

## Isolation and Purification of metalloprotease produced by *Aeromonas hydrophila*

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### Abstract

**Background:** The bacteria *Aeromonas hydrophila* was isolated from clinical specimens responsible of causing diarrhea in children and adults.

**Objective:** To purify metalloprotease enzyme produced by *Aeromonas hydrophila*.

**Patients and Methods:** This study involved 150 stool samples collected from patients suffering from diarrhea.

**Results:** Eight isolate of *Aeromonas hydrophila* was detected and metalloprotease was purified with 11.06 U/ml of enzyme activity.

**Conclusion:** The bacteria have ability to produce metalloprotease and enzyme was fully purified in three steps of purification.

**Key words:** *Aeromonas hydrophila*, Metalloprotease.

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### Introduction

Species of *Aeromonas* spp are Gram-negative, Non spore forming, the shape like rod, anaerobic bacteria in everywhere and especially in aquatic environments. *Aeromonas* widely range in aquatic environments such as surface waters (fresh, estuarine and marine), groundwater, drinking water systems, wastewater, soil, as well as food, such as vegetables, meat, offal, sausage, raw milk, and fish [1].

The versatility movement of *Aeromonas* spp. caused many diseases in man and animals is related to an array of virulence factors. The factors of virulence are widely divided in structural components, extracellular factors and cells associated characters, a number of studies mention the

Genetic distribution of virulence in isolates from different regions of the world [2]. A number the factors of virulence have been investigated and have been identified in *Aeromonas hydrophila*, which include pili and adhesions, exotoxins and a number of external enzymes such as proteases, amylases and lipases [3].

The assistance of the proteases to the pathogenicity it may be due to the capabilities to overcome the host defenses and supply nutrients to bacterial cells, then promoting invasive and establishment of infected [4]. Aims of this study:

1-Isolation and identification of *A. hydrophila* from patients suffering diarrhea.

2- Screening ability of the local isolates of metalloprotease production and select the most efficient one.

3- Purification of metalloprotease enzyme produced by the selected isolated.

## Patients and Methods

### Subjects and Samples

A total 150 clinical samples of stool were collected with aseptic technique in sterile cups from patients suffering diarrhea who had certain clinical symptoms. These samples were collected from Fatima-Alzahraa Teaching Hospital, Ibn-albaldi and Teaching laboratory in medical city Hospital in Baghdad, during the period from October 2016 to January 2017.

**Identification:** After first selection based on growth density, the selected isolate was purified by repeated streaking plate methods. When a plate yielded only one type of colonies the organisms considered to be pure. The collected swabs were streaked aseptically on Blood agar and MacConkey agar. The plates were incubated at 37 °C for 18-24 h. Preliminary identification of bacteria was based on colony characteristics of the tested bacteria such as colony description, hemolytic activity on blood agar and enzyme activities of the tested isolates [5].

**Vitek 2 system:** The Vitek-2 System is a phenotypic system of identification, which depends on the biochemical reactions between the bacterial isolates suspended in their respective tubes of solution, and the media in the VITEK2 Identification Cards, to identify the isolates. In the first stage of the

experiment, 8 sample isolates were inoculated onto MacConkey agar and then incubated for 24 h. at 37°C. A single colony was taken and suspended in solution. The turbidity of the suspension of bacteria was modified with VITEK Densi chek (bioMerieux) to match the McFarland 0.5 standard in 0.45% sodium chloride.

Then the VITEK 2 ID-GN (Gram negative) card and suspension of the bacteria tubes were manually loaded in the VITEK-2 system. Following steps on the software were done according to the manufacturer's instructions.

Biochemical tests for identification of Gram-negative bacteria were conducted, in the same context, Gram-positive bacteria were identified based on catalase and coagulase tests. All bacterial isolates were confirmed by using VITEK 2 (Biomérieux, France) and the results were interpreted by using VITEK software (version 06.01).

**Screening isolates for metalloprotease production:** Each isolate of *Aeromonas hydrophila* streaked on nutrient agar and was incubated at 30 °C for 24h. A single colony was taken and put on the center of skim milk agar plate. The plate was incubated for 24h. at 30°C. The ability of Tested in metalloprotease production have been measured based on appearance of the clear halo zone around the colony [6].

### Metalloprotease purification

**Enzyme production and cell free filtration:** The over producer *Aeromonas hydrophila* was grown in the production medium under the optimum conditions. Crude filtrate (crude

metalloprotease) was collected through centrifugation amount of enzyme for 15 min. at 4°C in order to take out a cell free filtrate, 100 ml of the cell free filtrates contains metalloprotease were collected, the activities of proteolytic and protein concentration have been determined.

