CHEMICAL CHARACTERIZATION OF CATHEPSIN D INHIBITOR FROM POTATOES

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The cathepsin D inhibitor, isolated in one of our earlier studies, has been subjected to chromatography on SE-Sephadex C-25 at pH 3 and thus resolved from a trypsin inhibitor lacking anticatheptic activity. The cathepsin D inhibitor purified by this procedure was homogeneous as evidenced by disc electrophoresis, sedimentation analysis in the ultracentrifuge ($s_{20,w} = 2{\cdot}9$ S), and N-terminal end group analysis showing glutamic acid to be the only N-terminal amino acid. The amino acid composition of the inhibitor was determined; the molecular weight of the inhibitor calculated from this composition (21 790) is in agreement with the data of sedimentation analysis (20 830 $\pm$ 420). The N-terminal 17-residue sequence of the inhibitor determined by Edman stepwise degradation is the following: Glu-Ser-Pro-Leu-Pro-Lys-Pro-Val-Leu-Asp-X-Asn--Gly-Lys-Glu-Leu-Gln.

The isolation of a cathepsin D inhibitor from potatoes and its partial characterization have been described in our earlier studies[1,2]. This inhibitor is the only naturally occurring high molecular weight cathepsin D inhibitor isolated so far. It inhibits in the acid pH-range the proteolytic activity of cathepsin D, an intracellular protease of the carboxyl type, yet is without any effect on other proteases of this type, such as cathepsin E, hog and chicken pepsin, and chymosin (rennin). The inhibitor also inhibits two serine proteases, trypsin and chymotrypsin in the alkaline pH-range. This specificity of inhibition in both the acid and alkaline pH-range is retained in preparations of potato cathepsin D inhibitor obtained by dissociation of its complex with either cathepsin D or trypsin.

This study was designed to purify further the inhibitor and to provide its chemical characteristics. We endeavored to determine whether the inhibitor isolated by us is either an entirely new inhibitor so far unknown or whether we merely discovered cathepsin D inhibiting activity in one of the potato trypsin inhibitors described earlier.

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EXPERIMENTAL

Material

Potatoes (*Solanum tuberosum*) were of common commercial origin. Cathepsin D (EC 3.4.23.5) was prepared from bovine spleen in this Laboratory according to ref.[3] or by affinity chromatography on a haemoglobin-Sepharose column according to Smith and Turk[4]. Trypsin (EC 3.4.21.4) was purchased from Léčiva, Prague. N-α-benzoyl-D,L-arginine-*p*-nitroanilide was synthetized in this Institute. Sephadex G-25 and SE-Sephadex C-25 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Chemicals used for sequential degradation were supplied by the manufacturer of the instrument (Beckman Instruments, Spinco Div., Palo Alto, California, USA). Coomassie Brillant Blue G-250 was from Serva, Heidelberg, FRG. All the remaining chemicals used were of analytical purity grade. Silufol, thin-layer chromatography sheets were from Kavalier-Sázava, Czechoslovakia.

Methods

Assay of enzymatic activities. The proteolytic activity of cathepsin D was assayed with 2% hemoglobin as substrate at pH 3·5 and 40°C for 30 min, trypsin activity was determined in terms of cleavage of N-α-benzoyl-D,L-arginine-*p*-nitroanilide. The modification of both methods was described earlier[1]. The inhibition of the enzymic activity was effected as described in the preceding paper[1]. The enzyme solution with the inhibitor solution were preincubated 10 min at ambient temperature in a buffer whose pH corresponded to the pH optimum of the enzyme used. McIlvain buffer pH 3·5, was used for cathepsin D and 0·1M Tris-HCl buffer, pH 8·0 containing 0·001M-$CaCl_2$ for trypsin.

Amino acid analysis. Amino acid analyses were performed on a Beckman Spinco, Model 120 B Amino acid analyzer according to Benson and Peterson[5]. Samples were hydrolyzed in 5·7N-HCl at 110°C in evacuated sealed tubes for 20 and 70 hours. Tryptophan was determined according to Liu and Chang[6]. Cysteine was determined as cysteic acid after oxidation by performic acid[7,8]. The dry weight, ash and nitrogen content (Kjeldahl) of samples of potato cathepsin D inhibitor were determined.

Automatic sequential degradation. Native potato cathepsin D inhibitor (6 mg, ~0·25 μmol) and the performic acid oxidized sample of the inhibitor were subjected to Edman degradation[9] in Beckman Model 890 C Sequencer. The degradation proceeded according to the "Fast Quadrol Program" recommended by the manufacturer. The phenylthiohydantoins of the amino acids obtained by the conversion of the thiazolinones were identified by thin-layer chromatography on Silufol sheets[9,10] and by gas chromatography in Beckman Model GC-65 Gas Chromatograph, either as such of after their sililation[11].

