Sinapine and Related Esters in Seed Meal of *Crambe abyssinica*

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The presence of sinapine in *Crambe abyssinica* seed has been established by isolation of its thiocyanate salt from extracts of the defatted meal freed of natural thioglucosides. Assay of a series of representative defatted crambe meals showed an average content of 0.46% sinapine, reported as thiocyanate salt, or about half the amount present in commercial rapeseed meals. The occurrence in crambe seed meal of 1-sinapoyl-β-D-glucose was also demonstrated. Sinapine (as the bisulfate salt) fed to rats at levels normally encountered in crambe meal had no effect on growth rate, feed intake, or feed efficiency.

Phytochemical investigations have frequently been directed to the various thioglucosides present in members of Cruciferae, the seed meals of which usually provide the most convenient source. These substances evoked interest initially because of their presence in mustard and their ready conversion to the pungent compounds characteristic of this condiment. However, the scope of these investigations has since expanded greatly, as evidenced by Kjaer's review (1963).

Other constituents of crucifer plants have received less attention than the thioglucosides, even though they may have considerable biochemical significance. The longest known and best characterized of these minor components is sinapine (I), the choline ester of 3,5-dimethoxy-4-hydroxycinnamic acid, or sinapic acid (II) (Figure 1). Both hydrolysis products of sinapine appear to have metabolic importance in higher plants—sinapic acid for biosynthesis of lignin and flavonoids (Neish, 1960) and choline for its function in the methylation cycle (Byerrum and Wing, 1953). Despite this possibility, the only published indication sinapine may serve as a reservoir for sinapic acid and choline was given by Tzagoloff (1963a), who found that it was rapidly degraded in germinating mustard seedlings.

Sinapine occurs rather widely among species of Cruciferae (Schultz and Gmelin, 1952, 1953), although its presence is mainly indicated by paper chromatography of plant extracts, and quantitative information on its distribution is meager (Schwarze, 1949). The bitter nature of sinapine has been mentioned (Schultz and Gmelin, 1952), but incorporation of the bisulfate salt in feed at levels normally encountered in rapeseed did not adversely affect the growth rate of chicks (Clandinin, 1961).

As part of a continuing program on determining characteristics of *Crambe abyssinica* Hochst ex R. E. Fries, the occurrence of sinapine and related compounds in the seed meal of this crucifer was investigated. Beforehand, presumptive evidence for the presence of sinapine had been obtained by paper chromatography of seed extracts, but proof by isolation of the compound or a derivative had not been achieved. Initially, extracts of crambe resembled those from *Iberis amara* L., from which Shultz and Gmelin (1954) were unable to crystallize the substance, even though the extracts exhibited sinapinelike properties.

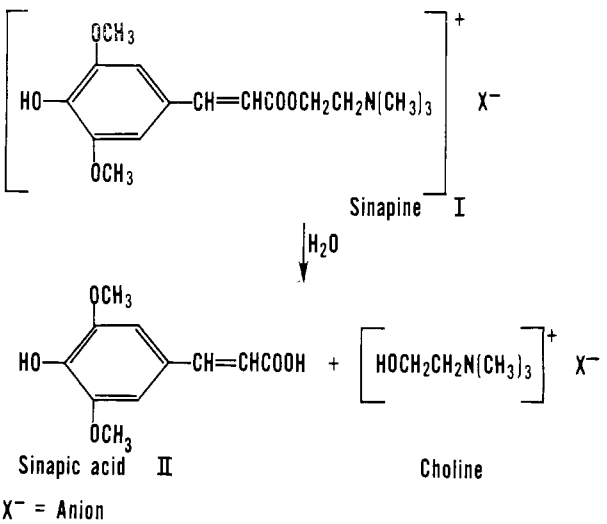

Figure 1. Sinapine and its hydrolytic cleavage products

In contrast to earlier work on crambe, we demonstrated that sinapine can be successfully isolated from this source in the form of its thiocyanate salt. In addition, we report further observations on the properties of sinapine and its quantitative assessment in several representative seed meals.

EXPERIMENTAL

Isolation of Sinapine from Crambe. Direct isolation of sinapine from various extracts of crambe meal, by methods suitable for rapeseed meal (Clandinin, 1961), was unsatisfactory. More productive was an examination of extracts freed of *epi*-progoitrin (Daxenbichler *et al.*, 1965), the major thioglucoside present. Mature, flaked crambe seeds, extracted at room temperature with pentane-hexane (total: 6 liters per kg. in nine portions), were ground and then extracted with acetone–water (76:24 w./w.; total 4 liters per kg.). Hydrolysis of thioglucoside (s) by endogenous enzymes is minimized under these conditions. Solids were removed in a continuous centrifuge and re-extracted with aqueous acetone (2 liters per kg.). On concentration of these combined extracts in vacuo to remove acetone and a portion of the water, followed by chilling, the polypeptide crambin separated (Van Etten *et al.*, 1965). Two liters of aqueous concentrate derived from about 2 kg. of defatted crambe seed were freed of crambin by filtration and then placed on a column

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consisting of 4 kg. of acidic alumina (Camag), scaled up from Daxenbichler *et al.* (1965). The alumina, which adsorbs most of the *epi*-progoitrin, was washed with a total of 9 liters of water.

