Biochimica et Biophysica Acta, 315 (1973) 421–429

BBA 66971

STUDIES ON MYROSINASES

II. PURIFICATION AND CHARACTERIZATION OF A MYROSINASE FROM RAPESEED *(BRASSICA NAPUS* L.)

BO LÖNNERDAL AND JAN-CHRISTER JANSON
Institute of Biochemistry, University of Uppsala, Box 531, S-751 21 Uppsala (Sweden)

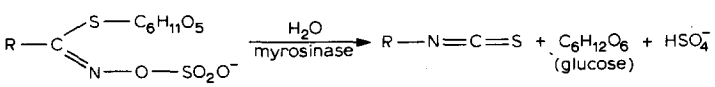
(Received February 21st, 1973)

SUMMARY

Myrosinases (EC 3.2.3.1) are a group of isoenzymes present in Cruciferaes which catalyze the hydrolysis of glucosinolates. The myrosinases from rapeseed (*Brassica napus* L.) have been purified and the main component isolated and characterized. It is a glycoprotein with a molecular weight of 135 000 which consists of two peptide chains with a molecular weight of 65 000 and contains about 14% carbohydrate. It exists in 3 forms with different carbohydrate compositions and the isoelectric points are 4.96, 4.99 and 5.06, respectively. This work describes the isolation and physicochemical characterization and makes a comparison between the myrosinases from rapeseed and white mustard (*Sinapis alba* L.).

INTRODUCTION

Myrosinases [thioglucoside (glucosinolate) glucohydrolase, EC 3.2.3.1] are a group of isoenzymes which catalyze the hydrolysis of glucosinolates to goitrogenic isothiocyanates, glucose and bisulfate:



The enzymes have been found in plants[1–4], fungi[5,6], bacteria[7] and mammals[8].

The myrosinases from white mustard (*Sinapis alba* L.) have been isolated and characterized by Björkman and Janson[9], who showed the existence of a number of isoenzymes, also found by Henderson and McEwen[10]. The separation of 4 myrosinase isoenzymes and the isolation and characterization of the main myrosinase component in rapeseed is described here. A comparative study on the enzymatic properties of the myrosinases from rapeseed and white mustard is to be published.

MATERIALS AND METHODS

Preparative methods

Preparation of crude extract. Seed from winter rape (cv. Panter) was obtained from AB Karlshamns Oljefabriker, Karlshamn, Sweden. The seeds were crushed in a roller mill and defatted with *n*-hexane, 2.5 kg seeds giving approximately 1.2 kg defatted meal. The enzymes were extracted as described earlier[9], but with 10 l 0.02 M imidazole–HCl buffer (pH 6.0).

Group fractionation. The proteins were separated from low molecular weight substances on a column packed with Sephadex G-50 fine (45.0 cm × 30.0 cm) (AB Pharmacia, Uppsala, Sweden) equilibrated with 0.02 M imidazole–HCl buffer (pH 6.0). The flow-rate was adjusted to 6 l/h and the fraction volume was 1 l. A sample volume of 6.5 l was applied.

Ion-exchange chromatography. The myrosinase isoenzymes were separated by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52). The column (12.0 cm × 8.0 cm) was equilibrated with 0.02 M imidazole–HCl buffer (pH 6.0). The size of the sample was 16 l (two combined G-50 runs) and the flow rate 600 ml/h. The sample was followed by 2 column volumes of starting buffer. The adsorbed proteins were displaced by a linear ionic strength gradient, made from 10 l 0.02 M (mixing chamber) and 10 l 0.1 M (reservoir) imidazole–HCl buffer (pH 6.0). The final separation of the myrosinase isoenzymes was performed on a smaller DE-52 column (3.2 cm × 10.0 cm) using a gradient made from 1500 ml 0.02 M and 1500 ml 0.07 M imidazole buffers.

