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# Fractionation of whey proteins and caseinomacropeptide by means of enzymatic crosslinking and membrane separation techniques

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**Abstract**

In this study a new method for the separation of caseinomacropeptide (CMP) from native whey proteins has been developed. This new method includes the pretreatment of whey protein concentrate with the enzyme transglutaminase followed by microfiltration. The amino acid sequence of CMP includes two glutamine and three lysine residuals, whereby this peptide can be crosslinked by tranglutaminase, whereas the native whey proteins show much less sensitivity to be crosslinked by this enzyme due to their globular structure. The covalent linked CMP aggregates can be removed be means of microfiltration or diafiltration. The results show that this technique can be successfully used to obtain a CMP-free whey protein concentrate from sweet whey, which can be further used for the fractionation of individual whey proteins after CMP removal.
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## 1. Introduction

Sweet whey is produced in large amounts worldwide from milk using the enzymatic action of chymosin (also called rennet enzyme) on the casein fraction. Casein represents about 80% of the total milk protein. It looses its colloidal solubility following chymosin treatment. Milk protein can thus be separated into a coagulum from the casein protein fraction, i.e. the cheese curd, and the whey. Whey, therefore, consists of milk serum, including low molecular weight solutes such as lactose, milk salts and other minor components, whey proteins and caseinomacropeptide (CMP). CMP stems from the outer layer of the micellar casein fraction. Its presence in whey is the result of the chymosin action during cheese making, which cuts CMP off from the κ-casein at the outer layer of the casein micelle, thus rendering the casein micelles sensitive to coagulation.

There is an increased interest in separation and fractionation of whey proteins which arises from the differences in their functional and nutritional properties.

β-lactoglobulin (β-lg) shows excellent gel formation and foaming properties and can be used as a structuring and stabilizing agent in a number of milk and non-milk products. β-lg is an interesting component because of its capability in structuring dairy product matrices such as yoghurt or cream cheese (Dannenberg, 1988). This protein is quite resistant to gastric digestion, as it is stable against acids and proteolytic enzymes (Reddy, Kella, & Kinsella, 1988). It also apparently remains mostly intact after having passed through the stomach. It is therefore suggested to have some specific function in the intestine of the newborn infant. Furthermore, β-lg has a similar three-dimensional structure to that of the lipocalin family (Pérez & Calvo, 1995), and thus, appears to

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be an interesting substance for medical purposes. It has the ability to bind small hydrophobic molecules. For instance, it is reported to have binding and transport capabilities for retinol (provitamin A) in newborn animals (Godovac-Zimmermann, Conti, Liberatori, & Braunitzer, 1985).

β-lg may also function as a fatty acid or lipid binding protein. It is a rich source of cysteine, an essential amino acid that appears to stimulate glutathione synthesis, and an anti-carcinogenic tripeptide produced by the liver as protection against intestinal cancer (Mcintosh, Regester, Lelue, Royle, & Smithers, 1995). Furthermore, β-lg is considered to be the most allergenic protein fraction in cow's milk. From this latter perspective, it seems interesting to remove it from products for allergic people.

α-la has a relatively high thermal stability and consequently low gel formation properties. Therefore, it can be used in fluid or viscous products to increase their nutritional value (Pearce, 1991). This protein is used commercially in infant formulas because it is similar in structure and composition to the main whey protein in human breast milk. α-la is also broadly used in sport food formulas. It contains a high amount of the branched-chained amino acids L-isoleucine, L-leucine and L-valine, which must be present in the muscle cells to enable protein synthesis (Walzem, Dillard, & German, 2002).

It has been observed that a folding variant of human α-la induces apoptosis in tumor cells and immature cells, but not in healthy cells. The apoptosis-induced form of α-la is a mixture of monomeric and multimeric forms of α-la with molten globule-like properties (Svenson et al., 1999). Markus et al. (2000) showed that this protein increases the plasma ratio of tryptophan, which in turn stimulates the serotonin activity of the brain. The increase of brain serotonin may improve the ability to cope with stress, because it reduces the cortisol concentration, which is responsible for stress.

