Biochimica et Biophysica Acta, 429 (1976) 912—924

BBA 67797

SPECIFICITY OF ACID PROTEINASE A FROM *ASPERGILLUS NIGER* VAR. *MACROSPORUS* TOWARDS B-CHAIN OF PERFORMIC ACID OXIDIZED BOVINE INSULIN

KUNIKO IIO and MAKOTO YAMASAKI

Department of Chemistry, College of General Education, University of Tokyo, Komaba, Meguro-ku, Tokyo (Japan)

(Received November 3rd, 1975)

Summary

1. A comparative study on the mode of action of two highly purified acid endopeptidases (EC 3.4.-) from *Aspergillus niger* var. *macrosporus*, acid proteinase A and B, on the B-chain of performic acid oxidized insulin was performed, putting emphasis on the quantitative analysis of the effects of enzyme A. Acid proteinase A behaved very specifically towards the substrate and hydrolyzed four peptide bonds exclusively: three major sites, where hydrolysis proceeded rapidly and almost completely, Asn^{3}-Gln^{4}, Glu^{13}-Ala^{14}, and Tyr^{26}-Thr^{27}; and a minor one, Gly^{20}-Glu^{21}, at which hydrolysis was much slower.

2. The effects of four protease inhibitors, pepstatin, diazoacetyl-D,L-norleucine methyl ester/Cu(II), di-isopropyl phosphorofluoridate, and 1,2-epoxy-3-(*p*-nitrophenoxy) propane on acid proteinases A and B were studied. Acid proteinase A preparations, treated with the former two inhibitors, were used to establish that the major sites of attack were really affected by enzyme A and not by contaminating proteinase B.

Introduction

Three acid proteases (EC 3.4.-) have been isolated and purified from a culture media of *Aspergillus niger* var. *macrosporus*: two acid endopeptidases, acid proteinases A and B (commercial names "Proctases A and B") [1] and an acid carboxypeptidase (Kumagai, I., Iio, K., Horiuchi, S. and Yamasaki, M., unpublished; and Refs. 2 and 3). The endopeptidases differ in such properties as activity and optimum pH towards protein substrates [1], susceptibilities to tempera-

Abbreviations: Cbzo, carbobenzoxy; DAN, diazoacetyl-D,L-norleucine methyl ester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; DFP, di-isopropyl phosphorofluoridate.

ture and organic solvents and optimum culture conditions (Ref. 1 and Koaze, Y. and Goi, H., unpublished, mol. wt and aminoa cid composition [4,5], susceptibilities to certain acid protease inhibitors (pepstatin [6,7], a few diazoacetyl derivatives (Ref. 7, Chang, W.-J. and Takahashi, K, personal communication, and Horiuchi, S. and Yamasaki, M., unpublished) and EPNP (a,2-epoxy-3-(*p*-nitrophenoxy) propane).

These results indicate that enzyme B may be classified as an acid protease similar to pepsin (EC. 3.4.23.1) but with much resemblance to aspergillopeptidase A (EC 3.4.23.6), whereas enzyme A seems to be unique and representative of a new subclass of acid endopeptidases. No quantitative information on the specificities of enzymes A and B towards larger peptide substrates or synthetic substrates is at present available. The present paper reports the results of quantitative analysis of the specificity of enzyme A towards the B-chain of performic acid oxidized bovine insulin.

Materials and Methods

Enzymes

Enzymatically distinct and disc gel electrophoretically homogeneous specimens of enzymes A and B were isolated and purified according to a procedure (Iio, K. and Yamasaki, M., unpublished), which was a combination of two kinds of ion-exchange chromatography on DEAE-Sephadex A-50 columns and gel filtration using Sephadex G-100, from crude enzyme powder prepared from water extracts of Kohji cultures of *Asp. niger* var. *macrosporus*. (The purification procedures will be reported in detail elsewhere). The powder (commercial name: Proctase, lot No. PTP 274) was a gift from Meijiseika Co. Ltd. Porcine pancreatic carboxypeptidase A was obtained by courtesy of Dr. T. Tobita of Chiba University and Dr. K. Suzuki of University of Tokyo. A 0.1% solution in 10 mM Tris · HCl buffer, pH 7.5, was treated with 5.1 mM DFP (BDH Chemicals Ltd., Poole England, lot No. 44084) for 4 h at 25° C and the mixture was dialyzed against the same buffer at 0° C overnight. The dialysate was stored frozen at −20° C.

Substrates

Crystalline zinc beef insulin (Connaught Medical Research Lab., Univ. Toronto; lot No. 1134, 24.0 units/mg) was oxidized by the method of Craig et al. [8]. The lyophilized oxidized powder was chromatographed according to Griffin et al. [9] to obtain oxidized A- and B-chains. The B-chain gave the expected amino acid composition and had phenylalanine as the sole NH_2-terminal residue. Carbobenzoxy-di-L-alanyl-L-lysyl-tri-L-alanine, a synthetic substrate for enzyme B [10], was a gift from Dr. K. Morihara of the Shionogi Research Laboratory.

Acid protease inhibitors and other reagents

DAN (diazoacetyl-D,L-norleucine methyl ester) was synthesized in our laboratory by Mr. S. Horiuchi: m.p. 63—65° C (literature value, 49—50° C [11]). Pepstatin and EPNP were gifts from Professor K. Takahashi of Kyoto University. Pyridine, used for ion-exchange chromatographic separation of peptides,

was purified according to the directions of Schroeder [12]. Glass-redistilled water was used in the Edman degradation.

