PRODUCTION AND PURIFICATION OF CHITINASE FROM *AEROMONAS SPP.* AND STUDY ITS EFFECT ON SOME CANCER CELL LINES *IN VITRO*

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ABSTRACT

Three hundred samples of stools were collected from children who are suffering from diarrhea. Twelve of them (4%) were positive for *Aeromonas* spp. test, the ability of *Aeromonas* isolates to produce chitinase enzyme was tested on medium supplemented with colloidal chitin. Chitinase enzyme was extracted, the crude extract activity and specific activity was 13 U/ml and 7.3 U/mg respectively. The enzyme was purified by precipitating with ammonium sulfate 70% saturation followed by ion exchange chromatography using DEAE- cellulose column and gel filtration chromatography using Sephadex G-200 column. The specific activity reached 154.5 U/mg with 21.16 purification fold and yield of enzyme was 52.3%. The molecular weight of the purified chitinase was 48 kDa when determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cytotoxicity effects of chitinase enzyme on different normal and cancer cell lines were investigated by MTT assay. The results showed that the chitinase has a cytotoxic effect on breast cancer cells line (MCF-7) at concentration of 300 µg /ml., and it was cytotoxic to prostate cancer cell line (PC-3) at 400 µg/ml. Whereas it showed no cytotoxic effect on normal embryonic liver cell line (WRL-68).

Key words: **diarrhea, chitinase, chromatography, cancer.**

مجلة العلوم الزراعية العراقية -2018: 49(3):438-444 هاشم ونعمه

انتاج وتنقية انزيم الكايتينيز من بكتريا *AEROMONAS SPP.* ودراسة تأثيره على بعض خطوط الخلايا السرطانية

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المستخلص

جمعت 300 عينة خروج من مرضى اطفال يعانون من اصابات الاسهال 12 منها (4%) أعطت نتيجة موجبة لبكتريا ***Aeromonas spp.*** . اختبرت قابلية عزلات ***Aeromonas*** **sp.** على انتاج انزيم الكايتينيز على وسط اكار مدعم بالكايتين الغروي. تم استخلاص الانزيم حيث كانت الفعالية والفعالية النوعية للإنزيم الخام 13 وحدة/مل و 7.3 وحدة/ملغم على التوالي. تم ترسيب الانزيم بواسطة كبريتات الامونيوم بنسبة اشباع 70% ثم نقي بواسطة كروماتوغرافيا التبادل الايوني باستخدام عمود المبادل الايوني –DEAE (Cellulose) وثم كروموتوغرافي الترشيح الهلامي بعمود (Sephadex G–200) . اذ تم الحصول على انزيم منقى بفعالية نوعية 154.5 وحدة/ملغم بعدد مرات تنقية 21.16 وحصيلة انزيمية 52.3%. كما تم تحديد الوزن الجزيئي للكايتينيز المنقى بتقنية –SDS PAGE حيث كان 48 KDa. تم الكشف عن التأثير السمي الخلوي لأنزيم الكايتينيز على الخلايا الطبيعية والسرطانية وباستخدام طريقة MTT. اظهرت النتائج التأثير السام لأنزيم الكايتينيز على خطوط خلايا سرطان الثدي (MCF–7) اذ بلغ التركيز المثبط 300مايكرو غرام/مل. بينما اظهر الانزيم تأثيرا سميا على خطوط خلايا سرطان البروستات (PC–3) اذ بلغ التركيز المثبط 400مايكرو غرام/مل، في حين لم يكن له تأثير يذكر مع خطوط خلايا الكبد الجنينية الطبيعية (WRL–68).

الكلمات المفتاحية: الاسهال، الكايتينيز، الكروماتوغرافيا، السرطان

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INRODUCTION

Aeromonas species got amassed attention as opportunistic pathogens because its link with diarrhea and extra intestine diseases in human especially, in children and adults with impaired immune system (19, 28). Isolation of *Aeromonas* from water and food sources, and the growing resistance of this bacteria to antibiotics and chlorination in drinking water, their spreading in the environment is considered as threating issue for public health because food and water are main sources of human infection (29). Among bacteria etiologies of diarrhea, *Aeromonas* is known progressively as a clinically important enteric pathogen (17). Chitinase is part of the glycosyl hydrolase group, which breaks the 1, 4 *β*-glycoside bond of *N*-acetyl D-glucosamine. Except for chitinase (3.2.1.14), which casually breaks glycosidic bond in chitin molecule a *β*-*N*-acetyl glucose aminidase has a significant role in the hydrolysis method. This enzyme has many important and various applications specially, in the medicine field and tumors researches (24). Cancer is a molecularly heterogeneous disease and one of the main reasons of death worldwide. The presence of several types of tumors with diverse histopathology, genetic and epigenetic differences, and clinical consequences, is difficult understand this disease the mechanisms of action of chemotherapeutics and the making of new therapies (30). The objectives of this study are isolation and identification of *Aeromonas spp.* from patients suffering from diarrhea and screening the ability of local *Aeromonas spp.* isolates for chitinase enzyme production, optimization conditions of chitinase production from selected isolate of *Aeromonas spp*. then studying the effect of purified chitinase enzyme *in vitro* against normal and cancer cell lines.

