Purification of Two Specific Antibodies against Drug and Carrier Protein Molecules

HIDEAKI TANIMORI, KENSEI YOSHIDA, HIDEAKI MOTOMURA, KAZUHIRO KITADA, SIROKI YAGISAWA, and TSUNEHIRO KITAGAWA*

Faculty of Pharmaceutical Sciences, Nagasaki University, 1–14 Bunkyo-machi, Nagasaki 852, Japan

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Affinity purification procedures for antibodies specific to a drug and to a carrier protein were examined in detail with the use of three enzyme immunoassays (EIAs). Many blasticidin S (BLS) molecules were coupled to an affinity ligand with pig serum albumin as a spacer protein. The influence of the spacer on the affinity purification of an antibody specific to BLS was quantitatively analyzed. The antibody showed higher binding to BLS bound to the solid matrix with a carrier protein than without. The stability of specific antibody in six representative eluents, used for affinity chromatography of specific antibodies, was examined, and 0.1 M potassium chloride–0.008 N hydrochloric acid buffer was selected as the preferred eluent based on the stability of the specific antibody and convenience in handling. The ability of the buffer to elute the specific antibody from an affinity column was studied, and was improved by modifying the concentration of potassium chloride in the buffer to 0.3 M. Complete purification of anti-BLS antibody was performed by affinity chromatography under the chosen conditions. The specific anti-BLS and anti-carrier protein antibodies were purified quantitatively. Formation of denatured specific antibody was hardly detected by a sensitive EIA, under the chosen conditions. The purity of the standard anti-BLS antibody was demonstrated by the affinity chromatographic method with the aid of two EIAs for rabbit immunoglobulin G and for BLS.

Keywords—affinity chromatography; affinity ligand; enzyme immunoassay; blasticidin S; drug–protein conjugate; drug-specific antibody

Introduction

Specific antibodies are key reagents in a variety of studies in the biochemical, immunological and pharmacological fields. Affinity chromatography is regarded as the best way to isolate specific antibodies from specific antisera. Isolation of highly purified specific antibodies is not easy; the elution of a purified specific antibody from its bound form on the affinity column usually involves drastic conditions which may partially inactivate unstable antibodies. The optimum conditions for elution have not been established so far. This may be the reason why a number of enzyme-linked immunosorbent assay (ELISA) procedures for specific antibodies use tentatively defined titers or dilutions of the antibody or antiserum as standards. It is difficult to determine accurately the content of an antibody specific to a drug in a specific antiserum, owing to the difficulty in preparing a standard specific antibody.

Previously, we developed a new method for preparing hapten immunogens, with the use of hetero-bifunctional reagents of maleimide succinimidyl ester type. Application of the method allowed us to prepare blasticidin S (BLS) and bovine serum albumin (BSA) conjugate as the hapten immunogen. Separation and purification of the two specific antibodies, anti-BLS and anti-BSA, from the rabbit antiserum to BLS were studied quantitatively, with the use of EIAs for BLS, BSA and rabbit immunoglobulin G (IgG). We report here the results of a detailed examination aimed at preparing standard samples of these two specific antibodies.
Materials and Methods

Reagents — Cyanogen bromide-activated Sepharose 4B was bought from Pharmacia Fine Chemicals (Uppsala). N-(Gamma-maleimidebutyryloxy)succinimide (GMBS)26 (Dojin Chemicals, Kumamoto), BLS (Kaken Kagaku Ind., Tokyo) and Amino-Dylark cylinders (6 mm diameter, 4 mm in height) (Sekisui Chem. Ind., Osaka) were commercial products. Rabbit antisera to BLS,20)  β-galactosidase-labeled BLS (Gal-BLS)20) and Gal-labeled goat anti-rabbit IgG antibody27) were prepared by the cited methods. EIA for rabbit IgG27) and BLS20) were performed by the cited methods. All other chemicals were of reagent grade.

Preparation of the Affinity Ligand for Anti-BLS Antibody — The scheme for a three-step synthesis of BLS–GMBS–pig serum albumin (PSA) conjugate is shown in Fig. 1.

