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CHARACTERIZATION OF ANTI-CHLORAMPHENICOL ANTIBODIES BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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Polyclonal antibodies against conjugates of chloramphenicol succinate and chloramphenicol base with proteins were obtained and characterized in direct ELISA. Antiserum against a conjugate of chloramphenicol (CAP) base with BSA (direct coupling) was very specific and showed cross-reactivity only with CAP succinate (11.3%) and CAP base (4.6%); whereas, antisera against a conjugate of CAP succinate with a protein recognized CAP succinate strongly as an initial compound. In direct ELISA, antisera against a conjugate of CAP succinate with KLH (homologous assay) and CAP base with BSA (heterologous assay) showed similar sensitivity: IC_{50} were 1.3 and 1.5 ng mL^{-1}, respectively. Applicability of the immunoreagents obtained was shown in the analysis of CAP residues in milk (3.5% fat content). Detection limit of 0.3 ng mL^{-1} was obtained for milk diluted 5 times.

Keywords: Antibodies; Chloramphenicol; Enzyme-linked immunosorbent assay

INTRODUCTION

Chloramphenicol (CAP) is a relatively cheap, highly effective antibiotic with broad-spectrum activity. It was widely used in veterinary practice because of its excellent antibacterial and pharmacokinetic properties. However, CAP is a hemotoxic substance for humans and can cause bone-marrow depression and as a consequence non-dose-related aplastic anaemia. Because of the uncertainty about CAP doses that can cause these pathologies, the use of CAP has been banned for the treatment of food-producing animals in some countries (the European community, the United States, Canada, and others). But, because of its low cost and excellent properties CAP is still in use, sometimes illegally. For instance, CAP residues were detected in shrimps and honey imported into the European Union from Asian countries. As a prohibited substance, zero tolerance applies (European

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Commission Regulations 1430/94). In Europe, the maximum required performance limit (MRPL) of 0.3 $\mu g\,kg^{-1}$ was established (2003/181/EC Commission Decision). Therefore, very sensitive analytical methods are required for monitoring food for CAP residues.

A variety of methods for the detection of CAP residues in food have been developed. Relatively cheap and rapid enzyme-linked immunosorbent assay (ELISA) is usually used for screening purposes, followed by more expensive and time-consuming physico-chemical confirmatory methods such as LC and HPLC (Impens, Peybroeck, and Vercammen 2003; Scortichini et al. 2005). Traditional ELISAs (Kolosova, Samsonova, and Egorov 2000; Gaudin, Cadieu, and Maris 2003), time-resolved fluoroimmunoassay (Shen et al. 2006), more sensitive ELISA with chemiluminescent detection (Zhang et al. 2006; Chuanlai et al. 2006), and meso-fluidic immunoassay system (Zhang, Zuo, and Ye 2008) were described. Development of miniaturized and high-throughput small-molecular microarray methods for simultaneous detection of a few chemicals including chloramphenicol are also reported (Zuo and Ye 2006; Gao et al. 2009). Recently, a range of fast and sensitive biosensor tests employing antibodies was developed. Among them are a surface plasmon resonance (SPR) biosensor assay (Ferguson et al. 2005; Dumont et al. 2006), quartz crystal microbalance system (Park et al. 2004), and chemiluminescent immunosensor (Park and Kim 2006). The latest achievement is an ultrasensitive SPR biosensor method with limit of detection as low as 0.74 $fg\,mL^{-1}$ in buffer and 17.5 $fg\,mL^{-1}$ in honey (Yuan et al. 2008). Among all variety of the developed methods, only a few ELISA kits and a kit for SPR biosensor system (Biacore®, Sweden) are available commercially.

The purpose of our work was to obtain a range of immunochemical reagents for sensitive and specific determination of CAP and characterize them in direct ELISA. The antibodies were evaluated for their sensitivity in homologous and heterologous assays in buffer. Cross-reactivity of each antibody with CAP derivatives and other phenicols was investigated.

