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Studies on Taramira Seed (*Eruca sativa* Lam.) Proteins

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The total extractable proteins of the defatted Taramira meal (*Eruca sativa* Lam.) have been fractionated into a number of components by gel filtration, ion-exchange chromatography, and polyacrylamide gel electrophoresis. A major globulin fraction (F1) which constituted 40% of total proteins has been isolated to homogeneity. Protein F1 was a typical storage glycoprotein containing 8% total carbohydrates. Its molecular weight was 2.5×10^5 as determined by gel filtration. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gave four subunits with molecular weights of 60 000, 35 000, 26 600, and 18 000, respectively. The apparent viscosity of the F1 protein was 0.038 dL/g, and the absorption maximum was at 280 nm. The amino acid acid composition of total protein and F1 protein has been determined.

Taramira (*Eruca sativa* Lam.), which belongs to the family Cruciferae, is commonly grown as an oilseed crop in northwest India. The seeds contain 20–25% protein and 30–35% oil. The oil is used for edible purposes but is considered inferior to rapeseed oil for industrial purposes (Vaughan, 1970). The cake (residue left after extraction of the oil) is mainly fed to cattle.

In view of the worldwide protein shortage, rapeseed has received a great deal of attention as a potential source of protein for edible purposes because of its balanced amino acid composition (Ballester et al., 1970; Appelqvist and Ohlson, 1972). Proteins from rapeseed have been fractionated and some of their properties studied (Bhatty et al., 1968; Lonnerdal and Janson, 1972; MacKenzie, 1975; Schwenke and Raab, 1979; Schwenke et al., 1980). However, a literature survey showed that no work has been reported on the study of the nature of proteins from Taramira seeds. In the present investigation, Taramira proteins have been isolated and partially characterized.

MATERIALS AND METHODS

Materials. Taramira seed (*Eruca sativa*) var. ITSA was obtained from the Department of Plant Breeding, Punjab Agricultural University, Ludhiana. Reagents used were of analytical grade.

Preparation of Taramira Meal. Taramira seeds were crushed in a mechanical grinder and defatted by repeated extraction with hexane at room temperature (25 °C) for 48 h. The defatted material was then ground to a powder of 85 mesh. The nitrogen content of the flour was 9.8% and the moisture content 6%. Nonprotein nitrogen (estimated from the 10% Cl_3AcOH extract of the defatted meal) accounts for 7–8% of the total nitrogen content of defatted meal.

Extraction of Proteins from Defatted Meal. Water, 0.1 M NaCl, and 1 M NaCl were employed as the extraction solvents for nitrogen solubility studies. Defatted flour

(2 g) was dispersed in the solvent (15 mL), and the pH of the suspension was adjusted to the desired value by the addition of 1 M HCl or 1 M NaOH. The extraction was carried out with mechanical shaking for 2 h at room temperature (25 °C). The extract was clarified by centrifugation at 5000 rpm for 15 min. The recovered pellet was then reextracted by shaking for 30 min with 10 mL of the solvent in the same manner. Both extracts and the supernatants were combined and the pH was noted. Extracts of pH range 1–12 were thus prepared. In each case, aliquots of 5 mL were taken for nitrogen estimation by the micro-Kjeldahl method. The nitrogen extracted was expressed as the percentage of the total meal nitrogen.

Ammonium Sulfate Fractionation. To 10 g of the meal 100 mL of 1 M NaCl solution was added, and the contents were shaken for 2 h at room temperature. This extract was centrifuged at 5000g for 15 min. The supernatant was dialyzed extensively with 1 M NaCl solution for 48 h and diluted to about 1% protein concentration. Solid $(NH_4)_2SO_4$ was added to the protein solution divided into 8 parts with a 10-mL volume in each case so as to obtain 10–80% $(NH_4)_2SO_4$ concentration. The $(NH_4)_2SO_4$ was mixed thoroughly for 1 h at room temperature and centrifuged at 5000g for 15 min to remove the precipitates. The protein in the supernatant was estimated by the method of Lowry et al. (1951) in each case.

