SEPARATION OF PROTEOLYTIC ENZYME INHIBITORS FROM THE POTATO AND THEIR CHARACTERISTICS

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Two homogeneous polypeptides and two homogeneous proteins with antiproteolytic activities were isolated from the Epoka variety of potato. These substances differ in their electrophoretic mobility, molecular weight, amino acid composition and specificity of their action on proteolytic enzymes.

The molecular heterogeneity of vegetable proteolytic enzyme inhibitors is a common phenomenon. It is found among others in inhibitors isolated from the bean, soya bean[1], the *Phaseolus lunatus*[2] and other plants[3-5]. Several authors have also found that various potato proteins possess an antiproteolytic activity[6-8]. In our investigation on the potato proteolytic enzyme inhibitors a crude preparation containing all the peptide and protein inhibitors present in the Epoka variety of potato was used as an initial material[9].

From this preparation a fraction was isolated previously which consisted of a mixture of four proteins of an antiproteolytic action[10]. This fraction inhibits trypsin, chymotrypsin, plasmin, cathepsin A activity and the activation of prothrombin by plasma thromboplastin. It does not inhibit the activity of cathepsin B_1, C and D, the activation of plasminogen by urokinase and streptokinase, the activation of prothrombin by tissue thromboplastin and thrombin activity[11,12]. The same crude preparation was used for the isolation of homogeneous inhibitors in the investigations presented in this paper. The chemical characteristics of these inhibitors were given and their specific action on various proteolytic enzymes were investigated.

EXPERIMENTAL

Materials

A crude preparation of the potato proteinase inhibitor was obtained by the method described earlier[9]. Bovine trypsin, crystallized twice and chymotrypsin A, crystallized 3-times, "carboxypeptidase A ex bovine pancreas" and N-α-carbobenzoxy-L-glutamyl-L-tyrosine, were produced

by Koch-Light Laboratories Ltd., England. Plasmin was a product of Novo Industri A/S, Copenhagen, Denmark, and casein of BDH, England. Cathepsin A was obtained from hen muscle according to the method given by Iodice and coworkers[13]. N-benzoylglycyl-L-phenylalanine, and ovalbumin were commercial products of Mann Research Laboratories, Inc., New York, USA, human blood serum albumin of Behringwerke, West Germany, cytochrome c of Biomed, Cracow, Poland, and Trasylol of Bayer, West Germany. Pletelet-poor plasma was obtained by centrifugation (3000 g for 20 min) of dog blood collected in 0·1M sodium citrate in proportion 9 : 1. SE-Sephadex C-50 and Sephadex G-100 were products of Pharmacia, Uppsala, Sweden: CM-cellulose of Whatman, England, and dialyser membranes of Kalle Aktiengesellschaft, Wiesbaden-Biedrich, West Germany.

Chromatography of the Crude Inhibitor Preparation on SE-Sephadex C-50

50 mg of the crude inhibitor preparation was dissolved in 2·5 ml 0·05M acetate buffer at pH 3·6 placed on a column filled with SE-Sephadex C-50 equilibrated with the same buffer (Fig. 1). Absorption at 280 nm was measured using Beckman DU (model 2400) spectrophotometer, the quantity of proteins was calculated from the formula given by Kalckar[14]: protein (mg/ml) = $= (A_{280} \cdot 1{\cdot}45) - (A_{260} \cdot 0{\cdot}74)$. The fractions containing proteins were dialysed against 100 volumes of distilled water for 6 hours and the antitrypsin activity was determined. Fractions forming peaks 4 and 5 (Fig. 1) containing antiproteolytic activity, were pooled and evaporated in a vacuum evaporator.

