SPECIAL GUEST EDITOR SECTION

Biosensor Analysis of β-Lactams in Milk Using the Carboxypeptidase Activity of a Bacterial Penicillin Binding Protein

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The applicability of a β-lactam receptor protein for detection of β-lactam antibiotics in milk using surface plasmon resonance (SPR) biosensor technology was investigated. The advantage of using a receptor protein instead of antibodies for detection of β-lactams is that a generic assay, specific for the active form of the β-lactam structure, is obtained. Two assays based on the enzymatic activity of the DD-carboxypeptidase from *Actinomadura* R39 were developed, using a Biacore SPR biosensor. The carboxypeptidase converts a tri-peptide into a di-peptide, a reaction which is inhibited in the presence of β-lactams. Polyclonal antibodies against the 2 peptides were developed and used to measure the amount of enzymatic product formed (di-peptide assay) or the amount of remaining enzymatic substrate (tri-peptide assay), respectively. The 2 assays showed similar performances with respect to detection limits (1.2 and 1.5 μg/kg, respectively) and precision (coefficient of variation <5%) for penicillin G in milk. Several other β-lactams were detected at or near their respective maximum residue limit. Furthermore, the 2 peptide assays were evaluated against 5 commercial kit tests in the screening of 195 producer milk samples. The biosensor assays showed 0% false-negative and 27% false-positive results, whereas the figures were 0% false-negative and 27–53% false-positive results for other screening tests investigated.

The β-lactam antibiotics, penicillins and cephalosporins, constitute the group of antimicrobial drugs most frequently used within veterinary medicine for treatment of infections caused by Gram-positive bacteria, e.g., mastitis in dairy cows. The antibacterial effect of β-lactams is due to their inhibition of the bacterial cell wall synthesis, interfering with the transpeptidases that perform the cross-linking of the peptides (1, 2). The resulting structural weakness of the cell wall is followed by activation of autolytic enzymes causing lysis of the bacteria (3).

Administration of antimicrobial drugs to lactating cows is always followed by a withdrawal period, during which the milk may not be delivered to the dairy. This period is necessary for residue levels to decline below legislative limits, and the length of the period varies depending on the drug used. Lack of awareness of withdrawal times or deliberate abuse may, however, lead to elevated levels of drug residues in the milk. Therefore, to ensure that the milk is free from residues, different control programs are performed within all European Union (EU) countries, both by authorities (4) and food producers and industry (5). The toxicities of various veterinary drug residues are continuously evaluated to establish EU maximum residue limits (MRLs) for different substances. A consolidated version of the Annexes I–IV of the Council Regulation 2377/90 can be found on the homepage of the European Commission (http://pharmacos.eudra.org/F2/mrl/index.htm).

Shortly after the introduction of antimicrobial drugs for treatment of infections within veterinary medicine, the first test for analysis of antibiotic residues in milk, a microbial inhibition test, was developed (6). The microbial inhibitor tests have, over the years, proven to be very suitable for screening purposes. Their main limitation is the time-consuming incubation, resulting in several hours before the result is obtained. To meet the demand of the dairy industry, the number of rapid tests on the market has increased during the last decade. One of the first rapid tests developed during the early 1980s was the enzyme-based Penzym test (UCB Bioproducts, Braine-l'Alleud, Belgium). This test is based on a soluble DD-carboxypeptidase from *Actinomadura* R39 (reclassified from *Streptomyces* R39), thoroughly studied by Frére et al. (2, 7–12). The natural substrates for the enzyme are peptides ending with D-alanyl-D-alanine (D-Ala-D-Ala) and the enzymatic activity of R39, i.e., hydrolysis of a tri-peptide into a di-peptide, has the following appearance (13):

$$\text{L-Lys-D-Ala-D-Ala} + H_2O \rightarrow \text{L-Lys-D-Ala} + \text{D-Ala}$$

Because penicillin is a structural analog to the di-peptide D-Ala-D-Ala (14, 15), the enzyme will also interact with the

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β-lactam structure, forming a very stable complex and, as a result, the enzymatic activity will be inhibited.

