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Review of methods for the analysis of protein hydrolysates

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Protein hydrolysates have been used for nutritional or technological purposes. Various methods are used for the quality control of these preparations. This paper reviews those used for the determination of the hydrolysis degree, the characterization according to the peptide size, the evaluation of the molecular weight distribution, and the estimation of the amino acid and peptide contents. The potential and limitations of different techniques are also described. © 1997 Elsevier Science Ltd

INTRODUCTION

Protein hydrolysates have been used for the nutritional management of individuals who cannot digest intact protein. The most prevalent use of such hydrolysates however, has been for feeding infants with food hypersensivity. Also, reports have documented the functional properties of enzymatically hydrolysed food proteins from different sources using various proteolytic enzymes. Those studies showed improved solubility and enhanced emulsifying properties or reduced emulsifying activity depending on the hydrolysis conditions and the starting protein materials (Mahmoud *et al.*, 1992).

For dietary use, it has been shown that protein hydrolysates should be rich in low molecular weight peptides, especially di- and tripeptides, with as little as possible free amino acids, for the hydrolysate to be of a high nutritional and therapeutic value (Vijayalakshmi *et al.*, 1986). On the other hand, large molecular weight peptides (more than 20 amino acid residues) are presumed to be associated with the improvement functionality of hydrolysates. Thus, irrespective of the use of the protein hydrolysates, it is always important to characterize them on the basis of their peptide size (Gauthier *et al.*, 1986; Turgeon *et al.*, 1991).

However, it is difficult to know the composition of protein hydrolysates because of a large number of possible constituents. This is due to multiple degrees of polymerization of the peptides, characterized by 20 natural amino acids.

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For the quality control of these preparations, several analysis may be done: the osmolality, the degree of hydrolysis, the total nitrogen, the amino acid composition and the presence of toxic compounds (e.g. biogenic amines). Among this great variety of methods, what will be described here are those related to the determination of the hydrolysis degree, the characterization according to the peptide size, the estimation of the amino acid and peptide composition and the evaluation of the molecular weight distribution.

DETERMINATION OF THE DEGREE OF HYDROLYSIS

Different methods are used to evaluate the degree of hydrolysis (DH) of the peptide bonds. They are based on three essential principles: the determination of the amount of nitrogen released by the protein hydrolysis that becames soluble in the presence of a precipitation agent (e.g. trichloroacetic acid), the determination of free α-amino groups and the titration of the released protons.

Determination of the released nitrogen

In the first case, the following methods may be used: Kjeldhal technique (A.O.A.C., 1995), spectrophotometric determination in the UV region of the peptides with aromatic groups (Pelissier, 1984) or the spectrophotometric determination in the visible region after colorimetric reaction (Biuret reaction, Hung *et al.*, 1984).

Determination of free α-amino groups

For the evaluation of free α-amino groups, the formol titration is widely used. The classical Sörensen method is based on the action of formic aldehyde over the amino acids, decreasing the basic character of the α-amino groups by the liberation of their protons. The great practical inconvenience of this method is related to a large number of interfering parameters making it difficult to set up an ideal technique. The France Pharmacopea method (1988) is based on the adjustment of the pH of both test and formaldehyde solutions to 7·0, followed by the titration of the mixture by an alkaline solution up to phenolftalein change in colour (pH 9·2).

Using the American Pharmacopea method (U.S.P., 1989) the pH of the test solution is regulated to 7·0 and that of the formol solution to 9·0. The titration by an alkaline solution goes on up to pH 9·0. Finally, the method of the Association of Official Analytical chemists (A.O.A.C., 1995) seems to be more logical, since the pH of 9·2 (change in colour of phenolphtalein) is chosen as well for the initial pH fitting as for the equivalence point. This diversity of methods for formol titration leads to different results, especially for the weak hydrolysates. The determination of the ratio between the α-amino nitrogen and the total nitrogen by one of the techniques described above gives an estimation of the degree of hydrolysis which rises with this ratio.

