



The effect of *Serratia marcescens* protease on human lymphocytes transformation

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Abstract:

Fifteen blood samples were collected from healthy males and females (6 males & 9 females), average age (21-34 years) in heparinized sterile tubes. The extracellular protease was extracted from a clinical isolate of *Serratia marcescens* that was isolated from a patient suffering from urinary tract infection taken from the Central Health Laboratory. The extracted protease was purified partial by two steps, precipitation with 30-55% saturation of ammonium sulfate following with dialysis and ion exchange chromatography DEAE-cellulose. The protease concentration was 0.15 mg/ml. Two concentration 0.25µg/ml and 0.5µg/ml of protease were prepared and applied in current study. Lymphocyte transformation test using whole blood was used in order to study the effect of protease concentrations on human lymphocyte transformation. The results show that these were highly significant differences ($P < 0.05$) between control samples and lymphocytes stimulating with Phytohemagglutinin (PHA) and between control sample and 0.5µg/ml protease,; while there was no significant differences ($P < 0.05$) between control sample and 0.25 µg/ml protease concentration . That is mean that the higher concentrations of protease have the same effect of PHA on human lymphocyte transformation and it is better in effect on human lymphocyte transformation than the lowest concentration.

Keyword: *Serratia marcescens*, Proteases, Lymphocyte transformation.

تأثير إنزيم البروتيز لبكتريا ال *Serratia marcescens* على تحول الخلايا للمفاوية لدم الإنسان

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الخلاصة

جمعت خمسة عشر عينة دم من ذكور و إناث أصحاء (6 ذكور و 9 إناث) معدل العمر يتراوح بين (21- 34 سنة) بواسطة أنابيب معقمة حاوية على مادة الهيبارين. تم استخلاص الانزيم الحال للبروتين (البروتيز) من عزلة مرضية لبكتريا ال *Serratia marcescens* من مريض يعاني من التهاب المجاري البولية من مختبر الصحة المركزي. و تم تنقيته بخطوتين تضمنت الترسيب باستخدام سلفات الامونيوم المشبعة بنسبة اشباع 30-55% و ديلزة و الترشيح باستعمال المبادل الأيوني DEAE- Cesolulle. وقد كان تركيز البروتين 0.15 ملغم/مل. ومن هذا التركيز تم تحضير التراكيز (0.25 مايكرو غرام/مل، 0.5 مايكرو غرام/مل). استعمل اختبار تحول الخلايا للمفاوية لدراسة تأثير تركيز الانزيمات الحالة للبروتين على تحول الخلايا للمفاوية لدم الإنسان، و قد اوضحت النتائج ان الفروقات كانت جدا معنوية ($P < 0.05$) بين عينات السيطرة و الخلايا للمفاوية المحفزة بالمشطر النباتي (PHA)، وبين عينات السيطرة و تركيز 0.5 مايكروغرام/مل لانزيم البروتيز، و في حين لا توجد فروقات معنوية ($P < 0.05$) ما بين عينات السيطرة و تركيز 0.25 مايكروغرام/مل من انزيم البروتيز مما يدل على ان التراكيز العالية لانزيم البروتيز لها فعل مشابه للمشطر النباتي (PHA) على تحول الخلايا للمفاوية لدم الإنسان كما ان لها تأثير أكبر من التراكيز الواطنة على تحول الخلايا للمفاوية لدم الإنسان.

Introduction:

The lymphocyte transformation test (LTF) development was based on the observation in 1960 that incubation of lymphocytes with phytohemagglutinin, a mitogen, leads to cell activation and proliferation. The principle of the LTF is based on the fact that lymphocytes, which have been sensitized by a certain antigen ("memory cells"), transform into blasts and proliferate when they are again exposed to this antigen [1]. Lymphocyte transformation assay potentially offers a sensitive indicator for several conditions involving lymphocyte dysfunction due, for instance, to immunologic deficiency [2]. *Serratia marcescens* is a common opportunistic pathogen in hospitalized patients. *Serratia* (usually non pigmented) cause pneumonia, bacteremia and endocarditis especially in narcotics addicts and hospitalized patients [3]. In the last 40 years, LTF has been proven to be useful especially in the diagnosis of drug-induced allergic disorders and allergic disorders associated with exposures at the workplace [1]. A huge amount of widespread microorganisms naturally existing in different environments are able to produce proteases [4]. *S. marcescens* produces a potent extracellular metalloprotease, which is widely used as an anti-inflammatory agent [5]. There are many categories of antitumor agents known today; most of them affect nucleic acid metabolism or impair nucleic acid functions directly or indirectly, and others act as alkylating T-cells and other leukocytes [6]. The previous studies found that certain bacterial proteases were potent antitumor agents when injected into tumor tissue in vivo [7]. Among these protease, the protease (molecular weight 56,000) from *Serratia marcescens*, subtilisin from *Bacillus subtilis*, and thermolysin from *Bacillus stearothermophilus* were effective anticancer agents when injected into the tumor [7]. The results recorded by [6] suggest that the proteases can follow either one of two antitumor mechanisms of action: (a) direct damage to the cell membrane, for which a high dose of protease is required to kill cells or (b) binding and internalization of proteases via the $\alpha 2M$ receptor, which requires a relatively low amount of protease to kill cells. The previous report [6] describes a new possible approach for the treatment of solid tumors with using proteases. Our research aims to study the effect of protease that extracted and purified from *Serratia*

