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Review

Chemical methods for the characterization of proteolysis in cheese during ripening

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Summary — Proteolysis is the principal and most complex biochemical event which occurs during the maturation of most cheese varieties. Proteolysis has been the subject of much study and a range of analytical techniques has been developed to assess its extent and nature. Methods for assessing proteolysis can be classified under two broad headings: non-specific and specific techniques, both of which are reviewed. Non-specific techniques include the quantitation of nitrogen soluble in various extractants or precipitants and the liberation of reactive groups. Specific techniques include those which resolve individual peptides or free amino acids, principally chromatography or electrophoresis. Techniques are often combined into a fractionation scheme for cheese nitrogen to facilitate the isolation of individual peptides. Methods for the identification of individual peptides are also reviewed. Strategies for assessing proteolysis in various cheese varieties are discussed.

proteolysis / cheese ripening / methodology

Résumé — Méthodes chimiques pour la caractérisation de la protéolyse des fromages durant l'affinage. La protéolyse, qui est le phénomène principal et le plus complexe intervenant dans la maturation de la plupart des fromages, a été le sujet de nombreuses études. Toute une gamme de techniques analytiques a été développée afin de permettre une meilleure compréhension de ce phénomène. Les méthodes de suivi de la protéolyse peuvent être classées en deux grands chapitres, traités dans cette revue : les techniques non spécifiques, et les techniques spécifiques. Les techniques non spécifiques incluent la quantification de l'azote soluble dans les divers extraits, et s'intéressent aussi à la libération des groupements réactifs. Les techniques spécifiques, principalement la chromatographie ou l'électrophorèse, sont celles permettant de séparer les peptides ou les acides aminés libres. Ces diverses techniques sont souvent combinées dans la stratégie de purification de la fraction azotée du fro-
mage afin de pouvoir isoler individuellement les peptides. Les méthodes d'identification des peptides, ainsi que les différentes stratégies de suivi de protéolyse dans diverses variétés de fromages sont discutées dans cette revue.

protéolyse / affinage du fromage / méthodologie

INTRODUCTION

The biochemistry of cheese ripening is normally considered under three general headings: proteolysis, lipolysis and glycolysis. Of these processes, proteolysis is the most important for most ripened cheese varieties (Fox et al, 1995a). Proteolytic agents in cheese originate from five sources: 1) the coagulant; 2) indigenous and endogenous proteinases from the milk; 3) the starter; 4) the adjunct starter; and 5) non-starter lactic acid bacteria.

The coagulant

Chymosin and bovine pepsin in the case of calf rennet or recombinant chymosin or fungal aspartyl proteinases in cheeses manufactured using rennet substitutes are principally responsible for the production of large peptides (Visser, 1977; McSweeney et al, 1994a).

Indigenous and endogenous proteinases from the milk

The principal indigenous proteinase in milk is plasmin, but somatic cell proteinases and enzymes from the native microflora of the milk (particularly heat-stable proteinases from psychrotrophs) are also present (Fox et al, 1995a).

The starter

Lactococcus spp, Lactobacillus spp and Streptococcus salivarius spp thermophilus used as starters for cheesemaking have extensive proteinase/peptidase systems (see Exterkate, 1995; Bockelmann, 1995) which contribute to proteolysis in cheese, particularly at the level of the production of short peptides and free amino acids (Visser, 1977).

The adjunct starter

Proteinases and peptidases from the adjunct starter are particularly important for proteolysis in certain cheese varieties. Penicillium spp and microorganisms from the smear of bacterial surface-ripened cheeses produce potent extracellular proteinases and peptidases (Gripon, 1993; Reps, 1993).

Non-starter lactic acid bacteria

The microflora of essentially all cheeses is characterized by the growth of adventitious non-starter lactic acid bacteria, NSLAB (typically Lactobacillus spp but also Pediococcus spp and perhaps other genera). The contribution of NSLAB to cheese quality has been the subject of much recent research (see Peterson and Marshall, 1990; Martley and Crow, 1993; McSweeney et al, 1995, for references). The contribution of NSLAB enzymes to proteolysis in Cheddar cheese appears to be relatively minor and at the level of the production of short peptides and free amino acids (McSweeney et al, 1994b; Lane and Fox, 1996).

The extent and nature of proteolysis show considerable intervarietal variation caused by differences in production protocols and ripening conditions (particularly whether a secondary microflora is encouraged). Proteolysis is very limited in Pasta Filata varieties (eg, Mozzarella) due to denaturation of enzymes during high-
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The products of proteolysis vary in size from large peptides comparable to intact caseins (eg, $\alpha_{s1}$-CN 24-199, the primary product of chymosin action on $\alpha_{s1}$-casein) to free amino acids and their degradation products. Proteolysis has important consequences for the development of cheese texture due to cleavage of the protein matrix (Creamer and Olson, 1982; Lawrence et al, 1983) and flavour through the production of the precursors of a number of volatile flavour compounds and directly by the liberation of amino acids and certain peptides (see Fox et al, 1995a).

Because of its central role in cheese ripening, techniques for the assessment of proteolysis have been reviewed (Rank et al, 1985; Fox, 1989; IDF, 1991; McSweeney and Fox, 1993; Fox et al, 1995b). These reviews suggest that techniques for the assessment of proteolysis in cheese fall into two general categories: non-specific and specific methods. Non-specific methods include determination of the nitrogen (N) soluble or extractable in various solvents or measurement of reactive groups, while specific techniques are those which resolve individual peptides, ie, electrophoretic and chromatographic methods. Non-specific methods provide adequate information on the overall extent of proteolysis and the general contribution of the various agents to proteolysis. Such techniques are generally simple and are valuable for the routine assessment of cheese maturity. The thorough study of proteolysis necessitates the isolation and identification of peptides; therefore techniques which resolve individual peptides, such as electrophoresis and chromatography, have received considerable attention in recent years. The objective of this review is to assess methodology for the study of proteolysis, but not to simply catalogue methods used by specific authors. In the case of widely-used techniques, only a few recent representative references are supplied. If further references are required, the reader is referred to the above reviews.

NON-SPECIFIC METHODS FOR ASSESSING PROTEOLYSIS

Extraction / solubility methods

The caseins are insoluble in many solvents, but peptides produced from them may be soluble and thus the proportion of soluble nitrogen will increase with proteolysis. This is the principle of a number of widely-used methods for assessing proteolysis in cheese during ripening. Such methods are particularly useful for determining the extent of proteolysis, since peptides of varying size can be precipitated by careful choice of solvents. A number of the solvent systems described below are also used to extract peptides as the first step in their purification.

Hydraulic pressure/centrifugation

The aqueous phase of cheese ('cheese juice') can be prepared by subjecting a cheese/sand mixture to hydraulic pressure (eg, 30 MPa). Preparation of cheese juice by hydraulic pressure has the advantage of not altering the ionic composition of the aqueous phase of cheese and has been exploited principally in studies on calcium phosphate in cheese, the buffering capacity of cheese and in studies on the lysis of starter cells. Authors who have used this technique include Morris et al (1988), Wilkinson et al (1992), Lucey et al (1993), Wilkinson et al (1994) and Salvat-Brunaud et al (1995). Preparation of the aqueous phase of cheese by centrifugation has the same advantages. Guo and Kindstedt (1995) prepared the aqueous fraction of Mozzarella by centrifugation at 12 500 g for 75 min at 25 °C.

Urea

The proteins and large polypeptides in cheese are completely soluble in 4 to 6 mol/L urea, which is used to prepare samples for analysis by chromatography or electrophoresis (see below).
Water

Water is perhaps the most commonly used extractant for cheese N. The level of water-soluble N (WSN) is a widely-used index of cheese ripening (see Rank et al, 1985; Fox et al, 1995b) and several procedures have been developed (see Christensen et al, 1991). Water is frequently used as the primary extractant in the isolation of amino acids and peptides.

Kuchroo and Fox (1982a) compared various extraction techniques for Cheddar cheese. All but one of the several homogenization techniques studied yielded essentially similar results; a stomacher was chosen for routine work. Homogenization temperature (in the range 5–40 °C) had little effect on the level of extractable N, which increased with the ratio of water to cheese; a ratio of 2:1 was recommended, and 90% of the potentially water-extractable N was recovered in two extractions. The procedure recommended is: grated cheese is homogenized in a stomacher at 20 °C for 10 min with twice its weight of water; the slurry is held at 40 °C for 1 h, centrifuged and the supernatant filtered through glass wool and Whatman no 113 filter paper. The residue can be re-extracted to increase the yield of extract. This and similar procedures have been used by a number of workers (eg, González de Llano et al, 1991; Tieleman and Warthesen, 1991; Wilkinson et al, 1992; Bütikofer et al, 1993; Singh et al, 1995, 1996).

The water-soluble extract (WSE) of Cheddar cheese contains numerous small- and medium-sized peptides, free amino acids and their degradation products, organic acids and their salts; extraction with water efficiently separates the small peptides in cheese from proteins and large peptides. In many cheeses, the principal proteolytic agents responsible for the production of WSN are the coagulant (chymosin) and, to a lesser extent, plasmin (Visser, 1977; Fox et al, 1995a). Starter proteinases and peptides play a relatively minor role in the hydrolysis of intact caseins and large polypeptides to water-soluble peptides, although they are active on many of the peptides released by chymosin or plasmin. Water-insoluble peptides in Cheddar cheese have been studied by McSweeney et al (1994a), who found that the principal peptides in this fraction were produced from $\alpha_{s1}$-casein by chymosin or pepsin or from $\beta$-casein by plasmin and therefore that the complementary primary water-soluble peptides must be produced via the action of these enzymes. This hypothesis has been strengthened by the results of Singh et al (1994, 1995, 1996) who isolated and identified a large number of water-soluble peptides from Cheddar cheese; with a few exceptions, these peptides corresponded directly to or were in the immediate vicinity of cleavage sites for lactococcal cell envelope-associated proteinase but generally did not include the principal cleavage sites of chymosin on $\alpha_{s1}$ or plasmin on $\beta$-casein, suggesting that chymosin and plasmin act first.

