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ABSTRACT

THE effect of heat on trypsin inhibitor (TI) activity during steam-infusion cooking of soymilk was studied. At 154°C only 40 s process time reduced TI to the same level as conventional cooking at 99°C for 60 min. Between 99 and 154°C the kinetics of TI inactivation followed behavior exemplified by the summation of two first-order reactions. Spline fitting functions effectively modeled the data with r^2 ranging from 0.984 to 0.999. The heat-labile reaction was attributed to Kunitz inhibitor and the heat-stable reaction, to Bowman-Birk inhibitor. The former accounted for approximately 85 percent of the original TI activity. Arrhenius equation kinetic constants for each reaction were calculated.

INTRODUCTION

Their high protein content gives soybeans considerable potential as human food (Smith and Circle, 1972). For centuries, the Chinese have prepared aqueous extracts of whole soybeans for milk-like beverages. Recent increases in the cost of bovine milk and continuing insufficient supplies of dairy products in developing countries have rekindled interest in milk analogs from vegetable protein sources. Success largely depends on improving flavor and nutritional characteristics. The nutritional value of soymilk is improved by heat treatment until significant amino acid degradation occurs (Logenecker et al., 1964; and Rios Iriarte and Barnes, 1966). The improvement in nutritional value results from increased accessibility of protein to enzyme attack (Fukushima, 1968; and Boonvisut and Whitaker, 1976) and inactivation of proteolytic inhibitors, primarily trypsin inhibitors (Liener, 1969).

Pancreatic hypertrophy and reduced proteolytic digestion of soybeans have been attributed to soy trypsin inhibitors (TI). The effectiveness of heat treatment on the nutritional value of soymilk depends on pH, process temperature, and heating time. Maximum nutritional value or protein efficiency ratio (PER) of soymilk is reached when at least 90 percent of the native TI activity is inactivated (Hackler et al., 1965; and Wallace et al.,

1971), which is achieved after 60-70 min of atmospheric boiling (99°C) or retorting for 5-10 min at 121°C.

At the same time TI is inactivated, amino acids may degrade, which reduces the nutritional value and protein may be insolubilized, which reduces the yield of milk. In an attempt to minimize amino acid degradation and protein insolubilization while providing adequate inactivation of TI, a high-temperature (121-154°C), short-time (0-240 s) process involving direct steam infusion of soymilk was evaluated (Johnson, 1978). Optimization of that process necessitated establishing the kinetics of TI inactivation with heat. This report describes the modeling of the kinetics of TI inactivation in this high-temperature, short-time process.

MATERIALS AND METHODS

Slurry Preparation

Whole seed-grade Columbus soybeans were ground in a Model D-6 Fitzmill fitted with a 0.125-mm opening screen. One part soy flour was slurried with four parts tap water. The slurries were allowed to hydrate for about 10 min before processing.

Soymilk Processing

A modified Penick and Ford laboratory jet cooker was designed to accommodate soy flour slurries and to operate in both continuous and semi-batchwise fashions (Fig. 1). A control sample processed at 99°C was prepared by allowing the slurry to discharge immediately after steam injection and heating by conventional atmospheric cooking. Soy flour slurries were also cooked by hydro-thermal jet cooking (HTC) at 121, 132, 143 and 154°C. Under continuous fashion operation, the residence time varied from 4 to 40 s depending on back

T₁-T₈ Thermocouples
Valve 1 By-pass Throttle (Needle Valve)
Valve 2 Flow Control (3-way Ball Valve)
Valve 3 Discharge Pressure Control (Gate Valve)
Valve 4 Flow Control (2-way Ball Valve)
Valve 5 Back Pressure Control (Gate Valve)

FIG. 1 Steam-infusion cooking system.

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pressure and length of the holding tube. Longer process times were achieved by utilizing the unit in a semi-batchwise fashion (filling the hold tube and holding it for the desired process time). The cooked product discharged from the flash unit into an ice water cooler. Product temperature with respect to process time and position was controlled to within $\pm 1.5^{\circ}\text{C}$ from the desired temperature. Instantaneous heating to the desired temperature and cooling to 99°C were achieved. Cooling from 99°C to 30°C required about 120 s. Samples cooked at pH 9.5 were neutralized with HCl immediately after cooking. The cooked slurries were adjusted to 10 percent solids with distilled water after slurry solids were determined by AOAC procedure 16.032. The slurry was centrifuged at 5°C and $1050 \times g$ for 5 min. The supernatant was stored at 5°C for 7 days to allow maximum regeneration of TI before being analyzed for residual TI activity.

