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Research Article

Purification and Characterization of Agarase from *Bacillus* sp., H12

Ghazi M. Aziz and Hala M. Ali
Department of Biotechnology, College of Science, University of Baghdad, Iraq

Abstract: The present study was conducted to study the Purification and Characterization of agarase from local isolate *Bacillus* sp., H12 to use in some industrial and pharmaceutical application. The agarase produced from local isolate *Bacillus* sp., H12 was purified by precipitation with 70% saturation ammonium sulphate, followed by ion-exchange chromotography and Gel filtration. Results showed appearance of two protein peaks, once in the wash step without enzyme activity and the other in the elution step have enzyme activity at this step by using Ion-exchanger DEAE-cellulose and separate one protein peak contain enzyme activity at gel filtration step by using the gel Sephadex G-100, the enzyme was purified to 5.67 times with an enzymes yields of 21.45%. Enzyme characterization of the enzyme indicated that the optimum pH for the enzyme activity and stability was 7. The maximum activity for enzyme appeared at 45°C and stable for 15 min at 35-45°C and lost approximately 60% of its activity at rang above 65°C. Enzyme characterization results showed that the chlorides of silver and mercury had inhibitory effect on enzyme activity, the remaining enzyme activity for the enzyme was 25%, for silver ions and 12.5% for mercury ions at 5 mM and 13.75% for silver ions and 7.5% for mercury ions at 10 mM. The enzyme was affected by chelating agent Ethylene Diamine Tetra Acetic Acid (EDTA) at concentration 2, 5 mM the remaining activity 43.75 and 25%, respectively and the enzyme referred to metalloenzyme the enzyme was kept their activity when treated. by reducing agent (2-mercaptoethanol) at 2 mM while the enzyme kept 83.75% of its activity at 5 mM of (2-mercaptoethanol). The enzyme was kept their activity when treated by Phenyl Methyl Sulphonyl Fluoride (PMSF) at concentration 1, 5 mM, the remaining activity was 97.5 and 91.25%, respectively and this indicated that this enzyme did not refer to serine enzyme group.

Keywords: Agarase, *Bacillus* sp., characterization, gel filtration, ion-exchange, purification

INTRODUCTION

Agarases are the enzymes which catalyze the hydrolysis of agar. They are classified into α-agarase (E.C.3.2.1.158) and ß-agarase (E.C.3.2.1.81) according to the cleavage pattern. The basic structure of agar is composed of repetitive units of ß-D-galactose and 3, 6-anhydro-α-L-galactose (Fu and Kim, 2010). Several agarase have been isolated from different genera of bacteria found in seawater, marine, sediment and other environments. Agarase have a wide variety of applications. They have been used to hydrolyze agar to produce oligosaccharides, which exhibit important physiological and biological activities beneficial to health of human being (Wang *et al*., 2004). Besides that, agarase also have other uses as tools to isolate protoplast from seaweeds (Araki *et al*., 1998). And to recover DNA from agarose gel (Sugano *et al*., 1993), Recent progress in cloning and sequencing of these enzymes has led to structure and function analyses of agarase (Fu *et al*., 2009). This information will provide valuable insight into the use of this enzyme. The purification procedure of ammonium sulphate fractionation followed by anion exchange chromatography and gel filtration chromatography has often been used in purification of several agarase (Sugano *et al*., 1993). The α-Neoagarooligosaccharide hydrolase was purified from *Bacillus* sp., MK03 by using ammonium sulphate fractionation with 70% saturation followed by ion exchange by using DEAE-Toypearl 650M then gel filtration by using Superdex 200, the enzyme was purifird 49.7 fold with a yield of 3.6% and the specific activity of purified enzyme was 22.2 U/mg protein (Suzuki *et al*., 2002). The effect of pH and temperature on the enzyme activity and stability was studied, the enzyme purified from *Bacillus cereus* ASK 202 showed more than 80% of its activity at optimum pH 5.8-10 and temperature 4-50ºC (Kim *et al*., 1999). The effect of different metal such as Mg^{+2}, Sn^{+2}, Zn^{+2}, Ag^{+}, Ba^{+2}, Ni^{+2}, Cu^{+2}, Sr^{+2}, Hg^{+2} (2 mM) on α-NAOS hydrolase purified from *Bacillus* sp., MK03 was examined , the enzyme activity was enhanced by Mg^{+2}, Sn^{+2} and Zn^{+2}, while the enzyme activity was strongly inhibited by Ag^{+}, Ba^{+2}, Ni^{+2}, Cu^{+2}, Sr^{+2} and completely inhibited by Hg^{+2}. The organic compound tested P-Chloromercuri Benzoate (PCMB), SDS and EDTA also showed relatively strong inhibitory effects on the enzyme activity (Suzuki *et al*., 2002).

