Purification of recombinant human prostromelysin

Studies on heat activation to give high- M_r and low- M_r active forms, and a comparison of recombinant with natural stromelysin activities

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Recombinant human prostromelysin was purified in a single step using Procion Red–Sephacrose chromatography. The purified prostromelysin was self-activated to high- M_r (45 000) and low- M_r (28 000) forms by incubation at 55 °C without the addition of extraneous activators. The two forms of stromelysin were subsequently separated, again using Procion Red–Sephacrose. Both of the heat-activated recombinant forms demonstrated similar specific activities (for the macromolecular substrates casein, gelatin, elastin, proteoglycan and type IV collagen) when compared with either heat- or trypsin-activated natural stromelysin. The heat-activated recombinant stromelysins both showed similar abilities to potentiate activation of human procollagenase when compared with trypsin-activated natural stromelysin.

INTRODUCTION

Stromelysin, otherwise named proteoglycanase and matrix metalloproteinase-3, is one member of a family of metalloendopeptidases implicated in the turnover of connective tissue (Galloway *et al.*, 1983; Okada *et al.*, 1986; Wilhelm *et al.*, 1987; Murphy & Docherty, 1988). It is secreted as a latent proenzyme (M_r 57 000) and a minor glycosylated form (M_r 60 000), and once activated exhibits a broad substrate specificity acting on proteoglycans, gelatin, laminin, fibronectin, elastin, type IV collagen and immunoglobulins. This degradative ability with its broad pH optimum (Galloway *et al.*, 1983) indicates a possible key role for this enzyme in the regulation of the turnover of connective tissue matrix. Extensive structural studies of stromelysin have been hampered by a scarcity of the natural protein and the complexity of the procedure for its purification. Recently, the gene for prostromelysin has been cloned and expressed in mammalian cells (Docherty & Murphy, 1990) in order to provide larger quantities of the protein for such studies. In this paper, we report a one-step procedure for the purification of recombinant prostromelysin.

The mode of activation of prostromelysin (PSL) *in vivo* is unknown, although methods for activation *in vitro* are documented which involve treatment with trypsin or 4-aminophenylmercuric acetate (APMA) (Chin *et al.*, 1985; Okada *et al.*, 1986). The proteinase or organomercurial treatment of PSL and other related metalloproteinases is thought to effect conformational changes in the latent enzyme molecules, ultimately resulting in a self-cleavage (Springman *et al.*, 1990), with a concomitant loss of M_r of 10 000. Under some conditions conversion to lower- M_r forms is observed (Chin *et al.*, 1985; Okada *et al.*, 1986, 1988). In this paper we present a novel and facile method for the generation of high- M_r (HM, 45 000) and low- M_r (LM, 28 000) forms of stromelysin using heat activation. The heat-activated recombinant forms were purified and their action on a range of substrates was compared with both APMA- and trypsin-activated natural stromelysin.

MATERIALS AND METHODS

Sources

Mouse EHS sarcoma type IV collagen was kindly provided by Professor K. Kuhn, Martinsried, Germany, and [3 H]elastin by Dr. M. Banda, San Francisco, CA, U.S.A. A mouse fibroblast C127 cell line expressing recombinant human PSL (Docherty & Murphy, 1990) was used to generate culture supernatants containing PSL (obtained from G. Renner, Fermentation Technology Department, Celltech).

Purification of PSL

PSL was purified by dye-ligand chromatography as described below. Procion Red HE3B–Sephacrose CL6B was prepared according to the method of Angal (1988). Crude culture supernatant was adjusted to pH 7.9 and supplemented with CaCl_2 to 5 mM, Brij 35 to 0.05 % (v/v) and NaN_3 to 0.05 % (w/v). The supernatant was applied to a Procion Red–Sephacrose column (13 cm $\times$ 5 cm) equilibrated in buffer A (25 mM-Tris/HCl, pH 7.9, 5 mM- CaCl_2 , 0.05 % NaN_3 and 0.05 % Brij 35) containing 150 mM-NaCl. The column was washed with 5 column vol. of the same buffer. PSL was eluted with buffer A containing 750 mM-NaCl. The purified PSL was stored at -70 °C, after dialysis against 25 mM-Tris/HCl (pH 7.5)/10 mM- CaCl_2 containing 0.05 % Brij 35 and 0.05 % NaN_3 . The column was cleaned with buffer A containing 2 M-KCl and then stored in buffer A.

