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REVERSIBLE AND IRREVERSIBLE IMMOBILIZATION OF CARBOXYPEPTIDASE Y USING BIOSPECIFIC ADSORPTION

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SUMMARY

To find optimal biospecific hydroxyalkyl methacrylate (Spheron) derivatives for reversible and irreversible immobilization of carboxypeptidase Y, we elaborated the procedure for the biospecific sorption of carboxypeptidase Y to Spheron—Gly-Gly-*p*-aminobenzylsuccinic acid, mercury—Spheron and concanavalin A—Spheron. The highest yield was achieved in the case of concanavalin A—Spheron, which was also used for the irreversible, oriented immobilization by cross-linking of carboxypeptidase Y and concanavalin A with glutaraldehyde.

INTRODUCTION

The combination of many complementary binding sites on the surface of enzymes makes possible the formation of a number of biospecific complexes that can be used not only for efficient isolation but also for oriented immobilization. Fig. 1 shows the surface of an enzyme covered with several complementary binding sites. For example, this could be carboxypeptidase Y (CPY) containing a complementary binding site for the specific inhibitor Gly-Gly-*p*-aminobenzylsuccinic acid and one free SH group, which is placed in the active site of the enzyme. CPY also contains a carbohydrate moiety, which is a complementary binding site for the active site of lectin concanavalin A (Con A). The antigenic sites of the protein can be identified by determination of the

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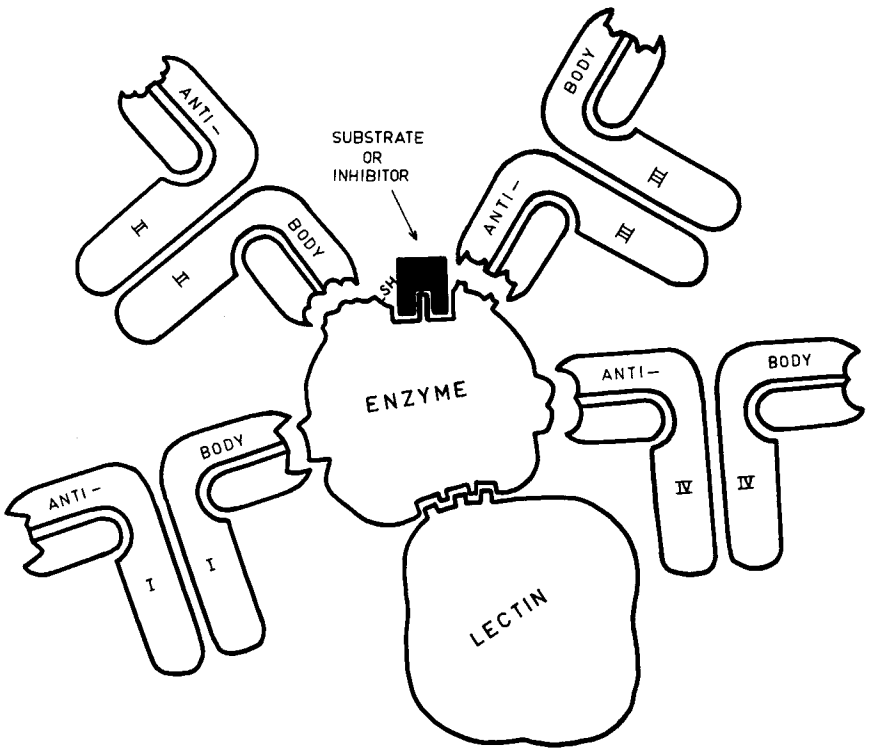

Fig. 1. Possibilities of the enzyme to form biospecific complexes.

antigenic structures of the peptide chain after its interaction with specific antibodies according to Attasi [1] who described, for example, five precise antigenic sites in the molecule of myoglobin.

