

The effect of pH on the digestion of proteins *in vitro* by pepsin

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The significance of peptic digestion in the utilization of dietary protein is not fully elucidated. Pepsin is active in the proteolysis of protein substrates over a pH range extending from below 2 to above 4 and has optimum activity for most proteins at about pH 1.8 (Northrop, 1922-3). There is little or no pepsin in the gastric mucosa of newborn pigs (Langendorff, 1879), but after birth the secretion of pepsin increases rapidly (Kvasnitskii & Bakeeva, 1940; Lewis, Hartman, Liu, Baker & Catron, 1957; Dollar, 1958). There is sufficient secretion of hydrochloric acid in many species of animal to lower the pH of the gastric contents to around 2, but it has been found (Lewis *et al.* 1957; Dollar, 1958) that the pH of the stomach contents of young pigs is approximately 6 during the first few days of life and that, though it decreases subsequently, it is never below 3.5 during the first 7 weeks of life. During these first few weeks young pigs cannot use soya-bean protein effectively, whereas they can utilize satisfactorily the mixed proteins of milk or casein (see review by Dollar, 1958). It was of interest, therefore, to determine whether there is a basic difference in the influence of pH on the rate of digestion by pepsin of milk proteins and soya-bean protein, and to examine the further possibility of a difference in the products of the digestion by pepsin of the same food protein at different pHs.

Although casein is the milk protein most often used in 'synthetic' milk diets for young animals, we preferred to study the proteolysis of α -casein, the major constituent of casein, as a homogeneous substrate was likely to yield a less complicated pattern of proteolytic products than the mixture of proteins comprising casein.

EXPERIMENTAL

Materials

Three sources of soya-bean protein were used: commercially prepared solvent-extracted soya-bean flakes (McMillan Feed Company, Gibson City, Illinois), α -protein (Glidden Company, Chicago) and C-1 assay protein (Archer, Daniels Midland Company, Minneapolis). α -Casein, prepared by the urea method of Hipp, Groves, Custer & McMeekin (1952), was kindly supplied by Dr R. Aschaffenburg of this Institute. Roller-dried whey (Genatosan Ltd, Loughborough) was used as a source of whey proteins.

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Methods

Digestion. The protein (200 mg) was added to each of a series of twelve flasks each containing 10 ml buffer made by mixing appropriate amounts of 0.1 M-sodium citrate and 0.1 N-HCl to give twelve stepwise increases in pH from about 1.5 to 4. After further addition of 1 ml of a pepsin solution containing 80 μ g crystalline pepsin (L. Light & Co, Colnbrook, Bucks) per ml, the mixture was incubated at 37° with constant shaking. The pH was measured with a Pye pH meter before and after incubation and the mean value was used in presenting the results. After 24 h, proteolysis was stopped by adding 11 ml of 50% (w/v) trichloroacetic acid. The mixture was filtered and the amino nitrogen in the filtrate determined by the ninhydrin method of Moore & Stein (1954). The results are expressed in terms of leucine equivalents.

In several experiments, in addition to measuring the amino nitrogen content of the trichloroacetic acid filtrate by the ninhydrin method, the nitrogen content was determined by the micro-Kjeldahl procedure.

Peptide separation. Two-dimensional paper chromatography did not give satisfactory resolution of the components of the protein-free filtrate, but electrophoresis followed by chromatography at right angles to the direction of current flow gave good separation.

Portions of the protein-free filtrates containing equal amounts of amino nitrogen were concentrated to small volume (1–2 ml) under reduced pressure and then extracted three times with successive 30 ml portions of diethyl ether to remove most of the trichloroacetic acid. The residual solution was applied to a single spot on a 57 cm \times 46 cm sheet of Whatman no. 4 filter-paper moistened with 0.05 M-veronal buffer at pH 8.6, and a potential of 8 V/cm was applied to the paper for 16 h. The paper was then dried and developed by descending chromatography with a mixture of butan-1-ol-acetic acid-water (100:20:80 by vol.) for about 9 h. The paper was then dried and sprayed with a solution of ninhydrin (0.5%, w/v) in acetone and heated at 90°–100° for 15 min to develop the colour.

Composition of peptide. The segment of paper containing the peptide was cut out and eluted with water. The eluate was concentrated and again applied to a sheet of Whatman no. 4 paper and the electrophoresis and chromatography were repeated to purify the material. The peptide was eluted and hydrolysed by refluxing with 5.8 N-HCl for 18 h. The hydrolysate was concentrated under reduced pressure and applied to a sheet of Whatman no. 4 paper for two-dimensional chromatography with butan-1-ol-acetic acid-water for development in one direction and s-butanol-triethylamine-water (75:1:24) for development in the other. After drying, the paper was sprayed with ninhydrin and heated to develop the colour.

