Journal of Chromatography, 168 (1979) 133–138

CHROM. 11,265

AFFINITY CHROMATOGRAPHY AT SUB-ZERO TEMPERATURES

A MODEL STUDY WITH PORCINE PANCREATIC ELASTASE

CLAUDE BALNY, CHRISTIAN LE DOUCEN and PIERRE DOUZOU
INSERM U 128, B.P. 5051, 34033 Montpellier Cédex (France)
and
JOSEPH G. BIETH
Laboratoire d'Enzymologie, Université Louis Pasteur, 67083 Strasbourg Cédex (France)
(Received June 22nd, 1978)

SUMMARY

A new variety of affinity chromatography of enzymes is described which consists of building up an affinity adsorbent composed of a real substrate. The chromatography is performed at a sub-zero temperature where the turnover of the enzyme is very low or stopped.

As a model system Sepharose-bound L-trialanine *p*-nitroanilide was for used the affinity binding of porcine pancreatic elastase, which was adsorbed to the column in a hypersaline medium at −14° and eluted from the column at the same temperature using 50% (v/v) ethylene glycol.

The affinity adsorbent proved to be very specific as it did not retain trypsin, chymotrypsin and ovalbumin and retained only 20% of cytochrome *c*.

INTRODUCTION

Affinity chromatography is a useful method for the isolation of biological materials[1]. If enzymes are to be purified, the affinity adsorbents are usually made up of specific inhibitors. Such inhibitors are not always available, however. In such instances, it may be desirable to perform affinity chromatography by using true substrates under non-turnover conditions. The turnover of an enzyme–substrate system can be slowed down either by chosing a pH that is far from the optimum for the enzyme[2] or by using sub-zero temperatures in fluid media[3–6].

The latter principle was used in this work. As a model system to demonstrate the practicability of sub-zero temperature affinity chromatography, we used the porcine pancreatic elastase–L-trialanine *p*-nitroanilide system[7].

EXPERIMENTAL

Materials

Porcine pancreatic elastase was prepared as described by Shotton[8]. Bovine

1-chloro-3-tosylamido-7-amino-2-heptanone-treated α-chymotrypsin was obtained from Merck (Darmstadt, G.F.R.) and bovine trypsin (type III), cytochrome *c* (type VI) and ovalbumin from Sigma (St. Louis, Mo., U.S.A.).

Succinyl-L-trialanine *p*-nitroanilide and L-trialanine *p*-nitroanilide were prepared according to Bieth *et al.*[9].

N-Acetyl-L-tryptophan ethyl ester was prepared in this laboratory. Benzoyl-L-arginine ethyl ester (highest quality grade), ethylene glycol and Sepharose 6 B CL were obtained from Fluka (Buchs, Switzerland), Riedel-de-Haën (Seelze, G.F.R.) and Pharmacia (Uppsala, Sweden), respectively.

Solutions

The buffer used was 0.1 *M* Tris–hydrochloric acid (pH 8) in all instances. Hypersaline solution was made by using 4 *M* sodium chloride in buffer, allowing a freezing point of −17°[10]. Ethylene glycol was in an aqueous buffer at a volume concentration of 50% ,and the pa_H (where a_H is the protonic activity in mixed solvent) was 8.0 at 20°[11,12]. The ΔH value of this Tris–hydrochloric acid buffer, both in hypersaline solution and in mixed solvent, was 11 kcal · l · mole^{-1}, giving a pH or pa_H of 9.2 at −14°[11].

Kinetic parameters

The K_m and k_{cat} values of the elastase–L-trialanine *p*-nitroanilide system were determined in the presence of the three media reported in Table I, at 20°, by using Lineweaver–Burk plots. The release of *p*-nitroaniline was followed at 410 nm using an Aminco DW 2 spectrophotometer. The molar absorption coefficients of *p*-nitroaniline at 410 nm were 8540, 10,200 and 10,400 l · mole^{-1} · cm^{-1} in 0.1 *M* Tris buffer, 0.1 *M* Tris + 4 *M* sodium chloride solution and 0.2 *M* Tris + 50% (v/v) ethylene glycol, respectively, at pH or pa_H 8.0 at 20°. A molar absorption coefficient of $5.2 \cdot 10^4$ mole · l^{-1} · cm^{-1} of elastase[8] was used to calculate k_{cat}.

