Comparison of Cibacron Blue Affinity-Based Formats for Depletion of Albumin From Samples Prior to Two-Dimensional Gel Electrophoresis

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Abstract

Serum albumin is an abundant interfering constituent in many samples of animal origin to be analyzed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in proteomic studies. It constitutes 50–70% of serum and is present in high levels in many other biological fluids, including urine, bronchoalveolar lavage fluid, sputum, follicular fluid, cerebrospinal fluid, and culture supernatants containing fetal calf serum constituents. Furthermore, it is present over a wide pI and molecular weight range, reducing resolution and masking proteins of potential interest.

A number of protocols for depleting albumin have been reported. These protocols include: ion exchange, size exclusion (gel filtration), and reverse-phase chromatography; ammonium sulfate precipitation; and affinity-based methods using anti-BSA antibodies or Cibacron Blue dye. These methods allow researchers to load more total protein for 2-D PAGE, but all show limitations in capacity or specificity of albumin removal.

We investigated Cibacron Blue-mediated albumin removal to determine whether this approach can be improved and incorporated into a simple protocol for robust depletion of serum albumin from samples. Such a protocol would increase the resolution and representation of proteins of interest in 2-D PAGE profiles. Three methods were compared: two solely employing Cibacron Blue resin, and one employing a Cibacron Blue resin in combination with a protein A resin. This latter mixture comes in the Aurum™ serum protein mini kit, which offers simultaneous removal of albumin and IgG. Analyses are presented and results compared for albumin removal, 2-D PAGE resolution, representation of low-abundance proteins, and nonspecific binding.

Methods

Accuset liquid calibrator, a human serum preparation (Sigma-Aldrich, Inc.), was used in all experiments. Albumin concentration was 44 µg/µl; total protein was 60 µg/µl.

Albumin Reduction

Batch binding — Accuset liquid calibrator (10 µl) and 30 µl 25 mM phosphate buffer (pH 7.0) were added to 30 µl Cibacron Blue 3G-A agarose (Sigma-Aldrich, Inc). The mixture was incubated for 3 hr at room temperature with intermittent vortexing. After incubation, the mixture was centrifuged for 3 min at 9,000 x g, the supernatant was collected, and 30 µl 25 mM phosphate buffer (pH = 7.0) was added to the pellet. After vortexing and centrifugation, the supernatant was collected and combined with the first supernatant. This unbound fraction (100 µg protein) was analyzed by 2-D PAGE.

Gel-loader mini column — A gel-loader pipet tip was packed with 30 µl Cibacron Blue 3G-A dye, and the mini column was washed with 30 µl 25 mM phosphate buffer (pH 7.0). Serum was then loaded on the column, and the eluate was collected. The column was again washed, and this fraction was collected and combined with the first eluate to form the unbound fraction. The total eluate (30 µg protein) was analyzed by 2-D PAGE.

Aurum serum protein mini kit — The Aurum serum protein mini column (200 µl Affi-Gel® Blue and Affi-Gel protein A) was washed with 25 mM phosphate buffer (pH 7.0). Affi-Gel Blue is a crosslinked agarose bead with covalently attached Cibacron Blue F3G-A dye. Affi-Gel protein A is a crosslinked agarose bead with covalently attached protein A. The serum sample (50 µl) was loaded on the column and incubated for 30 min, with intermittent vortexing. The column was centrifuged for 3 min at 9,000 x g, and the eluate was collected. The column was then washed with one column volume of 25 mM phosphate buffer (pH 7.0), vortexed, and centrifuged. The eluate was collected and combined with the first eluate to form the unbound fraction. Protein determination was carried out by amino acid analysis. Total protein (30 µg) from the unbound fraction was analyzed by 2-D PAGE.

Sample Preparation

Serum samples treated with the three albumin-reduction protocols were solubilized in 5 M urea, 2 M thiourea, 2 mM TBP, 65 mM DTT, 2% (w/v) CHAPS, 2% (w/v) sulfobetaine 3-10, 40 mM Tris, and 1% carrier ampholytes. Samples were ultrasonicated for 3 min and centrifuged at 15,000 x g for 5 min. They were then loaded by in-gel rehydration on pH 4–7 and pH 5–8 IPG strips.

