ELSEVIER

Journal of Chromatography A, 881 (2000) 1–21

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Application of chromatography and mass spectrometry to the characterization of food proteins and derived peptides

Joëlle Léonil*, Valérie Gagnaire, Daniel Mollé, Stéphane Pezennec, Saïd Bouhallab

INRA Laboratoire de Recherches de Technologie Laitière, 65 Rue Saint Brieuc, 35042 Rennes Cedex, France

Abstract

The following review describes the development of mass spectrometry off-line and on-line coupled with liquid chromatography to the analysis of food proteins. It includes the significant results recently obtained in the field of milk, egg and cereal proteins. This paper also outlines the research carried out in the area of food protein hydrolysates, which are important components in foodstuffs due to their functional properties. Liquid chromatography and mass spectrometry have been particularly used for the characterization of food peptides and especially in dairy products.

Keywords: Reviews; Food analysis; Milk; Cereals; Eggs; Cheese; Liquid chromatography–mass spectrometry; Interfaces, LC–MS; Proteins; Peptides

Contents

*Corresponding author. Fax: +33-223-485-350.
E-mail address: jleonil@rennes.inra.fr (J. Léonil)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00071-6

1. Introduction

Proteins are important components in food, for both their nutritional and functional values. Dietary proteins provide amino acids and nitrogen necessary for organisms. In addition, they are believed to have other specific functions owing to the presence of bioactive peptides in their primary sequences making them potential health-promoting ingredients [1,2]. Aside from this biological function, protein components play a major role in determining the sensory and textural characteristics of food products. These functional properties are related to their ability to form viscoelastic networks, to bind water, to entrap flavors, to emulsify fat and oil, and to form stable foams. The molecular basis of the functionality of food proteins is closely related to their intrinsic attributes that are size, amino acid composition, sequence and their physico–chemical characteristics (hydrophilicity/hydrophobicity, charge distribution), all of which vary with external environment. A certain number of food proteins such as milk proteins or gluten prolamins are characterized by a great heterogeneity with the presence of several molecular forms, more or less phosphorylated or glycosylated combined with a high degree of polymorphism. Increasingly, it is shown that each molecular species exhibits its proper functional behavior and certain proteins may have a greater contribution to functional properties in foodstuffs than others. This behavior can be modified during technological processing due to chemical modifications such as glycation via Maillard reaction occurring during heat treatments. It is, therefore, of paramount importance to relate structural and physico–chemical properties of food proteins and their functional properties so as to elucidate the molecular basis of their functionality. For this purpose, focus is now directed towards a better structural characterization of the food proteins.

In food protein analysis, liquid chromatography is the most widely used methods for analytic and preparative separations, commonly coupled with conventional UV and fluorescence detectors. Structural characterization of protein was gained by alternative methods such as amino acid composition, molecular spectroscopy, whereas SDS–electrophoresis or ultracentrifugation were used for mass determination with an accuracy for SDS–PAGE, ranging from about 0.5% for globular protein to about 20–30% for a heavily glycosylated protein. The recent advent of ''soft'' ionization techniques, starting in 1981 with the introduction of fast atom bombardment (FAB), followed by electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) has made such analyses by mass spectrometry possible for proteins and peptides, with (0.01%) high accuracy. The last two methods have quickly become important tools for the detection and characterization of large biomolecules, because of their sensitivity, high mass range, and the capacity of analyzing unseparated complex mixtures. In addition, mass spectrometry (MS) analysis can be extended by collision-induced fragmentation as processed by tandem instruments (as a triple quadrupole system) or newly design interfaces (as MALDI source) to give structural information and characterize and locate molecular events such as post-translational or chemically induced modifications.

Amongst food proteins, numerous researches in MS have been reported on milk proteins during the last 10 years and to a lesser extent on egg proteins. Comparatively few studies were found on other food proteins arising from plants and meat. Several reasons related to characteristics of these proteins can explain this lack, amongst them, their insolubility, their high molecular mass, a great heterogeneity due to the presence of several isoforms, a poor ionization due to the low content of charged amino acid residues.

This review will deal with the application of liquid chromatography off-line with ESI, MALDI, FAB and on-line with ESI and flow-FAB in the area related to research and development of food proteins and derived peptides. Applications will concern proteins and derived peptides of milk as well as some studies reported on egg and cereal proteins to

date. Using MS in combination with other biochemical methods, substantial progresses have been realized in the analysis of hydrolysis products arising from breakdown of caseins in cheese, allowing to better understand the ripening phenomenon. This example is given as an illustration of the performance of MS in analysis of complex food samples.

A brief survey of the different techniques of ionization (FAB, ESI and MALDI) is included since excellent reviews have recently been published thereon and will be mentioned here. A comprehensive review on the innovative techniques and instrumentations in MS has been recently written by Burlingame et al. [3].

2. Mass spectrometry

This section is not intended to be a detailed description of all the characteristics of FAB-, ESI- and MALDI-MS. We will place emphasis on aspects related to liquid chromatography when on-line coupling is achievable and on the analytical and instrumental improvements making these techniques indispensable tools for the characterization of food proteins.

2.1. Fast atom bombardment mass spectrometry

FAB-MS is one of the pioneering desorption techniques that has contributed to substantial progress in the mass determination of peptides and small proteins with masses larger than 10 000 on magnetic sector instruments [4]. The general characteristics of FAB have been extensively discussed in detail and recent reviews about the technology and analytical aspects are referenced here: [5–7].

Briefly, the analyte of interest is mixed with a suitable matrix, usually glycerol. Ionization is made by bombardment with an energetic beam of atoms or ions (typically Xe or Cs^+ at 8–40 keV). FAB has been successfully interfaced to liquid chromatography for analysis of peptides. In continuous-flow (CF) FAB, the sample is continuously introduced, via a split effluent steam, at a flow-rate of 3 up to 10 μl/min into the source of the mass spectrometer. The sample is dissolved in volatile solvents (mixture of water, acetonitrile or methanol) containing a few liquid matrix-FAB. CF-FAB has the advantage to decrease the ion suppressing effect often observed for hydrophilic peptides, and the matrix-derived background. FAB-MS and related techniques such as CF-FAB and (stainless steel frit) frit-FAB have some drawbacks that have been recently reviewed by Nguyen et al. [8]. Today, the development of ESI-MS, more easily interfaced to liquid chromatography, has made the latter technique an attractive alternative to CF-FAB-MS.

2.2. Electrospray ionization mass spectrometry

The development of electrospray ionization has been covered in detail in recent reviews [9–11].

In electrospray, analytes are ionized at atmospheric pressure directly from a flowing liquid stream, and the ions produced are then directed into the mass spectrometer. Liquid from either an infusion pump or HPLC effluent enters into the atmospheric pressure ionization source through a capillary restriction held to high voltage (±3–5 kV). The high electric field at the tip of the needle causes the solution to disintegrate into an aerosol plume of very small electrically charged droplets, a process referred to as electrospray. Eluent usually used is water containing an organic solvent (such as acetonitrile, methanol or propanol) and few of a weak volatile acid (1–5%, trifluoroacetic, acetic acid or formic acid) or base (ammonium hydrogencarbonate, ammonia solution) to promote ionization of sample constituents as positive or negative ions, respectively. Flow-rate range from 0.5 to 200 μl/min is usually employed in a conventional electrospray source. The highest flows require the assistance of pneumatic nebulization to promote the formation of uniform small charged droplets, a process known as pneumatically-assisted electrospray (atmospheric pressure chemical ionization, APCI). Desolvation of charged droplets is achieved through the controlled use of heating, differential pumping or countercurrent gas flow, usually N_2 or pure air. By this procedure, ions are emitted directly from these liquid droplets and are subjected to mass analysis. At the present time, the theoretical investigation into the ESI mechanism has led to the development of nanoelectrospray, in which the flow-rate is established by the electrostatic force and therefore is independent of the solvent delivery

[12]. In this way, the stability of flow is obtained at flow-rates of 20–40 nl/min into the source.

The electrospray process produces multiply-charged molecular species. Whether positively or negatively charged species are detected, depends on the instrument polarity. Most proteins can gain a high number of charges due to the presence of charged amino acid residues [9]. Hence a mass analyser that can measure m/z values in the range 1–2500/3000 as a quadrupole mass spectrometer is usually sufficient to analyse molecules with absolute masses as large as 150 000. The molecular mass of the protein may be calculated by deriving the charge states of any two adjacent ions in the series. The mass of any multiply charged molecular ions is determined by multiplying the m/z ratio at which the ion is observed by the derived charge, z. A comprehensive review on the algorithms used for the mass calculation has been published by Mann et al. [13].

Mass assignment accuracies of 0.01% are commonly obtained. Electrospray can also be interfaced to other mass analyzers allowing to gain higher resolving powers such as Fourier transform ion cyclotron resonance [14], magnetic sector [15], time-of-flight [16] and ion trap mass spectrometers [17]. Accuracies of 0.001% or better should be obtainable with such mass spectrometers.

Because ESI requires a constant delivery of liquid, it can be easily coupled with liquid-based separation systems such as HPLC. Due to the limited amount of solvent which can be tolerated for a good efficiency of the ionization process, a splitter is commonly incorporated for conventional (2.1 or 4.6 mm I.D.) columns that allows a suitable flow-rate (2–40 μl/min) of the mobile phase to form small droplets. Splitting of the flow into the mass spectrometer can be accomplished with either a calibrated length of deactivated fused-silica [7] or a low dead volume T-connector [18]. In both cases, the splitting of flow is done from the HPLC column effluent, just prior to MS allowing one to connect a UV detector and/or fraction collector on the split line. Le et al. [19] have described the use of a commercial splitter, which can generate a steady split flow-rate down to a few nanoliters per minute from an eluent flow-rate of up to 0.5 ml/min. Another arrangement was proposed by Davis et al. [20], who use preformed gradients to circumvent the limitations of flow splitting.

The development of small-bore and capillary liquid chromatography columns allows one to decrease the split ratio and even to suppress the splitter in the last case [21]. Very recently, newly designed interfaces have been developed that are able to handle eluent flows of up to 2 ml/min [22–24].

The advantages of on-line LC–MS include an increase of signal-to-noise ratio by removing background signals and increasing the ion signal due to the concentration of species in a single peak. In addition, complete chromatographic resolution is not required to determine molecular mass. On-line LC–ESI-MS allows a rapid mass determination feasible without the need for laborious and time-consuming fractionation and purification steps. ESI mass determination is usually carried out in a few minutes. On-line LC–ESI-MS includes the time necessary for LC separation, which may range from 15 to 60 min or more.

Liquid chromatography techniques can be combined with tandem mass spectrometry (MS–MS) for enhancing the capacity of electrospray ionization to elucidate the structure of protein by sequence assignment from peptide mapping. An overview of emerging techniques and methodologies for identification of proteins and peptides by MS–MS has been published recently by Dongré et al. [25].

