Removal of Glucosinolates from Rapeseed Meal Using Bowl-Shaped Tetrameric Molecules in Apolar Solvent

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A novel approach for the removal of toxic glucosinolates from rapeseed meal has been successfully tried. Synthetic bowl-shaped tetrameric macromolecule **2** from cetanal and resorcinol has been synthesized. These lipophilic molecules have been used in CCl_4 at various concentrations (0.03−0.05 mM) to check the effect on the total glucosinolates content (TGC) by using the Croft method. Glucosinolates were found to decrease linearly with increases in concentration of **2** in CCl_4. The overall reduction of TGC was up to 66%. The results were also complimented by electronic absorption of CCl_4 containing **2** after extraction.

Keywords: *Tetrameric molecules; host−guest complexation; glucosinolates; extraction; rapeseed meal*

INTRODUCTION

Rapeseed protein has a well-balanced amino acid pattern and high biological value. However, the nutritive value of rapeseed meal is reduced by the presence of several antinutritional factors. Glucosinolates and their degradation products (Figure 1) formed by reaction catalyzed by myrosinase (EC 3.2.3.1) have attracted particular attraction due to the difficulty of their removal. A lot of work has been done in various parts of the world to treat rapeseed meal (RSM) so that glucosinolate content and its effects may be reduced to allow RSM to be used freely as source of dietary protien. Chemical, heat, and microwave treatments have been tried, but mice feeding trials still indicate toxicity (Maheshvari et al., 1980; Schwenke et al., 1994). In heat treatment, along with the deactivation of myrosinase, there is a decrease in the essential amino acid content of the meal (Bille et al., 1983). Cold extraction by alkaline and saline solutions also fails due to protein losses in the solution (Rutkowski et al., 1965). Hydrothermal treatment of the intact seed in sodium sulfate has also been tried (Mothadi-Nia et al., 1986). Extraction of ground seeds with a two-phase solvent system (Diosady et al., 1985) and soaking of intact seeds with citric acid or ammonium carbamate (Schwenke et al., 1990) were some of the methods investigated. Water−ethanol mixtures and ammonia spray have also been used in the presence of steam. Alkanol−water−ammonia treatment removed up to 55% of glucosinolates (Meith et al., 1984). Ultrafiltration and diafiltration for the isolation of protein have been used but are not feasible on a large scale (Kroll et al., 1989). Binding of glucosinolates to protein was prevented by adding heavy metal salts such as $CuSO_4$. Recently, the effect of supercritical CO_2 on myrosinase activity has been investigated (Dunford et al., 1996). The effect of processing on the antinutritive factors has shown that the production of protein isolates and concentrates reduces antinutritional factors but also affects vitamin and mineral contents (Mansur et al., 1993).

In the present study a new approach is used employing an emerging idea in the field of biomimetic chemistry to use synthetic macromolecular hosts (**2**) from resorcinol and cetanal. **2** is soluble in CCl_4 and has been shown to bind glucose in its cavity quite strongly, and **2** has been shown to selectively extract glucosinolates out of the protein mesh without altering the hydrophilic amino acid and mineral content.

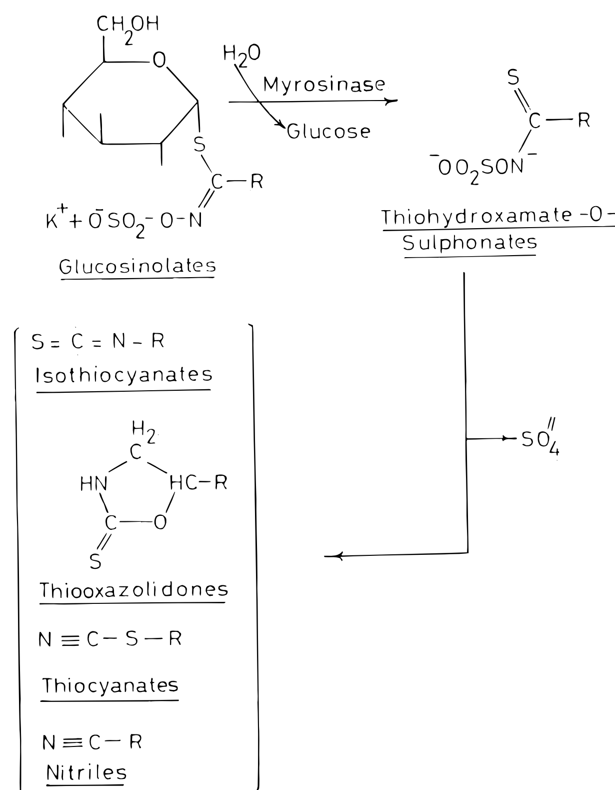

Figure 1. Products of enzymatic hydrolysis of glucosinolates.

