ELSEVIER

Chemical Engineering Journal 65 (1997) 105–108

Chemical Engineering Journal

Discrimination between α-amylase isozymes with anti-peptide antibodies

Shigeo Katoh, Masaaki Terashima, Keiji Yamamoto, Mituhiro Kouno

Department of Synthetic Chemistry and Biological Chemistry, Kyoto University, Sakyo-ku Yoshida, Kyoto 606-01, Japan

Received 4 December 1995; accepted 29 August 1996

Abstract

Anti-peptide antibodies against the C-terminal region of α-amylase were originated by immunizing two kinds of peptide antigen and were used to discriminate between two isozymes of α-amylases. The anti-peptide antibodies only reacted with α-amylase that had the C-terminal region corresponding to the peptide antigens, and could purify the target α-amylase from the mixture of the isozymes. The adsorption and elution behaviors of the anti-peptide antibodies differed depending on the characteristics of the peptide antigen used for immunization. Peptide immunization can provide suitable affinity ligands for the purification and detection of target proteins from contaminants that exhibit high homology with the targets. © 1997 Elsevier Science S.A.

Keywords: Anti-peptide antibody; Purification; Isozyme; α-Amylase

1. Introduction

Immuno-affinity chromatography, which depends on the specific interaction between the antigen and antibody, is very effective for the purification of bioactive proteins. However, the following problems may arise. First, in some cases, it is difficult to obtain a sufficient amount of an antigen of suitable purity for immunization. Secondly, because of the high affinity, the elution of a target protein from an antibody ligand often requires extreme conditions which cause denaturation of the target protein. Thirdly, the cross-reactivity of antibody ligands may co-purify proteins that exhibit high homology, such as isozymes and proforms, as well as processed forms of the target protein.

In previous work [1], we proposed the utilization of an anti-peptide antibody as an affinity ligand to resolve the first problem. The anti-peptide antibody was obtained by immunizing a synthesized peptide that corresponded to the C-terminal region of recombinant α-amylase, and it was used successfully for the purification of the enzyme. In this work, we demonstrate the usefulness of anti-peptide antibodies in discriminating between isozymes of α-amylases and also for controlling the elution conditions for immuno-affinity chromatography by selecting suitable peptide antigens.

2. Materials and methods

2.1. Fermentation of rice-α-amylases-secreting yeast

The genes of rice-α-amylase (Amy1A) and a chimeric α-amylase (Amy1A/3D), which consisted of 158aa of one isozyme (Amy1A) and 252aa of another (Amy3D), were subcloned into the yeast expression vector pMAC101 to give the yeast expression plasmids as reported previously [1]. These enzymes were expressed in *Saccharomyces cerevisiae* LL20 under the yeast enolase promoter and were secreted to a fermentation broth with a signal peptide. Amy1A and Amy1A/3D show about 80% homology in their amino acid sequences.

The yeast was grown in 300 cm^3 of YNBDH medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 20 $\mu g\ cm^{-3}$ histidine, 5 mM $CaCl_2$) and inoculated to 2 l of YEPD media (1% yeast extract, 2% Bacto peptone, 8% glucose, 5 mM $CaCl_2$). The inoculated culture was grown in a jar fermenter (Mitsuwa KMJ-5C) for 24 h at 30 °C with agitation at 300 rev min^{-1} and aeration of 2 l min^{-1}. The values of the pH and DO were not controlled. After cells were separated by centrifugation (6500 rev min^{-1} for 10 min), the supernatant that contained α-amylase was concentrated to various concentrations by ultrafiltration with a hollow fiber module (Asahi Kasei ACP-0013, with 13 kD molecular cut-off).

