Original article

Sulphated proteins secreted by rat mammary epithelial cells

Eric CHANAT*

Institut National de la Recherche Agronomique, Laboratoire de Génomique et Physiologie
de la Lactation, Jouy-en-Josas, 78352, France

Abstract – The main sulphated proteins secreted by rat mammary gland tissue have Mr of approximately 32 000, 27 000 and 25 000 Da. In addition, there are high Mr components which have a diffuse electrophoretic mobility (Mr > 200 000) and most likely corresponded to proteoglycans. The sulphate groups in the proteins with discrete Mr are most likely all linked to carbohydrates. These sulphated molecules were partially purified and identified to isoforms of rat α-lactalbumin for the 25−27 kDa bands and to κ-casein for the 32 kDa band. This pattern of protein sulphation is, as far as we know, quite specific to rat mammary epithelial cells.

mammary gland / epithelial cell / milk protein / caseins / alpha-lactalbumin / sulphation

1. INTRODUCTION

During lactation, mammary epithelial cells synthesise and secrete huge quantities of proteins and other components such as lipids and lactose in a polarised fashion: from their apical surface into the alveolar lumen that they surround. Milk protein secretion is obviously of interest due to its physiological and economical importance but also from the point of view of the study of high-efficiency intracellular transport pathways. The trafficking events leading to the secretion of milk proteins are known in general outline (for review see [1, 2]) but relatively little is known of the molecular cell biology of milk protein transport in the secretory pathway of mammary epithelial cells.

Caseins, the major milk proteins in most mammals, are phosphorylated and κ-casein is O-glycosylated (for review see [3]). Another well-known post-translational modification of milk proteins is the formation of intra and intermolecular disulphide bridges, a modification which affects primarily whey proteins such as ß-lactoglobulin, α-lactalbumin or whey acidic proteins, but also caseins [4]. In the present study, we searched for the presence of sulphated proteins in the proteins secreted by rat mammary epithelial cells.

Protein sulphation has proved invaluable to monitor the transport of secretory proteins from the trans-Golgi network and to study the molecular machinery involved in the formation of secretory vesicles from this compartment [5, 6]. This approach has been largely used to investigate protein sorting in the regulated pathway of secretion in neuroendocrine cells (for review see [7]). Since sulphation is a

* Corresponding author:
  eric.chanat@jouy.inra.fr

ubiquitous trans-Golgi network specific for post-translational modification of proteins passing through the secretory pathway (for review see [8,9]), short pulse labelling with [35S]sulphate can be used to label proteins selectively in this compartment. The transport of such markers to the next compartment, secretory vesicles to the plasma membrane or transport vesicles to the endosomal compartment, for example, can then be specifically monitored [10]. We characterised and identified the sulphated proteins expressed in the rat mammary gland, in an attempt to develop an assay to investigate the molecular mechanisms of secretory vesicle formation in mammary epithelial cells.

2. MATERIALS AND METHODS

2.1. Animals, milk, and antibodies

Animal welfare and conditions for animal handling were in accordance with French guidelines (May 2001). The animals used in the present study were Wistar rats at mid-lactation. To collect milk samples, rats were injected intraperitoneally with oxytocin a few minutes before anaesthesia and manually milked.

Rabbit antiserum against goat α-lactalbumin was obtained from Dr. J.-C. Mercier (INRA, Jouy-en-Josas, France). Protein A sepharose CL 4B was from Amersham Biosciences.

Unless otherwise indicated, chemicals were from Sigma-Aldrich.

