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STUDIES ON MYROSINASES

I. PURIFICATION AND CHARACTERIZATION OF A MYROSINASE FROM WHITE MUSTARD SEED *(SINAPIS ALBA*, L.)

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SUMMARY

Myrosinases (EC 3.2.3.1) are glucosinolate (thioglucoside) hydrolases primarily occurring in plants of the *Cruciferae* family.

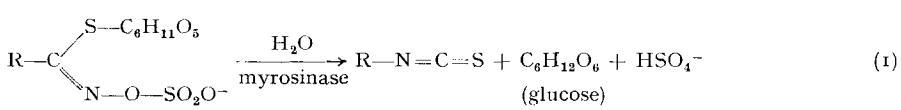
1. The separation of three myrosinase isoenzymes in *Sinapis alba* seed was achieved by DEAE-cellulose chromatography on Whatman DE-52.

2. The main myrosinase component was completely purified by DEAE-cellulose chromatography followed by gel chromatography on Sephadex G-200 and isoelectric focusing in an LKB-column.

3. The isolated myrosinase was found to be a glycoprotein with a molecular weight of 151 000, consisting of two identical polypeptide subunits with a molecular weight of 62 000 each and a carbohydrate part. The electrophoretic mobility was determined to $7.0 \cdot 10^{-4}$ cm^2/s per V and the isoelectric point was found to be pH 5.08.

INTRODUCTION

Myrosinases (thioglucoside (glucosinolate) glucohydrolase, EC 3.2.3.1) are a group of isoenzymes which catalyze the hydrolysis of glucosinolates (natural thioglucosides) to goitrogenic isothiocyanates, glucose and bisulfate, according to the following reaction:



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The glucosinolates occur in all *Cruciferae*[1,2] and seem always to be accompained by myrosinases. Chemotaxonomic studies on some *Cruciferae* by gel electrophoresis of protein extracts have demonstrated a variation in the distribution of the myrosinase isoenzymes among different plant species and parts[3,4]. Enzymes possessing myrosinase activity have also been found in fungi[5,6], bacteria[7] and mammals[8].

The myrosinase from *Brassica juncea* has been partly purified by means of ion-exchange chromatography. The separation of two myrosinase components was reported[9]. The present work comprises chromatographic separation of three myrosinase isoenzymes from *Sinapis alba* seed, purification of the main myrosinase component, and some chemical and physicochemical properties.

A study of the enzymatic properties of the three myrosinase isoenzymes will be published later.

METHODS AND MATERIALS

(I) Preparative methods

Preparation of crude extract. The enzymes were prepared from white mustard (*Sinapis alba*, L.) seed, purchased from AB Slotts Industrier, Uppsala, Sweden. 250 g of seed were crushed with a roller-mill and defatted by extraction with hexane at room temperature. The proteins were extracted at 4 °C by stirring the meal with 900 ml of 0.01 M imidazole–HCl (pH 9.0) for 10 min. The non-soluble material was removed by centrifugation. After changing the pH to 6.0 by the addition of 1 M HCl and leaving the extract overnight at 4 °C a precipitate, possessing no myrosinase activity ,was formed. After centrifuging the extract at 10 000 × *g* for 30 min, 650 ml of yellow transparent extract was obtained.

Group fractionation. The crude extract was dialyzed for two days against 4 × 5 l of 0.01 M imidazole–HCl (pH 6.0). The precipitate formed was removed by centrifugation.

The dialyzed protein solution was then fractionated by gel chromatography on a 12 cm × 130 cm column, packed with Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was equilibrated and run in 0.01 M imidazole–HCl (pH 6.0). The flow rate was about 1000 ml/h and the eluate was collected in 500-ml fractions. The fractions showing myrosinase activity were combined and concentrated to about 400 ml by ultrafiltration (Diaflo PM-10, Amicon Corp.).

Ion-exchange chromatography. Chromatographic experiments were carried out on a 3.2 cm × 7.0 cm column, packed with DEAE-cellulose powder (Whatman DE-52). The absorbent was equilibrated in 0.01 M imidazole–HCl (pH 6.0). Generally about 400 ml samples were applied. The enzymes were eluted with a linear concentration gradient of 0.01 to 0.1 M imidazole–HCl (pH 6.0) (700 + 700 ml) at a flow rate of about 50 ml/h. Fractions of 10–15 ml were collected. The column was washed with 0.1 M and regenerated with 0.01 M buffer. The fractions obtained containing myrosinase activity were combined and concentrated by ultrafiltration to a final volume of 5 ml.

