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# Effects of Nonprotein Substances on Protein Hydrolysis and Plastein Formation

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*ABSTRACT*

*Effects of various naturally occurring nonprotein substances (carbohydrates, polysaccharides, fats and salts) on enzymatic hydrolysis of soy protein isolate and plastein formation from hydrolyzed soy protein were investigated. Relative extent of hydrolysis and plastein formation were measured as protein solubility in 10% trichloroacetic acid (TCA) since this method was found suitable for analysis of turbid, viscous and/or low protein samples. The presence of guar, xanthan, locust bean and arabic gums, arabinogalactan, unsaturated fatty acids (2%), salt mixture and xylan were found to enhance soy protein peptic hydrolysis at 0·5% enzyme/substrate; unsaturated fatty acids (1%) inhibited hydrolysis. At enzyme/substrate of 3·5%, hydrolysis was enhanced by xanthan gum, unsaturated fatty acids and sodium chloride but inhibited by gum karaya, salt mixture, starch, cellulose, and saturated fatty acids. Plastein synthesis was inhibited by xanthan, locust bean and guar gums but stimulated by arabinogalactan. Several nonprotein substances were found to interfere with the TCA solubility assay. Positive interference was noted for systems containing saturated and unsaturated fatty acids and magnesium, but negative interference was observed for systems containing guar gum, xanthan gum, calcium chloride and gum arabic.*

## INTRODUCTION

Enzymatic modification of food proteins by proteolytic breakdown and plastein formation have been shown to be suitable methods for improving

the functional and nutritional properties of food proteins. Enzymatic proteolysis, for example, can be used to substantially increase the solubility of food proteins over a wide pH range (Nair *et al.*, 1976), decrease the tendency for soy proteins to gel (de Rham *et al.*, 1978), increase emulsification properties of soy proteins (Puski, 1975), improve protein whipping characteristics (Gunther, 1979), increase the nutritional value of soy protein (de Rham *et al.*, 1978) and to produce flavor-potentiating peptides from fish protein (Noguchi *et al.*, 1975). The plastein reaction has likewise been found potentially useful for the improvement of functional and nutritional properties of food proteins and food protein hydrolysates (Fujimaki *et al.*, 1970; Watanabe *et al.*, 1981; Yamashita *et al.*, 1971).

Presently, limited information is available on the effects of nonprotein substances on the enzymatic modification of proteins and, in general, on the effects of nonprotein substances on the chemistry and state of proteins. Some results have been reported on the inhibitory effects of sugars on protein hydrolysis as a result of the Maillard reaction (Lowry & Thiessen, 1950), as well as on the inhibition of protein hydrolysis in heat-treated soy flour due to the presence of starch (Boonvisut & Whitaker, 1976). Others have also found soy flour to be considerably more resistant to enzymatic hydrolysis than soy isolate (Fukushima, 1959).

This investigation was intended as a preliminary study of the effects of a wide range of naturally occurring nonprotein substances on the relative extent of peptic hydrolysis and plastein formation through measurement of changes in 10% trichloroacetic acid (TCA) solubility of substrate protein upon enzymatic modification. Several of the nonprotein substances used in these experiments were selected on the basis of their chemical or functional similarity to naturally occurring nonprotein substances reportedly found in soybeans and their products (Whistler & Saarnio, 1957; Aspinall *et al.*, 1967; Yasumatsu *et al.*, 1972); other substances are commonly used ingredients in food systems.

For this study, the TCA solubility test was selected as the method for determining relative extent of proteolysis, since the assay shows high precision, and also because TCA solubilities were previously found to correlate strongly with degree of hydrolysis (measured as the change in free amino groups by reaction with 2,4,6-trinitrobenzene sulfonate) of pepsin- and papain-modified soy proteins (Hagan *et al.*, 1985). Furthermore, the TCA solubility test appears suitable for analysis of samples containing high levels of nonprotein substances which may interfere, due to turbidity, with colorimetric assays such as the TNBS and ninhydrin methods. The 10% TCA solubility test has also been used previously in studies for determining plastein yields (Arai *et al.*, 1975).

## MATERIALS AND METHODS

### Enzymes

Pepsin (Sigma Chemical Co., No. P-7000) was used in preparing all protein hydrolysates, including the substrate for the plastein reaction. Papain (Sigma Chemical Co., No. P-4762) was used as the catalyst for the plastein reaction.

