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A Competitive ELISA for Quantification of Protein A in Culture Medium

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ABSTRAK

Satu asai imunterjerap enzim berangkai secara bertanding untuk pengukuran protin A yang dihasilkan oleh Staphylococcus aureus *A676 (rintang terhadap metisilin) adalah berasaskan kepada pertandingan untuk terikat kepada molikul IgG arnab antara protin A yang ditandakan dengan enzim fosfatase beralkali dan protin A yang tidak bertanda. Kepekatan IgG yang optima bagi tujuan adalah diantara 2 dan 4 µg/ml. Masa pengeraman yang optima untuk pembentukan warna dengan penggunaan substrat ρ-nitrofenol fostat adalah diantara 20 dan 30 minit. Kepekatan protin A yang terendah dapat diukur dengan menggunakan asai imunterjarap enzim berangkai secara bertanding yang telah dioptimakan adalah 20 ng/ml dan kepekatan maxima adalah 2 µg/ml. Jumlah protin A yang dihasilkan didalam medium infusi otak-hati telah bertambah secara eksponen ketika fasa log pertumbuhan sel dan mencecah kepekatan maksima pada of 22.5 µg/ml selepas pengkulturan selama 15 jam.*

ABSTRACT

A competitive enzyme-linked immunosorbent assay (ELISA) for quantification of protein A produced by Staphylococcus aureus *A676 (a methicillin-resistant strain) was based on competitive binding to rabbit IgG molecules between alkaline phosphatase-labelled protein A and unlabelled protein A. The optimum IgG concentration required for coating was 2-4 µg/ml. The optimum incubation time for colour development using (ρ-nitrophenol phosphate substrate was 20-30 min. The lowest protein A concentration that could be measured using the optimized competitive ELISA was 20 ng/ml, and the maximum 2 µg/ml. The amount of protein A produced in brain-heart infusion medium increased exponentially during log phase of cell growth, reaching a maximum concentration of 22.5 µg/ml after 15 h cultivation.*

INTRODUCTION

Protein A is a 42 kDA polypeptide protein produced by most *Staphylococcus aureus* strains (Lind *et al.* 1970; Hjelm *et al.* 1972). This protein is capable of binding to human immunoglobulin and of forming precipitin lines in gel diffusion serological test (Lofkvist and Sjoquist 1962). Protein A has four homologous binding sites for Fc receptors; each site consists of approximately 60 amino acid residues. These binding sites are present at the N-terminus, which does not bind to the peptidoglycan of the *S. aureus* cell wall (Sjodahl 1977). However, due to the structural configuration only two binding sites can bind simultaneously to the complementary receptor sites (Langone *et al.* 1978).

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Protein A reacts mainly with the Fc receptor of IgG (Forsgen and Sjoquist 1969). Among IgG subclasses, IgG_1, IgG_2 and IgG_4 have a high affinity to protein A, but IgG_3 does not (Kronvall and Frommel 1970; Arkerst *et al.* 1974). Other immunoglobulin classes to which protein A binds are IgA, IgM and polyclonal IgE (Heremans 1974; Harboe and Folling 1974; Brunda *et al.* 1977). Due to their high binding affinity towards the Fc region of human, rabbit and guinea pig immunoglobulins, protein A and staphylococci-bearing protein A have been used in a wide variety of immunoassays such as agglutination, radioimmunoassay (O'Keefe and Bennett 1980; Richman *et al.* 1982) and enzyme-linked-immunosorbent assay (ELISA) (Engvall 1976; Buchanan *et al.* 1981; Ahmad *et al.* 1988; Zainal-Abidin *et al.* 1992). Protein A has also been used on agarose as ligand in affinity chromatography for purification of immunoglobulins (Goding 1978; Jaton *et al.* 1979; Gentile *et al.* 1984).

Langone (1982) reported that more than 95% of *S. aureus* strains produce protein A in varying amounts. This protein was reported as a cell wall constituent, which is covalently linked to the peptidoglycan (Sjoquist *et al.* 1972). Some *S. aureus* strains are capable of secreting protein A into culture medium (Forsgen and Sjoquist 1969; Masuda *et al.* 1975). For example, the strain Cowan I secretes 30% of protein A, and methicillin-resistant strains secrete almost all protein A synthesized by the cell into the culture medium (Forsgen and Sjoquist 1969; Masuda *et al.* 1975). The-cell wall bound protein A can be isolated by using enzymes such as lysozyme, DNAase and lysostaphin (Yoshida *et al.* 1963; Sjoquist *et al.* 1972). However, the yields produced by lysozyme and DNAase are variable and heterogeneous compared to lysostaphin (Bjork *et al.* 1972).