Hydrolysis of B-chain of oxidized insulin by acid proteinases A and B

Hydrolysis was carried at 30° C with 2.4 mM B-chain dissolved in 50 mM sodium acetate buffer pH 1.5 for enzyme A and pH 2.6 for enzyme B. Unless otherwise stated, the enzyme/substrate molar ratio was 1 : 2500.

Finger printing

Finger printing was performed by the method of Richmond and Hartley [13] using Toyo Roshi filter paper No. 51. High-voltage paper electrophoresis was carried out at pH 3.6 [14] with a voltage gradient of 33 V per cm. Paper chromatography, i.e. the second run, was developed in 1-butanol/pyridine/acetic acid/water (15 : 10 : 3 : 12, v/v) [15]. Peptides were detected by spraying 0.2% ninhydrin in water-saturated 1-butanol.

Amino acid analysis

Amino acid compositions of peptides and proteins were determined according to Spackman et al. [16] using a Hitachi KLA-3B amino acid analyzer or a JEOL JLC-6AH amino acid analyzer. Specimens were hydrolyzed in evacuated sealed tubes with 0.5 ml of 6 M HCl at 110° C for 24 h.

Sequence study of NH_2-terminal regions of peptides

Amino-terminal residues were detected by the dansylation methods of Hartley [17] and Weiner et al. [18]. Quantitative analyses of NH_2-terminal amino acids were performed by applying the dinitrophenylation method (in triethylamine-ethanol media [19]) to digestion mixtures corresponding to 0.26 μmol of B-chain.

Edman degradations [20] were carried out on digestion mixtures corresponding to 0.26 μmol of B-chain, under the guidance of Dr. J.-P. van Eerd of Professor Ebashi's laboratory (University of Tokyo). Phenylthiohydantoin derivatives of amino acids were separated and identified by gas-liquid chromatography using a JGC-20K (JEOL) gas chromatograph according to the method of Pisano and Bronzert [21] and by thin layer chromatography on silica-gel coated plastic plates with a fluorescent indicator (Eastman Chromagram 6060) according to the directions of Inagami and Murakami [22] and Inagami [23] and on double-faced micropolyamide sheets (Seikagaku Kogyo, Tokyo) by the method of Kulbe [24].

COOH-Terminal analysis of isolated peptides

Specimens were digested at 25° C with DFP(di-isopropyl phosphorofluoridate)-treated carboxypeptidase A in 10 mM Tris · HCl buffer, pH 8.0. Substrate concentration was 1.5 mM and enzyme/substrate molar ratio was 1 : 18 000. Aliquots of 1- and/or 2-h digests, corresponding to 0.1 μmol of B-chain, were subjected to amino acid analysis.

Assays of acid proteinases A and B

Caseinolytic activity was assayed at 30° C as described by Horiuchi et al. [4]

TABLE I

MOLECULAR WEIGHT AND ABSORBANCE OF ACID PROTEINASES A AND B AND PORCINE CARBOXYPEPTIDASE A

Enzymes	Mol. wt.	$A^{0.1\,\%}_{280\ nm,\ 1\ cm}$	Reference
Acid proteinase A	$19._2 \times 10^3$	1.3_3	4
Acid proteinase B	$34._0 \times 10^3$	1.1_8	4
Carboxypeptidase A	34.0×10^3	1.96	38

using approx. $8 \cdot 10^{-10}$ (pH 1.5) and $1.6 \cdot 10^{-10}$ (pH 2.6) μmol of enzymes A and B, respectively, in the incubation mixtures.

Other assay methods and inhibition studies using DAN/Cu(II) and EPNP

Amounts of free amino groups and arginine residue were determined by applying the ninhydrin colorimetry of Yemm and Cocking [25] and Sakaguchi colorimetry by the procedure of Ishii [26]. Inhibition studies of enzymes A and B by DAN/Cu(II) and EPNP, respectively, were carried out according to the procedures of Rajagopalan et al. [11] (50 mM sodium-acetate buffer, pH 5.6, 14°C) and Tang [27] (100 mM sodium-citrate buffer, pH 4.6, 25°C) with slight modifications.

Calculations

The net contents of oxidized B-chain and peptides in incubation mixtures were estimated by performing amino acid analysis with aliquots of individual specimens after acid hydrolysis. The amounts of enzymes in solutions were calculated using the values indicated in Table I.

Designation of peptide bonds

In this paper, the designation proposed by Desnuelle [29] was followed: thus, for an example, "phenylalanine bond" and "phenylalanyl bond" mean the peptide bond which involves the amino and carbonyl groups, respectively, of a phenylalanine residue.

