Minimizing Protein Insolubilization during Thermal Inactivation of Lipoxygenase in Soybean Cotyledons

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ABSTRACT

The objective was to develop a procedure for inactivating lipoxygenase in soybean cotyledons without losing protein solubility. The approach was to moisten cotyledons to one of 2 levels in soft water or carbonate buffers, steam for a short time, hold for a definite period at a known temperature, cool and analyze for enzyme activity and protein solubility. Temperature dependence of both inactivation and insolubilization kinetics was determined. Increasing temperature of steaming and holding favored our objective. At 16.3% moisture, the pH 9.8 buffer was beneficial but the pH 10.8 buffer was not. The holding period was not beneficial compared to steaming alone. Recommended conditions were adjustment of cotyledon moisture to 16.3% with pH 9.8 buffer and then heating in steam for about 10 sec; at temperatures of 91 C and above, 99% of the lipoxygenase could be inactivated with retention of over 70% protein solubility. The effect of buffers on kinetics of heat inactivation and insolubilization appeared to be related to the states of hydration water, i.e., the presence of solute water at the higher moisture content.

INTRODUCTION

Soybeans have been used in preparing various types of food in the Orient for many centuries (1). However, off-flavors described as painty or beany have limited the acceptability of these foods in other parts of the world. Using heat to inactivate lipoxygenase in the soybean greatly improved the flavor of soymilk (2-4). Rice et al. (5) found that steam-heat treatment of soybeans prior to oil extraction was beneficial to the quality of both oil and flakes. Unfortunately, concomitant protein insolubilization also occurred with heat inactivation of lipoxygenase, limiting usefulness of the resulting product.

Inactivation of lipoxygenase without significant protein insolubilization has been studied. Working with the steaming of cotyledons, Wapinski (6) demonstrated the existence of a critical initial moisture range of 19-20 g H_2O/100 g solids favorable to this goal, suggesting that water activity may be affecting enzyme inactivation in one way and protein insolubilization in another. Borhan and Snyder (7) showed that addition of carbonates to the hydration water helped to minimize protein insolubilization during heat inactivation of lipoxygenase in soybeans. Lazar et al. (8) and Lund et al. (9) indicated that adding a holding period after a short steaming to inactivate enzymes in vegetables gave better quality than a longer steaming alone.

The purpose of this investigation was to determine the combined effects of carbonate soaking and moisture content of soybean cotyledons on the temperature dependence of both lipoxygenase inactivation and protein insolubilization kinetics. The ultimate objective was to learn how to inactivate lipoxygenase without losing protein solubility. The approach used was to steam-heat premoistened cotyledons for short times and then hold for various times before cooling and analyzing.

EXPERIMENTAL

Preparation

Williams variety, certified, seed-grade soybeans which had been stored at 1 C were used for all experiments. The soybeans were dehulled to give intact cotyledons (10). Moisture content was adjusted by soaking the cotyledons for up to 12 min in soft water (3 grains hardness/gal) or 1 of 2 carbonate alkaline buffers. Buffer 1, pH 9.8, contained 0.115 M KOH and 0.250 M $NaHCO_3$ whereas buffer 2, pH 10.8, contained 0.164 M KOH and 0.186 M $NaHCO_3$.

Equilibration of the moisture within and between the cotyledons was accomplished by holding at 1 C. The moisture content of cotyledons was determined by air-oven drying (11) and expressed on a dry wt basis. Water activity was determined by a method developed by McCune et al. (12).

Heat Treatment

Three-hundred-g batches of cotyledons, prepared with and without moisture adjustment, were heated in atmospheric steam for 9-12 sec and transferred to an insulated, closed box, which was placed in a constant-temperature air cabinet. The box was made of styrofoam having 4.5 in. × 4.5 in. × 5.5 in. external dimensions with a 0.5-in. wall thickness on all sides. The temperature of the atmosphere in equilibrium with the cotyledons was determined by a thermocouple in the geometric center of the insulated box. Readings between 60 and 95 C were obtained. The air temperature of the cabinet was adjusted to that of the cotyledons to establish isothermal conditions in the box. The cotyledons were held in the insulated box for various times to 60 min and then cooled by immersion in ice-water for 30 sec. The cooled cotyledons were dried at 40 C for 8 hr with high air flow and then at 25 C for 60 hr with minimal air flow. Finally, they were ground to 49 mesh by a laboratory hammer mill. The moisture content of the ground samples was determined by vacuum oven (13).

