Characterization of an Immobilized Digestive Enzyme System for Determination of Protein Digestibility

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Porcine pepsin, trypsin, α-chymotrypsin, and intestinal mucosal peptidases, covalently immobilized on porous glass beads, were used to determine the digestibilities of a number of proteins from both plant and animal sources. Immobilized pepsin was used in a separate reactor maintained at low pH, whereas trypsin, chymotrypsin, and intestinal peptidase were placed in a single reactor operated at pH 7.5. Digestibilities determined with pepsin alone were lowest for those proteins having high structural stability such as lysozyme, ovalbumin, and β-lactoglobulin. Nevertheless, combination of pepsin pretreatment with proteolysis by pancreatic and intestinal enzymes indicated more rapid initial digestion rates by the latter enzymes as a result of the pretreatment of structurally stable proteins. The complete system gave digestibilities in agreement with in vivo studies. Correlation (r^2) of digestibilities with FAO and literature values for in vivo digestibilities for a number of plant and animal proteins was 0.83. Moreover, the system was stable to repeated use without significant loss of activity.

The nutritional quality of a protein depends on its amino acid content and on the bioavailability of the amino acids. A major factor determining bioavailability is the digestibility of a protein.

Standard methods of food processing can produce modifications such as lysinoalanine cross-linking, racemization, and the formation of Maillard reaction product (Kies, 1981), which may decrease a protein's digestibility. Thus, a convenient method for assessing protein digestibility is desirable.

Since rat bioassays for digestibility are expensive and time consuming, several in vitro assays for protein digestibility have been developed. Both single enzyme systems, involving pepsin (Sheffner et al., 1956), trypsin (Maga et al., 1973), or papain (Buchanan, 1969), and multienzyme systems, pepsin–pancreatin (Mauron et al., 1955; Akeson and Stahmann, 1964; Gauthier et al., 1982), pepsin–trypsin (Saunders et al., 1973; Rhinehart, 1975), pepsin–chymotrypsin (Rhinehart, 1975), and trypsin–chymotrypsin–peptidase (Rhinehart, 1975; Hsu et al., 1977), have been developed. The multienzyme systems involving pepsin are performed as two-stage processes because of the differing pH requirements for pepsin and other enzymes used.

These systems fall into two groups depending on how the extent of digestibility is estimated. In one group, "digested" material was separated from "undigested" material by boiling and filtration (Sheffner et al., 1956), acid precipitation and centrifugation (Akeson and Stahmann, 1964), centrifugation and filtration (Saunders et al., 1973), or dialysis (Mauron et al., 1955; Gauthier et al., 1982; Steinhart and Kirchgessner, 1973). In the other group, the fractions were not separated and digestibility was correlated with the pH change after a 10-min incubation with enzymes (Hsu et al., 1977). The results of these methods have been encouraging. However, the systems requiring separation of fractions by procedures other than simple dialysis tend to be complicated and time consuming. The method of Hsu et al. (1977) is convenient but its short periods of incubation with enzymes will result in underestimation of the digestibility of structurally stable proteins (this paper). The methods involving dialysis are convenient, but measurement of the rate of hydrolysis is difficult because the rate of separation of "digested" and "undigested" protein depends on the rate of dialysis. Also, in all of these systems correction for enzyme autolysis may be required.

We have developed a system of immobilized enzymes for the determination of protein digestibility. The system includes pepsin, trypsin, chymotrypsin, and intestinal peptidase. The procedure is convenient, prevents enzyme autolysis, and permits reuse of digestive enzymes.

The digestibility is determined by using the reaction of orthophthaldialdehyde (OPA) and 2-mercaptoethanol with the α-amino groups freed by hydrolysis of peptide bonds. Thus, in contrast to other methods, we determine directly the number of peptide bonds hydrolyzed. The OPA reaction is useful for measuring hydrolysis of proteins because (a) OPA is soluble and stable in aqueous solution, (b) the reaction proceeds essentially to completion (Simons and Johnson, 1978) within several seconds at room temperature (Chen et al., 1979; Trepman and Chen, 1980), and (c) the extinction coefficient is the same for OPA adducts of amino acids, peptides, and ε-amino groups (Rowlett and Murphy, 1981; Church et al., 1983).

