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PRODUCTION OF EXTRACELLULAR PROTEIN A FROM A METHICILLIN-RESISTANT STRAIN OF STAPHYLOCOCCUS AUREUS

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PRODUCTION OF EXTRACELLULAR PROTEIN A FROM A METHICILLIN-RESISTANT STRAIN OF *STAPHYLOCOCCUS AUREUS*

By

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LIST OF ABBREVIATIONS

BHI	:	Brain Heart Infusion
CNBr	:	Cyanide bromide
DNS	:	Dinitrosalicylic acid
ELISA	:	Enzyme linked immunosorbent assay
IgG	:	Gamma immunoglobulin
KLa	:	Mass transfer coefficient
O.D	:	Optical density
PBS	:	Phosphate buffered saline
PYK	:	Peptone/yeast/potassium
rpm	:	Revolutions per minute
SAS	:	Saturated ammonium sulphate
SDS	:	Sodium dodecyl sulphate
v/v	:	Volume/volume
YE	:	Yeast extract

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Protein A was produced extracellularly by methicillin-resistant *Staphylococcus aureus*, strain A676. A competitive-ELISA technique based on a competitive binding between unlabelled protein A and conjugated protein A was employed to measure the concentration of protein A. A study was also conducted to develop this technique.

Rabbit IgG was first purified before it was used to coat a microtiter plate. IgG purified using ammonium sulphate precipitation in combination with DEAE-cellulose chromatography gave purer IgG than the caprylic acid method combined with saturated ammonium sulphate or single-step DEAE-cellulose ion exchange chromatography. The molecular weight of the purified IgG was 140,000 dalton based on SDS gel electrophoresis. The minimum detectable amount of protein A was in the range of 0-20 ng/ml. The optimum concentration of IgG required for coating was 2 µg/ml and the incubation time for the substrate was 20 minutes.

In shake flask culture, the optimal temperature and pH for growth of *S. aureus*, strain A676 and protein A production was found to be 40°C and pH 7.4, respectively. Production of protein A correspond to growth. Protein A was released extracellularly during lysis of cell, and reached the maximum level, with a concentration of 24.06 µg/ml after 36 hours of incubation. The presence of yeast extract and peptone in Brain Heart Infusion (BHI) medium enhanced protein A production. The production of protein A was found to be inhibited by the addition of 0.8-3.0% glucose and 7% NaCl. The yield of protein A increased when strain A676 was cultured in a 2-litre fermenter where a maximum yield of 28.04 µg/ml was obtained

culture was grown for 30 hours and stirred at a impeller speed of 600 rpm with dissolved oxygen concentration maintained at 40% saturation.

Protein A was purified by two separate methods, namely the DEAE-Sephadex in combination with gel filtration of Sephadex G-100 and affinity chromatography on CNBr-activated Sepharose gel. The percentage recovery of protein A purified by affinity chromatography was 43% higher than the gel filtration method (37.1%). By using SDS-PAGE, protein A was shown to be homogeneous and the molecular weight of the purified protein A was estimated to be about 41,000 dalton.

impeler 600 rpm dan ketepuan oksigen terlarut 40% iaitu pada kepekatan 28.04 μg/ml.

Penulenan protein A dilakukan melalui dua kaedah yang berasingan iaitu DEAE-Sephadex yang digabungkan dengan gel penurasan Sephadex G-100 dan satu lagi kaedah kromatografi keafinan gel Sepharos CNBr-teraktif. Peratus pemulihan protein A yang ditulenkan menggunakan kaedah kromatografi keafinan adalah 43% iaitu lebih tinggi daripada kaedah gel penurasan (37.1%). Berdasarkan kepada gel SDS, protein A yang ditulenkan adalah homogenous dan berat molekul dianggarkan 41,000 dalton.

CHAPTER I

INTRODUCTION

Protein A is a single polypeptide chain found in most *Staphylococcus aureus* (Sjodahl, 1977; Boisnard, 1988) and the production of protein A is restricted to only this species (Cox *et al.*, 1986; Winblad and Ericson, 1973). It exists either covalently linked to peptidoglycan of bacterial cell walls (cell-bound protein A) or freely in the culture medium (extra-cellular protein A).