2.2. Preparation of explants and metabolic labelling

Mammary glands were collected immediately after death and dissected free from connective tissue and muscles on an ice-cold plastic pad. The samples were cut manually into fragments of about 1 mm^3,

or using a home-made multi-mounted razor blade device. Metabolic labelling with [35S]sulphate or [35S]methionine/cysteine mix, both from Amersham Biosciences, were as previously described [11, 12]. To accumulate [35S]sulphate-labelled proteins in the medium or to allow the exit of [35S]methionine/cysteine-labelled proteins out of the endoplasmic reticulum, explants were chased in complete DMEM for the time indicated in the figure legends. At the end of the labelling period or of the chase, mammary fragments were collected and extensively washed with ice-cold Tris buffer saline (TBS; 25 mM Tris-HCl pH 7.4, 4.5 mM KCl, 137 mM NaCl, 0.7 mM Na_2HPO_4). Explants were further washed once in homogenisation buffer (HB; 10 mM HEPES buffer pH 6.8 containing 250 mM sucrose, 1 mM EDTA, 1 mM magnesium acetate) containing 0.5 mM PMSF and homogenised at 4 °C in HB/PMSF supplemented with an aliquot (10 μL.mL^{-1}) of a protease inhibitor cocktail (Sigma Chemicals), with three strokes of a tissue grinder (AA2 Teflon/glass, Thomas Scientific). The homogenate was centrifuged for 10 min at 1000 g, and 4 °C, and the resulting supernatant, referred to as the post-nuclear supernatant (PNS), was collected. The medium was centrifuged for 5 min at 1200 g at room temperature and the proteins contained in the resulting supernatant were either precipitated with 10% trichloracetic acid (final concentration) or stored at −20 °C. Proteins from the medium and from an aliquot of the PNS (1:10th) were analysed by SDS-PAGE or two-dimensional electrophoresis (2D-PAGE).

2.3. Analytical methods

Heat-stable protein fractions, highly enriched in caseins, were prepared from either [35S]sulphate- or [35S]methionine/cysteine-labelled explants. A PNS was

prepared, diluted 5–10 times with H_2O and a volume of 2 times concentrated TNTE buffer (final concentrations: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.3% Tween 20, 20 mM EDTA) was added. After incubation for 30 min at 4 °C under gentle mixing, the samples were boiled for 5 min and centrifuged at approximately 13 000 g for 15 min. The resulting supernatant, referred to as the heat-stable fraction, was collected and the heat unstable proteins were resuspended in 1 × TNTE. Proteins from both fractions were precipitated with 10% trichloracetic acid and analysed by either SDS-PAGE or 2D-PAGE, followed by protein staining and autoradiography.

We tested the presence of potential acid labile tyrosine O-sulphate in proteins produced by rat mammary gland tissue by HCl treatment of [35S]sulphate-labelled proteins in polyacrylamide gel [13]. For affinity purification of sulphated proteins with lectin, an aliquot of secretion medium obtained from [35S]sulphate-labelled explants was centrifuged for 10 min at 13 000 g, and at 4 °C, to pellet casein micelles, and the supernatant was diluted (1/4, v/v) in TBS. The sample was loaded onto a concanavalin A Sepharose column and bound proteins were eluted with 0.5 M α-methyl-mannose in 20 mM Tris-HCl pH 7.3, 150 mM NaCl. Fractions containing [35S]sulphate-labelled proteins (mainly fractions 1 to 4) were subjected to TCA precipitation and analysed by SDS-PAGE.

Sialidase treatment was performed either directly with proteins from medium or with concanavalin A purified proteins. In the former case, the pH of the medium was reduced to approximately 6.0 with 1 N HCl. In the second case, fractions containing [35S]sulphate-labelled proteins were pooled, proteins were precipitated with acetone (80% final) at −20 °C and resuspended in 50 mM MES pH 6.0, 1 mM $CaCl_2$. Incubation in the absence or the presence of α2-3, 6, 8-Neuraminidase from

Vibrio cholerae (≥ 0.1 unit.mL^{-1}, Calbiochem) was for approximately 15 h at 30 °C. The reaction was stopped by TCA precipitation and samples were analysed by SDS-PAGE or 2D-PAGE.

