Journal of Dairy Research (2007) **74** 374–380. © *Proprietors of Journal of Dairy Research 2007*
doi:10.1017/S0022029907002646 *First published online 9 July 2007 Printed in the United Kingdom*

Differential expression and secretion of α_1-acid glycoprotein in bovine milk

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Received 19 October 2006 and accepted for publication 17 April 2007

α_1-Acid glycoprotein (AGP) is a lipocalin that is produced mainly by the liver and secreted into plasma in response to infections and injuries. In this study, we evaluated AGP isoforms that can be detected in bovine milk. We found that milk-AGP content is made up of at least two isoform groups, a low MW group (44 kDa) that is produced in the mammary gland (MG-AGP), and a higher MW group (55–70 kDa), that is produced by somatic cells (SC-AGP). Identical SC-AGP isoforms can be found both in milk and blood PMN cells. Analysis of the mammary tissue cDNA showed that the sequence of the MG-AGP isoform is identical to that of plasma AGP. Each group contains several proteins with different MWs and different isoelectric points, as shown by 2D-electrophoresis. The glycosylation patterns of these isoforms were analysed by means of specific lectin binding, to evaluate the degree of sialylation, fucosylation and branching. The MG-AGP glycan pattern was identical to plasma AGP produced by the liver. Several differences were detected, however, between plasma and SC-AGP isoforms, the most evident being the strong degree of fucosylation and the elevated number of di-antennary glycans in SC-AGP. Immunohistochemistry showed that AGP is found in all tissues that make up the mammary gland, but that it is most likely produced for the main part by the alveoli.

Keywords: α_1-acid glycoprotein, acute phase reaction, glycosylation, udder defense.

List of Abbreviations: AGP: bovine α_1 acid glycoprotein; MG-AGP: mammary gland AGP; SC-AGP: somatic cells AGP; SC: somatic cells; PMNC: polymorphonuclear cells.

α_1-Acid glycoprotein (AGP, orosomucoid) belongs to the structurally unrelated group of acute phase proteins (Petersen et al. 2004). AGP is a lipocalin (Lodberg & Wester, 2000) with immunomodulatory and anti-inflammatory activity, and plays an important role in the regulation of local inflammatory reactions, for example by reducing the damage to tissues due to uncontrolled complement activation (Tilg et al. 1993), or due an excessive rate of apoptosis (Van Molle et al. 1997). Moreover, AGP can downgrade the activation state of neutrophils and increase the expression of anti-inflammatory cytokines by macrophages (Hochepied et al. 2003).

AGP is produced mainly by the liver, but extra-hepatic expression has been reported in other tissues, such as stimulated human alveolar macrophages (Fournier et al. 1999) and endothelial cells (Sorensson et al. 1999). Bovine AGP is one of the acute phase proteins that are expressed by mammary gland epithelium (Ceciliani et al. 2005), together with Haptoglobin, M-SAA3 and albumin (Rainard et al. 2006). Plasma bovine AGP is a single polypeptide with 185 amino acid residues (Ceciliani et al. 2005), the mature isoform weighs 33·8 kDa (Nakano et al. 2004) and over 40% of the protein mass is made up of glycan chains. Notwithstanding its potentially important role as regulator of mammary gland innate immunity, basic information on milk-AGP is still lacking. For example, neither the primary structure nor the post-translational modifications of milk-AGP isoforms are known. Therefore, it is still not clear if milk-AGP is the product of the same gene as plasma-AGP or if different post-transcriptional mechanisms of regulation result in a different protein, as has been shown for M-SAA3 (Mc Donald et al. 2001). Furthermore, data

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concerning the localization of the protein in mammary gland tissue, as well as the stimuli that regulate its local expression, is not available.

We have decided to focus attention on milk-AGP for several reasons. Firstly, AGP is a minor acute phase protein in cattle (Tamura et al. 1989) and improved knowledge of its expression in the mammary gland may improve its utilization for early diagnosis in mastitis. Moreover, AGP exhibits a very wide range of anti-inflammatory activities, in particular against PMN cells, whose uncontrolled activation is considered to be the major cause of damage to the mammary gland during mastitis, therefore, any insights into this protein might provide useful information about the regulation of innate immunity of the bovine mammary gland.