Gel chromatography. Gel chromatographic experiments were carried out on a 3.2 cm × 90 cm column, packed with Sephadex G-200. The column was equilibrated and run in 0.03 M imidazole–HCl (pH 6.0). 5 ml of sample were applied to the column. The flow rate was about 15 ml/h and the eluate was collected in 5-ml fractions. The fractions containing myrosinase were combined and concentrated to about 15 ml by ultrafiltration.

Isoelectric focusing. Isoelectric focusing experiments were performed in the LKB apparatus type 8101 according to the manufacturer's instructions[10]. A glycerol density gradient was used instead of one containing sucrose. The reason is that the

acid anode solution causes inversion of the disaccharide, producing glucose which will interfere in the myrosinase assay based upon the glucose oxidase reagent. The anode solution was placed at the lower electrode and the cathode solution at the upper. The electrofocusing experiments were carried out at 10 °C for about 80 h with a final voltage of 1 kV. The column was drained from the bottom in 2-ml fractions. The pH values of the fractions were immediately measured at 10 °C with a Radiometer PM 26 pH-meter.

(II) Analytical methods

Polyacrylamide gel electrophoresis. Electrophoresis in polyacrylamide gel according to Ornstein and Davis[11] was performed in the manner described by Hjertén *et al.*[12]. Gels (0.8 cm × 10 cm) with compositions T = 6, C = 5 and T = 4, C = 2 were used. For explanations of the symbols, see ref. 12. The runs were carried out for 3 h and 2 mA per gel at pH 2.5 (0.8 M acetic acid) and pH 10.0 (0.05 M glycine–NaOH buffer) with and without 6 M urea present in the gel. The gels were stained with Amido Black B.

Isoelectric focusing in polyacrylamide gel. Isoelectric focusing experiments were carried out in 0.8 cm × 10 cm polyacrylamide gels, T = 7, C = 2.5. The gels were prepared according to Wrigley[13] by photopolymerization using a pH gradient of 4–6 or 5–8. The experiments were run at 4 °C for 16 h at 350 V.

Free zone electrophoresis. Determination of electrophoretic mobility and test for homogeneity was made by free zone electrophoresis in a rotating quartz tube according to Hjertén[14]. The electrophoresis was carried out in 0.05 M Tris–HCl (pH 8.0) at 22 °C for 96 min at a voltage of 1.2 kV producing a current of 6 mA. The electrophoretic pattern was recorded every 12 min by scanning the quartz tube with an ultraviolet-light beam.

Ultracentrifugation. Sedimentation-equilibrium experiments were performed according to Yphantis[15] modified by Chervenka[16] in 0.2 M NaCl at 12 000 rev./min. The centrifuge, a Spinco Model E, was equiped with an analytical D-rotor and interference optics.

Sedimentation-velocity measurements were made in a Spinco Model E ultracentrifuge in 0.05 M Tris-HCl buffer at 59 780 rev./min. An analytical D-rotor and a phase-plate schlieren optical system was used.

Reduction, alkylation and gel chromatography in 6 M guanidine–HCl according to Fish et al.[17,18] *modified by Rydén*[19]. About 3 mg of lyophilized enzyme was reduced under nitrogen with 10 μl of β-mercaptoethanol in 200 μl of 0.1 M Tris–HCl (pH 8.5) and 6 M guanidine–HCl. The mixture was shaken for 4 h and then 10 μl of acrylonitrile (alkylating agent) was added .After 2 h reaction the solution was applied to a 1.5 cm × 85 cm column packed with Sepharose 4B in 0.1 M Tris–HCl (pH 8.5) and 6 M guanidine–HCl. The column, which was carefully calibrated with peptides of known molecular weights, was eluted at a flow rate of 4 g/h. About 1-g fractions were collected. The effluent volume was accurately measured by weighing each fraction.

