INTRODUCTION

The PRM/Alf inbred strain is characterized by a huge intestinal lengthening (1). The lengthening is harmonious and homogenous in all parts of the intestine. The length of the small and large intestines of adult PRM/Alf mice is approximately 75 cm, compared to 55 cm in classical laboratory strains, such as DBA/2J, C3H/He, 129/Sv, and C57BL/6J. Surprisingly, this intestinal lengthening induces no functional digestive defect in PRM/Alf mice. Actually, the intestinal transit is accelerated, and thus compensates for the intestinal lengthening. The contractility and slow waves frequency are also increased. Accordingly, the number of interstitial cells of Cajal, responsible for the slow waves production, is increased in the intestine of PRM/Alf mice (2). The determinism of this intestinal lengthening is multifactorial, including both genetic and environmental factors (1). Indeed, the intestinal lengthening displays a strong maternal effect, in agreement with the fact that it develops throughout the lactating period, before weaning. The maternal effect accounts for approximately 40% of the length difference between PRM/Alf and other inbred strains of mice (1). Two non-mutually exclusive hypotheses could explain this maternal effect. First, the milk of PRM/Alf females would contain intestinotrophic factor(s) transmitted from the nurse-dam to its pups. Second, the intestinal microbiota, also transmitted from the nurse-dam to its pups, would contain micro-organism(s) capable of inducing a lengthening of the intestine, either directly or indirectly.

The PRM/Alf inbred mice exhibit a huge intestinal lengthening. Since milk contains bioactive factors implied in numerous biological processes, one hypothesis is that PRM/Alf milk contains intestinotrophic factors contributing to this remarkable phenotype. A comparison between the milk from PRM/Alf and C57BL/6J (as a control) strains could be helpful in the identification of such factors, including proteins. However, a complete description of the mouse milk major protein fraction is still missing. Hence we adapted a reliable technique to separate and identify the major mouse milk proteins. This approach was achieved through the protein study of milk from C57BL/6J and PWK/Pas strains representative of two Mus musculus subspecies, M. m. domesticus and M. m. musculus respectively. C57BL/6J milk samples were first skimmed and fractionated by reverse phase-HPLC (RP-HPLC). The protein content of each chromatographic peak was analysed by SDS-PAGE and identified by mass spectrometry. This methodological approach allowed characterization of nine major mouse milk proteins: αs1, β, γ, ε and κ-caseins, Whey Acidic Protein, lactoferrin, Serum Albumin, Fatty Acid Binding Protein, as well as an αs1-casein isoform. Then, RP-HPLC patterns of C57BL/6J milk proteins were compared with those obtained starting from the milk of PWK/Pas females. This comparison revealed a protein polymorphism for the αs1-casein.

Key words: caseins, whey proteins, RP-HPLC, SDS-PAGE, Mus musculus musculus, Mus musculus domesticus, mouse resources
transposed the bidimensional approach, combining RP-HPLC followed by SDS-PAGE, already validated and applied in our laboratory to characterize the milk protein fraction from several domestic mammals, including cow, goat and pony (7).

In this report, we describe the ability of proteomic tools to achieve the identification and characterization of mouse milk proteins. In addition, we have compared the RP-HPLC profiles and SDS-PAGE: patterns of milk proteins from two mouse strains: C57BL/6J and PWK/Pas. These two strains belong to Mus musculus subspecies that split less than one million years ago (8). This comparison was performed to show that our approach is valuable to detect milk protein polymorphisms between different mouse milks.

MATERIALS AND METHODS

Animals

The original genetic background of the PRM/Alf inbred strain is not defined. However, the history of its derivation reveals a backcross with C57BL/6J inbred mice. Hence, C57BL/6J is the only known mouse strain sharing a part of its genome with the PRM/Alf strain. C57BL/6J M. m. domesticus mice were bred at the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). PWK/Pas M. m. musculus mice were bred at the Institut Pasteur (Paris, France). All mice were kept at stable temperature (21°C), with regulated humidity. They had free access to water and were fed with standardized diets (M25 pellets; Dietex, Saint-Gratien, France). Animal care and use were conformed to the International Guiding Principles for Biomedical Research.

