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Journal of Chromatography B, 803 (2004) 337–343
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Concanavalin A chromatography coupled to two-dimensional gel electrophoresis improves protein expression studies of the serum proteome

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Received 8 August 2003; received in revised form 23 December 2003; accepted 12 January 2004

Abstract

In the present study, we show a simple method to analyse human serum proteins using Concanavalin A (Con A) chromatography coupled to two-dimensional gel electrophoresis. Serum samples were separated into two fractions, one mainly containing non-glycosylated and *O*-glycosylated proteins and the other enriched in *N*-glycosylated proteins. Both fractions were subjected to two-dimensional gel electrophoresis, and the obtained maps were analysed. The method presented here improves the resolution of the serum proteome, increasing the number of visualized spots over two times and allowing the detection of proteins with lower abundance in serum. We have proved the feasibility of the method comparing the *N*-glycoprotein fraction of serum from donors and colorectal cancer (CRC) patients.

Keywords: Glycosylation; Concanavalin A; Albumin

1. Introduction

One of the Human Proteome Organization's (HUPO) highest priorities is to research into the human blood plasma/serum proteome, because of its outstanding potential for diagnostics. Unfortunately, plasma contains the most proteins of any human proteome version. The complete plasma proteome contains not only the protein forms that function in plasma—bearing in mind splicing and other modifications—but also forms released from tissues, clonal forms of immunoglobulins, aberrant secretions from disease states, temporary plasma passengers such as lysosomes, and foreign products from viruses and other sources [1].

An interesting approach to the analysis of altered protein expression is the basic proteomic technique of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), combined with the use of computer-assisted programmes and mass spectrometric analysis [2–4]. Most two-dimensional gel-based proteomic studies employ a 'one-extract–one-gel' approach, and thus the majority of proteins identified in these studies are high-abundance proteins [5]. In the case of plasma or serum, low-abundance proteins are rarely seen on traditional two-dimensional maps, because of the overwhelming quantities of abundant soluble proteins such as albumin, which represents more than 50% of the total protein content in serum [6,7]. However, the identification of the low-copy-number gene products may be more decisive in, for example, discovering novel markers or drug targets. As other authors have previously described [5,8], to improve the visualization of low-abundance proteins in serum, further fractions should be made prior to 2D-PAGE.

Here, we have used the lectin Concanavalin A (Con A) from *Canavalia ensiformis*, which can recognise and bind Glcα1→, Manα1→ and GlcNAcα1→ residues occurring at the non-reducing termini of sugar chains, and also interacts with →2Glcα1→ and →2Manα1→ residues located in internal positions of a carbohydrated chain. It has been demonstrated that this is an effective method to fractionate *N*-glycoproteins. Con A binds proteins with complex

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doi:10.1016/j.jchromb.2004.01.019

and high-mannose-type oligosaccharide chains, obtaining a fraction enriched in *N*-glycoproteins and free of albumin and other serum proteins mainly non-glycosylated or *O*-glycosylated [9]. Thus, the use of this chromatography coupled to 2D-PAGE allows the separation of the whole proteome into two subproteomes on the basis of the glycosylation pattern, increasing the concentration of proteins in the sample, and therefore, meaning a significant improvement in the resolution of serum proteins.

2. Experimental

2.1. Chemicals and reagents

Con A-Sepharose 4B was purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Analytical grade of sodium di-hydrogen phosphate-2-hydrate, di-sodium hydrogen phosphate anhydrous (PANREAC Quimica, Barcelona, Spain), and methyl-α-D-mannopyranoside (Sigma) were used as reagents. ReadyStrip™ IPG Strips (4%T; 3%C) were purchased from Bio-Rad (Hercules, California, USA). PhastGel™ Blue R-350 (Coomassie brilliant blue) was obtained from Amersham Biosciences (Uppsala, Sweden). Silver nitrate was purchased from Sigma.

Lysis buffer was prepared with 7 M urea, 2 M thiourea and 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Rehydration buffer contained 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.3% (w/v) dithiothreitol (DTT) and 0.5% (v/v) Bio-Lytes 3/10 ampholytes. SDS-PAGE equilibration buffer was prepared with 6 M urea, 50 mM Tris pH 8.8, 2% (w/v) SDS, and 30% (v/v) glycerol, and afterwards 1% (w/v) DTT and 2.5% (w/v) iodoacetamide (IAA) were added. Thiourea was purchased from Sigma, IAA was obtained from Merck (Schuchardt, Germany), and the other chemicals were purchased from Bio-Rad.

