Salt Responses of Enzymes from Species Differing in Salt Tolerance

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ABSTRACT

Enzymes which are affected by the addition of inorganic salts during *in vitro* assay were extracted from salt-sensitive *Phaseolus vulgaris*, salt-tolerant *Atriplex spongiosa*, and *Salicornia australis* and tested for sensitivity to NaCl. In each case malate dehydrogenase, aspartate transaminase, glucose 6-phosphate dehydrogenase, and isocitrate dehydrogenase showed NaCl responses similar to those found for commercially available crystalline enzymes from other organisms. Enzymes extracted from plants grown in saline cultures showed no important changes in specific activity or salt sensitivity. Interaction of pH optima and NaCl concentrations suggests that enzymes may differ in the way they respond to salt treatment.

Enzymes isolated from halophytic bacteria require high salt concentrations during assay for maximal activity (2, 3). It may be inferred that enzymes from these cells are adapted to high cytoplasmic ion concentration. In higher plants, similar adaptations of the metabolic machinery of halophytic species have been proposed to account for salt tolerance (1, 5, 7) as well as for a chloride-induced stimulation of growth in salt-accumulating halophytes (5). Optimal growth of these halophytes is attained with leaf sap concentrations of 200 to 300 mM Cl⁻ (5). Nonhalophytic species are much more salt sensitive, and growth is severely inhibited at these same concentrations.

This paper examines the effect of salts on the *in vitro* activity of a number of enzymes from salt-sensitive and salt-tolerant plants. Also, some of these species were grown in saline cultures, and the effect of this salt treatment on the level and salt response of these enzymes was examined. Each of the four enzymes studied showed a different pattern of sensitivity to salts during assay. However, there were no significant differences in salt sensitivity of enzymes isolated from different species. Moreover, the enzyme response to salts did not change after plants had been grown at high NaCl concentrations.

MATERIALS AND METHODS

Leaf and root material was sampled from plants grown in solution cultures described earlier (15). *Atriplex spongiosa* F.v.M. cultures contained 5 mM NaCl and those of *Phaseolus*

vulgaris L. (var. Hawkesbury Wonder) contained 1 mM NaCl. *Salicornia australis* Banks et Sol was collected from the shores of a saline river estuary (Pelican Point, Nedlands, W. A.). In some experiments the salinity of culture solutions was increased, and this was done at the rate of 50 mM NaCl every 2 days.

Tissues were extracted in 20 or 50 mM Bicine buffer, pH 7.5, with 10 or 50 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and 2% Polyclar-AT. Crude extracts prepared in this way did not inhibit the activity of crystalline enzymes added as internal standards. These extracts were used as enzyme sources during the following assays, all of which were coupled to pyridine nucleotide oxidation or reduction. In each case a 3-ml reaction mixture contained 0.083 mM NADH, NADP, or NADPH. The control assay system for NADH malate dehydrogenase (EC 1.1.1.37) also contained Bicine, pH 7.5, 50 mM; $MgCl_2$, 5 mM; and freshly prepared oxaloacetate, 0.1 mM. Aspartate transaminase (EC 2.5.1.1) control assays contained 5 units of crystalline malate dehydrogenase; Bicine buffer, pH 8.5, at 50 mM; $MgSO_4$, 1.3 mM; aspartate, 5 mM; pyridoxal phosphate, 0.40 μM; and α-ketoglutarate, 0.67 mM. NADPH glucose 6-phosphate dehydrogenase (EC 1.1.1.47) control assays contained Bicine buffer, pH 7.5, at 50 mM; $MgCl_2$, 5 mM; and glucose 6-phosphate, 1.67 mM. NADP isocitrate dehydrogenase (EC 1.1.1.42) control assays contained Bicine buffer, pH 7.5, at 50 mM; $MgCl_2$, 5 mM; and freshly prepared sodium isocitrate, 1.67 mM.

Additions of salts and mannitol were made prior to pH adjustment of buffers. The graphs are constructed with data for a single extract from each plant source, assayed over a range of salt or pH conditions, or both. In all cases the data are representative of several experiments, carried out on extracts from plants grown at different times of the year. Crystalline enzymes from the following sources were used: pig heart malate dehydrogenase (Sigma), aspartate transaminase and isocitrate dehydrogenase (Boehringer), and yeast glucose 6-phosphate dehydrogenase (Boehringer).

RESULTS

Enzyme Response to Salt *in Vitro*. Preliminary experiments showed that Bicine concentrations between 10 and 50 mM did not influence salt response of the enzymes.

