17

GENETIC ENGINEERING OF MILK PROTEINS

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17.1 INTRODUCTION

Changes in the productivity, composition or appearance of plants and animals have, traditionally, been achieved by means of selective breeding, drawing upon naturally occurring variations within the gene pool of a species. Many commercial plant species such as cereals and members of the brassica family bear very little resemblance to their ancestral forms and their productivity is very substantially higher. Changes achieved with domesticated animals are less dramatic but, in conjunction with improved animal husbandry, selective breeding has been so successful in increasing the milk yield of dairy cattle that within the European Union, quotas have had to be imposed in order to limit surpluses. By the use of artificial insemination and embryo transplants, an individual bull with the desired characteristics can produce thousands of offspring.

However, in contrast to the dramatic increases which have been achieved in yield, changes in the actual composition of the milk, such as the ratios of fat to protein and whey protein to casein and in the overall solids content, have been much more limited. In addition, within a species, the amino acid sequences of the milk proteins, which largely dictate their functionality as components in processed foods, are generally well conserved. In cattle, for example, with the exception of α_{s1}-casein (α_{s1}-CN) where there is a deletion of a sequence of 13 amino acids in the A variant, variation is restricted to single amino residues, severely limiting the scope for changing the functionality of individual proteins.

These restrictions can, potentially, be overcome by the application of a relatively new area of biotechnology, transgenesis. This technique utilises recent advances in molecular biology to provide an alternative method for

Advanced Dairy Chemistry Volume 1 Proteins, 3rd edn.
Edited by P.F. Fox and P.L.H. McSweeney, Kluwer Academic/Plenum Publishers, 2003.

producing much more dramatic, but equally much more specific, genomic changes. Genes from one species can be introduced into the genome of organisms of the same or different species and subsequently transmitted to their progeny in a stable fashion. Transgenic techniques were used originally to modify microorganisms, such as yeasts and bacteria, in order to produce large quantities of valuable, pharmacologically-active proteins and peptides usually present in only minute amounts in higher organisms. Palmiter *et al.* (1982) extended this technique to mammals and reported the successful

TABLE 17.1
Expression of some pharmaceutical proteins in transgenic milks

Protein	*Regulatory sequence*	*Species*	*Maximum level* ml^{-1}	*Reference*
Human α_1-antitrypsin	ovine βLG	sheep mouse	33 mg 21 mg	Carver *et al.* (1993)
Human factor IX	ovine βLG	sheep	25 ng	Clark *et al.* (1989)
Human tissue plasminogen activator	bovine αs1-cn	mouse, rabbit	0.05 mg	Riego *et al.* (1993)
Human gamma interferon	ovine βLG	mouse	1800 I.U.	Dobrovolsky *et al.* (1993)
Cystic fibrosis transmembrane conductance regulator	goat β-cn	mouse	1 mg	Ditullio *et al.* (1992)
Human superoxide dismutase	mouse WAP ovine βLG	mouse		Hansson *et al.* (1994)
Human protein C	mouse WAP	mouse, pig	0.7 mg 1 mg	Drohan *et al.* (1994) Velander *et al.* (1992)
FSH	rat β-cn	mouse	60 I.U. (15ng)	Greenberg *et al.* (1991)
Human CD6 antibody	rabbit WAP	mouse		Limonta *et al.* (1995)
Human glycosyltransferase	mouse WAP	mouse		Prieto *et al.* (1995)
Human parathyroid hormone	mouse WAP	mouse		Rokkones *et al.* (1995)
Human insulin-like growth hormone	bov α_{s1}-cn	rabbit	1 mg	Brem *et al.* (1994)
Human growth hormone	rabbit WAP rabbit WAP bov α_{s1}-cn	mouse mouse mouse	10 mg 22 mg 11 μg	Stinnakre *et al.* (1993) Devinoy *et al.* (1994) Ninomiya *et al.* (1994)
Bovine growth hormone	rabbit WAP	mouse	16 mg	Thepot *et al.* (1995)
Ovine trophoplastin	bovine αLA	mouse	1 μg	Stinnakre *et al.* (1991)

expression of rat growth hormone in mice. This was achieved by the direct microinjection of rat DNA, fused to the regulatory sequence of the metallothionein gene, into fertilized mouse eggs. The growth hormone was produced in the liver, and plasma levels up to 100-fold higher than normal were detected. However, the foreign protein interfered with the normal development of the host and transgenic mice were significantly larger than normal. It was quickly realised that a better option would be to target expression specifically to the mammary gland.

