Thermal Interaction of Lysine and Triglycerides

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Mixtures of lysine and either tributyrin or tricaproin were heated under vacuum for 1 h at 250 °C, and the volatile decomposition products were analyzed by gas chromatography and mass spectrometry. Interaction products included the fatty acid amide, secondary amides, a tertiary amide, and a series of pyridine compounds not previously isolated. Most of the identified products can be accounted for by well-known reactions.

Evidence of lipid–protein interactions in foods has been reported by Lea et al. (1958, 1960), Carpenter et al. (1962), and Tooley and Lawrie (1974). In studies involving the chemical and nutritional changes in stored herring meal, Lea et al. (1958, 1960) and Carpenter et al. (1962) reported a decrease in the available lysine determined by measuring the ϵ-amine of lysine when herring meal was stored in air. No change was observed if the meal was stored in nitrogen.

Tooley and Lawrie (1974) reported that the state of thermal oxidation of oil used to fry fish fillets effected the loss of available lysine. Three different oils and six different fish were studied. From the results, they concluded that the loss of available lysine was approximately 10% greater if oxidized oil was used.

Due to the complexity of proteins and lipids found in foods, model systems were used to study these interactions. Severin and Ledl (1972) studied the thermal interaction of cysteine and tributyrin at 200 °C. Lien and Nawar (1974) studied thermal interaction of valine and tricaproin at 270 °C. Sims and Fioriti (1975) heated fatty esters with α-amino acids and isolated N-substituted amides as the major products.

In earlier publications we reported on the decomposition of lysine (Breitbart and Nawar, 1979) and that of tricaproin when heated separately (Lien and Nawar, 1973).

In the present study, the thermal interactions between lysine and tributyrin and lysine and tricaproin were examined. Lysine was chosen since it has a free amino group when present in proteins and since nutritionally it is the limiting essential amino acid in many of the proteins found in foods. The short-chain triglycerides were used to simplify the separation and identification of decomposition and interaction products.

EXPERIMENTAL SECTION

Materials. L-Lysine, free base, was purchased from Sigma Chemical Co. (St. Louis, MO). Tributyrin and tricaproin were purchased from Eastman Kodak (Rochester, NY). The purity of these compounds was confirmed by paper chromatography and by gas chromatographic analysis of the distillate after cold-finger distillation for 1 h at 60 °C. Reference compounds and reagents were purchased commercially at the highest purity available. Secondary amides were synthesized by acylation of the appropriate primary amine with the acid chloride according to Kent and McElvain (1955).

Heat Treatment. One gram of one of the saturated triglycerides and 0.5 g of lysine were sealed under vacuum (1 μm) inside a Pyrex ampule (8 in. long by 1 in. o.d.) and heated at 250 ± 10 °C in a muffle furnace (Thermolyne Corp., Dubuque, IA) for 1 h.

Analytical Techniques. The heated lysine–triglyceride mixtures were subjected to cold-finger distillation as described by Nawar et al. (1969). Distillation was carried out for 1 h at 60 °C in vacuum. The cold finger was then raised with 15 mL of diethyl ether and the distillate was concentrated at room temperature under a continuous stream of N_2 gas prior to analysis.

The concentrated ether extracts were separated by gas chromatography using an F & M Model 810 dual-column gas chromatograph (F & M Scientific Corp., Avondale, PA) equipped with temperature programming, sample splitter, and flame ionization detector. Two approaches were used to analyze the distillates. First, the compounds were trapped from a 6 ft × 1/8 in. s/s 28% Pennwalt 223 Amine plus 4% KOH on 80–100-mesh Gas-Chrom R (Applied Science Laboratories, Inc., State College, PA) column and then rechromatographed on either a 6 ft × $^1/_8$ in. s/s 10% Carbowax plus 2% KOH on 80–100-mesh Chromosorb W AW (Supelco, Inc., Bellefonte, PA) column or a 500 ft × $^1/_{16}$ in. s/s Carbowax 20M capillary column.