MATERIALS AND METHODS

1. Samples collection: A total of 300 stool samples were collected between October and December 2016 from children who suffering from diarrhea, before starting their antibiotic therapy. Samples were collected from Fatima Al Zahra Hospital and Shaheed Al-Sadr Hospital. Stool samples were taken from the patients and inoculated on specific media by direct streaking method.

2. Isolation and Identification of bacteria: Isolated bacteria was tested and recognized by cultural, microscopic, biochemical testes, and Vitek 2 system.

3. Screening *Aeromonas spp*. isolates for chitinase production: 3.1. Semi quantitative screening

For screening of chitinase producing bacteria, the agar medium was supplied with the used colloidal chitin. The medium contains: Na_2HPO_4 6 g, KH_2PO_4 3 g, NH_4Cl 1 g, NaCl 0.5 g, yeast extract 0.05 g, agar 15 g, and colloidal chitin 1% w/v in 1L deionized water (D.W.) The isolated bacteria were screened by inoculate the isolates on the medium, and incubated at 37 ºC for 96 h., the positive isolates will produce clear zones (25).

3.2. Quantitative screening

Screening was achieved with bacterial isolates on the colloidal chitin agar medium incubated at 37°C. Positive isolates were selected after 96 h. of incubation and screened for maximum enzyme production in nutrient broth media supplemented with colloidal chitin. The cultures were centrifuged at 10,000 rpm for 15 min. at 4 °C and the crude extract was used for chitinase assay (25).

4. Preparation of colloidal chitin: Chitin powder (40 g) was gradually added to 600 ml of HCl and kept for 60 min. at 30 °C with continuous stirring. Chitin was settled as a colloidal suspension by adding 2 L of water slowly. The suspension was filtrated and washed by suspending in about 5 L of D.W. Washing was repeated 3 times until the pH of the suspension became neutral (10).

5. Measurement of chitinase activity: The reaction mixture contained 100 µl of supernatant and 100 µl of substrate 1% of colloidal chitin, it was incubated at 40 °C for 10 min. 400 µl of 3,5- Dinitro salicylic acid (DNS) regent was added to the mixture and boiled for 15 min. 4.4 ml with D. W. was added and the colour was measured at 535 nm. The chitinase activity was then reported due to a standard curve of N-acetyl-D-glucosamine prepared from a standard solution. One unit is defined as the amount of chitinase, which releases one µmol of N-acetyl-D-glucose amine in 1 min. (18).

6. Determination of protein concentration: The protein content was determined by using Bradford method with bovine serum albumin as the reference protein, (3).

7. Purification of chitinase enzyme: The enzyme crude extract collected was subjected to different steps of purification including ammonium sulfate precipitation, dialysis, and passed through DEAE-cellulose ion- exchange chromatography column with dimensions (2.5×30 cm) at a flow rate of 5ml/3min., then Sephadex G-200 gel filtration column with dimensions (2.5×25cm) at a flow rate 5ml/3min, both columns were equilibrated with Tris-HCl buffer pH 7.

8. Determination of molecular weight of chitinase: The molecular weight was determined by SDS- Poly acrylamide gel electrophoresis (SDS-PAGE) as mentioned by Lameli (14).

9. Effect of chitinase on tissue cultures *in vitro:* Effect of different concentration of chitinase enzyme on the adherent cells in 96-well microtiter plate has been achieved by MTT-assay according to method of (2). The cell lines that used in this study were MCF-7 (Human breast cancer cells), WRL-68 (Normal embryonic liver cell line), and PC-3 (Human prostate cancer cells), these cell lines were supplied by the Biotechnology Research Center, Al-Nahrain University.

10. Statistical Analysis: The Statistical Analysis System- SAS program, (27) was used to notice the effect of different factors (Concentrations, lines) in viability. Least significant difference –LSD test (ANOVA) was used for comparison between means.