Analysis of Samples

The nitrogen solubility index of the ground sample was determined (11) using semi-micro-Kjeldahl nitrogen analysis (14). The pH of a 2% soy solids slurry in distilled, deionized water was determined after 2 hr of stirring. Other samples were defatted by Soxhlet extraction with petroleum ether (bp 30-60 C) in preparation for lipoxygenase activity determination. The lipoxygenase was extracted from the defatted samples by a procedure described by Klein (15); it was modified by extracting for 1 hr at 1 C with 0.05 M borate buffer. The lipoxygenase activity was determined using the procedure of Rice et al. (5). Controls were unheated cotyledons that had received the same preparatory soak as the heated cotyledons.

CALCULATIONS

Because we were treating soybean cotyledons, rather than a powder or a liquid, a major difficulty in analyzing the effect of temperature was that no isothermal studies could be done, i.e., no individual cotyledon temperature could be duplicated. To circumvent this difficulty, we combined the first-order rate equation with the Arrhenius equation to relate time and temperature of cotyledon treatment with either enzyme inactivation or protein insolubilization.

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Several assumptions had to be made: (a) due to the small size of the cotyledon, ca. 4 mm × 5 mm × 7 mm, steam heating was postulated to uniformly and "instantly" heat the entire mass of the cotyledon; (b) the holding period in the insulated box was postulated as being isothermal; (c) any pH change within the cotyledon would not affect the kinetics of inactivation and insolubilization; and (d) the moisture gain by the cotyledon from contact with the steam would not affect these reaction kinetics.

Lipoxygenase inactivation and decrease in nitrogen solubility index (NSI) have been shown to follow a first-order rate of degradation (6). The energy of activation was determined using Equation I:

$$\ln[\ln(C_o/C)/t] = \ln k_1 = \ln A + (-Ea)/(RT), \quad [I]$$

where k_1 = first-order rate constant; C_o = initial lipoxygenase activity or NSI; C = final lipoxygenase activity or NSI; t = time of holding; Ea = energy of activation, kcal/mol; R = gas law constant, 1.987 kcal/mol K; T = temperature; and A = pre-exponential factor.

Equation I relates 3 measured variables: (a) decrease in C, either loss of activity or of solubility; (b) temperature; and (c) time.

The relationship of enzyme inactivation and protein insolubilization with temperature of treatment was determined by Equation II:

$$\ln[\ln(C_o/C)_N/\ln(C_o/C)_E] = \ln A_N/A_E + (-Ea_N + Ea_E)/RT, \quad [II]$$

where A_N, A_E = pre-exponential factors for temperature dependence of k_{1N} and k_{1E} which are rate contants for protein insolubilization and enzyme inactivation, respectively. Ea_N, Ea_E = energy of activation for each reaction; $(C_o/C)_N$ = ratio for NSI; and $(C_o/C)_E$ = ratio for enzyme activity.

RESULTS AND DISCUSSION

In order to study thermal inactivation of lipoxygenase and concomitant protein insolubilization, cotyledons of 9.7% moisture were soaked to various moisture contents in each of 3 solutions: soft water, pH 9.8 carbonate buffer and pH 10.8 carbonate buffer.

The general procedure followed for preparation, heat treating and subsequent analysis of samples is outlined in Figure 1.

