pigments played a greater role in the nitrosating reaction than nitrite did. We hope our study will be useful in the prevention of the nitrosamine contaminations.

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Adsorption onto an Oil Surface and Emulsifying Properties of Bovine α_{s1}-Casein in Relation to Its Molecular Structure

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When coconut oil is emulsified with an α_{s1}-casein solution, the α_{s1}-casein is adsorbed onto the surface of the oil globules and stabilizes the emulsion. This adsorption on the emulsified oil, as well as the emulsifying properties of α_{s1}-casein, was investigated by using some of the large peptides derived from α_{s1}-casein by limited proteolysis. The adsorbability of α_{s1}-casein onto the emulsified oil surface was reduced by removal of the N-terminal segment (1–23 residues) with pepsin but not by removing the C-terminal segment (145–199 residues) with papain. The emulsifying properties (emulsifying activity, emulsion capacity, creaming stability) were changed by the removal of the N-terminal segment (1–23 residues). α_{s1}-Casein adsorbed on the emulsified oil was barely extracted from the oil surface with 0–5 M urea and was not hydrolyzed by pepsin. These results suggest that α_{s1}-casein is tightly adsorbed onto the oil globule surfaces, principally by its hydrophobic N-terminal region, and that it stabilizes the oil globules.

Basic studies on protein structure and functionality are essential for the utilization of new protein sources into many functional food systems. The various properties of proteins, namely, molecular size, shape, conformation, net charge, hydrophobicity, and protein–protein interactions, have been noted as factors that influence such functional properties as emulsification and foamability of these proteins (Horiuchi et al., 1978; Chou and Morr, 1979; Kinsella, 1981).

In previous papers (Yamauchi et al., 1980; Shimizu et al., 1981), we have studied the emulsifying properties of bovine whey proteins in relation to their structures. During these investigations we found that the adsorption of proteins onto an oil surface during emulsification depends on various structural properties of the proteins, including the hydrophobicity and such conformational factors as the rigidity of the molecule. Since the adsorption of proteins is essential for stabilizing the oil globules and affects the properties of the emulsion (Tornberg, 1979), the adsorption mechanism is of great interest. However, the general relationship between the structure and the adsorbability of proteins is still obscure.

Caseins, the major proteins of bovine milk, are known to be flexible proteins without a rigid conformation and their primary structures have already been established (Mercier et al., 1971; Ribadeau-dumas et al., 1972; Jollès et al., 1972; Mercier et al., 1973). Furthermore, caseins have amphiphilic properties in their primary structures. The hydrophobic and hydrophilic areas are distinguishable. Studies using caseins, therefore, may provide valuable information on lipid–protein interactions. Such studies can be expected to elucidate the relationship between the

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protein structure at a molecular level (especially the primary structure) and the emulsifying properties. The present study was undertaken to advance this knowledge, using α_{s1}-casein and its peptides that were formed by limited proteolysis.

MATERIALS AND METHODS

Preparation of α_{s1}-Casein. Crude α_{s1}-casein (Variant B) was prepared from fresh raw skim milk according to the procedure of Zittle et al. (1959). The crude α_{s1}-casein was purified on a DEAE-Sephadex A-50 column (2.5 × 45 cm), eluting with 0.02 M phosphate buffer (pH 6.5) containing 4 M urea under a linear gradient of NaCl (0.1–0.6 M).

Limited Proteolysis of α_{s1}-Casein. The limited cleavage of this purified α_{s1}-casein was carried out by pepsin or papain.

Pepsin Digestion. To a 2% α_{s1}-casein solution (w/w) in 0.1 M sodium acetate buffer (pH 6.4) were added pepsin crystals (Sigma, from porcine stomach) to give an enzyme/substrate ratio of 1/330 (w/w), and the mixture was incubated at 30 °C for 30 min. Under these conditions, the Phe–Phe (residues 23–24) linkage of α_{s1}-casein is selectively cleaved and most of the α_{s1}-casein is converted into α_{s1}(24–199) peptide by a splitting off of the *N*-terminal residues (1–23) (S. Kaminogawa et al., unpublished data). The pH level of the digest was adjusted to 8.0 with 1 N NaOH to terminate the enzyme action and then adjusted to 4.6 with 1 N HCl. The pH 4.6 insoluble material was collected by centrifugation at 1870*g* for 10 min. The precipitate was solubilized at pH 7.0, dialyzed against water, and freeze-dried.

