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Peptide Composition of Enzyme-Treated Cheddar Cheese Slurries, Determined by Reverse Phase High Performance Liquid Chromatography

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ABSTRACT

Semi-liquid slurries of Cheddar cheese curd were used to investigate enzyme-accelerated cheese ripening. Addition of a neutral microbial proteinase from Bacillus subtilis *induced bitter off-flavours. When this was followed with an intracellular peptidase extract from* Streptococcus lactis *(NCDO 712) the bitter flavour disappeared leaving behind a normal Cheddar flavour. Associated with this last observation a group of bands eluting late from a reverse phase column were dramatically reduced in peak height. It is thought that these bands with long retention times are hydrophobic and contain bitter-tasting peptides. Peptidase addition also increased the amounts of fast-eluting bands presumed to be products of bitter peptide breakdown.*

INTRODUCTION

The manufacture of cheese is a capital intensive activity. Hard cheeses in particular need long ripening periods; for example, up to 12 months is required to produce a mature Cheddar cheese. During this period, interest must be paid on capital invested in the cheese itself, and running costs for the storage warehouses must be provided. It therefore follows that any method that shortens storage time may be of interest to manufacturers. Methods used for the acceleration of cheese ripening have been reviewed extensively by Law (1978, 1980, 1984*a*).

Based on an increased understanding of the importance of proteolysis

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(Law, 1981, 1982, 1984*b*) in the ripening of cheese, Law & Wigmore (1983) proposed the addition of proteinase and peptidase enzymes in the cheesemaking process to achieve balanced increases in the production of savoury flavour notes. A neutral proteinase from *B. subtilis* and an intracellular peptidase extract from the starter *S. lactis* NCDO 712 were added to accelerate the ripening process. The proteinase from B. subtilis was used to supplement starter proteinase but, at high concentrations, cheese containing bitter-tasting peptides is produced (Law & Wigmore, 1982*a*,*b*). Use of an intracellular extract from *S. lactis* NCDO 712, which contains a range of peptidases, was assumed to degrade these to smaller non-bitter peptides. A development of this enzyme system is now available commercially ('Accelase', Imperial Biotechnology Ltd, London, UK) as an effective method of shortening cheese ripening times.

Although enzyme addition to accelerate cheese ripening is now a commercial reality, the mechanism is not fully understood. Detailed investigations are required to determine the interrelationships between enhanced proteolysis of intact proteins by proteinase on the one hand, and enhanced peptide breakdown by the peptidases on the other.

A procedure for the isolation of bitter and astringent fractions from Cheddar cheese was developed by Harwalkar & Elliot (1971) but only the astringent flavour fraction was characterised (Harwalkar, 1972). Three peptides, isolated from a bitter Cheddar cheese by Richardson & Creamer (1973) using permeation and ion-exchange chromatography, were found to have been derived from α_{s1}-casein. The peptides found were Glu-Val-Leu-Asn (designated as PI), Asn-Glu-Asn-Leu-Leu (PII) and Ala-Pro-Phe-Pro-Gln-Val-Phe (PIII). In contrast, Hamilton *et al.* (1974) isolated one bitter peptide from a bitter Cheddar cheese using gel permeation chromatography, paper chromatography and high voltage paper electrophoresis and concluded that it originated from β-casein. The bitter peptide isolated (Peptide I) corresponded to residues 46–67 of β_{A2}-casein with one Gln substituted for Pro and one Ile substituted for Val. Champion & Stanley (1982) were probably the first researchers to fractionate bitter peptides extracted from Cheddar cheese using reverse phase high performance liquid chromatography. Unlike the previously mentioned methods, reverse phase high performance liquid chromatography offers the possibility of fractionating the water-soluble nitrogen fraction of Cheddar cheese by one technique. However, the run time used by Champion & Stanley (1982) is too long.

The present paper describes the use of rapidly-ripened Cheddar cheese slurries (Kristoffersen *et al.*, 1967) to investigate the feasibility of using a recently-developed peptide profiling technique (Cliffe *et al.*, 1989) to gain a deeper understanding of the action of 'Accelase' during accelerated ripening.

