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Utilization of antipeptide antibodies as affinity ligands in immunoaffinity purification

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Abstract

Anti-peptide antibodies against the C-terminal regions of chimeric α-amylase, recombinant CD2 and insulin B-chain were obtained by using peptides corresponding to the C-terminal regions as immunogens. These anti-peptide antibodies adsorbed the native proteins, as well as the antigen peptides. The proteins were purified to high purity using the anti-peptide antibodies as affinity ligands. These ligands could discriminate the target proteins having different C-terminal regions. The adsorbed proteins were specifically eluted by the eluents containing the antigen peptides.

Keywords: Anti-peptide antibodies; Peptides; Proteins

1. Introduction

For purification of high-value bioproducts produced using genetically engineered microorganisms or cells, immunoaffinity chromatography is very effective because of the high affinity and specificity between antigens and antibodies. However, the following problems may occur in immunoaffinity purification. It is often difficult to obtain suitable antibody ligands for immunoaffinity purification, because a sufficient amount of antigen (target protein) of suitable purity for immunization is not available. Because of the high affinity, elution of the target proteins from antibody ligands sometimes requires extreme conditions which may cause their denaturation. To solve these problems, we proposed utilization of cross-reactive antibody and anti-peptide antibodies, which were obtained without use of a target protein as antigen, for the purification of α-amylases secreted from yeast cells in one step of immunoaffinity chromatography [1,2]. We also showed that specific elution method using an antigen peptide as an eluent from anti-peptide antibodies is effective to avoid denaturation of a target protein during the elution step [3]. In this work, the feasibilities of anti-peptide antibodies as ligands for immunoaffinity purification and the specific elution method were studied using anti-peptide antibodies against the C-terminal regions of several bioactive proteins.

2. Experimental

2.1. Materials

Fig. 1 shows the antigen peptides corresponding to the C-terminal regions of chimeric α-amylase

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PC-Am3D (C-terminal region of Chimeric α-amylase Amy1A/3D)

Arg-Val-Pro-Ala-Gly-Arg-His-Leu

PC-CD2-12 (C-terminal region of human soluble CD2)

Glu-Ser-Ser-Val-Glu-Pro-Val-Ser-Cys-Pro-Glu-Lys

PC-InB12P (C-terminal region of bovine insulin B-chain)

Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

PC-InB11P

Fig. 1. Peptides used as antigens.

(Amy1A/3D), bovine insulin B-chain and recombinant soluble human CD2 antigen (sCD2). Chimeric α-amylase consists of 158 aa of one isozyme (Amy1A) of rice α-amylases and 252 aa of another (Amy3D), and soluble human CD2 lacking transmembrane domain consists of 182 aa. Chimeric α-amylase and sCD2 were produced by secretion from recombinant yeast cells, as stated below, and bovine insulin B-chain (oxidized) was obtained from Sigma–Aldrich (St. Louis, MO, USA). PC-InB12P is a peptide which represents residues 19–30 of insulin B-chain and was used for immunization to obtain an anti-peptide antibody (anti-PC-InB12P). For measurement of adsorption characteristics of obtained anti-PC-InB12P antibody, PC-InB11P was used. These peptides were synthesized by the solid-phase method (431A, Applied Biosystems, Foster City, CA, USA) and purified by an HPLC system (LC-10A, Shimadzu, Kyoto, Japan) and a reversed-phase column. The sequences of the synthesized peptides were confirmed by a peptide sequencer (Procise 492, Applied Biosystems). The chemicals used were of reagent grade.

2.2. Preparation of anti-peptide antibody ligands

The antigen peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde [4]. Rabbits were immunized with a mixture of the antigen (1 mg/ml) and Freund's complete adjuvant (1 ml each). Booster injections were repeated twice in a similar manner at 10-day intervals. In some cases booster injections were further repeated to obtain anti-peptide antibodies with high affinity. Specific antibodies (anti-PC-Am3D, anti-PC-InB12P and anti-PC-CD2-12) were purified from pooled sera by adsorption on antigen-coupled Sepharose 4B and elution with 0.1 *M* HCl. The binding specificities of the purified antibodies against the C-terminal regions were confirmed by remarkable decreases in the adsorption capacities of these antibodies against synthetic peptides lacking one or two residues in the C-terminal regions of the original antigen peptides. The immunoadsorbent was prepared by coupling the purified specific antibody to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden).