EXPERIMENTAL SECTION

Materials. Porcine crystalline pepsin, trypsin, chymotrypsin, and crude intestinal peptidase were obtained from Sigma Chemical Co. Whole casein was prepared in our laboratory and ANRC casein was obtained from U.S. Biochemicals. Soy isolate (Supro 620) was obtained from Ralston Purina and wheat gluten (composite sample) was obtained from the International Wheat Gluten Association. All other protein substrates and controlled pore glass were purchased from Sigma Chemical Co. Orthophthaldialdehyde was from Pierce and sodium dodecyl sulfate (SDS) was electrophoresis grade from Bio-Rad. All other chemicals were reagent grade. Solutions were made with water prepared by reverse osmosis and deionization.

Assay for Proteolysis. OPA reagent was made as described by Porter et al. (1982). The reagent was prepared by combining the following components and diluting to 100 mL with water: 50 mL of 0.1 M sodium borate, 80 mg of OPA (dissolved in 2 mL of 95% ethanol), 200 μL of 2-mercaptoethanol, and 5 mL of 20% SDS. The reagent was prepared fresh daily.

A 20-μL aliquot of the solution to be assayed was added to 1 mL of OPA reagent and was incubated for 2 min at

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room temperature. Absorbance was measured at 340 nm. For substrate solutions containing a single protein, digestibilities were calculated from

$$\text{digestibility} = \frac{n}{n_0} = \frac{M_r}{d\epsilon n_0}\frac{\Delta A_{340}}{[\text{P}]} \quad (1)$$

where n is the average number of peptide bonds hydrolyzed per protein molecule, n_0 is the number of peptide bonds in the protein, M_r is the molecular weight of the protein, d is the dilution factor (1/51 for our case), ΔA_{340} is the increase in absorbance at 340 nm, [P] is the initial protein concentration in mg/mL, and ϵ is 5850 M^{-1} cm^{-1}.

For casein, soy isolate, and wheat gluten, which are mixtures of proteins, digestibilities were calculated from

$$\text{digestibility} = \sum_i f_i\frac{n_i}{n_{0,i}} = \frac{\bar{m}_{0,w}}{d\epsilon}\frac{\Delta A_{340}}{[\text{P}]} \quad (2)$$

where f_i is the fraction of the total protein concentration resulting from component i, $\bar{m}_{0,w}$ is the average amino acid residue weight, taken to be 115, and n_i and $n_{0,i}$ are analogous to n and n_0, respectively, but refer only to component i of the protein mixture (see Appendix for derivations of eq 1 and 2).

Protein Concentration. Protein concentrations for soy isolate and wheat gluten were estimated from amino acid analysis. Concentrations of whole casein or ANRC casein were determined from absorbance at 280 nm corrected for light scattering (Sliwkowski et al., 1980) and the weighted average of the absorptivities of the individual casein components (0.88) or by weight and volume, respectively. Concentrations of other proteins were determined from absorbance at 280 nm and absorptivities appropriate for the pH of the solution being measured.

Glass Derivatization. Controlled-pore glass (2000-Å pore, 80–120 mesh, Sigma) was acid-cleaned and silanized by treatment with (3-aminopropyl)triethoxysilane as described by Swaisgood et al. (1976). The derivatized glass was dried and stored at room temperature. Aminopropyl glass was succinylated in acetone containing 10% triethylamine by adding 5 g of succinic anhydride/g of glass and allowing the reaction to proceed for 1 h at room temperature. The succinylation procedure was performed twice. Succinylated beads were washed extensively with water, dried, and stored at room temperature.

Enzyme Immobilization. Pepsin was immobilized on aminopropyl-glass by a modification of the procedure of Line et al. (1971). One gram of glass beads was degassed and added to a column made from a plastic 5-mL pipet tip and a plastic snap-on cap for a 10-mL volumetric flask (see Figure 1A). Pepsin solution, 75 mg dissolved in 15 mL of HCl, pH 4.0, at room temperature, was recirculated through the aminopropyl-glass (fluidized beads) by using a peristaltic pump at a flow rate of 2.5 mL/min. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide, metho-*p*-toluenesulfonate (60 mg) obtained from Sigma Chemical Co. (St. Louis, MO) was added, and the pH of the stirred reservoir was maintained at 4.0 by titration with HCl. After 30 min the system was transferred to the cold room (4 °C) and recirculated overnight. The immobilized pepsin was washed consecutively at pH 2.0 with 350 mL of HCl, 100 mL of 4 M urea, and 100 mL of HCl and was stored at pH 2.0 in HCl containing 0.06% NaN_3 at 4 °C. The immobilized pepsin was used no sooner than 24 h after the urea wash to allow for complete structural reequilibration. The activity, assayed with Gly-Gly-Phe-Phe ethyl ester as the substrate (Inouye et al., 1966), was 0.39 μmol min^{-1} (g of beads)$^{-1}$.