Expressing therapeutic proteins and peptides in milk is an attractive proposition. The mammary gland itself is capable of very high rates of protein expression and secretion and, since the stored milk is relatively isolated from the general circulation of the animal, transgenic proteins are much less likely to influence the physiology of the host. Milk is a well defined substance, relatively free from proteolytic enzymes and infective organisms such as viruses and prions and can be harvested in a non-invasive fashion using normal milking procedures and subsequently processed on a very large scale, if necessary.

In this article, after briefly considering changes achieved by site-directed mutagenesis in the structure and functionality of milk proteins expressed in microorganisms, we will concentrate mainly on the use of transgenic techniques to modify the composition and properties of milk both as a food and also as a means of producing valuable pharmaceutical proteins.

17.2 Expression of milk proteins in non-mammalian systems

17.2.1 Design of expression systems

The prokaryotic bacterium, *Escherichia coli*, and the eukaryotic yeasts, *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Pichia pastoris*, are the principal microorganisms in which native and mutated milk proteins have been expressed. Typically, the gene coding for the milk protein is fused to an inducible promoter sequence (although constitutive expression is becoming more common) and inserted into a suitable vector such as a plasmid or phage. The vector is then used to transform the host and after screening, transformed cells are cultured. After the cell mass reaches a sufficiently high level, the appropriate inducer is added and expression of the milk protein gene begins. By incorporating a secretion signal peptide, it is possible to direct export of the gene product into the medium.

17.2.2 Whey proteins

Bovine β-lactoglobulin (β-lg) has been expressed in both prokaryotic and eukaryotic organisms. In *E. coli*, levels of 8 to 10 mg L^{-1} of culture were

reported (Batt *et al.*, 1990). However, the protein was deposited within the cells in the form of inclusion bodies and required solubilisation and refolding. The same protein was expressed using the glyceraldehyde-3-phosphate promoter in the eukaryotic yeast, *S. cerevisiae*, as a soluble, secreted product at a concentration of about 1.1 mg L^{-1} (Totsuka *et al.*, 1990). Ovine β-lg has also been reported to be expressed in *S. cerevisiae* at 3 to 4 mg L^{-1} but was secreted in cultures of another yeast, *K. lactis*, using the phosphoglycerate kinase promoter, at a concentration of 40 to 50 mg L^{-1} (Rocha *et al.*, 1996). Introducing an additional disulphide bridge into bovine β-lg by site-directed mutagenesis in *E. coli*, increased the thermal stability of the protein by 8 to 10°C (Cho *et al.*, 1994). In contrast, introducing two additional thiol groups into the protein decreased its thermostability by more than 15°C, increased gel strength and decreased syneresis in yogurts made from milk fortified with this novel β-lg (Lee *et al.*, 1994).

Human lactoferrin has been expressed in *S. cerevisiae* and in the Baculovirus expression system. In *S. cerevisiae*, using the yeast chalatin promoter, the recombinant protein was secreted at levels up to 2 mg L^{-1} in a glycosylated form which bound iron and copper (Liang *et al.*, 1993). The protein was purified from the Baculovirus expression system at a concentration of 10 to 15 mg L^{-1} of culture supernatant (Salmon *et al.*, 1997). The iron-binding properties of this transgenic protein were identical to those of the native protein, but the two differed in the extent of glycosylation. Recombinant truncated variants of bovine lactoferrin have been expressed in *E. coli* at a level of 10 mg L^{-1} (Sitaram *et al.*, 1998). By determining the ability of these truncated proteins to bind to the Ca^{2+}-dependent receptors on hepatocytes, it was established that lactoferrin does so *via* its C-lobe.

Recombinant α-lactalbumin (α-la) expressed in *E. coli* was shown to have an additional methionine at the N-terminus. The effects of this modification on the physical properties of recombinant caprine (Chaudhuri *et al.*, 1999) and human (Ishikawa *et al.*, 1998) α-la were very pronounced, with the Met-α-la form of the protein being considerably more heat labile than the native form. Comparison of the X-ray structures of the caprine Met-α-la with that of the native protein showed the structural differences were confined to the N-terminal region.

17.2.3 Caseins

Human β-CN expressed in *E. coli* at a level of 300–500 mg L^{-1}, using an inducible T7-based expression system (Hansson *et al.*, 1993), was shown to be intracellular and to co-migrate with authentic full-length, unphosphorylated human β-CN. Co-expression of human casein kinase II in the same

plasmid resulted in *in vivo* phosphorylation of the transgenic protein (Thurmond *et al.*, 1997).