Results

Preliminary study of the reactions of acid proteinases A and B on oxidized B-chain

Fig. 1 indicates the time courses of liberation of free amino groups from oxidized B-chain by acid proteinases A and B at two enzyme/substrate molar ratios (1 : 2500 and 1 : 500). In the case of enzyme A digests, white flocculant precipitates appeared about 60 min (with 1 : 2500 digests) or 20 min (1 : 500 digests) after the start of incubations and they remained apparently unchanged on further prolonged incubation. However, digestions with enzyme B remained clear. Finger prints, performed with digests corresponding to 0.1 μmol of B-chain, are shown in Fig. 2. Digestions were also run for 2 h and for 24 h with enzyme A. Aliquots of these, corresponding to 0.1 or 0.26 μmol of B-chain,

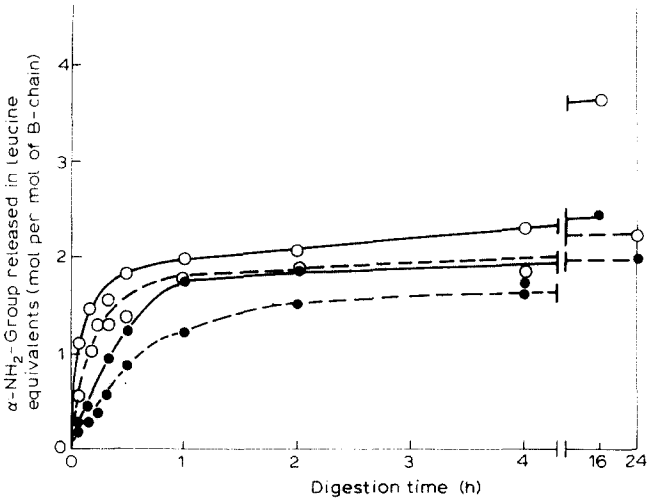

Fig. 1. Hydrolysis of B-chain of performic acid oxidized insulin with acid proteinases A and B. Enzyme A, - - - - - -; B, ———; molar ratio of enzyme/substrate 1 : 2500, ●; 1 : 500, ○.

were subjected to amino acid analysis without previous acid hydrolysis. No free amino acids were detected.

Separation and characterization of peptides in 2-h digestion mixtures of the B-chain of oxidized insulin by acid proteinase A

Fig. 3 shows typical elution patterns obtained by chromatographing a 2-h digest on Dowex 50W-X4 columns. In this case, 5 ml of a 2-h digest, corre-

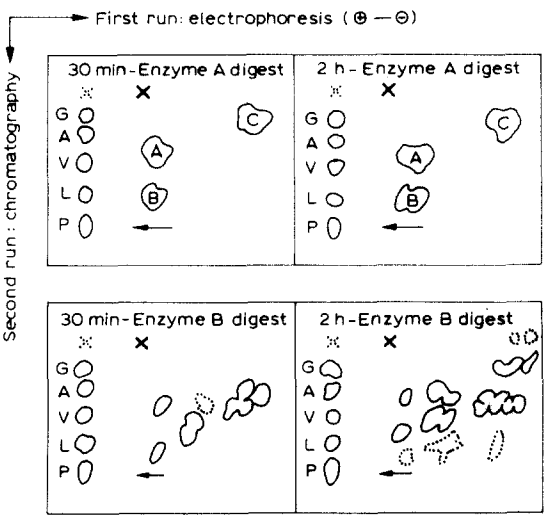

Fig. 2. Finger prints of digests obtained by hydrolyzing oxidized B-chain with acid proteinases A and B respectively (X, point of application). G, A, V, L and P indicate glycine, alanine, valine, leucine and phenosafranine, respectively, applied in the second run as markers (point of application: dotted cross). Control experiments indicated that spots A, B and C, respectively, corresponded to the peptides found in Peaks 2, 3 and 4 in Fig. 3. No intact oxidized B-chain was detected with ninhydrin: the B-chain would appear at the position indicated by the arrows.

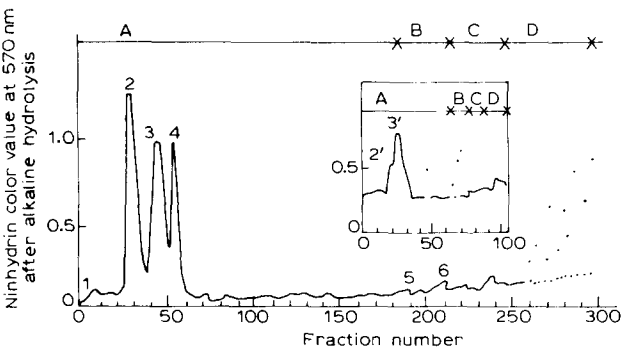

Fig. 3. Elution patterns obtained by chromatographing a 2-h digest of oxidized B-chain with acid proteinase A on Dowex 50W-X4 columns. The first and second extracts of the lyophilized specimen of a 2-h digest were chromatographed on large and smaller columns of Dowex 50W-X4 at 42°C with flow rates of 25 ml/h (smaller column, 10 ml/h) respectively. Eluant system: A, a gradient elution using the gradient vessels recommended by Schroeder [30], a mixer containing 280 ml (48 ml) of 0.2 M pyridine/acetate buffer, pH 3.1, and a reservoir with 560 ml (96 ml) of 2.0 M pyridine/acetate buffer, pH 5.0; B, 2.0 M pyridine/acetate buffer, pH 5.0; C, 2.0 M pyridine-acetate buffer, pH 6.9; D, 2.0 M ammonium acetate. Fraction size: 4.5 ml/tube (1.9 ml). Aliquots (0.2 ml) from alternate effluent fractions were subjected to ninhydrin assay [25] after alkaline hydrolysis [31]. For other details, see text.

sponding to 12 μmol of B-chain, was lyophilized. The dried powder was treated with 10 ml of 0.2 M pyridine/acetate buffer, pH 3.1. The extract was centrifuged and 9.8 ml of the supernatant was applied on a column (1.5 × 42 cm) of Dowex 50W-X4 (200-400 mesh) equilibrated with the buffer mentioned above. A small amount of precipitate was again treated with 2.6 ml of the same buffer to obtain a clear solution and 2.5 ml of the extract was similarly chromatographed separately using a smaller column (0.9 × 19 cm; elution pattern in the inset) of Dowex 50W-X4. Both columns were developed using the eluant system described in the legend of Fig. 3.

