

Identification of damaged proteins in human serum using modified Ehrlich's reagent to target protein-bound pyrroles

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Short Title: Detecting damaged proteins with ER-B

Category Assignment: Article

Abstract

Protein-bound pyrroles are a sign of oxidative damage. Here we report a specific method for detecting pyrrole-containing proteins using biotin-labeled Ehrlich's reagent (ER-B). After treatment of either human serum or isolated human serum proteins with various oxidising agents, damaged, biotin-labeled components could be detected by blotting. Combining the use of ER-B with proteomic techniques allowed human serum proteins susceptible to oxidative damage to be detected and then identified by LC/MS/MS. Identification of such proteins in different human conditions such as obesity, diabetes and cardiovascular disease should lead to the discovery of new biomarkers and the development of specific assays to monitor health status.

Key Words: protein oxidation, protein-bound pyrroles, modified Ehrlich's reagent, detection of damaged proteins, proteomics

Introduction

Pyrroles are not present in newly-formed proteins but occur when lipid oxidation products such as 4,5-Epoxy-2-alkenals, e.g. 4,5(E)-epoxy-2(E)-heptenal (EH) which is produced from oxidation of n – 3 polyunsaturated fatty acids, react with free amino groups such as lysine residues on proteins [1;2]. Sugars such as glucose can also react non-enzymatically with free amino groups. This non-enzymic glycosylation is known as the Maillard reaction and is well known in food science. Both the Maillard reaction and lipid peroxidation follow similar reaction pathways, producing carbonyl derivatives which then form advanced glycation end products (AGE) or advanced lipid peroxidation end products (ALE) by means of carbonyl-amine reactions and aldol condensations forming protein cross-links [3]. Pyrrole cross-links have been identified in long lived proteins such as lens crystallins and skin collagen and implicated in the stiffening of arteries and joints associated with aging [4;5]. Increased (carboxyalkyl) pyrrole immunoreactivity was detected in plasma from patients with renal failure and atherosclerosis compared with healthy volunteers [1]. AGE and ALE increasingly accumulate during aging and in chronic diseases [6], suggesting that detecting pyrroles in proteins should be a good way to develop biomarkers for early stage disease.

Pyrroles in proteins can be measured spectrophotometrically using Ehrlich's reagent (p-dimethylamino benzaldehyde) [7]. This method has been used to detect pyrroles in tail tendon collagen of streptozotocin-diabetic rats [8;9] and in human plasma proteins following treatment with hydrogen peroxide [10] but these assays lack sufficient sensitivity for wide application. This paper describes a proteomic technique to identify serum proteins susceptible to oxidative and glycation damage using a modified Ehrlich's reagent, first used to investigate pyrrole cross-links in bone collagen [11]. The application of this methodology

will aid in the discovery of novel biomarkers and further the aim of developing specific assays to monitor health status.

Materials and Methods

Synthesis of biotinylated Ehrlich's reagent (ER-B)

Biotinylated Ehrlich's reagent was synthesised as described previously [11] with minor modifications. Briefly N-Methyl-N-propionic acid-4-amino benzaldehyde (100 mg) was dissolved in 5ml of dimethyl formamide: dichloromethane (1:5 v/v) and 300 mg of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma-Aldrich, UK) was added and the solution was stirred at room temperature for 5 min. Biotin pentyl-amine (50 mg; Thermo Fischer, UK) was dissolved in 0.4 ml of methanol and added slowly to the above mixture giving a molar ratio of 3:1 for N-Methyl-N-propionic acid-4-amino benzaldehyde to biotin pentyl-amine. After stirring at room temperature for 8 h. the mixture was concentrated to a volume of 2 ml using a rotary evaporator and then 18 ml of 2% v/v tifluoroacetic acid (TFA) was added to bring the volume to 20 ml. The ER-B was then purified by HPLC by applying aliquots (2.5 ml) of the mixture to 8 separate chromatographic runs using a preparative C-18 VYDAC column (0.9 x 25 cm) pumped at 4 ml min⁻¹. The buffers used were 0.1% TFA (buffer A) and 70% acetonitrile, 0.1% TFA (buffer B). The gradient applied was 25 % buffer B for 5 min followed by a linear increase to 70% buffer B over 40 min and the eluent was monitored at 330 nm to detect biotin. The chromatogram (Fig.1, left panel) showed two major peaks, with the peak eluting after 15 min corresponding to the unreacted acid. The second major component eluting around 25 min was analyzed by MALDI TOFF mass spectrometry and showed [M+H]⁺ of 518.2 and [M+Na]⁺ of 540.2, (Fig. 1, right panel) corresponding to the calculated M_r of 517.7 for ER-B. The peak containing the ER-B was