The following may serve as another example: about 30% of the approximately 2000 enzymes found in the cell contain nucleotide coenzymes participating in the reaction with nucleotides. The specific recognition of certain nucleotide sequences by proteins is one of the reasons for studying peptide—nucleotide interactions [2].

It is generally known that many enzymes in the form of biospecific complexes exhibit higher stability. Well oriented attachment of enzymes to solid supports can result in a higher relative activity of the immobilized enzyme. Hsiao and Royer [3] compared several ways of attaching CPY to agarose and glass. Immobilized CPY with the highest activity was prepared by adsorption of the enzyme to Con A—Sepharose and subsequent cross-linkage by glutaraldehyde. Hsiao and Royer [3] also proved that the carbohydrate moiety can be used for oriented immobilization of enzymes, and oriented immobilization guarantees high activity of immobilized enzymes. Practically the same kinetic constants of peptidase and esterase activities of native and immobilized carboxypeptidase A were determined by Solomon et al. [4] when the enzyme was attached to the immobilized monoclonal antibody. Monoclonal antibodies were also used for specific immobilization of guinea pig liver transglutaminase by Ikura et al. [5].

The aim of the work reported here was to find optimal biospecific hydroxy-

alkyl methacrylate derivatives for reversible and irreversible immobilization of CPY. The first step was purification of CPY by biospecific sorption via different complementary binding sites. The results of these purification experiments served for the determination of the optimal hydroxyalkyl methacrylate derivative for immobilization. Con A—Spheron was used for oriented immobilization of CPY.

EXPERIMENTAL

CPY (EC 3.4.16.1) was obtained from Biolar (Olain, U.S.S.R.). N-Carboxybenzoxy-L-phenylalanyl-L-alanine (*Z*-Phe-Ala) was a product of Sigma (St. Louis, MO, U.S.A.), ribonuclease (EC 3.1.26.1) and 2-(N-morpholino)ethanesulphonic acid (MES) were from Reanal (Budapest, Hungary) and 2-mercaptoethanol and glutaraldehyde were from Fluka (Buchs, Switzerland). Sodium dodecyl sulphate (SDS) was from Serva (Heidelberg, F.R.G.). Spheron 1000 (100—200 μm), DEAE—Spheron 1000 (25—40 μm) and the remaining chemicals of analytical purity were supplied by Lachema (Brno, Czechoslovakia). The mercury derivative of the hydroxyalkyl methacrylate gel (mercury—Spheron) was prepared according to Turková et al. [6]; Gly-Gly-*p*-aminobenzylsuccinic acid—Spheron was prepared according to Turková et al. [7]. Con A—Spheron was kindly donated by Dr. K. Filka (Department of Biochemistry, Charles University, Prague, Czechoslovakia).

Purification of CPY from a yeast autolysate on Spheron—Gly-Gly-p-aminobenzylsuccinic acid

The autolysate from yeast *Saccharomyces cerevisiae* (50 ml) prepared according to Johansen et al. [8] was applied to a column (9 × 0.8 cm I.D.) of Spheron—Gly-Gly-*p*-aminobenzylsuccinic acid (content of immobilized inhibitor: 2.6 μmol/g of dry carrier) equilibrated with 0.01 *M* sodium acetate (pH 5.0). The column was washed with 1 l of 1 *M* sodium chloride—0.01 *M* sodium acetate (pH 4.3). CPY was desorbed with 0.01 *M* phosphate buffer (pH 7.0). The flow-rate was 50 ml/h; fraction volume 4.0 ml (see Fig. 2A).

Chromatography of CPY on mercury- Spheron

A solution (10 ml) of CPY (0.1 mg/ml) was applied to a column (8 × 0.8 cm I.D.) of mercury—Spheron (prepared with 15% mercury derivative of methacrylanilide, 93 μmol Hg per ml gel) equilibrated with 0.1 *M* sodium acetate (pH 6.0). The column was washed with 15 ml of starting buffer and CPY was desorbed with 0.05 *M* 2-mercaptoethanol—0.1 *M* sodium acetate (pH 6.0). The flow-rate was 15 ml/h; fraction volume 1 ml (see Fig. 2B).