Aliquots of the pooled fractions under each peak (Fig. 3) were subjected to amino acid analysis. The remaining material in each pool was lyophilized for later experiments. Table II shows amino acid analyses done with material from Peaks 1—6 and also Peak 3′ (inset curve). Further analyses confirmed that no appreciable amounts of peptides were contained in effluent fractions outside the peak regions. Material from each of the major peaks (2, 3 and 4) gave rise to single spots when examined paper chromatographically. The NH_2-terminal amino acids and the COOH-terminal sequences of the individual peptides in these peaks are also indicated in Table II. Thus Peaks 2, 3 and 4, respectively, were identified as a tridecapeptide H-Phe^{1}—· · ·—Glu^{13}-OH, a tridecapeptide H-Ala^{14}—· · ·—Tyr^{26}-OH, and a tetrapeptide H-Thr^{27}—· · ·—Ala^{30}-OH. The contribution of Peak 1 to the total recovery of amino acids on chromatography was not negligible. From the data in Table II, it is reasonable to suppose that Peak 1 is a 1 : 1.1 (molar ratio) mixture of Peak 2 peptide and the decapeptide, H-Gln^{4}—· · ·—Glu^{13}-OH, derived from Peak 2 peptide by release of an NH_2-terminal tripeptide. The intermediate between-peak fractions 14—28, 33—43 and 49—53 were also examined by amino acid analysis and thin layer chromatography. It was concluded that these grouped fractions contained only those peptides that were present in adjacent peaks. A control experiment had shown that

TABLE II

AMINO ACID COMPOSITION AND STRUCTURE OF PEPTIDES ISOLATED BY ION-EXCHANGE CHROMATOGRAPHY FROM A 2-H DIGEST OF B-CHAIN OF OXIDIZED INSULIN BY ACID PROTEINASE A AT 30°C

Amino acid	Peaks and fraction numbers analyzed *						
	1 Nos. 1—13	2 29—32	3 44—48	4 54—57	5 183—190	6 206—210	3′ 29—31
Lysine	0	0	0	0.062 (1)	0.002	0	0
Histidine	0.038	0.10_6 (2)	0	0	0.001	0.001	0
Arginine	0	0	0.059 (1)	0	0.001	0.002	0.019 (1)
Cysteic acid	0.026	0.068 (1)	0.090 (1)	0.009	0	0	0.020 (1)
Aspartic acid	0.007	0.057 (1)	0	0	0.004	0.002	0
Threonine	0	0	0	0.061 (1)	0.002	0.001	0
Serine	0.019	0.060 (1)	0	0	0.006	0.003	0
Glutamic acid	0.042	0.12_5 (2)	0.086 (2)	0	0.007	0.003	0.024 (1)
Proline	0	0	0	0.067 (1)	0	0	0
Glycine	0.024	0.062 (1)	0.137 (2)	0	0.008	0.006	0.043 (2)
Alanine	0.005	0	0.093 (1)	0.071 (1)	0.002	0	0.020 (1)
Valine	0.022	0.10_5 (2)	0.070 (1)	0	0	0	0.018 (1)
Leucine	0.037	0.13_8 (2)	0.16_1 (2)	0	0.002	0.001	0.039 (2)
Tyrosine	0	0	0.12_8 (2)	0	0	0	0.037 (2)
Phenylalanine	0.010	0.056 (1)	0.14_6 (2)	0	0.003	0	0.041 (2)
Methionine	0	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	0	0
NH_2-Terminal amino acid	Phe, Glu and/or Gln	Phe	Ala	Thr	—	—	—
COOH-Terminal sequence		-His-Leu-Val-Glu-OH	-Phe-Phe-Tyr-OH	-Ala-OH	—	—	—
Structure of peptide **	1—13 + 4—13	1—13	14—26	27—30	—	—	14—26

* See Fig. 3 and text. The values of amino acid composition indicate the assayed amounts in μmol and those in parentheses denote the theoretical number of residues in the peptide.

** Residue numbers in the B-chain.

intact oxidized B-chain would be eluted in the region between Peaks 2 and 3 with 70% recovery. However, no significant amount could be detected. The amounts of peptides in Peaks 5 and 6 were too minute to make a significant contribution to the total recovery of amino acid, and their identities are uncertain. By applying the same procedure, it was concluded that Peak 2′ (fractions 23—24 of inset curve) contained the peptides of Peaks 2 and 3, while Peak 3′ (29—31) contained the peptide of Peak 3. Recoveries of the four peptides are shown in Fig. 5.

The time courses of release of new NH_2-terminal amino acids during digestion of oxidized B-chain by acid proteinase A are shown in Fig. 4. The amount of dinitrophenyl-phenylalanine recovered remained essentially constant during a digestion period of 24 h. This suggests that the phenylalanine bonds in the sequence · · —Gly[23]-Phe-Phe-Tyr— · · were unaffected by the enzyme. This was verified by Edman degradation of the enzyme A digest: the phenylthiohydantion spots of neither phenylalanine nor tyrosine were detected at the second cycle of the degradation. Besides dinitrophenyl-phenylalanine, only dinitrophenyl-alanine, dinitrophenyl-threonine and dinitrophenyl-glutamic acid were detected in appreciable amounts. The patterns of the release of these dinitrophenyl-amino acids are expressed in Fig. 4 as molar percents relative to dinitrophenyl-phenylalanine.

