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INHIBITION OF GLUCAN PHOSPHORYLASE IN THE LEAVES OF *DENDROPHTHOE FALCATA*

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Abstract—The activity of α-glucan phosphorylase was either not demonstrable or was very low in the leaves of *Dendrophthoe falcata* infecting *Mangifera indica*. On mixing a homogenate of the parasite leaves with a standard source of glucan phosphorylase, the activity of the latter was powerfully inhibited, suggesting the presence of inhibitor(s) in the former. The inhibitor(s) was present in the parasite growing on all trees examined and was identified as phenolic. Maximal enzyme activity and simultaneous solubilization were achieved by developing a novel technique of grinding the parasite tissue in two stages with different media. The first stage was aimed at preferential solubilization of phenolics, with minimum solubilization of enzyme or protein and was effected with an 'elimination medium' made up of Tris-cysteine-EDTA. In the second stage, the tissue residue was ground with an 'extraction medium' made up of Tris-cysteine-EDTA-Triton X-100, which resulted in solubilizing 85 per cent of the enzyme activity and 60 per cent of tissue protein.

INTRODUCTION

TISSUES of angiosperm parasites are characterized by an accumulation of starch,[1] formed either as a result of increased synthesis, or decreased utilization, or both. Enzymes involved in starch metabolism, therefore, assume special significance in the biochemistry of angiosperm parasites. Of these, the degrative enzymes have special importance since they may play a role also in the process of parasitization and absorption of carbohydrates from the host.

Whelan[2] listed a number of inhibitors of phosphorylase in crude biological systems. In addition, some general inhibitors such as vacuolar acids, phytic acid and tannin[3,4] and certain cell wall components[5] are known to hamper the isolation of active enzymes from tissues. Our present researches show that endogenous phenolics are responsible for the inability to demonstrate α-glucan phosphorylase activity in the leaves of *D. falcata*. A novel technique has been developed for overcoming the inhibition by phenolics and for obtaining optimally active cell-free preparations of glucan phosphorylase with simultaneous solubilization of the enzyme.

RESULTS

Demonstration of Phosphorylase Inhibitor and its Preliminary Characterization

Homogenates of leaves of *D. falcata* in water and buffer were devoid of α-glucan phosphorylase activity. Progressive inhibition of the phosphorylase activity of homogenates

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of *Amorphophallus* leaves and of potato tuber on mixing with increasing amounts of a homogenate of *D. falcata* suggested the presence of an inhibitor in the preparation from *D. falcata* leaves. The inhibitor was found to be more or less evenly distributed between the 1600 *g* residue and supernatant of homogenates prepared in water. Only 8% of the inhibitor was found to be precipitable on addition of ammonium sulfate to 0·9 saturation to the supernatant. The inhibitor was partly dialysable (57 per cent of the inhibitor activity was lost when homogenate of leaves in water was dialysed against water for 48 hr) and was stable to heating for 30 min at 100°. The reaction between the enzyme and the inhibitor did not require a period of preincubation but was almost instantaneous. The inhibitor was not a metal since ashed leaves did not cause any inhibition of potato phosphorylase. Furthermore, it accumulated in the ethanol soluble fraction of fresh leaf extracts.

Phenolic Compounds as the likely Inhibitor

Qualitative colour tests indicated the presence of phenolic compounds in an ethanol soluble fraction of the parasite leaves. These could be precipitated with basic lead acetate and also be absorbed onto Norit-A.

Effect of Loranthus *Inhibitor on the Activity of Phosphorylase from Different Sources*

A comparison of the sensitivity of phosphorylase from different leaves (*Ipomoea batatas*, *Melia azedarach*, *Solanum tuberosum*, *Manihot utilissima*), tubers (*Amorphophallus campanulatus*, *I. batatas*, *M. utilissima*, *S. tuberosum*) and phylloclades of *Nopalea dejecta* towards *Loranthus* inhibitor was made using the ethanolic extract as source of inhibitor. Phosphorylase preparations from all sources were inhibited, though the extent of inhibition differed. The leaf enzyme was much less sensitive than the tuber enzyme. At the same time, the higher protein content of the former in relation to the latter has to be borne in mind.

Relationship Between Total Phenolics and the Inhibitory Activity in Leaves of Parasite and Hosts

The total phenolics and the inhibitor content of the leaves of parasite varied from host to host (Table 1). With the exception of *A. marmelos* all the hosts possessed inhibitory phenolics. The absence of a direct relationship between the host and parasite in phenolic content and inhibitor and the fact that even when a host was devoid of inhibitor the parasite possessed the inhibitor, suggested that the inhibitory phenolics were a special characteristic of this parasite irrespective of the host.

