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ANALYSIS OF TRYPTIC DIGESTS OF BOVINE β-CASEIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to separate the tryptic peptides of β-casein. A gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid at a flow-rate of 0.8 ml/min resolved ten of the thirteen peptides. A modified gradient resolved the three peptides eluted at *ca.* 25% acetonitrile. RP-HPLC proved superior to high-voltage paper electrophoresis in analysis time, resolution and flexibility. The methods developed for the analysis of proteolysis of the milk protein, β-casein, are now being applied to study the action of extracellular proteases from dairy bacteria on milk proteins.

INTRODUCTION

It is well documented that proteolysis of caseins may lead to the release of bitter peptides, particularly from the most hydrophobic casein, β-casein[1–4]. Separation of these fragments has normally been carried out by conventional methods, such as liquid chromatography, thin-layer chromatography and high-voltage paper electrophoresis (HVPE). In order to separate and identify the peptides released by proteolysis of caseins, it is preferable to have a method that is both rapid and sensitive. High-performance liquid chromatography (HPLC) is proving to be such a method and has become an increasingly useful tool for the separation of amino acids, peptides and proteins. HPLC has been applied to the study of milk proteins. In most cases, size-exclusion HPLC has been the method of choice. Several groups of researchers[5–7] have used size-exclusion chromatography to resolve and quantify milk proteins, whilst others[8,9] determined the degree of total protein breakdown in milk by this method. Whey proteins have been analysed by both size-exclusion and reversed-phase (RP) HPLC[10,11]. Bican[12] utilised RP-HPLC to resolve the products of a tryptic digest of total casein and concluded that this method had great potential for use in the food industry. However, he was unable to resolve completely or identify any of the proteolytic fragments. Recently, Carles[13] successfully resolved some tryptic fragments of genetic variants of β-casein, whilst Monnet *et al.*[14] have employed RP-HPLC to determine the specificity of a cell wall proteinase from *Streptococcus lactis* towards β-casein. There is a fairly large body of published work on the preparation and identification of bitter peptides released from the caseins as result of proteolysis.

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TABLE I

AMINO ACID SEQUENCE OF β-CASEIN A^2 VARIANT[17]

Z denotes phosphoserine residues.

H_2-N-R-E-L-E-E-L-N-V-P-G-E-I-V-E-Z-L-Z-Z-Z-E-E-S-I-T-R-I-N-K-K-I-E-K-F-Q-Z-E-E-Q-Q-Q-T-E-D-E-L-Q-D-K-I-H-P-F-A-Q-T-Q-S-L-V-Y-P-F-P-G-P-I-P-N-S-L-P-Q-N-N-I-P-P-L-T-Q-T-P-V-V-V-P-P-F-L-Q-P-E-V-M-G-V-S-K-V-K-E-A-M-A-P-K-N-E-M-P-F-P-K-Y-P-V-Q-P-F-T-E-S-Q-S-L-T-D-V-E-N-L-H-L-P-P-L-L-L-Q-S-W-M-H-Q-P-H-Q-P-L-P-P-T-V-M-F-P-Q-S-V-L-S-L-S-Q-S-K-V-L-P-V-P-E-K-A-V-P-Y-P-Q-R-D-M-P-I-Q-A-F-L-L-Y-Q-Q-P-V-L-G-P-V-R-G-P-F-P-I-I-V-COOH

Examples of this work include the isolation and identification of bitter peptides from cheese[15], purification of bitter peptides from rennet-treated casein[16] and the isolation of such peptides from casein digested by a bacterial proteinase[2].

In this study, a method is described for the rapid separation by RP-HPLC of proteolytic fragments of bovine β-casein released by trypsin. A model system was chosen to enable methods to be developed for the fingerprinting of proteolytic fragments of caseins. β-Casein was selected because it is well characterised (the sequence of genetic variant A^2 is shown in Table I) and is readily susceptible to hydrolysis. Trypsin was chosen as the standard protease as a wide range of fragments, both in terms of size and hydrophobicity, should be released from β-casein hydrolysed by this enzyme[18]

MATERIALS AND METHODS

Preparation of β-casein

Homogeneous β-casein was prepared from raw milk by the method of Andrews and Alichanidis[19].