An Edman degradation of 2, 8 and 24-h digests gave very clear-cut results (Fig. 5). We concluded that the dinitrophenyl-glutamic acid in Fig. 4 had arisen mainly from the dinitrophenyl-glutaminyl peptide. Thus, at each first cycle we detected appreciable amounts of glutamine phenylthiohydantoin, and also of glutamic acid-phenylthiohydantoin which was presumably mostly derived from

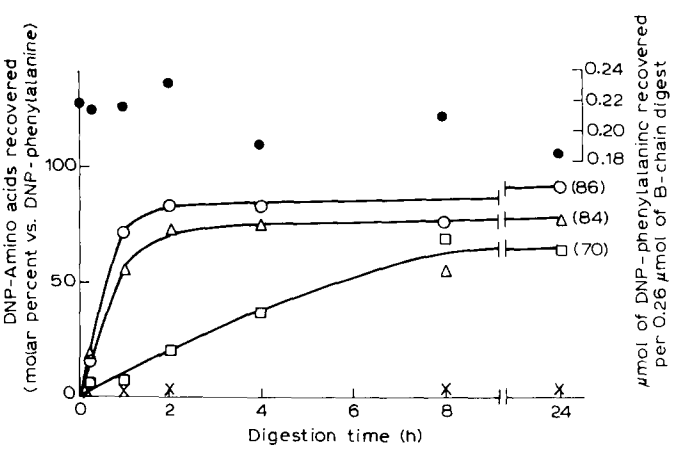

Fig. 4. Rates of release of new NH_2-terminal amino acids during the digestion of B-chain of oxidized insulin with acid proteinase A. Right ordinate: dinitrophenyl (DNP)/phenylalanine derived from the NH_2-terminal of 0.26 μmol of the B-chain. Left ordinate: newly released NH_2-terminals (as DNP-amino acids) expressed as percentages of the amount of DNP-phenylalanine in each set of analyses. No values were corrected for losses during dinitrophenylation, acid hydrolysis and two-dimentional paper chromatographic separation except for values in parentheses which were corrected for chromatographic loss. DNP-phenylalanine, ●———●; DNP-threonine, ○———○; DNP-alanine, △———△; DNP-glutamic acid, □———□; an unidentified DNP-spot always recovered in approximately 0.02 mol per mol of B-chain nearby DNP-valine, X- - - - -X.

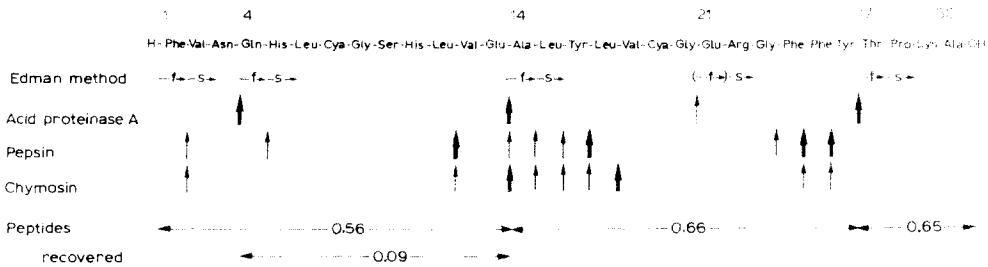

Fig. 5. Summary of the results of Edman degradations carried out on digestion mixtures of oxidized B-chain by acid proteinase A and comparison of the specificaties of three kinds of acid endopeptidases towards the B-chain. Cya, cysteic acid. Edman method: horizontal arrows indicate the amino acids whose phenylthiohydantoin derivatives were detected at the first (*f*) and second (*s*) cycle of the degradation respectively. Dotted arrows indicate residues recovered in low yield. Bold, thin and dotted vertical arrows indicate bonds with respectively, very high, moderate and very low susceptibilities to the individual enzymes. The data on pepsin and chymosin (or rennin) were taken from the literatures [32,33]. Recoveries of peptides: the values (in mol per mol of B-chain) are those of individual peptides isolated by ion-exchange chromatography from 2-h digest of oxidized B-chain as shown in Fig. 3.

glutamine during Edman degradation. There was some cleavage of the glutamic acid bond in the sequence, · · —Gly^{20}-Glu-Arg— · ·. Since small amounts of arginine phenylthiohydantoin were detected at each second cycle, the extent of hydrolysis of this bond by enzyme A was estimated to be about 0.04 mol per mol of B-chain by the following procedure. After the first cycle of Edman degradation, the products were dinitrophenylated. From the acid hydrolysates of dinitrophenylated specimens, dinitrophenyl-arginine was separated and identified by paper chromatography [19]. Individual dinitrophenyl-arginine spots were eluted with a 1% sodium bicarbonate solution and the amounts of dinitrophenyl-arginine were determined by Sakaguchi colorimetry [26].

Effects of inhibitors on digestion of B-chain of oxidized insulin by acid proteinase A

Acid proteinases A (14 μM) and B (9 μM) were each incubated with DFP (3.6 mM) at 30° C in 50 mM sodium acetate buffer of pH 3.5 and 5.0 respectively. After 0, 30, 90 and 180 min of incubation, aliquots of the incubation mixtures were assayed. No losses in caseinolytic activity were detected.

