COMPARISON OF α-CHYMOTRYPSIN AND SUBTILISIN BPN': SIZE AND SPECIFICITY OF THE ACTIVE SITE

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It is a well-known fact that there are mechanistic similarities between the proteolytic enzymes α-chymotrypsin and subtilisin BPN' (an alkaline proteinase of Bac. subtilis). For example, an active center serine residue reacts with DFP* and the essential role of a histidine has been clarified in both the enzymes (Shaw and Ruscica, 1968). They exhibit a high specificity against aromatic amino acid residues such as L-tyrosine, L-phenylalanine, etc. (Morihara and Tsuzuki, 1969). However, there are some conflicts between them as follows. Subtilisin BPN' was insensitive to TPCK, a specific inhibitor of α-chymotrypsin, but has recently been shown by Shaw and Ruscica (1968) to be inhibited by ZPCK and ZPBK. Morihara and Tsuzuki (1969) have also found that Bz-Tyr-NH_2, a suitable substrate for α-chymotrypsin, was hydrolyzed only a small amount by subtilisin BPN'.

To clarify the conflict, a systematic study was undertaken using various synthetic peptides such as Z-A-Tyr-NH_2, Z-A-Gly-Tyr-NH_2, Z-Tyr-B-NH_2, and Z-Tyr-Gly-B (A or B = various amino acid residues) as substrates. These synthetic peptides should be split at the peptide bond containing the carboxyl group of L-tyrosine by both α-chymotrypsin and subtilisin BPN'. Therefore, we should be able to examine the effects of the

* The abbreviations used are: DFP, diisopropyl fluorophosphate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; ZPCK, benzyloxycarbonyl-phenylalanine chloromethyl ketone; ZPBK, benzyloxycarbonyl-phenylalanine bromomethyl ketone. Abbreviated designations of peptides or the derivatives obeyed the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature.

neighboring residues surrounding the sensitive L-tyrosine of the peptide substrate by varying the kinds of A or B in the peptides. This would provide an information on the sizes and the specificities of the active sites in both the enzymes, if the assumptions previously made concerning papain by Schechter and Berger (1967) are true. These assumptions are; 1) the substrates are lined up on the enzyme in such a way that the CO-NH linkage to be hydrolyzed always occupies the same location (the catalytic site) and 2) the amino acid residues occupy adjacent subsites, those towards the NH_2-end occupying subsites S_1, S_2, etc., and those towards the COOH-end occupying subsites S_1', S_2', etc.

Our results indicated that both enzymes have a considerably large active site which covers at least 18 Å and can be divided into at least 5 "subsites", each accomodating one amino acid residue of peptide substrate. Further, it was ascertained that the properties of the large active sites of the enzymes except subsite S_1, which lies adjacent to the tyrosine residue of the peptide substrate, are considerably different from one another.

MATERIALS AND METHODS

Some peptides were obtained commercially, and the others were prepared in our laboratory, the procedures of which will be reported in an another paper. Their purity was checked by elementary analysis and by thin layer chromatography. Except when specified, the constituent amino acids were all of the L-configuration. A crystalline α-chymotrypsin was obtained from Worthington Biochemical Corporation, New Jersey, and a crystalline alkaline proteinase of Bac. subtilis (subtilisin BPN') was obtained from Nagase Co., Osaka. The peptidase activity was determined as follows: A reaction mixture containing 0.05 M Tris-buffer of pH 8.5, 3 mM substrate, 15% dimethylformamide (for low solubility of most peptides), and a suitable amount of enzyme, in a final volume of 1 ml (or 5 ml), was incubated at 30° (for α-chymotrypsin) or 40° (for subtilisin BPN'). The enzyme concentration was suitably adjusted to determine the initial

rate of proteolysis. At every 3 minutes, 0.1 ml (or 1 ml) each of the reaction mixture was withdrawn and put into a test tube which contained 0.1 ml (or 1 ml) of 0.1 N HCl to stop further hydrolysis. The extent of hydrolysis was measured by the ninhydrin method of Yemm and Cocking (1955), in which calculations were made by using authentic compounds as a standard. The sites of action of enzymes upon substrates were deduced by a paper chromatogram of the hydrolysates comparing authentic compounds, or by the usual DNP-method. Ammonia released was measured using Conway's apparatus.

RESULTS AND DISCUSSION

Table I shows the proteolytic activities of both enzymes against various diastereoisomeric peptides, in which the linkages containing the carboxyl group of L-tyrosine were hydrolyzed as had been expected from their specificity. The positions of the amino acid residues (P) in the peptide substrate are numbered according to the subsites they occupy, i.e., the residues which are adjacent to subsites S_1, S_2, etc. are referred to as P_1, P_2, etc., respectively, and those adjacent to subsites S_1', S_2', etc. are referred to as P_1', P_2', etc., respectively, as shown in the table. It indicates that a stereospecificity is present in both the enzymes at all the subsites, such as S_2-S_3 and S_1'-S_2' which surround the central subsite S_1. The specificity, however, decreases as the distance from the catalytic site increases except in the case of subsite S_3 of subtilisin BPN'. The latter case may indicate that the enzyme has a larger active site than that of α-chymotrypsin in the region corresponding to the N-terminus of the peptide substrate.

