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ISOLATION AND PURIFICATION COMMERCIAL AEROLYSIN ENZYME FROM AEROMONAS HYDROPHILA IN NATURAL FLORA OF GUT SYSTEM IN WARM WATER FISH

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ABSTRACT

An important pathogenic factor for bacteria is secretory toxins. Aerolysin is a virulence factor of A.hydrophila. hemolytic activity of aerolysin destroys the cell membrane of eukaryotic cells by pore-forming. The receptor of this enzyme is GPI-anchored proteins. These two properties (hemolytic activity, GPI-anchored protein receptor) caused used aerolysin for different goals. There is recombinant form of aerolysin commercially but this is very expensive. This study determines a simple method for aerolysin isolation from A.hydrophila.

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1. Introduction

Aerolysin is a channel-forming cytolytic toxin secreted by a pathogen bacterial, Aeromonas hydrophila, and exerted as an inactive dimeric precursor. Aerolysin was identified 1975 by Bernheimer and Avigad, who gave the protein its name. Aerolysin was purified by Buckley et al. This enzyme is a virulence factor for A.hydrophila [1-3]

A.hydrophila is one of the normal bacterial flora of the all species of freshwater fish that is a no spore-forming, Gram-negative, pleomorphic bacillus with a monotrichous flagellum [4].

Like many other microbial toxins, the protein changes in a multistep processes from a completely water-soluble form to produce a trans membrane channel that ruins sensitive cells by breaking their permeability barriers. Any precursor is water soluble 52KD protoxin monomer [5]. In the inner cell membrane of the bacterial aerolysin is hydrophobic preprotoxin form that contain a secreted signal sequence, which is cleaved cotranslationally. 25 amino acids near the C- terminus within the mobile loop of protoxin removed by proteolytic removal that is required for oligomerization and pore forming because mobile loop contains the KVRARRA sequence that Mammalian proprotein convertase enzymes recognize it [5-7].

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Aerolysin uses amphipathic ß-hairpins from 6 to 7 individual monomers that insert into the lipid bilayer, and subsequently form the pore that rich in ß-sheets [8, 9].

The cytolytic toxin, aerolysin, was found to form ion channels which displayed slight anion selectivity in planar lipid two layers [10, 11]. In voltage-clamp experiments the ion current flowing through the channels was homogeneous that indicating a defined conformation and a uniform size. The channels remained open between −70 to +70 mV, but outside this range they underwent voltage-dependent inactivation which was observed as open-closed fluctuations at the single-channel level [12-14].

- **Receptor of aerolysin**

The receptor of aerolysin on cell membrane of host is glycosylphosphatidylinositol (GPI)-anchored proteins. The structure and the condition between host - the 52-kDa protoxin monomer was recognized by X-ray crystallography 1996 and shows that the protoxin composed of four domain, Domains 1 and 2 are responsible for the dual binding to N-glycosylated GPI-anchored proteins, 4 with domain 2 binding directly to the glycan core of the GPI-anchor, while domain 1 is responsible for binding the N-linked sugar modifications present on the receptor. Domain 3 consists of a five-stranded ß -sheet and a prestem loop, which is curled up against the ß-sheet. Previous experiments have shown that the prestem loop immediately refolds into one hairpin of the final transmembrane ß-barrel, and is responsible for driving both the insertion and the anchoring of the ß -barrel. Domain 4 is a prolongation of the domain 3 ß -sheet, but the sheet is split open by the C-terminal peptide (CTP) into a twisted doubl ß -sheet fold.

Investigation about pathogensis of some bacteria is related to toxin production of them. Some enzymes like aerolysin form pore in cell membrane of host so study about aerolysin aid to figure out the other enzymes[15-17]. On the other hand, GPI-proteins of eukaryotic cell membranes are targets of many researches and aerolysin is the best gadget for these researches.

Organelle isolation from eukaryotic cells and parasite purification from host cell in protozoan, require the suitable material to unstable the cell membrane of host without any injuries to organelle or parasite and able the researcher to control time of effect and concentration of material. Aerolysin is a suitable for these goals.

In this research, aerolysin isolated from cell culture of A.hydrophila. Nonuse of ultracentrifuge is the most important difference among the method of this study and the other studies so this method is feasible in any lab with a simple centrifuge.

2. Material and methods

2.1. Aerolysin isolation:

A.hydrophila gifted from bacteriology lab of veterinary faculty of ferdowsi university that isolated from aquarium fish in ichthyology lab of veterinary faculty. Inoculated A.hydrophila in 5-10 ml of triptic soy broth (TSB) (sigma,WKG,Germany) in a 125 Erlenmeyer flask and grow overnight. Next, use 2 mls of A.hydrophila of prior stage to inoculate 200 ml of TSB in a 2 Litre Erlenmeyer flask and grow overnight. Next day centrifuged the culture at 6000 rpm, and poured the supernatant and cooled it on ice. Placed the supernatant in a bucket with ice around it, on a magnetic stir plate. While stirring constantly, added 36.1 grams of ammonium sulphate (merck Millipore)/100 mls of supernatant. This should take about 5-15 minutes. Then solvent Centrifuged at 6000 rpm, at 4 degrees Celsius. In this stage poured off the supernatant carefully. Added 5 mls of cold phosphate buffered saline, pH 7.4 (PBS) and resuspended the pellet gently. Centrifuged the resuspended material at 6000 rpm to remove any precipitates. This solution now has a lot of hemolytic activity.

