Evaluation of Alkaline Conversion of Sinapic Acid to Thomasidioic Acid

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Conditions which promote the alkaline conversion of sinapic acid (SA), the main phenolic acid in canola, to the lignan thomasidioic acid (TA) were investigated as the presence of TA could affect nutritional and functional properties of canola products. Reaction rates were studied using ultraviolet spectroscopy under oxygen, nitrogen, and air and in the presence of antioxidants. High-pressure liquid chromatography was used to quantify the conversion of SA to TA. This reaction appears to involve an oxidative coupling of SA molecules which can be controlled by purging with nitrogen. By including ascorbic acid, the reaction was slowed but not completely controlled, and the presence of sodium bisulfite accelerated the reaction. In the presence of air, there was complete conversion of SA to TA at pH 8.5 and 30% conversion at pH 7.

Keywords: *Phenolic acids; sinapic acid; thomasidioic acid; reaction rate; alkali*

INTRODUCTION

There is an interest in the development of canola meal as a source of protein for animal feed and also for human consumption. However, the presence of certain antinutritional compounds including fiber and glucosinolates in the meal limits its use. Phenolic compounds are also antinutritional compounds, with sinapic acid (SA) being the main phenolic acid in canola. The choline ester of SA, sinapine, is the principal phenolic ester in canola (Krygier et al., 1982a).

The dark color of canola products is usually attributed to the presence of phenolic compounds, and phenolics present in the hull free flours are also responsible for the brown coloration that develops when aqueous slurries of canola flours or protein isolates are adjusted to alkaline pH (Sosulski and Bakal, 1969).

Unprocessed canola meal has a bitter taste resulting in poor palatability. This bitter taste is generally associated with the phenolic coumpounds, mainly SA and sinapine. Maga and Lorenz (1973) have shown that the taste threshold of free SA is 1 ppm as compared to other phenolic acids such as ferulic acid (90 ppm) and *p*-coumaric acid (40 ppm). This low threshold value, and the large amount of SA present in canola meal, flours, and isolates, presents a serious taste problem in food products when canola is included. In addition to the color and palatability problems, phenolic compounds and their derived products may also form complexes with essential amino acids, enzymes, and other proteins, thereby lowering the nutritional value of the canola products (Sosulski, 1979; Kozlowska et al., 1975).

Presently protein isolation procedures often require a phenolic removal step in which the canola meal is exposed to solvent systems containing ammonia or other bases (Naczk et al., 1986; Shahidi et al., 1989; Naczk and Shahidi, 1989). A primary goal of an initial study was to examine the fate of SA during processing, mainly during the process of extraction, purification, and isolation of protein. In previous work, it was discovered that SA was readily converted to a lignan, thomasidioic acid (TA), when exposed to alkaline aqueous buffer (Rubino et al., 1995). On the basis of NMR data, it appeared that virtually total conversion was achieved when SA was left for 24 h in an aerated aqueous buffer at pH 8.5. There were also indications from the previous work that TA was formed in solutions of lower pH.

The presence of lignans in food has been of interest to food scientists as it has been suggested that lignans can be converted by intestinal microflora into hormone-like compounds which might protect against hormone dependent cancer (Ward, 1993). Extensive research in this area has been carried out on the natural lignans in products such as flaxseed (Thompson et al., 1994). In the case of TA, no one has yet assessed whether it is formed and retained during processing of canola meal, and neither its toxicity nor beneficial properties have been investigated.

A detailed study of the conversion of SA to TA has been undertaken in order to more fully understand the factors which affect the rate of this reaction. The goal of such a study was to develop a method for removal of SA from canola meal without affecting protein integrity. To this end the relative rates of conversion of SA to TA were measured in the presence of oxygen, air, nitrogen, and two different antioxidants. The yield of the reaction at two pH levels was also assessed.

MATERIALS AND METHODS

Materials. Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co (Milwaukee, WI). All solvents and chemicals used for high-pressure liquid chromatography (HPLC) were HPLC grade.