In this paper we report the electrophoretic pattern of the various AGP fractions purified from bovine milk, with the aim of studying the structural basis of their differences by determining the cDNA sequence of the AGP produced by the mammary gland, and eventual post-translational modifications. Moreover, possible sources of the various isoforms found in milk were investigated by immunohistochemical localization of the protein in mammary gland tissues and in somatic cells. Finally, an in vitro model was evaluated to study the expression of mammary gland AGP isoform.

Material and Methods

Source of experimental material

Six healthy mid-lactating Holstein cows were selected as milk and serum sample donors. Somatic cell content was less than 100×10^3 cells/ml. Mammary gland (secretory epithelium, gland cistern, teat canal from two quarters) and liver specimens were obtained from two healthy cows during routine slaughter. Samples were removed within 10 min of slaughter and snap frozen in liquid nitrogen.

Purification and structural characterization of AGP from milk

Structural characterization of AGP in bovine milk was carried out following different approaches:

(a) Analysis of the plasma- and milk-AGP isoforms was carried out following SDS-PAGE of purified plasma- and milk-AGP (Ceciliani et al. 2005) and electrotransferring onto nitrocellulose membrane. AGP isoforms were immunostained with two anti-bovine AGP antibodies: the polyclonal anti-AGP, that was raised in rabbit against purified bovine AGP (Ceciliani et al. 2007) and the monoclonal 5AG1 (Hytest Ltd – Finland). Purified plasma- and milk-AGPs (10 μg) were further analysed by 2D-electrophoresis followed by silver staining (Gorg et al. 1988).

(b) Analysis of the primary structure of AGP produced by the mammary gland was carried out after isolation of AGP mRNA from the mammary gland and sequencing of the corresponding cDNA. Total RNA for RT (reverse-trancription)-PCR was isolated from mammary glands using TRIZOL (Sigma Aldrich, Milan, Italy) and iScript cDNA Synthesis Kit (BIO-RAD, Segrate, Italy) according to the manufacturer's instructions. Sequencing of the corresponding cDNA was carried out as previously reported (Ceciliani et al. 2005).

(c) The analysis of the carbohydrate moiety of plasma and milk-AGP was carried out on 2 μg proteins purified from plasma and milk, or immunoprecipitated from SC, from six healthy cows. Proteins were analysed after SDS-PAGE and electrotransferring onto nitrocellulose. Four biotinylated lectins (Vector Laboratories, Segrate, Italy) were used: *Sambucus nigra* agglutinin (SNAI; 5 μg/ml), specific for α(2-6)-linked sialic acid, *Maackia amurensis* agglutinin (MAA; 5 μg/ml) specific for α(2-3)-linked sialic acid, Concanavalin A (ConA; 2 μg/ml), which reacts with glycoproteins exposing diantennary N-linked glycans, and *Aleuria aurantia* lectin (AAL; 10 μg/ml), a fucose-specific lectin. The incubation with biotinylated lectins was carried out for 1 h at room temperature and the detection and the densitometric analysis of AGP glycoforms that react with biotin-conjugated lectins was carried out as previously described (Ceciliani et al. 2004).

Detection of AGP in mammary gland and milk cells

Mammary gland tissue (50 μg) was washed extensively with PBS and proteins were extracted following homogenization in 1 ml lysis buffer (10 mM-Tris-HCl pH 7·4, 10 g Nonidet-P40 (NP-40)/l, 150 mM-NaCl, 1mM EDTA and Protease Inhibitor cocktails (Sigma-Aldrich)) at 4 °C for 1 h. The supernatant was centrifuged at 10 000 ***g*** for 30 min at 4 °C, and 1 μl was directly loaded onto SDS-PAGE.

