AFFINITY CHROMATOGRAPHY PURIFICATION OF BETA-THIOGLUCOSIDASE FROM RAPESEED.

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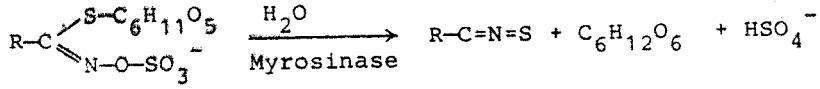
ABSTRACT:

A rapid procedure that permits large scale purification of myrosinase (beta-thioglucosidase, E.C. 3.2.3.1) from crude extracts of rapeseed is presented. Centrifuged and dialyzed crude extracts were run on a Con-A-Sepharose column. The specific activity after elution with α-methyl-D-mannopyranoside gave an average specific activity of 2.95 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, a recovery of 94.5% and a 15-fold purification of the enzyme. Isoelectric focusing and SDS-PAGE of the combined fractions after affinity chromatography showed 8 and 10 bands, respectively.

INTRODUCTION:

Myrosinase appear in different forms and catalyze the hydrolysis of glucosinolates. The hydrolysis generally results in the production of isothiocyanates, sulphate and glucose:



Isothiocyanates may deteriorate the oil quality and can together with other possible hydrolytic products also have undesirable effects when rape products are used in animal feeding. Myrosinase has been found in all Brassicaceae species examined, but have also been found in other plant families, in fungi, bacteria and mammals (ref. see Iversen and Baggerud, 1980).
There have been many efforts to characterize myrosinase biochemically. The separation and characterization have mainly been carried out using myrosinase isolated from mustard seeds (Tsuruo *et al.*, 1967; Bjørkman and Janson, 1972; Ohtsuru and Hata, 1972; Pihakaski and Iversen, 1976; Palmieri *et al.*, 1986). Myrosinase from rape seeds have been studied by Lønnerdal and Janson (1973) and Kozlowska *et al.* (1983). The occurence and distribution of myrosinase at different stages of development and in various organs of rape is reported by Bones (1987).

MATERIALS AND METHODS:

Materials

Seeds of *Brassica napus* L. cv. Niklas (rape) were kindly provided by Svalöf AB, Sweden. Con A-Sepharose, PhastGels and standards for electrophoresis were obtained from Pharmacia. Sinigrin for the myrosinase assay was from Sigma. Bio-Gel 200 Fine was from BioRad.

Preparation of crude extract

A 200 g sample of rape seed was crushed in a coffee mill and homogenized with rinsed sea sand and a small volume of distilled water in a mortar. Water was added to a total volume of 1 litre, and myrosinase extracted during continous stirring for one hour. The nonsoluble material was removed by centrifugation at 17 000 g for 30 min, and the supernatant thoroughly dialyzed against distilled water. The precipitations were removed by centrifugation at 17 000 g for 30 min, and the supernatant further dialyzed against 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl. All steps were carried out at 4°C.

Affinity chromatography

The crude, dialyzed extract (740 ml) was loaded on a Con A-Sepharose column (1.5 x 14 cm) and then thoroughly washed with starting buffer (20 mM Tris-HCl, pH 7.4) until zero absorbance at 280 nm was reached. The myrosinase was then eluted from the column with starting buffer containing 0.5 M α-methyl-D-mannopyranoside. Fractions of 5.5 ml were collected, and the flow stopped for 30 min after each fraction. After 10 fractions, the flow was stopped and elution continued the next day.

Gel filtration

The active fractions were ultrafiltrated to 10 ml with a PM-10 membrane (Amicon), and applied to a column of Bio-Gel P200 fine. Flow rate was 5.3 $ml.h^{-1}$, and fractions of 2.7 ml were collected.

Electrophoresis and isoelectric focusing

Sodium dodecyl sulphate and native polyacrylamide gel electrophoresis and isoelectric focusing were performed with a PhastSystem (Pharmacia) on PhastGel 10-15, 8-25 and IEF 4-6.5 and 3-9, respectively. All gels were silver stained. For SDS-page, the samples were reduced with 2-mercaptoethanol (5%) in a 10 mM Tris-HCl buffer (pH 8.0) containing 2.5% SDS and 1 mM EDTA on a boiling water bath for 5 min.

Enzyme assay

Myrosinase activity was tested using GOD-Perid reagent to determine the amount of glucose liberated from hydrolysis of sinigrin (2-propenyl-glucosinolate). GOD-Perid contains buffer, peroxidase, glucose oxidase and a chromogen. The absorbance at 420 nm was measured in a Perkin Elmer λ 7 spectrophotometer against a blank solution prepared by mixing one sample without enzyme and one without sinigrin

Protein assay
Protein was measured with the Bio-Rad protein assay using ovalbumin as standard reference.