Moreover, various compounds that react specifically with the amino groups are used to determine the DH, such as: ninhydrine (Moore & Stein, 1948), trinitrobenzene sulfonic acid (Fields, 1971; Spadaro *et al.*, 1979; McKellar, 1981; Valles *et al.*, 1985; Polychroniadou, 1988; Humbert *et al.*, 1990), fluorescamin (Weigele *et al.*, 1972) and orthophtaldehyde – OPA (Goodno *et al.*, 1981; Church *et al.*, 1983, 1985; Touati *et al.*, 1992).

The oldest technique is the ninhydrin that produces a heavy blue product with the primary amines. This method is very sensitive but it has a lot of disadvantages such as the oxygen sensitivity of the reagent, the interference of ammonia and the high values of the blank. Futhermore, the analysis duration is quite long due to the heating and cooling phases required to the chromophore formation (Turgeon *et al.*, 1991).

The utilization of a specific reagent for primary amino groups, the trinitrobenzenesulfonic acid (TNBS), was proposed by Adler-Nissen (1979) for analysing protein hydrolysates. After incubation of samples for 1 h at 37°C, the absorbance is measured at 420 nm. Among the disadvantages of this method are the analysis duration, the reagent contamination by picric acid giving high blank values, the interference of reducing sugars and ammonia, and the lack of reactivity of proline and hydroxyproline. Moreover, the ε-amino group of lysine is also capable of reacting with the TNBS, altering the results.

The fluorescamine and the orthophtaldialdehyde (OPA) may also be used for measuring the free amino groups. These fluorimetric methods have a great sensitivity but their accuracy is highly influenced by the variability of the fluorescence yield. The low stability of the amino acid derivatives is another disadvantage of this type of analysis (Turgeon *et al.*, 1991).

On the other hand, the OPA reagent was used by Church *et al.* (1983, 1985) for measuring spectrophotometrically the proteolysis in milk and in isolated milk proteins. The reaction with primary amines takes place quickly at room temperature. However, cystein reacts slightly and proline does not react with OPA.

Determination of the released protons

The titration of the protons released during the hydrolysis is the pH-stat technique (Adler-Nissen, 1979). The reaction occurs in neutral or slightly alkaline medium, favourable to the dissociation of the amine groups and to the liberation of protons in reactional medium. This method is generally used for continuous measurement of DH, due to its simplicity, rapidity and reproducibility. Another advantage is that this technique is not denaturant (Adler-Nissen, 1979). However, the DH values obtained by this method are relative and their exactitude needs to be checked by other methods (OPA or TNBS).

QUALITATIVE ANALYSIS

Separation and identification of peptides and amino acids

Besides information about the degree of hydrolysis, the separation and the identification of peptides and amino acids has to be studied in order to obtain better knowledge about the composition of protein hydrolysates. Several methods may be used for this purpose.

The high performance liquid chromatography (HPLC), especially at the reverse phase mode (RP-HPLC), showed to be efficient to separate peptides from protein hydrolysates and also to give some indications about their hydrophylicity and hydrophobicity (Lemieux *et al.*, 1991).

Some works were carried out using different experimental conditions of RP-HPLC for separating the peptides from tryptic hydrolysates of casein, from β-lactoglobulin A, and from human growth hormone (Kalgahati & Horvath, 1987; Maa & Horvath, 1988).

In order to obtain more information about peptide precipitation, Yvon *et al.* (1989) used the RP-HPLC for separating the peptides from tryptic and chymotriptic hydrolysates of α_{s1}-casein and β-casein, and also from κ-casein hydrolysed by the rennet. The retention times of the peptides were then correlated with their solubility in TCA, at different concentrations.

