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FRUCTOSE 1,6-BISPHOSPHATASE IN SEEDS OF *CORYLUS AVELLANA*

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Key Word Index—*Corylus avellana*; Corylaceae; hazel; dormancy breakage; fructose 1,6-bisphosphatase; gluconeogenesis.

Abstract—Fructose 1,6-bisphosphatase from cotyledons of hazel (*Corylus avellana*) seeds was partially purified and characterized. Change in the total enzyme activity was monitored in seeds stratified at 5° or moist incubated at 20°. A steady increase in the enzyme activity was found during stratification while the level remained constant in seeds held at 20°, showing a similar developmental pattern to those of lipase and isocitrate lyase as well as reducing sugars. The present results provide additional evidence of increased activity of the gluconeogenesis pathway during dormancy breakage treatment.

INTRODUCTION

During germination of oil-seeds there is a substantial conversion of reserve lipid to sugars via the gluconeogenesis pathway [1]. The final stage of this conversion involves the synthesis of hexose from phosphoenolpyruvate, which reverses the flow of glycolysis. Fructose 1,6-bisphosphatase (EC 3.1.3.11) is an important and characteristic enzyme of gluconeogenesis. It catalyses the irreversible hydrolysis of fructose 1,6-bisphosphate into fructose-6-phosphate. Considerable evidence is available indicating that fructose 1,6-bisphosphatase (FBPase) is a metabolic control point in this pathway [2–5]. FBPase of both chloroplastic and cytoplasmic forms has been isolated and characterized in great detail from spinach leaves [6–10]. However, in oil-seeds during mobilization of storage lipid, apart from studies on the FBPase from castor bean endosperm [11–14], few reports have appeared on enzyme studies. In particular, little is known about the FBPase properties in seeds that require cold after-ripening prior to germination.

Hazel seed (*Corylus avellana* L.) develops an innate embryo dormancy during the period of drying out; this is alleviated naturally by a period of cold moist incubation (stratification). The seed of hazel contains large quantities of storage lipid which accounts for about 60–68% of the dry weight [15]. Previous work in this laboratory has shown that an apparent reduction in both size and number of lipid bodies occurs during the dormancy-breakage stratification at 5° [16]. In separate studies (to be published elsewhere), we have demonstrated an increase in the activities of lipase, isocitrate lyase and the level of reducing sugars from the embryonic axes and cotyledons of hazel seeds. The present paper describes an investigation of the total FBPase activity in response to moist incubation at 5° and warm control treatment at 20°. The aim of this study was to establish evidence of the operation of the gluconeogenesis pathway at the dormancy-breakage stage prior to radical growth. Also in this study, FBPase from cotyledons of five-week chilled seeds was partially purified and studied. The properties of this enzyme are compared with those reported in other species.

RESULTS AND DISCUSSION

After centrifugation of the osmotically buffered preparation (extraction buffer plus 0.5 M sucrose) by centrifugation at 10 000 *g* for 30 min over 90% of the FBPase activity was detected in the supernatant. Also the activity did not undergo DTT activation unlike chloroplastic FBPase [7, 9]. These results suggest that the FBPase assayed was the cytoplasmic FBPase rather than that associated with the plastids. Following seed germination and the utilization of lipid reserves, the cytoplasmic FBPase is the predominant form, as demonstrated in castor bean endosperm [12, 13] and marrow cotyledon [17]. The changes of the total FBPase activity in the crude extracts from embryonic axes and cotyledons of hazel seeds during dormancy-breaking stratification at 5° and moist control incubation at 20° are shown in Fig. 1. It is apparent that the crude extracts from dry seeds contained an initially high level of FBPase activity. This activity increased progressively in the embryonic axes of stratified seeds, while the level remained nearly constant in those of 20° control samples (Fig. 1a). In the cotyledons, the FBPase activity rose steadily during the stratification period in comparison with only a slight rise during the moist control treatment at 20° (Fig. 1b).

