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# Pig leukocyte cysteine proteinase inhibitor (PLCPI), a new member of the stefin family

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A new stefin type low-$M_r$ cysteine proteinase inhibitor (PLCPI) was isolated from pig polymorphonuclear leukocytes as a contaminant of the cathelin sample. The inhibitor consists of 103 amino acids, and its $M_r$ was calculated to be 11,768. The inhibitor exhibits considerable sequence identity with inhibitors from the stefin family, particularly with human stefin A. The PLCPI is a fast acting inhibitor of papain and cathepsins L and S ($k_{ass} \geq 1 \times 10^6$ $M^{-1} \cdot s^{-1}$) and forms very tight complexes with these enzymes ($K_i \leq 190$ pM). The affinity for cathepsins B and H ($K_i \geq 125$ nM) was lower. These results also show that the inhibitory activity previously ascribed to cathelin was due to the presence of PLCPI.

Stefin; Papain; Cathepsin; Cathelin; Amino acid sequence; Kinetics

## 1. INTRODUCTION

Stefins are a group of cysteine proteinase inhibitors which may (as well as the members of the other two families of the cystatin superfamily, cystatins and kininogens) be involved in the control mechanism responsible for protein degradation. They act as tight and reversibly binding inhibitors of the papain-like intracellular cysteine proteinases [1]. Although it was suggested that stefins are intracellular proteins, there are some indications that they may also function extracellularly [2]. So far, mammalian stefins have been isolated and characterized from human, rat [1] and bovine tissues [3,4]. All stefins are acidic proteins with $M_r$ of about 11,000 and without carbohydrates and disulphide bonds [1,5,6]. Three regions of the inhibitors form close contacts with papain: two $\beta$ hairpin loops and the amino terminal part [7,8]. The central QVVAG region (first $\beta$ hairpin loop) was found to be highly conserved within most of the stefins [6]. In addition, the inhibitory properties were not significantly changed if slight modifications were present in either of the contact regions [9–12].

An inhibitor of cysteine proteinases, named cathelin, has also been isolated and characterized from pig polymorphonuclear leukocytes [13,14]. Its amino acid sequence exhibited remarkable similarity to the pro-regions of several antibacterial peptides, such as bovine Bac5 [15], rabbit CAP-18 [16], bovine indolicidin [17], rabbit 15-kDa protein [18], and to the homolog of porcine proline/arginine-rich antibacterial peptides [19]. These peptides are also present in large quantities in the cytoplasmic granules of polymorphonuclear leukocytes and are released after the elastase action [20]. Recently, it was shown that pro-Bac5 efficiently inhibits cysteine proteinase cathepsin L activity [21].

In this paper, we report the isolation and determination of the amino acid sequence of the cysteine proteinase inhibitor (PLCPI) obtained from pig polymorphonuclear leukocytes. Using a modified isolation procedure which was previously applied to the isolation of cathelin [13], we found that the cathelin sample was composed of two proteins, a stefin type cysteine proteinase inhibitor (PLCPI) and a non-inhibitory protein similar to pro-regions of antibacterial peptides [15–19]. In addition, the kinetics of the interaction of the PLCPI with some papain-like cysteine proteinases was studied.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Cm-papain Sepharose was prepared as described [22]. Bz-DL-Arg-2 naphtylamide, dithiothreitol and papain (2 × crystallized; EC 3.4.22.2) were purchased from Sigma (Germany), Z-Phe-Arg AMC from Serva (Germany) and Ep-475 from Peptide Research Foundation (Japan). Papain was further purified according to [23]. Sequencing reagents were obtained from Applied Biosystems (USA), clostripain (EC 3.4.22.8) from Sigma (Germany) and *Staphylococcus aureus* V-8 pro-

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*Abbreviations:* PLCPI, pig leukocyte cysteine proteinase inhibitor; Bz-, benzoyl; Cm-, carboxymethyl-; EDTA, ethylenediamine tetraacetic acid; Ep-475, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(3-guanidino)butane; -AMC, 4-methyl-7-coumarylamide; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PITC, phenylisothiocyanate; PTH, phenylthiohydantoin; Z-, benzyloxycarbonyl.

teinase from Miles Scientific (UK). ChromSpher C8 column (100 × 3 mm) was from Chrompack (Netherlands). Human cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15) were purified as described [24,25] as were bovine cathepsins H (EC 3.4.22.16) and S (EC 3.4.22.27) [26,27]. All other chemicals used were of analytical or sequential grade.

