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ESSENTIAL GROUPS AT THE ACTIVE SITE OF *TRAPAEOLUM* INVERTASE

MARÍA INÉS ISLA,* MARTA AMELIA VATTUONE* and ANTONIO RODOLFO SAMPIETRO*†

Cátedra de Fitoquímica, Instituto de Estudios Vegetales, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 461 4000–San Miguel de Tucumán, Argentina

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Key Word Index—*Tropaeolum majus*; Tropeolaceae; invertase; active site; carboxylic groups; imidazole groups; histidyl groups; thiol groups.

Abstract—Amino acid residues involved at the active site of *Tropaeolum majus* invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) were determined. Photo-oxidation and Woodward's reagent K revealed the presence of histidyl and carboxylic groups at the active site of the enzyme. These groups were protected by sucrose and trehalose but not by fructose. Thiol groups were determined with sodium *p*-chloromercuribenzoate and with 5,5′-dithio-*bis*(2-nitrobenzoic acid). These groups were protected by fructose and to a lesser degree by sucrose, but were not protected by trehalose. The pH dependence of V_{max} and K_m at 27° and 37° is consistent with the presence of carboxyl and histidyl residues at the invertase active site. The pK_a values obtained correspond to ionization heats of 1700 cal mol^{-1} and 6820 cal mol^{-1}. The results were consistent with the presence of imidazole, carboxylic and thiol groups at the active site. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Acid soluble invertases (β-D-fructofuranoside fructohydrolases, EC 3.2.1.26) from higher plants are, in general, localized in vacuoles [1]. These enzymes were classified in two groups according to the regulation of their activity, *in vitro*, by the reaction products [2]. The first group of invertases shows complex inhibition of their activity by fructose being fructose an allosteric competitive inhibitor [3], while glucose is a classical non-competitive inhibitor. Proteins are activators of these invertases [4] and activation occurs without suppression of the inhibitory action by the reaction products. As fructose and glucose are present, together with the invertase in the vacuole [1, 5, 6], this type of inhibition by the reaction products may occur in plants. The second group of invertases has simple inhibition kinetics by the reaction products, glucose being a classical non-competitive inhibitor and fructose a classical competitive inhibitor [7]. Proteins are also activators of these enzymes but the activation is accompanied by the suppression of the activity inhibition by the reaction products. Thus, the inhibition by products appears to be without physiological meaning in this invertase group.

This paper is the first study of the functional groups at the active site of the invertase from *Tropaeolum majus* leaves. This enzyme belongs to the second invertase group from higher plants.

RESULTS AND DISCUSSION

The demonstration of histidyl groups in the acid soluble invertase from *T. majus* was performed by photo-oxidation in presence of methylene blue (Fig. 1). The enzyme activity decays *ca* 60% after 40 min of photo-oxidation. Photo-oxidation of *T. majus* invertase in the presence of 60 mM sucrose resulted in only a minor reduction of inactivation rate. On the other hand, fructose, glucose and trehalose did not protect the enzyme even though, according to kinetic studies, fructose and glucose form inhibitory complexes with this invertase [8]. Under the conditions used, a restrained histidine oxidation was found, and tryptophan and tyrosine invertase residues were not affected by the photo-oxidation [21]. In conformity with thiol group estimations, these residues were not affected by the photo-oxidation (not shown). The involvement of histidyl groups in the active site of *T. majus* invertase was confirmed by the ethoxyformylation reaction in the chosen conditions. Figure 2 shows that after 30 min of incubation the invertase activity fell to 40% of its original level. Trehalose and

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*Researchers from the Consejo Nacional de Investigaciones Cientificas y Técnicas, Buenos Aires, Argentina.

†Author to whom correspondence should be addressed.