### Experimental nonprotein substances

All nonprotein substances tested were of either food grade or reagent grade. Most of the nonprotein materials, which were investigated to determine their influence on the degree of protein hydrolysis or plastein formation, were studied individually; however, several tests were conducted using combinations of two or more nonprotein substances. Included among these combinations were a simple sugar treatment consisting of a mixture of sucrose and glucose (2:1 ratio, w/w), an unsaturated fatty acid treatment consisting of linoleic, oleic, and linolenic acids (3:2:1 ratio, w/w/w), a saturated fatty acid treatment consisting of stearic and palmitic acids (2:1 ratio, w/w) and a total salts mixture composed of equimolar amounts of the chloride salts of sodium, calcium, magnesium and potassium. Other nonprotein materials tested were soluble potato starch and microcrystalline cellulose. All carbohydrates and lipids were added to reaction mixtures on a weight basis but salts were added on a molar basis.

### Substrates

A freeze-dried soy isolate of 92% protein (N × 6·25) was prepared from a defatted soy flour (A. E. Staley Co., I-200) by a standard alkaline solubilization/isoelectric precipitation method. All protein hydrolysates were produced from this soy isolate substrate. All plasteins were synthesized from a pH-adjusted, freeze-dried soy protein peptic hydrolysate assayed at 65% protein (N × 6·25) and 88·7% TCA solubility. Nonenzyme receiving 'assay controls' contained a soy protein peptic hydrolysate assayed at 65% protein and 85·6% TCA solubility. These soy protein hydrolysates were produced from reaction mixtures containing soy isolate at a substrate concentration of 1% (w/w) and pepsin at an enzyme/substrate ratio of 4·0/100 and 3·5/100 (w/w) to produce the 88·7 and 85·6% TCA soluble products, respectively. Reaction mixtures were incubated at pH 1·6 for 24 h and 37°C with constant mixing. Following

incubation, the hydrolysate mixtures were adjusted to pH 6·0 and heated to 70°C for 15 min to inactivate the enzyme prior to freeze-drying.

**Hydrolysates, plasteins and controls**

Experimental hydrolysates were produced from a soy protein isolate substrate (1% substrate concentration, protein basis, w/v) using pepsin at enzyme/substrate ratios of 0·5:100 and 3·5:100 (w/w). Thus, two hydrolysates were produced for each nonprotein treatment tested. Experimental nonprotein substances were blended into respective hydrolysate mixtures at concentrations of either 2%, or 2 and 4% (w/w), except for salt treatments which were added at cation concentrations of 0·3M. Blending was performed at very low speed with the use of a Waring blender equipped with a semi-micro jar. In addition, soy flour was tested by addition to reaction mixtures at a 1% substrate concentration (protein basis), but no soy isolate was added. A control hydrolysate mixture, designated as 'experimental control', was also prepared at each of the two enzyme concentrations. These controls were prepared similarly to the experimental mixtures except that they did not contain any experimental nonprotein substance. All incubation mixtures were preincubated at 37°C for 2 h to allow escape of air bubbles. All enzyme-containing reaction mixtures were then adjusted to pH 1·6 and incubated at 37°C for 24 h with constant mixing.

A complete set of 'assay controls' (one per nonprotein treatment) was incubated and assayed so as to ascertain whether or not the nonprotein treatments would interfere with the proper determination of the relative extent of hydrolysis by the TCA solubility assay. These controls were formulated using soy protein peptic hydrolysate (88·7% TCA sol.) in place of soy isolate with addition of each nonprotein substance or substances at the same concentrations used for experimental mixtures. Assay controls were also incubated in the same manner as experimental mixtures except that the controls did not receive the enzyme. Following incubation, all experimental hydrolysates, assay controls, and the soy isolate control were adjusted to pH 7·0 to inactivate the enzyme and stored at 2°C until assayed.

Plasteins were synthesized from a soy peptic hydrolysate at a substrate concentration of 30% (w/w, protein basis) using papain at an enzyme/substrate ratio of 1:100 (w/w). Each reaction mixture contained a nonprotein treatment at a 15% concentration (w/w), based on the total weight of the reaction mixture. A set of assay controls (prepared for determination of nonprotein interference with the TCA solubility test) was incubated in the presence of each nonprotein treatment while containing an 85·6% TCA-soluble soy protein peptic hydrolysate as the 'substrate' and

without any added enzyme. An experimental control without a nonprotein treatment was also prepared.

All experimental and control reaction mixtures were incubated in the presence of 10 mM L-cysteine (papain activator) at pH 6·0 and 37°C for 24 h without agitation. Following incubation, all plasteins and controls were quick-frozen in liquid nitrogen and stored at −40°C until assayed.

**Sample analysis**

Hydrolytic and synthetic activities were determined by a 10% trichloroacetic acid solubility test (TCA test) which was performed as follows.

