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Characterization of β-galactosidase in the Crude Plant Extract of *Artemisia judaica* L. in Presence and Absence of Some Heavy Metals

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Abstract: β-galactosidase (EC 3.2.1.23) is important in the formation of a medicinal plant *Artemisia judaica* (al-ba'atharan) aroma. The crude plant extracts of *Artemisia judaica* were used to characterize the enzyme in the term of pH, temperature, enzyme kinetic and effects of some heavy metals on its activity. The enzyme activity was measured by its ability to hydrolyze the substrate 2-nitrophenyl β-D-galactopyranoside (ONPG). The enzyme activity was reached maximum at 50°C and at pH 6.0. The K_m and V_{max} values of the enzyme were 3.6 mM and 1.67 µmol/min, respectively. Uncompetitive inhibition was observed in presence of Hg^{+2}, Fe^{+3} and Zn^{+2} for the enzyme β-galactosidase in the crude extract through the decrease in the K_m and V_{max} values. Pb^{+2} and Cu^{+2} were found to act as a noncompetitive inhibitors on the enzyme β-galactosidase in the crude extract due to increase in the K_m values and decrease in V_{max} values. The study showed that Hg^{+2} was the most potent inhibitor while Cu^{+2} exhibited the least inhibition degree on β-galactosidase activity in the *Artemisia judaica*. These finding indicated that the enzyme β-galactosidase in the crude leaves extract of *Artemisia judaica* can be used in industrial and medical applications.

Keywords: Al-ba'atharan, β-galactosidase, Enzymatic Kinetics, Heavy Metals

1. Introduction

β-galactosidase (also β-D-galactohydrolase) called lactase and transglycosylases [1] are group of enzymes able to cleave β linked galactose residues from various compounds and is commonly used to cleave lactose into galactose and glucose [2], it was widely distributed in nature and found in many microorganisms, plant and animal tissues [3, 4, 5, 6]. β-galactosidases have many biological roles include degradation of structural polysaccharides in plant cell walls; thereby can promote their loosening and the consequent elongating of the cell [7, 8]. They have many medical and industrial applications include treatment of lactose malabsorption and production of lactose hydrolyzed milk [9, 10, 11]. These enzymes have two important applications: the removal of lactose from milk products for lactose intolerant people and production of galactosylated products [12, 13, 14, 15]. β-galactosidases have been detected in a wide range of plant organs and tissues and are described by their ability to hydrolyze terminal non-reducing β-D-galactosyl residues from β-D-galactosides [16]. It has been purified from various plant sources, like chick pea [17], almond [6], apricots [18], *Vigna unguiculata* [19], apricot seed [20]. β-galactosidase play key roles in fruit ripening. β-galactosidase activity was reported during fruit development and ripening for rice [21], pepper [22] and Arabidopsis [23]. Many studies have indicated remarkable increase in expression level of mRNA β-galactosidase during fruit ripening in many fruits [24, 25]. It was reported that β-galactosidases are widely distributed in many plant tissues, like seeds [6, 20], stems [19], and meristem zones of roots, trichomes, cotyledons, vascular tissues, and pollens [26, 27]. On the other hand, it also participates in the cell wall modification during elongation and differentiation of plant cells [28, 29]. Plant β-galactosidase would be best suited for industrial applications because of its easy availability, cost effectiveness and easy adaptability [30]. β-galactosidase from almond seeds was used for the preparation of delactosed milk for those lactose-intolerant individuals [6].

Heavy metals are essential and important for plants growth, and play a great role in many vital compounds [31]. Some of these metals are micronutrients necessary for plant growth, such as Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+}, while others have unknown biological function, such as Cd^{2+}, Pb^{2+} and Hg^{2+} [32]. At high concentrations, all heavy metals have strong toxic effects and are regarded as environmental pollutants [33, 34]. They may alter the reaction rates and influence the kinetics properties of enzymes which cause changes in metabolism of plant, or any excessive amount of heavy metals may drive the oxidative stress [35].

Artemisia judaica L. (Wormwood), known al-ba'atharan in Arabic, is a perennial fragrant shrub, bushy herbs, grows in the valley bottoms in the desert areas particularly at the Southern part nearest Saudi-Jordan borders (Al-Mudawarah) and in Sinai Peninsula in Egypt [36, 37, 38]. It is used traditionally as a medicinal herb in Jordan and Egypt. *Artemisia* has multiple beneficial bioactivities such as antiviral, antipyretic, antihemorhagic, anticoagulant, antitumor, anti-anginal, anti-ulcerogenic and anti-hepatitis [39, 40]. In this original study, the crude plant extracts of leaves of Artemisia *judaica* L. were used as a source for the enzyme β-galactosidase. The enzyme activities, kinetics and the effects of heavy metals were investigated as a primary step for the use of β-galactosidase in industrial, biotechnological and medical applications in future.