MATERIALS AND METHODS

General. Melting points were determined in capillaries and were uncorrected. Proton NMR spectra were recorded on a Bruker FTNMR AC200 instrument with TMS as an internal standard. ^{13}C NMR spectra were recorded on a Bruker FTNMR AC 200. IR spectra were recorded on a SP-3-300 Pye Unicam infrared spectrophotometer, and electronic absorption spectra were recorded on a Shimadzu UV/160A. pH was measured on Systronics pH system 361. For studying the

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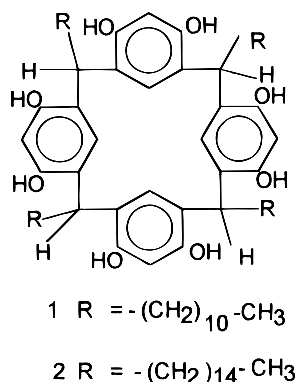

progress of reactions, TLC plates were coated with silica gel G and eluted with ethyl acetate, benzene, and benzene–hexane mixtures, and after elution spots were developed in iodine.

Solvent used for optical spectroscopy was spectral grade carbon tetrachloride (Sisco). Anhydrous pyridine (Glaxo) was obtained by distillation of reagent grade material after drying over KOH and was stored over 4 Å molecular sieves. Dichloromethane (S.d fine) was purified by shaking with sulfuric acid, aqueous sodium hydroxide, and water and drying over calcium chloride and distilling. Chromium trioxide of analytical grade (Nice) was stored in a phosphorus pentoxide desiccator.

Preparation of 2,8,14,20-Tetracetylpentacyclo-{19.31.1^{3,7},1^{9,13},1^{15,19}}octacosa-1(25), 3, 5, 7 (28), 9, 11, 13, (27), 15, 17, 19, (26), 21, 23 dodecaene-4,6,10,12,16,18,22,-24-octol (2). To a solution of resorcinol (19.8 g, 0.18 mol) in 95% ethanol/hydrochloric acid (3:1) at 20 °C was added a solution of cetanal (43.2 g, 0.18 mol) in 9% ethanol (50 mL) dropwise, stirred over a period of 2 h. The resulting solution was allowed to slowly warm to 25 °C and then heated at 75 °C for 24 h. The separated precipitates were washed repeatedly with cold methanol, dried, and recrystallized twice from methanol to yield the product (**2**): yield 70%; ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 12 H, CH_3), 1.23 (br s, 104 H, CH_2) (m, 8H, CH_2-CHO), 4.09 (t, 4H, methine), 6.84 (sH Ar), 8.88 (8, OH); ^{13}C NMR ($CDCl_3$) δ 14/164 (q, CH_3), 20.477–31.813 [overlapping triplets of cetyl $(CH_2)_{14}$], 37.115 (>CH methine), 96.034 (d, C_g), 124 (d, C_h), 150 (s, C_f); inept 20.277–31.813 (-ive).

Preparation of Rapeseed Meal. Untreated seeds of *Brassica napus* were procured from the local market. RSM was obtained by grinding the seeds (25 g) in a grinder. The oil was extracted from the seeds by Soxhlet apparatus using hexane, and detailed proximate composition was studied. The meal was dried to evaporate trapped hexane at 35 °C and weighed. Crude protien (N × 6.25) was determined according to the Kjeldahl procedure using a Kjeltec auto 1030 analyzer (Tecator, Sweden).