2.2. Preparation of anti-peptide antibodies

In this work, two peptides were synthesized using a solid-phase method and were used for immunization. One is a peptide (RVPAGRHL, PC-Am3D) against the eight amino acids of the C-terminal region of Amy1A/3D (chimeric α-amylase produced from two rice-α-amylase isozymes), while

PII S1385-8947(96)03160-0

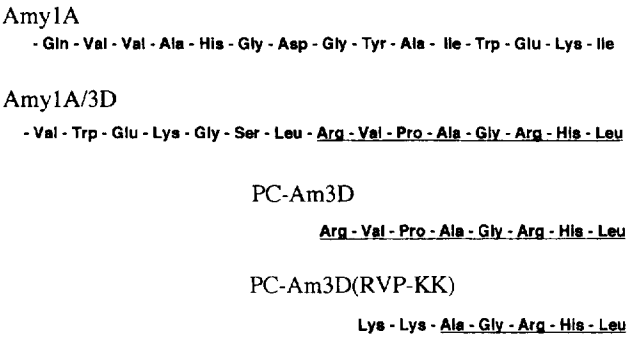

Fig. 1. Amino acid sequences of peptide antigens and C-terminal regions of α-amylases.

the other is a peptide (KKAGRHL, PC-Am3D(RVP-KK)) in which three amino acids (RVP) of PC-Am3D were replaced with two lysine residues. Fig. 1 shows the sequences of the C-terminal regions of Amy1A, Amy1A/3D and the peptide antigens. These peptides were synthesized using a protein synthesizer (Applied Biosystems, 431A), purified using a high pressure liquid chromatography (HPLC) system (Shimadzu, LC 10A) with a reverse-phase column, and coupled to keyhole limpet hemocyanin [2]. A mixture of the antigen and Freund's complete adjuvant (1 cm^3 each) was immunized in rabbits. Booster injections were repeated twice in a similar way at intervals of 10 days. Specific antibodies (anti-PC-Am3D and anti-PC-Am3D(RVP-KK) antibodies) were purified from pooled sera by affinity chromatography using peptide-coupled Sepharose 4B. An anti-barley α-amylase antibody was also prepared as previously reported [3].

2.3. Purification of rice-α-amylases by immuno-affinity chromatography

Immuno-adsorbents were prepared by coupling the antibodies to CNBr-activated Sepharose 4B (Pharmacia LKB Biotech). The amounts of the coupled antibodies were 0.76 $mgIgG \cdot cm^{-3}$ per bed for both the antibodies. The immunoadsorbent was packed in an adsorption column of diameter 1.26 cm and the column was equilibrated with an equilibration buffer (50 mM Tris-HCl + 5 mM $CaCl_2$, pH 7.6 or five-times-diluted buffer) at a flow rate of 1 $cm^3\ min^{-1}$ and temperature of 23 ± 2 °C. The fermentation broth (adjusted to pH 7.6) was applied to the column. After washing with the equilibration buffer, adsorbed α-amylase was eluted by an eluent (2.5 M NaSCN containing 5 mM $CaCl_2$, pH 5.0, or 50 mM sodium acetate containing 5 mM $CaCl_2$ and 0.5 M NaCl, pH 4.5). The absorbance of the effluent solution at 280 nm was continuously measured using a spectrophotometer, and the activity of α-amylase in the samples collected was determined as described below.

The adsorption behavior of the peptides to the anti-peptide antibodies was also examined in a similar manner. A peptide solution in phosphate buffer (pH 7.6) adjusted to a desired ionic strength using NaCl or NaSCN was pumped into the column, and the breakthrough curve was obtained by measuring the absorbance of the effluent at 215 nm.

The total amount of adsorbed α-amylases or peptides was obtained by numerical integration of the breakthrough curves, assuming that the total void fraction of the packed bed was 0.96 [2].

2.4. Enzyme-linked immunosorbent assay of α-amylases

Wells of a micro-titer plate (96 flat-bottom wells, Falcon 3912) were coated with 50 μl per well of various concentrations of α-amylase solution in phosphate buffer saline (PBS) for 20 h at 4 °C. The α-amylases were purified using the method described above. After washing with the PBS, all the wells were coated for 1 h with 200 μl per well of a bovine serum albumin solution (1% BSA in PBS) to decrease non-specific adsorption. After washing with a washing buffer (PBS that contained 0.05 vol.% Tween 20 (PBST)), an antibody solution (50 $\mu g\ ml^{-1}$) was added to the wells (50 μl per well) and incubated for 1 h at room temperature. After washing with PBST, each well was incubated with 100 μl of × 1000 diluted anti-rabbit IgG-horseradish peroxidase antibody (Organon Teknika Corp.) in PBS for 1 h. After washing with PBST, color was developed using 100 μl per well of an 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution (0.3 $mg\ ml^{-1}$ in 0.1 M citrate buffer that contained 0.01% H_2O_2, pH 4.1). The absorbance of each well was recorded by the microplate reader (Bio-Rad, Model 450) at 405 nm.