The detection of AGP in milk cells was carried out after immunoprecipitation. The starting material for immunoprecipitation of AGP was:

(a) milk somatic cells (SC) purified as previously described (Dosogne et al. 2001)
(b) milk PMNC, purified as described (Mehrzad et al. 2001)
(c) blood PMNC, purified as described (Mehrzad et al. 2001)

Cells (2×10^7) were washed twice in PBS and the pellet was resuspended in 1 ml lysis buffer. Nuclei were removed after centrifugation at 3000 ***g*** for 5 min at 4 °C, and the supernatant was centrifuged at 10 000 ***g*** for 30 min at 4 °C. In order to rule out an aspecific interaction between the activated Protein G-Sepharose and the AGP, 50 μl 20% GammaBind Protein G-Sepharose (GE-Healthcare,

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Nerviano, Italy) were added to 1ml cell lysate for 1 h at RT. The Sepharose was removed by centrifugation at 200 ***g*** for 1 min at 4 °C. Meanwhile, specific antibody-coupled GammaBind Protein G-Sepharose was prepared by adding 10 μg anti-AGP polyclonal antibody (Ceciliani et al. 2007) to 50 μl 20% Protein G-Sepharose and incubated for 1 h at RT. The antibody-coupled Sepharose was washed twice with 1 ml lysis buffer and finally added to 0·5 ml pre-cleaned lysate. Immunoprecipitation was carried out at 4 °C for 2 h. The AGP bound to Sepharose was collected by spinning at 200 ***g*** (1 min). The pellet was washed once with 1 ml lysis buffer plus 0·5 M-NaCl and, successively, with lysis buffer plus 1 g SDS/l and 10 mM-Tris-HCl pH 7·4, 1 g NP-40/l to remove proteins that do not bind directly to the Sepharose. The pellet containing AGP bound to the Sepharose was resuspended in 100 μl SDS sample buffer, boiled for 5 min, centrifuged at 200 ***g*** (5 min) and the supernatant was removed. Supernatant (1 μl) was used for SDS-PAGE. Both monoclonal antibody 5AG1 (Hytest Ltd – Finland) (dilution 1 : 1000–7·2 μg/ml) and polyclonal antibody poly anti-AGP (dilution 1 : 10 000–0·33 μg/ml) were used as primary antibodies. The immunoreactive bands were visualized by using enhanced chemiluminescence assay (GE-Healthcare, Nerviano, Italy) and were exposed to X-ray film.

Immunohistochemistry and RT-PCR in mammary gland samples

Both immunohistochemistry and RT-PCR were carried out on mammary gland tissue. The detection of mRNA expression in mammary gland tissue was performed as previously described (Ceciliani et al. 2005). For immunohistochemistry, 5 μm-cryosections were air dried, fixed in acetone (3 min) and stored at −70 °C. Sections were stained using a streptavidin-biotin peroxidase complex method with anti-bovine AGP polyclonal antibody (33 μg/ml). Negative controls consisted of substitution of specific antibodies with an isotype-matched, irrelevant monoclonal antibody or omission of the primary antibody. Frozen sections of bovine liver were used as positive controls and to check for normal distribution of the antigen.

The amount of AGP positive cells was evaluated as a percentage of the total and the intensity of the staining was scored as a low (+), moderate (++) and high (+++) intensity.

