Toasted Soybean Flour Components with Trypsin Inhibitor Activity[1]

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Compounds in toasted soybean flour having trypsin inhibitor (TI) activity were isolated and characterized. Sodium hydroxide (0.01N) extracts of toasted soybean flour had an average of 2.59 mg TI/g sample. These extracts, after trichloroacetic acid (TCA) precipitation and dialysis, yielded supernatant and precipitate fractions. Addition of polyvinylpyrrolidone to eliminate free tannins and phenolics in the extracts, which may lead to overestimation of TI activity, was unnecessary. Material balance studies revealed 91% protein recovery and 92% recovery of TI activity in the TCA supernatant (1.1% protein, 2.0% TI) and precipitate (89.8% protein, 90.0% TI) fractions. Column chromatography and electrophoresis showed the TCA supernatant and precipitate fractions to contain proteins, including those having TI activity. Kunitz type TI and Bowman-Birk type protease inhibitors accounted for most residual TI activity of toasted soybean flour, as verified by column chromatography, isoelectric focusing, sodium dodecyl sulfate polyacrylamide electrophoresis, and size-exclusion high performance liquid chromatography, using the two similarly treated protease inhibitors as standards. Immunoblotting was also used to detect and identify Kunitz type TI's in toasted soybean meal extracts. This study established the proteinaceous nature of residual trypsin inhibitor activity in toasted soybean flour and the presence of both Kunitz and Bowman-Birk inhibitors.

Long term (2-yr) rat feeding studies, referred to as the USDA-Trypsin Inhibitor Study, were initiated to investigate the physiological effects on rats of trypsin inhibitors (TIs) in edible soybean flour and protein isolates processed under different conditions (1). Prevalent pancreatic lesions, both nodular hyperplasia and acinar adenoma, were found in Wistar rats fed soybean flour or soybean protein isolate diets. Diets containing raw, commercially toasted and over-toasted soybean flour, as well as raw and heated soybean isolates, yielded positive linear dose response relationships between diet and incidence of pancreatic lesions. In general, heat treatment of soybean flour at 100 C for 20 min inactivates 90% of TI (2); all commercial processes retain some residual TI activity, however. The low TI activity of commercially processed soybean meals may represent residual native TI of the raw meal or modified types; in addition, artifacts may arise during processing or isolation, and tannins, phenolics or phytates may appear to inhibit trypsin in the colorimetric assay (3). Toasted soybean flour still caused pancreatic lesions, though at a much lower incidence than did raw soybean flour in the long term feeding study (1). Therefore, characterization of protease inhibitors in these products is imperative.

There are two major types of soybean protease inhibitors: Kunitz inhibitors have activity against trypsin, and Bowman-Birk inhibitors inhibit both trypsin and chymotrypsin. Orf and Hymowitz (4) and Freed and Ryan (5) reported three genetic variants of soybean Kunitz inhibitors; Stahlhut and Hymowitz (6) and Tan-Wilson et al. (7) also have reported 5–10 Bowman-Birk isoinhibitors in seven soybean strains. In addition, Hwang and coworkers (8) isolated five low molecular weight protease inhibitors from "Tracy" soybeans which possess TI activity and weak chymotrypsin inhibitor activity. Inhibitor TI:V of Hwang et al. (8), inhibitor E of Tan-Wilson et al. (7), and inhibitor III of Stahlhut and Hymowitz (6) have been identified as the Bowman-Birk inhibitor.

Using trichloroacetic acid (TCA) as a precipitant for soybean proteins, Hafez and Mohamed (9) reported that 20 to 57% of the total TI activity resulted from non-protein components. Such high non-protein TI activity appears questionable, however, when total TI activity due to proteins and distribution of soybean protease inhibitors (2) are considered. The purpose of this work was to better characterize components having TI activity in the commercially toasted soybean flour used in the USDA Trypsin Inhibitor Study.

