Digestibility of Technologically Treated Lamb Meat Samples Evaluated by an in Vitro Multienzymatic Method

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Digestion is the first step in the metabolic fate of ingested food proteins; in view of its significant physiological and pathological consequences, the digestion process frequently has to be taken into consideration. In this paper, an improved in vitro multienzymatic digestibility assay has been used to evaluate protein degradation in lamb meat samples, that is, raw, steam-cooked, homogenized (strained), and commercially available freeze-dried meat, all from the same processing chain. Our data indicate that enzymatic attack is strongly affected by heat treatment as shown in steam-cooked meat samples. On the other hand, homogenization and freeze-drying processes are able to partially reverse the phenomenon, thus improving peptide and amino acid release. Data on modification of SDS–PAGE protein pattern during the multienzymatic assay are also reported.

INTRODUCTION

Digestion is the first step in the metabolic fate of ingested food proteins. Protein digestion starts in the stomach where pepsin, the most abundant gastric protease, performs a selective cleavage of proteins into smaller peptidic fragments. The peptic digestion produces mainly polypeptides with a small quantity of oligopeptides and amino acids (Neurath et al., 1976; McNeish, 1984). In the intestine, the products of peptic degradation undergo a complicated process of breakdown by pancreatic enzymes (trypsin, chymotrypsin, elastase, and carboxypeptidases A and B) which liberate small peptides (two to six amino acid residues) and free amino acids.

Oligopeptides and amino acids are then absorbed by the brush-border membrane via their distinct transport mechanisms (Adibi and Kim, 1981; Matthews, 1975; Matthews and Adibi, 1976). Thus, they can reach the portal circulation and finally the sites of protein synthesis.

The uptake of undigested protein and/or polypeptides is low in adult organisms, but it is considerable in the new immature gut of the newborn and in some pathological situations where mucosal integrity is disrupted (Dannaeus et al., 1979).

In view of the physiological and pathological consequences, digestion is a very important function to investigate. Considering the obvious difficulties involved in carrying out in vivo enzymatic studies, the availability of an in vitro method represents a valid approach for a better understanding of proteolytic degradation of food proteins. Several in vitro methods to evaluate protein digestibility are reported in the literature (Bodwell et al., 1980; Brule and Savoie, 1988; Buchanan, 1969; Eid and Matty, 1989; Floridi and Fidanza, 1975; Satterlee et al., 1977, 1979; Swaisgood and Catignani, 1991); some are described as multienzymatic assays able to simulate physiological conditions (Akeson and Stahmann, 1964; Buchmann, 1977; Gauthier et al., 1982; Semino et al., 1985). In this paper, data obtained by a modification of Buchmann's and Semino's multienzymatic methods are presented. In particular, four different lamb meat samples were subjected to enzymatic attack to evaluate digestibility changes associated with structural protein changes.

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MATERIALS AND METHODS

Lamb Meat Products. Lamb meat products were kindly supplied by STAR, Mellin Division, Carnate, Milan.

Products used in the digestion studies were (1) raw and chopped lamb meat; (2) steam-cooked (steam injection at 110–120 °C, 150 s) and minced lamb meat; (3) homogenized (strained) lamb meat containing 4% rice flour (homogenization was performed with a crossed-toothing colloid mill and a corundum grinder colloid mill); and (4) freeze-dried (from a semicontinuous freeze-drying unit) lamb meat (baby food) containing 31% rice flour (corresponding to a protein addition of 0.04%). Meat samples came from successive stages of the same processing chain for the production of lamb meat freeze-dried as a baby food.

Chemicals. Chemicals were of the highest purity grade available commercially, and when unspecified, they were from Merck (Darmstadt, Germany).

Protein Digestion. *Peptic Digestion.* Protein digestion was performed as shown in Figure 1. Samples of meat products were weighed into 50-mL screw-cap flasks and suspended in 0.01 N HCl, pH 2, containing 0.05 mg/mL of pepsin. The final protein concentration was 3.0 ± 0.1 mg/mL and the enzyme/protein ratio was 1/60 (w/w). Protein concentrations were determined in each product according to both Kjeldahl and Lowry methods.

