Extraction and Characterization of Glucosinolates and Isothiocyanates from Rape Seed Meal

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Abstract: While some isothiocyanate (ITCs) are attractive targets for the agricultural and pharmaceutical industries, the presence of goitrin and ITCs has hampered the widespread utilization of rapeseed meal. ITCs are the products of the myrosinase-mediated hydrolysis of glucosinolate (GSLs). As such, a study was conducted in order to gain a better understanding into the identity of the GSLs contained in rapeseed meal. Extraction of the GSLs was carried out with 20% ethanol, affording 3.0% GSL content. The resulting GSL extracts were purified via silica gel column chromatography resulting in the isolation of main three pure GLSs (GSL A, B, and C) and a final GSL content of 39.8%. The indirect-identification of the GSLs in rapeseed meal was also carried out via GC/MS analysis of ITCs. The GSLs, progoitrin and gluconapin, were present in the highest concentration in these extracts. Interestingly, only goitrin was produced when GSL A was the substrate for the defatted rapeseed meal mediated hydrolysis reaction. This indicates GSL A is a progoitrin. Conversely, 3-butenyl ITC was produced only when GSL B was used as substrate, indicating GSL B is gluconapin. These results will be helpful for opening the doors for the use of rapeseed meal in the agricultural or pharmaceutical sectors.

Key words: rape seed meal, glucosinolate, isothiocyanate, myrosinase

1 INTRODUCTION

Rapeseed meal is a by-product formed during the process of rapeseed oil (canola oil) production. In 2012, Japan produced the 1.3 and 1.1 million tons of rapeseed meal and canola oil¹⁾. The high nutritional value of rapeseed meal (ca. 40% protein, 35% fiber, 8.5% minerals, 1% lipid and others) makes it an ideal feedstock and/or fertilizer²⁾. However, the goitrin (Fig. 1) and isothiocyanates (ITCs) synthesized via myrosinase mediated hydrolysis of its constituent glucosinolates (GSLs) limits the utilization of rape seed meal. Goitrin, a sulfur-containing oxazolidine, is known to reduce the production of certain thyroid hormones leads to goiter^{3,4)}.

On the other hand, several ITCs and GSLs exert desirable physiological properties. For example, the gluconapin is known to have a suppressive effect on postprandial hypertriglyceridemia in mice¹³⁾. While the allyl ITC shown in Fig. 1 possesses irritant properties and initiates an acute inflammatory response⁵⁾, some of the allyl ITCs contained in wasabi have beneficial anti-microbial properties. Isshiki et al. reported that vapor of allyl ITC suppressed the microbial growth of *Staphylococcus aureus* IFO-12732 and *Escherichia coli* JCM-1649, the yeast *Hansenula*

anomala NFRI-3717 and *Candida albicans* IFO-1061, and the fungi *Penicillium chrysogenum* IFO-6223 and *Fisarium solani* IFO-9425⁶⁾. Anti-cancer properties against breast, lung, and stomach cancers have also been reported⁷⁾. Furthermore, sulforaphane (mainly from broccoli sprouts), 6-methylsulfinylhexyl ITC (wasabi), and phenethyl ITC (watercress) all exhibit strong anti-cancer effects in addition to good bioaccumulation and favorable toxicological profiles (Fig. 1)^{8,9)}. Epidemiological research has also showed that carcinogenic risk can be reduced by the intake of cruciferous vegetables such as cabbage, napa, and broccoli^{10,11)}.

GSLs are distributed mainly in the leaves, stems and roots of plants such as *Capparales Brassicaceae* (especially in *Brassica*, *Sinapis*, *Raphanus*, *Wasabia*), *C. Resedanceae*, *C. Capparaneae*, and *C. Caricaceae*. One or more GSLs derived can be found within each species. For example, only sinalbin is contained in *Sinapis alaba* L. while *V. oleracea* L. is known to contain Sinigrin, glucoiberin, progoitorin, glucoraphanin and *gluconasturtiin*. *B. napus* L. (rapeseed), gluconapin, progoitrin, glucoiberin, glucobrassicinapin, and sinalbin are all known to exist¹²⁾. It is noteworthy, that the main point of diversity between

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Fig. 1 Chemical structures of goitrin, ITCs and GSL.

many of these related compounds lies with the alkyl(R) functionality (Fig. 1).