The RP-HPLC was used to monitor the peptides produced at different stages of the hydrolysis process of whey proteins with commercial proteinases (Perea *et al.*, 1993). Imbert & Nicolas (1993) proposed a technique of RP-HPLC for separating the two peptides obtained from κ-casein hydrolysis by rennet, A and B caseinomacropeptides, that are important for identifying the adulteration of milk or milk powder with whey.

Also, the RP-HPLC technique has been shown useful in separating active peptides from protein hydrolysates. Thus, the separation of a bioactive peptide prepared by a continuous hydrolysis of β-casein in a membrane reactor was carried out by RP-HPLC (Bouhallab *et al.*, 1993). The elution profiles on a RP-HPLC column was also used in a collaborative study about the antioxidative activity of peptides from protein hydrolysates (Chen *et al.*, 1995). The formation of opioid peptides by *in-vitro* proteolysis of bovine caseins was investigated by RP-HPLC (Pihlanto-Leppälä *et al.* 1994).

Another utilisation of RP-HPLC technique in protein hydrolysate analysis is related to the identification of the specificity of different proteases. Thus, the characteristic RP-HPLC patterns of κ-casein cleavage were shown to be useful for characterizing the types of proteinases produced by different bacteria strains (Reid *et al.*, 1994). The action of a milk-clotting enzyme toward k-casein in the flowers of cardoon (*Cynara cardunculus* L.) was investigated by Macedo *et al.* (1993), using the elution profiles of the peptides obtained on a RP-HPLC column. Gallagher *et al.* (1994) showed the difference in the action of two proteases on bovine caseins using the peptide maps of RP-HPLC of the hydrolysates.

The isolation of bitter peptides from enzymic hydrolysates is generally carried out by RP-HPLC. Minagawa *et al.* (1989) used this technique for isolating the bitter peptide fraction present in a tryptic casein hydrolysate.

However, despite its high resolution capacity, the RP-HPLC gives only a partial characterization of protein hydrolysates (Vijayalakshmi *et al.*, 1986). In fact, the separation criteria of these phases, based on the hydrophobicity or on the charge of peptides, are not good for characterising protein hydrolysates from a nutritional point of view, where their quality is associated with the peptide size.

The micro columns having a diameter below 1 mm may be of interest for separating peptides by HPLC, especially in the cases where the available amount of the sample is quite small. One of the applications of this technique, named capillary-HPLC, was shown by Davis and Lee (1992), using three different diameter columns for the separation of peptides released by the hydrolysis of cytochrome c using an endopeptidase (endo Lys C). According to the authors, since no attempt was made to optimize the separations, they were unable to explain with certainty the better resolution obtained on the 0·5 mm diam column. However, they could say with certainty that the there has never been a compromise in the quality of the chromatography by using capillary-size columns.

The ion-exchange chromatography may also be used for analysing protein hydrolysates. In fact, Dizdaroglu (1985) showed the efficiency of this technique in separating peptides from tryptic hydrolysates of cytochrome c and lyzosyme. According to the authors, the number of detected peaks corresponds nearly to the fragment number expected for a tryptic digestion of lysozyme.

The size-exclusion chromatography (SEC) or gel permeation chromatography, essentially in high performance liquid mode (SE-HPLC), is one of the attractive techniques allowing the study of the chromatographic profiles of protein hydrolysates. Different materials have been elaborated as a support for separating peptides on the basis of their size. Some soft gels such as Sephadex G-25 (Amiot & Brisson, 1980; Zhang *et al.*, 1992), Sephadex G-10 (Landry *et al.*, 1988) and Bio-gel P-2 (Iliev & Tchorbanov, 1992) have been used for this purpose, essentially in cases where the determination of tryptophan is the main interest, due to its absorption on the gelified matrix (Kowalska, 1969; Landry *et al.*, 1988; Iliev & Tchorbanov, 1992). Pellerin *et al.* (1985) used different gels of Sephadex type, with varied fractionation range (G-10 to G-200), in order to study the relative composition in peptides and in amino acids of various milk products such as acidic and enzymic hydrolysates of milk proteins. Visser *et al.* (1992) tested the efficiency of an agarose gel, superose-12 HR 10/30, and an agarose modified gel, Superdex-75 HR 10/30, when studying the distribution of molecular mass of milk protein hydrolysates.