Sorption Isotherm Data

The water activity at 20 C of cotyledons adjusted to moisture contents of 9.7-35% in each of the 3 solutions was determined. The data are presented as a Smith plot (16) in Figure 2. All the points fell on a common line, showing that soak solution had no effect. However, the breakpoint at 19.8% moisture and 0.82 Aw resulted in 2 straight lines with slopes statistically different (5% level). Lang and Steinberg (17) showed that, in a heterogeneous material, one state of water exists below the breakpoint as polymer water and an additional state of water exists above it as solute water. The lower line of the sorption isotherm is in agreement with the data for whole soybeans (18). Based on this result, 2 moisture contents were chosen for the enzyme inactivation studies; one below the break, 16.3%, and one above the break, 32.0%.

Heating at 16.3% Moisture

Cotyledons that were adjusted to this moisture in each of the 3 solutions were steam-blanched to a range of temperatures.

As shown in Figure 3, Arrhenius plots of the rate constants for lipoxygenase inactivation indicate that adjusting the cotyledon moisture content to 16.3 ± 1.0% with either

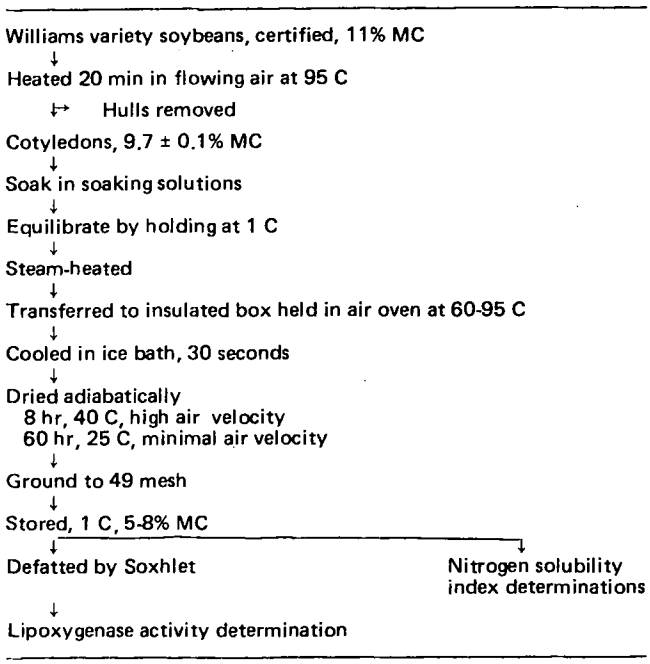

FIG. 1. General experimental procedure for enzyme inactivation in soybean cotyledons and preparation of samples for analysis. MC, moisture content.

pH 9.8 or pH 10.8 buffer increased the rate of inactivation at a given temperature compared to adjusting with soft water. From the slopes of the lines for soft water, pH 9.8 buffer and pH 10.8 buffer, activation energies for the inactivation rates were calculated to be 127.4 ± 9.4, 97.0 ± 4.7 and 103.3 ± 13.1 kcal/mol, respectively. The temperature dependence for the rates of lipoxygenase inactivation was decreased significantly (10% level) by the use of the buffers. There was no difference between the 2 buffer solutions.

Figure 4 presents Arrhenius plots of the rate constants for protein insolubilization. From the slopes of the lines for soft water, pH 9.8 buffer and pH 10.8 buffer, the activation energies were calculated to be 104.2 ± 5.9, 67.6 ± 5.9 and 71.8 ± 6.5 kcal/mol, respectively. Two interesting effects are observed. First, the lines for soft water and the pH 9.8 buffer intersect at 91 C; at temperatures above this, the pH 9.8 buffer gave a relative decrease in the rate of protein insolubilization. Second, the pH 10.8 line paralleled

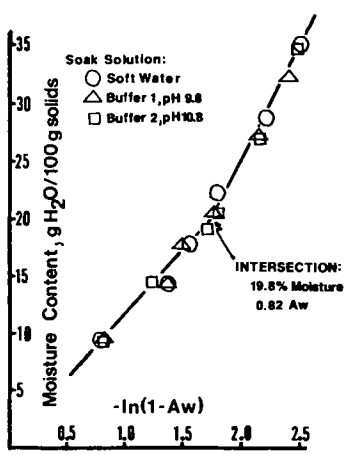

FIG. 2. Water activity (Aw) at 20 C for cotyledons adjusted in moisture content by soaking in soft water or a carbonate buffer.

the pH 9.8 line, indicating relatively higher insolubilization rates for a given temperature. Both buffers reduced temperature dependence of the insolubilization reaction compared to the soft-water control.

