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Isolation and identification of some major peptides in the ethanol-soluble fraction of the pH 4·6-soluble extract from Manchego cheese

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Proteolysis is one of the major biochemical events which takes place during cheese ripening and its degradation products, amino acids and peptides, have a considerable influence on the sensory characteristics of cheese (Urbach, 1993). Primary proteolysis leads to the formation of large water-insoluble peptides and smaller water-soluble peptides. Several peptides from bovine milk cheeses have been isolated and identified, particularly from Cheddar cheese (e.g., McSweeney et al. 1994; Singh et al. 1994, 1995, 1997; Gouldsworthy et al. 1996; Fernández et al. 1998). However, there are few data available on the identification of peptides from ewes' milk cheeses, although Michaelidou et al. (1998) identified some major peptides in the water-soluble fraction of Feta cheese (ewe's milk cheese).

Water-soluble peptides are characteristic of particular varieties and are related to the specificity of the proteinases and peptidases of the microflora of the cheese (Fox & McSweeney, 1996). The ethanol-soluble fraction of the pH 4·6-soluble extract of the cheese contributes to cheese flavour as it contains the smallest peptides from the pH 4·6-soluble extract.

The objective of this work was to isolate and identify the main peptides from the ethanol-soluble fraction of Manchego cheese, in order to understand better the proteolytic pathways during ripening of this cheese made from ovine milk.

Materials and Methods

Manchego cheeses were manufactured using a defined-strain starter culture of lactic acid bacteria and *Lactobacillus plantarum* as starter adjunct as described by Poveda et al. (2003).

The pH 4·6-soluble fraction of the cheese samples was obtained and fractionated into 700 ml/l ethanol-soluble (small, hydrophilic peptides) and -insoluble (large, hydrophobic peptides) fractions as described by Poveda et al. (2003). Peptide profiles of the ethanol-soluble fraction of the pH 4·6-soluble extract were determined by RP-HPLC as described previously (Poveda et al. 2003). Samples were prepared before analysis as described by Sousa & McSweeney (2001). Peptides were isolated from the 700 ml/l ethanol-soluble fraction of the 90 day-old cheese. Fractions of individual peaks were collected manually as they eluted and those from 6 successive RP-HPLC runs were pooled and freeze-dried.

The isolated peptides were subjected to N-terminal amino acid sequencing by automated Edman degradation and mass analysis using a plasma desorption time of flight mass spectrometer as described Singh et al. (1995). Peptides were identified from the sequence of their six N-terminal residues (no six residue sequence is duplicated in any of the caseins) and by reference to the amino acid sequence of ovine α_{s1}- (Ferranti et al. 1995) and β-CNs (Richardson & Mercier, 1979).

Results and Discussion

The RP-HPLC peptide profile of the 700 ml/l ethanol soluble fraction of the pH 4·6-soluble fraction of Manchego cheese at 90 d ripening is shown in Fig. 1. Ten major peaks (indicated by numbers) were selected for peptide isolation and identification. Some peaks contained more than one peptide (from the results of the N-terminal amino acid sequencing) and a total of 14 peptides were identified completely, except peptides 8 II and 9 I, for which no mass value was obtained (Table 1).

Peptides from α_{s1}-casein

According to the peptides isolated and identified in the present work, two main cleavage sites in ovine α_{s1}-CN

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Table 1. Identity of some major peptides isolated from the 700 ml/l ethanol-soluble fraction of the pH 4·6-soluble extract from Manchego cheese

HPLC peak no.	N-terminal sequence	Experimental mass (Da)	Theoretical mass (Da)	Peptide
1	KHPIKH	1371·8	1370·8	α_{s1}-CN (f3-14)
2	RPKHPI	1310·0	1310·8	α_{s1}-CN (f1-11)
3	RPKHPI	1623·1	1623·6	α_{s1}-CN (f1-14)
4	VAPFPE	756·7	757·8	α_{s1}-CN (f25-31)
5	VVAPFP	989·5	1004·1	α_{s1}-CN (f24-32)
6	ENLLAF	791·3	791·0	α_{s1}-CN (f18-23)
7 I	LNENLL	1017·1	1018·3	α_{s1}-CN (f16-23)
7 II	APFPEV	945·8	962·1	α_{s1}-CN (f26-33)
8 I	VLNENL	1118·1	1117·4	α_{s1}-CN (f15-23)
8 II	VAPFPE	not found	—	α_{s1}-CN (f25-?)
9 I	VVAPFP	not found	—	α_{s1}-CN (f24-?)
9 II	DVPSER	1043·6	1035·2	α_{s1}-CN (f85-93)
10 I	YQEPVL	1879·9	1881·3	β-CN (f191-207)
10 II	EPVLGP	1589·2	1590·0	β-CN (f193-207)