Immunoprecipitation of α-lactalbumin was carried out with conditioned medium from both [35S]sulphate or [35S]methionine/cysteine labelled explants. Unless otherwise indicated, all steps were performed at 4 °C. Medium was diluted in two volumes of immunoprecipitation buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 2 mM EDTA, 2% Triton X-100, 1% sodium deoxycholate, 2.5 mM PMSF), incubated for 30 min and centrifuged at approximately 13 000 g for 15 min to remove insoluble material. The supernatant was supplemented with rabbit anti-goat α-lactalbumin and incubated overnight with gentle mixing. Fifty microliters of protein A Sepharose in the form of a 1:1 slurry in buffer A (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100) were added and the samples were further incubated for 2 h. Beads were pelleted by centrifugation at approximately 6000 g and washed twice in buffer A, twice in buffer B (10 mM Tris-HCl pH 8.0, 450 mM NaCl, 2 mM EDTA, 0.2% Triton X-100), and once in buffer C (10 mM Tris-HCl pH 8.0, 450 mM NaCl, 2 mM EDTA, 0.1% Triton X-100). Immunoprecipitated material was solubilised in one volume of H_2O and one volume of three times concentrated Laemmli sample buffer, and analysed by SDS-PAGE.

SDS-PAGE analysis (13% gels) was performed according to the method of Laemmli, except that the sample buffer contained 10 mM EDTA. In some experiments, proteins were subjected to 2D-PAGE according to O'Farrell [14], using a mixture of Ampholines (Amersham Biosciences), preblended ampholytes, pH 3.5–9.5 (2.0% v/v), pH 3.5–5.0 (2.5% v/v) and pH 5.0–7.0 (2.5% v/v) in
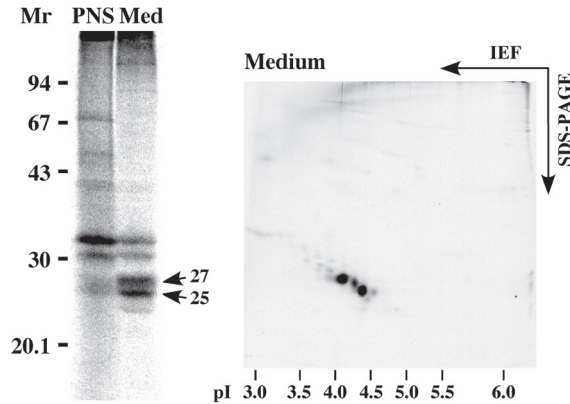
Figure 1. Sulphated proteins secreted by rat mammary gland explants. Mammary gland explants were prepared from the rat at mid-lactation and labelled for 90 min with [^{35}S]sulphate and either collected (SDS-PAGE) or chased for 90 min (2D-PAGE). Aliquots of the tissue proteins (PNS, 1:10 as compared to medium) and of the labelling medium (Med) were analysed by SDS-PAGE, the chase medium was analysed by 2D-PAGE (Medium); the two types of analysis being followed by autoradiography.

the first dimension and 13% gels in the second dimension. The gels were stained, destained, dried and exposed to storage phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) and/or to Kodak Biomax MR-1 films.

N-terminal sequencing was performed on proteins passively eluted from polyacrylamide gel pieces. Proteins in a fraction enriched in [^{35}S]sulphate-labelled proteins were resolved by 2D-PAGE. The gel was stained, destained, dried and exposed to storage phosphor screen. The phosphor screen was scanned, and Coomassie blue-stained proteins associated with radioactivity were identified. Relevant spots were excised from the gel and gel pieces were swollen in a minimal amount of 10 mM Tris-HCl pH 8.4, 2% SDS. The sample was further diluted 5−10 times with H$_2$O. A small piece ($\approx$ 3 mm^2) of Problott^{TM} membrane (Applied Biosystems, Inc., CA, USA) was added to the tube and passive transfer of a substantial part of the protein occurred during a 24−48 h incubation period at room temperature. Protein on the

membrane was processed for N-terminal sequencing by automated Edman degradation.

