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Extraction, Purification and Characterization of β-Galactosidase from Apricot(*Prunus armeniaca kaisa*) Fruit for lactose intolerance treatment

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Abstract : β-Galactosidase (β-D-galactoside-galactohydrolase, EC 3.2.1.23) was extracted by eight solutions from apricot fruit (*Prunus armeniaca kaisa*), purified in several protein purification steps and characterized biochemically. It was found that 10% sodium chloride pH 5 was the best solution for extraction the enzyme and that 20-60% ammonium sulfate saturation was the best method for partially purification of enzyme with a purification fold 4.31 and enzymatic recovery 61.6%. After that, it was purified by ion-exchange chromatography column using DEAE-Cellulose (Diethyl amino ethyl Cellulose) and gel filtration column with Sephadex G-100 as a final purification step with 2000U/mg proteins specific activity, 4.5 purification fold and 40.7% enzymatic yield. The molecular weight of the enzyme was estimated to 165.95 KDa by gel filtration using SephadexG-100 column and the isoelectric point for enzyme was 4.4. Carbohydrate concentration of enzyme was found to be 25.9% by employing phenol-sulfuric acid method. Optimum enzymatic activity was found at 50^{o}C and pH 5 and its activity was stable at 25-55 ^{o}C and pH 5-6 for 15 min. Activation energy (Ea) was estimated by Arrhenius equation to be 7.56kcal/ mol, while deactivation energy was found to be 48.46kcal/mol. The existence of metal ions like Na^{+1}, Mg^{+2} and Mn^{+2} at 0.1and 1.0mM concentrations had an positively effect on enzyme activity, on the other hand, enzyme activity was inhibited when incubated with Cd^{+2}, $Ca^{+2,}$ $Fe^{+2,}$ Pb^{+2} and Hg^{+2}. The average values of Michalis-Menten constant (Km), maximum velocity (Vmax), as well as, catalytic constant (K_{cat})were evaluated to be 1.43mg/ml, 1.36U/min and 13.6min^{-1}, respectively using ONPG as substrate. In addition to the ability of purified enzyme to hydrolyze 24.5% of lactose after one hour and about 89.6% after four hours.
Key Words : β-Galactosidase, Apricot, Lactose Intolerance, Purification.

Introduction

Lactose intolerance is a phenomenon common among more than 70 % of the world's adult population and it is caused by the intestinal deficiency of lactase. [1-3] Deficiency of lactase leads to accumulation of undigested lactose in the small intestine leading to increase the influx of fluids inside the intestinal lumen. Then, unabsorbed lactose is crossed to the large intestine where in addition to excess fluid volume of gastrointestinal content due to generate a difference in the osmotic pressure on both sides of bowel tissue causing diarrhea, is metabolized by colonic microflora leading to the production of short chain fatty acids, carbon dioxide hydrogen and methane gases, and causing one or more of the following symptoms: diarrhea, abdominal pain, flatulence, bloating, cramps and nausea. [4-5] However, people with these syndromes can consume lactose free milk and

dairy products which prepared by removing lactose from the diet through addition of exogenous β-galactosidase enzyme. [6]

β-Galactosidase (β-D-galactoside-galactohydrolase, EC 3.2.1.23), commercially known as lactase, is an extracellular and hydrolytic enzyme which catalyzes the hydrolysis of terminal non-reducing β-D-galactose moieties from β-D-galactoside polymers include carbohydrates, glycolipids and glycoproteins. [7]It is widely distributed in many natural sources including: plants, animals and microorganisms. [8-9]In plants, the biological functions of lactase involve degradation of structural polysaccharides present in the cell walls thereby controlling on the fruit softening during ripening, germintation of seed and evalution of vegetative organs. Whereas, in mammals it hydrolyzes of dietary lactose, degradation of proteoglycans and glycolipids; and metabolism of lactose and another galactosides in microorganisms. [10]As well as, it can catalyze transgalactosylation reaction and form galactooligosaccharides. [11-12]

The aim of the present investigation was to extract, purify andcharacterize Beta-galactosidase from apricot fruit which has nutritional and industrial importance for individuals who suffering from lactose intolerance.

2. Materials and Methods:

2.1 Materials: Apricots were obtained from local markets of Baghdad, Iraq. All chemicals utilized in the present investigation were supplied from BDH, England; Himedia, India; Pharmacia fine chemicals, Sweden and Riedel-de Haën, Germany.

