Izabela Sokolowska[1]
Mary Ann Gawinowicz[2]
Armand G. Ngounou Wetie[1]
Costel C. Darie[1]

[1]Biochemistry & Proteomics Group, Department of Chemistry & Biomolecular Science, Clarkson University, Potsdam, NY, USA
[2]Protein Core Facility, College of Physicians & Surgeons, Columbia University, New York, NY, USA

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Research Article

Disulfide proteomics for identification of extracellular or secreted proteins

The combination of SDS-PAGE and MS is one of the most powerful and perhaps most frequently used gel-based proteomics approaches in protein identification. However, one drawback of this method is that separation takes place under denaturing and reducing (R) conditions and as a consequence, all proteins with identical apparent molecular mass (Mr) will run together. Therefore, low-abundant proteins may not be easily identified. Another way of investigating proteins by proteomics is by analyzing subproteomes from a total proteome such as phosphoproteomics, glycoproteomics, or disulfide proteomics. Here, we took advantage of the property of secreted proteins to form disulfide bridges and investigated disulfide-linked proteins, using SDS-PAGE under nonreducing (NR) conditions. We separated sera from normal subjects and from patients with various diseases by SDS-PAGE (NR) and (R) conditions, followed by LC-MS/MS analysis. Although we did not see any detectable difference between the sera separated by SDS-PAGE (R), we could easily identify the disulfide-linked proteins separated by SDS-PAGE (NR). LC-MS/MS analysis of the disulfide-linked proteins correctly identified haptoglobin (Hp), a disulfide-linked protein usually found as a heterotetramer or as a disulfide-linked heteropolymer. Western blotting under NR and R conditions using anti-Hp antibodies confirmed the LC-MS/MS experiments and further confirmed that upon reduction, the disulfide-linked Hp heterotetramers and polymers were no longer disulfide-linked polymers. These data suggest that simply by separating samples on SDS-PAGE under NR conditions, a different, new proteomics subset can be revealed and then identified.

Keywords:
Disulfide proteomics / Fractionation / Mass spectrometry / Serum biomarkers
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1 Introduction

The combination of SDS-PAGE and MS is one of the most powerful and perhaps most frequently used gel-based proteomics approaches for protein identification [1]. However, one drawback of this method is that separation takes place under denaturing and reducing (R) conditions, and all proteins with identical apparent molecular mass (Mr) will run together. As a consequence, primarily the abundant proteins will be identified by MS. For example, if two proteins with identical mass are separated by SDS-PAGE and one is abundant but the other one is an indicator of a disease or disorder (but less abundant), the second protein will rarely be identified by MS (and if it is identified, it will be with great effort), unless the protein sample was a priori depleted of the most abundant proteins or prefractionated using other biochemical approaches. Depletion of abundant proteins is primarily used in the preparation of sera for proteomics experiments for biomarker discovery.

Biochemical fractionation or prefractionation of a protein mixture is usually performed using almost any possible and logical biochemical approach [1–7]. One of the most used prefractionation method in proteomics is SDS-PAGE. A variation of this method is isoelectric focusing followed by SDS-PAGE. As such, the combination of a biochemical approach with MS allows one to investigate and identify proteins from a whole proteome. Another way of investigating proteins via proteomics is by using specialized biochemical approaches to analyze subproteomes from a total proteome such as phosphoproteomics or glycoproteomics. Therefore, taking advantage of the physiochemical properties of the amino acids comprising proteins, the proteins themselves, their noncovalent interaction, and/or of their posttranslational modifications may be good options for identifying low-abundant proteins or proteins that are not easy to identify by classical approaches such as SDS-PAGE and MS. One such option is by varying

Correspondence: Dr. Costel C. Darie, Biochemistry and Proteomics Group, Department of Chemistry and Biomolecular Science, Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5810, USA
E-mail: cdarie@clarkson.edu
Fax: +1-315-268-6610

Abbreviations: Ab, antibodies; **Hp**, haptoglobin; **NR**, nonreducing conditions; **R**, reducing conditions; **WB**, Western blotting

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the separation/fractionation conditions, without performing an additional prefractionation step.

One important property of extracellular proteins is to form disulfide bridges through oxidation of their cysteine residues [8]. Two main types of extracellular proteins are of high interest: membrane-bound extracellular proteins involved among others in cell–cell interactions or ligand–receptor interactions in signal transduction pathways and secreted proteins in the bodily fluids such as blood or spinal fluids [3, 7, 9–12]. Human sera or plasma are good indicators of the health state of the whole organism and monitoring serum or plasma proteins may give us clues about the disease state of the organism. Sera and plasma contain a large number of proteins and many of them have cysteine residues that are either intra- or intermolecularly bridged. For example, albumin forms intramolecular disulfide bridges, while immunoglobulins form intermolecular disulfide bridges.

Historically, disulfide bridges in proteins have been identified by diagonal paper electrophoresis [13], and more recently by diagonal gel electrophoresis [14, 15]. Using these methods, the nonreduced and reduced protein samples are separated by SDS-PAGE under nonreducing (NR) and then R conditions. While the proteins that are not disulfide linked will run in diagonal, the disulfide-linked proteins or peptides could be easily identified, because they do not migrate the same way as the rest of the proteins (do not run in diagonal). Diagonal gel electrophoresis is also particularly useful for differentiating between the disulfide-linked proteins and proteins that are not disulfide linked that have identical or similar molecular mass. A different way of investigating the disulfide-linked proteins is by separating them under NR conditions, followed by either Coomassie staining, Western blotting (WB), or MS (the current study). While these two approaches (direct identification of the disulfide linked proteins by SDS-PAGE (NR) and LC-MS/MS or identification of the proteins that are disulfide linked by diagonal gel electrophoresis) are somehow different (diagonal gel electrophoresis reveals and identifies the reduced proteins/peptides that were involved in a disulfide bridge, while SDS-PAGE (NR) reveals and identifies only the disulfide linked proteins) the outcomes of these methods complement with each other.