pooled from all 8 runs, freeze dried and stored at -20°C. The yield estimated gravimetrically was >80%.

Oxidation of Proteins and Reaction with ER-B

Albumin from human serum (essentially fatty acid free) (HSA), human ApoA1 and serum from human male AB plasma were obtained from Sigma-Aldrich (UK) and were treated with different oxidising agents to produce pyrroles. Solutions of 1 mg/ml HSA, 1 mg/ml Apo A1 and a 1:1 dilution of human serum in 0.9 M NaCl, 50 mM sodium phosphate, pH 7.2 (PBS) were incubated in the presence of different concentrations of 4,5(E)-epoxy-2(E)-heptenal, (EH) as described previously for bovine serum albumin [2]. EH (0.25 µl), synthesised as described by Zamora & Hidalgo [12;13], was added to 0.5 ml of PBS and incubated with shaking for 10 min at 45 °C, then spun and portions of the supernatant were added to protein and serum samples. Unless otherwise stated, EH treatment comprised adding 10 µl of the supernatant per ml of sample and incubating at 37 °C for 16 h. HSA and human serum were oxidised in a FeSO₄ (200mg/L) and H₂O₂ (100mg/L) mixture for 30 min at room temperature. To produce non-enzymic glycation, protein and serum samples were incubated in the presence of 10 mM glucose or 10 mM ribose for 7 d at 37 °C. Control samples of HSA and serum were incubated under identical conditions where the oxidising reagents were replaced with PBS. The protein and serum samples were passed through Hi Trap desalting columns (GE Biosciences, UK) and concentrated using Amicon Centricon concentrators (Millipore, UK). Protein concentrations were determined using the Pierce 660 protein assay (Thermo Fischer, UK) following the manufacturer's recommended protocol.

The protein and serum samples were reacted with ER-B to label the pyrroles on the proteins as described [11]. Briefly ER-B was dissolved in methanol to give a 10 mg/ml solution which was diluted 1:4 with 3.5 M HCl and 50 µl was added to 450 µl of protein sample, to give a final reagent concentration of 0.039 mM. The samples were incubated for

15 min at 45 °C. The reaction was stopped by adding 50 µl of 2 M NaOH and excess non-protein-bound ER-B was removed from the samples using Hi Trap desalting columns.

SDS-PAGE and Blotting

Following treatment with ER-B, protein samples were separated by electrophoresis using precast Criterion XT gels. All Blue Precision Protein™ Standards (Bio-Rad) were run alongside the samples. Following electrophoresis the gels were either stained using Simply Blue Safe Stain (Invitrogen, UK) to visualise the proteins or the proteins were transferred onto PVDF membranes. Transfer buffer was 25 mM Tris; 192 mM glycine pH 8.3; and 20% (v/v) methanol. Following protein transfer the membranes were blocked using Western Blocker solution (Sigma-Aldrich, UK), The membranes were then incubated for 1 h with Extra Avidin peroxidase (Sigma-Aldrich, UK) 1/5000 in TTBS (0.1 % v/v Tween 20 in 100 mM Tris-HCl, 150 mM NaCl) pH 7.4 washed with TTBS three times and then developed using the Opti-4CN Substrate Kit (Bio-Rad, UK).