### 2.2. *Purification procedure*

The PLCPI was purified from the cytosol of pig leukocytes using a method that was previously described for the isolation of cathelin [13].The isolated sample was further applied to Cm-papain Sepharose. The non-bound proteins were removed with Tris buffer (0.1 M, 0.5 M NaCl) pH 7.8, and the bound proteins were eluted with 0.01 M NaOH, pH 11.5. The pH of eluted fractions was immediately adjusted to pH 7.5 with 3 M HCl.

All steps, unless otherwise indicated, were performed at 4°C.

### 2.3. *Analytical isoelectric focusing*

Analytical isoelectric focusing was performed on Desaga apparatus (Pharmacia, Sweden) following the instructions of the manufacturer.

### 2.4. *Inhibition assay and determination of kinetic constants*

For the routine assays, the papain and cathepsin L inhibitory activities of the samples were determined using Bz-DL-Arg-2 naphtylamide or Z-Phe-Arg-AMC as substrate [28].

Enzymes were active-site titrated by cysteine proteinase inhibitor Ep-475. Active-site titrated papin was then used to determine the molarity of the inhibitor [4].

The equilibrium dissociation constants ($K_i$) for the interaction between the inhibitor and the cathepsins B and H were determined using a stopped assay. Cathepsin B (130 nM final concentration) and cathepsin H (25 nM final concentration) were incubated for 20 min at 25°C with PLCPI at various concentrations (50–750 nM final concentrations) in 0.1 M phosphate buffer, containing 2 mM dithiothreitol and 1.5 mM EDTA, pH 6.0. Residual activities were determined under the same conditions as described previously [28] using Bz-DL-Arg-2 naphtylamide as substrate. The $K_i$ values were then calculated using the modified Ackermann-Potter equation [29].

Continuous rate assays using Z-Phe-Arg-AMC as a substrate were used for kinetic analysis of papain and cathepsins L and S with PLCPI. Variable concentrations of PLCPI and substrate (5 $\mu$M for papain, 10 $\mu$M for cathepsin L and 20 $\mu$M for cathepsin S) were dissolved in 1.97 ml of the same buffer, as described above. For cathepsin L 0.34 M acetate buffer, containing 2 mM dithiothreitol and 1.5 mM EDTA, pH 5.5, was used. The reaction was started by the addition of 30 $\mu$l of activated papain (380 pM final concentration), cathepsin L (60 pM final concentration) or cathepsin S (200 pM final concentration), respectively. All experiments were done under pseudo-first-order conditions with PLCPI concentrations at least 10-fold higher than the enzyme concentrations. Data were analysed by non-linear-regression analysis according to Morrison [30].

### 2.5. *Amino acid analysis*

Samples were hydrolysed in 6.0 M HCl at 110°C for 24 h. Analysis of the hydrolysates was performed on Applied Biosystems amino acid analyser (USA) with pre-column PITC derivatization.

### 2.6. *Amino acid sequence determination*

Amino acid sequence analyses were performed on an Applied Biosystems 475A liquid-pulse sequencer fitted with an on-line model 120A phenylthiohydantoin (PTH) analyzer.

The PLCPI, dissolved in 0.02 M Tris buffer, containing 0.01 M $CaCl_2$, 0.05% (w/w) Brij 35 and 2.0 M urea, pH 7.0, was digested by clostripain, which was added in an enzyme-to-substrate mass ratio of 1:50. The total incubation time was 3 h at 40°C.

*S. aureus* V-8 proteinase cleavage was performed in 0.5 M sodium lactate buffer, pH 4.0, at 37°C for 10 h with 2% (w/w) of proteinase.

Peptides were purified by HPLC (Milton Roy) using a reverse-phase ChromSpher C8 column, equilibrated with 0.1% trifluoroacetic acid and acetonitrile for gradient elution. Absorbance was monitored at 215 nm.

## 3. RESULTS AND DISCUSSION

PLCPI was isolated using the purification scheme for cathelin [13]. In addition, the affinity chromatography on Cm-papain Sepharose was introduced as a last step. The majority of the protein sample (~95%) was eluted from the column, while the minor part (~5%) was tightly bound to the carrier and could be eluted from the column only with 0.01 M NaOH. The non-adsorbed protein did not inhibit either papain or cathepsin L. Only the Cm-papain Sepharose bound protein exhibited inhibitory activity toward papain and cathepsin L. On analytical isoelectric focusing, both samples had the same acidic p$I$ value of 4.6 (Fig. 1) and SDS-PAGE revealed that the two proteins have the same $M_r$ of about 12,000 (not shown). These findings may explain why the two different proteins could not be separated previously [13]. Affinity chromatography proved to be an essential step in this purification procedure, as it efficiently separated the inhibitor from the non-inhibitory protein.

