Biochimica et Biophysica Acta, 659 (1981) 334—343
Elsevier/North-Holland Biomedical Press

BBA 69289

ISOLATION, PURIFICATION AND SOME CHEMICAL PROPERTIES OF AN ACID CARBOXYPEPTIDASE FROM *ASPERGILLUS NIGER* VAR. *MACROSPORUS*

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(Received August 18th, 1980)
(Revised manuscript received January 21st, 1981)

Key words: Acid carboxypeptidase; Glycoprotein; Subunit structure; (Asp. niger)

Summary

Acid carboxypeptidase (peptidyl-L-amino-acid hydrolase, EC 3.4.16.1) was purified to a homogeneous state from the water extracts of Koji cultures of *Aspergillus niger* var. *macrosporus*. The molecular weight of the enzyme was determined to be 136 000 by sedimentation equilibrium method. The denatured specimen of the enzyme exhibited a molecular weight of 60 000 in the sedimentation equilibrium in 6 M guanidinium chloride, suggesting that the native enzyme is composed of two identical subunits. However, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the enzyme showed an anomalous Ferguson plot, which may account for the inconsistent values of apparent molecular weights obtained by this method. The acid carboxypeptidase was found to be an acidic glycoprotein (p*I*, 4.1), composed of 955 amino acid, 140 mannose, 14 galactose and 30 glucosamine residues/molecule.

Introduction

Carboxypeptidases, which display maximal activity in an acidic pH range and are sensitive to diisopropylphosphorofluoridate, have been isolated mainly from plants and microorganisms [1—10]. They were originally termed 'acid carboxypeptidase' by Zuber and Matile [1] or recently 'serine carboxypeptidase' by Hayashi [11] (peptidyl-L-amino-acid hydrolase, EC 3.4.16.1). In

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contrast to pancreatic metalloenzymes, carboxypeptidases A and B [12,13], these carboxypeptidases are completely inactivated by diisopropylphosphorofluoridate but do not require divalent ions for activity. On the other hand, the chemical nature and certain enzymatic properties reported for the enzymes vary depending upon the biological source [2—4]. Their molecular weights fall within the range of 50 000—150 000 [4—10], and several are reported to be glycoproteins [2—4].

Acid carboxypeptidases were also isolated from *Aspergillus saitoi* [8] and *Aspergillus oryzae* [14]. These enzymes from the genus *Aspergillus* exhibit carboxypeptidase activity in a more acidic pH range compared with the enzymes from other sources. In contrast to the considerably abundant information on the enzymatic properties of these enzymes, little has been reported about their chemical natures.

This paper describes a purification method for an acid carboxypeptidase from *Aspergillus niger* var. *macrosporus*, which enabled us to prepare a highly homogeneous enzyme in gram quantities, and to characterize its molecular properties.

Materials and Methods

Materials. The supernatant of $(NH_4)_2SO_4$ fractionation at 60% saturation of water extracts of Koji cultures of *Aspergillus niger* var. *macrosporus* was prepared by Meiji Seika Co., Ltd. [15] and kindly given to us. Bz-Arg-OEt and Z-Glu-Tyr-OH were obtained from the Protein Research Foundation, Minowa, Osaka, Japan. Proteins used as standards for molecular weights were from Mann Res., Lab., New York, except for phosphorylase *a* (from Boehringer Mannheim), and carrier ampholyte (pH range 3—6) was the product of LKB; DEAE-Sephadex A-50 and Sephadex G-100 were from Pharmacia Fine Chemicals. Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) was from Sigma Chemical Co.

Porcine pancreatic carboxypeptidase A, a gift from Dr. T. Tobita of Chiba University and Dr. K. Suzuki of the University of Tokyo, was used following diisopropylphosphorofluoridate treatment as described by Iio and Yamasaki [16].

Enzyme assay and definition of units. Peptidase activity was routinely followed by measuring the release of tyrosine from 5 mM Z-Glu-Tyr-OH in 50 mM sodium acetate buffer, pH 3.0, at 30°C [8]. 1 peptidase unit for Z-Glu-Tyr-OH was defined as the amount of the enzyme which liberated 1 μmol tyrosine/min. Esterase activity toward Bz-Arg-OEt was followed by measuring the increase of absorbance at 253 nm accompanied by the hydrolysis of 1 mM Bz-Arg-OEt in 50 mM sodium acetate buffer, pH 5.0, at 30°C as described by Schwert and Takenaka [17] with slight modifications. 1 esterase unit for Bz-Arg-OEt was defined as the amount of the enzyme which resulted in the increase of absorbance unit/min.