Two-dimensional gel electrophoresis (2DE)

Human serum samples which had been reacted with ER-B were run in 2 DE. Bio-Rad immobilized pH gradient (IPG) strips (pH 3–10) were used for the separation of the proteins in the first dimension. Strips were rehydrated in 340 µl of rehydration buffer (7M urea; 2M thiourea; 4% w/v CHAPS; 2% w/v Biolyte; and 50mM DTT) containing 300µg of protein sample at 20 °C for 1 h without applied voltage in a Bio-Rad IEF cell. Then focusing was carried out as detailed previously[14].

Following completion of the first dimension IPG strips were incubated in fresh equilibration buffer (6 M urea; 2% w/v SDS; 0.375 M Tris-HCl, pH 8.8; 20% v/v glycerol; and 130 mM DTT) for 10–15 min at room temperature before transfer to a second equilibration buffer (6 M urea; 2% w/v SDS; 0.375 M Tris-HCl, pH 8.8; 20% v/v glycerol;

and 135 mM iodoacetamide) for 10–15 min at room temperature. The strip was then applied to the top of an 18 × 18 cm gel cassette. Gels were run at 200V for 9.5 h or until the bromophenol blue had reached the bottom of the gel. After the second dimension run, the gels were fixed and stained with Coomassie Blue as described before [14].

Duplicate gels were run for blotting and the proteins were transferred from the polyacrylamide gels onto PVDF membranes at 0.8 mA/cm² membrane for 3 h. Following transfer the ER-B labeled proteins were detected using Extra avadin peroxidase as described above for the 1D blots.

Identification of Serum Proteins Tagged with ER-B

Spots which aligned with those detected on the blots were excised from the duplicate SDS-PAGE gels. Gel plugs were trypsinized using the MassPrep Station (Waters, Micromass, UK) protocol. Identity of spots was analysed by LC/MS/MS essentially as described previously [15] with an ‘Ultimate’ nanoLC system (LC Packings, UK) using a C18 PepMap 100 nanocolumn, 15 cm x 75 µm id, 3 µm, 100 Å (LC Packings). The MS was performed using a Q-Trap (Applied Biosystems/MDS Sciex, UK) triple quadrupole mass spectrometer fitted with a nanospray ion source. The total ion current (TIC) data were submitted for database searching using the MASCOT search engine (Matrix Science, UK) using the MSDB database.

Results

Blotting to detect ER-B tagged proteins

In order to determine whether ER-B tagged proteins could be detected by blotting, 1D SDS-PAGE was carried out with purified protein and serum samples treated with different oxidising agents as described in the Methods Section. Fig. 2a and 2b show blots and the

corresponding Coomassie Blue stained gels of human serum albumin, Apo A1, and human serum. All of the treated samples show more intense bands in the blots compared to the control samples, indicative of increased formation of pyrroles in the treated samples. It is also notable that the gel of Apo A1 treated with EH shows the presence of two higher molecular weight bands not present in the control which are also detected in the western blot. This may be due to cross-links produced by the EH treatment. The bands for Apo A1 were fainter than those for HSA (Fig. 2b), possibly due to the lower proportion of lysine residues in Apo A1 resulting in a smaller number of pyrroles per molecule of protein. HSA treated with various concentrations of ribose was blotted (Fig. 3a) and different protein loadings of HSA treated with EH (Fig. 3b). This shows increasing band intensity with higher concentrations of ribose and increased protein loading producing broader, darker bands.

Proteomics to identify ER-B tagged proteins in human serum treated with oxidising agents

2DE and blotting was carried out as described in the Methods. All gels and blots were done in duplicate and spots corresponding to the blot were cut from two gels. Fig. 4 shows representative 2DE Coomassie-stained gels and corresponding blots of human serum samples treated with different reagents. Although there is little difference in appearance between the stained gels the blots show many more spots in the samples treated with the oxidising agents compared to the control blots. The spots which corresponded to those on the blots were cut and identified using mass spectrometry as described above. Fig. 5 shows the identities of the spots cut from the gel A with reference to corresponding blot B for serum treated with EH. The identities of the protein spots as determined by LC/MS/MS are listed in Table 1. In order to demonstrate the reproducibility of the protein pattern identified by blotting, 2DE blots were carried out with human serum samples prepared on four different days (Fig. 6).