Bovine mammary gland epithelial cell cultures

The BME-UV1 cell line used in this study (Zavizion et al. 1996) was obtained from the University of Vermont. Cells were cultured in 75 cm^2 tissue culture flasks (Costar, Corning, NY, USA), in a mixture of 50% DMEM-F12, 30% RPMI-1640 and 20% NCTC-135), supplemented with 10% foetal calf serum (FCS), 0·1% lactose, 0·1% lact-albumin hydrolysate, 1·2 mM-glutathione, 1·0 μg insulin/ml, 5·0 μg transferrin/ml, 1·0 μg hydrocortisone/ml, 0·5 μg progesterone/ml, 10·0 μg L-ascorbic acid/ml and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml). The cells were maintained at 37 °C in a humidified 5% CO_2 incubator until confluence. Cells from the 39th passage were cultured for 4–5 d, until cells reached the stage of sub-confluent monolayer. Cells were then harvested following trypsinization (Trypsin-EDTA 0·05%) and approximately $0{\cdot}2 \times 10^6$ cells/ml were seeded into 60 mm dishes (Costar) for the experiments. Each dish received 3 ml complete medium with 10% serum, containing phorbol 12-myristate 13-acetate (PMA) (100 ng/ml), dexamethasone (1 M, 1 μM, 100 nm, 10 nM and 1 nM) or increasing concentrations of all-*trans* retinoic acid (10^{-7}–10^{-5} M). Dexamethasone and retinoic acids have been reported to induce the hepatic expression of AGP (Hochepied et al. 2003). PMA and all-*trans* retinoic acid were dissolved in ethanol (96%) and serially diluted in complete culture medium to yield the final concentrations tested. Dexamethasone was dissolved in methanol. Final ethanol and methanol concentrations did not exceed 1 ml/l. Media were changed after 24 h of culture with appropriate supplementation of each tested factor. After 48 h, cells were removed from the tissue culture dishes by complete trypsinization and the total number was counted using a hemocytometer. Then, the cells were harvested by centrifugation (1200 ***g*** for 10 min) and washed with PBS to remove all medium. The cells were resuspended in 200 μl PBS and stored frozen in RNA later stabilizing reagent (Quiagen, Milan, Italy) until RNA isolation or protein immunoprecipitation. Unless otherwise specified, all supplements and reagents were purchased from Sigma-Aldrich.

Results and Discussion

Structural characterization of milk-AGP isoforms: milk contains more than one AGP isoform group

The first part of the study was carried out in order to determine if the plasma and milk-AGP isoforms were similar. The analysis of AGP cDNA obtained from mammary gland tissue revealed an open reading frame of 658 bp, that coded for an amino acid sequence of 219 residues. Both cDNA and deduced protein sequences were identical to those of the plasma-AGP isoform (data not presented). These results suggest that MG-AGP and plasma AGP isoforms are likely expressed by the same gene. However, several differences were detected after immunostaining of the AGP positive fraction purified from milk. Fig. 1a shows SDS-PAGE and WB on nitrocellulose of milk- and plasma-AGP isoforms after immunostaining using a polyclonal anti-AGP antibody. Several immunoreactive bands were observed in purified milk-AGP: a low MW band of 44 kDa, that corresponds to the expected MW of bovine AGP, and a group of bands with high MW, ranging from 55 to 70 kDa. The 44 kDa band was present in both milk and plasma fractions, with a similar MW. An AGP dimer

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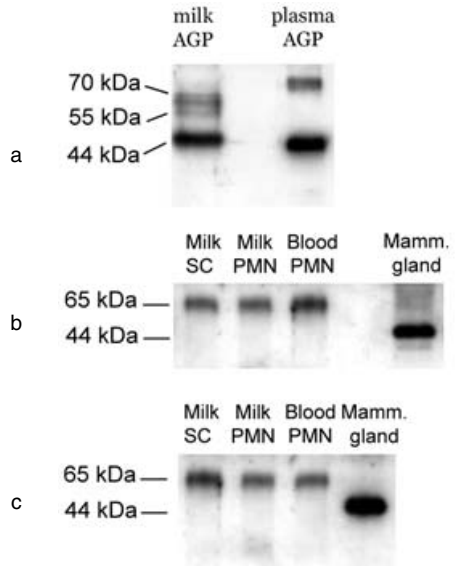

Fig. 1. SDS-PAGE and WB on nitrocellulose of milk and plasma AGP isoforms. (a) SDS-PAGE and WB on nitrocellulose of purified milk and plasma AGP isoforms after immunostaining using anti-AGP polyclonal antibody and ECL detection. 1 ng of total protein was loaded onto each lane. (b) Detection of AGP from milk somatic cells, milk PMNC cells, blood PMNC and mammary gland. Bands were detected after immunostaining with anti-AGP polyclonal antibody and ECL detection. Approximately 1 ng of total immunoprecipitated proteins (1 µg for the mammary gland extract) was loaded onto each lane. (c) Detection of AGP from milk somatic cells, milk PMNC, blood PMNC and mammary gland. Bands were detected after immunostaining with 5AG1 anti-AGP monoclonal antibody and ECL detection.