To four conical flasks (100 mL) host 2 solution (different concentrations) in CCl_4 (15 mL) was added, and the flasks were labeled A, B, C, and D. Defatted RSM (1 g) was added into each flask. All of the flasks were stoppered and were shaken (169 rpm) at 30 °C for 0.5 h. This was followed by filtration through Whatman No. 4 filter paper. Glucosinolates bound with **2** were obtained as filtrate, and the residue was washed with 20 mL of CCl_4 and preserved in 50 mL measuring flasks for further studies. Filter paper containing RSM was dried in oven maintained at 303 K (to evaporate carbon tetrachloride). Similarly, RSM was treated with **2** [four concentrations and shaken for different times (2, 3.5, and 5 h). The resultant meal was kept to study total glucosinolates content (TGC).

Croft Method for Calculation of TGC. Croft's method (Croft, 1979), based on enzyme-liberated acid titration, has been used for calculating TGC.

Reagents. (1) For Carrez reagent I zinc acetate (219 g) and acetic acid (30 mL) were mixed, and the volume was made up to 1 L with distilled water. (2) Carrez reagent II was potassium ferrocyanide [K_4{$Fe(CN_6)\cdot 3H_2O$, 10.6% w/v]. (3) Sodium hydroxide I (0.1 N). (4) Sodium hydroxide II (0.025 N).

Preparation of Extracts. (a) Enzyme Extract. An active enzyme extract of white mustard seeds was prepared from defatted crushed seeds (25 g) blended with distilled water (200 mL) for 2 min. The mixture was filtered through Whatman No. 1 filter paper. The filtrate was kept at 303 K for 2 h and cooled, and the acid produced was neutralized by adjusting the pH to 6 with 0.1 or 0.025 NaOH. The enzyme extract was prepared fresh every time.

(b) RSM Filtrate. RSM (1 g) obtained after treatment with **2** was put in 19 mL of boiling water for 5 min (to inactivate myrosinase) and the volume was maintained. Carrez reagent I (0.5 mL) was added to it, followed by Carrez reagent II (0.5 mL). The solution was mixed after each addition and cooled. Finally, it was filtered and the pH of the filtrate was adjusted to 6 with 0.1 or 0.025 N NaOH.

Titrations. To the RSM filtrate (*b*) (20 mL) was added white mustard seed enzyme extract (*a*) (8 mL) and the mixture was kept at 30 °C for 2 h. On cooling, it was titrated with 0.025 N NaOH to pH 6.0 (end point), and the volume of alkali used was recorded. During the monitoring of the pH, all solutions were kept at the same temperature.

Calculations. Glucosinolate (%) = $Y \times 0.025 \times 400 \times 100/1000 \times 1$, where 400 is the average molecular weight of glucosinolates, Y is the milliliters of alkali used, 0.025 is the normality of alkali, and 1 is the weight (g) of RSM in the filtrate.

General Procedure for Recording NMR. (i) The CCl_4 filtrate of host and glucosinolate obtained by shaking was evaporated to 1 mL and the NMR spectrum was recorded. (ii) The host **2** solution (15 mL) was shaken with glucose (AR) (1 g) for 15 min and filtered twice through Whatman No. 1 paper, and the filtrate was evaporated to 1 mL.

The NMR spectra of **2** with glucose and with glucosinolate were compared.

RESULTS AND DISCUSSION

Separation of glucosinolates from rapeseed meal has been a problem. Treatments to remove glucosinolates resulted in loss of protein in the aqueous system, and in nonpolar environments glucosinolates were not soluble and could not be extracted to any appreciable extent (Schwenke, 1994). An intermediate solvent system was required that could selectively catch these molecules and extract them. An answer to this problem was found using a novel approach based on recent molecular recognition studies (Aoyama et al., 1989) of sugars via hydrogen bonding interactions with a synthetic polyhydroxy macrocycle. It was found that resorcinol dodecanal cyclotetramer **1** is a lipophilic host and soluble in CCl_4. The presence of this molecule increases the solubility of polar molecules such as glycerol and water (neat liquids) and ribose (in an aqueous solution) as polar guests in CCl_4 upon formation of monomeric complexes in the ratio 1:4 (glycerol), 1:4 (H_2O), and 1 (ribose):2(H_2O), where ribose is bound highly selectively in pyranose form. A pair of hydrogen-bonded OH groups on adjacent benzene rings in **1** provide the essential binding site for a guest OH group, while glycerol and water are singly bound with such a binding site via hydrogen bonding, ribopyranose in doubly bonded with two binding sites separated by a metaphenyl bridge. The glucosinolates contain glucose units as a glycon part, which could bind with **1** in the same manner. This work has been carried out for the removal of glucosinolates on the basis of this observation.

