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Immobilized Carboxypeptidase N

A Potent Bioreactor and Specific Adsorbent for Peptides

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ABSTRACT

Carboxypeptidase N-Sepharose was prepared by covalent immobilization of purified human plasma carboxypeptidase N. More than 98% of the carboxypeptidase N was immobilized; 42% of the applied activity can be detected on the support. The column has excellent capabilities to quantitatively remove carboxy-terminal basic amino acids from peptides, as is demonstrated using the synthetic peptide substrate hippuryl-L-arginine and the nonapeptide bradykinin, and remains stable for several months. In contrast with apocarboxypeptidase B-Sepharose, apocarboxypeptidase N-Sepharose poorly binds its substrates.

Index Entries: Carboxypeptidases; carboxypeptidase N; immobilized enzymes; bioreactor; bradykinin.

Abbreviations: CPN, carboxypeptidase N; Hip-Arg, hippuryl-L-arginine; GEMSA, guanidinoethylmercaptosuccinic acid.

INTRODUCTION

Carboxypeptidase N (CPN, arginine carboxypeptidase, EC 3.4.17.3) is a Zn-metallopeptidase that is synthesized in the liver (*1*) and circulates in plasma as a 280 kDa tetrameric complex. It is composed of two identical

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high-mol-wt subunits (M_r 83,000) that are heavily glycosylated and lack enzymatic activity, and two identical low-mol-wt subunits (M_r 55,000) that contain the active center (*2,3*). Carboxypeptidase N specifically cleaves C-terminal basic amino acids from different peptides and proteins. Physiologically interesting substrates for this enzyme are the kinins bradykinin and kallidin (*1*), the anaphylatoxins C_{3a} and C_{5a} (*4,5*), the fibrinopeptides 6A and 6D (*6*), the creatine-kinase MM-isoenzyme (*7,8*), the hexapeptide enkephalins (*9*), and the atrial natriuretic peptide atriopeptin II (*10*). Its most likely physiological function is to protect the organism from the actions of potent peptides that may escape from tissues or be released in the circulation. Here we used CPN purified from human plasma to covalently immobilize on a Sepharose support. Immobilized CPN can be utilized as a stable bioreactor for removing C-terminal basic amino acids quantitatively from different peptides.

MATERIALS AND METHODS

Chemicals

Hippuryl-L-arginine (Hip-Arg) was from Bachem Feinchemicalien (Bubendorf, Switzerland). Guanidinoethylmercaptosuccinic acid (GEMSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Calbiochem (La Jolla, CA). Human serum albumin was obtained from Sigma Chemical Company (St. Louis, MO), and $CoCl_2 \cdot 6H_2O$ was from Aldrich Chemical Company (Milwaukee, WI). Bradykinin and des-Arg^9-bradykinin were obtained from Novabiochem (Laüfelfingen, Switzerland). Trifluoroacetic acid was from Janssen Chimica (Beerse, Belgium). Arginine-Sepharose 4B and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden), *o*-Methylhippuric acid was synthesized from glycine and *o*-methylbenzoylchloride (UCB, Drogenbos, Belgium) by a procedure analogous to that used for the synthesis of hippuric acid (*11*). 1,10-phenanthroline and all other reagents used were of high purity grade and were from Merck (Darmstadt, FRG).

Instruments

The pipeting of serum samples and reagents was performed with a Dilutrend dispenser (Boehringer, Mannheim, FRG). For colorimetric determinations a Hewlett-Packard 8540 ultraviolet/visible diode-array spectrophotometer was used with a quartz flow cell (10 mm optical pathway). The high-pressure liquid chromatography system consisted of a 303 solvent delivery system, an 802 C manometric module, a model 231-401 autosampling injector (all from Gilson, Paris, France), an LKB 2140 diode-array UV detector (LKB, Bromma, Sweden), and a 100 × 8 mm (id) C18 reversed-phase μ-Bondapak column fitted in a radial compression module

(Millipore, Brussels, Belgium). Dialysis sacks (30 × 2.1 cm) were from Sigma Chemical Company (St. Louis, MO). Proteins were concentrated with Centriprep-10 concentrators (Amicon, Danvers, MA), for centrifugation a Sorvall RC-5 (Dupont, Newton, CT) was used.

