Research Article

Journal of PeptideScience

Received: 26 June 2008 Revised: 2 October 2008 Accepted: 8 October 2008 Published online in Wiley Interscience: 26 November 2008

(www.interscience.com) DOI 10.1002/psc.1093

Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey‡

Carmela De Simone,[a] Gianluca Picariello,[b] Gianfranco Mamone,[b] Paola Stiuso,[c] Alessandra Dicitore,[c] Daniela Vanacore,[c] Lina Chianese,[a] Francesco Addeo[a] and Pasquale Ferranti[a]*

Bioactive peptides are present in a latent state, encrypted within the amino acid sequence of milk proteins, requiring enzymatic proteolysis for their release. They can be produced by gastrointestinal digestion or food processing, thus they can be present in fermented milks, cheese and also in the by-products of dairy industry such as waste whey. The spectrum of biological activity covered by milk-derived peptides is extremely wide, including antibacterial, immunostimulating, antihypertensive, antithrombotic and opioid actions. However, the characterisation of milk-derived peptides with classical analytical methodologies is severely challenged by the complexity of the milk protein fraction and by the wide dynamic range of relative peptide abundance in both dairy products and by-products. Here we report the characterisation of the peptide fraction released in the whey during the different production stages of Mozzarella di Bufala Campana cheese. The peptide extracts were separated by RP HPLC and analysed by MS in order to identify the peptides produced and to trace the pathway of formation of potential bioactive peptides. The antioxidant properties and the modulatory effect on the cell cycle exerted by the peptide extracts were also studied in CaCo2 cell line. We found that a significant antiproliferative effect on CaCo2 was exerted by Mozzarella di Bufala waste whey peptides.

Supporting information may be found in the online version of this article

Keywords: river buffalo milk; Mozzarella di Bufala Campana cheese; bioactive peptides; cytomodulatory activity; MS; cheese whey

Introduction

During the last two decades it has progressively emerged that milk proteins are a source of biologically active peptides [1–3], which remain inactive within the sequence of the parent protein until they are released in consequence of either gastrointestinal digestion or food processing [4]. Once bioactive peptides are liberated, they may act as regulatory compounds with hormone-like activity [5].

The peptides generated from milk proteins can influence a wide number of physiological processes as they exhibit antibacterial [6,7], immunomodulating [8–10], antihypertensive [11], antithrombotic [12], cytomodulant [2] and opioid [13] activities. Most of the known bioactive peptides are composed of short amino acid sequences. Oligopeptides present several advantages in comparison with large-sized molecules in view of a possible therapeutic use; they can more easily pass through the digestive tract and reach the blood circulation to be transported to their target organs.

A number of studies have been carried out on peptides produced *in vitro* from bovine milk proteins, mainly by use of purified proteases or microbial cell-wall proteinases [14]; on the contrary, peptides naturally occurring in milk or in cheese whey have not been exhaustively characterised, and their potential bioactive properties have not been studied so far [15]. It must be underlined that even though bioactive peptides have been isolated *in vivo* in individuals following ingestion of caseins (CNs) or of milk-containing foods [16,17], their pharmacodynamic and pharmacocynetic parameters, as well as their bioavailability, remain to be established. In particular, no study has been carried out on peptides in buffalo milk.

The need for the characterisation of the peptide fraction in buffalo milk arises from the fact that the production of Mozzarella di Bufala Campana Protected Denomination of Origin (PDO) cheese has recorded a strong increase in Regione Campania in the

* *Correspondence to: Pasquale Ferranti, Department of Food Science, University of Naples 'Federico II', I-80055 Parco Gussone, Portici (NA), Italy. E-mail: ferranti@unina.it*

a *Department of Food Science, University of Naples 'Federico II', I-80055 Parco Gussone, Portici (NA), Italy*

b *Istituto di Scienze dell'Alimentazione del CNR, Via Roma 52, I-83100 Avellino, Italy*

c *Dipartimento di Biochimica e Biofisica, II Università di Napoli, 80100 Napoli, Italy*

‡ *11th Naples Workshop on Bioactive Peptides*

Abbreviations used: *CNs, caseins; PDO, protected denomination of origin; BSW, buffalo sweet whey; BWW, buffalo waste whey; BS, buffalo scotta; BM, buffalo milk; PMSF, phenyl-methyl-sulfonyl fluoride; AcN, acetonitrile; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate buffer saline; MEM, minimum essential medium; FBS, foetal bovine serum; HE, hydroethydine; DHB, 2,5-dihidroxybenzoic acid; CHCA, ciano-4-hydroxycinnamic acid; PSD, post-source decay; PI, propidium iodide; HE-O, hydroethidine-superoxide anion; CMP, k-casein macropeptide.*

last years. A large amount of whey has to be disposed off yearly, as about 80% milk volume remains as whey after transformation. Three kinds of whey are produced: sweet whey, formed immediately after or simultaneously to milk clotting; waste whey – the acid whey formed through curd maturation; 'scotta', the liquid fraction remaining after removal of thermally coagulated whey proteins for the production of 'ricotta' from the sweet whey. After separation of the valuable whey proteins, the residue remaining is putrescible and has high chemical oxygen demand. Therefore, whey has to be considered as a by-product in milk processing and, according to the Italian legislation (D. L. 22/97), it is a 'special waste' and its disposal engraves highly on costs.

On the other hand, during the production of Mozzarella, it is expected that a large number of peptides can be released in the whey, for the combined proteolytic effect on milk caseins endogenous enzymes (plasmin, cathepsin D) [18], rennet enzymes (chymosin, pepsin) and lactic acid bacteria exo- and endo-peptidases [19–22]. The mild manufacturing conditions employed in the production of Mozzarella di Bufala cheese could effectively preserve the bioactive molecules in whey, particularly in sweet whey and waste whey.

In this study we characterised the soluble peptide extract from river buffalo sweet whey (BSW), waste whey (BWW) and 'scotta' (BS), tracing the breakdown pathway generating potential bioactive peptides, starting from raw river buffalo milk (BM). The aim of this investigation was to identify biologically active peptide candidates which could be recovered from the whey of Mozzarella cheese. The peptide fractions were characterised with MS techniques, such as MALDI-TOF and electrospray ionisation (ESI) MS; the latter is either off-line or on-line with RP-HPLC. In addition, we investigated the antioxidant and cytomodulatory effects of the peptide extracts on hydrogen-peroxide-induced oxidative damage in CaCo2 cell lines [23].