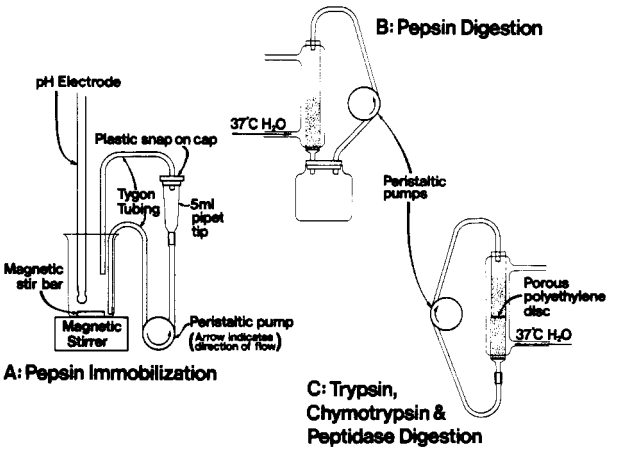

Figure 1. Schematic diagram of the apparatus used for (A) immobilization of pepsin, (B) digestion of protein by pepsin, and (C) digestion of protein by trypsin, chymotrypsin, and peptidase.

Trypsin was immobilized by the procedure of Taylor (1979). Trypsin solution, 14 mL of 5 mg/mL trypsin–20 mM $CaCl_2$, pH 7.0, was cooled to 0 °C and adjusted to 10 mM in 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Sigma Chemical Co., St. Louis, MO) immediately prior to recirculation through a column containing 0.6 g of degassed succinamidopropyl-glass. After 20 h of reaction at 4 °C, the glass beads were washed with 100 mL of 20 mM $CaCl_2$–2 M NaCl, pH 7.0, and were stored in 20 mM $CaCl_2$–0.02% NaN_3, pH 7.0, Chymotrypsin was immobilized by an identical procedure except that the starting chymotrypsin solution contained 100 mM $CaCl_2$ instead of 20 mM $CaCl_2$. The activities for immobilized trypsin and chymotrypsin were 45.8 and 17.9 μmol min^{-1} (g of beads^{-1}, assayed with benzoylarginine ethyl ester (Schwert and Takenada, 1955) and benzoyltyrosine ethyl ester (Hummel, 1959), respectively.

Intestinal peptidase was partially purified by elution from DEAE-Sephacel with 10 mM sodium phosphate, pH 8.0, 0.5 M in sucrose and 0.115 M in NaCl (Porter et al., 1982). Partially purified peptidase (8–16 mL) was dialyzed for 4 h against 2 L of 10 mM sodium phosphate, pH 7.0, containing 0.2 M NaCl. The peptidase solution was cooled to 0 °C and adjusted to 10 mM in EDC immediately prior to recirculation through a column containing 0.4–1.0 g of degassed aminopropyl-glass beads. After 18 h at 4 °C, the glass beads were washed with 340 mL of 10 mM sodium phosphate, pH 7.0, 0.2 M in NaCl, then 100 mL of 10 mM sodium phosphate, pH 7.0, 1 M in NaCl, then 100 mL of 10 mM sodium phosphate, pH 7.0, 4 M in urea, and finally 100 mL of 10 mM sodium phosphate, pH 7.0. Glass beads were stored in 10 mM sodium phosphate (pH 7.0)–0.06% NaN_3 at 4 °C. The immobilized peptidase was used no sooner than 24 h after the urea wash. The activity, assayed with leucylglycine as the substrate (Porter et al., 1982), was 0.39 μmol min^{-1} (g of beads)$^{-1}$.

Protein Digestion. Pepsin digestion was performed by using a recirculation system consisting of a peristaltic pump, a circulating water bath at 37 °C, and a jacketed 15 cm long, 5 mm inner diameter column containing 1.04 mL of immobilized pepsin–glass beads (Figure 1). The recirculating flow rate was maintained at 10 mL/min to ensure that the rate was not inhibited by external diffusion and that the per pass conversion would be less than that required for complete backmixing so the reactor would operate according to initial rate kinetics (Taylor and Swaisgood, 1980). Substrate solutions (25 mL) were made by dissolving the desired protein in HCl (pH 2.0)–0.06% NaN_3 to give a protein concentration of 1 mg/mL or less.