Molecular-sieve chromatography. The main myrosinase peak from the ion-exchange chromatography experiment was concentrated in an ultrafiltration cell with a PM-10 membrane (Amicon Corp.) to 100 ml and applied to a column of Sepharose 6B (AB Pharmacia, Uppsala, Sweden). The agarose gel was cross-linked with divinylsulfone (Porath *et al.*, unpublished) to improve flow characteristics and separation range. The column (7.0 cm × 95.0 cm) was equilibrated with 0.02 M imidazole–HCl buffer (pH 6.0) at a flow-rate of 125 ml/h. The sample volume was 90 ml and the fraction volume 18 ml.

Isoelectric focusing. The method described by Vesterberg and Svensson[11] was performed on the 110-ml LKB-column (LKB Produkter, Stockholm, Sweden), with carrier ampholytes in the pH range 4–6 and 4.78–5.12. The density gradient was formed from glycerol (sucrose is not good in this case since the glucose produced by inversion at the acid anode disturbs the enzyme assay used). The ampholytes in the pH range 4.78–5.12 were prepared from pH 4–6 ampholytes in a pre-run without sample on a 440-ml LKB column. Experimental data have been given earlier[9].

Analytical methods

Electrophoresis and isoelectric focusing in polyacrylamide gels. The electrophoretic homogeneity of the preparations was tested by polyacrylamide gel electrophoresis as described by Hjertén *et al.*[12]. The concentration of acrylamide was 6% and of bisacrylamide 5% of the total acrylamide concentration (T_6C_5).

Analytical isoelectric focusings were performed in the same gel as above in the pH range 3–10 according to Wrigley[13], using photopolymerization. The gel size and experimental data have been described[9].

Free zone electrophoresis. Free zone electrophoresis was carried out according to Hjertén[14] in a rotating quartz tube which was scanned with a ultraviolet detector every 6 min. The voltage was 1100 V and the current 5 mA. The protein concentration of the sample was 1 mg/ml and the buffer used was 0.05 M Tris–HCl (pH 8.0). The sample volume was 7 μl and the temperature 17 °C.

Ultracentrifugation. Sedimentation-velocity measurements were made in a Spinco Model E in 0.05 M Tris–HCl buffer (pH 8.0) at 52 000 rev./min. An analytical D-rotor and a phase-plate schlieren optical system were used.

Sedimentation-equilibrium experiments were performed according to Yphantis[15] modified by Chervenka[16] in 0.2 M NaCl at 18 000 rev./min. The centrifuge, a Spinco Model E ultracentrifuge, was equipped with an analytical D-rotor and interference optics.

The partial specific volume was calculated from the amino acid and carbohydrate composition according to the methods of Cohn and Edsall[17] and Gibbons[18].

Reduction, alkylation and molecular-sieve chromatography in 6 M guanidine hydrochloride according to Fish et al.[19,20]. The sample [3 mg protein in 250 μl 0.1 M Tris–HCl buffer (pH 8.5) containing 6 M guanidine hydrochloride] was reduced with β-mercaptoethanol under N_2 for 4 h. The alkylating agent, acrylonitrile (10 μl), was added and the reaction was complete after 2 h. The reaction mixture was run on a Sepharose 6B (AB Pharmacia, Uppsala, Sweden) column (1.5 cm × 84.5 cm) in 6 M guanidine·HCl, 0.1 M Tris–HCl buffer (pH 8.5). The tubes were weighed before and after the fractions were collected. The flow-rate was 4 g/h and the fraction weight was about 1 g. The column was calibrated as described by Rydén[21]. This method gives a measure of the peptide chain length under reducing and dissociating conditions. To find out whether the molecular weights of the enzymes in dilute salt solutions correspond to this value or were multiples thereof, the proteins were analyzed by molecular sieving on Sephadex G-200.

