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The pH-Stat Method for Assessing Protein Digestibility: An Evaluation

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With the use of a microprocessor controlled pH stat, factors affecting the digestibility of proteins were studied. Linear hydrolysis was obtained with a substrate concentration of 3 mg/mL and an enzyme–substrate ratio of 1:128 and 1:100 for pancreatin and trypsin, respectively. Salt ($>10^{-3}$ M) reduced proteolysis of soy glycinin (11S) but had little effect on bovine serum albumin (BSA) or casein digestibility. Under optimum conditions the pH stat was sensitive to trypsin inhibitor (TI), and linearity in rates of digestion of 11S was sensitive to the TI–trypsin ratio rather than total trypsin or TI concentration. This may complicate the use of the pH stat as a routine method for determining TI activity. Extensive reduction of the disulfide bonds in bovine serum albumin enhanced proteolysis of BSA but upon reoxidation digestibility was reduced.

INTRODUCTION

Many proteins, e.g., oilseed proteins, are thermally treated to desolventize and to improve their potential nutritional value (Rackis, 1974; Anderson et al., 1979; Liener, 1979), and proteins may also be modified to improve specific functional properties (Kinsella and Shetty, 1979). Because digestibility is an important factor affecting amino acid availability (Kakade, 1974), in vitro digestibility tests are useful in evaluating the effect of such treatments on bioavailability. The important aspects of in vitro digestibility tests were summarized by Mauron (1973), Hackler (1975), Evans and Witty (1978), and Milhalyi (1978).

Several in vitro digestibility studies of proteins are based on the initial proteolysis rates (Saunders et al., 1973; Maga et al., 1973; Hsu et al., 1977). Hsu et al. (1977) reported good correlations between the pH drop after 10 min of proteolysis and apparent in vivo digestibility. However, significant discrepancies due to buffering and differing pK values of amino groups may invalidate this approach (O'Hare et al., 1984). Proteolysis can also be monitored continuously in a pH stat at constant pH, as proposed by Milhalyi (1978), and thus, enzymatic activity is not affected by pH changes during proteolysis. The number of peptide bonds cleaved are directly proportional to alkali consumption, and the results generally agree with results obtained from chemical tests, e.g., ninhydrin and DTNB (Von Hippel et al., 1960; Samuelson and Li, 1964). Theoretical and experimental aspects of the pH-stat method were discussed in detail by Milhalyi (1978).

Nutritional evaluations of proteins based on the pH-stat method have been reported (Richardson and Catsimpoolas, 1979; Lynch et al., 1977; Stinson and Snyder, 1980; Hill et al., 1982). Richardson and Catsimpoolas (1979) used the pH-stat method to monitor the effects of heat treatment on the initial rates of trypsin proteolysis of soy protein. They observed decreased digestibility of a minimally heat treated soy protein sample. Lynch et al. (1977) determined the effects of chemical modification on the tryptic hydrolysis of soy proteins fractions. Stinson and Snyder (1980) and Hill et al. (1982) measured the initial rate of tryptic hydrolysis of heat treated soy meal samples at several trypsin concentrations to assess residual trypsin inhibitor (TI) activity. Several experimental problems and inconsistencies were encountered, e.g., the initial proteolysis rate showed an unusual decrease with soy meal protein concentration (Stinson and Snyder, 1980) and when initial rates were plotted against added trypsin, shifts in intercepts were not proportional to the amount of TI activity present in the samples.

Emanating from an interest in monitoring the effects of protein modification and conformational changes on digestibility of proteins (Shetty and Kinsella, 1982, 1982a) and a need to assess the sensitivity of the in vitro proteolysis method to modifications of quaternary and tertiary structure of proteins, we investigated the experimental and practical aspects of the pH-stat method in more detail. The effects of protein concentration, substrate–enzyme ratio, pH, ionic strength, and interfering food components (e.g., TI and fatty acids), and protein modification on

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digestibility were examined.

MATERIALS AND METHODS

The 11S and 7S soy protein, free of trypsin inhibitor, were isolated from defatted, minimally heat treated soy flour (Central Soya, Fort Wayne, IN) according to the method of Thanh and Shibasaki (1976). Crystallized and lyophilized bovine serum albumin (BSA, fatty acid free, fraction V), casein (sodium caseinate, low protease contamination), pancreatin (porcine pancreatin, grade III), trypsin from bovine pancreas, porcine pepsin type III, and soy trypsin inhibitor type I-S were purchased from Sigma Chemical Co., St. Louis, MO. All the chemicals used in the experiments were of analytical grade from Sigma Chemical Co., St. Louis, MO, Eastman Kodak Co., Rochester, NY, or Fisher Scientific Co., Rochester, NY.

