Digestibilities of casein and soya-bean protein in relation to their effects on serum cholesterol in rabbits

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1. Diets containing isolated soya-bean protein induce lower levels of serum cholesterol in animals than diets containing casein. Experiments were conducted to investigate whether differences in digestibility of the proteins might explain this effect.

2. At pH 8 with pancreatic enzymes or intestinal peptidase, soya-bean protein was hydrolysed in vitro much less rapidly than casein. However, with pepsin (*EC* 3.4.23.1) at acid pH, soya-bean protein was hydrolysed more rapidly than casein.

3. These differences in digestibility may be due to pH-dependent changes in solubility of the proteins. Casein and soya-bean protein were most soluble at alkaline and acid pH respectively.

4. Heat treatment of the proteins resulted in lower solubilities and digestibilities. Sonication of soya-bean protein at pH 7·8 increased solubility but only slightly raised digestibility.

5. When fed to rabbits, enzymically hydrolysed soya-bean protein induced a 2·3-fold higher concentration of serum cholesterol than did intact soya-bean protein. The hypocholesterolaemic effect of soya-bean protein may be partly attributable to its low solubility and digestibility at alkaline pH.

Dietary plant proteins induce lower levels of serum cholesterol than do animal proteins (Carroll, 1982; Gibney & Kritchevsky, 1983). This difference mainly reflects alterations in the low-density-lipoprotein fraction, and eventually results in divergent degrees of atherosclerosis.

One characteristic of the proteins which appears to influence serum cholesterol concentration is their amino acid composition. It is known that mixtures of amino acids resembling animal or plant proteins elicit respectively high and low levels of serum cholesterol (Yadav & Liener, 1977; Huff & Carroll, 1980; Nagata *et al.* 1981). However, attempts to relate specific amino acids to cholesterolaemia and atherogenesis have not produced any consensus (Huff & Carroll, 1980; Kritchevsky, 1983; West *et al.* 1983).

There is increasing evidence that, in addition to amino acid composition, the conformation of dietary proteins affects serum cholesterol. Thus intact plant proteins are more hypocholesterolaemic than are amino acid mixtures of the same composition (Yadav & Liener, 1977; Huff & Carroll, 1980; Nagata *et al.* 1981). Moreover, amino acid supplementation of casein and soya-bean protein, carried out so as to reverse overall amino acid composition, did not substantially modify their effects on plasma cholesterol concentration (Huff & Carroll, 1980). Finally, diets having the same overall amino acid composition, but containing different proteins and amino acids, gave dissimilar concentrations of serum cholesterol (Hermus *et al.* 1979).

The digestibility of dietary protein is a property which may affect cholesterol levels (Hufff & Carroll, 1980; Carroll & Woodward, 1982). The present experiments were designed to test this hypothesis. The digestibilities of casein and soya-bean protein were studied initially in vitro, and subsequently an experiment was carried out in vivo.

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MATERIALS AND METHODS
Chemicals

Pancreatin 1X (hog pancreas), pepsin 1–10,000 (hog stomach mucosa, EC 3.4.23.1), peptidase (hog intestine, partially purified), α-chymotrypsin (beef pancreas, 45 units/mg, EC 3.4.21.1) and trypsin (beef pancreas, 180 units/mg, EC 3.4.21.4) were purchased from ICN Pharmaceuticals, Life Sciences Group, Cleveland, OH. Sephadex G 25-150, bovine serum albumin, insulin A-chain and bacitracin were from Sigma Chemical Co., St Louis, MO. Other chemicals were supplied by Fisher Scientific Co., Fair Lawn, NJ.

Dietary constituents

Supro 610 soya-bean-protein isolate was provided by the Ralston Purina Co. Inc., St Louis, MO. High-nitrogen casein was obtained from United States Biochemical Corporation, Cleveland, OH. Hydrolysed soya-bean protein, Soy Peptone Type III, came from Sigma Chemical Co. The manufacturer's analysis showed that it contained (g/kg): 135 total N and 63 amino-N. Vitamins and salt mixture (Phillips & Hart, (1935), salt mixture IV plus cobalt) were purchased from ICN Pharmaceuticals. Glucose monohydrate was supplied by Teklad Test Diets, Madison, WI, and Celluflour by Chicago Dietetic Supply House, Chicago, IL.