### 1.1. Novel aspects of whey utilisation

As a result of these physiological insights, whey is more and more considered a novel raw material for many purposes beyond physiologically motivated concepts, but also related to its technologically useful properties. Next to the whey proteins, the CMP present in sweet whey is a valuable constituent for both its functional properties as well as its specific nutritive value. CMP is considered a potential ingredient for functional food concepts (Brody, 2000; Neeser, 2000). However, it also has interesting properties related to its technological functions due to its amphiphilic nature, which results from a partial glycosylation. The sugar residues are highly hydrophilic, whereas the peptide chain is more hydrophobic.

On the other hand, rennet (or sweet) whey is a desired raw material for baby food manufacture. In this case, however, CMP is a less desirable component due to its high content of the amino acid threonin. Threonin has certain disadvantages in particular concerning the brain development of premature or very new born infants, causing hyperthreoninemia (Rigo et al., 2001), whereas for all other consumers the positive nutritional value of CMP would enhance the quality of food products produced with CMP addition. From this perspective, it appears lucrative to separate CMP from whey, both in order to offer CMP free rennet whey as a raw material for baby food manufacture and to obtain CMP concentrates for extended use in food matrix design and for functional food concepts directed towards adult nutrition. In addition, the individual whey proteins are emerging candidates for being used as single fractions.

There are numerous methods described to obtain pure α-la or β-lg fractions from either whey or milk. Different techniques are used for fractionation such as high performance liquid chromatography (HPLC) (Bobe, Beitz, Freeman, & Lindberg, 1998), fast protein liquid chromatography (FPLC), isoelectric focusing (IEF) (Godovac-Zimmermann & Klostermeyer, 1996) as well as other chromatographic methods (Basak, Velayudhan, Kohlmann, & Ladisch, 1995; Manji, Hill, Kakuda, & Irvine, 1985) and membrane separation techniques (Konrad, Lieske, & Faber, 2000; Maubois, Fauquant, Famelart, & Caussin, 2001). Membrane techniques appear to be especially suitable for the food industry, because of the relatively easy scale up and low processing costs in comparison to chromatographic techniques.

Nowadays, there are two major methods known to separate the CMP from whey proteins by means of ultrafiltration. The first method described in Kawasaki et al. (1996) is based on the ability of CMP to form non-covalent linked polymers with a molecular weight up to 50 kDa at neutral pH which dissociate at acid conditions. The dissociated form of CMP permeates through the ultrafiltration membrane with MWCO 20–50 kDa at pH 3.5, whereas the majority of whey proteins such as β-lg, α-la, immunoglobulin and bovine serum albumin are held back. After pH adjustment to 7.0 the permeate containing CMP can be concentrated by means of the same membrane. This technique guarantees a good separation of CMP and native whey proteins, but due to the low permeation rate of CMP at least two diafiltration steps are necessary, to obtain the fractions with a high degree of purity and high yield.

The second method described in Martín-Diana and Fontecha (2002) uses the high thermal stability of CMP in comparison to that of the whey proteins. According to this technique, the heat treatment of a rennet whey or WPC at 90 °C for 1 h leads to a complete denaturation and aggregation of whey proteins. The denatured proteins can be removed by centrifugation at 5200*g* and 4 °C for 15 min and the supernatant

containing CMP can be concentrated by ultrafiltration with MWCO 10 kDa after pH adjustment to 7.0. This method guarantees a good product yield, but the whey proteins loose a part of their functionality due to the denaturation. For a better understanding of the approach chosen in this study the special functional and molecular features of CMP and whey proteins shall be briefly reviewed.

### 1.2. Molecular structure of CMP and whey proteins

CMP consists of a backbone of 64 amino acids, including 3 glutamic acid and 2 lysine residues (Brody, 2000). Up to four sugar residues per molecule create a partially hydrophilic character, whereas the peptide chain possesses more hydrophobic properties. Depending on the degree and type of glycosylation and genetic variants, CMP exists as a variety of subfractions which may also be influenced by processing conditions. It is therefore of great interest to both food engineers and product developers to make extended and more specific use of whey proteins and CMP, provided these substances are available as pure components, more economic to use and reproducibly available. This approach is currently being investigated as reported by Thomä and Kulozik (2004).

