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# Redox options in two-dimensional electrophoresis

*Review Article*

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**Summary.** Two-dimensional electrophoresis is usually run on fully reduced samples. Under these conditions even covalently bound oligomers are dissociated and individual polypeptide chains may be fully unfolded by both, urea and SDS, which maximizes the number of resolved components and allows their pI and $M_r$ to be most accurately evaluated. However, various electrophoretic protocols for protein structure investigation require a combination of steps under varying redox conditions. We review here some of the applications of these procedures. We also present some original data about a few related samples – serum from four species: *Homo sapiens, Mus musculus, Rattus norvegicus, Bos taurus* – which we run under fully unreduced and fully reduced conditions as well as with reduction between first and second dimension. We demonstrate that in many cases the unreduced proteins migrate with a better resolution than reduced proteins, mostly in the crowded '$\alpha$-globulin' area of pI 4.5–6 and $M_r$ 50–70 kDa.

**Keywords:** Cysteine – Cystine – Reduction – Alkylation – Oxidation – Two-dimensional electrophoresis – Serum

**Abbreviations:** 1DE, 2DE, one- and two-dimensional electrophoresis; apo, apolipoprotein; CA, carrier ampholytes; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; DTT, dithiotreitol; EDTA, ethylendiaminotetracetic acid; -eeo, electroendosmotic flow; Gc, Gc globulin, or vitamin D-binding protein; GGE, gradient gel electrophoresis; Ig, immunoglobulin; IPG, immobilized pH gradient; MS, mass spectrometry; NL, non-linear; PAA, polyacrylamide; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS

## 1 Introduction

In separation science *two-dimensional* is a broad term encompassing many combinations of analytical procedures. One of the oldest such examples was sequential electrophoresis and chromatography by which the proteolytic peptides from a protein could be separated to produce a diagnostic pattern – the so-called *fingerprint* of the protein (Ingram, 1959). The most recent developments are the sequential chromatographic separations before MS identification to inventory all protein components in a complex sample (multidimensional protein profiling technology such as in (Fujii et al., 2004)).

The highest resolution is obtained by use of independent separation principles in the two steps of an analytical protocol. A further requirement is the opportunity of evaluating relevant chemico-physical parameters of the resolved components. For proteins these considerations have led to the generalized use of the sequence between separation according to charge (pI) by isoelectric focusing and separation according to size ($M_r$) by SDS-PAGE. In order to resolve all gene products vs homo- and heteropolymeric assemblies and to correctly assess pI and $M_r$ it is customary to run fully unfolded, fully reduced samples.

In this report we review literature data on alternative approaches to the 'fully reduced' option and present our data on the parallel analysis of a few samples under different redox conditions.

## 2 Redox conditions in electrophoresis: A survey

### *2.1 Elucidation of structural aspects*

#### 2.1.1 1DE

Running SDS-PAGE in parallel under non-reducing and reducing conditions and comparison of the SDS-PAGE data with results from gel permeation experiments used to be 'the' standard procedure for elucidation of the quaternary structure and the pattern of S–S bonding in purified proteins. Recent examples of this approach (using 2DE techniques as well) include (Wearsch and Nicchitta, 1996) and (Nair et al., 2000). Figure 1A shows schematically the possible results for a hypothetical protein run under non-reducing (left) and under reducing conditions (middle to right), and of their interpretation. Two proteins with the same apparent $M_r$ in non-reducing electrophoresis may migrate as a double band under reducing conditions (for instance, (Vollmer et al., 1995) with a single chain and a homodimeric protein). Homologous proteins may display different disulfide polymerization (redox forms) (such as in (Bromage et al., 2004)) and are easily compared with this approach. As explained in the discussion, $M_r$ can only be correctly assessed in the absence of disulfide bridges. However, the offset of band migration between non-reducing and reducing conditions enables estimation of the number of such bridges or, more precisely, about the restraints such bridges impose on protein unfolding (Fig. 1B). Creighton

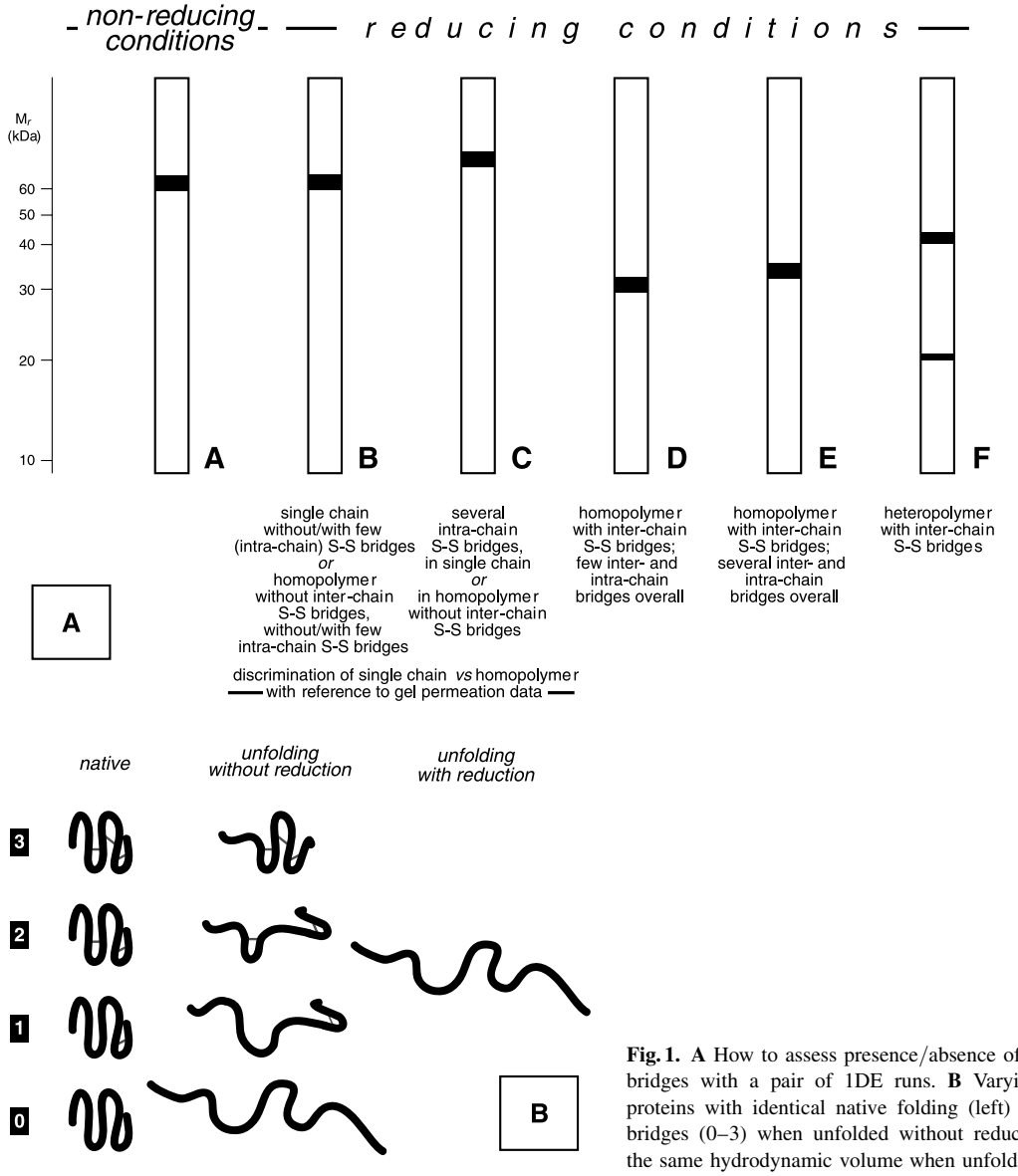


**Fig. 1. A** How to assess presence/absence of intra- or inter-chain disulfide bridges with a pair of 1DE runs. **B** Varying hydrodynamic volume of proteins with identical native folding (left) but different number of S–S bridges (0–3) when unfolded without reduction (middle). They all have the same hydrodynamic volume when unfolded after reduction (right)

(Creighton, 1980) introduced a method of counting the number of cysteine residues in proteins, based on thiol alkylation with mixtures of iodoacetic acid and iodoacetamide. Varying the ratio between the neutral and the acidic blocking reagent enables the formation of N + 1 derivatives (where N = number of cysteines) with from zero to N additional acidic groups, which may be resolved by isoelectric focusing and counted. Each reaction mixture contains a subset of the products, and the various mixes and their pool need to be run side by side.

At the glomerular level the kidney creates a protein ultrafiltrate: functional impairment of the basal membrane results in the escape of higher $M_r$ proteins, including $\alpha_2$-macroglobulin, haptoglobin, IgA, IgG and transferrin, into the urine. The kidney tubules reabsorb low $M_r$ proteins from the ultrafiltrate; impairment of tubular function results in increased levels of $\alpha_1$-microglobulin, retinol binding protein, immunoglobulin light chains, lysozyme and $\beta_2$-microglobulin in urine. Assessment of glomerular versus tubular impairment is based on the size of the two populations of proteins – those with $M_r$ higher or lower than albumin; therefore for diagnostic purposes the best experimental conditions require the use of non-reduced samples. (It should be noted that routine hospital procedures are based on zonal agarose electrophoresis rather than on SDS-PAGE, although the latter technique affords higher resolution and better definition of the stained bands.) Electrophoresis of urinary proteins is reviewed in (Schiwara et al., 1986; Weber, 1988; Bianchi-Bosisio et al., 1991; Marshall and Williams, 1991), and specific applications to Bence-Jones paraproteins are described by (Marshall and Williams, 1999; Miller et al., 2004).

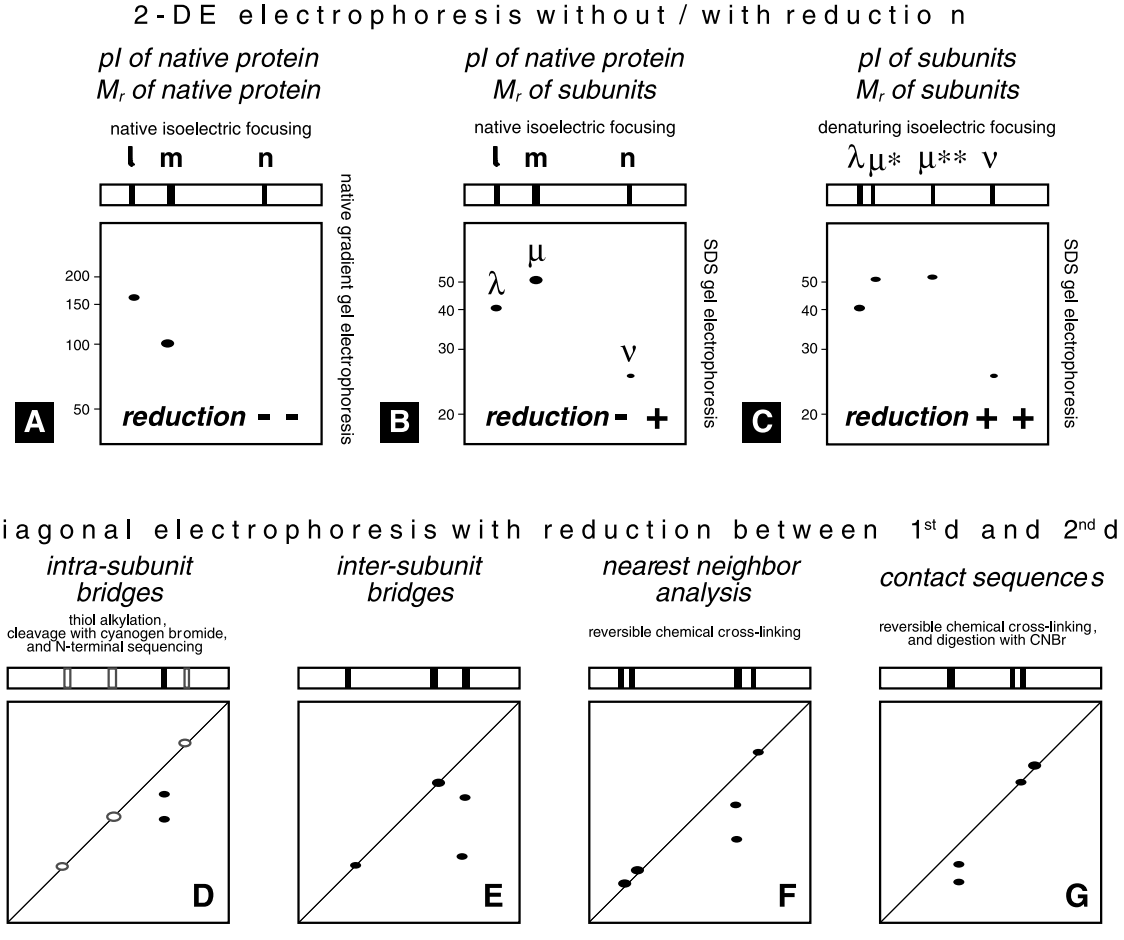


**Fig. 2. A–C** How to assess the quaternary structure of a protein while evaluating pI and $M_r$ of the native structure and of its constituent subunits – with a series of 2DE runs. In the hypothetical protein mixture in figure, *l* is a homotetramer (of $\lambda$ chains), *m* is a heterodimer ($\mu^*$ and $\mu^{**}$ subunits differing by charge) and *n* is a single-chain, low $M_r$ protein (lost from GGE). In **A**, native isoelectric focusing is followed by native GGE, both steps without sample reduction ($-\ -$); in **B**, native isoelectric focusing without reduction is followed by SDS-PAGE with reduction ($-\ +$); in **C** denaturing isoelectric focusing is followed by SDS-PAGE, both steps being run under reducing conditions ($+\ +$). **D–G** Different ways to use diagonal electrophoresis either to locate intra- or inter-subunit native disulfide bridges in individual protein molecules or to define the topology of supramolecular assemblies after reversible chemical cross-linking

2.1.2 Possible combinations in 2DE (Fig. 2)

*– 2.1.2.1 Runs with fully native, non-reduced samples*

This combination addresses the evaluation of charge and size of proteins with intact quaternary structure – whether or not stabilized by inter- or intra-chain disulfide bridges. For the second dimension electrophoresis on polyacrylamide gradient gels is virtually the only choice, the first dimension may be either (native) isoelectric focusing or zonal agarose electrophoresis. The latter assesses charge densities rather than pI, but is less prone to problems due to protein solubility and matrix sieving (agarose isoelectric focusing is not easily driven to equilibrium due to -eeo (electroendo-osmotic flow)).

There are three main fields of application of these methods. One is the analysis of serum/plasma samples using a general protein stain for visualization, which detects proteins of $M_r \geq$ transferrin. A miniaturized gel format could enable rapid clinical diagnostic analyses with higher resolving power than routine 1DE methods, including zymography (Manabe et al., 1979, 1980, 1981, 1982a, b, 1987a, b; Visvikis et al., 1987; Shimazaki et al., 1998; Manabe et al., 1999; Shimazaki et al., 1999, 2000; Manabe et al., 2003; Shimazaki et al., 2003, 2004a, b). A second field of application of native 2DE is the analysis of lipoproteins (Miida et al., 1998, 2000, 2003, 2004). Although considered a reference procedure for research purposes, it is not routinely applied diagnostically. Whole plasma is loaded without lipoprotein purification or enrichment and the apolipoprotein(s) or accessory protein(s) of interest are usually detected by immunoblotting. This provides data on composition of the lipoprotein particles, enabling assessment of protein–protein interactions, which is at the core of structural proteomics. A third area of application of native 2DE is the analysis of supramolecular assemblies of integral membrane proteins, such as mitochondrial (Tiranti et al., 1999; Jesina et al., 2004) and chloroplast complexes. The protocol is described as 'Blue native' (Schägger and von Jagow, 1991). Pelleted organelles are solubilized with dodecyl maltoside (1 g of detergent per 1 g of protein). After centrifugation Serva Blue G dye is added to the supernatant at a concentration of 0.1 g/g of detergent and is also included in the cathode buffer at a concentration of 0.02% w/v. The dye induces a charge shift on the proteins, while the buffer used for the gel phase (aminocaproic acid titrated with Bistris) improves solubilization of the membrane proteins.

Another reason to prefer native conditions during the electrophoretic separation is for the evaluation of the immunoreactivity of potential antigens and allergens. Many epitopes are spatial rather than sequential and may not be recognized once protein is unfolded e.g. by SDS. Neutral or zwitterionic detergents are used instead in order to recover at least some of the hydrophobic proteins (Hird et al., 2000).

Urine analysis by native zone electrophoresis in combination with non-reducing SDS-PAGE (Lapin and Feigl, 1991) is a related methodology which also lacks a reducing step.