2-D PAGE

IPG strips were equilibrated in 6 M urea, 2% (w/v) SDS, 375 mM Tris-HCl (pH 8.8), 20% (v/v) glycerol, 5 mM TBP, and 2.5% (w/v) acrylamide. First-dimension isoelectric focusing was carried out for 40,000 V-hr.

Strips were embedded on Criterion™ 10–20% Tris-HCl precast SDS-polyacrylamide gels using 0.5% agarose in cathode buffer. Second-dimension electrophoresis was carried out at 4ºC at 10 mA/gel for 1 hr, then 20 mA/gel for 3 hr. Gels were fixed in 10% (v/v) methanol: 7% (v/v) acetic acid. Proteins were stained overnight using SYPRO Ruby protein gel stain (Molecular Probes, Inc.). Gels were destained in 10% (v/v) methanol: 7% (v/v) acetic acid for 1 hr, imaged with a Molecular Imager FX™ system, and analyzed.

Mass Spectrometry

Spots of interest were excised from the gel and subjected to an in-gel, 16 hr tryptic digest at 37ºC. The resulting peptides were extracted from the gel with 10% (v/v) acetonitrile, 1% (v/v) TFA, and cleaned up and concentrated with a ZipTip C18 tip (Millipore Corp.) A 1 µl aliquot of sample and matrix (α-cyano-4-hydroxycinnamic acid) was spotted on a sample plate and allowed to air-dry. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was carried out with a TofSpec 2E time-of-flight mass spectrometer (Waters Corp.). A nitrogen laser (337 nm) was used to irradiate the sample. The spectra were acquired in reflection mode across the mass range 600–3,500 Da. A near-point calibration was applied with a mass accuracy of ~100 ppm or less. ProteinLynx software (Waters Corp.) was used for automated database searching against *Homo sapiens*. For manual database searches, the peptide masses were searched against *H. sapiens* using Mascot (http://www.matrixscience.com) and PeptIdent (http://www.expasy.ch/tools/peptident.html) software packages.

Results

Reduced Serum Albumin

All three methods significantly reduced serum albumin (Table 1 and Figure 1). Figure 1A shows the gel results for the neat serum control. With the batch-binding method, a significant amount of albumin remained in the sample (Figure 1B); 45% of albumin was removed, but 6% of nonalbumin proteins also lost compared to the serum control.

Table 1. Comparison of spot numbers and percent albumin depletion for the different Cibacron Blue albumin depletion methods.

Test Sample	Spot Number	% Change	% Albumin Depletion
Control	670	N/A	N/A
Batch binding	620	–6%	45%
Gel loader columns	650	–3%	36%
Aurum serum protein mini kit	700	+4%	>80%

