Inactivation of Trypsin Inhibitors in Aqueous Soybean Extracts by Direct Steam Infusion[1]

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ABSTRACT

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A means of direct steam-infusion cooking of aqueous soybean extracts for milk analogs was developed. The effect on trypsin inhibitor (TI) activity of heat in continuous processing was quantified over the temperature range 99–154°C. As process temperature increased each 11°C at pH 6.7, the rate of TI inactivation doubled. At 154°C, pH 6.7, 40 sec of heat treatment produced TI inactivation equivalent to that in 60 min at 99°C (7.6% residual

TI activity). Increasing the pH of the slurry to 9.5 substantially increased the rate of TI inactivation. Inactivation of TI activity by high-temperature, short-time processing exhibited reaction kinetics similar to the summation of two first-order reactions with different heat stabilities. We speculate that the heat-labile inhibition stemmed from the Kunitz inhibitor and the more heat-stable inhibition, from the Bowman-Birk inhibitor.

The nutritional value of soybeans is improved by heat treatment until the point at which significant amino acids are degraded (Logenecker et al 1964, Rios Iriarte and Barnes 1966, Smith and Circle 1972). The increased feeding efficiency has been attributed to increased accessibility of protein to enzyme attack as a result of changes in protein conformation (Boonvisut and Whitaker 1976, Fukushima 1968) and inactivation of proteolytic inhibitors, primarily trypsin inhibitors (TI) (Liener 1969).

The effectiveness of heat treatment on nutritional characteristics of soy protein largely depends on water activity, pH, heating time, and process temperature. Conventional heat treatment of soymilk involving atmospheric cooking or retorting for 60–70 min at 99°C or 5–10 min at 121°C reportedly destroys 90% of the native TI activity and yields the maximum protein efficiency ratio (PER) of processed soymilk (Badenhop and Hackler 1970, Hackler et al 1965, Wallace et al 1971). In an attempt to reduce amino acid degradation and protein insolubilization yet provide adequate inactivation of TI activity, we studied high-temperature, short-time processing of aqueous soybean extracts by direct steam infusion (Johnson 1978).

MATERIALS AND METHODS

Soy Flour Slurry Preparation

Whole seed-grade Columbus soybeans were ground in a laboratory hammermill through a 0.125-mm opening screen. One part soy flour was slurried in four parts water. Slurries were processed at pH 6.6 and 9.5 after 30 min of hydration.

Soymilk Processing

A Penick and Ford laboratory jet cooker was modified to handle soy flour slurries and to operate in continuous or semibatchwise fashion (Fig. 1). A control sample processed at 99°C (210°F) was prepared by allowing the material to discharge immediately from the steam injection valve into a preheated 2,000 ml, round-bottomed flask, then refluxing it and cooling it in an ice bath after the desired cooking. Soy flour slurries were also cooked by steam-infusion cooking at 121, 132, 143, and 154°C (250, 270, 290, and 310°F). Under continuous operation, the residence time varied between 4 and 40 sec, depending on the back pressure and length of

the holding tube. Residence time was measured by timing a small slug of dye through the system. Back-mixing was believed to be small because the dye exited the system largely as a slug. Longer process times were achieved, using the unit in a semibatchwise fashion, by filling the holding tube and holding the material for the desired processing time. The cooked product discharged the flash unit into a stainless steel container in an ice bath. Product temperature with respect to process time and position was controlled to within ±1.5°C from the desired processing temperature. Instantaneous heating (< 0.5 sec) to the desired temperature and cooling to 99°C were achieved. Cooling from 99 to 30°C required about 120 sec. Samples cooked at pH 9.5 were neutralized with HCl. All samples were adjusted to 10% solids with distilled water after slurry solids were determined by AOAC procedure 16.032. The slurry was centrifuged at 5°C and 1,050 × g for 5 min. Because some proteins may renature after denaturation with heat, the soymilk (supernatant) was stored at 5°C for seven days before being analyzed for TI; this allowed regeneration of 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 × g for 30 min at 5°C. The supernatant was diluted from 1:1 to 1:4, depending upon TI activity. We assayed TI activity by the procedure of Swartz et al (1977), observing the rate at which DL-benzoylarginine p-nitroanilide (BAPA) was hydrolyzed by a standard solution of bovine pancrease Type II trypsin (twice crystallized) in pH 8.2 tris buffer with a known quantity and dilution of soymilk extract. The rate of hydrolysis was spectrophotometrically quantified against a substrate/distilled-water blank. One trypsin inhibitor unit (TIU) was defined as the amount of trypsin inhibitor activity that produced 50% inhibition of BAPA hydrolysis in the standard assay solution. All determinations of TI activity were from assays in which 35–60% inhibition of BAPA hydrolysis was observed. The fraction of residual TI activity was calculated as the number of TIU per gram of processed soymilk divided by the number of TIU per