*– 2.1.2.2 Sample redox between first and second dimension*

– 2.1.2.2.1 Identical protocol in first and second dimension except for redox conditions (diagonal electrophoresis, Fig. 2, panels D–G)

In diagonal electrophoresis an identical separation protocol is applied in both dimensions but the sample is chemically treated between first and second dimension: unmodified components migrate along a diagonal, whereas modified components are observed off the diagonal. This experimental approach allows the investigation of a single structural feature per run – most often the redox state of the protein. The first protocols were devised in connection with 'fingerprinting' i.e. mapping by paper electrophoresis the peptides from enzymatic or chemical proteolysis of a test vs a reference protein. Exposure of the first dimension electropherogram to performic acid vapour oxidizes cysteines and cystines to cysteic acid and results in a change in number and/or position of the spots when rerun orthogonally (Brown and Hartley, 1966). Another approach for isolation with this procedure of sulfhydryl peptides is alkylation with N-ethylmaleimide (Gehring and Christen, 1983). Parallel protocols have been devised for carboxyl-terminal (Bietlot et al., 1989), phosphoserine- (Milstein, 1967), arginine- (Patthy and Smith, 1975) and tryptophan-containing peptides (Veronese et al., 1975).

Diagonal electrophoresis may be used for defining disulfide pairing i.e. the position of intrachain cystines in combination with thiol alkylation, cleavage with cyanogen bromide, and N-terminal sequencing (Leary et al., 1979; Wei et al., 2000).

More frequently it is used for analyzing composition and topology of supramolecular complexes. Nearest-neighbor analysis requires reversible chemical cross-linking of the components with cleavable reagents (Bloxham and Sharma, 1979; Hultin, 1986). The analysis may address identification of subunits or definition of contact sequences in neighboring proteins after digestion e.g. with cyanogen

bromide (Hultin and Nika, 1986). Diagonal electrophoresis has been applied to the analysis of virus capsid (Takemoto et al., 1977), ATPase (Hermolin et al., 1983), RNA polymerase II (Cervoni et al., 1994), photosystem I (Jansson et al., 1996) and II (Harrer et al., 1998; Zhang and Scheller, 2004) holocomplexes. A physiological process investigated this way was the assembly of fibrous proteins (collagen (Colombatti et al., 1987), keratins (Hatzfeld and Weber, 1990)); pathological issues studied were age- or oxidative stress-related *in vivo* and *in vitro* protein cross-linking (cataract (Kodama and Takemoto, 1988), UVA light irradiation (Giblin et al., 2002), hydrogen peroxide, S-nitroso-N-acetylpenicillamine, doxorubicin, simulated ischemia, or metabolic inhibition (Brennan et al., 2004)).

Radioactive alkylation and diagonal electrophoresis are useful for localization (e.g. intra- vs inter-chain) of the susceptible disulfide bridges and also for partial reduction experiments, such as in (Cunningham-Rundles and Lamm, 1975). The most easily reduced half-cysteines may be identified with an approach similar to limited proteolysis, which targets solvent-exposed peptide bonds in flexible loops.

– 2.1.2.2.2 First and second dimension with different electrophoretic techniques (Fig. 2, panels A–C)

The possible combinations are extensive and many of them have been attempted several times – including the reverse order to standard in 2DE, i.e. SDS-PAGE as first dimension and IEF as second dimension, for stubbornly insoluble proteins (Stan-Lotter and Bragg, 1986) – but usually not in connection with alternative redox approaches. The most meaningful combinations are indeed, for the first dimension: a) native, zonal or disc, electrophoresis either on homogeneous concentration or gradient PAA gel, or native isoelectric focusing, or b) electrophoresis or isoelectric focusing in urea, followed by SDS-PAGE in second dimension. Sample reduction between first and second dimension allows to connect non-covalently and covalently bound quaternary structures (as existing in water and in urea, respectively) with their constituent subunits. Results presented and discussed in Section 4 demonstrate that there may be further, although non-systematic, effects from these procedures, with specific advantages in some separation protocols.

– 2.1.2.2.3 Sample reduction at the outset ..... i.e. standard 2DE

With this 'standard' approach the aim is to resolve individual polypeptides under dissociating and denaturing conditions as with first dimension in the presence of urea and second dimension in the presence of SDS. This results in the maximization of the number of resolved components and allows the pI and the $M_r$ of each of them to be evaluated with the maximum of accuracy. These physicochemical parameters are direct consequences of the primary structure and may be computed from the amino acid sequence. High concentrations of urea, plus or minus thiourea and various non-ionic and zwitterionic detergents, in a reducing milieu, are a fairly effective solubilization medium for at least some of the integral membrane structures and most of the cytoplasmic proteins. This allows a representative sample of the protein components of a tissue specimen to be extracted and subjected to fractionation and identification – which is the basis to proteomic investigation. It is then most rational that the outlined (and well-known) protocol has become 'standard'. However, at least in terms of resolution, and for some specific groups of proteins, the standard conditions may be inferior to non-conventional protocols – as demonstrated in Section 4.

### *2.2 Artefacts*

The conditions required for complete and stable reduction of disulfide bridges in proteins during electrophoresis has been an issue since the introduction of SDS-PAGE. Boiling for a short time, or incubating at above room temperature for longer periods in the presence of SDS, enhances access and facilitates the action of the reducing agent on the target disulfides. However tightly folded proteins or those with an exceptionally high number of disulfides may be difficult to react (Fig. 3). Increasing the concentration of 2-mercaptoethanol or DTT above standard levels may sometimes afford a solution. Thiols are weak acids and their thiolate anions are mobilized from the sample wells during electrophoretic runs in alkaline media such as the trailing zone of discontinuous buffer systems (Gianazza and De Ponti, 1993). The high protein concentration at decreased absolute and relative reducing agent concentration in the stacking gel phase may result in thiol reoxidation, often producing artefactual patterns. These considerations apply more severely under non-denaturing conditions and to separations, e.g. by isoelectric focusing, in the presence of 8 M urea. In these protocols boiling/heating are impracticable and high amounts of thiols heavily buffer the alkaline region and distort the course of the gradient (Righetti et al., 1982). Essentially three kinds of remedies have been proposed for these problems of incomplete reduction and re-oxidation. The first protocol to be applied was the formation of a curtain of reducing agent through the separation medium, from a cathodic

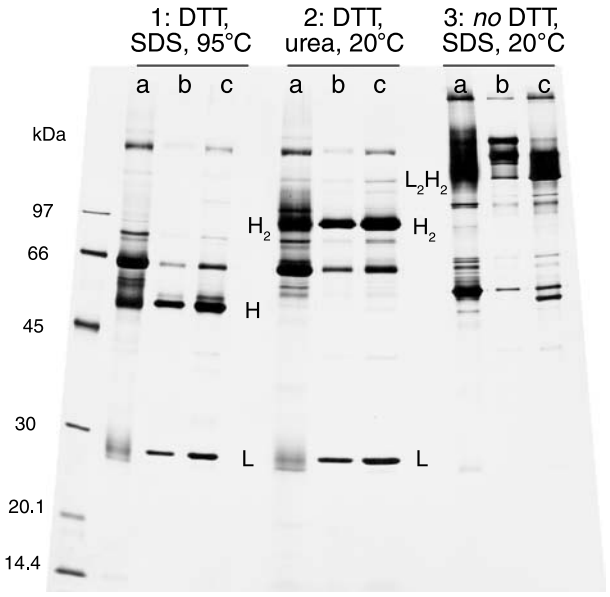


**Fig. 3.** SDS-PAGE on 10–15% T PAA and silverstaining. Leftmost lane: LMW Amersham; middle-right lanes: horse serum samples, a normal animal (a = 1:100) and two different dilutions of a sample with monoclonal gammopathy (b = 1:500, c = 1:200), differently treated: 1) reduced with DTT in SDS buffer, 5 min 95°C, addition of glycerol and BPB after cooling; 2) diluted with IPG sample mix (urea, CHAPS, DTT, alkaline ampholytes), left overnight at 10°C (corresponding to IPG run), then addition of glycerol and BPB; 3) unreduced samples, diluted with SDS buffer with glycerol and without DTT, incubated 15 min at room temperature. Light (*L*-) and heavy (*H*-) chains of IgG are found in different "combinations"

reservoir (thiolic acid being used in SDS-PAGE (Nakamura et al., 1989), DTT on IPG (Altland et al., 1988)). The second is the use of reducing agents with more favorable redox potential than thiols. One such reagent, tributylphosphine (Ruegg and Rudinger, 1977), has found its way to a commercial kit for sample preparation in provision for isoelectric focusing on immobilized pH gradients and 2DE. The third is making reduction irreversible through S-alkylation with iodoacetamide or N-ethylmaleimide (e.g. (Fratelli et al., 2002)). Organic disulfides in the focusing medium effectively prevent sulfide reoxidation in alkaline media thus preventing multiple banding and/or horizontal streaking in 2-DE maps (Olsson et al., 2002) (hence the commercial name of DeStreak® to hydroxyethyl disulfide as commercialized by Amersham-Pharmacia).

## 3 Examples of 2DE of heteropolymeric proteins under different redox conditions: Immunoglobulins

Immunoglobulins (Ig) are complex molecules comprising four polypeptides, two identical heavy (G, A, M, D, or E) and two identical light chains (kappa or lambda), with different degree of polymerization (mono-, di-, pentamers). They are produced by the body as humoral defence against foreign antigens, and secreted into the bloodstream. Being specifically directed against those antigens, they show high sequence variability, which contributes to heterogeneity in electrophoretic pattern. Perturbations of the immune system may result in formation of aberrant peptides and proteins e.g. production of monoclonal (all deriving from a single B-cell clone) whole antibodies or single heavy or light chains (Stites et al., 1987). Classical 2DE of serum or urine, under reducing conditions, resolves Ig into their constituent chains which are found at different positions of the gels (Tracy et al., 1982; Büeler et al., 1995) and http://www.expasy.org/ch2d/. By omitting reduction in all 2DE steps, overproduction or asynchronous production of single chains, both heavy and light, can be detected (Miller et al., 2004; Miller and Goldfarb, 2005).

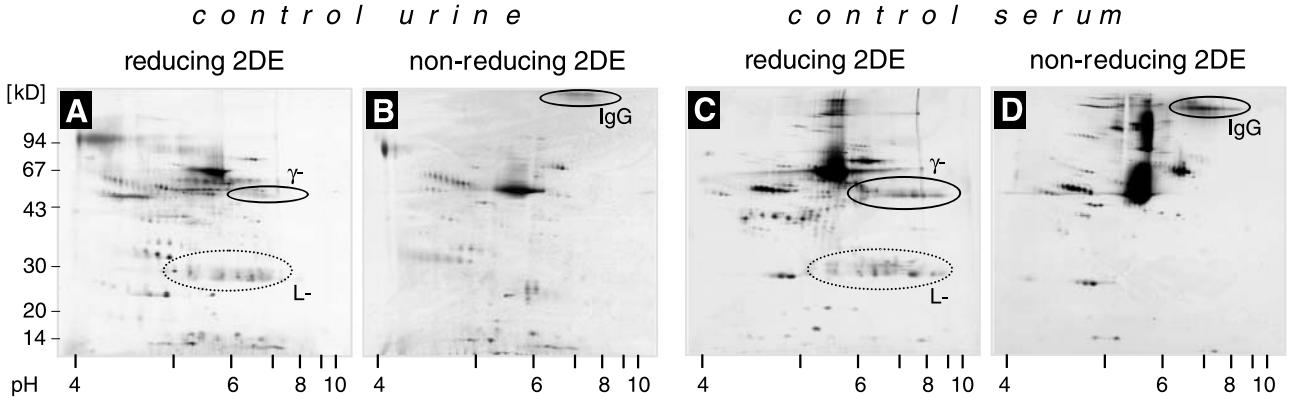


**Fig. 4.** 2DE under different redox conditions: Serum and urine of a healthy human volunteer. **A**, **C** Sample reduction before first dimension and reduction/alkylation between first and second dimension; **B**, **D** omission of DTT in all steps of the protocol. IPG 4–10 NL, 10–15% T, silverstaining. 12 $\mu$g protein load

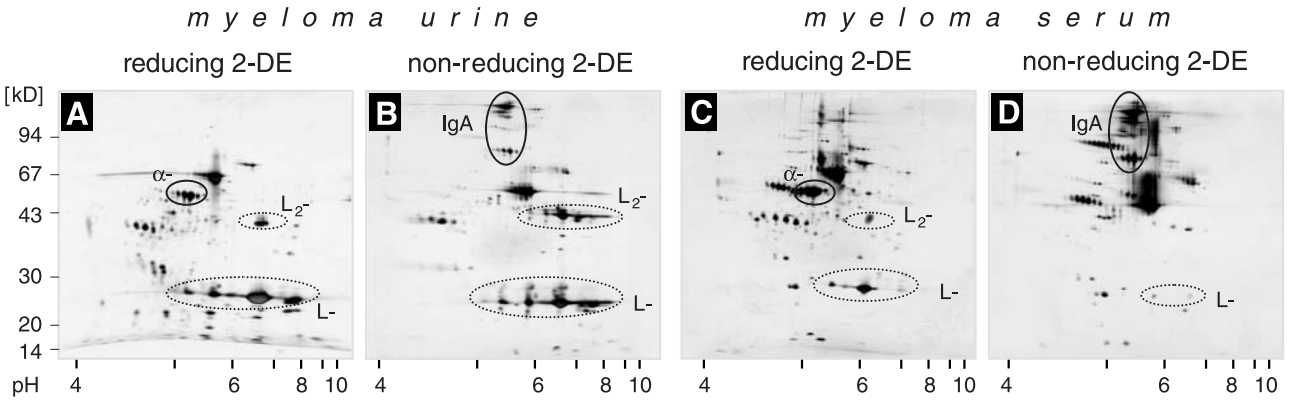


**Fig. 5.** 2DE under different redox conditions: Serum and urine of a patient with monoclonal gammopathy of IgA-type. **A**, **C** sample reduction before first dimension and reduction/alkylation between first and second dimension; **B**, **D** omission of DTT in all steps of the protocol. IPG 4–10 NL, 10–15% T, silverstaining. Protein load = 12 $\mu$g

Figures 4 and 5 show serum and urine of a healthy person separated under reducing and non-reducing conditions (Fig. 4). In serum IgG predominates, but only trace amounts are found in urine (as high molecular weight proteins are filtered out from urine by the intact kidneys). It is detected as single chains ($\gamma$-, L-) or as complete molecule, depending on the electrophoretic conditions. Figure 5 in contrast displays samples from a patient with plasmacytoma. In this type of disease, large amounts of monoclonal antibody are found in serum, whereas in urine there is usually an excess of light chains. Accordingly, classical 2DE detects light and heavy chains (in this case, IgA and $\alpha$-chain are predominant in serum), both in serum and urine. Only in non-reducing 2DE (Fig. 5, panels B, D) it becomes apparent that esp. in urine there are excess light chains, present mainly as monomer (L-), but also as dimer ($L_2$-). This is not revealed in reduced samples, although one might guess when there are gross differences in the distribution of single chains.

## 4 Examples of 2DE of monomeric proteins under different redox conditions: Sera from various animal species

### *4.1 Samples*

Sera were obtained from four species:

- *Homo sapiens* (one 30 year old male)
- *Mus musculus* (five adult males, CD1 strain)
- *Rattus norvegicus* (five adult males, Sprague-Dawley strain)
- *Bos taurus* (one Frisian cow).

The human healthy volunteer gave informed consent to blood collection. Mice and rats were housed and treated at Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, in compliance with local rules, which conform to national and international guidelines. Bovine blood was obtained during routine veterinary tests on herds.

### *4.2 Reduction*

**Table 1.** Procedures for sample reduction

| | A: no reduction | B: intermediate reduction | C: reduction |
|---|---|---|---|
| first dimension | dilution with distilled water | dilution with distilled water | dilution with 2% $\beta$-mercapto-ethanol |
| second dimension | equilibration in 3% SDS | equilibration in 2% $\beta$-mercapto-ethanol/ 3% SDS | equilibration in 2% $\beta$-mercapto-ethanol/ 3% SDS |

### *4.3 Reduction and alkylation*

**Table 2.** Procedures for sample reduction and alkylation

| Reducing agent | Passive in-gel rehydration | Active in-gel rehydration | Lay-on cathodic sample application |
|---|---|---|---|
| tributyl-phosphine | √ | √ | |
| $\beta$-mercapto-ethanol in water | | √ | √ |
| $\beta$-mercapto-ethanol in alkaline buffer/SDS | | √ | √ |

4.3.1 Tributylphosphine as reducing agent

Aliquots of 5 $\mu$L of serum were diluted with 300 $\mu$L in 7 M urea/2 M thiourea/4% w/v CHAPS/1 mM EDTA/2 mM Tris and reduced with tributylphosphine and alkylated with iodoacetamide in the ProteoPrep$^{TM}$ kit (Sigma) according to the manufacturer's instructions. At the end of the procedure the CA (4 $\mu$L) and bromophenol blue (1.5 $\mu$L saturated solution in water) were added to the samples.