Purification of HM stromelysin (M_r 45 000)

Heat-activated (for conditions, see later) PSL was applied to a Procion Red–Sephacrose column (2.6 cm $\times$ 2 cm) equilibrated with buffer A. The column was washed with several column volumes of buffer A until the A_{280} reached baseline. Stromelysin (M_r 45 000 and 48 000) was eluted using a 0.15–2.0 M-NaCl gradient (6.25 column vol.).

Purification of LM stromelysin (M_r 28 000)

Heat-activated purified PSL was applied to a Procion Red–

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; PSL, prostromelysin; APMA, 4-aminophenylmercuric acetate; PTH, phenylthiohydantoin; PVDF, poly(vinylidene difluoride); HM, high- M_r ; LM, low- M_r .

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Sephacrose column (1.2 cm $\times$ 0.8 cm) equilibrated with buffer A. The column was washed with buffer A. The flow-through and wash fractions were collected and pooled after analysis by SDS/PAGE.

Natural stromelysin purification

PSL was purified from the culture medium of human gingival fibroblasts stimulated with partially purified pig mononuclear cell cytokines and phorbol 12-myristate 13-acetate (10 ng $\cdot$ ml⁻¹) by the method of Galloway *et al.* (1983) with minor modifications. Culture medium (6 litres) was initially concentrated by adjusting to 25 mM-Tris/HCl, pH 8.5, and binding to dextran sulphate beads equilibrated with buffer A (pH 8.5) containing 0.15 M-NaCl. All metalloproteinases bound to this matrix were eluted with column buffer containing 1 M-NaCl. The eluate was adjusted to a conductivity of 2 mS by dialysis against buffer A, pH 7.3, and chromatographed on DEAE-Sephacrose. Gelatinase bound to this matrix, whereas collagenase and stromelysin did not. The column flow-through was loaded directly on to heparin-Sephacrose (Pharmacia) equilibrated in buffer A. PSL did not bind or was lightly retarded by this matrix, whereas collagenase bound strongly. The pool containing PSL activity was chromatographed on Procion Red-Sephacrose (Pharmacia) equilibrated with buffer A, pH 7.2. The enzyme was in a fully latent form and bound to this matrix (LM human stromelysin as described above and LM rabbit stromelysin are only slightly retarded; Galloway *et al.*, 1983). PSL was eluted with a gradient of 0–1 M-NaCl in buffer A. Fractions containing PSL were finally chromatographed on Ultrogel AcA 44, from which PSL was eluted as a single protein and activity peak.

Recombinant human tissue inhibitor of metalloproteinase (TIMP)

Recombinant human TIMP expressed in C127 mouse fibroblast cells was purified as described in Williamson *et al.* (1990).

Heat activation of PSL

Samples of purified PSL (100 μ l) were incubated at 55 °C in 25 mM-Tris HCl (pH 7.5)/10 mM-CaCl₂ containing 0.05 % (v/v) Brij 35 and 0.05 % (w/v) NaN₃ for various time periods.

Activity assays

Assays using ¹⁴C-labelled casein, ¹⁴C-labelled type 1 collagen and ¹⁴C-labelled gelatin were carried out as described by Galloway *et al.* (1983). The proteoglycan bead assay of Nagase & Woessner (1980) and the ³H-labelled insoluble elastin assay of Banda & Werb (1981) were used. The ability of the stromelysin preparations to potentiate the trypsin activation of collagenase was assayed using recombinant procollagenase, as described by Murphy *et al.* (1987).