Chromatography of the Material from the 4th and 5th Peak on CM-cellulose

40 mg of proteins from the 4th or 5th peak obtained in the first stage were placed on the column filled with CM-cellulose equilibrated with 0·05M acetate buffer at pH 3·6 (Figs 2, 3). The proteins were eluted from the column with linearly increasing concentrations of NaCl (0·0—1·0M). 3 ml fractions were collected at 10 minute intervals. The absorption at 280 nm and electrical conductivity of the various fractions were determined in order to check the NaCl gradient. The fractions containing proteins were dialyzed against 100-times volume of distilled water for 6 hours and the

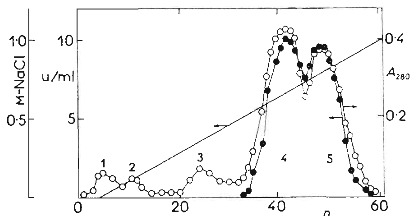

Fig. 1

Chromatography of the Crude Preparation Potato Inhibitor on SE-Sephadex C-50 (2 . 8 cm)

Linear NaCl-gradient in 0·05M acetate buffer, pH 3·6 is designated by a slanting line. Fractions 5 ml in 10 minute intervals. ● optical density, ○ antitrypsin activity.

antitrypsin activity was determined. The fractions with antiproteolytic activity were pooled and represent active material of peaks 4a, 4b and 5a, 5b, respectively (Fig. 2, 3).

Characterization of the Substances Obtained

Electrophoresis on polyacrylamide gel (Fig. 4) was carried out by the method of Reisfeld and coworkers[15]. 7·5% separating gel was used. 0·5 mg protein in 100 μl of the solution was put on the gel. The electrophoresis was carried out in a β-alanine and acetic acid buffer at pH 4·5. The current intensity on the tube was 10 mA and the separation time was 20 minutes. The electropherograms were dyed with 1% amide black solution in 7% acetic acid for 1 hour. 7% acetic acid was used for decolorization.

Determination of molecular weights on Sephadex G-100 followed the Andrews method[16]. The Sephadex was suspended in 0·05M-NH_4HCO_3 (pH 8·2) and filled to form chromatographic columns 1·5 . 50 cm. One ml samples containing 3 mg of standard proteins and the proteins investigated were placed on the column. 1·5 ml fractions were collected at 10 minute intervals. The elution of the proteins from the column was followed by measuring the absorption at 280 nm and in the case of cytochrome c by measuring the absorption at 540 nm. A calibration curve of the dependence of the elution volumes on the logarithm of the molecular weight was prepared and the molecular weights of the various fractions of the proteinase inhibitor were found by using interpolation (Fig. 5).

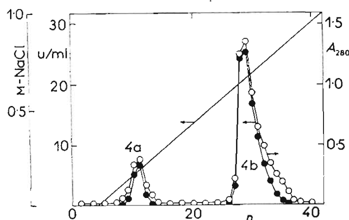

FIG. 2

Chromatography of Fraction 4 Obtained from SE-Sephadex on CM-Cellulose Column

For explanation see Fig. 1.

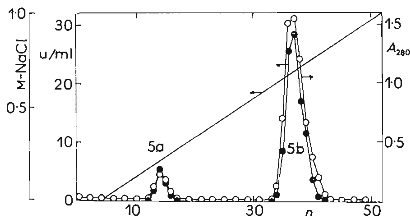

FIG. 3

Chromatography of Fraction 5 Obtained from SE-Sephadex on CM-Cellulose Column

For explanation see Fig. 1

Absorption spectrum of the fractions isolated was determined in a ultraviolet light at 215 to 360 nm on a Unicam SP 800 spectrophotometer. The protein concentration was 0·25 mg/ml. A 0·05M-NH_4HCO_3 (pH 8·2) was used as a solvent.

Paper chromatography of hydrolyzed (for 12 hours in 6M-HCl at 110°C) and unhydrolyzed fractions was carried out on Whatman No 3 paper in a solvent system[17] of n-butyl alcohol–pyridine–acetic acid water (15 : 10 : 3 : 12). 20 μl samples containing 0·1 mg of the material investigated or of the standard amino acid mixture (0·05 μmol of each amino acid) were placed on the paper. The migration speed of this material was checked by means of the spot of neutral red. The chromatogram was detected with 0·2% ninhydrin in acetone and fixed[18] in 1% solution of $Cu(NO_3)_2$ in acetone.

The quantitative amino acid composition was determined after 20 hours' hydrolysis in 6M-HCl at 110°C in sealed evacuated tube by means of an automatic amino acid analyzer Beckman--Spinco, type 120, according to Spackman and coworkers[19].