2.2. Equipment

Chromatographic separation was carried out on an Econo Column from Bio-Rad. Fractions were collected with a Microfraction Collector 203 from Gilson (Middleton, Wisconsin, USA), and optical density measured in an UVIKON Spectrophotometer 930 from Kontron Instruments (Milan, Italy). Samples were lyophilised in a Christ Alpha 2-4 freeze drier and re-suspended in a Sanyo Gallenkamp orbital shaker incubator, both purchased from B. Braun Biotech (Leicester, UK). The Protean IEF Cell for isoelectric focusing (IEF), Mini Protean II and Protean II xi Cell for electrophoresis, the power supply Power PAC 1000, and the PDQuest software package were all purchased from Bio-Rad.

2.3. Sample preparation

Eight blood samples (four male and four female) from healthy donors, provided by the Galician Transfusion Centre, and five blood samples from colorectal cancer (CRC) patients, provided by the Hospital Xeral-Cíes of Vigo, were allowed to coagulate at room temperature and centrifuged at 2000 × *g* for 15 min. The resultant sera were stored at −85 °C until used.

2.4. Concanavalin A chromatography

To obtain the chromatographic profiles, 1 ml of filtered serum was applied to a Con A-Sepharose column (0.8 cm × 7 cm) and equilibrated in 10 mM sodium/di-sodium phosphate buffer, pH 6.0. Flow was stated at 0.3 ml/min. After applying the serum, the column was first washed with 30 ml of equilibrating buffer, releasing fractions enriched in non-glycosylated and *O*-glycosylated proteins. Then the column was washed with 0.5 M methyl-α-D-mannopyranoside in equilibrating buffer, eluting fractions constituted mainly by *N*-glycoproteins. Optical density at 280 nm was measured along the chromatographic process. Fractions obtained after chromatography were dialysed against Milli-Q water, lyophilised, and then stored at −85 °C until gel electrophoresis was performed.

2.5. One-dimensional electrophoresis

For one-dimensional gel electrophoresis studies, 20 μg of protein from total serum and from the chromatographic fractions were re-suspended in sample buffer and resolved by SDS-PAGE in 12% polyacrylamide (30%T; 2.6%C) denaturing minigels, according to Laemmli [10]. Gels were stained with 0.1% PhastGel™ Blue R-350 in 40% methanol and 10% acetic acid for 1 h. For destaining, 40% methanol and 10% acetic acid (3 × 1 h) were used. Gels were left in Milli-Q water overnight, digitised and analysed.

2.6. Two-dimensional electrophoresis

For 2D-PAGE, lyophilised total serum and chromatographic fractions were solubilized by suspension in lysis buffer for 3 h at 30 °C with vigorous shaking. For isoelectric focusing, 150 μg of protein sample were mixed with rehydration buffer up to 350 μl. This mixture was used to rehydrate 17-cm, pH 4–7 linear ReadyStrip™ IPG Strips (4%T; 3%C) for 12 h at 20 °C, with a constant voltage (50 V) applied across the gel strips, which were placed in the Protean IEF Cell focusing tray. The rehydrated gels were electrophoresed at 250 V for 15 min, subjected to a linear voltage ramp from 250 to 10,000 V for 5 h, and then focused until 60,000 Vh. The temperature was maintained at 20 °C [11]. The IPG strips were then incubated in SDS-PAGE equilibration buffer with 1% (w/v) DTT with gentle shaking. After 15 min, the procedure was repeated with SDS-PAGE equilibration buffer with 2.5% (w/v) IAA for 15 min [12]. After equilibration, the IPG gel was transferred onto a 9–16% gradient polyacrylamide (30%T; 2.6%C) gel [13] and SDS-PAGE was performed in

a Protean II xi Cell at a constant temperature of 15 °C [14], 20 mA per gel for 15 min, and then 40 mA per gel until the bromophenol blue marker had reached the bottom of the gel. For computer analysis of 2D-PAGE patterns, ammoniacal silver-stained gels (modified from [15]) were digitised and protein patterns were analysed with the PDQuest 7.1.1 software package.

The reproducibility of the method presented here has been tested measuring the relative volume of the spots detected in samples from different subjects in experiments done in different days. A correlation coefficient between experiments of 0.92 has been obtained. Similar results of reproducibility have been described for 2D-PAGE by other authors [16–18].