In recent years, different silica gels chemically bonded with hydrophilic compounds have been commercially found, and have been used for steric size-exclusion chromatography (SSE-HPLC). The TSK-SW (Vijayalakshmi *et al.*, 1986; Lemieux *et al.*, 1991) and the Protein Pack 125 (Visser *et al.*, 1992) have been used for the SSE-HPLC of milk protein hydrolysates. Also, Barth (1982) used a silica support bonded with glyceropropylsilyl residues to determine the molecular mass of plant proteins hydrolysed. On the other hand, the use of this technique is limited by various factors such as secondary interactions, electrostatic or hydrophobic, with the stationary phase (Kopaciewicz & Regnier, 1982; Golovchenko *et al.*, 1992) and the inefficiency in separating the small peptides (Lemieux *et al.*, 1991).

Considering that the peptide retention on a reverse-phase column depends on the amino acid composition, on the ionisation state, on the charge and on the hydrophobicity (Lemieux & Amiot, 1989), while the molecular volume is the determining factor of their separation on a size-exclusion support, the peptides fractionated by SE-HPLC may be then isolated and purified by RP-HPLC in order to obtain more information about peptide and amino acid composition of protein hydrolysates. This process was used for analysing casein hydrolysates (Lemieux & Amiot, 1989;

Lemieux *et al.*, 1991) and bovine hemoglobine hydrolysates (Piot *et al.*, 1992).

The ligand exchange chromatography (LEC), involving the complex formation with the metalic ions, essentially copper (II), is another method used for separating peptides and amino acids of protein hydrolysates. Salmona *et al.* (1982) separated the amino acids and peptides added to different casein hydrolysates, using a copper–chelex resin. After a derivative reaction with the 5-dimethylaminonaphtalene-1-sulphonylchloride (Dns-Cl), the peptides were hydrolysed and fractionated by HPLC in order to determine their amino acid composition. The distribution of peptides, according to their molecular size, of casein and egg white hydrolysates, was determined by Ford *et al.* (1986) on a copper (II)–Sephadex column. Verneuil *et al.* (1990) fractionated a bovine serum albumin hydrolysate in four families, by high-performance ligand exchange chromatography (LE-HPLC) using silica gel bonded with copper (II): amino acids, no-basic dipeptides, tripeptides and larger size peptides. Considering the low retention of the peptides on immobilized metal ion stationary phases compared to the amino acids, Aubry *et al.* (1992) successfully employed the LEC on an iminodiacetate bonded polymer in the Cu(II) form, for separating dipeptides from hydrolyze bovine brain.

It is interesting to point out that sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) may be used for characterizing protein hydrolysates, notably in the cases of large peptide detection. In general, small peptides are not detected by this technique since they are eliminated from the gel during the colouring and washing steps. Serrano *et al.* (1984) studied the controlled proteolysis of tubulin by subtilisin using this technique. Schmidt and Poll (1991) showed the relevance of this method for differing the peptide profiles of protein milk serum hydrolysates, obtained by various enzymes. Another kind of PAGE technique, the urea-PAGE analysis, was able to confirm the formation of adstringent compounds during proteolysis of pasteurized skim milk by different proteinases (Harwalkar *et al.*, 1993).

The capillar electrophoresis (CE), having high sensitivity and resolution may be used for studying the profiles of hydrolyzed proteins. Castangola *et al.* (1991) used the CE coupled with the RP-HPLC to analyse a tryptic myoglobin hydrolysate. According to the authors, these two techniques give complementry results because the separation on an RP-HPLC column depends on the polarity, while that on CE is associated with the peptide charge.