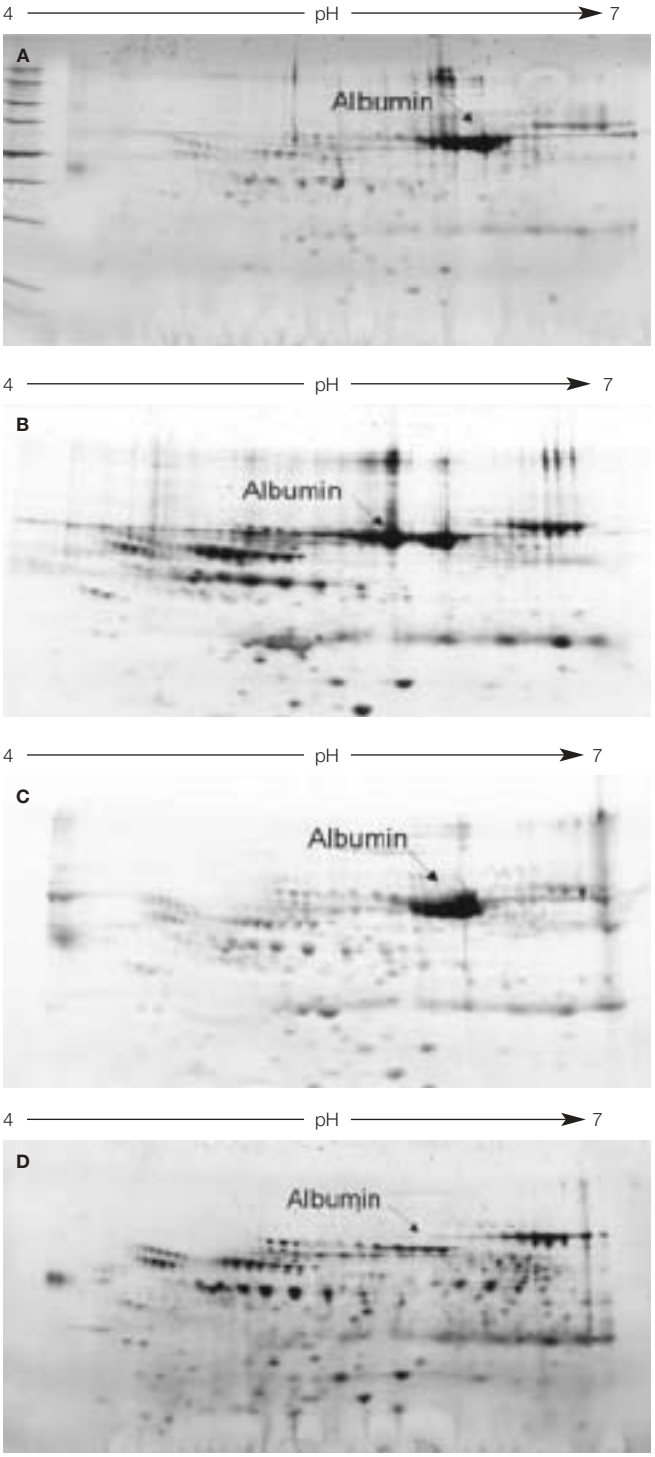

Fig. 1. Comparison of methods used to remove serum albumin. A, neat serum control; B, batch-binding method; C, gel-loader mini column; D, Aurum serum protein mini kit. Protein samples (30 µg, except for batch-binding method, which used 100 µg) were separated by 2-D PAGE across a pH 4–7 gradient as described in Methods.

