Two different molecular species of pig calpastatin

Structural and functional relationship between 107 kDa and 68 kDa molecules

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Calpastatin, the inhibitor protein acting specifically on calpain (EC 3.4.22.17; Ca^{2+} -dependent cysteine proteinase), is known to be widely distributed in mammalian and avian cells. Two different molecular species of calpastatin were isolated and purified to homogeneity from pig heart muscle and from pig erythrocytes, and shown to be of 107 kDa and 68 kDa respectively on SDS/polyacrylamide-gel electrophoresis. Both calpastatins had very similar amino acid compositions when expressed as mol per cent of the residues, differed by only 0.1 pH unit in their isoelectric points, and showed immunological cross-reactivity. One molecule of the 107 kDa species could bind approx. 8 calpain molecules, whereas the 68 kDa inhibitor could bind approx. 5 calpain molecules. These findings suggest similar protein structures of the 107 kDa and 68 kDa calpastatins, each being composed of extended multidomains, with unit inhibitor domains aligned along the polypeptide chain of the molecule. The present study does not conclude, however, whether or not the 68 kDa calpastatin found in erythrocytes is a derived product from the 107 kDa species, which is present as such in heart muscle.

INTRODUCTION

Calpain (EC 3.4.22.17; Ca^{2+} -dependent cysteine proteinase) and calpastatin, calpain-specific inhibitor protein, are known to coexist in a very wide variety of mammalian and avian cells (for review, see Murachi *et al.*, 1981; Murachi, 1983, 1984; Kay, 1983; Goll *et al.*, 1985). Despite the apparent importance of a regulatory system involving calpain and calpastatin, and in contrast with rapid progress in research on calpain (e.g., Sakihama *et al.*, 1985; Ohno *et al.*, 1984; Sasaki *et al.*, 1984), the characterization of the inhibitor calpastatin has been greatly hampered by the seemingly complex nature of its molecular species. For example, the molecular-mass values reported for calpastatin have ranged from 24 kDa (Nakamura *et al.*, 1984) to 400 kDa (Otsuka & Goll, 1980), which may have included those for somehow fragmented molecules and associated oligomers. Even for those isolated as intact monomeric proteins, the reported values are still as diverse as 68 kDa (Ishiura *et al.*, 1982), 70 kDa (Takano & Murachi, 1982), 125 kDa (DeMartino & Croall, 1984), 145 kDa (Mellgren & Carr, 1983) and 172 kDa (Lepley *et al.*, 1985). We have previously reported that such molecular diversity of calpastatin does exist even in one single species of animal, and that the molecular forms encountered most frequently in humans and pigs are the 70 kDa and 100 kDa calpastatins (Takano *et al.*, 1984b). Thus the present study was undertaken to isolate both of these molecular species to homogeneity and to compare them in both structural and functional aspects.

Pig erythrocytes were chosen as the starting material for 70 kDa calpastatin, which was actually found to be

of molecular mass 68 kDa on SDS/polyacrylamide-gel electrophoresis, and so is referred to as 68 kDa calpastatin in the present paper. Pig heart was used for isolation of a calpastatin species which had been shown to migrate on SDS/polyacrylamide-gel electrophoresis as a 100 kDa protein (Takano *et al.*, 1984b). The latter, after isolation, was found to behave as having molecular mass 107 kDa; hence it is called 107 kDa calpastatin throughout the present paper. A 70 kDa calpastatin that had been isolated from human erythrocytes and characterized (Takano & Murachi, 1982) was also used for comparison.