2.3. Matrix-assisted laser desorption mass spectrometry

MALDI is a laser desorption mass spectrometry technique introduced in 1988 by Karas and Hillenkamp [26]. Briefly, the sample is first mixed in solution, with a large excess of a suitable matrix, typically a low-molecular-mass organic acid and introduced on a target, into the mass spectrometer source. When the matrix is a crystalline compound, a good co-crystallization of sample and matrix, occurring during the evaporation of the solvent, is essential to have an efficient ionization of the sample. The sample and matrix mixture is irradiated with a nitrogen laser beam operating usually at 337 nm. The function of the matrix is to absorb the laser energy and ionize the sample. Each pulse from the laser

vaporizes both sample and matrix from the surface of the target and provokes a chemical reaction, which is not yet clearly understood but results in the cationization of the sample with a proton $[M+H]^+$ or an alkali metal atom (Na^+, K^+) in the positive ion mode, whereas removal of a proton $[M-H]^-$ is produced in the negative ion mode. Proper choice of the matrix is crucial to obtaining good MALDI-MS spectra. The choice of the matrix is function of the analyte structure, and numerous studies have been devoted to find appropriate matrices for each type of molecules. For proteins, the choice also depends on their molecular mass [27]. The most commonly matrices for MALDI analysis of peptides are cinnamic acid derivatives (α-cyano-4-hydroxycinnamic) and benzoic acid derivatives (2,5-dihydroxybenzoic acid), whereas sinapinic acid (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid) is preferentially used for proteins [28,29].

Sample sizes are usually in low pmol range (e.g., 0.1–10 pmol/μl) and sample-matrix volume of about 1–2 μl with a large excess (about 500–50 000-fold) of the matrix. To promote its ionization, the sample is dissolved in a mixture of acetonitrile and 0.1% trifluoroacetic acid. Alkali metals such as silver salts can also be added to the sample solution. For food protein analysis, a great advantage of this ionization technique is its tolerance to the presence of salts and other additives such as detergent in the buffer, in contrast to electrospray ionization technique. However, excessive amounts of salts can also cause signal suppression. To circumvent this problem, several solutions have been proposed to remove contaminants, as the loading of samples onto synthetic membranes [30], RP beads [31] or microseparation on a C_{18} RP chromatographic bed [27].

Because of the pulsed nature of MALDI, this technique is most conveniently coupled to a time-of-flight (TOF) mass spectrometer, abbreviated generally as MALDI-TOF. The sample ions are accelerated in an electric field and allowed to drift through a field-free region to a detector. MALDI usually produces singly charged ions, but higher charge states can also be observed. The time of flight for the ions is measured to yield the mass spectrum. A recording of the detector signal as a function of time constitutes a TOF mass spectrum. The difference between the start time common to all ions, and the arrival time of an individual ion at the detector is proportional to $(m_i/z_i)^{1/2}$ and therefore can be used to calculate the ion mass. The sample is bombarded with laser pulses (1–200 ns range). The time to accumulate a spectrum for a protein is based on the accumulation of laser shots (typically 10–100). The typical mass resolution for protein expressed in units of full width at half maximum ($m/\Delta m$) is in the range of 200–500 for with accuracies of 0.01% for linear TOF instruments. Proteins with molecular masses over 100 000 are able to be desorbed/ionized by MALDI [32,33].

Recent instrumental improvements of MALDI-TOF have introduced time-lag focusing (or delayed extraction, DE) and reflectron allowing to gain in both resolution and mass accuracy with respect to other mass analyzers. The procedure of delayed extraction [34,35] can be described as follows: after desorption/ionization, the ions are kept in the ion source under a field-free conditions for a short period of time, i.e., 100–400 ns, before they are extracted with a high electrical field and accelerated towards the detector. During the brief time between ion desorption and the extraction pulse, for the peptides for example, peptide ions can undergo prompt fragmentation within the source, process known as ion source decomposition (ISD). Delayed extraction allows one to reduce the initial velocity distribution of ions produced into the source. Thereby, this technology considerably improves instrument resolution by separating the ion desorption process from the ion accelerating one. It will be noted that fragment ions are also produced after leaving the ion source during the travel in the first field-free drift path of the instrument, process known as post-source decay (PSD) [36]. The reflectron added to the DE technology allows to discriminate ions as a function of their kinetic energies through a curved arc using an electrostatic field or curved-field reflector. Large ions with a greater kinetic energy penetrate further into the field than smaller fragment ions and therefore had a longer path to the detector. In reflectron MALDI-TOF-MS performed under DE conditions, a mass resolving power better than 10 000 has been observed, and mass accuracies below 5 ppm. With such an accuracy, the PSD ion spectra are able to

give structural information on peptides up to mass 3000 [37]. An account on these instrumental improvements has been recently published by Chaurand et al. [38].

Continuous-flow (CF)-MALDI has been tempted but the sole reports concern communications in congresses (41st and 42nd ASMS Conferences on Mass Spectrometry). As an alternative to on-line HPLC–MS that is not applicable to MALDI analysis to date, studies have demonstrate its ability to analyze proteins separated from two-dimensional electrophoresis directly by scanning of gels without the need for membrane blotting [39].

3. Applications

3.1. Milk proteins

Milk is a complex biological fluid including water (87.3%), proteins (3.2%), carbohydrates specially lactose (4.6%), fat (3.9%) and a mineral fraction (0.7%). Proteins in cow's milk are usually divided into two major groups: about 79.5% of caseins characterized by their insolubility at pH 4.6 and 20°C, and about 19.3% of whey proteins. Another class exits as a minor fraction (about 1.2%), which are proteins associated with membrane of fat globules, enzymes and proteins arising from blood and a polypeptide fraction (named proteose–peptone) [40].

Almost all caseins in milk are organized in casein micelles with an average diameter of about 120 nm containing 93% proteins and 7% inorganic salts, mainly calcium and phosphate. The structure of the micelles is not yet well established and numerous models have been proposed (for recent review, see Ref. [41]). The casein micelles contain the four caseins: α_{s1}-, α_{s2}-, β- and κ-caseins in an approximate ratio of 4:1:3.5:1.2. Caseins are phosphoproteins characterized by extensive polymorphism, hence the occurrence of numerous genetic variants. Polymorphism induces several molecular forms of the same protein sequence that differ by either a single or a limited number of amino acid residues [42]. Great heterogeneity of the caseins is also associated with the variation in number and location of phosphate and glycosyl groups attached to the polypeptide chain.

Whey contains proteins soluble at pH 4.6. The main components in bovine milk are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), serum albumin (BSA), immunoglobulins (Igs) and low-molecular-mass peptides derived by proteolysis of some caseins, which represent approximately 3.2, 1.2, 0.4, 0.7 and 0.8 g/l, respectively. There are many other minor proteins, including lactoferrin (Lf), and several enzymes (lipoprotein lipase, acid and alkaline phosphatases, lysozyme, lactoperoxydase . . .), growth factors and hormones. The primary sequences of the most of these proteins are known [43].

Many amongst milk proteins exhibit biological activities such as iron (Lf), fatty-acid binding (β-Lg) properties as well as protective (Ig, lysozyme) effects in newborn. Otherwise, milk proteins are known to be precursors of biologically active peptides as illustrated in Section 3.4 [1].

Milk proteins are usually separated into classes before chromatographic analysis. Acid precipitation at pH 4.6 or rennet coagulation allows to keep whey proteins in solution. However, other methods such as ultra-centrifugation, gel filtration as well as membrane technologies (ultrafiltration, microfiltration) on a large scale can be used to separate whole caseins from whey proteins [44]. All the chromatographic methods applied to the separation of milk proteins have been reviewed by Strange et al. [45].

3.1.1. Mass spectrometry applied to milk proteins

There is a broad diversity in the objectives for which MS analysis has been applied to studies on these food proteins, including the determination of molecular mass for complete identification, ligand binding, peptide sequencing from enzymatic mapping for structural characterization. Most of the reports concern the determination of molecular masses of chromatographically purified proteins analyzed by direct infusion in ESI-MS.

ESI-MS analyses have been successfully applied to all bovine major milk proteins including caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) and whey proteins (α-La, β-Lg). ESI-MS analysis of caseins takes advantage of the fact that these proteins generally give strong signals both in negative and positive ion mode based on their high contents in acid (aspartic

and glutamic acids and phosphoric groups) and basic (arginine, lysine, histidine) amino residues, respectively. Nonetheless, ESI-MS analysis of bovine α_{s1}-casein showed a better sensitivity in the negative ion mode [46].

Even if the initial interest of ESI-MS lies in the confirmation of identity of proteins, whose primary structures are known, numerous genetic variants have been fortunately evidenced, based on the mass difference between the predicted and measured M_r. Two new genetic variants of bovine β-casein named F [47] and G [48] have been recently characterized using ESI-MS combined along automated Edman degradation chemistry after proteolytic digestion and chromatographic separation of peptides. By the same approach completed by FAB, five α_{s1}-casein variants have been determined in ovine milk [49,50]. Post-translational modifications occurring in ovine β-casein were characterized by Chianese et al. [51] from the ESI-MS analysis of whole casein. Based on a mass difference of 80 u between M_r values calculated from several ion series, Chianese et al. [51] revealed several degrees of phosphorylation for ovine β-casein with a number of phosphate groups per molecule of casein ranging from 1 to 5. The growing interest for the research of genetic variants using MS analysis is easily explainable by the extensive genetic polymorphism occurring in most major milk proteins and its impacts on the composition and functional properties of milk [52].