? : C-terminal not determined

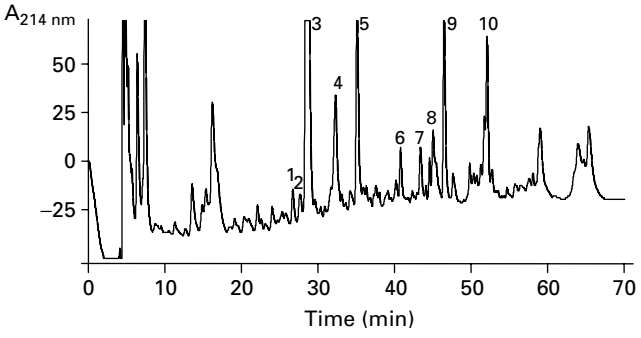

Fig. 1. RP-HPLC profile of the 700 ml/l ethanol-soluble fraction of the pH 4·6-soluble extract from 90 day-old Manchego cheese.

from Manchego cheese are observed, i.e., Phe_{23}-Val_{24} and Glu_{14}-Val_{15}. The first one is the primary site of chymosin action on bovine α_{s1}-CN, so it is likely that chymosin cleaves the bond Phe_{23}-Val_{24} in ovine α_{s1}-CN, resulting in the formation of the peptides 6, 7 I and 8 I (Table 1). These peptides correspond to the C-terminal region of α_{s1}-CN(f1-23). Similar peptides have been found in the diafiltration retentate of the water-soluble fraction of Cheddar (Singh et al. 1997) and other cheese varieties and are produced from α_{s1}-CN(f1-23) by the action of lactocepin (cell envelope-associated proteinase of *Lactococcus*), and the peptides found in Manchego are likely to have been produced in a similar fashion. It has been reported that lactocepin hydrolyses bovine α_{s1}-CN(f1-23) at the bond Glu_{14}-Val_{15} (Kaminogawa et al. 1986), so this could be the enzyme responsible for the formation of peptide 8 I. The N-terminus of peptide 6 derived from the hydrolysis of the bond Asn_{17}-Glu_{18}, which is a reported cleavage site for lactocepin III proteinase from strains including *Lc. lactis* ssp. *cremoris* SK11 on bovine α_{s1}-CN (Reid et al. 1991; Reid & Coolbear, 1999). Peptide 7 I originated from the cleavage of Val_{15}-Leu_{16}, which is a reported cleavage site for endopeptidases from strains including *Lc. lactis* ssp. *cremoris* AM1, *Lc. lactis* ssp. *lactis* NCDO 763 and *Lc. lactis* ssp. *lactis* MG 1363 in bovine α_{s1}-CN (Fox et al. 1994).

The hydrolysis of the bond Glu_{14}-Val_{15} may have been due to the action of starter proteinases and may have been involved in the formation of peptides 1, 2 and 3. Hence, it seems that the N-terminus of α_{s1}-CN(f1-23) has been hydrolysed during the ripening of Manchego cheese. In bovine casein, the hydrolysis of the bond Glu_{14}-Val_{15} has been attributed to the action of the proteinase from *Lc. lactis* ssp. *cremoris* H61 (Kaminogawa et al. 1986) and also from *Lc. lactis* ssp. *cremoris* C13 (Baankreis, 1992). It can be observed in Fig. 1 that α_{s1}-CN(f1-14) (peptide 3) is the major peak in the HPLC-chromatogram of the 700 ml/l ethanol-soluble fraction of the pH 4·6-soluble extract from Manchego cheese; other authors have also reported the accumulation of this peptide in other cheese varieties, including Feta (Michaelidou et al. 1998), Cheddar (Singh et al. 1994; Gouldsworthy et al. 1996) and Gouda (Kaminogawa et al. 1986).

The N-terminus of ovine α_{s1}-CN includes one phosphorylated residue (Ser_{12}) and one amino acid substitution in genetic variants. The genetic variants A, C and D of the ovine α_{s1}-casein, differ from each other by a few amino acid substitutions and degree of phosphorylation (Ferranti et al. 1995, 2001). In the present work, peptides 1 and 3 were found to contain a dephosphorylated seryl residue at position 12 (based on molecular mass) and were found to be the genetic variants C, D of ovine α_{s1}-CN (Ferranti et al. 1995). Peptide 1 (α_{s1}-CN f3-14) probably originated from the hydrolysis of the bond

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Pro_2-Lys_3 of the peptide 3 or α_{s1}-CN(f1-23) by the action of X-prolyldipeptidyl aminopeptidase (PepX) as this enzyme is capable of releasing X-Pro dipeptides from the N-terminal of oligopeptides (Upadhayay et al. 2004). Michaelidou et al. (1998) found a similar peptide, α_{s1}-CN(f4-14), in Feta cheese.

