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Bioactive peptides in long-time ripened open texture semi-hard cheese

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Abstract

Bovine milk is an excellent source of bioactive peptides. The bioactive peptides are in an inactive state in the protein and are released during enzymatic activity, e.g. during cheese ripening. Specific properties of bioactive peptides from ripened cheese have been reported, e.g. antihypertensive, antioxidative, antimicrobial, immunomodulatory and mineral binding.

This work was made to screen for bioactive peptides in the Swedish long time ripened open-texture cheese, Präst cheese from Skånemejerier. Cheeses with three different ripening times available at the market were investigated; Prästost (ripened for 8 months), Allerum (1 year) and XO (2 years).

A solution of the pH 4.6-soluble N-compounds was made using a citrate dispersion of the cheese and then lower the pH to below 4.6. Separation of the peptides in the pH 4.6 soluble fractions was conducted by reverse phase high performance liquid chromatography (RP-HPLC). For identification of the peptides, on-line electrospray ionisation mass spectrometry (ESI-MS) was monitored. Suggested identities of the peptides were given by using MS/MS analysis of the most intense ions in the mass spectra.

All three kind of cheese contained similar profiles of bioactive peptides. Peptides from the *N*-terminal part of α_{S1}-casein were a group of bioactive peptides (ACE-inhibitory and antioxidative) accounting for a high presence in Präst cheese. Caseinphosphopeptides (CPPs) were another group of bioactive peptides found in Präst cheese, e.g. β-casein f(8-28), f(29-93)A1 and f(29-93)A2.

The most dominating peaks in the medium ripened Präst cheese and Allerum (long ripened) were identified as α_{S1}-casein f(1-6), f(1-7), f(1-9), f(1-13) and f(1-14) as well as β-casein f(8-28),(29-93)A1 and f(29-93)A2. In XO (extra-long ripened) the most dominating peaks were identified as α_{S1}-casein f(1-6), f(1-7), f(1-9) as well as β-casein f(29-93)A1 and f(29-93)A2.

Sammanfattning

Komjölk är en utmärkt källa av bioaktiva peptider. De bioaktiva peptiderna i mjölk är inaktiva så länge de är en del av ett mjölkprotein och blir aktiva först då proteinet klyvs under enzymatisk aktivitet, vilket sker vid t.ex. lagring av ost. Bioaktiva peptider med olika egenskaper har identifierats i lagrad ost; t.ex. blodtryckssänkande, antioxidativa, antimikrobiella, immunomodulerande och mineralbindande.

Detta projekt har genomförts för att screena den svenska, lagrade ostsorten Präst från Skånemejerier för bioaktiva peptider. Prästost med tre olika lagringstider som finns på den svenska marknaden undersöktes; Mellanlagrad Prästost (8 månaders lagring), Allerum (1 år) och XO (2 år).

För att undersöka N-föreningar som är lösliga vid pH 4,6 gjordes en citratdispersion av osten, i vilken pH sänktes till under 4,6. Peptiderna i lösningen separerades med hjälp av reverse phase high performance liquid chromatography (RP-HPLC). On-line electrospray ionisation mass spectrometry (ESI-MS) användes för att identifiera peptiderna. Genom MS/MS analys av de mest intensiva jonerna i de uppkomna mass-spektrumen gavs förslag på peptidsekvens.

Alla tre Prästost-sorterna innehöll liknande proportioner av bioaktiva peptider. ACE-hämmande och antioxidativa peptider från N-terminalen av α_{S1}-kasein var en betydande grupp av bioaktiva peptider som detekterades i Prästost. Exempel på dessa är: α_{S1}-kasein f(1-6), f(1-7), f(1-9), f(1-13) och f(1-14). Mineralbindande peptider var en annan grupp av bioaktiva peptider i Prästost, t.ex. β-kasein f(8-28), f(29-93)A1 and f(29-93)A2.

De mest dominerande topparna i Mellanlagrad Prästost och Allerum var identifierade som α_{S1}-kasein f(1-6), f(1-7), f(1-9), f(1-13) och f(1-14) liksom β-kasein f(8-28),(29-93)A1 och f(29-93)A2. I XO var de mest dominerande topparna identifierade som α_{S1}-kasein f(1-6), f(1-7), f(1-9) liksom β-kasein f(29-93)A1 och f(29-93)A2.

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1. Introduction

1.1 Background

It is a fact that diet contributes to our health. It is also known that milk protein possesses values apart from nutritional, such as biological and biochemical values (Korhonen & Pihlanto-Leppälä, 2004). According to present knowledge, bovine milk is the greatest source of bioactive peptides derived from food (Möller et al., 2008). These bioactive peptides are in an inactive state in the protein and are released during enzymatic digestion *in vivo* or *in vitro*. Many dairy starter cultures, used in cheese manufacture, possess complex proteolytic enzymes. Thus, fermented milk products can itself be a source of bioactive peptides. Bioactive peptides can also be released by further digestion of oligopeptides in the gastrointestinal tract. When liberated in the gastrointestinal tract the bioactive peptides may act locally or be transported by the blood stream to the target organ (Korhonen & Pihlanto-Leppälä, 2004).

Presence of bioactive peptides in fermented milk products and ripened cheese has been reported, including specific activities such as antihypertensive, antioxidative, antimicrobial, immunomodulatory and mineral binding. Due to the wide range of functions, bovine milk derived peptides are regarded as potent ingredients in health-promoting functional foods or even in pharmaceutical preparations (Korhonen & Pihlanto-Leppälä, 2004). The target groups for foods enriched with bioactive peptides are likely to be health conscious people and people with specific demands (Möller et al., 2008).

The production of bioactive peptides in cheese is affected by the starter culture and ripening conditions (O´Brien & O´Connor, 2004). Thus, the peptide composition reflects the ripening process of the cheese. Many factors during the ripening time affect the outcome of the peptide profile. Differences in peptide composition can be of significant value for the cheese producers as well as for the consumer when deciding which cheese to consume.

Cheese consumption has increased tremendously during the last decades in Sweden. From 1960 to 2006 the consumption has more than doubled, from seven kilos to almost 18 kilos per person and year (Swedish board of Agriculture, 2011). This fact makes cheese an interesting field to investigate as a natural source of bioactive components.

1.2 Objective

The objective of this project was to screen for bioactive peptides in the common Swedish long time ripened open-texture cheese, Präst cheese from Skånemejerier. Cheeses with three different ripening times that are available at the market were investigated and compared; Mellanlagrad Präst cheese (ripened for 8 months), Allerum (1 year) and XO (2 years).

Differences in protein breakdown between the three Präst cheese types were studied with focus on the presence of bioactive peptides.

2. Literature review

2.1 Protein composition of milk and cheese

Milk is an aqueous solution of protein, lactose, vitamins, minerals, casein micelles and emulsified globules of fat. Bovine milk contains about 3-5 % protein, of which approximately 20 % are whey proteins and 80 % are caseins. The two types of milk proteins are defined by their solubility at pH 4.6. At this pH the caseins precipitate whereas the whey proteins are soluble. (Sindayikengera et al., 2006; Farrell et al., 2004)

Milk contains proteins of high biological value. During cheese manufacture much of the whey proteins are lost, and of the total protein in cheese only about 2-3 % may be whey proteins. The remaining proteins are caseins. Caseins are not as high in sulphur amino acids as whey proteins. (O'Brien & O'Connor, 2004)

The protein content in cheese correlates inversely with the fat content, which approximately is 30 w/w. Cheese protein is almost 100 % digestible. During the ripening phase the casein is degraded to water-soluble peptides and free amino acids. Thus, much degradation has taken place before the cheese is consumed and exposed to the gastrointestinal proteolytic actions. (O'Brien & O'Connor, 2004)

The amino acid composition, bioavailability, and the digestibility of the protein are important factors when defining protein quality. Moreover, an important aspect of the nutritive value of bovine milk protein is its source of biologically active peptides with specific physiological properties. (Sindayikengera et al., 2006)

2.2 Casein

Casein is a main protein component of milk. An important physiological role of casein is as a very potent source of amino acids. Another important role of the casein micelle system is to prevent pathological calcification of the mammary gland while supporting enough Ca to the calf. Partly due to increasing awareness regarding the impact of food on health, much focus is today on hidden and inactive peptides in casein. Many of these peptides possess bioactivity, and knowledge regarding their release and efficiency is of great interest. (Silva & Malcata, 2005)

Bovine caseins are a family of four phosphoproteins, α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, which are found in the approximate proportions 4:1:4:1. The caseins are synthesised in the mammary gland and undergo post-translational modifications such as phosphorylation and glycosylation. In bovine milk, only κ-casein is glycosylated. Phosphorylation on the other

hand occurs at serine residues in all caseins. α_{s1}-casein, α_{s2}-casein, β-casein exhibit clusters of phosphorylated residues, κ-casein does not have theses clusters and is often only phosphorylated at one residue per molecule. The phosphoserine residues of the caseins bind calcium, which in turn bind colloidal calcium phosphate (CCP). These bonds highly contribute to binding the casein molecules together and to form a colloidal structure, which is called the casein micelle. The high content of proline residues and the amphiphilic structure of casein also contribute to the micelle formation. The roughly spherical micelle structure enables fluidization of the caseins and solubility of calcium and phosphate. (Horne & Banks, 2004)

The degree of phosphorylation determines the molecules sensitivity to calcium-induced precipitation. α_{s2}-casein is the most calcium sensitive molecule, whereas κ-casein is calcium insensitive and prevents precipitation when present with other caseins. κ-casein is localized near the micelle surface and stabilizes the structure, which means that the calcium in milk is unable to precipitate the caseins. (Horne & Banks, 2004)

The fundamental process of cheese and yoghurt making is the precipitation of the casein micelle to form a curd. In yoghurt production this is obtained by lowering the pH to the isoelectric point. In cheese making a common method to precipitate casein is by adding rennet, which enzymatically precipitates the casein. The enzyme chymosin effectively cleaves κ-casein at Phe_{105}-Met_{106}. The part of κ-casein, the glycomacropeptide, that carries the carbohydrate part of the molecule is lost in the whey. The loss of this carbohydrate coating means that strong cross-links between the micelles appear and a curd is rapidly developed. (Coultate, 2009)

2.3 Präst cheese

Präst cheese is an old Swedish cheese variety. The name Präst (Eng. Priest) originates from the days when the cheese was made on the rectory. Präst cheese has an intense and aromatic flavour with a weak bitter note. The cheese has an open texture with plenty of small irregular eyes (2-6 mm). The cheese is semi-hard and has a smooth body that can easily be melted and sliced. When extra ripened, the texture gets more brittle and the flavour stronger with a pungent note (Swedish Dairy Association, 2011). Präst is a common cheese on the Swedish market.

Mesophilic DL-starter is used for production of semi-hard cheeses in northern Europe. The starter is composed of *Lactococcus lactis* subsp. *lactis* (*Lc. lactis*) *Lc. lactis* subsp. *cremoris* (*Lc. cremoris*) *Lc. lactis* subsp. *lactis biovar diacetylactis* (*Lc. diacetylactis*) and different subspecies of *Leuconostoc mesenteroides* (*Leuconostoc*). A mesophilic DL-starter often consists of an undefined mixture of strains of the mentioned species (Rehn et al., 2010). Präst cheese is a variety made with mesophilic DL-starter culture.

Proteolysis in Präst cheese has not been thoroughly investigated. Two other common Swedish varieties are Västerbottensost and Herrgård, which have similarities with Präst cheese. The Swedish variety Västerbottensost is a full fat hard to extra-hard cheese, which is produced with mesophilic DL-starter and cooked for several hours. During proteolysis, accumulation of *Lactococcus* derived peptides from α_{s1}-casein f1-23 and β-casein f29-93 was found in Västerbottensost (Rehn et al., 2010).

Herrgård is a Swedish semi-hard cheese variety made with mesophilic DL-starter culture. The phosphopeptide profile of this cheese variety has previously been determined by Lund & Ardö (2004). High activity from rennet enzymes, as wells as relatively high plasmin activity was observed in the variety. It was observed that accumulation of β-casein derived peptides takes place, especially β-casein f29-93 (Ardö et al., 2007; Rehn et al., 2010).

2.4 General mechanisms of cheese ripening

During cheese ripening several complex biochemical events takes place that leads to characteristic texture and taste for each cheese variety. Proteolysis is an important event. The development of cheese texture is formed via hydrolysis of the casein matrix, which leads to softening of the cheese (McSweeney, 2004). When carboxy and amino groups are liberated, the water in the cheese binds to these groups, which means that the water binding capacity increase (decreased a_w) due to the hydrolysis. The cheese texture is also affected by the increase in pH caused by liberation of ammonia and amino acids produced during proteolysis. The flavour, and off-flavour formation, is influenced by the release of short peptides and amino acids. Some of the released amino acids do not have flavour but act as precursors for different catabolic reactions, which generate volatile flavour compounds (McSweeney, 2004; Upadhyay et al., 2004).

During cheese ripening, proteolysis is caused by mechanisms derived from the following sources: residual coagulant from rennet (often chymosin), milk enzymes (often plasmin), starter and non-starter microflora, and in some varieties a ripening culture (e.g. *Penicillium* sp.) (Sousa et al., 2001; McSweeney, 2004; Upadhyay et al., 2004).

The enzymatic degradation of the coagulant is dependent on the type of coagulant used. In traditional cheese production the coagulant is extracted from the abomasa of calves. The extract is called calf rennet and consists mainly of the proteolytic enzyme chymosin, but also of bovine pepsin. Other coagulants that sometimes are used in cheese making are *e.g.* porcine pepsin and fungal or plant acid proteinases. After manufacture most chymosin is lost in the whey, approximately 0-15 % of the rennet activity added to the milk is in the curd (Sousa et al., 2001). Nevertheless, residual coagulant represents a major proteolytic activity in semi-hard cheeses. The primary action of chymosin is to hydrolyse the micelle-stabilising protein κ-casein in order to coagulate the milk (Upadhyay et al., 2004; Sousa et al., 2001).

The most important proteinase in milk is the heat-stable enzyme plasmin. This enzyme is of particular importance in cheese varieties with high cooking temperatures. Plasmin may also be stimulated in varieties with long cooking periods at lower temperatures (Ardö, 2001). Plasmin activity is important in Präst cheese.

From somatic cells, entered into the milk to prevent mastitic infection, lysosomes containing the acid proteinase Cathepsin D are present. The specificity of this enzyme is similar to that of chymosin, but Cathepsin D´s contribution to ripening is poor when rennet is used in the manufacturing. (Sousa et al., 2001)

The first important action of the starter lactic acid bacteria (LAB) is to acidify the cheese, and decreasing the pH. The starter cultures used in cheese manufacturing contain lactic acid bacteria, which hold a cell envelope-associated proteinase (lactocepin) and intracellular peptidase enzymes. This enzyme-system hydrolyses intermediate-sized and short peptides to smaller peptides and amino acids. (Sousa et al., 2001)

Most cheeses contain an adventitious secondary microflora of lactic acid bacteria; non-starter lactic acid bacteria (NSLAB). This population is initially small, but often becomes the dominating microflora during ripening. The proteolytic effect of NSLAB is similar to that of the starter, producing similar small peptides and free amino acids. (Sousa et al., 2001)

In several cheese varieties a ripening culture is used (e.g. *Propionibacterium*). The role of this culture is not to acidify the cheese, but to produce e.g. CO_2, propionate and acetate. The culture contributes to ripening by the production of peptidases. Thus, in varieties with an added secondary microflora their enzymes may also contribute to proteolysis and the release of peptides and amino acids. (Sousa et al., 2001)

2.5 Casein breakdown in semi-hard cheese

Two mechanisms dominate the primary hydrolysis of casein of semi-hard cheeses, i.e. plasmin activity on β-casein and chymosin activity on α_{s1}-casein (Ardö, 2001). The structure of casein does not allow the enzymes to hydrolyse at all possible cleavage sites. In solution, β-casein is cleaved on seven sites; many are located near the hydrophobic C-terminal of β-casein and give rise to short hydrophobic peptides, e.g. by cleaving Leu_{192}-Tyr_{193}. The primary cleavage site of chymosin on α_{s1}-casein is Phe_{23}-Phe_{24}, which results in the formation of the peptide α_{s1}-casein f(1-23). This peptide rapidly undergoes hydrolysis by the starter proteinases. Another common cleavage site of chymosin on α_{s1}-casein is Leu_{101}-Lys_{102}. Chymosin action on α_{s2}-casein is restricted to the hydrophobic regions, i.e. in sequence 90-120 and 160-207 (McSweeney, 2004).