Digestibility tests at fixed pH

The digestibilities of casein and soya-bean protein were measured initially by the decrease in pH due to peptide-bond hydrolysis. The procedure was modified from that of Hsu et al. (1977). Pancreatin suspension (3·0 mg/ml in water) was adjusted to pH 8·0 using dilute hydrochloric acid and sodium hydroxide, and centrifuged at 1200 g for 10 min. Supernatant fraction (1 ml) was mixed with 10 ml protein solution (3·1 mg/ml in water; previously adjusted to pH 8·0). The pH was followed for 6 min at room temperature. For studies with purified enzymes, 1·6 mg trypsin, 3·0 mg chymotrypsin or 1·2 mg peptidase in 1 ml water were added to the protein solution, and the pH was followed as before.

The buffering capacities of casein and soya-bean protein were measured by adjusting protein suspensions (125 mg/50 ml water) to pH 8·0 and then titrating to pH 6·4 with dilute HCl.

The validity of results given by pH readings was verified by determination of the low-molecular-weight trichloroacetic acid (TCA)-soluble peptides before and after hydro-lysis. Incubations were performed as described previously, except that 0·1 M-boric acid–NaOH buffer, pH 7·8, was used in place of water. Portions of the hydrolysate were taken at intervals and mixed with TCA solution (100 g/l). After centrifugation at 1200 g for 10 min, the protein concentration of the supernatant fraction was measured by the method of Lowry et al. (1951). Bovine serum albumin was used as standard.

Digestibility by pepsin was measured as the accumulation of TCA-soluble peptides during incubation of casein or soya-bean protein suspension (2·5 mg/ml in 0·1 M-HCl) with 100 μg enzyme/ml. TCA-soluble peptides were determined as described previously. For all incubations, the components were mixed at room temperature using either a magnetic stirrer or metabolic shaker.

Solubility and digestibility at different pH values

Studies over the pH range 2·6–7·6 were carried out in 0·1 M-citric acid–sodium hydrogen phosphate buffer. Protein (2·5 mg/ml) was suspended in the buffer using a Teflon-and-glass homogenizer. Solubility was arbitrarily defined as the protein concentration of the supernatant fraction after centrifugation of a portion at 1200 g for 10 min.

The remainder of the suspension was incubated with enzyme for 10 min at room temperature. Digestibility was defined as the increase in TCA-soluble peptides over this

Table 1. *Composition of semi-purified diets containing intact or hydrolysed soya-bean protein*

Ingredient*	g/kg
Intact or hydrolysed soya-bean protein	250
Glucose	600
Celluflour	50
Salt mixture	40
Molasses (500 ml/l water)	30
Water-soluble vitamins†	10
Fat-soluble vitamins (in maize oil)‡	10
L-Lysine hydrochloride	5
L-Methionine	5

* Molasses and maize oil were obtained locally; origins of the other ingredients are given on p. 356.

† Providing (mg/kg diet): thiamin hydrochloride 15·0, pyridoxine hydrochloride 37·5, biotin 0·3, myo-inositol 150, riboflavin 15, nicotinic acid 190, calcium pantothenate 80, folic acid 3·0, choline chloride 1500.

‡ Providing (/kg diet): retinyl acetate 0·7 mg, α-tocopheryl acetate 110 mg, menadione 30 mg, cholecalciferol 25 μg.

interval. For studies at pH 5·6–7·6, pancreatin suspension (10 mg/ml) was centrifuged and 1 ml supernatant fraction was added to 10 ml protein suspension. For incubations at pH 2·6–4·6, 100 μl pepsin solution (10 mg/ml 0·1 M-HCl) were added.

Heating and sonication of proteins

For studies of heated proteins, casein and soya-bean protein were autoclaved at 138 kPa (20 psi) for 1 h. Digestibilities and solubilities over the pH range 2·6–7·6 were assayed as described previously. For experiments involving sonication, protein suspensions (2·5 mg/ml) in 0·1 M-HBO$_3$–NaOH buffer, pH 7·8, were treated using a W350 Sonifier Branson Sonic Power Co., Danbury, CT) on setting 5. Protein solubilities and digestibilities using pancreatin were determined as described previously.

