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An approach to remove albumin for the proteomic analysis of low abundance biomarkers in human serum

Proteomic technologies are being used to discover and identify disease-associated biomarkers. The application of these technologies in the search for potential diagnostic/prognostic biomarkers in the serum of patients has been limited by the presence of highly abundant albumin and immunoglobulins that constitute approximately 60–97% of the total serum proteins. The purpose of the study was to evaluate whether treatment of human serum with Affi-Gel Blue alone or in combination with Protein A (Aurum serum protein mini kit, Bio-Rad) before two-dimensional gel electrophoresis (2-DE) analysis removed high abundance proteins to allow the visualization of low abundant proteins. Serum samples were treated with either Affi-Gel Blue or Aurum kit and then subjected to 2-DE using 11 cm, pH 4–7 isoelectric focussing strips for the first dimension and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for second dimension. Protein spots were visualized using a fluorescent protein dye (SYPRO Ruby, Bio-Rad). Comparison between treatment methods showed significant removal of albumin by both Affi-Gel Blue and Aurum kit and considerable differences in the protein profile of the gels after each treatment. Direct comparison between treatments revealed twenty-eight protein spots unique to Affi-Gel Blue while only two spots were unique after Aurum kit treatment. Unique spots in Affi-Gel Blue and Aurum kit treated serum were not visualized in untreated serum. Sixteen hours of Affi-Gel Blue treatment resulted in enhanced visualization of fifty-three protein spots by two-fold, thirty-one by five-fold, twelve by ten-fold and six by twenty-fold. In parallel after Aurum kit treatment two-, five-, ten- and twenty-fold enhancements of thirty, thirteen, eight and five protein spots, respectively, were observed. The pattern of increased visualization of protein spots with both treatment methods was similar. In conclusion, treatment of serum samples with Affi-Gel Blue or Aurum kit before 2-DE analysis can be used to remove high abundance proteins in order to increase the detection sensitivity of proteins present in low abundance.

Keywords: Affi-Gel Blue / Albumin / Aurum kit / Biomarkers / Immunoglobulin / Two-dimensional gel electrophoresis
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1 Introduction

The introduction of new technologies for the detection of specific biomarkers in the serum of patients will have an important impact on public health. This need is particularly urgent in cancer and other related diseases where early diagnosis dramatically improves patient outcome [1].

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Abbreviations: BPB, bromophenol blue; **PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Serum proteins are useful diagnostic tools and alteration of the expression of some serum proteins is an early sign of an altered physiology and may be indicative of disease [2]. In routine diagnostic laboratories identification of specific low abundant disease-associated proteins in serum relies heavily on time consuming and expensive radiolabelled or enzyme-linked immunoassay methods (RIA or ELISA) that only have the ability to evaluate a single protein component at a time. Due to the heterogenous nature of most physiological disorders there is a common belief that no single marker is likely to be sufficiently predictive [3] and a few studies have emphasized the need for more than one candidate biomarker to enhance already available diagnostic/prognostic tests [4]. The necessity to develop a panel of multiple diagnostic/prognostic mark-

ers can be met by utilizing proteomic approaches to serum specimens that have the capacity to profile multiple biomarkers [5]. The application of current proteomic technologies is limited by the presence of high abundance 'common housekeeping' proteins like albumin and immunoglobulins that constitute approximately 60–97% of the total serum protein [6]. Such proteins hinder the detection of hundreds of low abundance proteins, some of which might potentially be relevant to a particular disease-state. If proteomic technologies are to be used routinely for diagnostic purposes, a rapid, inexpensive and a simple method is required to remove the high abundant proteins. The aim of the study was to determine whether treatment of human serum by Affi-Gel Blue or Aurum kit could be used to efficiently remove albumin prior to proteomic analysis.

2 Materials and methods

This study was approved by the Royal Women's Hospital, Melbourne, Research and Human Ethics Committee (Human Ethics Committee #98/28). Human blood was collected from healthy volunteers (n = 6) at the Royal Women's Hospital, Melbourne, after the provision of a participant information statement and with informed consent. Whole blood (10 mL) was collected by venepuncture into plain collection tubes for serum (blood was allowed to clot at room temperature for 30 min). Samples were centrifuged at $2000 \times g$ for 10 min after which serum was collected. An aliquot (100 μL) was removed for the determination of total protein. Serum was stored at −80°C until analyzed.

