COMMERCIAL BENZIMIDAZOLE DERIVATIVES AS IMMUNIZING HAPTENS FOR THE DEVELOPMENT OF A POLYCLONAL ANTIBODY RECOGNIZING THE FUNGICIDE CARBENDAZIM

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EXTENDED ABSTRACT

Carbendazim [methyl 2-benzimidazole carbamate] is a fungicide widely used for controlling a broad range of fungi affecting fruits, vegetables, field crops etc. Determination of carbendazim in water, soil and various crops -especially cereals- is frequently required to assure compliance with national/European laws and regulations. An anti-carbendazim polyclonal antibody was developed against a combination of three commercially available benzimidazole derivatives, namely 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid and 2-mercaptopbenzimidazole; this considerably accelerated the antibody development procedure and reduced cost. The above derivatives were conjugated to keyhole limpet hemocyanin, following well-established conjugation chemistry protocols, and a mixture of the conjugates was administered to New Zealand white rabbits; the IgG fraction of the antisera collected was isolated and its purity was verified with SDS-PAGE. Immunochemical functionality of the antisera and the isolated antibody was evaluated with in-house developed titer- and displacement ELISAs. In these ELISA systems, the commercially available chemical 2-mercaptobenzimidazole was coupled through 3-maleimidopropionic acid to an in-house synthesized lysine-dendrimer, prepared as previously described by our team (Papasaratostos et al., 2010), and was finally used as the immobilized hapten. The antisera ELISA-titer values were in all cases higher than 1:10,000. ELISA-displacement curves were obtained in the presence of carbendazim at various concentrations (1 - 20 μg/mL). As shown by ELISA-displacement experiments -and probably due to the variety of the immunizing haptens used for its development, the anti-carbendazim antibody developed was capable of recognizing not only carbendazim, but also the fungicide benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate] as well as the benzimidazole molecule and various benzimidazole derivatives. Considering its highly promising immunochemical profile, especially its broad specificity, the anti-carbendazim antibody reported here may be used in the development of immunoassay systems for analyzing samples of environmental interest. The above antibody is being currently tested as a specific biorecognition element in a cell biosensor platform for pesticide residue detection based on the Bioelectric Recognition Assay (BERA) technology, which has been developed in the framework of the EU Capacities Project FOODSCAN.

Keywords: Benzimidazole; carbendazim; fungicides; polyclonal antibody; ELISA; biosensors
1. INTRODUCTION

Carbendazim [methyl 2-benzimidazole carbamate] is applied to a wide range of cereals, fruits, vegetables, field crops etc. as a broad spectrum benzimidazole fungicide (Davidse, 1986), being one of the most commonly used pesticides in modern agriculture. Despite their great contribution to the improvement of crop production yields, pesticides - including benzimidazole fungicides - have been recognized as being possibly hazardous to human health (McCarroll et al., 2002). Analytical methodologies that enable fast and accurate determination of pesticide levels in water, soil and various food commodities are therefore needed and frequently required to assure compliance with national/European legislation. Instrumental analysis including LC-MS has been used for the determination of carbendazim in various samples. As an alternative, immunoenalytical methodology (Chan et al., 2008) has also been used, due to rather low cost, simple assay protocols and high sample throughputs. A challenging step in pesticide immunoanalysis is the development of specific antibodies, since most pesticides are small molecules (haptens) that should be conjugated through a suitable, active chemical group to a carrier protein in order to elicit an immune response (Singh et al., 2004). If the parental pesticide does not contain any suitable active groups in its molecule, then special derivatives should be synthesized, following often laborious and skill-demanding organic chemistry protocols; these special synthetic derivatives should be then coupled to the carrier protein.

In the present work we describe the development of a polyclonal antibody for carbendazim by using a combination of commercially available benzimidazole derivatives as immunizing haptens, thus avoiding the need for extended hapten derivative synthesis.

2. MATERIALS AND METHODS

2.1 Immunizing Haptens

Commerially available benzimidazole derivatives, namely 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid and 2-mercaptopbenzimidazole, all products of Sigma-Aldrich, were used as immunizing haptens. The aforementioned reagents (stock solutions in H$_2$O, DMSO, and 0.1 M HCl$_{aq}$/ethanol, respectively) were conjugated to the carrier protein keyhole limpet hemocyanin (KLH, product of Thermo Scientific), through glutaraldehyde (product of Sigma-Aldrich), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC, product of Sigma-Aldrich) and sulfo-succinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sSMCC, product of Thermo Scientific), respectively, following well-established conjugation chemistry protocols (Avrameas & Ternynck, 1969; Mattson et al., 1993; Singh et al., 2004).