Apparatus. In vitro digestibility of protein samples was determined with a radiometer pH stat (London Comp., Cleveland, OH) which consisted of a titration assembly (type TTA 60), a pH meter (PHM 84), a titrator (TTT 60), a 250-μL autoburette (ABU 12), a recorder (Rec 61 Servograph), a glass electrode (G 2020B), and a calomel electrode (K 4020C). A specially designed microprocessor interface connected the autoburette electronically to the recorder and to a computer for recording, evaluating, and storing data. The enzymatic reactions were carried out in a thermostatted cell equipped with a magnetic stirrer. A flow of water vapor saturated nitrogen excluded carbon dioxide from the reaction vessel thereby preventing pH drop due to carbon dioxide dissolution. All solutions were prepared from freshly distilled CO_2-free water. Titrations with freshly prepared 0.02 N NaOH standardized immediately before use were performed at a constant pH 8.00 and constant temperature of 37 °C. The proportional band setting was 0.05 pH units, while the speed setting was 20 on the pH stat.

Preparation of Protein and Enzyme Solutions. The protein solutions used were either fresh or deep-frozen samples thawed immediately before use. To eliminate small errors in preparing protein solutions daily we usually made up large volumes of protein and enzyme solutions and stored aliquots in deep frozen state. Aliquots (5 mL) of protein solutions were quick-frozen in liquid nitrogen and stored at −70 °C. Before use, they were quickly thawed in a water bath at 50 °C. The thawing process was stopped shortly before all the ice had been melted. The samples were then degassed and used. The digestibility of fresh and deep-frozen samples were compared periodically to make sure that freezing did not alter protein digestibility. Enzyme solutions were prepared in a similar manner. Aliquots (0.2 mL) of enzyme solution were adjusted to pH 8 and frozen and stored at −70 °C. The thawing procedure was the same as used for the protein solutions. This procedure had no effect on enzyme activity and insured that enzyme solutions of constant activity were available for a large number of experiments. Protein was determined by the micro-Kjeldahl method (AOAC, 1970).

In Vitro Digestibility. In a standard run, the aqueous protein solution (5 mL) was pipetted into the thermostatted reaction vessel maintained at 37 °C. The vessel was inserted into the titration assembly, flushed with a stream of nitrogen, and continuously stirred. The pH of the solution was adjusted to approximately 7.9 by injecting 1 N NaOH with a microsyringe and then the pH was automatically adjusted to 8.00. Once the system was equilibrated, 100 μL of pancreatin (1.17 mg/mL) or trypsin (1 mg/mL) was injected and hydrolysis was continuously monitored.

Reduction of Disulfide Bonds. In order to determine the effect of modification and concomitant conformational changes on in vitro digestibility, proteins were modified by disulfide bond reduction. Lyophilized bovine serum albumin (50 mg) was dissolved in 25 mL of 0.03 M tris-HCl buffer containing 1 mM EDTA and 0.02% NaN_3. Urea (7.5 g) in 25 mL of tris buffer was added and then dithiothreitol (3.85 mg) dissolved in 1 mL water was pipetted into the solution. The mixture was shaken for 6 h at 37 °C and dialyzed against water (pH 8, 4 °C) for 48 h to remove all reagents, and the reduced BSA was then lyophilized. The same reaction was also repeated in the presence of urea without DTT and with DTT only. To eliminate the possibility of reoxidation of reduced BSA, the free sulfhydryl groups were reversibly blocked with sodium tetrathionate (Work and Work, 1975). Reduced BSA (50 mg) was dissolved in 50 mL of 0.02 M tris-HCl buffer, pH 7, containing 1 mM EDTA and 0.02% NaN_3. Sodium tetrathionate (1 mL of a 0.23 mM (70 mg/mL) solution) was pipetted into the solution at 25 °C. The reaction was stopped after 1 h and the mixture was dialyzed against distilled water to remove residual reagents and then lyophilyzed.