The determination of sedimentation coefficient was effected by the schlieren method in Spinco Model E Ultracentrifuge. The molecular weight was determined by the sedimentation equilibrium method in the long column according to Chervenka[12] in the same instrument.

Disc electrophoresis was carried out in 7·5% polyacrylamide gel in Tris-glycine buffer, pH 8·3, or in β-alanine-acetic acid buffer, pH 4·0 in the instrument (GE 4) manufactured by Pharmacia Fine Chemicals, Sweden. Protein zones were detected by Coomassie Brillant Blue G-250 (ref.[13]).

Chromatography on SE-*Sephadex* C-25. SE-Sephadex C-25 was equilibrated with 0·01M sodium formate buffer pH 3·0 (adjusted by formic acid). The inhibitor sample (30 mg, obtained by chromatography on DEAE-Sephadex) was dissolved in 5 ml of the same buffer and applied to the SE-Sephadex column. After application of the sample a linear elution gradient of NaCl was

used (0—0·5M-NaCl, 100 ml + 100 ml). The chromatography was monitored by absorbance measurement at 280 nm. Aliquots of 100 and 10 µl were taken for the determination of inhibition of cathepsin D and trypsin, respectively.

RESULTS

In this study an additional purification of the inhibitor isolated earlier was achieved by chromatography on SE-Sephadex C-25 in 0·01M formate buffer, pH 3·0; a linear concentration gradient of NaCl was used for elution. The course of the chromatography is shown in Fig. 1. By using a higher molarity of NaCl we were able to separate from the cathepsin inhibitor another inhibitor which was without effect on cathepsin D. Fractions containing the cathepsin D inhibitor were pooled, desalted on a Sephadex G-25 column equilibrated with dilute acetic acid, and lyophilized.

The homogeneity of this preparation of cathepsin D inhibitor was proved by several methods. The preparation gave only one discrete zone when examined by disc electrophoresis at pH 4. One peak only was observed upon sedimentation analysis in the ultracentrifuge. The preparation was shown to contain only one N-terminal amino acid, glutamic acid, by the phenyl isothiocyanate method. All the remaining studies were carried out with the preparation characterized as described above.

Sedimentation analysis in the ultracentrifuge in 0·1M phosphate buffer at pH 7·7

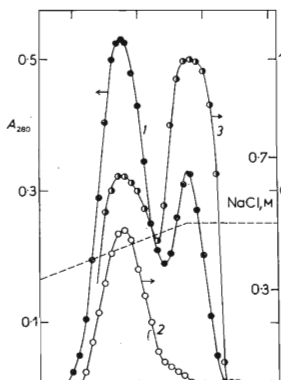

FIG. 1

Chromatography on SE-Sephadex C-25

The cathepsin D inhibitor from potatoes (30 mg) was applied onto a 0·9 × 12·5 cm column. Flow rate 3 ml/15 min. Initial buffer 0·01M Na-formate pH 3·0 Elution by a linear gradient of increasing concentration of NaCl (0—0·5M-NaCl, 100 + 100 ml). *1* Absorbance at 280 nm, *2* inhibition of proteolytic activity of cathepsin D in %, *3* inhibition of tryptic activity in %, *n* tube number, ------ molarity of NaCl (0—0·5M).

gave a molecular weight of 20880 ± 420 and a sedimentation coefficient $s_{20,w}$ = = 2·9 S. When the sedimentation coefficient of cathepsin D inhibitor was examined in the same buffer yet in the presence of 6M urea, the $s_{20,w}$ value decreased to 1·5 S.

The results of amino acid analysis are given in Table I. The cathepsin D inhibitor contains 199 amino acid residues according to this analysis and the molecular weight calculated from these data is 21 790.

Since the phenyl isothiocyanate method showed the presence of one N-terminal amino acid only, the cathepsin D inhibitor both native and oxidized in performic acid, was subjected to automatic sequential degradation. The following 17-residue sequence was determined: Glu-Ser-Pro-Leu-Pro-Lys-Pro-Val-Leu-Asp-X-Asn-Gly--Lys-Glu-Leu-Gln...