Proteolysis was carried on at 37 °C in a Dubnoff water bath shaken at 100 beats/min.

Pancreatic Digestion. After 2 h of peptic attack, a solution (1 M boric acid, 0.5 N NaOH corrected to pH 6.8 with 5 N HCl) containing 0.5 mg/mL of pancreatin and 25 mM $CaCl_2$ was added to each sample in the ratio 1/3.5 (v/v). The final pH value was 7.6, and the pancreatin/protein ratio was 1/24 (w/w).

Sample Analyses. The reaction was stopped at different times (0, 10, and 30 min, and 1 and 2 h of pepsin attack and 5 and 30 min and 1, 3, 6, and 24 h of pancreatic attack) either by precipitating the protein in final 10% trichloroacetic acid (TCA) or by diluting with the same volume of sample dilutor (0.25 M Tris-HCl buffer, pH 6.8, containing 7.5% glycerol, 2% SDS, and 0.01% β-mercaptoethanol) and then heating for 10 min at 100 °C. The latter samples were analyzed by SDS–PAGE. Those precipitated with TCA were centrifuged (J2-21 centrifuge, Beckman Instruments, Inc., Palo Alto, CA) for 20 min at 4 °C and 10 000 rpm. Supernatants were collected; pellets were washed with 0.1% TCA, centrifuged as described previously, and then solubilized in a volume of 0.5 N NaOH equivalent to the volume of HCl used at the start of the digestion.

Protein Determination. Pellets solubilized in 0.5 N NaOH were assayed for the protein content. Determination was performed according to the method of Lowry et al. (1951), using BSA as a standard protein. Data were expressed as a percentage of the initial protein value (time = 0).

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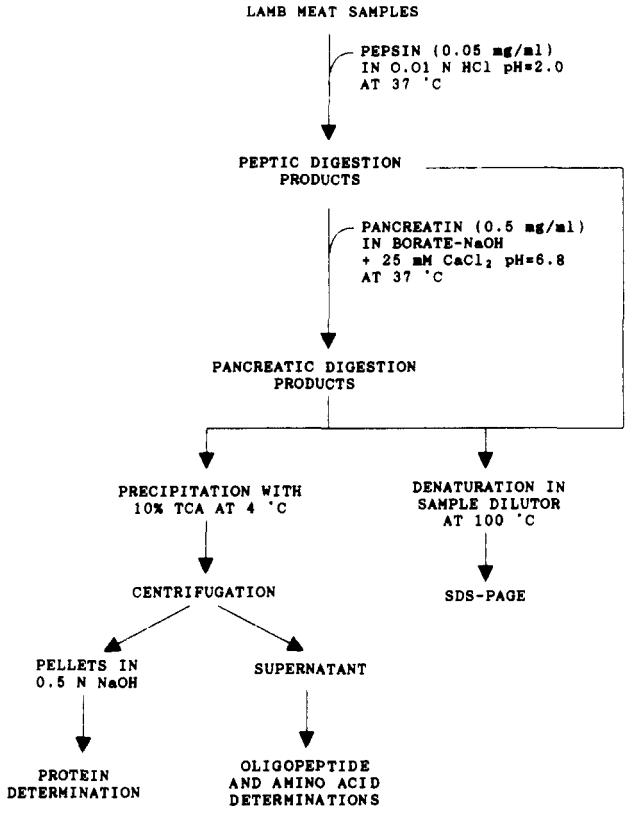

Figure 1. Experimental design of the in vitro multienzymatic digestion.

Oligopeptide Determination. Oligopeptides liberated during the digestion were measured in the supernatant obtained after precipitation with 10% TCA. Determination was carried out according to the method of Itzhaki and Gill (1964) (microbiuret method), using BSA as a standard protein. Data were expressed as milligrams of oligopeptides liberated per 100 mg of initial protein.