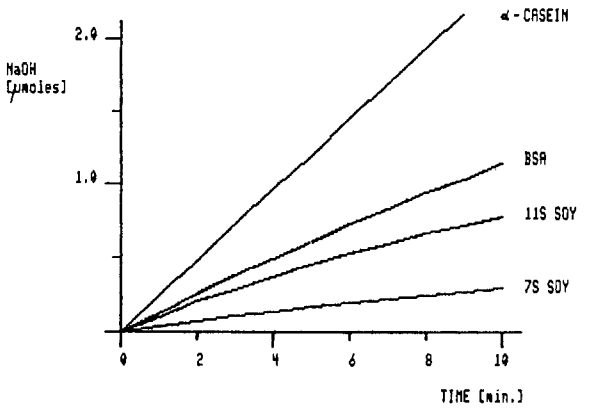

Figure 1. Time course showing relative digestibility of different proteins by pancreatin with the pH stat. Conditions: sample 5 mL; substrate concentration 3 mg/mL; protein–enzyme ratio 1:128; pH 8.0; temperature 37 °C.

In order to examine the effects of reoxidation of the disulfide modified BSA on digestibility, the blocking groups were removed. The *S*-sulfenyl sulfonate derivative of BSA (50 mg) was dissolved in 50 mL of 0.03 M tris-HCl buffer, pH 7.6, containing 1 mM EDTA and 0.02% NaN_3 (Work and Work, 1975). The mixture was warmed to 37 °C and 350 μL of 2-mercaptoethanol (0.5 mmol) was added. After 15 min, the reaction was stopped and the reactants were dialyzed against water and lyophilyzed.

The concentration of the sulfhydryl groups was determined by a modified Ellman test (1959) (Janatova et al., 1968). The sample, having a sulfhydryl group concentration between 25 and 100 mM, was dissolved in a mixture of 0.8 mL of tris-HCl buffer, pH 8, and 0.2 mL of a 1% sodium dodecylsulfate solution in tris-HCl buffer. Then 5,5′-dithiobis(2-nitrobenzoic acid) (20 L of 10 mM (4 g/mL)) solution in methanol was added. After 5 min, the absorbance at 412 nm was read against the reference (cysteine).

RESULTS AND DISCUSSION

Digestibility curves for four proteins at a substrate concentration of 3 mg/mL, an enzyme concentration of 0.12 mg/mL, and 10 mM NaCl are shown in Figure 1. The rates of proteolysis appeared to reflect the complexity and compactness of structure of the respective proteins. Thus, casein with little tertiary and limited secondary structure was most digestible, whereas soy glycinin (11S),

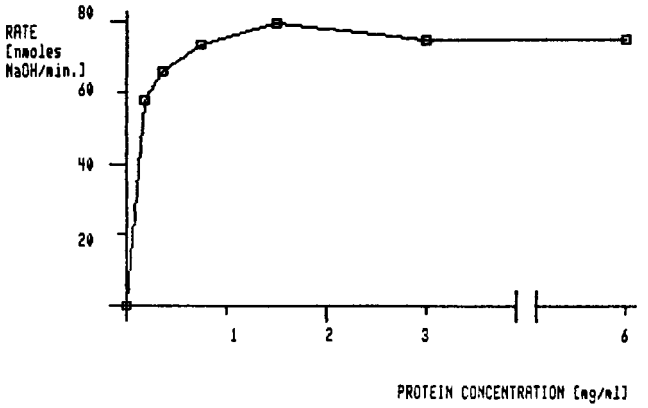

Figure 2. The effect of substrate concentration on initial digestibility rate of 11S soy protein with pancreatin. (Conditions as in Figure 1.)

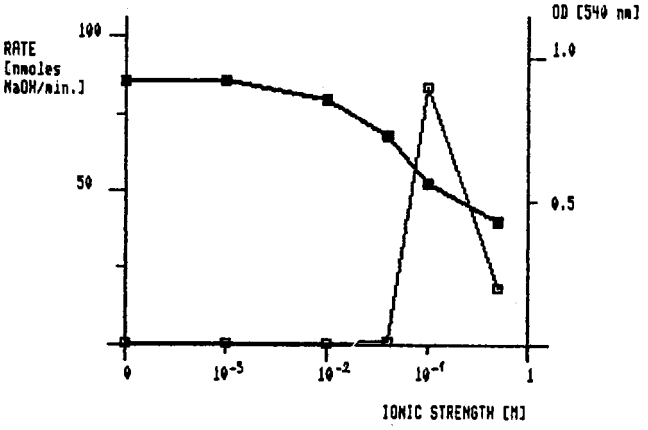

Figure 3. The effects of increasing ionic strength on initial digestability rate (■) and protein solubility (□) of 11S soy protein.