Trypsin digest

β-Casein (50 mg) was incubated with 2 mg N-tosyl-l-phenylalanylchloromethyl ketone (TPCK)-treated trypsin in 0.1 *M* ammonium acetate, pH 6.5, at 37°C for 4 h. After freeze-drying, the digest was dissolved in 0.1 *M* ammonia and aliquots were removed and redried for analysis by RP-HPLC. The remainder was used for the preparation of fragments by conventional methods.

High-voltage paper electrophoresis

Individual fragments were isolated by HVPE[20] using the following solvent systems: (1) pyridine–acetic acid–water (10:1:89) (pH 6.5); coolant, 8% (v/v) pyridine in toluene; (2) pyridine–acetic acid–water (1:10:89) (pH 3.5); coolant, white spirit; (3) formic acid–acetic acid–water (10:35:355) (pH 2.1); coolant, white spirit.

Detection of peptides and amino acids was by staining with 0.2% (w/v) ninhydrin in acetone containing 5% (w/v) colidine acetate.

N-terminal analysis

N-terminal analysis was carried out by the dansylation technique[20]. Separation of the dansyl amino acids was in a flat-bed electrophoresis system at pH 4.4 using pyridine–acetic acid–water (9:15:976).

Dansyl–Edman degradation

Edman degradation was carried out by the dansyl-phenylisothiocyanate (PTC) method[20].

Total hydrolysis

Samples of pure peptide were hydrolysed in 6 *M* hydrochloric acid overnight at 105°C. After vacuum-drying, 50 μl 0.1 *M* ammonia was added and the amino acid components were separated by HVPE, using buffer system 3 on Whatman No. 1 paper. Detection was by staining, as described above.

High-performance liquid chromatography

The HPLC system (LKB, Bromma, Sweden) consisted of two HPLC pumps, gradient mixer and controller. Detection was by a Uvicord fixed-wavelength detector (LKB) at 206 nm and fractions were collected using a Superrac fraction collector (LKB). An Apex C_8 120 Å column and C_8 guard column (Jones Chromatograpohy, Llanbradach, U.K.) was used for all separations.

Solvent A was 0.1% (v/v) aq. trifluoroacetic acid (TFA) (pH 2.1), and solvent B was 0.08% (v/v) TFA in 50% (v/v) aq. acetonitrile (pH 2.3). All solvents were filtered through 0.45-μm Durapore membrane filters (Millipore, Bedford, MA, U.S.A.) and degassed prior to use. All solvents were of HPLC grade, including HPLC water (May and Baker, Dagenham, U.K.).

Sample preparation: complete tryptic digest samples and individual peptides were dissolved in either solvent A or in solvent containing 10% (v/v) aq. acetonitrile (pH 2.1). All samples were filtered prior to injection through 0.45-μm HV4 filter units (Millipore).

Running conditions were: flow-rate, 0.8 ml/min; pressure, 30–35 bar; sample size, 20 or 100 μl; gradient 1, 10–50% aq. acetonitrile at 1%/min; gradient 2, 0–25% aq. acetonitrile at 1%/min, followed by 10 min at 25% acetonitrile.

RESULTS

Preparation and identification of individual tryptic peptides from β-casein

A tryptic digest of β-casein (the sequence of genetic variant A^2 is shown in Table I) under the conditions described above resulted in more than the theoretical number of fragments. The theoretical fragments are listed in Table II. Sixteen peptides were separated by HVPE methods and subsequently identified by N-terminal analysis, total hydrolysis, and Edman degradation. These are shown in Table III. It is clear that some bonds are less readily hydrolysed than others, and this has resulted in the incomplete degradation of β-casein. Undigested β-casein and the large expected fragment EF12 did not redissolve after freeze-drying and, as a consequence, EF12 was not isolated. Thirteen of the sixteen peptides thus identified were singly subjected to RP-HPLC under the conditions described above. The three remaining peptides, namely EF7a, EF8/9, and EF10, were not available in sufficient quantities to permit further analysis by RP-HPLC. Several of the peptides isolated as a single spot by HVPE were shown to be impure by RP-HPLC, indicating that HPLC is considerably more sensitive than conventional methods in terms of resolution and detection.

TABLE II

THEORETICAL PRODUCTS OF TRYPTIC DIGEST OF β-CASEIN

EF = expected fragment. * Denotes bond which is hydrolysed under certain conditions[18].

