Purification and characterization of extra cellular Pectin lyase from Erwinia carotovora isolate from spoilt potatoes

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Abstract

Erwinia carotovora (7) isolates were obtained out of 20 spoilt potato samples from local market of Baquba city .The isolates that gave higher Pectinolytic activity was selected to purify pectin lyase through three stages of purification including (ethanol precipitation, ion- exchange chromatography by DEAE – Sepharose and gel filtration by Sephadex G50 with 63.9- fold purification , 69.5U / mg specific activity and 30% recovery.The purified enzyme was characterized :the molecular weight was about 29 KDa by gel filtration chromatography .The temperature for maximum activity was 60c° and maximal activity was observed at pH 8.5 .Some metallic ions such as Ca+ and Mg+2 increased Pectin lyase activity to 140 and 133 % respectively .while the other metals such as Co+2 ,Hg+2 ,Ni+2 Zn+2and Sn+2 inhabited enzyme activity.Therefor ,This research leads to increase interest by using Pectin lyase in the current biotechnological application .

الخلاصة:

تنقية وتوصيف انزيم بكتين لايز Pectin lyase من العزلات المحلية لبكتريا Erwinia carotovora المعزولة من البطاطا المصابة بالتعفن الطري تم الحصول على 7 عزلات تعود لبكتريا Erwinia carotovora من مجموع 20عينةبطاطا تالفة ماخوذة من الاسواق المحلية في مدينة بعقوبة التي اعطت اعلى انتاجية لانزيم البكتين لايز لاجراء

تجارب التنقية .تم استخلاص الانزيم وتنقيته باستخدام ثلاث مراحل تضمنت (الترسيب بالايثانول ، التبادل الايوني باستخدام DEAE-Sepharose والترشيح الهلامي باستخدام Sephadex G-50) بعدد مرات تنقية 63.9 وفعالية نوعية 89.5 وحدة/ملغم بروتين وحصيلة نهائية 30%. تم توصيف الانزيم المنقى ووجد ان وزنه الجزيئي حوالي 29 كيلو دالتن باستخدام كروماتوغرافيا الترشيح الهلامي ، كما درست درجة الحرارة المثلى لفعالية الانزيم هي حوالي 29 كيلو دالتن باستخدام كروماتوغرافيا الترشيح الهلامي ، كما درست درجة الحرارة المثلى لفعالية الانزيم هي 60 م° بينما الرقم الهيدروجيني الامثل للفعالية الانزيمية كان 8.5 درس تاثير بعض العناصر الفلزية في فعالية الانزيم حيث وجد ان Ca+2, Mg+2 ادى الى زيادة الفعالية الانزيمية الى 140و 133% على التوالي بينما العناصر الاخرى مثلCo+2 , Hg+2, Ni,+2 ,Zn+2, Sn+2 ادت الى تثبيط الفعالية الانزيمية .وان هذه النتائج تعطي اهمية للانزيم في استخدامات التقانة الاحيائية الحديثة .

Introduction

Erwinia caratovora is a Gram- negative, facultative anaerobic, rod- shaped bacteria and belongs to Enterobacteriaceae (1).This bacterium is widely spread in the environment and commonly present in lakes, streams, rain and ground water(2) . It is able to survive in the soil (3). Erwinia caratovora is a plant pathogen which can cause diseasesin awide range of plants, including tomatoes ,carrots ,onions ,but is beast known in temperature regions for causing blackleg and soft rot in potatoes .Its success partly lies in its ability to produce exoenzymes (Pectinase ,Pectate lyase ,Cellulases and Proteases) which break dowin its hosts cell walls.The degraded cell walls provide nutrients to the bacterium , and so aid its survival and growth. Tubers are an important source of the disease for potatoes.(3,4,5,6). Pectins are high molecular weight acid polysaccharides primarily made up of α-(1→4) linked D- galacturonic acid residues with a small number of rhamnose residues in the main chain and arabinoses , galactose and xylose on it is side chain (7,8). Pectinase is a generic name for a family of enzymes that catalyase hydrolysis of the glycocidic bonds in the pectic polymers. Pectinases are one of the most widly enzymes in bacteria , fungi and plants(8).