Anti-human CD2 antibody TS2/18 (monoclonal antibody recognizing D1 domain of CD2) produced by hybridoma ATCC HB195 was purified from ascetic fluid by salting-out with ammonium sulfate and ion-exchange chromatography with a DEAE column.

2.3. Production of chimeric α-amylase and sCD2 and their immunoaffinity purification

Chimeric α-amylase (Amy1A/3D) was produced from the cloned genes of the two isozymes, Amy1A and Amy3D, kindly provided by Dr. R.L. Rodriguez [5]. The chimeric enzyme was expressed in *Saccharomyces cerevisiae* LL20 under the yeast enolase promoter and secreted to fermentation broth with a signal peptide [6].

The sCD2 gene was inserted downstream of the alcohol oxidase 1 promoter and signal genes, and expressed using *Pichia pastoris*. The selected strain secreting sCD2 was first grown in the MD medium (glucose 1%, yeast nitrogen base w/o amino acids 1.34%, biotin 0.001%) for 24 h in a 1-l shaking flask. Then, cells were centrifuged (6700 *g* for 10 min), resuspended in the MM medium (methanol 0.5%, yeast nitrogen base w/o amino acids 1.34%, biotin 0.001%) and grown again for 24 h. The production of sCD2 was induced in the latter medium containing methanol.

The immunoadsorbent was packed into an adsorption column of 1.26 cm diameter and equilibrated with an equilibration buffer (50 m*M* Tris–HCl+5 m*M* $CaCl_2$, pH 7.6, for Amy1A/3D, 10 m*M* Tris–HCl, pH 7.0, for sCD2, PBS, pH 7.6, for insulin B-chain) at a flow-rate of about 1.0 ml/min and 23±2°C. The fermentation broth containing secreted target protein was obtained by centrifuga-

tion, concentrated by ultrafiltration (molecular mass cut-off, 10 000 Da) and applied to this column. After washing with the equilibration buffer, the adsorbed solute was eluted by nonspecific eluents (2.5 *M* NaSCN+5 m*M* $CaCl_2$, pH 5.0, for Amy1A/3D, 60 m*M* glycine buffer, pH 2.28, for sCD2, 0.1 *M* HCl for insulin B-chain) or by specific eluents containing the peptide (PC-Am3D in 50 m*M* sodium acetate buffer+5 m*M* $CaCl_2$, pH 5.0, PC-InB11P in PBS, pH 7.6). The absorbance of the effluent solution at 280 nm was continuously measured by using a spectrophotometer. The activity of α-amylase in the effluent from the column was determined as previously reported [2]. The concentrations of sCD2 and insulin B-chain in the effluent from the column were measured by direct ELISA using anti-PC-CD2-12 antibody and TS2/18 antibody, and by an HPLC system (Shimadzu LC-10A) equipped with a reversed-phase column, respectively. The total amount of the adsorbed protein was obtained by numerical integration of breakthrough curves. The peptide adsorbed on the immunoadsorbent at the specific elution step was eluted by 0.1 *M* HCl, and the column was re-equilibrated with the equilibration buffer.

The protein concentration in the collected samples was determined by the dye method (BioRad protein assay kit, BioRad Lab, Hercules, CA, USA) and the purity of eluted proteins was examined by SDS–PAGE stained with silver (Bio-Rad silver stain kit, Bio-Rad Lab).

2.4. ELISA

Direct ELISA protocol is as follows. Wells were coated with 100 μl/well of 10 μg/ml of antigen solution in phosphate-buffered saline (PBS, pH 7.6) for 20 h at 4°C. After washing with PBS, all wells were coated for 1 h with 200 μl/well of 4-fold diluted Block Ace (Snow Bland Milk) to decrease nonspecific adsorption. After washing with PBST (PBS+0.05% Tween 80), various dilutions of antiserum or various concentrations of antibody were added to the wells (50 μl/well) and incubated for 1 h at room temperature. After washing with PBST, each well was incubated with 100 μl/well of ×2000 diluted anti-rabbit IgG–horseradish peroxidase or anti-mouse IgG–horseradish (Organon Teknika Co.) solution in 10-fold diluted Block Ace for 1 h. After washing with PBST, color was developed using 100 μl/well of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution (0.3 mg/ml in 0.1 *M* citrate buffer containing 0.01% H_2O_2, pH 4.1). The absorbance of each well was recorded by the microplate reader at 415 nm.