The inhibitor (PLCPI) was further subjected to detailed kinetic analysis for the interaction with papain and cathepsins B, H, L and S. The dependence of the observed first order rate constant, $k_{obs}$, over the range of inhibitor concentration was linear, suggesting that

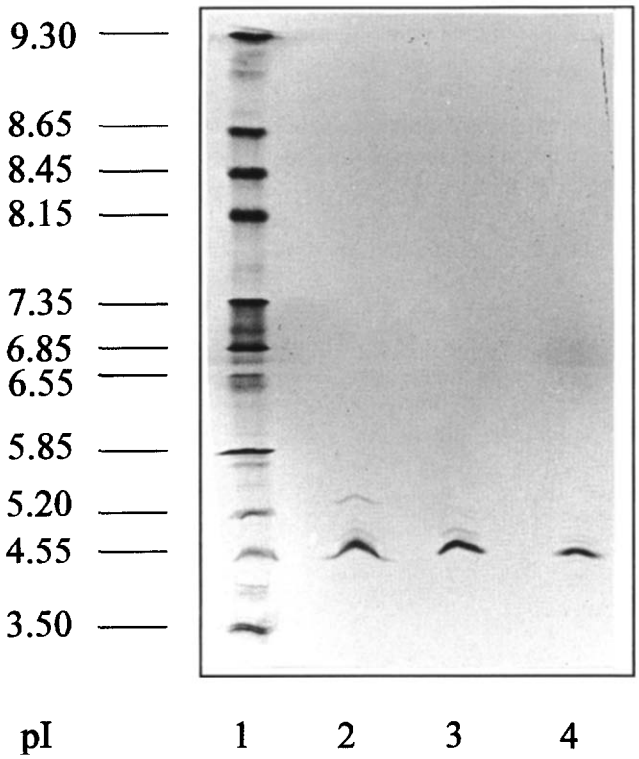


Fig. 1. Isoelectric focusing of the proteins (1) standards, (2) cathelin sample, (3) non-inhibitory protein, (4) PLCPI.

inhibition of papain and cathepsins L and S by PLCPI occurs in a single step. The $k_{ass}$, $k_{diss}$ and $K_i$ values are presented in Table I. The inhibition of papain and cathepsins L and S by PLCPI exhibits $k_{ass}$ values between $1 \times 10^6$ and $1.35 \times 10^7$ $M^{-1} \cdot s^{-1}$ and $k_{diss}$ values between $1.9 \times 10^{-4}$ and $6.3 \times 10^{-4}$ $s^{-1}$, yielding $K_i$s ranging from 46 to 190 pM. The determined $k_{ass}$ values are comparable to those for the binding of cystatins to the same enzymes [1]. Also, the $K_i$ values are similar to those previously determined for some other cystatin–cysteine proteinase interactions [1,2,4,11]. The PLCPI is shown to be a fast-acting and tight binding inhibitor of papain and cathepsins L and S. In contrast, the inhibition of cathepsins B and H by PLCPI is weak with $K_i$ values of 335 nM and 125 nM, respectively. The former constant is in good agreement with the $K_i$ values for the inhibition of human cathepsin B by human and bovine stefins B and C [1,12,4]. However, the inhibition of cathepsin H by PLCPI is significantly weaker than that of any of known stefins [1].

The amino acid sequence, as well as the strategy of the sequence determination of PLCPI, is presented in Fig. 2. Native inhibitor gave an unequivocal sequence up to the 40th sequencing cycle (uncleaved molecule in Fig. 2). The low initial yield of the run (~10%) indicated that a part of the applied material might be N-terminally blocked. The first set of peptides was obtained by clostripain hydrolysis. The sequences of the three CL peptides covered almost the whole protein chain (CL2-CL4 in Fig. 2). Peptide CL1 could not be sequenced, but its amino acid composition corresponds to the N-terminal sequence of the native molecule with a non-blocked terminus. Overlaps of these peptides were provided by the second set of peptides generated by the action of *S. aureus* V-8 proteinase (E1-E6 in Fig. 2). The C-terminal part of the inhibitor was established from CL4 and E6 peptides. Both peptides ended with Phe, which is not a cleavage site of highly specific clostripain or *S. aureus* V-8 proteinase.

