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Isolation of Cathepsin B and α-*N*-Benzoylarginine-β-naphthylamide Hydrolase by Covalent Chromatography on Activated Thiol Sepharose

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Cathepsin B and α-*N*-benzoylarginine-β-naphthylamide (BANA) hydrolase have been isolated from bovine lymph nodes using a novel procedure that includes besides gel filtration and ion exchange chromatography also covalent chromatography as the essential step. Both enzymes were selectively bound to the activated thiol-Sepharose and afterwards eluted with cysteine. The homogeneity of enzymes was proved by polyacrylamide gel electrophoresis.

Intracellular proteinase cathepsin B (EC 3.4.22.1) has been isolated from various animal tissues using ion exchange and gel chromatography, often in combination with affinity chromatography on organomercurial agarose[1]. In this paper a new method for the isolation of cathepsin B is described using covalent chromatography on activated thiol-Sepharose as the essential step for obtaining thiol enzymes. By this method it is possible to isolate not only pure cathepsin B but also another BANA hydrolase which is probably identical with cathepsin H[2].

MATERIALS AND METHODS

Freeze dried activated thiol Sepharose 4B (Pharmacia, Sweden) was reswollen and washed as suggested by the manufacturer. Sephadex G-75 was also the product of Pharmacia and CM-cellulose was obtained from Sigma, USA. All reagents used were of analytical grade.

Enzymatic assay. Cathepsin B activity was measured according to the method of Barrett[3] using α-*N*-benzoyl-DL-arginine-β-naphthylamide. HCl (Bz-Arg-2-NNap, Sigma, USA) as substrate. Free naphthylamine was determined colorimetrically after coupling with Fast Garnet GBC (Sigma, USA). One unit of activity is defined as the amount of the enzyme that will hydrolize 1 nmol of substrate per minute under reaction conditions.

Proteolytic activity was determined by the method of Anson with hemoglobin as substrate[4].

Protein content was determined by the method of Lowry[5], or by measuring absorbance at 280 nm.

Polyacrylamide gel electrophoresis was carried out in a Canalco apparatus (Canalco USA) using 7% gel in Tris glycine buffer of pH 8.4 and 15% gel in 0.34 M β-alanine buffer of pH 4.4 Gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was run according to the method of Weber and Osborn[6]. Samples were dissolved in 1% SDS containing 1% β-mercaptoethanol.

Covalent chromatography. Prior to application on thiol Sepharose, the samples were incubated with 5 mM cysteine for 30 min at room temperature and subse-

quently dialyzed overnight toward approximately 100-times larger volume of 0.1 M Na acetate buffer of pH 5.0, containing 0.3 M NaCl and 1 mM EDTA. After application to thiol Sepharose, the column was washed with the same buffer and the absorbances at 280 nm and 343 nm were followed until their values fell to less than 0.03 absorbance units. Covalently bound material was eluted from thiol Sepharose by the addition of 5 mM cysteine to the starting buffer. The contribution of A_{280} due to protein in protein-2-thiopyridone mixtures was calculated from the known extinction coefficients of 2-thiopyridone:

$$\varepsilon_{343} = 7.0 \cdot 10^3 \text{ mol}^{-1} \text{ dm}^3 \cdot \text{cm}^{-1} \text{ and } \varepsilon_{280} = 7.7 \cdot 10^3 \text{ mol}^{-1} \text{ dm}^3 \cdot \text{cm}^{-1}\ ^{7}.$$

Eluted active fractions were pooled, concentrated on an Amicon UM-10 ultrafilter (Amicon, Holland) and dialyzed against 0.02 M Na acetate buffer of pH 5.0 containing 1 mM EDTA.

Initial purification. Bovine lymph nodes were used as the source of the enzyme. They were obtained fresh in the slaughterhouse and brought on ice to the laboratory. After being cleaned of fat and connective tissues, they were stored at —20 °C until required.

A 33% homogenate was prepared in 1 mM EDTA containing 5 g NaCl per dm^3 using a Sorvall Omnimixer (Sorvall, USA). The homogenate was acidified to pH 4.0 with 3 M HCl and centrifuged for 25 min at 4200 *g* in a Sorvall RC 2B centrifuge at 0 °C. The acid extract was used for further purification.

The entire procedure was carried out in the cold room at 4 °C.