Plastein reaction mixtures were thawed and suspended in distilled water (30 ml $g^{-1}$ plastein). Hydrolysate and suspended plastein incubation mixtures were homogenized in a Waring blender at low speed for 10 sec followed by adjustment of pH to 7·0. Aliquots (25 ml) from hydrolysate or plastein homogenates were combined with equal volumes of 20% TCA, mixed vigorously for 30 sec and allowed to stand 1 h at room temperature prior to centrifugation (2500 *g* for 15 min). Nitrogen content was determined for both the precipitate (10% TCA insoluble fraction) and the supernatant (10% TCA soluble fraction). The % TCA solubility was calculated by the equation:

$$\% \text{ TCA solubility} = \frac{10\% \text{ TCA soluble N}}{\text{Total N}} \times 100\%$$

All determinations were performed in duplicate.

Plastein yields were expressed in terms of percentage decrease in 10% TCA solubility as a result of the reaction as calculated by the equation:

$$\% \text{ plastein yield} = \% \text{ TCA solubility hydrolyzed substrate prior to incubation} - \% \text{ TCA solubility of product after incubation}$$

All determinations were performed in duplicate.

Nitrogen was determined by macro-Kjeldahl (AACC, 1969).

**Viscosities**

Relative viscosities were measured for hydrolysate reaction mixtures just prior to incubation. Measurements were performed at 37°C and shear stress readings were recorded at a shear rate of 539 $sec^{-1}$ using a Haake Rotovisco RV3 equipped with an NV rotor head.

**Data analysis—hydrolysates**

Since nonprotein substances may directly affect the enzymatic breakdown of protein as well as interfere with the TCA solubility assay, it was

**TABLE 1**
Hydrolysates[a]

| *Nonprotein substances* | *TCA sol. (%) experimental 0·5% E/S*[b] | *TCA sol. (Δ) absolute 0·5% E/S*[c] | *TCA sol. (%) experimental 3·5% E/S*[b] | *TCA sol. (Δ) absolute 3·5% E/S*[c] | *TCA sol. (Δ) control TCA test (%)*[d] | *Relative viscosity (Cp)* |
|---|---|---|---|---|---|---|
| Control | 71·9 | — | 88·6 | | — | 27·6 |
| Guar gum | 81·1$^{pp}$ | 13·6 | 83·4 | 0·8 | −4·4$^{nn}$ | 409·5 |
| Xylan (1%) | 75·3$^{p}$ | 5·3 | 87·5 | 0·8 | −1·9 | 42·5 |
| Xylan (2%) | 72·9 | 3·7 | 87·3 | 1·4 | −2·7 | 66·9 |
| Xanthan gum (1%) | 86·8$^{pp}$ | 20·5 | 95·2$^{pp}$ | 12·2 | −5·6$^{nn}$ | 346·5 |
| Xanthan gum (2%) | 81·9$^{pp}$ | 17·4 | 91·8$^{p}$ | 10·6 | −7·4$^{nn}$ | 716·6 |
| Gum karaya | 73·7 | 3·9 | 82·1$^{nn}$ | 4·4 | −2·1 | 89·8 |
| Locust bean gum | 79·8$^{pp}$ | 11·8 | 85·1 | 0·4 | −3·9$^{n}$ | 444·9 |
| Gum arabic | 81·4$^{pp}$ | 10·3 | 89·6 | 0·2 | +0·8 | 28·4 |
| Arabinogalactan | 76·2$^{pp}$ | 4·9 | 90·2 | 1·6 | −0·6 | 25·2 |
| Sugar (1%) | 70·7 | 0·2 | 91·2 | 3·6 | −1·0 | 29·1 |
| Sugar (2%) | 71·9 | 1·8 | 89·9 | 3·1 | −1·8 | 30·7 |
| Raffinose | 71·6 | 0·1 | 86·9 | 1·5 | −0·2 | 32·3 |
| Starch (1%) | 70·3 | 1·6 | 86·6 | 2·0 | 0 | 27·6 |
| Starch (2%) | 71·4 | 0·1 | 85·1$^{n}$ | 3·1 | −0·4 | 36·2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Cellulose | 71·1 | 0·7 | 84·6$^{n}$ | 3·9 | −0·1 | 41·0 |
| C-M cellulose | 73·9 | 3·3 | 87·1 | 0·2 | −1·3 | 37·8 |
| Saturated fatty acids (1%) | 73·4 | 1·3 | 87·4 | 4·4 | +2·8 | 28·1 |
| Saturated fatty acids (2%) | 71·6 | 4·8 | 84·6$^{n}$ | 8·5 | +4·5$^{pp}$ | 29·6 |
| Unsaturated fatty acids (1%) | 63·6$^{nn}$ | 12·9 | 97·8$^{pp}$ | 4·6 | +4·6$^{pp}$ | 26·7 |
| Unsaturated fatty acids (2%) | 95·0$^{pp}$ | 12·0 | 97·4 | 2·3 | +11·1$^{pp}$ | 28·4 |
| Potassium | 73·7 | 1·2 | 89·2 | 2·4 | +3·0 | 27·9 |
| Sodium | 75·8 | 2·9 | 94·3$^{pp}$ | 4·6 | +1·1 | 28·1 |
| Magnesium | 76·0 | 1·2 | 88·3 | 3·2 | +2·9$^{p}$ | 26·8 |
| Calcium | 65·3 | 2·3 | 86·9 | 7·2 | −8·9$^{nn}$ | 31·6 |
| Combined salts | 76·7$^{p}$ | 3·1 | 85·2$^{n}$ | 5·1 | +1·7 | 27·8 |
| Soy flour | 65·2 | — | 81·6 | — | — | 28·4 |