2.2 Methods:

2.2.1 Enzyme assay: The activity of *β*-galactosidase was estimated by method which described by [13] usingortho-nitrophenyl *β*-D-galactopyranoside ONPGas a substrate. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the liberation of 1 µmol of ONP per minute under assay condition of the reaction (37°C).

2.2.2 Protein Assay: Protein concentrations were estimated either by UV at 280nm or colorimetrically as performed by Lowry et alemploying Bovine Serum Albumin (BSA) as a standard.[14]

2.2.3 Extraction: 25gm of apricot tissue were homogenized in a blender with 25ml of anyone of extraction solutions which mentioned in table (1), then centrifuged at 9,000 rpm for 15 min and filtered through 4 layers of cheese cloth. The supernatant was utilized for further purification ofenzyme as a crude extract after loading it into sephadex G-10 gel filtration for removing dyes, salts and polysaccharides.

2.2.4 Purification: Five methods including cold acetone, ammonium sulfate, sephadex G-25, ethanol alcohol and ultrafiltration were used at 4^{o}C as a partial purification step and selected the best one for precipitation of protein,then precipitate was loaded on ion-exchange chromatography column using DEAE-Cellulose (Diethyl amino ethyl Cellulose) and then the highly active elution reactions were passed through gel filtration column with Sephadex G-100 as a final purification step as mention by [15].

2.2.5 Molecular Weight Estimation: Molecular weight was determined by sephadex gel filtration chromatography. The sephadex G-100 column was pre-equilibrated with standard proteins.

2.2.6 Determination of Isoelectric point (pI): Method that described by [16] in preparation of solutions and pI determination by isoelectric focusing, was used.

2.2.7 Estimation of Carbohydrate content Phenol-sulfuric acid method which described by Dubois et al was used. [17]

2.2.8 Effect of pH on Enzyme Activity: Optimum pH of apricot *β*-galactosidases was estimated by incubation purified enzyme with solutions of 12mMONPG which prepared in buffer solutions with various pH ranging from 3-8 including 0.2M glycine HCl buffer, pH 3- 3.5, 0.2 M NaoAc buffer pH (4-5.5)and 0.2 M phosphate buffer pH (6-8). The enzyme assay was performed separately in each buffer system.

2.2.9Effect of Temperature on Enzyme Activity: Optimal temperature of enzyme activity was determined by incubating purified enzyme at a temperature ranging from 25 -80°C for 15min. The residual enzyme activity was determined.

2.2.10Determination Kinatic Constants of Enzyme: Maximum velocity Vmax, Michaelis Menten constant Km and catalytic constant $K_{cat}$of β galactosidase for ONPG were estimated bythe method adopted by [18].

2.2.11Activation energy determination: Activation energy of enzyme for converting ONPG to product was determined using Arrhenius equation.

2.2.12 Hydrolysis of Lactose: One ml of purified enzyme was added to 10ml of 5% lactose solution which prepared in 0.1M sodium acetate buffer pH 5. The mixture was incubated at 50^{o}C and hydrolysis of lactose was monitored at various intervals (1-7h) by taking an amount of interaction solution and handling it thermally for 10minutes in boiling bath for stopping the activity of enzyme. The concentration of hydrolyzed lactose is equaled to the concentration of glucose which measured by enzyme colorimetric method (GOD-POD).The percentage of lactose hydrolysis was calculated by an equation described by [19].

3.Results and Discussion:

3.1 Extraction of β-Galactosidase: Enzymatic extract was obtained by homogenizing of apricot and regarded as a crude enzyme. As shown in table (1) Sodium chloride solution was the more efficient solution used for extraction of lactase with a specific activity 494.08U/mg protein due to the high ionic strength of NaCl solution which acts to dissociate the linkages between enzyme and other cellular constituents. These results agree with [20]