2. Materials and Methods

2.1. Materials

Fresh plant sample of *Artemisia judaica* L. was collected from Wadi Rum (South Jordan) during February-August, 2014. Characterization of β-galactosidase was conducted at the Biochemistry lab in Mutah University.

2.2. Crude Plant Extract Preparation

Crude plant extract was prepared from leaves of *Artemisia judaica* and used as source for β-galactosidase. Plant leaves were homogenized in 100 mM sodium-phosphate buffer (pH 6.0) in a blender for 4 min. The homogenate was filtered using cloth sheet and then was centrifuged for 20 min at 10,000 rpm. The supernatants were used for β-galactosidase assay as crude enzyme solution [22].

2.3. Protein Estimation

Protein concentration was determined by Lowry *et al*., [41] using Bovine Serum Albumin (BSA) as standard.

2.4. Enzyme Assay

Crude plant extract was prepared from leaves of *Artemisia judaica* and used as source for β-galactosidase. β-galactosidase activity was assayed by measuring the rate at which it hydrolyzes ONPG by the method described by Sekimata *et al*. [42]. In the presence of β-D-galactosidase, ONPG is hydrolyzed to D-galactose (colorless) and *o*-nitrophenol (ONP) (yellow). The reaction mixture of β-galactosidase contained 0.4 ml of 0.1 M acetate buffer (pH 4.0), 0.5 ml of 2 mM of substrate and 0.1 ml of enzyme solution. After incubation for 15 min at 37°C, the reaction was terminated by addition of 1 ml of 0.1 mM Na_2CO_3 and monitored at 420 nm. One unit of enzyme activity is defined as the amount of enzyme that liberates 1.0 µmol of ONP per minute under the assay conditions.

2.5. Determination of Kinetic Parameters

To determine the maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) of β-galactosidases, ONGP was used as substrate, and the effect of substrate concentration on enzyme activity were studied at pH 6.0 and at temperature 50°C. The concentration of ONGP was increased from 1 mM to 9 mM. The enzyme activity was assayed by monitoring the absorbance at 420 nm. Line weaver-Burk Plot (Reciprocal plots) used to determine Vmax, and Km values [43].

2.6. Effect of pH on Enzyme Activity

The optimal pH of β-galactosidases was determined by incubating it at 50°C in various buffers with different pH values ranging from 2.0 to 9.0 according to Gulzar and Amin [18]. The enzyme assay was performed separately in each buffer system. Relative activity (%) was calculated.

2.7. Effect of Temperature on Enzyme Activity

The optimal temperature of β-galactosidases was determined by incubating the reaction mixtures at various temperatures ranging from 20°C to 90°C, and the activity was expressed by relative activity (%).

2.8. Effect of Different Heavy Metals on Enzyme Activity

The effect of various metal ions (Hg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+3} and Pb^{+2}) on β-galactosidase activity was determined through incorporated them in the standard assay mixture at different concentrations ranging from 100-900 µM. The activity was expressed as relative activity (%) compared to control.

2.9. Statistical Analysis

All experiments were implemented in triplicates and the results are expressed as mean values ± standard deviations (SD) using Microsoft excel 2007.

3. Results and Discussion

3.1. Protein Content

The protein content in the crude extract of leaves of *Artemisia judaica* was measured by Lowry method using BSA as standard protein (Figure 1). The result showed that the crude extract of leaves of *Artemisia judaica* has (0.56 mg/ml) amount of protein.

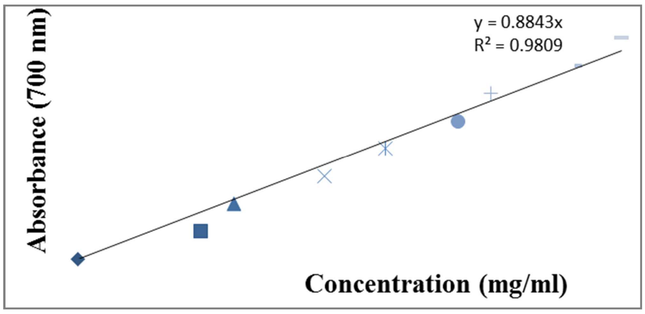

Figure 1. Determination of protein content (mg/ml) in crude extract of leaves Artemisia judaica using BSA as standard.

