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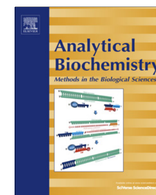

Penicillin-binding protein 3 of *Streptococcus pneumoniae* and its application in screening of β-lactams in milk

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ABSTRACT

The soluble form of penicillin-binding protein 3 (sPBP3*) from *Streptococcus pneumoniae* was expressed in *Escherichia coli* as a six-histidine fusion protein. The protein was purified and used to develop a microplate assay in direct competitive format for the detection of penicillins and cephalosporins in milk. The assay was based on competitive inhibition of the binding of horseradish peroxidase-labeled ampicillin (HRP–Amp) to the sPBP3* by free β-lactam antibiotics in milk. Under optimized conditions, most of the β-lactam antibiotics (11 penicillins and 16 cephalosporins) could be detected at concentrations corresponding to the maximum residue limits (MRLs) set by the European Union. Analysis of spiked milk samples showed that acceptable recoveries ranged from 74.06 to 106.31% in skimmed milk and from 63.97 to 107.26% in whole milk, with coefficients of variation (CVs) less than 16%. With the high sensitivity and wide-range affinities to penicillins and cephalosporins, the developed assay based on sPBP3* exhibited the potential to be a screening assay for fast detection of β-lactam antibiotics in milk.
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The β-lactam antibiotics are among the oldest and most important group of antibiotics, basically consisting of penicillins and cephalosporins that contain a bulky side chain attached to 6-aminopenicillanic acid and 7-aminocephalosporanic acid nuclei, respectively (Fig. 1) [1]. Currently, they are still frequently used in veterinary practice for the prevention and treatment of bacterial infections, especially in mastitis therapy [2]. The β-lactam antibiotics presented in milk may result in a potential hazard to consumers and technological problems in the dairy industry [3]. To minimize the high risk to public health, the maximum residue limits (MRLs)[1] of β-lactams in different animal tissues and milk were established in European Council regulation 2377/90 and subsequent modifications [4,5]. For milk, MRL values ranged from 4 μg kg^{-1} for benzylpenicillin to 125 μg kg^{-1} for cefacetrile.

Physicochemical methods for β-lactams residue usually provide reliable and quantitative results for confirmation and cannot be suitable for high-throughput screening [6]. Immunoassays based on the use of antibodies were developed rapidly as an alternative to the traditional microbial inhibitor tests, being simple, convenient, and reliable. Apparently, for efficient surveillance purposes, an immunoassay that can detect multi-β-lactams instead of each individual β-lactam would be preferable. To achieve this goal, the generation of antibodies with broad specificity is necessary. Nevertheless, due to the chemical reactivity of the β-lactam ring, production of sensitive group-specific antibodies against intact β-lactams seems to be quite difficult, which may be the bottleneck problem for immunoassay [7,8]. In such a case, the receptor binding assays based on bacterial proteins could be a solution to this problem.

Receptor proteins defined as penicillin-binding proteins (PBPs) are typically both generic and highly specific for the active form of β-lactams [9], which displayed a significant advantage over antibodies. Based on their molecular mass, PBPs can be classified into two groups [10]. High-molecular-mass (HMM) PBPs exhibit transpeptidase and glycosyltransferase activities, whereas low-molecular-mass (LMM) PBPs are thought to control the degree of cross-linking of the peptidoglycan as D,D-carboxypeptidases [11]. In general, the HMM PBPs were mainly used in β-lactam-specific receptor binding assays, and the LMM PBPs (D,D-carboxypeptidase from *Actinomadura* R39) were exploited on the basis of the enzymatic activity in enzyme assay [12–14].

Over the years, commercially available receptor binding assays, such as the BetaStar, Twinsensor, SNAP MRL, and Rosa MRL3 test for β-lactams, have been employed in a dipstick format, but only for qualitative screening in milk [4,15]. Currently, receptor-based

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[1] *Abbreviations used:* MRL, maximum residue limit; PBP, penicillin-binding protein; HMM, high-molecular-mass; LMM, low-molecular-mass; sPBP3*, soluble PBP3; HRP–Amp, horseradish peroxidase-labeled ampicillin; IPTG, isopropy-β-D-thiogalactoside; LB, Luria–Bertani; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TMB, 3,3′,5,5′-tetramethylbenzidine; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT, room temperature; LOD, limit of detection; CB, carbonate buffer; TB, Tris buffer; BSA, bovine serum albumin; CV, coefficient of variation.

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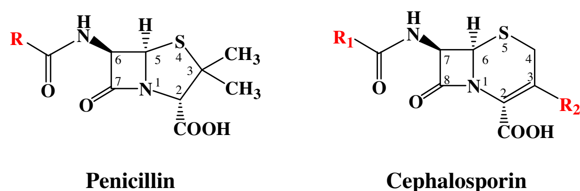

Fig.1. Structures of penicillins and cephalosporins.

biosensor and microplate assays have emerged in the quantitative detection of β-lactams in different food matrices using PBP2x* [16–18]. Our group also has expressed PBP2x* of *Streptococcus pneumoniae* and developed a rapid multiresidue assay for detecting 16 β-lactams in milk [19]. However, the affinities of PBP2x* to some β-lactams were relatively low, which might restrict the application in detection.

In this study, a soluble PBP3 (sPBP3*) belonging to LMM PBP in *S. pneumoniae* was obtained and first applied in binding assay to screening 27 β-lactams (penicillins and cephalosporins) in milk. This assay was based on the direct competitive binding to sPBP3* by horseradish peroxidase-labeled ampicillin (HRP–Amp) and free β-lactams in milk. The optimization of the assay and structural implications on the affinity of the sPBP3* are described.