Materials and Methods

Materials

River buffalo raw milk and whey samples from manufacturing of Mozzarella di Bufala Campana PDO cheese were provided by a local dairy farm of the Salerno area (Southern Italy). To prevent undesired peptide hydrolysis, immediately after collection, samples were added with 1-mM phenyl-methyl-sulfonyl fluoride (PMSF, Sigma St. Louis, MO) and stored at −20 °C until used.

All chemicals and reagents were of analytical grade or better from Sigma. HPLC-grade acetonitrile (AcN) was purchased from Carlo Erba (Milano, Italy). Dulbecco's Modified Eagle's Medium (DMEM), PBS (phosphate buffer saline, composed of 0.1 M phosphate buffered saline containing NaCl 0.138 M, KCl 0.0027 M, pH 7.4), minimum essential medium (MEM) non-essential amino acids, streptomycin, penicillin, L-glutamine, FBS (foetal bovine serum), and Nonidet P40 were obtained from Gibco-BRL (Grand Island, NY). Tissue culture plasticware was furnished from Becton Dickinson (Lincoln Park, NJ, USA). Hydrogen peroxide (H_2O_2) 1 M solution was prepared immediately before use. Hydroethydine (HE) was purchased from Invitrogen Srl (Milan, Italy).

Extraction of Peptides from Milk and Whey Samples

BM, BSW, BWW and BS samples were collected at the proper stage of the technological process of Mozzarella manufacturing. The cream was separated from milk or whey by centrifugation at 4500 *g* at 4 °C for 30 min (Haereus Biofuge, Kendro, Germany). To harden the cream, the tubes were kept at −20 °C for 10 min and finally the cream was scraped off.

Skimmed whey samples (25 ml) were ultra-filtered on Centriprep® cartridges having a 3-KDa cut-off membrane (Millipore, Bedford, MA). The permeates, lyophilised and solubilised in 6 ml 0.1% (v/v) aqueous TFA, were purified from saline contaminants and lactose with a Sep-pak C_{18} cartridge (Waters, Milford, MA), previously equilibrated in 0.1% TFA and eluted with 70/30/0.1 AcN/water/TFA (v/v/v). The peptide extracts were dried using a Savant concentrator (Speed-Vac, Milan, Italy) and stored at −20 °C either for structural analysis or for biological assays. Fresh milk was processed as above after skimming and isoelectric precipitation of casein as previously reported [24].

MALDI-TOF-MS Analysis

MALDI-TOF-MS experiments were carried out on a Voyager DE-PRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with an N_2 laser (337 nm, 3 ns pulse width) operating both in the linear and reflector positive ion modes using the Delayed Extraction. Samples were co-cristallised with 2,5-dihidroxybenzoic acid (DHB) and α-ciano-4-hydroxycinnamic acid (CHCA) used as the matrices, both prepared at concentration 10 mg/ml by dissolving the powder in 50% AcN (v/v)/0.1% (v/v) TFA. External calibration was performed by acquiring separate spectra of a mixture of standard peptides (Perseptive Biosystems). Post-source decay (PSD) fragment ion spectra were obtained after isolation of the appropriate peptide precursor using timed ion selection. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflector and the individual segments were combined using the software developed by PerSeptive BioSystems furnished with the instrument. Signals detected in the mass spectra were associated with the corresponding peptides on the basis of the expected molecular weight deduced from the sequence of the casein fractions [25] by using a suitable computer programme (GPMAW 5.1, Lighthouse data, Odense, Denmark).

LC/ESI-MS Analysis of Peptides

The BWW peptide extract was fractionated by HPLC using an HP 1100 modular system (Agilent Technology, Palo Alto). Sample was loaded onto a 218TP52, 5-µm reversed-phase C_{18}, 250 × 2.1 mm column (Vydac, Hesperia, CA). Solvent A was water containing 0.1% TFA (v/v) and solvent B was AcN containing 0.1% TFA (v/v). A linear gradient from 5 to 70% solvent B was applied over 90 min, after 5 min of isocratic elution at 5% solvent B, at a constant flow rate of 0.2 ml/min. UV detection was carried out at 220 nm. The effluents were directly injected, through a 100-µm i.d. fused silica capillary into the electrospray source of a Platform single quadrupole mass spectrometer (Waters, Manchester, UK). The ESI mass spectra were scanned in the positive ion mode, from 1600 to 400 *m/z* at a scan cycle of 4.9 s/scan and 0.1 s inter-scan delay. The source temperature was 180 °C and the capillary and orifice voltages were 3.6 kV and 40 V, respectively. Mass scale calibration was performed using the multiple charged ions from a separate injection of horse heart myoglobin. Mass spectra were elaborated using the software MassLynx 2.0, furnished with the spectrometer.

Tandem MS (MS-MS) data were obtained by using a Q-STAR PULSAR (Applied Biosystems, Foster City, CA) equipped with

nanospray interface (Protana, Odense, Denmark). Dried samples were resuspended in 0.1% TFA, desalted by using ZipTip C_{18} microcolumns (Millipore, Bedford, MA), and sprayed from gold-coated 'medium-length' borosilicate capillaries (Protana). The capillary voltage used was 800 V. Double-charged ion isotopic clusters were selected by using the quadrupole mass filter and then induced to fragment by collision. The collision energy was 20–40 eV, depending on the size of the peptide. The collision-induced dissociation was processed by using Analyst 5 software (Applied Biosystem). The deconvoluted MS-MS spectrum was manually interpreted with the help of Analyst 5 software.

Cell Culture and Cell Proliferation Assay

CaCo2 cells (American Type Culture Collection, Rockville, MD) were grown at 37 °C in h-glucose MEM containing: 1% (v/v) MEM non-essential amino acids and supplemented with 10% (v/v) decomplemented FBS (Flow, McLean, VA), 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate. The cells (17–21 passages) were grown in a humidified atmosphere of 95% air/5% CO_2 at 37 °C and were plated in 12 multi-well plates at different densities. After incubation for 4 h in DMEM with 10% FBS, the cells were washed with 1% PBS to remove unattached dead cells.