Since the rice flour, present in homogenized and freeze-dried products, interfered with oligopeptide determination in the samples coming from pancreatic attack, the interference value (due to starch hydrolysis by amylase) was deducted from the affected measurements. The interference values were calculated by performing a parallel digestion on the rice flour only.

Amino Acid Determination. Amino acids were measured in the 10% TCA supernatant with ninhydrin reagent (McCaldin, 1960; Moore and Stein, 1954). A standard solution containing 17 amino acids (Sigma Chemical Co., St. Louis, MO) was used for the calibration curve. In our conditions, oligopeptide amino groups did not interfere with amino acid determinations, even when the amino acid/oligopeptide ratio was 1:1 (w/w).

Data were expressed as milligrams of free amino acids per 100 mg of initial protein.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). All chemicals were purchased from Bio-Rad (Richmond, CA). The gels had the following final concentrations.

Gradient Running Gel: 9–17% acrylamide; 0.24–0.45% bis(acrylamide); 0.36 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% *N,N,N′,N′*-tetramethylenediamine (Temed). Bromophenol Blue was added to the gel.

Stacking Gel: 3.5% acrylamide; 0.09% bis(acrylamide); 0.125 M Tris-HCl buffer, pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% Temed.

Samples (20 μL, prepared as described under Protein Digestion) were loaded onto the gel (Bio-Rad Mini 2D-cell, Bio-Rad). Run was performed at 100 mV (Bio-Rad 200/2 power supply, Bio-Rad) and lasted approximately 3 h. The run was stopped when Bromophenol Blue reached the bottom of the gel.

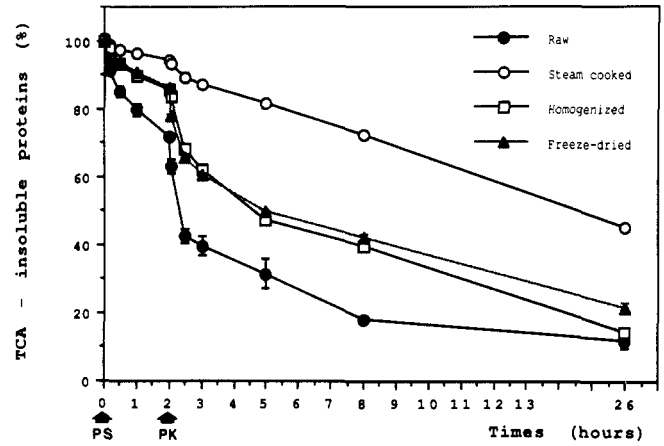

Figure 2. TCA-insoluble protein in different lamb meat products. PS, pepsin; PK, pancreatin. Data are means ± SE (n = 3).

In these conditions, polypeptides having molecular weights ranging between 8000 and 100 000 can be separated and quantified.

Gels were dyed with Coomassie Blue G-250 according to the method of Neuhoff et al. (1988) and analyzed with a laser densitometer (Ultroscan XL, LKB, Bromma, Sweden). Densitometries were elaborated with the LKB 2400 Gel Scan XL software package, using an Olivetti M24 personal computer.

Protein molecular weights were calculated by using a calibration curve. Low molecular weight (MW) marker solution included phosphorylase B (MW = 97 400), bovine serum albumin (MW = 66 200), ovalbumin (MW = 42 700), carbonic anhydrase (MW = 31 000), soybean trypsin inhibitor (MW = 21 500), and lysozyme (MW = 14 400).

RESULTS

In vitro digestion studies were performed on four lamb meat samples, one raw and three processed by steam-cooking, homogenization, and freeze-drying, respectively. In Figure 2 TCA-insoluble protein (TIP) percentages (initial value = 100) are reported; the proteins of raw meat are rapidly hydrolyzed after both pepsin and pancreatin addition. On the contrary, steam-cooked meat is digested very slowly, and this is more evident during the peptic action. After 2 h of pepsin attack, TIP is 71.8% of initial protein for the raw product, whereas it is 94.1% for steam-cooked meat. Homogenized and freeze-dried product behavior when subjected to pepsin action are comparable, and TIP values are 85.3% and 86.1%, respectively.