2.1 Protein assay

Total protein content was determined using a commercial protein assay kit with BSA standards according to the manufacturer's instruction (Pierce, Rockford, IL, USA).

2.2 Serum sample processing for the removal of high abundance proteins

2.2.1 Affi-Gel Blue treatment

Affi-Gel Blue (Bio-Rad, Hercules, CA, USA) is a cross-linked agarose bead with covalently attached Cibacron Blue F3GA dye. The attached dye functions as ionic, hydrophobic, aromatic, or sterically active binding sites for protein. Affi-Gel Blue has high affinity for albumin and is suited for albumin removal. Serum samples were thawed at room temperature and incubated with five volumes of Affi-Gel Blue® and incubated for 1 h or 16 h at 4°C on a rotary platform. Samples were centrifuged at $2000 \times g$ for 10 min. The supernatants were recovered and aliquots (100 μL) were removed for the determination of total protein after correcting for dilution factors.

2.2.2 Aurum kit treatment

Human serum samples were treated with the components of the Aurum serum protein mini kit (Bio-Rad). This kit utilizes spin columns containing a mixture of Affi-Gel Blue and Affi-Gel Protein A to selectively bind and remove albumin and immunoglobulin. The Aurum matrix (Bio-Rad) in a Micro Bio-Spin Column was washed twice with 1 mL of binding buffer (20 mM phosphate buffer, pH 7.0) by centrifugation for 20 s at $1000 \times g$. Sixty μL of serum was added to 180 μL of binding buffer and mixed by vortexing. Two hundred μL was added to the Aurum matrix. Following incubation at room temperature for 15 min, 1 h, 5 h or 16 h, the column was centrifuged for 20 s at $1000 \times g$ to collect the eluate. The column was washed with 200 μL of binding buffer and combined with the first eluate to form the depleted serum sample. The total protein concentration of the combined eluate was determined after taking into account the dilution factors. The eluate was stored at −80°C until further analysis.

2.3 Two-dimensional electrophoresis

2.3.1 First dimension separation

Fifty μL of neat serum was diluted in sample preparation buffer (62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue (BPB) and 2.3% DTT) was incubated at 95°C for 5 min. Fifteen μg of treated serum protein or diluted neat serum protein were mixed with IEF solvent (7 M urea, 2 M thiourea, 100 mM DTT, 4% CHAPS, 0.5% carrier ampholytes pH 4–7, 0.01% BPB and 40 mM Tris) to a final volume of 200 μL and incubated for 1 h at room temperature. This mixture was then applied to a Ready Strip® (11 cm, pH 4–7; Bio-Rad) and actively rehydrated at 50 V at 20°C for 16 h. Serum proteins were isoelectrically focused at 250 V for 15 min and then 8000 V for 150 min and then maintained at 8000 V for a total of 35 000 Vh/gel (*i.e.,* a total of 42 000 Vh *per* gel). Ready Strips were then stored at −80°C until second dimension processing.

2.3.2 Second dimension separation

Ready Strips from the first dimension separation were equilibrated in 6 mL of equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.01%

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BPB, 2 mM tributyl phosphine (TBP)). Strips were rinsed in Tris glycine SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS pH 8.3) and then applied to the top of a 10% Tris-HCl precast Criterion gel (Bio-Rad). Low melting point agarose (0.5% in running buffer containing BPB) was layered on top of the strip. Gels were electrophoresed at 10 mA/gel for 1 h, 20 mA/gel for 2 h and then 30 mA/gel for 30 min. Gels were then fixed in methanol/acetic acid (40%/10% in distilled H_2O) for 1 h at room temperature and then incubated in SYPRO Ruby® (Bio-Rad) for 16 h at room temperature on a rocking platform. Gels were destained for 1 h in methanol/acetic acid (10%/7% in distilled H_2O), imaged using a Bio-Rad FX imager at 100 nm resolution and analyzed using PDQuest V6. The computer program identified protein spots from the digitalized images of the gel. Each serum sample was repeated three times and variability between the experiments was assessed on three different gels.