Discussion and Conclusions

This study has demonstrated the feasibility of using a specially designed form of Ehrlich's reagent to target pyrroles in oxidised proteins in human serum. A wide range of mechanisms have been proposed to explain pyrrole formation in proteins, but the most common involves reaction with lipid oxidation products, including 4,5 epoxy-2-alkenals, 4-hydroxy-2-alkenals, unsaturated epoxyxoxo fatty acids and lipid hydroperoxides [3;16;17]. The initial stages of many glycation reactions are also mediated by pyrrole intermediates[18] and these may progress further to AGE products that may constitute cross-links within and between proteins. In the present study, the mechanisms of pyrrole formation in the isolated components have not yet been investigated, although further examination by mass spectrometry and other techniques will be used in future to provide information on the sources of protein damage.

Lipid oxidation products generally interact with nucleophilic amino acid residues in proteins, including lysine, histidine, free cysteine and arginine. The predominant protein labelled by the ER-B in human serum treated with oxidising agents was human serum albumin reflecting both its abundance in serum and its relatively high lysine content of 9.5% per mole [19]. Recent studies by mass spectrometry have characterized a number of compounds, including pyrroles, resulting from the reaction of proteins with oxidised linoleic acid [20]. Ehrlich's reagent specifically reacts with pyrroles and not with other moieties present in proteins such as carbonyls: this reagent has been used in many studies to detect pyrroles [7;21;22]. For reaction with the modified Ehrlich's reagent used in the present study, the only requirement is that either the 2 or the 5 position of the heterocyclic ring has to be available (see Fig. 7 for reaction scheme). This would be the case, for example, where

monoalkylpyrrole derivatives of lysyl residues are formed [3], as well as for pyrrole-mediated cross-linking involving additional lysyl, cysteinyl or histidyl residues, such as histidino-threosidine, a cross-link between lysine and histidine described by Dai et al. [23]. Earlier studies with model systems indicated that pyrrole-mediated cross-linking proceeds through an initial Michael addition mechanism and not by autoxidation of monoalkylpyrrole derivatives [24;25].

In summary, this study has shown that pyrrolized proteins in human serum can be labeled specifically using a modified Ehrlich's reagent and identified using proteomic techniques. The aim of our future studies will be to apply these techniques to define patterns of damaged proteins which characterise disease states such as diabetes and obesity. The detailed characterisation of such pyrrole adducts will provide mechanistic information on the type of damage that has occurred thus allowing the development of specific biomarkers and assays to monitor health status.

Acknowledgements

We are indebted to the Scottish Government Rural and Environment Research and Analysis Directorate and to Immunodiagnostic Systems Ltd, Boldon, UK for support

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Figure Legends

Figure 1: Preparation of Biotinylated Ehrlich's Reagent (ER-B)

Left panel, typical chromatograph showing the purification of ER-B from the reaction mixture as described in the methods section.

Right panel, MS trace of purified ER-B

Figure 2: Detection of pyrroles in human serum proteins by ER-B using western blotting

a: Left Panel, representative SDS PAGE of human serum albumin (HSA) and Apo A1 following treatment with various oxidising or glycation agents and ER-B as described in the methods section. **Right panel**, blot of the same samples.

Lane: **1.** All blue mwt markers, **2.** HSA control, **3.** HSA control 7 days 37°C, **4.** HSA 10mM glucose 7 days 37°C, **5.** HSA 10mM ribose 7 days 37°C, **6.** HSA ferric sulphate/H₂O₂, **7.** HSA EH, **8.** Apo A1 control, **9.** Apo A1 EH. Protein loaded 10 µg per lane.

b: Left panel, representative SDS PAGE of human serum following treatment with various oxidising or glycation agents and ER-B as described in the methods section. **Right panel**, blot of same samples.

1. mwt. markers **2.** Control, **3.** EH, **4.** Fe SO₄/H₂O₂, **5.** 10mM ribose 7 days 37°C. Protein loaded 10 µg per lane.

Figure 3: a: Representative SDS PAGE (left panel) and blot of HSA (right panel) incubated with different concentrations of ribose.

