Hofmeister Effects in Biology: Effect of Choline Addition on the Salt-Induced Super Activity of Horseradish Peroxidase and Its Implication for Salt Resistance of Plants

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The effect of choline addition on the salt-induced super activity of horseradish peroxidase (HRP) is investigated. HRP is presented in the literature as an efficient H_2O_2 scavenger, and choline is the precursor of glycine betaine, a strong osmoprotectant molecule. Both the regulations of H_2O_2 and of osmoprotectant concentrations are implicated in plants in order to counteract salt-induced cell damage. For the oxidation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sulfate anions were found to play a crucial role in the increase of HRP activity. This induced super activity can be strongly reduced by adding choline chloride. The phenomena provide an example of physicochemical Hofmeister effects playing a central regulatory role in an important biological system.

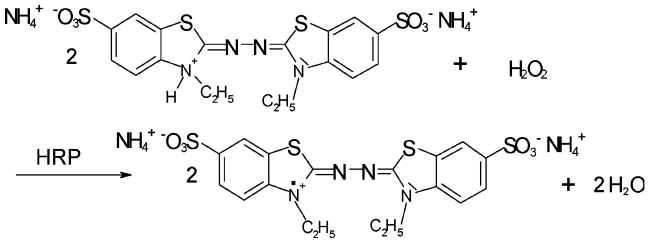
Introduction

In plants, seeds, or vegetable cells, osmotic and oxidative stresses are often related. In the culture media of these organisms it was observed that the addition of salt induced an increase in H_2O_2 concentration and a subsequent increase of peroxidase activity.[1−3] Peroxidases are enzymes that serve as efficient H_2O_2 removers.[4] A comparison between salt-nontolerant tomato and salt-tolerant tomato has shown a relation between damaged membrane lipids and the activities of peroxidases on one hand and the salt concentration outside the cells on the other hand.[5] Thus, salt tolerance appears as a recurrent important problem in the optimization of transgenic plants that have to be modified to improve specifically their tolerance to both biotic (pathogens, wounding, etc.) and abiotic (light, ozone, temperature, drought, salinity, flooding, heavy metals, etc.) stresses. Some considerable work has been done in order to develop salt-tolerant plants. Recently, for example, overexpressions of peroxidase genes of tomato have been shown to confer increased salt-tolerance to tobacco seeds.[6]

Plants may also counteract osmotic stresses by producing osmoprotectants such as zwitterionic compounds (proline), sugar alcohols (mannitol), or quaternary ammonium compounds (glycine betaine and choline-O-sulfate). It has been shown that the last mentioned category of osmoprotectants develop particularly as a consequence of salinity stress, see, for example, ref 7.

It is well recognized that not only salinity, expressed as ionic strength, but also the type and the concentration of buffers, pH, and specific ion effects influence enzymatic activity. In addition, specific ion effects are often observed to follow the Hofmeister series of anions.[8]

SCHEME 1: Oxidation of ABTS by HRP and Hydrogen Peroxide



In a recent work[9] the influence of sodium salts (Na_2SO_4, NaCl, NaBr, and $NaNO_3$) on horseradish peroxidase (HRP) activity was investigated through the classical oxidation reaction of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) by H_2O_2 according to Scheme 1.

In that paper it was found that the catalytic efficiency trend followed the usual Hofmeister series for anions (that is, as posed from the cosmotropic (salting-out) to the chaotropic (salting-in) direction: $SO_4^{2-} > OH^- > F^- > CH_3COO^- > Cl^- > Br^- > NO_3^- > I^- > ClO_4^- > SCN^-$). All added salts changed the pH of the buffer. But in addition to this "bulk" effect, other effects of the ions on the catalytic efficiency were found with opposite deviations from the pure pH effect for cosmotropic and chaotropic anions, whereas negligible deviations from the pure pH effect due to the addition of the salt was observed for the "Hofmeister-neutral" NaCl salt.

In the present work the focus is on the super activity of HRP, acting as a H_2O_2 remover, induced by adding different ion pairs. Those chosen for comparison were Na_2SO_4, which contains the highest cosmotropic anion, tetramethylammonium sulfate (TMAS), tetramethylammonium bromide (TMAB), and choline chloride (CH). This choice was made since it is known that quaternary ammonium salts are active as osmoprotectants or precursors of osmoprotectants.[7] The choice of the choline chloride instead of the sulfate salt arose from the observation that Cl^- anions appear to be more or less "neutral" Hofmeister

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ions. Only at ionic strength $I \gg 1$ mol/L does chloride have a moderate inhibitory effect on HRP enzymatic activity.