Electrophoresis

SDS/PAGE was carried out by the method of Laemmli & Favre (1973), with either silver staining according to Merrill *et al.* (1981) or Coomassie Blue staining. Protein purity was determined by quantitative microdensitometry of Coomassie Blue-stained gels.

N-Terminal amino acid sequencing

Purified PSL was desalted into distilled water (Pharmacia, PD10) and applied to a Biobrene-treated glass-fibre disc which was dried at 45 °C. The disc was loaded on to an Applied Biosystems 470A Gas Phase Sequencer and N-terminally sequenced by Edman degradation. The resulting phenylthiohydantoin (PTH)-derivatized amino acids were analysed with an on-line Applied Biosystems 120A HPLC.

Heat-activated HM and LM stromelysins were electrophoresed on SDS/PAGE using the methods of Laemmli (1970) and Hunkapiller *et al.* (1983), and electroblotted on to poly(vinylidene difluoride) (PVDF) membranes (Matsudaira, 1987). Membranes were stained using 0.1 % (w/v) Ponceau S dye in 1 % aqueous acetic acid (Aebersold *et al.*, 1987), and the relevant bands of *M_r* 45000 and 28000 were removed and sequenced as above.

Protein determinations

*A*₂₈₀ values were measured and an *A*^{0.1%} of 1.0 assumed for pure protein samples.

RESULTS

Purification of recombinant PSL

Procion Red-Sephacrose chromatography gave an effective one-step purification of PSL from the C127 cell culture supernatant. The PSL was eluted as a single peak and the pooled eluate was dialysed and analysed by SDS/PAGE. The purified PSL (Fig. 1, lane 2) appeared as two bands (*M_r* 57000 and 60000 in a 3:1 ratio by microdensitometry) with a combined purity of 98 %. The major contaminants of crude culture supernatants (*M_r* 200000) were washed off the column with 2 M-KCl (Fig. 1, lane 3).

Characterization of purified PSL

The N-terminal sequence of the purified PSL was determined to be Tyr-Pro-Leu-Asp-Gly-Ala, which is identical to that reported for the cDNA-derived amino acid sequence of human PSL (Whitham *et al.*, 1986; Saus *et al.*, 1988).

Activation of PSL using heat

Samples of purified recombinant PSL (0.5 mg $\cdot$ ml⁻¹) were incubated at 55 °C for various periods of time, then boiled with Laemmli sample buffer with or without β -mercaptoethanol and subjected to SDS/PAGE in 7.5–15 % polyacrylamide gradient gels. Gels were stained with Coomassie Blue (Fig. 2).

After 25 min of incubation the majority of PSL had been converted to lower-*M_r* forms (HM, *M_r* 45000 and 48000; LM, *M_r* 28000) and a number of peptides (*M_r* < 14000). Under non-reducing conditions both PSL and the HM form exhibited faster mobility, suggesting the presence of a disulphide-bonded molecule (results not shown).

Fig. 1. Purification of recombinant PSL

Samples of recombinant PSL before and after purification were analysed by SDS/PAGE under reducing conditions and by Coomassie Blue staining. Lane 1, C127 cell culture supernatant containing PSL; lane 2, pooled 0.75 M-NaCl eluate from Procion Red-Sephacrose column; lane 3, pooled 2 M-KCl wash from Procion Red-Sephacrose column.

Fig. 2. Activation of recombinant PSL by heat

Purified recombinant PSL was incubated at 55 °C for various periods of time (as described in the Materials and methods section): lane 1, 0 min; 2, 25 min; 3, 45 min; 4, 90 min; 5, 180 min; 6, 4.5 h; 7, 6 h. Lane 8 contains M_r markers. The samples were mixed with reducing sample buffer, electrophoresed on a 7.5–15% gradient polyacrylamide gel and stained with Coomassie Blue.