Haptoglobin (Hp), a hemoglobin-binding serum protein, is composed of two alpha chains and two beta chains, all intermolecularly disulfide linked [16–19]. These proteins are synthesized from three common alleles, Hp^{1F}, Hp^{1S}, and Hp^{2}, whose combination leads to three major Hp phenotypes – Hp (1–1), Hp (2–1), and Hp (2–2). Hp is an acute-phase protein, acts as an antioxidant, has antibacterial activity, and has also been proposed as a biomarker for the detection of various diseases [10, 20–24]. In addition, possession of a particular Hp phenotype has been associated with a variety of common disorders such as cardiovascular disease, autoimmune disorders, or malignancy [18, 19, 25–28].

Here, we took advantage of the property of secreted proteins to form disulfide bridges and investigated whether we can use this property for specific identification of disulfide-linked proteins, using SDS-PAGE (NR). We separated sera from normal subjects and from patients with various diseases by SDS-PAGE (NR) and (R), followed by LC-MS/MS analysis. Although we did not see any detectable difference between the sera separated by SDS-PAGE (R), we could easily identify the disulfide-linked proteins separated by SDS-PAGE (NR). LC-MS/MS analysis of the disulfide-linked proteins correctly identified Hp, a disulfide-linked protein usually found as a heterotetramer or as a disulfide-linked heteropolymer. WB under NR conditions using anti-Hp antibodies (Ab) confirmed the LC-MS/MS experiments and identified Hp as a protein ladder. WB under NR and R conditions using anti-Hp Ab confirmed that upon reduction, the disulfide-linked Hp heterotetramers and polymers were reduced and no longer identified as polymers. When assessed as a potential biomarker, we did not find any pattern for Hp. These data suggest that simply by separating samples on SDS-PAGE under NR conditions, a different, new proteomics subset (disulfide proteomics) can be revealed and then identified.

2. Materials and methods

2.1 Materials

Most materials were purchased from Fisher (Waltham, MA, USA), VWR (Bridgeport, NJ, USA), Sigma (St. Louis, MO, USA), Invitrogen (Carlsbad, CA, USA), Bio-Rad (Hercules, CA, USA), or Waters (Milford, MA, USA), unless otherwise specified. Human sera were purchased from ProMedDx (Norton, MA, USA) or Bioreclamation (Hicksville, NY, USA). The work with human sera was reviewed and approved by the Institutional Review Board/IRB and determined as human subject exempt. Upon arrival, the sera were aliquoted and stored at –80°C either undiluted or diluted 1:10, 1:20, and 1:50 with 20 mM Tris, 150 mM NaCl.

2.2 SDS-PAGE and WB

SDS-PAGE (NR) was performed as previously described [8]. Briefly, the sera were combined with a self-made SDS-PAGE sample buffer from which the reducing agent (DTT or beta-mercaptoethanol) was omitted and then run by regular SDS-PAGE. Gradient SDS-PAGE (NR) was performed in-house using a Bio-Rad gravity gradient maker. The starting solutions contained 4% and a 13% acrylamide/bisacrylamide. The gels were 4–13% linear gradient of acrylamide/bisacrylamide. 2D SDS-PAGE was performed as previously described [8], except that the first-dimension (1D) SDS-PAGE gel was a 4–13% linear gradient of acrylamide/bisacrylamide. For the second-dimension SDS-PAGE (R), the gel lanes from 1D SDS-PAGE (NR) were excised and reduced by incubation in 1% beta-mercaptoethanol for 60 min at room temperature under moderate shaking, followed by separation on a 2D self-made SDS-PAGE gel and staining with Coomassie. WB was performed as previously described [29]. Depending on

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the gel size (minigel or maxigel, Coomassie staining or WB), we tested different volumes of sera at different dilutions for optimal results. For example, for SDS-PAGE NR minigels, we loaded 10 μL/lane at 1:20 dilution. For WB, we loaded 4 μL/lane at a dilution of 1:50. For large-scale 1D SDS-PAGE gels, we loaded 20 and 40 μL at a dilution of 1:20. For the 2D SDS-PAGE, we loaded 100 μL/lane at a dilution of 1:10.

2.3 Protein digestion and peptide extraction

The Coomassie-stained SDS-PAGE gel pieces were digested according to a published procedure [30], with some modifications as previously described [29]. Briefly, the gel pieces were washed in high-purity HPLC grade water under moderate shaking and then cut into very small pieces. The gel pieces were then dehydrated in 50 mM ammonium bicarbonate, in 50 mM ammonium bicarbonate/50% ACN, and in 100% ACN. These three steps were performed for 20 min each under moderate shaking at room temperature. After the last incubation step, the gel pieces were dried in a Speed-vac concentrator and then rehydrated with 50 mM ammonium bicarbonate. The washing procedure was repeated twice. The dried gel bands were then rehydrated with a solution containing 10 mM DTT and 50 mM ammonium bicarbonate, and incubated for 45 min at 56°C. DTT solution reduced the disulfide bridges in the proteins from the gel. The DTT solution was then replaced by a solution containing 100 mM iodoacetamide and 50 mM ammonium bicarbonate, and further incubated for 45 min in the dark, with occasional vortexing. In this step, the cysteine residues were irreversibly modified by iodoacetamide to form carbamydomethyl-cysteine. The initial washing procedure was then repeated one more time, and then the gel pieces were dried in the Speed-vac concentrator and then rehydrated using 10 ng/μL trypsin in 50 mM ammonium bicarbonate, and then incubated overnight at 37°C under low shaking. The resulting peptides were extracted from the gel pieces by incubation with 5% formic acid/50 mM ammonium bicarbonate/50% ACN (twice) and with 100% ACN (once) under moderate shaking. Solutions containing peptide mixture were then combined and then dried in a Speed-vac concentrator. The peptides were then solubilized in 20 μL of 0.1% formic acid/2% ACN/HPLC water, placed in UPLC vials and further used for LC-MS/MS analysis.