3 Results

The serum protein yield in untreated and Affi-Gel Blue or Aurum kit treated specimens are summarized in Table 1. Both the treatment methods removed 96–98% of total serum protein in 16 h, but with equal protein loading (~15 μg), there was no significant change in the total number of detectable protein spots by 2-DE analysis (Table 1). A SYPRO Ruby stained 2-DE gel of serum samples revealed a typical 2-DE serum profile [7] (Fig. 1a). The albumin smear at around 68 kDa was present in untreated but was absent in 16 h Affi-Gel Blue and Aurum kit treated serum samples (Figs. 1a, b and c). Concomitant to the removal of albumin there was a significant enhancement in the staining intensity of several protein spots (Table 2 and Figs. 2a and b). Affi-Gel Blue treatment resulted in the enhancement of 53 protein spots by two-fold, 31 by five-fold, 12 by ten-fold and 6 by twenty-fold (Table 2 and Fig. 2a).

Table 1. Serum protein concentration and the number of protein spots obtained before and after treatment with Affi-Gel Blue or Aurum kit

Treatment	Total protein (mg/ml)			Total number of spots		
	Gel 1	Gel 2	Gel 3	Gel 1	Gel 2	Gel 3
Untreated	58.9	60.6	70.9	163	174	190
Mean		63.46 ± 3.8			175 ± 7	
Affi-Gel Blue	2.05	2.26	2.7	210	222	232
Mean		2.34 ± 0.33			220 ± 5	
Aurum kit	0.8	0.83	1.26	142	151	160
Mean		0.96 ± 0.26			151 ± 5	

Values are mean ± SEM of three different gels run on three different days.

Table 2. Increased protein spot counts in Affi-Gel Blue treated serum compared to untreated serum

Spots increased	Number of spot counts
Two-fold	53 ± 6
Five-fold	31 ± 5
Ten-fold	12 ± 3
Twenty-fold	6 ± 1

Values are mean ± SEM of six different serum samples.

In parallel, 16 h Aurum kit treatment resulted in 2, 5, 10 and 20-fold enhancement of 30, 13, 8 and 5 protein spots respectively (Table 3 and Fig. 2b). The pattern of enhanced visualization of protein spots with both treatment methods was similar with more protein spots being detected by Affi-Gel Blue treatment. Further analysis revealed that compared to untreated serum, 28 protein spots were unique to Affi-Gel Blue treatment and were not visualized in untreated serum (Fig. 3a). In contrast, only two protein spots were unique to Aurum kit treatment consistent with the loss of more protein with this treatment method (Fig. 3b). These results suggest that Affi-Gel Blue or Aurum kit treatment of human serum results in the removal of high abundant albumin, thereby not only increasing the detection of low abundance proteins but also allowing the detection of some unique proteins, which in the presence of albumin would have remained obscured.

Table 3. Increased protein spot counts in Aurum kit treated serum as compared to untreated serum

Spots increased	Number of spot counts
Two-fold	30 ± 6
Five-fold	13 ± 4
Ten-fold	8 ± 4
Twenty-fold	5 ± 2

Values are mean ± SEM of six different serum samples.

To determine if the removal of albumin by Affi-Gel Blue or Aurum kit with increasing time affects concomitant removal of protein spots other than albumin, a time course study was performed. Human serum sample was treated with Affi-Gel Blue for 1 and 16 h and with Aurum kit for 15 min, 1 h, 5 h and 16 h respectively. Fifteen μg