4.3.2 $\beta$-Mercaptoethanol as reducing agent

Aliquots of serum were diluted 1:10 with either distilled water or 1% w/v SDS in 100 mM Tris/HCl pH 8.8, then made 0.5% 2-mercaptoethanol and incubated for 1 h at 37°C. The solution was then added with iodoacetamide to a final concentration of 100 mM and incubated 1 h at room temperature, in the dark. The proteins were precipitated with 9 volumes of acetone at −20°C and washed three times. The pellet was let dry at room temperature and dissolved with either 8 M urea or the urea/thiourea/CHAPS/EDTA/CA mixture (see 4.3.1).

*4.4 Electrophoretic procedures*

Samples were run in 2DE by the IPG-DALT procedure with laboratory-made IPGs (NL 4–10) for the first dimension and 7.5–17.5% T PAA gels for the second dimension. Slab size was $18 \times 18\,cm^2$.

4.4.1 Cathodic sample application

The whole IPG gel was reswollen in 8 M urea/0.5% CA. Gel strips, 7 mm wide, were cut on an intact GelBond$^{TM}$ backing. Application (25–50 $\mu$L) was by absorption in 1–2 paratex pads (cat. 80-1129-46 from Amersham-Pharmacia).

This procedure was used for all reduced samples (listed in 4.2) and for two instances of reduced/alkylated samples (as in 4.3.2, 8 M urea as solvent).

The first dimension run included a ramp from 300 to 800 V in 5 hours, followed by 800 V overnight and 3,500 V for 1 h. Equilibration before the second dimension was for 15 min with 3% SDS in Tris/glycine buffer (Gianazza et al., 1985; Gianazza et al., 2002).

4.4.2 In-gel rehydration

Slabs (gel + backing) were cut into 3.5 mm wide strips and were rehydrated either passively or actively (for >12 h) with 300 $\mu$L of sample (reduced and alkylated, see 4.3.1 and 4.3.2). The rehydrated gels were then run overnight in a Protean IEF$^{TM}$ cell (BioRad) according to the 'rapid ramp' protocol as defined in the manufacturer's instructions. Equilibration before second dimension was for 15 min with 2% SDS/6 M urea in Tris/glycine buffer.

*4.5 MS identifications*

Spots of interest were excised from the gel slabs. In-gel trypsinolysis was performed using an Investigator Progest (Genomic Solutions) robotic digestion system (Wait et al., 2001). The resulting mixtures of peptides were characterized by tandem electrospray high-performance liquid chromatographic mass spectrometry using a Q-TOF spectrometer interfaced with a Waters CapLC chromatograph (Waters). Uninterpreted tandem mass spectra were correlated to entries in SwissProt/TREMBL using ProteinLynx Global server (version 1.1, Waters) (Wait et al., 2002). Candidate identifications were verified by manual interpretation of the spectra.

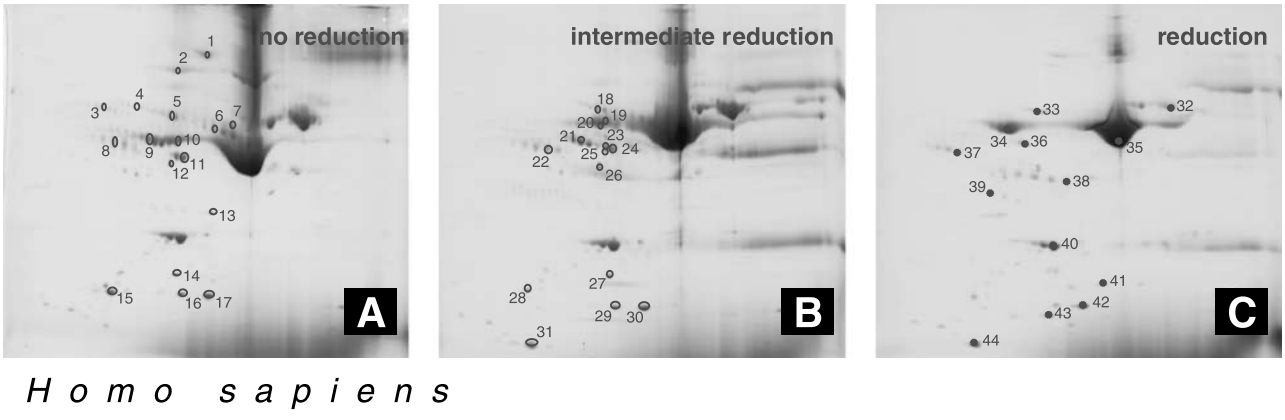


**Fig. 6.** 2DE of a human serum sample under different redox conditions. **A** No sample reduction; **B** reduction between first and second dimension; **C** sample reduction before first dimension. IPG 4–10 NL, 7.5–17.5% T, Coomassie staining

*4.6 Comparative serum patterns*

4.6.1 Sample reduction

The patterns of the four test sera run under varying redox conditions are shown in Figs. 6–9. Figure 6 corresponds to human samples, Fig. 7 to mice, Fig. 8 to rats and Fig. 9 to bovines. In all series, Panel A corresponds to 'no reduction', Panel B to 'intermediate reduction' and Panel C to 'reduction'.

In Panels A and B, the spots excised for MS processing are marked by numbers. The same numbers apply to the entries in Tables 3–6 with MS identification and list

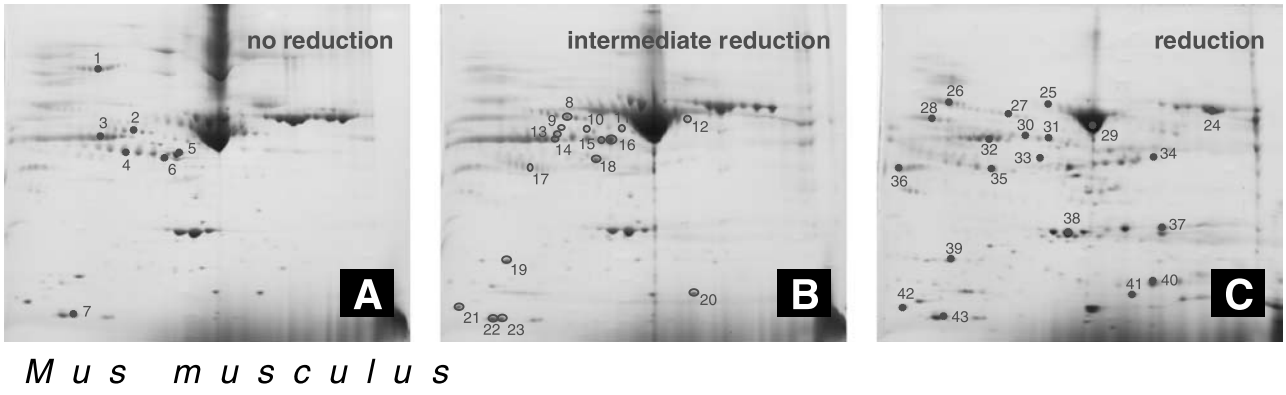


**Fig. 7.** 2DE of a mouse serum sample under different redox conditions. **A** No sample reduction; **B** reduction between first and second dimension; **C** sample reduction before first dimension. IPG 4–10 NL, 7.5–17.5% T, Coomassie staining

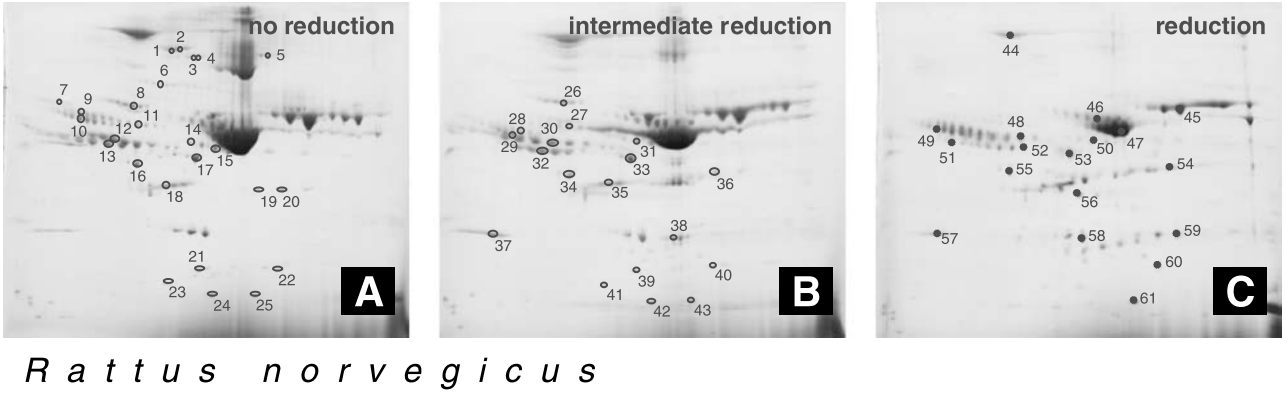


**Fig. 8.** 2DE of a rat serum sample under different redox conditions. **A** No sample reduction; **B** reduction between first and second dimension; **C** sample reduction before first dimension. IPG 4–10 NL, 7.5–17.5% T, Coomassie staining

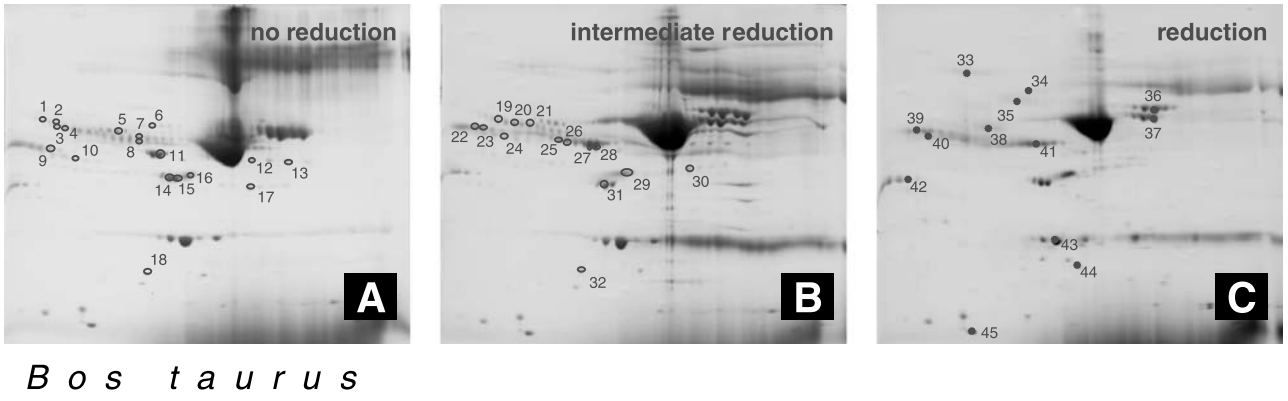


**Fig. 9.** 2DE of a bovine serum sample under different redox conditions. **A** No sample reduction; **B** reduction between first and second dimension; **C** sample reduction before first dimension. IPG 4–10 NL, 7.5–17.5% T, Coomassie staining

**Table 3.** Human serum

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| *1st dimension and 2nd dimension under non-reducing conditions, in Fig. 6A* | | |
| 1 | ALBU_HUMAN Serum albumin/ | (K)LVNEVTEFAK(T)<br>(K)KVPQVSTPTLVEVSR(N)<br>(K)VFDEFKPLVEEPQNLIK(Q)<br>(K)ALVLIAFAQYLQQCPFEDHVK(L)<br>(K)SHCIAEVENDEMPADLPSLAADFVESK(D) |
| | A1AT_HUMAN Alpha-1-antiproteinase | (K)ITPNLAEFAFSLYR(Q)<br>(K)ELDRDTVFALVNYIFFK(G) |
| 2 | ALBU_HUMAN Serum albumin | (K)AVMDDFAAFVEK(C)<br>(K)TCVADESAENCDK(S)<br>(K)QNCELFEQLGEYK(F)<br>(R)HPYFYAPELLFFAK(R)<br>(K)VFDEFKPLVEEPQNLIK(Q)<br>(K)ALVLIAFAQYLQQCPFEDHVK(L)<br>(R)MPCAEDYLSVVLNQLCVLHEK(T)<br>(K)QNCELFEQLGEYKFQNALLVR(Y)<br>(K)SHCIAEVENDEMPADLPSLAADFVESK(D) |
| 3 | CLUS_HUMAN Clusterin | (R)ELDESLQVAER(L)<br>(R)ASSIIDELFQDR(F)<br>(R)EILSVDCSTNNPSQAK(L)<br>(R)VTTVASHTSDSDVPSGVTEVVVK(L) |
| 4 | CLUS_HUMAN Clusterin | (R)ELDESLQVAER(L)<br>(R)ASSIIDELFQDR(F)<br>(R) ASSIIDELFQDRFFTR(E) |
| 5 | A1BG_HUMAN Alpha-1B-glycoprotein | (R)SGLSTGWTQLSK(L)<br>(R)CEGPIPDVTFELLR(E)<br>(R)IFFHLNAVALGDGGHYTCR(Y)<br>(R)TPGAAANLELIFVGPQHAGNYR(C) |
| 6 | ALBU_HUMAN Serum albumin | (R)HPDYSVVLLLR(L)<br>(K)QNCELFEQLGEYK(F)<br>(R)HPYFYAPELLFFAK(R)<br>(K)VFDEFKPLVEEPQNLIK(Q)<br>(R)MPCAEDYLSVVLNQLCVLHEK(T)<br>(K)SHCIAEVENDEMPADLPSLAADFVESK(D) |
| | HEMO_HUMAN Hemopexin | (R)YYCFQGNQFLR(F)<br>(R)GECQAEGVLFFQGDR(E)<br>(K)SGAQATWTELPWPHEK(V)<br>(K)LLQDEFPGIPSPLDAAVECHR(G) |
| 7 | ALBU_HUMAN Serum albumin | (K)LDELRDEGK(A)<br>(R)HPDYSVVLLLR(L)<br>(K)AVMDDFAAFVEK(C)<br>(R)RHPDYSVVLLLR(L)<br>(K)QNCELFEQLGEYK(F)<br>(R)HPYFYAPELLFFAK(R)<br>(K)VFDEFKPLVEEPQNLIK(Q) |
| 8 | ALBU_HUMAN Serum albumin | (K)DVFLGMFLYEYAR(R)<br>(K)VFDEFKPLVEEPQNLIK(Q)<br>(K)EFNAETFTFHADICTLSCK(E)<br>(K)FSVVYAK(C) |
| | A2HS_HUMAN Alpha-2-HS-glycoprotein | (K)CDSSPDSAEDVRK(V) |
| 9 | A1AT_Human alpha-1-antiproteinase | (K)LSITGTYDLK(S)<br>(K)ITPNLAEFAFSLYR(Q)<br>(K)VFSNGADLSGVTEEAPLK(L)<br>(K)FNKPFVFLMIEQNTK(S)<br>(K)ELDRDTVFALVNYIFFK(G)<br>(R)TLNQPDSQLQLTTGNGLFLSEGLK(L)<br>(R)HPYFYAPELLFFAK(R) |

(*continued*)

