Vol. 166, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1990 Pages 330-335

APOCARBOXYPEPTIDASE B-SEPHAROSE: A SPECIFIC ADSORBENT FOR PEPTIDES

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Received November 30, 1989

Summary: Apocarboxypeptidase B-Sepharose was prepared by immobilization of porcine carboxypeptidase B, followed by treatment of the column with o-phenanthrolin. This column efficiently adsorbed Met-enkephalin-Arg-Arg (YGGFMRR) in an optimum pH range of 6.5-7.5. The adsorbed Met-enkephalin-Arg-Arg was eluted at pH 4.0 and confirmed to be unaltered. In the apocarboxypeptidase B-Sepharose chromatography, Met-enkephalin-Arg-Arg or dynorphin 1-13 (YGGFLRRIRPKLK), substrates of carboxypeptidase B, was separated from Met-enkephalin (YGGFM), dynorphin B 1-9 (YGGFLRRQF), and B-neo-endorphin (YGGFLRKYP) which do not react with the immobilized enzyme.

It has been known for some time that enzyme, especially protease, functions of substrate binding and peptide hydrolysis are independent. The specific residues of a protein responsible for binding of substrates and enzyme catalysis are not identical and can be separately manipulated. For example, amino acid substitution at the active site's serine of subtilisin by genetic engineering (1) or chemical modification of the active serine of trypsin (2) affected turnover rate but did not significantly alter the Michaelis constant of the enzymes.

Carboxypeptidase A (CPA) is a metalloprotease containing a zinc atom at its active site which is essential for catalytic function (3). This protease cleaves neutral C-terminal residues of substrate peptides (4). ApoCPA, produced by treating the active metalloCPA with chelating reagent, o-phenanthrolin, is catalytically inert but retains binding affinity to substrates (5). Here we used carboxypeptidase B (CPB) which acts with high specificity to peptides with basic C-terminal residues (6). This report shows that the retained binding affinity of immobilized apoCPB to the substrates of the active metalloenzyme can be utilized as a method for absorbtion of peptides which have basic C-terminal residues.

Abbreviations used: CPA, carboxypeptidase A; CPB, carboxypeptidase B; Met-enk, Met-enkephalin; Met-enk-RR, Met-enkephalin-Arg-Arg; HPLC, high-performance liquid chromatography.

0006-291X/90 $1.50

Materials and Methods: Preparation of Carboxypeptidase B-Sepharose -- 10 mg of CPB (from porcine pancreas, PMSF-treated, Worthington Biochemical Co.,USA) was dissolved in 2 ml of 0.5 M NaCl-0.1 M $NaHCO_3$,pH 8.3 and coupled to 1.5 ml wet volume of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals Co.) for 24 hr at 4 °C, followed by blocking the residual reactants with 0.5 M Tris-HCl,pH 8.5.
Enzyme assay -- Activity of CPB was measured by using the 0.5 mM N-(2-Furanacryloyl)-Ala-Lys (Bachem AG,Switzerland) as a substrate in 0.5 ml assay system which was composed of 0.5 M NaCl-0.1 M Tris-HCl,pH 7.5 (7).
Protein concentration -- Protein concentration of CPB was measured by a spectrophotometer at 278 nm, using the absorption coefficient $E^{1\%}_{278nm} = 21.4$ (6).
ApoCPB-Sepoharose chromatography -- CPB-Sepharose column (5 x 60 mm) was connected to the High Performance Liquid Chromatography (HPLC) system. The column was pretreated with 2 mM o-phenanthrolin-1 mM EDTA-0.5 M NaCl-50 mM Tris-citrate,pH 7.5 (50 ml). The chromatography was carried out at varying pH with 1 mM EDTA-0.5 M NaCl-50 mM Tris-citrate at a flow rate of 1 ml/min at 4°C 2 nmoles of each peptide was applied on the column and the eluate was recorded on a fluorescence HPLC monitor with exitation at 275 nm and emission at 304 nm to detect the fluorescence of tyrosine residues (8). HPLC analysis with reversed phase column -- HPLC analysis with reversed phase column (4 x 50 mm, Superspher RP-8, Merck Co.) was carried out in starting buffer of 50 mM citrate-NaOH,pH 4.0 (Buffer A) and gradient buffer of 25 % acetonitrile-50 mM citrate-NaOH,pH 4.0 (buffer B). Application of each sample (1 ml) was followed by the washing with 4 ml of buffer A, elution from the column was performed by gradual increase in proportion from buffer A to B over 15 min. The eluate was monitored fluorophotometrically as in apoCPB-Sepharose chromatography. The flow rate was 1 ml/min. Peptides -- The peptides were purchased as follows. Met-enkephalin (Met-enk) and Dynorphin 1-13 from Peptide Institute Inc.,Osaka, Japan; Met-enkephalin-Arg-Arg (Met-enk-RR) from Bachem AG, Switzerland; β-Neo-endorphin and Dynorphin B 1-9 from Peninsula Laboratories Inc.,USA. Met-enk-RR methyl ester was prepared by esterification of Met-enk-RR by incubation in 5 %-hydrogen chloride-methanol solution (Nacalai Tesque, Japan) overnight at room temperature.