2.7. Protein concentration measurement

Protein concentration was measured by Biuret reaction [19] for total serum, according to Bradford [20] for chromatographic fractions, and with a modification of this method [21] for IEF.

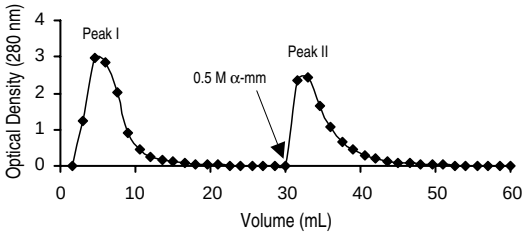
2.8. Protein identification

Gel spots corresponding to proteins differentially expressed were excised from Coomassie-stained gels and destained with 50 mM ammonium bicarbonate and 50% (v/v) acetonitrile. Then, proteins were dried and digested with 10 μg/ml trypsin in 25 mM ammonium bicarbonate at 37 °C overnight. Peptides were eluted with 5% (v/v) trifluoroacetic acid and 75% (v/v) acetonitrile. Finally, 0.7 μl of the samples were mixed with 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile, and analysed on a MALDI-time-of-flight (TOF) M@LDI-HT™ mass spectrometer (Micromass). Data processing was performed with MASSLYNX (Micromass), and database searching to identify the proteins of interest from their peptide fingerprint was done with MASCOT Daemon search engine (Matrix Science Ltd., London, UK) against NCBInr and Swiss-Prot databases.

3. Results and discussion

Serum samples were processed by affinity chromatography through the lectin Con A. A representative example of the chromatographic profile of serum is shown in Fig. 1A, in which Peak I represents the first fraction of eluted proteins, enriched in non-glycosylated proteins and *O*-glycoproteins (hereinafter Fraction I), while Peak II represents the fraction enriched in *N*-glycoproteins (Fraction II). The procedure was repeated with serum samples from eight donors and five CRC patients, obtaining similar chromatographic profiles in all the experiments. Fig. 1B shows the amount of protein measured in total serum from donors and patients, before the Con A chromatography, and in Fractions I and II after the separation. There was almost no loss of protein during the chromatography and results were consistent among the experiments.

(A)



(B)

		Serum	Peak I	Peak II
Healthy donors	N = 8			
	Median (mg)	93.20	80.35	14.82
	Mean ± SD (mg)	94.90 ± 6.20	73.62 ± 14.63	15.22 ± 6.24
	Percentage (%)	100	77.6	16.0
CRC patients	N = 5			
	Median (mg)	75.09	52.54	14.93
	Mean ± SD (mg)	76.96 ± 8.01	49.90 ± 13.77	16.54 ± 6.47
	Percentage (%)	100	64.8	21.5

Fig. 1. (A) Representative profile of a Con A chromatography of serum. One millilitre of human serum was applied to a 5.5 ml column of Con A-Sepharose. Peak I: proteins mainly non-glycosylated and *O*-glycosylated. Peak II: fraction enriched in *N*-glycoproteins. (B) Median, mean ± standard deviation and percentage of the protein quantity measured in eight samples from healthy donors and five from colorectal cancer patients, and in Peaks I and II after Con A chromatography. α-mm: methyl α-D-mannopyranoside.

Serum albumin, as well as other predominantly non-glycosylated or *O*-glycosylated proteins, does not bind to the column matrix, eluting in Fraction I. To verify this separation, 20 μg of protein fractions from a donor serum, corresponding to each peak, were resolved by one-dimensional gel electrophoresis (Fig. 2). Total serum was electrophoresed in Lane 1, where there is an intense band about 67 kDa, corresponding to serum albumin. This protein is also visible in Fraction I (Lane 2) representing

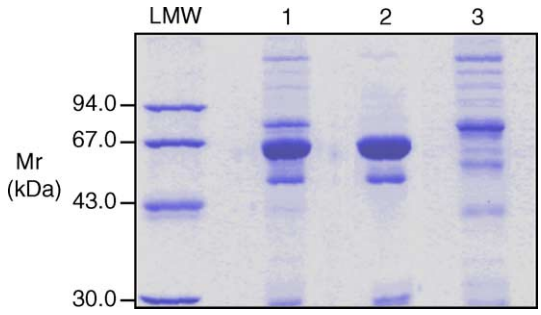


Fig. 2. One-dimensional SDS-PAGE of a donor total serum (Lane 1), chromatographic Fraction I (Lane 2) and Fraction II (Lane 3). Twenty micrograms of protein were electrophoresed at 90 V (4%T stacking gel) until the samples entered the 12% polyacrylamide gel, and then at 200 V until the bromophenol blue marker reached the bottom of the gel. LMW: low molecular weight markers.