EXPERIMENTAL PROCEDURES

Materials. Toasted Nutrisoy®, a commercially toasted soybean flour (protein, 51.8%; moisture, 7.5%; ash, 6.3%; TI, 4.1 mg/g), was purchased from Archer Daniels Midland, Decatur, Illinois. Heat treatment of this flour is described elsewhere (1).

Specialty chemicals and electrophoretic and chromatographic materials were obtained as follows: DEAE-BioGel A and Immuno-Blot Assay Kit—Goat Anti-Rabbit IgG Horseradish Peroxidase Conjugate, Bio-Rad Laboratories, Richmond, California; α-N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA), ICN Nutritional Biochemicals, Cleveland, Ohio; low pI range isoelectric focusing calibration kit, Pharmacia Fine Chemicals, Piscataway, New Jersey; Fast Blue B salt and Servalyt Precotes 3-6 and 4-7, Serva Fine Biochemicals Inc., Garden City Park, New York; bovine serum albumin, α-chymotrypsin Type VII, Folin & Ciocalteu phenol reagent, N-acetyl-DL-phenylalanine β-naphthyl ester, SDS-Dalton Mark VII-L molecular weight standards, type 1-S lyophilized soybean trypsin inhibitor, and trypsin, type III-s from bovine pancreas, Sigma Chemical Co., St. Louis, Missouri; Spectrophor 3 dialysis membrane (molecular weight cutoff 3500), VWR Scientific, Bellwood, Illinois.

Inhibitor isolation. To determine the nature of TI activity in extracts of commercially toasted soybean flour and to examine non-protein constituents which have TI activity, we used a modified version of the Hafez and Mohamed (9) TCA method, and performed a material balance study to account for TI activity with each treatment and extraction step. Our fractionation scheme, Figure 1, included extraction of protease inhibitors from toasted Nutrisoy (10 g) with 0.01 N NaOH at a meal to solvent ratio of 1:20 (w/v). To optimize TCA concentration for precipitation of proteins, we used bovine serum albumin and commercially purchased soybean TI as standards. Free tannins and phenolics, which may interfere with the colorimetric TI assay (3), were eliminated by including 5% polyvinylpyrrolidone with TCA in the protein precipitation step. A portion of the original extract was

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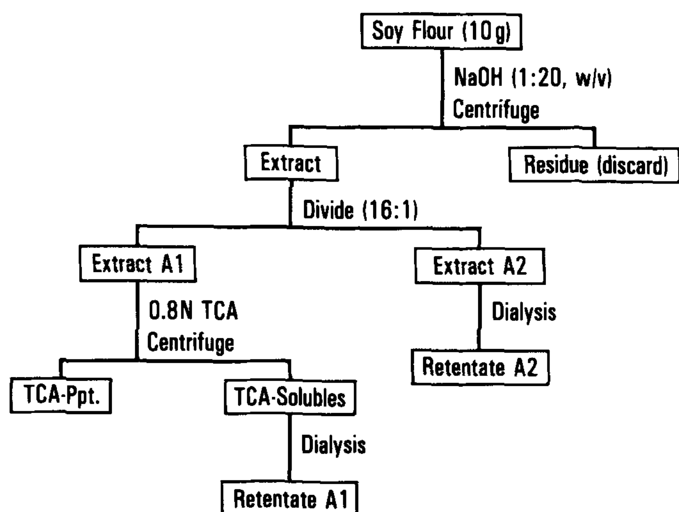

FIG. 1. Fractionation of toasted soybean flour extract with trichloroacetic acid (TCA).

dialyzed before TCA treatment (i.e. retentate A2) to determine whether low molecular weight constituents with TI activity and no TCA treatment were lost upon dialysis.

Components of TCA-soluble and insoluble fractions from toasted soybean flour (100 g) were fractionated on DEAE-Bio Gel A columns by the gradient elution scheme of Frattali (10). Column eluates consisting of 5.6 ml/tube were monitored at 280 nm absorbance and for TI activity. Eluate fractions having both 280 nm absorbance and TI activity were pooled and dialyzed extensively against deionized, distilled water; retentates were lyophilized.

