Kinetics of phenylalanine and tyrosine ammonia-lyase enzymes activity of banana fruit (*Musa cavendishii* L., cv. Enana).

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**ABSTRACT**

**Objective:** This investigation was carried out to study the characterization and Kinetics of crude Phenylalanine and Tyrosine Ammonia-lyase enzymes activity (PAL and TAL) of banana fruit.

**Methodology:** Optima and stability of pH and temperature, optimum substrate concentration and metal ions as inhibitors were determined for the PAL and TAL enzymes activity in banana fruit.

**Results:** Results evidenced that the stability of enzyme activity was constant to 80 °C and its activity retained over a large range of pH (6.7 – 8.8). Also, 30°C was the optimum temperature at pH 8.8. While, 11mM was an optimum L-phenylalanine and L-Tyrosine substrate concentration for PAL and TAL enzyme activity. The enzyme activity was increased in the metal ions presence like PI, NaCl and PCl, but decreased in the presence of calcium chloride (CaCl), Ferric chloride (FeCl) and Zink chloride (ZnCl) ions. Also, the results indicated that the Vmax and Km values were 0.15 and 1.45 for PAL enzyme activity and 0.101 and 0.618 for TAL enzyme activity, respectively.

**Conclusion:** The present study exposed that the banana fruit is a prolific source of PAL and TAL enzyme activity having high pH and thermo stability and it can be used for commercial scale operations.

**Key words:** Banana, L-phenylalanine, L-Tyrosine, ammonia-lyase, enzyme, stability, Kinetics, metal ions

**Introduction**

Banana (*Musa cavendishii* L., cv. Enana) fruits are generally changes in texture, flavor and color in fresh fruit by ripening. Although, total phenolic content was relatively high in the edible fruit part compared with many other plant parts. It is well recognized that different phenolics are obtained from phenylalanine by cinnamic and coumaric acids. In ripening banana, phenolic compound accumulation is ready visible and easily measured change. In general, a fruit change in color was associated with ripening and processing. Phenolic compounds were derived from flavonoid compounds and synthesized by the aromatic amino acid phenylalanine. Phenylalanine ammonia-lyase (PAL) is the most important of the enzymes which in controlling biosynthesis of phenolic compounds from phenylalanine, and to be synthesized de novo in many plant tissues by UV light and mechanical damages (Tucker, 1993; Teresa et al., 1998). The high difference in phenylalanine concentration between treated and untreated food especially in egg white and mushroom that due to the presence of TAL and PAL enzymes activity in the extracted solution (Hesham et al., 2017).

Phenylalanine ammonia lyases (PAL) and tyrosine ammonia lyases (TAL) are enzymes which seem to be confined to certain fungi and to higher plants. PAL and TAL enzymes in higher plants are link between aromatic amino acids metabolism and a lot of the secondary biosynthetic activity of the plant. Phenylalanine ammonia lyase (PAL; E.C.4.3.1.5) catalyzes the removal of ammonia from L-phenylalanine to produce trans-cinnamic acid. This reaction is the first step in the formation of phenylpropanoid in plants (Koukol and Conn, 1961; Jones, 1984). In all higher plants and in some fungi analyzed to date PAL enzyme activity has been found, but not in animals or bacteria. This enzyme is the most important of the best research in plants because of the importance of PAL in the lignin pathway and other secondary metabolities, (Campbell and Ronald 1996; Hyun et al., 2011; Kyndt et al., 2002; Andrea and Christine, 2013). L-tyrosine is stimulates to produce trans-p-hydroxycinnamic acid by using tyrosine ammonia lyase (TAL; E.C.4.3.1.23). Some results provided that the enzyme activity of PAL in dicot and monocot plants, but the enzyme activity of TAL was linked with the enzyme from

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monocots (Rössler et al., 1997; Khan et al., 2003). As pointed out by Rössler et al. (1997), PAL and TAL activities dwell on the same polypeptide and have very similar catalytic efficiencies, despite large differences in Km and Vmax.

It has been showed PAL enzyme activity increases during the minimum processing due to the injuries which happen in the plant cells (Saltveit, 1997). Consequently, increasing of PAL enzyme activity leads to increment substrate concentration (phenolic compounds) for oxidative enzymes, such as PPO and POD. According to Saltveit, (2000), enzyme activity increase of PAL, PPO and POD is a response to the compress undergone by cut the tissue, which caused reduced shelf-life. However, PAL enzymes regulate the phenolic pathway through activating conversion of phenylalaine to transcinnamate (Ke and Saltveit, 1989).