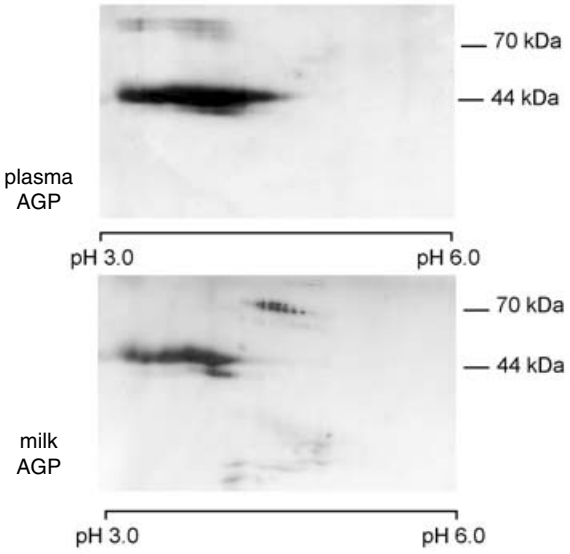

Fig. 2. 2D-Electrophoresis analysis of milk and plasma AGP isoforms. Approximately 10 µg of purified AGP samples were first loaded onto home-made strips of immobilized pH gradient, range 3–6 linear, 7 cm long. Second dimension was performed using homemade 12·5% acrylamide homogeneous vertical SDS slab gels. The bands were identified with silver staining.

(90 kDa) was present in the plasma isoform. In order to evaluate the possible source of milk-AGP, different cell types were analysed for the presence of the protein. Results are shown in Fig. 1b: the high MW isoforms, but not the 44 kDa isoform, were detected in both SC and purified milk PMNC. Since it has been reported that human AGP is produced by mielocytes and stored in secondary granules of PMNC (Theilgaard-Monch et al. 2005; Poland et al. 2005), we extended our study to blood neutrophils and found that these cells also contain the high MW AGP. Remarkably, the high MW isoforms were only detected in milk somatic cells and PMNC, while the mammary gland contained only the 44 kDa isoform. The same experiments were replicated using the monoclonal antibody 5AG1 (Fig. 1c), that recognizes immunoprecipitated AGP, although with substantially lower sensitivity. The data presented in Fig. 1 showed that bovine milk contains at least two isoforms groups that derive from two different sources: an abundant, low MW, 44 kDa isoform (MG-AGP) that is expressed by mammary gland epithelium, and an high MW isoform (MW 55–70 kDa), that is produced by somatic cells (SC-AGP) and that is apparently identical to the isoform found in blood PMNC. We did not detect the plasma AGP isoform, that may be endocytosed by neutrophils (Theilgaard-Monch et al. 2005) and carried into mammary glands, or leaked into milk from systemic circulation through the hemato-mammary barrier during inflammation. We may conclude that the plasma AGP isoform is scarce in healthy milk, but that it might increase during mastitis.

Post-translational modification analysis of milk-AGP isoform

2D-Electrophoresis was carried out on purified plasma- and milk-AGP isoforms in order to study the difference in the glycan pattern among AGP isoforms. Figure 2 shows how the SC-AGP group could be fractionated into at least eighteen isoforms, whereas the low MW group was made up of at least six major isoforms with a similar MW but a different isoelectric point. Due to the very low amount of available material, it was not possible to analyse the 2D-electrophoresis pattern of the AGP that was immunoprecipitated from somatic cells and PMNC.

The terminal oligosaccharide residues of AGP isoforms were evaluated by means of specific lectin binding. No differences were observed between the plasma-AGP and MG-AGP (the low MW milk isoform). It is possible that, due to the unique and novel sialic acid-containing diantennary oligosaccharides of bovine AGP which is composed of only N-glycolylneuraminic acid (Nakano et al. 2004), a MALDI-TOF structural approach will help to

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identify differences between the two isoforms. On the contrary, some difference was detected between the MG-AGP and the SC-AGP isoforms that make up milk-AGP, as reported in Fig. 3. Figure 3a shows the typical terminal glycosylation pattern using the lectin binding technique and the corresponding densitometric analysis plot (Fig. 3b). The results of these experiments carried out on six healthy cows are presented in Fig. 3c.