3. RESULTS

3.1. Characterisation of the sulphated proteins secreted by rat mammary gland explants

We used metabolic sulphate labelling of rat mammary gland fragments to identify the subset of sulphated secretory proteins produced by rat mammary epithelial cells. Long-term labelling of explants with [^{35}S]sulphate revealed the production of four major newly synthesised sulphated proteins with discrete Mr by the tissue, as shown in Figure 1. In addition, components with diffuse electrophoretic mobility, between the top of the gel and Mr of approximately 200 000 were found in both the post nuclear supernatant (PNS) and the medium. These molecules most likely correspond to sulphated glycosaminoglycans. All of the sulphate-labelled proteins
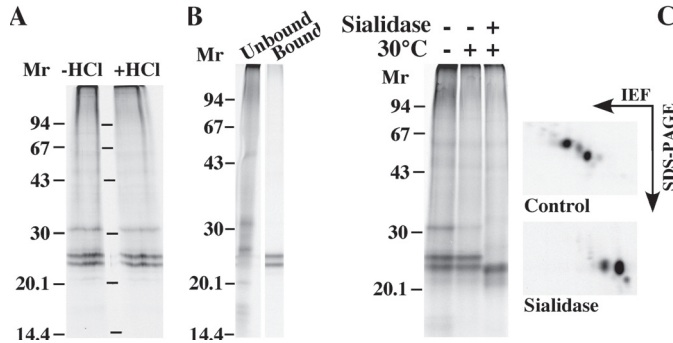
Figure 2. Sulphation of the main sulphated proteins produced by rat mammary gland mostly occurs on carbohydrate residues. Mammary gland explants from the rat at mid-lactation were labelled for 90 min with [^{35}S]sulphate and chased for 90 min. (**A**) Aliquot of the proteins contained in the chase medium were analysed by SDS-PAGE and the gel was fixed. Gel pieces were incubated for 5 min at ≥ 90 °C, in the absence or the presence of 1 M HCl, and processed for autoradiography. (**B**) Aliquot of the chase medium was applied to a column of concanavalin A sepharose. Proteins recovered in the flow-through (1:4 of total; Unbound) or eluted by 0.5 M α-methyl-mannose (content of one of the fractions is shown, Bound) were analysed by SDS-PAGE, followed by autoradiography. (**C**) Aliquots of the chase medium (left panel, SDS-PAGE) or of concanavalin A purified proteins (right panels, 2D-PAGE) were incubated for 15 h at 30 °C and pH 6.0, in the absence or the presence of sialidase, and analysed by SDS-PAGE or 2D-PAGE, followed by autoradiography. Identical regions of the 2D-PAGE gels are shown.

were also found in the medium, consistent with the notion that sulphation is a post-translational modification which occurs on secretory proteins. In addition, a protein with an Mr approximately 23 000 was faintly labelled. The release of these proteins in the secretion medium proceeded at different rates. Although only 1:10 of the PNS was analysed as compared to the medium, the majority of Mr 30 and 32 000 bands were recovered within the PNS fraction whereas the Mr 25 and 27 000 proteins were mostly found in the medium. Notably, the Mr 30 000 molecule was not observed after a 90 min chase (See Fig. 1, 2D-PAGE and Figs. 2 and 3). In pulse/chase experiments, the 25 and 27 kDa proteins were secreted with identical kinetics (data not shown), approximately 50% of these being recovered in the medium after a 75 min chase. In contrast, only a few percents of the 32 kDa protein were observed in the chase medium and

the molecule was difficult to identify when analysed by two-dimensional gel electrophoresis (2D-PAGE; Fig. 1, Medium). This was in striking contrast to the behaviour of the 25 and 27 kDa species which were nicely resolved by 2D-PAGE, giving 2 well-defined spots of pI ≈ 4.4 and 4.1, respectively. This analysis also revealed that the other sulphated molecules present in this part of the SDS-PAGE gel, which gave a smear behind the 2 main bands, could be resolved. A rather well-labelled spot was present between the two main ones, and at least two faint spots were detected around pH 4.5, one having a slightly higher electrophoretic mobility.