Purification of Carboxypeptidase N

Carboxypeptidase N (CPN) was purified from human serum by a two-step affinity chromatography on Arginine-Sepharose 4B as described before (*2,12*). Briefly, the CPN was first eluted from Arginine-Sepharose with 600 m*M* NaCl in 10 m*M* Tris buffer (pH 7.4). The eluate was dialyzed overnight against Tris buffer (Tris 10 m*M* containing 100 m*M* NaCl, pH 7.4), and subsequently rechromatographed on the Arginine-Sepharose affinity column by a more specific elution with 150 m*M* NaCl combined with 1 m*M* of the CPN inhibitor GEMSA. GEMSA was removed by washing five times with coupling buffer (0.1*M* $NaHCO_3$ containing 0.5*M* NaCl, pH 8.3) in a Centriprep-10 concentrator. The CPN isolated by this procedure was purified an average 1000-fold in specific activity (*12*).

Preparation of CPN-Sepharose

One gram of CNBr-activated Sepharose 4B was swollen in 5 mL of 1 m*M* HCl for 15 min and washed with 200 mL of 1 m*M* HCl on a sintered glass filter. The washed gel was reswollen in 5 mL coupling buffer (0.1*M* $NaHCO_3$ containing 0.5*M* NaCl, pH 8.3). 1.8 mL of this gel suspension (equivalent with 0.35 g of CNBr-activated Sepharose 4B) was immediately mixed in a test tube with 5.5 mL of a solution of 11 mg purified CPN (1 U/mg) in the coupling buffer. The coupling was allowed to proceed for 24 h at 4°C with constant mixing in an end-over-end mixer. After coupling, the nonadsorbed protein was washed away with coupling buffer. The gel was transferred into a Tris buffer (0.1*M* Tris, pH 8.0) and mixed for 16 h at 4°C to block remaining active groups on the gel. The gel was then poured into a column (1.0 × 20 cm) and washed alternately with coupling buffer and acetate buffer (0.1*M* sodium acetate containing 0.5*M* NaCl, pH 4) five times. The column was stored at 4°C.

The coupling efficiency was evaluated by comparing the rate of hydrolysis of hippuryl-L-arginine of both the CPN solution before application onto the column and the CPN coupled to the column. To do so, the CPN-Sepharose column was kept at 37°C by cycling water from a constant temperature water bath. A solution containing 20 m*M* Hip-Arg (in HEPES 50 m*M*, pH 8.0) was continually pumped through the column at a flowrate of 0.5 mL/min. Fractions of 1 mL were collected and 50-μL aliquots of the fractions were acidified by 50 μL of 1 m*M* HCl. Hippuric acid was determined using the standard procedure (*13*). The fractions in which the concentration of hippuric acid reached a steady state concentration were used to calculate the carboxypeptidase N activity.

Enzyme Activity Assay

The enzyme activities with the substrate hippuryl-L-arginine were determined by a high-pressure liquid chromatography (HPLC)-assisted assay described elsewhere (*13*), with the following modifications: The buffered substrate solutions were prepared at pH 8.0 and the incubation time was 30 min.

The conversion of bradykinin to des-Arg^9-bradykinin by carboxypeptidase N was followed by HPLC. Calculation of peak area was under computer control using the 714 V 1.1 Controller Software (Gilson, Paris, France). Peptide products were applied to a Waters Novapack C18 stainless steel reversed-phase column (3.9 mm id × 15 cm) of 4-μm particle size (Millipore, Brussels, Belgium). The separations were carried out with an isocratic system of 17% (v/v) acetonitrile in 60 m*M* phosphate buffer, pH 3.2, using a flowrate of 2 mL/min at ambient temperature. The products detected at 214 nm were identified by coelution with peptide standards. Substrate degradation was calculated by comparing the integrated peak area to the peak area of a known amount of authentic standard. The assay procedure was as follows: To 70 μL of a solution of bradykinin (2.3 m*M* during incubation) in 50 m*M* HEPES, pH 8.0, was added 10 μL of the enzyme sample; this mixture was incubated for 30 min. The reaction was stopped with 20 μL of 0.5% (v/v) trifluoroacetic acid in water and the mixture was centrifuged for 5 min (12,000*g*); 60 μL of the supernatant was injected into the HPLC-system. For the determination of Michaelis-Menten constants, we measured initial velocities with bradykinin concentrations ranging from 5 to 500 μ*M* and incubation times from 3 to 5 min.

