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RESEARCH ARTICLE

The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence

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Abstract

The serum amyloid A protein is one of the major reactants in the acute-phase response. Using representational difference analysis comparing RNA from normal and involuting quarters of a dairy cow mammary gland, we found an mRNA encoding the SAA3 protein (M-SAA3). The M-SAA3 mRNA was localized to restricted populations of bovine mammary epithelial cells (MECs). It was expressed at a moderate level in late pregnancy, at a low level through lactation, was induced early in milk stasis, and expressed at high levels in most MECs during mid to late involution and inflammation/mastitis. The mature M-SAA3 peptide was expressed in *Escherichia coli,* antibodies made, and shown to have antibacterial activity against *E. coli*, *Streptococcus uberis and Pseudomonas aeruginosa*. These results suggest that the mammary SAA3 may have a role in protection of the mammary gland during remodelling and infection and possibly in the neonate gastrointestinal tract.

Keywords: *serum-amyloid-A3; SAA3; milk; mammary; antimicrobial; bovine*

Introduction

The serum amyloid A protein (SAA) family is one of the major reactants in the acute-phase response (Pepys & Baltz 1983, Schultz & Arnold 1990, Sellar & Whitehead 1993, Uhlar & Whitehead 1999). These proteins are synthesised mainly in the liver and concentrations of SAAs in the plasma can rise up to 1000-fold in response to inflammation or infection (Rienhoff Jr et al. 1990, Jensen & Whitehead 1998, Bing et al. 1999). The mRNA encoding SAA1, 2 and 4 proteins has been detected by *in situ* hybridisation in a range of human tissues including some epithelial cells of the breast, pancreas and intestine (Urieli-Shoval et al. 1998). The SAA proteins are conserved and some have been shown to be effective markers for the presence and severity of a range

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of diseases and health conditions (Whicher et al. 1985, Liuzzo et al. 1994, Casl et al. 1995, 1996, Hartmann et al. 1997, Eckersall 1999, 2004, Gruys et al. 2005a, b). Levels of SAA proteins have also been shown to be elevated up to 110 $\mu g\,ml^{-1}$ in milk during mastitis (Eckersall et al. 2001, Grönlund et al. 2003, Nielsen et al. 2004, Eckersall et al. 2006), and hence they are viable biomarkers for the presence of this disease in cows and possibly other species (Petersen et al. 2005, Akerstedt et al. 2007, 2008, O'Mahony et al. 2006). To use M-SAA3 as a marker it is important to know how and during what biological conditions this protein arrives in the milk and whether or not it is made locally or elsewhere. Initially milk SAAs were thought to arise from blood leakage into the milk during mastitis but now it is thought that the synthesis of SAA3 specifically also occurs in mammary epithelial cells (Molenaar et al. 2000, Eckersall et al. 2001, McDonald et al. 2001, Larson et al. 2005, Eckersall et al. 2006, Weber et al. 2006). The exact role of the serum amyloid proteins in the acute-phase response is unclear, but they are multifunctional and include immunological and other functions (Jensen & Whitehead 1998). Recent data suggest that various SAA proteins may have direct (Hirakura et al. 2002, Wang et al. 2002, Hari-Dass et al. 2005, Shah et al. 2006) and indirect (McDonald et al. 2001, Larson et al. 2003a, Mack et al. 2003) antimicrobial activities. It is thought that locally expressed serum amyloid proteins may play a role related to the site of expression and be produced under conditions that do not initiate the systemic acute-phase response (Urieli-Shoval et al. 1998). An extramammary protective role has been suggested for M-SAA3 in neonates, and possibly adults, against gastrointestinal infections by inducing mucin production (Mack et al. 2003).

The mRNA encoding a SAA3 protein variant we isolated and reported previously from bovine mammary tissue (Molenaar et al. 1999, 2000), was named M-SAA3.2 (GenBank accession number AY943216) due to the presence of another slightly differing sequence in the database. The objective of the present study was to characterise the expression of the M-SAA3 in the bovine mammary gland, investigate its possible role in mammary gland function and test the activity of the recombinant protein. Our results show that the mammary gland expresses M-SAA3 and suggest that M-SAA3 may function to protect mammary tissue during stress and in defence against mammary gland and milk infection.

Materials and methods

All experiments involving animal manipulations were approved by the Ruakura Animal Ethics Committee (Hamilton, New Zealand).

Tissue collections and processing

Dairy cows at various stages during the lactation cycle and some with mastitis were slaughtered at a commercial abattoir. Representative mammary and other tissue samples were immediately excised and snap-frozen in liquid nitrogen. Similarly located tissues were also harvested, fixed in formalin overnight, and embedded in wax for histological examination and *in situ* hybridisation analysis. Approximately 50-g pieces of frozen tissue were powdered in liquid nitrogen using a specially designed metal crucible (http://www.agresearch.co.nz/agr/agrsci/crusher/tissue_crush.asp) and subsequently total RNA was extracted from tissue using TRIzol reagent (Chomczynski 1993) (Invitrogen, Auckland, New Zealand) and stored at –80°C. Specific details of tissues accompany each section.

Northern analysis

Mammary RNA was isolated from tissue taken from cows in the following states: virgin, pregnant (195 days), first lactation non-pregnant heifers (mid-lactation, six per time point) 0, 12, 24, 36, 72, 192 h after abrupt termination of milking, and 20 days natural involution ($n = 1$), subclinical mastitis, chronic mastitis and acute mastitis ($n = 1$ each) (defined by SCC of 1 million cells ml^{-1} per quarter or 400 000 cells per combined udder milk and bacterial counts). Liver tissue from a healthy cow was also taken. To generate the M-SAA3 probe, the partial cDNA (300 bp) for the M-SAA3 gene, isolated in the course of a representational difference analysis (RDA) (Hubank & Schatz 1994) comparing lactating and involuting quarters from the mammary gland of a 2-month lactating Friesian cow, was subcloned into the pGEM3 vector (Promega, Madison, WI, USA), amplified, and used to isolate a full-length M-SAA3 sequence from a bovine lactating mammary gland cDNA library constructed in the Lambda Zap Express vector (Stratagene, La Jolla, CA, USA), with cDNA derived from the mammary alveolar tissue of a 12-year-old Jersey cow in late lactation using standard methods. Equal amounts of these samples and RNA from bovine liver as determined by methylene blue staining of the filters, were subjected to northern blotting using standard protocols (no. 53) (Sambrook et al. 1989), and hybridisation buffer (Church & Gilbert 1984), but without bovine serum albumin (BSA). Replicate blots were probed with ^{32}P (Amersham Biosciences, Amersham, UK)-labelled M-SAA3, α-lactalbumin (M18780) and lactoferrin (X54801). The blots were washed to a stringency of 1 × SSC (150 mM NaCl, 15 mM Na_3 citrate/0.1 × SDS at 65°C). The signal was detected either with XAR-5 film (Kodak, NY, USA) or a storage phosphor HD screen (Kodak).