While the two groups of isoforms apparently share a similar content of sialic acid α(2-3)- and α(2-6)-linked to galactose, as determined by reaction with MAA and SNAI lectins, Fig. 3a shows that there is a strong reaction of SC-AGP with AAL, which is completely lacking in MG-AGP. SC-AGP isoforms also appeared to be more reactive to ConA than plasma-AGP. These results showed that somatic cells produce AGP isoforms that are strongly fucosylated and contain an elevated number of di-antennary glycans. This finding is remarkable because the immunomodulatory function of AGP is dependent on both its fucosylation and its branching degree. For example, it is known that the prevalent function of hyperfucosylated AGP is anti-inflammatory, since it can reduce the local inflammatory reaction by both ameliorating neutrophils-mediated damage and by reducing the complement activation (Williams et al. 1997). We may therefore envisage differential roles for the various milk isoforms during mastitis: the concentration of milk-AGP in healthy conditions is low (1–2 μg/ml; Ceciliani et al. 2005), and probably depends only on the MG-AGP produced by the mammary gland epithelium. This physiological amount may be increased during mastitis, when high numbers of PMNC migrate from blood to milk and release the hyperfucosylated SC-AGP isoforms. We may hypothesize that these hyperfucosylated SC-AGP glycoforms that are locally released by milk somatic cells into the inflamed area contribute to the dampening of neutrophil-mediated damage by antagonizing the adhesion of newly recruited PMNC, and by competing with these cells in their interactions with E-selectins.

The expression of milk AGP isoform cannot be induced by glucocorticoids or retinoic acid in BME-UV cells

Once it was established that the expression of the two AGP isoforms is the result of the same gene activation, we investigated whether the same molecules that regulate expression of the protein in the liver also regulate expression in mammary epithelial cells. MG-AGP expression was not observed either with RT-PCR or by SDS-PAGE analysis followed by immunostaining. These results confirm that, at least in BME-UV1 cells, the protein is not present in resting conditions and that the expression of AGP is not regulated by the same molecules that regulate the expression of AGP in mouse and human hepatocytes. Therefore, the regulation pathways of AGP expression in bovine mammary gland epithelium remain unknown.