EXPERIMENTAL

Reagents

Horse radish peroxidase (HRP, RZ 3.3) was obtained from Byozyme (Blaenaron, UK). Inorganic salts, methanol, and dimethyl formamide (DMF) were purchased from "Chimmed" (Russia). Ready-to-use substrate solution was from "Immunotek" (Russia). Chloramphenicol, chloramphenicol succinate, chloramphenicol base, and all other reagents were supplied by "Sigma" (St. Louis, MO, USA).

The following buffers were used: PBS – 0.01 M KH_2PO_4-$KHPO_4$ 0.15 M NaCl buffer, (pH 7.4); PBST – PBS containing 0.1% Tween 20 v/v (pH 7.4); CB – 0.01 M sodium carbonate/bicarbonate buffer (pH 9.6). All buffers were prepared with deionized water (Milli-Q).

Milk (3.5% fat content) was bought in local supermarkets and used as CAP-free samples.

CAP standard solutions in the range 0.05–100 $ng\,mL^{-1}$ were prepared by dilution of the stock solution in methanol (1 $mg\,mL^{-1}$) with PBS.

Apparatus

Measurements of optical density for 96-wells microtiter plates were performed on a microtiterplate reader (Molecular Devices, Palo Alto, CA, USA).

Procedures

Synthesis of CAP Succinate-Protein Conjugates. A conjugate of CAP succinate with bovine serum albumin (BSA) (Immunogen 1) was synthesized as described earlier (Kolosova et al. 2000). A conjugate of CAP succinate with keyhole limpet hemocyanin (KLH) (Immunogen 2) was synthesized as follows: 8 mg CAP succinate, 10.3 mg N,N′-dicyclohexylcarbodiimide, and 5.75 mg N-hydroxysuccinimide were dissolved in 500 μL DMF. Then the mixture was stirred for 2 hours at room temperature. To the solution of 30 mg KLH in 6 mL 0.01 M borate buffer (pH = 8.6), 30 μL of activated CAP succinate was added. The mixture was incubated with stirring for 2 hours at room temperature followed by overnight incubation at 4°C. Then, the dialysis against PBS was carried out for 2 days at 4°C.

Synthesis of CAP Succinate-Enzyme Conjugate. The CAP was coupled to HRP by a similar method. The 100 μL of activated CAP succinate was added drop-wise to the chilled solution of 2 mg HRP in 900 μL CB under continuous stirring. The mixture was incubated for 2 hours at room temperature and then purified by gel filtration (Sephadex G-25).

Synthesis of CAP Base-Protein Conjugate (Immunogen 3). The 50 mg BSA, 10 mg N-hydroxysuccinimide, and 20 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were dissolved in 4 mL 0.01 M 2-(N-morpholino)ethane sulphonic acid (pH 4.7) and stirred for 15 min. Then, a solution of 20 mg CAP base in 1 mL DMF was added. The reaction mixture was mixed for 3 hours at room temperature. The conjugate was purified by dialysis against PBS at 4°C.

Production of Polyclonal Antibodies. Chinchilla rabbits (each weighs 2–3 kg) were immunized in a dose of 2 mg per animal (two animals per an immunogen). A mixture (1:1) of an immunogen solution (1 mg mL^{-1} in PBS) and Freund's complete adjuvant was subcutaneously injected into rabbits once a week during a month. Then, rabbits were intravenously injected with 1 mL immunogen solution (1 mg mL^{-1} in PBS) for 3 days. Blood was collected in 7–9 days. Booster injections were repeated every 5–6 weeks. Separated sera were checked for binding with CAP-ovalbumin conjugate by indirect ELISA as described (Kolosova et al. 2000). The IgG fraction of selected sera was precipitated with anhydrous sodium sulphate and then desalted by extensive dialysis against PBS.