Digestion during pancreatin attack shows a similar pattern. Degradation of raw meat is rapid, and after 6 h (8 h from the start of the experiment) TIP is 17.8%, whereas it is 72.3% for steam-cooked meat, 39.3% for homogenized meat, and 41.8% for freeze-dried product. After 24 h of pancreatin digestion, TIP values for homogenized and freeze-dried products are close to the value for raw meat (14.1% and 21.5% vs 11.9%, respectively).

Oligopeptides and amino acids are the most abundant products of proteolysis. Oligopeptides (expressed as percent of the initial protein) liberated during pepsin and pancreatin attacks are reported in Figure 3. Since heat treatment causes release of a small quantity of oligopeptides and amino acids, their values measured at time = 0 were deducted, when necessary, to evaluate the true enzymatic action on different products.

Digestion of raw meat produces a rapid release of oligopeptides; in fact, at the end of pepsin attack they represent 23.4% of initial protein. When pancreatin is added, a further rapid release is shown, and after 30 min,

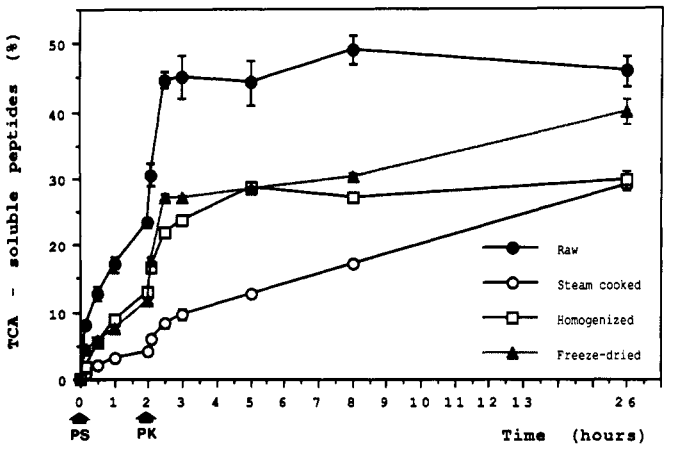

Figure 3. Oligopeptides (mg/100 mg of initial protein) released during in vitro digestion. Data are means ± SE (n = 3).

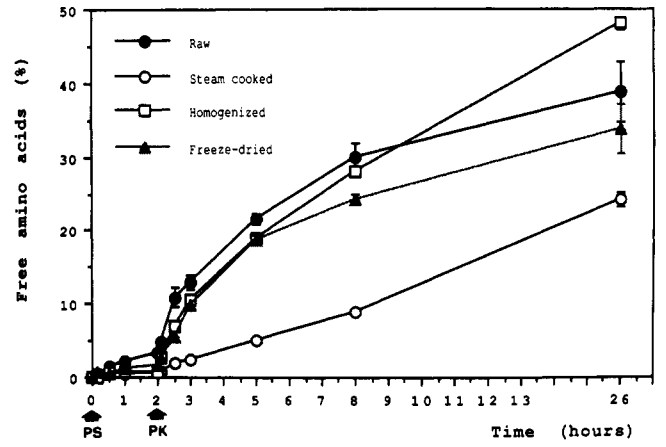

Figure 4. Free amino acids (mg/100 mg of initial protein) liberated during in vitro digestion. Data are means ± SE (n = 3).

oligopeptides increase to 44.7%, where a plateau is reached. Oligopeptide production is extremely slow when steam-cooked meat is digested. After 2 h of pepsin action, oligopeptides are 4.3% of initial protein. They continue to increase slowly under pancreatin attack, and a plateau is not seen even after 24 h, when oligopeptides are 28.9% of initial protein.

Homogenized and freeze-dried samples show an intermediate pattern. After 2 h of pepsin action on homogenized meat, the oligopeptide value is 13%; it increases during the first period of pancreatin proteolysis and reaches a plateau of 28.6% after 3 h.

Finally, at 24 h, the value is close to that obtained with steam-cooked meat (29.7% vs 28.9%). Freeze-dried product behavior is similar to that of homogenized meat, but a plateau is not reached and oligopeptide release continues even after 24 h of pancreatin attack, when the value is 39.8%.