**Table 3** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)VFDEFKPLVEEPQNLIK(Q) |
| | | (K)SHCIAEVENDEMPADLPSLAADFVESK(D) |
| 10 | ALBU_HUMAN Serum albumin | (R)RHPDYSVVLLLR(L) |
| | | (K)DVFLGMFLYEYAR(R) |
| | | (K)QNCELFEQLGEYK(F) |
| | | (R)RPCFSALEVDETYVPK(E) |
| | | (K)SLHTLFGDKLCTVATLR(E) |
| | | (R)MPCAEDYLSVVLNQLCVLHEK(T) |
| | | (K)QNCELFEQLGEYKFQNALLVR(Y) |
| | | (K)SHCIAEVENDEMPADLPSLAADFVESK(D) |
| | ANGT_HUMAN Angiotensinogen/ | (R)ADSQAQLLLSTVVGVFTAPGLHLK(Q) |
| | ANT3_HUMAN Antithrombin-III | (R)VAEGTQVLELPFKGDDITMVLILPKPEK(S) |
| 11 | RETB_BOVIN Plasma retinol-binding protein | (K)ELPEHTVK(L) |
| | | (K)FEDCCQEK(T) |
| | | (K)HLSLLTTLSNR(V) |
| | | (R)VCSQYAAYGEK(K) |
| | | (K)EDFTSLSLVLYSR(K) |
| | | (K)FPSGTFWQVSQLVK(E) |
| | | (R)KFPSGTFEQVSQLVK(E) |
| | | (K)SCESNSPFPVHPGTAECCTK(E) |
| | | (K)VPTADLEDVLPLAEDITNILSK(C) |
| | | (K)ELSSFIDKGQELCADYSENTFTEYK(K) |
| | VTDB_HUMAN Vitamin D-binding protein | (K)KDPEGLFLQDNIVAEFSVDENGHMSATAK(G) |
| 12 | APA4_HUMAN | (R)ISASAEELR(Q) |
| | Apolipoprotein A-IV | (R)LAPLAEDVR(G) |
| | | (K)VNSFFSTFK(E) |
| | | (R)LEPYADQLR(T) |
| | | (K)SELTQQLNALFQDK(L) |
| | | (K)LGPHAGDVEGHLSFLEK(D) |
| | | (K)SLAELGGHLDQQVEEFR(R) |
| | | (K)SLAELGGHLDQQVEEFRR(R) |
| 13 | TTHY_HUMAN Transthyretin | (R)GSPAINVAVHVFR(K) |
| | | (K)AADDTWEPFASGK(T) |
| | | (K)ALGISPFHEHAEVVFTANDSGPR(R) |
| | | (K)TSESGELHGLTTEEEFVEGIYK(V) |
| | | (R)RYTIAALLSPYSYSTTAVVTNPK(E) |
| | | (K)TSESGELHGLTTEEEFVEGIYKVEIDTK(S) |
| 14 | RETB_HUMAN Plasma retinol-binding protein | (R)QEELCLAR(Q) |
| | | (R)FSGTWYAMAK(K) |
| | | (R)DPNGLPPEAQK(I) |
| | | (K)YWGVASFLQK(G) |
| | | (R)LLNLDGTCADSYSFVFSR(D) |
| | | (K)GNDDHWIVDTDYDTYAVQYSCR(L) |
| | | (R)LLNNWDVCADMVGTFTDTEDPAKFK(M) |
| 15 | APA2_HUMAN Apolipoprotein A-II | (K)EQLTPLIKK(A) |
| | | (K)SKEQLTPLIK(K) |
| | | (K)AGTELVNFLSYFVELGTQPATQ |
| 16 | TTHY_HUMAN Transthyretin | (R)GSPAINVAVHVFR(K) |
| | | (K)AADDTWEPFASGK(T) |
| | | (R)GSPAINVAVHVFRK(A) |
| | | (R)YTIAALLSPYSYSTTAVVTNPK(E) |
| | | (R)ALGISPFHEHAEVVFTANDSGPR(R) |
| | | (R)RYTIAALLSPYSYSTTAVVTNPK(E) |
| 17 | TTHY_HUMAN Transthyretin | (R)GSPAINVAVHVFR(K) |
| | | (R)GSPAINVAVHVFRK(A) |
| | | (R)YTIAALLSPYSYSTTAVVTNPK(E) |
| | | (K)ALGISPFHEHAEVVFTANDSGPR(R) |

(*continued*)

**Table 3** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)TSESGELHGLTTEEEFVEGIYK(V)<br>(R)RYTIAALLSPYSYSTTAVVTNPK(E)<br>(K)TSESGELHGLTTEEEFVEGIYKVEIDTK(S) |
| *1st dimension under non-reducing conditions, 2nd dimension under reducing conditions, in Fig. 6B* | | |
| 18 | A1BG_HUMAN Alpha-1B-glycoprotein | (R)LETPDFQLFK(N)<br>(R)SGLSTGWTQLSK(L)<br>(R)CEGPIPDVTFELLR(E)<br>(K)VTLTCVAPLSGVDFQLR(R)<br>(R)TPGAAANLELIFVGPQHAGNYR(C) |
| 19 | ALBU_HUMAN Serum albumin | (K)LDELRDEGK(A)<br>(R)HPDYSVVLLLR(L)<br>(K)AVMDDFAAFVEK(C)<br>(R)RHPDYSVVLLLR(L)<br>(K)QNCELFEQLGEYK(F)<br>(K)SLHTLFGDKLCTVATLR(E) |
| 20 | ALBU_HUMAN Serum albumin | AEFAEVSK<br>TYETTLEK<br>LDELRDEGK<br>HPDYSVVLLLR<br>AVMDDFAAFVEK<br>RHPDYSVVLLLR<br>DVFLGMFLYEYA<br>VFDEFKPLVEEP |
| 21 | A1AT_HUMAN Alpha-1-antitrypsin | (R)SASLHLPK(L)<br>(K)LSSWVLLMK(Y)<br>(K)ITPNLAEFAFSLYR(Q)<br>(K)TDTSHHDQDHPTFNK(I)<br>(K)LQHLENELTHDIITK(F)<br>(K)ELDRDTVFALVNYIFFK(G)<br>(K)LSITGTYDLKSVLGQLGITK(V) |
| | IDHA_HUMAN Isocitrate dehydrogenase subunit alpha | (K)GTEAAGAMFLEAIPMSIPPEVK(F)<br>(R)IAEFAFEYAR(N) |
| 22 | A2HS_HUMAN Alpha-2-HS-glycoprotein | (K)HTKNQIDEVK(V)<br>(K)CDSSPDSAEDVRK(V)<br>(R)AQLVPLPPSTYVEFTVSGTDCVAK(E) |
| | A1AT_HUMAN Alpha-1-antitrypsin<br>KNG_HUMAN Kininogen | (K)ITPNLAEFAFSLYR(Q)<br>(K)ELDRDTVFALVNYIFFK(G)<br>(K)YFIDFVAR(E)<br>(R)DIPTNSPELEETLTHTITK(L) |
| 23 | VTDB_HUMAN Vitamin D-binding protein | (R)THLPEVFLSK(V)<br>(K)HLSLLTTLSNR(V)<br>(R)VCSQYAAYGEK(K)<br>(K)EDFTSLSLVLYSR(K)<br>(R)KFPSGTFEQVSQLVK(E)<br>(K)VPTADLEDVLPLAEDITNILSK(C)<br>(K)ELSSFIDKGQELCADYSENTFTEYK(K) |
| 24 | VTDB_HUMAN Vitamin D-binding protein | (R)THLPEVFLSK(V)<br>(K)HLSLLTTLSNR(V)<br>(K)EDFTSLSLVLYSR(K)<br>(K)FPSGTFEQVSQLVK(E)<br>(R)KFPSGTFEQVSQLVK(E)<br>(K)EFSHLGKEDFTSLSLVLYSR(K)<br>(K)VPTADLEDVLPLAEDITNILSK(C)<br>(K)ELSSFIDKGQELCADYSENTFTEYK(K) |
| 25 | VTDB_HUMAN Vitamin D-binding protein | (K)HLSLLTTLSNR(V)<br>(K)EDFTSLSLVLYSR(K)<br>(R)KFPSGTFEQVSQLVK(E) |

(*continued*)

**Table 3** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)VPTADLEDVLPLAEDITNILSK(C) |
| | | (K)ELSSFIDKGQELCADYSENTFTEYK(K) |
| | A1AT_HUMAN Alpha-1-antitrypsin | (K)ELDRDTVFALVNYIFFK(G) |
| 26 | APA4_HUMAN Apolipoprotein A-IV | (R)LLPHANEVSQK(I) |
| | | (R)DKVNSFFSTFK(E) |
| | | (K)SLAELGGHLDQQVEEFR(R) |
| | | (K)SLAELGGHLDQQVEEFRR(R) |
| | | (R)SLAPYAQDTQEKLNHQLEGLTFQMK(K) |
| 27 | RETB_HUMAN Plasma retinol-binding protein | (R)FSGTWYAMAK(K) |
| | | (R)DPNGLPPEAQK(I) |
| | | (K)YWGVASFLQK(G) |
| | | (K)GNDDHWIVDTDYDTYAVQYSCR(L) |
| 28 | APA1_HUMAN Apolipoprotein A-I | (R)QKLHELQEK(L) |
| | | (R)DYVSQFEGSALGK(Q) |
| | | (R)VKDLATVYVDVLK(D) |
| | | (K)LLDNWDSVTSTFSK(L) |
| | | (K)DSGRDYVSQFEGSALGK(Q) |
| | | (K)LREQLGPVTQEFWDNLEK(E) |
| 29 | TTHY_HUMAN Transthyretin | (R)GSPAINVAVHVFR(K) |
| | | (K)VLDAVRGSPAINVAVHVFR(K) |
| | | (K)ALGISPFHEHAEVVFTANDSGPR(R) |
| | | (K)TSESGELHGLTTEEEFVEGIYKVEIDTK(S) |
| 30 | TTHY_HUMAN Transthyretin | (R)GSPAINVAVHVFR(K) |
| | | (K)AADDTWEPFASGK(T) |
| | | (R)GSPAINVAVHVFRK(A) |
| | | (K)VLDAVRGSPAINVAVHVFR(K) |
| | | (K)ALGISPFHEHAEVVFTANDSGPR(R) |
| | | (K)TSESGELHGLTTEEEFVEGIYK(V) |
| | | (R)RYTIAALLSPYSYSTTAVVTNPK(E) |
| | | (K)ALGISPFHEHAEVVFTANDSGPRR(Y) |
| | | (K)TSESGELHGLTTEEEFVEGIYKVEIDTK(S) |
| 31 | TTHY_HUMAN Transthyretin | (K)ALGISPFHEHAEVVFTANDSGPR(R) |
| | | (K)TSESGELHGLTTEEEFVEGIYK(V) |
| | | (K)TSESGELHGLTTEEEFVEGIYKVEIDTK(S) |
| | APA2_HUMAN Apolipoprotein A-II | (K)SPELQAEAK(S) |
| | | (K)SKEQLTPLIK(K) |
| | | (K)VKSPELQAEAK(S) |
| | | (K)AGTELVNFLSYFVELGTQPATQ |

| Spot number | Spot identification |
|---|---|
| *1st dimension and 2nd dimension under reducing conditions, in Fig. 6C (identifications as published in Anderson and Anderson, 1977; Anderson et al., 1984; Hughes et al., 1992; Anderson et al., 2004 and in ExPASy)* | |
| 32 | Transferrin |
| 33 | Alpha-1-B-glycoprotein |
| 34 | Alpha-1-antitrypsin |
| 35 | Albumin |
| 36 | Gc-globulin |
| 37 | Alpha-2-HS-glycoprotein |
| 38 | Haptoglobin, chain beta |
| 39 | Apolipoprotein J/clusterin |
| 40 | Apolipoprotein A-I |
| 41 | Haptoglobin, chain alpha-2 |
| 42 | Transthyretin |
| 43 | Haptoglobin, chain alpha-1 |
| 44 | Apolipoprotein A-II |

**Table 4.** Mouse serum

| Spot number | Spot identification |
|---|---|
| *1st dimension and 2nd dimension under non-reducing conditions, in Fig. 7A (Wait et al., in press)* | |
| 1 | Carboxylesterase |
| 2 | Kininogen |
| 3 | Alpha-1-antitrypsin |
| 4 | Alpha-2-HS-glycoprotein |
| 5 | Vitamin D-binding protein |
| 6 | Apolipoprotein A-IV |
| 7 | Apolipoprotein A-II |

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| *1st dimension and 2nd dimension under non-reducing conditions, in Fig. 7A* | | |
| 8 | TTHY_HUMAN Transthyretin | (K)ALGISPFHEHAEVVFTANDSGPR(R)<br>(R)RYTIAALLSPYSYSTTAVVTNPK(E) |
| | KNG_MOUSE Kininogen | (K)TSESGELHGLTTEEEFVEGIYKVEIDTK(S)<br>(R)RPPGFSPFR(S)<br>(R)ENEFFIVTQTCK(I)<br>(R)DIPVDSPELKEVLGHSIAQLNAENDHPFYYK(I) |
| 9 | TTHY_HUMAN Transthyretin | (K)TSESGELHGLTTEEEFVEGIYKVEIDTK(S) |
| 10 | VTDB_MOUSE Vitamin D-binding protein | (R)SLSLILYSR(K)<br>(K)VPTANLENVLPLAEDFTEILSR(C)<br>(K)LAQKVPTANLENVLPLAEDFTEILSR(C) |
| 11 | FETB_MOUSE Fetuin-B | (K)LVVLPFPGK(E)<br>(R)HVPLIQPVEK(S) |
| 12 | HEMO_MOUSE Hemopexin | (K)LFQEEFPGIPYPPDAAVECHR(G)<br>GGNNLVSGYPK<br>FNPVTGEVPPR<br>FNPVTGEVPPRYP |
| 13 | A2HS_MOUSE Alpha-2-HS-glycoprotein | (R)AQNVPLPVSTLVEFVIAATDCTAK(E) |
| 14 | Q8VC20 Serine (Or cysteine) proteinase inhibitor, clade A, member 1b | (K)ELDQDTVFALANYILFK(G)<br>(K)DQSPASHEIATNLGDFAISLYR(E)<br>(K)SFQHLLQTLNRPDSELQLSTGNGLFVNNDLK |
| 15 | VTDB_MOUSE Vitamin D-binding protein | (R)SLSLILYSR(K)<br>(K)FSSSTFEQVNQLVK(E)<br>(R)KFSSSTFEQVNQLVK(E)<br>(K)VPTANLENVLPLAEDFTEILSR(C)<br>(K)LAQKVPTANLENVLPLAEDFTEILSR(C) |
| 16 | A2HS_MOUSE Alpha-2-HS-glycopppppprotein<br>A1T3_MOUSE Alpha-1-antitrypsin 1–3 | (R)HAFSPVASVESASGETLHSPK(V)<br>(R)AQNVPLPVSTLVEFVIAATDCTAK(E)<br>(K)KLDQDTVFALANYILFK(G)<br>(K)DQSPASHEIATNLGDFAISLYR(E)<br>(R)FDHPFLFIIFEEHTQSPLFVGK(V) |
| 17 | APA4_MOUSE Apolipoprotein A-IV | (R)SLAPLTVGVQEK(L)<br>(K)NLAPLVEDVQSK(V)<br>(K)TDVTQQLSTLFQDK(L)<br>(K)LVPFVVQLSGHLAKETER(V)<br>(R)QQLGPNSGEVESHLSFLEK(S) |
| 18 | HPT_MOUSE Haptoglobin | (R)VGYVSGWGR(N)<br>(R)HGLTTGATLISDQWLLTTAK(N) |
| 19 | MUP_MOUSE Major urinary protein 1 | (R)EPDLSSDIK(E)<br>(R)EKIEDNGNFR(L)<br>(R)ENIIDLSNANR(C)<br>(K)INGEWHTIILASDKR(E) |

(*continued*)

**Table 4** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)TDYDNFLMAHLINEK(D)<br>(R)LFLEQIHVLENSLVLK(F)<br>(K)AGEYSVTYDGFNTFTIPK(T) |
| 20 | TTHY_MOUSE Transthyretin | (R)GSPAVDVAVK(V)<br>(K)TAESGELHGLTTDEKFVEVGYR(V)<br>(K)TLGISPFHEFADVVFTANDSGHR(H)<br>(R)HYTIAALLSPYSYSTTAVVSNPQN |
| 21 | APC3_MOUSE Apolipoprotein C-III | (K)TVQDALSSVQESDIAVVAR(G) |
| 22 | APA2_MOUSE Apolipoprotein A-II | (K)THEQLTPLVR(S) |
| 23 | APA2_MOUSE Apolipoprotein A-II | (K)TSEIQSQVK(A)<br>(K)THEQLTPLVR(S) |

| Spot number | Spot identification |
|---|---|
| *1st dimension and 2nd dimension under reducing conditions, in Fig. 7C (Wait et al., in press)* | |
| 24 | Transferrin |
| 25 | Hemopexin |
| 26 | Carboxylesterase |
| 27 | Kininogen |
| 28 | Contrapsin |
| 29 | Albumin |
| 30 | Alpha-2-HS-glycoprotein |
| 31 | Vitamin D-binding protein |
| 32 | Alpha-1-antitrypsin |
| 33 | Apolipoprotein A-IV |
| 34 | Albumin, C-terminus fragments |
| 35 | Haptoglobin chain beta |
| 36 | Alpha-1-acid glycoprotein |
| 37 | Albumin, N-terminus fragments |
| 38 | Apolipoprotein A-I |
| 39 | Major urinary protein |
| 40 | Albumin, fragments |
| 41 | Transthyretin |
| 42 | Apolipoprotein C |
| 43 | Apolipoprotein A-II |

of sequenced peptides. In Panels C (and in Panel A of Fig. 7), numbers refer to spots or spot chains already identified either by other research groups (human samples) or by us (mouse, rat and bovine samples) as referenced in Tables 3–6.

Some proteins are present in the sera of all species under investigation, interspecific sequence divergence varying between orthologs. Conversely, some proteins are species-specific or are present at greatly varying level between species. Accordingly, some trends along the redox scale are similar in all samples, whereas others are peculiar to one species.

Under non-reducing conditions *albumin* of all species migrates to a much lower $M_r$ than under reducing conditions: 55 vs 68 kDa, consistent with the presence of 17 S–S bonds. Either because of better recovery or higher affinity for CBB stain, the spot for non-reduced albumin is larger and darker. Polymeric forms on top of a diffuse vertical streaking are also observed. No reduction is the best condition for the resolution between albumin and hemopexin in human, mouse and rat serum samples. Albumin fragments are present in different amount in various species. Where they are prominent (rat and mouse serum) detection is possible only after reduction – which implies the presence in rat and mouse circulation of a substantial amount of cleaved but undissociated albumin.

