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Invertase of Germinated Barley

Neville Prentice

Invertase was present in soluble and insoluble form in barley axis, scutellum, shoot, and rootlet, but was absent or at a low level in the degermed caryopsis. The shoot tissue contained two soluble invertases which were isoelectric at pH 4.1 and pH 10.0, but the axis, scutellum, and rootlets contained only one soluble invertase, and this was isoelectric at pH 9.5. The soluble invertases have been examined also for pH optimum and stability, thermostability, molecular weight, and Michaelis constants; the insoluble enzymes have been examined for all these properties except molecular weight and isoelectric pH. The apparent molecular weights of all soluble invertases as determined by gel filtration were 92,000 ± 3000. K_m values for soluble and insoluble enzymes were in the range 2×10^{-3} *M* to 9×10^{-3} *M*.

One of the important processes in the germination of grain is the formation of sugars from endosperm carbohydrates for use by the developing seedling. In germinating wheat and barley seed, glucose is converted to sucrose in the scutellum (Edelman *et al.*, 1959) and sucrose is then transported to the axis, where it is rapidly utilized (Palmer, 1969).

Jones and Armstrong (1971) have shown that in germinating barley, α-amylase increases with increasing levels of applied gibberellic acid until a maximum level of enzyme activity is reached. This maximum level was determined by the level of maltose, glucose, and other low molecular weight substances near the aleurone cells. Removal of these sugars by way of sucrose synthesis in the scutellum and sucrose utilization in the rootlets and shoots should stimulate enzyme formation in the aleurone cells.

Radley (1969) has shown that if sucrose accumulates in barley scutellum, further breakdown of carbohydrate in the endosperm declines, since gibberellin production in the scutellum, and hence gibberellin-dependent enzyme synthesis in the aleurone cells, stops or continues at a low level.

Invertase (D-fructofuranoside fructohydrolase, E.C. 3.2.-1.26) of the barley kernel, an enzyme likely to be involved in sucrose utilization in the axis, has received little attention. Hoffmann and Günzel (1955) demonstrated increasing activity in aqueous extracts of tissue obtained at various times during germination. Most of the enzyme was in the seedling tissue. Nolte and Kirchdorfer (1954) viewed the increase in the water-soluble invertase activity to arise from the release of an extracellular enzyme by a hydrolytic cleavage of an intracellular one. Data obtained with three barley varieties indicated that activity of the soluble enzyme is influenced by nitrogenous fertilization of the plant, and that the level of activity is a varietal character. The results of Nolte and Kirchdorfer and of Radley suggest that invertase activity of a variety may be meaningful in relation to good malting quality for which optimal modification of endosperm constituents is necessary. Before such a relationship can be properly examined, a better characterization of the enzymes would be appropriate.

This paper describes some properties of soluble and insoluble invertase and the distribution of these enzymes in tissues of two barley varieties.

MATERIALS AND METHODS

Barleys and Malt Rootlets. The barleys were Larker, a midwest 6-row type, and Piroline, a 2-row type. Malt rootlets from kilned Larker malt were furnished by the Kurth Malting Co., Milwaukee, Wis.

Germination Procedures. (a) One-hundred-and-seventy grams (dry basis) of each barley was steeped in running tap water at 16°C to 45% moisture and germinated at 16°C in the dark until rootlet length was about 1 cm. This required 7 days for Larker and 6 days for Piroline. (b) Larker barley was washed thoroughly with sterile distilled water. Fifty kernels were placed in a sterile Petri dish which contained two sheets of Whatman No. 1 filter paper moistened with 5 ml of the water, and germinated as before for 96 hr. Twelve-hundred kernels were germinated and stored at −25°C.

Preparation of Tissue from Germinated Barleys. For the separation of component tissues, Larker and Piroline barleys were germinated by method (a). Kernels were thawed and separated into rootlet, shoot, degermed caryopsis, and embryo tissues. Piroline embryo was separated into axis and scutellum. All tissues were lyophilized.

