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Isolation and characterisation of cathepsin-B from bovine pancreas

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Abstract. A simple procedure for the isolation of cathepsin-B from bovine pancreas employing ammonium sulphate fractionation, DEAE cellulose chromatography and Sephadex G-200 gel filtration is described. The purified enzyme gave a single band on polyacrylamide gel electrophoresis. The molecular weight as determined by gel filtration of the enzyme was 26,850. Its K_m and V_{max} values were 12·8 mM and 0·303 μmol/min/mg, respectively. The K_i for iodoacetamide was 0·16 mM.

Keywords. Lysosomes; cathepsin-B; purification; bovine pancreas.

Introduction

Lysosomal cathepsins hydrolyse various native proteins and play a key role in turnover or modification of cellular proteins. Cathepsin-B (EC 3.4.22.1), a thiol-dependent lysosomal protease, is ubiquitously distributed in mammalian tissues (Bouma and Gruber, 1964; Otto, 1971). Presence of cathepsin-B has been demonstrated in lysosomal fraction of many tissues including spleen, heart, liver, brain, lung, muscle, kidney, adrenal, thymus, etc., and attempts have also been made to isolate and characterise this enzyme from different sources (Greenbaum and Fruton 1957; Shibko and Tappel, 1965; Bouma and Gruber, 1964, 1966; Otto, 1967, 1971, 1975; Snellman, 1969; Franklin and Metrione, 1972; Keilova and Tomasek, 1973; Barret, 1973; Matsuda and Misaka, 1974; Swanson *et al.,* 1974; Etherington, 1972, 1974, 1976; Evans and Etherington, 1978; Bayleess and Yousuf Ali, 1978). However, no attempt has so far been made to purify and characterise cathepsin-B from pancreas, where this enzyme is supposed to convert proinsulin to insulin (Aronson and Barret, 1978; Puri *et al.,* 1978).

In the present paper, we report a simple method for purification of cathepsin-B and some of its kinetic characteristics from bovine pancreas.

Materials and methods

Materials

Bovine pancreas was collected within an hour after slaughter and stored at – 20°C. Triton X-100 and human haemoglobin were obtained from Centron Research Laboratories, Bombay. L-Cysteine hydrochloride hydrate was procured from

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Aldrich Chemicals Co., Milwaukee, Wis., USA. Sephadex G-100, G-200 and DEAE cellulose were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Iodoacetamide was a product of Eastman Kodak Rochester, NY, USA. All other chemicals used were of the analytical grade.

Methods

Isolation of cathepsin-B : All the steps were carried out at 0-5°C unless otherwise stated. The enzyme was extracted according to the procedure which was essentially similar to that of Etherington (1976). Fresh, chilled pancreas was trimmed of the outer fascia and fat, cut into small pieces and was homogenised in a Waring blendor with 10 volumes of 10 mM acetate buffer, pH 5·0 containing 1 mM EDTA. The homogenate was filtered through double layer of muslin cloth and the filtrate was centrifuged at 5,000 g for 20 min (IEC-model B-20 Rotor No. 872). The sediment was resuspended in 10 ml of homogenising medium and centrifuged again. Addition of Triton X-100 (final concentration of 0·1%) to the pooled supernatants under constant stirring for 20 min resulted in the complete disruption of lysosomes.

Crystalline ammonium sulphate (0-30% saturation) was added to lysosomal lysate with constant stirring for 30 min; the precipitate was removed by centrifugation. Ammonium sulphate was added to the supernatant to obtain 80% saturation. The mixture was stirred for 1 h. The precipitate was collected by centrifugation, resuspended in 1 mM acetate buffer, pH 5·0 and dialysed against the same buffer for 20 h and centrifuged; the supernatant was lyophilised and suspended in 1 mM acetate buffer, pH 5·0.

Column chromatography : Ion exchange chromatography of the lyophilised material was carried out on DEAE-cellulose column (2·5 × 26 cm), pre-equilibrated with 10 mM acetate buffer, pH 5·0. Elution was carried out by increasing concentration of sodium chloride (0·005–0·2 M). Cathepsin-B activity was assayed in all the fractions.

The fractions containing enzyme activity were pooled and lyophilised. The lyophilised material was suspended in a minimum volume of 1. mM acetate buffer, pH 5·0, dialysed against the same and chromatographed on a Sephadex G-200 column (1·5 × 55 cm) previously equilibrated with 10 mM acetate buffer, pH 6·0. The fractions containing enzyme activity were pooled, dialysed and lyophilised.

Enzyme assay : Cathepsin-B was assayed according to Anson (1938) as modified by Mycek (1970). The reaction mixture (10 μl enzyme in 0·4 M citrate buffer, pH 5·0 and cysteine hydrochloride 0·07M, pH 5·0) was preincubated for 5 min prior to the addition of haemoglobin (15·5 mM). The enzyme reaction was stopped after 10 min of incubation at 37° C by trichloroacetic acid (10% w/v). Amino acids released in to the acid filtrate were estimated by the method of Lowry *et al.* (1.951). One unit of enzyme activity is defined as that amount which liberated one μmol of tyrosine/min. The specific activity is expressed as units of enzyme/mg of protein.