EXPERIMENTAL

Materials

Hammarsten-grade casein and monoiodoacetic acid were obtained from E. Merck, Darmstadt, Germany. DEAE-cellulose (DE 52) was purchased from Whatman, Maidstone, Kent, U.K. Sephacryl S-300, Pharmalyte, M_r standards for gel filtration, and isoelectric-point standards were products of Pharmacia, Uppsala, Sweden. High- and low- M_r protein standards were products of Bio-Rad Laboratories, Richmond, CA, U.S.A. The Centricon-30 microconcentrator was a product of Amicon Corp., Danvers, MA, U.S.A. The TSK-Gel G 3000 SWG column and h.p.l.c. apparatus were products of Toyo Soda, Tokyo, Japan. Nitrocellulose filters were purchased from Schleicher and Schüll, Dassel, Germany. Other reagent-grade chemicals were obtained from Wako Pure Chemicals, Osaka, or Nakarai Chemicals, Kyoto, Japan.

The buffers used were: buffer A, 20 mM-Tris/HCl containing 1 mM-EDTA and 1 mM-EGTA, pH 7.5;

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buffer B, buffer A containing 0.1 mM-phenylmethanesulphonyl fluoride; buffer C, 50 mM-Tris/acetate containing 5 mM-EDTA, 1 mM-EGTA and 0.1 mM-phenylmethanesulphonyl fluoride, pH 8.0; buffer D, buffer A containing 6 M-guanidine hydrochloride, pH 7.0.

Analysis

Low- Ca^{2+} -requiring calpain I and high- Ca^{2+} -requiring calpain II were purified to homogeneity from pig erythrocytes and kidney, respectively, by the method of Kitahara *et al.* (1984). Specific activities were 190 and 285 units/mg for calpains I and II respectively. The hydrolytic activities of calpains I and II on casein were assayed at 0.1 mM- and 5 mM- Ca^{2+} respectively, and the inhibitory potency of calpastatin was determined by the method of Murakami *et al.* (1981). One unit of calpain was defined as the amount of the enzyme that caused a change of 1.0 in A_{750} for the trichloroacetic acid-soluble products after incubation for 30 min at 30 °C. One unit of calpastatin was defined as the amount of inhibitor that inhibited 1 unit of pig erythrocyte calpain I under the standard assay conditions. The protein concentration of calpastatin was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Polyacrylamide-slab-gel electrophoresis in the presence of SDS was performed by the method of Laemmli (1970), with 7.5–10% resolving gel and 3% stacking gel. The isoelectric focusing was carried out in thin layers of agarose gel with 7% Pharmalyte, with a pH gradient from 4.5 to 6.5 (Burdent, 1981). The anode electrolyte was 0.05 M- H_2SO_4 and the cathode electrolyte was 1 M-NaOH. The proteins were applied and the gel was focused at 15 W for 1.5 h at 10 °C. After focusing, the proteins were fixed in 10% (w/v) trichloroacetic acid/5% (w/v) sulphosalicylic acid and stained with 0.2% Coomassie Brilliant Blue. The isoelectric point was estimated by using a pI Calibration Kit from Pharmacia.

Amino acid analysis of calpastatin samples (50 µg each), which had been hydrolysed in 0.2 ml of constant-boiling HCl at 110 °C for 24, 48 and 72 h, was carried out with a Hitachi model 835 amino acid analyser. For tryptophan content, the hydrolysis was conducted with methanesulphonic acid. Reduction and carboxymethylation of proteins was performed by the method of London *et al.* (1971).

Purification of calpastatins

Pig heart was removed immediately after slaughter and chilled in ice. All the subsequent steps of purification were carried out at 4 °C. The cardiac muscle (1.0 kg) was minced, and homogenized in 2.5 vol. of buffer C with a Waring Blendor. To the centrifuged (10000 g for 20 min) supernatant of the homogenate was added solid $(\text{NH}_4)_2\text{SO}_4$ to 45% saturation, and the precipitates were dissolved in 400 ml of buffer B and dialysed against the same buffer overnight. The dialysed residue, after centrifugation, was applied to a column (2.1 cm × 45 cm) of DEAE-cellulose DE-52 in buffer B and the adsorbed proteins were eluted with a linear gradient of 0–150 mM-NaCl in buffer B. The calpastatin activity appeared between 70 and 100 mM-NaCl. These fractions were pooled, concentrated by 30–45% saturation with $(\text{NH}_4)_2\text{SO}_4$, dialysed against buffer B, and then heated at 100 °C for 20 min. The centrifuged (10000 g for 30 min) supernatant was further fractionated on a column

(3.2 cm × 95 cm) of Ultrogel AcA 34 in buffer A containing 50 mM-NaCl to obtain the final product.