Fast protein liquid chromatography (FPLC) is another technique used for separating peptides from protein hydrolysates. Harwalkar *et al.* (1993) correlated the proteolysis and the astringent off-flavor in milk by analysing the astringent compounds of hydrolysates using an FPLC system. This technique was also used for investigating the influence of heating and cooling on the trypsinolysis of caseins in bovine milk (Leaver & Thomson, 1993).

Finally, the efficiency of five chromatographic systems for the separation of commercial casein hydrolysates was tested in a work of Lemieux & Amiot (1990): size-exclusion, reversed-phase and ion-exchange HPLC; gel filtration and immobilized metal affinity. HPLC systems showed several advantages over other chromatographic methods, like SEC and metal afinity chromatography. Among them is the short time required for the analysis. The best system for molecular weight distribution was the SE-HPLC. It was also shown that tryptic digests and closely related and neutral peptides could be successfully resolved by HPLC on a weak anion-exchange bonded phase (AE-HPLC). The combination of SE-HPLC and RP-HPLC has proven to be successful in separating casein hydrolysates phosphorylated and dephosphorylated into more than 200 identified peptides.

After fractionation, the identification of peptides in protein hydrolysates is normally carried out using the following methods: the determination of the amino acid composition (Roland *et al.*, 1978; Umetsu *et al.*, 1983; Yvon *et al.*, 1989; Lemieux & Amiot, 1989; Minagawa *et al.*, 1989; Adachi *et al.*, 1991; Macedo *et al.*, 1993; Pihlanto-Leppälä *et al.*, 1994; Chen *et al.*, 1995), the N-terminal analysis using a protein sequencer (Macedo *et al.*, 1993; Pihlanto-Leppälä *et al.*, 1994), the mass spectrometry (Reid *et al.*, 1994; Chen *et al.*, 1995), the electro-spray mass spectrometry (Bouhallab *et al.*, 1993), the gas chromatography coupled with the mass spectrometry (electronic impact - Ford *et al.*, 1986; Aubry *et al.*, 1992) or the mass spectrometry by fast atom bombardement (FAB) (Pucci *et al.*, 1992). This last technique may also be used for determining the molecular mass of peptides in the chromatographic fractions of protein hydrolysates (Lemieux *et al.*, 1991).

All these observations showed that the separation of small peptides was not yet satisfactory, despite the progress in developing new techniques for fractionating protein hydrolysates. So, it would be important to continue searching for an efficient method able to separate these peptides because the nutritional quality of protein hydrolysates is partly related to their di- and tripeptide contents.

More recently, however, a new chromatographic support was tested (Silvestre *et al.*, 1994*a*). This is a poly (2-hydroxyethyl-aspartamide)-silica column (PHEA). Using a 0·05 M formic acid mobile phase, it was possible to fractionate by SE-HPLC amino acids and small peptides (molecular mass lower than 1000 Da.). The PHEA showed good SEC properties although some non-ideal size-exlusion behavior suggested it had a low cationic character. The technique was applied for analysing several casein hydrolysates and proved useful for separating peptides with molecular mass less than 1000 Da.

Estimation of the molecular weight distribution

In some cases, the interest in characterizing protein hydrolysates is not related with the separation and identification of peptides, and the determination of their molecular weight (MW) distribution may be useful. In fact, the peptide size profiles have been correlated with the antigenic and functional properties of different protein hydrolysates, and used for characterizing protein cleavage using various proteinases and for showing differences in pretreatments of the substrate.

The method of choice for MW determination, because of its simplicity and rapidity, has been most frequently SDS-gel electrophoresis. The alternative method of gel filtration requires longer times for a single run. However, recent developments in supports for HPLC have made more rapid separations possible, and thus gel permeation or size-exclusion chromatography (SE-HPLC) have become widely used techniques for MW determinations. These techniques allow easier quantification, better recovery and resolution than that achieved by gel filtration with conventional materials (Mayes, 1984).