2.4 LC-MS/MS

The peptide mixture was analyzed by reversed-phase LC-MS/MS using a 2695 Alliance HPLC (Waters, Milford, MA, USA) or a NanoAcquity UPLC (Micromass/Waters, Milford, MA, USA) coupled to a Q-TOF Micro MS (Micromass/Waters, Milford, MA, USA). The procedure using 2695 Alliance HPLC coupled with the QTOF Micro MS was previously described [29]. Briefly, peptides were loaded onto a XBridge^{TM} C18 3.5 μm, 2.1 × 100 mm column (Waters) and eluted over a 60 or 120 min gradient of 2–100% ACN in 0.1% formic acid at a flow rate of 200 μL/min. MS data acquisition involved survey MS scans and automatic data dependent analysis (DDA) of the top three ions with the highest intensity and with the charge of 2+, 3+, or 4+. The MS/MS was triggered when the MS signal intensity exceeded 10 counts/s. In survey MS scans, the three most intense peaks were selected for CID and fragmented until the total MS/MS ion counts reached 10 000 or for up to 20 s each. Additional experiments were performed using a NanoAcquity UPLC (Waters) coupled to the Q-TOF Micro MS, but with a nanoelectrospray source. The procedure used was previously described [3]. Briefly, the peptides were loaded onto a 100 μm × 10 mm NanoAcquity BEH130 C18 1.7 μm UPLC column (Waters) and eluted over a 120 min gradient of 2–85% ACN in 0.1% formic acid at a flow rate of 400 nL/min. The aqueous solvent was HPLC water containing 0.1% formic acid. The column was coupled to a Picotip Emitter Silicatip nano-electrospray needle (New Objective, Woburn, MA, USA). The MS parameters were unchanged from the previously described settings, except that the voltages were adjusted to nanoflow rate and the ions detected in the MS mode and selected for CID were fragmented until the total MS/MS ion counts reached 10 000 or for up to 6 s each. Full description of the nanoLC-MS/MS analysis and data processing is described elsewhere [29]. Calibration was performed for both precursor and product ions using either 1 pmol or 100 fmol GluFib standard peptide (Glu1-Fibrinopeptide B) with the sequence EGVNDNEEGFFSAR, with the singly charged monoisotopic m/z of 1570.68. The precursor ion monitored was the double charged peak of Glu-Fib, with m/z of 785.84. Optimization of the nanospray was performed using direct infusion of 100 fmol/μL GluFib at a flow rate of 400 nL/min. The preliminary experiments were performed using a CapLC (Waters) coupled with a QTOF Micro MS, described in [8, 31].

2.5 Data processing and protein identification

The raw data were processed using published procedures from our laboratory [29]. Briefly, the raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software with the following parameters: background subtraction of polynomial order 5 adaptive with a threshold of 30%, two smoothings with a window of three channels in Savitzky–Golay mode, and centroid calculation of top 80% of peaks based on a minimum peak width of four channels at half height. The resulting pkl files were submitted for database search and protein identification to the public Mascot database search (http://www.matrixscience.com, Matrix Science, London, UK) using the following parameters: human databases from NCBI and SwissProt, parent mass error of 1.3 Da, product ion error of 0.8 Da, enzyme used: trypsin, one missed cleavage, and carbamidomethyl-cysteine as fixed modification and methionine oxidized as variable modification. The pkl files were also analyzed using an in-house, custom-created Hp database in PLGS software

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(http://www.waters.com), with search parameters similar to the ones used for Mascot search. Hp isotypes were identified through customized database search using the in-house PLGS 2.4 software. We used two databases to search our data: one contained the Hp isotypes and the other one contained the SwissProt database. To identify the false-negative results, we varied our database search parameters and compared the results with each other. To eliminate false-positive results, we manually checked the MS/MS spectra.

3 Results and discussion

3.1 Proteins that are undetectable in SDS-PAGE (R) can be identified by SDS-PAGE (NR) and Coomassie staining

The rationale for initiating this study was that if two proteins known to be disulfide linked are separated by SDS-PAGE under R conditions, they may not be easily observed in the Coomassie-stained gels due to their mass (low mass) or because many other proteins run in the same molecular mass range, or both. As such, identification of these proteins by LC-MS/MS may also be difficult, for the same reasons mentioned earlier. However, if these proteins are disulfide linked, they will migrate at a higher molecular mass, where there are fewer proteins in the gel and therefore, it is easier to identify these disulfide-linked proteins in the Coomassie-stained gel or by LC-MS/MS. In addition, by comparing the SDS-PAGE under R and NR conditions, it could be relatively easy to identify the disulfide-linked proteins. A schematic of the principle of identifying disulfide-linked proteins by either Coomassie staining or LC-MS/MS is shown in Fig. 1A.

To test our rationale, we separated sera from normal subjects and from patients with various types of cancer by SDS-PAGE under R and NR conditions (Fig. 1B). No difference between the proteins patterns of these two types of sera (C1 and C2 versus N) could be observed in Coomassie-stained SDS-PAGE gels separated under R conditions (Fig. 1B). Also, no differences between these samples can be identified by LC-MS/MS (data not shown). However, in the Coomassie-stained SDS-PAGE gels separated under NR conditions, differences between the gel lanes could be observed, specifically around 110 and 150 kDa. LC-MS/MS analysis of the 110-kDa band identified this protein as Hp (45 kDa, Fig. 1C and D), a well-known marker for cancer and inflammation. Examples of MS/MS spectra in which fragmentation of a triple-charged peak with m/z of 729.97 in sample C1 and fragmentation of a double-charged peak with m/z of 720.29 in sample C2 led to identification of two peptides with amino acid