The preferred substrate for plasmin is β- and α_{S2}-casein. Plasmin cleaves β-casein primarily at three sites: i.e. Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}. α_{s2}-casein is also susceptible to plasmin activity, and the common degradation of this protein during semi-hard cheese ripening is caused by plasmin. Plasmin is a relatively heat stable enzyme, and its activity is most significant in cheese with high cooking temperature, when much chymosin activity is lost. (Sousa et al., 2001)

The primary role of the *Lactococcus* proteinase system (Lactocepin) in cheese is to degrade intermediate-sized peptides, produced from casein by chymosin or plasmin, to small peptides. The peptidases from the microflora are intracellular and are, after the cells have lysed, responsible for the degradation of small peptides into free amino acids (Sousa et al., 2001). *Lactobacillus* proteinases are especially important in long-time-ripened cheeses using thermophilic starters and manufacture temperatures that inhibit rennet activities in cheese. Peptides that typically accumulate in semi-hard cheese varieties are derived by microbial enzymatic activity of the chymosin produced peptide α_{s1}-casein f(1-23), and plasmin derived peptides from β-casein (Ardö et al., 2007).

2.6 Bioactive peptides encrypted in bovine casein

One definition for bioactive peptides is "*components (genuine or generated) of ready-to-eat foods which may exert a regulatory activity in the human organism, irrespective of their nutritive functions*" (Gobbetti et al., 2004). Thus, bioactive peptides can be consumed in conventional foods, dietary supplements and in functional foods.

Bovine milk proteins are a source of a wide range of peptides with bioactive properties. In fact, cheese and dairy products seem to be the richest source of bioactive peptides of all foods (Möller et al., 2008). During proteolysis a variety of peptides are released from the milk proteins. Some of these have bioactive properties, which are inactive while encrypted in the milk proteins. The proteolysis takes place during the food processing e.g., milk fermentation and cheese maturation or during gastrointestinal transit. Several milk-derived peptides possess more than one bioactive property. These multifunctional peptides consist of so-called strategic zones of the casein amino acid sequence (Korhonen & Pihlanto-Leppälä, 2004). The casein derived bioactive peptides can e.g. affect the cardiovascular system, the immune system and bone and teeth mineralization (Silva & Malcata, 2005).

Dairy starter cultures possess proteolytic activity, which often is controlled and exploited to achieve desired flavour, aroma and texture of the fermented product. The proteolytic actions of e.g. lactic acid bacteria (LAB) are well known. Some of the LAB-formed oligopeptides will be broken down, either by intracellular peptidases or by enzymes in the gastrointestinal tract to bioactive peptides. Thus, microbial enzymes have a great capacity to produce bioactive peptides from milk proteins. The types and amount of peptides are mainly regulated by the starter cultures and the rate of proteolysis. Highly proteolytic bacteria, such as *L. helveticus* produce short peptides with different bioactive effects. Cultures used to ferment milk products is not as often known to produce bioactive peptides, however, bioactive peptides can be released by enzymes in the intestine instead (Korhonen & Pihlanto-Leppälä, 2004). Furthermore, the bioactivity can be greater after gastrointestinal degradation than after microbial proteolysis solely (Ardö et al., 2009).

The bioactive peptides may act locally in the intestine or be transported by the blood stream to the target organ. Several studies have found bioactive peptides in both small intestine and plasma of human subjects after intake of bovine milk. However, the physiological role of most bioactive peptides in human health is still unknown (Korhonen & Pihlanto-Leppälä, 2004). The future for bioactive peptides will probably not be as substitutes for drugs. However, in health-promoting dairy products they possess values important for both consumers and producers (Gobbetti et al., 2004). Examples of bioactive peptides previously found in cheese are summarized in Table 1.

Table 1. Examples of bioactive peptides from bovine milk casein previously found in cheese

Bioactivity	Source	Pepetide sequence	Reference
Angiotensin converting enzyme (ACE) inhibitory	Parmigiano-Reggiano, Gouda, Cheddar, Comté	α_{s1}-casein f(1-13), β-casein f(60-68)A2	Hayes et al., 2007

Antimicrobial	Caviocavallo, Semi-hard cheese variety	α_{s1}-casein f(1-7), f(1-9)	Hayes et al., 2007
Antioxidative	Semi-hard cheese variety	α_{s1}-casein (f1-9), (f1-13	Ardö et al., 2009
Caseinophosphopeptide (CPP)	Herrgård	β-casein f(29-93), f(29-105)	Ardö et al., 2007
Cytomodulatory	Gouda	β-casein f(60-66)	Gobbetti et al., 2004
Opioid	Edam	β-casein f(60-63)	Hayes et al., 2007
Immunomodulatory	Feta, Camembert	β-casein f(193-209), f(192-209)	Hayes et al., 2007

2.6.1 Hypertension and angiotensin-converting enzyme inhibitors (ACE-I)

Hypertension is a state of a sustained increase in blood pressure (BP) and an indicator of development of cardiovascular diseases, such as stroke and coronary infarction. A small reduction of BP has a large impact on the development of cardiovascular diseases, i.e. 15 mm Hg reduction in diastolic BP reduces the risk of heart disease with 16 % in hypertensive patients (Hayes et al., 2007).

The regulation of blood pressure is associated with the hormone system "renin angiotensin system" (RAS). Central in RAS is the angiotensin-converting enzyme (ACE). ACE converts the peptide angiotensin I to the vasoconstrictor angiotensin II, which narrows the blood vessel and increase the BP. ACE-inhibitors are competitive substrates for ACE. The C-terminal of the inhibitor is the primary feature governing the inhibition of ACE. ACE prefers hydrophobic amino acid residues in the three C-terminal positions. In the penultiamte position aliphatic, basic and aromatic amino acids induce inhibition. In the ultimate position aromatic, proline (Pro) or aliphatic residues are found. ACE inhibitors with Trp, Tyr, Phe or Pro residues in the three C-terminal positions are the most efficient. Bovine milk peptides with ACE-inhibitory (ACE-I) effect usually contain up to 10 amino acids. (Hayes et al., 2007)

An important factor for the release of ACE-I peptides is the microbial strain. Fermentation using LAB or their proteinases has been shown to release ACE-I peptides from milk protein and especially from casein. The presence of ACE-I peptides has been reported in several cheeses, e.g. Parmigiano-Reggiano, Gouda, Cheddar and Comté. (Hayes et al., 2007)

Fermented milk products have for a long time been consumed in e.g. Japan, East Asia and France, as a way to maintain good health and a stable blood pressure. Two of the most potent and well-studied ACE-inhibitory casein peptides are β-casein f(84-86) (Val-Pro-Pro) and β-casein f(74-76) (Ile-Pro-Pro). These peptides are ingredients in the *Lactobacillus helveticus* and *Saccharomyces cerevisiae* fermented sour milk Calpis from Japan. Calpis is a popular product due to its anti-hypertensive properties. The anti-hypertensive effect of Calpis has been shown in human clinical trials. A significant decrease of BP was observed in mildly hypertensive patients after consuming Calpis during eight weeks (Silva & Malcata, 2005; Hayes et al., 2007). Evolus is another product on the market with antihypertensive claims, which have been determined in human clinical trials. The active peptides in this product are,

just as Calpis, f(84-86) (Val-Pro-Pro) and β-casein f(74-76) (Ile-Pro-Pro) (Möller et al., 2008).

The presence of ACE-I peptides is influenced by the degree of proteolysis. It increases until a certain level and then the amount starts to decrease, e.g., the antihypertensive peptide α_{S1}-casein f(1-22) was identified in a 6-months old Parmigiano-Reggiano, but was not present after 15 months of ripening. It has been reported that short to medium-ripened cheese has the most potent ACE-I activities (Hayes et al., 2007). In one study the most ACE-I potent of six cheeses (with different ripening times) was an eight-months ripened Gouda, containing i.e. the peptides α_{s1}-casein f(1-13) and β-casein f(60-68)A2 (Saito et al., 2000).

In a study by Ardö et al., (2009) the presence and activity of ACE-I peptides from the amino end of α_{s1}-casein (α_{s1}-casein f(1-6), f(1-7), f(1-9)) was found to increase during ripening. However, after a certain point the ACE-I activity decreased again. It was also found that the ACE-inhibitory effect becomes significantly higher after gastrointestinal proteolysis than after only cheese ripening proteolysis (Ardö et al., 2009).

2.6.2 Antimicrobial peptides

Peptides with antimicrobial properties are found throughout nature. In mammals these kinds of peptides are found at the surface of epithelial cells as well as within phagocytic cells. Antimicrobial peptides often also have properties to modulate immune responses. (Gobbetti et al., 2004)

It is believed that milk can prevent and limit microbial infection. The antimicrobial effect may be due to a synergistic effect of immunoglobulins, lysozymes and other defence peptides and proteins. Antimicrobial peptides can be released from its parent protein by heat or alkali treatment, but also from dairy fermentation. The action of antimicrobial peptides derived from milk proteins is likely to be membrane-lytic, with specificity for prokaryotic cell membranes. The mechanism of the membrane rupturing is not fully understood. However, it is believed that the peptides may assemble and form channels in the bacterial membrane. Charge interactions between the membrane and the peptides are believed to facilitate the peptide to enter the membrane interior. (Gobbetti et al., 2004)

One important antimicrobial peptide, isracidin (α_{s1}-casein f1-23), is derived from chymosin action on α_{s1}-casein (Hayes et al., 2007). Isracidin may inhibit the *in vitro* growth of lactobacilli and other Gram-positive bacteria, at relatively high concentrations. *In vivo*, isracidin has shown inhibition of *S. aureus* and *Listeria monocytogenes* growth, with efficiency comparable to antibiotics (Hayes et al., 2007). Many of the peptides with reported antimicrobial effect are intermediate fragments of isracidin. The Italian cheese Caviocavallo was found to contain the peptides α_{s1}-casein f(1-7) with possible antimicrobial activity. The peptide α_{s1}-casein f(1-9) was isolated from a yoghurt product and was reported to have antimicrobial effects (Hayes et al., 2007). The milk derived peptide β-casein f(193-209) has been reported to have convincingly antimicrobial activity against *Escherichia coli* (Birkemo et al., 2008).

2.6.3 Opioid peptides

The active ingredient in opium; morphine, was isolated in 1803. Since then, compounds of many chemical classes have been found to possess morphine-like properties and are termed opioid drugs. These classes include e.g. phenylpiperidines and benzomorphans and are defined as "*agents that bind to or otherwise influence opioid receptors*" (Hayes et al., 2007). Opioid receptors are membrane proteins of the central nervous system, when binding its substrate the receptors mediate effects such as pain relief, euphoria and changes in the endocrine immune system. At least three types of opioid receptors are known; μ-receptor, δ-receptor and κ-receptor. The κ-receptor mediates emotional behaviour, the μ-receptor mediates emotional behaviour and intestinal motility and the δ-receptor is responsible for sedation, analgesia and food intake (Hayes et al., 2007). Many people claim that it is easier to fall asleep after a glass of milk and that babies are calmed after breastfeeding. It is a fact that casein-derived peptides have the ability to affect the nervous system. Peptides derived from milk proteins that act as opioid receptor ligands can have both agonistic and antagonistic effect on the opioid receptors (Silva & Malcata, 2005).

The bovine caseins are potential sources of opioid peptides. The first identified and most thoroughly studied opioid peptides in β-casein are called β-Casomorphins, and correspond to fragments of β-casein f(60-70) (Hayes et al., 2007). A common structural feature of opioid peptides is the presence of a Tyr-residue at the amino terminal end of the peptide. In the third or fourth position another aromatic residue is found (Phe, Tyr). These structural features are crucial to fit the binding site of the opioid receptor (Gobbetti et al., 2004).

The β-Casomorphine amino acid sequence is analogous in bovine, sheep, water buffalo and human. Thus, the well-preserved sequence of the β-Casomorphins indicates a biologically important function of these peptides (Hayes et al., 2007). β-Casomorphins are resistant to gastrointestinal degradation and have been detected in the small intestine of human. However, no passage for β-Casomorphins through the epithelial layer has been observed. Thus, the physiological effects of β-Casomorphins may be limited to the gastrointestinal tract, effects such as gastrointestinal transit time and antisecretory action. The situation is different for neonates in which a passive transport of β-Casomorphins through the intestinal membranes is possible. Therefore effects such as analgesia and calmness are conceivable in infants (Korhonen & Pihlanto-Leppälä, 2004).

The β-Casomorphins β-casein f(60-62) has been detected in Edam cheese and the β-casein f(60-63) in Australian vintage cheese (Hayes et al., 2007). Furthermore, β-Casomorphins can have other effects than opioid, e.g. anti-hypertensive, immunomodulatory and antidepressant effects. Opioid peptides are very potent and low concentrations (μ-molar) can be enough to induce physiological effects (Hayes et al., 2007).

2.6.4 Immunomodulatory peptides

One of the main functions of the immune system is to recognise pathogens or foreign substances, and subsequently eliminate those. The first time a pathogen or a foreign substance is identified the innate (non-specific) immune system is activated. Cytokines, that inhibit pro-inflammatory substances, are released. The other part of the immune system is called the adaptive (specific) immune response. It recognises pathogens or foreign substances,

like an immunological memory, and mounts an attack each time the antigen comes into the host. The tissue of the human gut is a well-organized immune network, which protects the host from infection. Foreign proteins are broken down in the gut and suppressor T-cells are activated. (Hayes et al., 2007)

Peptides derived from milk proteins have been reported to regulate the immune system. Milk derived immunostimulatory peptides affect cells of the immune system and subsequently affect responses and cellular functions of the system. A human β-casein peptide induced phagocytosis and resistance to *Klebsiella pneumoniae* in mice. *Lb. helveticus*-fermented bovine milk has been shown to effect the proliferation of lymphocytes (white blood cells) *in vitro* and to stimulate phagocytic activity (Gobbetti et al., 2004; Hayes et al., 2007). The number of IgA-secreting cells in mice was also found to increase significantly by intake *Lb. helveticus*-fermented milk. Bovine casein, fermented by *Lb. rhamnosus,* has been reported to have positive effect on human blood lymphocytes and cytokine production. The immunomodulatory peptides β-casein f(193-209) and f(192-209) have been identified in yoghurt and in several types of cheeses, e.g. Feta and Camembert (Hayes et al., 2007). Caseinphosphopeptides are also believed to have immunoenhancing properties. The phosphoseryl residues of these peptides seem to activate receptors on lymphocytes and macrophages. An example of this kind of peptide is β-casein f(1-28) (Gobbetti et al., 2004).

2.6.5 Cytomodulatory peptides

Cytomodulatory peptides inhibit cancer cell growth, and this kind of peptides has been identified in milk fermented with different kinds of bacteria. Inhibition of *in vitro* growth has been reported in human breast, colon and leukaemia cancer cell lines. In a mouse model, it was demonstrated that seven days of feeding with milk fermented with *Lb. helveticus* resulted in delayed tumour development. The delay was found to be caused by a decrease in cytokine IL-6, a molecule involved in oestrogen synthesis. (Hayes et al., 2007)

Cytomodulatory peptides have been identified in a wide range of fermented dairy products, like yoghurt and cheese. A peptide extract from a Gouda cheese inhibited growth of leukaemia cells at a very low concentration. The cancer cell line was more susceptible to the peptide-induced apoptosis than the non-malignant cells (Gobbetti et al., 2004; Hayes et al., 2007). Peptides with opioid properties often also exert cytomodulatory properties, e.g. the β-Casomorphine β-casein f(60-66) (Gobbetti et al., 2004).