In addition, PAL and TAL enzymes activity after external treatment and processing of pH, heat and kinetics have not been studied in banana before. There is very little documentation on the kinetics and characterization of Phenylalanine and Tyrosine Ammonia-lyase activities in fruit. This investigation was carried out to study the characterization and Kinetics of crude Phenylalanine and Tyrosine Ammonia-lyase enzymes activity (PAL and TAL) of banana fruit.

Materials and Methods

Banana Fruit Samples

Banana (Musa cavendishi L., cv. Enana) fruits grown at Egypt were obtained from a local supermarket in Cairo. These were placed in refrigerator at 3-4°C for 4 hours until used. The banana fruits were washed, peeled, and pulped using a blender.

Extractions of PAL and TAL enzymes

PAL and TAL were extracted by the method of Lister et al. (1996). The homogenate was filtered through four layers of cotton cloth and then centrifuged at 5000 rpm (HERMLE z323k, Germany) for 20 min at 4°C. The collected supernatant was used as a crude enzyme extract.

Assay of PAL and TAL enzymes activity

PAL and TAL enzymes activity was assayed by a little modification on the method of Peixoto et al. (1999) and Nita-Lazar et al. (2002) using a reaction mixture of 0.06M sodium borate buffer (pH 8.8) containing 11mM L-phenylalanine for PAL or 11mM L-tyrosine for TAL and 0.4 mL of PAL or TAL crude enzyme, with a final volume of 2.4 mL. Tubes were incubated at 30°C for 2 h, and the reaction was stopped by 35 g trichloro acetic acid (0.6 mL) /100mL. After the tubes were centrifuged for 5 min at 5000rpm to pellet the denatured proteins, the absorbance at 290nm for PAL and 333nm for TAL was measured by a Shimadzu spectrophotometer UV-2401PC, UV-VIS recording spectrophotometer, Japan. One unit of the PAL or TAL enzyme activity was explained as the amount of enzyme that precedes a change of 0.001 in absorbance per hour.

Properties of PAL and TAL crude enzymes activity of banana pulp

pH optima of crude PAL and TAL enzymes activity

The optimum pH was determined through incubation of the enzyme in a group of different concentrations of sodium borate buffers of pH from 5.7 to 10.8 and the reaction was completed at 25 °C for 2 hr.

Optimum temperature of crude PAL and TAL enzymes activity

The optimum temperature was determined for enzyme activity of the reaction mixture through incubation temperature changes from 20 to 80 °C.
**pH stability of crude PAL and TAL enzymes activity**

Incubation of crude enzyme extract (0.4 ml) at 30 °C for 30 min in an buffer solution (0.8 ml) at different pH values. pH was differed in the range of 5.7 and 10.8 of 0.06M sodium borate buffer (pH 8.8). L-phenylalanine and L-Tyrosine (11mM) used as a substrate. PAL and Tal enzyme activity was determined as a residual percentage of enzymes activity at the optimum pH.

**Temperature stability of crude PAL and TAL enzymes activity:**

Eppendorf tubes with the crude enzyme solutions incubated in a water bath for 5 min at different temperatures (20, 30, 40, 50, 60, 70, 80 °C). PAL and TAL activity was determined at 30°C, using L-phenylalanine and L-Tyrosine (11mM) as a substrate. The residual percentage of PAL and TAL enzyme activity was calculated compared with unheated enzyme.

**Effect of substrate concentration of crude PAL and TAL enzymes activity:**

The different L-phenylalanine and L-Tyrosine substrate concentrations (from 5.5mM to 60mM) were used to determine the optimum substrate concentration.

**Effect of metal ions as inhibitors on PAL and TAL enzymes activity:**

The enzymes activity assayed through incubation of the enzymes with different metal ions at a concentration of 0.18Mole/L of assay buffer. Each metal ions experimental was repeat for 3 times (n=3) for PAL and TAL enzymes activity.

**Results**

A study of crude PAL and TAL enzymes preparations extracted from banana pulp leads to the current results. A combination of isoenzymes may be resulted in enzyme production, which considered from the properties of the crude, as opposite a purified enzyme. Also probably were found interactions with non-enzymatic proteins. Due to PAL and TAL enzymes complex nature, it is habitual to indicate to the characteristics of crude enzyme preparations as `apparent' quantities. The characteristics and kinetics explained below are as long as apparent quantities even where the apparent has not been used. However, the characteristics and kinetics of a crude enzyme production can be as related to the Food manufacture as those of the purified or isolated enzyme.