[a] Effects of nonprotein substances on relative extent of peptic hydrolysis of soy protein isolate.

[b] Values of percentage TCA sol. for hydrolysates produced at enzyme/substrate ratios of 0·5 and 3·5%. Experimental significance indicated as follows: *p*—one-sided significant ($P < 0·05$) positive effect (nonprotein-induced increases in mean percentage TCA sol. compared with the control); *pp*—two-sided positive effect; *n*—one-sided significant ($P < 0·05$) negative effect (nonprotein-induced decrease in mean percentage TCA sol. compared with the control); *nn*—two-sided negative effect.

[c] Calculated as the absolute value of the difference between Δ TCA solubility experimental and Δ TCA solubility control.

[d] Calculated as the difference in percentage between values of percentage TCA sol. values for nonprotein-containing TCA controls and the nonprotein-free TCA control. Negative values indicate nonprotein-induced decreases in percentage TCA sol. (negative interference), but positive values confer the opposite. Experimental significance indicated as follows: *p*—one-sided significant ($P < 0·05$) positive interference with the TCA test compared with the control; *pp*—two-sided positive interference; *n*—one-sided significant ($P < 0·05$) negative interference with the TCA test compared with the control; *nn*—two-sided negative interference.

necessary to determine whether or not significantly high 'Δ TCA solubility experimental' values were obtained due to assay interference. For this purpose, assay controls were set up for each treatment. Values for Δ TCA solubility control were calculated as the difference between the percentage TCA solubility values for the assay controls and the percentage TCA solubility of the nonprotein-free assay control. Significantly high Δ TCA solubility control values were taken to indicate that positive nonprotein interference with the TCA test occurred in response to the presence of the experimental substance, thereby resulting in a higher-than-actual degree of hydrolysis as determined by the TCA solubility method. Significantly negative values for Δ TCA solubility control indicated negative nonprotein interference with the TCA test; hence lower-than-actual percentage values of TCA solubility were obtained. To determine any effect on the relative extent of enzymatic hydrolysis or synthesis, sample data were analyzed as follows. Differences between each experimental percentage TCA solubility value and the percentage TCA solubility value for the experimental control were calculated as Δ TCA solubility experimental. These values were then compared to respective values of Δ TCA solubility control. The absolute values between respective Δ TCA solubility experimental and Δ TCA solubility control were then calculated for each nonprotein-containing system. These values will be referred to as Δ TCA solubility absolute. If values of Δ TCA solubility absolute were found significantly large, it was inferred that significant corresponding values for percentage TCA solubility for a given system were due to direct nonprotein-induced effects on the actual modification reaction aside from interfering effects on the TCA test. However, if a significant value of percentage TCA solubility was not associated with a corresponding significant value for Δ TCA solubility absolute, then it could not be inferred whether or not that particular nonprotein treatment had acted directly on the modification reaction or whether it interfered with the TCA test. The data analysis scheme also allowed for where a nonprotein treatment could be determined as affecting both the actual degree of hydrolysis as well as interfering with the TCA test; in this case, values for Δ TCA solubility experimental, Δ TCA solubility control and Δ TCA solubility absolute were all significantly large.

**Data analysis–plasteins**

Results on the effects of nonprotein substances on the plastein reaction were analyzed as for hydrolysate data. Plastein yield was calculated as the difference between the percentage TCA solubility of the initial hydrolyzed substrate (88·7%) and the percentage TCA solubility of the plastein reaction product. Values of Δ TCA solubility experimental were calculated

as the difference in percentage between values of percentage TCA solubility for experimental nonprotein-containing plastein reaction products and percentage TCA solubility of the nonprotein-free control. Values of Δ plastein yield absolute were calculated as the absolute difference between Δ TCA solubility experimental and Δ TCA solubility control (TCA test). If values of Δ plastein yield absolute were found to be significantly large, it was inferred that corresponding significant values of Δ TCA solubility experimental were attributed to direct nonprotein-induced effects on the plastein reaction aside from interfering effects on the TCA test.