2.6.6 Mineral binding properties of caseinphosphopeptides (CPP)

Bone is built up by a calcium rich collagen matrix. Bone tissue is maintained by a constant process that forms or removes the bone tissue. Bone resorption takes place when there is a demand for calcium, thus calcium is released from the bone to the blood stream. Osteoblasts are cells that are associated with the formation of bone and osteclasts are cells associated with the removal of bone tissue. Osteoporosis is a skeletal disease characterised by low bone mass and leads to an increased risk of fractures. The best way of avoiding osteoporosis is by an adequate intake of dietary calcium. Despite the fact that milk is a rich source of calcium, the absorption of calcium may be enhanced when associated with caseinphosphopeptides (CPP) (Hayes et al., 2007). CPPs are α_{s1}-, α_{s2}-, and β-casein-derived phosphorylated peptides, which are released by enzymatic hydrolysis. CPPs contain a serine phosphate cluster; often three phosphoryalted serine residues followed by two glutamic acid residues (SerP-SerP-SerP-Glu-Glu). The high content of negative charges makes the CPPs to bind divalent cations very

efficiently. The CPPs can therefore act as bio carriers of minerals such as Ca, Fe, Mn, Cu and Se across the small intestine (Hayes et al., 2007). Most minerals from food are dissociated at the low pH of the stomach and transported to the duodenum. The CPPs have the ability to form soluble complexes with e.g. Ca^{2+}, which prevents Ca^{2+} from precipitation (Korhonen & Pihlanto-Leppälä, 2004).

A number of animal studies have reported the positive effect of CPPs on calcium absorption *in vivo*. CPPs may also prevent cariogenic damages and demineralization of the teeth (Gobbetti et al., 2004; Hayes et al., 2007). Cheese consumption has been shown to have an anti-cariogenic effect. A significant correlation was found between cheese intake and caries-protective effect (Silva & Malcata, 2005). However, controversy regarding whether CPPs enhance mineral absorption exist. Some animal studies claim that CPPs only enhance absorption if the subject's intake of calcium is very low. Thus, the dietary intake of calcium is likely to be an important factor of the CPPs efficiency (Hayes et al., 2007).

CPPs are used as a fortifier or functional ingredient in a variety of products, for example in breakfast cereals and toothpaste. Several CPPs derived from α_{s1}-casein, α_{s2}-casein β-casein have been identified in Cheddar and Comté. In Parmigiano-Reggiano, CPPs derived from β-casein have been identified (Hayes et al., 2007). Peptides containing much phosphate groups have been identified in ripened semi-hard cheeses made with mesophilic starter (Ardö, et al., 2009). The content of phosphopeptides was found to differ between a semi-hard and an extra-hard cheese. In a semi-hard cheese (Herrgård) a large number of small phosphopeptides derived from α_{s1}-casein, α_{s2}-casein and β-casein was identified in a considerably higher amount than in an extra-hard cheese (Parmigiano Reggiano) (Lund & Ardö, 2004). Ardö et al (2007) showed that the Swedish semi-hard variety Herrgård during ripening accumulate large hydrophobic phosphopeptides, which are derived from plasmin activity on β-casein (e.g. f29-93, f29-105, f1-107).

2.6.7 Antioxidative peptides

Oxidative stress is a condition of imbalance between reactive oxygen species (contains unpaired electrons) and the body's ability to detoxify and repair the damage of the reactive components. Oxidative stress is involved in many diseases associated with aging, e.g. Parkinson's disease and Alzheimer's disease. (Hayes et al., 2007)

Milk protein derived peptides are among the natural dietary sources of antioxidants. Peptides from β-casein and α_{s1}-casein have been found to be potent anion radical scavenger. The antioxidative mechanism of milk-derived peptides is not fully understood, but oxidation of amino acid residues in the peptides is thought to quench free radicals. Furthermore, the amino acid combination Pro-His-His has been identified as an active centre in many antioxidative peptides. (Hayes et al., 2007)

The primary cleavage site of chymosin on α_{s1}-casein is Phe_{23}-Phe_{24}, thus the peptide α_{s1}-casein f(1-23) is rapidly released in semi-hard cheese. Subsequently bacterial proteases cleave this peptide into e.g. α_{s1}-CN (f1-9) and (f1-13) which often accumulate during cheese ripening. These peptides have a high histidine content and are positively charged, therefore they have the potential to act as antioxidants. A variety of antioxidant peptides of α_{s1}-casein f(1-23) have been detected in semi-hard cheese. (Ardö, et al., 2009)

3. Materials and Methods

3.1 Cheese samples

Cheese samples of the semi-hard cheese variety Präst (55% fat in dry matter) from Skånemejerier, Kristianstad, Sweden were selected at three different ages, about 8 months (Mellanlagrad Präst cheese), 12 months (Allerum Präst) and 24 months (XO). During organoleptic quality control at the dairy plant the selected cheeses were judged to be representative for the variety in the matter of appearance, texture and flavour.

Cheeses were collected at two different occasions, i.e. cheeses from different batches were analysed. In total four Mellanlagrad (Mell 1-4), six Allerum (All 1-6) and six XO (XO 1-6) were analysed. Cheeses for each cheese type were assigned a number from 1 to 6. Number 1 and 2 were collected at the first occasion and produced from the same batch. The cheeses assigned number 3-6 were collected on the second occasion and were likely produced at the same day.

3.2 Gross composition

Moisture content and pH were measured according to standard method procedures. The pH was measured by an electrode inside a small beaker with tightly packed grated cheese, after that s small volume of deionised water was applied on the surface of the packed cheese (Rehn et al., 2010).

The moisture of the cheeses was measured by drying approximately 15 g of clean pumice in a beaker at 100˚C. An amount of 5 g of grated cheese was mixed with the pumice, and dried at 100˚C for approximately 24 hours. From the weight difference the moisture content was calculated (Ardö & Polychroniadou 1999).

3.3 Preparation of samples for peptide analysis

A solution of the pH 4.6-soluble N-components was made using a citrate dispersion of the cheese and then lower the pH to below 4.6 (Ardö & Polychroniadou 1999). To 10 g of grated, room-tempered cheese, 40 ml of tri-sodium-citrate (40-45°C) were added and the mixture was stirred at 45°C for approximately 1.5 hours. When the solution was room tempered, water was added to a volume of 200 ml. Subsequently, a portion of 40 ml was transferred to a beaker and pH was adjusted to approximately 4.5 by adding 1 M HCl. Water was added to a final volume of 50 ml, and 35-40 ml of it was centrifuged at 3400 rpm (~ 1396 *g*) and 4 °C for 30 minutes. 2 ml of the clear phase were filtered through a 0.2 μm filter and stored at - 20 °C until analysis.

3.4 Peptide analysis and identification by liquid chromatography – mass spectrometry

Separation of the peptides in the pH 4.6 soluble fractions was conducted by reverse phase high performance liquid chromatography (RP-HPLC). RP-HPLC separates the analytes based

on hydrophobic interactions between the analytes and the hydrophobic stationary phase. When the hydrophobicity of the mobile phase increases the analytes elute. The analyses were operated by an Agilent 1100 LC-MSD Trap (Agilent Technologies A/S, Naerum, Denmark).

The thawed samples were vortexed and centrifuged at 10000 rpm for three minutes prior to injection. 20 µl were injected on a Zorbax C18 column (2.1 × 150 mm, 5 µm, Agilent Technologies A/S) and the operation temperature was 40 ºC. Eluent A was 0.1% trifluoroacetic acid, and eluent B was 0.1% trifluoroacetic acid in 10/90 water/acetonitrile. The components were separated using a flow rate of 0.25 $mL \cdot min^{-1}$ and a linear gradient of 2-55% eluent B for 60 minutes. Prior to injection the column was rinsed with eluent A for 20 minutes and subsequently eluent B for at least 5 minutes. Between each injection the column was rinsed with eluent B for 6 minutes, and re-equilibrated with 8% eluent B for 10. The eluting peptides were UV-detected at 210 nm and 280 nm.

For identification of the peptides, on-line electrospray ionisation mass spectrometry (ESI-MS) was used. The mass spectrometer was operated in a positive mode with a nebuliser pressure of 345 kPa, nitrogen flow of 9 L min^{-1} and a drying temperature of 300 ºC. Mass spectra were recorded in the scan mode 100-2200 m/z with the target mass set to 1521 m/z. The RP-HPLC was repeated one to three times per cheese to ensure that the chromatograms were repeatable. Data were processed by the software Bruker Daltonics Data Analysis (Bruker, Billerica, MA, USA). Peptides were identified by MS/MS analysis of the most intense ions in the mass spectra of one cheese of each type. The chromatograms from the cheeses with identified peptides were subsequently compared with the chromatograms from cheeses within the same type.

The General Protein Mass Analysis for Windows (GPMAW, Lighthouse Data, Odense, Denmark) was used to assign proposed peptide masses to specific sequences of α_{S1}-casein (with/without 8P), α_{S2}-casein (12 P) and β-casein A1 and A2 (with/without 5 P). Usually GPMAW proposed a number of peptides. Peptides with low probability (a difference of more than ± 1 in molecular weight) were sorted out. Peptides that were unlikely to exist due to the patterns of the proteolytic actions were also sorted out. E.g. a proposed peptide with the sequence α_{s1}-casein Phe_{23}-Phe_{24} intact was regarded as unlikely to be present in high amounts due to the actions of chymosin in Präst cheese.

Protein Prospector (University of California, San Fransisco, USA) was used for theoretical fragmentation of the selected peptides proposed by GPMAW. By comparing the theoretical fragmentation with the MS/MS fragmentation it was possible to conclude which peptide that had the best correlation. During this comparison a couple of factors were regarded as important, in order to enable the most reliable identification possible, e.g., the most intense daughter ions in the MS/MS spectra had to be present in the theoretical fragmentation. Also, when a series of b- or y-ions were found in both the theoretical and the MS/MS fragmentation it was regarded as very likely that the two fragmentations correlated. Furthermore, the peptides proposed by GPMAW were compared with results of previous studies with the same analysis method as the one used in this study. If the mass of a previously identified peptide appeared at the same retention time in this study, it was regarded as likely that it was this peptide. As with the other peptides, the MS/MS fragmentation was compared with the theoretical fragmentation.

4. Results and Discussion

4.1 Gross composition

The results indicated some variation in gross composition among the three groups of Präst cheese of different age. The mean values and standard deviation of pH were 5.66 ± 0.02, 5.54 ± 0.10 and 5.54 ± 0.06 for Mellanlagrad, Allerum and XO, respectively. According to Skånemejerier standards, the pH of Präst should be 5.6 ± 0.2. The variation was rather high, also between the results for cheeses from the same batch, and the analyses were not repeated, which makes the differences in the mean values unreliable. The mean values for the moisture content of the three groups of cheeses were rather stable 35.0% ± 2.32, 35.1% ± 0.33 and 35.3% ± 0.63 for Mellanlagrad, Allerum and XO. According to Skånemejerier standards the moisture content of Präst should be 36% ± 2. However, the moisture content was expected to decrease with cheese age, and, again, the variation was high between the results, likely because of insufficient repetitions of the analyses.

4.2 Peptide profiles of Präst cheese

The RP-HPLC chromatograms (210 nm) of the cheeses were analysed to investigate the variation in peptide composition within and between the three cheese types. In total, four Mellanlagrad, six Allerum and six XO were analysed. One cheese from each ripening age was selected for the identification analysis. Subsequently, the MS/MS results of one cheese of each kind (Mell2, All2 and XO2) were analysed in order to suggest peptide identities of selected peaks.

4.2.1 Peptide profile of Mellanlagrad

The most dominating peaks in the chromatograms of Mellanlagrad Präst cheese were peaks 5, 7, 10, 14, 15, 42, 54 and 55 (Fig. 1). The high peaks at retention time (RT) 6 and 40 were not given a suggested identify in this work. The two cheeses Mell1 and Mell2 had similar HPLC chromatograms, and so had Mell3 and Mell4. Some obvious differences existed between the chromatograms of Mell1/Mell2 and Mell3/Mell4, but overall the Mellanlagrad cheese chromatograms were similar. For example; peak 42, 54 and 55 had approximately the same height in all four cheeses. Peak 5 at RT 13 min was clearly higher in Mell1/Mell2 than in Mell3/Mell4. Peak 7 was found to be higher in Mell3/Mell4 than in Mell1/Mell2. Furthermore, in Mell1/Mell2 peak 5 was higher than peak 7, while in Mell3/Mell4 the relation between the peaks was the opposite. Peak 10 was higher in Mell1/Mell2 than in Mell3/Mell4. A clear difference between the chromatograms was found for peaks 14 and 15. In Mell1/Mell2 peak 14 was higher than peak 15, while in Mell3/Mell4 the relation between the two peaks was the opposite. Mell2 was thoroughly analysed for the distribution of bioactive peptides, which is described in section 4.3.

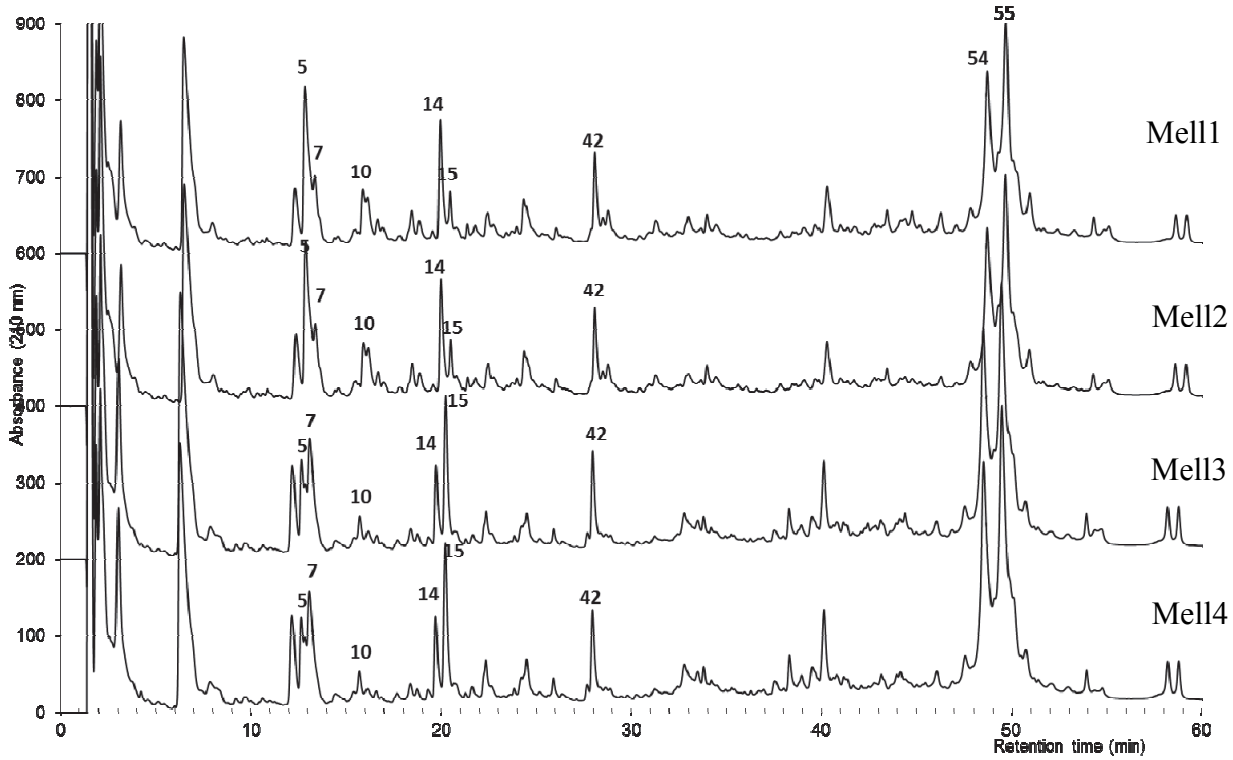

Figure 1. Peptide profile obtained by RP-HPLC of four cheeses of the type Mellanlagrad Präst. Mell2 was subsequently analysed by MS/MS in order to suggest identifications of the peptides.