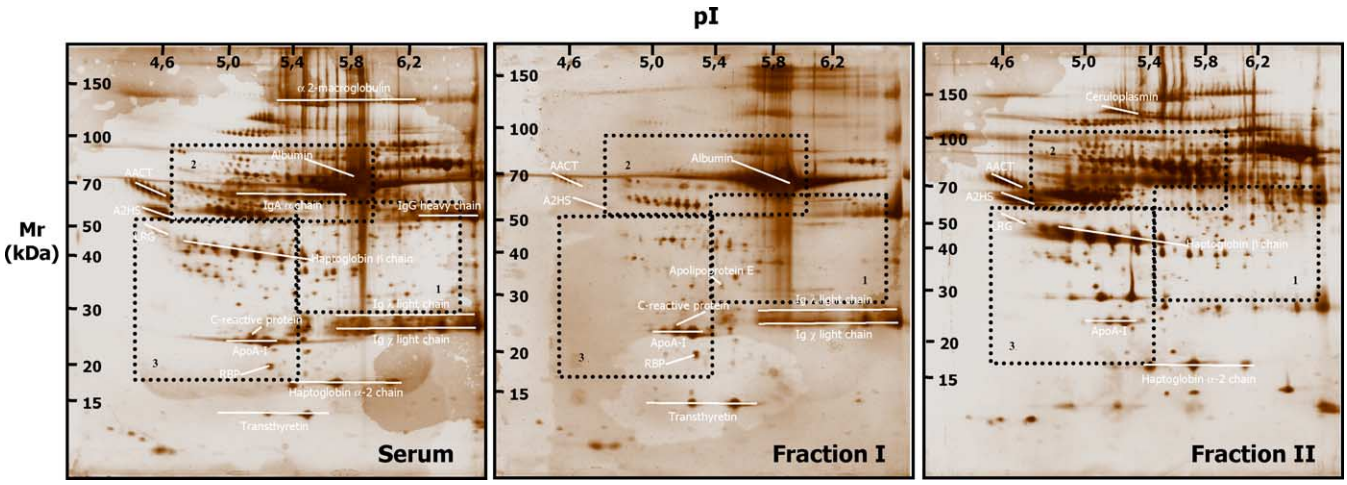


Fig. 3. Representative silver-stained 2D-PAGE of total serum and Con A chromatographic Fractions I (mainly non-glycoproteins and *O*-glycoproteins) and II (enriched in *N*-glycoproteins). In these 2D-gels, we detected 1376 spots in serum, 841 in Fraction I and 1185 in Fraction II. Some major proteins are highlighted in all the three maps. Boxes 1, 2 and 3 are enlarged in Fig. 4. Mr: relative molecular mass; p*I*: isoelectric point; AACT: α-1-antichymotrypsin; A2HS: α-2-HS-glycoprotein; LRG: leucine-rich α-2-glycoprotein; Ig: immunoglobulin; ApoA-I: apolipoprotein A-I; RPB: plasma retinol-binding protein.

more than 50% of the sample, but not in Fraction II (Lane 3). Thus, apparently, all the albumin has been eluted in the chromatographic Fraction I. Moreover, in Lane 3 removal of albumin allows the visualization of a number of bands that were not seen in Lane 1, either by a mass effect or by overlapping. Furthermore, other two bands of about 25–30 and 55–60 kDa, also visible in total serum and in Fraction I, are hardly visualized in Fraction II. These molecular masses correspond with those observed for immunoglobulin light and some heavy chains [22].

Fig. 3 shows two-dimensional electrophoretic maps of proteins corresponding to total serum and chromatographic Fractions I and II from a donor. Using the serum map as a standard, the spot pattern in the three maps was analysed, counting 1376 spots in serum, 841 in the first fraction and 1185 in the second one. Broadly, considering the sum of the spots found in both chromatographic fractions in relation to the spots found in the serum, we achieved an increase of over 45% in the number of proteins resolved by 2D-PAGE. As in one-dimensional electrophoresis, serum albumin appears in the serum and chromatographic Fraction I maps, but it is not detected by silver-staining in the map of Fraction II.

