M ost of us think of milk as a good source of nutrition, especially in early childhood. Advances in dairy technology have continuously improved milk yields without altering the product’s composition. But the use of transgenics as an expression technology for producing recombinant proteins in milk is beginning to transform that traditional industry. A number of companies are actively engaged in preclinical and clinical trials with therapeutic proteins derived from the milk of transgenic mammals (e.g., antithrombin III, certain monoclonal antibodies, and Factor VIII).

Transgenic mammals offer the ability to produce significant amounts of protein at greater expression levels (1 g/L has been achieved) and volume output than traditional fermentation and cell culture systems. The unique nature of the mammary gland for production of complex molecules allows it to express human-like proteins. And a significant reduction of the cost-per-unit is possible because the transgenic “bioreactor” requires fewer inputs (raw materials) and less complex monitoring and support systems than a stainless steel fermentation system.

Although gene expression and heterologous protein production is possible in many different animal tissues and fluids (e.g., blood, urine, or semen), this article highlights transgenic milk production because it is at present the most feasible and farthest along in development and regulatory processes. With that in mind, I am focusing on the composition of milk and the approaches taken to purify a recombinant protein from such a complex mixture.

DEFINING THE ISSUE
Transgenic companies working with milk production systems have used a number of animal models. Most small-scale studies have been completed in mice and rabbits, but such small animals are usually inappropriate for production scale. The most popular transgenic bioreactors to date have been pigs, cows, goats, and sheep. The genetic construct consists of both a DNA sequence encoding the protein of interest and a milk whey protein gene sequence to direct expression of that protein to mammary glands in adult females. Obtaining commercially viable amounts of a recombinant product from the milk of transgenic livestock is challenging because milk is a complex, multiphasic, and colloidal
Milk Composition: Cow milk is about 87% water, 4–5% fat, 5% carbohydrate, and 3–4% protein. Goat’s milk and sheep’s milk have lower fat content but higher protein content. Lactose is the major carbohydrate in the milk of most species — and the least variable component of milk.

The fat component is a complex mixture of lipids secreted as globules primarily composed of a triglyceride surrounded by a lipid bilayer membrane, which helps to stabilize those fat globules in an emulsion within the aqueous environment of milk. More than 95% of total milk lipids are in the form of globules ranging from 0.1 to 15 µm in diameter. These liquid fat droplets are covered by a thin membrane, 8–10 nm thick, with properties completely different from both milk fat and plasma. The native fat globule membrane (FGM) is an apical plasma membrane of the secretory cell that continually envelopes the lipid droplets as they pass into the lumen. The major components of that native FGM, therefore, are protein and phospholipids.

The major milk protein is casein. The principal casein fractions are \( \alpha(s1) \) and \( \alpha(s2) \) caseins, \( \beta \)-casein, and \( \kappa \)-casein. The distinguishing property of all caseins is their low solubility at pH 4.6. A common compositional factor is that caseins are conjugated proteins, most with phosphate group(s) esterified to serine residues. Most if not all are found within a structure called a micelle. Its biological function is to carry large amounts of highly insoluble calcium phosphate to mammalian young in liquid form and to form a clot in the stomach for more efficient nutrition. Micelles are colloidal molecules with hydrophobic cores and casein-enriched surfaces held loosely together by calcium phosphate molecules. They form large aggregates with diameters of 90–150 nm. These aggregates are porous structures occupying about 4 mL/g and 6–12% of the total volume fraction of milk. The micelle structure also contains minerals, amino acids, and bioactive peptides.

Submicelle Models: Several researchers and technicians ascribe to what has been dubbed the “casein submicelle” model (Figure 1), but it has not yet received universal acceptance. Evidence is mounting to suggest that there is no defined submicellar structure to the micelle at all (1). In the submicelle model, the core of a micelle is built of submicelles: roughly spherical units of about 14-nm diameter, which are fairly tightly aggregated. Small aggregates containing 10–100 whole casein molecules are thought to be of two different types: those with and without \( \kappa \)-casein. The submicelles contain a hydrophobic core covered by a hydrophilic coating, which at least partly comprises the polar moieties of \( \kappa \)-casein. The hydrophilic part of the \( \kappa \)-casein is thought to exist as a flexible hair.

An alternative model describes a more open structure, suggesting that there are more and less dense regions within the micelle, but there is a less well-defined structure. In this model, calcium phosphate nanoclusters bind caseins and provide for the differences in density within the casein micelle. The calcium phosphate acts as cement among hundreds or even thousands of submicelles forming the casein micelle. Binding may be covalent or electrostatic. Submicelles rich in \( \kappa \)-casein occupy a surface position, whereas those with less of it are buried in the interior. The resulting hairy layer, at least 7 nm thick, acts to prohibit further aggregation of submicelles by steric repulsion.

Casein micelles and fat globules behave as separate phases, preventing filtration of the milk and interfering with the usual separation methods. A typical first stage in milk purification is removal of the fat by low-speed centrifugation (about 5,000–10,000 g), resulting in a layer of cream at the top, an aqueous supernatant middle layer, and a small pellet of leukocytes and other debris at the bottom of the centrifuge tube. That aqueous supernatant is sometimes called the plasma phase. Ultracentrifugation (usually about 50,000 g or greater) will cause pelleting of the casein micelles and produce a supernatant called whey, which contains the water, lactose, and soluble noncasein proteins.