The ratio of peptidase activity to the esterase activity remained almost constant throughout all steps of purification. This observation suggested that both activities were intrinsic properties of the enzyme.

Determination of protein. Protein concentrations in crude materials were

estimated by absorbance at 280 nm. The amount of the purified enzyme was determined by its absorbance at 280 nm using the value of $E^{1\%}_{1cm}$ = 14.0, which was calculated with specimens desalted on a column of Bio-Gel P-6 and dried to constant weight in vacuo over P_2O_5 at 105°C.

Electrophoretic procedures. Electrophoresis in 7.5% polyacrylamide gel at pH 8.3 was performed according to the procedure of Davis [18] either in the absence or in the presence of 8 M urea. The method for SDS-polyacrylamide gel electrophoresis was based on those of Weber and Osborn [19]. After electrophoresis, gels were treated as described by Fairbanks et al. [20] either with 0.05% Coomassie brillant blue R-250 to stain protein moieties or with periodic acid-Schiff's staining to detect carbohydrate moieties. Isoelectric focusing was carried out as described by Vesterberg et al. [21] using an LKB smaller column of 110 ml capacity.

Ultracentrifuge analyses. Sedimentation velocity analysis was performed using a Hitachi UCA-1A ultracentrifuge, equipped with schlieren optics, at 25.7°C in 0.1 M NaCl/50 mM sodium acetate buffer, pH 5.0.

Sedimentation equilibrium experiments were carried out using a Beckman-Spinco model E analytical ultracentrifuge equipped with a photoelectric scanner. The distribution of protein within centrifuge cells was measured by the scanner operated at 280 nm. Instead of using the scanner chart provided by the manufacturer, amplified photomultiplier pulses were directly measured and processed by an on-line computer system (Ui, N., unpublished data). Experiments in nondenaturing solvents were performed by the procedure of Edelstein and Schachman [22]. Five samples (protein concentrations: 0.022—0.043%) in 0.18 M NaCl/0.02 M sodium acetate buffer, pH 5.4, with or without ^2H_2O were centrifuged simultaneously in an An-G Ti rotor. Runs were made at a speed of 7200 rev./min for 48 h and then 17 000 rev./min for 24 h. For experiments in 6 M guanidinium chloride, a reduced-carboxymethylated enzyme specimen was subjected to sedimentation equilibrium analyses in 6 M guanidinium chloride/0.05 M sodium acetate buffer, pH 5.4. Sedimentation runs were carried out at 11 000 rev./min for 42 h and then at 28 000 rev./min for 26 h.

Amino acid and carbohydrate analyses. For amino acid analyses, samples were hydrolyzed in 6 N HCl at 110°C for 24 h. Quantitative analyses were performed by the method of Spackman et al. [23] with a JEOL (Type JLC-6AH, one-column system) automatic amino acid analyzer. Half-cystine was determined as cysteic acid after performic acid oxidation [24] and as carboxymethyl-cysteine after reduction and alkylation [25]. Tryptophan was determined spectrophotometrically [26].

For analyses of neutral sugars, the method of Spiro [27] using gas-liquid chromatography, was applied to 8-h hydrolysates in 1 N H_2SO_4 at 100°C. The amino sugar constituent was identified and quantified with 4-h hydrolysates in 4 N HCl at 100°C, using a short column (0.90 × 19 cm) of a Hitachi KLA-3B amino acid analyzer.

End-group analyses. The dinitrophenylation-method was carried out according to the procedure of Fraenkel-Conrat et al. [28]. The Edman method was also applied to the purified enzyme as described by Sauer et al. [29]. Acid carboxypeptidase specimens, inactivated by phenylmethanesulfonyl fluoride,

were subjected to carboxypeptidase A digestion at substrate/carboxypeptidase A molar ratio of 200 : 1 according to the method of Ambler [30].