β-lg, α-la and CMP have molecular weights of approximately 18.7, 14.2 and 7 kDa, respectively. β-lg and α-la are known as globular proteins with relatively little exposure of the protein chain to the exterior. This statement also applies to the glutamine and lysine residues present in whey proteins which are buried inside of their globular structure (Aboumahmoud & Savello, 1990).

In order to obtain the whey protein fractions in high degrees of purity and CMP as a separated fraction, a novel approach is being investigated in this study for the separation of CMP from whey. It is based on the use of the enzyme transglutaminase (Tgase), which specifically the crosslinks proteins or peptides between their glutamine and lysine residues.

### 1.3. Application and specificity of action of transglutaminase

Tgase (code E.C. 2.3.2.13) is a microbial enzyme that forms inter- and intramolecular crosslinks in different proteins. The enzyme catalyses an acyl transfer reaction between the γ-carboxamide group of peptide-bound glutamine residues as acyl donors and primary amines as acceptors. When protein-bound lysine residues act as acyl receptors, intra- and intermolecular-(γ-glutamyl)-lysine isopeptide crosslinks are formed by the enzyme reaction (Folk & Cole, 1966; Motoki & Seguro, 1998; Seguro, Nio, & Motoki, 1996). The catalysed reactions

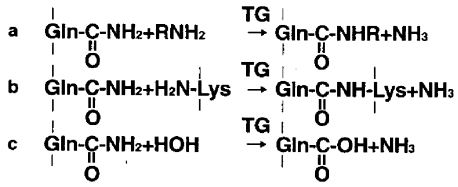



Fig. 1. Transglutaminase catalysed reactions (Katsuya, 1999).

are depicted in Fig. 1. Reaction b is the one prevalent in the crosslinking reaction of proteins.

Tgase is being applied in various fields of food matrix design. Products such as yoghurt or meat based sausages gain in texture and can eventually be produced with a higher serum binding capacity on a lower raw material level. In dairy products the preferred substrate of Tgase is the casein fraction with its various sub-fractions ($\alpha_s$; β; κ) (Ikura, Kometani, Yoshikawa, Sasaki, & Chiba, 1980), whereas native whey proteins show much less sensitivity to Tgase due to their globular structure (Aboumahmoud & Savello, 1990; Traoré & Meunier, 1992). Also, CMP is a candidate for being crosslinked by Tgase due to its amino acid composition including glutamine and lysine.

However, little is known about the binding sites in the complex structure of the casein micelle, as the individual casein fractions all possess the amino acids required for crosslinking reaction. It is unclear, whether CMP as a single fraction can be crosslinked by Tgase.

It is the purpose of this study to demonstrate a new way of separating whey proteins from CMP in rennet whey by means of Tgase in order to allow purer fractions of β-lg and α-la to be obtained further downstream. Crosslinked CMP can be separated by means of membrane technology from the native whey proteins due to the difference in size.

## 2. Materials and methods

### 2.1. Trial materials and experimental procedure

*Whey protein concentrate* (*WPC*) was obtained from locally sourced *sweet cheese whey*. The total protein content of whey was 5–6 g/l of which approximately 12% relate to the CMP fraction. The whey also contained traces of casein curd particles which are unavoidably carried over in traces into the whey during the casein curd/whey separation process. The whey was concentrated by means of ultrafiltration (nominal cut-off 25 kDa), diafiltrated six times and a 20-fold WPC with 10% total protein was obtained. For further experiments total protein content of WPC was set to 6% by dilution with deionized water.

*Enzymatic treatment* of the whey was done with the enzyme transglutaminase (Activa MP, supplied by

Ajinomoto, Kawasaki-Ku., Japan) in a thermostatically controlled reactor (40 °C, 100 $cm^3$). The dosage was 3 U/g protein, the treatment time was varied between 0 and 120 min in order to track the progress of the enzyme action. After each point in time a sample was taken from the reactor and the enzyme reaction was immediately stopped by means of the addition of perchloric acid (Merck, 60%) with a final concentration of 3%.

### 2.2. Analytical measurements

#### 2.2.1. Total protein content

The protein content was assessed with a nitrogen analyser using the Dumas method (Leco FP-528, Leco Instrumente GmbH, Moenchengladbach, Germany). The principle of the method is that the sample is flushed with pure oxygen for combustion (850 °C). Aliquot amounts of gas are swept through hot copper to remove oxygen and to change $NO_x$ to $N_2$. Nitrogen in a helium carrier is measured by thermal conductivity.