The starved cells (DMEM without serum) were pre-treated for 30 min with 50 µM H_2O_2 (H-CaCo2) and then incubated with the peptide extracts. To this aim, the dry peptide extracts from BM, BSW, BWW and BS were weighed, dissolved in DMEM without serum and added to the H-CaCo2 cells at the final concentrations of 0.1 or 1 mg/ml. 1.0×10^5 H-CaCo2 cells treated with the peptides extracts were seeded in 12 multi-well plates and incubated at 37 °C. After 12 h the cells were counted with a hemocytometric counter and cell proliferation was determined through CyQuant Cell Proliferation Assay Kit (Invitrogen, Milan, Italy) with dye fluorescence measurement at 480 and 520 nm excitation maximum.

The control sample was constituted by H-CaCo2 cells not added of peptide extract (untreated cells). Cell proliferation was expressed as percentage of proliferation compared with the control sample, which was therefore assumed as 100%. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.

Flow Cytometry Analysis of H-CaCo2

H-CaCo2 cells were seeded in six multi-well plates at the density of 25×10^5 cells/plate. After 12-h incubation with 0.08 mg/ml of peptide extracts from BM and BWW in DMEM without serum at 37 °C, cells were washed in PBS, pelleted in centrifuged and directly stained in a propidium iodide (PI) solution (50 mg PI in 0.1% sodium citrate, 0.1% Nonidet P40, pH 7.4) for 30 min at 4 °C in the dark. Flow cytometric analysis was performed using an FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). To evaluate cell cycle PI fluorescence was collected as FL2 (linear scale) by the ModFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least 20 000 events for each point were analysed in at least three different experiments giving an SD lower than 5%.

The mitochondrial superoxide anion production was analysed by hydroethidine (HE) staining. The cells were incubated for 1 h at the end of treatment with 20 ng/ml HE. At the time of processing, cells were scraped, washed twice with PBS and the pellet was added to 1-ml PBS. The hydroethidine-superoxide anion (HE-O) accumulation was determined using the CellQuest software (FACScan, Becton Dickinson) furnished with the cytometer. For each sample, 2×10^4 events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

Results and Discussion

Characterisation of Peptides in Buffalo Milk and in Mozzarella PDO Cheese Whey

The peptides of river buffalo raw milk.

The peptide extracts from raw BM were analysed in mixture by MALDI-TOF-MS (Figure 1). The mass spectrum clearly showed the presence of two families of casein fragments, those deriving from the *N*-terminal end of α_{s1}-CN and those from the *C*-terminal end of β-CN. The simultaneous occurrence of peptides progressively shortened in the same mixture suggested the possible identity of the signals in the spectrum, as it was possible to reconstruct entire sequences on the basis of the differences in molecular masses corresponding to amino acids (Figure 1, inset). For unambiguous assignment of peptide identity,

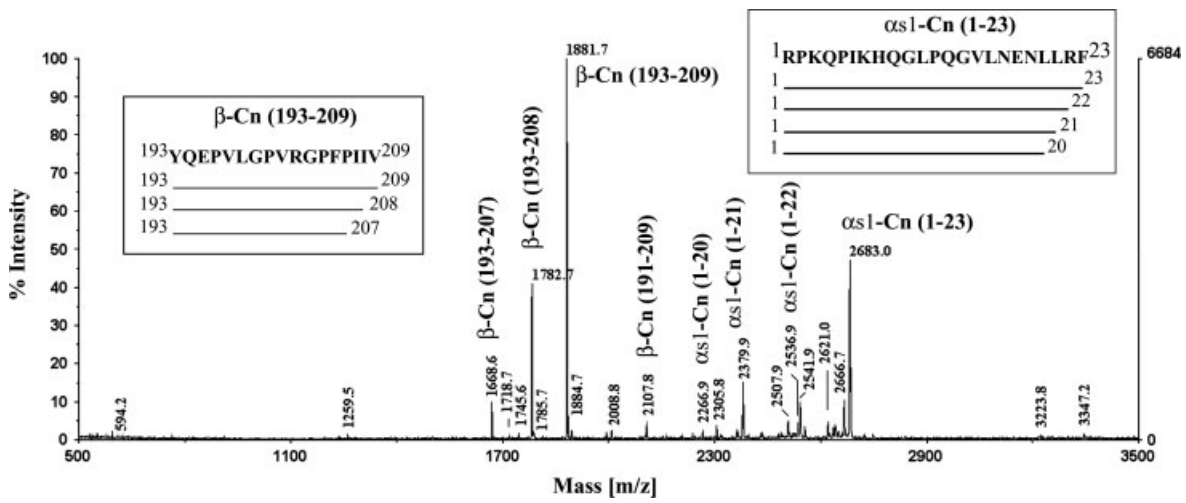

Figure 1. MALDI-TOF mass spectrum of the low-molecular-mass peptide fraction extracted from river buffalo raw milk (BM). In the insets the peptides derived from the main fragments β-CN f193–209 and $\alpha s1$-CN f1–23 are schematised.

