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Comparison of in-vitro proteolysis of casein and gluten as edible films or as untreated proteins

C. L. Garcia-Rodenas,* J. L. Cuq

Laboratoire de Génie Biologique et Sciences des Aliments, Université de Montpellier II, 34095 Montpellier, France

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Ch. Aymard

Unité GTA, CIRAD-SAR, Montpellier, France

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In-vitro trypsin hydrolysis of two protein edible packaging films was studied and compared with that of their raw materials: casein and gluten. Both initial reaction rates and whole progress curves were considered for this comparison. Michaelis parameter values showed only small differences between casein film and untreated protein. The progress curve of the hydrolysis of these samples was described by the semi-empirical Chrastil–Wilson model and no significant differences between the parameters of the correlative equation were obtained for the casein solutions or casein films. The K_m value of gluten film was nearly 10 times higher than that of raw gluten. In these samples, a good fit of data of the reaction time course was achieved by a first-order equation. Overall rate constant (k) values were greater for untreated samples; however, film samples seemed to yield a higher whole broken peptide bond concentration.

INTRODUCTION

During the last 30 years, considerable research work aimed at the development of edible packaging films and coatings has been conducted and appears to have been intensified recently (Guilbert, 1986; Kester & Fennema, 1986; Gontard, 1991). A number of important advantages that these films offer over other currently employed materials anticipate their great future utilization in food packaging (Gennadios & Weller, 1990).

A wide variety of edible compounds may have film-forming ability; among them, proteins have been widely employed because of their interesting properties. Many original research papers and reviews on the manufacture and technological properties of edible protein films have been published (Guilbert, 1986; Kester & Fennema, 1986; Gennadios & Weller, 1990, 1991; Gontard, 1991). However, although these materials are conceived to be eaten together with the packaged food, no comprehensive studies exist on their digestibility and biological utilization. Depending on the nature of the protein and the treatments applied, the process of film elaboration can induce more or less important changes in protein conformation, which are responsible for the molecular structure and rheological characteristics of the film (Gontard, 1991). Taking into account that the susceptibility of proteins to hydrolysis by proteinases is very sensitive to structural changes in protein molecules (Swaisgood & Catignani, 1991), it seems likely that these treatments could alter protein digestibility.

The objective of this work was to study the effects of film elaboration on the susceptibility of casein and gluten to proteolysis. The films obtained from these starting materials and different elaboration processes were already known to have very different rheological characteristics and water solubilities (Guilbert, 1985; Gontard, 1991). Trypsin, which is the major intestinal proteinase, was used to monitor the effects of molecular alterations on digestibility.

MATERIALS AND METHODS

Materials

Wheat gluten was supplied by Ogilvie Aquitaine, Bordeaux (France). Casein (according to Hammarsten; no. 2242) and trypsin (pancreatic proteinase, 2 Anson-Units/g; no. 8214) were obtained from Merck. Stock

*Present address: Department of Nutrition, Nestle Research Centre, Vers-Chez-les-Blancs, CH-1000 Lausanne 26, Switzerland.

solutions of both enzyme (5 mg/ml, pH 3) and casein (20 mg/ml, pH 8) were prepared and stored at −18°C until use. 2,4,6-Trinitrobenzene sulfonic acid (TNBS) and L-leucine were purchased from the Sigma Chemical Co., St Louis, MO. All other reagents and chemicals were of analytical grade.

Elaboration of films

Casein film solution was made according to the method of Guilbert (1985). Casein (10 g) was added slowly to approximately 50 ml distilled water, previously heated at 50°C. pH was kept constant (pH 8) by adding 5 N NaOH. After total casein dissolution, 5 g glycerol was added and mixed. The solution was made up to 100 ml with water and heated at 80°C for 20 min. The film was made by pouring and spreading 10 ml of the film solution into Petri plastic dishes of 8 cm diameter. They were placed on a flat horizontal surface and the solution was allowed to dry at 30°C for 48 h.

Gluten film solution was made according to the procedure of Gontard (1991). Gluten (10 g) and 0·02 g sodium sulfite were mixed with roughly 15 ml distilled water to form an homogeneous dough. After that, an homogeneous slurry was obtained by adding and mixing 40 ml absolute ethanol. Glycerol (2 g) was added to the slurry and its pH adjusted to 4 (by adding glacial acetic acid). The solution was made up to 100 ml with water and heated to 45°C. The film casting method was similar to that used for casein.

