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Note

One-step purification of human leukocyte elastase by biospecific affinity chromatography at subzero temperatures

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We have previously shown[1] that it is possible to perform biospecific affinity chromatography with a "true" substrate under non-turnover conditions, by using subzero temperatures in fluid media. The enzyme system used was porcine pancreatic elastase, which can be selectively absorbed on and desorbed from an affinity column supporting the artificial specific substrate, L-trialanine-*p*-nitroanilide.

In the present paper we show that this affinity column may be used for the one-step purification of elastase from a crude extract of granules from human leukocytes.

EXPERIMENTAL

Reagents

Porcine pancreatic elastase, succinyl-L-trialanine-*p*-nitroanilide, L-trialanine-*p*-nitroanilide, *N*-acetyl-L-tryptophan ethyl ester, Sepharose 6B CL, 1,4-butanediol-diglycidyl ether (Bioxirane), and ethylene glycol were prepared or purchased as previously described[1]. Bovine α-chymotrypsin was obtained from Worthington (Freehold, N.J., U.S.A.).

The granule extract of human leukocytes was obtained by the procedure of Baugh and Travis[2] and was stored at $-20°$ in 0.2 *M* sodium acetate buffer, pH 4, until used. The procedure of Baugh and Travis was also used to obtain the mixture of human leukocyte elastase iso-enzymes using affinity chromatography on Trasylol–Sepharose**.

When buffers (Tris–hydrochloric acid or sodium acetate) were used in mixed hydro-organic solvents at various temperatures, the pa_H was calculated as described previously[3,4] (a_H being the protonic activity in mixed solvents).

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** Trasylol is the trade name for basic pancreatic trypsin inhibitor.

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The Sepharose 6B CL was activated with diglycidyl ether and L-trialanine-*p*-nitroanilide was coupled as described before[1]. The chromatographic column[1] had an inner diameter of 6 mm, and the temperature was regulated with a Colora bath (from $+20$ to $-30 \pm 0.2°$). Usually 1 ml of affinity adsorbent was used for each experiment. During the elution, the flow-rate through the column was 5 ml/h. The eluted fractions (1 ml) were collected at 4°.

Assay procedure

Enzyme activities were measured spectrophotometrically at 25° with a Beckman Acta III spectrophotometer equipped with a thermostated cell compartment[5]. Elastase activity was determined at 410 nm with succinyl-L-trialanine-*p*-nitroanilide[6]. Chymotrypsin activity was measured with N-acetyl-L-tryptophan ethyl ester (2 m*M*) at 300 nm and pH 8.5, in 0.1 *M* Tris–HCl containing 5% (v/v) of ethylene glycol. This method can detect α-chymotrypsin at concentrations as low as 1 n*M* (ΔOD/min = $6 \cdot 10^{-4}$). Protein concentration was measured by the micromethod of Bradford[7] using porcine pancreatic elastase as a standard.

RESULTS AND DISCUSSION

The conditions used to adsorb pancreatic elastase on the column (*i.e.* Tris–HCl pH 9.2, 4 *M* NaCl, $-14°$) could not be used for the binding of human leukocyte elastase, because 5–10% of the substrate bound on the column was hydrolysed during the adsorption of the latter enzyme. This result is rather surprising for the leukocyte enzyme, which is usually considered a poorer catalyst than pancreatic elastase. Indeed, Twumasi and Liener[8] have shown that the former hydrolyses succinyl-L-trialanine-*p*-nitroanilide 40 times slower than does the latter. We therefore determined the kinetic parameters of the Michaelis-Menten equation (k_{cat} and K_m)[1] for the hydrolysis of L-trialanine-*p*-nitroanilide by leukocyte elastase. At pH 8 and 25° we found k_{cat} and K_m values of 0.045 sec^{-1} and 1.4 m*M*, respectively. The substrate bound on the affinity columns has thus a k_{cat} value with leukocyte elastase which is five times greater than that observed with pancreatic elastase[9]. This higher turnover number does not solely account for the very rapid hydrolysis of the substrate during the chromatographic adsorption. For this reason, we considered the possibility that the NaCl present in our adsorption buffer enhances the enzyme activity. We discovered that ionic strength indeed stimulates markedly the elastase-catalysed hydrolysis of L-trialanine-*p*-nitroanilide: at pH 8, 25°, the rate is increased by a factor of *ca.* 12 in the presence of 4 *M* NaCl. These effects are now under investigation[11].

