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Purification of External Invertase from Brewers Yeast

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The present work describes a preparation of β-D fructofuranoside fructohydrolase (invertase) from brewers yeast. The enzyme obtained has a very high specific activity and a low content of carbohydrate. The procedure consists of three steps, a gelfiltration on Sephadex G-50, an ion exchange on DEAE-Sephadex A-50 and a precipitation with ammonium sulphate. Comments on each step are given.

The enzyme obtained has a specific activity of 2630 U/mg enzyme, a nitrogen content of 11.9 % and a polymannan content of 13 %. The amino acid composition corresponds to that known for external invertase.

A survey of some of the more successful preparations described in the literature is given. It includes the published values of the specific activities re-calculated in the same internationally recommended units.

Brewers yeast invertase (β-D fructofuranoside fructohydrolase, E.C.: 3.2.1.26) has been investigated with increasing frequency with respect to the formation of oligosaccharides,[1,2] the intermediate formation of a fructosyl-enzyme,[3] the relations between the external cell wall invertase and the internal invertase,[4] and the purification of the cell wall enzyme. Reports have been presented discussing purification of the cell wall invertase by chromatography on DEAE-cellulose,[5–7] on CM-Sephadex and hydroxylapatite,[8] on Sephadex molecular sieves G-100 and G-200,[9] and on a combination of SE- and DEAE-Sephadex.[10] Besides these experiments preparative iso-electric focusing has been applied.[11] All the preparations contain significant amounts of carbohydrate, generally about 50 %, and the specific activities are varying. Gascón, Neumann and Lampen [4] have proposed that the internal enzyme is chemically bound to the carbohydrate in the formation of the external cell-wall bound invertase and Neumann and Lampen [10] thought that pure cell wall invertase should contain about 50 % carbohydrate.

In the present work a simple method is presented for the preparation of yeast invertase in good yield. The resulting enzyme has a very high specific activity and the carbohydrate content is well below the usual.

Until now it has been difficult to make comparisons between the values of specific activities found in the literature. Many different units have been used

Table 1. Yield, purity and relative purity for the discrete steps in the procedure. For explanation of symbols, see under Experimental.

	ml	mg	U	% yield	U/P_{280}	relative purity	U/F-C	relative purity	U/C-A	relative purity	U/mg	C-A/mg	% N
Crude invertase	130		155 000	—	18	1	96	1	90	1	—	—	—
Filtered on Sephadex G-50	160		181 000	117	500	28	760	8	135	2	—	—	—
Ion exchange on Sephadex A-50	90		138 300	89	1760	98	2100	22	2 790	31	—	—	—
Desalted and lyophilized		65	83 700	54	1420	79	2460	26	3 200	36	1290	0.40	8.4
Precipitated with $(NH_4)_2SO_4$ and lyophilized		31	81 500	53	1350	75	3220	34	20 900	232	2630	0.13	11.9

and the temperature has been different from assay to assay. We have recalculated most of these activities in the same unit and for the same temperature. The results of this are given in Table 2.

Table 2. Comparisons of different highly active invertase preparations. For explanation of symbols, see under Results.

	% Carbohydrate	% Nitrogen	Stated activity	Recalculated activity in U/mg at 25°C
1926 Euler and Josephson [15]		10.8	If=320	860
1951 Fischer and Kohtès [16]	70–80	4–5	4000 A_{20}/mg N	160– 200
1951 Fischer, Kohtès and Fellig [17]	about 0		inactive	
1960 Andersen [5]	30	11.0	22 000 A_{25}/mg N	2240
1965 Myrbäck and Schilling[6]		11.5	14 300 A_{20}/mg N	1650
1966 Berggren [7]	40–60	8.0	If=300–320	810– 860
1967 Berggren [8]	40–60		If=400	1080
1967 Berggren [9]	40–60		If=400–450	1080–1220
1967 Vesterberg and Berggren [11]	30		If=300–320	810– 860
1967 Neumann and Lampen [10]	50		2700–3000 U_{30}/mg prot.	1080–1200
1968 Gascón and Lampen * [18]	< 3		2900 U_{30}/mg	2310
1968 This work	13	11.9	2630 U_{25}/mg	2630

* This enzyme is an internal invertase with a quite different amino acid composition to the normal external invertase (Table 3).

EXPERIMENTAL

Enzyme activity. In accordance with the statement of the Enzyme Commission the unit activity U is the amount of enzyme hydrolysing 1 μmol sucrose pr. min at 25°C assuming optimal conditions, *i.e.* the optimum pH and substrate saturation.