RESULTS AND DISCUSSION

The purification steps of cathepsin B and BANA hydrolase are listed in Table I. The acid extract was fractionated by ammonium sulphate precipitation. The first fraction between 0—40% of saturation contained no BANA hydrolase activity and was discarded. All enzyme activity was found in the second fraction that precipitated between 40—70% of saturation. This fraction was further purified by gel chromatography on Sephadex G-75. A typical separation is shown in Figure 1. BANA hydrolase activity was separated from the bulk of proteolytic activity due to cathepsin D. However, it still contained proteolytic activity that was insensitive toward pepstatin and can be ascribed to cathepsin S [8] and cathepsin H [2]. Pooled active fractions were concentrated and covalently attached to activated thiol Sepharose 4B through the active groups of thiol enzymes. After elution of non-thiol proteins, the thiol enzymes were eluted

TABLE I

Purification of cathepsin B and BANA hydrolase from bovine lymph nodes

Step	Total prot. mg	Total act. units	Spec. act. units/mg	Yield %	Purificat. -fold
Acid exctract	63 600	572 400	9	100	1
Am. sulph. ppt.	3 364	447 320	130	76	14
Sephadex G-75	225	350 775	1 559	61	131
Thiol Sepharose	28	132 440	4 730	23	664
CM-cellulose					
— cathepsin B	5	22 440	4 488	4	520
— CM II	3	10 968	3 656	2	423

The data presented are the results of a typical experiment starting with 3.9 kg of tissue. Enzyme and protein assays were carried out after each step as described under Material and methods.

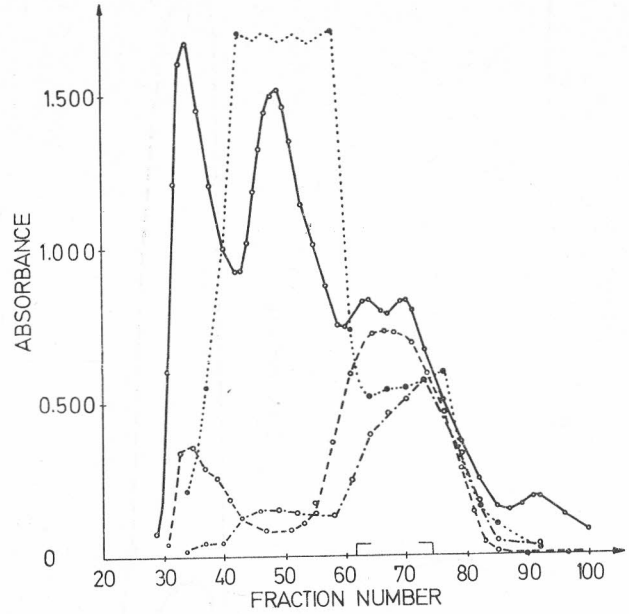

Figure 1. Gel filtration of ammonium sulphate fraction on Sephadex G-75. Approximately 70 cm³ of ammonium sulphate precipitate solution in 1 mM EDTA was applied to a column (4 × 110 cm) of Sephadex G-75 and eluted with 0.02 M Na acetate buffer pH 5.0, containing 1 mM EDTA and 0.2 M NaCl. Flow rate was 25 cm³/h, fraction volume vas 8 cm³. Active fractions were pooled as indicated and concentrated on Amicon UM-10 ultrafilter. —— A_{280}; - - - - activity toward Bz-Arg-2-NNap at pH 6.0, expressed as $\Delta A_{520}/0.05$ cm³ of eluate in 10 min; . . . . . activity toward hemoglobin expressed as $\Delta A_{750}/0.1$ cm³ of eluate in 2 hrs; - · - · - activity toward hemoglobin at pH 3.5 in the presence of 10^{-7} M pepstatin, expressed as $\Delta A_{750}/0.1$ cm³ of eluate in 2 hrs.

with 5 mM cysteine (Figure 2). Polyacrylamide gel electrophoresis showed that the eluate contained 2 major protein components (Figure 3). They were extracted from gel and had approximately the same specific activity toward Bz-Arg-2--NNap.

The final separation of the two amidohydrolases was achieved by CM-cellulose chromatography at pH 5.0 (Figure 4). Cathepsin B emerged from the column after the concentration of NaCl was increased to 0.02 M. Above 0.05 M NaCl the second thiol amido hydrolase (named CM II) was eluted. The homogeneity of both enzymes was proved by gel electrophoresis at pH 4.4 (Figure 3) and at pH 8.6. Electrophoresis in the presence of SDS also showed one band for either proteinase (Figure 5). From Table I which gives quantitative data on the purification of cathepsin B it is evident that the enzyme was purified 520 fold if compared with acid extract with the yield of 4%, and 2% for the second enzyme. The yields are calculated, based on the total activity in acid extract. From SDS electrophoresis, the apparent molecular weight for cathepsin B was 26 300, and 30 800 for the other thiol enzyme. Cathepsin B was completely inhibited by iodoacetic acid, 4-chloromercuribenzoate and 2,2'-dipyridysulphide at 1 mM concentration of inhibitor and with $5 \cdot 10^{-5}$ M leupeptin. Results of further investigations that are in progress will be published elsewhere.