In order to slow down this high turnover, we decided to adsorb the enzyme at pH 5. Under these conditions (0.1 *M* sodium acetate buffer, 4 *M* NaCl) only 0.5% and 0.05% of substrate is hydrolysed at $+4°$ and at $-14°$, respectively. When elution was started immediately after the sample was poured on to the column, only 80% of the total elastase activity was bound to the adsorbent. Column flow was therefore stopped for 1 h before elution was initiated, in order to achieve complete adsorption of the enzyme.

The experiments described lead to the following technique for the one-step purification of human leukocyte elastase. 100 μl of crude granular extract (1 mg of

protein) are mixed with 500 μl of 0.1 *M* sodium acetate buffer, pH 5, containing 4 *M* NaCl. This mixture is cooled to $-14°$ and applied to the chromatographic column previously equilibrated with the same buffer at the same temperature. This sample is applied to the column at a flow-rate of 1 ml/h. After 1 h, impurities are eluted with 20 ml of the same buffer. Elastase is then desorbed from the column with a mixture of equal volumes of ethylene glycol and 0.2 *M* sodium acetate buffer, pa_H 5.0, without NaCl, at the same temperature. The pa_H is adjusted after mixing[4].

The elution profile contained a single peak with good coincidence of protein and activity (Fig. 1). The elastase activity is recovered with a yield of *ca.* 85%. Activity measurements of the combined fractions showed that this preparation had the same specific activity at $\pm 5\%$ (measured with succinyl-L-trialanine-*p*-nitroanilide) as the enzyme prepared following the procedure of Baugh and Travis[2]. In addition, no cathepsin G activity (*i.e.* chymotrypsin-like activity) could be detected. The isolation procedure of Baugh and Travis uses Trasylol–Sepharose and yields elastase contaminated by the chymotrypsin-like enzyme cathepsin G; this may be subsequently removed by CM cellulose chromatography[2]. In order to achieve a one-step purification with Trasylol–Sepharose, cathepsin G must first be removed by precipitation at low

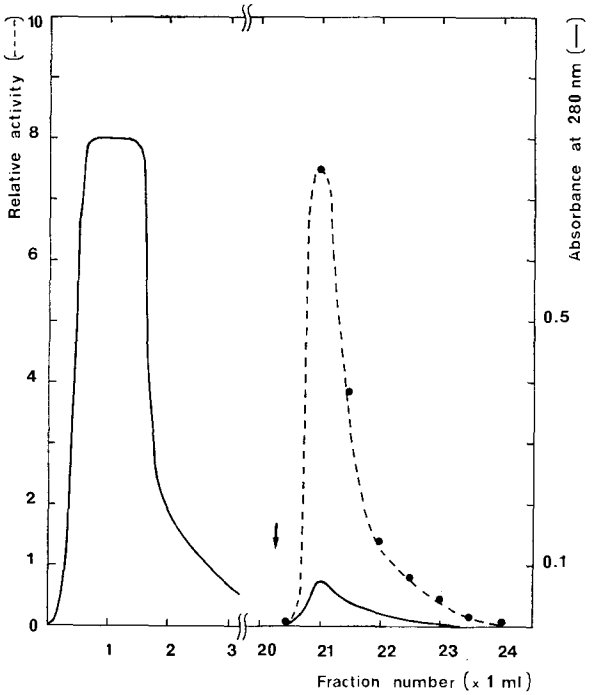

Fig. 1. Elution pattern of human leukocyte elastase from the affinity column (1 × 0.6 cm I.D.). Elastase was fixed with 0.1 *M* sodium acetate (pH 5.0) and 4 *M* NaCl, and eluted with 50% (v/v) ethylene glycol, 0.1 *M* sodium acetate (pa_H 5.0). During the chromatography the temperature was maintained at $-14°$. The arrow indicates the introduction of the elution buffer. The eluted activity was measured at 25° in 0.2 *M* Tris-HCl (pH 8.0) and 5 *M* NaCl in presence of 2% (v/v) ethylene glycol. At the eluted peak tube, the corresponding activity in 0.2 *M* Tris buffer (pH 8.0) at 25° is 20 mU. 1 U of elastase activity corresponds to the hydrolysis of 1 μmole of substrate per minute under the above conditions.

ionic strength[2]. This additional step is not necessary with the affinity chromatography system described here. However, we do not wish to stress the relative merits of the two procedures. We wish to emphasize that we have been able to demonstrate that affinity chromatography at subzero temperatures using a true substrate[1] is a feasible procedure for a real purification problem.

Our technique may be particularly suited for purifying trace amounts of elastases from other sources. In this connection, it is worthwhile noticing that macrophage elastase, which has never been purified, reacts with succinyl-L-trialanine-*p*-nitroanilide[10].

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