Determination of the Hydrolysis of Hip-Arg and Bradykinin by CPN-Sepharose

The hydrolysis of Hip-Arg and bradykinin by CPN-Sepharose was carried out at 37°C in the column. The CPN-Sepharose column was equilibrated with 50 m*M* HEPES buffer (pH 8.0). For Hip-Arg, a solution of 5 m*M* of Hip-Arg in HEPES buffer (50 m*M*, pH 8.0) was continually pumped through the column at a flow rate of 0.1 mL/min. After 5 mL of this solution passed through the column, the flowrate was increased to 0.25 mL/min and further to 0.5 mL/min when another 5 mL had passed. Fractions of 250 μL each were collected and 50-μL aliquots of these fractions were used for the determination of the concentrations of hippuric acid by the standard procedure (*13*).

For bradykinin, a concentration of 0.5 m*M* bradykinin in HEPES buffer (50 m*M*, pH 8.0) was pumped through the column at a flowrate of 0.05 mL/min. The flowrate was stepwise increased to 0.10, 0.25, and 0.80 mL/min, respectively, each time after 5 mL of the substrate had passed the column. Fractions of 250 μL were collected and 10 μL of each fraction was injected into the HPLC-system to measure the concentrations of des-Arg^9-bradykinin as described above.

Determination of the Substrate Binding Affinity of Immobilized CPN

The binding affinity of immobilized CPN for hippuryl-L-arginine was examined at 4°C. A solution of 2 mL of substrate (containing 3 nmols of Hip-Arg in 50 m*M*, pH 8.0) was loaded onto the CPN-Sepharose column at a flowrate of 0.25 mL/min. The column was washed with 4 mL of start buffer (50 m*M* HEPES containing 0.1*M* NaCl, pH 8.0) at the same flowrate. The bound substrate was eluted from the column with acetate buffer (20 m*M* Na-acetate containing 0.1*M* NaCl, pH 4.0) at a flowrate of 0.5 mL/min. Fractions of 1 mL were collected during the washing and the elution and subsequently assayed for the presence of Hip-Arg.

The amount of Hip-Arg present in each fraction was determined by incubating the fractions with a high concentration of CPN, followed by measuring the concentration of enzyme-produced hippuric acid, equivalent with the concentration of Hip-Arg. The assay procedure is based on the standard method for the arginine carboxypeptidase activity assay (*13*). To 1 mL of each fraction eluted with acetate buffer, 200 μL of a 1*M* HEPES buffer (pH 8.0) is added subsequently in order to neutralize the pH. 200 μL of 50 m*M* HEPES buffer (pH 8.0) is added to the fractions eluted with start buffer in order to have the same assay volumes as the other fractions. Three hundred microliters of human serum (containing 65 U/L of arginine carboxypeptidase activity) is incubated overnight with each fraction at 37°C. The reaction is stopped by adding 200 μL of 6*M* HCl. After adding 10 μL of the internal standard (*13*), the hippuric acid and internal standard are extracted into 5 mL of ethylacetate, mixed and centrifuged for 15 min at 1000*g*. 4.6 mL of the supernatant is evaporated. The dry residue is dissolved in 50 μL mobile phase, and 20 μL is injected into the HPLC-system. By using a control sample, in which 300 μL serum was incubated with 3 nmol of Hip-Arg under the same assay conditions, we confirmed that more than 98% of Hip-Arg present in a fraction can be hydrolyzed and that the produced hippuric acid can be accurately measured by this assay procedure. The determination of hippuric acid in these fractions was performed by using the same procedure as for Hip-Arg, except without incubating the fractions with serum. Three hundred microliters of a human serum albumin solution (30 mg/mL in 50 m*M* HEPES buffer, pH 8.0) was used instead of serum to minimize adsorption to the test tubes.