Gel Filtration. Two milliliters of 1 M NaCl extract containing nearly 100 mg of protein was chromatographed on a Sephadex G-200 column (60 × 2.2 cm) equilibrated with 0.01 M sodium borate buffer, pH 8.2, containing 1 M NaCl solution. The proteins were eluted at a flow rate of 0.4 mL/min, and 5-mL fractions were collected. Protein in the column effluents was determined by a UV spectrophotometer at 280 nm. A Sephadex G-150 column (60 × 2.2 cm) equilibrated with same buffer was also used for gel filtration studies.

DEAE-cellulose Ion-Exchange Chromatography. DEAE-cellulose was activated by washing first with 1 M NaOH and distilled H_2O and then with 1 N HCl and distilled H_2O. Washed DEAE-cellulose was equilibrated

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with 0.025 M borate buffer (pH 8.0) and packed into a column (2 × 30 cm). Samples containing 100 mg of protein in the same buffer were chromatographed on the column by using a linear NaCl gradient (0.0–1.0 M NaCl) in borate buffer. Four-milliliter fractions were collected at a flow rate of 0.5 mL/min and monitored for protein content by measuring absorbance at 280 nm. The major fraction was designated as F1 protein.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed by the method of Davis (1964) in 7% gels using 0.02 M Tris–glycine buffer of pH 8.3. Tubes of the size 0.5 × 7.5 cm were used, and electrophoresis was carried out for 2 h at 3 mA/tube in a disc electrophoresis apparatus. The gels were stained with 0.1% Coomassie blue in 7.5% acetic acid, and the excess dye was subsequently removed by washing with 7.5% acetic acid.

Determination of Molecular Weight by Gel Filtration. A Sephadex G-200 column (60 × 2.2 cm) was equilibrated with 0.05 M borate buffer (pH 8.2) containing 1 M NaCl solution. The molecular weight of F1 protein (major purified fraction) was determined by using apoferritin (440 000), aldolase (150 000), bovine serum albumin (68 000), pepsin (35 500) and ribonuclease A (13 600) as standards. The value of V_e/V_0 for each protein was plotted against the logarithm of the molecular weight of the standard protein from which the molecular weight of F1 protein was calculated.

Determination of Subunits and Their Molecular Weight by NaDodSO$_4$-Polyacrylamide Gel Electrophoresis. The determination of the number of subunits and their molecular weight was carried out by the method of Weber and Osborn (1969) using bovine serum albumin, pepsin, α-chymotrypsin (23 800), and ribonuclease A as standards. Electrophoresis was carried out for 7 h at 6 mA/tube. The gels were stained with 0.1% coomassie blue in 7.5% acetic acid overnight. Destaining was done by using 7.5% acetic acid.

Apparent Viscosity. Intrinsic viscosity was determined at 30 ± 0.1 °C in an Ubbelohde viscometer having a flow time of 250 s with 1 M NaCl solution.

Absorption Spectrum. The absorption spectrum of the protein in 1 M NaCl was recorded in a Gilford spectrophotometer in the range 220–300 nm.

Amino Acid Analysis. Total proteins and F1 protein were hydrolyzed in 6 M HCl for 24 h at 110 °C in sealed tubes. The acid was removed by evaporating in a vacuum desiccator, and the hydrolysate was analyzed in Beckman amino acid analyzer for amino acid composition.

Phosphorus Estimation. Phosphorus was estimated by the method of Ames (1966) using protein solution in 1 M NaCl.

Carbohydrate Content. Total sugars in F1 protein were estimated by the method of Dubois et al. (1956). For qualitative analysis of neutral sugars, 50 mg of F1 protein was hydrolyzed in 1 M HCl for 4 h at 100 °C in a sealed tube. The hydrolysate was filtered, the filtrate after diluting with an equal amount of distilled water was passed through a Dowex I (acetate) column, and the eluate was concentrated to dryness under vacuum and subjected to chromatography on Whatman No. 1 paper by using butanol–acetic acid–H$_2$O (BAW), 4:1:5, as the developing solvent. The dried chromatogram was sprayed with benzidine–trichloroacetic acid reagent.