Corresponding Author: Ghazi M. Aziz, Department of Biotechnology, College of Science, University of Baghdad, Iraq

MATERIALS AND METHODS

This study has been conducted during May 2011 to February 2012, in Biotechnology Department, Baghdad University.

Extraction of agarase enzyme: Agarase enzyme was extracted from local isolate *Bacillus* sp., H12 identified in previous study (Ali and Aziz, 2010). After inoculating the isolate in the production media composed of the 0.3% agarose as carbon source, 0.5% beef extract and peptone as nitrogen sources, 0.5% sodium chloride, at initial pH 8 and inoculums size 1×10^8 cell/mL at 37°C for 24 h. Using shaker incubator at 150 rpm/min., cultures was centrifuged at 6000 rpm for 20 min.

Enzyme activity and protein concentration were then estimated in the crude filtrates. Activity of agarase was estimated according to reducing sugar liberated after hydrolysis of agarose by the enzyme (Suzuki *et al*., 2002; Kirimura *et al*., 1999). Protein concentration was estimated according to the procedure of Bradford (1976). The unit of enzyme activity was defined as the amount of enzyme that liberates 1 mM of reducing sugars (galactose) in one minute at reaction conditions (Suzuki *et al*., 2002; Kim *et al*., 1999).

Purification of agarase:

Precipitation by ammonium sulphate: The supernatant (crude extract) was fractionated with ammonium sulphate at 40, 50, 60, 70, 80, 90%, respectively saturation then the precipitate obtained by centrifugation at 10000 rpm for 30 min was suspended in 5 mL of 0.05 M phosphate buffer (pH7) and the enzyme activity and protein concentration were measured.

Ion exchange chromatography: DEAE-celllulose ionic exchange was prepared according to the procedure of Whitaker and Bernard (1972). The sample obtained after ammonium sulphate precipitation with saturation ratio (70%) after dialysis the precipitate in distilled water for 24 h. then the concentrated enzyme solution was applied to a DEAE-cellulose column (2.5×18 cm) previously equilibrated with 50 mM phosphate buffer (pH7). The column was washed with the same buffer and eluted with a linear salt gradient with the same buffer containing 0.1-1 M NaCl. The fractions were collected in test tubes at flow rate 30 mL/h. Protein concentration in each fraction was monitored spectrophotometrically at 280 nm. Fractions of the protein peaks were assayed for agarase activity. Fractions containing enzymatic activity were collected and concentrated for further experiment.

Gel filtration chromatography: The gel (Sephadex G-100) was prepared according to the instruction of the manufacturer (Pharmacia Fine Chemical). The fractions collected from DEAE-cellulose column chromatography were applied to a Sephadex G-100 column (2×40 cm) previously equilibrated with 0.2 M phosphate buffer (pH7). Elution was performed with the same buffer. The fractions were collected in test tubes at flow rate 30 mL/h. Protein concentration in each fraction was monitored spectrophotometrically at 280 nm. Fractions containing enzymatic activity were collected and stored in refrigerator for further characterization.

Enzyme characterization:

Effect of pH on agarase activity: Buffer solutions of different pHs (4-10) were prepared. (0.1 mL) of partially purified agarase was added to 0.9 mL. Of a buffer solution containing 0.5% agar as a substrate and the enzyme activity was assayed for each pH.

Effect of pH on agarase stability: Equal volumes of partially purified enzyme and buffer solution with pH ranging from 4 to 10 were incubated in a water bath at 35°C for 30 min. Then transferred immediately into an ice bath. The remaining activity of enzyme for each pH was measured.

