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Separation and identification of hydrophilic peptides in dairy products using FMOC derivatization

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Abstract

Small hydrophilic di- and tripeptides from food products are not separated by reversed-phase high-performance liquid chromatography (RP-HPLC). A simple method using precolumn derivatization with 9-fluorenylmethoxycarbonyl chloride (FMOC) of hydrophilic peptides followed by RP-HPLC separation is presented. Peptides can subsequently be identified by Edman degradation after deprotection of the peptide derivatives with piperidine. Fifteen peptides (ten dipeptides, four tripeptides and one tetrapeptide) were sequenced from the water-soluble fraction of an enzyme-modified cheese model. Some synthetic peptides (Ile–Asn, Val–Thr, Ala–Pro, Val–Gln, Thr–Gln and Gly–Gly) corresponding to purified peptides were sensory tested.

1. Introduction

The importance of the non-volatile water-soluble fraction in cheese flavour has been recognized by several workers [1–4]. McGugan *et al.* [1] reported that the water-soluble fraction, containing mainly salts, lactic acid, amino acids and peptides, contributes most to the flavour intensity. Biede and Hammond [2] assigned the brothy–nutty, sweet flavours to the amino acids and dipeptides, whereas bitter and burned flavours were attributed to larger peptides (tri- to hexapeptides). Aston and Creamer [3] also observed that the water-soluble fraction containing peptides is responsible for the taste intensity with bitter and brothy notes.

However, little information is available concerning the structure of these small "savoury" peptides. Mojarro de Guerra et al. [5] purified seven peptides from a Swiss cheese containing proline, namely Gly–Pro–Val–Arg, Arg–Pro, Leu–Pro, Lys–Pro and Tyr–Pro. Roudot-Algaron and co-workers [6,7] isolated three γ-glutamyl dipeptides (γ-Glu–Phe, γ-Glu–Tyr, γ-Glu–Leu) and five diketopiperazines [cyclo(Pro–Pro), cyclo(Pro–Val), cyclo(Pro–Phe), cyclo(Pro–Ala) and cyclo(Pro–Leu)] from Comté cheese. All peptides containing Pro have a bitter taste. The taste of γ-Glu–Phe was described as umami and slightly sour, salty and metallic. The peptide γ-Glu–Tyr was reported as sour and slightly salty. Other peptides isolated from cheese are longer and have been reported to be bitter owing to their hydrophobic properties. Some polar hydrophilic peptides from other food sources were shown to have flavour properties. Glutamyl peptides isolated from proteinase-modified soy bean protein, fish hydrolysate or

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gravy of beef meat have brothy taste properties [8–10].

The aim of this study was to identify such peptides in the non-volatile water-soluble fraction of an enzyme-modified cheese model. Peptides are classically separated by reversed-phase chromatography [11–14]. However, small hydrophilic peptides cannot be studied using this method because they interact poorly with the hydrophobic chains of the stationary phase in reversed-phase chromatography. To overcome this difficulty, we used precolumn derivatization with 9-fluorenylmethoxycarbonyl chloride (FMOC) to allow the separation of hydrophilic peptides on a reversed-phase column. The identification was achieved by Edman degradation after deprotection of the peptide derivatives.

2. Experimental

2.1. Enzyme-modified cheese model

Enzyme-modified cheese model was supplied by SOREDAB (Société de Recherche et Développement Alimentaire Bongrain). This product was the result of the action of lipases, peptidases and proteases on a dairy pasteurized slurry. After 6 days of incubation under aseptic conditions, the product had a strong cheesy flavour and a bitter taste.

2.2. Water-soluble extract

The water-soluble fraction was extracted from an enzyme-modified cheese model with the method described by Aston and Creamer [3] with minor modifications. The enzyme-modified cheese model (16 g) was homogenized in water (30 ml) with an Ultra-Turrax T25 (IKA, Staufen, Germany) for 2 min at room temperature. The resulting slurry was centrifuged at 8650 *g* for 20 min using a JA-21 rotor in a J2-21 centrifuge (Beckman, Palo Alto, CA, USA) at 4°C. The fat and the aqueous layers were collected separately. The pellet was homogenized twice in water (30 ml) and centrifuged. Each time, the fat layer and the water fraction were recovered. The three fat layers were then combined, homogenized in 20 ml of water and centrifuged. The four water fractions were pooled and filtered at 4°C through a Whatman No. 42 paper and a 0.45-μm filter (Millipore, Bedford, MA, USA).