Preparation of the affinity adsorbent[13]

Sepharose (6 g) was activated for 12 h with 6 ml of diglycidyl ester and 6 ml of 0.6 *M* sodium hydroxide solution containing 12 mg of sodium borohydride. The gel was then washed with 1 l of water. A 200-mg amount of L-trialanine *p*-nitroanilide was dissolved in 4 ml of water and the pH of this solution was adjusted to 8.0 with solid sodium carbonate. This solution was then added to the activated gel. After stirring for 48 h at 35°, the affinity adsorbent was washed successively with 250 ml of 0.5 *M* sodium carbonate solution (pH 8.0) that was 1 *M* in sodium chloride, 250 ml of water and 250 ml of 0.05 *M* glycine–hydrochloric acid buffer (pH 3.0) that was 1 *M* in sodium chloride. The gel was then stored at 4° in the dark.

The yield of the coupling reaction was determined by alkaline hydrolysis of the bound substrate. A given volume of gel was poured into 2 *M* sodium hydroxide solution. After centrifugation, the absorbance of the supernatant was read at 400 nm and converted into *p*-nitroaniline concentration by using a molar absorption coefficient of 11,000 l · mole^{-1} · cm^{-1} (ref. 7).

Chromatographic procedure

The sub-zero temperature column (I.D. 2 cm) was as described previously[14]. The temperature was regulated with a Colora thermostated bath and measured inside

the gel with a thermocouple associated with a millivoltmeter. About 2 ml of gel were used to fill the column. Elution was monitored at 280 nm using an ISCO UA-5 apparatus. The flow-rate of the column (30 ml · h^{-1}) was regulated with an ISCO Dialagrad pump. Fraction tubes were collected at 4° (ISCO fraction collector) and stored at this temperature before being analysed.

The detection of ovalbumin and cytochrome *c* was performed spectrophotometrically at 280 and 410 nm, respectively. The detection of α-chymotrypsin, trypsin and elastase was performed by enzymatic rate measurements with N-acetyl-L-tryptophan ethyl ester[15], benzoyl-L-arginine ethyl ester[16] and succinyl-L-trialanine *p*-nitroanilide [9].

The sodium chloride concentration of each fraction was measured with a CDM 3 Radiometer conductivity meter calibrated with standard solutions.

RESULTS AND DISCUSSION

Coupling yield

About 40% of the substrate reacted with the activated gel was fixed (36 μmole of substrate bound per gram of Sepharose). This low yield is probably due to the low pH used to perform the coupling[13]. A higher pH could not be used because of autolysis of the substrate.

Choice of the temperature and the medium used for the affinity chromatography of elastase

As our affinity adsorbent contained a true substrate of elastase, it was essential to perform the chromatography under non-turnover or very slow turnover conditions. In previous work[17] we have shown that the elastase-catalysed hydrolysis of succinyl-L-trialanine *p*-nitroanilide can be completely stopped at −38° in a mixed solvent system containing 50% (v/v) ethylene glycol. The initial idea was therefore to use these conditions for affinity chromatography. Measurement of the kinetic parameters revealed, however, that this was not an advantageous choice. As shown in Table I, the introduction of 50% (v/v) ethylene glycol increases K_m* by a factor of 5.6 at 20°. This is in agreement with previous data obtained with other organic solvents[7,17,18] and

TABLE I

KINETIC PARAMETERS DESCRIBING THE ELASTASE-CATALYSED HYDROLYSIS OF L-TRIALANINE-*p*-NITROANILIDE AT A pH OR A pa_H (*i.e.* PROTONIC ACTIVITY[16]) OF 8.0 AND 20°

Elastase concentration: 0.76 μ*M*.

Buffer no.	*Buffer composition*	K_m *(mM)*	k_{cat} *(sec^{-1} · 10^{-3})*
1	0.1 *M* Tris–HCl	2.3 ± 0.2	12 ± 2
2	0.1 *M* Tris–HCl + 4 *M* NaCl	2.3 ± 0.2	23 ± 2
3	0.2 *M* Tris–HCl + 50% (v/v) ethylene glycol	13 ± 2.0	14 ± 2

* For such a type of substrate for which acylation of the enzyme is the rate limiting step, K_m represents the true dissociation constant of the enzyme substrate complex.

demonstrates that hydrophobic interactions play a considerable role in the enzyme–substrate binding[15]. Moreover, from the well known effect of temperature on hydrophobic interactions, we can infer that at sub-zero temperatures the affinity is even less than that measured at room temperature. The value thus obtained, above 10 m*M*, is not suitable for affinity conditions.

It was therefore decided to try to exploit the anti-freeze property of high salt concentrations[19]. On the other hand, such a high salt concentration does not affect the enzyme–substrate affinity (Table I). In addition, high salt concentrations are thought to strengthen hydrophobic interactions[20–22] and are commonly used in hydrophobic chromatography [23].