2.2. hemolysis test:

For hemolysis test, provided a series 50µl of ½ dilutions from the solution in PBS in the final stage in 96 wall plate, and mixed some of each dilution it with an equal volume of diluted cattle blood (about 1/50 dilution of whole heparin treated blood in PBS), and incubated it at 37 degrees Celsius for 1 hour.

2.3. SDS PAGE

SDS-PAGE is an ideal procedure for confirmation aerolysin isolation and purification from cell culture of A.hydrophila. SDS-PAGE was performed in 12% separating gel according to Laemmli's method, after the electrophoresis, gels were stained with brilliant coomassie blue R250 (sigma,WKG,Germany) to visualize proteins.

2.4. Bradford technique

Bradford technique used for Aerolysin quantification. Albomin used for protein standard.the amount of aerolysin.

3. Result and discussion

After aerolysin isolation from cell culture of A.hydrophila, hemolysis activity is the best way to evaluate existence of this enzyme in PBS.

In hemolysis test, more than 1/64 titer, hemolysis is completely obvious (fig1). This result in hemolysis test showed, hemolysis activity of a component of cell culture.

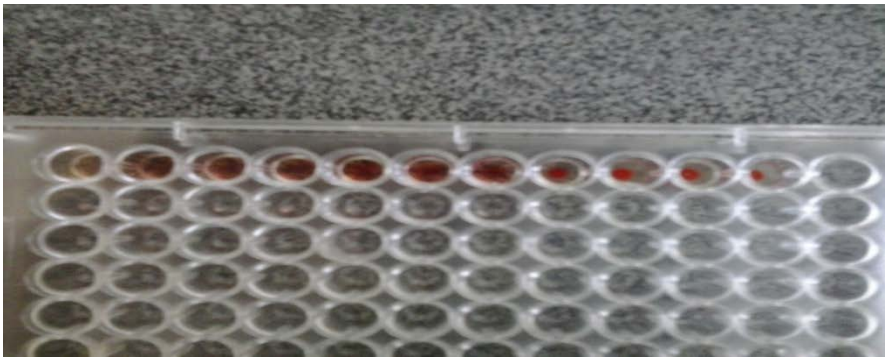

Fig 1. hemolysis test of PBS content by cattle red blood cells.hemolysis is completely obvious in more than 1/64 titres.

In SDS-PAGE test of cell culture after aluminum sulphate precipitation, a sharp band existed between 50KD and 70KD bands of ladder. This result determined the presence of a protein with hemolytic activity that its weight was between 50-70KD (fig2).

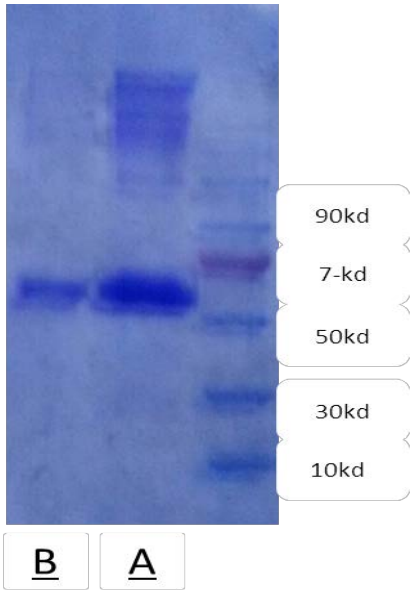

Fig 2. SDS-PAGE analysis of PBS content. Aerolysin was separated using 12% SDS–PAGE according to the method of Laemml. The protein ladder purchased from Eurx.Lane A: 4µl of aerolysin in PBS loaded on the SDS-PAGE pageLane B:2 µl of aerolysin in PBS loaded on the SDS-PAGE page.

By bradford technique, the amount of aerolysin determined 3.5mg/5ml PBS in 595 nm wave length.
Now aerolysin sold out recombinant by Cusabio company and by Pinewood scientific services in Canada an inactive form of aerolysin that labeled by Alexa 488. An inactive aerolysin- Alexa 488 binds very specifically to GPI-anchored proteins on mammalian cells.
Aerolysin is an active enzyme that used in different forms and in different experiments. Aerolysin isolation and purification with the simple method is the consequence of this study.

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Declarations

Ethics approval

Animal care and all experimental protocols were performed after approval by the guideline of animal welfare committee of Ferdowsi University of Mashhad (permit number: 1392–27816) based on the national guidelines from Ministry of Science, Research and Technology of Iran, adopted from the 86/609/EEC Directives of European Community.

Consent for publication
Not applicable.
Availability of data and materials
All data are disclosed as figures in the article.
Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
AH conceived and designed the study. TA performed the experiments and assisted in writing the manuscript. AH, TA and AA analyzed the data. AH, AA and GHH contributed reagents/materials/analysis tools. AH wrote, revised and edited the final version of the manuscript. All authors read and approved the final manuscript.
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