Figure 1 shows that enzymes in crude extracts of *Atriplex*, *Salicornia*, and *Phaseolus* leaves responded to NaCl in much the same way as did the crystalline enzymes from other tissues. The pattern of response varied with each enzyme. Low concentrations of NaCl usually stimulated activity whereas
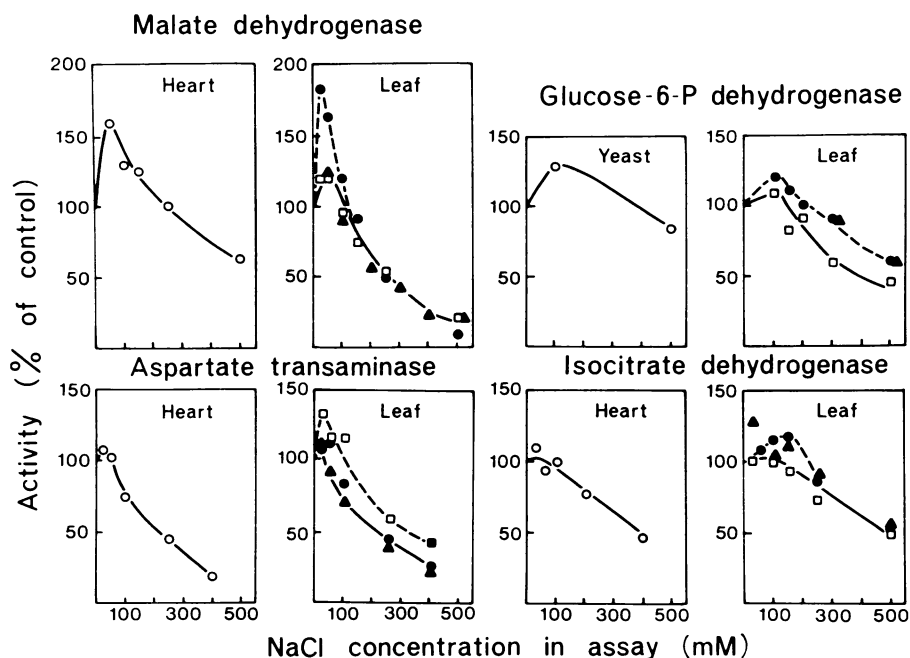
Malate dehydrogenase

FIG. 1. The effect of NaCl during assay on the activity of enzymes. Crystalline enzyme (O); *A. spongiosa* leaf extract (●); *P. vulgaris* leaf extract (□); *S. australis* (▲).
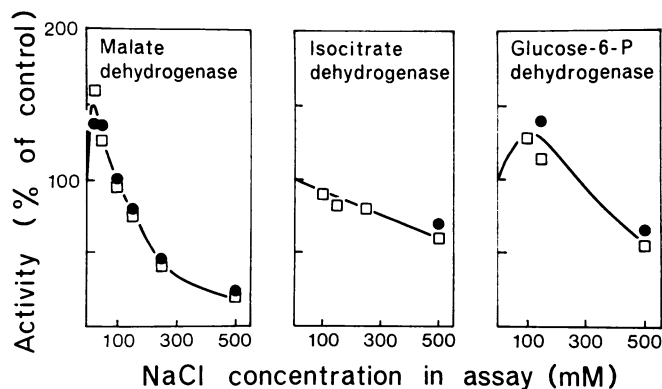

FIG. 2. The effect of NaCl during assay on the activity of enzymes extracted from roots of *A. spongiosa* (●) and *P. vulgaris* (□).

high concentrations were invariably inhibitory. Extracts of salt-tolerant *Atriplex* and *Salicornia* showed marginally higher stimulation of dehydrogenase activity by NaCl than those of salt-sensitive *Phaseolus*. However, there were no striking differences in NaCl response of leaf enzymes from these species. There were also no major differences in the response of enzymes from mature root tissues of salt-tolerant *Atriplex* and salt-sensitive *Phaseolus* (Fig. 2). Glucose 6-phosphate dehydrogenase from *A. spongiosa* roots did show slightly higher stimulation at intermediate salt concentrations and lower inhibition at high concentrations than the enzyme from the roots of *Phaseolus* (Fig. 2).