Few examples on on-line coupling between liquid chromatography and MS have so far been reported. This methodology becomes attractive when complex biological fluid such as milk is analysed. The first study of this type was conducted by Léonil et al. [18]. Milk diluted in a buffer containing 6 *M* urea and a reducing agent (dithiothreitol), both added for optimizing the separation, was injected on a RP-HPLC C_4 column (150×2.1 mm) at a flow-rate of 0.3 ml/min. The effluent was split in a 1:10 ratio before introduction into the mass spectrometer. Urea and contaminants, which hampered the efficiency of the ionization process, were eliminated by a bypass into the source according an arrangement described elsewhere [18]. Approximately 75 pmol per component was injected in order to have a practical full-scan MS. Fig. 1 illustrates the total ionization current obtained in the positive ion mode. Several

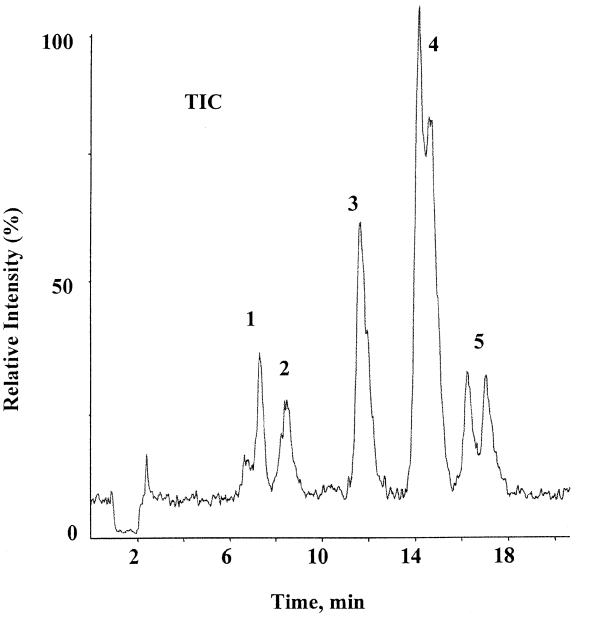

Fig. 1. Analysis by on-line RP-HPLC and ESI-MS of skim milk proteins. Chromatographic peaks noted 1 to 5 were detected by total ion current (TIC). Peak 1: one calculated mass of 19 038 corresponding to unglycosylated κ-casein variant A with 1P; Peak 2: two calculated masses of 19 007 corresponding to unglycosylated κ-casein variant B with 1P and 25 230 corresponding to α_{s2}-casein variant A with 11P; Peak 3: one calculated mass of 23 617 corresponding to α_{s1}-casein variant B with 8P; Peak 4: three calculated masses of 24 092, 24 025 and 23 985 corresponding to β-casein variants, B, A^1 and A^2 respectively; Peak 5: two calculated masses of 19 365 and 18 278 corresponding to β-lactoglobulin A and B. Figure adapted from Ref. [18].

masses were determined with a close correspondence with the theoretical masses. Several masses were detected in a single peak in RP-HPLC. It is noteworthy that all the major milk proteins except α-La were detected. The failure to detect α-La was a consequence of constraints imposed by the set conditions including the ''tuning'' conditions (i.e., interface conditions such as orifice tension as well as selection and scanning of mass range). This together with sample preparation conditions are known to be very dependent on analyzed proteins. Hence, the best conditions of ionization are not always achievable for each species in the mixture as illustrated by the case of α-La. Bovine α-La (M_r 14 177) has 17 basic residues and four disulfide bridges. The distribution of charge states of this protein in the mass range of the quadrupole analyser (up to 2500) was rather low,

with 4 (up 6^+ to 9^+) and 7 (up 6^+ to 12^+) charge states at m/z greater than 1500 using 0.1% TFA and 0.05% formic acid, respectively. After reduction of the four disulfide bonds, up to 14 charge states can be obtained in positive mode due to unfolding of the protein [9,18]. Therefore, the charge distribution observed for α-La can provide useful information on the folding state of the molecule. Negative-ion ESI-MS has been evaluated for determining the calcium-binding stoichiometry of α-La [53]. α-La in aqueous solution yielded intense ion signals in the 1400–3000 mass range and specifically bound one Ca^{2+} ion per protein molecule, in agreement with previous results obtained with other physical methods.

The ability of ESI-MS to characterize chemical modifications of proteins during industrial process (separation, heating, concentration, drying) has been illustrated by several examples. Among them, it was shown that many reducing sugars can react with the ε-amino group of the lysine residue via the well-known Maillard reaction. Using ESI-MS, a specific glycation of β-Lg with lactose occurred during mild heat treatment of milk before whey separation [54,55]. With the same technique, the heterogeneity of β-Lg glycoforms was demonstrated with respect to the number of lactose residues linked per protein molecule. Such a chemical modification affected both the conformation of β-Lg and its association state, hence functional properties of the protein [56,57]. Ward and Bastian [58] identified by using ESI-MS in conjunction with ^{31}P-NMR, a large proportion of β-casein modified by dephosphorylation (up to 27% of the total protein) during industrial process of caseinate manufacture.

In contrast to ESI-MS widely used as seen above for milk proteins, the number of studies using MALDI is still very limited in spite of potentiality of this technique. However, the recent results obtained on MALDI-MS analyses of either chromatographically purified milk proteins or raw milk are promising. All major milk proteins including α_{s1}-, α_{s2}-, β- and κ-caseins as well as α-La, β-Lg and the polypeptide fraction ''proteose peptone'' were detected. MALDI provided a significant advantage over ESI to analyze such a unseparated mixture containing aside proteins, a high content (about 110 mmol/kg) of salt including potassium, sodium, calcium, phosphore and other minor mineral ions. A milk sample (200 μl corresponding to 250 pmol of total proteins) was dried and solubilized in an acid aqueous solution (0.1% TFA). After mixing with sinapinic acid used as matrix and drying in a cold air stream, the sample deposited on a stainless steel sample holder was introduced into the mass spectrometer. Marsilio et al. [59] and Catinella et al. [60,61] showed the ability of MALDI to rapidly evaluate the effects of heat treatment on these proteins from a single analysis. The mass measurement was carried out in a few minutes. A limitation of the analysis was the low resolution ($m/\Delta m<100$) of the used instrument. The recent improvement of the MALDI ion source design introducing delayed extraction allows one to considerably increase the ion resolution and the mass accuracy as discussed in Section 2.3.

3.2. Egg proteins

The hen egg proteins constitute about 10%, 16% and 13% of albumen, yolk and whole egg, respectively [62]. Proteins are the main constituents of egg albumen on a dry matter basis. Most of known egg-white proteins are glycoproteins, with the exception of lysozyme. Many of them exhibit biological activities such as iron- (ovotransferrin) or vitamin-binding properties (avidin, ovoflavoprotein, thiamin-binding protein), proteinase inhibitory activities (ovomucoid, ovoinhibitor, ovomacroglobulin, cystatin) or antimicrobial properties (lysozyme, E.C. 3.2.1.17). Lysozyme, from different species or of recombinant origin, is the foremost model in the research on protein structure and folding. The study of lysozyme structure gives rise to abundant literature which is beyond the scope of this review. Yolk contains lipoproteins (lipovitellin, low- and very low-density lipoproteins) and water-soluble proteins (phosvitin, livetin). Some of the minor components possess biological activities (immunoglobulin Y, cobalamin-, riboflavin- and biotin-binding proteins) [62–64].

The applications of liquid chromatography to the fractionation of egg-yolk and egg-white proteins have been recently reviewed [64].

3.2.1. Mass spectrometry applied to egg proteins

ESI-MS has been applied for the off-line characterization of egg-white proteins (lysozyme, ovotran-

sferrin) after separation by size-exclusion and anion-exchange liquid chromatography [65]. The homogeneity of lysozyme purified from egg white in a single step was unambiguously shown, the determined mass corresponding to the theoretical one deduced from lysozyme sequence. In the case of ovotransferrin (conalbumin), ESI-MS analysis revealed the microheterogeneity of the purified protein [65]. The presence of three major forms in the purified protein, most probably differing by their glycan components, was evidenced. It should be noticed that ovotransferrin, of molecular mass 77 000, is one of the model proteins which showed the potential of ESI-MS in mass spectrometry of high-molecular-mass, glycosylated macromolecules [66,67]. In a similar way, the characterization by ESI-MS of A1-ovalbumin purified from egg white by anion-exchange chromatography suggested that the ovalbumin fraction, although chromatographically and electrophoretically pure, consisted of four major ovalbumin glycoforms with a main form of mass 44 339, as shown in Fig. 2 (S. Pezennec and D. Mollé, unpublished results).

The interaction of size-exclusion-purified egg-white lysozyme with the divalent cations Cu^{2+} and Zn^{2+} has also been studied by using ESI-MS [68]. It was shown that the lysozyme ionization was not modified in the presence of a large molar excess of metal ions, and that lysozyme was able to form reversible complexes with up to eight copper ions and six zinc ions per molecule, without losing its enzymatic activity.

The irradiation treatment is employed in some countries for the elimination of bacteria from egg-products. Mass spectrometry can also constitute the mean of detecting the irradiation history of industrial egg products. As a matter of fact, it has been recently reported that the MS profile of purified major egg white proteins, ovalbumin and ovotransferrin, obtained by ESI-MS, was altered by prior γ irradiation [69]. However, in the same experiment, the lysozyme mass spectra were not significantly modified by irradiation.

As in the case of proteins from other sources, FAB and MALDI techniques have been used to characterize post-translational modification of egg proteins. FAB-MS has been used in conjunction with the known primary structure to identify the phosphorylation sites in the egg yolk riboflavin-binding protein [70]. Oligosaccharides, such as those found in the glycan moieties of glycoproteins, have been intensively studied in recent years due to their potential biological activity and pharmacological value. Mass spectrometry has been used in combination with other analytical techniques such as ^{1}H-NMR to

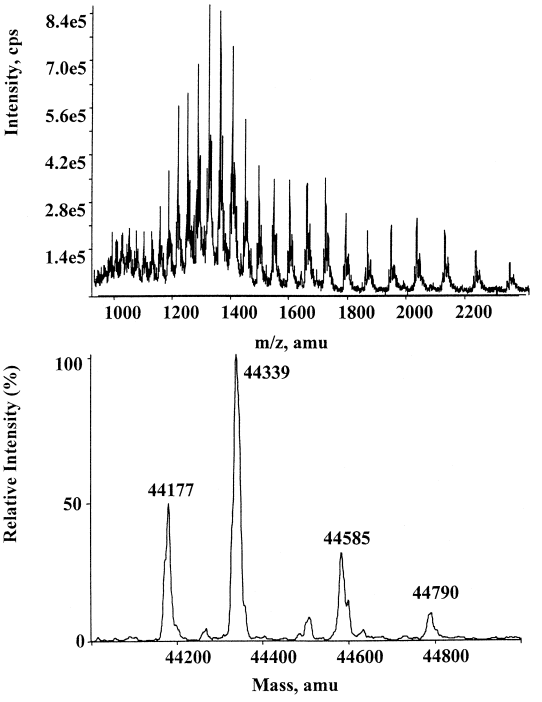

Fig. 2. ESI-MS spectra of ovalbumin purified by anion-exchange HPLC (S. Pezennec and D. Mollé, unpublished results). Egg albumen was diluted 10-fold in 50 m*M* Tris–HCl, pH 8.0 (buffer A) and gently stirred overnight at 4°C. Precipitated material was discarded. Proteins were separated on a Q HyperD column (10× 0.46 cm) equilibrated with buffer A by a linear gradient of NaCl concentration in the same buffer at a 1.0 ml/min flow-rate. Fractions corresponding to the peak of the main, diphosphorylated A1 form of ovalbumin were collected, and purified ovalbumin was dialyzed against ultrapure water. The mass spectrometry system was a API III^{+} triple quadrupole (Perkin-Elmer Sciex Instruments, Thornill, Canada) equipped with an articulated atmospheric pressure electrospray ionization source. Samples were diluted with 50% acetonitrile and 0.1% (v/v) TFA and the infusion ESI-MS analysis was performed at a flow-rate of 5 μl/min in the positive-ion mode with 4800 V ion spray voltage and a 70 V orifice voltage. The spectra of ovalbumin were obtained by accumulation of 25 individual scans. The molecular masses were determined from these data using the package software supplied by Sciex (Tune 2.5 Multiview 1.2).

characterize the glycan oligosaccharides of egg glycoproteins. The glycan structures of yolk immunoglobulin [71] and egg-white ovalbumin [72–74] have been studied by FAB-MS. In the case of hen egg-white ovalbumin [73,74], oligosaccharides released from tryptic glycopeptides by *N*-glycosidase F were separated by ion-exchange chromatography and derivatized to form tyrosinamide-oligosaccharides, suitable for subsequent radiolabeling and biological studies. After RP-HPLC purification, FAB-MS measurements were used to calculate a sugar composition of the different oligosaccharides, prior to the complete determination of their structure by ^{1}H-NMR. Using the same approach with MALDI, a sialylglycopeptide, isolated from hen egg yolk by successive steps of size-exclusion and ion-exchange chromatography, was characterized and proposed to originate from proteolysis of an egg yolk vitellogenin [75].