No.	*Sequence*	*Residues*
EF1	R	1
EF2	E-L-E-E-L-N-V-P-G-E-I-V-E-Z-L-Z-Z-Z-E-E-S-I-T-R	2–25
EF3	I-N-K	26–28
EF4	K	29
EF5	I-E-K	30–32
EF6	F-Q-Z-E-E-Q-Q-Q-T-E-D-E-L-Q-D-K	33–48
EF7a/b	I-H-P-F-A-Q-T-Q-S-L-V-Y-P-F-P-G-P-I-P-N-*-S-L-P-Q-N-I-P-P-L-T-Q-T-P-V-V-V-P-P-F-L-Q-P-E-V-M-G-V-S-K	49–97
EF8	V-K	98–99
EF9	E-A-M-A-P-K	100–105
EF10	H-K	106–107
EF11	E-M-P-F-P-K	108–113
EF12	Y-P-V-Q-P-F-T-E-S-Q-S-L-T-L-D-V-E-N-L-H-L-P-P-L-L-L-Q-S-W-M-H-Q-P-H-E-P-L-P-P-T-V-M-F-P-P-Q-S-V-L-S-L-S-Q-S-K	114–169
EF13	V-V-P-V-P-E-K	170–176
EF14	A-V-P-Y-P-Q-R	177–183
EF15	D-M-P-I-Q-A-F-L-L-Y-E-Q-P-V-L-G-P-V-R	184–202
EF16	G-P-F-P-I-I-V	203–209

TABLE III

ACTUAL TRYPTIC FRAGMENTS OF β-CASEIN

Hydrophobicities calculated after Bigelow[21].

No.	*Residues*	*HPLC*	*Acetonitrile (%)*		*Average hydrophobicity*
			Single peptide	*Total digest*	
EF1/2	1–25	+	30.0	30.0	0.84
EF2	2–25	+	32.5	32.0	0.85
EF3	26–28	+	19.0	18.0	1.48
EF4/5	29–32	+	24.0	23.0	1.49
EF6	33–48	+	28.2	27.2	0.44
EF7a	49–68	–	–	–	–
EF7b	69–97	+	44.0	45.0	1.49
EF8	98–99	+	27.0	26.0	1.60
EF8/9	98–105	–	–	–	–
EF9	100–105	+	20.5	21.5	1.15
EF10	106–107	–	–	–	–
EF11	108–113	+	35.7	36.5	1.78
EF13	170–176	+	312.5	31.2	1.69
EF14	177–183	+	29.0	29.5	1.61
EF15	184–202	+	40.5	40.2	1.56
EF16	202–209	+	48.7	48.5	2.21

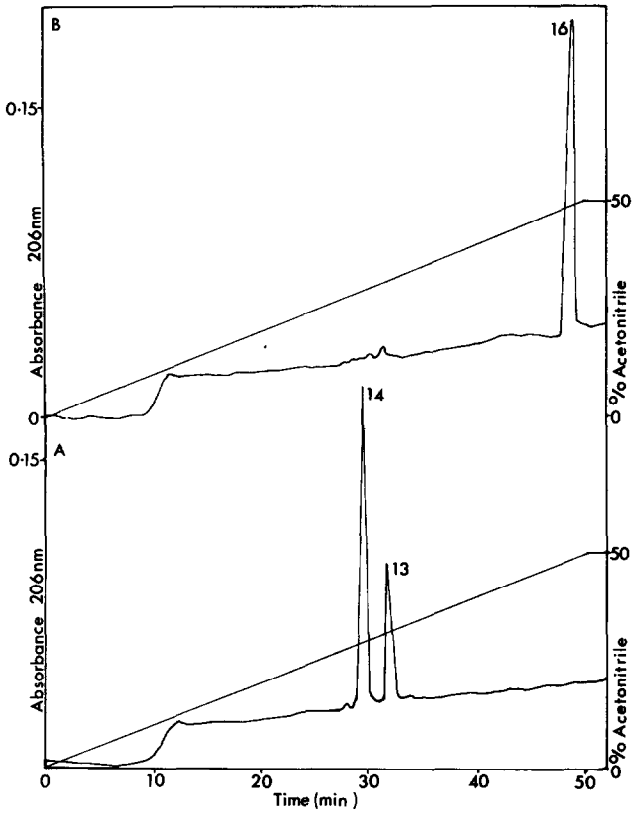

Fig. 1. Analysis of individual tryptic peptides (20 μg) of β-casein by RP-HPLC after preparation by HVPE. (A) EF13 and EF14 (20 μg); (B) EF16 (20 μg).

