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REPETITIVE REGION OF CALPASTATIN IS A FUNCTIONAL UNIT OF THE PROTEINASE INHIBITOR*

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SUMMARY: A cDNA portion coding for one of the repetitive regions of pig heart calpastatin (107 kDa) was subcloned into E. coli plasmid pUC119 to express the portion of the proteinase inhibitor gene in bacteria. The expressed protein was a chimaeric protein whose calpastatin segment (130 amino acid residues) was fused with an amino-terminus portion (7 amino acid residues) of β-galactosidase. The chimaeric protein could inhibit proteolytic activity of calpain (Ca^{2+}-dependent cysteine proteinase), and maintained properties of the authentic calpastatin concerning inhibition specificity and heat stability. These findings led us to conclude that the repetitive region is a functional unit of the proteinase inhibitor.

Calpastatin is an endogenous inhibitor protein acting specifically on calpain (EC. 3.4.22.17) which is a typical intracellular non-lysosomal cysteine proteinase requiring calcium ion (1). Both calpain and calpastatin are known to be widely distributed in mammalian and avian cells (2), and involvement in cellular functions coupled with calcium mobilization has been suggested (3, 4). Previously we reported a partial primary structure of pig heart calpastatin (107 kDa) analyzed both by Edman degradation of the purified inhibitor and by nucleotide sequencing of a cloned cDNA (5).

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Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.

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The finding of the repetitive amino acid sequences at interval of 140 residues substantiated the multidomain structure of the inhibitor which had earlier been suggested by stoichiometrical analysis of calpain binding to calpastatin, and by comparison of biochemical properties of the pig heart calpastatin (107 kDa) with a smaller inhibitor (68 kDa) isolated from pig erythrocytes (6). Similar repetitive domain structure of a rabbit calpastatin has also been reported (7). However, it was not clear whether the inhibitory function of calpastatin could be attributed to each domain. In the present study attempts were made to produce a calpastatin fragment containing only one of the repetitive regions (130 amino acid residues) of pig heart calpastatin in E. coli to examine the inhibitory capability of the unit domain.

MATERIALS AND METHODS

Materials: Restriction endonucleases, T4 DNA ligase, plasmid pUC119 and its E. coli strain MV1304, and helper phage M13KO7 were obtained from Takara Shuzo Co. (Kyoto, Japan). A 25-mer oligodeoxyribonucleotide primer containing Hind III restriction site was synthesized by the phosphoramidite method (8) using an automatic DNA synthesizer (Applied Biosystems, model 381A), and phosphorylated by T4 polynucleotide kinase as described (9). E. coli strain 545πHR1 defective in an aberrant polypeptide degradation system (10) was kindly supplied by Dr. M. Imai (Kyoto University).

Site-directed mutagenesis: A 0.8-kb Pst I fragment of pOBCS1 (5) was subcloned into Pst I site of pUC119 (11). A single-stranded DNA was produced by infecting E. coli MV1304 transformant with helper phage M13KO7 as described by Vieira and Messing (11). Oligonucleotide-directed in vitro mutagenesis was performed using a mutagenesis kit supplied by Amersham International (Amersham, U.K.) as described by Taylor et al. (12), except employing a single-stranded DNA of pUC119 derivative as a template and using M13/pUC reverse primer (Takara Shuzo Co.) for repair synthesis after Nco I nicking and Exonuclease III digestion.

Preparation of E. coli cell extracts: E. coli 545πHR1 transformed with pUC-119 or its derivative was grown to late log-phase at 37°C. The culture was supplemented with cAMP and isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM each, and the incubation was continued for 4 hours. At the end of the incubation, sodium azide was added to the culture to 0.02%. Cells were harvested by centrifugation at 4°C, and washed with Buffer L (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride). The cells were lysed by incubation with one twentieth volume of lysozyme solution, 1 mg/ml in Buffer L, at 4°C for 10 min, followed by 3 cycles of freeze-thawing. After 2-mercaptoethanol was added to 5 mM, the lysed cells were centrifuged at 14,000 rpm for 30 min at 4°C to obtain the supernatant as cell extracts. Alternatively, the lysed cells were boiled for 5 min, and then centrifuged at 14,000 rpm for 5 min at 4°C. The cell extracts were stored at -20°C until use.