Effect of temperature on agarase activity: Agarase activity was assayed at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C, respectively). Agarase activity was plotted against the temperatures to determine the optimal temperature for agarase activity.

Effect of temperature on agarase stability: Equal volumes of partially purified enzyme were incubated for 15 min at different temperature (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80ºC, respectively) then immediately transferred into an ice bath. Enzymatic activity was measured and the remaining activity (%) was plotted against the temperature.

Effect of PMSF on enzyme activity: Equal volumes of partially purified enzyme were mixed with 1 and 5 mM of PMSF (dissolved in methanol) and incubated in water bath at 35ºC for 15 min. The remaining activity of enzyme (%) was estimated.

Effect of metal ions, reducing and chelating agents on enzyme activity: Equal volumes of partially purified enzyme were mixed with 5 and 10 mM of different metal salts ($CuSO_4$, $MnCl_2$, KCl, AgCl, $HgCl_2$ and $CaCl_2$) and incubated in water bath at 35°C for 15 min. These metal ions were prepared by dissolving these metal salts in phosphate buffer (pH7).

Equal volumes of partially purified enzyme were mixed with 2 and 5 mM of chelating agent Ethylene Diamine Tetra Acetic acid (EDTA) and reducing agent (ß-mercaptoethanol) and incubated in water bath at

Table 1: Purification steps of agarase produced by *Bacillus* sp., H12

Steps of purification	Volume (mL)	Activity (U/mL)	Protein conc (mg/mL)	Specific activity (U/mg)	Total activity (U)	Fold of purification	Yield (%)
Crude extract	25	9.6	0.80	12	240	1	100
Precipitation by 70% saturation ammonium sulphate	5	20	0.60	33.3	100	2.80	41.7
Ion exchange chromatography by DEAE-cellulose (elution)	15	5	0.10	48.5	75	4.04	31.3
Gel filtration chromatography by Sephasex G-100	18	2.9	0.04	68.1	51.5	5.70	21.5

35°C for 15 min. These compounds were prepared by dissolving these compounds in phosphate buffer (pH7). The remaining activity (%) was estimated.

RESULTS AND DISCUSSION

Precipitation of enzyme by ammonium sulphate: In order to concentrate the crude extract of agarase and remove a much of water and some protein molecules as possible, ammonium sulphate were used at (40, 50, 60, 70, 80, 90%, respectively) saturation, the saturation ratio (70%) was selected. It achieved specific activity 33.3 U/mg protein, 2.8 purification fold with 41.7% yield. Protein precipitation by ammonium sulphate depends on the salting out phenomenon. Since ammonium sulphate has the ability to neutralize charges at the surface of the protein and to disrupt the water layer surrounding the protein, it will eventually cause a decrease in the solubility of the protein which, in turn will lead to the precipitation of the protein by the effect of salt (Segel, 1976; Englard and Seifter, 1990). Ammonium sulphate are widely used because of its availability, high solubility and low cost and for the more it stabilizes the proteins (Volesky and Loung, 1985).

Ion-exchange chromatography: Purification of agarase was done by ion-exchange chromatography by (DEAE-cellulose). Results in Table 1 showed agarase purified by using anion-exchange column chromatography using DEAE-cellulose. Figure 1 showed the wash and elution steps of DEAE-column and the wash step of DEAE-column contained one protein peak without agarase activity, while the eluted fractions of DEAE-cellulose revealed one peak for protein had agarase activity. This result indicates that *Bacillus* sp., H12 agarase have negative charge. The fractions which had agarase activity were collected together giving specific activity of 48.5 U/mg proteins with 4.04-fold of purification and 31.25% yield (80-96) for the agarase enzyme. These results are similar to those found by Suzuki *et al.* (2003) and Suzuki *et al.* (2003) who purified ß-agarase from *Bacillus* sp., MKO3 by DEAE-Toyopearl 650S, and the purified agarase showed specific activity 1.57 U/mg protein and