Lane: 1. 0, **2.** 20 mM ribose, **3.** 100 mM ribose. HSA loaded 5 µg per lane, incubated for 18 hours at 37°C followed by treatment with ER-B as described in methods section.

b: Representative SDS PAGE (left panel) and blot (right panel) of HSA treated with EH.

Lane: **1.** 0.25 µg, **2.** 0.5 µg, **3.** 1 µg, **4.** 2.5 µg, **5.** 5 µg, **6.** 10 µg , **7.** 15 µg

Figure 4: Representative images of 2DE gels and blots of human serum showing ER-B labeled proteins.

Top: Coomassie-stained gels, **Bottom:** blots. Experimental procedures are as described in the methods section.

Figure 5: Representative 2DE gel and blot of human serum treated with EH showing protein spots identified by LC/MS/MS.

Top: Coomassie-stained gel, **Bottom:** blot. Experimental procedures are as described in the methods section.

Numbered spots indicate protein spots identified by LC/MS/MS. Spots inside dotted lines have been identified as the same protein. See table 1 for protein identification.

Figure 6: 2DE blots of Human Serum treated with EH prepared on 4 different days

Experimental procedures were as described in the methods section.

Figure 7: Reaction of Biotinylated Ehrlich's Reagent(ER-B) with protein bound pyrroles.

Either the 2 or the 5 position of the heterocyclic ring of the protein bound pyrrole needs to be available for the reaction to take place.