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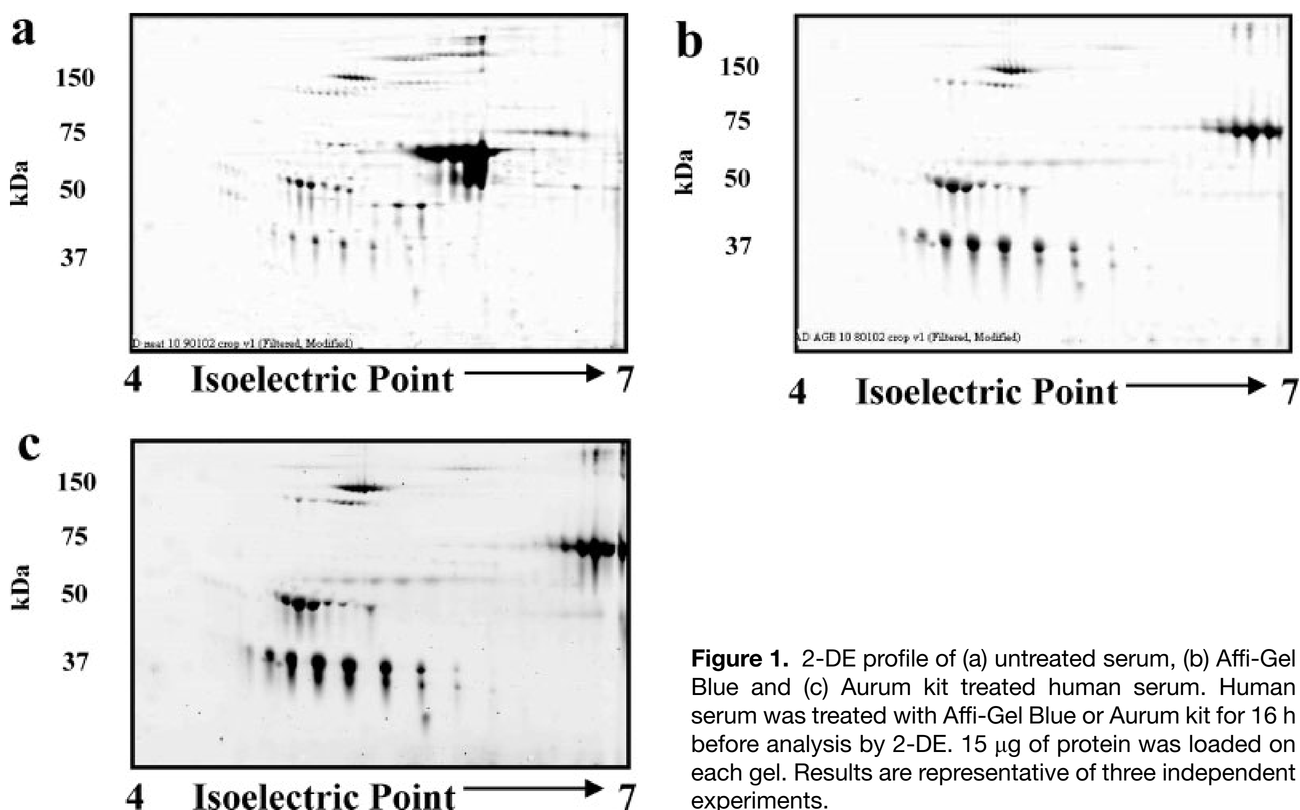

Figure 1. 2-DE profile of (a) untreated serum, (b) Affi-Gel Blue and (c) Aurum kit treated human serum. Human serum was treated with Affi-Gel Blue or Aurum kit for 16 h before analysis by 2-DE. 15 μg of protein was loaded on each gel. Results are representative of three independent experiments.

Table 4. Time course of serum protein concentration and number of protein spots following Aurum kit treatment

Treatment	Protein concentration (mg/mL)	Reduction in protein concentration (%)	Number of spots
Untreated	62.3	0	174
15 min	1.45	98	365
1 h	1.02	99	337
5 h	1.02	99	287
16 h	0.92	99	221

Table 5. Time course of serum protein concentration and number of protein spots following Affi-Gel Blue treatment