This paper describes a competitive ELISA technique for quantification of extracellular protein A in culture medium produced by a methicillin-resistant strain of *Staphylococcus aureus*, strain A676. Competitive ELISA is a heterogeneous enzyme immunoassay where the antigen-antibody complexes physically separate an antibody from free antigen using a solid phase system (Engvall 1976). This assay system is very specific and sensitive, and can be used for measuring either antigen or antibody. A competitive ELISA system, which is based on a competitive binding between enzyme-labelled protein A and unlabelled protein A with human IgG, was first described by Goding (1978). Human or rabbit IgG was selected because of its high binding affinity to the Fc region by protein A through extensive hydrophorbic interaction to the binding sites CH_2 and CH_3 at the constant regions of heavy-chain (Endresen and Grov 1978; Zikan 1980; Gentile *et al.* 1984). This ELISA alkaline phosphatase system was chosen to be conjugated with protein A because it is stable and the activity has a linear relationship with substrate concentrate compared with horseradish peroxidase enzyme (Voller *et al.* 1976).

MATERIALS AND METHODS

Cultivation of Bacteria and Source of Protein A

Staphylococcus aureus strain A676, a methicillin-resistant strain, was provided by Prof. C. Brown of Heriot-Watt University, Edinburgh, UK. The bacterium, isolated from a single colony, was sub-cultured on nutrient agar containing 0.2% methicillin (Sigma, St. Louis, MO) at 37°C for 24 h, and then transferred to 10 ml of nutrient broth in a 100-ml flask for 10 h at 37°C with continuous shaking at 150 rpm (Centromat, B. Braun, Germany). The culture was centrifuged at 3000 rpm for 10 min and the pellet was resuspended in phosphate-buffered saline (pH 7.4) and

optical density (OD) adjusted to 0.6 at 600 nm. The brain-heart infusion medium (pH 7.4) was inoculated with the bacterial suspension at a final concentration of 1% (v/v) and incubated at 37°C in a shaking water bath at 150 rpm. A 5-ml sample was removed at 2-h intervals and centrifuged at 10,000 xg for 5 min.

Preparations of IgG and Purification of IgG

IgG was purified according to the method of Clark and Adam (1977). Blood from white New Zealand rabbits was allowed to clot at room temperature for 60 min and kept overnight at 4°C. Serum was separated from blood cells by centrifugation at 3000 rpm for 10 min. An equal volume of 40% saturated ammonium sulphate was added and the mixture was again centrifuged at 2000 rpm for 10 min. The precipitate was dissolved in 5 mM phosphate buffer (pH 7.4) and dialysed with three changes of the same buffer. Immunoglobulin G in serum was chromatographed on DEAE-cellulose which was pre-swollen in 5 mM phosphate buffer (pH 7.4) and packed into a column (1.6 × 30 cm) and equilibrated with 1 l of the same buffer. One m of partially purified IgG was applied and eluted with a gradient of increasing ionic strength of phosphate buffer (5-50 mM). Fractions from the first peak were collected and pooled. The concentration was determined at 280 nm and stored at -20°C.

Competitive ELISA (CELISA)

Optimization of the ELISA method was developed with respect to IgG concentration and incubation time for colour development. To determine the optimum concentration of IgG, solutions of various concentrations (1, 2, 4, 6 and 8 µg/ml) in carbonate buffer (pH 9.6) were dispensed at 150 µl per well of a 96-well microtitre plate (Nunc). The plate was incubated overnight at 4°C and then washed three times with PBS containing 0.05% Tween 20 (Merck) PBS-T). The wells were blocked with 150 µl of 3% bovine serum albumin (BSA) in PBS and incubated at 37°C for 1 h after a subsequent washing with PBS-T.

For the assay, 200 µl of unlabelled protein A (Sigma) at various concentrations (0.005 - 4 µg/ml) in PBS or BHIB complex medium (Oxoid) with the addition of 200 µl protein A alkaline phosphate (Sigma) at a concentration of 1 mg/ml was used. The plate was incubated at 37°C for 2 h and washed three times with PBS-T. Fresh para-nitrophenyl phosphate (Sigma) substrate prepared at a concentration of 1 mg/ml was added to each well and kept in the dark for 15, 20, 25 or 30 min. The reaction was stopped by the addition of 50 µl of 3 M NaOH. The OD value was read using an ELISA reader (Bio-Tek Instruments, USA) at λ = 405 nm. The standard curve was established by plotting the OD values at Y axis and protein A at X axis. The concentration of samples was then estimated by using the Kinetic-Calc program on a computer linked to the ELISA reader (Bio-Tek Instruments, USA). For logit-log plot, the logit values for Y axis were calculated by using the formula: Logit $[(^{OD}\text{maximum})/^{OD}\text{maximum}-^{OD}\text{standard or sample})]$, versus log standard protein A in X axis.