Amino acid analysis. Amino acid analyses were carried out in a Biocal automatic amino acid analyzer. 1.0 ml samples containing about 0.8 mg enzyme were hydrolyzed in 6 M HCl for 24 and 72 h. One sample was subjected to performic acid oxidation prior to hydrolysis.

Carbohydrate analyses. Hexose assay was performed by the orcinol–sulfuric acid method as described by Svennerholm[20] with mannose as a standard.

Pentose content was determined according to Dische and Shettles[21] using rhamnose as a standard.

Enzyme assay. Myrosinase activities were measured by determining the glucose released from the substrate sinigrin. The glucose was determined with a specific glucose reagent, "Glox" (AB Kabi, Stockholm, Sweden) containing glucose oxidase, peroxidase, and *o*-dianisidine. The enzyme assays were carried out according to the following procedure: 200 μl of enzyme solution were mixed with 300 μl of 0.05 M, citrate (pH 5.5) and 500 μl of 1% sinigrin in citrate buffer. After 30 min at 40 °C the reaction was stopped by heating the solution in boiling water for 5 min. After cooling, 2 ml of a "Glox" solution (1 g "Glox" in 61 ml of distilled water) was added and the mixture was kept at 40 °C for another 30 min. The absorbance at 450 nm was measured in a spectrophotometer against a blank solution prepared in a parallel run with heat-inactivated myrosinase.

Definition of myrosinase unit and specific activity. One myrosinase unit is defined as that amount of enzyme which causes the liberation of 1 μmole of glucose per min under the conditions described above. The glucose concentration is linearly proportional to absorbance values below 1.1. From a calibration curve (based on 1 ml glucose and 2 ml "Glox" solution) it was determined that an absorbance at 450 nm of 1.0 corresponds to 0.31 μmole of glucose. The specific activity of the myrosinase solutions is calculated from the following equation:

$$\text{Specific activity} = A_{450\text{ nm}} \cdot 0.31/m \cdot t \ \mu\text{mole/min per mg} \qquad (2)$$

where t is the incubation time (min) and m is the amount of protein (mg).

The "Glox" reagent has the disadvantage that it is inhibited by ascorbic acid. The assay of myrosinase in the presence of ascorbic acid may therefore be performed spectrophotometrically. This method will be presented in detail in the next paper in this series.

RESULTS AND DISCUSSION

(I) Purification procedure

Group fractionation. The crude extract was fractionated by gel chromatography on Sephadex G-50. This method gave a far better purification than dialysis or precipitation procedures with ammonium sulfate, alcohol or acetone. Another advantage over the dialysis method is the possibility of rapid large scale preparation of the enzyme. In our laboratory material from 2 kg mustard seed has been fractionated in one step on a 45 cm × 27.5 cm column[22] at room temperature.

However, when the chromatography was performed at a low temperature (4 °C) the proteins tended to precipitate in the column due to the change in ionic strength. This was overcome by a short dialysis of the extract prior to application on the column. The precipitate formed in the dialysis tubings showed no myrosinase activity and was discarded.

In the gel chromatography step (Fig. 1) the extract was separated into two major protein fractions and a low molecular weight part. The enzyme activity was associated with the high molecular weight protein fraction. In most of the separation

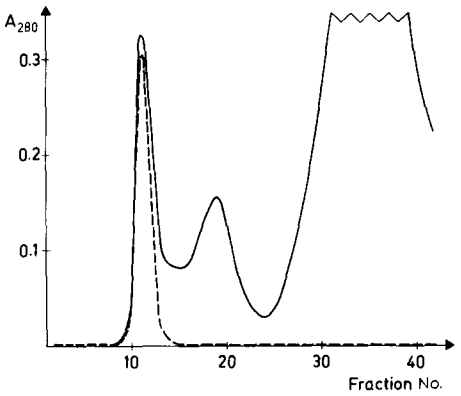

Fig. 1. Group fractionation on Sephadex G-50 of dialyzed *Sinapis alba* seed extract. ———, absorbance at 280 nm; — — —, myrosinase activity.

steps imidazole–HCl buffer was chosen as solvent. Preliminary experiments indicated that the enzymic activity is better preserved in this buffer than in other buffers tested (*e.g.* phosphate, Tris–HCl and citrate). For the same reason all the experiments were performed at 4 °C unless otherwise stated in the text. The yield and degree of purification are presented in Table I.

