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Application of two-dimensional electrophoresis for monitoring gastrointestinal digestion of milk

Short Communication

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Summary. Two-dimensional electrophoresis (2-DE) was used for tracing in vivo gastrointestinal digestion of milk proteins in a rapid model system with rats. Contents of stomach and small intestine from digestion trials with rats given a single dose of milk powder were recovered after 1 hour. They were then subjected to 2-DE (IEF and SDS-PAGE). 2-DE showed undigested proteins in a MW range 13.0–66.0 kDa in stomach and 13.0–25.0 kDa in the small intestine, thus indicating that milk proteins are slowly digested. This approach may shed light on pattern of protein digestion and mechanism of amino acid and peptide assimilation.

Keywords: 2-DE – In vivo digestion – Milk

Introduction

In the proteomic era, the systematic analysis of proteins has recently been extended to the study of food quality. Analysis of complex food protein mixtures by two-dimensional electrophoresis (2-DE) and advanced techniques, such as capillary electrophoresis and matrix assisted laser desorption ionization-time of flight mass spectrometry, has been performed (Ferranti, 2004). Electrophoretic and mass spectrometric techniques have been established as an efficient tool for protein and peptide analysis in several food matrices, providing accurate estimation of molecular weight and structural assessment (Dreger, 2003). This allowed to gain information on quality, authenticity and safety of foods, properties of food allergens and other aspects of nutritional sciences and food technology, including the effect of new technologies on food products (Iwahashi and Hosoda, 2000; Kvasnicka, 2003; Carbonaro, 2004). On the other hand, application of these approaches to bioavailability studies is still very limited (Carbonaro et al., 2003).

Protein digestibility and amino acid bioavailability are strictly dependent on interaction that may occur during digestion (protein–protein interaction or interaction with non-protein food components), with consequence on absorption of essential aminoacids or of biologically active peptides (Kilara and Panyam, 2003).

Milk contains a variety of proteins with different properties. Although digestibility of major milk proteins is quite high, several proteins, such as immunoglobulins, kappa-casein, lysozyme, lactoferrin, haptocorrin, alpha-lactalbumin, and lactoperoxidase, are relatively resistant against proteolysis in the gastrointestinal tract (Lonnerdal, 2003).

Understanding mechanisms of digestion of milk proteins is very important because both caseins and whey proteins are known to contain within their sequence peptides with relevant bioactivities (mineral-binding properties, opioid, antihypertensive or immunomodulatory activities) (Meisel, 2004).

A method that employs rats in short-term (1 hour) experiments was previously developed for monitoring gastrointestinal processing of food components and measuring percentages of absorption of nutrients specifically in the small intestine (Carbonaro et al., 2000). It was successfully applied in bioavailability studies of essential amino acids, minerals and trace elements (Carbonaro et al., 2003).

In this study, a 2-DE approach was applied in studying bioavailability of proteins from milk. For this purpose,

in vivo digestion pattern of whole milk in both stomach and small intestine was analyzed by 2-DE (IEF and SDS-PAGE).

Materials and methods

Materials

Pasteurized milk was obtained from the local market. Milk was freeze-dried and stored at −20 °C until use. Protein content (N × 6.38) was determined by the Kjeldahl method (AOAC, 1990).

Water was purified by a Milli-Q purification system (Millipore, Bedford, MA, USA).

In vivo digestion system

In vivo digestion was performed as described by Carbonaro et al. (2000) in an acute (1 h) experiment with growing rats. Male rats of the Hooded Lister strain (40 days of age), weaned at 19 days of age, were adapted to experimental conditions by feeding them a semisynthetic lactalbumin-control diet for 7 days. Rats (140 ± 1 g) were then housed individually in polypropylene and stainless-steel cages and fasted overnight.

