(Pfleiderer et al., 1967). Figure 2 indicates that the cunner enzymes acted to destroy the activity of RNase more so than bovine trypsin. Approximately 12% of RNase activity was lost with bovine trypsin while cunner trypsin destroyed about 58% and trypsin-like enzyme about 72% of RNase activity after 4-h incubation at 23 °C.

CONCLUSIONS

The trypsin and trypsin-like enzymes from stomachless cunner appear to be better suited for digestion of native proteins than is bovine trypsin. This conclusion is supported by the higher initial rates and/or degree of hydrolysis of native protein substrates by the cunner enzymes.

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Effect of Preliminary Thermal Treatment on the Digestion by Trypsin of Lupin Seed Protein

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The degradation by trypsin of lupin seed proteins (conglutin γ, globulin 6, globulin 8) of BSA and of casein is increased by preliminary heating of the protein (30 min at 100 °C). The effect differs with the protein considered. SDS–PAGE, TCA-soluble peptides, and amino acids liberated indicate rapid and massive breakdown of globulin 8 (a legumin) and of casein; degradation is less prevalent with BSA and develops even more slowly with globulin 6 (a vicilin). Least effect is with conglutin γ. Results are interpreted in terms of modifications of the protein structure.

The nutritional quality of a protein depends on bioavailability of essential amino acids; this in turn is affected to a large extent by the action of digestive enzymes. The efficiency of the latter is influenced by the treatments the protein undergoes, the most common of which is heating. Information on these items is still lacking for new food proteins as those of lupin seed.

Legume seed proteins are less susceptible to tryptic digestion than animal proteins (Kakade, 1974; Lynch et al., 1977a,b; Restani et al., 1983; Romero and Ryan, 1978). Several factors are involved, beside the presence of trypsin inhibitors. The primary structure, protein conformation, and presence of bound oligosaccharide all may contribute in decreasing the efficiency of proteolysis (Boonvisut and Whitaker, 1976; Fukushima, 1968; Kakade, 1974; Kakade et al., 1969; Lynch et al., 1977b; Romero and Ryan, 1978;

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Table I. Effect of Heat Treatment on Biuret- and Ninhydrin-Reactive Compounds Liberated by Trypsin[a]

incubn with trypsin at 37 °C	conglutin γ				globulin 6				globulin 8			
	as such		heat treated		as such		heat treated		as such		heat treated	
	μb	nin	μb	nin	μb	nin	μb	nin	μb	nin	μb	nin
15 min	7.0	1.8	19.3	15.1	18.2	7.8	32.7	8.9	14.9	n.d.	35.8	3.6
1 h	8.1	1.3	22.3	16.2	19.9	7.7	37.1	12.7	18.3	1.4	44.5	4.2
3 h	8.7	2.1	24.9	16.2	24.3	8.8	53.5	14.4	22.3	4.4	50.0	5.3
6 h	15.5	1.9	26.7	16.1	43.1	11.6	51.7	14.4	36.6	6.1	53.8	7.1
24 h	18.3	2.9	47.9	19.3	44.2	12.8	53.0	14.1	47.6	6.9	60.4	9.4

	bovine serum albumin				casein			
	as such		heat treated		as such		heat treated	
	μb	nin	μb	nin	μb	nin	μb	nin
15 min	16.3	nd	21.1	1.2	35.7	4.0	40.1	5.3
1 h	27.9	1.7	37.4	2.8	44.6	4.8	44.3	6.8
3 h	31.2	2.9	45.9	3.4	50.0	6.6	57.1	9.4
6 h	32.5	3.0	63.2	4.8	58.8	8.8	59.4	12.2
24 h	38.5	4.6	62.7	7.1	67.1	11.3	68.4	12.0

[a] Values of microbiuret- (μb) and ninhydrin- (nin) reactive compounds in the TCA supernatant are given as percent of the original protein. Blanks are deduced. nd = below detection limit.

Seidl et al., 1969; Semino et al., 1985).