#### 2.2.2. Whey proteins

The whey protein content was determined by HPLC according to a method described in detail by Beyer (1990). The samples were diluted with distilled water, pH was adjusted to 4.6 by hydrochloric acid (Merck) and the samples were filtered to remove coagulated aggregates and injected into HPLC column. A gradient of solvent A (water: acetonitrile 1000:0 vol/vol + 1% (vol) trifluoroacetic acid) and solvent B (water: acetonitrile 200:800 vol/vol + 0.555% (vol) trifluoroacetic acid) was applied. The gradient started with 43% of solvent B and increased to 47% B over 8 min, to 52% over next 8 min, to 57% over 6 min and to 58% over 1 min and then kept at 100% B for 5 min before returning to the initial conditions. Analysis was carried out at 40 °C, flow 1 ml/min with a PLRP-S 300 Å 8 μm column (Latek, Eppelheim, Germany). Peaks detection followed at 226 nm.

#### 2.2.3. Caseinomacropeptide (CMP)

CMP was analysed by HPLC using a modified method described in Minkiewicz et al. (1996). The same solvents and conditions were applied as for whey protein detection. The gradient started with 10% of solvent B then increased to 49% B during 20 min and then kept at 100% B for 5 min before returning to the initial conditions. Peaks detection followed at 226 nm.

### 2.3. Membrane separation techniques

Microfiltration was carried out with a ceramic tubular membrane system with an average pore diameter of 0.1 μm and an active layer of α-Aluminium oxide (SCT, Bazet, F). To keep the transmembrane pressure and the wall shear stress at a constant level along the membrane retentate/permeate flow paths, a UTP (uniform transmembrane pressure) based pilot plant was used (APV, Silkeborg, DK) as described in Kulozik and Kersten (2002). Ultrafiltration was performed using a polysulfon plate-frame membrane with a nominal cut-off of 25 kDa (DSS AS, Nakskov, DK).

A sample of 100 l WPC was subjected to a microfiltration (MF)/diafiltration process in order to quantitatively separate the soluble native whey proteins from the crosslinked CMP. The MF permeate was concentrated by means of UF. The UF permeate recirculated to the MF unit as a diafiltration medium. Fig. 2 shows the overall experimental sequence of the processing steps.

The decrease of concentration of individual whey proteins in the MF retentate can be modelled according to Eq. (1) (van Reis & Saksena, 1997):

$$C_t = C_o \exp(-p \cdot n_{DS}), \tag{1}$$

where $C_t$—concentration of individual whey proteins during diafiltration process; $C_0$—initial concentration of individual whey proteins; $p$—permeation of individual whey proteins through the MF membrane or observed sieving coefficients calculated as a ratio of concentration of individual whey proteins in the MF permeate $C_{PER}$ to concentration in the MF retentate $C_{RET}$:

$$p = \frac{C_{PER}}{C_{RET}}; \tag{2}$$

$n_{DS}$—number of diafiltration steps or diavolumes, defined as ratio between volume of added permeate for diafiltration $V$ to the hold-up volume of MF unit $V_U$:

$$n_{DS} = \frac{V}{V_U}, \tag{3}$$

Diafiltration experiments were carried out applying the following process parameters: temperature $\upsilon = 55$ °C, transmembrane pressure $\Delta p_{TM} = 40$ kPa, wall shear stress $\tau_W = 150$ kPa (crossflow velocity 7.2 $ms^{-1}$). Permeation flux $J$ was almost constant during the whole diafiltration experiment ($J = 48.8$ l/($hm^2$)) Diafiltration was performed up to 14 diafiltration steps. After each

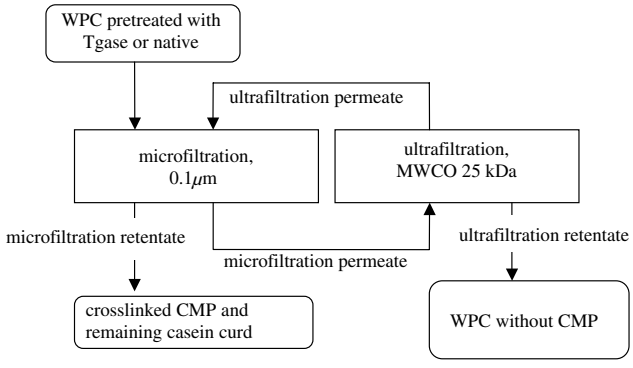



Fig. 2. Experimental scheme.

diafiltration step a sample of the MF retentate, MF permeate and UF retentate was taken for protein content analysis.