Assay for calpastatin activity: The calpastatin activity was measured by the method of Murakami et al. (13), using low-(or μM)Ca^{2+}-requiring calpain I purified from pig erythrocyte or high-(or mM)Ca^{2+}-requiring calpain II purified from pig kidney (14).

Partial purification of calpastatin segment produced in E. coli: The cell extract obtained after boiling treatment was dialyzed against Buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol), and then subjected to DEAE-cellulose chromatography as described previously (15), except that the column of 1.5-ml bed volume was previously equilibrated with Buffer A and the absorbed proteins were eluted stepwise with Buffer A containing different concentrations of NaCl (25 mM, 50 mM, and 75 mM).

SDS-polyacrylamide gel electrophoresis: Gel electrophoresis was performed according to the procedure of Laemmli (16), using a 15% polyacrylamide gel. Extraction of calpastatin activity from SDS-gel was performed as described previously (17).

RESULTS

Construction of recombinant plasmid for expression of calpastatin cDNA in E. coli: Figure 1 illustrates the strategy for the construction of the recombinant plasmid which expressed one of the repetitive regions of the calpastatin cDNA in E. coli. To isolate a DNA fragment covering the region Y, Hind III restriction site was created by in vitro site directed mutagenesis using a 25-mer oligonucleotide primer containing the Hind III recognition sequence as described in Materials and Methods. Hind III/Pst I fragments isolated from the mutated plasmid pCSPst(-)H2 were subcloned into pUC119 and one of the clones which contained the region Y was designated pCSD3. The calpastatin polypeptide segment (amino acid residue Nos. 123-252; numbering of the residues is tentative, see ref. 5) was preceded by eight non-calpastatin residues, seven of which were derived from the amino-terminal portion of E. coli β-galactosidase, Lac Z', and one of which was created by the mutagenesis. The translation termination codon TAG following Arg-Leu was created by frameshift insertion of the calpastatin cDNA fragment (Hind III/Pst I) to the multiple cloning site of pUC119.

Inhibitory activity of the calpastatin segment produced in E. coli: E. coli cell crude extracts were tested for the production of the calpastatin segment possessing the inhibitory activity. Calpain II was inhibited in a dose-dependent manner by the extract from E. coli harboring pCSD3, whereas no inhibition was observed in the case of the control plasmid pUC119 (Table