Step 1. GMBS-Acylated BLS: A solution of BLS (10 μmol) in 1 ml of 0.05 M sodium phosphate buffer (pH 7.0) was incubated at 30 °C for 30 min with 2.8 mg of GMBS (10 μmol) dissolved in 0.5 ml of tetrahydrofuran under stirring. Tetrahydrofuran was removed by flushing with nitrogen, then excess GMBS was extracted from the reaction mixture three times with 5 ml of methylene chloride. The aqueous layer was immediately used for step 3.

Step 2. Sodium Borohydride Reduction of Disulfide Bonds in PSA: NaBH₄ (total 20 mg) and n-butanol (0.2 ml) were alternately added portionwise to 10 mg of PSA (0.15 μmol) dissolved in 2 ml of 6 M urea-0.1 M ethylenediaminetetraacetic acid (EDTA). The mixture was incubated at 30 °C for 30 min and then excess NaBH₄ was decomposed by adding 1 ml of 0.1 M sodium phosphate (monobasic) and 0.4 ml of acetone. The solution was immediately used for the next step.

Step 3. Conjugation of GMBS-Acylated BLS to the Reduced PSA: The solution of the reduced PSA (step 2) was incubated at 25 °C for 2 h with the aqueous layer of GMBS-acylated BLS (step 1) and the mixture was then loaded onto a 2.5 × 57 cm column of Sephadex G-100 and eluted with 3 M urea.

BSA and BLS–GMBS–PSA conjugate, in the pooled fraction, were coupled with cyanogen bromide activated Sepharose 4B according to the manual of Pharmacia Fine Chemicals. A 3 M urea solution did not disturb the coupling reaction.

Preparation of BLS–GMBS–PSA–Loaded Amino-Dylark Cylinders — Amino-Dylark cylinders were immersed at 25 °C for 1 h in 1% glutaraldehyde under mechanical shaking. The cylinders were washed with 0.01 M phosphate-buffered saline, pH 7.0 (PBS), and then immersed at 25 °C for 20 min in a 0.01% solution of BLS–GMBS–PSA in PBS, and then at 4 °C for 2 h. After successive washing with PBS and buffer A (0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% BSA and 0.1% NaN₃), Amino-Dylark cylinders loaded with BLS–GMBS–PSA were stored in buffer A at 4 °C. BSA- and BLS-loaded Amino-Dylark cylinders were prepared in a similar way.

Isolation of Rabbit Anti-BLS Antibody — Rabbit antisera to BLS (10 ml) was fractionated with 50% saturated ammonium sulfate. The precipitate was washed with 33% saturated ammonium sulfate solution and dissolved in Tris–HCl buffer, pH 8.5 (4 ml). The electric conductivity was adjusted to 1 mU, and the solution was then dialyzed overnight against the same buffer. The IgG solution was preincubated overnight at room temperature with 1 ml of buffer B (0.06 M sodium phosphate-buffered saline, pH 7.4, containing 0.1% BSA and 0.01 M EDTA). The solution was loaded onto the affinity column (1.5 × 5 cm) of the BLS–GMBS–PSA-coupled Sepharose 4B previously
swollen with 0.02M Tris–HCl buffered saline, pH 8.5. The column was eluted with 100ml of the same buffer containing 1% BSA, and non-specific IgG and anti-BSA were eluted. BSA was then eluted with 300ml of 0.05M sodium phosphate-buffered saline, pH 7.0. The eluent was changed to 0.3M KCl-0.008N HCl buffer, pH 2.3. Each fraction was immediately neutralized with 0.1M glycine-0.1N NaOH buffer, pH 10.3. IgG content and immune reactivity in each fraction were assayed by means of EIAs for rabbit IgG and BLS.