Purification of the acid carboxypeptidase. The starting material was the supernatant of water extracts from Koji cultures of *A. niger* var. *macrosporus*, previously fractionated with 60% $(NH_4)_2SO_4$. Unless otherwise mentioned, all steps were performed at 4°C. Step 1: To about 40 l of the starting material, 9 kg $(NH_4)_2SO_4$ were slowly added with stirring to bring the concentration of $(NH_4)_2SO_4$ up to 100% saturation. After adjusting the pH to 4.2, the suspension was left undisturbed overnight following which the suspension was stirred for several hours with 225 g Hyflo Super-Cel. The suspension was then filtered with suction. The filtrate was discarded, and the filter cake was stored at −20°C for further use. Step 2: The precipitated proteins were extracted three times with 50 mM sodium citrate buffer, pH 5.0. The combined extracts thus obtained, having a total volume of 2700 ml, were mixed with 1350 ml of 60% (w/v) polyethylene glycol-6000 in the same buffer. After 30 min of stirring, the suspension was centrifuged. The supernatant was discarded and the precipitate was used for further purification. Step 3: The precipitate of polyethylene glycol-6000 fractionation was dissolved in 600 ml of 0.1 M NaCl/50 mM sodium citrate buffer, pH 5.0. After readjusting the pH to 5.0, the solution was applied to a column (4.6 × 32.5 cm) of DEAE-Sephadex A-50 previously equilibrated with the same buffer. After washing the column with the starting buffer, the column was equilibrated with 0.2 M NaCl/50 mM sodium citrate buffer, pH 5.0, followed by the application of a linear gradient of NaCl (0.2—0.45 M). Active fractions were pooled (670 ml) and proteins were precipitated by adding 514 g $(NH_4)_2SO_4$. Step 4: The precipitate was taken up in 100 ml of 0.1 M NaCl/50 mM sodium citrate buffer, pH 5.0. The solution was divided into two equal parts and each of them was applied to a column (5.0 × 80 cm) of Sephadex G-100 previously equilibrated with the same buffer. The elution pattern is shown in Fig. 1.

Results and Discussion

Purification of the acid carboxypeptidase. Table I summarizes the purification procedures of the enzyme. By this method, approx. 1.7 g of highly purified enzyme could be obtained from 40 l of the starting supernatant with a yield of 34%. In the last step of purification on a column of Sephadex G-100 (Fig. 1), there is a shoulder on the ascending side of the eluted peak. As to the specific activity toward Bz-Arg-OEt, the values remained constant over the entire region of the eluted peak including the shoulder region. When the specimens from the shoulder region (fraction No. 53) and the main peak region (fraction No. 71) were examined by SDS-polyacrylamide gel electrophoresis, no differences could be detected throughout the peak region (Fig. 1, right inset), suggesting an artifactual basis for the shoulder caused by the column operating on a large scale.

Purity of the purified enzyme. The symmetrical nature of the schlieren pattern of the purified enzyme (Fig. 1, left inset) suggests a high degree of homogeneity and s value of 6.6 S (0.71% of the enzyme). The purified specimen gave a single but a rather diffuse band on polyacrylamide gel electro-

