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Alpha1-acid glycoprotein post-translational modifications: a comparative two dimensional electrophoresis based analysis

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ABSTRACT: Alpha1-acid glycoprotein (AGP) is an immunomodulatory protein expressed by hepatocytes in response to the systemic reaction that follows tissue damage caused by inflammation, infection or trauma. A proteomic approach based on two dimensional electrophoresis, immunoblotting and staining of 2DE gels with dyes specific for post-translational modifications (PTMs) such as glycosylation and phosphorylation has been used to evaluate the differential interspecific protein expression of AGP purified from human, bovine and ovine sera. By means of these techniques, several isoforms have been identified in the investigated species: they have been found to change both with regard to the number of isoforms expressed under physiological condition and with regard to the quality of PTMs (i.e. different oligosaccharidic chains, presence/absence of phosphorilations). In particular, it is suggested that bovine serum AGP may have one of the most complex pattern of PTMs among serum proteins of mammals studied so far.

Key words: Fluorescent staining, Immunoblotting, Post-translational modifications (PMTs), Two-dimensional gel electrophoresis (2-DE).

INTRODUCTION – The concentration of α1-Acid Glycoprotein (AGP) increases in plasma from two to five fold in humans and from ten to twenty folds in some animal species as a consequence of the systemic response to the inflammation (the so called acute phase reaction) (Hochepied *et al.*, 2003). AGP has a molecular weight of 40-44 kDa, the carbohydrate moiety accounting for approximately 45% of its overall mass (Fournier *et al.*, 2000), depending on the species. AGP belongs to the lipocalins family, a group of proteins deputed to the binding and transport of small hydrophobic molecules (Flower *et al.*, 2000). AGP has been further classified in a subset of lipocalins, the so called immunocalins, a subfamily of proteins that may also modulate the inflammatory reaction. A definitive knowledge of AGP function is still elusive: all the several AGP's activities so far described converge toward an immunomodulatory activity that may reduce the "collateral damages" caused by inflammation. Depending on the species, the protein exposes on its surface four to five N-linked complex oligosaccharide groups, and exhibit a high degree of subtle structural variations resulting in the expression in blood of several isoforms which have identical amino acid sequence but different glycosylation patterns. The relative occurrence of these glycoforms is strictly dependent on the pathophysiological status, that is determined by cytokines and hormones expressed during the inflammation (Fournier *et al.*, 2000). The precise pattern of the various AGP glycoforms is still unknown. This protein presents several glycoforms, that have been fractionated only by lectin affinity. It should be pointed out that from the data available in the literature (Nakano *et al.*, 2004), the glycan composition of AGP from various species is very different, and several isoforms do not interact with lectins. Moreover, AGP has 8 potential phosphorylation sites. This report describes a proteomic approach to investigate the differences

in glycan blood serum AGP post-translational pattern of three different species: human (hAGP), cow (bAGP) and sheep (sAGP). Several different isoforms were separated on two-dimensional electrophoresis, and the glycan pattern was identified by means of specific antibodies and lectins that identified several differences of terminal sialic acid distribution in the analyzed species.

MATERIAL AND METHODS – AGP purification. Human and ovine serum AGP were purified to homogeneity from blood serum as previously described (Ceciliani *et al.*, 2005). Bovine serum AGP was purified and quantified as previously decribed (Ceciliani *et al.*, 2005). 2D-PAGE. Purified serum AGP samples were first loaded on home made strips of immobilized pH gradient, range 3-6 linear, 7 cm long with cathodic cup-loading. Second dimension was performed using homemade 12.5% acrylamide homogeneous vertical SDS-PAGE slab gels (dimensions 80 x 70 x 1 mm).Gel staining. Control analytical gels were stained with silver nitrate. Glycosylations and phosphorylations were detected using respectively Pro-Q Emerald 300 Glycoprotein Gel Stain and Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes) according to manufacturer's instructions. Gel scanning. Gel scans have been performed using a ProXPress 2d Proteomic Imaging System (Perkin Elmer) and a FX100 laser scanner (BioRad). Pro-Q Emerald 300 stained gels (Figure 3) were scanned using the ProXPress System at a resolution of 100 nm, bottom illumination, excitation with UV light, emission with 530/30 nm filter and 100 ms of exposure; Pro-Q Diamond stained gel (Figure 4) was scanned using the FX100 laser scanner at a resolution of 100 nm, excitation with 532 nm laser and emission with 555 nm long pass filter. Western blotting. Western blotting was performed onto nitrocellulose. Human AGP was detected with an anti-hAGP monoclonal antibody (Sigma). Bovine and ovine AGP was detected using a polyclonal antibody raised in rabbit against purified bovine AGP. Secondary antibodies were alkaline phosphatase-conjugated anti-mouse and anti-rabbit respectively. Lectin staining with biotinylated *Sambucus nigra* agglutinin (SNAI) (Roche) and *Maackia amurensis* agglutinin (MAAI) (Roche) has been performed as previously described (Ceciliani *et al.*, 2005). SNAI is specific for α(2-6)-galactose linked sialic acid, while MAAI reacts with α(2-3)-galactose linked sialic acid. The blots were developed using the Amplified AP Immun-Blot Kit (BioRad).

Figure 1. Analytical silver stained 2D-PAGE gels of 2 μL of serum AGP purified from human a), bovine b) and ovine c) blood.

Figure 2. Immunoblots of 2D-PAGE gels of 2 μL of serum AGP purified from human a), bovine b) and ovine c) blood.

Figure 3. Glycoprotein-specific stained 2D-PAGE gels of 10 μL of AGP pur fied from human from human a), bovine b) and ovine c) blood.