Erwinia caratovora pectinases include polygalacturonase ,pectatelyase, pectinlyase and pectin esterase, classified on the basis of their mode of action (7,9).Pectinolytic enzymes are of

significant importance in the current biotechnological era with their all- embracing applications in fruit juice extraction and its clarification, scouring of cotton ,de gumming of plant fibers ,waste water treatment ,vegetable oil extraction ,tea and coffee fermentations ,bleaching of paper,in poultry feed additives and in the alcoholic beverages and food industries(10,11,12). For these reasons , the aim of this experiment was to purify pectin lyase from Erwinia caratovora and characterize this enzyme by detection the molecular weight for it , the optimum conditions for its activity and the effect of some cationic ions on its activity.

Materials and methods

Samples collection:

Twenty spoilt potatoes samples were collected from local markets in Baquba city .These samples were analyzed according to the method that described by (13) . Briefly ,25 g of potatoes sample was blended with 200 ml in peptone water o.1% by using gab lender for 2 min and incubated at 30 c° for 18- 24 hr.Isolation and identification of Erwinia caratovora :

One loop full of potatoes was plated on blood ager and MacConkys agar , then atincubated at 30 c° for 18-24 hr . Erwinia cells were grown on PT medium that containing the following [g/ml] (polygalactonic acid ,5;NaNO3 ,1 ;K2HPO4 ,4;MgSO4.6H2O,O.2 ;1NNaOH ,1ml and agarose,18)(14).Several biochemical tests were done to differentiate Erwinia caratovora from other species .These include the following testes : negative indole test inability to phosphatase and lecithinnase production and ability to produce acid from trehalose and maltose (15,16,17).In addition to these biochemical tests ,API 20 E identification was used to differentiate Erwinia caratovora from the other type .

Pectionlytic activity on Media:

10 ml of Erwina carotovora cultures were placed into wells (5 mm In diameter) in Luria– Bertani agar plates supplemented with 0.4% (w/v) polygalacturonic acid .After growth ,plates were flooded with 1M CaCl2 and pectinase-producing colonies were detected by the appearance of a halo around them (18,19).

Pectin lyase assay:

Pectin lyase activity was assayed by mixing 2ml of 0.2% poly galacturoni acid solution in 50mM glycine – NaOH buffer (pH=8.5) with 1 ml in 50 mM glycine-NaOH buffer solution .The mixture was incubated at 60c° for 30 min after incubation , 3ml dinitro salicylic acid (DNSA) reagent added and the solution was boiled for 5min to stop the reaction .The absorbance was measured at 232nm and the galacturonic acid contend was obtained by using calibration curve relating galacturonic acid centration (0 – 2.5 mM) to 232 nm . one unit of pectin lyase activity was defined as amount of enzyme that released 1µ mol of reducing sugars (galacturonic acid) from polygalactur onic acid Per minute .(10,18,20)

Protein assay :

Analysis for protein were carried out by the method .(21) by spectrophotometer assay at 600n min each stage chitinase Purification .

Purification of pectin lyase from Erwina carotovora:

Erwina carotovora extracellular pectin lyase was purified by a modification of the method (22). Cells were grown in basal medium containing per littre:2g(NH4)2SO4,20.4KH2PO4,3 g yeast extract , 5g glucose , 4g poly galacturonic acid and 2.5 g urea and incubated at ◌̊3oC° in shaking incubator for 18.24h .(7,20) .Supernatant were carefully removed after centrifugation at 10000xg for 30 min at and pectin lyase activity in supernatant was assayed .