Pig erythrocyte 68 kDa calpastatin was purified by a method essentially the same as that for human erythrocyte 70 kDa calpastatin (Takano & Murachi, 1982). As the final step of purification, h.p.l.c. in buffer D was used in place of the Sephacryl S-200 chromatography used in the cited paper.

Anti-calpastatin antibodies

Three antibodies were raised in rabbits, directed respectively against calpastatins that had been purified from pig erythrocytes (68 kDa) and heart (107 kDa) and from human erythrocytes (70 kDa). They were affinity-purified by the method of Takano *et al.* (1984a). Immunoelectrophoretic-blot analysis ('Western' blot) was carried out as described by Towbin *et al.* (1979), by using nitrocellulose membranes, on which proteins were stained with Amido Black 10 and the antigens were located with affinity-purified antibody and peroxidase-conjugated goat anti-(rabbit IgG) (Hawkes *et al.*, 1982).

RESULTS

Purification of 107 kDa and 68 kDa calpastatins

Table 1 summarizes the purification of two different calpastatin species. From 1 kg of pig heart or from 2 litres of pig blood, 1.95 mg of the 107 kDa calpastatin or 0.44 mg of the 68 kDa calpastatin respectively was obtained. The specific activity of the final product (units/mg) was similar for both species. Inclusion of phenylmethanesulphonyl fluoride in the buffer was necessary to avoid fragmentation of 107 kDa calpastatin from heart muscle during the purification procedures. For both species of calpastatin, the heat treatment eliminated contaminating proteins effectively.

Homogeneity and molecular mass

Purified calpastatins were shown to be homogeneous on SDS/polyacrylamide-gel electrophoresis, with calculated molecular masses of 107 kDa and 68 kDa (Fig. 1). These values are those for their monomers in the presence of SDS, but the monomers tend readily to self-associate to form oligomers in the absence of the detergent. As shown in Fig. 2, 107 kDa calpastatin is present in buffer A containing 150 mM-NaCl as oligomers, whose molecular masses are distributed rather widely, with a maximum corresponding to 550 kDa, and likewise the 68 kDa species forms oligomers with a peak molecular mass of 360 kDa. The self-associability of calpastatin from other tissues has been well documented (Murachi, 1983).

Amino acid composition

Table 2 shows the amino acid compositions of pig 107 kDa and 68 kDa calpastatins, and also contains the composition of human erythrocyte 70 kDa calpastatin (Takano & Murachi, 1982). The data are shown in terms of mol per cent of the residues so as to permit direct comparison, regardless of different molecular sizes. The similarity of composition between 107 kDa and 68 kDa calpastatins is obvious. Both contain high amounts of lysine, with much less arginine. Both contain only few, even in total, residues of phenylalanine, tyrosine and histidine, and no tryptophan, showing $A_{280}^{1\%}$ values less than 2. Both contain more than 10% aspartic acid and

107 kDa calpastatin from pig heart				68 kDa calpastatin from pig erythrocytes						
Step	Total protein (mg)	Total activity		Specific activity (units/mg of protein)	Purification (fold) ^a	Total activity		Specific activity (units/mg of protein)	Purification (fold) ^b	
		(units)	(%)			(units)	(%)			
Crude extract	27700	(5540) ^c	(0.20) ^d	1		Haemolysate	68000	(4760) ^c	(0.070) ^d	1
0-45% -satn. - (NH ₄) ₂ SO ₄ precipitation	6320	(5540) ^c	(0.88) ^d	4.4		DEAE-cellulose	1710	(4760) ^c	(2.78) ^d	39.7
DEAE-cellulose	454	5540	(100)	61		Ultrogel Aca 34	219	4760	(100)	310
30-45% -satn. (NH ₄) ₂ SO ₄ precipitation	198	5210	(94.0)	132		DEAE-cellulose	39.0	4060	(85.3)	1490
Heat treatment	7.4	4840	(87.4)	654		Heat treatment	5.8	3280	(68.9)	8060
Ultrogel Aca 34	1.95	1890	(34.1)	970		H.p.l.c.	0.44	435	(9.1)	14100