TABLE I
Process Conditions for Optimum Reduction in Trypsin Inhibitor Activity

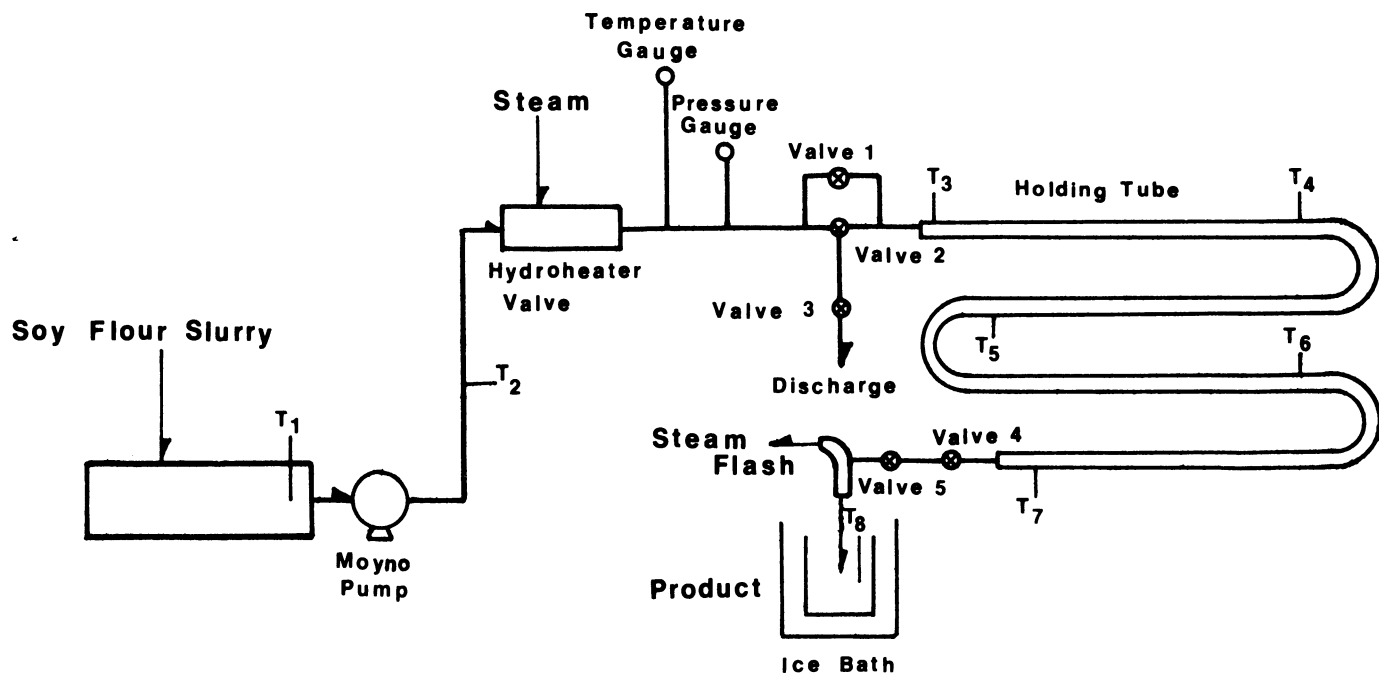| pH	Temperature °C (°F)	Cooking Time[a] (sec)
6.6	99 (210)	3,600
	121 (250)	282
	132 (270)	165
	143 (290)	100
	154 (310)	40
9.5	99 (210)	150
	121 (250)	80
	143 (290)	40

[a] Time to 7.6% residual TI activity.

