A NEW APPROACH FOR THE ISOLATION OF BIOLOGICALLY ACTIVE COMPOUNDS BY AFFINITY CHROMATOGRAPHY: ISOLATION OF TRYPSIN

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Received 30 January 1973

1. Introduction

Affinity chromatography has recently been introduced as a method for purification of biologically active compounds [1]. The method depends on the affinity of a protein towards its specific hapten or inhibitor covalently coupled to an insoluble matrix. In contrast to other chromatographic methods which separate proteins according to their size and charge, affinity chromatography separates proteins on the basis of their functional specificity and has been applied to the isolation of enzymes [1, 2], antibodies [3], antigens [4, 5] and receptors [6].

Several laboratories have reported the purification of trypsin by using a protein inhibitor covalently attached to Sepharose [7, 8]. We report here a new approach for the isolation of trypsin as its soluble complex with dinitrophenylated soybean trypsin inhibitor (DNP-STI) on an antidinitrophenyl antibody column [4, 5]. The procedure involves the following steps: i) Dinitrophenylation of STI with dinitrobenzene sulfonate. ii) Formation of the complex between the DNP-STI and trypsin. iii) The complex is adsorbed to the anti-DNP-column and eluted from the column under conditions which dissociate antigen–antibody complexes. iv) Separation of the complex to its components to give pure trypsin.

Although this paper describes the isolation of trypsin, the principles and techniques are clearly applicable to virtually all interacting systems comprised of two or more components, such as hormone–receptor interactions.

2. Materials and methods

2.1. *Materials*

Soybean-trypsin inhibitor, and trypsin were obtained from Worthington Biochemical Corp., Freehold, N.J. Sepharose 4B and Sephadox G-75 were purchased from Pharmacia, Uppsala, Sweden. Dinitrobenzenesulfonate was a product of Fluka, A.G., Buchs, Switzerland. N-Benzoyl-L-arginine ethyl ester (BAEE) was a product of Miles-Yeda, Rehovot, Israel.

2.2. *Methods*

Goat anti-DNP antibodies were prepared from goat serum by affinity chromatography on DNP-ovalbumin-Sepharose column [3]. Coupling of antibodies to Sepharose was performed as previously described [4]. Amino acid analysis was performed essentially as described by Moore and Stein [9] using a one column system. No corrections were made for the destruction of amino acids during acid hydrolysis (6 M HCl 110°, 24 hr). Absorbance of proteins or their derivatives was read at appropriate wavelengths using an Acta V Beckman spectrophotometer. Tryptic activity was determined spectrophotometrically following the hydrolysis of BAEE at pH 8.0 [10].

2.3. *Dinitrophenylation of soybean trypsin inhibitor*

A solution of 24 mg STI in 1 ml of 1 M sodium bicarbonate was reacted with 40 mg dinitrobenzenesulfonate at 37° for 2 hr. The protein was then dialyzed against 0.02 M sodium acetate pH 5.0. The precipitate formed was dissolved in 0.1 M ammonium bicarbonate pH 8.4. The protein emerged as a single peak when chromatographed on Sephadex G-75.

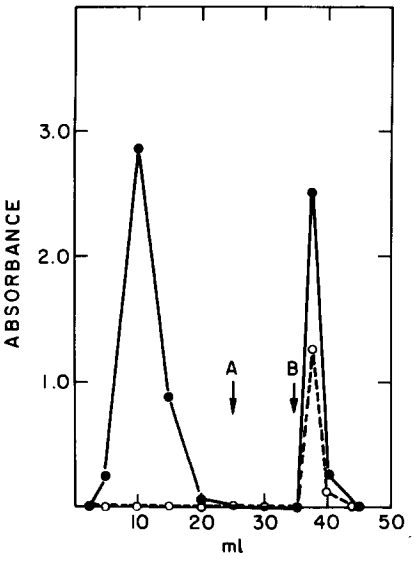

Fig. 1. Fractionation of DNP-STI on anti-DNP-Sepharose column. DNP-STI (10 mg) was applied to a 0.5 × 10 cm anti-DNP-Sepharose column. After washing with 0.1 M NH_4HCO_3 the column was washed with water (A). The DNP-STI was eluted with 10% formic acid (B). Absorbance at 280 nm (•—•—•), absorbance at 335 nm (o—o—o).

