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Hydrolysis Kinetics of Secoisolariciresinol Diglucoside Oligomers from Flaxseed

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Flaxseed is the richest dietary source of the lignan secoisolariciresinol diglucoside (SDG) and contains the largest amount of SDG oligomers, which are often hydrolyzed to break the ester linkages for the release of SDG and the glycosidic bonds for the release of secoisolariciresinol (SECO). The alkaline hydrolysis reaction kinetics of SDG oligomers from flaxseed and the acid hydrolysis process of SDG and other glucosides were investigated. For the kinetic modeling, a pseudo-first-order reaction was assumed. The results showed that the alkaline hydrolysis of SDG oligomers followed first-order reaction kinetics under mild alkaline hydrolytic conditions and that the concentration of sodium hydroxide had a strong influence on the activation energy of the alkaline hydrolysis of SDG oligomers. The results also indicated that the main acid hydrolysates of SDG included secoisolariciresinol monoglucoside (SMG), SECO, and anhydrosecoisolariciresinol (anhydro-SECO) and that the extent and the main hydrolysates of the acid hydrolysis reaction depended on the acid concentration, hydrolysis temperature, and time. In addition, the production and change of *p*-coumaric acid glucoside, ferulic acid glucoside and their methyl esters and *p*-coumaric acid, ferulic acid, and their methyl esters during the process of hydrolysis was also investigated.

KEYWORDS: Secoisolariciresinol diglucoside (SDG); SDG oligomers; hydrolysis; kinetics; flaxseed

INTRODUCTION

Flaxseed (*Linum usitatissimum* Linn., Linaceae) contains a large amount of the lignan secoisolariciresinol diglucoside (SDG) (*1*−*7*), which belongs to the group of phytoestrogens (*8*). The content of SDG varies between 6 and 29 mg/g in the defatted flaxseed powder (*1, 4, 5, 9, 10*), corresponding to a secoisolariciresinol (SECO) content of 3.2−15.3 mg/g. A number of studies have shown that SDG and its metabolites might contribute to potential health benefits (*11*), such as inhibiting the development of breast, prostate, and colon cancers (*12*−*14*) and acting as an antioxidant better than vitamin E (*15*). Furthermore, other phenolic compounds accumulated at high concentrations in flaxseed such as *p*-coumaric acid and ferulic acid glucosides were found to possess antioxidant properties and be of special interest in dermatology (*5*).

In flaxseed, SECO is present as a diglycoside, SDG, which is further ester-linked with 3-hydroxy-3-methylglutaric acid (HMGA) and other phenolic compounds such as *p*-coumaric acid and ferulic acid glycosides to form SDG oligomers of unknown structure (*2*−*4, 6, 7, 10, 16*). A straight-chain oligomeric structure (**Figure 1**) composed of five SDG residues interconnected by four HMGA residues was assigned to the main lignan of flaxseed (*2, 4*), in which *p*-coumaric acid and ferulic acid glucosides as a terminal unit were linked directly via ester linkage of their carboxyl groups to the glucosyl moiety of SDG (*16*). Therefore, SECO and SDG could not be directly determined in the flaxseed extract. To determine the content of SDG or SECO, SDG oligomers had to be hydrolyzed to obtain the glucoside or aglycone (*7, 17, 18*). It had been established that SDG and *p*-coumaric acid and ferulic acid glucosides could be completely liberated from SDG oligomers following alkaline hydrolysis (*3, 7, 10, 19*). Although it was suggested that an alkaline hydrolysis step was not necessary because the acid hydrolysis was able to efficiently break both ester linkages and glycosidic bonds (*9*), acid treatment was destructive to some lignans or caused transformation reactions from one lignan to another lignan (*20*), indicating that the cleavage of the conjugates was the crucial point of the analysis method (*17*). The instability of SECO under acidic conditions resulted in the conversion of SECO to anhydro-SECO as an artifact by elimination of a water molecule from the diol structure of SECO (*7, 9, 17, 18, 21*−*23*). Anhydro-SECO is chemically identical to shonanin (3,4-divanillyltetrahydrofuran) (*24*), a naturally occurring monoepoxylignan (*25*−*27*), which has a very high binding affinity to human sex hormone-binding globulin (*25, 26*) and potential inhibitory effects on the