Table (1): Extraction methods of β-galactosidase from local apricot fruits

No.	Extraction solution	Volume (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Total protein (mg)
1	Distilled water	21.5	282.51	0.720	392.37	6073.96	15.48
2	Sodium chloride 10%	14.5	412.07	0.834	494.08	5974.87	12.093
3	Potassium chloride 0.2%	16	291.08	0.717	405.96	4657.28	11.472
4	Calcium chloride 0.2%	18.5	403.17	0.910	443.04	7458.64	16.835
5	Sodium carbonate 0.5%	22.5	278.55	0.837	332.79	6267.37	18.832
6	Glycerol 20%	30.5	198.12	0.481	411.89	6042.66	14.670
7	Phosphate buffer 0.2M, pH7	17	282.46	0.719	392.85	4801.82	12.223
8	Acetate buffer 0.2M, pH5	15	328.99	0.758	434.02	4934.85	11.37

3.2 Purification of β-Galactosidase

Crude extract was subjected to several techniques for partial purification and concluded that 60% ammonium sulfate saturation was the best method for concentration of enzyme with a specific activity 2169.57U/mg proteins, 5.02 purification fold and 71.6% enzymatic yield. (Table 2) When applied the purified enzyme by ammonium sulfate into DEAE-Cellulose column, three protein peaks with one lactase activity peak located in the second protein peak appeared in the eluted fractions. (fig.1) Specific activity of this step was 2083.3U/ml with a purification fold and enzymatic yield were 4.72 and 58.2%, respectively. The results approach to those reported by [21] and differ to those obtained by [22].

Table (2): Precipitation techniques of β-galactosidase enzyme from crude extract "partial purification"

Method	Vol. (ml)	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (Unit)	Purification fold	Yield %
Crude enzyme	25	401.4	0.928	432.5	10035	1	100
Cold acetone	6	635.6	0.436	1457.8	3813.6	3.37	38
Ethanol 30% 60%	8 8	269.4 618.2	1.68 0.228	160.36 2711.4	2155.2 4945.6	0.37 6.26	21.5 49.3
Ammonium sulfate 20% 60%	8 8	66.5 898.2	1.66 0.414	40.06 2169.57	532 7185.6	0.09 5.02	5.3 71.6
Ultrafiltration	8	361.7	0.626	577.8	2893.6	1.34	28.8
Poly ethylene glycol	5	506	0.434	1165.9	2530	2.69	25.2

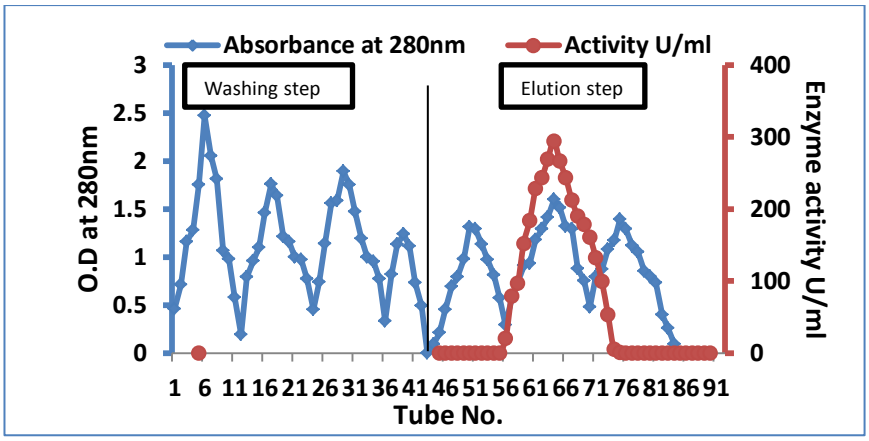

Figure (1): Ion exchange chromatography of β-galactosidase produced from local apricot using DEAE-Cellulose column (40× 1.6cm) equilibrated with potassium phosphate buffer pH7, lactase was eluted by a linear gradient of sodium chloride from (0.2-1.0)M, and volume of a fraction was 3ml at flow rate of 30ml/hr.

Furthermore, the eluted fractions from sephadex G-100 gel column appeared one protein with one peak of lactase activity, so this step is considered as an evidence of purity of lactase enzyme. (Fig. 2) Specific activity which obtained from this step was 2000U/mg with a purification fold 4.5 and an enzymatic enzyme was 40.7% as shown in table (3).The results agree satisfactorily with those reported by [13] and are different with [23].