2.3. Gel filtration

The water-soluble fraction was fractionated on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (200 cm × 4 cm I.D.). Elution was carried out with water purified with a Milli-Q system (Millipore) at 84 ml/min. Detection was performed at 206 nm and 84-ml fractions were collected for sensory analysis and for purification. The exclusion volume (728 ml) was determined using bovine serum albumin (M_r = 66 000).

2.4. Derivatization

Samples and standards were reacted with FMOC according to the method of Einarsson et al. [15]. A volume of 0.2 ml of gel filtration fraction was dried with a Speed-Vac system (Savant, Farmingdale, USA). The sample was resuspended in 0.2 ml of sodium borate buffer (0.5 *M*, pH 7.8) and 0.2 ml of FMOC reagent (5.8 m*M* in acetone) were added. The vial was vortex mixed and after 45 s the mixture was extracted with 0.4 ml of pentane–ethyl acetate (80:20). The aqueous phase containing the FMOC derivatives was then ready for reversed-phase high-performance liquid chromatography (RP-HPLC).

2.5. HPLC conditions for the separation of non-derivatized peptides

Peptides were separated by RP-HPLC using a Model 600E gradient system (Waters, Bedford, MA, USA) fitted with a Nucleosil C_{18} (5 μm) column (250 mm × 46 mm I.D.) (SFCC, Neuilly Plaisance, France). Peptides were eluted with a linear gradient of solvent A decreasing from 80% to 20% in 30 min at 25°C. Solvent A

consisted of 0.115% trifluoroacetic acid (TFA) in water and solvent B consisted of 0.1% TFA in acetonitrile (ACN)–water (60:40). The flow-rate was 1 ml/min and the absorbance was read at 214 nm.

2.6. HPLC conditions for the separation of FMOC derivatives of peptides and amino acids

The solvent system used for separation consisted of solvent A, ACN–100 m*M* ammonium acetate buffer (pH 3.8) (20:80), and solvent B, ACN–100 m*M* ammonium acetate buffer (pH 4.2) (80:20). Chromatography was carried out at a flow-rate of 1 ml/min at 40°C. Samples were eluted with a gradient from 30 to 80% solvent B in 50 min and isocratic at 100% B for 10 min. The gradient was started 10 min after injection. The absorbance was read at 260 nm.

The main hydrophilic peaks were collected and a second purification step was applied with isocratic elution with ACN–100 m*M* ammonium acetate buffer (pH 3.3) (30:70).

2.7. Deprotection

The peptide derivative collected after RP-HPLC separation was dried with a Speed Vac system. Piperidine (10 μl) was added and the sample was dried again to remove excess of piperidine. The sample was resuspended in 200 μl of 20% TFA and 400 μl of pentane–ethyl acetate (80:20) were added. The aqueous fraction was recovered, extracted again with 400 μl of pentane–ethyl acetate (80:20) and dried. The sample was then ready for sequencing.

2.8. Peptide sequencing

The peptide sequence was determined by automatic Edman degradation using an Applied Biosystems (San-Jose, CA, USA) Model 477A protein sequencer.

2.9. Sensory test

Each fraction (10 ml) from gel filtration was lyophilized, weighed and resuspended in 10 ml of Milli-Q-purified water. The fraction was then evaluated by an eight-member panel, ranging in age from 25 to 35 years. Panellists were selected for their sensitivity to salty, sweet, sour, bitter and umani tastes. Each panellist evaluated the flavour and taste of every sample (1 ml) using a small plastic teaspoon. Between samples they rinsed their mouths with mineral water. Samples were presented to the panellists in random order and not more than four samples were presented at each session.

After identification, some peptides were synthesized by Neosystem (Strasbourg, France), dissolved in Milli-Q-purified water, lyophilized to remove solvent odour and tasted. These synthetic peptides were resuspended in Milli-Q-purified water at a concentration of 5 mg/ml. The panellists were asked to describe each sample for taste and flavour.