Unfortunately, a complete understanding of these biologically relevant compounds is lacking due in part to the relatively few reports describing the process of GSL, extraction and the preparation of ITCs from rapeseed meal. To satisfy this demand, we describe herein processes for the isolation and characterization of GSLs from rapeseed meal.

2 EXPERIMENTAL

2.1 Materials

Rapeseed meal was donated by Nisshin OilliO Group, Ltd (Tokyo, Japan). (-)-Sinigrin hydrate was utilized as a GSL standard and was purchased from Sigma-Aldrich (Tokyo, Japan). Allyl ITC which was employed as an ITC standard, and silica gel utilized for column chromatography (63-210 μm) were both purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 3-Butenyl ITC and phenethyl ITC were both purchased from Tokyo chemical industry Co., Ltd (Tokyo, Japan). ODS (Cosmosil[®], C_{18} -75OPN, 75 μm) was purchased from Nacalai tesque (Kyoto, Japan). All other reagents and solvents were of analytical grade and utilized directly and without manipulation.

2.2 Extraction of GSLs from rapeseed meal

Rapeseed meal (2.0 g) was suspended in 20% ethanol (20 mL). After stirring at 300 rpm for 30 min, the solution was filtered with paper. This extraction process was repeated twice. Next, the proteins were removed from the mixture by precipitation. To this end, the solvent was stirred in 70% ethanol (60 mg of extract/mL) for 30 min prior to centrifugation (4,000 rpm, 10 min). The supernatant was next evaporated then reconstituted in methanol (4 mg of extract/mL). After briefly vortexing, the soluble fraction was collected and concentrated. Following purification (Section 2.3) the identity of the methanol soluble GSLs were analyzed by the method described in Section 2.4.

2.3 Fractionation of GSLs – silica gel and ODS column chromatography

The first step in the purification of GSLs from the extracts obtained in Section 2.2 is silica gel column chromatography. To do so, the silica gel must be pre-activated by heating at 130°C for 3 h. At thirty-fold volume of silica gel to the extract was loaded into a glass column. The separation was carried out with an isocratic elution system composed of an acetone-water (30:1, v/v) mobile phase. Each fraction was eluted with 50 mL of solvent.

GSL extracts (Section 2.2) were also purified via ODS column chromatography. At twenty fold volume of ODS was loaded into glass column and ammonium sulfate (200

mM) served as the mobile phase. The fractions were eluted with 20 mL of solvent.

The GSL content obtained by each of these methods was quantified as described in the next section.

2.4 High performance liquid chromatography (HPLC)

GSLs in extracts were analyzed as follows: Pump (TRI ROTAR-V, JASCO Corporation, Tokyo, Japan) equipped with degasser (DG-2080-53, JASCO), ultra violet detector (875-UV, JASCO) and reversed phased column (Crest Pak C18S, 4.6 mm × 150 mm, JASCO). The mobile phase was ammonium sulfate (200 mM), and the flow rate was 1.0 mL/min. The GSLs were detected at 227 nm, which is the λ_{max} of (-)-sinigrin. The resulting data was processed using a Chromatocorder 21 (System Instruments Co., Ltd, Tokyo, Japan), and the GSL concentration was extrapolated from (-)-sinigrin hydrate standard curve.

The ITCs were analyzed under the same conditions as described above for the GSLs except that the mobile phase was acetonitrile-water (1:1, v/v) and the detection was carried out at 247 nm. The total concentration was calculated from a 3-butenyl ITC standard curve.

2.5 Preparation of defatted rape seed (dRS)

dRS was used as the myrosinase source for the ITC preparation. Briefly, 20.6 g of rapeseed was powdered in food processor prior to addition of *n*-hexane (100 mL). After 30 min of stirring, the solution was filtered. This extraction process was repeated three times, ultimately yielding 12.0 g of residual powder (dRS).

