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Qualitative and Quantitative screening of alkaline protease production from some pathogenic bacteria

الغربلة النوعية والكمية لانتاج انزيم الببروتييز القاعدي من بعض انواع البكتيريا المسببة للامراض

Sind Shamel Omer and Hamid Gehad Humadi
Kerbala University/ College of science / Dep.of biology

Abstract

Four different isolates were isolated from different sites of infection, these include 2 isolates of G +ve bacteria (*Staphylococcus aureus* from urine and *Bacillus cereus* from tooth) and G-ve bacteria (*Pseudomonas aeruginosa* from burns and *Klebsiella pneumoniae* from sputum).To detect the ability of these bacteria to produce alkaline protease enzyme in one hand and to correlate the production yield to site of infection from other side, it had been performed the qualitative screening on solid media by using skim milk agar and quantitative screening by measuring the specific activity of each isolates.The results of qualitative screening showed the inhibition zone of *Staph aureus*and *Bacillus cereus* were higher than *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (2.8 , 2.1 , 1.3 and 0.5 cm respectively). When these results compared with quantitative results , it had nearly same, in which specific activity of *Staph aureus* and *Bacillus cereus* were higher than *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (1955.7 ,1902.8 , 100.8 and 65.4)**U/mg** respectively.

الخلاصة

تم عزل أربعة عزلات مختلفة من مواقع مختلفة من العدوى، وتشمل عزلتين من البكتيريا الموجبة لصبغة الغرام (المكورات العنقودية الذهبية من البول والعصوية الشمعية من الأسنان) وعزلتين من البكتيريا السالبة لصبغة الغرام (الزائفة الزنجارية من الحروق والكلبسيلة الرئوية من البلغم). لغرض الكشف عن قدرة هذه البكتيريا على انتاج انزيم البروتييز القاعدي من جهة وبيان علاقة الانتاجية مو موضع الاصابه , فانه تم اجراء الغربلة النوعية باستخدام وسط الحليب الخالي من الدسم الصلب والغربلة الكمية من خلال قياس الفعالية النوعية لكل عزلة. اظهرت نتائج الغربلة النوعية ان قطر منطقة التثبيط للـ *Staph aureus* و *Bacillus cereus* كانت اكبر من قطر منطقة التثبيط لكل من *Pseudomonas aeruginosa* و *Klebseilla pneumonia* (2.8، 2.1، 1.3 و 0.5 سم على التوالي) وعندما قورنت نتائج الغربلة النوعية مع نتائج الغربلة الكمية وجدت انها متقاربة . حيث كانت نتائج الفعالية التخصصية للـ *Staph aureus* و*Bacillus cereus* اعلى من *Pseudomonas aeruginosa* و *Klebseilla pneumonia* (1955.7، 1902.8، 100.8 و65.4) U / ملغ على التوالي.

Introduction

Pathogenic bacteria need to interact with their host to establish an infection and to maintain it successfully afterwards. This requires to "understand" signals of the immune-system to "respond" appropriately when host tries to defence against its. Part of response of successful pathogens is secretion of so called virulence factors which manipulate or even destroy defense lines of the host. An important part of the arsenal of bacterial virulence factors are proteases. Proteases are enzymes that hydrolyze peptide bonds and can therefore degrade proteins and peptides. As proteins are one of the basic building blocks in nature, proteases can influence a broad range of biological functions including the infection process which is not just a simple and rapid multiplication of bacterial cells in the human body. The opportunistic human pathogen *Pseudomonas aeruginosa k.pneumoniae ,Staph aureus and B.cereus* have an arsenal of impressively efficient proteases that helps establishing and maintaining an infection and thereby controlling and modifying the environment according to the needs of the bacterium within the host tissue.Also *Pseudomonas aeruginosa , Klebsiella pneumoniae, Staph aureus and Bacillus cereus* are the epitome of an opportunistic pathogen of human and animals which causes a

secondary bacterial infections particularly to patients suffering from neutropenic cancer undergoing chemotherapy, immunodeficiency relating to AIDS, diabetes mellitus, severe burns, cystic fibrosis, paranasal sinus, meningitis, brain abscesses and devastating infections in the human eye (1,2).

In spite of the availability of different type of antibiotics the mortality rate of these bacteria still high and there is no effectiveness vaccines against it (3)

The pathogenicity of these bacteria may related to ability for production of various virulence factors, which some of these were produced extracellular such as alkaline protease, hemolysin and elastase and another's were somatic antigen such as lipopolysaccharide and alginate (4).

Protease enzyme considered as one of the enzyme that play an important role in bacteria by aid the cells to remove abnormal proteins and supply with amino acid, needs in proteins synthesis, also can help bacteria to invade tissues by resistance to phagocytosis and breaking immunoglobulin and inhibit their activity.