Papain Digestion. To a 2% α_{s1}-solution (w/w) in 1 M carbonate buffer (pH 9.0) containing 10 mM 2-mercaptoethanol and 20% acetone (Arai and Watanabe, 1980) was added papain (Sigma, 2× crystallized) to give an enzyme/substrate ratio of 1/2500 (w/w), and the mixture was incubated at 37 °C for 60–90 min. Under these conditions, papain is known to specifically cleave the 145–146 linkage of α_{s1}-casein and to produce a large peptide corresponding to α_{s1}(1–145) (Toiguchi et al., 1982). After being heated at 100 °C for 5 min to stop the reaction, the digest was adjusted to pH 4.6. The precipitate collected by centrifugation was resolubilized at pH 7.0, dialyzed against water, and freeze-dried.

Purification of the α_{s1}-Casein Peptides. The pH 4.6 insoluble fraction of the pepsin digest was dissolved in 0.02 M phosphate buffer (pH 6.5) containing 4 M urea and applied to a column of DEAE-Sephacel that had been equilibrated with the same buffer. The α_{s1}(24–199) peptide was eluted under a linear gradient of NaCl (0.1–0.35 M). No contamination of the other proteins or peptides in the α_{s1}(24–199) fraction was observed on polyacrylamide gel electrophoresis. The pH 4.6 insoluble fraction of the papain digest was chromatographed on DEAE-Sephacel by the same procedure as used for the pepsin digest. A fraction that contained α_{s1}-casein and α_{s1}(1–145) in the ratio of approximately 1:1 was recovered and used for the adsorption study.

Preparation of the Emulsion. A protein solution (1% protein, pH 7.0) was homogenized with 20% (w/w) coconut oil (Nakarai Chemicals) by using Polytron PT-20 or PTA-7 (Kinematica) at full speed (19 500 rpm) for 8 min at 50 °C.

Isolation of Protein Adsorbed on the Oil Globule Surface during Emulsification. This emulsion (20 g) was diluted with 20 mL of water (pH adjusted to 7.0) and centrifuged at 25000*g* for 10 min at room temperature. The aqueous portion was removed by aspiration. To the floating cream was added 40 mL of water (pH 7.0, 50 °C), and the diluted cream was gently mixed and centrifuged as before. A washed cream was obtained by twice repeating this step. The protein adsorbed on the oil globule surface was recovered by delipidating the washed cream with a methanol–chloroform mixture (1:2 v/v) (Yamauchi et al., 1980).

Extraction of Adsorbed Proteins from the Oil Globule Surface with Urea. To the washed cream was added urea to give a final concentration of 1, 3, or 5 M. The mixture was incubated at 30 °C for 30 min and then centrifuged at 25000*g* for 10 min. The floated cream and aqueous fractions were dialyzed against water. The proteins were solubilized from these fractions with 2% SDS and determined by the procedure of Lowry et al. (1951).

Enzyme Digestion of α_{s1}-Casein Adsorbed on Oil Surfaces. *Pepsin Digestion.* Washed cream was prepared from the emulsion (1% α_{s1}-casein–20% coconut oil) and suspended in 0.02 M phosphate buffer (pH 6.0). To the washed cream was added pepsin to give an adsorbed protein/enzyme ratio of 500/1 (w/w). The mixture was incubated at 37 °C for 40 min and then adjusted to pH 8.0 to terminate the reaction.

Papain Digestion. Washed cream was suspended in 1 M carbonate buffer (pH 9.0) containing 10 mM 2-mercaptoethanol. To the suspension was added papain to give an adsorbed protein/enzyme ratio of 2500/1 (w/w). The mixture was incubated at 37 °C for 40 min and then heated at 100 °C for 5 min to stop the reaction. The protein in both mixtures was recovered by removing the oil with a methanol–chloroform mixture (1:1 v/v).

Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was performed according to the procedure of O'Farrell (1975) using 7.5% acrylamide gel containing 5 M urea and Tris–glycine (pH 8.6) as the electrode buffer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was according to Weber and Osborn (1969) using 10% acrylamide gel. The proteins were stained with Coomassie blue R-250, and the gels were scanned at 550 nm by using a Gelman DCD-16 densitometer.