The supernatant was treated with ethanol at ratio of saturation 50% .The mixture was centrifuged , then to the supernatant pectin lyase activity was assayed .The supernatant was dialyzed a against distilled water and the pectin lyase activity was assayed .A two steps chromatographic procedure was employed to purify pectin lyase . for the first mentional step , the supernatant was loaded onto a DEAE-Sepharose fast flow anion .exchange column (1.5 by 17 cm) previously equilibrated in 20 Mm Tris –HCl by buffer (ph=8) . The pectin lyase was eluted 20m M Tris- HCl buffer (pH=8) with 0.5- 1.3 M NaCl gradient . The fractions (5 ml) containing the highest pectin lyase activity were pooled and used in gel filtration step .Gel filtration was carried out in sephadex G-50 column (2.5 by 40 cm) which had been equilibrated and washed with 20 mM phosphate buffer (pH = 7.0) and the elution done by the same buffer.The fractions (5 ml) containing the highest pectin lyase activity were pooled and used in further studies .

Characterization of pectin lyase:

1.Evaluation of the molecular weight :

The molecular weight was evaluated by gel filtration according to the principles described by (23) .Gel filtration was carried out in sephadex G-50 column .this column was equilibrated in 20 mM phosphate buffer (PH=7.0).the void volume (Vo) was determined by using blue dextran .Elution volumes(Ve) of proteins of known molecular weight (Bovine serum albumin){66k Da} ,ovalbumin {45kDa}, chemotrypsinogen A{25kDa}and ribonuclease A {13kDa},dissolved in 20 mM phosphate buffer were measured and used as reference standards in pectin lyase native molecular mass determination .The relation ship between(Ve/ Vo) and log molecular weight for standard proteins was plotted to obtain the standard curve . The molecular mass for pectin lyase was evaluated from incidence (Ve/Vo)value for pectin lyase on the standard cave .

2.Effect of temperalure on pectin activity:

The optimum temperature of the purified pectin lyase was evaluated at temperatures ranging from 20 to 70˚C under standard conditions at 50m M glycine _NaOH buffer (pH=8.5)

3.Effect of pH on pectin lyase activity:

The influence of enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 5 to 10 at 60˚C using different suitable buffers , 50 mM sodium acetate (pH 5,5.5 and 6) 50mM sodium phosphate (pH6.5,7,7.5 and 8)and50 mM glycine_NaOH (8,5,9,9.5and 10),respectively.

4.Effect of some cationic metals on pectin lyase activity:

The purified enzyme was diluted with cations Ca+2, Mg+2,Sn+2,Hg+2,Co+2,Ni+2 and Zn+2 in the following concentrations (0.5mM.1mM .and 10mM). After 1 h of incubation with constant shaking at 60Cº ,pectin layse activity was measured .

Result and Discussion:

Erwinia caratovora 7(%35) isolates were obtained out of 20 samples. Erwinia caratovora survived for 5 months at temperatures of 10c° and 20 c°and relative hemidities of 81 and 93%,3-4 months at relative hemidities51-62% and temperatures of 30 and 35 c°(24).In addition , This bacterium cause blacking of potatoes of which it is the chief if not only cause in cool temperature climates. Howeve, in warmer climates Erwinia caratovora and Erwinia chrysanthemi can cause similar or identical symptoms (25) .In a study done by (26) reported that Erwinia caratovora strains were isolated from potato ,cucumber ,broccoli, radish ,tomato and sweet pepper.

Pectinolytic activity on media

Erwinia caratovora (7)isolates were tested for measuring the pectin lyase activity by detection the diameter of clear zone of lysis in Luria –Bertani agar plates supplemented with Polygalactouronic acid (fig.1) In this figure see that Erwinia caratovora P2 produced pectin lyase in higher level , therefore this isolate was chosen for purification experiment .The pectinase are inducible enzymes that require the presence of the inducer to be synthesized . Although pectin is the natural inducer for pectinase production ,its elevated cost makes difficult its use at industrial level (8). In a study done by (27) found that the addition of glysine betaine to the media containing NaCl increased the extracelullar enzyme activity (pectate lyase)and reduced the activity of the cell associated enzyme. (7) revealed that pectinase production was optimal when a combination of glucose and citrus pectin was added along with urea in the basel medium devoid of yeast extract and peptone . Also found that amino acids and vitamins greatly induced pectinase production . while (8) showed that the agriculture product containing pectin and other poly saccharides have been used for pectinase production .