Competitive ELISA Procedure. Microtiter plates (Costar, USA) were coated with anti-CAP antibodies (150 μL per well) in CB overnight at 4°C. After washing, (PBST, 3 × 200 μL) CAP standard solutions (50 μL) and CAP-HRP conjugate (50 μL) in PBST were added in duplicates into wells and incubated for 1 hour at 37°C. After washing the substrate solution, (100 μL) was added to each well. The reaction was stopped with 100 μL of 0.2 M H_2SO_4 in 10–15 min. The absorbance was measured at 450 nm. The values of optical densities were normalized relative to the optical density of "zero" standard into %B/Bo values.

Cross-reactivity values were determined by comparing IC_{50} for different compounds as follows:

$$\%\ \text{cross-reactivity} = \frac{IC_{50}\ \text{for CAP}}{IC_{50}\ \text{for cross-reacting compound}} \times 100.$$

The IC_{50} is a concentration of analyte that causes a 50% inhibition of maximum binding (at analyte concentration $0\,\text{ng}\,\text{mL}^{-1}$).

RESULTS AND DISCUSSION

Antibody Sensitivity

Since Hamburger (1966) first reported on chloramphenicol-specific antibodies, a range of polyclonal and monoclonal antibodies were produced against CAP. Polyclonal anti-CAP antibodies were obtained in rabbits, sheep, and such unusual animals as camels, donkeys, and goats (Fodey, Murilla, and Cannavan 2007). Usually, the CAP succinate with a free carboxyl group is used to produce conjugates with proteins (enzymes). This derivative is easily activated and then conjugated with the amino group of a protein. In this case the nitrophenyl and dichloroacetamido groups, which account for a large portion of the immunological reactivity of the hapten (Hamburger and Douglass 1969), are well displayed for antibody recognition. To produce polyclonal antisera we used conjugates of CAP succinate with BSA (Immunogen 1) and KLH (Immunogen 2). Enzyme labeled CAP was also produced from CAP succinate. As an alternative, a conjugate of another CAP derivative—CAP base, merely CAP without dichloroacetamido group—with BSA was used (Immunogen 3). The CAP base was directly coupled to BSA with activated carboxylate groups. In a few early works, a modified CAP base was used for the production of polyclonal (Arnold et al. 1984; Märtlbauer and Terplan 1987) and monoclonal antibodies (van de Water et al. 1987; Hack, Martlbauer, and Terplan 1989). The first CAP base was modified by a bifunctional agent then conjugated to a carrier protein. As a result, the CAP base was conjugated to a protein not directly but via a bridge. Later, CAP base was also used for direct immobilization on a surface of a chip in a SPR biosensor assay (Gaudin and Maris 2001; Dumont et al. 2006; Fodey, Murilla, and Cannavan 2007).

As expected, the big and more immunogenic carrier protein (KLH) helped to produce antisera, not only with higher titers compared to an immunogen with BSA (data not shown), but also with better characteristics. Figure 1 presents calibration curves for CAP determination in direct ELISA using best antisera in optimized conditions. It is well seen that the most sensitive was the homologous ELISA employing antibodies against CAP succinate conjugated with KLH ($IC_{50} = 1.3\,\text{ng}\,\text{mL}^{-1}$, limit of detection [as 90% inhibition] $= 0.04\,\text{ng}\,\text{mL}^{-1}$); and the least sensitive was homologous ELISA using antisera against a conjugate of the same derivative with BSA ($IC_{50} = 4.9\,\text{ng}\,\text{mL}^{-1}$, limit of detection $= 0.2\,\text{ng}\,\text{mL}^{-1}$). At the same time, heterologous ELISA with antibodies against a protein conjugate with CAP base was also