3.2. Sulphation of the main sulphated proteins secreted in milk mostly occurs on carbohydrates

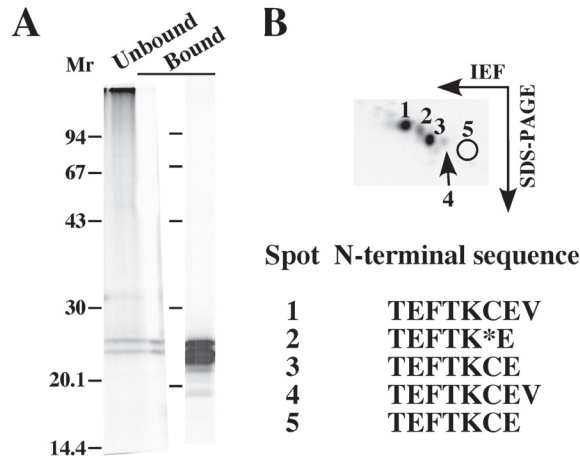Sulphation of a protein can occur on tyrosine residues, carbohydrate moieties

Figure 3. Rat α-lactalbumin is sulphated. (**A**) Mammary gland explants from the rat at mid-lactation were either labelled with [^{35}S]sulphate (two left tracks) or [^{35}S]methionine/cysteine (right track) and chased. Aliquots of the chase media were processed for immunoprecipitation with a rabbit anti-goat α-lactalbumin and the bound as well as 1:20 of the unbound material were analysed by SDS-PAGE, followed by autoradiography. (**B**) Proteins contained in a fraction enriched in [^{35}S]sulphate-labelled proteins were resolved by 2D-PAGE and the numbered spots were processed for N-terminal sequencing. The circle area of the gel contained a Coomassie blue-stained protein (numbered 5) which was not sulphate-labelled. N-terminal sequences are shown. * Unidentified residue.

or both. Sulphate is linked to tyrosine residues as an O-sulphate ester which was found to be largely more acid labile than most carbohydrate sulphate esters. Hence, a short acid treatment of sulphated proteins allows identifying those proteins likely to contain tyrosine sulphate [13]. Figure 2A shows that in gel treatment of the [^{35}S]sulphate-labelled proteins released in the chase medium with 1 N HCl had no significant effect on their labelling. As previously shown [11], such treatment leads to the complete loss of [^{35}S]sulphate from secretogranin II, a protein known to be primarily sulphated on tyrosine residues [15]. We concluded from this experiment that the 25–27 kDa proteins are glycoproteins mainly sulphated on carbohydrates. Consistent with this, the [^{35}S]sulphate-labelled 25–27 kDa proteins were quantitatively retained on a concanavalin A column and were specifically eluted with α-methyl-mannose (Fig. 2B, Bound). This was ap-

parently not the case for the 32 kDa protein and the proteoglycans.

Since the 25–27 kDa proteins behave in a quite similar manner when subjected to the various analytical methods described above, but having distinct pI, we wondered whether these molecules only differed by their sialic acid content. [^{35}S]sulphate-labelled proteins recovered in the chase media were therefore subjected to treatment with neuraminidase from *Vibrio cholerae* at slightly acidic pH. There was a substantial reduction of the 32 kDa band in the resulting autoradiogram when proteins were incubated overnight at 30 °C in the absence of sialidase and analysed by SDS-PAGE, (Fig. 2C, left panel). Such an effect was not observed for the 25–27 kDa proteins. However, the pattern of [^{35}S]sulphate-labelled proteins was clearly modified by treatment with the enzyme. Both the 32 kDa and 27 kDa bands disappeared and more material migrated

around 25 and 23 kDa. As expected, the 2D-PAGE pattern (Fig. 2C, right panels) was also profoundly modified, with a reduction of the number of [35S]sulphate-labelled proteins detected, and a shift of those molecular species toward slightly more basic pH. These results suggest that at least part of the 25−27 kDa sulphated molecules might be isoforms of the same protein.

3.3. The 25–27 kDa sulphated proteins produced by rat mammary gland explants are isoforms of α-lactalbumin

In the course of our efforts to identify the sulphated proteins produced by rat mammary gland, we tested whether the 25−27 kDa proteins were recognised by antibodies against α-lactalbumin. A rabbit antiserum against bovine α-lactalbumin recognised a band that co-migrated with the 27 kDa band in SDS-PAGE (data not shown), but we were unable to immunoprecipitate the corresponding sulphated molecules with this antibody. We were, however, able to immunoprecipitate these molecules with a rabbit antiserum against goat α-lactalbumin, although immunoprecipitation was not quantitative (Fig. 3A, Bound). In chase medium prepared from [35S]methionine/cysteine-labelled rat mammary gland explants, immunoprecipitation yields three bands, the additional protein having a Mr of approximately 23 000 (Fig. 3A, Bound, right track).