Acid proteinase A was not inhibited by pepstatin, whereas pepstatin completely inhibited both caseinolytic and peptidase activities of enzyme B. If acid proteinase A (16 μM) was preincubated in 120 μM pepstatin (almost saturation concentration)/30 mM sodium acetate buffer, pH 1.5, at 30° C and if the activity of the enzyme was then assayed at appropriate intervals (0, 10, 120 and 240 min) using solutions of casein in 120 μM pepstatin, no losses in activity were observed. By thin layer chromatography, it was also confirmed that enzyme A did not degrade pepstatin with formation of non-inhibitory products. On the other hand, the activity of enzyme B was completely inhibited in solutions of casein (pH 2.6 and 1.5) in 11 μM pepstatin. In addition, the presence of pepstatin (45 μM) completely inhibited the splitting by enzyme B ([B] = 0.25 μM, pH 4.5 and pH 1.5, 30° C, for 0—24 h) of Cbzo-di-L-alanyl-L-lysyl-tri-L-alanine ([substrate] = 4.2 mM) to give Cbzo-di-L-alanyl-L-lysine and tri-L-alanine [10].

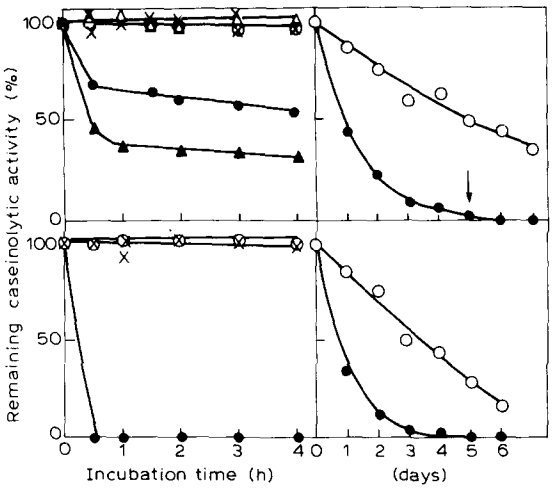

Fig. 6. Effects of DAN/Cu(II) and EPNP on caseinolytic activity of acid proteinases A and B. For incubation conditions, see Methods. At intervals, appropriate aliquots of incubation mixtures were withdrawn to assay the remaining caseinolytic activity. Enzymes : A (upper), B (lower). DAN (left): ○, enzyme control, [A] = 22 μM, [B] = 14 μM; x, 1.2 mM DAN added to the incubation mixtures; ●, 1.2 mM DAN/1.1 mM $CuCl_2$; △, 2.5 mM DAN; ▲, 2.5 mM DAN/2.2 mM $CuCl_2$. EPNP (right): ○, enzyme control, [A] = 41 μM, [B] = 27 μM; ●, 51 mM (i.e. 10 mg/ml) EPNP in the incubation mixtures; the arrow indicates further addition of EPNP (5 mg/ml) to the remaining incubation mixture.

Fig. 6 shows the effect of DAN/Cu(II) and EPNP, respectively, on the caseinolytic activity of enzymes A and B.

The time courses of the release of new NH_2-terminal amino acids on digestions of oxidized B-chain by enzyme A preparations treated with pepstatin and DAN/Cu(II), respectively, were studied as in Fig. 4. In the case of pepstatin, three new NH_2-terminals (as dinitrophenyl-amino acids) were released essentially in the same way, either in the absence and presence (11 μM, examined with 2- and 24-h digests) of the compound. Using the enzyme A preparations, partially inactivated by DAN/$CuCl_2$ treatment (2.5/2.2 mM, 14°C, 4 h: under the conditions which must completely inactivate enzyme B; see Fig. 6), qualitatively the same newly released amino terminals were detected with 15- and 120-min digests of the B-chain as was the case with intact enzyme A. However, the results were not so clear-cut quantitatively as those with the pepstatin-treated enzyme A. The ambiguity might be caused by the presence of Cu(II), as control experiments indicated that enzyme A-catalyzed rates of hydrolysis of the susceptible bonds in the B-chain were differently affected by 0.1 mM Cu(II) in the reaction mixtures.

Discussion

Mode of action of acid proteinases A and B on B-chain of oxidized insulin

The effect of enzyme A on the substrate was very specific. The enzyme hydrolyzed only four peptide bonds. There were three major sites of attack, Glu^{13}-Ala(I), Tyr^{26}-Thr(II) and Asn^{3}-Gln(III), and a minor site, Gly^{20}-Glu. Under the experimental conditions, hydrolysis at the major sites proceeded almost to completion and essentially no overlapping peptide fragments were detect-

able. (Putting together the results of finger printing (Fig. 2), ion-exchange chromatographic separation, and digestion products characterization (Fig. 3), and of the analysis using the dinitrophenylation method (Fig. 4), it may be safe to say that the observed values with enzyme A digests in leucine equivalent (Fig. 1) were somewhat lower than the real amounts of hydrolysis by a factor of approx. 0.8.) The four sites of attack in the oxidized B-chain are shown in Fig. 5: the so-called primary specificity [34] of acid proteinase A towards the substrate is not immediately obvious. Enzyme A behaved like a typical endopeptidase, as no free amino acid was detected even after digestion for 24 h.