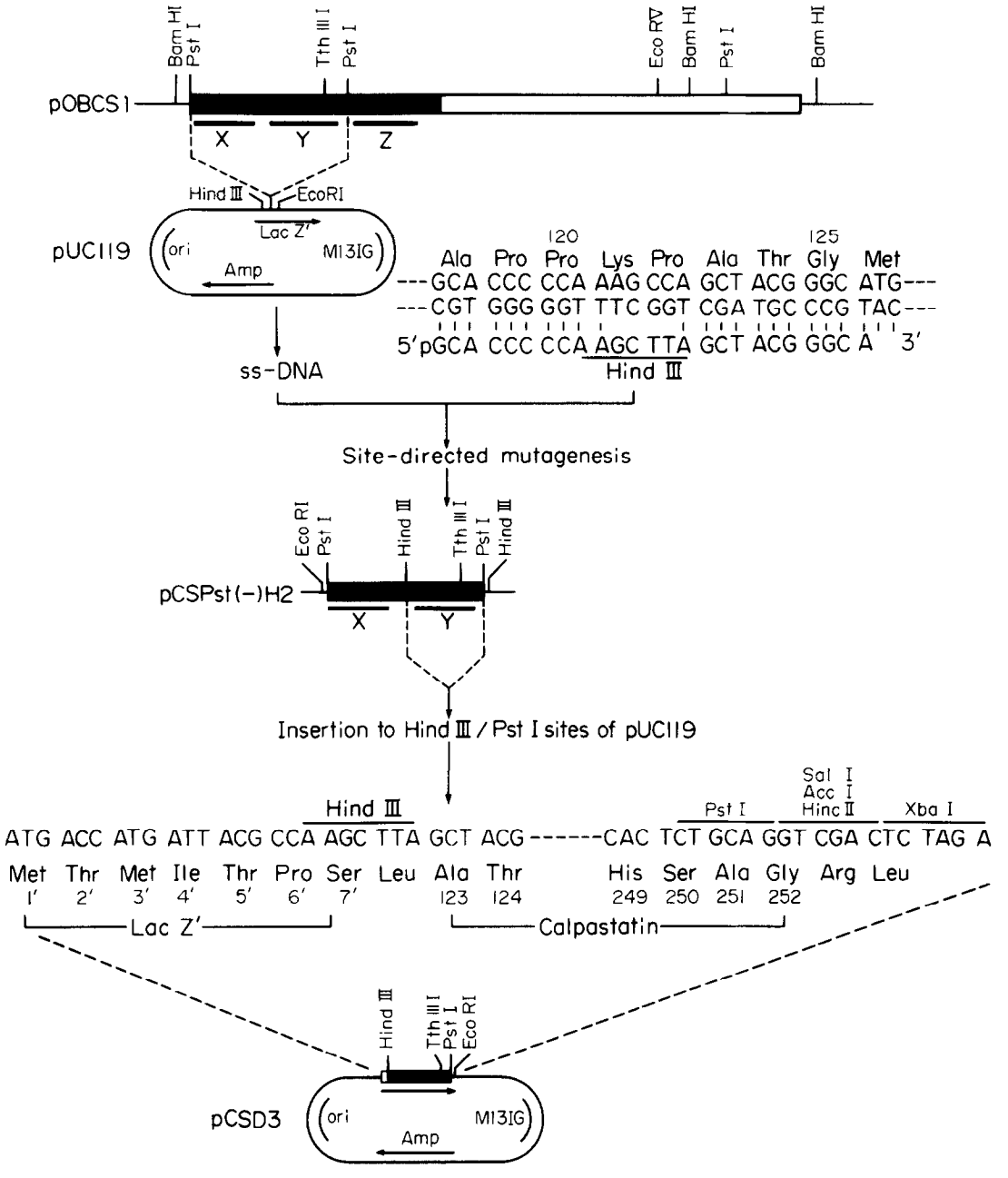

Fig. 1. Scheme for the construction of plasmid pCSD3. Closed box and open box of pOBCS1 indicate translated and 3'-untranslated regions, respectively. Repeated homologous regions are indicated by bold underlines (X, Y, Z). Details are described in the text.

I). The inhibitor activity was not lost by heat treament of the cell lysate (boiling for 5 min) during the extraction procedure. The heat treatment was found to be an efficient procedure for the purification of the calpas-

Table I. Inhibitory activity of E. coli cell extract

Cell extract		Remaining calpain II activity (%)	
Plasmid	Volume added[a] (μl)	Without heat treatment[b]	With heat treatment[b]
None	0	100	100
pUC119	1.0	100	107
	10.0	102	104
pCSD3	0.1	82.0	88.2
	0.5	12.6	12.4
	1.0	6.2	5.3

[a] Cell extract was diluted with Buffer A. The values indicate the volume before dilution.

[b] Heat treatment was performed by boiling the cell lysates for 5 min, and the resultant aggregates were removed by centrifugation at 14,000 rpm for 5 min.

tatin segment produced in E. coli (Fig. 3, lanes 3 and 5). As shown in Fig. 2, the calpastatin segment produced in E. coli inhibited both calpain I and calpain II; the inhibition being stronger on calpain II than on calpain I. An authentic (68 kDa) calpastatin preparation also exhibited stronger inhibition on calpain II than on calpain I under the same conditions. The

