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THE ACTION OF PROTEINASE A_2 OF *ACTINIDIA CHINENSIS* ON THE B-CHAIN OF OXIDIZED INSULIN

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SUMMARY

The specificity of action of the major anionic mercaptoproteinase (A_2) from *Actinidia chinensis* (Chinese gooseberry) against the B-chain of oxidized insulin was investigated. This specificity closely resembled that of papain (EC 3.4.4.10) and could best be explained by a multiple sub-site model for the active centre of the enzyme like that proposed for papain in 1970 (Berger, A. and Schechter, I. (1970) *Phil. Trans. R. Soc., London*, B257, 249). Thus, proteinase A_2 cleaved most readily the carboxyl peptide bonds of those residues of the B-chain acylated by large hydrophobic residues—valyl, leucyl or phenylalanyl, but not tyrosyl— and also the 22–23 Arg–Gly bond as expected from its known action against N^1-acylated esters of L-arginine and L-lysine.

INTRODUCTION

The mercaptoenzyme isolated from the previously described crystalline preparation[1] of the proteinase from the Chinese gooseberry, *Actinidia chinensis*, has been fractionated (McDowall, M. A., unpublished) into two active components, proteinases A_1 and A_2. The unfractionated crystalline preparation had been found to catalyze the hydrolyses of amino-substituted esters of L-glutamine and L-arginine[1] and of glycine (McDowall, M. A., unpublished), thus resembling the action of papain (EC 3.4.4.10) against these esters or the corresponding amides[2–6]. Moreover, as with papain, the rate of hydrolysis of benzoyl L-arginine ethyl ester was found to be near-maximal from pH 5 to 7. On the other hand, the rate of hydrolysis of gelatin catalyzed by the unfractionated extracts of the Chinese gooseberry fruit was found by Arcus[7] to be maximal at pH 4.0.

Abbreviation: –Cya–, residue of cysteic acid in peptide sequences.

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In this study of the specificity of the major component, proteinase A_2, against the standard proteinase substrate, the B-chain of oxidized insulin[8], proteolysis was performed at pH 4.65 because the substrate had low solubility between pH 5 and 7.

MATERIALS AND METHODS

Insulin and most reagents (A.R. grade) were obtained from B.D.H.

Preparation of proteinase A_2

Crystallized anionic proteinase[1] was resolved by recycling gel chromatography[1] on Sephadex G-50F into an active mercaptoenzyme fraction (1.0 mole SH per 24 200 g protein) and an inactive non-mercaptoprotein fraction. The mercaptoenzyme, after conversion to the inactive *S*-2-aminoethylsulphenyl derivative, was resolved by chromatography at pH 5.0 on DEAE-Sephadex A-25 into two components, which could be reactivated by dithiothreitol, each homogeneous on polyacrylamide gel electrophoresis at pH 5.0 (ref. 1), and designated A_1 and A_2 in order of elution and in order of increasing electrophoretic mobility toward the anode. This preparation will be described in detail elsewhere (McDowall, M. A., unpublished).

Performic acid oxidation of insulin

Bovine insulin was oxidized by performic acid[10], prepared from redistilled formic acid (laboratory grade, 99%) and H_2O_2 (30%), and the two chains of the oxidized insulin were then fractionated on DEAE-cellulose (Whatman DE-52) by the method of Griffin *et al.*[11]. Amino acid analysis of the B-chain agreed well with the known composition and showed no contamination with the A-chain.

Digestion of oxidized insulin B-chain by A. chinensis proteinase A_2

Oxidized insulin B-chain (4 μmoles, 17 mg) was dissolved in dilute acetic acid containing EDTA and the pH was adjusted to 4.65 with dilute pyridine, the final volume of the solution being 1.66 ml. *S*-2-Aminoethylsulphenyl-proteinase A_2 in 5 mM sodium citrate–1 mM EDTA, 17.5 mM dithiothreitol (Sigma), pH 6.8, was kept for 10 min at 20 °C to reactivate the enzyme; then 40 μl of this solution containing 40 munits[1] (31 μg) of the reactivated enzyme was added to the vial containing the substrate. The final concentrations (in 1.7 ml) were: insulin B-chain, 10 mg/ml; proteinase A_2, 23.5 munits/ml; dithiothreitol, 0.4 mM; pyridine, 20 mM; acetic acid, 40 mM; EDTA, 1 mM. The vial was flushed with nitrogen, capped, and kept at 30 °C for 10 h with occasional mixing. A precipitate formed during the first hour of digestion but redissolved after about 6 h. The enzyme was inactivated (ref. 15 and McDowall, M. A., unpublished) after 10 h by the addition of excess potassium tetrathionate (kindly supplied by Dr P. A. Trudinger) and the product was dried on the rotary evaporator.