On the other hand, some major proteins have been highlighted in all three maps. Their glycosylation pattern has been confirmed by data searching in the Swiss-Prot protein knowledgebase [23]. The number in brackets after each protein corresponds to its primary accession number in this database. In the serum gel we have marked some non-glycosylated proteins, such as albumin (P02768) and C-reactive protein (P02741), which are also present in the Fraction I gel. In it, we have located other well-known non-glycosylated proteins, such as the transthyretin (P02766) and the plasma retinol-binding protein (P02753); and mostly *O*-linked glycoproteins, such as apolipoprotein E (P02649), which presents one *O*-linked oligosaccharide. The third map, containing predominantly *N*-linked glycoproteins, features some spots such as ceruloplasmin (P00450), which contains four *N*-linked oligosaccharides, and therefore, is retained by the Con A matrix; haptoglobin (haptoglobin-1: P00737; haptoglobin-2: P00738), which contains four *N*-linked oligosaccharides in the β chain, the α chain appearing also in the map because the denaturation is performed after the chromatography; and leucine-rich α-2-glycoprotein, that despite having one *O*-linked oligosaccharide is retained by the Con A because of other four *N*-linked chains.

Nevertheless, the separation of some spots is not so clear as in the previous examples. For instance, α-2-HS-glycoprotein or fetuin-A (P02765) has three *O*-glycosylation and two *N*-glycosylation sites, these being sufficient for binding to the lectin and eluting with the second fraction; however, some spots can also be faintly visualized in the Fraction I gel, maybe due to a conformational eclipse of the *N*-oligosaccharide residues interacting with the column matrix. Alpha-1-antichymotrypsin (AACT) (P01011) is another exception. Its sequence contains two *N*-linked oligosaccharides, the majority of the protein appearing in Fraction II as expected. However, some spots are visible in its predicted focusing point in the Fraction I gel, raising the possibility of their being different proteins masked by the AACT in 2D-maps of total serum, or forms with aberrant glycosylation. Finally, other remarkable issue is the apolipoprotein A-I (P02647), which is thought to be non-glycosylated but appears both in Fraction I and in Fraction II gels. Therefore, the separation achieved through the Con A chromatography is consistent with most of the available data about the glycosylation pattern of serum proteins, although further studies could provide more information on certain particular spots as the three mentioned.

Areas with the highest improvement in resolution have been marked in Fig. 3 and enlarged in Fig. 4 for comparison.

