COMPOSITION OF INVERTASES FROM GERMINATED BARLEY[1]

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ABSTRACT

The soluble acidic invertase from barley shoots and the soluble basic invertase from shoots and roots were purified by ion-exchange chromatography and gel filtration. The purified proteins were homogenous by the gel electrophoretic criterion. The amino acid ratios of the acidic invertase from shoots and the basic enzyme from roots reflect their isoelectric behavior. The acidic enzyme contains about 49% glucan and 0.7% glucosamine.

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Previous work (1) has shown that the invertases of germinated barley are located in the tissues of the embryo. The shoot contains two soluble invertases; one, an acidic enzyme, is isoelectric at pH 4.1, and the other, a basic enzyme, is isoelectric at pH 9.8. The roots, scutellum, and axis node have only one soluble invertase which is a basic enzyme isoelectric at pH 10.1. In addition, all these tissues have an invertase tightly bound to the cellular material. The kinetic parameters have been described as well as the importance of these enzymes during the germination of barley (1,2). This report describes the purification and composition of the soluble enzymes.

MATERIALS AND METHODS

Preparation of Roots and Shoots

Barley (*Hordeum vulgare* L.) variety Larker was germinated for 96 hr in petri dishes as described previously (1) and frozen immediately. The roots and shoots were separated while frozen and lyophilized.

Extraction of Invertase

Detailed procedures have been described (1). All operations were done at 0°–4°C. Briefly, 3 g of shoots was ground with sand in a mortar and extracted with 0.2*M* citrate, pH 5.0. After centrifugation, the extract was dialyzed exhaustively against 0.005*M* citrate, pH 5.0. A total of 6 g of shoots was extracted. Twenty grams of roots was ground and extracted with citrate buffer at pH 4.1 and dialyzed against 0.005*M* buffer at this pH. The extracts were concentrated to about 30 ml by ultrafiltration.

Assay for Invertase

Qualitative Assay of Fractions from Columns. To 0.2 ml of 1.25% (w/v)

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sucrose in 0.2*M* citrate, pH 5.0 for the acidic invertase and pH 4.1 for the basic invertase, 50 μl of enzyme solution was added. The solutions were incubated at 30°C for 30 min and then examined for the presence of glucose by the glucose oxidase-peroxidase method with Clinistix (Ames Co., Elkhart, Ind.).

Quantitative Assay. This was done by the copper reduction method described previously (1). A unit of activity is defined as the production of 1 μg of reducing sugar as glucose per min.

Qualitative Analyses for Sugars

The acidic invertase solution containing 25 μg of protein was made 1*N* with H_2SO_4 and sealed in a tube. This was hydrolyzed at 100°C for 3 hr, then neutralized with AG 50 W X8 cation exchange resin (Bio-Rad Laboratories) in the basic form. The resin was removed and the solution evaporated to dryness with a stream of nitrogen. The residue was dissolved in 10% isopropanol and chromatographed in single dimension on Whatman No. 1 filter paper with water-saturated phenol and with 1-butanol-pyridine-water in the ratio of 2:2:1 (3), and on thin layers of Kieselguhr with ethyl acetate-isopropanol-water in the ratio of 65:24:12 (4). Reducing sugar was located with ammoniacal silver nitrate, and glucosamine was sought with the Elson-Morgan reagent (3).

Quantitative Carbohydrate Assay

Carbohydrate was determined quantitatively in duplicate by the phenol-sulfuric acid method of Dubois *et al.* (5). Glucosamine was determined in quantitative amino acid chromatograms (see below).

Protein and Nitrogen Analyses

Protein was determined by the method of Lowry *et al.* (6) with bovine serum albumin as a standard, and nitrogen by the method of Johnson (7).

Amino Acid Analyses

For amino acids other than tryptophan (and for glucosamine) enzymes were hydrolyzed for 22 hr in 6*N* HCl in an evacuated sealed tube at 110°C. For tryptophan, the enzymes were hydrolyzed with mercaptoethane sulfonic acid (8). Analyses were made with a Beckman 121 amino acid analyzer (9).

Disc Electrophoresis

Electrophoresis was carried out using a modification of the method and apparatus of Davis (10). For the basic enzymes, the system used stacked at pH 5.0 and separated at pH 4.0 (11); the enzymes migrated toward the cathode. Solutions of enzyme containing 25 to 90 units and 100 μg of protein were concentrated to about 50 μl by exosmosis with sucrose, and applied to the column. About 12 μg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) in 40 μl of 25% sucrose solution was applied to a separate column as a reference. Electrophoresis was carried out for 120 min at 5 mA per column for the basic enzymes. Electrophoresis of 80–200 μg of the acidic enzyme containing 350 to 900 units was carried out similarly, except that the system stacked at pH 8.3 and separated at pH 9.5. In this case, the enzymes migrated toward the anode and the electrophoresis continued for 80 min. The gels were stained for protein with aniline blue black (Amido Schwarz).

