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Preparative Isolation and ^{35}S-Labelling of Glucosinolates from Rapeseed (*Brassica napus* L.)

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A procedure for preparative isolation of the main glucosinolates, *viz.* progoitrin, gluconapin, glucobrassicanapin, and glucoalyssin from rapeseed is described. The procedure consists of ion-exchange chromatography on DEAE-Sephadex A-25 followed by chromatography on arginine coupled to Sephadex G-10. Application of the procedure to the preparation of ^{35}S-labelled glucosinolates is also described, along with a method for the quantitative assay of glucosinolates by determination of enzymically liberated glucose with a specific glucose oxidase reagent.

The glucosinolates are a group of natural thioglucosides that occur in all Cruciferae.[1,2] These substances are converted to antinutritional isothiocyanates by the enzyme myrosinase (a thioglucosidase), which is also present in the plant material, according to the following reaction:

$$R\text{-}C(\text{=}N\text{-}O\text{-}SO_2O^-)\text{-}S\text{-}C_6H_{11}O_5 \xrightarrow[\text{myrosinase}]{H_2O} R\text{-}N\text{=}C\text{=}S + \underset{\text{(glucose)}}{C_6H_{12}O_6} + HSO_4^- \qquad (1)$$

Oxazolidinethiones, which are also antinutritional, are formed spontaneously by cyclization of the 2-hydroxy isothiocyanates.

In rapeseed (*Brassica napus* L.), the major glucosinolate is progoitrin, the precursor of the goitrogenic L-5-vinyloxazolidine-2-thione. The predominant isothiocyanate precursor is the glucosinolate gluconapin. Four or five minor glucosinolate components are also present.[3]

The goal of our program is the production of rapeseed protein isolate for human consumption. Removal of the antinutritional factors or the prevention of their formation are the major obstacles to the preparation of acceptable protein isolate. Feeding experiments on rats with rapeseed proteins isolated by gel filtration on Sephadex G-25 have shown residual toxicity in the proteins.[4,5] This indicates that association or reaction between protein and glucosinolate or its aglucon occurs. The conditions for and the nature of such interactions is not fully known, and might differ among diverse kinds of

glucosinolates. To investigate this it is necessary to follow the glucosinolate and aglucon distribution in various separation stages, even when they are present in very low concentrations. We found radioactive-labelled glucosinolates very useful for this purpose.

The labelled glucosinolates will also be used to study the distribution of glucosinolates or aglucons within animals, by performing autoradiography on slices of animals fed with the labelled material.

The present paper describes a procedure for the preparative isolation of the four main glucosinolates of rapeseed. Application to the production of ^{35}S-labelled glucosinolates is demonstrated.

METHODS AND MATERIALS

Seed material. Seed from winter rape (cv. Panter) was obtained from AB Karlshamns Oljefabriker, Karlshamn, Sweden, and seed from summer rape (cv. Gylle) was purchased from AB Hammenhögs Frö, Hammenhög, Sweden.

Radioactive-labelled glucosinolates were prepared by cultivating summer rape with ^{35}S-sulphate added to the soil. 10 mCi of ^{35}S-sulphate (R. C. C. Amersham, England) was added to a soil area of 1 m^2 in two portions, the first one month and the second two months after sowing the seed.

Extraction of glucosinolates. The seed (100 g) was crushed in a roller-mill and defatted by extraction with ether or hexane. The glucosinolates were extracted by boiling the meal in 750 ml of 80 % methanol for 20 min. After filtering on a Büchner funnel with suction the extract was concentrated nearly to dryness in a rotatory evaporator at 40°. The brown syrup was dissolved in 40 ml of distilled water and centrifuged at 18 000 r.p.m. for 30 min. After filtering on glasswool a clear, brown crude extract was obtained.