According to those mentioned above, this study was aimed to:-

Quantitative and qualitative screening of isolates for the production of protease enzyme from different clinical samples.

Materials and methods

1. Bacterial strains

The bacterial isolates used in this study are list in Table (1)

Table (1) Types and sources of bacterial isolates

Bacteria	Gram stain	Source
Pseudomonas aeruginosa	G -ve	Burns
Klebsiella pneumoniae		sputum
Staphylococcus aureus	G+ve	Urine
Bacillus cereus		Gingivitis tooth

Identification of test isolates

The following tests were employed to identify the presumptive colonies of *P. aeruginosa* , K.*penumoniae* , *Staph aureus and B. cereus*from nutrient agar plates:

- Gram's stain: A single colony was transferred by a loop to a clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with absolute alcohol, and counterstained with safranine, then examined by a microscope.(5)
- Catalase test

This test was performed by adding drops of hydrogen peroxide (H_2O_2) 3% on a single colony grown on trypticase soya agar. The productions of gaseous bubbles indicate the presence of catalase.(6)

- Growth on manitol salt agar

This test was used to examine the ability of isolates to tolerate high concentration of salt (NaCl 15%) . In this test, a colony was inoculated on to the surface of medium and incubated overnight at 35ºC. The appearances of colony indicate the positive result.(7)

- Growth on MacConkey agar

This test was used to show the ability of isolates to ferment the carbohydrate as a sole source of carbon. In this test, a colony was inoculated on to the surface of MacConkeyagar plateand the medium was incubated overnight at 35ºC. The appearances of growing colony indicate the positive result.(7)

- Citrate utilization

This test was used to examine the ability of isolates to utilize citrate as a sole source of carbon. In this test, a colony was inoculated on to the surface of simmon citrate slant and the medium was incubated overnight at 35ºC. The appearances of blue color indicate the positive result.(7)

- Motility test

This test was performed to demonstrate the ability of bacteria motility . The tubes of soft agar media were inoculated with bacteria by stabbing, and then the tubes were incubated at 25^{o}C for two days.(5)

Screening the test isolates for production of alkaline protease

1. The Qualitative Screening on Solid Media

To detect the ability of bacteria to produce the alkaline protease enzyme, skim milk agar was used as described by (5).

It was performed by placing aseptically loopful of culture on the center of the plate and spread it in a circular fashion to cover an area about (5 to 18mm in diameter) then incubate the plate in a inverted position at 37ºC for 24 to 48hrs. Clearing the cloudy agar (zone of proteolysis) indicates the positive result.

2. The Quantitative Screening in Liquid Media

The liquid media used in this test was trypticase soya broth as described by (8).

Twenty-milliliter aliquots of this media were dispensed in to 100ml Erlenmeyer flasks. Following sterilization by autoclaving, 0.2ml of each isolate fresh (18 hrs) culture were inoculated per flask, and then incubated in shaker incubator (130 rpm, 35ºC for 24 hrs). The production of alkaline protease was determined by using casein as a substrate working solution at pH 8 as will be described.

Assay of enzymatic activity

A. CrudeEnzyme Extraction

Alkaline protease enzyme was extracted from liquid culture media by a centrifugation at 5000 rpm at 4ºC for 20min. the supernatant was used as a crude enzyme for the following steps.

B. Assay of protease activity

Alkaline protease activity was determined according to the method originally described by (9) and modified by (10)) as following:

Casein (0.8 ml, 0.5%, pH 8) was preincubated in a water bath at 37ºC for 10min then 0.2ml of crude enzyme was added to the substrate working solution and incubated for 20min.The reaction was stopped by adding 3ml of TCA (5%). The blank was prepared using the same steps apart of TCA (5%) were added before the addition of the crude enzyme. Both reactant and blank were centrifuged at 5000rpm for 20min.

The amount of TCA-soluble products formed can be determined by measuring the absorbency of the supernatant at 280nm.

Unit of activity was defined as the amount of the enzyme, which gives 0.001 increases in the absorbance (280nm) per min. under the determination condition. The protease activity was determined by using this equation.

$$\text{Alkaline protease activity U/ml} = \frac{\text{Absorbance at 280 nm}}{0.001 \times 20\,\text{min} \times 0.2\,\text{ml}}$$

C. Determination the protein concentration

The protein concentration was estimated by absolute method as described by (11) as following:

The protein was precipitated by adding 3ml of TCA (5%) to each 2ml of extracted enzyme, and then centrifuged at speed 5000rpm for 20 min.The precipitation was dissolved by adding NaOH

(0.05M), then measuring the absorbance at wavelengths 280nm and 235nm by using NaOH (0.05M) as a blank.