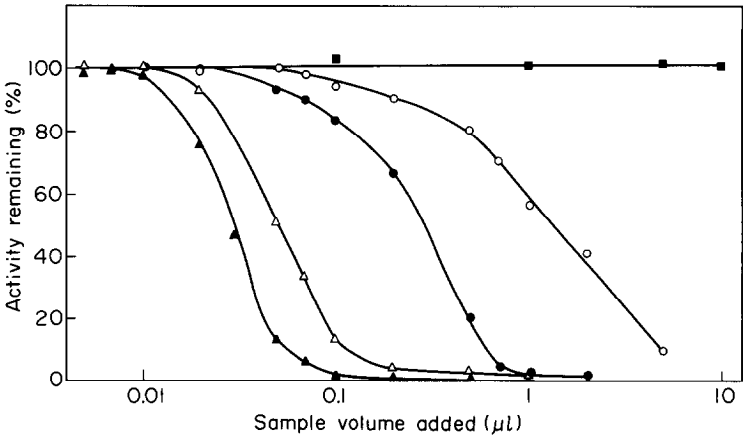

Fig. 2. Inhibition profiles of calpains I and II. Proteolytic activities of calpain I (-○-, -△-) and calpain II (-●-, -▲-, -■-) were assayed in the presence of various amounts of inhibitor samples: purified pig erythrocyte calpastatin (-△-, -▲-), and heat-treated extracts of E. coli cells harboring pCSD3 (-○-, -●-) or pUC119 (-■-). Stock solutions of inhibitor samples were diluted with Buffer A for the inhibition assay. Numbers on abscissa (logarithmic scale) indicate the sample volume (μl) before dilution.

Table II. Inhibition specificity of calpastatin

Inhibitor	(μg)	Remaining proteolytic activity (%)		
		Calpain II	Trypsin	Papain
None	0	100	100	100
Authentic calpastatin[a]	0.12	33.9	106	102
	0.24	3.8	106	101
	0.60	1.6	108	102
Calpastatin segment[b]	0.27	70.5	102	101
	0.54	15.3	103	104
	2.70	0.5	104	102

[a] Purified from pig erythrocytes as described in ref. 6.

[b] Partially purified by DEAE-cellulose chromatography from the extract of E. coli cells harboring pCSD3.

calpastatin segment produced in E. coli showed no inhibition on papain and trypsin (Table II).

Molecular size analyzed by SDS-polyacrylamide gel electrophoresis: The calpastatin segment produced in E. coli was partially purified by DEAE-cellulose chromatography after heat treatment of the cell lysate. Most of the activities were recovered by the elution from the column with Buffer A containing 50 mM NaCl (data not shown). As shown in Fig. 3, the purifi-

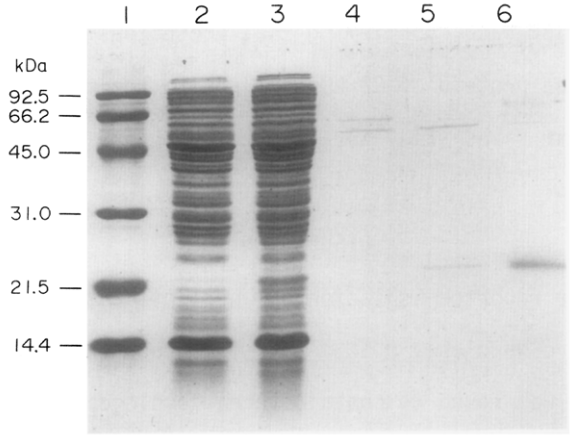

Fig. 3. SDS-polyacrylamide gel electrophoresis of calpastatin segment produced in E. coli. Lane 1, molecular weight marker proteins including phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa); lane 2, crude extract of clone pUC119; lane 3, crude extract of clone pCSD3; lanes 4 and 5, heat-treated and aggregate-removed extracts of clone pUC119 and pCSD3, respectively; lane 6, calpastatin segment partially purified by DEAE-cellulose chromatography.

