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Current Opinion in Biotechnology

Can recombinant milk proteins replace those produced by animals?

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The consumption of animal proteins in general, and dairy proteins in particular, is associated with sustainability and animal welfare issues. Recombinant synthesis of milk proteins is therefore receiving increasing interest, with several studies showing synthesis of milk proteins using a wide range of expression systems. Achieving a high yield and purity is essential for economic production. Besides the synthesis, also the construction of the specific structure in which milk proteins are present in animal milks, casein micelles, is needed. Looking at the current state-of-the-art, the steps to produce recombinant dairy products are technically feasible, but whether it can be implemented at low cost, with the process being environmentally friendly, remains to be seen in the coming years.

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Current Opinion in Biotechnology 2022, **75**:1–6

This review comes from a themed issue on **Food biotechnology**

Edited by **Ilje Pikaar**, **Tim Huelsen** and **Lars Angenent**

https://doi.org/10.1016/j.copbio.2022.102690

Introduction

Animal source food products play an important role in diets worldwide, supplying on average 17% of the energy and 35% of the protein in the diet [1] with a higher intake in Western countries. This high intake of animal source food products is an important sustainability issue, as livestock is responsible for 18% of global greenhouse gas (GHG) emissions [2]. Milk production is an important contributor to the overall GHG emissions [3] and land use [4] within the livestock sector. Therefore, in both academia and food industry there are many initiatives that aim at replacing dairy foods by plant-based products. [5]. These plant-based products are, however, generally unable to replace the nutritional quality of dairy products, as dairy products are a source of high quality protein and many micronutrients [6]. In addition, the physicochemical functionality (e.g. solubility, gelling ability, and emulsifying properties) of plant-based proteins is often inferior to those of milk proteins [7]. Because of these difficulties in replacing milk proteins by plant-based proteins, synthesizing nature-identical milk proteins would be a possible solution. Different companies have recently started to research the production of dairy products based on recombinant milk protein synthesis [8]. This review will provide an overview of the properties of relevant milk proteins, the current state-of-the-art in recombinant milk protein synthesis, as well as future research challenges before animal-free dairy products can be produced.

Milk protein composition and functionality

Bovine milk contains hundreds of different proteins among which six are the most abundant; four types of caseins and two whey proteins. The two whey proteins (α-lactalbumin and β-lactoglobulin) represent approximately 15% of the total milk protein. Both these whey proteins are globular proteins that are highly soluble and sensitive to heat treatment. In milk, they play an important nutritional role by providing essential amino acids. Approximately 80% of the total bovine milk protein content is composed of the four caseins (α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein) [9]. These caseins occur in many genetic variants, where specific amino acid replacements occur that may change protein functionality [10,11], as shown in Table 1. These four caseins are assembled into supramolecular protein structures called casein micelles (Figure 1), consisting of thousands of individual casein molecules together with calcium salts in the form of calcium phosphate nanoclusters [12,13].

The different caseins are phosphorylated and/or glycosylated to different extents with variability in the extent of modification (Table 1). Phosphorylation of caseins is catalyzed by kinase enzymes that attach a phosphate group to the amino acids residues Ser and Thr, in a Ser/Thr-Xxx-Glu/pSer/Asp motif. Whereas glycans can be linked at Thr residues of κ-casein by glycosyltransferase enzymes via *O*-glycosidic bonds, with mono-saccharides, di-saccharides, tri-saccharides and tetra-saccharides being attached [10]. The phosphorylation plays an important role in the interaction with calcium salts, whereas the glycosylation plays an important role in the stabilization of the micelles [10].

The internal structure of the casein micelle consists of a matrix of all caseins with calcium phosphate nanoclusters. The calcium phosphate plays an important role in the

Table 1

Overview of the number of known milk protein genetic variants, and the variation in the degree of phosphorylation and glycosylation of the four different caseins that exist in bovine milk [11]

Protein	Genetic variants	Phosphorylation	Glycosylation
α_{s1}-casein	7	9–10	
α_{s2}-casein	3	10–13	
β-casein	12	5	
κ-casein	11	1–2	0–6

structural integrity of the casein micelle [13,14•]. The surface of the casein micelle consists of a hairy layer of κ-casein, that provides stabilization of the micelles in milk. During cheese making, the hairy layer of κ-casein is proteolytically cleaved, which destabilizes the micelles and thereby causes aggregation into a protein network [9]. To make animal-free dairy products, it would thus be required to synthesize caseins, have them post-translationally modified, as well as assembled into micelles.