4.2.2 Peptide profile of Allerum

The most dominating peaks in the chromatograms of Allerum Präst cheese were peaks 5, 7, 10, 14, 15, 42, 54 and 55 (Fig. 2). The high peaks at retention time (RT) 6 and 40 were not given a suggested identify in this work. The two chromatograms of All1 and All2 were rather similar. The chromatograms of All3 - All6 were also rather similar. Examples of peaks with similar height in all six Allerum were 54, 55 and 42. However, differences between the Allerum chromatograms existed and the main differences were found when comparing All1-All2 to the other chromatograms. At RT 12.2-13.5 min two high peaks (5 and 7) were present in all chromatograms. Peak 5 was highest in All4. Peak 7 was considerably smaller in All3-All6 than in All1-All2. Peak 10 at RT 15.8 min was somewhat higher in the chromatograms of All1-All2 than in the other chromatograms.

Peak 14 showed clear differences in height between the chromatograms. In All3 and All4 the peak was considerably higher compared to the other chromatograms. The peak 15 differed somewhat in height between the cheeses, but was clearly present in all chromatograms. The peak 42 at RT 28 differed somewhat in height between the chromatograms. The highest peak was observed in All3. All2 was thoroughly analysed for the distribution of peptides, which is described in section 4.3.

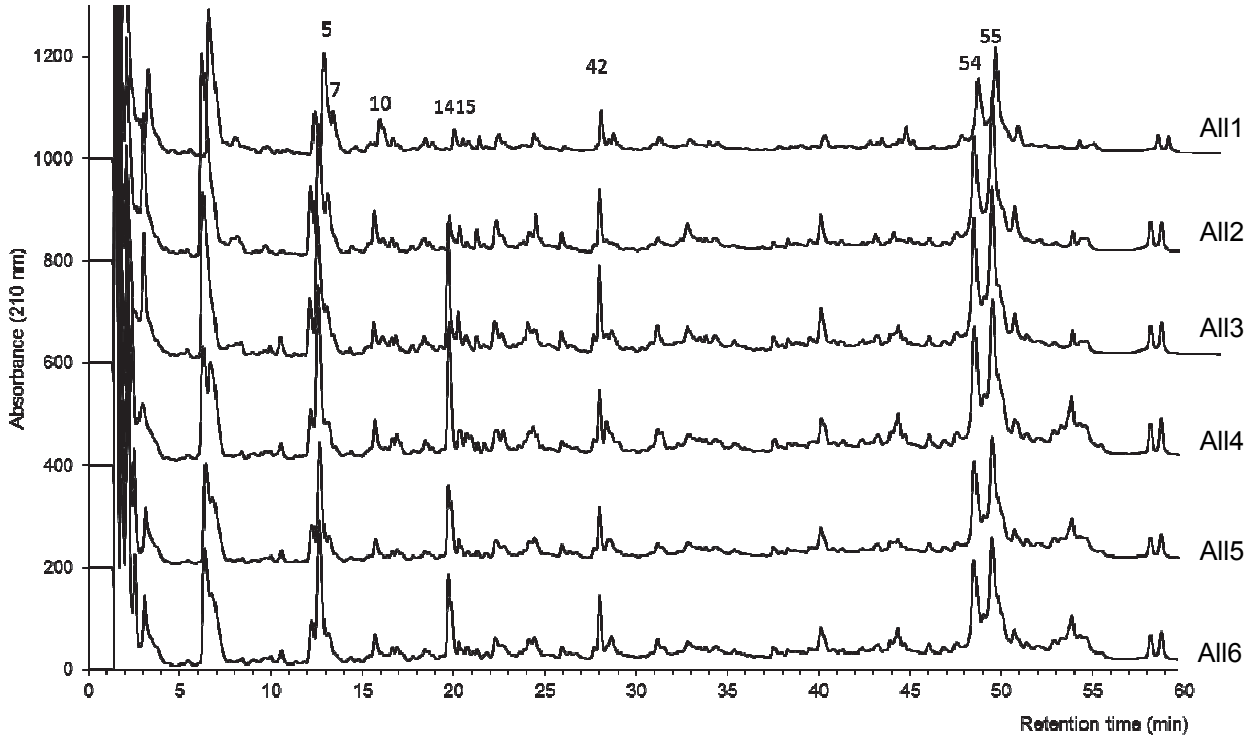

Figure 2. Peptide profile obtained by RP-HPLC of six cheeses of the type Allerum Präst. All2 was subsequently analysed by MS/MS in order to suggest identifications of the peptides.

4.2.3 Peptide profile of XO

The most dominating peaks in the chromatograms of XO Präst cheese were peaks 5, 7, 10, 54 and 55 (Fig. 3). The high peak at retention time (RT) 6 was not given a suggested identity in this work. More differences between the chromatograms were found within the group of XO cheeses, compared to Allerum and Mellanlagrad. However, just as with Allerum and Mellanlagrad, it was possible to find similarities in the cheeses from the same batch (XO1-XO2). Peak 5 at approximately RT 12.7 was represented by a high peak in all 6 chromatograms. The peak 7 had the most clear and defined peak in X01, XO2 and XO3. Peak 8 and peak 10 was found in all chromatograms with approximately the same height. The peaks 14 and 15 were found in lowest height in XO2 and XO4. Overall, XO4 had not as well defined peaks as the other cheeses. The peak 42 was highest in XO3, followed by XO1 and XO2. Peak 54 and 55 was found in all chromatograms with somewhat different height. XO2 was thoroughly analysed for the distribution of peptides, which is described in section 4.3.

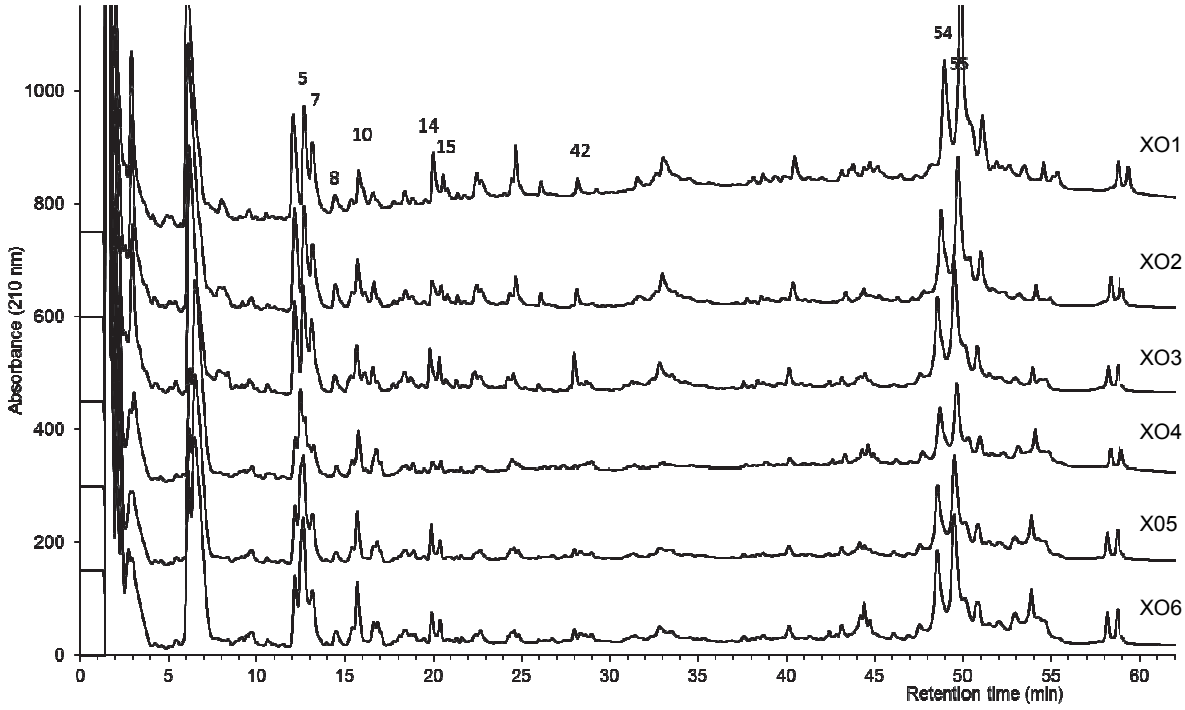

Figure 3. Peptide profile obtained by RP-HPLC of six cheeses of the type XO Präst. XO2 was subsequently analysed by MS/MS in order to suggest identifications of the peptides.

4.2.4 Peptide profile comparison of Mellanlagrad, Allerum and XO

The mass spectrum of XO2, All2 and Mell2 was analysed in order to identify as many peptides as possible. As described in previous sections, differences within the same cheese type existed when the chromatograms were compared. In this section the most important differences between the cheese types are evaluated (Figs. 1-3).

The peaks 5 and 7 were dominating peaks in all the investigated Präst cheeses. Peak 5 was higher than peak 7 in all cheeses, except in Mell3/Mell4. Peak 8 was higher in XO2 (Fig. 4) than in All2, and was not detected in Mell2. The height of peaks 10 and 20 did not differ very much between the cheese types. Peak 11 was somewhat higher in XO2 compared to All2 and Mell2.

The peaks 14 and 15 are high in all investigated cheeses. In Mellanlagrad these peaks are among the dominating ones. In Allerum the peaks 14 and 15 are somewhat smaller than in Mellanlagrad, especially in All1/All2. In XO cheeses the peaks 14 and 15 are relatively high, but much smaller compared to Mellanlagrad. Peak 37 was higher in Mellanlagrad than in Allerum and XO. Peak 38 diluting directly after, was not detected in Mell2, but found in similar height in All2 and XO2. Peak 42 had approximately the same height in Mellanlagrad and Allerum, but was considerably lower in the XO cheeses. Peak 51 was not observed in Mell2, but found with low height in XO2 and All2. Peak 54 and 55 was high in all investigated cheeses.

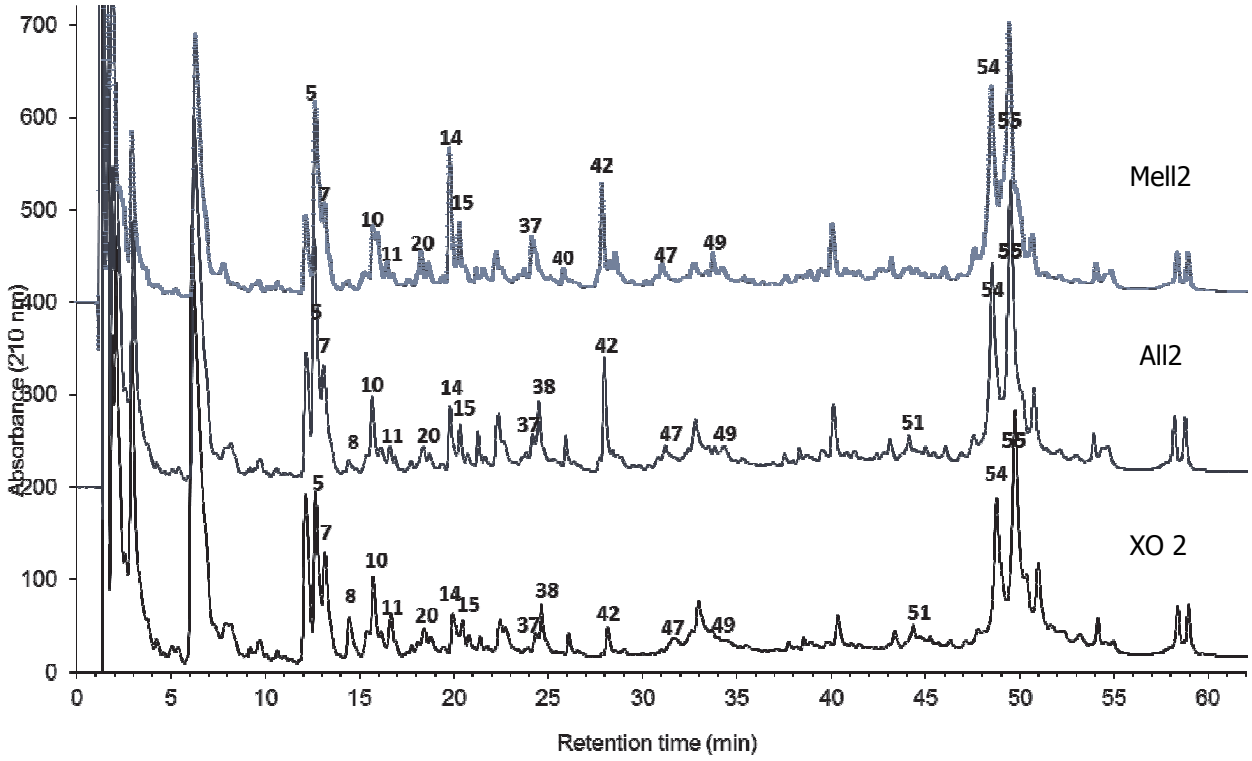

Figure 4. RP- HPLC curves presenting the peptides of the casein breakdown in the cheeses XO2, All2 and Mell2. These three cheeses were analysed by MS/MS in order to identify the peptides in the chromatograms.

4.3 Identification of peptides in Präst cheese of different ripening ages

In total, identity of 55 peptides were suggested from the LC-MS analyses (Table 2) of the three analysed Präst cheeses; Mell2, All2 and XO2. Of the 55 analysed peptides, the suggested identification was supported for 31 of the peptides by comparing LC-MS results from previous experiments of cheese (Ardö et al., 2007; Ardö et al., 2009; Andersen et al., 2009; Lund & Ardö 2004; Rehn et al., 2010).

Peptides that typically accumulate in semi-hard cheeses were tentatively identified in the Präst cheeses. For example, α_{s1}-casein f(1-23) is a chymosin-derived peptide that rapidly undergoes hydrolysis in semi-hard cheeses (Ardö et al., 2007; Rehn et al., 2010). In the Präst cheeses, ten different peptides derived from α_{s1}-casein f(1-23) was found. Chymosin activity on β-casein was detected by the presence of β-casein f(193-209) in Allerum and XO. The plasmin derived peptide, β-casein f(1-28) was identified in all cheese types and in a somewhat higher peak in Mellanlagrad (Table 3). Microbial activity on β-casein f(1-28) resulted in presence of the peptides β-casein f(7-28), f(8-28) and f(11-28).

The peptides β-casein f(29-93) A1 and A2 were found in all investigated cheeses. These peptides indicate a cheese with significant plasmin and chymosin activity and limited microbial proteolysis. When using thermophilic starter, these peptides normally undergo

further hydrolysis. A high presence of these two peptides has previously been observed in Herrgård and Västerbottensost (Ardö et al., 2007; Rehn et al., 2010).

