ISOLATION OF CARBOXYPEPTIDASE N BY AFFINITY CHROMATOGRAPHY ON COLUMN OF CNBr-ACTIVATED SEPHAROSE WITH IMMOBILIZED ANTIBODY

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The isolation of rat serum carboxypeptidase N (E.C.3.4.2.2.) by affinity chromatography on a column of CNBr-activated Sepharose with immobilized antibody is described. The ligands used were either rabbit antiserum to rat carboxypeptidase N or the IgG fraction prepared from this serum. The coupling of the isolated antibodies to CNBr-activated Sepharose increased the capacity of the column approximately three times. The specific activity of the enzyme prepared by this method was 109-times higher than the activity of the serum. Analysis of the final product by polyacrylamide gel electrophoresis showed carboxypeptidase N and traces of albumin.

The introduction of affinity chromatography has provided new possibilities of purification of proteins based on differences in their biological activity. Since the reaction of the antigen with the antibody belongs – like the reaction of the enzyme with the inhibitor or the substrate – to highly specific reactions, antigens and haptens coupled to various supports have extensively been utilized[1–15] for the isolation of complementary antibodies (or, *vice versa*, immobilized antibodies have been used for the isolation of antigens) even though affinity chromatography has gained wider application during the past few years only.

In this study rat carboxypeptidase N was isolated by affinity chromatography on columns of CNBr-activated Sepharose 4B to which rabbit antiserum to the enzyme or the IgG fraction isolated from the serum were covalently coupled. We endeavored to develop a simple and time-saving method of separation of rat carboxypeptidase N to be used in studies on this enzymes and related problems.

EXPERIMENTAL

Material

Rat serum was stored at −35°C. The euglobulin fraction of rat serum was prepared by twenty-fold dilution of the serum by distilled water and adjustment of pH to 5·2. The precipitate was separated after 90 min of standing at +4°C by centrifugation and dissolved in 0·1M sodium phosphate buffer, pH 8·0, to 1/3–1/4 of the volume of the original serum.

The synthetic substrate alpha-N-carbobenzoxy-L-phenylalanyl-L-arginine (CboPheArg) was prepared and kindly provided by Dr E. Kasafírek of the Research Institute for Pharmacy and

Biochemistry, Prague. CNBr-activated Sepharose 4B was from Pharmacia, Uppsala. Rabbit antiserum to rat carboxypeptidase N was prepared in our laboratory. The preparation of the antigen and the immunization are described in our preceding paper[16].

Methods

Preparation of IgG fraction of rabbit antiserum to rat carboxypeptidase N. The immunoglobulins of rabbit antiserum were isolated by column chromatography on DE 52 cellulose according to Levy and Sober[17]. The first protein effluents emerging from the column in 17·5 mM phosphate buffer at pH 6·3 and containing unadsorbed IgG were pooled, dialyzed against distilled water, and lyophilized. The IgG fraction obtained by this procedure was immunochemically homogeneous.

Preparation of CNBr-activated Sepharose with immobilized antibody: CNBr-activated Sepharose 4B (3 g) was washed with 1 mM-HCl according to the manufacturer's directions. Rabbit antiserum (2 ml) to rat carboxypeptidase N or the lyophilized IgG fraction (150 mg) prepared from this antiserum were mixed with 10 ml of 0·1M-$NaHCO_3$ in 0·5M-NaCl, pH 8·8 and added to the washed gel in a test tube. The coupling was allowed to proceed 2 h at room temperature with constant stirring. After the coupling the gel was set aside for 16 h at 4°C. Subsequently the proteins adsorbed were washed off with four 100 ml portions of buffer at pH 4 (0·1M acetate buffer containing 1M-NaCl) and pH 9 (0·1M borate containing 1M-NaCl). The gel was then washed with distilled water and the starting buffer. The determination of the protein content of the filtrate showed that the proteins were adsorbed from the antiserum completely and from the IgG fraction by about 83%.

Affinity chromatography on CNBr-activated Sepharose with the immobilized antibody was carried out on a cooled 1·2 × 10 cm column. Rat serum (2 ml) or the corresponding quantity of the euglobulin fraction, which had been dialyzed against the starting buffer, were applied to the equilibrated column. After the application of the sample to the support the protein was eluted by 0·1M Tris-HCl buffer in 0·5M-NaCl, pH 8·0. Proteins not adsorbed were washed off and the column was then eluted by the same buffer yet in 2M-NaCl and the protein fraction showing carboxypeptidase activity emerged. Finally, the column was washed with 0·1M Tris-HCl buffer in 2M-NaCl, pH 5·0. Fractions (3 ml) were automatically collected at a rate of 36 ml/h. The protein content of the fractions was determined by absorbance measurement at 280 nm in Unicam SP 500 spectrophotometer and the carboxypeptidase activity of the fractions was assayed with CboPheArg as substrate.

The activity of carboxypeptidase N was assayed by the ninhydrin method[18] with CboPheArg as substrate.