In vivo experiment

Intact and enzymically hydrolysed soya-bean proteins (Supro 610 and Sigma Soy Peptone Type III) were characterized by chromatography on a column of Sephadex G25-150 (500 × 25 mm). Samples of approximately 30 mg protein were suspended in 5 ml 0·1 M-potassium dihydrogen phosphate–potassium hydrogen phosphate buffer, pH 7·8. The soya-bean protein suspension was centrifuged at 10000 *g* for 30 min before loading the supernatant fraction on the column. The peptone was fully soluble. Protein concentration in the eluate was measured by monitoring absorbance at 280 nm. The column was standardized with bovine serum albumin, insulin A-chain, bacitracin and L-tryptophan. Void volume was measured using Dextran Blue.

Male, New Zealand White rabbits were purchased at a weight of 1·5 kg and housed individually in air-conditioned quarters (24°) with a 12 h light–12 h dark cycle. A stock diet (Master Feed Rabbit Pellets, Maple Leaf Mills, Toronto, Ont.) and tap water were available *ad lib.* At 10 d after arrival, six animals were transferred to each of two semi-purified diets, containing intact or hydrolysed soya-bean protein respectively (Table 1). The diets were made up weekly, stored at 4° and provided *ad lib.*

Blood samples were obtained by venesection of the marginal ear vein before the diet change, and 2 and 4 weeks thereafter. After allowing the blood to clot, serum was separated by centrifugation at 1200 *g* for 15 min. Cholesterol was extracted (Mann, 1961) and
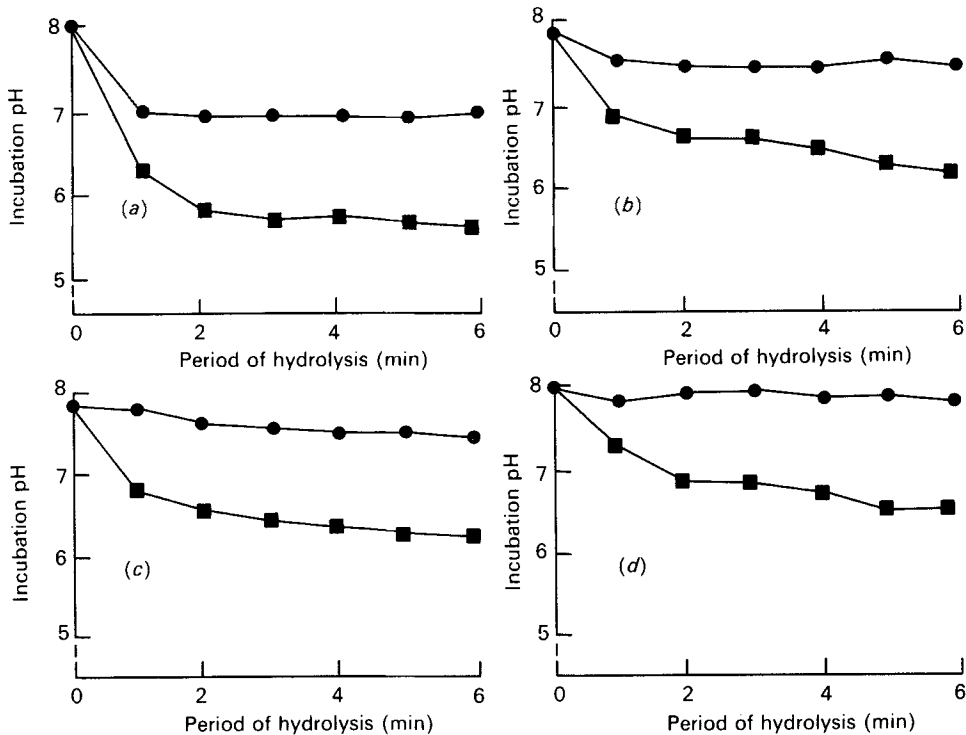
Fig. 1. In vitro digestibilities of casein (■) and soya-bean protein (●) solutions measured as the decrease in pH after adding enzyme. Incubations with (a) pancreatin, (b) trypsin (EC 3.4.21.4), (c) chymotrypsin (EC 3.4.21.1) and (d) peptidase. Points are means of duplicate incubations. Pooled standard errors were: pancreatin 0·035 pH units, trypsin 0·102 units, chymotrypsin 0·097 units and peptidase 0·101 units.

determined colorimetrically (Zlatkis & Zak, 1969). At the end of the experiment, serum pools were prepared and pre-stained with Sudan Black before lipoprotein analysis by density-gradient centrifugation (Terpstra et al. 1981). Cholesterol was determined in each visible lipoprotein fraction by the methods referred to previously.