WSN as a percentage of total N varies with variety and increases throughout ripening (fig 1); a typical value for mature Cheddar cheese is ~25%. Water is a suitable extractant for internal bacterially-ripened cheese varieties, the pH of which changes little during ripening. However, mould and bacterial surface-ripened varieties are characterized by an increase in pH during ripening; and since the extractability of N varies with pH, an alternative method should be adopted.

Extraction with solutions containing salts

Extraction and quantification of cheese N soluble at pH 4.6 (pH 4.6–SN) is widely used as an index of proteolysis. Since the pH of many internal bacterially-ripened varieties is ~5.2, there is little difference between the levels extracted by water or pH 4.6 buffers. However, in the case of cheeses characterized by an increase in pH during ripening, WSN may be considerably higher than pH 4.6–SN. Like WSN, pH 4.6–SN is produced mainly by rennet (O’Keeffe et al, 1976) and increases during ripening (eg, fig 1). Whey proteins and proteose peptones (from plasmin action) are also soluble at pH 4.6, but their contribution to pH 4.6–SN is relatively small;
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![Fig 1. Formation of pH 4.6-soluble N (■), 12% trichloroacetic acid-soluble N (●) and 5% phosphotungstic acid (PTA)-soluble N (▲) in an Irish artisanal blue cheese and formation of water-soluble N (□) and PTA-soluble N (○) in a commercial Cheddar cheese. Modified from Folkertsma et al (1996) and Zarmpoutis et al (1996).](image)

γ-caseins are insoluble at pH 4.6 (see Rank et al, 1985). According to Kuchroo and Fox (1982a), this method gives slightly lower values for soluble N than extraction with water; and although it is more difficult to perform, it may be easier to standardize (Fox, 1989). Recent studies in which pH 4.6–SN has been used as an index of ripening include those by Lau et al (1991), Addeo et al (1992), Bütikofer et al (1993), Picon et al (1994), Yun et al (1995) and Zarmpoutis et al (1996).

Precipitation of casein-derived peptides by CaCl₂ has been used by some investigators. Solutions containing CaCl₂ have been used as the primary extractant or as a method to subfractionate WSN (Lau et al, 1991; Addeo et al, 1992; Bütikofer et al, 1993). Noomen (1977a) and Venema et al (1987) homogenized cheese in 0.037 mol/L CaCl₂, adjusted the homogenate to pH 7.5, followed by centrifugation. Visser (1977) homogenized cheese in 4% NaCl containing 0.55% Ca (as CaCl₂). Fat was removed and the pH of the homogenate was adjusted to 7.5, followed by centrifugation. Noomen (1977b) used a method based on sensitivity to coagulation by pH and heat to fractionate cheese N. An extract was prepared by homogenizing cheese in a CaCl₂ (0.137 mol/L)/NaCl (0.684 mol/L) solution; the homogenate was adjusted to pH 5.1. The extract was fractionated by acidification with 0.25 mol/L HCl to a final pH of ~1.6 and holding at 55 °C for 30 min, and then overnight at room temperature before filtration.

Kuchroo and Fox (1982a) found that only 40% of the WSN was soluble in 0.1 mol/L CaCl₂. Increasing the concentration of CaCl₂ above 0.05% at or above pH 7.0 has little influence on extractability (Noomen, 1977b). The increase in CaCl₂-soluble N correlates with the age of cheese and the extracts contain whey proteins, peptides and amino acids, while the CaCl₂-insoluble fraction contains caseins and large peptides, similar to those in the water-insoluble fraction (Christensen et al, 1991).

NaCl has also been used to fractionate cheese N (eg, Chakravorty et al, 1966; Gupta et al, 1974), but Reville and Fox (1978) claimed that > 90% of Cheddar cheese N was soluble in 5% NaCl, which was thus not sufficiently discriminating, except perhaps for very young cheeses. NaCl (5%)-soluble N and unfractionated cheese are indistinguishable electrophoretically, indicating that the parent caseins, as well as peptides, are extracted (Rank et al, 1985; Bican and Spahni, 1991). Inclusion of CaCl₂ in the NaCl solution should reduce the percentage N extracted and thus increase the selectivity of the method (see Fox, 1989).

**Extraction/fractionation with organic solvents**

Harwalkar and Elliott (1971) developed an extraction technique with chloroform/methanol...
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Freeze-dried cheese was extracted using this solvent, the extract filtered and water added. The chloroform layer contained lipids and was discarded. Removal of methanol from the aqueous methanol layer by evaporation resulted in the formation of a precipitate; the supernatant was very bitter and astringent. This method has also been used by a number of authors (eg, Lemieux et al, 1989, 1990; Puchades et al, 1989a, b; Tielemans and Warthesen, 1991) using different fractions of cheese as the starting material.

Chloroform/methanol extracts more N than water, which is understandable considering the relatively hydrophobic nature of many casein-bound peptides, but chromatograms of both extracts on Sephadex G-25 were similar (Rank, et al, 1985). However, Tielemans and Warthesen (1991) found that extracts prepared by the method of Harwalkar and Elliott (1971) were significantly less heterogeneous, as indicated by HPLC, than extracts prepared by the methods of McGugan et al (1979) or Kuchroo and Fox (1982a).

McGugan et al (1979) developed a fractionation scheme using methylene chloride: methanol:water which has also been used by Smith and Nakai (1990).

Fractionation of proteins and peptides by ethanol is a classical technique, and has been used extensively to fractionate peptides in cheese (Christensen et al, 1991; McSweeney and Fox, 1993; Fox et al, 1995b). Ethanol concentrations used have varied from 30 to 80% (Fox et al, 1995b); 70% has been used widely (eg, Abdel Baky et al, 1987). Trichloroacetic acid, TCA (12%, see below) and 70% ethanol yield approximately equal extraction levels (Reville and Fox, 1978) and soluble fractions contain similar peptides (Kuchroo and Fox, 1982b), although differences are apparent in the peptides in the insoluble fractions.

An ultrafiltration (UF) retentate (10 kDa) of a WSE of Cheddar was fractionated by Breen et al (1995) using increasing concentrations of ethanol (30–80%); fractionation of the UF retentate containing 30% ethanol by stepwise adjustment of pH in the range 5.5–6.5 was found to be quite effective.

Precipitation with ethanol may be used to fractionate cheese or to sub-fractionate WSEs, and should be preferred to the largely equivalent 12% TCA owing to the ease of removal of ethanol by evaporation (Christensen et al, 1991). Fractionation of WSEs of Cheddar using 70% ethanol is used routinely in the authors’ laboratory.

Poznanski et al (1966) fractionated 2 or 12% TCA-soluble extracts with 75% acetone acidified to pH 4.6, while Aston and Creamer (1986) used methanol to fractionate a WSE of Cheddar. Butan-1-ol has been used to extract bitter peptides from casein hydrolysates (McSweeney and Fox, 1993) but does not appear to have been applied to the fractionation of cheese. This extractant may have potential for the isolation of bitter and/or hydrophobic peptides.

**Fractionation using trichloroacetic or trifluoroacetic acids**

Trichloroacetic acid (CCl$_3$COOH; TCA) is a classical protein precipitant which has been used widely for this purpose. Concentrations ranging from 2 or 2.5% (eg, Reville and Fox, 1978; O’Sullivan and Fox, 1990) to 12% (eg, Bican and Spahni, 1991; Folkertsma and Fox, 1992; Addeo et al, 1992; Bütikofer et al, 1993; Katsiarie and Voutsinas, 1994; Picon et al, 1994; Yun et al, 1995) have been used, depending on the degree of fractionation required (larger peptides being soluble at the lower TCA concentrations). There is no ‘precipitation threshold’ relating peptide size to solubility in TCA, but all peptides studied by Yvon et al (1989) containing less than seven amino acid residues were soluble in 12% TCA; peptide solubility is related to hydrophobicity. The majority of 12% TCA-soluble peptides isolated and identified from Parmigiano–Reggiano cheese by Addeo et al (1994) were phosphopeptides. Typically, in a mature Cheddar about 15% of the total N (Reville and Fox, 1978) and ~90% of the WSN (Kuchroo and Fox, 1982b) is soluble in 2.5%
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TCA; while 50–60% of a UF retentate (10 kDa membranes) and 100% of the UF permeate (O’Sullivan and Fox, 1990) is soluble in 2% TCA.

Rennet is responsible for the production of some of the 12% TCA-soluble N, but starter proteinases and peptidases make a substantial contribution (Reiter et al., 1969; O’Keeffe et al., 1976) to the formation of 12% TCA-soluble N. Venema et al. (1987) reported that the level of 12% TCA-soluble N is a better index of maturity than WSN. The development of 12% TCA-soluble N in an Irish artisanal blue cheese is shown in figure 1.

A major disadvantage of TCA for peptide fractionation is the need to remove it prior to further analysis of the fractions. Since small peptides and free amino acids will be lost on dialysis, other methods for removal of TCA, e.g., repeated ether extraction or some form of chromatography, are required and are laborious procedures (Fox, 1989). The use of 70% ethanol, which gives similar precipitation levels, is preferable because ethanol can be readily removed by evaporation (Fox, 1989).

Trifluoroacetic acid (CF₃COOH; TFA) as a precipitant or extractant for cheese peptides may be a useful alternative to TCA, and it has the major advantage of being easily removed by evaporation. Perhaps surprisingly, there are few references to its use in the fractionation of cheese. Bican and Spahni (1991, 1993) used a buffer containing TFA as an extractant for Appenzeller cheese. The procedure involved homogenization at 4 °C in TFA (1%, v/v)/formic acid (5% v/v)/1% (v/v) NaCl/1 mol/L HCl at 4 °C, followed by centrifugation and application to a Sep-Pak C₁₈ cartridge. The use of TFA as an ion-pair reagent in HPLC buffers is widespread (see below).

**Phosphotungstic acid and similar techniques**

It is often desirable to precipitate all peptides from a cheese extract in order to quantify free amino acids in the cheese since nitrogen soluble in such precipitants is a crude measure of free amino acids. Samples for amino acid analysis (see below) must also be rendered free of peptides.

