Multiple Peak Formation of Avidin on an Iminobiotin Affinity Support

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Abstract

When pure avidin is chromatographed on a homemade iminobiotin column, two distinct peaks are observed using a gradient solvent system starting with a pH 9 buffer. Proteins in both peaks are shown to be identical by nondenatured polyacrylamide gel electrophoretic experiment. The weak interaction of the protein with the stationary phase at pH 9 could cause this unusual behavior.

Introduction

Although chemically pure proteins lead, in many cases, to a single peak in chromatographic separation, multiple peaks have been reported in a number of cases. For example, in the reversed-phase liquid chromatography (LC) of papain, two major peaks widely separated from one another in gradient elution were observed (1). In this study, it was noted that a later-eluting peak grew at the expense of the early-eluting peak when the column temperature was raised. Analysis of these two peaks showed that one peak contained papain in its active form, whereas the other did not. These results have been interpreted in terms of the irreversible denaturation of papain on the chromatographic phase. Because the denaturation seemed to occur at a slow rate, papain could be isolated rapidly in an active state under reversed-phase chromatographic conditions. In another study, it was reported that peptides containing proline residues may yield multiple peaks in reversed-phase LC if the proline is not at the *N*-terminus (2). The phenomena is caused by slow kinetic of *cis*-trans isomerization that are on the same time scale as the chromatographic separation. In this case, the multiple peaks are often not completely resolved, but rather they are bridged by a shoulder. Multiple peak formation in the reversed-phase chromatography of a recombinant human platelet-derived growth factor has also been attributed to the possible isomeric structures arising from the presence of pro–pro bonds within the primary structure of the protein (3). In this case, the relative areas of the peaks depend on the gradient time. There are also reports regarding the observation of multiple peaks in the reversed-phase chromatography of bovine serum albumin (BSA) (4). In this case, the multiple peaks are thought to be caused by the existence of oligomers of BSA. Such multiple peak formation has also been reported in the hydrophobic interaction chromatography of β-lactoglobulins (5,6). Multiple peak formation observed in these reversed-phase chromatographic experiments is often thought to be the results of secondary dynamic effects (7).

All the examples of multiple peak formation discussed above were observed in reversed-phase or its closely related hydrophobic-interaction chromatography. In our research on the development of affinity ligands for protein purification, two peaks were observed in the affinity chromatography of a pure avidin using a homemade iminobiotin column. Interestingly, by nondenatured [non-sodium dodecyl sulfate (SDS)] gel electrophoresis, the peaks are of identical proteins. The relative peak areas of these two peaks depend on the amount of samples injected. Our observation of this multiple peak formation in affinity chromatography is described in this paper.

Experimental

General supplies and equipment

Iminobiotin, avidin, and cyanogen bromide activated, 4% cross-linked beaded agarose were purchased from Sigma (St. Louis, MO). All other chemicals and solvents were purchased from Aldrich (Milwaukee, WI), Fluka (Ronkonkoma, NY), or Fisher Scientific (Pittsburgh, PA). An HR 5/5 empty glass column (5 × 50 mm) was purchased from Pharmacia (Piscataway, NJ). Chromatographic analyses were completed with a Beckman analytical gradient HPLC system (software: system Gold, Beckman Coulter, Fullerton, CA). Gel electrophoreses were accomplished using a Mini-Protean 3 system and ready gels from Bio-Rad (Hercules, CA). The gel images of electrophrograms were obtained with the Eagle Eye system from Stratagene (La Jolla, CA).

Synthesis of agarose–NH$(CH_2)_3$NHGlyGly–iminobiotin

The commercially available, cyanogen bromide activated, 4% cross-linked beaded agarose (500 mg) was washed first with 1mM HCl (5 × 4 mL) and then with a pH 8.5 buffer solution (0.1M $NaHCO_3$, 0.5M NaCl) (1 × 3 mL). To it, 1,3-diamino-

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propane (445 mg, 6.00 mmol) in the same pH 8.5 buffer solution (3 mL) was added. After stirring at room temperature for 2 h, the amino functionalized agarose beads were collected by filtration and washed with the pH 8.5 buffer solution and then with water.

The above resin (300 mg) was then added into a solution of Fmoc-Gly-OH (66 mg, 0.23 mmol), PyBOP (117 mg, 0.23 mmol), and *N,N*-diisopropylethylamine (39 mg, 0.3 mmol) in 5 mL of dimethylformamide. After shaking gently for 1 h, the resin was filtered and washed with DMF, methanol, and dichloromethane to yield agarose–$NH(CH_2)_3NHGlyFmoc$. The surface Gly loading for this resin was 0.25 mmol/g, as determined by the Fmoc cleavage method (8).