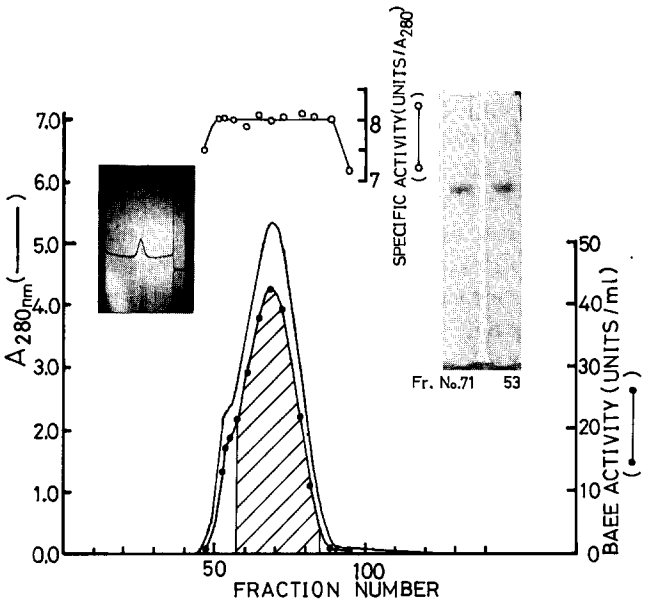

Fig. 1. Purification of acid carboxypeptidase (Step 4: gel filtration on a column of Sephadex G-100). Column size, 5.0 × 80 cm; fraction volume, 12.3 ml; eluant, 0.1 M NaCl/50 mM sodium citrate buffer, pH 5.0. BAEE; Bz-Arg-OEt. In the figure, SDS-polyacrylamide gel electrophoresis patterns of effluent fractions No. 53 and No. 71 (Right inset) and a sedimentation pattern of the enzyme recovered from the main peak fractions in the figure (No. 57—No. 85) (Left inset) are also shown. In the latter case, the pattern was obtained after 45 min centrifugation at 60 000 rev./min, 25.7°C; bar angle, 70°.

phoresis at pH 8.3 (Fig. 2A). However, in the presence of 8 M urea, the band became far sharper (Fig. 2B). This specimen also gave a single sharp band on SDS-polyacrylamide gel electrophoresis (Fig. 2C). By applying specific stainings for protein and carbohydrate on electrophoresed gels (Fig. 2D and E, respectively), the stained bands showed that both types of residues comigrated, suggesting the presence of a glycoprotein. As shown in Fig. 3 the purified enzyme showed only one peak on isoelectric focusing at pH 4.1.

Stability of the acid carboxypeptidase. The purified enzyme was stored for 2 h at pH 3—6 (37°C) and for 12 h at pH 3—8 (4°C). No decrease in residual

TABLE I

SUMMARY OF PURIFICATION OF ACID CARBOXYPEPTIDASE

Step	Volume (ml)	Total A_{280}	Total Activity (units)	Specific Activity (units/A_{280})	Yield (%)	Purification (-fold)
60% $(NH_4)_2SO_4$ supernatant	40 000	3690 000	34 400	0.009	—	—
100% $(NH_4)_2SO_4$ precipitate	5 040	244 000	53 900	0.22	100	1
20% polyethylene glycol-6000 ppt.	600	16 884	38 130	2.26	70.9	10.3
DEAE-Sephadex A-50	670	3 109	24 013	7.72	44.7	35.1
Sephadex G-100	715	2 280 (approx. 1.7 g)	18 394	8.07	34.2	36.7