RESULTS AND DISCUSSION

1. Isolation and Identification of bacteria: Out of three hundred (300) stool samples analyzed, only 12 (4%) samples were positive for *Aeromonas* bacteria. This finding agrees with the result of Lobna (15) that reported 3.15% (11 isolates) out of 349 stool samples in Babylon province was *Aeromonas spp*. Also agreed with other studies, Obaid (20) stated 2.7% (13 isolates) from 479 stool samples in Babylon province, Rogo (23) found 3.12% (4 isolates) of 128 stool samples in Nigeria, and Guerra (9) stated 6.6% (27 isolates) from 408 stool samples in Brazil were *Aeromonas spp*. The isolated colonies of *Aeromonas* that were grown on culture media looked 2 mm, rounded, smooth, convex, and small colonies on agar medium (4). The *Aeromonas* agar (Hi Media) supplied with ampicillin was used as a selective and differential medium. The microscopical examination of positive isolates of *Aeromonas spp*. revealed Gram negative rod bacteria, that looked single, or in pairs slide this finding agreed with the result that was realized by (4, 26). The positive isolates of *Aeromonas spp.* gave a positive result for oxidase, catalase, indole, methyl red, vogues - proskauer, citrate utilization. This bacteria can ferment glucose on kligler iron agar and give red color on the top while the bottom seemed yellow without H_2S production. It gave a negative result for urease test. This is agreed with the standard characteristics of *Aeromonas spp*. That were cited by (7, 15). Vitek 2 System was used to confirm the positive results that was obtained from the former tests. Other studies (5, 11) were used Vitek 2 System as effective biochemical test for identification of *Aeromonas spp*.

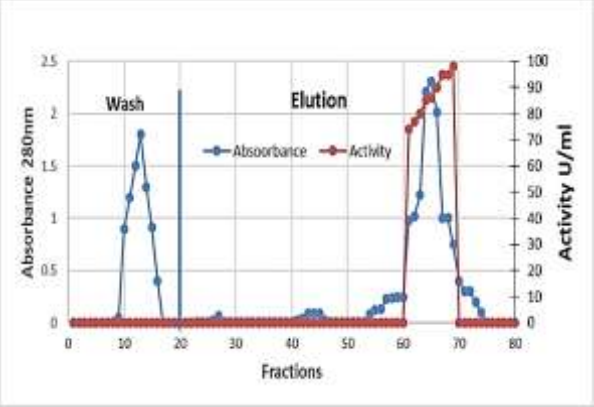
2. Screening and selection the chitinase producing isolates: All isolates were screened on the colloidal chitin agar medium. Producing isolates showed clear zone surrounding the colonies. The results showed that three isolates were chitinase producer while nine isolates were not able to produce this enzyme. Selection of the isolates were done upon the diameter of the clear zone around the colonies.

3. Extraction of the enzyme: The selected isolates were grown in Luria Bertaini broth medium, (pH 8), incubated at 37^{o}C for 48 h. into shaking incubator then centrifuged under refrigeration, and the supernatant was used as crude enzyme extract. The chitinase activity and specific activity was 13 U/ml and 7.3 U/mg respectively, (Table 1).

4. Purification of chitinase enzyme: After extraction, the pellet was taken for 70% ammonium sulfate precipitation. The chitinase activity and specific activity was 35.3 U/ml and 29.9 U/mg respectively. The sample was exposed to dialysis against Tris- HCl buffer pH 7. The results in Table 1 display increasing in both activity of chitinase (52.2 U/ml) and the specific activity (46.6 U/mg protein). The dialyzed chitinase was applied to DEAE-cellulose column with dimensions (2.5 × 30)

cm equilibrated with Tris-HCl buffer pH 7. The column was washed with an equal volume of Tris-HCl buffer and eluted by using gradient concentrations of NaCl 0.5, 1, and 1.5 M. Figure 1 shows one protein peak in the washing step, and another protein peak appeared after elution by the gradient concentrations of NaCl. The protein peak obtained after elution step shows that the eluted protein fractions (61 to 69) contained the most chitinase activity that reaches 98.2 U/ml. Results in Table 1 show that the protein concentration, chitinase activity, and specific activity in this concentrate were 0.821 mg/ml, 98.2 U/ml, and 119.6 U/mg protein respectively, with purification fold of 16.29 and enzyme yield of 75.5 %.