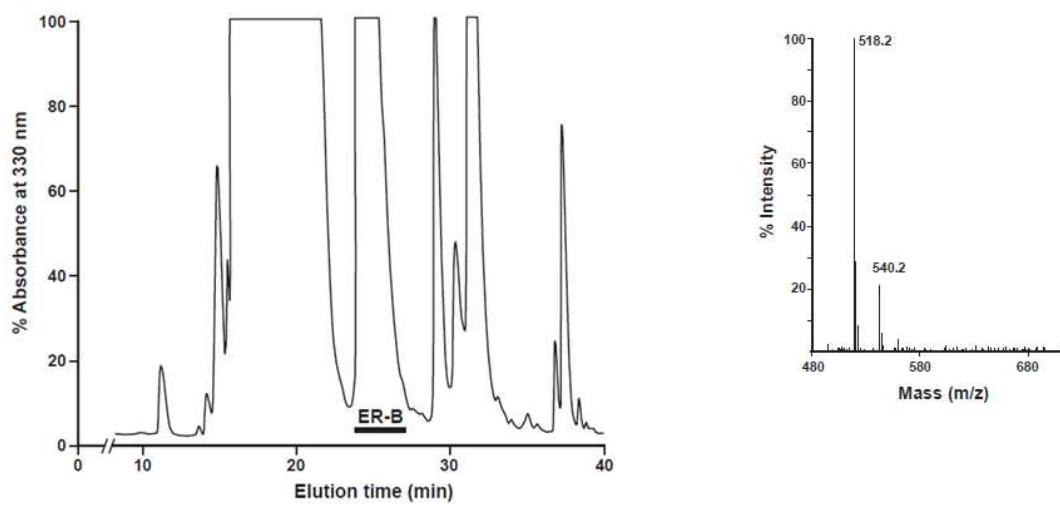


Figure 1

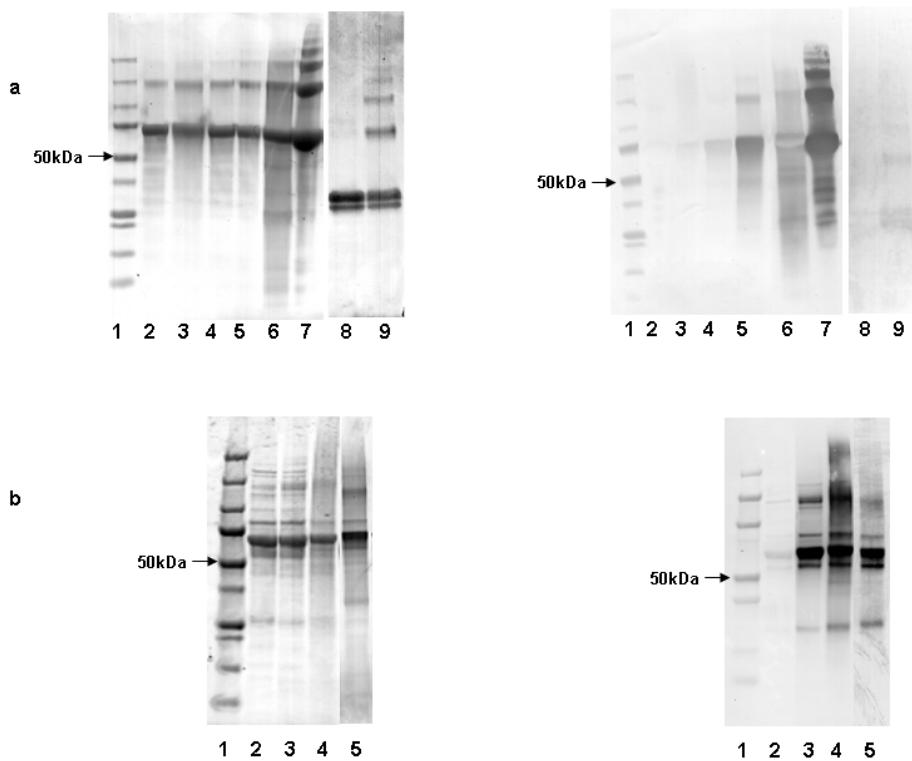


Figure 2

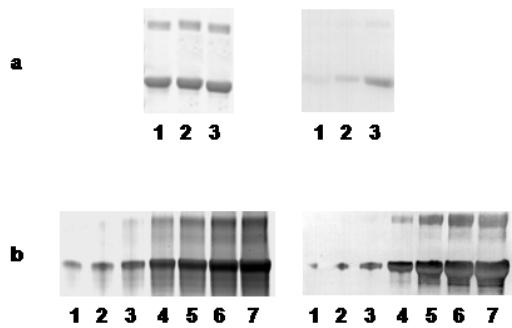


Figure 3

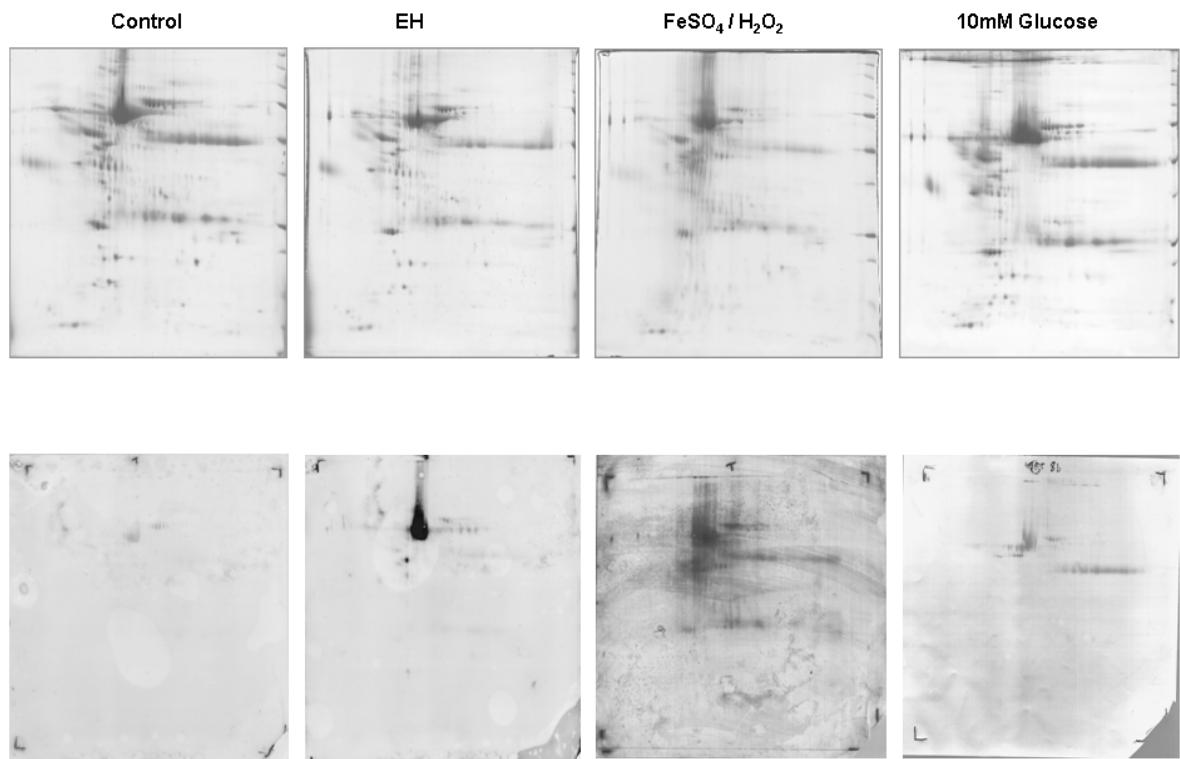


Figure 4

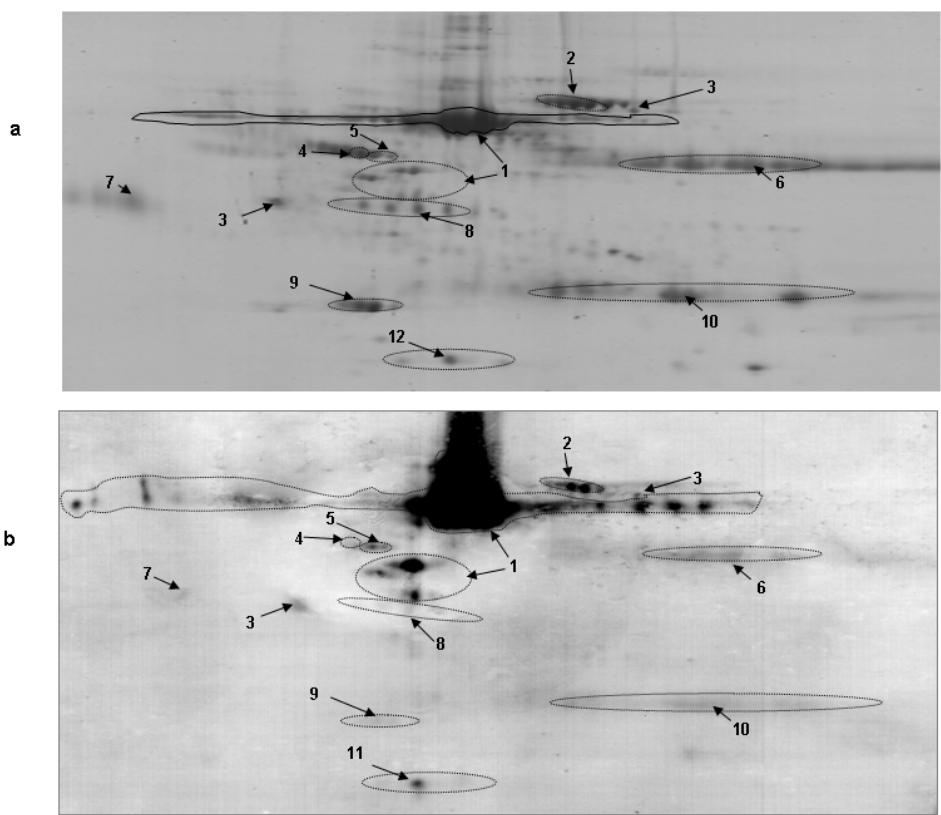


Figure 5

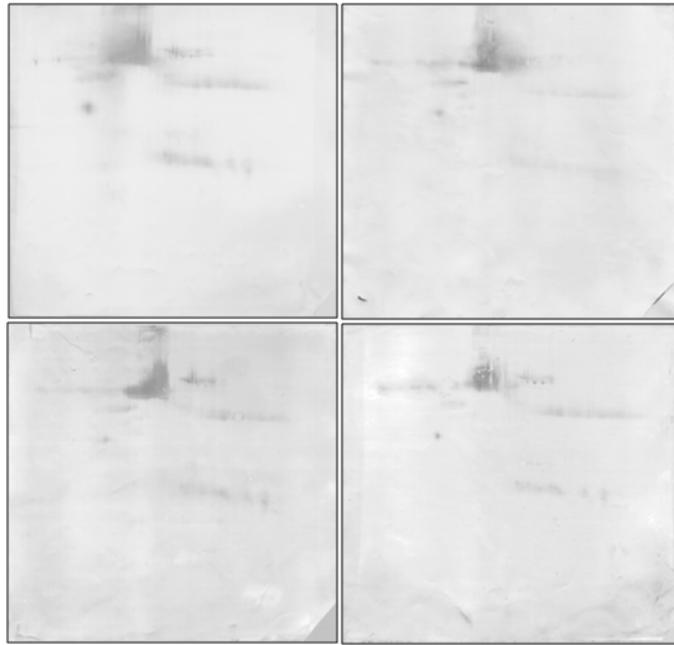


Figure 6

