The Substrate Specificity of Thermomycolase, an Extracellular Serine Proteinase from the Thermophilic Fungus *Malbranchea pulchella* var. *sulfurea*

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The specificity of thermomycolase toward glucagon and the oxidized A and B chains of insulin was investigated. Extensive digestion of glucagon occurred when conducted at pH 7.0 and 45°C for 40 min, whereas hydrolysis of only three peptide bonds occurred at pH 7.0 and 28°C for 5 min. A similar situation was observed for the oxidized B chain of insulin, which exhibited only a single major cleavage after 5 min at 25°C. No well-defined specificity for particular amino acid residues was evident, but ready hydrolysis of peptide bonds occurred within sequences containing non-polar residues. This endoproteinase must therefore possess an extended hydrophobic binding site for polypeptides. Thermomycolase hydrolysed acetylalanylalanylalanine methyl ester and elastin-Congo Red at 22 and 8.5 times the rate of porcine elastase respectively. A limited degradation of native collagen and significant hydrolysis of benzyloxycarbonyl-Gly-Pro-Leu-Gly-Pro were suggestive of some collagenase-like activity. No keratinase activity was apparent.

The production of proteolytic enzymes by thermophilic fungi was examined by Ong & Gaucher (1973) in a search for an extracellular proteinase whose biosynthesis, structure and properties would be both interesting and amenable to investigation.

Thermophilic fungi have been defined as fungi that have maximum and minimum growth temperatures at or above 50° and 20°C respectively (Cooney & Emerson, 1964). Various other studies of extracellular enzymes produced by thermophilic fungi have dealt with amylases, cellulases, β -1,3-glucanases, a lipase, aminopeptidases and an acid proteinase. *Malbranchea pulchella* var. *sulfurea*, one of ten thermophiles investigated, was selected because it produced a significant amount of a single thermostable extracellular proteinase. Inactivation of this alkaline proteinase with Dip-F* at pH 8 indicated that it belonged to the 'serine family' of proteolytic

enzymes (Hartley, 1960). Its production, purification and partial characterization have been investigated (Ong & Gaucher, 1972, 1975), and the trivial name thermomycolase has been adopted. Thermomycolase has a molecular weight of 33000 (Voordouw *et al.*, 1974a,b) and is remarkably thermostable. In the presence of 10 mM-CaCl₂ at pH 7.4 and 73°C, one-half of the proteinase activity is lost in 110 min. In contrast, one-half of the activity is lost in 7.5 min in the absence of added Ca²⁺ ions (Ong & Gaucher, 1972). The hydrolysis of *p*-nitrophenol esters of *N*-benzyloxycarbonyl-L-amino acids by thermomycolase has revealed a preference, alanine > tyrosine > phenylalanine > glycine > leucine > tryptophan > valine residues (Ong & Gaucher, 1975). The D isomers of the alanine and phenylalanine esters were only hydrolysed to a very limited extent.

Studies with *M. pulchella* var. *sulfurea* have also shown that thermomycolase is only produced in the presence of a protein nutrient such as casein. Readily catabolized metabolites such as glucose and amino acids support growth, but strongly repress proteinase production even when casein is present in the growth medium (Ong & Gaucher, 1972; J. M. Dunham & G. M. Gaucher, unpublished work).

In the present paper, the specificity of thermomycolase towards polypeptide substrates of known sequence is reported. With respect to its proteolytic activity, the enzyme is particularly elastase-like, as well as being similar to chymotrypsin and perhaps a collagenase.

* Abbreviations: Dip-F, di-isopropyl phosphofluoridate; Boc, t-butoxycarbonyl; Ac, acetyl; Cbz, benzyloxycarbonyl; Cbz-Gly-ONp, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester; Ac-Ala₃-OMe, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; Cys(O₃H), Cys, cysteic acid; *m*_A⁺*T*₃, the mobility of a peptide relative to aspartic acid during high-voltage electrophoresis at pH 6.5. Measurements were taken from the neutral point denoted by alanine at pH 6.5 and by taurine at pH 2.0 and pH 3.5. Similar notations denote the mobility of a peptide relative to an amino acid during high-voltage electrophoresis at a particular pH. Symbols have been presented according to IUPAC-IUB Commission on Biochemical Nomenclature [Biochem. J. (1972) 126, 773-780].

Experimental

Materials

Glucagon and elastin-Congo Red were purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Ac-Tyr-OEt and Bz-Arg-OEt were purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Ac-Ala₃-OMe, Cbz-Gly-Pro-Gly-Gly-Pro-Ala, Cbz-Gly-Pro-Leu-Gly-Pro, Cbz-Gly-Gly-Phe, Cbz-Gly-Gly-Leu, Cbz-Pro-Leu-Gly, Ac-Ala-Ala-Ala-Ala, Gly₆, Cbz-Gly-ONp, Cbz-Ala-ONp and collagen (bovine achilles tendon) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Keratin was purchased from Calbiochem, Los Angeles, Calif., U.S.A. Oxidized A and B chains of bovine insulin were purchased from Schwarz-Mann, Orangeburg, N.Y., U.S.A. Phenanthrenequinone was purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Methods

Isolation of thermomycolase. The production and purification of thermomycolase was carried out as described by Ong & Gaucher (1975) and care was taken to avoid significant autolysis (Voordouw *et al.*, 1974b).

Digestion of glucagon with thermomycolase. A solution of thermomycolase was dialysed at 0°C for 24h against 1mM-CaCl₂, adjusted to pH7.0 with 10mM-NH₃. The concentration of the non-diffusible proteinase was determined by using the $E_{1\text{cm}}^{1\%} = 13.5 \pm 0.2$ at 280nm, 25°C and pH7.0 (Voordouw *et al.*, 1974b). A portion of this solution was added to 10mg of glucagon dissolved in 1.0ml of water maintained at pH7.0 with 10mM-NH₃ by using a Radiometer type SBR2C pH-stat. The molar ratio of glucagon to thermomycolase was 50 and the temperature was maintained at 45°C. The digestion was allowed to proceed for 5 or 40min and the pH of the digest was then adjusted to 3 with 0.1M-HCl and the solution was placed in ice. To ensure that all peptides arose from the digestion of glucagon, controls were prepared from which glucagon or thermomycolase was omitted.