For chromatographic and electrophoretic standards, Bowman-Birk protease inhibitor was isolated from raw Nutrisoy 7B, a commercial raw soybean flour, and purified according to the methods of Frattali (10). Commercial soybean Kunitz TI was purified on DEAE-BioGel A, equilibrated with 76 mM TRIS-HCl, pH 8.6, by elution with a 0–0.3 M NaCl gradient in the same buffer used as a standard.

Assays for protein and TI activity. Protein content was estimated by the Lowry method (11) using bovine serum albumin as a standard.

TI activity was assayed and quantified as described by Hamerstrand et al. (3), using BAPA as substrate for trypsin.

Polyacrylamide gel electrophoresis (PAGE). Discontinuous PAGE was performed on a vertical slab apparatus (model 2001LKB) with gels (13% T, 3.0% C) using TRIS-glycine electrode buffer (0.038 M glycine, 0.005 M TRIS, pH 7.8) at both electrodes. Electrophoresis was performed at 10 C with 60 mA until bromophenol blue tracking dye reached the bottom of the running gel. Gels were stained for protein with 0.023% Coomassie brilliant blue R-250 dissolved in a solution containing 19.5 g TCA in 460 ml acetic acid/methanol/water (30:85:345, v/v/v), and destained by gentle rocking in acetic acid/methanol/water (115:685:1600, v/v/v).

Isoelectric focusing was performed on Servalyt Precotes 3-6 and 4-7 at 10 C using a flat bed FBE-3000 apparatus (Pharmacia Fine Chemicals) and a constant power supply (3 watts, 3 milliamps, and final voltage 1700). Focused gels were stained for protein by the formaldehyde:Coomassie brilliant blue G-250 procedure of Trah and Schleyer (12). For ultrathin gels, replacement of formaldehyde with 5% glutaraldehyde better fixed low molecular weight peptides, which tend to solubilize along with ampholytes during fixing and staining. pI's were estimated by comparing mobilities of proteins from column fractions to purified proteins of known pI. Components with protease inhibitor activity were detected on focused gels by the methods of Chavan and Hejgaard (13), except that gels were developed at room temperature.

Molecular weight estimation. Molecular weights of isolated weights of isolated protease inhibitors were estimated by SDS-PAGE (14) by comparison to known standards, using log Rf vs molecular weight plots for 11, 13 and 15% T gels, with an acrylamide to methylene bisacrylamide ratio of 39:1 (w/w).

Solutions of protease inhibitors, diluted 1:1 v/v with either 1% SDS in 0.2 M sodium phosphate buffer pH 7 (native) or 1% SDS in buffer containing 0.1% β-mercaptoethanol (reduced) and incubated 2 hr at 50–60 C, were evaluated by size-exclusion HPLC (15) on a TSK 2000 SW column (500 mm by 7.5 mm I.D., Varian Associates, Walnut Creek, California), monitoring the column effluent at 210 nm. Molecular weights were estimated from plots of standards (log molecular weight versus elution volume) run under identical conditions.

Immunological detection of Kunitz TI. Protein components in fractions from DEAE-BioGel A column eluates, which had been separated first by PAGE on gels containing SDS (14), were transferred onto nitrocellulose membranes according to operating instructions for the TE 42 transphor electrophoresis cell (Hoefer Scientific Instruments, San Francisco, California). Membrane bound antigen was then incubated with rabbit antibody for Kunitz TI, washed with 0.05 M Tris-HCl, pH 7.5, containing 0.2 M NaCl, to remove unbound antibody, incubated with goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate and washed again. HRP bound substrate was then detected with 4-chloro-1-naphthol. Details of the procedure are outlined in the BIO-Rad IMMUN-BLOT™ (GAR-HRP) ASSAY KIT (Bio-Rad Laboratories, Richmond, California).

