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ACTIVITY, EXTRACTION AND STABILITY OF ENZYMES INVOLVED IN POLYSACCHARIDE BIOSYNTHESIS IN *CITRUS*

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Key Word Index—*Citrus aurantifolia*; Rutaceae; polysaccharide biosynthesis; hexokinase; phosphoglucomutase; UDPglucose pyrophosphorylase; 1,3-β-glucan synthase.

Abstract—The effects of EDTA, 2-mercaptoethanol and bovine serum albumin on the extraction and stability of hexokinase, phosphoglucomutase, UDPglucose pyrophosphorylase and 1,3-β-glucan synthase from Mexican lime bark have been examined. The activity of these enzymes was generally increased and stability was tested in refrigerated and frozen extracts. Bovine serum albumin was the best stabilizing agent for phosphoglucomutase and 1,3-β-glucan synthase, but the former was more stable in refrigerated and the latter in frozen extracts. UDPglucose pyrophosphorylase stability was strongly dependent on the presence of 2-mercaptoethanol. The fact that the activity of 1,3-β-glucan synthase is the lowest of the four enzymes even in the presence of optimal concentrations of its activator strongly suggests that it is the key enzyme in the regulation of the metabolic pathway.

INTRODUCTION

Several compounds have been used in extraction media in order to avoid loss of enzyme activity in extracts from higher plants. The effects of these compounds are mainly a) to remove phenolics [1], b) to inhibit phenolase activity, either complexing the enzyme copper with chelating agents or reducing quinones with reducing agents [2], and c) to increase osmotic strength and to maintain enzyme solubility [3].

EDTA, 2-mercaptoethanol (EtSH) and bovine serum albumin (BSA) are frequently used as stabilizing agents but their effects on metabolically related plant enzymes have not been systematically studied. Only the effects of some sequestering and reducing agents have been examined on plant dehydrogenases [2].

In extracts from *Citrus* we have observed a rapid loss in the activity of hexokinase (EC 2.7.1.1), phosphoglucomutase (EC 2.7.5.1), UDP glucose pyrophosphorylase (EC 2.7.7.9) and 1,3-β-glucan synthase (EC 2.4.1.34), enzymes of polysaccharide biosynthesis pathway. In order to minimize these losses in enzyme activities the effects of EDTA, EtSH and BSA on the extraction and stability of these enzymes have been tested. We have also examined the effect of refrigeration and freezing on the storage properties of the extracts.

RESULTS

Hexokinase

The extraction of hexokinase was increased when the medium contained EDTA or BSA (Table 1) and the stability of the enzyme depends on the storage method (Fig. 1). A more rapid fall in activity was noted in frozen extracts than in refrigerated extracts. In the latter, hexokinase was more stable in the presence of EtSH or BSA. Under these conditions the activity after 120 hr was about 85–90% of the original activity.

Phosphoglucomutase

An increase in the extracted activity was observed when the extraction medium contained any one of the tested stabilizing agents. The activity was increased more than three fold in media containing EDTA or EtSH (Table 1).

Table 1. Activities of enzymes related to polysaccharide metabolism and effect of EDTA, EtSH and BSA on their extraction

	Additions to the extract medium				
	None	None	EDTA 2 mM	EtSH 10 mM	BSA 1%
	Activity (nkat/g fr. wt)	Relative activity (%)			
Hexokinase	0.4	100	180	100	140
Phosphoglucomutase	40	100	380	330	220
UDPglucose pyrophosphorylase	160	100	75	210	155
1,3-β-Glucan synthase	0.07	100	235	145	400

The extract medium without additions was 50 mM Tris–HCl pH 7.5. Assays were carried out immediately after the preparation of the extracts. Extraction and assays are as described in Experimental.