RESULTS AND DISCUSSION

The optimum concentration of rabbit IgG at which unlabelled protein A effectively competed with the enzyme-labelled protein A was 2-4 µg/ml (*Fig. 1*). The binding of protein A-alkaline phosphate to the IgG molecules adsorbed to the wells was reduced when the concentration of unlabelled protein A in the standard or samples was increased. The absorbance values of alkaline-phosphate-protein A bound to the IgG were measured 20 min after additions

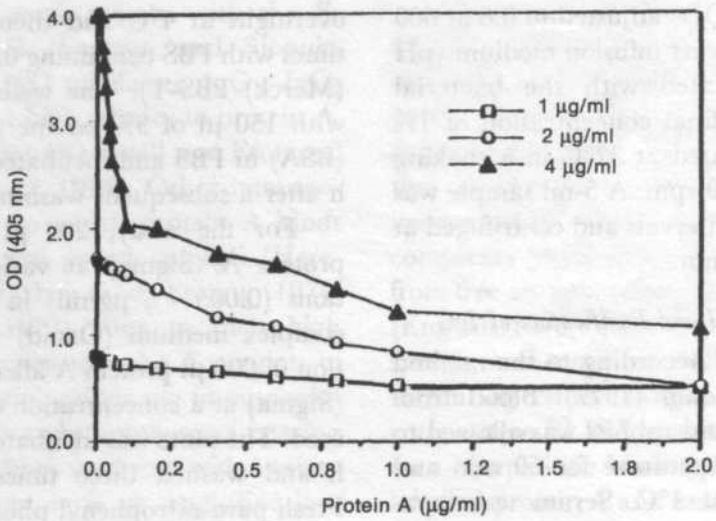

Fig. 1. Standard curves of protein A (Wells were coated with IgG at 1, 2 or 4 µg/ml and the competitive ELISA performed as described in the text)

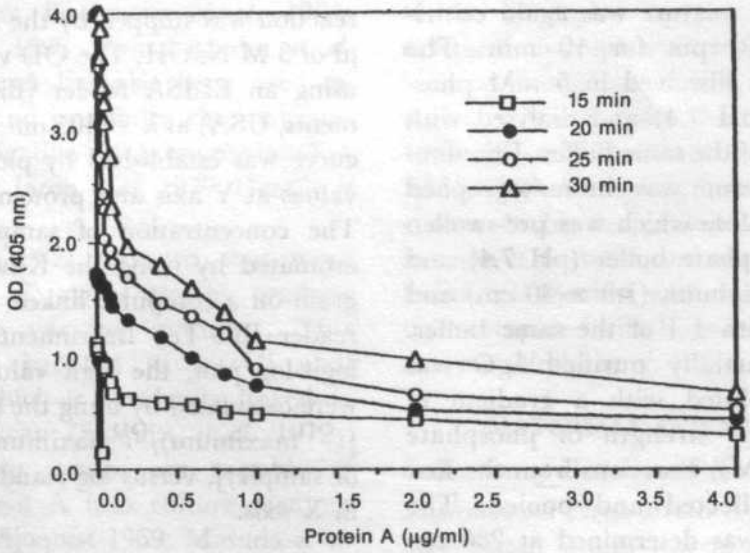

Fig. 2. Determination of optimum incubation time with IgG coated at 2 µg/ml (The IgG at 2 µg/ml was coated on the microtitre plate and the competitive ELISA performed; absorbance was measured every 5 min)

of the substrate. The absorbance at zero concentration of protein A was increased with increasing concentrations of IgG coated. The significant reduction of absorbance values was observed with the increase in unlabelled protein A for the wells coated with 2 and 4 µg/ml IgG. At higher IgG concentrations (4 µg/ml or above), no competition occurred because the binding sites for protein A were not limited. Conversely, at IgG concentrations lower than 1 µg/ml the number of protein A receptors was too small to allow any competition to occur between alkaline phosphatase-protein A and the unlabelled protein A molecules (Goding 1978).

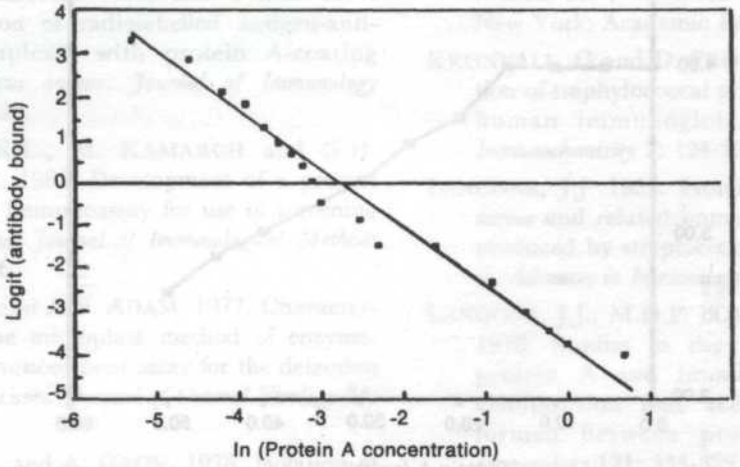

Fig. 3. Logit-log plot of standard curve using IgG coating at 2 μg/ml with a 25-min substrate incubation time