The first reported surface plasmon resonance (SPR) biosensor assay for detection of β-lactams in milk was an antibody-based assay described by Gaudin et al. (16). In that method, the active β-lactam structure was hydrolyzed, chemically or enzymatically, and the inactive form was detected. Because established safe levels and MRLs only include the active forms of β-lactams, the assay is of limited practical use. More recently, Cacciatore et al. (17) described an SPR biosensor assay based on a penicillin-binding protein derivate (PBP2x*) from *Streptococcus penumoniae*, expressed in *E. coli.* The assay is based on inhibition of the binding of digoxigenin-labeled ampicillin (DIG-AMPI) to the PBP2x* by β-lactam residues in the milk. After a first incubation step, the DIG-AMPI:PBP2x* complex is detected using a sensor surface with bound digoxigenin antibodies. If the sample contains β-lactams, less complex will be formed and bind to the immobilized antibodies on the surface. Samples free of β-lactams thus produce a higher response than positive samples due to the difference in molecular mass between the DIG-AMPI:PBP2x* complex and free DIG-AMPI. Bulk tank milk with β-lactam antibiotics added at levels below MRLs (penicillin G, ampicillin, amoxicillin, cloxacillin, cephalexin, and cefoperazone) could be differentiated from a blank milk sample. The assay was used to analyze a limited number of field samples, but additional studies have to be conducted to further assess the influence of matrix components on the assay.

The application presented in this manuscript is a summary of studies that were performed during 1998–2003 within a research project at the Swedish University of Agricultural Sciences (Uppsala). The objective of our work was to explore the possibilities to develop an SPR biosensor assay based on the carboxypeptidase from *Actinomadura* R39 (R39), i.e., the same receptor protein that is used in the Penzym test, for generic detection of β-lactam antibiotics in milk. A first version of a receptor protein-based biosensor assay was based on a conjugate between an antibody raised against a small organic molecule (H1), and cephalosporin (18). To perform the assay, conjugate is first injected and its antibody part will bind to H1 immobilized on the surface. The receptor protein is added to the milk and the mixture is injected over the surface. If the protein is not inhibited by free β-lactams in the milk, it will bind to the cephalosporin part of the conjugate on the sensor surface; however, if the milk contains β-lactams no binding will take place. Because the assay suffered from problems related to nonspecific binding, an alternative approach based on the enzymatic activity of the receptor protein was investigated (19). The characteristics of 2 resulting assays (20, 21) are summarized.

METHOD

Instrumentation

Biacore Q (Biacore AB, Uppsala, Sweden) was used for assay development and Biacore Q Control software (Version 3.0.1) for instrument operation and data handling. A test tube heater (GTF, Göteborg, Sweden) was used for incubation at 47°C.

Special Reagents

Sensor chip CM5 (research grade), 10 mM 4-[2-hydroxyethyl] piperazine-lethane-sulfonic acid (HEPES), 3.4 mM EDTA, 150 mM NaCl, 0.005% (v/v) surfactant P-20 HBS-EP buffer, pH 7.4, and amine coupling kit were obtained from Biacore AB.

The di-peptide (diacetyl-L-lysyl-D-alanine; Ac-L-Lys-D-Ala) used in the initial studies was obtained from the Veterinary Sciences Division at the Department of Agriculture and Rural Development for Northern Ireland (DARDNI, Belfast, UK) and later synthesized by QCB Inc., Biosource International (Hopkinton, MA). The tri-peptide (diacetyl-L-lysyl-D-alanyl-D-alanine; Ac-L-Lys-D-Ala-D-Ala) was purchased from Bachem AG (Bubendorf, Switzerland).

R39 was obtained from UCB Bioproducts (Braine-l'Alleud, Belgium). Polyclonal antibodies against the di- and tri-peptides, respectively, were kindly produced by the Veterinary Sciences Division at DARDNI.