Aqueous effluents from the column were spot tested at 500-ml. intervals with alkali (yellow color) and ferric chloride (red color). Tests were carried out on 0.2-ml. samples of effluent to which was added one drop of reagent (0.2*N* sodium hydroxide or 10% aqueous ferric chloride). The visual intensity of the colors was used as an indicator for the presence of sinapine. Fractions that gave the strongest color tests were combined (3 liters) and concentrated in vacuo to 250 ml. To a 40-ml. aliquot of this concentrate were added 100 ml. of a 20% aqueous solution of potassium thiocyanate. The precipitate that formed after 3 days' refrigeration was removed by filtration and recrystallized twice from 95% ethanol. There were recovered 619 mg. of sinapine thiocyanate, which melted at 179.5–81° C. The literature reports m.p. 179° C. (Gadamer, 1896). A sample of sinapine thiocyanate obtained in simpler fashion from rapeseed meal (*Brassica napus*) (Clandinin, 1961) was identical with that from crambe by m.p. and mixed m.p. Final confirmation of the identity of the compound from crambe was achieved by hydrolysis with 0.1*N* barium hydroxide (Schultz and Gmelin, 1952). The insoluble barium salt of sinapic acid was decomposed with dilute hydrochloric acid, and free sinapic acid was isolated by filtration and recrystallized from 95% alcohol, m.p. 193.2–94.5° C. The literature reports m.p. 193.6–95.6° C. (Kung and Huang, 1949).

Spectroscopic Properties. Ultraviolet and visible absorption data for sinapine and sinapic acid were obtained with a Beckman DK-2 recording spectrophotometer (Table I). Solvents used were distilled water and 0.2*M* sodium carbonate–bicarbonate buffer at pH 10.0. Significant bathochromic shifts occurred when the solutions were made alkaline.

For comparison, the molar absorptivity (ϵ) for sinapine was determined at its absorption minimum at 290 mμ at pH 10. The value found, $\epsilon = 2.65 \times 10^3$, is in agreement with the value ($\epsilon = 2.7 \times 10^3$) reported by Tzagoloff (1963b).

Freshly prepared solutions were used for the spectrophotometric studies. At pH 10, facile hydrolysis of sinapine is expected, although nonenzymatic hydrolysis of the ester may not be important within a short time period (Tzagoloff, 1963b). Even in aqueous solution at pH 3.0, deterioration of sinapine was observed. After a week, only three quarters of the original amount remained in a dilute solution of the compound.

Table I. Spectral Data for Sinapine and Sinapic Acid from Crambe Meal

	Distilled Water		pH 10 Buffer	
Compound	λ_{max}, mμ	Log ϵ	λ_{max}, mμ	Log ϵ
Sinapine	326	4.33	395	4.43
thiocyanate	235	...	257	...
			233	...
Sinapic acid	318	4.28	355	4.34
	235	...	305	...

Assay of Seed Meals for Sinapine. An assay procedure for sinapine in mustard has been reported by Tzagoloff (1963a), but the method had to be modified before it was applied to crambe meals. The procedure finally adopted involved three extractions with hot methanol (25 + 15 + 15 ml. per extraction) of a 1-gram sample of ground, defatted crambe meal, followed by gravity filtration of the extracts. Solvent was removed from the combined extracts in vacuo and the residue suspended in successive portions of water and filtered through analytical grade Celite. A clear aqueous filtrate was essential at this point to avoid difficulties in subsequent steps of the assay. Chloroform extraction was eliminated because of its propensity to form stable emulsions. Aliquots of the aqueous filtrate were promptly treated with Reinecke salt to precipitate sinapine and the remainder of the assay was carried out by the Tzagoloff method. The absorbance of the alkalinized methanol solution was determined at 400 mμ with a Beckman Model B spectrophotometer. Comparison was made with a standard curve derived from freshly prepared solutions of sinapine thiocyanate, since Tzagoloff's so-called "stock solution" was unstable on storage. The optical response was linear in the range 0 to 5 μg. per ml. of methanol solution. Replicate samples checked within ± 0.02% absolute.

The assay values for 10 oil-free crambe meal samples of diverse age and from several geographical areas ranged from 0.27 to 0.56% on an air-dry basis, calculated as sinapine thiocyanate. The majority of these samples gave values near the upper limit of this range (av. 0.46%), or about half as much as found by Schwarze (1949) for rapeseed meals.

