THE NATURE OF THE BACTERIOLYTIC PROTEASES OF SORANGIUM SP.

D. R. Whitaker, L. Jurášek and C. Roy

Division of Biosciences, National Research Council,

Ottawa, Canada

Received June 6, 1966

Culture filtrates of a soil bacillus of the genus Sorangium are intensely lytic towards several groups of bacteria (Gillespie and Cook, 1965; Whitaker, Cook and Gillespie, 1965a). Two proteolytic enzymes, which have been given the trivial names "α- and β-lytic proteases", are responsible for most of this activity. Isolation procedures for the two enzymes, some of their physical and proteolytic properties, and their action on mucopeptides from bacterial cell-walls have been described (Whitaker, 1965b; Jurášek and Whitaker, 1965; Whitaker et al., 1965c; Tsai et al., 1965).

This report gives evidence of the following:-

(1) The α-lytic protease is a "serine-protease" with the sequence: -Asp-Ser-Gly- at the reactive serine residue. Thus it belongs to the same group as all mammalian serine proteases of known sequence and not to the group, characterized by the sequence: -Thr-Ser-Met- which hitherto has included all microbial serine proteases of known sequence (Dixon, 1966).

(2) The α-enzyme contains only one residue of histidine and thus cannot operate by a cyclic mechanism involving two imidazole groups such as that proposed for chymotrypsin by Bender and Kézdy (1964).

(3) The β-lytic protease cannot be placed in any of Hartley's

four classes of protease (Hartley, 1960): it contains one atom of zinc per molecule but it is not a metal protease; nor is it an acid protease or a thiol protease and, if indifference to diisopropyl phosphorofluoridate (DFP) and to isopropyl methylphosphonofluoridate (sarin) disqualifies it, it is not a serine protease.

Materials and Methods

The α- and β-enzymes were purified by procedures described previously (Whitaker, 1965b). Other enzymes were obtained from commercial sources. ^{32}P-labelled sarin was prepared in the Suffield laboratories of the Defence Research Board of Canada.

Amino acids were analysed with a modified Technicon analyser using Hamilton's (1963) sequence of buffers; hydrolysates were prepared by Moore and Stein's (1963) procedure. The values for half-cystine in Table I were determined from the cysteic acid produced by oxidation with performic acid (Moore, 1963). Thiol groups were estimated by the method of Klotz and Carver (1961). Semi-quantitative spectrographic analyses scanned each enzyme for about 40 metals; zinc was determined by the method of Rush and Yoe (1954) after combustion by the procedure of Vallee and Gibson (1948). Lytic activity was measured from the rate of change in absorbance of a suspension of Arthrobacter globiformis cells at pH 9 (Whitaker, 1965b). N-terminal amino acids were determined by the "Dansyl" method of Gray and Hartley (1963a) with electrophoresis (Gray and Hartley, 1963b) and thin-layer chromatography (Cole, Fletcher and Robson, 1965) as separation procedures.

Results

The amino acid compositions of the two enzymes require that each has a minimum molecular weight of approximately 20,000. This value is within 5% of the molecular weights estimated by the

Archibald method (Jurášek and Whitaker, 1965). The data which are most relevant to this report are in Table I.

TABLE I. Moles of Various Components per Mole of Enzyme[1]

Component	α-Lytic Protease		β-Lytic Protease	
	Average value	Nearest integer	Average value	Nearest integer
Histidine	1.06±.09	1	8.05±.20	8
Lysine	2.03±.03	2	2.99±.13	3
Arginine	12.08±.12	12	5.03±.06	5
Half cystine	5.95	6	3.96	4
Thiol groups	<0.1	0	<0.1	0
Zinc	<0.1	0	0.9	1

[1] The estimate of the weight of enzyme preparation which contains one mole of enzyme was made with an I.B.M. 360 computer from the data (averages and their standard deviations) for all acid-stable amino acids. The method will be described elsewhere.

The β-enzyme was almost completely freed of zinc by washing the enzyme in an ultrafiltration cell with 10^{-3} M o-phenanthroline in 0.01 M acetate buffer of pH 5.5. This treatment had no effect on lytic activity and did not increase the titer for thiol groups.

Inhibition by DFP was tested on solutions of enzyme in 0.2 M phosphate buffer of pH 7.7. Treatment of the β-enzyme for 4 hours with a tenfold excess of DFP had no effect on lytic activity; the zinc-free enzyme was equally insensitive. Treatment of the α-enzyme with 2.5 mole of DFP per mole of enzyme gave a 95% inhibition of lytic activity within 10 minutes and complete inhibition within an hour. The inhibited enzyme was dialysed and then hydrolysed for 20 hours with 2 N HCl at 100°. The yield of serine phosphate was 0.35 moles/mole of enzyme. Essentially the same yield was obtained by Schaffer et al. (1953, 1954) by similar hydrolyses of

DFP-treated chymotrypsin and cholinesterase. It was concluded that DFP esterifies only one serine residue of the α-enzyme.

The type of sequence at the reactive serine residue was indicated by comparisons with trypsin and chymotrypsin. The methods were the same as those of Naughton et al. (1960) except that $DF^{32}P$ was replaced by sarin containing about 11 μc of ^{32}P/μmole. The enzymes, 0.1 μmole of each, were dissolved in 0.1 M phosphate buffer of pH 8, incubated with 0.5 μmoles of sarin for five hours, dialysed, freeze-dried and partially digested with acid. Fig. 1 shows the electrophoretic pattern of ^{32}P-labelled peptides.