an oligomeric protein made of compact extensively disulfide-linked polypeptide subunits (Smith and Circle, 1978; Kinsella et al., 1985), was quite resistant to pancreatin hydrolysis. Linear regression coefficients (r) for all the samples shown were better than 0.96 when the initial digestibility rates were calculated from the linear (first 10 min) part of the reaction curves by using linear regression analyses. The reproducibility of triplicate experiments was within 2%. In subsequent experiments, some of the factors which could affect the validity of in vitro digestibility of food proteins using the pH stat were studied.

When the initial rates of proteolysis were plotted against the concentration of 11S soy protein (between 0 and 6 mg/mL), it was observed that at higher concentrations, the initial rate was practically independent of the substrate concentration (Figure 2). Thus, in subsequent studies, a substrate protein concentration of 3 mg/mL was used in most experiments.

A linear relationship between enzyme concentration and initial rate of proteolysis was obtained with BSA and 11S soy protein with correlations of 0.98 and 0.99 being obtained for pancreatin and trypsin, respectively. In subsequent studies, concentrations of 24 and 20 μg/mL of pancreatin and trypsin corresponding to enzyme–substrate ratios of 1:128 and 1:100, respectively, were used.

Ionic strength (KCl from 0 to 10^{-2} M) had little effect on the initial rates of digestibility of soy 11S (Figure 3); however, at higher salt concentrations, a significant decrease in hydrolysis occurred. In the case of soy 11S the effects with $CaCl_2$ were more pronounced, i.e., 10 mM $CaCl_2$ decreased digestibility by more than 25%. Significantly, the 11S protein showed lowest solubility above 0.1

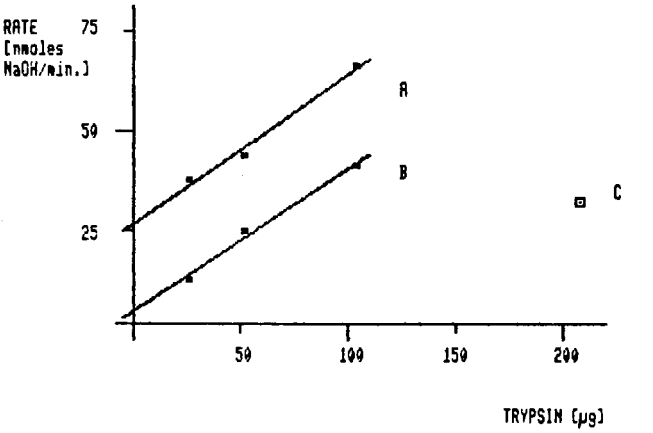

Figure 4. Effects of trypsin inhibitor on initial digestibility rate of 11S soy protein at different trypsin concentrations. (A) Trypsin inhibitor (50 mg) added after enzyme addition. (B) Trypsin inhibitor added to enzyme before addition of soy protein. (C) Sample containing 200 μg of trypsin and 50 mg of trypsin inhibitor, i.e., TI–trypsin 1:5.

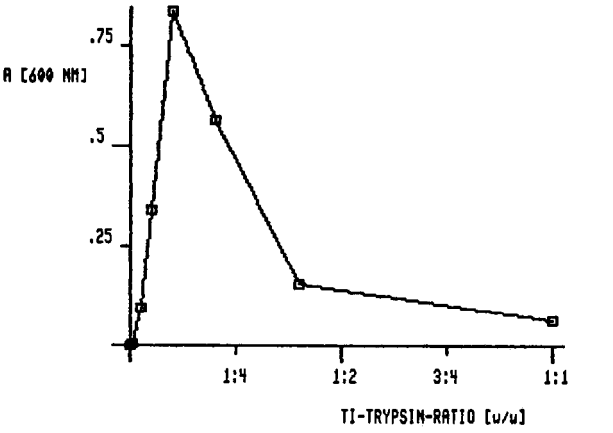

Figure 5. Optical density (A) showing solubility of trypsin–trypsin inhibitor mixtures at different concentration ratios. Trypsin concentration 2 mg/mL; wavelength 600 nm.