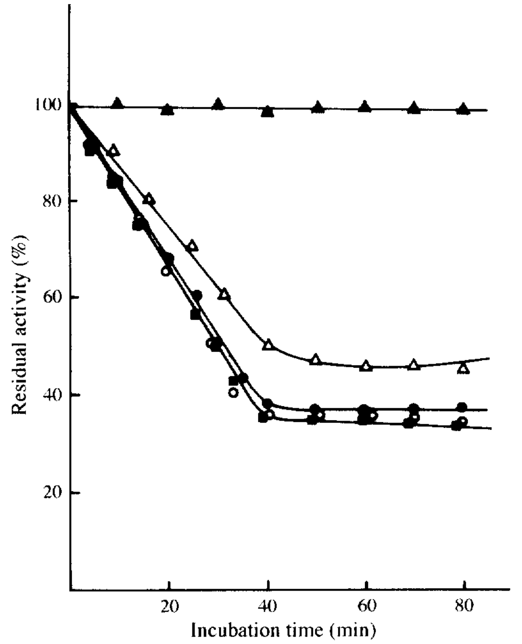

Fig. 1. Effect of photo-oxidation on the activity of the acid soluble invertase from *T. majus* leaves. The reaction was made in the absence (■), and presence (○) of 60 mM fructose; (△) 60 mM sucrose or (●) 60 mM trehalose. Invertase activity in the absence of methylene blue (▲).

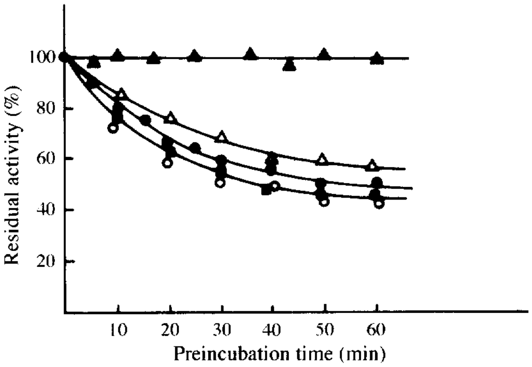

Fig. 2. Effect of diethylpyrocarbonate on *T. majus* invertase activity. The enzyme was preincubated with diethylpyrocarbonate at 0° for different times in absence (■), and presence (△) of 60 mM sucrose; (○) fructose or (●) trehalose. Invertase activity determined after preincubation in absence of diethylpyrocarbonate (▲).

sucrose protected the invertase activity but fructose did not, under the same conditions. According to these results the active site of the invertase from *T. majus* appears to involve the histidyl group. The histidine ethoxyformylation reaction can be reversed by hydroxylamine at neutral pH (Fig. 3). Values were corrected for the effect of hydroxylamine on the original invertase activity.

Figure 4 shows the effect of Woodward's reagent on the invertase activity. Sucrose acts as a protective agent of invertase activity, suggesting the presence of carboxyl groups in the enzyme active site. Trehalose protected invertase activity but fructose again did not.

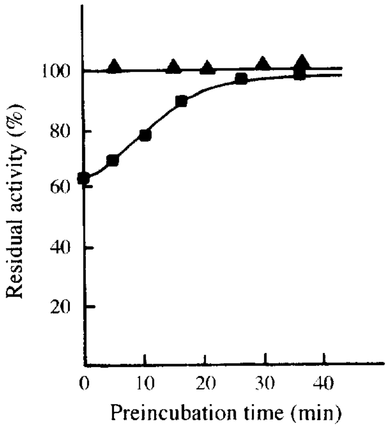

Fig. 3. Decarbethoxylation of the invertase from *T. majus* leaves by hydroxylamine. Invertase was preincubated with diethylpyrocarbonate until the residual invertase activity was 64%. Then, hydroxylamine was added and the preincubation was continued for 40 min. Samples (20 μl) were taken each 10 min for invertase activity determinations. Control of the effect of hydroxylamine on invertase activity (▲).