In order to relate the degree of protein insolubilization to enzyme inactivation under different conditions, the ratio of NSI after heating to 99% inactivation of lipoxygenase was calculated as a percentage of NSI before heating. These values are plotted against the reciprocal of absolute temperature in Figure 5. Two interesting effects are observed. First, the use of the pH 9.8 buffer significantly increased the remaining soluble protein; above 91 C, at least 70% of the soluble protein was retained. Second, the use of the pH 10.8 buffer gave no improvement over the soft water. It should be noted that the pH 9.8 buffer contained more carbonate and less hydroxyl than pH 10.8 buffer. Borhan and Snyder (7) found that, for the same amount of enzyme inactivation in whole soybeans soaked in 10% ethanol at 45 C, the addition of Na_2CO_3 to the soak solution resulted in a higher retention of soluble protein than equimolar amounts of NaOH. Therefore, we conclude that a higher carbonate concentration is more valuable here than a higher pH.

Adjustments with soft water to 16.3% moisture resulted in a maximal NSI retention of about 55-60% of the initial as a result of steaming and holding at 95 C (Fig. 5). This agrees with Wapinski (6) who found maximal NSI retention of around 60% after inactivation of lipoxygenase in cotyledons adjusted to 16.3% moisture with soft water by steaming alone. Thus, the holding period was of no advantage with lower moisture.

Heating at 32.0% Moisture

As shown in Figure 6, Arrhenius plots of the rate constants for lipoxygenase inactivation in cotyledons adjusted to 32.0 ± 1.0% moisture indicated that the 2 carbonate buffers affected the inactivation rates differently. The pH 9.8 buffer did not appreciably change the rate of lipoxygenase inactivation over the soft-water control. In contrast, the pH 10.8 buffer caused a marked increase in the rate of inactivation at a given temperature. From the slopes of the lines for soft water, pH 9.8 buffer and pH 10.8 buffer, the activation energies for the inactivation of lipoxygenase were calculated to be 104.6 ± 10.1, 107.4 ± 11.5 and 105.7 ± 5.5 kcal/mol, respectively. This shows the buffers did not alter the temperature dependence of the inactivation rate.

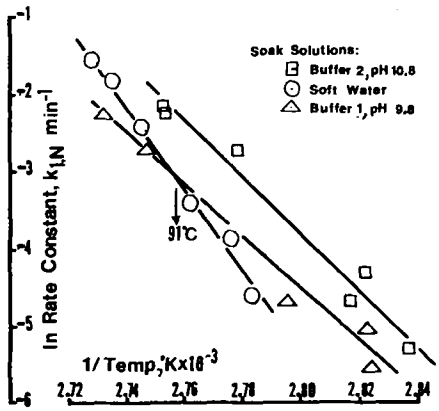

FIG. 4. Arrhenius plots of rate constants for thermal insolubilization of protein in cotyledons adjusted to an initial moisture content of 16.3% by soaking in soft water or a carbonate buffer.

The effects of the buffers compared to soft water on the rate of inactivation here (Fig. 6) were different in 2 ways from that at 16.3% moisture (Fig. 3). First, at 16.3%, the use of either pH 9.8 or pH 10.8 buffer increased the rate of inactivation at lower temperatures, whereas at 32.0% moisture, only the pH 10.8 buffer significantly increased the rate of inactivation. Second, at 16.3% moisture, the use of either pH 9.8 or pH 10.8 buffer decreased the temperature dependence of the rate of inactivation, whereas at 32.0%, neither buffer altered the temperature dependence of this rate.