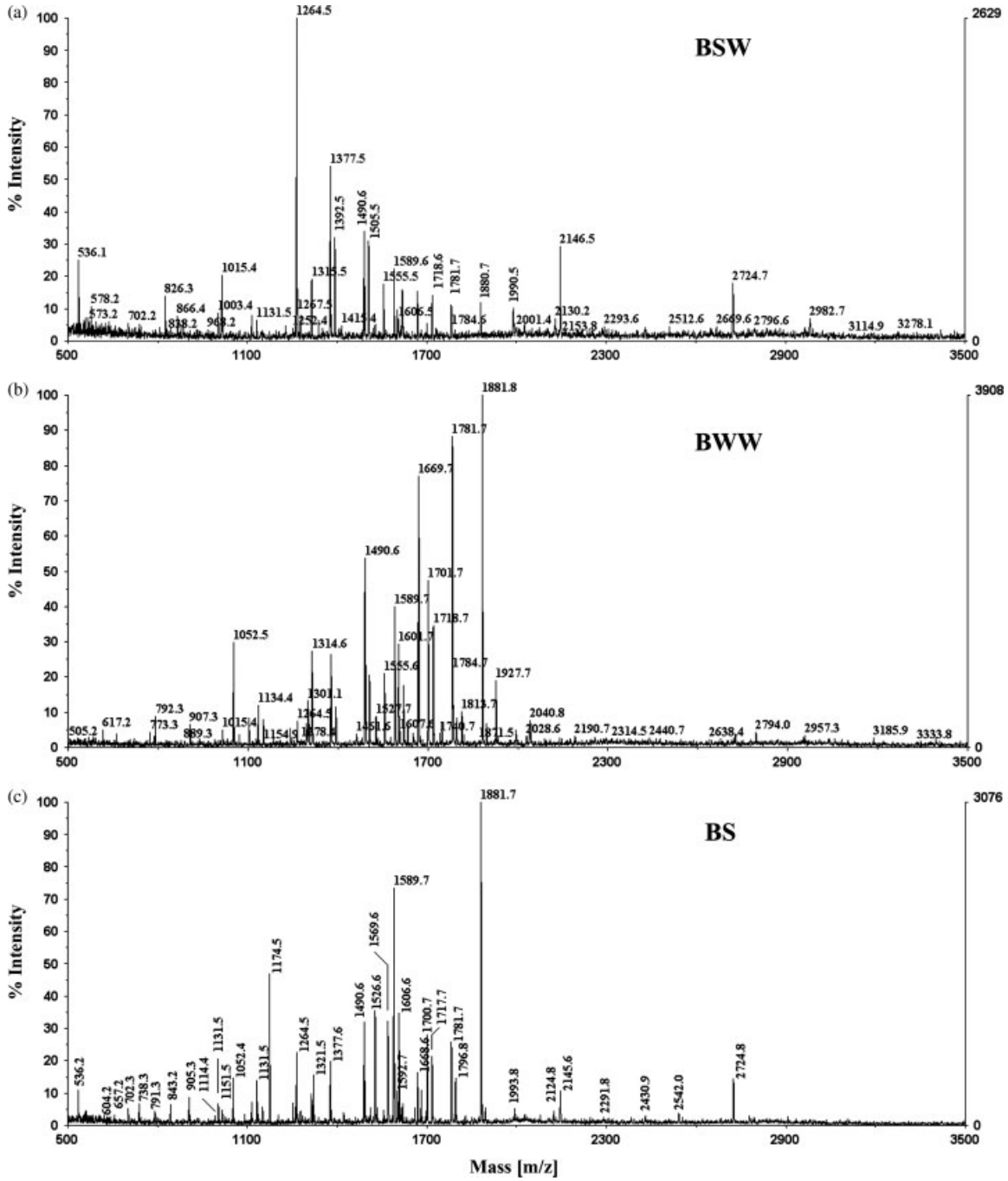

Figure 2. MALDI-TOF mass spectra of the low-molecular-mass peptide fractions extracted from whey at the different stages of Mozzarella di Bufala Campana production: (a) sweet whey (BSW); (b) waste whey (BWW); (c) 'scotta' (BS). Assignment of the main mass signals is given in Table S1 in the Supporting Information.

MALDI-TOF-MS PSD and ESI-MS/MS tandem MS analyses were carried out.

The peptide α_{s1}-CN f1-23 arose from primary cleavage at the level of the Phe^{23}–Phe^{24} bond, a well-known chymosin proteolytic cleavage site [26]. As chymosin is only added at the beginning of the cheese-making process and is not present in raw milk, the peptide bond Phe^{23}–Phe^{24} was probably hydrolysed by the milk endogenous enzyme cathepsin D [18]. Furthermore, Oomen [27] showed that also the proteolytic action of *L. delbrueckii* spp. *bulgaricus* on α_{s1}-CN produced peptide 1–23 in cow milk. This finding, combined with a possible carboxypeptidase-like enzyme activity producing the shorter peptides α_{s1}-CN f1–22 and f1–21, suggested that α_{s1}-CN *N*-terminal fragments could be generated by action of extracellular P_I- and P_{III}-proteinases of lactic acid bacteria similarly to what occurs in bovine α_{s1}-CN [28].

The bovine peptide α_{s1}-CN f1-23 presents an antimicrobial activity towards *Staphylococcus aureus* and *Candida albicans* [6]. However, the sequence of cow α_{s1}-CN f1–23 differs from its buffalo homologue in two amino acid substitutions: His^4 → Pro and

Journal of PeptideScience

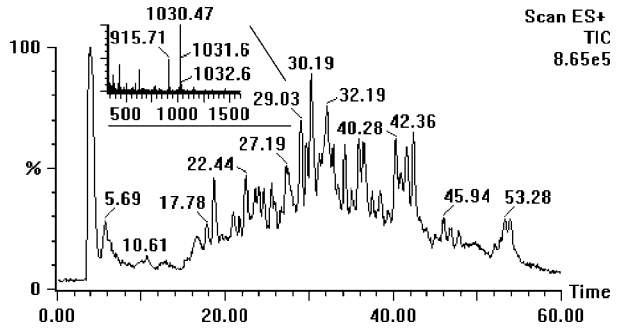

Figure 3. HPLC/ESI-MS chromatogram of the BWW peptide extract. The total ion current (TIC) chromatogram is shown. Co-elution of several peptides in each chromatographic peaks (inset) emphasises the great complexity of the peptide fraction.

$Glu^{14} \rightarrow Gly$ [29]. Therefore a possible antimicrobial activity for buffalo milk-derived peptide should be confirmed experimentally.

Similarly, for the *C*-terminal portion of buffalo β-CN, the finding of the precursor peptide β-CN f193–209, followed by the shorter peptides f193–208 and f193–207, supported the hypothesis of a carboxypeptidase-like activity in BM. Furthermore, β–CN f193–209 is non-specifically produced by isolated proteolytic enzymes (unpublished data) indicating that the entire Leu^{192}-Leu-Tyr^{194} sequence is particularly susceptible to hydrolysis, possibly because it is located in an exposed protein region.

A multifunctional activity has already been described for the 193–209 region of bovine β-CN, which contains the bioactive sequence β-casokinin-10. Since the C-terminal sequence of β-CN is conserved between river buffalo and cow species, precursors of peptides with immunostimulatory and angiotensin converting enzyme (ACE)-inhibitory activities [11] are therefore possibly present also in BM.