Table I. Digestibility of Selected Proteins by Immobilized Digestive System Enzymes[a]

protein	enzyme system: pepsin[b]	trypsin, chymotrypsin, intestinal peptidases[c]	pepsin–trypsin, chymotrypsin, intestinal peptidases[d]
ANRC casein	0.094	0.430	0.470
W casein	0.075		0.514
β-lactoglobulin	0.038 ± 0.012 (4)	0.522	0.499 ± 0.013 (2)
α-lactalbumin	0.085 ± 0.011 (4)	0.650 ± 0.019 (2)	0.583 ± 0.033 (3)
BSA	0.075 ± 0.010 (3)		0.623
lysozyme	0.007 ± 0.002 (4)	0.576	0.521 ± 0.037 (3)
ovalbumin	0.019	0.293	0.271
soy isolate	0.069 ± 0.004 (2)		0.495 ± 0.057 (2)
wheat gluten	0.047		0.359

[a] Values are average ± SEM. The number of replicate determinations is given in parentheses. [b] Digestibility after 18 h of pepsin treatment. [c] Digestibility after 24 h of trypsin, chymotrypsin, and peptidase treatment and no pepsin pretreatment. [d] Total digestibility after 18 h of pepsin treatment and 24 h of trypsin, chymotrypsin, and peptidase treatment.

Soy isolate and wheat gluten were solubilized by incubation at 37 °C for 4 h with stirring followed by filtration through Whatman No. 3 filter paper. Whole casein and ANRC were dissolved in water at pH 7.0 and 10.0, respectively, and were titrated to pH 2.0 with HCl. NaN_3 was added to 0.06%. All protein solutions were filtered through a 0.45-μm Gelman Acrodisc immediately prior to use.

A 1-mL portion of the filtered protein solution was set aside as the zero time blank and for determination of protein concentration. The digestion apparatus was washed with 10 mL of protein solution and the wash discarded and the remaining 14 mL was recirculated from a sealed reservoir made from a 30-mL screw-cap polypropylene bottle with entrance and exit tubing going through holes drilled in the bottle top. Hole size and tubing diameter were chosen to give a tight seal (see Figure 1B). Samples (50 μL) were taken from the top of the column at appropriate times and were stored in sealed tubes at 4 °C until the assays for proteolysis were performed. After 18 h the recirculating protein solution was collected in the reservoir bottle. The immobilized pepsin was washed with 100 mL of 4 M urea, pH 2.0, at room temperature and then with 100 mL of HCl, pH 2.0, and was stored in HCl (pH 2.0)–0.06% NaN_3 at 4 °C for at least 24 h prior to reuse.

Digestion by immobilized trypsin, chymotrypsin, and intestinal peptidase (TCP) was performed in the same apparatus described above except that no separate reservoir was used (see Figure 1C). One milliliter of a trypsin–chymotrypsin mixture (0.5 mL of each) and 1 mL of peptidase were used. A porous polyethylene disk separated the trypsin–chymotrypsin mixture from the peptidase.

The volume of the protein solution from the pepsin hydrolysis was determined (typically about 13 mL), $NaH_2PO_4 \cdot H_2O$ was added to a 50 mM concentration, and the pH was adjusted to 7.5 with NaOH. The solution was filtered through a 0.45-μm Acrodisc immediately prior to use. A portion (about 200 μL) was set aside as the zero time blank. The apparatus was washed with approximately 10 mL of the substrate solution, the wash was discarded, and the remainder was recirculated. Samples (50 μL) were taken and treated as described above prior to assaying for proteolysis. After 24 h the system was drained and washed with 100 mL of 10 mM sodium phosphate, pH 7.0, 4 M in urea, followed by 100 mL of 10 mM sodium phosphate, pH 7.0. The immobilized enzymes were removed from the column and stored in 10 mM sodium phosphate (pH 7.0)–0.06% NaN_3 for at least 24 h prior to reuse.