3.2. Effect of pH on β-galactosidase Activity

Each enzyme has an optimum pH at which it performs best. Any changing in pH will cause alteration in the enzyme structure and affecting their activity. As pH increases or decreases, certain amino acids are deprotonated or protonated, thereby changing the proteins conformation and activity [44].

The β-galactosidase activity was found to vary with pH values (Figure 2). The optimum pH of the enzyme activity was 6.0 and the enzyme was stable at pH from 2.0 to 9.0. The relative activities of β–galactosidase at pH 2.0, 3.0, 4.0, 5.0, 7.0, 8.0 and 9.0 were 28%, 45%, 62%, 87%, 67%, 37% and 24%, respectively.

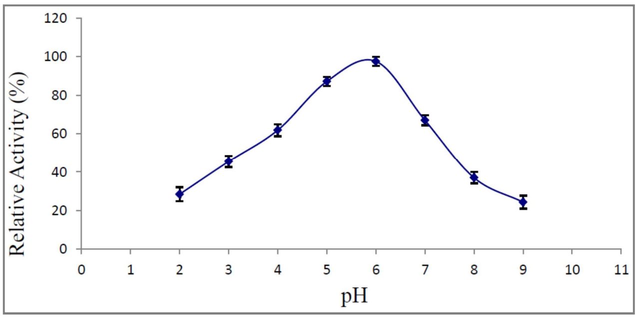

Figure 2. Relative activity (%) of the enzyme β-galactosidase in the crude extract of Artemisia judaica at different pH values. Mean ± SD (n=3).

These results agreed with the observations reported that the optimum pH of plant β-galactosidase lie in the acidic range [45]. It has been found that the extraction of three isoenzymes of β-galactosidase from apricots had an optimum pH between 4.0 and 6.0 [18] and in almond was 5.5 [6] but the optimum pH value of β-galactosidase from peach was 3.0 [46] *Hymenaea courbaril* 3.5 [47] and kidney beans 4.0 [48]. The results indicated that the enzyme is suitable for hydrolysis of lactose present in whey or milk where pH varies from 4.5 to 6.8 [6].

3.3. Effect of Temperature on β-galactosidase Activity

Each enzyme has an optimum temperature at which reaction reaches V_{max}. The reaction velocity increases with temperature until a peak velocity is reached, where maximum number of molecules having sufficient energy to pass over the energy barrier and form the products of the reaction [49]. Further increase in temperature will lead to decrease the reaction velocity as a result of temperature-induced denaturation of the enzyme due to changing the native folded structure of proteins to uncoil into random configuration. At high temperature, the hydrogen bonds are broken, therefore, the molecular conformation of the enzyme becomes altered.

The effect of temperature on β-galactosidase activity in the crude plant extract was investigated by measuring enzyme activity at different temperature values ranging from 20°C to 80°C. The optimum temperature was found to be 50°C as shown in figure (3). The enzyme was stable at temperature from 20°C to 60°C and at 80°C the enzyme exhibited 4% of the maximum activity. The relative activities of β - galactosidase at 20°C, 30°C, 40°C, 60°C, 70°C, 80°C and 90°C were 40%, 63%, 84, 72%, 13% and 4%, respectively.

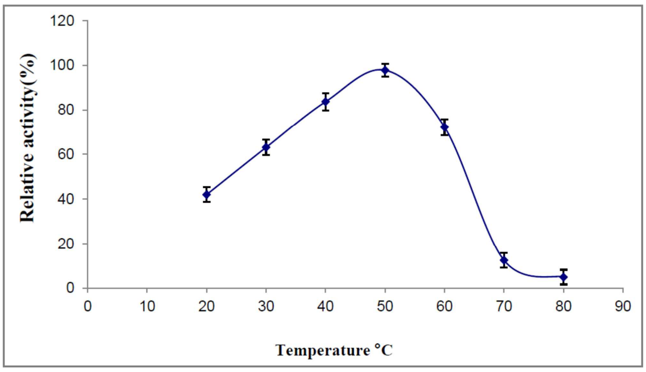

Figure 3. Relative activity (%) of the enzyme β-galactosidase in the crude extract of Artemisia judaica at different temperature degrees. Mean±SD (n=3).