The second approach was direct analysis of the total distillate using either a 6 ft × $^1/_8$ in. s/s 10% silicone gum rubber, SE-30, on 80–100 WHP (Hewlett-Packard, Palo Alto, CA) column or a 500 ft × $^1/_{16}$ in. s/s Carbowax 20M capillary column in conjunction with a 6 ft × $^1/_8$ in. s/s 10% Carbowax plus 2% KOH subtractive loop used to remove the free fatty acid from the sample.

The isolated compounds were identified by combined gas chromatography–mass spectrometry (GC–MS). The mass spectrum and gas chromatographic retention time of the unknown compound and of the suspected authentic compound were compared when the authentic compound was available. A positive identification consisted of obtaining the same mass spectrum and GC retention time of the unknown compound and the authentic compound on at least one column. An identification was classified as tentative when based solely on the interpretation of the mass spectrum or by matching the mass spectrum with that of a compound reported in the literature.

Two GC–MS systems were used: (1) a Varian Aerograph Model 1200 gas chromatograph coupled to a Perkin-Elmer Hitachi Model RMU-6A mass spectrometer via a heated line and a Bieman separator (both maintained at 200 °C), and (2) a Varian Mat 111 GC–MS system. In both cases, the ion source was operated at 80 eV.

RESULTS AND DISCUSSION

Thirteen compounds were positively identified and four tentatively identified in the heated lysine–tributyrin sample (Table I). Of these, pyridine, 2-methylpyridine, 3-ethylpyridine, 2-piperidone, and 2-oxohexamethylenimine were produced from lysine when heated alone (Breitbart and Nawar, 1979). Methyl butyrate, 4-heptanone, and butyric acid were isolated when tributyrin was heated alone. The latter compounds are analogous to those found

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Table I. Thermal Decomposition Products from Lysine-Tributyrin Mixtures Heated at 250 °C for One Hour under Vacuum

compound	identifn GC	identifn MS	μmol/150 g[a] of mixture
methyl butyrate	b	e	g
butyronitrile		f	65210
4-heptanone	b	e	350
pyridine	b	e	g
2-methylpyridine	b	e	g
3- or 4-methylpyridine	b	e	1720
3-ethylpyridine	b	e	1215
3-(*n*-propyl)pyridine		e	1240
4-(*n*-butyl)pyridine		f	1111
N,*N*-dimethylbutyramide		f	1913
N-methylbutyramide	b	e	3168
N-(*n*-propyl)butyramide	b	e	2636
butyramide	d	e	51034
N-(*n*-butyl)butyramide	b	e	2727
2-piperidone	b	e	2424
2-oxohexamethylenimine	b	e	5133
butyric acid	c	e	546591

[a] Micromoles of decomposition product per 100 g of triglyceride plus 50 g of lysine mixture. [b] Retention time agreed with that of the authentic compound on Carbowax plus KOH. [c] Retention time agreed with that of the authentic compound on SE-30. [d] Retention time agreed with that of the authentic compound on two columns: Carbowax plus KOH and Pennwalt Amine. [e] MS fragmentation agreed with that of authentic compound. [f] Identification by MS interpretation only. [g] Compounds were not quantitated due to poor separation from other compounds present in the sample.

Table II. Thermal Decomposition Products from Lysine-Tricaproin Mixtures Heated at 250 °C for One Hour under Vacuum

compound	identifn GC	identifn MS	μmol/150 g[a] of mixture
methyl hexanoate	b	d	7260
pyridine	b	d	f
2-methylpyridine	b	d	f
caproic nitrile	b	d	45670
3-ethylpyridine	b	d	308
3-(*n*-propyl)pyridine		e	107
4-(*n*-butyl)pyridine		e	74
2-(*n*-hexyl)pyridine		e	74
N,*N*-dimethylcaproic amide		e	1608
N-methylcaproic amide		e	2674
N-(*n*-propyl)caproic amide		e	1395
caproic amide	b	d	95826
2-oxohexamethylenimine	b	d	4425
caproic acid	c	d	87931

[a] Micromoles of decomposition product per 100 g of triglyceride plus 50 g of lysine mixture. [b] GC retention time agreed with that of the authentic compound on Carbowax plus KOH. [c] Retention time agreed with that of the authentic compound on SE-30. [d] MS fragmentation agreed with that of the authentic compound. [e] Identification by MS interpretation only. [f] Compounds were not quantitated due to poor separation from other compounds present in the sample.

