P_3 and P_4 in peptide substrates. The table further indicates that the hydrolysis by all the subtilisins is remarkably inhibited when charged α-amino groups at P_2, P_3 and P_4 in peptide substrates are not blocked with a Z residue, possibly owing to the repulsion of the charged group corresponding to the respective subsite in the subtilisin. By elongation of the peptide chain to the N-terminus in peptide substrates, a remarkable increase of hydrolysis is observed with all the subtilisins, as seen in the correlation among Z-Tyr-NH_2, Z-Gly-Tyr-NH_2 and Z-Gly-Gly-Tyr-NH_2. This well reflects the results with chloromethyl ketone derivatives of the peptide substrates shown in Table I.

Table III indicates that hydrolysis of Z-Gly-Leu-B by all the subtilisins is negligible irrespective of the kind of B, while hydrolysis of either Z-Gly-Leu-NH_2

Table III
Hydrolysis of Z-Gly-Leu-$(Gly)_n$-B

Reaction was carried out in the absence of dimethylformamide, and the other conditions were described in MATERIALS AND METHODS.

Peptide $P_3-P_2-P_1\downarrow P_1'-P_2'$	Enzyme: BPN'	Novo	Carlsberg	Fukumoto	SO4
	Rate of hydrolysis, (μM substrate/mg enzyme/min)				
Z—Gly—Leu—NH_2	0.83	0.92	4.23	0.60	0.51
Z—Gly—Leu—Gly Z—Gly—Leu—Ala Z—Gly—Leu—Leu Z—Gly—Leu—Phe	≤0.01	<0.01	≤0.02	≤0.01	<0.01
Z—Gly—Leu—Gly—Gly	2.05	2.16	9.07	2.59	2.08
Z—Gly—Leu—Gly—Ala	2.48	3.70	10.58	2.65	2.53
Z—Gly—Leu—Gly—D-Ala	0.66	0.72	1.52	0.80	0.74
Z—Gly—Leu—Gly—Phe	3.15	3.95	12.95	4.24	3.15

or Z-Gly-Leu-Gly-B is considerable. This suggests that the presence of charged α-carboxyl group at P_1' in peptide substrates disturbs the enzyme-substrate interaction, possibly by repulsion with the corresponding subsite in the subtilisin. At P_2', stereo-specificity is considerable but sidechain-specificity is small in all the subtilisins. The effect of the kind of amino acid residue at P_1' on hydrolysis by subtilisins cannot be deduced from Table III. An experiment using Z-Tyr-B-NH_2 (B = various amino acid residues) as substrates indicated that these subtilisins hydrolyzed the peptides at the peptide bond (Tyr-B) but did not at the amide bond (B-NH_2) when glycine and L-alanine were used as B, but the reverse was observed when L-leucine and L-phenylalanine were used as B. This might suggest that a bulky residue occupying the P_1' position rather inhibits the hydrolysis. Stereo-specificity was also observed at the position.

Some differences have previously been observed between Carlsberg and Novo (or BPN') enzymes in their enzymatic properties, as seen in hydrolysis of clupein (5). Our present study, however, indicates that all five subtilisins have a large active site corresponding to at least six amino acid residues (P_1-P_4 and P_1'-P_2') in peptide substrates and that the properties of these active sites (except for the subsite corresponding to P_1) are qualitatively alike. This is not inconsistent with the previous assumption (3) that both subtilisins Carlsberg and BPN' possess an identical or very similar three-dimensional structure.

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