Table 1. The nucleotide sequences or RT-PCR primers[1].

mRNA	Forward primer 5′–3′	Reverse primer 5′–3′	Product size (bp)
M-SAA3	ACATTCCTCAAGGAAGCTGGTCAA	CAGGCCAGCAGGTCTGAAGTG	287
ubiquitin	GGCAAGACCATCACCCTGGAA	GCCACCCCTCAGACGAAGGA	201

[1]The PCR primer sequences were designed using vector NTI (Invitrogen).

In situ hybridisation analysis

After histological examination, representative sections of mammary tissue were selected for examination. Lactating mammary alveolar and cisternal tissue, sampled at 2 and 40 h after the last milking and inflamed or mastitic (SCC >5 270 000 cells ml^{-1} of milk) tissue sections (6 μm), were mounted in duplicate onto 3-aminopropylthriethoxy-silane (A3648, Sigma Chemical Company, St Louis, MO, USA)-coated slides (Rentrop et al. 1986). The 300-bp RDA product in pGEM3 was used to generate ^{35}S-UTP-labelled antisense RNA probes and hybridised as previously described (Molenaar et al. 1992). Sense transcripts from the opposite strand were used as negative controls. The sections were hybridised with approximately 10 000 cpm μl^{-1} of each probe, washed, coated with Amersham Hypercoat LM1 emulsion (GE Healthcare Ltd., Auckland, New Zealand) and allowed to autoradiograph for 2.5 months prior to development and staining. The results were compared with those obtained and previously reported for the α-lactalbumin and lactoferrin genes (Molenaar et al. 1992, 1996).

Real-time polymerase chain reaction analysis

Involution of the bovine mammary gland was induced by abrupt termination of milking in 48 non-pregnant Friesian dairy heifers at mid-lactation (57–113 days), as previously reported (Singh et al. 2005). Briefly, alveolar mammary tissue was obtained following slaughter at 0, 6, 12, 18, 24, 36, 72 and 192 h (n = 6 per group) after the last milking and stored at –80°C. Real-time polymerase chain reaction (RT-PCR) analysis was performed essentially as previously described (Singh et al. 2005) using SYBR Green I PCR Master Mix (Applied Biosystems, Foster City, CA, USA) (Table 1).

The primers were used at 100 nM. Real-time PCR was performed using 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C, for 30 s, 72°C for 30 s, 78°C for 10 s on an ABI Prism 7900HT cycler (Applied Biosystems). Dissociation curve analysis confirmed that only one product was amplified. The amplified products were verified by sequencing. The threshold cycles (C_T) generated by real-time RT-PCR were used to quantify the relative abundance of M-SAA3 using the relative standard curve method (Applied Biosystems, Sequence Detection System, Chemistry Guide, 2003). The values for each gene were $\log_{10}$ transformed and normalized to ubiquitin $\log_{10}$ transformed values. The differences between means were analyzed using ANOVA in the Minitab software package (Minitab Inc.). The means for each group were back-transformed and expressed as the fold change ± SEM relative to the 6 h mean. Data were expressed relative to the 6 h mean time point. The 6 h time point was selected over the 0 h time point because the latter milking follows approximately 16 h non-milking overnight and hence the tissue lactational activity is likely to be lower. Least significant differences identify the means significantly different from each other (p <0.001).

Measurement of M-SAA3 levels by ELISA

An ELISA kit for amyloid A protein measurement was obtained from Tridelta Development Ltd (Maynooth Business Campus, Co. Kildare, Ireland) and used to compare SAA3 levels in milk from cows with experimentally induced mastitis. Ten healthy, mid-lactation dairy cows were used. The rear quarter of each cow was infused with 1000 colony-forming units (CFU) of *Streptococcus uberis* in 1 ml of Ringer's solution (quarter strength) to induce mastitis. Milk samples were taken before infusion, and antibiotics (sodium cloxacillin) (Orbenin L.A., Beecham Veterinary Products, Auckland, New Zealand) were given after the onset of clinical mastitis or after 72 h. Six of the cows showed symptoms of mastitis within 24–48 h. A subset of the foremilk samples taken from these cows was assayed.

Recombinant M-SAA3 protein expression

The segment of the M-SAA3 cDNA encoding the mature protein in the Lambda Zap Express vector was amplified by 30 cycles of PCR and cloned, via a transitional cloning vector, pGEM-T Easy (Promega), into the pET-23b prokaryote expression vector (Novagen, Madison, WI, USA) utilising *Nde*I and *Xho*I restriction enzyme recognition sites in the vectors. DNA bands from PCR and plasmid extractions (Riggs & McLachlan 1986) and digestions were separated on 1% agarose gels, visualised under UV illumination then extracted from the gels by freeze-squeeze (Thuring et al. 1975). The primer sequences were 5′-catatgCAGAGATGGGGGACATTC-3′(forward) and 3′-ctcgagGTACTTGTTAGGCAGGCC-5′ (reverse). The insert was ligated in-frame ahead of the vector encoded 6 His-tag sequence which, on translation, added a

histidine tag to the protein. A non-His-tag construct was made using a 3′ primer 5′- ctcgagTCAGTACTTGTTAGGCAG-3′ (reverse) that included a stop codon just prior to the vector-encoded histidine tag, so that the translated protein would not have the tag. The constructs were transformed into in DH5α *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA). After verification of the sequence, the constructs were transformed into either isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible BL21 *E. coli* cells (Stratagene) or arabinose-inducible BL21AI *E. coli* cells (Invitrogen). Transformed cells were grown in terrific broth (Tartoff & Hobbs 1987) or LB broth containing 20 μg ml^{-1} ampicillin at 37°C until $OD_{600} = 0.6–1.0$. Protein expression was induced by adding IPTG to a final concentration of 250 μg ml^{-1} or arabinose at a final concentration of 0.2% (w/v) to the appropriate cells, and shaking for 3–6 h at 37°C. The cells were then harvested by centrifugation at 1600 *g* for 15 min, and the pellet stored at –80°C until required. The level of induction was checked on a SDS-PAGE MES 10% Bis–Tris Gel (NuPage; Invitrogen) performed with uninduced and induced culture subsamples resuspended and boiled in cracking buffer (6 M urea, 4% SDS, 0.1 M Tris–HCl, pH 6.8).