Cell-bound protein A is found in most *S. aureus*, for example the Cowan I strain was reported by Sjoquist *et al.* (1972a) as a high producer of protein A. This type of protein A can be extracted from bacteria cell wall by lysostaphin digestion. Other enzymes such as lysozyme and DNAase (Yoshida *et al.*, 1963) can also be used for the extraction. According to Lind (1972) and Lindmark *et al.* (1977), over 95% of the *S. aureus* strains isolated from several animal species are positive for cell-bound protein A. Only small amounts are found extracellularly, except in certain methicillin-resistant strains which secrete all their proteins into medium.

The unique property of protein A is its ability to bind specifically to the 'Fc' region of gamma-globulin (IgG) and gamma-globulin sub-classes from various species such as humans (Live and Rau, 1968), rabbits and guinea pigs (Stalenheim and Sjoquist, 1970). However, some researchers have reported that it has low binding affinity to IgG of hamsters, horses and rats, and could not bind to goats and chickens (Kronvall *et al.*, 1970; Richman *et al.*, 1982).

Due to this binding ability to IgG, protein A is widely used in affinity chromatography as a ligand for isolation and purification of IgG (Hjelm *et al.*, 1972; Goding, 1978). Protein A is also conjugated to various enzymes such as ß-galactosidase, horseradish peroxidase and alkaline phosphatase (Buchanan *et al.*, 1981), and is widely used in enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies or antigens.

Protein A from this strain is easy to isolate because it produces extracelllar protein A. Although protein A can be purchased easily, for example from Sigma, Company, it is very expensive. The price of protein A is approximately RM100.00 per 1 mg. Thus it is necessary to produce protein A. The main objective of this study is to maximise the production of

extracellular protein A from a methicillin-resistant strain of *S. aureus*, strain A676 and to purify the protein. In this study, a competitive-ELISA technique was developed for the quantification of protein A.

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CHAPTER II

LITERATURE REVIEW

Discovery of Protein A

In an effort to classify strains of Staphylococci on the basis of serological tests, Verwey (1940) separated the cellular constituents of bacteria into five fractions by using the trichloroacetic acid extraction procedure. One of these fractions, designated as fraction B, was found to be a protein which was highly antigenic when determined on gel precipitation technique. Similarly, Jensen (1959) found that *Staphylococcus aureus* extracted a component which gave strong precipitation lines with serum from rabbits, humans and mice. The extracted component was called antigen A and classified as a polysaccharide although Verwey's fraction B was shown to be a protein. In order to clarify this confusion, antigen A from an extract prepared according to Jensen's procedure by Grov *et al.* (1964). This clearly demonstrated that it was a protein which was identical to the major component of Verwey's fraction B. Furthermore, Grov and his co-workers (1964) found that sugar was not present in this extract.

This result clearly suggested that antigen A was a protein and not a polysaccharide as defined by Jensen (1959). Lofkvist and Sjoquist (1962) also supported this finding. Oeding and Hukenes (1963) and Grov *et al.* (1964) suggested that in order to stress the protein structure and clarify the ambiguous nomenclature, both Verwey's and Jensen's ideas be compromised. The name protein A was then adopted and it has generally been referred to by this name ever since.

Reactivity of Protein A with IgG

Protein A was shown to be capable of binding to serum globulin where precipitation lines were found when it was incubated with serum using the gel diffusion technique (Lofkvist and Sjoquist, 1963). However, the reaction was not an antigen-antibody reaction. Forsgren and Sjoquist (1966) have shown that the reaction of protein A with IgG is a "pseudoimmune" reaction. In their experiments, the mixtures of protein A either with Fab or Fc or Fc' were incubated for 2 hours and the supernatant, after centrifugation, was tested against normal IgG. They found that the precipitation lines were inhibited in gel diffusion when protein A was mixed with the Fc or Fc' fragments of IgG. However, when protein A was incubated with Fab (antigen binding site) fragment, the precipitation lines were formed. This phenomenon occurred because protein A had reacted with Fc and Fc' fragments and thus did not react with added IgG to form a precipitation.

Lind and Mansa (1968) have also shown that the fluorescent-labelled Fc fragment of IgG is able to bind to most *S. aureus* strains. Furthermore, in double diffusion gel method, Kronvall (1970) showed that the reaction of protein A is mediated by sites on the Fc region; by detecting the Fab, Fc and Fc′ fragments against a rabbit anti-human IgG. These observations suggest that the binding of protein A to IgG is at the Fc domain but not at Fab domain of the IgG molecules.