Materials and Methods

Chemicals. The enzyme, peroxidase type VI from horseradish peroxidase (HRP) was purchased from Sigma; citric acid monohydrate >99.5% ($C_6H_8O_7 \cdot H_2O$) and trisodium citrate dihydrate 99% ($C_6H_5O_7Na_3 \cdot 2H_2O$) were purchased from Acros; 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) >99% was from Fluka; hydrogen peroxide solution 30% (H_2O_2) and sodium sulfate >99% (Na_2SO_4) were from Merck; tetramethylammonium bromide >98% ($C_4H_{12}NBr$) was from Lancaster; tetramethylammonium sulfate hydrate, purum ($(C_4H_{12}N)_2SO_4-H_2O$) was from Fluka; choline chloride, minimum 98%, was from Sigma. Purified water with an electrical conductivity of 0.054 μS/cm was used to prepare all aqueous solutions. Tetramethylammonium sulfate hydrate and choline chloride were dried before using.

pH Measurements. The pH measurements of buffer and salt solutions were performed with a combined glass electrode and a pH meter Consort C835.

The water used to prepare the solutions was purified by passage through a Millipore Milli-Q system. All readings were made at the constant temperature of 25 ± 0.1°C.

Peroxidase Assay. A typical experiment was performed using 10 μL of a 0.012 M ABTS solution in citrate buffer (25 mM, pH 5) that was added to 2.9 mL of the buffer solution with or without salt. Then, 10 μL of a buffer solution containing 0.0262 M H_2O_2 was added. The reaction was started by adding 10 μL of a buffer solution containing HRP (31.25 mg). The kinetics of ABTS oxidation was studied via UV spectroscopy at 25 ± 0.1°C using a Varian Cary 3E spectrometer. The progress of the reaction was estimated measuring the absorbance, at 414 nm, of the oxidized form of ABTS that was detected during the first 4 min after the addition of HRP.

The initial velocity, V, in mol L^{-1} s^{-1}, of the enzymatic reaction was inferred from the slope of the absorption intensity versus time in s. A linear trend was observed, at least during the first few minutes.

To reduce the uncertainty of the activity measurements due to variations in the reproducibility of the initial enzyme solutions, a relative activity (A) was calculated according to the relation $A = V/V_0$, where V is the initial velocity measured in the actual experiment (different pH of the citrate buffer or different amounts of the various additives) and V_0 is the initial velocity measured in the corresponding enzymatic preparation in citrate buffer (5 mM, pH 5) without any added salt. For each of the different reaction compositions at least three samples were prepared, and the reaction velocities of these samples were measured independently at 25 ± 0.1 °C to check the reproducibility. The estimated reproducibility in the velocity is within ±5%.

Results and Discussion

Effect of pH on the Enzymatic Activity of HRP. A series of measurements were performed to ascertain the dependence of the catalytic activity on the pH. To avoid modifications of the ionic strength and possible effects due to small amounts of added strong acids and bases, different citrate buffers, always 25 mM, were prepared. The HRP activity assay was performed at different pH values. The initial velocity, V_0, measured in the buffer at pH = 5 was chosen as a reference to evaluate the relative activity $A = V/V_0$ at each pH value. Figure 1 shows the results obtained in the pH range 3.8−5.5. Data can be described

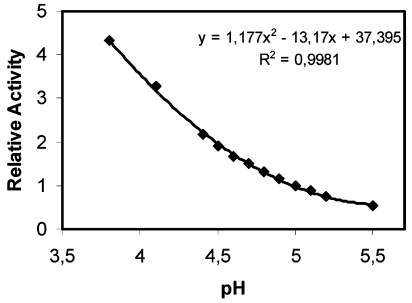

Figure 1. Effect of pH (citrate buffers 25 mM prepared at different citric acid/sodium citrate ratios) on the enzymatic activity of HRP.

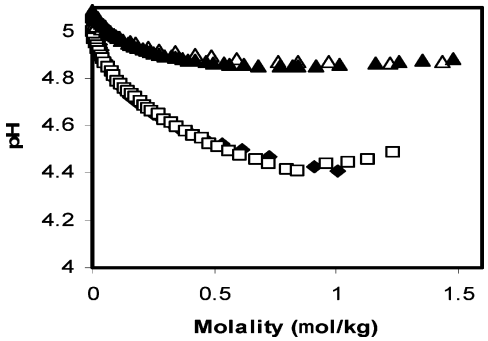

Figure 2. Effect of the concentration of different salts on the pH of a citrate buffer (25 mM) at initial pH = 5: (◆) Na_2SO_4, (□) TMAS, (△) TMAB, and (▲) CH.

by a second-order equation that will be useful to predict expected pH effects (see below). Clearly a decrease of the pH of the buffer medium favors a marked increase of the activity of HRP. Once the effect of pH had been established, the initial condition of pH = 5 was chosen as the reference medium to investigate the effect of salt addition. There are two advantages in this choice. One is the observation that the addition of some sodium salts to a citrate buffer at pH 5 showed a marked decrease of pH with increasing ionic strength;[9] hence, super activity of HRP may here be expected upon salt addition due to this pH effect. The second point is the fairly obvious one that extreme pH values should be generally avoided when dealing with enzymes. Indeed, deactivation, denaturation, or hydrolysis phenomena of protein easily occur particularly at low pH values.