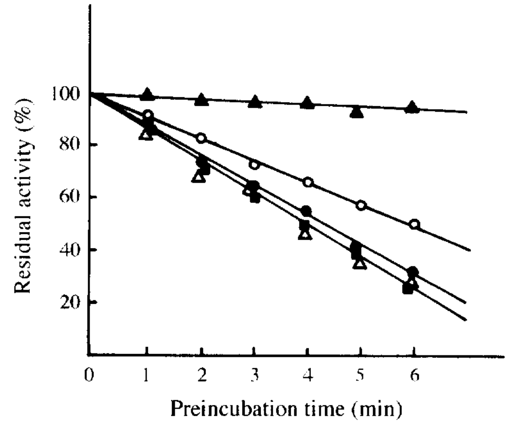

Fig. 4. Effect of Woodward's reagent K on invertase activity. Invertase was preincubated in absence (▲), and presence (■) of Woodward's reagent K, with 40 mM fructose (△); 60 mM trehalose (●), and 60 mM sucrose (○). Then, invertase activity was determined as described in Experimental.

Sodium *p*-chloromercuribenzoate was a competitive inhibitor of the invertase from *T. majus* (Fig. 5). Consequently, the inhibitor is most probably acting at the invertase active site. Sucrose and trehalose did not preserve the invertase activity from the action of this reagent, but fructose had a protective action. The presence of thiol groups at the invertase active site was confirmed by the enzyme inhibition with 5,5′-dithio-*bis*(2-nitrobenzoic acid). Here fructose behaved as a protecting agent of the enzyme activity (residual activity was 50% after 5 min of preincubation), sucrose gave lower protection (42% of residual activity after 5 min preincubation) and trehalose did not. The residual invertase activity after 5 min of

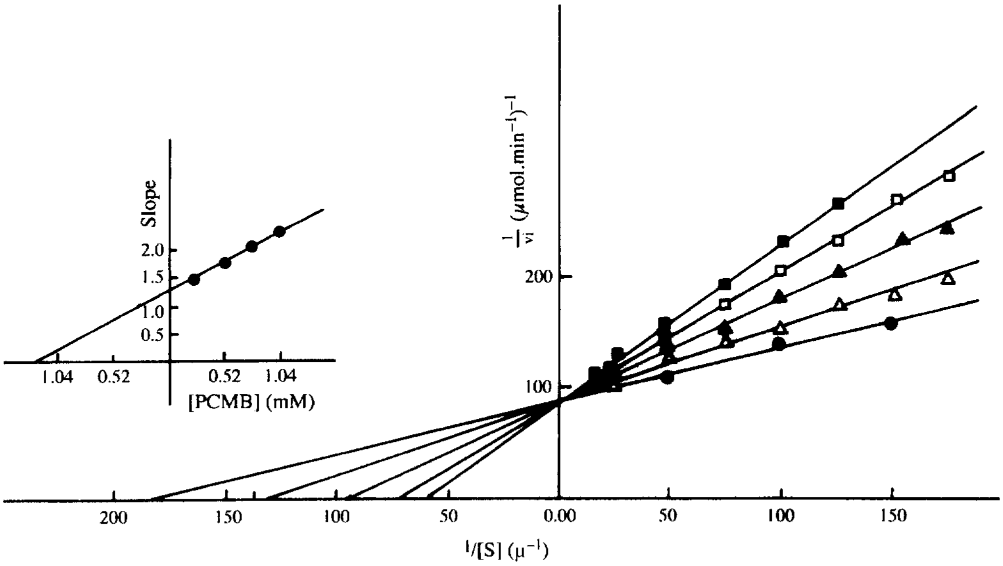

Fig. 5. Lineweaver–Burk plot of the effect of sodium *p*-chloromercuribenzoate on invertase activity and replot of slopes vs sodium *p*-chloromercuribenzoate concentration. Control (●); 0.26 mM (△); 0.52 mM (▲); 0.78 mM (□), and 1.04 mM (■) sodium *p*-chloromercuribenzoate.

preincubation at 4° with 5,5′-dithio-*bis*(2-nitrobenzoic acid) was *ca* 37% (Fig. 6).

Figure 7(A) shows the pH effect on invertase activity (Dixon plot). A pK_a of 6.10–6.30 and a pK_a of 4.45–4.40 were determined from the plots at different temperatures. Approximately the same values for pK_a were obtained when log K_m was plotted against pH [Fig. 7(B)]. These values correspond to ionization heats of 6820 cal mol^{-1} and 1700 cal mol^{-1} that belong to histidyl and carboxylic groups, respectively [20].