Rats (4 per treatment) were given a single oral dose of 1.0 g of milk powder (240 mg protein) added to 1.0 g of basal diet (maize starch, 500 mg; glucose, 300 mg; glycerol, 200 mg). All animals consumed the meal within 10 min. Basal groups were obtained by giving rats basal diet only, in the same amount that was given to the corresponding treated rats. One hour after feeding the rats were killed by anaesthetic overdose. The time of 1 h was chosen because it has previously been calculated that digesta would not have reached yet the large intestine by this time (Sgarbieri et al., 1982). Stomach and small intestine were taken out separately from the abdomen. The stomach and intestinal contents were washed out with ice-cold water and centrifuged (4000 × *g*, 20 min). The protein content of the supernatants was determined by the method of Lowry et al. (1951). Protein contents in stomach and small intestine were corrected for non-dietary protein and digestibility (%) was calculated by the following expression:

$$\text{P.D. } (\%) = \frac{P_{ing} - P_{st} - P_{int}}{P_{int} - P_{st}} \times 100$$

where P.D. = protein digestibility; P_{ing} = mg of protein ingested; P_{st} = mg of protein in the stomach; P_{int} = mg of protein in the intestine.

All management and experimental procedures were carried out in strict accordance with the requirements of UK Animals (Scientific Procedures) Act 1986 by staff licensed under this Act to carry out such procedures.

2-DE

First dimension IEF was performed on a IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech, Uppsala, Sweden) with ready-to use Immobiline Dry-Strips, (pH 3–10, 18 cm), according to the manufacture's instructions. Strips were placed in the IPGphor strip holder. Sample (25 µg) and rehydration buffer were dropped onto the strips. IEF run was conducted at 20 °C at 500 V for 1 h, 1000 V for the next hour and 8000 V for 4 h. After IEF, the strips were stored at −80 °C.

The second dimension (SDS-PAGE) was carried out with a Multiphor II Apparatus (Amersham Pharmacia Biotech) on precast Excel Gels (12–14% gradient) at a costant corrent of 20 mA for 45 min and then of 40 mA for 2.5 h. LMW marker proteins 97.0–14.4 kDa (Sigma) were used. After protein fixation overnight, the gel was stained with Comassie Brilliant Blue G-250. The gel was destained, gel image was taken and processed using the software Image Master 2D Elite 4.01 (Amersham Pharmacia Biotech).

Results

Digestibility in the small intestine of whole milk measured by this method was 92.0% while that of isolated