### Statistical analysis

All TCA solubility determinations were performed in duplicate, and the coefficient of variation for all assay pairs was found to be no greater than 1%. The interassay coefficient of variation for the TCA solubility test was calculated as 0·46% based on data for 8 control samples. The null hypothesis, stating that any difference between control and experimental percentage TCA solubility values could be accounted for by chance, was rejected if mean Δ TCA solubility values exceeded set 95% confidence limits. Confidence limits were calculated assuming a common maximal standard deviation of 1% for each assay pair. This statistical scheme was adopted so as to minimize the probability of committing type I error (rejecting null hypothesis when in fact it is true) in favor of type II error (accepting null hypothesis when in fact it is false). For the sake of comparison, both one-sided and two-sided testing of the null hypothesis were performed with results indicated in Tables 1 and 2.

## RESULTS AND DISCUSSION

### Nonprotein substances and the TCA solubility test

Results on the effects of nonprotein substances on the calculation of percentage hydrolysis by the 10% trichloroacetic acid solubility test (TCA test) are given in Table 1 (hydrolysate TCA controls) and Table 2 (plastein TCA controls). Due to the possibility that nonprotein substances could affect enzymatic modification reactions while also affecting the assay for determination of the extent of modification, it was necessary to set up TCA control samples to ascertain how and to what extent nonprotein substances would interfere with the TCA test. Proper interpretation of TCA test results is important in assessing the actual extent of modification by the TCA solubility method, especially with regard to plastein synthesis which,

**TABLE 2**
Plasteins[a]

| *Nonprotein substances* | *Plastein yield (%)*[b] | *TCA sol. (Δ) experimental (%)*[c] | *TCA sol. (Δ) control TCA test (%)*[d] | *Plastein yield absolute (Δ)*[e] |
|---|---|---|---|---|
| Control | 8·3 | — | — | — |
| Guar gum | 4·1* | +4·2[n] | −0·1 | 4·3 |
| Xylan | 8·1 | +0·2 | −3·2[n] | 3·4 |
| Xanthan gum | −0·2* | +8·5[nn] | +1·7 | 6·8 |
| Gum karaya | 9·7 | −1·2 | −4·6[n] | 3·4 |
| Locust bean gum | 4·0* | +4·3[nn] | +0·3 | 5·3 |
| Gum arabic | 7·6 | +0·7 | −0·7 | 1·4 |
| Arabinogalactan | 13·2 | −4·9[pp] | −1·0 | 5·2 |
| Sugar | 6·4 | +1·9 | −1·8 | 3·7 |
| Raffinose | 6·5 | +1·8 | −2·0 | 3·8 |
| Starch | 8·0 | +0·3 | −1·7 | 2·0 |
| Cellulose | 7·7 | +1·4 | −3·5[n] | 4·9 |
| C-M cellulose | 8·8 | −0·5 | +0·1 | 0·6 |
| Saturated fatty acids | 8·7 | −0·4 | +0·6 | 1·0 |
| Unsaturated fatty acids | 10·7 | −2·4 | 0·0 | 2·4 |
| Potassium | 6·9 | +1·4 | −0·8 | 2·2 |
| Sodium | 6·2 | +2·1 | −1·3 | 3·4 |
| Magnesium | 5·8 | +2·5 | +2·5 | 0 |
| Calcium | 9·2 | −0·9 | −8·4[nn] | 9·3 |

[a] Effect of nonprotein substances on papain-catalyzed plastein synthesis.

[b] Plastein yield calculated as: percentage TCA sol. of initial hydrolyzed substrate (88·7%)—percentage TCA sol. of plastein following incubation. The symbol * indicates that significant ($P < 0{\cdot}05$) net plastein synthesis did not occur.

[c] Calculated as the difference in percentage between values of percentage TCA sol. for experimental nonprotein-containing plastein systems and the nonprotein-free control. Positive values of Δ TCA sol. experimental indicate lower plastein yields compared with the control, but negative values confer the opposite. Experimental significance indicated as follows: *n*—one-sided significant ($P < 0{\cdot}05$) negative effect (nonprotein-induced decrease in plastein yield compared with the control); *nn*—two-sided negative effect; *pp*—two-sided significant ($P < 0{\cdot}05$) positive effect (nonprotein-induced increase in plastein yield compared with the control).

[d] Calculated as the difference in percentage between values of percentage TCA sol. for nonprotein-containing TCA controls and the nonprotein-free TCA control. Negative values indicate nonprotein-induced decreases in percentage TCA sol. (negative interference), but positive values confer the opposite. Experimental significance indicated as follows: *n*—one-sided significant ($P < 0{\cdot}05$) negative interference with the TCA test compared with the control; *nn*—two-sided negative interference.