#### **Purification steps**

##### **The Ammonium sulfate precipitation:**

Ammonium sulfate precipitation was achieved by adding ammonium sulfate to the crude enzyme gradually with continuous mixing on ice in saturation percentage of 30%-70%, the Mixture was centrifuged for 20 min at 4°C. The pellet was dissolved in 50 ml of 0.05M Tris-HCl buffer (pH8). The proteolytic activity and protein concentration were determined.

**Dialysis of crude enzyme:** The obtained ammonium sulfate precipitates (in solution) was dialyzed in dialysis tube with 12000 MW cutoff against 0.05M Tris- HCl buffer pH8, for 3h., followed by dialysis against the same buffer overnight. The obtained protease enzyme preparation was kept at 4°C for further purification steps.

**Purification through ion exchange chromatography:** The dialysis of metalloprotease was moreover purified through ion-exchange chromatography technique by using DEAE Cellulose column by dissolving 20 g of resin in 1L of distilled water. Then beads were left to settle down and washed several times with D.W until getting clear appearance. The suspension was filtered throughout Whatman No.1 using Buchner funnel under discharging. The resin

was suspended in 0.25 M sodium chloride and sodium hydroxide solution. The suspension was filtered again as mentioned above and washed several times with 0.25 M hydrochloric acid solution and next by distilled water before it was equilibrated with 0.05 M Tris-HCl pH8.

After preparation, the dialyzed enzyme was then applied to ion exchange chromatography column equilibrated previously with 0.05M Tris-HCl buffer pH8. The column was washed with an equal volume of the same buffer, while attached proteins were eluted with gradual concentrations of sodium chloride (0.1-0.9 M respectively). Enzyme was eluted at flow rate of 30ml/h (3ml/fraction), absorbance of each fraction has been measured at 280 nm. using spectrophotometer. The enzyme activity was determined in each fraction as described, Fractions presents metalloprotease activity were pooled and kept for further steps of purification.

##### **Purification by Gel filtration chromatography:**

The next step in purification of metalloprotease was achieved by gel filtration chromatography technique through Sephadex G-200. It was prepared as recommended by Pharmacia Fine Chemicals Company [7]. A quantity of Sephadix G-200 was suspended in 0.1 M Tris-H Cl buffer pH8, degassed, and packed in a glass column 20x2.5 cm. and calibrated with 0.1 M Tris-HCl buffer pH8. A volume of concentrated metalloprotease obtained from the ion exchange step was applied onto the matrix,

equilibrated previously with 0.1 M Tris-HCl buffer pH8.

Elution was achieved at a flow rate of 5ml/3min using the same buffer for equilibration, Absorbance of each fraction has been measured at 280 nm. enzyme activity was also determined in each fraction.

## Results

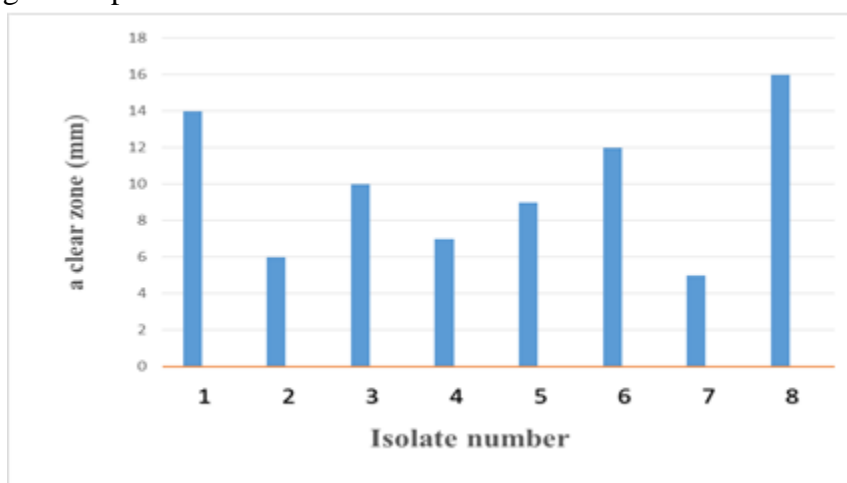
### Identification of bacteria

Colonies appearance on blood agar was appeared a large flat- gray colonies have been shown to detect beta-type  $\beta$ -hemolysis, whereas, appeared The colonies appeared pale pigmented, not having the ability to ferment lactose sugar on MacConkey agar. There are eight samples were identified as

(*Aeromonas hydrophila*) by the VITEK-2 system.