VanEtten *et al.* (1966) showed that age and storage conditions, among other factors, affect the nature of the products formed from the thioglucoside when crambe meals are autolyzed in the presence of native enzyme. Parallel determinations of sinapine displayed no corresponding variations (Table II).

Additional studies demonstrated that autolysis of the meals by conventional procedures (VanEtten *et al.*, 1966) did not produce any concomitant degradation of the sinapine. A blend of solvent-extracted meals, unheated, contained 0.45% sinapine before autolysis and 0.42% after. On the other hand, a process to remove autolysis products from meal by wet acetone extraction (Tookey *et al.*, 1965) also reduced residual sinapine to negligible levels (0.05%).

Data on the sinapine content of crambe were compared with those from several other species of Cruciferae which possess in common a high erucic acid content in their seed oil. Table III summarizes these results. The particular *Brassica napus* and *Brassica campestris* varieties reported

Table II. Sinapine Content of Crambe Meals in Relation to Age and Storage Conditions

	Sinapine (%) under Storage Condition	
Seed	Controlled temperature at 5° C.	Uncontrolled temperature (barn)
Stored 1 year	0.36	0.39
Stored 4 years	0.42	0.38

Table III. Sinapine Content of Crucifer Seed Meals

Species	Sinapine, %
Crambe abyssinica (range for 10 accessions)	0.27–0.56
Brassica napus	0.94
Brassica campestris	0.92
Brassica carinata	1.17

are rapeseeds of commerce, known in Canada as Argentine and Polish rape, respectively.

Chromatography of Crambe Meal Extracts. Paper chromatography of aqueous extracts derived from crambe was carried out with the solvent system butanol–acetic acid–water (4:1:5). The mixture of substances in the extract was resolved into four spots which could be visualized as a blue fluorescence under long-wave ultraviolet light. The major spot had R_f = 0.51 and was the same as sinapine run concurrently. Two minor fluorescent spots remained near the origin (R_f = 0.09 and 0.15, respectively), while one migrated faster (R_f = 0.58) than sinapine. Sinapic acid (R_f = 0.81) was not observed in fresh extracts of crambe but was present after prolonged standing.

Sinapine Fraction. 1-Sinapoyl Glucose. Twelve samples of aqueous extract (100 μl. per sample) were streaked on paper (Whatman No. 1) and developed with the butanol–acetic acid–water solvent system. The major band corresponding to sinapine was cut out and eluted with 70% methanol. The extracts were combined and the methanol was evaporated in vacuo. An aliquot of the residue was examined spectroscopically in both neutral solution and at pH 10. The curves were indistinguishable from those of sinapine under these conditions and corresponded to a total of 15.3 mg. of sinapine (calculated as thiocyanate salt). The sinapine from another aliquot was precipitated with Reinecke salt and analyzed as the crambe meal extracts were. The assay value was 9.1 mg. total sinapine (calculated as thiocyanate), or only 60% of the amount determined spectroscopically. Obviously, the chromatographic fraction comprised sinapine and at least one other component that possessed the chromophoric function of sinapine, but lacked the basic group required for precipitation by Reinecke salt.

Components comprising the "sinapine spot" were separated by treatment of an aqueous extract (5 ml.) derived from crambe meal with excess Reinecke salt at 0° C. After an hour the precipitate was separated by centrifugation. The aqueous supernatant was free of sinapine as demonstrated by TLC on silica gel G (solvent: ethyl acetate–isopropyl alcohol–water; 53:30.5:16.5); nevertheless, on paper chromatography in butanol–acetic acid–water it showed a major fluorescing component at the same R_f value as sinapine. Three minor fluorescent substances of lower R_f values also appeared.

The same separation was carried out on a larger scale, and the soluble fraction from the Reinecke salt precipitation was streaked on a series of papers developed in butanol–acetic acid–water. The major fluorescent band was eluted with 70% methanol, and the process of chromatography and elution repeated, once with the same developing solvent and again with a mixture of ethyl acetate–isopropyl alcohol–water (53:32.5:16.5). Eluting solvent remained the same. Only one fluorescent component was evident in the final eluate, either by paper or thin-layer chromatography. The residue from the final eluate, dissolved in methanol (0.5 ml.), was precipitated by the addition of chloroform (2 ml.). The separated solid was then dissolved in ethanol (1 ml.) to which was added ethyl acetate (1 ml.). Chilling this mixture caused the separation of a dark substance which was removed by centrifugation. Evaporation of solvents in a stream of nitrogen gave a light yellow product (11 mg.) having chromatographic mobility and spectral characteristics unchanged by the purification procedure. It contained no free sinapic acid or glucose.