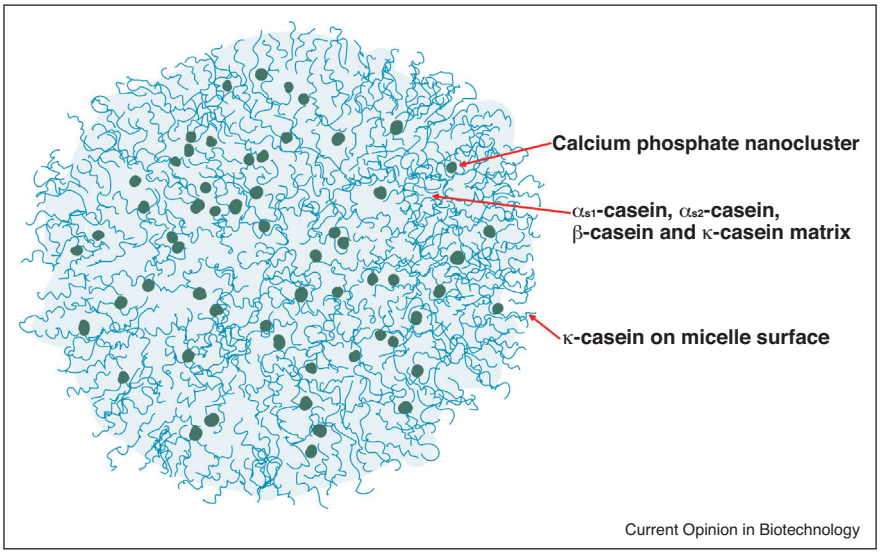
Production of recombinant milk proteins

Recombinant proteins are products of recombinant DNA technology which encompasses the insertion of a genetically modified protein gene into the cell of a host organism. This technology is already extensively used for many recombinant proteins for medical purposes [15], for example, to human-identical proteins and hormones, as well as for use in food processing, for example, to produce specific enzymes [16]. However, such recombinant protein synthesis has not been used to produce the bulk proteins constituting food products. With cost declining and technology improving, mass production of the major food proteins, such as milk proteins, is expected to become feasible. For example, with current prices for casein being around €10 per kg [17], which is similar to recombinant proteins produced for industrial purposes, such as cellulases by Trichoderma [18,19], recombinant synthesis of milk proteins may indeed become economically feasible. This economic feasibility is confirmed by modelling studies on recombinant fungal production of enzymes [19], and on bacterial production of the whey protein α-lactalbumin [20].

Recombinant production of proteins has been shown for all classes of micro-organisms, including, for example, the bacterium *Escherichia coli* [21•], the yeast *Pichia pastoris* [22], and the filamentous fungus *Rhizopus* [23]. The choice of micro-organism for protein production has important implications for yield, purity, and post-translation modifications of the synthesized protein, as reviewed elsewhere [24]. For producing milk proteins, all these aspects are relevant, as a low yield and/or low purity would make mass production of food proteins uneconomical, whereas the post-translation modifications are important to achieve the correct techno-functional properties.

Previous studies on recombinant milk protein synthesis were mostly based on the bacterium *E. coli*. For example the whey proteins α-lactalbumin [25] and β-lactoglobulin [21•,26] as well as α_{s1}-casein [27,28], β-casein [29], and κ-casein [30] have all been synthesized this way. Modifying bacteria is generally cheap and easy to perform, but leads to relatively low yield and complex purification [31••]. Bacterial recombinant protein synthesis generally leads to proteins that are not post-translationally modified

Figure 1



Schematic representation of a casein micelle.