MATERIALS AND METHODS

Salted curd was made (Chapman & Burnett, 1972) with a single strain starter (*S. lactis* NCDO 924) using standard commercial rennet extract, then stored overnight at 4°C prior to use. Accelerating enzymes were added at 20 times the recommended rate (Law & Wigmore, 1983). The procedure of Kristofferson *et al.* (1967) was used for the slurry preparation. It was not considered necessary to add potassium sorbate to prevent mould growth (Dulley & Taylor, 1972) or store the slurries in the absence of oxygen because of the short duration of the experiment. A sample of untreated curd (84 g equivalent to about 80 g of cheese) was frozen and stored at −20°C to allow future evaluation of peptide content (sample A). Curd (200 g) was blended with 4·5% NaCl (100 ml) (preheated to 48°C) using an Atomix homogeniser (MSE Scientific Instruments, Crawley, West Sussex, UK) at the lowest speed for 2 min. Neutrase 1·5 S, 1·5 Anson Units/g (50 mg) (Novo Industri AS, Copenhagen, Denmark) was added, and homogenisation was continued at the lowest speed for 2 min. The sample was then homogenised at the highest speed for 2 × 15 s. The homogeniser jar was sealed and incubated at 30°C. The contents were tasted at 1·5 h, and at 5 h, when bitterness was noted. A sample (128 g, equivalent to 84 g of curd) was removed at 7·5 h for later peptide analysis (sample B) after which a peptidase extract 'Accelase' from *S. lactis* NCDO 712, 4·7 units/g (2·30 g) was added. Incubation was continued for 21·5 h more when it was noted that bitterness had disappeared to be replaced by a Cheddar-like flavour, so a sample (128 g) was taken for later peptide analysis (sample C). In a control experiment the above process was repeated, incubating the curd slurry with Neutrase alone for 29 h at 30°C. A sample was taken (128 g) for later peptide analysis (sample D, results not shown). All four samples were stored at −20°C and processed by the method of Cliffe *et al.* (1989) to extract and analyse the peptides.

RESULTS

As found previously (Cliffe *et al.*, 1989), reverse phase solvent systems, containing the ion-pairing agent trifluoroacetic acid (TFA), produced chromatograms with the most detailed features (peptide and amino acid bands) and will be considered first. The untreated curd sample (A) (Fig. 1a) produced very few peaks; the bands shown are probably the result of limited proteolysis of the curd by the chymosin milk coagulant. Treatment of the curd with Neutrase, a proteinase possessing broader bond specificities than chymosin, produced a chromatogram consisting of many bands (Fig. 1b). The appearance of these bands was associated with the formation of a bitter taste.

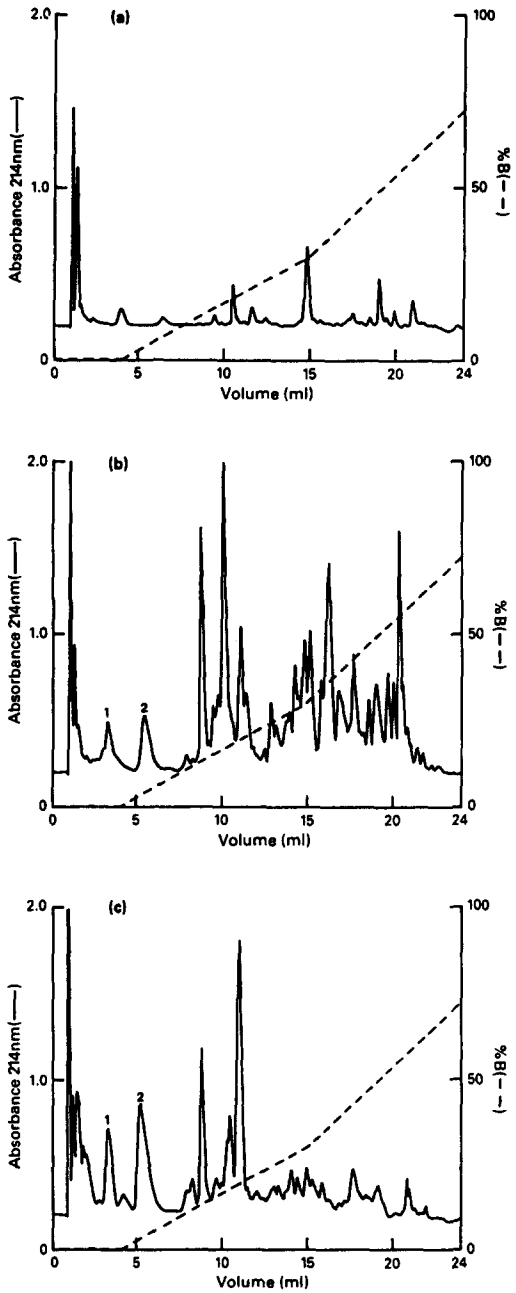

Fig. 1. Reverse phase chromatography of peptide fractions from curd slurries on a C_{18} (50 × 5 mm) column. Curd only (a and d). Neutrase added (b and e), followed by starter peptidase (c and f). Elution was with the following solvent systems. Solvent A, 0·1% v/vTFA in H_2O; solvent B, 0·1% v/v TFA in methanol (a, b and c). Solvent A, H_2O; solvent B, methanol (d, e and f).