2.2 Immunization of Rabbits

New Zealand white rabbits (2-month old) were immunized with a mixture of the KLH-conjugates of the aforementioned benzimidazole derivatives. The mixture of the KLH-conjugates had been emulsified with an equal volume of Complete Freund’s Adjuvant and then administered subcutaneously to the host-animals, according to the method of Vaitukaitis (Vaitukaitis, 1981). The animals were boosted initially 6 weeks after first exposure and subsequently every 4 weeks. Incomplete Freund’s Adjuvant was used in boosting immunizations. Blood was collected 2 weeks after each booster injection. Antisera were obtained with low speed centrifugation of whole blood. Care of animals was in accordance to the corresponding European legislation.

2.3 Isolation of antibody (IgG-fraction) from the antisera

Isolation of antibody (IgG fraction) from the antisera was accomplished through sequential precipitation with caprylic acid and ammonium sulphate, as previously described (Perosa et al., 1990) with slight modifications. Briefly, 1 mL of antisera was added to 3 mL of 60 mM acetate buffer and the pH adjusted to 4.5 with 1 N NaOH. One
hundred μL of caprylic acid was then added drop-wise while stirring at room temperature. After 30 min of stirring at room temperature, the mixture was centrifuged for 45 min at 10,000 x g at room temperature. The supernatant was then harvested, filtered through a 0.45 μm filter to remove fine precipitates and the pH was adjusted to 7.4 with 1 N NaOH. The sample was then cooled on ice and, while stirring vigorously, 1.1 g of ammonium sulfate was added to it very slowly. After stirring at 0 °C for 30 min, the mixture was centrifuged (10,000 x g, 30 min, 4 °C) and the precipitated IgGs were resuspended in 0.01 M phosphate-buffered saline (PBS), pH 7.4. The IgGs were dialysed for 72 h against 0.01 M PBS, pH 7.4. IgG purity was tested with sodium dodecyl sulphate polyacrylamide gel electrophoresis (Chevalier, 2010). Protein concentration was measured using the BCA method (Sorensen & Brodbbeck, 1986).

2.4 ELISA evaluation
The ELISA-titer and ELISA-displacement systems were developed in-house and used for the evaluation of both the antisera and the isolated antibodies.

2.4.1 ELISA Coating:
One of the commercially available benzimidazole derivatives used as immunizing haptns, i.e. 2-mercaptobenzimidazole, was also used as the immobilized hapten in the ELISA systems. More specifically, 2-mercaptopbenzimidazole was dissolved in dimethylformamide and coupled, through 3-maleimidopropionic acid, to an in-house synthesized lysine-dendrimer by following a protocol based on solid-phase peptide synthesis (Amblard et al., 2006), properly modified. The lysine-dendrimer was synthesized with solid phase chemistry as well, as previously described by our team (Papasarantos et al., 2010).

2.4.2 ELISA Buffers:
Coating buffer: 0.01 M phosphate buffer (PB), pH 7.4; Washing buffer (PBS-T): 0.01 M PBS, pH 7.4, containing 0.05 % (v/v) Tween-20; Diluting Buffer 1: PBS-T containing 0.2 % (w/v) bovine serum albumin (BSA) and 5 % (v/v) ethanol; Diluting Buffer 2: PBS-T containing 0.2 % (w/v) BSA; Diluting Buffer 3: PBS-T containing 0.2 % (w/v) BSA and 10 % (v/v) ethanol;

2.4.3. ELISA Titration experiments:
ELISA microtiter plates were coated with the above described benzimidazole-bearing lysine-dendrimer (1 μg/mL in coating buffer, 100 μL/well, overnight, 37 °C). The following day, the solution was discarded, and the wells were washed once with 0.01 M PB, pH 7.4 (250 μL/well). Blocking was performed with a 2% BSA solution in PBS-T (200 μL/well, 1 h, room temperature). After blocking, the solution was discarded, the wells were washed three times with PBS-T and then incubated with serial dilutions of the anti-carbendazim antisera (or the corresponding antibody solution) in diluting buffer 1 (100 μL/well, 2 h, 37°C). After incubation, the solution was discarded, the wells were washed three times with PBS-T and then incubated with a commercially available anti-rabbit IgG coupled to horseradish peroxidase (anti-rabbit IgG/HRP, product of Sigma-Aldrich), at 1:3,000 dilution in diluting buffer 2 (100 μL/well, 2 h, 37 °C). Afterwards, the solution was discarded, the wells were washed three times with PBS-T and finally incubated with an ABTS (1 mg/mL)/H₂O₂ (0.003%) solution in 0.1 M citrate/phosphate buffer, pH 4.5 (100 μL/well, 30 min, 37 °C). After color development, the OD was measured (405 nm) in a microtiter plate reader (Sirio S, SEAK) and the corresponding titer curves were plotted.