Digestion of the oxidized A and B chains of insulin. A portion of the thermomycolase solution described above was added to 10mg of the oxidized A chain dissolved in 4ml of water adjusted to pH7.0 with 10mM-NH₃ by using the pH-stat. The molar ratio of A chain to thermomycolase was 50 and the temperature was 25°C. After proceeding for 5min, the digestion was terminated by lowering the pH to 3 and placing in ice-water. The digestion of the oxidized B chain of insulin (10mg) was conducted as outlined above for the oxidized A chain. To solubilize the oxidized B chain, the pH was raised to 9.0. Digestions for 5min were carried out at both 25°C and 45°C.

Purification of peptides. The thermomycolase digests were spotted in the centre of a sheet of Whatman 3MM paper and subjected to high-voltage electrophoresis at pH6.5 and 60V/cm for 1h. Side strips (1cm) were removed to detect the peptide bands with cadmium-ninhydrin. The 'neutral' band was removed, sewn on to a second sheet of Whatman 3MM paper at a distance of 12cm from the anode and subjected to high-voltage electrophoresis at pH2.0 and 60V/cm for 50min.

The purification procedure for each peptide present in the digest was determined by preparing a series of two-dimensional peptide 'maps'. Side strips of the basic and acidic peptides (initial pH6.5 ionogram) were sewn on to Whatman 3MM paper at 12cm or 15cm from the anode and subjected to high-voltage electrophoresis at pH2.0 and 60V/cm for 1h or pH3.5 and 60V/cm for 1h respectively. Similarly, a side strip from the pH2.0 ionogram of the 'pH6.5 neutrals' was subjected to high-voltage electrophoresis at pH3.5 and 60V/cm for 1h. Two-dimensional peptide 'maps' were also prepared by sewing side strips on to Whatman 3MM paper and subjecting to descending chromatography in butan-1-ol-acetic acid-water (3:1:1, by vol.) for 24h or until N⁶-2,4-dinitrophenyl-lysine migrated to within 7cm of the bottom. All chromatograms were stained with cadmium-ninhydrin.

Digestion of peptides. The peptides Cbz-Gly-Pro-Gly-Gly-Pro-Ala, Cbz-Gly-Pro-Leu-Gly-Pro, Cbz-Gly-Gly-Phe, Cbz-Gly-Gly-Leu, Cbz-Pro-Leu-Gly, Ac-Ala-Ala-Ala-Ala and Gly₆ were dissolved separately in 50mM-N-ethylmorpholine adjusted to pH8.0 with 1M-acetic acid to yield 2.2mM solutions. Thermomycolase (2nmol in 10μl of 0.1mM-CaCl₂) was added to 45μl portions of each peptide solution to yield a molar ratio of peptide to proteinase of 50 to 1. The digestions were conducted at 45°C for 30min and 24h, after which the mixtures were acidified to about pH3 with 10μl of 1M-acetic acid and spotted on to Whatman 3MM paper as 1cm bands on a line 10cm from the anode. High-voltage electrophoresis was conducted at pH2.0 and 60V/cm for 1h and the ionogram was stained with cadmium-ninhydrin. Digests were scaled-up 15-fold to isolate peptides for characterization. Control incubations of each digest from which either peptide or thermomycolase were omitted were prepared and handled as described above.

Digestion of keratin and collagen. Keratin and collagen fibres were washed with water and ethanol, refluxed in ethanol for 5h, washed again with ethanol and air-dried. The fibres (2.2mg) were suspended in 100μl of 0.1M-N-ethylmorpholine buffer adjusted to pH8.0 with acetic acid. Thermomycolase (2nmol) was added and the digestion allowed to proceed at 37°C for 18h. Each digest was spotted on to Whatman 3MM paper as 1cm bands at

a distance of 12cm from the anode and subjected to high-voltage electrophoresis at pH2.0 and 60V/cm for 1h. The ionogram was stained with cadmium-ninhydrin. Controls of each digest minus enzyme or fibres were handled identically.

High-voltage electrophoresis. The vertical strip high-voltage electrophoresis apparatus was similar to that described by Michl (1951) as modified by Ryle *et al.* (1955). The buffer systems and coolants used were as described by Smillie & Hartley (1966). Peptides were detected with the cadmium-ninhydrin dip reagent of Heilmann *et al.* (1957) by developing guide strips of chromatograms or the entire two-dimensional peptide 'maps'. Amino acids were detected with a dip reagent consisting of 0.7ml of collidine in 100ml of 0.5% (w/v) ninhydrin in acetone. The presence of tryptophan-containing peptides was investigated with Ehrlich reagent (*p*-dimethylamino-benzaldehyde) as described by Bailey (1968*a*). The suitability of the Ehrlich reagent was confirmed by obtaining positive results with peptides known to contain tryptophan. The presence of histidine- and tyrosine-containing peptides was detected with the Pauly reagent (diazotized sulphanilic acid) as described by Bailey (1968*b*) and arginine-containing peptides were detected with phenanthrenequinone (Yamada & Itano, 1966). Visual dye markers were used throughout high-voltage electrophoresis (Stevenson, 1971).

Detection of amino acids in proteolytic digests of polypeptides. α -Chymotrypsin (30mg) was dissolved in 0.1M-*N*-ethylmorpholine buffer adjusted to pH7.0 with acetic acid. Thermomycolase (0.5mg) was added and the digestion allowed to proceed at 45°C for 5h. The digest was spotted on a sheet of Whatman 3MM paper at a concentration of 1mg/cm at a distance of 12cm from the anode and subjected to high-voltage electrophoresis at pH1.6 and 60V/cm for 45min. The buffer used was 8.6% formic acid. A 3cm side strip was removed, sewn on to a second sheet of Whatman 3MM paper at a distance of 12cm from the anode, and subjected to high-voltage electrophoresis at pH2.4 and 40V/cm for 1h. The buffer used was 4% formic acid adjusted to pH2.4 with pyridine (final pyridine content was 0.58%). The two-dimensional peptide 'map' was stained with cadmium-ninhydrin. Amino acids, if present, were found lying below the peptide 'diagonal'. Various proteins were digested with proteolytic enzymes and subjected to two-dimensional high-voltage electrophoresis as outlined. In each case ninhydrin-positive spots appearing below the peptide 'diagonal' were identified as free amino acids.