*Transferrin* also contains a large number of S–S bridges (19) and its $M_r$ shifts from 71 to 77 kDa between non-reducing and reducing conditions. Bovine serum contains two different molecular forms of transferrin, which differ by the presence or absence of a C-terminal peptide of

**Table 5.** Rat serum

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| *1st dimension and 2nd dimension under non-reducing conditions, in Fig. 8A* | | |
| 1 | ALBU_RAT Serum albumin | (K)QTALAELVK(H)<br>(K)LVQEVTDFAK(T)<br>(R)FPNAEFAEITK(L)<br>(K)LGEYGFQNAVLVR(Y)<br>(K)TCVADENAENCDK(S)<br>(K)DVFLGTFLYEYSR(R)<br>(R)LPCVEDYLSAILNR(L) |
| | CPI1_RAT Contrapsin-like protease inhibitor 1 | (R)TSMVLVNYLLFK(G)<br>(K)NVVFSPLSISAALAILSLGAK(D)<br>(K)AVLDVDETGTEGAAATAVTAALK(S) |
| 2 | ALBU_RAT Serum albumin | (K)SIHTLFGDK(L)<br>(K)LVQEVTDFAK(T)<br>(R)FPNAEFAEITK(L)<br>(K)LGEYGFQNAVLVR(Y)<br>(K)TCVADENAENCDK(S)<br>(K)DVFLGTFLYEYSR(R)<br>(R)LPCVEDYLSAILNR(L)<br>(K)CCAEGDPPACYGTVLAEFQPLVEEPK(N)<br>(K)SQCLAETEHDNIPADLPSIAADFVEDK(E) |
| | CPI3_RAT Contrapsin-like protease inhibitor 1 | (K)SQCLAETEHDNIPADLPSIAADFVEDKEVCK(N)<br>(K)DLYVSQVVHK(A)<br>(K)IAELFSDLEER(T) |
| 3 | ALBU_RAT Serum albumin | (R)FPNAEFAEITK(L)<br>(R)DNYGELADCCAK(Q)<br>(K)DVFLGTFLYEYSR(R)<br>(R)LPCVEDYLSAILNR(L)<br>(K)SQCLAETEHDNIPADLPSIAADFVEDK(E) |
| 4 | ALBU_RAT Serum albumin | (K)DVFLGTFLYEYSR(R)<br>(R)LPCVEDYLSAILNR(L)<br>(K)SQCLAETEHDNIPADLPSIAADFVEDK(E) |
| | A1AT_RAT Alpha-1-antiproteinase | (K)TLLSSLGITR(V)<br>(K)ISSNLADFAFSLYR(E) |
| 5 | CO3_RAT Complement C3 | (K)TVLTGATGHLNR(V)<br>(R)YYQTIEIPPK(S)<br>(K)SGSDEVQAGQER(R)<br>(R)IFTVDNNLLPVGK(T)<br>(R)SGIPIVTSPYQIHFTK(T)<br>(K)EYVLPSFEVLVEPTEK(F)<br>(K)NVDGTAFVIFGVQDEDKK(I)<br>(R)SHFPESWLWTIEELKEPEK(N) |
| 6 | Q7TP23 Ba1-647 (Haptoglobin isoform) | (K)CELHYEK(S)<br>(K)DIAPTLTLYVGK(N)<br>(K)GAVSPVGVQPILNK(H) |
| 7 | CLUS_RAT Clusterin | (R)ASGIIDTLFGDR(F)<br>(K)LFDSDPITVVLPEEVSK(D) |
| 8 | AFAM_RAT Afamin | (K)TLNSINIAVFSK(K)<br>(R)CLADSTLPECSK(I)<br>(R)NPFAFAPVLLNVAAR(F)<br>(K)AAPQLPMEELVSLSK(E)<br>(K)IANDAIQDMLCDMK(G)<br>(K)ADTTYALPSVSALVSALR(A)<br>(K)KANVGFLPPFPTLDPEEK(C)<br>(K)FLAAGEECGNIQKPEACFSPESSK(T) |
| 9 | CPI3_RAT Contrapsin-like protease inhibitor 3 | (K)VFSQQADLSR(I)<br>(K)DLYVSQVVHK(A) |

(*continued*)

**Table 5** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)IAELFSDLEER(T)<br>(K)DSTMEEILEGLK(F)<br>(R)DEELSCSVLELK(Y)<br>(R)TSMVLVNYLLFK(G)<br>(R)ALYQAEAFIADFK(Q)<br>(R) ALYQAEAFIADFKQPNEAK(K)<br>(K)AVLDVDETGTEATAATGVATVIR(R)<br>(R)NPDKNVVFSPLSISAALTILSLGAK(D) |
| 10 | CPI1_RAT Contrapsin-like protease inhibitor 1 | (R)KIFSQQADLSR(I)<br>(R)DTLPHEDQGKGR(Q)<br>(R)DEELSCSVLELK(Y)<br>(K)NVVFSPLSISAALAILSLGAK(D)<br>(K)AVLDVDETGTEGAAATAVTAALK(S)<br>(R)LSQPEDQAEINTGSALFIDK(E)<br>(R)NPDKNVVFSPLSISAALAILSLGAK(D) |
| 11 | KNT1_RAT T-kininogen I | (R)EIPVDSPELK(E)<br>(K)FSVATQICNITPGK(G)<br>(K)YNAELESGNQFVLYR(V)<br>(K)ATSQVVAGVIYVIEFIAR(E)<br>(R)CQALDMMISRPPGFSPFR(L)<br>(K)KTEEDLCVGCFQPIPMDSSDLKPVLK(H)<br>(K)AVLDVDETGTEGAAATAVTAALK(S) |
| | CPI1_RAT Contrapsin-like protease inhibitor 1 | (K)FSISTDYNLEEVLPELGIR(K) |
| 12 | A1AT_RAT Alpha-1-antiproteinase | (K)AVLTLDER(G)<br>(K)ISSNLADFATSLYR(E)<br>(R)VFNNDADLSGITEDAPLK(L)<br>(K)QLDEDTVFALVNYIFFK(G)<br>(K)LSISGTYNLKTLLSSLGITR(V)<br>(K)FDHPFIFMIVESETQSPLFVGK(V)<br>(K)AFHHLLQTLNRPDSELQLNTGNGLFVNK(N) |
| 13 | A2HS_RAT Alpha-2-HS-glycoprotein | (R)LGGEEVSVACK(L)<br>(R)QQAEHAVEGDCDFHILK(Q)<br>(R)HAFSPVASVESASGEVLHSPK(V)<br>(R)ELACDDPETEHVALIAVDYLNK(H)<br>(R)AQNVPFPVSTLVEFVIAATDCTGQEVTDPAK(C) |
| 14 | FETB_RAT Fetuin-B | (K)ATTQWVVGPSYFVEYLIK(E)<br>(R)QEDMGSLFYLTLDVLETGCHVLSR(K) |
| | Q02495 Homeodomain protein? | (R)LAGVRHGK(V) |
| 15 | FETB_RAT Fetuin-B | (K)LVVLPFPGK(E)<br>(R)IFYETVHGQCK(A)<br>(K)ATTQWVVGPSYFVEYLIK(E)<br>(R)QEDMGSLFYLTLDVLETGCHVLSR(K)<br>(K)IHSMCPDCPHPVDLSAPSVLEAATESLAK(F) |
| 16 | APA4_RAT Apolipoprotein A-IV | (K)ATIDQNLEDLR(S)<br>(K)NLAPLVEDVQSK(L)<br>(K)TDVTQQLNTLFQDK(L)<br>(R)QQLGSDSGDVESHLSFLEK(N) |
| 17 | VTDB_RAT Vitamin D-binding protein | (R)SLSLILYSR(K)<br>(K)CCSINSPPR(Y)<br>(K)FPSSTFEQVSQLVK(E)<br>(R)KFPSSTFEQVSQLVK(E)<br>(K)SCESDAPFPVHPGTSECCTK(E)<br>(K)LAQKVPTANLEDVLPLAEDLTEILSR(C) |
| 18 | TTHY_RAT Transthyretin | (K)ALGISPFHEYAEVVFTANDSGHR(S) |
| 19 | ALBU_RAT Serum albumin | (K)KYEATLEK(C)<br>(R)FPNAEFAEITK(L) |

(*continued*)

**Table 5** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)TVMGDFAQFVDK(C) |
| | | (K)LGEYGFQNAVLVR(Y) |
| | | (K)DVFLGTFLYEYSR(R) |
| | | (R)LPCVEDYLSAILNR(L) |
| | | (K)CCAEGDPPACYGTVLAEFQPLVEEPK(N) |
| | | (K)SQCLAETEHDNIPADLPSIAADFVEDK(E) |
| | | (K)SQCLAETEHDNIPADLPSIAADFVEDKEVCK(N) |
| 20 | ALBU_RAT Serum albumin | (K)KYEATLEK(C) |
| | | (R)FPNAEFAEITK(L) |
| | | (K)TVMGDFAQFVDK(C) |
| | | (K)LGEYGFQNAVLVR(Y) |
| | | (R)LPCVEDYLSAILNR(L) |
| | | (R)RPCFSALTVDETYVPK(E) |
| | | (K)CCAEGDPPACYGTVLAEFQPLVEEPK(N) |
| | | (K)SQCLAETEHDNIPADLPSIAADFVEDK(E) |
| | | (K)SQCLAETEHDNIPADLPSIAADFVEDKEVCK(N) |
| 21 | RETB_RAT Plasma retinol-binding protein | (R)DPNGLTPETR(R) |
| | | (R)FSGLWYAIAK(K) |
| | | (R)DPNGLTPETRR(L) |
| | | (R)QRQEELCLER(Q) |
| | | (R)LQNLDGTCADSYSFVFSR(D) |
| | ALBU_RAT Serum albumin | (K)DVFLGTFLYEYSR(R) |
| 22 | RETB_RAT Plasma retinol-binding protein | (R)FSGLWYAIAK(K) |
| | | (R)LQNLDGTCADSYSFVFSR(D) |
| | | (K)KDPEGLFLQDNIIAEFSVDEK(G) |
| | ALBU_RAT Serum albumin | (K)LVQEVTDFAK(T) |
| 23 | MUP_RAT Major urinary protein | (R)TKDLSSDIK(E) |
| | | (K)LNGDWFSIVVASNKR(E) |
| | | (R)VFMQHIDVLENSLGFK(F) |
| 24 | TTHY_RAT Transthyretin | (R)GSPAVDVAVK(V) |
| | | (K)TADGSWEPFASGK(T) |
| | | (K)ALGISPFHEYAEVVFTANDSGHR(H) |
| 25 | TTHY_RAT Transthyretin | (R)GSPAVDVAVK(V) |
| | | (K)TAESGELHGLTTDEK(F) |
| | | (K)ALGISPFHEYAEVVFTANDSGHR(H) |
| *1st dimension under non-reducing conditions, 2nd dimension under reducing conditions, in Fig. 8B* | | |
| 26 | AFAM_RAT Afamin | (K)ALSSYQR(N) |
| | | (K)TLNSINIAVFSK(K) |
| | | (R)NPFAFAPVLLNVAAR(F) |
| | | (R)RNPFAFAPVLLNVAAR(F) |
| | | (K)KANVGFLPPFPTLDPEEK(C) |
| | TTHY_MOUSE Transthyretin | (K)TLGISPFHEFADVVFTANDSGHR(H) |
| 27 | KNT1_RAT T-kininogen I | (K)TELTADCETK(H) |
| | | (K)YNAELESGNQFVLYR(V) |
| 28 | CPI3_RAT Contrapsin-like protease inhibitor 3 | (K)DLYVSQVVHK(A) |
| | | (K)IAELFSDLEER(T) |
| | | (R)TSMVLVNYLLFK(G) |
| | | (R)ALYQAEAFIADFKQPNEAK(K) |
| | | (K)AVLDVDETGTEATAATGVATVIR(R) |
| | | (R)NPDKNVVFSPLSISAALTILSLGAK(D) |
| 29 | CPI1_RAT Contrapsin-like protease inhibitor 1 | (K)LALRNPDK(N) |
| | | (K)IFSQQADLSR(I) |
| | | (R)KIFSQQADLSR(I) |
| | | (R)DEELSCSVLELK(Y) |
| | | (R)TSMVLVNYLLFK(G) |

(*continued*)

**Table 5** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)AVLDVDETGTEGAAATAVTAALK(S)<br>(R)LSQPEDQAEINTGSALFIDK(E)<br>(K)FSISTDYNLEEVLPELGIRK(I)<br>(R)NPDKNVVFSPLSISAALAILSLGAK(D) |
| 30 | A2HS_RAT Alpha-2-HS-glycoprotein | (R)HAFSPVASVESASGEVLHSPK(V)<br>(R)ELACDDPETEHVALIAVDYLNK(H) |
| 31 | FETB_RAT Fetuin-B | (R)GSIQHLPEQEEPEDSKGK(S)<br>(K)ATTQWVVGPSYFVEYLIK(E) |
| 32 | A1AT_RAT Alpha-1-antiproteinase | (K)AVLTLDER(G)<br>(R)SAILYFPK(L)<br>(K)TLLSSLGITR(V)<br>(K)LSISGTYNLK(T)<br>(R)KISSNLADFAFSLYR(E)<br>(R)VFNNDADLSGITEDAPLK(L)<br>(K)QLDEDTVFALVNYIFFK(G)<br>(K)FDHPFIFMIVESETQSPLFVGK(V)<br>(R)VFNNDADLSGITEDAPLKLSQAVHK(A)<br>(K)AFHHLLQTLNRPDSELQLNTGNGLFVNK(N) |
| 33 | VTDB_RAT Vitamin D-binding protein | (R)SLSLILYSR(K)<br>(K)CCSINSPPR(Y)<br>(K)FPSSTFEQVSQLVK(E)<br>(R)KFPSSTFEQVSQLVK(E)<br>(K)SCESDAPFPVHPGTSECCTK(E)<br>(K)LAQKVPTANLEDVLPLAEDLTEILSR(C) |
| 34 | APA4_RAT Apolipoprotein A-IV | (R)LAPLAEGVQEK(L)<br>(K)TDVTQQLNTLFQDK(L)<br>(R)QQLGSDSGDVESHLSFLEK(N) |
| 35 | TTHY_RAT Transthyretin | (K)TAESGELHGLTTDEK(F)<br>(K)ALGISPFHEYAEVVFTANDSGHR(H) |
| 36 | ALBU_RAT Serum albumin | (K)KYEATLEK(C)<br>(K)KQTALAELVK(H)<br>(R)RHPDYSVSLLLR(L)<br>(K)LGEYGFQNAVLVR(Y)<br>(R)LPCVEDYLSAILNR(L)<br>(K)TNCELYEKLGEYGFQNAVLVR(Y)<br>(K)CCAEGDPPACYGTVLAEFQPLVEEPK(N) |
| 37 | CRP_RAT C-reactive protein | (R)SFSIFSYATK(T)<br>(K)TSFNEILLFWTR(G)<br>(K)YETHGDVFIKPQLWPLTDCCES |
| 38 | ALBU_RAT Serum albumin | (K)CPYEEHIK(L)<br>(K)LVQEVTDFAK(T)<br>(K)GLVLIAFSQYLQK(C)<br>(K)TCVADENAENCDK(S)<br>(K)SIHTLFGDKLCAIPK(L)<br>(R)HPYFYAPELLYYAEK(Y)<br>(R)RHPYFYAPELLYYAEK(Y) |
| 39 | RETB_RAT Plasma retinol-binding protein | (R)DPNGLTPETR(R)<br>(R)FSGLWYAIAK(K)<br>(K)YWGVASFLQR(G)<br>(R)QRQEELCLER(Q) |
| 40 | RETB_RAT Plasma retinol-binding protein | (R)DPNGLTPETR(R)<br>(R)FSGLWYAIAK(K) |
| 41 | MUP_RAT Major urinary protein | (K)LCEAHGITR(D)<br>(K)LNGDWFSIVVASNKR(E) |
| 42 | TTHY_RAT Transthyretin | (R)GSPAVDVAVK(V)<br>(K)TADGSWEPFASGK(T) |

(*continued*)

**Table 5** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)VLDAVRGSPAVDVAVK(V)<br>(K)ALGISPFHEYAEVVFTANDSGHR(H)<br>(R)HYTIAALLSPYSYSTTAVVSNPQN |
| 43 | TTHY_RAT Transthyretin | (R)GSPAVDVAVK(V)<br>(K)TADGSWEPFASGK(T)<br>(K)ALGISPFHEYAEVVFTANDSGHR(H) |
| | HBB2_RAT Hemoglobin beta chain | (R)LLVVYPWTQR(Y) |

| Spot number | Spot identification |
|---|---|
| *1st dimension and 2nd dimension under reducing conditions, in Fig. 8C (identifications as published in Haynes et al., 1998; Eberini et al., 1999; Miller et al., 1999; Eberini et al., 2000; Wait et al., 2001; Gianazza et al., 2002)* | |
| 44 | Alpha1-inhibitor 3 |
| 45 | Transferrin |
| 46 | Hemopexin |
| 47 | Albumin |
| 48 | Alpha-2-HS-glycoprotein |
| 49 | Kallikrein-binding protein |
| 50 | Fetuin beta |
| 51 | Serine proteaseinhibitor (SPI) 3 |
| 52 | Alpha-1-antitrypsin |
| 53 | Vitamin D-binding protein (Gc) |
| 54 | Albumin fragments (C-terminus) |
| 55 | Apolipoprotein A-IV |
| 56 | Alpha-1-macroglobulin |
| 57 | C-reactive protein |
| 58 | Apolipoprotein A-I |
| 59 | Albumin fragments (N-terminus) |
| 60 | Retinol-binding protein |
| 61 | Transthyretin |

about 6 kDa (Tsuji et al., 1984). The resolution between the two is minimal under non-reducing conditions but increases on reduction. The charge microheterogeneity of native transferrin is connected to both covalent post-transcriptional modifications (glycosylation, implying up to 8 sialic acid residues) and non-covalent interactions (binding of up to 2 $Fe^{++}$ ions). The susceptibility to urea denaturation varies depending on iron saturation and redox status. The diferric protein is unaffected by urea, and in the monoferric transferrin only the iron-free domain is assumed to unfold (Evans and Williams, 1980); in ovotransferrin, the molecule containing 4 S–S bridges is still able to bind iron, and the corresponding complex doesn't unfold in 8 M urea, whereas the 3 S–S and 2 S–S proteins exist only as apoforms (Yamashita et al., 1995). Accordingly more isoforms are resolved before S–S reduction.