RESULTS

Digestibility tests at fixed pH

In initial experiments, digestibility was measured from pH decreases resulting from the release of amino acids during hydrolysis. After all incubations, the pH of casein hydrolysates was at least 1·3 units lower than the corresponding values for soya-bean protein (Fig. 1). Comparing the different enzymes, greater pH decreases were observed with crude pancreatin (Fig. 1 (a)) than with the purified preparations of trypsin, chymotrypsin and peptidase (Fig. 1 (b–d)).

Measurements were taken to check whether differences in buffering capacity rather than digestibility between the proteins might affect the previously quoted results. In order to decrease the pH of solutions containing 125 mg casein or soya-bean protein from pH 8·0 to pH 6·4, 6·5 and 14·8 ml dilute HCl were required respectively. Because of the different buffer capacities indicated by these results, further studies of digestibility were made using another technique.

Fig. 2 (a) shows the amounts of TCA-soluble peptides released from the substrate proteins
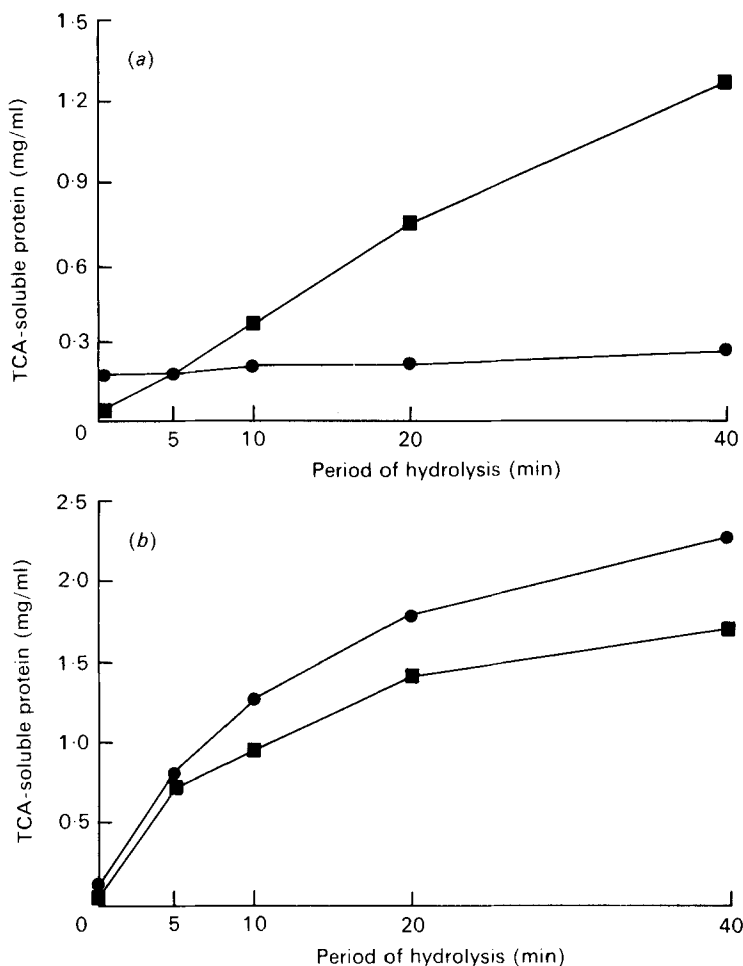
Fig. 2. In vitro digestibilities of casein (■) and soya-bean protein (●) solutions measured as the accumulation of trichloroacetic acid (TCA)-soluble protein after adding enzyme. Incubations with (*a*) pancreatin in 0·1 M-boric acid–sodium hydroxide buffer, pH 7·8, and (*b*) pepsin (*EC* 3.4.23.1) in 0·1 M-hydrochloric acid. Points in (*a*) are means of duplicate samples with a pooled standard error of 0·014 mg/ml; (*b*) shows a typical example of three replicates.

by pancreatin. At all time-points after the initial reading, TCA-soluble peptide levels were higher for casein than for soya-bean protein. The initial reading at 1 min was slightly higher for soya-bean protein; a similar difference was noted in subsequent experiments where the initial reading was taken before hydrolysis.

Incubations with pepsin revealed different digestibilities from those found at alkaline pH (Fig. 2(*b*)). Both substrates were readily digested, soya-bean protein showing slightly more rapid hydrolysis than casein. After 40 min incubation, both proteins were over 60% digested into TCA-soluble peptides.