3. Results

3.1. Adsorption equilibrium

Fig. 2 shows the adsorption equilibria of the peptides and the proteins to the anti-peptide antibodies. The native proteins, as well as the corresponding antigen peptides, were adsorbed by these anti-peptide antibodies against the C-terminal regions of the proteins. Thus, the native proteins were adsorbed by the anti-peptide antibodies obtained without use of the proteins. The adsorption of the peptides and proteins by these antibodies showed adsorption isotherms of the Freundlich type.

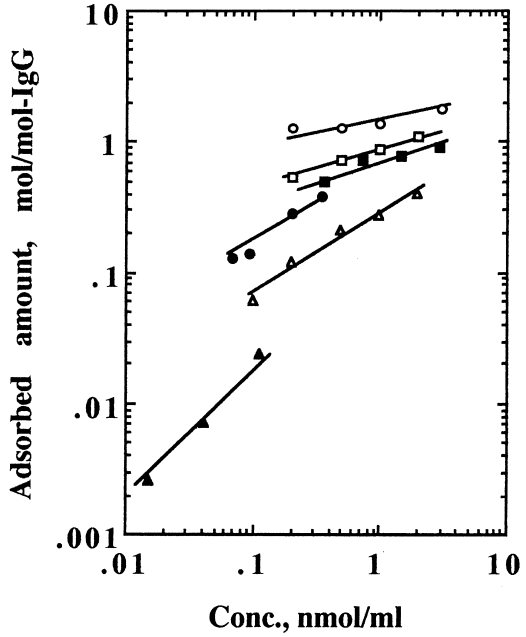

Fig. 2. Adsorption equilibria of antigen peptides and proteins to antipeptide antibodies: (△) PC-CD2-12; (▲) sCD2; (□) PC-InB11P; (■) insulin B-chain; (○) PC-Am3D; (●) Amy1A/3D.

From the adsorption equilibria, the effective association constant K_0 and the heterogeneity index σ for the antibodies were estimated by assuming that the free energy of antigen–antibody combination can be described by the normal distribution function, according to Pauling et al. [7]:

$$y = 1 - \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 C\, e^{\alpha\sigma}}\, d\alpha \quad (1)$$

$$\alpha = \ln(K/K_0)/\sigma \quad (2)$$

where C is the liquid phase concentration of free antigen; K is the association constant; σ is the heterogeneity of K; and y is the fractional saturation of antibody.

The values of K_0 and σ were calculated using Eq. (1) and Eq. (2). These parameters are summarized in Table 1.

Depending on the association constants of anti-peptide antibodies against the antigen peptides, the molar ratios of the adsorbed peptide to IgG varied from almost 2 (the maximum molar ratio) to about 0.1.

The adsorption capacity of insulin B-chain to anti-PC-InB12P antibody was almost the same that of PC-InB11P (11 aa), because B-chain has a size equivalent to an oligopeptide (30 aa) in comparison with α-amylase and sCD2. On the other hand, the adsorption capacities of α-amylase and sCD2 were in the range of from one-third to one-tenth of those of the antigen peptides. This may be caused by differences in steric structures of the C-terminal region between the native proteins and the antigen peptides and also in molecular sizes.

3.2. Immunoaffinity purification of native proteins

The supernatant of fermentation broth containing secreted α-amylase (Amy1A/3D) was concentrated by ultrafiltration and was purified by immunoaffinity chromatography, as stated in Section 2.

Table 1
Average association constant and heterogeneity index

Antibody	Antigen	K_0 (M^{-1})	σ
Anti-PC-Am3D	PC-Am3D	8.0×10^6	2
Anti-PC-Am3D	Amy1A/3D	2.0×10^6	2
Anti-PC-InB12P	PC-InB11P	3.2×10^6	2
Anti-PC-InB12P	Insulin B-chain	2.3×10^6	3
Anti-PC-CD2-12	PC-CD2-12	5.9×10^5	2
Anti-PC-CD2-12	sCD2	1.1×10^5	2