According to these results, the active site of *T. majus* invertase appears to involve carboxyl, histidyl and sulphydryl groups. The presence of these three groups has been reported for the invertase active sites of *Phaseolus vulgaris* and *Solanum tuberosum* [22]. The invertase from *P. vulgaris* has not been classified in any invertase group, but the enzyme from *S. tuberosum* tubers has been classified as an invertase from the first group [4] and the invertase from *T. majus* to the second group [8]. Consequently, the same amino acid residues would presumably be present in the active site of both groups of higher plant invertases. The difference between these invertases probably involves structural differences other than the chemical groups at the active site. It is interesting to note the differences between the chemical residues at the active site of these invertases and the invertase from *Schizophyllum commune*, containing carboxyl and probably sulphydryl, but no histidyl groups in the active site of the enzyme [23]. These differences among invertases show the danger of extrapolation of the results of studies on a given enzyme to the same activity in other organisms.

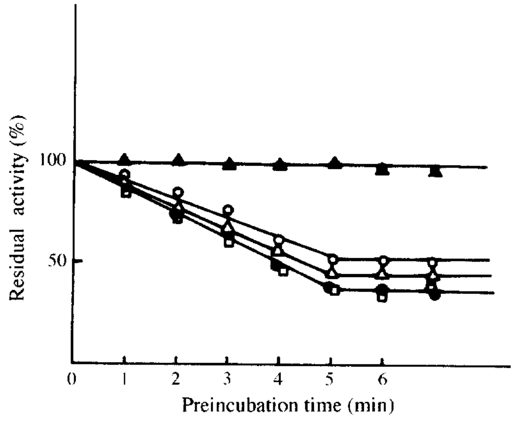

Fig. 6. Effect of invertase preincubation with 1.1 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid) in absence (●), and presence (○) of 60 mM fructose; (▲) sucrose or (□) trehalose. Effect of preincubation time on invertase activity (▲).

EXPERIMENTAL

Plant material. Fully expanded leaves from *Tropaeolum majus* were used [8].

Enzyme purification. The invertase was purified as described in ref. [8]. One enzyme unit was defined as the enzyme amount which yields 1 μmol of product per min. at 37° and pH 5.25. The final prepn contained 4.9 EU ml^{-1} (0.96 mg of protein ml^{-1}) in 10 mM NaOAc buffer, pH 5.25, containing 50 mM NaCl and 1 mM 2 mercaptoethanol (Buffer A).

Enzyme assays. The reaction mixt. consisted of 10 μl of 0.6 M sucrose, 40 μl of 0.2 M NaOAc buffer, pH 5.25, 20 μl of enzyme and H_2O in a final vol. of 100 μl. The reactions were run at 37° and stopped by the