All β-lactam antibiotics (amoxicillin, ampicillin, cefalexin, cephapirin, cloxacillin, oxacillin, penicillin G) were purchased from Sigma-Aldrich Co. (St. Louis, MO) except ceftiofur, which was obtained from Pharmacia Animal Health (Puurs, Belgium).

Preparation of Peptide Surface

Briefly, separate di- and tri-peptide surfaces were prepared in the instrument using a surface preparation unit accessory and amine coupling kit. The carboxymethylated dextran was activated by injection of *N*-hydroxysuccinimide/carbodi-imide (NHS-EDC) mixture (1:1) and then the appropriate peptide, dissolved in 50 mM borate buffer, pH 8.0, was injected and coupled to the activated surface. Remaining surface-active esters were blocked with ethanolamine.

Principle of Peptide Assays

Two assays based on the enzymatic reaction whereby tri-peptide is hydrolyzed into di-peptide were developed. In both assays, a milk sample is mixed with tri-peptide and R39, and incubated for 5 min at 47°C to allow the enzymatic reaction to proceed. If the sample is β-lactam-free, R39 will hydrolyze the tri-peptide into di-peptide. In the presence of β-lactams, the enzymatic activity of R39 is inhibited and less di-peptide will be formed, as illustrated in Figure 1 and described below.

The di-peptide assay.—Following incubation, the sample is mixed with antibodies against the di-peptide and the mixture is injected over a di-peptide sensor surface. With a β-lactam-free sample (negative), the antibodies will be inhibited by di-peptide produced in the sample, whereas with a β-lactam-contaminated sample (positive), the antibodies will bind to the di-peptide surface. The response obtained is thus directly proportional to the amount of β-lactam antibiotics in the sample.

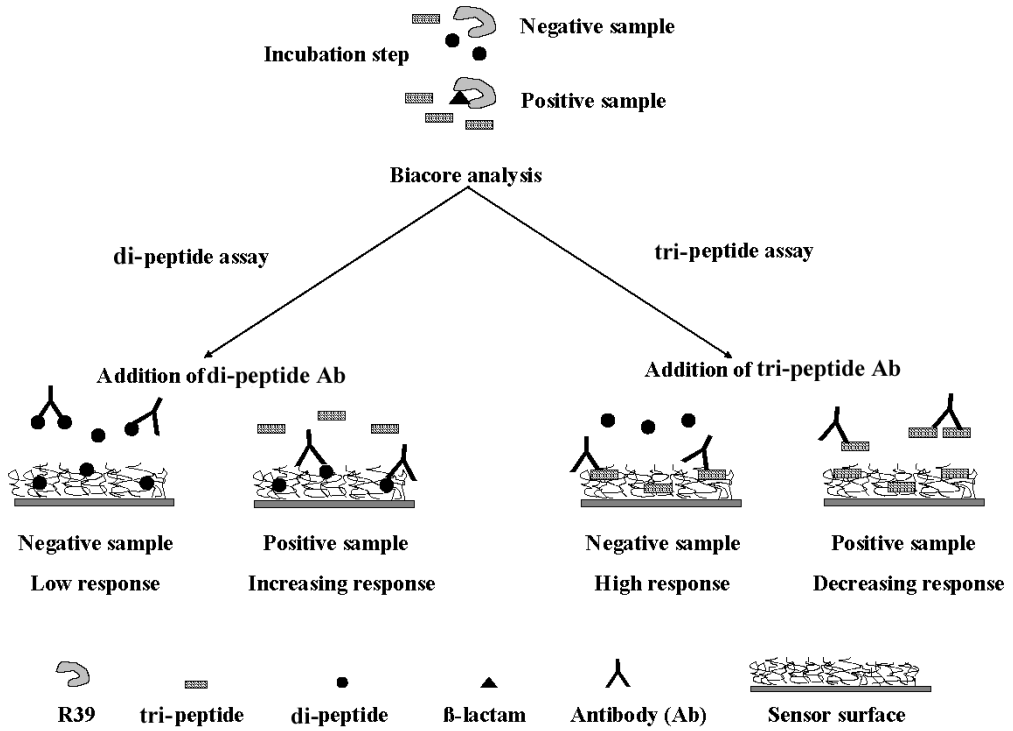

Figure 1. Illustrations of the principles of the di- and tri-peptide assays, both of which are based on the enzymatic activity of R39. During the incubation step, R39 catalyzes hydrolysis of tri-peptide into di-peptide. However, in the presence of β-lactam antibiotics, the enzymatic activity of R39 is inhibited. Antibodies are added and the amount of di-peptide formed, or the amount of remaining tri-peptide is measured.