The peptides of Mozzarella PDO sweet whey.

k-CN is the main substrate of chymosin in cheese-making, but natural calf rennet contains traces of other proteases, such as pepsin or gastricsin (pepsin C) [30]; furthermore, the microflora of 'starter' cultures significantly contributes to proteolysis. Accordingly, the MALDI-TOF-MS peptide pattern of BSW (Figure 2(a)) was more complex than that observed for BM peptide extract. Several peptides produced from the *C*-terminal portion of β-CN, from k-CN (in particular from the k-casein macropeptide, CMP) and from α_{s1}-CN were identified in BSW peptide extract (Table S1 in the Supporting Information).

The precursor peptide α_{s1}-CN f1–23 previously detected in BM disappeared completely in BSW, although its increase was expected because of the known susceptibility of the Phe^{23}–Phe^{24} bond to chymosin action [31,32]. This finding could be justified by the secondary proteolysis on the early casein fragment f1–23 which sequentially produced α_{s1}-CN f1–22, f1–21, f1–19; the identification of α_{s1}-CN f24–37 confirmed the cleavage at sites Phe^{23}–Phe^{24} and Val^{37}–Asn^{38}, the latter by action of chymosin [28].

The fragments derived from β-CN *C*-terminal region were still present in the MALDI-TOF spectrum of BSW peptide extract, although at a lower relative intensity. The precursor peptide f193–209 found in milk was also present in BSW. Identification of β-CN f193–206, f193–207, f193–208 and f195–206, f195–207, f195–208, f195–209 supported the occurrence of secondary proteolysis by action of aminopeptidase and carboxypeptidase removing amino acids sequentially from both the ends of primary peptides.

The peptide β-CN f161–177 was produced by hydrolysis at the level of the residues Gln^{160}–Ser^{161}, as already known for bovine β-CN [33]. Similarly β-CN f57–68 and f58–68 were produced, supporting the hypothesis that, since β–CN is resistant to chymosin action [15], its proteolysis can be ascribed to bacterial hydrolytic enzymes.

Para-k-CN (the *N*-terminal peptide 1–105 of k-CN generated by chymosin action on casein during milk curding) was scarcely sensitive to proteolysis, whereas several CMP fragments were detected (Table S1 in the Supporting Information), confirming the CMP sensitivity to proteolysis [15,34]. As expected by the specificity of chymosin action, significant amount of intact CMP (k-CN f106–169) either glycosylated or non-glycosylated (data not shown) was also found. CMP-derived peptides could have a beneficial role in modulating the gut microflora, as the casein macropeptide is known to promote the growth of bifidobacteria mainly because of its carbohydrate content (mainly sialic acid) [35]. Furthermore, CMP and CMP-derived peptides (casoplatelins) may have important biological value as in bovine they exhibited antithrombotic activity [36].

Only the peptides f185–206 ($MH^+ = 2723.8$) and f185–207 ($MH^+ = 2836.1$), both with the *N*-terminal Gln converted to pyroglutamic acid (p-Glu) were identified from α_{s2}-CN (Table S1 in the Supporting Information).

The peptides of Mozzarella PDO waste whey

As evidenced by MALDI-TOF-MS analysis (Figure 2(b)), the BWW peptide pattern was very complex because of secondary protease and peptidase action during curd maturation under whey. A multiplicity of peptides from β-, k- and α_{s1}-casein were identified. In the MALDI-TOF spectrum, the most prominent signals arose from the *C*-terminus of β-CN, probably also because of their high ionisation efficiency in MALDI. For this reason, the BWW peptide mixture was also characterised by HPLC/ESI-MS (Figure 3).

A number of peptides derived from β-CN, not present in the BSW peptide extract, were identified in peptide extracted from BWW (Table S1 in the Supporting Information). A drastic progress in the proteolysis of the primary peptide α_{s1}-CN f1–23 was also observed. Several fragments of this peptide were likely produced by the lactic acid bacteria cell-wall envelope enzymes [34]. Furthermore, k-CN fragments either from CMP (f116–127, already identified in BSW) or from para-k-CN, such as f25–40 and f25–45, occurred in BWW. The region 66–79 of para-k-CN was particularly exposed to proteolytic attack, as several fragments derived from it. It has to be underlined that para-k-CN following the increase of bacterial proteolytic activity and the pH lowering during curd maturation, becomes sensitive to proteolysis. In bovine, this was already reported by Visser *et al*. [34] who noted that peptides from para k-CN are generated by action of lactic acid bacteria belonging to *Lactococcus lactis* ssp. *cremoris* AM1.

The peptides from 'Scotta'.

'Scotta' is the residual whey resulting from the thermal coagulation of whey proteins during the production of 'ricotta' starting from sweet whey. Figure 2(c) shows the MALDI-TOF spectrum of peptide extracted from BS and the peptides identified are reported in Table S1 in the Supporting Information. The signals $MH^+ = 2723.8$ and $MH^+ = 2145.5$ dominating the MALDI spectrum, assigned to α_{s2}-CN f185–206 and α_{s1}-CN f180–199,

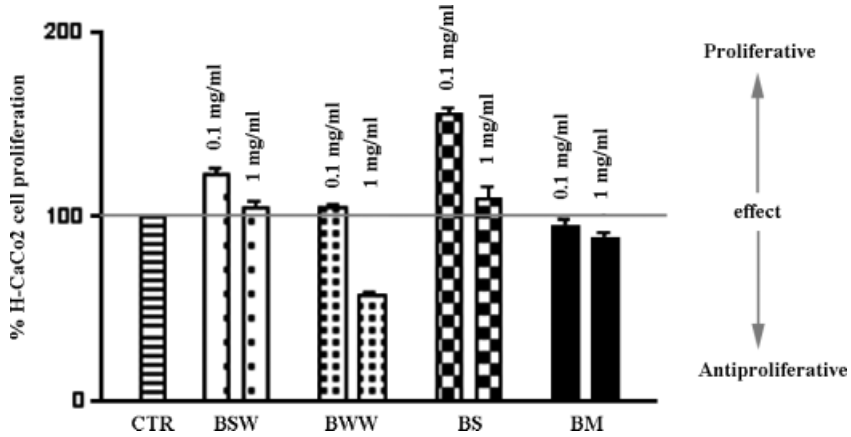

Figure 4. H-CaCo2 cell proliferation assay. Starved cells were pre-treated for 30 min with H_2O_2 (50 μM) and then incubated with 0.1 and 1 mg/ml of peptides extracted from BM, BSW, BWW and BS at 37 °C for 12 h. The control sample was constituted by H-CaCo2 cells untreated with the peptide extracts. Cell proliferation measured in treated samples was expressed as percent increase (proliferative effect) or decrease (antiproliferative effect) compared with that of the control sample, assumed as 100%. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.