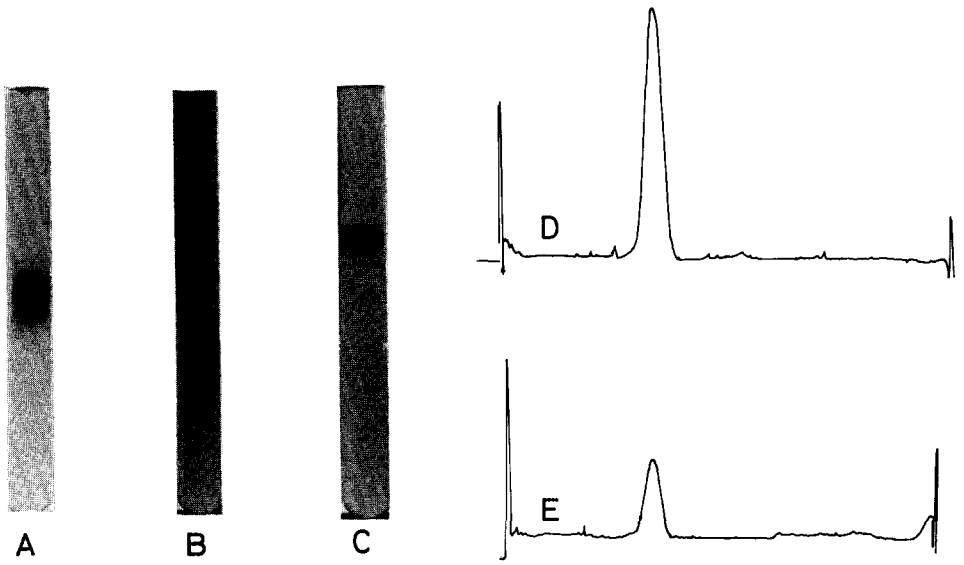

Fig. 2. Polyacrylamide gel electrophoresis of the final preparation of acid carboxypeptidase. Polyacrylamide gel electrophoresis was performed in 7.5% gels at pH 8.3 in the absence (A) and presence of 8 M urea (B). SDS-polyacrylamide gel electrophoresis was carried out in 5% gels with enzyme preparations treated with 2% SDS in the presence of 5% β-mercaptoethanol at 37°C overnight (C). One of the two separate and parallel runs of SDS-polyacrylamide gel electrophoresis was stained by Coomassie brillant blue R-250 to reveal a protein band (D) and another gel was treated with periodic acid-Schiff's reagent to detect carbohydrate band (E), and the densitometric scans at 570 nm were compared to demonstrate that the enzyme is a glycoprotein.

activity could be detected. In addition, the enzymatic activity appeared stable after incubation for 10 min at 55°C and pH 5.0; however, at 60°C, rapid inactivation occurred under the same conditions. The acid carboxypeptidase activity could be recovered following lyophilization, but some loss in specific activity was evident.

Molecular weight of the acid carboxypeptidase. Fig. 4A shows a representative result obtained by low-speed sedimentation equilibrium in both H_2O and ^2H_2O. The molecular weight and the partial specific volume of the enzyme were calculated to be 136 000 and 0.701 (ml/g), respectively. The results obtained by high-speed sedimentation equilibrium gave a molecular weight of 137 000, although the value is a little less accurate than that obtained in low-speed runs. The partial specific volume calculated from the amino acid and carbohydrate compositions was 0.704.

In order to determine the molecular weight of the enzyme in denaturing solvents, a reduced-carboxymethylated enzyme specimen was subjected to sedimentation equilibrium analyses in 6 M guanidinium chloride. The results of low-speed centrifugation, typically shown in Fig. 4B, again indicate the homogeneous nature of the specimen. The partial specific volume of the enzyme in 6 M guanidinium chloride was calculated by the method of Lee and Timasheff [31], assuming that each residue of carbohydrate binds 1 molecule guanidinium chloride. This calculated value of 0.688 yielded a molecular weight of

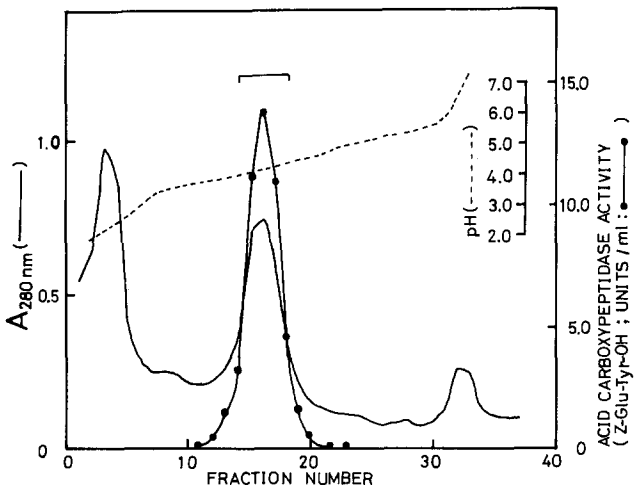

Fig. 3. Isoelectric focusing of acid carboxypeptidase. The purified enzyme (6.7 mg) was subjected to isoelectric focusing in 1% Ampholine of pH range 3—6. After electrophoresis for 40 h at 600—750 V and 1.2°C, 3.2-ml fractions were collected and each fraction was monitored by absorbance at 280 nm and peptidase activity toward Z-Glu-Tyr-OH.