RESULTS

In Fig. 1 the measured amino nitrogen contents of typical series of pepsin digests, expressed as μ moles leucine equivalent per ml of the trichloroacetic acid filtrate, are plotted against the mean pH of the digest. (Essentially similar curves were obtained

when the nitrogen content of the trichloroacetic acid filtrates was plotted against the pH of the digest.) It is apparent that there was no clearly defined optimum pH for the digestion of α -protein, α -casein or C-1 assay protein, for the rate of digestion progressively decreased as the pH was raised from 1.3 to 3.7. However, there was a slight increase in the rate of digestion of whey proteins and soya-bean flakes as the pH was raised to 1.7–1.8, and thereafter a progressive fall. Comparisons of the extent of digestion of the individual proteins at pHs 1.8 and 3.8 indicate that the digestion

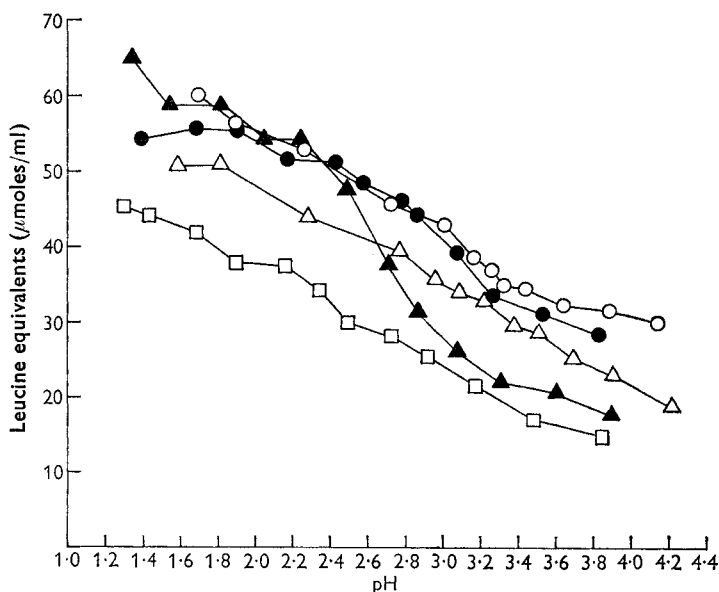


Fig. 1. Amino nitrogen contents of trichloroacetic acid filtrates of pepsin digests of proteins at different pHs. Δ — Δ , soya-bean flakes; \blacktriangle — \blacktriangle , α -protein; \square — \square , C-1 assay protein; \circ — \circ , α -casein; \bullet — \bullet , lactalbumin.

of α -casein and whey proteins decreased rather less rapidly as the pH was raised than that of any of the soya-protein preparations. Thus, for the two former, the ratio between the amounts of amino nitrogenous material in the trichloroacetic acid filtrates at these two pHs was 1.8:1, whereas the ratios for α -protein, C-1 assay protein and soya-bean flakes were 3.2, 2.8 and 2.2:1 respectively.

Typical examples of the pattern of ninhydrin-positive zones evident after separation of the peptides in the digest of α -casein at pHs 1.7 and 4.1 are shown in Pl. 1. The conditions chosen for electrophoresis caused most of the peptides to migrate towards the anode, and the combination of electrophoresis and chromatography allowed the separation of a large number of peptides. The general distribution of these peptides was similar in the digests prepared at pH 1.7 or 4.1, though rather more zones were evident at the lower pH, and one major component (peptide A) at pH 1.7 was consistently almost absent at pH 4.1. Peptide A moved slightly towards the anode during electrophoresis at pH 8.6 and had an R_F of 0.12 on chromatography with butan-1-ol-acetic acid-water. This peptide was isolated, purified and hydrolysed. Six ninhydrin-positive zones were evident after two-dimensional chromatography of the hydrolysate;

the R_F values of these zones corresponded to those of lysine, glutamic acid, aspartic acid, glycine, proline and alanine.

Unfortunately it has not yet proved possible to obtain a satisfactory separation of peptides from any of the digests of the soya-protein samples by either electrophoretic or chromatographic methods.