DEAE-cellulose chromatography. By chromatography on Whatman DE-52 the separation of three enzymes, all possessing myrosinase activity was achieved (Fig. 2). These enzymes were named myrosinase A, B, and C, and were recovered in Fractions I, II, and III, respectively. Component A passed through the column without retention in 0.01 M buffer together with the main part of the inactive material. After rechromatography of this fraction, the specific activity was 1.5 μmoles/min per mg. Components B and C were adsorbed to the exchanger eluting at the buffer concentrations 0.022 and 0.034 M, respectively. In this step the specific activity of Component C, forming the major myrosinase component, was highly increased. Rechromato-

TABLE I

PURIFICATION OF MYROSINASE (250 g white mustard seed)

Step	*Volume (ml)*	*Protein (mg)*	*Total act. (μmoles/min)*	*Spec. act. (μmoles/min per mg)*	*Purification*	*Yield (%)*
Dialysis	637	12700	2800	0.22		
Sephadex G-50	1140	685	3020	4.4	1	100
DEAE-cellulose 1						
Myrosinase A	370	203	300*	1.5*		
Myrosinase B	180	72	280**	3.9**		
Myrosinase C	220	59	2460	42	9.6	82
DEAE-cellulose 2						
Myrosinase C	189	38	1900	50	11.4	63
Sephadex G-200						
Myrosinase C	35	20	1100	55	12.5	36
Isoelectric focusing						
Myrosinase C	12	10	610	60	13.6	20

* The enzyme assay was carried out at pH 4.25 (citrate).
** The enzyme assay was carried out at pH 5.13 (citrate).

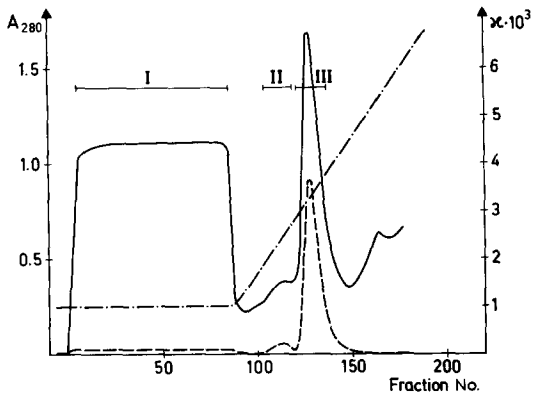

Fig. 2. DEAE-cellulose chromatography on Whatman DE-52 of *Sinapis alba* proteins, after group fractionation on Sephadex G-50. Fractions I, II and III contain myrosinase A, B and C, respectively. ———, absorbance at 280 nm; — — —, myrosinase activity; — · —, conductivity.

graphy of Fraction III gave a single peak in the same position, with a specific activity of 50 μmoles/min per mg. Analytical polyacrylamide gel electrophoresis at pH 2.5 in 6 M urea showed two strong and a few weak bands. Fraction II, having a specific activity of 3.9 μmoles/min per mg contained at least 6 proteins, including myrosinase B.

Comparative gel electrophoresis runs on Components A, B and C were performed at pH 2.5 (no urea present). The enzymes were detected according to the method described by MacGibbon and Allison[4]. After removing the gels from their tubes, they were treated with a solution of sinigrin, $BaCl_2$ and acetic acid. Within 30 min white bands of insoluble $BaSO_4$ were formed indicating the position of the enzymes. It was thereby found that the three enzymes migrated at a slightly different velocity. Co-electrophoresis of myrosinase A, B and C showed three distinct bands.

The following steps comprise the isolation of myrosinase C and some further purification of Components A and B.

Gel chromatography. After dialysis against 0.03 M buffer the concentrated Fractions I, II and III containing myrosinase A, B and C, respectively, from the ion-exchange chromatography step were further fractionated on a Sephadex G-200 column. The chromatographic patterns are shown in Fig. 3. This step gave about 3-fold purification of myrosinase A, which was found in the high molecular peak at a K_{av} value of 0.29 ± 0.005. Fraction II, containing myrosinase B, gave an asymmetric protein peak, having the enzyme activity displaced to the rear part at aK_{av} value of 0.28 ± 0.005. Myrosinase C eluting at K_{av} 0.27 ± 0.003 was purified from some minor protein material. Polyacrylamide gel electrophoresis of this peak showed that only the two strong bands still remained. The yields and specific activities are shown in Table I.