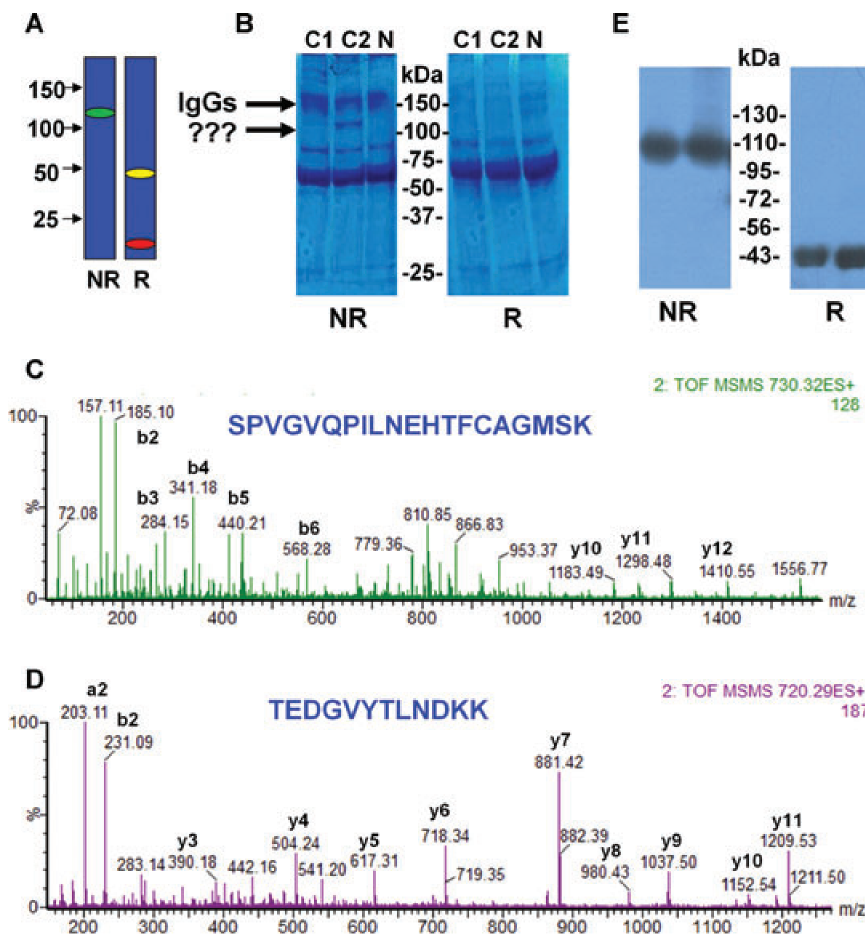

Figure 1. Analysis of sera by SDS-PAGE (NR) and (R). (A) Schematic of the principle of identifying disulfide-linked proteins. The disulfide-linked proteins that are separated by SDS-PAGE in NR (120 kDa band) are reduced and dissociate into individual subunits when separated by SDS-PAGE in R (45 and 15 kDa bands). (B) Sera from patients with cancer (C1 and C2) and from normal subjects, when separated by SDS-PAGE (NR), reveal protein bands with different intensities that are not observed in the regular SDS-PAGE (R). The 150 kDa band in all three samples in SDS-PAGE (NR) are IgGs. (C) and (D) LC-MS/MS analysis of the 110 kDa band from sample C1 and C2: MS/MS spectra of a triple-charged peak with m/z of 730.32 that corresponds to a peptide with sequence SPVGVQPILNEHTFCAGMSK (C) and of a double-charged peak with m/z of 720.29 that corresponds to a peptide with sequence TEDGVYTLNDKK (D); both peptides were part of Hp. (E) Validation of the LC-MS/MS analysis of the SDS-PAGE (NR): the sera were analyzed by SDS-PAGE (NR), followed by WB using anti-Hp Ab. The molecular weight markers are indicated.

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Table 1. Identification of Hp by LC-MS/MS. Hp peptides identified from LC-MS/MS analysis of the 110 kDa Hp from samples C1, C2, and N separated by SDS-PAGE (NR) (shown in Fig. 1B).