Chromatography of CPY on Con A—Spheron

A solution (200 ml) of CPY (0.1 mg/ml) was applied to a column (18 × 0.8 cm I.D.) of Con A—Spheron (content of immobilized Con A: 0.4 μmol/g of dry carrier) equilibrated with 0.4 *M* sodium chloride—0.1 *M* sodium acetate (pH 7). The column was washed with 20 ml of starting buffer and CPY was eluted with 0.1 *M* borate buffer (pH 6.5). The flow-rate was 10 ml/h; fraction volume 1 ml (see Fig. 2C).

Chromatography of CPY on DEAE—Spheron

A solution (4 ml) of CPY (3 mg/ml) was applied to a column (35 × 0.6 cm I.D.) of DEAE—Spheron (1.5 mequiv./g) equilibrated with 0.1 *M* sodium chloride—0.1 *M* sodium acetate (pH 7.0). A gradient of sodium chloride was used for elution. The pressure was 1 MPa, flow-rate was 130 ml/h and fraction volume was 3.5 ml (see Fig. 3).

Immobilization of CPY on Con A—Spheron by cross-linking with glutaraldehyde

The immobilization of CPY was performed according to Hsaio and Royer [3], with some modifications. A fraction of CPY from DEAE—Spheron chromatography (10 ml) was applied to a column (6 × 0.8 cm I.D.) of Con A—Spheron (content of immobilized Con A: 0.4 μmol/g of dry carrier) equilibrated with 0.4 *M* sodium chloride—0.1 *M* sodium acetate (pH 7.0). The carrier with adsorbed CPY was then put into a 25-ml flask and after sucking out the buffer was suspended in 10 ml of the equilibrating buffer with 0.5% (w/w) glutaraldehyde. The suspension was shaken at laboratory temperature for 45 min, then the carrier was washed with 1 l of 0.1 *M* borate buffer (pH 6.5).

Peptidase activity of CPY

Peptidase activity was measured according to Hayashi [9] in 0.05 *M* MES buffer (pH 6.75) with 2 m*M* *Z*-Phe-Ala as substrate.

Rate of release of amino acids from ribonuclease by CPY-Con A—Spheron

The reaction was carried out at 25°C with a substrate concentration of 0.3 μmol/ml and substrate to enzyme ratio (mol/mol) of 2000:1 in 0.1 *M* sodium acetate—0.5% SDS buffer (pH 6.0).

RESULTS AND DISCUSSION

For several years we have used Spheron—Gly-Gly-*p*-aminobenzylsuccinic acid for the isolation of CPY from yeast autolysate. Fig. 2A shows the purification of the enzyme under the conditions described by Johansen et al. [8]. Chromatography of yeast autolysate on Spheron—Gly-Gly-*p*-aminobenzylsuccinic acid has already been used for large-scale preparation of CPY by the Latvian company Biolar. Since the specific activity and homogeneity of the isolated CPY are largely influenced by the quality and conditions of yeast fermentation [10], we used only the enzyme prepared by Biolar for our study of enzyme stabilization by immobilization, rather than our own preparations. To compare the individual specific adsorbents, the yield of active CPY isolated was determined by its rechromatography under identical conditions; the yield of CPY isolated on Spheron—Gly-Gly-*p*-aminobenzylsuccinic acid was 68%.

Fig. 2B shows the chromatography of CPY from Biolar on mercury—Spheron. We [6] described the preparation of hydroxyalkyl methacrylate copolymers containing various amounts of mercury derivatives of *p*-acetaminophenoxyethyl methacrylates or of methacrylanilide. The derivative best suited for isolation of SH-proteinases was the one containing 15% mercury derivative of methacrylanilide. The yield of the CPY isolated was 25%.