For large quantities of rootlets, Larker was germinated by method (a) and lyophilized. For large quantities of shoots the Larker was germinated by method (b), and the shoots were excised and lyophilized.

Barley and Malt Laboratory, Plant Science Research Division, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin 53705.

Extraction of Invertase. One gram of tissue was ground finely with 1 g of acid-washed quartz sand in a mortar. It was extracted at approximately 4°C with 10 ml of 0.2 *M* sodium citrate, pH 5.0 for shoots and 4.1 for other tissues, for 1 hr, and then centrifuged at 54,000 × *g*. The supernatant was removed and the precipitate was washed with 6 ml of buffer. The suspension was centrifuged as before. The precipitate was washed twice more in this way. In each case the washing was combined with the original extract. After the final washing, the precipitate was suspended in 20 ml of the buffer.

The solution and suspended precipitate were dialyzed at 4°C against three changes of 1300 ml of 0.02 *M* sodium citrate, pH 5.0 for preparations from shoot tissue, and pH 4.1 for the preparations from the other tissues.

In some experiments attempts were made to solubilize the bound enzyme of commercial Larker rootlets with the following compounds added to the sodium citrate extraction buffer at the levels indicated: 5% Tween 20, 5% Carbowax 4000, 5% Triton X 100, 5% dimethyl sulfoxide (DMSO), 2.5% dimethyl formamide (DMF), 1% β-mercaptoethanol, 1% bovine serum albumin, 5% polyvinylpyrrolidone, and 20% glycerol. In one experiment 0.2 *M* sodium borate, pH 8.5, was used instead of the citrate buffer.

Assay for Invertase. One milliliter of enzyme preparation was added to 4 ml of 0.2 *M* sodium citrate containing 50 mg of sucrose. The pH of the citrate buffer was as follows: pH 4.1 for all rootlet enzymes, for the shoot-insoluble enzyme, for the shoot basic enzyme, and for enzymes from scutellum, axis, and endosperm; pH 5.0 for shoot acidic enzyme and for mixtures of the shoot acidic and basic enzymes. The solutions or suspensions were incubated at 35°C for the rootlet enzymes and 25°C for the shoot enzymes. Insoluble enzymes were kept in suspension by agitation during incubation. One-milliliter samples were removed at 0-, 10-, 20-, and 30-min intervals, and the reaction in each was terminated by adding the sample to 1 ml of 5% $ZnSO_4$. The pH was adjusted to 7.0 with 0.2 *M* NaOH and the volume brought to 10 ml with water. The inactivated insoluble enzyme was removed by centrifuging at 54,000 × *g*.

Blanks consisted of 0.8 ml of the substrate solution and 1 ml of $ZnSO_4$, to which was added 0.2 ml of enzyme preparation. The NaOH and water were added as before.

A 30-min incubation time was used and quantities of substrate enzyme, and other reagents were adjusted accordingly for monitoring fractions from electrofocusing or from gel filtration.

Reducing sugar in 2-ml samples of these solutions was determined by the colorimetric method of Nelson, as described by Hestrin *et al.* (1955), and the results were expressed as μg of glucose per min incubation per g of tissue extracted or per ml of enzyme solution.

Determination of Optimum pH. The following 1.25% sucrose substrate solutions buffered at 0.2 *M* were used: sodium citrate, pH 3.5, 3.8, 4.0, 4.2, 4.5, and 5.0; sodium succinate, pH 6.0; and TES (*N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, sodium salt), pH 7.0 and 7.5.

Invertase activities were determined as described previously except that quantities of NaOH and water were adjusted appropriately.

Determination of Moisture. Approximately 0.3 g of finely ground material was heated at 95°C for 1 hr and reweighed.