Protein name	Mascot score	Identified peptide	Peptide *m/z*	Peptide charge
Hp coverage for band C1				
Haptoglobin OS = *Homo sapiens*; GN = HP; PE = 1; SV = 1	489	DYAEVGR	405.18	2
		QLVEIEK	429.73	2
		VGYVSGWGR	490.72	2
		VM*PICLPSK	530.75	2
		HYEGSTVPEK	573.75	2
		HYEGSTVPEK	382.84	3
		HYEGSTVPEKK	425.53	3
		LPECEAVCGKPK	694.32	2
		TEGDGVYTLNDKK	720.30	2
		YVMLPVADQDQCIR	854.39	2
		YVM*LPVADQDQCIR	862.35	2
		YVM*LPVADQDQCIR	862.85	2
		VVLHPNYSQVDIGLIK	897.95	2
		AVGDKLPECEAVCGKPK	619.94	3
		SPVGVQPILNEHTFCAGM*SK	729.98	3
Haptoglobin-related protein OS = *Homo sapiens*; GN = HPR; PE = 1; SV = 2	213	NYAEVGR	405.18	2
		QLVEIEK	429.73	2
		VM*PICLPSK	530.75	2
		LPECEAVCGKPK	694.32	2
		TEGDGVYTLNDKK	720.30	2
		AVGDKLPECEAVCGKPK	619.94	3
Hp coverage for band C2				
Haptoglobin OS = *Homo sapiens*; GN = HP; PE = 1; SV = 1;	405	DYAEVGR	405.21	2
		QLVEIEK	429.74	2
		QLVEIEK	430.24	2
		VGYVSGWGR	490.73	2
		VGYVSGWGR	490.73	2
		VM*PICLPSK	530.79	2
		HYEGSTVPEK	382.85	3
		HYEGSTVPEK	573.78	2
		HYEGSTVPEK	573.82	2
		HYEGSTVPEKK	425.55	3
		SCAVAEYGVYVK	673.85	2
		LPECEAVCGKPK	694.32	2
		LPECEAVCGKPK	463.22	3
		TEGDGVYTLNDKK	720.32	2
		YVMLPVADQDQCIR	854.39	2
		YVM*LPVADQDQCIR	862.38	2
		YVM*LPVADQDQCIR	862.40	2
		VVLHPNYSQVDIGLIK	598.99	3
		SPVGVQPILNEHTFCAGMSK	724.68	3
		SPVGVQPILNEHTFCAGM*SK	730.00	3
Haptoglobin-related protein OS = *Homo sapiens*; GN = HPR; PE = 1; SV = 2	121	NYAEVGR	405.21	2
		QLVEIEK	429.74	2
		QLVEIEK	430.24	2
		VM*PICLPSK	530.79	2
		SCAVAEYGVYVK	673.85	2
		LPECEAVCGKPK	694.32	2
		LPECEAVCGKPK	463.22	3
		TEGDGVYTLNDKK	720.32	2
Hp coverage for band N				
Haptoglobin OS = *Homo sapiens*; GN = HP; PE = 1; SV = 1	88	DYAEVGR	405.18	2
		QLVEIEK	429.74	2
		HYEGSTVPEK	382.83	3
		HYEGSTVPEK	573.75	2
		TEGDGVYTLNDKK	720.31	2
		YVMLPVADQDQCIR	854.42	2
		YVM*LPVADQDQCIR	862.41	2
		SPVGVQPILNEHTFCAGM*SK	729.99	3
		NYAEVGR	405.18	2
		QLVEIEK	429.74	2
		TEGDGVYTLNDKK	720.31	2

M* represents oxidized methionine.

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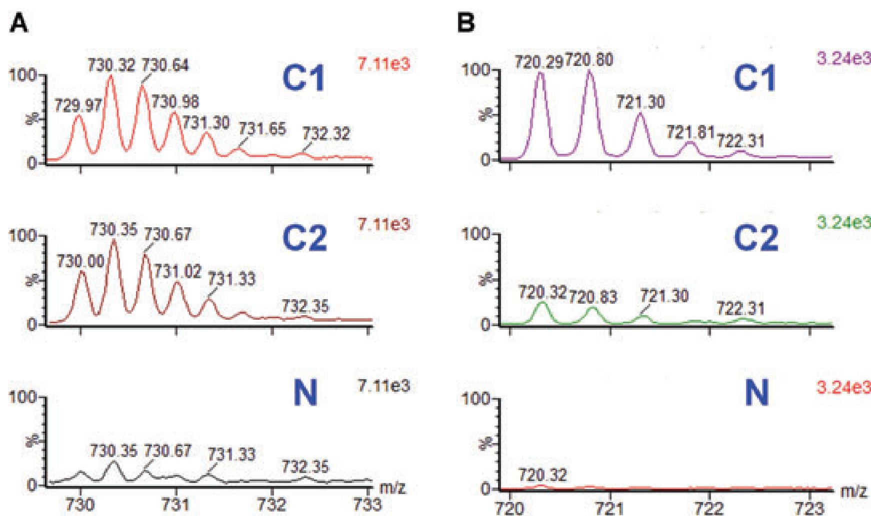

Figure 2. Comparison of the intensities of MS spectra for peaks corresponding to peptides that are part of Hp found by SDS-PAGE (NR) and LC-MS/MS. (A) The peak with *m/z* of 729.97 (3+) that corresponds to an Hp peptide with sequence SPVGVQPILNEHTFCAGMSK had a higher intensity of this peptide in the sera from cancer patients C1 and C2, compared with sera from normal subjects N. (B) The peak with *m/z* of 720.29 (2+) that corresponds to an Hp peptide with sequence TEDGVYTLNDKK had a higher intensity of this peptide in the sera from cancer patients C1 and C2, compared with sera from normal subjects N. The intensity scale for the spectra from both types of sera for each individual peptide was identical.

sequences SPVGVQPILNEHTFCAGMSK (*m/z* of 729.97) and TEDGVYTLNDKK (*m/z* of 720.29) that were part of Hp are shown in Fig. 1C and D. The full list of the peptides identified by LC-MS/MS from the 110-kDa bands from samples C1, C2, and N are shown in Table 1.

Hp is an intensely processed protein that splits into alpha and beta subunits. These two subunits form a heterotetramer that contains two alpha and two beta subunits, all of them disulfide linked. Therefore, Hp identified by LC-MS/MS is indeed a multisubunit, disulfide-linked protein that was correctly identified by SDS-PAGE (NR) but not SDS-PAGE (R), simply by staining the gel with Coomassie. The other band observed in SDS-PAGE (NR) but not (R) at about 150 kDa was correctly identified by LC-MS/MS as immunoglobulin (IgG, data not shown). Again, IgG is a good example of disulfide-linked proteins (it is a heterotetramer of two 50-kDa heavy chains and two 25-kDa light chains) that can be identified by SDS-PAGE (NR) and Coomassie stain or by LC-MS/MS. To validate our findings from the LC-MS/MS analysis of the SDS-PAGE in NR conditions, we reanalyzed the sera by SDS-PAGE under NR conditions followed by WB using anti-Hp Ab. We found Hp as a 45-kDa band in SDS-PAGE (R) and as 110 kDa in SDS-PAGE (NR) (Fig. 1E). These data suggest that proteins that form disulfide-based protein complexes and may not be detected by SDS-PAGE (R) can be easily identified by SDS-PAGE (NR).