Effect of Salt Addition on pH Measurements. It is first necessary to check the influence of the different salts on the pH of the reaction medium, that is, the citrate buffer 5 mM at pH = 5. We recall that the salts considered are Na_2SO_4, TMAS, TMAB, and CH. It is also worth noticing that, in terms of the language of Hofmeister series, the sulfate anion and sodium cation are classified as cosmotropic ions (especially the sulfate ion), whereas quaternary ammonium cations can be considered as chaotropic ions, and bromide and chloride anions reside in the intermediate range of the series. By analogy with the other quaternary ammonium ions, a chaotropic role can be expected for the choline cation.

As it can be seen from Figure 2, all added salts decrease the pH = 5 of the initial buffer solution. The origin of this decrease is not certain, and its cause is usually attributed mainly to the electrostatic interactions between the different ions, as explained elsewhere.[9] Clearly, the "cosmotropic power" of the sulfate ions decreases the pH values much more than choline chloride and tetramethylammonium bromide. The reason for that is again a matter of debate, but these uncertainties in theories of electrolytes do not affect our main conclusions. Most remarkable, in

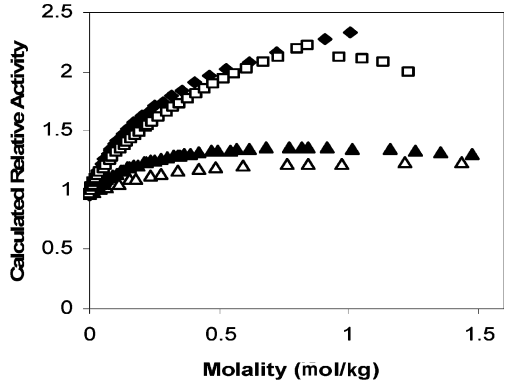

Figure 3. Calculated relative activity of HRP as a function of salt concentration using the pH−activity relation from Figure 1 and the pH−salt concentration relation from Figure 2: (◆) Na_2SO_4, (□) TMAS, (△) TMAB, and (▲) CH.

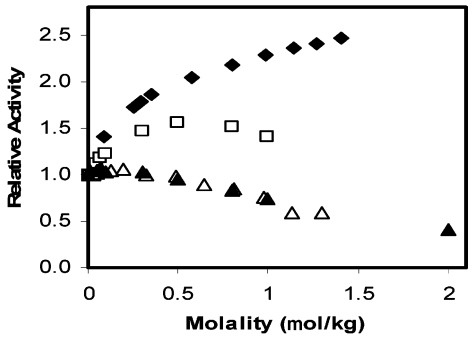

Figure 4. Experimental relative activity of HRP as a function of salt concentrations: (◆) Na_2SO_4, (□) TMAS, (△) TMAB, and (▲) CH.

the view of our aims, is that at high concentrations the pH goes up again, except for the sodium sulfate solutions.

Effect of Salt Addition on the Enzymatic Activity of HRP. Now, if the pH changes were the only effect of the salts on enzymatic activity, it would be straightforward to take the results obtained in Figure 2 to predict the enzymatic activities with the help of data in Figure 1. This is done in Figure 3. As Na_2SO_4 and TMAS decrease the pH values more significantly, they are expected to cause the largest increase of enzymatic activity.

To check the validity of this hypothesis, the enzymatic activities of HRP were determined in the presence of the four salts at different concentrations. The results are given in Figure 4. The differences between the calculated curves (Figure 3) and the experimental results (Figure 4) are significant, except for the Na_2SO_4 solutions. From data of Figures 1−3, that is, on the basis of pH decrease, the super activity in the case of sulfate solutions is nearly quantitatively predicted, whereas for TMAS salt it is significantly lower, particularly at high ionic strength, and goes through a maximum at concentrations that are lower than expected from the pH variation, see Figure 2.

In agreement with the predictions of Figure 3, in the presence of TMAB and CH the activity of HRP should not have been changed significantly. Eventually, a slight increase of activity could have been expected for these halogen salts when added at concentrations around 0.5 m. Experiments clearly indicate that both TMAB and CH induce a significant decrease of the activity at $c > 0.5$ m and that their influence on the relative enzymatic activity is similar.

Two important conclusions can be deduced from these results.