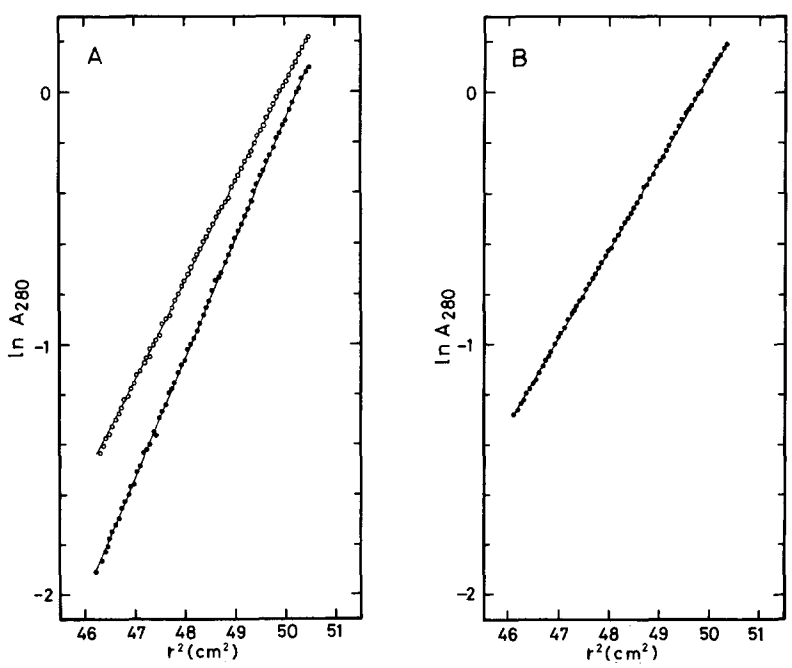

Fig. 4. Sedimentation equilibrium of acid carboxypeptidase. (A) Sedimentation equilibrium run in a non-denaturing solvent. A_{280} is absorbance at 280 nm; r denotes the distance from the axis of rotation; ●——●, plot in H_2O; ○——○, plot in 81.8% ^2H_2O. Centrifugation was performed at 16.6°C at an enzyme concentration of 0.029% (in H_2O) or 0.038% (in ^2H_2O). An An-G Ti rotor was operated at the speed of 7200 rev./min for 48 h. (B) Sedimentation equilibrium run in 6 M guanidinium chloride. Centrifugation was performed at 16.8°C with a 0.040% protein at a speed of 11 000 rev./min for 42 h.

60 000 at low-speed centrifugation and a value of 61 000 at high-speed centrifugation.

Behavior of the acid carboxypeptidase in SDS-polyacrylamide gel electrophoresis. An estimation of the molecular weight of the enzyme was also attempted using SDS-polyacrylamide gel electrophoresis. On 5% gels, the enzyme migrated as a single band with an apparent molecular weight of 82 000 (Fig. 5). The mobilities remained essentially the same, even if β-mercaptoethanol was omitted during incubation or the enzyme was reduced and carboxymethylated before electrophoresis. On the other hand, with 10% gels, the estimated molecular weight was 73 000. Thus, it was clear that the apparent molecular weight varied depending on the gel concentrations. Fig. 5 shows plot of the relative mobilities (R_F) of four proteins vs. the gel concentration (T) (Ferguson plot). Extrapolation of R_F values to $T = 0$ for three typical marker proteins demonstrated that they share a nearly common and unique R_F value at $T = 0$ (R_{F0}) as reported by Hayashi et al. [32]. On the other hand, the plot for the enzyme exhibited a quite different line giving a much lower R_{F0} value.

Segrest and Jackson [33] have pointed out that the apparent molecular weights of some glycoproteins determined by SDS-polyacrylamide gel electrophoresis method are often unreliable. Since the enzyme is a glycoprotein of high carbohydrate content as described below, it seems likely that an anomalous Ferguson plot was obtained.

Amino acid and carbohydrate analysis. The amino acid and carbohydrate composition of the enzyme are summarized in Table II. The total amino acids were estimated to be 955. Carbohydrates account for 22% of the dry weight of the enzyme. No free sulfhydryl group was titrated, when examined by the use of Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) as described by Habeeb [35].

End-group analyses. In NH_2-terminal amino acid analyses of the enzyme by the dinitrophenylation-method and the Edman method, 0.69 mol leucine and