3. Results

3.1. Preparation of cheese extract

A total of 4.8 g of lyophilized water-soluble compounds was obtained from 33 g of enzyme-modified cheese model. The amino acid analysis was carried out before (to determine the free amino acid content) and after acid hydrolysis. The difference indicated amino acids involved in peptide linkages. The freeze-dried water-soluble fraction consisted of peptides (35.9%) and free amino acids (6.2%), mainly Glu, Phe, Leu and Lys.

3.2. Gel filtration

Gel filtration was performed in order to fractionnate the extract into less complex fractions. Fig. 1 shows the chromatogram obtained by gel filtration with the water-soluble fraction of the enzyme-modified cheese model. The separation, performed with water as eluent to allow sensory testing, was not strictly on a molecular size basis: aromatic amino acids such as Phe and Tyr were considerably retarded owing to interactions with the Sephadex G-25 matrix.

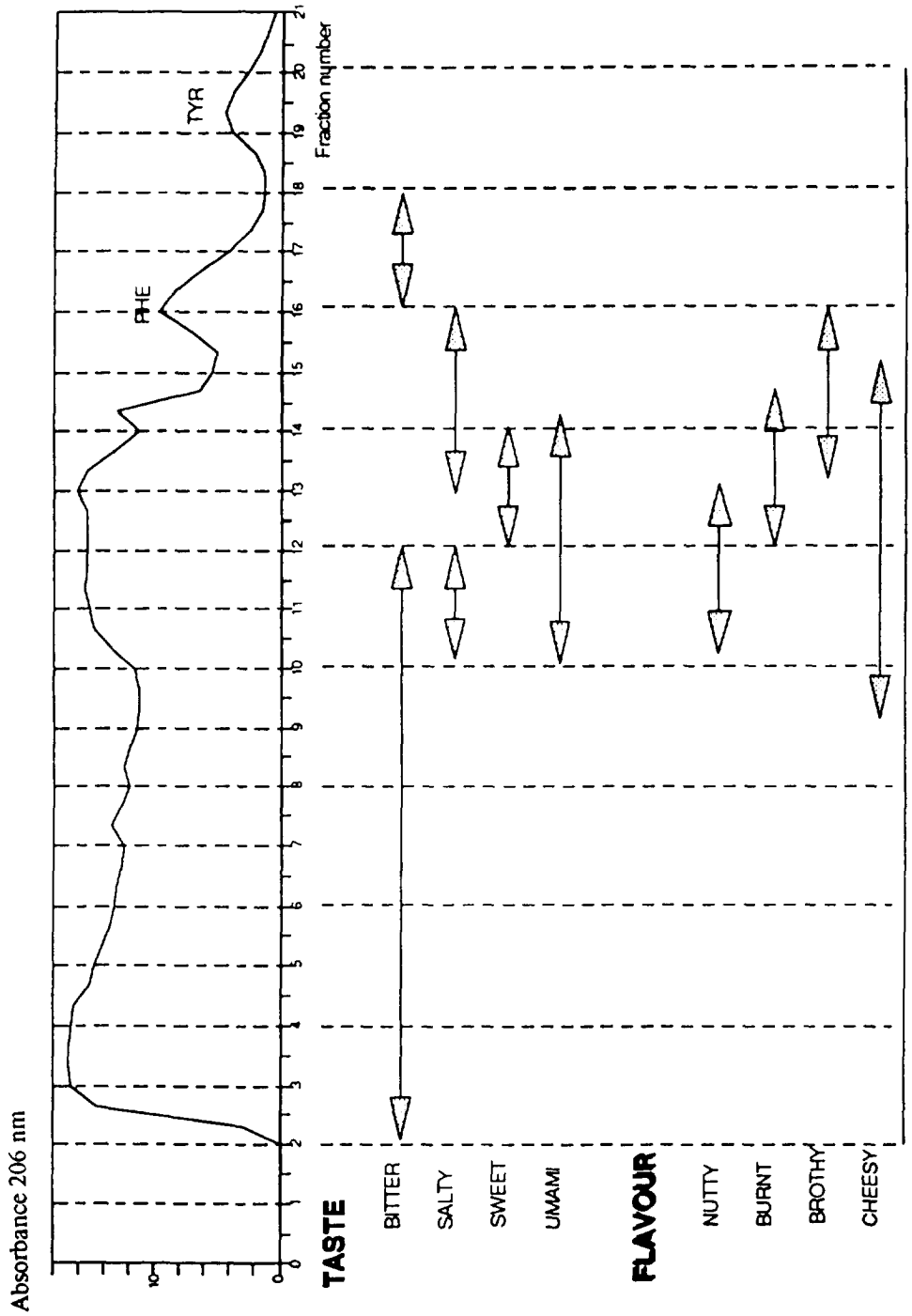

Fig. 1. Gel filtration chromatogram and sensory characterization of the collected fractions.

Fractions were collected and tasted (Fig. 2). Fractions 3–10 were described as bitter. More intense and complex tastes were perceived from fractions 11–14. According to their elution volume, these compounds had low molecular masses.

The comparison of the RP-HPLC traces obtained from each gel filtration fraction revealed that more hydrophilic compounds were present in the later fractions. The separation and identification of short hydrophilic peptides was consequently performed on two of those fractions (12 and 13).

3.3. Separation of peptides derivatives

The separation of peptide derivatives from fractions 12 and 13 is shown in Fig. 4. These chromatograms were compared with a chromatographic profile of amino acid derivatives (Fig. 3) in order to identify the retention times of the amino acid derivatives. Most hydrophilic derivatives were individually collected and dried. Further purification of peptide derivatives was carried out in a second step by decreasing the pH of solvent A from 3.8 to 3.3 (see Experimental). At a lower pH we observed an increase in the retention times of the amino acid and peptide derivatives. A better resolution of most hydrophilic derivatives was also observed. Similar properties had been reported by Einarsson et al. [15] for amino acid derivatives.

3.4. Identification

After deprotection with piperidine as described under Experimental, fifteen peptides (eleven dipeptides and four tripeptides) were sequenced (Table 1) with an automatic Edman degradation sequencer. The retention times of