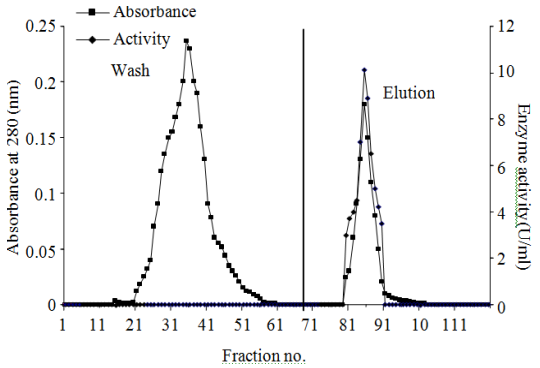

Fig. 1: Purification of agarase from local isolate *Bacillus* sp., H12 by DEAE-celluloseion-exchange chromatography column (2.5×18 cm) equilibrated with 0.05 M phosphate buffer pH 7; enzyme was eluted with linear salt gradient 0.1-1 M Nacl, flow rate 30 mL/h

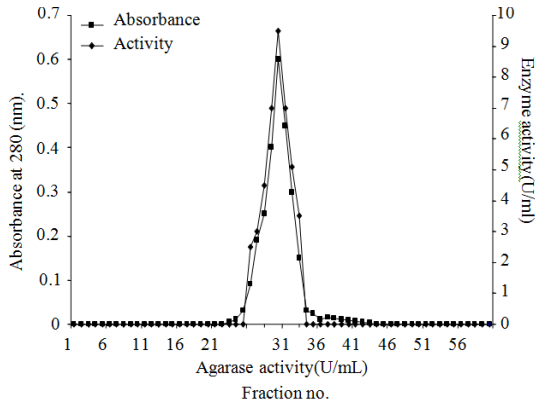

Fig. 2: Gel filtration chromatography of agarase from local isolate *Bacillus* sp., H12 by Sephadex G-100 column (2×40 cm) equilibrated with 0.2 M phosphate buffer pH 7, flow rate 30 mL/h

14.3-fold of purification with a yield 21.5%. The purified an extracellular α-Neoagarooligosaccharide Hydrolase from *Bacillus* sp., MKO3 by DEAE-Toyopearl 650S showed specific activity 3.26 U/mg protein and 7.3 fold of purification with a yield 6.3% (Suzuki *et al*., 2002).

Gel filtration chromatography: The fractions of peak containing agarase activity from DEAE-cellulose

column chromatography were applied to a Sephadex G-100 column. Figure 2 showed that there was one peak have agarase activity with specific activity 68.09 U/mg protein, fold of purification 5.67 and yield 21.45%. These results are similar to those found by Suzuki *et al.* (2003) who purified agarase from *Bacillus* sp., MKO3 by gel filtration chromatography using Sephadex G-150 and Superdex 200HR columns as third step of purification, the enzyme was purified 129-fold and 14.2 U/mg proteins with a yield of 7.0%. ß-agarase purified from marine bacterium *Bacillus cereus* ASK202 by using DEAE Sepharose CL-6B and the purified enzyme showed specific activity 3250 U/mg protein and 27-fold of purification with a yield 30% (Kim *et al*., 1999).

Enzyme characterization:

Optimum pH of agarase activity: Figure 3 showed the effect of pH on the activity of partially purified agarase from *Bacillus* sp., H12. Was studied in pH range from 4-10. Results showed that the maximum activity of agarase was at pH 7 and the agarase activity was 5.5 U/mL. pH has effect on the ionic state of enzyme by effecting on the amino acid chains necessary for tertiary structure of enzyme and hence its activity may varies. Higher or lower pH from the optimum will lead to denature the enzyme and losing its activity (Whitaker and Bernard, 1972; Segel, 1976). The optimum pH value for the agarase activity from *Agarivorans* sp., JAMB-A11 was 7.5 to 8 (Ohta *et al*., 2005). While the optimum pH for the activity of agrase enzyme from *Bacillus* sp., MKO3 was 6.1 (Suzuki *et al*., 2002). The optimum pH for the activity of agarase enzyme from marine bacteria *Altermonas* sp., SY 37-12 was 7 (Wang *et al*., 2006).

