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A SURVEY OF THE MULTIPLE FORMS OF INVERTASE IN THE LEAVES OF WINTER WHEAT, *TRITICUM AESTIVUM* L. EMEND THELL. SSP. *VULGARE*

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SUMMARY

Wheat leaf invertases (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) were separated by gel filtration into three size groups with molecular weights greater than 10^7, around 100 000, and between 12 000 and 13 000, respectively. Isoelectric focusing revealed that a few of the invertases were basic proteins (pI approx. 8.0–8.7) but most were acidic (pI 3.2–5.0). The enzyme molecules in the 10^7-size group were a mixture of basic protein with pI = 8.7 and acidic proteins with isoelectric points usually in the range 3.5–4.8. Most enzyme proteins in the medium-size group had isoelectric points 4.2, 4.3 and 4.8 while the smallest enzyme molecules mostly had a pI of 8.5.

The proportions of invertases of different sizes in wheat plants grown at 6 °C were different from those in plants grown at 20 °C or in the greenhouse.

INTRODUCTION

The energy of activation of crude invertase from the leaves of Kharkov 22 MC wheat decreases when this very cold-hardy winter wheat is subjected to cold hardening conditions[1]. The difference in properties between invertase from Kharkov 22 MC grown under hardening conditions (6 °C) and that from plants grown at 20 °C persists even after the enzymes are purified by $(NH_4)_2SO_4$ precipitation and dialysis. Rescue, a cold-sensitive spring wheat, does not show this change in the energy of activation of its invertase. Since invertases from other organisms apparently exist in several forms[2–5], one explanation for the above observations on Kharkov 22 MC may be that crude wheat leaf invertase consists of several forms whose proportions in the leaves vary with the temperature of growth. The present paper is a first step in assessing this hypothesis and describes the resolution of the wheat leaf invertase enzyme complex into several forms by means of gel filtration and isoelectric focusing.

MATERIALS AND METHODS

Some plants of Kharkov 22 MC wheat were grown in vermiculite at 20 °C as described earlier[1]; some in vermiculite in the greenhouse; and some in vermiculite in a plant growth cabinet with a 16-h day of 10 500 lux supplied by a bank of 16 cool-white fluorescent tubes (F96.T12.CW) and 10, 25-W incandescent bulbs. Day temperature was 6 °C and night temperature, 4 °C in the growth cabinet. Plants were harvested at the 3–4-leaf stage.

Juice was prepared from the tops (mostly leaf blades) of these plants with a mechanical vegetable juicer at room temperature. The juice (50–400 ml) was treated with an equal weight of purified Polyclar AT[6], which was removed by filtration under vacuum followed by squeezing the filter cake in the Buchner funnel with a beaker. The filtrate was diluted with one fifth of its volume of 1.0 M sodium acetate buffer, pH 4.4, and the precipitate removed by centrifugation at 5 °C. The supernatant liquid was concentrated to 5–10 ml by dialysis against 30% polyethyleneglycol 20 000 (Fisher Scientific) in 0.01 M sodium maleate buffer, pH 6.5. The resulting precipitate was removed by centrifugation and the supernatant was chromatographed at 5 °C on a Sephadex G-200 column (bed dimensions 85 cm × 5 cm) eluted with 0.01 M sodium maleate buffer, pH 6.5, at an upward flow rate of 7.3 ml/h. Dilute buffer was used to facilitate subsequent analysis for reducing sugars. Fractions of approx. 10 ml were collected and stored in the presence of thymol at 0–1 °C. The fractions containing invertase were identified by incubating 0.3 ml of each of the fractions with 0.2 ml water and 0.5 ml of 0.02 M sodium acetate buffer, pH 4.7, containing 5% (w/v) sucrose for 1–15 min and then determining the reducing sugar liberated[7].

Prior to electrofocusing, fractions from the chosen peak from gel filtration were pooled and concentrated by dialysis against polyethyleneglycol 20 000 and then dialysed against several changes of 1% glycine. Electrofocusing[8,9] was carried out in a 110-ml column (LKB Produkter, Stockholm) at 5 °C using an "Ampholine" for the pH range 3–10 or 3–5, depending on the separation required. The density gradient was prepared with glycerol[2] in a density gradient mixer[10] using 56% (w/v) glycerol for the dense solution. Fractions (2 ml) were collected. Portions (0.5 ml) of the fractions were incubated with 0.5 ml of 0.5 M acetate, pH 4.7, containing 8% (w/v) sucrose. Since Ampholines interfere with the determination of reducing sugar and identification of the samples containing invertase, 10-μl samples from these digests were spotted directly on Whatman No. 1 filter paper and irrigated overnight with the upper layer from butanol–acetic acid–water (4:1:5, v/v/v). The sugars were detected with naphthoresorcinol–benzidine[11].