Table 2. Suggested identities of the peptides found in Mellanlagrad (Mell2), Allerum (All2) and XO2. The peaks are given a number and sorted after their retention time, and are presented one group at a time, starting with α_{s1}-casein. The experimental $M+H^+$ from the MS/MS were matched with a theoretical molecular weight. Subsequently a peptide identity is suggested. The reference is a source that previously has identified the peptide with the same experimental procedure

Peak number	**$M+H^+$ (experimental)**			**RT LC-MS (min)**	**z (+)**	**Suggested Casein fragment**	**MW**	**Reference [a]**
	Mell2	**All2**	**XO2**					
						α_{s1}-casein		
1	355.4	355.2	355.2	4.4-4.7	1	f(128-130)	354.4	
2	494.6	494.3	494.3	7.7-8.4	1	f(158-162)	493.5	
3	634.6	634.4	634.4	9.2-9.6	1	f(1-5)	633.8	
4		851.1	851.0	10.7	1	f(187-194)	850.5	
5	1141.0	1140.9	1140.9	12.5-13.1	1	f(1-9)	1140.4	3,5
6			1012.6	13.1	1	f(1-8)	1012.2	3,5
7	876.0	875.6	875.7	13.1-14.1	1	f(1-7)	875.1	3,5
8		1486.4	1486.6	14.6-14.9	1	f(72-83)1P	1486.6	
9	679.5		679.5	15.5-15.6	1	f(8-13)	678.7	3
10	747.7	747.6	747.5	15.8-16.6	1	f(1-6)	746.9	3,5
11	1571.8	1570.7	1570.6	16.6-16.8	1	f(42-54)2P	1570.4	2,3,4
12	890.5	890.5	890.5	17.0-17.3	1	f(102-108)	889.7	3
13		1154.7	1154.7	18.7	1	f(4-13)	1154.3	3
14	1536.5	1536.1	1536.1	19.8-20.4	2	f(1-13)	1535.8	3,5
15	1665.4	1665.2	1665.2	20.4-20.7	2	f(1-14)	1664.9	3.5
16	560.5			21.0	1	f(26-30)	559.0	3
17		1992.1		26.7	2	f(1-17)	1991.3	3
						α_{s2}-casein		
18	374.4	374.4	374.4	3.8-4.0	1	f(85-87)	373.5	
19	485.5	485.4		8.1-8.3	1	f(194-197)	484.5	3
20	1751.7	1751.7	1751.6	18.4-18.5	1	f(6-18)4P	1751.4	2,3,4
21	627.3	627.3	627.4	18.3-18.7	1	f(117-122)	626.8	3
22	2273.6			22.6	2	f(5-21)4P	2273.0	2,4
23		1186.8	1186.9	26.2-26.4	1	f(194-203)	1186.5	
						β-casein		
24		517.3		6.6	1	f(29-32)	516.5	
25	428.5	428.3	428.3	6.7-7.3	1	f(199-202)	427.5	
26	476.5		476.0	8.8-8.9	1	f(22-25)	475.5	
27	312.4	312.1	312.3	7.7-10.0	1	f(84-86)	311.4	
28	423.5	423.1	423.2	9.6-10.4	1	f(64-67)A1	422.5	
29	449.2	449.2	449.2	11.9-12.0	1	f(166-169)	448.7	
30	746.8		746.2	12.2-12.4	1	f(94-100)	745.9	
31		744.3		14.5	1	f(23-28)	743.6	3
32	497.4	497.3	497.3	15.4	1	f(64-68)A2	496.6	

33		1857.6	1856.8	20.9-21.0	1	f(85-101)	1856.2	
34	742.7	742.7	742.7	20.9-21.0	1	f(8-14)	741.8	
35	607.7	607.4	606.9	22.5-23.7	1	f(81-86)	606.8	3
36	708.6	708.3		23.8-24.5	1	f(80-86)	707.9	3
37	756.4	756.5	756.4	24.4-24.5	1	f(47-52)	755.9	3
38		459.3	459.3	24.6-25.0	1	f(84-87)	458.4	
39	867.0	867.6	867.0	25.4-26.2	1	f(74-81)	866.0	3
40	2341.6			26.1	2	f(11-28)4P	2341.1	2,4,5
41	523.5	523.5		27.3-27.4	1	f(60-63)	522.4	3
42	2595.6	2594.3	2595.2	28.0–28.4	2	f(8-28)4P	2594.4	2,3,4,5
43	2709.2		2709.0	28.8-29.3	2	f(7-28)4P	2708.5	2,3,5
44		1126.7	1126.7	28.9-29.0	1	f(44-52)	1126.3	3
45		2482.6		30.0	2	f(144-165)	2482.0	
46	891.8	891.6	891.5	30.8–31.8	1	f(70-77)	891.1	3
47	1001.8	1001.6	1001.7	31.4–31.6	1	f(60-68)A2	1001.2	
48	3055.2	3055.2	3055.2	32.9–33.1	2	f(133-158)	3055.7	
49	3479.4	3477.7	3477.7	33.9–34.5	2	f(1-28)4P	3478.4	3,5
50	3658.4	3658.5	3658.4	43.1-43.6	2	f(38-69) A2	3654.0	
51		1881.4	1882.1	44.0-44.6	1	f(193-209)	1881.2	3,5
52		3787.2		48.0-48.3	2	f(63-98) A2	3788.5	
53		3864.7		48.5-48.7	2	f(29-60)1P	3866.2	
54	7531.2	7531.2	7530.4	48.3-49.0	4	f(29-93)1P A1	7530.4	1,5
55	7491.6	7491.6	7491.6	49.6–49.8	4	f(29-93)1P A2	7490.4	1,5

[a] Suggested peptide identification supported with LC-MS results from previous analysis of cheese: 1) Ardö et al., (2007), 2) Ardö et al., (2009), 3) Andersen et al., (2009), 4) Lund & Ardö (2004), 5) Rehn et al., (2010)

4.4 Bioactive peptides in Präst cheese

In the three Präst cheeses, 21 peptides with known bioactivity (described in scientific literature) were tentatively identified (Table 3). Furthermore, 10 peptides with amino acid sequences similar to peptides with known bioactivity were suggested (Table 4).

Peptides with the following bioactivities were identified: ACE-inhibitory, antioxidative, antimicrobial, immunomodulatory, cytomodulatory, phosphopeptides and opioid (Silva & Malcata 2005; Hayes et al., 2007). Peptides with more than one bioactive property, multifunctional peptides, were identified in the Präst cheeses. An example is β-casein f(193-209) that can act ACE-I, antimicrobial and immunomodulatory (Birkemo et al., 2007; Hayes et al., 2007; Ong et al., 2007).

The relative content of each peptide in the three cheeses was estimated from the 210 nm UV-chromatograms (Fig. 4). In several cases the height of the peaks differed between the cheeses of different age. However, most of the peptides were identified in similar proportions in all three cheeses, i.e. the chromatograms of the three cheeses revealed relatively similar chromatograms. The most dominating peaks in the chromatograms of Mellanlagrad Präst and Allerum Präst were identified as α_{s1}-casein f(1-6), f(1-7), f(1-9), f(1-13) and f(1-14), as well as β-casein f(8-28)4P and β-casein f(29-93)1P A1 and f(29-93)1P A2. The most dominating

peaks in the chromatograms of XO were identified as α_{s1}-casein f(1-6), f(1-7), f(1-9) and β-casein β-casein f(29-93)1P A1, f(29-93)1P A2.

4.4.1 ACE-Inhibitory peptides (ACE-I)

The ACE-I peptides β-casein f(47-52) and f(193-209) previously identified by Ong et al., (2007), were found in the Präst cheeses. β-casein f(193-209) was found in Allerum and XO, but not in Mell2. β-casein f(47-52) was found in all of three investigated cheeses. The ACE-I peptides α_{s1}-casein f(1-6), f(1-7) and f(1-9) were found in all three investigated cheeses. The peaks of this group of co-eluting peptides were relatively high in all cheeses. The ACE-I peptides α_{s1}-casein f(1-13) and β-casein f(60-68) have previously been identified in Gouda cheese (Saito et al., 2000). These peptides were found in all three cheeses.

One of the most potent and well-studied ACE-inhibitory casein peptide is β-casein f(84-86) (Val-Pro-Pro). This peptide is an active component in the fermented sour milk Calpis from Japan (Hayes et al., 2007). β-casein f(84-86) was found in a small peak in Allerum and XO. This suggested identification was supported by the correlation between the theoretical fragmentation of Val-Pro-Pro and the MS/MS fragmentation.

Some peptides with similarity to previously identified ACE-I peptides were found in the investigated Präst cheeses. β-casein f(84-87) (Val-Pro-Pro-Phe) had a high peak in the investigated XO. Other peptides with similarity to known ACE-I peptides are β-casein f(8-14), f(64-67) and α_{s1}-casein f(26-30). Gobbetti et al., (2004) found that β-casein f(7-14) had ACE-I properties. Hayes et al., (2007) reports ACE-I properties of β-casein f(63-67). FitzGerald et al., (2000) reported that α_{s1}-casein f(27-30) had antihypertensive effect on rats. These three peptides were found in small peaks in the investigated cheeses.

4.4.2 Antimicrobial peptides

Peptides with reported antimicrobial activity, α_{s1}-casein f(1-7) and f(1-9), were found in the investigated cheeses (Hayes et al., 2007). Both α_{s1}-casein f(1-7) and f(1-9) was found with high peaks in the three cheeses Mell2, All2 and XO2. α_{s1}-casein f(1-9) and α_{s1}-casein f(1-7) had a relatively equal distribution in all three cheeses. Birkemo et al., (2008) reported β-casein f(193-209) to have convincingly antimicrobial activity. This peptide was found in Allerum2 and XO2, but not in Mellanlagrad2.

4.4.3 Opioid peptides

Proteinases of *Lc. Lactis* degrade β-casein and the peptide β-casein f(60-68) is often generated. The peptide β-casein f(60-63) has been identified in Australian cheddar; this peptide is likely to be an opioid agonist (Hayes et al., 2007). The peptide β-casein f(60-68) was present in all three cheeses, Mell2, All2 and XO2. The peptide β-casein f(60-63) was identified in Mell2 and in All2, but not in XO2.

Gobbetti et al., (2004) listed β-casein f(158-164) as an opioid antagonist. In the investigated Präst cheeses, the peptide β-casein f(158-162) was detected in low amounts. Since the *N*-terminal of the peptide is described as the most important for the receptor binding (Gobbetti et al., 2004), it is a possibility that also this peptide could be an opioid.

4.4.4 Cytomodulatory peptides

Peptides with opioid properties may also exert cytomodulatory properties, e.g. the β-casomorphins. In the investigated cheese two β-casomorphins were identified. The peptide β-casein f(60-68) was present in all three cheeses, Mell2, All2 and XO2. The peptide β-casein f(60-63) was found in small peaks in Mell2 and All2, but was not found in XO2.

4.4.5 Immunomodulatory peptides

The immunomodulatory peptide β-casein f(193-209) has been reported to enhance proliferation of blood lymphocytes (Gobbetti et al., 2004). This peptide was identified in All2 and XO2. The peptide was not found in the Mell2. Gobbetti et al., (2004) list β-casein f(63-68) as an immunomodulatory peptide. In the investigated Präst cheeses the similar peptide β-casein f(64-68) was detected.

Caseinphosphopeptides are also believed to have immunoenhancing properties (Gobbetti et al., 2004). β-casein f(1-28)4P was found in Mell2, and in slightly lower peaks in All2 and XO2. See next paragraph for more details regarding identified CPPs.

4.4.6 Caseinphosphopeptides (CPP)

Eight mineral binding caseinphosphopeptides were identified in the investigated cheeses. α_{s1}-casein f(42-54) 2P was found in high peaks in all three cheeses. However, XO2 had a somewhat higher peak. α_{s2}-casein f(5-21) 4P was detected in a small peak in Mell2 and not at all in the All2 and XO2. α_{s2}-casein f(6-18) 4P was identified in all three cheeses. Six CPPs from β-casein was identified in the cheese. β-casein f(1-28)4P was identified in all three cheeses and in somewhat higher peak in Mell2. β-casein f(7-28)4P was found in Mell2 and in a small peak in XO2, but not identified in All2. β-casein f(8-28)4P was identified in high peaks in all three cheeses. β-casein f(11-28) 4P was identified in Mell2, but not in All2 or XO2. β-casein f(29-93)1P A1 and A2 was identified in high peaks in all three cheese types.
Apart from the above mentioned CPPs, which are described frequently in the literature, two more CPPs were identified in the investigated Präst cheeses. Namely, β-casein f(29-60)1P and α_{s1}-casein f(72-83)1P. These peptides were not found or found in small peaks in the cheeses, except α_{s1}-casein f(72-83)1P that had a high peak in XO.

4.4.7 Antioxidative peptides

Seven potential antioxidative peptides from α_{s1}-casein were identified in the investigated cheeses. α_{s1}-casein f(1-6), f(1-7), f(1-9) and f(1-13) were found in relatively high peaks in all three cheeses. α_{s1}-casein f(1-8) was identified in a small peak in XO2, but not at all in All2 and Mell2. α_{s1}-casein f(1-14) was found in high peaks in all three cheeses, but with a slightly smaller peak in Mell2. α_{s1}-casein f(4-13) was found in very small peaks in All2 and XO2.
Some peptides of α_{s1}-casein f(1-23), were identified in small peaks in the Präst cheese, but was not found in the literature that describes antioxidant peptides. These peptides with high similarity to antioxidant peptides are: α_{S1}-casein f(1-5) and f(1-17). α_{S1}-casein f(1-5) was found in all three cheeses, whereas α_{S1}-casein f(1-17) was detected in All2.

Table 3. Suggested bioactive peptides in the investigated Präst cheeses (Mell2, All2 and XO2) based on the tentatively identification by LC/MS. The references refer to the source that describes the peptide as bioactive

Casein Fragment	**Relative concentration**			**Bioactivity**	**Previously found in**	**Reference** [b]
	Mell2	**All2**	**XO2**			
α_{S1}-casein						
f(1-6)	[a]++	++	++	ACE-I, Antioxidative	Festivo, Cheddar, Semi-hard cheese	2, 10, 12
f(1-7)	++	++	+++	ACE-I, Antioxidative, Antimicrobial	Festivo, Cheddar, Semi-hard cheese	2, 10, 12
f(1-8)	-	-	+	Antioxidative	Semi-hard cheese	2
f(1-9)	+++	+++	+++	ACE-I, Antioxidative, Antimicrobial	Gouda, Festivo, Cheddar, Semi-hard cheese	2, 5, 6, 10, 12, 13
f(1-13)	+++	++	++	ACE-I, Antioxidative	Semi-hard cheese	13
f(1-14)	++	++	++	Antioxidative	Semi-hard cheese	1, 2
f(4-13) 2P	-	+	+	Antioxidative	Semi-hard cheese	2
f(42-54) 2P	+	+	++	CPP	Herrgård	7
α_{S2}-casein						
f(5-21) 4P	+	-	-	CPP	Herrgård	7
f(6-18) 4P	+	+	+	CPP	Herrgård	7
β-casein						
f(1-28)4P	++	+	+	CPP, Immunomodulatory	Herrgård	1, 9
f(7-28)4P	++	-	+	CPP	Herrgård	1, 7
f(8-28)4P	+++	+++	++	CPP	Herrgård	1, 7
f(11-28)4P	++	-	-	CPP	Herrgård	1, 7
f(47-52)	++	++	++	ACE-I	Cheddar	10
f(60-68)A2	++	++	++	Cytomodulatory, ACE-I, Opioid	Gouda	5, 13
f(60-63)	+	+	-	Cytomodulatory, Opioid		14
f(84-86) (VPP)	-	+	+	ACE-I	Evolus (milk drink)	4,5, 6
f(193-209)	-	++	++	ACE-I, Antimicrobial, Immunomodulatory	Cheddar	3, 8, 10,14
f(29-93)1P A1	+++	+++	++	CPP	Herrgård	1
f(29-93)1P A2	+++	+++	+++	CPP	Herrgård	1

[a]) The grading system is based on the relative height of the specific peak in the 210 UV-chromatograms obtained from LC-MS. Interpretation is made as follows: -: no detectable peptide, +: small peak , ++: medium sized peak, +++: large peak.