Anion-exchange chromatography on DEAE-Sephadex. The crude extract was fractionated on a DEAE-Sephadex A-25 anion-exchange column (Pharmacia Fine Chemicals, Uppsala, Sweden). 25 g of the exchanger was swelled overnight in 0.2 M NH_4HCO_3 buffer at pH 8.0. After washing with distilled water to remove excess carbonate the gel was packed in a 5 × 9 cm column. About 50 ml of sample was applied. Large molecular size compounds, uncharged molecules and coloured substances were eluted with distilled water. The glucosinolates were desorbed with 0.1 M NH_4HCO_3 buffer at pH 8.0. The flow rate was about 100 ml/h and the eluate was collected in 20 ml fractions. The fractions containing glucosinolates were combined in two portions, I and II, as indicated in Fig. 1. After freeze-drying, 2.5 – 3 g pale yellow material containing about 85 % glucosinolates was obtained.

Chromatography on arginine-Sephadex dipolar adsorbent. The individual glucosinolates were separated by chromatography on columns packed with arginine-Sephadex G-10. This amphoteric gel [6] was prepared by attaching arginine to Sephadex G-10 according to the cyanogen bromide method.[7]

200 g of dry Sephadex G-10 was stirred into a solution of 200 g cyanogen bromide in 1.5 l of distilled water. The pH was kept at 11.0 for 8 min by addition of 5 M sodium hydroxide. The gel was then rapidly washed on a Büchner funnel with ice-cold 0.5 M bicarbonate solution.

The activated gel was slurried in 500 ml distilled water and 64 g of solid arginine was added. After stirring the reaction mixture at room temperature for 20 h the gel was washed thoroughly with distilled water. The arginine content of the gel as determined by conventional amino acid analysis was 200 μequiv./g dry gel (corr.), which indicates that smaller amounts of cyanogen bromide and arginine could have been used.

The gel was packed in two 2.5 × 42 cm columns which were coupled in tandem. The columns were equilibrated and run in 0.1 M ammonium acetate solution (pH 6.9). The flow rate was about 50 ml/h. A maximum of 0.5 g of crude glucosinolates dissolved in 5 ml buffer was applied. The eluate was collected in about 20 ml fractions, which were pooled as shown in Figs. 2 and 3. The volatile buffer salts were removed by lyophilization, leaving the glucosinolates as white to yellowish solids.

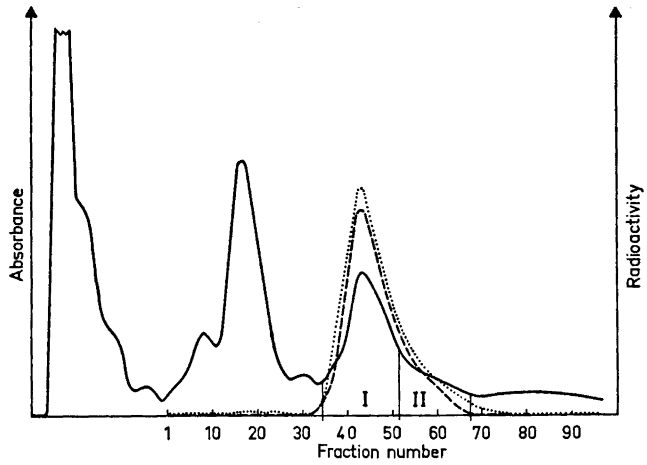


Fig. 1. Ion-exchange chromatography of ^{35}S-glucosinolate extract on DEAE-Sephadex A-25 in ammonium bicarbonate pH 8.0. ——— A_{254} from monitor, – – – glucosinolate concentration, ... radioactivity.

Glucosinolate assay. Glucosinolates were quantitatively assayed by determining the glucose released upon enzymic hydrolysis. The glucose was determined with a specific glucose reagent, "Glox" (Kabi, Sweden) containing glucose oxidase, peroxidase, and *o*-dianisidine. The glucosinolate assays were carried out according to the following procedure: 50 µl of sample was incubated with 10 µl of myrosinase solution (prepared from white mustard seed) in 1 ml 0.05 M phosphate buffer at pH 6.8. The mixture was kept at 40° for 30 – 60 min. 2 ml of a "Glox" solution (1 g of "Glox" dissolved in 61 ml of distilled water) was then added and the mixture was kept at 40° for another 30 min. The absorbance at 450 nm was measured in a spectrophotometer against a blank prepared in a parallel run with heat-inactivated myrosinase.

