A simple two-step purification of protease nexin

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This paper describes a simple purification procedure for protease nexin, a serine proteinase inhibitor secreted by cultured human fibroblasts that regulates proteinase activity at and near the cell surface. The first step in the procedure takes advantage of the high-affinity binding of protease nexin to dextran sulphate–Sephadex. This step eliminates the need for prior concentration of the serum-free fibroblast-conditioned medium, since protease nexin binds to the resin in the presence of physiological saline. The use of dextran sulphate also provides an affinity resin with considerably less variability than the heparin-based resins previously used. Final purification to homogeneity involves a combination of DEAE–Sephadex in-line with dextran sulphate–Sephadex to simultaneously purify and concentrate the protein. Purified protease nexin is shown by Ouchterlony analysis and peptide mapping to be immunologically and structurally distinct from antithrombin III and heparin cofactor II, two plasma proteinase inhibitors with similar properties.

INTRODUCTION

Protease nexin (PN) is a serine proteinase inhibitor first described by Baker *et al.* (1980) as a secreted protein from human fibroblasts. PN inhibits certain proteinases by forming a covalent complex with the proteinase, apparently via the active-site serine residue. The complexes then bind back to the fibroblasts and are rapidly internalized and degraded (Low *et al.*, 1981). These features suggest that PN may regulate proteinase activity in the immediate extracellular environment. In particular, PN inhibits thrombin, urokinase and plasmin with rapid second-order kinetics. Interestingly, heparin has been shown to increase the rate at which PN inhibits thrombin by over 100-fold (Scott *et al.*, 1985). This property is shared by two plasma thrombin inhibitors, antithrombin III (ATIII) and heparin cofactor II (HCII) (Rosenberg & Damus, 1973; Tollefsen & Blank, 1981). Comparisons of the amino acid compositions of PN with ATIII and HCII in fact indicate that PN is closely related to these inhibitors (Scott *et al.*, 1985).

Structural analysis of PN was made possible by its purification by Scott & Baker (1983), and later by a modified procedure by Scott *et al.* (1985). This most recent procedure involves concentration of serum-free human fibroblast-conditioned medium by use of a hollow-fibre filter, then chromatography on heparin–Sephadex, followed by concentration against Aquacide II before Bio-Gel P-100 chromatography. A limitation of this procedure has been that the batch of heparin used for the affinity column is critical to the purification, since there is variability of heparin preparations. We found that contaminants of different M_r values were eluted from the heparin–Sephadex column, depending on the source of the heparin. The present investigation was therefore undertaken to devise a more reproducible method for PN purification that does not involve the use of heparin. In addition, in view of the similarities among PN, ATIII, and HCII, it was decided to determine whether purified

PN has immunological or structural homology to ATIII or HCII.

MATERIALS AND METHODS

Cell culture

Human foreskin fibroblasts were isolated by mincing neonatal foreskins in 15 mM-Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 100 units of penicillin/ml, 100 µg of streptomycin/ml, 50 µg of gentamicin/ml, 0.1% collagenase (Millipore) and 0.1% Dispase (Boehringer Mannheim). Minced foreskins were kept at 4 °C for 1 h to allow diffusion of the proteinases into the tissue, then warmed at 37 °C for 1 h. Cells were then triturated and resuspended in DMEM containing 10% (v/v) foetal-calf serum (Gibco) and plated in two 100 mm plates (Corning) per foreskin. Cells were passaged at a 1:4 split ratio in DMEM containing 10% (v/v) foetal-calf serum but without gentamicin.

Roller-bottle (1750 cm²) (Falcon) cultures were initiated by trypsinizing four confluent 100 mm plates with 0.05% trypsin and adding this to 150 ml of DMEM containing 10% (v/v) foetal-calf serum. After reaching confluence, roller-bottle cultures were incubated for 1 day with 150 ml of serum-free DMEM containing 0.1% bovine serum albumin (radioimmunoassay grade; Sigma Chemical Co.). This medium was discarded and replaced with 150 ml of fresh medium, and the cultures were incubated for 2 days. This medium was collected, and 150 ml of fresh medium was added and collected for 3 more days. Cells were returned to DMEM containing 10% (v/v) foetal-calf serum for 1 week before collection of serum-free medium was resumed.

Electrophoresis

Protein samples were electrophoresed on 10% T/2% C SDS/polyacrylamide gels at 20 mA according to the

Abbreviations used: PN, protease nexin; ATIII, antithrombin III; HCII, heparin cofactor II; DMEM, Dulbecco's modified Eagle's medium.