Milk has many whey proteins, and the specific set found in mammary secretions varies with the species and the stage of lactation. The major whey proteins in cow’s milk are \( \beta \)-lactoglobulin and...
α-lactalbumin. Others are serum albumin and the immunoglobulins. Whey proteins also include a long list of enzymes, hormones, growth factors, nutrient transporters, and disease-resistance factors. If the product protein tends to associate with either the fat or micelles, purification may be simplified, but this scheme is rare. Casein molecules can be separated from whey by precipitating out the casein with acid (a slow addition of 0.1-N HCl to lower the milk pH to 4.6) or by disrupting the micellar structure using partial hydrolysis of the protein molecules with a proteolytic enzyme such as chymosin. However, those methods can result in product losses as high as 40–60%, leading to significantly lower overall yields (5–25%) and low biological activity.

**Solutions to the Problem**

A new tool that could assist in tackling the association of protein molecules with casein micelles is BioSante’s calcium phosphate-based (CAP) particle technology. It claims to produce a casein-free product stream with >90% yield of the target protein available for further chromatographic processing. The process consists of four steps: removal of fat by centrifugation (>90% yield); dilution and clarification of the skimmed milk using ethylenediaminetetraacetic acid (EDTA); addition of CAP particles in clarified skim milk to reform casein micelles away from the target protein; and removal of casein complexes and some endogenous milk proteins by centrifugation. The company claims that 65–75% overall yields of pharmaceutical-grade purity can be achieved.

BioSante believes that its newly patented technology offers a less expensive initial purification process for transgenically produced therapeutic proteins, producing a casein-free and low-protein mixture in two steps with 90% yield of the drug protein. This suggests a need for fewer subsequent chromatography steps to achieve the desired product purity. The system is designed to be easily scalable up to large pharmaceutical operations. And the company maintains that a greater product yield at initial purification steps will contribute to higher overall yields of the drug protein.

**PPL Therapeutics Ltd.**’s purification method (2) for the purification of its lead transgenic product α-1 proteinase inhibitor (α1-AT) incorporates nine steps. The recombinant protein is expressed at a concentration of 10–12 mg/mL in sheep’s milk, and the overall process yield is 44%. First, fat is removed by centrifugation (90% yield). PEG precipitation of the skimmed milk removes caseins by centrifugation (68% yield). And PEG precipitation is also performed on the casein-free whey fraction (volume reduction), which contains whey proteins and α1-AT (65% yield). Five sequential chromatography steps follow: cation- and anion-exchange, immunoaffinity with immobilized Protein G, immobilized metal (Ni) affinity (IMAC), and hydrophobic interaction chromatographies (HIC) (44% yield).

Notably, 30% of the overall product loss occurs during casein precipitation. When the same method was applied to fibrinogen (3), the recombinant product was found to coprecipitate with the casein and was very quickly proteolytically damaged as well, probably due to coprecipitation with protease enzymes. The company’s solution was to disrupt association between the casein micelles and proteolytic enzymes using lysine, an analogue (e.g., 6-aminohexanoic acid), or other basic amino acids in the 10–200 mM range.

**GTC Biotherapeutics** developed a tangential-flow filtration method (4) to isolate its target protein from transgenic goat’s milk. Unlike with previous isolation methods, this one eliminates the need for a fractionation step to remove fat and casein micelles, thereby simplifying the process and avoiding costly losses of recovery and bioactivity. The procedure can be operated as a closed-loop continuous extraction system by adding a sufficient volume of solution to the retentate continually, maintaining a constant volume as the permeate is removed.

The GTC process consists of optional dilution of raw milk with a chelating agent (such as EDTA), which improves permeate flow; clarification by tangential flow filtration across an ultrafiltration membrane to remove caseins, fat, viruses, bacteria and somatic cells; collection of permeate; a series of chromatography steps for further purification; and a final clean-up step by ultrafiltration to prepare a pharmaceutical-grade therapeutic product.

In practice, recovery rates for antithrombin III after seven sample passes were 75–90%. GTC expects to file for European market authorization in the first half of 2004 for the use of recombinant human antithrombin III derived from transgenic milk using this process to treat patients with hereditary antithrombin deficiency. If approved, this would be the first transgenically derived product to be approved in Europe.

| Table 1: Breakdown of three purification processes for transgenic milk |
|------------------|------------------|------------------|------------------|
| **Step**         | **BioSante**     | **PPL**          | **GTC**          |
| Fat removal      | Centrifugation   | Centrifugation   | EDTA             |
| Casein removal   | EDTA             | PEG precipitation| Tangential-flow   |
|                  | CAP particles    | Centrifugation   | filtration       |
|                  | Centrifugation   | PEG precipitation|                   |
|                  | centrefugation   |                   |                   |
| Product clean-up | Chromatography   | Chromatography   | Chromatography   |
MORE CHALLENGES AHEAD

Other issues affect the overall yield of any manufacturing process involving transgenic mammals: stability of constructs, control of expression, and seasonal variations in lactation, to name a few. A sound understanding of the health and physiology of the livestock species used is essential because a transgenic animal may live for 7–10 years and will experience physiological changes and various environments throughout its life as it develops, gives birth, and ultimately lactates (5). Recovery processes must be robust enough to handle those changes, but if the advances in our understanding of milk composition and bioprocessing techniques continue, such challenges should be overcome as well.

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Merlin Goldman runs Magnetical ETTC, Mayfield Road, King’s Buildings, Edinburgh, Midlothian, EH9 3JL, +44-131-472-4704, info@magnetical.com. He is a member of the European Society for Animal Cell Technology (newsletter coeditor), a founding member of the Scottish Branch of the Society of Chemical Industries, and an associate member of the Institute of Chemical Engineers.