Treatment	Protein concentration (mg/mL)	Reduction in protein concentration (%)	Number of spots
Untreated	62.3	0	174
1 h	2.65	96	330
16 h	2.20	96	220

of these samples was analyzed after resolving by 2-DE. Within 15 min of Aurum kit treatment there was 98% loss of total serum protein with accompanying decrease in albumin staining and two-fold increase in the number of protein spots (Table 4, Fig. 4). Within 1 h of Affi-Gel Blue or Aurum kit treatment, significant loss of albumin was achieved with no significant loss of protein profile or number of protein spots (Tables 4 and 5, Figs. 4a and 4b). However, 16 h treatment of Affi-Gel Blue or Aurum kit resulted in the loss of albumin with approximately 35% loss of total number of protein spots compared to 1 h treatment (Table 4 and Fig. 4). These observations suggest that even though 16 h exposure of human serum to Affi-Gel Blue or Aurum kit can result in significant loss of albumin and consequent enhancement of several low abundance proteins, it is also associated with nonspecific removal of serum protein other than albumin.

Individual variations in the protein profile of the same serum samples prepared on three different days and repeated three times were investigated to eliminate confounding factors that may arise from sample handling. No substantial variation in the profile of protein spots of the same sample repeated on different days was detected (Table 1).

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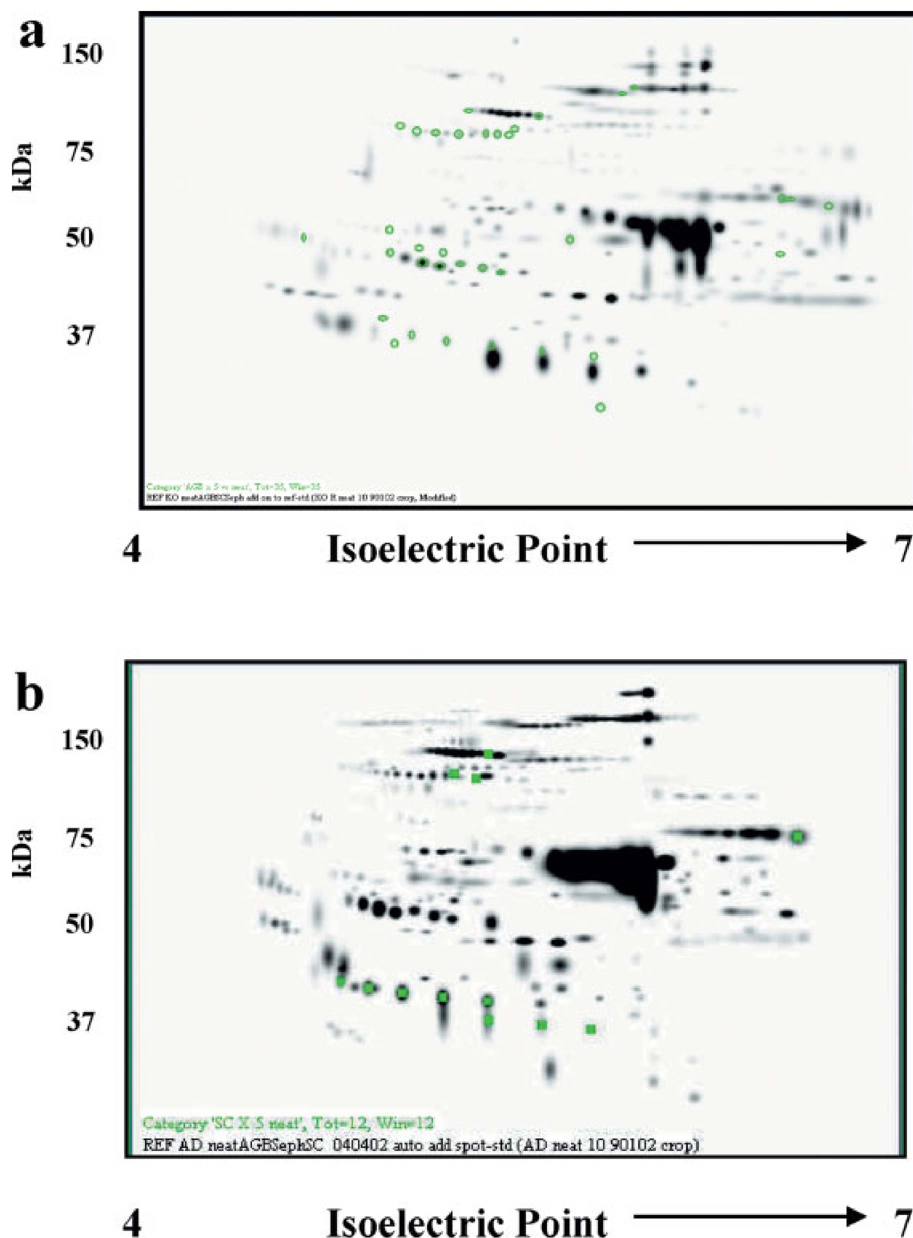