The lowest temperature possible in 4 *M* sodium chloride hypersaline solution of −17° seems low enough to slow considerably the turnover of elastase. Previous work[17] had shown that the activation energy of the catalysis is relatively high (15–20 kcal · 1 · mole^{-1}) so that the k_{cat} is expected to be about 2 · 10^{-4} sec^{-1} at −15 °C. Indeed, as will be shown in the next section, negligible amounts of *p*-nitroaniline were formed in the course of the chromatography.

Sub-zero temperature affinity chromatography of elastase

The technique used to adsorb and desorb elastase was established as follows. A 2-ml volume of affinity adsorbent was poured at room temperature into the column and equilibrated with buffer 2 (Table I). The temperature was then decreased to −14° at the rate of 30° · h^{-1}. Under these conditions the pH was 9.2, a value at which the enzyme has still its maximal activity [18]. A 1-ml volume of a 0.2 mg · ml^{-1} elastase solution dissolved in buffer 2 (Table I) was brought to −14° and applied to the column, the temperature being maintained at −14° during manipulation. Elution was performed with the same buffer (at −14°). As shown in Fig. 1, elastase is not eluted under these conditions.

For the desorption of elastase, the property of the mixed solvent (buffer 3 in Table I) to decrease the enzyme substrate affinity was exploited. This solvent was first cooled to −14° and then applied to the column. Fig. 1 shows that elastase is then eluted. The recovery of enzyme was 90–98% in different experiments. The turnover of elastase during the sub-zero temperature chromatographic procedure was very low. Only about 0.1 μM of *p*-nitroaniline was present in the recovered elastase solution. This compound (and ethylene glycol) could be easily removed by dialysis.

As the ionic strength increases the hydrophobic interaction between proteins and matrix[22], even at sub-zero temperatures[23], it was important to study the specificity of the adsorbent used toward elastase. To this end, we tested four other proteins, including proteases, with our chromatographic system.

Specificity of the affinity adsorbent

The specificity was checked with ovalbumin, cytochrome *c*, trypsin and α-chymotrypsin under conditions identical with those used for elastase. With ovalbumin and cytochrome *c*, the column was loaded with 1 mg of protein dissolved in 1 ml of buffer 2. The two compounds were eluted with buffer 2 in a position corresponding to the void volume of the Sephadex column (2–4 ml). No further desorption could be detected after elution with buffer 3. Whereas the recovery was 100% with ovalbumin, it was only 80% with cytochrome *c*, indicating that part of this

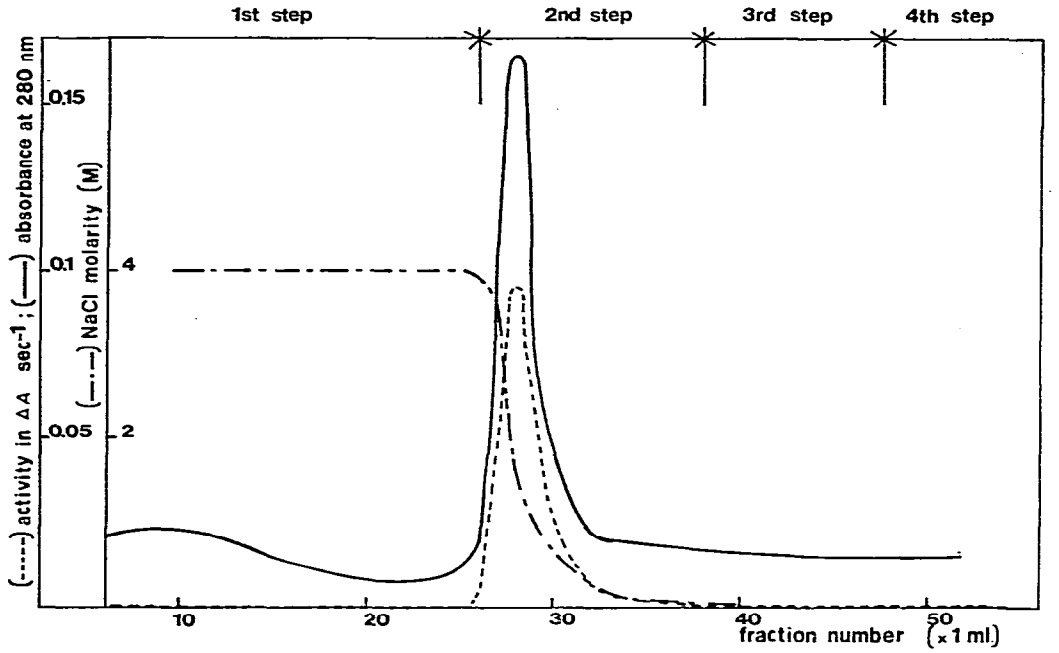

Fig. 1. Elution pattern of elastase (0.2 mg applied) from the affinity column (2 × 1 cm). First step, 0.1 *M* Tris buffer containing 4 *M* NaCl, pH = 9.2, temperature −14°; second step, 0.1 *M* Tris buffer containing 50% (v/v) ethylene glycol, pa_H = 9.2, temperature −14°; third buffer ,same as the second but temperature = 0°, pa_H = 8.6; fourth buffer, same as the second but temperature = 20°, pa_H = 8.0. (———), Absorbance at 280 nm; (·····), activity in $\Delta A \cdot sec^{-1}$ measured at 410 nm (20°) using succinyl-L-trialanine *p*-nitroanilide as substrate; (—·—·—), molarity of the fractions.

