protecting crude oils from oxidation is not entirely clear, and it is not known whether they act as true antioxidants, as metal inactivators, or as synergists in conjunction with naturally occurring tocopherols. Nonetheless, results in Figure 1 show that degumming of hexane-extracted crude oil lowers oxidative stability and that addition of soy phosphatides to $SCCO_2$-extracted crude oil markedly improves oxidative stability. Although further work is required to elucidate the mechanism by which phosphatides protect crude oils, they may act as oxygen barrier at the oil/air interface and thus reduce the rate of oxygen uptake by the sample. However, it should be pointed out that a high level of tocopherol in the absence of phosphatides is not sufficient to protect crude $SCCO_2$-extracted oils and that they should not be stored for extended periods.

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⁂Development of a Pilot Plant Process for the Preparation of a Soy Trypsin Inhibitor Concentrate[1]

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ABSTRACT

A pilot-plant procedure was developed to prepare a soy trypsin inhibitor (TI) concentrate in sufficient quantities to support a life-time (2-yr) feeding trial in which diets containing varying amounts of TI would be fed to rats to assess the physiological effects on the pancreas and other organs. Starting with water dispersions of commercial defatted soy flour, separation of TI (MW<21,500) from non-TI protein (MW 180,000-350,000) by virtue of their MW difference was attempted using ultrafiltration techniques but was not successful. However, good separation was obtained when selective acid precipitation coupled with "salting in" of the TI with 0.1 N sodium chloride was employed. Low MW components were separated successfully by ultrafiltration using a 1,000 MW cutoff membrane. The final soy TI concentrate obtained by freeze drying exhibited a 9-fold increase in TI activity.

INTRODUCTION

Naturally occurring proteinase inhibitors are substances that exist in a wide variety of food crops and other foods, such as soybeans, potatoes, lima beans and egg whites (1,2). Many short-term animal feeding studies have shown that raw soybean meal and purified soybean trypsin inhibitors (TI) inhibit growth and enlarge the pancreas in certain monogastric animals (3,4). Very limited information (5) is available on the effect of various levels of TI in the diets of rats over a life-time (2-yr) feeding trial because of the considerable costs generated over the 2-yr period and the general unavailability of sufficient quantities of TI concentrate from various food sources. Previous work by the USDA involved processing 24,000 lb of potatoes at two locations (ERRC and NRRC) to produce 34 lb of a potato TI concentrate (6). It appeared desirable to test, at the same time, the effects of TI isolated from soybeans. The purpose of this study was to develop pilot-plant procedures to prepare a sufficient quantity of TI concentrate from soy to sustain a 2-yr rat feeding trial.

MATERIALS AND METHODS

Commercial defatted soy flour Nutrisoy 7B (Archer Daniels Midland, Decatur, Illinois) was used as the starting material in all experiments. The commercial process uses a mild heat treatment and results in minimal denaturation of protein as evidenced by the nitrogen solubility index of 82.6 (Table I). Most of the TI activity of the raw beans is retained in the soy flour. Chemicals used in cleaning and sanitizing process equipment surfaces were Foam-nox, Status, AC-101 and XY-12 (Klenzade Division, Economics Laboratories, St. Paul, Minnesota). Ultrafiltration modules were cleaned with Ultrazyme (Osmonics Inc., Minnetonka, Minnesota).

Trypsin inhibitor was determined by the method of Hamerstrand et al. (7), which evolved primarily from the work of Kakade et al. (8). Total solids were determined by evaporation. Bacterial plate counts were made on standard methods agar (Baltimore Bacteriological Laboratories, Baltimore, Maryland) and incubated 3 days at 28 C. Nitrogen, ash and nitrogen solubility index were determined by official AOCS methods (9).