Isoelectric focusing. Electrophoretic molecular sieving in polyacrylamide gels demonstrated that the two proteins in the myrosinase C peak had a slight difference in charge. Separation by preparative zone electrophoresis on cellulose in 0.05 M Tris–HCl buffer at pH 8.0 was, however, unsuccessful. Therefore, preparative isoelectric focusing in a 110 ml LKB-column with a carrier pH gradient 4.5–5.0 (Am-

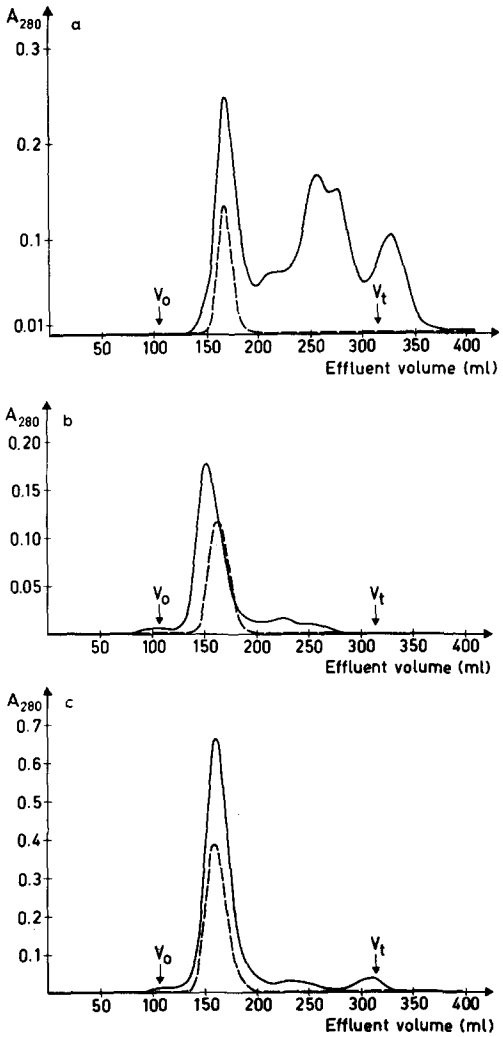

Fig. 3. Gel chromatography on Sephadex G-200 of Fractions I, II and III (a, b and c, respectively) from DEAE-cellulose chromatography. Void volume (V_0) and total volume (V_t) are indicated. ———, absorbance at 280 nm; — — —, myrosinase activity.

pholine type 8163) was attempted. As is shown in Fig. 4c the enzyme activity was located in the protein peak with an isoelectric point at pH 5.08. (The same isoelectric point was obtained in a pH-gradient 4–6 and 5–7.) Polyacrylamide gel electrophoresis of this material now showed a single band. The missing band was found in the material focusing at about pH 6. The irregularities in the base line, as well as the material outside the pH gradient were found to be due to ultraviolet absorption of the carrier ampholytes and the electrolytes. The electrofocusing behaviour of myrosinase A and B at pH 5–7 (Ampholine type 8158) is shown in Fig. 4a and 4b, respectively. Runs performed in pH-gradient 4–6 gave exactly the same isoelectric points