earlier in heated tricaproin (Lien and Nawar, 1974). The remaining compounds were classified as interaction products. These were butyronitrile, 3- or 4-methylpyridine, 3-(*n*-propyl)pyridine, 4-(*n*-butyl)pyridine, *N*,*N*-dimethylbutyramide, *N*-methylbutyramide, *N*-(*n*-propyl)butyramide, butyramide, and *N*-(*n*-butyl)butyramide. Table II gives the decomposition products identified in lysine–tricaproin mixtures. Of these, caproic nitrile, 3-(*n*-propyl)pyridine, 4-(*n*-butyl)pyridine, 2-(*n*-hexyl)pyridine, *N*,*N*-dimethylcaproic amide, *N*-methylcaproic amide, *N*,*N*-propylcaproic amide, and caproic amide were attributed to interaction between the triglyceride and the amino acid. Both Severin and Ledl (1972) and Lien and Nawar (1974) reported finding the primary fatty acid amide as well as a secondary amide as interaction products when an α-amino acid and a saturated triglyceride were heated together. These workers suggested that the primary amides may arise from acylation of ammonia by the fatty acid resulting from hydrolysis of the triglyceride, acylation of ammonia by the acid anhydride resulting from the thermal decomposition of the triglyceride, and ammonolysis of the triglyceride itself.

The secondary amides isolated from the interaction samples may result either by acylation of the appropriate amine by the fatty acid or the acid anhydride or by the reaction of the primary amide with the appropriate amine (Beckwith, 1970). Ammonolysis of the triglyceride by primary amines would also produce the secondary amides. As in the case of the primary amides, total ammonolysis of the triglyceride can occur.

The amines required for the formation of the secondary amides could result from the thermal decomposition of lysine. Winter and Arbro (1964) reported isolating ammonia, methylamine, dimethylamine, ethylamine, tripropylamine, dipropylamine, and tributylamine from pyrolysates of lysine. They proposed that the presence of di- and triamines suggested that interaction and recombination of decomposition products occur. The formation of propylamine and butylamine from lysine by thermal decomposition may therefore be possible.

The reaction involving methylamine yields the *N*-methyl secondary amide, propylamine, the *N*-propyl secondary amide, and butylamine, the *N*-butyl secondary amide. Only the lysine–tributyrin sample contained an *N*-butyl secondary amide, *N*-(*n*-butyl)butyramide. *N*-(*n*-Butyl)-caproic amide was expected from the lysine–tricaproin but was not isolated. Similar mechanisms would explain the production of *N*-methylcaproic amide and *N*-(*n*-propyl)-caproic amide in the lysine–tricaproin sample.

A tertiary amide was isolated from each interaction sample. *N*,*N*-Dimethylbutyramide and *N*,*N*-dimethylcaproic amide were isolated from the lysine–tributyrin and the lysine–tricaproin, respectively. The following reactions may be considered: acylation of *N*,*N*-dimethylamine by the fatty acid primary amide or acid anhydride. *N*,*N*-dimethylamine may be produced by the reaction of methylamine with formaldehyde to form the imine, followed by hydrogenation to yield the secondary amine.

The formation of butyronitrile and caproic nitrile is consistent with the mechanisms discussed earlier by Davidson and Karten (1956) and Lien and Nawar (1974).

Several pyridine compounds not isolated from the heated lysine alone were found in the interaction samples. In addition, the amounts of 3-ethylpyridine in the heated lysine–tributyrin and lysine–tricaproin mixtures were greater than those in the lysine alone (Breitbart and Nawar, 1979).

The formation of the secondary amides is relevant to the mechanism of pyridine production. The same reactions which produce methylamine, propylamine, and butylamine, formed from lysine, may lead to the formation of butanal, acetaldehyde, and acetone, respectively (Ratcliff et al., 1974). Such carbonyl compounds, as well as those produced from the triglycerides themselves, can react with ammonia to yield pyridine derivatives. For example, 4-methylpyridine may result from the reaction of acetaldehyde and ammonia or of acetone, acetaldehyde, formaldehyde, and ammonia.