Antibody production

The recombinant protein was extracted from the cells using standard methods (Novagen). As the M-SAA3 was equally divided between the soluble and insoluble *E. coli* fractions, both denaturing (8 M urea) and non-denaturing protocols were used to purify the proteins using a nickel affinity matrix by the Novagen pET His-tag system or the Invitrogen ProBond system using an 8 cm high × 0.8 cm wide column. The eluted fractions were tested for the presence and purity of M-SAA3 by SDS-PAGE on a 10% Bis–Tris NuPAGE gel (Invitrogen), alongside a SeeBlue protein marker (Invitrogen). Western blot analysis using an alkaline phosphate-labelled monoclonal antibody to a SAA3 protein, (Tridelta Development Ltd) was employed as described below to check that the transcribed protein was in the correct reading frame. The imidazole eluant was removed from the protein by dialysis or a 10DG column (Bio-Rad, Hercules, CA, USA) buffer exchange column. The protein was reconcentrated by freeze-drying. As a further check, the protein was purified using two-dimensional gel electrophoresis (Criterion 10–20% gradient gel; BioRad), the band excised, trypsin digested and directly infused into a LCQ Deca ion trap mass spectrometer fitted with a nanospray ESI interface (ThermoQuest, Finnigan, San Jose, CA, USA). Spectra were searched against the bovine/ovine subdatabase of the public non-redundant protein database (NRPD) of the National Center for Biotechnology Information (NCBI). Two rabbits were inoculated three times according to standard immunization procedures, with a total 0.125 mg of a mixture of 'denatured' and non-denatured proteins over a period of 9 weeks, after which the rabbit serum was collected, diluted 1:1 in glycerol and stored at –20°C.

Western blot analysis

The rabbit antibodies generated against M-SAA3 were characterised by Western blot analysis using samples of mammary secretions (colostrum, mastitic and standard homogenized milk) and His-tag column purified recombinant protein. The samples were mixed with an equal volume of 2x sample loading buffer (4% SDS, 20% glycerol, 125 mM Tris–HCL pH 8.6, 0.4 mg ml^{-1} Bromophenol Blue Powder, 1% 2-mercaptoethanol) and boiled for 5 min in a water bath, then run on a 10% Bis–Tris NuPAGE gel. The gel was transferred onto a nitrocellulose (BiotraceNT, Pall Corporation, Pensacola, FL, USA) or PVDF membranes (Biotrace PVDF Pall Corporation) for 1.5 h at 30 V in the miniblot Xcell II Blot module (Invitrogen) using NuPAGE (Invitrogen NP0006-1) or CAPS (3-(cyclohexylamino)-1-propanesulfonic acid)/10% methanol (Sigma C-2632) transfer buffers. The clarity of separation and extent of the transfer was examined by staining the membrane with Ponceau S stain (Sigma P-3504), and the gel with Coomassie blue R-250 gel stain (Bio-Rad). The membrane was blocked in a solution of 1X IGEPAL (Sigma)–Tris buffered saline (TBS), pH 7.9 (western buffer) containing 1% polyvinylpyrrolidone (PVP) (Aldrich) or Roti Block (No. A151.1, Carl Roth, Karlsruhe, GmbH) and latterly, 0.1% immunoglobulin free BSA (ABGF-001 ICP_{bio} Ltd, Auckland, New Zealand) (this particular reagent had the advantage of not causing a cross-reaction with a marker band) for at least 2.5 h at room temperature. Additional blocking was achieved by using a streptavidin/biotin blocking kit (Vector SP-2002, CA, USA or Dako X0590, CA, USA) but modified to incubate first with 0.8% streptavidin for 10 min, washed for 3 min with TBS then incubated with 0.8% biotin for 10 min, washed for 3 × 1 min in TBS before incubating in antisera diluted to 1:80000 with western buffer for 2 h at room temperature or overnight at 4°C. The membrane was washed in western buffer for 3 × 10 min and the remaining primary antibody detected with biotinylated goat antirabbit secondary antibody (Lot HK3260596) diluted to 1:20 000, washed in western buffer for 3 × 10 min, incubated with a streptavidin horseradish peroxidase conjugate (Lot HK320D896) diluted to 1:80 000 (Biogenex, San Ramon, CA, USA; stored in 1:1 glycerol at –20°C for long term stability) then washed in western buffer for 3 × 10 min prior to visualisation by enhanced chemiluminescence (ECL) and X-ray film (Kodak). The membrane was covered in ECL solution (221 μg ml^{-1} Luminol (Sigma no. A8511), 11.2 μg ml^{-1} *p*-coumaric acid (Sigma no. 9008), 0.01% H_2O_2 in 100 mM Tris, pH 8.6) and exposed to X-ray film

for seconds to minutes as required to achieve a suitable signal (Figure 1).

Bacterial killing activity of recombinant M-SAA3

BL21 AI *E. coli* containing the 'non-tagged' construct were grown, induced and harvested as described above. For cell lysis, the harvested pellet was redissolved in 1 ml lysis buffer (50 mM Tris-HCl), per ~ 70 mg wet pellet and then sonicated as above. After centrifugation at 1600 *g* for 15 min the supernatant was collected, stored on ice and then purified using a Octyl Sepharose 4 Fast Flow column (Amersham Biosciences). Supernatant (10 ml) was loaded on the column and eluted with a gradient of 50 mM Tris-HCl buffer pH 7.0 as solvent A and solvent B which was solvent A plus 6 M ≥98% pure guanidinium HCL (Fluka, Switzerland), pH 7.0 at a flow rate of 8.0 ml min^{-1}. The peaks were collected (approximately 30 ml each). The collected fractions were thoroughly ultrafiltered and diafiltered against MilliQ (Millipore) filtered water using a 5-kDa membrane (Millipore Pellicon XL PLCC 5), aliquoted and freeze dried. The fractions were analysed by western using PVDF transfer membrane and the rabbit polyclonal anti-M-SAA3 antibody.