Table (3): Purification steps of β-galactosidase from apricot fruit

Method	Vol. (ml)	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (Unit)	Purification fold	Yield %
Crude enzyme	25	412.2	0.934	441.3	10305	1	100
Sephadex G-10	15	620	0.833	744.3	9300	1.68	90.2
Ammonium sulfate 20-60%	8	792.9	0.417	1901.43	6343.2	4.31	61.6
Ion exchange by DEAE	24	250	0.12	2083.3	6000	4.72	58.2
Gel filtration	21	200	0.1	2000	4200	4.5	40.7

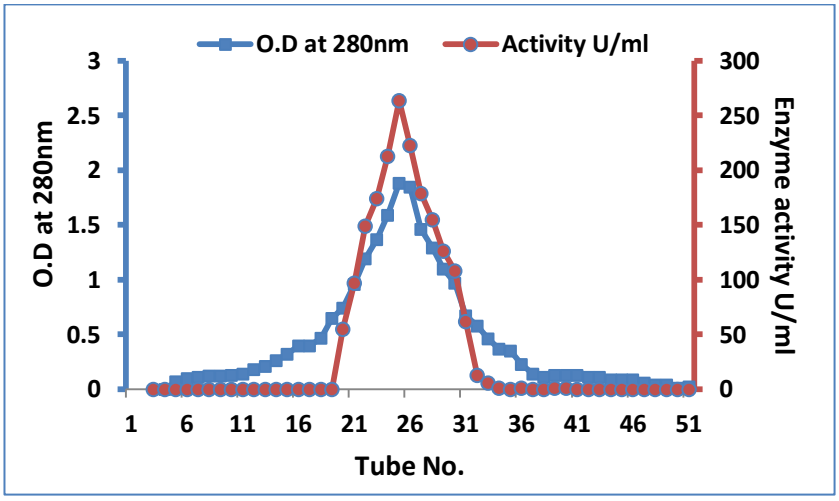

Table (2): Gel filtration chromatography of lactase purified from local apricot utilizing Sephadex G-100 column (60×1.6cm) equilibrated with potassium phosphate buffer pH7, lactase were eluted with the same buffer, volume of a fraction was 3ml at flow rate of 30ml/hr.

3.3 Characterization of purified enzyme:

3.3.1 Enzyme molecular weight:

The molecular weight of purified β-galactosidase was found to be 165.95kDa using sephadex G-100 gel filtration column. (fig. 3) Molecular weight of enzyme purified from almond was 62kDa using gel filtration chromatography, whereas itwas 335kDa from *psychrotolerant* yeast.[23, 24] Differences in the molecular weight of a specific enzyme belongs to the enzyme source, the method used in estimation and extraction method,accuracy of purification and type of enzyme, e.g. extra or intracellular, additionally some references indicate that the genetic and environment situation can also effect on the molecular weight value. [25]

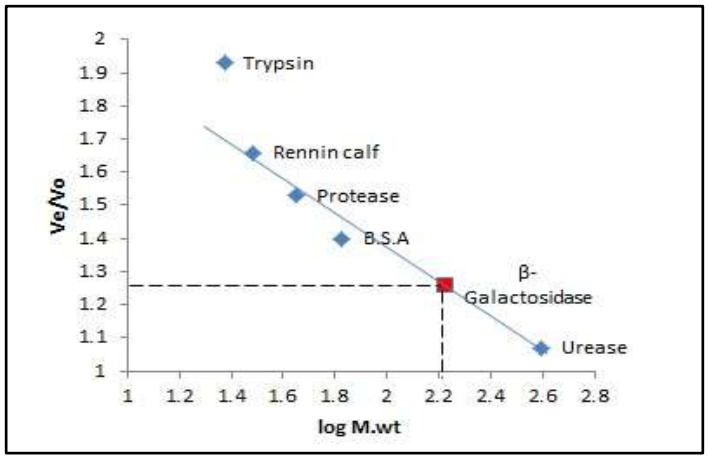

Figure (3): Standard curve for estimation the molecular weight of β-galactosidase purified from apricot employing Sephadex G-100 gel filtration (60×1.6 cm)

3.3.2 Isoelectric Point of β-Galactosidase:

One protein band was appeared after coloring the gel with Commassie Brilliant Blue R-250 as shown in figure 4. Isoelectric point of β-galactosidase was found to be 4.4 when estimated by Isoelectric focusing which depends on progressive hydrogen number (pH) of the gel that is sustainable and stable due to the charged small particles (Ampholytes) and that means the existence of higher percentage of acidic amino acids in the protein as compared to basic amino acids. Our result is in agreement with [26].