[b]) Bioactivity of the suggested peptides are supported with results of the following authors: 1) Ardö et al., (2007), 2) Ardö et al., (2009), 3) G.A. Birkemo et al., (2008), 4)Hayes et al., (2007), 5) Hernández-Ledesma et., (2011), 6) Korhonen & Pilantho, (2006), 7) Lund & Ardö (2004), 8) Meisel, (1997), 9) Meisel, (2004), 10) Ong et al., (2007), 12) Ryhänen et al., (2001), 13) Saito et al., (2000), 14) Silva & Malcata (2005)

Table 4. Peptides in the analyzed Präst cheeses (Mell2, All2 and XO2) whose tentative identity is similar to a bioactive peptide described in previous research. The references in the table refer to the source that describes a similar peptide as bioactive

Casein fragment	Relative concentration			Suggested bioactivity and Reference
	Mell2	All2	XO2	
α_{S1}-casein				
f(1-5)	[a]+	+	+	Antioxidative (1) [b]
f(1-17)	-	+	-	Antioxidative (1)
f(26-30)	+	-	-	ACE-I (4)
f(72-83)1P	-	+	++	CPP (1)
f(158-162)	+	+	+	Opioid (2)
β-casein				
f(8-14)	+	+	+	ACE-I (2)
f(29-60)1P	-	+	-	CPP(1)
f(64-67)	+	+	+	ACE-I (2, 3)
f(64-68)	+	+	++	Immunostimulatory (2, 3)
f(84-87)	-	+++	+++	ACE-I (3)

a) The grading system is based on the relative height of the specific peak in the UV-chromatogram obtained from the LC-MS. Interpretation is made as follows: -: no detectable peptide, +: small peak, ++: medium sized peak, +++: large peak.

b) 1) Ardö et al., (2009), 2) Gobbetti et al., (2004), 3) Hayes et al., (2007), 4) Ong et al., (2007)

5. Conclusions

Most of the bioactive peptides found in Präst cheese belonged to the groups ACE-inhibitory, antioxidative and CPPs. The content of bioactive peptides differed somewhat between the three cheese types. However, since differences also exist within the cheese types it is hard to argue on a specific bioactive profile for each of the cheese types. The three analysed cheeses had relatively similar chromatograms. The cheese ripened for the longest period, XO, were not very much more degraded than the other two cheeses.

The most dominating peaks in Mellanlagrad and Allerum were identified as α_{S1}-casein f(1-6), f(1-7), f(1-9), f(1-13) and f(1-14) as well as β-casein f(8-28),(29-93)A1 and f(29-93)A2. In XO, the most dominating peaks were identified as α_{S1}-casein f(1-6), f(1-7) and f(1-9) as well as β-casein f(29-93)A1 and f(29-93)A2. It appears that α_{S1}-casein f(1-13), f(1-14) and β-casein f(8-28) had been further broken down in XO compared to Allerum and Mellanlagrad. Peptides from the *N*-terminal part of α_{S1}-casein were a group of bioactive peptides accounting for high presence in Mell2, All2 and XO2. The peptides from α_{S1}-casein were found in peaks with similar height in the three cheese types. Similar variation was found both within and between cheese types. Furthermore, the observed differences were not large, e.g., if a peptide could not be identified in one cheese type, the concentrations in the other cheese types were very low. Peptides from the *N*-terminal part of α_{S1}-casein are reported to be ACE-inhibitory and antioxidative. α_{S1}-casein f(1-9) is also reported to be antimicrobial.

Caseinphosphopeptides (CPPs) were another group of bioactive peptides that were dominating in Präst cheese. Many of the identified peptides originate from the *N*-terminal part of β-casein. The peaks of these peptides were relatively high in all three cheese types. The result indicated, however, that the presence of CPPs from the *N*-terminal part of β-casein was somewhat higher in the Mellanlagrad cheeses. The large CPPs β-casein f(29-93) A1 and A2 were found in large peaks in all three cheese types. These peptides have previously been found to accumulate in the cheese variety Herrgård (Ardö., et al 2007).

The multifunctional peptide β-casein f(193-209) were detected in All2 and XO2, but not in Mell2. This peptide can have antimicrobial, immunomodulatory and ACE-inhibitory effects. The two peptides β-casein f(47-52) and β-casein f(60-68) were found equally distributed and with relatively high peaks in the three types of cheeses. Both of these peptides can act ACE-inhibitory and β-casein f(60-68) are also reported to act cytomodulatory and as an opioid.

It is important to state that even though some peaks in the chromatograms were very small, it is not certain that the level of the bioactive peptide is too low to have an effect. Also, it is not certain that the bioactive peptides with the highest peak have the desired effect after normal consumption. Further research is needed on the relationship between concentration and bioactivity before any estimations can be made whether any of the peaks are high enough to exert bioactivity.

Previous research has reported that the content of ACE-I peptides decrease after a certain point during the ripening (Gobbetti et al., 2004). This is not apparently seen in the Präst cheeses from the age of 8 months. Since the investigated cheeses were not analysed throughout the ripening period, detected differences between the cheese types can be caused by other factors than the actual ripening time, e.g. the raw milk, temperature and other factors during the cheese making.

The results of this study indicate a high content of ACE-inhibitory peptides and an interesting field for further investigations is the ACE-inhibitory potential and the effect on blood pressure of Präst cheese. The ACE-I activity of Präst cheese can be measured spectrosphotometrically as described by Ardö et al., (2009). If high ACE-I activity is revealed, a further step can be to investigate possible antihypertensive effect by *in vivo* studies in hypertensive rats, which is an accepted model for studying hypertension in humans (Ricci et al., 2010).

Another potential bioactivity of Präst cheese to further investigate is the effect of CPPs. It is important to note that this study did not give the full picture of bioactive peptides in Präst cheese. To do so, multi-step purification techniques are required e.g., to study the presence of CPPs more thoroughly by selecting a purification method that utilizes the interaction between phosphopeptides and polyvalent metal ions (Lund & Ardö, 2004).

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References

Andersen, L. T., Sorensen, N.K., Ardö, Y. (2009). A comparison of peptide and free amino acid composition of seven commercial available Cheddar cheeses with different sensory characteristics (*Manuscript*)

Ardö, Y, Lilbaek, H., Kristiansen, K.R, Zakora, M, Otte, J. (2007) Identification of large phosphopeptides from β-casein tahat characteristically accumulate during ripening of the semi-hard cheese Herrgård. *International Dairy Journal,* 17: 513-524

Ardö, Y., Polychroniadou, A. ed. (1999) Laboratory Manual for Chemical Analysis of Cheese. European Communities, Office for Official Publications of the European Communities, Luxembourg (ISBN 92-828-6599-1)

Ardö, Y., Pripp, A. H., Lillevang, S. K. (2009) Impact of heat-treated Lactobacillus helveticus on bioactive peptides in low-fat, semi-hard cheese. *The Australian journal of dairy technology*, 64: 58-62

Birkemo, G.A., O´Sullivan, O., Ross, R.P., Hill, C. (2008) Antimicrobial activity of two peptides casecidin 14 and17, found naturally in bovine colostrum. *Journal of Applied Microbiology,* 106: 233-240

Coultate, T (2009) Food – The chemistry of its components. 5th ed. Cambridge: The Royal Society of Chemistry, 2009, pp. 176-184

FitzGerald, R.J. & Meisel, H. (2000). Milk protein derived inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition*, 84: 33-37

Gobbetti, M., Minervini, F., Rizzello, C.G. (2004) Angiotensin I-converting enzyme-inhibitory and antimicrobial bioactive peptides. *International Journal of Dairy Technology,* 57: 173-188

Hernández-Ledesma, B., del Mar Contreras, M., Recio, I. (2011) Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science,* 165: 23-35

Hayes, M., Stanton, C., Fitzgerald, G.F., Ross, R.P. (2007) Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. *Biotechnology Journal*, 2: 435-449

Horne, D.S., & Banks J.M. (2004) Rennet-induced Coagulation of Milk. In PF. Fox. Ed: Cheese: Chemistry, Physics and Microbiology. Vol 1: General Aspects, 3d ed. London: Elsevier Applied Science, 1987, pp. 47-50

Farrell, H. M., Jimenez-Flores, R., Hollar, C. M., Ng-Kwai-Hang K. F., Swaisgood, H. E. (2004). Nomenclature of the Proteins of Cows' Milk—Sixth Revision *Journal of Dairy Science,* 87: 1641-1674

Korhonen, H. & Pihlanto-Leppälä, A. (2004) Milk-derived bioactive peptides: Formation and prospects for health promotion. Handbook of Functional Dairy Products CRC Press LLC

Korhonen, H. & Pihlanto-Leppälä, A. (2006) Bioactive Peptides: Production and functionality. *International Dairy Journal,* 16: 945-960

Lund, M. & Ardö, Y. (2004) Purification and identification of water-soluble phosphopeptides from cheese using Fe(III) affinity chromatography and mass spectrometry. *Journal of Agricultural Food Chemistry*. 52: 6616-6622

McSweeney, P.L.H, Ottogalli, G., Fox, P.F., (2004) Diversity of Cheese Varieties: An Overview . In PF. Fox. Ed: Cheese: Chemistry, Physics and Microbiology. Vol 2: General Aspects, 3d ed. London: Elsevier Applied Science, 1987, pp 7

Meisel, H. (1997) Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers,* 43: 119-128

Meisel, H. (2004) Multifunctional peptides encrypted in milk proteins. *BioFactors*, 21: 55-61

Möller, N.P., Scholz-Ahrens, K.E., Roos, N., Schrezenmeir, J., (2008) Bioactive peptides and proteins from foods: indication for health effects. *European Journal of Nutrition*, 47: 171-182

O'Brien, N.M., O'Connor; T.P. (2004) Nutritional Aspects of Cheese. In PF. Fox. Ed: Cheese: Chemistry, Physics and Microbiology. Vol 1: General Aspects, 3d ed. London: Elsevier Applied Science, 1987, pp. 573-575

Ong, L., Henriksson, A., Shah, N.P. (2007) Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic *Lactobacillus casei* sp. *Le Lait*, 87: 149-165

Rehn, U., Petersen, M.A., Hallin Saedén, K., Ardö, Y. (2010) Ripening of extra-hard cheese made with mesophilic DL-starter. *International Dairy Journal*, 20: 844-851.

Ricci, I., Artacho, R., Olalla, M. Milk protein peptides with Angiotensin I-converting enzyme inhibitory (ACEI) activity. *Food Science and Nutrition*, 50: 390-402

Ryhänen, E.L., Pihlanto-Leppälä, A., Pahkala, E. (2001) A new type of ripened, low fat cheese with bioactive properties. *International Dairy Journal*, 11: 441-447

Saito, T., Nakamura, H., Kitazawa, H., Kawai, Y., Itoh, T., (2000) Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *Journal of Dairy Science*, 83: 1434-1440

Silva, S.V. & Malcata X. (2005) Caseins as source of bioactive peptides. *International Dairy Journal*, 15: 1-15

Sindayikengera, S., Xia, W., (2006) Nutritional evaluation of caseins and whey proteins and their hydrolysates from Protamex. *Journal of Zhejiang University,* 7: 90-98

Sousa, M. J., Ardö, Y., McSweeney, P.L.H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, 11: 327- 345

Upadhyay, V.K., McSweeney, P.L.H.,. Magboul, A.A.A., Fox, P.F. (2004) Proteolysis in cheese during ripening. In PF. Fox. Ed: Cheese: Chemistry, Physics and Microbiology. Vol 1: General Aspects, 3d ed. London: Elsevier Applied Science, 1987, pp. 391-392

Internet:

Swedish board of Agriculture:

Consumption of Food 1960-2006. (2009) Available from: (http://www.sjv.se/webdav/files/SJV/Amnesomraden/Statistik%2C%20fakta/Livsmedel/2009%3A2/20092_amk_ihopb.pdf) [2011-11-10]

Swedish Dairy Association:

Sensoriska beskrivningar för fem lagrade hårdostar 2011-10-25. Available from: (http://www.svenskmjolk.se/Global/Document/Nyheter/Publikt/Sensoriska_beskrivningar_f%c3%b6r_fem_lagrade_h%c3%a5rdostar.pdf?epslanguage=sv) [2011-12-04]

Präst® - hårdost som hårdvaluta. Available from: (www.svenskmjolk.se/Mjolk-smor-och-ost/Vara-varumarken/Prast---hardost-som-hardvaluta/) [2011-12-04]

Appendix

All2 and XO2 was extracted at two occasions (occasion *a* and *b*) and subsequently analyzed by RP-HPLC at two occasions. The obtained chromatograms are presented in Fig. 1 and Fig. 2. The chromatograms of All2 were similar. However, All2a had lower peaks than All2b. The chromatograms of XO2 were also very similar; again XO2a had lower peaks than XO2b. It is likely that the samples from extraction occasion *a* were somewhat more diluted than the samples from occasion *b*.

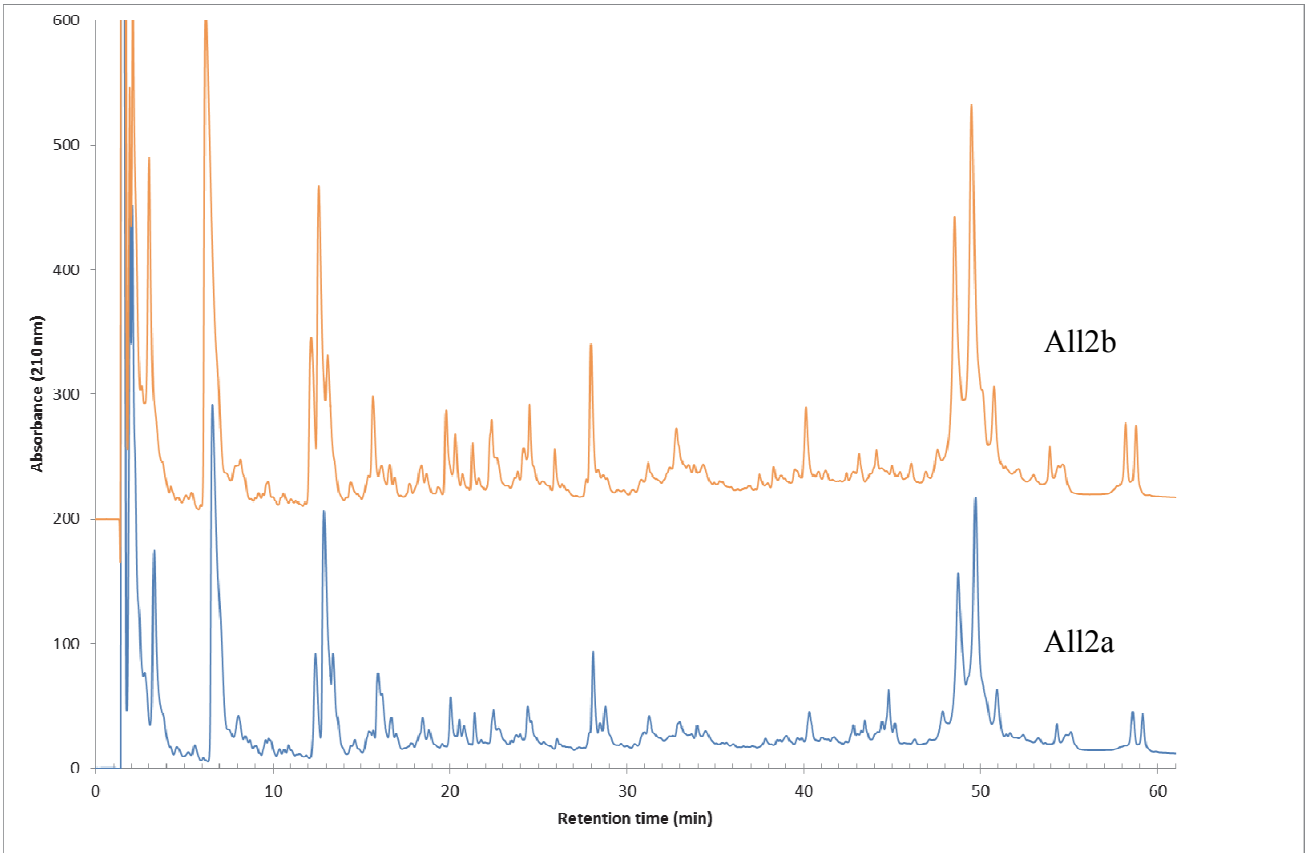

Figure 1. Peptide profile of All2 from RP-HPLC. All2 was the Allerum cheese that was analyzed thoroughly for peptide identification. The two chromatograms show different extractions and different RP-HPLC runs of All2.