RESULTS AND DISCUSSION

Inhibitor characterization. Concentration of TCA needed to optimize precipitation of soybean protein and Kunitz TI activity was 0.8 N. Under these conditions, TCA solubles from commercial Kunitz TI contained 4% of the total protein and 5% of total TI activity. PAGE revealed that TCA-soluble TI activity resided in low molecular weight protein contaminants, and did not result from incompletely precipitated Kunitz TI. The proteinaceous nature of these contaminants was evident from the coincidence of bands stained with Coomassie blue and of bands negatively stained for TI activity.

A material balance for protein content and TI activity from TCA-treated toasted soybean flour extracts is shown in Table 1. Little loss of protein and no loss of TI activity occurred after dialysis of the original extract (retentate A2). Thus, dialyzable non-protein components in toasted soybean flour extracts contribute little to TI activity. We recovered 97.7% of the protein and 91.5%

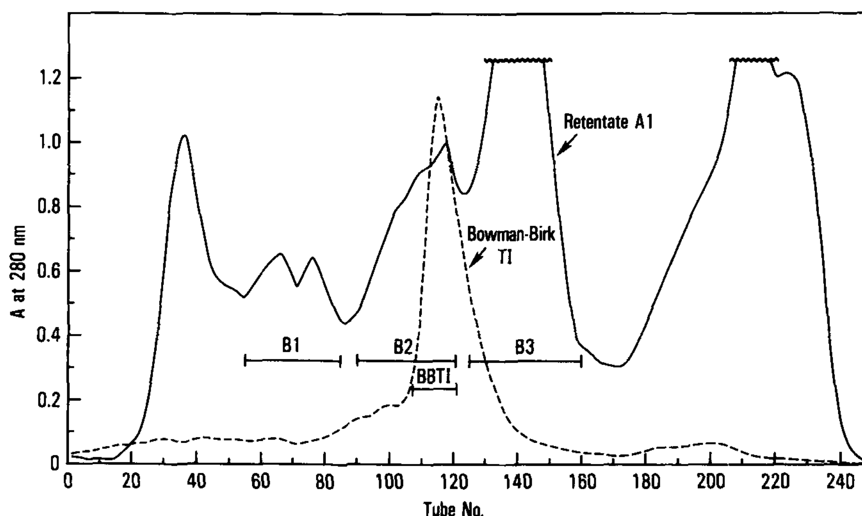

FIG. 2. Elution profiles of TCA-soluble retentate A1 from toasted soybean flour (complex elution pattern) and soybean Bowman-Birk protease inhibitor (profile with one major peak) on DEAE-BioGel A. The 250 ml (2.0 cm id.) column resin bed was eluted with a salt, pH gradient (10) at 32 ml/hr, collecting fractions of 5.6 ml.

TABLE 1

Protein Content and TI Activity of Fractions from TCA Treated Toasted Soy Flour[a]

Fraction	mg Protein/ 10 g Sample	mg TI/ 10 g Sample
Extract	2451 (100)[b]	25.9 (100)[b]
Retentate A2	2319 (94.6)	27.8 (107)
TCA ppt.	2201 (89.8)	23.3 (90.0)
TCA solubles	194 (7.9)	0.4 (1.5)
TCA retentate A1	27 (1.1)	0.5 (1.0)

[a]See Fig. 1 for identification of fractions.