As shown in Figure 7, Arrhenius plots for the rates of insolubilization of protein in cotyledons at 32.0% moisture indicated that the 2 carbonate buffers increased the protein insolubilization rates differently, compared to soft water, at a given temperature. The pH 9.8 buffer increased the insolubilization rate over that observed for soft water; the pH 10.8 buffer increased the rate over that observed for pH 9.8 buffer. From the slopes of the lines for soft water, pH 9.8 buffer and pH 10.8 buffer, the activation energies for the insolubilization of protein were calculated to be 66.9 ± 8.1, 66.3 ± 5.8 and 46.7 ± 3.8 kcal/mol, respectively. From these activation values, it can be seen that the pH 10.8 buffer significantly decreased the temperature dependence

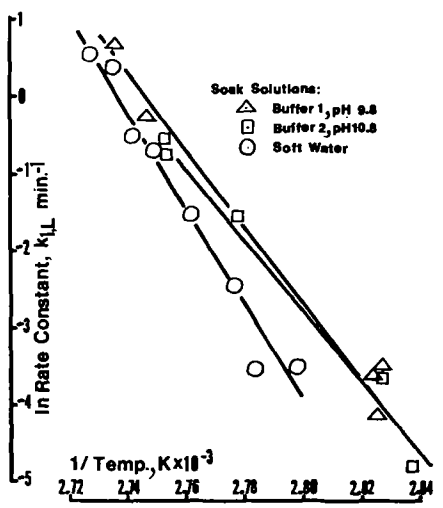

FIG. 3. Arrhenius plots of rate constants for thermal inactivation of lipoxygenase in cotyledons adjusted to an initial moisture content of 16.3% by soaking in soft water or a carbonate buffer.

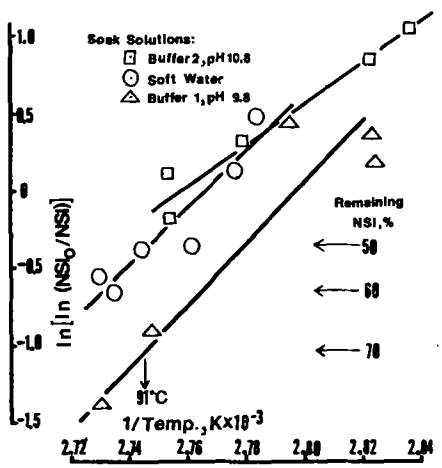

FIG. 5. Arrhenius plots showing remaining soluble protein after a 99% reduction of lipoxygenase activity in cotyledons adjusted to an initial moisture content of 16.3% by soaking in soft water or a carbonate buffer.

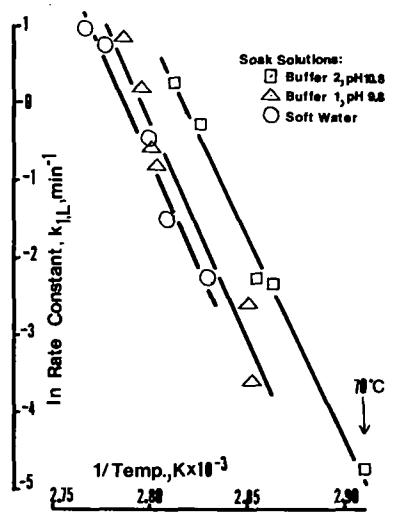

FIG. 6. Arrhenius plots of rate constants for thermal inactivation of lipoxygenase in cotyledons adjusted to 32.0% moisture by soaking in soft water or a carbonate buffer.

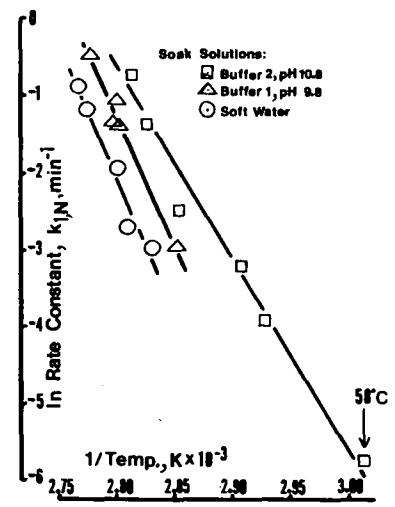

FIG. 7. Arrhenius plots of rate constants for thermal insolubilization of protein in cotyledons adjusted to an initial moisture content of 32.0% by soaking in soft water or a carbonate buffer.