DISCUSSION

It is evident from our results that all the proteins studied were most rapidly digested at pHs near 2 and that, although some proteolysis occurred at pH 4, there was no indication of a second peak of proteolytic activity at pH 3.5-4. There is general agreement that pepsin causes optimal proteolysis of protein substrates at pHs near 2, but it has also been claimed (cf. Taylor, 1959*a*) that it shows a second peak of activity at pH 3.5-4 in the digestion of certain substrates, such as, for example, plasma proteins. Extracts of human and pig's gastric mucosa frequently also show two peaks of activity one at pH 1.8-2 and the other at pH 3.5-4. This latter peak has been attributed to the presence in such extracts of cathepsin (Buchs, 1953-4), or gastricsin (Richmond, Tang, Wolf, Trucco & Caputto, 1958), but Taylor (1959*a*) was unable to demonstrate the presence of enzymes other than pepsin. However, Ryle & Porter (1959) isolated from crude pig's pepsin two minor components, parapepsins I and II. These enzymes, which were similar to pepsin in many of their properties, accounted for only a small percentage of the total proteolytic activity of the starting material, but it is possible that parapepsin II, which has a pH optimum at 3, may contribute to the proteolytic activity of mucosal extracts at pH 3.4-4.

On the basis of his results Taylor (1959*b*) has postulated that pepsin has two sites for enzyme substrate interaction, each of which attacks maximally a different type of substrate at a different pH. Such a concept is in line with the findings that pepsin hydrolyses optimally some synthetic substrates at pH 1.8 (Baker, 1951) and others at pH 4 (Fruton & Bergmann, 1939). It would also favour the view that proteolysis at pH 1.8 would result in the formation of products different from those produced by proteolysis at pH 4, and Taylor (1959*b*) showed differences in the products resulting from the action of human gastric juice on plasma proteins at pH 2.2 and 4. Our results can be interpreted as providing further support for this view in that, although only one peak of activity was observed, differences were apparent between the products of proteolysis of α -casein at pH 1.7 and 4.1 (Pl. 1), and in particular in the formation of peptide A at the lower, but not at the higher, pH. Peptide A contained lysine, glutamic acid, aspartic acid, glycine, proline and alanine, but no aromatic amino acids which would make it susceptible to further digestion (Fruton & Bergmann, 1939; Baker, 1951). Neither did it contain any serine, which would suggest that it was not a phosphorus-containing peptide related to those described by Mellander (1950).

A simpler and perhaps more acceptable alternative explanation of the different products at pH 1.7 and 4.1 can be sought by postulating, according to the views of Christensen (1955) and Schlamowitz & Peterson (1959) that the effect of the lower pH might be to cause the peptide chains to unfold and make a particular substrate bond available to digestion. At the higher pH the site of this bond might be sterically blocked

and not susceptible to enzyme attack. Pepsin has a wide specificity for the hydrolysis of peptide bonds; it is quite possible that it has only one active site which acts on different peptide bonds in the substrate at different pHs according to the accessibility of these bonds to the active site of the enzyme. This accessibility will be governed by steric factors which, in turn, will be influenced by the different distribution of charges in the substrate, and enzyme, molecules at different pHs. If this explanation is correct incubation of a solution of α -casein at pH 1.7 before the pH is raised to 4 and pepsin added might be expected to increase the rate and extent of digestion and lead to the formation of the products normally formed at the lower pH. In fact an initial pre-incubation for 4 h at pH 1.7 was found to have no effect whatsoever on the products of the subsequent digestion at pH 4.1. (It is possible that this finding may be the result of refolding of the peptide chains when the pH is raised.)

The indication from our findings that raising the pH of digestion causes a more rapid fall-off in the rate of digestion of soya protein than of milk proteins implies that milk or synthetic-milk diets based on milk proteins may be more rapidly digested by pepsin at the pH of the stomach contents of young pigs than similar diets based on soya proteins. These implications are being investigated.

SUMMARY

1. α -Casein, whey proteins, soya-bean flakes and two isolated soya proteins, α -protein and C-1 assay protein, were digested by pepsin with a pH optimum at 1.8–2.0.
2. Raising the pH of digestion to 4 caused a greater decrease in the rate of digestion of the soya than of the milk proteins.
3. Electrophoretic and chromatographic separations showed that different products were formed during the digestion of α -casein at pHs 1.7 and 4.1.
4. A peptide containing lysine, glutamic acid, aspartic acid, glycine, proline and alanine was produced from α -casein at pH 1.7, but hardly at all at pH 4.1.
5. Possible explanations of the findings are discussed.

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EXPLANATION OF PLATE

Pl. 1. Peptide zones separated by two-dimensional electrophoresis and chromatography of trichloroacetic acid filtrates from pepsin digests of α -casein at (a) pH 1.7 and (b) pH 4.1.