Results

Preparation of BLS–GMBS–PSA Conjugate

BLS–GMBS–PSA conjugate, with covalent bonds between the amino groups of BLS and the thiol groups of chemically modified PSA, was prepared with the use of the heterobifunctional cross-linker GMBS (Fig. 1). BLS–GMBS–PSA conjugate was isolated by chromatography on a Sephadex G-100 column; the elution profiles are shown in Fig. 2.

Effect of a Spacer Protein

The effect of a spacer on the binding of anti-BLS antibody to BLS was studied. The amino group (groups) in free BLS or BLS–GMBS–PSA conjugate was coupled covalently to amino groups of Amino-Dylark cylinders by using glutaraldehyde as the coupling reagent. Binding activities of anti-BLS antiserum in variously diluted solutions to BLS-coupled to Amino-Dylark cylinders with or without the spacer GMBS–PSA were examined. Gal-labeled goat anti-rabbit IgG antibody was used as the tracer, in the presence of an excess amount of BSA to prevent the cross-reactive binding of anti-BSA antibody to PSA (Fig. 3).

Stability Test of Anti-BLS Antibody in Various Eluents

An aliquot of anti-BLS antiserum was incubated at 30°C for 3 h in 10 solutions, 6 of which are common eluents for affinity chromatography of specific antibodies. Every solution was then diluted 100 times with buffer C. The binding activity of anti-BLS antibody in an aliquot of the diluted solution against BLS–GMBS–PSA-coupled Amino-Dylark cylinders

Fig. 2. Elution Profile of BLS–GMBS–PSA Conjugate from a 2.5 x 57 cm Sephadex G-100 Column with 3M Urea as an Eluent

Closed circles; optical density at 280 nm.

Fig. 3. Effect of Spacer Protein on the Binding of Rabbit Anti-BLS Antiserum to BLS-Linked Amino-Dylark Cylinders

Enzyme activity of the bound Gal-labeled anti-rabbit IgG was plotted against the dilution of rabbit anti-BLS antiserum. BLS–GMBS–PSA– (open circles) and BLS– (closed circles) loaded Amino-Dylark cylinders were used as the solid-phase antigens.
was measured by using Gal-labeled anti-rabbit IgG antibody as the tracer. The anti-BLS activities remaining after the incubation are summarized in Table I. A marked reduction in the immuno-reactivity of anti-BLS was observed only in 6 M guanidine. Three solutions, 0.2 M glycine-HCl buffer, 4.5 M MgCl₂ and 0.1 M KCl-0.008 N HCl buffer, were chosen as candidates for the best eluent, based on the results of the stability test.

### Table I. Recovery of Immuno-Reactive Rabbit Anti-BLS Antibody after Incubation in Buffer Solutions at 25 °C for 3 h

<table>
<thead>
<tr>
<th>Solution</th>
<th>Recovery percent of anti-BLS activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (buffer C)</td>
<td>100.0 ± 8.1</td>
<td>11</td>
</tr>
<tr>
<td>0.2 M Glycine-HCl buffer (pH 2.3) (I)</td>
<td>97.2 ± 5.4</td>
<td>12</td>
</tr>
<tr>
<td>50% Ethylene glycol (II)</td>
<td>91.7 ± 10.6</td>
<td>12</td>
</tr>
<tr>
<td>10% Dioxane (III)</td>
<td>89.2 ± 3.7</td>
<td>13</td>
</tr>
<tr>
<td>6 M Guanidine (IV)</td>
<td>22.4 ± 3.7</td>
<td>14</td>
</tr>
<tr>
<td>4.5 M MgCl₂ (V)</td>
<td>99.8 ± 12.3</td>
<td>29</td>
</tr>
<tr>
<td>8 M Urea (VI)</td>
<td>87.0 ± 3.7</td>
<td>30</td>
</tr>
<tr>
<td>3.0 M KSCN (VII)</td>
<td>94.7 ± 8.1</td>
<td>31</td>
</tr>
<tr>
<td>0.1 M KCl-0.008 N HCl (pH 2.3) (VIII)</td>
<td>94.0 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>10% Dioxane-0.1 M KCl-0.008 N HCl (pH 2.3) (IX)</td>
<td>92.3 ± 12.3</td>
<td></td>
</tr>
<tr>
<td>0.3 M KCl-0.008 N HCl (pH 2.3) (X)</td>
<td>105.9 ± 11.9</td>
<td></td>
</tr>
</tbody>
</table>