Mass spectrometry techniques have been used to study structural aspects of some egg proteins. The existence, in ESI-MS spectra of chicken lysozyme, of a bimodal charge state distribution, depending on the physico–chemical conditions and the ionization mode, showed the coexistence of different conformational states in the sample, unfolded conformers giving higher charge states (lower m/z) [76,77]. The lysozyme folding has also been studied by monitoring the hydrogen–deuterium exchange kinetics by ESI-MS [78]. Structural informations about egg proteins have also been obtained by the combination of mass spectrometry with other techniques including proteolysis and peptide mapping. Mizutani et al. [79] have used enzymatic digestion and mass profiling to identify the initial conformational changes of the isolated ovotransferrin *N*-lobe which lead to gelation upon extensive disulfure reduction, as in the case of the whole protein. After purification by ion-exchange chromatography, in combination with biochemical analyses, MALDI-TOF-MS was used to identify a chymotrypsin-resistant M_r 8000 fragment arising from the *N*-lobe part. This fragment, corresponding to the N-terminal region of the ovotransferrin *N*-lobe, keeps a native-like disulfide pattern and three-dimensional structure upon extensive reduction.

In a similar approach, the structural state of ovalbumin at acidic pH was investigated [80]. The protein was shown to be resistant to moderate pepsin digestion at pH 2.2. The mass of a small peptide fragment released under more drastic proteolytic conditions was measured by MALDI-TOF-MS after purification by RP-HPLC. The cleavage site was thus identified, and the protein was shown to exhibit a native-like fragmentation pattern. These results show that ovalbumin has a highly ordered structure at pH 2.2. Recently, the products of the digestion of wild-type ovalbumin and site-directed mutants by elastase have also been studied by ESI-MS, and the sites of proteolytic cleavage thus located in the primary structure [81].

3.3. Cereal proteins

Gliadins, hordeins, secalins and avenins constitute the alcohol-soluble prolamin fractions from wheat, barley, rye and oats endosperm, respectively. The prolamin fraction is a complex mixture of proteins differing by molecular mass (ranging from 30 000 to 80 000), isoelectric point and amino acid composition [82]. Hence, the complete separation and identification of the prolamin fraction are very difficult. Most of biochemical data on these storage proteins has been achieved through the extensive use of electrophoretic and chromatographic techniques. More recently, the introduction of mass spectrometry in the analysis of these proteins has allowed to obtain precise measurements of their molecular mass. Besides their functional properties, the alcohol-soluble proteins of the gluten fraction are toxic to patients with coeliac disease. In this research line, MALDI-MS and ESI-MS have been used to determine the molecular masses of purified avenins [83], gliadins [84], and high-molecular-mass fraction of glutenins [85] previously chromatographed by HPIEC and RP-HPLC. An interesting work was recently published allowing to evaluate the potential of MALDI-MS in the analysis of the unseparated prolamin fraction. Direct MALDI-MS analysis of unfractionated alcohol-soluble prolamin fractions extracted from four cereals (wheat, barley, rye, oat) yielded characteristic mass patterns, thus allowing the clear identification of each of the cereal prolamin fractions [86]. The MALDI-MS analysis was performed in a few minutes and required small amounts (0.05–0.1 mg) of sample. On the basis of these results, a procedure was developed to detect gluten gliadins directly in food by observing the characteristic gliadin mass pattern ranging from M_r 25 000 to

40 000 [87]. A quantitative procedure using MALDI-MS has been recently published [88]. In this study, the detection by MALDI-MS was compared with that obtained from enzyme-linked immunosorbent assay (ELISA) method. The procedure allowed the micro-quantification of gluten in food samples below levels toxic for coeliac patients, with a linear response in the 0.4–10 mg per 100 g of food range and a high detection sensitivity similar to that of ELISA system. It is noteworthy that a chemically related internal standard was integrated into the quantitative analysis to better monitor, amongst others, the reproducibility of mass spectra. The spot-to-spot variations due to an heterogeneous crystallization of sample and matrix can greatly affect quantitative data. The main critical points of the quantitative procedure have been discussed by the authors. Camafeita et al. [88] have found that the heat treatment led to dramatic changes in the MALDI-MS spectra of gliadins. They suggested that these modifications could be used to evaluate the alteration of gliadins in food during the baking process.

The high-molecular-mass (HMW) subunits of breadwheat glutenin are the most intensively studied group of cereal prolamins because of their association with bread-making quality. MALDI-MS was used to compare known protein sequences with those calculated from gene sequences. It was shown that the HMW subunits were not extensively glycosylated. In addition, MALDI allowed the detection of low-molecular-mass components in highly purified HMW subunit preparations, probably associated through strong non-covalent forces [89].

3.4. Food protein hydrolysates

Protein hydrolysates are made by breaking the peptide bonds in proteins to form mixtures of peptides, with various size and amino acids. This is achieved by the use of proteolytic enzymes. The properties of generated hydrolysates depend on the original proteins, the type of protease used for preparing the hydrolysate and the extent of hydrolysis [90]. Food hydrolysates can have functional, nutritional and biological applications. Commercially available hydrolysates are usually derived from milk proteins such as caseins and whey proteins because of their high nutritional value, and their commercial availability in large quantities with moderate cost. Functional applications include improved whipping, gelling, solubility or acid stability of formulated products [91]. Nutritional applications include increased digestibility in vivo and reduced allergenicity [91]. During the last decade, several studies have shown that food protein hydrolysates or peptides possess also biological properties such as the regulation of the immune system, the gastrointestinal functions, blood pressure or mineral absorption [1,92].

Whatever the target function, it must be always important to characterize protein hydrolysates on the basis of their physico–chemical properties: peptide size, free amino acids content, post-translational modifications (e.g., phosphorylation, glycosylation), chemical modifications occurring during food processing (e.g., glycation, oxidation, dephosphorylation).

Besides information on their composition, a better characterization of the hydrolysate needs the separation and identification of its peptides and amino acids. For the separation purpose, several methods may be used including electrophoretic (PAGE, capillary electrophoresis) and chromatographic techniques (size exclusion, ion-exchange, hydrophobic, and RP chromatography) [93]. After separation, peptides were identified by amino acid composition, sequence analysis by Edman degradation and mass spectrometry analysis.

3.4.1. Mass spectrometry applied to food hydrolysates

The application of HPLC and MS to food protein hydrolysates can be classified according to four different objectives: (1) determination of size distribution profile, (2) extraction and identification of bioactive components, (3) determination of the specificity of different proteases used in food industry, (4) localization of chemical or post-translational modifications in the primary structure. Whatever the objective, the introduction of MS in the analysis of food hydrolysates is linked to the great advantage, that components of a chromatographic peak may be resolved by mass even though they are not completely resolved by time. Fig. 3A–C shows an example of the on-line LC–MS analysis of tryptic hydrolysate of bovine β-Lg. The total ion chromatogram (TIC)

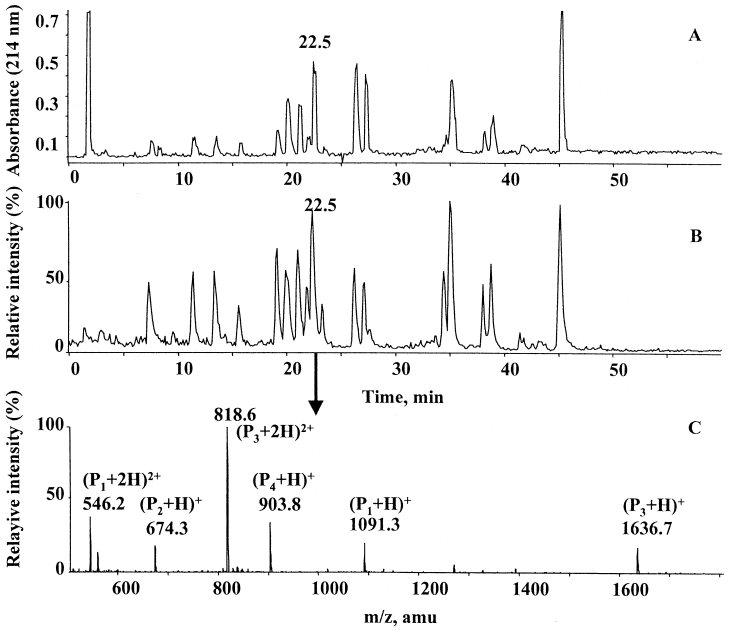

Fig. 3. A typical spectrum of four coeluting peptides from an on-line HPLC–MS analysis of a tryptic digest. (A) HPLC–UV chromatogram. (B) Corresponding HPLC–ESI-MS chromatogram in full-scan mode. (C) ESI-MS spectrum from peak with retention time 22.5 min showing the presence of four peptides: singly and doubly charged P1, singly charged P2, singly and doubly charged P3 and singly charged P4.

(Fig. 3B) is qualitatively similar to the UV trace in Fig. 3A. Each UV peak may contain several peptides which can be discriminated by their masses as illustrated in Fig. 2C for the peak eluted at 22.5 min. The corresponding mass spectrum indicates the co-elution of four different and identified peptides, i.e., M_1 (m/z 1091.3, assigned sequence Leu_{133}–Lys_{141}), M_2 (m/z 674.3, Ile_{78}–Lys_{83}), M_3 (m/z 1636.7, Thr_{125}–Lys_{138}) and M_4 (m/z 903.8, Thr_{76}–Lys_{83}).

3.4.1.1. Size distribution profile of whole hydrolysates

The main nutritional applications for hydrolysates are reduction of allergenicity or improvement of digestibility. For both applications, small peptides are needed because they have low antigenicity and are more rapidly absorbed from the small intestine than whole proteins and individual amino acids. A number of different methods are used to determine the nature and molecular mass distribution of peptides in a hydrolysate, and the best results are generally obtained from HPSEC (for a review, see Ref. [93]). However, whatever the chromatographic support used, the problems arising from secondary interactions between the peptides and the matrix have not been resolved [94,95]. Working on pepsin hydrolysate of bovine hemoglobin, Piot et al. [96] underlined important differences between the accurate molecular mass of the peptides determined by HPSEC and those obtained by FAB-MS analysis of collected fractions. Similarly, Lemieux et al. [95] found that the molecular mass of peptides in casein hydrolysate determined by FAB-MS did not correspond to the values determined experimentally by HPSEC. Hence, the combination of MS and RP-HPLC has emerged as a powerful technique for the determination of size distribution in a complex proteolytic digest of proteins. Further developments in the accurate determination of molecular masses distribution in food protein hydrolysates include the combination of HPSEC on-line with MS as a detection technique, as already applied in the biomedical field [97,98].