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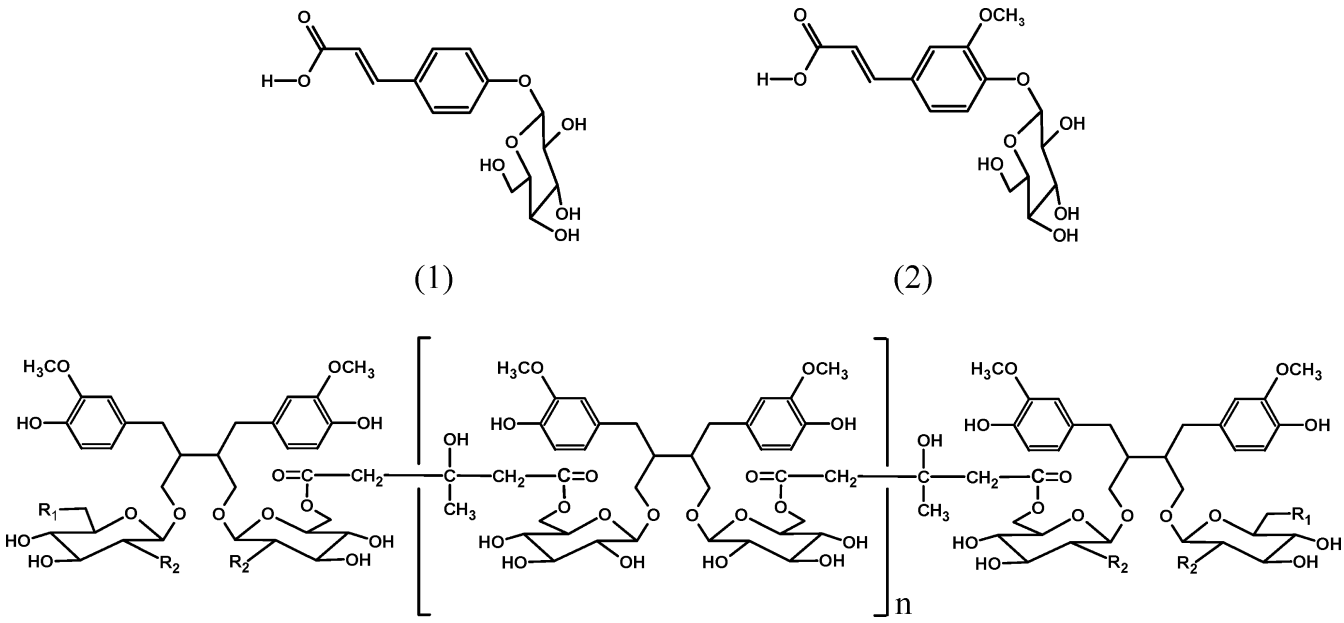

Figure 1. Proposed structure of a SDG oligomer from flaxseed (average value of $n = 3$): R_1 = OH or *p*-coumaric acid glycoside (**1**); R_2 = OH or ferulic acid glycoside (**2**).

development of prostate cancer (*24*). Therefore, it is necessary to investigate the production of anhydro-SECO during the process of acid hydrolysis of SDG.

Although it had been well-known that the breaking of ester and glycosidic bonds had been used to analyze lignans in flaxseed products for years, it could be interesting to study in depth the alkaline and acid conditions needed to destroy these bonds, with the minimum concentration of degradation products. In our previous study, a gradient reversed-phase HPLC method has been developed for the simultaneous separation and determination of SDG oligomers and their alkaline and acid hydrolysates (*7*). This HPLC method is suitable to monitor the alkaline and acid hydrolysis processes of SDG oligomers. The major objective of the present work is to study the alkaline hydrolytic reaction kinetics of SDG oligomers to SDG, the production process of other glucosides, and the acid hydrolysis processes of SDG and other glucosides.

MATERIALS AND METHODS

Chemicals and Reagents. HPLC-grade methanol was purchased from Merck KGaA (Darmstadt, Germany). SECO (95% purity), *p*-coumaric acid (98% purity), ferulic acid (98% purity), and 5-hydroxymethyl-2-furfural (HMF, 99% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO). SDG (96% purity), secoisolariciresinol monoglucoside (SMG, 97% purity), and anhydro-SECO (95% purity) were separated and purified as described previously (*7*). Acetic acid, sodium hydroxide, and hydrochloric acid were of analytical grade. Water was purified using a Millipore Simplicity system (Billerica, MA).

Flaxseed Sample and the Extraction of SDG Oligomers. The commercial flaxseed lignan (40% lignan) for the purification of SDG was obtained from Hunan Deray Biological Industry Group Co., Ltd. (Huaihua, Hunan, China). The flaxseed sample, grown in Wulanchabu, Inner Mongolia, was purchased from Inner Mongolia Flaxseed Oil Health Promotion Center (Hohehot, Inner Mongolia, China), ground into powder with the help of a mortar and a pestle, and then defatted with petroleum ether for 8 h in a Soxhlet apparatus. A 50 g amount of the milled and defatted flaxseed powder was accurately weighed and extracted with 300 mL of 70% aqueous methanol solution with continuous stirring for 4 h followed by sonication for 10 min and centrifugation at 10000*g* for 5 min (*7*). The extract was sampled for HPLC analysis to detect SDG oligomers from flaxseed.

Alkaline Hydrolysis and Hydrolysis Kinetics of SDG Oligomers. The flaxseed extract (70% aqueous methanol solution) was subjected to an alkaline hydrolysis with the final NaOH concentrations of 3, 3.5, 4, 4.5, 5, 10, and 20 mM at 30, 40, 50, and 60 °C for hydrolyzing SDG oligomers. The reaction mixture was directly sampled to HPLC for monitoring the progress of hydrolysis of SDG oligomers and the release of SDG and other glycosides during the process of alkaline hydrolysis. Aliquots of 20 μL sampled from the reaction mixture by manual injector on time were immediately injected into the HPLC. Experiments were repeated three times, and the data were expressed as mean values of three replicates.