This method is not applicable when the solutions to be measured are strongly coloured or contain substances that disturb the "Glox" reagent, for instance high concentration of ascorbic acid.

The amount of myrosinase added must be large enough to quantitatively hydrolyze the glucosinolates within the chosen incubation time.

With 50 µl of sample glucosinolate concentrations in the range 0.4 – 2.5 mg/ml were measured. By increasing the sample volume to 1 ml it was possible to determine glucosinolate concentrations of 0.02 mg/ml. The glucosinolate content was calculated from a calibration curve prepared with glucose or sinigrin. The curve was linear up to an optical density of 1.1.

Glucosinolates were also determined from their ultraviolet absorption spectra. The concentration was calculated according to Beer's law from the absorbance at the absorption maximum of about 227 nm.

Radioactive measurements. Radioactive material was assayed with a Nuclear-Chicago model D-47 Gas Flow Counter. The samples (usually 0.5 ml) were pipetted into steel planchets. After addition of a wetting agent (triethylamine) the samples were dried under an infra-red lamp.

Identification of glucosinolates. Identification of the glucosinolates was mainly done by spectrometric and chromatographic methods.

The spectrometric methods comprised UV-spectrophotometry on the glucosinolates and their aglucons, IR-spectrophotometry on the ammonium salts of the glucosinolates in pressed KBr pellets, and in a certain cases NMR and masspectrometry on glucosinolate aglucons.

Thin-layer chromatography was performed on silica gel plates (Merck F_{254}). Intact glucosinolates were run in butanol:ethanol:water, 4:1:4 (upper phase). Oxazolidinethiones and thioureas were run in chloroform:methanol, 9:1.

Paper chromatography (on Whatman No. 1) was performed on thioureas and oxazolidinethiones in water-saturated chloroform (ascending technique).[8] Oxazolidinethiones were also run in benzene:methanol:water, 2:1:1 (descending technique).[9] Glucosinolate spots were detected in UV light and with anisaldehyde-sulphuric acid reagent. Thioureas and oxazolidinethiones were detected in UV light and with Grote's reagent.[10]

RESULTS

The isolation experiments with non-radioactive glucosinolates were mainly performed on seed from winter rape (cv. Panter). Comparative isolations from the summer type (cv. Gylle) indicated that the glucosinolate patterns were qualitatively and quantitatively very similar in the two cultivars.

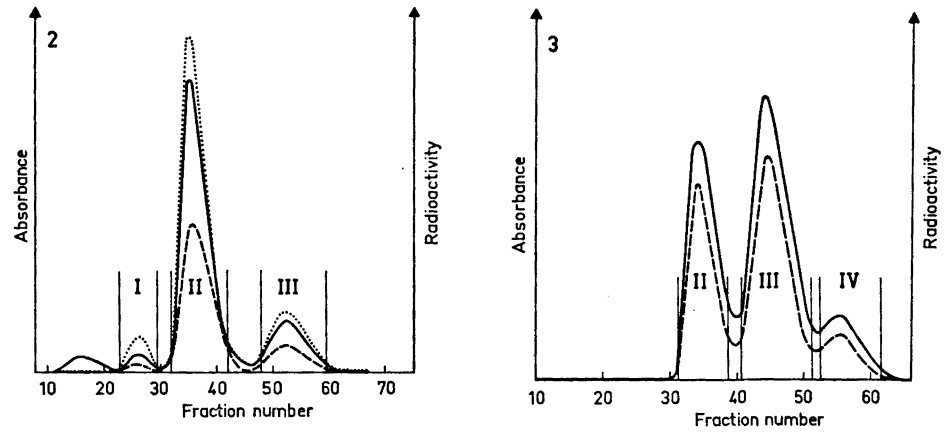
The preparation of ^{35}S-glucosinolates was done with the summer rape (cv. Gylle) because of its shorter period of cultivation.