Emulsifying Properties. The emulsifying activity was measured according to the procedure described by Pearce and Kinsella (1978) with slight modifications. An emulsion was prepared by homogenizing a solution of 1% protein and 20% coconut oil at 50 °C with Polytron PTA-7 for 3 min. The emulsion was immediately diluted with 0.1% SDS and its turbidity measured at 500 nm. The emulsifying activity was expressed as its emulsifying activity index (EAI).

The emulsion capacity was measured according to Webb et al. (1970) by using a 0.5% protein solution and coconut oil. The rate of oil addition was 0.3–0.4 mL/s, while mixing with a Polytron PT-20 at 19 500 rpm. The transition point was detected by electrical resistance measurement and emulsion capacity (EC) was expressed in mL of oil/mg of protein.

Creaming stability (CS) was measured as follows: The emulsion (1% protein–20% oil) was allowed to stand in a test tube (12 × 105 mm) at 30 °C for 30 min, and the height of the cream layer was measured. CS was expressed as (height of the cream layer/total height) × 100.

Surface Hydrophobicity. The surface hydrophobicity of the proteins was determined by the procedure of Kato and Nakai (1980), using *cis*-parinaric acid (Molecular Probe, Inc.).

RESULTS

Effect of Limited Proteolysis of α_{s1}-Casein on the Adsorption onto the Oil Globule Surface. The pure α_{s1}-casein and pure α_{s1}(24–199) were mixed in the ratio of

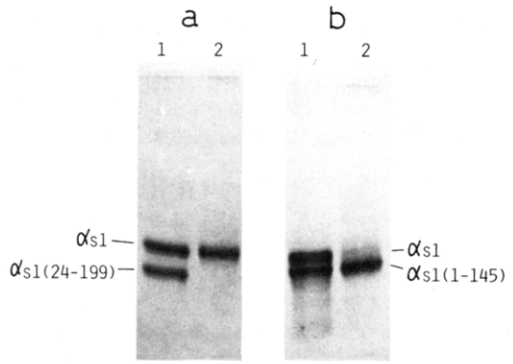

Figure 1. Slab gel electrophoretic patterns of proteins adsorbed on oil globule surfaces during emulsification. Emulsification was carried out with a mixture of α_{s1}-casein and α_{s1}(24–199) peptide (1:1 w/w) (a) or with α_{s1}-casein and α_{s1}(1–145) peptide (1:1) (b). (1) Proteins in the bulk phase; (2) proteins adsorbed on the oil surface.

Table I. Hydrophobicities of α_{s1}-Casein and α_{s1}(24–199) Peptide

	average hydrophobicity[a]	relative surface hydrophobicity
α_{s1}-casein	1170	100
α_{s1}(24–199)	1157	47.6

[a] Calculated by the procedure of Bigelow (1967).

1:1 (w/w), and coconut oil was emulsified with this mixture. The proteins adsorbed on the oil globule surface were isolated. Slab-gel electrophoresis showed that the protein adsorbed after emulsification with α_{s1} plus α_{s1}(24–199) was only α_{s1}-casein (Figure 1a). This indicates that the adsorbability of α_{s1}-casein is reduced by removal of the N-terminal residues (1–23).

As a control experiment, the adsorbability of α_{s1}(1–145) was compared with that of α_{s1}-casein. Coconut oil was emulsified with the papain digest, which contained α_{s1}-casein and α_{s1}(1–145) in the ratio of approximately 1:1 (determined by densitometry of SDS–PAGE gels), and the proteins adsorbed on the oil globule surface were isolated. As shown in Figure 1b, the adsorbability of α_{s1}-casein was not reduced but increased by the removal of the C-terminal residues (146–199). This confirms the dominant contribution of the N-terminal hydrophobic region to the adsorption of α_{s1}-casein.

Effect of Removing the N-Terminal Residues on the Surface Hydrophobicity of α_{s1}-Casein. Removal of the N-terminal residues decreased the surface hydrophobicity of α_{s1}-casein by approximately 50% (Table I), although the average hydrophobicity calculated by the procedure of Bigelow (1967) was only slightly decreased.