Purification of Shoot Enzymes

Carboxymethyl Cellulose (CMC) Chromatography. The CMC (Whatman CM 52) was treated successively with 15 vol of 0.5*M* NaOH and, after standing 30 min, fines were removed. The CMC was washed thoroughly with distilled water, treated similarly with 15 vol of 0.5*M* HCl, and washed with water. It was equilibrated with 0.005*M* citrate pH 5.00 and used to form a column (2.5 × 50 cm) which was washed with the buffer until washings from the column contained no carbohydrate when examined by the phenol-sulfuric acid method (5).

The dialyzed extract containing about 2 mg protein/ml was pumped on the column at 20 ml/hr by the upward flow technique, and the column was eluted at this rate with a linear citrate concentration gradient at pH 5.00. Two hundred and fifty grams of 0.5*M* citrate was drawn into 250 g of 0.005*M* citrate as the latter was pumped on the column. The effluent was monitored at 280 nm and fractions of 5 ml were collected and examined qualitatively for enzyme activity. Fractions containing the resolved enzymes were pooled and assayed quantitatively for activity.

Gel Filtration

The pooled fractions from CMC were concentrated to about 20 ml by ultrafiltration, dialyzed against a buffer consisting of 0.05*M* citrate, 0.1*M* NaCl, 0.0027*M* NaN_3, pH 4.50, and applied to gel columns (2.5 × 50 cm) at 20 ml/hr by the upward flow procedure. For shoot enzymes, the gels used consecutively were Bio-Gel P-10, P-60, and P-300 (Bio-Rad Laboratories, Richmond, Calif.). Three hundred to 500 mg of protein was applied. Five-milliliter fractions were collected and invertase activity was located as for the CMC treatment.

Purification of the Root Enzyme

The crude extract was gel-filtered prior to CMC treatment as described for the shoot enzymes, except that columns of Sephadex G-75 and G-200 were used (Pharmacia Fine Chemicals, Piscataway, N.J.). The enzyme was filtered twice through each of these columns. Twenty-four to 240 mg of protein was applied. The final product from this treatment (11 mg of protein) was treated with the CMC column as described previously.

RESULTS AND DISCUSSION

Previous work (1) showed that the acidic and basic invertases can be easily separated by isoelectric focusing over the range pH 3–10. Since the isoelectric pH values of the basic enzymes are close to 10, these enzymes are concentrated close to the highly alkaline cathode solution and, on removal from the column, there was some evidence of inactivation caused by mixing of the enzyme region with a small amount of the cathode solution. For the present work, the CMC ion exchanger was chosen to avoid this disadvantage.

The separation of the shoot enzymes is shown in Fig. 1A. The acidic enzyme did not adsorb on the ion exchanger and emerged with the solvent front in fractions 20–30. The basic enzyme was adsorbed on the column until the citrate concentration became 0.38*M* at fraction 75 (Fig. 1A).

Subsequent purification of the acidic enzyme by filtration Bio-Gel P-10, P-60, and P-300 gel columns is shown in Fig. 1, B–D, respectively. Inasmuch as this

enzyme is a glycoprotein (see below) the polyacrylamide gel was used in preference to Sephadex in order to avoid the contamination with carbohydrate from the column and to avoid adsorption of glycoproteins by Sephadex (12). Similar patterns (not shown) were obtained for the filtration of the basic enzymes from the shoot.

Shown in Fig. 2, A and B, are the first Sephadex G-75 and G-200 filtration patterns of the basic invertase from roots. Only one peak of activity appeared in each of the second chromatographic procedures. When the product from gel filtration was chromatographed on CMC, the elution pattern in Fig. 2C was obtained. Activity appeared at fraction 55 when the citrate concentration became 0.28*M* and elution continued through fraction 63.

The purification achieved for all three enzymes at the various stages of purification is shown in Table I. A very large increase in specific activity of the shoot enzymes resulted from CMC treatment, particularly for the acidic enzyme. The gel filtration with polyacrylamide increased the specific activity of both enzymes (approximately fivefold for the basic enzyme and twofold for the acidic enzyme). The basic enzyme from roots was purified about eightfold by the gel filtration and about fourfold by CMC treatment.

