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Analytica Chimica Acta 586 (2007) 296–303
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Development of a receptor-based microplate assay for the detection of beta-lactam antibiotics in different food matrices

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Received 21 June 2006; received in revised form 16 August 2006; accepted 18 September 2006
Available online 26 September 2006

Abstract

The penicillin-binding protein PBP 2x* from *Streptococcus pneumoniae* has been utilised to develop a novel microplate assay for the detection and determination of penicillins and cephalosporins with intact beta-lactam structure in milk, bovine and porcine muscle juice, honey and egg. In the assay, the receptor protein is immobilised to a microplate in the first step. To each sample a bifunctional reagent is added, with ampicillin and digoxigenin as functional groups (DIG-AMPI). The amount of bifunctional reagent, which is bound via its ampicillin part to the receptor protein, decreases with increasing beta-lactam concentration in the sample. The detection step uses anti-digoxigenin F_{ab} fragments marked with horseradish peroxidase. The more bifunctional reagent is bound to the receptor protein, the more antibody fragments are bound via the digoxigenin part of the reagent. A maximum colour development with tetramethylbenzidine as chromogen for the peroxidase reaction is achieved, when no beta-lactam residues are present.

A fractional factorial design was applied to detect chemometrically effects and interactions of the assay parameters. For optimisation of the significant parameters a Box-Behnken design was used.

The assay has been developed for various food matrices as screening test with the option for a quantitative assay, when the identity of the residual beta-lactam is known (e.g. elimination studies). Cefoperazon, cefquinome, cefazolin, cloxacillin, ampicillin and benzylpenicillin could be detected at levels corresponding to 1/2 EU maximum residue limit (MRL) in milk, meat juice from muscle tissue of different species, egg and honey (where applicable) without needing lengthy and elaborate sample pre-treatment. Matrix calibration curves are presented, which show that quantitative analyses are possible.

Keywords: Penicillins; Cephalosporins; Penicillin-binding protein; Chemometric optimisation; Residues; Food

1. Introduction

Antibiotics of the beta-lactam group are widely used for treatment of bacterial infections and are the preferred drugs for the treatment of clinical mastitis in dairy cows. Correspondingly, beta-lactams constitute the major source of antibiotic residues in milk [1]. The knowledge of their presence in foodstuffs is of great importance for food industry as their presence can inhibit the growth of starter cultures used for food technological processes, cause allergic reactions in sensitised individuals or affect the intestinal flora of consumers [2]. Therefore, their use in veterinary practice is regulated by the EU Council Regulation 2377/90 [3]. For example, the maximum residue limit (MRL) for both, benzylpenicillin and ampicillin, in milk is 4 $\mu g\,kg^{-1}$, in muscle 50 $\mu g\,kg^{-1}$ [4] and for cefacetril in milk 125 $\mu g\,kg^{-1}$ [5].

Nowadays, there are numerous screening tests commercially available which are able to detect beta-lactam antibiotics in milk and other tissues and indicate the presence at or above the maximum residue limits. These screening tests are enzyme assays, immunoassays or receptor binding assays [6–8]. Receptor-based tests in dipstick format like the Beta-Star, Twinsensor, or Charm ROSA MRL beta-lactam/tetracycline test can only be used for milk and no quantitative determination is possible [9,10]. Receptor-based biosensor assays [11,12] require high cost equipment for surface plasmon resonance (SPR) measurements and labour-intensive sample pre-treatment because of non-specific binding of matrix compounds to the sensor chip surface.

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doi:10.1016/j.aca.2006.09.032

The mechanism of action of beta-lactams is based upon the inhibition of membrane-bound enzymes involved in the final stages of bacterial peptidoglycan synthesis [13]. Due to their ability to covalently bind penicillins and other beta-lactam antibiotics via the active-site serine, these enzymes are named penicillin-binding proteins (PBPs) [14,15]. One classification is the division into two groups on the basis of their molecular mass, the high molecular mass PBPs (≈50–100 kDa) and the low molecular mass PBPs (≈30–40 kDa). In addition, the high molecular mass PBPs are subdivided into two classes. Class A forms are bifunctional enzymes that catalyse both the transpeptidation and transglycosylation during cell wall synthesis and class B forms with only transpeptidation activity. The low molecular mass PBPs are mainly D-alanyl D-alanine carboxypeptidases and are thought to control the degree of cross-linking of the peptidoglycan [16,17].

The described PBPs are used as binding reagents in beta-lactam specific receptor binding assays and enzyme assays. In enzyme assays the inhibition of the enzymatic DD-carboxypeptidase activity is measured [18].