M ionic strength. In many studies, an ionic strength of 0.1 M is usually recommended to ensure constant ionic strength (Milhalyi, 1978; Richardson and Catsimpoolas, 1979; Stinson and Snyder, 1980). However, for certain proteins, this may result in protein precipitation, e.g., the 11S proteins of legumes (Figure 4), and thereby invalidate experimental data. Decreased digestibility rates with increasing ionic strength were also reported by Kamata and Shibasaki (1979) and may be attributed to impaired solubility, more compact conformation, or decreased interaction between enzyme and substrate.

The pH-stat method may provide a sensitive method for detecting trypsin inhibitor (TI) activity in food especially legume proteins; hence, we assessed the effects of TI on proteolysis. When TI (50 mg) was added to the system containing soy 11S immediately after enzyme addition, a positive intercept reflecting hydrolysis that occurred prior to addition of the TI was obtained (Figure 4 part A). When TI was mixed with the trypsin prior to addition to the protein solution, the intercept was almost eliminated (Figure 4 part B). However, as the concentration of trypsin was subsequently increased, proteolysis increase linearly in both cases.

Significantly, the addition of TI to the trypsin solution resulted in partial precipitation as observed by the development of turbidity (Figure 5) with a maximum in the turbidity (i.e., absorbance of 0.85) occurring at a TI–trypsin ratio of 1:5 w/w. This observation may be of practical

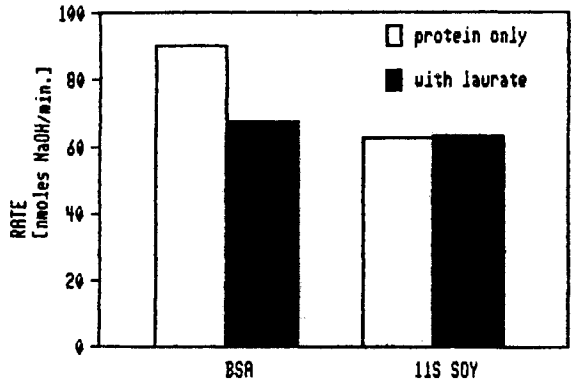

Figure 6. The effects of sodium laurate on the initial digestibility rate of BSA and 11S soy protein with pancreatin with the pH stat. Molar ratio of protein to sodium laurate: (a) BSA 1:2; (b) 11S soy 1:12. (Conditions as in Figure 1.)

interest; thus, in the presence of TI, the increase in proteolysis rates may not always be proportional to increasing amounts of trypsin, being dependent upon the actual TI–trypsin ratio. When the amount of trypsin was increased from 25 to 50 μg, and the corresponding TI–trypsin ratios were 2:1 and 1:1, no precipitation was observed, and the hydrolysis rate was doubled (Figure 4 part B). When the amount of trypsin was doubled again, the hydrolysis rate did not increase proportionally, and at a trypsin content of 200 μg, it actually decreased (Figure 4 part C). This corresponded to a TI–trypsin ratio of 1:5, at which the highest turbidity was also observed (Figure 5). Thus depending on the actual trypsin–TI ratio, a linear regression of the rate of proteolysis vs. concentration may lead to an erroneous intercept with respect to TI content in sample being studied. Experiments with TI-free 11S soy protein samples showed a more linear relationship in the rate–time diagram (Figure 1) than those of samples containing TI, which showed nonlinear curves. Samples with a very low or very high TI–trypsin ratio showed the best linearity (Rothenbuhler and Kinsella, 1985). Stinson and Snyder (1980) and Hill et al. (1982) suggested using the pH stat for determining residual TI activity in soy protein meals. However, because of the anomalous behavior depending on the TI–enzyme ratio, this may not be totally reliable unless the investigator has a prior estimate of the TI content of the sample and adjusts the trypsin concentration accordingly.