Further evidence for the degree of purity of the final products is indicated by

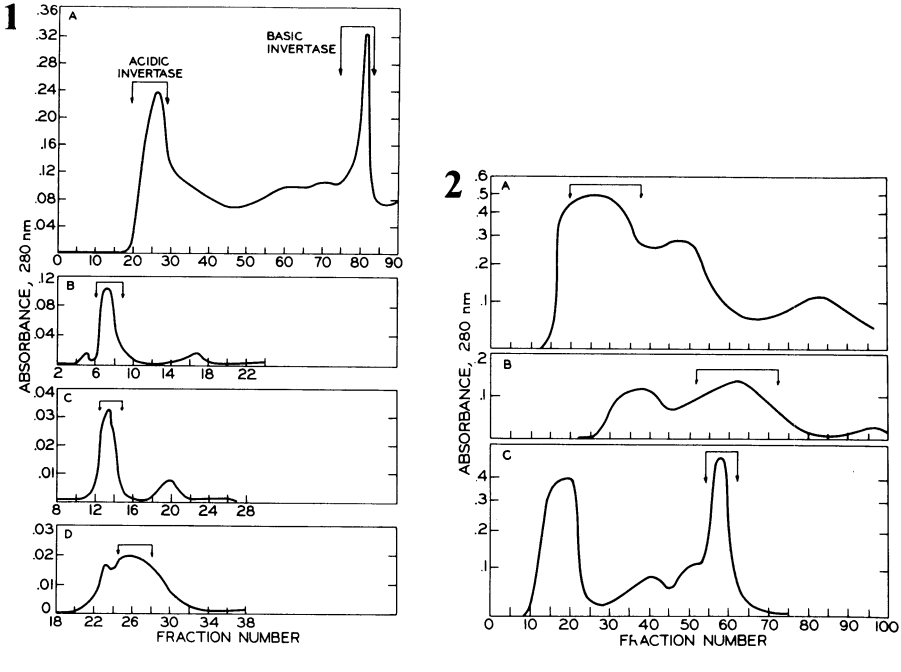

Fig. 1. Purification of invertase from shoots. A = CMC chromatography with increasing ionic strength of eluant; and B, C, and D = gel filtration of the acidic invertase through Bio-Gel P-10, P-60, and P-300, respectively. Fig. 2. Purification of basic invertase from roots. A and B = Gel filtration through Sephadex G-75 and G-200, respectively; and C = CMC chromatography.

TABLE I
Invertase Purification

Source and Treatment	Extract vol ml	Protein Applied to Columns mg	Eluate vol ml	Protein μg/ml	Activity[a] U/ml	Activity[a] U/μg Protein
Shoot invertases						
Crude extract from 3-g shoots	105			680	135	0.198
CMC		71				
Acidic invertase			39	94	194	2.06
Basic invertase			24	240	107	0.45
P10, P60, P300						
Acidic invertase						
P10		3.7	...	...	...	...
P60		2.9	...	...	...	...
P300		1.7	34	17	64	3.77
Basic invertase						
P10		5.8	...	...	...	...
P60		4.3	...	...	...	...
P300		2.7	18	9	20	2.22
Root invertases						
Crude extract from 20 g roots	237			1040	91	0.088
G75—1st treatment		250	86	480	155	0.323
G75—2nd treatment		41	81	300	144	0.480
G300—1st treatment		24	215	76	49	0.642
G300—2nd treatment		16	115	100	83	0.830
CMC		11	33	70	200	2.86

[a] μg reducing sugars (as glucose) per min.

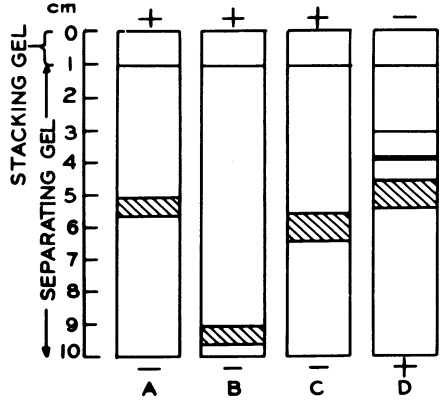

Fig. 3. Disc-gel electrophoretic patterns. A = Basic invertase from roots; B = lysozyme; C = basic invertase from shoots; and D = acidic invertase from shoots.

the disc electrophoretic patterns of Fig. 3. In all cases, an appreciable quantity of protein was applied, and only the acidic invertase appeared to have impurities which seemed to be in only trace amounts.