### Screening ability of *A. hydrophila* for in metalloprotease production

Results was showed that eight isolates of bacteria were have ability to hydrolyze enzyme production medium to forming a clear zone with variable degrees. The diameters of halos was variable from 5 to 16mm depending in the isolate. The isolate number eight was the most effective to produce metalloprotease with the highest diameter (16 mm) on the enzyme production medium.



**Figure (1):** The diameter of clear zone around colonies of *Aeromonas hydrophila* on metalloprotease production medium.

### Enzyme purification

#### Ammonium sulfate precipitation and dialysis

Ammonium sulfate, used at different saturation rates (0-30 and 30-70%), were used to determine the best range for metalloprotease precipitation, the results showed that the best range of the enzyme

was precipitated in the saturation range between (30-70%). The dialysis was done by using 12000 molecular weight cutoff dialysis bag against 0.05M Tris/ HCl buffer pH8. Results showed increasing in both activity of enzyme (8.68 U/ml) and the specific activity (41.3 U/mg protein).

**Table (1):** Purification steps of enzyme.

Steps	Volume (ml)	Enzyme activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (units)	Fold	Yield (%)
Crud extract	100	7.3	0.35	20.8	730	1	100
Ammonium Sulfate	50	7.8	0.23	33.9	390	1.6	53.4
Dialysis	30	8.64	0.21	41.3	260	1.9	35.6
Ion exchange chromatography	25	9.12	0.16	57	228	2.7	31.2
Gel-filtration Sephadex G-200	20	11.06	0.03	368.6	221	17.7	30.2

### Ionic Exchange Chromatography

The fractions pooled and tested for specific activity (57 unit/mg) a fold purification of (2.7) and enzyme yield of (31.2%).

### Gel filtration chromatography

Furthermore purification done through a gel filtration chromatography by using Sephadex G-200. The Enzyme fractions from DEAE-cellulose were pooled and passed through gel filtration column. The fractionation yielded one protein peak as absorbance reading at 280nm (wave length), the peak (fraction tubes 17–21) contained enzyme activity (11.06 unit/ml), protein concentration (0.03mg/ml) with specific activity (368.6 unit/mg) and the purification fold was (17.7) with yield of enzyme (30.2%).

### Discussion

Due to the fact which *Aeromonas* are usually isolated from water environment and are linked with gastroenteritis and wound infections in humans and studies concerning

the presence of these organisms in raw and drinking water distribution in systems are so important to be sure the good quality of the water which leads to protection and promotion the public health. The World Health Organization at present proposed that *Aeromonas hydrophila* is consider one of the contaminants of concern in waterborne diseases [8]. The bacteria Infect digestive system of the fish as well as the human range of infection from inflammation of the intestines into the blood infection and are found in foods such as fish and milk as well as meat, therefore these bacteria classified as the pathogenesis of man and fish [9].

The infection increased in the warm months of the year as the number of the bacteria frequently in the environment, and in cases of diarrhea related to the existence *Aeromonas* be more common in children and in particular at least three years abound injury in developing countries compared to the infection in the industrialized countries and this is proof that the health factor is the

specific epidemiological factor [Rey *et al.*, 2009], *Aeromonas* was increasing their persistence over many years and make it harmful for public health consumers of distribution pipe water, especially who immunocompromised [10].

Metalloproteases were been a proteolytic enzymes produced by bacteria, they are widely distributed in *Aeromonas* species. This enzymes could have a potential effect due to play a role in infected and establishment through overcoming host defenses and providing nutrients, [11].

Purification of the protein is a chain of processing aims to isolate a one type of protein from a mixture. The purification protein is very important for the description of the function, structure and interactions among the protein of interest. Different steps in the purification operations leads to free the protein from a matrix that contain it, the separate of protein and non-protein parts of the mix, and at final separate the selected protein from all other proteins. The protein separation from others is usually the hardest side of protein purification. The purification steps benefit of differences in the size of protein, physico-chemical characters and binding affinity. There are many methods for separation according to molecular weights such as Ultra filtration; Dialysis and Gel filtration, while the Ion-Exchange Chromatography and Electrophoresis are dependent on net charges of protein molecules to be purified [12].

## Conclusion

1-The best method to diagnose the bacteria is Vitek2 GN system.

2-*Aeromonas hydrophila* clinical isolate produce metalloprotease at high percentage and efficient in the production of metalloprotease.

3-Metalloprotease produced by AH8 *A. hydrophila* was fully purified in three steps of purification.

4-*A. hydrophila* has one pattern of Metalloprotease.

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