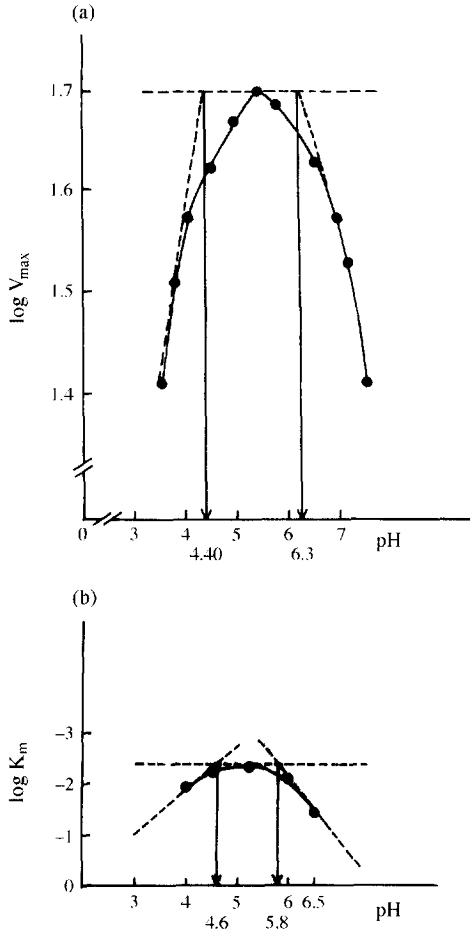

Fig. 7. pH dependence of invertase kinetic parameters. A: effect of pH on V_{max} and K_m. The buffers used were 0.2 M glycine-HCl, pH 3–3.5; 0.2 M NaOAc, pH 3.5–5.5 and 0.2 M sodium cacodylate-HCl, pH 6–7.5. The assay procedure is described under Experimental.

Cu alkaline reagent of ref. [9]. Reducing power was measured by the method of ref. [10].

Protein determinations. Proteins were determined by the method of ref. [11] using BSA as standard.

Polyacrylamide gel electrophoresis. Vertical gel electrophoresis was performed according to the method of ref. [12, 13]. Proteins were stained with Coomassie Brilliant Blue R-250. Invertase activity was revealed with 2,3,5,-triphenyltetrazolium chloride [14].

Photo-oxidation. The method of ref. [15] was used. Assays were also run in the presence of 60 mM fructose, sucrose or trehalose. Controls for enzyme activity with these sugars with and without illumination and in the presence and absence of methylene blue were run for corrections. The method of ref. [16] was used to measure glucose production in the presence of fructose.

Reaction of carbethoxylation. The method of ref. [17] was applied. The reaction of carbethoxylation was made on 0.4 ml (0.38 mg) of enzyme in mercaptoethanol free Buffer A. This reaction mixt. was incubated at 0°, and 20 μl aliquots were taken each 15 min over 1 hr. Controls for the effect of the incubation time, presence of EtOH and effect of K-P_i buffer with and without EtOH on the enzyme activity were included. Invertase activity of these frs was determined as described in *Enzyme assays*.

Reactivation of the carbethoxylation reaction. The reactivation of the enzyme activity was made according to ref. [18]. After hydroxylamine addition the mixt. was maintained at 0° for 40 min and then 20 μl aliquots were taken each 10 min over 40 min. Invertase activity was measured as described in *Enzyme assays*.

Reaction with Woodward's reagent K. The enzyme was filtered through a 1.2 × 7 cm Bio-Gel P6 column equilibrated and eluted with 10 mM MES buffer, pH 5.25, containing 1 mM 2 mercaptoethanol. The reaction mixt. consisted of 0.61 ml of 0.2 M MES buffer, pH 4.7, containing 1 M NaCl. 0.250 ml invertase, 65 μl H_2O and 75 μl of Woodward's reagent K (0.156 M in 1 mM HCl) [19]. The reaction mixt. was incubated at 30° for 10 min. Frs of 75 μl were taken each min and invertase activity was determined. A similar reaction was run in presence of 40 mM fructose and 60 mM sucrose or trehalose. Controls for effect of reagent composition on the invertase activity were run.

Determination of the active site groups by effects of pH and temp. on kinetic parameters. V_{max} and K_m were graphically determined at 27° and 37° and pH values from 3 to 7.5 [20].

Sulphydryl group determinations. The reagents Na *p*-chloromercuribenzoate and 5,5′-dithio-*bis*(2-nitrobenzoic acid) were used for sulphydryl group determinations. The incubation mixt. (final vol. 0.1 ml) consisted of 20 μl (0.019 mg) of enzyme prepn, 40 μl of 0.2 M NaOAc buffer, pH 4.5 or 5.5 for 5,5′-dithio-*bis*(2-nitrobenzoic acid) and Na *p*-chloromercuribenzoate, respectively. The sulphydryl reagents in a final concn of 1.1 mM were used in separate experiments. The mixts were pre-incubated at 4° for 1, 2, 3, 4 or 5 min and invertase activity was determined after substrate addition. Parallel experiments were run including 60 mM fructose, trehalose or sucrose in the pre-incubation mixt.

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