The hydrolysis kinetics of SDG oligomers was investigated on the basis of the following reaction:

$$\text{SDG oligomers} + \text{NaOH} \rightarrow \text{SDG} + \text{other glycosides}$$

Because adequate NaOH was added, the concentration of NaOH remained almost constant throughout. The hydrolysis reaction was assumed to follow pseudo-first-order kinetics. Suppose that at the beginning of the hydrolysis reaction, the concentration of SDG oligomers was C_0 and that at time t the amount of SDG oligomers which had been hydrolyzed per unit volume was x, then the rate of the hydrolysis reaction of SDG oligomers might be expressed by the following equation:

$$dx/dt = k(C_0 - x) \quad (1)$$

Separation of the variables x and t in eq 1 and integration give

$$\ln[C_0/(C_0 - x)] = kt \quad (2)$$

Equation 2 can be rewritten as

$$\ln(1 - x/C_0) = -kt \quad (3)$$

In eq 3, x/C_0, the ratio of the concentration of SDG oligomers hydrolyzed to the initial concentration of SDG oligomers, may be evaluated from the ratio of the concentration of SDG (y) at time t to the concentration of SDG (C_{SDG}) at the end of the hydrolysis reaction, that is

$$x/C_0 = y/C_{SDG} \quad (4)$$

Substitution of this equation into eq 3 leads to

$$\ln(1 - y/C_{SDG}) = -kt \quad (5)$$

Graphical methods can be employed to test the first-order equation and obtain the rate constant k. If the hydrolysis reaction of SDG oligomers is first order, a plot of $\ln(1 - y/C_{SDG})$ versus time t will give a straight line. The slope of this plot is the rate constant, that is, slope $= -k$.

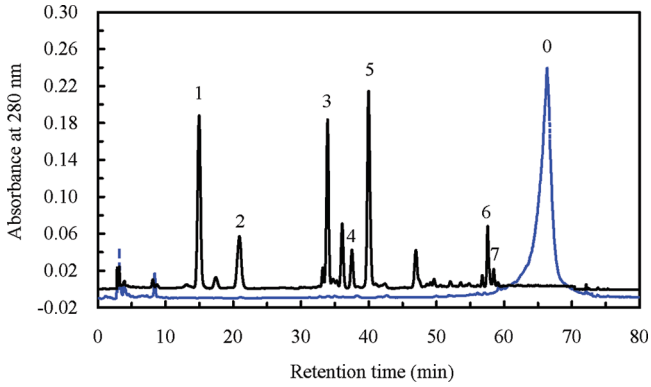

Figure 2. HPLC chromatograms of the intact flaxseed extract (dashed line) and the alkaline hydrolysates (solid line) of SDG oligomers in the flaxseed extract after 185 min of alkaline hydrolysis with the final NaOH concentration of 20 mM at 50 °C. Peaks: 0, SDG oligomers; 1, *p*-coumaric acid glucoside; 2, ferulic acid glucoside; 3, methyl ester of *p*-coumaric acid glucoside; 4, methyl ester of ferulic acid glucoside; 5, SDG; 6, methyl ester of ferulic acid; 7, methyl ester of *p*-coumaric acid.

Absolute temperature (T) dependence of the rate constant (k) of the hydrolysis reaction of SDG oligomers can be described by the Arrhenius equation:

$$\ln k = -E_a/RT + \ln k_0 \quad (6)$$

Activation energy (E_a) can be estimated on the basis of the linear regression analysis of ln k versus $1/T$. In eq 6, R is the universal gas constant (8.314 J K^{-1} mol^{-1}).

Acid Hydrolysis of SDG and Other Glycosides. After SDG oligomers were completely hydrolyzed with the final NaOH concentration of 20 mM at 50 °C for 15 min (*7*), SDG in the alkaline hydrolytic solution was subjected to an acid hydrolysis with the final HCl concentrations of 1, 2, and 3 M at 95 °C. The SDG standard solution was also hydrolyzed with the final HCl concentrations of 1, 2, and 3 M at 95 °C to investigate the effects of HCl concentrations on the acid hydrolysates of SDG. The reaction mixtures were directly sampled to HPLC for monitoring the progress of hydrolysis of SDG and other glycosides during the process of acid hydrolysis.

High-Performance Liquid Chromatography (HPLC). SDG oligomers and their alkaline and acid hydrolysates were analyzed using HPLC as developed previously (*7*). HPLC analysis was conducted on an HPLC system (Waters, Milford, MA) with a 1525 binary pump and a 2996 photodiode array detector. A Waters XTerra RP18 column (4.6 × 250 mm, 5 μm) and a guard column (4.6 × 12.5 mm, 5 μm) at 30 °C were used for the analysis. A binary solvent system was used consisting of 0.5% of acetic acid in water as solvent A and methanol as solvent B. A linear gradient program was used, at 1.0 mL/min, as follows: 15% of B for 20 min; 15−28% of B within 10 min; 28% of B for 10 min; 28−55% of B within 20 min; 55−85% of B within 10 min; 85% of B for 10 min; and back to 15% of B within 1 min (total run time of 81 min). The detecting wavelength was set between 210 and 350 nm, and the chromatographic peaks were measured at a wavelength of 280 nm.

RESULTS AND DISCUSSION

Alkaline Hydrolysis and Hydrolytic Kinetics of SDG Oligomers. The addition of NaOH was necessary to break the ester linkages of SDG oligomers (**Figure 1**) to release SDG. The HPLC method was used to measure the changes in the contents of SDG and other glycosides in the process of alkaline hydrolysis. **Figure 2** shows the HPLC chromatograms of the intact flaxseed extract and the alkaline hydrolysates of SDG oligomers in the flaxseed extract after 185 min of alkaline hydrolysis. As can be seen from **Figure 2**, SDG (peak 5) could be released from SDG oligomers (peak 0) following alkaline hydrolysis.