3.2 Proteins that are undetectable in SDS-PAGE (R) can be identified and relatively quantified by SDS-PAGE (NR) and LC-MS/MS analysis

We were wondering whether we can both identify and relatively quantify the proteins that are specifically observed in SDS-PAGE (NR), but not (R). We therefore analyzed the 110-kDa gel bands observed in SDS-PAGE (NR) for protein identification and relative quantitation. In our experiments, we identified Hp in the cancer samples C1 (Mascot score 645) and C2 (Mascot score 345), as well as in the normal N sample (Mascot score 70). For relative quantitation, we compared the relative intensity of the peaks that corresponded to Hp peptides identified in both normal and cancer sera. Such an analysis is shown in Fig. 2. One triple-charged peak with *m/z* of 729.97 in sample C2 and 730.00 in sample C2 had a much higher intensity than the peak with *m/z* of 729.99 from normal sample N (Fig. 2A). Similarly, one double-charged peak with *m/z* of 720.29 in sample C1 and 720.32 in sample C2 had a much higher intensity than the peak with *m/z* of 720.32 from normal sample N (Fig. 2B). These data suggest that the relative quantitation of Hp in particular can be done at the level of disulfide-linked heterotetramer.

3.3 Customized gradient SDS-PAGE (NR) can find higher order, disulfide-linked Hp multimers

It is well documented that Hp, in addition to forming heterotetramers, also forms multimers of heterotetramers. Depending of the subunit composition of these proteins, Hp can form Hp 1–1, 1–2, and 2–2 disulfide-linked multimers. Therefore, we wondered whether we can identify multimers of the Hp heterotetramers and if so, to identify their subunit composition. To do so, we made SDS-PAGE gradient gels and separated various sera under NR conditions, stained them by Coomassie, and then inspected them visually. As observed, additional bands of higher molecular mass could be detected (Fig. 3A). The gel bands that are observed at about 110 kDa, 180 kDa, and at higher molecular mass are mostly disulfide-linked Hp heterotetramers and multimers (Fig. 3A–C), because when the same serum samples were separated under SDS-PAGE (R) these bands were no longer observed (data not shown). To confirm that these bands indeed contain disulfide-linked Hp, we separated sera by SDS-PAGE (NR) and analyzed the gel bands that could be disulfide linked by

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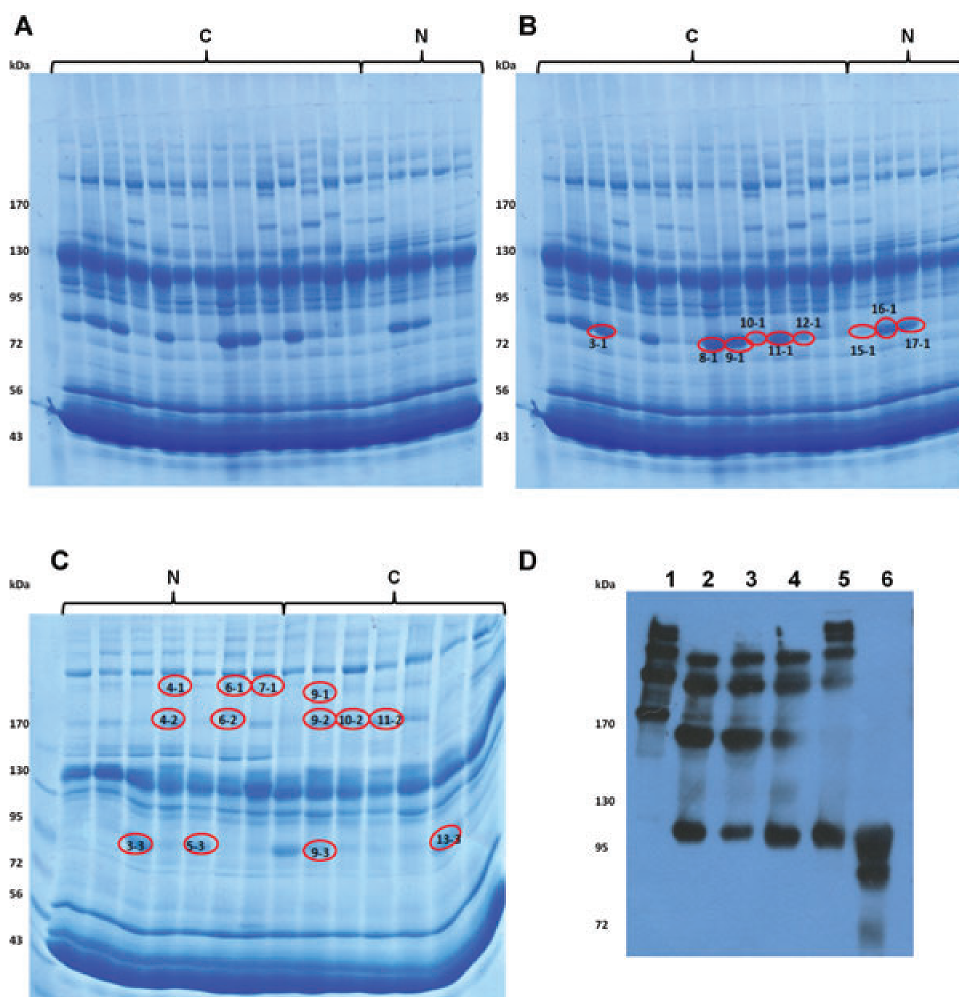

Figure 3. Gradient SDS-PAGE (NR) for visualization and identification of the Hp heterotetramers and multimers. (A) Sera from patients with cancer (C) and from normal (N) subject were separated on 4–13% linear acrylamide/bisacrylamide SDS-PAGE (NR) and the gels were stained by Coomassie (A) and the gel bands were excised and digested and then the resulting peptide mixture was analyzed by LC-MS/MS (B) and (C). The gel bands that contained Hp, as determined by LC-MS/MS, are circled and described in Table 1. (D) The LC-MS/MS experiments were validated by SDS-PAGE (NR) and WB using anti-Hp-Ab. The molecular weight markers are indicated.