An aliquot of 100-fold diluted anti-BLS antiserum in buffer C (BSA in buffer B was replaced by the corresponding amount of horse serum albumin) was further diluted 100 times with a test solution. After the 3 h incubation the test solution was diluted 100 times with buffer C. Anti-BLS activity in the test solution was measured in terms of binding to BLS-GMBS-PSA coupled Amino-Dylark cylinders using Gal-labeled goat anti-rabbit IgG as the indicator. <sup>a</sup> Mean ± S.D. (number of assays was 5).

Fig. 4. Elution Profiles of Anti-BSA Antiserum from a 1.5 × 5 cm BSA-Coupled Sepharose 4B Column

Each column was first eluted with 100 ml of 0.02 M Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl to remove non-specific IgG. Anti-BSA antibody was then eluted with three different eluents: A, elution profile with 0.2 M glycine-HCl buffer, pH 2.5; B, elution profile with 4.5 M MgCl₂, pH 7.0; C, elution profile with 0.1 M KCl-0.008 N HCl buffer, pH 2.3. The OD at 280 nm of each fraction was measured; open circles, the scale for OD values is shown on the left (0—20); closed circles, the scale for OD values is shown on the right (0—0.4).

was measured by using Gal-labeled anti-rabbit IgG antibody as the tracer. The anti-BLS activities remaining after the incubation are summarized in Table I. A marked reduction in the immuno-reactivity of anti-BLS was observed only in 6 M guanidine. Three solutions, 0.2 M glycine-HCl buffer, 4.5 M MgCl₂ and 0.1 M KCl-0.008 N HCl buffer, were chosen as candidates for the best eluent, based on the results of the stability test.

**Selection of the Eluent for Affinity Chromatography**

Anti-BSA antibody contained in anti-BLS–MBS–BSA antiserum was adsorbed on a BSA-coupled Sepharose 4B column. The eluting abilities of the above 3 eluents in affinity...
Improvement of Eluent for Affinity Chromatography

Anti-BSA antibody, adsorbed on the BSA-coupled Sepharose 4B column, was eluted with 0.008 N HCl Buffer, pH 2.3, Containing Either 0.1 M KCl (Open Circles) or a Gradient of 0.1 to 0.3 M KCl (Closed Circles) as the Second Eluent.

Open squares, optical density at 280 nm; open and closed circles, anti-BSA activities determined by EIA for anti-BSA antibody.

Fig. 5. Elution Profiles of Anti-BSA Antibody from a BSA-Coupled Sepharose 4B Column (1.5 x 5 cm) Using 0.008 N HCl Buffer, pH 2.3, Containing Either 0.1 M KCl (Open Circles) or a Gradient of 0.1 to 0.3 M KCl (Closed Circles) as the Second Eluent.

IgG fraction, separated from anti-BLS antiserum by the ammonium sulfate method, was applied to an affinity column. The column was first eluted with 100 ml of 0.02 M Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl and 0.1% BSA to remove non-specific and anti-BSA antibodies. BSA was then eluted with 0.05 M sodium phosphate-buffered saline, pH 7.0 (300 ml). Anti-BLS antibody was eluted with 0.3 M KCl-0.008 N HCl buffer, pH 2.3. The elution profile was measured in terms of OD at 280 nm (open circles), anti-BSA antibody activity (binding activity against the BSA-coupled Amino-Dylark cylinders; open triangles), sandwich EIA for rabbit IgG (open squares) and anti-BLS activity (binding activity against BLS-GMBS-PSA conjugate-coupled Amino-Dylark cylinders; closed circles) using Gal-labeled anti-rabbit IgG antibody as the common indicator.