Bovine β-CN expressed in *S. cerevisiae* under the control of the hexokinase P1 promoter at levels as high as 10 mg L^{-1} was believed to be located either in the periplasmic space or in the cytoplasm (Jiminez-Flores *et al.*, 1990). Detailed analysis of the protein showed that, in addition to being multiply phosphorylated, some had also undergone *O*-glycosylation. Bovine β-CN has also been expressed in *E. coli*, again using the T7 expression system (Simons *et al.*, 1993). The level of expression was estimated to be 20% of the total soluble proteins, with most of it being located in the periplasmic fraction. Amino acid sequence analysis showed that the protein had an additional methionine at the N-terminal, and the glutamine residue at position 175 had been mutated to an arginine residue. The β-CN cDNA was modified in order to remove the principal chymosin cleavage site at position 192–193, which gives rise to bitter peptides during cheese maturation. This was achieved by changing the Leu-Tyr sequence at this point to a chymosin-insensitive Pro-Pro bond, which was not cleaved, or to Leu-stop which produced a β-CN molecule truncated at position 192.

κ-CN has been expressed in *E. coli* at 2 mg mL^{-1} using the secretion vector pIN-III-ompA and mutated to increase both its nutritional quality (Oh and Richardson, 1991a) and rate of proteolysis by chymosin (Oh and Richardson, 1991b). In the first instance, three methionine residues were inserted between Ala_{167} and Thr_{168} in order to increase the content of sulphur-containing amino acids. In the latter instance, the Phe_{105}–Met_{106} bond was changed to Phe–Phe and the rate of chymosin-catalysed hydrolysis of the novel protein was shown to be 80% higher than that of the wild-type.

17.3 Genetic engineering of milk proteins in animals

17.3.1 Production of transgenic animals

Currently, the method most commonly used to incorporate foreign genes into host animals is pro-nuclear injection. DNA is microinjected into the pro-nucleus of one-cell embryos obtained from superovulated females and the eggs are then surgically implanted into hormonally-primed foster mothers. This technique, originally developed for the generation of transgenic mice, has been extended, with minor adaptations, to larger animals, including ruminants (see review by Clark, 1998). Generally, the success rate of this technique, as measured by the number of live births of transgenic animals, is about 1 to 2%.

An alternative method for generating transgenic animals is by modifying the genome of embryonic stem (ES) cells. These are obtained early in the

development of the embryo and can be cultured in a suitable medium whilst retaining their totipotency. Following the introduction of foreign DNA, usually including a marker gene in order to enable positive cells to be identified, these cells can be injected into a host blastocyst. Here, the cells become integrated into the tissues of the developing embryo, including the germline, producing transgenic animals capable of transferring the transgene to their progeny. While this technique has been used widely to produce transgenic mice, this is the only species from which embryonic stem cells have been obtained.

Nuclear transfer has caused enormous excitement in the popular press in recent years. A review of this technique and an assessment of its potential for modifying livestock has been published recently (Woolliams and Wilmut, 1999). It has great potential to produce cloned copies of mammals, including dairy animals, whose milk composition has been modified in specific ways. Basically, this technique involves fusing cultured cells obtained either from embryonic, fetal or adult tissues with enucleated, unfertilized egg cells, using electrical pulses. The reconstructed embryos are cultured further and those that develop to morula or blastocyst are implanted into recipient females and allowed to develop to full-term. An obvious extension to this technique is to use cloned transgenic cell lines as donors in order to produce cloned transgenic offspring. Transgenic sheep, produced by nuclear transfer, expressing human factor IX in their milk were reported by Schnieke *et al.* (1997). The authors estimated that the efficiency of production of these animals was more than twice as high as could be attained by pro-nuclear microinjection.

TABLE 17.2
Expression of milk proteins in transgenic milks

Protein	*Host species*	*Max. Level (mg ml^{-1})*	*Reference*
Ovine βLG	mouse	23	Simons *et al.* (1987)
Ovine βLG (phosphorylated)	mouse	c. 30	unpublished
Caprine κ-casein	mouse	3	Persuy *et al.* (1995)
Bovine αLA	mouse	1.5	Bleck & Bremel (1994)
Bovine β-casein	mouse	10	Bleck *et al.* (1995)
Bovine β-casein	mouse	5	Hitchin *et al.* (1996)
Caprine β-casein	mouse	40	Persuy *et al.* (1992)
Human serum albumin	mouse	10	Hurwitz *et al.* (1994)
Human lactoferrin	mouse	0.04	Platenburg *et al.* (1994)
Human α-lactalbumin	mouse	1.4	Stacey *et al.* (1995)
Mouse WAP	pig	1.5	Shamay *et al.* (1991)
	sheep	0.5	Wall *et al.* (1996)