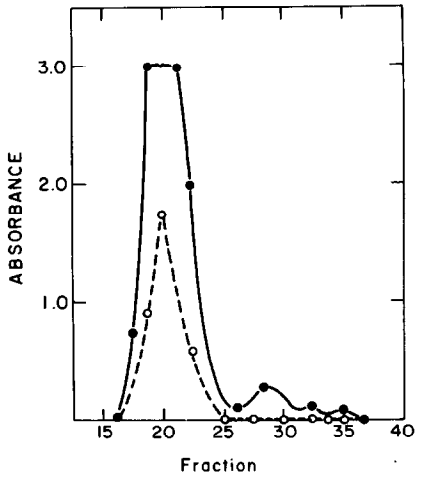

Fig. 2. Gel filtration of dinitrophenylated STI–trypsin complex. A mixture of 18 mg of trypsin and 10 mg DNP-STI in 1 ml of 0.05 M NH_4HCO_3, pH 8.4 was applied to column (1.5 × 90 cm) of Sephadex G-75 previously equilibrated with the same buffer. Column was eluted at a flow rate of 12 ml/hr and monitored at 280 nm (•—•—•) and at 335 nm (o—o—o). Volume of fractions 5 ml/tube.

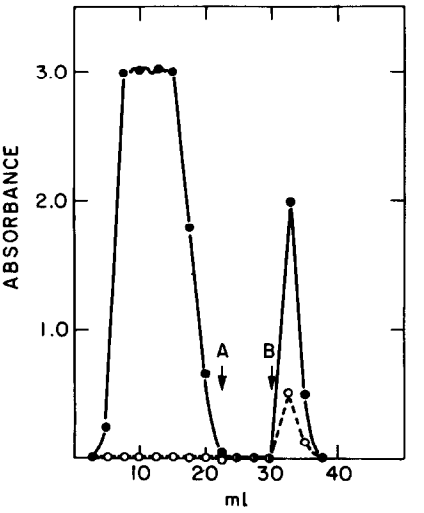

Fig. 3. Isolation of DNP-STI–trypsin complex on anti-DNP-Sepharose. A mixture of 3 mg of DNP-STI and of 10 mg of trypsin in 1 ml of 0.1 M NH_4HCO_3 was applied to 0.5 × 10 column of anti-DNP-Sepharose. After washing with 0.1 M NH_4HCO_3 and water (A) elution was started with 10% formic acid (B). Absorbance at 280 nm (•—•—•). Absorbance at 335 nm (o—o—o).

3. Results

The reaction of STI with dinitrobenzene sulfonate modified 2 to 4 lysines out of the eleven present in the protein [10]. The separation of dinitrophenylated STI (with at least one DNP group per mole of protein) from the protein that remained unmodified was accomplished by chromatography on anti-DNP-Sepharose column. All the DNP-STI was adsorbed, while native unmodified STI passed unretarded (fig. 1).

It was found that the DNP-STI still inhibits trypsin and 1 mg of DNP-STI binds 1.8 mg of trypsin. Passage of a solution of 10 mg DNP-STI and 18 mg of trypsin through a column of Sephadex G-75 permitted the isolation of the DNP-STI–trypsin complex. The yellow coloured complex which corresponds to the first peak (fig. 2) represented 92% of the protein applied to the column.

Isolation of the complex by chromatography on Sephadex G-75 column as mentioned above is non-specific. It would not ensure the homogeneity of complex isolated when using biological material

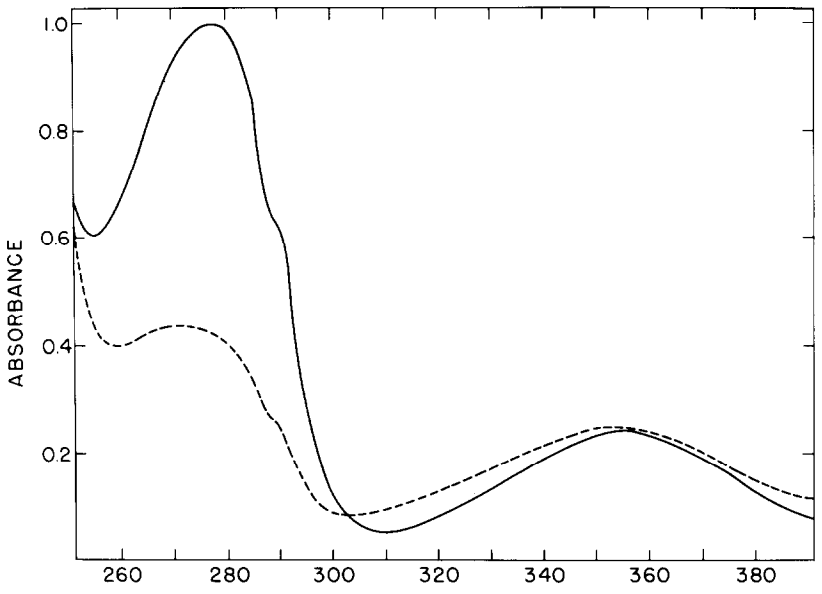

Fig. 4. Absorption spectra of DNP-STI (---), and of DNP-STI–trypsin complex (—) isolated from the anti-DNP–antibody column.