To test microbicidal activity of recombinant M-SAA3, the percentage CFUs remaining after exposure to the peptide was measured according to the method of Hooper et al. (2003) on separate occasions by different investigators, using recombinant material prepared as described above on two different occasions. On the first occasion the protein precipitated and after testing a number of agents including 0.6% Tween and 2% urea, the latter agent was used to solubilise the precipitate. The protein concentration was estimated by the Bradford assay (Bradford 1976) and tested at a concentration of 18 μg ml^{-1}. Bacteria used were *E. coli* strain ATCC 25922, *S. uberis* strain 01405 and *Pseudomonas aeruginosa* strain ATCC 25668. Each well was inoculated with 10 μl bacteria, at a range of 106 CFU ml^{-1} and incubated for 2 h at 37°C. On the second occasion the protein remained soluble in MQ water. The protein concentration was estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at an absorbance of 280 nm and was derived by multiplying the absorbance at 280 nm with the extinction coefficient of M-SAA3 (2.288) calculated using http://au.expasy.org/tools/protparam.html (Wilkins et al. 1999). The protein solution was then titrated in 100 μl phosphate-buffered saline in a polypropylene 96-well plate and duplicate 2-fold dilutions from 0 to 500 μg ml^{-1} protein were made and tested against *E. coli*. After incubation, viable bacteria were quantified by plating 1-, 10- and 100-fold dilutions. As controls, similar dilutions of the bacteria without added recombinant M-SAA3 but with buffer, were used. The results were expressed as % CFU = number of colonies in test/number of colonies in control × 100.

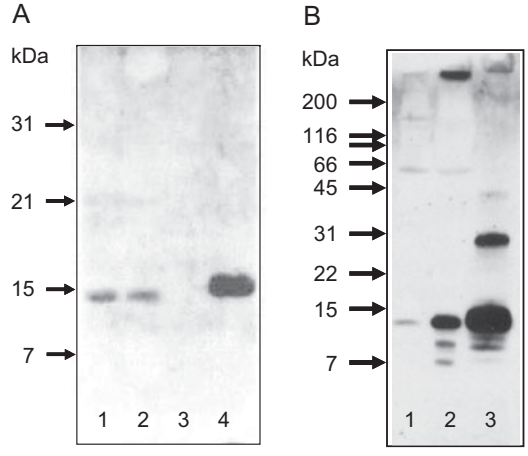

Figure 1. Western analysis. Composite western blots of various normal and mastitic milks and recombinant protein samples tested with various methods. (A) Western method 1 with nitrocellulose transfer membrane and 0.1% BSA/PVP blocking. Lane 1 contained 0.5 μl colostrum, lane 2 contained 0.5 μl mastitic milk, lane 3 contained 0.5 μl normal milk, lane 4 contained approximately 4 μg of recombinant His-tagged protein. (B) The same method as in (A) but twice as much material was loaded. Lane 1 was colostrum, 2 was mastitic milk, 3 was recombinant His-tagged protein. The BioRad Broad Range size marker was used.

Results

M-SAA3 expression in the mammary gland epithelium

In order to examine the relative temporal expression profiles in the mammary gland through the lactation cycle and during mastitis, northern analysis was performed with M-SAA3, α-lactalbumin and lactoferrin probes. The M-SAA3 was present in liver tissue and mammary tissue from pregnant and immediately post-parturient animals, not detected in lactating mammary glands, increasingly expressed from 24 h after milk removal had ceased and strongly expressed in mammary glands with mastitis (Figure 2). This was opposite to the expression pattern of α-lactalbumin, a lactation-associated milk protein, and similar to that of lactoferrin, a protein associated with involution, except that lactoferrin was not as strongly induced in mastitis as M-SAA3 was, relative to its induction in involution (Figure 2).

As northern analysis does not identify which type of cells express the M-SAA3 mRNA an *in-situ* hybridisation analysis was performed. The *in situ* studies revealed that the M-SAA3 mRNA was expressed in the mammary epithelium at a low level in cells with a histologically apparent lactation phenotype but at high levels in ductal epithelial cells and vesicle engorged alveoli (defined in Molenaar et al. 1996) but absent from stromal and connective tissue and leukocytes. It was strongly induced in

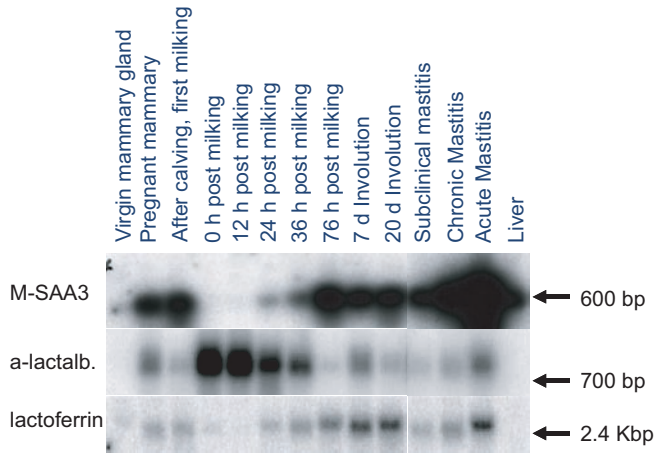

Figure 2. Representative northern analysis of replicated M-SAA3, **α**-lactalbumin and lactoferrin mRNA in bovine mammary alveolar tissue during different physiological states and in liver tissue. Post-milking time points were after abrupt termination of milking.

secretory mammary epithelial cells (MEC) during mastitis, notably where white blood cells were present in the alveoli (Figure 3).