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method of Ornstein (1964) and Davis (1964), with 0.05% SDS in the reservoir buffer, modified from Laemmli (1970). The sample buffer contained 1% (w/v) SDS, 10% (v/v) glycerol, 2 mM-EDTA, 5% (v/v) 2-mercaptoethanol, 0.1 mg each of Phenol Red and Bromophenol Blue/ml and 15 mM-Tris/HCl buffer, pH 6.8. It was diluted 1:1 with sample, and boiled for 3 min. Gels were silver-stained by the method of Merril *et al.* (1981). For autoradiography, gels were stained for 2 h in 0.05% Coomassie Brilliant Blue R (Sigma Chemical Co.) in methanol/acetic acid/water (5:1:5, by vol.) and destained in 5% (v/v) ethanol/7% (v/v) acetic acid, dried, and exposed to XAR-5 film (Kodak) at -70°C with a Cronex Lightening Plus intensifying screen (Du Pont).

Assay for PN

The SDS/polyacrylamide-gel-electrophoretic assay described above was employed to identify fractions containing PN and to quantify PN activity. Highly purified human thrombin (about 3000 N.I.H. units/mg, generously supplied by Dr. John W. Fenton, II, New York State Department of Health, Albany, NY, U.S.A.) was iodinated to $2.6 \times 10^4 - 3.9 \times 10^4$ Ci/mmol by using the Iodogen (Pierce Chemical Co.) method as previously described (Baker *et al.*, 1980). ^{125}I -thrombin was diluted to 0.167 ng/ml in 150 mM-NaCl/20 mM-imidazole (grade III; Sigma Chemical Co.) buffer, pH 7.4, containing 0.3 unit of heparin (167.8 units/mg; Calbiochem)/ml. A 15 μl portion of this mixture was added to 10 μl of PN for 10 min at room temperature (approx. 24°C); the reaction was then quenched with 25 μl of electrophoresis sample buffer. The samples were not boiled, to avoid dissociation by the Tris of the PN- ^{125}I -thrombin complexes. Samples were then electrophoresed as described above. PN activity was detected by drying the gels and performing autoradiography; PN-containing samples showed a 78000- M_r band corresponding to PN- ^{125}I -thrombin complexes. For quantification, bands containing the PN- ^{125}I -thrombin complexes (which migrate as a doublet) were excised and their radioactivities counted in a γ -radiation counter (Beckman). One unit of activity is defined as the quantity of the inhibitor required to form the same amount of complex with ^{125}I -thrombin as 10 pmol of purified PN.

Synthesis of dextran sulphate-Sephacrose

Sephacrose CL-6B (Sigma Chemical Co.) was coupled to dextran sulphate after epoxide activation of the resin. Briefly, 300 ml of Sephacrose was washed with 4 litres of water and aspirated to near-dryness on a Buchner funnel. The resin was resuspended in 150 ml of 1 M-NaOH containing 2 mg of NaBH_4 /ml; 150 ml of butane-1,4-diol diglycidyl ether (Aldrich Chemical Co.) was then added. The resin was gently mixed for 7 h at room temperature, then washed with 4 litres of water and dried on a Buchner funnel. A 10 g portion of dextran sulphate (Pharmacia) in 250 ml of 0.2 M- NaHCO_3 , pH 11, was added, and the resin was gently mixed for 18 h at 37°C (Scouten, 1981). Sites on the resin that had not reacted were blocked by adding 27 ml of ethanolamine to the mixture and mixing for 24 h at 37°C . The resin was washed with 2 litres of 0.2 M- NaHCO_3 , pH 11, 2 litres of 0.1 M-sodium acetate buffer, pH 4, and then with 4 litres of phosphate-buffered saline (137 mM-NaCl/2.7 mM-

KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4), and stored in phosphate-buffered saline containing 0.02% NaN_3 (Pharmacia Fine Chemicals, 1983).

Purification of PN

A 5-litre volume of serum-free conditioned medium was made 0.2 mM with respect to phenylmethanesulphonyl fluoride and 0.02% with respect to NaN_3 and chilled to 4°C . All subsequent steps were performed at 4°C . The conditioned medium was then loaded at 70–100 ml/h on a 2.5 cm $\times$ 40 cm column of dextran sulphate-Sephacrose equilibrated in phosphate-buffered saline. The column was washed with phosphate-buffered saline until the A_{280} of the effluent returned to baseline, then eluted with a 1.5-litre linear gradient of 0.15–1.2 M-NaCl in the same phosphate buffer at 75 ml/h; 20 ml fractions were collected. Fractions containing the major ^{125}I -thrombin-complexing activity were pooled, and protein content was measured by the method of Bradford (1976) as modified by Spector (1978), with bovine γ -globulin as the standard.