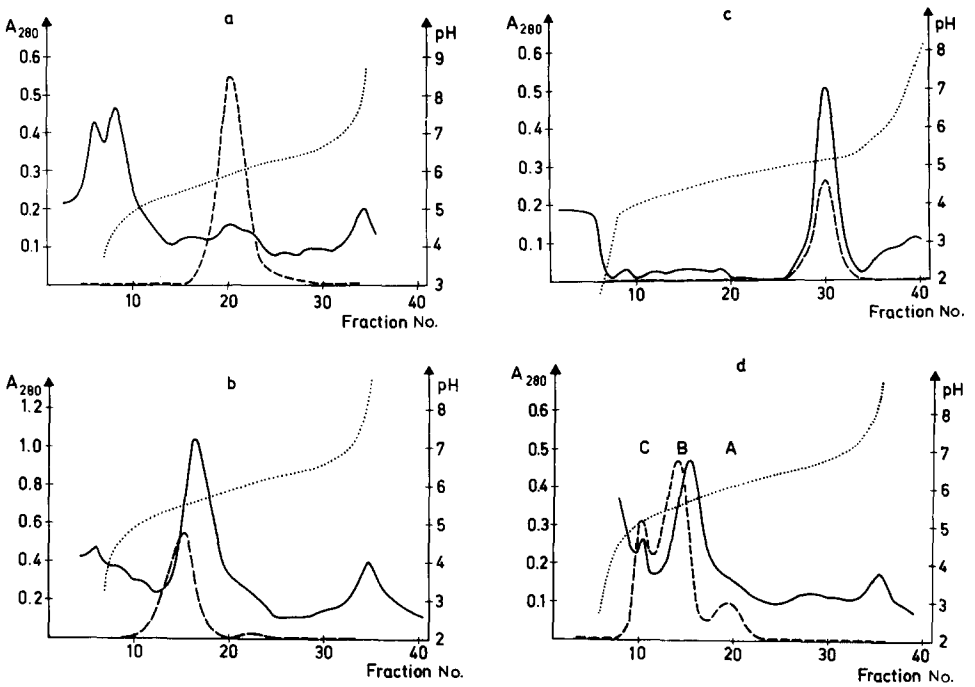

Fig. 4. Preparative isoelectric focusing of myrosinase containing fractions after Sephadex G-200 chromatography. a. Myrosinase A containing material. Carrier ampholytes pH 5–7. b. Myrosinase B containing material. Carrier ampholytes pH 5–7. c. Myrosinase C containing material. Carrier ampholytes pH 4.5–5.0. d. An artificial mixture of material containing myrosinase A, B and C. Carrier ampholytes pH 5–7. ———, absorbance at 280 nm; — — —, myrosinase activity; ·····, pH.

of the enzymes. Co-electrofocusings of myrosinases A, B and C (Fig. 4d) also support the existance of three myrosinase isoenzymes, having different isoelectric points.

(II) Criteria of homogeneity (myrosinase C)

Electrophoresis in polyacrylamide gel at pH 2.5 and 10.0 of the isolated myrosinase C indicates a homogenous protein.

Isoelectric focusing in polyacrylamide gel with a carrier gradient of pH 4–6 of the enzyme also shows a single band.

Free zone electrophoresis according to Hjertén[14] is also a sensitive method for testing homogeneity. A single peak was registrated during the entire run.

Ultracentrifugation. The linear relation between log fringe displacement and r^2 obtained from the sedimentation-equilibrium experiments (Fig. 5) is another evidence of a pure protein.

(III) Physicochemical and chemical characterization (myrosinase C)

Molecular weight determination. In the preparative molecular sieving experiments on Sephadex G-200 myrosinase C was eluted at a K_{av} value of 0.27 (Fig. 3c). For globular proteins this value corresponds to a molecular weight of about 150 000. By sedimentation-equilibrium centrifugation (Fig. 5) the molecular weight was

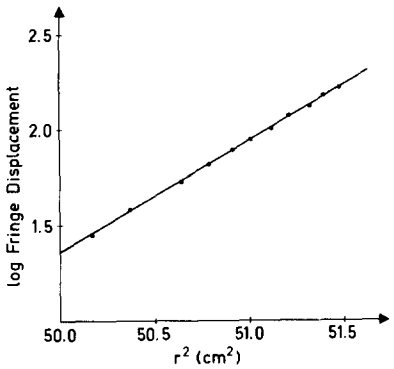

Fig. 5. Sedimentation-equilibrium centrifugation of myrosinase C. The concentration, expressed as log fringe displacement, was measured as a function of the square of distance from the center of rotation, r^2, at a speed of 12 000 rev./min and a temperature of 20 °C. The partial specific volume was 0.72 and the density of the medium was 1.0065 g/ml.

determined to be 151 000. The Svedberg constant of the enzyme was determined to be 7.41 S by sedimentation-velocity studies.

