MILK COMPONENTS IN FOOD, NOVEL FOOD AND NON-FOOD

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Proceedings of the symposium "Milk components in food, novel food and non-food", February 27, 1991, Wageningen

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Agricultural Research
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SYMPOSIUM

MILK COMPONENTS IN FOOD, NOVEL FOOD AND NON-FOOD *)

(Under the auspices of the Netherlands National Council for Agricultural Research)

Date : February 27, 1991
Location: International Agricultural Center (I.A.C.)
Lawickse Allee 11, 6701 AN Wageningen,
the Netherlands (phone 08370-90133)
Chairman: Dr. J.E. Mellema

ORDER OF THE DAY

09.30 h. a.m. : ***RECEPTION*** of participants

10.00 h. a.m. : ***OPENING*** by Dr. J.E. Mellema, Coberco Zutphen (NL).

10.05 h. a.m. : ***THE NEED FOR AGRIFICATION IN THE DAIRY FIELD***
Prof. dr. C.P. Veerman, President of the National Coöperative Council for Agriculture and Horticulture of the Netherlands, Rijswijk, the Netherlands.

10.35 h. a.m. : ***MODIFYING THE COMPOSITION OF RAW COW MILK BY CHANGES IN THE GENETIC MATERIAL***
Prof. dr. R.D. Bremel, University of Wisconsin, Madison, U.S.A.

11.05 h. a.m. : ***REFRESHMENTS***

11.30 h. a.m. : ***MODIFICATIONS OF MILK PROTEINS AND THE POSSIBLE APPLICATIONS***
Dr. R.J. Siezen, NIZO, Ede, the Netherlands.

12.00 h. p.m. : ***BIOLOGICALLY ACTIVE PEPTIDES FROM MILK PROTEINS***
Prof. dr. P. Jollès, Université de Paris V, Paris, France.

12.30 h. p.m. : ***LUNCH*** at the canteen of the I.A.C.

13.30 h. p.m. : ***FUNCTIONAL PROPERTIES OF MILK PROTEINS***
Prof. dr. C.V. Morr, Ohio State University, Columbus, Ohio, U.S.A.

14.00 h. p.m. : ***FERMENTATIVE PRODUCTION AND APPLICATIONS OF LOW-MOLECULAR COMPOUNDS FROM LACTOSE (WHEY)***
Drs. H. Veringa, NIZO, Ede, the Netherlands.

14.30 h. p.m. : ***THE POTENTIAL OF INDUSTRIAL BIOPOLYMERS FROM MILK AND WHEY***
Prof. dr. B. Witholt, Rijksuniversiteit Groningen, Groningen, the Netherlands.

15.00 h. p.m. : ***REFRESHMENTS***

15.20 h. p.m. : ***A FUTURE FOR MILKFAT***
Dr. G. Page, New Zealand Dairy Research Institute, Palmerston North, New Zealand.

15.50 h. p.m. : ***TRENDS IN PRODUCTION AND UTILIZATION OF MILK CONSTITUENTS; IMPRESSION OF STUDY TOURS***
Dr. W.IJ. Aalbersberg, NIZO, Ede, the Netherlands.

16.20 h. p.m. : ***CLOSING*** of the symposium by an informal gathering of the participants at I.A.C.

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Organizing Committee:

Dr. J.E. Mellema, Chairman of the Program Advisory Committee on Dairy Research of the Netherlands National Council for Agricultural Research, chairman.

Dr. W.IJ. Aalbersberg, General Director of the Netherlands Institute for Dairy Research (NIZO).

Ir. P.L. Slis, Secretary of the Department Processing and Marketing of the Netherlands National Council for Agricultural Research.

Ir. S.A. de Vlaming, Secretary of the Program Advisory Committee on Dairy Research of the Netherlands National Council for Agricultural Research, secretary.

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The need for non-food applications in the dairy field

Prof.Dr. C.P. Veerman and Dr.Ir. T.J.H.M. Hutten

Paper presented at the symposium:
"Milk components in food, novel food and non-food"

27-02-1991
I.A.C.
Wageningen, The Netherlands.

Introduction

The title of is paper suggests that there is a need for non-food applications in the dairy field. However, the Common Agricultural Policy of the EC in the eighties and especially the recent proposals of EC-commissioner McSharry make it likely that in political circles in Brussels the development of new markets in the dairy field is of marginal importance. Therefor, the question that have to be answered first in this paper, is: **Is there a need for non-food applications in the dairy field at this moment and in the future?**
In our opinion the recent proposals of McSharry suggest that the answer is no. Let us explain this thesis by examining the proposals and the effects on dairy production and on development of dairy farming and dairy industry.
Although there are more versions of the proposals going around, we only discuss the most far-reaching one, because this version reflects the ideas in the inner circle of DG-VI in Brussels.

Commissioners proposals on milk

First the proposal on milk establishes that on one hand the production of milk is still rising despite the quota system and on the other hand the consumption of milk is dropping. As a result stocks in the beginning of 1990 stand at 350 kilotons skimmed milk-powder and 240 kilotons of butter. It is not expected that this trend will alter in the near future. It is concluded that the quota systems and the tax on additional production - called superlevy - has to be maintained. However, the volume of quotas have to be reduced by at least 5 percent. So far nothing new. The split between the current system and the new proposals is the unequal distribution of the quota reduction, a simultaneous reduction of the intervention price and a compensatory allowance to compensate for an income loss of small farmers. The income allowance has to diminish the effect of a reduction of the intervention price by 10 % (butter 15% and skimmed milkpowder 5 %). It is meant for farmers with lesser than 15 cows and no more than 1 cow/ha. The quota reduction is not applied to small- and medium-sized firms producing lesser than 200 kilotons milk per year. This means a 10% reduction at quotas for firms producing more than 200 kiloton.

The main purpose of this policy is to avoid new explosions of spendings and to save as many small farmers as possible. At the establishment of the EC in 1958 (conference of Stresa) the income allowance has been linked to production volume in order to supply a parity income to efficient farmers. However, in the new proposals the Common Agricultural Policy has been translated to rural policy and to a purely political income distribution amongst farmers. The justification is found in the hugh differences in income (a factor 3), rural depopulation and protection of the natural environment and maintenace of landscapes.

However, the question is if these goals are reached as the link between farmers' income and their production volume is abolished. Income allowance is not a garantee for rural development, neither it is an incentive for environmental protection and maintenance of landscapes. On the contrary, it takes away every incentive to new developments and it punishes farmers with a high productivity like in the Netherlands, Danmark and Northern Germany, Northern France and the UK. This policy also is contrary to economic theory and practice. Since the beginning of the eighties it is commonly recognized that employment simply cannot be replaced by social security, due to structurally economic and social as well as phychological consequences. In the case of agriculture one should realize oneself that one is taking a one-way route that looks attractive, but leads to the abolisment of agriculture as an economic activity.

Consequences of the commissioners' proposals

On one hand the consequence of the commisioners proposal will be capital destruction at farm level, in dairy industries and in infrastructure and will lead to loss of infrastructure and of high skilled human resources. On the other hand reduction of quota reduces the basis for new investments.
And lastly, but not at least the international competitiveness of a major export sector in the Netherlands is severely weakened. Among the reasons are: increased overcapacity and increased production costs, separation of originally common interests between farmers and their co-operatives, ceased interest in product- and processinnovation and increased

uncertainty about future cuts and developments in the sector.
Besides, the new proposals will lead to more burocracy and complex control systems. The control is very difficult because of the many family members at the farms in great parts of the EC and because of the many part-time farmers. One third of the farmers in the EC is part-timer.

Dairy sector in the Netherlands

The consequences for Dutch dairy farmers become clear if the current structure and developments are taken into account.

The introduction of the quota system in 1984 have had important consequences for the dairy sector. In the period 1984-1990, the volume of quotas had reduced by 20% and Dutch dairy sector made great efforts to adapt to this development. The following examples may show this.

In period 1984-1990 the number of dairy farms in the Netherlands decreased with 19% to 47.000. The number of specialized dairy firms declined even more (23%, to 39.000). In 1984 the average quotum was 230 ton and 40 % of the dairy farms had a quotum of 200 tons and more. About 6 % of the quotum-holders is a part-time dairy farmer with in most cases a quotum of lesser than 200 tons. In 1990 51 % of the dairy farmers had a quotum of more than 200 tons. They produce 78 % of the Dutch milk pool. Therefor proposals of McSharry would cut this trend.
Moreover, in the next decade the development strongly depends on the age-distribution at the dairy farms and the share of firms with a successor. About 60 % of the firms have a farm head older than 50 years and about 50 % of these firms donot have a successor. Therefor it is likely that onefourth of the firms will end within 10 years. The number of the small firms that ends the production within about 10 years, will be relative high because 60 % of the firms without a successor is smaller than 20 cows (about 100 tons of milk).
The reduction of quota also effects quota prices and productivity. The development of the

number of farms had been accompagnied by a decrease of land productivity. Between 1985 and 1989 the number of milk- and calfcows per ha diminished from 1,76 to 1,46 , but the productivity per cow increased. However, in the Netherlands pollution abatement will cause a considerable reduction of intensity of land use and make a further processing and distribution of dung necessary. The only way for the Dutch dairy farming to meet these challenges as an economic activity, is increase in scale. Production costs may cease and the labour productivity may increase by the use of firm guidance systems and automation. Again, the proposals of the EC strike with these developments.
The effects of the EC-proposals on Dutch dairy industries are also very adverse.
Since the second world war the concentration and scaling-up of the dairy industry has been considerable. In the period 1949-1979 the number of co-operatives decreased drastically from 600 to about 150 firms. In 1979 67% of the milk had supplied in 13 cooperatives and in 1990 74% had supplied by 3 cooperatives (DMV/Campina/Melkunie, Coberco, Noord Nederland) and 14 % by some private enterprises. The 3 co-operatives respectively specialized at different productgroups (industrial products/ice-cream/desserts, cheese, cheese). None of these co-operatives mainly produce supported products like butter and skimmed milkpowder. Therefor, cutting the quota of their members doesnot seem the right way to solve the overproduction of butter and skimmed milkpowder.

The fast concentration of dairy industries have been accompagnied by high investments in modernized production capacity at a moment that the production volume still increased. However, within 5 years the introduction of the quota system and the superlevy caused a 29% decrease in the production of skimmed milk powder and butter. This is an indication for the problems, with which the industries have been confronted. Since 1984 they cannot grow by increasing the milk supply and many of them face overcapacity. The management policy must now think in terms of adding value to the existing milk pool. A second drastic reduction of quotas - as proposed by Mr McSharry - will cause serious damage to these firms.

After the initial adaptation to the quota system a new growth trend developed. This was mainly due to a relative shortage at the world market in 1988 and 1989 and to changes in storage policy of the EC. Together with the lower world market prices of 1990 the budget

burden grows very rapidly.

What may be the conclusion ?

The evaluation of the structure of the Dutch dairy sector and the consequences of the commisioners proposal for the Dutch sector lead to the following conclusions.

- An adaptation of the existing quota system and superlevy for excess production is necessary,
- the proposals of EC-commissioner McSharry nor meet the initial goals of the CAP, neither contribute to solutions for the major problem: **lack of new markets and the grading-up and maintenance of existing markets,**
- At least in the Netherlands the proposals donot contribute to solution of the pollution problem in the Netherlands. At best there will be a shift to other sectors of agriculture.

If we return to our initial question, the answer is clear: The proposals of McSharry have to be rejected. A further development of markets and of the sector is necessary. Non-food applications may be one of the few possiblities for meeting new markets. Firms have to be challenged by an EC-policy to meet these markets. Besides, the EC policy have to solve the environmental problems. But first we have to examine what the possibilities of agrification may be.

Non-food applications: challenge or illusion?

Usually, non-food applications has been connected to arable land farming. It is generally thought that dairy products are too expensive. However, this statement doesnot hold out. In the past the industrial use of agricultural products hadnot been restricted to fibers, starch and oils. A Dutch co-operative like DMV/Campina has produced caseïns and lactose for industrial purposes for more than 30 years and is still involved in R&D for the development of new products and markets in this field. However, the current market is

restricted to very specific applications like special coatings and adhesives, light sensitive emulsions, cathode-ray tubes, polyurethane foams, fine chemicals as e.g. lactulose and lactose for the preparation of tablets and pills. For commodity applications the components are too expensive.

In our opinion industrial uses for dairy products seem to be subject to the same rules and restrictions as products from arable land farming. A further examination of successively industrial markets, milk as a resource, available technology and distribution problems support this.

Industrial markets

The supply of products to an industry is subject to other rules than supply to consumers. The following 4 points are of great interest:

1. At the industrial markets products are purchased on specification with narrow ranges of deviation. Constant quality and sufficient and year around availability are presumed. At these criteria dairy products score perhaps better than many other agricultural products. Nevertheless, it is one of the weakest points if agricultural products are compared with chemical products.

2. In many cases agricultural products have to compete with chemical products, produced from cheap oil and gas. The price/performancy of agricultural products lag behind those of chemical products. Dairy products are not an exception to this rule. The best opportunities have products with specific characteristics, like biodegradability, stereospecificity or complexity, especially if these products cannot or very expensively can be made from basic chemicals.

3. After World War II agriculture have directed almost completely to food security. The process - and chemical industry lost the knowledge of agricultural products. Chemical products are thought to be superior in all aspects. This means that dairy industry has to explore and to conquer the market of their products.

4. Purchasing industries are only interested in their markets. They buy at world market prices as is recently shown at the casein market. This means that they donot want to solve agricultural problems or the problems of their suppliers at these markets. Even more, many industries donot like to be dependent of agricultural policy and political

determined prices.

Milk as resource

In comparison with other agricultural products the industrial use of milk or dairy products has some advantages, but also some disadvantages. They will be summarized in short.
Advantages are:

1. Milk is of constant and high quality.
2. Dairy industry is able to decompose milk in its substituants in an economicly efficient way. All components, except water, are applied. However, many experts donot see a future for milk fats or its derivatives. Perhaps this symposium provide some counterbalance.
3. In contrary to e.g. fibers, milk donot need to be rendered soluble. This is a great advantage. Separation, purification and modification of components are the main elements of its processing.
4. There is plenty of milk available during all seasons and it can be transported easily in cooled tanks.

Disadvantages are:

1. Milk is highly perishable and has a very high water content.
2. The water removal costs plenty of energy.
3. Milk contains relatively low levels of interesting components, like lactose, proteins and fats. Besides, the diversity within the fat and protein fraction is considerable. Because of this separation and purification are complicated.
Fats contains only minor fractions of interesting fatty acids. Industries are mostly interested in short chain fatty acids. Other acids can be prepared more easily and cheaper from other materials. Maybe biotechnology brings some improvements in the composition of milkfat. Otherwise the fat component is from industrial point of view of little value.
4. At this moment agrification of milkcomponents has been limited to byproducts of mainly the butter and cheese production. Untill now, these applications compete with supported products like (skimmed) milk powders.

5. The longer foodchain and the strong link to foodproduction make it very unlikely that within 10 years dairy products will be used for industrial purposes on large scale. This may not hold out for components that cannot be made by chemical or microbial routes and that cannot be substituted by qualitatively equal vegetable products.

Technology

A strong point for the use of dairy products is the highly developed separation and purification technology of milk and milkcomponents, like caseins. However, this isnot a garantee for industrial applications. New applications have to be developed. The more specialized they are, the more the help of the purchasers is necessary. Relationships based on mutual trust and mutual benefit must be build. A prerequisite is a sufficient level of R&D efforts. At this moment many dairy industries lag behind and spent lesser than 1 % of their sales at R&D.
Perhaps in future biotechnology will serve with new applications or made it possible the manipulate the milk composition in a proper way. However, the development of such a technology takes more than 10 years. As is shown in the theory of the product life cycle, the commercialisation of a new product takes even more time. Besides, two serious problems may arise. The first one is the consumer acceptance of dairy products from biotechnological origin. The second one is the separated processing that will be necessary for milk with a different or manipulated composition.

Distribution

The main problem in distribution is how to meet your industrial purchaser. Many other problems like the political and trade restrictions, are discussed before.

Conclusion with respect to the economic feasibility of non-food applications

When the foregoing evaluation of the strong and week points of milk components with

respect to non-food applications are summarized, the following conclusion can be made: From technical point of view agrification may contribute to the development of new markets for dairy products. However, as well as basic research as application research have to be intensified. The efforts of dairy industries must be supported and not be restricted by dairy policy or uncertainty about future EC-policy.

New markets may only be developed if non-food applications are not the final piece. The goal may be to supply specific resources for products with a high added value. High quality, proper specifications and high level of service to the purchaser are key-elements of the marketing strategy. In many cases, e.g. for lactose, fats , fatty acids and caseins, availability at worldmarketprices is a prerequisite. This means a substantial lower price level than the current EC levels for dairy products. To determine an indicative range: At 30 to 50% of the current levels dairy products become interesting.

In a period of at least 10 years non-food applications will only be possible if byproducts of the milkprocessing for food can be used. Thus non-food applications donot contribute to the solution of current problems with respect to overproduction.

But it is a long term challenge that contributes to an economically efficient processing of milk. However, the Common Agricultural Policy has to be adapted. It is shown that nor the current quotasystem, nor the recent proposals of McSharry support the development of new markets. This conclusion creates obligations and leads to a last question: **In which way can the CAP be adapted in order to meet new markets?**

We like to defend a two-price system, which in our opinion

1. makes it possible to maintain the goals, determined at the conference of Stresa (reasonable income, growth of productivity, stable markets and stable prices for farmers and consumers and reasonable pricelevels for consumers) and
2. serves the development of new markets under the limitations of current and future pollution control.

Pollution control has to be an essential part of the policy because of its effects on production and structure of the sector. Although this isnot the place to discuss the pollution problem in detail, it is necessary to made some general remarks. In the next decade it seems to be unevitable to restrict the use of fertilizers and pesticides and to find other applications for the use of dung. Broadly speaking, the dairy farming have to

become more extensive and a proper technology has to be developed. This goal may be met by well-choosen taxes, subsidies and by restrictions to the agricultural production. However, such a policy is only successful if dairy farming is still economicly attractive at farm level.

An economicly attractive dairy farming cannot be achieved by leavig sairy farming to the rules of a liberal market mechanism. Competition at the market doesnot take environment, farm income and farm structure into account. Therefor, a complete abolition of the quota system seems irrelevant. A reasonable milkprice for the quotum share also has to be a key element of a Common Dairy Policy in the future. A higher milk price for the quotum share may be justified if it serves pollution control. The question is how to stimulate a further development of the sector and how to manage additional production, the inlimited intervention and the export restitutions. I our opinion the two price system may provide an answer.

Two-price system

The difference between the current quota system and its superlevy and the two price system is the abolition of the superlevy, the exportrestitutions and the intervention for unrestricted amounts of butter and milkpowder. The income obtained from the quotum share must garantee a basic income. This means the volume of quota has to be choosen at the level of the internal consumption of milk and dairyproducts. The internal EC-price is a dynamic factor that is manipulated to supply a basic income level. Byproducts are not supported and are subject to world market prices.

Additional production at farm level is stimulated within the limits of pollution control. It has to compete at the world market. Only farmers who are able to produce additional milk at world market prices are encouraged. The development of new markets is a challenge for as well as farmers as dairy industries, while at world market price levels new applications for dairy products become into sight. At the opposite site, industrial purchasers donot face political determined price levels because they buy products at world market prices.

Such a two price system creates on one hand a basis for the development of new markets under politically and economicly preferred conditions, but the development of new

markets doesnot depend on income policy alone. On the other hand the quotum share supplies a basic income and is by that a tool in the income policy for farmers in the EC. The quotum part of the two price system can also be used as a tool in pollution abatement and maintenance of landscape. It is essential that a further development of agriculture and especially the dairy sector, is ensured. Not only because of the survival of a economic viable agriculture, but also because of the renewable character of agricultural products and the environmental advantages of agricultural products.

Genetic Engineering of Milk: Modification of Milk Proteins and Fat Composition in Milk of Transgenic Animals

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Abstract

Transgenic animal biotechnology has become relatively a routine process over last decade, allowing the introduction of recombinant genes into the germline of many species of animal . These animals transmit the foreign genes to their offspring at 50%. Milk protein gene expression is so tightly controlled that they are only expressed in the mammary gland over the period of lactation. Experience has shown that with appropriate promoter elements and flanking regions, expression of the transgenes for milk proteins are controlled in a tissue-specific and/or in a stage-specific manner. Thus, the alteration of the composition of milk is now possible through this biotechnology. Various foreign proteins have been secreted in milk of many animal species and a variety of molecular methodology has been developed to increase the efficiency of production of transgenic animals. In principle, any protein genes of interest, which can be placed under the control of the cis-acting promoter and enhancer elements of a milk protein gene, can be expressed in mammary glands and secreted into milk. These modification of milk composition can also be extended, by manipulation of key-metabolic enzymes, to fat, lactose, and other minerals in milk.

Introduction

Gene regulation of milk proteins invloves synergistic effects of many peptide and steroid hormones, growth factors, and interactions of cell to cell and cell to substratum (3). The use of transgenic animals is an attractive model in vivo for the study of regulation and expression of milk protein genes. It has been demonstrated that rat whey acidic protein (WAP) was expressed in mice (4), rat β-casein in mice (5,6), sheep β-lactoglobulin in mice (7), bovine α-lactalbumin in mice (8), bovine α-S1 casein in mice, and human blood proteins in the milk of either mice (9,10), rabbits(11) or sheep (12,13). Advantages of the use of mammary glands as a bioreactor for the production of foreign proteins include

the mass production of proteins at low cost (7,14,15), and the ability to carry out the necessary post-translational modifications, such as phosphorylation, glycosylation required to obtain a biologically active form (14).

The transgenes incorporated into the germline of animals are transmitted in a Mendelian dominant pattern. Transgenic rabbits (11,16), pigs (16,17,18), and sheep (12,17), and recently transgenic cattle (19) have been reported. Mammary expression of transgenes were reported in mice (4-10), rabbits (11), sheep (12,13) and recently pigs (57) . Further advances in the efficiency of gene transfer and knowledge in regulatory elements of milk protein genes will allow the production of transgenic cattle and the propagation of transgenes in the dairy cattle population by use of embryo transfer and artificial insemination.

Based on functionality of food proteins, future food scientists will be able to obtain certain characteristics of food materials at the production level in either plants or animals. The computer modeling of three dimensional structures of proteins is advancing rapidly and is widely adapted in pharmaceutical development (2). For food proteins it is often possible to predict useful structural and functional information at a less refined level with less concern for detailed molecular structure (1).

In this article, we will review examples of mammary expression where novel milk proteins were secreted in milk of various animals, and will discuss characteristics and structure of milk protein genes, potential modification of milk proteins and fat in transgenic animals, and prospects for genetic engineering of milk. Also we will address what types of technological potentials currently exist and are being developed and how they will affect dairy industry.

Organization of Milk Protein Genes

The major regulatory elements which confer mammary- or lactation-specificity reside in 5'- and 3'- flanking regions and internal regions of a milk protein gene (4,6) such as some segments of introns or structural gene itself. It is interesting that among milk protein genes across various species of animals the high conservative homology was observed in the corresponding 5'- and 3'-flanking sequences of the genes (22-24). For example the amino acid sequence of signal peptides for caseins from various species is so highly conserved that their

inter-species homology is as high as 100% at amino acid level (25) although somewhat lower at nucleotide level (22) due to silent mutations where a change in a base does not give rise to an amino acid substitution. The signal sequences of the various lactoproteins share common charateristics such as high hydrophobicity and clustered hydrophobic amino acid residues (25).