The ammonium salts of the following glucosinolates were isolated (approximate yields from 100 g of seed in parenthesis. Concerning R, see eqn. 1.):

I.	Glucoalyssin (50 mg)	R = $CH_3-SO-(CH_2)_5-$
II.	Progoitrin (1700 mg)	R = $CH_2=CH-CHOH-CH_2-$
III.	Gluconapin (550 mg)	R = $CH_2=CH-(CH_2)_2-$
IV.	Glucobrassicanapin (50 mg)	R = $CH_2=CH-(CH_2)_3-$

The glucosinolates are numbered according to their elution order in the arginine-Sephadex chromatography as shown in Figs. 2 and 3.

When lyophilized preparations of the glucosinolates progoitrin and gluconapin were left in a sealed flask overnight clusters of needle-shaped crystals occasionally formed. Several needles were more than two centimeters long.



Figs. 2 and 3. Chromatography of fraction I and II respectively from DEAE-Sephadex (Fig. 1) on arginine-Sephadex G-10 dipolar ion adsorbent in 0.1 M ammonium acetate pH 6.9. ——— A_{254} from monitor, – – – glucosinolate concentration, ... radioactivity.

Unfortunately it was impossible to collect the crystals, since they were immediately destroyed when the flasks were opened. Crystallization of the progoitrin from benzene gave small stable white needles, m.p. 137 – 139° (*cf.* Ref. 11).

Unsuccessful attempts were made to detect 2-hydroxy-4-pentylglucosinolate, a higher homolog of progoitrin.[9] Descending paper chromatography in benzene : methanol : water, 2 : 1 : 1, on the oxazolidinethione from the progoitrin gave only one spot.

Besides the glucosinolates mentioned, rapeseed contain traces of glucoraphanin and gluconasturtiin,[3] which we did not attempt to isolate.

DISCUSSION

The crude aqueous glucosinolate extract from the methanol extraction was freed of most of the proteins, carbohydrates, and coloured and uncharged compounds by anion-exchange chromatography. Previously Amberlite IR-4B and similar resins have been used for the purification of glucosinolate extracts.[11,12] In our hands DEAE-Sephadex proved more convenient and efficient than the resins.

Chromatographic separation of the individual glucosinolates on highly crosslinked dextran gels was attempted. Chromatography on Sephadex LH-20 gave a partial separation of some glucosinolates. After extensive washing of Sephadex G-10 with 1 M pyridine solution to change its adsorption properties [13] some separation of the glucosinolates was attained.

Improved fractionation was obtained on an arginine derivative of Sephadex G-10. The gel was prepared by coupling arginine to Sephadex G-10 by the cyanogen bromide method.[7] Chromatography on this gel gave a complete separation of the main glucosinolates in rapeseed. The separation on these gels is likely to depend on adsorption or affinity phenomena rather than molecular sieving or ion-exchange chromatography.

The four glucosinolates isolated represent the precursors to three different types of aglucons. Glucoalyssin (I) yields a sulfoxy isothiocyanate on enzymic hydrolysis. Progoitrin (II) gives rise to an oxazolidinethione, while gluconapin (III) and glucobrassicanapin (IV) form unsaturated alkyl isothiocyanates. Under certain conditions thiocyanates and nitriles also may be formed.

The various products presumably interact with rapeseed proteins in different ways. For instance, isothiocyanates can react with the amino groups or the sulfhydryl groups of a protein to form thio- and dithiocarbamates, respectively. Oxazolidinethiones and glucosinolates may adsorb to proteins in weaker complexes. It is also possible that the glucosinolate aglucons have different reactivities toward different kinds of proteins in rapeseed.

The elucidation of these interactions should greatly facilitate investigations on preparation of nutritious and innocuous protein isolate. We consider it important to study the protein reactions of each glucosinolate type individually. This can be performed by addition of radioactive labelled glucosinolates to a rapeseed meal extract. After incubation under certain conditions the material is fractionated and analyzed. A detailed account of these studies will be published elsewhere.

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