The protease concentration was estimated by using this equation:

$$\text{Protein concentration (mg/ml)} = \frac{\text{Abs at 235 nm} - \text{Abs at 280 nm}}{2.51} \times \text{D.F.}$$

where D.F.: Dilution factor.

$$\text{Specific activity (U/mg of protein)} = \frac{\text{Enzymatic activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

Results and discussion

1. Identification of bacterial isolates

Four isolates from different clinical samples (*Pseudomonas aeruginosa , Klebsiella Pneumoniae, Staph aureus and Bacillus cereus*) were selected randomly to make a comparison for production of protease enzyme between different site of infection , these isolates were submitted to some of biochemical test according to(12) .

Table (1): Morphological, Physiological and Biochemical characterization of isolated bacteria

Character	*K.pneumonia*	*P.aeruginosa*	*Staph.aureus*	*B.cereus*
Gram stain	G-ve rod	G-ve rod	G+ve spherical	G +ve rod
Catalase production	-	-	+	+
Citrate utilization	+	+	-	+
MacConkey's agar	+	+	-	-
Growth on Mannitol salt agar	-	-	+	+
Motility	-	+	-	+

2. Screening the isolates for alkaline protease production

A. The qualitative screening on solid media

All the isolates used in this study were alkaline protease producer when they had been grown on skim milk agar and incubated at 37^{o}C for 24-48 hrs. The proteolyticzone around the colonies was very clear and could be simply detected. The diameters of zone were varied from one isolates to another, *Staph aureus* was given a very large zone (2.8 cm); while the others were about (0.5- 2.1 cm) as shown in Table (2).

It was shown that there were clear variations in the efficacies of the production of alkaline protease from different species and different source, these variants were due to differences of genetic ability among isolates and site of infection. (13).

Table (2). Qualitative screening of isolates for alkaline protease production on skim milk agar (pH 7.2) at 37^{o}C for 24-48hrs

Bacterial strain	Source	Diameter of zone inhibition (cm)
K.pneumoniae	**Burn**	0.5
P.aeruginosa	**Sputum**	1.3
Staph aureus	**Urine**	2.8
B.cereus	**Gingivitis Tooth**	2.1

B. The Quantitative screening on liquid media

For further detection and accurate selection of efficient isolate to produce alkaline protease, the 4 isolates were tested again in a liquid media which was casein broth (pH7) as described by (8) and incubated at 37^{o}C for 24 hrs.The production of alkaline protease and estimation of its activity and specific activity were detected .

As shown in Table (3), the *Staphaureus* gave the highest enzymatic activity in which the specific activity was 1955.7 U/mg protein while *B.cereus* had specific activity 1902.8 U/mg . the specific activity of *K.pneumoniae* was 100.8 U/ mg and*P.aeruginosa* was 65.4 U/mg. When these results were compared with qualitative detection of enzyme Table (2) they were nearly identical.

*Staphylococcus aureus*was the highest enzyme producer and this may be related to site of infection (i.e urine). In this site, it need protease enzyme to invade tissue , due to role of alkaline protease enzyme to resist phagocytosis by cleaving the lgG, degrading gamma interferon and inhibited neutrophil function. (14) onother hand high enzymatic activity in tooth specimens may be attributed the surface of teeth , may sustains growth of *B.cereus to* adhesion(15).

Also *Staph.aureus*have ability to produce several toxins and digestive enzymes, as well as a large number of proteins on the bacterial surface that bind to extracellular matrix and plasma proteins . In vitro studies have shown that staphylococcal proteases can cleave and degrade a number of important host proteins, including the heavy chains of all human immunoglobulin classes, plasma proteinase inhibitor, indicating that they are important virulence factors. Proteasesenzyme alsoplay a role in the transition *Staph. aureus* cells from an adhesive to an invasive phenotype by degrading bacterial cell surface proteins, such as fibronectin binding protein and protein A .(16)

On the other hand, the specific activity of *B.cereus* show high ratio of protease production may due to ability of these bacteria to colonized surface of teeth depending primarily on the biofilm formation and then help bacteria to digest remaining food especially protein by ability to produce protease enzyme.(17,18)

Table (3): Quantitative screening of isolates for alkaline protease production on casein broth at pH7 and 37^{o}C for 24 hrs.

Name of isolate	Source	Activity U/ml	Specific activity U/mg protein
P. aeruginosa	**burn**	**23.75**	**65.4**
K.pneumoniae	**sputum**	**26.75**	**100.8**
Staph. aureus	**urine**	**57.5**	**1955.7**
B.cereus	**Gingivitis Tooth**	**47**	**1902.8**

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