Thermal denaturation increases the tryptic hydrolysis of many foods proteins of either animal or plant origin (Marquez and Lajolo, 1981). Native ovalbumin and Maillard-reacted ovalbumins resist tryptic hydrolysis on the basis of their rigid conformation, whereas their heat-denatured counterparts are well hydrolyzed (Kato et al., 1983). The action of trypsin on soybean seed proteins is increased after heat denaturation at low pH values (Boonvisut and Whitaker, 1976) whereas heat treatment at neutral pH increases significantly the digestion of bovine serum albumin (BSA) and of G1, the major storage protein of the bean (Bradbear and Boulter, 1984; Liener and Thompson, 1980; Romero and Ryan, 1978). The mechanism of this effect is as yet not completely understood. It has been suggested that the native protein has a hydrophilic surface while the amino acids recognized by trypsin are in its interior and become exposed upon heat treatment (Fukushima, 1968). In the case of legume seed globulins the situation is complicated by the fact that they are oligomeric proteins and each subunit contains more than one polypeptide: only some are linked by disulfide bridges, the subunit itself being held together by noncovalent interactions (Larkins, 1981).

Lupin seed globulins, which represent 87% of the total seed proteins in *Lupinus albus* and are glycoproteins (Duranti et al., 1981; Eaton-Mordas and Moore, 1978), are digested by trypsin less than casein and BSA (Restani et al., 1983). In previous work the effect of bound carbohydrate on the action of trypsin on the various globulins has been considered (Semino et al., 1985). In the present study the effect of heat denaturation on the tryptic hydrolysis of purified conglutin γ, of globulin 6 (one major vicilin-like protein), and of globulin 8 (the major legumin-like protein of the seed) is examined in parallel with the behavior of two animal proteins, casein and BSA.

MATERIALS AND METHODS

Chemicals were of the highest purity commercially available. Trypsin (EC 3.4.21.4.) from bovine pancreas type III, crystallized twice, dialyzed, and lyophilized, was from Sigma Chemical Co.

Universal buffer was prepared as described by Long (1968). When used it was 28.75 mM.

L. albus was of the sweet Multolupa variety. Seed globulins were extracted and purified as previously described (Duranti et al., 1981).

Proteins were determined by the microbiuret method (Itzaki and Gill, 1964) after precipitation with trichloroacetic acid (TCA), 10% final concentration, and 30-min centrifugation at 13000*g*: bovine albumin (BSA) A4378 of Sigma was used as standard. Colorimetric assays after tryptic digestion were done on the supernatant of samples added with TCA and centrifuged as above.

The ninhydrin reaction was carried out as described by McCaldin (1960). The amino acid standard was from Sigma Chemical Co.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed as previously described (Restani et al., 1981) under nonreducing conditions unless otherwise specified, using a 5–22% (w/v) polyacrylamide gradient in the gels. Aliquots (100 μL) of the reaction mixtures were used: the proteolysis was terminated by diluting 1:1 (v/v) with SDS–PAGE sample buffer [15% glycerol and 2% SDS in 0.5 M Tris-HCl (pH 6.8)] followed by heating 10 min at 100 °C. The scale of apparent M_r shown in the figures was determined with standard proteins.

Heat Treatment and Tryptic Digestion. The proteins (3 mg/mL) were dissolved in universal buffer, pH 7.9. The sealed digestion tubes were heated in boiling water for 30 min. They were then cooled in water and ice. Trypsin (1:60, w/w) was added where required, and the tubes were incubated at 37 °C under continuous shaking. Aliquots were withdrawn after 15 min and 1, 3, 6, and 24 h and analyzed by the microbiuret and the ninhydrin methods and by SDS–PAGE.

RESULTS

Colorimetric Assays. The action of trypsin in producing low-M_r TCA-soluble polypeptides is shown in Table I. Proteolysis was sizable and comparable in native globulins 6 and 8 and was quite small in conglutin γ. Preliminary heat treatment considerably increased the amount of peptide liberated from all the globulins. The change was particularly evident in conglutin γ. With globulins 6 and 8 the most conspicuous effect was a more rapid breakdown. Highest hydrolysis in native proteins was measured in casein. It was less in BSA. In both proteins it developed more rapidly than in lupin globulins. The effect of thermal denaturation was evident mainly for BSA: here the degradation reached on prolonged incubations was similar to the one of casein. This level was not attained with lupin globulins.

Ninhydrin-reactive compounds liberated by trypsin from native proteins were very scarce except for casein and globulin 6. Heat treatment had no noticeable effect except for conglutin γ (Table I).