The cyclic tetramer **2** obtained from long-chain aldehyde, i.e. cetanal, and resorcinol under acidic conditions has been synthesized, and the structure was established by ^{1}H NMR, ^{13}C NMR, and elemental analysis. **2** contains eight OH groups of resorcinol and four apolar cetyl chains, and hence it was soluble in carbon tetrachloride. Solutions of four different concentrations were

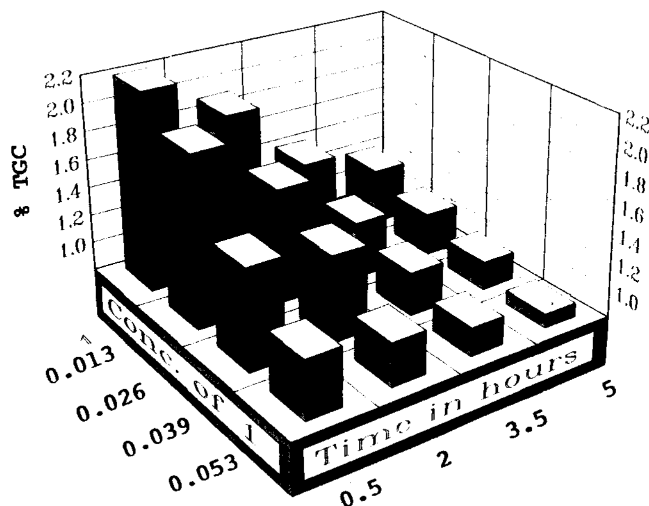

Figure 2. Change in TGC with change in concentration of **2** (mM) and time (h).

prepared as A (0.013 mM), B (0.026 mM), C (0.039 mM), and D (0.053 mM).

GC was determined using Croft's method at 30 °C, which is based upon enzyme-liberated acid titration. When myrosinase hydrolyzes glycosinolates, the acid is produced as a byproduct and is titrated against a standard solution of NaOH (0.025 N) using a pH meter to a fixed pH, in our case pH 6. For this purpose a standard enzyme extract was prepared using white mustard seeds according to the established procedures. The RSM was first treated with hot water for 5 min to inactivate myrosinase and was then treated with a standard enzyme extract at pH 6. Enzyme-treated RSM was incubated for 2 h at 37 °C. The acid produced was neutralized to pH 6 using 0.025 N NaOH solution.

The TGC determined was found to be 2.9%, and after extraction with pure CCl_4 at 169 shakes/min, the loss was only 0.1%. The extractions of glucosinolates from rapeseed were carried out with different concentrations of host **2** (A–D) and for different time intervals (0.5, 2, 3.5, and 5 h) to see the effects of concentration and time of shaking (Figure 2). After shaking all solutions were filtered through Whatman No. 4 paper. The residue was checked for TGC after drying overnight at 30 °C by Croft's method, and the filtrate was kept for ^{1}H NMR studies. The percentage of glucosinolates in samples was calculated from the milliliters of alkali (0.025 N) used in titration. As shown from (Figure 2) the results were beautifully along the lines expected; that is, the percentage TGC decreased as the concentration of **2** increased from 0.013 to 0.053 mM from 2.2% to 1.2% for 0.5 h of shaking, from 1.9% to 1.1% for 2 h of shaking, from 1.4% to 1.0% for 3.5 h of shaking, and from 1.2% to 0.9% for 5 h of shaking. Hence, the maximum decrease was ~66% with 5 h shaking and at 0.053 mM of host **2** solution.