2.5. Measurement of enzyme activity and protein concentration

The activity of the recombinant α-amylase was determined from the rate of increase in reducing ends from soluble potato starch, as previously reported [4]. One enzyme unit was defined as the activity to liberate 1 μmol of maltose per minute.

The protein concentration was determined using the dye method (Bio-Rad protein assay kit, Bio-Rad Lab). BSA was used as a standard. The purity of the α-amylases was examined using sodium dodecyl sulfate in polycylamide gel electrophoresis (SDS-PAGE) stained with silver (Bio-Rad silver stain kit, Bio-Rad Lab).

3. Results and discussion

3.1. Enzyme-linked immunosorbent assay of two α-amylases with different C-terminal sequences

Fig. 2 shows the results of enzyme-linked immunosorbent assay (ELISA) of Amy1A and Amy1A/3D using anti-barley α-amylase antibody (Fig. 2(a)), anti-PC-Am3D antibody (Fig. 2(b)) and anti-PC-Am3D(RVP-KK) antibody (Fig. 2(c)). The concentrations of the α-amylases were calculated from the specific activities of these enzymes purified by the method described above (220 U mg^{-1} for Amy1A

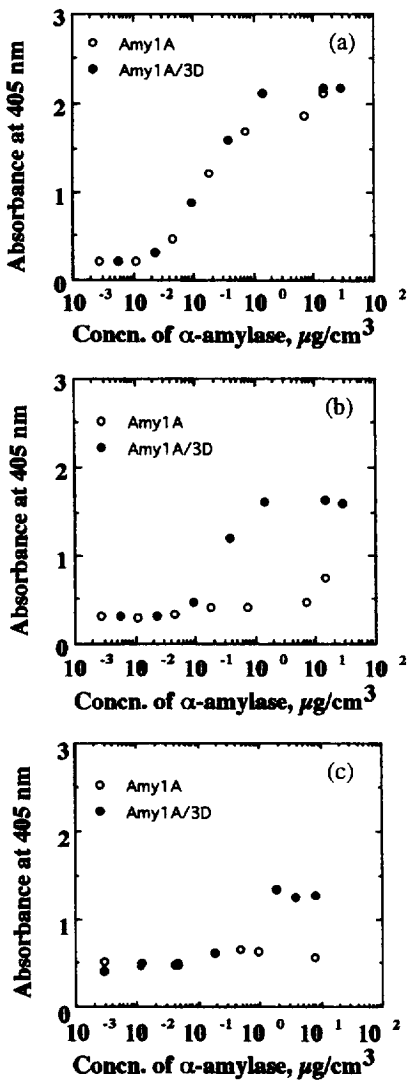

Fig. 2. Enzyme-linked immunosorbent assay of Amy1A and Amy1A/3D using (a) anti-barley α-amylase antibody, (b) anti-PC-Am3D antibody and (c) anti-PC-Am3D(RVP-KK) antibody.

and 100 U mg^{-1} for Amy1A/3D). The anti-PC-Am3D and anti-PC-Am3D(RVP-KK) antibodies reacted only with the Amy1A/3D, while the anti-barley α-amylase antibody reacted with the Amy1A and the Amy1A/3D to the same extent. These results show that the anti-peptide antibodies can discriminate between the C-terminal sequences of these two α-amylases.

3.2. Separation of two rice-α-amylases using anti-PC-Am3D antibody

A mixture of Amy1A and Amy1A/3D (with an activity ratio of 1:2) in a fermentation broth was purified using anti-PC-Am3D antibody and anti-barley α-amylase antibody. Fig. 3 shows the results of SDS-PAGE of fractions eluted by 2.5 NaSCN from these antibodies. The fraction purified by the anti-PC-Am3D antibody showed a single band (lane A), while that purified by the anti-barley α-amylase antibody showed another band with a higher molecular weight (lane