RESULTS AND DISCUSSION

Solubility. The relative solubility of Taramira meal proteins as a function of pH in different solvents is shown in Figure 1. Higher extraction of meal nitrogen in water was obtained at pH 6.5 and pH 11.0 where 55% and 90%
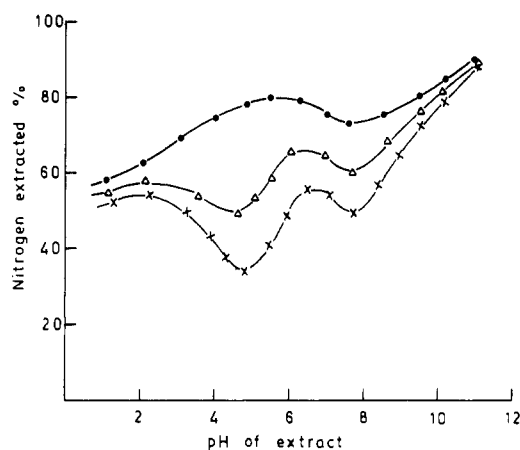

Figure 1. Extractability of Taramira meal nitrogen as a function of pH. (×) In water; (△) in 0.1 M NaCl; (●) in 1 M NaCl.
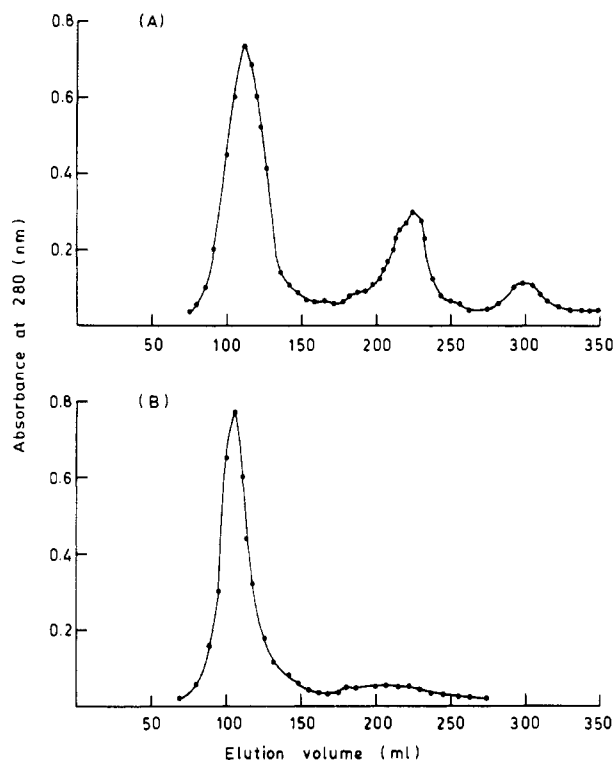

Figure 2. Gel filtration pattern on Sephadex G-200. (A) Total proteins; (B) F1 protein.

of the meal nitrogen was extracted, respectively. Minimum solubility was observed at pH 4.8 and 7.8. Solubility increased rapidly as pH was increased beyond 8.