Bovine casein genes are located in a single chromosome as a gene cluster (26) on chromosome 6 where they were, recently, identified within 200 Kb of DNA in the oder of αS1, β, αS2, and K by pulsed field gel electrophoresis (58,59). For the species examined, the caseins are homologous of one or other of four archtypal bovine caseins (22). Some consensus sequences have already been determined from sequencing studies of bovine and rat caseins (27,28). The regulation of all casein genes is highly controlled because their expression is tissue-, stage-, sex-, and age-specific. The mutation rate in the flanking regions is lower than that in the coding regions (29) which implies that a stronger functional requirements were necessary for these regions throughout evolution than for the coding regions of casein genes themself. Signal peptides and phosphorylation sites of three Ca-sensitive caseins (αS1-, αS2-, and β-) are conserved at the amino acid and nucleotide levels. The mRNAs of untranslated regions are short at the 5' end and long at the 3' end, with the 5' untranslated region being conserved within each casein (22). The sequence homology between bovine αS1-casein and guinea pig β-casein is 90.5% for 5'-nontranslated region, 82.2% for signal peptide region, 64% for coding region, and 72% for 3'-noncoding region (23). The conserved sequences in 5' flanking region of bovine β-casein are identical or homologous to the potential binding sites for nuclear factors and for glucorticoid and progesterone receptors (30). The comparison of casein mRNA sequences of different animals has indicated that the 3'-uncoding region is more conserved when compared to the coding portion (28,31).

The sequences responsible for a tissue-specific and developmental stage-specific expression reside in not only 5'- and 3'-flanking region but also certain intragenic sequences including introns (4,6). Evidence seems to indicate that transgenes containing genomic sequences are expressed more efficiently than those with cDNA as compared in Table 1. However, the results from our laboratory show that a simple genetic construct of bovine αS1 casein cDNA can be expressed at 0.21 mg/ml in heterozygotes and at 0.40 mg/ml in homozygotes.

These results are encouraging because they show that cDNA constructs can be expressed at high level as well. Nevertheless, the effects of introns on gene expression have been demonstrated in certain cases where 10- to 100-fold more mRNA was produced from the intron-containing construct (32). An example of this type of control has been shown in the human collagen α1(I) gene where the first intron contains several positively and negatively acting elements (33). As more detailed information of gene sequences becomes available, it is likely that more consensus sequences will be found between the sequences in the different species.

A new method of introduction of a fragment of chromosomal DNA into mouse eggs has been developed recently. Rather than linear fragments of DNA which get inserted into the genome chromosome fragments are injected. This technique facilitates incorporation of very large (more than 10 megabases) pieces of DNA fragments into cells and embryos without the need for cloned sequences (35). This can be feasible in cases where it is desirable to introduce a gene cluster or a gene that spans over a great distance. Recent progresses in cloning technology using yeast artificial chromosome (YAC), now allow one to clone 400 or more kilo base pairs (34). Since it is known that the four casein loci are located within a tightly linked multi-gene complex (58), it may be possible to clone all the casein genes in a single vector through adaptations of YAC technology, and to produce transgenic animals carrying the multiple copies of casein genes.

Expression of Foreign Proteins in Milk

The example of expression of foreign proteins in milk of transgenic animals are summerized in Table 1. Most of proteins are either milk proteins from other species or human blood proteins with pharmaceutical values. A variety of studies with murine whey acidic protein (WAP) promoter have shown a considerable variation of expression level (9,20). However, the expression levels in the studies where promoters from sheep β-lactglobulin (7) and bovine α-S1 casein genes (10) were used are promising and may be appropriate for use in livestock species. The transgenic mice with genomic sheep β-lactoglobulin genes expressed sheep β-lactoglobulin into their milk at 3-23mg/ml (7). However, high level expression was only observed in mice, much higher level of expression in farm animals is required. The variation of expression level of the transgenes seems coming from many factors which include cis-acting sequences,

site of integration of transgene on a chromosome, presence of introns, and possibly as yet unidentified trans-acting factors. We have also observed by repeated sampling of milk from a variety of individuals that the variation between full siblings within lines of mice carrying bovine α-lactalbumin constructs is as large as the between line variation (Bleck & Bremel, unpublished) . To ultimately apply this technology in livestock, the areas in which technological progress is required include efficiency of microinjection, embryo culture and transfer, isolation of embryo-derived stem (ES) cells of domestic animals, *in vitro* maturation of embryos, development of viral vectors, development of strong mammary-specific vectors, more defined information on promoters and enhancers or their interaction, and development of a mammary cell culture system *in vitro* to mimic expression level of a construct *in vivo*. Since the technology to express foreign proteins in milk rely on results of many areas described above, improvement in any of these areas will increase the efficiency of production of animals or the level of expression and ultimately permit permit the application of this technology in farm animals.

Modification of Milk

Proteins

Figure 1 shows the composition of milk proteins in cow milk. Milk proteins are broadly classified by caseins and whey proteins, resulting from their relationship to cheese manufacturing (36). The caseins are important in dairy industry because they form the curds in cheese formation and together compose approximately 80% of the total protein in milk. The rest are whey proteins which do not form the curds in cheese formation. All milk proteins provide several critical functions for processing and handling of fluid milk and manufactured milk products. These functionalities include fat globule emulsification, ionic and colloidal mineral stabilization, pH buffering, cheese curd formation, regulation of heat stability, development of viscosity and gelation in cultured and sterile milk products, foam expansion and stabilization of frozen dairy products, and control of ice and lactose crystallization in frozen milk and ice cream products (37). Since a single change in amino acid composition of a protein can change its functionality remarkably, any well-defined modification in one of milk protein system is likely to make changes in some of the functionalities of food system described above.

Although there are hundreds of proteins in a cows' milk, the proteins with economic value are quite limited. Figure 1 shows that bovine caseins comprise four main classes: αS1 (40%), αS2 (10%), β (40%), and κ (10%). They are all phosphorylated to varying degree: αS2 (10-13P), αS1 (8-9P), β (5P) and κ (1P) (38). Caseins are rich in proline, thus resulting in a random coil conformation that is resistant to heat-induced denaturation but imparts a strong tendency for them to undergo polymerization by hydrophobic, ionic, and Ca^{++} bonding (37), and have never been crystallized. Their uneven distribution polar and apolar domain makes them amphiphilic, giving them useful properties as an emulsifier (1).

An obvious change in cows' milk would be to selectively increase one of milk proteins that is already present in milk. One way achieve this end would be by inserting extra copies of existing genes into bovine genome. For example, one would anticipate that an increased proportion of normal and engineered αS1-casein would result in larger micelles, which, in turn, might change the cheese curd characteristics in addition to enhancing rate of desirable textural development (1). Another conceivable modification would be a production of modified αS1-casein by introduction of chymosin-sensitive regions through site-directed mutagenesis (1), which can be achieved easily by application of polymerase chain reaction (PCR) with synthetic oligonucleotides. Another modification might be brought about by incorporation of addtional κ-casein genes, From this alteration one might anticipate a reduction in the casein micelle size and perhaps to increase the thermal stability thereby decreasing the degree of coagulation during sterilization process. Additionally, removal of some phosphate groups of casein peptide may result in production of a softer cheese (1).

Figure 1 shows the whey fraction which consists of β-lactoglobulin (50%), α-lactalbumin (20%), serum albumin (10%) and other minor proteins . Whey proteins have a nutritional value in bottled milk but represent a waste product for the cheese industry. Heat processing of milk prior to rennet treatment (the initial step in cheese manufacturing when coagulation takes place) causes sufficient whey protein denaturation and whey protein interaction with casein micelles to inhibit the action of rennin. This reduced susceptibility of κ-casein to rennin is due to its formation of intermolecular disulfide bonds between β-lactoglobulin and κ-casein (37). For this reason it might be desirable to selectively

eliminate β-lactoglobulin fraction from milk. It is only present in the milk of ruminant animals and other sea mammals such as dolphin and manatee (39), and is apparently not required for lactation *per se*. Its presence in milk confers some undesirable manufacturing properties (37), and thus its elimination could provide an opportunity for new types of manufacturing practices and development of novel milk products.

Fat

As discussed above, the genetic engineering of milk components is not limited to the commodity proteins. A variety methodologies are being tested for the elimination of undesireable genes with medical implications. This technology can also be adapted for the modification of milk composition. Through either reduction or extinction of key enzymes in the synthesis of milk fat, it should be possible to modify the fat percentage of milk. An example, which we are pursuing (Bremel and Kim, unpublished), is the extinction of acetyl CoA carboxylase, which regulates the rate of *de novo* fat synthesis from 2 and 4 carbon precursors within the mammary gland. It is a large and highly complex enzyme whose genetic sequence has recently been determined (40). Since approximately 50% of milk fat is synthesized from these rumen volatile fatty acid precursors in the mammary gland one might expect this modification to maximally reduce the fat percentage by this one-half. An interesting side effect of potential importance to dairy producers is that reduction in the amount of this enzyme leading to a reduction in fat percentage of milk is expected to result in a concomitant reduction in the energy requirements of the animal producing the milk. In Table 2 is summarized the economic impacts and effects on dietary nutrition requirements for dairy cattle for milk production if fat content were reduced from 3.8% to 2.0% by a specific inhibition of mammary fat synthesis. Similar strategies can be applied to other milk constituents such as reduction of α-lactalbumin which will be discussed in the next section.

Lactose

Lactose content of cows' milk is approximately 5% , it has limited solubility and its crystal is responsible for sandiness defect in ice cream (41). Because of lactose intolerance resulting from absence of its hydrolyzing enzyme, β-galactosidase, many people in the world can not consume milk (41). The

problems associated with lactose might be overcome either by reduction of α-lactalbumin or by introduction of an enzyme such as β-galactosidase into milk. Either of these strategies might be useful.

However, unlike β-lactoglobulin and acetyl CoA carboxylase, α-lactalbumin is requisite to the secretory process because lactose is critical for the movement of water and thus most probably other milk constituents through secretory cells (42). It is one subunit of lactose synthetase which catalyses the synthesis of lactose. Once secreted, lactose cannot permeate the luminal membrane of the mammary alveolus and thereby establishes an osmotic gradient across the secretory cells.

There are several possible strategies through which these components might be removed. A premature stop codon might be inserted into the α-lactalbumin gene leading to abortive synthesis of the gene product through site-specific mutagenesis within the eukaryotic genome. A second strategy is by the introduction of an anti-sense gene (43) coupled with a mammary specific promoter. The transcriptional product of an anti-sense gene of α-lactalbumin is an mRNA whose complementarity with endogenous α-lactalbumin mRNA would give rise to an RNA/RNA hybrid blocking translation of the endogenous mRNA.

Antimicrobials

Milk provides a variety of immunological protections for young mammals. It is possible to consider the intervention in human and animal health problems through the modification of milk. As Figure 1 indicates, bovine milk normally contains a complete complement of antibodies (IgG1, IgG2, IgA, and IgM) (38) . The presence of pathogenic organisms in milk and other parts of the food supply continues to be a problem and the transgenic techniques described can be envisioned to have application to this area as well. A gene coding for a monoclonal antibody against a certain antigen has been expressed in other (non-antibody producing) cells under the control of the appropriate promoter. Fab fragments of IgG were expressed in E. Coli to give specific binding to antigen and recently anti-phosphorylcholine antibody has been produced in transgenic mice (44). Therefore production of monoclonal antibodies in the mammary gland might be reasonably achieved by introduction of the genes coding for an

antibodies under the control of a mammary-specific promoter. Likewise, antibodies against enteric pathogens such as salmonella, lysteria or others could be produced.

With the increased interest in bacterial peptides such as nicin with antimicrobial activity one can envision utility for the production of these agents directly by the mammary gland as well. Most likely for this application one would have to engineer genetic contructs with codons more appropriate to the mammal where the proteins are to be expressed. This is because bacteria tend to use different codons to code for certain amino acids than to higher mammals.

Human Milk Proteins

An area which has some interest is in the production of a milk which contains certain human milk proteins. The composition of human milk is significantly different from cow milk. It has a protein content among the lowest (0.9%) in mammals and whey proteins are the major protein, in contrast to bovine milk where caseins predominate (45). Currently, whey protein concentrates and modified whey products are used as functional ingredients in infant formula (37). For this purpose, a similar strategy to bovine caseins might be applied to exclusively increase bovine whey fraction in milk or to produce a human whey protein such as α-lactalbumin. By use of a transgenic mammary gland model, human milk proteins can be produced by the mammary gland of farm animals to substitute the milk for human milk or to make components for an improved infant formula. Another example for production of a human milk protein component might be lactoferrin (46).

Implementation

While chemical modifications of milk protein have provided the food scientist with insights on structure-function relationships in a number of food systems (47), genetic engineering of milk proteins can provide new insights on the production of novel food products. However, many challenges remain if the composition of milk is to be effectively altered. More detailed information on the interactions of promoters and enhancers in eukaryotic gene must be accumulated, and cis-acting and trans-acting elements must be defined. These will permit transgene constructs with strong, highly efficient, mammary-specific

promoters and enhancers which will direct their high expression in mammary gland. The promoters that are currently used for the production of transgenic animals and cell culture transfection experiments are well characterized and are effectively used various cell culture systems. Rather limited information is available on interaction among mammary-specific promoters, enhancers, trans-acting elements, and possibly intragenic sequences. As shown in Table 1, the high expression level for sheep β-lactoglobulin in mouse milk using regulatory elements of sheep β-lactoglobulin (7) is encouraging since it demonstrated the development of mammary-specific targeting under the control of milk protein promoters. Other work, human urokinase expression using long 5' upstream control sequences of bovine casein to drive mammary-specific expression demonstrates that this technique may have general applicability for proteins foreign to mammary glands (10).

One of the greatest barriers to the immediate application of the transgenic technology in farm animals is the relatively low efficiency of production of transgenic founder (mice 1-2%. sheep 0.5%). The efficiency might be improved either by an improvement of the efficiency of gene transfer itself or by the ability to determine whether the embryo has the gene incorporated before it is transferred. It is clear that an increase in efficiency is required for the process to be economically practical in animal husbandry where techniques involved are much more complicated than those of experiments in laboratory animal models. An attractive alternative would utilize the methodology for introduction of DNA constructs into somatic cells. In this way it might be possible to produce mammary cells with modified functions (transgenic mammary glands) without the necessity of producing transgenic animals carrying genes in their germs cells. For example, if DNA constructs with viral sequences could be introduced directly into the mammary cells in a non-lactating animals it would then obviate the necessity to produce transgenic animals. A limiting factor in this regard is the generally low titers of virus vectors that are obtained. The mammary gland of a cow has ~10^{11} cells and thus to obtain a system where the probability of each cell being infected one would need to introduce a comparable number of viral vectors.

There is a need for improved specificity of gene insertion. This can be also accomplished by gene targeting through homologous recombination where viral

promoters can be flanked by sequences of multiple copy gene at which transgenes can be inserted by loop-in mechanism. For commercial application in domestic livestock the improvement in the efficiency of transgenic production must occur concurrently with an increased specificity of gene insertion. Both the location of insertion into the genome and level of expression are critical to any scenario for introduction of genes into a population of animals. One case has supported such an argument where human β-globin gene was expressed tissue-specifically in transgenic mice at a level directly related to its copy number yet independent of its position of integration (48)

Transgenes segregate as Mendelian dominants. A useful system of genetic constructs in agricuture must be correlated with quantitative traits. A transgenic cow with a significantly lower production of milk would be of little economic value to a commercial dairy farmer. If one assumes that only one founder animal would be produced, then breeding schemes must be developed so that the gene can be propagated and maintained in the population. The need for elaborate breeding schemes to maintain a gene in the population would be eliminated if a simple and reliable way of continually introducing gene(s) of interest could be developed. If it were possible to continually introduce gene(s) of interest then the agricultural producers could continue to take advantage of advances made through traditional animal breeding practices while at the same time utilizing transgenic technology.

One of barriers in the production of transgenic animals lies in the current inability to quickly screen potential transgenic offspring with perhaps only one copy of the gene of interest in their genome. The use of polymerase chain reaction (PCR) in which 2 oligonuclotides are used to amplify specific sequences between them (49) is encouraging because it allows one to quickly screen the positive transgenic animals. Also it allows one to obtain large amount of DNA without cloning in a vector once sequences are known. It only takes two to three days instead of weeks to screen a large number of animals.

As mentioned earlier, considerable progress has been reported for cloning of a big fragment of genomic DNA using YAC (34). Recent advance in generation of a large combinatory phage library of immunoglobulin repertoire in phage lamda has demonstrated that genes for antibodies primed by specific antigen can be

easily cloned (50). All of these technology can be applied to facilitate engineering of transgenes to be expressed in mammary glands of transgenic animals.

Other Possibilities

There are other ways that we might be able to utilize molecular genetic technology along with a more traditional animal breeding scheme. Within the past several years milk analysis has shifted from the traditional Babcock test to the use of multi-spectral infrared analyzers which make it possible to estimate the various individual components in milk. Widespread use of this technology in the management of dairy cattle has made it possible to screen several million animals and to select animals in the population which have milk with unique characteristics. An example of this is shown in Figure 2. This is the result of a preliminary screen of approximately 650,000 Holsteins in Wisconsin whose milk was tested for both fat and protein content. In order to be included in the data set the animal had to have been tested on three separate occasions. The points in the graph represent the unusual animals in the population. Traditional animal breeding programs have not evaluated these animals. Since the protein composition in milk is limited to a few genes careful analysis of the genes either by restriction fragment polymorphism studies (51) or gene sequencing should be of considerable interest. It is likely that a mutation in one of the genes or a potentially usefull combination of alleles might give rise to the high and low levels of expression.

Impacts

The protein content of the milk of laboratory animals is typically in excess of 10%. If the protein content of the milk of dairy animals could be increased to similar levels through genetic engineering while at the same time maintaining the same volume of milk production then changes would be required in dairy cattle diets. It should be pointed out that this situation is not limited to dairy cattle. Laboratory animal diets may have to be evaluated for protein sufficiency if additional proteins are to be expressed in their milk. Were it possible to specifically inhibit fat synthesis, an example of how the nutritional requirements might be expected to change can be seen in Table 2. Approximately half the fat found in milk is synthesized *de novo* from 2C and 4C precursors in the mammary gland. If we were to inhibit acetyl CoA carboxylase through gene

targeting what would happen to the nutritional requirements of the animals? The ration formulation systems used to formulate cattle rations are based on the assumption that fat and protein in milk are highly coupled and therefore need not be considered independently. To simulate a situation the rations shown. The fat and protein coefficients were uncoupled and rations generated with the help of a ration-balancing program from normal feedstuffs using linear programming techniques with the criteria that maintain the protein content at a similar value to that normally found but decreasing only the fat content (52). What emerges from this simulation is that the diet of the animal will change dramatically. The model predicts that the same level of milk volume and milk protein production could be maintained while simultaneously suppressing fat on a diet consisting predominantly of forage. The modelling predicts that the forage level in the diet could exceed 80%. We recognize that this predictions are totally hypothetical, but the potential impact could be enormous.

Any system introducing new genes into the population will have potential ramifications on animal breeding. The high productivity level of the dairy cattle population is testament to the breeding strategies that have developed and implemented over the past several decades. A number of scenarios for the introduction of new proteins into milk have been outlined above. If the method of introduction of these genes is via transgenic animals then it will be necessary to consider how these genes will be propagated in the population. It has been pointed out that considerable resources will be required to evaluate, introduce, and maintain desirable traits into the population (53). Scenarios, such as those which they describe depend very heavily on the technological capabilities considered to be available when the scenario is developed. It will be possible (if not essential) to evaluate some of the changes in milk in laboratory animals so that there is a higher probability of a desirable outcome when introduced into lactating animals. Advances in genetic medicine and the production of rare biologicals with sufficiently high economic values will most likely drive the development of the technological capabilities described above. Following this it will become feasible to develop small, specialized populations of dairy cattle, sheep and goats for the production of specialty milks.

Conclusions

Prospects of genetic engineering of milk and various aspects for the modification of milk through the use of lactating transgenic animals have been discussed. At present, the efficiency of production is low and the level of expression has limited feasibility in domestic animals. However, we predict that as further information at gene level and in transfer technology evolves, the application of mammary expression in transgenic livestock will have substantial effect on protein biotechnology and related industries such as dairy farming , food processing, and feeding practice. The major challenge for scientists from related areas is to economically produce modified or engineered milk proteins with improved functionality that poses no health hazard to producing animals and consuming public, and retains a high nutritional quality.

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References

1. Jimenez-Flores R, Richardson T. Genetic engineering of the caseins to modify the behavior of milk during processing: A review. J Dairy Sci 1988;71:2640-54.

2. Computer-assisted modeling: Contributions of computational approaches to elucidating macromolecular structure and function. National Academy Press. Washington, D.C. 1987.

3. Eisenstein RS, Rosen JM. Both cell substratum regulation and hormonal regulation of milk protein gene expression are exerted primarily at the posttranscriptional level. Mol Cell Biol 1988;8:3183-90.

4. Bayna EM, Rosen JM. Tissue-specific, high level expression of the rat whey acidic protein gene in transgenic mice. Nucl Acids Res 1990;18:2977-85.

5. Lee K-F, DeMayo J, Atiee SH, Rosen JM. Tissue-specific expression of the rat β-casein gene in transgenic mice. Nucl Acids Res 1988;16:1027-41.

6. Lee K-F, Atiee SH, Henning SJ, Rosen JM. Relative contribution of promoter and intragenic sequences in the hormonal regulation of rat β-casein transgenes. Mol Endo 1989;3:447-53.

7. Simons JP, McClenaghan M, Clark AJ. Alteration of the quality of milk by expression of sheep β-lactoglobulin in transgenic mice. Nature 1987;328:530-2.

8. Vilotte J-L, Soulier S, Stinnakre M-G, Massoud M, Mercier J-L. Efficient tissue-specific expression of bovine α-lactalbumin in transgenic mice. Eur J Biochem. 1989;186:43-8.

9. Gordon K, Lee E, Vitale JA, Smith AE, Westphal H, Hennighausen L. Production of Human Tissue plasminogen activator in transgenic mouse milk. Bio/technology 1987;5:1183-7.

10. Meade H, Gates L, Lacy E, Lonberg N. Bovine αS1-casein gene sequences direct high level expression of active human urokinase in mouse milk. Bio/technology 1990;8:443-6.

11. Buehler TA, Bruyere T, Went DF, Stranzinger G, Buerki K. Rabbit β-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits. Bio/technology 1990;8:140-3.

12. Simons JP, Wilmut I, Clark AJ, Archibald AL, Bishop JO, Lathe R. Gene Transfer into Sheep. Bio/technology 1988;6:179-83.

13. Clark AJ, Bessos H, Bishop JO et al. Expression of human anti-hemophilic factor IX in the milk of transgenic sheep. Bio/technology 1989;7:487-92.

14. Bremel RD, Yom H-C, and Bleck GT. Alteration of milk composition using molecular genetics. J Dairy Sci 1989;72: 2826-33.

15. Pittius CW, Hennighausen L, Lee E et al. A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. Proc Natl Acad Sci 1988;85:5874-8

16. Brem G, Brenig B, Goodman HM et al. Production of transgenic mice, rabbits and pigs by microinjection. Zuchthygiene 1985;20:251-2.

17. Hammer RE, Pursel VG, Rexroad CE Jr et al. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 1985;315:680-3.

18. Petters RM, Shuman RM, Johnson BH, Mettus RV. Gene transfer in swine embryos by Injection of Cells infected with retrovirus vectors. J Exp Zool 1987;242:85-8.