As northern and *in situ* analyses are only semi-quantitative, quantifiable real-time PCR analysis was performed. The RT-PCR analysis of mammary tissue taken at various times after non-milking showed that a measurable but non-significant increase occurred by 36 h after milking had ceased. By 72 h after milking there was a significant 25-fold increase compared to the 6 h time point ($p<0.001$) and by 8 days after the last milking this had increased by approximately 160-fold compared with the 6 h time point ($p<0.001$) (Figure 4).

As the RNA studies do not prove the presence of the protein, the level of SAA3 was measured by ELISA in the milk of dairy cows with induced mastitis (Figure 5). At 24 h, SAA3 levels in milk from the quarters with clinical mastitis were significantly higher than the samples taken before mastitis occurred (24.6 ± 5.1 vs $9.7 \pm 0.1\,\mu g\,ml^{-1}$, $p<0.05$), and were also seen to be increased compared with the control quarters ($9.8 \pm 0.2\,\mu g\,ml^{-1}$, $p<0.01$). Levels increased even further after 60 h (108.1 ± 28.2 vs $10.5 \pm 1.0\,\mu g\,ml^{-1}$ for control quarters, $p<0.01$).

Recombinant protein expression

In the arabinose-inducible cells, prominent correctly sized bands were visible by SDS-PAGE and Coomassie staining, which appeared to be about half of the protein in the bacteria (Figure 6). The higher molecular weight size of the major band in lane 7 is due to the presence of the histidine tag. While expression of the His-tagged protein was successful using IPTG-inducible cells (lanes 6 and 7), attempts to transform and grow the non-tagged M-SAA3 construct with IPTG or salt-inducible BL21 cells were unsuccessful, and was only successful in arabinose-inducible BL21 cells. There was a tendency of the purified protein to precipitate out of solution when the solubilising buffer was removed by dialysis, or if the protein in imidazole was frozen overnight prior to buffer exchange and a considerable proportion (80%) lost on the buffer exchange column. Once precipitated the material was difficult or unable to be resuspended. The supernatant contained predominantly monomeric M-SAA3 (12.7 kDa) but on occasions Laemelli/SDS-resistant bands corresponding to dimers and multimers were seen, particularly after storage (data not shown).

Electrospray ionisation mass spectrometry analysis identified four tryptic peptides of 6–13 amino acids which matched the predicted M-SAA3 sequence (data not shown). The two-dimensional PAGE indicated a protein with a pI of between 9 and 10, in good agreement with the theoretical monomer pI of 9.56. These results indicated that recombinant M-SAA3 had been produced correctly.

Western blot analysis

Western blot analysis was performed to check that the antibody generated could detect both its antigen and the native M-SAA3 (Figure 1). The recombinant protein was detected by the antibody and was a little larger than the native protein due to the addition of the histidine tag. Approximately 12.7 kDa bands were detected in colostrum, mastitic milk, skim and whey from mastitic milk but not in normal milk. Immunoreactive bands of differing sizes were also detected at times (example panel B).

Purification of non-tagged M-SAA3 for the bacterial killing assay

To test the antimicrobial activity, the non-tagged recombinant M-SAA3 was chosen to avoid potential interference from the histidine tag. Hence, hydrophobic interaction chromatography (HIC) was used to purify the

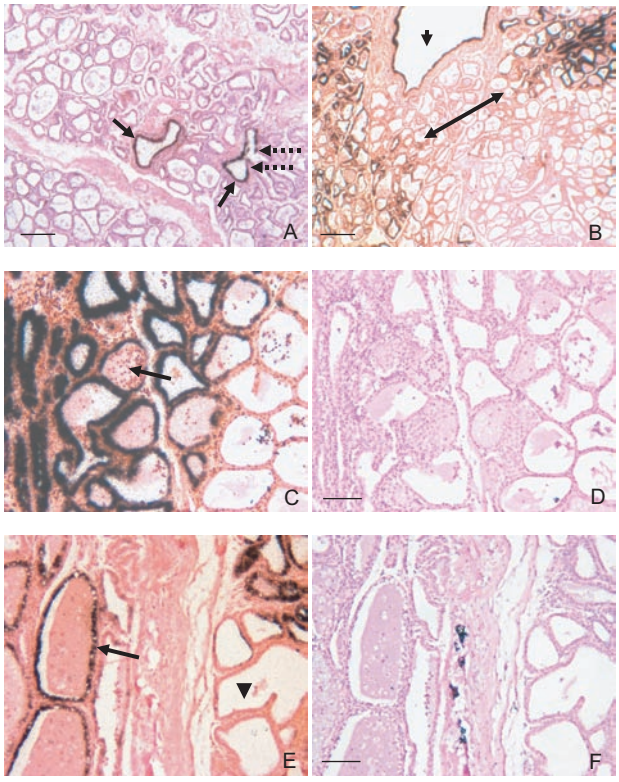

Figure 3. *In situ* hybridisation on tissue from late lactating, healthy and mastitic mammary tissue with sense and antisense ^{35}S-labelled riboprobes encoding a mammary serum amyloid A3. Positive hybridisation is revealed as black grains decorating the cells. Cells and alveoli with an appearance consistent with normal lactation are not decorated. (A) Alveolar secretory tissue from the mid-mammary gland taken 40 h after last milking and with a somatic cell count of 117 000 cells ml^{-1} in the milk from that quarter. The secretory alveoli have a histological appearance consistent with normal lactation and do not contain M-SAA3 mRNA. Solid arrows indicate ductal epithelial tissue containing M-SAA3 mRNA and dashed arrows indicate transition points of M-SAA3 mRNA in the ductal epithelial cells. (B–F) Various sites and magnifications of tissues from a similar cow but with a high somatic cell count of 5 270 000 cells ml^{-1} in the milk of that quarter. (D, F) The result with control sense probes. The small arrow in (B) indicates a large duct or cistern. The epithelial cells lining it are M-SAA3 mRNA positive. The double-headed arrow indicates areas of alveolar tissue that are inflamed and are M-SAA3 mRNA positive. (C) A higher magnification of the upper right corner of (B) showing neutrophil invasion of the area more clearly (arrow). (E) An alveolus with epithelial cells engorged with vesicles (arrow) and a nearby alveolus with a normal lactational appearance. The bar in (A) is 200 μm, (B) 300 μm, (C–F) 80 μm. All sections were stained with rapid haematoxylin and eosin.

protein as described in the materials and methods. The chromatogram showed two peaks in the guanidinium gradient, one at a concentration of 2.7 M and another at 3.0 M guanidinium HCL (data not shown). Western analysis showed that only the 3.0 M peak was immunoreactive to the M-SAA3 antibody (data not shown) and this fraction was subsequently used for the activity assays.