Sampling

Pups were separated from their mothers 2 h before milking. Dams were injected with 0.2 U of synthetic oxytocin (CEVA SANTE ANIMALE, Libourne, France) and then anesthetized by intraperitoneal injection (0.01 ml/kg of body weight) of a solution containing 1ml Imalgene 1,000 (MERIAL, Lyon, France), 0.5 ml Rompun (Bayer Pharma, Puteaux, France) in a final volume of 10 ml water. Approximately 200 µl of milk were collected at mid-lactation (10 days after birth) into a sterile tube, kept on ice and eventually stored at -80°C. For RP-HPLC analyses, milk samples containing 1ml Imalgene 1,000 (MERIAL, Lyon, France), 0.5 ml Rompun (Bayer Pharma, Puteaux, France) were fed with standardized diets (M25 pellets; Dietex, Saint-Gratien, France). Animal care and use were conformed to the International Guiding Principles for Biomedical Research.

RP-HPLC analysis

For RP-HPLC analysis, diluted skimmed milk samples were first clarified by the addition of 7 volumes of 0.1 M bis-Tris buffer, pH 8.0, containing 8 M urea, 1.3% trisodium citrate and 0.3% DTT (7). Reduction of disulfide bonds present in the mouse casein micelles. Gradient conditions were also slightly modified in comparison with the method described by Miranda et al. (7), to obtain appreciable improvement of peaks separation. Fig. 1A shows the elution pattern of the principal milk proteins from clarified diluted and skimmed milk from the C57BL/6J mouse. Identical elution patterns were recurrently obtained with several

Mass spectrometry MALDI-TOF

One microliter of each supernatant was mixed to an equal volume of a matrix solution, consisting in alpha cyano-4-hydroxyxycinnamic acid dissolved in 0.3% TFA/acetonitrile (1:1, v/v) at a 5 mg/ml concentration. Then, 1 microliter of the mix was spotted onto a stainless steel Maldi plate and allowed to air dry. The Maldi target was inserted into a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems Inc., Framingham, MA). Peptides were desorbed and ionized with a nitrogen laser at 337 nm in the positive ion mode and delayed extraction. One thousand laser shots were accumulated and averaged for each spectrum. Peak masses were internally calibrated using trypsin autolysis peaks (m/z = 842.509 and 2211.104). After deisotoping, the peptide mass lists were used for identification of proteins in the SwissProt 2007 and UniProt 2007 Databases with the Aldente software (http://www.expasy.org/tools/aldente/).

RESULTS

Optimization of a chromatographic method for separation and identification of the major mouse milk proteins

To separate and characterize the major mouse milk proteins, we adapted a RP-HPLC method previously described by Miranda et al. (7). Preliminary tests were performed with milk from our control strain (C57BL/6J), collected on the 10th day of lactation.

The clarification of milk samples is a crucial step for the chromatographic analysis of the milk protein fraction. It permits to alleviate the protein-protein interactions and thus to dismantle the micellar organization of caseins. Indeed, an overnight clarification at 4°C with a final concentration of 0.3% DTT in the reaction buffer was sufficient to achieve a complete reduction of disulfide bonds present in the mouse casein micelles. Gradient conditions were also slightly modified in comparison with the method described by Miranda et al. (7), to obtain appreciable improvement of peaks separation. Fig. 1A, shows the elution pattern of the principal milk proteins from clarified diluted and skimmed milk from the C57BL/6J mouse. Identical elution patterns were recurrently obtained with several
milk samples obtained from individuals at the same stage of lactation (data not shown). Fractions of interest were collected and pooled under each peak (denoted from a to i).

The protein content of each chromatographic peak was then analysed by SDS-PAGE (Fig. 1B). Proteins were identified using Peptide Mass Fingerprinting (PMF). The major milk proteins of C57BL/6J being referenced in SwissProt and UniProt databases, the relevant fractions were unambiguously identified. All the bands excised from the gel were identified except bands 7 and 10 (Fig. 1B). Identified proteins were eluted from the C5 column, in the following order: αs1-casein (peak b), γ-casein (peak h) and β-casein (peak i). The RP-HPLC revealed the occurrence of a minor αs1-casein (peak b, band 5, Fig. 1A and 1B) that was eluted before the major αs1-casein (peak f, band 9, Fig. 1A and 1B). The SDS-PAGE revealed the presence of two bands (bands 1 and 3) identified as κ-casein by mass spectrometry. Band 1 with the highest apparent molecular weight corresponded to the glycosylated κ-casein, as revealed by Schiff coloration (data not shown). Thus, the proteomic approach described above, allows an almost complete separation of the major proteins from mouse milk and their subsequent identification by mass spectrometry.