Extraction of α_{s1}-Casein from the Oil Globule Surface with Urea. Washed cream prepared from the α_{s1}-casein–coconut oil emulsion was incubated with different concentrations of urea (0–5 M). Urea is known to solubilize proteins by breaking various interactions between their molecules (e.g., hydrophobic interaction and hydrogen bonding) and has been utilized for the solubilization of membrane proteins (Steck and Fox, 1972) and lipoproteins (Kurisaki and Yamauchi, 1977). However, urea is not effective for the solubilization of those proteins associating tightly with lipids by hydrophobic interactions (Hatefi and Hanstein, 1969). The extent of extraction of proteins with a urea solution has been measured to investigate the mode of interaction between protein and lipids in various lipid–protein complexes including protein-stabilized emulsions (Shimizu et al., 1981).

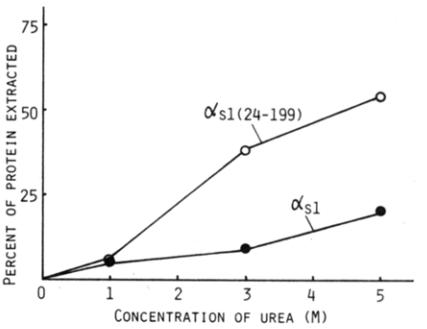

Figure 2. Extraction of α_{s1}-casein and α_{s1}(24–199) peptide from oil globule surfaces with different concentrations of urea. Proteins adsorbed on the oil surface were extracted with urea solutions as described under Materials and Methods and determined by the procedure of Lowry et al. (1951).

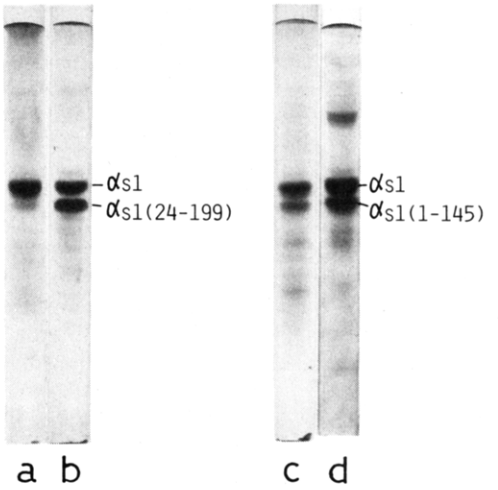

Figure 3. SDS–PAGE patterns of α_{s1}-casein adsorbed on oil globule surfaces followed by treatment with proteases. (a) α_{s1}-Casein in washed cream incubated with pepsin at pH 6.0 and at 37 °C for 40 min; (b) α_{s1}-casein incubated with pepsin in an aqueous solution under the same conditions as in (a); (c) α_{s1}-casein in washed cream incubated with papain at pH 9.0 and at 37 °C for 40 min; (d) α_{s1}-casein in an aqueous solution incubated with papain under the same conditions as in (c).

As shown in Figure 2, α_{s1}-casein adsorbed on the oil surface was barely extracted with 1 and 3 M urea and only about 20% was extracted with 5 M urea. On the other hand, α_{s1}(24–199) adsorbed on the oil surface was more easily extracted with urea. These results suggest that the α_{s1}-casein was more tightly bound to the oil surface than the α_{s1}(24–199).

Enzyme Digestion of α_{s1}-Casein Adsorbed on the Oil Surfaces. α_{s1}-Casein samples as washed cream (α_{s1}-casein adsorbed on the oil globule surfaces) were treated with pepsin or papain. No proteolysis was observed by pepsin in the case of the washed cream (Figure 3a) while α_{s1}-casein in the aqueous solution was cleaved into α_{s1}(24–199) by similar treatment (Figure 3b). The fact that the peptide 23–24 bond of α_{s1}-casein, which is most susceptible to pepsin action (S. Kaminogawa et al., unpublished data), was not broken down in the washed cream suggests that the N-terminal hydrophobic region, including the portion of 23–24 residues, tightly interacts with the oil or buries itself into the oil phase.