The solubility profiles in 0.1 M NaCl and 1 M NaCl solutions showed that nitrogen extractability was sensitive to changes in NaCl concentration. Higher yields of nitrogen were obtained with 1 M NaCl solution specifically between pH 4 and pH 8 compared to those obtained with water. This may be due to the fact that the bulk of proteins of Taramira consists of globulins which are highly soluble in 1 M NaCl solution. Solubility in 0.1 M NaCl was similar and showed intermediate values between those of water and 1 M NaCl. Similar solubility profiles have been reported in rapeseed (Quinn and Jones, 1976; Gillberg and Tornell, 1976), whereas in other oil seeds, such as soybeans, peanuts, and sunflower, U-shaped solubility profiles have been reported (Fontaine et al., 1944; Fan and Sosulski, 1974). In comparison to these seeds, in Taramira relatively high dissolution of nitrogen was obtained even at its pH minima. Evidently, the protein structure of
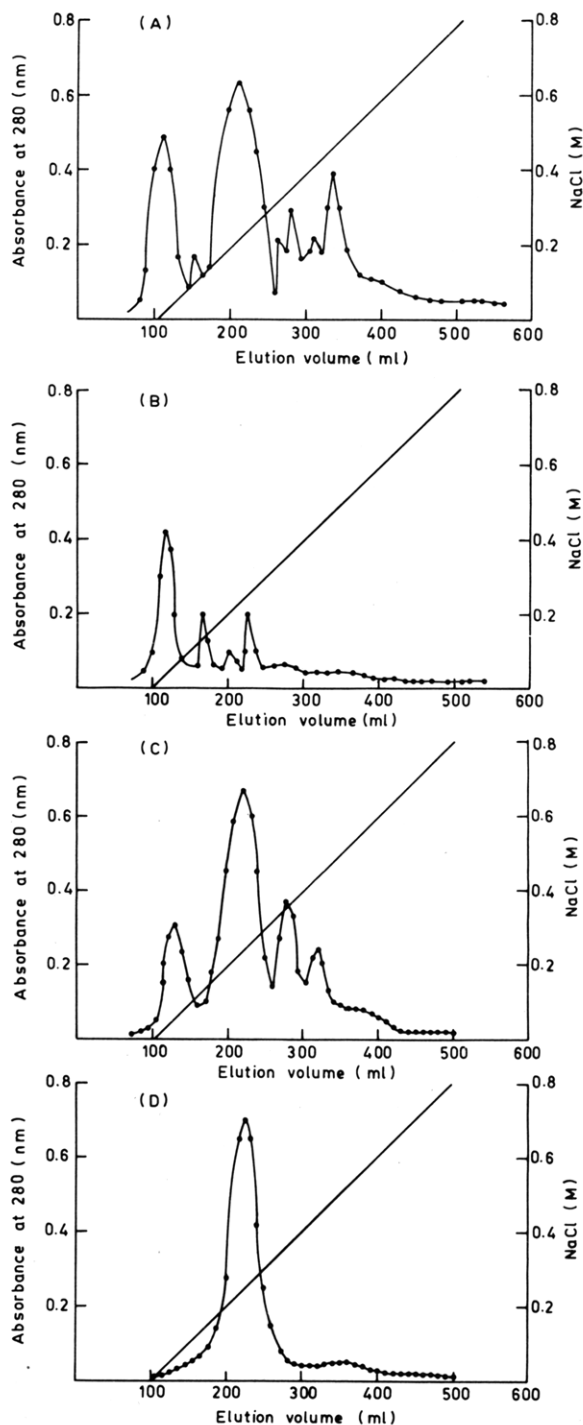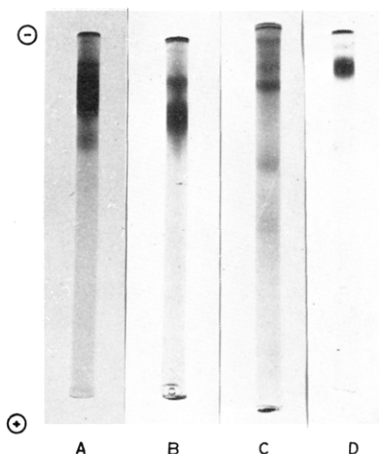
Figure 4. Polyacrylamide gel electrophoretic pattern of Taramira proteins in 0.025 M Tris–glycine buffer, pH 8.3. (A) Total proteins; (B) globulins; (C) albumins; (D) F1 protein.

(results not shown). These fractions were again found to be heterogeneous by electrophoresis. From these results, it is evident that first peak obtained by gel filtration of total proteins is a mixture of high molecular weight albumins and globulins.

