THE SPECIFICITIES OF VARIOUS NEUTRAL AND ALKALINE PROTEINASES FROM MICROORGANISMS

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It is well-known (Hagihara, 1960) that most of the neutral proteinases (peptide peptidohydrolase) of microorganisms are inhibited by EDTA but not by either DFP or potato inhibitor, while most of the alkaline proteinases from the same origin are not affected by the former but are completely inhibited by the latter two agents. The former enzymes can also be called metallo-enzymes and the latter serine-enzymes. However, classification according to their side-chain specificities has not been investigated, it being assumed that microbial proteinases possess specificities too broad to be distinguishable from each other.

Five neutral and four alkaline proteinases of homogeneous nature were obtained from various origins of either bacteria or mold, and their specificities were comparatively determined using several synthetic peptides and esters as substrates. The results showed that neutral enzymes possess specificity toward amino acid residues which contain the amino group to be hydrolyzed, while alkaline proteinases possess specificity toward amino acid residues which contain the carboxyl group to be hydrolyzed.

MATERIALS AND METHODS

Enzymes: The neutral proteinase of Bac. subtilis was prepared from the crude protease preparation which was kindly supplied by Nagase, Co. Ltd., Osaka. The purification procedures were exactly the same as described by McConn et al. (1964). Neutral and alkaline proteinases of St. griseus were prepared by column chromato-

graphy on CM-cellulose and DEAE-Sephadex, as developed by Narahashi and Yanagita (1966). A commercial preparation of "Pronase" (Kaken kagaku Co. Ltd., Tokyo) was used as the starting material. Neutral and alkaline proteinases of Asp. oryzae OUT 5038 were prepared as follows: solid cultures were grown on wheat bran, extracted with distilled water, precipitated with ammonium sulphate (80% saturation), chromatographed on DEAE-cellulose and further chromatographed on Sephadex G-100 in case of the former enzyme or rechromatographed on DEAE-cellulose in case of the latter enzyme. Special care was taken to prevent contamination by peptidase activities in the latter two cases. A thermostable proteinase of Bac. thermoproteolyticus Rokko (thermolysin) and an elastase of Ps. aeruginosa were regarded as a neutral proteinase from their optimum pH and their behavior against inhibitors (Endo, 1962, Morihara et al., 1965, Morihara and Tsuzuki, 1966). The former enzyme (crystalline) was kindly donated by Dr. Endo of Daiwa kasei Co. Ltd., Osaka, and the latter one was crystallized according to a method described previously (Morihara et al., 1965). "Nagarse" (crystalline) which was obtained from Nagase Co. Ltd., Osaka, was used as an alkaline proteinase of Bac. subtilis. Crystalline proteinase of St. fradiae which showed the typical characteristics of a usual alkaline proteinase was prepared according to a method described previously (Morihara et al., 1966). The purity of the above enzymes were determined by the proteolytic activity of each enzyme remaining after treatment with EDTA (10^{-3} M) or DFP (10^{-3} M) at 40°C for 30 minutes at pH 7.4. The proteolytic activity was determined using casein as a substrate as described in a previous paper (Morihara, 1963). All the neutral proteinases as mentioned above were inhibited completely by the former inhibitor but not by the latter, while the alkaline proteinases were not affected by the former inhibitor but were completely destroyed by the latter one.

Determination of activity against synthetic peptides or esters: Various synthetic peptides or esters were kindly supplied by Dr. Sakakibara of Osaka University. Except

specified, the constituent amino acids were all L-configurations. Either peptidase or amidase activity was measured by the ninhydrin method of Moore and Stein (1954), in which the percentage of hydrolysis was calculated using authentic compounds as a standard. Esterase activity was determined by the alcoholic titration method (Willstätter and Waldschmidt-Leitz, 1921). The bonds hydrolyzed were identified either by paper chromatography of the hydrolysate compared to authenticly known compounds or by the usual dinitrophenylation technique. The release of ammonia from the substrate was deduced by the reduction of the ninhydrin value after drying in a vacuum over P_2O_5.