Validation of the bidimensional method and comparison of the major milk proteins between C57BL/6J and PWK/Pas strains of mice

Could our method be useful and discriminative enough to detect protein polymorphism and subtle differences in the
Table 1. * Protein concentrations were estimated starting from 11 (C57BL/6J) and 8 (PWK) milk samples. Relative proportions of the different major milk proteins from C57BL/6J (n=3) and PWK/Pas (n=3) mice are given as percentages (+/- SE), calculated as described in § RESULTS. Statistical differences were assessed using a Student's t-test. NS, not significant; FABP, fatty acid binding protein.

<table>
<thead>
<tr>
<th>Protein concentration (g/l)*</th>
<th>C57BL/6J</th>
<th>PWK/Pas</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative proportion (%) of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_s$-casein major</td>
<td>21.6 +/- 0.5</td>
<td>18.3 +/- 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>$\alpha_s$-casein minor</td>
<td>1.8 +/- 0.3</td>
<td>2.0 +/- 0.8</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-casein</td>
<td>28.1 +/- 1.8</td>
<td>20.4 +/- 0.9</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>$\gamma$-casein</td>
<td>15.7 +/- 1.1</td>
<td>15.7 +/- 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>$\kappa$- and $\epsilon$-caseins and FABP</td>
<td>11.3 +/- 0.1</td>
<td>11.3 +/- 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Whey acidic protein</td>
<td>12.2 +/- 0.2</td>
<td>11.6 +/- 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>4.8 +/- 0.2</td>
<td>10.2 +/- 2.1</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>5.7 +/- 0.5</td>
<td>5.6 +/- 0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

![Image](image_url)

Fig. 2. SDS-PAGE analysis of skimmed milk from C57BL/6J and PWK/Pas mice on the 10th day of lactation. The name of the main proteins and their corresponding theoretical molecular weight are indicated (Lf: lactoferrin, SA: serum albumin, $\alpha_s$-: $\alpha_s$-casein, glyc $\kappa$: glycosylated $\kappa$-casein, $\beta$: $\beta$-casein, $\gamma$: $\gamma$-casein, $\epsilon$: $\epsilon$-casein, $\kappa$: $\kappa$-casein, WAP: whey acidic protein).

protein fraction of milk from another mouse strain? To address this question we first apply our analytical method to another mouse subspecies: PWK/Pas (M. m. domesticus), to yield a bidimensional profile which was finally compared with that obtained with C57BL/6J (M. m. musculus). For comparison purpose, the protein concentration of both milks was first determined at the same stage of lactation (10th day). The average values of protein concentration (g/l) are presented in Table 1. Surprisingly, the protein concentration was approximately four times lower in PWK/Pas milk compared to C57BL/6J milk (32 ± 6 g/l and 125 ± 12 g/l, respectively; $P<0.0001$, Student's t-test).

The comparative analysis of whole milks (adjusted at the same protein concentration) by SDS-PAGE (Fig. 2) revealed rather similar electrophoretic patterns between the two subspecies. The major proteins identified in C57BL/6J milk are also present in PWK/Pas milk, with the same apparent molecular weights. However, relative amounts of some proteins seem different between the two strains of mice.

The milk from PWK/Pas was analyzed using RP-HPLC under the same chromatographic conditions as those used previously with C57BL/6J milk (Fig. 1C). Major peaks were collected and analyzed by SDS-PAGE. Protein identification was performed by PMF, using C57BL/6J SwissProt and UniProt databases. Major proteins occurring in each chromatographic peak were identified (Fig. 1D). The protein elution profiles of PWK/Pas and C57BL/6J milks were very similar (from $\epsilon$ and $\kappa$-caseins, minor $\alpha_s$-casein, Lf, WAP, major $\alpha_s$-casein, SA, $\gamma$-casein, to $\beta$-casein) with one exception concerning $\epsilon$-casein that co-eluted with $\kappa$-casein in PWK/Pas. However, $\alpha_s$-caseins showed different retention times. As a matter of fact, the two $\alpha_s$-casein isoforms from PWK/Pas milk showed a longer retention time compared to C57BL/6J milk.