Results

By coupling of CPB to the CNBr activated Sepharose, 6.3 mg of CPB were immobilized per 1 ml of Sepharose. Although 95 % of CPB was immobilized, only 2.7 % of expected CPB activity was detected on the CPB-Sepharose.

CPB-Separose was converted to apoCPB-Sepharose by treating the column with 2 mM o-phenanthrolin-1 mM EDTA-0.5 M NaCl-50 mM Tris-citrate,pH 7.5 to remove the zinc from the active site of the enzyme and abolish its activity. The chromatography was carried out at each pH level of 1 mM EDTA-0.5 M NaCl-50 mM Tris-citrate. The eluted peptides were monitored by fluorescence derived from tyrosine residues which change little in intensity between pH 4 and 8 (8). Figure 1 shows the behavior of peptides in apoCPB-Sepharose chromatography at pH 4.0 and 7.5. Unreactive Met-enk (YGGFM) passed through the column at both pH 4.0 and 7.5 (Fig. 1 A). Substrate peptide Met-enk-RR (YGGFMRR) also passed through the column at pH 4.0, but was adsorbed to the column at pH 7.5 (Fig. 1 B). The adsorption of Met-enk-RR could be greatly reduced by esterification of its C-terminus (Fig. 1 C). This esterified peptide seemed to retain some enzyme affinity as demonstrated by tailing in its elution pattern.

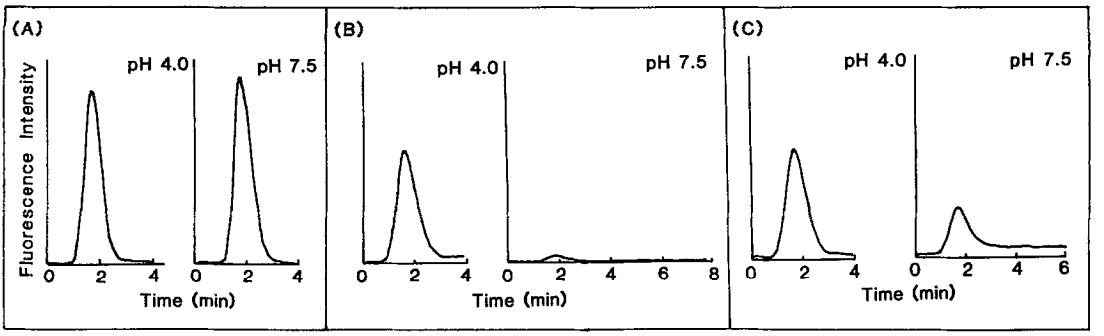

Fig. 1 Behaviors of peptides on the apoCPB-Sepharose chromatography. Various peptides (2 nmoles) were applied on the apoCPB-Sepharose column(25 x 60 mm) in 1 mM EDTA-0.5 M NaCl-50 mM Tris-citrate,pH 4.0 or 7.5. (A), Met-enkephalin; (B), Met-enkephalin-Arg-Arg; (C), Met-enkephalin-Arg-Argmethylester. The eluates were evalated by fluorescence HPLC monitor with excitation and emission points at 275 nm and 304 nm, respectively. Flow rate was 1 ml/min.

The effect of pH on specific affinity between apoCPB-Sepharose and Met-enk-RR was examined (Fig. 2). After applying the peptide and washing the column in each pH, the adsorbed material was eluted at pH 4.0. The passed part was minimized and the amount of adsorbed Met-enk-RR which eluted at pH 4.0 was maximized between starting pH of 6.5 and 7.5. The results show that the optimum pH of apoCPB-Sepharosse / Met-enk-RR affinty falls within this range.