Fig. 6. Elution Profile of Anti-BLS Antiserum from a BLS-GMBS-PSA-Coupled Sepharose 4B Column (1.5 x 5 cm)

The chromatography of anti-BSA antibody were compared. The elution profiles determined by the optical density measurement at 280 nm are shown in Fig. 4.

Improvement of Eluent for Affinity Chromatography

Anti-BSA antibody, adsorbed on the BSA-coupled Sepharose 4B column, was eluted
with a KCl concentration gradient from 0.1 to 0.3 M in 0.008 N HCl buffer, pH 2.3 (Fig. 5). The peak fraction of anti-BSA antibody was at 0.15 M KCl concentration. Consequently, 0.3 M KCl was deduced to be a sufficient concentration for the improved eluent, and a stability test in this solution was carried out (Table I).

Affinity Chromatography of Anti-BLS Antibody

An aliquot of the crude IgG, separated from anti-BLS antiserum by the ammonium sulfate method, was incubated overnight at 25 °C with buffer B which contained a large excess of BSA. The mixture was then applied to affinity column of the BLS-GMBS-PSA-coupled Sepharose 4B. The column was eluted with Tris–HCl buffered saline, pH 8.5, followed by elution with 0.3 M KCl–0.008 N HCl buffer, pH 2.3. The elution profile was measured by means of sandwich EIA for rabbit IgG.

Fig. 7. Elution Profile of the Purified Anti-BLS Antibody on the 2nd Affinity Chromatography Using the Same Affinity Column as in the 1st Purification of the Antibody

The elution conditions were slightly modified since the purified antibody did not contain BSA. The column was first eluted with 100 ml of 0.02 M Tris–HCl-buffered saline, pH 8.5, followed by elution with 0.3 M KCl–0.008 N HCl buffer, pH 2.3. The elution profile was measured by means of sandwich EIA for rabbit IgG.

Affinity Chromatography of Anti-BLS Antibody

An aliquot of the crude IgG, separated from anti-BLS antiserum by the ammonium sulfate method, was incubated overnight at 25 °C with buffer B which contained a large excess of BSA. The mixture was then applied to affinity column of the BLS–GMBS–PSA-coupled Sepharose 4B. The column was eluted with Tris–HCl buffered saline, pH 8.5, containing 0.1% BSA. Non-specific IgG and anti-BSA antibody bound to BSA were eluted under these conditions. BSA was washed with 0.05 M sodium phosphate-buffered saline, pH 7.0. The bound anti-BLS antibody was eluted with 0.3 M KCl–0.008 N HCl buffer, pH 2.3. Elution profiles in terms of the optical density at 280 nm, and the immune specificities against BSA and BLS-Gal are shown in Fig. 6.
Amounts of non-specific IgG and specific antibody to BLS were determined by sandwich EIA for rabbit IgG (Table II).

**Purity of Anti-BLS Antibody**

The purity of the isolated anti-BLS antibody was examined by an affinity chromatographic analysis. The purified anti-BLS antibody (100 µg) was chromatographed again on the same affinity column under the same conditions used for purification of the antibody. The elution profiles were followed by means of EIAs for rabbit IgG and BLS. Non-specific IgG was not detected by the sensitive EIA for rabbit IgG (less than 1 ng/ml; Fig. 7, Table II).

**Discussion**

Affinity purification of antibodies specific to a drug BLS and to the carrier protein BSA was examined in detail with use of a combination of three enzyme immunoassays. The effect of spacer protein on affinity chromatography of an anti-drug antibody was first studied. PSA was chosen as a protein able to bind many BLS molecules.

BLS–GMBS–PSA conjugate was prepared applying the method of Kitagawa et al.\(^{18-22}\) with the use of the heterobifunctional cross-linker, GMBS\(^{26}\) (Fig. 1). GMBS differs from MBS in the substitution of n-butyric acid for benzoic acid, to avoid cross-reaction of anti-BLS antibody with MBS, which was used for preparation of the BLS-immunogen.\(^{20}\) The BLS–GMBS–PSA conjugate gave a homogeneous band on sodium dodecyl sulfate electrophoresis and the number of BLS molecules coupled per PSA was calculated as 15 based on the molecular weight of the conjugate, determined by the electrophoresis (data not shown; \(\text{cf. references}\)\(^{20,21}\)).