First of all, it can be stated that the addition of salts produces effects that can partly be explained by pH changes. But there are additional specific effects. Whereas for the specific ion pair

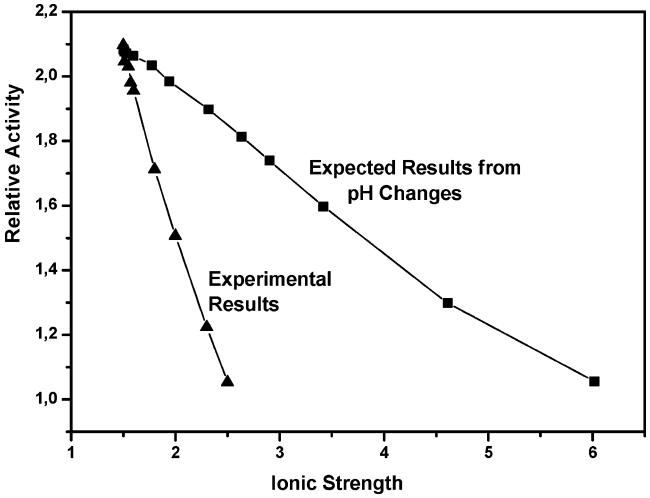

Figure 5. Relative activity of HRP in a buffer solution containing 0.5 m Na_2SO_4 as a function of added choline chloride from 0 to 1 m. The triangles show the measured results, whereas the squares indicate hypothetical results in the case where only the measured pH values of the mixtures were responsible for the relative enzymatic activity (as inferred from Figure 1).

Na_2SO_4 the pH effect is dominant, this is not so for TMAS. It seems that TMA is a powerful chaotropic "Hofmeister antagonist" of sulfate, in contrast to Na^+.

Choline as a Specific Inhibitor of the H_2O_2 Remover Activity of HRP? To look closer on these antagonistic roles of quaternary ammonium ions and sulfate, we used Na_2SO_4 to induce super activity in HRP and CH to act in the opposite direction. To this purpose we measured again the relative HRP activity $A = V/V_o$ in the presence of 0.5 m Na_2SO_4 in the citrate buffer 25 mM, initially prepared at pH = 5, but this time as a function of increasing CH concentration. In parallel, we measured the pH values of the mixtures and used again the data shown in Figure 1 to infer the expected curve, if only pH effects were present. Figure 5 shows the results.

It is remarkable that the relative activity decreases linearly with increasing CH concentration. For CH at 1 m (corresponding to a total ionic strength of 2.5 m) the initial activity, measured in 25 mM citrate buffer at pH 5, is restored. Interestingly, this is one charge of choline per one charge of sulfate. The effect is much more pronounced than what could be expected from a pure pH effect.

Possibly, Figure 5 reflects an inhibitor role of choline on salt-induced super activity of peroxidases in living cells. When a high salt concentration, especially of cosmotropic ions, induces a super activity in enzymes, an increasing choline concentration in the cell can reduce or even prevent such a super activity. It is probable that sulfate ions have a strong interaction with proteins due to the double charge on the anion. But obviously, the organic ions have a more pronounced direct influence on the enzymatic activity. Probably, the active site is more hydrophobic (as with most of the enzymes), and therefore the interactions with the more hydrophobic ions, although expected to be less pronounced, have a bigger effect on the enzymatic activity than the direct electrostatic interactions of the protein with sulfate ions.

The chaotropic nature of the choline can of course also influence other enzymes and could be at the origin of the enhanced salt tolerance of transgenic plants that have choline oxidase genes in their genome and are cultivated in a medium containing added choline.[10]

Concluding Remarks

From the experiments, the following points can be inferred:

(i) The addition of salts to protein buffer solutions induces pH changes that depend on the nature and the concentration of the ions.

(ii) The resulting influence on HRP enzymatic activities is not only a consequence of the pH change, but also there are specific ion effects acting directly on the protein. If there are such effects acting on the protein, then there might well be such effects affecting pH due to specific ion effects acting on the glass electrode. That problem remains unresolved.

(iii) The super activity induced by sulfate ions can be balanced by a stoichiometric amount of choline counterions.

(iv) As HRP, a typical peroxidase, and choline show antagonistic behaviors concerning H_2O_2 global consumption, the addition of choline chloride cannot be considered as a good choice of an osmoprotectant agent, because choline has the unwanted side effect of reducing the activity of HRP. This may therefore increase the oxidative stress in a cell. However, in a living cell, other enzymes (e.g., the choline oxidase in bacteria and fungi) may rapidly decrease the concentration of choline or transform it to the betaine that may be less inhibitory to HRP. However, these phenomena are beyond the scope of the present paper.

(v) Whatever the ultimate causes of the specific ion phenomena we have observed, being due to surface adsorption near enzyme surface or bulk water effects, it is clear that Hofmeister effects are not just an esoteric matter, but play an important direct regulatory role in plant biology, see also ref 11.

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