LC-MS/MS. Initially, we started with the 110-kDa gel bands (Fig. 3B), then the 180-kDa gel bands (Fig. 3C), and so on. The circled gel bands shown in Fig3.B and C were analyzed by LC-MS/MS and were confirmed to contain Hp (data not shown). However, we could not distinguish between Hp 1–1, 1–2, or 2–2 isoforms. To further confirm that the polymers are made by the disulfide-linked Hp, we analyzed the gels by SDS-PAGE (NR) and WB using anti-Hp Ab. As observed, the 110- and 180-kDa bands, as well as the higher molecular mass disulfide-linked Hp heteromultimers are immunopositive with anti-Hp Ab. Important to note is that some of the sera contain no 110-kDa Hp, but only higher order polymers (Fig. 3D, lane 1), some other sera contain both 110- and 180-kDa heteropolymers, as well as higher order polymers (Fig. 3D, lanes 2–4), only 110-kDa and higher molecular mass polymers bands, but no 180-kDa band (Fig. 3D, lane 5) band, while other sera contain only the 110-kDa band, but no other polymeric forms of Hp (Fig. 3D, lane 6). Taken together, these data suggest that customized gradient could identify all disulfide-linked Hp multimers.

3.4 Proteins that are detectable in 1D SDS-PAGE (NR) but not by SDS-PAGE (R) and Coomassie staining can also be identified by 2D SDS-PAGE (NR) and SDS-PAGE (R) and LC-MS/MS analysis

To further confirm that the gel bands observed in SDS-PAGE (NR) but not in SDS-PAGE (R) contain Hp, we separated the sera by SDS-PAGE (NR) and then excised the gel lanes and reduced them and further separated them by SDS-PAGE (R) and analyzed them by LC-MS/MS. We called this SDS-PAGE (NR) and SDS-PAGE (R) procedure 2D SDS-PAGE (NR–R) or 2D (Fig. 4). We expected that the gel spots from 2D with a molecular mass around 45 kDa that correspond to the 110- and 180-kDa bands from SDS-PAGE (NR) are Hp. Indeed, as observed, when we analyzed the bands circled in Fig. 4 as Hp 45-kDa chain, we correctly identified them as Hp. Additional bands can also easily be identified simply by visual inspection. For example, the gel spots marked as IgG heavy chain and IgG light chain were easily identified since they had the expected molecular mass (50 kDa for heavy chain and 25 kDa

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for the light chain) in the 2D, but also corresponded to a protein with a mass of 150 kDa. In addition, contaminant serum proteins such as albumin could be used as markers in our 2D experiments, since they run as monomer (67 kDa), dimer (130 kDa), and trimer (190 kDa). These albumin spots are in agreement with the Hp protein bands from SDS-PAGE (NR), which ran at about 110 kDa and 180 kDa, as well as with IgG band from SDS-PAGE (NR), which ran at about 150 kDa. Taken together, these data suggest that the gel bands observed in SDS-PAGE (NR) in Coomassie-stained gels and by WB were correctly identified as Hp, confirming the previous experiments.

3.5 Hp isotypes (1–1, 1–2, and 2–2) can be distinguished by SDS-PAGE (NR) and WB

Since we knew that the gel bands that are specifically observed in Coomassie-stained SDS-PAGE (NR) gels, but not SDS-PAGE (R), are Hp, as demonstrated by LC-MS/MS, we were wondering whether we can distinguish between Hp isotypes (1–1, 1–2, and 2–2). Hp 1–1 is composed of two beta Hp subunits (45 kDa each) and two alpha 1S subunits (9 kDa each), and the heterotetramer is 110 kDa. Hp 2–2 is composed of two beta Hp subunits (45 kDa each) and two alpha-2 subunits (16 kDa each). Compared with Hp 1–1 heterotetramer, the Hp 2–2 heterotetramer should run in SDS-PAGE (NR) at around 120 kDa. At the heterohexamer level, the Hp 1–1 hexamer should run in SDS-PAGE (NR) at around 165 kDa, while the Hp 2–2 hexamer should run at around 180 kDa. Therefore, a clear distinction between Hp 1–1 and Hp 2–2 should be observed by SDS-PAGE (NR). As observed in the WB from Fig. 3D, we could clearly distinguish between the 180 kDa Hp heterohexamer (Fig. 3D, lane 1) and the 160 to 165 kDa heterohexamer (Fig. 3D, lanes 2–5). While it is not clear to us which band corresponds to Hp 1–2 heterotetramer and heterohexamer, it is clear that the 180 kDa band is the Hp 2–2 heterohexamer (Fig. 3D, lane 1), while the Hp heterotetramers and multimers (from lanes 2–6) are either Hp 1–1, Hp 1–2, or a combination of both. A closer examination of the 170 kDa bands from lanes 2–6 (Fig. 3D) reveals that at least gel bands from lanes 2 and 3 (Fig. 3D) contain an extra 180 kDa band, suggesting that these gel lanes contain a mixture of Hp 1–1 and 2–2, the Hp1–2 phenotype. Taken together, these data suggest that SDS-PAGE (NR) and WB could be a good method for identification of disulfide-linked Hps and determination of their isotype.