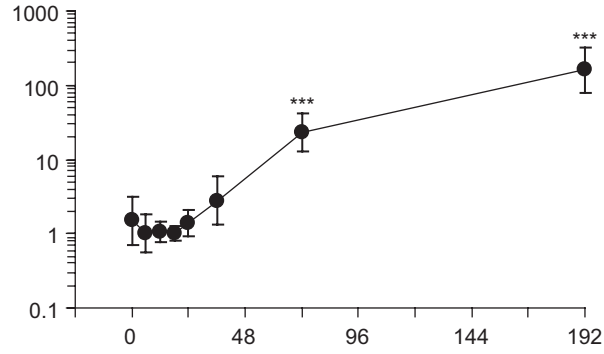

Figure 4. Real-time PCR measurement of the change in M-SAA3 mRNA levels in up to 8 days post-milking compared with 6 h post-milking, $n=6$ per time point (means ± SEM) (p <0.001 for the 72 h compared with the 6 h time point and p <0.05 for the 8 days compared with the 72 h time point).

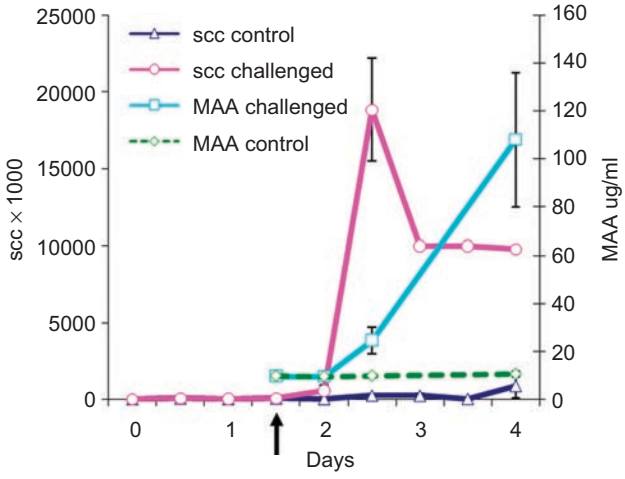

Figure 5. ELISA. Concentration of MAA/M-SAA3 and SCC in milk before and after a *Streptococcus uberis* mastitis challenge. Arrow indicates the start of the challenge (means ± SEM).

Antimicrobial activity

To determine if M-SAA3 had antimicrobial activity, the activity of recombinant M-SAA3 was tested against three species of bacteria. *E. coli, S. uberis* and *P. aeruginosa* bacteria were treated with an 18 μg ml^{-1} dose of recombinant protein in 10 mM sodium phosphate buffer containing 2% urea. Urea alone had no effect on the bacteria. There was a significant microbicidal activity, ranked in killing activity from highest to lowest, against *E. coli, S. uberis* and *P. aeruginosa* (Figure 7).

This activity was further investigated by carrying out a dose–response assay from 0 to 500 μg ml^{-1} protein on *E. coli* using a separate preparation of recombinant M-SAA3. Near complete killing of *E. coli* occurred at concentrations from ≥63 μg ml^{-1} of protein; at lower concentrations the cell killing declined to that of the untreated bacteria control (Figure 8).

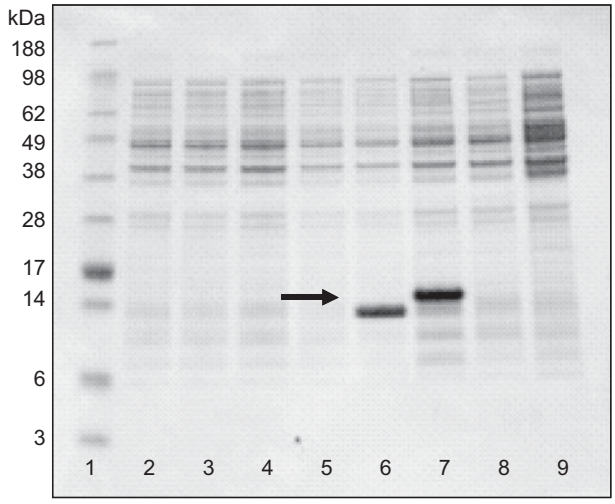

Figure 6. Recombinant protein expression. An example of a Coomassie blue-stained SDS-PAGE gel of 0.5 ml culture pellet, resuspended in cracking buffer and separated on an SDS-PAGE 10% Bis–Tris gel (Novagen). Lane 1 is the SeeBlus Plus 2 marker (Invitrogen). Lanes 2–5 were not induced and were loaded with extracts from bacteria containing MSAA-3 no-tag, MSAA-3 His tag, empty pET23b vector and no vector, respectively. Lanes 6–9 were the corresponding arabinose-induced samples. The arrow indicates the expressed proteins. The slight difference in size is due to the histidine tag.

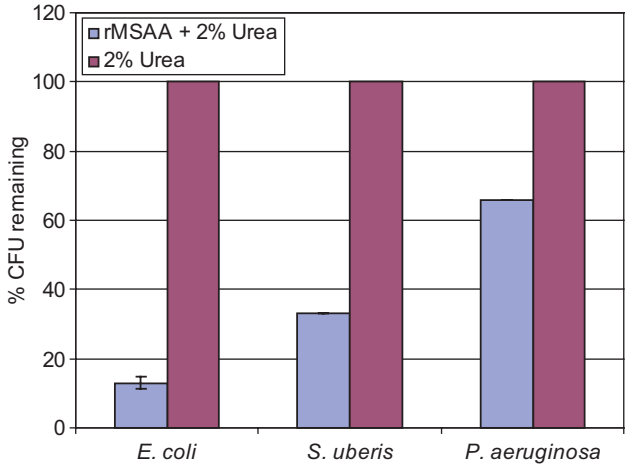

Figure 7. Effect of recombinant recombinant M-SAA3. *Echerichia coli, Streptococcus uberis* and *Pseudomonas aeruginosa* were treated with 18 μg ml^{-1} (~1.5 μM) recombinant M-SAA3 in 10 mM phosphate buffer containing 2% urea (means ± SEM).