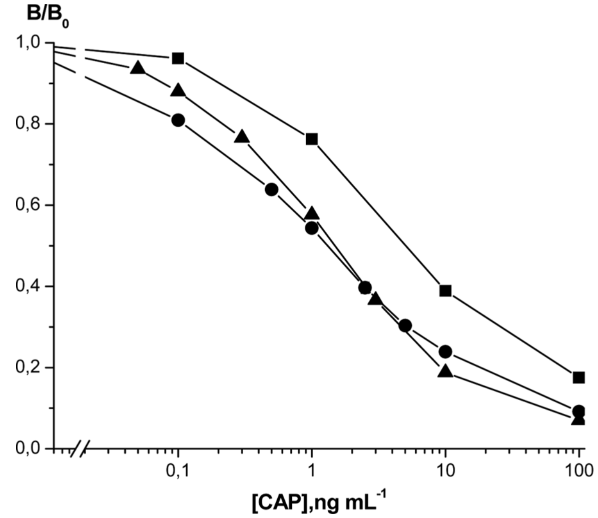


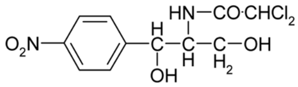
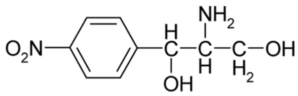
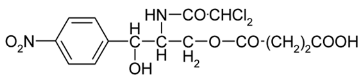
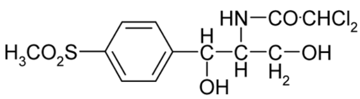
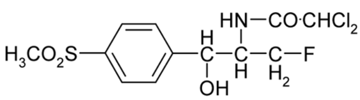
Figure 1. ELISA calibration curves for different polyclonal antibodies. Antibodies were produced against a conjugate: CAP-succinate-BSA (■), CAP succinate-KLH (•), CAP base-BSA (▲).

sensitive ($IC_{50} = 1.5\,ng\,mL^{-1}$, limit of detection $= 0.07\,ng\,mL^{-1}$). To our knowledge this is the first communication of antibodies produced against a conjugate where the CAP base was directly coupled to a protein and used successfully in an assay. Similar immunogen (a conjugate of CAP base with KLH) was briefly mentioned earlier (Freebain, Crosby, and Landon 1988), but the produced antisera did not recognize ^{14}C-labelled CAP in RIA.

Antibody Specificity

The specificity of antisera was assessed in direct ELISA (Table 1). Antisera were specific to CAP and none of them reacted with antibiotics of other groups. Among CAP derivatives, antisera produced against Immunogen 1 and 2 strongly recognized CAP succinate as an initial compound. It is a usual finding for polyclonal and monoclonal antibodies produced against conjugates of CAP succinate with proteins (Gaudin and Maris 2001; Shen et al. 2006; Zhang et al. 2006). Again, antiserum against a conjugate with KLH showed lower cross reactivity towards CAP succinate (251%) compared to antiserum obtained against a conjugate with BSA (1056%). It explains lower sensitivity of ELISA with the latter (Fig. 1). These findings also indicate that this antiserum contains a large portion of antibodies recognizing CAP succinate with part of protein molecule. Both antisera did not recognize other relative compounds where immunologically important nitrophenyl (thiamphenicol and florfenicol) and dichloroacetamido (CAP base) groups are lacking. Some researchers were able to change specificity of similar antisera by employing heterologous competing antigen. Dumont et al. (2006) used florfenicol amine to immobilize on the surface of a SPR biosensor chip. In such a system, florfenicol was recognized at 107%

Table 1. Cross-reactivity values (%) in direct ELISA for polyclonal antibodies produced against different conjugates*

Substance	Immunogen 1 CAP-succinate-BSA	Immunogen 2 CAP succinate-KLH	Immunogen 3 CAP base-BSA
 Chloramphenicol	100	100	100
 Chloramphenicol base	<0.1	<0.1	4.6
 Chloramphenicol succinate	1056	251	11.3
 Thiamphenicol	<0.1	<0.1	<0.1
 Florfenicol	<0.1	<0.1	<0.1

*Cross reactivity values for other antibiotics (ampicillin, gentamicyn, penicillin, streptomicyn, erithromicyn) were less then 0.1%.

and thiamphenicol at 26% (CAP was as 100%). Later Fodey, Murilla, and Cannavan (2007) used enzyme labeled thiamphenicol in ELISA and a chip with immobilized thiamphenicol modified with disuccinimidyl carbonate in a SPR biosensor assay. Cross reactivity values up to 53%/56% for thiamphenicol and 82%/129% for florfenicol in ELISA/biosensor assay, correspondingly, were obtained.