The changes in SDG concentrations during alkaline hydrolysis with different concentrations of NaOH at different temperatures are shown in **Figure 3**. As can be seen from **Figure 3**, the different concentrations of NaOH and temperatures could result in obvious differences in the hydrolysis reaction rate. A higher concentration of sodium hydroxide and a higher temperature can make the rate of the hydrolysis reaction increase and shorten the required hydrolysis time. As shown in **Figure 3**, during the initial stage at a higher concentration of sodium hydroxide (10−20 mM), alkaline hydrolysis resulted in a rapid hydrolytic cleavage of SDG oligomers with a concomitant increase in SDG concentration, which kept increasing up to 10−30 min and then leveled off (C_{SDG} = 679.1 ± 4.3 mg/L), indicating that SDG was stable to NaOH even for 10 h of alkaline hydrolysis with the final NaOH concentration of 20 mM at 60 °C (**Figure 3d**). While the concentration of NaOH was low, for example, 3−4 mM, a long reaction time was necessary for the complete hydrolysis of SDG oligomers, especially at a lower hydrolysis temperature.

The graphical results (**Table 1**) showed that there was a linear relationship between $\ln(1-y/C_{SDG})$ and time with the exception of temperature of 60 °C and NaOH concentrations of 10 and 20 mM, indicating that the hydrolysis of SDG oligomers was a first-order reaction under mild alkaline hydrolytic conditions. The temperature dependence of the rate constants of the hydrolysis reaction of SDG oligomers could adequately be described by the Arrhenius equation (eq 6). A linear relationship between ln k and reciprocal absolute temperature ($1/T$) and the corresponding activation energy for the hydrolysis reaction of SDG oligomers at NaOH concentrations of 3, 3.5, 4, 4.5, and 5 mM is shown in **Table 2**. The activation energy for the hydrolysis reaction of SDG oligomers was found to increase at low concentrations of NaOH. The results indicated that the hydrolysis reaction of SDG oligomers became particularly temperature-dependent at low concentrations of NaOH; that is, the temperature was a very critical parameter for the hydrolysis reaction of SDG oligomers at low concentrations of NaOH.

It had been suggested that *p*-coumaric acid and ferulic acid glucosides were linked directly via ester linkage of their carboxyl groups to the glucosyl moiety of SDG as a terminal unit in SDG oligomers (*16*) and were easily released by alkaline hydrolysis (*3, 7, 10, 19*). As can be seen from **Figure 2**, the methyl esters of *p*-coumaric acid glucoside (peak 3) and ferulic acid glucoside (peak 4) could be released from SDG oligomers following alkaline hydrolysis, and further alkaline hydrolysis resulted in the releases of *p*-coumaric acid glucoside (peak 1), ferulic acid glucoside (peak 2), and the methyl esters of *p*-coumaric acid (peak 7) and ferulic acid (peak 6) from their respective precursors. **Figure 4** shows the changes in the concentrations of the methyl esters of *p*-coumaric acid and ferulic acid glucosides during the process of alkaline hydrolysis with the final NaOH concentration of 20 mM at different temperatures. After the hydrolysis reaction began, alkaline hydrolysis resulted in hydrolytic cleavage of SDG oligomers with a rapid increase in the concentrations of the methyl esters of *p*-coumaric acid and ferulic acid glucosides. While the concentrations of NaOH were higher (e.g., 20 mM), the concentrations of the methyl esters of *p*-coumaric acid and ferulic acid glucosides decreased markedly, especially at a higher temperature, after reaching a maximum value (**Figure 4**).

Luyengi et al. (*28*) first isolated a cinnamic acid methyl ester from the defatted flaxseed. The methylation/transmethylation of their carboxylic groups (*1*) resulted in the formation of the methyl esters of *p*-coumaric acid and ferulic acid glucosides in a reaction medium containing a large amount of methanol. The