The tri-peptide assay.—The sample is mixed with antibodies against the tri-peptide and the mixture is injected over a tri-peptide surface. With a positive sample, the antibodies will be inhibited by nonhydrolyzed tri-peptide, whereas with a negative sample, the antibodies will bind to the tri-peptide surface. The response obtained is therefore inversely proportional to the amount of β-lactam antibiotics in the sample.

Assay Procedure

Briefly, milk is pipetted into a test tube and mixed with tri-peptide and R39 in HNM-buffer according to Gustavsson et al. (20). The mixture (80% milk, 10% tri-peptide, 10% R39) is incubated for 5 min at 47°C and then transferred to a microtiter well. In the automated instrument, the sample is mixed with di- or tri-peptide antibody and the mixture (10% sample, 90% antibody) is injected for 2 min across the sensor surface. Finally, the surface is regenerated by injection of 0.5 M NaOH with 10% acetonitrile. HBS-EP is used as running buffer with a constant flow of 30 μL/min. The procedures for the 2 assays are virtually identical, but the concentrations of reagents and regeneration time differ between the 2 assays as described previously (20).

Results and Discussion

Specificity of R39 and Cross-Reactivity of Peptide Antibodies

Because existing legislation concerning residue limits is only applicable for the active form of β-lactams, the specificity of R39 was investigated. Milk samples were spiked with penicillin G concentrations of 4 and 8 μg/kg and treated with β-lactamase to hydrolyze the β-lactam structure (16). These samples, together with nontreated samples, were analyzed using the di-peptide assay. The results showed that there were no detectable residues in the β-lactamase-treated samples, whereas the concentrations of the nontreated samples were determined to be 4.0 and 7.7 μg/kg, respectively. That is, β-lactams with a hydrolyzed ring structure will not be detected by the assay.

To ensure secure detection with the respective assay, it was crucial that the antibody to the di-peptide did not cross-react with tri-peptide and vice versa. The cross-reactivities of di- and tri-peptide antibodies for tri- and di-peptide were determined to be <0.1%, respectively.

Performance of Di- and Tri-Peptide Assays

The di- and tri-peptide assays were comparable with respect to detection limit and precision (Table 1); however, the calibration curves of the assays differed in appearance (Figure 2). The di-peptide assay showed a calibration curve in which the response increased with the concentration of β-lactam, whereas the calibration curve of the tri-peptide assay had a very sharp decrease in response within a low concentration range, offering a very distinct threshold level, with high sensitivity at the MRL, for classification of positive and negative samples.

Table 1. Detection limits and precisions at 4 μg/kg penicillin G (MRL) for the di- and tri-peptide assays

		Precision within day (n = 10)		Precision between days (n = 3)	
	LOD, μg/kg	Mean, μg/kg	CV, %[a]	Mean, μg/kg	CV, %
Di-peptide assay	1.2	4.3	3.1	4.1	2.2
Tri-peptide assay	1.5	3.7	4.8	3.7	1.8

[a] CV = Coefficient of variation.

The peptide assays were tested for their abilities to detect different β-lactam antibiotics at their respective MRL levels. Milk samples were spiked with 7 different substances at concentrations corresponding to 0.5 × MRL, 1.0 × MRL, and 1.5 × MRL of the respective β-lactam, and the obtained responses were compared with a penicillin G calibration curve. Both assays detected all investigated β-lactams at their respective MRL, except cloxacillin, which was not detected by either of the assays. Ceftiofur was detected at MRL by the di-peptide assay in one of 2 analyses. These results were compared with the claimed detection limits of the Penzym test. Because the Penzym test and the biosensor assays utilize the same enzymatic reaction, the similarities in the abilities to detect different β-lactams were rather expected (Table 2).