Amino acid analyses. The amino acid analyses of peptides were performed by hydrolysing the peptide in 6M-HCl at 110°C for 24h. The acid hydrolysates were evaporated to dryness on an Evapomix and were analysed on a Beckman 121 or Phoenix M7800 amino

acid analyser by the method of Spackman *et al.* (1958) as extended by Spackman (1967).

Enzyme assays. The esterolytic activity of thermomycolase was assayed with Ac-Ala₃-OMe (Gertler & Hofmann, 1970), Cbz-Gly-ONp and Cbz-Ala-ONp (Kirsch & Igelstrom, 1966). Elastolytic activity was assayed by the elastin-Congo Red method (Gertler & Hofmann, 1967; Gertler & Birk, 1970).

Results

The substrate specificity of thermomycolase was investigated by analysing peptides derived from digests of glucagon and insulin (oxidized A and B chains). These digestions were carried out at 25°C or 45°C and at close to the optimum pH in the presence of CaCl₂. The conditions chosen were based on studies by Ong & Gaucher (1975) on the pH stability and thermostability of thermomycolase and on the stabilizing effect of CaCl₂. The characterization of 27 peptides isolated from a 40min thermomycolase digest of glucagon is presented in Table 1. In total, however, about 40 peptides were present; those not reported were isolated in very low yield. The complexity of the digest was a result of extensive partial hydrolysis of peptide bonds under the conditions used.

Thermomycolase possesses only endopeptidase activity, since amino acids were essentially absent from the glucagon digest. This observation is in keeping with the low peptidase activity of fungal proteinases (Moriyama *et al.*, 1971; Oka & Moriyama, 1973). Additional support for this lack of exopeptidase activity was obtained from the observation that amino acids were not detected on a two-dimensional peptide 'map' (pH1.6/pH2.4) of a thermomycolase digest of α -chymotrypsin (cf. under 'Methods'). Amino acids, if present, would appear below the peptide 'diagonal' because of an increase in their negative charge arising from the ionization of their α -carboxyl groups (pK approx. 2). This procedure is selective for amino acids, since peptides remain on the 'diagonal' because their α -carboxyl groups have higher pK values (near 3).

The peptides isolated from the digest of glucagon are aligned with the known amino acid sequence of the hormone in Table 2. The assignment of major, intermediate and minor cleavages occurring along the polypeptide was based on the recovery and sequential assignment of each peptide as determined from their amino acid composition (Table 1). No simple specificity characteristics exist for thermomycolase. The large number of peptide bonds completely or partially hydrolysed (15 out of 28 in glucagon) suggests that the proteinase has a low specificity at its temperature and pH optima. Digestion conditions which allowed fewer cleavage of glucagon assisted in determining the nature of those

Table 1. *Amino acid composition of peptides isolated from a digest of glucagon by thermomycolase*

The digestion of glucagon occurred at pH7.0 and 45°C for 40min with a molar ratio of glucagon to thermomycolase of 50:1. The peptides were purified on high-voltage electrophoresis at pH6.5 (60 V/cm for 1h), pH2.0 (60 V/cm for 1h), pH3.5 (60 V/cm for 1h) and descending chromatography in butan-1-ol-acetic acid-water (3:1:1, by vol). Peptide nomenclature and purification: acidic peptides (A), numerals refer to $m_{2.5}^{ser}$, with A1 having the slowest migration ($m_{2.5}^{ser}$ also presented for A1, A2 and A6), a, b and c refer to R_{XCF} , the migration of a peptide relative to xylene cyanol FF during chromatography. Basic peptides (B), numerals refer to $m_{2.5}^{ser}$ with B1 having the slowest migration ($m_{2.5}^{ser}$ and $m_{3.5}^{ser}$ also given for B1-B3 and B6-B9). Neutral peptides (N), refers to $m_{2.5}^{ser}$, numerals refer to $m_{2.5}^{ser}$ values with N1 having the slowest mobility, a, b, and c refer to $m_{2.5}^{ser}$ values with 'a' representing a peptide with the slowest mobility relative to Crystal Violet at pH3.5 in high-voltage electrophoresis. 'Sequential position' refers to the glucagon sequence shown in Table 2.

Peptide	Yield (nmol)	Cadmium-ninhydrin		$m_{2.5}^{a.p.}$	$m_{2.5}^{b.p.}$	$m_{3.5}^{c.v.}$	$m_{3.5}^{d.p.}$	R_{XCF}	Other stains	Composition	Sequential position
		colour	colour								
A1	176	Orange	Orange	0.17	1.15	-	-	-	-	Asp (1.00), [†] Thr (1.00), Gly (0.41)	28 29 Asn-Thr
A6c	124	Red	Red	0.47	0.62	-	-	1.09	Tyr	Asp (1.18), Glu (0.19), Ala (0.14), Leu (1.00), [†] Tyr (0.53)	13 15 Tyr-Leu-Asp
A6b	116	Red	Red	0.47	0.62	-	-	1.30	Tyr	Asp (1.21), Ser (0.93), Leu (1.00), [†] Tyr (0.72)	13 16 Tyr-Leu-Asp-Ser
A6a	86	Red	Red	0.47	0.62	-	-	1.39	-	Asp (1.00), [†] Glu (1.00), Val (0.93), Phe (1.12)	21 24 Asp-Phe-Val-Gln
A2a	22	Red	Red	0.26	0.88	-	-	-	Tyr	Lys (1.00), Asp (2.00), Ser (1.27), Leu (1.00), [†] Tyr (0.63)	9 15 Asp-Asp
B6	166	Red	Red	-0.43	1.97	-	-	0.72	Arg	Arg (1.20), Glu (1.00), Ala (1.00) [†]	18 20 Arg-Ala-Gln
B7	100	Yellow	Yellow	-0.51	1.97	-	-	0.83	Arg	Arg (1.00), [†] Ser (0.86)	16 17 Ser-Arg
B3	66	Red	Red	-0.31	1.54	-	-	0.50	Arg	Arg (2.14), Asp (1.18), Ser (1.09), Glu (1.03), Ala (1.00), [†] Leu (0.45)	14 20 Leu-Gln
B9	58	Red	Red	-0.61	1.93	-	-	0.92	Arg	Arg (2.31), Glu (1.00), Ala (1.00) [†]	17 20 Arg-Arg-Ala-Gln
B1	54	Red	Red	-0.23	1.34	-	-	0.47	Arg Tyr	Arg (2.30), Asp (1.00), Ser (0.88), Glu (0.96), Ala (0.92), Leu (1.00), [†] Tyr (0.41)	13 20 Tyr-Gln
B8	52	Red	Red	-0.54	20.4	-	-	1.05	-	Lys (1.00), [†] Ser (0.65)	11 12 Ser-Lys
B2	36	Red	Red	-0.28	1.54	-	-	0.58	Arg	Arg (2.20), Asp (1.00), [†] Ser (0.83), Glu (0.33), Ala (0.16), Leu (0.77), Tyr (0.22)	14 18 Leu-Asp-Ser-Arg-Arg