Instrumental Methods. The ^{1}H and ^{13}C NMR spectra were recorded on a Bucker AM-300 spectrometer using tetramethylsilane as internal standard [residual protonated dimethyl sulfoxide (DMSO), δ 2.5, was used as internal standard in DMSO-d_6]. Ultraviolet (UV) spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer with MS-DOS UV−vis software.

HPLC was used as a mean of rapid identification and quantification of TA and SA. The chromatograph was equipped with a UV detector at 330 nm. A reverse phase column (Supelcosyl, 3 μm particle size, 33 nm length, and 4.6 nm i.d.)

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was used. The buffer (buffer A) used for elution was 1:100 (v/v) water dilution of stock pH 4.7 acetate buffer, prepared by adjusting 5 M acetic acid to pH 4.7 with sodium hydroxide (NaOH) and filtering through a 0.45 μm Millipore filter (Krygier et al., 1982b). The initial elution was 15% methanol and 85% buffer A. After 8 min of isocratric flow at 1.7 mL/min, a 3 min linear gradient was used to change the solvent to 100% methanol, thereby cleaning the column. The column was maintained at a constant temperature of 37 °C and run at a constant flow rate of 1.7 mL/min. For standards, 2 mg of TA, synthesized as outlined elsewhere (Rubino et al., 1995) and confirmed by ^{1}H and ^{13}C NMR (Rubino et al., 1995; Ahmed et al., 1973a,b), and 2 mg of SA were dissolved separately in 10 mL of 15% methanol and 85% buffer A. Volumes ranging from 1 to 5 μL of this mixture were injected by using a 20 μL sample loop to determine standard curves.

UV Absorption Spectra. A solution of 5 mg of SA in 40 mL of aqueous buffer of NH_4HCO_3/NH_4OH (pH 8.5) was prepared. The solution was placed in a cuvette and the sample scanned at 15 min intervals for 6 h. The sample was scanned again after 24 h. A spectrum of the TA standard was also recorded for comparison purposes.

UV Kinetic Study. A solution containing 10 mg/100 mL SA in aqueous buffer (NH_4HCO_3/NH_4OH at pH 8.5) was used. The absorbance of the solution was measured at 310 nm, and after the first absorbance measurement the solution was divided in three equal parts. The solutions had either N_2 (nitrogen), O_2 (oxygen), or air bubbled directly into them. For each solution, absorbance was read at 310 nm every 10 min for 110 min.

Treatment with Antioxidants. Several experiments were undertaken to determine the effect of the presence of antioxidants on the oxidation of SA to TA. As the exact mechanism (including intermeditate compounds) responsible for the conversion of SA to TA in an aqueous alkaline environment has not yet been established, the choice of antioxidants was somewhat arbitrary. For each experiment, a single solution of SA at a concentration of 3 mg/100 mL in NH_4OH/NH_4HCO_3 buffer at pH 8.5 was prepared and subdivided for further experimentation. Four experiments were conducted using the following combinations of antioxidant in NH_4OH/NH_4HCO_3 buffer and gas: (1) ascorbic acid (550 ppm) and air, (2) ascorbic acid (2750 ppm) and air, (3) ascorbic acid (550 ppm) and oxygen, and (4) sodium bisulfite (550 ppm) and air. In each experiment a reference solution containing no antioxidant was used for comparison. Absorbances were recorded at 310 nm every 10 min for 110 min. At the completion of the experiment samples from experiments 1−3 were lyophilized and their ^{1}H NMR spectra recorded. The sample containing sodium bisulfite was acidified to pH 2 with 2 M HCl and extracted three times with ethyl acetate; the extract was dried with sodium sulfate (Na_2SO_4) and then evaporated under vacuum prior to analysis by ^{1}H NMR.

