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Adsorption and Elution Characteristics of Anti-Peptide Antibodies in Immunoaffinity Chromatography

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Anti-peptide antibodies against the N- and C-terminal regions of chimeric α-amylase, recombinant CD2 and insulin B-chain were obtained by using synthesized peptides corresponding to the parts of target proteins as immunogens. These anti-peptide antibodies, which should be obtained without the use of target proteins, adsorbed the target proteins and the antigen peptides; therefore, they could be used for detection and purification of those proteins. The adsorbed proteins were specifically eluted by the eluants containing the antigen peptides under mild conditions.

INTRODUCTION

For purification of high-value bioproducts produced using genetically engineered microorganisms or cells, immunoaffinity chromatography is very effective due to 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, since a sufficient amount of antigen (target protein) of suitable purity for immunization is not available. Due to high affinity, elution of the target proteins from antibody ligands sometimes requires extreme conditions which may cause their denaturation. To solve these problems, in previous work, utilization of cross-reactive antibody and anti-peptide antibodies is proposed, which were obtained without use of a target protein as an antigen, for the purification of chimeric α-amylases secreted from yeast cells in one step immunoaffinity chromatography [1,2]. It has also been shown that α-amylase adsorbed by the anti-peptide antibodies was eluted by an eluant containing an antigen peptide and that this specific elution method was effective in avoiding denaturation of a target protein during the elution step [3].

In this work, adsorption and elution characteristics of several anti-peptide antibodies against proteins, as well as antigen peptides, in immunoaffinity chromatography are studied and feasibility of the specific elution method by use of antigen peptides is discussed.

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EXPERIMENTAL

Materials

Figure 1 shows the antigen peptides corresponding to the C-terminal regions of chimeric α-amylase (Amy1A/3D), recombinant soluble human CD2 antigen (sCD2) and the N- and C-terminal regions of bovine insulin B-chain. To the peptide corresponding to 7 amino acids in the N-terminal region of insulin B-chain, lysine was added at the C-terminal of the peptide for conjugation to a carrier protein. Chimeric α-amylase consists of 158aa of one isozyme (Amy1A) of rice α-amylases and 252aa of another (Amy3D); 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 and B-chain (oxidized) were obtained from Sigma-Aldrich (St. Louis, USA). These peptides were synthesized by the solid-phase method (431A, Applied Biosystems, Foster City, USA) and purified by an HPLC system (LC-10A, Shimadzu, Kyoto, Japan) with a reverse-phase column. The sequences of

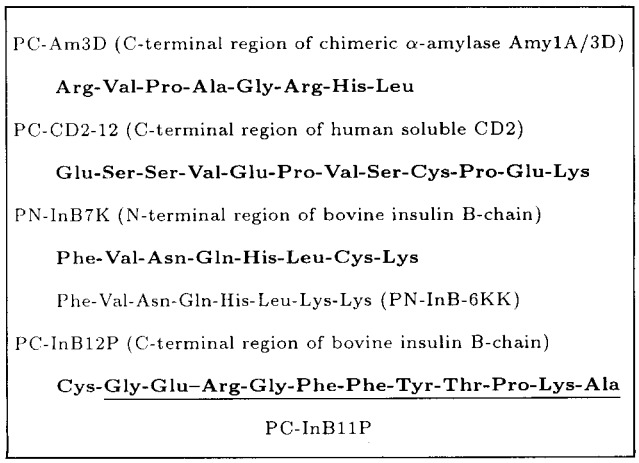


Figure 1. Peptides used as antigens for measurement of adsorption equilibrium.

the synthesized peptides were confirmed by a peptide sequencer (Procise 492, Applied Biosystems). The chemicals used were of reagent grade.

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-CD2-12, anti-PC-InB12P and anti-PN-InB7K) were purified from pooled sera by adsorption on antigen-coupled Sepharose 4B, eluted with 0.1 N HCl. The binding specificities of the purified antibodies against the N- or 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 N- or C-terminal regions of the original antigen peptides. The immunoadsorbent was prepared by coupling the purified specific antibody to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, 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.