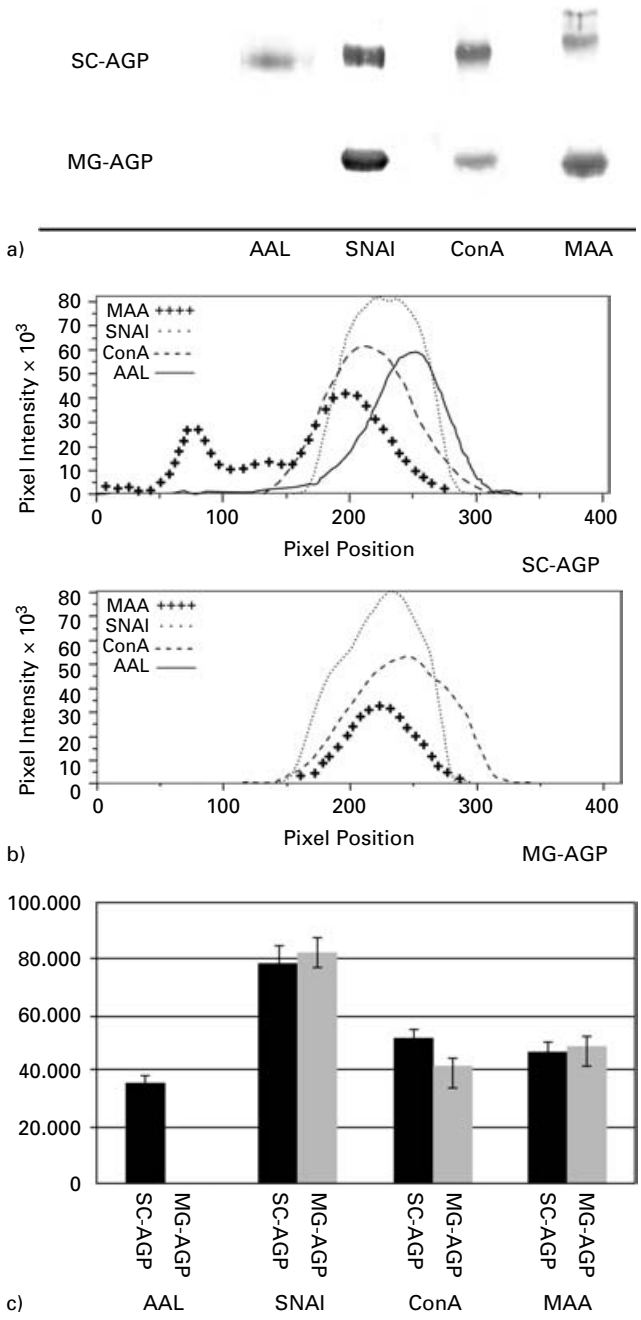

Fig. 3. Comparison analysis of the glycosylation pattern of low molecular weight, mammary gland-AGP (MG-AGP) and somatic cells (SC-AGP) isoforms. (a) Detection of oligosaccharides chains after WB and staining with specific lectins. (b) Density profile plot displaying the peaks corresponding to AAL (———), SNAI (------), ConA (·····) and MAA (++++++). Values are arbitrary units expressed in relative pixel intensity. (c) Histograms displaying the average volume of density profiles of MG-AGP and SC-AGP, purified from 6 cows, after reaction with specific lectins. Values are arbitrary units expressed in relative pixel intensity. Results are expressed as mean values ± standard deviation.

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Table 1. Immunohistochemical evaluation of AGP expression in normal bovine mammary gland

	Mammary parenchyma			Cistern		Teat
	Ducts	Secretory Alveoli	Resting Alveoli	Epithelium	Alveoli	Epitelium
% positive cells	70–100%	20–40%	60–70%	80–100%	30–40%	100%
Intensity*	++	+/++	++	+++	++/+++	+++