Equipment and Procedure

Acid precipitation of protein curd. Defatted soy flour (50 lb) was suspended in 10 to 15 parts of water in a 130-gallon stainless steel agitated tank fitted with an internal coil for heating or cooling. Slurry temperature was varied from 7 C to 49 C. TI was "salted in" by the addition of sodium chloride or magnesium chloride to 0.1 N. Protein curd was precipitated by the addition of dilute sulfuric acid (pH range 3.7 to 5.0). The protein curd was separated from

TABLE I

Composition and Microbial Population of Commercial Defatted Soy Flour

Component		Plate count per gram	
Moisture, %	6.6	Aerobic bacteria	37,500
Ash, %	6.0	Anaerobic bacteria	1,400
Nitrogen, %	8.0	Molds	120
Protein (N X 6.25), %	49.8	Yeast	0
NSI	82.6		
Trypsin inhibitor mg/g	27.6		

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the whey on a 26-in.-diam solid bowl (2,400 rpm) centrifuge (Tolhurst, Ametek Inc., Temecula, California). The whey was polished on a 4-in. solid bowl (17,000 rpm) centrifuge (Sharples, Pennwalt Corp., Warminister, Pennsylvania). The polished whey was pH adjusted (range 2.4 to 4.2) and ultrafiltered over a pair of 55-sq.-ft polysulfone, spiral wound modules (Osmonics Inc., Minnetonka, Minnesota) with a molecular-weight cutoff (MWCO) of 1,000. The permeate was discarded and the retentate, which contained the TI activity, was freeze-dried in a 5-shelf, 50-lb-capacity freeze dryer (Virtis Co., Gardiner, New York).

Membrane separation of non-TI protein. Defatted soy flour (30 lb) was suspended in 10 parts of water, adjusted to pH 8.5 with dilute sodium hydroxide and stirred 1 hr at ambient temperature. The residue was separated in a 4-in.-diam solid bowl (17,000 rpm) centrifuge and discarded. The supernatant was ultrafiltered over a 100,000 MWCO spiral-wound module. The -100,000 permeate was ultrafiltered and concentrated over a 1,000 MWCO module, and the -1,000 permeate from this step was discarded. The +1,000 retentate was freeze-dried and analyzed for TI.

RESULTS AND DISCUSSION

Separation of TI (MW<21,500) from non-TI protein (MW 180,000-350,000) by virtue of the difference in molecular size was attempted using an ultrafiltration module with a 100,000 MWCO. The non-TI protein, because of its larger molecular size, was rejected by the membrane as expected, but the much smaller TI molecule, which was expected to pass through the membrane, was for the most part also rejected. If the TI was being retained because of association with the non-TI protein, the separation might be improved by varying the pH or the concentration of the feed to the UF. When this was attempted, the TI was retained as before. Likewise, varying the ionic strength of the feed solution by addition of sodium chloride up to 1.0 N did not improve the separation.

Solute retention in ultrafiltration occurs at the surface of a very thin skin, which determines the selectivity of the membrane. As in filtration, the size and shape of the particle being filtered determines the ability of the filter to retain it. Fallick (10) has shown dextran of 110,000 MW to permeate a 10,000 MWCO membrane. He explained that apparent contradiction by the fact that the membranes usually are characterized with globular protein, having a larger molecular cross section than the higher MW, but linear dextran molecule. Although our problem was just the opposite, i.e., no penetration where penetration was expected, perhaps the TI molecule behaves as though it were presenting a larger cross-sectional area to the membrane.

On the other hand, using UF techniques to separate the TI from low MW impurities (sugars, ash, etc.) presented no particular problem using a 1,000 MWCO module, and good separations were obtained by this method.

Separation by Selective Acid Precipitation

Anderson et al. (11) have shown that extraction of soybean protein increased with sodium chloride concentration. Previous unpublished work at this Center had shown that when precipitating protein from water dispersions of defatted soy flour, TI was effectively "salted in" to the whey by addition of sodium chloride to 0.1 N. In our current experiments we found that by adding sodium chloride to 0.1 N, we increased the yield of TI in the whey from 48 to 71% using a 15:1 water-to-meal ratio and precipitating at pH 4.2.

In our early experiments, we precipitated the non-TI protein at pH 5.0. The whey then was passed over a 1,000 MWCO UF module, which rejected both the TI and non-TI protein but allowed the low MW impurities, such as sugars and ash, to pass into the permeate. The retentate was freeze-dried to obtain a crude concentrate that contained 10-14% TI. It was hoped that the soy TI concentrate would have a purity at least as good as the potato TI concentrate (25%) prepared previously (6).