2.6 Preparation of ITCs from GSL extracts

GSL extracts (2.1 g, 80 μmol) were dissolved in 25 mL of 0.2 M citrate-phosphate buffer (pH 6.0). Once dissolved dRS (0.125 g) and *n*-hexane (10 mL) were added. The biphasic solution was permitted to reaction at 37°C for 120 min followed by centrifugation at 4,000 rpm for 20 min. Due to mobile phase compatibility issued the ITCs were transferred from hexane acetonitrile prior to HPLC analysis. The resultant water and organic fractions were analyzed by HPLC as described in Section 2.4.

2.7 Gas-chromatography/mass spectrometry (GC/MS) analysis

Aliquots from the dRS mediated hydrolysis reaction (Section 2.6) were analyzed by GS/MS. A GC-MS QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) equipped with UA⁺-65 column (0.25 mm ID × 30 m, film thickness = 0.10 μm , Frontier Laboratories Ltd, Fukushima, Japan) was used. The column temperature was 50–300°C (ramp = 10°C/min) and the helium served as the carrier gas at 69.4 kPa. Samples were ionized by electron ionization at 280°C.

3 RESULTS AND DISCUSSION

3.1 Extraction of GSLs from rapeseed meal

From 2.0 g of rapeseed meal, 0.27 g of extract (brown solid) was obtained according to the method described in Section 2.2. The GSL content in the extract was determined to be 3.0%. In the extract, three main peaks (GSL A, B, and C) were observed by HPLC (Fig. 2A). These three main peaks were not detected in the water phase after the

Fig. 2 HPLC chromatogram of extract of rape seed meal. (A) before and (B) after dRSM treatment. New peak was detected after dRSM treatment (black arrow X). HPLC conditions were described in Section 2.4.

Fig. 3 HPLC chromatogram of hexane layer after dRSM treatment of extract of rape seed meal. HPLC conditions were described in Section 2.4.

extract was treated by dRS. This is consistent with the hypothesis that these compounds were converted to ITC upon myrosinase hydrolysis (Fig. 2B). In addition, a new peak (X) was detected in the water phase after dRS treatment. This is also consistent with the expulsion of a hydrophilic compound during the myrosinase mediated hydrolysis (Fig. 2B).

3.2 Preparation of ITCs from GSL extract

Upon reaction of the GSL extract obtained (Section 3.1) with dRS, several peaks were detected in the hexane layer (Fig. 3) via HPLC. These peaks were not detected when the reaction was conducted in the absence of dRS. Taken together, a total of six peaks (6.8, 8.8, 12.3, 13.9, 14.9 and 17.8 min) were identified as corresponding to ITCs. In support of this, the peaks that eluted at 8.8 min and 14.9 min had the same RT as the 3-butenyl and phenethyl ITC standards, respectively. Furthermore, GS/MS confirmed that those two peaks had the same RT and m/z values as the 3-butenyl and phenethyl ITC standard. Under the reaction conditions — 1.25 g of GSL extracts (80 μ mol of GSL in sinigrin equivalent)/25 mL of 0.2 M citrate-phosphate

Fig. 4 Elution curve of silica gel column chromatography of extract of rape seed meal.

buffer (pH 6.0), 0.125 g of dRSM, 10 mL of hexane at 37°C for 120 min—, 18.8 mol% of the ITCs was synthesized as the 3-butenyl ITC equivalent. Interestingly, although has been reported that ITCs are generated in a pH dependent manner^{11–13}, in our hands, there was no correlation between the yields and pH over a broad range (3.0–8.0, data not shown). Variations in the reaction temperature (23, 37 and 60°C) did not significantly improve the yield (data not shown).

3.3 Identification of GSLs in rapeseed meal

The extract (2.1 g, 2.5% of GSL) was purified via silica gel column chromatography. This process revealed that the GSL amount (mg/content %) of GSL increased with elution volume. As such fraction 9 was found to contain highest concentration of GSLs (39.8%) as brown solid (38.8 mg, Fig. 4).