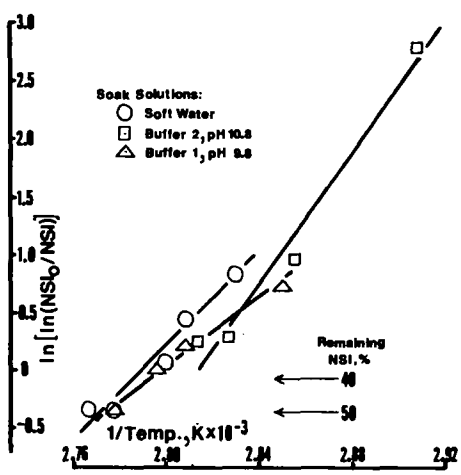

FIG. 8. Arrhenius plots showing remaining soluble protein after a 99% reduction in lipoxygenase activity in cotyledons adjusted to an initial moisture content of 32.0% by soaking in soft water or a carbonate buffer.

whereas the pH 9.8 buffer did not decrease this temperature dependence compared to soft water.

It is interesting to compare protein insolubilization in pH 9.8 buffer vs in soft water at 32% moisture (Fig. 7) and at 16.3% moisture (Fig. 4). There are 2 differences. First, at 16.3%, the insolubilization rate was decreased above 91 C as opposed to an overall increase in the rate at 32.0% moisture. Second, at 16.3% moisture, the temperature dependence for the pH 9.8 buffer in comparison to soft water was significantly decreased whereas it remained the same at 32.0% moisture.

The remaining soluble protein after a 99% inactivation of lipoxygenase is plotted against the inverse of the absolute temperature in Figure 8. This shows that neither buffer improved the retention of soluble protein compared to soft water. At the higher temperatures of steaming and holding, essentially the same amount of soluble protein remained for soft water and pH 9.8 buffer after a 99% inactivation of lipoxygenase. This is in agreement with Wapinski (6) who found that steaming (without holding) of cotyledons adjusted to 37% moisture by soaking in either a pH 9.6 carbonate buffer or water resulted in approximately the same amount of soluble protein remaining after lipoxygenase inactivation. Thus, our holding period had no advantage. The pH 9.8 buffer displayed a dramatic difference in its effect on the retention of the soluble protein at 16.3% moisture compared to that at 32.0% moisture. At 16.3% moisture, the use of the pH 9.8 buffer resulted in a large increase in the remaining soluble protein compared to soft water; above 91 C, over 70% of the soluble protein was retained after a 99% inactivation of lipoxygenase. However, at 32.0% moisture, no difference in retained soluble protein was observed between the pH 9.8 buffer and soft water.

It is interesting to compare the effects of the carbonate buffers at 16.3 and 32.0%. They displayed differences in (a) the rates of enzyme inactivation, (b) the rates of protein insolubilization and (c) the amount of soluble protein remaining after a 99% enzyme inactivation. We concluded, based on the sorption isotherm of cotyledons (Fig. 2), that these differences in the buffer effects on the reaction rates were primarily related to the absence, at 16.3% initial moisture, and the presence, at 32.0% initial moisture, of an additional state of water called solute water (17) as already discussed.

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A Rapid Synthesis of Fatty Acyl Urea Derivatives

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ABSTRACT

Fat-based aliphatic amides react with aromatic isocyanates in refluxing xylene generally within 1 hr to form crystalline acylaryl urea compounds in 50-80% yields. The reaction appears to be general with either component being aliphatic or aromatic. Although the reaction occurs in various solvents, xylene is the preferred solvent in which the reactions were completed within 4 hr for the chosen amides and isocyanates. The progress of the reaction was conveniently monitored by infrared through the disappearance of the intense isocyanate band at 2258 cm^{-1}.