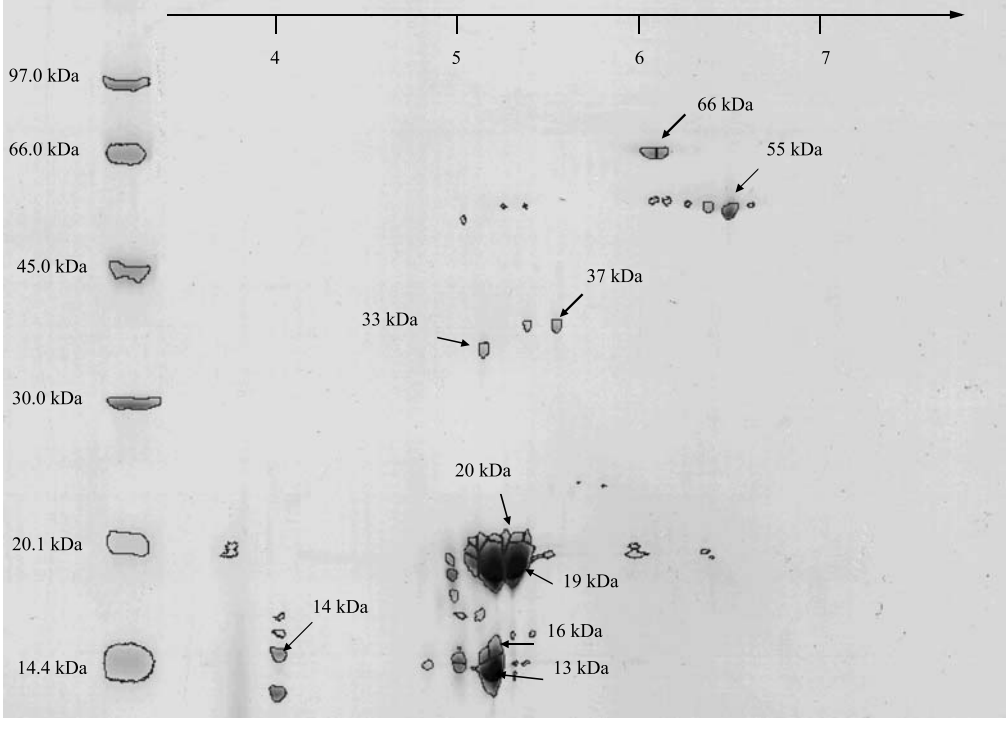

Fig. 1. 2-DE pattern of milk digestion in the stomach of rat after 1 h from feeding (25 µg of protein was loaded on the strip)

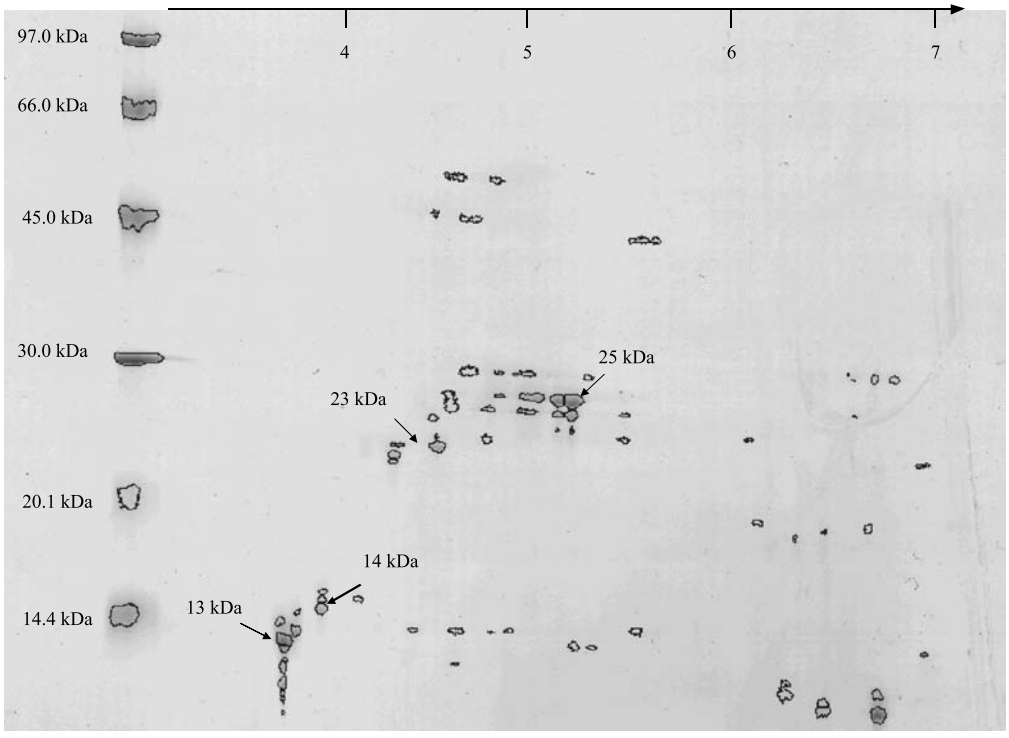

Fig. 2. 2-DE pattern of milk digestion in the small intestine of rat after 1 h from feeding (25 μg of protein was loaded on the strip)

caseins was 98.0% (Carbonaro et al., 2003), in good agreement with values calculated for the same proteins by other methods (Friedman, 1996). Digestibility of bovine serum albumine, that was used as a reference protein, was 98%.