Amino acid analysis. The amino acid pattern of myrosinase C is shown in Table II. Glucosamine was the only amino sugar detected. The total amino acid content of the sample was found to be 0.693 mg/ml and the glucosamine content 0.033 mg/ml. The absorbance of the sample solution was 1.26 at 278 nm (1 cm).

Carbohydrate analysis. In order to establish whether the enzyme was a glycoprotein, the carbohydrate content was determined. The hexose and pentose contents

TABLE II

AMINO ACID COMPOSITION OF MYROSINASE C

The values are expressed as residues per 100 residues. The total number of amino acid residues per subunit was calculated to be 550–560.

Amino acid	
Tryptophan*	2.05
Lysine	6.60 ± 0.01
Histidine	2.48 ± 0.00
Arginine	3.91 ± 0.01
Cysteic acid**	1.62
Aspartic acid + amide	13.80 ± 0.07
Threonine	5.69
Serine	6.72
Glutamic acid + amide	7.85 ± 0.01
Proline	5.08 ± 0.09
Glycine	8.32 ± 0.03
Alanine	4.22 ± 0.04
Valine	4.42
Methionine	1.42 ± 0.02
Isoleucine	6.34
Leucine	7.25 ± 0.00
Tyrosine	7.15 ± 0.04
Phenylalanine	5.07 ± 0.05

* The tryptophan value was determined spectrophotometrically in 0.1 M NaOH.
** The cysteic acid value was determined after performic acid oxidation of the sample.

of a 1 ml sample solution ($A_{278\ nm} = 1.26$) were found to be approximately 100 μg and 16 μg, respectively. From these results the extinction of a 1% myrosinase solution was calculated to be 15 at 278 nm. The total composition of myrosinase C would thus be: protein 82, glucosamine 4, hexose 12 and pentose 2%.

Micro-Kjeldahl analysis. The nitrogen content of moisture-free enzyme was determined by micro-Kjeldahl analysis to about 12.5%. This value is in fair accordance with the value calculated from the amino acid and carbohydrate analyses.

Quaternary structure (subunits). Chromatography of the reduced and alkylated enzyme on Sepharose 4B in guanidine–HCl gave a single protein peak at a K_d value of 0.308. From a calibration curve the number of amino acid residues was estimated to be 592 $\pm$ 36, which corresponds to a molecular weight of 66 000 $\pm$ 4000. This indicates that the myrosinase molecule is composed of either two equivalent or two rather similar polypeptide chains and a non-protein component. Analytical polyacrylamide gel electrophoresis in 6 M urea, pH 2.5 of the reduced and alkylated enzyme from the Sepharose 4B run showed only one sharp band, having a migration velocity different from that of the non-treated enzyme. This supports the theory of two identical peptide chains.

From the amino acid and carbohydrate analyses the protein part of the enzyme makes 82% of the total weight. Since the total molecular weight is 151 000 the two polypeptide entities should have a molecular weight of approximately 62 000 each. The number of amino acid residues per subunit was calculated to be 550–560. The somewhat higher value obtained from the Sepharose 4B run is probably due to influence from the carbohydrate part.

Electrophoretic mobility. The electrophoretic mobility of the enzyme was determined by free zone electrophoresis according to Hjertén[14] to be $7.0 \cdot 10^{-4}$ cm^2/s per V (in 0.05 M Tris–HCl, pH 8.0, at 6 mA).

Isoelectric point. From several preparative isoelectric focusing experiments of 5 different preparations the isoelectric point of myrosinase C was determined to be pH 5.08 $\pm$ 0.02. Fig. 4c shows a typical elution diagram.

(IV) Some properties of myrosinases A and B

No attempts have yet been made to completely purify the myrosinases A and B. They were, however, partly purified by the methods used for the isolation experiments on myrosinase C.

Molecular weights. From the elution positions in the molecular sieving experiments on Sephadex G-200 the molecular weights of myrosinases A and B were estimated to be slightly lower than that of myrosinase C. The K_{av} values presented are the mean values from 5 runs on each myrosine. However, the differences in K_{av} between the three myrosinases are small, and might be within experimental errors.

Isoelectric points. From preparative isoelectric focusing experiments the isoelectric points of myrosinase A and B were determined to be pH 5.9 and 5.45 respectively (Fig. 4a and 4b).

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