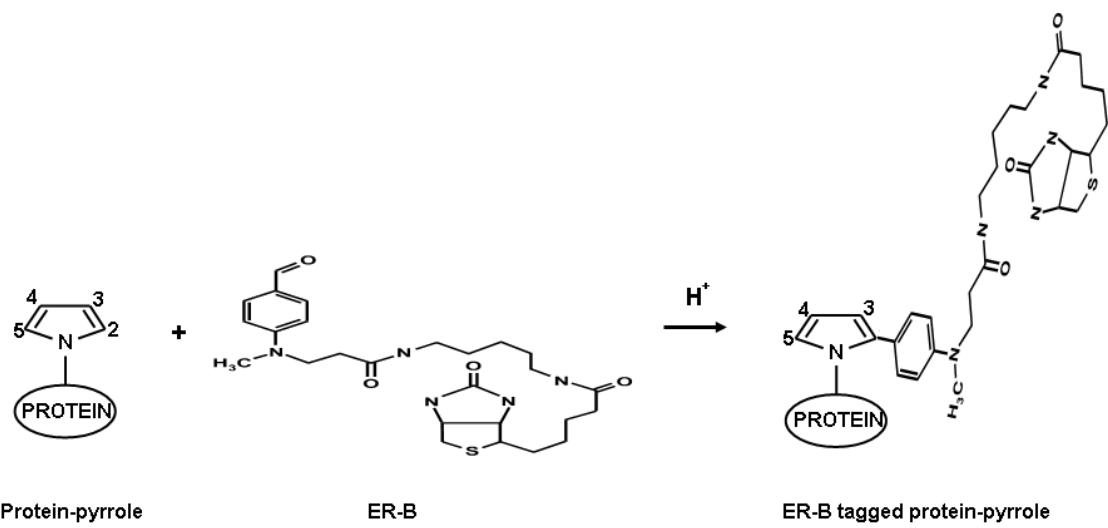


Figure 7

Table 1: Protein Identification by LC/MS/MS

<i>Spot</i>	<i>Accession No.</i>	<i>Protein</i>	<i>Peptides matched</i>
1	1AO6A	Serum albumin, chain A -human	34
2	TFHUP	Transferrin precursor-human	29
3	C3HU	Complement C3 precursor-human	8
4	ITHU	Alpha-1-antitrypsin precursor-human	12
5	Q6GTG1_HUMAN	Vitamin D-binding protein-human	8
6	GHHU	Ig gamma-1 chain C region	5
7	OMHU2	Alpha-1-acid glycoprotein 2 precursor	3
8	Q6NSB4_HUMAN	Haptoglobin	10
9	LPHUA1	Apolipoprotein A-I precursor	5
10	1B2WL	Antibody v domain and c domain, chain L	6
11	HPHU2	Haptoglobin precursor, allele 2	2