Fig. 5. Purification of HM and LM stromelysin

The preparations of stromelysin were electrophoresed on 5–15% polyacrylamide gradient gels under non-reducing conditions and stained with Coomassie Blue. Lane 1, PSL purified using Procion Red-Sepharose; lane 2, heat-activated PSL; lane 3, LM stromelysin (M_r 28000) purified from heat-activated PSL using Procion Red-Sepharose; lane 4, HM stromelysin (M_r 45000 and 48000) purified from heat-activated PSL using Procion Red-Sepharose.

Fig. 3. Comparison of natural PSL activation by alternative methods: effect of TIMP

Natural PSL (lanes a–d) and recombinant PSL (e–h) were analysed by SDS/PAGE after no treatment or activation (a,e), incubation at 55 °C for 1 h (b,f), incubation with 1.5 mM-APMA at 37 °C for 2 h (c,g), or by treatment with 10 μ g of trypsin/ml at 37 °C for 2 h, or treatment with 10 μ g of trypsin/ml at 37 °C for 30 min (d,h). In the presence of an approx. 2-fold excess of TIMP (determined by titration with the trypsin-activated stromelysin; lanes i–l), activation was considerably slowed (i,j as b,c and k,l as f,g), but not totally inhibited. Protein bands were visualized by silver staining. The mobilities of standard proteins are indicated. The TIMP band is marked with an arrow.

Fig. 4. A_{280} profile for Procion Red-Sepharose chromatography used to purify HM stromelysin (as described in the Materials and methods section)

After prolonged incubation (3 h or greater) all of the PSL had been converted to the LM form or peptides. The LM form appeared to be converting to a slightly smaller version (M_r 26000). This conversion was a much slower process than the generation of the M_r -28000 form, and only about 50% had been converted after 24 h of incubation (results not shown). The peptides of M_r < 14000 were also further degraded during the incubation period.

Comparison of natural and recombinant PSL activation

Natural PSL could be activated by incubation at 55 °C over a similar time course to that for the recombinant enzyme (Fig. 3). Both natural and recombinant PSL could be activated by exposure to 1.5 mM-APMA at 37 °C for 2 h, or by digestion with trypsin (10 μ g $\cdot$ ml⁻¹), yielding HM stromelysin. The presence of an excess of TIMP markedly slowed the conversion of the proenzymes to lower- M_r forms, during either activation at 55 °C or APMA-mediated activation.

Purification of HM stromelysin

HM stromelysin (M_r 45000 and 48000) was isolated from a sample of heat-activated PSL (0.18 mg $\cdot$ ml⁻¹, incubated for 15 min) using Procion Red-Sepharose. The HM stromelysin was eluted as the first of two peaks using a linear NaCl gradient (Fig. 4). The second peak contained both PSL and HM stromelysin by analysis on SDS/PAGE. The HM stromelysin peak fractions were pooled and re-analysed on SDS/PAGE. The sample comprised a doublet of protein bands (M_r 45000 and 48000 in a 3:1 ratio) typical of HM stromelysin (Fig. 5, lane 4).

Purification of LM stromelysin

LM stromelysin (M_r 28000) was isolated from a sample of heat-activated PSL (0.12 mg $\cdot$ ml⁻¹, incubated for 2 h and 30 min) using Procion Red-Sepharose. The LM stromelysin was slightly retarded on Procion Red-Sepharose (Fig. 4) and was collected in the flow-through fractions. The flow-through samples were analysed by SDS/PAGE (Fig. 5, lane 3) and LM-stromelysin-containing fractions were pooled. The purified LM stromelysin comprised approximately four protein bands around M_r 28000.

N-Terminal sequences of HM and LM stromelysins

The N-terminal sequences of HM and LM stromelysin determined after SDS/PAGE and blotting were both Phe-Arg-Thr-

Phe-Pro-Gly. LM stromelysin did not bind concanavalin A-Sepharose (results not shown); therefore the heterogeneity of LM stromelysin can be attributed to variable C-terminal processing.