The protein concentration for the TCP hydrolysis ([P]) was determined from the protein concentration for the pepsin hydrolysis ($[P]_p$), the absorbance of the OPA reagent (x), the OPA assay of the zero time blank for the TCP hydrolysis (y), and the OPA assay of the final pepsin hydrolysate (z) by using

$$[P] = [P]_p \frac{y - x}{z - x}$$

In cases where no pepsin pretreatment was used, protein, except ANRC casein, was dissolved in 50 mM sodium phosphate (pH 7.5)–0.06% NaN_3. ANRC casein was dissolved in water at pH 10.0 and titrated to pH 2.0 with HCl; $NaH_2PO_4 \cdot H_2O$ was added to 50 mM concentration, the solution was titrated to pH 7.5 with NaOH, and NaN_3 was added to 0.06%. This procedure was used with ANRC casein to ensure that it remained soluble under the conditions desired for the digestion. Other than these changes, digestion with no pepsin pretreatment was performed as described above.

Control experiments included OPA analysis of substrate proteins that had been incubated or circulated through succinamidopropyl-glass under conditions for protein digestion without exposure to immobilized enzymes and of buffer solutions that had been recirculated through immobilized enzyme beads under the conditions for protein digestion.

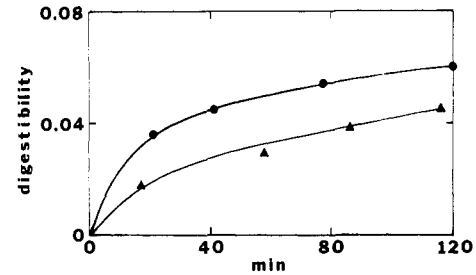

Figure 2. Time course for the hydrolysis of ANRC casein (●) and BSA (▲) by immobilized pepsin.

RESULTS

Time courses for pepsin hydrolysis of ANRC casein and bovine serum albumin (BSA) show digestibilities of 0.06 and 0.045, respectively, after 2 h (Figure 2). Similar results were obtained for α-lactalbumin. Time courses were not determined for pepsin hydrolysis of other proteins. Digestibilities by pepsin after 18 h range from a high of 0.094 for ANRC casein to a low of 0.007 for lysozyme with the average being 0.056 (Table I). Proteins that were poorly digested by pepsin tend to be those that have the greatest structural stability, such as lysozyme. Digestibilities at 2 h for ANRC casein, BSA, and α-lactalbumin are approx-

Table II. Ratio of Digestibility to the Average for ANRC and Whole Casein at the Indicated Times

time:	10 min[a]	30 min[a]	60 min[a]	120 min[a]	24 h[b]
casein digestibility:[c]	0.054	0.078	0.098	0.128	0.492
protein	digestibility ratio (relative to casein)				
ANRC	0.89	0.86	0.88	0.89	0.96
W casein	1.11	1.14	1.12	1.11	1.04
β-lactoglobulin	0.50	0.61	0.71	0.82	1.01
α-lactalbumin	1.09	1.14	1.14	1.15	1.19
BSA	1.20	1.34	1.40	1.47	1.27
lysozyme	0.28	0.31	0.41	0.58	1.06
ovalbumin	0.08	0.17	0.23	0.30	0.55
soy isolate	1.30	1.29	1.33	1.40	1.01
wheat gluten	0.43	0.49	0.53	0.57	0.73

[a] These values are based on the digestibility by trypsin, chymotrypsin, and peptidase after a pepsin pretratment. [b] These values are based on the total digestibility by pepsin, trypsin, chymotrypsin, and peptidase. [c] The average value of digestibility of ANRC and whole casein at the indicated times.

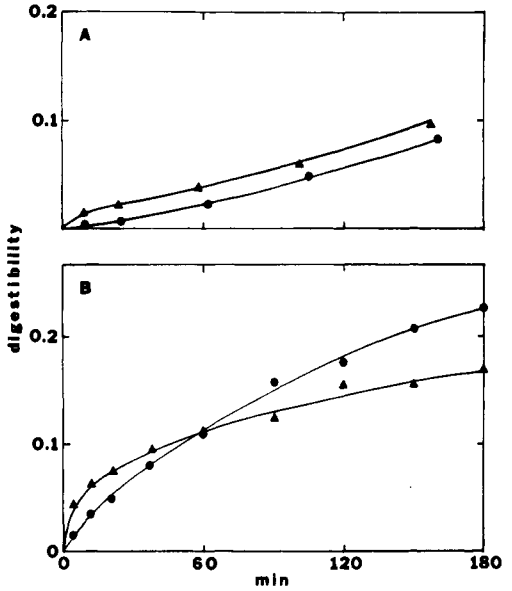

Figure 3. Time course for hydrolysis by immobilized trypsin, chymotrypsin, and peptidase with (▲) and without (●) a pepsin pretreatment. (A) Lysozyme. (B) α-Lactalbumin.