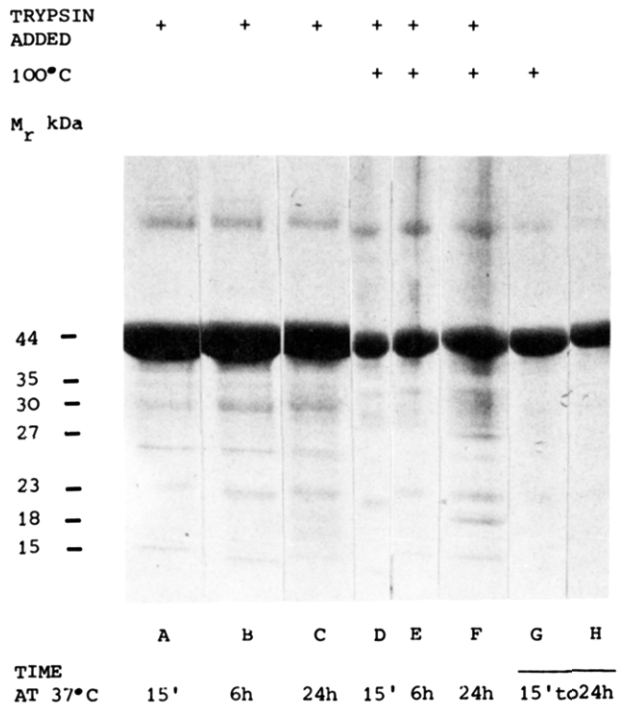

Figure 1. SDS–PAGE of conglutin γ unheated (A–C, H) or heated 30 min at 100 °C (D–G) and incubated with trypsin (A–F) or without it (G, H) for the time indicated. Where indicated 15 min–24 h samples were unchanged over that span of time.

SDS Electrophoresis. The breakdown of proteins was monitored also by determining their constituent polypeptides and the products formed. Samples with and without trypsin were run in parallel. In the absence of enzyme the peptide patterns remained unchanged over the incubation period tested.

The pattern of breakdown and the effect of preliminary heating were typical for each protein. In native conglutin γ the subunit (44 kDa in Figure 1) was very slowly split by trypsin. Main products were peptides of 30, 27, and 23 kDa. Proteolysis became more relevant in the heat-treated protein, and components accumulated (e.g., at 33, 28, and 18 kDa) that did not form from the native globulin. Heat-treated samples formed, especially after enzyme action, a continuous streak in the high-M_r region of the gel that did not appear after addition of 2-mercaptoethanol (not shown).

Globulin 6 and to a lesser extent globulin 8 display in their native state a number of peptides (Figures 2A and 3A). Heat treatment alone did not change the peptide pattern in globulin 6 (Figure 2B). When trypsin acted on the native protein, the high-M_r polypeptides slightly decreased in 6 h and then remained constant (Figure 2C, D) whereas in the denatured protein they disappeared and only components in the range 14–30 kDa were left (Figure 2F).

Globulin 8 was considerably affected by heat treatment alone: high-M_r polypeptides disappeared and new material accumulated in the regions of 50 and 30 kDa (Figure 3B). Streaking was noted in the upper part of the gel. When SDS was present during heat treatment (Figure 3H), part of the high-M_r peptides were preserved and polypeptides of 20 and of 40 kDa were formed that did not appear in the absence of SDS. Less streaking was apparent. In the presence of 2-mercaptoethanol the basic polypeptide of 20 kDa that in the native globulin is disulfide bonded to acidic ones (Restani et al., 1981) became separated in both the heat-treated and the -untreated protein (Figure 3I,L). The heat-treated globulin displayed new polypeptides in the

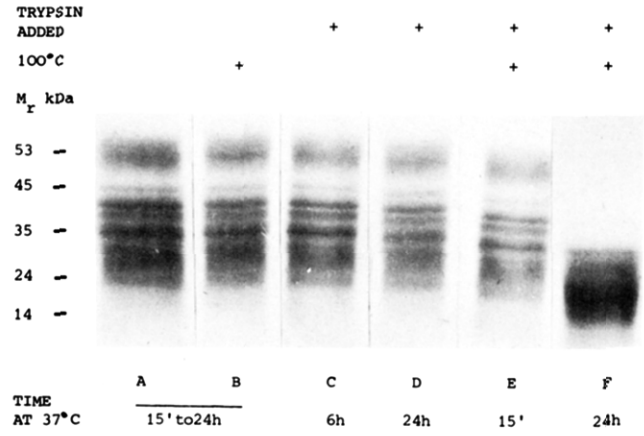

Figure 2. SDS–PAGE of globulin 6 unheated (A, C, D) or heated 30 min at 100 °C (B, E, F) and incubated with trypsin (C–F) or without it (A, B) for the time indicated, 15 min–24 h, as in Figure 1.