## 3. Results and discussion

### 3.1. Analytical assessment of the crosslinking reaction of CMP

According to the procedure described above the WPC was subjected to the Tgase crosslinking reaction. The first question was whether the theoretical pathway of crosslinking of CMP by Tgase can be realised in practice and whether all CMP fractions react during the enzymatic treatment. Fig. 3 depicts a comparative chromatogram of CMP from three samples taken at different reaction times (untreated, 10 and 120 min). As can be seen, CMP consists of a number of fractions. The diversity of the CMP molecule can be explained by the fact that CMP exists in two main genetic variants A and B. It further consists of a non-glycosylated fraction (Lieske & Konrad, 1996), which makes up about 70% of the CMP fraction and a glycosylated fraction (30%). The glycosylated fraction in itself is quite diverse due to the many variants in the degree of glycosylation (up to 4 sugar residues per molecule) and type of glycosylation. The prevalent sugar residues are galactose (Gal), *N*-acetylgalactosamin (GalNAc), *N*-acetylneuramic acid (NeuNAc) and sialic acid. The molecular weight and hydrophobic properties of CMP vary accordingly to the degree of glycosylation.

Fig. 3 shows that all peaks of both the glycosylated and the unglycosylated CMP A and B are getting smaller as the reaction time proceeds. The diverse nature of the CMP obviously does not have an impact on the availability of the amine acids glutamine and lysine to the enzyme Tgase, but it cannot be excluded that glycosylation of CMP has an influence on the velocity of the

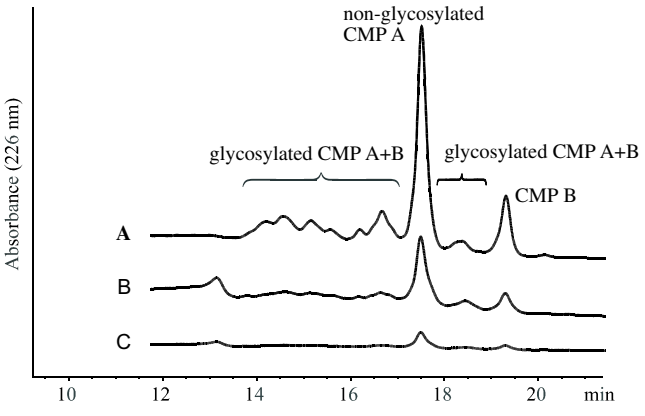



Fig. 3. Comparative assessment of chromatograms of CMP as a function of Tgase reaction time: (A) 0 min, (B) 10 min, (C) 120 min.

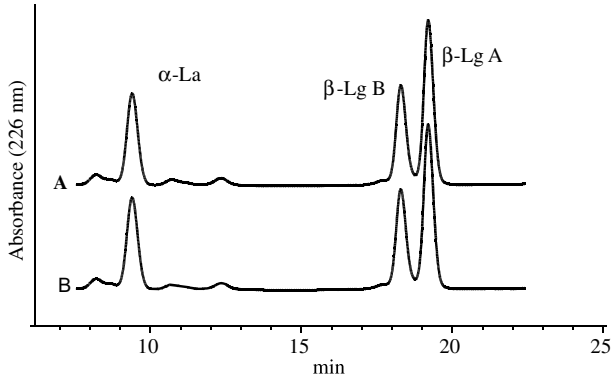



Fig. 4. Comparative assessment of chromatograms of whey proteins as a function of Tgase reaction time: (A) 0 min, (B) 120 min.

crosslinking reaction. The concentration of native whey proteins (α-la and β-lg A and B), on the other hand was found to be unchanged, as was expected because native whey proteins are known not to be a substrate for Tgase (Fig. 4). These results are in accordance with Nielsen (1995), who reported that whey proteins can be crosslinked only either after thermal denaturation, or after splitting intramolecular disulphide bonds by chemical reagents. From the results shown in Figs. 3 and 4 it can be concluded that the pretreatment of whey or whey protein concentrate by Tgase can be a useful tool for the separation of whey proteins from CMP.

