Inhibition of cysteine proteinases by a protein inhibitor from potato

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The inhibitory specificity of a protein from potato tubers that inhibits cysteine proteinases (potato cysteine proteinase inhibitor, PCPI) has been compared with that of chicken egg-white cystatin. Most proteinases that are inhibited by cystatin were also inhibited by PCPI, but the potato inhibitor inhibited stem bromelain and fruit bromelain, which are not inhibited by cystatin, and for which no protein inhibitor of comparable potency has previously been described. In contrast, papaya proteinase IV was unaffected by PCPI as it is by the cystatins, and the exopeptidase, dipeptidyl peptidase I, is inhibited by cystatins, but was unaffected by PCPI. The differences in inhibitory specificity between these proteins may well reflect differences between superfamilies of cysteine proteinase inhibitors.

Cysteine proteinase; Enzyme inhibition; Potato; Cystatin

1. INTRODUCTION

Potato tubers are known to contain a number of protein inhibitors of endopeptidases, including inhibitors of serine proteinases [1], and an inhibitor of the aspartic proteinase, cathepsin D, which has been found to be structurally related to soybean trypsin inhibitor [2]. Several inhibitors of cysteine proteinases which also belong to the same structural superfamily [3] have recently been isolated and partially characterized from potato peelings [4]. These proteins all had M_r values of about 23000, but differed in pI. They also differed in their effectiveness of inhibition of various cysteine proteinases [3]. The potato cysteine proteinase inhibitor variant of pI 8.3 (PCPI 8.3), which was isolated as a homogeneous protein lacking any inhibitory activity for trypsin or chymotrypsin [3,4], has now been more fully characterized as an inhibitor of cysteine proteinases, and the results are here compared to those for an inhibitor from the cystatin superfamily, chicken egg-white cystatin.

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Abbreviations: Boc, *t*-butyloxycarbonyl; Bz, benzoyl; E-64, L-3-carboxy-2,3-trans-epoxypropionyl-leucylamido(4-guanidino)butane; $K_i(_{app})$, apparent dissociation constant of inhibitor in the presence of substrate; K_i, the inhibition constant; K_m, the Michaelis constant; NHMec, 7(4-methyl)-coumarylamide; $NHPhNO_2$, *p*-nitroanilide; [S], total substrate concentration; Z, benzyloxycarbonyl.

2. EXPERIMENTAL

2.1. *Materials*

Z-Arg-NHMec, Z-Arg-Arg-NHMec, Z-Phe-Arg-NHMec, Bz-Phe-Val-Arg-NHMec and Gly-Phe-NHMec were from Bachem Feinchemikalien, Bubendorf, Switzerland. Boc-Ala-Ala-Gly-NHMec was the gift of Dr C.G. Knight (Strangeways Research Laboratory).

PCPI 8.3 was prepared as described elsewhere [4]. The active molar concentration of PCPI 8.3 was determined by titration with ananain, which itself had been previously standardized by titration with Compound E-65 [5], using Bz-Phe-Val-Arg-$NHPhNO_2$ as substrate according to the method of [6]. Chicken egg-white cystatin was purified [7] and titrated as described elsewhere [5].

Clostripain (cat. no. C0888), dipeptidyl peptidase I (cat. no. C8511), papain (cat. no. P3152) and ficin (cat. no. F4125) were from Sigma. Ananain and stem bromelain [5], fruit bromelain and pinguinain [8], actinidin [9], papaya proteinase III [6], papaya proteinase IV [10], and sheep cathepsin L [11,12] were purified as previously described. Human liver cathepsin B was prepared as described [13] except that the affinity ligand used was phenylalanylaminoacetaldehyde semicarbazone. Chymopapain was prepared as described [14].

2.2. *Determination of K_i values*

$K_i(_{app})$ values for the interaction between PCPI 8.3 and the various cysteine proteinases were determined by use of continuous fluorimetric assays. Fluorescence (excitation 360 nm, emission 460 nm) was monitored in a fluorimeter (Perkin-Elmer LS-3B) fitted with a temperature-controlled cell with magnetic stirrer, and connected to an IBM-compatible computer running the FLU software system [15]. Enzyme was allowed to activate in 0.75 ml of assay buffer (see below) for 5 min at assay temperature, and then prewarmed 0.01% Brij 35 was added, followed by substrate (see below), to a final volume of 3.0 ml. The rate of the reaction in the absence of inhibitor (V_o) was recorded, and inhibitor (ranging from 2.5×10^{-11} to 5×10^{-7} M final concentration of active PCPI 8.3, depending on the enzyme) was added in a negligible volume. The initial rate was allowed to relax to a new steady state (v_i). Non-linear regression analysis of the replot of fractional activity (v_i/v_o) versus inhibitor concentration [16] gave $K_i(_{app})$. The K_i value was obtained from the relationship:

$$K_i = K_i(_{app})/(1 + [S]/K_m)$$

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Table I

Substrate concentrations used for the determination of K_i with PCPI 8.3

Substrate	Enzyme	[S] (μM)
Z-Arg-NHMec	clostripain	5
Z-Arg-Arg-NHMec	cathepsin B	10
	stem bromelain	5
Z-Phe-Arg-NHMec	actinidin, ficin	5
	papain, cathepsin L, papaya proteinase III,	2
	chymopapain	1
Bz-Phe-Val-Arg-NHMec	ananain	5
	fruit bromelain	4
	pinguinain	2
Boc-Ala-Ala-Gly-NHMec	papaya proteinase IV	5
Gly-Phe-NHMec	dipeptidyl peptidase I	250

The substrate concentrations[S] were chosen such that [S] < K_m, in all cases, K_m values (not shown) having been determined in preliminary experiments.

For the plant proteinases, the assay buffer was 0.40 M sodium phosphate buffer, pH 6.8. With cathepsin B, a 0.25 M 2-(N-morpholino)ethanesulphonic acid buffer, pH 5.5, was used, and for cathepsin L, 0.40 M sodium acetate buffer, pH 5.5, was used. All the above assay buffers contained 16 mM cysteine and 4 mM EDTA. Clostripain and dipeptidyl peptidase I were assayed as described previously [17] at 25 and 37°C, respectively, apart from the use of Z-Arg-MHMec as substrate for clostripain. The plant enzymes were assayed at 40°C, the cathepsins at 30°C. The substrates were used at final concentrations given in Table I.

3. RESULTS AND DISCUSSION

The inhibitory spectrum of PCPI 8.3 for cysteine endopeptidases (Table II) is remarkably broad, including two enzymes, stem bromelain and fruit bromelain, that are not inhibited by chicken egg-white cystatin, and for which no protein inhibitor of comparable potency has previously been described. On the other hand, the cysteine-dependent exopeptidase, dipeptidyl peptidase I, which is well inhibited by several cystatin-type inhibitors [18], was not detectably inhibited by PCPI. Also, papaya proteinase IV, perhaps the most specific known member of the papain superfamily [19] was unaffected by PCPI 8.3, as it is by the cystatins [20].

The cystatin-related inhibitors have invariably been found to show more potent inhibition of papain than of cathepsin L [18], whereas our present results establish the stronger inhibition of cathepsin L by PCPI 8.3, consistent with that previously reported for the pI 6.6 and 9.4 variants of the protein [4].

In conclusion, the inhibitory spectrum of PCPI 8.3 shows a number of clear differences from those of chicken egg-white cystatin and its homologues. Since the available amino acid sequence data [3; M. Drobnic-Kosorok, A. Ritonja and J. Brzin, unpublished results] reveal no relationship of PCPI to the cystatins, the differences of inhibitory spectrum may well be those of distinct superfamilies of cysteine proteinase inhibitors.

Table II

Comparison of K_i values for inhibition of cysteine proteinases and dipeptidyl peptidase I by PCPI 8.3 and chicken cystatin

Proteinase	K_i (nM)		
	PCPI 8.3	Cystatin	
Ananain	0.06	1.1	[5]
Cathepsin L	0.07	0.02[1]	[18]
Papain	3.3	0.005	[18]
Actinidin	27	5	[21]
Fruit bromelain	33	≫1100	[8]
Pinguinain	35	1.6	[8]
Papaya proteinase III	150	73[2]	
Stem bromelain	190	>36000	[5]
Ficin	210[3]	0.00005	[21]
Chymopapain	210	0.33	[10]
Cathepsin B	320	0.81	[22]
Papaya proteinase IV	≫500	≫1130	[10]
Clostripain	≫500	>500[4]	
Dipeptidyl peptidase I	≫500	0.35	[18]

Values of K_i for PCPI 8.3 were determined as described in the Experimental section. The enzymes are ranked in order of increasing K_i with PCPI 8.3, and literature values for chicken cystatin are given for comparison.
[1]For human cathepsin L; [2]D.J. Buttle and R. Feltell, unpublished results; [3]this value should be regarded as an estimate only, since the enzyme preparation used was heterogeneous (results not shown); [4]approx. 50% inhibition of clostripain was obtained at cystatin concentrations from 0.5–1.5 μM, as observed previously [23], suggesting heterogeneity of the commercial enzyme preparation.

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