Analysis

Nitrogen content of samples was assayed by the Kjeldahl method and protein content calculated as $6{\cdot}38 \times N$ for casein samples and $5{\cdot}7 \times N$ for gluten samples (Adler-Nissen, 1986).

The particle size distribution in the untreated gluten sample was determined by a micrometric method; specific area was calculated by assuming that this material consisted of perfect spheres of diameter equal to the average particle size.

Study of hydrolysis kinetics

Procedure I

Enzymatic hydrolysis was performed with trypsin in a thermostated 100 ml vessel, at 37°C and pH 8. The pH was kept constant by using 0·05 N NaOH, in a pH-stat system (702 SM Titrino titrator, Metrohm Ltd, Switzerland). The volume of NaOH was recorded continuously and initial rates (V_0) were calculated as the slope at the origin of the alkali consumption curves. Enzyme concentrations (E) were 10 and 20 μg/ml for casein and gluten samples, respectively, and the enzyme to protein ratio (E/S) ranged over 0·1–25% for casein and 0·3–10% for gluten materials. The proteins were suspended in 50 ml distilled water; untreated casein was added from stock solution, untreated gluten in powder form and films as 140 cm^2/g fragments. After an equilibrium period, a volume of enzyme solution was added and samples were digested for 15 min.

Procedure II

The procedure was slightly modified to study the time course of hydrolysis. Protein concentration (S) was 1·8 mg/ml; enzyme concentrations ranged over 5–60 μg/ml in casein samples and over 20–60 μg/ml in gluten samples. Time of digestion was extended to 10 h (several drops of hexane were added to prevent microbial growth) and, during hydrolysis, 2×250 μl samples were withdrawn at specified times (5, 10, 20, 30, 40, 60, 80, 100, 120, 160, 200 and 240 min and 6, 8 and 10 h of hydrolysis time). To inactivate the enzyme, samples were mixed rapidly with 1% sodium dodecyl sulfate and kept at 75°C for 10 min. Terminal amino nitrogen concentration was determined by the TNBS method (Adler-Nissen, 1979). A 1·5 mM L-leucine solution was used as standard.

Statistical treatment of data

The analyses were performed at least in duplicate. A computer program was used for non-linear regression analysis. This program estimated the parameter values of tested models by the least-squares criterion and allowed the calculation of their standard deviations. Correlation coefficient (r) between experimental and estimated data was used to evaluate the global goodness of the fit. Comparisons between parameter values were made by Student's t-test; t values were calculated as in eqn (1) and compared with the critical values of t for $n_1 + n_2$ (number of points in each experiment) degrees of freedom (Henderson, 1993).

$$t = \frac{P_1 - P_2}{\sqrt{\sigma_1^2 + \sigma_2^2}} \quad (1)$$

where P_1 and P_2 are the estimated values of parameters; and σ_1, σ_2 their standard deviations.

RESULTS AND DISCUSSION

Two different approaches were used to compare the susceptibilities of untreated and film materials to hydrolysis: the study of initial rate–substrate concentration relationships and the temporal development of the reactions.

Relationship between initial rate and substrate concentration

Initial rates of proteolysis as a function of initial substrate concentration were determined in untreated and film samples (Figs 1 and 2). The initial reaction rate was described by the Michaelis–Menten model. It must be stressed that this relationship was obeyed, at least in a limited range of concentrations, for the hydrolysis kinetics of all samples, including the insoluble and

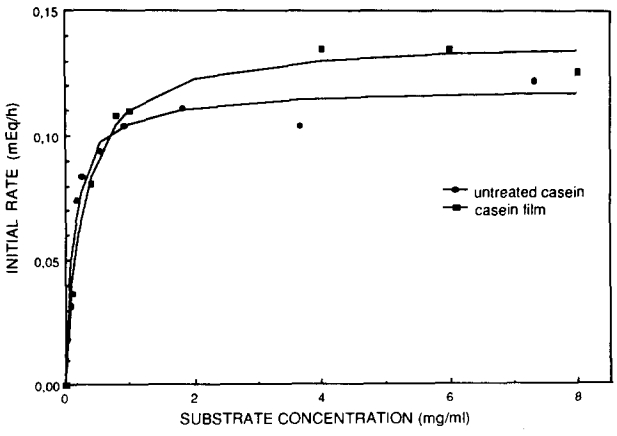

Fig. 1. Michaelis–Menten plots of casein samples; S = 0·04–8 mg/ml, E = 10 μg/ml.