3.3.3 Carbohydrate content:

Figure (5) shows that the carbohydrate peak at 490nm is approximately identical to the protein peak at 280nm and activity peak at 420nm, this is an evidence of presence carbohydrates in the protein structure of lactase and this means that apricot lactase is a glycoprotein. Carbohydrate concentration of apricot β-galactosidase was found to be 25.9% by employing phenol-sulfuric acid method and this result approximately agree with [27]. Carbohydrate percentage in lactase enzyme differs from one source to another.

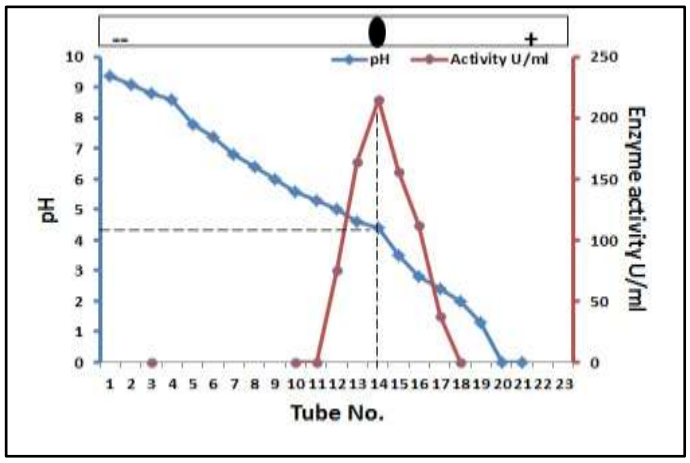

Figure (4): Isoelectric point pI of β-galactosidase purified from apricot.

3.3.4 Optimum pH of β-galactosidase activity:

Activity of lactase was increased with increasing pH and observed that the maximum activity was 218 U/ml at pH 5 as shown in figure (6). Also, it can be seen that the enzyme had low activity below pH 3 and completely disappeared at pH 8. Decline in lactase activity is due to protonation or deprotonation of ionizable catalytic groups present in the active site of the enzyme, substrate, enzyme substrate complex [ES] and enzyme-product complex [EP]. [28-29]The results are agreement with those reported by [30-31].

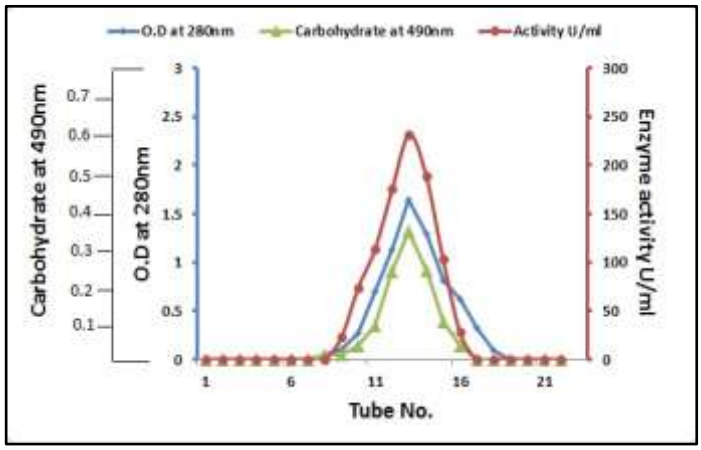

Figure (5):Carbohydrate content of local apricot β-galactosidase estimated by phenol-sulfuric acid method after eluted from Sephadex G-100 gel column (60 x 1.6 cm).

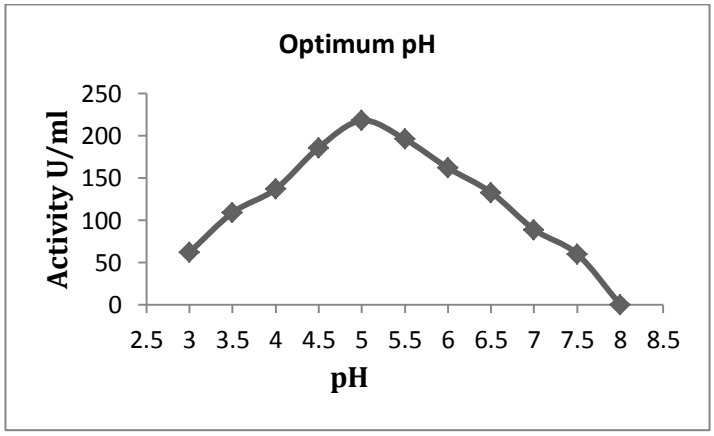

Figure (6): Effect of pH values on β- galactosidase activity purified from apricot fruit.