The pooled protein was made 0.2 mM with respect to phenylmethanesulphonyl fluoride and 0.02% with respect to NaN_3 and diluted with 10 mM-sodium phosphate buffer, pH 7.0, to lower the conductivity to that of the starting buffer of the next columns, 50 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.0. The protein was then loaded at 45 ml/h on to a 10 ml column of DEAE-Sephacrose (Sigma Chemical Co.) whose effluent line was connected directly to a 1 ml column of dextran sulphate-Sephacrose. PN flowed through the DEAE-Sephacrose and bound to the dextran sulphate-Sephacrose, whereas the contaminants were adsorbed on the DEAE-Sephacrose. The columns were washed with 100 ml of starting buffer; the dextran sulphate-Sephacrose column was then eluted with 1 M-NaCl/20 mM-imidazole buffer, pH 7.0, at 10 ml/h, and 1 ml fractions were collected. Fractions containing homogeneous PN were quantified for protein content by their absorbance at 280 nm, by using the specific absorption coefficient $A_{280}^{1\%} = 14.2$ (Scott *et al.*, 1985). The DEAE-Sephacrose was also eluted with 1 M-NaCl/20 mM-imidazole buffer, pH 7.0, to recycle it for re-use.

Purification of ATIII and HCII

ATIII and HCII were purified by using a modification of the procedure of Tollefsen *et al.* (1982). Imidazole (20 mM) was substituted for Tris in all the buffers used. Briefly, human plasma was chromatographed on sulphated dextran as described by Tollefsen *et al.* (1982). However, a larger (5 cm $\times$ 21 cm) column of DEAE-Sephacrose was used for the second step and eluted with a 2-litre gradient of 0.05–0.2 M-NaCl. The fractions containing ATIII and HCII were applied to heparin-Sephacrose, and the HCII was eluted with a 1.6-litre gradient of 0.05–0.2 M-NaCl. The ATIII was eluted as a homogeneous preparation by using a 1-litre gradient of 0.2–1.5 M-NaCl. The HCII was further purified by the use of a 10 ml column of dextran sulphate-Sephacrose in 0.15 M-NaCl, replacing the DEAE-Sephacrose concentration step and Sephadex G-150 step. HCII was eluted with a 200 ml gradient of 0.3–0.5 M-NaCl. By use of this procedure both ATIII and HCII were isolated from the same batch of plasma.

Fig. 1. Chromatography of conditioned medium on dextran sulphate-Sepharose

A 5-litre volume of conditioned medium was loaded on a 2.5 cm × 40 cm column at 70–100 ml/h. The effluent was monitored for u.v.-absorbing material at 280 nm (—). The column was eluted with a 0.15–1.2 M-NaCl gradient (-----); 20 ml fractions were collected and pooled as shown by the black bar.

Ouchterlony analysis

Ouchterlony plates (Cooper Biochemical) were incubated for 2 days at room temperature with 5 μ l of antiserum and 2 μ g of each protein. Polyclonal antiserum to ATIII was a gift from Dr. Robert Rosenberg (Massachusetts Institute of Technology, Boston, MA, U.S.A.) and antiserum to HCII was a gift from Dr. Douglas Tollefsen (Washington University, St. Louis, MO, U.S.A.). PN used in this analysis and for peptide mapping was generously provided by Dr. Joffre Baker (University of Kansas, Lawrence, KS, U.S.A.). Ouchterlony plates were washed 4 days in phosphate-

buffered saline containing 0.02% NaN_3 at room temperature and stained with 0.05% Coomassie Brilliant Blue R in methanol/acetic acid/water (5:1:5, by vol.) for 30 min. Plates were destained in 5% (v/v) ethanol/7% (v/v) acetic acid and photographed.

Peptide mapping

A 10 μ g portion of each inhibitor was dialysed against 1 M-acetic acid and freeze-dried. The dried proteins were resuspended in 100 μ l of 70% (v/v) formic acid; 2 mg of CNBr (Aldrich Chemical Co.) in acetonitrile (2 g/ml) was then added (Gross, 1967). This digestion mixture was kept under N_2 for 24 h at room temperature in the dark. The samples were then evaporated under N_2 , resuspended in 100 μ l of water, frozen and freeze-dried. The digested proteins were analysed by electrophoresis in the urea/SDS/polyacrylamide-gel system of Swank & Munkres (1971), with digested myoglobin (Pharmacia) as standard. Gels were silver-stained as described by Rubin *et al.* (1982).