3.4.1.2. Extraction and identification of bioactive components

The formation of opioid peptides by in vitro proteolysis of bovine hemoglobin by pepsin was

investigated by RP-HPLC and subsequent off-line identification of collected fractions by FAB-MS [92]. The same technique was used to isolate opioid peptide from milk fermented with a strain of *Lactobacillus helveticus* [99] and to identify some bioactive peptides released by pepsin and trypsin digestion of UHT milk fermented by *Lactobacillus casei* [100]. Using RP-HPLC off-line with ESI-MS allowed Dionysius and Milne [101] to identify peptides with antimicrobial activity in tryptic hydrolysate of bovine lactoferrin. Working with the same protein, Shimazaki et al. [102] identified a heparin binding peptide after pepsin hydrolysis, RP-HPLC separation and MALDI-TOF-MS measurements. RP-HPLC–ESI-MS was also reported to be useful to monitor the kinetics of a bioactive production during continuous hydrolysis of β-casein with chymosin in a membrane reactor [103].

Beside its use as a tool for mass determination related by above various studies, MS and tandem MS start to be used in food science for peptide sequencing in sample as well as in complex medium. Thus, the presence of bioactive peptides in water-soluble extracts of 13 commercial cheeses as determined by combination of RP-HPLC–ESI MS and tandem MS was reported [104]. Peptides with antibacterial, immunomodulatory or anti-hypertensive activities were thus identified. RP-HPLC–ESI-MS and MS–MS were recently used to evaluate the digestibility of the β-casein phosphopeptide, i.e., β(1–25), known as a mineral carrier, during its duodenal transit in rats [105]. In this study, the luminal content of rats perfused with β(1–25) were directly analyzed by RP-HPLC–ESI-MS and β(1–25) derived fragments were detected at the pmol level. The primary structures of the assigned peptides were confirmed by subsequent MS–MS. Such study aimed to determine the mechanism by which the phosphopeptide improved iron absorption in rat. This strategy opens possibilities for sensitive detection of food peptides in biological fluids and hence to determine the nutritional properties of these components.

3.4.1.3. Determination of the specificity of different proteases

Another advantage of RP-HPLC–MS in protein hydrolysate analysis is related to the identification of the specificity of different proteases on food proteins. Thus, RP-HPLC on-line with ESI-MS and RP-HPLC off-line with MALDI-MS were applied to determine the action of trypsin on casein micelles [106,107]. RP-HPLC off-line with plasma desorption–MS was used to study the action of chymosin and plasmin on α_{s1}-casein [108,109]. Reid et al. [110] used RP-HPLC and ESI-MS to determine the action of chymosin on both κ-casein and its derived caseinomacropeptide. ESI-MS was also used on-line with RP-HPLC to study the effect of high pressure on the tryptic hydrolysis of β-Lg [111]. In addition to the information on the effect of processes on the action of known enzymes on a given food protein, LC–MS starts to take place in other research areas such as the determination of aspecific cleavage sites not easily detected by conventional methods or in the study of the action of novel enzymes.

3.4.1.4. Localization of chemical or post-translational modifications in the primary structure

One of the most important areas in food analysis is the detection, characterization and localization of modifications on peptidic sequences, which occur during protein synthesis or during processing of food components. Localization of these modifications in protein sequence is of special interest because they affect the functional as well as nutritional properties of proteins and derived peptides. However, such location in a primary structure is tedious and not possible in all cases by classical chemical methods. With the introduction of MS into protein chemistry, it has become possible to determine the modified groups by rapid, accurate and non-chemical means. MS has been demonstrated to be useful for the analysis of phosphopeptides or glycopeptides that result from digests of whole proteins with biomedical interest [112–114]. Further, elegant methods for sensitive and selective identification of phosphopeptides or glycopeptides have been developed. Briefly, phosphopeptide detection involves the generation under high potentials and detection of diagnostic ions at m/z 63 (PO_2^-) and 79 (PO_3^-) during negative ion LC–ESI-MS analysis of protein digests [115]. Similarly, the detection of glycopeptides is based on the observation that glycopeptide produce diagnostic fragment ions when subjected to high collision voltages [116].

Post-translational modification of polypeptidic chains: Several recent studies reported the utility of

coupling HPLC on-line or off-line with MS for better knowledge and localization of modified sites.

Caseinomacropeptide (CMP), the C-terminal moiety of κ-casein, i.e., residues 106–169, cleaved by chymosin during cheese making, is known to be a mixture of highly heterogeneous glycomacropeptides. CMP was reported to have diverse nutritional and biological significances due to its unique amino acid composition and sialic acid content [117]. In particular, since CMP does not contain the aromatic amino acid residues, several reports suggested the use of CMP as a protein source for the treatment of phenylketonurea, a hereditary disorder in which aromatic amino acids cannot be metabolized [118]. Also, other activities such as inhibition of pathogens or stimulation of a digestive hormone release were attributed to the carbohydrate moiety of CMP [119,120]. Consequently, the promising industrial potential of CMP mixture has induced studies on its better biochemical characterization. Thus, the use of RP-HPLC on-line with ESI-MS allowed a better characterization of this mixture with a least 18 different molecular species identified in one genetic variant of CMP [121]. These species differ in the carbohydrate chains but also by the presence of three phosphorylated sites. The occurrence of a new triphosphorylated and diphosphorylated forms, with one disialated chain, not previously detected by classical methods were then evidenced. The authors concluded that ESI-MS has proved to be a powerful method which allowed the accurate identification of individual components in the mixture in spite of the lower resolution in separating various forms by RP-HPLC. Furthermore, using FAB-MS and ESI-MS analysis of CMP and products of its proteolysis by Glu-specific endopeptidase allowed Minkiewicz et al. [122] to identify CMP digestion products including peptides containing phosphate, carbohydrate moieties and methionine sulfoxide.

Phosphopeptides: Several ionization methods were used to monitor phosphorylated peptides in complex hydrolysates. MALDI-MS and ESI-MS have been shown to be more effective methods for efficient ionization of phosphorylated peptides than FAB due to the lower surface activity of these components in FAB matrices [113]. The negative ion LC–ESI-MS with collision energy scanning was used to identify phosphopeptides from enzymatically digested proteins. This approach allows a rapid and selective identification of all phosphorylated peptides in a complex hydrolysate as shown in the case of tryptic hydrolysate of bovine β-casein (Fig. 4). Among the

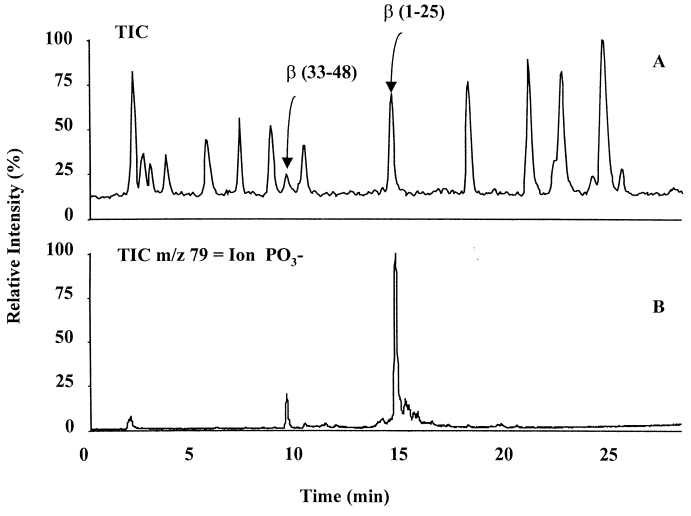

Fig. 4. Selective detection of phosphorylated peptides in a tryptic digest. (A) HPLC–ESI-MS chromatogram in full-scan positive mode (+80 V). (B) ESI-MS chromatogram generated at high negative voltage (−250 V) which allows the detection of peptides with a loss of m/z 79 (ion PO_3^-).

mixture of 17 peptides (Fig. 4A) only the phosphorylated ones produced a response (loss of m/z 79) after fragmentation under −250 V (Fig. 4B).

Chemical changes of polypeptidic chains: The preparation of protein hydrolysates at industrial scale is done in various steps with the potential for changing their properties and functionality through physico–chemical modifications such as heat, alkali and acid treatments. The main changes that occur during processing include serine and tyrosine dephosphorylation, lysine glycation, methionine oxidation and desamidation of asparagine or glutamine residues. ESI-MS allows not only the determination of dephosphorylated sites but also to discriminate between the two classes of dephosphorylation mechanisms: alkali hydrolysis that proceeds through β-elimination reaction leading to the production of a dehydroalanine residue with a loss of 97 u, and enzymatic, alkali, or acid hydrolysis that results in the release of the phosphate group but the serine residue is preserved giving a loss of 80 u [58]. Another chemical change that occur during food processing is the glycation of proteins by reducing sugars throughout the Maillard reaction. Focusing on glycation of food proteins with lactose, Mollé et al. [123] have reported a selective and sensitive identification of glycated sites after tryptic digestion of chemically glycated β-Lg. The method, illustrated in Fig. 5, is based on RP-HPLC–ESI-MS and RP-HPLC–MS–MS using neutral loss scanning for detection of marker ion. The marker ion $[M+H]^+-216$ corresponded to the glycated peptide with a characteristic neutral loss of 216 u following cleavage of the O-glycosidic bond and dehydration during the fragmentation process [123]. Fig. 5A shows the total current chromatogram in the normal LC–MS mode, with numerous peaks corresponding to glycated and non glycated peptides. Using tandem mass spectrometer set up in the neutral loss scanning mode in a second run, only glycated peptides (loss of 216 u) were selectively detected (Fig. 5B). This elegant and sensitive approach can be easily extended to monitor the occurrence of this chemical covalent reaction in food products where proteins and sugar naturally coexist.

3.5. Cheeses

Cheese is given in this review as an application area where the LC and MS allow great advances in the understanding of the ripening process for such a complex foodstuff.