**pH optima of crude PAL and TAL enzymes activity:**

The activity of pH profiles for crude Phenylalanine Ammonia-lyase (PAL) and crude Tyrosine Ammonia-lyase (TAL) are seen in Figure 1. A maximum activity of PAL was around pH 8.8, but for TAL was pH 8.8 with a narrow plateau between pH 8.4 and 9.8. As showed from the Figures, the PAL and TAL enzymes activity extracted from banana pulp were nearly decrease at pH 10.8. The optimum pH be based on variety, kind of phenolic substrates and extraction methods. The pH optimum of PAL and TAL enzymes did not represent to have been mention before.

The enzyme Phenylalanine and Tyrosine Ammonia-lyase activity towards Phenylalanine and Tyrosine were demonstrated as a function over a wide range of pH 5.7-10.8 (Figure 1). Results showed that pH optimum was found 8.8. About 98.4% activity, relative to that determined was detected at pH 8.8; however reduced activity was found at pH 10.8. The optimum pH for banana PAL and TAL, was reported to be dependent on cultivars and experimental factors used during the determinations, range from 5.7 – 10.8 (Janovitz-Klapp and Nicolas, 1989). These results were found to be in accordance with Oktay et al. (1995) who mentioned the maximum activity of PAL and TAL from banana fruit at pH 8.8 with Phenylalanine and Tyrosine as a substrate. The acidic pH is common for banana PAL and TAL. However, some investigators showed a single pH optimum at pH 5.5-6.0 (Janovitz-Klapp and Nicolas 1989). Whereas, others indicated the presence of two pH optima, one at about pH 8.8 and the other at about pH 6.0 (Stelzig et al., 1972).
Fig. 1: Effect of pH on PAL and TAL enzymes activity (unit/hour).

In general, pH is an important agent that effectiveness the catalytic activity of Phenylalanine and Tyrosine Ammonia-lyase. Ionization changes of prototropic groups in the active site of an enzyme might prohibit proper conformation of the active site, binding of substrates, and/or catalysis of the reaction was at higher alkali and lower acid pH values (Whitaker, 1994). In addition, the catalytic activity of enzymes affected by the protein denaturation and lowering in the stability of substrate.

**Temperature optima of crude PAL and TAL enzymes activity**

The temperature activity profiles for crude Phenylalanine Ammonia-lyase (PAL) and crude Tyrosine Ammonia-lyase (TAL) are seen in Table 1. 30°C for 1 minute was the optimum temperature of PAL and TAL enzyme activity. 21.6% of PAL activity was lost whereas was 29.4% of TAL activity by increasing the optimum temperature. Obviously, crude TAL enzyme is more sensitive to the temperature increase in enzyme examination than crude PAL enzyme.

The temperature effect between 20 and 80 °C at different times were assayed and the results are cited in Table 1. The optimum temperature for activity of banana PAL and TAL enzyme activity was 30 °C. The enzyme activity decreased with increasing temperature and time showing less activity at 70 and 80 °C. Results findings is in agreement with Duangmal and Owusu-Apenten, (Duangmal and Apenten 1999) they found that high temperature heat-denaturation of the enzyme placed after incubation at 60 °C for 10min., the descent in enzyme activity is indeed due to the evident tertiary structure of the enzyme to form the secondary structure. Differences in the pH and temperature optimum for PAL and TAL have been reported by several workers (Kyndt et al., 2002; Andrea and Christine, 2013; Lister et al. 1996; Peixoto et al. 1999; Nita-Lazar et al. 2002; Moffitt et al., 2007; Vannelli et al., 2007; Amy et al., 2008). However, Andrea and Christine, (2013) showed that 8.0–8.5 pH optimum, 32 °C temperature optimum and metal reliance on the chloride salts of potassium, magnesium, ferrous and sodium of PAL-enzyme extracted from *Trichosporon cutaneum*.

**pH stability of crude PAL and TAL enzyme:**

The relative percentage activity of PAL and TAL enzymes from banana pulp with various pH values (5.7 - 10.8) are seen in Figure 2. Both enzymes were quite similar of the pH-stability profiles and not stable at pH below 8.8. The retention of PAL and Tal enzyme activity was > 95% at pH 8.8.