The Fmoc group was then removed by treatment with 25% piperidine in DMF for 20 min to yield agarose–$NH(CH_2)_3NHGlyNH_2$. The second Gly unit was then coupled to the resin following the same procedure as described previously for the coupling of the first Gly unit to yield agarose–$NH(CH_2)_3NHGlyGlyFmoc$ with a surface loading of 0.24 mmol/g. Removal of the Fmoc group with piperidine yielded agarose–$NH(CH_2)_3$–$NHGlyGlyNH_2$.

The resin was then added to a solution of iminobiotin (110 mg, 0.45 mmol) in water (5 mL) of which pH had been adjusted to 4–5 with 0.4M HBr. To the mixture, coupling reagent 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) (990 mg, 2.25 mmol) was added. The mixture was shaken gently for 5 h. After that, the desired stationary phase was collected by filtration and washed with a Bis-tris buffer (0.04M Bis-tris • HCl, 1M NaCl, pH = 6), methanol, water, and Bis-tris buffer again.

Gel electrophoresis analysis

Ten microliters of the protein sample was mixed with 20 mL of bromophenol blue sample buffer. The gel electrophoresis was run on a 12-well gradient (4–20%) non-SDS polyacrylamide gel electrophoresis (PAGE) tris-HCl-ready gel. Each well was loaded with 10 µL of the mentioned prepared solution. The running power was 200V, and the current was 100mA. For visualization, the gel was stained with Coomassie Blue R-250 solution for approximately 3 h.

Column packing and chromatographic measurements

The column was packed using a slurry method. The outlet tubing of the column was connected to a water aspirator pump. A slurry of 0.3 g stationary phase in 5 mL of tris-HCl buffer (0.05M, pH 9.0) was added to the column. An additional amount of buffer (5–10 mL) was pumped through the column with the aid of the water pump until the column bed volume no longer changed. The inlet cap was then placed, and the column was ready to be tested.

For chromatographic measurement, a flow rate of 1.0 mL/min was used. The elution process was monitored with a UV detector at 280 nm. The solvent gradient employed was: 0–10 min, 100% buffer A; 10–11 min, 100% buffer A to 100% buffer B; 11–31 min 100% buffer B; and 31–32 min, 100% buffer B to 100% buffer A. Buffer A was 0.04M Tris-HCl and 1M NaCl at pH 9. Buffer B was 0.04M Bis-tris-HCl and 1M NaCl at pH 6. The column size was 50 × 5-mm i.d.

Results and Discussion

The avidin-biotin complex is characterized by an extremely low dissociation constant. The tightness of this interaction has formed the basis for the use of the complex in several cytochemical techniques in membrane and molecular biology. Although the essentially covalent character of the avidin-biotin complex makes it an ideal choice for many studies, it severely restricts its use in affinity chromatography. For this reason, iminobiotin was developed as an alternative specific binder for avidin (9). Unlike biotin, iminobiotin binds avidin weaker, and its binding is also more sensitive to pH changes. Generally speaking, its binding strength with avidin increases with increasing pH values. Consequently, one could modulate the retention of avidin on an imino-biotin column by varying the pH value of the mobile phase. Avidin has a pI value of approximately 10, and its molecular weight is approximately 67 kd.

For a model study associated with our combinatorial library project, we prepared a stationary phase by immobilizing iminobiotin onto agarose and studied its chromatographic behavior towards avidin.

Synthesis of iminobiotin–agarose stationary phase

For stationary phase synthesis, the preactivated, commercially available agarose resin was first functionalized with an aminopropyl group by treatment with 1,3-diaminopropane (Figure 1). Subsequently, two Gly units were introduced as a linker group. The desired stationary phase was then synthesized by attaching iminobiotin to the solid support. The two Gly units were introduced to ensure hydrophilicity.

Syntheses on the solid resin were monitored by Fmoc cleavage reaction, as well as a ninhydrin test. For all those reactions involving free amino groups on the resin as the starting materials, negative ninhydrin tests were observed, which indicates the complete reaction of the amino group. According to the Fmoc cleavage reaction, the concentration of the first introduced Gly unit was 0.25 mmol/g. The concentration of the second Gly unit was 0.24 mmol/g, as determined by the same method. Adjusting for the weight increase of the resin, the coupling yield of the second Gly to the first Gly unit was approximately 98%. These results demonstrate the reliable, direct solid-phase synthesis of the ligand on this agarose resin.