Proteolysis is the most important biochemical event for most ripened cheeses, because it is responsible for their typical textural and flavor characteristics [124]. The products of proteolysis vary from

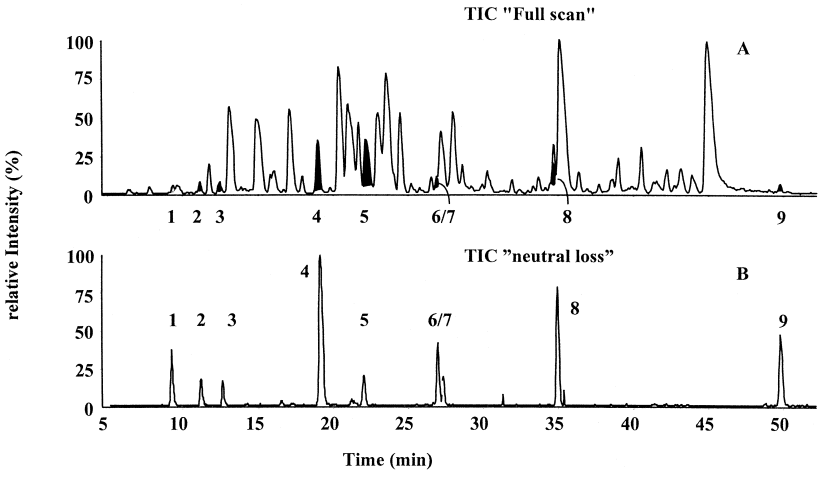

Fig. 5. Selective detection of glycated peptides from a tryptic digest of β-lactoglobulin. The mixture was resolved and analyzed by LC–ESI-MS. (A) HPLC–ESI-MS chromatogram in full scan mode (first run). (B) HPLC–ESI-MS–MS chromatogram in the neutral loss scanning mode (second run) for selective detection of glycated peptides: the spectrometer was scanned for mass losses of 216 u allowing the detection of nine $[M+H]^+-216$ ions. Figure adapted from Ref. [123].

large polypeptides, to free amino acids throughout the ripening time [124]. The peptide system, which contains at least 200 different peptides in most cheese varieties, is extremely complex since they are embedded in an heterogeneous multiphase matrix containing not only proteins and peptides but also fat, mineral components, organic acids . . . [125].

Therefore, the detailed study of proteolysis in cheese requires that peptides are first extracted from the cheese and further fractionated with different chromatographic or electrophoresis methods before being characterized (for a review of the different methods used see Ref. [126]). Identification of the peptides present in cheese was initially performed either by amino acid composition of the purified peptides by different chromatographic systems and/or by N-terminal sequencing with automated Edman degradation [127–129]. At the present time, MS determination is widely used for identifying the numerous peptides, a less time-consuming method compared to amino acid analysis.

A typical fractionation scheme was established and applied to Cheddar cheese [130,131] in order to obtain a systematic approach to the proteolysis. It was based on: (1) water extraction of peptides present in Cheddar according to the method of Kuchroo and Fox [132]; (2) preliminary separation of peptides according to the molecular size by diafiltration through membranes of cut-off M_r 10 000; (3) further fractionation by chromatographic methods (anion-exchange, gel filtration or RP chromatography) or urea–PAGE followed by electroblotting on nitrocellulose membranes, to obtain single pure or a mixture of components; and (4) the identification of peptides by mass spectrometry. Most of the peptide fractions were characterized with the use of PD- or MALDI-TOF or FAB- or ESI-MS.

At the present time, more than 100 peptides are completely or partly identified in cheeses, with the use of PD- or MALDI-TOF-MS in Cheddar [131,133–137] and with FAB-MS in Grana Padano [138–140]. These results allowed one to determine the first steps of degradation of the four main caseins, i.e., α_{s1}-, α_{s2}-, β- and κ-caseins giving information about the prominent proteolytic agents active in cheese: milk coagulant, milk endogenous and microbial enzymes. However, even if the major cleavage sites of the proteinases found in vitro are hydrolyzed in cheeses, their selectivity was shown to be affected by the cheese environment. This was especially observed for the coagulating agent of the milk, i.e., chymosin [141] and for the cell envelope proteinases (CEPs) of *Lactococcus lactis*, exhaustively characterized in solution of pure fractions of β-, α_{s1}- and κ-caseins with the use of mass spectrometry (for a review see Ref. [142]). This microbial proteinase seems to have a preferred action at the bonds Gln_{56}–Ser_{57} and Asn_{68}–Ser_{69} of the β-casein sequence, under the conditions prevailing in Cheddar cheese, whereas it has a broader one in solution [135]. Recently, Broadbent et al. [143] showed that different types of CEP from single-strain lactococci starter could be directly assessed in Cheddar cheese, using MALDI-TOF-MS and Edman degradation. Attempts were made to correlate the production of some peptides arising from the N-terminal end of α_{s1}-casein with bitterness, which is a default of cheese flavor.

It appears that the products of casein hydrolysis can be considered as markers of the technological processes (pH, temperature, salting . . .) applied to the cheese or a cheese family. Thus, in fresh cheese, such as Quarg, the predominant action arose from lactic acid bacteria [139]. In Cheddar cheese, most of the primary proteolysis of the caseins can be explained first by the action of chymosin on α_{s1}-casein and of endogenous milk proteinase, i.e., plasmin on β-casein and second by CEP of *Lactococcus lactis* spp. on both caseins [131,133–137]. Finally, in Italian cheeses (Parmigiano Reggiano, Grana Padano) and in Swiss-type cheeses (Emmental, Comté), most of the oligopeptides identified arose from β-casein with Lys or Arg residues at C-terminal end, indicating that the primary proteolysis is mainly due to plasmin, and few from α_{s2}-casein also sensitive to plasmin [138–140,144–147].

The identification of the peptides by the different MS detection methods allows one to determine other types of enzymes implied in the proteolytic breakdown of the casein throughout the ripening process. Thus, the presence of aminopeptidase activity was deduced from the presence of a number of peptides of different lengths with the loss of one or more residues from the N-terminal end, dipeptidyl peptidase with the loss of a dipeptide such as X-Pro and intracellular proteinases with splitting of bonds in-

side polypeptide chain, whereas the progressive hydrolysis of the C-terminal end reflected carboxypeptidase activity. For the latter, this is only by the observation of peptides with truncated C-terminal end that such activity has been proved whatever the technological process performed, whereas no carboxypeptidase activity was ever found in lactococci and only few carboxypeptidases were found or even partially characterized in lactobacilli and propionibacteria [135,142,148,149].

Among the peptides produced during cheese production, one type, i.e., phosphopeptides, is accumulated in great amounts throughout ripening of the Italian and Swiss-type cheeses and to a lesser extent of Cheddar cheese. These peptides enhance the functional properties of the cheese since they have biological activity of mineral carrier and anticariogenicity [140]. In most studies, they were selectively precipitated with barium salts, further analysed by RP-HPLC and easily characterized whatever the ionization source used: FAB-MS in Grana Padano [140] or ESI-MS for Swiss-type cheese, Emmental [146] or both methods of mass detection in Comté depending on the peptide molecular mass [147]. Most of the methods (MALDI, FAB, PD) used in cheese were off-line with LC. Until now, there have been few studies on on-line analysis between chromatography and ESI-MS. Roudot-Algaron et al. [147], Léonil et al. [146] and Alli et al. [150] used on-line LC–ESI-MS for analysing cheese peptides and further characterized them with tandem mass spectrometry. Thanks to the fine analysis of the phosphopeptides with MS it was observed that some phosphopeptides were partly dephosphorylated, and especially when SerP residue was present at the N-terminal end of the peptide but never or almost never when SerP was present in a cluster of three in all types of cheese. In this case the phosphopeptides were more resistant to further degradation, in contrast to the dephosphorylated peptides.

However, the sequential action of the peptidases and their origin is currently unknown throughout ripening of cheese, but can be obtained only if the peptidase pool of each genus of bacteria is completely characterized and its specificity of action determined. Very recent studies have fulfilled this challenge by deleting one or more genes encoding key enzymes/components of the proteolytic system in lactococci and using mass spectrometry to analyze the complex peptide mixture. Thus, they have completely reconstructed the proteolytic pathway for the use of β-casein in *Lactococcus lactis* [151]. In other bacterial genera, studies are not so complete but intracellular peptidases of propionic acid bacteria [152] and thermophilic lactic acid bacteria [153] were characterized using hydrolysate of β-casein as substrate and ESI-MS and tandem MS under conditions prevailing in Swiss-type cheese.

4. Conclusion

One of the most important research areas concerning food proteins and peptides is to establish the relationships between structure and functionality of these components. Without doubt, it can be stated that the combination of liquid chromatography with the available panel of mass spectrometry methods has been shown to be a powerful tool in obtaining structural information in complex medium without the need of individual purification of proteins and peptides prior to their identification. Until now, such a combination has proved to be an invaluable qualitative approach for the detection of new minor genetic variants; identification and location of post-translational and/or chemical modifications of the primary sequence and the study of non-covalent interaction of proteins with other components of foods (minerals, vitamins . . .). The instrumental advances and rapid development of the interface/ionization technologies will allow further advances in the analysis of food proteins and peptides. Particularly, the improved resolution and sensitivity of both MALDI and nanoelectrospray MS offer new possibilities for the characterization of high molecular mass proteins, identification of trace endogenous proteins or peptides and quantitative analyses of a specific protein in food samples.

5. Nomenclature

APCI	Means Atmospheric pressure chemical ionization
BSA	Means Bovine serum albumin

CEP	Means Cell-envelope proteinase
CF-FAB	Means Continuous-flow fast atom bombardment
CMP	Means Caseinomacropeptide
DE	Means Delayed extraction
ELISA	Means Enzyme-linked immunosorbent assay
ESI	Means Electrospray source ionization
FAB	Means Fast atom bombardment
HMW	Means High molecular mass
HPLC	Means High-performance liquid chromatography
HPIEC	Means Ion-exchange high-performance liquid chromatography
Ig	Means Immunoglobulin
ISD	Means Ion source decomposition
α-La	Means α-Lactalbumin
LC	Means Liquid chromatography
Lf	Means Lactoferrin
β-Lg	Means β-Lactoglobulin
MALDI	Means Matrix-assisted laser desorption/ionization
MS	Means Mass spectrometry
MS–MS	Means Tandem mass spectrometry
NMR	Means Nuclear magnetic resonance
PD	Means Plasma desorption
PSD	Means Post source decay
RP-HPLC	Means Reversed-phase high-performance liquid chromatography
SDS	Means Sodium dodecyl sulfate
SDS–PAGE	Means Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
HPSEC	Means Size-exclusion high-performance liquid chromatography
SerP	Means Phosphorylated serine
TFA	Means Trifluoroacetic acid
TIC	Means Total ion chromatogram
TOF	Means Time-of-flight
UHT	Means Ultra high temperature
UV	Means Ultraviolet