The substrate binding affinity of the apoCPN-Sepharose column was tested in a similar way.

Preparation of apoCPN-Sepharose

CPN-Sepharose was converted to apoCPN-Sepharose by treating the column with 1,10-phenantroline and EDTA in 50 m*M* HEPES buffer (pH 8.0) containing 0.5*M* NaCl. *o*-Phenantroline was used at concentrations of 2, 4, and 10 m*M* combined with EDTA in a concentration of 1, 2, and 20 m*M*, respectively. The above solutions were passed through the column

under the conditions described in Table 3. In the experiment to restore the enzymatic activity, a solution of 1 m*M* $ZnCl_2$ (in 20 m*M* HEPES buffer pH 7.0) was passed through the apoCPN column at a flowrate of 6 mL/h during 15 h. In a similar experiment, the enzyme activity was restored using 1 m*M* $CoCl_2$ (in 20 m*M* HEPES, pH 7.0).

RESULTS AND DISCUSSION

Immobilization of CPN on the CNBr-Activated Sepharose

We used 9.8 mg of purified CPN (with a specific activity of 1000 U/g) to couple onto 1.8 mL of CNBr-activated Sepharose 4B. We could not detect any CPN activity in the eluates from the different steps in the coupling procedure (coupling, washing, blocking, and washing). This means that almost 100% of CPN was immobilized. Passing the substrate Hip-Arg through the CPN-Sepharose column, we could detect 16.4% of the expected CPN activity. When an aliquot of the CPN-Sepharose is taken out of the column and assayed for CPN activity using the standard reference assay in a test tube (*13*), we can detect 42% of the original applied activity. The CPN-Sepharose column used here contains 11.7 nmol of the 280 kDa enzyme; 1.9 nmol of CPN (16.4%) effectively cleaves the Hip-Arg. This coupling efficiency is considerably higher than that of pancreatic carboxypeptidase B (EC 3.4.17.2), which was only 2.7% (*14*).

Hydrolysis of the Synthetic Substrate Hip-Arg by CPN-Sepharose

The hydrolysis of the synthetic substrate Hip-Arg by CPN-Sepharose was measured at 37°C. The K_m of CPN for this substrate is 1.8 m*M* (*13*). A solution of 5 m*M* of Hip-Arg was passed through the column packed with 1.8 mL of CPN-Sepharose at flowrates of 0.1, 0.25, and 0.5 mL/min. Consequently, the contact times between substrate and immobilized enzyme were 18, 7.2, and 3.6 min, respectively. At steady-state conditions, 60% of the substrate is converted to its products by one passage through the column at a flowrate of 0.1 mL/min (Table 1).

Conversion of Bradykinin to des-Arg^9-Bradykinin by CPN-Sepharose

The bradykininase activity of CPN was determined at substrate concentrations ranging from 5 to 500 μM. Incubation times varied from 3 to 5 min to determine initial velocities. The Michaelis-Menten contant K_m, calculated using a direct linear plot (*15*), was 130 μM (Fig. 1). Thus, CPN has a low K_m using bradykinin compared with the K_m using Hip-Arg as

Table 1
Determination of the Hydrolysis of Hippuryl-L-Arginine by CPN-Sepharose

Flowrate, mL/min	Reaction time, min	Hippuric acid[a] μmol/mL	conversion of Hip-Arg, %
0.1	18	3.00	60.0
0.25	7.2	2.03	40.6
0.5	3.6	1.44	28.8

[a]Concentration of hippuric acid in the substrate solution that has passed the column (steady state concentration).