Molecular-sieve chromatography. The molecular weight of the enzymes was estimated by molecular-sieve chromatography on a Sephadex G-200 (AB Pharmacia, Uppsala, Sweden) column (3.2 cm × 90.0 cm). The sample volume was 5 ml and the flow-rate was 15 ml/h. The column was calibrated with blue dextran, human serum albumin (dimer and monomer), ribonuclease and glucose.

Amino acid analysis. The sample was hydrolyzed in 6 M HCl at 110 °C for 24 and 72 h. The hydrolyzates were analyzed with a Biocal-200 analyzer equipped with CRS-12 integrators. Total half-cystine and cysteine was determined after performic acid oxidation. The tryptophan content was estimated spectrophotometrically in 0.1 M NaOH (refs 22 and 23).

Carbohydrate analysis. Hexose assay was performed by the orcinol–H_2SO_4 method as described by Svennerholm[24] with mannose as standard. Pentose content was determined according to Dische and Shettles[25] using rhamnose as standard.

Gas chromatography. Analysis of the carbohydrates in the isoenzymes was performed on a Pye-104 gas chromatograph with a 3.8% SE-30 column and N_2 as carrier gas (50 ml/h). A temperature gradient from 120 to 220 °C (1°/min) was used. Mannitol was added as internal standard. Before application to the column the sample was lyophilized followed by methanolysis and acetylation with acetic anhydride. Silylation was carried out with pyridine–HMDS (hexamethyldisilizane)–TMCS (trimethylchlorosilane) (5:1:1, by vol.).

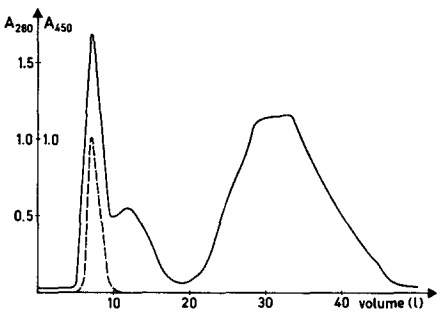
Enzyme assay. The enzymatic activity was determined by the release of glucose from the substrate sinigrin (Koch-Light, Bucks., England), with the glucose reagent "Glox" (AB Kabi, Stockholm, Sweden), as described by Björkman and Janson[9]. The pH in the buffer used was 4.25, at which the rapeseed myrosinases have their maximum activity.

Definition of myrosinase unit and specific activity. The definitions according to Björkman and Janson[9] were used.

RESULTS AND DISCUSSION

Purification procedure

Group fractionation. To minimize the interaction between myrosinase and the glucosinolates, the dry defatted meal was extracted with ice-cold buffer. Molecular-sieve chromatography on Sephadex G-50 gave 3 peaks (Fig. 1). The first peak contained high molecular weight proteins, the second low molecular weight proteins (isolated and characterized by Lönnerdal and Janson[26]) and the third peak low molecular weight non-protein substances (among them the glucosinolates). The first peak contained all myrosinase activity. The buffer used, imidazole–HCl, has been shown to have a stabilizing effect on the enzymes[9].



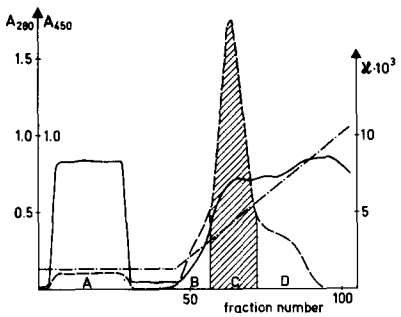


Fig. 1. Molecular-sieve chromatography of crude extract from defatted rapeseed meal on Sephadex G-50 in 0.02 M imidazole–HCl buffer (pH 6.0). ———, protein concentration (A_{280}). — — —, myrosinase activity (A_{450}).

Fig. 2. Ion-exchange chromatography of the myrosinase fraction from Fig. 1 on DEAE-cellulose (Whatman DE-52) in 0.02 M imidazole–HCl buffer (pH 6.0). Column volume: 905 ml. Linear ionic strength gradient: 10 l 0.02 M–10 l 0.1 M. ———, protein concentration (A_{280}). — — —, myrosinase activity (A_{450}). —·—·—, conductivity (κ).