Analysis of N-terminal amino acid were performed by the dansylation method[20–22].

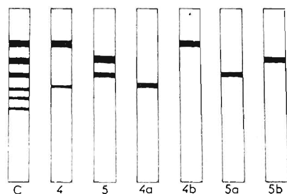

FIG. 4

Electrophoretic Patterns of the Inhibitors Investigated

C crude potato proteinase inhibitor, 4 and 5 fractions obtained by SE-Sephadex C-50 chromatography (Fig. 1), 4a, 4b, 5a and 5b fractions obtained by CM-cellulose chromatography (Fig. 2, 3).

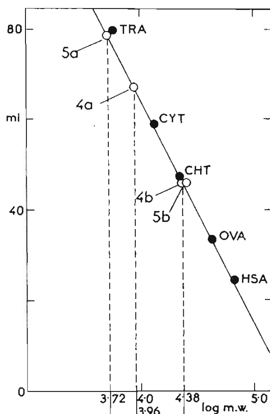

FIG. 5

Determination of the Molecular Weight According to[16] Using Sephadex G-100 (1 . 100 cm) Column

HSA human serum albumin, OVA ovalbumin, CHT chymotrypsin, CYT cytochrome and TRA trasylol. 4a, 4b, 5a and 5b potato proteinase inhibitor fractions obtained by CM-cellulose chromatography (Fig. 2, 3). The resulting molecular weights are 5200 (5a), 9100 (4a) and 24000 (4b, 5b).

Measurement of Inhibition

The antitrypsin, antichymotrypsin and antiplasmin activity was determined by using casein as substrate. 0·5 ml of the tested fraction (or 0·5 ml of the borate buffer, pH 7·6, for blank tests) were added to 0·5 ml of the enzyme solutions (0·005% trypsin, 0·01% chymotrypsin or 0·004% plasmin), then incubated 3 minutes at a temperature of 37°C and 1 ml of the 2% casein solution was added. The incubation time for determination of the activity of trypsin and chymotrypsin was 10 minutes and for determination of the plasmin activity 60 minutes. Undegraded proteins were then precipitated by means of 5 ml trichloroacetic acid (20%), and tyrosine was determined in the clear filtrate using the Folin-Ciocalteu reagent[23].

The effect of isolated fractions of the potato proteinase inhibitors on cathepsin A activity was tested using N-α-carbobenzoxy-L-glutamyl-L-tyrosine as substrate[13]. 0·5 ml of inhibitor fraction (or 0·5 ml of 0·1M acetate buffer (pH 5·0) in the blank test) was added to 0·5 ml of the enzyme (2·5 mg/ml) and the mixture was incubated for 3 minutes at 37°C. 1 ml of 0·0125M N-α-carbobenzoxy-L-glutamyl-L-tyrosine in 0·1M acetate buffer (pH 5·0) was then added and incubated for one hour. Hydrolysis was stopped by adding 1 ml of 10% trichloroacetic acid after which the samples were heated for 10 minutes at 55°C. After removing the precipitate by centrifugation (1 000 *g*, 10 minutes) the supernatant was diluted 1 : 10 with distilled water and the α-amino-nitrogen was determined by means of a ninhydrin reagent[24].

The effect of the potato proteinase inhibitors on carboxypeptidase A activity was tested by means of the spectrophotometric methods of Folk and Schirmer[25] using N-benzoylglycyl-L-phenylalanine as a substrate. 0·5 ml of the fraction investigated (or 0·5 ml 0·9% NaCl in the blank experiments) was added to 0·5 ml carboxypeptidase A (0·01%), incubated for 3 minutes at a temperature of 25°C, 1 ml of a 1 mM solution of N-benzoylglycyl-L-phenylalanine in 0·02M Tris-HCl buffer containing 0·5M-NaCl (pH 7·5) was added and after 30 minutes' incubation the absorption at 254 nm was measured.