Effect of pH on agarase stability: In order to determine the optimum pH for agarase stability, the enzyme was incubated in buffer solution with pH range (4-10) at 35°C for 15 min. Results indicated in Fig. 4 that agarase had high stability at pH range between 6.5-7.5 the activity of agarase was 6.5 U/mL, in which it kept more than 90% of its total activity. The effect of pH on the enzyme stability could be explained in the formation of ionic form of enzyme or the active sites, irreversible inactivation. The stability of the enzyme depends on many factors such as temperature, ionic strength, chemical nature of buffer, concentration of various preservatives, concentration of metal ions, substrate and enzyme concentration (Segel, 1976). Suzuki *et al*. (2002) demonstrated that the optimum pH value for agarase enzyme stability from *Bacillus* sp., MKO3 was 6.4 to 8.6 (Suzuki *et al*., 2002). While the

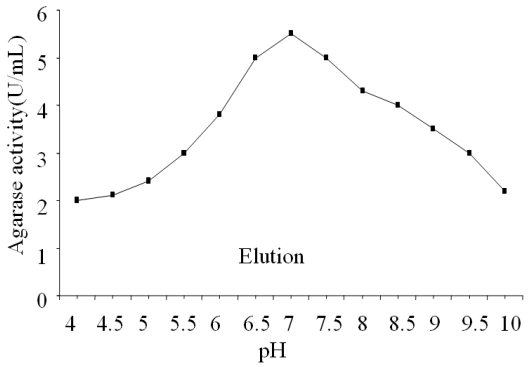

Fig. 3: Effect of pH on partially purified agarase from local isolate *Bacillus* sp., H12

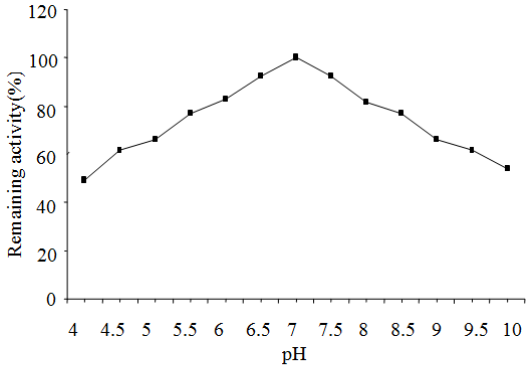

Fig. 4: Effect of pH stability on partially purified agarase from local isolate *Bacillus* sp., H12

optimum pH value for agarase enzyme stability from *Bacillus* sp., ASK202 was 5.8 to 10 (Kim *et al*., 1999). The agarase produced from *Pseudomoas* sp., SK38 has a pH value for stability was 8-9 (Kang *et al*., 2003).

Effect of temperature on agarase activity: The results in Fig. 5 indicated that the activity of agarase reaches the maximum 7.0 U/mL at 45ºC and decreased to 1.1 U/mL at 75ºC, which may be attributed to the denaturation of the enzyme after incubation at high temperature due to structural and conformational changing of the protein molecule, this will influence the binding of enzyme and substrate (Wang, 1999). The optimum temperature for agarase activity from *Altermonas* sp., SY37-12 was 35ºC (Wang *et al*., 2006). While The optimum temperature for agarase activity from *Bacillus cereus* ASK202 was 50ºC (Kim *et al*., 1999).

Effect of temperature on agarase stability: The thermal stability of agarase from *Bacillus* sp., H12 was examined by enzyme incubation at various temperature

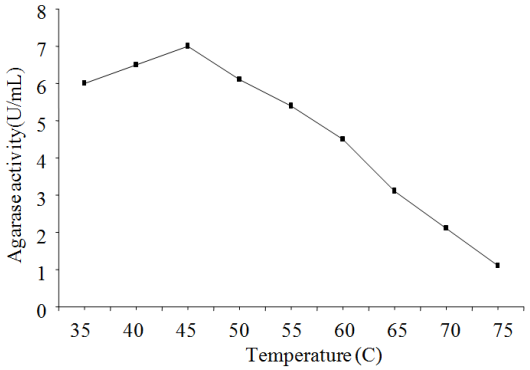

Fig. 5: Effect of temperature on partially purified agarase from local isolate *Bacillus* sp., H12