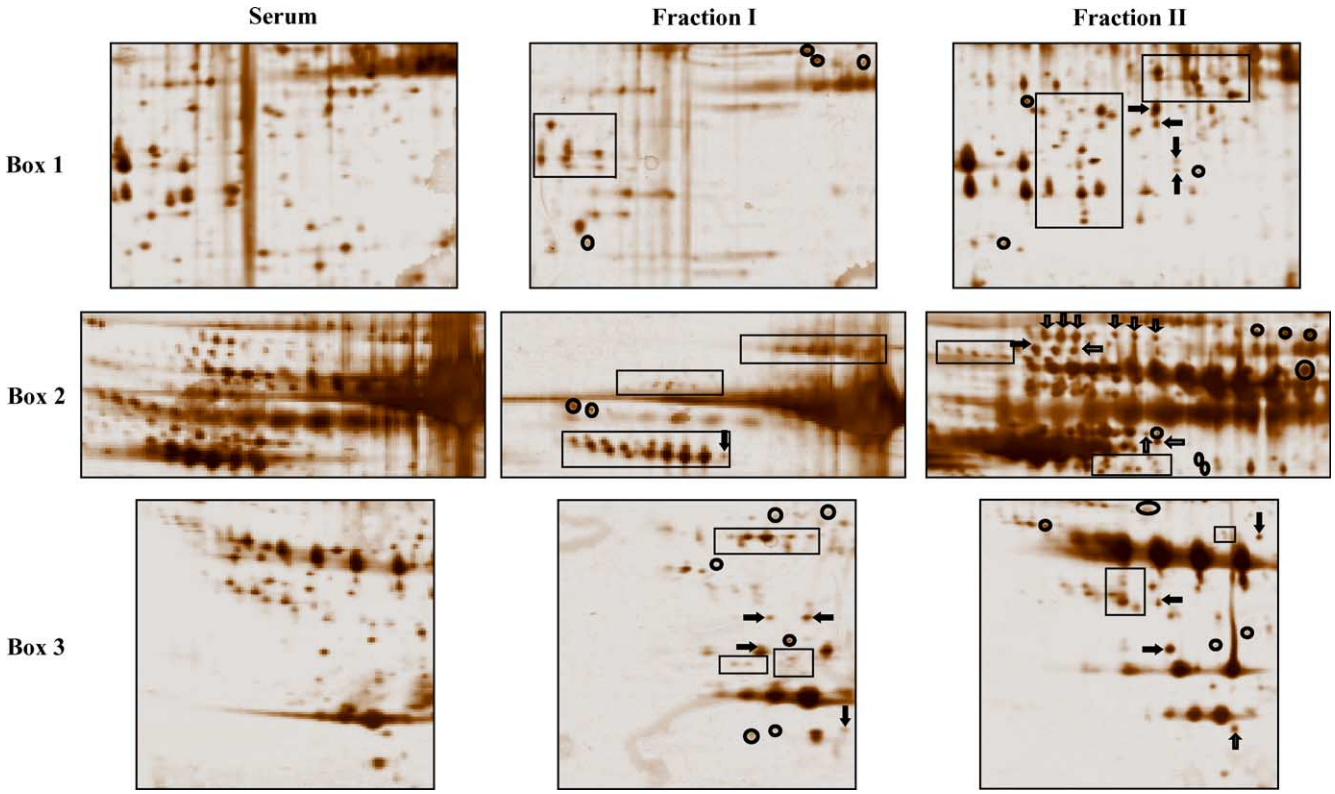


Fig. 4. Enlargement of Boxes 1, 2 and 3 (see Fig. 3 for reference) from representative 2D-maps of serum and chromatographic Fractions I and II. Spots highlighted correspond to: proteins not visualized in a total serum map, visible in Peak I, Peak II or both, depending on the glycosylation pattern (circles); spots increased over two-fold (arrows); and areas where better separation was achieved (rectangles). Box 1 accounts for 91 spots in total serum, 50 spots in Peak I and 113 in Peak II. Box 2 presents 105 spots in total serum, 43 in Peak I and 121 in Peak II. Box 3 shows 89 spots in total serum, 75 in Peak I and 105 in Peak II.

In these sections, we have detected spots visible in Fractions I and II, but not in the total serum gel, and marked some of them with circles in the Boxes 1–3. Other proteins, marked with arrows, correspond to proteins with an increased relative volume (>2-fold). Finally, sectors where a better separation was achieved are shown in rectangles. In all the three boxes, the number of spots detected in Fraction I plus the number of spots recorded in Fraction II is higher than the number of spots present in the total serum, proving an increased overall resolution achieved by the Con A chromatographic separation prior 2D-PAGE (see data in the figure legend). Therefore, certain proteins with lower abundance, either proteins with glycosylation mainly by *O*- or *N*-links, or non-glycosylated proteins, not visualized in 2D-maps of total serum, could be readily analysed.

As an example of the utility of the method, we have compared the *N*-glycoprotein enriched fraction subproteome of three healthy donors and three CRC patients looking for differences in protein expression. In order to avoid the variability among patients, only the spots present in all gels were included in the analysis. On average, 715 ± 140 and 655 ± 17 spots were considered in gels from healthy donors and CRC patients, respectively. After the analysis, 27 altered spots were submitted to MALDI-TOF protein identification. Seven spots did not produce valid peptide mass fingerprintings (PMFs). Ten protein species, despite having a good fingerprinting, could not be identified using databases. Finally, we summarize in Tables 1 and 2 the identity found for increased and decreased proteins, respectively, in CRC patients with regard to donors. The experimental relative molecular mass and isoelectric point for those spots are also shown.

Antithrombin-III (P01018), coagulation factor XIII B chain (P05160), and beta-2-glycoprotein I (P02749), were found to be related with the blood coagulation cascade. It is known that cancer is related with coagulation disorders which can contribute to the metastatic spreading of the primary tumour [24]. Honegger et al. [25] proved that patients with cancer of the colon, ovary and prostate showed a deficiency of antithrombin-III (P01018) more frequently than other common tumours.

On the other hand, complement factor H (P08603) and complement factor H-related protein I (Q03591) are plasma proteins with regulatory functions in the alternative pathway of complement activation [26]. Junnikkala et al. [27], using human H2 glioblastoma cells, have shown that production and binding of both factors is a novel mechanism those cells utilize to escape complement-mediated killing.