protein remains on the gel. A plausible explanation for this behaviour is that non-specific adsorption of this protein occurs because chromatography is performed at pH 9.2 which is not far from its isoelectric point[24].

With trypsin and α-chymotrypsin, the column was loaded with 0.4 mg of enzyme dissolved in 1 ml of buffer 2. As for ovalbumin, these two proteases were eluted in a position corresponding to the void volume of the column. No further elution was detected after passage of buffer 3 and the recovery was 100%.

These results demonstrate the specificity of the affinity adsorbent for porcine pancreatic elastase.

CONCLUSION

This is the first report describing the use of a true substrate for the isolation of an enzyme by sub-zero temperature affinity chromatography under conditions of very low turnover of the enzyme. Owing to the specificity of the adsorbent for elastase, this method can probably be used for the single-step isolation of this protease from crude extracts of pancreatic powder, leukocyte granular extracts or homogenates of macrophages. The last two extracts are worth mentioning because our method is particularly suitable for the purification of micro-amounts of elastase.

Other enzymes (*e.g.*, α-chymotrypsin) may also be isolated after coupling a specific substrate to an insoluble matrix. Work along these lines is now in progress.

ACKNOWLEDGEMENTS

The authors thank K. Andersson for helpful discussions. This work was supported by grants from the D.G.R.S.T. (convention No. 75.7.045) and the Fondation pour la Recherche Médicale Française.

REFERENCES

1 K. Mosbach, *Methods Enzymol.*, 44 (1976) 3.
2 S. C. March, I. Parisk and P. Cuatrecasas, *Advan. Exp. Med. Biol.*, 42 (1974) 3.
3 P. Douzou, *Cryobiochemistry, an Introduction*, Academic Press, New York, 1977.
4 J. W. Hastings, C. Balny, C. Le Peuch and P. Douzou, *Proc. Nat. Acad. Sci. U.S.*, 70 (1973) 3468.
5 A. L. Fink, *Biochemistry*, 15 (1976) 1580.
6 P. Douzou, *Advan. Enzymol.*, 45 (1977) 157.
7 J. G. Bieth and C. G. Wermuth, *Biochim. Biophys. Res. Commun.*, 53 (1973) 383.
8 D. M. Shotton, *Methods Enzymol.*, 19 (1970) 113.
9 J. G. Bieth, B. Spiess and C. G. Wermuth, *Biochem. Med.*, 11 (1974) 350.
10 R. C. Weast (Editor), *Handbook of Chemistry and Physics*, CRC Press, Cleveland, Ohio, 54th ed., 1974.
11 G. Hui Bon Hoa and P. Douzou, *J. Biol. Chem.*, 248 (1974) 4649.
12 C. Larroque, P. Maurel, C. Balny and P. Douzou, *Anal. Biochem.*, 73 (1976) 9.
13 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87.
14 C. Balny, C. Le Peuch and P. Debey, *Anal. Biochem.*, 63 (1974) 321.
15 P. Maurel, *J. Biol. Chem.*, 253 (1978) 1677.
16 P. Maurel, G. Hui Bon Hoa and P. Douzou, *J. Biol. Chem.*, 250 (1975) 1376.
17 C. Balny and J. G. Bieth, *FEBS Lett.*, 80 (1977) 182.
18 J. G. Bieth and C. G. Wermuth, *Proc. IVth FEBS Meeting, Budapest, 1974.*
19 P. Douzou, G. Hui Bon Hoa and G. A. Petsko, *J. Mol. Biol.*, 96 (1975) 367.
20 C. J. Morris, *Trends Biochem. Sci.*, 1 (1976) N207.
21 J. Rosengren, J. Pahlman, M. Glad and S. Hjerten, *Biochim. Biophys. Acta*, 412 (1975) 51.
22 J. L. Ochoa, *Biochimie*, 60 (1978) 1.
23 C. Le Peuch and C. Balny, *FEBS Lett.*, 87 (1978) 232.
24 R. J. Yon, *Biochem. J.*, 126 (1972) 765.