RESULTS AND DISCUSSION

Lønnerdal and Janson (1973) demonstrated that myrosinase from rapeseed is a glycoprotein containing 14% carbohydrate. Palmieri *et al.* (1986) recently reported that interaction between the enzyme and the plant lectin concanavallin A, could serve as basis for the purification of myrosinase from white mustard seeds. The enzyme was adsorbed to a column of concanavallin A covalently linked to agarose (Con A-Sepharose), followed by elution with different sugars. In the purification experiment reported in table 1, myrosinase from 740 ml crude extract was adsorbed to a column of Con A-Sepharose (1.5 x 14 cm). The combined fractions after elution with α-methyl-D-mannopyranoside, showed a specific activity of 2.95 $\mu mol \cdot min \cdot mg^{-1}$, which corresponds to a 15-fold purification of myrosinase. As shown in Table 1, a yield of more than 90% could be obtained when 0.5 M α-methyl-D-mannopyranoside was used as eluent.

Table 1: Purification of myrosinase.

Step	Volume (ml)	Protein (mg)	Tot. act. ($\mu mol \cdot min^{-1}$)	Spec. act. ($\mu mol \cdot min \cdot mg^{-1}$)	Purif.	Yield (%)
Crude extract	740	2491	568	0.19	1	100
Con A-Sepharose	81	177	536	2.95	15	94.5

The chromatographic profiles obtained by the present method (Figure 1), were typical of an affinity chromatography, with an initial broad peak, corresponding to non-adsorbed material, and a second narrow peak containing the myrosinase activity.

Native polyacrylamide gel electrophoresis of the active fractions, showed two bands corresponding to 150 000 and 300 000 daltons (Figure 2A), of which myrosinase probably is the smallest protein. This is somewhat larger than reported by Lønnerdal and Janson (1973), who obtained a molecular weight of 135 000.

In contrast, SDS-PAGE gave 10 bands (Figure 2B), ranging from 23 000 to 86 000 daltons. In comparision, Lønnerdal and Janson (1973) detected

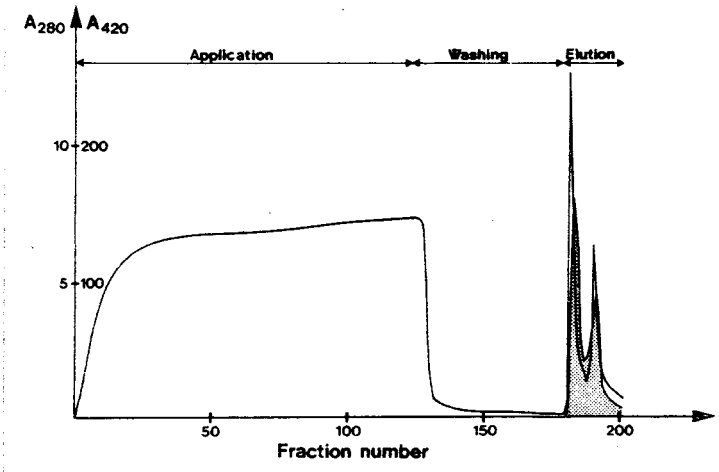


Figure 1: Separation of myrosinase on Con A-Sepharose. The enzyme was eluted as indicated (Shaded area). ——; A_{280}.

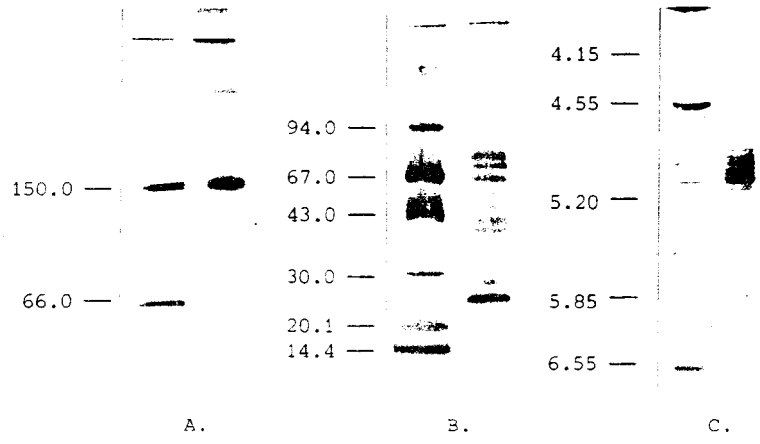


Figure 2: (A) Native and (B) SDS polyacrylamide gel electrophoresis on PhastGel gradient 8-25 and 10-15, respectively. (C) Isoelectric focusing on PhastGel 4-6.5.
1: Molecular weight and pI standards
2: Samples from the combined fractions after Con A-Sepharose purification.

eleven bands in the fractions B to D after separation on DEAE-cellulose. It should be stressed that all forms of myrosinase was adsorbed to the Con A-Sepharose.

As shown in Figure 2C, isoelectric focusing on a PhastGel IEF 4-6.5 (Pharmacia) gave 8 bands within a narrow pI range, of which three were located at the same positions as bands from highly purified myrosinase (cf. Bones and Espevik, 1987).

Gel filtration of the combined fractions on a Bio-Gel P200 fine column (1.6 x 90 cm) did not remove the main impurities from the affinity purification step. The myrosinase was eluted near the void volume, but was contaminated by protein of higher molecular weight, as shown by native polyacrylamide gel electrophoresis.

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