Each determination was performed in 10 mM sodium acetate buffer at pH 4.75 containing 5.00 % sucrose. After 3 min the reaction was stopped with 0.5 M Na_2CO_3. The degree of reaction was determined in a Zeiss Lichtelektrisches Polarimeter, which allows an accuracy of 0.005°. In each test an amount of enzyme leading to a final degree of reaction of about 5 % was selected.

Protein determinations. Absorption of light: P_{280} is the amount of protein in 1 ml buffer solution giving unit extinction at 280 mμ in 1 cm cells. *Folin-Ciocalteau:* The method used was described by Lowry *et al.*[12] Standard was bovine serum albumin in H_2O. 1 F–C is the amount of protein giving the same extinction at 500 mμ as 1 mg albumin.

Determination of carbohydrate. The anthrone method [13,14] was used. 1 C–A is the amount of carbohydrate giving the same extinction at 585 mμ as 1 mg mannose.

Elementary analysis. Nitrogen, carbon, and hydrogen were determined with a Perkin Elmer 240 Elemental Analyser.

Amino acid composition. The amino acid analyses were performed on a Beckmann Spinco Amino Acid Analyser model 120 (modified to 3½ h developing time) which used principles developed by Spackman *et al.*[20] The amino acid contents were determined from samples hydrolysed 24, 48, and 72 h.

Procedure

All preparative procedures were performed at 4—6°C. The following describes the course of a typical preparation.

Desalting. 130 ml of commercial invertase "Invertan. K.B." was desalted on a Sephadex G-50 column (90.0 × 2.6 cm). The enzyme solution was eluted in 0.1 M Na/HAc buffer, pH 4.75. Fractions of 10 ml were collected. The active protein is leaving with void volume.

Ion-exchange. In the next step the pooled fractions containing the active protein in a volume of 160 ml were lead on a Sephadex A-50 anion exchange column 24.0 × 2.6 cm previously equilibrated with 0.1 M Na/HAc buffer, pH 4.75. After this 400 ml 0.1 M Na/HAc buffer, pH 4.75 was added. During this step 50 % of the protein and about 80 % of the carbohydrate were eluted (Fig. 1 a and b). The active protein was eluted after application of a linear gradient of sodium chloride obtained from a solution of 0.5 M NaCl in the acetate buffer, adjusted to pH 4.75. 10 ml fractions were collected. In the fractions immediately before the protein, a great deal of the remaining carbohydrate was eluted (Fig. 2 c). The first part of the protein eluted was inactive but, immediately after, the invertase was eluted in a sharp peak. (Fig. 2, b and d.) The active fractions, *i.e.* 84 to 92, were pooled, desalted on Sephadex G-50 and freeze-dried.

Precipitation. In this step the freeze-dried material was dissolved in 0.05 M Na/HAc buffer, pH 4.75, and solid $(NH_4)_2SO_4$ was added until 75 % saturation. After spinning 15 min at 20 000 *g* the precipitate was removed and resuspended in 5 ml 85 % $(NH_4)_2SO_4$. After spinning 15 min at 20 000 *g* the precipitate was removed and washed once more with 85 % $(NH_4)_2SO_4$. The remaining solid was dissolved in 10 ml 0.1 M buffer and dialyzed against running ion-exchanged water at 4°C for 24 h. The resulting dialyzed solution was freeze-dried.

Comments on the procedure

Desalting by dialysis and by gelfiltration leads to the same degree of purity. Here it is preferable to use gelfiltration because the enzyme is eluted in the buffer and so can be lead directly on the ion exchange column.

Concerning the ion exchange, experiments have clearly shown that the best result is obtained in the concentration 0.1 M Na/HAc buffer and at a pH of 4.75. Using 0.005 M buffer at pH 4.75 most of the carbohydrate adhered to the column and was eluted together with the active protein. Use of a 0.005 M acetate buffer at pH 7.5 resulted in the inactive protein being fixed at the column and eluted together with the active.

In one experiment the enzyme preparation obtained from the ion exchange was passed through another column of the same dimensions, the experiment being carried out in 0.1 M, pH 6.50 phosphate buffer. No change in the proportion between the carbohydrate and the active protein and no further purification was obtained.

In another experiment simultaneous changes of the ionic strength and pH were made. The concentration of NaCl was varied from 0.1 to 0.5 M and the pH from 4.75 to 6.50. In this way it was possible to elute the invertase in two separate peaks, both with high activity, but the yield was very low.