Ion-exchange chromatography. Fig. 2 shows a chromatographic profile which is representative of the large-scale DEAE-cellulose step. Part of the myrosinase activity (18%) passed through the column and this material was not adsorbed when re-run on a fresh column. This myrosinase fraction was called A. The enzymes eluted with the ionic-strength gradient were dialyzed and re-run on a smaller column using a less steep gradient to improve the separation (Fig. 3). The enzyme fractions were named B, C and D of which Fraction C is the largest. Polyacrylamide gel electrophoretic analysis of Fraction A showed 8–10 bands, Fraction B 3 bands, Fraction C 2 bands and Fraction D 4 bands. The enzymes were detected with the method de-

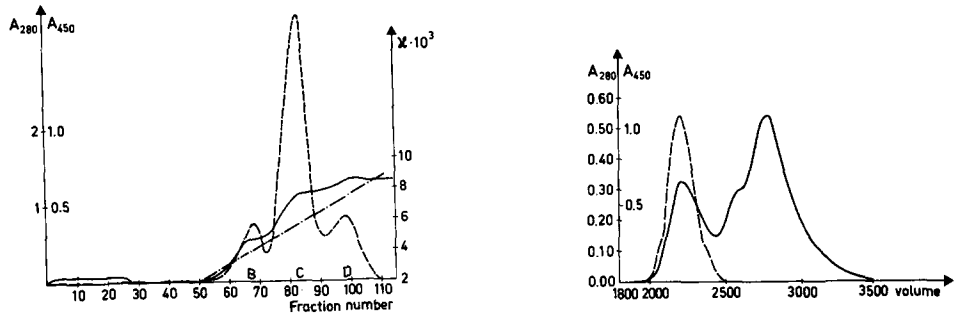


Fig. 3. Ion-exchange chromatography of myrosinases B, C and D from Fig. 2 on DEAE-cellulose (Whatman DE-52) in 0.02 M imidazole–HCl buffer (pH 6.0). Column volume: 80 ml. Linear ionic strength gradient: 1500 ml 0.02 M–1500 ml 0.07 M. ———, protein concentration (A_{280}). — — —, myrosinase activity (A_{450}). —·—·—, conductivity (κ).

Fig. 4. Molecular-sieve chromatography of myrosinase C on Sepharose 6B (cross-linked with divinylsulfone) in 0.02 M imidazole–HCl buffer (pH 6.0). Column volume: 3465 ml. ———, protein concentration (A_{280}). — — —, myrosinase activity (A_{450}).

scribed by MacGibbon and Allison[4], using sinigrin, $BaCl_2$ and acetic acid. Fractions A, B, C and D showed 1 $BaSO_4$ band each while coelectrophoresis gave 4 bands. This shows that Fractions A, B and D are isoenzymes but they are not as pure as Fraction C.

Molecular-sieve chromatography. The enzyme Fraction C was run on a column of cross-linked Sepharose 6B (this gel had better separation properties than Sephadex G-200 in this case). 3 peaks were obtained of which the first one eluted contained all the activity (Fig. 4).

Isoelectric focusing. Isoelectric focusing of the myrosinase C was performed in a pH gradient from 4.78 to 5.12 (Fig. 5). 3 active peaks called C_1, C_2 and C_3 were obtained, containing about equal amounts of protein.

Purification and recovery data. Data giving the percentage of enzymatic activity recovered and the degree of purification in the individual steps of this purification procedure, and for the procedure as a whole, is recorded in Table I.