Electrofocusing. The soluble enzymes from Larker rootlets and shoots, and from Piroline axis and scutellum, as extracted from the tissue in 0.2 *M* sodium citrate buffer, were dialyzed against 1% glycine and electrofocused (Burger *et al.*, 1970) with sucrose gradient and ampholyte of pH range 3–10 (LKB Instruments Inc., Rockville, Md.) for 3 days at a potential of 300 V. Seven to 20 mg of protein (0.5 to 2 mg of N, were applied to the column. Two-milliliter fractions were collected and were dialyzed against 0.02 *M* sodium citrate, pH 5.0 for the shoot enzymes and pH 4.1 for the rootlet enzymes, to remove glucose which had formed during electrofocusing. The fractions were assayed for invertase activity.

Fractions containing the enzyme from rootlets were pooled, dialyzed against glycine again, and electrofucused for 5 days at 400 V with ampholyte of pH range 8–10. Two-milliliter fractions from this electrofocusing were collected as before, dialyzed against 0.02 *M* sodium citrate, pH 4.1, and assayed for activity.

Fractions containing the basic enzyme from shoots were pooled, as were those containing the acidic enzyme. These were dialyzed against 1% glycine and electrofocused again. For the basic enzyme the procedure was as for the enzyme from rootlets. For the acidic enzyme from shoots the ampholyte of pH range 3–5 was used with 450 V for 2 days. Two-milliliter fractions were collected, dialyzed against 0.02 *M* sodium citrate, pH 5.0, and assayed.

Stability of Enzymes to pH. Two milliliters of soluble enzyme solutions and of insoluble enzyme suspensions were dialyzed for 6 hr against three successive changes of 130 ml of the following buffers at 0.02 *M:* sodium citrate, pH 4.5; sodium succinate, pH 5.5 and 6.5; TES (sodium salt) pH 7.5; Tris chloride (tris(hydroxymethyl)aminomethane), pH 8.0; and sodium borate, pH 8.5 and 9.0. The activities of the dialyzed solutions and suspensions were compared with the controls dialyzed against 0.02 *M* sodium citrate, pH 4.1 for the basic enzymes and the insoluble enzymes and pH 5.0 for the acidic enzyme.

Stability of Enzymes to Temperature. Two milliliters of the enzyme solutions and suspensions were held at various temperatures from 25°C to 50°C for 1 hr prior to assay for activity. Results were compared to those for the corresponding enzyme preparations held at 4°C.

Determination of Michaelis Constants. Rootlet enzymes were prepared from commercial Larker malt rootlets and from Larker barley that had been germinated by method (a). Shoot enzymes were obtained from Larker shoots from barley germinated by method (b). The following molar concentrations of sucrose in 0.2 *M* sodium citrate were prepared as substrate: 1×10^{-3}, 2×10^{-3}, 3×10^{-3}, 5×10^{-3}, 8×10^{-3}, 1×10^{-2}, 2×10^{-2}, 3.6×10^{-2}, 5×10^{-2}. The pH's were as described previously. The Michaelis constants were determined by plotting the reaction velocity against the ratio of the reaction velocity to the substrate concentration (Dowd and Riggs, 1965).

Determination of Protein and Nitrogen. Protein was determined by the method of Lowry *et al.* (1951) and nitrogen by the procedure of Johnson (1941).

Determination of Molecular Weights. The molecular weights were determined by gel filtration with Sephadex G-200 equilibrated with 0.05 *M* sodium succinate, 0.1 *M* NaCl, 0.02% NaN_3, pH 6.0. The standard proteins were aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.).

RESULTS

Extraction Procedure. Table I shows the effects of the variations in procedure for extracting commercial rootlets. None of the reagents was effective in freeing the insoluble en-