Discussion

Acute-phase proteins play an important role in the inflammatory response to infection. Previous literature indicated that acute-phase proteins, including SAAs, leaked into the milk from the serum in times of infection or other stress (Salonen et al. 1996). Within the last decade it has been shown that the bovine mammary gland produces a number of acute-phase proteins such

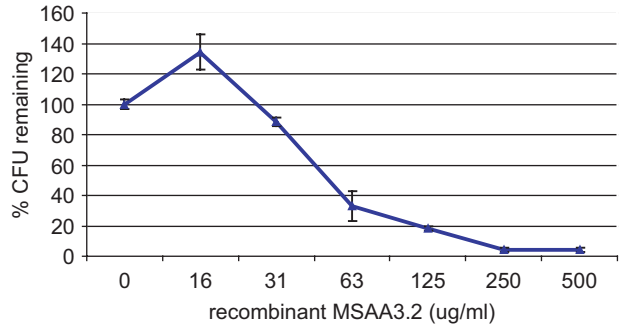

Figure 8. Dose–response of recombinant M-SAA3 on antimicrobial activity against *Escherichia coli* (means ± SEM).

as albumin (Shamay et al. 2005), haptoglobin (Hiss et al. 2004, Eckersall et al. 2006, Thielen et al. 2007), mammary serum amyloids (Urieli-Shoval et al. 1998, Molenaar et al. 2000, Eckersall et al. 2001, McDonald et al. 2001, Eckersall et al. 2006) and other immune-related factors (Rainard & Riollet 2006). The present study examined the expression of M-SAA3 in the bovine mammary gland and its possible role in mammary host defence.

Northern analysis confirmed that the M-SAA3 transcript was present in mRNA extracted from mammary gland tissue. Furthermore it exhibited an unusual expression profile for a mammary-expressed gene in that it was elevated in tissue from pregnant animals, low in lactation, elevated in involuting tissue and strongly elevated in mastitic mammary tissue. It was also expressed in liver tissue. With the exception of liver expression, this profile was similar to what we had previously seen with lactoferrin, a gene shown to be associated more with udders in prelactation pregnancy, involution and infection than lactation (Goodman & Schanbacher 1991, Molenaar et al. 1996) and hence implies a role for the M-SAA3 in the defence and maintenance of the mammary gland integrity during remodelling when the glands are more susceptible to infection (Oliver & Mitchell 1983, Burvenich et al. 1994).

As northern blots do not indicate which particular cells make the mRNA, *in situ* hybridisation analysis was performed. This analysis revealed that the transcript was present in the mammary secretory epithelial cells in a spacially restricted pattern. Generally, in tissue that had a histologically lactational appearance, the M-SAA3 mRNA was not detected. However, the epithelial cells lining ductal tissues were consitutitively M-SAA3 mRNA-positive except where the morphology of the ducts more closely resembled alveolar tissue. As the ductal cells are closer to the external environment (the teat) it makes sense that defence proteins are synthesised there where potential colonisation of that area by bacteria can occur during milking or suckling (Capuco et al. 1992). Local defence protein mRNA expression in this site has also been observed for another gene, lactoferrin (Molenaar

et al. 1996). In contrast to the situation in tissue with a 'lactating' appearance, the M-SAA3 mRNA was strongly induced in inflamed alveoli as indicated by the presence of invading neutrophils, most likely as a result of a mastitis causing infection, as inferred from the high SCC counts. These results indicate that the M-SAA3 mRNA is expressed also in cells that are under microbial attack and are supported by the observation that mammary epithelial cells in culture respond to exposure to microbial components by producing serum amyloid A mRNA (Wellnitz & Kerr 2004, Weber et al. 2006). The M-SAA3 mRNA was also present in engorged alveoli as shown by the presence of vesicles, presumably consisting of fats, within the epitheilial cells. These cells are thought to be in milk stasis (Molenaar et al. 1992). A separate function in mammary gland involution may be indicated by the association of M-SAA3 with the vesicle or fat globule membranes as demonstrated by immunohistochemistry using the antibody used in the westerns (data not shown). This association has also been reported by others (Nguyen et al. 2003) and is consistent with a role in the removal of accumulated lipid which becomes trapped in the alveoli during milk stasis (Molenaar et al. 1995).

To characterise the physiological conditions under which M-SAA3 was induced, the mRNA's induction was studied in an involution model by real-time PCR, it and the closely related SAA3 protein's induction in a mastitis model by ELISA, and, in colostral, normal, and mastitic milks by western analysis. Other workers have shown that M-SAA3 can be induced before and after the rise in SCC associated with clinical and subclinical mastitis. In our study the M-SAA3 rose at the same time as the SCC and continued to rise after the concentration of the latter had plateaued. Intramammary antibiotics were administered after the 24-h sampling which may have affected subsequent M-SAA3 concentrations. The M-SAA3 was essentially at or below the level of detection in the uninfused quarters. The western supported these results by showing essentially a single band at around 13 kDa in colostrum, mastitic milk, and whey and skim milk from mastitic milk. No M-SAA3 protein was detected in normal milk. Faint immunoreactive bands of differing sizes were also detected at times depending on the storage conditions of the samples. Human SAA forms multimers on storage and freeze-thawings (John Raynes, pers. comm. 2005). A similar event may have occurred with the M-SAA3 as the band sizes detected could indicate the presence of various SDS-resistant multimers and degradation products. The samples in Figure 1 panel B had undergone some freeze-thawings. The bands just below the 66-kDa marker could be 5-unit multimers of the M-SAA3. Other factors that can affect the formation of monomeric or multimeric structures of SAAs are *in vivo* by factors such as calcium concentration (Wang & Colon 2007), hence influencing possibly influencing the M-SAA3 structure in the milk, and *in vitro* with urea concentration (Wang et al. 2005) partially altering its complexity during the analysis under denaturing conditions.