[b]Percent of original extract. All values represent average of three replicates with an error of 9.9% for protein and 14.4% for TI.

of the TI activity in the TCA precipitate and solubles. Addition of 5% (by weight) polyvinylpyrrolidone (PVP) during TCA precipitation did not change protein recovery or TI activity. Therefore, free tannins and phenolics in toasted soybean flour extracts do not significantly interfere with the colorimetric assay for TI activity or contribute to residual TI activity of toasted flour using our extraction conditions. In subsequent preparations of TI active components, we therefore did not add PVP. Upon dialysis of the TCA solubles, loss of 86% Lowry positive components occurred, with no loss of TI activity. This observation is contrary to that of Hafez and Mohamed (9), who reported high TI activity in TCA solubles from raw meal extracts and no Lowry positive components. Our TCA soluble retentates from toasted meal possessed both Lowry positive components and TI activity. Dialysis casings used in our study have a 3500 mol wt cutoff and would therefore retain most low mol weight proteins and large peptides. The good correlation of protein estimates with TI activity in all fractions from toasted soybean flour extracts, plus retention of TI activity in retentate A2 (Fig. 1), suggest that both TCA-soluble and precipitated TI activity reside in proteins.

Protein fractionation. Retentates from dialysis of TCA solubles were separated by DEAE-BioGel A chromatography with a salt gradient (10) (Fig. 2). Two minor 280 nm absorbing peaks combined as fraction B1 (tubes 55–85), and one major peak B2 (tubes 90–121), which elutes at the position of Bowman-Birk inhibitor standard chromatographed under the same conditions, possessed TI activity. A third peak, B3 (tubes 125–160), had low TI activity and eluted similarly to soy Kunitz TI (results not shown). Fractions were labeled *B* for Bowman-Birk type versus *K* for Kunitz type inhibitors. Pooled tube fractions B1, B2 and B3 were dialyzed against distilled water and lyophilized. Contents of tubes 170–250, unlabelled and representing a large UV absorbing peak, possessed no TI activity and were discarded.

TCA precipitates, upon DEAE-BioGel A chromatography, gave complex elution patterns (not shown) having numerous peaks in tubes 1–200 and a major broad peak with no TI activity in tubes 250–350. Pooled eluates from tubes 250–350, upon PAGE, yielded bands with mobilities of 7S and 11S storage protein standards. TI activity was detected in tubes 56–102 and 105–155, which were pooled into fractions K1 and K2, respectively.

Immunochemical detection of Kunitz TI. Fractions B3 and K2 were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were then treated with antisera for Kunitz TI (Fig. 3). TCA precipitate fraction K2 contained major amounts of Kunitz TI (20,000 Da) along with smaller amounts of higher molecular weight (40,000–60,000 Da) components which may be polymeric forms of Kunitz TI. TCA-soluble fraction B3 does not react with Kunitz TI antisera, showing that the low TI activity in fraction B3 is therefore not due to Kunitz TI.

pI determination. Column fractions with TI activity were subjected to isoelectric focusing and negatively stained for TI activity (Fig. 4). TCA precipitated fractions K-1 and K-2 contained components with the isoelectric points of Kunitz (pI 4.55) and Bowman-Birk (pI 4.20) pro-

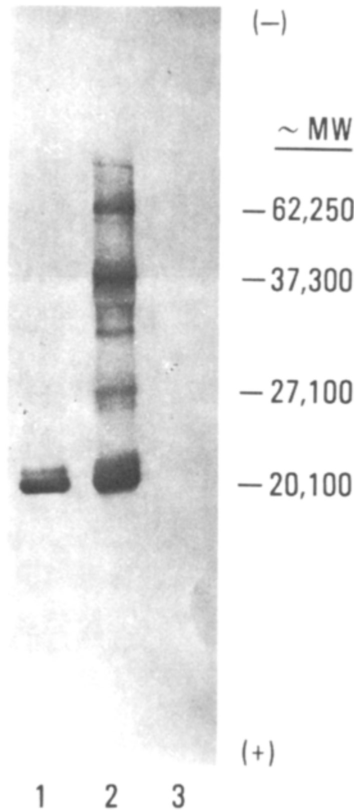

FIG. 3. Electrophoretic transfer of DEAE-BioGel A column fractions of soybean protease inhibitors from TCA-treated, toasted soybean flour extracts from SDS-PAGE gels blotted onto nitrocellulose membranes. The membrane was reacted with soybean Kunitz TI rabbit antiserum followed by goat anti-rabbit IgG HRP conjugate and a chromagenic agent for HRP. Lane 1, soybean Kunitz TI; 2, K-2; 3, B-3.