Free amino acid production (expressed as percent of the initial protein) during enzymatic attack is shown in Figure 4. As expected, pepsin liberates only a small amount of amino acids, which increase only after pancreatin addition due to its content of exopeptidases. Amino acid release from steam-cooked meat is very slow, whereas the other three products show a similar behavior with a rapid and continued increase of values. Only at 24 h of pancreatin attack does the homogenized meat seem to be more digested (48%) than raw meat and freeze-dried product (38.7% and 33.7%, respectively).

SDS–PAGE results of the four meat products at different times of digestion are shown in Figure 5. In general, raw meat protein bands are more clearly defined than those of processed products. Moreover, the protein patterns coming from the digestion of the four meat samples are quite similar; during pepsin action, the only interesting differences regard raw meat gel, where low molecular weight proteins are already present after a short time of digestion (30 min) and where the myoglobin band (MW = 16 900) is less evident.

More differences can be seen in the protein pattern deriving from pancreatin digestion. The protein with MW = 35 000 (one of the two monomers of tropomyosin) is present in all raw meat lines and is still visible at the last time considered (24 h). On the contrary, the protein disappears in the three processed samples after 1 h of pancreatin digestion. Actin (MW = 43 000) shows a rapid degradation in raw meat, while it lasts longer in the processed lamb meat products.

Finally, at 24 h of pancreatin attack the raw meat pattern presents only a few protein bands, distributed over a large range of molecular weights, whereas several undefined protein bands, ranging from 25 000 to 8000, can be seen in the three processed samples.

Differences are more evident when densitometries of the four SDS–PAGE gels are overlapped. As an example, densitograms of the four meat products after 24 h of pancreatin digestion are shown in Figure 6.

DISCUSSION

Among the available in vitro digestibility assays, we chose and modified a multienzymatic method which may simulate the physiological process of protein digestion. To mimic human digestion more closely, mammalian gastric and pancreatic enzymes were used. The limitation of the method is the persistence of proteolysis products that in vivo are removed by absorption. This fact could theoretically slow down the progress of digestion, but our results show that, when structural characteristics of the substrate permit it, digestion can continue until most of the protein is degraded (90% in raw meat samples).

The use of TCA, as a precipitating agent, allows not only the inactivation of enzymatic action (Alfred and Narasinga Rao, 1971) but also the separation of oligopeptides and amino acids from undigested protein. The average molecular weight of 10% TCA soluble peptides ranges from 330 to 380, indicating that most of the peptide content left in solution contains three or four amino acid residues (Greenberg and Shipe, 1977). The selectivity of TCA precipitation permits interesting in vivo correlations. In fact, oligopeptides of this size may possess a remarkable physiological significance because of their selective absorption via the intestinal peptide carrier system as reported by Adibi and Kim (1981). Amino acids follow a similar physiological fate; therefore, the measure of oligopeptides and amino acids released during proteolysis indicates how proteins can be broken down into small molecules that are more easily absorbed and utilized for synthetic purposes.

The strong reduction of enzymatic action observed in steam-cooked meat digestion, when compared to that of raw samples, can be justified by the protein structural changes due to heat denaturation (Privalov, 1990). This change can improve (Mauron, 1982; Restani et al., 1983) or reduce (Nufur-Chopra and Hira, 1986; Seidler, 1987) the enzymatic attack on proteins. After 26 h, at the end of digestion, the TCA-insoluble proteins in homogenized and freeze-dried samples tend to approach the raw meat value. This is probably a consequence of the increased surface of enzymatic attack due to the homogenization