17.3.2 Targeting expression to the mammary gland

To direct the expression of transgenic proteins specifically to the mammary gland, a gene construct is formed by coupling the gene coding for the foreign protein to a milk protein regulatory sequence. Promoter elements which have been used for this purpose include those from various caseins, whey acidic protein (WAP) from both mouse and rabbit, ovine β-lg and α-la genes. Both the efficiency of expression and tissue specificity are affected by the nature of the promoter. WAP expressed in transgenic sheep under the control of the mouse WAP promoter was detected in the liver, lung, heart, kidney and bone marrow as well as in the milk, but not in skeletal muscle or intestine (Wall *et al.*, 1996). Ectopic expression of transgene products from constructs containing the ovine β-lg 5′-regulatory sequences has also been detected but usually in the salivary gland (Carver *et al.*, 1992) and at very low levels (0.1% of that in milk). The caprine β-CN transgene with 3 kb of 5′ and 6 kb of 3′ flanking region has been expressed in mice (Persuy *et al.*, 1992). High levels of caprine β-CN were detected in 5 lines but analysis of the mRNA from a variety of tissues by Northern blotting showed that caprine β-CN mRNA was present only in mammary gland. The exception to this was the detection of some caprine β-CN mRNA in the skin of a few animals but this may have been due to contamination with mammary tissue.

17.3.3 Post-translational processing of transgenic proteins

One of the major benefits of expressing transgenic proteins in mammalian cell cultures and milk is the ability of these systems to perform correctly a variety of post-translational modifications such as glycosylation, phosphorylation, γ-carboxylation, proteolytic processing and assembly of multi-component complexes.

(a) Glycosylation

Human extracellular superoxide dismutase, a glycosylated, tetrameric metalloprotein, has been expressed in mouse milk under the control of the mouse WAP promoter, at a maximum concentration of 3 mg mL^{-1} (Stromqvist *et al.*, 1997). Analysis of the purified protein showed that it had the correct polypeptide backbone and glycosylation pattern and was tetrameric. In contrast, human bile salt-stimulated lipase, also expressed in mouse milk under the control of murine WAP regulatory elements at a concentration up to 1 mg mL^{-1}, was found to have a significantly lower degree of glycosylation than either the native protein or recombinant protein isolated from mouse cell cultures (Stromqvist *et al.*, 1996). However, the lipolytic activity and the physical properties of the recombinant protein isolated from milk were found to be very similar to those of the native protein. Human erythropoietin has been expressed in transgenic mouse and

rabbit milks as a bovine β-lg fusion protein (Korhonen *et al.*, 1997). After specific proteolytic cleavage of the purified fusion protein, the erythroprotein portion of the molecule was found to have a lower molecular mass than the native protein. The difference in molecular mass was shown to be due to different types of glycosylation. Human granulocyte-macrophage colony-stimulating factor expressed in mouse milk was found to have the same glycosylation-derived size heterogeneity as the native protein. Expressing a human glycosyltransferase enzyme in mouse milk resulted in the production of not only the active enzyme but also of a series of novel glycoconjugates and milk oligosaccharides resulting from its *in vivo* activity (Prieto *et al.*, 1995). By varying the specificity of the glycosyltransferase it may be possible to use the mammary gland to synthesise different series of glycoconjugates and oligosaccharides.

(b) Phosphorylation

Bovine β-CN expressed in mouse milk was shown by tryptic digestion and mass spectrometry to have the correct polypeptide backbone and to be fully phosphorylated (Hitchin *et al.*, 1996). Phosphatase digestion was also used to demonstrate that transgenic bovine β-CN had the correct degree of phosphorylation (Jeng *et al.*, 1997). Bovine α_{s1}-CN expressed in mouse milk was indistinguishable from the native protein on the basis of specific immunoblotting and radioimmunoassay (Rijnkels *et al.*, 1997). Caprine κ-CN expressed in mouse milk was found to have four additional amino acid residues at its N-terminus, presumably as a result of incorrect cleavage of the signal peptide (Persuy *et al.*, 1995). A modified form of ovine β-lg in which the native gene had been altered to encode a casein kinase recognition

TABLE 17.3
Peptides detected by MALDI-TOF mass spectrometry in tryptic digests of transgenic bovine β-casein and authentic bovine β-casein, phenotypes A^1 and B