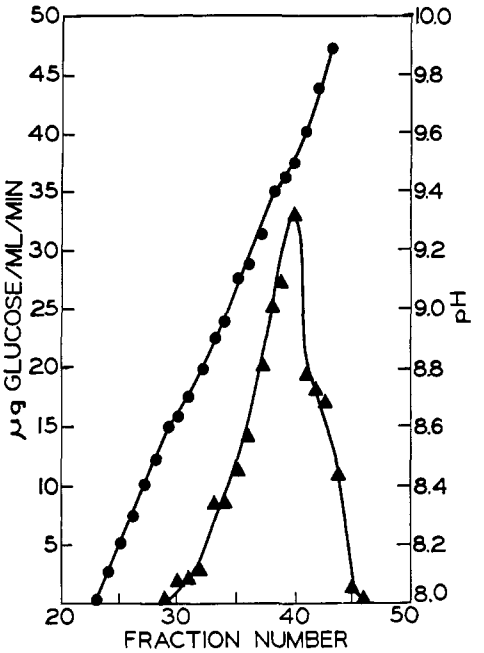

Figure 1. Electrofocusing soluble invertase from Larker rootlets. pH range 8–10. ▲——▲, invertase activity, μg glucose/ml/min; ●——●, pH

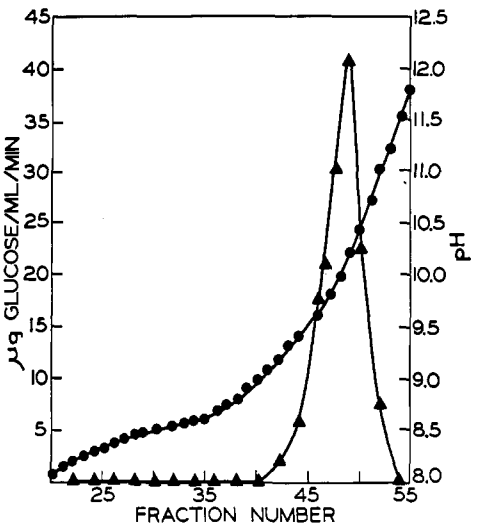

Figure 2. Electrofocusing basic invertase from Larker shoots. pH range 8–10. ▲——▲, invertase activity, μg glucose/ml/min; ●——●, pH

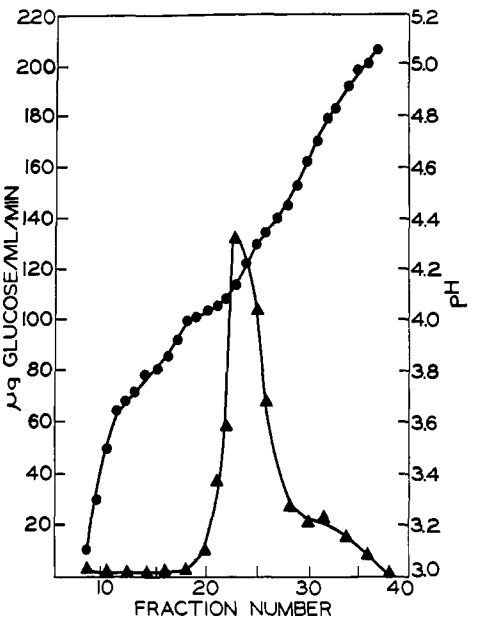

Figure 3. Electrofocusing acidic invertase from Larker shoots. pH range 3–5. ▲——▲, invertase activity, μg glucose/ml/min; ●——●, pH

Table I. The Effect of Reagents on the Solubility of Invertase of Larker Rootlets

Treatment	μg glucose/g tissue/min Soluble	Insoluble	Soluble, % of total
Commercial rootlets			
None	770	1060	42
Tween 20	973	1760	36
Carbowax 4000	431	1600	21
Triton X-100	591	1270	32
DMSO	499	1350	27
DMF	509	1000	33
β-Mercaptoethanol	592	1065	36
Bovine serum albumin	575	1675	34
Polyvinylpyrrolidone	674	1199	36
0.2 *M* Borate pH 8.5	552	1620	25
20% glycerol	850	1510	36
Lyophilized rootlets			
None	1414	2000	42
Tween 20	1899	3860	33

zyme. Tween 20 and glycerol allowed a higher yield of both enzymes, although the proportion of soluble to insoluble was less than for the untreated extract.