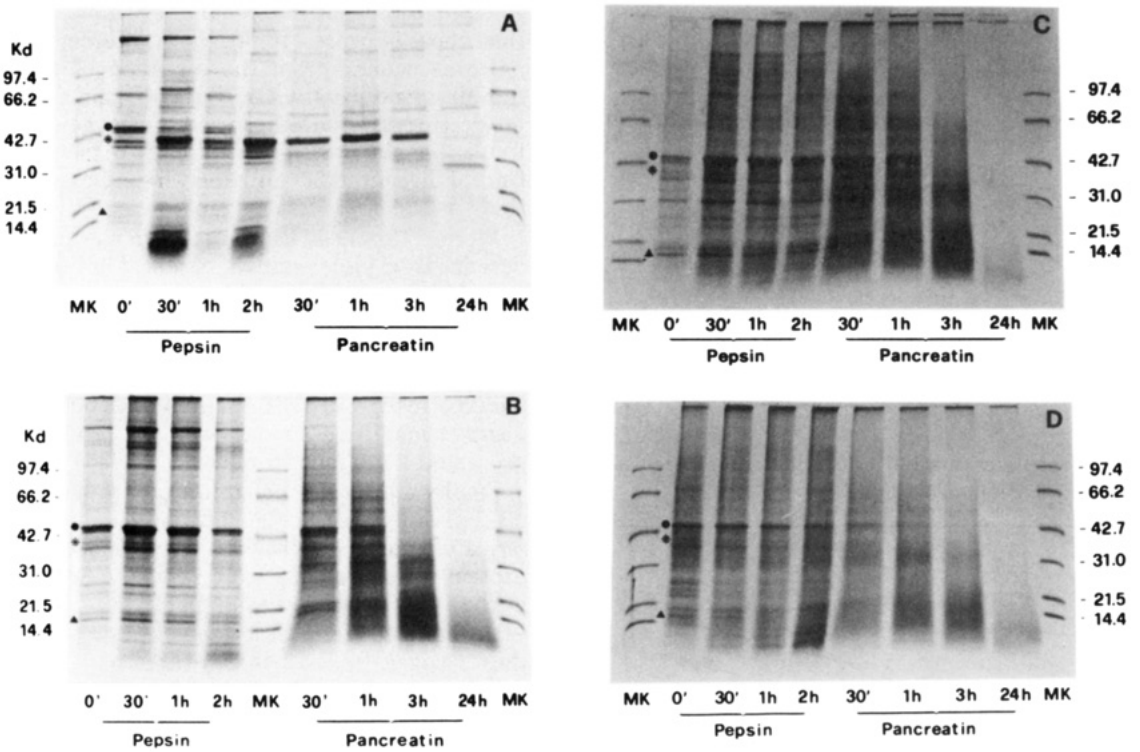

Figure 5. SDS–PAGE of lamb meat products after enzymatic degradation. A, raw meat; B, steam-cooked meat; C, freeze-dried meat; D, homogenized meat. *, tropomyosin; ●, actin; ▲, myoglobin.

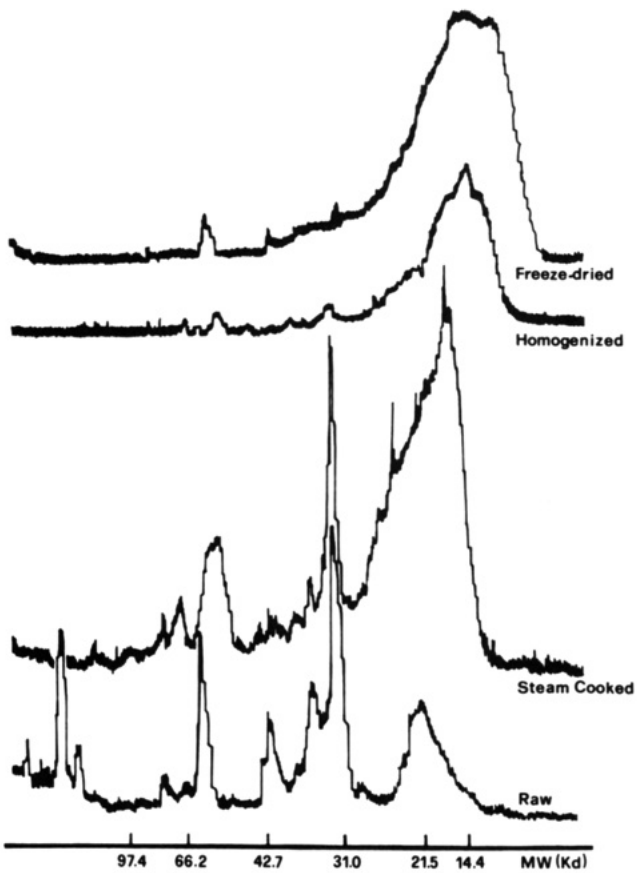

Figure 6. SDS–PAGE densitograms of lamb meat products after 24 h of pancreatin attack.