References

[1] J.L. Maubois, J. Léonil, Lait 69 (1989) 245.
[2] D. Tome, H. Debabbi, Int. Dairy J. 8 (1998) 383.
[3] A.L. Burlingame, R.K. Boyd, S.J. Gaskell, Anal. Chem. 70 (1998) 647R.
[4] M. Barber, B.N. Green, Rapid Commun. Mass Spectrom. 1 (1987) 80.
[5] P.J. Arpino, G. Guiochon, J. Chromatogr. 251 (1982) 153.
[6] K.B. Tomer, C.E. Parker, J. Chromatogr. 492 (1989) 189.
[7] S.A. Carr, M.E. Hemling, M.F. Bean, G.D. Roberts, Anal. Chem. 63 (1991) 2802.
[8] D.N. Nguyen, G.W. Becker, R.M. Riggin, J. Chromatogr. A 705 (1995) 21.
[9] R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barinaga, H.R. Udseth, Anal. Biochem. 62 (1990) 882.
[10] J.B. Fenn, J. Am. Soc. Mass Spectrom. 4 (1993) 524.
[11] R.B. Cole, Electrospray Ionization Mass Spectrometry – Fundamentals, Instrumentation and Applications, Wiley, New York, 1997.
[12] M.S. Wilm, M. Mann, Anal. Chem. 68 (1996) 1.
[13] M. Mann, C.K. Meng, J.B. Fenn, Anal. Chem. 61 (1989) 1702.
[14] K.D. Henry, E.R. Williams, B.H. Wang, J. McLafferty, J. Shabanowitz, D.F. Hunt, Proc. Natl. Acad. Sci. USA 86 (1989) 9075.
[15] M.L. Alexandrov, L.N. Gall, N.V. Krasnov, V.I. Nikolaev, V.A. Pavlenko, V.A. Shkurov, J. Anal. Chem. USSR 40 (1986) 1127.
[16] O.E. Mirgorodskaya, A.A. Shevchenko, I.V. Chernushevich, A.F. Dodonov, A.I. Miroshnikov, Anal. Chem. 66 (1994) 99.
[17] J.F. Todd, A.D. Penman, Int. J. Mass Spectrom. Ion Processes 106 (1991) 1.
[18] J. Léonil, D. Mollé, F. Gaucheron, P. Arpino, P. Guenot, J.L. Maubois, Lait 75 (1995) 193.
[19] J.C. Le, J. Hui, M. Haniu, V. Katta, M.F. Rohde, J. Am. Soc. Mass Spectrom. 8 (1997) 703.
[20] M.T. Davis, D.C. Stahl, T.D. Lee, J. Am. Soc. Mass Spectrom. 6 (1995) 571.
[21] E.C. Huang, J.D. Henion, J. Am. Soc. Mass Spectrom. 1 (1990) 158.
[22] G. Hopfgartner, T. Wachs, K. Bean, J.D. Henion, Anal. Chem. 65 (1993) 439.
[23] A.P. Bruins, T.R. Covey, J.D. Henion, Anal. Chem. 59 (1987) 2642.
[24] C. Molina, H. Honing, D. Barcelo, Anal. Chem. 66 (1994) 4444.
[25] A.R. Dongré, J.K. Eng, J.R.I. Yates, TIBTECH 15 (1997) 418.
[26] M. Karas, F. Hillenkamp, Anal. Chem. 60 (1988) 2299.
[27] F. Rusconi, J.M. Schmitter, Analusis 26 (1998) M13.
[28] R.C. Beavis, T. Chandhary, B.T. Chait, Org. Mass Spectrom. 27 (1992) 156.
[29] R.C. Beavis, B.T. Chait, Rapid Commun. Mass Spectrom. 3 (1989) 233.
[30] T.A. Worral, R.J. Cotter, A.S. Woods, Anal. Chem. 70 (1998) 750.
[31] K. Gevaert, H. Demol, M. Puype, D. Broekaert, S. De Boeck, T. Houthaeve, J. Vandekerckhove, Electrophoresis 18 (1997) 2950.
[32] F. Hillenkamp, M. Karas, R.C. Beavis, B.T. Chait, Anal. Chem. 63 (1991) 1193A.
[33] M. Karas, U. Bahr, Trends Anal. Chem. 9 (1990) 321.
[34] B. Spengler, R.J. Cotter, Anal. Chem. 62 (1990) 793.
[35] R.S. Brown, J.J. Lennon, Anal. Chem. 67 (1995) 1998.
[36] B. Spengler, D. Kirsch, R. Kaufmann, E. Jaeger, Rapid Commun. Mass Spectrom. 6 (1992) 105.

[37] R. Kaufmann, P. Chaurand, D. Kirsch, B. Spengler, Rapid Commun. Mass Spectrom. 10 (1996) 1199.
[38] P. Chaurand, F. Luetzenkirchen, B. Spengler, J. Am. Soc. Mass Spectrom. 10 (1999) 91.
[39] R.R. Ogorzalek, T.I. Stevenson, C. Mitchell, J.A. Loo, P.C. Andrews, Anal. Chem. 68 (1996) 1910.
[40] P. Walstra, R. Jenness, Dairy Chemistry and Physics, Wiley, New York, 1984.
[41] H.S. Rollema, in: P.F. Fox (Ed.), Advanced Dairy Chemistry, Elsevier, London, 1992, p. 111.
[42] International Dairy Federation, Milk Protein Polymorphism, International Dairy Federation, Brussels, 1997.
[43] W.N. Eigel, J.E. Butler, C.A. Ernstrom, H.M. Farrell Jr., V.R. Harwalkar, R. Jenness, R.M. Withney, J. Dairy Sci. 67 (1984) 1599.
[44] J.L. Maubois, G. Ollivier, in: S. Damodaran, A. Paraf (Eds.), Food Proteins and Their Applications, Marcel Dekker, New York, 1997, p. 595.
[45] E.D. Strange, E.L. Malin, D.L. Vanhekken, J. Chromatogr. 624 (1992) 81.
[46] J.A. Loo, R.R. Loo, K.J. Light, C.G. Edmonds, R.D. Smith, Anal. Chem. 64 (1992) 81.
[47] S. Visser, C.J. Slangen, F.M. Lagerwerf, W.D. Vandongen, J. Haverkamp, J. Chromatogr. 711 (1995) 141.
[48] C. Dong, K.F. Ng-Kwai-Hang, Int. Dairy J. 8 (1998) 967.
[49] P. Ferranti, A. Malorni, G. Nitti, P. Laezza, R. Pizzano, L. Chianese, F. Addeo, J. Dairy Res. 62 (1995) 281.
[50] L. Chianese, G. Garro, R. Mauriello, P. Laezza, P. Ferranti, F. Addeo, J. Dairy Res. 63 (1996) 49.
[51] L. Chianese, G. Garro, P. Ferranti, A. Malorni, F. Addeo, A. Rabaco, P.M. Pons, J. Dairy Res. 62 (1995) 89.
[52] R.J. Fitzgerald, in: R.A.S. Welch, D.J.W. Burns, S.R. Davis, A.I. Popay, C.G. Prosser (Eds.), Milk Composition, Production and Biotechnology, CAB International, Wallingford, 1997, p. 153.
[53] P. Hu, C.Z. Ye, J.A. Loo, Anal. Chem. 66 (1994) 4190.
[54] J. Léonil, D. Mollé, J. Fauquant, J.L. Maubois, R.J. Pearce, S. Bouhallab, J. Dairy Sci. 80 (1997) 2270.
[55] R. Burr, C.H. Moore, J.P. Hill, Milchwissenschaft 51 (1996) 488.
[56] F. Morgan, D. Mollé, G. Henry, A. Vénien, J. Léonil, G. Peltre, D. Levieux, J.L. Maubois, S. Bouhallab, Int. J. Food Sci. Technol. 34 (1999) 429.
[57] F. Nacka, J.M. Chobert, T. Burova, J. Léonil, T. Haertlé, J. Prot. Chem. 17 (1998) 495.
[58] L.S. Ward, E.D. Bastian, J. Agric. Food Chem. 46 (1998) 77.
[59] R. Marsilio, S. Catinella, R. Seraglia, P. Traldi, Rapid Commun. Mass Spectrom. 9 (1995) 550.
[60] S. Catinella, P. Traldi, C. Pinelli, E. Dallaturca, R. Marsilio, Rapid Commun. Mass Spectrom. 10 (1996) 1629.
[61] S. Catinella, P. Traldi, C. Pinelli, E. Dallaturca, Rapid Commun. Mass Spectrom. 10 (1996) 1123.
[62] W.D. Powrie, S. Nakai, in: W.J. Stadelman, O.J. Cotterill (Eds.), Egg Science and Technology, AVI, Westport, CO, 1986, p. 97.
[63] L. Stevens, Comp. Biochem. Physiol. 100 (1991) 1.
[64] A.C. Awadé, Z. Lebensm. Unters. Forsch. 202 (1996) 1.
[65] A.C. Awadé, S. Moreau, D. Mollé, G. Brule, J.L. Maubois, J. Chromatogr. A 677 (1994) 279.
[66] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[67] R.D. Smith, J.A. Loo, C.J. Barinaga, C.G. Edmonds, H.R. Udseth, J. Chromatogr. 480 (1989) 211.
[68] S. Moreau, A.C. Awadé, D. Mollé, Y. Le Graet, G. Brule, J. Agric. Food Chem. 43 (1995) 883.
[69] N. Deighton, S.M. Glidewell, B.A. Goodman, Food Sci. Technol. Today 9 (1995) 144.
[70] C. Fenselau, D.N. Heller, M.S. Miller, H.B.I. White, Anal. Biochem. 150 (1985) 309.
[71] M. Ohta, J. Hamako, H. Hatta, M. Kim, T. Yamamoto, S. Oka, F. Matsuura, Glycoconjugate J. 8 (1991) 400.
[72] T.H. Wang, T.F. Chen, D.F. Barofsky, Biomed. Environ. Mass Spectrom. 16 (1988) 335.
[73] M.L. Corradi Da Silva, H.J. Stubbs, T. Tamura, K.G. Rice, Arch. Biochem. Biophys. 318 (1995) 465.
[74] M.L. Corradi Da Silva, H.J. Stubbs, T. Tamura, K.G. Rice, Arch. Biochem. Biophys. 318 (1995) 465.
[75] A. Seko, M. Koketsu, M. Nishizono, Y. Enoki, H.R. Ibrahim, L.R. Juneja, M. Kim, T. Yamamoto, Biochim. Biophys. Acta 1335 (1997) 23.
[76] J.A. Loo, R.R. Loo, H.R. Udseth, C.G. Edmonds, R.D. Smith, Rapid Commun. Mass Spectrom. 5 (1991) 101.
[77] L. Konermann, D.J. Douglas, J. Am. Soc. Mass Spectrom. 9 (1998) 1248.
[78] S.K. Kulkarni, A.E. Ashcroft, M. Carey, D. Masselos, C.V. Robinson, S.E. Radford, Protein Sci. 8 (1999) 35.
[79] K. Mizutani, H. Yamashita, H. Oe, M. Hirose, Biosci. Biotech. Biochem. 61 (1997) 641.
[80] E. Tatsumi, M. Hirose, J. Biochem. 122 (1997) 300.
[81] J.A. Huntington, B. Fan, K.E. Karlsson, J. Deinum, D.A. Lawrence, P.G. Gettins, Biochemistry 36 (1997) 5432.
[82] P.R. Shewry, M.J. Miles, A.S. Tatham, Prog. Biophys. Mol. Biol. 61 (1994) 37.
[83] T.A. Egorov, A.K. Musolyamov, A.A. Kochergin, J.S. Andersen, P. Roepstorff, J. Cereal Sci. 20 (1994) 107.
[84] T.A. Egorov, A.K. Musolyamov, J.F. Bazbashov, O. Zolotykh, J. Andersen, P. Roepstorff, Y. Popineau, Proceedings of the International Meeting on Wheat Kernel Proteins, Molecular and Functional Aspects, Vinterbo (Italy), 1994.
[85] D.R. Hickman, P. Roepstorff, A.R. Clarke, A.S. Tatham, P.R. Shewry, Proceedings of the International Meeting on Wheat Kernel Proteins, Molecular and Functional Aspects, Vinterbo (Italy), 1994.
[86] E. Mendez, E. Camafeita, J.S. Sebastian, I. Valle, J. Solis, F. Mayer-Posner, D. Suckau, C. Marfisi, F. Soriano, J. Mass Spectrom. Rapid Commun. Mass Spectrom. (1995) S123.
[87] E. Camafeita, P. Alfonso, B. Acevedo, E. Mendez, J. Mass Spectrom. 32 (1997) 444.
[88] E. Camafeita, P. Alfonso, T. Mothes, E. Mendez, J. Mass Spectrom. 32 (1997) 940.
[89] D.R. Hickman, P. Roepstorff, P.R. Shewry, A.S. Tatham, J. Cereal Sci. 22 (1995) 99.
[90] J. Adler-Nissen, Enzymic Hydrolysis of Food Proteins, Elsevier, London, 1986.
[91] P.M. Nielsen, Food Proteins and Their Applications, Marcel Dekker, New York, 1997.
[92] J.M. Piot, Q. Zhao, D. Guillochon, G. Ricart, D. Thomas, FEBS Lett. 299 (1992) 75.