N9a	360	Red	0	1.31	0.92	-	-	Tyr	Lys(1.12), Asp(1.00), [†] Ser(0.86), Tyr(1.36)	⁹ Asp-Tyr-Ser-Lys ¹²
N9c	200	Red	0	1.31	1.52	-	-	His	His(0.83), Thr(1.04), Ser(0.77), Glu(1.00), [†] Gly(0.97)	¹ His-Ser-Gln-Gly-Thr ⁵
N7a	134	Orange	0	1.14	0.75	-	-	-	Lys(0.11), Thr(1.00), [†] Ser(0.74), Tyr(0.14)	⁷ Thr-Ser ⁸
N8	90	Red	0	1.21	1.19	-	-	Arg, Tyr	Arg(0.73), Asp(1.00), [†] Ser(1.13), Leu(0.93), Tyr(1.27)	¹³ Tyr-Leu-Asp-Ser-Arg ¹⁷
N6c	80	Orange	0	1.10	1.25	-	-	His	His(1.05), Thr(2.07), Ser(2.00), Glu(1.00), [†] Gly(1.10), Phe(1.42)	¹ His-Ser-Gln-Gly-Thr- ⁸ Phe-Thr-Ser
N1a*	78	Yellow	0	0.68	0.56	-	-	Trp	Thr(2.00), Ser(1.05), Gly(1.00), [†] Met(0.32), Leu(0.35), Phe(1.26)	⁴ Gly-Thr-Phe-Thr-Ser ⁸
N9bI	52	Red	0	1.31	1.31	-	-	Arg	His(0.45), Arg(0.81), Asp(1.00), [†] Thr(0.54), Ser(0.81), Glu(1.38), Gly(0.65), Ala(0.85), Phe(1.38)	¹⁸ Arg-Ala-Gln-Asp-Phe ²²
N7bI	38	Orange	0	1.14	0.81	-	-	His	His(1.12), Asp(0.47), Thr(0.35), Ser(0.88), Glu(1.00), [†] Gly(0.29), Ala(0.71), Tyr(0.47)	¹ His-Ser-Gln ³
N6bI	36	Red	0	1.10	1.06	-	-	Arg, Trp	Arg(1.88), Asp(2.00), [†] Thr(0.76), Ser(1.00), Glu(3.76), Gly(0.54), Ala(1.32), Val(1.00), Phe(0.95)	¹⁴ Leu-Trp ²⁵
N9bII	34	Red	0	1.31	1.31	-	-	His	As for N9bI	¹ His-Phe ⁶
N3b	34	Red	0	0.88	0.75	-	-	Tyr	Asp(1.00), [†] Thr(1.18), Ser(0.35), Glu(0.23), Gly(0.29), Met(1.00), Leu(0.41), Phe(0.29)	²⁶ Leu-Met-Asn-Thr ²⁹
N6a	33	Yellow	0	1.10	0.72	-	-	Tyr	Lys(2.00), Asp(1.00), [†] Thr(0.81), Ser(1.81), Tyr(2.81)	⁷ Thr-Ser-Asp-Tyr-Ser- ¹³ Lys-Tyr
N4a	30	Yellow	0	0.94	0.72	-	-	-	Thr(1.13), Gly(1.00), [†] Phe(1.13)	⁴ Gly-Thr-Phe ⁶
N1b*	28	Yellow	0	0.68	0.56	-	-	Trp	as for N1a, Met(0.93), Leu(1.00) [†]	²⁵ Trp-Leu-Met ²⁷
N6bII	28	Red	0	1.10	1.06	-	-	Arg	as for N6bI	³ Gln-Gly-Thr ⁵

* Peptides N1a and N1b were non-separable and were deduced from amino acid analyses. The composition of N1b is as for N1a.

† Arbitrarily taken as 1.00 or 2.00 residues as indicated with recoveries of other amino acids relative to this amino acid.

Table 5. Amino acid composition of peptides isolated from a low-temperature digest of the oxidized B chain of insulin by thermomycolase
The conditions were pH9.0 and 25°C for 5min at a molar ratio of substrate to proteinase of 50:1.

Pep- tide	Yield (nmol)	Cadmium- ninhydrin colour	Sequential position	
			Composition	Sequential position
B1	10	Red	Lys (1.10), Thr (0.81), Pro (1.00), *Ala (0.83), Tyr (0.82), Phe (0.87)	25 30 Phe-Ala
N1	70	Red	Lys (0.81), Arg (0.77), Cys (0.3H) (0.96), Thr (0.80), Glu (1.40), Pro (1.00), Gly (2.00), Ala (1.00), *Val (0.95), Leu (1.30), Tyr (1.50), Phe (2.00)	16 30 Tyr-Ala
A1	114	Red	His (1.09), Cys (0.3H) (1.09), Asp (1.04), Ser (0.90), Glu (2.27), Gly (1.44), Ala (1.00), Val (1.71), Leu (2.99), Tyr (0.23), Phe (1.10)	1 15 Phe-Leu
A2	15	Red	Arg (0.87), Cys (0.3H) (0.78), Glu (1.10), Gly (1.90), Val (0.84), Leu (1.00), * Tyr (0.77), Phe (0.87)	16 24 Tyr-Phe

* Arbitrarily taken as 1.00 residue with recoveries of other amino acids relative to this amino acid.