Carbohydrate analysis. Carbohydrate analysis of myrosinase C from the second

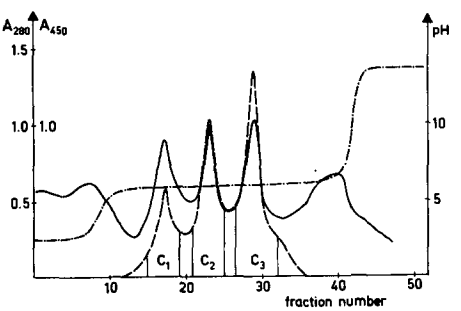


Fig. 5. Isoelectric focusing of myrosinase C in the pH range 4.78–5.12. The experiment was performed in a 110-ml LKB column. ———, protein concentration (A_{280}). — — —, myrosinase activity (A_{450}). —·—·—, pH.

TABLE I

PURIFICATION OF MYROSINASE (4000 g SEED)

Step	*Volume (ml)*	*Protein (mg)*	*Total act. (μmoles/ min)*	*Spec. act. (μmoles/ min per g)*	*Purification*	*Yield (%)*
Sephadex G-50	16 000	27 500	9320	0.34	1	100
DEAE-cellulose I						
Myrosinase A	16 000	15 500	1570	0.10		
Myrosinase						
B+C+D	10 200	7 400	7600	1.03	3.1	82
DEAE-cellulose II						
Myrosinase C	930	620	3410	5.50	16	37
Sepharose 6B						
Myrosinase C	90	121	2760	22.8	67	30
Isoelectric focusing						
Myrosinase						
$C_1+C_2+C_3$	40	24	1540	64.0	188	16

DEAE-cellulose chromatography step showed a hexose and pentose content of 12 and 2%, respectively.

Tests of homogeneity (myrosinase C)

The tests were made on myrosinase C from the molecular sieving on Sepharose 6B (Fig. 4).

Electrophoresis. Electrophoresis in polyacrylamide gel at pH 2.5 gave 1 sharp band and free-zone electrophoresis gave 1 single symmetrical peak during the whole run. This indicates a homogeneous protein.

Ultracentrifugation. The linear relationship between log fringe replacement and r^2 obtained from the sedimentation-equilibrium experiments (Fig. 6) is also evidence of a pure protein.

Isoelectric focusing. Isoelectric focusing in polyacrylamide gel in the pH range 3–10 gave 1 broad band. The material was therefore subjected to isoelectric focusing in a column using a very narrow pH gradient (4.78–5.12), which is described in the analytical part.

Although conventional methods for homogeneity tests indicated a pure enzyme

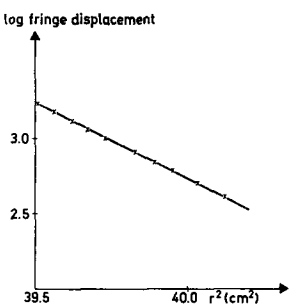


Fig. 6. Sedimentation-equilibrium centrifugation of myrosinase C. The concentration, expressed as log fringe displacement, was measured as a function of the square of distance from the center of rotation, r^2, at a speed of 18 000 rev./min.

we were able to separate 3 individual isoenzymes by isoelectric focusing. The 3 isoenzymes were designated C_1, C_2 and C_3. The difference between these three is very small in contrast to the isoenzymes A, B and D.

Physicochemical and chemical characterization (myrosinase C)

Molecular weight determination. The molecular weight was estimated from a Sephadex G-200 run. The K_{av} value was 0.30 ± 0.005 which corresponds to a molecular weight of 135 000 ± 10 000. The molecular weight calculated from ultracentrifugation data and with a partial specific volume of 0.712 was 133 000.

Subunit structure. The reduced and alkylated myrosinase C was analyzed by molecular sieving on a calibrated Sepharose 6B column in 6 M guanidine·HCl. The enzyme gave only 1 peak with a K_{av} value of 0.117, consistent with a polypeptide of 588 ± 36 amino acid residues. This suggests a peptide chain of 65 000 ± 4000. Since polyacrylamide gel electrophoresis of the reduced and alkylated enzyme gave only 1 band, the enzyme probably consists of 2 identical chains giving a molecular weight of about 130 000. To this value the contribution from the carbohydrate part must be added, which depends on its localization in the chain. The total molecular weight with respect to all the mentioned methods will be around 135 000.