Phosphotungstic acid (tungstophosphoric acid; 12WO₃·H₃PO₄·xH₂O; PTA)-H₂SO₄ is a very discriminating protein precipitant; only free amino acids (apart from lysine and arginine), and peptides less than about 600 Da are soluble in 5% PTA (Jarrett et al., 1982). PTA (1, 2.5, 5, 6 or 6.5%)-soluble N has been used widely as an index of free amino acids in cheese (e.g., Wilkinson et al., 1992; Büttikofer et al., 1993; Picon et al., 1994; Guinee et al., 1995). The most widely used PTA concentration is 5%. The short PTA-soluble peptides in some cheese varieties have been studied (Jarrett et al., 1982; Bican and Spahni, 1991; González de Llano et al., 1991). The development of 5% PTA-soluble N in Cheddar and an Irish artisanal blue cheese is shown in figure 1.

5-Sulphosalicylic acid (2-hydroxy-5-sulphobenzoic acid; SSA), a discriminating protein precipitant, has been used at 3% to prepare extracts of cheese for amino acid analysis (Reiter et al., 1969; Ramos et al., 1987), or as an index of amino acid N (Cliffe and Law, 1991). However, Kuchroo and Fox (1982a) found that 2.5% SSA precipitated only 10% of the WSN. The water-soluble peptides in the fractions obtained with SSA do not appear to have been characterized.

Picric acid (2, 4, 6-trinitrophenol) is also a very discriminating protein precipitant (Fox, 1989). Reville and Fox (1978) found that of the methods examined 0.85% picric acid-soluble extracts of cheese contained the lowest levels of N. It was considered to be the most suitable extractant for amino acids, but small peptides are also soluble in picric acid (Salji and Kroger, 1981). Picric acid interferes with the determination of N by Kjeldahl or spectrophotometric methods (Revilie and Fox, 1978; Fox, 1989).

Free amino acids were extracted from Cheddar by Hickey et al. (1983) using Ba(OH)₂/ZnSO₄. Cheese (10 g) was macerated in 33 mL 0.15 mol/L Ba(OH)₂ and 33 mL 0.14 mol/L ZnSO₄, filtered and residual fat removed by extraction with chloroform/ethanol (1:1). The aqueous phase was filtered and the filtrate freeze-
dried and dissolved in a sodium citrate buffer prior to amino acid analysis. No data were given on extraction levels or whether peptides were soluble. This reagent has not been used extensively to fractionate cheese N nor to prepare samples for amino acid analysis.

Miscellaneous techniques for fractionating peptides

Phosphopeptides (or other peptides with a high negative charge) can be precipitated from a water extract of cheese by addition of BaCl₂ to a final concentration of 10 to 50 mmol/L and adjusting the pH to 6.7–7.0. Ba²⁺ complex with serine phosphate residues of phosphopeptides (and potentially other negatively charged groups) which can then be precipitated by adding ethanol (20% v/v) and recovered by centrifugation (3,000 g, 30 min, 4 °C). The pellet can be redissolved in water and adjusted to pH < 2.0 using H₂SO₄ to precipitate BaSO₄. This method, based on that of Peterson et al (1957), has been used in the authors' laboratory to prepare phosphopeptides from Cheddar. Kuchroo and Fox (1982b) fractionated a WSE of Cheddar with 0.1 mol/L ethylenediamine tetraacetic acid (EDTA); approximately 30% of the WSN was precipitated. However, EDTA does not appear to have been used by other authors for this purpose.

Fernández and Fox (1997) fractionated peptides in the water-soluble and -insoluble fractions of Cheddar cheese using chitosan (a polymer of N-glucosamine). Chitosan is polycationic at acid pH and thus can complex with negatively-charged molecules, leading to their flocculation. Chitosan (0.02%) was unsuitable for the fractionation of water-insoluble peptides but fractionated water-soluble peptides quite effectively at pH 4.0. Chitosan–peptide complexes were dissociated at pH 7.0.

Fractionation techniques based on molecular mass

Techniques for the fractionation of peptides based on their molecular mass, ie, dialysis, ultrafiltration and size-exclusion chromatography have been used as an alternative to techniques based on peptide solubility in various reagents.

Kuchroo and Fox (1982b) found that exhaustive dialysis (96 h, four changes) was a simple and effective method for partitioning water-soluble peptides, and that it was suitable for relatively large samples. About 50% of the WSN in a mature Cheddar was dialysable; the diffusate was completely soluble and the retentate 50% soluble in 70% ethanol. This technique was included by Kuchroo and Fox (1983b) in their fractionation scheme. Sciancalepore and Alviti (1987) dialysed unfractinated, grated cheese (1 g), dispersed in H₂O (1 mL), against H₂O (10 mL) at 30 °C for 30 min. The A₂₈₀ of the dialysate correlated well with age and with 12% TCA-soluble and pH 4.6-soluble N. This method was also used by González de Llano et al (1993) to study proteolysis in blue cheese.

While dialysis is a simple and useful technique for peptide fractionation, it has been superseded by ultrafiltration (UF) which is faster, capable of handling larger samples, uses membranes of known molecular mass cut-off and reduces the problem of recovering peptides from a large volume of diffusate. Many authors have used diafiltration to improve resolution. Membranes with nominal molecular weight cut-offs ranging from 0.5 to 10 kDa (most commonly) have been used. Most authors have used UF to fractionate the larger peptides in cheese, although Visser et al (1983a, b) isolated very low molecular weight peptides from a bitter extract of cheese by UF on 0.5 kDa membranes, and Aston and Creamer (1986) used ultrafiltration with 1 kDa cut-off membranes to fractionate WSN. All peptides in a 10 kDa UF permeate are soluble in 2% TCA, but some peptides in the retentate are insoluble (O'Sullivan and Fox, 1990); the permeate contains no peptides detectable by polyacrylamide gel electrophoresis (PAGE) when stained using Coomassie blue G250, and 40–50% of the WSN is permeable. UF was used by Singh et al (1994, 1995) as the principal fractionation technique for water-soluble peptides.
Rejection of hydrophobic peptides by UF membranes and aggregation of small peptides are potential disadvantages of this method. However, UF allows fractionation of large samples and does not require solvents, both of which facilitate taste panel work (Fox, 1989). Despite some studies, the potential of UF membranes with low molecular weight cut-offs (< 1 kDa) to fractionate the smaller peptides from cheese has not been extensively investigated.

The use of size-exclusion chromatography to fractionate cheese peptides is discussed below.

Comparison of techniques to quantify nitrogen in fractions

The majority of studies described above have quantified nitrogen in various fractions by the macro-Kjeldahl method (Wallace and Fox, 1994). Although this method is highly repeatable, it is a slow and potentially dangerous technique. Alternative methods have also been used for this purpose, most notably by absorbance of ultraviolet light at 280 nm (Vakaleris and Price, 1959) or the Folin-Ciocalteau reagent. The latter method was used by Citti et al (1963) to monitor TCA-soluble peptides. The Lowry method (a combination of the Folin-Ciocalteau and biuret reagents), which is widely used in biochemistry and is simple and rapid, has been used to quantify peptides in various cheese fractions (e.g., Kaminogawa et al, 1986). In the hands of a competent analyst, the Lowry method is suitable for the routine analysis of protein/peptides in cheese fractions (Wallace and Fox, 1994). According to González de Llano et al (1993), the Kjeldahl and Lowry methods gave similar results for protein/nitrogen in water-soluble extracts from blue cheeses. Protein can also be estimated by the Bradford method (which is based on a colour change in Coomassie blue on binding to protein) or by using erythrosin, but preliminary results from this laboratory suggest that these methods are unsuitable for analysis of cheese fractions.

Methods based on the liberation of reactive compounds or groups

Most of the techniques described above for the preparation of various fractions and the quantification of N which is soluble therein are quite time-consuming, and it is desirable to develop more rapid methods to estimate proteolysis. Such techniques are based on the liberation of specific compounds or reactive groups which are easily measured. A wide range of such techniques is available, and includes the estimation of Tyr and Trp in cheese fractions by absorbance at 280 nm or by the Folin–Ciocalteau reagent, protein–dye binding, reaction of liberated amino groups with various reagents or the quantification of ammonia or glutamic acid. Because of their relative simplicity, there is a growing demand for such rapid methods for assessing cheese maturity (Ardö and Meisel, 1991).

Formation of ammonia

Ammonia, formed in cheese on the deamination of free amino acids, is an important product of proteolysis in certain cheese varieties (e.g., Camembert or smear-ripened varieties; Fox et al, 1995a) where it contributes to characteristic flavour and texture (by neutralization of the cheese). Several authors (e.g., Furtado and Chandan, 1985; Alonso et al, 1987; Zarmpoutis et al, 1996) have indirectly monitored ammonia production by measuring the increase in the pH of cheeses. However, it is perhaps surprising that the development of ammonia per se has not been used more widely as an index of proteolysis. Ammonia levels in a non-protein fraction of Ulloa cheese were measured by Ordonez and Burgos (1977) using the reaction of ammonium salts with the Nessler reagent (alkaline solution of K$_2$HgI$_4$) with the formation of a red-brown colloidal precipitate (NH$_2$Hg$_{2}I_3$) which was measured spectrophotometrically; the concentration of ammonia was ~ 4 mg g$^{-1}$ cheese dry matter and increased slightly during ripening. Fernandez-Salgueiro et al (1989) quantified ammonia in a number of blue cheese varieties by isothermal distillation from a sample dispersed in a
borate buffer, pH 10, followed by trapping in boric acid and back titration. An enzymatic assay for ammonia is available (Anonymous, 1989) which quantifies NH₃ by the glutamate dehydrogenase-catalyzed conversion of 2-oxoglutarate to L-glutamic acid, with the concomitant stoichiometric oxidation of NADH to NAD⁺ which is determined spectrophotometrically. However, this technique does not appear to have been used in cheese analysis.

**Formation of soluble tyrosine and tryptophan**

Measurement of the ‘soluble’ tyrosine and tryptophan in cheese is a well-established method for assessing proteolysis. These amino acids can be quantified by the use of Folin–Ciocalteau reagent (acidic solution of Na tungstate and Na molybdate) or by measurement of absorbance at 280 nm. It is necessary to fractionate the cheese (eg, preparation of a water- or TCA-soluble extract) before using either of these techniques. Hull (1947) used the Folin-Ciocalteau reagent to assess proteolysis in milk. This reagent was also used by Singh and Ganguli (1972) to quantify peptides in the TCA-soluble fraction of cheese, but it appears to be less sensitive than 2, 4, 6-trinitrobenzenesulphonic acid, TNBS (Samples et al, 1984). As described above, Vakaleris and Price (1959) measured the A₂₈₀ of a sodium citrate–HCl extract (pH 4.6) of cheese as an index of proteolysis.