Measured peptide mass (Da)	*Identity of peptide (calculated mass: Da)*	*Transgenic β-casein*	*β-casein A^1*	*β-casein B*
1138.3	114–122 (1137.3)	–	–	+
1382.7	191–202 (1382.7)*	+	+	+
1768.1	53–68 (1769.0)*	+	–	–
2107.7	191–209 (2107.6)*	+	+	+
2184.5	184–202 (2185.5)	+	+	+
2910.3	184–209 (2909.5)	+	+	+
3123.1	1–25 (3123.3)§	+	+	+
5306.7	123–169 (5312.2)	–	–	+
5359.3	49–97 (5359.3)	+	+	+
6361.7	114–169 (6361.7)	+	+	–
7396.3	33–97 (7402.2)§	+	+	+

+ = detected; * = non-specific clevages; § = phosphopeptides.

site on an exposed loop of the molecule, was expressed in mouse milk and shown to be partially phosphorylated *in vivo* (McClenaghan *et al.*, 1999).

(c) γ-Carboxylation

In order to bind calcium, which is an essential cofactor, some plasma proteins possess clusters of carboxylated glutamic acid residues. The ability of the mammary gland to perform γ-carboxylations of these proteins is therefore crucial if they are to be expressed in milk in an active form, particularly since none of the endogenous milk proteins are modified in this fashion. Human protein C (hPC) has been expressed in transgenic mice but only trace amounts were γ-carboxylated and therefore active (Drohan *et al.*, 1994). In contrast, Yull *et al.* (1995) reported that most of the human factor IX recovered from transgenic mouse milk was active and therefore the γ-carboxylation process was relatively efficient.

(d) Proteolytic processing

Maturation of hPC precursor requires endoproteolytic cleavage at two sites in the molecule to remove the propeptide from the N-terminal and to convert the single chain to a heterodimer by excising a dipeptide. Most of the recombinant hPC expressed in the milk of transgenic swine was shown to be inefficiently processed by the endoproteases of the mammary epithelial cells (Lee *et al.*, 1995).

(e) Assembly of complexes

Assembly of transgenic protein complexes has also been achieved in milk. Fibrinogen is a heterodimeric glycoprotein composed of two subunits of three different polypeptide chains. Three expression cassettes, each encoding one of the polypeptide chains under the control of ovine β-lg promoter sequences, were co-injected into fertile mouse eggs (Prunkard *et al.*, 1996). The fibrinogen subunits were expressed in the milk at levels up to 2 mg mL^{-1} and in some animals up to 100% of the individual subunits were incorporated into active, fully-assembled hexamers.

17.4 Modification of the nutritional properties of milk

In industrialised countries, milk proteins are increasingly being consumed not in liquid milk but as components in processed foods ranging from cheese and yoghurt to low-fat spreads and sauces. Since the composition of bovine milk has evolved to enable it to be consumed directly, from the mammary gland by neonatal calves, it is not surprising that food technologists can suggest ways in which its processing behaviour could be improved (see Table 17.1 and reviews by Karatzas and Turner, 1997; Batt, 1997; Wall *et al.*, 1997; Murray, 1999).

Despite the technology being available to produce ruminants with modified milks, very few actual modifications have been made. This is largely due to the expense of producing large transgenic animals given the low success rate of the current transgenic techniques, their long gestation period and the time required for them to reach sexual maturity. Much of the reported research has therefore been performed on small animals such as the mouse, which produce milk very different from that of dairy animals. The amino acid sequences of the caseins of mouse milk, for example, are different from those of the corresponding proteins in ruminants and the protein content of mouse milk is two to three times higher than that of bovine milk. In addition, mouse milk contains no β-lg and very little α-la. The major whey protein in mouse milk, WAP, is not present in ruminant milks. While over-extrapolation of the effects of changes in mouse milk to the effect of similar changes in the milk of dairy animals is not wise, some of the changes which have been achieved have had interesting effects on the properties of the milk (Table 17.2).

17.4.1 Altering protein content and casein/whey protein ratio

It may be possible to increase the protein content of milk by inserting multiple copies of milk protein genes. Obviously, this depends on the full protein synthetic capacity of the mammary gland not already being utilised. Expression of high levels of ovine β-lg in mouse milk was achieved at the expense of endogenous protein production with the production of WAP being more suppressed than that of the caseins. No significant increase in the total protein content of the transgenic milk was detected (McClenaghan *et al.*, 1995). The suppression of endogenous protein production was matched by a decrease in the corresponding steady-state mRNA levels. In contrast, expression of human α-antitrypsin (hAAT) in ovine milk at a level up to 50 mg mL^{-1} was not achieved at the expense of endogenous milk proteins (Colman, 1996).