Figure 1. Ion exchange chromatography for purification of chitinase produced by *Aeromonas spp.* using DEAE-cellulose column (2.5×30 cm) equilibrated with Tris-HCl buffer pH7 at flow rate 5ml/3min



The enzymic fractions were pooled and applied to Sephadex G-200, which was previously equilibrated with 0.1 M Tris-HCl buffer (pH 7). Figure 2 shows two peaks with chitinase activity appeared after elution with Tris- HCl buffer. Fractions representing chitinase activity were pooled and concentrated. Protein concentration, activity and specific activity of chitinase were measured, and the results in Table 1 show that there is a decrease in the activity and protein concentration of the purified enzyme (68 U/ml) and (0.44 mg/ml) respectively, but there were an increases in both specific activity (154.5U/mg) and Purification fold (21.04). Park (22) reported 33.1 purification fold for purified chitinase produced by marin *Vibrio* sp. 98CJ11027 with specific activity of 43 U/mg after applying of the enzyme on the Sephadex G-200 column, while purification of chitinase with Sephadex G-100 achieved 3.23-purification folds with specific activity of 114.74 U/mg (8).

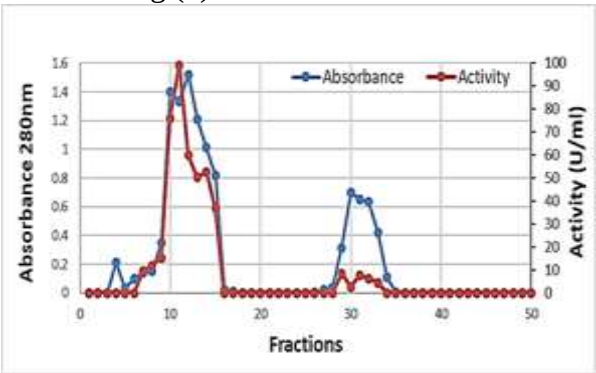


Figure 2. Gel filtration chromatography of chitinase produced by *Aeromonas spp.* using sephadex G-200 column (2.5×25cm) equilibrated with Tris-HCl buffer pH7 at flow rate 5ml/3min

5. Determination of molecular weight of chitinase: Figure 3 shows the SDS-PAGE gel analysis for chitinase molecular weight determination. The results revealed that the molecular weight of chitinase was 48 kDa. This result agrees with other studies that reported the molecular weight of chitinase from *Bacillus subtilis* was 45 kDa when determined by SDS PAGE technique (1).

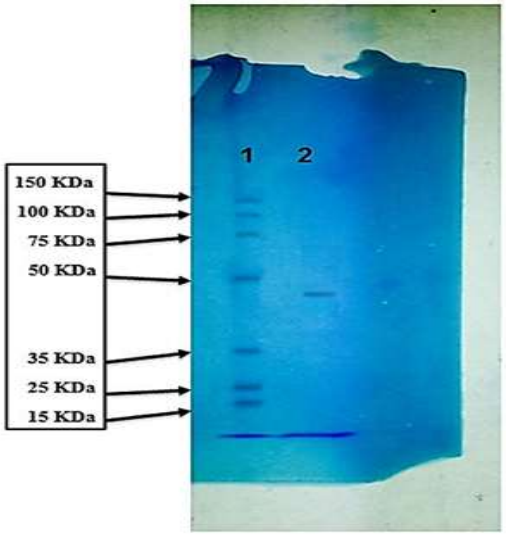


Figure 3. SDS- PAGE gel (15%poly acrylamide) analysis of chitinase Lane l: ladder protein Lane 2: sample

Table 1. Purification steps for chitinase produced by *Aeromonas spp.*

steps of purification	volume (ml)	Activity (U/ml)	protein concentration (mg/ml)	Total activity (U)	Total protein (µg)	specific activity (U/mg)	purification fold	yield %
crude	100	13	1.77	1300	177	7.3	1	100
Ammonium sulfate	30	35.3	1.18	1059	35.4	29.9	4.1	81.5
Dialysis	20	52.2	1.12	1044	22.4	46.6	6.35	80.3
Ion exchange	10	98.2	0.821	982	8.21	119.6	16.3	75.5
Gel filtration	10	68	0.44	680	4.4	154.5	21.0	52.3

6. **Effect of chitinase on tissue cultures *in vitro:*** Results in Figure 4 and Table 2 show that the purified chitinase has cytotoxic effect on MCF-7 cells and the half-maximal inhibition concentration (IC_{50}) values of purified chitinase-treated MCF-7 cells after 24 h. of incubation at 37 ºC was 300 µg /ml.