Molecular weights were estimated from a plot of the log of molecular weight against K_{av} (refs 12–13) determined by gel filtration using Sephadex G-50 or Sephadex G-200 under the conditions outlined above. Cytochrome *c*, myoglobin, chymotrypsinogen, albumin, ovalbumin, and γ-globulin were used as standards. This method assumes that the different forms of invertase are globular proteins, an assumption that may not be true for all the wheat leaf invertases just as it is not true for yeast and *Neurospora* invertases derived from cell walls[14–17] or for some invertases from tomato fruit[18]. However, these determinations do give an approximate idea of the size of the various forms of invertase.

Chromatography on columns (3.2 cm × 58 cm) of Sepharose 4B or 6B was performed with an upward flow (6.2 ml/h) of 0.1 M maleate buffer, pH 6.5. Fractions were collected every 30 min.

Nitrogen determinations were made by a micro-Kjeldahl method[19].

RESULTS

Gel filtration

Chromatography of invertase preparations from the leaves of greenhouse-grown Kharkov wheat revealed a major multi-component peak (II) of activity together with two or three smaller peaks (I, III, and IV) of enzyme activity (Fig. 1A). The multicomponent peak (II) varied considerably in shape and frequently showed two quite distinct peaks (Fig. 1A) and one or more minor peaks or shoulders. Sometimes (Fig. 1B) Peak II appeared as a single peak with one or more shoulders. The cause of this variability is not known but apparently was not the result of the difference in growing conditions of the plants used to prepare the samples for these figures. Peak IV was frequently absent in preparations from Kharkov 22 MC wheat leaves. The elution patterns obtained for invertases from warm-grown wheat leaves were similar regardless of whether 0.1 M or 0.01 M maleate buffer was used for their elution from the Sephadex G-200 column.

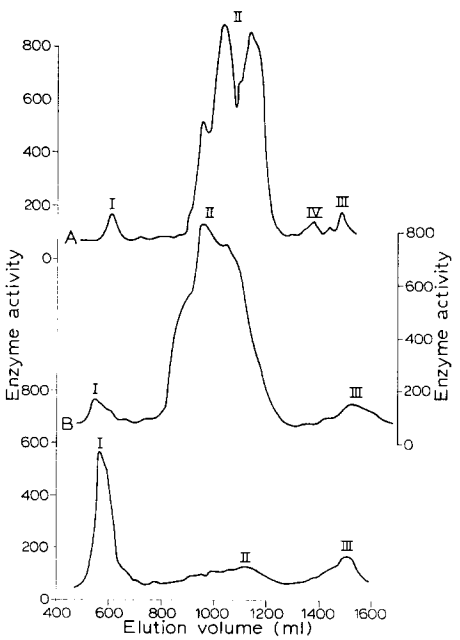

Fig. 1. Sephadex G-200 column chromatography of invertases extracted from wheat leaves grown under different conditions. Assay based on hydrolysis of sucrose. Sephadex column (bed volume 85 cm × 5 cm) eluted as described in text. Curves were not derived from similar weights of leaf material and are not quantitatively comparable. Curve A, invertase preparation from plants grown in greenhouse; Curve B, from plants grown at 20 °C; Curve C (bottom) from plants grown at 4–6 °C. Curves B and C from experiment with same column packing with Sephadex. Curve A from different column packing with Sephadex. Peak of activity in Peak I corresponded to void volume as determined with blue dextran.

When invertase preparations from the leaves of cold-hardened Kharkov 22 MC wheat grown at 4–6 °C were chromatographed the same peaks of activity were observed but their relative proportions were dramatically altered (Fig. 1C). Peak I was consistently found to be the major peak and Peak III sometimes had greater activity than Peak II.

Attempts were made to determine the molecular weights of the invertases from Peaks I, II, and III. The bulk of Peak-I activity eluted in the void volume of Sephadex G-200 and Sepharose 6B. When this material was chromatographed on Sepharose 4B its K_{av} was 0.04. Peak-I material behaved as if it were a globular protein of high molecular weight ($> 10^7$). When rechromatographed, Peak-II invertase gave variable results for its molecular weight. It probably consisted of more than one component. The molecular weights of its components were in the range 50 000–130 000 if it is a globular protein. Peak-III invertase had a molecular weight around 12 000–13 000; it eluted from a Sephadex G-50 column in nearly the same volume as cytochrome *c*. It was unstable and was not always recovered when rechromatographed.