### 3.2. Crosslinking of CMP as a function of the duration of the enzymatic treatment

To assess the optimal conditions of pretreatment of whey protein concentrates, the change of free CMP in the serum during the incubation time with Tgase was measured. The starting level of the CMP concentration (8 g/l) was set to 100%. A whey protein concentrate with 6% total protein content was used. From Fig. 5 it can be

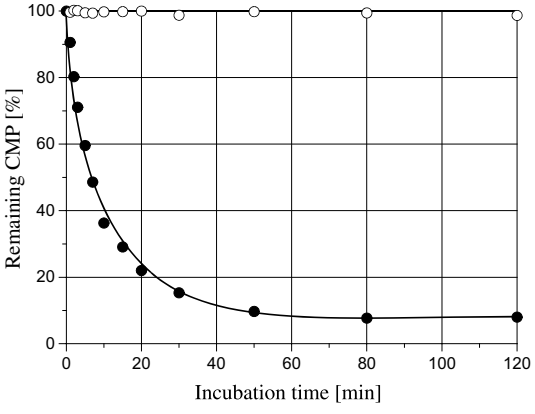



Fig. 5. Changes of free CMP and whey proteins content in the serum of WPC during incubation with Tgase: (●) CMP, (○)whey proteins (α-la + β-lg).

seen that the amount of CMP decreases during the whole incubation time. After 40 min treatment time the remaining content of the peptide was not higher than approximately 12%. After a pretreatment time of 80 min 7% of the CMP was not crosslinked. The crosslinked CMP could be removed from the WPC leading to an almost complete removal of CMP from the whey protein concentrate.

The asymptotic shape of the curve indicates that other substances than CMP (i.e. *para*-κ-casein, non-micellar β-casein, products of unspecific hydrolysis of caseins etc.) were present in the whey and could be crosslinked by Tgase between themselves or CMP. The majority of these components of diverse nature could be suspected to be eluted from the HPLC-column at the same time as different CMP-fractions and explains why 8% of CMP cannot be detected by HPLC as crosslinked. In fact, the remaining 8% of peptides detected by HPLC as not crosslinked CMP could then be attributed to non-CMP components.

On the other hand, due to the high variety of the CMP structure, some of the glycosylated molecules of CMP could be crosslinked only slowly or not at all. The crosslinked glycosylated dimers and trimers can have elution times similar to that of some of the monomers. This can lead to the remaining non-crosslinked CMP being detected by HPLC.

It appears that both of the above-mentioned reasons are responsible for the remaining non-crosslinkable CMP detected by HPLC. For practical application regarding the separation of crosslinked CMP from native whey proteins it can be assumed that the CMP is completely crosslinked after 60 min reaction time. The content of native whey proteins in WPC was not affected by Tgase during the whole incubation time (Fig. 5).

### 3.3. Diafiltration to purify the WPC from CMP

For the separation of crosslinked CMP and native whey proteins, a diafiltration experiment involving MF (0.1 μm) and UF (25 kDa) was carried out (see Fig. 2). 100 l of pretreated whey protein concentrate (60 min) was microfiltered and the permeate (containing the native whey proteins, lactose and salts) was transferred to the UF unit. The whey proteins were collected in the UF retentate. The UF permeate (containing lactose and salts) was transferred back to the MF retentate. During diafiltration the native whey proteins were concentrated in the UF retentate and both the crosslinked CMP plus the remaining casein curd (about 5–10% of total protein) were collected in the MF retentate. A control experiment without pretreatment with Tgase was also performed.

Fig. 6 depicts the decrease in concentration of the whey proteins in the MF retentate and increase in the UF retentate during diafiltration of pretreated WPC.