Kinetic Analysis. Both SA and TA absorb at 310 nm with TA having a smaller extinction coefficient. During the experiment the absorption begins at an initial value which is proportional to the concentration of SA and decays to a limiting value proportional to that of TA formed. Assuming that the conversion is quantitative (two SA molecules dimerize to form one TA), then the absorption of the solution as a function of time can be described by the equation

$$(\mathrm{Ab})_t = (\mathrm{SA})_0(\epsilon_S - \epsilon_T/2)e^{-kt} + (\mathrm{SA})_0\epsilon_T/2 \qquad (1)$$

where $(\mathrm{Ab})_t$ is the absorbance at time t, $(\mathrm{SA})_0$ is the concentration of SA at time zero, ϵ_S and ϵ_T are the molar extinction coefficients for SA and TA, respectively, and k is the rate constant for the reaction. Substituting $(\mathrm{SA})_0 = (\mathrm{AB})_0/\epsilon_S$ gives

$$(\mathrm{Ab})_t = (\mathrm{Ab})_0(1 - \epsilon_T/2\epsilon_S)e^{-kt} + (\mathrm{Ab})_0(\epsilon_T/2\epsilon_S) \qquad (2)$$

The values of ϵ_S and ϵ_T were determined at 310 nm, and using these, the values of k were iteratively determined for each experiment using the curve-fitting routines of Sigma Plot 4.2.

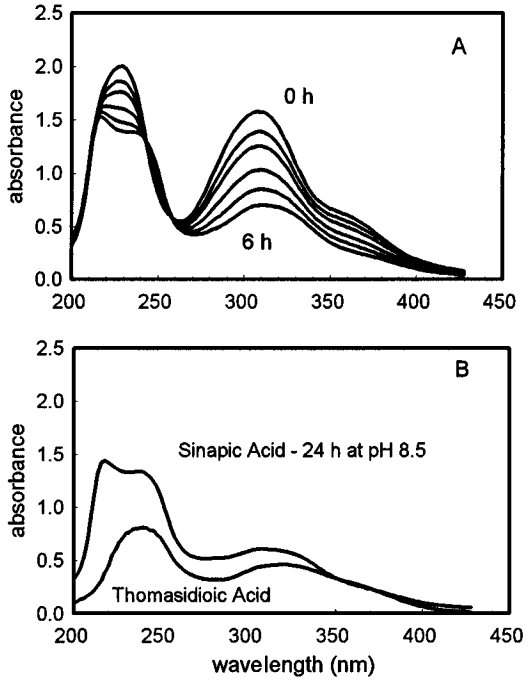

Figure 1. Absorbance spectra indicating continual changes in sinapic acid spectrum with time (0, 0.5, 1.0, 2.0, 3.0, and 6.0 h) during exposure to oxygenated base (A) and comparison of spectra of sinapic acid in base for 24 h to that for thomasidioic acid (B).

Sample Preparation for HPLC Analysis. Solutions of SA with a concentration of ca. 7 mg in 40 mL of water were prepared. An aliquot was adjusted to pH 7 and another aliquot to pH 8.5 using 0.2 M NaOH. After the solutions reached their respective pH values, they were left stirring at room temperature under air for 24 h. The solutions were then analyzed by HPLC for TA and SA.

RESULTS AND DISCUSSION

Changes in UV Spectra during Conversion of SA to TA. A solution of SA in aqueous buffer at pH 8.5 was monitored by periodically recording a UV/vis spectrum (Figure 1A). There are two clear isosbestic points at 248 and 258 nm indicating that the conversion of SA to TA probably resulted in the formation of a single end product. This is consistent with earlier NMR observations where only TA was seen in the ^{1}H NMR spectrum (Rubino et al., 1995) and analysis by HPLC reported in this paper. The absorption spectrum of the SA solution was recorded after 24 h and is shown in Figure 1B along with a spectrum of synthetic TA. Except for a peak at 215 nm which may be due to a very strongly absorbing minor product, the two spectra appear identical. During the initial reaction of the SA solution, the greatest change in absorption occurred at 310 nm, and therefore this wavelength was subsequently used for kinetic studies.