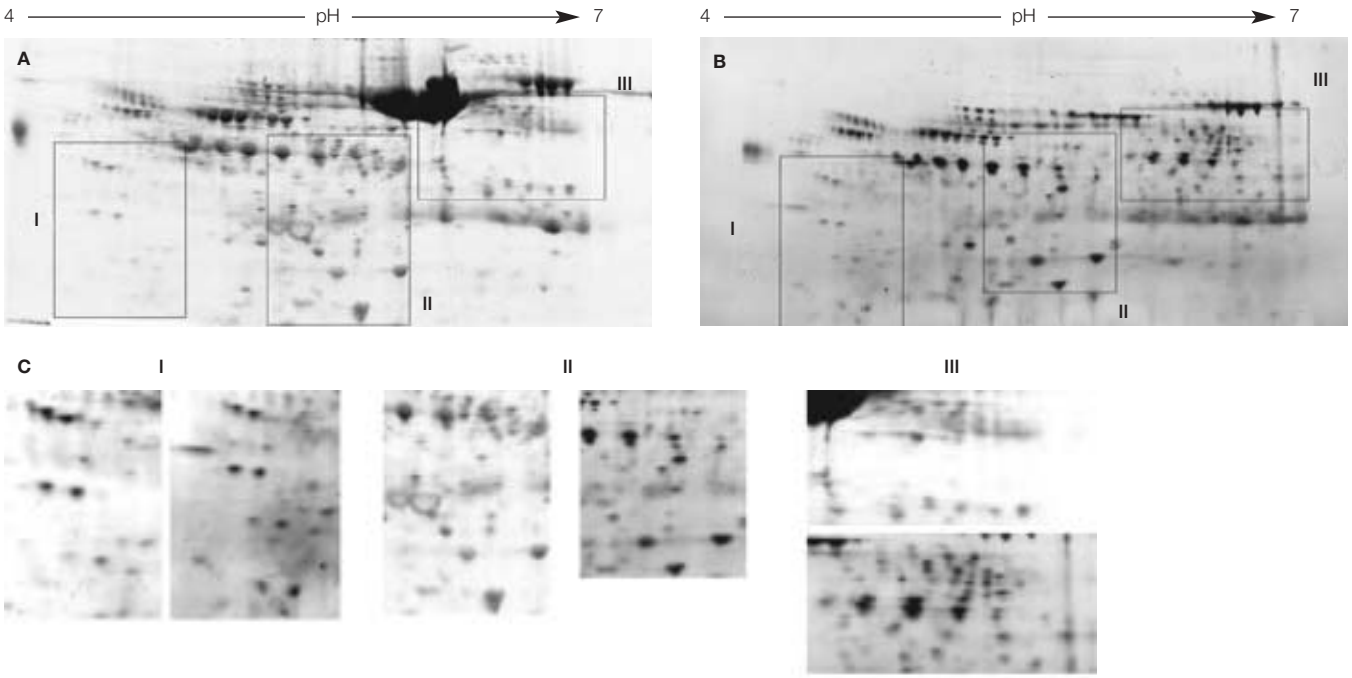

Fig. 2. Serum albumin depletion with the Aurum serum protein mini kit. A, neat serum control (600 µg protein); B, after treatment with the Aurum kit (30 µg protein). Samples were separated by 2-D PAGE across a pH 4–7 gradient. C, Enlarged regions highlight increased spot number and spot intensity after treatment. Panel I shows similar protein patterns between the samples with several new or higher-intensity proteins in the region after treatment. Panel II shows similar patterns between the samples. Panel III shows the most significant changes on the 2-D gel. A number of proteins were visualized only in the clean sample.

The gel-loader mini column method removed some albumin and increased resolution of low-abundance proteins as well as proteins with a similar molecular weight to albumin; 36% of albumin was removed from the sample, but spot number decreased by 3% (Table 1). Furthermore, a significant albumin smear was still present on the gel (Figure 1C).

The Aurum serum protein mini kit removed IgG and most of the albumin; more than 80% was removed from the sample, and spot number increased by 4%. Albumin was reduced to tiny spots on the 2-D gel (Figure 1D), allowing better detection of surrounding protein spots.

Increased Concentration of Low-Abundance Serum Proteins

The initial aim of this work was to increase resolution of spots and improve visualization of low-abundance proteins isolated from serum and resolved by 2-D PAGE. The Aurum serum protein mini kit reduced serum albumin by more than 80%. Figure 2A shows the results for untreated serum separated by 2-D PAGE. Figure 2B shows serum cleaned up with the Aurum serum protein mini kit. Figure 2C compares specific, identical areas from Figures 2A and 2B. The albumin-depleted serum resulted in better visualization of low-abundance proteins because albumin interference was virtually eliminated. The reduction of albumin from the serum samples allowed an increase in the complexity of the overall protein profile and better spot resolution on 2-D gels. An increase in the protein load of neat serum does not allow the complete representation of lower-abundance proteins on the gel. Preparative loads of the albumin-depleted samples would allow lower-abundance proteins to be visualized in sufficient quantity using current detection limits of protein stains.