19. Roschlau K, Rommel P, Andreewa L et al. Gene transfer experiments in cattle. J Reprod Fert Suppl 1989;38:153-60.

20. Andres A-C, Schoenenberger C-A, Groner B, Hennighausen L, LeMeur M, Gerlinger P. Ha-Ras oncogene expression directed by a milk protein gene promoter: Tissue specificity, hormonal regulation, and tumor induction in transgenic mice. Proc Natl Acad Sci 1987; 84:1299-303.

21. Simons JP, Land RB. 1987. Transgenic Livestock. J Reprod Fert Suppl 1987;34:237-50.

22. Bonsing J, Mackinlay AG. Recent studies on nucleotide sequences encoding the caseins. J Dairy Res 1987;54:447-61.

23. Gorodetskii SI, Zakhar'ev VM, Kyarshulite DR, Kapelinskaya TV, Skryabin KG. Cloning of nucleotide sequence of cDNA for bovine α S1-casein. Biokhimya 1986;51:1641-48.

24. Stewart AF, Bonsing J, Beattie CW, Shah F, Willis IM, Mackinlay AG. Complete nucleotide sequence of bovine αS2- and β-casein cDNAs: Comparisons with related sequences in other species. Mol Biol Evol 1987;4:231-41.

25. Gaye PP, Mercier JC. The functional significance of signal sequence: their role in the transfer of lactoproteins across the endoplasmic reticulum membrane. Repro Nutr Develop 1981; 21:199-208.

26. Matyukov VS, Urmysher AP. Linkage of α S1-. β-. κ- casein loci in cattle. Genetika 1980;16:884-6.

27. Hobbs AA, Rosen JM. Sequence of α- and γ-casein mRNAs: evolutionary comparion of the calcium-dependent rat casein multigene family. Nucl Acids Res 1982;10:8079-98

28. Stewart AF, Willis IM, Mackinlay AG. Nucleotide sequences of bovine αS1- and κ-casein cDNAs. Nucl Acids Res 1984;12:3895-907.

29. Yu-Lee L, Richter-Mann L, Couch CH, Stewart AF, Mackinlay AG, Rosen JM. Evolution of the casein multigene family: conserved sequences in the 5' flanking and exon regions. Nucl Acids Res 1986;14:1883-902.

30. Gorodetsky SI,Tkach TM, Kapelinskaya TV. Isolation and characterization of the Bos taurus β-casein gene. Gene 1988;66:87-96.

31. Bayev AA, Smirnov IK, Gorodetsky SI. The primary structure of bovine β-casein cDNA. Mol Biol(USSR) 1987;21:255-65.

32. Brinster RL, Allen JM, Behringer RR, Gelinas RE, Palmiter RD. Introns increase transcriptional efficiency in transgenic mice. Proc Natl Acad Sci 1988;85:836-840.

33. Bornstein P, Mckay J, Liska DJ, Apone S, Devarayalu S. Interactions between the promoter and first intron are involved in transcriptional control of α1(I) collagen gene expression. Mol Cell Biol 1988;8:4851-57.

34. Brownstein BH, Silverman GA, Little RD et al. Isolation of single-copy human genes from a library of yeast artificial chromosome clones. Science 1989;244:1348-51.

35. Richa J, Lo CW. Introduction of human DNA into mouse eggs by injection of dissected chromosome fragments. Science 1989;245:175-7.

36. It should be pointed out that classification of milks from various animals is based on the fraction of the total proteins which precipitate at pH 4.6. The precipitability of caseins from various species differs from pH 4.6 and thus the categorization as 'casein' and 'whey' must be viewed with some skepticism. For example, it is known that the optimum pH for precipitation of casein from rodent milk is pH 4.0.

37. Morr CV. Functionality of heated milk proteins in dairy and related foods. J Dairy Sci 1985;68:2773-81.

38. Eigel WN, Butler JE, Ernstrom CA et al. Nomenclature of proteins of cow's milk: fifth revision. J Dairy Sci 1984;67:1599-631.

39. Pervaiz S, Brew K. Purification and characterization of the major whey proteins from the milks of the bottlenose dolphin (Tursiops truncatus), the Florida manatee (Trichechus manatus latirostris), and the beagle (Canis familiaris). Arch Biochem Biophys 1986;246:846-54.

40. Lopez-Casillas F., Bai DH, Luo X, Kong IS, Hermondson MA, Kim KH. Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase. Proc Nat Acad Sci 1988;85:5784-8.

41. Kang Y, Jimenez-Flores R, Richardson T. Casein genes and genetic engineering of the caseins. In:Genetic Engineering of Animals: An Agricultural Perspective. J.W. Evans and A. Hollaender, ed. Plenum Press, New York, N.Y. 1986:95-111.

42. Kuhn NJ. The biosynthesis of lactose. In:Biochemistry of Lactation. T.B. Mepham, ed. Elsevier Science publishers B.V. 1983:351.

43. Nishikura, K. and J.M. Murray JM. Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells. Mol Cell Biol 1987;7:639-49.

44. Pinkert CA, Manz J, Linton P-J, Klinman NR, Storb U. Elevated PC responsive B cells and anti-PC antibody production in transgenic mice harboring anti-PC immunoglobulin genes. Vet Immunol Immunopathol 1989;23:321-32.

45. Bounous G, Kongshavn PAL, Taveroff A, Gold P. Evolutionary traits in human milk proteins. Med Hypo 1988;27:133-40.

46. Mercier J-C, Genetic engineering applied to milk producing animals: some expectations. In Exploiting new technologies in animal breeding. Eds Smith C, King JWB, Mckay JC. Oxford University Press. 1986;121-31

47. Richardson T. Chemical modification and genetic engineering of food proteins. J Dairy Sci 1985;68:2753-62.

48. Grosveld F, van Assendelft GB, Greaves DR, Kollias G. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell 1987;51:975-85.

49. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487-91.

50. Huse WD, Sastry L, Iverson SA et al. Generation of a large combinatorial library of the immunoglobulin repertoiri in phage lambda. Science 1989;246:1275-81.

51. Cowan CM, Dentine MR, Ax RL, Schuler,LA. Structural variation around prolactin gene linked to quantitative traits in an elite Holstein sire family. Theor Appl Genet 1990;79:577-82.

52. Howard WT. Total mixed ration. Dept of Dairy Science University of Wisconsin, Madison, WI

53. Land RB, Wilmut I. Gene transfer and animal breeding. Theriogenology. 1987;27:169-79.

54. Schoenenberger C-A, Andres A-C, Groner B, van der Valk M, LeMeur M, Gerlinger P. Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription. EMBO J 1988;7:169-75.

55. Tomasetto C, Wolf C, Rio M-C et al. Breast cancer protein PS2 synthesis in mammary gland of transgenic mice and secretion into milk. Mol Endo 1989;3:1579-84.

56. Lee KF, Atiee SH, Rosen JF. Differential regulation of rat β-casein-chloramphenicol acetyltransferase fusion gene expression in transgenic mice. Mol Cell Biol 1989;9:560-5.

57. Wall RJ, Pursel VG, Shamay A, Mcknight R, Pittius C, Hennighausen L. Production of a foreign milk protein in the mammary glands of transgenic pigs. J Cell Biochem 1991;Supp15A:p175

58. Threadgill DW, Womack JE. Genomoc analysis of the major bovine milk protein genes. Nucl Acid Res 1990;18:6935-42

59. Ferretti L, Leone P, Sgaramella V. Long range restriction analysis of the bovine casein genes. Nucl Acid Res 1990;18:6829-33

Figure 1. Composition and concentration of cow's milk proteins. CN: casein, BLG:β-lactoglobulin, ALA:α-lactalbumin, BSA: bovine serum albumin, IgGs:immunoglobulins. The concentration of each protein was from the nomenclature of proteins of cow's milk: fifth revision by Eigel et al (38).

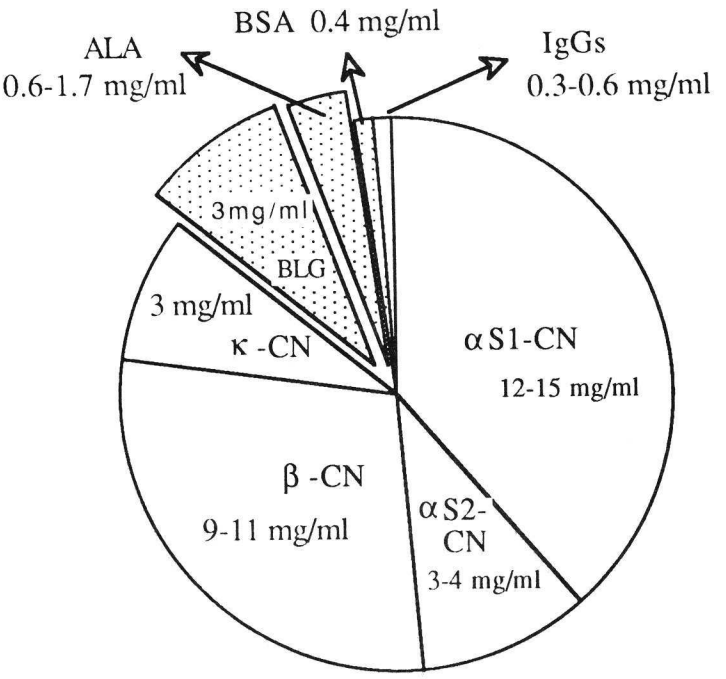

Figure 2. Protein and fat percentages of milk from unusual Holstein cows in Wisconsin population.

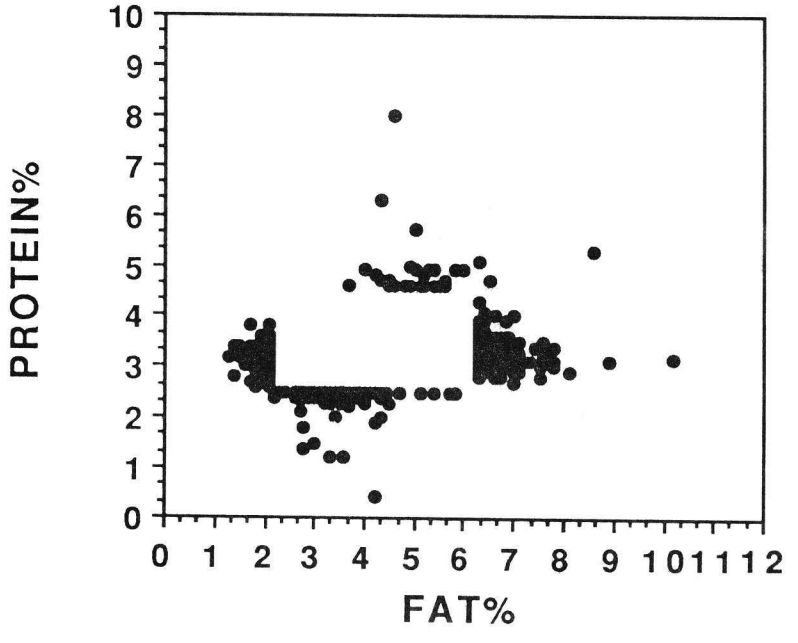

Table 1. Summary of various proteins expressed in mammary glands of transgenic animals.

Promoter Size (kb)	Signal Peptide	Gene(Coding Region) Size (kb)	3' Region Size (kb)	Transgene Size (kb)	Transgenic Animal	Expression Level	References
Mouse WAP[1] 2.5		Mouse c-myc 4.8 (including 3')	Mouse c-myc	7.3	Mouse	1/10 of endogenous	54
Mouse WAP		Human Ha-ras (genomic) 4.9	Human Ha-ras	7.4	Mouse	1/50 of endogenous	20
Mouse WAP 2.5	Human PS2[2]	Human PS2 (cDNA) 0.49	Mouse WAP	8.0	Mouse	2.5% of endogenous or 1.5 µg/ml	55
Mouse WAP 2.6	Human tPA[3]	Human tPA (cDNA)	SV40	4.9	Mouse	114-460ng/ml 20-50,000ng/ml or 5% of endogenous	9 15
Rat β-casein 2.3 or 0.5		Bacterial CAT[4] including exon I and intron A (0.49 kb) of rat β-casein	SV40		Mouse	Expressed in mammary extract and thymus	6 56
Rat β-casein 3.5	Rat β-casein	Rat β-casein (genomic) 7.5	Rat β-casein 3.0	14.0	Mouse	0.01-1% of endogenous	5 6
Rat WAP 0.95	Rat WAP	Rat WAP (genomic) 1.95	Rat WAP 1.4	4.3	Mouse	1-95% of endogenous	4
Rabbit β-casein 2.0	Human IL2[5]	Human IL2 (genomic) 5.3	Human IL2	7.3	Rabbit	50-430 ng/ml	11
Sheep BLG[6] 4.0	Sheep BLG	Sheep BLG (genomic) 4.9	Sheep BLG 7.3 or 1.6	16.2 or 10.5	Mouse	3-23mg/ml	7

Sheep BLG 4.0	Human FIX[7]	Human FIX (cDNA) 1.55+4.9(BLG)	Sheep BLG 1.6	12.05	Sheep	1/250 of human plasma or 25ng/ml	12 13
Sheep BLG 4.0	Human α1AT[8]	Human α1AT (cDNA) 1.3+4.9(BLG)	Sheep BLG 1.6	11.8 or 14.0	Sheep	5μg/ml	12 13
Bovine ALA[9] 0.75	Bovine ALA	Bovine ALA (genomic) 2.0	Bovine ALA 0.336	3.1	Mouse	0.0025-0.45 mg/ml	8
Bovine αS1-CN[10] 21	Human UK[11]	Human UK (genomic) 7.5	Bovine αS1-CN 2.0		Mouse	1-2mg/ml	10

---

[1]Whey Acidic Protein
[2]Breast cancer protein, estrogen-inducible secretory polypeptide
[3]Tissue Plasminogen Activator
[4]Chloramphenicol Acetyltransferase
[5]Interleukin-2
[6] β-lactoglobulin
[7]Anti-hemophilic Factor IX
[8]α1-anti-trypsin
[9]α-lactalbumin
[10]α S1-casein
[11]Urokinase

Table 1

Table 2. Dietary requirements for production of a hypothetical low-fat milk by reduction of fat synthesis in the mammary gland of dairy cattle.

Characteristic	Fat Composition Assumed	
	3.8%	2.0%
Forage, % of D.M.	58.8%	83.2%
Concentrate, % of D.M.	41.2%	16.8%
Forage, % of Feed Cost	44.4%	74.7%
Grain, As % of Feed Cost	48.9%	20.8%
Protein Supp.,% of Feed $	4.4%	0.0%
Feed Cost % of Milk	38.8%	37.1%
Milk Price $/cwt	$10.49	$8.45
Feed Cost$/cwt Milk	$4.07	$3.14

MODIFICATIONS OF MILK PROTEINS AND THE POSSIBLE APPLICATIONS

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Milk components in food, novel food and non-food

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MODIFICATION OF MILK PROTEINS AND THE POSSIBLE APPLICATIONS

Introduction

Despite the ubiquitous use of milk proteins in the food industry, there is a continuing effort to modify further some of their functional attributes. Such modifications are undertaken to improve physical, sensory and nutrional properties, to block deteriorative reactions, and to develop novel products for use in the food and non-food industries. Modification methods can be divided into physico-chemical methods (physical, noncovalent chemical and covalent chemical modification), used extensively in the past, and biotechnological methods (enzymatic and genetic modification), with present and future applications (Figure 1). In order to understand the choice and effects of different modification procedures of milk proteins, a brief description is required of the structure-function relationship of proteins in general, and milk proteins in particular [1].

Proteins are macromolecules which are made up of linear chains of various amino acids, known as the polypeptide chain. Each different type of protein has its own characteristic number and unique order of amino acids, the primary structure. This unique order ensures that each polypeptide chain is spatially folded up in a very well-defined manner due to the formation of highly specific interactions between amino acid side chains. Through these interactions the polypeptide chain first acquires local order, the secondary structure units, which can then interact to generate the specific three-dimensional order, the tertiary structure (Figure 2). Functionally important areas of the chain are brought together within this spatial structure in such a way that the protein acquires its specific properties. The large variety of protein structures which are possible leads to major differences in functional properties of native proteins.

Noncovalent forces that stabilize the native conformation of proteins (and hence influence their functional properties) are predominantly hydrogen bonds and hydrophobic interactions; additional stabilization is provided by van der Waals and electrostatic interactions, solvation effects and occassionally the specific binding of bivalent ions [1]. Disulfide bonds can provide further stabilization by covalent crosslinking of the polypeptide chain. Any physical or chemical changes in the environment may lead to weakening or strengthening of these stabilizing forces (Table 1) and concomitant changes in protein structure and function. The unfolding, or denaturation, of proteins can occur to various extents, leading to a variety of structures (as in Figure 1) with corresponding functional properties.

Milk proteins

Bovine milk contains about 34 g/liter of protein, of which 80% is classified as the casein fraction and the remaining 20% as the whey protein fraction (Table 2). Caseins are, by definition, the proteins which precipitate out when milk is acidified to pH 4.6. It is also the protein fraction which forms the coagulum upon the addition of rennet to milk during cheesemaking. The major milk proteins are αs1-casein, αs2-casein, ß-casein, k-casein, and the whey proteins α-lactalbumin and ß-lactoglobulin. The properties and modification of other minor milk proteins, including enzymes, will not be discussed (for details see reviews [2,3].

The functional properties of milk proteins in foods are numerous (Table 3). Well-known examples are proteins with physiological, foaming, gelling, emulsifying or water/fat absorbing properties. Milk proteins have a high nutrional value [4] and they can have a significant effect on the sensory properties of flavor, appearance and texture. Their functionality in products is often dependant on interactions with other components such as fats, carbohydrates and other proteins. Extensive reviews are available on the functionality of milk proteins [1,5-7] and the applications of caseins and caseinates [8] and whey proteins [9] in food and non-food products.

It is important to note that there are large differences in the chemical, structural and physical properties of caseins and whey proteins (Table 4). The caseins, with their high content of proline and hydrophobic residues and low content of disulfides, have a very open, flexible structure (as in Figure 2b). They are extensively phosphorylated and combine with calcium phosphate to form large micellar aggregates in milk, as illustrated in Figure 3b,c [10]. The whey proteins are quite the opposite, with compact and rigid globular structures which normally do not aggregate, as illustrated in Figure 4 for ß-lactoglobulin. These structural differences are reflected in their functional properties. Caseins and caseinates (Ca-phosphate-depleted caseins) are characterized by their ability to bond with water or fat, to control rheology and to act as emulsifiers. In addition to being highly flexible, caseins are amphiphilic molecules with localized regions of high hydrophilicity and high hydrophobicity (Figure 3a), which provides them with excellent surface activity. Whey proteins are used in food products where solubility and foaming are important. In some cases their favorable foaming characteristics are used to replace superior, but more expensive egg proteins.

Physical modification

The effects of physical modification on milk proteins, due to environmental variations in temperature, pH, pressure, drying, irradiation, etc. (Table 5), have been extensively studied in the past [7,11]. These modifications are of particular relevance because such extreme processing conditions are often encountered in food production. Physical modifications often lead to irreversible denaturation and insolubilization of the whey proteins [11].

Noncovalent chemical modification

Chemical variations in the environment of milk proteins, such as those listed in Table 6, can lead to structural modifications (and hence functional modification) without directly affecting the covalent structure of the proteins. These noncovalent chemical modifications of milk proteins have also been extensively studied in the past, often in combination with physical modifications [1,7,11,12]. Many of these chemical compounds induce unfolding of the proteins since they weaken hydrophobic interactions (detergents, organic solvents) or hydrogen bonds and electrostatic interactions (salts, chaotrophs). Stabilizing effects have been ascribed to specific metal ions, large anions, sugars, polyols and polymers [1,13].

Covalent chemical modification

Many chemical reactions can be used to covalently modify the side chains of amino acid residues in proteins [14,15]. The main reaction types and reagents are listed in Table 7. The

type of side chains available for chemical modification is rather limited: the most commonly modified residue is lysine, followed by the negatively charged residues and cysteine. Many chemical modification studies of milk proteins have been performed, either of the individual purified proteins or mixtures thereof, and excellent reviews are available [1,14-16]. Only a few examples of such modifications are listed in Table 8, together with the observed effects on functionality of caseins or whey proteins. Generally speaking, the introduction of additional charges decreases aggregation and enhances solubility and water binding, while the introduction of long alkyl groups decreases solubility and enhances aggregation and surfactant properties.

In theory, covalent chemical modification has great advantages over physical and noncovalent modification methods in that a large variety of coupling reactions and corresponding coupled compounds or chemical groups can be applied, leading to a large variety of functional modifications. Cheap reagents can be employed to affect permanent modifications, often without protein denaturation.

In practice however, commercial application of chemically modified proteins in foods is very limited, mainly due to the hazards of potentially toxic reagents, products and byproducts. In addition, chemical modification often leads to reduced digestibility and nutrional value (loss of essential amino acids) [16].

Enzymatic modification

Enzymes catalyse many post-translational modifications of proteins, including crosslinking of polypeptide chains, phosphorylation, dephosphorylation, glycosylation, hydroxylation, dehydratation, methylation and hydrolysis (Table 9). Hydrolysis is a unique modification method in that it produces smaller fragments of the polypeptide chain, called peptides, which may have entirely new properties.Although the specificity of enzyme-catalysed reactions may influence the modification, the properties of the enzymatically modified derivatives are probably similar to those prepared by covalent chemical modification. Advantages of using enzymatic modification are mild reactions conditions (e.g. pH, temperature, pressure), high specificity of enzymes, less byproducts, and low toxicity, particularly if acceptable "food-grade" enzymes are used. Some disadvantages are the limited stability of many enzymes under industrial processing conditions, and the requirement to inactivate and/or remove the enzymes from the final product. Although purified enzymes can be very expensive, it is generally adequate to use cheaper crude enzyme preparations, microbial cell extracts or whole cells.

There has been increasing interest in recent years in the enzymatic modification, and particularly hydrolysis, of milk proteins [15,17]. Several examples of modifications and applications are listed in Table 10. Enzymatic phosphorylation and dephosphorylation are important modifications of milk proteins, since phosphate groups play an important role in calcium binding and stability of micelles, and hence in casein digestibility and cheese texture. Transglutaminase-catalysed substitution of primary amines can be used to incorporate amino acids or long chain alkyl groups to improve nutritional and functional properties, respectively.

Partial and complete hydrolysis of milk proteins by proteases (both endo- and exo-peptidase) has been widely used to alter functional properties. Hydrolysis results in an increase of charged groups and hydrophilicity, a decrease in molecular weight and alterations in

molecular configuration. Commonly, solubility increases and viscosity decreases with degree of hydrolysis. Other effects that are frequently observed include altered gelation properties, enhanced thermal stability, increased emulsifying and foaming abilities, but decreased emulsion and foam stabilities [15,17]. Caseins are particularly susceptible to proteolysis due to their open flexible structure. The specific properties of ripened cheese are a speciale example of the changes in casein micelles that are initiated by the specific action of rennet enzymes, especially chymosin, and continued by the multiple proteolytic enzymes of lactic acid bacteria. The cheese ripening process can be accelerated by the addition of appropriate proteases [18]. Peptides with a large number of hydrophobic amino acids, as derived from the caseins, can have a bitter taste, and care must be taken to control the enzymatic degradation in such a way that either formation of bitter peptides is limited, or that they are further broken down.