marcescens clinical isolate on human lymphocyte transformation.

Materials and Methods:**1- Bacterial isolate:**

Serratia marcescens was isolated from a patient suffering from urinary tract infection taken from the Central Health Laboratory. The isolate was identify by biochemical tests [8] and API 20 E system (bio Merieux).

2- Protease enzyme:**A- Extraction And Purification of protease:**

Protease was extracted and purified according to slandered method reported previously [9, 10, and 11].

B- Preparation of protease concentrations:

The protease concentrations (0.25 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$) that were used in order to study the effect of protease on lymphocyte transformation were prepared from the extracted and purified protease.

3- Collection of blood samples:

Five ml of venous blood was collected healthy from 15 persons, (6 male & 9 female), average age 25.13 years, range, 21-34years, in a sterile tubes contained heparin, as demonstrated in the following table:

Table1- The age and sex, for the 15 blood sample

Sample no.	Age(Year)	Sex
1	21	female
2	21	female
3	21	female
4	34	female
5	34	female
6	30	male
7	24	male
8	24	male
9	24	male
10	24	male
11	24	female
12	24	female
13	24	female
14	24	female
15	24	male

4- Lymphocyte transformation assay using whole blood

This assay was done according to [12]; it was carried out as follow:

❖ A volume of 250 μl of heparinized blood was cultured in 2.5 ml of complete RPMI-1640 medium (Sigma) in sterile silicon coated tubes, using four tubes to each test

(control, PHA, protease 0.5 µg/ml and 0.25 µg/ml).

❖ A volume of 250 µl of mitogen (Phytohemagglutinin (PHA) from the Iraqi center for Cancer and Molecular Genetics Research) was added to one of these tubes 0.5 µg/ml and 0.25 µg/ml to other tubes while the last tube (control) left without any addition of mitogen.

5- Viability test

The viability was determined by trypan blue dye exclusion test (13) as follows:

5 µl of cells suspension was added to 200 µl of 0.2 % trypan blue dye, mixed, waited for 3 minutes and then the cells were counted by hemocytometer until having a minimum of 100 cells. Living cells were not be staining by trypan blue dye.

$$\text{Viability} = \frac{\text{Viable cells counted}}{\text{Total cells count(viable \& dead cells)}} \times 100$$

The viability was always judged to be greater than 95% [14].

6- 7-The procedure was completed for all tubes (whole blood) as follow:

❖ At the end of incubation time (72 hr) the cells were sedimented by centrifugation at 2000 rpm for 10 min.

❖ Five ml of hypotonic solution (2.85 gm of potassium chloride (KCl) was dissolved in 500 ml of sterile D.D.W.) was added to the pallets, and then incubated at 37 °C for 50 min.

❖ Centrifugation at 2000 rpm for 10 min.

❖ Five ml of fixative solution (Three volumes of absolute methanol were mixed with one volume of glacial acetic acid) was added to the sediment cells and kept in refrigerator at 4 °C for 10 min.

❖ Washing up by fixation solution 3-4 times with centrifugation which repeated until colorless suspension of sediment cells formed.

❖ One drop of sediment cells were dropped on two clean slides by using Pasteur pipette, left to dry at room temperature, stained by giemsa stain for 10 min., washed by D.W. and examined microscopically under oil immersion lens by counting 200 cells.

❖ The ratio of transformed cells was calculated by the following equation:

$$\text{Transformed cells\%} = \frac{\text{No. of transformed cells}}{\text{Total No. of lymphocyte and lymphoblast cells count}} \times 100$$

Statistical analysis:

The t test for significance at ($P < 0.05$) was adopted for the comparison and calculation of association in quantitative data according to contingency tables method within the SAS (2001) program [15].