TABLE I

Amino Acid Composition of Cathepsin D Inhibitor from Potatoes

Amino acid	Amino acid μmol	Residue/mol	Nearest integer	g Amino acid residue/mol
Lysine	0·1865[a]	9·74	10	1 281·7
Histidine	0·0	0·0	0	0
Arginine	0·1565[a]	8·17	8	1 249·5
Aspartic acid	0·5047[a]	26·36	26	2 992·3
Threonine	0·1645[c]	8·59	9	910·0
Serine	0·2748[c]	14·35	14	1 219·0
Glutamic acid	0·2250[a]	11·75	12	1 549·4
Proline	0·3060[b]	15·97	16	1 553·8
Glycine	0·3642[a]	19·01	19	1 084·0
Alanine	0·1332[a]	6·95	7	497·6
Half-cystine	0·1160[b]	6·06	6	612·7
Valine	0·3060[e]	15·97	16	1 586·1
Methionine	0·0187[a]	0·98	1	131·2
Isoleucine	0·2340[e]	12·22	12	1 357·9
Leucine	0·4120[a]	21·51	22	2 489·5
Tyrosine	0·1535[d]	8·01	8	1 305·4
Phenylalanine	0·2275[d]	11·88	12	1 766·0
Tryptophan	0·0162	0·85	1	186·2
			199	21 772·4
				18
				21 790·4

[a] Mean value of two determinations of 20 h and two determinations of 70 h hydrolysates; [b] determined as cysteic acid on a sample oxidized in performic acid; [c] extrapolated to zero time of hydrolysis; [d] mean value obtained with 20 h hydrolysate; [e] mean value obtained with 70 h hydrolysate.

DISCUSSION

In spite of the fact that in earlier experiments we used a number of separation techniques for the isolation of the cathepsin D inhibitor (such as fractionation with ammonium sulfate, chromatography on Sephadex G-50, on DEAE-Sephadex A-25 at pH 7·6, on SE-Sephadex C-25 at pH 5, or by chromatography on Sepharose 4B with covalently coupled cathepsin D or trypsin as affinants), the resulting preparations always gave multiple zones[1] on disc electrophoresis at pH 8·3. When the preparation obtained by chromatography on DEAE-Sephadex was subjected to additional purification by chromatography on SE-Sephadex at pH 3, we were able to separate the cathepsin D inhibitor from a very potent inhibitor of trypsin; this inhibitor was inactive on cathepsin D. The inhibition of trypsin by the cathepsin D inhibitor prepared by chromatography on SE-Sephadex at pH 3 was markedly weaker than by the preparation not subjected to this purification step. The purity tests of this preparation show that it is homogeneous. The results of disc electrophoresis in polyacrylamide gel at pH 4 were confirmed by sedimentation analysis in the ultracentrifuge at pH 7·7. The homogeneity of the preparation is also evidenced by the presence of only one N-terminal end group, glutamic acid, and by the results of Edman sequential degradation In spite of that this homogeneous preparation gives one to two weak zones in addition to the main zone on disc electrophoresis at pH 8·3. We assume that this fact may be accounted for by the presence of other, minority forms of the cathepsin D inhibitor, forms which can be separated electrophoretically in the alkaline pH-range.

A great number of inhibitors showing different specificities have been isolated from potatoes[14]. The molecular weights of most of these inhibitors were originally determined as 20000 – 25000. It was shown later that the molecules of most of these inhibitors consist of two or four subunits of molecular weight 5000 – 10000 (ref.[15,16]). In certain cases the inhibitors dissociate to subunits in 6M guanidine[15], in other cases the subunits are formed as a result of formation of a complex of the inhibitor and the enzyme[16–18].

It has not been shown as yet whether the molecule of the cathepsin D inhibitor from potatoes also consists of subunits or not. We have merely observed that upon sedimentation analysis in the ultracentrifuge the sedimentation coefficient drops in the presence of urea from $s_{20,w} = 2{\cdot}92$ S to $s_{20,w} = 1{\cdot}5$S. This drop could be caused, among others, by dissociation of the cathepsin D inhibitor to subunits in the presence of urea. This problem as well as the isolation of the subunits (if any) will be dealt with in one of our future studies.

The amino acid composition of the cathepsin D inhibitor is given in Table I. The molecule of the inhibitor has three disulfide bonds and does not contain histidine. The molecular weight calculated from the amino acid composition is 21790, *i.e.* in good agreement with the value of 20880 ± 420 determined by sedimentation

TABLE II

Amino Acid Composition of Protease Inhibitors from Potatoes

The inhibitors bear the designation used by the authors in the papers cited. Inhibitor 1 is a tetra-