Hydrolysates of milk proteins are used in foods to provide specific flavors, reduce allergic reactions and as predigested fragments in diet foods [8,15]. Small peptides are absorbed better by the digestive tract than free amino acids. Hydrolysates low in phenylalanine have been developed for phenylketonuria patients. Large peptides can have good emulsifying properties, especially those fragments that are more amphiphilic than the parent caseins (see Figure 3a). Casein fragments rich in phosphate groups can be used for calcium complexation and suppression of calcium phosphate crystal formation, with possible applications in the technological and medical fields. Certain peptides isolated from milk protein hydrolysates have special physiological effects such as opioid activity (e.g. casomorphins), growth stimulation, blood pressue regulation and immunomodulation [19]. It seems important therefore to develop economically attractive procedures for the production and isolation of biologically or otherwise interesting fragments (or mixtures of these) of milk proteins through enzymatic hydrolysis.

Genetic modification

Genetic modification methods of milk proteins can be divided into two categories. First, there are those that can be employed to control the gene dosage and regulation of protein synthesis in the milk gland, and thereby allow for changes in the ratios of proteins in milk [20,21]. In the present context, the second category, in which the milk proteins themselves can be modified [20,22,23], is more relevant. In recent years new genetic engineering techniques, using recombinant DNA, have been developed to replace one or more specific amino acid residues in the primary structure of a protein. Site-specific genetic modification has the great advantage that any targetted amino acid residue can be very specifically modified into any of the 19 other residues. At the same time this limits the type of chemical groups that can be introduced to those of normal amino acids. Since the genes of all major milk proteins and most of their natural genetic variants have been isolated and sequenced [2,3], and most of the post-translational modifications are known, it is now possible to design and introduce highly specific alterations at the primary sequence level, at least in laboratory model systems.

An entirely different but extremely important consideration for the future is that the application of site-specific modification of milk proteins will require modification of existing genes or introduction of new genes in the cow [20,21]. This technology is still in a developmental stage, and at the present time definitely raises ethical questions, which will not be addressed here. A possible limitation to keep in mind is that if existing genes cannot be

blocked or suppressed, there will be a normal baseline level of milk proteins upon which will be superimposed the modified or new protein. Only if sufficient quantities of the novel protein are synthesized may the functional properties of this "enriched" milk protein system be modified. Alternatively, the modified protein might be isolated from milk for use separately as a food ingredient or otherwise.

Nevertheless, it is important to identify the potential of specific genetic modification of milk proteins, and some ideas are given in Table 11. Many of these examples relate directly to cheese production. For instance, cheese yield could be improved by reduction of plasmin sensitivity of ß-casein, cheese texture could be modulated by introduction or removal of phosphorylation sites, and cheese ripening could be accelerated by introduction of new proteolytically sensitive sites. Heat stability of k-casein and/or ß-lactoglobulin could be improved by eliminating the cysteine residues that are susceptible to disulfide linkage upon heating. An interesting application, presently being pursued in Japan, is the specific engineering of known antigenic epitopes in ß-lactoglobulin in order to reduce suspected allergenicity in humans of this bovine protein [24].

Conclusions

Physico-chemical modifications of milk proteins, which can be largely classified as empirical, have been so exhaustively studied and applied in food and non-food products in the past that new applications have become increasingly difficult to find. At present, enzymatic modification still has considerable potential and continues to generate new and often unanticipated products with immediate applications.

Although all of the modifications of milk proteins I have described above could be considered as protein engineering, the modern meaning relates only to genetic modification, which may be the doorway to future applications, even for food proteins. A long-term goal of modern biotechnologists is to be able to rationally design (milk) protein structures based upon a desired function, that can vary from enzymatic activity, to surface activity, to physiological activity, to sensory properties.

REFERENCES

[1] J.E.Kinsella, D.M.Whitehead, J.Brady & N.A.Bringe. Milk proteins: possible relationships of structure and function. In: Developments in Dairy Chemistry, Vol.4: Functional Milk Proteins (P.F.Fox, Ed.), 1989, Elsevier Applied Science, London, pp. 55-95.

[2] H.E.Swaisgood. Chemistry of milk protein. In: Developments in Dairy Chemistry, Vol.1 (P.F. Fox, Ed.), 1982, Applied Science, London, pp. 1-59.

[3] P.F.Fox. The Milk protein system. In: Developments in Dairy Chemistry, Vol.4: Functional Milk Proteins (P.F.Fox, Ed.), 1989, Elsevier Applied Science, London, pp.1 - 53.

[4] L.Hambraeus. Nutrional aspects of milk proteins. In: Developments in Dairy Chemistry, Vol.1 (P.F. Fox, Ed.), 1982, Applied Science, London, pp. 289-313.

[5] C.V.Morr. Functional properties of milk proteins and their use as food ingredients. In: Developments in Dairy Chemistry, Vol.1 (P.F. Fox, Ed.), 1982, Applied Science, London, pp.

375-399.

[6] D.M.Mulvihill & P.F.Fox. Physico-chemical and functional properties of milk proteins. In: Developments in Dairy Chemistry, Vol.4: Functional Milk Proteins (P.F.Fox, Ed.), 1989, Elsevier Applied Science, London, pp. 131-172.

[7] J.N. de Wit. Functional properties of whey proteins. In: Developments in Dairy Chemistry, Vol.4: Functional Milk Proteins (P.F.Fox, Ed.), 1989, Elsevier Applied Science, London, pp. 285-321.

[8] C.R.Southward. Uses of caseins and caseinates. In: Developments in Dairy Chemistry, Vol.4: Functional Milk Proteins (P.F.Fox, Ed.), 1989, Elsevier Applied Science, London, pp. 173-244.

[9] J.N. de Wit. The use of whey protein products. In: Developments in Dairy Chemistry, Vol.4: Functional Milk Proteins (P.F.Fox, Ed.), 1989, Elsevier Applied Science, London, pp. 323-345.

[10] D.G.Schmidt. Association of caseins and casein micelle structure. In: Developments in Dairy Chemistry, Vol.1 (P.F. Fox, Ed.), 1982, Applied Science, London, pp. 61-86.

[11] D.M.Mulvihill & M.Donovan. Whey proteins and their thermal denaturation- a review. Irish J. Food Sci.Technol. 11 (1987) 43-75.

[12] H.E.Swaisgood. Structural changes in milk proteins. In: Milk Proteins. Nutrional, Clinical, Functional and Technological Aspects (C.A.Barth & E.Schlimme, Eds.), 1989, Steinkopff Verlag, Darmstadt, pp. 192-210.

[13] C.H.Schein. Solubility as a function of protein structure and solvent components. Bio/Technology 8 (1990)308-317.

[14] J.E.Kinsella & D.M.Whitehead. Emulsifying and foaming properties of chemically modified proteins. In: Advances in Food Emulsions and Foams (E.Dickinson & G.Stainsby, Eds.), 1988, Elsevier, Amsterdam, pp. 163-188.

[15] S.Nakai & E.Li-Chan. Chemical and enzymatic modification of milk proteins. In: Developments in Dairy Chemistry, Vol.4: Functional Milk Proteins (P.F.Fox, Ed.), 1989, Elsevier Applied Science, London, pp.347-376.

[16] H.W.Modler. Functional properties of nonfat dairy ingredients - a review. Modification of products containing casein. J.Dairy Sci. 68 (1985) 2195-2205

[17] S.Arai & M.Watanabe. Emulsifying and foaming properties of enzymatically modified proteins. In: Advances in Food Emulsions and Foams (E.Dickinson & G.Stainsby, Eds.), 1988, Elsevier, Amsterdam, pp. 189-220.

[18] C.J.Kirby & B.A.Law. Recent developments in cheese flavour technology: application of enzyme microencapsulation. Dairy Indust. Intern. 52 (1987) 19-21.

[19] H.Meisel & H.Frister. Chemical characterization of bioactive peptides from in vivo digests of casein. J.Dairy Res. 56 (1989) 343-349

[20] R.Jimenez-Flores & T.Richardson. Genetic engineering of the caseins to modify the behavior of milk during processing: J.Dairy Sci. 71 (1988) 2640-2654.

[21] R.D.Bremel, H.-C.Yom & G.T.Bleck. Alteration of milk composition using molecular genetics. J.Dairy Sci. 72 (1989) 2826-2833.

[22] L.K.Creamer, R.Jimenez-Flores & T.Richardson. Genetic modification of food proteins. Trends Biotech.6 (1988) 163-169.

[23] C.A.Batt. Biotechnology and its implication for the future design and production of food ingredients. In: Biotechnology and Food Process Engineering (H.G.Schwatzberg & M.A.Rao, Eds.), 1990, Marcel Dekker, Amsterdam, pp. 1-17.

[24] T.Takahashi, K.Yamauchi & S.Kaminogawa. Comparison between antigenicity of native and unfolded ß-lactoglobulin. Agric.Biol.Chem. 54 (1990) 691-697.

[25] M.Z.Papiz, L.Sawyer, E.E.Eliopoulos, A.C.T.North, J.B.C.Findlay, R.Sivaprasadarao, T.A.Jones, M.E.Newcomer & P.J.Kraulis. The structure of ß-lactoglobulin and its similarity to plasma retinol-binding protein. Nature 324 (1986) 383-385.

LEGENDS

Figure 1: Modifications of milk proteins

Figure 2: Stages in the protein folding and unfolding process from (a) completely unfolded, to (e) completely folded. Whey proteins are best described by state (e), whereas caseins are presumably more like state (b).

Figure 3: Schematic representation of (a) casein primary structures, (b) a casein submicelle, and (c) a casein micelle. Adapted from [10].

Figure 4: Schematic representation of the compact tertiary structure of ß-lactoglobulin [25], showing the secondary structure elements as a spiral (α-helix) and arrows (ß-sheet strands). Proposed binding of a small hydrophobic molecule (retinol) is shown.

Table 1
Stabilizing forces in proteins

force	weaken	strengthen
non-covalent:		
hydrogen bonds	high temperature denaturants	low temperature
hydrophobic	low temperature detergents organic solvents	high temperature
electrostatic	high salt	low salt
hydration	non-aqueous solv.	aqueous solvent
bivalent ions	chelators	bivalent ions
covalent:		
disulfide bonds	reductants oxidants	-

Table 2
Milk proteins (cow)

proteins	proportion of total protein (%)	phosphate groups	disulfide bonds
CASEINS			
α_{s1}-casein	32	8	0
α_{s2}-casein	8	10-13	1
β-casein	28	5	0
κ-casein	10	1-2	1
γ-casein	2	0-1	0
	80		
WHEY PROTEINS			
β-lactoglobulin	10	0	2
α-lactalbumin	4	0	4
immunoglobulins	3	0	15
proteose peptones	2	4-5	0
serum albumin	1	0	17
lacto-/trans-ferrin	1	0	16-20
enzymes	< 1	0	-
	20		

Table 3
Functional properties of proteins in foods

property	function
sensory	colour, taste, aroma, texture
surface-activity	emulsifying, foaming, film-forming
molecular interaction	binding of fat/flavour components
structure forming	fibrous, network, sticky, elasticity, extrudable
rheological	viscosity, gelation, adhesion
water interaction	solubility, swelling, water absorption, thickening

Table 4
Properties of milk proteins

property	caseins	whey proteins
chemical:		
non-polar amino acids	high	low
proline	high	low
cysteine, methionine (sulfur)	low	high
phosphorylation	yes	no
glycosylation	yes	no
hydration	high	low
physical:		
compact structure	no	yes
aggregation state	high	low
heat stability	high	low
pH stability	low	high
proteolytic stability	low	high
viscosity	high	low
emulsion capacity	high	reasonable
emulsion stability	high	high
foam stability	reasonable	high

Table 5

Physical modification of proteins

- temperature
- pH (acid, alkali)
- pressure, extrusion
- drying, evaporation
- mixing, pumping, aeration
- light, radiation

Table 6

Non-covalent chemical modification of proteins

(environmental factors)

- salt concentration
- specific cations (Ca^{2+}, Zn^{2+}, heavy metals)
- specific anions
- chelators
- chaotrophs (urea, guanidine hydrochloride
  trichloroacetic acid)
- detergents
- organic solvents
- sugars, polyols
- polymers

Table 7

Covalent chemical modification of proteins

reaction type	reagent	amino acid residue
acylation	anhydrides	Lys
red. alkylation	carbonyls	Lys
phosphorylation	phosphooxychloride	Lys
glycosylation	sugars	Lys
lipophilization	anhydrides, alcohols	Lys, Glu, Asp
guanidination	methylisourea	Lys
esterification	alcohols	Glu, Asp
amidation	amines	Glu, Asp
deamidation	alkali, acid	Gln, Asn
crosslinking	various	Lys, Cys
alkylation	haloacetates	Cys
oxidation	peroxides	Cys, $(Cys)_2$, Met
reduction	thiols, sulfite	$(Cys)_2$
mixed disulfide	thiols	Cys,$(Cys)_2$

Table 8: Examples

Covalent chemical modification of milk proteins

protein	reaction type	altered property	
Caseins	succinylation	aggregation	↓ ↑
	red. alkylation	solubility	↓
		emulsifying	↑
	glycosylation	viscosity	↑
		aggregation	↑
	phosphorylation	viscosity	↑
		Ca-binding	↑
		hydration	↑
		emulsifying	↓
	lipophilization	foaming	↑
		emulsifying	↑
Whey proteins	succinylation	solubility	↑
		emulsifying	↑
	alkylation	stability	↑
	thiolation	aggregation	↑
	guanidination	stability	↑
	phosphorylation	viscosity	↑
		hydration	↑
	glycosylation	stability	↑
		solubility	↑
		foaming	↑
	amino acid-coupling	nutrional value	↑
	reduction	stability	↓
		proteolysis	↑
		emulsifying	↑

Table 9

Enzymatic modification of proteins

enzyme type	reaction type
proteases	hydrolysis synthesis
phosphatases	dephosphorylation
phosphokinases	phosphorylation
transglutaminases	crosslinking amine coupling deamidation
hydroxylases	hydroxylation
dehydratases	dehydratation
methylases	methylation
glycosylases	glycosylation

Table 10: Examples

Enzymatic modification of milk proteins

Hydrolysis	
- hydrolysis rate	accelerated cheese ripening
- hydrolysates	hypoallergenic foods diet foods flavors emulsifiers pharmaceutics cell culture media
- specific peptides	growth stimulation blood pressure regulation immuno-modulation opioid (ant)agonists Ca complexation emulsifiers
Dephosphorylation	infant foods cheese texture
Crosslinking	high nutrition foods surfactants

Table 11: Examples
Genetic modification of milk proteins

milk protein	modified property	anticipated effect on
α_{s1}-casein	phosphorylation	micelle stability
	proteolytic sensitivity	cheese ripening
β-casein	phosphorylation	micelle stability
	proteolytic sensitivity	cheese ripening
	hydrophobic residues	bitter peptides
	lysines	plasmin sensitivity
κ-casein	cysteines	heat stability
	hydrophobic residues	emulsion capacity
	chymosin sensitivity	milk renneting
β-lactoglobulin	cysteine	heat stability
	hydrophobic residues	flavour binding
	antigenic epitopes	allergenicity

Modification of milk proteins

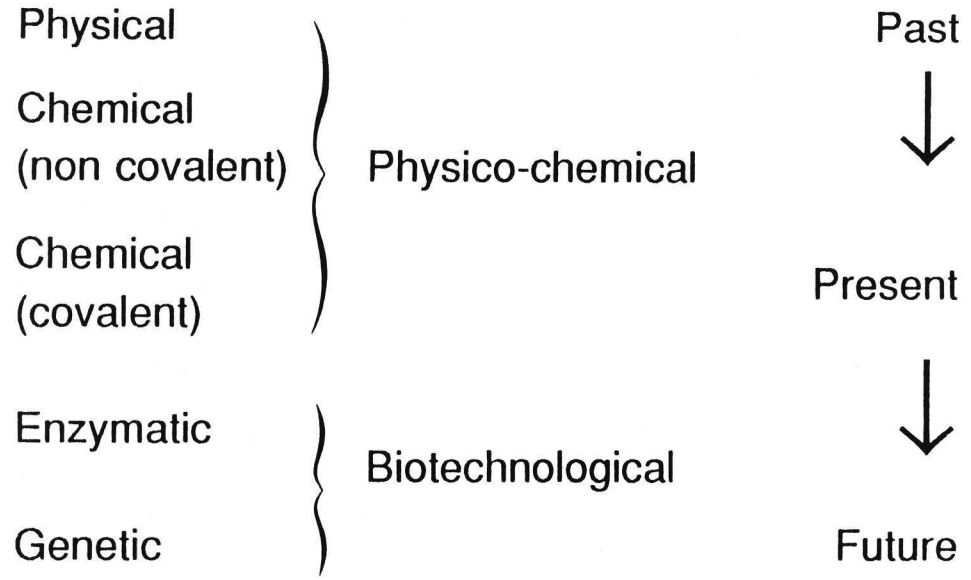


Figure 1

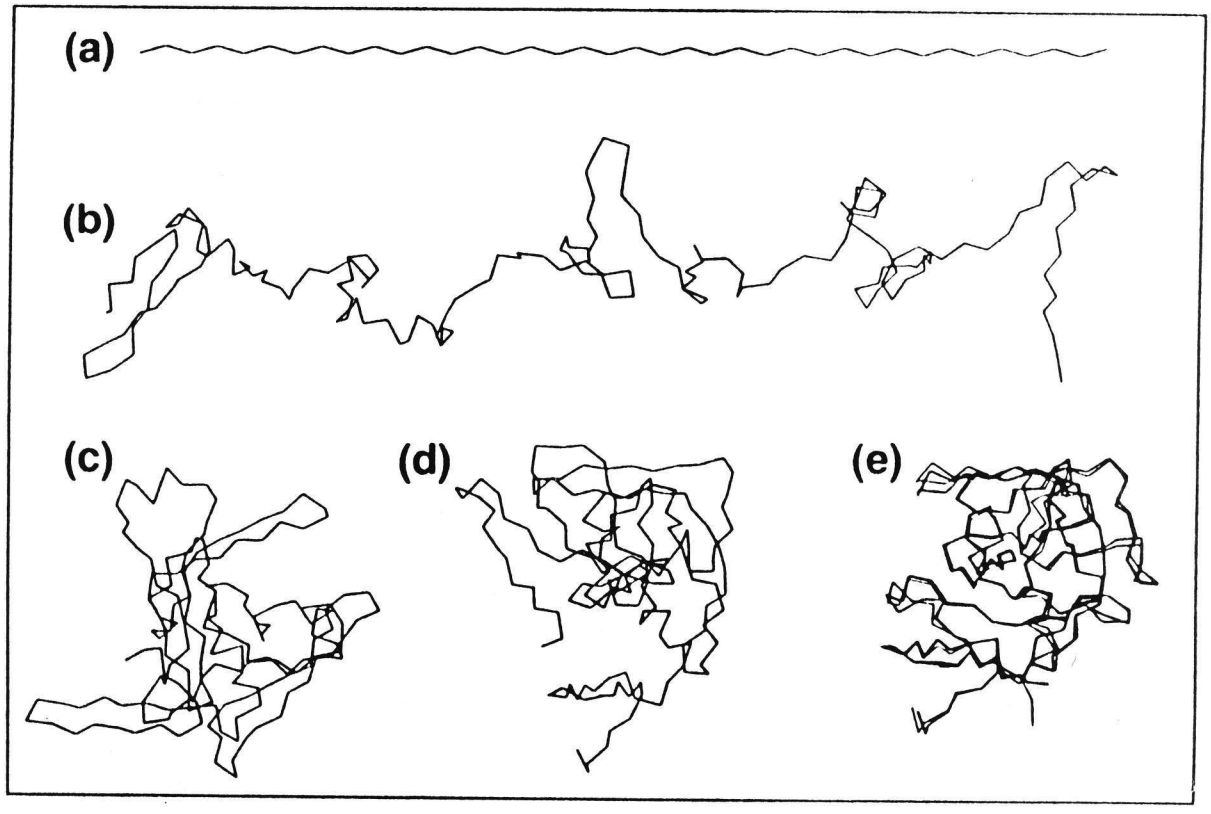
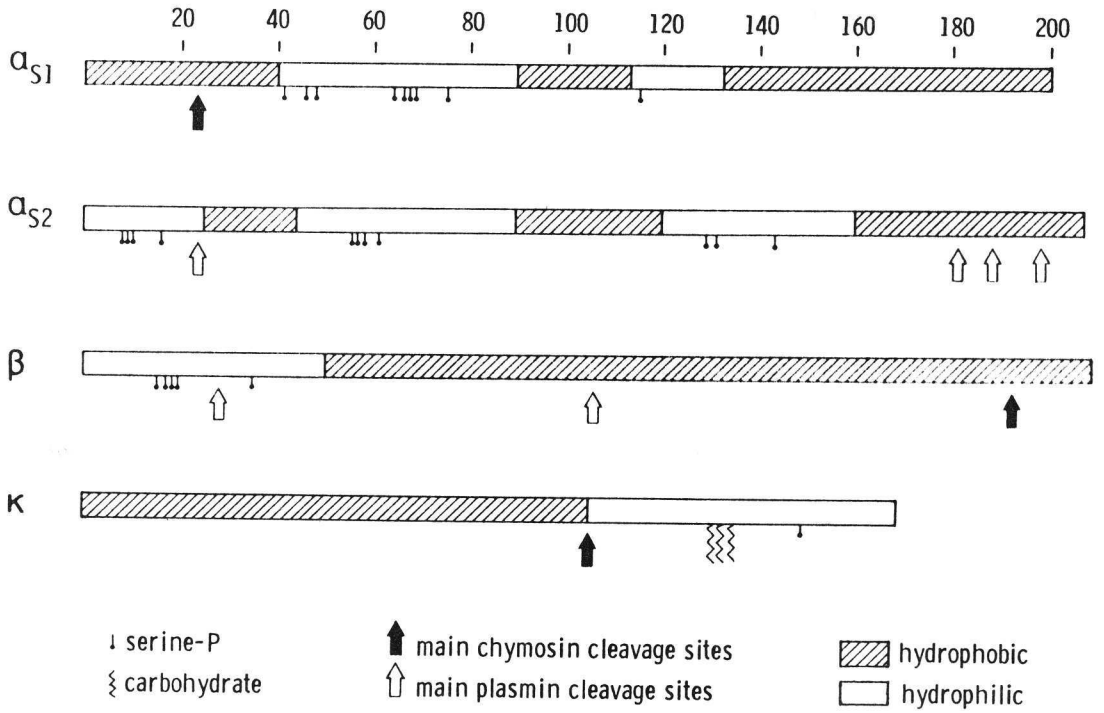
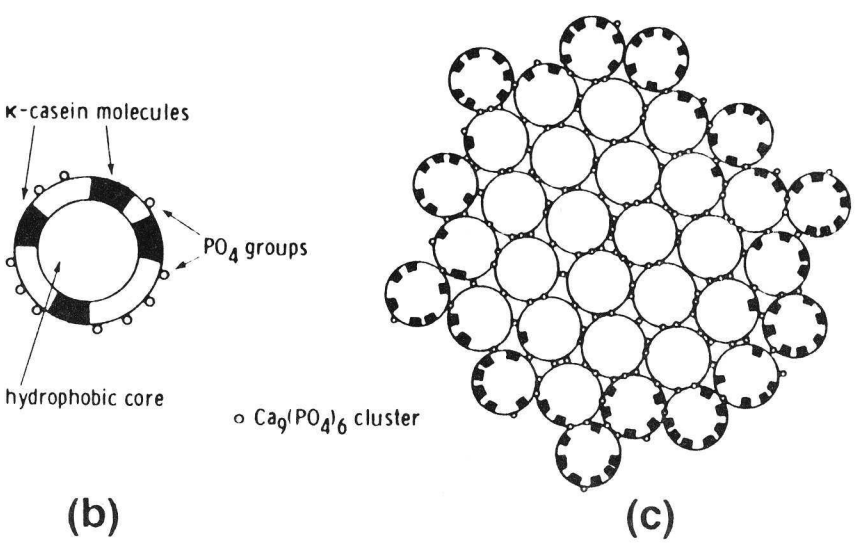


Figure 2


20
40
60
80
100
120
140
160
180
200
α_{S1}
α_{S2}
β
κ
serine-P
carbohydrate
main chymosin cleavage sites
main plasmin cleavage sites
hydrophobic
hydrophilic

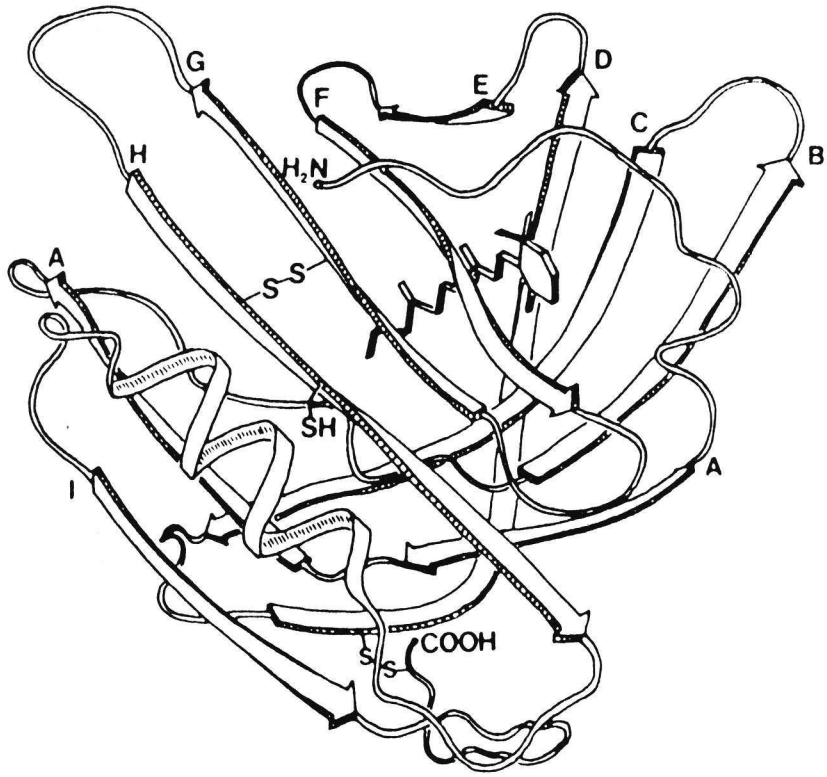
(a)


κ-casein molecules
PO_4 groups
hydrophobic core
o $Ca_9(PO_4)_6$ cluster

(b) (c)

Figure 3

Figure 4



BIOLOGICALLY ACTIVE PEPTIDES FROM MILK PROTEINS

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SUMMARY OF THE LECTURE

My apporoach to the topic "Milk components in food" has its origin in fundamental, structural and phylogenetic studies devoted to a certain number of proteins we performed many years ago.