The loss of activity of the enzyme at higher temperatures could be attributed to its unfolding and subsequent loss of active site due to denaturated proteins [50]. The same optimum temperature obtained (50°C) for nasturtium, peach and *Hymenaea courbaril* [51, 46, 47]. It has been found the extraction of three isoforms of β-galactosidase from mung bean seedlings have the optimum temperature between 50°C to 53°C [51]. In many plants, The optimum temperature was slightly different, such as in chick pea, cowpea and almond, it was 60°C [52, 17, 6], apricots 40°C [18] and for apricot seed was 70°C [20]. Most of above studies provided that the optimum temperature of most β-galactosidase in the range 40-60°C.

Determination of optimum temperature is an important factor for the selection of enzymes for industrial, biotechnological and medical applications. It has been reported that most of industrial enzymes have V_{max} at 40-50°C [54]. *Artemisia judaica* has optimum temperature 50°C, that means it can be used in industrial and medical applications.

3.4. Kinetics Analysis

To determine the enzyme kinetic parameters (K_m and V_{max} of β-galactosidase), initial reaction rates at different ONPG concentration ranging from 1 mM to 9 mM were measured. The data were analyzed according to Line weaver Burk plot by plotting 1/V value against 1/[S] value and kinetic parameters were calculated from the graph. The results of K_m and V_{max} values of the enzyme were 3.6 mM and 1.67 µmol/min, respectively (Figure 4).

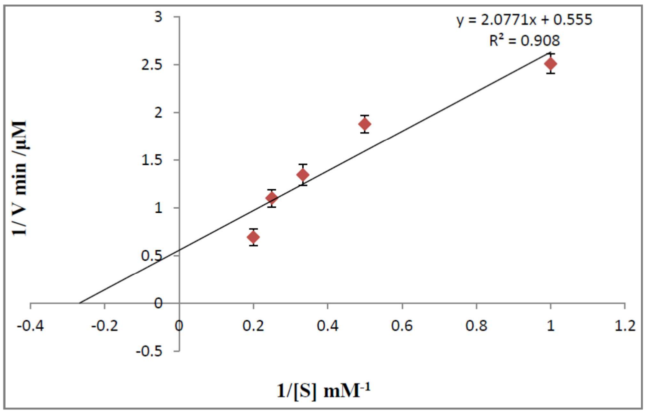

Figure 4. *Determination of V_{max} and K_m values of the enzyme β-galactosidase in the crude extracts of Artemisia judaica using ONPG as a substrate. Mean±SD (n=3).*

K_m is the concentration of substrate at which the reaction rate is half- maximum. K_m is important in enzyme kinetic because its value includes not only the affinity of substrate for the enzyme but also the rate at which the enzyme-bound substrate is converted to the product in the catalytic reaction. Thus, K_m value can be interpreted as a crude measurement of the affinity of the substrate for the enzyme [55].

The K_m value of the enzyme was higher than reported earlier, 1.67 mM for carrot [56], 1.77 mM for tomato fruit [57], 1.85 mM for apricot β-galactosidase I [18], 1.73 mM for chick pea [17] and 1.19 mM for radish seed [42], but it was lower than that of other plants such as 5.16 mM for peach [46] and 10.53 mM for almond [6].

The rate or velocity of a reaction (V) is the number of substrate molecules converted to product per unit time. The rate of an enzyme-catalyzed reaction increases with substrate concentration until a maximal velocity (V_{max}) is reached. The leveling off of the reaction rate at high substrate concentrations reflects the saturation with substrate of all available binding sites on the enzyme molecules present [49].

However, the V_{max} value of the enzyme β-galactosidase in the crude extract was higher than reported earlier. The V_{max} value for β-galactosidase I, β-galactosidase II and β-galactosidase III isolated from apricots was found to be 0.52, 0.70 and 0.38 µmol/min, respectively [18], but it was lower than that of other plants such as 5.2 µmol/min for rice [56].

3.5. Effect of Heavy Metals on β-galactosidase Activity in Artemisia judaica Leaves Extract

Heavy metals can be stimulatory, inhibitory, or even toxic in biochemical reactions depending on their concentrations and type of heavy metals [44]. All results are analyzed according to their effects: uncompetitive inhibition and noncompetitive inhibition.

The apparent effect of an uncompetitive inhibitor is to decrease V_{max} and to actually decrease K_m. Once the inhibitor has bound to enzyme, it will prevent it from turning the substrate into product by direct interaction, or due to a change in conformation of the active site [58].

The V_{max}/K_m ratio is called "catalytic power" and it is a good parameter for finding the most effective or ineffective heavy metal (Table 1) [59].