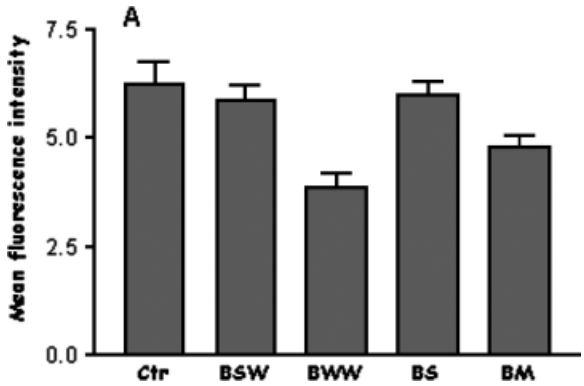

Figure 5. Flow cytometry analysis of oxidative stress in H-CaCo2 after incubation with peptide extracted from buffalo milk (BM), sweet whey (BSW), waste whey (BWW) and 'scotta' (BS). The control represented H-CaCo2 cells untreated with peptide extract. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.

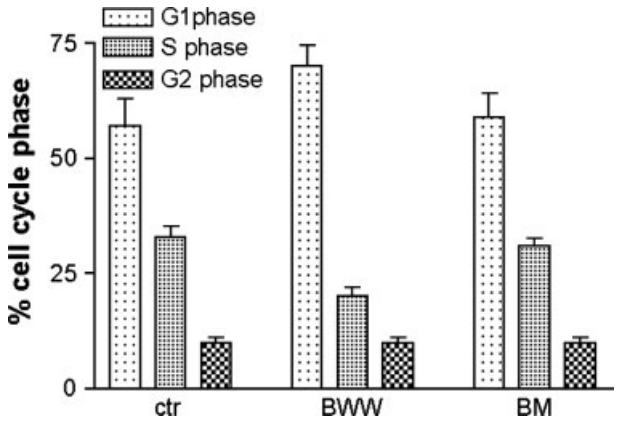

Figure 6. BM and BWW effects on G1 and S phase of H-CaCo2 cell cycle. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.

respectively, were found unchanged from BSW to BS, most likely because of proteolytic enzyme inactivation due to thermal treatment applied in the production of 'ricotta'. The finding that the BSW peptide pattern is practically conserved in BS allowed us to conclude that most of the peptides of BSW are not included in 'ricotta' whey protein network and that the peptide content of whey is scarcely affected by mild thermal treatment.

Functional effects of peptides extracted from buffalo milk on CaCo2 cells

Recent studies, carried out on several human cancer cell lines, have shown that milk-derived peptides may act on the regulation of cell growth, differentiation and apoptosis [37–40]. On the basis of these considerations, we decided to evaluate the protective effects of peptides derived from Mozzarella whey samples against H_2O_2-induced DNA damage in CaCo2 cell lines. Starved cells were exposed to 50 μM H_2O_2 for 30 min (H-CaCo2) and then treated for 24 h with 0.1 and 1 mg/ml of peptide extracted from BM, BSW, BWW and BS. Interestingly, we observed that only exposure of H-CaCo2 cells to BWW resulted in a 43% reduction in cell proliferation (Figure 4) and decreased mitochondrial superoxide CaCo2 anion production analysed by FACS. The adduct mean fluorescence intensity decreased from 6.2% (H-CaCo2 cells) to 3.7% with BWW peptide extract fraction (Figure 5). To further investigate the effect of peptide extracted from BWW on cell proliferation, the distribution in each phase of the cell cycle was determined by flow cytometric analysis of DNA content. As reported in Figure 6, a significant reduction ($p < 0.05$) of cells in S phase and an accumulation in the G1 phase were observed in CaCo2 cells treated with 0.08 mg/ml BWW for 24 h. Concomitantly a sub-G1 peak in CaCo2 cells (data not shown) was detected. These results suggest that, at the experimental concentration, BWW peptide extract is able to inhibit cell proliferation, interfere with cell cycle and exert a possible pro-apoptotic activity in CaCo2 cancer cells.

It is worth noting that the difference in the biological activity between the peptide extracted from BWW and the other three samples finds a correspondence in the different peptide composition defined by the structural study (compare the MALDI spectra in Figures 1–2). This means that the components responsible for the biochemical effects are specifically produced in the transition from BSW to BWW during curd maturation.

Journal of PeptideScience

Conclusions

The proteolysis of milk proteins during the production of Mozzarella di Bufala Campana PDO cheese is the result of a complex series of events because of the combined action of native or endogenous milk proteases, milk clotting enzymes, starter culture and contaminating microflora. During the maturation of curd under whey, proliferation of bacterial microflora results in a rapid acidification of whey and in a prominent secondary proteolysis performed by endo- and exo-cellular peptidases and proteinases. Accordingly, we found that composition of low-molecular-mass peptides in BWW was highly heterogeneous, with a large number of different oligopeptides. Thermal treatments also influence the peptide composition in BS, by activating or more likely inhibiting the activity of peptidases and proteases. This means that the production of peptides with potential bioactivity occurs mainly in BWW. This hypothesis is also supported by the biochemical assays carried on CaCo2 cell cultures.

In agreement with the structural characterisation, we found that peptides in BWW exerted the greater reduction in H-CaCo2 cell proliferation. The absence of this effect in the peptide extract from original milk (BM) suggests that the production of specific bioactive compounds occurs specifically during the production process of Mozzarella cheese. Previous studies [41,42] have suggested protective activity of peptides originating either from the bacterial cell-wall or by hydrolysis products of the lactic acid bacteria.