[29,31••], as was shown in a study by the absence of phosphorylation on recombinant human β-casein and bovine β-lactoglobulin [29,32]. The synthesis of recombinant β-lactoglobulin, which is not post-translationally modified in its bovine form, has been shown to lead to a protein with similar folding and functionality [21•,32].

Yeast-based secretion of milk proteins has been performed with both *Saccharomyces cerevisiae* and *P. pastoris*. Only one study reported the production of α_{s1}-casein [33], whereas other studies were on the whey proteins α-lactalbumin [34] and β-lactoglobulin [35,36]. Although yeast-based secretion generally leads to higher yields compared to bacterial protein production [31••], it often also leads to additional post-translational modifications that do not occur in regular milk proteins, such as extensive mannosylation of β-lactoglobulin [34,36], changing the functionality of the synthesized milk proteins. For example, the formation of casein micelles may be hindered by the presence of glycosylation on the caseins, although the specific glycosylation of the C terminal domain of κ-casein is important for its functionality [10]. *P. pastoris* usually leads to lower levels of glycosylation than *S. cerevisiae* [31••].

Synthesis of milk proteins by filamentous fungi has hardly been reported, with the only example being production of lactoferrin [37]. In general, fungal synthesis usually leads to the highest yields, although this strongly depends on protein to be expressed [31••]. One disadvantage of fungal synthesis is the common co-secretion of proteases that may lead to proteolytic damage to the produced proteins [23]. Knowing that the caseins are very sensitive to proteolytic cleavage [9], this may be a possible challenge of using fungal synthesis for milk proteins. Still, taking into account that for food proteins the production of high yields at low cost is required, fungal synthesis may be the most economical route [18,38•].

Most of these published studies on recombinant milk protein synthesis have been performed between 10 and 30 years ago. That work was mostly based on scientific curiosity, without the aim of application in food production. However, due to tremendous progress in protein expression technologies, leading to lower cost of recombinant proteins, this has become of interest for food production. Recent research in this field is mainly driven by start-ups such as Perfect Day, New Culture, Formo, Those Vegan Cowboys, and others [8]. Of these, only Perfect Day has regulatory approval for its whey protein and has an actual product on the market. What all these companies have in common is that they aim to synthesize milk proteins, with most focusing specifically on caseins. Both information provided by companies, as well as some of their patents [39,40•] show that they focus on several hosts for recombinant protein synthesis, but especially yeast and fungi.

For recombinant milk protein synthesis, independent of the microbial specie used, both the human and bovine milk protein genes have been used as the DNA template. The use of human genes may allow the synthesis of proteins for infant formula, as these proteins may lead to a product that is more similar to human milk [41]. Using bovine genes as a DNA template allows the production of animal-free dairy products similar to those based on bovine milk. This also allows the selection of specific genetic variants of the milk proteins, which may be advantageous for the making of for example cheese, where specific genetic variants have been shown to lead to better properties [42].

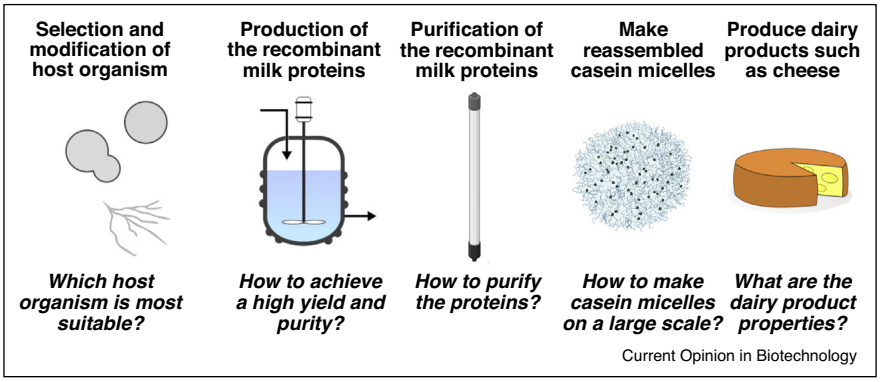
The DNA template for the chosen milk protein then needs to be included in a host-specific vector. This vector is composed of a promotor, which can either be inducible or constitutive, a signal peptide, that is either host-specific or part of the milk protein gene, then the DNA encoding the milk protein itself, and finally a termination element [38•,43,44•,45]. An often used vector is a plasmid, which can either be self-replicating or be integrated in the host genome at a desired location, with the last option often being preferred [43,44•,46]. As mentioned earlier, the post-translational modification of caseins is essential to its functionality. For the specific phosphorylation patterns, co-expression of casein kinase would be the most logical approach to achieve this, with other recombinant kinases being possible alternatives [47]. Replicating the specific glycosylation pattern of animal κ-casein may be more challenging, although site-directed mutagenesis may be used to achieve this [48]. Finally, for the prevention of proteolytic cleavage of the produced protein, which is a high risk for the unstructured casein molecules, knocking-out proteases in the host organism may be required [38•,43,45,49].