Protein determination : Protein in eluants was monitored by absorption at 280 nm in a Beckman DU Spectrophotometer model-24 and estimated according to Lowry *et al.* (1951), using bovine serum albumin as a standard.

Gel electrophoresis : Polyacrylamide disc gel electrophoresis was conducted using 7·5% gel in 0·1 M tris-glycine buffer as described by Davis (1964). The gels were stained with 0·25% Coomasie Brilliant Blue in methanol : acetic acid: water (5:1:5 v/v) and destained by 7% acetic acid.

Molecular weight determination : A Sephadex G-100 column (1 × 100 cm) pre-equilibrated with 10 mM acetate buffer pH 5·0 was calibrated with blue dextran-2000 (200,000), bovine serum albumin (66,500), D-amino acid oxidase (37,000), trypsin (23,300), cytochrome C (14,700) and ribonuclease (13,700). NADH was used for bed volume (V_t) measurement (absorbance at 340 nm.) The ratio of elution volume (V_e) and (V_t) was plotted against log molecular weight.

Results

Table 1 shows purification of cathepsin B at different steps. On DEAE-cellulose chromatography of the 30-80% ammonium sulphate fraction, 85% of the total enzyme activity was eluted at 0·1 M and 0·2 M NaCl concentration. By this procedure a 330-fold enrichment of the enzyme was achieved, which revealed four bands on polyacrylamide gel electrophoresis (figure 1). Gel filtration of this partially purified cathepsin-B on Sephadex G200 resulted in appearance of four protein peaks (figure 1) but the enzyme activity peak coincided with the protein

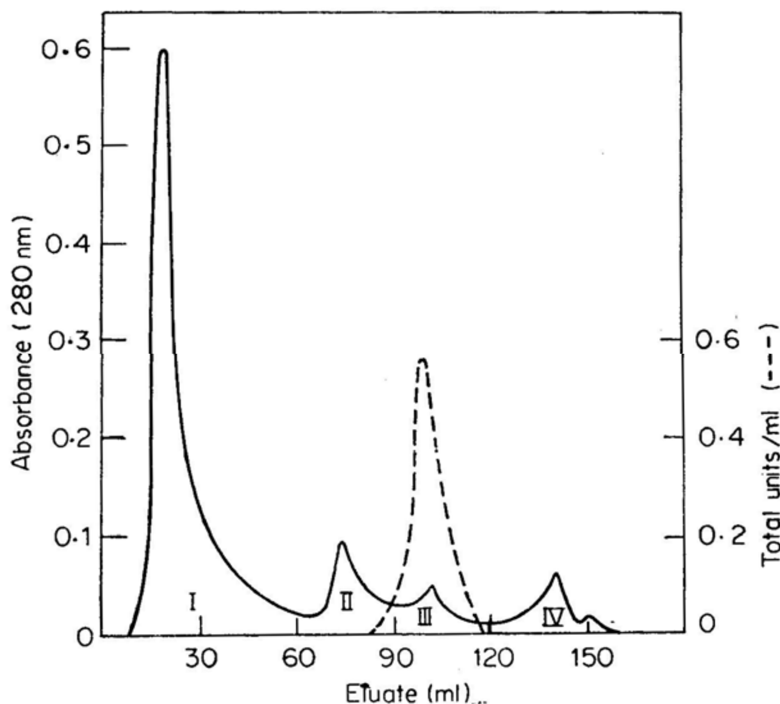

Figure 1. Sephadex G-200 chromatography of lysosomal cathepsin B (DEAE-cellulose fraction).
Flow rate was 15ml per h and fraction volume 3ml (——), absorbance at 280nm; (– – –) cathepsin-B activity.

peak obtained between 80-120 ml of the eluant. This procedure led to an 855-fold purification of the enzyme with a recovery of 83%. This enzyme preparation was found to be homogeneous, as revealed by a single protein band on polyacrylamide gel electrophoresis (figure 2c).

The molecular weight of the protein was found to be 26,850 by chromatography on Sephadex G-100. The Michaelis constant was 12·8 mM (figure 3) and V_{max} was 0·303 μmol/min/mg protein. The inhibitor constant (K_i) for inhibition of cathepsin-B by iodoacetamide was 0·16mM (figure 4). It is evident from the figure that iodoacetamide was a competitive inhibitor.

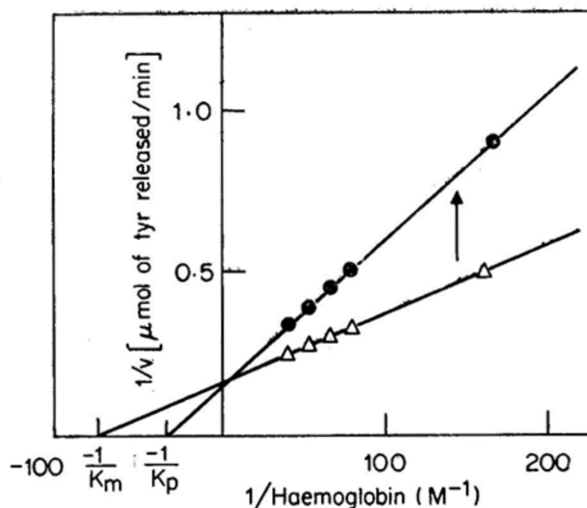

Figure 3. Effect of varying substrate concentration on cathepsin-B
The concentration of enzyme solution used was 0·3 mg/ml and 0·05 ml (15 μg) was used in each assay. Δ–Δ without inhibiton, ●–● in presence of iodo-acetamide (0·2mM)

Table 1. Purification of cathepsin-B from bovine pancreas.