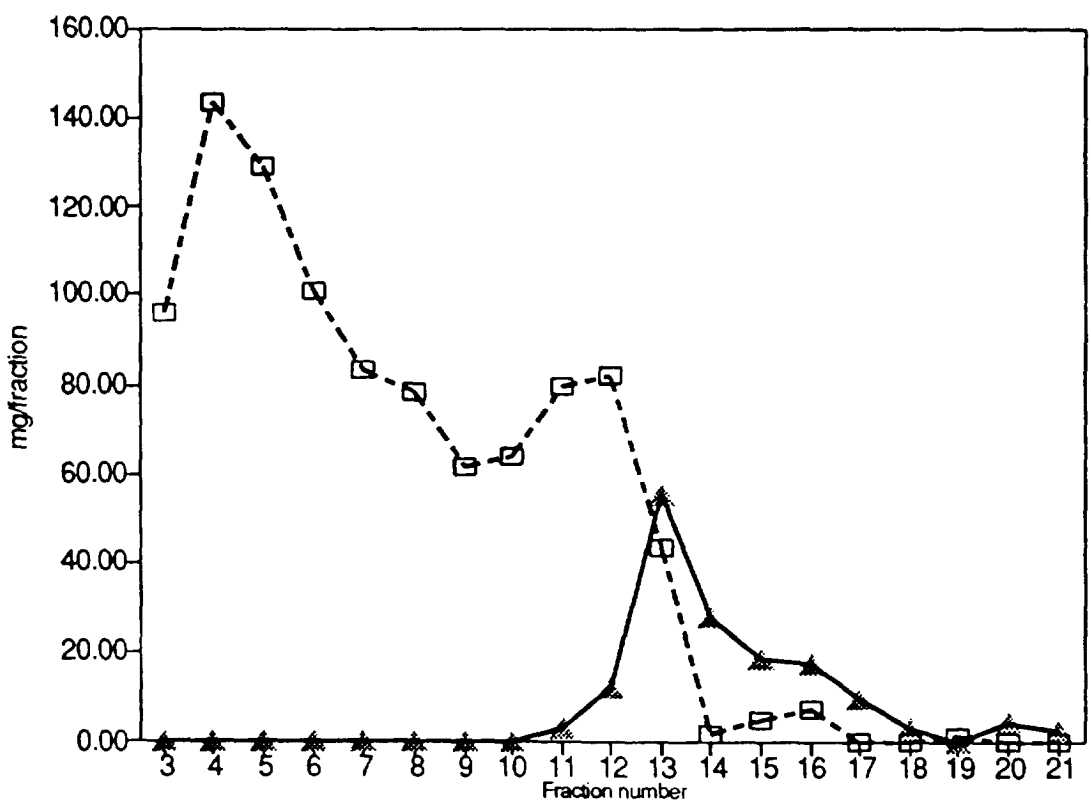

Fig. 2. (▲) Amino acid and (□) peptide concentrations in gel filtration fractions.

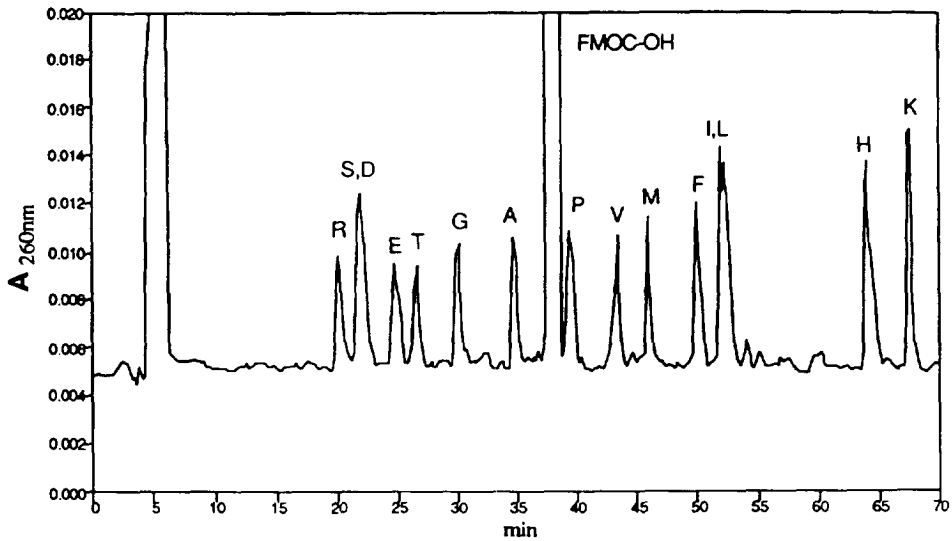

Fig. 3. Separation of derivatized standard mixture of amino acids containing 2.5 μmol of each amino acid. See Experimental for chromatographic conditions.

the purified peptide derivatives were compared with those of synthetic peptides (Fig. 5) (Ile–Asn, Val–Thr, Ala–Pro, Val–Gln, Ala–Gln, Thr–Gln, Gly–Val–Ser and Ala–Gln–Thr) in order to confirm some of the sequences.

3.5. Sensory evaluation of synthetic peptides

Synthetic dipeptides (Ile–Asn, Val–Thr, Ala–Pro, Val–Gln, Ala–Gln and Thr–Gln) were tasted at a concentration of 5 mg/ml. Most of the peptides were described as flat. Only Val–Gln and Ala–Pro were slightly sweet and slightly bitter, respectively. Ala–Gln was tasted at a concentration of 20 mg/ml and was found to be slightly bitter and umami.

4. Discussion

Separations of short hydrophilic peptides were carried out with FMOC derivatization followed by RP-HPLC. This method has certain advantages: FMOC-Cl reacts rapidly with both primary and secondary amino acids to form very stable derivatives [15–18]. Moreover, this acylation is less sensitive to salts than other reagents, e.g., phenyl isothiocyanate (PITC) [18]. In order to identify peptides, we developed a simple method for peptide derivative deprotection with piperidine, classically used in peptide synthesis. Piperidine allows the liberation of peptides by reacting with the methylfluorenyl group of the derivative. However, the resulting molecule leads to an artefact peak during the RP-HPLC step of sequencing. This problem was solved by resuspending the sample in 20% TFA. This procedure induces FMOC-OH formation (detected by RP-HPLC), suggesting cleavage of the molecule formed by reaction of piperidine with the methylfluorenyl group. The FMOC-OH is removed by pentane–ethyl acetate extraction and piperidine is eliminated by drying. One limitation of the FMOC method is the ratio between the amino acid and small peptide concentrations in the extract. A high concentration of amino acid derivatives could mask peptide derivative peaks in RP-HPLC. Prior partial separation of peptides and amino acids must be done by gel permeation.