The effects of NaCl during assay were closely reproduced by KCl treatment (Fig. 3). Sodium sulfate concentrations equivalent to NaCl were less effective in stimulating malate dehydrogenase activity and more inhibitory to aspartate transaminase (Fig. 3). Glucose 6-phosphate dehydrogenase showed a similar response with KCl and NaCl, but Na₂SO₄ stimulated less than NaCl at 150 meq/liter and inhibited more strongly at 500 meq/liter (Table I). These results demonstrate some
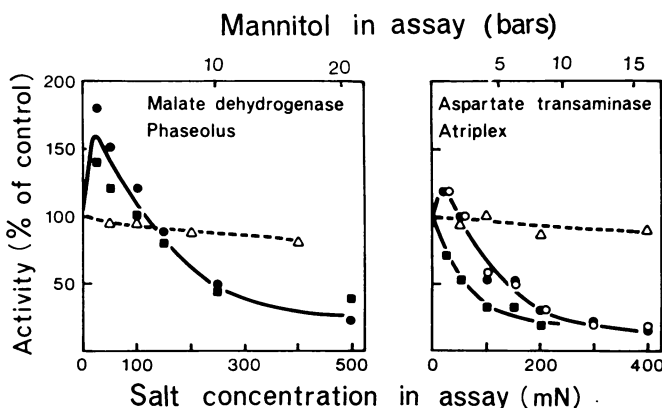
Mannitol in assay (bars)

FIG. 3. The effects during assay of NaCl (●), KCl (O), Na₂SO₄ (■), and mannitol at isosmotic concentrations with chloride salts (△) on the activity of enzymes in leaf extracts. Malate dehydrogenase response to NaCl and KCl was identical.

Table I. *Effect of Some Salts on Activity of Glucose 6-Phosphate Dehydrogenase Isolated from Atriplex spongiosa Leaves*

Activity is expressed as percentage of activity in assay not containing inorganic salts.

Concn	NaCl	KCl	Na₂SO₄
meq/liter			
150	163	153	119
500	70	72	46

specificity of the inorganic anion. The inhibition is not due to low water potentials, as is shown by the insensitivity of the enzymes to mannitol solutions isosmotic with NaCl (Fig. 3).

Nonspecific anion responses are known for several enzymes and are frequently associated with a shift in the pH optima (4). Such shifts might account for the stimulations and inhibitions
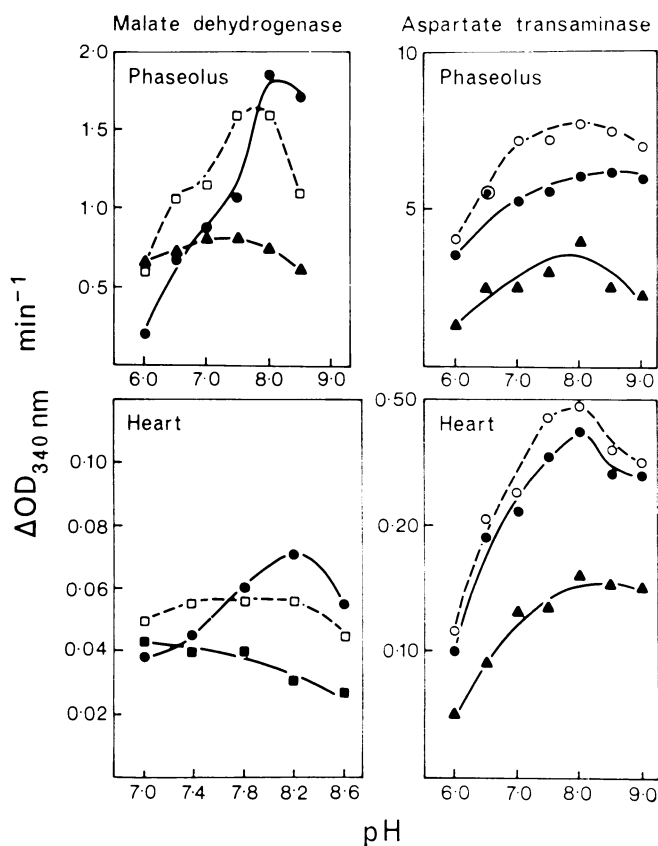
FIG. 4. The interaction of salt level and pH during assay of enzymes in leaf extracts and crystalline enzymes from heart muscle. NaCl concentrations during assay were nil (●), 20 mM (○), 50 mM (□), 200 mM (■), and 250 mM (▲).

Table II. *Effect of NaCl Concentration during Plant Growth in Culture Solutions on the Specific Activity of Some Enzymes*

Specific activity is expressed as a percentage of that for plants grown at 1 mM NaCl. *A. spongiosa* was grown for 12 days, and *Atriplex nummularia* for 3 months, in cultures high in NaCl.