[e] Calculated as the absolute value of the difference between Δ TCA sol. experimental and Δ TCA sol. control TCA test.

by definition, is the amount of 10% TCA-insoluble proteins formed during the plastein reaction (Arai *et al.*, 1975). The TCA test is primarily based on the fact that higher molecular weight proteins precipitate in TCA solutions while smaller enzymatically modified proteins or peptides remain soluble although other factors, such as chain length or amino acid composition, may influence the solubility of peptides in TCA. Nonprotein interference with the TCA test could be expected to result if the solubilities of proteins or peptides in TCA solutions are affected by the presence of nonprotein substances, or if the separation of TCA-soluble from insoluble proteins is hindered.

For hydrolysate systems, nonprotein substances resulting in higher-than-actual values of % TCA-soluble proteins (positive nonprotein interference) were saturated fat (2%), unsaturated fatty acids (1 and 2%) and magnesium. The presence of fatty acids in reaction systems appeared to hinder the separation of TCA-soluble from TCA-insoluble protein by centrifugation since these substances tended to float to the top of the centrifuge tubes, thereby entrapping or pulling TCA-insoluble proteins into the upper (TCA-soluble) layer.

Negative interference with the TCA test, resulting in lower values for percentage TCA solubility, were observed in control systems containing guar gum, xanthan gum (1 and 2%), calcium chloride and gum arabic. The abovementioned food gums tended to precipitate, centrifuge out, or form gels in TCA solutions, hence entrapping otherwise TCA-soluble protein in the lower (TCA-insoluble) layer. Such occlusion of soluble nitrogen in the TCA-insoluble fraction may simply relate to the volume of the precipitate; however, specific protein-nonprotein interactions may also have occurred. Calcium ions may have induced negative interference by causing the precipitation of otherwise TCA-soluble protein, thereby increasing the amount of nitrogen in the lower (TCA-insoluble) layer. The ability of calcium to promote the precipitation of food proteins, i.e. tofu or cheese manufacture, is well known.

Due to major physical differences between plastein and hydrolysate incubation mixtures, separate controls were set up to determine TCA interference in plastein systems. Results showed negative nonprotein interference by the presence of calcium chloride, cellulose, gum karaya and xylan, but no positive nonprotein interference was noted. Nonprotein substances in plastein reaction mixtures generally showed less pronounced effects on TCA test results compared with hydrolysate systems; this is perhaps due to the lower relative nonprotein/protein ratio in the plastein reaction systems.

It should be noted that TCA test interference was determined using only 88·7 and 85·6% TCA-soluble controls for the hydrolysate and plastein

systems, respectively; some deviation in the degree of TCA-interference may occur upon variation in the extent of protein hydrolysis.

## Hydrolysates

Table 1 gives results on the effects of nonprotein substances on the relative extent of peptic hydrolysis of soy isolate-containing model systems incubated at an enzyme-to-substrate ratio of 0·5% (0·5 E/S). For these reaction systems, nonprotein substances found to induce increases in the relative extent of hydrolysis (after 24 h incubation), as compared to a soy isolate control, included guar gum, xanthan gum (at addition levels of both 1 and 2%), locust bean gum, gum arabic, arabinogalactan, unsaturated fatty acids (only at the 2% level), combined salts, and xylan. Decreases in the final extent of hydrolysis were noted only for the unsaturated fatty acid (at the 1% level) treatment.

Table 1 also gives results for hydrolysates produced at a 3·5% enzyme-to-substrate ratio (3·5 E/S). As expected, the extent of hydrolysis was higher for hydrolysates produced at 3·5 E/S than for those produced at the lower enzyme concentration of 0·5 E/S. In the 3·5 E/S systems, enhancement of hydrolysis compared to the control was observed in systems containing xanthan gum (at both 1 and 2% levels), unsaturated fatty acids (at 1% only) and sodium chloride. Inhibition of hydrolysis occurred in systems containing gum karaya, combined salts, starch (at 2% only), cellulose, and saturated fatty acids (at the 2% level).

Results from the 0·5 and 3·5 E/S experiments suggested that nonprotein-induced alterations in the extent of protein hydrolysis were at least partly dependent on the enzyme concentration used. Compared to the 3·5 E/S hydrolysates, soy protein hydrolysis in the 0·5 E/S systems was enhanced by a greater number of nonprotein substances, particularly the plant gums. Conversely, protein hydrolysis in the 3·5 E/S systems, compared to the 0·5 E/S hydrolysates appeared more readily inhibited by the presence of nonprotein substances. It was observed that those substances which induced the highest increases in protein hydrolysis in the 0·5 E/S systems, also resulted in the development of the most highly viscous reaction mixtures as well as causing negative interference with the TCA test. In the 3·5 E/S experiment, however, only the high viscosity-elevating xanthan treatments caused significant increases in the extent of hydrolysis. It should also be noted that enhancement of hydrolysis of 0·5 E/S occurred in the presence of a combined salt preparation but individual salts tested showed no significant effects on hydrolysis. Among all preparations tested, only xanthan gum and unsaturated fatty acids caused increased hydrolysis at both enzyme concentrations.