The degree of inhibition by equivalent amounts of phenolics from the various hosts and the parasite was significantly different. Tannin, tested simultaneously, was more inhibitory than the tissue extracts on the basis of equivalence in color intensity with the Folin and Denis reagent. The ethanolic extract of *Loranthus* was inhibitory also to aspartate aminotransferase of cactus phylloclades and UDPglucose:α-1,4-glucan α-4-glucosyltransferase of tapioca tubers.

Demonstration and Solubilization of Glucan Phosphorylase

Extraction of the enzyme by a wide range of standard procedures, with and without additives such as Polyclar AT and PVP, failed to yield active preparations. However, grinding of tissue in two steps was more successful. Data for enzymic activity and protein when tissue was subjected to extraction in two stages are recorded in Table 2.

TABLE 1. TOTAL PHENOLICS AND INHIBITOR CONTENT OF LEAVES OF THE PARASITE (*Dendrophthoe falcata*) AND VARIOUS HOSTS

	Total phenolics (mg tannic acid equivalent/g fr. wt.)		Inhibitor content (units/g fr. wt.)	
	Host	Parasite	Host	Parasite
Aegle marmelos	8·5	7·8	0	205
Barringtonia acutangula	45·6	10·6	2428	302
Mimusops elengi	29·0	22·1	1156	557
Grevillea robusta	28·3	24·5	748	805
Mangifera indica	44·4	55·0	2165	2036

Grinding of the leaves with buffered cysteine and EDTA ('elimination medium') yielded a dispersion with significant activity. The 1600 *g* supernatant fraction of this suspension was only slightly active and was discarded. The residue was now ground with the 'extraction medium'. On centrifugation a deep green extract was obtained with considerable enzymic activity and protein. The residual fraction also contained activity, but this was only a fifth of the activity in the supernatant. The combined activity in the extract and residue was nearly 4-fold that of the activity of homogenate prepared in a single stage with the 'extraction medium'. In relation to the tissue residue resulting on the first stage of grinding, about 40% of protein and 80% of enzyme activity were solubilized in the second stage.

A Comparison of the Action of Anionic, Cationic and Nonionic Detergents

Different lots from a large batch of randomized leaves were subjected to grinding in two stages, employing different detergents in the 'extraction medium' (pH adjusted to near neutrality) and in varying concentration. Use of the anionic detergent deoxycholate (0·5, 1·0, 2·0%, w/v) resulted in a progressive increase in the activity with increasing concentration of detergent and using 2% deoxycholate, about 65% of enzyme was solubilized. Using sodium lauryl sulfate, only low activity was demonstrable at 0·5 and 1% (w/v) level of detergent, while 2% concentration resulted in complete inhibition. Considerably higher

TABLE 2. EXPOSURE OF ENZYME AND SOLUBILIZATION BY GRINDING THE LEAVES IN TWO STAGES

Fraction*	Protein (mg/g fr. wt. or equiv.)	Enzyme activity (units/g fr. wt. or equiv.)
Elimination Medium used with Fresh Leaves		
Whole dispersion	34·4	4·2
Extract	1·4	1·6
Extraction Medium used with Above Residue		
Extract	12·7	17·1
Residue	16·2	3·6

* See Experimental for details of media used in fractionation.

activities were obtained by the use of the cationic detergent cetrimide (0·5, 1·0 and 2·0% w/v). There was no significant effect due to the change in the concentration of detergent on the activity of homogenate, but there was increased solubilization with increasing detergent concentration. The maximum solubilization achieved was only 50% at 2% concentration of detergent. Homogenates prepared in the presence of Triton X-100 had nearly the same activity as those with cetrimide, but a higher order of solubilization was achieved. The optimum concentration of Triton X-100 was 1% (v/v), when 85% of the enzyme was solubilized.

DISCUSSION

Enzyme isolation from *D. falcata* was hampered by the presence of inhibitory phenolics and by the peculiar texture of the leaves, which led to the retention of practically the entire protein in the cell debris, resembling in this respect pine leaves.[6] Special agents such as alkaline buffer, or reducing agents, Norit A, Polyamide, Polyclar AT, soluble PVP, gelatin or detergents, used singly or as mixtures, failed to elicit enzymic activity and to affect adequate solubilization of protein. This suggested that the conditions for their action were not the optimum.[7] It is known that PVP may not be effective with some tissues, depending on the nature of the phenolics.[8–11] A mixture of alkaline Tris, cysteine, Triton X-100 and EDTA was effective in exposing considerable enzymic activity.