40- and 31-kDa regions (Figure 3L). Streaking was not manifest.

Trypsin had a visible effect on the native protein since it gradually degraded the high-M_r polypeptides, forming a number of products of intermediate and small size (Figure 3C–E): some larger polypeptides however remained also upon long incubations. The pattern of decay differed from the one produced by heating alone. On the other hand when the heat-denaturated globulin was acted upon, the breakdown was very rapid and only peptides of 20 kDa or smaller were evidenced from very early times (Figure 3F,G). Where the enzyme had acted, streaking in the upper part of the gel was very scarce or nil.

The digestion of BSA was slow: the unmodified protein decreased upon long incubation, but no product of intermediate size appeared (Figure 4B). Proteolysis increased after heat treatment of the protein. In this condition the BSA dimer disappeared from the very beginning (Figure 4C). Heat treatment alone did not visibly induce protein breakdown but produced some streaking (Figure 4E). Also with casein, heating alone had no effect. The native protein was rapidly digested (Figure 5C,D), and the action of trypsin was increased by preliminary thermal treatment (Figure 5E).

DISCUSSION

Since lupin seed does not contain antitryptic factors (Gallardo et al., 1974), it seems likely that the structure of the native proteins was responsible for the reduced action of trypsin. Preliminary thermal treatment had an effect both on the rate and on the final yield of degradation as evidenced by small TCA-soluble peptides formed and by SDS–PAGE.

Thermal treatment did not consistently affect interpeptide disulfide bonds. In native globulin 8 it destroyed the high-M_r polypeptides, giving rise to smaller fragments, but it did not separate the basic and the acidic polypeptides that are bound through an interchain disulfide. Basic and acidic polypeptides were resolved in the presence of 2-mercaptoethanol: the new polypeptides appearing in the heated globulin under reducing conditions matched the M_r of the presumed acidic component of those evidenced under nonreducing conditions. Also in conglutin γ the subunit of 44 kDa is formed by two smaller disulfide-linked polypeptides (Restani et al., 1981), and they were not separated by heating.

Nonetheless heat-treated cyst(e)ine-containing proteins, globulin 8, BSA, and conglutin γ, showed a peculiar streaking in the upper part of the gel on SDS–PAGE. In