On the other hand, conversion of the α_{s1}-casein to α_{s1}-(1–145) by papain did occur in the washed cream, although the reaction seemed to be relatively slow compared to that in the aqueous solution (Figure 3c,d). On the oil surface,

Table II. Emulsifying Properties of α_{s1}-Casein and α_{s1}(24-199) Peptide

	emulsifying activity, m^2/g	emulsion capacity, mL of oil/mg of protein	creaming stability, %
α_{s1}-casein	32.8 ± 2.1	0.76 ± 0.06	100
α_{s1}(24-199)	26.6 ± 0.3	0.70 ± 0.01	39.4 ± 1.4

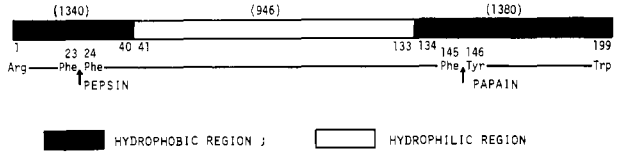

Figure 4. Amphiphilic structure of α_{s1}-casein. Values in parentheses are the average hydrophobicities of the polypeptide regions. The arrows indicate the positions hydrolyzed by pepsin and papain.

the C-terminal hydrophobic region, including the peptide 145–146 bond, might be more accessible to the enzyme than the N-terminal region. Thus, the C-terminal residues of α_{s1}-casein seemed to be relatively weak in interacting with the oil phase.

Emulsifying Properties of α_{s1}-Casein and α_{s1}(24–199). Three parameters, EAI, EC, and CS, were determined to elucidate the emulsifying properties of α_{s1}-casein and α_{s1}(24–199). The results are shown in Table II. The removal of the N-terminal residues decreased the values of the emulsifying properties. This result suggests that the N-terminal hydrophobic residues are important for α_{s1}-casein to act as a good emulsifier. Since the isolation of the α_{s1}(1–145) peptide was not successful, the emulsifying properties of α_{s1}(1–145) were not measured in the present study.

DISCUSSION

In the interaction between proteins and lipids, the hydrophobic property of the molecules generally plays an important role. For example, integral membrane proteins that associate to the lipid matrix of the membranes have hydrophobic sites in their structures (Capaldi, 1977).

A protein-stabilized emulsion is also considered to be one of the systems containing lipid–protein complexes, in which the ability of proteins to interact hydrophobically with lipids, usually triglycerides, is essential. The significance of the hydrophobicity of proteins, especially surface hydrophobicity, has been noted (Kato and Nakai, 1980; Nakai et al., 1980). However, the contribution of protein hydrophobic structures to the formation of stable emulsions remains obscure.

The amphiphilic property in the primary structure of α_{s1}-casein is shown in Figure 4. Such sequential amphiphilic structures are often observed in such integral membrane proteins as glycophorin of the erythrocyte membrane (Marchesi et al., 1976) or cytochrome b_5 of the liver microsomal membrane (Tajima and Sato, 1980). α_{s1}-Casein, which has a hydrophobic area in both the N- and C-terminal regions, was used in the present study to reveal the relationship between the amphiphilic structures of the proteins and their emulsifying properties or adsorbability on oil surfaces.

The surface hydrophobicity and the adsorbability of α_{s1}-casein onto an oil surface were markedly decreased by removal of the N-terminal residues. At the same time, the emulsifying activity and creaming stability were reduced. Kaminogawa et al. (1980) have reported that the calcium sensitivity of α_{s1}-casein was lost by splitting off the N-terminal residues by the action of chymosin and suggested that the hydrophobic N-terminal region played an important role in its precipitation (intermolecular association) by Ca^{2+}. Recently, Creamer et al. (1982) have reported that α_{s1}(25–199), which is formed by limited proteolysis with chymosin, has a lower surface hydrophobicity than α_{s1}-casein and that the peptide segment involving residues 14–24 of the α_{s1}-casein plays an important role in the formation of a network of hydrophobically bonded α_{s1}-casein molecules in fresh cheese. The present findings also suggest that the N-terminal region of α_{s1}-casein contributes to the hydrophobic property of the surface of the protein molecule and is important for the interaction with lipids. The contribution of the N-terminal region to adsorption on the oil globule surface seemed to be greater than that of the C-terminal region, although the latter is more hydrophobic than the former (average hydrophobicities of segments 1–23 and 146–199 are 1270 and 1371 cal/residue, respectively). This might indicate that hydrophobicity alone does not account for the functional properties of the proteins, as pointed out by Kinsella (1981).

The urea extraction and pepsin digestion of washed cream also demonstrated that α_{s1}-casein tightly interacts with lipids similar to the integral proteins of the biomembranes and that the N-terminal region probably takes part in this interaction. It is, therefore, considered that α_{s1}-casein is adsorbed onto oil globule surfaces dominantly by its hydrophobic N-terminal region and hence stabilizes the oil globules.