Using the FMOC method, we were able to identify fifteen peptides. As expected, most of these peptides are particularly hydrophilic owing to their chain length and their amino acid

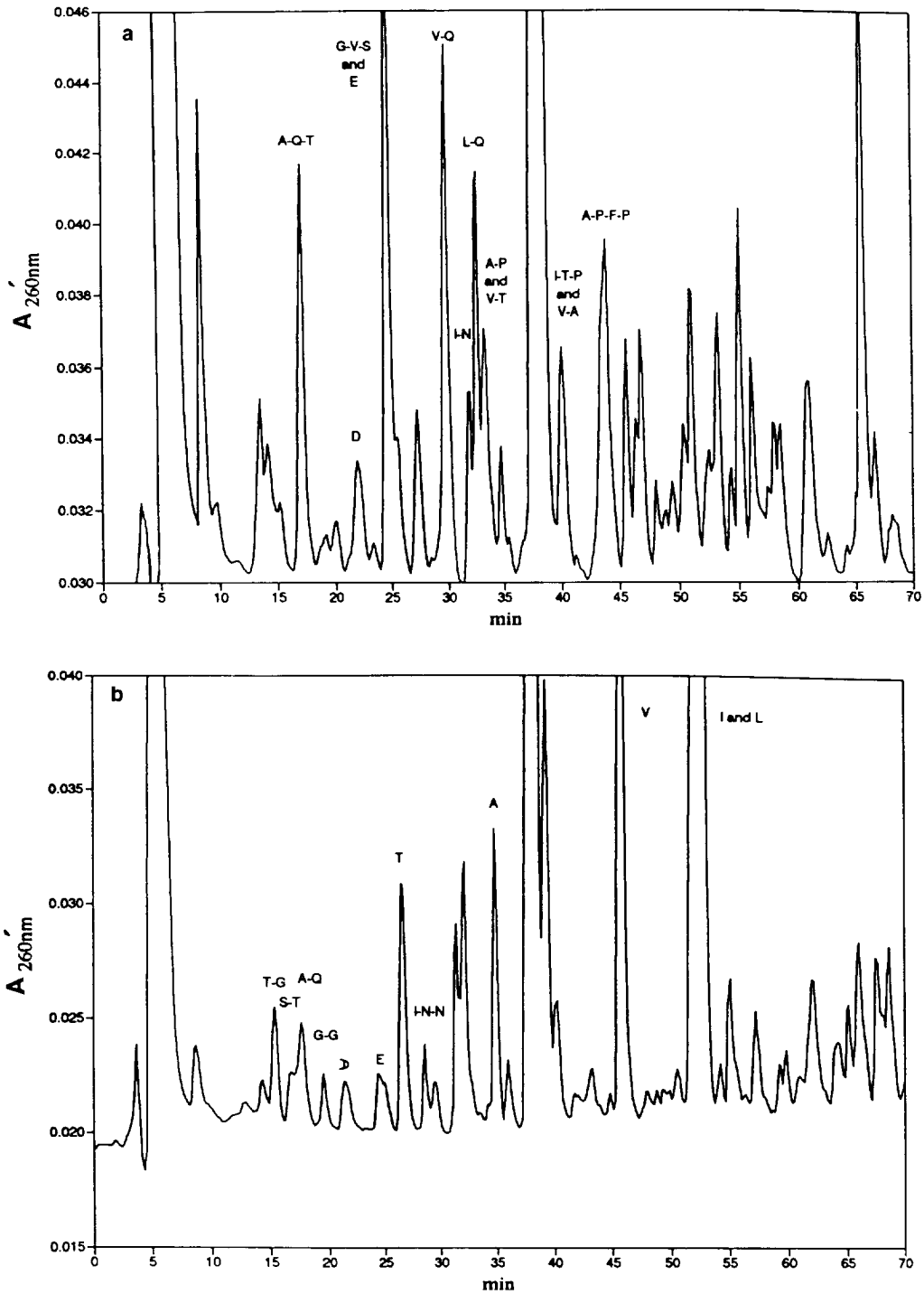

Fig. 4. Separation of peptide derivatives of gel filtration fractions (a) 12 and (b) 13. See Experimental for chromatographic conditions.