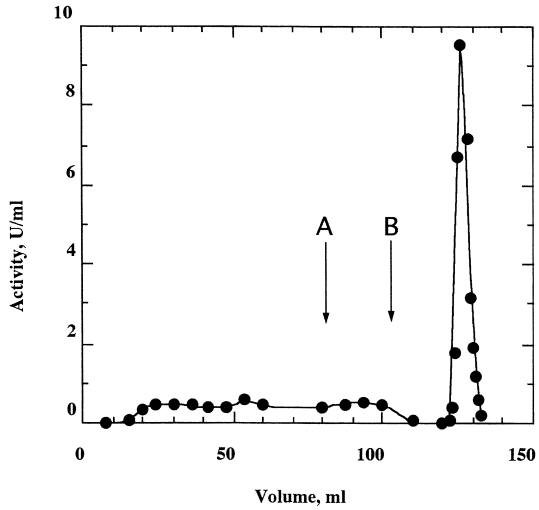

Fig. 3. Adsorption and elution profiles of Amy 1A/3D from anti-PCAm3D column. Sample: crude Amy1A/3D (1.42 U/ml); (A) 50 m*M* Tris–HCl, pH 7.6+5 m*M* $CaCl_2$; (B) 2.5 *M* NaSCN, pH 5.0+5 m*M* $CaCl_2$.

Fig. 3 shows the activity of α-amylase in the effluent solution from the anti-PC-Am3D column. As shown by the flat portion of the activity profile in the figure, a part of α-amylase, which was about one-third of the total activity in all fermented lots, was not adsorbed and was considered to have different characteristics with adsorbable α-amylase. It may be said that a part of Amy1A/3D secreted from yeast lost some residues in its C-terminal region. The column was washed, and Amy1A/3D adsorbed was eluted as a sharp peak by 2.5 *M* NaSCN containing 5 m*M* $CaCl_2$, pH 5.0. The eluted Amy1A/3D had a specific activity of 100 U/mg and showed a single band by SDS–PAGE, which indicates the high purity of obtained Amy1A/3D. Other rice α-amylase isozymes with different C-terminal sequences, for example GYAIWEKI for Amy1A, were not adsorbed by the anti-PC-Am3D column.

Fig. 4 shows the concentrations of sCD2 in the effluent solution from the anti-PC-CD2-12 column measured by direct ELISA using both anti-PC-CD2-12 and TS2/18 antibodies. The recognition sites of these antibodies are the C-terminal regions in the D2 and D1 regions of sCD2, respectively. Immediate breakthrough of sCD2 was observed by measurement with TS2/18 antibody. On the other hand, sCD2 was

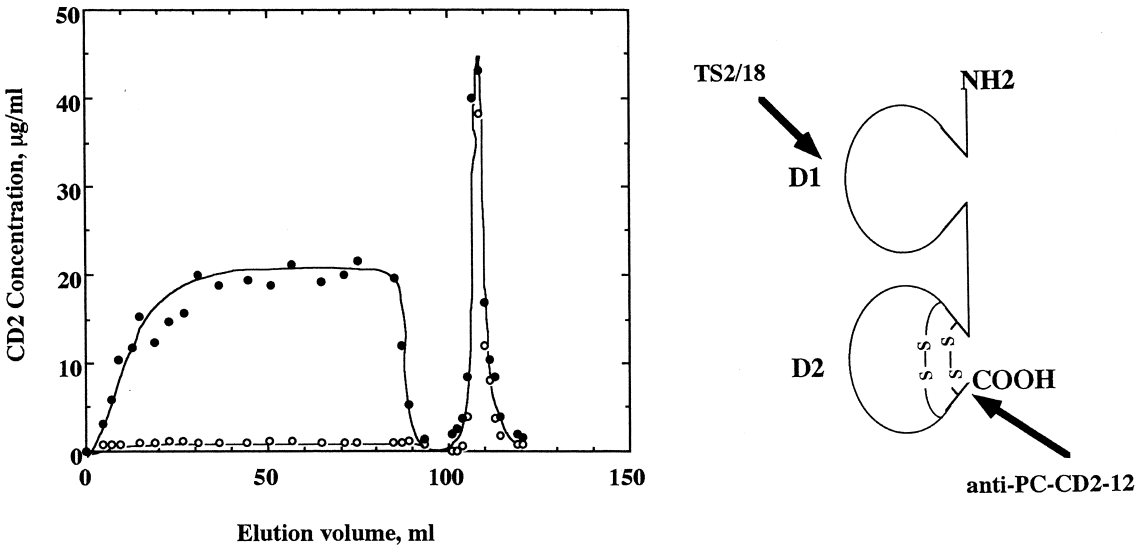

Fig. 4. Adsorption and elution profiles of sCD2 with anti-PC-CD2-12–Sepharose 4B column: (○) anti-PC-CD2-12; (●) TS2/18 (monoclonal).

completely adsorbed by the column according to the results obtained by the ELISA with anti-PC-CD2-12 antibody. This is again caused by the degradation of the C-terminal amino acids in secreted sCD2, because TS2/18 antibody should detect sCD2 molecules losing some of the C-terminal amino acids in the D2 domain.