Purification of pectin lyase from Erwinia carotovora: Purification procedure consisting of ethanol precipitation ,ion exchange ,chromatography and gel filtration chromatography was developed to obtain a highly purified pectin lyase from Erwin carotovora.The effectiveness of each purification step is given in table (1) .Ethanol and solution at 50% saturation on the crude extract lead to rise in the specific activity to 6.1 U/mg and reveled 4.3 fold of purification with 47% pectin lyase recovery before the dialysis .

Ion exchange chromatography by DEAE-Sepharose column was the second purification step .When pactein lyase solution where passed through DEAE-Sephrose column and eluted

with NaCl solutions(0.5-1.3M) , Two peaks of proteins in the eluted fractions with one peak of pectin lyase activity located in the first protein peak (fig . 2) .Fold of purification was 19.7in this step with 33% recovery .The last purification was performed by gel filtration chromatography on sephadex G-50 column (fig. 3) .The eluted fractions on this step contained tow proteins peaks , only the first peak contained the pectin lyase activity .This procedure yielded a 63.9 fold purification and 30 % recovery of the enzyme with specific activity 89.5 U/mg . Pectate lyase was purified from Erwinia carotovora subsp. atroseptica with 32% recovery by ion exchange chromatography on a S-Sepharose fast flow column (22) . In contrast ,pectin lyase was purified from Erwin carotovora on a S-Sepharose column with 42% recovery (28). Acrophialophora nainlana pectinase was purified by ultra filtration and a combination of gel filtration and ion- exchange chromatography procedure (29). Also (30) found that pectate lyase was purified from Amycolata sp. By anion –and cation exchnge chromatograies followed by hydrophobic in traction chromatography with 37% recovery .

Characterization of pectin lyase:

1-Evaluation on the molecular weight of pectin lyase

The molecular weight of purified pectin lyase was evaluated by gel filtration with Sephadex G-50 .The result showed that purified pectin lyase of approximately 29kDa (fig.4).Many studies done by (8,28) reported that the molecular weight of pectin lyase that purified from Erwinia caratovora and Erwinia areideae were 31 and 28 kDa , respectively .Also (31) found that Erwinia carotovora strains had molecular weight range of 28 -33 kDa

In addition , an extra cellular pectinase that purified from Achrophialophora nainiane had a molecular weight 35,500 dalton by SDS-PAGE (29) .

2- Effect of temperature on pectin lyase activity :

The effect of temperature on the activity of purified pectin lyase was determined at various temperature ranging from 20 to 70 c° at pH = 8.5 (fig .5) .The enzyme showed agood activity between 50 to 70 c° with maximum activity at 60 °. While in 20,30 and 40 c° pectin lyase lost most its activity .The optimum for activity of pectin lyase produsedby Erwinia

carotovora was 50c° (31) .In contract ,(28) found that Erwinia carotovora pectin lyase had maximal activity at 35c° .In another study done by (8) found that Erwinia areideae pectinase had optimum temperature at 40c°.

3-Effect of Ph on pectin lyase activity

The pectin lyase activity was evaluated at different pH values at 60c° using polygalacturonic acid as substrate (fig.6). The optimum activity for polygalacturronic hydrolysis of 188 U/ml was reached at pH = 8.5 . This enzyme has also aboard range of pH activity (pH 8-10) .On the other hand , pectin lyase showed very low activity in the acidic pH values . Bacillus sp. Pectate lyase was most active in a narrow alkaline pH values ,showing the highest activity at pH 10 (18) . (8) reported that Erwinia areideae pectinase was reached to its optimum activity at pH= 8.0.