Fig. 1A shows the separation of EF13 and EF14 by RP-HPLC which ran as a single spot on HVPE. The hydrophobic C-terminal peptide EF16 was, in contrast, relatively pure after preparation by HVPE and this can be seen in Fig. 1B.

Table III shows the percentage acetonitrile at which the individual fragments were eluted from the C_8 column. Generally, the peptides behaved predictably according to their average hydrophobicity. However, peptides containing phosphoserine residues, *e.g.*, EF1/2, EF2, and EF6. and large peptides, *e.g.*, EF7b and EF15 were more strongly retained than expected. From these results, it was possible to predict where in the chromatogram of a sample of total tryptic digest an individual peptide would be eluted.

Analysis of total tryptic digest by RP-HPLC

Tryptic digest supernatant (100 μg) was loaded onto the C_8 column and a gradient of acetonitrile increasing at 1%/min from 10% up to 50% was applied. Fig. 2A shows the chromatogram obtained. The peak numbers refer to the expected fragments listed in Table III. There are seven major peaks, eluted between 25 and 50% acetonitrile, and a large number of smaller peaks. The identity of all the major peaks

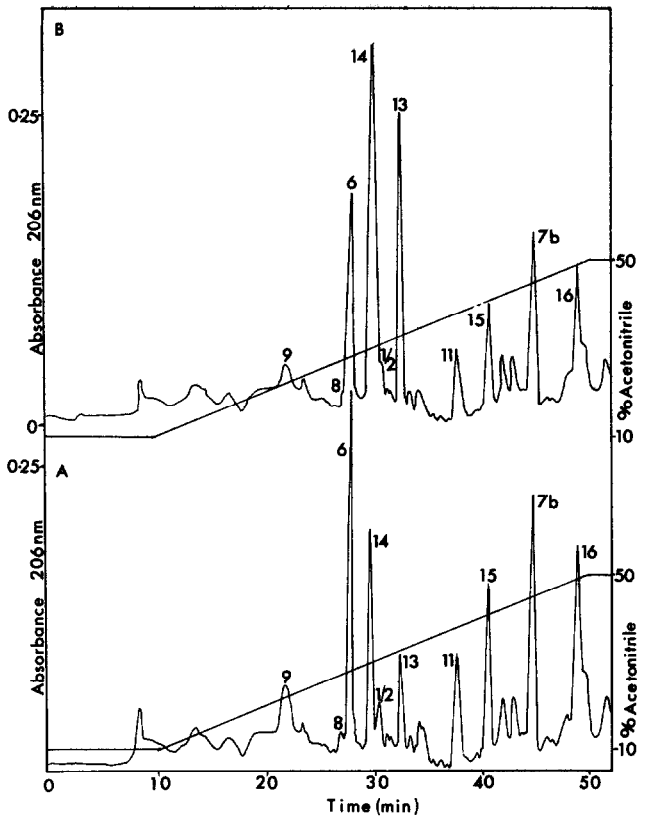

Fig. 2. Analysis of tryptic digest of β-casein by RP-HPLC. (A) Total trypotic digest (100 μg); (B) peak enhancement (80 μg tryptic digest + 20–30 μg individually prepared peptides EF13 and EF14).

was established by two methods: (A) collection and identification by N-terminal analysis and in some cases partial Edman degradation, and (B) peak enhancement. The position at which the remaining six fragments eluted was established by peak enhancement alone.

(A) Collection of peaks with the peak detection system on the Superrac fraction collector from a total of ten consccutive analyses of 100 μg digest per run yielded sufficient material to allow the identity of the seven major peaks to be established by N-terminal analysis and partial Edman degradation

(B) The second method employed to determine the identity of the peaks was by peak enhancement. Complete tryptic digest and aliquots of the conventionally purified peptides were injected together and it was observed which peaks in the digest were enhanced. In this way the identity of the major peaks was confirmed. The enhancement of the second and third major peaks corresponding to fragments EF13 and EF14 can clearly be seen in Fig. 2B when compared to the unenhanced chromatogram in Fig. 2A. The resolution of peptides eluted by less than 25% acetonitrile was not particularly good, and for this reason a modified gradient was employed to separate these fragments. A gradient of 0–25% acetonitrile at 1%/min was applied.