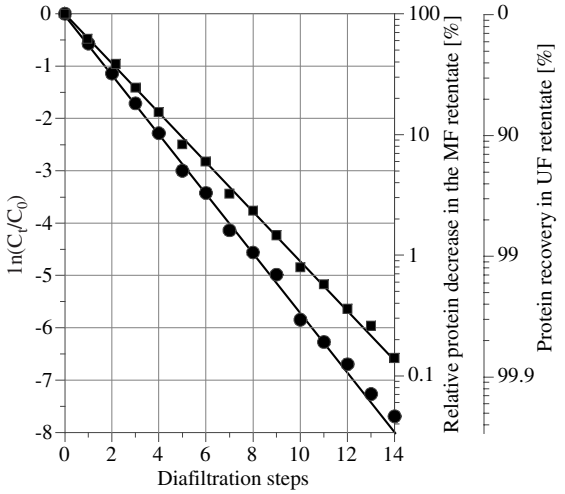



Fig. 6. Changes of the whey protein concentration in the MF and UF retentate during the diafiltration process: (●) α-la, (■) β-lg.

The permeation of α-la, β-lg in treated WPC through MF membrane was 57% and 43%, respectively, and can be read as the slope of each corresponding line according to Equation (1). As can be seen from Fig. 6 more than 10 diafiltration steps are necessary to recover 99% of β-lg in the UF retentate. The exact number of diafiltration steps can be calculated from Equation (1) and was found to be 10.71.

Fig. 7 shows a CMP chromatogram of UF retentate with and without Tgase treatment, which shows that virtually no CMP remains in the UF retentate after the diafiltration procedure.

The protein composition of both resulting concentrates is shown in Table 1. As can be seen from the result of the Tgase treated sample, the CMP level decreased through this procedure to a level below the detection limit, whereas in the control experiment without Tgase treatment the composition of the whey proteins and the CMP essentially remained unchanged. The total protein content of untreated and treated diafiltrated WPC

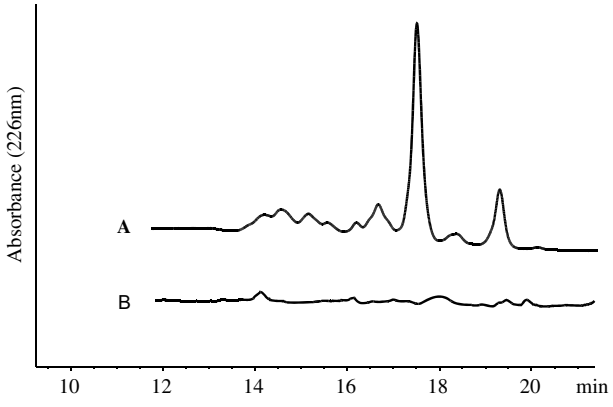



Fig. 7. Chromatogram of CMP in the WPC after diafiltration: (A) without pretreatment by Tgase, (B) with pretreatment by Tgase.

Table 1
Composition of proteins in raw and diafiltrated WPC with and without pretreatment by Tgase

| | Whey protein concentrate | Diafiltrated whey protein concentrate (with pretreatment by Tgase) | Diafiltrated whey protein concentrate (without pretreatment by Tgase) |
|---|---|---|---|
| Total protein, % | 6.02 | 4.83 | 5.42 |
| α-La, % | 0.78 | 0.75 | 0.76 |
| β-Lg, % | 2.90 | 2.88 | 2.89 |
| CMP, % | 0.80 | – | 0.78 |

was 10% and 19.7% respectively lower than in raw concentrate. These values are in the same range as those found in the literature for the relative contents of casein curd and CMP remaining in whey after cheese making (Maubois et al., 2001).

## 4. Conclusions

It can be concluded from these results that CMP appears to be a good substrate for the enzyme Tgase as it was assumed from its molecular structure (presence of glutamine and lysine amino acid residuals, absence of secondary structure). The difference between native whey proteins and CMP in the ability to be enzymatically crosslinked could be used for its separation by means of microfiltration. Furthermore, CMP seems to play an important role in the enzymatic crosslinking of native casein by Tgase as a fraction located on the surface of the casein micelle. The casein micelle appears to be fixed by crosslinked conjugates of κ-casein via CMP on the surface of the micelle. This leads to changes of functional properties of milk regarding rennet characteristics, high pressure or heat stability (O'Sullivan, Kelly, & Fox, 2002a; O'Sullivan, Kelly, & Fox, 2002b; O'Sullivan et al., 2001).

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