The complexity of BWW peptide extract makes almost impossible to identify the peptide(s) responsible of the cytomodulatory activity. However, in BWW, we have identified two peptides β-CN f57–68 and f60–68, which are precursors of the agonist opioid β-casomorphin 7 and β-casomorphin 5 [11]. Recently, it has been reported that casomorphin-agonist peptides derived from the limited proteolysis of caseins, interacting with both opioid [43,44] and somatostatin receptors [45], acted in decreasing cell proliferation. In particular, Hatzglou *et al*. [46] have proved that in T47D human breast cancer cells *in vitro,* α- and β-casomorphins inhibit cell proliferation in a dose-dependent manner, by interaction with δ - and κ-opioid receptors. Opioid and somatostatin receptors are present in the cells of nervous, endocrine and immune system as well as in the intestinal tract of mammals [47], including CaCo2 cells. Therefore, the proliferation decrease we observed in colon cells could be mediated by a direct interaction between opioid precursors contained in BWW peptide extract and the specific opioid and somatostatin receptors [48,49] expressed on CaCo2 cells. Work is in progress in our laboratory to confirm this hypothesis by analysis of the effect of homogeneous synthetic peptides corresponding to the opioid peptides found in BWW. These assays will lead to a deeper definition of the structure-activity relationship (SAR) in milk-derived peptides. Another, not a secondary issue under investigation, is the confirmation of the effects observed on CaCo2 cells on other cell systems. The information obtained could also drive research in the utilisation of industrial by-products for the development of dietary supplements for functional foods and of novel drugs for pharmaceutical industry.

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgements

This research was not supported by any specific grant or funding from public or private institutions. The authors gratefully acknowledge the '*Centro di Competenza per la Produzioni Agroalimentari della Regione Campania*' for permitting use of its MS facilities. We gratefully acknowledge Prof. Antonio Malorni for the use of MS facilities from Rete di Spettrometria di Massa (CNR, Avellino, Italy), acquired in part with contracts FERS No. 94.05.09.013 and ARINCO No. 94.IT.16.028.

References

1. Meisel H. Biochemical properties of peptides encrypted in bovine milk proteins. *Curr. Med. Chem.* 2005; **12**: 1915–1919.
2. Meisel H, FitzGerald J. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Curr. Pharm. Des.* 2003; **9**: 1289–1295.
3. Ferranti P, Traisci MV, Picariello G, Nasi A, Boschi V, Siervo M, Falconi C, Chianese L, Addeo F. Casein proteolysis in human milk: tracing the pattern of casein breakdown and the formation of potential bioactive peptides. *J. Dairy Res.* 2004; **71**: 74–87.
4. Meisel H, FitzGerald RJ. Opioid peptides encrypted in intact milk protein sequences. *Br. J. Nutr.* 2000; **84**: S27–S31.
5. Meisel H. Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livest. Prod. Sci.* 1997; **50**: 125–138.
6. Lahov E, Regelson E. Antibacterial and immunostimulating casein-derived substances from milk: Casecidin, isracidin peptides. *Food Chem. Toxicol.* 1996; **34**: 131–145.
7. Recio I, Visser S. Identification of two distinct antibacterial domains within the sequence of bovine αs2-casein. *Biochim. Biophys. Acta* 1999; **1428**: 314–326.
8. Jollés P, Parker F, Floch F, Migliore D, Alliel P, Zerial A, Werner GH. Immunostimulating substances from human casein. *J. Immunophar.* 1981; **3**: 363–369.
9. Migliore-Samour D, Floch F, Jollés P. Biologically active casein peptides implicated in immunomodulation. *J. Dairy Res.* 1989; **56**: 357–362.
10. Sandre C, Gleizes A, Forestier F, Gorges-Kergot R, Chilmonczyk S, Leonil J, Moreau MC, Labarre C. A peptide derived from bovine β-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice. *J. Nutr.* 2001; **131**: 2936–2942.
11. Meisel H, Schlimme E. Inhibitors of angiotensin converting- enzyme derived from bovine casein (casokinins). In *β-Casomorphins and Related Peptides: Recent Developments*, Brantl V, Teschemacher H (eds). Weinheim, VCH: Germany, 1994; 27–33.
12. Schlimme E, Meisel H. Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung* 1995; **39**: 1–20.
13. Teschemacher H. Opioid receptor ligands derived from food proteins. *Curr. Pharm. Des.* 2003; **9**: 1331–1344.
14. Hebert EM, Mamone G, Picariello G, Raya RR, Savoy G, Ferranti P, Addeo F. Characterization of the pattern of αs1- and β-Casein breakdown and release of a bioactive peptide by a cell envelope proteinases from *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. *Appl. Environ. Microbiol.* 2008; **74**: 3682–3689.
15. Chianese L, Caira S, Ferranti P, Laezza P, Malorni A, Mucchetti G, Garro G, Addeo F. The oligopeptides of sweet and acid cheese whey. *Lait* 1997; **77**: 699–715.
16. Kuwata H, Yip TT, Yip CL, Tomita M, Hutchens TW. Bactericidal domain of lactoferrin: detection, quantitation, and characterization of lactoferricin in serum by SELDI affinity mass spectrometry. *Biochem. Biophys. Res. Commun.* 1998; **245**: 764–773.
17. Chabance B, Marteau P, Rambaud JC, Migliore-Samour D, Boynard M, Perrotin P, Guillet R, Jollés P, Fiat AM. Casein peptide release and passage to the blood in humans during digestion of milk and yogurt. *Biochemistry* 1998; **80**: 155–165.
18. Larsen LB, Benfeldt C, Rasmussen LK, Petersen TE. Bovine milk procathepsin D and cathepsin D: Coagulation and milk protein degradation. *J. Dairy Res.* 1996; **63**: 119–130.