Because of concerns about effects of other incidental contaminants on protein digestibility, we examined the effects of fatty acid on in vitro digestibility. Lauric acid caused a 33% decrease in initial rate of proteolysis of BSA whereas even at high concentration (12 mol:1 mol of protein) it exerted little effect on the pancreatin digestion of soy 11S (Figure 6). This may reflect the high affinity of BSA for lauric acid (K_a 10^{-8}, Hansen, 1981) and the knowledge that binding of low amounts of aliphatic compounds stabilizes the BSA structure (Damodaran and Kinsella, 1980). Fatty acid induced conformational change may account for the reduced digestibility of BSA. Soy 11S appears to have limited affinity for binding of aliphatic ligands (Damodaran and Kinsella, 1981) and its digestibility was unaffected by the added lauric acid indicating little interaction.

The effects of chemical, enzymatic, and physical modification on the susceptibility of proteins to proteolysis was assessed by the pH-stat method. Bovine serum albumin is a compact globular protein the tertiary structure of which is maintained by 17 disulfide (S–S) bonds. Reduction of six S–S bonds actually reduced initial rate of

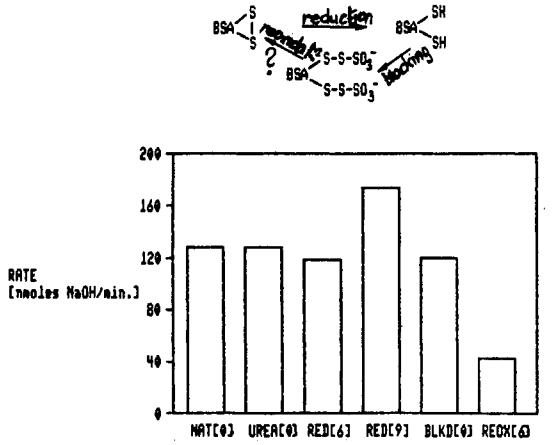

Figure 7. The initial digestibility rate by pancreatin of chemically modified bovine serum albumin (BSA) with the pH stat. Nat, BSA native conformation; urea, BSA modified with 3 M urea; Red(6), BSA modified with 10 mM DTT (6 disulfide bonds reduced); Red(9), modified with 3 M urea and 10 mM DTT (9 disulfide bonds reduced; Blkd, modified with 3 M urea and 10 mM DTT, blocked with tetrathionate; Reox, BSA tetrathionate modified, deblocked with β-mercaptoethanol and then reoxidized in air.

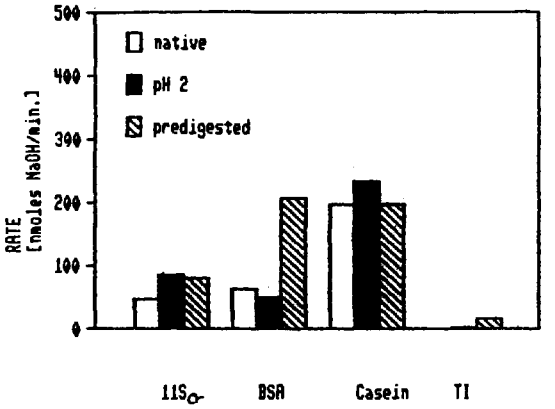

Figure 8. Comparison of initial digestibility rates of native, acid modified (HCl, pH 2, 30 min), and pepsin (1:100 pepsin–protein, 30 min) modified proteins using pancreatin.

digestibility but when nine S–S bonds were reduced, the rate increased by 35% (Figure 7). When the nine free thiol groups were blocked with tetrathionate, proteolysis was reduced to below the rate for the native molecule. Following reductive deblocking, the initial rate of proteolysis was significantly impeded. This may reflect some intermolecular polymerization of the BSA via disulfide linkages or hydrophobic interactions following deblocking. Prior treatment with urea (3 M), a reagent required in all the reduction experiments, had a negligible effect on initial hydrolysis rates reflecting its lack of effect on BSA structure or reversal of its effects during subsequent dialysis. This is in contrast to 11S soy protein where urea both reduced and enhanced digestibility presumably by causing dissociation of the dimeric subunits and release of acidic and basic subunits, respectively (Rothenbuhler and Kinsella, 1985).