Comparative substrate activities

The relative abilities of trypsin-activated natural and recombinant stromelysins (HM form; Fig. 3) and the purified heat-activated HM and LM forms of the recombinant enzyme to degrade a number of protein substrates were assessed. Table 1 shows that all forms of the enzyme were able to degrade casein, gelatin, elastin and proteoglycan beads with comparable specific activities. Activity generated by APMA treatment gave similar results (results not shown). The variously activated forms of both natural and recombinant stromelysin showed a similar action on soluble type IV collagen at 26 °C (Fig. 6). At 35 °C the enzyme

Table 1. Comparison of the specific activities of various stromelysins on macromolecular substrates

Data shown are average of duplicates from a single experiment. All activities were measured on at least four occasions and always gave similar results. Rec. SL, recombinant stromelysin; CS, chondroitin sulphate.

Substrate	Specific activities			
	Trypsin-activated	Rec. SL		Native SL (trypsin-activated)
		Heat-activated HM	LM	
Casein ($\mu\text{g}/\text{min}$ per mg)	294	342	318	366
Gelatin ($\mu\text{g}/\text{min}$ per mg)	26	35	37	30
Elastin ($\mu\text{g}/\text{h}$ per mg)	129	130	134	130
Proteoglycan beads ($\mu\text{gCS}/15$ h per mg $\times 10^{-3}$)	521	650	450	837

Fig. 6. Degradation of soluble type IV collagen by activated stromelysins

Type IV collagen prepared from the EHS sarcoma was incubated at 26 °C for 15 h as follows: lane a, alone; b, with trypsin-activated recombinant stromelysin; c, with trypsin-activated natural stromelysin; d, with purified heat-activated high- M_r recombinant stromelysin; and e, with purified heat-activated low- M_r recombinant stromelysin. The products were analysed by SDS/PAGE under reducing conditions, with Coomassie Blue staining. The mobility of the α and β chains of type I collagen and of standard proteins ($M_r \times 10^{-3}$) are indicated.

Fig. 7. Potentiation of procollagenase activation by forms of activated stromelysin

Latent procollagenase (300 ng) could be activated ($\blacktriangle$) to a limited extent by treatment with trypsin ($10 \mu\text{g} \cdot \text{ml}^{-1}$) at 37 °C for 30 min (followed by addition of a 10-fold excess of soya bean trypsin inhibitor). Trypsin activation in the presence of increasing concentrations of trypsin-activated recombinant stromelysin ($\square$), trypsin-activated natural stromelysin ($\circ$), purified heat-activated HM recombinant stromelysin ($\blacklozenge$) and purified, heat-activated LM recombinant stromelysin ($\bullet$) gave up to an 8-fold increase in collagenase activity relative to trypsin alone. Addition of maximal amounts of any form of stromelysin to collagenase pre-activated with trypsin had no effect on collagenase activity ($\triangle$).

forms totally degraded the collagen. As described previously for rabbit stromelysin (Galloway *et al.*, 1983), human stromelysins can solubilize cross-linked type IV collagen that would not normally penetrate the polyacrylamide gel, giving an apparent increase in high- M_r material on the gel (Fig. 6).

Both purified heat-generated HM and LM stromelysins were equipotent with trypsin-activated recombinant PSL or trypsin-activated natural PSL in the potentiation of recombinant procollagenase activation in the presence of trypsin (Fig. 7). By the use of much higher concentrations of procollagenase in these experiments (5 – $10 \mu\text{g} \cdot \text{ml}^{-1}$) than described previously (Murphy *et al.*, 1987), it was found that as little as 0.1 – 1.0 mol of stromelysin/10 mol of trypsin-activated collagenase gave a marked increase in activity.