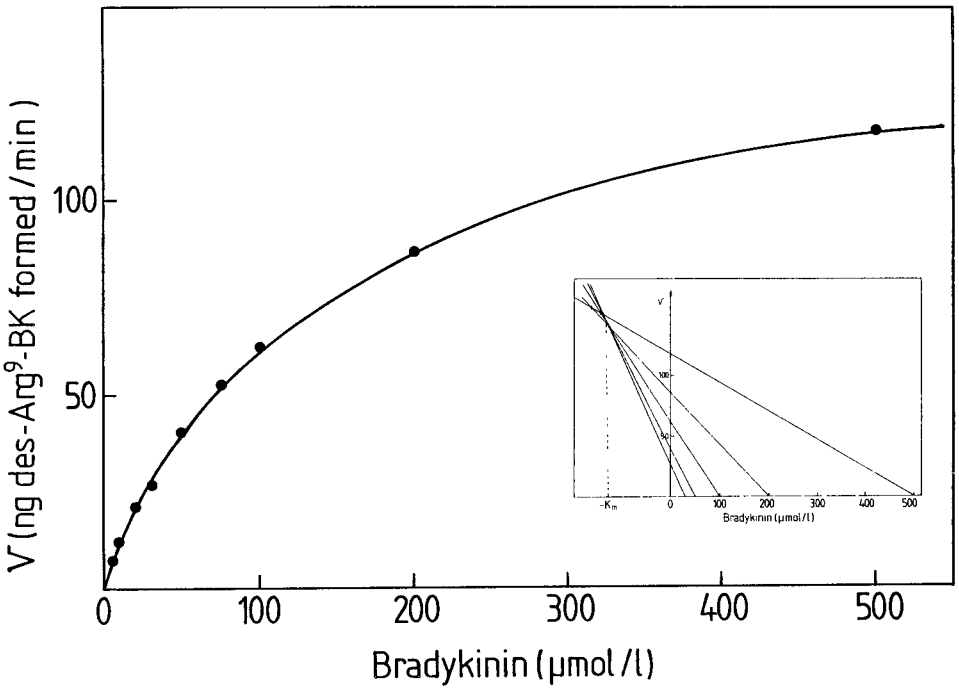

Fig. 1. Conversion rate of bradykinin to des-Arg⁹-bradykinin by purified carboxypeptidase N as a function of substrate concentration (each point presents the mean of triplicate determinations). Inset: Determination of the K_m for bradykinin by means of the direct linear plot of enzyme velocity (v) vs substrate concentration.

the substrate (1.8 mM) (*13*). Consequently, to compare the kinetics of the immobilized CPN on the substrate bradykinin with the results on the Hip-Arg substrate, we used a bradykinin concentration of 0.5 m*M*. The rate of conversion of bradykinin to des-Arg⁹-bradykinin by immobilized CPN is summarized in Table 2. 85% of the bradykinin can be converted to des-Arg⁹-bradykinin in 7.2 min at 37°C by this CPN-Sepharose column.

Table 2
Determination of the Conversion of Bradykinin to des-Arg^9-Bradykinin by CPN-Sepharose

Flowrate, mL/min	Reaction time, min	des-Arg^9-BK, μmol/mL	conversion of BK, %
0.05	36	0.410	81.9
0.10	18	0.423	84.6
0.25	7.2	0.427	85.4
0.82	2.2	0.316	63.1

We compared the rate of degradation of bradykinin to des-Arg^9-bradykinin by free CPN (CPN in solution) and immobilized CPN (CPN-Sepharose) with the rate of degradation of the substrate Hip-Arg using both these enzyme preparations. Both CPN in solution (70.9 U/L vs 10.2 U/L) and immobilized CPN (1008 U/L vs 144 U/L) cleave Hip-Arg 7 times faster than bradykinin.

Substrate Binding Affinity of CPN-Sepharose

In order to determine if the immobilized enzyme can be used to detect substrates, we investigated the substrate binding affinity of immobilized CPN by placing the CPN-Sepharose column at 4°C, which decreases the rate of cleavage of potential substrates considerably. The CPN-Sepharose column contains 1.9 nmol of enzymatically active CPN. Because CPN is composed of two active and two inactive subunits (2,3), theoretically a maximum of 3.8 nmol of substrate could be bound on this column. We loaded 3 nmol of Hip-Arg onto the column and subsequently washed the column with the starting buffer. The eluates were assayed for the presence of both Hip-Arg and hippuric acid. These eluates contain both the unbound substrate and the hydrolyzed substrate (which is the hippuric acid). The adsorbed but unaltered substrates were eluted with low pH buffer (pH 4.0). Of the 3 nmol Hip-Arg that were loaded onto the column, 1.5 nmol substrate and 0.9 nmol hippuric acid were detected in the eluate at pH 8.0, whereas 0.6 nmol unaltered substrate was detected in the eluate at pH 4.0. This means that 50% of the substrate passes through the column, and 50% is bound. Of the bound substrate, 60% is hydrolyzed and 40% is recovered as unaltered substrate.