Since relative amounts of the major proteins seemed different between the two strains of mice, as was revealed by SDS-PAGE and confirmed by RP-HPLC, we performed a semi-quantitative analysis by integrating HPLC peak areas of identified proteins. The areas calculated for the major proteins were summed and the relative proportion of each protein was expressed as a percentage of this value in the milk from C57BL/6J and PWK/Pas mice (Table 1). The statistical comparison revealed a significant difference for the relative proportion of $\beta$-casein and SA between the two subspecies. The relative proportion of $\beta$-casein was higher in C57BL/6J milk than in PWK/Pas milk (28.1% ± 1.8 vs. 20.4% ± 0.9, respectively; $P<0.05$, Student's t-test). By contrast, the relative proportion of SA was lower in C57BL/6J milk than in PWK/Pas milk (4.8 ± 0.2 vs. 10.2% ± 2.1, respectively; $P<0.05$, Student's t-test).

Finally, comparison of RP-HPLC profiles between the milks from the two Mus musculus subspecies, suggests the existence of a protein polymorphism, which was not highlighted in SDS-PAGE analysis. Both $\alpha_s$-casein isoforms appeared to be different in the C57BL/6J and PWK/Pas strains, with distinct retention times during RP-HPLC analysis. Preliminary cDNA...
sequence data indicate the existence of mutations in the coding region of $\alpha_s^1$-casein transcript from PWK/Pas (manuscript in preparation), thus confirming the hypothesis of genetic polymorphisms. Moreover, the milk proteins were around four times less concentrated in the PWK/Pas inbred strain, but except for the $\beta$-casein and SA, the relative proportion of the major proteins was identical in both PWK/Pas and C57BL/6J milk.

**DISCUSSION**

*Perfecting a method to describe the different major proteins in mouse milk*

Providing a clear and precise description of the milk major protein fraction is a complex task. It usually requires different techniques and it is always time consuming. In this paper, we have described a simple and reliable method, based on RP-HPLC coupled to SDS-PAGE and followed by mass spectrometry, to identify the major proteins in mouse milk. After checking the repeatability and reproducibility of the RP-HPLC profiles, we observed a good resolution of this chromatographic approach, which allows a better separation of the two $\alpha_s^1$-casein isoforms in comparison with SDS-PAGE. Moreover, our analysis reveals a protein polymorphism of the two $\alpha_s^1$-casein isoforms between two inbred strains of mice (C57BL/6J and PWK/Pas).

Thus, the described method is reliable and should allow the identification of variants in various milks. RP-HPLC coupled to SDS-PAGE has already been applied to mare, bovine and goat milk proteins (7, 11 and 12), highlighting the existence of different sources of polymorphisms.

The implemented proteomic approach has allowed the identification of nine major mouse milk proteins: $\kappa$, $\beta$, $\gamma$, $\delta$, and $\alpha_s$, caseins, lactoferrin, SA, FABP and WAP. The caseins exhibited a lower mobility on SDS-PAGE, and hence a higher apparent molecular weight, than expected from their known molecular weight. This characteristic, already reported for caseins from other species, is probably due to the unusual extended nature of the molecules and to an abnormally low binding of SDS (13). Another striking result is the identification of two isoforms of $\alpha_s$-caseins, already mentioned by Bouguyon (14). Mouse $\alpha_s$-casein contains 298 amino acid residues. This protein is significantly larger in comparison with $\alpha_s$-casein from the other species studied so far, such as guinea-pig and cattle (183 and 199 amino acid residues, respectively). The difference is due to the insertion in the mouse protein of the tandem repeated hexapeptide sequence QASLAQ (15).