The amount of *immunoglobulins* varies among species, being much higher in humans and *Bovidae* than in *Rodentia*. As expected, without sample reduction Igs form horizontal streaks at high $M_r$ whereas after reduction heavy and light chains are resolved at lower $M_r$.

Baseline levels of *haptoglobin* also vary among species, being higher in humans and mice. In these serum samples, $\alpha$ and $\beta$ chains are resolved after sample reduction but no intact haptoglobin is observed in the two-dimensional map, probably as a result of aggregation. In contrast, in rat serum a distinct faint series of high $M_r$ haptoglobin spots is seen under non-reducing conditions. Possibly due to their low abundance once separated, neither $\alpha$ nor $\beta$ chains are detectable in rat serum after sample reduction. In rats and bovines, noticeable levels can only be detected in case of inflammation (Miller et al., 1998; Wait et al., 2002). In addition, haptoglobin appears in different phenotypes and polymorphism, depending on the species (Meier et al., 1980).

*Apolipoprotein A-II* is a homodimer in humans and a monomeric protein in the other species. Accordingly, the $M_r$ of apoA-II in human serum is twice as high under non-reducing than under reducing conditions.

**Table 6.** Bovine serum

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| *1st dimension and 2nd dimension under non-reducing conditions, in Fig. 9A* | | |
| 1 | KNL2_BOVIN Kininogen, LMW II/<br>APA2_HUMAN Apolipoprotein A-II | (K)ATVQVVGGLK(Y)<br>(J)SLSSGDTGECTDK(A)<br>(K)AGTELVNFLSYFVELGTQPATQ |
| 2 | TTHY_RAT Transthyretin<br>KNL2_BOVIN Kininogen, LMW II | (K)ALGISPFHEYAEVVFTANDSGHR(H)<br>(K)ATVQVVGGLK(Y) |
| 3 | KNH2_BOVIN Kininogen, LMW II | (K)ATVQVVGGLK(Y)<br>(K)SLSSGDTGECTDK(A)<br>(K)SCEINIHGQILHCDANVYVVPWEEK(V) |
| 4 | KNH2_BOVIN Kininogen, HMW II/ | (K)YSIVFIAR(E)<br>(K)ATVQVVGGLK(Y)<br>(K)RPPGFSPFR(S)<br>(K)SLSSGDTGECTDK(A)<br>(K)EEFSFLTPDCK(S)<br>(K)SCEINIHGQILHCDANVYVVPWEEK(V) |
| | A1AT_BOVIN Alpha-1-antiproteinase | (K)SVLGDVGITEVFSDR(A) |
| 5 | Gil 29252782; gil29264268 (Bovine ESTs homologous to alpha-1B-glycoprotein, A1BG_Human) | |
| 6 | A1AT_BOVIN Alpha-1-antiproteinase | (R)ADLSGITK(E)<br>(R)DFHVDEQTTVK(V)<br>(K)SVLGDVGITEVFSDR(A)<br>(K)VLDPNTVFALVNYISFK(G)<br>(K)LVDTFLEDVKNLYHSEAFSINFR(D) |
| 7 | gil 47523270 alpha-a-antichymotrypsin 2 | GPTLTELLEGLK |
| 8 | A1AT_BOVIN Alpha-1-antiproteinase/ | (R)DFHVDEQTTVK(V)<br>(K)SVLGDVGITEVFSDR(A)<br>(K)VLDPNTVFALVNYISFK(G) |
| | KNH1_BOVIN Kininogen, HMW I | (K)YSIVFIAR(E)<br>(J)SLSSGDTGECTDK(A) |
| 9 | A2HS_BOVIN Alpha-2-HS-glycoprotein | (K)CNLLAEK(Q)<br>(K)QDGQFSVLFTK(C) |
| 10 | A2HS_BOVIN Alpha-2-HS-glycoprotein | (K)QDGQFSVLFTK(C)<br>(R)HTFSGVASVESSSGEAFHVGK(T) |
| 11 | Bovine ESTs Gil28302525 gil29396925;<br>gil28151360 (Bovine homologues<br>of VTDB_HUMAN Vitamin D-binding protein) | (K)GQELCADYSENTFTEYK(K) |
| 12 | APOH_BOVIN Beta-2-glycoprotein I | (R)FTCPLTGLWPINTLK(C)<br>(K)CSYTEDAQCIDGTIEIPK(C)<br>(K)WSPDLPVCAPITCPPPPIPK(F)<br>(K)STATFGCHETYSLDGPEEVECSK(F)<br>(K)CTEEGKWSPDLPVCAPITCPPPPIPK(F) |
| 13 | APOH_BOVIN Beta-2-glycoprotein I | (R)FTCPLTGLWPINTLK(C)<br>(R)TYEPGEQIVFSCQPGYVSR(G)<br>(R)YTTFEYPNTISFSCHTGFYLK(G)<br>(K)CTEEGKWSPDLPVCAPITCPPPPIPK(F) |
| 14 | APA4_PIG Apolipoprotein A-IV<br>TTHY_BOVIN Transthyretin | (R)LLPHATEVSQK(I)<br>(K)SELTQQLNTLFQDK(L)<br>(R)HYTIAALLSPYSYSTTALVSSPK(A) |
| 15 | APA4_PIG Apolipoprotein A-IV<br>TTHY_BOVIN Transthyretin | (K)SELTQQLNTLFQDK(L)<br>(K)TSESGELHGLTTEDKFVEGLYK(V)<br>(K)SLGISPFHEFAEVVFTANDSGPR(H) |
| 16 | TTHY_BOVIN Transthyretin | (K)FVEGLYK(V) |
| 17 | ALBU_BOVIN Serum albumin | (K)QTALVELLK(H)<br>(K)SHCIAEVEK(D)<br>(K)TVMENFVAFVDK(C) |

(*continued*)

**Table 6** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (R)RHPEYAVSVLLR(L)<br>(K)LGEYGFQNALIVR(Y)<br>(K)DAFLGSFLYEYSR(R)<br>(K)LFTFHADICTLPDTRK(Q) |
| 18 | RETB_BOVIN Plasma retinol-binding protein | (R)DPSGFSPEVQK(I)<br>(K)YWGVASFLQK(G)<br>(K)YWGVASFLQK(G)<br>(R)LLNLDGTCADSYSFVFSR(D)<br>(K)KDPEGLFLQDNIVAEFSVDENGHMSATAK(G) |
| *1st dimension under non-reducing conditions, 2nd dimension under reducing conditions, in Fig. 6B* | | |
| 19 | KNH2_BOVIN Kininogen HMW II | YSIVFIAR<br>(K)ATVQVVGGLK(Y) |
| 20 | KNH1_BOVIN Kininogen HMW I | (K)SGNQFVLYR(I)<br>(K)SCEINIHGQILHCDANVYVVPWEEK(V) |
| 21 | KNH1_BOVIN Kininogen HMW I | (K)YSIVFIAR(E) |
| 22 | A2HS_BOVIN Alpha-2-HS-glycoprotein | (K)ALGGEDVR(V)<br>(K)QDGQFSVLFTK(C)<br>(R)HTFSGVASVESSSGEAFHVGK(T)<br>(R)AQFVPLPVSVSVEFAVAATDCIAK(E) |
| 23 | A2HS_BOVIN Alpha-2-HS-glycoprotein | (K)ALGGEDVR(V)<br>(K)QDGQFSVLFTK(C)<br>(R)GYKHTLNQIDSVK(V) |
| 24 | A2HS_BOVIN Alpha-2-HS-glycoprotein | (K)ALGGEDVR(V)<br>(K)QDGQFSVLFTK(C) |
| 25 | A1AT_BOVIN Alpha-1-antitrypsin | (K)SVLGDVGITEVFSDR(A)<br>(K) SVLGDVGITEVFSDRADLSGITK(E) |
| 26 | A1AT_BOVIN Alpha-1-antitrypsin | (K)VLDPNTVFALVNYISFK(G) |
| 27 | Q66RQ0 Vitamin D-binding protein | (K)VLDQYIFELSR(K) |
| 28 | A1AT_BOVIN Alpha-1-antitrypsin | (K)SVLGDVGITEVFSDR(A)<br>(K)VLDPNTVFALVNYISFK(G)<br>(K) SVLGDVGITEVFSDRADLSGITK(E) |
| | VTDB_BOVIN Vitamin D-binding protein | (K)GQELCADYSENTFTEYK(K) |
| 29 | TTHY_BOVIN Transthyretin | (K)AADETWEPFASGK(T)<br>(K)TSESGELHGLTTEDKFVEGLYK(V)<br>(K)SLGISPFHEFAEVVFTANDSGPR(H) |
| 30 | ALBU_BOVIN Serum albumin | (K)QTALVELLK(H)<br>(K)HLDVEPQNLIK(Q)<br>(K)LGEYGFQNALIVR(Y)<br>(K)DAFLGSFLYEYSR(R)<br>(K)KVPQVSTPTLVEVSR(S)<br>(K)QNCDQFEKLGEYGFQNALIVR(Y) |
| 31 | APA4_PIG Apolipoprotein A-IV | (K)SELTQQLNTLFQDK(L) |
| 32 | RETB_BOVIN Plasma retinol-binding protein | (R)QEELCLAR(Q)<br>(R)DPSGFSPEVQK(I)<br>(K)YWGVASFLQK(G)<br>(R)LLNLDGTCADSYSFVFAR(D) |

| Spot number | Spot identification |
|---|---|
| *1st dimension and 2nd dimension under reducing conditions, in Fig. 9C (identifications as published in (Wait et al., 2001))* | |
| 33 | Alpha-1-antichymotrypsin |
| 34 | Amino oxidase |
| 35 | Immunoglobulin M |
| 36 | Transferrin |

(*continued*)

**Table 6** (*continued*)

| Spot number | Spot identification |
|---|---|
| 37 | Transferrin, C-truncated |
| 38 | Kininogen |
| 39–40 | Alpha-2-HS-glycoprotein |
| 41 | Alpha-1-antitrypsin |
| 42 | Alpha-1-acid glycoprotein |
| 43 | Apolipoprotein A-I |
| 44 | Retinol-binding protein |
| 45 | Apolipoprotein C |

The most crowded area in serum 2DE patterns is the one where several "$\alpha$-*globulins*" migrate – pI ca. 4.5–6 and $M_r$ ca. 50–70 kDa. In human and rat samples the intrinsic differences in pI and $M_r$ between adjacent spot rows under reduced conditions are sufficient for discrimination of all components with minimal overlaps. Full-range IPG and SDS-PAGE are adequate for quantitation of all serum proteins and zooming is an aid, not a requirement, for resolution in the "$\alpha$-globulin" area of these species. With mouse serum the reduced proteins migrate in closer proximity, and zooming becomes more valuable. In reduced bovine serum a number of proteins concentrate in an extremely narrow $M_r$ range while forming a continuum of charge distribution with few sharply defined spots: even with specific PAA gradients for finer size resolution the discrimination among proteins is thus very poor.

For all serum samples the situation in terms of overall resolution over the specified "$\alpha$-globulin" area is at least as good under non-reducing as under reducing conditions – and in some cases it is more favorable to run the proteins unreduced or partially reduced. This is especially the case with bovine serum, for analysis of which it seems appropriate to use a two-step strategy, namely a run under reducing conditions on a wide range 2DE gel followed by a run under non-reducing conditions on a narrow-range 2DE gel (*e.g.* 4–6 IPG, SDS-PAGE on 6–12% PAA). The second step is adequate for the evaluation of the "$\alpha$-globulins", the first step for the evaluation of the other proteins (low $M_r$, or alkaline pI).

### 4.6.2 Sample reduction and alkylation

A peculiar feature in the 2DE pattern of mouse serum is the series of bands/spot rows with pI 4–4.5 and $M_r > 70$ kDa that occupy the upper left quadrant of the map. These bands are present with similar but not identical features when running both unreduced and reduced samples. Since all the bands seem to contain contrapsin and $\alpha_1$-antitrypsin 1–3 with or without some kallikrein binding protein, and since the apparent $M_r$ is higher than expected for each of the specified proteins, a likely explanation is for them to be hetero-polymers. It seems unlikely that mixed disulfides (re)form during the separation procedure: this type of artefact has also been described for heteropolymers in 1DE (Nakamura et al., 1989) but only for homopolymers in 2DE (Miller et al., 1998). More drastic reducing conditions than attained with 2% $\beta$-mercaptoethanol might be required. Access of the reducing agent to all sensitive bonds is made easier by protein unfolding. With our current procedure for the 2DE analysis of biological fluids (Gianazza et al., 2002) samples are exposed to reducing agent alone for a short time, at room temperature, before loading, then to urea and reducing agent, at 15°C, during the first part of isoelectric focusing, eventually to detergent and reducing agent, at room temperature and in an alkaline medium, before SDS-PAGE. Procedures for reduction and alkylation need to ensure both completeness and irreversibility of S–S breakage. A popular protocol, now available in a reagent kit, uses tributylphosphine as the reducing agent; the procedure is carried out at room temperature in the urea/thiourea/detergent mix used as focusing medium (Herbert et al., 2001). In a procedure we developed and tested, samples are reduced at 37°C in the presence of SDS and at alkaline pH; proteins are concentrated and washed free of detergent by acetone precipitation (Gianazza and De Ponti, 1993). We applied some variants of the above protocols as for the mode of sample application – detailed in Table 2 – to two sera: mouse serum as the test sample for difficult fractionation problems and rat serum as the reference specimen.