A great variety of Sephadex gels has been described in the literature for characterizing protein hydrolysates by their MW range. In this way, gel chromatography on a Sephadex G-15 column has been used. The determination of MW distribution by gel chormatography on a Sephadex G-15 column was used for evaluating the wheat carboxypeptidase action in debittering proteins hydrolysates in food application (Umetsu *et al.*, 1983). The course of the hydrolysis of casein by consecutive thermolysin and papin treatments was studied on a Sephadex G-15 column (Adachi *et al.*, 1991).

Another type of Sephadex column, the G-25, has also been used by various authors for different purposes. Thus, Takase *et al.* (1979) prepared a low antigenic product from cow's milk, hydrolysing casein by different proteinases. The MW patterns of these hydrolysates were correlated with their antigenicity. Vallejo-Cordoba *et al.* (1986) correlated the peptide size to the effectiveness of soy protein hydrolysates in reducing water activity in meat products. Behnke *et al.* (1989) estimated the MW distribution of peptides from maize gluten hydrolysates. This investigation showed that small peptides were preferably found as hydrolysis products using different proteinases. To characterize the antioxidative peptides derived from soybean β-conglycinin hydrolysis, the hydrolysate was separated by SEC on Sephadex G-25 and a fraction estimated to be about 1400 Da was shown to have the most important antioxidative activity (Chen *et al.*, 1995).

The Sephadex G-50 has been the choice of other researchers. Thus, for studying the influence of enzymatic hydrolysis on structure and emulsifying properties of sardine protein hydrolysates, the MW distribution was carried out by gel chromatography on a Sephadex G–50 column (Quaglia & Orban, 1990). The elution profiles of trypsin hydrolysates from casein on Sephadex G-50 column were studied by Tchorbanov and Iliev (1993).

Deeslie and Cheryan (1988) used two kinds of Sephadex for studying the peptide distribution of soy protein hydrolysates produced in a continuous ultrafiltration reactor and correlated it with the foaming and emulsification properties. The peptide eluate was obtained by gel permeation chromatography using Sephadex G-15 and G-50.

When the HPLC technique is used for determining MW distribution of protein hydrolysates, some TSK columns (Toko Soda, Tokyo, Japan) have been described. Thus, Mahmoud *et al.* (1992) used a TSK-2000SW column in investigating hydrolysis of casein for potential use in a hypoallergenic infant formula. Two columns of this type were connected in series for determining the MW range of whey protein hydrolysates.This method revealed the effectiveness of different hydrolysates for inclusion in special enteric diets (González-Tello *et al.*, 1994). Working with the same proteins, Nakamura *et al.* (1993*a*) estimated the MW distribution of hydrolysates in an HPLC system using two TSK gel G3000PWxl columns arranged in series. This technique contributed to showing the antigenic superiority of high pressure over heat as a pre-treatment of enzymatic hydrolysis for preparing hypoallergenic formulae. The same technique was used by Nakamura *et al.* (1993*b*) to analyse casein hydrolysates for enzymatic production of hypoallergenic peptides.

The SDS-PAGE patterns have been used for characterizing some proteins hydrolysates in different aspects. Thus, Desphande & Nielsen (1987) used the SDS-PAGE pattern of phaseolin hydrolysates, the major storage protein of *Phaseolus vulgaris* L., for comparing the peptide size distribution obtained by different hydrolysis conditions (pre-heat treatment and a number of proteinases). The monitoring of trypsin-catalyzed hydrolysis of the various micellar casein fractions in whole bovine milk was conducted by analysing the SDS-PAGE patterns of the hydrolysates, (Leaver & Thomson, 1993). The MW profiles of the peptides produced at different stages of the hydrolysis process of whey proteins with commercial proteinases was monitored by SDS-PAGE (Perea *et al.*, 1993). The influence of heat treatment in preparing enzymatic hydrolysates of whey proteins was investigated by characterizing the products using SDS-PAGE patterns (Schmidt & van Markwjk, 1993). Parrado *et al.* (1993) used this technique for studying the MW distribution of sunflower protein hydrolysates in order to characterize them as potentially useful in the food industry for dietic purposes or as special food ingredients. The MW of the samples was also investigated by gel filtration using an FPLC system (fast protein liquid chromatography). The peptide maps obtained by SDS-PAGE from casein hydrolysates were used in a comparative study to ascertain the application of two different enzymes in