RESULTS AND DISCUSSION

The preparation of rat serum carboxypeptidase N by affinity chromatography was effected on CNBr-activated Sepharose with attached rabbit antiserum. The adsorption to the column was allowed to proceed in 0·1M Tris-HCl buffer containing 0·5M-NaCl, pH 8·0, and the enzyme emerged as a sharp peak after the concentration of sodium chloride in the eluting buffer had been increased to 2M (Fig. 1). We found that the quantity of the active fraction obtained increases with the increasing volume of serum applied (Table I).

TABLE I

Quantity of Protein Eluted by 2 M-NaCl as Function of Volume of Serum Applied
The enzymatic activity was determined with CboPheArg as substrate.

Volume of serum ml	Quantity of protein mg	Specific activity μmol Arg/mg protein
0·5	4·2	4·7
1·0	4·6	5·6
2·0	8·6	6·3
3·0	10·4	6·0
4·0	11·2	6·5

TABLE II

Purification of Carboxypeptidase N

Fraction	Volume ml	Protein mg/ml	Activity μmol Arg/ml	Specific activity μmol Arg/mg protein	Purification step
Rat serum	20	70	30	0·43	1
Euglobulin fraction	6	32	80	2·5	5·8
Effluent from column of CNBr-activated Sepharose + IgG fraction	3	3·3	155	46·9	109

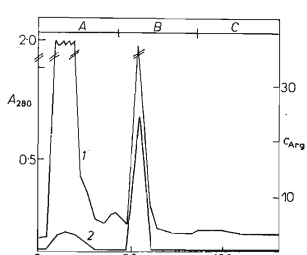

FIG. 1

Affinity Chromatography of Rat Serum on CNBr-Activated Sepharose with Immobilized Antiserum to Carboxypeptidase N
1 Absorbance at 280 nm, *2* carboxypeptidase activity hydrolyzing CboPheArg, elution volume in ml, A_{280} absorbance at 280 nm, C_{Arg} enzymatic activity expressed in μmol of arginine liberated in 1 ml.

After the application of 2 ml of rat serum to the column a part of the carboxypeptidase activity was contained in the proteins not adsorbed to the column (Fig. 1) yet the quantity of the fraction eluted by 2M-NaCl was almost the double. The further increase of the emerging protein showing carboxypeptidase activity was not proportional, however, to the volume of the sample applied. Likewise, the specific activity of this protein fraction remained approximately constant.

Since the yield of carboxypeptidase N depends both on the binding capacity of the support and on the binding capacity of the antiserum, we made an effort to increase the efficiency of the method by attachment of the isolated antibody prepared from the antiserum by column chromatography on DEAE-cellulose.

Rat serum, equilibrated with the first buffer and the support in the column, was used as starting material for the adsorption to CNBr-activated Sepharose to which the IgG fraction of rabbit antiserum had been coupled. The quantity of the fraction showing carboxypeptidase activity and emerging from the column in the buffer containing 2M-NaCl, increased with the volume of rat serum applied from 1 ml to 6 ml. The elution profile corresponded to the profile shown in Fig. 1. The effluents showing carboxypeptidase activity were pooled, dialyzed and lyophilized, and the protein content was analyzed by polyacrylamide gel electrophoresis and immunoelectrophoresis. Since this material contained in addition to carboxypeptidase a considerable quantity of albumin which could not be decreased either by increasing the ionic strength of the buffer or by decreasing the pH, we adsorbed to the column the euglobulin fraction of rat serum prepared at pH 5·2. The purification procedure is summarized in Table II.

The specific activity of carboxypeptidase N prepared by this procedure increased 109 times compared to the original serum. Polyacrylamide gel electrophoresis showed the zone of carboxypeptidase N and traces of albumin. The activity of carboxypeptidase N was estimated after cutting the gel to pieces and its homogenization using CboPheArg as substrate.

After attachment of the IgG fraction of the antiserum the capacity of the column increased approximately three times. To obtain maximum yields of the active fraction the column could be charged with up to 6 ml of rat serum whereas only 2 ml could be applied to the column with the immobilized antiserum. The increase in the capacity of the column, however, was not proportional to the quantity of the IgG fraction attached; this could be ascribed to unfavorable sterical parameters or to the absence of an equilibrium between the antigen and the antibody.

The increase of the quantity of the antibody eluted after the increase of the volume applied has been noted by Anderson and coworkers[19] in their experiments with cyclic affinity chromatography on an immobilized antigen. They observed an almost linear dependence over a certain range.

The preparation of carboxypeptidase N by the method described is very little time-consuming. Its main advantage lies in the fact that one column can be repeatedly

used without an impairment of its function. In order to obtain the pure enzyme it would be necessary to adsorb to the support a material freed beforehand of albumin or to find a method of decreasing its adsorption to the column. The rabbit antiserum to rat carboxypeptidase N attached to the support in this study did not contain any antibodies to rat serum albumin which could account for the considerable adsorption of this protein. The cause of the relatively strong adsorption of albumin are most likely not only electrostatic forces but also simultaneously proceeding hydrophobic interactions[20,21]. We have met with a similar phenomenon when isolating human and rat serum carboxypeptidase N by affinity chromatography on CNBr-activated Sepharose with immobilized hippuryl-L-lysine, a synthetic substrate[22]. Neither in the latter case we were able to prevent albumin from adsorption to the column by increasing the ionic strength of the buffer.

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