Comparison with Existing Screening Methods for β-Lactams

The 2 biosensor assays were used to analyze 195 producer milk samples (21). These samples were selected on the basis of their results in the control of inhibitory substances, one of the parameters in the dairy cooperatives quality program for milk payment. The inhibitor test was conducted at the laboratory of Steins AB (Umeå and Jönköping, Sweden). The samples were obtained frozen, and upon arrival they were randomized and recoded in order to perform blind-coded analyses. For comparison with the biosensor assays, all milk samples were also analyzed with a set of commercially available screening tests, including the Delvotest SP (DSM Group, Delft, The Netherlands), Parallux and SNAP (IDEXX Laboratories, Inc., Lawrence, MA), Penzym test and Beta-STAR (UCB Bioproducts). Of these tests, Delvotest SP

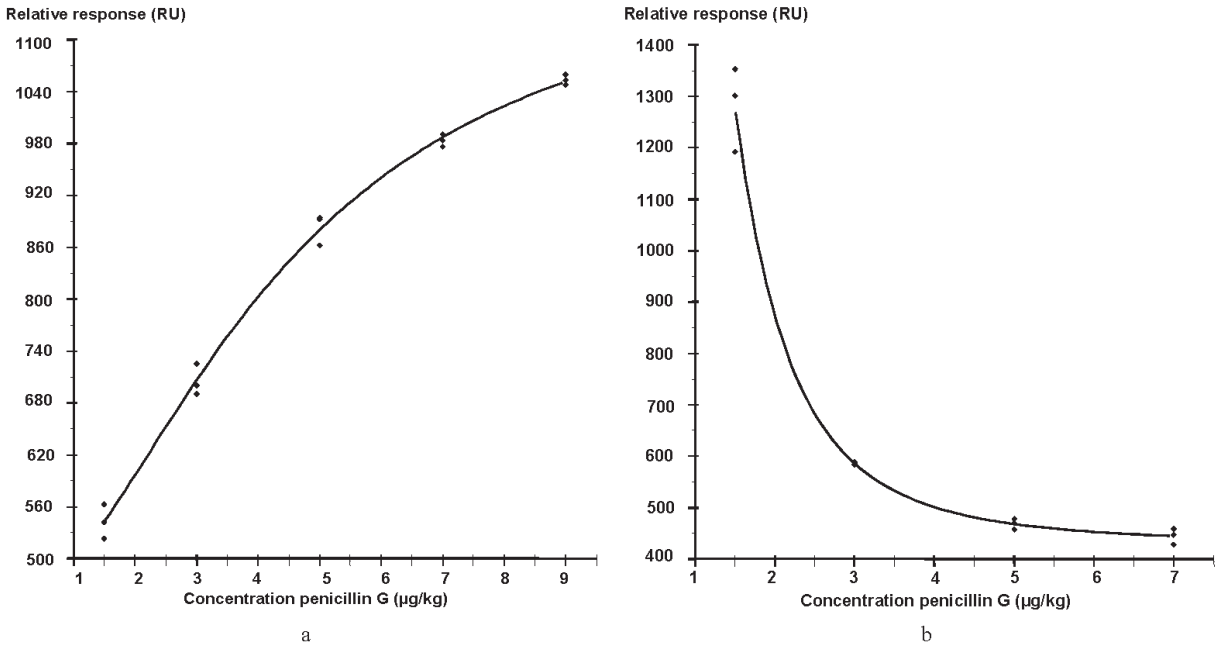

Figure 2. Illustration showing typical calibration curves from one run (n = 3) for the (a) di-peptide and (b) tri-peptide assays. The di-peptide curve shows an increasing response with increasing β-lactam concentration; the more β-lactam in the sample, the less di-peptide is formed, resulting in more binding from the di-peptide antibody to the surface. The response for the tri-peptide calibration curve decreases with increasing β-lactam concentrations, i.e., the more β-lactams present in the sample, the more tri-peptide remains nonhydrolyzed in the sample, resulting in inhibition of the tri-peptide antibody.