NaCl in Culture Solution	Specific Activity of Malate De-hydrogenase in:		Specific Activity of Glucose-6-P Dehydrogenase in:			Specific Activity of Isocitrate Dehydro-genase in:		
	A. spongiosa leaves	*A. spongiosa* roots	*A. spongiosa* shoots	*A. spongiosa* roots	*A. num-mularia* shoots	*A. spongiosa* shoots	*A. spongiosa* roots	*A. num-mularia* shoots
meq/liter								
10	100	74						
50	97	102	74	142	100	100	123	87
100	89	67	76					
250	84[1]	132[1]	80[1]	44	142	77	124	96
400	114	87		57		84	124	

[1] Mean of two different experiments.

shown in Figures 1 to 3. The effect of NaCl on the pH optima for two enzymes was examined in detail in *Phaseolus* leaf extracts (Fig. 4) and was reproduced in experiments with crystalline enzymes. Substantial shifts in the pH optimum for malate dehydrogenase activity in the presence of NaCl, shown in Figure 4, have also been reported by Weimberg (19). Figure 4 shows that NaCl did not stimulate the activity of this enzyme at its optimal pH. The stimulation of malate dehydrogenase activity by 50 mM NaCl during assay at pH 7.4, for example, is due only to displacement of the pH curve. Higher concen-

trations of NaCl are inhibitory at all but the lowest pH values. On the other hand, 20 mM NaCl causes a true stimulation of aspartate transaminase activity and little change in the pH optimum (Fig. 4). Again, high NaCl concentrations were inhibitory throughout the pH range. Glucose 6-phosphate dehydrogenase showed a NaCl *versus* pH response similar to that of aspartate transaminase, whereas the response of isocitrate dehydrogenase resembled that of malate dehydrogenase. None of these pH-salt interactions change the previous conclusion, that enzymes isolated from very salt-sensitive and from very salt-tolerant species had about the same sensitivity to salt.

Enzyme Response in Plants Grown at High Salinity. A number of experiments were conducted with *Atriplex* seedlings grown in culture solutions containing up to 400 mM NaCl. These plants showed markedly enhanced growth in the cultures containing up to 200 mM NaCl. *Phaseolus*, when treated with as little as 150 mM NaCl, grew very poorly. In all experiments, these long term salt treatments, which lasted between 5 days and 3 months, did not induce major changes in the specific activity of the malate dehydrogenase, glucose-6-P dehydrogenase, and isocitrate dehydrogenase (Table II), or of aspartate transaminase. Furthermore, the pH optima of these enzymes were unaltered (results not reported here), and the response to added NaCl during assay was unchanged (Fig. 5).

DISCUSSION

Enzymes isolated from salt-sensitive *Phaseolus* and salt-tolerant *Atriplex* and *Salicornia* were equally sensitive to NaCl during assay. This was established for four different enzymes, which included the rather salt-sensitive aspartate transaminase as well as salt-tolerant glucose-6-P dehydrogenase. Furthermore, growth of *Phaseolus* and *Atriplex* in saline cultures failed to alter the specific activity, or NaCl sensitivity, of the enzymes studied. These data thus support and extend the observations of Weimberg (20, 21). Weimberg found that different isozymes of malate dehydrogenase, isolated from various plant species, responded similarly to NaCl (20). Moreover, Weimberg (21) showed that a variety of salinity treatments failed to alter the specific activity of many enzymes and the isoenzyme patterns of malate dehydrogenase in pea leaf, stem, and root. The data conflict with those of Porath and Poljakoff-Mayber (6, 16, 17), who reported that two of the
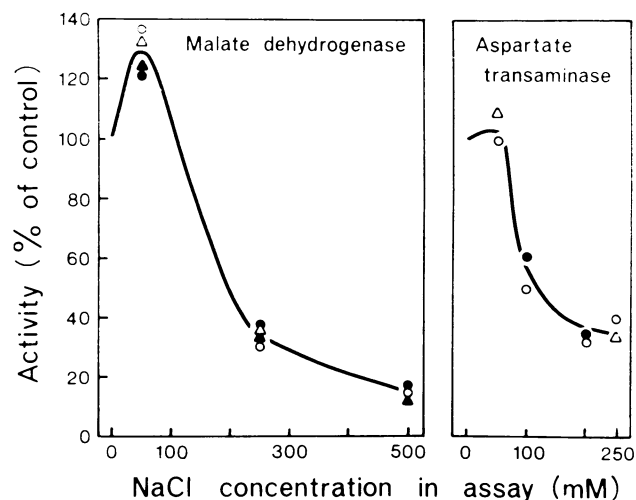

FIG. 5. The effect of NaCl in culture solutions during plant growth on the sensitivity to NaCl during assay of enzymes from *A. spongiosa* leaves (12 days growth at 1 mM [○], 50 mM [●], 250 mM [△], and 400 mM [▲] NaCl).

enzymes tested in the present study showed marked response in specific activity to NaCl and Na₂SO₄ salinity in pea root. However, in the experiments reported here and in those of Weimberg (21), whole roots were used, whereas Porath and Poljakoff-Mayber used only the root tips (16, 17).