Kinetics of Conversion of SA to TA. The presence of oxygen (O_2) had an important effect on the transformation of SA to TA under aqueous alkaline conditions as shown in Figure 2A. As the level of oxygen in the system increased with the use of N_2, air, and pure O_2, reaction rate constants (k) also increased. There was little change in the absorption of SA solutions in the absence of oxygen (nitrogen purging) indicating that the transformation must be dependent on oxygen. The ^{1}H NMR spectrum of the lyophilized nitrogen-purged solution corresponded to that for SA (Table 1) confirming that the SA was unchanged. The ^{1}H NMR spectra of

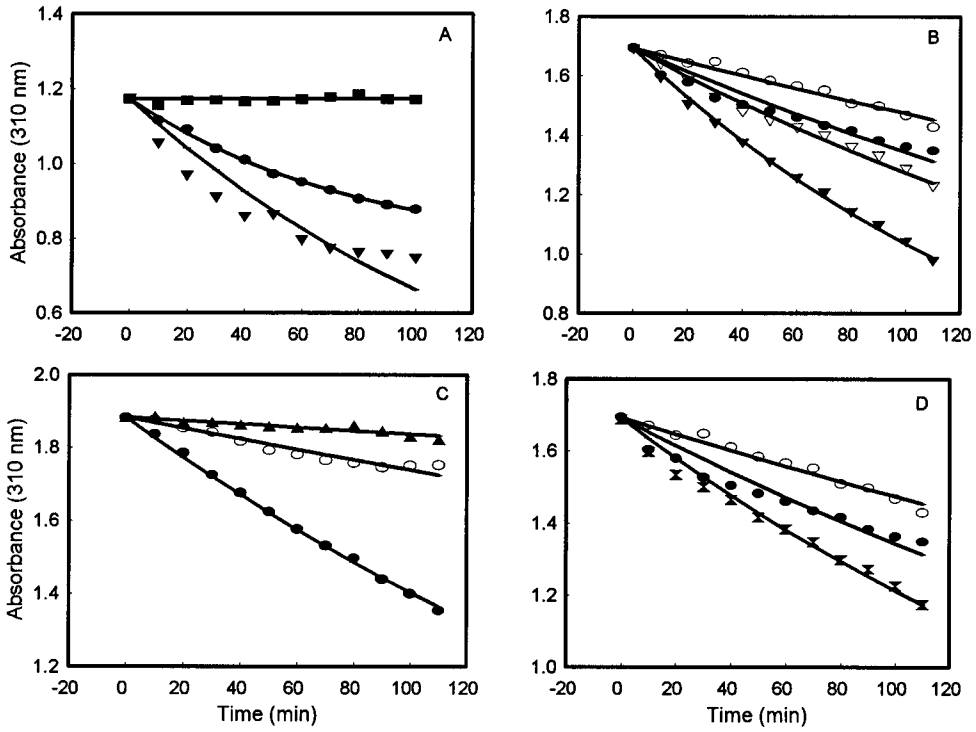

Figure 2. Effect of atmospheres and antioxidants on the conversion of sinapic acid to thomasidioic acid. (A) Change in absorbance (310 nm) of sinapic acid with time in different atmospheres. Calculated *k* values for these curves are nitrogen (■), 7.8×10^{-6}; air (●), 0.0039; and oxygen (▼), 0.0069. (B) Effect of 550 ppm ascorbic acid on the absorbance (310 nm) of sinapic acid with time in air and oxygen. Calculated *k* values for these curves are air/ascorbic acid (○), 0.0016; air/no antioxidant (●), 0.0028; oxygen/ascorbic acid (▽), 0.0034; and oxygen/no antioxidant (▼), 0.0061. (C) Effect of ascorbic acid concentration on the absorbance (310 nm) of sinapic acid with time in air. Calculated *k* values for these curves are 2750 ppm ascorbic acid (▲), 0.000 29; 550 ppm ascorbic acid (○), 0.000 94; and air/no ascorbic acid (●), 0.0035. (D) Effect of antioxidant type (550 ppm) on the absorbance (310 nm) of sinapic acid with time in air. Calculated *k* values for these curves are ascorbic acid (○), 0.0016; sodium bisulfite (filled hourglass), 0.0040; air/no antioxidant (●), 0.0028.