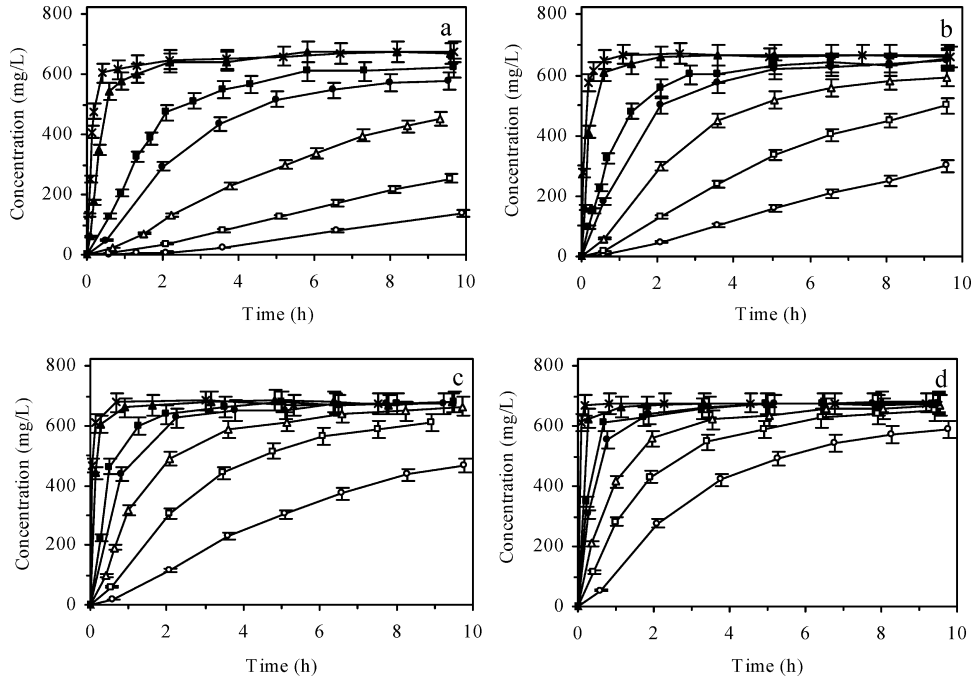

Figure 3. Changes in SDG concentrations during alkaline hydrolysis with final NaOH concentrations of 3 (○), 3.5 (□), 4 (△), 4.5 (●), 5 (■), 10 (▲), and 20 mM (×) at 30 (**a**), 40 (**b**), 50 (**c**), and 60 °C (**d**). The mean values of three experiments (RSDs < 5%) are presented.

Table 1. Rate Constant k (h^{-1}) for First-Order Reaction Kinetics of the Alkaline Hydrolysis of SDG Oligomers with Different Concentrations of NaOH at Different Temperatures

NaOH	30 °C		40 °C		50 °C		60 °C	
concn (mM)	k	r	k	r	k	r	k	r
3.0	0.0199	0.9815	0.0567	0.9902	0.1198	0.9973	0.2253	0.9917
3.5	0.0456	0.9877	0.1345	0.9967	0.2738	0.9937	0.3721	0.9790
4.0	0.1163	0.9963	0.2586	0.9906	0.4195	0.9817	0.7520	0.9797
4.5	0.2683	0.9936	0.5010	0.9869	0.8047	0.9586	1.2235	0.9352
5.0	0.4888	0.9894	0.8034	0.9940	1.2149	0.9483	1.8276	0.8156
10.0	2.2532	0.9799	2.4134	0.8906	4.4608	0.8911		
20.0	5.6650	0.9807	6.0915	0.9423	9.5363	0.9042		

Table 2. Corresponding Activation Energy for the Hydrolysis Reaction of SDG Oligomers at Different Concentrations of NaOH ($n = 3$)

NaOH concn (mM)	linear relationship of ln k and ($1/T$)	r	E_a (kJ/mol)
3.0	$\ln k = -8129.7/T + 22.985$	0.9960	67.6 ± 1.5
3.5	$\ln k = -7123.3/T + 20.573$	0.9771	59.2 ± 2.4
4.0	$\ln k = -6154.2/T + 18.203$	0.9964	51.2 ± 1.9
4.5	$\ln k = -5086.0/T + 15.500$	0.9978	42.3 ± 1.1
5.0	$\ln k = -4412.1/T + 13.851$	0.9997	36.7 ± 0.9

linear regression equation: $E_a = -15.751 C_{NaOH} + 114.39$, $r = 0.9977$

present study indicated that, when SDG oligomers were dissolved in methanol and hydrolyzed by adding a methanolic NaOH, alkaline hydrolysis resulted in the production of methyl esters of *p*-coumaric acid and ferulic acid glucosides, which are stable in methanol; when SDG oligomers were dissolved in water and hydrolyzed by adding an aqueous NaOH solution, alkaline hydrolysis resulted in the immediate production of *p*-coumaric acid and ferulic acid glucosides; when SDG oligomers were dissolved in aqueous methanol solutions (i.e., 70% aqueous methanol solution), the alkaline hydrolysis process might be divided into two stages: (a) the releases of SDG and the methyl esters of *p*-coumaric acid and ferulic acid glucosides and (b) the release of *p*-coumaric acid and ferulic acid glucosides from their respective precursors (*7*). In stage a, the production of the methyl esters of *p*-coumaric acid and ferulic acid glucosides was inevitable in a reaction medium containing a large amount of methanol, even in the 70% (**Figure 2**) and 30% (data not shown) aqueous methanol solutions, indicating that the presence of water would not prevent the formation of methyl esters. However, these methyl esters would not be stable in an alkaline aqueous alcohol mixture and, thus, in stage b, *p*-coumaric acid and ferulic acid glucosides would be gradually released (**Figure 4**). The rate of release of *p*-coumaric acid and ferulic acid glucosides from their respective methyl esters would depend on the percentage of water in the aqueous methanol solutions; that is, the release rate in 70% methanol was slower than that in 30% methanol (data not shown).

Recently, Struijs et al. (*16*) suggested that, within the SDG oligomers, ferulic acid is also linked to the glucosyl moiety of SDG via its carboxyl group. However, our results showed that, in stage a, only a very small amount of ferulic acid methyl ester was detected in the alkaline hydrolytic reaction mixture (*7*). In contrast, in stage b, as can be seen from **Figure 2**, a larger amount of ferulic acid methyl ester (peak 6) was found, indicating that ferulic acid methyl ester might be released from the methyl esters of ferulic acid glucosides instead of from SDG oligomers. The results suggested that the methyl esters of hydroxycinnamic acid glucosides, especially ferulic acid glucoside, could be partly deglycosylated by alkaline hydrolysis. The fact that only a very small amount of *p*-coumaric acid methyl ester was detected indicated that 3-substitution by the methoxy group in a cinnamic acid methyl ester was favorable to the deglycosylation, whereas the methyl ester of *p*-coumaric acid glucoside without methoxy substitution was more difficult to deglycosylate by alkaline hydrolysis.