19. Pellissier JP. Proteolysis of caseins. *Sci. Aliments* 1984; **4**: 1–35.
20. Bockelmann W. The proteolytic system of starter and non-starter bacteria: component and their importance for cheese repening. *Lait* 1995; **73**: 97–108.
21. Poolman B, Kunji ERS, Hagting A, Juillard V, Koning WN. The proteolytic pathway of *Lactococcus lactis*. *J. Appl. Bacteriol. Symp. Supp.* 1995; **79**: 65S–75S.
22. Law J, Haandrikman A. Proteolytic enzymes of lactic acid bacteria. *Int. Dairy J.* 1997; **7**: 1–11.
23. Welling PG. Effect of food on drug absorption. *Ann. Rev. Nutr.* 1996; **16**: 383–415.
24. Ashaffenburg R, Drewry J. New procedure for the routine determination of the various non-casein protein of milk. In *15th International Dairy Congress*, London, vol. 3, 1959; 1631–1637.
25. Swaisgood H. Chemistry of caseins. In *Advanced Dairy Chemistry – I. Proteins*, Fox P, Mc Sweeney P (eds). Kluwer Academic/Plenum Publishers: New York, 2003; 139–201.
26. Tsong-Yong Y, Norihiro A, Shuichi K, Kunio Y. Purification and characterization of a substrate-size-recognizing metalloendopeptidases from *Streptococcus cremoris* H61. *Appl. Environ. Microbiol.* 1987; **53**: 2296–2302.
27. Oomen BS, McMahon DJ, Oberg CJ, Broadbent JR, Strickland M. Proteolytic specificity of *Lactobacillus delbrueckii* subsp. *bulgaricus* influences functional properties of mozzarella cheese. *J. Dairy Sci.* 2002; **85**: 2750–2758.
28. Péllisier JP, Mercier JC, Ribadeau Dumas B. Étude de la protéolyse des casein αs1 et β par la présure. Spécificité d'action. Peptides amers libérés. *Ann. Biol. Anim. Biochim. Biophys.* 1974; **14**: 343–363.
29. Ferranti P, Scaloni A, Caira S, Chianese L, Malorni A, Addeo F. The primary structure of water buffalo alpha (s1)- and beta-casein identification of phosphorylation sites and characterization of a novel beta-casein variant. *J. Protein Chem.* 1998; **17**: 835–844.
30. Rampilli M, Larsen R, Harboe M. Natural heterogeneity of chymosin and pepsin in extracts of bovine stomach. *Int. Dairy J.* 2005; **15**: 1130–1137.
31. McSweeney PL, Olson NF, Fox PF, Healy A, Højrup P. Proteolytic specificity of chymosin on bovine alpha s1- casein. *J. Dairy Res.* 1993; **60**: 401–412.
32. Carles C, Ribadeau Dumas B. Kinetics of the action of chymosin (rennin) on a peptide bond of bovine αs1- casein. Comparison of the behavior of this substrate with that of beta- and kappa o- caseins. *FEBS Lett.* 1985; **185**: 282–286.
33. Tan PST, Poolman B, Konings WN. Proteolytic enzymes of *Lactococcus lactis*. *J. Dairy Res.* 1993; **60**: 269–286.
34. Visser S, Slangen CJ, Robben AJPM, Donjen WD, van, Heerma W, Haverkamp J. Action of a cell-envelope proteinases (CEP_{III}-type) from *Lactococcus lactis* subsp. *cremoris* AM^{I} on bovine k-casein. *Appl. Microbiol. Biotechnol.* 1994; **41**: 644–651.
35. Manso MA, Lopez-Fandino R. Angiotensin I converting enzyme-inhibitory activity of bovine; ovine; and caprine kappa-casein macropeptides and their tryptic hydrolysates. *J. Food Prot.* 2003; **66**: 1686–1692.
36. Qian ZY, Jolles P, Migliore-Samour D, Schoentgen F, Fiat AM. Sheep kappa-casein peptides inhibit platelet aggregation. *Biochim. Biophys. Acta* 1995; **1244**: 411–417.
37. Roy MK, Wanatabe Y, Tamai Y. Induction of apoptosis in HL-60 cells by skimmed milk digested with a proteolytic enzyme from the yeast *Saccharomices cerevisiae*. *J. Biosci. Bioeng.* 1999; **88**: 426–432.
38. Roy MK, Kuwabara Y, Hara K, Wanatabe Y, Tamai YJ. Peptides from the N-terminal end of bovine lactoferrin induce apoptosis in human leukemic (HL-60) cells. *J. Dairy Sci.* 2002; **85**: 2065–2074.
39. Hatzglou A, Bakegeorgou E, Hatzglou C, Martin PM, Castanas E. Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line. *Eur. J. Pharmacol.* 1996; **310**: 217–223.
40. Svensson M, Hakansson A, Mosseberg AK, Linse S, Svanborg C. Conversion of α- lactoalbumin to a protein inducing apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 2000; **97**: 4221–4226.
41. Bodana AR, Rao DR. Antimutagenic activity of milk fermented by *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. *J. Dairy Sci.* 1990; **73**: 3379.
42. Hosoda M, Hashimoto H, Morita H, Chiba M, Hosono A. Antimutagenicity of milk cultured with lactic acid bacteria against N-methyl-N'-nitro-Nnitrosoguanidine. *J. Dairy Sci.* 1992; **75**: 976.
43. Maneckjee R, Biswas R, Vonderhaar BK. Binding of opioids to human MCF-7 breast cancer cells, and their effects on growth. *Cancer Res.* 1990; **50**: 2234.
44. Hatzglou A, Bakogeorgou E, Castanas E. The antiproliferative effect of opioid agonists on the T47D human breast cancer cell line, is partially mediated through opioid receptors. *Eur. J. Pharmacol.* 1996; **296**: 199.
45. Hatzglou A, Bakogeorgou E, Thermos K, Castanas E. Morphine cross-reacts with somatostatin receptor SSTR2 in the T47D human breast cancer cell line, and decrease cell proliferation. *Cancer Res.* 1995; **65**: 5632.
46. Hatzglou A, Bakogeorgou E, Hatzglou C, Martin PM, Castanas E. Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line. *Eur. J. Pharmacol.* 1996; **310**: 217–223.
47. Teschemacher H, Brandtl V. Milk protein derived atypical opioid peptides and related compounds with opioid antagonist activity. In *β-Casomorphins and Related Peptides: Recent Developments*, Brantl V, Teschemacher H (eds). Weinheim, VCH: Germany, 1994; 3–17.
48. Laws SA, Gough AC, Evans AA, Bains MA, Primrose JN. Somatostatin receptor subtype mRNA expression in human colorectal cancer and normal colonic mucosae. *Br. J. Cancer* 1997; **75**: 360–366.
49. Reubi JC, Laiussue J, Waser B, Horisberger U, Schaer JC. Expression of somatostatin receptors in normal, inflamed, and neoplastic human gastrointestinal tissues. *Ann. N.Y. Acad. Sci.* 1994; **733**: 122–137.