Figure 2. Depiction of the reference profile of all protein spots identified by 2-DE. (a) Protein spots enhanced by five-fold after Affi-Gel Blue treatment (in green); and (b) enhanced five-fold after Aurum kit treatment (in green).

4 Discussion

Proteomic approaches have been utilized by different laboratories to discover and identify novel proteins that can be used as potential diagnostic/prognostic biomarkers [8]. The technology has been limited by the presence of high abundance proteins such as albumin and immunoglobulin that can mask or considerably decrease the sensitivity of detection of several low abundant potential biomarkers. Moreover, the widely spread pattern of albumin and immunoglobulin in the 2-DE gel can also obscure proteins with a similar p*I* and molecular weight. Theoretically, by removing albumin and immunoglobulin, which together constitute 60–97% of the total serum protein, three- to five-fold more protein can be analyzed. The aim of the study was to determine if the removal of albumin and immunoglobulin would improve the pattern of serum protein profile by increasing the resolution of low abundant serum proteins. Our results demonstrate that Affi-Gel Blue and Aurum kit treatment results in the removal of highly abundant albumin and simultaneous enhancement in the detection of several other proteins.

Within 16 h both treatment methods removed 96–98% of total protein content of the serum, but there were no significant differences in the total number of protein spots

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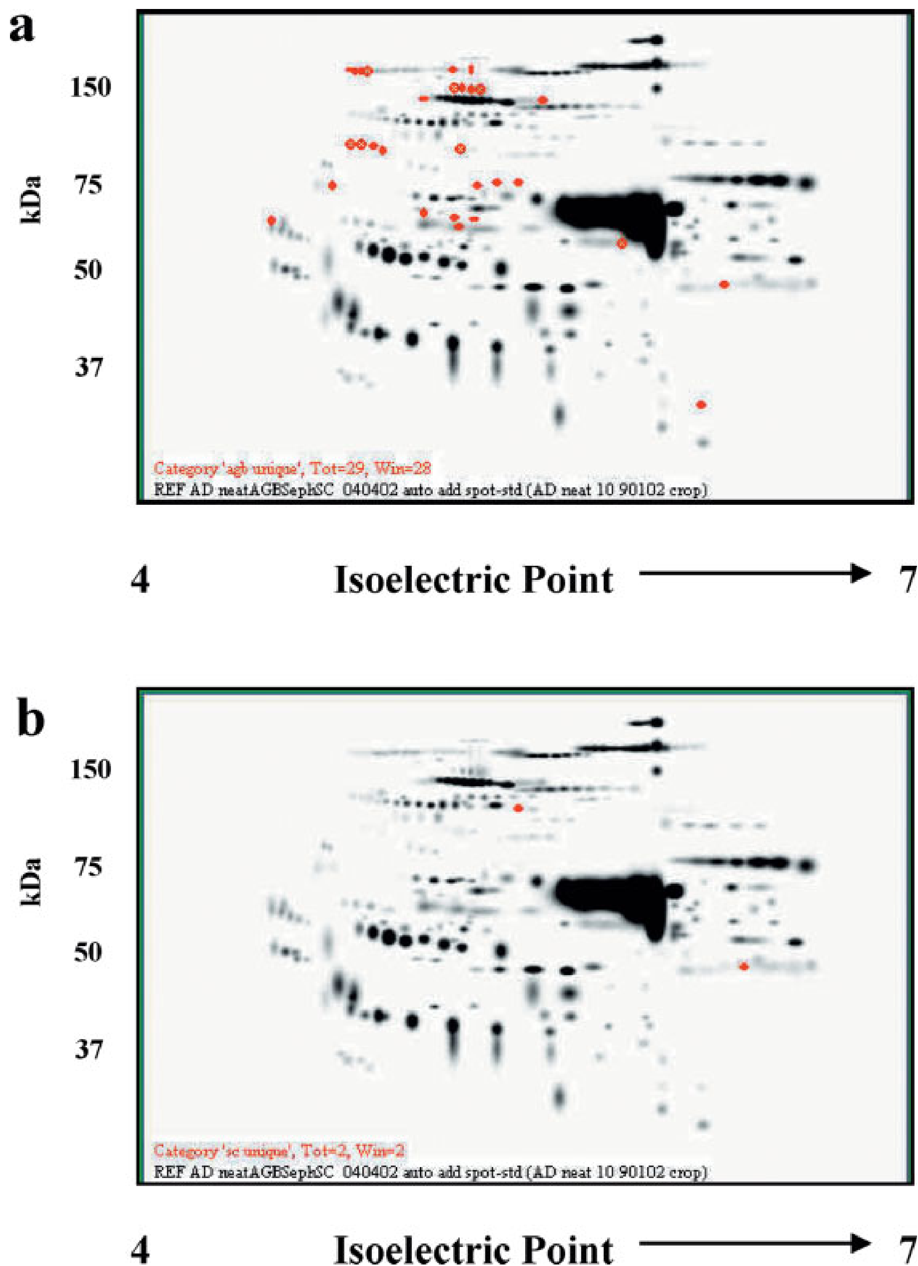