Haptoglobin and inter-alpha-trypsin inhibitor are acute-phase reactant proteins. Haptoglobin (P00738) has been associated with the implantation and angiogenesis of

Table 1
N-Glycoproteins increased in colorectal cancer patients in comparison with healthy donors

SSP	p*I*	Mr (kDa)	Fold variation	*P* (*U* Mann–Whitney)	Swiss-Prot identification
3205	5.3	43.5	5.7	<0.05	Haptoglobin (P00738)
3507	5.3	77.8	3.4	n.s.	Hemopexin (P02790) + alpha-1B-glycoprotein (P04217)
5708	5.6	182.0	26.4	n.s	Complement factor H (P08603)

SSP, standard spot number; n.s., not significative.

Table 2
N-Glycoproteins decreased in colorectal cancer patients in comparison with healthy donors

SSP	p*I*	Mr (kDa)	Fold variation	*P* (*U* Mann–Whitney)	Swiss-Prot identification
1214	4.9	38.7	8.4	<0.05	Clusterin (P10909)
2412	5.2	63.3	3.6	<0.05	Antithrombin-III (P01008)
2708	5.1	126.6	6.3	<0.05	Inter-alpha-trypsin inhibitor heavy chain H4 (Q14624)
5606	5.6	84.3	15.0	<0.05	Coagulation factor XIII B chain (P05160)
6606	5.8	84.3	9.1	n.s	Coagulation factor XIII B chain (P05160)
7304	6.1	46.6	12.5	n.s.	Complement factor H-related protein I (Q03591)
8402	6.4	59.5	10.3	<0.05	Beta-2-glycoprotein I (P02749)

SSP, standard spot number, n.s., not significative.

the carcinoma, and several authors have stated that haptoglobin is increased in CRC [28–30]. In fact, haptoglobin had been connected to CRC as a serum tumour marker, even together with carcinoembryonic antigen [28,29], and it was also described as a prognostic tumour marker for CRC [30]. The inter-alpha-trypsin inhibitor heavy chain H4 (Q14624) was shown to be involved in malignant transformation of lung tissue [31]. In relation to hemopexin or beta-1B-glycoprotein (P02790), Salas-Valdes et al. [32] described an increase of the hemopexin band in 1D gels of serum of cancer patients, and afterwards other authors have studied its expression in different tumours [33,34].

Clusterin or apolipoprotein J (P10909) is a heterodimeric highly conserved secreted glycoprotein, expressed in a wide variety of tissues and found in all human fluids, that seems to be able to bind to cells, membranes and hydrophobic proteins [35]. Its function is not clear, however, it has been specifically related to cancer, apoptosis [35–37], and recently Chen et al. [36] have shown that its levels seem to be increased in colonic precancerous lesions and in tumour neighbouring normal cells. To explain this result, the authors suggest that secreted clusterin is endocytosed by surrounding normal cells. This hypothesis would be in accordance with lower levels of clusterin in serum from CRC patients. Moreover, in oesophageal squamous cell carcinoma a loss of clusterin both in serum and tissue has been detected using proteomic approaches [37].

4. Conclusions

In conclusion, Con A-Sepharose chromatography combined with 2D-PAGE is a consistent, reproducible and reliable method, becoming an excellent tool for comparative studies of serum. The main advantage of performing Con A-Sepharose chromatography is that it is a simple and inexpensive method for separation of proteins with different glycosylation pattern. When the chromatographic fractions are resolved by electrophoresis, either in one or two dimensions, a greater resolution is achieved, be it an improvement in the visualization of low-abundance proteins or an increase in the quantity of protein analysed. We have confirmed that this method allows the detection of differentially expressed proteins in serum from cancer patients.

Acknowledgements

The authors would like to thank Professor G. Hansson and T. Larson (Gothenburg University) for MS identification, supported by The Swedish Research Council (No. 7461) and Ms B. Otero and G. Otero for her technical assistance. We would also like to thank ‘Centro de Transfusión de Galicia’ and ‘Hospital Xeral-Cíes de Vigo’ for providing samples. This work was supported by grants from ‘Xunta de Galicia’ (PGIDIT02BTF30103PR, PGIDIT03PX130103IF) and ‘Universidad de Vigo’. A.M. Rodríguez-Piñeiro and D. Ayude were supported by a predoctoral fellowship from ‘MECD’ and a contract from ‘Programa Torres Quevedo, MCYT’, respectively.

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