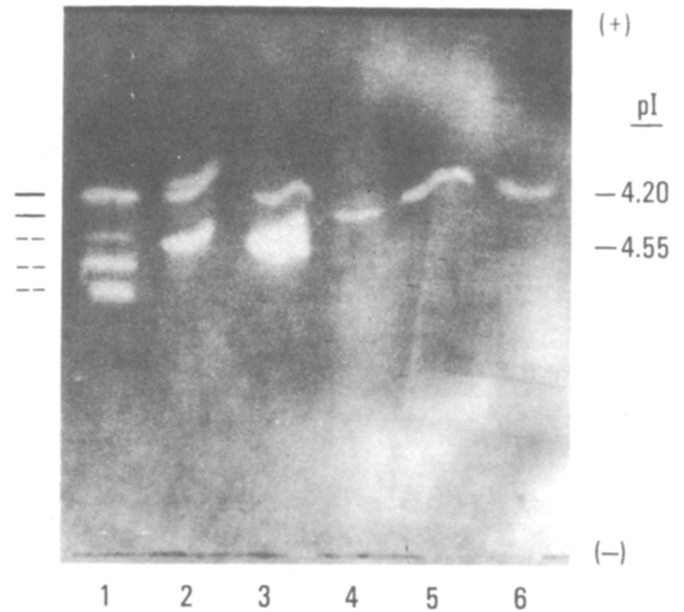

FIG. 4. Isoelectric focusing of DEAE-BioGel A column fractions of soybean protease inhibitors from TCA-treated, toasted soybean flour extracts. TI's were visualized by negative staining (13). Lane 1, K-1; 2, K-2; 3, Bowman-Birk inhibitor (BBI) + Kunitz TI (KTI); 4, B-1; 5, B-2; 6, B-3; 7, BBI + KTI. Designation: — BBI; − − KTI.

tease inhibitors. K-1 in addition to Bowman-Birk and a small amount of Kunitz protease inhibitors contained two additional TI-positive components. When a duplicate gel was stained for chymotrypsin activity, only those components having mobility of the Bowman-Birk inhibitor stained positively (results not shown). The TCA-soluble fractions B-1 and B-2 possessed only components corresponding to Bowman-Birk type inhibitors. Apparently 0.8 N TCA incompletely precipitates constituents with the mobility of Bowman-Birk inhibitors, which react with both trypsin and chymotrypsin. Thus, isoelectric focusing indicates at least three Kunitz type inhibitors and two Bowman-Birk type inhibitors.

Molecular weight estimation. Based on amino acid sequence, the mol wt of Kunitz TI is 20,083 (16), and that of Bowman-Birk protease inhibitor is 7,848 (17). Upon SDS-PAGE, Kunitz TI had an apparent mol wt 2.2% lower than the literature value, while HPLC of Kunitz TI in native or reduced state gave molecular weights 14 to 17% lower than reported (Table 2). Bowman-Birk inhibitor aggregation was observed by both SDS-PAGE and HPLC, which suggested that both dimeric and monomeric forms were present. The monomeric form observed by HPLC had an apparent molecular weight 2–10% lower than reported (17). Foard et al. (18) also noted dimeric and trimeric aggregates of soybean

TABLE 2

Molecular Weight Estimates of Soybean Protease Inhibitors via SDS-PAGE and Size-Exclusion HPLC

Inhibitor	SDS-PAGE Reduced	HPLC Native	HPLC Reduced
Kunitz	19633	17200	16700
Bowman-Birk	14850	12000; 7700	11800; 7100
K-2	19675; 14925	16500; 8100	17133; 8975
B-2	14700	12667; 7750	14100; 8250

Bowman-Birk protease inhibitor upon SDS-PAGE. Despite the self association of the Bowman-Birk protease inhibitor upon SDS-PAGE or HPLC (Table 2), column fraction K-2 showed evidence for both Kunitz and Bowman-Birk protease inhibitors, while B-2 showed strong evidence for only the Bowman-Birk inhibitor (Table 2).