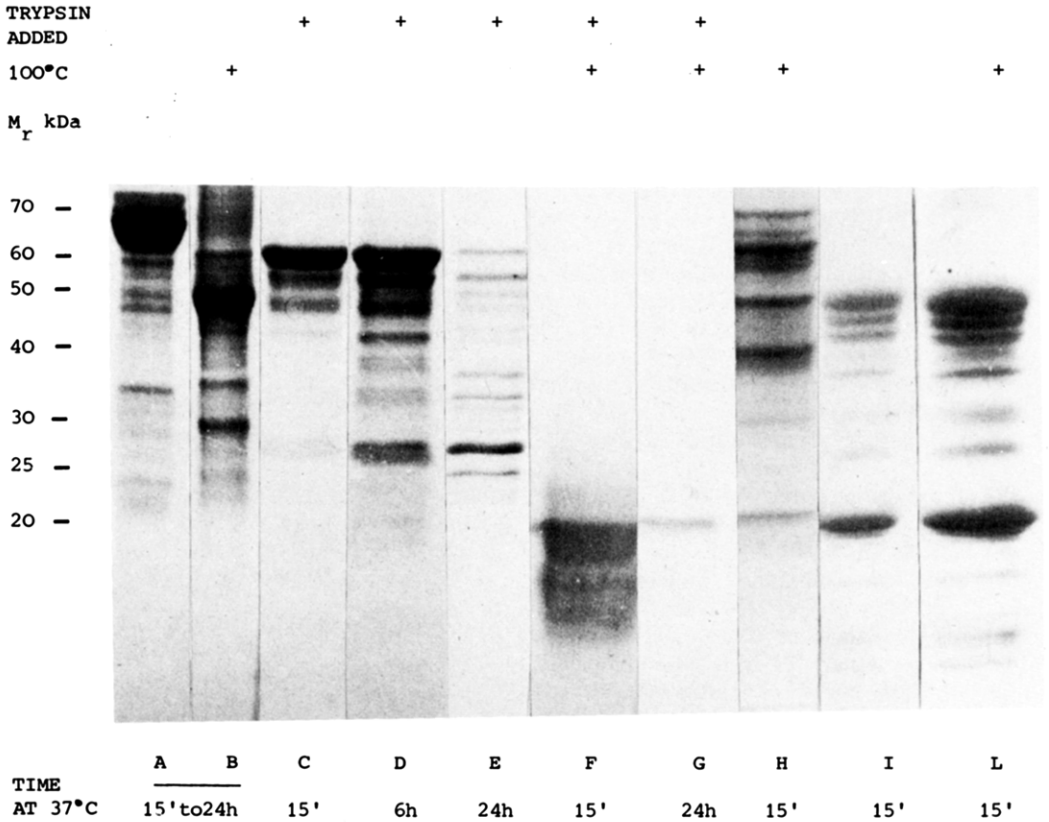

Figure 3. SDS–PAGE of globulin 8 unheated (A, C–E, I) or heated 30 min at 100 °C (B, F, G, L) and incubated with trypsin (C–G) or without it (A, B, H–L) for the time indicated. Sample H had 5% SDS during heating, samples I and L received before SDS–PAGE 2-mercaptoethanol (5% final concentration), and the electrophoresis was run under reducing conditions.

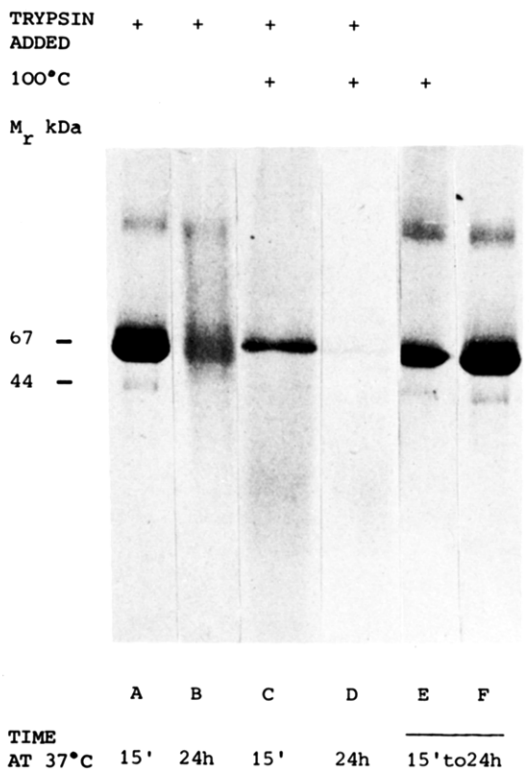

Figure 4. SDS–PAGE of BSA unheated (A, B, F) or heated 30 min at 100 °C (C–E) and incubated with trypsin (A–D) or without it (E, F) for the time indicated, 15 min–24 h, as in Figure 1.

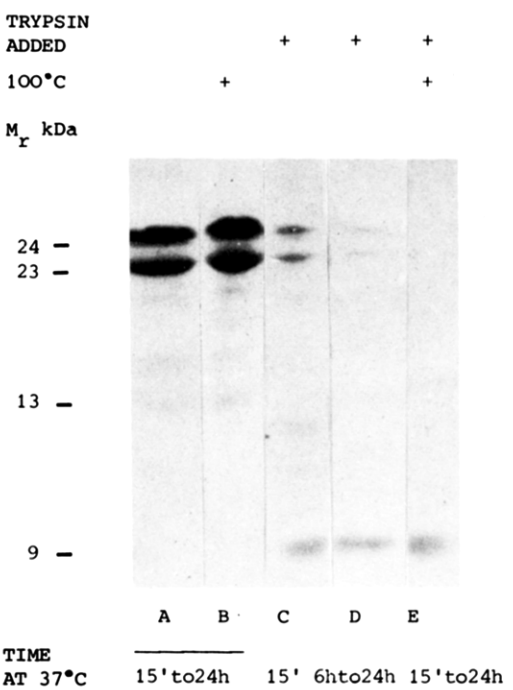

Figure 5. SDS–PAGE of casein unheated (A, C, D) or heated 30 min at 100 °C (B, E) and incubated with trypsin (C–E) or without it (A, B) for the time indicated, 15 min–24 h, as in Figure 1.