**Dye-binding**

At pH values below their isionic point, proteins have a net positive charge and can interact with anionic dyes (eg, amido-black, acid orange 12 or orange G) leading to the formation of an insoluble protein–dye complex. This complex can be removed by centrifugation or filtration and the excess dye in the supernatant or filtrate measured spectrophotometrically. The amount of dye bound by the protein is proportional to the concentration of protein (and thus the excess dye is inversely proportional to the protein concentration). However, low molecular weight proteins and peptides react only slowly, leading to poor separation and turbid filtrates or centrifugal supernatants.

A decrease in the dye-binding capacity of a dispersion of Cheddar cheese with age was demonstrated by Ashworth (1966). Kroger and Weaver (1979) reported that a dye-binding method could be used as an index of proteolysis in cheese, but Kuchroo et al (1983) concluded that the dye-binding method of McGann et al (1972), using amido black, is not suitable for this purpose.

The Bradford method for protein determination (Bradford, 1976) is based on the colour change in Coomassie blue G250 when it reacts with proteins. However, Wallace and Fox (1994) found that this technique was not suitable for quantifying water-soluble peptides.

**Acid/base titration**

The formol titration technique is a simple method for estimating amino groups in milk and may be used to measure the extent and rate of proteolysis. The method involves titration of a sample with NaOH to the phenolphthalein end-point, followed by the addition of formaldehyde which converts free amino groups to less basic secondary and tertiary amines which dissociate at a lower pH; therefore the solution becomes more acidic, and it is necessary to re-titrate to the phenolphthalein end-point (Davis, 1965). This procedure has been used to estimate proteolysis in cheese (eg, Vakaleris et al, 1960; El Soda et al, 1981) but it is now considered obsolete owing to variations in the buffering capacity of cheese (Ardó and Meisel, 1991). Sciancalepore and Longone (1988) compared a dialysis method (Sciancalepore and Alviti, 1987) with formol titration and the method of Inikhow (1951). The latter method involved homogenization of finely ground cheese in water, followed by filtration. Samples of the filtrate were diluted and titrated with NaOH to thymolphthalein and phenolphthalein end points, the index of proteolysis being the difference in the titration values.

The buffering capacity of cheese increases during ripening owing to the formation of
ammonia, amino, imino and carboxyl groups. This principle was used by Ollikainen (1990) to assess proteolysis in a Swiss-type cheese; it was claimed that the method is rapid and convenient and as accurate as colorimetric methods. Changes in the buffering capacity of Emmental around pH 9 have been attributed to proteolysis (Lucey et al, 1993).

Free amino groups-colorimetric and fluorimetric methods

The cleavage of a peptide bond results in the liberation of a new amino group which can react with several chromogenic or fluorogenic reagents. A number of techniques have been developed to assay proteolysis in cheese based on this principle.

2,4,6-Trinitrobenzenesulphonic acid (TNBS) is one such reagent which reacts stoichiometrically with primary amines, producing a chromophore which remains attached to the amino acid, peptide or protein and absorbs maximally at 420 nm (fig 2a). The reaction is performed at an alkaline pH and stopped by lowering the pH. TNBS also reacts slowly with OH⁻, a reaction which is catalyzed by light. Since ammoniacal nitrogen produces only 20% of the absorbance of amino groups when reacted in equimolar concentrations with TNBS (Clegg et al, 1982), this reagent may underestimate proteolysis in cheeses which have undergone significant deamination. Clegg et al (1982) concluded that although TNBS is not as sensitive as ninhydrin for assaying proteolysis in cheese, it is preferable owing to the simple analytical procedure; they proposed a correction factor for ammoniacal nitrogen. A disadvantage of the TNBS method is that the dry powder is explosive, and prolonged storage leads to high blank values (Ardö and Meisel, 1991).

![Fig 2. Reaction of (a) 2, 4, 6-trinitrobenzenesulphonfonic acid, (b) ninhydrin, (c) fluorescamine and (d) o-phthalaldehyde with the α-amino group of an amino acid.

Réaction de (a) l’acide 2,4,6-trinitrobenzénesulphonique, (b) de la ninhydrine, (c) de la fluorescamine et (d) de l’o-phthaldialdéhyde avec le groupe α-aminé d’un acide aminé.
Habeeb (1966) and Adler-Nissen (1979) measured the absorbance of the TNBS–amino group complex at 340 nm, whereas in the procedure of Fields (1971) the absorbance of a sulphite–TNBS–amino group complex was measured at 420 nm, thus avoiding the maximum of absorbance of TNBS itself at 335–340 nm. Barlow et al (1986) reported that the latter is preferable to the method of Habeeb (1966) in which an unstable coloured complex is formed and the procedure is liable to operator error. Alder-Nissen (1979) developed a procedure to assess the degree of hydrolysis of food proteins using TNBS; a linear relationship was found between the concentration of ε-amino groups and $A_{420}$ but the relationship varied between proteins owing to variations in the concentration of lysine.

The method of Hull (1947) was compared with the TNBS method by Samples et al (1984), who found that the latter was a better index of proteolysis in cheese. Jarrett et al (1982) used the TNBS assay to quantify free amino acids in 5% PTA-soluble fractions of cheese. The TNBS method was found to be reproducible for monitoring proteolysis in cheese (Kuchroo et al, 1983) and while unfraccionated cheese could be assayed, it is more sensitive if applied to pH 4.6- or 12% TCA-soluble extracts owing to a lower background colour caused by reaction of TNBS with ε-amino groups. A strong correlation was found by Madkor et al (1987) between WSN and the $A_{420}$ of the TNBS complex for water-soluble extracts of Stilton cheese.

Barlow et al (1986) claimed that analysis of a water-soluble extract prepared according to Kuchroo and Fox (1982a) by the TNBS method of Fields (1971) would provide a simple routine method for quantifying soluble N in cheese. Whole cheese, dispersed in 0.1 mol/L Na$_2$B$_4$O$_7$, 0.1 mol/L NaOH, pH 9.5, was analyzed by Polychroniadou (1988), who found a good correlation between the results of the TNBS method and WSN determined by the Kjeldahl procedure; fractionation of the cheese prior to analysis was considered to be unnecessary. The TNBS method was also used by Lemieux et al (1990), who compared a flow-injection technique for the TNBS and o-phthalaldehyde (see below) assays with classical methods. Bouton and Grappin (1994) found a good relationship between PTA-soluble N values (by Kjeldahl) and the TNBS method.

Ninhydrin is also widely used to monitor the liberation of free amino groups in cheese during ripening. The principle of the ninhydrin reaction is the spectrophotometric estimation of a chromophore formed when ninhydrin reacts with free amino groups (fig 2b). The purple chromophore, named Ruhemann’s purple after its discoverer, does not remain attached to the protein or peptide and therefore is not precipitated during procedures necessary to clarify the sample prior to spectrophotometric analysis at 570 nm (Ardö and Meisel, 1991), which is a major advantage of the technique (Pearce et al, 1988). The $\lambda_{\text{max}}$ of the chromophore is shifted to a shorter wavelength (~507 nm) when the water content of the reaction mixture is reduced (Doi et al, 1981). Ninhydrin reacts with ammonia almost as readily as with amino groups and therefore levels of proteolysis found by ninhydrin assays are consistently higher than those found by the TNBS procedure, the discrepancy being due to ammonia (Clegg et al, 1982). Ninhydrin is more sensitive than TNBS, but the latter was preferred by Clegg et al (1982) because the analytical procedure is simpler. Ninhydrin is widely used to quantify amino groups in chromatographic eluates, particularly in amino acid analysis by ion-exchange chromatography followed by post-column derivatization (see below). Cliffe et al (1989) used ninhydrin to monitor fractions obtained by reversed-phase chromatography.

Pearce et al (1988) developed a Li-ninhydrin assay for proteolysis in cheese based on that of Friedman et al (1984): an aliquot of cheese dispersed in a citrate buffer was mixed with aqueous solutions of dimethyl sulphoxide, ninhydrin and lithium acetate at pH 5.2, heated, diluted and $A_{570}$ determined. Total free amino acids determined by this method correlated well with conventional amino acid analyses.
Doi et al (1981) described a number of ninhydrin-based assays for peptidase activity; two were modifications of the methods of Moore and Stein (1948, 1954b) and two were based on the Cd-ninhydrin assay of Tsarichenko (1966). The Cd-ninhydrin reagent was found to be more selective for the amino group of free amino acids than for the amino groups of peptides or proteins and was the most sensitive of several ninhydrin reagents, including Sn-ninhydrin. Folkertsma and Fox (1992) applied the Cd-ninhydrin assay of Doi et al (1981) to the assessment of proteolysis in cheese; the reagent was found to be about five times as sensitive as TNBS for the measurement of amino acid nitrogen and could be performed on citrate-, water- or PTA-soluble fractions but not on TCA-soluble fractions, as the latter appeared to interfere with colour development. The release of total free amino acids in cheese, as determined by the Cd-ninhydrin technique, is normally expressed as mg Leu g⁻¹ cheese (by reference to a leucine standard curve) and increases during ripening (fig 3). The Cd-ninhydrin procedure of Folkertsma and Fox (1992) is a simple, useful method for estimating total free amino acids in WSEs of cheese and is used routinely in this laboratory; for best results, a set of samples should be analyzed together.

Fluorimetric reagents are also available for quantifying free amino groups. Such techniques are more sensitive than the colorimetric methods described above but have not been used as widely. The HPLC separation of fluorescent derivatives of amino acids and amines (pre-column derivatization) is common (see below).

Fluorescamine (4-phenylspiro [furan-2 (3H), 1'-phthalan]-3,3'-dione) is used to quantify amino acids and peptides in the picomole range. Fluorescamine, introduced by Weigele et al (1972), reacts with primary amino groups to produce a fluorophore which is assayed at 390 nm excitation and 475 nm emission (fig 2c). It reacts at room temperature with water or primary amines, the latter reaction being far faster (Udenfried et al, 1972). Fluorescamine has been used to monitor the hydrolysis of κ-casein by chymosin (Pearce, 1979; Beeby, 1980) and to quantify acid-soluble proteins, peptides and amino acids in cheese extracts (Creamer et al, 1985). These authors claimed that this reagent gave more consistent results than TNBS.