Table 1. Spectral Properties of Sinapic Acid, Thomasidioic Acid Produced from Sinapic Acid, and Authentic Thomasidioic Acid

description	^{1}H NMR (DMSO) spectra (δ)
sinapic acid	8.90 (s, 1H), 7.54 (d, 1H, J =15.8 Hz), 6.91 (s, 2H), 6.44 (d, 1H, J = 15.8 Hz), 3.80 (s, 6H, OCH_3)
lyophilized product of alkaline treatment of sinapic acid in air or O_2—identified as thomasidioic acid	9.15 (s, 1H), 8.20 (s, 1H), 7.54 (s, 1H), 6.98 (s, 1H), 6.21 (s, 2H), 4.81 (s, 1H), 3.82 (s, 3H, OCH_3), 3.74 (s, 1H), 3.60 (s, 6H, OCH_3) 3.49 (s, 3H, OCH_3)
thomasidioic acid[a]	12.45 (br s, 2H), 9.13 (s, 1H), 8.17 (s, 1H), 7.54 (s, 1H), 6.98 (s, 1H), 6.21 (s, 2H), 4.82 (s, 1H), 3.83 (s, 3H, OCH_3), 3.75 (s, 1H), 3.61 (s, 6H, OCH_3), 3.49 (s, 3H, OCH_3)

[a] 7-Hydroxy-6,8-dimethoxy-1-(4′-hydroxy-3′,5′-dimethoxyphenyl)-*trans*-1,2−dihydronaphthalene-2,3-dicarboxylic acid.

the lyophilized air- and oxygen-purged solutions corresponded to those for the standard TA (Table 1). This identification confirms an earlier observation when SA was treated with air only (Rubino et al., 1995). The conversion of SA to TA, however, can be controlled by purging with nitrogen.

Role of Antioxidants in Controlling the Conversion of SA to TA. When exposed to air or O_2, the presence of ascorbic acid (550 ppm) in solutions of SA slowed, but did not halt, the conversion of SA to TA (Figure 2B). This is reflected in the rate constants calculated from the data. As in the absence of antioxidants, samples exposed to air had lower *k* values than those samples exposed to oxygen. The ^{1}H NMR spectra of the lyophilized samples showed that the sample containing antioxidant and purged with air contained mainly but not completely unchanged SA, while the sample containing antioxidant and purged with oxygen was largely converted to TA (Table 1).

Effect of Ascorbic Acid Concentration on Conversion of SA to TA. The level of ascorbic acid has an important influence on the rate of the oxidation of SA to TA in the presence of air. Initially ascorbic acid at 550 ppm was used since this is the amount allowable in some foods, and this seemed to be a reasonable starting point as no specific food application was identified. It was found that this amount decreased the rate of transformation of SA to TA significantly. When this amount was increased 5-fold to 2750 ppm, the rate of the conversion dropped further (Figure 2C). The TA formed in this experiment was too small to be detected by ^{1}H NMR analysis of the lyophilized sample, and the spectrum corresponded to that for SA, while experiments using 550 ppm ascorbic acid yielded easily detectable amounts of TA (Table 1).

Effect of Sodium Bisulfite on Conversion of SA to TA. Sodium bisulfite was also considered as a possible inhibitor of the reaction. Surprisingly, it was

discovered that the sodium bisulfite was totally ineffective in inhibiting the reaction and actually increased the rate of reaction (Figure 2D). The ^{1}H NMR spectrum of the extracted solution showed the presence of TA only (Table 1). The exact mechanism of this rate acceleration is unknown.

Effect of pH on Conversion of SA to TA. The degree of conversion of SA to TA at pH 8.5 was 100% after 24 h, whereas at pH 7 there was only partial converison to TA (30%) after 24 h. It is clear that pH is an important factor in controlling the reaction.

There are several aspects of this reaction that need further attention. This investigation was conducted using aqueous solutions of SA as a model for SA in canola meal. Further studies will have to be conducted to determine if the results obtained here will also be observed when canola meal itself is processed under basic conditions. It would also be desirable to determine the exact mechanism of the reaction. Only with a full understanding of the mechanism will it be possible to confidently predict and rationalize the effects of pH, temperature, and inhibitors.

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