3.3.5 Optimum temperature of β-Galactosidase activity:

The activity of purified lactase from apricot increased with temperature rising until reached to its maximum activity 216U/ml at 50˚C, and then the activity was gradually decreased to reach 40U/ml at 70 ˚C and completely disappeared at 75-80 ˚C as observed from figure (7). This result is in agreement with those reported by [32]Lactase activity reduces due to denaturation of enzyme as a result of that energy breaks down the weak bonds of enzyme leading to change the configuration of an active side and becomes less complementary to substrate shape.

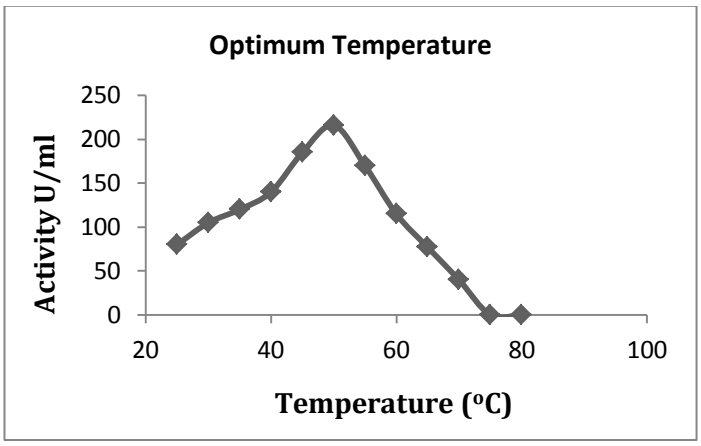

Figure (7): Optimum temperature of β-galactosidase purified from apricot fruit.

3.3.6 Activation energy of β-galactosidase:

Activation energy (Ea) of lactase purified from apricot for conversion of ONPG to product was measured to be 7.56kcal/ mol; and this value is within the range that stated by [33] which is located between 6-18Kcal/mol. Low value of Ea is considered as an indicator of the catalytic efficiency of the enzyme to convert substrate to products. Whereas, it was found that inactivation energy of purified enzyme from apricot equals 48.46kcal/mol, this value gives an idea about un-stability of enzyme at high temperatures. Inactivation energy of enzymes ranged 40-175 Kcal/mol. [34]

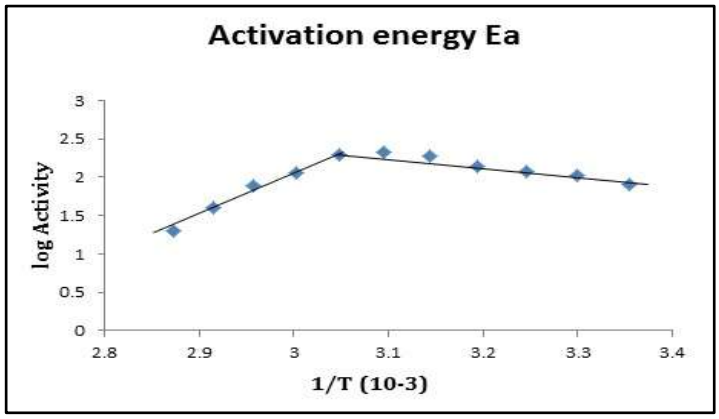

Figure (8): Arrhenius plot for determining activation energy of apricot β-galactosidase.