Assay for TI Activity

Approximately 4.0 g of soymilk was diluted to 100 mL with distilled water and centrifuged at $30,000 \times g$ for 30 min at 5°C . The supernatant was diluted 1:1 to 1:4 depending upon TI activity. TI activity was assayed by the procedure of Swartz et al. (1977). The rate of DL-benzoylarginine p-nitroanilide (BAPA) hydrolysis by a standard solution of bovine pancreas Type II trypsin (2X crystallized) in pH 8.2 tris buffer and a known quantity of soymilk was observed. The rate of hydrolysis was spectrophotometrically quantified against a substrate, distilled-water blank. One trypsin inhibitor unit (TIU) was defined as the TI activity producing 50 percent inhibition of BAPA hydrolysis in the standard assay solution. All determinations of TI activity were from assays with 35 to 60 percent inhibition observed. The fraction of residual TI activity was calculated as the number of TIU/g processed soymilk divided by the number of TIU/g raw soymilk. The fraction, multiplied by 100 percent, represented the percent residual TI activity.

Curve Fitting

Linear-quadratic-linear spline functions (Perneski et al., 1973) were fit to the TI inactivation data. These are segmented functions: the first segment is linear; the second, quadratic; and the third, linear. The segments are joined together at points in such a way that the slope and concavity of each segment are equal at join points. In some cases a quadratic-linear spline function was used due to the lack of data in early time periods. In those cases, the tail of the quadratic segment was approximately linear in the region of interest and thus is referred to as the linear segment. Spline functions were estimated by the modified Gauss-Newton procedure.

RESULTS AND DISCUSSION

Many enzymes and enzyme inhibitors follow first-order kinetics during inactivation with heat. Therefore plots of residual concentration versus time of heat treatment are curvilinear, while plots of log concentration versus time of heat treatment are linear. The denaturation of TI in the control sample of soymilk was observed at 99°C at pH 6.7 (inherent pH of soybeans) and 9.5 (Fig. 2). The plots of TI inactivation did not conform to a single reaction following first-order kinetics because plots of log [TI] versus time were curvilinear. Wallace et

FIG. 2 Effect of cooking soymilk at 99°C on inactivation of trypsin inhibitors

al. (1971), studying the kinetics of TI inactivation in canned soymilk processed by retort cooking, found a linear relationship between cooking time at 99°C and the percentage of TI inactivated in soymilk prepared from pre-soaked soybeans. The rate of TI inactivation was much faster in carbonate-soaked beans (pH 8.7). As pH increased, TI inactivation at 99°C approached first-order kinetics. Heat transfer lags and temperature stratification would be expected to be high in retorting canned soymilk compared with steam-infusion processed soymilk. HTC processing facilitated instantaneous heat transfer with no temperature stratification.

Soy milk produced by wet grinding traditionally is heated for 60 min at 99°C and pH 6.7 to improve flavor, inactivate TI, and maximize feeding efficiency. However, our data (Fig. 2) indicate that residual TI activity was 7.6 percent of the original activity after cooking by steam infusion at conventional times and temperatures. Increasing the pH from 6.7 to 9.5 greatly reduced the time required for equivalent residual TI activity. Approximately 150 s at 99°C and pH 9.5 were required to reach 92.4 percent reduction in TI activity. The pH of 9.5 is significant since lipoxigenase activity, which is responsible for raw, green beany flavors, is minimized during slurring. That soy TI was more heat-labile in moderately alkaline conditions was consistent with observations by Wallace et al. (1971) and Obara and Watanabe (1971). Its instability in heated alkali has been attributed to intrapeptide disulfide bonds, which stabilize the conformational structure of TI.