DISCUSSION

Recombinant human PSL was purified to homogeneity from C127 cell culture supernatants using Procion Red-Sepharose. Procion Red-agarose has previously been used in purification procedures for human PSL from skin fibroblasts (Wilhelm *et al.*, 1987) and for stromelysin from rabbit bone and synovium (Galloway *et al.*, 1983; Chin *et al.*, 1985), but purification of PSL from natural sources involves multiple chromatographic steps to remove contaminants, including other metalloproteinases. Okada *et al.* (1988) reported the use of an anti-(human collagenase) immunoabsorbent column as a further purification step for PSL. The recombinant PSL offers a source of material free of other human metalloproteinases. Additionally, as only one chromatographic step is used, the likelihood of inadvertent activation of the PSL is minimized. The purified recombinant PSL consists of two forms (M_r 57 000 and 60 000). The higher- M_r form has been previously reported to be a glycosylated variant by its removal on concanavalin A-Sepharose (Okada *et al.*, 1988) and by removal of the carbohydrate moiety with endoglycosidase F (Wilhelm *et al.*, 1987). The proportions of the two variants are similar to those found in natural human stromelysin.

A novel activation procedure using heat is described for purified recombinant PSL from which different activated forms can be derived. The proportion of HM:LM stromelysin was dependent upon length of the incubation. The heat activation

method is simply performed and effective, without the need to introduce extraneous molecules into the sample. Traditional methods of activating metalloproteinases involve incubations with proteinases (e.g. trypsin, chymotrypsin, plasmin, plasma kallikrein and thermolysin), or treatment with APMA (Murphy *et al.*, 1987; Wilhelm *et al.*, 1987; Okada *et al.*, 1988). The HM and LM forms of stromelysin were purified on Procion Red-Sepharose to remove residual PSL and the contaminating active form respectively. Both heat-activated forms of recombinant stromelysin displayed similar specific activities with respect to many different substrates. Thus a large part of the C-terminal portion is not necessary for enzyme activity. Both forms also showed similar substrate activities compared with trypsin-activated recombinant and natural stromelysins.

The contribution of stromelysin to the full activation of collagenase has been clarified by Murphy *et al.* (1987) and Suzuki *et al.* (1990). Stromelysin is thought to carry out a further cleavage of trypsin-activated collagenase which renders it fully active. Both heat-activated forms of stromelysin also showed this activation property concomitant with a loss in M_r , as is seen with trypsin-activated stromelysin (results not shown). Thus the C-terminal portion of stromelysin is shown not to be required for full activation of collagenase.

Both HM and LM stromelysins were shown to have the N-terminal sequence Phe-Arg-Thr-Phe-Pro-Gly. This indicates that LM stromelysin is generated by the loss of approximately 150 amino acids from the C-terminus of stromelysin. APMA-activated rabbit stromelysin is also reported to have a phenylalanine at its N-terminus (Whitham *et al.*, 1986). In contrast, trypsin-activated stromelysin is reported to start two residues later (Wilhelm *et al.*, 1987). These differences do not apparently affect enzyme activity with high- M_r substrates. The low- M_r form of stromelysin purified from rabbit bone (Galloway *et al.*, 1983) was shown to represent the N-terminal portion of a larger molecule (Whitham *et al.*, 1986). Removal of the C-terminal portion of rat stromelysin (transin) by a protein engineering method also yielded an active proteinase (Sanchez-Lopez *et al.*, 1988). These data support the concept that the connective tissue metalloproteinases, including stromelysin, consist of a propeptide that is lost on activation, an N-terminal proteinase domain, and a C-terminal domain whose function remains to be discovered. Collagenase has been shown to lose the C-terminal portion in some circumstances and to retain some proteolytic activity, but with a loss of specificity for collagen (Birkedal-Hansen *et al.*, 1988; Clark & Cawston, 1989; G. Murphy, unpublished work). In contrast, the LM form of stromelysin appears to mimic the activities of the HM form on macromolecular substrates. This mini-enzyme molecule may be well suited to biophysical studies.

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