O-Phthaldialdehyde (OPA) reacts with 2-mercaptoethanol and primary amines to form a fluorescent complex (1-alkylthio-2-alkylisoindole; fig 2d), which also absorbs strongly at 340 nm. Church et al (1983), who used OPA to quantify proteolysis in milk protein systems, reported that the method is more accurate than the measurement of A₂₈₀ and is more convenient than the ninhydrin, TNBS or fluorescamine procedures. Lemieux et al (1990) considered the OPA method to be fast and simple for estimating free amino acids in cheese and if the reaction time was strictly controlled, was less variable than the TNBS method which was considered to be tedious, required heating and the reagent was
light-sensitive. 9-Fluorenymethoxy-carbonyl (FMOC) may also have potential for use in cheese analysis as a fluorescent labelling reagent for amino groups.

**Enzymatic techniques**

l-Glutamic acid is an important free amino acid in many cheese varieties. An enzyme assay for Glu using flow injection analysis and glutamate dehydrogenase immobilized on activated glass was developed by Puchades et al (1989b); enzyme activity was determined based on the reduction of NAD+. These authors measured free Glu in Cheddar cheese extracts, prepared by the method of Harwalkar and Elliott (1971), which were freeze-dried, resuspended in a phosphate buffer, sonicated, filtered and passed through a Sep-Pak C18 cartridge prior to analysis. This technique was reported to be sensitive, rapid and accurate.

McSweeney et al (1993a) measured free Glu in water extracts of Cheddar using a commercially available assay kit containing glutamate dehydrogenase (Boehringer–Mannheim, Mannheim, Germany). Unlike the procedure of Puchades et al (1989b), in which NADH was measured directly, this procedure uses pig-heart diaphorase to catalyze the reaction of iodonitrotetrazolium chloride with NADH, forming formazan which is assayed spectrophotometrically at 492 nm. The free Glu content of the cheeses increased with age and varied between cheeses with different non-starter populations.

Puchades et al (1990) developed an enzymatic assay with flow injection analysis for total free amino acids in water-soluble extracts of Cheddar cheese, prepared by the method of Harwalkar and Elliott (1971) and filtered through Sep-Pak C18 cartridges. l-Amino acid oxidase (immobilized on glass beads) acted on free amino acids, producing stoichiometric amounts of H2O2 which then reacted with luminol, a chemiluminescent agent, in the presence of K3Fe(CN)6, emitting light, which was quantified. This technique was reported to be rapid and reproducible and specific for free amino acids, since peptides, which react to a greater or lesser extent in most other direct-assay procedures for free amino acids, are not degraded by l-amino acid oxidase. However, this enzyme is not equally active on all amino acids; it exhibits high activity on Leu, Phe and Trp but His, Ile and Cys are poor substrates (Puchades et al, 1990).

**TECHNIQUES WHICH RESOLVE PEPTIDES**

Although the above non-specific techniques can provide valuable information about the extent of proteolysis and the activity of proteolytic agents, they provide little information on which peptides accumulate or are degraded during ripening. For this reason, techniques which resolve individual peptides have received much attention in recent years.

**Electrophoresis**

Since only proteins and large peptides can be visualized by staining, electrophoresis in gel media is limited to monitoring hydrolysis of the parent caseins and the formation and subsequent hydrolysis of the primary proteolytic products of caseins and thus it has been used widely to monitor the primary proteolysis of caseins in cheese. More recently, it has been shown that capillary electrophoresis (CE) has considerable potential for resolving casein-derived peptides with a wide range of molecular masses.

Although classical free-boundary techniques have been used (eg, Lindqvist and Storgards, 1959), the overwhelming majority of electrophoretic analyses of cheese and other dairy products involve zonal electrophoretic techniques in which the electrophoresis buffer is stabilized in various media and the proteins and peptides migrate as discrete bands under the influence of an electric field. Zonal electrophoresis on paper (eg, Lindqvist et al, 1953; Kuchroo and Fox, 1982b), porous cellulose
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Acetate or in agar or agarose gels are now rarely used.

Electrophoresis in starch gels, which was first applied to cheese by Melachouris and Tuckey (1966), has been used by some workers to separate caseins or their degradation products in cheese or other products (e.g., Vreeman and van Reil, 1990; van den Berg and de Koning, 1990; de Koning et al., 1992) and is quite effective. Although starch gel electrophoresis gives far better results than earlier zonal techniques and Mott (1971) claimed that it gave superior resolution of lower-mobility and cationic peptides than polyacrylamide gels, starch gels are brittle and opaque after staining (Creamer, 1991) and have been largely replaced by polyacrylamide gels.

Electrophoresis in polyacrylamide gels, first applied to cheese by Ledford et al (1966), has become the standard technique. The literature was reviewed by Shalabi and Fox (1987), Fox (1989), Creamer (1991), Strange et al (1992), McSweeney and Fox (1993) and Fox et al (1995b). Continuous buffer systems have been used to separate caseins and are reported to have certain advantages over discontinuous buffers (see Creamer, 1991). However, nearly all one-dimensional PAGE techniques used in recent studies on cheese have employed discontinuous buffer systems containing urea or sodium dodecylsulphate (SDS) as a dissociating agent. Non-denaturing buffers are not suitable for caseins, although they are used for the analysis of whey proteins.

Sample preparation usually involves dissolving cheese in a buffer (which usually contains a reducing agent, usually 2-mercaptoethanol) prior to electrophoresis; the solution may be centrifugally defatted, and sucrose or glycerol added to increase the density of the sample to facilitate loading into gel slots (Creamer, 1991).

Direct or indirect staining (followed by destaining using acetic acid; e.g., Laemmli, 1970) using Coomassie blue is usually used. Shalabi and Fox (1987), who compared a number of staining techniques for PAGE gels, recommended the direct staining procedure of Blakesley and Boezi (1977) with Coomassie blue G250 in the presence of TCA, although amido black was preferred for larger peptides. However, since only relatively large peptides stain under these conditions, the procedure is limited to the detection of the primary degradation products in cheese. O’Sullivan and Fox (1990) found that the peptides in a 10 kDa UF permeate did not stain with Coomassie blue on urea–PAGE, but the retentate of the WSE contained several detectable peptides, as did 2% TCA-soluble and insoluble fractions of the retentate. Low molecular mass peptides can be visualized using a silver staining technique involving extensive fixing with glutaraldehyde, although such stains have not been used widely to study cheese peptides.

Although urea-containing buffers at acid pH have been used (e.g., Creamer, 1991), the majority of authors have used urea-containing buffers at alkaline pH. Shalabi and Fox (1987) compared several electrophoretic procedures for the analysis of cheese and strongly recommended the stacking gel system of Andrews (1983) in Tris buffers (pH 8.9) containing 4.5 mol/L urea. Electrophoresis in alkaline urea-containing gels with direct staining by Coomassie blue G250 is widely used in this and other laboratories to monitor proteolysis in various cheese varieties (fig 4).

Electrophoresis in SDS-containing buffers is a standard technique for protein analysis in general biochemistry; electrophoretic mobility in the presence of SDS is inversely proportional to the logarithm of the molecular weight of the peptide. However, it is less widely used for cheese since the caseins have similar molecular weights (20 000 to 25 000) and are therefore not as well resolved by SDS–PAGE as by alkaline urea–PAGE. Shalabi and Fox (1987) concluded that SDS–PAGE was inferior to urea–PAGE for cheese analysis, and did not recommend its use. However, Creamer (1991) and Strange et al (1992) considered that SDS–PAGE provides valuable information on cheese ripening, and has been widely used (e.g., Marshall et al,
Fig 4. Alkaline urea–polyacrylamide gel electrophoretograms (Andrews, 1983) of casein (lane 1), Cheddar cheeses made from high heat-treated milk (90 °C x 10 min) at one day and 1, 2, 4 and 6 months of age (lanes 2–6) and water-soluble extracts therefrom (lanes 7–11). Direct staining using Coomassie brilliant blue G250 was by the method of Blakesley and Boezi (1977); (Folkertsma and Fox, unpubl results).

1988; Basch et al, 1989; Bhowmik et al, 1990; Tunick et al, 1993). Shalabi and Fox (1987) considered that the 20% acrylamide gel system of Tegtmeyer et al (1975) was more satisfactory than the more widely used method of Laemmli (1970), in which 12% acrylamide gels are used. However, Creamer (1991) reported that 20% acrylamide gels give poor resolution of the caseins (all caseins migrate as two bands), but are very satisfactory for peptides with molecular weights in the region of 10 000.

Two-dimensional electrophoresis has been used by some authors; eg, Trieu-Cuot and Gripon (1982) used SDS–PAGE in one dimension and isoelectric focusing in the other to study proteolysis in Camembert. Although 2-D electrophoresis may give good resolution, it is time-consuming and there are difficulties with reproducibility and in obtaining quantitative data (Creamer, 1991). Bican and Spahni (1991) separated peptides in extracts from Appenzeller cheese by thin-layer chromatography in one dimension, followed by electrophoresis at 90°.

Isoelectric focusing (IEF) is a powerful electrophoretic technique for resolving proteins and peptides based on separation according to differences in their isoelectric points. It has been particularly valuable in studies on genetic polymorphism in milk proteins (see Creamer, 1991; and Strange et al, 1992, for references). Some of the applications of IEF in dairy chemistry include the detection of adulteration of ovine milk cheeses with bovine or caprine milks or the study non-bovine caseins (Addeo et al, 1983, 1990a,b; Conti et al, 1988; Moio et al, 1989, 1992; Amigo et al, 1992). Trieu-Cuot and Gripon (1982) used IEF to study the pH 4.6-insoluble fraction of Camembert. Kim and Jimenez-Flores (1994) obtained excellent resolution of milk proteins by preparative IEF followed by urea- or SDS–PAGE. This technique could easily be adapted for the study of proteolysis in cheese. Electrophoresis of cheese peptides has been facilitated by the recent introduction of pre-poured gels and by rapid semi-automated systems such as the Phast system™ (Pharmacia, Uppsala,
Methods used to assess proteolysis in cheese

Sweden; Strange et al, 1992; Van Hekken and Thompson, 1992).