HTC facilitated pressurized steam-infusion for cooking at temperatures ranging from 121 to 154°C . Fig. 3 shows effects of cooking temperature and time on inactivation of TI. Process times that resulted in equivalent inactivation of TI to 60 min at 99°C (7.6 percent residual TI activity) were 280 s at 121°C , 165 s at 132°C , 100 s at 143°C and 40 s at 154°C .

Under all process conditions, plots of log [TI] with respect to time of heat treatment were curvilinear, in-

FIG. 3 Effect of temperature on inactivation of trypsin inhibitor at pH 6.7.

dicating that inactivation of TI activity in soymilk processed by steam infusion did not behave according to simple first-order kinetics. Initial and latter portions of the curves were linear, each having distinctly different slopes. Between the two linear segments was a transitional period in which the relative importance of the two reactions switched. Assays for TI activity measure total TI activity without discriminating between different TI reactions. Wolf (1977) reported that TI activity largely

results from two different inhibitors, the Kunitz inhibitor and the Bowman-Birk inhibitor. The Kunitz inhibitor is moderately heat-labile and accounts for most TI activity. The Kunitz inhibitor is a low molecular-weight protein consisting of 181 amino acid residues with two disulfide bridges. The Bowman-Birk inhibitor is a smaller protein consisting of 71 amino acid residues with seven disulfide bridges. The Bowman-Birk inhibitor is considerably more heat stable because its high proportion of disulfide bridges stabilize the molecular conformation required for biological activity (Wolf, 1977). Although the kinetics of heat inactivation of these two inhibitors have not been separated before, the inactivation curves (Figs. 2 and 3) probably are the sum of two separate first-order reactions representing the inactivations of the Kunitz and Bowman-Birk inhibitors.

Spline regression functions were fit to the TI inactivation data. The initial parts of the curves were fit with a linear model; the transitional period, with a quadratic model; and the last portion, with another linear model (Table 1). Multiple correlation coefficients (r^2) ranged from 0.984 to 0.999 so the estimated models fit the data well.

The hypothesis that the model results from the sum of reaction kinetics for two TI activities provides a basis for estimating the reaction kinetics of both inhibitors in biological inactivation during HTC processing of soymilk. The most satisfactory method for expressing the influence of temperature on reaction velocity is the Arrhenius equation (Charm, 1971).

$$2.303 \log K = 2.303 \log A - \frac{\Delta H_a}{RT} \quad [1]$$

TABLE 1. MODEL PARAMETERS RELATING INACTIVATION OF TI TO PROCESS TIME AT VARIOUS TEMPERATURES

HTC processing at 99 °C, pH 6.7	
Transitional period:	$Y = 2.000 - 0.00272X + 0.0000020X^2$
Join point:	$X = 666.2 \text{ sec}$
Final linear period:	$Y = 1.112 - 0.00006442X$
Model fit:	$r^2 = 0.984$
HTC processing at 99 °C, pH 9.5	
Transitional period:	$Y = 2.000 - 0.07325X + 0.001115X^2$
Join point:	$X = 32.70 \text{ sec}$
Final linear period:	$Y = 0.8075 - 0.0003143X$
Model fit:	$r^2 = 0.999$
HTC processing at 121 °C, pH 6.7	
Initial linear period:	$Y = 2.000 - 0.01393X$
First join point:	$X = 22.87 \text{ sec}$
Transitional period:	$Y = 2.043 - 0.01770X + 0.00008245X^2$
Second join point:	$X = 99.91 \text{ sec}$
Final linear period:	$Y = 1.220 - 0.001223X$
Model fit:	$r^2 = 0.996$
HTC processing at 132 °C, pH 6.7	
Initial linear period:	$Y = 2.000 - 0.02044X$
First join point:	$X = 17.48 \text{ sec}$
Transitional period:	$Y = 2.049 - 0.02606X + 0.0001607X^2$
Second join point:	$X = 76.87 \text{ sec}$
Final linear period:	$Y = 1.099 - 0.001351X$
Model fit:	$r^2 = 0.994$
HTC processing at 143 °C, pH 6.7	
Initial linear period:	$Y = 2.000 - 0.03019X$
First join point:	$X = 16.21 \text{ sec}$
Transitional period:	$Y = 2.105 - 0.04319X + 0.0004011X^2$
Second join point:	$X = 52.17 \text{ sec}$
Final linear period:	$Y = 1.0134 - 0.001335X$
Model fit:	$r^2 = 0.999$
HTC processing at 154 °C, pH 6.7	
Initial linear period:	$Y = 2.000 - 0.04227X$
First join point:	$X = 11.59 \text{ sec}$
Transitional period:	$Y = 2.090 - 0.0577X + 0.0006681X^2$
Second join point:	$X = 41.69 \text{ sec}$
Final linear period:	$Y = 0.9287 - 0.002049X$
Model fit:	$r^2 = 0.995$