17.4.2 Changing lactose content

It has been estimated that approximately 70% of the adult human population has a deficiency of the intestinal enzyme, lactase, and therefore cannot digest lactose. This leads to a variety of intestinal problems arising from the malabsorption of lactose and an understandable tendency to avoid consuming milk. Reducing the lactose content of milk should therefore enable its consumption by some people who suffer from lactose intolerance. Since lactose also plays a major role in regulating the osmolarity of milk, reducing its concentration should also reduce the water content of the modified milk, increasing the solids contents and reducing transport and processing costs.

Two different genetic engineering approaches have been used in an attempt to reduce the lactose content of milk. Since α-la is involved in the

synthesis of lactose in milk, the most obvious method for reducing lactose content is by disrupting the α-la gene, thus preventing the expression of this protein. This has been achieved by homologous recombination in mouse embryonic stem cells (Stinnakre *et al.*, 1994). Homozygous mutant mice produced very viscous milk, rich in proteins and fat. Unfortunately, due to its viscosity, pups were unable to remove this milk from the mammary gland. Heterozygous mutant mice showed a 40% decrease in the level of α-la but only a 10–20% decrease in the lactose content of the milk. Stacey *et al.* (1995) observed the same effect when they deleted the murine α-la gene. However, when the endogenous murine α-la gene was replaced by the human α-la gene, the level of α-la detected in homozygous females, which is very low in the native strains, was found to be 14 times greater than in the native mice and these animals lactated normally.

An alternative method for obtaining low-lactose milk is to produce transgenic mice expressing rat intestinal lactase in both the apical side of mammary alveolar cells and the outer membrane of fat globules in the milk (Jost *et al.*, 1999). This enzyme, which hydrolyses lactose to glucose and galactose, reduced the lactose content of the milk by up to 85% with no effect on fat or protein content or on pup development. The authors proposed that if the same technique was applied to dairy cattle, the low-lactose milk would satisfy the needs of lactose maldigesters since a 50 to 70% decrease in lactose content is sufficient to prevent the intestinal disorders associated with this condition.

Bovine α-la has been expressed in the milk of transgenic pigs in order to determine the effect of over-production of α-la on milk production and piglet growth (Bleck *et al.*, 1998). Levels of bovine α-la as high as 0.9 g L^{-1} were attained, resulting in a 50% increase in the total level of α-la produced throughout a lactation. The concentration of bovine α-la varied, being highest in the early stages of lactation; the ratio of the bovine and porcine α-la also changed throughout the lactation; indicating that the transgene and the endogenous gene were under different control elements. Although there was a 3.8% increase in the lactose content of the transgenic milks at day 0 of lactation (which may indicate an increase in milk output), by days 5 and 10, the difference in lactose content between transgenic and control milks was no longer significant. The authors speculated that if the increase in lactose concentration at the start of lactation was accompanied by an increase in milk production, this might lead to more efficient growth of offspring.

17.4.3 Changes in micellar properties

Milk is not simply a solution of proteins and salts. Under physiological conditions, the caseins and much of the calcium and phosphate are

organised into colloidal particles, the casein micelles. The micelles in milk are stabilised by a layer of κ-CN and there is a negative correlation between κ-CN content and micellar size. It has been proposed that if the relative concentration of κ-CN could be increased by inserting multiple copies of this gene, the effect would be to reduce the mean diameter of the micelles. This should increase the heat stability of the milk during processing and produce cheese with a firmer texture. Bovine κ-CN has been expressed in mouse milk with the mean concentration in individual lines being as high as 3.8 mg mL^{-1} (Gutierrez-Adan *et al.*, 1996). This resulted in a decrease in the range of mean micelle diameters from 250–274 nm in the controls to 141–211 nm in homozygous lines. There was no effect on rennet coagulation time, but the curd formed from the transgenic milks was significantly stronger than that produced from control milk. The total protein content of the milk was not affected, indicating that the synthesis of the bovine κ-CN was at the expense of other endogenous proteins. A relative increase in the κ-CN content of mouse milk was achieved by disrupting the β-CN gene by gene targeting in embryonic stem cells (Kumar *et al.*, 1994). This resulted in a decrease in both the total casein and total protein content of the β-CN-deficient milks. However, since β-CN was absent from the milk of homozygous animals, the proportion of κ-CN increased and the mean micelle diameter decreased by 10%. The antimicrobial enzyme, lysozyme, is present in human milk at a concentration of 400 μg mL^{-1} compared to 0.13 μg mL^{-1} in cows' milk. Expressing human lysozyme in mouse milk at a concentration ranging from 0.25 to 0.71 μg mL^{-1} caused a decrease in mean micelle size from 172 to 157 nm, a 35% decrease in rennet clotting tune and a 2.5 to 5-fold increase in gel strength (Maga *et al.*, 1995). The authors speculated that these changes were due to the association of the positively charged lysozyme with the negatively charged micelles which would reduce electrostatic repulsion between individual micelles.