The indifferent response of enzyme levels *in vivo* to salt treatment during growth raises the question of the significance of salt effects on plant cell metabolism. There are few direct metabolic effects of salinity which have been studied in sufficient detail to assess the interaction at the level of individual enzymes. In pea root tips an apparent increase in pentose phosphate pathway activity was associated with increased specific activity of glucose-6-P dehydrogenase (16). In many tissues Cl⁻ treatment decreases the incorporation of ¹⁴CO₂ into organic acids, relative to amino acids (13, 18). It has been suggested that this decrease in malate synthesis is induced by a Cl⁻ inhibition of malate dehydrogenase (19). However, the data presented in this paper show that it would be difficult to ascribe this shift from malate to aspartate labeling to a differential effect of NaCl on activity of malate dehydrogenase and aspartate transaminase.

The failure of plant enzymes to respond significantly to long term NaCl treatment of intact plants contrasts with very definite responses to Ca^{2+}. Striking parallels between the adaptation of *Lemna minor* to calcium status of the growth media and the calcium response of malate dehydrogenase have been reported (8).

It is possible that the inorganic ion interaction with enzymes is an artifact of the isolated protein in aqueous solution. In other words, the organization of the cytoplasm is such that enzymes do not respond to inorganic anions as they do *in vitro*. If so, it would be an interesting artifact in that isoenzymes do not differ in NaCl response (20), but different enzymes may show quite different types of responses. Alternatively, the cell may regulate ionic environment of the cytoplasm so that enzymes are not normally exposed to high salt levels. The latter suggestion presumes that species differ in the degree to which the ion concentration can be regulated within cell compartments, in the face of increasing ionic supply to the tissue as a whole. When this regulation breaks down, components of the metabolic machinery may respond to salts as described above.

In the following discussion only the chloride ion is considered, because in most natural habitats this is the principal anion which accumulates in vascular plants. If, for example, *Phaseolus* tissue is unable to regulate the distribution of 300 mM NaCl within the leaf tissue, this level of ions could interact with enzymes as it does *in vitro*. In *Atriplex*, the same tissue concentration of NaCl has no inhibitory effect on growth (5), possibly because the tissue is able to regulate cytoplasmic ionic concentration. For example, in *A. spongiosa*, grown in 250 mM NaCl, the leaf lamina contained approximately 130 mM chloride. In contrast, the chloride level was 720 mM in epidermal bladders, which function as a salt-secreting system (14). Judging from the data presented in this paper and by Weimberg (19), cytoplasmic Cl⁻ concentrations in the region of 50 to 100 mM would not lead to severe inhibitions of enzyme activity. The ionic status of the cytoplasm is difficult to specify, but the control exerted by the cell membranes responds very rapidly to changes in external concentration (11). Presumably similar events occur at other cytoplasmic membranes.

Chloride exclusion from specific cell compartments in turn raises problems of osmotic adjustment. In the following we assume that Cl⁻ accumulation in the vacuole has achieved osmotic adjustment with the saline environment. At equilibrium, the water potential of cellular compartments which exclude Cl⁻ must be the same as the water potential of the rest of the

cell. Preferentially high Cl⁻ accumulation in the vacuole may be compensated by increased concentrations of other solutes in the cytoplasm. These solutes might be either neutral compounds or ions which inhibit enzyme activity less severely than the Cl⁻ and SO_4^{2-} salts tested in the present experiments (*cf.* Ref. 19).

Little is known of ionic activity in the cytoplasm of plant cells. Studies with giant algal cells suggest that the streaming cytoplasm has a lower ionic content than the chloroplasts and the vacuoles (9). Aqueous and nonaqueous preparations of chloroplasts from a variety of tissues (9) show high ionic activities (0.1–1.3 M). Chloroplasts in *Limonium* leaves increase in salt content when salt excretion from the leaf by glands is inhibited (10), suggesting dynamic regulation of the ion content of different cellular compartments. Whether these changes in ionic status have any metabolic effects has yet to be determined.

Response of some carboxylating enzymes to salt treatment *in vitro* is reported in the following paper (12).

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