Solubility and digestibility at different pH values

During the previously described incubations, visual differences in protein solubility were apparent. Casein seemed more soluble at alkaline than at acid pH, while the converse was
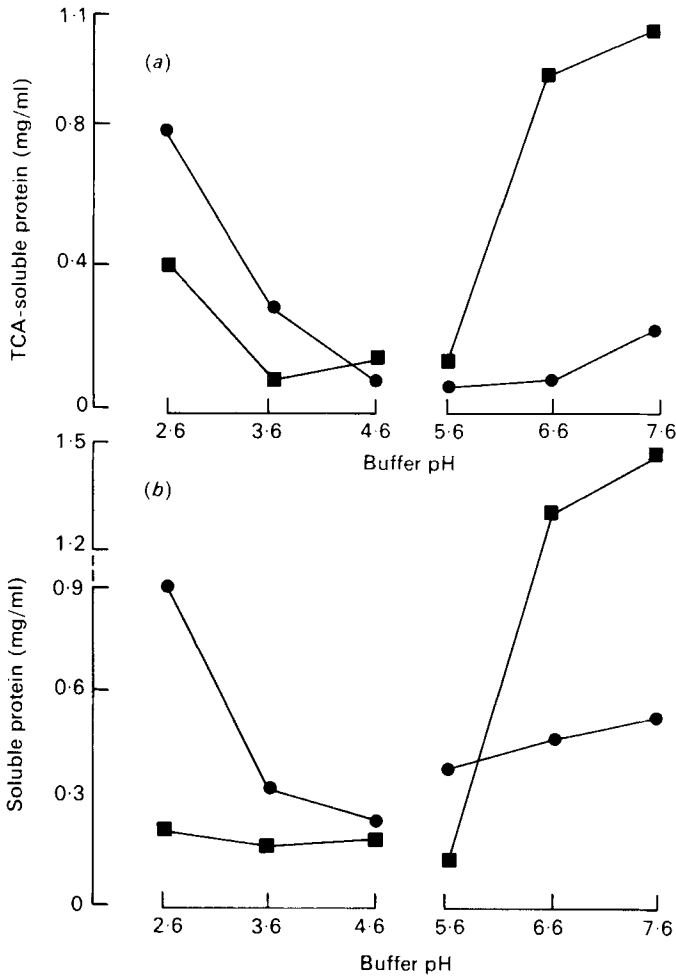
Fig. 3. In vitro digestibilities and solubilities of casein (■) and soya-bean protein (●) in 0·1 M-citric acid–sodium hydrogen phosphate buffer at various pH values. (a) Digestibility, measured as accumulation of trichloroacetic acid (TCA)-soluble protein during 10 min incubation with pepsin (EC 3.4.23.1) (pH 2·6–4·6) or pancreatin (pH 5·6–7·6). (b) Solubility, measured as the protein concentration in the supernatant fraction after centrifuging at 1200 g for 10 min. Points are means of duplicate incubations, with pooled standard errors of 0·014 mg/ml and 0·040 mg/ml for digestibility and solubility respectively.

true for soya-bean protein. The possible influence of this factor on digestibility in vitro was therefore investigated.