The effect of predigestion with a pepsin–protein ratio of 1:100 or exposure to acid (HCl, pH 2) for 30 min on subsequent proteolysis by pancreatin following adjustment of pH was examined (Figure 8). With the exception of casein, pepsin pretreatment increased subsequent pancreatin proteolysis of all the proteins particularly BSA (3-fold) and trypsin inhibitor. Acid treatment greatly enhanced the digestibility of soy 11S and of casein to a lesser degree. The increased hydrolysis of 11S may reflect

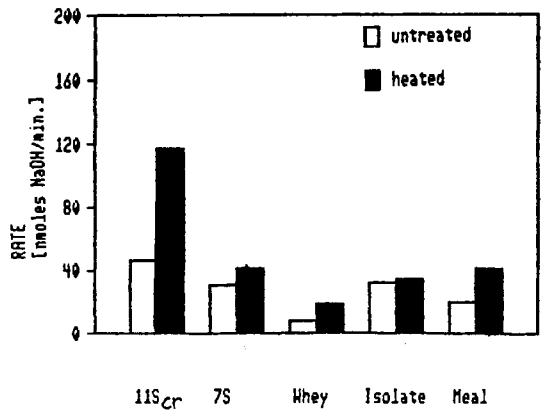

Figure 9. Initial digestibility rates of heat treated (80 °C, 20 min) and untreated soy protein fractions by pancreatin with the pH stat.

dissociation of the oligomeric structure following acid treatment.

The pH-stat method may be useful in examining the effects of heat treatments on proteins and thus in monitoring thermal processing of soy and other proteins. The crude 11S globulins showed the most significant response to heat treatment, i.e., heating (80 °C for 20 min) doubled the initial rate of proteolysis (Figure 9). The soy isolate sample showed the least change in digestibility rates conceivably because of prior heat treatments. The increase in the digestibility rate of the soy meal sample was approximately comparable to that observed for the respective mixture of 11S, 7S, and whey. The crude 11S soy faction exhibited some residual TI activity, i.e., digestibility rate was only about 45 nmol/min compared to 70 nmol/min observed for the pure 11S protein (Figure 1). The increase from 45 nmol/min to approximately 115 nmol/min following heat treatment is, therefore, due to TI destruction and to protein denaturation, which accounted for about 60% of the change. This is consistent with the observation of Boonvisut and Whitaker (1976) for soy proteins but contrary to the report of Richardson and Catsimpoolas (1979) that heating glycinin (87 °C for 30 min) decreased the initial rate of proteolysis.

These results demonstrate the sensitivity of the pH-stat method in monitoring changes in protein digestibility resulting from chemical, physical, or enzymatic modification. Because the experimental conditions simulate the situation in vivo, there are less restrictions than with other methods (Mauron, 1973; Hsu et al., 1977; Evans and Witty, 1978). Reliable results are obtained when important parameters e.g., substrate concentration, enzyme–substrate ratio, and ionic strength, are controlled. Working at a protein concentration of 3 mg/mL and an enzyme–substrate ratio in ranges of 1:100–1:130 ensures linearity in the digestibility rate. Studies using enzyme–substrate ratios of 1–10 (Maga et al., 1973; Hsu et al., 1977) are working in the steep part of the rate–concentration diagram (Figure 3) where small errors in the substrate protein concentration can cause considerable deviations in the initial rates. Stinson and Snyder (1980) observed that trypsin hydrolysis rates with defatted soy meal decreased by more than 50% by increasing protein concentration from approximately 5–6.6 mg/mL. They attributed this unusual decrease to increasing trypsin inhibitor concentration and increased the buffering capacity. Hill et al. (1982) conducted a similar experiment but could not reproduce this observation. Such an effect was not detected during our experiments with pure 11S protein.

Autolysis of the enzyme in solution and subsequent decrease in pH can be a problem in the pH-stat method. Methods have been proposed to avoid this problem; e.g., Stinson and Snyder (1980) maintained enzyme solutions at a pH 5.5. However, this caused an initial jump in the time–rate diagram. Other authors have added calcium chloride to stabilize the enzyme (Milhalyi, 1978); however, as shown with 11S protein, calcium salt may cause precipitation and apparent decreased digestibility. The use of deep-frozen enzyme preparations circumvented these problems and greatly facilitated consistent experimentation. When conditions are standardized, i.e., conducting assays under nitrogen to eliminate CO_2 effects, preparing fresh alkali solutions daily, having the correct enzyme–substrate ratio, ionic strength for the protein system being studied, and good temperature control, the pH-stat method is reliable as shown by Friedman et al. (1981) for casein and is sensitive to changes in quaternary and tertiary structure of soy proteins (Rothenbuhler and Kinsella, 1985) and to the effects of ligand binding on protein digestibility in vitro (Barbeau and Kinsella, 1985).