Fig. 1. Modified hydrothermal jet cooker. T_1–T_8, thermocouples. Valves: **1,** by-pass throttle (needle valve); **2,** flow control (three-way ball valve); **3,** discharge pressure control (gate valve); **4,** flow control (two-way ball valve); **5,** back pressure control (gate valve).
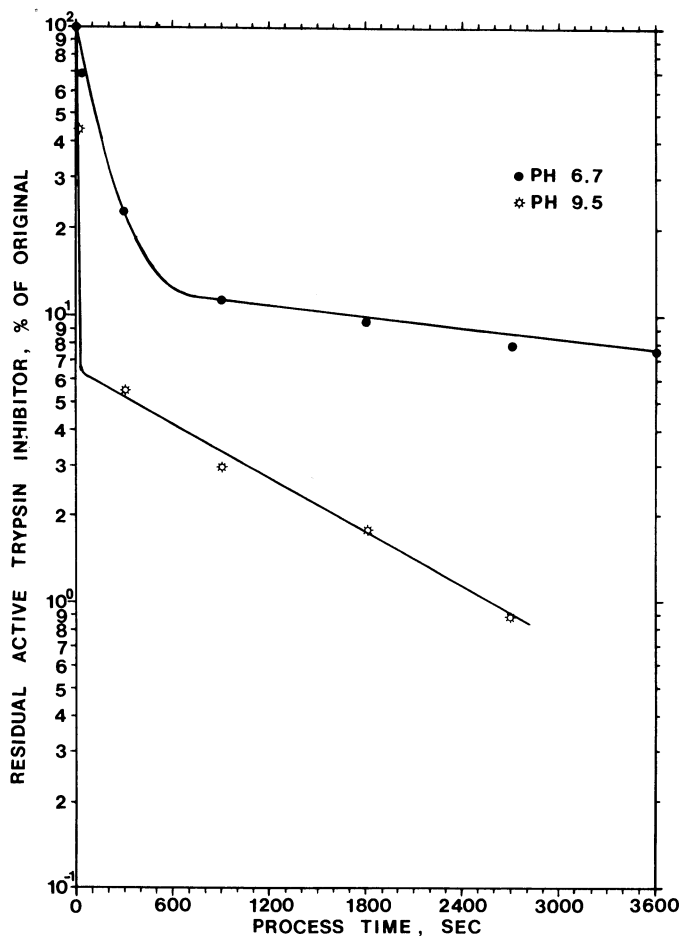

Fig. 2. Residual trypsin inhibitor activity in soymilk processed at 99°C.

Fig. 3. Effect of process temperatures and times on residual trypsin inhibitor activity in soymilk.

gram in raw soymilk. That fraction times 100 represented the percent residual TI activity in processed soymilk.

Curve Fitting

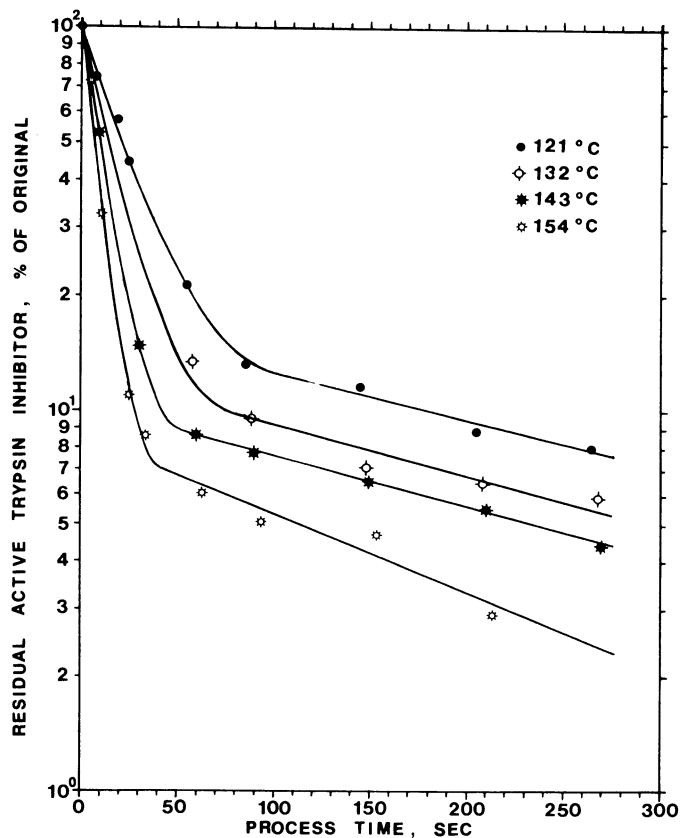All curves were fit by least squares analysis. Curves of TI inactivation during processing were fit with spline functions where the curve was fit in three segments (Perneski et al 1973). The first segment was fit by a linear model; the second segment, a quadratic model; and the third segment, another linear model.