4- Effect of cationic metals on pectin lyase activity :

Erwinia carotovora pectin lyase was treated with many bivalent cationic metals. Ca+2 increased pectin lyase activity to 140 % at o.5 mM concentrations ;while its activity deceased at higher concentrations and only 9% of maximum activity was found at 10 mM CaCl2 (Table 2). Also Mg+2 increased pectin lyase activity to 133 % at o.5 mM concentration ,but in the higher concentration the activity was decreased and reached to 62 % at 10 mM MgCl2. In contrast , the other metals such as Co+2 ,Hg+2,Ni+2 and Zn+2 caused only low levels of inhibition (88,77,75 and70%).Sn+2 caused no table inhibition of the enzyme (36% residual activity) .(31)revealed that Erwinia caratovora pectinase activity increased by 50-70% with o.5 mM Ca+2 concentration .In other study by (8) found that Erwinia areideae pectinas stimulated by Ba+2,Ca+2,Co+2 ,Mg+2 ,Mn+2 and Sr+2. In addition ,(18) showed that maximum activity of Bacillus sp. Pectate lyase was found at o.5 –o.75 mM CaCl2.

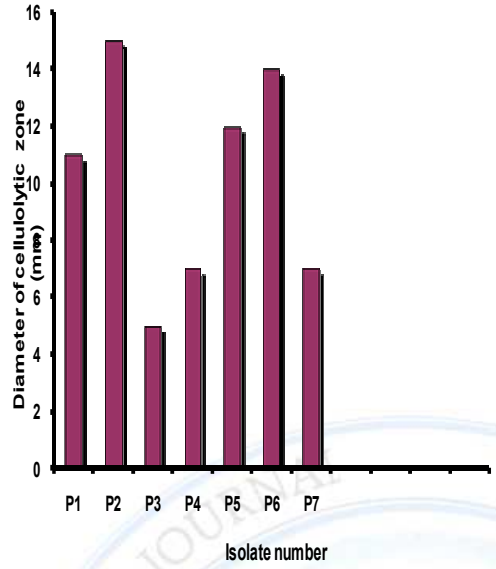

Fig.(1) :Diameter of clear zones of lysis for all Erwinia carotovora isolates.