RESULTS

Purification of PN

As previously mentioned, the variability of commercial heparin preparations has limited its usefulness as an affinity ligand for purification of PN. In order to circumvent this problem, dextran sulphate-Sepharose was used instead of heparin-Sepharose. This change in affinity resin had the additional benefit that the PN was eluted from dextran sulphate-Sepharose at much higher concentrations of NaCl than from the heparin-Sepharose. This allowed the conditioned medium to be loaded directly on to the column, thus eliminating the need for a costly and time-consuming hollow-fibre filtration step.

PN was eluted from the column at 0.7–0.9 M-NaCl, as shown in Fig.1. This first step achieved over a 1000-fold purification (Table 1). PN in the fractions was identified by its ability to form complexes with ^{125}I -thrombin; the

Fig. 2. SDS/polyacrylamide-gel-electrophoresis assay of dextran sulphate-Sepharose fractions for PN activity

Samples (10 μ l) from the column fractions shown in Fig. 1 were incubated with 15 μ l of mixture containing 0.3 unit of heparin/ml and 0.167 ng of ^{125}I -thrombin/ml for 10 min at room temperature; 25 μ l of SDS sample buffer was then added. Samples were electrophoresed on 10% SDS/polyacrylamide gels at 20 mA, stained, dried and autoradiographed as described in the Materials and methods section. Fractions 42–50 were pooled.

Fig. 3. Chromatography of the pooled fractions from dextran sulphate-Sepharose on DEAE-Sepharose + dextran sulphate-Sepharose

The dextran sulphate-Sepharose fractions pooled as shown in Fig. 1 were diluted to the conductivity of 50 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.0, and loaded on a 10 ml DEAE-Sepharose column connected to a 1 ml dextran sulphate-Sepharose column. After being washed with the above buffer, the dextran sulphate-Sepharose column was eluted with 1 M-NaCl/20 mM-imidazole buffer, pH 7.0, as described in the Materials and methods section, and the effluent was monitored for u.v.-absorbing activity at 280 nm (—). The DEAE-Sepharose column was recycled by eluting it with 1 M-NaCl/20 mM-imidazole buffer, pH 7.0.

complexes were located in autoradiographs from SDS/polyacrylamide gels after electrophoresis (Fig. 2). As can be seen, a form of PN was eluted earlier in the gradient and peaked at fraction 39. This may be related to the low-affinity heparin-binding form of PN previously seen (Scott *et al.*, 1985). This molecule was not purified, as it was co-eluted with a number of contaminating proteins.

The second step of the purification took advantage of the fact that PN did not bind to DEAE-Sepharose at pH 7.0 in 50 mM-NaCl/10 mM-sodium phosphate buffer. Those fractions that contained the high-affinity form of PN and a series of high- M_r contaminants were pooled for the final step (fractions 42–50 in Fig. 1). Fractions containing lower- M_r contaminants, which were eluted earlier in the gradient, were not included, since the contaminants co-purified with PN. The pooled fractions were diluted to the conductivity of the starting buffer for the DEAE-Sepharose and were loaded on to the column. By simply attaching a 1 ml dextran sulphate-Sepharose column immediately after the DEAE-Sepharose, the unadsorbed PN was concentrated after it was purified. This column was then eluted with 1 M-NaCl/20 mM-imidazole buffer, pH 7.0, as was the DEAE-Sepharose to prepare it for re-use (Fig. 3). As shown in Fig. 4, the high- M_r contaminants were retained on the DEAE-Sepharose, and only the PN flowed through to the dextran sulphate-Sepharose (Fig. 4), resulting in approximately a 4-fold purification (Table 1). The PN eluted from the dextran sulphate-Sepharose was homogeneous, as judged by silver-stained SDS/polyacrylamide-gel electrophoretograms (Fig. 4).

The apparent losses of PN from the first dextran sulphate-Sepharose column (Table 1) were due in part to the low-affinity form of PN, which was not recovered, as well as loss of activity of PN in dilute solutions, as previously reported (Scott & Baker, 1983). Interestingly, PN activity was lost even in the presence of 0.1% bovine serum albumin, suggesting that it was not simply

Fig. 4. SDS/polyacrylamide-gel electrophoresis of PN from DEAE-Sepharose + dextran sulphate-Sepharose

Samples (10 μ l) from the column fractions shown in Fig. 3 were electrophoresed on 10% SDS/polyacrylamide gels and silver-stained. The left half of the gel shows the high- M_r contaminants eluted from the DEAE-Sepharose. The right half of the gel shows PN eluted as a homogeneous peak from the dextran sulphate-Sepharose.