I always was fascinated by the numerous common points I suspected to exist on a molecular level between the blood and milk clotting processes. Thus the comparative study of these two clotting phenomena was the starting point of our research concerning biologically active milk peptides, mainly casein peptides. The first part of my talk will be devoted to a rapid comparison of the milk and blood clotting processes.

The relationship we observed between both coagulation processes and the structural homologies we characterized between fibrinogen and κ-casein prompted us to describe a series of active casein peptides, more particularly casein peptides, but also peptides from other milk proteins, involved in platelet function, or antithrombotic peptides. These results will constitute the second part of my talk.

In a third part I will discuss some immunomodulating peptides we isolated from casein fractions.

In the conclusion, I will briefly mention many many others peptides from milk proteins endowed with interesting biological activities.

FUNCTIONAL PROPERTIES OF MILK PROTEINS

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THE NETHERLANDS NATIONAL COUNCIL FOR
AGRICULTURAL RESEARCH SYMPOSIUM

MILK COMPONENTS IN FOOD, NOVEL FOOD AND NON-FOOD

INTERNATIONAL AGRICULTURAL CENTER, WAGENINGEN, THE NETHERLANDS

FEBRUARY 27, 1991

SUMMARY

Milk proteins, caseins and whey proteins, provide important functionality to dairy products, i.e., emulsification of milkfat globules, stabilization of foam air cells in whipped cream and frozen desserts, heat stability in sterile milk products, gelation in cheese curd formation, viscosity, colloidal phosphate stabilization, heat-induced gelation and water binding. Isolated caseins, caseinates, whey protein concentrates and whey protein isolates are manufactured and used in large quantities as functional ingredients in dairy, bakery, confectionery, pasta, whipped topping and other formulated food products.

This paper provides a brief overview of the fundamental properties of the major milk proteins, i.e., their percentages in milk and whey; molecular weights; hydrophobicity; sulfur-containing amino acid residues; acidic groups; phosphoserine groups; and their ability to form submicelles and micelles. The role of key reactions that enable milk proteins to provide desireable functionality in milk and other food products are also considered.

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INTRODUCTION

In addition to excellent nutritional value, milk proteins provide critically important functionality to processed milk and dairy products. An important segment of the dairy industry has developed in recent years to isolate, concentrate and dry the proteins from skim milk and cheese whey for use as functional ingredients in a wide variety of forumlated food products.

This paper will consider the general and functional properties of milk protein ingredients, the fundamental properties of milk proteins, the manufacture of milk protein ingredients, and the functionality of milk proteins in formulated food products. A listing of key literature references is included for those who desire more information on this topic.

GENERAL AND FUNCTIONAL PROPERTIES OF MILK PROTEINS

General requirements of milk protein ingredients are listed in Figure 1. Milk protein ingredients are free of toxic chemical, biochemical and microbiological substances and are therefore generally recognized as safe (GRAS substances) by governmental regulatory agencies. Milk protein ingredients are highly digestible and nutritious. They are generally compatible with other ingredients in the food formulation, although care must be taken to select the proper processing conditions for providing the desired functionality of milk protein ingredients. For example, the heat susceptibility of whey proteins may limit their functionality in certain product

applications, but may be essential for other applications. Similarly, the strong dependence of solubility and functionality of casein and caseinates on pH and ionic composition of the food product can be used to advantage in certain food products, but may also be detrimental for other applications. Variations in the composition and functionality of whey protein concentrates (WPC) has limited their utilization in certain food products. Milk protein ingredients are readily available at a competitive price for use by the industry. The inclusion of certain organic chemical compounds that are derived from the oxidation of residual lipids and/or Maillard browning reactions impart off-flavors and off-colors that limit the value of WPC as food ingredients.

The functional requirements of milk protein ingredients are listed in Figure 2. In addition to sensory property requirements, milk protein ingredients must be highly dispersible, soluble and provide adequate viscosity to the food product. In order to meet these and other functionality requirements the protein ingredients must by manufactured, stored and utilized under proper conditions so that they can be fully rehydrated. The proteins may also be required to bind specific mineral ions, function as a surfactant in a variety of product applications that require oil emulsification or foam structure development and stability.

Milk proteins function differently in each food product application. These differences are due to variations in pH, ionic composition and processing treatment conditions. Their functionality is quite predictable in milk and most dairy products due to the more uniform composition of the proteins and mineral salts and ions in milk and to the use of more standardized processing conditions for their manufacture. However, milk protein functionality exhibits a much greater degree of variability in most formulated food product applications due to the wide range of composition and processing treatment conditions. Variations in processing conditions used for manufacturing casein, caseinate and WPC are also responsible for major differences in their functionality.

FUNDAMENTAL PROPERTIES OF CASEINS

There are four major casein subunits, i.e., α_{s1}-Cn, α_{s2}-Cn, β-Cn and κ-Cn (Tables 1 & 2) in approximate weight ratios of 3 α_s-, 2 β- and 1 κ-. Each of these casein subunits contain variable numbers of serine phosphate, proline and CYS/2 groups that strongly affect their chemical and functional properties. The high concentration and uniform distribution of proline groups impart a random coil secondary structure to the casein subunits that renders them highly reactive and subject to interaction via hydrophobic and ionic bonding. Thus, although their monomer molecular weights range from about 19,000 to 25,000, they occur in milk as large, complex structures

in milk, termed micelles, that range in molecular weight up to 2-18 x 10^8 daltons (Figure 3). The size of the micelles is a function of pH, temperature and ionic composition. The caseins are subject to pH-dependent precipitation at their isoionic points that range from about 5 to 5.4. In addition, the casein subunits exhibit an amphiphilic nature due to their uneven distrubution of serine phosphate, carboxyl and hydrophobic amino acid residues along their polypeptide chains. This latter property strongly affects their chemical and functional properties. These fundamental compositional differences result large differences in Ca sensitivity.

The caseins exist in milk as roughly spherical submicelle particles ranging in diameter from about 10 to 20 nm in which up to about 25 subunits are arranged under biological control to provide a hydrophobic core consisting largely of Ca-sensitive α_s-and β-Cn and a hydrophilic outer layer enriched with Ca-insenstive, κ-Cn (Figure 4). These casein submicelles are further assembled into roughly spherical, porous, but stable micelles ranging in size from about 50 to 250 nm. These micelles contain a major portion of the Ca, Mg, phosphate and citrate of the milk as "colloidal phosphate" that provides them with a highly stable structural framework. The micelles interact with mineral ions and soluble casein subunits from the milk serum by an equilibrium mechanism that is affected by pH, temperature and ionic conditions (Figure 5). In addition, κ-Cn with its strongly hydrophilic 106-169 amino acid glycomacropeptide (GMP) segment provides colloidal stability to the micelles in milk. Enzymatic cleavage of κ-Cn subunit chain at the 105-106 peptide bond position by rennet (chymosin) reduces the micelle's zeta potential sufficiently to result in their rapid polymerization to form a gel structure (curd) during the manufacture of cheese.

Heat-induced interaction of the CYS/2 groups in the κ-Cn subunits with CYS/2 groups on denatured β-Lg molecules reduces the susceptibility of casein micelles to the hydrolytic action of rennet, alters their pH-dependent coagulation and syneresis mechanism as in the manufacture of yogurt, and improves the heat stability of sterile milk concentrates.

MANUFACTURE OF CASEIN AND CASEINATES

Acid casein is manufactured by adjusting the pH of skim milk with acid to pH 4.5-4.6, the effective isoelectric point of casein. The caseins precipitate to form a curd which is processed to control particle size, washed to remove minerals and lactose, dried, ground, sieved and packaged. This acid treatment completely destroys the micellar structure and results in the formation of a strongly complexed casein curd of unknown

structure. Rennet casein is manufactured by treating skim milk with rennet to remove the protective GMP groups from the κ-Cn subunits on the micelle to form a curd as for the manufacture of cheese. Caseinates are manufactured by neutralizing acid casein to pH 6.5-7 with NaOH, KOH, or $Ca(OH)_2$ and drying the resulting solution.

FUNCTIONALITY OF CASEINS AND CASEINATES

The functionality of the acid casein and the caseinates is quite different from that of the casein micelles. A major portion of this difference may be due to their derived particle size distribution and structure which may generally resemble casein submicelles in milk. The casein subunits in acid casein and the caseinates are probably more susceptible to dissociation and interaction with other proteins and mineral ions than in the more highly structured casein submicelles and micelles in milk. Acid casein and Ca caseinate are virtually insoluble unless the pH and ionic compositions are properly adjusted. The Na and K caseinates are dispersed in the form of smaller, soluble aggregates in water, the size of which is highly dependent on pH, temperature and ionic environment. Rennet casein lacks solubility in the presence of Ca ions. Ca-caseinates form highly viscous suspensions of casein curd particles which are larger those formed by K and Na caseinates. The caseins generally exhibit excellent heat stability under solution conditions that are removed from their isoelectric point and that contain low Ca ion concentrations. The amphiphilic nature of the casein subunits allows them to function very effectively as emulsifiers and foaming agents.

FUNDAMENTAL PROPERTIES OF WHEY PROTEINS

The fundamental properties of the four major whey proteins are summarized in Table 3 . These proteins have molecular weights that range from 14,000 to ≥ 145,000 daltons and isoionic points ranging from 4.2 to 8.3. β-lactoglobulin (β-Lg), the major whey protein, and α-lactalbumin (α-La) contain only 5 and 1 proline residues per mole, respectively. Bovine serum albumin (BSA) contains 35 CYS/2 residues per mole. The whey proteins therefore exist largely as compact, globular proteins with considerable secondary and tertiary structure. They also contain a fairly uniform distribution of hydrophobic and hydrophilic amino acid residues along their polypeptide chains, which is in contrast to the major casein subunits. However, their relatively high abundance of CYS/2 amino acid residues make them susceptible to heat-induced denaturation, aggregation and interaction with κ-Cn present in the casein micelles. One of the most important properties of the whey proteins is their high degree

of solubility at all pH values. This property makes it much more difficult to isolate them from whey for the purpose of manufacturing whey protein concentrate (WPC) and isolate (WPI). Upon denaturation, however, their molecular structures are unfolded and they behave more like the caseins, i.e., they are much more prone to intermolecular interactions, are relatively insoluble at pH 4.5-5 and become more sensitive to Ca ions.

MANUFACTURE OF WHEY PROTEIN CONCENTRATE AND ISOLATE

Sweet cheese whey is subjected to a series of processing treatments (Figure 6) for the manufacture of whey protein concentrates (WPC) that contain 35-75% w/w protein. The proteins are fractionated from the whey by ultrafiltration (UF). The protein-enriched retentate fraction is usually further purified by diafiltration against demineralized water, concentrated and dried. The whey is suitably pretreated to remove phospholipoprotein complex material that is believed to be derived from from milkfat globule membrane before UF in order to improve the flavor and functionality of the WPC.

Whey protein isolate (WPI) that contains ≥ 90% w/w protein is manufactured by ion exchange adsorption (Figure 7). The whey proteins are adsorbed onto an ion exchanger and the deproteinized whey fraction fraction is eluted from the reactor. The pH of the reactor is adjusted with alkali and the proteins are released and eluted, concentrated and dried.

FUNCTIONALITY OF WHEY PROTEINS

Whey protein concentrates (WPC) and isolates (WPI) are particularly well suited for product applications that require a high protein solubility at acid pH. They also function well in those food product applications that require a protein subject to heat-induced gelation, coagulation and texturization. One example of this application that appears to have considerable promise is the recent development of Simplesse®, which reportedly functions well as a low calorie fat substitute. This product is manufactured by a patented process that utilizes a combination of heating and high shear to provide spherically shaped aggregate protein particles in the size range of 0.1-3 mμ. These whey protein products can also be used to manufacture surimi type structured meat products, baking and other product applications that provide adequate heating to form heat denatured whey protein polymers.

SELECTED MILK PROTEIN FUNCTIONALITY REFERENCES

MORR, C.V. 1979. Utilization of milk proteins as starting materials for other foodstuffs. J. Dairy Research 46: 369.

MORR, C.V. 1979. Functionality of whey protein products. New Zealand J. Dairy Science and Technology 14: 185.

MORR, C.V. Functional properties of milk proteins and their use as food ingredients. In, Developments in Dairy Chemistry-2. Applied Science Publishers. 1982.

MULLER, L.L. Manufacture of casein, caseinates and co-precipitates In, Developments in Dairy Chemistry-2. Applied Science Publishers. 1982.

MARSHALL, K.R. Industrial isolation of milk proteins: whey proteins. In, Developments in Dairy Chemistry-2. Applied Science Publishers. 1982.

MORR, C.V. 1984. Production and use of milk proteins. Food Technology 38: 39.

MORR, C.V. Manufacture, functionality and utilization of milk-protein products. In, Milk Proteins'84. Proceedings of the International Congress on Milk Proteins, Luxemburg, 1984. Pudoc Wagenignen. Wageningen, The Netherlands. 1985.

de WIT, J.N. New approach to the functional characterization of whey proteins for use in food products. In, Milk Proteins'84. Proceedings of the International Congress on MIlk Proteins, Luxemburg, 1984. Pudoc Wageningen. Wageningen, The Netherlands. 1985.

KIRKPATRICK, K. & WALKER, N.J. Casein and caseinates: manufacture and utilization. In, Milk Proteins'84. Proceedings of the International Congress on Milk Proteins, Luxemburg, 1984. Pudoc Wageningen. Wageningen, The Netherlands. 1985.

VISSER, F.M.W. Milk proteins in meat products and soups or sauces. In, Milk Proteins'84. Proceedings of the International Congress on Milk Proteins. Luxemburg. 1984. Pudoc Wageningen. Wageningen, The Netherlands. 1985.

HUYGHEBAERT, A. Applications in confectionery and bakery products. In, Milk Proteins'84. Proceedings of the International Congress on Milk Proteins, Luxemburg, 1984. Pudoc Wageningen. Wageningen, The Netherlands. 1985.

MULLVIHILL, D.M. & FOX, P.F. Assessment of the functional properties of milk protein products. Bulletin No. 209/1987. International Dairy Federation. Bruxelles, Belgium. 1987.

JENSON, G.K., IPSEN, R.H. & IISOE, C. 1987. Functionality and application of dairy ingredients in dairy products. Food Technol. 41: 66.

van den HOVEN, M. 1987. Functionality of dairy ingredients in meat products. Food Technol. 41: 72.

KIRKPATRICK, K.J. & FENWICK, R.M. 1987. Manufacture and general properties of dairy ingredients. Food Technol. 41: 58.

COCUP, R.O. & SANDERSON, W.B. 1987. Functionality of dairy ingredients in bakery products. Food Technol. 41: 86.

McDERMOTT, R.L. Functionality of dairy ingredients in infant formula and nutritional speciality products. Food Technol. 41: 91.

KINSELLA, J.E., et al. Milk proteins: possible relationships of structure and function. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

MULVIHILL, D.M. Caseins and caseinates: manufacture. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

MULVIHILL, D.M. & FOX, P.F. Physico-chemical and functional properties of milk proteins. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

SOUTHWARD, C.R. Uses of casein and caseinates. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

MORR, C.V. Whey proteins: manufacture. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

de WIT, J.N. Functional properties of whey proteins. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

de WIT, J.N. The use of whey protein products. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

NAKAI, S. & Li-CHAN, E. Chemical and enzymatic modification of milk proteins. In, Developments in Dairy Chemistry-4. Elsevier Applied Science. 1989.

MANGINO, M.E. Functionality of whey protein concentrates. In, Proceedings Dairy Products Technical Conference. American Dairy Products Institute. Chicago, IL, USA. 1990.

MORR, C.V. Whey protein functionality: current status and the need for improved quality and functionality. In, Proceedings Dairy Products Technical Conference. American Dairy Products Institute. Chicago, IL, USA. 1990.

HOWELL, J. et al. Manufacture and use of high protein whey products. In, Proceedings Dairy Products Technical Conference. American Dairy Products Institute. Chicago, IL, USA. 1990.

SINGER, N.S. Simplesse® all natural fat substitute and the dairy industry. In, Proceedings Dairy Products Technical Conference. American Dairy Products Institute. Chicago, IL, USA. 1990.

PUHAN, Z. Manufacture and use of milk protein concentrates. In, Proceedings Dairy Products Technical Conference. American Dairy Products Institute. Chicago, IL, USA. 1990.

MORR, C.V. & FOEGEDING, E.A. 1990. Composition and functionality of commercial milk protein concentrates and isolates: A status report. Food Technology 44: 100.

TABLE 1. FUNDAMENTAL PROPERTIES OF MAJOR CASEIN SUBUNITS[a]

	%[a]	SER P[b]	PROLINE RESIDUES[b]	CYS/2[b]
α_{s1}-	44	8	17	0
α_{s2}-	11-12	10-13	10	2
β-	11-12	5	35	0
κ-	27-32	1	20	2

TABLE 2. FUNDAMENTAL PROPERTIES OF MAJOR CASEIN SUBUNITS[a]

	MW (DALTONS)	pI	HYDRO-PHOBICITY (kJ/RESIDUE)	CHARGE (pH 6.6)
α_{s1}-	23,600	4.96	4.87	-20.9
α_{S1}-	25,300	5.2-5.4	4.64	-13/-16
β-	23,980	5.19	5.58	-12.3
κ-	19,000	5.43	5.12	- 3.9

[a] MODIFIED FROM FOX, 1982

TABLE 3. FUNDAMENTAL PROPERTIES OF MAJOR WHEY PROTEINS[a]

	%	pI[b]	MW[c]	CYS/2[d]
α-Lactalbumin	25	4.2	14,000	8
β-Lactoglobulin	55	5.1	18,000	5
Bovine serum albumin	12	5.1	66,000	35
Immunoglobulins	8	5.5-8.3	≥145,000	-

[a] J. Dairy Sci. 67: 1599. 1984
[b] based on amino acid composition
[c] daltons
[d] mole basis

FIGURE 1. MILK PROTEIN INGREDIENT GENERAL REQUIREMENTS

FREE OF TOXIC & ANTI-NUTRITIONAL FACTORS
- PATHOGENIC MICROORGANISMS
- ENZYME INHIBITORS
- NATURAL AND MICROBIAL TOXINS

FREE OF OFF-FLAVORS & OFF-COLORS

HIGH DIGESTIBILITY AND NUTRITIONAL VALUE

COMPATIBLE WITH OTHER INGREDIENTS AND PROCESSING CONDITIONS

CONSISTENT COMPOSITION AND FUNCTIONALITY

READILY AVAILABLE AT A COMPETITIVE COST

FIGURE 2. MILK PROTEIN INGREDIENT FUNCTIONAL REQUIREMENTS

SENSORY (FLAVOR, ODOR & COLOR)

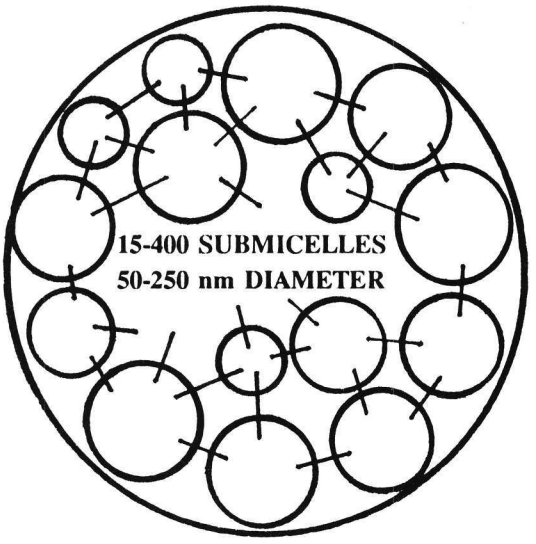
HYDRATION (DISPERSIBILITY, SOLUBILITY & VISCOSITY)

MINERAL ION BINDING

SURFACTANT (EMULSIFICATION OF OIL/FAT AND FOAM FORMATION/STABILITY)

pH AND/OR HEAT-INDUCED POLYMERIZATION (EXTRUSION, BAKING, COAGULATION & GELATION

ADHESION & COHESION (REFORMED, STRUCTURED MEAT PRODUCTS)