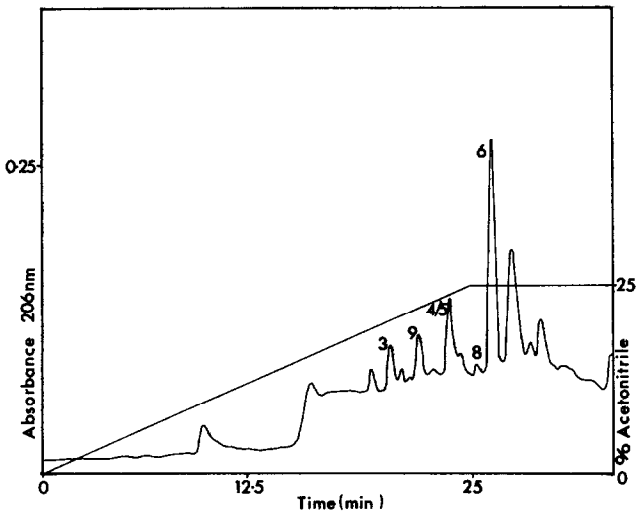

Fig. 3. Analysis of tryptic digest of β-casein (50 μg) using a modified gradient to resolve peptides co-eluting between 10 and 25% acetonitrile.

The result is shown in Fig. 3. There is improved separation of the peptides eluted between 10 and 25% acetonitrile, and use of this gradient allowed the establishment of the elution position of three tryptic peptides, namely EF3, EF4/5, and EF9, by peak enhancement.

DISCUSSION

The number of theoretical fragments from a tryptic digest of β-casein is sixteen, including two single amino acids, arginine and lysine, and the large fragment EF12. The actual number of peptides identified was sixteen, but this excluded the single amino acids and the large fragment. A short digestion time was selected to minimise the possibility of autolysis and non-specific hydrolysis of peptide bonds. This resulted in an incomplete digestion after 4 h. Trypsin hydrolyses arginyl and lysyl peptide bonds, but the rates of hydrolysis can vary considerably. The presence of an acidic residue lowers the rate of hydrolysis[20]. This could account for the failure to hydrolyse completely the bond between residues 1 and 2 (arginine and glutamic acid), giving rise to both fragments, EF1/2 and EF2.

Preparation of proteolytic fragments by conventional methods was time-consuming (around five days) and in some cases did not yield pure peptides. An example of this is the electrophoresis of a mixture of fragments EF13 and EF14 in HVPE. Determination of the N-terminal residue revealed the presence of two dansyl amino acids. However, these two peptides were well separated by RP-HPLC (Fig. 1A). Some fragments were not resolved by the reversed-phase column, but separation was achieved by modifying the elution gradient as shown in Fig. 3. This demonstrates the flexibility of HPLC; a major advantage in separations of complex mixtures of peptides. Method development is very rapid, given that single runs take around 60 min

for the gradients described above. Separating conditions were developed with the aim of obtaining a method by which the major proteolytic fragments of β-casein could be rapidly fingerprinted, and therefore it was not essential to achieve complete separation of all fragments. Better separations could have been achieved by using slower gradients and varying the flow-rate; the gradients chosen represent a compromise between the best possible separation and running time. The reproducibility of this method was excellent, thus allowing the repeated collection of material for further analyses.

Identification of the fragments subsequent to separation by RP-HPLC was carried out by two methods, N-terminal analysis and a partial Edman degradation. In all cases, sequencing the terminal three residues was sufficient to confirm the identity of the fragment. Recoveries of the individual fragments separated by conventional methods varied considerably in that in some cases the amount of pure peptide available was sufficient for further analyses by RP-HPLC, whilst in other cases there was only sufficient material to carry out identification of the peptide. Losses during preparation by conventional methods can be primarily attributed to absorption on the preparative HVPE papers. In contrast, the recoveries from the HPLC column were high. It was unclear why some peptides were present in larger quantities than others in the complete tryptic digest. Seven major peaks were observed and a large number of smaller peaks indicating that only small amounts of peptides such as EF1/2 were present. This could be due to losses occurring prior to chromatography, such as during freeze-drying and sample preparation.