Acid Hydrolysis of SDG and other Glucosides. After SDG oligomers in the flaxseed extract were completely hydrolyzed, SDG released from SDG oligomers in the alkaline hydrolytic solution was subjected to an acid hydrolysis to release SECO from SDG. The acid hydrolytic reaction mixtures were directly sampled to HPLC to monitor the acid hydrolysates of SDG and

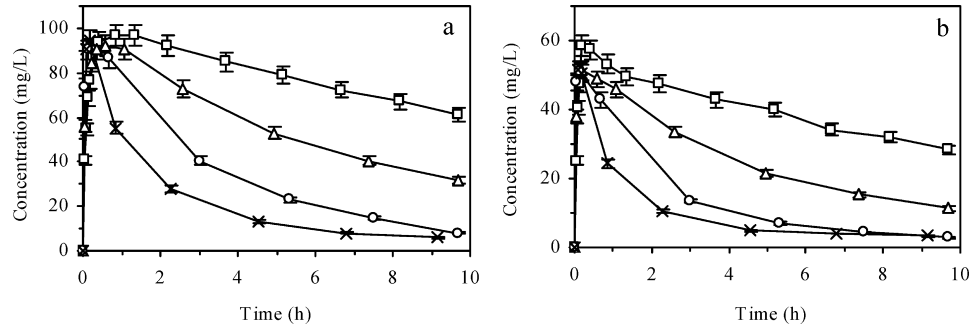

Figure 4. Changes in the concentrations of the methyl esters of *p*-coumaric acid glucoside (**a**) and ferulic acid glucoside (**b**) at 30 (□), 40 (△), 50 (○), and 60 °C (×) during alkaline hydrolysis with the final NaOH concentration of 20 mM. The mean values of three experiments (RSDs < 5%) are presented.

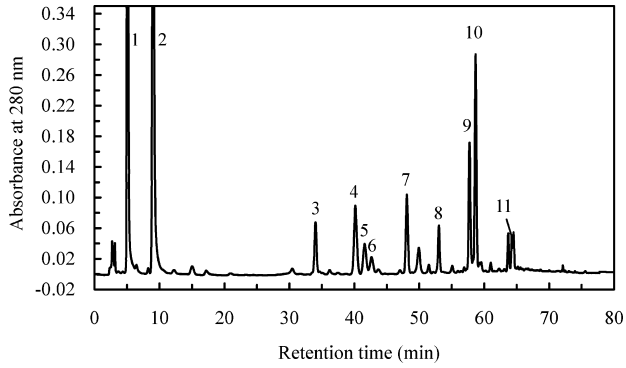

Figure 5. HPLC chromatogram of the acid hydrolysates after 60 min of acid hydrolysis with the final HCl concentration of 1 M at 95 °C. Peaks: 1, HMF; 2, degradation product of HMF; 3, methyl ester of *p*-coumaric acid glucoside; 4, SDG; 5, *p*-coumaric acid; 6, ferulic acid; 7, SMG; 8, SECO; 9, methyl ester of ferulic acid; 10, methyl ester of *p*-coumaric acid; 11, anhydro-SECO.

other glycosides during the process of acid hydrolysis. The typical HPLC chromatogram of acid hydrolysates in the acid hydrolytic reaction mixture is shown in **Figure 5**. As shown in **Figure 5**, the acid hydrolysates of SDG and other glycosides included SMG, SECO, anhydro-SECO, *p*-coumaric acid and its methyl ester, ferulic acid and its methyl ester, and HMF and its degradation product (*7*).

During acid hydrolysis, one of two glucose molecules of SDG was first removed from the SDG molecule to produce SMG, and then, SMG was deglycosylated to produce SECO. Under acid conditions, SECO was unstable and would be dehydrated to produce anhydro-SECO. As shown in **Figure 5**, the main acid hydrolysates of SDG (peak 4) included SMG (peak 7), SECO (peak 8), anhydro-SECO (peak 11), and some unknown compounds (e.g., peaks with retention times of ~72 min). The UV spectra of SDG and its acid hydrolysates were recorded between 210 and 350 nm, and the results indicate that SDG, SMG, SECO, and anhydro-SECO present maximum absorptions at ~280 nm, which is attributed to the aromatic chromophore and the substituents of −OH and $-OCH_3$ on an aromatic ring (*29*).