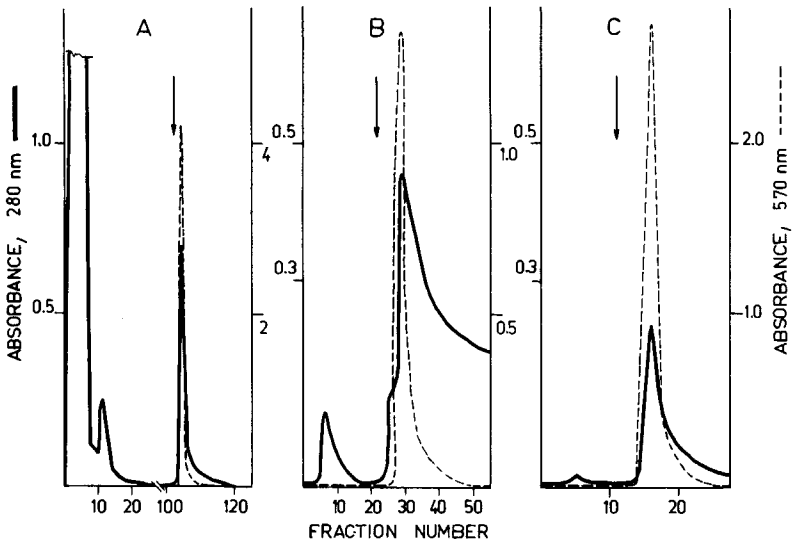

Fig. 2. (A) Gly-Gly-*p*-aminobenzylsuccinic acid–Spheron: the column (5 ml) was equilibrated with 0.01 *M* sodium acetate (pH 5.0). The sample of autolysate (50 ml) was added and then the column was washed with 1 *M* sodium chloride–0.01 *M* sodium acetate (1 l), pH 4.3. The elution (arrow) was performed with 0.01 *M* phosphate buffer (pH 7). (B) Mercury–Spheron: the column was equilibrated with 0.1 *M* sodium acetate (pH 6). After the application of sample, elution (arrow) was performed with 0.05 *M* mercaptoethanol–0.1 *M* sodium acetate (pH 6.0). (C) Con A–Spheron: the column (10 ml) was washed with 0.1 *M* sodium acetate and 1 m*M* manganese chloride–calcium chloride–magnesium chloride and equilibrated with 0.4 *M* sodium chloride–0.1 *M* sodium acetate (pH (pH 7). After the application of sample, elution (arrow) was performed with 0.1 *M* borate buffer (pH 6.5).

Another biospecific adsorbent for CPY is immobilized Con A. Fig. 2C shows the chromatogram of CPY on a column of Con A–Spheron. This specific adsorbent was prepared by the modification of Spheron with glucose [11], to which Con A was attached after periodate activation [12]. CPY was desorbed by borate buffer; the yield of CPY isolated was 89%.

The results of bioaffinity chromatography experiments show that the best yields of active CPY were obtained with Con A–Spheron. The homogeneity of all the enzymes isolated was assayed by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide gel, pH 7.0). None of the enzymes were completely pure.

Fig. 3 shows the chromatography procedure that yielded the purest enzyme, namely ion-exchange chromatography of CPY on DEAE–Spheron. A gradient of sodium chloride was used for the elution and the yield of the enzyme isolated was 72%. The enzyme was homogeneous when assayed by SDS-PAGE; the specific activity was 100 U/mg.

The fraction of CPY isolated by chromatography on DEAE–Spheron was used for irreversible immobilization on Con A–Spheron according to Hsiao and Royer [3]. CPY after adsorption on Con A–Spheron was cross-linked with Con A by glutaraldehyde. The amount of immobilized CPY determined by amino acid analysis was 1.6 mg/g of dry support; the peptidase activity was 130 U/g of dry derivative.