As to the precipitation with $(NH_4)_2SO_4$ no precipitation was obtained before the concentration reached 65 %. Redissolution and reprecipitation of the washed pellet did not result in any further purification.

RESULTS

Fig. 1 and Fig. 2 show details of the ion exchange on DEAE-Sephadex A-50 and Table 1 shows the yield and the relative purity of the discrete steps in the procedure. With respect to yield, the greatest waste occurs with desalting on Sephadex G-50 after ion exchange. As discussed in the comments on procedure, dialyses result in about the same loss. The yield of 117 % after the

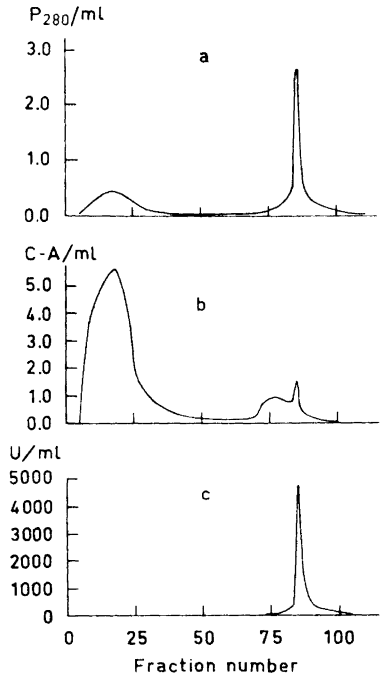

Fig. 1. Ion exchange of invertase on DEAE-Sephadex A-50. The NaCl gradient was established from fraction 65. Each fraction has a volume of 10 ml. a: Protein concentration as P_{280}/ml. b: The content of carbohydrate as C—A/ml. c: The activity U/ml.

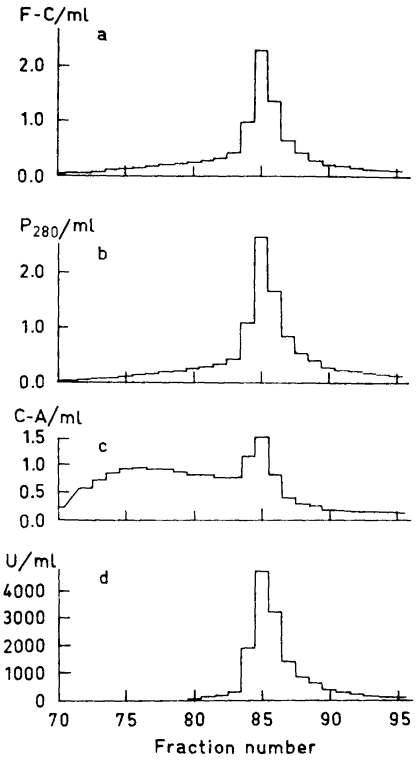

Fig. 2. Ion exchange of invertase on DEAE-Sephadex A-50. In this figure the elution of the active protein is enlarged. a: Protein concentration as determined in F—C/ml. b: Protein concentration determined as P_{280}/ml. c: Carbohydrate content C—A/ml. d: The activity U/ml.

first filtering on Sephadex G-50 is probably due to disappearance of an inhibitor in the crude invertase. From Table 2 is seen that the highest relative purity with respect to protein (P_{280} or F—C) is obtained with the ion exchange step. Highest relative purity with respect to carbohydrate (C—A) is obtained after the precipitation with ammonium sulphate.

Table 2 gives values for nitrogen content, carbohydrate content and specific activity for most of the preparations in the preceding years. To make a comparison possible the specific activities have been recalculated in the same units. The unit used is U/mg enzyme where U means the amount of enzyme hydrolysing 1 μmol sucrose pr. min at 25°C assuming optimal conditions. This unit is in accordance with the statements of the Enzyme Commission. In the recalculation from Fischer-Kohtès units 1.081 $U_{25°}=A_{25°}$ was used. In the recalculation

from If values data from Berggren [7] 320 If=820 $A_{20°}$/mg were used. Temperature corrections to 25°C were based on a series of experiments, which indicate that the conversion factor from 20°C to 25°C is 1.128 and from 30°C to 25°C 0.797.

In Table 3 the amino acid composition for this preparation is compared with that for internal and external invertase. It shows that the present preparation is external invertase.

Table 3. The amino acid composition of this invertase preparation calculated for molecular weight 135 000 of the protein moiety. The composition is compared with the values stated for internal and external invertase.