To identify the GSLs, four fractions with varied content of GSLs were obtained by silica gel column chromatography (Lot. 1–4, Table 1). Lots. 1–4 were the fractions obtained randomly by silica gel column chromatography. Using these fractions, a relationships between the generation of the hydrophilic compound (area of peak X, Fig. 1B) and the amount of GSLs consumed during the reaction was identified. There were positive correlation ($R^2 = 9.9747$)

Table 1 GSL extracts with variety content of GSLs.

	GSL A [μ mol]	GSL B [μ mol]	GSL C [μ mol]	Total GSL [μ mol]	Content of GSL [%]
Lot. 1	8.6	32.2	13.7	54.5	21.0
Lot. 2	11.7	12.6	18.0	42.3	23.3
Lot. 3	29.8	9.5	23.2	62.5	14.4
Lot. 4	46.8	3.8	21.1	71.7	14.0

Fig. 5 Elution curve of ODS column chromatography of extract of rape seed meal.

between the peak X and the amount of GSL A, while there was no relation between the peak X and GSL B/GSL C (data not shown). This data suggested that GSL A is precursor of peak X under the reaction condition. On the other hand, there was positive relationship ($R^2 = 0.9996$) between the amount of ITC prepared in the hexane layer and the amount of GSL B consumed, while connection between the amount of ITC and GSL A/GSL C was identified (data not shown). As the main ITC generated during the course of the reaction was 3-butenyl ITC, this suggested that GSL B is a 3-butenyl ITC precursor.

To confirm these hypotheses, three GSLs from rapeseed meal were condensed and used as substrates for the myrosinase reaction. Briefly, 2.0 g of the extract (GSL A, B and C were 14.6 mg, 4.8 mg and 36.3 mg, respectively) was subjected to ODS column chromatography. As shown in Fig. 5, each of the purified GSLs was isolated (fractions 4-7, 10-13 and 15-21) with 65, 68, and 30% recovery. The GSL content of each fraction containing GSL A, GSL B and GSL C was 9.6 (100% GSL A), 17.7 (100% GSL B) and 19.2% (100% GSL C), respectively. These fractions were named as hGSL A, hGSL B and hGSL C, respectively.

Peak X was detected in the water phase only when hGSL A was utilized for the hydrolysis reaction. Indeed, the myrosinase reaction of hGSL B or hGSL C did not afford peak X. In order to identify the molecule that corresponded to peak X, it was extracted with chloroform and the m/z was identified by GS/MS. Here, chloroform was chosen as extraction solvent due to its median polarity between water and hexane. From this experiment an $m/z = 129$ was identified. This fragment coincided with the fragmentation of goitrin. As such, GSL A was identified as progoitrin (a precursor of goitrin)¹⁴. Finally, 3-butenyl ITC was only detected when hGSL B served as the substrate for the myrosinase reaction. Based on this data, GSL B was identified as gluconapin, which is a precursor of 3-butenyl ITC¹⁵.

The identification of GSL C is beyond the scope of this

study. We postulate that this is either due to the stability of the resultant ITC under the conditions described herein or due to no ITC being formed in this case. It is possible that the aglucon generated from GSL C might be a nitrile or oxanoldine-thione, instead of an ITC¹⁴.

4 CONCLUSION

The solvent used in this study for extraction of GSLs was 20% ethanol, which is readily available and widely utilized in the food industry. Using silica gel column chromatography, the GSL content in the extract was condensed up to 39.8%. Progoitrin and gluconapin were identified as the main GSLs contained in the extract. Progoitrin is a precursor of the nutritionally harmful goitrin. Gluconapin is a precursor of 3-butenyl ITC, a compound showing potential antibacterial or anticancer effects^{16, 17}. The results of this study provide a foundation to improve our understanding of the GSLs composition in rapeseed meal. The processes described in this report provide an economical and environmentally friendly way to harvest these- and other- biologically relevant compounds from rapeseed meal. Ready access to these materials will undoubtedly facilitate wide range of know and as yet unidentified uses for these unique compounds.

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