The basic enzymes contained no carbohydrate, but the acidic one consisted of carbohydrate and protein in approximately equal proportion. This carbohydrate-protein combination was obtained when the enzyme was isolated by the electrofocusing procedure (1) as well as from the CMC column. It does not seem likely, therefore, that the two components are joined by a weak affinity. Perhaps the carbohydrate is esterified with acidic amino acids in the protein, as has been suggested for yeast invertase (13). Qualitative filter paper chromatography of sugars in the hydrolytic products from the acidic invertase showed only glucose to be present, but glucosamine was clearly evident in the quantitative chromatograms from the Beckman Analyzer. Thus, the acidic invertase consists of 0.7% glucosamine (w/w) and approximately 49% glucan (w/w). Other examples of invertases with a carbohydrate constituent have been reported. One of the invertases of *Saccharomyces cerevisiae* has up to 50% mannan and 2–3% glucosamine (13,14), and the activity of the preparation was linearly related to the carbohydrate content. This enzyme, the so-called heavy invertase, appeared to be associated with the interior of the vacuole and the exterior of the cell membrane. A carbohydrate-free enzyme was located on the outside of the vacuole. *Neurospora crassa* has been shown to produce a soluble invertase which contains about 2.4% hexosamine and is associated with the cell wall (15). The behavior of this enzyme with regard to CMC purification, gel electrophoresis, kinetic constants, and pH optimum is very similar to the acidic enzyme from the barley shoots.

The amino acid compositions for the acidic invertase and the basic enzyme from roots are shown in Table II. The quantity of the basic invertase from shoots

TABLE II
Amino Acid Composition of Invertase

	Moles per 100 moles from Hydrolysate	
	Shoot Acidic	**Root Basic**
Tryptophan	2.6	0.55
Lysine	5.3	5.0
Histidine	1.5	1.8
Arginine	3.2	4.4
Aspartic Acid	11.0	9.8
Threonine	6.0	6.4
Serine	6.5	6.6
Glutamic Acid	10.0	5.2
Proline	3.0	11.9
Half-Cystine	0.41	1.6
Glycine	8.8	9.3
Alanine	11.0	11.0
Valine	8.1	7.2
Methionine	1.4	1.5
Isoleucine	4.5	3.3
Leucine	9.2	7.8
Tyrosine	3.2	2.5
Phenylalanine	4.2	4.6

that was available after samples were taken for electrophoresis and carbohydrate analysis was insufficient to allow accurate amino acid analyses. As expected, the acidic enzyme has a high level of the acidic amino acids and a low level of the basic ones in comparison with the root basic invertase. According to Robel and Crane (16) the hydrolytic method used for amino acid analyses would result in the following losses in theoretical yield: serine, 10%; threonine, 5%; methionine, 1.6%; and lysine, 2%.

Literature Cited

1. PRENTICE, N. Invertase of germinated barley. J. Agr. Food Chem. 20: 764 (1972).
2. PRENTICE, N. Invertase activities during the germination of barleys that differ in malting properties. Cereal Chem. 50: 346 (1973).
3. SMITH, I. Chromatographic techniques. Interscience: New York (1958).
4. RANDERATH, K. Thin layer chromatography. Academic Press: New York (1963).
5. DUBOIS, M., GILLIS, K., HAMILTON, J. K., REBERS, P. A., and SMITH, F. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350 (1956).
6. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and CRANDALL, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265 (1951).
7. JOHNSON, M. J. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. 137: 575 (1941).
8. PENKE, B., FERENCZI, R., and KOVACS, K. A new acid hydrolysis method for determining tryptophan in peptides and proteins. Anal. Biochem. 60: 45 (1974).
9. POMERANZ, Y., and ROBBINS, G. S. Amino acid composition of maturing barley. Cereal Chem. 49: 560 (1972).
10. DAVIS, B. J. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121: 404 (1964).
11. REISFELD, R., LEWIS, U. J., and WILLIAMS, D. E. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 194: 281 (1962).
12. WHITAKER, J. R. Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem. 35: 1950 (1963).
13. WAHEED, A., and SHALL, S. The relationship between carbohydrate content and enzymic activity of yeast invertase. Enzymologia 41: 291 (1971).
14. BETETA, P., and GASCON, S. Localization of invertase in yeast. FEBS Lett. 13: 297 (1971).
15. METZENBERG, R. L. The purification and properties of invertase of *Neurospora*. Arch. Biochem. Biophys. 100: 503 (1963).
16. ROBEL, E. J., and CRANE, A. B. An accurate method for correcting unknown amino acid losses from protein hydrolysates. Anal. Biochem. 48: 233 (1972).

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