At pH 6·6–7·6, casein was more readily digested than soya-bean protein by pancreatin (Fig. 3(a)) and its solubility was also substantially higher (Fig. 3(b)). Conversely, at pH 2·6–3·6, soya-bean protein was more readily digested by pepsin and was more soluble than casein. Both proteins had isoelectric points between pH 4·6 and 5·6, where both digestibility and solubility were at a minimum. Changes in the relative digestibilities of the two proteins thus paralleled their solubilities. In addition, for each protein individually, patterns of digestibility and solubility were comparable. Only pancreatin digestibility of soya-bean protein was lower than expected on the basis of its solubility.
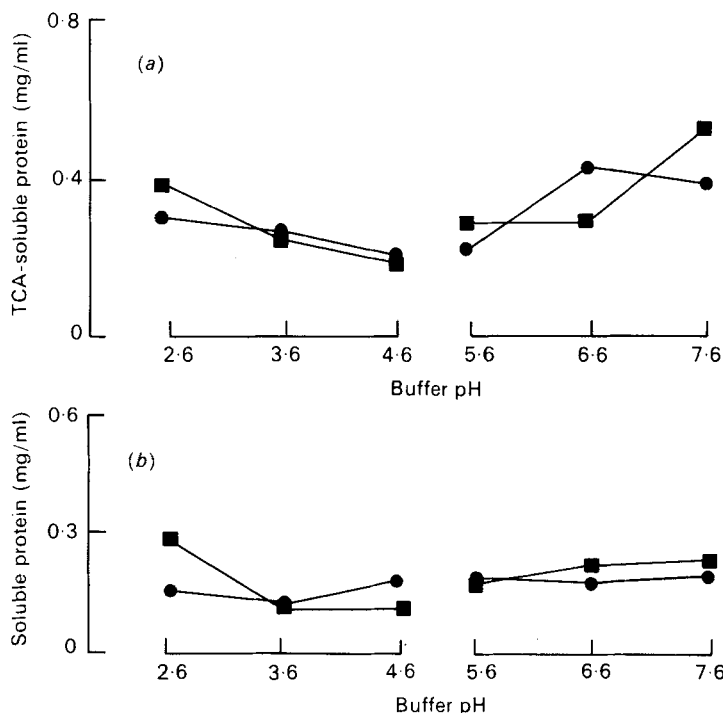
Fig. 4. In vitro digestibilities and solubilities of casein (■) and soya-bean protein (●) after autoclaving for 1 h. (a) Digestibility and (b) solubility. For details, see Fig. 3 and p. 356. Two replicate experiments were carried out at pH 2·6–4·6 only. TCA, trichloroacetic acid.

Table 2. *Effects of sonication on solubilities and digestibilities of casein and soya-bean protein**

Protein	Period of sonication (min)			
	0	1	5	10
	Solubility (mg protein/ml)			
Soya-bean protein	0·62	1·15	1·38	1·35
Casein	1·35	1·46	1·52	1·48
	Digestibility (mg TCA-soluble protein/ml)			
Soya-bean protein	0·67	—	0·74	—
Casein	1·59	—	1·75	—

TCA, trichloroacetic acid.
* Results are means of duplicate incubations with pooled standard errors of 0·067 mg/ml and 0·022 mg/ml for solubility and digestibility respectively.

Effects of heating and sonication

After heat treatment, there were no consistent differences between casein and soya-bean protein for either digestibility or solubility (Fig. 4). Compared with unheated proteins (Fig. 3), heat treatment tended to decrease both digestibility and solubility especially for casein at alkaline pH and soya-bean protein at acid pH respectively.

Sonication of the proteins at pH 7·8 increased the solubility of soya-bean protein more than that of casein (Table 2). When digestibility using pancreatin was measured after 5 min
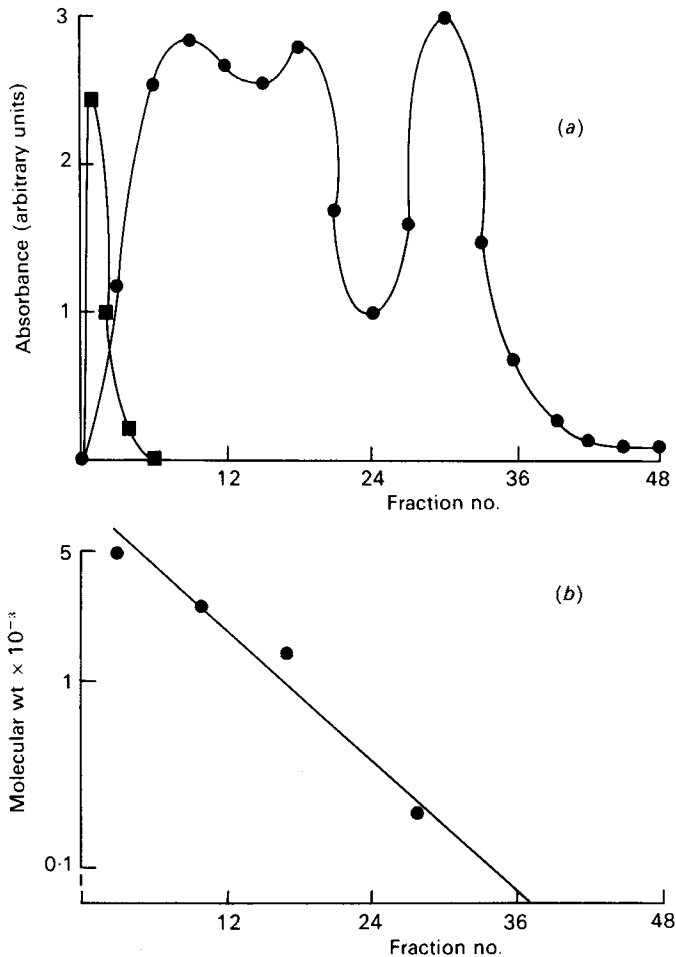
Fig. 5. Column chromatography of intact (■) and hydrolysed (●) soya-bean protein, using Sephadex G25-150 and 0·1 M-potassium dihydrogen phosphate–dipotassium hydrogen phosphate buffer, pH 7·8. (a) Elution profile. (b) Standard line obtained by plotting elution volume v. log (molecular weight) for three calibration proteins and L-tryptophan. The diagram shows typical results out of at least two replicates for each protein preparation.