Procedure	Total units	Total protein (mg)	Specific activity*	Purification	% Recovery
Homogenate	217	4,927	0·044	..	100
Ammonium sulphate fractionation (30–80%)	193	608	0·315	7	88
DEAE-cellulose chromatography	184	12·7	14·50	330	84
Sephadex G–200	181	4·87	37·21	846	83

* μmol tyrosine liberated/min/mg protein.

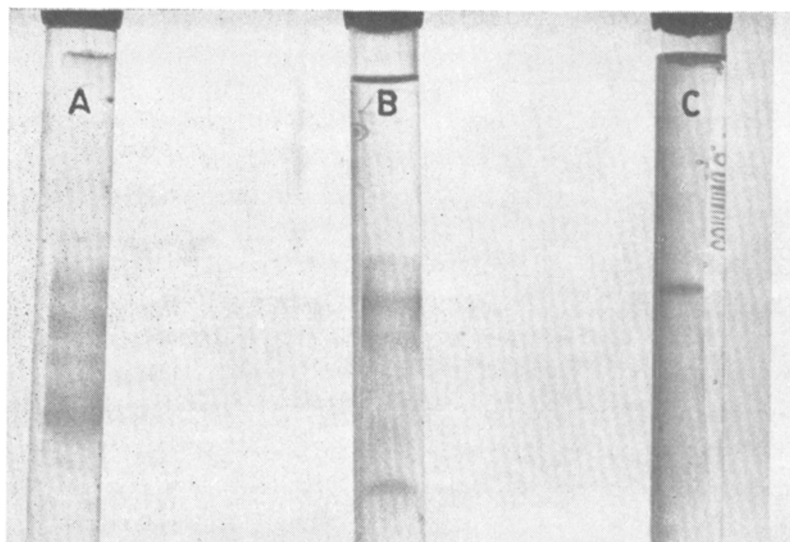

Figure 2. Polyacrylamide disc gel electrophoretic pattern of (A) crude homogenate; (B) DEAE-cellulose eluate and (C) Sephadex G-200 chromatographed enzyme.

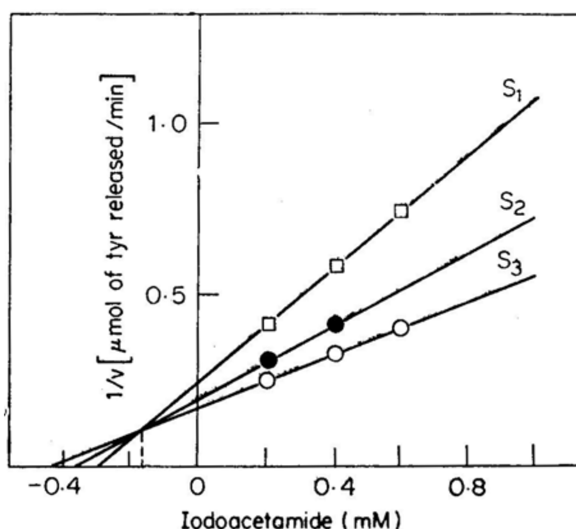

Figure 4. Dixon plot for the inhibition of bovine cathepsin-B by iodoacetamide at three concentrations of haemoglobin.
S_1 = 12·4mM; S_2 = 15·5mM; S_3 = 24·8 mM.

Discussion

Barret (1973) has reported a 320-fold purification with 6% yield of cathepsin-B from human liver by a method involving autolysis, fractional precipitation with acetone, organomercurial adsorption and chromatography on CM-cellulose, while Evans and Etherington (1978) have reported a 1040-fold purification with 65% recovery of this enzyme from human placenta using a six-step purification procedure.

The purification procedure for cathepsin-B presented here consists of compara tively simple steps. Most of the proteins are resolved by DEAE-cellulose chromato-graphy and a pure enzyme sample is obtained after Sephadex G-200 column which gives a single band on polyacrylamide gel electrophoresis.

The molecular weight of cathepsin-B is in agreement with other reported values (Otto, 1971; Barret, 1973; Franklin and Metrione, 1972; Etherington, 1976). The inhibitor constant is 0·16 mM and the enzyme inhibition is of competitive nature. The K_m and $V_{\max}$ values of the purified enzyme reported here are in agreement with earlier reports (Tesser *et al.,* 1964; Otto, 1971).

The availability of pure cathepsin-B would enable a detailed study of its specificity of action and its role in the *β*-cells of islets of Langerhans.

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