DEAE-cellulose Ion-Exchange Chromatography. Data pertaining to the composition of total proteins and its globulin and albumin fractions as determined by DEAE-cellulose chromatography are shown in Figure 3. Total proteins were resolved into seven components. The first fraction was almost unadsorbed and eluted at 0.00–0.02 M NaCl concentration, the next three fractions were obtained in a weakly bound region eluting at 0.05–0.3 M NaCl concentration, and the remaining three minor fractions were strongly bound and eluted between 0.35 and 0.55 M NaCl concentration. Globulins were resolved into four fractions eluting at 0.05, 0.25, 0.35, and 0.45 M NaCl concentration. Albumins were also resolved into four fractions which eluted at 0.04, 0.16, 0.25, and 0.30 M NaCl concentration. The major fraction of globulin was eluted at 0.25 M NaCl concentration (Figure 3C), whereas albumin was eluted at 0.04 M NaCl concentrations (Figure 3B). Albumins were very weakly bound and eluted much earlier than globulins. The major fractions of both albumins and globulins were found to be homogeneous by electrophoresis. Since major fraction of globulins designated as F1 protein constituted about 40% of the total extractable proteins, this fraction was taken up for detailed characterization. F1 protein could also be obtained in a homogeneous state by 20% $(NH_4)_2SO_4$ fractionation, but in this case there was a slight contamination. Final purification was acheived by either Sephadex G-200 (Figure 2B) filtration or DEAE-cellulose chromatography (Figure 3D).

Gel Electrophoresis. The polyacrylamide gel electrophoretic pattern of total proteins, globulins, albumins, and F1 protein is shown in Figure 4. Total proteins gave an intense diffuse band and a heterogeneous band. Globulins were resolved into three bands with one major broad globulin band. Similar electrophoretic behavior of vicilin has been reported by Bailey and Boulter (1972). They attributed this property to complex association–dissociation reactions between the protein molecule and its subunits during electrophoresis. Albumins were resolved into four bands and were well separated. F1 proteins gave a single band and it is very comparable to the major band of globulins. Thus, Taramira proteins are complex, consisting of at least three globulins and four albumin fractions.

Figure 3. DEAE-cellulose ion-exchange chromatography of (A) total proteins, (B) albumins, (C) globulins, and (D) F1 protein.

Taramira differs from that of the other oilseed proteins.

Gel Filtration. Gel filtration of total proteins gave three peaks eluted with V_e/V_0 values of 1.20, 2.44, and 3.44 in the fraction proportion of 62%, 28%, and 10%, respectively (Figure 2). The first fraction was turbid and eluted immediately after the void volume. None of these fractions were found to be electrophoretically homogeneous. For further resolution of the proteins, 1 M NaCl meal extract was extensively dialyzed against distilled water at 4 °C for 48 h. The globulins were precipitated and the supernatants contained the water-soluble albumins. The concentration of globulins and albumins was found to be in the ratio of 80:20. On gel filtration, globulins gave two peaks eluted with V_e/V_0 values of 1.23 and 2.42, whereas albumins eluted with V_e/V_0 values of 1.32 and 2.45

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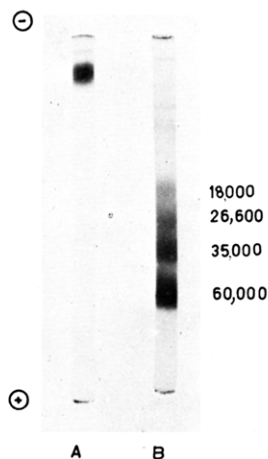Kaushal, Sital, and Bhatia

Figure 5. NaDodSO$_4$–polyacrylamide gel electrophoresis of F1 protein. (A) F1 protein; (B) subunits of F1 protein.
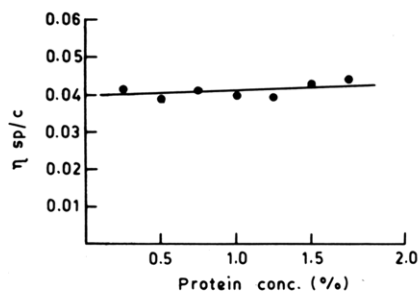

Figure 6. η_{rel} as a function of protein concentration.

Molecular Weight of F1 Protein and Its Subunits.

Molecular weight of F1 protein was determined by using both Sephadex G-200 and Sephadex G-150 molecular-sieve chromatography. The molecular weight in Sephadex G-200 was found to be 2.5×10^5, and in Sephadex G-150 it was found to be 2.4×10^5.