2.4.4. ELISA Displacement experiments:
ELISA microtiter plates were coated, washed, blocked and washed again as described above. Then, the wells were incubated (2 h, 37 °C) with 50 μL of an anti-carbendazim antibody solution, suitably diluted in diluting buffer 2, and 50 μL of a series of standard
solutions (1 - 20 µg/mL) of the substances to be tested (carbendazim, benomyl, benzimidazole, 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid, 2-mercaptopbenzimidazole), which had been preincubated (18 h, 4 °C) before added to the wells. All standard solutions were prepared by diluting a 10 mg/mL stock solution, in 0.1 N HCl/aq/ethanol (carbendazim, benomyl, 2-mercaptopbenzimidazole), H2O (2-(2-aminoethyl) benzimidazole), or 0.01 M PB, pH 7.4 (2-benzimidazole propionic acid), with diluting buffer 3. Afterward, the procedure described in the previous paragraph was followed. Finally, the OD (405 nm) was measured and the displacement curves were plotted.

3 RESULTS

In this work we present the development of a polyclonal antibody for carbendazim, by using a combination of commercially available benzimidazole derivatives as immunizing haptons. Immunochemical functionality of the antisera (four consecutive bleedings) and the corresponding isolated antibody was evaluated with in-house developed titer- and displacement ELISAs. As shown by the ELISA titer experiments, the antisera developed were able to recognize the immobilized hapten in the ELISA-system. The ELISA-titer values of all antisera (four consecutive bleedings) were higher than 1:10,000. The ELISA titer-curves for the IgG-antibodies isolated from the antisera of 2nd, 3rd and 4th bleedings are shown in Figure 1.

![ELISA Titer curves - Isolated IgG Antibodies](image)

**Figure 1.** ELISA-titer curves for the IgG-antibodies isolated from the antisera of 2nd, 3rd and 4th bleedings.

As shown by the results of the ELISA-displacement experiments (Figure 2), the antibodies developed against the commercially available benzimidazole derivatives were able to recognize carbendazim in standard solutions (1 - 20 µg/mL). It was also revealed that the antibodies were able to recognize the fungicide benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate], as well as the benzimidazole-core molecule and the other benzimidazole derivatives tested (Table 1).
<table>
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<tr>
<th>Chemical Structure</th>
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<td>2-benzimidazole propionic acid</td>
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*Table 1. Chemical structures of carbendazim, benomyl, benzimidazole and benzimidazole derivatives used in the ELISA-displacement experiments*
4 CONCLUSIONS - FUTURE PERSPECTIVES

The development of a polyclonal antibody for carbendazim by using a combination of commercially available benzimidazole derivatives as immunizing haptens is presented in this work. By using commercially available benzimidazole derivatives, the synthesis and purification of new carbendazim derivatives for use as immunizing haptens were avoided; this approach considerably accelerated the antibody development procedure and reduced the overall cost.

From ELISA-displacement experiments, it was found that the antibody developed was able to recognize carbendazim in standard solutions (1 - 20 µg/mL). Moreover, the antibody was also able to recognize the fungicide benomyl, as well as the intact benzimidazole molecule.

Due to the variety of the immunizing haptens used for its development, the anti-carbendazim antibody may recognize not only carbendazim and benomyl, but other benzimidazole-bearing molecules, as well; therefore, cross-reactivity with various bioactive benzimidazole carbamates will be thoroughly studied.

Considering its highly promising immunochemical profile, and especially its potential ability to recognize several bioactive substances bearing the benzimidazole group, the anti-carbendazim antibody reported here may be a useful tool in the development of immunoassay systems for analysing samples of environmental interest. Detection limits of these assays may be considerably improved by optimizing the assay format, the detection system, etc. The above antibody is being currently tested as a specific biorecognition element in a cell biosensor platform for pesticide residue detection based on the Bioelectric Recognition Assay (BERA) technology, which has been developed in the framework of the EU Capacities Project FOODSCAN, with promising results.

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REFERENCES