^aHA deduced specific activity of 0.20 unit/mg of protein for the first step was taken as unity.

^bP/A deduced specific activity of 0.070 unit/mg of protein for the first step was taken as unity.

^cTotal activity in these fractions could not be directly determined, owing to the presence of calpain, and was therefore assumed to be equal to the total activity found after the next step of the purification.

^dThe value was calculated from the deduced total activity.

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Lanes 1 and 4, M_r -marker proteins from Bio-Rad, which include myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa) and soya-bean trypsin inhibitor (21.5 kDa); lane 2, 8 μ g of calpastatin purified from pig heart; lane 3, 2 μ g of calpastatin purified from pig erythrocyte. Marker proteins were stained with 0.2% Coomassie Brilliant Blue.

●, Purified pig heart calpastatin (80 μg); ○, purified pig erythrocyte calpastatin (50 μg). A 1.7 cm $\times$ 96 cm column was used with buffer A containing 150 mM-NaCl at 4°C. M_r -marker proteins used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa). V_0 is the exclusion volume.

glutamic acid and proline, with 1 % or less methionine and half-cystine. All these features are exactly the same as those reported for human erythrocyte calpastatin (Takano & Murachi, 1982).

Table 2. Amino acid composition of calpastatin

Amino acid	Composition (residues/100 residues) ^a		
	107 kDa calpastatin from pig heart	68 kDa calpastatin from pig erythrocytes	70 kDa calpastatin from human erythrocytes ^b
Aspartic acid	10.3	11.4	12.6
Threonine	6.4	5.3	5.0
Serine	9.0	8.5	9.6
Glutamic acid	14.3	15.7	14.9
Proline	11.1	11.8	10.1
Glycine	6.7	5.3	5.3
Alanine	7.6	8.2	9.5
Half-cystine	0.5 ^c	0.5 ^c	0.1
Valine	5.2	4.2	3.3
Methionine	0.8	0.4	1.1
Isoleucine	2.7	1.9	2.2
Leucine	7.5	7.7	7.9
Tyrosine	0.7	0.5	0.9
Phenylalanine	1.4	1.3	1.2
Lysine	10.8	13.7	10.7
Histidine	1.0	0.9	1.0
Arginine	4.0	2.7	2.5
Tryptophan	0 ^d	0 ^d	0

^aAverage of determinations after 24, 48 and 72 h hydrolysis.

^bRecalculated from the data reported by Takano & Murachi (1982).

^cDetermined as S-carboxymethylcysteine.

^dDetermined after methanesulphonic acid hydrolysis.

Isoelectric point

The pI values for 107 kDa and 68 kDa calpastatins were estimated to be 4.7 and 4.8 respectively. These values are not very different from the value 4.55 reported for human erythrocyte 70 kDa calpastatin (Takano & Murachi, 1982).

Inhibition profile

The dose-dependence of the inhibition of calpains I and II by 107 kDa and 68 kDa calpastatins is shown in Fig. 3. For comparison the results obtained with 70 kDa calpastatin from human erythrocyte are also shown. The values of ID₅₀ (the dose of calpastatin that caused 50% inhibition) were calculated to be 0.70 nM and 1.34 nM on calpain I, and 0.50 nM and 0.83 nM on calpain II, for 107 kDa and 68 kDa calpastatins respectively. Human erythrocyte 70 kDa calpastatin exerted similar inhibition on heterologous (pig) calpains: ID₅₀ values were 1.57 and 0.96 nM on calpains I and II respectively. Note that a higher enzyme concentration was employed for calpain I than for calpain II, since calpain I had lower specific activity than calpain II (see the Experimental section, and Kitahara *et al.*, 1984).