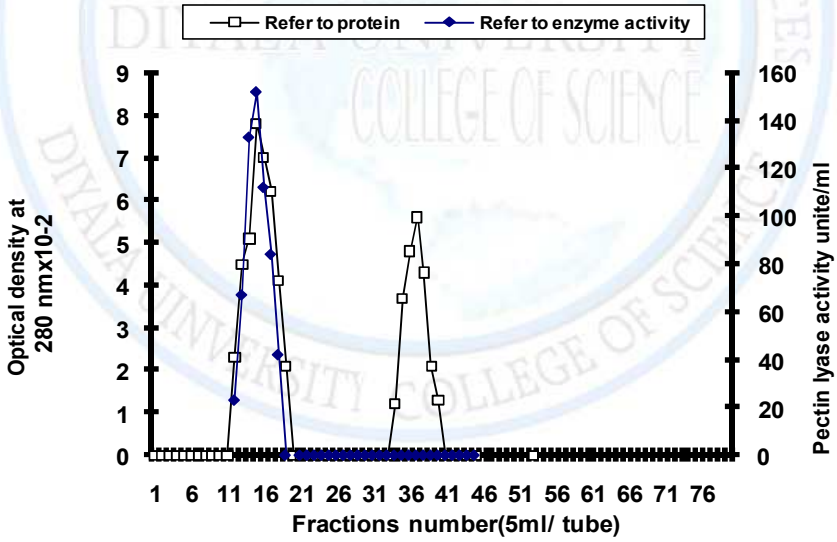

Fig.(2)): DEAE-Sepharose chromatography of Erwinia carotovora Pectin lyase

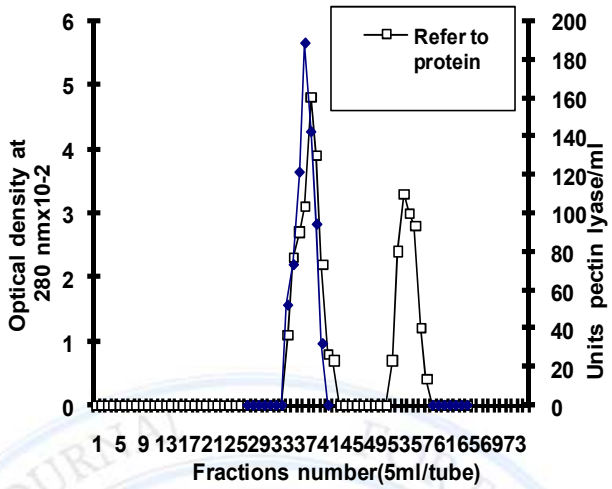

Fig. (3): Sephadex G- 50 chromatography of Erwinia carotovora Pectin lyase

Fig (4) : The standard curve of determination of molecular weight for pectin lyase by gel filtration on Sephadex G-50.

Table (1) : Purification of Pectin lyase erom Erwinia carotovora

Purification step	Size (ml)	Protein Conc. (mg/ml)	Pectinlyase lyase activity U/ml	Specific Activity U/mg	Total activity	Purification fold	Yield (%)
Crude extract	90	42	62	1.4	5580	1	100
(NH4)2SO4	25	17	105	6.1	2625	4.3	47
DEAE-Sepharose	12	5.5	152	27.6	1824	19.7	33
Sephadex G-150	9	2.1	188	89.5	1692	63.9	30

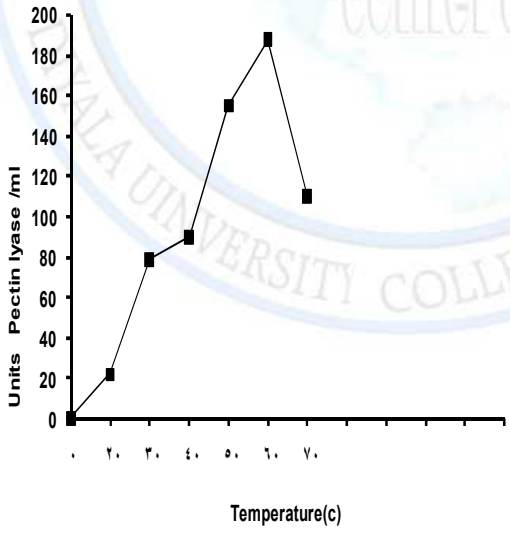

Fig. (5): Effect of Temperature on Pectin lyase activity

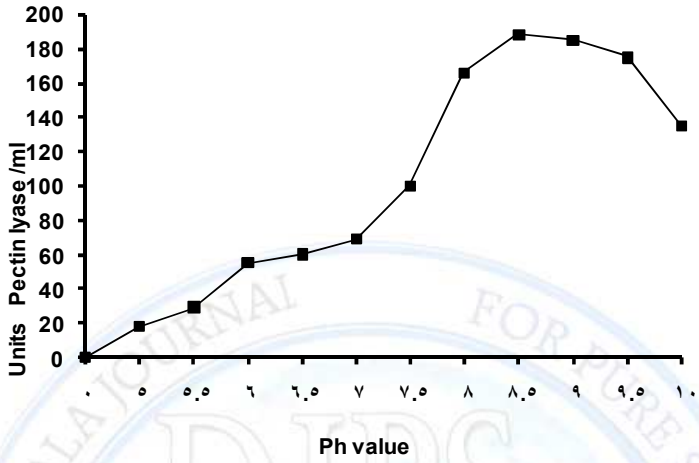

Fig. (6): Effect of pH on Pectin lyase activity

Table (2) Effect of cationic metals on pectin lyase activity

	Cation concentraction (Mm)			
	0.5	1	5	10
Metals	Remaining activit (%)			
Ca^{+2}	140	125	55	9
Co^{+2}	88	62	0	0
Ni^{+2}	75	35	18	0
Hg^{+2}	77	65	30	8
Zn^{+2}	70	32	23	18
Sn^{+2}	36	22	14	6
Mg^{+2}	133	110	88	62

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