In order to confirm that the binding of the substrate Hip-Arg was specific, hippuric acid—the product of hydrolysis of Hip-Arg—was used for examining the nonspecific binding affinity of the CPN-Sepharose. Three nmoles of hippuric acid were loaded onto the CPN-Sepharose column. Over 98% of the hippuric acid was recovered from the column by washing it with the pH 8.0 buffer. This indicates that the CPN-Sepharose has a very limited nonspecific binding affinity for this type of products.

Table 3
Transformation of CPN-Sepharose to apoCPN-Sepharose

Conc. of *o*-phenanthroline	2 m*M*	4 m*M*	10 m*M*
Conc. of EDTA	1 m*M*	2 m*M*	20 m*M*
Time of treatment	2 h	15 h	15 h
Solution volume	50 mL	110 mL	110 mL
Remaining arginine carboxypeptidase activity	15%	10%	10%

Preparation of apoCPN-Sepharose

CPN is a metalloenzyme with an active site zinc atom (*2*). In previous tests, we demonstrated that 1 m*M* of *o*-phenanthrolin abolished more than 96% of the catalytic activities of soluble CPN in 1 h at 4°C (*12*). By passing solutions containing different concentrations of *o*-phenanthrolin and EDTA through the CPN-Sepharose column, we tried to strip the zinc from the immobilized CPN to form an apoCPN-Sepharose. The results, represented in Table 3, indicate that it is difficult to completely strip the zinc from immobilized CPN. Even when the immobilized CPN is treated with 10 m*M* of *o*-phenanthrolin and 20 m*M* of EDTA for 15 h at room temperature, still about 10% of the catalytic activity of the immobilized CPN remains intact. This is compatible with the idea that the spatial fixation of the enzyme on the support creates a steric hindrance, which limits the diffusion of chemical reagents and increases resistance of enzyme toward its inactivation.

Substrate Binding Affinity of apoCPN

It was reported that apoCPN and apoCPB cannot hydrolyze substrates but have about the same substrate binding affinity as the active metalloenzyme (*14,16*). We loaded 3 nmol of Hip-Arg onto the apoCPN-Sepharose column (prepared as described above) under the same conditions as we did for the CPN-Sepharose. Only 0.1 nmol of Hip-Arg was bound to the apoCPN-Sepharose column. One has to consider that 10% of the CPN in the apoCPN-Sepharose column is still in its active form as stated before. Theoretically, since the CPN-Sepharose column can bind and elute 0.6 nmol of intact Hip-Arg, the remaining 10% of active CPN on the apoCPN column would bind 0.06 nmol. This means that the apoCPN shows only a minor binding affinity for this substrate.

Effect of Zinc and Cobalt on the Catalytic Activity of apoCPN

We tried to restore the catalytic activity of apoCPN by the addition of 1 m*M* of $ZnCl_2$ or $CoCl_2$, respectively. Zinc did not significantly restore the catalytic activity of apoCPN: The CPN activity of the apoCPN column

increased from 10 to 13% only (compared to the original activity of the CPN-Sepharose column). Treatment of the apoCPN-Sepharose column with 1 m*M* $CoCl_2$, however, enhanced the arginine carboxypeptidase activity on the column dramatically. The restored activity was 194% of the original activity of the CPN-Sepharose column. When soluble CPN is preincubated with 1 m*M* $CoCl_2$, the arginine carboxypeptidase activity increases to 282% (*12*).

Stability of CPN-Sepharose

The CPN-Sepharose column that was used for the experiments described above (both at 37 and at 4°C, and at pH 4 and 8) was each time stored at 4°C and at pH 7.0. The carboxypeptidase activity of the column remained stable during the 2 mo experiment period.

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