production of bitter peptides (Gallagher *et al.*, 1994). Also, the specificity of hydrolysis of bovine κ-casein by proteinases from *Lactococcus lactis* strains was studied using the SDS-PAGE patterns of the hydrolysates (Reid *et al.*, 1994).

The FPLC system in SEC mode was also used for determining the number and size of peptides from casein hydrolysates, and the results were used to evaluate the effect of limited hydrolysis and phosphorilation for improvement of functional and nutritional properties (Chobert *et al.*, 1987).

UV analysis: classical and second order derivative

Another method for the qualitative analysis of protein hydrolysates was recently described by Silvestre *et al.* (1993*a*): the UV spectrophotometry, classical and second order derivative. Different casein hydrolysates (commercial and laboratory-prepared) were analysed and the results were compared with those obtained by other authors using denaturated casein (Ragone *et al.*, 1984). It was shown that at the initial stage of protein hydrolysis, the decrease in absorbance measurements revealed the occurrence of similar phenomena to those described during protein denaturation (Creighton, 1989), probably due to the rupture of hydrophobic bonds. However, contrary to denaturation, the enzymic action leads to a cleavage of the protein molecule producing a more pronounced effect on the hydrolysate spectra. This technique was also used to estimate the degree of hydrolysis and the homogeneity (the presence of a real protein hydrolysate or a mixture of peptides and amino acids) of such preparations and proved to be a fast and a simple analytical method in characterizing casein hydrolysates, parallel to the cuprimetric technique described above. The tryptophan addition, used to compensate for the losses during the manufacture of protein hydrolysates, could also be detected by this technique. Moreover, this technique proved to be effective in identifying some difference between tryptic and pancreatic hydrolysis.

QUANTITATIVE ANALYSIS: EVALUATION OF PEPTIDE AND AMINO ACID CONTENTS

The techniques used for the quantitative analysis of protein hydrolysates are based on two essential principles: the direct quantification or the preliminary fractionation of the different components followed by a quantifying method.

The direct quantification

The determination of nitrogen content according to the Kjeldahl procedure and amino acid composition using an amino acid analyser, are very widespread processes for studying protein hydrolysates (Chang *et al.*, 1973; Mont & Jost, 1978; Cogan *et al.*, 1981; Helbig *et al.*, 1980; Umetsu *et al.*, 1983; Vallejo-Cordoba *et al.*, 1986; Deeslie & Cheryan, 1988; Hardwick & Glatz, 1989; Adachi *et al.*, 1991; Armstead & Ling, 1991; Zhang *et al.*, 1992, Parrado *et al.*, 1993).

A cuprimetric assay described by Lati *et al.* (1992) for analysing amino acids and peptides was used for analysing casein hydrolysates (Silvestre *et al.*, 1993*b*). It was shown that copper (II) ion consumption by these hydrolysates increased with the degree of hydrolysis. This opened the way to a new method for protein hydrolysate analysis which is complementary to the official methods (total and α-amino nitrogen determination), because the cuprimetric assay can predict the di- and tripeptide contents of these preparations. The proposed technique proved to be better than the classical α-amino nitrogen assay for diferentiating between homogeneous hydrolysates and mixtures consisting of amino acids and poorly hydrolysed proteins. Moreover, the cuprimetric assay showed no interference by lipids and carbohydrates and could be advantageously used for analysing commercial preparations that generally contain these compounds.