The real-time PCR analysis of mammary tissue undergoing induced involution showed that M-SAA3 mRNA was strongly induced by up to 160-fold at 8 days post-milking compared with 6 h post-milking. Taken together the expression results show that the M-SAA3 mRNA is induced in non-lactation phases of pregnancy, involution and with mastitis, when the gland is being remodelled or being stressed by mastitis. As the mammary gland clearly expresses M-SAA3 in a spatially and temporally restricted manner that is similar to that of other defence proteins such as lactoferrin (Molenaar et al. 2002) defensins (Goldammer et al. 2004, Swanson et al. 2004, Yang et al. 2006) and haptoglobin (Thielen et al. 2007), these results are consistent with the idea that M-SAA3 has roles in host defence and in innate immunity.

Moreover, given the reports (Hirakura et al. 2002, Wang et al. 2002, Shah et al. 2006) that SAAs from other species have antimicrobial activities or interactions (McDonald et al. 2001, Larson et al. 2003b, Mack et al. 2003, Hari-Dass et al. 2005), we decided to investigate this possibility by expressing M-SAA3 recombinantly. The M-SAA3 sequence we expressed (GenBank accession number AY943216) has a 92.8% identity to the Bos taurus serum amyloid A 3 (SAA3), mRNA NM_181016 (Kho et al. 2000, McDonald et al. 2001). Bioinformatic translation revealed that it differs from NM_181016 by 4 amino acids; hence it is denoted in the NCBI database as Mammary Serum Amyloid A3.2 (M-SAA3.2). Both histidine tagged and non-tagged versions of the mature protein were designed and made as determined by alignments of M-SAA3 to those sequences reported in other species (data not shown). Interestingly, while the synthesis of both constructs was straightforward, the transformation and growth of the non-tagged construct into inducible BL21 cells proved difficult. Several unsuccessfull attempts were made with IPTG- and salt-inducible cells but arabinose-inducible cells were used sucessfully. The manufacturer claims that unlike the former two hosts, there is no 'leaky' synthesis of proteins from recombinant constructs in arabinose-inducible cells until the arabinose is added. This suggested that the small amount of expression from the construct may have killed the former cells on their transformation. This was supported by an observation that when LB media was lightly innoculated with BL21 cells containing no vector, empty vector, His-Tag vector and non-tagged recombinant M-SAA3 constructs and 'induced' with arabinose at ranges from 2 to 0.02% arabinose, the latter two hosts did not grow, in contrast to the former two

which were measuably turbid by 10 h and fully turbid by 24 h (data not shown). A similar observation has also been reported for recombinant human SAA (Yamada et al. 1994).

Some amyloid proteins have been reported as difficult to work with (Zagorski et al. 1999). Purification of both the His-tagged and non-tagged recombinant M-SAA3 was problematical. Both forms had a tendency to precipitate when their elution agent (imidazole or guanidinium) was removed by buffer exchange, dialysis or dia-filtration, and there was also a tendency for aggregation or multimerisation to occur as indicated by immunoreactive bands at around 62 kDa (mer 5 = 63.5 kDa) and other multiples. The method of purification and storage of the protein is important as monomeric and multimeric forms of SAAs are thought to have different types of activities (J. Raynes, pers. comm. 2005).

Bacterial activity assays showed that non-tagged recombinant M-SAA3 had antimicrobial activity against both Gram-negative and Gram-positive bacteria, namely *E. coli, S. uberis* and *P. aeruginosa* with the greatest activity against *E. coli*. This offers an explanation as to why the protein is expressed in the gland at times where defence against bacteria is required. The more effective killing of the Gram-negative *E. coli* is consistent with the observation of Hari-Dass et al. (2005) that SAA binds to Gram-negative organisms possessing the OmpA protein. While this study did not address the recruitment of neutrophils by this SAA, it is possible that M-SAA3 may additionally act as an innate recognition and signalling molecule by opsonising Gram-negative bacteria (Shah et al. 2006). It was interesting that the antimicrobial activity of the recombinant M-SAA3 survived treatment with the denaturant guanidinium, indicating that the protein is either not denatured by the guanidinium or easily refolded when resuspended in water or low ionic strength buffer. There was an apparent difference in the amount of recombinant M-SAA3 required to kill the test organism *E. coli* in the two bacterial killing experiments shown. The concentration of the protein in the first experiment was measured by the Bradford method, while in the second it was measured using a nanodrop spectrophotometer and an adjustment factor of SAA proteins. The Bradford measurement gives 1/3 of the recombinant M-SAA3 concentration that the spectrophotometer method gives (data not shown) so the 18 $\mu g\ ml^{-1}$ concentration shown in Figure 7 is very similar to the 63 $\mu g\ ml^{-1}$ concentration shown in Figure 8 and within assay-to-assay variation. The M-SAA3 protein on a weight-to-weight comparison has a potency approximately equivalent to that of lactoferricin peptides (Yamauchi et al. 1993, Shin et al. 1998). Literature reports (Eckersall et al. 2001, Grönlund et al. 2003, Nielsen et al. 2004, Eckersall et al. 2006) and our own data indicate that M-SAA3 can be found in bovine mastitic milks at levels up to 110 $\mu g\ ml^{-1}$. These levels are very similar to the concentrations where the recombinant M-SAA3 had antimicrobial activity and supports the suggestion that the major role for M-SAA3 in mastitis is antibacterial and that this is the biological reason for M-SAA3 being induced during mastitis.

Summary and conclusions

Understanding why, where and under what conditions the mammary gland produces an acute phase protein such as M-SAAs strengthens the rational for using it as a biomarker. Serum amyloid A has been reported as a potentially useful mastitis biomarker in other species such as sheep (Winter et al. 2006), but not goats (Winter et al. 2005). As M-SAA3 has been shown to be expressed in the human breast and induced by lipopolysaccharide (Larson et al. 2003b), it may also have value as a biomarker for mastitis during lactation in humans. We have shown that the bovine mammary gland epithelium expresses the serum amyloid protein M-SAA3. M-SAA3 was induced at times of gland remodelling or infection. Recombinant M-SAA3 had an antimicrobial activity against *E. coli*, *S. uberis* and *P. aeruginosa*. These results support the notion that M-SAA3 has a role in mammary innate immunity, illustrate the likely reasons why the protein is expressed biologically and underscore the importance of M-SAA3 as a biomarker for mastitis.

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