Electrofocusing Separation. The soluble enzyme from Larker rootlet had a peak of activity isoelectric at pH 9.8 when electrofocused over the range pH 3–10. Electrofocusing over the range pH 8–10 showed the more accurate isoelectric pH to be 9.5 (Figure 1). Piroline axis and scutellum had the same enzyme as the Larker rootlets based on isoelectric pH.

When electrofocused over the range pH 3–10, the soluble enzyme preparation from Larker shoots showed the presence of two enzymes, one isoelectric about pH 4.2 and the other isoelectric about pH 9.8.

The soluble enzyme with the high isoelectric point will be referred to as "basic" shoot invertase and the one with the low isoelectric point as "acidic" shoot invertase.

Electrofocusing the basic enzyme from Larker shoots over the range pH 8–10 provided the pattern in Figure 2 in which the enzyme was isoelectric at about pH 10.0. Similarly, Figure 3 shows that the acidic enzyme from shoots was isoelectric at pH 4.1.

Optimum pH. Figure 4 shows the variation in activity of the soluble and insoluble enzymes from commercial Larker rootlets with change in pH of the reaction mixture. Both enzymes have an optimum pH of approximately 4.1. Essentially the same results were obtained for the basic shoot enzyme and the insoluble enzyme from Larker shoots. The acidic enzyme from shoots, however, and a mixture of acidic and basic enzymes as extracted from this tissue displayed a broad peak with a higher optimum pH, *i.e.*, about pH 5, as shown in Figure 5.

pH Stability. The stability of rootlet and shoot enzymes

Table II. pH and Temperature Stabilities of Larker Invertase

	% of activity at pH 4.5							% of control activity			
	pH 4.0	5.5	6.5	7.5	8.0	8.5	9.0	25°C	25°C	40°C	50°C
Rootlet (commercial)											
Soluble basic	100	100	96	96	104	108	99		100	86	11
Insoluble	100	95	95	106	93	102	105		100	61	33
Shoot											
Soluble acidic	56	133	120	110					100	0	0
Soluble basic	105	90	110	94				100	100	82	0
Insoluble	105	100	110	100					70	51	0

Table III. Reaction Velocities and Michaelis Constants

		Substrate concentrations, millimolar									
		1	2	3	5	8	10	20	36	50	
Tissue	Enzyme	Reaction velocity[a]									K_m, M
Commercial Larker rootlet	Soluble	9	13	15	17	19	19	20	21	22	2×10^{-3}
	Insoluble	5	8	10	13	15	15	19	19	20	4×10^{-3}
Larker rootlet	Soluble	7	11	15	20	25	29	42	52	57	9×10^{-3}
	Insoluble	10	18	23	32	39	42	47	59	57	6×10^{-3}
Larker shoot	Soluble, acidic	40	55	74	108	136	154	176	180	198	5×10^{-3}
	Basic	10	16	19	24	31	33	34	35	34	3×10^{-3}
	Insoluble	10	15	19	26	29	33	32	35	37	3×10^{-3}
Piroline axis	Soluble	...	...	28	30	35	36	38	43	42	2×10^{-3}
	Insoluble	...	...	15	17	24	28	34	39	40	6×10^{-3}
Piroline scutellum	Soluble	17	...	29	34	39	40	45	49	49	3×10^{-3}
	Insoluble	6	...	...	12	14	16	17	18	18	3×10^{-3}

[a] μg glucose formed per min per ml of enzyme solution or suspension.