Our study defines residual TI activity in toasted soybean flour extracts as both Kunitz and Bowman-Birk type protease inhibitors. Other proteins with protease inhibitory activity were also noted, and currently are being characterized. Additional immunological methods for detection, identification and quantitation will be performed after antibodies to Bowman-Birk protease inhibitor are prepared. Since long term rat feeding studies with raw, toasted and overtoasted soybean flour treatments show a linear dose relationship for pancreatic lesion formation (1), our results are consistent with the hypothesis that attributes hyperplasia and tumor formation to the proteinaceous TI's. Methods will be developed

to inactivate the protease inhibitors, both in the purified state and in food systems.

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♣Heat Capacity of Tristearin in the Presence of Food Emulsifiers

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The specific heats of tristearin in the presence of some food emulsifiers were determined by differential scanning calorimetry. Solid emulsifiers show Cp curves different from those of pure tristearin, indicating that a new mixed crystal has been obtained through the incorporation of the surfactant within the fat.

The specific heat (Cp) of simple saturated triglycerides has been determined in previous investigations by differential scanning calorimetry (1). The heat capacities of the different polymorphic forms were recorded, and it was evident that in the unstable α form the Cp values are significantly higher than in the β stable form. Simpson, Hockett and Harris (2), comparing the specific heats of tristearin and trimargarin, showed a change in the slope of the heat capacity curves at 300 K for both α and β forms.

Following previous works (3), it was postulated that this break point represents the beginning of two-dimensional melting, which is involved in an increase of heat capacity rather than latent heat. This proposed mechanism is based on the existence of different bond strengths in the three dimensions of the lattice and is applied to lipids and liquid crystals because of their structure of layers which are composed of alkyl chain molecules with lateral strong bonds, but weaker interactions between the layers themselves. According to this theory, prior to melting, a gradual increase in dislocations is possible inside the layer and leads to defects, with no latent heat involved, but rather an increase in the heat capacity. It is well known from previous work that addition of fatty acid sorbitan esters at low percentages affects polymorphic behavior of fats and triglycerides; because of this kind of action, these surfactants are called "crystal structure modifiers." It was shown that in tristearin the α-β transition was prevented (4); similar results were obtained during aging of unstable forms in fats (5) and cocoa butter (6). From these works it is apparent that solid emulsifiers are efficient in affecting the polymorphic behavior of solid fats, while liquid emulsifiers are ineffective.

The present work uses similar food emulsifiers which previously were tested for their ability to prevent the α-β transition in tristearin; their effect on the heat capacity of tristearin also was checked. The purpose of this work is to extend and deepen our comprehension of the mechanism of action of the emulsifiers as crystal structure modifiers through understanding their mode of incorporation among the fat molecules.

MATERIALS AND METHODS

Tristearin was purchased from Sigma Chemical Co., St. Louis, Missouri, and was 99% pure. The emulsifiers were commercially available from Grindsted Products, Denmark, and Atlas Europol A.p.S., Italy.

The following types of additives were tested: sorbitan monostearate (Span 60), sorbitan tristearate (Span 65), sorbitan monolaurate (Span 20), ethoxylated sorbitan monostearate (Tween 60), glycerol monostearate, citric acid ester of monoglyceride (Acidan), triglycerol ester of stearic acid (3G1S), stearyl alcohol and stearic acid. All the additives were introduced at the level of 10 wt %, and the mixtures were heated to above melting point and blended well outside the DSC prior to any experiment.

The thermal measurements were performed on a Mettler Differential Scanning Calorimeter (TA3000) calibrated with indium, lead and zinc for accuracy of caloric data and temperature readings. The samples were