Fig. 2 shows the standard curve, which was plotted using absorbance values taken at time intervals of 15, 20 and 30 min after the addition of substrate. At 20 and 30 min the absorbance values were inversely proportional to a wide range of protein A concentrations. *Fig. 3* shows a linear standard curve using IgG coating at 2 μg/ml with incubation time of 25 min transformed into logit-log plot. This plot allows approximation of sample concentration in the region which is approaching saturation (Peterman and Butler 1989). The minimum detectable limit of protein A in this competitive ELISA system was determined by constructing the standard curve with a concentration range of 0-5 ng/ml using 4 μg/ml IgG coating. *Fig. 4* shows that the minimum detectable limit was 20 ng/ml.

The production of protein A from methicillin-resistant *S. aureus* A676 cultured in brain-heart infusion medium in the shake flask was measured using the competitive ELISA as described above. The supernatant of samples was obtained every 2 h for the first 10 h and subsequently every 10 h until 48 h of total incubation time. The sample was then diluted 10 × in PBS before measuring the protein A concentration. Using this competitive ELISA measurement, absorbance of the protein A standard and samples was performed in the same 96-well plate. *Fig. 5* shows concentration of protein A in the medium measured for a period of 48 h using competitive ELISA technique. The concentration of protein A produced in this experiment was correlated with increase in cell number. The production increased exponentially during log phase and reached maximum concentration of 22.5 μg/ml after 15 h cultivation. Thus, this techniques is successful in measuring protein A concentration in *S. aureus* cultures.

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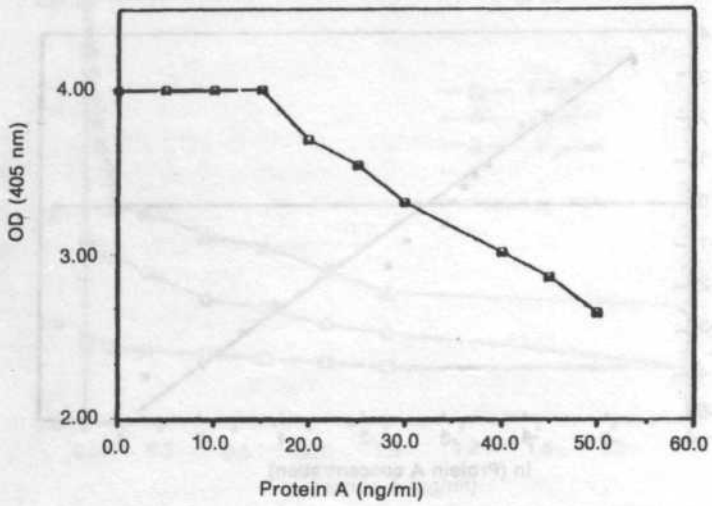

Fig. 4. Standard curve of protein A with a concentration range of 0-50 ng/ml using IgG coating at 4 µg/ml. (The OD was measured 25 min after substrate was added)

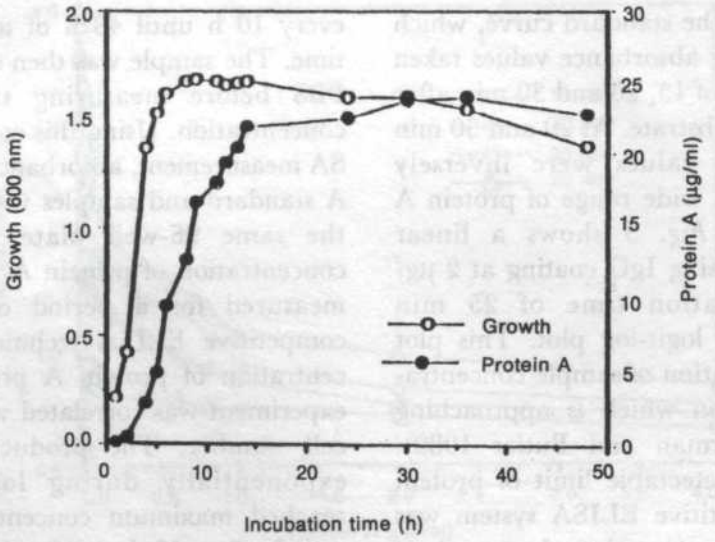

Fig. 5. Growth of S. aureus *A676 in BHIB medium and concentration of protein A produced when cultured in shake flask at 37°C for 48 hours*

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