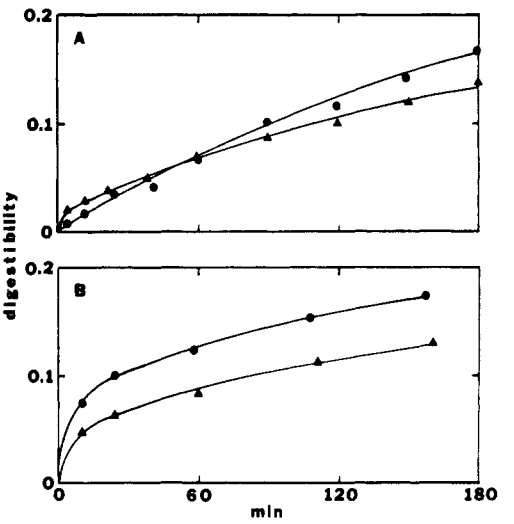

Figure 4. Time course for hydrolysis by immobilized trypsin, chymotrypsin, and peptidase with (▲) and without (●) a pepsin pretreatment. (A) β-Lactoglobulin. (B) ANRC casein.

imately half of the values obtained at 18 h.

Time courses for hydrolysis of several proteins by immobilized TCP, both with and without a pepsin pretreatment, are shown in Figures 3 and 4. These experiments were used to determine the effect of pepsin pretreatment, if any, on subsequent hydrolysis of substrate protein by TCP. Where pepsin pretreatment was used, the final OPA assay value following hydrolysis of the protein by pepsin was used as the zero time (reference cuvette) value for TCP hydrolysis. Thus, the total digestibility curves for the case of pepsin pretreatment could be obtained by adding a constant to the TCP curves shown (total digestibilities are given in Table I). Digestibilities at 2.5–3 h range from about 0.09 for lysozyme to 0.20 for α-lactalbumin (parts A and B of Figure 3). For all proteins tested, except casein, pepsin pretreatment increased the initial rate of digestion by TCP. It was also observed that, except for lysozyme and ovalbumin (not shown), the rate of hydrolysis decreased with time for the first 3 h. Determination of fractional hydrolysis from periods up to 48 h indicated that the reaction was complete within 24 h.

Digestibilities, both with and without a pepsin pretreatment, are shown in Table I. Repetitions were performed 2–3 weeks apart with the enzymes being used 2–3 times between each repetition. The values for pepsin pretreatment represent the sum of digestibilities by pepsin and by TCP and range from a high of 0.62 for BSA to a low of 0.27 for ovalbumin with an average of 0.48. No large differences were seen for digestibilities with and without a pepsin pretreatment.

Table II shows the ratio of protein digestibility by TCP treatment after pepsin pretreatment to the average for ANRC casein and whole casein. The ratio for 24 h is based on the sum of the digestibilities by pepsin and TCP. Two basic patterns occur: in one, the ratio is essentially constant with time and, in the other, the ratio increases with time. This latter pattern occurs with proteins having relatively high structural stability, i.e., lysozyme, ovalbumin, and β-lactoglobulin. These same proteins had relatively low pepsin digestibilities (see Table I).

The correlation between the ratio of protein digestibility to that of casein determined with the immobilized enzyme system (pepsin pretreatment in each case) and that ratio determined from published values of digestibility is shown in Figure 5. Literature values for the digestibility ratio are from the FAO (1970) except for that of the soy isolate, which is from Hsu et al. (1977), and ovalbumin, which is from Hopkins (1980). The FAO value for casein digestibility is 0.963. A digestibility ratio was used to allow comparison of results from different studies. Literature values for the digestibility of β-lactoglobulin, lysozyme, and BSA could not be found.

Control experiments with α-lactalbumin and β-lactoglobulin showed that no hydrolysis occurred in the absence of immobilized enzymes. Recirculation of buffer through the immobilized enzymes or substrate protein through the succinamidopropyl-glass did not release any OPA-positive