The antitrypsin activity of the inhibitors was expressed in these units: The amount of the inhibitor which caused the 50%-inhibition of the release of 1 μg of tyrosine for one minute under standard conditions was taken to be one inhibitor unit. The unit was calculated as described in our previous work[26]. In the remaining cases, the results were expressed in percentage of the activity-diminishing of the enzymes investigated. The percentage was calculated on the basis of the difference in quantity of products released in the experiments and blank tests, assuming the enzyme activity in the latter to be 100%.

The effect of the potato proteinase inhibitors on the blood plasma recalcination time was investigated in a system consisting of 0·1 ml plasma and 0·1 ml inhibitor fractions (or 0·1 ml 0·9% NaCl in the blank experiment). After adding 0·2 ml of 0·025M-$CaCl_2$ the clotting time at 37°C was measured in water bath.

RESULTS

From the crude preparation of the potato proteinase inhibitor five protein peaks were obtained by means of ion-exchange chromatography on SE-Sephadex C-50 (Fig. 1). Only the 4th peak, which eluted with 0·65M-NaCl, and the 5th peak, eluted with 0·74M-NaCl, possessed antitrypsin activity. Electrophoresis on polyacrylamide gel (Fig. 4) showed that the proteins of these peaks were non-homogeneous. The 4th peak had two protein zones with a mobility corresponding to the 1st and 4th protein spot of the crude preparation. The 5th peak was also non-homogeneous and consisted

of two protein zones of an electrophoretic mobility corresponding to the 2nd and 3rd spot of the crude preparation. The proteins in the 4th peak were separated by chromatography on CM-cellulose into 2 fractions 4a and 4b (Fig. 2). The proteins of the 5th peak also separated on CM-cellulose into 2 fractions 5a and 5b (Fig. 3).

The pooled fractions 4a, 4b, 5a and 5b obtained by chromatography on CM-cellulose were found to possess antitrypsin activity. The protein balance and antitrypsin activity of the fractions obtained by the first chromatography and rechromatography are given in Table I. The pooled fractions listed in the Table do not differ essentially in their antitrypsin specificity. All fractions obtained by rechromatography on CM--cellulose were found to be homogeneous in electrophoresis on polyacrylamide gel at pH 4·5 (Fig. 4). The results of the gel chromatography on Sephadex G-100 (Fig. 5) also indicate the homogeneity of these fractions since single, symmetrical protein peaks differing from each other in their elution volume were obtained. The molecular weights were calculated from the elution volumes and were about 9100 for fraction 4a, about 5200 for fraction 5a about 24000 for fraction 4b and 5b (Fig. 5). The absorption maxima for the inhibitor fractions in ultraviolet were within the range of 270 to 280 nm usual in proteins and peptides and do not indicate unusual UV-chromophore.

Paper chromatography of the unhydrolyzed inhibitors 4a and 5a gave single spots with a localization corresponding to aspartic acid and arginine. After acid hydrolysis a mixture of free amino acids has been obtained. These findings indicate the peptide character of these substances. Inhibitors 4b and 5b, on the other hand, gave one

TABLE I

Potato Proteinase Inhibitors Obtained from the Crude Preparation by Means of Chromatography on SE-Sephadex C-50 and Rechromatography on CM-Cellulose Columns

After separation on	Pooled fractions	Volume ml	Protein		Antitrypsin activity[a]	
			mg/ml	total, mg	u/ml	protein, u/mg
SE-Sephadex C-50	4	50·0	0·25	12·50	5·2	20·8
	5	55·0	0·27	14·80	6·8	25·2
CM-cellulose	4a	15·0	0·14	2·10	3·8	27·1
	4b	27·0	0·88	23·80	22·6	25·6
	5a	12·0	0·08	0·96	2·2	27·5
	5b	24·0	1·00	24·00	26·8	26·8

[a] The definition of the inhibition unit u see in the text.

ninhydrin spot on the starting line, characteristic for proteins. After hydrolyis, many spots with a localization corresponding to single amino acids were obtained.

Table II presents the specificity of the antiproteolytic activity of the inhibitor fractions obtained. All the fractions inhibit the activity of trypsin, chymotrypsin and plasmin. The carboxypeptidase A activity is inhibited only by fraction 5a. Cathepsin A activity is inhibited by fractions 4b and 5b. Recalcination time, on the other hand, was extended by fractions 5a and 5b.

