Acta Biochimica Polonica

Vol. 43 No. 3/1996

503–506

QUARTERLY

The use of sequential affinity chromatography for separation of human neutrophil elastase, cathepsin G and azurocidin*

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Received: 5 June, 1996

Key words: human neutrophil elastase, cathepsin G, azurocidin, squash trypsin inhibitor, bovine pancreatic trypsin inhibitor, affinity chromatography

Elastase, cathepsin G and azurocidin from human neutrophils are key components of body inflammatory defense. Perturbations in regulation of their activities lead to many serious pathological states. The paper describes a simple, fast and efficient method of joint purification of these proteins with the use of sequential affinity chromatography on squash trypsin inhibitor (CMTI I) and bovine pancreatic trypsin inhibitor (BPTI).

Human neutrophil elastase (HNE) and cathepsin G are serine proteinases able to hydrolyse a variety of proteins, including elastin, proteoglycan, collagens and other connective tissue proteins [1]. They express also some microbicidal activity independently from their enzymatic action [2]. Azurocidin, which has 45% sequence homology with HNE, lost in evolution its catalytic center [3], preserving microbicidal [3] and chemotactic activity [4]. These features make these entities a crucial factor in the body's inflammatory response, allowing neutrophils to migrate easily to the site of inflammation [5] and participate in the degradation of invading microorganisms by proteolytic and microbicidal mechanisms.

All three proteins reside in azurophilic granules — the important tool of neutrophil phagocytic activity [6].

Enzymatic activity of HNE and cathepsin G outside the neutrophil (after degranulation) is controlled by proteinase inhibitors such as α_1-proteinase inhibitor, mucus proteinase inhibitor, α_1-antichymotrypsin and α_2-macroglobulin [7]. Disturbances in proteinase-inhibitor balance lead to such serious pathological states as emphysema, adult respiratory distress syndrome, rheumatoid arthritis, gout or glomerulonephritis [8].

Purification methods for HNE and cathepsin G are usually multistep procedures; that most often used, described by Travis and his collegues [9, 10], consists of two chromatographic steps (affinity followed by ion exchange). In the present paper we propose a simpler and faster procedure based on simultaneous adsorption on a tandem of CMTI I- and BPTI-Sepharose columns. Lowering of pH releases cathepsin G from CMTI I, and HNE and azurocidin from BPTI column.

MATERIALS AND METHODS

Materials. Neutrophils were obtained from "buffy coats" provided by Wrocław District

*This investigation was supported by the State Committee for Scientific Research grant KBN-PO4A 01308.

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CMTI I, squash trypsin inhibitor; HNE, human neutrophil elastase.

Blood Bank. Granules were isolated from neutrophils by the procedure of Baugh & Travis [9]. CMTI I was purified according to Polanowski *et al.* [11]. BPTI was purified according to Wilusz *et al.* [12]. *N*-Methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide were from Sigma Chemical Company (U.S.A.).

Affinity column preparation. Inhibitors were coupled to Sepharose 4B by the divinyl sulphone method [13]. The size of the column was 5 cm × 1cm and 15 cm × 1.6 cm for CMTI I- and BPTI-Sepharose, respectively.

Purification procedure. Frozen granules were thawed and homogenised according to [9]. The homogenate was centrifuged for 30 min at 30000 × *g*, pH of the supernatant was adjusted to 8.0 with solid Tris and the preparation was loaded on the tandem of CMTI I-Sepharose and BPTI-Sepharose columns equilibrated with 0.05 M Tris/HCl buffer, pH 8.0, containing 1 M NaCl. After elution of unadsorbed proteins with the equilibration buffer the columns were disconnected. To elute protein adsorbed on CMTI I column 0.05 M citrate buffer, pH 3.5, with 1 M NaCl was applied. Application of 0.05 M citrate buffer, pH 4.0, with 1 M NaCl followed by 0.1 M glycine buffer, pH 3.0, eluted proteins from BPTI column. Fractions of 5 ml were collected into tubes containing 1 M CH_3COONa adjusted to pH 6.0 (0.1 of the fraction volume) to stabilise enzymatic activity.

Protein determination. Concentration of purified proteins was determined from the absorbance at 280 nm using the absorption coefficient ($A^{1\%}_{280}$) of 9.85 for HNE [9] and 6.64 for cathepsin G [10]. For azurocidin the absorption coefficient was calculated as 5.1 according to Gill & von Hippel [14].