However, the results obtained by all these direct estimations represent only a partial knowledge of the composition of these complex mixtures. For better characterizing protein hydrolysates, it is important to make a prior separation of peptides and amino acids using a chromatographic method followed by a quantifiation method. The fractionation by chromatographic methods have been described above (qualitative analysis).

The quantification following a fractionation step

Among the available methods for quantifying different components in the chromatographic fractions of protein hydrolysates, the amino acid analysis is the most commonly used (Verneuil *et al.*, 1990). The continuous determination of nitrogen compounds appearing in the chromatographic elution of protein hydrolysates was also proposed by Amiot & Brisson (1980). However, despite their precision, these techniques are time-consuming and laborious. Considering the high complexity of protein hydrolysates, it is desirable to use rapid and simple techniques.

The measure of the UV absorbance at 280 nm, a fast technique, could be a solution (Hernandez & Asenjo, 1982; Keohane *et al.*, 1985). However, in this case the free-aromatic amino acid peptides are not detected. This problem cannot be solved by measuring the absorbance at 206 or 220 nm, since both aromatic residues and peptide linkages absorb at low wavelengths. Some similar inconveniences are present when using the Lowry method (Hernandez & Asenjo, 1982), since the peptide linkages and the tyrosyl residues are detected.On the other hand, the absorbance measurement may be useful in the case of determining aromatic amino acids

previously separated on a chromatographic column (Iliev & Tchorbanov, 1992).

These comments showed that not only are there a restricted number of available methods for the evaluation of peptide and amino acid contents in protein hydrolysates, but also this analysis step still represented a tricky problem. Thus, it would be necessary to develop new alternative quantifying methods capable of being at the same time precise and quick to allow industrial use.

For this purpose, Silvestre *et al.* (1994*b*) proposed a rapid method for quantifying peptides in SE-HPLC fractions of casein hydrolysates, eluted from the PHEA column. It was based on UV absorbance measurement at 230 nm, with correction for aromatic amino acid absorbance using multidetection at 3 wavelengths (230, 280 and 300 nm). This quantitative technique proved to be a valuable tool for characterizing unknown protein hydrolysates.

CONCLUSIONS

A partial characterization of protein hydrolysates may be achieved by the evaluation of the hydrolysis degree of the peptide bonds. Despite the simplicity of the methods used for this purpose, they show a lot of practical inconveniences leading to discordant results.

For qualitative analysis, different techniques have been used for the separation and identification of peptides and amino acids in protein hydrolysates, based on chromatographic or electrophoretic behavior. However, the problems related to the interactions between the solutes and the matrix have not been completely resolved. The estimation of the molecular weight distribution of these preparations also uses chromatographic or electrophoretic techniques, and the best results are obtained when the SE-HPLC is the method chosen. UV spectrophotometry, classical and second order derivative, was recently used for analysing protein hydrolysates and proved to be a useful tool in estimating the hydrolysis degree, the homogeneity and the tryptophan addition.

The quantitative analysis of protein hydrolysates involves direct and indirect methods. In the first case, the cuprimetric assay, the nitrogen determination and the amino acid analysis have been used. However, the best characterization is obtained when the quantification procedure follows a fractionation step. Among the different methods described for quantifying the components in chromatographic fractions of protein hydrolysates, the UV measurement has largely been used. Nevertheless, this technique shows several disadvantages associated especially with the presence of aromatic amino acids. To overcome these and other problems related to the time-consuming and laborious techniques, a new rapid quantifying method was developed recently, based on UV absorbance measurement at 230 nm, with correction due to the presence of aromatic amino acids.

As a rapid quantifying technique now exists, the improvement of the analytical process for characterising protein hydrolysates, especially when the interest is related to the small peptide contents, depends in the future on the availability of a chromatographic support completely free of secondary interactions, where only separations based on size occur.

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