RP-HPLC separates peptides on the basis of hydrophobicity. There is a general correlation between bitterness and hydrophobicity and, therefore, RP-HPLC may offer a method for rapidly separating bitter from non-bitter peptides. The C-terminal peptide, EF16, released by the action of trypsin, has been previously identified as a bitter peptide[2]. This peptide was readily isolated by RP-HPLC being eluted at high concentrations of the organic solvent. Champion and Stanley[22] used a two-stage purification scheme with either conventional gel chromatography or RP-HPLC as the step following extraction with organic solvents to separate bitter peptides from cheese. These investigators concluded that separation of the components of the bitter extract from cheese by RP-HPLC was effective, despite the complexity of the mixture (over 71 components), although none of the fragments were identified. They also concluded that the retention of peptides in this complex mixture was influenced not only by hydrophobicity but also by molecular weight. In our study it has been observed that larger peptides (> 1500 dalton) were more strongly retained than would be predicted by their hydrophobicity, thus indicating that other factors, such as pore size of the column and size of the peptides may also influence separation. However, there was a reasonable correlation between retention and hydrophobicity of most of the individual peptides, and this is consistent with results of chromatography of both single peptides and complex mixtures.

We have shown that RP-HPLC offers a rapid and sensitive means of fingerprinting proteolytic fragments when compared with conventional methods*.

It is hoped that RP-HPLC separations of proteolytic fragments can be used in studies of bacterial proteinases of unknown specificity and that it may be useful technique for the food industry.

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* Similar results have been reported very recently by Carles and Ribadeau-Dumas[23].

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REFERENCES

1 T. Matoba, R. Hayashi and T. Hata, *Agric. Biol. Chem.*, 34 (1970) 1235–1243.
2 N. Minamiura, Y. Matsumura, J. Fukumoto and T. Yamamoto, *Agric. Biol. Chem.*, 36 (1972) 588–595.
3 J. J. Sullivan and G. R. Jago, *Austr. J. Dairy Techn.*, 27 (1972) 98–104.
4 K. M. Clegg, C. L. Lim and W. Manson, *J. Dairy Res.*, 41 (1974) 283–287.
5 G. P. Dimenna and H. J. Segall, *J. Liq. Chromatogr.*, 4 (1981) 639–649.
6 P. Bican and B. Blanc, *Milchwissenschaft*, 37 (1982) 592–593.
7 B. B. Gupta, *J. Chromatogr.*, 282 (1983) 463–475.
8 A. C. M. van Hooydonk and C. Olieman, *Neth. Milk Dairy J.*, 36 (1982) 153–158.
9 J. Mottaar, R. Van Renterghem and J. De Vilder, *Milchwissenschaft*, 40 (1985) 717–721.
10 L. L. Diosady, I. Bergen and V. R. Harwalkar, *milchwissenschaft*, 35 (1981) 671–674.
11 R. J. Pearce, *Austr. J. Dairy Techn.*, 38 (1983) 114–117.
12 P. Bican, *J. Dairy Sci.*, 166 (1983) 2195–2197.
13 C. Carles, *J. Dairy Res.*, 53 (1986) 35–41.
14 V. Monnet, D. Le Bars and J.-C. Gripon, *FEMS Microbiol. Lett.*, 36 (1986) 127–131.
15 L. Huber and H. Klostermeyer, *Milchwissenschaft*, 29 (1974) 449–454.
16 S. Visser, K. J. Slangen and G. Hup, *Neth. Milk Dairy J.*, 29 (1975) 319–334.
17 W. N. Eigel, J. E. Butler, C. A. Ernstrom, H. M. Farrell, V. R. Harwalkar, R. Jenness and R. McL. Whitney, *J. Dairy Sci.*, 67 (1984) 1599–1631.
18 J.-P. Pelissier, *Sciences des Aliments*, 4 (1984) 1–35.
19 A. T. Andrews and E. Alichanidis, *J. Dairy Res.*, 50 (1983) 275–290.
20 G. Allen, in T. S. Work and R. H. Burdon (Editors), *Sequencing of Proteins and Peptides. Laboratory Techniques in Biochemistry and Molecular Biochemistry*, Elsevier, Amsterdam, New York, Oxford, 1981, pp. 109–116, 168–188.
21 C. C. Bigelow, *J. Theor. Biol.*, 16 (1967) 187–211.
22 H. M. Champion and D. W. Stanley, *Can. Inst. Food Sci. Techn. J.*, 15 (1982) 283–288.
23 C. Carles and B. Ribadeau-Dumas, *J. Dairy Res.*, 53 (1986) 595–600.