The insets of Fig. 3 show plots of inhibition as a function of the molar ratio of calpastatin to calpain. By extrapolation to zero activity, the maximum binding ratios can be estimated to be 8.0 and 7.9 for calpains I and II to 107 kDa calpastatin, and 4.7 and 4.9 for calpains I and II to 68 kDa calpastatin, respectively. Thus 107 kDa calpastatin has a calpain-binding capacity 1.6–1.7 times that of 68 kDa calpastatin.

Immunological cross-reactivity

Immunoelectrophoretic-blot analysis was performed: (i) to find if the two antibodies directed, respectively, against 107 kDa and 68 kDa calpastatins cross-react with each other's antigens; and (ii) to determine how many different antigens are present in the respective crude cell extracts. As shown in Fig. 4(a), crude extract from pig heart gave heavy staining of the band at a position near 107 kDa when either anti-(107 kDa calpastatin) antibody or anti-(68 kDa calpastatin) antibody was used. Staining, though very much weaker, can also be seen at a position near 68 kDa. Anti-(70 kDa calpastatin) antibody gave a similar result. By contrast, haemolysate of pig erythrocytes gave a major 68 kDa band, as located by any one of the three different antibodies used, with several minor bands, including those at 90 and 107 kDa positions (Fig. 4b). In no case, any immunoreactive band corresponding to 145 or 172 kDa could be detected.

DISCUSSION

In a previous paper, we reported distinct differences in gel-electrophoretic pattern for calpastatins between pig erythrocytes and heart (Takano *et al.*, 1984b). The patterns were made visible as immunoreactive bands on nitrocellulose membranes, and the co-migration of such a band with calpain-inhibiting ability was demonstrated. The results of the present isolation studies substantiate conclusively the predominant presence of 68 kDa calpastatin in erythrocytes, with a 107 kDa species being the major calpastatin component in heart tissues (Table 1 and Fig. 1). The value 68 kDa is almost equal to that reported for human erythrocytes by Takano & Murachi (1982), and not very much different from the value 60 kDa reported by Melloni *et al.* (1982). The value 107 kDa for pig heart calpastatin coincides with that reported for human liver calpastatin (Imajoh *et al.*, 1985), but it is lower than the 125 kDa reported for rat liver (DeMartino & Croall, 1984) and 145 kDa reported for bovine heart calpastatin (Mellgren & Carr, 1983). However, such a difference could have arisen from different techniques for *M_r* estimation (Shannon & Goll, 1985), suggesting that all of these calpastatins in heart and liver tissues are similar in molecular size, which is distinctly larger than erythrocyte calpastatin (approx. 70 kDa). A still larger molecule of 172 kDa was reported to be the intact calpastatin in pig skeletal muscle (Lepley *et al.*, 1985), but we were unable to demonstrate such molecular species in pig heart and erythrocytes (Fig. 4).

Several different results have so far been presented concerning the stoichiometry of the calpain inhibition. Thus a complete inhibition was reported to occur at such diverse ratios of calpain to monomeric calpastatin as 2:1 (Takahashi-Nakamura *et al.*, 1981), 1:1 (Ishiura *et al.*, 1982), 3:1 for calpain I and 9:1 for calpain II (Cottin *et al.*, 1983), more than 1:1 (Mellgren & Carr, 1983), 4:1 (DeMartino & Croall, 1984), 5:1 (Imajoh *et al.*, 1985) and 8:1 (Lepley *et al.*, 1985). Besides, the binding study by gel filtration gave ratios of one molecule of 80 kDa calpain to one of 60 kDa calpastatin (Melloni *et al.*, 1982) and one 108 kDa calpain molecule to one of 125 kDa calpastatin (DeMartino & Croall, 1984). Our results from the inhibition study are approx. 8 and 5 calpain molecules per molecule of 107 kDa and 68 kDa calpastatins