Table 7. *Thermomycolase digest of peptides*

The conditions of the digest were pH 8.0 and 45°C at a substrate to proteinase molar ratio of 70:1. The arrows indicate complete (↓) and partial (↓) cleavages. 'Composition' shows the amino acid analyses after acid hydrolysis. Use of abbreviations only indicates that the amino acids were detected on high-voltage electrophoresis at pH 2.0 and 60 V/cm for 50 min.

Peptide substrates	Digestion period	Characterization of degradation products		
		Cadmium-ninhydrin colour	$m_{2.0}^{ser}$	Composition
Cbz-Gly-Pro-Gly-Gly-Pro-Ala ↓ ↓	30 min	—	—	—
	24 h	Trace yellow	1.10	Pro(1.03), Gly(1.00), Ala(1.03)
Cbz-Gly-Pro-Leu-Gly-Pro ↓	30 min	Trace red	1.22	Ala
	24 h	Yellow	1.26	Pro(0.99), Gly(1.00)
Ac-Ala-Ala-Ala-Ala ↓ ↓ ↓	30 min	Strong yellow	1.26	Pro(0.99), Gly(1.00)
		Red	1.26	Ala*
Cbz-Gly-Gly-Phe ↓ ↓	24 h	Weak red	1.16	Ala†
		Weak red	0.90	Ala‡
Cbz-Gly-Gly-Leu ↓ ↓	24 h	Weak red	0.78	Phe
		Weak red	0.97	Leu
Cbz-Pro-Leu-Gly ↓	30 min	—	—	—
	24 h	Yellow	1.37	Gly
Gly ₆	24 h	Yellow	0.87	(starting peptide)

* Before acid hydrolysis recovery of alanine 3 nmol; after, recovery of alanine 36 nmol

† The amount of alanine did not increase after acid hydrolysis.

‡ Alanine only present after acid hydrolysis.

peptide bonds most susceptible to the proteinase. To this end, digestion of glucagon was carried out under more restrictive conditions. The peptides produced and the proteolytic cleavages involved are shown in Table 3. Proteolytic cleavage was confined to the C-terminal region of glucagon, with the major cleavages being glutamine-24-tryptophan-25 and methionine-27-asparagine-28.

The acidic nature of the dipeptide Asn-Thr observed during high-voltage electrophoresis at pH 6.5 reflected the low pK of the α -amino group. This observation is in keeping with the anomalously low pK for the amino group of the dipeptide Asn-Gly ($pK = 7.2$) and peptides with N -terminal asparagine (Leach & Lindley, 1954). A similar situation was found in the present study with Met-Asn-Thr (Table 3).

To compare the specificity of thermomycolase with that of other proteinases and to delineate further its preference for amino acid side chains, the oxidized A

and B chains of insulin were digested under conditions of near optimum pH, at 25°C or 45°C, for only 5 min. Interestingly, only two peptide bonds, leucine-13-tyrosine-14 and glutamic acid-17-asparagine-18, were cleaved in the A chain of insulin (Table 4). The cleavages occurred in an amino acid sequence between cysteic acid residues 11 and 20 containing predominantly non-polar amino acids. The digestion of the oxidized B chain of insulin at pH 9.0 and 25°C resulted in a single major cleavage at leucine-15-tyrosine-16, with a very minor hydrolysis of phenylalanine-25-phenylalanine-26 also occurring (Table 5). Again the hydrolysis of the polypeptide occurred in a very hydrophobic sequence. Repeating the digestion at the optimum temperature (45°C) for thermomycolase caused major cleavages at the two positions mentioned above and also at glutamic acid-13-alanine-14. Minor cleavages were observed at histidine-5-leucine-6 and tyrosine-26-threonine-27 (Table 6).

The proteolytic activity of thermomycolase was further investigated by using a series of Cbz-peptides, Ac-Ala-Ala-Ala-Ala and Gly₆ (Table 7). Cleavage of Cbz-peptides occurred most readily at the peptide bond in which the carbonyl group was contributed by amino acids possessing hydrophobic side chains.

Hydrolysis products of Cbz-Gly-Pro-Leu-Gly-Pro and Ac-Ala-Ala-Ala-Ala present after a 30 min digestion at 45°C and pH 8.0 were identified as Gly-Pro and Ala-Ala respectively. A much lower yet significant hydrolysis rate was observed with Cbz-

Pro-Leu-Gly and Cbz-Gly-Pro-Gly-Gly-Pro-Ala. Since no hydrolysis of Gly₆ by thermomycolase was detected, the necessity of some side chain interaction(s) was evident. The hydrolysis products of Ac-Ala-Ala-Ala-Ala also support the view that multiple interactions between apolar side chains of the substrate and the active site of the proteinase are more important than simply the total number of residues in the substrate.

Peptide bonds involving proline (X-Pro or Pro-X) are not readily hydrolysed by thermomycolase. Exhaustive digestion of Cbz-Gly-Pro-Gly-Gly-Pro-Ala by thermomycolase yielded only a trace of alanine resulting from a cleavage of the Pro-Ala bond. An attempt to detect *N*-terminal proline residues, by using the specific isatin stain (Monier & Jutisz, 1954), on peptides produced by exhaustive digestion of α -chymotrypsin with thermomycolase was negative. This further suggests that X-proline bonds are not readily susceptible to this proteinase.

The significant esterolytic, elastolytic and collagenolytic activity of thermomycolase is a more

specific expression of the general proteolytic capacity of this proteinase. The enzyme has been shown readily to hydrolyse a series of Cbz-amino acid-ONp ester substrates (Ong & Gaucher, 1975) with Cbz-Gly-ONp or Cbz-Ala-ONp being best for spectrophotometric assays. Esterolytic activity may also be readily assayed by using the elastase substrate Ac-Ala₃-OMe in a pH-stat. This latter substrate was hydrolysed by thermomycolase at a rate 22-fold higher than with porcine elastase. The elastolytic activity of thermomycolase was further substantiated by the finding that elastin-Congo Red was hydrolysed 8.5-fold faster than by porcine elastase (Table 8).