From the obtained sequence results, it is evident that PLCPI consists of 103 amino acid residues with $M_r$ of 11,768 (Fig. 2). In Fig. 3 the amino acid sequence of

Table I

Equilibrium constants ($K_i$) and rate constants ($k_{ass}$ and $k_{diss}$) for the inhibition of cysteine proteinases by PLCPI

| Enzyme | $10^{-6} \times k_{ass}$ ($M^{-1} \cdot s^{-1}$) | $10^4 \times k_{diss}$ ($s^{-1}$) | $K_i$ (nM) |
|---|---|---|---|
| Papain | 1.0 ± 0.31 | 1.9 ± 0.39 | 0.19 ± 0.097 |
| Cathepsin B | ND | ND | 335 ± 50 |
| Cathepsin H | ND | ND | 125 ± 7 |
| Cathepsin L | 5.5 ± 0.65 | 3.7 ± 1.64 | 0.067 ± 0.036 |
| Cathepsin S | 13.5 ± 1.06 | 6.3 ± 1.25 | 0.046 ± 0.013 |

$K_i$ for the papain and cathepsins L and S were calculated from $k_{ass}$ and $k_{diss}$ values ($K_i = k_{diss}/k_{ass}$). $K_i$ for the cathepsins B and H were determined by equilibrium inhibition experiments. ND = not determined.

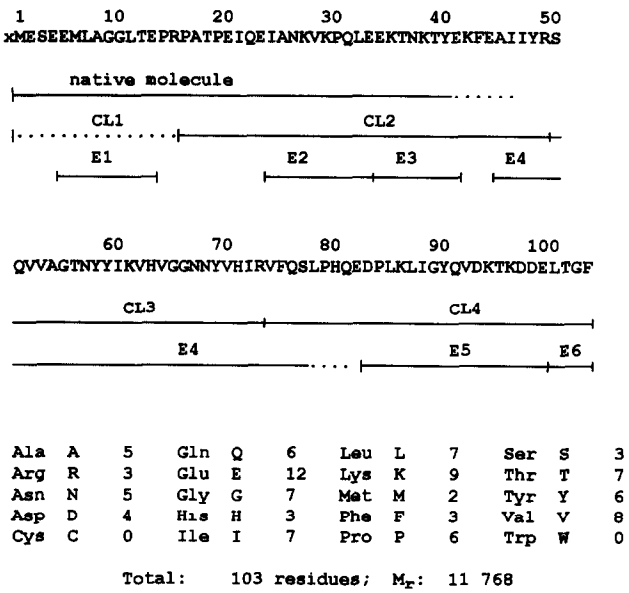


Fig. 2. The amino acid sequence and composition of PLCPI. CL-peptides were derived from clostripain fragmentation and E-peptides from *S. aureus* V-8 proteinase hydrolysis.

PLCPI is aligned with other mammalian stefin-type amino acid sequences. PLCPI has 70.4% sequence identity with human stefin A, 57.1% with human stefin B and rat cystatin $\beta$ and 53.1% with rat cystatin $\alpha$ and bovine stefins B and C. The level of identity with other members of the cystatin superfamily is much lower.

The partial amino acid sequence of the non-inhibitory protein was also determined and identified as cathelin (data not shown) [14]. It has been shown that cathelin shares significant similarity with the N-terminal regions of several antibacterial peptides, such as pro-Bac5, proindolicidin, CAP-18, 15-kDa protein [15–18] and homolog of proline/arginine-rich antibacterial peptides from porcine bone marrow [19], indicating that this protein is probably the pro-region or the N-terminal part analog of pig antibacterial peptide. The sequence alignments of these five proteins show high similarity in the N-terminal parts of the proteins, while the C-terminal part possesing the antibacterial activity is completely different. From the in vitro experiments of the processing of precursors, such as pro-Bac5 and pro-Bac7, it has become evident that the elastase is responsible for the release of antibacterial active peptides Bac5 and Bac7 [20].

From these studies, it is evident that previously described cathelin [14], which very likely corresponds to the N-terminal region of pig analog of antibacterial pro-peptides [19], does not show any inhibitory activity against cysteine proteinases. The observed inhibitory activity of the pig cathelin sample [13] is proved to be due to the presence of PLCPI. Therefore, the name cathelin should be excluded from the terminology of the cysteine proteinase inhibitors. The function of a puta-


Fig. 3. Alignment of amino acid sequences of PLCPI with those of human stefin A (HSTA), rat cystatin α (RCA), human stefin B (HSTB), rat cystatin β (RCB), bovine stefin B (BSTB) and bovine stefin C (BSTC). Residues identical to those in porcine inhibitor are marked with 'shading', numbering is according to human stefin A.

tive pro-region of mammalian leukocyte antibacterial peptides therefore has yet to be elucidated.

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