Our previous results on milk digestion (Carbonaro and Iametti, 2002) indicated that a consistent amount of protein was present in the stomach and small intestine of rat after 1 h from the beginning of digestion to allow further characterization. Therefore, stomach and small intestinal contents were recovered and subjected to 2-DE.

Figure 1 shows the 2-DE pattern of milk digestion in the stomach of rat after 1 h from feeding 240 mg of protein. Protein components in a MW range 12–66 kDa and pI range 4.0–6.5 were well resolved by 2-DE. Major spots were identified at MWs 19 and 13 kDa (pI 5.0–5.5). This is consistent with the presence of caseins and major whey protein β-lactoglobulin in this MW and pI range and in agreement with the results of other studies on digestion of milk proteins in the stomach of rat (Miranda and Pelissier, 1981). A minor spot at MW 19.0 kDa, but pI 6.0, may correspond to k-casein, the most basic protein among caseins. A spot at MW 14 kDa and pI 4.0 could be assigned to α-lactalbumin. In addition, protein components at MW 66, 55, 37 and 33 kDa were detectable in the electrophoretic profile. As far as protein species at MW 55 kDa is concerned, at least five spots were separated in the first dimension. Proteins at MW higher than 66 kDa, that corresponds to MW of undigested BSA, were not detectable in the 2-DE map (Fig. 1), thus indicating that minor milk proteins were already digested at the time of digest analysis.

2-DE pattern of digestion of milk proteins in the small intestine of rat is depicted in Fig. 2. Most of protein species identified in the stomach were no longer present after digestion in the small intestine. However, protein spots at MW 25 and 23 kDa and around 14 kDa were detectable, within a pI range 3.5–5.5. The presence of the protein species at 14 kDa assigned to α-lactalbumin is consistent with the reported high stability of this protein in several conditions (De Wit, 1990). Again, in the electrophoretic pattern of Fig. 2, some protein spots at a same MW could be identified (i.e. at 25 kDa).

Discussion

The aim of the present study was the application of 2-DE to bioavailability studies. Therefore, in vivo gastrointestinal digestion of proteins from food, such as milk, that contains high-value proteins and bioactive sequences, was monitored. In vivo short-term experiments with rats were

carried out to measure in vivo digestibility in the small intestine and to obtain gastric and small intestinal digests for further characterization.

2-DE digestion pattern of milk in the stomach showed protein spots in a MW range (13–66 kDa) that corresponded to that expected for undigested major milk proteins. This suggests that proteins in pasteurized milk are slowly digested in the stomach, likely because of coagulation of casein at low pH, a well-known phenomenon (Alaimo et al., 1999). It was also possible that some protein/peptide aggregation had occurred after 1 h of in vivo proteolytic cleavage. Indeed, the formation of aggregated species has also been observed in the course of in vivo gastric digestion of caseins in the mouse by other researchers (Yoneda et al., 2001). As far as protein species at MW 55 kDa is concerned, at least five spots were separated in the first dimension, suggesting the presence of protein aggregates, possibly of caseins with a different degree of phosphorylation or glycosylation. Moreover, protein species at MW 33–37 kDa may correspond to heteropolymers of whey proteins and κ-casein, whose presence in milk and high stability properties have been described in the course of several studies (Dalgleish, 1990; Hurley et al., 1993; Carbonaro et al., 1998). A contribution from endogenous proteins to the electrophoretic pattern was ruled out on the basis of the results of our previous experiments on characterization of proteins from stomach and gut lumen of overnight-fasted rats or rats fed with a protein-free diet (Carbonaro et al., 2005).

Although the extent of digestion of proteins in the small intestine was considerably higher than in the stomach, undigested material could still be detected, consisting in part of α-lactalbumin.

Further characterization of gastric and small intestinal digests of milk and isolated milk proteins by mass spectrometric techniques is currently being performed to obtain complementary information to those provided by 2-DE on in vivo gastrointestinal digestion of milk proteins, with special concern to the release of bioactive peptides.