Purification of invertase attained with Sephadex G-200

Crude invertase preparations from greenhouse-grown plants hydrolysed about 3.3 μmoles of sucrose/min per mg N at 25 °C. The best preparation of Peak-II invertase obtained from such plants by gel filtration of acetate-treated juice hydrolysed about 330 μmoles of sucrose/min per mg N at 25 °C and represented about 25% of the activity in the initial crude preparation. This represents about 100-fold purification on a nitrogen basis.

Electrofocusing

The invertase fraction with the lowest molecular weight consistently contained an active fraction with an isoelectric point near 8.5. Attempts to refocus this invertase in a narrower range Ampholine have not been successful. Traces of invertase with acidic isoelectric points were also frequently noted in this Peak-III material.

Before they were electrofocused with Ampholine 3–10, Peak-II invertases were divided into two fractions corresponding as nearly as possible to the two major sub-peaks often found in this fraction. The fraction of these invertases with the higher molecular weights contained enzyme with isoelectric point near 4.2 and occasionally small amounts of enzyme with isoelectric point near 4.8. If these two fractions of enzyme were combined and refocused in Ampholine 3–5, three active fractions were often obtained with isoelectric points 4.2, 4.3 and 4.8. The extra fraction obtained by electrofocusing with Ampholine 3–5 was probably the result of better resolution with the Ampholine covering the narrower pH range. The fraction of Peak-II invertases of lower molecular weight consisted mostly of enzyme with an isoelectric point near 4.3. Small amounts of enzyme with an isoelectric point near 8.4 were also usually present in this low molecular weight fraction. Refocusing the acidic enzyme protein on Ampholine 3–5 sometimes revealed the presence of a component with an isoelectric point near 4.6 in addition to the material with a pI of 4.3.

Electrofocusing of the invertases of high molecular weight (Peak I) consistently showed the presence of several acidic invertases with isoelectric points in the range 3.5–5.0 and an invertase with pI = 8.7. Other minor components were sometimes

found. The results of fractionating and refocusing Peak-I invertases were more variable than those obtained with the other two peaks. Refocusing the acidic invertases was successful chiefly when Kharkov wheat leaves grown at low temperature were the enzyme source. This refocusing on Ampholine 3–5 often showed more fractions than the initial electrofocusing. Fractions with isoelectric points near 3.5, 3.8, 3.9, 4.1, 4.3 and 4.8 were often found. The basic invertase from Peak I has not been successfully refocused.

Effect of pretreatment of juice

Wheat leaf juice had to be concentrated and fractionated to remove the large particulates before it was applied to a Sephadex column. Several methods of doing this were tried on preparations from greenhouse-grown leaves. Exploratory experiments were performed to ascertain whether these methods caused the loss of any major invertase fraction or drastically changed the proportions of the major components present.

During the concentration of the invertase preparations by dialysis against 30% polyethyleneglycol a precipitate formed that was later discarded in most experiments. Once the precipitate was extracted with a volume of 0.1 M acetate, pH 5.5, containing 2% Triton X-100 equal to the volume of the original supernatant liquid. The supernatant liquid and the dissolved precipitate were chromatographed on similar columns of Sephadex G-200. Both preparations yielded a small amount of Peak I- and a large amount of Peak-II invertase. The Peak-II invertase from the precipitate had an isoelectric point near 4.2 like the bulk of the Peak-II invertase from greenhouse-grown plants.

Separate Polyclar AT and acetate treatments of crude juice from greenhouse-grown leaves were compared. The acetate treatment caused greater losses of total invertase than Polyclar AT treatment and gave a 4-fold increase in specific activity whereas Polyclar AT alone gave a 2-fold increase. Both preparations contained small amounts of Peak-I invertase and large amounts of Peak-II invertase.

Experiments were conducted with greenhouse-grown plants to determine the isoelectric point of the invertases of Polyclar-treated wheat-leaf juice that had not been acidified with acetate buffer or run through a column of Sephadex. After Polyclar treatment this juice was centrifuged for 20 min at 16 000 $\times$ *g*, concentrated by dialysis against polyethyleneglycol and finally dialysed against 1% glycine before electrofocusing. The isoelectric point of most of the invertase was 4.1 and of a much smaller amount it was 8.0–8.3. When the acidic protein from electrofocusing was chromatographed on a Sephadex G-200 column it eluted as Peak-II material.

The difference in energy of activation between invertases from unhardened and cold-hardened leaves of Kharkov wheat has been shown to persist after $(NH_4)_2SO_4$ precipitation[1]. So invertase precipitated between 20 and 40% saturation with $(NH_4)_2SO_4$ from greenhouse-grown Kharkov leaf preparations was chromatographed on Sephadex G-200. It consisted mostly of Peak-II type invertase with small amounts of Peak-I type invertase.