The formation of 3-ethylpyridine from the heated lysine alone was discussed earlier. This compound, however, was produced in larger quantities from the interaction samples. This may be due to the formation of butanal and acetaldehyde from the triglyceride. The ethylpyridine could be formed by condensation of acetaldehyde, formaldehyde, and ammonia (Millar and Springall, 1966) and/or butanal, formaldehyde, acetaldehyde, and ammonia. The reaction of butanal, formaldehyde, acetaldehyde, and ammonia could yield 3-(*n*-propyl)pyridine, while 4-(*n*-butyl)pyridine may result from the reaction of butanal, formaldehyde, acetaldehyde, propanal, and ammonia. Similar reactions can explain the formation of 2-(*n*-hexyl)pyridine.

The absence or presence of the compounds isolated in this study in actual food systems has yet to be determined. However, the knowledge that these interactions may occur in food is important in (1) preserving the nutritional quality of foods, (2) generation of flavor compounds, and (3) identifying potentially toxic compounds in the food supply.

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Effect of Cysteine on Heat Inactivation of Soybean Trypsin Inhibitors

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Cysteine facilitated the heat inactivation of trypsin inhibitor activity with both purified soybean Kunitz inhibitor and soybean extracts. Effects of cysteine concentration, pH, temperature, and length of treatment on inactivating trypsin inhibitor were studied. Extent of trypsin inhibitor inactivation was determined spectrophotometrically by measuring reductions in rates of production of *p*-nitroaniline from a synthetic trypsin substrate, *N*-benzoyl-DL-arginine-*p*-nitroanilide. Attractive conditions for inactivating soybean trypsin inhibitor were 2.5 mM cysteine, pH 9.0, and 80 °C for 10 min. Under those conditions, inhibitor activity of soybean extract was reduced by 80–90%. In the absence of cysteine, activity was reduced by 29%. Inhibitors inactivated by heat in the presence of cysteine showed no significant reactivation after 60 h at 4 °C or 6 h at room temperature. The treatment results in retention of protein solubility; its nitrogen solubility index after neutralization and lyophilization was 99.7%.

There are several obstacles to realizing the full nutritional potential of soybean protein (Rackis, 1974). Among those are heat-inactivatable antinutritional factors, including trypsin inhibitors. Soybean trypsin inhibitors fall into two classes (Laskowki and Kato, 1980) represented by the well-studied Kunitz and Bowman-Birk inhibitors. The most direct evidence that one or both classes of soybean trypsin inhibitors are antinutritional was provided by Kakade et al. (1973), who found that removing them from water extracts by affinity chromatography on trypsin–agarose columns substantially increased the protein efficiency ratio of the soybean protein. More conventionally, soybean trypsin inhibitors are inactivated by heat (Rackis, 1974).

The Kunitz and Bowman-Birk inhibitors contain two and seven disulfide bonds, respectively (Wolf, 1977). Since disulfide bonds stabilize the native conformations of proteins (Liu, 1977), their cleavage would be expected to make the inhibitor proteins more susceptible to heat denaturation. At least some disulfide bonds in soybean trypsin inhibitors are accessible to reducing agents at room temperature in the absence of denaturants (DiBella and Liener, 1969). Disulfide bonds not readily accessible under such conditions would become accessible during heat denaturation. Their reduction would be expected to facilitate the further denaturation of the protein molecules.

We explored the use of cysteine as a reducing agent to enhance the heat inactivation of soybean trypsin inhibitors. The influence of several variables (cysteine concentration, pH, temperature, and length of treatment) was investigated. We found conditions for inactivating trypsin inhibitors that are considerably milder with added cysteine than those without cysteine. The milder treatment gave excellent retention of protein solubility, as assessed by the nitrogen solubility index.

MATERIALS AND METHODS

Preparation of Soybean Water Extract. Fifty grams of soybeans (Williams variety, grown in Riley County, KS, 1978) was washed with water and then soaked in 150 mL of distilled water for 4 h at 18–21 °C to increase the efficiency of extraction of proteins (Lo et al., 1968). The soaked beans were drained, rinsed, drained again, and

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