Table 1
Amino acid sequence of peptides isolated from gel filtration fractions 12 and 13 and sensory evaluation of some synthetic peptides

Peptide identification	Taste of synthetic peptides	Concentration of synthetic peptides for tasting (mg/ml)
Gly–Gly	Tasteless	5
Thr–Gln	Tasteless	5
Ser–Thr	Tasteless	5
Ala–Gln	Slightly bitter and umami	20
Ala–Gln–Thr		
Gly–Val–Ser		
Val–Gln	Slightly sweet	5
Ile–Asn–Asn		
Val–Thr		
Leu–Gln		
Val–Ala	Tasteless	5
Ile–Asn		
Ala–Pro	Slightly bitter	5
Ile–Thr–Pro		
Ala–Pro–Phe–Pro		

composition. Among these peptides, Ala–Gln–Thr, Gly–Val–Ser, Val–Gln, Val–Thr, Ile–Asn–Asn and Gly–Gly could be assigned to the residues β-casein 53–55, β-casein 94–96, κ-casein 161–162, κ-casein 164–165, κ-casein 51–53 and α-lactabumin 19–20, respectively. The other peptides could originate from several localizations in casein sequences.

None of these peptides contained glutamic acid in their sequence. This is surprising as caseins are particularly rich in glutamic acid and potentially could liberate numerous small glutamic peptides. On the other hand, eight peptides contained Gln or Asn residues. As far as we know, no information is available concerning the taste of these peptides. As mentioned, Ile–Asn, Ala–Gln and Thr–Gln were tasted and described as flat. Val–Gln is slightly sweet. The concentration of the peptides (estimated by amino acid analysis of peptide derivatives after the first step of purification) was lower than 0.1 mg/ml in the tasted samples. This indicates that these peptides individually could

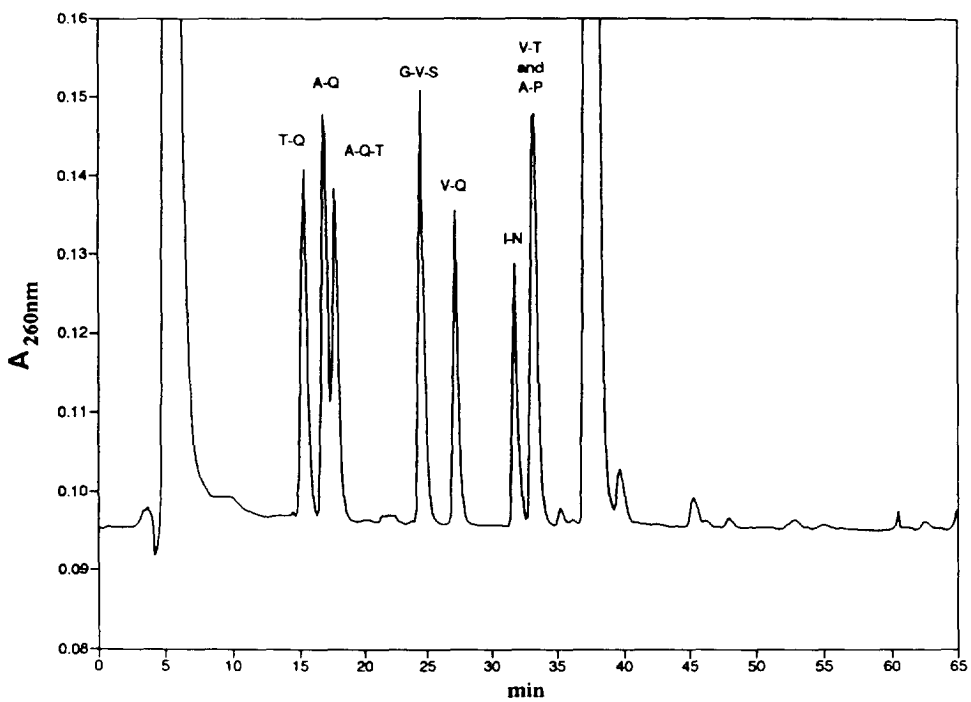

Fig. 5. Separation of synthetic peptide derivatives (T–Q, A–Q, A–Q–T, G–V–S, V–Q, I–N, V–T, A–P). See Experimental for chromatographic conditions.

not explain the enzyme-modified cheese model flavour. If they play a role in flavour, synergic effects or interaction phenomena must occur.