Increased FBPase activity from oil-storing endosperm of castor bean, marrow and cotton seeds has been shown to be correlated with mobilization of storage lipids *via* gluconeogenesis during germination and seedling growth [12, 18, 19]. During a dormancy-breakage stratification period of embryos of Norway maple seeds, an increase in FBPase activity was observed in conjunction with an increase in the amounts of sugars [20]. In the present study, the developmental patterns of FBPase activity from the embryonic axes and cotyledons are quite similar

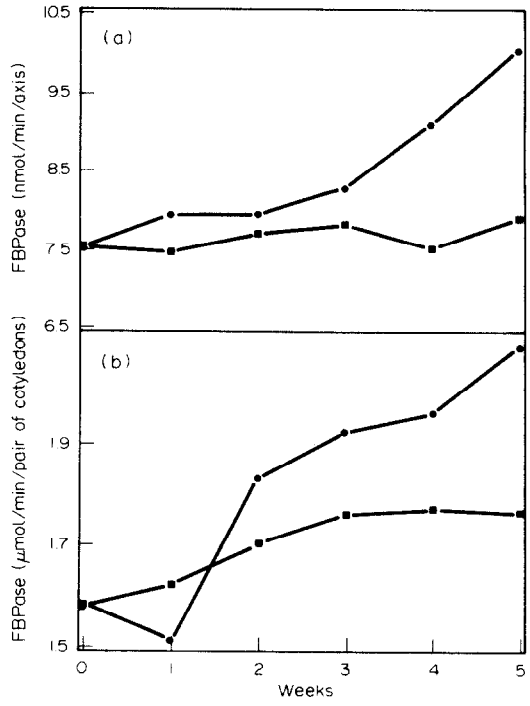

Fig. 1. Changes in the total activity of FBPase in hazel embryonic axes (a) and cotyledons (b) during cold stratification at 5° (●) and warm incubation at 20° (■). Each value represents the mean of three determinations.

to those of lipase and isocitrate lyase (results to be published elsewhere). Isocitrate lyase is commonly used as a marker enzyme of the glyoxylate cycle in the gluconeogenesis pathway [21]. Additionally, the rise in the level of reducing sugars in hazel seeds coincided with the increase of the FBPase activity (results not shown here). These combined results are the first indication that, in the case of hazel seeds, the mobilization of storage lipid and subsequent conversion to sugar occurred during the stratification period prior to radical emergence. Although the activation of the gluconeogenic pathway may not be directly coupled to the fundamental control of the loss of dormancy, it does seem to be related to the process as the increased activity occurred concomitantly with this period of dormancy release [22]. Thus, mobilization of storage lipid may be one part of an essential metabolic preparation for germination.

As FBPase is a characteristic enzyme of gluconeogenesis and has a regulatory role in the synthesis of sugars [2, 5], the enzyme extracted from the cotyledons of five-week chilled seeds was partially purified and studied. A summary of the partial purification procedure is presented in Table 1. Studies on the 10-fold purified FBPase showed an optimal activity at around pH 6.5 (Fig. 2a), which was similar to the optimal pH obtained from the crude extract of cotyledons (Fig. 2b). The pH optimum of FBPase was reported to be largely dependent on the concentration of Mg^{2+} and on the nature of the buffers [23]. However, in this case different buffer systems with the same concentration of Mg^{2+} showed the same pH optimum, but the reported buffer system gave higher enzyme reaction rates.

Mg^{2+} has been demonstrated to be an essential cofactor for FBPase activity [8, 10, 24]. Similarly, hazel FBPase is also a Mg^{2+}-dependent enzyme since the omission of Mg^{2+} from the assay mixture reduced the enzyme activity.

Activity of the partially purified FBPase shows a hyperbolic relationship to substrate concentration, with no inhibition at high(2.0 mM) substrate concentration. This finding is similar to that observed in *Peltigera rufescens* [25], but is different from the results obtained with castor bean endosperm [11, 13]. The Lineweaver–Burk plot of the data gave a value of 0.25 mM for the apparent K_m value for fructose 1,6-bisphosphate.