These results show that the anti-peptide antibodies against the peptides in the C-terminal regions of proteins can discriminate molecules whose C-terminal regions are processed from native ones, which may be of great advantage in obtaining high purity of protein with higher physiological activity.

3.3. Specific elution with antigen peptides

Fig. 5 shows elution profiles of Amy1A/3D using acetate buffer containing 0.153 and 0.48 mg/ml of PC-Am-3D from the anti-PC-Am3D column (1.5 mg IgG/ml-bed). In Fig. 5, C/C_0, where C_0 is an activity of adsorbable α-amylase in feed solutions, is plotted against the elution volume. Adsorbed Amy1A/3D was eluted by competition with the peptide in the buffer solution adjusted at a pH value stabilizing α-amylase. Thus, it was possible to elute under mild conditions without addition of chaotropic ions. The profiles of elution curves were not affected by the peptide concentration in this concentration range, which indicates the limiting step is external liquid film and intraparticle mass transfer of desorbed Amy1A/3D. The recovery of adsorbed α-amylase was 50–60% (almost the same value as in the case of elution by 2.5 *M* NaSCN). This high purity was attained because contaminating proteins nonspecifically adsorbed on the immunoadsorbent

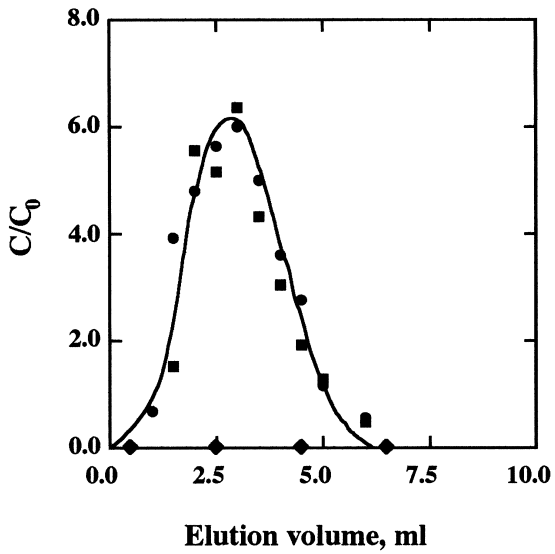

Fig. 5. Specific elution of Amy1A/3D by eluent containing PC-Am3D (mg/ml): (♦) 0; (●) 0.153; (■) 0.48.

were rarely desorbed under this specific elution condition. No activity of α-amylase was detected in the eluted fractions by the acetate buffer without the peptide, as shown in Fig. 5.

Insulin B-chain adsorbed by anti-PC-InB12P column was also eluted by the PBS buffer containing from 0.04 to 0.15 mg/ml of PC-InB11P (data not shown). Again, the profiles of elution curves were not affected by the peptide concentration in this concentration range.

Using the anti-peptide antibodies which bound native proteins, the target proteins of high purity was specifically eluted by the eluent containing the antigen peptide under mild conditions. Because mass transfer of the desorbed proteins was the rate-limiting step, very low concentrations of the peptide could effectively elute the target proteins. Since the molecular mass of the peptide is quite different from that of proteins, the peptide contained in the eluted fraction may easily be separated with protein and recovered by ultrafiltration or gel permeation chromatography. Thus, this method is economically feasible and suitable for a high degree of purification of unstable bioactive proteins contained in a low concentration.

Using peptide immunization, antibodies of predetermined specificity can be obtained [8], and these are useful for immunoaffinity purification of bioactive proteins with specific elution [9,10]. Application of several methods to create combinatorial libraries expressed on the surface of phage [11,12], in combination with peptide immunization, may be promising to obtain suitable ligands for highly selective adsorption and elution.

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