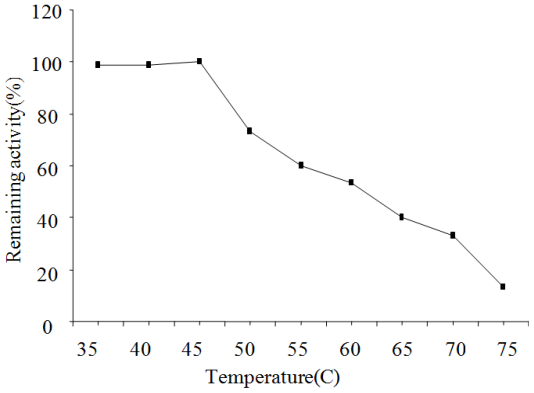

Fig. 6: Effect of temperature stability on partially purified agarase from local isolate *Bacillus* sp., H12

(35-75ºC) for 15 min. The results in Fig. 6 revealed that the agarase enzyme are stable at 35-45ºC, the activity declined at higher temperature, although at 65ºC about 40% of the activity remained. The enzyme was suppressed at 75ºC, about 13.3% of the activity remained. The agarase produced by *Pseudomonase* sp., SK38 was stable at temperature 37ºC (Kang *et al*., 2003), while the agarase produced by *Agarivorans* sp., JAMB-A11 keeps 85% of its activity when incubated at 45ºC for 15 min (Ohta *et al*., 2005).

Effect of some chemical compounds on enzyme activity:

Effect of metal ions on enzyme activity: The results of agarase treated with metal ions (Table 2) showed that the agarase was decreased when treated with 5 and 10 mM of $HgCl_2$, AgCl and $CuSO_4$. While the agarase activity was enhanced when treated with 5 and 10 mM of $CaCl_2$ and the agarase kept its activity when treated with 5 and 10 mM of $MnCl_2$ and KCl. The inhibition of agarase activity by $HgCl_2$, AgCl and $CuSO_4$ may

Table 2: Effect of metal ions on purified *Bacillus* sp., H12 agarase activity

Metal salt	Concentration (Mm)	Remaining activity (%)
Control	-	100
$CuSo_4$	5	37.50
	10	26.25
$CaCl_2$	5	100
	10	112.5
AgCl	5	25
	10	13.75
$HgCl_2$	5	12.50
	10	7.5
$MnCl_2$	5	90
	10	97.5
KCl	5	87.5
	10	92.5

Table 3: Effect of reducing and chelating agents on *Bacillus* sp., H12 agarase activity

Compound	Concentration (Mm)	Remaining activity (%)
Control	-	100
EDTA	2	43.75
	5	25
2-mercaptoethanol	2	97.50
	5	83.75
PMSF	2	97.50
	5	91.25

indicated the presence of SH group in the active site of the enzyme leading to oxidize them by $HgCl_2$, furthermore the presence of $HgCl_2$, AgCl and $CuSO_4$ in the substrate working solution may form a complex with the enzyme which prevent it from binding to the substrate and form the product. The activity of agarase enzyme produced by *Bacillus* sp., MKO3 enhanced by Mg^{+2}, Ca^{+2}, Rb^{+}, K^{+} at 2 mM, while the activity was inhibited by Ag^{+}, Hg^{+2} and Cu^{+2} at 2 mM (Suzuki *et al*., 2003).

Effect of reducing and chelating agents on enzyme activity: The results in the (Table 3) revealed the effect of reducing and chelating agents on agarase activity, the agarase activity was not inhibited when it was incubated with 2 and 5 Mm of 2-mercaptoethanol, these results indicated the presence of SH group in or near the active site. The reducing compounds aid the SH group to be stable. The agarase produced by *Bacillus* sp., MKO3 remained active when treated with reducing agent 2-mercaptoethanol at 2 mM (Suzuki *et al*., 2003). *Bacillus* sp., H12 agarase was inhibited by chelating agent (EDTA) at 2 and 5 mM, these results indicated that this agarase enzyme refered to metalloenzymes on which the activity of enzyme is dependent on some kinds of ions. Additional chelating agents to the reaction medium it forms complexes with the ions in the active site which cause inhibition of enzyme activity. The α-Neoagarooligosaccharide produced by

Bacillus sp., MKO3 was inhibited by chelating agent (EDTA) at 2 mM (Suzuki *et al*., 2002). The agarase enzyme did not affected by the present of PMSF in the reaction mixture at (1 and 5 mM) indicated that this agarase enzyme are not belong to serine enzymes.

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