AMP has been described as an allosteric inhibitor of FBPase in many species [e.g. 10, 12, 25, 26]. A study of the effect of AMP on this partially purified FBPase from cotyledons indicates that in this respect the hazel FBPase proved to be no exception. Enzyme activity was shown to be similarly inhibited by AMP. The double-reciprocal plot of the data for the inhibitor showed that the inhibition of hazel FBPase by AMP (0.2, 0.5 and 1.0 mM) was noncompetitive. The apparent K_i value from the Dixon plot was 2.2 mM (not shown).

FBPase from castor bean endosperm purified 64-fold, had an optimal activity at pH 7.5 to 7.7, an apparent K_m of less than 2.5 μM, and a mixed competitive and uncompetitive inhibition by AMP [13]. A previous report of the same enzyme with less purification (44-fold), however, showed a broad optimal pH (7.0 to 8.0), an apparent K_m *ca* 10-fold higher at 38 μM, and a non-competitive inhibition by AMP [12]. This apparent discrepancy may not reflect any fundamental difference, since in both papers the authors suggested that a considerable modification of the enzyme properties could occur during extraction. In the case of the hazel seeds, the partially purified FBPase was similarly inhibited by AMP, but showed a slightly acid pH optimum and a higher K_m value in comparison with that from castor bean endosperm. The K_m value was in the same range as those of chloroplast FBPase [10]. In a recent report, two enzymes

Table 1. Partial purification of fructose 1,6-bisphosphatase from hazel cotyledons

Isolation step	Total protein (mg)	Specific activity (μmol/min/mg prot)	Purification (-fold)
Crude extract	3643	0.028	1
$(NH_4)_2SO_4$ fraction	2167	0.036	1.3
Sephadex G-25	1067	0.057	2.0
Superose™6	111	0.283	10.1

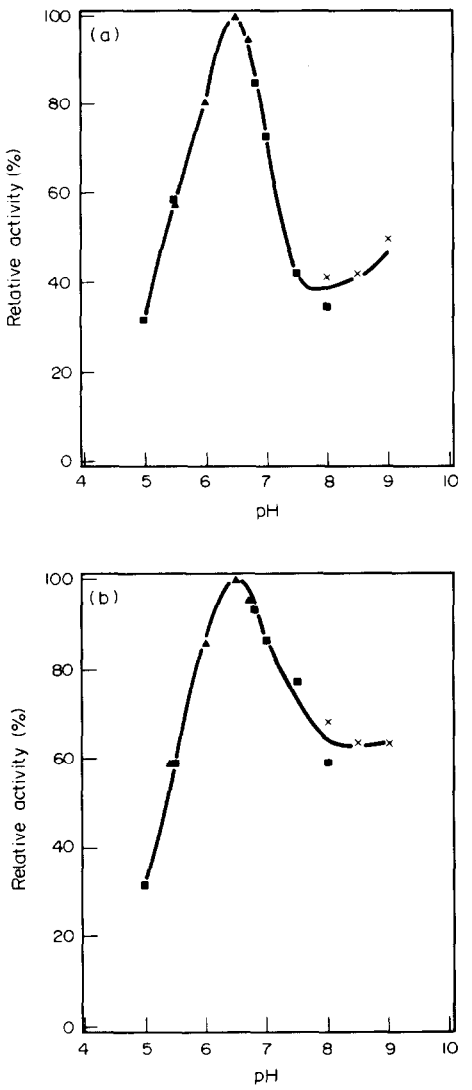

Fig. 2. Effect of pH on the activity of FBPase from cotyledons of five-week chilled seeds. A. FBPase activity in the partially purified preparation; B. FBPase activity in the crude extract. FBPase was measured in NaOAc–NOAc (●), Mes-NaOH (▲), HEPES–NaOH (■), and Tris–HCl (×) buffers, each at 100 mM.