Fractionation of peptides from the digest of the insulin B-chain

The digest was chromatographed at 40 °C on Bio-Rad AG 50W-X2 (0.9 cm × 186 cm) at 12 ml/h. A gradient between 1200 ml 0.2 M pyridine–4.8 M acetic acid, pH 3.1, and 800 ml 1 M pyridine–2 M acetic acid, pH 4.95, followed 350 ml of the first buffer. Tubes were assayed for peptides, after alkaline hydrolysis of samples,

by the colorimetric ninhydrin method. All peptides had been eluted from the column when the effluent pH had reached 4.5; no more were eluted by 300 ml of 3 M pyridine–2 M acetic acid, pH 5.34. Pooled fractions containing peptides were dried under vacuum over H_2SO_4 and NaOH pellets, and further purified where necessary, (a) by paper electrophoresis at 65 V/cm in pyridine–0.06 M acetic acid, pH 5.6, or (b) by paper chromatography in butanol–pyridine–acetic acid–water (15:10:3:12, by vol.) on Whatman 3 MM. Peptides thus purified by one of these procedures were checked for purity by the other procedure or by chromatography in butanol–water–acetic acid (40:15:6, by vol.), and if sufficiently pure, were then hydrolyzed 24 h in 3 times distilled HCl, 6.13 M, at 110 °C, for amino acid analyses. The composition of most peptides was within 10% of stoichiometry.

Amino-terminal analyses

Amino-terminal amino acid residues of most peptides were identified as the dansyl derivatives[12] by thin-layer chromatography on Gelman ITLC sheets type SA in modifications of published solvent systems[13]. (1) Toluene–pyridine–acetic acid (90:10:0.8, by vol.), (2) toluene–pyridine–acetic acid (80:20:0.8, by vol.), and (3) toluene–chloroform–*n*-butanol–acetic acid (50:30:15:5, by vol.). This sufficed to identify unequivocally the amino-terminal residues of the various peptides. Reference solutions of B.D.H. dansyl amino acids were used.

RESULTS AND DISCUSSION

The sequences of the peptides isolated from the proteinase A_2 digest of the insulin B-chain were inferred from the amino acid compositions and the amino-terminal residues of the peptides by comparison with the known sequence of the insulin B-chain (see ref. 8) and are listed in Table I together with the yields, corrected for sampling during purification. Peptide I was ninhydrin-negative but Pauly-positive and presumably had a blocked amino-terminal due to formation of a pyro-glutamyl residue from Gln-4 of the B-chain. A number of other column fractions contained small amounts of ninhydrin-negative, Pauly-positive acidic peptides which streaked very badly on paper electrophoresis and could not be effectively purified, no doubt accounting for the poor overall recovery of peptides from this part of the sequence.

The location of these peptides in the amino acid sequence of the insulin B-chain is shown in Fig. 1 together with the aggregate percentage recovery of each amino acid residue in all the peptides isolated from the digest, and the bonds shown to be hydrolyzed by papain[8,9]. Proteinase B shows marked similarity in specificity to papain but differs in its lack of action on the leucyl and alanyl peptide bonds of the insulin B-chain and its strong action on the 16–17 Tyr–Leu and on the 22–23 Arg–Gly peptide bonds. (The reported failure of papain to hydrolyze this last bond is rather surprising in view of the ease of hydrolysis of simple derivatives of arginine by papain).

There were some apparent inconsistencies in the action of proteinase A_2 on the B-chain. Thus, the peptide bond 5–6 His–Leu was hydrolyzed weakly, whereas bond 10–11 His–Leu was apparently not attacked. Similarly, the bond 12–13 Val–Glu of the sequence –Leu–Val–Glu–, and the 8–9 Gly–Ser and the 7–8 Cya–Gly bonds of

TABLE I

PEPTIDES ISOLATED FROM THE PROTEINASE A_2 DIGEST OF THE B-CHAIN OF OXIDIZED INSULIN

Amino-terminal residues were identified by the dansyl method[12,13]. Peptide I was ninhydrin-negative. Sequences were inferred by comparison with the known sequence of the B-chain. The percentage yields of the peptides are corrected for losses in sampling. Peptides are listed in order of elution from Bio-Rad AG 50W-x2.