Enzymatic activity. HNE activity was determined in 0.2 M Tris/HCl buffer, pH 8.0, with 1 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide as a substrate. Cathepsin G activity was determined in 0.1 M Tes buffer, pH 7.5, containing 0.5 M NaCl and 0.02% Triton X-100 with 0.2 mM *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide as a substrate. One unit of activity was defined as the amount of the enzyme releasing 1 µmol of *p*-nitroanilide per 1 min at 25°C.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS/PAGE) was performed in 15% (w/v) slab gels by the method of Laemmli [15] at pH 8.3. The gels were stained with Coomassie Brilliant Blue R-250. Molecular weight calibration kit from Pharmacia LKB Biotechnology AB (Uppsala, Sweden) (phosphorylase *b* – 94000, albumin – 67000, ovalbumin – 43000, carbonic anhydrase – 30000 and soybean trypsin inhibitor – 21100) was used.

Amino-acid sequence. *N*-Terminal sequence of azurocidin was determined in MilliGen ProSequencer (Millipore, U.S.A.) according to the manufacturer's procedure.

RESULTS AND DISCUSSION

Strong binding of cathepsin G ($K_a = 1.7 \times 10^8$ M^{-1}) [16] to CMTI I and binding of HNE [9] and azurocidin [17] to BPTI was the rationale for the presented separation procedure.

Loading of the neutrophil granules homogenate at pH 8.0, on connected CMTI I- and BPTI-Sepharose columns resulted in adsorption of cathepsin G on the first, and of HNE with azurocidin on the second column. A decrease of pH allowed for the desorption of separated proteins. Cathepsin G was eluted from CMTI I-Sepharose at pH 3.5. Elution of HNE at pH 4.0, from the BPTI-Sepharose column was followed by the elution of azurocidin at pH 3.0 (Fig. 1).

Determination of enzymatic activity in the eluted fractions showed no cross-contamination, which means that cathepsin G was bound exclusively to the CMTI I and HNE to the BPTI-Sepharose column.

Sequencing of the first 15 N-terminal residues of the protein eluted at pH 3 from BPTI-Sepharose column confirmed its identity with the published sequence of azurocidin [18].

SDS/PAGE revealed that the separated proteins are pure and show the characteristic microheterogeneity pattern (Fig. 2).

The applied method gave a good yield of the separated proteins (Table 1). By processing of 60 "buffy coats" from about 24 l of blood 25 mg of cathepsin G, 23 mg of HNE and 12 mg of azurocidin was obtained.

In summary, as a result of the applied procedure the three clinically important proteins were separated from a single batch of neutrophil granules in a fast, simple and efficient way.

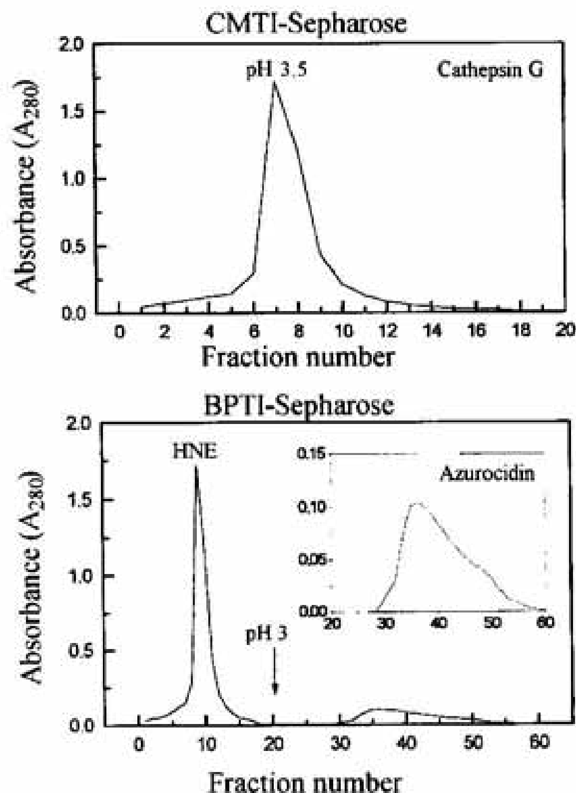

Fig. 1. Elution profiles of human neutrophil elastase, cathepsin G and azurocidin from CMTI I- and BPTI-Sepharose columns.
For details of chromatography see Materials and Methods.