NMR Studies To Check Solution A. **2** in CCl_4 was shaken with solid glucose (1 g) for 15 min and filtered twice through Whatman No. 4 paper. Filtrate was concentrated to 1 mL and its NMR spectrum was recorded. The ^{1}H NMR spectrum clearly indicates the characteristic peak of glucose at 6.46 due to C_1–H (Lemieux et al., 1958). The solution after shaking with defatted RSM was concentrated to 1 mL from 5 mL and checked for any extra peaks related to glucosinolates in ^{1}H NMR; a similar peak at 6.49 is observed in solution **2** in CCl_4 (0.05 mM), which is due to the glycon part of glucosinolates, indicating clearly that glucosinolates were extracted by the host solution.

Conclusion. This has been a preliminary investigation of a continuing study, and it clearly indicates that there is great potential in using the macromolecules for solving the complicated separation process with ease by employing host–guest chemistry, although much remains to be done before this technique can be used on a commercial scale. It is has a promising future.

LITERATURE CITED

Ayoma, Y.; Tanaka, Y.; Sugahara, S. Molecular recognition of sugars via hydrogen bonding interaction with a synthetic polyhydroxy macrocycle. *J. Am. Chem. Soc.* **1989**, *111,* 5397–5404.

Bille, N.; Eggum, B. O.; Jacobsen, I.; Olsen, O.; Sorensen, H. Antinutritional and toxic effects in rats of individual glucosinolates (± myrosinase) added to a standard diet 1. Effects on protein utilization and organ weights. *Z. Tierphysiol. Tierernahrg FuttermittelEde.* **1983**, *49*, 195–210.

Croft, A. G. The determination of total glucosinolates in rapeseed meal by titration of enzyme liberated acid and the identification of the individual glucosinolates. *J. Sci. Food Agric.* **1979**, *30*, 417–423.

Diosday, L. L.; Rubin, L. J.; Philips, C. R.; Naczk, M. Effect of alcohol-ammonia-water treatment on the glucosinolate content of rapeseed meal. *Can. Inst. Food Sci. Technol. J.* **1985**, *18*, 311–315.

Dunford, N. T.; Temelli, F. Effect of supercritical CO_2 on the pyrosinase activity and glucosinolate degradation in canola. *J. Agric. Food Chem.* **1996**, *44*, 2372–2376.

Kroll, J.; Kujawa, M.; Krock, R.; Schnaok, W. Glucosinolates free protein preparations from rapeseed. East German Pat. DD273-195, 1989.

Leemieux, R. U.; Kullning, R. K.; Bernstein, H. J.; Schneider, W. G. Configuration effects on the proton magnetic resonance spectra of six membered ring compounds. *J. Am. Chem. Soc.* **1958**, *80*, 6098–6105.

Maheshwari, P. N.; Stanley, D. W.; Vande Voort, F. R. Microwave treatment of dehulled rapeseed to inactivate myrosinase and its effect on oil and meat quality. *J. Am. Oil Chem. Soc.* 1980, 57, 194-199.

Mansour, E. H.; Dwoschak, E.; Lugasi, A.; Gaal, O.; Barmi, E.; Gergely, A. Effect of processing on the antinutritive factors and nutritive value of rapeseed products. *Food Chem.* **1993**, *47*, 247–252.

Meith, G.; Weigelt, E.; Bruecker, J. Production of protien concentrates from various rapeseed bareities. Part 1. Possibilities of eliminations of glucosinolates. *Nahrung* **1984**, *9*, 28.

Mothadi-Nia, D. J.; Bau, H. M.; Giannangeli, F.; Mejean, L.; Debry, G.; Evrard, J. Valorisation nutritionelle des proteins calza par on traitement hydrothermique des graines. *Can. Inst. Food Sci. Techol. J.* **1986**, *19*, 95–103.

Rutkowski, A.; Kozlowska, H.; Izaszek, J. *Rozzn. Technol. Chem. Zywn.* **1965**, *11*, 77–89.

Schwenke, K. D. Rapeseed proteins. In *New and Developing Sources of Food Protiens*; Hudson, B. J. F., Ed.; Chapman and Hall: London, U.K., 1994; pp 281–306.

Schwenke, K. D.; Kroll, J.; Lange, R.; Kujawa, M.; Schnaak, W.; Steinert, A. Preparation of detoxified high functional rapeseed flours. *J. Sci. Food Agric.* **1990**, *51*, 391–405.

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