Peptide	*Yield (%)*	*Sequence*
I	2	–Glu(His, Leu, Cya, Gly, Ser, His, Leu, Val, Glu)
II	47	Leu(Val, Cya, Gly, Glu, Arg)
III	35	Phe(Val, Asn)
IV	8	Glu(Ala, Leu, Tyr)
V	57	Ala(Leu, Tyr)
VI	20	Gly–Phe–Phe
VII	5	Gly(Ser, His, Leu, Val, Glu)
VIII	4	Phe(Val, Asn, Gln, His)
IX	60	Thr(Pro, Lys, Ala)
X	7	(Ser,His, Leu, Val, Glu)
XI	21	Tyr(Thr, Pro, Lys, Ala)
XII	36	Gly(Phe, Phe, Tyr)

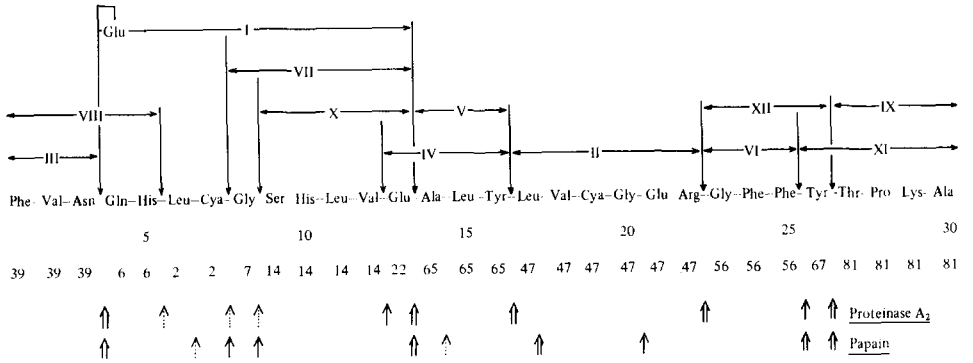

Fig. 1. Comparison of the action of proteinase A_2 of *A. chinensis* with that of papain on the B-chain of oxidized insulin. Peptides isolated from the proteinase A_2 digest are shown above the numbered sequence and the aggregate percentage recovery of each individual amino acid residue in the peptides isolated is given beneath. The extent of cleavage of the peptide bonds by proteinase A_2 and by papain[8,9] is indicated in the two rows of arrows below the sequence on a three-point scale with strong, intermediate and weak cleavage denoted by double, single and broken arrows, respectively.

the sequence–Leu–Cya–Gly–Ser– were hydrolyzed whereas there was no evidence for the hydrolysis of the corresponding bonds of residues 18, 19 and 20 of the sequence –Leu–Val–Cya–Gly–Glu–.

Berger and Schechter[14] have shown that the rates of hydrolyses of oligopeptides of L-alanine, catalyzed by papain, increased progressively with chain length up to seven residues. They postulated that the substrate-binding site of papain consists of seven subsites (S_4 ..... S'_3) thus:

$$S_4–S_3–S_2–S_1 \downarrow S_1'–S_2'–S_3'$$
$$P_4–P_3–P_2–P_1–P_1'–P_2'–P_3'$$

distributed about the catalytic site (↓) binding successive residues P_1 to P_4 on the carboxyl side of the bond being hydrolyzed (– – –) and residues P_1' to P_3' on the amino side, as shown. They demonstrated conclusively that the dominant feature of the specificity of papain against oligopeptide substrates was the nature of the residue P_2 of the peptide. Polyalanyl peptides, containing a single large hydrophobic residue near the middle, were always cleaved at the carboxyl peptide bond of the alanyl residue immediately following this residue, even if some other, normally susceptible bond, such as that of a lysyl residue was present elsewhere in the peptide. When the hydrophobic residue was next to the carboxyl-terminal residue of the peptide, the hydrophobic residue still bound to the subsite S_2, preferentially, so that the peptide was not hydrolyzed but could act as a good competitive inhibitor of the hydrolysis of some good substrate such as benzoyl-L-arginine ethyl ester.

It is at once evident that the action of the proteinase A_2 on the B-chain of insulin conforms closely to this pattern. For seven of the ten bonds shown to be hydrolyzed by proteinase A_2, the residue P_2 was one of the hydrophobic amino acid residues, leucyl, valyl or phenylalanyl, but not (as for papain) tyrosyl. For the other three bonds hydrolysis was presumably favoured by the nature of the P_1 residue (histidyl, glycyl or arginyl), for which the specificity of the enzyme papain has been well characterized[2–4]. Hydrolysis at the 5–6 His–Leu and the 22–23 Arg–Gly bonds may also have been favoured by the binding to subsite S_2 of the uncharged 4-glutaminyl residue and the undissociated form of the 21-glutamyl residue, respectively.

This model of the active centre of the anionic thiol proteinase A_2 from *A. chinensis*, is supported by some recent studies by Boland and Hardman[15] on the kinetics of hydrolysis of some benzyloxycarbonyl-L-aminoacyl-*p*-nitrophenyl esters, catalyzed by a pure preparation of the enzyme which had but a single electrophoretic component and which may be tentatively identified with proteinase A_2. Their results suggest that an aromatic N^1-acyl group contributes strongly to the interaction of the substrate with the enzyme, presumably by binding to the postulated hydrophobic subsite, S_2.

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