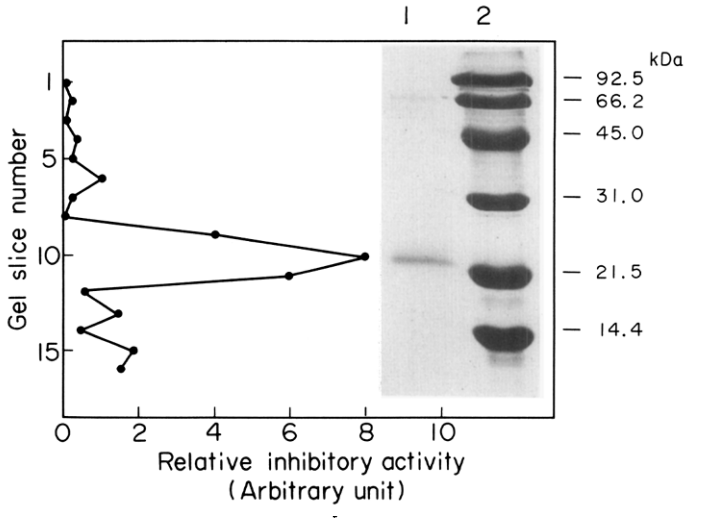

Fig. 4. Inhibitory activity of calpastatin segment extracted from SDS-polyacrylamide gel. After electrophoresis of the partially purified calpastatin segment on a 15% polyacrylamide gel, the inhibitor was extracted from the gel slices (0.5-cm segments) and assayed for calpain inhibition as described in ref. 17. For the detection of protein bands, a portion of the gel was stained with Coomassie Brilliant Blue. Lane 1, inhibitor; lane 2, molecular weight marker proteins.

cation procedures enriched the band of 22 kDa protein (lanes 3, 5, and 6) which was not detected in control cell extracts (lanes 2 and 4). The location of the inhibitory activity extracted from the electrophoresed gel coincided with the major band, confirming that the calpastatin activity was attributable to the protein of 22 kDa but not to the minor components of different molecular sizes (Fig. 4).

DISCUSSION

Previously we reported isolation and nucleotide sequence analysis of a pig calpastatin cDNA clone, pOBCS1 (5). Although the cDNA clone was not full-lengthed, it was found to contain three homologous regions, each being of approximately 140 amino acid residues (two of them were presented in ref. 5). In the present study, we selected the region Y of the cDNA for its expression in *E. coli* (Fig. 1). Unlike two other regions, X and Z, the region Y does not contain cysteine residues, whose sulfhydryl groups may form inter- or intra-molecular crosslinking of polypeptide. With the region

Y, the effects of such unexpected conformational changes of the calpastatin segment synthesized in bacteria on its function were thought to be excluded.

The calpastatin segment produced in E. coli was found to inhibit calpain I and calpain II (Fig. 2). The inhibition was calpain-specific, with no effect on papain and typsin (Table II). Heat treatment (boiling for 5 min) did not abolish the inhibition capability of the sample (Table I). These properties are entirely consistent with those of the authentic high-molecular-weight calpastatin (1, 15, 18), proving that the repetitive region is the functional unit of the proteinase inhibitor.

The calculated molecular weight of the inhibitor produced in E. coli is 14,852 excluding initiation methionine (19). The value contributed by calpastatin segment (130 amino acid residues) is 13,803. However, the molecular weight estimated by SDS-polyacrylamide gel electrophoresis was 22 kDa (Figs. 3 and 4). The discrepancy in the molecular weight values may be explained by anomalous electrophoretic behavior due to the deviated amino acid composition of calpastatin (6), as often explained for proline-rich proteins (20-22).

Specific inhibitory activity of the partially purified calpastatin segment produced in E. coli was estimated to be 292 units/mg. Considering the purity of the 22 kDa protein in the inhibitor sample (53.6%) which was estimated by densitometric analysis of the electrophoresed gel after Coomassie Brilliant Blue staining, the intrinsic activity of the inhibitor molecule was calculated to be 545 units/mg. This value was not greatly different from the values of pig calpastatins isolated from hearts and erythrocytes (900-1100 units/mg) which were previously shown to contain multiple binding sites for calpains (6). Therefore, the interactions among the repetitive regions of these naturally occurring calpastatin molecules may not be so much that they potentiate significantly the inhibitory function of each domain.

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