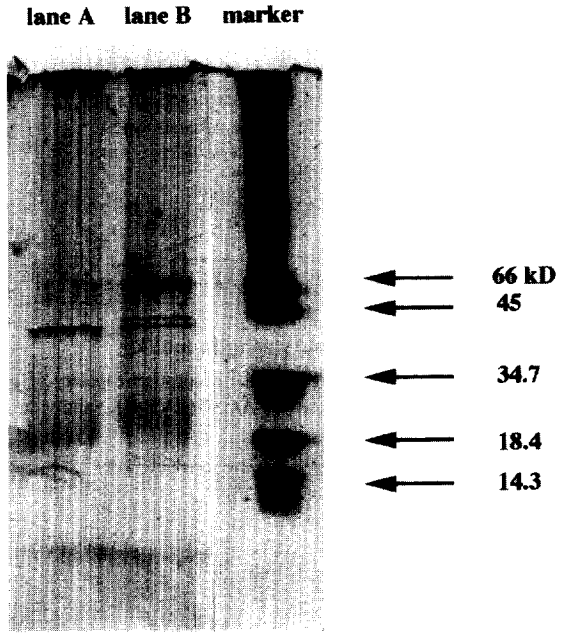

Fig. 3. Sodium dodecyl sulfate-polyacrylamidegel electrophoresis of α-amylases purified by anti-PC-Am3D antibody (lane A) and anti-barley α-amylase antibody (lane B).

B). Although Amy1A and Amy1A/3D consist of 403 and 410 amino acids, respectively, Amy1A has a carbohydrate chain, so has a higher molecular weight than that of Amy1A/3D [5]. Therefore, with the use of the anti-barley α-amylase antibody, both α-amylases were co-purified in the eluted fraction. In contrast, the anti-PC-Am3D antibody exhibited high specificity for Amy1A/3D and could purify it from the mixture of two enzymes that exhibit high homology with each other. This indicates the usefulness of the anti-peptide antibody for the purification of a specific protein contained in a mixture of proteins that exhibit high homology.

3.3. Elution characteristics of anti-peptide antibodies acting against different peptides

Two different anti-peptide antibodies (anti-PC-Am3D and anti-PC-Am3D(RVP-KK) antibodies) were obtained by immunizing the peptide (PC-Am3D) that corresponds to the C-terminal sequence of Amy1A/3D and its substitute (PC-Am3D(RVP-KK)). Fig. 4 shows the effects of the ionic strength (adjusted by the addition of NaCl) on the adsorption capacities of the peptide antigens to the anti-peptide antibodies. In this figure, the ratio of the amount adsorbed to the amount at an ionic strength of 0.055 mol kg^{-1} is plotted at a peptide concentration of 1.0×10^{-6} M. The adsorption capacity of the anti-PC-Am3D(RVP-KK) antibody decreased markedly with increasing ionic strength, while the adsorption capacity of the anti-PC-Am3D was affected only slightly by the ionic strength. Because two hydrophobic amino acids (VP) that corresponded to the C-terminal sequence of the Amy1A/3D were replaced by two lysine residues in the PC-Am3D(RVP-KK), the electrostatic interaction may become dominant in the interaction between the

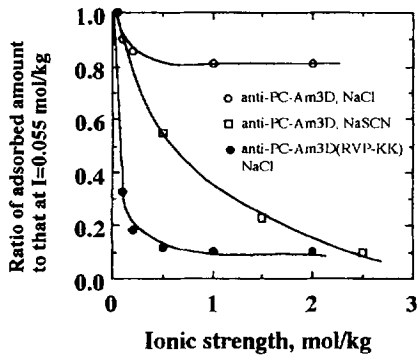

Fig. 4. Effect of the ionic strength on the adsorption capacity of peptide antigens to anti-PC-Am3D and anti-PC-Am3D(RVP-KK) antibodies.