Figure 10 panels $A^*$ and $A^{**}$ show the serum patterns obtained after tributylphosphine reduction, passive or active in-gel rehydration of 3-mm wide gel strips producing the same results. Identifications for the main spots are listed in Table 7. For rat serum, the pattern of acidic

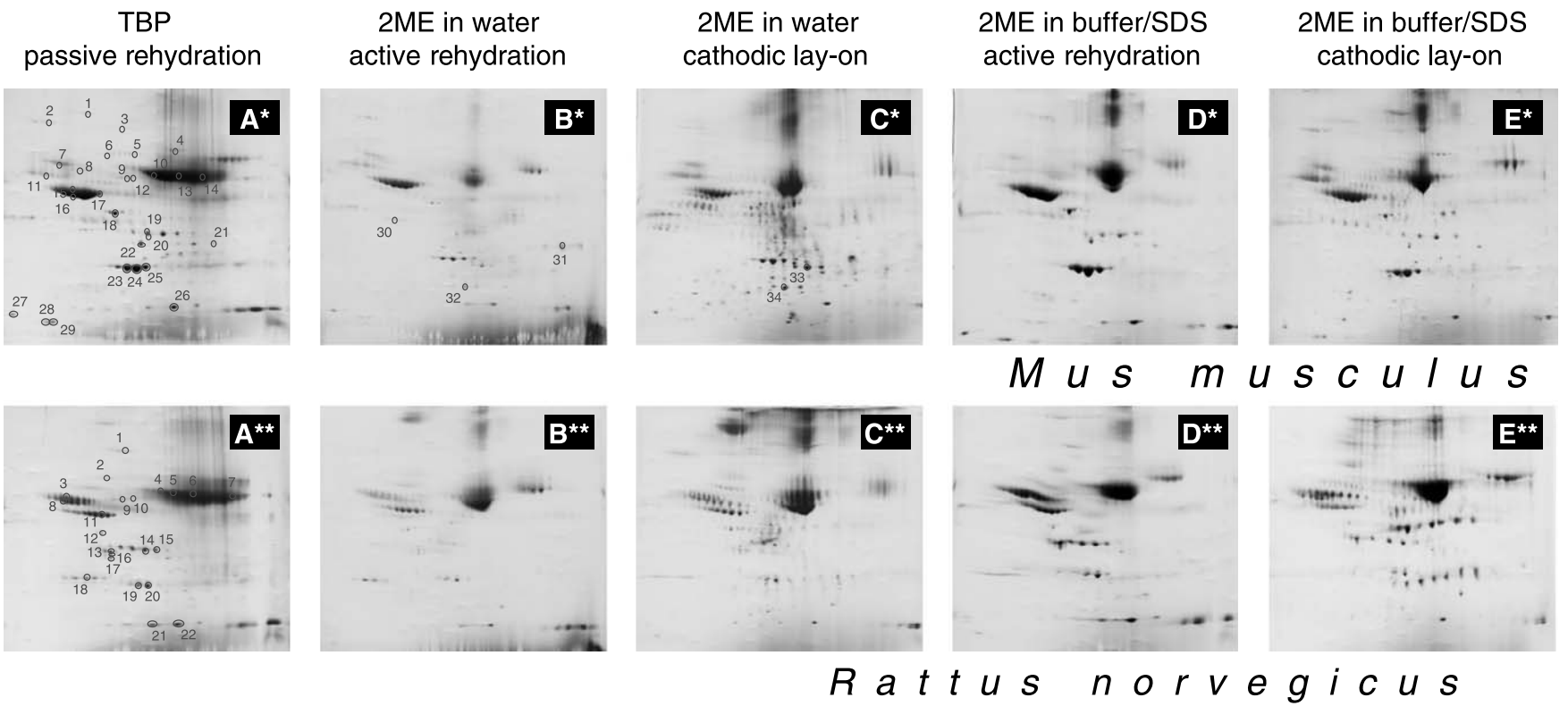


**Fig. 10.** 2DE of mouse (upper row) and rat serum (lower row) reduced/alkylated and run according to different experimental protocols. **A** Reduction with tributylphosphine (*TBP*), passive rehydration, sample load: 10 $\mu$L; **B** reduction with $\beta$-mercaptoethanol in water, acetone precipitation and active rehydration, sample load: 7.5 $\mu$L; **C** reduction with $\beta$-mercaptoethanol in water, acetone precipitation and lay-on cathodic application, sample load: 7.5 $\mu$L; **D** reduction with $\beta$-mercaptoethanol in alkaline buffer/SDS, acetone precipitation and active rehydration, sample load: 15 $\mu$L; **E** reduction with $\beta$-mercaptoethanol in alkaline buffer/SDS, acetone precipitation and lay-on cathodic application, sample load: 7.5 $\mu$L

**Table 7.** Mouse and rat serum, after reduction and alkylation

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| **A**. *Mouse serum run after reduction/alkylation with tributylphosphine (in Fig. 10 A*)* | | |
| 1 | A1T3_MOUSE Alpha-1-antitrypsin 1-3 | (R)IFNNGADLSGITEENAPLK(L)<br>(K)DQSPASHEIATNLGDFAISLYR(E)<br>(R)FDHPFLFIIFEEHTQSPLFVGK(V) |
| | TTHY_RAT Transthyretin | (K)ALGISPFHEYAEVVFTANDSGHR(H) |
| 2 | COTR_MOUSE Serine proteinase inhibitor A3K | (K)FSIASNYR(L)<br>(K)ELISELDER(T)<br>(K)KLSVSQVVHK(A)<br>(K)TMEEILEGLK(F)<br>(R)LEEDVLPEMGIK(E)<br>(R)TLMVLVNYIYFK(G)<br>(R)KTLFPSQIEELNLPK(F)<br>(K)ISFDPQDTFESEFYLDEK(R)<br>(K)AVLDVAETGTEAAAATGVIGGIRK(A)<br>(K)ISFDPQDTFESEFYLDEKR(S)<br>(K)NPDTNIVFSPLSISAALALVSLGAK(G)<br>(K)LALKNPDTNIVFSPLSISAALALVSLGAK(E) |
| 3 | O54882 PK-120 | (R)FAHTVVTSR(V)<br>(K)ITFELIYQELLQR(R)<br>(K)SQSEQDTVLNGDFIVR(Y) |
| 4 | GELS_MOUSE Gelsolin, plasma | (K)HVVPNEVVVQR(L)<br>(R)EVQGFESSTFSGYFK(S)<br>(R)QTQVSVLPEGGETPLFK(Q)<br>(R)SQHVQVEEGSEPDAFWEALGGK(T) |
| | ALBU_MOUSE Serum albumin | (K)LGEYGFQNAILVR(Y)<br>(K)DVFLGTFLYEYSR(R) |
| 5 | Q91W60 Inter alpha-trypsin inhibitor, heavy chain 4 | (K)QYSAAVGR(G)<br>(R)LGMYELLLK(V)<br>(K)LQDQGPDVLLAK(V)<br>(K)ITFELIYQELLQR(R)<br>(K0SQSEQDTVLNGDFIVR(Y)<br>(R)AHGGTNINNAVLLAVELLDR(S) |
| 6 | CO5_MOUSE Complement C5 | (K)YGVWTIK(A)<br>(R)FSVSIELER(T)<br>(K)VVPDAEVYAFFGLR(E)<br>(K)FQNAALLTLQPNQVPR(E)<br>(K)YVLSPYTLNLVATPLFVKPGIPFSIK(A) |
| 7 | ESTN_MOUSE Liver carboxylesterase N | (K)MNEETASLLLR(R)<br>(K)EGASEEETNLSK(M)<br>(R)DAGVSTYMYEFR(Y)<br>(K)SFNTVPYIVGFNK(Q)<br>(R)FAPPQPAEPWSFVK(N)<br>(K)QEFGWIIPMMLQNLLPEGK(M)<br>(R)LGIWGLFSTGDEHSQGNWAHLDQLAALR(W) |
| 8 | ESTN_MOUSE Liver carboxylesterase N | (K)EGASEEETNLSK(M)<br>(K)SFNTVPYIVGFNK(Q)<br>(R)FAPPQPAEPWSFVK(N) |
| 9 | CO3_MOUSE Complement C3 | (R)LPYSVVR(N)<br>(K)IGQQEVEVK(A)<br>(R)VMQDFFIDLR(L)<br>(R)SELEEDIIPEEDIISR(S)<br>(K)DSITTWEILAVSLSDKK(G)<br>(K)EADVSLTAFVLIALQEAR(D)<br>(R)IILQGSPVVQMAEDAVDGER(L) |
| 10 | ALBU_MOUSE Serum albumin | (K)TPVSEHVTK(C)<br>(K)CCSGSLVER(R)<br>(K)GLVLIAFSQYLQK(C)<br>(R)LPCVEDYLSAILNR(V) |

(*continued*)

**Table 7** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| 11 | COTR_MOUSE Serine proteinase inhibitor A3K | (K)DLQILAEFHEK(T)<br>(R)LEEDVLPEMGIK(E)<br>(R)TLMVLVNYIYFK(G)<br>(K)TLFPSQIEELNLPK(F)<br>(K)EVFTEQADLSGITETK(K)<br>(K)AVLDVAETGTEAAAATGVIGGIR(K)<br>(R)ALYQTEAFTADFQQPTEAK(N)<br>(K)AVLDVAETGTEAAAATGVIGGIRK(A)<br>(K)NPDTNIVFSPLSISAALALVSLGAK(G)<br>(K)LALKNPDTNIVFSPLSISAALALVSLGAK(E) |
| 12 | CO3_MOUSE Complement C3 | (K)IGQQEVEVK(A)<br>(K)GYTQQLAFK(Q)<br>(R)SELEEDIIPEEDIISR(S)<br>(K)DSITTWEILAVSLSDKK(G)<br>(K)EADVSLTAFVLIALQEAR(D) |
| 13 | ALBU_MOUSE Serum albumin | (K)TPVSEHVTK(C)<br>(K)TNCDLYEK(L)<br>(K)CCSGSLVER(R)<br>(R)YNDLGEQHFK(G)<br>(R)RHPDYSVSLLLR(L)<br>(K)LGEYGFQNAILVR(Y)<br>(R)LPCVEDYLSAILNR(V)<br>(R)LSQTFPNADFAEITK(L)<br>(R)RPCFSALTVDETYVPK(E)<br>(K)CCAEANPPACYGTVLAEFQPLVEEPK(N) |
| | HEMO_MOUSE Hemopexin | (R)CSPDPGLTALLSDHR(G)<br>(K)LFQEEFPGIPYPPDAAVECHR(G) |
| 14 | ALBU_MOUSE Serum albumin | (K)KYEATLEK(C)<br>(K)TPVSEHVTK(C)<br>(K)TNCDLYEK(L)<br>(K)CCSGSLVER(R)<br>(R)YNDLGEQHFK(G)<br>(R)RHPDYSVSLLLR(L)<br>(K)LGEYGFQNAILVR(Y)<br>(R)LPCVEDYLSAILNR(V)<br>(R)LSQTFPNADFAEITK(L)<br>(R)RPCFSALTVDETYVPK(E)<br>(K)ENPTTFMGHYLHEVAR(R)<br>(K)AETFTFHSDICTLPEKEK(Q) |
| 15 | A1T3_MOUSE Alpha-1-antitrypsin 1-3 | (K)KVINDFVEK(G)<br>(K)VINDFVEKGTQGK(I)<br>(K)KLDQDTVFALANYILFK(G)<br>(R)IFNNGADLSGITEENAPLK(L)<br>(R)FDHPFLFIIFEEHTQSPLFVGK(V)<br>(K)SFQHLLQTLNRPDSELQLSTGNGLFVNNDL(V) |
| | ALBU_MOUSE Serum albumin | (K)LGEYGFQNAILVR(Y) |
| 16 | A1T3_MOUSE Alpha-1-antitrypsin 1-3 | (R)LAQIHFPR(L)<br>(R)LSISGEYNLK(T)<br>(K)KLDQDTVFALANYILFK(G)<br>(R)IFNNGADLSGITEENAPLK(L)<br>(K)NHYQAEVFSVNFAESEEAKK(V)<br>(K)DQSPASHEIATNLGDFAISLYR(E)<br>(R)FDHPFLFIIFEEHTQSPLFVGK(V)<br>(R)IFNNGADLSGITEENAPLKLSQAVHK(A)<br>(K)SFQHLLQTLNRPDSELQLSTGNGLFVNNDL(V) |
| 17 | A1T3_MOUSE Alpha-1-antitrypsin 1-3 | (R)LAQIHFPR(L)<br>(K)KVINDFVEK(G)<br>(R)LSISGEYNLK(T) |

(*continued*)

**Table 7** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)KLDQDTVFALANYILFK(G) |
| | | (R)IFNNGADLSGITEENAPLK(L) |
| | | (K)NHYQAEVFSVNFAESEEAK(K) |
| | | (K)NHYQAEVFSVNFAESEEAKK(V) |
| | | (K)DQSPASHEIATNLGDFAISLYR(E) |
| | | (R)FDHPFLFIIFEEHTQSPLFVGK(V) |
| | | (R)IFNNGADLSGITEENAPLKLSQAVHK(A) |
| | | (K)SFQHLLQTLNRPDSELQLSTGNGLFVNNDL(V) |
| 18 | A1T3_MOUSE Alpha-1-antitrypsin 1-3 | (R)FDHPFLFIIFEEHTQSPLFVGK(V) |
| | APA4_MOUSE Apolipoprotein A-IV | (K)LQLTPYIQR(M) |
| | | (K)TDVTQQLSTLFQDK(L) |
| | | (K)LVPFVVQLSGHLAKETER(V) |
| | | (R)QQLGPNSGEVESHLSFLEK(S) |
| | | (K)TDVTQQLSTLFQDKLGDASTYADGVHNK(L) |
| 19 | Q6PEM2 Pzp protein | (K)YGAATFTR(S) |
| | | (R)DLSSSDLSTASK(I) |
| | | (R)LPDLPGNYVTK(G) |
| | | (K)GSGSGCVYLQTSLK(Y) |
| | | (K)APFALQVNTLPLNFDK(A) |
| | | (R)LLLQEVRLPDLPGNYVTK(G) |
| | | (K)APFALQVNTLPLNFDKAGDHR(T) |
| | | (K)QQNSHGGFSSTQDTVVALQALSK(Y) |
| | | (K)YNILPVADGKAPFALQVNTLPLNFDK(A) |
| 20 | Q6PEM2 Pzp protein | (K)TFHVNSGNR(L) |
| | | (K)LQDQPNIQR(T) |
| | | (R)LPDLPGNYVTK(G) |
| | | (K)GSGSGCVYLQTSLK(Y) |
| | | (K)EVLVTIESSGTFSK(T) |
| | | (K)APFALQVNTLPLNFDK(A) |
| | | (K)APFALQVNTLPLNFDKAGDHR(T) |
| | | (K)QQNSHGGFSSTQDTVVALQALSK(Y) |
| | | (K)GSGSGCVYLQTSLKYNILPVADGK(A) |
| | | (K)YNILPVADGKAPFALQVNTLPLNFDK(A) |
| 21 | Q6PEM2 Pzp protein | (K)LQDQPNIQR(T) |
| | | (R)DLSSSDLSTASK(I) |
| | | (R)LLLQEVRLPDLPGNYVTK(G) |
| | | (K)APFALQVNTLPLNFDKAGDHR(T) |
| | | (K)QQNSHGGFSSTQDTVVALQALSK(Y) |
| | | (K)GSGSGCVYLQTSLKYNILPVADGK(A) |
| | ALBU_MOUSE Serum albumin | (R)LSQTFPNADFAEITK(L) |
| 22 | Q6PEM2 Pzp protein | (K)EVLVTIESSGTFSK(T) |
| | | (K)QQNSHGGFSSTQDTVVALQALSK(Y) |
| | APE_MOUSE Apolipoprotein E | (R)LGKEVQAAQAR(L) |
| | | (K)ELEEQLGPVAEETR(A) |
| | | (K)IQASVATNPIITPVAQENQ |
| 23 | Q8BPD5 Apolipoprotein A-I | (R)QKLQELQGR(L) |
| | | (K)DFANVYVDAVK(D) |
| | | (K)VAPLGAELQESAR(Q) |
| | | (K)TQVQSVIDKAASETLTAQ |
| 24 | Q8BPD5 Apolipoprotein A-I | (R)QKLQELQGR(L) |
| | | (K)DFANVYVDAVK(D) |
| | | (K)SNPTLNEYHTR(A) |
| | | (K)VAPLGAELQESAR(Q) |
| | | (K)TQVQSVIDKAASETLTAQ |
| | | (R)DFWDNLEKETDWVR(Q) |
| 25 | APA1_MOUSE Apolipoprotein A-I | (R)QKLQELQGR(L) |
| | | (K)DFANVYVDAVK(D) |

(*continued*)