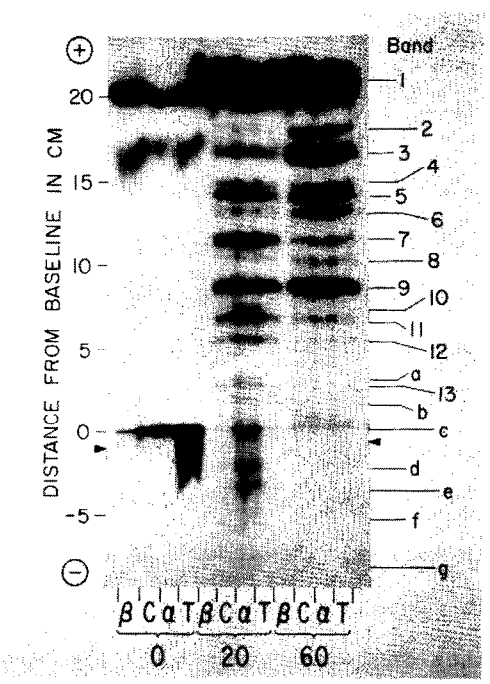

Fig. 1. Radioautograph after electrophoresis of acid digest of ^{32}P-sarin treated β-lytic protease (β), chymotrypsin (C) α-lytic protease (α) and tryps (T). The numbers at the bottc indicate the duration in minut of the digestion with 5.7 N HCl at 100°. The electrophore sis was for 90 min. at 40 volt cm. in SS 2043-B paper contair ing pyridine-acetic acid buffe of pH 3.5. The black arrow indicates the extent of electr osmotic and liquid flow. Banc which are common to C, α and ' are numbered; bands which are peculiar to α are lettered.

The major component of Bands 3, 5, 7 and 9 of the α-enzyme was isolated from a mixture of 20 minute and 60 minute acid digests

by ion-exchange followed by preparative electrophoresis at pH 3.5 and 6.5 (Naughton et al., 1960). Their compositions are in Table II. As Band 7, which gives an extract which is virtually homogeneous on electrophoresis at pH 6.5, is also produced at pH 8.5 by pronase digestion of sarin-treated α-enzyme, its aspartic acid is not derived from asparagine.

TABLE II. Analysis of the Major Component of Bands 3, 5, 7 and 9 of the α-Lytic Protease

Band	N-terminal amino-acid	Amino acid composition[1] relative to serine			Amino acid sequence
		Asp	Ser	Gly	
3	Ser		1.00	0.40	-Ser-
5	Asp	0.90	1.00	0.20	-Asp-Ser-
9	Ser		1.00	2.17	-Ser-Gly-Gly-
7	Asp	0.98	1.00	2.04	-Asp-Ser-Gly-Gly-

[1] After hydrolysis for 24 hr. with 6.1 N HCl at 110°.

Discussion

The α-enzyme is a more selective protease than pancreatic elastase but a functional affinity between the two enzymes is evident from their action patterns on the A and B chains of performate-oxidized insulin (Whitaker et al., 1965). For this reason, the above evidence of a structural affinity is not too surprising. The β-enzyme is a still more selective protease and may be a serine protease which is too selective to react with DFP or sarin.

Acknowledgements. The assistance of Dr. R. M. Heggie and Mr. G. A. Grant of the Defence Research Board of Canada is gratefully acknowledged.

REFERENCES

Bender, M.L. and Kézdy, F.J., J. Am. Chem. Soc., 86, 3704 (1964).
Cole, M., Fletcher, J.C. and Robinson, A., J. Chromatog., 20, 616, (1965).
Dixon, G.H., Essays in Biochemistry, Vol. 2, ed. by P.N. Campbell and G.D. Greville, Academic Press, London, p.190 (1966).
Gillespie, D.C. and Cook, F.D., Can. J. Microbiol., 11, 109 (1965).
Gray, W.R. and Hartley, B.S., Biochem. J., 89, 59P (1963a).
Gray, W.R. and Hartley, B.S., Biochem. J., 89, 379 (1963b).
Hamilton, P.B., Anal. Chem., 35, 2055 (1963).
Hartley, B.S., Ann. Rev. Biochem., 29, 45 (1960).
Jurášek, L. and Whitaker, D.R., Can. J. Biochem., 43, 1955 (1965).
Klotz, I.M. and Carver, B.R., Arch. Biochem. Biophys., 95, 540 (1961).
Moore, S., J. Biol. Chem., 238, 235 (1963).
Moore, S. and Stein, W.H., Methods in Enzymology, Vol. 6, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, N.Y., p.819 (1963).
Naughton, M.A., Sanger, F., Hartley, B.S. and Shaw, D.C., Biochem. J., 77, 149 (1960).
Rush, R.M. and Yoe, J.H., Anal. Chem., 26, 1345 (1954).
Schaffer, N.K., May, S.C. and Summerson, W.H., J. Biol. Chem., 202, 67 (1953).
Schaffer, N.K., May, S.C. and Summerson, W.H., J. Biol. Chem., 206, 201 (1954).
Tsai, C.S., Whitaker, D.R., Jurášek, L. and Gillespie, D.C., Can. J. Biochem., 43, 1971 (1965).
Vallee, B.L. and Gibson, J.G., J. Biol. Chem., 176, 435 (1948).
Whitaker, D.R., Cook, F.D. and Gillespie, D.C., Can. J. Biochem., 43, 1927 (1965a).
Whitaker, D.R., Can. J. Biochem., 43, 1935 (1965b).
Whitaker, D.R., Roy, C., Tsai, C.S. and Jurášek, L., Can. J. Biochem., 43, 1961 (1965c).