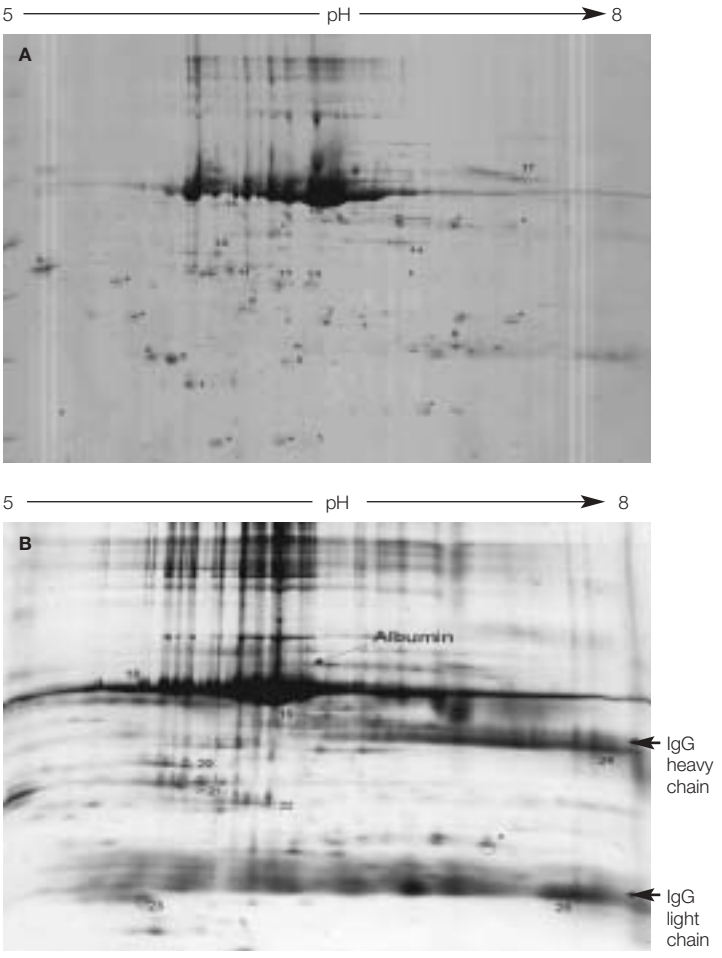

Fig. 3. Comparison of bound fractions obtained by two serum depletion methods. A, batch-binding method; B, Aurum serum protein mini kit. Samples were separated by 2-D PAGE across a pH 5–8 gradient.

Table 2. Bound-fraction analysis of serum treated with batch binding or the Aurum serum protein mini kit.

Spot No.	Entry	Peptides Matched	Coverage (%)	MW	pI	Description
1	ALBU HUMAN	8	11.49	69,387	6.2	Serum albumin
2	AAA51747	11	41.77	28,962	5.6	Apolipoprotein A1 protein (fragment)
3	AAA51747	12	41.37	28,962	5.6	Apolipoprotein A1 protein (fragment)
4	gil4557385	6	10.0	48,020	4.95	Complement component 3 precursor (fragment)
5	ALBU HUMAN	6	9.0	71,807	6.2	Serum albumin
6	APE HUMAN	9	30.6	36,154	5.7	Apolipoprotein E precursor
7	ALBU HUMAN	11	26.5	69,367	6.2	Serum albumin
8	ALBU HUMAN	7	11.4	69,367	6.2	Serum albumin
9	ALBU HUMAN	8	15.27	69,367	6.2	Serum albumin
10	ALBU HUMAN	11	19.87	69,367	6.2	Serum albumin
11	ALBU HUMAN	10	14.94	69,367	6.2	Serum albumin
12	ALBU HUMAN	5	9.0	71,807	6.2	Serum albumin
13	ALBU HUMAN	5	9.0	71,807	6.2	Serum albumin
14	ALBU HUMAN	9	15.6	69,367	6.2	Serum albumin
15	ALBU HUMAN	9	14.78	69,367	6.2	Serum albumin
16	ALBU HUMAN	20	39.24	69,367	6.2	Serum albumin
17	AAK77664	5	10.74	77,050	7.2	Transferrin
18	ALBU HUMAN	10	21.02	69,367	6.2	Serum albumin
19	ALBU HUMAN	13	23.81	69,367	6.2	Serum albumin
20	ALBU HUMAN	7	10.51	69,367	6.2	Serum albumin
21	ALBU HUMAN	8	18.37	69,367	6.2	Serum albumin
22	ALBU HUMAN	6	12.5	71,807	6.2	Serum albumin
23	APA1 HUMAN	3	12.73	30,778	5.7	Apolipoprotein A-1 (precursor)
24	GC2 HUMAN	4	18.1	35,888	7.66	IgG γ-2 chain C region, *H. sapiens* (human)
25	KAC HUMAN	3	48.1	11,600	5.58	IgG κ-2 chain C region, *H. sapiens* (human)

Bound-Fraction Analysis

Bound fractions were analyzed by 2-D PAGE and MALDI-TOF MS for nonspecific binding of serum proteins. Figures 3A and 3B show the bound fractions of serum treated with batch binding or the Aurum serum protein mini kit, respectively. The bound fraction from the batch-binding method (Cibacron Blue resin) contained mainly albumin or albumin fragments, while the bound fraction from the Aurum serum protein column (Cibacron Blue and protein A resins) contained IgG and IgG fragments along with albumin. The other major bound proteins were identified as apolipoprotein A1, apolipoprotein E precursor, and transferrin (Table 2).

Discussion and Conclusion

Serum samples have been used in numerous proteomic studies, but high levels of high-abundance proteins such as albumin and IgG interfere with the separation and representation of low-abundance proteins. Cibacron Blue-based methods were tested to evaluate their ability to remove serum albumin. All methods increased resolution and improved detection of low-abundance proteins. The Aurum serum protein mini kit was the most effective method, as it removed both albumin and IgG, and did the best job at leaving other proteins intact. The major proteins in the bound fractions were identified as albumin, IgG, and their degradation products. The other major proteins that bound to the resin were apolipoprotein A1, apolipoprotein E, and transferrin.

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