Fig. 3. Profiles of inhibition of calpains I and II by calpastatins

(a) 12.6 nM-calpain I; (b) 7.94 nM-calpain II. The incubation mixture (1 ml) contained 0.4% casein, 100 mM-imidazole/HCl buffer, pH 7.5, 5 mM-cysteine, 0.1 mM- CaCl_2 for calpain I and 5.0 mM- CaCl_2 for calpain II and various concentrations of 107 kDa calpastatin from pig heart (●), 68 kDa calpastatin from pig erythrocytes (○) or 70 kDa calpastatin from human erythrocytes (△). The reaction was terminated, after 30 min at 30 °C, by adding 1 ml of 5% trichloroacetic acid, and the acid-soluble products were determined by the method of Murakami *et al.* (1981). Insets show plots of the activity remaining (%) versus calculated molar ratios of calpastatin to calpain. Extrapolated intercept values on the abscissa are: 0.125 and 0.213 in (a) and 0.127 and 0.203 in (b).

respectively, with no appreciable difference between calpains I and II (Fig. 3). Although no reasonable explanation for these controversies can be found at present, the particular relevance of our present findings must be that the binding capacity of a calpastatin is proportional to its molecular size.

By contrast with their difference in molecular size, heart and erythrocyte calpastatins have strikingly similar amino acid compositions, as expressed in mol per cent (Table 2). The inhibition study indicates that a 107 kDa calpastatin molecule has the capacity to bind 1.6–1.7 times as many calpain I or II molecules as a 68 kDa calpastatin molecule does (Fig. 3). This ratio coincides with the ratio in molecular size between the two calpastatins, 1.57. The pI of 68 kDa calpastatin (4.7) differs from that of the 107 kDa species (pI 4.8) only by 0.1 pH unit. All these lines of information lend support to the notions (i) that a calpastatin molecule is composed of several structurally similar domains extending throughout the molecule, (ii) that the numbers of such

domains per molecule may be proportional to the size of the molecule, and (iii) that each of the domains may have similar inhibitory capacity towards the enzyme calpain. The notion may not be incompatible with, but not necessarily be supportive of, the postulate that 68 kDa calpastatin is in fact a product derived from 107 kDa molecule.

The immunological cross-reactivity found between 68 kDa and 107 kDa calpastatins (Fig. 4) do not provide any conclusion as to whether or not the 68 kDa protein is a portion of 107 kDa calpastatin. However, it is noteworthy that the crude extract from pig heart showed, besides the major 107 kDa calpastatin, a minor band of 68 kDa, whereas the erythrocyte cytosol gave minor bands of 90 kDa and 107 kDa in addition to the major one of 68 kDa (Fig. 4). This could be interpreted as suggesting extensive processing of the 107 kDa calpastatin molecule in erythrocytes during the course of their maturation and senescence, although much will have to be done to understand the implication of the existence of

Fig. 4. Immunoelectrophoretic-blot analysis for calpastatin

(a) Crude extract from pig heart (0.1 mg of protein); (b), pig erythrocyte cytosol (0.03 mg of protein). Proteins, after SDS/polyacrylamide-gel electrophoresis, were transferred to a nitrocellulose membrane. The membrane was cut into strips and immunoblotted with antibodies: lane 1, anti-(107 kDa pig heart calpastatin) IgG (2.5 µg/ml); lane 2, anti-(68 kDa pig erythrocyte calpastatin) IgG (1.9 µg/ml); lane 3, anti-(70 kDa human erythrocyte calpastatin) IgG (2.3 µg/ml). Lane M shows marker proteins, transferred and stained with Amido Black 10, which include phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soya-bean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

at least two different calpastatins in one species of animal. A genetic approach to the problem must be crucial.

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