⇌ β-Cn-ENRICHED SOLUBLE CASEIN

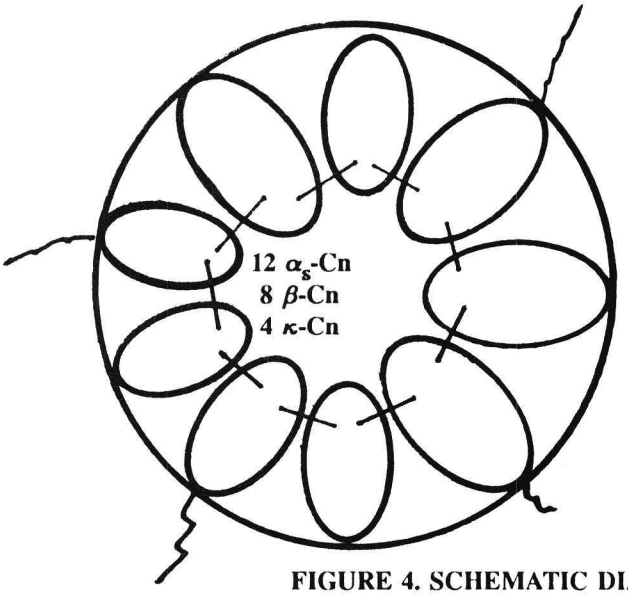
⇌ SOLUBLE Ca^{+2}, Mg^{+2}, PO_4^{-3} & $CITRATE^{-3}$

⇌ INTERACTION WITH HEAT DENATURED β-Lg

⇌ INTERACTION WITH RENNIN (CHYMOSIN)

FIGURE 3. SCHEMATIC DIAGRAM OF CASEIN MICELLES

GMP



α_s- & β- Cn-ENRICHED,
HYDROPHOBIC CENTER CORE ZONE

κ- Cn (GMP)-ENRICHED,
HYDROPHILIC LAYER

FIGURE 4. SCHEMATIC DIAGRAM OF CASEIN SUB-MICELLES

FIGURE 5. ASSOCIATION/DISSOCIATION OF CASEIN MICELLES

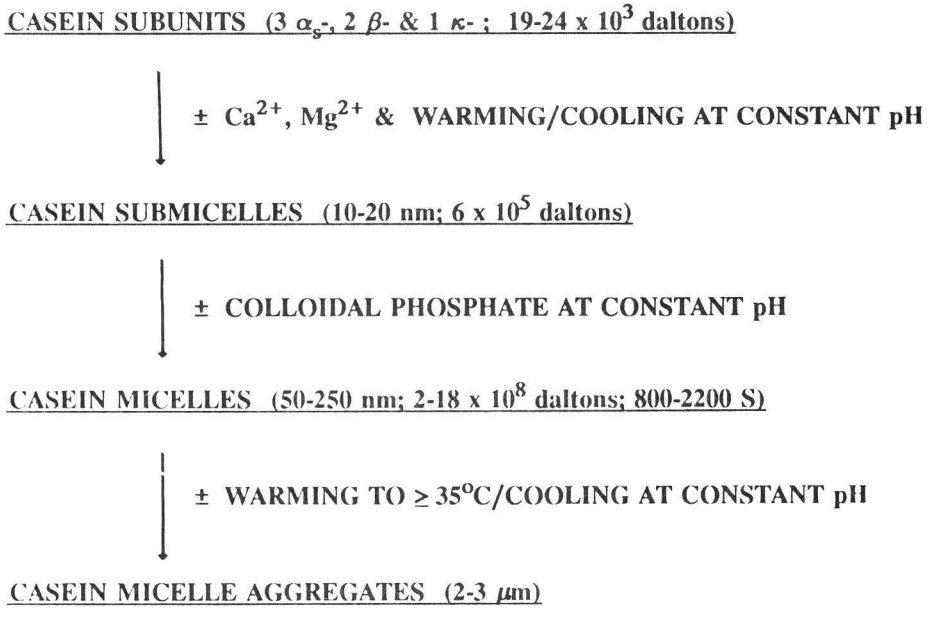


FIGURE 6. MANUFACTURE OF WHEY PROTEIN CONCENTRATE BY ULTRAFILTRATION

SWEET CHEESE WHEY (pH ≥ 6.2)

PASTEURIZE AND COOL TO 0-5°C

OPTIONAL STEP: ADD $CaCl_2$ TO PROVIDE 1.2 gL^{-1} (30 mM) Ca AND RAPIDLY HEAT TO 50°C

CENTRIFUGAL CLARIFICATION AND TANGENTIAL-FLOW MICROFILTER (MF) TO REMOVE RESIDUAL LIPIDS AND PHOSPHOLIPOPROTEIN COMPLEX MATERIALS

ULTRAFILTER MF PERMEATE TO VCR ≥ 20

DIAFILTER UF RETENTATE WITH ≥ 3 VOLUMES DEMINERALIZED WATER

CONCENTRATE AND/OR SPRAY DRY PROTEIN-ENRICHED RETENTATE

FIGURE 7. MANUFACTURE WHEY PROTEIN ISOLATE BY ION EXCHANGE PROCESS[a]

CHEESE WHEY ADJUSTED TO pH ~ 3.2 WITH ACID

ADSORB PROTEINS (+ CHARGE) ON REGENERATED CELLULOSE IN STIRRED-BED REACTOR

ADJUST pH TO ~ 9 TO DESORB PROTEINS

ELUTE DEPROTEINIZED WHEY CONTAINING LACTOSE, MINERALS AND LIPIDS

DESORB AND RECOVER WHEY PROTEINS IN A DILUTE (~ 1%) SOLUTION

CONCENTRATE PROTEIN SOLUTION BY UF AND VACUUM EVAPORATION AND DRY

[a] Food Processing 51(1): 1990.

Fermentative production and applications of low-molecular compounds from lactose (whey)

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1. Introduction

Since time immemorial man has employed micro-organisms in the preparation of such foods as cheese, fermented milks and alcoholic beverages. The production of gas and formation of alcohol which takes place in the brewing of beer was known as fermentation. In the course of time the term 'fermentation' took on a wider meaning, and today it covers all conversions in bioreactors which are due to the activity of micro-organisms. The micro-organisms convert a substrate, generally carbohydrates, into cell mass (biomass) and a number of secondary reaction products.

2. History

For many centuries fermentation was a process of trial and error in which a certain degree of efficiency merely was reached empirically, without knowing what was happening biologically. Only at the end of the last century efforts were made to control fermentation processes to manufacture products of standard quality on a large scale and at relative low costs.

So, from around 1875 (Table 1) true industrial microbiology set in with large-scale production of ethanol for technical and other uses, in 1881 of lactic acid for the food industry and in 1895 of mould amylase, the first enzyme, for the textile industry. At that time research was still in its infancy, and the attained process control was in general fairly primitive. As is often the case with technological advance the impetus was provided by war. At the start of the First World War the need of glycerol for explosives manufacture increased and in 1915 (Germany) the microbiological production of this compound from sugar with the aid of yeast was taken up. For economic reasons the fermentative production method was granted only a relative short life; from 1940 on glycerol was synthetically produced from propylene.
About the same time the aceton-butanol fermentation using Clostridium acetobutylicum begun (England). In addition to these solvents used for manufacture of munitions and artificial rubber respectively, also hydrogen gas is produced. Not surprisingly that in that time a number of manufacturing plants were destroyed by explosions. The process survived until the early 1950's, when many organic chemicals, including aceton and butanol, became readily available from by-products

of the petroleum industry. Meanwhile the First World War had initiated a rapid growth of research effort, not only in relation with the production of glycerol and aceton for explosives manufacture, but also outside this scope. This development continued in the inter-war period. As more knowledge was gathered on the growth conditions and the physiology of different kinds of micro-organisms, on (bio)reactor technology and on the physical operations which are needed to isolate and purify the final product, collectively known as downstream processing, one succeeded in raising the yield of existing processes. Moreover new fermentation products appeared.

The escalated price of citric acid, extracted from citrus fruits (Italy) gave rise to microbial production of this acid in 1923, using the obligate aerobic mould Aspergillus niger. Apparently it was the first aerobic industrial fermentation process at which the presence of sufficient oxygen initially was achieved by surface culturing in shallow metal pans. Later on this method was improved by absorbing the nutrient medium on an inert granular support.
Microbiological production methods for gluconic acid and sorbose were introduced in the thirties. The vitamin C (ascorbic acid)-manufacturing process consists of several chemical steps and a fermentative one. Sorbitol, an abundant raw material originating from the catalytic reduction of glucose, is microbiologically oxydized into sorbose using Acetobacter suboxydans. By chemical means sorbose is converted into the vitamin.
In 1940 the production of penicillins was realized, initially using the mould Penicillium notatum, yielding only 2 mg per liter of culture fluid. By screening many different Penicillium-species and by subjecting them to mutagenic influences together with other improvements in the process, a variant of P. chrysogenum was identified which produced 8 g per liter.
After the introduction of the penicillin fermentation there has been an ever-increasing contribution of fermentation technology to the production of therapeutic compounds. Some 50 different antibiotics useful in the treatment of infection diseases, but also compounds like vitamins B2 and B12, are now being produced on a relative large scale by microbial processes or by procedures involving these processes in the intermediary steps.
Gibberellic acid belongs to a group of 14 close-related compounds, the so-called gibberellins, which have vitamin-like effects on plants. They stimulate the germination and growth of seeds, on the other hand they have a retarding effect on the ripening of some kinds of fruit as bananas.
In the late 1960's considerable excitement was generated by the prospect of using microbial cells ("biomass") as a source of protein, the so-called single-cell protein (SCP). However by other than technological reasons (in the main economic) the introduction of SCP has not been a success.

3. Raw materials

Carbon sources used as raw materials for the industrial production of the compounds mentioned in Table 1 are in general sugars as glucose, sucrose and maltose, whether or not refined, or cheaper substances, rich in carbohydrates, as molasses. Nitrogenous sources such as malt extract or corn steep liquor and, if required yeast extract (vitamins) and minerals must supplement most carbohydrate sources to give fast and heavy growth of the used micro-organism. In practice minimal amounts of these substances are used in order to simplify the downstream processing and to decrease the cost of the process. Calcium carbonate and calcium hydroxide (limemilk) are often used to neutralize the acid produced during the fermentation.

3.1 Lactose (whey) as carbohydrate

Despite the very extensive literature on this subject, as far as we know, in the existing industrial fermentation processes lactose, whey or whey (ultrafiltration) permeate, by-products of the dairy industry, are only used as starting material on a small scale or are not used at all. The reason is that lactose in general is more expensive than sucrose. "Edible" and "refined edible" lactose, isolated from whey, find wide application in the manufacture of infant and other food formulations and in the pharmaceutical industry respectively. Whey with a reduced lactose content, so-called "delactosed" whey, which still has a relative high lactose content, is increasingly used in human food and moreover in cattle feed. In particular the use of "delactosed" whey and whey permeate as raw materials for fermentation processes, if neccessary supplemented with extra lactose and other components, should give these by-products a higher commercial value.

4. Fermentation products from lactose

In table 2 some compounds are summarized which in principle can be produced from lactose of from raw materials with lactose as base, like whey, whey permeate or "delactosed" whey. Some of them are already industrially produced from glucose or sucrose-containing raw materials (Table 1). The production of these compounds from lactose will be in general only attractive if the cost price works out favourable in comparison with that of the same products manufactured from glucose or sucrose. For ethanol, butanol/aceton and acetic acid this is in our country not the case; rough calculations have shown that the production costs of these compounds almost correspond with the current prices in the market, even when advanced fermentation technologies would be applied. Also for other reasons the development of production processes may fail to return a financial profit. Here I have in mind for example a low demand and a limited market volume, the competitive position, winning a position in the market for the product and so on.

In our oppinion the fermentative production of these compounds from lactose-containing raw materials does not appear promising.

Much attention has been paid at NIZO during the last 20 years to the fermentative production from lactose of some other non-dairy products, mentioned in Table 3.

4.1 Racemic lactic acid (starter permeate)

About 1975 the declining sale of buttermilk, the sour by-product of the manufacture of traditional cultured butter from cultured cream, gave rise to the development of a new method of buttermaking for which a lactic acid preparation was needed.
By that time the production of butter was becoming increasingly concentrated in specialised dairy factories, and particularly when the product was made in areas where there was no market for buttermilk, difficulties could arise because the number of dairy products, which can be made from or with the addition of cultured buttermilk, is limited.

According to the traditional process, milk is separated and the cream (35-40 % fat) is pasteurized. After inoculation with a starter, which contains, in addition to normal starter streptococci, so-called aroma bacteria, the cream is ripened. During this period lactic acid and aroma compounds, particularly diacetyl, are produced. By churning the ripened cream, fat globules are combined into butter granules and the sour buttermilk is liberated. Lactic acid and aroma compounds find their way into both the butter granules and the buttermilk. Finally the butter granules are worked to achieve a fine dispersion of moisture in the finished product, which contains max. 16 % (^{w}/w) moisture, min. 82 % fat and min. 1 mg diacetyl per kg. The pH of the serum of the butter ought to be 5.3 or lower.
In the modern method of buttermaking the traditional process is divided into three independent steps: (1) the production of sweet buttermilk and sweet butter with a low moisture content (13,5 %) by churning sweet cream; (2) the production of lactic acid in the form of "starter permeate"; (3) the production of aroma compounds in the form of a high diacetyl-producing starter. The new production method for cultured butter consists then in working the required quantities of starter permeate (lactic acid) and starter (aroma compounds), generally as aerated mixture of both components, into the sweet butter. With respect to the max. moisture content of the final product, 16 %, the quantities of these additives are limited (a margin of not more than 2,5 % is available). In this way butter is obtained with the characteristic aroma of butter made according to the traditional process from cultured cream.
By using this method the production of sour buttermilk is avoided; sweet buttermilk has better properties for the production of other dairy products. Moreover, as the three steps mentioned above proceed independently, a better process control is achieved and the "natural character " of

buttermaking is maintained. Meanwhile the method is applied on a large scale both in our and foreign countries.

For the production of the lactic acid preparation, starter permeate, used as additive in the modern way of buttermaking, a batch-wise fermentation is applied. The process is schematically shown in Figure 1.
A solution of 4 % "delactosed" whey powder in a volume of 60.000 l, containing 2 % lactose, is pasteurized and inoculated with a strain of L. helveticus. This species is known as a very acid-resistent micro-organism having a high acid-producing capacity, even below a pH of 4.5. Next the whey is soured during 48-60 hrs after which the lactose is almost completely fermented. To separate protein and bacteria the whey culture is submitted to ultrafiltration and the permeate is finally concentrated by evaporation until the lactic acid content amounts to 15 %. PH of the product amounts to 3.0. About 0.5 % of this final product together with 0.75 % starter is sufficient to lower the pH of sweet-cream butter to 5.3.

4.2 Diacetyl (starter distillate)
The starter used in buttermaking from sweet cream should generate sufficient aroma compounds, in particular diacetyl, to yield butter with a diacetyl content of min. 1 mg/kg. However, the availability of these high diacetyl-producing starters is limited. Moreover, they are generally sensitive to bacteriophages. In practice when no precautions are taken to prevent phage-infection the production of diacetyl can be inhibited.
These considerations gave rise to the development of a fermentative method for the production of diacetyl in the form of a distillate of the high aromatic starter.
Diacetyl can be produced by culturing the starter, containing Lactococcus lactis subsp. lactis var. diacetylactis (formerly Streptococcus diacetylactis) as aroma bacterium, in whey or whey permeate. After 18-20 hrs fermentation without neutralization the citric acid, naturally present in these starting materials and the substrate for diacetyl formation, is completely fermented. The whey culture is then intensively aerated by which the diacetyl content increases to 100 mg/l. The increase is due to a more complete oxidation during the spontaneous decarboxylation of α-acetolactic acid, the precursor of diacetyl. Next the aerated culture is submitted to ultrafiltration and diacetyl and some other volatile components present in the permeate are separated and concentrated by rectification. The diacetyl content of the final product amounts to 1800-2000 mg/l, dependent of the separation power of the rectification apparatus used. Moreover it contains small amounts of acetic, propionic, butyric, isobutyric and lactic acid. By the antimicrobial properties of diacetyl, particularly against gram negative bacteria and yeasts, and the low ph of the product, it can be stored almost unlimited at 5 °C. Also the chemical keeping quality is good; only after 8-10 months storage the diacetyl content showed a significant decrease. A great deal of practical experience was gained during buttermaking experiments using starter

distillate. The results have shown that the substitution of the aromatic starter by this additive leads to a further improvement of process control on the buttermaking from sweet cream. By using starter distillate the natural character of the method is unaffected.

4.3. D(-) lactic acid

Already a couple of years ago the D(-) isomer of lactic acid, or more exactly, esters of this isomer, attracted attention in literature as starting materials for the so-called chiral synthesis of biologically active compounds, particularly some kinds of herbicides and possibly pharmaceuticals. The biological activity of compounds is generally strictly linked to one particular isomer of these compounds. This means that the chemical synthesis of biologically active compounds from a racemic mixture of the starting material yields also a racemic final product. Only 50 % of it will have biological activity and the remaining 50 % is inactive and may be considered as "ballast" in this respect. The synthesis of this kind of herbicides from esters of D(-)lactic acid yields a product with 100 % biological activity. The use of this isomer for the production of herbicides gives it therefore a higher commercial value.
This consideration gave rise to the development of a fermentative production method for D(-)lactic acid from lactose as starting material.

According to Bergey's Manual of Systematic Bacteriology only L. delbrueckii subsp. lactis (formerly L.lactis) and L. delbrueckii subsp. bulgaricus (formerly L. bulgaricus) are able to ferment lactose homofermentatively into D(-)lactic acid. However, galactose is normally not fermented by these micro-organisms. This means that the acid production from lactose is max. 50 % (theoretically) and galactose remains unfermented.
We found in our collection some strains of lactic acid bacteria, identified as L. delbrueckii subsp. bulgaricus, which are capable of converting not only lactose, but also galactose into D(-) lactic acid. These strains have been deposited with the Centraal Bureau voor Schimmelcultures in Baarn, the Netherlands.
Fermentation experiments were carried out using these strains in whey (2000 l), supplemented with (extra) lactose, 10 % skim milk and some growth stimulants. In 72 hrs at 37 °C and constant pH 6,0 with limemilk as neutralizer, 11 % lactose was completely fermented. The total lactic acid yield amounted to 99 % of the theoretical value. The optical contamination with the L(+) isomer was < 1 %.
In aquaus lactose solution, supplemented with the additions above a similar conversion rate was observed.
The downstream processing includes a.o. precipitation and separation of Ca^{2+} ions as gypsum by addition of equimolar sulfuric acid, decoloration, reversed osmosis with selective membranes, ion-exchange and finally concentration by evaporation.

4.4 L(+)lactic acid

In general sucrose is the raw material for most of the industrially produced lactic acid (racemic and the L(+) isomer). As lactose is more expensive than sucrose the production costs of lactic acid produced from lactose will be higher.
Nevertheless the fermentative production of L(+) lactic acid from lactose using L.casei is within the possibilities.

4.5 Vitamin B12

You all know that vitamin B12 (cyanocobalamin) plays a part in the metabolism of higher animals and micro-organisms. It has not only a function in the synthesis of hemoglobin, but it is also a co-factor of enzyme systems. By this reason it is used as growth factor for poultry and other animals.
The vitamin is naturally present in animal tissues and in cow's milk and mother milk (4 and 300 µg/l). In higher plants it is not present. Vitamin B12 is a dark red compound, which is only produced by micro-organisms. It belongs to the so-called cobalamins; their structures are shown in Fig. 2. Not only vitamin B12, cyanocobalamin, but also the other cobalamins have physiological importance. By treatment with cyanide the other cobalamins are transformed into vitamin B12.
A large number of quite different micro-organisms are able to produce the vitamin, as can be seen in Table 4, which is taken from literature. The vitamin yields found in media containing different carbon sources vary strongly. Particularly the subspecies shermanii and freudenreichii of the species Propionibacterium freudenreichii are metioned as the proper micro-organisms for industrial production of the vitamin. In this case also propionic and acetic acid is produced. The main lines of the mechanism of vitamin B12 biosynthesis are known.
For optimal vitamin production the medium is not only supplemented with cobalt ions, but also with a number of vitamin B12 precursors, like a.o.5,6-dimethylbenzimidazol.
The vitamin B12 production process consist of two stages. In the first anaerobic period the bacteria are cultured and the sugar present, is completely fermented. In the second aerobic period the particular vitamin is produced.
In NIZO we screened a number of strains of the subspecies shermanii and freundenreichii on the capacity of producing the vitamin. Some strains were known as good vitamin B12 producers. In the experiments whey was used as medium, supplemented with all precursors known from literature. After the anaerobic period lactose in most cultures was completely fermented. The highest vitamin B12 concentration was found in a culture of a strain of the subspecies shermanii and amounted to 10 mg/l. In a similar medium containing glucose as carbon source almost the same vitamin content was detected. This level is however considerable lower than found in literature (Table 4). Efforts to optimize the vitamin production were not successful.

Apparently most manufacturers of vitamin B12 possess high yielding strains, possibly spontaneous or induced mutants, which are not available for outsiders.

From literature data it may be concluded that the isolation and separation of propionic and acetic acid, at the same time produced as the vitamin, hardly make the process economically more attractiv.

4.6 Lactobionic acid

Lactobionic acid is a white crystalline compound, which can be produced from lactose, with sequestrant and emulsifying properties. In literature these properties suggest a commercial potential for the product.
In food, a.o. milk, it combines a pH-decreasing effect with a sweet taste. Therefore it may be considered as a sweet acid and as such it could be of use as a special food acidulant and may have possibilities for use as a lactose replacement in specially health foods. Lactobionic acid is a solubilizing agent for calcium salts; i.e. it forms highly concentrated aqueous solutions. Solutions of calcium lactobionate containing up to 70 % salt have been prepared. This product may prove valuable in medicine a.o. as a source of calcium.

4.6.1. Structure and production

The structure as well as production methods for the acid are shown in Fig. 3. Lactobionic acid (4-O-β -D-galactopyranosyl-D-gluconic acid) is derived from lactose by conversion of the aldehyde group of the glucose part of the molecule into a carboxylic group (oxidation).
The oxidation of lactose can be carried out by chemical means, with the help of micro-organisms (fermentation) or enzymatically.

4.6.2. Chemical oxidation

Already in 1889 was found that by using bromine water, a mild oxidation reagent, lactobionic acid is produced from lactose. The conversion was slow and moreover the product was partly hydrolysed under these circumstances, resulting in a poor yield. In the presence of buffering salts the speed of the oxidation is greatly increased due to the maintenance of a relative high pH. Owing to this, the yield would be enhanced from 30 to nearly 100 % without hydrolysis of the product. Lactobionic acid may also be produced by electrolytic oxidation of lactose.

4.6.3. Microbiological oxidation

Certain aerobic micro-organisms of the genera Pseudomonas and Achromobacter accumulate lactobionic acid during fermentation of lactose. In particular Ps. taetrolens should produce the acid with the highest yield (77 %). The activity of this and other Pseudomonas species is not restricted to oxidation of lactose. Also maltose and cellobiose could be oxidized into the corresponding aldonic acids.

4.6.4 Enzymatic oxidation

Enzyme systems, present in so-called particulate fractions of bacteria of the genus Pseudomonas, for instance Ps. taetrolens, are able to oxidize lactose into lactobionic acid. Probably two enzymes are active in this conversion, one dehydrogenase, giving the δ-lacton of the acid, and another enzyme, lactonase, yielding the free acid.