Many factors concerning the structure of protein molecules have been reported to affect the functional properties of proteins. The present study suggested that, in case of amphiphilic proteins like α_{s1}-casein, their functional behaviors are more or less dependent on their amphiphilic structures. Similar studies using β-casein, another amphiphilic protein, will be described elsewhere.

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High-Performance Liquid Chromatographic Separation of Eastern Black Nightshade (*Solanum ptycanthum*) Glycoalkaloids

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The glycoalkaloids present in Eastern black nightshade were isolated, purified, and identified. An analytical system using high-performance liquid chromatography has been developed for their quantitation. The total glycoalkaloid content of nightshade berries was 12-14 mg/g and the alkaloids found were β-solamargine (2.4 mg/g), α-solamargine (3.3 mg/g), α-solasonine (4.9 mg/g), α-chaconine (0.5 mg/g), and α-solanine (1.0 mg/g).

A recent review (Rogers and Ogg, 1981) on the biology of weeds in the *Solanum nigrum* complex in North America describes four common species of nightshades. The four species are discussed in greater detail in another publication (Ogg et al., 1981). The nightshades have been considered minor weeds in most areas, but in recent years they have become pests in various crops in many parts of the world. The most recent crop to be infested has been the soybean. Since nightshade is a broad-leafed plant, its control by herbicides in soybeans is difficult (Jennings and Fawcett, 1977). Judicious use of herbicides and crop rotation is very important for nightshade control. Nightshade species are members of the Solanaceae family, which contains glycoalkaloids that can cause poisoning and even death when ingested in sufficient quantities.

In 1981, infestation of soybeans occurred in many of the growing areas of the United States. For 3 months in the fall of 1981, the Federal Grain Inspection Service of the U.S. Department of Agriculture conducted a survey on the incidence of eastern black nightshade berries (*Solanum ptycanthum* Dun) in soybeans (Hoy, 1982). On the basis of the results of the survey, the percentage of infested samples is small and the nightshade problem appears to be localized. In Mobile, AL, 0.3% of the soybean samples contained nightshade berries, whereas inspectors at Cedar Rapids, IA, and Fort Dodge, IA, found 0.6 and 1.9% infested samples, respectively.

We have identified the alkaloids present in eastern black nightshade, and this communication describes their isolation and purification. A high-performance liquid chromatographic (HPLC) procedure for quantitation was developed using methanol for the extraction and nicotine as an internal standard.

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MATERIALS AND METHODS

Materials. α-Solanine, α-chaconine, and nicotine standards were purchased from Sigma Co., St. Louis, MO. Solasonine and solamargine standards were generously supplied by Dr. Stanley Osman, Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia, PA. Dried nightshade berries separated from contaminated soybeans were supplied by Dr. Manjit Misra, Department of Plant Pathology, Iowa State University, Ames, IA. Plants grown from seeds separated from these berries were identified as Eastern black nightshade (*S. ptycanthum* Dun) by Dr. A. G. Ogg, Jr., Irrigated Agriculture Research and Extension Center, Prosser, WA, and Dr. E. E. Shilling, Jr., University of Tennessee, Knoxville, TN.

Dried nightshade berries were ground for 1-3 min in a Varco (Bellville, NJ) electric grinder, Model 228100. The full-fat flour from this grinder contained particles, 90% of which passed a 40-mesh screen. The full-fat powder was extracted for 6 h with *n*-hexane in a Soxhlet extractor to give a defatted material. After extraction, the hexane was stripped from the oil, and the oil was then extracted with methanol. No glycoalkaloids were detected in the methanol-soluble fraction, indicating a defatting process in which nearly all of the glycoalkaloids were left in the defatted residue.

HPLC Analysis. A Waters Associates (Milford, MA) HPLC system comprised of a M-45 solvent delivery system and a Model 450 variable-wavelength detector was used. All columns were protected with guard columns of Porasil μBondapak. The solvent flow rate was usually 2 mL/min and the absorption was measured at 200 nm. Solvents were spectral grade, and distilled water was deionized before use. All solvent ratios are on a volume basis.

HPLC conditions were according to Morris and Lee (1981), with some minor modifications. A 1-g defatted ground sample was extracted with 20 mL of methanol, containing approximately 0.20 mg of nicotine/mL as an internal standard, by refluxing on a steam bath for 2 h.