Production of Protein A

Staphylococcus aureus as a Producer of Protein

Protein A is only common to staphylococci and based on the results so far, the production of protein A is restricted to strains of *S. aureus* (Lind *et al.*, 1970; Hjelm *et al.*, 1975; Langone, 1982). Furthermore, Langone (1982) stated that protein A was found in over 95% of the *S. aureus* strains, with individual strains differing from one another in the amount of protein A produced. *S. aureus* strain Cowan I secreted 30% of protein A and the higher portion of protein A was covalently linked to peptidoglycan in cell walls (Movitz, 1976). Cox and his co-workers (1986) agree that the presence of protein A is a unique property of *S. aureus*. However, there have been some observations on the production ofprotein A from *Staphylococcus epidermis* (Kronvall *et al.*, 1971) and *Staphylococcus intermedius*

(Lachica *et al.*, 1979; Cox *et al.*, 1986), but the yield was very much lower.

Characterization of *Staphylococcus aureus*

Buchanan and Gibbons (1974) showed that *S. aureus* strains are gram-positive, non-motile and spherical-shaped with a diameter of 0.8 -1.0 µm. Colonies of these bacteria are smooth, glistening, low convex and with an entired edge. Most strains of *S. aureus* colonies are white (Parker, 1983) but certain antibiotic-resistant strains also possess yellowish pigmentation (Willis, 1964). *S. aureus* is also a facultative anaerobe. The optimum temperature and pH for growth are 30 - 37°C and 7.0 - 7.5, respectively. The major components of the *S. aureus* cell wall are peptidoglycan, ribitol teichoic acid and protein A (Schleifer and Kendler, 1972). These strains produce acid from mannitol and glucose in aerobic and also anaerobic conditions. All strains virtually release enzymes such as coagulase and hemolysins (α, β, γ-toxins) (Buchanan and Gibbons, 1974). However, Jensen *et al.* (1961) reported that the coagulase may be absent or low in quantity in the mutant strains of the *S. aureus*.

The coagulase content is also correlated to the phatogenicity of *S. aureus* (Lindmark *et al.*, 1977; Patel *et al.*, 1987). These pathogenic bacterium cause a variety of

infections in humans; including endocarditis, osteomyeditis, meningitis, wound sepsis and skin abscesses (Easmon and Adlam, 1983). The enterotoxins secreted from *S. aureus* can cause food poisoning, nausea and low blood pressure (Bergdoll, 1972). In 1982, Langone concluded that coagulase content should also give a strong correlation with protein A production, with protein A not being present in coagulase-negative strains. This conclusion was made by examining several hundred strains of staphylococci. Furthermore, since 57% of 30 protein A-negative strains of *S. hyicus* were coagulase positive, protein A production may therefore be the best criterion for identifying *Staphylococcus aureus*.

Mutant Strains of *Staphylococcus aureus*

Strains of the *S. aureus* can be distinguished based on drug resistance as it is related to the production of protein A (Langone, 1982). Some mutant strains produce extracellular protein A. As an example, Masuda *et al.* (1975) have isolated mutant strains from *S. aureus* 248ßH by cosedimentation with sensitized sheep red blood cells. In their experiments, 248ßH strain was mixed with IgG-sensitized red blood cells (S-SB) and the mixture was incubated at room temperature for cosedimentation. The protein A possessing cocci then adhered to the IgG molecules on the surface of S-SB during incubation and was released into the supernatant after centrifugation.

The supernatant was spread on antiserum agar (ASA) and the colonies formed were examined for protein A-deficient mutant by agglutinating with S-SB. By using this technique, they isolated several types of mutants; one was a nonproducer (haloless mutant), while the second type was deficient in both cell-bound and soluble protein A. They concluded that the deficient organisms present in the supernatant were free of cell-bound protein A. The other mutants produced extracellular protein A only in large amounts and did not contained cell-bound protein A (large halo mutant). They also found that the efficiency isolating protein A-deficient mutant was enhanced by repeating the cosedimentation procedure. From this experiment, it was concluded that not all the mutant strains which were isolated from the parental of protein A producer produce protein A. This information was also supported by Movitz *et al* (1979). They reported that about 50% of methicillin-resistant strains were nonproducers or yielded only extracellular protein A.

The mutants of Cowan I deficient in protein A have also been isolated by nitrosoguanidine and ethylmethane (Forsgren *et al.*, 1971). Sixty of such mutants were isolated and six classes were distinguished on the basis of production of cell-bound and extracellular protein A, nuclease, coagulase, α-hemolysin and fibrinogen activity. More than a third of them were negative for all activities, and two classes showed deficiencies of protein A production and α-hemolysin.