A series of experiments was conducted in which the curd was precipitated over a range of pH values that varied from 5.0 to 3.7. At pH 4.2, a significant increase in purity of the crude isolate was observed. Apparently, more of the non-TI protein was being precipitated at pH 4.2, which improved the ratio of TI to non-TI protein in the whey (Table II). Although good purity was obtained at pH 3.7, the yield was observed to decrease slightly, indicating perhaps that TI was beginning to be precipitated with the curd. When magnesium chloride was substituted for sodium chloride at 0.1 N, a considerable quantity of non-TI protein was "salted in"; in the resulting whey, the purity of the soy TI concentrate was reduced to only about 7%. No further experiments were run with magnesium chloride.

Effect of Slurry Concentration and Temperature on Yield

When defatted soy flour was slurried in 0.1 N sodium chloride at pH 4.2 at a water-to-flour ratio of 10:1, 38% of the TI was recovered in the whey. By simply increasing the water-to-flour ratio to 15:1, the recovery of TI was increased to 58%. When the curd was reslurried in 0.1 N sodium chloride at pH 4.2 at a 5:1 water-to-flour ratio, an additional 13% of the TI was recovered in either case, or 51% at 10:1 and 71% at 15:1.

Defatted soy flour was slurried in 0.1 N sodium chloride at 15:1 water/flour at pH 4.2 for 1 hr, at temperatures ranging from 7 C to 49 C. The curd was reslurried in 0.1 N sodium chloride at 5:1 water/flour ratio. The best yield (71%) was obtained at 27 C (Table III). At 49 C the yield fell off to 50%, although the purity was essentially the same. This would seem to indicate that the "salting in" effect is somewhat diminished at 49 C. However, at 7 C both yield and purity are adversely affected; the yield fell to 48% and the purity was 146 mg/g, which is only about half that obtained at the higher temperatures. The amount of non-TI protein appearing in the whey was increased significantly at the lower temperatures.

TABLE II

Effect of Slurry pH on TI Yield and Purity (Slurry 15:1 1 hr at 25 C, Reslurry 5:1 0.1 N NaCl)

pH	Yield %	TI purity mg/g
5.0	67	170
4.6	69	210
4.2	71	259
3.7	68	256

TABLE III

Effect of Slurry Temperature on Yield (Slurry 15:1 Water/Flour in 0.1 N NaCl for 1 hr, 5:1 Reslurry)

Temperature C	TI yield %	TI purity mg/g
7	48	146
27	71	259
49	50	246

TABLE IV

Effect of Processing Conditions on Bacterial Population in Soy TI Concentrate

pH			
Curd precipitated at	Whey adjusted to	UF technique	Aerobic bacteria per gram
4.2	4.2 (as is)	Overnight UF	9,000,000,000
4.2	4.2 (as is)	Short cycle UF	1,000,000
4.2	3.7	Short cycle UF	40,000
4.2	2.4	Short cycle UF	15,000

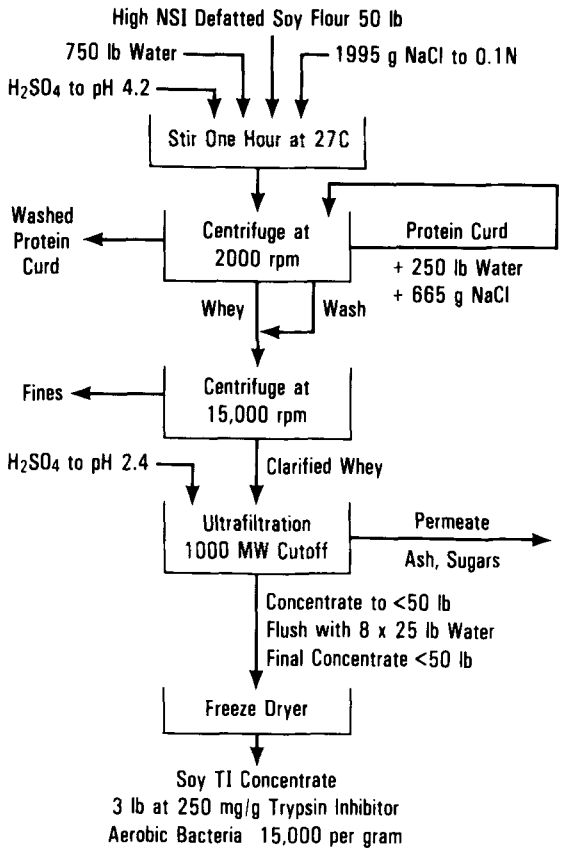

FIG. 1. Pilot-plant process for the preparation of a soy trypsin inhibitor concentrate.