Nonprotein-induced effects on the relative extent of hydrolysis also appeared to be significantly influenced by the concentration of nonprotein substances present in reaction mixtures. For example, in 0·5 E/S hydrolysate systems the presence of unsaturated fatty acids resulted in increased protein hydrolysis when added at a 2% level, but inhibited hydrolysis and interfered with the TCA test when added at the 1% level. Xanthan gum and xylan, when added to 0·5 E/S hydrolysate systems, appeared to exhibit nonproportional concentration dependency in that the 1% treatments enhanced hydrolysis to a greater extent than the 2% treatments. Xanthan gum also exhibited the same effect in 3·5 E/S hydrolysates. Conversely, for the 3·5 E/S systems, starch and unsaturated fatty acids inhibited hydrolysis slightly (only one-sided significance) when added at 2% but the lower 1% treatments produced no significant effects. Differences in nonprotein-induced effects between the 0·5 and 3·5 E/S systems may somehow relate to the initial rate of hydrolysis as influenced by the enzyme concentration.

Nonprotein substances present in hydrolysate incubation mixtures may influence protein hydrolysis by mechanisms involving changes in the stabilization, dispersion, conformation, and/or solubility of protein components of the system. Nonprotein-induced changes in soy protein conformation at pH 1·6 may, for example, allow for more extensive substrate/enzyme contact resulting in higher initial rates of hydrolysis. It has been reported that soy proteins in their native conformations are relatively resistant to proteolysis (Fukushima, 1959), but this resistance is thought to arise from the inability of the enzyme to penetrate and hydrolyze rigid, compact tertiary and quaternary protein structures (Fukushima, 1969). Native protein structure is, furthermore, believed to be stabilized by internal hydrophobic forces; disruption of these forces is necessary for protein denaturation and subsequent hydrolysis of the substrate (Fukushima, 1968). Nonprotein-induced increases in soy protein hydrolysis observed in the 0·5 E/S experiment may have resulted from increased disruption of native protein conformation by the presence of large amounts of hydrophilic nonprotein substances, particularly the plant gums and hemicelluloses. Such disruption may result from perturbations in the water structure of protein hydrogen bonding by the hydrophilic nonprotein substances, thereby altering the free energy of the system so as to decrease the activation energy required for denaturation of soy proteins. Other mechanisms may involve nonprotein-induced stabilization of dissociated soy proteins against aggregation and precipitation. Nonprotein substances may alter the association–dissociation–aggregation equilibria so as to allow proteins to remain in a more readily hydrolyzable configuration.

The presence of high viscosity-forming nonprotein substances, particularly food gums, could also enhance hydrolysis by increasing the solubility and/or dispersion of low-solubility soy protein fractions; such stabilizing effects of certain food gums on proteins are well known. In this study, xanthan gum appeared to clarify the protein dispersion when added to reaction mixtures while also enhancing relative hydrolysis when tested at concentrations of 1%. At the 2% level, lower relative hydrolysis may have been due to hindered enzyme/substrate diffusion caused by excessive viscosity in the reaction mixture. The effects of plant gums on viscosity and protein solubility are likely to be of considerable importance and more information on these effects is clearly needed.

The presence of fatty acids in reaction mixtures also appeared to significantly affect relative soy protein hydrolysis. Unsaturated fatty acids increased hydrolysis when present at 1% in 3·5 E/S systems, as well as at 2% in 0·5 E/S systems; conversely, unsaturated fatty acids inhibited hydrolysis when added at 1% to 0·5 E/S. Unsaturated fatty acids also dramatically interfered with the TCA test. The presence of saturated fatty acids induced slight decreases in hydrolysis when added at 2% to 3·5 E/S systems but the 1% preparation showed no significant effect. Effects of fatty acids on hydrolysis appeared to be concentration-dependent as well as influenced by enzyme concentration. The presence of large amounts of the amphipathic unsaturated fatty acids may have caused enhancement of the relative extent of hydrolysis in 0·5 and 3·5 E/S systems by disrupting protein hydrophobic bonding through interaction between the fatty acids and hydrophobic protein regions. Difference in fatty-acid-induced effects between the two enzyme concentrations may have been due to differences in the emulsification capacity of the two systems resulting from differences in the molecular weight distributions of peptides formed during hydrolysis.