Carbohydrate analysis. As myrosinase C was shown to be homogeneous by the methods used and as the differences in isoelectric points between the three varieties C_1, C_2 and C_3 are very small, the latter disparity could not be due to differences in the content of charged amino acids for example, but possibly to differences in carbohydrate content. Table II shows the results of a gas chromatographic analysis of the carbohydrates in myrosinases C_1–C_3. The differences are significant and would probably cause the slight shifts observed in the isoelectric point.

TABLE II

COMPOSITION OF THE CARBOHYDRATE CONTENT IN THE MYROSINASES C_1, C_2, C_3 (EXPRESSED IN NMOLES/mg PROTEIN)

Carbohydrate	C_1	C_2	C_3
Fucose	24	90	263
Mannose	348	602	522
Galactose	22	—	—
N-Acetylglucosamine	63	114	136
Glucose	61	44	50
Total carbohydrate (%)	9.3	15.2	17.4

Amino acid analysis. The results of the amino acid analyses are shown in Table III. The only amino sugar detected with this method was glucosamine (the acetyl group in *N*-acetylglucosamine is split off by the hydrolysis).

Electrophoretic mobility. The electrophoretic mobility determined by means of free zone electrophoresis was $7.0 \cdot 10^{-5}$ cm$^2 \cdot$s$^{-1} \cdot V^{-1}$ in 0.05 M Tris–HCl buffer (pH 8.0) at 17 °C.

Isoelectric point. The isoelectric points for C_1, C_2 and C_3 were 4.96 ± 0.01, 4.99 ± 0.01 and 5.06 ± 0.01 (2 determinations), respectively. The values obtained for myrosinases A, B and D were 6.2, 5.6 and 4.9, respectively, analyzed in the pH

TABLE III

AMINO ACID COMPOSITION OF MYROSINASE C

The values are expressed as residues per 100 residues. The total number of amino acids was calculated to be 580–590

Tryptophan*	1.79
Lysine	4.86 ± 0.03
Histidine	2.39 ± 0.02
Arginine	3.30 ± 0.00
Cysteic acid**	1.66
Aspartic acid + amide	12.50 ± 0.00
Threonine	5.79
Serine	8.57
Glutamic acid + amide	8.17 ± 0.08
Proline	6.27 ± 0.04
Glycine	9.31 ± 0.00
Alanine	6.16 ± 0.00
Valine	4.98
Methionine	1.81 ± 0.00
Isoleucine	5.44
Leucine	7.10 ± 0.02
Tyrosine	5.88 ± 0.02
Phenylalanine	4.95 ± 0.00

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

range 4–6. (These enzymes were not pure but their isoelectric points were determined with the enzyme assay used.)

Comparison between the myrosinases from rapeseed and white mustard[9]

The physicochemical and chemical data are similar for both species; the enzymes are apparently very much alike. The carbohydrate content is somewhat different and so are the isoelectric points (Table IV). The total amount of myrosinase in rapeseed is only one-fifteenth of that in white mustard. The number of different

TABLE IV

COMPARISON BETWEEN THE MYROSINASES FROM RAPESEED AND WHITE MUSTARD

	Rapeseed (B. napus)	*White mustard (S. alba)*
Molecular weight		
Whole enzyme	135 000	151 000
Peptide chain	65 000	62 000
Isoelectric point		
Myrosinase A	6.2	5.9
Myrosinase B	5.6	5.45
Myrosinase C	4.96, 4.99 5.06	5.08
Myrosinase D	4.9	
Carbohydrate content (%)		
Myrosinase C	14	18
Specific activity of the pure enzyme C	64	60
Purification	188	13.6

isoenzymes for each species may be due to genetic differences. A comparison of enzymatic properties will be published soon.