Bovine β-CN has been expressed in mouse milk under the control of both ovine β-lg and bovine α-la 5'-flanking regions. In the former case, the protein was shown to be incorporated into the micelles and the appearance of the milk and the development of the pups were normal (Hitchin *et al.*, 1996). In the latter case, the transgenic milk was very viscous and lactation stopped at between 1 and 18 days post-partum, depending on the concentration of the bovine protein (Bleck *et al.*, 1995). This may have been due to some, or all, of the transgenic protein being non-micellar, but this was not investigated.

17.4.4 Improving infant formulae

The composition of bovine milk has evolved to act as the optimum food for the development of calves, rather than for human neonates. A great deal of effort has therefore been applied to adapting the composition of infant

formulae based on bovine milk, in order to promote growth and development equivalent to that in infants fed on human milk. A variety of ways for improving the composition of infant formula have been suggested, particularly for the nutrition of premature infants and those with special dietary requirements as a result of allergic, inflammatory and metabolic disorders (see review by Lo and Kleinman, 1996). These include incorporation of antibodies and cytokines, increasing the level of lactoferrin, lysozyme and metallothionein and the removal of allergenic epitopes from proteins. Some of these changes may be achievable through the use of genetic engineering.

In an attempt to provide passive immunisation against enteric infections, transgenic mice secreting a monoclonal antibody (Mab) against transmissible gastroenteritis coronavirus (TGEV) were generated (Catilla *et al.*, 1998). The Mab was a chimera consisting of the constant modules of a human IgG and the variable modules of the murine TGEV-specific Mab 6A.C3 expressed under the control of WAP regulatory sequences. Antibody expression titers up to 10^6 that reduced TGEV infectivity by 10^6-fold were measured in some transgenic milks. Antibody levels were independent of transgene copy number but were related to the site of integration.

Human lysozyme has been expressed in mouse milk at a concentration up to 0.7 μg mL^{-1} (Maga *et al.*, 1995). The transgenic protein was shown to have antimicrobial activity. Human lactoferrin has been successfully expressed in mouse milk under the control of regulatory elements of the bovine α_{s1}-CN gene (Nuijens *et al.*, 1997). The transgenic protein was 90% saturated with iron whereas natural lactoferrin is only 3% saturated. The transgenic protein was immunologically identical to the natural protein, as were other properties such as iron release and ligand binding.

The hypothesis that milk-borne growth factors might stimulate gastrointestinal growth and development was tested by determining the growth of pups suckling from mice expressing des(1–3) Human Insulin-Like Growth Factor-1 (hIGF-1) in their milk (Burrin *et al.*, 1999). Although the concentration of des(1–3) hIGF-1 was 40- to 200-fold higher than mouse IGF, there was little evidence of stimulation of growth or of the transgenic protein being adsorbed into the circulation after ingestion of the milk.

17.5 THE MAMMARY GLAND AS A FACTORY FOR THE MANUFACTURE OF THERAPEUTIC PROTEINS

A pharmacologically active protein, human tissue plasminogen activator, has been produced in mouse milk (Gordon *et al.*, 1987). Since then, a variety of other proteins with potential pharmaceutical interest have been expressed in the milk of different species and a new industry has been created based on this technology (see reviews by Houdebine, 1994 and Clark, 1998).