**Temperature stability of crude PAL and TAL enzymes activity:**

Temperature-stability profiles (relative percentage activity) for crude of PAL and TAL enzymes are seen in Table 1. Although both enzymes (PAL and TAL) take identical temperature constant profiles, the decrease in percentage relative activity of crude PAL enzyme at high temperature was lower than that of crude TAL enzyme, which is may be the outcome of changes in the enzyme tertiary structure. Both PAL and TAL enzymes were inactivated to 85-88% by heating at 80 °C for 5 min and to partial (72%) inactivation by heating at 60°C for the same time. PAL and TAL enzymes are not rated
a heat-stable enzyme. Partial or total irreversible destruction of both enzymes catalytic activity were enough by short exposures to temperatures of 80°C (Vamos-Vigyazo, 1981). Whereas, Scott, (1975) found that, enzyme inactivation at low temperature was supposed to be due to the formation of intra molecular hydrogen bonds prevent adequate unfolding of the molecule.

![Graph showing pH activity profiles of PAL and TAL enzymes activity](image)

**Fig. 2:** pH activity profiles of PAL and TAL enzymes activity.

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<th>TAL (unit/hour)</th>
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**Table 1:** Effect of temperature and time on PAL and TAL enzyme activity (unit/hour) and relative activity profiles (%).

**Effect of different metal ions (inhibitors) on the crude PAL and TAL enzymes activity:**

The effects of different metal ions (CaCl, NaCl, PI, PCI, ZnCl, and FeCl) on crude PAL and TAL enzyme activity were seen in Figure 3. The control of 100% activity was compared with that of the percentage inhibition. FeCl and ZnCl were the most effective ion inhibitor on TAL enzyme followed by CaCl and NaCl, but FeCl and CaCl were the most effective ion inhibitor on PAL enzyme followed by ZnCl and NaCl.

The effect of sodium chloride ions on crude PAL enzyme activity was nearly the same pattern to that of crude TAL enzyme activity. The effect of sodium chloride ions on the inhibition of enzyme activity was greater than PI and PCI. However, the concentration of NaCl used, 0.18Mole/L, the relative inhibition demonstrated less than 48.5% for PAL and 42% for TAL compared to the control sample.
Results described that the inhibitory effect of NaCl was not enough; this may be related to the low concentration of NaCl. It is confirmed that, the inhibition was due to interaction between sodium chloride and copper at the active centre of the enzyme (Iyengar and McEvily, 1992; Sapers, 1993; Martinez and Whitaker, 1995).

**Fig. 3:** Effect of different ions on PAL and TAL enzymes activity (unit/hour).

**Effect of Substrate concentration of crude PAL and TAL enzyme activity:**

It was also found that the increased of PAL and TAL enzyme activity with increasing of substrate concentration up to 11 mM, following first order kinetics, then reduced the enzyme activity. This may be due to maximum saturation, as seen in Figure 4. Simple amino acids such as phenylalanine and tyrosine are commonly used as substrates for Phenylalanine and Tyrosine Ammonia-lyase enzyme activities assay in spite of that it may not always found in association with the enzyme (Dogan et al., 2002). The results obtained during Phenylalanine and Tyrosine oxidation by PAL and TAL enzyme are given in Figure 4.

**Fig. 4:** Effect of substrate concentrations (mM) on PAL and TAL enzymes activity (unit/hour).

The maximum activity was 11 mM Phenylalanine and Tyrosine with increasing the concentration a corresponding decline in activity was noticed. The low activity with high substrate concentration could be attributed to:

a)- Enzyme denaturation which can continuously cause a relative decrease of the enzyme concentration in the reaction system.

b)- The inhibition of the enzyme activity increased by the product formed.

C)- The reverse reaction converting the product back into the initial reactant after the concentration of the product increased or the oxidation products may change pH or temperature of PAL and TAL about the optimum values (Belitz and Grosch, 1999).

The results mentioned above concerning the effect of pH, temperature and substrate concentration derived from the study of crud enzyme collection of isoenzymes and the reaction with nonenzymatic
proteins. These characteristics comparatively expresses obvious quantities, even where the detailing has not been used. However, the characteristics of a crude enzyme preparation could be used in the food manufacture than those of the purified enzyme preparation (Duangmal et al., 1999). There is little work on the inhibition of PAL and TAL by its oxidation products, and the mechanism is not clear. In a recent attempt to substantiate the hypothesis of an inhibition of banana PAL and TAL by oxidation products.