The amino group of BLS was linked by glutaraldehyde directly or indirectly to amino groups of Amino-Dylark cylinders, with or without the use of the spacer. Gal-babeled anti-rabbit IgG antibody was used as the indicator. There was a large difference between the two preparations (Fig. 3), and BLS–GMBS–PSA conjugate was chosen for further study.

The best eluent for affinity chromatography of anti-BLS antibody was then investigated. The stability of the antibody was examined in 6 representative solutions, commonly used as eluents for affinity chromatography of specific antibodies. A period of 3 h was chosen for the test since most elutions for affinity chromatography would be finished within that time. Anti-BLS antibody was stable in these solutions except for 6 M guanidine (Table I).

The driving force of these solutions in eluting the specific antibody from its bound form would be a reversible change in molecular conformation of IgG. The antibody should revert its active form when the driving force is removed. High salt concentration and acidity of eluents should be effective for conformational change of the specific antibody, as judged from the constituents of the eluents shown in Table I: Eluates containing highly concentrated salts would require dialysis. For acidic eluents, pH adjustment should be enough to recover the native conformation of the eluted antibody.

Three eluents chosen on the base of stability results were then examined for ability to elute anti-BSA antibody bound to the BSA-coupled Sepharose 4B. The 4.5 M MgCl\(_2\) and 0.1 M KCl–0.008 N HCl buffers showed good eluting activities (Fig. 4). Improvement of the latter buffer was then attempted. When a KCl concentration gradient from 0.1–0.3 M in 0.008 N HCl solution was applied, the peak fraction of the specific antibody was observed at 0.15 M KCl. Thus, 0.3 M KCl–0.008 N HCl buffer was tested as the eluent and anti-BSA antibody was isolated effectively.

Anti-BLS–MBS–BSA antiserum contains anti-BSA antibody. Complete removal of the specific anti-BSA antibody from anti-BLS antibody is required during the affinity purification of anti-BLS antibody. Anti-BSA antibody in anti-BLS–MBS antiserum was reacted overnight.
with a large excess of BSA so that only anti-BLS antibody would be absorbed on the affinity ligand. BLS-GMBS-PSA-coupled Sepharose 4B. Under these conditions, most anti-BSA antibody was collected in the non-specific IgG fractions and very little anti-BSA antibody was eluted in the fractions obtained soon after those containing the non-specific IgG. After anti-BSA antibody had been eluted completely, the eluent was changed to phosphate buffered saline to wash out BSA completely (Fig. 6).

The bound anti-BLS antibody absorbed on the affinity column was then eluted with 0.3 M KCl-0.008 N HCl solution. No contamination by anti-BSA antibody in the isolated fraction was found in the binding test with the BSA-coupled Amino-Dylark cylinders. Amounts of anti-BLS were determined by sandwich EIA for rabbit IgG.27)

The purity of the isolated anti-BLS antibody was confirmed by the recovery test in rechromatography on the BLS-loaded affinity column; the antibody contained a negligible amount of non-specific IgG (Fig. 7 and Table II).

Very recently, we have developed a convenient new method for measuring the affinity constants of specific antibodies by the use of EIA.32) It was found that the binding of anti-BLS to BLS is among the strongest immuno-reactions, judging from its binding constant. The present purification method for specific antibody was successfully applied for several specific antibodies to antigens and haptens (unpublished data). A purified goat anti-rabbit IgG antibody, eluted from an affinity column with 0.3 M-0.008 N HCl buffer and then neutralized with 0.1 N NaOH (the specific IgG content was 1.5 mg/ml), could be stored for more than 1 year at −80 °C. Applications of the purified specific antibodies for enzyme immunoassay and for immunohistochemistry are under way.

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References and Notes