Amino acid	Calculated content		
	Internal [4]	External [10]	This preparation
Glycine	115	71	74
Alanine	84	68	72
Serine	151	114	129
Threonine	80	84	106
Proline	63	65	64
Valine	73	69	68
Isoleucine	38	40	39
Leucine	77	83	76
Phenylalanine	77	80	85
Tyrosine	31	65	71
Tryptophan	30	33	—
Half-cystine	0	5	>1
Methionine	14	21	19
Aspartic acid	165	178	171
Glutamic acid	124	115	116
Arginine	32	27	27
Histidine	29	16	16
Lysine	85	60	54
Glucosamine	0	38	—

DISCUSSION

Despite intensive preparative efforts an active cell-wall invertase free of carbohydrate has not been prepared. It seems that in every preparation of high activity described hitherto where ion exchange has been used the result is a relatively stable mixture of the active protein and 30—50 % polymannan; after the ion exchange step the present preparation is no exception. Neumann and Lampen [10] state that 50 % polymannan is an integral part of the external invertase. Fisher, Kohtès and Fellig [17] were able to remove all polymannan from this enzyme using absorption on bentonite but the preparation lost its activity.

It seems that precipitation with $(NH_4)_2SO_4$ is the only way to lower the carbohydrate content below 30 %. Adams and Hudson [19] were able to prepare an invertase containing only 7 % polymannan in this manner but the activity was small. In the present work precipitation from $(NH_4)_2SO_4$ leads to an

enzyme containing only 13 % carbohydrate but retaining a very high activity. Since it appears from these experiments that highly active external invertase with a relatively low content of carbohydrate can be obtained, it would be interesting to find relatively mild methods to remove further amounts of carbohydrate from the protein to determine if any amount is indispensable to the enzymatic activity.

The relative stable preparation containing about 50 % polymannan does not seem to be a homogeneous compound. Berggren [7] obtained three separate enzyme peaks after elution with a pH gradient in phosphate buffer. Sephadex filtration on G-200 resulted in a few active components [9] and using iso-electrical focusing Vesterberg and Berggren [11] described two main peaks and several small peaks. In the preparations described here the products from the separate peaks had nearly the same specific activity and carbohydrate content. Neumann and Lampen [10] obtained, from ultracentrifuging a main peak with a molecular weight around 270 000 ($s_{20,w}=10.4$ S) and in addition a smaller peak with a somewhat larger molecular weight. Ultracentrifuging the present enzyme preparation also gave a heterogeneous product with a main peak of $s_{20,w}=20$ S, *i.e.* a very high molecular weight.

From the present experiments it is reasonable to conclude that the active external invertase possibly exists in an association-dissociation equilibrium so that a low content of polymannan promotes the association of the protein molecules.

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REFERENCES

1. Bacon, J. S. and Edelman, J. *Arch. Biochem. Biophys.* **28** (1950) 467.
2. Blanchard, P. H. and Albon, N. *Arch. Biochem. Biophys.* **29** (1950) 220.
3. Andersen, B. *Acta Chem. Scand.* **21** (1967) 828.
4. Gascón, S., Neumann, N. P. and Lampen, J. O. *J. Biol. Chem.* **243** (1968) 1573.
5. Andersen, B. *Acta Chem. Scand.* **14** (1960) 1849.
6. Myrbäck, K. and Schilling, W. *Enzymologia* **29** (1965) 306.
7. Berggren, B. *Arkiv Kemi* **25** (1966) 555.
8. Berggren, B. *Arkiv Kemi* **26** (1967) 259.
9. Berggren, B. *Arkiv Kemi* **26** (1967) 415.
10. Neumann, N. P. and Lampen, J. O. *Biochemistry* **6** (1967) 468.
11. Vesterberg, O. and Berggren, B. *Arkiv Kemi* **27** (1967) 119.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
13. Kretschmer, K. *Z. Physiol. Chem.* **341** (1965) 146.
14. Hörmann, M. and Gallwitzer, R. *Ann.* **655** (1962) 178.
15. Euler, H. von and Josephson, K. *Ber.* **59** (1926) 1129.
16. Fischer, E. H. and Kohtès, L. *Helv. Chim. Acta* **34** (1951) 1123.
17. Fischer, E. H., Kohtès, L. and Fellig, J. *Helv. Chim. Acta* **34** (1951) 1132.
18. Gascón, S. and Lampen, J. O. *J. Biol. Chem.* **243** (1968) 1567.
19. Adams, M. and Hudson, C. S. *J. Am. Chem. Soc.* **65** (1943) 1359.
20. Spackman, D. H., Stein, W. H. and Moore, S. *Anal. Chem.* **30** (1958) 1190.

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