The inhibitors 4b and 5b appear to be the most interesting in view of their inhibiting cathepsin A activity. The amino acid composition of these inhibitors has been determined and the N-terminal amino acid has been identified (Table III). The amino acid composition of both the inhibitors has a high content of lysine, acid amino acid and leucine residues. The 4b inhibitor contains more cystein residues and less valine residues than the 5b inhibitor. The N-terminal amino acid of both inhibitors was found to be arginine.

DISCUSSION

In the potato of the Epoka variety there are two peptide and two protein proteinase inhibitors with antitryptic and antichymotryptic activity. They have been designated

TABLE II

The Specificity of the Potato Proteinase Inhibitors

Result are given in % of activity or in seconds of clotting time.

Enzyme	Substrate	Without inhibitor	With inhibitor[a]			
			4a	4b	5a	5b
Trypsin	casein	100·0	41·7	6·1	51·1	4·4
Chymotrypsin	casein	100·0	42·2	5·3	60·0	3·6
Plasmin	casein	100·0	31·0	8·0	44·2	3·4
Cathepsin A	N-carbobenzoxy--L-glutamyl-L-glutamyl-L-tyrosine	100·0	92·0	26·0	100·0	2·0
Carboxypeptidase A	N-benzoylglycyl--L-phenylalanine	100·0	100·0	100·0	24·6	100·0
Recalcination time[b]	platelet-poor plasma	80·0	75·0	86·0	320·0	380·0

[a] End concentration of inhibitor: 4a 0·035 mg/ml, 4b 0·22 mg/ml, 5a 0·02 mg/ml and 5b 0·25 mg/ /ml. [b] The results are given in seconds of clotting time.

4a, 4b, 5a and 5b in order of their elution from ion exchange columns and differ in their specificity toward other peptidases (cathepsin A, carboxypeptidase A and B) and in their action on the intrinsic thromboplastic generation system.

The findings of these investigations have confirmed the molecular heterogeneity of the proteolytic enzyme inhibitors of the Epoka potato demonstrated in our previous investigations[8,10]. Fraction 4a has molecular weight similar to that of the inhibitor isolated by Kiyohara and coworkers[27]. The molecular weight of inhibitor 5a is similar to that of the inhibitor described by Ryan and coworkers[28] an like that inhibitor it also inhibits carboxypeptidase A activity. Fractions 4b and 5b have

TABLE III
Amino Acid Composition of Inhibitor 4b and 5b

Amino acid	Number[a] of mol per mol of	
	inhibitor 4b	inhibitor 5b
Lysine	11	15
Histidine	2	3
Arginine	6	7
Aspartic acid	23	26
Threonine	9	10
Serine	15	12
Glutamic acid	12	14
Proline	10	16
Glycine	19	21
Alanine	7	7
Half-cystein	5	1
Valine	14	20
Methionine	1	2
Isoleucine	9	12
Leucine	17	20
Tyrosine	6	8
Phenylalanine	9	10
Tryptophan	1–2	1
Number of amino acid residues	176–7	205
N-Terminal amino acid	arginine	arginine

[a] Calculated on the basis of a molecular weight of 24000 for both inhibitors.

a molecular weight similar to that of the inhitors obtained by Hochstrasser and coworkers[7]. The N-terminal amino acid of the inhibitors 4b and 5b is arginine. Potato inhibitors containing N-terminal arginine were also reported by Belitz and coworkers[6]. The N-terminal amino acid of the inhibitors isolated from potato by Iwasaki and coworkers[29] was, however, found to be alanine and the inhibitor isolated by Kiyohara and coworkers[27] and Melville and Ryan[30] had glutamic acid molecules on the N-terminal. The 4b and 5b inhibitors differ in their amino acid composition, not only between themselves but also from the inhibitors isolated from other potato varieties by other authors[27,29,30]. This evidences the great molecular heterogeneity of the potato proteinase inhibitors both within the same variety of potato and among other varieties.

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