In order to identify unambiguously the 25−27 kDa proteins by N-terminal sequencing, [35S]sulphate-labelled proteins prepared as described above were further enriched and resolved by 2D-PAGE. Gel pieces corresponding to the indicated radioactive spots, as well as to a Coomassie Blue stained protein that migrated within the circle numbered 5 (Fig. 3B), were ex-

cised from the dried gel and processed for N-terminal sequencing. As shown in Figure 3, each sample gave an identical N-terminal amino acid sequence. Swiss-Prot database search revealed that, among mammals, this sequence was unique to rat α-lactalbumin. This result, as well as our observation that sialidase treatment was effective on the 25−27 kDa sulphated proteins (see above), were consistent with the observation that rat α-lactalbumin, a N-glycosylated protein, exists as multiple charged forms which differ in the number of sialic acid residues per molecules [16].

3.4. The 32 kDa sulphated proteins produced by rat mammary gland explants correspond to isoforms of κ-casein

The 32 kDa protein, although secreted, was clearly found mostly associated with the tissue. We therefore had to find a way to prepare it from the cell extract. Since prior results indicate that secretory proteins are often heat-stable [17, 18], we tried to enrich the sulphated 32 kDa protein in a heat-stable fraction. We tested this possibility using a PNS prepared from mammary gland explants, pulse-labelled for 3 min with [35S]methionine/cysteine and chased for 30 min. As expected, the majority of the cellular proteins were recovered in the pellet after boiling and centrifugation (Fig. 4A, Protein staining, P). In contrast, most of the labelled proteins remained quantitatively in solution (Fig. 4A, 35S-methionine, S). The highly radioactive proteins, including the 32 kDa band, had electrophoretic mobilities identical to rat caseins (data not shown). Similarly, a heat-stable protein fraction, obtained from a PNS prepared from rat mammary gland tissue pulse-labelled for 5 min with [35S]sulphate and chased for 30 min was analysed by 2D-PAGE, and this revealed that the 32 kDa band was resolved into 12
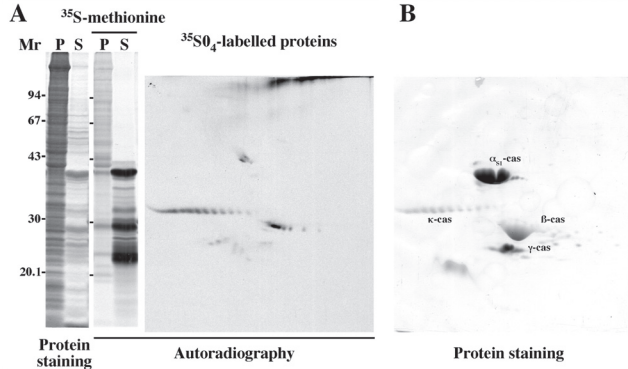
Figure 4. Rat κ-casein is sulphated. (**A**) A PNS was prepared from rat mammary explants which were either pulse-labelled for 3 min with [^{35}S]methionine/cysteine (^{35}S-methionine) or for 5 min with [^{35}S]sulphate (^{35}SO$_4$-labelled proteins), and chased for 30 min. Heat-stable protein fractions were prepared from the cells and analysed by either SDS-PAGE (S) or 2D-PAGE, followed by protein staining and autoradiography. P: heat unstable proteins. (**B**) A casein fraction was prepared from rat milk and the caseins contained in 2 μL of milk were analysed by 2D-PAGE, followed by protein staining.

or more spots of the same Mr, all differing by a fraction of pI (Fig. 4, ^{35}SO$_4$-labelled proteins). The analysis of a casein fraction prepared from rat milk by 2D-PAGE (Fig. 4B) showed that one of the caseins exhibited identical behaviour. This casein was identified as κ-casein ([4]; Lavialle and Chanat, manuscript in preparation). This comparison allowed us to conclude that the sulphated 32 kDa protein is rat κ-casein.