3.6 Use of Hp and Hp isotypes as serum biomarker for detection of cancer and acute inflammation

We further investigated whether we can take advantage of our method for identifying disulfide-linked Hp to validate it as a biomarker for either detection of various types of cancer or for acute inflammation. We analyzed various numbers of sera from healthy individuals and from patients with various

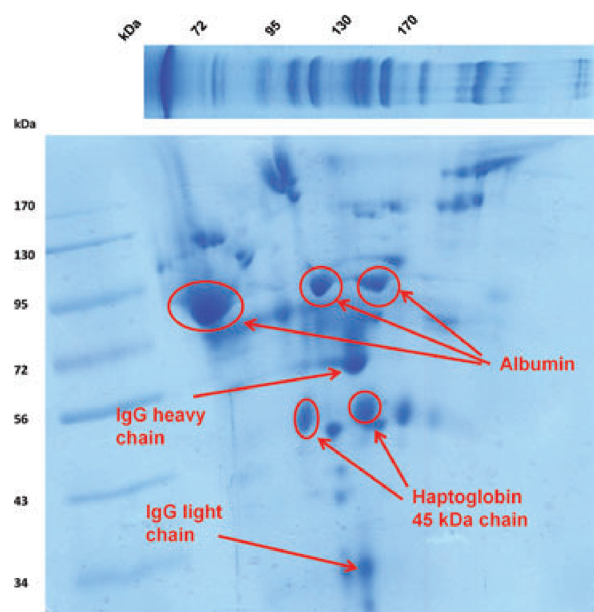

Figure 4. 2D SDS-PAGE. The sera from normal subjects were separated in SDS-PAGE (NR) in 1D and one of the gel lanes were excised, reduced, and denatured (by incubation in a solution containing DTT and SDS) and then oriented horizontally and polymerized in a second polyacrylamide gel and further separated in 2D by SDS-PAGE (R). The 2D gel was then stained by Coomassie and then the gel spots were analyzed by LC-MS/MS. To reveal the protein pattern in SDS-PAGE NR (1D), a second gel lane containing the same serum sample was stained by Coomassie; this gel lane is shown in 1D. The position of Hp as well as of other high-intensity proteins is indicated. The molecular weight markers are indicated. The direction of migration is also indicated.

types of cancer by SDS-PAGE (NR) and WB using anti-Hp Ab (data not shown). Overall, we found no particular pattern for Hp that could be exploited to use it as a biomarker. We also investigated sera from people with acute inflammation by SDS-PAGE (NR) and compared them with the sera from healthy individuals (Fig. 5). Again, we found that Hp cannot be used to detect inflammation in blinded experiments; at least not using our method. The only potential use for this method is to identify Hp in the sera of patients that contain only the 110-kDa band, but no other higher molecular mass oligomers. In this case, we did find that sera that contain only the 110-kDa Hp monomer have a stronger reaction in WB with anti-Hp Ab (Fig. 5B, lanes 1 and 7), compared with the Hp bands from other samples. Taken together, we believe that it is safe to conclude that our method cannot be used to relatively quantify Hp and as a biomarker for detection of cancer or inflammation. This conclusion is simply because not only there is more than one type of pattern made by Hp, as demonstrated by WB, but also different sera contain different types of Hp combinations (1–1, 1–2, or 2–2) that cannot be compared with each other. However, there may be plenty of other proteins that could be explored as potential serum biomarkers and that were not investigated in the current study. Therefore, this method is not intended to compete and replace other methods, but it is

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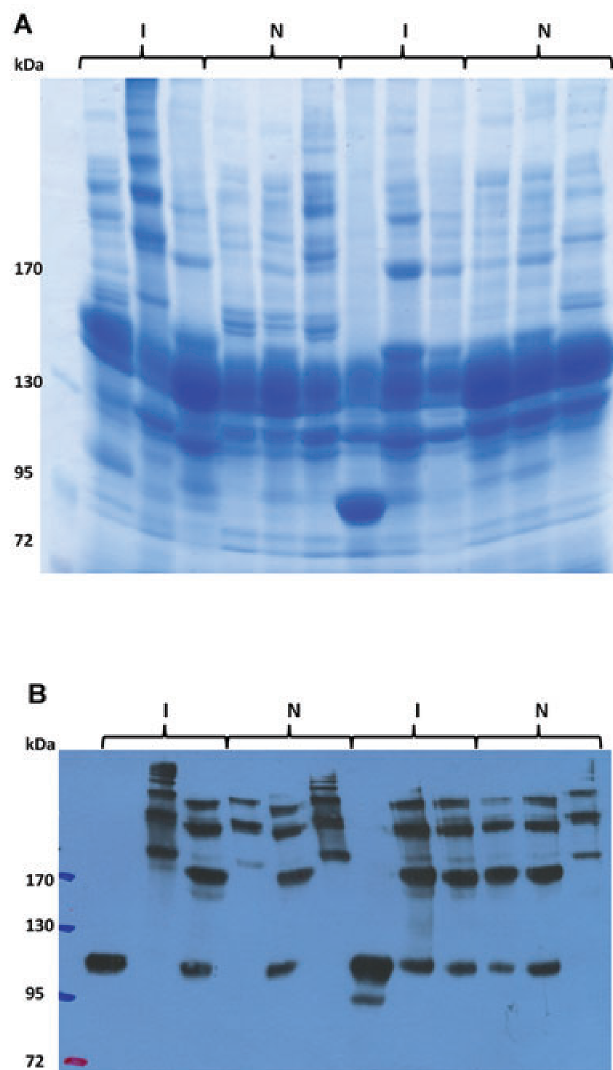

Figure 5. Use of Hp and Hp isotypes as serum biomarker for detection of cancer and acute inflammation. Sera from patients with acute inflammation (I) and from normal (N) subject were separated by 4–13% linear acrylamide/bisacrylamide SDS-PAGE (NR) and the gels were either stained by Coomassie (A) or further analyzed by WB using anti-Hp-Ab (B).

rather intended to reveal the disulfide-linked proteome for an easier proteomics analysis of the disulfide-linked proteins.

4 Concluding remarks

Overall, we presented a simple method that allows us to easily detect a subproteome composed of disulfide-linked proteins. This method consists on SDS-PAGE (NR) and Coomassie stain, followed by LC-MS/MS analysis and validation by WB. Although simple, this approach has not been used to investigate the disulfide-linked proteomes. This may be useful in proteomics analysis of secreted proteins (secretomes) or of the extracellular or membrane-linked extracellular proteins.

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