After staining, electrophoretograms are usually recorded photographically, although densitometry or excision and elution of the stained bands, followed by spectrophotometric quantitation have also been used. Difficulty in obtaining quantitative data is a serious limitation of electrophoresis. Creamer (1991) recommended that several control samples should be included in each gel and that comparisons should be made only between samples on the same gel. He emphasized that band dimensions are critical for densitometry, and that dye uptake is a function of the protein as well as staining and destaining protocol. However, de Jong (1975) reported that electrophoresis in alkaline urea gels (pH 8.9), stained with amido black 10B and scanned by densitometry, gave good quantitative results.

Identification of peptides produced during cheese ripening has posed difficulties. Peptides can be isolated from PAGE gels by excision of the bands or by electroblotting. The latter technique is preferable because of higher recoveries of protein and because the size of PAGE gels can change on staining which makes accurate excision of unstained regions difficult. Electroblotted peptides can be identified by N-terminal amino acid sequencing (see below), but they are more difficult to analyze by mass spectrometry (MS) because they cannot be stained prior to MS and must be eluted from the blotting matrix. However, the location of all the caseins and some major degradation products (eg, γ-caseins and αs1-CN 124-199) on most PAGE systems is known (Creamer, 1991; Strange et al, 1992). The majority of the peptides in a urea–PAGE electrophoretogram of Cheddar cheese were partially identified (fig 5) by McSweeney et al (1994a). Addeo et al (1995) used immunoblotting (rabbit polyclonal antibodies raised against the caseins and peroxidase-labelled immunoglobulins as secondary antibodies) to detect bands in electrophoretograms of a number of cheese varieties. This technique permitted the identification of the casein from which a number of peptides originated.

A technique described as high performance electrophoresis chromatography was used by Girardet et al (1994) to resolve component 3 glycoproteins from bovine milk. Proteins were separated in a gel-filled column under the influence of an electric field and separated proteins exiting the bottom of the gel tube were eluted by a continuous flow of buffer and passed through a UV spectrophotometric detector. To
date, this technique does not appear to have been used to study proteolysis but may have application in the fractionation of large peptides in cheese.

Capillary electrophoresis (CE) resolves peptides in a buffer-filled capillary under the influence of an electric field (fig 6) and as such is a free-boundary technique although, as described below, zonal CE is also possible. CE separates on the basis of the net charge on the peptides, their mass and Stokes' radius (Young et al, 1992) and sometimes on some other property of the peptide (see below). The use of CE in food analysis has been reviewed (Zeece, 1992; Lindeberg, 1996a,b).

Capillary electrophoresis is reported to have great potential for the resolution of complex mixtures of peptides. It has a number of advantages over traditional electrophoretic techniques, including the choice of running buffer and the use of automated, high-performance instrumentation. The composition of running buffer can be changed easily and separation times are relatively short, although only one sample at a time can be analyzed. Unlike conventional electrophoresis, in which proteins and peptides in a gel are visualized by staining, CE uses continuous monitoring by UV absorbance, and potentially by the range of other techniques used for detection in HPLC. UV absorbance allows the detection of small peptides which cannot be visualized by staining and also means that CE is quantitative, unlike other electrophoretic techniques. It can be used in a number of modes, including free solution capillary electrophoresis, FSCE (although problems have been encountered with interations between peptides and the capillary wall), micellar electrokinetic chromatography (where electrophoresis is performed in the presence of micelles of SDS and peptides partition between the micelles and buffer), capillary isoelectric focusing (where the analyte is 'sandwiched' between a leading and terminating electrolyte), capillary gel electrophoresis (which uses a molecular sieving effect), capillary electrochromatography (which uses a capillary packed or coated with a stationary phase) or isoelectric focussing (in which analytes may be eluted from the capillary by a pump after they have reached equilibrium at their pI) (Zeece, 1992; Lindeberg, 1996a). There are also CE techniques for resolving racemic mixtures by diasteriometric interaction of analytes with a chiral mobile or stationary phase (Gordon et al, 1988).

The capital cost of CE instrumentation at present is higher than that of equivalent high performance liquid chromatographs (HPLC), although costs are likely to fall. Sample size in CE is extremely small and therefore the technique is not suitable for preparative-scale work. CE may be limited to producing peptide profiles, although CE coupled with in-line mass

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Fig 6. Schematic diagram of a capillary electrophoresis unit.
Représentation schématique d'une unité d'électrophorèse capillaire.
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1. Methods used to assess proteolysis in cheese

**Fig 7.** Free solution capillary electrophoretogram of the water-soluble extract (WSE) from a 6-month-old Cheddar cheese made using Lactococcus lactis subsp cremoris SK11 as starter. WSE was dissolved in 1 mL 8% (v/v) CH$_3$CN in water containing 0.1% (v/v) trifluoroacetic acid. Electrophoresis was performed using a Beckman Model P/ACE 2050 capillary electrophoresis unit (Beckman, San Ramon, CA, USA) equipped with a 57 cm fused silica capillary (50 cm to detector; internal diameter, 75 μm). Electrophoretic buffer was 0.1 mol/L Na phosphate buffer, pH 2.5. Electrophoresis was performed at 20 kV and spectrophotometric detection was at 214 nm (Singh, unpubl results).

Capillaire en solution libre d’un extrait soluble dans l’eau (WSE) de fromage de cheddar de 6 mois fabriqué avec Lactococcus lactis subsp cremoris SK11 comme levain. WSE était dissous dans 1 mL de CH$_3$CN à 8% (v/v) dans l’eau, contenant 0,1% (v/v) d’acide trifluoracétique. L’électrophorèse était réalisée avec une unité d’électrophorèse capillaire Modèle Beckman P/ACE 2050 (Beckman, San Ramon, CA, États-Unis) équipé d’un capillaire en silice fondue de 57 cm (50 cm jusqu’au détecteur; diamètre intérieur, 75 μm). Le tampon pour l’électrophorèse était un tampon phosphate de Na 0,1 mol/L, à pH 2.5. L’électrophorèse était réalisée à 20 kV et la détection spectrophotométrique à 214 nm (Singh, non publié).

Spectrometry gives much information on the identity of peptides (Zeece, 1992). It is unlikely that CE will replace chromatographic and other electrophoretic methods, and it is now viewed as complementing other separation techniques (Lindeberg, 1996a).

CE has received considerable attention in general food analysis (Zeece, 1992; Lindeberg, 1996a,b). To date, FSCE has been the form of CE most widely used in dairy chemistry. Applications have included ion analysis in milk, milk powders and cheese (Morawski et al, 1993; Schmitt et al, 1993; Prestwell et al, 1995), measurement of hippuric and orotic acids in whey (Tienstra et al, 1992), fractionation of whey proteins (Cifuentes et al, 1993; Otte et al, 1994), measurement of sorbate and benzoate in cheese slices and dips (Pant and Trenerry, 1995), detection of chloramphenicol in milk (Blais et al, 1994) and quantification of seleno amino acids in human milk (Michalke, 1995).

The potential of this technique for isolating peptides from cheese was demonstrated by Zeece (1992), who separated peptides in a tryptic digest of native and dephosphorylated α$_s$- and β-caseins. The authors are aware of a number of laboratories investigating the potential of CE to study proteolysis in cheese, although no reports of capillary electrophoretograms of cheese peptides appear to have been published to date. A free-solution capillary electrophoretogram of a water extract from a mature Cheddar cheese is shown in figure 7 (Singh, unpubl results).
Chromatography of cheese peptides

Paper chromatography (PC) was used by many early workers to quantify free amino acids in cheese (eg, Kosikowsky, 1951a; Storgards and Lindqvist, 1953; Clemens, 1954) or to study cheese peptides (eg, O’Keeffe et al, 1978; Kuchroo and Fox, 1982b, 1983a). Paper chromatography is cheap and straightforward, but suitable only for assessing the complexity of systems containing only small peptides (Ardö and Gripon, 1991) and has been largely superseded by other techniques. Thin layer chromatography (TLC) on silica gel, using different solvents, eg, n-propanol/water (70:30, v/v) or n-propanol/acetic acid/water (5:1:3), has been used to characterize peptides in cheese fractions (Kuchroo and Fox, 1982b, 1983a, b; Edwards and Kosikowski, 1983; Visser et al, 1983b). Ninhydrin is usually used to develop the plates, although UV fluorescence of the spots was used by Edwards and Kosikowski (1983). Preparative TLC has been used to isolate bitter peptides from Cheddar (Edwards and Kosikowski, 1983). Bican and Spahni (1991) combined TLC on silica gel plates (chloroform/ammonia/ethanol as solvent) with electrophoresis in the perpendicular dimension to resolve peptides in Appenzell cheese: plates were developed by spraying with fluorescamine and viewed under UV light. TLC is a simple, straightforward method for separating peptides and is normally superior to paper chromatography, although Kuchroo and Fox (1983a) obtained better resolution of peptides by PC than by TLC. Both paper and TLC chromatographic techniques are now rarely used.

Column chromatography on silica gels (eg, Visser et al, 1975), metal chelating media (eg, Cu-Sephadex; Ney, 1985; Mojarro-Guerra et al, 1991) or on hydrophobic interaction media (eg, Kuchroo and Fox, 1983a; Visser et al, 1983b) have been used to fractionate peptides from cheese or casein hydrolysates. Mulvihill and Fox (1979) used phosphocellulose to fractionate peptides produced from αs-1-casein by chymosin, but this medium does not appear to have been used for cheese. A few authors have used the molecular sieving properties of cation-exchanger Dowex 50 resins to fractionate cheese peptides (eg, Tokita and Nakanishi, 1962; Huber and Klostermeyer, 1974; Biede and Hammond, 1979).

Mooney and Fox (unpubl results) fractionated peptides in the water-insoluble fraction of Cheddar cheese by high-performance hydrophobic interaction chromatography on a Phenyl-Sepharose column in a 0.48 mol/L Na phosphate buffer, pH 6.3, containing 2.5 mol/L urea; elution was by means of a gradient from 0.48 to 0.037 mol/L Na phosphate (fig 8).