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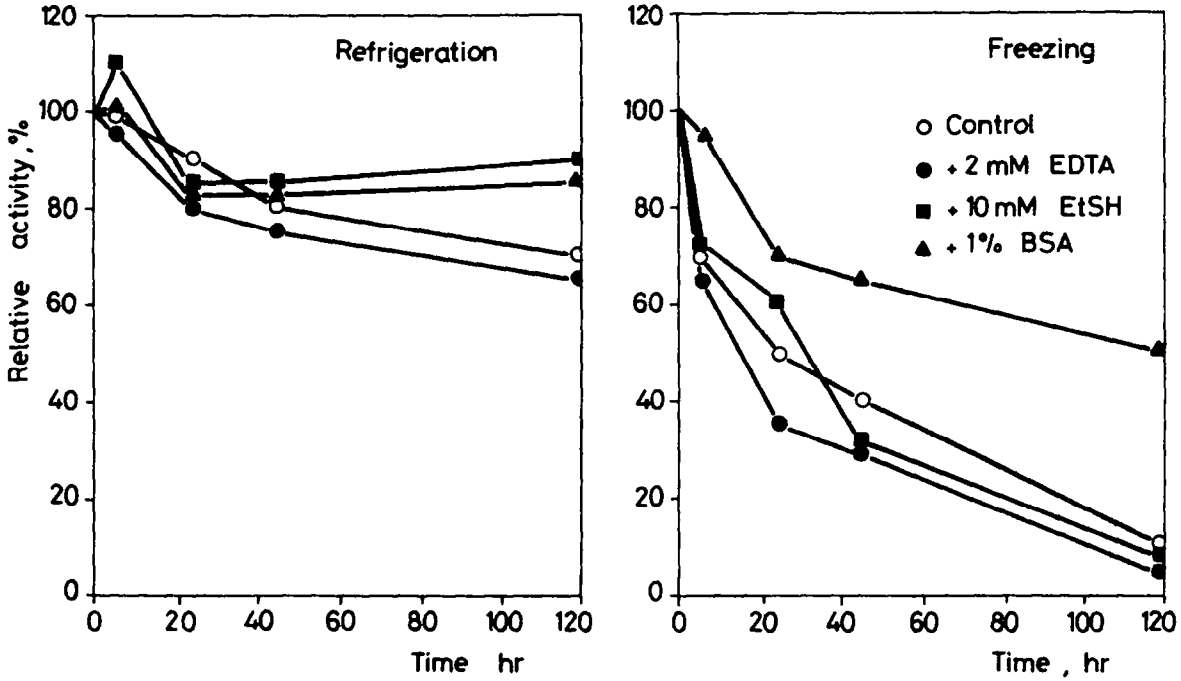

Fig. 1. Time course of the changes in hexokinase activity in several extracts. The composition of control buffer was 50 mM Tris–HCl pH 7.5. Activities are expressed as percentages of the activities measured in each extract immediately after their preparation. Extraction and assay are as indicated in Experimental.

The stability of phosphoglucomutase was markedly dependent on the storage method (Fig. 2) and activity decreased rapidly in frozen extracts. Even in the presence of BSA, less than 30% of original activity remained 20 hr after the preparation of the extracts. In refrigerated extracts the fall in activity is less rapid and depends on the added compound. Under refrigeration the medium containing BSA is the best for phosphoglucomutase stability. In the first hours, a rise in activity can be observed and this effect was consistently reproduced.

UDPglucose pyrophosphorylase

The extraction and stability of UDPglucose pyrophosphorylase were strongly dependent on EtSH (Table 1