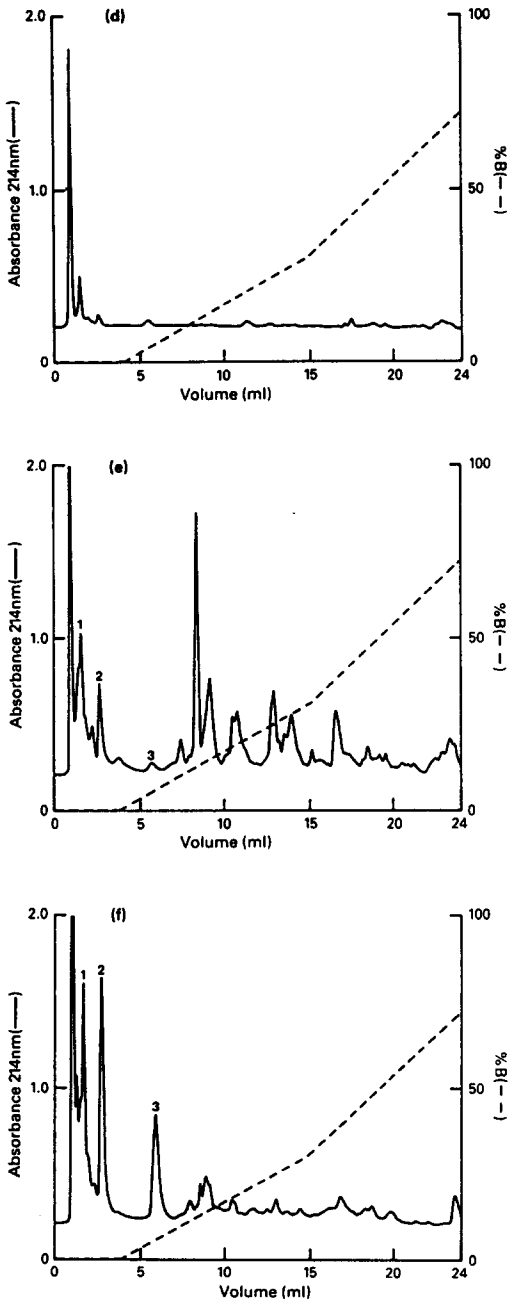
(d)
(e)
(f)
Absorbance 214nm(——)
%B(– –)
Volume (ml)
2.0
1.0
0
100
50
0
5
10
15
20
24
1
2
3

Fig. 1—*contd.*

When the partly degraded curd was treated with starter peptidase extract (Fig. 1c), many late-eluting bands (between 12 and 23 ml) were reduced in height relative to those in Fig. 1b. Two bands, numbered 1 and 2, increased in height and area.

In the solvent system containing methanol and water alone the curd yielded a chromatogram (Fig. 1d) almost devoid of bands beyond an elution volume of about 2·5 ml. Presumably some components observed in the presence of TFA are passing through the column with little or no retention when TFA is absent. Treatment of the curd with Neutrase (Fig. 1e) produced many bands which tended to decrease in size as the elution volume increased. A similar observation was made by Cliffe *et al.* (1989) when peptides extracted from Cheddar cheese were chromatographed using similar conditions. Subsequent treatment of the partially digested curd with starter peptidase extract (Fig. 1f) resulted in a considerable reduction of height of bands having elution volumes greater than 7 ml and an increase in height of bands numbered 1, 2 and 3.

Peaks showing high retention in both solvents persisted in slurries treated with Neutrase alone (not shown).

DISCUSSION

It is generally accepted that bitter peptides produced in cheese are relatively rich in hydrophobic residues (e.g. phenylalanyl, leucyl, prolyl, valyl; Matoba & Hata, 1972; Richardson & Creamer, 1973; Guigoz & Solms, 1976). This class of peptide should therefore exhibit higher retention on a reverse phase column, relative to more hydrophilic peptides. Even though peptides may not elute strictly in order of increasing hydrophobicity when ion-pairing agents are used (such as TFA), bands degraded by starter peptidase eluted later in methanol and water, whether or not these solvents contain TFA. This observaton, coupled with the removal of bitter taste from the slurry, directly supports the concept that the main function of starter peptidase is to further degrade the large peptides produced by Neutrase and chymosin to small peptides and free amino acids (Law, 1981). Not all of the bands reduced in size or removed will necessarily be bitter, of course. Fractionation in either solvent system shows an intensification of a limited number of hydrophilic bands with shorter elution volumes after treatment with starter peptidase. It is possible that the production of these bands is associated with desirable background flavours in Cheddar cheese and their composition requires further characterisation since they may be exploitable as flavour constituents if their production can be controlled and enhanced in protein hydrolysates.

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