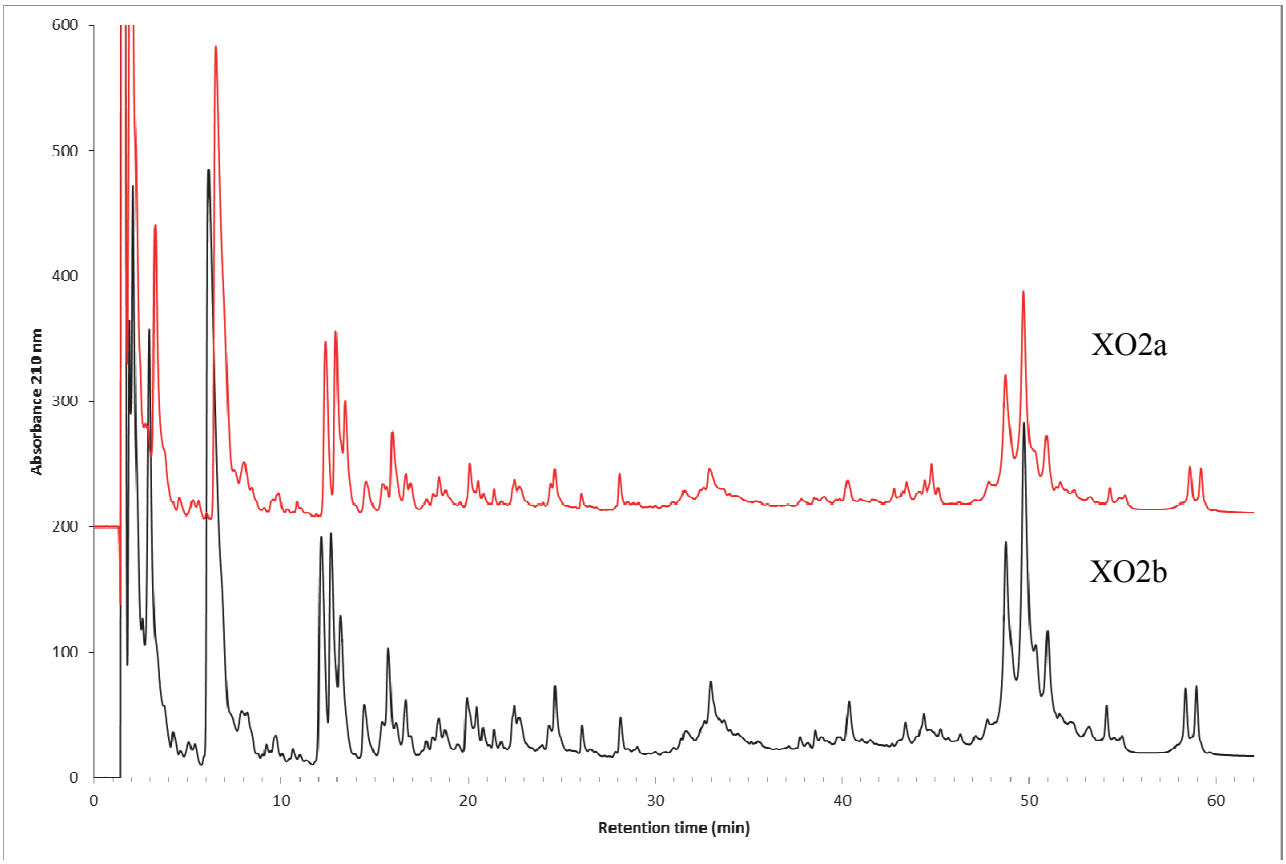

Figure 2. Peptide profile of XO2 from RP-HPLC. XO2 was the XO cheese that was analyzed thoroughly for peptide identification. The two chromatograms show different extractions and different RP-HPLC runs of XO2.

During RP-HPLC the molecules are detected at both 210 nm and 280 nm. In Fig. 3 the 280 nm chromatograms of the cheeses Mell2, All2 and XO2 are presented.

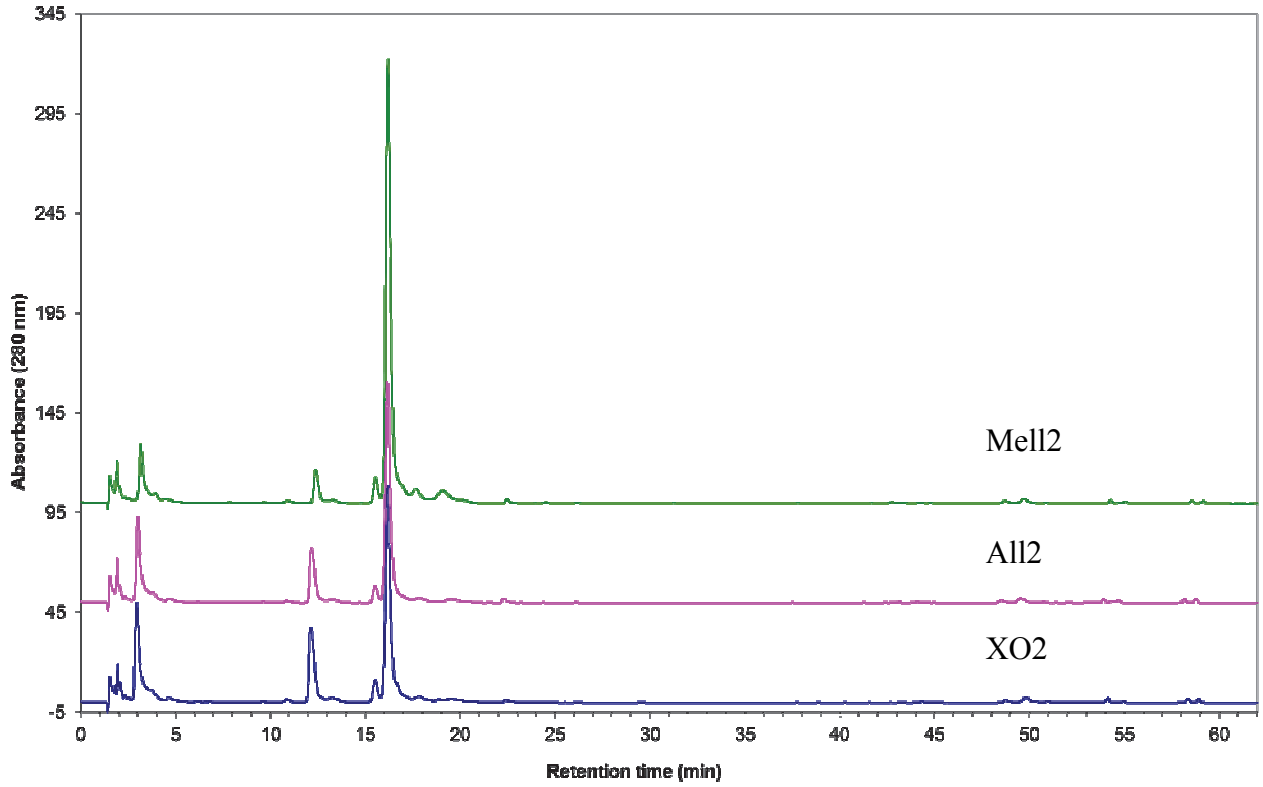

Figure 3. RP-HPLC chromatograms of the three Präst cheeses (Mell2, All2 and XO2) that were investigated thoroughly for peptide identification by MS/MS. The chromatograms are recorded at 280 nm.

Raw data from LC-MS. Analyzed peaks are presented, both those with suggested peptide identity and those which were not given a suggested identity.

MELLANLAGRAD2 (Mell2)

RT Range [min]	**Prec. m/z**	**Charge**	**[M+H]+**	**Amino Acid seq**	**Suggested Casein fragment**
	374,4	1+	374,4	INK alt INQ	Beta Casein26-28, alpha s2 85-87
4.4 - 4.7	355,4	1+	355,4	HAQ	alpha S1 128-130
	195,2				
	530,5	1+	530,5	111-114 [F] QVKR, 158-161 [F] KNRL, 25- 28 [A] RINK	
7.1 - 7.3	428,5	1+	428,5	GPVR	Beta-Casein 199-202
7.7 - 7.8	312,4	1+	312,4	VPP	Beta casein 84-86
8.0 - 8.4	494,6	1+	494,6		
8.2 - 8.3	485,5	1+	485,5	IQPK	alpha S2 Casein 194-197
8.4 - 8.4	485,5	1+	485,5	IQPK	alpha S2 Casein 194-197
8.8 - 8.9	476,5	1+	476,5	SITR	Beta-Casein (22-25)
	312,4	1+	312,4	VPP	Beta casein 84-86
9.6 - 9.6	634,6	1+	634,6	RPKHP	alpha S1 Casein 1-5
	423,5	1+	423,5	GPIH	Beta Casein 64-67
11.0 - 11.0	348,5	1+	348,5		
12.3 - 12.3	746,8	1+	746,8	GVSKVKE	Beta Casein 94-100
12.8 - 12.9	1141	1+	1141	RPKHPIKHQ	alpha S1 1-9
12.9 - 13.1	571,2	2+	1141,4	RPKHPIKHQ	alpha S1 1-9
13.0 - 13.1	1141	1+	1141	RPKHPIKHQ	alpha S1 1-9
13.1 - 13.2	571,2	?	1141	RPKHPIKHQ	alpha S1 1-9
13.3 - 13.4	1141	1+	1141	RPKHPIKHQ	alpha S1 1-9
13.4-13.8	876	1+	876	RPKHPIK	alpha S1 1-7
14.4 - 14.5	770,5	1+	770,5		

14.8 - 14.8	609,5	1+	609,5		
15.4 - 15.4	497,4	1+	497,4	GPIPN	Beta Casein 64-68
15.5 - 15.5	679,5	1+	679,5	HQGLPQ	AlphaS1 (8-13)
15.8 - 15.8	1515,9	1+	1515,9		
16-16.6	747,7	1+	747,7	RPKHPI	alpha S1 1-6
16,8	1571,8	1+	1571,8	KDIGZEZTEDQAM	alpha S1 (8p) (42-54)
17.1 - 17.1	602,8	2+	1204,6		
18.4 - 18.7	627,3	1+	627,3	VPITPT	alpha S2 (12p) (117-122)
20.1 - 20.3	1536,5	1+	1536,5	RPKHPIKHQGLPQ	alpha S1 1-13
20.1 - 20.4	769,2	2+	1537,5	RPKHPIKHQGLPQ	alpha S1 1-13
20.7 - 20.7	1665,4	1+	1665,4	RPKHPIKHQGLPQE	alpha S1 1-14
20.9 - 21.0	742,7	1+	742,7	VPGEIVE	Beta-Casein (8-14)
21.0 - 21.1	560,5	1+	560,5	APFPE	alpha S1 26-30
21.4 - 21.6	1871,2	1+	1871,2		
21.5 - 21.6	936,3	2+	1871,6		
21.6 - 21.7	1871,4	2+	1871,4		
22.5 - 22.6	1073,1	?			
22.6 - 22.7	1137,3	2+	2273,6		alphas2 5-21
22.8-23.3	607,7	1+	607,7	PVVVPP	Beta-Casein (81-86)
23.5 - 23.7	607,7	1+	607,7	PVVVPP	Beta-Casein (81-86)
23.8 - 24.5	708,6	1+	708,6	TPVVVPP	Beta-Casein (80-86)
25.1 - 25.2	439	2+	877		
25.4 - 25.5	1120,3	?			
26.0 - 26.2	1171,3	2+	2341,6	EIVEZLZZZEESITRINK	Beta-Casein (5p)(11-28)
26.5 - 26.6	1200	3+	3597,9		
26.8 - 26.9	741,7	1+	741,7		

27.3 - 27.4	523,5	1+	523,5	YPFP	Beta-Casein (60-63)
27.9 - 28.0	1362,1	?			
28.2 - 28.2	1298,3	2+	2595,6	VPGEIVEZLZZZEESITRINK	Beta-Casein (5P) 8- 28
28.0 - 28.4	1298,3	2+	2595,6	VPGEIVEZLZZZEESITRINK	Beta-Casein (5P) 8- 28
28.3 - 28.4	1298,3	2+	2595,6	VPGEIVEZLZZZEESITRINK	Beta-Casein (5P) 8- 28
28.6 - 28.7	1234,2	2+	2467,5		
28.8 - 29.2	1355,1	2+	2709,2	NVPGEIVEZLZZZEESITRINK	Beta-Casein (5P) 7- 28
29.0 - 29.3	1355,1	2+	2709,2	NVPGEIVEZLZZZEESITRINK	Beta-Casein (5P) 7- 28
30.8 - 31.1	891,8	1+	891,8	LPQNIPPL	Beta-Casein (70-77)
31.5 - 31.5	1001,8	1+	1001,8	YPFPGPIPN	Beta-Casein (60-68)
31.7 - 31.7	891,9	1+	891,9	LPQNIPPL	Beta-Casein (70-77)
31.7 - 31.8	891,8	1+	891,8	LPQNIPPL	Beta-Casein (70-77)
31.8 - 31.8	891,9	1+	891,9	LPQNIPPL	Beta-Casein (70-77)
32.9 - 33.0	1916	2+	3831		
33.0 - 33.1	1854,5	2+	3708		
33.1 - 33.1	1854,5	2+	3708		
34.1 - 34.1	1740,2	2+	3479,4	RELEELNVPGEIVEZLZZZEESITRINK	Beta-Casein 5 P(1-28)
34.1 - 34.1	1740,2	2+	3479,4	RELEELNVPGEIVEZLZZZEESITRINK	Beta-Casein 5 P(1-28)
34.2 - 34.5	1740,2	2+	3479,4	RELEELNVPGEIVEZLZZZEESITRINK	Beta-Casein 5 P(1-28)
34.4 - 34.4	855,6	1+	855,6		
36.1 - 36.2	1670,6	2+	3340,1		
40.0 - 40.1	1744,3	2+	3487,6		
40.2 - 40.3	1589,9	2+	3178,8		
40.4 - 40.4	1547,3	2+	3093,6		
40.5 - 40.5	1695,5	?			
40.6 -	1916	3+	5746		

40.6					
41.0 - 41.1	2043,5	2+	4086		
43.5 - 43.6	1829,8	2+	3658,4	QQQTEDELQDKIHPFAQTQSLVYPFPGPIPNS	Beta-Casein (38-69)
43.5 - 43.6	1829,8	2+	3658,4	QQQTEDELQDKIHPFAQTQSLVYPFPGPIPNS	Beta-Casein (38-69)
48.4 - 48.8	1883,8	2+	3766,6		
48.8 - 48.8	1883,8	2+	3766,6		Beta-Casein A1 1p (29-93)
49.0 - 49.0	1851,9	?			
49.0 - 49.0	1884,1	?			
49.6 - 49.7	1873,9	2+	3746,8		Beta-Casein A2 1p (29-93)
49.7 - 49.8	1873,9	2+	3746,8		
49.8 - 49.8	1873,9	2+	3746,8		
49.9 - 50.0	1484,6	?			
50.0 - 50.1	1873,9	?			
51.0 - 51.0	1342,8	2+	2684,6		
51.0 - 51.1	2013,5	2+	4025,9		
51.6 - 51.6	1638,9	?			
58.6 - 58.7	1662,7	3+	4986,2		
59.2 - 59.2	1670,4	?			
66.0 - 66.1	435,6	2+	870,2		
66.2 - 66.2	614,5	1+	614,5		
66.2 - 66.2	435,6	2+	870,2		
66.5 - 66.5	439,3	2+	877,6		

ALLERUM2 (All2)

Range [min]	**Prec. m/z**	**Charge**	**[M+H]+**	**Amino Acid sequence**	**Suggested Casein fragment**
3.0 - 3.1	213,1	1+	213,1		