The production of lactobionic acid from lactose by a fermentation process using Pseudomonas species will probably be most promising. The conversion of lactose will be limited by the produced acid (pH decrease). Therefore it will be advantageous to maintain a constant pH during the fermentation process by neutralization with limemilk.

In NIZO strains of Pseudomonas species were screened on lactobionic acid production in whey, supplemented with growth stimulants and minerals, by shaking at 30 °C. We found that particularly strains of Ps. fragi and Ps. taetrolens are the best producers of the acid.

Summary
In a historical survey the development of the fermentation industry is shown. For most of the existing industrial fermentative production processes sugars as glucose and sucrose, eventually as molasses, are generally used as starting materials. Because lactose is more expensive it is hardly used or is not used at all in these processes. The possibility to use lactose as starting material for the fermentative production of some "new" compounds was considered.

Consulted literature

M.M. Young, Comprehensive Biotechnology Vol. 3 (Pergamon Press, New York, 1984).

H.J. Peppler & D. Perlman, Microbial Technology Vol. 1, (Academic Press, New York, 1979).

A.L. Demain & N.A. Soloman, Manual of Industrial Microbiology and Biotechnology (American Soc. for Microbiology, Washington, 1986).

Table 1 - Industrial microbiological production of some compounds (rough historical survey)

year	product	micro-organism
1875	ethanol	Saccharomyces cerevisiae, other yeasts
1881	lactic acid	Lactobacillus delbrueckii, other lactic acid bact.
1894	(mould) amylase	Aspergillus oryzae
1914	glycerol[1])	Torulopsis magnoliae, other yeasts
1915	aceton/butanol[2)]	Clostridium acetobutylicum
1923	citric acid	Aspergillus niger
1930	gluconic acid	Aspergillus niger, Penicillium species
1937	sorbose (intermediate vit. C-production)	Acetobacter suboxydans
1940	penicillins	Penicillium notatum, chrysogenum
1947	riboflavin (vit. B2)	Clostridium acetobutylicum, yeasts
1954	glutamic acid	Micrococcus glutamicus
1957	vitamin B12	Propionibacterium shermanii, freudenreichii
1960	lysin	Micrococcus glutamicus
1961	xanthan	Xanthomonas campestris
1961	threonine	Escheria coli
1962	gibberellic acid	Fusarium moliniforme

1) until 1940
2) until 1950

Table 2 - Possibilities of producing non-dairy products from lactose (whey, whey permeate or "delactosed" whey) by fermentation

product	micro-organism
ethanol	Kluyveromyces fragilis, other lactose-fermenting yeasts
acetic acid	Clostridium thermoaceticum
butanol/aceton	Clostridium beyerinckii
Xanthan	Xanthomonas campestris
citric acid	Aspergillus niger
2.3-butanediol	Bacillus polymyxia, Klebsiella pneumoniae, Enterobacter cloacae
threonine	Escherichia coli
gibberellic acid	Fusarium moliniforme

Table 3 - Production of non-dairy products from lactose (whey, whey permeate or "delactosed" whey) by fermentation (some processes realized)

Product	micro-organism
racemic lactic acid ("starter permeate")	Lactobacillus helveticus
D(-) lactic acid	Lactobacillus bulgaricus
L(+) lactic acid	Lactobacillus casei
diacetyl (starter distillate)	Lactococcus lactis subsp. lactis var. diacetylactis
vitamin B12 + acetic-/propionic acid	Propionibacterium freudenreichii subsp. shermanii, freudenreichii
Lactobionic acid	Pseudomonas taetrolens, fragi a.o.

Table 4 - Production of vitamin B12 by various micro-organisms

micro-organism	carbon source	vitamin B12 content (mg/l)
Micromonospora sp.	Glucose	11.5
Nocardia rugosa	Glucose-cane molasses	14
Propionibacterium freudenreichii	Glucose	25
Propionibacterium shermanii	Glucose	23
Propionibacterium shermanii	Glucose	28
Propionibacterium shermanii	Glucose	39
Propionibacterium vannielli	Glucose	25
Pseudomonas denitrificans	Beet molasses	59
Streptomyces olivaceus	Glucose-lactose	8.5
Mixed methanogenic bacteria	Methanol	35
Bacterium FM-02T	Methanol	2.6
Methanobacillus omelianskii	Methanol	8.8
Protoaminobacter ruber	Methanol	2.5
Corynebacterium and Rhodopseudomonas	n-Paraffins	2.3
Nocardia gardneri	Hexadecane	4.5

Figure 1
Fermentative production of racemic lactic acid (starter permeate)
(schematic)

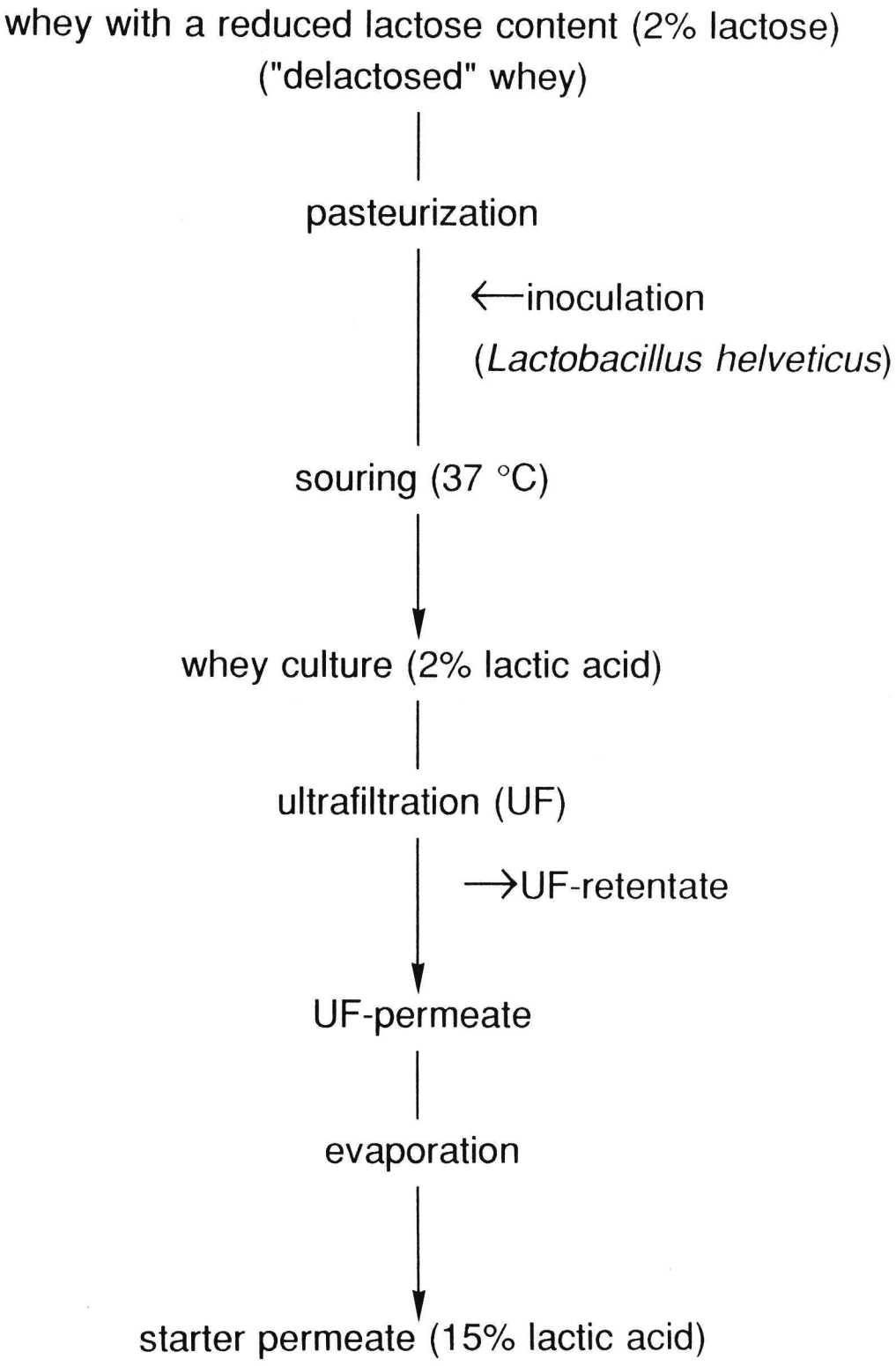
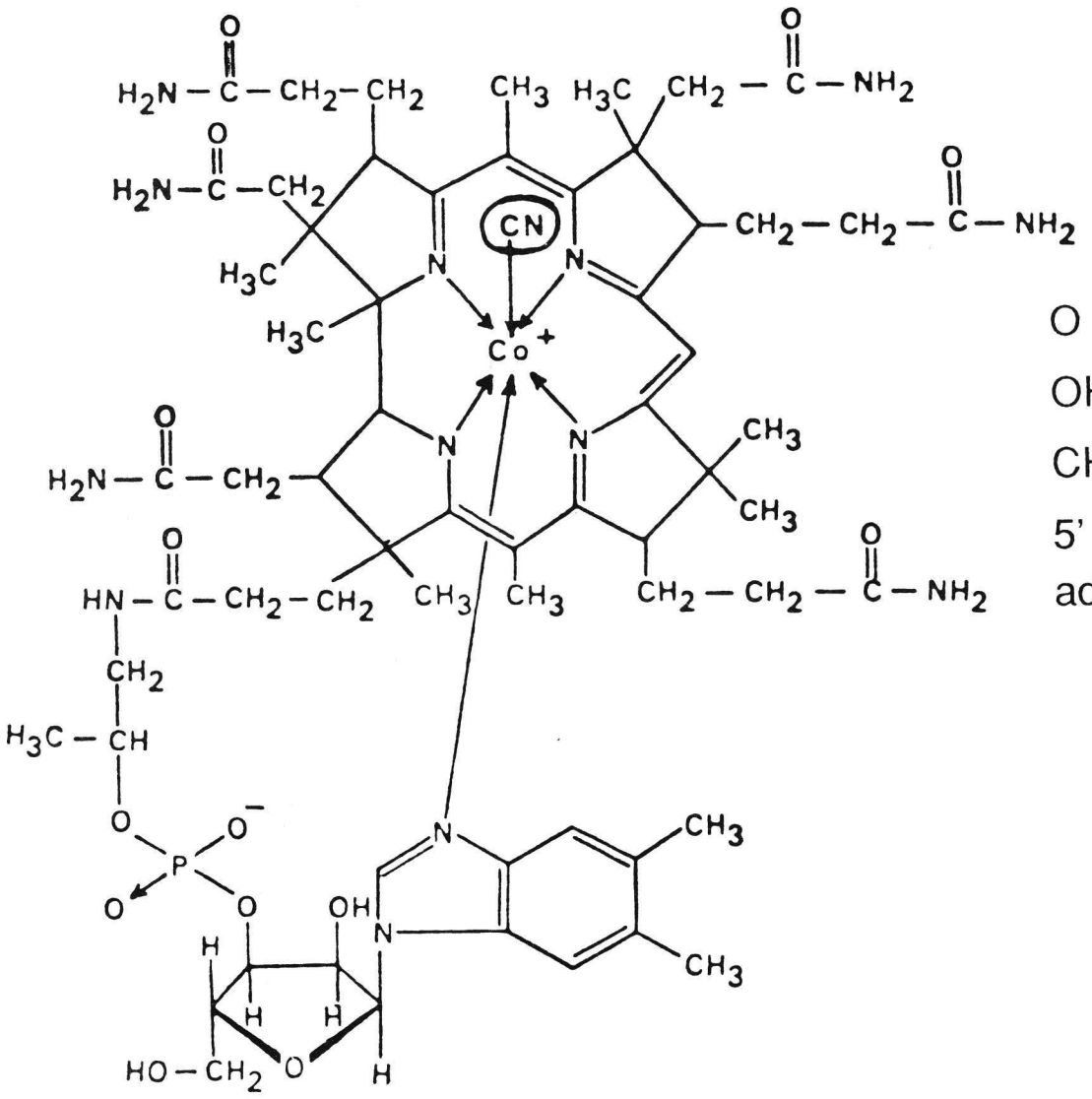


Figure 2

Structure of vitamin B12

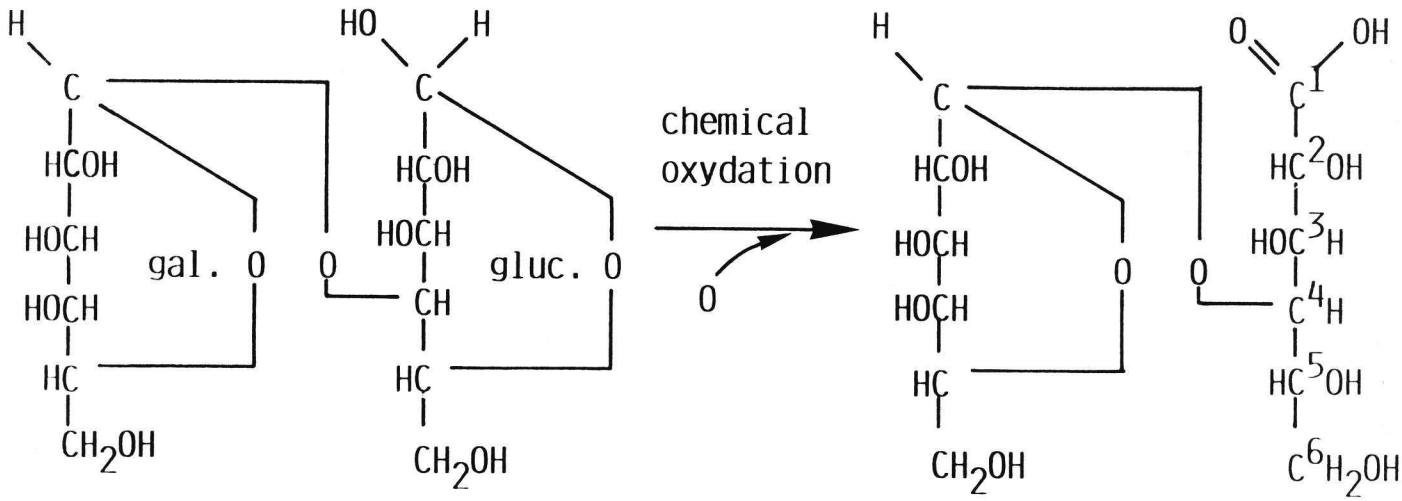
(cyanocobalamin)

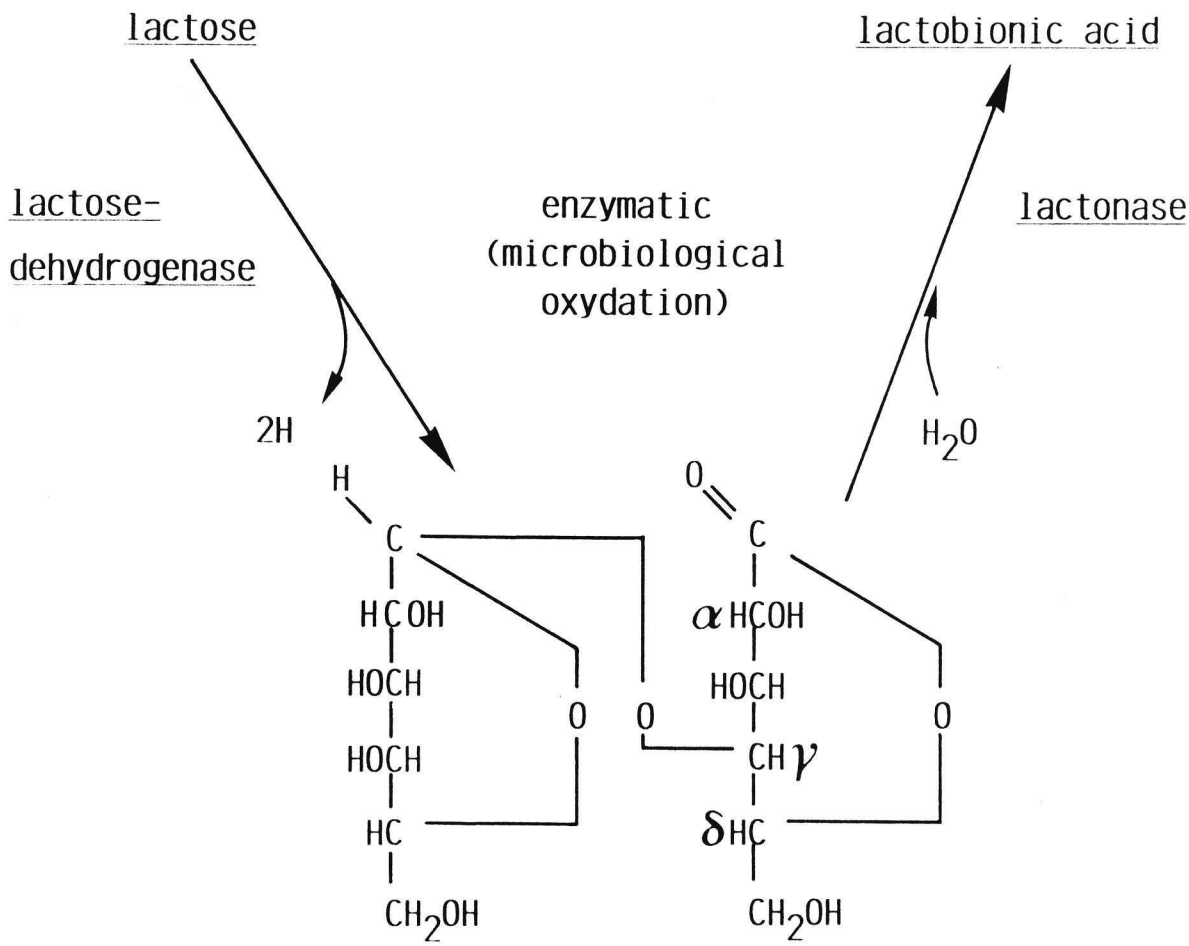


O	B12-derivatives
OH	vitamin B12
CH_3	mecobalamin
5' deoxy-adenosyl	coenzyme B12 (cobamamide)

Figure 3

Production of lactobionic acid from lactose





lactobionic acid - δ - lacton

THE POTENTIAL OF INDUSTRIAL BIOPOLYMERS FROM MILK AND WHEY

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Microorganisms produce a variety of biopolymers, including proteins, polyaminoacids, polysaccharides and polynucleotides. There are certain bacteria which can also make Poly-Hydroxy-Butyrate and Poly-Hydroxy-Alkanoates (PHB/PHAs). These polymers are polyesters composed of 3-hydroxy-fatty acid monomers. Since fatty acids containing up to 12 C-atoms can be incorporated into PHAs, these biopolymers can be considered as "polyfats", "polyparaffins", or "polylipids".

PHB was originally isolated more than 60 years ago and has since been seen in many microorganisms. Many studies of PHB have been carried out in Alcaligenes eutrophus. PHAs were first seen about 10 years ago in Pseudomonas strains. Their production by these and other microorganisms has been studied in some detail, and conditions have been developed which allow the production of 50 - 80% polymer relative to bacterial dry mass.

The characteristics of PHB and various PHAs have been determined. PHB is a brittle, hard plastic which has been used to make molded products. It is now being produced in ton quantities by ICI. Several other companies are gearing up to enter this area. PHAs are typically pliable and softer plastics. They have only been made in gram amounts in our laboratory and at the Polymer Research Center of the University of Massachusetts.

The genetics of polyester synthesis has been studied by several groups. Several major genes which are involved in the synthesis of these polymers have been cloned, sequenced and transferred to other bacteria.

Dennis and coworkers at the James Madison University in Virginia have shown that E. coli equipped with PHB synthesizing genes can produce substantial amounts of PHB. Since E. coli grows on various sugars, including lactose, and it produces PHB on these different carbon sources, lactose can be utilized for the production of PHB. The same is true for whey.

Given that several different polyester polymerases have been found, which differ in their monomer/precursor specificity, it is reasonable to expect that a variety of modified polymerases will be developed over the next few years. It will therefore be possible to produce a variety of biopolyesters from simple carbon sources such as lactose and crude media such as whey.

The uses of these bioplastics remain to be explored. Current possibilities include high-priced uses in the medical/pharmaceutical and optoelectronics sectors, and possibly in the luxury cosmetics market. Medium-priced uses might include food packaging, hospital wastes, and engineering plastics with built-in obsolescence, all of which take advantage of the biodegradability of these polyesters. Since the polymers consist of chiral monomers, they might also be interesting sources of synthons for the fine chemicals market.

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SYMPOSIUM IN WAGENINGEN, HOLLAND

27 FEBRUARY 1991

"MILK COMPONENTS IN FOOD, NOVEL FOOD AND NON-FOOD"

Dr G Page
NZ Dairy Research Institute

A FUTURE FOR MILKFAT

This symposium has been concerned about the future opportunities for dairy products, and in dealing with this other speakers have illustrated that both market and technical opportunities exist. In many areas the technical opportunities are both exciting and challenging, however, the importance of milkfat based products must not be forgotten. This importance is based on the simple fact that milkfat represents such a large proportion of the total solids in milk and the total solids mix needs to be marketed.

At this time, the future for milkfat is dependent on political events, particularly the gatt discussions. In order to make sense of a paper on the future of milkfat therefore, it is necessary to ignore the consequences which might flow from the outcome. To do otherwise would make this paper a catalogue of guesses based on a range of possible options.

The future opportunities of milkfat need us to consider carefully what have been the characteristics of the milkfat situation. Firstly, in a number of countries historically there have been restrictive regulations. Secondly, there has been a remarkable complacency about the problem of declining milkfat consumption and the dairy industry's response to it. That, coupled with the declining sales of yellow fat in general, has exacerbated the situation and, finally, the major technical advantages of milkfat lie in the areas of flavour, flavour development, and the functionality of the product as perceived by the user.

This paper presents comments on what may be required overall in order to ensure that the flavour characteristics and the physical performance characteristics of milkfat are maximised in future and pass some comments about a defensive strategy to repair some of the perceptions which have been developed through our inactivity.

As others in the symposium have demonstrated, milk is a complex mixture of useful and potentially useful components in greater or lesser amounts. Like any part of the food production industry, some of the components are the result of metabolic processes, and some are present as a function of the environment outside the cow herself. The significance of the fact that the external environment influences composition, offers both a threat and opportunity. On the adverse side, the transmission of minute agricultural chemical residues which then metabolise through the cows biochemical processes to the milk has long been known, and our industry is no different to many others in that respect. As an industry our reactions have quite properly been to minimise such effects. On the positive side the effects of varying feed have been amply demonstrated around the world. For example in new zealand where cows are fed largely on grass the milkfat tends to be much harder than say the european milkfat, particularly when the cows have been fed on grain based diets. Hannah research institute has published a lot of work on the effect of feeding on the physical properties of milkfat. However, such work is really a small amount of work when compared to the effects of feeding on the milkfat production as such. In the future therefore, the more the milkfat composition is linked to the generation of products with specific functional properties, the greater will be the opportunity to

control the composition right on the farm, by modifying feed regime. However, that clearly has an effect on the financial situation which is outside the scope of this paper. The point should, however, not be lost for if we go for market signals, which tell us the particular product characteristic required and can be produced more economically by changing the feeding regime of the cow on a farm, then so be it. Historically in our research into development activities we all tend to separate on farm research from production research. This area is extremely complex of course for feed modification to occur economically, we have to be clear about the effects on all aspects of cow metabolism, particularly where it affects animal health, as well as production level. Therefore, a much greater understanding of the link between the feed stuff and the composition of milkfat is desirable. This does not apply to the fatty acids, for while fatty acids are relatively easily measured and affect the physical characteristics, there are other effects on desirable product characteristics, such as flavour. This problem is considerably more difficult. But differences in flavour are well known between countries and therefore feeding regimes and the source of these effects, if any, have not been fully established. Therefore looking into the future, we must take on board that a considerable amount of research and development needs to take place in order to both establish and then utilise a possible tailor made range in composition of milkfat and that these modifications do not necessarily have to be carried out within the process plants. The future could therefore see our industry steering the composition of milkfat in a direction which minimise difficulties and maximises flexibility in product manufacture.