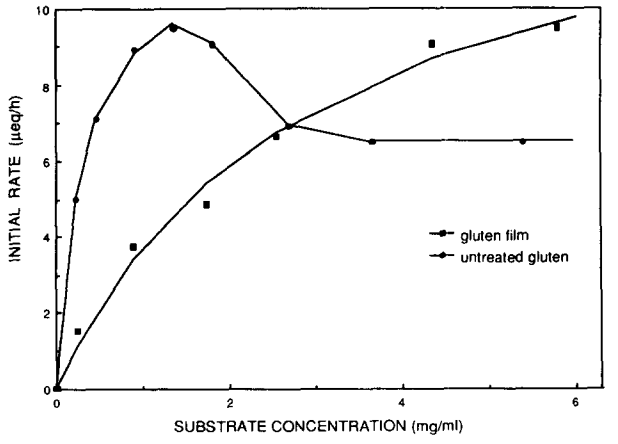

Fig. 2. Michaelis–Menten plots of gluten samples; S = 0·2–6 mg/ml, E = 20 μg/ml.

complex gluten materials. V_{max} and K_m values, determined by non-linear regression analysis from the Michaelis–Menten plot, are shown in Table 1.

Both V_{max} and K_m values were slightly higher in film than in untreated casein, although only the difference in K_m was significant. Treatments employed in film elaboration (alkalinization, heating, drying) may be responsible for this difference; a suppression of intramolecular interactions is produced during film solution elaboration, which is continued, after drying, by the formation of an intermolecular bond network (Kester & Fennema, 1986). Though casein film is readily soluble in water and the intermolecular bonds are broken as soon as the film is immersed in the reaction bath, the original protein conformation may not be recovered. Proteolysis kinetics, being very sensitive to protein conformational changes (Kato *et al.*, 1985), could have detected these alterations.

Departure from simple hyperbolic behavior predicted by the Michaelis–Menten equation was observed with untreated gluten (Fig. 2). The curve shape, suggesting a partial enzyme inhibition by excess of substrate, has restricted the range of experimental points that were used in the K_m and V_{max} determinations. However, a good correlation coefficient (r = 0·995) was obtained and confidence limits of estimated parameter values were similar to those of other samples.

No differences were observed between V_{max} values for untreated and film gluten; however, the K_m value was nearly 10 times higher for the latter. Both materials are very insoluble in the reaction medium. In a soluble enzyme/insoluble substrate system, where enzyme adsorbs to a limited number of sites, the exposed surface of protein material must be a determinant of proteolysis. Hardwick and Glatz (1989) have shown that the initial rate of gluten hydrolysis by alcalase is increased when gluten particle size is reduced. 'Specific area' of the film (140 cm^2/g) was smaller than that of untreated gluten (1480 cm^2/g); for the same protein concentrations, the number of bonds accessible to the enzyme would be clearly smaller in the film sample and, except for conditions of substrate saturation (V_0 = V_{max}), V_0 must always be higher in untreated gluten.

Time course of the hydrolysis

Casein

Figure 3 shows hydrolysis progression curves for untreated and film casein at different enzyme concentrations. In all cases, curves were characterized by an initial rapid increase in reaction rate, followed by an equally rapid decrease. This is a typical behavior of trypsin and may be attributed to its narrow specificity (Adler-Nissen, 1986). Usually, protein/trypsin systems

Table 1. Michaelis–Menten parameters for untreated protein and film/trypsin systems[a]

	Apparent maximum reaction velocity $V_{max} \pm 2\,\sigma$ (meq/h)	Apparent Michaelis constant $K_m \pm 2\,\sigma$ (mg/ml)	r
Casein:[b]			
untreated	0·119 ± 0·008	0·140 ± 0·044	0·986
film	0·139 ± 0·050	0·263 ± 0·058	0·994
Gluten:[c]			
untreated	0·012 ± 0·000 5	0·303 ± 0·038	0·995
film	0·014 ± 0·003	2·870 ± 0·276	0·983

[a] V_{max} and K_m were obtained by non-linear regression analysis (Michaelis–Menten equation).
[b] Enzyme concentration (E) was 10 μg/ml and substrate concentration (S) varied between 0·04 and 8 mg/ml.
[c] Enzyme concentration was 20 μg/ml and substrate concentration ranged between 0·2 and 6 mg/ml.