The addition of salt to reaction mixtures was also found to influence protein hydrolysis, which appeared to be dependent on the enzyme concentration as well as on the composition of the ionic species. Despite addition of salts to reaction mixtures at equivalent molarities, alterations in relative protein hydrolysis were not observed for all salt treatments tested. At 0·5 E/S, the combined salts preparation enhanced hydrolysis while inhibiting hydrolysis in the 3·5 E/S system. Sodium chloride, on the other hand, was found to enhance hydrolysis in the 3·5 E/S system while exhibiting no corresponding effect in the 0·5 E/S system. It thus appears evident that salt-induced effects on hydrolysis may be dependent on either the rate of reaction (as influenced by the enzyme concentration) or on the molecular weight distribution of peptides liberated during the hydrolysis reaction. The effects of calcium on hydrolysis are indeterminable, however,

since calcium-induced marked negative interference with the TCA test. Mechanisms of salt-induced alteration in proteolysis may involve changes in the physical state of the soy proteins in the reaction mixtures. Such changes may be responsive to altered stability of protein complexes as well as variation in protein/peptide solubility due to salting-in or salting-out reactions.

Results on the effects of nonprotein substances on hydrolysate formation may have significance in the formulation of reaction systems for the production of more complete protein hydrolysates while using lower enzyme concentrations. Studies of this type may also yield valuable information on the mechanisms of protein hydrolysis and provide a better understanding of the interactions between proteins and nonproteins during enzymatic modification reactions.

## Soy flour

Results of this investigation revealed that nonprotein substances present in reaction mixtures generally did not inhibit proteolysis of soy protein isolate. Observed decreases in the extent of hydrolysis of soy flour may or may not be due to the presence of nonprotein substances, *per se*. It is quite possible that other intrinsic factors associated with soy flour may be responsible for inhibiting peptic hydrolysis; such factors may involve the presence of native protein–nonprotein complexes which restrict protein-/enzyme contact. It is apparent, however, that under the reaction conditions used in this experiment, soy flour proteins were sufficiently denatured to allow for a considerable degree of proteolysis to occur.

## Plasteins

Results on the effects of nonprotein substances on plastein synthesis are given in Table 2. Net plastein synthesis, as determined by the increase in 10% TCA-insoluble protein during incubation, was found to be relatively unaffected by the presence of nonprotein substances in the reaction mixtures. Only the high viscosity-forming, high water binding xanthan, locust bean, and guar gum preparations were found to decrease plastein synthesis as compared to the control and to negatively interfere with the TCA test. Inhibition of plastein synthesis by these substances most likely resulted from hindered substrate/enzyme diffusion caused by high viscosity and lack of free water. Plastein reaction mixtures, due to high substrate concentrations, are quite viscous and contain relatively low amounts of free water even when prepared in the absence of nonprotein substances.

Consequently, addition of large amounts of nonproteins to the system can be critical if such substances bind excessive amounts of water, produce extreme viscosity (gelation etc.) or increase the solids content beyond what can be effectively hydrated. However, it cannot be ruled out that nonprotein substances inhibit plastein synthesis by other mechanisms, such as disruption of hydrophobic bonds which could hinder plastein formation, since hydrophobic forces are believed to be instrumental in plastein chain assembly (Aso *et al.*, 1974).

Stimulation of plastein production compared to the control was observed only for arabinogalactan. Such a finding may have implications in formulation of plastein reaction mixtures for increased plastein production and modification of functionality. In this study, however, salts were shown not to alter plastein synthesis, this being in disagreement with a previous study showing enhanced plastein formation in the presence of sodium chloride (Tanimoto *et al.*, 1975).

## SUMMARY

Several nonprotein substances, when added to incubation mixtures containing soy protein isolate, were found to enhance the relative extent of protein hydrolysis as determined by a TCA solubility assay. Nonprotein-induced enhancement of hydrolysis was most pronounced for systems incubated at a low enzyme concentration (0·5% enzyme/substrate) as opposed to a higher (3·5%) enzyme concentration. Relatively few of the nonprotein substances resulted in inhibition of hydrolysis despite their addition to incubation mixtures at high relative concentrations.

Several nonprotein substances were also noted to interfere with the performance of the TCA solubility test and, because of this, proper assay controls should be instituted when using the TCA test on samples containing high relative amounts of certain nonprotein substances.

Plastein formation, measured as a decrease in TCA-soluble protein nitrogen, was relatively unaffected by the presence of most nonprotein substances tested, although xanthan and locust bean gums significantly inhibited plastein formation.

Results from this study suggest that enzymatic protein modification reactions can proceed uninhibited or may even be enhanced by the presence of high amounts of various nonprotein substances, several of which may occur naturally in vegetable protein flours or as functional components in food systems.

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