sonication and compared with untreated samples, increases of only 8 and 12% were noted for soya-bean protein and casein respectively.

In vivo experiment

Soya-bean protein and hydrolysed soya-bean protein were characterized by Sephadex column chromatography before examining their effects in rabbits. The chromatogram of the hydrolysate showed three peaks, while soya-bean protein eluted in a single peak within the void volume (Fig. 5(a)). The plot of log (molecular weight) v. elution volume for the standard proteins produced a straight line (Fig. 5(b)). Using this, the molecular weights of peaks in the hydrolysate chromatogram were calculated as 2000, 600 and 350 approximately.

Table 3. *Growth and serum cholesterol concentrations of rabbits given intact or hydrolysed soya-bean protein*

(Means with their standard errors for six animals per group)

	Body-wt (g)				Serum cholesterol concentration (mmol/l)					
Week...	0		4		0		2		4	
Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Hydrolysate	1722	42	2235	52	1·62	0·12	2·42	0·22	2·81	0·40
Intact	1752	88	2497	81	1·60	0·15	1·46	0·27	1·17	0·29
Statistical significance of difference (Student's *t* test): *P*	NS		< 0·05		NS		< 0·02		< 0·01	

NS, not significant.

Animals given hydrolysate gained slightly less weight than did those receiving soya-bean protein (Table 3). Initial levels of serum cholesterol were similar for the two groups. Cholesterol concentration increased on feeding hydrolysate but was reduced in animals given soya-bean protein (Table 3). At 4 weeks, rabbits given hydrolysate had a 2·3-fold higher cholesterol level than did those given soya-bean protein. Most of this difference was due to the low-density-lipoprotein fraction (1·01 < density < 1·05). Cholesterol concentrations in this fraction were 1·53 and 0·54 mmol/l for rabbits fed on hydrolysate and soya-bean protein respectively. Cholesterol levels in very-low-density and high-density lipoproteins were increased to a lesser extent in animals fed on hydrolysate (values not shown).

DISCUSSION

Initial digestibility tests using the pH technique showed that soya-bean protein was less rapidly hydrolysed than casein by pancreatic or intestinal enzymes. The difference in final pH between soya-bean protein and casein digests was rather greater than that found by Hsu *et al.* (1977). This discrepancy might be explained by differing buffer capacities of the proteins used in the two studies. Hsu *et al.* (1977) concluded that differences in buffer capacity did not affect the method. However, in our study Supro 610 had a substantially greater buffering action than casein, suggesting that this property should be taken into account.

Further experiments were carried out using a TCA precipitation technique, which measures the hydrolysis of proteins to peptides of molecular weight less than about 10 000 daltons. Free amino acids and small peptides, which are the absorbable end-products of protein digestion, are not differentiated by this technique from peptides of molecular weight up to 10 000 daltons. The results showed that, with pancreatin, catabolism to medium-weight peptides proceeded more readily with casein than with soya-bean protein. However, at acid pH with pepsin, both proteins were readily digested. This latter finding is consistent with previous reports that soya-bean protein is quite susceptible to pepsin digestion (Steinhart & Kirchgessner, 1974; Amiot *et al.* 1981). To our knowledge, there is no previous study on digestibility of soya-bean protein using pancreatic enzymes alone. However, Camus *et al.* (1973) showed that vegetable proteins were generally less readily digested by trypsin compared with proteins of animal origin.

The incubations at different pH values suggested a causal relation between solubility and digestibility. Casein and soya-bean protein were most soluble at high and low pH respectively. The solubility patterns of casein and soya-bean protein are exploited in their isolation, which involves selective precipitation at pH 4·0–4·2 and pH 4·6 respectively (Brunner, 1977; Wolf, 1977). Precipitated proteins may be less digestible because they are physically removed from enzyme molecules. In interpreting the present results, it should be noted that low digestibilities at intermediate pH may have been due partly to the enzymes operating far from their optimal pH.