Taking advantage of the specificity of apoCPB-Sepharose to substrate molecules, peptides with basic residues at C-termini were easily separated from other peptides (Fig. 3). When a mixture of Met-enk and Met-enk-RR was applied onto the apoCPB-Sepharose column at pH 7.5, Met-enk passed through the

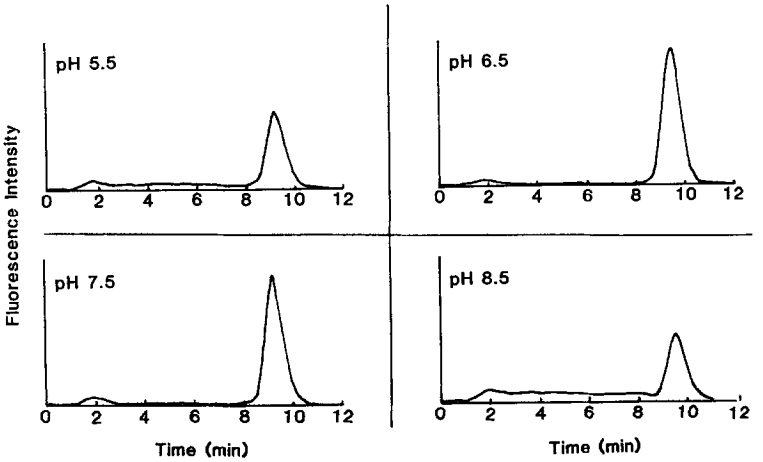

Fig. 2 Effects of pH on apoCPB-Sepharose / Met-enkephalin-Arg-Arg affinity. Conditions were the same as Fig. 1 except after 6 min in the indicated pH buffer, the adsorbed Met-enkephalin-Arg-Arg was eluted by changing to pH 4.0.

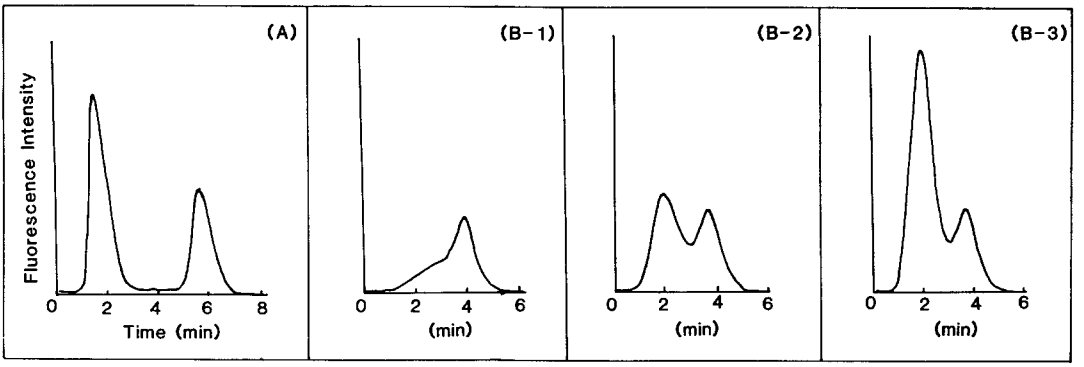

Fig. 3 Separation of peptides on apoCPB-Sepharose. The chromatography conditions were again as in Fig. 1 except the mixture of peptides was applied at pH 7.5 (A) or 6.5 (B-1,2,3,) and eluted at pH 4.0 after 2.5 min (A) or one min (B-1,2,3). (A),Met-enkephalin and Met-enkephalin-Arg-Arg; (B-1),Dynorphin 1-13; (B-2),Dynorphin 1-13 and Dynorphin B 1-9; (B-3),Dynorphin 1-13 and β-Neo-endorphin.

column while Met-enk-RR was bound (Fig. 3 A). Dynorphin 1-13 (YGGFLRRIRPKLK), which contains a C-terminal Lysine also had significant affinity to the column. Some of the bound dynorphin 1-13 leaked from the column at pH 6.5. The peptide still eluted as a peak by changing the pH 4.0 (Fig. 3 B-1) and could be distinguished from dynorphin B 1-9 (YGGFLRRQF) and β-neo-endorphin (YGGFLRKYP) which have neutral amino acids phenylalanine or proline in their C-terminals passed through the column (Fig. 3 B-2, 3).