From the initial slopes of the curves in Fig. 4, the turn-over numbers of hydrolysis at the major sites, I, II and III were calculated to be 0.48, 0.43 and 0.077 s^{-1}, respectively. (These values should be regarded as minimal, since the concentration of B-chain in the digestion mixtures was not high enough to saturate enzyme A fully). Compared to the reported k_{app} values of other acid proteinases towards synthetic substrates [10,35—40], the observed values of turnover numbers for sites I and II are quite high. Thus the study of synthetic peptides containing the moieties of these two sites may be fruitful in finding efficient substrates for enzyme A.

The specificity of acid proteinase A towards oxidized B-chain in general has little resemblance with that of pepsin [32,33] and chymosin (or rennin, EC 3.4.23.4) [33] indicated in Fig. 5. The specificity of acid endopeptidases from such various origins as bovine spleen [41] uterus [42] and fibroblast [43], *Aspergillus saitoi* [44], *Candida albicans* [45], *Rizopus chinensis* [46], *Penicillium janthinellum* [47], and *Mucor miehei* [48], towards oxidized B-chain, has been reported to resemble more or less that of pepsin and/or chymosin. In this respect, therefore, enzyme A may be a very unique acid endopeptidase.

Our preliminary study of acid proteinase B (Figs. 1 and 2) shows that it attacks the B-chain at many more sites than enzyme A, but that hydrolysis at individual sites in incomplete.

Effects of protease inhibitors on acid proteinases A and B and the use of inhibitor-treated enzyme A preparations for the digestion of oxidized B-chain

The so-called carboxypeptidases from non-pancreatic origins, which have optimal pH in acidic regions, have been reported to be very sensitive to DFP [49—57]. However, acid proteinases A and B were resistant to this reagent.

Umezawa and his colleagues [6] have reported that among the acid proteases, enzyme A behaved exceptionally towards pepstatin. Their observations were based on the comparison of the LD_{50} values, which are of physiological significance but have little enzymatic meaning. Enzyme A was fully active in media of 120 μM (almost saturation concentration) pepstatin. Because of the poor solubility of pepstatin, it is not clear whether this reagent has some inhibitory effect on enzyme A or not. Assuming that pepstatin inhibits an acid protease by forming a 1: 1-complex [6,58], the result with enzyme A means that the value of the inhibition constant of pepstatin towards enzyme A must be greater than 10^{-2} M. The major sites were attacked by enzyme A essentially in the same way, either in the absence or in the presence of 11 μM (enzyme B was completely inhibited at this concentration) of pepstatin respectively, indicating that these sites were really affected by enzyme A.

In the reaction with DAN/Cu(II), both enzymes A and B were inactivated. Enzyme B was far more sensitive than enzyme A to this reagent. Enzyme A preparations, partially inactivated by treatment with DAN/Cu(II), also split the same three major sites, although in this case the rate of hydrolysis at site III was far lower than those at sites I and II.

Enzymes A and B were inactivated by EPNP at similar rates. However the inhibition reactions proceeded in heterogeneous suspensions of the poorly soluble inhibitor and several days were required for complete inactivation.

Acknowledgements

The authors express their thanks to Mr. Yujiro Yamada of Central Research Laboratories of Meiji Seika Kaisha Ltd, Yokohama, for his interest and kind consideration to their study, to Dr. Tohru Tobita of Chiba University and Dr. Kohichi Suzuki of the University of Tokyo for the kind gift of carboxypeptidase A, to Professor Kenji Takahashi of Kyoto University for the kind gift of pepstatin and EPNP, and to Dr. Kazuyuki Morihara of Shionogi Research Laboratory, Osaka, for the kind gift of synthetic substrates for enzyme B. For amino acid analyses, the authors are indebted to Professor Toshio Ando, Dr. Tohru Tobita and Dr. Minoru Nakano of Chiba University, Dr. Masaru Shiraki and Mr. isao Yagi of the Research Institute for Polymers and Textiles, Yokohama, and Dr. Kohji Nomura of the Tokyo Metropolitan Institute of Gerontology. The authors are also indebted to Professor Setsuro Ebashi and Dr. Jean-Paul van Eerd of the University of Tokyo for excellent instruction of Edman method.

References

1 Koaze, Y., Goi, H., Ezawa, K., Yamada, Y. and Hara, T. (1964) Agric. Biol. Chem. 28, 216—223
2 Ichishima, E. (1973) Seikagaku 45, 267—288
3 Oka, T. and Morihara, K. (1973) Arch. Biochem. Biophys. 156, 543—551
4 Horiuchi, S., Honjo, M., Yamasaki, M. and Yamada, Y. (1969) Sci. Pap. Coll. Gen. Educ., Univ. Tokyo 19, 127—139
5 Horiuchi, S., Yamasaki, M. and Yamada, Y. (1969) Sci. Pap. Coll. Gen. Educ., Univ. Tokyo 19, 141—150
6 Umezawa, H. (1972) in Enzyme Inhibitors of Microbial Origins, pp. 34—52, University of Tokyo Press, Tokyo
7 Chang, W.-J. (1973) Ph. D. Thesis, University of Tokyo, Tokyo
8 Craig, L.C., Koningsberg, W. and King, T.P. (1961) Biochem. Prep. 8, 70—75
9 Griffin, T.B., Wagner, F.W. and Prescott, J.M. (1966) J. Chromatog. 23, 280—286
10 Morihara, K. and Oka, T. (1973) Arch. Biochem. Biophys. 157, 561—572
11 Rajagopalan, T.G., Stein, W.H. and Moore, S. (1966) J. Biol. Chem. 241, 4295—4297
12 Schroeder, W.A. (1967) in Methods in Enzymology, Vol. 11, pp. 351—361 Academic Press, New York
13 Richmond, V. and Hartley, B.S. (1959) Nature 184, 1869—1870
14 Ryle, A.P., Sanger, F., Smith, L.F. and Kitai, R. (1955) Biochem. J. 60, 541—556
15 Walley, S.G. and Watson, J. (1953) Biochem. J. 55, 328—337
16 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
17 Hartley, B.S. (1970) Biochem. J. 119, 805—822
18 Weiner, A.M., Platt, T. and Weber, K. (1972) J. Biol. Chem. 247, 3242—3251
19 Iwai, K. and Fujioka, H. (1963) in Proteins, Nucleic Acids, Enzymes (in Japanese) pp. 8, 5—26, Kyoritsu Shuppan, Tokyo
20 Sauer, R.T., Niall, H.D., Hogan, M.L., Keutman, H.T., O'Riordan, J.L.H. and Potts, Jr., J.T. (1974) Biochemistry 13, 1994—1999
21 Pisano, J.J. and Bronzert, T.J. (1969) J. Biol. Chem. 244, 5597—5607