ACKNOWLEDGMENTS

We are very grateful to our teacher, Professor Jerker Porath, for his interest and valuable support, to Dr Rune Björkman for frequent discussions and to Mrs Lise-Britt Wahlberg for skilful technical assistance. We also owe thanks to Professor Stellan Hjertén and Mrs Irja Blomqvist for the free zone electrophoresis runs, to Dr David Eaker for performing the amino acid analyses, to Mr Anders Bonnevier for the gas-chromatographic analyses of the carbohydrates and to Dr Håkan Pertoft for the ultracentrifugation analyses. Financial support has been given by the Swedish Board for Technical Development, AB Karlshamns Oljefabriker, Karlshamn, Sweden and Alfa Laval AB, Tumba, Sweden and is gratefully acknowledged.

REFERENCES

1 Kjaer, A. (1960) *Prog. Chem. Org. Nat. Prod.* 18, 122–176
2 Van Etten, C. H., Daxenbichler, M. E. and Wolff, J. A. (1969) *J. Agric. Food Chem.* 17, 483–491
3 Vaughan, J. G., Gordon, E. and Robinson, D. (1968) *Phytochemistry* 7, 1345–1348
4 MacGibbon, D. B. and Allison, R. M. (1970) *Phytochemistry* 9, 541–544
5 Reese, E. T., Clapp, R. C. and Mandels, M. (1958) *Arch. Biochem. Biophys.* 75, 228–242
6 Ohtsuru, M., Tsuruo, I. and Hata, T. (1969) *Agric. Biol. Chem.* 33, 1309–1314
7 Oginsky, E. L., Stein, A. E. and Greer, M. A. (1965) *Proc. Soc. Exp. Biol. Med.* 119, 360–364
8 Goodman, I., Fouts, J. R., Bresnick, E., Menegas, R. and Hitchings, G. H. (1959) *Science* 130, 450–451
9 Björkman, R. and Janson, J.-C. (1972) *Biochim. Biophys. Acta* 276, 508–518
10 Henderson, H. M. and McEwen, T. J. (1972) *Phytochemistry* 11, 3127–3133
11 Vesterberg, O. and Svensson, H. (1966) *Acta Chem. Scand.* 20, 820–834
12 Hjertén, S., Jerstedt, S. and Tiselius, A. (1965) *Anal. Biochem.* 11, 219–223
13 Wrigley, C. (1968) *Sci. Tools* 15:2, 17-23
14 Hjertén, S. (1967) *Chromatogr. Rev.* 9, 122–219
15 Yphantis, D. (1964) *Biochemistry* 3, 297–317
16 Chervenka, C. (1970) *Anal. Biochem.* 34, 24–29
17 Cohn, E. J. and Edsall, J. T. (1952) *Proteins, Aminoacids and Peptides as Dipolar Ions*, pp. 303–381, Reinhold Publishing Corp., New York
18 Gibbons, R. A. (1966) in *Glycoproteins* (Gottschalk, A., ed.), pp. 29–95, Elsevier, Amsterdam
19 Fish, W. W., Mann, K. G. and Tanford, C. (1969) *J. Biol. Chem.* 244, 4989–4994
20 Fish, W. W. (1971) *J. Agric. Food Chem.* 19, 660–664
21 Rydén, L. (1972) *Eur. J. Biochem.* 26, 380–386
22 Bencze, W. L. and Schmid, K. (1957) *Anal. Chem.* 29, 1193–1196
23 Goodwin, T. W. and Morton, R. A. (1946) *Biochem. J.* 40, 628–632
24 Svennerholm, L. (1956) *J. Neurochem.* 1, 42–53
25 Dische, Z. and Shettles, L. B. (1948) *J. Biol. Chem.* 175, 595–603
26 Lönnerdal, B. and Janson, J.-C. (1972) *Biochim. Biophys. Acta* 278, 175–183