process. This is even more evident when the amino acid release during pancreatin digestion is considered; in fact, pancreatin liberates from homogenized samples an amino acid amount equivalent to that of raw meat. Freeze-dried samples behave similarly; in fact, freeze-drying is a mild process that affects protein structure only slightly.

The improvement is less consistent when oligopeptide release is analyzed. The differences can be explained by the characteristic of enzymes involved in the digestion. Pepsin and some of the enzymes present in pancreatin (trypsin and chymotrypsin) are endopeptidases that break peptidic bonds inside the molecules. For these reasons, their action is significantly affected by protein structural changes such as those obtained after heat treatment. Their action improves, however, when the surface available to enzymatic attack increases after the homogenization process.

On the other hand, exopeptidases that are present in pancreatin (carboxypeptidases A and B) work only at the carboxy terminus of the protein chain; therefore, they act more actively after meat homogenization.

SDS–PAGE has been performed to monitor TCA-insoluble peptides; in fact, proteins can be partially attacked by proteolytic enzymes with the release of protein fragments which, having a molecular weight above 380, precipitate with 10% TCA. Hence, SDS–PAGE allows the evaluation of intermediate product release.

The lower definition of protein bands observed in processed samples could be justified by heat denaturation that causes a minor solubility of proteins.

Endopeptidases seem to be more affected than exopeptidases in their proteolytic action by heat treatment; this fact can be inferred both from the lower amount of oligopeptides liberated from processed samples and from the delayed release of low molecular weight proteins during digestion as shown in SDS–PAGE.

Protein having MW = 35 000 (tropomyosin) behaves differently; in fact, it seems to be more easily digested in processed samples than in raw meat. As a hypothesis, it can be suggested that heat denaturation is able to expose, in this particular protein, specific sites for enzymatic attack that are normally hidden inside the molecule.

In conclusion, our in vitro method is able to quantify

proteolytic changes associated with protein structure modifications resulting from food technological processes. The protein degradation decrease observed after heat denaturation is partially reversed by the technological processes used for the production of meat baby foods (homogenization and freeze-drying) which combine hygienic and long-lasting conservation with a better digestibility (and consequently a higher nutrient availability) as compared with steam-cooked product.

Finally, the method described can be suggested for the study of protein digestibility in other food preparations. In fact, although we used the method to evaluate meat protein digestibility, we believe that because of its high versatility this assay could be applied to a wide variety of food samples.

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LITERATURE CITED

Adibi, S. A.; Kim, Y. S. Peptide absorption and hydrolysis. In *Physiology of the gastrointestinal tract*; Johnson, L. R., Ed.; Raven Press: New York, 1981; pp 1073–1095.

Akeson, W. R.; Stahmann, M. A. A pepsin pancreatin digest index of protein quality evaluation. *J. Nutr.* **1964**, *83*, 257–261.

Alfred, G.; Narasinga Rao, M. S. A study of the inactivation of pepsin by trichloroacetic acid. *Indian J. Biochem. Biophys.* **1971**, *8*, 28.

Bodwell, C. E.; Satterlee, L. D.; Hackler, L. R. Protein digestibility of the same protein preparations by human and rat assays and by in vitro enzymatic digestion methods. *Am. J. Clin. Nutr.* **1980**, *33*, 677–686.

Brule, D.; Savoie, L. In vitro digestibility of protein and amino acids in protein mixtures. *J. Food Sci.* **1988**, *43*, 361–372.