Hydrophobic proline peptides (Ala–Pro, Ile–Thr–Pro and Ala–Pro–Phe–Pro) were also identified. The last two peptides could be assigned to the residues α_{s1}-casein 119–121 and α_{s1}-casein 26–29. Ala–Pro, previously described as bitter by Shiraishi *et al.* [19], was also found to be bitter by our panel. Ile–Thr–Pro and Ala–Pro–Phe–Pro are probably bitter owing to their high hydrophobicity.

5. Conclusions

We have demonstrated that the FMOC method permits the separation and identification of very hydrophilic peptides in a complex medium such as cheese water-soluble extract. Such a technique could be applied in combination with RP-HPLC to follow and understand protein hydrolysis mechanisms during the maturation of cheese, meat or any protein hydrolysate used in the food industry.

Acknowledgements

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References

[1] W.A. McGugan, D.B. Emmons and E. Larmond, *J. Dairy Sci.*, 62 (1979) 398.
[2] S.L. Biede and E.G. Hammond, *J. Dairy Sci.*, 62 (1979) 227.
[3] J.W. Aston and L.K. Creamer, *N.Z.J. Dairy Sci. Technol.*, 21 (1986) 229.
[4] J. Kowaleska, H. Zelakowska, A. Babochowski, E.G. Hammond and F. Ross, *J. Dairy Sci.*, 68 (1985) 2165.
[5] S.H. Mojarro de Guerra, R. Amado, E. Arrigoni and J. Solms, *J. Food. Sci.*, 56 (1991) 943.
[6] F. Roudot-Algaron, L. Kerhoas, D. Le Bars, J. Einhorn and J.C. Gripon, *J. Dairy Sci.*, 77 (1994) 1161.
[7] F. Roudot-Algaron, D. Le Bars, J. Einhorn, J. Adda and J.C. Gripon, *J. Food Sci.*, 58 (1993) 1005.
[8] J. Kirimura, A. Shimizu, A. Kimizuka, T. Ninomiya and N. Katsuya, *J. Agric. Food Chem.*, 17 (1969) 689.
[9] M. Fujimaki, S. Arai, M. Yamashita, H. Kato, and M. Noguchi, *Agric. Biol. Chem.*, 37 (1973) 2891.
[10] M. Tamura, T. Nakatsuka, M. Tada, Y. Kawasaki, E. Kikuchi and H. Okai, *Agric. Biol. Chem.*, 53 (1989) 319.
[11] H.M. Champion, and D.W. Stanley, *Can. Inst. Food Sci. Technol. J.*, 15 (1982) 283.
[12] M.P. Polo, D.G. De Llano and M. Ramos, in L.M.L. Nollet (Editor), *Food Analysis by HPLC*, Marcel Dekker, New York, 1992, pp. 117–139.
[13] D.G. De Llano, M.C. Polo and M. Ramos, *J. Dairy Res.*, 58 (1991) 363.
[14] K.F. Kaiser, H.D. Belitz and R.J. Fritsch, *Z. Lebensm.-Unters.-Forsch.*, 195 (1992) 8.
[15] S. Einarsson, B. Josefsson and S. Lagerkvist, *J. Chromatogr.*, 282 (1983) 609.
[16] I. Betner and P. Foldi, *LC·GC Int.* 3 (1988) 12.
[17] J.H. Kunte, A.E. Galnski and H.G. Truper, *J. Microbiol. Methods*, 17 (1993) 129.
[18] R. Cunico, A.G. Mayer, T. Wehr and T.L. Sheenan, *Biochromatography*, 1 (1986) 7.
[19] H.S. Shiraishi, K. Okuda, Y. Sato, N. Yamaoka and K. Tuzimura, *Agric. Biol. Chem.*, 37 (1973) 2427.