Result & Discussion:

1- Extraction and purification of protease:

The extracellular protease produced by *Serratia marcescens* was purified by gradient ammonium sulfate precipitation and ion exchange chromatography [16]. The protein concentration was done according to the absolute method [17]; it was 0.15 mg/ml. From this concentration, the protease concentrations (0.5 µg/ml, 0.25 µg/ml) were prepared.

2- Lymphocyte transformation assay by using whole blood:

The cells are isolated under strictly sterile conditions from heparinized blood by centrifugation. For the determination of unspecific lymphocyte function, mitogen (phytohemagglutinin (PHA)) is added to the cultures which activate nonspecifically T- and B-lymphocytes. For the detection of a specific sensitization, the protease is added in increasing concentrations. The different concentrations are necessary to obtain the optimal ratio between lymphocytes and mitogen in the cultures, which induces the strongest proliferation. The cells are incubated in suspension with the antigen for 4–7 days at 37 °C [1]. Lymphocyte transformation assay was measured by estimating the morphological changes of separated lymphocyte under oil emersion lens of the light microscope and the result were expressed as lymphoblast percentage from the total lymphocytes that had been calculated (figure 1). The LTF is now used for the evaluation of lymphocyte function (lymphocytes are incubated with mitogens, there by inducing a nonspecific transformation into blasts in vitro), as well as for the demonstration of specific sensitization of patients toward exogenous antigens (infectious agents, allergens) or autoantigens (autoimmune diseases) [1].

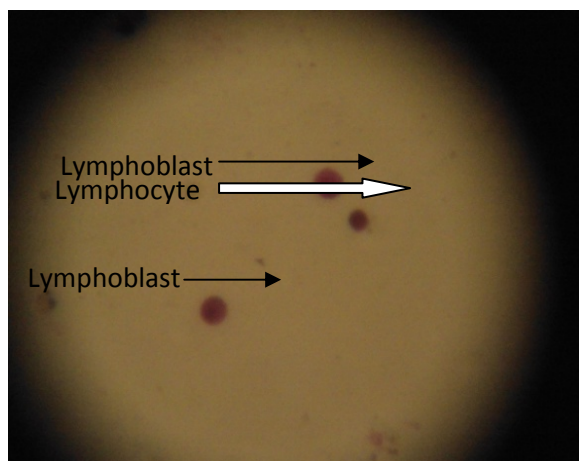


Figure 1- Lymphocyte transformation by *Serratia marcescens* protease (⇔) lymphoblasts (→) lymphocyte.

3- Effect of *Serratia marcescens* protease (0.5µg/ml and 0.25 µg/ml) on lymphocyte transformation:

The percentage of lymphocyte transformation of control blood sample, with PHA, with 0.5 µg/ml and 0.25 µg/ml of *Serratia marcescens* protease are listed in table [2]. The result show that there was a highly significant differences ($p < 0.05$) between control and lymphocyte stimulating with PHA, control and 0.5µg/ml protease, while there was no significant differences between control and 0.25µg/ml protease. This result show that 0.5 µg/ml of protease concentration have the same effect of PHA effect on lymphocyte transformation and better in effect than 0.25 µg/ml concentration.

Table 2-The percentage of lymphocyte transformation of control blood sample, with PHA, with 0.5 µg/ml and 0.25 µg/ml of *Serratia marcescens* protease.

Sample no.	Control (%)	PHA (%)	0.5µg/ml (%)	0.25 µg/ml (%)
1	48	88.3	47.5	77.1
2	46.6	80	59.3	71
3	45	87.5	86.7	78.5
4	52.6	71.4	75.8	84.2
5	28.5	60.9	64	66.6
6	40.9	74	53	58.8
7	22.3	77.1	53.8	47.8
8	23.5	70	69.2	54.5
9	56.5	52	66.6	38.09
10	50	77.2	62.5	73.3
11	43.2	65.8	69.3	71.8
12	33.3	80	42.3	52.3
13	56.25	75.3	55.5	71.4
14	50	71.4	60	83.7
15	17.6	76.9	73.9	51.8

There are many categories of antitumor agents known today; most of them affect nucleic acid metabolism or impair nucleic acid functions directly or indirectly, and others act as alkylating agents, antimetabolites, or antihormones. Thus far, however, proteases have received little attention as antitumor agents [18]. Recent interest in cancer immunology has included cytotoxic. Previously researchers found that certain bacterial proteases were potent antitumor agents when injected into tumor tissue in vivo [19]. This study show that the higher concentrations of protease better in effect on human lymphocyte transformation than the lower concentrations.

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