Multipeak formation of chromatographic behavior of avidin

The stationary prepared previously was packed into a glass column and evaluated with a Beckman HPLC system. When pure avidin was chromatographed using a gradient solvent system

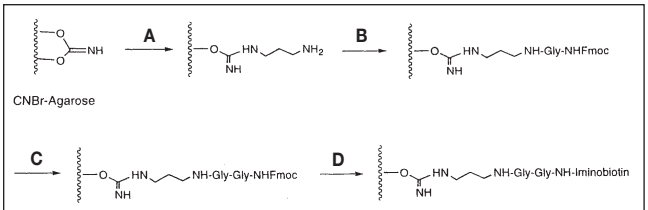

Figure 1. Preparation of the iminobiotin–agarose stationary phase. Conditions: (A) $NH_2(CH_2)_3NH_2$; (B) Fmoc-Gly-OH, PyBop; (C) (1) piperidine and (2) Fmoc-Gly-OH, PyBop; and (D) (1) piperidine, (2) iminobiotin, CMC.

starting with a pH 9 buffer, two distinct peaks were observed. The first fraction appeared shortly after injection, and the second fraction eluted after switching to a stronger eluting solvent (Figure 2). When the loading concentration was decreased, the later-eluting peak increased relative to the first eluting peak. The magnitude of the relative increase leveled off when the loading concentration became sufficiently low.

Both the first- and second-eluting peaks in Figure 2 arose from the same protein in the same form, as evidenced by chromatographic and gel electrophoretic experiments. In chromatographic experiments, when both peaks were reinjected into the column, they had similar chromatograms. More convincingly, in nondenatured (non-SDS) polyacrylamide gel electrophoretic experiments, both fractions had the same mobility (Figure 3). Therefore, it can be concluded that both fractions contain the same protein in the same form.

As suggested by one reviewer, a flow-through peak could be responsible for this multiple peak formation. Unlike examples cited in the introduction, protein denaturation is not involved in this case. Although avidin does contain proline residues, restricted cis-trans rotation of the proline bond is unlikely responsible for the observed phenomena, as multiple peak formation resulting from such restricted rotation should not be influenced by sample loading. We noticed that this multiple peak formation is somehow related to the binding strength of avidin to the stationary phase. Instead of starting the gradient system with a pH 9 buffer, a chromatographic experiment with a pH 11 starting buffer yielded only the later-eluting peak. As pointed out earlier, the binding strength of imino-biotin to avidin depends on the pH value. At higher pH, its binding strength with avidin is stronger. It seems that at pH 9, the binding of imino-biotin with avidin could be quite weak. This binding strength dependence is consistent with the reviewer's suggestion that the first peak could be a flow-through peak caused by unbound avidin. Supporting this suggestion is the chromatographic dependence on flowrate. If the flowrate was slowed from 1.0 to 0.5 mL/min, the first peak disappeared. With the slower flowrate, a flow-through peak is less likely, as the equilibrium between bound and unbound avidin can be maintained more readily.

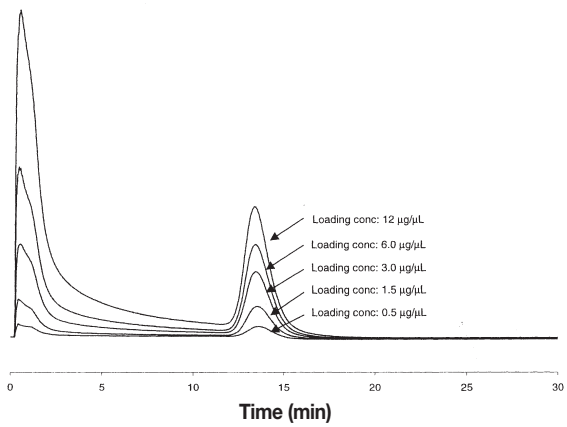

Figure 2. Multiple peak formation of avidin on the iminobiotin column. Mobile phase: 0–10 min, 100% buffer A; 10–11 min, 100% buffer A to 100% buffer B; and 11–30 min, 100% buffer B. See the Experimental section for details.

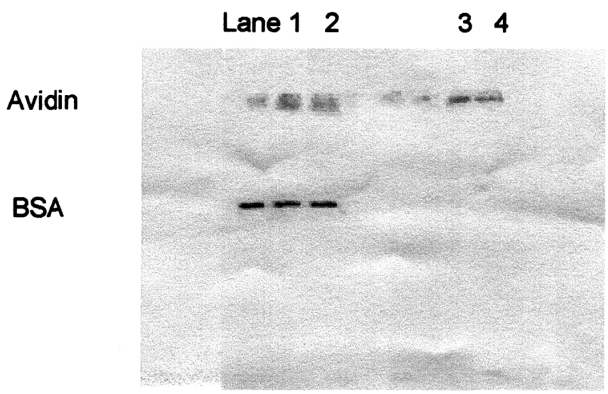

Figure 3. Non-SDS polyacrylamide gel electrophoresis of avidin. Lanes 1 and 2, mixture of avidin and BSA; lane 3, protein from first-eluting fraction; and lane 4, protein from second-eluting fraction.

Acknowledgments

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