22 Inagami, T. and Murakami, K. (1972) Anal. Biochem. 47, 501—504
23 Inagami, T. (1973) Anal. Biochem. 52, 318—321
24 Kulbe, K.D. (1974) Anal. Biochem. 59, 564—573
25 Yemm, E.W. and Cocking, E.C. (1955) Analyst 80, 209—215
26 Ishii, S. (1960) Nippon Kagaku Zasshi 81, 1586—1588
27 Tang, J. (1971) J. Biol. Chem. 246, 4510—4517
28 Folk, J.E. and Schirmer, E.W. (1963) J. Biol. Chem. 238, 3884—3894
29 Desnuelle, P. (1953) in The Proteins, Vol. I, Part A, pp. 87 (Neurath, H. and Bailey, K., eds.) Academic Press, New York
30 Schroeder, W.A. (1972) in Methods in Enzymology Vol. 25, Part B, pp. 203—213, Academic Press, New York
31 Hirs, C.H.W., Moore, S. and Stein, W.H. (1954) J. Biol. Chem. 219, 623—642
32 Sanger, F. and Tuppy, H. (1951) Biochem. J. 49, 481—490
33 Bang-Jensen, V., Foltmann, B. and Rombauts, W. (1964) C.R. Trav. Lab. Carlsberg 34, 326—345
34 Neil, G.L. and Niemann, C. (1966) Nature, 210, 903—907
35 Fruton, J.S. (1970) Adv. Enzymol. 33, 401—443
36 Voynick, I.M. and Fruton, J.S. (1971) Proc. Natl. Acad. Sci. U.S. 68, 257—259
37 Ferguson, J.B., Andrews, J.R., Voynick, I.M. and Fruton, J.S. (1973) J. Biol. Chem. 248, 6701—6708
38 Oka, T. and Morihara, K. (1973) Arch. Biochem. Biophys. 156, 552—560
39 Sampath-Kumar, P.S. and Fruton, J.S. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1070—1072
40 Oka, T. and Morihara, K. (1974) Arch. Biochem. Biophys. 165, 65—71
41 Press, E.M., Porter, R.R. and Cebra, J. (1960) Biochem. J. 74, 501—514
42 Woessner, Jr., J.F. and Shamberger, Jr., R.J. (1971) J. Biol. Chem. 246, 1951—1960
43 Schwabe, C. and Sweeny, S.C. (1972) Biochim. Biophys. Acta, 284, 465—472
44 Ichishima, E. and Yoshida, F. (1961) Agric. Biol. Chem. 25, 102—107
45 Remold, H., Fasold, H. and Staib, F. (1968) Biochim. Biophys. Acta, 167, 399—406
46 Tsuru, D., Hattori, A, Tsuji, H. Yamamoto, T. and Fukumoto, J. (1969) Agric. Biol. Chem. 33, 1419—1426
47 Mains, G., Takahashi, M., Šodek, J. and Hofmann, T. (1973) Can. J. Biochem. 49, 1134—1149
48 Sternberg, M. (1972) Biochim. Biophys. Acta 285, 383—392
49 Zuber, H. (1964) Nature, 201, 613
50 Shaw, D.C. and Wells, J.R.E. (1967) Biochem. J. 104, 5C
51 Shaw, D.C. and Wells, J.R.E. (1972) Biochem. J. 128, 229—235
52 Hayashi, R., Minami, Y. and Hata, T. (1972) Agric. Biol. Chem. 36, 621—629
53 Hayashi, R., Moore, S. and Stein, W.H. (1973) J. Biol. Chem. 248, 8366—8369
54 Ichishima, E. (1972) Biochim. Biophys. Acta 258, 274—288
55 Ichishima, E., Sonoki, S., Hirai, K., Torii, Y. and Yokoyama, S. (1972) J. Biochem. 71, 1045—1048
56 Jones, S.R. and Hofmann, T. (1972) Can. J. Biochem. 50, 1297—1310
57 Ihle, J.N. and Dure, L.S., III. (1972) J. Biol. Chem. 247, 5041—5047
58 McKown, M.M., Workman, R.J. and Gregerman, R.I. (1974) J. Biol. Chem. 249, 7770—7774