globulin 8 the process was most evident at heat treatment whereas in conglutin γ and in BSA it became relevant during the subsequent tryptic digestion. It was likely due to sulfhydryl–disulfide exchange, forming interpeptide disulfide and large-size and/or scarcely soluble products. Indeed it was not seen in samples treated with 2-mercaptoethanol nor in globulin 6, a protein that does not contain cyst(e)ine (Duranti et al., 1981). The size of the available polypeptides appeared to play a role: streaking was especially evident in samples with copious medium-

sized (40–30-kDa) fragments (conglutin γ, 24-h incubation, heat-treated globulin 8). In globulin 8 it also involved high-M_r polypeptides that disappeared after heating (Figure 3B). Streaking disappeared once fragmentation by the protease proceeded further (BSA, globulin 8). Formation of polymers due to interaction of sulfhydryl groups during heat treatment has been described for the soybean 11S globulin (Yamagishi et al., 1980).

The protein–SDS complex has been described as a series of SDS micelles surrounding amino acid nuclei with strings of hydrophylic or negatively charged residues joining them (Shirahama et al., 1974). A structure of this type may have stabilized the disulfide-linked polypeptides during heat treatment when SDS was present and may have prevented the interaction between sulfhydryl groups so that high-M_r protomers were in part preserved and disulfide-linked polymers did not form.

The action of the endopeptidase developed much more efficiently after thermal treatment, indicating a change in the substrate molecule that improved the affinity and/or made available new substrate sites.

Previous research had shown that removal of bound sugar improved the action of trypsin on lupin seed globulins and the effect was particularly evident for conglutin γ (Semino et al., 1985). Heat-treated conglutin γ appears less digested than the deglycosylated protein: this may in part be due to residual steric hindrance by the bound carbohydrate. When trypsin acted on heat-treated conglutin γ, it formed more readily TCA-soluble oligopeptides than large fragments evidenced by SDS–PAGE: this may be taken as a sign of structural compactness of the molecule.

Lack of covalent continuity in the subunits assembled in the oligomeric molecules is likely to have favoured splitting of small TCA-soluble peptides and free amino acids from native globulins 6 and 8. Also surface polarity may have contributed since globulin 6 has a more hydrophylic surface than globulin 8 (Bonomi et al., 1983) and trypsin acted more rapidly on it. A similar relationship between surface hydrophilicity and digestibility by trypsin has been suggested for 7S and 11S globulins of soybean (Lewis and Chen, 1979). However, when high-M_r products evidenced by SDS–PAGE were considered, the reverse situation was observed: globulin 8 underwent a more gradual and complete attack, greatly enhanced by heat treatment while globulin 6 was split only after thermal treatment and in extreme incubations. This may be interpreted as a more compact folding in globulin 6 than in globulin 8.

Degradation of BSA is "all or none" in that fragments formed escape detection by SDS–PAGE: the considerable number of intrachain disulfide linkages (Brown, 1975) is likely to prevent release of fragments until proteolysis has progressed. Casein is readily split by trypsin: this agrees with the relatively loose folding of this molecule (Mac Kinley and Wake, 1971; Creamer and Richardson, 1984).

A modified affinity between enzyme and substrate might in part be the cause of the activity changes brought about by heat treatment. However, no correlation can be established between the heat-induced modifications in proteolytic activity and the changes in amount and distribution of surface hydrophobic areas in the proteins considered, determined in a previous research (Bonomi et al., 1983). Since the native quaternary and tertiary structures of lupin proteins are modified at temperatures well below 100 °C (Bonomi et al., 1983), exposure of buried substrate sites is more likely the explanation of our results.

Protein digestion in a cooked and/or processed food depends on the treatment applied and on the medium. Nonetheless information about the behavior of the protein per se is of major help in understanding the system in vivo.

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