Measurement of Adsorption Equilibrium and Elution Characteristics

The immunoadsorbent (about 5 ml) was packed in an adsorption column of 1.26 cm diameter which was equilibrated with an equilibration buffer (50 mM Tris-HCl + 5 mM $CaCl_2$, pH 7.6 for Amy1A/3D, 10 mM Tris-HCl, pH 7.0 for sCD2, PBS, pH 7.6 for insulin) at a flow rate of 1-2 ml/min and temperature of 23 ± 2°C. The buffer solution containing the protein or the antigen peptide (0.07-3.0 nmol/ml) was applied to the column. In order to avoid dimerization of the peptides by formation of disulfide bonds, PC-InB11P and PN-InB6KK, which were lacking cystein residues from PC-InB12P and PN-InB7K, respectively, were used for measurement of adsorption equilibrium of these peptides (Figure 1). After washing with the equilibration buffer, the adsorbed protein or peptide was nonspecifically eluted by 0.1 N HCl. For specific elution of the proteins, the buffer solution containing 0.2 mg/ml of the antigen peptide was supplied to the column. After the specific elution, the remaining protein and adsorbed peptide were eluted by 0.1 N HCl. The absorbance of the effluent solution at 215 nm (peptide) or 280 nm (protein) was continuously measured by a spectrophotometer (Shimadzu SPD-6AV). The concentration of the antigen peptides, insulin and insulin B-chain in the effluent from the column were measured by an HPLC system (Shimadzu LC-10A) equipped with a reversed-phase column. The concentration of sCD2 was measured by direct ELISA and the activity of α-amylase was determined as previously reported [2].

The total amount adsorbed was obtained by numerical integration of breakthrough curves, assuming that the total void fraction of the packed bed is 0.96.

Production of Chimeric α-Amylase and sCD2

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). The chimeric enzyme was expressed in *Saccharomyces cerevisiae* LL20 under the yeast enolase promoter and secreted to fermentation broth with a signal peptide [5].

The sCD2 gene was inserted downstream of the alcohol oxidase 1 promoter and signal genes and expressed by use of *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 shaking flask of 1 l. Then, cells were centrifuged (6500 rpm 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 [6].

ELISA

Direct ELISA protocol is as follows. Wells were coated with 100 μl/well of antigen solution 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, Tokyo, Japan) to decrease non-specific adsorption. After washing with PBST (PBS + 0.05% Tween 20), various dilutions of anti-serum 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-fold diluted anti-rabbit IgG-horseradish peroxidase or anti-mouse IgG-horseradish (Cappel, Ohio, USA) 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 405 nm.

RESULTS AND DISCUSSION

Adsorption Equilibrium

Figure 2 shows the adsorption equilibria of the antigen 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 N- and C-terminal regions of the proteins. The adsorption of the peptides and proteins by these antibodies showed adsorption isotherms of the Freundlich type. Although the native proteins were adsorbed by the anti-peptide antibodies obtained without use of the proteins, the adsorption capacities of the proteins were lower than those of the corresponding antigen peptides. In anti-PC-InB12P antibody, however, the ratio of the adsorption capacity of the protein to that of the peptide was higher than those of the other two antibodies. These antibodies may show different elution characteristics.

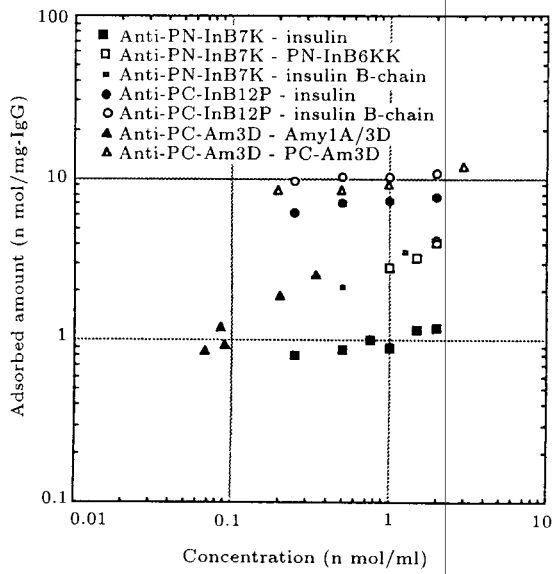
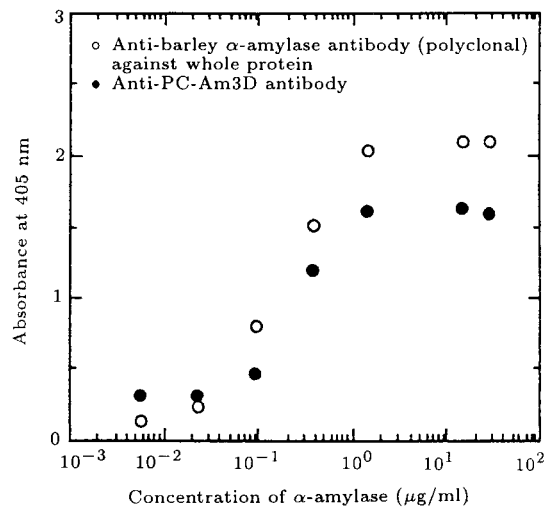