After the milk proteins have been produced using the above described recombinant production systems, they need to be isolated with sufficient purity before dairy products can be produced. This downstream processing may vary in complexity, which is relevant for the overall economic feasibility. It is currently unknown how high the purity of the proteins isolated during downstream processing needs to be, to achieve a proper balance between cost and functionality of the milk proteins.

Production of dairy products based on recombinant milk proteins

As explained in the section on milk protein composition and functionality, the casein micelle structure is essential for many dairy products. Casein would first need to be isolated, either as single caseins or as mixed casein

Figure 2



overview of the different stages of the production process of animal-free dairy products, as well as well as the associated research challenges.

fractions followed by a separate casein micelle formation process. Several procedures exist for the production of reassembled casein micelles from solubilized caseins [13,50,51]. These all have in common that calcium and phosphate, often in combination with other ions, are added to a solution of caseins.

Not all four caseins may be required as, for example, human milk lacks α_{s2}-casein [41] and elephant milk does not contain either of the α-caseins [52]. A previous study showed that using only (human) β-casein and (bovine) κ-casein was sufficient to produce reassembled casein micelles [53]. The precise number and concentration of the salts to be added is relevant, as next to calcium and phosphate also magnesium and citrate may be needed [54], as these are also present in regular casein micelles [9]. Tprocedure for pH control differs between direct pH adjustment by NaOH [55] whereas also urea combined with urease has been used for a more gradual pH change [51].

For the production of reassembled casein micelles, the cost and complexity of the process is relevant for its application. More complex procedures may lead to micelles that are more similar to those occurring in regular milk. However, if less complex procedures lead to reassembled casein micelles that are functionally sufficiently similar to bovine casein micelles, this may be preferred.

Future research perspective

As described in this review, the recombinant synthesis of recombinant milk proteins, the formation of reassembled casein micelles, and the production of dairy products have separately been shown to be possible. However, to be able to make such recombinant dairy products on an industrial level, these different elements need to be combined. For example, although the synthesis of reassembled casein micelles has been shown, the ability of making such micelles from recombinant caseins has not been shown. Figure 2 shows the different stages of the production process, as well as the research challenges that need to be solved before animal-free dairy products can be made.

In addition to studying the ability of linking all steps from microbial milk protein synthesis to dairy products, a number of specific issues can be studied to achieve optimal production of animal-free dairy products:

1) How can the post-translational modifications of the milk proteins be optimized to have the required functionality of the recombinant milk proteins?
2) What is the environmental impact of the overall process and how does this compare to regular bovine milk products?

Finally, the production of animal-free dairy products also depends on legislation and societal acceptance. Milk proteins produced using recombinant synthesis would be seen as novel food, and therefore require specific regulatory approval. In addition, the societal acceptance is also something that is unsure: how will consumers perceive this type of products?

In conclusion, looking at the current state-of-the-art, the separate steps of producing of animal-free dairy products using recombinant synthesis are technically feasible, but whether it can be achieved at such a low cost and at a low ecological footprint that it will be economically and environmentally sustainable is to be shown in the coming years.

Conflict of interest statement

Nothing declared.

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