Registry No. TI, 9035-81-8; pancreatin, 8049-47-6; trypsin, 9002-07-7; sodium chloride, 7647-14-5.

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Volatile Components of the Thermal Degradation of Cystine in Water

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Thermal degradation of cystine in aqueous solutions was studied by using a closed model system. The degradations were carried out at pH's of 5.5 and 2.3 and at 160 °C, representing roasting temperature. Forty-two volatile compounds were identified at pH 5.5 and twenty-three at pH 2.3. Three novel sulfides were found in the pH 5.5 volatile mixture: ethyl 1-(ethylthio)ethyl disulfide and the corresponding tri- and tetrasulfides. Only the disulfide was found at pH 2.3. The organoleptic character of the disulfide was sulfury, roasted, and oniony. A mechanism for the formation of these sulfides is proposed. Fifty-five percent of the volatile yield at pH 2.3 were 1,2,4-trithiolanes. Mechanisms for the formation of secondary products are discussed.

INTRODUCTION

Sulfur-containing amino acids are generally recognized as very important precursors of food flavors (Hurrell, 1982; Ching, 1979). Thermal degradation of amino acids produces the corresponding amines via degradation (Lien and Nawar, 1974; de Rijke et al., 1981). Sulfur-containing amino acids also form additional breakdown products which are highly reactive and can generate various types of heterocyclic compounds (Fujimaki et al., 1969; Boelens et al., 1974; Sakaguchi and Shibamoto, 1978).

Obata and Tanaka (1965) studied the photolysis of cystine and cysteine and observed that hydrogen sulfide, ammonia, carbon dioxide, and acetaldehyde were evolved. Fujimaki et al. (1969) pyrolyzed cystine and cysteine separately at 270–300 °C at reduced pressure under nitrogen. They identified several highly volatile compounds including ethylamine, mercaptoethylamine, hydrogen sulfide, ammonia, acetaldehyde, and 2-methylthiazolidine by using GC and classic derivatization methods. They also proposed the mechanism of the formations of those volatiles. Later, Kato et al. (1973) in a similar pyrolysis study identified additional compounds including thiazoles, pyridines, and several thiophenes. Ledl (1976) heated cystine/cysteine in soybean oil at 200 °C and formed various heterocycles including 1,2-dithiane, 3,5-dimethyl-1,2,4-trithiolane, and 3-methyl-1,2,4-trithiane.

Boelens et al. (1975) studied the degradation of cystine/cysteine in terms of the primary products and secondary products. They postulated a mechanism for the interaction between acetaldehyde and hydrogen sulfide which formed 2,4,6-trimethyl-1,3,5-trithiane, 2,4,6-trimethyl-1,3,5-dithiazine, 3,5-dimethyl-1,2,4-trithiolane, and various sulfides.

The volatile products of thermal degradation at 160 °C of aqueous cystine at pH 5.5 and pH 2.3 in a closed system were identified in this study. Mechanisms for the formation of the novel compounds generated are also presented.

EXPERIMENTAL SECTION

Sample Preparation by Parr Bomb, A Closed Model System. A mixture of 0.05 mol L-cystine (Ajinomoto Co., Tokyo, Japan) and 500 g of distilled water was placed in a 2-L Parr Bomb (Parr Instruments Co., Moline, IL) equipped with a magnetic stirrer, an internal cooling coil, and a temperature controller. The pH of the mixture was measured as 5.5. The reaction mixture was heated to 160 °C for $^1/_2$ h and then allowed to cool to room temperature. Another sample was prepared in a similar fashion, however, the initial pH was adjusted to 2.3 with 1% HCl.

Isolation of the Volatiles. The reaction mass (200 g) was diluted with 200 mL of distilled water and steam distilled under a vacuum (10–12 mmHg) at 15–18 °C for 1 h and 45 min. Approximately 500–550 mL of distillate was obtained. The total distillate was saturated with sodium chloride and extracted, in a 1-L separatory funnel, with 100 mL of methylene chloride three times. The combined extracts were washed twice with 50% NaCl saturated water, dried over anhydrous magnesium sulfate, and then filtered through Whatman no. 1 filter paper. After filtration, the extract was concentrated to about 5 mL with a Kurdena-Danish apparatus fitted with a Vi-

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