Buchanan, R. A. In vivo and in vitro methods of measuring nutritive value of leaf protein concentrates. *Br. J. Nutr.* **1969**, *23*, 533–545.

Buchmann, N. B. In vitro digestibility of barley protein. *Risoe Rep.* **1977**, *No. 351*.

Dannaeus, A.; Inganas, M.; Johanson, S. G. D.; Fouchard, T. Intestinal uptake of albumin in malabsorption and food allergy in relation to serum IgG antibody and orally administered sodium chromoglycate *Clin. Allergy* **1979**, *9*, 263–270.

Eid, A. E.; Matty, A. J. A simple in vitro method for measuring protein digestibility. *Aquaculture* **1989**, *79*, 111–119.

Floridi, A.; Fidanza, F. The enzyme-ultrafiltrate digest (EUD) assay for measuring protein quality. *Riv. Sci. Tecnol. Alimenti Nutr. Um.* **1975**, *5*, 13–18.

Gauthier, S. F.; Vachon, C.; Jones, J. D.; Savoie, L. Assessment of protein digestibility by in vitro enzymatic hydrolysis with simultaneous dialysis. *J. Nutr.* **1982**, *112*, 1718–1725.

Greenberg, N. A.; Shipe, W. F. Comparison of the abilities of trichloroacetic, picric, sulfosalicilic and tungstic acids to precipitate protein hydrolysates and proteins. *J. Food Sci.* **1979**, *44*, 735–737.

Itzhaki, R. F.; Gill, D. M. A micro-biuret method for estimating proteins. *Anal. Biochem.* **1964**, *9*, 401–410.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Matthews, D. M. Intestinal absorption of peptides. *Physiol. Rev.* **1975**, *55*, 537–608.

Matthews, D. M.; Adibi, S. A. Peptide absorption. *Gastroenterology* **1976**, *71*, 151–161.

Mauron, J. Effect of processing on nutritive value of food: Protein. In *Handbook of nutritive value of processed food*; Rechcigl, M., Ed.; CRC Press: Boca Raton, FL, 1982; Vol. 1, pp 429–471.

McCaldin, D. J. The chemistry of ninhydrin. *Chem. Rev.* **1960**, *60*, 39–51.

McNeish, A. S. Enzymatic maturation of the gastrointestinal tract and its relevance to food allergy and intolerance in infancy. *Ann. Allergy* **1984**, *53*, 643–648.

Moore, S.; Stein, W. H. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **1954**, *211*, 907–913.

Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **1988**, *9*, 255–262.

Neurath, H.; Walsh, K. A. Role of proteolytic enzymes in biological regulation (a review). *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3825.

Nufur-Chopra; Hira, C. K. Effect of roasting on protein quality of cereals. *J. Food Sci. Technol. India* **1986**, *23*, 233–235.

Privalov, P. L. Cold denaturation of proteins. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 281–305.

Restani, P.; Cerletti, P.; Semino, G.; Mariani, A. Nutritional value of proteins from lupine seed: factors hindering the attack by proteolytic enzymes. *Qual. Plant Plant Foods Hum. Nutr.* **1983**, *33*, 187–191.

Satterlee, L. D.; Kendrick, J. G.; Miller, G. A. Rapid in vitro assays for estimating protein quality. *Food Technol.* **1977**, *31*, 76–81.

Satterlee, L. D.; Marshall, H. F.; Tennyson, J. M. Measuring protein quality. *J. Am. Oil Chem. Soc.* **1979**, *56*, 103–109.

Seidler, T. Effects of additives and thermal treatment on the content of nitrogen compounds and the nutritive value of hake meat. *Nahrung* **1987**, *31*, 959–970.

Semino, G. A.; Restani, P.; Cerletti, P. Effect of bound carbohydrate on the action of trypsin on lupin seed glycoproteins. *J. Agric. Food Chem.* **1985**, *33*, 196–199.

Swaisgood, H. E.; Catignani, G. L. Protein digestibility: *In vitro* methods of assessment. *Adv. Food Nutr. Res.* **1991**, *35*, 185–236.

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