The present approach may be useful to clarify pattern of protein digestion and mechanism of amino acid and peptide assimilation.

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References

Alaimo MH, Wickham ED, Farrell HM Jr (1999) Effect of self-association of alphas1-casein and its cleavage fractions alphas1-casein(136-196) and alphas1-casein(1-197),1 on aromatic circular dichroic spectra: comparison with predicted models. Biochim Biophys Acta 18: 395–409

AOAC (1990) Official methods of analysis, 15th ed. Association of Official Analytical Chemists, Arlington, VA

Carbonaro M (2004) Proteomics: present and future in food quality evaluation. Trends Food Sci Technol 15: 209–216

Carbonaro M, Bonomi F, Iametti S, Cappelloni M, Carnovale E (1998) Aggregation of proteins in whey from raw and heat-processed milk: formation of soluble macroaggregates and nutritional consequences. Lebensm Wiss Technol 31: 522–529

Carbonaro M, Grant G, Cappelloni M (2005) Heat-induced denaturation impairs digestibility of legume (*Phaseolus vulgaris* L. and *Vicia faba* L.) 7S and 11S globulins in the small intestine of rat. J Sci Food Agric 85: 65–72

Carbonaro M, Grant G, Cappelloni M, Pusztai A (2000) Perspectives into factors limiting in vivo digestion of legume proteins: antinutritional compounds or storage proteins? J Agric Food Chem 48: 742–749

Carbonaro M, Iametti S (2002) Sensitivity of food allergens to proteolysis: in vivo and in vitro approaches. Pol J Food Nutr Sci 11: 141–144

Carbonaro M, Mattera M, Di Lullo G (2003) In vivo model systems and proteomics for traceability of nutritionally relevant compounds in the gastrointestinal tract. Ital J Biochem 52: 138

Dalgleish DG (1990) Denaturation and aggregation of serum proteins and caseins in heated milk. J Agric Food Chem 38: 1995–1999

De Wit JN (1990) Thermal stability and functionality of whey proteins. J Dairy Sci 73: 3602–3612

Dreger M (2003) Emerging strategies in mass-spectrometry based proteomics. Eur J Biochem 270: 569

Ferranti P (2004) Mass spectrometric approach for the analysis of food proteins. Eur J Mass Spectrom 10: 349–358

Friedman M (1996) Nutritional value of proteins from different food sources. A review. J Agric Food Chem 44: 6–29

Kilara A, Panyam D (2003) Peptides from milk proteins and their properties. Crit Rev Food Sci Nutr 43: 607–633

Kvasnicka FJ (2003) Proteomics: general strategies and application to nutritionally relevant proteins. J Chromatogr B 787: 77–89

Hurley WL, Zou S, Magura CE, Jimenez-Flores R (1993) Heating skim milk alters the migration of immunoreactive milk proteins in acrylamide gels. J Agric Food Chem 41: 280–282

Iwahashi Y, Hosoda H (2000) Effect of heat stress on tomato fruit protein expression. Electrophoresis 21: 1766–1771

Lonnerdal B (2003) Nutritional and physiologic significance of human milk proteins. Am J Clin Nutr 77: 1537S–1543S

Lowry OH, Rosebrough AN, Farr JL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

Meisel H (2004) Multifunctional peptides encrypted in milk proteins. Biofactors 21: 55–61

Miranda G, Pelissier J-P (1981) In vivo studies on the digestion of bovine casein in the rat stomach. J Dairy Res 48: 319–326

Sgarbieri V, Clarke EMV, Pusztai A (1982) Proteolytic breakdown of kidney bean (*P. vulgaris*) storage proteins: nutritional implications. J Sci Food Agric 33: 881–891

Yoneda M, Shiraishi J, Kuraishi T, Aoki F, Imakawa K, Sakai S (2001) Gastric proteinase digestion of caseins in newborn pups of the mouse. J Dairy Sci 84: 1851–1855

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