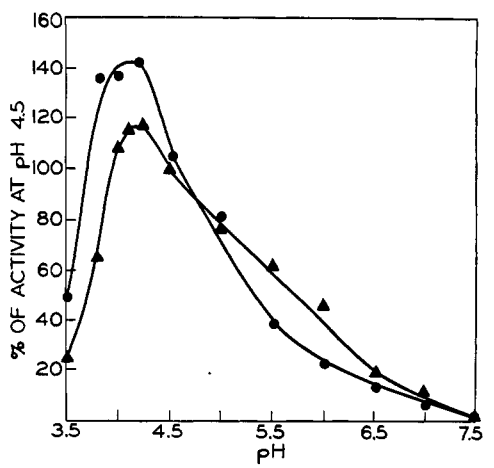

Figure 4. Optimum pH for invertase from Larker rootlets. ▲——▲, soluble enzyme. ●——●, insoluble enzyme

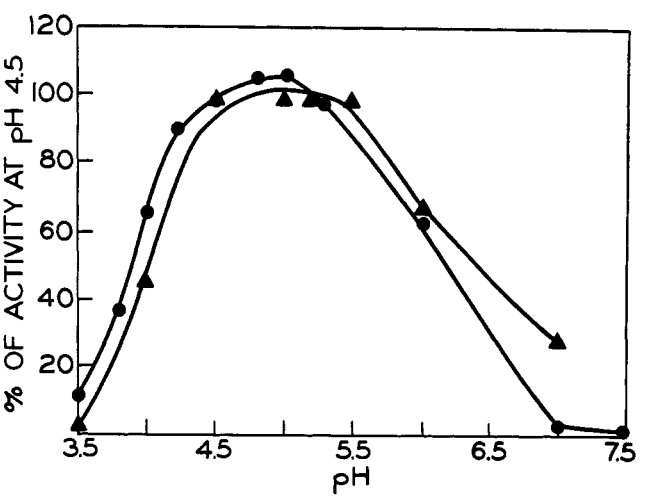

Figure 5. Optimum pH for soluble acidic invertase and a mixture of basic and acidic soluble invertase from Larker shoots. ▲——▲, acidic enzyme. ●——●, mixture of acidic and basic enzymes

to long exposure to a wide range of pH is shown in Table II. The rootlet enzymes showed no loss of activity from pH 4 to pH 9. While the shoot enzymes were examined over a more limited range, there appeared to be no loss of activity for the soluble basic enzyme and for the insoluble enzyme from pH 4 to 7.5. The soluble acidic invertase from shoots was labile at pH 4.0.

Temperature Stability. Table II shows the effect of treating the enzyme for 1 hr at temperatures from 25 to 50°C. The soluble acidic enzyme and the insoluble enzyme from shoots appeared to be particularly sensitive to heat. It was for this reason the assay temperature of 25°C was chosen for the shoot enzymes.

Michaelis Constants. The reaction velocities and K_m are shown in Table III. The affinities of enzymes for substrate appear to be of the same order of magnitude for all enzymes, except for the soluble invertase from lyophilized Larker rootlet, which had an inexplicably high K_m.

Molecular Weights. The molecular weights of all the soluble enzymes from the various tissues (rootlets, shoots, axis, scutellum) are essentially the same, *i.e.*, 92,000 ± 3000 as determined by gel filtration.

Distribution of Enzymes in the Germinating Kernel. The degermed caryopsis is the only part of the kernel that has little or no enzyme (Table IV). The insoluble enzyme is present in greatest proportion in the embryo tissue as a whole, in the separated axis and scutellum, and in the rootlets. However, in the shoots the soluble enzyme activity predominates, particularly for Piroline. The Piroline shoots were not examined for the presence of more than one soluble enzyme.

Table IV. Distribution of Enzymes in the Germinated Kernel

Barley	Tissue	Enzyme (μg glucose/g tissue/min) Soluble	Insoluble	Insoluble/soluble
Larker	Embryo (axis plus scutellum)	1350	2100	1.55
	Degermed caryopsis	0	0	...
	Shoots	2340	647	0.25
	Rootlets	975	2580	2.64
Piroline	Axis	1705	3530	2.07
	Scutellum	465	1000	2.15
	Degermed caryopsis	Trace	Trace	...
	Shoots	2460	1280	0.53
	Rootlets	1443	6100	4.23

Piroline, which displayed a more vigorous germination, has a higher invertase level in all tissues.