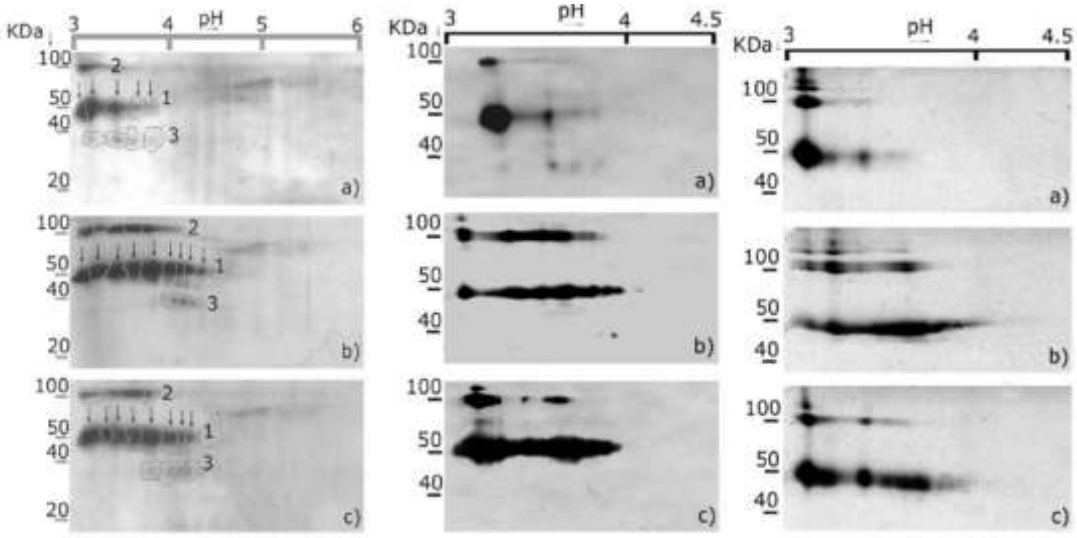


Figure 4. Phosphoprotein-specific stained 2D-PAGE gel of 5 μL of AGP purified from bovine blood.

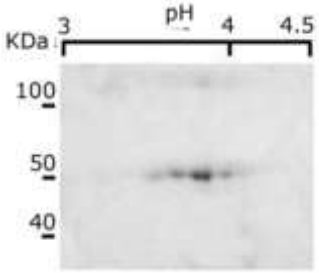
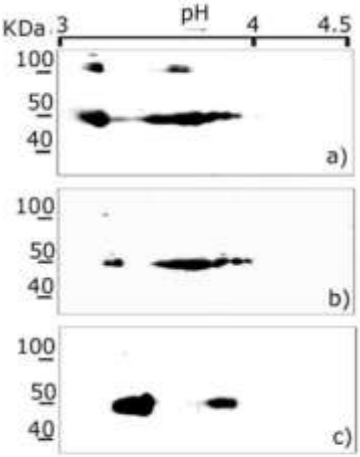


Figure 5. Western blots of 2D PAGE gels of: 5 μL of serum AGP purified from bovine blood stained with SNAI a); 10 μL of serum AGP purified from bovine blood stained with MAAI b); 5 μL of serum AGP purified from ovine blood stained with SNAI c).



RESULTS AND CONCLUSIONS – In order to investigate if this heterogeneous banding pattern was due to the non homogeneity of the purified AGP, an immunostaining of the three WB membranes was carried out using specific antibodies that directly reacts (human and bovine) or cross-reacts (anti-bovine AGP against ovine AGP). Results are shown in Figure 2. Figure 3 shows the same samples stained with glycosylation specificstaining. Separation of isoforms by 2-DE (Figure 1) has shown that in all the three species serum AGP is mainly present at an apparent molecular weight of ~ 45-50 kDa in the pH range of 3-4.5 (spot cluster marked as 1 in Figure 1). Noteworthy, hAGP is present in only five isoforms, while bAGP and sAGP display a much more rich pattern: at least 9 isoforms were distinguishable for the bovine sample and 8 for the ovine. AGP from all species seems to dimerize at 100 kDa (spot cluster marked as 2 in Figure 1). Moreover, some basic isoforms are present in low amount at ~ 35 kDa (spot cluster marked as 3 in Figure 1). This may be due to the loss of uncharged groups from the protein surface during HPLC or 2-DE processing, but it's not clear if this is caused by some physiological modification or by experimental treatment of samples. Remarkably, only bovine serum AGP displays clear phosphorylation referred to the 45-50 kDa isoforms (Figure 4), while hAGP and sAGP are not phoshorylated (data not shown). Human AGP did not show any specific reactivity with the lectins used in this study (data not shown). Bovine AGP has shown reactivity for both SNAI and MAAI, while ovine AGP reacted only with SNAI (Figure 5). Interestingly, the ruminant species here investigated showed to possess 8-9 different isoforms compared with human being who seems to have only five isoforms in the pH range of 3 – 4.5. Moreover AGP phosphorylation has been demonstrated for the first time in the bovine sample (Figure 4), while human and ovine AGP did not show this particular post-translational modification under physiological condition. The very low Ip of some AGP glycoforms depends on the sialic acid content, therefore we should not rule out the possibility that most of the SNAI- and MAAI-reacting human glycoforms have an Ip lower than 3. All these observations (high number of glycosylated isoforms, presence of phosphorylation and differential sialylation), together with those made about its peculiar oligosaccharide mojeties (Nakano *et al.*, 2004), suggest that bovine serum AGP may have one of the most complex patterns of post-translational modifications among serum proteins of mammals.

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