	Hojima and coworkers[18]		Melville and coworkers[15]				
	Ip 5·9	Ip 6·4	I[a]	A	B	C	D
Lysine	10	11	5·2	5·2	5·0	5·9	5·8
Histidine	2.	5	0·6	0·4	0·4	0·7	1·0
Arginine	7	6	3·6	3·9	3·6	4·6	3·9
Cysteic acid	—	—	—	—	—	—	—
Aspartic acid	23	23	9·1	11·6	10·4	10·5	8·4
Threonine	12	12	2·9	3·6	2·8	3·1	3·3
Serine	26	23	4·2	5·1	4·0	4·2	3·4
Glutamic acid	14	13	9·2	11·1	9·3	8·6	8·0
Proline	10	9	5·4	6·6	7·1	5·4	4·3
Glycine	21	21	6·3	6·9	5·5	7·1	8·1
Alanine	9	10	4·7	6·5	2·3	2·4	2·5
Half-cystine	4	3	2·1	2·5	2·1	2·6	2·1
Valine	16	15	7·6	9·6	8·0	8·7	6·1
Methionine	2	2	0·8	1·1	0·6	0·6	1·4
Isoleucine	11	10	5·9	7·7	6·3	7·7	5·6
Leucine	20	20	7·7	8·0	9·8	10·4	6·9
Tyrosine	7	7	1·3	2·3	0·6	1·0	1·3
Phenylalanine	10	10	2·4	3·1	3·2	2·3	1·9
Tryptophan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-terminal group	n.d.	n.d.	Glu	Glu	Glu	Glu	Glu
Mol.wt.	22 689	22 148	38 000	9 500	9 500	9 500	9 500

[a] Calculated on the basis of monomer of mol. wt. 9 500, [b] calculated on the basis of monomer

analysis. We found a value of 27000 in our previous experiments by the method of gel filtration. The latter value, obtained by gel filtration, is higher than the molecular weight calculated from the amino acid composition (21790) and determined by sedimentation analysis (20880); this could be ascribed to an anomalous behavior of the molecule of the cathepsin D inhibitor upon gel chromatography. We regard the value of 21000–22000 as more correct.

In order to elucidate the relation between the cathepsin D inhibitor and other inhibitors from potatoes, *i.e.* to show whether the inhibitor isolated by us is a new one or one of those already reported, we compared the amino acid composition

mer, A, B, C, D are monomers (Melville and coworkers), inhibitor A_5 is a tetramer (Belitz and coworkers). n.d. not determined.

Hochstrasser and coworkers[17]				Belitz and coworkers[16]		Iwasaki and coworkers[19]		This paper
K_{1a}	K_{1c}	K_2	K_3	A_5[b]	A_2	IIa	IIb	PDI
14	19	13	11	7·8	7·1	8	10·0	10
0	0	1	3	0·1	1·2	0	1·0	0
8	10	14	7	3·8	4·2	3	2	8
—	—	—	—	0·3	0·3	—	—	—
25	18	28	23	11·6	13·0	11	9	26
11	10	10	10	5·9	5·0	6	5	9
16	14	23	21	6·9	7·6	7	6	14
17	16	13	16	9·2	7·2	8	8	12
12	14	11	11	5·6	6·7	6	9	16
26	26	24	23	11·4	10·0	13	12	19
10	12	10	10	5·3	4·6	5	6	7
12	12	12	12	6·2	1·3	12	12	6
5	1	1	2	1·7	7·7	2	1	16
0	0	0	0	0	0	0	0	1
7	6	9	13	4·2	5·7	4	4	12
13	8	24	22	3·4	9·3	3	3	22
10	12	8	5	6·2	3·7	6	6	8
8	6	12	9	3·8	5·4	3	3	12
n.d.	n.d.	n.d.	n.d.	0	n.d.	0	0	1
Asp	Glu	Glu	Asp	Arg	Arg	Ala	Ala	Glu
(Ala)		(Val)	(Ala)					
23 500	23 500	24 000	23 000	22 000 26 000		10 350	10 350	21 790

of mol. wt. 6 400.

of our inhibitor with that of other inhibitors from potatoes in Table II. These data show that our cathepsin D inhibitor differs in amino acid composition from the remaining inhibitors isolated from potatoes, even though certain features in common with these inhibitors can be traced (such as N-terminal glutamic acid and the absence of histidine). This leads us to assume that our cathepsin D inhibitor is a new one.

The most objective appraisal of the similarities existing among the inhibitors from potatoes would require the knowledge of their amino acid sequences. As yet the primary structures of two inhibitors inhibiting both trypsin and chymotrypsin are known. In one case it is the structure of a subunit of molecular weight about

10000 (ref.[20,21]), in the other the structure of an active fragment of molecular weight 4500 (ref.[22]). The data required for a sound comparison are meagre and we can merely state that the 17-residue sequence determined by us is not analogous to any of the two known structures mentioned above.

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