The abundance of the two identified isoforms is different, with a minor and a major form, the minor form showing a lower molecular weight as revealed by the SDS-PAGE data. Thus, the minor mouse $\alpha_s$-casein isoform observed in RP-HPLC profiles could correspond to a splice variant of the $\alpha_s$-casein primary transcript. Exon-skipping events, first described for the goat $\alpha_s$-casein (16) and the human $\beta$-casein (17, 18) and afterwards reported in several species, are very frequent. As a result, the mature goat $\alpha_s$-casein exists as a mixture of at least four molecular species which differs in peptide chain length (19). Since it is eluted earlier from the RP-HPLC column (Fig. 1A and 1C), the minor mouse $\alpha_s$-casein isoform has probably a less hydrophobic nature than the major isoform.

Another interesting result came from the identification, in the same chromatographic fraction, of two $\kappa$-casein isoforms with different SDS-PAGE migrations. The retarded band corresponds to the glycosylated form, as revealed by Schiff coloration (data not shown). In Fig. 2 glycosylation of the $\kappa$-casein is evidenced by an extensive smear. This observation indicates the presence of considerable variation in the post-translational glycosylation level (20). The non-glycosylated form of $\kappa$-casein band 3 shown in Fig. 1B (lanes a and a') could correspond to the faint band observed above the WAP-distinctive band following SDS-PAGE analysis of the skimmed milk (Fig. 2).

Finally, $\alpha_l$-lactalbumin remains the only major milk protein which was not identified in our study. However, it could correspond to bands 10 and 8 (Fig. 1B and Fig. 1D respectively), with a molecular weight close to 14 kDa, whereas the $\alpha_l$-lactalbumin native mass is 13999.85 Da. This lack of identification does not come as a surprise since $\alpha_l$-lactalbumin accounts for less than 1% of the total protein in milk from mice (21). However, $\alpha_l$-lactalbumin was identified in the whey fraction of C57BL/6J milk by mass spectrometry (data not shown).

**Identification of a protein polymorphism in milk from two Mus musculus subspecies**

The protein concentration comparison between C57BL/6J and PWK/Pas milk brought a striking result. Although we found a protein concentration in milk from C57BL/6J females similar to the concentration previously reported for this inbred strain (22), i.e. 125 g/l compared with 132 g/l, the protein concentration was significantly lower (32 g/l) in milk from PWK/Pas females. It could be interesting to evaluate the milk production in C57BL/6J mice and PWK/Pas mice. Indeed, an increase in milk production can be hypothesized with PWK/Pas mice, to compensate its lower protein concentration. Conversely, one could expect a detrimental effect on the offspring growth or a lower litter size. This does not appear to be true in our PWK/Pas breeding population. This apparent dilution effect is consistent with the fact that the relative proportions of all milk proteins were identical between C57BL/6J and PWK/Pas inbred strains, with the exception of $\beta$-casein and SA. The difference in total protein concentration could be explained by a larger amount of lactose in milk from PWK/Pas since increased lactose concentration is usually associated with more diluted milk (23). However, preliminary results showed that there are no differences in lactose concentration between the two strains (data not shown).

Finally, our study revealed a protein polymorphism in the milk from C57BL/6J and PWK/Pas mice. Indeed, the comparison of RP-HPLC profiles showed that the two $\alpha_s$-casein isoforms exhibited different retention times in milk from C57BL/6J and PWK/Pas mice. This protein polymorphism likely reflects an amino acid substitution in the coding sequence of $\alpha_s$-casein gene in C57BL/6J and PWK/Pas genome.

To conclude, this study allowed us to validate a RP-HPLC method that will be useful to address the analysis of the PRM/Alf milk protein fraction and to start a comparative analysis to search for differences between PRM/Alf and C57BL/6J used as a control strain.

**Acknowledgments:** We thank Koenraad Duhem (and APIS-Geno) for his support. We are indebted to Isabelle Lancin for technical assistance. We thank Luc Reverbel, Christophe Auger and Bernard Cayron for mice breeding at the Unité d’Infectiologie Experimentale des Rongeurs et Poissons.

**Conflict of interest statement:** None declared.

**REFERENCES**


**Received:** November 5, 2008  
**Accepted:** March 25, 2009

**Author’s address:** Sylvain Bellier PhD, Ecole Nationale Vétérinaire d’Alfort, 7 Av. Du Gal. De Gaulle, Maisons-Alfort Cedex, France; Phone: +33 1 43 96 73 55; Fax: +33 1 34 65 29 26; e-mail: sylvain.bellier@jouy.inra.fr