The known collagenase substrates, Cbz-Gly-Pro-Leu-Gly-Pro and Cbz-Gly-Pro-Gly-Gly-Pro-Ala, are hydrolysed by thermomycolase (Table 7) to yield the same products as those produced by collagenases and various serine proteinases (Moriwaka *et al.*, 1971). These substrates have been commonly used to assay various proteinases for collagenase activity (Grassmann & Nordwig, 1960; Nordwig & Jahn, 1968; Morihara, 1967). Native collagen (bovine achilles tendon) was cleaved by thermomycolase with the slow release of ninhydrin-positive material as detected after high-voltage electrophoresis at pH 2.0. However, the denaturation of collagen by refluxing in ethanol increased the release of ninhydrin-positive material. Similar experiments were carried out with native and ethanol-denatured keratin as a substrate for thermomycolase, but no ninhydrin-positive material was observed in these digests after high-voltage electrophoresis at pH 2.0. Thus thermomycolase exhibits remarkable elastase activity, some 'collagenase' activity and no keratinase activity.

Table 8. *Elastolytic and esterolytic activity of thermomycolase and related alkaline proteinases*

The kinetic data for the hydrolysis of Ac-Ala₃-OMe by thermomycolase were determined at 30°C in 1.2 mM-Tris adjusted to pH 8.5 with HCl and containing 1.2 mM-CaCl₂ and 1.2 mM-KCl.

	Elastin-Congo Red ($\Delta E_{495}/h$ per μmol)	Ac-Ala ₃ -OMe		
		K_m (mM)	$k_{cat.}$ (s ⁻¹)	$k_{cat.}/K_m$ (mM ⁻¹ · s ⁻¹)
Control	0.04			
Thermomycolase	424	1.5	2050	1370
Porcine elastase	50	0.43	73.0	170*
		0.28	17.3	62†
Alkaline proteinase from	Not hydrolysed	21.6	2800	130
<i>Aspergillus sojae</i> †				
<i>Aspergillus sojae</i> †	—	32.1	3720	116
<i>Aspergillus melleus</i> †	—	6.7	1540	230
Subtilisin BPN†	—	0.45	255	566
Subtilisin Carlsberg	—	0.83	1046	1260

* Data of Gertler & Hofmann (1970). Conditions: 1.2 mM-Tris-HCl buffer, pH 8.0 and 25°C.

† Data of Morihara *et al.* (1974). Conditions: 0.1 M-KCl adjusted to pH 7.5 and 30°C.

‡ Data of Gertler & Hayashi (1971). Conditions: Tris-HCl containing CaCl₂ and KCl at pH 8.5 and 30°C.

Discussion

Schechter & Berger (1967) have proposed that the binding of polypeptides to the active site of proteinases is dependent on the interactions of several amino acid residues numbered from the *N*-terminal to the scissile bond (P_4-P_1), and from the scissile bond to the *C*-terminal ($P_1'-P_4'$). The series of corresponding subsites on the proteinase are identified as S_4-S_1 and $S_1'-S_4'$. Extended active sites, allowing multiple interactions with polypeptides, have been identified in papain (Schechter & Berger, 1967, 1968), elastase (Atlas & Berger, 1972, 1973; Thompson & Blout, 1970, 1973*a,b,c*), thermolysin (Moriwaka & Tsuzuki, 1970) and a neutral proteinase from *Bacillus subtilis* (Moriwaka *et al.*, 1969*a,b*). Fungal proteinases in particular are considered to have extended binding sites capable of interacting with large substrates (Moriwaka *et al.*, 1971).

Studies on the specificity of thermomycolase towards glucagon under optimum conditions of pH and temperature revealed a broad proteolytic activity (Tables 1 and 2) after a 40 min digestion. In comparison, Aspergillopeptidase C hydrolysed only five peptide bonds in glucagon during a 2 h digestion period under optimal conditions (Nordwig & Jahn, 1968).

Careful examination of the amino acids occupying positions P_3-P_1 and $P_1'-P_3'$ relative to the scissile bond P_1-P_1' did not indicate a well-defined pattern of hydrolysis in glucagon. The existence of one or more hydrophobic residues on each side of the scissile bond was, however, generally observed. Further, if an active site possessing six subsites, S_3-S_1 and $S_1'-S_3'$, is considered, then all major cleavages in glucagon occur in a sequence (P_3-P_3') of six amino acid residues, of which at least four are always non-polar. Charged amino acids were, however, not excluded from the active site probably because any unfavourable interaction is more than offset by the numerous favourable apolar interactions arising from neighbouring residues. In addition, the apolar portion of the charged residue itself (i.e. lysine, arginine and glutamic acid) could contribute a favourable interaction. There is in fact evidence to suggest that interactions at subsites as far removed as S_3 may contribute to optimal substrate binding. For example, phenylalanine-6 is two residues removed from the major cleavage between serine-8 and aspartic acid-9. Similarly, tyrosine-10 is in proximity to another major cleavage point at lysine-12. Tyrosine-13, leucine-14 and tryptophan-25 are also situated two residues removed from the carbonyl groups of aspartic acid-15, serine-16 and methionine-27 respectively.

A more selective degradation pattern of glucagon was obtained by shortening the digestion period to 5 min and lowering the temperature to 28°C (Table 3). Under these conditions cleavages were restricted

to the non-polar *C*-terminal region. X-ray-diffraction investigations of glucagon indicated that although the peptide is 75% helical, the *C*-terminal hexapeptide is not (Haugen & Lipscomb, 1969). Other studies (Epand, 1972) have also indicated that retention of biological activity is unimpaired by the loss of *C*-terminal residues. Thus it is possible that, at 28°C rather than 45°C, the *C*-terminal end of glucagon may possess a more random and proteolytically susceptible conformation than the remainder of the molecule. The peptide bonds hydrolysed lend further support to the proposal that thermomycolase prefers to bind to hydrophobic amino acids on both sides of the scissile bond. The favoured interaction of apolar residues with subsite S_3 was again indicated, since phenylalanine-22 and tryptophan-25 are two residues removed from the scissile bonds glutamine-24-tryptophan-25 and methionine-27-asparagine-28 respectively. Portions of the digest analysed after 1 min indicate that the latter peptide bond was the first to be hydrolysed. This also agrees with the observed favourable binding of methionine in the S_1 subsite of thermomycolase (Ong & Gaucher, 1975).