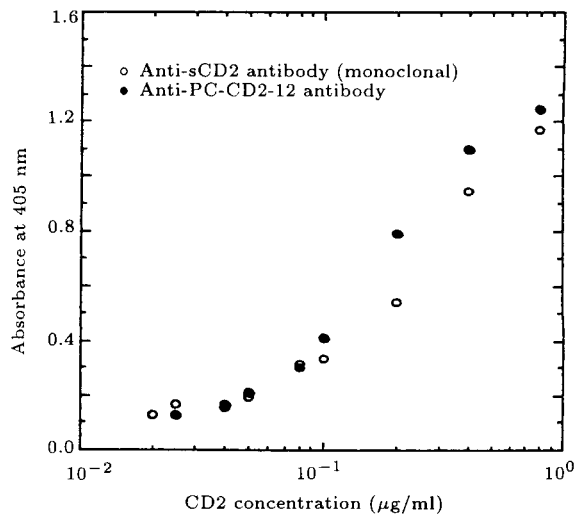
Figure 2. Adsorption equilibria of proteins and antigen peptides.

Detection of Proteins by ELISA with Anti-Peptide Antibodies

Figure 3 illustrates the results of ELISA of chimeric α-amylase (Amy1A/3D) and sCD2 with anti-peptide antibodies against the C-terminal region of these proteins and also with antibodies against whole proteins (polyclonal and monoclonal). The anti-peptide antibodies could detect these proteins with almost the same sensitivity as the antibodies against whole proteins. Since anti-peptide antibodies can be obtained by immunization of synthetic peptides conjugated with carriers, ELISA with anti-peptide antibodies possesses



(a) ELISA of Amy1A/3D



(b) ELISA of sCD2

Figure 3. ELISA using anti-peptide antibodies.

advantages that a detection system can be designed prior to expression and purification of target proteins and that it may discriminate a target protein among proteins having high homology.

Elution Characteristics of Anti-Peptide Antibodies

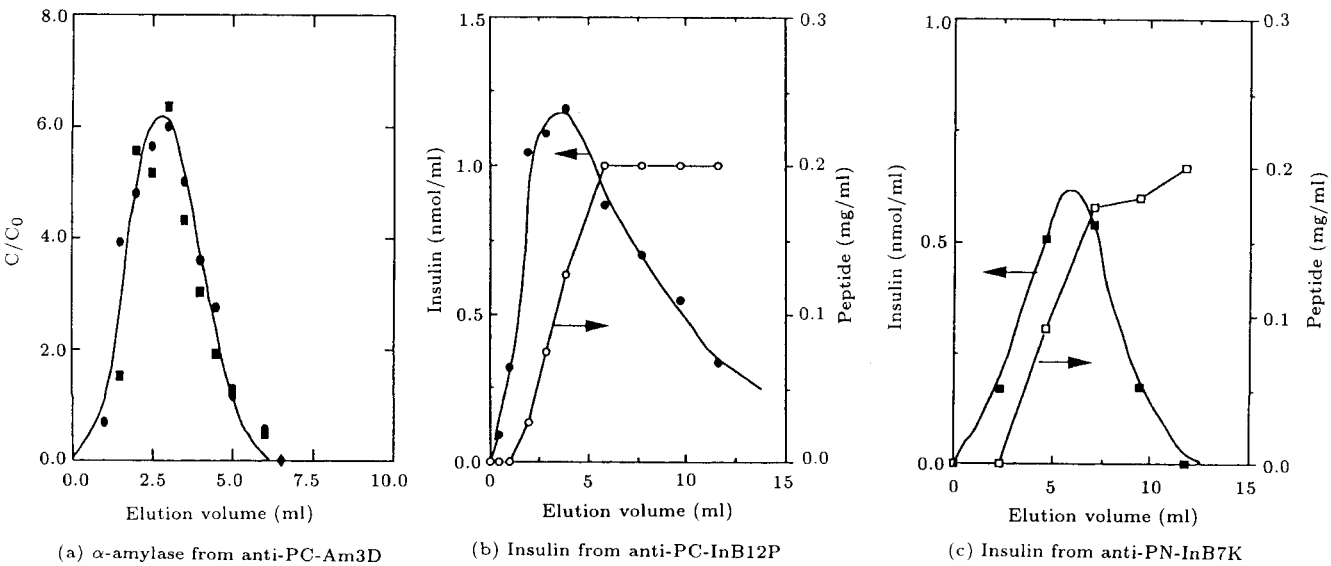
Figure 4 depicts the elution profiles of the proteins from the anti-peptide antibody columns, as well as the concentration changes of the antigen peptides. Alpha-amylase from anti-PC-Am3D column and insulin from anti-PN-InB7K column were specifically eluted with about 10 ml of the eluants containing the antigen peptides and the recoveries were about 50%. Table 1 summarizes the amount of insulin eluted non-specifically by 0.1 N HCl and specifically by 10 ml of the eluant containing the antigen peptides after saturation of the adsorbents with 2 nmol/ml of insulin. For anti-PC-Am3D and anti-PN-InB7K antibodies, less amount of α-amylase or insulin was eluted by 0.1 N HCl after the specific elution with the antigen peptides. Thus, it was possible to elute the adsorbed proteins under mild conditions without loss of their biological functions [3]. On the other hand, an elution profile on insulin from anti-PC-InB12P column showed tailing as well as low recovery.