Table 2. The abilities of biosensor assays to detect 7 different β-lactams at their respective MRLs[a]

β-Lactam	MRL, μg/kg	Di-peptide assay[b]	Tri-peptide assay	LOD Penzym test, μg/kg
Amoxicillin	4	+	+	4–6
Ampicillin	4	+	+	4–7
Cloxacillin	30	–	–	60–100
Oxacillin	30	+	+	30–50
Cefalexin	100	+	+	20–40
Cephapirin	60	+	+	5–7
Ceftiofur	100	±	+	40–70

[a] The responses were compared against a penicillin G calibration curve using the limit of detection (LOD) of penicillin (1.5 μg/kg) as the threshold limit. The LODs of the Penzym test for the β-lactams as claimed by the manufacturer are also presented.

[b] + = Substance was detected in both analyses; – = substance was not detected in any of the analyses; ± = substance was detected in 1 of 2 analyses.

is a microbial inhibitor test, SNAP; Beta-STAR, and Penzym test are rapid tests using β-lactam receptor proteins; and Parallux uses antibodies for detection.

Samples with a positive result in any of the screening tests were re-analyzed with the same method. Only samples showing positive results in 2 consecutive analyses were classified as positive in a method. Of the 195 milk samples analyzed, the results of 190 samples were in agreement in the different screening methods, i.e., 21 samples showed positive and 169 samples showed negative results (21). Of the samples that were positive in one or more of the screening assays, 23 samples were selected for liquid chromatography (LC) analysis. In addition, 7 samples with negative results in all screening tests were subject to LC analysis. The milk sample was applied on a solid-phase extraction column, eluted with acetonitrile (AcN)–water and methanol, and then derivatized with a 1,2,4-triazol and mercury chloride solution (22). The analysis was performed with a C-18 column and a mobile phase gradient (AcN, acetate buffer, and methanol) with UV-Vis detection at 323 nm.

The di- and tri-peptide biosensor assays, as well as the other screening tests, showed 0% false-negative results with 15 producer milk samples containing between 4.0 and 268 μg/kg penicillin G. The biosensor assays showed 27% false-violative results, with 15 producer milk samples containing penicillin G concentrations between 0 and 3.6 μg/kg penicillin G, i.e., levels below MRL. This figure varied between 27 and 53% for the other screening tests. Because the methods are based on different principles and most of them are applied at their detection limits, this may explain the difference in results. The use of a cutoff level typically reduces the risk of detecting samples with residue levels far below legislated limits. The biosensor assays were used with a cutoff level of 2.7 μg/kg penicillin G. For obvious reasons, the assay gives no information regarding the type of the β-lactam present in milk. Considering that penicillin G is the only β-lactam registered for treatment of mastitis in dairy cows in Sweden, we used penicillin G to construct calibration curves. It is, important to realize, however, that the response resulting from the presence of another type of β-lactam will not necessarily be identical with the response caused by penicillin G. Therefore, under circumstances where other types of β-lactam residues are likely to occur in the milk, the cutoff level may need to be adjusted to avoid false-negative results.

Conclusions

Our review of previous studies summarizes the use of a microbial receptor protein, specifically using the enzymatic activity of the protein as basis for detection, in SPR biosensor-based assays for β-lactam antibiotics in milk. The use of a penicillin-binding protein has several advantages over using antibodies for detection. The techniques are not only generic, allowing detection of both penicillins and cephalosporins, but also specific for the active form of the antibiotic. This is a crucial characteristic of the assays, because MRL legislation does not cover inactive derivatives, and both these aspects have so far been difficult to provide in assays based on antibodies. To make the technique an interesting future alternative to existing screening techniques, generic assay for a wide range of antimicrobial drugs will be required.

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