DISCUSSION

Differences in optimum reaction conditions and isoelectric behavior of the soluble invertases in the shoot of germinated barley suggest that distinct sucrose splitting systems exist. Possibly the enzyme in newly formed cells of the shoot differs from that of the relatively mature tissue, a situation that has been postulated to occur in broad bean root (Robinson and Brown, 1952). Although the acidic and basic enzymes from barley shoots were separated by electrofocusing, measurement of the relative amounts of them was not meaningful in view of the length of time (about 7 days) required between extraction of the tissue and the assay of the electrofocused products. However, the shift in the optimum pH from 4.1, the optimum for the basic enzyme, to about 5, the optimum for the acidic one, for the freshly extracted mixture of the two (Fig. 5) suggests that the acidic enzyme may predominate. The data for pH optima and stability and thermal stability indicate that the bound enzyme of shoots is not an immobilized modification of either soluble enzyme.

The two enzymes in the rootlets appear to be similar except for their solubilities, and may differ only in association with an insoluble carrier. Complete solubilization could have resolved the question if one or more of the treatments had broken the association of the enzyme with cellular material.

Several other plant tissues contain soluble and insoluble invertases that have similar properties, for example, carrot roots and corn coleoptile. The insoluble enzyme of grapes but not of carrot roots or corn coleoptile could be solubilized by extracting the tissue with solutions of Carbowax 4000 or Tween 20 (Hawker, 1969). Less solubilization occurred when the grape-insoluble fraction obtained without these reagents was subsequently extracted with them. Solubilization of the grape enzyme was accomplished by including bovine serum albumin in the extraction buffer. Presumably these treatments prevented the association of the enzyme during extraction but were less efficient in breaking the combination once formed. Arnold (1966) was able to prevent the insolubilization of the grape enzyme by extraction with borate buffer at pH 8.5. Hasegawa and Smolensky (1970) found soluble and insoluble invertases in dates but were unable to free the insoluble one with Carbowax 8000 or Tween 80. β-Mercaptoethanol released invertase from cell walls of *Saccharomyces fragilis* but not from the cell walls of *S. cerevisiae* (Kidby and Davies, 1970).

Invertase is associated with the growth and elaboration of cells, *e.g.*, in corn radicle (Hellebust and Forward, 1962) where it reached a maximum activity when the rate of cell elongation was greatest. Its presumed functions are to: (1) provide osmotically active substances to cell sap during water uptake; (2) supply substrate for respiration from which energy is derived for growth; and (3) provide substrates for synthesis of cell wall materials. In yeast (Hoshino *et al.*, 1964; Beteta and Gascon, 1971) and in corn coleoptile (Kivilaan *et al.*, 1961) the bound enzyme is attached to cell wall components, while the soluble form is located in the vacuole and cytoplasm.

An invertase called "alkaline" invertase because of its high pH optimum (about 7) and an invertase called "acid" invertase with pH optimum about 4 are often found in higher plants, for example in pea roots (Lyne and ap Rees, 1971) and in carrots (Ricardo and ap Rees, 1970). The "alkaline" invertase is located in the cytoplasm, particularly in cells which have low levels of the intracellular "acid" enzyme, while the latter seems to be involved with cell elongation. The "alkaline" invertase, however, is not present in germinating barley.

The distribution and levels of invertase in Larker and Piroline barleys indicate varietal differences that may be meaningful in relation to germination behavior. Subsequent investigations should accommodate the demonstrated temperature and pH requirements of the enzymes from the various regions of the kernel. In previous work (Hoffmann and Günzel, 1955; Nolte and Kirchdorfer, 1954) reactions were carried out at pH 4.7 and 37°C for up to 32 hr. At this pH much less than optimum activity would be obtained for all invertases except the acidic one from shoots. Furthermore, above 35°C some inactivation of all the invertases can be expected, particularly the acidic one from shoots, which is completely inactive after 1 hr at 40°C.

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