Figure 3. Depiction of the reference profile of all protein spots identified by 2-DE. (a) Profile of proteins unique to Affi-Gel Blue treatment compared to untreated serum (in red), (b) profile of proteins unique to Aurum kit treatment compared to untreated serum (in red). Results are representative of three independent experiments.

analyzed after 2-DE analysis. We have also shown that Affi-Gel Blue and Aurum kit treatment removes high abundant albumin and enhances the staining intensity of different spots by several-fold. In addition, with equal protein loading, 28 and 2 unique spots were detected by Affi-Gel Blue and Aurum kit treatment compared to neat serum. These spots remained obscured in the neat untreated serum samples. Aurum kit treatment results in a greater depletion of protein as its Protein A component also removes immunoglobulins. The serum pattern of IgG (heavy chain) is apparent over a p*I* range of 6.5–8.3. As this range falls in the borderline of the p*I* range used in this study, less defined pattern of IgG heavy chain was evident on the gels.

The 16 h treatment of Affi-Gel Blue and Aurum kit resulted in the removal of proteins other than albumin. Affi-Gel Blue and Aurum kit bind albumin with high affinity but other proteins can also bind to the planar ring structure of Cibracon Blue 3G dye through a complex combination of electrostatic, hydrophobic and hydrogen bonding interactions. Hence, nonspecific removal of protein other than