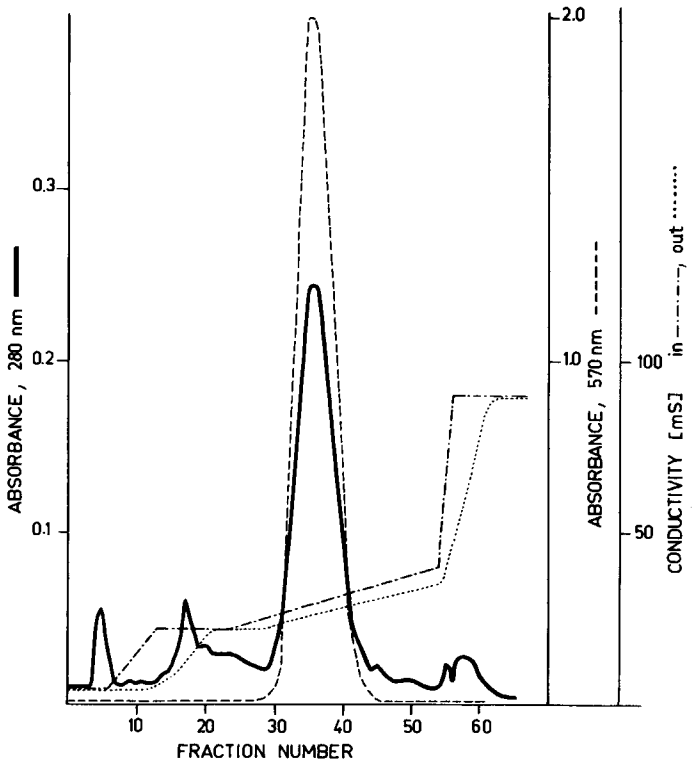

Fig. 3. Purification of CPY on DEAE–Spheron. The column (10 ml) was equilibrated with 0.1 *M* sodium acetate (pH 7.0). The flow-rate was 130 ml/h; fraction volume 3.5 ml; pressure 1 MPa. A gradient of sodium chloride was used for the elution.

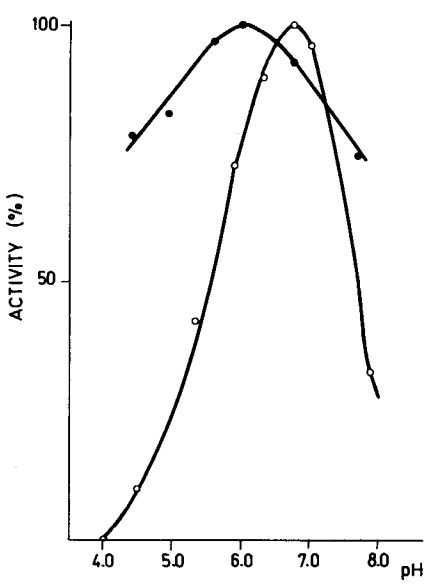

Fig. 4. The pH rate profiles of peptidase activities of immobilized (•) and native (○) CPY. The activity was masured in 50 m*M* MES buffer.

The pH profile of peptidase activity of native CPY and of CPY attached to Con A—Spheron is shown in Fig. 4. The pH optimum of the bound enzyme is shifted to the acid side with respect to the optimum of the native enzyme in the peptidase assay.

For determination of the C-terminal sequence of ribonuclease by CPY attached to Con A—Sepharose, an ultrafiltration cell was found very useful. Practically the same results as described by Hsiao and Royer [3] were achieved. The rates at which amino acids were released are in agreement with the known amino acid sequence of the C-terminal part of the ribonuclease chain [13].

CONCLUSIONS

Bioaffinity chromatography can be used for elaboration of the conditions for biospecific sorption of enzymes that can be used for their oriented immobilization. The formation of biospecific complexes with different parts of the enzyme surface could be very useful for the preparation of immobilized enzymes for use as active catalysts.

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