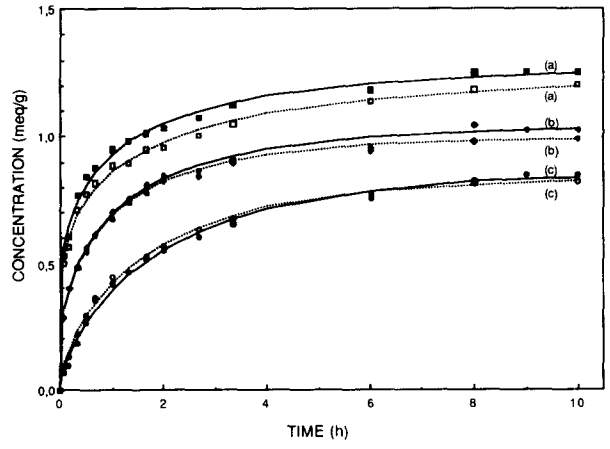

Fig. 3. Production of terminal amino groups during hydrolysis of untreated (·····) and film (——) casein samples at three enzyme concentrations (μg/ml): (a) 60, (b) 20, (c) 5; S = 1·8 mg/ml.

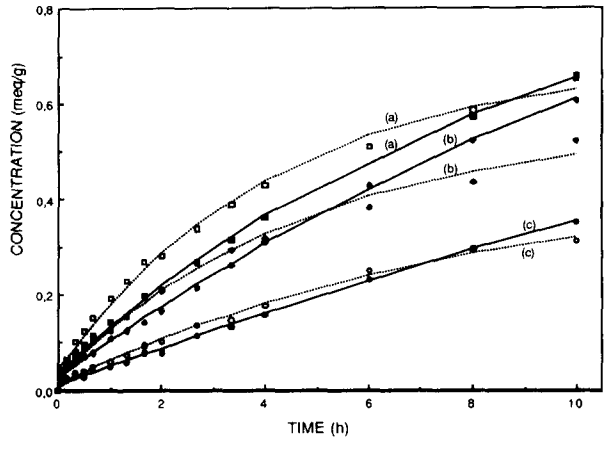

Fig. 4. Production of terminal amino groups during hydrolysis of untreated (·····) and film (——) gluten samples at three enzyme concentrations (μg/ml): (a) 60, (b) 40, (c) 20; S = 1·8 mg/ml.

show a relatively high initial reaction rate, but since the number of susceptible bonds is generally rather small (casein contains 11·9% lys + arg), the hydrolysis curve soon levels off. On the other hand, at the three enzyme concentrations used, rates were similar and close to 0 during the last phase of hydrolysis. In spite of this, the final number of peptide bonds broken was not the same, but increased with enzyme concentration. This behavior, which was observed in two assayed samples, could be attributed to two phenomena, common to many protein/protease systems: enzyme inactivation (including autolysis) and product inhibition (Adler-Nissen, 1986; Bombara *et al.*, 1992). Both phenomena, that may act together, would be responsible for the lower reaction efficiency at lower enzyme concentrations.

In order to compare statistically the hydrolysis curves of two materials (untreated casein and film), different mathematical models were tested. Best fit of data was obtained with the semiempirical Chrastil–Wilson model (Chrastil & Wilson, 1982):

$$P = P\infty\,[1-e^{-kt}]^n \qquad (2)$$

where P is the product concentration at time t; $P\infty$ is the product concentration when time tends to ∞, corresponding to the greatest product concentration that can be reached under the hydrolysis conditions; k is a rate constant proportional to the enzyme concentration; and n is a constant dependent on the sterical structure of the system and which governs the physiognomy of the curve (protein/protease system seem to have $n << 0$). These parameters were estimated by non-linear regression analysis and high correlations were obtained (Table 2) by applying eqn (2) to the experimental data. As expected from the preceding discussion, $P\infty$ values were reduced with enzyme concentration in both untreated and film casein systems. However, the maximum degree of hydrolysis did not seem to be affected by the process of film elaboration, since significant differences of $P\infty$ between the two materials were not found. The n values also remained unchanged and only small differences on k parameter values were observed between untreated and film casein. Moreover, these differences did not show a clear direction, but depended on enzyme concentration. Thus, film formation seemed to have no influence either on initial kinetics or the complete hydrolysis of native casein.