17.5.1 Choice of species

Whereas food proteins are high volume, low value products, therapeutic proteins are low volume and high value. Two factors need to be considered when deciding which species of animal to use for the expression of therapeutic proteins. These are the demand for the protein and the cost of producing the transgenic animal. The mouse, the species on which most research in this area has been performed, has the advantages of small size (reducing feeding and housing costs), short generation time and large numbers of pups in each litter. The major disadvantages are that a typical female produces less than 1 mL of milk per milking and repeated milking is very difficult. It has been estimated that the production of 100 kg of a transgenic protein which is expressed in milk at a concentration of 1 mg mL^{-1} would require 6,000,000 mice, 2000 rabbits, 200 sheep or 10 cows (Lee and de Boer, 1994). However, the cost of producing a transgenic cow has been estimated to be about $300,000, with the delay time from microinjection to first milking being 2 years compared to 3 months for the mouse. The world requirement for some therapeutic proteins is very low and large-scale production is unnecessary.

17.5.2 Expression of therapeutic proteins

Pharmaceutical proteins which have been expressed in milk are listed in Table 17.3, although this is not exhaustive. As expected, the most popular host is, again, the mouse, although the rabbit, sheep and pig have been used also. Most of the transgenic animals have been produced by pro-nuclear injection although antithrombin III and human factor IX have been produced in goats (Baguisi *et al.*, 1999) and sheep (Schnieke *et al.*, 1997), respectively, using the nuclear transfer cloning technique.

Expression levels of therapeutic proteins are very variable, ranging from approximately 3 μg to 33 mg mL^{-1} and higher in the case of hAAT expressed in sheep (Carver *et al.*, 1993). Generally, transgenic proteins were found to be active although some problems have been reported with respect to the authenticity of their post-translational modifications (see Section 17.3.3). Some proteins, such as erythropoietin, have been expressed as fusion proteins in order to reduce their activity and prevent side effects caused by ectopic expression or leakage from the mammary gland (Korhonen *et al.*, 1997). These proteins require specific proteolytic cleavage after collecting the milk to produce an active protein. As mentioned previously, hPC, a vitamin K-dependent anti-coagulant plasma protein, has been expressed in the milk of transgenic mice and pigs (Drohan *et al.*, 1994). The precursor protein undergoes a variety of post-translational modifications during maturation, including glycosylation, β-hydroxylation, γ-carboxylation and proteolytic processing prior to forming the active heterodimer. Since the mammary

epithelial cells perform the proteolytic processing inefficiently (Lee *et al.*, 1995), the hPC precursor was co-expressed with human furin in mouse milk (Paleyanda *et al.*, 1997). Furin is an enzyme which cleaves paired basic amino acids from proteins and is involved in proteolytic processing of proteins in the secretory pathway. Human furin, both as the expected intracellular form and also a smaller secreted form, was detected at levels more than 100 times greater than the corresponding endogenous mouse enzyme. Whereas most of the hPC in the original transgenic mice consisted of the unproteolysed precursor, the hPC in the mice expressing human furin was processed to the mature, active protein.

17.6 Future developments

While the dream of herds of transgenic cows producing modified milks for specific manufactured food products is still some way from reality, it is potentially achievable. The success rate of producing transgenic animals is increasing and costs are decreasing. However, the political problems associated with attaining this goal may well outweigh the scientific ones. Will the idea of genetically modified milks produced by cloned animals prove acceptable to the consumer? At the time of writing, genetically modified crops such as maize and soya bean are a topic of great debate within the UK and elsewhere. Some pressure groups are calling for their cultivation, even on well-defined tests sites, to be banned and foods containing material derived from such crops to be clearly labelled. An increasing number of manufacturers are seeking, under consumer pressure, guarantees from their suppliers that raw materials are not derived from these sources. Whether milk, a product that has long benefited from its natural, wholesome image, would continue to do so if it underwent any form of genetic modification of the types suggested is debatable.

The production of therapeutic proteins in milk is politically much less contentious. Producing these proteins in transgenic milk rather than by fermentation or cell culture is considerably less demanding, both financially and environmentally when feeding, waste disposal and product isolation costs are considered. A number of these proteins were at various stages of clinical evaluation in early 2000. Human lactoferrin, for heparin neutralisation, was expected to start Phase II clinical trials. hAAT, produced in kilogram-sized batches from milk obtained from flocks of sheep, had almost completed Phase II trials for the treatment of cystic fibrosis and congenital emphysema which affects one child in every 2000 in the Western Hemisphere. Human alpha-glucosidase was undergoing Phase II trials for the treatment of Pompe's disease, a lethal lysosomal storage disorder. Recombinant human antithrombin III, which helps regulate blood clotting, was in Phase III trials. If these products are successful, more will

undoubtedly follow. It will be interesting to see the status of both these areas by the time the next edition of this book is published.

ACKNOWLEDGEMENTS

Core funding for the Hannah Research Institute is provided by The Scottish Executive Rural Affairs Department.

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