[93] M.P.C. Silvestre, Food Chem. 60 (1997) 263.
[94] M.P.C. Silvestre, M. Hamon, M. Yvon, J. Agric. Food Chem. 42 (1994) 2778.
[95] L. Lemieux, J.M. Piot, D. Guillochon, J. Amiot, Chromatographia 32 (1991) 499.
[96] J.M. Piot, D. Guillochon, Q. Zhao, G. Ricart, B. Fournet, D. Thomas, J. Chromatogr. 481 (1989) 221.
[97] J. Silberring, F. Nyberg, J. Chromatogr. A 777 (1997) 41.
[98] M.T. Smoluch, P. Mak, J.P. Chervet, G. Hohne, J. Silberring, J. Chromatogr. B 726 (1999) 37.
[99] C. Matar, J. Amiot, L. Savoie, J. Goulet, J. Dairy Sci. 79 (1996) 971.
[100] R. Rokka, E.L. Syvaoja, J. Tuominen, H. Korhonen, Milchwissenschaft 52 (1997) 675.
[101] D.A. Dionysius, J.M. Milne, J. Dairy Sci. 80 (1997) 667.
[102] K. Shimazaki, T. Tazume, K. Uji, M. Tanaka, H. Kumura, K. Mikawa, T. Shimo-Oka, J. Dairy Sci. 81 (1998) 2841.
[103] S. Bouhallab, D. Mollé, J. Léonil, Biotechnol. Lett. 15 (1993) 697.
[104] H. Jiang, P.A. Grieve, R.J. Marschke, A.F. Wood, D.A. Dionysius, P.F. Alewood, Aust. J. Dairy Technol. 53 (1998) 119.
[105] S. Bouhallab, N. Aït Oukhatar, D. Mollé, G. Henry, J.L. Maubois, P. Arhan, D.L. Bouglé, J. Nutr. Biochem. 10 (1999) 723.
[106] V. Gagnaire, J. Léonil, Lait 78 (1998) 471.
[107] O. Diaz, A.M. Gouldsworthy, J. Leaver, J. Agric. Food Chem. 44 (1996) 2517.
[108] P.L.H. McSweeney, N.F. Olson, P.F. Fox, A. Healy, P. Hojrup, J. Dairy Res. 60 (1993) 401.
[109] P.L.H. McSweeney, N.F. Olson, P.F. Fox, A. Healy, P. Hojrup, Food Biotechnol. 7 (1993) 143.
[110] J.R. Reid, T. Coolbear, J.S. Ayers, K.P. Coolbear, Int. Dairy J. 7 (1997) 559.
[111] F. Maynard, A. Weingand, J. Hau, R. Jost, Int. Dairy J. 8 (1998) 125.
[112] W. Schroeder, T.C. Covey, F. Hucho, FEBS Lett. 273 (1990) 31.
[113] M. Busman, K.L. Schey, J.E. Aotis, D.R. Knapp, J. Am. Soc. Mass Spectrom. 7 (1996) 243.
[114] K.D. Greis, B.K. Hayes, F.I. Comer, M. Kirk, S. Barnes, T.L. Lowary, G.W. Hart, Anal. Biochem. 234 (1996) 38.
[115] M.J. Huddleston, R.S. Annan, M.F. Bean, S.A. Carr, J. Am. Soc. Mass Spectrom. 4 (1993) 710.
[116] M.J. Huddleston, M.F. Bean, S.A. Carr, Anal. Chem. 65 (1993) 877.
[117] M.H. Abdelsalam, S. Elshibiny, W. Buchheim, Int. Dairy J. 6 (1996) 327.
[118] S.C. Marshall, Food Res. Quart. 51 (1991) 86.
[119] Y. Kawasaki, S. Dosako, B.R.A.N. Snow, Process for Producing κ-Casein Glycomacropeptides, Patent EP.0.488.589.A1, 1 November 1992.
[120] S. Beucher, F. Levenez, M. Yvon, T. Corring, Reprod. Nutr. Dev. 34 (1994) 613.
[121] D. Mollé, J. Léonil, J. Chromatogr. 708 (1995) 223.
[122] P. Minkiewicz, C.J. Slangen, F.M. Lagerwerf, J. Haverkamp, H.S. Rollema, S. Visser, J. Chromatogr. A 743 (1996) 123.
[123] D. Mollé, F. Morgan, S. Bouhallab, J. Léonil, Anal. Biochem. 259 (1998) 152.
[124] P.F. Fox, T.P. O'Connor, P.L.H. McSweeney, Adv. Food Nutr. 39 (1996) 163.
[125] P.F. Fox, T.K. Singh, P.L.H. McSweeney, in A.T. Andrews, J. Varley (Eds.) Biochemistry of Milk Products, Royal Society of Chemistry, Cambridge, 1995, p. 1.
[126] P.L.H. McSweeney, P.F. Fox, Lait 77 (1997) 41.
[127] D.E.L. Gonzales, M.C. Polo, M. Ramos, J. Dairy Res. 58 (1991) 363.
[128] H.D. Belitz, K.P. Kaiser, Z. Lebensm. Unters. Forsch. 197 (1993) 118.
[129] A. Michaelidou, E. Alichanidis, H. Urlaub, A. Polychroniadou, G.K. Zerfiridis, J. Dairy Sci. 81 (1998) 3109.
[130] M. O'Sullivan, P.F. Fox, J. Dairy Res. 57 (1990) 135.
[131] T.K. Singh, P.F. Fox, P. Hojrup, A. Healy, Int. Dairy J. 4 (1994) 111.
[132] C.N. Kuchroo, P.F. Fox, Milchwissenschaft 38 (1982) 389.
[133] P.L.H. McSweeney, S. Pochet, P.F. Fox, A. Healy, J. Dairy Res. 61 (1994) 587.
[134] T.K. Singh, P.F. Fox, A. Healy, J. Dairy Res. 62 (1995) 629.
[135] T.K. Singh, P.F. Fox, A. Healy, J. Dairy Res. 64 (1997) 433.
[136] M. Fernandez, T.K. Singh, P.F. Fox, J. Agric. Food Chem. 46 (1998) 4512.
[137] J.S. Mooney, P.F. Fox, A. Healy, J. Leaver, Int. Dairy J. 8 (1998) 813.
[138] P. Ferranti, E. Itolli, F. Barone, A. Malorni, G. Garro, P. Laezza, L. Chianese, F. Migliaccio, V. Stingo, F. Addeo, Lait 77 (1997) 683.
[139] L. Chianese, S. Caira, P. Ferranti, P. Laezza, A. Malorni, G. Mucchetti, G. Garro, F. Addeo, Lait 77 (1997) 699.
[140] P. Ferranti, F. Barone, L. Chianese, F. Addeo, A. Scaloni, L. Pellegrino, P. Resmini, J. Dairy Res. 64 (1997) 601.
[141] F.A. Exterkate, F.M. Lagerwerf, J. Haverkamp, Int. Dairy J. 7 (1997) 47.
[142] E.R.S. Kunji, I. Mierau, A. Hagting, B. Poolman, W.N. Konings, Antonie Leeuwenhoek 70 (1996) 187.
[143] J.R. Broadbent, M. Stickland, B.C. Weimer, M.E. Johnson, J.L. Steele, J. Dairy Sci. 81 (1998) 327.
[144] F. Addeo, L. Chianese, A. Salzano, R. Sacchi, U. Cappuccio, P. Ferranti, A. Malorni, J. Dairy Res. 59 (1992) 401.
[145] F. Addeo, L. Chianese, R. Sacchi, S. Spagna Musso, P. Ferranti, A. Malorni, J. Dairy Res. 61 (1994) 365.
[146] J. Léonil, V. Gagnaire, D. Mollé, J.L. Maubois, in: Proceedings of the Symposium on Quality and Microbiology of Traditional and Raw Milk Cheeses, COST Action 95 – Improvement of the Quality of the Production of Raw Milk Cheeses, Dijon, 1998, Office for Official Publications of the European Communities, 1999, p. 344.
[147] F. Roudot-Algaron, D. Le Bars, L. Kerhoas, J. Einhorn, J.C. Gripon, J. Food Sci. 59 (1994) 544.
[148] D. Atlan, C. Gilbert, J. Frot-Coutaz, R. Portalier, in: E.L. Foo, H.G. Griffin, R. Mollby, C.G. Heden (Eds.), The Lactic Acid Bacteria, Horizon Scientific Press, London, 1993, p. 31.

[149] T. Langsrud, T. Sorhaug, G.E. Vegarud, Lait 75 (1995) 325.
[150] I. Alli, M. Okoniewska, B.F. Gibbs, Y. Konishi, Int. Dairy J. 8 (1999) 643.
[151] E.R.S. Kunji, G. Fang, C.M. Jeronimus-Stratingh, A.P. Bruins, B. Poolman, W.N. Konings, Mol. Microbiol. 27 (1998) 1107.
[152] V. Gagnaire, D. Mollé, T. Sorhaug, J. Léonil, Lait 79 (1999) 43.
[153] S.M. Deutsch, D. Mollé, V. Gagnaire, M. Piot, S. Lortal, in: Proceedings of the 6th Symposium on Lactic Acid Bacteria, Genetics, Metabolism and Applications, Veldhoven, Federation of European Microbiological Societies and Netherlands Society for Microbiology, 1999, p. K36.