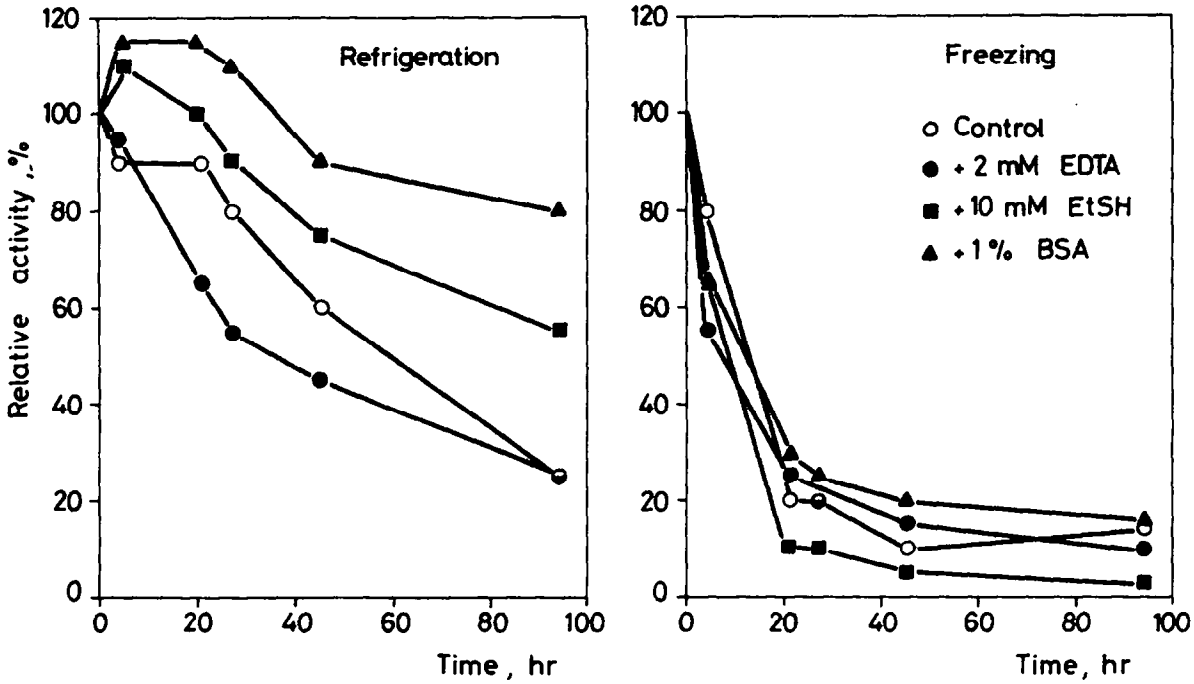

Fig. 2. Time course of the changes in phosphoglucomutase activity in several extracts. For details see legend to Fig. 1.

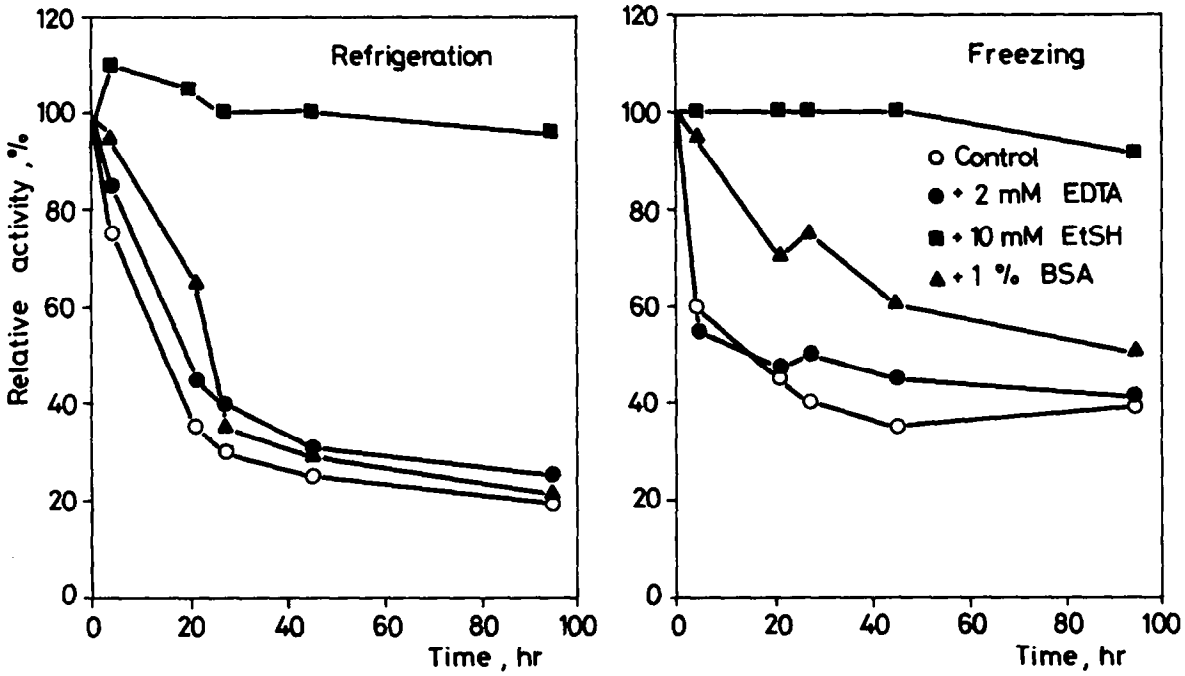

Fig. 3. Time course of the changes in UDPglucose pyrophosphorylase activity in several extracts. For details see legend to Fig. 1.

and Fig. 3) and the activity was maintained at above 90% of the original activity after 100 hr in both refrigerated and frozen extracts.