Lysosomal thiol proteinase cathepsin B was first purified from bovine spleen[9]. It was purified also from human liver[10] by a procedure involving

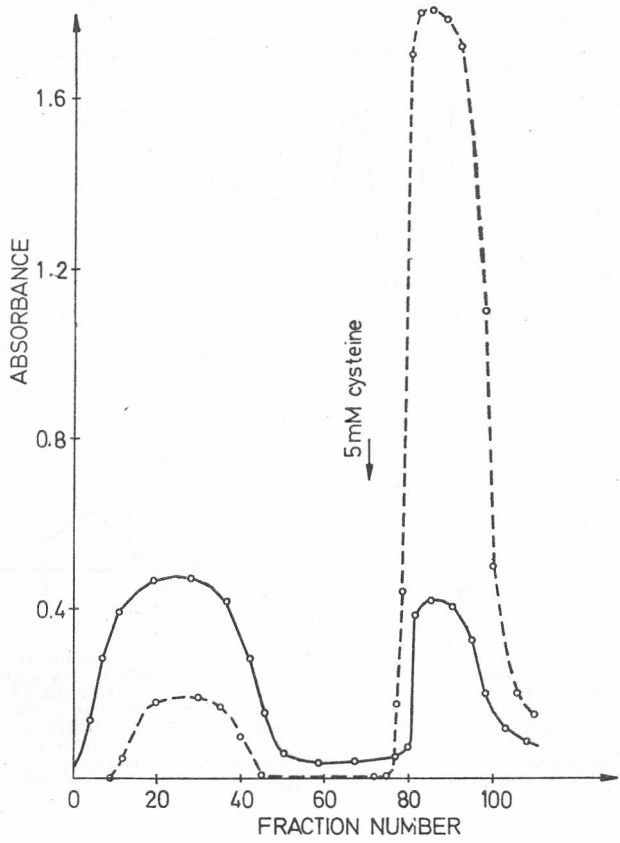

Figure 2. Covalent chromatography of active fractions from Sephadex G-75 on activated thiol Sepharose 4B. 140 cm³ of sample, containing 300 mg of protein was applied to a column of activated thiol Sepharose (3.5 × 14 cm) and eluted with the same buffer. Flow rate was 6 cm³/h. After absorbance at 280 nm fell to 0.03 absorbance units elution proceeded with the same buffer containing 5 mM cysteine. ——— A_{280} due to protein; - - - - activity toward Bz-Arg-2-NNap at pH 6.0, expressed as $\Delta A_{520}/0.025$ cm³ of eluate in 10 min.

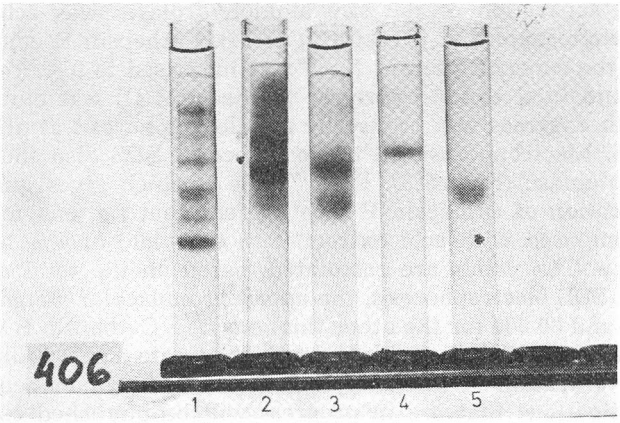

Figure 3. Polyacrylamide gel electrophoresis at pH 4.4 of enzymes at various purification steps. Gel 1: ammonium sulphate fraction; gel 2: Sephadex G-75 concentrate; gel 3: the second peak from thiol Sepharose; gel 4: cathepsin B (the first maximum from CM-cellulose); gel 5: CM II (the second maximum from CM-cellulose).