4. DISCUSSION

Previous work from our group allowed us to establish that the main sulphated proteins secreted by rabbit mammary gland tissue belong to polymeric IgA [11]. We pursued the study of protein sulphation in other species in our effort to identify secretion markers which could be used to monitor precisely the transport of milk proteins from the trans-Golgi network of mammary epithelial cells, and to study the molecular machinery involved in

the formation of secretory vesicles from this compartment. The present investigation demonstrates that at least two main milk specific proteins expressed by rat mammary epithelial cells are sulphated: κ-casein and α-lactalbumin. Such posttranslational modification of κ-casein and α-lactalbumin was neither observed in the rabbit [11] nor in the goat (data not shown). Although we obviously have not screened enough species to conclude definitively that these modifications are rat specific, it could well be the case, at least for α-lactalbumin.

Unlike other α-lactalbumin, rat α-lactalbumin has 17 extra amino acid residues which form an extension at the carboxyl terminus of the protein [19, 20]. The C-terminal sequence extension is hydrophobic and proline rich, two features which might explain the anomalous electrophoretic mobility of the protein, which migrated with an Mr of at least 23 000 in our conditions, compared with an Mr of approximately 14 000–15 000 in other species. Notably, the protein

with Mr 14 400 in the molecular weight calibration kit from Pharmacia, a kit that was used in the present study, is bovine α-lactalbumin. Another characteristic of the protein which might help explain its unexpected low electrophoretic mobility is the fact that, in contrast to other species, rat α-lactalbumin is efficiently glycosylated [16]. This is not the case for most α-lactalbumin molecules, with the exception of a minor form of bovine α-lactalbumin which contains carbohydrates. Prasad and collaborators have suggested that this peculiar high level of glycosylation of the rat molecule would be due to variation in its secondary structure around the carbohydrate attachment site (asparagine 45). In addition, this difference in carbohydrate composition provides an explanation as to why, as far as we know, only rat α-lactalbumin is sulphated.

As mentioned above, we also did not observe sulphation of rabbit and goat κ-casein. Species differences in the level of glycosylation of κ-casein may also explain, at least in part, why rat κ-casein is sulphated. In rat milk, κ-casein exists as multiple molecular forms, with distinct charges, each of them probably containing different amounts of sialic acid (see 2D-PAGE in Fig. 4). We were able to resolve up to 16 forms (Lavialle and Chanat, manuscript in preparation). Moreover, the relative proportion of the highly acidic forms was much higher, the five most acidic spots containing approximately $^3/_4$ of the total amount of κ-casein in rat milk. Using similar conditions for 2D-PAGE analysis, we observed at most 7 κ-casein forms in the goat, the major spots (the majority of the protein is confined to 2 spots) being the least charged (see Fig. 7 in [21]). In the rabbit, we observed an intermediate situation with 11 forms, the 7 central spots being equally represented (unpublished results). If the number of the different charged molecules and the relative proportion of the most acidic spots di-

rectly reflect the level of glycosylation, one can conclude that rat casein is by far more glycosylated than goat and rabbit κ-caseins. The lack of sulphation of milk specific proteins in the latter two species is unlikely to be due to the absence of expression of sulphotransferases since this post-translational modification occurs in essentially all animal cells (for review see [8, 9]). In addition, protein sulphation was detected in the secretory pathway of rabbit mammary epithelial cells by electron microscopy autoradiography (unpublished result).

In contrast to the sulphated proteins we previously identified in the secretion medium of rabbit mammary epithelial cells, the present molecules are true milk specific proteins expressed by mammary epithelial cells that can therefore be used as secretion markers. The fact that such markers undergo sulphation in the trans-Golgi network, and thus can be selectively labelled in this compartment, would greatly facilitate our studies on the biogenesis of secretory vesicles in mammary epithelial cells.

ACKNOWLEDGEMENTS

REFERENCES

[1] Boisgard R, Chanat E, Lavialle F, Pauloin A, Ollivier-Bousquet M. Roads taken by milk proteins in mammary epithelial cells. Livestock Prod Sci 2000, 70: 49–61.