RESULTS AND DISCUSSION

Table I shows the enzymatic activities of various neutral and alkaline proteinases upon several synthetic substrates. It indicates that all the peptides tested were attacked by both the neutral and alkaline enzymes but esters were hydrolyzed only by the latter enzymes. The peptide bonds hydrolyzed were determined, and it was found that all the neutral enzymes hydrolyzed the peptide bond containing the amino group of leucine, phenylalanine or tyrosine, while the alkaline enzymes commonly split the peptide, amide or ester bond involving the carboxyl group of leucine, phenylalanine or tyrosine.

The neutral proteinases presented here did not hydrolyze Z-Gly-Pro-D-Leu-Gly-Pro-OH, Z-Gly-D-Leu-NH_2 and Z-Gly-D-Phe-NH_2. It was further found that the activities of these enzymes upon peptides such as Z-Gly-Ala-NH_2, Z-Gly-Val-NH_2, Z-Gly-Ser-NH_2 and Z-Gly-Pro-NH_2 were negligible in comparison with those of suitable substrates as shown in Table I. Peptides such as Z-Ala-Leu-NH_2, Z-Leu-Leu-NH_2, Z-Pro-Leu-NH_2, Z-Phe-Leu-NH_2 and Z-Tyr-Leu-NH_2 were hydrolyzed by these enzymes releasing Leu-NH_2. These results lead to the consideration that neutral proteinases possess specificity to the amino acid residue which involved the amino group to be hydrolyzed. This is not inconsistent with the previous view of Matsubara et al. (1965 and 1966) concerning thermolysin and our findings (Morihara and Tsuzuki, 1966,

TABLE I

Hydrolysis[a] of Synthetic Substrates by Various Neutral and Alkaline Proteinases of Microorganisms

Substrate	Neutral Proteinase from					Alkaline Proteinase from			
	B. subtilis (0.74)[b]	*B. thermoproteolyticus* (0.14)	*P. aeruginosa* (0.24)	*S. griseus* (0.026)	*A. oryzae* (0.18)	*B. subtilis* (0.75)	*S. griseus* (0.08)	*S. fradiae* (0.6)	*A. oryzae* (0.28)
	(per cent of hydrolysis)								
Z-Gly-Pro-Leu-Gly-Pro-OH	18.3	21.5	25.6	33.5	35.9	32.3	25.9	87.1	34.1
Z-Gly-Pro-Leu-Gly-OH	7.0	8.7	29.1	3.9	9.4	18.1	11.8	15.4	8.1
Z-Gly-Leu-NH_2	18.5	18.0	21.5	38.7	0.9	10.6	11	8.8	0
Z-Gly-Phe-NH_2	1.8	18.5	35.2	65.0	2.6	7.1	5	19.4	4.6
Z-Gly-Tyr-NH_2	0	0	18.0	22.9	2.7	19.2	26	28.2	8.7
Ac-Tyr-OEt	0	0	0	0	0	64	47[c]	51	61
Ac-Phe-OEt	0	0	0	0	0	100	30[c]	98	100

[a] The reaction mixture contained 0.05 M Tris-buffer (pH 7 for neutral proteinase and pH 9 for alkaline proteinase), a suitable amount of enzyme and 4 mM of peptide or 20 mM of ester. In the latter case, 8% ethanol was involved in the reaction mixture for low solubility of the substrate. The reaction was carried out for 20 minutes or 16 hours at 40°C, in which the results in the latter case are shown by underline.

[b] The value in the parenthesis shows the proteolytic activity (x 10^{-3} [PU]) per ml of reaction mixture, in which [PU] was determined by mg of tyrosine released per minute per ml enzyme solution in a casein digestion at pH 7.4 and 40°C, as described previously (Morihara, 1963).

[c] Enzyme concentration used was 0.16 x 10^{-3} [PU] per ml of the reaction mixture.

Morihara and Ebata, 1966) about thermolysin and Ps. aeruginosa elastase, which have shown that these enzymes hydrolyzed the peptide bonds involving the amino group of isoleucine, leucine and phenylalanine in the digestion of cytochrome c and others.

In addition, the alkaline proteinases did not attack the above three synthetic peptides containing D-Leu or D-Phe. However, they showed a remarkably high esterase activity as seen in Table I, as well as the amidase activity mentioned above. These results indicate that alkaline proteinases possess specificity to the amino acid residue which contains the carboxyl group to be hydrolyzed. This is similar to other proteolytic enzymes such as trypsin, α-chymotrypsin etc.

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