Effect of UF Technique on Purity of Soy TI Concentrate

When the whey was fed to the 1,000 MWCO UF module to remove low MW impurities in the permeate, the feed tank was fitted with a level control; the feed volume was maintained constant by continuous water addition and allowed to run overnight. This overnight continuous water flushing produced a soy TI concentrate with a purity of 355 mg/g. However, the product was found to be heavily contaminated with microorganism (Table IV). The overnight flushing caused the pH to approach neutral and, together with the ambient temperature, provided an ideal environment for bacterial growth. Consequently, the UF cycle was shortened to approximately 4 hr by immediate concentration of the whey, followed by a series of short flushes. The microbial count was significantly reduced, although the purity of the soy TI concentrate was reduced to 259 mg/g. By reducing the pH of the whey below 4.2 after curd separation and before the UF step, the microbial population was reduced further (Table IV). The lowest bacterial contamination was observed when ultrafiltration was conducted at pH 2.4.

TABLE V

Composition of Freeze Dried Soy Trypsin Inhibitor Concentrate

Component		Plate count per gram	
Moisture, %	3.6	Aerobic bacteria	15,000
Ash, %	3.7	Anaerobic bacteria	3,500
Protein (N X 6.25), %	77.1	Molds	200
Trypsin inhibitor mg/g	255	Yeast	4,000

PILOT PLANT PREPARATION

Conditions found effective for the pilot-plant preparation of a soy TI concentrate from commercial defatted soy flour are: *Yield*—1. "Salt in" TI with 0.1 N sodium chloride; 2. Slurry defatted soy flour at 27 C; 3. Slurry soy flour with 15 parts water, and 4. Reslurry curd with 5 parts water; *Purity*—1. Precipitate curd at pH 4.2; 2. "Salt in" TI with 0.1 N sodium chloride, and 3. Overnight water flushing of whey on UF; *Bacterial Population*—1. Short cycle UF, and 2. Whey adjusted to pH 2.4 for UF.

The final procedure involved compromising the purity of the soy TI concentrate somewhat by employing the short-cycle UF procedures (Fig. 1). This, together with adjusting the pH of the whey to 2.4 before the UF step kept the microbial population at acceptable levels. The composition of freeze dried soy trypsin inhibitor concentrate from a typical run is given in Table V. Sufficient material to support a 2-yr rat feeding trial was produced using these procedures, and the feeding studies are in progress at this time.

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R.L. Brown conducted the pilot-plant experiments; L.T. Black, J.D. Glover, R.L. Haig, P.L. Milroy, I.M. Schulte and M.E. Riggen ran the analyses and R.F. Rogers and C. Featherston made the microbial plate counts.

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Photodecomposition of Gossypol by Ultraviolet Irradiation

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ABSTRACT

Decomposition of gossypol as a thin film or as a solution by ultraviolet irradiation was studied. The decomposition of gossypol followed monophasic exponential kinetics in which the rate of decomposition varied and depended upon the irradiation condition. The lowest rate of gossypol decomposition was observed as a thin film which showed a half-life of 97 min, while the highest rate was attained as a solution in acetone as indicated by a half-life of 4.5 min. Solutions in methanol and ethanol showed relatively lower rates of decomposition with similar half-lives of approximately 50 min. Acetonitrile and chloroform solutions showed intermediate rates of decomposition for gossypol with half-lives of 15 and 19 min, respectively. Although the degradation products of gossypol were not identified, their HPLC profiles were characteristic of the solvent used. HPLC profiles of gossypol degradation products in methanol, ethanol, acetone and acetonitrile were similar, each exhibiting two peaks with variable ratios depending on the solvent and the time of exposure. The degradation products of gossypol when irradiated as a thin film and as a solution in chloroform were different from those in other solvents. In all cases, when gossypol and/or its degradation products were continuously exposed to ultraviolet radiation, they decomposed to products no longer having an aromatic structure.