Furthermore, a considerable amount of insulin was eluted by 0.1 N HCl after the specific elution with 10 ml of the eluant containig 0.2 mg/ml of PC-InB11P. As shown in Figure 2, this anti-peptide antibody demonstrates more favorable recognition against the native protein (insulin) than the other two anti-peptide antibodies. This might be the cause of insufficient elution by the antigen peptide PC-InB11P.

The competition between insulin and the antigen peptide for the antigen binding sites of anti-PC-InB12P antibody was checked by applying a mixture of insulin (2 nmol/ml) and antigen peptide (PC-InB11P, 50 nmol/ml) in PBS. As shown in Figure 5, insulin flowed out from the column immediately after the elution volume corresponding to the dead space of the column and was not adsorbed. No insulin was eluted by 0.1 N HCl after saturation with the mixture. These results show that large excess of the antigen peptide reject adsorption of insulin. Once insulin was adsorbed, however, it might be difficult to conduct elution by antigen peptides in some cases.

Table 1. Specific and non-specific elution of insulin from anti-peptide antibodies (unit: nmol).

Antibody	Non-Specific Elution 0.1 N HCl	Specific Elution 0.2 mg/ml Antigen Peptide	Elution After Specific Elution 0.1 N HCl	Total Amount Adsorbed
Anti-PC-InB12P	13.7	8.1	5.3	24
Anti-PN-InB7K	3.1	2.7	0.6	5.9



(a) α-amylase from anti-PC-Am3D (b) Insulin from anti-PC-InB12P (c) Insulin from anti-PN-InB7K

Figure 4. Specific elution of α-amylase and insulin from anti-peptide antibodies.

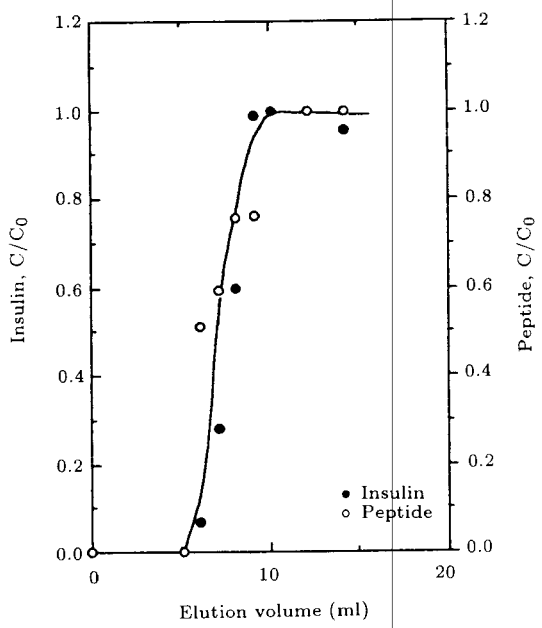


Figure 5. Competitive adsorption between insulin and antigen peptide.

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