Fig. 2. SDS/PAGE of proteins separated by affinity chromatography on CMTI I- and BPTI-Sepharose columns.
Lanes 1 and 5, molecular weight standards; lane 2, HNE; lane 3, azurocidin; lane 4, cathepsin G. For details of electrophoresis see Materials and Methods.

Table 1
The yield of affinity chromatography separation of cathepsin G and human neutrophil elastase.
For details of enzymatic activity determination see Materials and Methods.

Enzyme	Total enzymatic activity (U)		Yield %
	in homogenate	after chromatography	
HNE	847600	642500	76
Cathepsin G	22960	10550	46

REFERENCES

1. Rest, R. (1988) Human neutrophil and mast cell proteases implicated in inflammation. *Methods Enzymol.* **163**, 309–327.
2. Oderberg, H. & Olsson, I. (1975) Antibacterial activity of cationic proteins from human granulocytes. *J. Clin. Invest.* **56**, 1118–1124.
3. Campanelli, D., Detmers, P., Nathan, C.F. & Gabay, J.E. (1990) Azurocidin and a homologous serine protease from neutrophils. *J. Clin. Invest.* **85**, 904–915.
4. Pereira, H.A., Shafer, W.M., Pohl, J., Martin, L.E. & Spitznagel, J.K. (1990) CAP37, a human neutrophil-derived chemotactic factor with monocyte specific activity. *J. Clin. Invest.* **85**, 1468–1476.
5. Owen, C.A., Campbell, M.A., Sannes, P.L., Boukedes, S.S. & Campbell, E.J. (1995) Cell surface-bound elastase and cathepsin G on human neutrophils: A novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J. Cell Biol.* **131**, 775–789.
6. Klebanoff, S.J. & Clark, R.A. (1978) *Neutrophil: Function and Clinical Disorders;* pp. 217–280, North-Holland, Amsterdam.
7. Travis, J. & Salvesen, G. (1983) Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52**, 655–709.
8. Havemann, K. & Gramse, M. (1984) Physiology and pathophysiology of neutral proteinases of human granulocytes; in *Proteases: Potential Role in Health and Disease* (Hörl, W.H. & Heidland, A., eds.) pp. 1–20, Plenum Press, New York.
9. Baugh, R.J. & Travis, J. (1976) Human leukocyte granule elastase: Rapid isolation and characterization. *Biochemistry* **15**, 836–841.

10. Travis, J., Bowen, J. & Baugh, R. (1978) Human α-1-antichymotrypsin: Interaction with chymotrypsin-like proteinases. *Biochemistry* **17**, 5651–5656.

11. Polanowski, A., Cieślar, E., Otlewski, J., Nienartowicz, B., Wilimowska-Pelc, A. & Wilusz, T. (1987) Protein inhibitors of trypsin from the seeds of *Cucurbitaceae* plants. *Acta Biochim. Polon.* **34**, 395–406.

12. Wilusz, T., Łomako, J. & Mejbaum-Katzenellenbogen, W. (1973) An improved method of isolation of crystalline basic trypsin inhibitor from bovine tissues. *Acta Biochim. Polon.* **20**, 25–31.

13. Pepper, D.S. (1992) Some alternative coupling chemistries for affinity chromatography; in *Practical Protein Chromatography* (Kenney, A. & Fowell, S., eds.) pp.181–183, The Humana Press, Totova, N.J.

14. Gill, S.C. & von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326.

15. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.

16. Otlewski, J., Zbyryt, T., Krokoszyńska, I. & Wilusz, T. (1990) Inhibition of serine proteinases by squash inhibitors. *Biol. Chem. Hoppe-Seyler* **371**, 589–594.

17. Petersen, L.C., Birktoft, J.J. & Flodgaard, H. (1993) Binding of bovine pancreatic trypsin inhibitor to heparin binding protein/CAP37/ /azurocidin. Interaction between a Kunitz-type inhibitor and a proteolytically inactive serine proteinase homologue. *Eur. J. Biochem.* **214**, 271–279.

18. Pohl, J., Pereira, H.A., Martin, N.M. & Spitznagel, J.K. (1990) Amino acid sequence of CAP37, a human neutrophil granule-derived anti-bacterial and monocyte-specific chemotactic glycoprotein structurally similar to neutrophil elastase. *FEBS Lett.* **272**, 200–204.