[2] Burgoyne RD, Duncan JS. Secretion of milk proteins. J Mammary Gland Biol Neoplasia 1998, 3: 275−286.

[3] Swaisgood HE. Chemistry of the caseins. In: Fox PF, McSweeney PLH (Eds), Advanced dairy chemistry, Kluwer academic/Plenum publishers, 2003, p 139−201.

[4] Bouguyon E, Beauvallet C, Huet JC, Chanat E. Disulphide bonds in casein micelle from milk. Biochem Biophys Res Commun 2006, 343: 450−458.

[5] Fernandez CJ, Haugwitz M, Eaton B, Moore HP. Distinct molecular events during secretory granule biogenesis revealed by sensitivities to brefeldin A. Mol Biol Cell 1997, 8: 2171−2185.

[6] Tooze SA, Huttner WB. Cell-free protein sorting to the regulated and constitutive secretory pathways. Cell 1990, 60: 837−847.

[7] Glombik MM, Gerdes HH. Signal-mediated sorting of neuropeptides and prohormones: secretory granule biogenesis revisited. Biochimie 2000, 82: 315−326.

[8] Huttner WB. Tyrosine sulfation and the secretory pathway. Annu Rev Physiol 1988, 50: 363−376.

[9] Moore KL. The biology and enzymology of protein tyrosine O-sulfation. J Biol Chem 2003, 278: 24243−24246.

[10] Chanat E, Dittie AS, Tooze SA. Analysis of the sorting of secretory proteins to the regulated secretory pathway. A subcellular fractionation approach. In: Clegg RA, Methods in Molecular Biology, Humana Press, Totowa, New Jersey, 1998, pp 285−324.

[11] Boisgard R, Charpigny G, Chanat E. Polymeric IgA are sulfated proteins. FEBS Lett 1999, 463: 250−254.

[12] Pechoux C, Boisgard R, Chanat E, Lavialle F. Ca(2+)-independent phospholipase A2 participates in the vesicular transport of milk proteins. Biochim Biophys Acta 2005, 1743: 317−329.

[13] Huttner WB. Determination and occurrence of tyrosine O-sulfate in proteins. Methods Enzymol 1984, 107: 200−223.

[14] O'Farrell PH. High resolution two-dimensional gel electrophoresis of proteins. J Biol Chem 1975, 250: 4007−4021.

[15] Lee RW, Huttner WB. Tyrosine-O-sulfated proteins of PC12 pheochromocytoma cells and their sulfation by a tyrosylprotein sulfotransferase. J Biol Chem 1983, 258: 11326−11334.

[16] Prasad R, Hudson BG, Butkowski R, Hamilton JW, Ebner KE. Resolution of the charge forms and amino acid sequence and location of a tryptic glycopeptide in rat alpha-lactalbumin. J Biol Chem 1979, 254: 10607−10614.

[17] Chanat E, Weiss U, Huttner WB, Tooze SA. Reduction of the disulfide bond of chromogranin B (secretogranin I) in the trans-Golgi network causes its missorting to the constitutive secretory pathways. EMBO J 1993, 12: 2159−2168.

[18] Rosa P, Hille A, Lee RWH, Zanini A, De Camilli P, Huttner WB. Secretogranins I and II: two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway. J Cell Biol 1985, 101: 1999−2011.

[19] Dandekar AM, Qasba PK. Rat alpha-lactalbumin has a 17-residue-long COOH-terminal hydrophobic extension as judged by sequence analysis of the cDNA clones. Proc Natl Acad Sci USA 1981, 78: 4853−4857.

[20] Prasad RV, Butkowski RJ, Hamilton JW, Ebner KE. Amino acid sequence of rat alpha-lactalbumin: a unique alpha-lactalbumin. Biochemistry 1982, 21: 1479−1482.

[21] Chanat E, Martin P, Ollivier-Bousquet M. Alpha(S1)-casein is required for the efficient transport of beta- and kappa-casein from the endoplasmic reticulum to the Golgi apparatus of mammary epithelial cells. J Cell Sci 1999, 112 (Pt 19): 3399−3412.