containing trypsin without prior purification. Several other proteins, similar in size to the complex, could be expected as contaminants. Hence to isolate a pure complex, the solution containing the complex DNP-STI–trypsin was applied to the anti-DNP-Sepharose column. The amount of complex adsorbed was equivalent to the amount of bound antibody. Free trypsin or complex not containing dinitrophenyl groups was not adsorbed by the antibody column. Fig. 3 depicts the use of the anti-DNP-Sepharose columns for the isolation of the DNP-STI–trypsin complex. A mixture of DNP-STI (3 mg) and excess trypsin (10 mg) was passed through the column at room temp. Most of the yellow material was adsorbed to the column and could be eluted with 10% formic acid, or ϵ-DNP-lysine. After treatment with formic acid the antibody Sepharose column was immediately washed with water and 0.1 M NH_4HCO_3 and can be used again but with reduced capacity. The eluted yellow DNP-STI–trypsin complex (fig. 4) which did not possess any tryptic activity was lyophilized, dissolved in 0.1 M KCl pH 2 and applied to the Sephadex G-75 column equilibrated with the same solvent. The first peak was collected and found to contain 55% of the original tryptic activity.

4. Discussion

In this study trypsin was used as a model compound in the development of a new approach for the purification of proteins present in minute amounts which possess the ability to complex with other compounds.

The 4-step method described in this communication takes advantage of the biological specificity of antibodies. It is based on the affinity of the antibody to a certain ligand attached to a complex of two or more proteins and is independent of the chemical, physical and biological properties of the complex itself. Only the complexes which possess such a ligand will be specifically sequestered by the antibodies and thereby removed from the entire extract of proteins.

The introduction of Sepharose as a convenient support for insolubilizing antibodies [4] allows the employment of small columns for the isolation of micromole amounts of the complex. The antibody Sepharose column can also serve as a means for concentrating the complex. Thus, the adsorbed complex can be recovered in approximately the volume of the antibody Sepharose column, regardless of the volume

in which the complex was applied to this column. The adsorption of the DNP-STI–trypsin complex was accomplished under mild conditions. The elution of the complex from the antibody column required more drastic conditions such as 10% formic acid. The complex was also eluted with the hapten ε-DNP-lysine but after this treatment the removal of the ε-DNP-lysine from the column required 6 M guanidine hydrochloride. The use of 6 M GnHCl causes substantial inactivation of the antibodies, and the column could not be used again efficiently. Since the elution of DNP hapten from the antibody column requires drastic conditions, attempts are now being made to modify the proteins with other haptens such as arsanilic acid, which dissociate from the antibody column under mild conditions, e.g. 0.1 M acetic acid or 0.8 M NH_4OH. After such treatment the column can be regenerated to full capacity, and can be used many times.

The use of antibodies as a tool for the isolation of complexed molecules can be extended to many other instances, since antibodies can be raised against almost any hapten. Attempts to isolate specific proteins from membranes may be divided into two types: i) The protein is specifically labelled in situ, solubilized and then purified. The preparation of antibodies to the labeling molecule will provide means for the isolation of the labeled protein in one step. ii) In the other approach the membrane is solubilized, and the protein identified and purified. This approach was recently used for the isolation of insulin receptor of liver cell membranes on an insulin Sepharose column [6]. Studies are in progress in the use of anti-DNP-Sepharose columns for the isolation of the complex containing DNP-[I^{125}]-insulin–specific receptor from human lymphocytes.

References

[1] P. Cuatrecasas, M. Wilchek and C.B. Anfinsen, Proc. Natl. Acad. Sci. U.S. 61 (1968) 636.
[2] M. Wilchek and M. Gorecki, European J. Biochem. 11 (1969) 491.
[3] Y. Weinstein, M. Wilchek and D. Givol, Biochem. Biophys. Res. Commun. 35 (1969) 694.
[4] D. Givol, Y. Weinstein, M. Gorecki and M. Wilchek, Biochem. Biophys. Res. Commun. 38 (1970) 825.
[5] M. Wilchek, V. Bocchini, M. Becker and D. Givol, Biochemistry 10 (1971) 2828.
[6] P. Cuatrecasas, Proc. Natl. Acad. Sci. U.S. 69 (1972) 1277.
[7] G. Feinstein, FEBS Letters 7 (1970) 353.
[8] N.C. Robinson, R.W. Tye, H. Neurath and K.A. Walsh, Biochemistry 10 (1971) 2743.
[9] S. Moore and W.H. Stein, Methods in Enzymology, Vol. VI (Academic Press, 1963) p. 319.
[10] G.W. Schwert and Y. Takenaka, Biochim. Biophys. Acta 16 (1955) 570.
[11] Y.V. Wu and H.A. Scheraga, Biochemistry 1 (1962) 698.