It is well known that heat damage reduces the nutritive value of proteins (Friedman, 1977; Vachon et al. 1982). The mechanism is thought to involve the reaction of amino acids to form products that are resistant to digestion and absorption. In the present experiments, heated proteins displayed both low digestibility and solubility, confirming the relation between these properties. Reduction of solubility could thus contribute to the lowering of nutritional value found after heat treatment.

In contrast with the previous results, sonication produced a large increase in solubility of soya-bean protein, but only a small change in digestibility. Sonication is known to aggregate soya-bean protein molecules as well as increasing their solubility (Wang, 1981). This could explain why, in this case, the susceptibility to enzymic hydrolysis was not raised. Presumably the energy imparted by sonication allows the molecules to assume a state of lower potential energy, in which hydrophobic amino acid residues in one molecule are shielded from the aqueous medium by similar residues in neighbouring molecules (Tanford, 1978). Wang (1981) found that the soya-bean protein aggregates were more stable at concentrations above 0·8 mg/ml, a range which includes the concentration used in the present study. Sonication promotes aggregation in a number of other circumstances, for example the interaction of immunoglobulins with liposomes (Huang & Kennel, 1979).

In addition to the considerations of solubility discussed previously, it is possible that trypsin inhibitors might contribute to the low digestibility of soya-bean protein. However, these substances are unlikely to play a major role, since most of the trypsin inhibitor activity in whole soya beans is lost during isolation of the proteins (Anderson et al. 1979). Moreover, when added to a diet containing casein, purified trypsin inhibitor did not significantly affect cholesterol levels in mice, despite a stimulation of pancreatic weight and enzyme content (Roy & Schneeman, 1981).

Attempts to mimic in vivo digestion, by means of sequential treatment with pepsin and pancreatic enzymes, have shown less (Amiot et al. 1981) or not consistently different (Gauthier et al. 1982) hydrolysis for soya-bean protein compared with casein. Such studies are questionable because the relative contribution of pepsin to proteolysis in vivo is not known. Overall digestibility in vivo, measured as the difference between ingested and faecal N, is relatively high for soya-bean protein (Gillberg, 1977). In this case microbial degradation in the colon may disguise preceding differences in digestibility between proteins. Rats given soya-bean protein have more N in the small gut than those given casein (Zebrowska, 1968). Moreover, a greater flow of protein to the ileum was noted in pigs given soya-bean protein than in those given casein (Leibholz, 1981). Such evidence suggests that soya-bean protein is indeed relatively slowly digested in vivo.

In the present experiments, hydrolysed soya-bean protein caused a hypercholesterolaemia due to accumulation of cholesterol in low-density lipoproteins. An earlier study showed similar cholesterol levels in rabbits given intact or hydrolysed soya-bean proteins (Huff et al. 1977). This discrepancy might be explained by different degrees of digestion of the hydrolysates during preparation. In the present case, total and free amino-N contents of the hydrolysate were 13·5 and 6·3 % respectively. The ratio, free:total amino-N is 0·47, and this is an index of the degree of hydrolysis. The equivalent values for the product used by

Huff *et al.* (1977) are 12·7 and 4·7%, giving a ratio of 0·37. Alternatively, it is possible that the differing weight gains of the two groups in the present study contributed to their divergent cholesterol levels. However, it is unlikely that this factor wholly accounts for the hypercholesterolaemia. Lacombe *et al.* (1983) found that a 50% food restriction was necessary to induce a twofold elevation of serum cholesterol concentration in rabbits.

Lefevre & Schneeman (1982) have previously studied the relation between digestibility and serum cholesterol. They found that heat-treated casein was poorly digested by rats and induced higher levels of cholesterol than untreated protein. A link between low digestibility and reduced serum cholesterol levels, as found here, is supported by experiments in which formaldehyde-treated casein was fed to rabbits (West *et al.* 1984). Digestibility might affect serum cholesterol concentration by means of delays in the release and absorption of amino acids from the gut (Carroll & Woodward, 1982). Alternatively, poorly digested proteins may bind bile acids and inhibit reabsorption of the latter from the ileum (Woodward & West, 1984). Further experiments could usefully evaluate these mechanisms in animals and man (Redgrave, 1984).

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