Table 1. Purification of PN

For experimental details see the text.

Step	Total protein (mg)	Total PN activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Conditioned medium	5500	3140	0.571	100	1
Dextran sulphate-Sepharose	1.62	1060	654	33.8	1150
DEAE-Sepharose + dextran sulphate-Sepharose	0.133*	310	2330	9.9	4080

*Based on A_{280} , where $A_{1\text{cm}}^{1\%} = 14.2$. **Fig. 5. Ouchterlony analysis of PN, ATIII and HCII**

Portions ($2\ \mu\text{g}$) of PN, ATIII and HCII and $5\ \mu\text{l}$ of anti-ATIII and anti-HCII antibodies were incubated in agarose plates for 2 days at room temperature, washed for 4 days in phosphate-buffered saline containing 0.02% NaN_3 , stained with Coomassie Brilliant Blue R for 30 min and destained as described in the Materials and methods section.

adsorbing on vessel surfaces, but perhaps was due to some other effect, such as oxidation or proteolysis. The losses seen in the final step, on the other hand, may reflect adsorptive losses on the vessel, since the protein concentration was quite low. It was not possible to compare the recoveries directly with prior published procedures, since the total activity of PN in the starting conditioned medium has not previously been quantified.

Immunological comparison and peptide mapping

Ouchterlony analysis with polyclonal rabbit antisera against ATIII and HCII failed to show any cross-reactivity between these antisera and PN (Fig. 5), suggesting that PN did not share major antigenic determinants with ATIII or HCII. This finding was supported by the pattern of CNBr-cleavage fragments generated from the three inhibitors. Few, if any, common peptides were seen between PN and either ATIII or HCII, even on a one-dimensional separation (Fig. 6). Taken together, these data suggest that, although PN shares some functional homology with ATIII and HCII, neither of them is precursor to PN.

DISCUSSION

The data show that PN has been purified to apparent homogeneity from serum-free human fibroblast-conditioned medium by a two-step procedure, resulting in over a 4000-fold purification and a recovery of 9.9% . The procedure described simplifies previous methods in

Fig. 6. Peptide maps of the CNBr-digested inhibitors

Portions ($10\ \mu\text{g}$) of PN, ATIII and HCII were digested for 24 h in 70% (v/v) formic acid containing $20\ \text{mg}$ of CNBr/ml at room temperature. The digested and freeze-dried peptides were separated by electrophoresis on a urea/SDS/polyacrylamide gel, and silver-stained as described in the Materials and methods section. Electrophoresis standards (myoglobin peptides) are shown in the left lane, and ATIII, PN and HCII are in the other lanes as shown.

several ways. With the present method, it was not necessary to concentrate the conditioned medium before the first chromatographic column, saving time and expense in the use of a hollow-fibre filtration apparatus. The use of dextran sulphate-Sepharose instead of heparin-Sepharose for the first step also eliminates the problem of variability in commercial heparin preparations. This resin may in fact find general use as a replacement or adjunct step to heparin-Sepharose in other purifications, such as in the isolation procedure for HCII described in the Materials and methods section.

The combination of DEAE-Sepharose and dextran sulphate-Sepharose used for the second step replaces Aquacide II concentration and Bio-Gel P-100 chromatography used previously. Besides eliminating the separate concentration step, the dextran sulphate-Sepharose also allows recovery of more highly concentrated PN than from the Bio-Gel column, which is advantageous for the stability of the PN. Presumably, the use of large micro carrier cultures with the present procedure could increase the total amount of PN

isolated, but the amount recovered with the use of roller-bottle cultures is quite sufficient for structural and enzymic studies.

Purified PN was compared with ATIII and HCII to see whether either of these molecules was related to PN. Ouchterlony analysis showed no apparent immunological cross-reactivity between PN and ATIII or HCII. One-dimensional peptide maps of the three inhibitors showed little, if any, similarity in the CNBr-digestion fragments. These results confirm that no precursor-product relationship exists between PN and ATIII or HCII. However, this does not preclude the possibility of homologous regions shared between PN and these inhibitors. Indeed, Griffith *et al.* (1985) have shown that ATIII and HCII have striking homologies in their reactive-site regions, although their *N*-termini show no similarities. A definitive comparison of PN with ATIII and HCII by sequence analysis will eventually reveal the extent of conservation among these three functionally related inhibitors.

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