Materials and methods

Chemicals and instruments

The β-lactam antibiotics—benzylpenicillin, ampicillin, amoxicillin, cloxacillin, dicloxacillin, oxacillin, nafcillin, penicillin V, piperacillin, methicillin, azlocillin, cefalexin, ceftiofur, cefalonium, cefquinome, cefazolin, cefoperazone, cefalothin, cefotaxime, ceftriaxone, cefadroxil, cefaclor, cefixime, cephradine, cefuroxime, cefapirin, and moxalactam—were bought from Sigma–Aldrich (St. Louis, MO, USA) and the National Institutes for Food and Drug Control (Beijing, China). *S. pneumoniae* R6 was used as a source of chromosomal DNA for amplification of *pbp3**. The pET-28b vector and His-Bind columns were purchased from Novagen (Madison, WI, USA). DNA polymerase and restriction enzymes were purchased from TaKaRa (Dalian, China). BL21(DE3) Chemically Competent Cell, Trans5α Chemically Competent Cell, anti-6×His monoclonal antibody, isopropy-β-D-thiogalactoside (IPTG), and pEASY-T1 Simple Cloning Vector were obtained from TransGen (Beijing, China). Luria–Bertani (LB) medium and LB agar were obtained from Beijing Aoboxing (Beijing, China). Goat anti-mouse IgG–HRP conjugate was supplied by Bio-Rad (Hercules, CA, USA). T4 DNA ligase was purchased from Promega (Madison, WI, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), HRP, 3,3′,5,5′-tetramethylbenzidine (TMB), and kanamycin were purchased from Sigma–Aldrich. Primer synthesis and DNA sequencing were performed by Invitrogen (Shanghai, China). DNA and proteins were quantified using a NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA). Enzyme-linked immunosorbent assay (ELISA) plates (96 wells) were purchased from Costar (Cambridge, MA, USA). A SpectraMax M5 microplate reader from Molecular Devices (Downingtown, PA, USA) was coupled with an IBM PC for data acquisition and analysis.

Buffers and solutions

Binding buffer consisting of 20 mM Tris–HCl (pH 7.9), 500 mM NaCl, and 5 mM imidazole was prepared for protein purification according to the manufacturer's directions. Wash buffer and elution buffer were the binding buffer with the imidazole concentration up to 20 and 500 mM, respectively.

Stock solutions of β-lactam antibiotics

Stock solutions of β-lactam antibiotics were prepared in 0.01 M phosphate-buffered saline (PBS, pH 7.4) at a concentration of 1 mg ml^{-1} and stored at −80 °C. Before experiments, standard solutions of β-lactams in the range 0.1 to 1000 ng ml^{-1} were freshly prepared by dilution of stock solution with PBS.

Construction of expression plasmid

The *pbp3** gene of *S. pneumoniae* R6 encoding a truncated form of PBP3 (Gly15 to Lys394) was amplified by polymerase chain reaction (PCR) using the primers 5′-GGGA-ATTCCATATGGGGGTGTTTCTACTGCT-3′ (*Nde*I site underlined) and 5′-CCGCTCGAGTTATTTTTCAATTTTCTTGTCTGC-3′ (*Xho*I site underlined). The PCR products were first cloned into a pEASY-T1 Simple Cloning Vector and further inserted as an *Nde*I/*Xho*I-digested fragment into pET28b to generate an expression plasmid, pET28b–sPBP3*. Following confirmation of composition of the expression plasmid by DNA sequencing, pET28b–sPBP3* was transformed into *Escherichia coli* BL21(DE3).

*Expression and purification of sPBP3**

Transformed bacteria were first grown overnight at 37 °C in LB medium supplemented with 30 μg ml^{-1} kanamycin. The culture was inoculated into fresh medium with a dilution of 1:100 for further incubation at 37 °C. Until an optical density at 600 nm up to 0.6, expression was induced by adding 0.2 mM IPTG for 16 h at 20 °C. Cells were collected, washed once, and suspended in binding buffer. The resulting suspension was subjected to sonication on ice. After centrifugation and filtration with a 0.45-μm filter, the supernatant was loaded on a His-Bind column equilibrated with binding buffer. Finally, His-tagged sPBP3* was eluted with elution buffer and dialyzed against PBS overnight at 4 °C. The concentrations of the purified proteins were determined on the NanoDrop spectrophotometer at 280 nm. After adding 50% (v/v) glycerol, proteins were stored at −20 °C.

Purified proteins and flow-through samples in purification were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting with the anti-6×His monoclonal antibody according to standard protocols.

Labeling of ampicillin with HRP

Ampicillin was coupled to HRP according to the procedure using EDC [20]. Briefly, 4 mg (0.1 μmol) of HRP dissolved in 1 ml of PBS was added dropwise to 4 mg (11 μmol) of ampicillin, followed by 4 mg (20 μmol) of EDC in 1 ml of PBS. The reaction mixture was stirred for 2 h at room temperature (RT) and then overnight at 4 °C. Subsequently, dialysis against 2 L of PBS was performed over 3 days.

Development and optimization of a microplate receptor assay

A microplate receptor assay was developed in a competition format. The whole procedure was carried out as described below. The appropriate sPBP3* in coating buffer was pipetted into microplates and incubated for 16 h at 4 °C. The plates were blocked at 37 °C for 2 h with 150 μl of blocking buffer. After the blocking, 50 μl of the standards or samples and 50 μl of HRP–Amp (1:150 diluted in 0.01 M PBS, pH 7.4) were added successively to each well and incubated at 37 °C for 30 min. After washing four times with

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