To confirm that the eluted peptides were never hydrolyzed on the apoCPB-Sepharose column, HPLC analysis with reversed phase column was carried

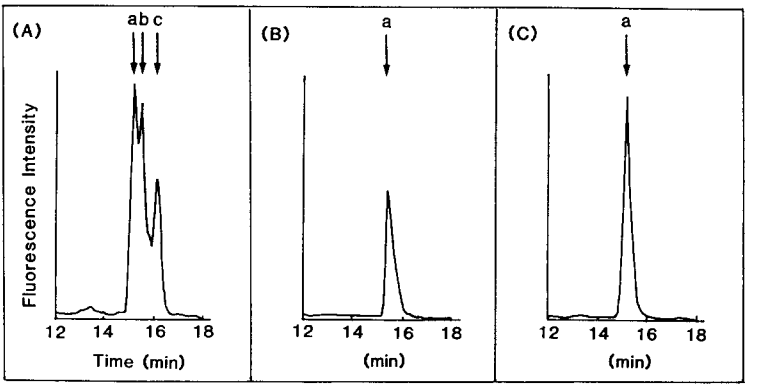

Fig. 4 HPLC-analysis of eluate from apoCPB-Sepharose with reversed phase column. The samples were applied in 50 mM citrate-NaOH,pH 4.0 and eluted with linearly increasing acetonitrile at a flow rate of 1 ml/min. (A) Markers made by hydrolysis of 4 nmoles of Met-enkephalin-Arg-Arg with carboxypeptidase B. Arrows show eluate position of Met-enkephalin-Arg-Arg,a; Met-enkephalin-Arg,b; and Met-enkephalin,c. (B) Eluate from apoCPB-Sepharose when 2 nmoles of Met-enkephalin-Arg-Arg was applied. (C) Same sample as (B) mixed with 2 nmoles of Met-enkephalin-Arg-Arg. The flow rate was 1 ml/min.

out (Fig. 4). The substrate peak in Fig. 2-C which eluted from apoCPB-Sepharose after applying Met-enk-RR as test peptide was pooled and applied on a reversed phase column. The retention time of this eluate was in accord with that of Met-enk-RR (Fig. 4 B). A mixture of the eluate and Met-enk-RR was also analysed (Fig. 4 C). The single additive height of the peak confirmed positively that the eluate from apoCPB-Sepharose was unaltered Met-enkephalin-Arg-Arg.

Discussion

It has been reported that substrate binding and hydrolysis of the zinc metalloprotease CPA can be separated. ApoCPA can not hydrolyze substrates but has about the same substrate binding affinity of the active metalloenzyme (5). The zinc of CPB is essential for its hydrolytic activity (9). CPB is very homologous to CPA except in its substrate specificity (10). Specific binding of peptides with C-terminal basic residues to apoCPB-Sepharose shows that apoCPB retains its binding affinity to the substrates. Esterification of Met-enk-RR caused the substrate to pass through the column (Fig. 1 B), although some residual binding was observed. This may reflect that esters are also possible substrates of CPB (8).

Alteration of the serine on the active site of subtilisin did not destroy affinity to substrates but catalytic activity (1). Similarly, anhydrotrypsin in which the active serine modified chemically to dehydroalanine retains affinity to its substrates (2). Unexpectedly, anhydrotrypsin had much higher affinity towards product-type ligands, contain arginine at the C-termini, than substrate-type ligands. Thus, immobilized anhydrotrypsin can be used as adsorbent for peptides with basic residues in their C-terminals. Another unexpected feature of anhydrotrypsin was its stronger affinity to product-type ligands in acidic pH 5.0 than the optimum pH 8.0 for catalytic activity of native trypsin (2). The optimum pH range 6.5-7.5 of apoCPB-Sepharose was a little below that of metalloCPB (pH 7-9)(8). The apoCPB-Sepharose, like the anhydrotrypsin-Sepharose, is a specific adsorbent for the peptides with basic residues with a free carboxyl group. Advantageously, the preparation of apoCPB-Sepharose has no need for the chemical modification and the following purification.

In order to make this method more efficient, it is necessary to improve immobilization of CPB on the Sepharose gel. Only a 2.7 % of CPB activity was found on the CPB-Sepharose. We calculated that the apoCPB-Sepharose column used here contains 220 nmoles of the 34.3 K dalton enzyme, less than 6 nmoles of which were effectively binding molecules.

There exist endo-metalloproteases as well as exo-metalloproteases, aminopeptidases and carboxypeptidases which distinguish both amino and

carboxyl termini. The apo-form of these metalloproteases may possibly be used for convenient peptide separation with a variety of residue-specificities.

Acknowledgment: We are thankful to Ann Yonetani for her critical reading of the manuscript.

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