BSA increased the extraction of UDPglucose pyrophosphorylase but did not have a significant effect on the enzyme stability. The extracted activity was less in the extracts containing EDTA than it was in extracts without additives.

1,3-β-Glucan synthase

BSA stimulated strongly 1,3-β-glucan synthase extraction and the extracted activity was four times that of the control. Minor increases were observed with EDTA and EtSH in the extraction medium (Table 1).

The stability of the enzyme was dependent on both the presence of BSA in the extraction medium and on the freezing of the extract (Fig. 4). Less than 20% of

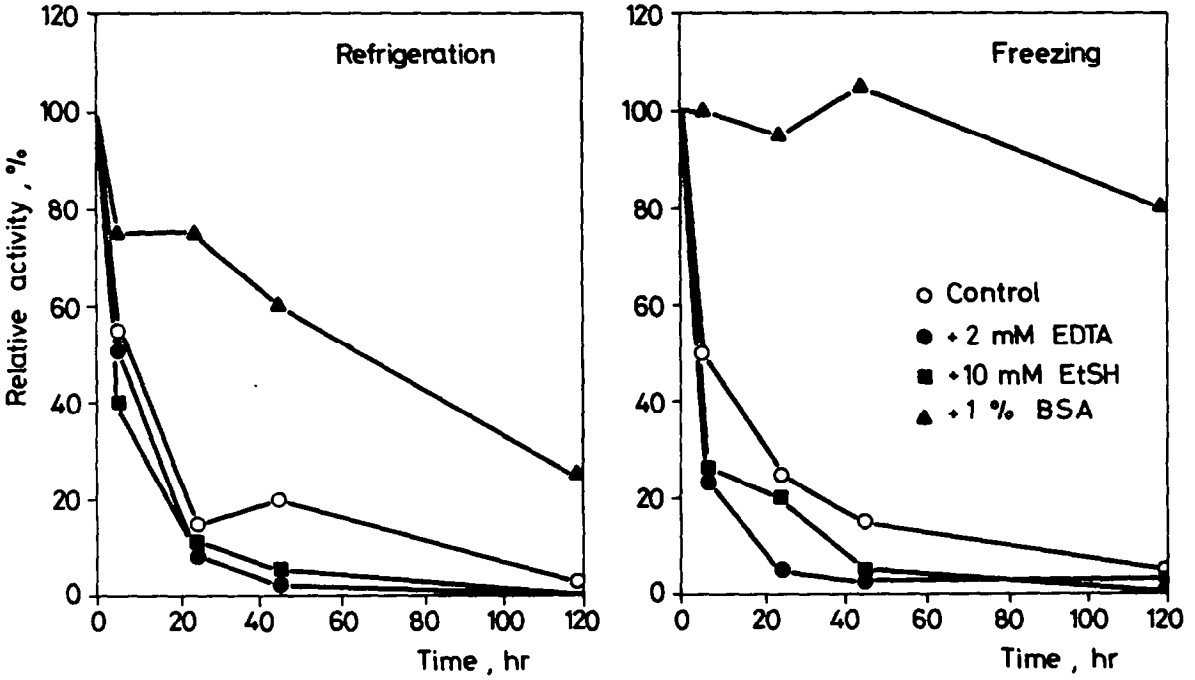

Fig. 4. Time course of the changes in 1,3-β-glucan synthase activity in several extracts. For details see legend to Fig. 1.

the original activity is lost after 120 hr. In refrigerated extracts, BSA again was the best stabilizing agent, although only 25% of original activity was present after 120 hr.

DISCUSSION

An examination of the effects of EDTA, EtSH and BSA on enzyme extraction and stability of all four metabolically related enzymes showed a great variety of effects on each one of the enzymes tested. The most significant effects were those of EtSH on UDPglucose pyrophosphorylase and those of BSA on 1,3-β-glucan synthase, mainly in frozen extracts, and on phosphoglucomutase, only in refrigerated extracts. The increases in enzyme extraction and stability produced by mixtures of EDTA, EtSH and BSA are the same as those produced when only the most effective compound was present.