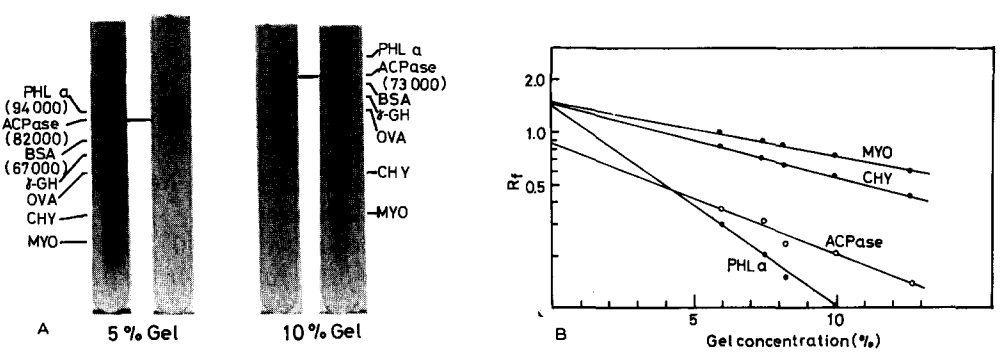

Fig. 5. SDS-polyacrylamide gel electrophoresis of acid carboxypeptidase and marker proteins. The electrophoretic patterns of the enzyme were compared with those of marker proteins in 5 and 10% gels (left). The values in parentheses denote molecular weights. Comparison of the Ferguson plot of the enzyme with those of three marker proteins (right). PHL *a*, phosphorylase *a*; (M_r = 94 000); BSA, bovine serum albumin (M_r = 68 000); γ-GH, heavy chain of γ-globulin (50 000); OVA, ovalbumin (45 000); CHY, chymotrypsinogen (25 000); MYO, myoglobin (17 800). ACPase; acid carboxypeptidase.

TABLE II

AMINO ACID AND CARBOHYDRATE COMPOSITION OF ACID CARBOXYPEPTIDASE FROM *Aspergillus niger* var. *macrosporus*

Residues	Content (g/100 g glycoprotein)	Residues * molecule
Asp	10.53	124.4
Thr **	4.02	58.7
Ser **	4.68	77.2
Glu	6.61	69.6
Pro	5.94	83.2
Gly	3.32	79.2
Ala	2.91	55.6
Half-Cys	0.83	11.0
Val	3.62	49.6
Met	1.26	13.1
Ile	4.24	51.0
Leu	6.97	83.8
Tyr	7.29	60.8
Phe	6.24	57.7
Lys	2.10	22.3
His	1.46	19.9
Arg	1.93	1.69
Trp	2.86	20.9
Total	77.35	954.9
GlcN	3.5	29.5
Man	16.6	140.1
Gal	1.6	13.6
Total	21.6	183.2

* Calculations were based on a molecular weight of 136 000.
** Values of Thr and Ser were corrected for losses of 5% and 10% during hydrolysis [34].

0.19 mol threonine/mol enzyme (M_r = 136 000) were identified. In the case of carboxyl-terminal amino acid analyses by carboxypeptidase A digestion, 2 mol serine and about 1.6 mol tyrosine and threonine/mol enzyme were rapidly released during the first 1 h. Even after prolonged incubation, further release of amino acids was found to be very slow.

The results of physicochemical studies on the acid carboxypeptidase suggest that the enzyme is composed of two identical subunits. Carboxyl-terminal analyses supported this idea. In our accompanying paper [36], we show the presence of two diisopropylphosphorofluoridate-reactive sites per molecule of the enzyme (M_r = 136 000), strongly suggesting the dimeric structure for the enzyme. In the case of NH_2-terminal analysis, a small degree of heterogeneity and low recovery of NH_2-terminal amino acid were observed.

Acid carboxypeptidases with molecular weight above $1 \cdot 10^5$, have been reported. The enzymes from *A. saitoi* (M_r = 139 000, monomer 51 000) [8] and from *Penicillium janthinellum* (penicillocarboxypeptidase II, M_r = 128 000—140 000, monomer 65 000) [9] possess a molecular nature which may be similar to the acid carboxypeptidase reported in our study.

Acknowledgements

The authors (I.K. and M.Y.) express their thanks to Mr. Y. Yamada and Mr. K. Yoshida of Central Research Laboratory of Meiji Seika Ltd., Yokohama, for

their considerations to this work and the kind supply of crude enzyme materials. The authors are indebted to Professor K. Iso and Dr. K. Watanabe of University of Tokyo for sedimentation velocity analyses, to Professor T. Osawa and Dr. S. Toyoshima of University of Tokyo for their instruction in neutral sugar analyses and to Dr. K. Nomura of the Tokyo Metropolitan Institute of Gerontology for amino acid analyses.

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