The side-chain-specificity of each subsite in both the enzymes was determined as shown in Table II. As to subsite S_2, a remarkable difference is observed between the enzymes: An aromatic residue seems to act as a promoter of hydrolysis for α-chymotrypsin (the poor amidase activity against Z-Tyr-Tyr-NH_2 can be ascribed to the sensitivity of Tyr-Tyr bond, as will be seen later), but the same residue rather acts as a depressor for subtilisin BPN'. The reactivity of subtilisin BPN' is greatly promoted by L-alanine at the subsite, while the reverse is seen in α-chymotrypsin. A more striking effect is

Table I. Stereo-specificity

The arrow shows the bond split. Peptides (a) were obtained from Cyclo Chemical Corporation, and those (b) were prepared in this laboratory.

Peptide P_4—P_3——P_2——P_1—↓—P_1'——P_2'	α-Chymotrypsin (μM/min·mg enzyme)	Subtilisin BPN' (μM/min·mg enzyme)
Z —— L-Ala —Tyr —NH_2 (a)	0.031	0.920
Z —— D-Ala—Tyr —NH_2 (b)	0.005	0.008
Z – L-Ala —Gly——Tyr —NH_2 (b)	0.177	15.200
Z – D-Ala—Gly——Tyr —NH_2 (b)	0.034	0.067
Z ——Tyr —L-Ala— NH_2 (a)	0.358	0.005
Z ——Tyr —D-Ala— NH_2 (b)	0.010	0.000
Z ——Tyr —Gly——L-Ala (b)	0.014	0.010
Z ——Tyr —Gly——D-Ala (b)	0.004	0.004

Table II. Side-chain-specificity

Peptides (a) and (b) were described in Table I. Peptides (c) were obtained from "Peptide Center" at Osaka University.

Peptide P_4— P_3 ——P_2 ——P_1 —↓—P_1' ——P_2'	α-Chymotrypsin (μM/min·mg enzyme)	Subtilisin BPN' (μM/min·mg enzyme)
Ac —Tyr —NH_2 (c)	0.011	0.011
Bz —Tyr —NH_2 (c)	0.186	0.003
Z —— Gly — Tyr —NH_2 (c)	0.067	0.098
Z —— Ala — Tyr —NH_2 (a)	0.031	0.920
Z —— His — Tyr —NH_2 (a)	0.020	0.017
Z —— Tyr — Tyr —NH_2 (a)	0.000	0.033
Z —Gly — Gly — Tyr —NH_2 (a)	0.048	5.000
Z —Ala — Gly — Tyr —NH_2 (b)	0.177	15.200
Z —— Tyr —Gly — NH_2 (a)	0.051	0.004
Z —— Tyr —Ala — NH_2 (a)	0.358	0.005
Z —— Tyr —Ser — NH_2 (a)	0.297	0.000
Z —— Tyr —Leu — NH_2 (c)	0.488	0.016
Z —— Tyr —Tyr — NH_2 (a)	1.092	0.000
Z —— Tyr —Phe — NH_2 (a)	0.208	0.000
Z —— Tyr —Gly — Gly (a)	0.007	0.010
Z —— Tyr —Gly — Ala (b)	0.014	0.010
Z —— Tyr——Gly — Phe (b)	0.015	0.050

observed by elongation of the peptide chain to the N-terminus in subtilisin BPN' as seen in the correlation between Z-Gly-Tyr-NH_2 and Z-Gly-Gly-Tyr-NH_2, but this is not observed in α-chymotrypsin.

The specificity of subsite S_1' is much more strict in α-chymotrypsin than in sub-

tilisin BPN'; L-tyrosine promotes hydrolysis by about twenty times higher degree than does glycine in α-chymotrypsin, while no such promotion of hydrolysis by any specific amino acid residue is observed in subtilisin BPN'. The peptide bonds that are split in Z-Tyr-Tyr-NH_2 are different by both the enzymes; α-chymotrypsin hydrolyzes the peptide bond of Tyr-Tyr and subtilisin BPN' splits the Tyr-NH_2 bond. This might be ascribed to the difference of the specificity of subsite S_1' in both the enzymes.

In this communication, we reported that both α-chymotrypsin and subtilisin BPN' have a considerably large active site which can be divided at least into 5 "subsites" such as S_1-S_3 and S_1'-S_2' at both sides from the catalytic site, each accomodating one amino acid residue of peptide substrate. The sizes are thus calculated as about 18 Å taking 3.5 Å per one amino acid residue of peptide substrate. The specificity of subsite S_1 in both the enzymes were comparatively determined using Ac-X-OEt (X = various L-amino acids), as reported in an another paper (Morihara and Tsuzuki, 1969). It indicated that subtilisin BPN' showed a somewhat broader specificity than that of α-chymotrypsin. The present study indicated that there are remarkable differences between the enzymes in their enzymatic properties of the large active site except subsite S_1. The degree of the difference may be greater than that of subsite S_1. The X-ray analysis of subtilisin BPN' may clarify the difference of the enzymatic properties of the large active site from them of α-chymotrypsin.

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