The number and molecular weight of subunits of F1 protein were determined by NaDodSO$_4$–polyacrylamide gel electrophoresis. It consisted of mainly four subunits having molecular weights of 60 000, 35 000, 26 600, and 18 000, respectively (Figure 5). Some minor bands of very low molecular weight also appeared on the gel which may be due to some artifacts. From a comparison of the molecular weights of F1 protein and its subunits, it appears that at least some of the subunits are repeated in the F1 protein.

Apparent Viscosity. The intrinsic viscosity of F1 protein was found to be 0.039 dL/g (Figure 6). Most globular proteins have intrinsic viscosity values between 0.03 and 0.04 dL/g (Tanford, 1961). The value 0.039 dL/g indicates that it is a globular protein with a good degree of compactness.

Absorption Spectrum. The ultraviolet absorption spectrum of F1 protein showed maximum absorption at 280 nm and minimum absorption at 255 nm, which shows that the spectrum is that of a typical protein. the ratio of absorbance at 280/260 was 1.48. this suggested very little cotamination with nucleic acids (Layne, 1957).

Carbohydrate and Phosphorus Contents. The total carbohydrate content in F1 protein was found to be 8%. The neutral carbohydrates were identified as glucose and arabinose by paper chromatography by comparisons of their chromatographic behavior with that of authentic samples of hexoses and pentoses. Phosphorus was found to be absent in F1 protein.

Amino Acid Analysis. The amino acid composition of total proteins and F1 protein is given in Table I. Both

Table I. Amino Acid Composition (Grams per 100 Grams of Recovered Amino Acids) of Total Proteins and F1 Proteins from Taramira[a]

	total proteins	F1 protein	12S globulin from *B. napus*[b]
Lys	7.2 ± 0.2	5.4 ± 0.2	5.6
His	3.2 ± 0.1	2.9 ± 0.4	2.2
NH$_3$	2.6 ± 0.3	3.6 ± 0.1	2.2
Arg	7.5 ± 0.0	7.3 ± 0.4	7.1
Asp	8.0 ± 0.4	10.7 ± 0.4	10.8
Thr	2.7 ± 0.1	3.2 ± 0.0	4.6
Ser	2.3 ± 0.0	2.8 ± 0.1	4.6
Glu	17.0 ± 0.1	17.0 ± 0.1	21.5
Pro	6.9 ± 0.5	4.4 ± 0.5	5.8
Gly	6.1 ± 0.1	5.6 ± 0.0	5.8
Ala	5.0 ± 0.2	5.5 ± 0.2	4.8
Cys[c]	3.9 ± 0.1	2.9 ± 0.1	0.4
Val	6.1 ± 0.1	6.4 ± 0.2	5.8
Met	1.6 ± 0.1	1.3 ± 0.1	1.8
Ile	4.5 ± 0.1	4.6 ± 0.1	5.0
Leu	8.3 ± 0.0	9.0 ± 0.2	8.8
Tyr	2.0 ± 0.1	2.2 ± 0.1	3.2
Phe	4.7 ± 0.2	4.9 ± 0.0	5.1
recovery, %	83	84	80

[a] Values are mean ± SD of three determinations. [b] Data calculated from Goding et al. (1970). [c] Cys equals cysteine and cystine.

total proteins and F1 protein contained large amounts of glutamic acid, arginine, leucine, and aspartic acid residues. These proteins also contained high concentrations of lysine which is the first limiting amino acid in cereals and other vegetable proteins. The F1 protein showed the characteristic amino acid composition of a storage protein (Derbyshire et al., 1976). A comparison of the amino acid compn. of F1 protein with that of 12S globulin isolated from *Brassica napus* (Goding et al., 1970) indicates that the values are very close to each other (Table I). Considering the good amino acid compn. of taramira proteins, these proteins have good potential as a protein source for edible purposes.