A portion of the isolated substance was hydrolyzed with 2*N* potassium hydroxide (4 ml.) at room temperature for 4 hours. Neutralization with dilute hydrochloric acid and extraction with ether gave 2.4 μmoles of sinapic acid (spectroscopic assay). Identity was also confirmed by isolation and mixed m.p. with an authentic sample. The aqueous portion was assayed for glucose by the phenol–sulfuric acid method (Dubois *et al.*, 1956; Marier and Boulet, 1959) and also by means of glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J.). Respective values were 2.6 and 2.5 μmoles. Thus glucose and sinapic acid were obtained in a 1:1 molar ratio by hydrolysis. The absorption spectra of the colored products in the unsulfonated resorcinol reaction (Devor *et al.*, 1958), as well as paper chromatography, confirmed glucose as the sugar component. Hydrolysis was also achieved with β-glucosidase (EC 3.2.1.21; Pierce Chemical Co., Rockford, Ill.) on incubation at 35° for ½ hour at pH 5.2. Products were the same as those obtained with alkali.

The results fit the criteria of Harborne and Corner (1961) for the attachment of D-glucose from its 1-position by a β-linkage to the carboxyl group of the parent sinapic acid. These pertinent observations are (a) easy hydrolysis by β-glucosidase and by dilute alkali at room temperature; (b) color reactions and spectral characteristics (including the strong bathochromic shift at pH 10) strongly resembling those of an authentic ester of sinapic acid, in this instance sinapine; and (c) chromatographic properties. The substance is therefore the 1-sinapoyl-β-D-glucose, previously found in leaves of *Brassica oleracea* (Harborne and Corner, 1961). More recently, the glucose ester of sinapic acid was claimed by Maksyutina (1965) as a component of complex glycosides from seeds of several species of crucifers.

Sinapine Bisulfate. Sinapine bisulfate required for animal feeding was prepared from rapeseed because the sinapine content is higher than in crambe and it is more easily isolated. Defatted and ground seed of *Brassica napus* in 200-gram portions was extracted with hot ethanol (2000 ml.). After the filtered extracts were concentrated in vacuo, the residue was suspended in water (150 ml.) and then transferred to a centrifuge bottle. The mixture was extracted with chloroform (100 ml.), the layers were separated by centrifuging, and the clear aqueous solution was withdrawn. Additional water was added in 50-ml. portions and the process repeated until

the aqueous layer became colorless. To the combined aqueous portion was added 20% potassium thiocyanate (30 ml.) and the mixture refrigerated. Recoveries of crystalline sinapine thiocyanate ranged from 70 to 80% of that present in the meal. The thiocyanate salt was converted to sinapine bisulfate, m.p. 187–89° C. by the procedure of Clandinin (1961). A total of 10 grams of the bisulfate was prepared.

Rat Feeding of Sinapine Bisulfate. A Purina diet was supplemented with 0.6% sinapine bisulfate and fed to five Sprague-Dawley male rats for 7 days. Mean growth rate, mean feed intake, and feed efficiency were the same for animals fed the supplemented diet as for control animals.

DISCUSSION

Endogenous enzyme(s) of *Crambe abyssinica* degrade the native thioglucosides when water is added to the crushed seeds. The degradation by thioglucosidase (EC 3.2.3.1) is formally regarded as a hydrolytic cleavage, but esters such as sinapine are unaffected by the action of this enzyme system. Therefore, the sinapine esterase described by Tzagoloff (1963b) is not present in the seed meal, and seed germination must be a requisite condition for the production or activation of esterase. Evidently, sinapine is protected during storage of the seed until presumably its biochemical function as a source of sinapic acid and choline is called for by the germinating plant.

The observations of Clandinin (1961) with chicks, together with the rat-feeding results reported here, raise serious doubts whether sinapine plays any significant role in the consumption or feeding values of crambe-containing feedstuffs. This view gains additional support because the sinapine content of crambe meal is only half that of rapeseed and about one third that reported for white mustard seeds (Tzagoloff, 1963a). Furthermore, the thioglucosides of crambe, which adversely affect the feeding quality of the meal (Tookey *et al.*, 1965), exceed the sinapine concentration by 15- to 20-fold. Likewise, analogy with the rat-feeding trial with sinapine bisulfate suggests that the low level of sinapoyl glucose observed is also of little consequence in the feeding of crambe meals.

Although a number of additional minor constituents of crambe have been detected chromatographically, no detailed study of them has been undertaken. The possibility has not been excluded that they may have a relationship to the complexes of sinapic acid, D-glucose, and a steroid aglycone reported by Maksyutina (1965) to be present in several crucifer seeds. The question, then, whether these trace components play a role in palatability or consumption of crambe meals remains open, although at present these possibilities may be regarded as unlikely.

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