Kinetics Assay of Kinnow Serum Acid Phosphatase

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Summary: Acid phosphatase activity has been measured spectrophotometrically for the first time, in Kinnow serum by the release of phenol from phenyl phosphate esters in buffer of pH 5.0. Various salts were added in the reaction mixture to observe their effect on the activity of enzyme.

Introduction

It has been known and well established for a long time that along with tartaric, citric, ascorbic acids and other constituents, there is varying amounts of phosphomonoesterase activity present in the tissues, seeds and juices of almost all citrus fruits[1]. This groups of enzymes are often enzymes of comparatively low speceficity, although some in the group, especially those acting on sugar phosphates, are quite highly specific.

Depending on their pH optima, phosphomonoesterases are divided into acid or alkaline phophoesterases. Acid phosphatases are widely distributed in plants, bacteria and animals. Resolution of the details of the tertiary structure by x-ray crystallography has not yet been achieved for any of the phosphatases. Despite the fact that glucose-6-phosphatase has not yet been purified to homogenity, since it has been studied in detail, the kinetic studies with this enzyme has indicated that the reaction proceeds by a two step transfer mechanism in which a phosphoryl enzyme is formed as an intermediate and this phosphate group has been shown to be bound to a histidine residue in the protein[2]. Lately there has been developed a great interest in studying this group of enzymes due to their role as indicators of various pathological conditions[3].

Citrus fruits production in Pakistan has increased significantly and among them, Kinnow has become very popular due to its adoptation to conditions of Pakistan. In fact among tangrines family, Kinnow has been fully developed into its present shape by Pakistani agriculture scientists and throughout the world. Kinnow is only grown in Pakistan. We have undertaken a research project to obtain information concerning the quantitative distribution of acid phosphatase at various stages during the growth of Kinnow fruit, the factors involved in its activation and inhibition and to kinetically measure the rate of enzyme catalyzed hydrolysis of organic phosphate monoesters and report our findings in this paper. Information regarding quantitative measurements of various nutrients in Kinnow juice could be found in ref. 4 and 5 .

Experimental

Materials

Fresh Kinnow serum sample was obtained by extracting the juice and centrifuging it to remove all suspended tissues and pigments. Its pH was noted and stored for the day at cold temperature. Solutions were prepared in distilled/deionized water. The substrates, phenyl phosphate (sodium salt) was purchased from E. Merck Inc. and m-

nitrophenyl phosphate (potassium salt) was a gift from Dr. K.R. Lynn, Division of Biological Sciences, National Research Council of Canada.

Kinetic measurements

These were made on a Pye-Unicam SP-8400 spectrophotometer at 20°C by the following method to determine first order rate constants for the enzyme catalysed hydrolysis reactions.

One ml of Kinnow serum solution was diluted to ten ml by adding 0.1 M acetate buffer of pH 5.0. To 1.90 ml of acetate buffer (pH5.0) was added 1.0 ml of above mentioned diluted serum solution containing acid phosphatase enzyme. A 0.1 ml aliquot of the substrate (2.5×10^{-2} M in buffer) was added at zero time to one cuvette, the other containing the reference sample, which was identical in all respects except that no substrate was added. Successive spectra were recorded at measured times thereafter, and the "infinity" spectrum ($t\infty$) was recorded generally after the reaction had proceeded over night ($>8\ t^{\frac{1}{2}}$).

Results and Discussion

The mechanism of action of acid phosphatases from various sources has been investigated in detail by number of workers using kinetics and other techniques[6,7,8]. The following scheme has been suggested for the catalyzed reactions where

$$E + S \underset{k_{-i}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P_1 + E^*S \xrightarrow{k_3} E + P_2$$

E,S,P, and P_2 are respectively, enzyme, substrate, released first product (alcohol or phenol) and second product (phosphate). The slowest or rate determining step being k_3, where N-phosphoryl histidine species has been detected [9] on the intermediate ES*.

Effect of Substrate and Substrate Concentration

The following figure records the successive spectra during the progress of hydrolytic reaction of phenyl phos-

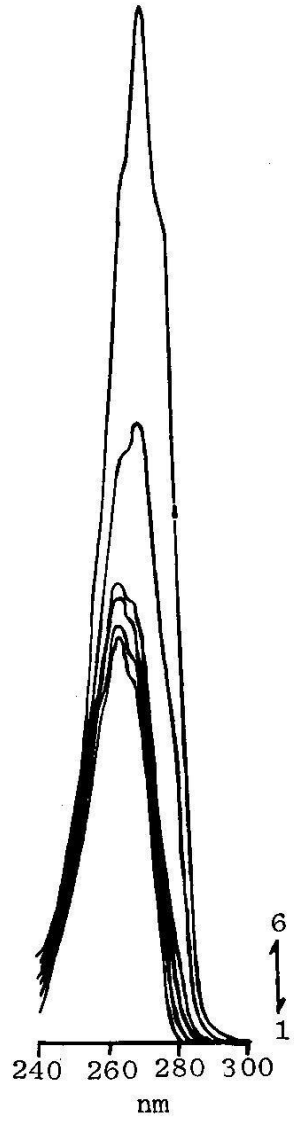

Fig.1: Spectra recorded during acid phosphatase-catalysed hydrolysis of phenyl phosphate under the conditions described in the text. Successive measurements were made at (1) 1.5 m, (2) 10 m, (3) 24 m, (4) 51 m, (5) 242 m, (6) t_∞

phate to release phenol. Under the similar condtions, there was almost no hydrolysis of substrate in the absence of enzyme.

The observed pseudo-first order rate constant k_2 (at subsaturating conditions) was calculated as $k_2 = 4.08 \times 10^{-4}$ sec^{-1} from the slope of the plot between time and log $(A_\infty - A_t)$. When m-nitrophenyl phosphate was employed as substrate, an activation effect on catalysed reaction was noted from the observed rate $k_2 = 4.55 \times 10^{-4}$ sec^{-1}. This might be due to the better fit of the substrate at the active site of the enzyme.

The reaction was also studied under varying amounts of substrate concentrations. It was observed that the reaction velocity increased with raising the substrate concentration and was in conformity with the behaviour displayed by the enzyme acting on a single substrate.

Inhibition and Activation

The following table summarizes the effects of various salts upon phosphatase activity.

Table-1:Effect of Various Salts on Phosphatase Activity

Salt Added	Conc	Relative Activity
—	—	100
Nacl	1 mM	110
$MgCl_2.6H_{20}$	3 mM	200
$(NH_4)_2\ SO_4$	5 mM	87
$Na_2C_2O_4$	1 mM	126

It has already been mentioned by some workers that the acid phosphatase enzyme appears to require a divalent metal cation for its maximum activity [10]. The metal ion may bind to the negative charges of the phosphate group of the substrate, and then the histidine residue may make a nucleophilic attack upon the phosphorus atom of the substrate.

As recorded in the above table, Mg^{+2} ion caused a 100 percent increase in activity as compare to the control reaction (containing no added salt) whereas ammonium sulfate inhibited the reaction velocity by about 13%. Since ammonium sulfate is most commonly employed salting out reagent in protein chemistry, the enzyme may be somewhat denatured by its presence resulting in lower activity of enzyme.

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