No important differences were observed among the various extracts in hexokinase activity, but the activity was more effectively maintained in refrigerated extracts than it was in frozen ones. For three of the four enzymes studied the correct storage method was essential in order to maintain the activity.

The increase in phosphoglucomutase activity in extracts containing BSA (Fig. 2) could be due to the initial formation of a more active form or to a progressive liberation of the bound or latent enzyme; the activity reached a maximum and then it began to fall. Rapid losses in phosphoglucomutase activity have been described in extracts of some higher plants [4,5]. EDTA increased enzyme extraction but had no effect on its stability.

The effects of EtSH and BSA could be explained by their capacity to counteract the oxidative action of phenolic compounds [6]. Cysteine, a reducing agent, has been successfully tested in nitrate reductase extraction from *Citrus* [7].

The marked effect of BSA on 1,3-β-glucan synthase extraction and stability is similar to that observed by Tsai and Hassid [8] on the enzyme from oat coleoptiles.

1,3-β-glucan synthase seems to be the key enzyme in the regulation of the pathway, being the limiting activity (Table 1) even at optimal concentrations of the enzyme activator, cellobiose. When the cellobiose was absent from the assay mixture the activity was reduced to 5% of the optimal activity. On the other hand, 30 mM glucose produced the same activation as 10 mM cellobiose. These effects support the hypothesis of the regulatory function of 1,3-β-glucan synthase.

EXPERIMENTAL

Plant material. Mexican limes [*Citrus aurantifolia* (Christm.) Swing.] 4 yr old, grown in a greenhouse.

Extraction. Bark from 3–4 month old stems was homogenized in a cold mortar with 9 ml of extraction buffer per gm of bark. Composition of the extraction buffers was 50 mM Tris–HCl pH 7.5 with or without one of the following stabilizing agents: 2 mM EDTA, 10 mM EtSH or 1% BSA. Homogenates were centrifuged at 30000 *g* for 15 min. The pellet resuspended in 1 ml of buffer per gm of initial bark was the *particulate fraction*. The supernatant of the last centrifugation was the *soluble fraction*. The particulate fraction was used in the hexokinase and 1,3-β-glucan synthase assays and the soluble fraction in the phosphoglucomutase and UDP glucose pyrophosphorylase assays. All operations were carried out at 4°.

Enzyme assays. Soon after the extracts were obtained enzyme activities were assayed and extracts were distributed in several tubes. One half was kept at 0° and the other at −8°. Hexokinase, phosphoglucomutase and UDPglucose pyrophosphorylase activities were assayed at 25° in a coupled system by following NADPH formation at 340 nm. The reaction mixtures contained in a final vol of 3 ml, Hexokinase: 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.5 mM NADP, 3 units glucose-6-phosphate dehydrogenase (G6PD), 2 mM MgATP, 50 μl of particulate fraction and 2 mM glucose. No activity was observed without glucose. Phosphoglucomutase: 50 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 0.5 mM NADP, 3 units G6PD, 10 μM glucose-1,6-diphosphate (G1,6P_2), 1.33 mM histidine, 50 μl of soluble fraction and, after 3 min, 2 mM glucose-1-phosphate (G1P). No activity was observed without G1P. UDPglucose pyrophosphorylase: 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.5 mM NADP, 3 units G6PD, 10 μM G1,6P_2, 1 mM UDPglucose, 3 units phosphoglucomutase (prepared in 0.16 M acetate pH 5), 50 μl of soluble fraction and 2 mM NaPPi. No activity was observed without NaPPi. 1,3-β-Glucan synthase activity was determined by the incorporation of radioactivity into a polymer which is insoluble in H_2O and also in 95% EtOH, obtained by the incubation of UDPglucose-[^{14}C] in the presence of enzymatic extract [9]. The reaction mixture contained in a final vol of 0.4 ml, 50 mM Tris–HCl pH 7.5, 10 mM cellobiose, 1 mM UDPglucose, *ca* 20000 cpm UDPglucose-[^{14}C] and 50 μl of particulate fraction. The mixture was incubated at 37° for 1 hr. The subsequent steps were carried out as described in ref. [9]. The radioactivity on the filter was measured in a liquid scintillation counter. The conditions that were used involved the absence of Mg^{2+} and a high UDPglucose concn and they produced a glucan with a high proportion of β-1.3 bonds (unpublished results) as indicated in ref. [9].

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