RESULTS AND DISCUSSION

Many enzymes and some enzyme inhibitors follow first-order kinetics in denaturation due to heat treatment, so plots of residual

TI concentration versus time of heat treatment are curvilinear, whereas plots of log concentration versus time are linear. The denaturation of TI activity in soymilk at pH 6.7 and 9.5 during cooking by direct steam infusion at 99°C did not conform to only a single reaction following first-order kinetics (Fig. 2). Initial rate of loss in TI activity was rapid and the final rate was much lower. Increasing the pH to 9.5 significantly increased the rate of TI inactivation by heat, but the same two-phase pattern was observed. Decreased heat stability of TI in steam-infusion cooking at pH 9.5 has practical significance because above pH 9.5, lipoxygenase activity, which results in "green beany" flavors during flour slurrying, is minimal.
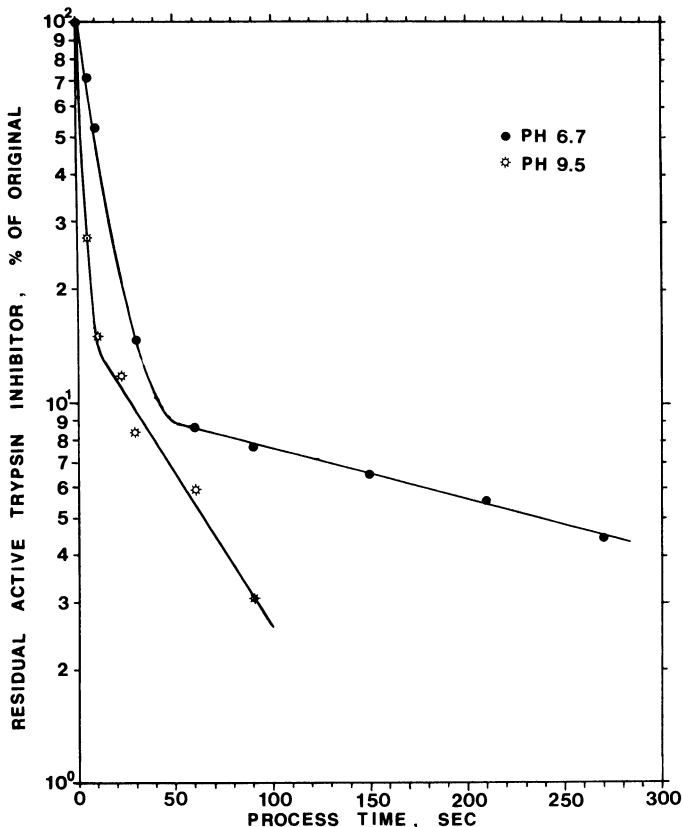
TI activity in soymilk processed by steam-infusion cooking decreased 90% after either 29 min at pH 6.7 and 99°C or 2 min at pH 9.5 and 99°C. Soymilk produced by wet grinding is traditionally heated for 60 min at pH 6.7 and 93–99°C to produce maximum PER (Hackler et al 1965, Hackler and Stillings 1967). Heat treating soymilk for 60 min under the same conditions resulted in 7.6% residual TI activity.

Steam-infusion cooking also facilitates pressurized steam infusion for cooking at temperatures from 121 to 154°C. We quantified effects of cooking temperature and time steam-infusion processing (Fig. 3). Every 11°C increase in process temperature from 121 to 154°C decreased the cooking time for 90% inactivation of TI by approximately 50%. Process times that resulted in TI activity equivalent to that achieved in 60 min at 99°C (7.6% residual activity, shown to produce maximum PER), were determined (Table I). About 282 sec at 121°C, 165 sec at 132°C, 100 sec at 143°C, and 40 sec at 154°C was required to adequately inactivate TI, with 7.6% residual TI activity as the acceptable criterion.