The results (Table 1) indicated that Hg^{2+}, Zn^{2+} and Fe^{3+} have an uncompetitive inhibition on the β-galactosidase activity in the crude extract of *Artemisia judaica* by decreasing both V_{max} value from 1.67 µmol/min (control) to 0.676, 1.25 and 1.12, and K_m value from 3.6 mM (control) to 1.75, 2.77 and 2.32, respectively. Figure (5) showed that the relative activities of Hg^{2+}, Zn^{2+} and Fe^{3+} were 40.4%, 74.8% and 67.0%, respectively.

A compound may have unequal affinity for both free enzyme and the enzyme-substrate complex. This mixture of competitive and noncompetitive phenotypes is called mixed inhibition, so K_m value is increased. These inhibitors will decrease the V_{max}/K_m ratio (Table 1) [55].

The results which are shown in figures (5) and table (1) indicated that that Pb^{2+} and Cu^{2+} acted as a mixed noncompetitive inhibitors on the enzyme β-galactosidase in the crude extract of *Artemisia judaica* by decreasing V_{max} value from 1.67 umol/min (control) to 1.28 and 1.61 umol/min, relative activities to 76.6% and 96.4%,, and increasing K_m value from 3.6 mM (control) to 4.54 and 5.55 mM, respectively.

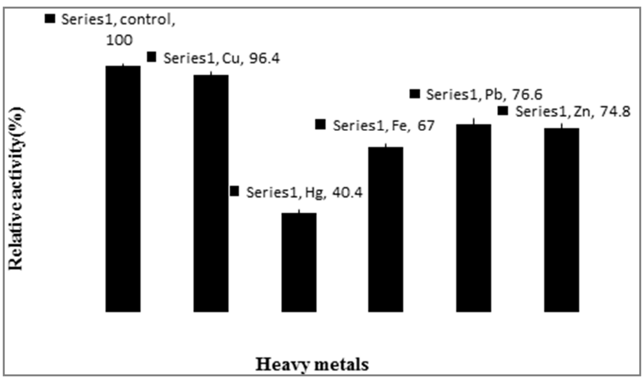

Figure 5. *Relative activity (%) of β- galactosidase in the crude extract of Artemisia judaica in control and in the presence of different heavy metals using ONPG as substrate. Mean±SD (n=3).*

Table 1. *Kinetic values for the enzyme β-galactosidase in the crude extract of Artemisia judaica in control and in the presence of different heavy metals. Mean±SD (n=3).*

Heavy metals	K_m (mM)	V_{max} (umol/min)	V_{max}/K_m	Effects
Control	3.6	1.67	0.461	Normal
Hg^{2+}	1.75	0.676	0.386	Uncompetitive
Zn^{2+}	2.77	1.25	0.451	Uncompetitive
Fe^{3+}	2.32	1.12	0.482	Uncompetitive
Pb^{2+}	4.54	1.28	0.369	Mixed noncompetitive
Cu^{2+}	5.55	1.61	0.281	Mixed noncompetitive

β-galactosidases from different sources like cotyledon of cowpea, chick pea, mung bean seedlings were found to be inhibited by Hg^{+2}, Zn^{+2}, Cu^{2+} and Fe^{+3} [60, 53], while Cu^{2+} and Zn^{+2} had no effect on β-galactosidase activity from peach [46]. Recent reports revealed that heavy metals such as Hg^{2+} and Fe^{3+} as well as some other heavy metals and organic compounds are well-known inhibitors of β-galactosidases from peach and rice shoots, While Zn^{2+} had no effect on the enzyme activity [56, 46].

4. Conclusions

Crude plant extracts of leaves of *Artemisia judaica* L. were analyzed for β-galactosidase activity and kinetics. The results demonstrated that the enzyme β-galactosidase in the crude extract of *Artemisia judaica* have a potential activities according to the K_m and V_{max} values. The β-galactosidase of *Artemisia judaica* had its maximum activity at pH 6.0 and was stable at pH values from 2.0 to 9.0, and the optimum temperature for was 50°C. The results of K_m and V_{max} values of the enzyme were 3.6 mM and 1.67 μmol/min, respectively. According to these findings, the enzyme β-galactosidase in the crude extract of *Artemisia judaica* can be used in industrial, biotechnological and medical applications. Presence of heavy metals altered these activities as uncompetitive or mixed noncompetitive inhibitors. For example, Hg^{2+}, Zn^{2+} and Fe^{2+} acted as uncompetitive inhibitors by decreasing K_m and V_{max} values, while Pb^{2+} and Cu^{2+} acted as mixed noncompetitive inhibitors.

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