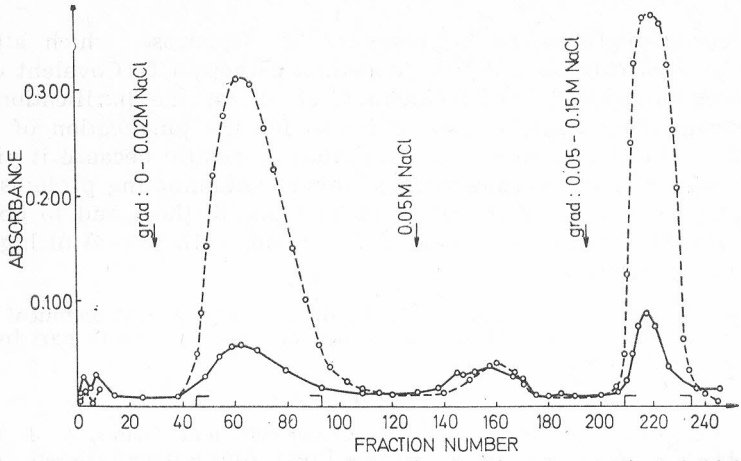

Figure 4. CM-cellulose chromatography of pooled active fractions from activated thiol Sepharose 4B. To a column of CM-cellulose (1 × 13 cm) equilibrated with 0.02 M Na acetate buffer pH 5.0, containing 1 mM EDTA, protein solution (20 mg) was applied. Elution started with the same buffer. After A_{280} fell to 0.01 absorbance units a linear gradient to 0.02 M NaCl in starting buffer was applied. Elution continued with 0.05 M NaCl in starting buffer and finally with the gradient from 0.05 to 0.15 M NaCl in starting buffer. Flow rate was 9.6 cm³/h, fraction volume was 4 cm³. ——— A_{280}; - - - - activity toward Bz-Arg-2-NNap at pH 6.0, expressed as $\Delta A_{520}/0.025$ cm³ of eluate in 10 min.

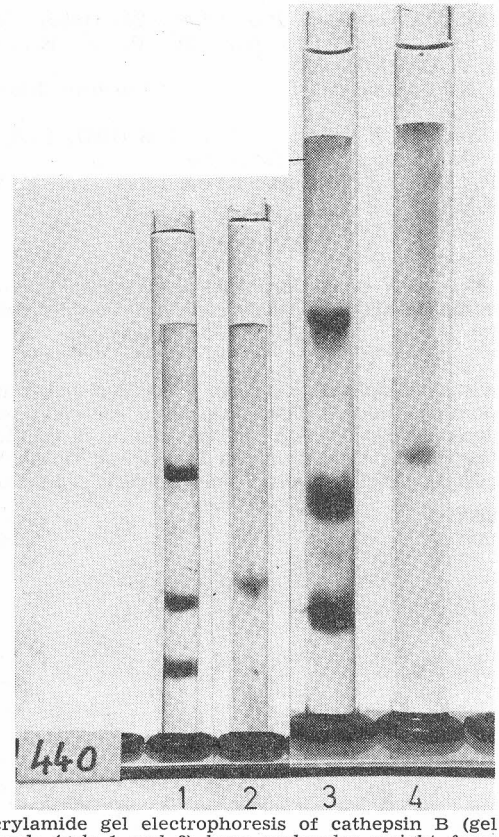

Figure 5. SDS polyacrylamide gel electrophoresis of cathepsin B (gel 2) and CM II fraction (gel 4). Protein standards (gels 1 and 3) have molecular weight from top to bottom: bovine serum albumin (67 000), chymotrypsinogen A (25 000) and lysosyme (14 000).

affinity chromatography on organomercurial Sepharose, which allowed separation of thiol cathepsin B from non-thiol cathepsin D. Covalent chromatography was introduced by Brocklehurst et al. for the purification of fully active papain from Papaya latex and also for the purification of urease[7,11]. It was also an efficient step in our isolation procedure because it yielded the mixture of both thiol enzymes without other contaminating proteins. Only an additional step was necessary in order to separate them and to obtain pure cathepsin B. The method is very fast and yields cathepsin B of high specific activity in a single form.

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IZVLEČEK

Izolacija katepsina B in α-*N*-benzoilarginin-β-naftilamid hidrolaze s kovalentno kromatografijo na aktivirani tiolni sepharozi

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Iz govejih bezgavk smo izolirali katepsin B in α-*N*-benzoilarginin-β-naftilamid (BANA) hidrolazo s pomočjo novega postopka, ki je vseboval poleg gelske filtracije in kromatografije na ionskem izmenjalcu tudi kovalentno kromatografijo kot bistveno stopnjo. Oba encima se vežeta selektivno na aktivirano tiolno Sepharozo, nakar ju eluiramo s cisteinom. Homogenost encimov smo potrdili z gelsko elektroforezo.

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