INTRODUCTION

Gossypol [1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl (2,2′-binaphthalene)-8,8′-dicarboxaldehyde] is a yellow coloring matter present in various parts of cotton plants (1-3). Gossypol causes many deleterious effects to nonruminant animals, a characteristic which limits the use of cottonseed meal as a source of protein for animals and humans (1-3). It uncouples oxidative phosphorylation of rat liver mitochondria in vitro (4).

Recently, gossypol was found to cause reversible male infertility (5). This was first observed in humans, and later it was found that some animal species are sensitive (e.g. rats and hamsters) while others are not (e.g. mice and rabbits) (5-7). The compound is being tested as a male contraceptive (5) and as a vaginal spermicide (8). It has been suggested that gossypol might be used to lower plasma cholesterol levels (9) and for treatment of Chagas disease, which is caused by *Trypanosoma cruzi* (10). Furthermore, gossypol inactivated influenza, parainfluenza-3, and herpes simplex viruses (11,12) and showed general antifungal (13), antibacterial (14) and antitumor activity (15).

Recent studies from this laboratory showed that gossypol was highly unstable when stored in solution at room temperature or upon refrigeration at 4 C. The instability was highly dependent on both the storage temperature and the solvent used (16). The rate of decomposition of gossypol increased in the following order with respect to the solvent: acetone < acetonitrile < chloroform < ethanol < methanol. This communication reports on the continued investigation of the stability of gossypol upon irradiation by ultraviolet light when the gossypol is a thin film or a solution in any of the five solvents used in the previous investigation.

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EXPERIMENTAL

Reagents

Gossypol acetic acid was purchased from Sigma (St. Louis, Missouri). Its purity was determined by high performance liquid chromatography (HPLC) to be 99.5%. All solvents and reagents used were HPLC grade and obtained from Fisher Scientific (Raleigh, North Carolina).

Ultraviolet Lamp

An Ace-Hanovia medium pressure mercury lamp (approximately 100 watts) was purchased from Ace Glass Inc. (Louisville, Kentucky). The lamp was reported by the manufacturer to emit ultraviolet enery at the following wavelengths: 185, 238, 248, 254, 265, 280, 297, 302, 313 and 366 nm.

Exposure of Gossypol to Ultraviolet Radiation

Gossypol solutions were made freshly at a concentration of 1 mg/ml in methanol, ethanol, chloroform, acetonitrile and acetone. Solutions were placed in a 3 ml quartz spectrophotometric cuvette (Fisher Scientific Products, Raleigh, North Carolina) mounted on a 30 × 10 cm glass plate by pieces of styrofoam and white sticking tape. The glass plate was inserted into a 29 × 25.5 × 9.5 cm glass thin layer chromatography (TLC) jar in which the ultraviolet lamp also was inserted in a position directly facing the cuvette with a constant distance of 1.4 cm between the lamp and the cuvette. Only one side of the cuvette was exposed to the ultraviolet radiation. Water was circulated in the TLC jar (by means of a water tube in the bottom of the jar and a water vacuum pump hose mounted on the internal side of the jar just below the top level of the quartz cuvette) to maintain the temperature below 20 C. The TLC jar was heavily wrapped in aluminum foil, and the experiment was run inside a highly efficient hood. At various time intervals (Fig. 1 and 2), the lamp was turned off, and 100 μl of gossypol solution was taken from the cuvette from which volumes of 1-5 μl were analyzed by HPLC. Because of acetone's high absorbance at 254 nm, this solvent was first evaporated by a gentle stream of dry nitrogen, and the residue was dissolved in 100 μl of methanol prior to HPLC analysis. Six determinations were made from three separately exposed cuvettes for each solvent at each time interval.

Gossypol was spread as a thin film on the surface of 3 × 1 cm glass plates at a concentration of 100 μg/cm^2. The compound was first dissolved in a mixture of ethanol/ether 1:1 (v/v) at a concentration of 300 μg/10 μl. From the resulting solutions, 10 μl was spread as evenly as possible on the glass plate by a capillary tube, then the solvent