**Table 7** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (K)SNPTLNEYHTR(A)<br>(K)VAPLGAELQESAR(Q)<br>(K)AQSVIDKASETLTAQ<br>(R)DFWDNLEKETDWVR(Q) |
| 26 | TTHY_MOUSE Transthyretin | (K)TSEGSWEPFASGK(T)<br>(K)TAESGELHGLTTDEKFVEVGYR(V)<br>(K)TLGISPFHEFADVVFTANDSGHR(H) |
| 27 | APC3_MOUSE Apolipoprotein C-III | (K)TVQDALSSVQESDIAVVAR(G) |
| 28 | APA2_MOUSE Apolipoprotein A-III | (K)TSEIQSQVK(A)<br>(K)THEQLTPLVR(S) |
| | APC3_MOUSE Apolipoprotein C-III | (K)AYFEKTHEQLTPLVR(S)<br>(K)TVQDALSSVQESDIAVVAR(G) |
| 29 | APA2_MOUSE Apolipoprotein A-III | (K)TSEIQSQVK(A)<br>(K)THEQLTPLVR(S) |
| ***B***. *Mouse serum after reduction/alkylation with β-mercaptoethanol/iodoacetamide in water (active rehydration) (in Fig. 10B*)* | | |
| 30 | APA4_MOUSE Apolipoprotein A-IV | (K)LQLTPYIQR(M)<br>(K)NLAPLVEDVQSK(V)<br>(K)TDVTQQLSTLFQDK(L)<br>(K)LVPFVVQLSGHLAKETER(V)<br>(R)QQLGPNSGEVESHLSFLEK(S)<br>(K)TDVTQQLSTLFQDKLGDASTYADGVHNK(L) |
| 31 | TRFE_MOUSE Serotransferrin | (K)TVLPPDGPR(L)<br>(K)TSYPDCIK(A)<br>(R)DQYELLCLDNTR(K)<br>(R)SAGWVIPIGLLFCK(L)<br>(K)NNGKEDLIWEILK(V)<br>(R)SAGWVIPIGLLFCKLSEPR(S)<br>(K)LCQLCPGCGCSSTQPFFGYVGAFK(C) |
| 32 | ALBU_MOUSE Serum albumin | (K)LVQEVTDFAK(T)<br>(R)ENYGELADCCTK(Q)<br>(K)GLVLIAFSQYLQK(C)<br>(K)CSYDEHAKLVQEVTDFAK(T) |
| **C**. *Rat serum run after reduction/alkylation with tributylphosphine (in Fig. 10A**)* | | |
| 1 | O35802 Inter-alpha-inhibitor H4 heavy chain | (K)LALDNGGLAR(R)<br>(K)IPAQGGTNINK(A)<br>(K)AVLSAVELLDK(S)<br>(K)VTISLLSLDDPQR(G)<br>(R)LWALLTIQQQLEQR(I)<br>(K)TGLLQLSGPDKVTISLLSLDDPQR(G) |
| 2 | O35802 Inter-alpha-inhibitor H4 heavy chain | (K)IPAQGGTNINK(A)<br>(R)LWALLTIQQQLEQR(I) |
| 3 | CPI3_RAT Contrapsin-like protease inhibitor 3 | (K)DLYVSQVVHK(A)<br>(K)IAELFSDLEER(T)<br>(R)DEELSCSVLELK(Y)<br>(R)TSMVLVNYLLFK(G)<br>(R)ALYQAEAFIADFK(Q)<br>(K)NVVFSPLSISAALTILSLGAK(D)<br>(R)ALYQAEAFIADFKQPNEAK(K)<br>(K)AVLDVDETGTEATAATGVATVIR(R)<br>(R)NPDKNVVFSPLSISAALTILSLGAK(D) |
| 4 | TTHY_MOUSE Transthyretin<br>ALBU_RAT Serum albumin | (K)TLGISPFHEFADVVFTANDSGHR(H)<br>(K)KYEATLEK(C)<br>(R)FPNAEFAEITK(L)<br>(K)LGEYGFQNAVLVR(Y)<br>(R)LPCVEDYLSAILNR(L) |

(*continued*)

**Table 7** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| | | (R)RPCFSALTVDETYVPKEFK(A)<br>(K)SQCLAETEHDNIPADLPSIAADFVEDK(E)<br>(K)SQCLAETEHDNIPADLPSIAADFVEDKEVCK(N) |
| 5 | ALBU_RAT Serum albumin | (K)KYEATLEK(C)<br>(R)FPNAEFAEITK(L)<br>(K)LGEYGFQNAVLVR(Y)<br>(K)DVFLGTFLYEYSR(R)<br>(R)LPCVEDYLSAILNR(L)<br>(R)HPYFYAPELLYYAEK(Y)<br>(R)RHPYFYAPELLYYAEK(Y)<br>(K)SQCLAETEHDNIPADLPSIAADFVEDKEVCK(N) |
| 6 | A1T1_MOUSE Alpha-1-antitrypsin 1-1<br>ALBU_RAT Serum albumin | (K)KYEATLEK(C)<br>(K)NHYQAEVFSVNFAESEEAKK(V) |
| 7 | ALBU_RAT Serum albumin | (K)KYEATLEK(C)<br>(R)FPNAEFAEITK(L)<br>(K)LGEYGFQNAVLVR(Y)<br>(R)LPCVEDYLSAILNR(L)<br>(K)SQCLAETEHDNIPADLPSIAADFVEDKEVCK(N) |
| 8 | CPI1_RAT Contrapsin-like protease inhibitor 1 | (K)IFSQQADLSR(I)<br>(R)TSMVLVNYLLFK(G)<br>(K)NVVFSPLSISAALAILSLGAK(D)<br>(K)AVLDVDETGTEGAAATAVTAALK(S)<br>(R)LSQPEDQAEINTGSALFIDK(E)<br>(K)FSISTDYNLEEVLPELGIRK(I)<br>(R)NPDKNVVFSPLSISAALAILSLGAK(D) |
| 9 | CO3_RAT Complement C3 | (K)IGLQEVEVK(A)<br>(R)YYQTIEIPPK(S)<br>(R)SDVDEDIIPEEDIISR(S)<br>(K)EADVSLTAFVLIALQEAR(D)<br>(R)SHFPESWLWTIEELKEPEK(N)<br>(K)AGEYLEASYLNLQRPYTVAIAGYALALMNK |
| 10 | CO3_RAT Complement C3 | (R)YYQTIEIPPK(S)<br>(K)DSITTWEILAVSLSDKK(G)<br>(K)EADVSLTAFVLIALQEAR(D)<br>(R)ILLQGTPVAQMAEDAVDGER(L)<br>(R)SHFPESWLWTIEELKEPEK(N) |
| 11 | A1AT_RAT Alpha-1-antiproteinase | (K)AVLTLDER(G)<br>(K)TLLSSLGITR(V)<br>(K)LSISGTYNLK(T)<br>(K)RPFNPEHTR(D)<br>(K)ISSNLADFAFSLYR(E)<br>(R)VFNNDADLSGITEDAPLK(L)<br>(R)GTEAAGATVVEAVPMSLPPQVK(F)<br>(K)FDHPFIFMIVESETQSPLFVGK(V)<br>(K)AFHHLLQTLNRPDSELQLNTGNGLFVNK(N) |
| 12 | APA4_RAT Apolipoprotein A-IV | (K)EAVEQLQK(T)<br>(R)QLTPYIQR(M)<br>(R)LAPLAEGVQEK(L)<br>(K)LVPFAVQLSGHLTK(E)<br>(K)TDVTQQLNTLFQDK(L)<br>(K)SLEDLNKQLDQQVEVFR(R)<br>(R)QQLGSDSGDVESHLSFLEK(N)<br>(R)AVEPLGDKFNMALVQQMEK(F) |
| 13 | giΩ37805241 Apolipoprotein E<br><br>*really omega*? | DRLEEVR<br>LQAEIFQAR<br>ELEEQLGPVAEETR<br>IQASVATNSIASTTVPLENQ |

(*continued*)

**Table 7** (*continued*)

| Spot number | Spot identification | Sequenced peptides |
|---|---|---|
| 14 | Q63041 Alpha-1-macroglobulin | (K)LQDQSNIQR(T)<br>(K)VNTLPLNFDK(A)<br>(R)DLSSSDLTTASK(I)<br>(K)VSGSGCVYLQTSLK(Y)<br>(R)TEVNTNHVLIYIEK(L)<br>(K)YNILPEAEGEAPFTLK(V)<br>(K)EVSVTIESSGTVSGTLHVNNGNR(L)<br>(K)QQNSHGGFSSTQDTVVALQALSK(Y)<br>(K)SNKEVSVTIESSGTVSGTLHVNNGNR(L) |
| 15 | Q63041 Alpha-1-macroglobulin | (K)NLKPAPVK(V)<br>(K)LQDQSNIQR(T)<br>(K)VNTLPLNFDK(A)<br>(R)LADLPGNYITK(V)<br>(R)TEVNTNHVLIYIEK(L)<br>(K)YNILPEAEGEAPFTLK(V)<br>(K)QQNSHGGFSSTQDTVVALQALSK(Y) |
| 16 | APE_RAT Apolipoprotein E | (R)LGPLVEQGR(Q)<br>(R)TANLGAGAAQPLR(D)<br>(K)GWFEPLVEDMQR(Q)<br>(R)IKGWFEPLVEDMQR(Q) |
| 17 | APE_RAT Apolipoprotein E | (R)DRLEEVR(E)<br>(R)LGPLVEQGR(Q)<br>(R)TANLGAGAAQPLR(D) |
| 18 | CRP_RAT C-reactive protein | (R)SFSIFSYATK(T)<br>(K)TSFNEILLFWTR(G) |
| 19 | APA1_RAT Apolipoprotein A-I | (K)VVAEEFR(D)<br>(R)DYVSQFESSTLGK(Q)<br>(K)QLNLNLLDNWDTLGSTVGR(L)<br>(K)AKPALDDLGQGLMPVLEAWK(A)<br>(R)LQEQLGPVTQEFWANLEKETDWLR(N) |
| 20 | APA1_RAT Apolipoprotein A-I | (K)VVAEEFR(D)<br>(K)DFATVYVDAVK(D)<br>(R)QKLEPLGTELHK(N)<br>(R)DYVSQFESSTLGK(Q)<br>(K)QLNLNLLDNWDTLGSTVGR(L)<br>(K)AKPALDDLGQGLMPVLEAWK(A)<br>(K)MQPHLDEFQEKWNEEVEAYR(Q)<br>(R)LQEQLGPVTQEFWANLEKETDWLR(N) |
| 21 | TTHY_RAT Transthyretin | (K)ALGISPFHEYAEVVFTANDSGHR(H) |
| 22 | TTHY_RAT Transthyretin | (K)TAESGELHGLTTDEK(F)<br>(K)ALGISPFHEYAEVVFTANDSGHR(H) |

spots is similar to 'standard' reducing conditions (see 4.2). In contrast some neutral proteins (transferrin and albumin) display greater heterogeneity and poorer resolution than 'controls'. CRP extensively shifts in pI, but its resolution improves from a diffusely stained area to a defined Gaussian spot train. $\alpha_2$-HS-glycoprotein, Gc, albumin fragments and apoC are missing from the pattern of reduced/alkylated serum. Most of these findings are replicated with mouse serum samples, however the intensity of haptoglobin $\beta$-chains falls below detection limit after treatment. The pattern of acidic high $M_r$ proteins obtained is similar in tributylphosphine treated samples and 'standard' reducing conditions.

Figure 10 panels B–E show the serum patterns obtained after $\beta$-mercaptoethanol reduction and either active in-gel rehydration or cathodic sample application. Reduction in water or alkaline buffer/SDS has similar effects although yield is higher in water for high $M_r$ proteins (specifically, $\alpha_1$-inhibitor 3 in rat serum), in alkaline buffer/SDS for neutral to basic proteins (transferrin and albumin

fragments in both sera). Cathodic sample application appears to provide better recovery of alkaline components and more resolved spots overall compared to in-gel rehydration. No high $M_r$ band is seen under these conditions in mouse samples.

## 5 Discussion

Shifts in pI and $M_r$ can be expected between unreduced and reduced proteins. The effects are obvious for inter-chain S–S bridged homo- and hetero-polymeric assemblies, the contribution of each subunit to the size of the native protein being linearly correlated to its molecular mass (Fig. 1A). Conversely, the contribution of each subunit to the pI of the native protein depends on the number and pKs of its charged aminoacids and to partial *vs* total buffering power. The pI of a homopolymeric assembly is similar to the pI of its subunits whereas the pI of a heteropolymeric assembly is intermediate between the pIs of its subunits though the relationship is not linear (Fig. 11).

The effects of reduction of intra-chain S–S bridges are usually more subtle. Only the release of this structural constraint allows complete unfolding when the protein is exposed to chaotropes. Complete unfolding to a random coil together with a close-to-stoichiometric binding of detergent – resulting in a constant charge density – is the prerequisite for molecular size evaluation in SDS-PAGE. When intra-chain S–S bridges are not reduced, unfolding is partial, the hydrodynamic volume of the protein is closer to native and the apparent $M_r$ is lower than actual $M_r$. The difference between actual and apparent value roughly depends on the number of intra-chain S–S bridges (Fig. 1B). Folded *vs* incompletely or completely unfolded structure usually imply a different micro-environment for some charged aminoacid side chains, which results in different pK values for the specific dissociating groups and in different pIs for the protein (Fig. 12). As a hydrophobic environment better accommodates neutral than charged structures, the pK of (partially) buried acids is higher and the pK of (partially) buried bases is lower than for their solvent-exposed counterparts. The pK of an acid is also higher when in a folded protein structure the protonated form (COOH) is able to form a stable H-bond with a neighboring acceptor (e.g. (Eberini et al., 2004)). The relative contributions of cysteine and cystine to a protein titration curve differ only at very alkaline pH; accordingly, SH/S–S redox affects the pI of just the most alkaline proteins (Fig. 13). The pKs of aminoacid side chains can be computed under various conditions (e.g. absence/presence of chaotropic agents) for proteins of experimentally determined three dimensional structure, or for which homology modeling is possible. The influence of protein redox state on variation in hydrodynamic volume can also be estimated from known or modeled structures by computation of propensity to S–S bridge formation between cysteine pairs. However, because of the complexity of biological samples, the changes in resolution due to redox state are more easily determined experimentally as in the electrophoretic data in Section 4.

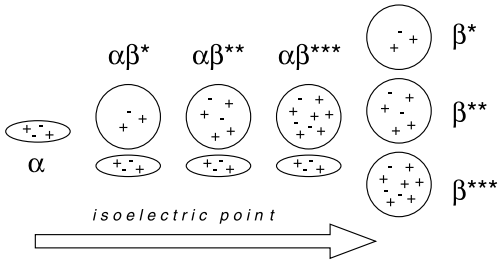


**Fig. 11.** Relationship between pI of a heterodimeric protein and pI of its subunits. Three examples are shown. $\alpha$ subunit is common to the three heterodimers, subunits $\beta^{*}$, $\beta^{**}$ and $\beta^{***}$ have identical pI but increasing (1×, 2× and 3×) buffering power

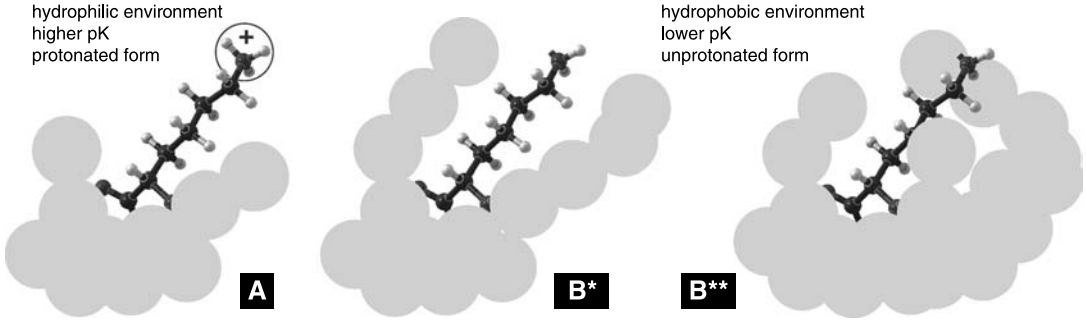


**Fig. 12.** Changes in pK of a dissociable group (e.g. a lysine $\varepsilon$ $NH_2$) are caused by burying in a hydrophobic environment, whether apolar aminoacid residues are adjacent in primary (**B***) or in tertiary structure (**B****)

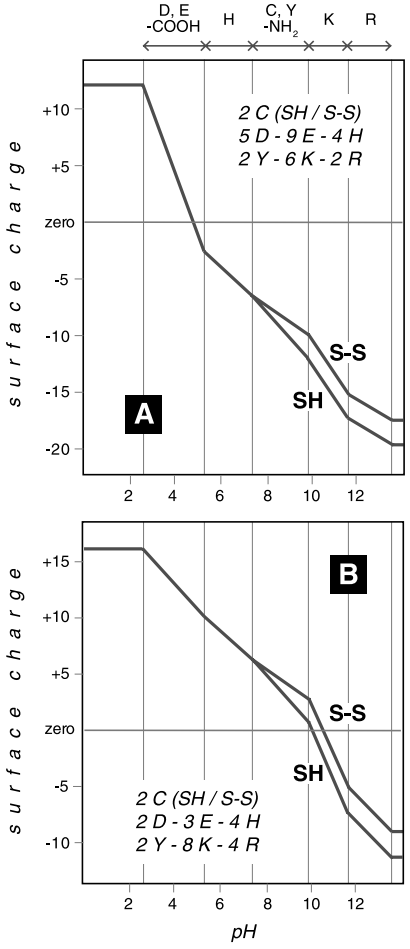


**Fig. 13.** Charge vs pH curves (titration curve) of proteins containing cysteine (*SH*) vs cystine (S–S) differ at alkaline pH. This results in a change in pI between different redox states only for basic proteins. In the examples, **A** and **B** both contain 2 C but **A** is an acidic protein with 6 D, 9 E, 4 H, 2 Y, 6 K and 2 R while **B** is a basic protein with 2 D, 3 E, 4 H 2 Y, 8 K and 4 R (as marked in the panels)

Since the effects of the redox condition on protein migration are sequence-dependent rather than protocol-dependent (i.e. since the effects are not systematic) optimal resolution is seldom if ever achieved for all proteins in a sample under a specific redox condition. Rather, quality of resolution may vary among various areas (pI and $M_r$ ranges) or between various difficult protein pairs. Although multiple runs of the same samples are laborious, from our results the alternative of zooming over different areas while changing the redox conditions of the samples seems worth considering.

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