INTRODUCTION

In the course of a screening program to develop potential bacteriostats for use in soap or nonionic surfactants, it was found necessary to prepare some acyl urea derivatives. Disubstituted acyl ureas of the type RCONHCONHR', where R and R' may be aliphatic or aromatic, are generally prepared by reacting an acyl urea with an amine at 160-170 C for 2-4 hr or with an acid chloride (1-3). The high temperatures used with the amine tend to decrease yields and complicate the reaction by dissociation of the product into an amide and an isocyanate. The reaction of isocyanates with amines or alcohols is well known as a standard approach to the formation of solid derivatives. The reaction between isocyanates and amides to form acyl urea derivatives according to the following equation is not so familiar:

$$RCONH_2 + R'NCO \underset{<150}{\overset{\geq 160\ C}{\rightleftharpoons}} RCONHCONHR' \qquad [1]$$

Reaction temperatures below 160 C lead to formation of the product whereas temperatures of 160-200 C lead to dissociation (1,2,4). The initial work on this synthesis was done by B. Kühn (1884) who studied the action of phenyl isocyanate with benzamide, acetamide and propionamide to yield the corresponding acyl ureas (4). He also investigated the reaction of phenyl isocyanate with anilides derived from aniline and naphthylamine. He found that acetanilide was the only secondary amide to react according to the Equation I giving N-acetyl N-phenyl N'-phenyl urea (4,5). Subsequent investigators utilized this reaction on occasion, principally to prepare aroylaryl ureas (6-9) with no details of experimental conditions. Other investigators described syntheses of various acylaryl ureas using sealed tube techniques or prolonged heating at 110 C for 18-24 hr (10-12).

An approach was developed in which an isocyanate and a fatty amide are heated in xylene with the reaction frequently being complete in 1 hr or less.

[1] Agricultural Research Service, USDA.

EXPERIMENTAL

Materials

Benzamide, 1,2-dichloroethane and ethyl acetate were white label from Eastman Organic Chemicals, Rochester, NY, whereas the isocyanates were obtained from Aldrich Chemical Co., Milwaukee, WI, or Pfaltz-Bauer, Stamford, CT. The compounds were used as received. (Caution is required when handling some of the solvents, such as 1,2-dichloroethane, as this substance has carcinogenic properties.) All other solvents used were reagent grade. The amides were prepared by the low-temperature addition of an acid chloride to concentrated ammonia (13).

Synthesis of N-Stearoyl N'-3-nitrophenyl Urea

A solution of 3-nitrophenyl isocyanate was prepared by heating 1.64 g (0.01 mol) of the isocyanate with 30 mL of dry xylene and filtering off the small amount of yellow amorphous solid under a blanket of dry nitrogen. The filtrate was added to a dry, 100-mL R.B. flask equipped with a stirrer, reflux condenser and drying tube. The system was flushed with nitrogen, 2.84 g (0.01 mol) of stearamide was added and the mixture was heated to reflux. Infrared (IR) spectra of the reaction mixture taken after 30 min and 1 hr showed the complete absence of the intense NCO absorption band at 2258 cm^{-1} after 1 hr. The turbid, pale yellow reaction mixture was filtered. The filtrate was evaporated in the rotovac at 0.5 mm/50 C to constant weight. The crude product was crystallized 2 times from absolute ethanol to give 3.8 g (85% yield) of crystalline N-stearoyl N'-3-nitrophenyl urea, mp 105-106 C.

Where the isocyanate was completely soluble in the solvent, indicating the absence of contaminating diaryl urea, the amide was added to an excess of xylene and the entire system dried by azeotropic distillation of a portion of the solvent. The isocyanate was added and the synthesis was performed as indicated above. Diaryl ureas are normal contaminants of isocyanates which have been exposed to moisture in the air. In this study, the low solubility of the diaryl ureas permitted their separation from the desired products.

Infrared Spectra

IR spectra of the acyl urea derivatives were obtained as Nujol Mulls with a P.E. 257 spectrophotometer. The spectra were quite consistent for all the samples in Table I. NH-stretching bands appeared at 3220 and 3120 cm^{-1} with deviations from these values of 5-10 cm^{-1} for only a few samples. Carbonyl bands frequently were partially resolved