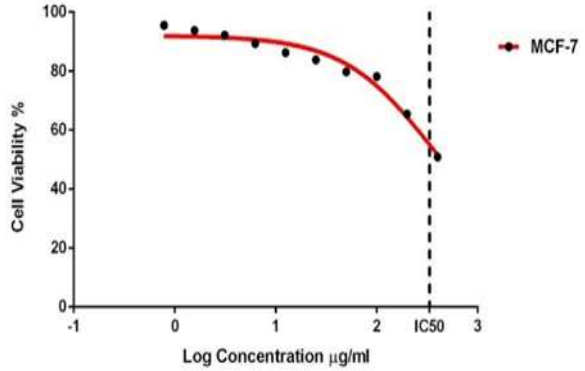


Figure 4. Cytotoxicity effect of purified chitinase on MCF-7 cell line

Results from Figure 5 and Table 2 show that the purified chitinase was cytotoxic to PC-3 cells and the IC_{50} of purified chitinase-treated PC-3 cells after 24 hr. was 400 µg/ml.

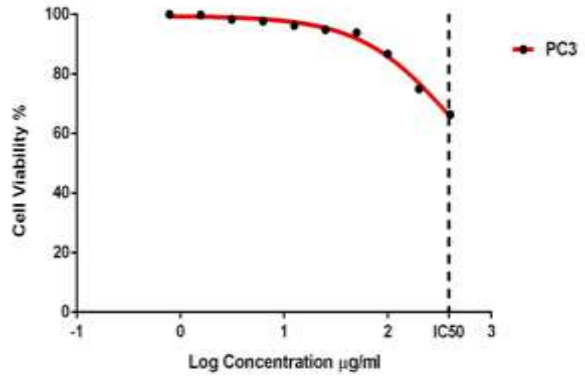


Figure 5. Cytotoxicity effect of purified chitinase on PC-3 cell line

While the results in Figure 6 show that the purified enzyme was not or less cytotoxic on WRL-68 cells.

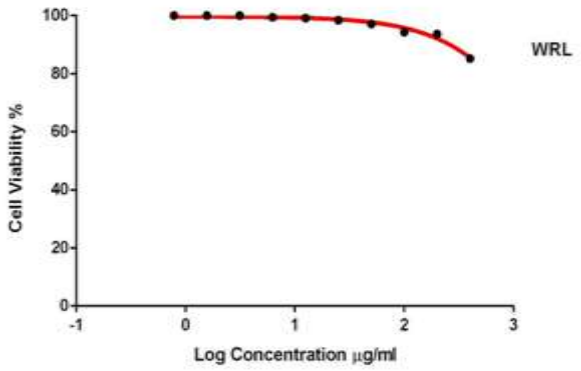


Figure 6. Cytotoxicity effect of purified chitinase on WRL-98 cell line

Table 2. Cytotoxic activity of Chitinase on WRL-68, MCF-7, and PC-3 cell lines by using MTT assay

Concentration (µg/ml)	Mean ± SD of viability (%) WRL68	MCF-7	PC-3	LSD value
400	85.23 ± 2.78	50.87 ± 7.07	66.38 ± 6.66	9.644 *
200	93.63 ± 1.45	65.40 ± 10.78	75.01 ± 2.47	9.261 *
100	94.28 ± 1.30	78.16 ± 8.54	86.74 ± 7.21	8.633 *
50	97.16 ± 1.70	79.75 ± 8.73	93.88 ± 2.40	7.912 *
25	98.45 ± 0.76	83.79 ± 7.45	94.90 ± 3.13	8.022 *
LSD value	8.177*	11.405 *	10.315 *	---

* (P<0.05)

Krithika and Chellaram (12) and Fadhil (6) findings were confirmed that the chitinase plays a great role in the medical field as an anticancer. Lam and Ng (13) recorded specificity of chitinase cytotoxicity against breast cancer cells. Pan (21) observed that 0.5 U/ml of family 18 chitinases could induce surface lysis of several kinds of cultural cancer cells as a results of chitinase induced lysis cancer cells killed during 24 h. whereas

normal cells were not killed. Xing (31) suggests that new polycarbohydrates, which react with chitinase, will appear on the surface of many cancer cells. These carbohydrates will have digested by chitinase, so their original function is damaged, and the tumor cells die. As normal mammalian cells do not appear these carbohydrates on their surfaces, so the treatment with chitinase will not hurt them.

The real mechanism of the anticancer effect of chitinase is not clear yet, thus future studies are necessary.

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