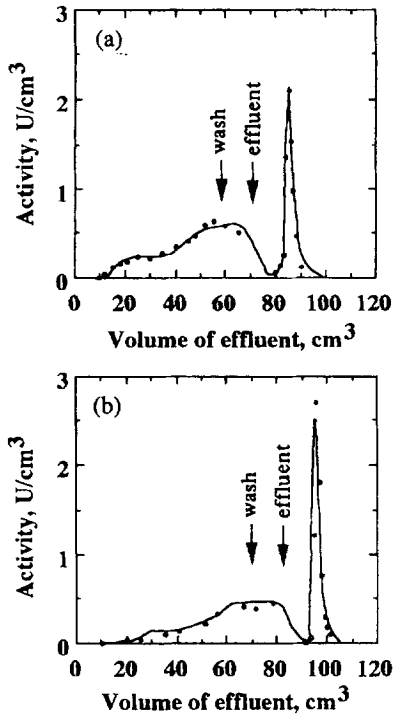

Fig. 5. Adsorption and elution profiles of Amy1A/3D from (a) anti-PC-Am3D(RVP-KK) antibody and (b) anti-PC-Am3D antibody columns.

anti-PC-Am3D(RVP-KK) antibody and the PC-Am3D-(RVP-KK).

In Fig. 4, the effect of the ionic strength (adjusted using NaSCN) on the adsorption capacity of PC-Am3D to anti-PC-Am3D antibody is also shown. At 0.5 M NaCl, the adsorption capacity of the anti-PC-Am3D(RVP-KK) decreased to the level of the adsorption capacity of the anti-PC-Am3D antibody at 2.5 M NaSCN, which was used for elution of the Amy1A/3D from the anti-PC-Am3D antibody. Because α-amylases are unstable at acidic pH values below 4, the chaotropic electrolyte NaSCN was used as an eluent.

This suggests that it is possible to elute adsorbed Amy1A/3D from anti-PC-Am3D(RVP-KK) antibody using an eluent that contains 0.5 M NaCl, which has less harmful effects on the protein activity in comparison with the use of 2.5 M NaSCN. Because the adsorption capacity strongly depends on the ionic strength, a fermentation broth that contained Amy1A/3D was dialyzed against 10 mM Tris-HCl + 1 mM $CaCl_2$ (pH 7.6) and supplied to the anti-PC-Am3D(RVP-KK) column equilibrated with the same buffer solution. After washing, the α-amylase was eluted using 50 mM sodium acetate that contained 5 mM $CaCl_2$ and 0.5 M NaCl (pH 4.5).

Fig. 5 compares adsorption and elution profiles of the Amy1A/3D from the anti-PC-Am3D(RVP-KK) and anti-PC-Am3D columns. In both cases, similar profiles were observed, and the α-amylase was effectively eluted from the PC-Am3D(RVP-KK) column by the eluent that contained 0.5 M NaCl. As shown by the first flat portion of the breakthrough curves, a portion of the Amy1A/3D was not adsorbed and was considered to have processed the C-terminal [1]. These results show that the characteristics of peptide antigens affect the adsorption behaviors of anti-peptide antibodies, and that the selection of peptide antigens can change the elution conditions of target proteins to form anti-peptide antibodies.

4. Conclusions

Anti-peptide antibodies that act against short peptides that consist of the surface portion of proteins have the advantage in discrimination of a target protein from contaminants that show high homology with the target protein and/or from processed forms of the target. Furthermore, it is possible to change the adsorption and elution characteristics of anti-peptide antibodies by selecting suitable peptides for immunization. Thus, by utilizing anti-peptide antibodies as ligands, one can attain high degree of purification in immuno-affinity chromatography.

Acknowledgements

The authors are very grateful to Prof. Raymond L. Rodriguez (University of California at Davis) for supplying the plasmids of cloned rice-α-amylases. The partial support of a Grant-in-Aid for Scientific Research (C) (Grant 06650912) from the Ministry of Education, Science and Culture of Japan, is gratefully acknowledged.

References

[1] S. Katoh, M. Terashima and M. Kouno, *Appl. Microbiol. Biotechnol.*, *43* (1995) 871.
[2] A. Kondo, H. Takamatsu, S. Katoh and E. Sada, *Biotechnol. Bioeng.*, *35* (1990) 146.
[3] S. Katoh and M. Terashima, *Appl. Microbiol. Biotechnol.*, *42* (1994) 36.
[4] B. Bernfeld, *Methods in Enzymology*, Academic, Florida, 1955, pp. 149–158,
[5] M. Terashima, A. Kubo, M. Suzawa, Y. Itoh and S. Katoh, *Euro. J. Biochem.*, *226* (1994) 249.