The results of the exploratory experiments suggested that precipitating crude wheat leaf juice with Polyclar AT, acetate pH 4.0, or $(NH_4)_2SO_4$ did not cause the complete loss of any major fraction of invertase activity nor did it drastically change the proportion of the forms of invertase present. Gel filtration and electrofocusing

did not introduce any major artefacts. However, there were indications of changes in the isoelectric points of some fractions when electrofocusing was repeated and minor changes in molecular weight after repeated gel filtration.

Products of sucrose hydrolysis

Unlike yeast[20–23] and mould[24] invertases but like grape[25] and coffee bean[26] invertases, crude wheat leaf invertase synthesized only one detectable trisaccharide from sucrose at room temperature. This finding disagrees with that of some earlier workers[27,28] but confirms that of Lopatecki[29] who tentatively identified the trisaccharide as F^1-β-fructosylsucrose. Disaccharide synthesis was not detected. During the experiments on invertase fractionation Peak-II enzymes were observed to form trisaccharide.

pH optimum

The pH optima were checked for the invertase of high molecular weight and pI of 8.7, and the invertases of medium molecular weights and isoelectric points of 4.2 and 4.8. In all cases a pH optimum in the range 4.5–5.0 was obtained. None of these enzymes were neutral invertases such as have been found in some other higher plants[30–36]. No search was made for an invertase with a very low pH optimum like one that is bound to the cell walls of sugar cane[34].

DISCUSSION

The data show that Kharkov 22 MC wheat leaves contain a number of proteins with invertase activity. These enzymes may be divided into three groups based on size and two groups based on isoelectric points (acidic and basic). These observations make it attractive to postulate that the decrease in energy of activation of wheat leaf invertase that occurs during cold-hardening of Kharkov 22 MC wheat is the result of a change in the proportion of the different forms of invertase present in the leaves (see also Figs 1A, 1B and 1C).

Invertases of three different sizes have been found in sugar cane[37] and commercial preparations of yeast invertase[38]. Corn grains contain invertases of different molecular weights at different stages of development[39]. Size differences that have been observed in the invertases of *Neurospora* apparently result from the enzyme dissociating into active subunits[40].

Yeast invertase appears to be a mixture of glycoproteins with isoelectric points between pH 3.4 and 4.4 (ref. 2). The bulk of the wheat leaf invertases have isoelectric points in the same range as yeast invertases. However, wheat leaves do also contain basic as well as acidic proteins with invertase activity. Wheat is therefore similar to sugar cane in which invertases migrating in opposite directions during electrophoresis at pH 8.0 have been observed[5].

In wheat the isoelectric points of the invertases of high molecular weights correspond in part to the isoelectric points of the invertases of medium or low molecular weight. Like some other plant and fungal invertases[14–16,18] the invertases of high molecular weight (Peak I) in wheat are probably glycoproteins. The protein portions of some of the invertases with similar isoelectric points but of widely different molecular weights may be similar. The differences in molecular weight may result

from differences in the amount of covalently-bonded or adsorbed carbohydrate. Because they may differ only in the carbohydrate associated with them or be polymeric, I have avoided designating the various forms of wheat leaf invertase as isozymes although the invertases with widely differing isoelectric points probably are isozymes in the strict sense of the term[41].

A similar situation exists in yeast. Yeast invertases[38] differ in carbohydrate content, the larger molecules containing more carbohydrate. In brewer's yeast[42] the internal invertase lacks bound carbohydrate and appears on the basis of genetic and immunological evidence to be related to the external invertase. Nevertheless, the best preparations of internal and external invertases from brewer's yeast differ appreciably in amino acid composition.

Before we can conclude that forms of wheat leaf invertase differ in carbohydrate content rather than in the structure of the protein portion we must first purify them. Our preparation hydrolyzed approximately 330 μmoles of sucrose/min per mg N at 25 °C and represented approx. 100-fold purification. This preparation was much less active than the better preparations of yeast invertase that hydrolyse about 33 000 μmoles sucrose/min per mg N at 25 °C (ref. 38). However, it was more active than the one from potatoes obtained by Frost *et al.*[43] who achieved roughly 100-fold purification over the crude extract and an activity hydrolysing 9 μmoles sucrose/min per mg N at 25 °C. Arnold[25] achieved approx. 55-fold purification of invertase from grapes. His preparation appears to have been three or four times more active on a nitrogen basis than my preparation from wheat leaves.

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