Increasing the pH from 6.7 to 9.5 reduced cooking time to give 7.6% residual TI activity from 3,600 to 150 sec at 99°C (Fig. 2), from 282 to 80 sec at 121°C (Fig. 4), and from 100 to 40 sec at 143°C (Fig. 5). The logarithm of time for 90% reduction in TI activity was not quite linear with process temperature. The rate of reduction in process time slightly decreased with increases in process temperature. Also, the rate of reduction in process time
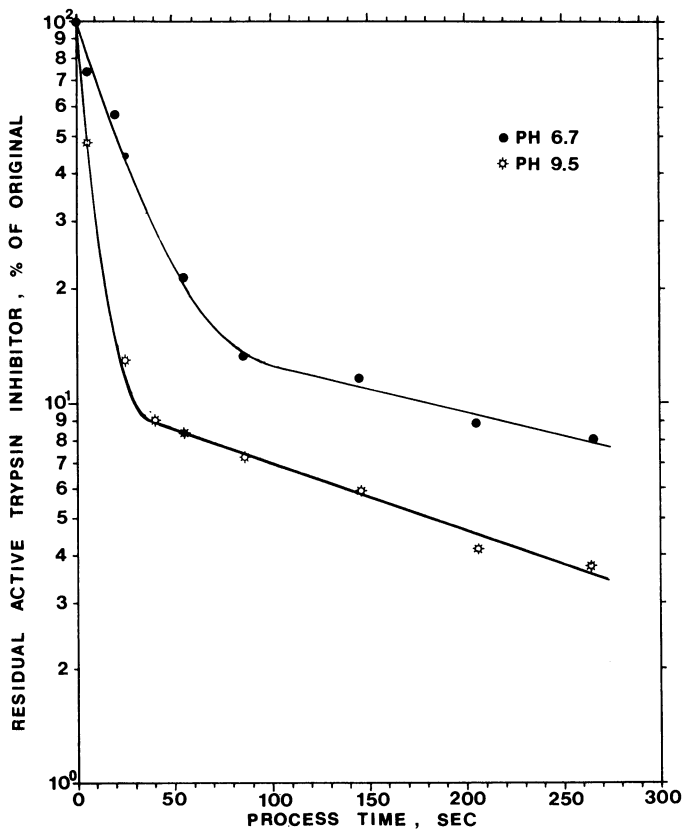

Fig. 4. Residual trypsin inhibitor activity in soymilk processed at 121°C.

Fig. 5. Residual trypsin inhibitor activity in soymilk processed at 143°C.
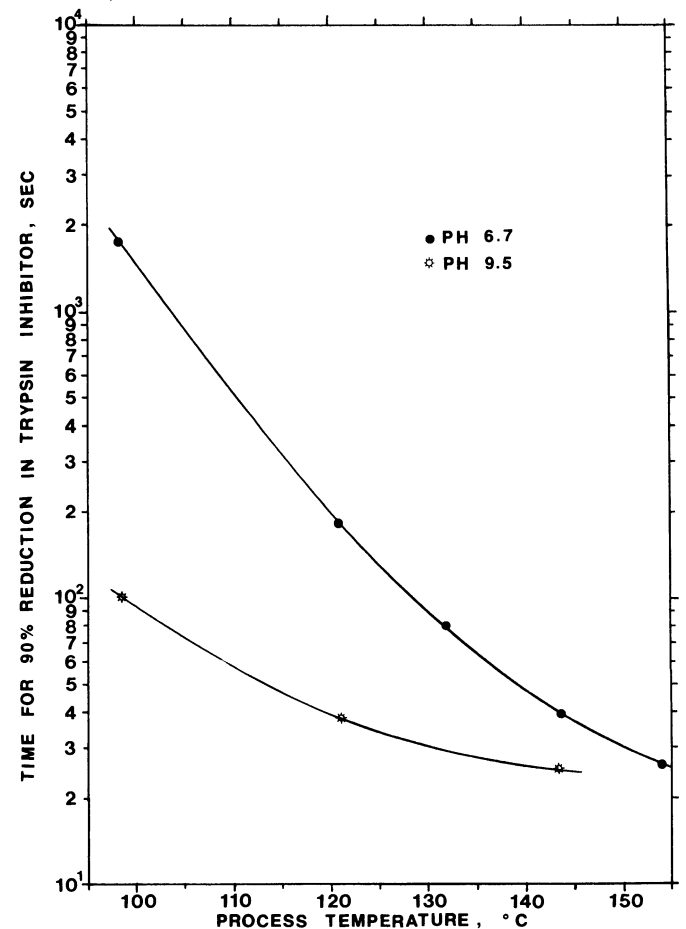