Fig 8. Hydrophobic interaction chromatogram of the water-insoluble fraction of a 20-week-old Cheddar cheese on Phenyl-Sepharose High Performance™ (Pharmacia, Uppsala, Sweden). Elution was by a gradient of Na phosphate buffer from 0.48 (buffer A) to 0.037 mol/L (buffer B), pH 6.3 to 6.6, containing 2.5 mol/L urea and 0.1% dithiothreitol. Flow rate was 1 mL/min and UV spectrophotometric detection was at 280 nm (Mooney and Fox, unpublished).
The most popular forms of chromatography for analysis of cheese peptides have been ion-exchange and size-exclusion techniques and reverse-phase high performance liquid chromatography (RP-HPLC).

Molecular weights may be estimated by size-exclusion chromatography on a calibrated column. The majority of workers have used Sephadex™ gels (Pharmacia, Uppsala, Sweden) of various pore sizes. Cheese preparations chromatographed have included cheese or high molecular weight peptides (Lindqvist, 1962; Tokita and Hosono, 1968; Creamer and Richardson, 1974; Foster and Green, 1974; Gripon et al, 1975), water-soluble or similar extracts (Nath and Ledford, 1973; Desmazeaud et al, 1976; O’Keeffe et al, 1976; Santoro et al, 1987), dialyzable or UF permeable fractions from water extracts (Kuchroo and Fox, 1983b; Singh et al, 1994, 1995) or PTA-soluble peptides (González de Llano et al, 1987, 1991).

Numerous eluents have been used, although water and dilute buffers are most common and chromatograms are usually quantified by spectrophotometry at UV wavelengths. Generally, 280 nm is used but for fractions containing smaller peptides, absorbance of the carbonyl group in the peptide bond at a lower wavelength, eg, 220 nm (Foster and Green, 1974; Green and Foster, 1974) is preferable, since small peptides may not contain aromatic residues. Measurement of the amino group by reaction with ninhydrin (eg, Gripon et al, 1977) or some other reagent is an alternative.

Low-pressure, preparative HPLC techniques (eg, FPLC™, Pharmacia, Uppsala, Sweden) on size-exclusion media have simplified this form of chromatography. Wilkinson et al (1992) resolved press juice from Cheddar by high-performance size-exclusion chromatography (HPSEC) on a Superose-12 column; changes in the peptide profile during ripening were evident. HPSEC on a Superose-12 column is also valuable for characterizing peptides in various fractions from Cheddar (Breen et al, 1995) and is suitable for preparing peptide fractions. Haasnoot et al (1989) fractionated peptides in Gouda cheese solubilized in urea by HPSEC on TSK 3000SW and 2000SW columns in series. This technique gave some information about primary and secondary proteolysis during ripening, but these authors considered that the size-exclusion columns were not suitable for the separation of the components present in water- or TCA-soluble fractions. Vijayalakshmi et al (1986) reported an interesting method for resolving peptides of molecular mass between 250 and 6000 Da on a TSK-SW 2000 column using 50 mmol/L phosphate buffer containing 0.1% trifluoroacetic acid and 35% methanol as mobile phase. HPSEC has also been used to characterize caseins, whey proteins and peptides derived therefrom (van Hooydonk and Olieman, 1982; Bican and Blanc, 1982; Vreeman et al, 1986).

Ion-exchange chromatography is used widely to fractionate milk proteins but has only had limited use in cheese analysis (Fox, 1989). Anion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose was used to isolate α$_{s1}$-CN f24–199 (α$_{s1}$-I casein) from Cheddar (Creamer and Richardson, 1974) and to fractionate water-insoluble peptides (Breen, 1992; McSweeney et al, 1994a; Mooney and Fox, unpubl results) and the 2% TCA-soluble and insoluble fractions of a 10 kDa UF retentate of a water-soluble extract of Cheddar (O’Sullivan and Fox, 1990). High-performance ion-exchange columns (eg, Mono-S™ or Mono-Q™, Pharmacia) give better resolution of bovine milk proteins than classical chromatography on DEAE-cellulose (Barrefors et al, 1985; St Martin and Paquin, 1990). Anion exchange chromatography of a urea solution of Gouda cheese on a Mono-Q™ column was used by Haasnoot et al (1989) to study proteolysis. It was concluded that the ratio γ- to β-casein peak areas is a good indicator of the maturity of Gouda cheese ripened for up to about 10 months, whereas the ratio of the peak areas representing α$_{s1}$-casein and α$_{s1}$-CN f 24–199 was a suitable index for very young cheeses (< four weeks). Urea-containing buffers have usually been used to dissolve cheese prior to chromatography and chromatograms are monitored by A$_{280}$, although
ninhydrin has been used by some workers (Fox et al, 1995b).

In our experience, anion-exchange chromatography on DEAE-cellulose or equivalent high-performance medium (eg, Mono-Q™) in urea-containing buffers is very suitable for the fractionation of large casein-derived peptides. This form of chromatography is used routinely in this laboratory to fractionate the water-insoluble peptides from cheese (fig 9; Mooney and Fox, unpubl results). Fractions from the anion-exchange chromatogram can be collected and analyzed by urea-PAGE.

The introduction of high performance ion-exchange chromatography (HPIEC) and HPSEC has greatly reduced the workload involved in these forms of column chromatography, while increasing speed and reproducibility. Thus, it seems likely that these techniques will achieve more widespread application in the future for monitoring proteolysis in cheese.

Reverse phase-HPLC has been used extensively to characterize peptides in casein hydrolyzates (eg, Le Bars and Gripon, 1989; McSweeney et al, 1993b) and the technique is also very valuable for resolving shorter peptides in cheese fractions. Water-soluble extracts have usually been used for RP–HPLC analysis (eg, Cliffe and Law, 1991; González de Llano et al, 1991; McSweeney et al, 1993a) but other fractions have also been studied, including pH 4.6-soluble and -insoluble extracts (eg, Kamino-gawa et al, 1986; Christensen et al, 1989), 10 kDa UF permeate (Singh et al, 1994), 70% ethanol soluble and insoluble fractions (Zarm-poutis et al, 1996) and fractions from size-exclusion chromatography (Cliffe et al, 1993).

Gradient elution with water/acetonitrile (eg, Amantea et al, 1986) or water/methanol (eg, Cliffe et al, 1993) is usually used; but isocratic conditions using a phosphate buffer as eluent have also been used by some workers (eg, Pham and Nakai, 1984). TFA is the most widely used ion-pair reagent. Detection is generally by UV spectrophotometry, usually at a wavelength in the range of 200–230 nm (which measures the carbonyl in the peptide bond), although 280 nm has been used in cases where larger peptides, which are more likely to contain aromatic residues, are expected (eg, Christensen et al, 1989). Fluorescence detection has also found limited use (eg, González de Llano et al, 1991). Reverse-phase chromatography on a semipreparative C₂/C₁₈-coated silica was used by Cliffe et al (1989) to fractionate cheese peptides.

Although RP–HPLC essentially remains a research tool, Smith and Nakai (1990) discussed its potential for the routine assessment of cheese quality. Difficulties remain with the interpretation of RP–HPLC data and the development of a solvent system which will permit tasting of the fractions (Ardö and Gripon, 1991). As var-
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Various columns, elution conditions and detection wavelengths have been used, comparison of chromatograms from different studies is difficult. Although RP–HPLC is widely used to purify individual peptides from cheese (eg, Singh et al, 1994, 1995, 1996), the authors caution against the identification of most peptides in chromatograms based solely on their retention time. However, two major peptides, \( \alpha_\text{s1}-\text{CN f1–9} \) and \( \alpha_\text{s1}-\text{CN f1–13} \) (produced by the action of Lactococcus cell envelope-associated proteinase on \( \alpha_\text{s1}-\text{CN f1–23} \), which is produced rapidly by chymosin) are characteristic of the chromatograms of Cheddar cheese (fig 10a).

RP–HPLC is a very valuable technique for the resolution of water-soluble peptides in cheese. It has been our experience that water-soluble extracts resolve well on C8 wide-pore (300 Å) columns with a shallow acetonitrile/water gradient with TFA as the ion-pair reagent (eg, McSweeney et al, 1994b). However, due to the complexity of the WSE from many cheeses, it is often desirable to fractionate the WSE. Chromatography of 10 kDa UF permeates is adequate but time-consuming. For this reason, we now fractionate with 70% ethanol prior to RP–HPLC. Typical chromatograms of the ethanol-soluble and insoluble fractions from Cheddar are shown in figure 10a, b (Tobin et al, unpubl results).

Elution of peptides from a C18-reverse phase cartridge using a stepwise acetonitrile gradient was used by Singh et al (1994, 1995) to resolve peptides in a fraction obtained by size-exclusion chromatography of a 10 kDa UF permeate of a WSE of Cheddar cheese, and by Bican and Spahni (1993) to sub-fractionate extracts of Swiss-type cheeses. This technique gave considerably simpler sub-fractions from which individual peptides were isolated.

Two-dimensional HPLC (2D-HPLC) was used by Lagerwerf et al (1995) to fractionate the peptides in the WSE of Cheddar cheese; the eluate from the first dimension (ion-exchange) was directed to a reverse-phase column (C18). The resolution obtained was impressive, although increased equipment requirements and long analysis times (~ 10 h) are likely to militate against the widespread use of 2D-HPLC.

QUANTIFICATION OF FREE AMINO ACIDS AND THEIR DEGRADATION PRODUCTS

Ultimately, the hydrolysis of caseins by the combined action of proteinases and peptidases in cheese leads to the liberation of free amino acids which are considered to be important flavour compounds in many cheeses; their concentration varies widely with variety. In addition, volatile compounds formed from amino acids by decarboxylation, deamination, transamination and other transformations can make substantial contributions to cheese flavour (see Fox et al, 1995a).

It is generally necessary to deproteinize samples prior to amino acid analysis to reduce interference from peptides; reagents used have included sulphosalicylic acid (Reiter et al, 1969; Omar, 1984), ethanol (Visser, 1977; Polychroniadou and Vlachos, 1979), picric acid (Weaver et al, 1978; Shindo et al, 1980), TCA (Zarmpoutis et al, 1996) or Ba(OH)₂/ZnSO₄ (Hickey et al, 1983). An internal standard (eg, norleucine) is normally added to facilitate quantitation of amino acids.