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Gas–Liquid Chromatographic Method for Analysis of Di- and Polyamines in Foods

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A gas–liquid chromatographic method for the quantitative determination of putrescine, cadaverine, spermidine, and spermine in foods has been developed. The amines were separated from foods by eluting through a cation-exchange resin column and then converted to their (ethyloxy)carbonyl derivatives by the reaction with ethyl chloroformate in aqueous medium before application to the gas chromatograph with a flame ionization detector. 1,8-Diaminooctane was used as an internal standard. Separation and determination of the resulting derivatives were performed on a 1.5% SE-30/0.3% SP-1000 on Uniport HP column (0.5 m) under the temperature-programmed condition. The calibration curves for the amines in the range of 12.5–125 nmol were linear and sufficiently reproducible for quantitative determination. The overall recovery rates were satisfactory. Putrescine and spermidine were present in all of the foods investigated. Relatively large amounts of spermidine occurred in the mushrooms and beans investigated.

The polyamines spermidine and spermine as well as the diamines putrescine and cadaverine are widely distributed in nature and have recently attracted considerable attention in the field of food chemistry from a public health standpoint since some of these amines may be nitrosated or act as potential precursors for other amines capable of forming nitrosamines. Lijinsky and Epstein (1970) postulated that putrescine and cadaverine might be converted by heating or cooking to pyrrolidine and piperidine, respectively. These amines formed are the precursors of the corresponding nitrosamines which are highly carcinogenic. Bills et al. (1973) demonstrated that *N*-nitrosopyrrolidine is produced from putrescine and spermidine when heated in the presence of nitrite. It is well-known that nitrite is widely present in nature and is also produced in human saliva. The possibility that nitrosamines can be formed in vivo, particularly in the gastrointestinal tract, has been reported (Spiegelhalder et al., 1976; Tannenbaum et al., 1974). Both spermidine and spermine contain secondary amino groups which may react with nitrite, and nitrosation might occur upon ingestion of foods containing these components. In fact, Hildrum et al. (1975, 1977) isolated 3-butenyl-2-propenyl-*N*-nitrosamine as the principal volatile product formed in the nitrosation of both spermidine and spermine and later identified three other volatile nitrosamines from spermidine. Recently, Hotchkiss et al. (1977) identified four nonvolatile nitrosamines, bis(hydroxyalkyl)-*N*-nitrosamines, formed upon nitrosation of spermidine. More recently, mutagenicity of these nonvolatile nitrosamines together with 3-butenyl-2-propenyl-*N*-nitrosamine was confirmed by using various strains of *Salmonella typhimurium* in the presence and absence of S9 mix (Hotchkiss et al., 1979).

On the other hand, although these di- and polyamines might be present in significant amounts in food systems, investigations on quantitative methods as well as their contents in foods and food materials are relatively few. It has been reported that these amines are present in germs such as barley, rice, and wheat (Moruzzi and Caldarera, 1964) and in soybean seeds (Wang, 1972). In these papers, determinations were carried out by ion-exchange chromatographic separation, followed by spectrophotometry of the derivatives formed by the reaction with 2,4-dinitrofluorobenzene. Smith (1975) has reviewed the distribution, biosynthesis, and metabolism of di- and polyamines in higher plants, but food materials are not included. Spinelli et al. (1974) and Lakritz et al. (1975) determined the concentrations of di- and polyamines in porks with other amines, in which the amines extracted with organic solvents were dansylated and the resulting derivatives were quantified spectrofluorometrically after separation by thin-layer chromatography. However, these methods are not satisfactory because of time requirements and lack of resolving power and selectivity.

During a study of the determination of tyramine in fermented food products by gas–liquid chromatography (GLC) (Yamamoto et al., 1980), we found that putrescine and cadaverine in foods could be also derivatized with ethyl chloroformate to the corresponding (ethyloxy)carbonyl (EOC) derivatives, which showed good and reasonable peaks on the gas chromatogram. The present study, based on this observation, was undertaken in order to explore the possibility of carrying out the GLC determination of not only diamines but also polyamines such as spermidine and spermine in foods as their EOC derivatives.

EXPERIMENTAL SECTION

Chemicals and Solvents. All di- and polyamines for standards were obtained, as hydrochloride salts, from Nakarai Chemicals (Kyoto, Japan) and, prior to use, dried

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