FIG. 4 Reaction kinetics of trypsin inhibitor inactivation. First first-order reaction model $\log K = 4.7567 - 4439.4T^{-1}$, $r_2 = 0.999$. Second first-order reaction model $\log K = 0.04765.1868.7T^{-1}$, $r_2 = 0.719$.

where:

- K = reaction velocity constant
- A = frequency factor
- R = gas constant
- ΔH_a = energy of activation
- T = absolute temperature

The reaction velocity constant K (for a constant temperature T) is given by

$$K = \frac{2.303}{\theta} \log \frac{[TI]_0}{[TO]_1} \dots \dots \dots [2]$$

where:

- K = reaction velocity constant
- θ = process time
- $[TI]_0$ = initial concentration of TI
- $[TI]_1$ = concentration of TI at time θ

Treating the data of residual TI activity as two first-order reactions yielded two linear plots of log K versus T^{-1} with different slopes (Fig. 4). The slopes were equal to $\Delta H_a/2.303R$. The reaction kinetic constants were calculated as follows:

Initial first-order reaction (probably Kunitz inhibitor) at pH 6.7:

$$\begin{aligned} A &= 14,806 \text{ s}^{-1} \\ \Delta H_a/2.303R &= 2531^\circ\text{K} \\ \Delta H_a &= 11,581 \text{ cal/mole} \end{aligned}$$

Second first order reaction (probably Bowman-Birk inhibitor) at pH 6.7:

$$\begin{aligned} A &= 0.151 \text{ s}^{-1} \\ \Delta H_a/2.303R &= 980^\circ\text{K} \\ \Delta H_a &= 4,484 \text{ cal/mole} \end{aligned}$$

By extrapolating the second linear segment of the inactivation curves to zero time, this reaction (presumed to be the Bowman-Birk inhibitor) accounts for 9 to 17 percent of the total TI activity and the remaining activity is the Kunitz inhibitor. The preponderance of TI activity (83 percent or more) in soymilk processed by HTC is

readily inactivated, but the remaining TI activity is difficult to inactivate. All TI activity could not be eliminated without severe heat treatment resulting in considerable damage to the nutritional quality of soy protein.

CONCLUSIONS

Soymilk is generally heated for 60 min at 99°C at pH 6.7 to sufficiently inactivate soy TI and produce maximum PER. That much heat treatment left about 7 to 8 percent of the original activity as a residual. At pH 9.5 only 150 s at 99°C were required to achieve the same level of residual TI. Soymilk was treated by hydrothermal jet cooking (steam injection) to facilitate continuous cooking at higher temperatures (121 to 154°C) for shorter periods. Cooking for 280 s at 121°C , 165 s at 132°C , 100 s at 143°C , or 40 s at 154°C reduced the TI activity to 7 to 8 percent of the original.

The kinetics of TI inactivation with heat followed behavior indicative of the sum of two first-order reactions. Modeling the data with two, separate linear segments separated by a curvilinear transitional period resulted in multiple correlation coefficients ranging from 0.984 to 0.999. The initial linear phase was attributed to the Kunitz inhibitor, which is moderately heat labile. The slower reaction was attributed to the Bowman-Birk inhibitor. The initial reaction had Arrhenius kinetic constants of $A = 14,806 \text{ s}^{-1}$ and $\Delta H_a = 11,581 \text{ cal/mole}$ while the heat stable reaction had $A = 0.151 \text{ s}^{-1}$ and $\Delta H_a = 4,484 \text{ cal/mole}$. The preponderance of TI activity in soymilk (83 to 91 percent) was attributed to the heat labile inhibitor.

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