If phenolics could be mechanically removed without affecting the protoplasmic mass, the residual tissue would constitute a good source for enzyme isolation. Optimum phosphorylase activity in parasite tissue was obtained in the present experiment when the phenolics were removed as a first step. Hydraulic pressure proved to be inefficient in achieving this objective, but a Waring blendor used with an elimination medium consisting of Tris buffer, cysteine and EDTA was highly satisfactory. Since the eliminated extract contained very little protein, but large amount of phenolics, it appeared that either (a) the intracellular phenolics were preferentially released, leaving the enzyme unaltered in the protoplasmic matrix, or (b) that simultaneously with release of phenolics, enzyme precipitation with or without partial inactivation also occurred. When the residue obtained in the first stage of grinding with elimination medium was reground with medium which contained also Triton X-100 (the extraction medium), the enzyme was exposed and at the same time solubilized to a considerable extent. A combination of factors, reversal of enzyme inhibition, breaking of latency and enzyme solubilization, might have operated in the 3- to 4-fold increase in the activity on grinding the residue with the extraction medium. The operation of one or more of these factors was possible only after the elimination of the major part of the phenolics in solution as evidence by the fact that the single stage grinding with the 'extraction medium' containing all the components added together elicited only a third or fourth of the total activity and solubilization of protein.

The technique of homogenization in two stages developed during the present investigation may have general application in enzyme isolation from plant tissues rich in phenolics or phenolases. The technique, however, does not permit a simultaneous study of enzyme localization.

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EXPERIMENTAL

Plant Tissues

The leaf tissue of angiospermic parasite *Dendrophthoe falcata* (L.F.) Ettingsh, a mistletoe parasitizing *Mangifera indica* L. was used in most of the studies. The sampling was carried out as described earlier.[12]

The parasite from a number of other hosts, namely, *Aegle marmelos* Corr., *Barringtonia acutangula* (L.) Gaertn. *Grevillea robusta* (Cunn.) and *Mimusops elengi* (L.) along with the host tissues, was used in others.

As the source of standard phosphorylase, use was made of leaves or tubers, as specified, of *Amorphophallus campanulatus* (Roxb.) Blume and *Solanum tuberosum* (L.); in some experiments *Ipomoea batatas* (Lam.), *Manihot utilissima* (Pohl), *Melia azedarach* (L.) and phylloclades of *Nopalea dejecta* (Salm-Dyck) were used.

Means of Disruption of Tissue

The homogenates were 20% (w/v) in strength, unless otherwise specified and the grinding period 3 min, at full speed of Waring blendor. The homogenates were tested without straining through cloth.

Media Employed

The control medium was water. The other media were simple media, special media with the components used singly and mixed media. Polyclar AT (purified according to McFarlane and Vadar[13]) and Polyamide were used in amounts equivalent to the fresh weight of tissue and Norit A (activated according to Crane and Sols[14]) in one fourth the amount. These agents were added along with water during cell dispersion, or were tested with tissue extract in water. Three different samples of soluble PVP were employed, with average molecular weights of 10,000, 40,000 and 360,000. They were dissolved in water to give 1 and 2% solutions.

The mixed medium had the composition: 0·05 M Tris-HCl buffer, pH 8, 0·04 M cysteine, 0·01 M EDTA and 1% Triton X-100. This medium has been referred to as the extraction medium. In some experiments, this medium was further supplemented with 1% PVP and Polyclar AT used in amount equivalent to the weight of tissue.

Grinding of Tissue in Two Stages in a Waring Blendor

Ten g leaf tissue was ground in a Waring blendor for 3 min operated at full speed, allowing twice an interval of half min each in between, with 100 ml of 'elimination medium' consisting of 0·05 M Tris-HCl buffer, pH 8, containing 0·04 M cysteine and 0·01 M EDTA. The dispersion, without straining through cloth, was centrifuged at 1600 *g* for 45 min. The pale yellow supernatant (pH 7·2) was kept aside for the determination of protein, phosphorylase activity and total phenolics. The residual solid was ground in a Waring blendor for 3 min with 100 ml of 'extraction medium', which differed from the above in containing also 1% (v/v) Triton X-100. Ninety ml of the homogenate was centrifuged in the cold at 1600 *g* for 45 min, the residue reground with 65 ml chilled 'extraction medium' for 3 min and the suspension centrifuged. The main supernatant and the wash fluid were mixed to give 150 ml of the extract. The final residue was suspended in 50 ml of the 'extraction medium'.

Phosphorylase Assay

The assay system, after Green and Stumpf,[15] was essentially the same as described earlier.[12] One unit of enzyme was equivalent to the liberation of 1 μmole orthophosphate in 30 min at 30° and pH 6·2, in the presence of added fluoride. The orthophosphate color development was according to LePage[16] using the reagents of Fiske and Subbarow.[17] About 50 mg of Norit A was used to remove the interference due to tissue phenolics and/or Triton X-100.[18]

Inhibitor Assay

The method of assay was essentially the same as described earlier.[12] A unit of inhibitor was defined as the amount which produced inhibition of phosphorylase by half a unit under the above enzyme assay conditions.

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Other Estimations

Protein estimation was carried out as described earlier,[18] using the method of Lowry *et al.*[19] Total phenolic content was determined using the reagent of Folin and Denis[20] as modified by Swain and Hillis[21] and Goldstein and Swain.[22] Tannic acid was employed as reference compound.

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