3.3.7 Kinetic constants of β-Galactosidase:

The values of Michalis-Menten constant (Km), maximum velocity (Vmax), as well as, catalytic constant (K_{cat}) of purified lactase from local apricot were evaluated utilizing Line weaver–Burk plot to be 1.43mg/ml, 2.5U/min and 25min^{-1}, respectively.Fig. (9)In addition to line weaver-Burk plot, four methods were also used for estimation Km, Vmax and K_{cat} values including; Woolf- Augustinsson- Hafstee plot, Hanes-Woolf plot, Edaie- Scatchar plot and Estesthal-Cornish-Bowden plot and found that the average values of these constant equal 1.43mg/ml, 1.36Umin and 13.6min^{-1}. Km and Vmax values of β-galactosidase purified from sweet cherry employing PNPG were 0.42 mM and 4.12 mmol min^{-1} $mg^{-1,}$ respectively.[35] K_{cat} of lactase purified from *Paracoccus sp*. was stated to be 71.81 S^{-1}. [36]

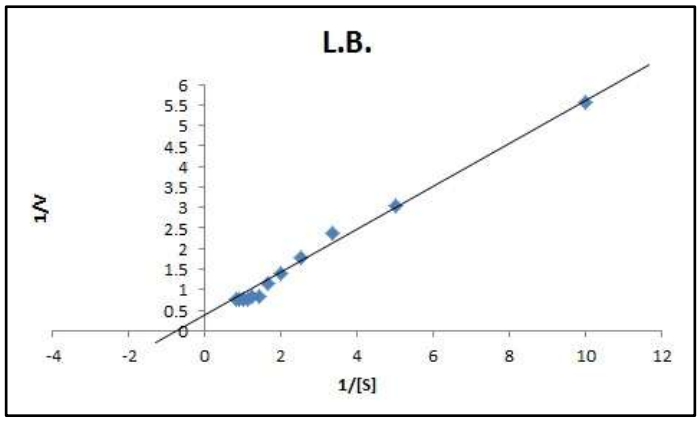

Table (9): Kinetic constants of lactase purified from apricot according toLinweaver- Burk

3.3.8 Effect of metal ions on β-Galactosidase activity:

Metal ions $MnCl_2$, $MgCl_2$ and NaCl had an activation effect on enzymatic activity. It was found that $MnCl_2$ increased 26 and 38.6% of its remaining activity compared with reference assay, at 0.1 and 1mM concentration, respectively and reduced when treated with $FeCl_2$, $CaCl_2$, $CoCl_2$ and $CuCl_2$, while $HgCl_2$ considered as a cation which highly inhibited lactase activity in this study which reduced the remaining activity to 78.2 and 14.8% at 0.1 and 1Mm, respectively. The reduction in lactase activity maybe returns to the influence of these cations on an enzyme conformation, active site and on the other hand, it effects on the substrate by formation complexes which prevent binding enzyme with substrate. These results agree with those recorded by [37].

3.3.9 Lactose Hydrolysis:

Apricot lactase has the ability to efficiently hydrolyze of lactose; it can be observed that enzyme had ability to hydrolyze 24.5% of lactose after one hour and about 89.6% after four hours. Fig (10) Lactase purified from the brain of new born goat had ability to hydrolyze 91% after 3h. [38]

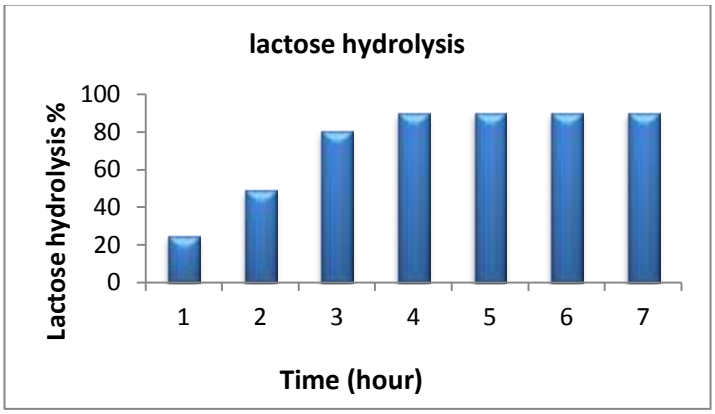

Figure (9): Hydrolysis of lactose by β-galactosidase purified from apricot through incubation it at 50^{o}C for (1-7) hours.

4. Conclusion

In this investigation, β- galactosidase was extracted and purified from apricot using several methods and techniques. The specific activity and enzymatic yield was found to be high at the end of purification steps as compared to other β-galactosidase enzyme purified from plant sources.The ability of the enzyme to hydrolyze lactose indicates the possibility of using lactase purified from apricot in reducing lactose percentage which presents in the milk and dairy products which is necessary for individuals who suffering from lactose intolerance.

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