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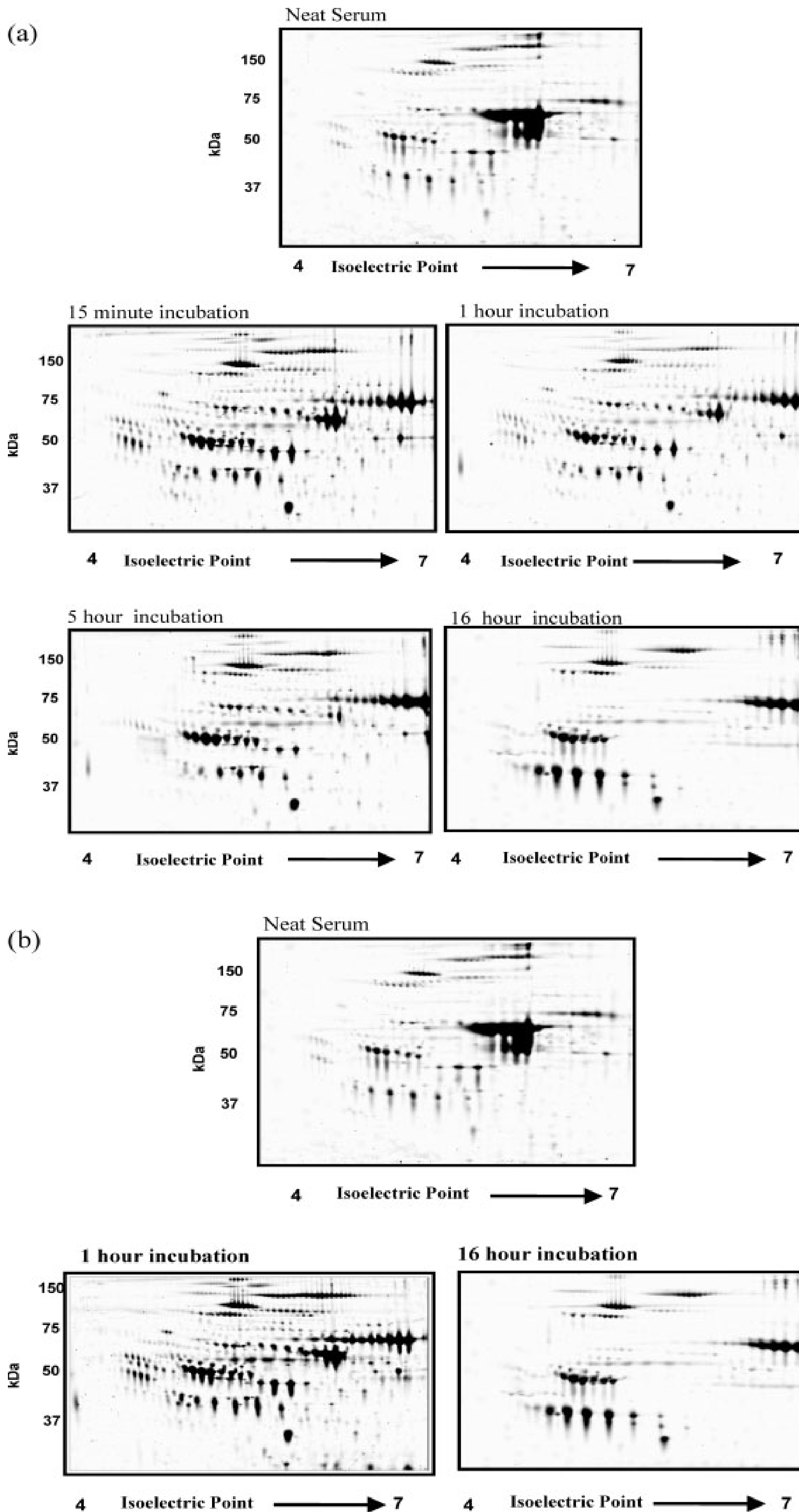

Figure 4. Time dependent removal of albumin after treatment of human serum with Affi-Gel Blue or Aurum kit. (a) Serum sample was treated with Aurum kit for 0 min (untreated serum), 15 min, 1 h, 5 h and 16 h; (b) serum sample was treated with Affi-Gel Blue for 0 min (untreated serum), 1 h and 16 h. 15 μg of protein was loaded on each gel.

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albumin after 16 h of treatment with Affi-Gel Blue or Aurum kit is not surprising and has been reported previously [7]. However, by using these protein stripping techniques for only 1 h, significant removal of albumin can be achieved with minimal nonspecific protein removal. While both Affi-Gel Blue and Aurum kit are effective reagents for the removal of highly abundant albumin, greater sensitivity in protein profiling without any significant loss of potential serum biomarker can be achieved only if the samples are exposed to the reagents for a period of 1 h.

5 Concluding remarks

This approach can generate a pattern of protein profiles independent of the identity of individual proteins and can be used as a discriminator of a particular disease-state. Our data suggests that by using such technique for sample processing before 2-DE analysis one can increase the likelihood of discovery of novel biomarkers of high sensitivity and specificity.

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