The bond Leu_{11}-Ser_{12} in ovine α_{s1}-CN corresponds to the Leu_{11}-Pro_{12} in bovine α_{s1}-CN and may have been hydrolysed by lactocepin, resulting in the formation of peptide 2 (α_{s1}-CN f1-11). This peptide has not been found in the water soluble fraction of Cheddar cheese (Singh et al. 1997).

Further hydrolysis of peptides could have given peptides 4, 5, 7 II, 8 II and 9 I. Peptide 5 (α_{s1}-CN f24-32) probably originated from α_{s1}-CN (f24-199) *via* hydrolysis by chymosin. The bond Phe_{32}-Arg_{33} of ovine α_{s1}-CN corresponds to Phe_{32}-Gly_{33} in bovine α_{s1}-CN, and the latter is a probable chymosin cleavage site at pH 5·2 in the presence of 50 g NaCl/l (McSweeney et al. 1993). Michaelidou et al. (1998) also found this peptide in Feta cheese, and it was formed early in the ripening process, being present in 4 d-old Feta cheese. Two peptides (4 and 8 II) were found originating at Val_{25}. This N-terminus could originate from the action of an aminopeptidase after the hydrolysis of the bond Phe_{23}-Val_{24} by chymosin in α_{s1}-CN. Singh et al. (1995) isolated the peptide α_{s1}-CN(f25-31) and several other peptides with the same N-terminus in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. The N-terminus of peptide 7 II (Ala_{26}) may originate through the action of chymosin on the cleavage site Phe_{23}-Val_{24} followed by the repeated action of an aminopeptidase on the N-terminus Val_{24}, giving the Ala_{26}. The C-terminus of peptide 7 II probably originated by the action of lactocepin on the bond Arg_{33}-Lys_{34}. Singh et al. (1995) found a similar peptide, α_{s1}-CN(f26-35), in Cheddar cheese.

Peptide 9 II corresponded to α_{s1}-CN(f85-93). Some authors have found peptides with Asp_{85} as N-terminus in the diafiltration permeate and retentate of the water-soluble fraction of Cheddar cheese, e.g., α_{s1}-CN(f85-91), α_{s1}-CN(f85-92), and α_{s1}-CN(f85-95) (Fox et al. 1994; Singh et al. 1994, 1997). Glu_{84}-Asp_{85} is a reported cleavage site for lactocepin on bovine α_{s1}-CN (Reid et al. 1991), and thus, the peptide 9 II found in Manchego may have been produced in a similar way.

Peptides from β-casein

In ovine β-CN, the residues Pro_{179} and Tyr_{180} are deleted, therefore, Leu_{190}-Tyr_{191} in the primary sequence of ovine β-CN corresponds to Leu_{192}-Tyr_{193} of bovine β-CN, the latter being the primary cleavage site of chymosin in bovine β-CN in solution (Visser & Slangen, 1977). Hence, peptide 10 I (β-CN f191-207) may have been produced by chymosin action. Michaelidou et al. (1998) found in Feta cheese, a peptide (β-CN f191-205) originating from this cleavage site in ovine β-CN. The peptide β-CN(f193-209) from bovine casein, that corresponds to ovine β-CN(f191-207), has been found in the water-soluble fraction of Cheddar cheese (Kelly et al. 1995; Gouldsworthy et al. 1996) and it is very hydrophobic and bitter (Visser et al. 1983), so peptide 10 I in the present study has similar hydrophobic characteristics and thus, if present at high concentrations, may impart a bitter flavour to Manchego cheese.

The N-terminus of the peptide 10 II found in Manchego cheese originates from cleavage at the bond Gln_{192}-Glu_{193}, which corresponds to the Gln_{194}-Glu_{195} in bovine β-CN; this bond has not been found to be cleaved in other cheese varieties. This peptide could originate from the action of exopeptidases from the starter or the non-starter lactic acid bacteria (NSLAB) on the peptide 10 I.

It can be concluded that most of the peptides isolated and identified from the 700 ml/l ethanol-soluble fraction of the pH 4·6 soluble-extract of Manchego cheese have been found also in other cheese varieties, and their production can be attributed to the action of chymosin or lactococcal enzymes. Most of these peptides possess a very low molecular weight (between approximately 700 and 1800 Da), therefore they probably contribute directly to the flavour of this type of cheese, or indirectly through the liberation of amino acids which acts as substrates for a range of catabolic reactions which generate important volatile flavour compounds.

Further work is necessary to study the action of enzymes of the starter lactic acid bacteria and NSLAB on ovine caseins and particularly in Manchego cheese to gain a better understanding of the proteolysis in this cheese variety.

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