Gluten

Figure 4 shows hydrolysis progression curves for untreated and film gluten at three enzyme concentrations. When the casein (Fig. 3) and gluten curves are compared, it can be seen that the initial rates were much smaller for the latter, although E/S ratios were comparable. The different susceptibilities of gluten and casein to trypsin hydrolysis have already been reported in the literature (Camus & Laporte, 1980) and can be

Table 2. Kinetic parameters of hydrolysis progression in casein samples[a] (mean ± 2 σ)

Sample	E (μg/ml)	$P\infty$ (mg/ml)	k (h^{-1})	n	r
Film	60	1·259 ± 0·090	0·283 ± 0·019	0·220 ± 0·022	0·996
Untreated		1·222 ± 0·089	0·213 ± 0·012	0·215 ± 0·009	0·996
Film	20	1·028 ± 0·077	0·396 ± 0·020	0·356 ± 0·013	0·997
Untreated		0·984 ± 0·078	0·461 ± 0·020	0·364 ± 0·008	0·998
Film	5	0·848 ± 0·101	0·354 ± 0·029	0·644 ± 0·020	0·996
Untreated		0·828 ± 0·092	0·393 ± 0.020	0.611 ± 0.010	0.998

[a] Substrate concentration (S) was 1·8 mg/ml.

Table 3. Kinetic parameters of hydrolysis progression in gluten samples[a] (mean ± 2 σ)

Sample	E (μg/ml)	$P\infty$ (mg/ml)	k (h^{-1})	r
Film	60	0·945 ± 0·133	0·111 ± 0·026	0·993
Untreated		0·668 ± 0·051	0·240 ± 0·044	0·997
Film	40	1·226 ± 0·295	0·066 ± 0·022	0·998
Untreated		0·549 ± 0·058	0·207 ± 0·049	0·994
Film	20	1·487 ± 0·993	0·026 ± 0·020	0·996
Untreated		0·452 ± 0·069	0·119 ± 0·030	0·998

[a] Substrate concentration (S) was 1·8 mg/ml.

explained not only by different amino acid compositions, but mainly by differences in tertiary structure and solubility of the substrate (Adler-Nissen, 1986). The content of predicted susceptible peptide bonds, higher in casein (11·9% lys + arg) than in wheat gluten (4·8% lys + arg), should influence the sensitivity of these proteins to trypsin, this factor being principally related to the final extent of hydrolysis. Proteins with ordered tertiary structure are less susceptible to proteinase attack. Native casein has very little tertiary structure (Sen *et al.*, 1981; Farrell *et al.*, 1993), whereas gluten is a very complex mixture of molecules with a high degree of structural order (Batey, 1980; Masson *et al.*, 1986; Popineau *et al.*, 1990). Moreover, it has been estimated that, under the reaction conditions, the solubility of gluten is several times lower than that of casein (data not shown).

The differences in the shapes of the curves for casein and gluten materials prevented the use of the Chrastil–Wilson model in this case. Good fit of data (Table 3) was obtained with a first-order equation, which has already been adopted by Bombara *et al.* (1992) to describe the hydrolysis of gluten by a fungal protease:

$$P = P\infty\,(1 - e^{-kt}) \qquad (3)$$

where P refers to product concentration at time t; $P\infty$ is the product concentration when time tends to ∞, and k is an overall rate constant. Table 3 shows the estimated values of these parameters for the different samples. For untreated gluten, $P\infty$ values depended on enzyme concentration. As has been discussed for casein materials, this fact can be explained by a reduced enzyme efficiency, due to enzyme inactivation and/or product inhibition. For film curves, the hydrolysis reaction was far from completion after 10 h of hydrolysis, so that $P\infty$ was not determined accurately. However, Fig. 4 shows that at the end of the hydrolysis period, both reaction rate and concentration of peptide bonds broken were higher in film samples. This fact should also imply a higher final hydrolysis degree in film. Because of the treatment that gluten proteins suffered during film elaboration, a larger number of accessible peptide bonds is expected in film samples. It has been stated that gluten has a resistant core which remains after proteolysis (Shewry *et al.*, 1984; Galili & Feldman, 1985; Hardwick & Glatz, 1989). Thebaudin (1990) has shown that a high molecular weigh fraction resists proteolysis by a number of proteinases and that this fraction is composed of peptides branched by interchain disulphide bonds. Film elaboration causes profuse changes in structure of native gluten proteins. Intra- and intermolecular interactions (disulfide and non-covalent bonds) are broken during the solubilization treatment and new bonds, responsible for film structure, are formed during the drying step (Gennadios & Weller, 1990). These changes could make the normally resistant fraction of native gluten susceptible to enzyme attack.

These conformational alterations could contribute to differences in the overall rate constant values (k), that were always higher in untreated gluten. Moreover, as already discussed, the differences in exposed surface to enzyme (higher in untreated gluten) should have a major influence on enzyme accessibility to substrate and therefore on the hydrolysis rate.

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