**Kinetic parameters (Km and Vmax values) of crude PAL and TAL enzyme:**

The kinetic parameters of the crude PAL and TAL enzymes were determined by using Phenylalanine and Tyrosine and calculated from Lineweaver and Burk plots. However, the Michaelis-Menten plot and Lineweaver-Burk plot are used for kinetic analyzes of data. While a plot of v as a function of CS yields a hyperbolic curve, the double reciprocal plot provides a straight line that is suitable for the estimation of the kinetic constants by linear regression (Alev Bayındır, 2010). The line relationship between 1/V vs. 1/[S] resulted in a straight line with 1/Vmax (intercept) and KM/Vmax (slope). This linear relationship was titled a Lineweaver-Burke. Plot 1/V vs. 1/[S] to obtain a straight line with a y-intercept = 1/Vmax and a slope =KM/Vmax. This plot is titled a Lineweaver-Burke plot. Then, maximum velocity of reaction (Vmax) and Michaeli’s constant (Km) values were 0.15 and 1.45 for PAL enzyme activity and 0.101 and 0.618 for TAL enzyme activity, respectively. Vannelli et al. (2007) reported a PAL/TAL ratio ((Vmax/Km) Phe /(Vmax/Km)Tyr) of 0.8, while we observed a ratio of 0.63. This implies that the enzyme is intrinsically a PAL and not a TAL enzyme

**Discussion**

The current discussions were derived from a study of crude enzyme preparations extracted from banana fruits. However, the properties of a crude enzyme preparation can be as relevant to the food industry as those of the purified or isolated enzyme. The results showed that the optima and stability of pH and temperature, optimum substrate concentration and metal ions as inhibitors for the PAL and TAL enzymes activity in banana fruit.

The general properties of the crude PAL and TAL enzyme were studied. The studies on the effect of different temperatures and pH on the activity of PAL and TAL enzymes revealed that with the rise in temperature and pH. The results showed that PAL and TAL enzymes are very abundant in pulp of banana fruit. Also, PAL, PPO and POD enzymes activity was increased result to the stress undergone through cutting of tissues, which leads to reduced shelf-life (Saltveit, 2000; Maria et al., 2011). Also, PAL, POD and PPO, and reduced disease incidence caused by Alternaria alternata in pears (Tian, et al., 2006) However, it was found that the enzyme is very stable and effective at pH 8.8 of PAL enzyme and it is also stable of pH from 5.7 to 10.8 of TAL enzyme. Results signified that pH has very little influence on the activity and stability of enzyme, as seen in figures 1 and 2. This result is similar to that reported about the good stability of enzyme in slightly acidic pH values (Tian et al., 2006). Generally PAL and TAL enzymes are thermo stable up to 30°C. The PAL and TAL enzymes extracted from banana pulp has also shown activity up to 50°C, with 50% loss activity. It was also found that the increased of PAL and TAL enzymes activity with increasing of substrate concentration up to 11mM, the enzyme activity also increased up to 0.177 (unit/hour) for PAL and 0.183 (unit / hour) for TAL enzyme, following first order kinetics, then reduced the enzyme activity. This may be due to maximum saturation, as seen in Figure 4. These results are similar to that reported by Zouari-Mechichi et al. (2006) about the substrate specificity of enzymes showed the highest activity toward a non-phenolic heterocyclic compound, while the activity with the phenolic substrates was much lower (Ibrahim et al., 2011; Sadak et al., 2015; Dogbo et al., 2012). The results investigated that the effect of metal ions on PAL and TAL enzyme activity, as seen in figure 3. Results indicated that PI, NaCl and CaCl have very low effect, but FeCl and ZCl have high effect on the PAL and TAL enzymes activity. Also, PCI have clear effect in increasing the enzyme activity. It was found that Na+, Cl− and K+ have low effect on the enzyme activity or clear effect in increasing the enzyme activity. However, Ca++, Fe++ and Zn have clear effect in reducing the enzyme activity. Mabrouk et al. (2013), found that the metal ions KCl and NaCl have similar effects on the enzyme activity where the enzyme retained about 71% of its initial activity, the other metal ion CaCl2 have little effect on the enzyme activity.
Conclusion

Results concluded that the optimum pH was 8.8 and optimum temperature was 30 °C for PAL and TAL enzymes from banana fruit. Results revealed that the FeCl and CaCl were the most effective ion inhibitor on PAL and TAL enzymes activity followed by ZnCl and NaCl. Also, the results indicated that the Vmax and Km values were 0.15 and 1.45 for PAL enzyme activity and 0.101 and 0.618 for TAL enzyme activity, respectively.

The present study exposed that the banana fruit is a prolific source of PAL and TAL enzymes activity having high pH and thermo stability. Also, it can be applied for commercial scale operations.

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References