On the contrary, antiserum against a conjugate of a CAP base with BSA (Immunogen 3) showed low cross-reactivity with a CAP succinate (11.3%) and recognized a CAP base to some extent (4.6%) (Table 1). Similar findings were obtained for monoclonal (van de Water et al. 1987; Hack, Martlbauer, and Terplan 1989) and polyclonal (Arnold et al. 1984; Märtlbauer and Terplan 1987) antibodies produced against a conjugate of a modified CAP base with BSA. The monoclonal antibody obtained by van de Water et al. (1987) was the most specific towards CAP (cross-reactivity: CAP base – 0,05%, CAP succinate – 3,13%, p-nitrobenzyl alcohol – 0,02%). On the basis of these data, we came to conclusion that for the production of sensitive anti-CAP antibodies, the CAP base can be directly coupled to free carboxyl groups of a carrier protein in one step without preliminary modification. It seems that for antibody recognition, the presence of an acylamido bond (−NH−CO−) is important but not the length of chemical bridge between the CAP molecule and a protein. However, Märtlbauer and Terplan (1987) modified the CAP base with a bifunctional imidoester (dimethyl adipimidate) and introduced an imidoamide (imidine) bond (−NH−C(NH)−). With this approach, highly specific antibodies were obtained also (cross reactivity towards CAP base 0.5%, thiamphenicol 0.05%).

Assay in Milk

Applicability of characterized immunoreagents was checked in an milk assay. For the assay, we used antiserum produced against Immunogen 2 as it showed the highest sensitivity in the buffer assay. Our preliminary experiments showed that to overcome the matrix influence, milk should be diluted but should not be defatted (results are not shown). To keep the sensitivity of assay as high as possible, milk was diluted 5 times with a buffer and used in ELISA. Analytical characteristics for ELISA in buffer and milk were comparable (Table 2). The detection limit of ELISA in milk was 0.3 ng mL^{-1} (calculated as the CAP concentration equivalent to the mean optical density of 20 milk samples minus two standard deviations). More detailed information about applicability of the immunoreagents obtained for the analysis of CAP residues in food of animal origin (milk, muscle, eggs) will be published elsewhere.

Table 2. Analytical characteristics of ELISA for CAP in buffer and milk (3.5% fat content)

CAP concentration, ng mL^{-1}			
Added	Found	SD	CV, %
Assay in buffer			
Intra-assay (N = 10, P = 0.95):			
0.5	0.53	0.03	5.7
3.0	3.50	0.2	5.7
10	120	0.8	6.7
Inter-assay (N = 3, P = 0.95):			
0.5	0.51	0.07	13.7
3.0	3.40	0.4	11.7
10	11.00	1.9	14.3
Assay in milk			
Intra-assay (N = 10, P = 0.95):			
0.5	0.49	0.04	8.2
3.0	2.60	0.1	3.8
10	7.80	0.2	2.6
Inter-assay (N = 3, P = 0.95):			
0.5	0.47	0.06	12.8
3.0	2.80	0.4	14.3
10	8.10	0.5	6.2

CONCLUSIONS

Sensitive ELISAs were developed employing antibodies obtained against a conjugate of a CAP succinate with a big carrier protein KLH and a conjugate of a CAP base with BSA. The antibodies produced against ''CAP base-BSA'' conjugate were very specific to CAP in an heterologous assay. On the basis of the reagents obtained, CAP residues in milk can be determined at the MRPL level.

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