The brief digestions of the oxidized A and B chains of insulin by thermomycolase at 25°C (below optimum) led to the ready hydrolysis of two peptide bonds in the oxidized A chain (Table 4) and only a single bond in the oxidized B chain (Table 5). Conducting the digestion of the B chain of insulin at 45°C led to the ready hydrolysis of two additional peptide bonds and another two partial cleavages (Table 6). An observed restriction in specificity at a lower temperature was also reported for the digestion of the oxidized chains of insulin at 0°C and 25°C by aspergillopeptidase B produced by the mesophilic fungus, *Aspergillus oryzae* (Moriwaka & Tsuzuki, 1969; Spadari *et al.*, 1974).

Again the presence of apolar residues on both sides of the scissile bond was observed during the digestion of the oxidized A and B chains of insulin by thermomycolase at 25°C (Tables 4 and 5). A similar situation was observed for the oxidized B chain of insulin when digested at 45°C (Table 6). The extensive hydrolysis of leucine-15-tyrosine-16 undoubtedly preceded the hydrolysis of glutamic acid-13-alanine-14 in the sequence -His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-.

The inhibition of thermomycolase with Dip-F, *p*-nitrobenzenesulphonyl fluoride, diphenylcarbamoyl chloride and tosyl fluoride suggests that at least part of the binding site is hydrophobic in nature. The absorption of thermomycolase on to a 4-phenylbutylamine-Sepharose affinity column during the purification further substantiates this view (Ong & Gaucher, 1972, 1975). This affinity column has been utilized to isolate chymotrypsin-like enzymes (Stevenson & Landman, 1971) as well as in 'hydrophobic' chromatography (Hofstee, 1973).

Table 9. Hydrolysis of the oxidized B chain of insulin by various alkaline 'serine' proteinases

Solid arrows (↑) denote major cleavage, broken arrows (↑) represent minor cleavages. The conditions of the digests differ in each case.

	1	5	10	15
	$\begin{array}{c} \text{O}_3\text{H} \\ \\ \text{H}_3\text{N}^+-\text{Phe}-\text{Val}-\text{Asn}-\text{Gln}-\text{His}-\text{Leu}-\text{Cys}-\text{Gly}-\text{Ser}-\text{His}-\text{Val}-\text{Leu}-\text{Glu}-\text{Ala}-\text{Leu} \end{array}$			
Thermomycolase (25°C, pH 9.0, 5 min)		↑		↑
Thermomycolase (45°C, pH 9.0, 5 min)		↑		↑
Aspergillopeptidase B (0°C, pH 10.2, 30 min) (Spadari <i>et al.</i> , 1974)		↑		↑
Aspergillopeptidase B (25°C, pH 9.2, 2 h) (Moriwara & Tsuzuki, 1969)		↑		↑
Aspergillopeptidase C (35°C, pH 7.2, 2 h) (Nordwig & Jahn, 1968)		↑		↑
Alkaline proteinase from <i>Cephalosporium</i> sp. (37°C, pH 8.5, 1 h) (Turková & Mikes, 1970)		↑		↑
Alkaline proteinase from <i>Cephalosporium</i> sp. (37°C, pH 10.5, 10 h) (Yagi <i>et al.</i> , 1974)		↑		↑
Mammalian elastases [porcine (25°C, pH 8.8, 15 min) (Narayanan & Anwar, 1969) moose (37°C, pH 8, 15 min) (Stevenson & Voordouw, 1975)]		↑		↑
α-Lytic proteinase from <i>Sorangium</i> (25°C, pH 9, 1 h) (Whitaker <i>et al.</i> , 1965)		↑		↑
<i>Streptomyces fradia</i> lb (25°C, pH 9, 2-3 h) (Moriwara & Tsuzuki, 1969)		↑		↑
Streptococcal proteinase (25°C, pH 7.6, 2.5 h) (Gerwin <i>et al.</i> , 1966)*		↑		↑
Alkaline proteinase from <i>Pseudomonas aeruginosa</i> (27°C, pH 8.0, 2 h) (Moriwara & Tsuzuki, 1966)		↑		↑
Elastase from <i>Pseudomonas aeruginosa</i> (27°C, pH 8.0, 5 h) (Moriwara & Tsuzuki, 1966)		↑		↑
Alkaline proteinase from marine psychrophilic <i>Pseudomonas</i> sp. No. 548 (37°C, pH 8, 3 h) (Kato <i>et al.</i> , 1974)		↑		↑

* Streptococcal proteinase is a thiol proteinase and the substrate was the S-carboxymethylated B chain of insulin.

	20	25	30
	$\begin{array}{c} \text{O}_3\text{H} \\ \\ \text{Tyr}-\text{Leu}-\text{Val}-\text{Cys}-\text{Gly}-\text{Glu}-\text{Arg}-\text{Gly}-\text{Phe}-\text{Tyr}-\text{Thr}-\text{Pro}-\text{Lys}-\text{Ala}-\text{CO}_2\text{H} \end{array}$		
Thermomycolase (25°C, pH 9.0, 5 min)		↑	
Thermomycolase (45°C, pH 9.0, 5 min)		↑	
Aspergillopeptidase B (0°C, pH 10.2, 30 min) (Spadari <i>et al.</i> , 1974)		↑	
Aspergillopeptidase B (25°C, pH 9.2, 2 h) (Moriwara & Tsuzuki, 1969)		↑	
Aspergillopeptidase C (35°C, pH 7.2, 2 h) (Nordwig & Jahn, 1968)		↑	
Alkaline proteinase from <i>Aspergillus flavus</i> (37°C, pH 8.5, 1 h) (Turková & Mikes, 1970)		↑	
Alkaline proteinase from <i>Cephalosporium</i> sp. (37°C, pH 10.5, 10 h) (Yagi <i>et al.</i> , 1974)		↑	
Mammalian elastases [porcine (25°C, pH 8.8, 15 min) (Narayanan & Anwar, 1969) moose (37°C, pH 8, 15 min) (Stevenson & Voordouw, 1975)]		↑	
α-Lytic proteinase from <i>Sorangium</i> (25°C, pH 9, 1 h) (Whitaker <i>et al.</i> , 1965)		↑	
<i>Streptomyces fradia</i> lb (25°C, pH 9, 2-3 h) (Moriwara & Tsuzuki, 1969)		↑	
Streptococcal proteinase (25°C, pH 7.6, 2.5 h) (Gerwin <i>et al.</i> , 1966)*		↑	
Alkaline proteinase from <i>Pseudomonas aeruginosa</i> (27°C, pH 8.0, 2 h) (Moriwara & Tsuzuki, 1966)		↑	
Elastase from <i>Pseudomonas aeruginosa</i> (27°C, pH 8.0, 5 h) (Moriwara & Tsuzuki, 1966)		↑	
Alkaline proteinase from marine psychrophilic <i>Pseudomonas</i> sp. No. 548 (37°C, pH 8, 3 h) (Kato <i>et al.</i> , 1974)		↑	