* *Score*:
- High intensity +++
- Moderate intensity ++
- Low intensity +

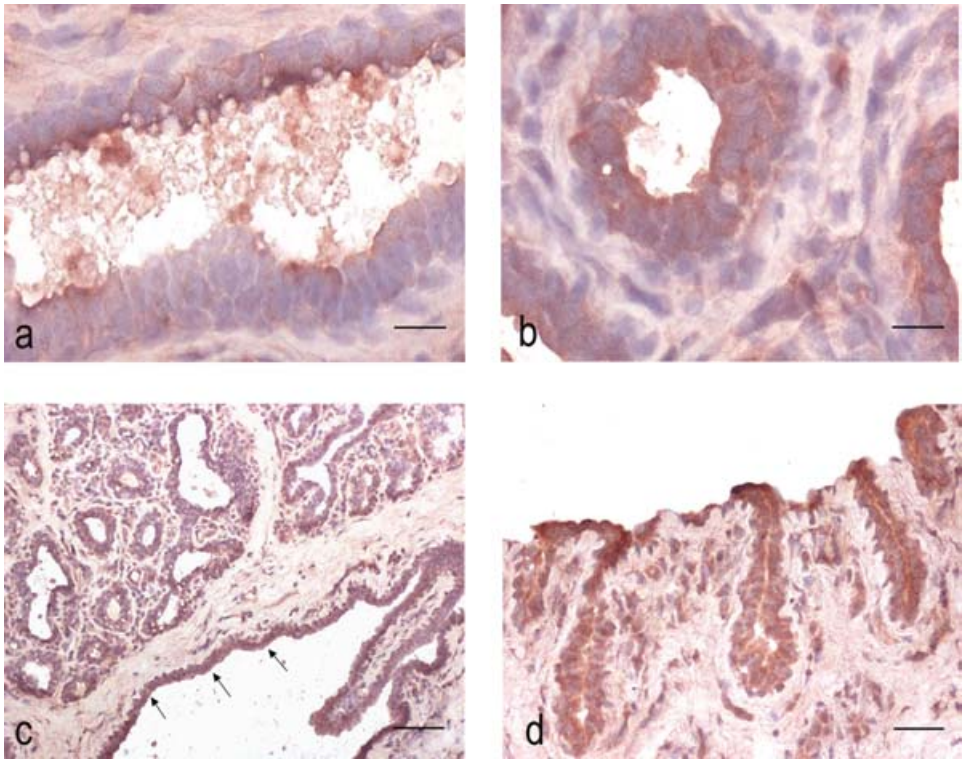

Fig. 4. Immunolocalization of AGP in mammary gland. Anti-AGP immunoperoxidase stain, hematoxylin counterstain. **(a)** Mammary gland. Secretory acini characterized by low intensity cytoplasmic membrane expression of AGP in approximately 40% of epithelial cells. Bar = 30 μm. **(b)** Mammary gland. Resting acini characterized by intense cytoplasmic expression of AGP in the majority of the cells. Bar = 30 μm. **(c)** Mammary cistern. Intense and diffuse cytoplasmic expression of AGP by epithelial lining (arrows). Bar = 120 μm. **(d)** Mammary teat duct epithelial lining. Intense and diffuse cytoplasmic expression of AGP. Bar = 60 μm.

AGP expression in mammary gland: RT-PCR and immunohistochemistry studies

Immunostaining of the normal components of the mammary gland was characterized by exclusive cytoplasmic positivity (Fig. 4). The expression of AGP showed variation among the different epithelial structures and functional status of the gland (Table 1). Secretory alveoli were characterized by low to moderate intensity of expression (Fig. 4a) with only 20–40% of the epithelial cells staining positive. Compared with secretory alveoli, resting alveoli were characterized by increased intensity and amount of AGP expression (Fig. 4b). Epithelial cells of the cistern (Fig. 4c) and of the teat duct (Fig. 4d) were characterized by high intensity expression of 70–100% of the cells. Results suggest that MG-AGP is present in all tissue of the mammary gland, but that it is probably produced for the most part in the alveoli.

Reverse-transcription PCR analysis of AGP expression in mammary gland tissues confirmed that AGP mRNA is expressed in all tissues examined (data not reported). On the contrary, RT-PCR analysis of milk somatic cells and PMNC, where the protein was detected by means of immunoblotting, gave negative results (data not shown),

https://doi.org/10.1017/S0022029907002646 Published online by Cambridge University Press

thus confirming that, as reported in human PMNC (Theilgaard-Monch et al. 2005), the protein is expressed early during the mieloproliferative phase and carried into the mammary gland by mature PMNC. The next step will be the confirmation by in situ hybridizaton of the exact correspondence beween the presence and the expression of the protein.

In this article, the structure of milk-AGP isoforms, including cDNA sequencing of the gene and its glycan moiety analysis, and the localization in the mammary tissue of lactating cow are reported. Results show that the source of milk-AGP isoforms is far more complex than thought at the beginning of our investigation and that two isoforms can be detected in healthy conditions. One of these, SC-AGP, presents a glycan pattern moiety that is associated with anti-inflammatory activity. While it is evident that this SC-AGP can dramatically increase during mastitis, thus acting as a regulator of somatic cell-induced damage in the mammary gland, it is still unknown whether MG-AGP increases as well. It would therefore be very interesing to study how pathophysiological challenge influences the expression of AGP by mammary epithelium. Molecules that usually induce hepatic expression of AGP did not work in our in vitro model, and therefore it is very probable that its expression depends on different stimuli. AGP is an acute phase protein, and therefore any further knowledge about its expression in the mammary gland would contribute to better understanding the modifications of its concentration during mastitis.

We thank Prof Paola Roccabianca, Dr Laura Kramer and Dr Paolo Moroni for their invaluable support in the critical reading of this paper. Thanks also go to Dr Alessio Scarafoni for help in the densitometric analysis with Imagemaster 1D Software (GE Healthcare), and to Dr Lorenzo Sommariva and Mr Luciano Molina for graphics. This work was financed by Grant FIRST/2003 founded to Dr Ceciliani, and by Grant PRIN 2006, founded to Prof Sartorelli. The cDNA sequence of bovine AGP from mammary glands has been deposited in the EMBL database under the accession number of AM403243.

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