The MRLs are defined for the intact beta-lactams and not for their metabolites or degradation products. According to that, the antibody used for immunochemical methods (e.g. enzyme immunoassay) should be specific for the intact beta-lactam ring. Until recently it was difficult to obtain antibodies against intact beta-lactam antibiotics because the immunisation procedure led to the open-ring forms of the beta-lactams and consequently to antibodies against hydrolysed penicillins. In the last years antibodies without open-ring specificity have been raised against intact ampicillin [19] and a group-specific antibody against intact penicillins [20]. The latter was used in an enzyme immunoassay, which was successfully validated with violative milk samples from routine control [21]. In 2006, a first commercial enzyme immunoassay for penicillins was launched with the claim of being specific for the intact compounds [22]. However, these test systems can only detect penicillins and not the whole group of beta-lactams including cephalosporins. The use of PBPs makes this detection possible because they interact with intact beta-lactams exclusively and have high affinities for both, penicillins and cephalosporins. These features were the driving force for the development of surface plasmon resonance (SPR) biosensor assays using the penicillin-binding proteins DD-carboxypeptidase from *Actinomadura* R39 [23] or PBP 2x* [12] for the detection of β-lactam residues in milk.

The purpose of the present study was to develop a receptor-based microplate assay for detection of penicillins and cephalosporins with intact beta-lactam structure in different matrices. A soluble PBP 2x derivative (PBP 2x*) of *Streptococcus pneumoniae* was used. This is a high molecular mass class B PBP which possesses high affinities to beta-lactams [24,25]. The principle of the described receptor assay is based on the non-competitive binding by beta-lactam antibiotics to the available PBP–beta-lactam binding sites. Digoxigenin-labelled ampicillin (DIG-AMPI) [26] binds to the remaining PBP–beta-lactam binding sites and the formed PBP 2x*/DIG-AMPI-complexes were detected by using horseradish peroxidase (HRP)-marked anti-digoxigenin F_{ab} fragments.

2. Experimental

2.1. Instrumentation and reagents

The microplate washer was from BioTek Instruments GmbH (Bad Friedrichshall, Germany); the microplate reader was obtained from Bio-Rad Laboratories GmbH (München, Germany). MaxiSorp F96 immunoplates were obtained from Nunc (Wiesbaden, Germany). Phenylmethylsulfonyl fluoride, benzylpenicillin, ampicillin, cloxacillin and cefazolin were from Fluka (Taufkirchen, Germany). Dithiothreitol, cefoperazon, tetramethylbenzidine (TMB), bovine serum albumin (BSA), glutathione–agarose and reduced glutathione were obtained from Sigma–Aldrich (Taufkirchen, Germany). Cefquinome was a gift from Intervet (Schwabenheim, Germany). Hydrolysed penicillins were prepared by solving 10 mg of the respective penicillin in 9.8 mL methanol and 0.2 mL 1 M potassium hydroxide and reaction overnight at 4 °C in the refrigerator. Digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (DIG-NHS), anti-digoxigenin F_{ab} fragments marked with horseradish peroxidase and β-isopropyl-D-thiogalactopyranoside (IPTG) were purchased from Roche Diagnostics (Mannheim, Germany). Bocillin FL was obtained from Mobitec (Göttingen, Germany). Prescission protease was from Amersham Biosciences (Freiburg, Germany). All other reagents were of reagent grade or better and purchased from Sigma–Aldrich or Carl Roth (Karlsruhe, Germany).

2.2. Expression and purification of recombinant PBP 2x*

The expression and purification of recombinant PBP 2x* was performed as described by Cacciatore et al. [12] with some modifications. The soluble derivative of PBP 2x was expressed as a glutathione *S*-transferase (GST)-fusion protein in *E. coli* BL 21 harbouring plasmid pGEX-tet-PBP 2x* coding for the GST-fusion protein linked with a prescission protease proteolytic site. The expression and purification consists of six major steps: the growth of *E. coli* BL 21 (pGEX-tet-PBP2x*) in 2xTYT broth (16 g tryptone, 10 g yeast extract, 5 g NaCl and 15 mg tetracycline per litre), expression of PBP 2x* induced by the addition of IPTG, cell disruption by two passages through a French Press at 18,000 psi (ca. 125 MPa) followed by purification of GST–PBP 2x* by glutathione affinity chromatography where the first 500 μL of the eluate was discarded. Afterwards, prescission protease was added to remove the GST-tag followed by dialysis against buffer. Removal of GST-tag and prescission protease (also a GST-fusion protein) was done by another step of glutathione affinity chromatography where PBP 2x* was in the non-adsorbed fraction.

The purification process was controlled by SDS-PAGE and the protein concentration was determined by the method of Bradford [27]. The purified PBP 2x* was stored in 10% aqueous glycerol at −20 °C. The protein concentration was 1226 μg mL^{-1}. The low protein concentration reported by Cacciatore et al. (190 μg mL^{-1}) [12] was due to the fact that too much eluate had been discarded (1 mL). The highest amount of PBP 2x* eluated with the first millilitre from the col-