* Streptococcal proteinase is a thiol proteinase and the substrate was the S-carboxymethylated B chain of insulin.

A comparison of the specificity of thermomycolase with selected mammalian, bacterial and fungal proteinases is shown in Table 9. In nearly all cases the conditions of the digests differ and thus exact comparisons of the specificity of the proteinases are difficult. Of particular note, however, were comparisons of thermomycolase with other extracellular proteinases produced by *Aspergillus*. The specificities of thermomycolase and Aspergillopeptidase B were markedly enhanced by performing digests at sub-optimal temperatures.

The rapid hydrolysis of elastin-Congo Red, a substrate for mammalian elastases, indicated the elastase-like activity of thermomycolase. A comparison of the esterolytic activity towards Ac-Ala₃-OMe of thermomycolase and other alkaline proteinases from bacterial, fungal and mammalian sources is presented in Table 8. The ability to bind alanine to subsite S₁ and also to subsites S₂ and S₃, was supported by the finding that alanine residues predominate in the interchain cross-links of elastin (Gerber & Anwar, 1974). Moreover, of the numerous synthetic amino acid esters investigated (Ong & Gaucher, 1975), Cbz-Ala-ONp was hydrolysed at the greatest rate, with Cbz-Tyr-ONp next. Of note was the excellent growth of *M. pulchella*, and the attendant high production of thermomycolase, when elastin (1% w/v) was the sole nutrient source in the fermentation medium. Casein and bovine serum albumin were inferior to elastin under the same conditions (Ong & Gaucher, 1975).

The moderate 'collagenase-like' activity of thermomycolase is implied, but not confirmed, by our studies. The ready hydrolysis of the synthetic peptide

Cbz-Gly-Pro-Leu-Gly-Pro has been utilized as an assay for collagenase activity (Mori-hara, 1967). By this criterion, thermomycolase and alkaline 'serine' proteinases from *Aspergillus mellus* and *Streptomyces fradiae* (Mori-hara *et al.*, 1971) have 'collagenase-like' activity. Although thermomycolase yielded peptides from a digest of native collagen, it is possible that the digestion was limited to the small non-helical portion of the three-chain collagen molecule. Since a true collagenase (e.g. clostridial collagenase) yields hydrolysis along the helical segments of native collagen (Seifter & Harper, 1971) and readily hydrolyses Cbz-Gly-Pro-Gly-Gly-Pro-Ala, the *Malbranchea* and *Aspergillus* proteinases are probably not true collagenases.

The failure of thermomycolase to liberate ninhydrin-positive material from keratin suggests that the enzyme does not possess 'keratinase' activity. Very poor growth of *M. pulchella* and low production of thermomycolase were observed when keratin was used as the sole nutrient in the growth medium. Considering the amino acid sequence of a feather keratin (O'Donnell & Inglis, 1974), it is possible that thermomycolase caused limited hydrolysis but that

the existence of numerous cystine residues prevented the release of peptides. The ready degradation of keratin has, however, been reported for an extracellular 'serine' proteinase (Protease K) produced by the fungus *Tritirachium album* Liber (Ebeling *et al.*, 1974). Further studies of the specificity of Protease K have not been reported.

It is difficult to identify the best experimental method of determining the detailed substrate specificity of a proteinase. The use of small synthetic substrates, which occupy two to three subsites, has the advantage of simplicity of interpretation if one assumes that the binding sites are indeed the same as those used in the binding of larger peptides and proteins. The use of larger substrates such as casein, followed by analysis of the C-terminal residues of the peptide products, may overcome this difficulty, but inevitably lead to rather complex specificity patterns for residue P₁ without a clear knowledge of how adjacent residues are important. The use of smaller peptides of known sequence, such as glucagon or the oxidized A and B chains of insulin appears to improve on both of the above problems, but in fact the overall complexity of the co-operative binding of six to seven amino acid residues is even more striking. With most proteinases of rather broad specificity the unambiguous 'mapping' of subsite specificity is not possible. This is most probably due to the fact that conformational changes in subsites are induced by the binding of substrates and are also influenced by changes in neighbouring subsites. Hence, as emphasized by Fruton (1974), subsites must be considered to be adaptable to a certain variation in the structure and properties of amino acid residues and must be considered capable of responding to interactions occurring at neighbouring subsites. Despite this complexity, however, it is possible to delineate some of the more prominent preferences of some proteinase subsites. Thus alkaline (Dip-F-sensitive) proteinases are more specific for amino acid residues, P₁ (Mori-hara & Tsuzuki, 1969), whereas neutral (EDTA-sensitive) proteinases are specific for amino acid residues P₁' (Mori-hara, 1967), and acid proteinases are specific for amino acid residues P₁ and P₁' (Oka & Mori-hara, 1973).

Thermomycolase undoubtedly possesses an extended active site which preferentially hydrolyses peptide bonds situated within stretches of predominantly non-polar amino acid residues. Like other fungal alkaline proteinases (especially those of *Aspergillus* sp.), this proteinase exhibits a composite 'chymotrypsin-elastase'-type specificity as well as some collagenase-like activity and no keratinase activity. Preliminary structural investigations (V. Dorian & K. J. Stevenson, unpublished work) indicate an 'active-serine' peptide sequence which resembles that of the subtilisin family of 'serine' proteinases.

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