Fig. 6. Effects of pH and temperature on process time required for 90% reduction in trypsin inhibitor activity of soymilk.

attributable to pH 9.5 decreased with increases in process temperature (Fig. 6), which indicates that a minimum cooking time may be required at process temperatures exceeding 160°C and that processing under alkaline conditions does not reduce the minimum. The fact that soy TI activity was much more heat-labile under moderately alkaline conditions was consistent with observations by Wallace et al (1971) and Obara and Watanabe (1971). The suggestion has been made that the intrapeptide disulfide bonds that stabilize the conformation of TI are unstable in heated alkali. Moderate alkaline conditions (pH 9.5) seem to be a practical way to reduce process time to inactivate TI activity. However, the effect on lysinoalanine production of alkaline conditions in steam-infusion processing requires study.

Under all process conditions, plots of log TI activity over time of heat treatment were curvilinear, indicating that inactivation of TI in soymilk did not behave according to simple first-order kinetics. The initial and latter portions of the curve were linear, each having distinctly different slopes. The relative importance of the two reactions switched during a transitional period between the two linear segments. Assays for TI activity measure total TI activity but do not discriminate between different TI activities. Obara and Watanabe (1971) have shown that soy TI can be fractionated by diethylaminoethyl cellulose chromatography into five fractions, each exhibiting a different rate of inactivation at 70°C. Data presented here indicate that only two reaction rates differed distinctly in steam-infusion cooking of soymilk. Although the total number of trypsin inhibitors is unknown, two predominate—the Kunitz inhibitor and the Bowman-Birk inhibitor. The curvilinear relationship between log TI activity and process temperature may result from differences in heat stability between the two inhibitors. The primary structure of the Kunitz inhibitor has 181 amino acid residues that give rise to a molecular weight of about 21,500 (Wolf 1977). Native Kunitz inhibitor largely consists of randomly oriented regions stabilized by two disulfide bonds, plus a small region of helical coil. The three-dimensional protein structure, therefore, is reasonably susceptible to heat treatment. The Bowman-Birk inhibitor consists of a single polypeptide chain of 71 amino acids, giving rise to a molecular weight of 7,861 (Wolf 1977), and seven disulfide crosslinks that impart remarkable stability. The preponderance of TI activity in soymilk is the result of a relatively heat-labile inhibitor, probably the Kunitz inhibitor. However, residual levels of TI activity may remain after heat treatment, and these are probably the Bowman-Birk inhibitor.

SUMMARY

A steam-infusion process was developed for continuous processing of soymilk to reduce TI and improve protein utilization. The effect of process temperature (99–154°C) on inactivation rate of TI activity was quantified. Inactivation of TI in this high-temperature short-time process exhibited reaction kinetics similar to the summation of two first-order reactions with significantly different heat stabilites. We speculate that the initial rapid reduction in TI activity stemmed from inactivation of the Kunitz inhibitor and the slow secondary reaction, from inactivation of the Bowman-Birk inhibitor. Cooking by steam-infusion for 60 min at pH 6.7 and 99°C left only 7.6% of the original TI activity. Such cooking has been reported to result in maximum PER. Cooking for 40 sec at pH 6.7 and 154°C or at pH 9.5 and 143°C also produced soymilk with 7.6% residual TI activity. Alkaline (pH 9.5) cooking reduced required cooking times more at low process temperature (99°C) than at high (154°C).

When soymilk is processed by steam-infusion cooking, the kinetics of heat inactivation of both inhibitors are independent, separable, and important for maximum nutritional value. The more heat-stable inhibitor, Bowman-Birk, should not be discounted when determining adequacy of heat treatment in high-temperature, short-time processing of soymilk.

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