Any discussion of manipulating the composition of the milkfat raises the question of genetics. Many of the genetic opportunities in the milkfat area have to be considered as highly speculative at the moment and therefore long term. But as an industry we already have a large genetic pool in our existing animals, and studies of the composition of milk from individual animals reveals a very wide range of fat level and fat composition. Very little has been published concerning individual variations within herds, or attempts to deliberately breed animals with specific fat characteristics. Again, such opportunities require considerable research and development effort in order to establish what may be feasible commercially.

Coupled with the chemical composition of milkfat is the production of fat containing products. We all know that the margarine industry has been successful over the years for many reasons, but which include their ability to use their raw material flexibly on the basis of minimising material costs within desired product attributes. Those attributes are appreciated by consumers either through conscious desires, as in spreadability or flavour, or subconsciously, as in the product's ease of use, for example in home baking. Their industry finally constantly promotes a product's positive nutritional and health attributes, rather than traditionally negative attributes.

As an industry we have to adopt some flexibility in thinking, not withstanding the economics of the dairy business, we must know how much we can modify and exploit milkfat just as the margarine industry does its own raw material. This comes back to the point that I made earlier, about restrictive regulations, they have arguably been to

our industry's long-term detriment. In general, I would venture to say that we are a long way from knowing our own raw material, the subsequent interaction with processes and the resulting product characteristics which the consumer perceives as important. If we take the view of maximizing flexibility then we open up our thinking to standard margarine processing techniques, such as inter-esterification, hydrogenation and fractionation techniques. Ralph timms gave an excellent paper on these subjects at the 'fats for the future' conference in auckland in 1989. Thus, for example, inter esterification of a butter olein, will result in quite different physical characteristics because it simply creates a different range of triglycerides. Such technical opportunities of course create debate surrounding the desirability to do this related to what the consumer perceptions and needs of dairy products are.

However, other processing techniques, such as fractionation, using supercritical gases offer new opportunities. Are we, for example, with this technology at the start of a new technology offering a new range of milkfat characteristics which might open up new possibilities, particularly as it has a different scientific mechanism from the traditional fractionation from a melt? We may also include several new processes for decholestoralization being developed around the world. All of these processes should not be thrown out for we will rely on our ability to exploit the products produced, and we should know the significant attributes of these processed fats in different types of products, not just using milkfat, and not just in the traditional fat continuous ones.

The whole question of what processing should we adopt for the future is affected by the consumer perception of our products. In many countries the problem or perception

of the nutritional value of milkfat is as influenced by speculation as by substantiated fact. But the industry must not sit by and let our products be damned by implication. This means that we have to consciously recognise that we have to overcome the current inertia in order to change the present situation.

In this nutrition discussion it is necessary to distinguish between three issues:

1 the cholesterol issue

2 the saturated fat issue

3 the total energy intake through fat consumption.

Recently a canadian paper has given a detailed review of the dietary cholesterol fat relationship. There is the general feeling that for the normal population there is little direct association between dietary cholesterol and heart disease. If this conclusion is right, then a situation exists where the issue is the actual cholesterol intake per head of the population. Taking the current recommendation in the united states of a maximum dietary intake of 300 mg of dietary cholesterol, then a normal diet of about 2 500 calories, containing about 30% of the calories coming from fat, then you can estimate that the diet in which the fat is milkfat, will contribute of the order of 70-100 mg of cholesterol a day. This means that the message which needs to be got across to consumers is that dairy products do not, per se, constitute a threat in respect to dietary cholesterol. However, even if this logic is correct. The public's perception of cholesterol is now so well ingrained that trying to bring about change using a head on

clash by refuting scientific studies is just a waste of time. The true situation will only be recognised after a significant period of time requiring a very large effort to get the message across to the public that there is significant doubt to the validity of the evidence which actually exists for the dietary cholesterol and heart disease link. Furthermore, consumers eliminating dairy products from a diet run into other potential problems, resulting from a reduction in the protein and calcium intake. So dairy products have positive nutritional aspects along with the cholesterol, a material which may not in fact be significant in the end.

On the second issue on the saturated fatty acid issue, the dairy industry internationally has been remarkably complacent. If you ask the normal population whether milkfat is saturated the answer would be yes, but in fact as we all know the level of mono-unsaturated fatty acids in milk is about 30%. In addition the short chain fatty acids are not, from a nutritional point of view, in the same league as the long chain saturated fatty acids. Therefore, the short train plus the mono-unsaturated fatty acids about 50% of the total composition of milkfat. A positive view of the future needs to be taken, and the industry needs to start educating the public particularly on the fact not just that the milkfat is only just 50% saturated fat of possible nutritional significance sense significance and that many of our competitive products have greater saturated fat levels than that. The dairy industry therefore needs to establish what the facts are, but also consider an aggressive education programme focusing on the facts of the nutritional claim being made, particularly where those claims are either false or based on uncertain facts as the relate to milkfat. Such an education process is the only way to

reverse current consumer perceptions. Indeed, in looking at the scientific literature on the effects on nutrition, fat products which are rich in mono-unsaturated acids seem to be quite clear, *i.E.* They resulted in relatively low blood serum cholesterol levels. As for the total energy intake from fat products, in north america and europe this energy intake is generally above the recommended who level, which is also an added spur to consumers drive toward low fat products. This is a common issue with all fat product and is not specific to milkfat. However, it does effect the future of milkfat in exactly the same way as vegetable oils and fats. The new products which are being demanded by the consumer must have an acceptable functionality be it, spreadability, baking performance *etc*. Consequently many of these low fat products require specific physical characteristics of the fat phase. Put quite simply the aqueous phase volume, is so high, that the product stability requirements to shear *etc* of the products are such that the physical characteristics of milkfat or the vegetable oil are far more critical than in traditional 80% fat products.

The ability of the industry to recognise consumer needs requires us to approach the new product development from a position of knowing a considerable amount about our raw material, which brings me back to the problem I raised earlier. It was with the implied damming of milkfat by association that led the new zealand dairy research institute to set up a joint programme with the department of scientific & industrial research at palmerston north which is looking at small animals - rats - and dr kerry james and alison maccoll are looking at the various effects of feeding a balanced diet with only the fat type as the variable. Accepting that such a study is no substitute for

the use of human subjects, it represents a small careful entre into this area where we are interested in the facts - not establishing whether a given hypothesis is true or false. The future opportunities for milkfat, therefore hinges on a commitment to research & development in order to establish a thorough understanding of our raw material, its interaction with processes and consequences in the consumer perceptions of functionality - be at a physical or nutritional functionality. There is evidence that this commitment is beginning to grow and the article by les lamb last year about the dairy industry activities in the usa as an identical approach to this paper can be read as the basis of this talk, we all see the same problems and research & development goals.

In conclusion therefore if we put on one side the national political issues surrounding milkfat and agricultural policies, its future is clearly dependent on research & development both in increasing our detailed understanding of the raw material, the manipulation of the physical characteristics, be it on farm or in processing and on the industry developing strategy (and if necessary research) into nutritional effects of milkfat, is essential. The consistent sensible education of the public on the benefits of milkfat goes hand in hand with this approach. To do this the separate national dairy industries must find bilateral or better multilateral ways to co-operate in key long term research studies. The future is not all doom and gloom, but demands a commitment for more concerted research in order to make the most of the future opportunity.

Dr. W.IJ. Aalbersberg

TRENDS IN UTILIZATION OF MILK CONSTITUENTS; IMPRESSIONS OF STUDY TOURS

1. Introduction

From May 19 till June 2, 1990 a study tour to Japan took place. From June 18 till June 28, 1990 a short study tour to the United States followed. The first-mentioned study tour was an official mission supported financially by the Ministry of Economic Affairs and was devoted to the utilization of animal raw materials for non-food applications. The number of participants to this mission was four. The second study tour, that to the United States, was carried out individually, annexed to the Annual Meeting of the American Dairy Science Association in Raleigh (N.C.), where I presented a lecture.

Before some impressions obtained during these study tours are presented, I like to make the following remarks:

1. Notwithstanding a tight travelling schedule allowing full use of available time and notwithstanding a thorough preparation allowing to meet the right experts for the indicated subjects, study tours of seven working days to the US and of ten working days to Japan are insufficient to obtain a full view of the actual situation. This was most severe in Japan as language and culture differences did not allow that discussions evolved rapidly.
2. Both study tours took place nine months ago. Since then new developments may have taken place. This means that the impressions I am going to present may have been surpassed by these developments.

2. Milk fat

In Japan the production and consumption of dairy products per capita is very low compared to those in the United States of America and Europe. Also the total consumption of fat per capita is very low. As a consequence of this in general in Japan neither the nutritional significance of milk fat in the diet nor the utilization of milk fat for alternative applications seem to be a subject of great concern. One is inclined to conclude that in Japan the relative small amount of milk fat produced per capita is consumed with the regular dairy products. However, it should be mentioned that in Japan one milk company gives attention to the fractionation of milk fat and that this company developed a product based upon triglycerides of caprylic acid. Very probably another milk company also gives attention to the fractionation of milk fat and to the enzymatic modification of milk fat. Furthermore the interestification of fats by lipases is investigated in the National Food Research Institute.

In the United States of America the situation is totally different. The production and consumption of dairy products per capita is much higher. During the last decade a growing preference for light products arose.

It seems that the preference has been enhanced recently by the recommendations of several official bodies, such as the American Health Association, the US Department of Agriculture and the Surgeon General (Kanto, 1990). As a consequence of this increasing preference for light products a dramatic surplus of milk fat is expected.

Faced with this development the National Dairy Promotion and Research Board, which annually spend US $ 5.000.000 to product and process research, devotes a substantial part to the research of milk fat and alternative applications of milk fat. The major part of this research is carried out at the Center for Dairy Research, Department of Food Science at the University of Wisconsin. Here attention is given to extraction of milk fat with supercritical carbon dioxyde, fractionated crystallization from the melt and from solutions and characterization of these fractions. The objective is to find milk fat fractions that can be utilized for special applications such as the replacement of cocoa butter in chocolate products, etc. One has also begun to investigate the enzymatic modification of milk fat dissolved in supercritital carbon dioxyde and in oil-in-water emulsions.
Furthermore it should be mentioned that in the United States of America the phobia for exogenous cholesterol led major dairies to manufacture and sell cholesterol-free products. Three methods are available: extraction by supercritical carbon dioxyde, steam distillation and absorption by coated glass beads or by cyclo-dextrins. The product I came across seemed to be prepared by steam distillation. No butter flavour was left at all.

3. Lactose and lactose derivatives

In Japan and the United States of America not much attention is given to the utilization of lactose and the regular lactose derivatives. However, in Japan attention is given to the enzymatic synthesis of oligo- and polysaccharides from lactose and to their utilization. Deya (1990) reported about the formation of four trisaccharides, two tetrasaccharides and one pentasaccharide during lactose hydrolysis using ß-galactosidase from Aspergillus oryzae. The oligosaccharides were isolated and the structures were assigned. One of the trisaccharides was 6'-galactosyllactose (GaL) (=0-ß-D-galactopyranosyl - (1-6)-o-ß-D-galactopyranasyl, - (1 → 4) - D-glucose). The maximum yield of GaL. was obtained when a reaction mixture of 40% lactose at pH = 5.0 and 3.3 mg % ß-galactosidase was incubated at 40° C for 4 hours. Amongst 20 species of human intestinal microorganisms only bifidobacteria were able to assimilate easily to GaL. Since the results suggested that GaL. is a sugar promoting the growth of bifidobacteria, GaL. is used in infant formula as a physiologically functional sugar. In a trial piglets fed a commercial piglet feed containing GaL. displayed more weight gain than control piglets. Based upon the results obtained, GaL. is used in animal feed as a physiologically functional sugar.
For this purpose the production of whey powder containing oligosaccharides has been developed (Yananira, 1990).

During the study tour to Japan it was observed that a dairy company produces a fermented milk by fermentation of skim milk to which polydextrose, GaL., pectine, aspartame and vitamins have been added, by Bifidobacterium brevis and Streptococcus thermophilus. The oligosaccharide, GaL., is said to improve the adherence of bacteria to the intestinal wall.

Recently Yokoi et al. (1990) reported the isolation of five polysaccharide-producing lactobacilli from Kefir grains. These lactobacilli are able to produce the polysaccharides in high yields so that they could be utilized for the industrial production of polysaccharides.

During the study tour to Japan it was also observed that a company produces a fine assortment of cosmetics. One of the main ingredients of these cosmetics is a polysaccharide, produced by a lactobacilli culture. The polysaccharide is said to have skin protecting properties.

4. Milk proteins

In the United States of America and Japan the utilization of milk proteins ranges from rather traditional to very advanced. On the one hand ultrafiltrated cheese is simply used as skim milk replacer, whereas on the other hand single milk proteins, such as lactoferrin, are utilized for specific purposes. On the one hand the ripening of cheese is accelerated in the well known ways by the addition of enzymes or an increase of the ripening temperature, whereas on the other hand very specific enzymatic hydrolysis of proteins is effectuated.
In the Center for Dairy Research, Department of Food Science, at the University of Wisconsin, for the acceleration of cheese ripening Lactobacillus helveticus has been investigated already in 1983. A similar investigation at NIZO gave rise to the new Proosdij cheese in 1988. Furthermore the first mentioned research center studies the effect of the addition of spray dried cultures to the cheese milk. In this way the proteolytic activity is increased without increasing the acidification. So far the results obtained did not give rise to much enthousiasm. A Japanese company applies proteases of P.caseolyticum and L.helveticus. Within 20 days a flavour corresponding to a normal cheese at 3 months was said to be obtained.

Much attention is given to the production and utilization of lactoferrin. Some companies in Japan add lactoferrin obtained from acid whey (Yoshida, 1989) to infant formula. They claim that by the additon of lactoferrin the iron transport in the body is facilitated, the cell growth in the epidermis is promoted and infection with escherichiae and staphylococci is prevented. However, other companies expressed as their opinion that in order to meet these objectives much higher, uneconomical concentrations should be applied.

This objection should not be valid anymore if human lactoferrin, which is said to be more active, would be applied. Recently the production and utilization of lactoferrin and lactoperoxydase from skim milk and whey has also been started in Europe (Burling, 1990).

As far as could be observed during the study tours, lactoperoxydase is not utilized very frequently in the two countries. The recent observation of Denis and Ramet (1989) that lactoperoxydase is effective in the elimination of viable cells of Listeria monocytogenes may give rise to the utilization of lactoperoxydase in some dairy products as a safety factor to assist in the inhibition of L.monocytogenes.

A Japanese company produces sialic acid from acid whey. Several properties are attributed to this product. It is said to control the life cylce of blood cells, to neutralize choleratoxin and influenza virus, to clean the mucosa of the throat once infected and to prevent from possible infections. From sialic acid and cytidyl monophosphate CMP-N-acetylneuraminic acid is made enzymatically. This product is used in clinics for renal dialysis.

Furthermore, hydrolysed casein is used in Japan as a nutrient in agarcount plates to count specific lactobacilli. Hydrolysed casein from colostrum, combined with 1 or 2% fetal calf serum, is offered as a medium for the growth of animal cell cultures for the production of interferon. A similar utilization has been published recently by French research workers (Derouiche et al., 1990). They claim the utilization of a non-modified specific whey fraction as such, combined with 1 or 2% fetal calf serum, for the same application.

Finally specific diet foods prepared by enzymatic hydrolysis of casein were observed. An example is a food with a phenylalanine content below 0.3%. This food is prepared by enzymatic hydrolysis of a whey concentrate followed by removal of bitter peptides and salt and is used by patients suffering from phenyl-ketonuria.

5. Milk and health aspects

The interest in health aspects of food in general and of dairy products in particular appeared to be very pronounced in Japan. Also the interest is very diverse. Therefore, in this presentation it is not possible to give a complete survey of all health aspects which receive attention. In the following we will mention some selected subjects.

Health aspects of fermented milks

After repeated inquiries Japanese investigators admitted that not sufficient scientific evidence is available to prove possible health claims regarding milks fermented by Lactobacillus casei and milks fermented by Bifidobacterium brevis and Streptococcus thermophilus.

The new food regulation does not allow to claim health benefits of food prepared with bifidobacteria. For this reason scientific research to collect additional evidence to prove health claims is pushed very much by some companies. In general these companies are well equipped. In most cases they have good laboratory facilities and stables with test animals available.

Associated health aspects

From the study of health aspects of fermented milks associated health aspects are easily approached. Examples of such associated health aspects are

- the immunogenicity of Bifidobacterium brevis and the change in antibody production after oral administration (Yasui et al., 1989);
- the binding properties of lactic acid bacteria isolated from Kefir milk with mutagenic amino acid pyrolyzates (Hosono et al., 1990);
- the probiotic action of L.casei (Yakult product);
- the growth-inhibiting action on lung sarcoma of L.casei (under investigation);
- the mechanism of antibody production against casein peptides (Prof. Kaminogawa, University of Tokyo);
- the interaction of casein and ß-lactoglobulin peptides with T-cells, B-cells and antibodies (Prof. Kaminogawa, University of Tokyo);
- the growth stimulating effect of lactoferrin and casein peptides (Prof. Kaminogawa, University of Tokyo);
- the antagonistic action of peptides from casein against opioids (Prof. Yoshikawa, Kyoto University).

Other health aspects

Modern molecular biology research is very well established in Japan. For instance the National Chemical Laboratory for Industry developed an expression and secretion system for human lysozyme in Saccharomyces cerevisiae. By means of protein engineering an enzyme with increased activity at higher ion strength and higher pH is obtained. In contrast to chicken lysozyme it would not cause allergenic reactions.

Another example is the cloning and expression of milk proteins in Saccharomyces cerevisiae by Prof. Kaminogawa, Univeristy of Tokyo with the objective to modify epitopes and to decrease the interaction with T- and B-cells.

The thorough knowledge of cell biology, immunology and molecular biology including protein engineering are the basis for a very wide interest in life sciences of companies like Snow Brand. This is obvious when one reads the Technical Research Institute, Snow Brand Milk Products Co. Ltd, Reports of Research Laboratory (1990). Some titles are:

- oligosaccharides from lactose and their utilization;
- polymerase chain reaction: its application to transgenic animals;

- studies on t-PA production by microcarrier cultivation on human normal diploid fibroblast;
- expression and characterization of erythropoietin receptors on normal human bone marrow cells;
- microbial production of two new dihydroxylated androstene dione derivatives by Acremonium strictuum.

This selection of titles clearly indicates that diversification is directed to new products and new markets based upon steadily growing know-how.

Another health approach

Before I finish my presentation I would like to mention another health approach based upon milk. Tacket and co-workers (1988) reported on the protection by milk immunoglobulin concentrate against oral challenge with enterotoxigenic Escherichia coli. Lyophylized milk immunoglobulins were prepared from the colostrum of cows immunized with several E.coli serotypes. From the results it was concluded that milk immunoglobulin concentrate may be an effective prophylaxis against traveler's diarrhoea. Since then, based upon the same idea, Land O'Lakes in the United States of America introduced to the market a veterinary care product. It is a pathogen specific antibody concentrate for the prevention of diarrhoea in new born calves.

6. Concluding remarks

Were the study tours to Japan and the United States useful to trace any important new utilization of milk constituents? When one would expect major new outlets, the answer is: no! However, usually new developments begin slowly and on a small scale. When one takes this for granted the study tours, in particular that to Japan, has brought to light several new utilizations of dairy ingredients or of scientific know-how present in the dairy industry.

In this context I like to refer to the report made by Arthur D. Little (1989) by order of the Netherlands Ministry of Economic Affairs. From this report I quote: "One of the most serious misinterpretations in the early period of biotechnology was that biotechnology would be applied broadly and deeply in a large number of industries. At present it looks like that only the pharmaceutical industry will have a high earning potential in this sector, though much lower than anticipated. For agricultural and bulk chemistry the application will only be fruitful if it will help the customer to increase his net profit. However, as the price these customers are prepared to pay is only a minor part of the value added to the product by biotechnology, it will require a long time to achieve a commercial sucess in these sectors".

I believe that the Japanese dairy industry did understand this, witness its involvement in immunology, cell biology and molecular biology related to the pharmaceutical sector.

Table 1 from Biotech Forum Europe (anonymous, 1990) gives an impression of the development stages of some R-DNA products in Japan and of the involvement of Japanese dairy companies in it.

As said before new developments begin slowly and on a small scale. Maybe the developments indicated in this table and in my paper in general will contribute to the profit of the companies involved in future years.

REFERENCES

- Anonymous (1990) Biotech Forum Europe, International Journal of Biotechnology 7 (3), 194
- Burling, H. (1990), Scandinavian Dairy Information 1, 46
- Denis, F. and Ramet, J.P. (1989), J.Food Protection, 52, 706
- Derouiche, A.F., Legrand, C., Bour, J.M., Capiaumont, J., Gelot, M.A., Bousset, B., Bellville, F., Nabet, P. and Linden, G. (1990), Lait 70, 313
- Deya, E. (1990), Technical Research Institute, Snow Brand Milk Products Co. Ltd., Reports of Research Laboratory, 92, 1
- Hosono, A., Tanabe, T. and Otani, H. (1990), Milchwissenschaft 45 (10), 647
- Jananira, ... (1990), Technical Research Institute, Snow Brand Milk Products Co. Ltd., Reports of Research Laboratory, 92, ...
- Kanto, M.A. (1990), Food Technology 44, 81
- Arthur D. Little (1989), Biotechnologie in de Nederlandse Industrie
- Tacket, C.O., Losonsky, G., Link, H., Hoang, Y., Guesry, P., Hilpert, H. and Levine, M. (1988), New England Journal of Medicine 318 (19), 1240
- Yasui, H., Mike, E. and Ohwaki, M. (1989), J.Dairy Science 72, 30
- Yokoi, H., Watanabe, T., Fujii Y., Toba, T. and Adachi S. (1990), J.Dairy Science 73, 1684
- Yoshida, S. (1989), J.Dairy Science 72, 1446

TABLE 1 - DEVELOPMENT STAGES OF SOME R-DNA PRODUCTS IN JAPAN

Product	Number of companies involved in the development	Stage of development	Activity of dairy companies
α-interferon	5	2 companies sell this product	---
ß-interferon	3	3 companies obtained registration	---
j-interferon	10	1 company obtained registration	Meiji finished development
tumornecrosis factor (TNF)	8	1 company performs clinical trials	---
tissue plasminogen activator (TPA)	13	3 companies filed registration	Meiji has started clinical trials. Snow Brand finished development
erythropoietin (EPO)	4	2 companies filed registration	Snow Brand started clinical trials
hepatitus B-vaccin (HB)	8	2 companies filed registration	Meiji is in stage 3 of clinical trials
lymphotoxin	5	3 companies perform clinical trials	Snow Brand performs pre-clinical tests
M-CSK	2	1 company performs clinical trials	Morinaga performs pre-clinical tests
staphylokinase	1	1 company performs pre-clinial tests	Yakult

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