capable of hydrolysing fructose 1,6-bisphosphate were isolated from *P. refesens* and a broad response to pH was shown in the crude extract. One of the enzymes had a pH optimum of 6.5, a K_m of *ca* 0.228 mM, was Mg^{2+}-independent and inhibited by AMP [25]. The authors suggested that the enzyme may not be specifically involved in fructose 1,6-bisphosphate metabolism because of the high K_m value in comparison with that of the alkaline photosynthetic FBPase. The hazel FBPase also showed a higher K_m value, however, it differed from the enzyme of *P. refesens* since hazel FBPase was Mg^{2+}-dependent and the enzyme from the crude extract only showed a single narrow pH optimum (Fig. 2b). Although the substrate specificity for the enzyme has not been investigated, it seems likely that the isolated FBPase is the native enzyme and the only enzyme responding for the hydrolysis of fructose 1,6-bisphosphate in the cytosol. The difference between the properties of FBPase in hazel seed and other species may reflect a variation among species or be the result of different treatments, e.g. increasing temperature has been reported to decrease the sensitivity of FBPase to AMP inhibition [27], and K_m values can vary with the temperature and pH of the assay [28].

EXPERIMENTAL

Seed material. Fruits of hazel (*Corylus avellana* L.) were obtained from a commercial source in Sept. 1984. The fruits were air-dried at room temp. and the lobed involucra removed. The nuts were stored in airtight tins under dry, cool conditions at 5° until required.

For the time-course experiments, dry fruits were layered in wet vermiculite and incubated at either 5 or 20°. At weekly intervals, samples of embryonic axes and cotyledons from each treatment were dissected and used in the preparation of the enzyme extracts. The unincubated dry seeds were designated as the week 0 sample.

FBPase for characterization was purified from extracts of the cotyledons of five-week chilled seeds.

Enzyme extraction. All procedures were carried out at 0–5°. 40 embryonic axes from each treatment were ground with a mortar and pestle for 2 min in 3 ml of extraction buffer containing 0.15 M Tricine–KOH (pH 7.5), 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, and 2 mM DTT [12]. The homogenate was centrifuged at 11 000 *g* for 30 min. The lipid bodies which formed a cake on the top were carefully removed and the resulting fat-free supernatant, referred to as the crude extract, was used directly for the assay of total enzyme activity.

In the preparation of cotyledonary extracts, 15 pairs of cotyledons, with testa removed, were first chopped to small pieces and homogenized for 2 min with a Silverson homogenizer in 2 vol of the extraction buffer. The extract was filtered through two layers of nylon cloth and centrifuged at 11 000 *g* for 30 min. The defatted supernatant was used for assay of the total activity.

Partial purification of FBPase. Purification procedures were essentially similar to that described in ref. [12]. Cotyledons (100 g fr. wt) from five-week chilled seeds were extracted in 200 ml of the extraction buffer as described above. The crude extract was sequentially fractionated with $(NH_4)_2SO_4$. The 45 to 65% saturation cut was pptd by centrifugation (20 000 *g* for 20 min) and the resulting pellet was taken up in a small vol of buffer containing 50 mM Mes–NaOH (pH 6.5), 1 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT and 10 mM KCl. This soln was then desalted by passage through a Sephadex G-25 column (12.5 × 3.0 cm) and the protein eluate, detected by a UV monitor at 280 nm, was collected. The resulting fraction was separated by FPLC (Pharmacia) on a 30.0 × 1.0 cm Superose 6 column (previously equilibrated with the same Mes–NaOH buffer) at a flow rate of 0.5 ml/min. Fractions showing high FBPase activity were collected and pooled. The course of the purification procedure is outlined in Table 1. The enzyme was stable for at least one month during storage at -20°.

Enzyme assay. FBPase activity was measured at 30° using a modification of a procedure described in [13, 29]. The standard reaction mixture contained in a total vol of 1.0 ml: 100 mM Mes–NaOH buffer (pH 6.5), 30 μM EGTA, 5 mM $MgCl_2$, 0.5 mM NADP, 1.0 mM fructose 1,6-bisphosphate, 2.5 units phosphoglucose isomerase (EC 5.3.1.9), 1.0 unit glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and the enzyme preparation. The reaction was initiated by the addition of fructose 1,6-bisphosphate and the change in absorbance at 340 nm was

recorded over 6 min. Reaction rates of FBPase for all the assays were shown to be linear with time and directly proportional to the enzyme concentration added to the mixture.

Protein determination. Protein was estimated by the Coomassie Blue dye-binding method, using bovine serum albumin (BSA) as the standard [30].

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