To investigate the effect of HCl concentration on the acid hydrolysates of SDG, the purified SDG was also hydrolyzed with the final HCl concentrations of 1, 2, and 3 M at 95 °C. **Figure 6** shows the changes in the concentrations of SDG, SMG, SECO, and anhydro-SECO in the reaction mixture during acid hydrolysis of the purified SDG. As shown in **Figure 6**, a higher concentration of HCl would increase the hydrolysis reaction rate of SDG (**Figure 6a**) and the production rate of anhydro-SECO (**Figure 6d**). The concentrations of SMG and SECO decreased markedly after reaching a maximum value (**Figure 6b**,**c**) because SMG and SECO could be further degraded to produce SECO and anhydro-SECO, respectively. While the initial concentration of SDG was 895 mg/L, the concentration of SMG reached a maximum value (~324 mg/L) after 1.7 h of acid hydrolysis (1 M HCl, 95 °C), the concentration of SECO reached a maximum value (~146 mg/L) after 3 h of acid hydrolysis (1 M HCl, 95 °C), and the concentration of anhydro-SECO reached a maximum value (~303 mg/L) after 3 h of acid hydrolysis (3 M HCl, 95 °C). The extent and the hydrolysates of the acid hydrolysis reaction depended on the acid concentration and hydrolysis time. Under mild acid conditions (e.g., 1 M of HCl for <1.6 h), SMG was the major acid hydrolysate of SDG (**Figure 6**). Under drastic acid conditions (e.g., 3 M of HCl for >1.6 h), anhydro-SECO was the major acid degradation product of SDG, and almost no SDG, SMG, and SECO were detected, which is similar to the data published by Charlet et al. (*9*), who found that the highest amount of anhydro-SECO was obtained without any trace of SECO after 2.5 h of 2 M HCl hydrolysis at 100 °C.

Although the content of SDG oligomers, the major fraction in the intact flaxseed extract, in the defatted flaxseed powder has been determined (*7*), due to the structural diversity of SDG oligomers and the lack of appropriate standards (*10, 23*), SDG oligomers with a broad peak could not be determined accurately. To determine accurately the content of SDG or SECO, SDG oligomers have to be hydrolyzed to get the glucoside or aglycone (*7, 17, 18*). The quantification of SDG requires an alkaline hydrolysis to break ester linkages for the transformation of the complex SDG oligomers into glucoside SDG, and the quantification of SECO requires an acid hydrolysis to break glycosidic bonds for the release of aglycone SECO from the glycosylated derivatives (*7, 9, 17, 18, 23*). However, SECO is unstable under acidic conditions and easily converted to anhydro-SECO. Consequently, Charlet et al. (*9*) developed an HPLC quantification method of SECO from flaxseed through the complete transformation of SECO into its anhydrous form obtained by a total acid hydrolysis. Although the previous studies indicated that anhydro-SECO was relatively stable in a hot acid solution (*9, 24*), with 80% or more remaining after 5 h in 2 M HCl (*24*), as shown in **Figure 5**, some unspecified peaks (e.g., peaks with retention times of ~72 min) were found to be the degradation products of SDG during the acid hydrolysis process. This study suggests that, because SDG is stable in a hot alkaline solution, the HPLC method for the quantification of SDG should be applied to the evaluation of flaxseed lignan content through the complete transformation of SDG oligomers into SDG obtained by an alkaline hydrolysis.

SMG, found first by Clavel et al. (*30*) during the deglycosylation of SDG by the newly isolated strain *Clostridium* sp. SDG-Mt85-3Db, was also found to be an intermediate product

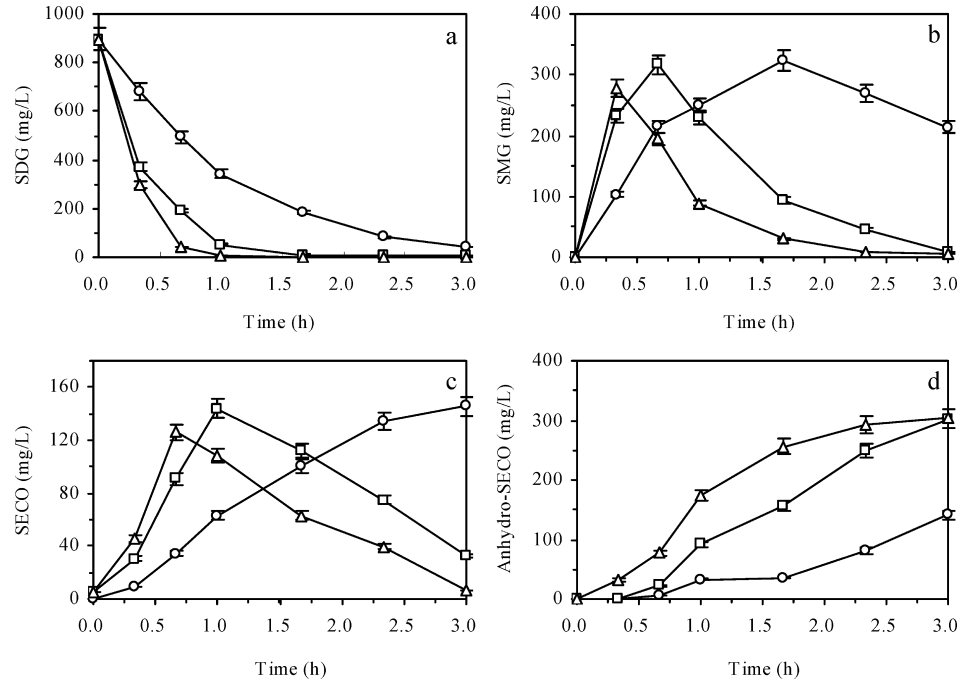

Figure 6. Changes in the concentrations of SDG (**a**), SMG (**b**), SECO (**c**), and anhydro-SECO (**d**) during acid hydrolysis with final HCl concentrations of 1 (○), 2 (□), and 3 M (△) at 95 °C. The mean values of three experiments (RSDs < 5%) are presented.