3.1 - 3.2	214,9	1+	214,9		
	374,4	1+	374,4	INK alt INQ	Beta Casein26-28, alpha s2 85-87
4.4 - 4.7	355,2	1+	355,2	HAQ	alpha S1 128-130
5.1 - 5.1	473,2	?			
6.0 - 6.2	254,1	1+	254,1		
	530,2	1+	530,2		
6.6 - 6.6	517,3	1+	517,3	KIEK alt KKIE	beta casein 29-32 alt 28-31
	428,3	1+	428,3	GPVR	Beta-Casein 199-202
7.6 - 7.7	644,8	1+	644,8		
7.7 - 7.7	623,2	1+	623,2		
8.0 - 8.1	494,3	1+	494,3	AYPSG	alpha s1 158-162
8.1 - 8.1	485,4	1+	485,4	IQPK	alpha S2 Casein 194-197
8.3 - 8.3	759,5	1+	759,5		
8.5 - 8.7	624,3	1+	624,3		
9.0 - 9.1	462,2	1+	462,2		
9.1 - 9.1	440,3	1+	440,3		
9.2 - 9.4	634,4	1+	634,4	RPKHP	alpha S1 Casein 1-5
9.6 - 9.6	423,1	1+	423,1	GPIH	Beta Casein 64-67
9.8 - 9.8	423,1	1+	423,1	GPIH	Beta Casein 64-67
	312,1	1+	312,1	VPP	Beta casein 84-86
10.6 - 10.7	689,5	1+	689,5		
10.8 - 10.9	366,1	1+	366,1		
11.0 - 11.1	326,1	1+	326,1		
12.5 - 13.1	1140,9	1+	1140,9	RPKHPIKHQ	AlphaS1 (1- 9)
12.8 - 12.9	1162,8	1+	1162,8	RPKHPIKHQ	AlphaS1 (1- 9)

12.9 - 13.1	1012,7	1+	1012,7	RPKHPIKH	AlphaS1 (1- 8)
13.1 - 13.5	875,6	1+	875,6	RPKHPIK	AlphaS1 (1- 7)
13.6 - 13.7	875,6	1+	875,6	RPKHPIK	AlphaS1 (1- 7)
13.7 - 13.8	875,6	1+	875,6	RPKHPIK	AlphaS1 (1- 7)
	1486,4	1+	1486,4	VPNZVEQKHIQK	alpha s1 (12P) 72-83
15.3 - 15.3	679,5	1+	679,5	HQGLPQ	AlphaS1 (8-13)
	497,3	1+	497,3	GPIPN	Beta Casein 64-68
15.5 - 15.6	474,3	2+	947,6		
15.8 - 16.0	747,6	1+	747,6	RPKHPI	AlphaS1 (1- 6)
16.6 - 16.7	1570,7	1+	1570,7	KDIGzEzTEDQAM	AlphaS1 8p (42-54)
	1818,9				
	627,3		627,3	VPITPT	ALPHA-S2 (12P) 117-122
18.4-18.5	1751,7		1751,7	HVZZZEESIIZQE	AlphaS2 12p (6-8)
18.6 - 18.6	627,2	1+	627,2	VPITPT	ALPHA-S2 (12P) 117-122
	1154,7	1+	1154,7	HPIKHQGLPQ	AlphaS1 (4-13)
	1509				
19.8 - 20.0	1536,1	1+	1536,1	RPKHPIKHQGLPQ	alpha S1 (1-13)
20.4 - 20.5	1665,2	1+	1665,2	RPKHPIKHQGLPQE	alpha S1 (1-14)
20.9 - 20.9	997,4	2+	1994,8		
	1857,6	1+	1857,6	PPFLQPEVMGVSKVKEA	Beta Casein 85-101
21.4 - 21.4	1870,9	1+	1870,9		
21.7 - 21.8	1871,9	1+	1871,9		
22.6 - 22.9	607,4	1+	607,4	PVVVPP	Beta-Casein (81-86)

23.3 - 23.4	788,5	1+	788,5		
23.6 - 23.7	708,3	1+	708,3	TPVVVPP	Beta-Casein (80-86)
23.8 - 23.9	708,3	1+	708,3	TPVVVPP	Beta-Casein (80-86)
24.4 - 24.4	756,5	1+	756,5	DKIHPF	Beta-Casein (47-52)
25.0 - 25.0	439,2	1+	439,2	IPPL, LPPL	Beta-Casein (74-77) Beta-Casein (135-138)
25.4 - 25.5	867,6	1+	867,6	IPPLTQTP	Beta-Casein (74-81)
26.2-26.4	1186,8	1+	1186,8	IQPKTKVIPY	ALPHA-S2 12P (194-203)
26.5 - 26.5	1199,5	?			
26.6 - 26.8	997,8	2+	1992,1	RPKHPIKHQGLPQEVLN	alpha s1 1-17
27.8 - 27.9	1128,6	1+	1128,6		
28.0 - 28.4	1297,6	2+	2594,3	VPGEIVEZLZZZEESITRINK	Beta-Casein A1 5P (8-28)
28.2 - 28.3	1297,6	2+	2594,3	VPGEIVEZLZZZEESITRINK	Beta-Casein A1 5P (8-28)
28.4 - 28.5	1234,5	2+	2469		
28.5 - 28.6	1234	?			
28.9-29.0	1126,7	1+	1126,7	ELQDKIHPF	Beta-Casein (44-52)
30	1241,8	2+	2482,6	MHQPHQPLPPTVMFPPQSVLSL	Beta-Casein (144-165)
30.8 - 31.3	891,6	1+	891,6	LPQNIPPL	Beta-Casein (70-77)
31.4 - 31.4	1001,6	1+	1001,6	YPFPGPIPN	Beta-Casein (60-68) A2

31.5 - 31.6	1001,3	?			
32.0 - 32.0	891,4	1+	891,4		
32.9 - 33.1	1528,1	2+	3055,2	LHLPLPLLQSWMHQPHQPLPPTVMFP	Beta-Casein (133-158)
32.9 - 33.1	1528,1	2+	3055,2	LHLPLPLLQSWMHQPHQPLPPTVMFP	Beta-Casein (133-158)
32.9 - 33.1	1528,1	2+	3055,2	LHLPLPLLQSWMHQPHQPLPPTVMFP	Beta-Casein (133-158)
33.9 - 34.1	1739,4	2+	3477,7	RELEELNVPGEIVEZLZZZEESITRINK	Beta-Casein 5 P(1-28)
34.3 - 34.7	855,3	?			
37.6 - 37.6	1558,6	2+	4116,2	AQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGA	Alpha S1 (129-163)
39.1 - 39.1	1993,7	?			
40.1 - 40.2	1854,3	?			
40.3 - 40.4	1915,5	2+	3829,9		
40.4 - 40.5	1916	2+	3831,1		
41.2-41.4	1624,3	2+	3247,6		
41.9-42.2	1805				
43.1 - 43.2	1829,7	2+	3658,5	QQQTEDELQDKIHPFAQTQSLVYPFPGPIPNS	Beta-Casein (38-69)
43.2 - 43.2	1829,7	2+	3658,5	QQQTEDELQDKIHPFAQTQSLVYPFPGPIPNS	Beta-Casein (38-69)
43.1 - 43.3	1829,7	2+	3658,5	QQQTEDELQDKIHPFAQTQSLVYPFPGPIPNS	Beta-Casein (38-69)
43.7-43.9	1843,4	2+	3685,8		
44.0-44.1	1881,4	1+	1881,4	YQQPVLGPVRGPFPIIV	Beta-Casein (193-209)
47.6 - 47.7	1805,7	?			
48.0 - 48.1	1894,1	2+	3787,2	PGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKV	Beta Casein (63- 98)
48.2 -	1894,	2+	3788,4		Beta

48.3	7			PGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKV	Casein (63- 98)
48.5 - 48.7	1932,9	2+	3864,7	KIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY	Beta Casein (29-60)
48.6 - 48.7	1943,7	2+	3886,3		
49.4 - 49.6	2033,3	2+	4065,5		
49.5 - 49.7	2033,3	2+	4065,5		
49.7 - 49.8	2032,7	2+	4064,4		

XO2

RT Range [min]	Prec. m/z	Charge	[M+H]+	Amino Acid seq	Suggested Casein fragment
1.5 - 1.5	450	2+	899,1		
2.2 - 2.3	450,8	1+	450,8		
2.8 - 3.0	213,1	1+	213,1		
	374,4	1+	374,4	INK alt INQ	Beta Casein26- 28, alpha s2 85-87
4.4 - 4.5	355,2	1+	355,2	HAQ	alpha S1 128-130
6.0 - 6.0	254,1	1+	254,1	ingen træff	
7.7 - 7.8	494,3	1+	494,3	AYPSG	alpha s1 158-162
7.9 - 8.0	494,3	1+	494,3	AYPSG	alpha s1 158-162
8.1 - 8.2	494,3	1+	494,3	AYPSG	alpha s1 158-162
8.2 - 8.3	781,5	1+	781,5		
8.3 - 8.4	645	1+	645		
8.5 - 8.6	312,2	2+	623,4		
8.8 - 8.9	312,3	1+	312,3	VPP	Beta casein 84- 86
9.1 - 9.2	462,1	1+	462,1		
9.3 - 9.5	634,4	1+	634,4	RPKHP	alpha S1 Casein 1-5

9.7-9.8	423,2	1+	423,2	GPIH	Beta Casein 64-67
	851	1+	851	GSENSEKT	alpha S1 187-194
11.5 - 11.6	326,2	1+	326,2		
11.9 - 12.0	449,2	1+	449,2	SQSK	Beta Casein 166-169
12.2 - 12.3	746,2	1+	746,2	GVSKVKE	Beta Casein 94-100
	422,5	1+	422,5		
12.7 - 13.0	1162,7	1+	1140,9	RPKHPIKHQ	alpha S1 Casein 1-9
12.6 - 13.1	1140,9	1+	1140,9	RPKHPIKHQ	alpha S1 Casein 1-9
13.0 - 13.2	1012,6	1+	1012,6	RPKHPIKH	alpha S1 Casein 1-8
13.2 - 13.3	1140,8	1+	1140,8	RPKHPIKHQ	alpha S1 Casein 1-9
13.2 - 13.6	875,7	1+	875,7	RPKHPIK	alpha S1 Casein 1-7
13.8 - 13.8	875,6	1+	875,6	RPKHPIK	alpha S1 Casein 1-7
14.5 - 14.5	785,3	1+	785,3		
14.6-14.9	1486,6	1+	1486,6	VPNZVEQKHIQK	alpha s1 (12P) 72-83
14.8 - 14.8	1486,6	1+	1486,6	VPNZVEQKHIQK	alpha s1 (12P) 72-83
15.4 - 15.4	497,3	1+	497,3		
15.7 - 15.7	1399,4	1+	1399,4		
15.8 - 15.8	1515,5	2+	3030	RPKHPI	alpha S1 Casein 1-6
15.8 - 16.2	747,5	1+	747,5	RPKHPI	alpha S1 Casein 1-6
16.3 - 16.4	374,3	2+	747,5	RPKHPI	alpha S1 Casein 1-6
	1570,6			KDIGZEZTEDQAM	AlphaS1 8p (42-54)
17.8-17.9	1819				
	1751,6			HVZZZEESIIZQE	AlphaS2 12p (6-8)
18.6 - 18.7	627,4	1+	627,4	VPITPT	ALPHA-S2 CASEIN (12P) 117-122
18.6 - 18.7	1481,3	?			
19.8-20.2	1536,1	1+			
18.9 -	1681,	2+	3362,		

19.0	7		3		
20.6 - 20.7	833,1	2+	1665, 2	RPKHPIKHQGLPQE	alpha S1 Casein 1-14
	1856, 8	1+	1856, 8	PPFLQPEVMGVSKVKEA	Beta Casein 85-101
21.6 - 21.7	1870, 8	1+	1870, 8		
21.7 - 21.9	997,4	1+	997,4		
22.5 - 22.6	606,9	1+	606,9	PVVVPP	Beta-Casein (81-86)
22.6 - 22.7	606,9	1+	606,9	PVVVPP	Beta-Casein (81-86)
23.7 - 24.0	607,1	?			
24.4- 24.5	756,4	1+	756,4	DKIHPF	Beta-Casein (47-52)
24.6- 25.0	459,3	1+	459,3	VPPF	Beta Casein (84- 87)
	867	1+	867	IPPLTQTP	Beta-Casein (74-81)
	1186, 9	1+	1186, 9	IQPKTKVIPY	ALPHA-S2 12P (194-203)
28.0- 28.1	1128, 7				
28.2 - 28.3	1298, 1	2+	2595, 2	VPGEIVEZLZZZEESITRINK	Beta-Casein A1 5P (8-28)
28.5 - 28.5	1298	?			
28.9 - 29.0	1354, 4	?			
30.9 - 31.3	891,5	1+	891,5	LPQNIPPL	Beta-Casein 70-77
31.1 - 31.4	891,6	1+	891,6	LPQNIPPL	Beta-Casein 70-77
31.5 - 31.6	1001, 7	1+	1001, 7	YPFPGPIPN	BETA CASEIN 60-68 A2
32.8 - 32.9	1880, 8	?			
32.9 - 33.0	1810, 7	2+	3620, 4		
33.0 - 33.0	1923, 4	2+			
33.1 - 33.4	1853, 9	2+	3706, 6		
	1739	2+	3477, 7	RELEELNVPGEIVEZLZZZEESITRINK	Beta-Casein 5 P(1-28)
34.3 - 34.4	754,4	1+	754,4		
37.8 -	1558,	2+	4116,	AQQKEPMIGVNQELAYFYPELFRQFYQLDAY	Alpha S1 (129-163)

37.9	4		2	PSGA	
39.3 - 39.4	1993, 8	?			
40.3 - 40.4	1596, 4	3+	4787, 1		
40.6 - 40.7	1850, 5	?			
	1624, 5	2+	3248		
	1786, 6	2+	3572, 2		
43.4 - 43.5	1829, 7	2+	3658, 4	QQQTEDELQDKIHPFAQTQSLVYPFPGPIPN S	Beta-Casein (38-69)
43.4 - 43.6	1829, 7	2+	3658, 4	QQQTEDELQDKIHPFAQTQSLVYPFPGPIPN S	Beta-Casein (38-69)
44.3 - 44.3	1882, 1	1+	1882, 1	YQQPVLGPVRGPFPIIV	Beta-Casein (193-209)
44.5 - 44.5	1882, 1	1+	1882, 1	YQQPVLGPVRGPFPIIV	Beta-Casein (193-209)
44.5 - 44.6	1882, 1	1+	1882, 1	YQQPVLGPVRGPFPIIV	Beta-Casein (193-209)
47.8 - 47.9	1804, 6	2+	3608, 3		
48.0 - 48.1	1850, 6	?			
48.3 - 48.7	1883, 6	2+	3766, 1		
48.7 - 48.8	1883, 4	2+	3765, 8		
48.8 - 48.9	1883, 4	2+	3765, 8		
48.9 - 49.0	1883, 4	2+	3765, 8		
48.9 - 49.0	1883, 4	2+	3765, 8		
49.0 - 49.1	1850, 6	2+	3700, 2		
49.7 - 50.0	2032, 8	2+	4064, 6		
49.9 - 50.0	2033, 4	2+	4064, 6		
	1924	2+	3847		
50.4 - 50.6	2099, 2	2+	4198, 4		
50.8 - 50.8	1912, 7	?			
51.0 - 51.0	1991, 8	2+	3983, 2		
50.9 - 51.2	2024, 1	2+	4047, 2		

51.1 - 51.2	2024, 1	2+	4047, 2		
51.0 - 51.3	2024, 1	2+	4047, 2		
51.5 - 51.6	1638, 8	2+	3276, 6		
51.7 - 51.8	1705, 4	2+	3409, 8		
51.8 - 51.9	2025, 8	2+	4050, 6		
58.4 - 58.4	1828, 4	3+	5483, 3		
59.0 - 59.1	2041, 2	?			
59.0 - 59.1	1837, 3	?			

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