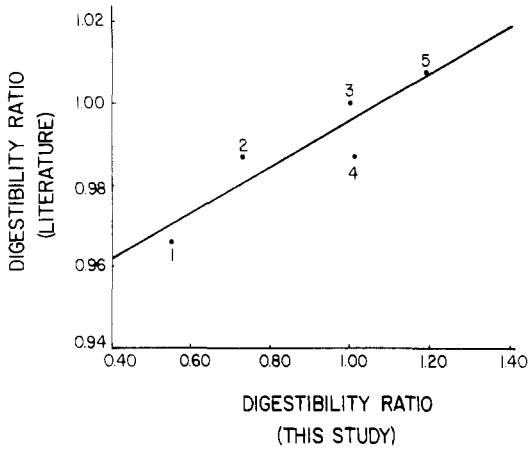

Figure 5. Plot of the ratio of digestibility of a protein to that of casein (pepsin pretreatment in each case) vs. the same ratio determined from published values of digestibility. Values for our data are from the 24-h digestibility in Table II. The data fit the linear relationship $Y = 0.0563X + 0.939$ ($r^2 = 0.83$). The proteins are (1) ovalbumin, (2) wheat gluten, (3) casein, (4) soy isolate, and (5) α-lactalbumin.

material. Also, after an immobilized enzyme preparation was used 8 times over a period of 35 days, no detectable loss of activity was seen as measured by protein digestibility at 18 and 24 h for pepsin and TCP, respectively. Furthermore, assay of immobilized enzyme with synthetic substrates indicated that significant loss of activity had not occurred. It was concluded that the results were not compromised by anomalies due to the presence of the matrix, the presence of soluble proteinases in the protein substrates, the occurrence of autolysis, or significant loss of activity.

DISCUSSION

The in vitro digestibility assay described here is convenient, prevents enzyme autolysis, and permits reuse of digestive enzymes. The results correlate well with published values of digestibility for the proteins tested. While we have not performed assays on an extensive collection of proteins, the results that we have obtained do include proteins from both animal and plant sources, viz., milk, egg, soy, and wheat.

The OPA–mercaptoethanol adduct of peptides and amino acids is not stable (Chen et al., 1979; Rowlett and Murphy, 1981; Simons and Johnson, 1976) over long periods of time due to a slow intramolecular rearrangement (Simons and Johnson, 1978). However, absorbances of the OPA adducts are proportional to concentration and are additive (Rowlett and Murphy, 1981; Church et al., 1983). Thus, as long as the interval of reaction with OPA reagent is constant for all samples (2 min for our data), the OPA reaction gives an accurate measure of the number of peptide bonds hydrolyzed. Accordingly, the molar absorptivity for the adduct given under Experimental Section was also determined by using a 2-min incubation time in OPA reagent.

For all proteins tested, except casein, pepsin pretreatment increased the initial rate of digestion by immobilized TCP. Even though pepsin digestion should eliminate some of the sites available for hydrolysis by chymotrypsin, the destabilization of protein structure that results from pepsin hydrolysis appears to be the dominant factor causing an increase in the initial rate of hydrolysis by TCP. However, for casein, which has a low structural stability, loss of sites for hydrolysis by chymotrypsin appears to be the dominant factor resulting in a decrease in the initial rate of hydrolysis of this protein.

Structurally stable proteins (lysozyme, ovalbumin, and β-lactoglobulin) have very low digestibilities at short hydrolysis times (Table II). Thus, a short time assay such as that of Hsu et al. (1977) will seriously underestimate the digestibility of these proteins. It is interesting to note that these same proteins exhibit relatively low digestibilities by pepsin (Table I). Consequently, use of single enzymes may also underestimate digestibility.

Measurement of digestibility on the basis of rate of digestion has certain limitations: (a) the unusually low initial rate of digestion of a structurally stable protein and (b) the difference in rate observed with and without a pepsin pretreatment. In the latter case, pretreatment with pepsin would be preferred since that corresponds to the in vivo situation. Moreover, use of extent of hydrolysis as a measure of digestibility as recommended here has the advantage of minimizing error due to loss of small amounts of individual enzyme activities.

Systems that measure digestibility by separating "digested" and "undigested" material by dialysis are compromised by the difficulty in differentiating between rate of hydrolysis and rate of dialysis. Thus, the apparent product inhibition of enzyme observed by Gauthier et al. (1982) could be explained by inhibition of diffusion of hydrolysis products, the degree depending on the rate of dialysate replacement that would determine the concentration gradient.

An in vitro digestibility system that contains a broad range of digestive enzymes should give a better simulation of the in vivo process than a system with a restricted complement of enzymes. The most sophisticated of the previously developed in vitro digestibility systems omit either gastric or intestinal enzymes. The system described in this paper includes gastric, pancreatic, and intestinal enzymes. This broad range of enzyme specificities should yield a greater extent of hydrolysis and should be able to detect changes in digestibility resulting from a broader range of protein modifications.