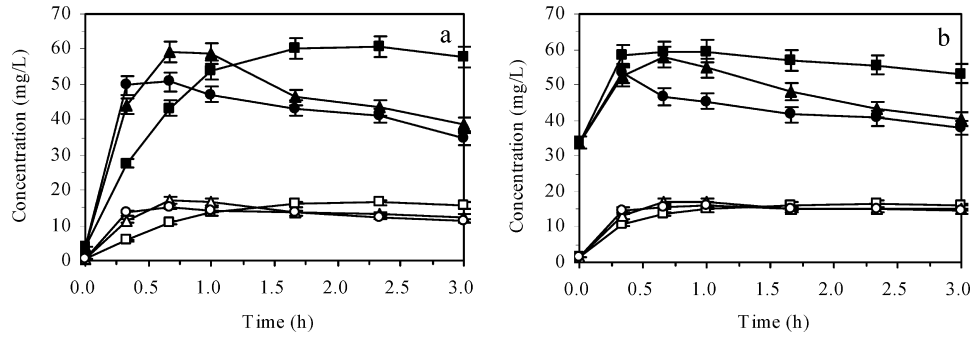

Figure 7. Changes in the concentrations of *p*-coumaric acid (**a**) and ferulic acid (**b**) (open symbols) and their methyl esters (solid symbols) during acid hydrolysis with final HCl concentrations of 1 (squares), 2 (triangles), and 3 M (circles) at 95 °C. The mean values of three experiments (RSDs < 5%) are presented.

during the deglycosylation of SDG to SECO by acid hydrolysis (*7*). In the previous studies (*9, 21, 22*), the acid hydrolysates were extracted with ethyl acetate/*n*-hexane (1:1, v/v), and the organic phases were collected and only the lipophilic compounds were extracted from the acid hydrolysates after acid hydrolysis, whereas SMG remained in the water phase. Therefore, there was no previous information concerning the detection of SMG under acid hydrolytic conditions (*7*).

As shown in **Figure 5**, after acid hydrolysis, comparable high amounts of *p*-coumaric acid (peak 10) and ferulic acid methyl esters (peak 9) could be produced, and only small amounts of free *p*-coumaric acid (peak 5) and ferulic acid (peak 6) were found. This indicates that a majority of *p*-coumaric acid and ferulic acid released from their glucosides have been esterified in a reaction medium containing methanol and hydrochloric acid (*7*) and the methyl esters of *p*-coumaric acid and ferulic acid were the main acid hydrolysates of the *p*-coumaric acid and ferulic acid glucosides in the reaction medium containing methanol. **Figure 7** shows the changes in the concentrations of *p*-coumaric acid, ferulic acid, and their methyl esters during acid hydrolysis with 1, 2, and 3 M of HCl at 95 °C. As shown in **Figure 7**, before acid hydrolysis, there had been a smaller amount of the methyl ester of *p*-coumaric acid (3.9 mg/L) and a larger amount of the methyl ester of ferulic acid (33.7 mg/L), which were produced by deglycosylation during alkaline hydrolysis, in the alkaline hydrolytic solution.

Different concentrations of HCl could result in obvious differences in the rate of the deglycosylation reaction of *p*-coumaric acid and ferulic acid glucosides. A higher concentration of HCl would increase the rate of the deglycosylation reaction, but the final concentrations of the methyl esters of *p*-coumaric acid and ferulic acid were lower than at a lower concentration of HCl. While the concentrations of HCl in reaction mixtures were higher (2 and 3 M), the concentrations of the methyl esters of *p*-coumaric acid and ferulic acid decreased significantly after reaching a maximum value, which was lower than the maximum concentrations of *p*-coumaric acid (60.6 mg/L) and ferulic acid methyl esters (59.5 mg/L) while the concentration of HCl in reaction mixtures was lower (1 M). In comparison with the methyl esters of *p*-coumaric acid and ferulic acid, free *p*-coumaric acid and ferulic acid were stable to HCl. The concentrations of *p*-coumaric acid and ferulic acid increased initially, then became constant for 1 M HCl or began to fall slightly along with the progress of hydrolysis for 2 and 3 M HCl (**Figure 7**). It had been found that two unspecified peaks eluted respectively before and after anhydro-SECO (peak 11 in **Figure 5**) were the derivatives of the methyl esters of *p*-coumaric acid and ferulic acid, respectively, and were not found in the methanol-free medium (*7*).

In conclusion, SDG oligomers are often hydrolyzed to break the ester linkages and the glycosidic bonds for the release of SDG and SECO. The hydrolysates of SDG oligomers are complicated because of the production of esters of hydroxycinnamic acids and their glucosides in an alcohol-containing medium. While SDG oligomers were dissolved in aqueous methanol solutions, the alkaline hydrolysis process might be divided into two stages: the release of SDG and the methyl esters of *p*-coumaric acid and ferulic acid glucosides and the release of *p*-coumaric acid and ferulic acid glucosides from their respective methyl esters. The studies showed that the release of SDG followed first-order reaction kinetics under mild alkaline hydrolytic conditions. The concentration of sodium hydroxide had a strong influence on the activation energy of the alkaline hydrolysis of SDG oligomers, and temperature was a very critical parameter for the hydrolysis reaction of SDG oligomers at low concentrations of NaOH. The studies also showed that SMG, SECO, and anhydro-SECO were the main acid hydrolysates of SDG, and their relative contents depended on the acid concentration, hydrolysis temperature, and time.

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