Early attempts to quantify free amino acids in cheese used paper chromatography developed using ninhydrin (eg, Kosikowsky, 1951a, b) but such techniques were only semi-quantitative. Likewise, classical ion-exchange column chromatography (eg, Mabbutt, 1955, who used Dowex-50 resin) is now obsolete and has been superseded by automated amino acid analyzers, which are less laborious.

Dedicated amino acid analyzers based on ion-exchange chromatography are used widely and have greatly facilitated the analysis of free amino acids in cheese, which is now relatively simple, accurate, quantitative and requires little sample preparation. Amino acids are usually detected by post-column derivatization, often using ninhydrin. Recent studies in which this
Fig 10. Reverse-phase (C8) high-performance liquid chromatograms of the 70% ethanol-soluble (a) and insoluble (b) fractions of the water-soluble fraction of a 9-month-old Cheddar cheese. The locations of the peptides αs-CN f1–9 (X) and f1–13 (Y) are indicated (Tobin et al, unpubl results).

Profil obtenu par chromatographie liquide haute performance en phase inverse (C8) des fractions (a) solubles dans l'éthanol à 70% et (b) insolubles de la fraction soluble dans l'eau d'un fromage de cheddar de 9 mois. Les localisations des peptides αs-CN f1–9 (X) et f1–13 (Y) sont signalées (Tobin et al, non publié).
Methods used to assess proteolysis in cheese

Reverse-phase HPLC of pre-column-derivatized fluorescent-labelled amino acids is increasingly being used to measure free amino acids in cheese, and has the advantage of using standard equipment and being amenable to automated derivatization. Amino acids have been separated as their dansyl (eg, Polo et al, 1985), OPA (eg, Ramos et al, 1987; Bütkofer et al, 1991) or FMOC (Bütikofer et al, 1991) derivatives. Bütkofer et al (1991) compared the results of amino acid analysis of an acid hydrolysate of cheese proteins by pre-column derivatization with OPA or FMOC, followed by RP-HPLC, with the results obtained using an amino acid analyzer fitted with an ion-exchange column and using post-column derivatization with ninhydrin. HPLC gave rapid, simple and sensitive determination of amino acids and yielded narrower, better-resolved peaks with shorter retention times and a more stable baseline. Although the HPLC method used had a slightly lower repeatability for some amino acids, it had good reproducibility and correlated well with ion-exchange chromatography.

Amino acids can be also quantified by gas chromatography (GC) after derivatization with heptfluorobutyric anhydride (HFBA) to yield n-heptafluorobutyryl isobutyl derivatives. Capillary GC is most suitable (eg, Wood et al, 1985; Laleye et al, 1987; Bertacco et al, 1992). This technique is reported to give good recovery of added amino acids; all protein amino acids can be resolved on a single chromatogram, and speed and accuracy are comparable to those of automated amino acid analyzers and at far lower equipment costs. The technique has good reproducibility and high accuracy, although the coefficients of variation for Met and Gly are somewhat high (Bertacco et al, 1992). However, GC has not been used as widely as alternative techniques.

Ammonia is produced by deamination of amino acids, and techniques for its quantification have been discussed above. Amines in cheese may be quantified by RP-HPLC using pre-column derivatization (eg, Bütkofer et al, 1990). Gas chromatography with mass spectrometric detection (GC-MS) allows the quantification and identification of volatiles from cheese (eg, Urbach, 1993), including those resulting from amino acid catabolism. A discussion of GC-MS is outside the scope of this review.

A FRACTIONATION SCHEME FOR CHEESE PEPTIDES

The peptide system in most cheese varieties is extremely complicated. For example, > 200 peptides have been isolated and identified from the WSE of Cheddar (Singh et al, 1994, 1995, 1996). The detailed study of proteolysis in cheese often requires that peptides in the initial extract be fractionated by a combination of the techniques discussed above. A number of fractionation schemes have been proposed (Kuchroo and Fox, 1983b; Aston and Creamer, 1986; Fox, 1989; O’Sullivan and Fox, 1990; Cliffe et al, 1993; Singh et al, 1994, 1995, 1996; Breen et al, 1995). The scheme used at present in this laboratory is shown in figure 11. This fractionation scheme was developed for Cheddar cheese and may need to be modified for other varieties.

ISOLATION AND IDENTIFICATION OF INDIVIDUAL PEPTIDES

Individual small peptides are usually isolated by RP-HPLC. Fractions of eluate may be collected and freeze-dried. It is desirable to check the homogeneity of fractions by re-chromatography under the same or preferably different elution conditions. Peptides eluted in salt-containing buffers may be desalted by re-chromatography using an acetonitrile or methanol gradient, the components of which are easily volatilized.

In our experience, RP-HPLC does not adequately resolve larger casein-derived peptides. Such peptides may be resolved by PAGE and isolated by electroblotting onto a suitable mem-
brane (eg, polyvinylidene difluoride; McSweeney et al, 1994a; Singh et al, 1995). Alternatively, unstained regions of the gel may be excised, and peptides extracted from the macerated gel pieces by electrophoresis. However, the precise location of peptides on unstained gels may be difficult since they cannot be compared directly with stained gels, the dimensions of which change on staining. The N-terminal sequence (see below) of electroblotted peptides can be determined readily, but it is necessary to remove peptides from the blotting membrane prior to mass spectrometric analysis.


The amino acid composition of a peptide, determined after hydrolysis, can provide useful information as to its identity (eg, Kaminogawa et al, 1986). Computer software can be written (eg, Petrilli, 1982) or is available from commercial sources to aid in locating a peptide of a given amino acid composition in the protein chain. However, more than one casein-derived peptide may have the same amino acid composition and thus identification of peptides in a complex mixture from their amino acid composition, alone is not recommended. Identification of the N-terminal residue or sequence (eg, Visser et al, 1977; González de Llano et al, 1991) improves the probability of correct identification of peptides of a given composition.

Amino acid sequencing (either manual, eg, Mojarro-Guerra et al, 1991 or automated, eg, McSweeney et al, 1994a; Singh et al, 1994, 1995, 1996) by Edman degradation can also be used to identify peptides. However, unless the peptide is relatively short, it may not be possible to determine its full sequence. The N-terminal sequence (5-7 residues) is normally adequate to locate the origin of a peptide in the caseins, but other techniques must be used to determine its length. C-terminal sequencing (eg, liberation of amino acids over time by carboxypeptidase Y, eg, McSweeney et al, 1993b) could be used for this purpose but is not popular. Mass spectrometry (MS) is more convenient for identifying peptides of known N-terminal sequence (eg, Singh et al, 1994, 1995, 1996), although mass analysis alone is often insufficient to identify peptides since different casein-derived peptides can have the same amino acid composition (and hence mass). An interesting application of MS involves determining the residual mass of a peptide after each cycle of a manual Edman degradation. The mass of the residue \( M_f \) released can be calculated from \( M_f = M_n - M_{n-1} \), where \( M_n \) and \( M_{n-1} \) are the masses of the peptide before and after removal of the N-terminal residue, which can thus be identified. Sequencing of the first few amino acids, coupled with its mass, permits precise identification of the peptide. Such an approach has been used to identify peptides in Cheddar cheese (Gouldsworthy et al, 1996). Most MS techniques used to identify peptides involve the production of ions of the intact peptide. However, if high energies are used, the peptide is fragmented and the masses obtained are those of the resulting fragments. Cyclic dipeptides and \( \gamma \)-glutamyl peptides have been identified using this technique (Roudot-Algaron et al, 1993, 1994), which yields more information on the structure of the compound under study than just its mass; it is likely to be useful for studying very short peptides or those with an unusual structure.

### STRATEGIES FOR ASSESSING PROTEOLYSIS

The choice of a technique for assessing proteolysis in cheese depends on a number of factors, including availability of equipment and resources, cheese variety and objectives of the study. Since proteolysis is one of the principal biochemical events during cheese ripening, it is desirable to include an assay for proteolysis in most cheese ripening studies. The formation of soluble N or liberation of reactive groups may be adequate if a detailed investigation of proteolysis is not warranted. However, if a thorough study of proteolysis is deemed worthwhile, it is desirable to consider the entire process from the initial hydrolysis of the caseins to the development of free amino acids and to use techniques which resolve individual peptides.

Certain cheese varieties have characteristics which influence the choice of method. The increase in pH of mould-ripened varieties means that pH 4.6 buffers are more suitable as primary extractants than water. Likewise, since ammonia is a major product of proteolysis in mould and bacterial surface-ripened varieties, it may be desirable to quantify ammonia. The rate and/or pattern of proteolysis may be influenced by location within a cheese, eg, surface-ripened or young, brine-salted cheeses. A suitable sampling scheme should take account of such differences.
If one or more of the proteolytic agents is modified in a cheese, then the methods used to study proteolysis should be chosen so as to emphasize the level of proteolysis caused by that agent. For example, if the influence of coagulant is studied, the formation of WSN and peptide profiles by urea-PAGE are suitable methods. Since the coagulant does not produce free amino acids, their determination does not directly measure the contribution of the coagulant. In contrast, since the contribution of cheese microflora (natural or modified) to cheese ripening is mainly in the production of short peptides and free amino acids, RP-HPLC and amino acid analysis are likely to be most effective. However, it is also important to include some index of primary proteolysis (eg, PAGE) to ensure that it is typical.

In this laboratory, routine assessment of proteolysis in Cheddar cheese during ripening involves the determination of WSN (Kuchroo and Fox, 1982a) and peptide profiles of the cheese and WSEs therefrom by alkaline urea–PAGE (Blakesley and Boezi, 1977; Andrews, 1983). These assays provide important information on primary proteolysis. Water-soluble extracts are fractionated using 70% ethanol and the soluble and insoluble fractions analyzed by RP–HPLC (Cs; Singh et al, 1995). The 70% ethanol-insoluble fraction can also be analyzed by urea–PAGE. Total free amino acids are determined using the Cd–ninhydrin assay (Folkertsma and Fox, 1992) and individual amino acids by an automated amino acid analyzer with post-column derivatization using ninhydrin. This strategy provides information on the level and type of proteolysis from the initial hydrolysis of the caseins to the liberation of free amino acids; and is suitable for most internal, bacterially-ripened cheese varieties.

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