We expect that the immobilized enzyme system described here will be able to detect changes in protein digestibility that may occur during processing. The soluble enzyme systems of Hsu et al. (1977) and Gauthier et al. (1982) are capable of detecting such changes. In the case of complex foods, we expect that extraction with urea followed by removal of the urea will yield a protein preparation that can be used with the immobilized enzyme system.

APPENDIX

Calculation of Digestibility. When the OPA assay for quantitation of liberated α-amino groups is used, the digestibility is defined as the fraction of the total peptide bonds hydrolyzed, i.e.

$$\text{digestibility} = n/n_0 \tag{1}$$

where n is the average number of peptide bonds hydrolyzed per molecule and n_0 is the total number of bonds in that molecule. The increase in A_{340} resulting from hydrolysis will be given by

$$\Delta A_{340} = d\epsilon\frac{[\mathrm{P}]}{M_r}n \tag{2}$$

where d is the dilution factor in the OPA assay, ϵ is the molar absorptivity at 340 nm, [P] is protein concentration in mg/mL or g/L, and M_r is the molecular weight of the protein. Thus, the digestibility would be

$$\frac{n}{n_0} = \frac{M_r}{d\epsilon n_0}\frac{\Delta A_{340}}{[\mathrm{P}]} \tag{3}$$

For a mixture of proteins the increase in A_{340} would be

$$\Delta A_{340} = d\epsilon\sum_i([P]_i/M_{r,i})n_i = d\epsilon[P]\sum_i(f_i/M_{r,i})n_i$$

where f_i is the fraction of the total protein corresponding to protein i. Since $M_{r,i} = \bar{m}_{0,i}(n_{0,i} + 1)$, the above equation becomes

$$\Delta A_{340} = d\epsilon[P]\sum_i f_i n_i/m_{0,i}(n_{0,i} + 1)$$

where $\bar{m}_{0,i}$ is the mean residue weight for protein i. Since the mean residue weight does not vary substantially among proteins, it is convenient to introduce the relationship $\bar{m}_{0,i} = \bar{m}_{0,w} + \Delta\bar{m}_{0,i}$, where $\bar{m}_{0,w}$ is the mean residue weight for a large group of proteins and $\Delta\bar{m}_{0,i}$ is the deviation from $\bar{m}_{0,w}$ for protein i. Substituting this relationship for $\bar{m}_{0,i}$ and rearranging gives

$$\frac{\bar{m}_{0,w}\Delta A_{340}}{d\epsilon[P]} = \sum_i f_i \frac{n_i}{n_{0,i}} \frac{1}{1 + \Delta\bar{m}_{0,i}/\bar{m}_{0,w}} \frac{1}{1 + 1/n_{0,i}}$$

Using the binomial expansion to approximate terms in parentheses yields, after dropping terms in $O(x^2)$

$$\frac{\bar{m}_{0,w}\Delta A_{340}}{d\epsilon[P]} = \sum_i f_i(n_i/n_{0,i})(1 - \Delta\bar{m}_{0,i}/\bar{m}_{0,w})(1 - 1/n_{0,i})$$

Multiplying the terms in parentheses and dropping the cross term gives, after rearrangement

$$\sum_i f_i(n_i/n_{0,i}) = \frac{\bar{m}_{0,w}\Delta A_{340}}{d\epsilon[P]} + \sum_i f_i(n_i/n_{0,i})(1/n_{0,i} + \Delta\bar{m}_{0,i}/\bar{m}_{0,w})$$

However, the last term is negligible, yielding for the digestibility of a mixture of proteins

$$\text{digestibility} = \sum_i f_i(n_i/n_{0,i}) = \frac{\bar{m}_{0,w}\Delta A_{340}}{d\epsilon[P]} \quad (4)$$

It can easily be shown that the term neglected is negligible, which is equivalent to assuming $\Delta\bar{m}_{0,i} = 0$ and $1/n_{0,i}$ is very small. The maximum error resulting from this assumption is $\sum f_i(1/n_{0,i} + \Delta\bar{m}_{0,i}/\bar{m}_{0,w})$. For whole casein, for which f_i, $n_{0,i}$, and $\Delta\bar{m}_{0,i}$ are known, and taking $\bar{m}_{0,w} = 115$, the maximum error is 0.0288.

Registry No. Pepsin, 9001-75-6; trypsin, 9002-07-7; α-chymotrypsin, 9004-07-3; peptidase, 9031-96-3; lysozyme, 9001-63-2.

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