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Commercially available chemicals as immunizing haptens for the development of a polyclonal antibody recognizing carbendazim and other benzimidazole-type fungicides

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ABSTRACT

Carbendazim is a fungicide widely used for controlling fungi affecting fruits, vegetables, field crops etc. Determination of carbendazim in water, soil and various crops is frequently required to assure compliance with national/European regulations. A polyclonal antibody recognizing carbendazim was developed by using commercially available 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid and 2-mercaptobenzimidazole as immunizing haptens; each of the above derivatives was directly conjugated to the carrier protein keyhole limpet hemocyanin and a mixture of the conjugates was administered to New Zealand white rabbits. Immunochemical functionality of the antisera and the corresponding isolated antibody (whole IgG fraction) was evaluated through titer and displacement curves in an in-house developed ELISA, which employed a 2-mercaptobenzimidazole – functionalized lysine-dendrimer as the immobilized hapten. As shown with ELISA-displacement curves, the above antibody could recognize carbendazim as well as other benzimidazole-type fungicides, i.e. benomyl and thiabendazole, and also intact benzimidazole, while it did not cross-react with the structurally different pesticides carbaryl and imazalil. Considering the rather simple approach which has led to its development and its highly promising immunochemical profile, the new antibody may be exploited in immunoanalytical systems for detecting benzimidazole-type pesticides e.g. in samples of environmental interest. The above antibody is being currently tested as a biorecognition element in the novel FOODSCAN cell biosensor platform for pesticide residue detection based on the Bioelectric Recognition Assay technology.

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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). Maximum residue limits (MRLs) have been established in the EU for benzimidazole residues in food commodities, in order to protect public health according to Commission Regulation 2010/37/EC, while annual surveillance

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BCA, bicinchoninic acid; BERA, bioelectric recognition assay; BSA, bovine serum albumin; DCM, dichloromethane; DIC, N,N′-diisopropyl carbodiimide; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; eq, equivalent; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IgG, γ-immunoglobulin; KLH, keyhole limpet hemocyanin; LC–MS, liquid chromatography–mass spectrometry; MRL, maximum residue limit; OD, optical density; oxyma, ethyl 2-cyano-2-(hydroxyimino)acetate; PB, phosphate buffer; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sSMCC, sulfo-succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate; TFA, trifluoroacetic acid.

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programs are carried out in member states under Council Directive 96/23/EC (Keegan et al., 2011). 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 (Hiemstra and deKok, 2007). As an alternative, assays based on the immunoanalytical methodology have also been developed and used (Gough et al., 2010), due to rather low cost, simple assay protocols and a high sample throughput capacity (Chan et al., 2008).

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 (Erlanger, 1980; 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 a rather simple experimental approach to develop a polyclonal antibody for carbendazim; more specifically, we employed a combination of commercially available benzimidazole derivatives as immunizing haptens, thus avoiding the need for extended synthesis of special carbendazim-hapten derivatives. Each of the above derivatives was directly conjugated to the carrier protein KLH and a mixture of the protein-conjugates was administered to New Zealand white rabbits for antibody development. As shown experimentally, the new antibody could recognize other benzimidazole-type fungicides as well.

2. Materials and methods

2.1. Immunizing haptens

Commercially available benzimidazole derivatives, namely 2-(2-aminoethyl) benzimidazole, 2-benzimidazole propionic acid and 2-mercaptobenzimidazole, all products of Sigma–Aldrich, were used as immunizing haptens. The aforementioned reagents (stock solutions in H_2O, DMSO, and 0.1 M $HCl_{(aq)}$/ethanol, respectively) were conjugated to the carrier protein KLH (product of Thermo Scientific), through glutaraldehyde (product of Sigma–Aldrich), EDC (product of Sigma–Aldrich) and sSMCC (product of Thermo Scientific), respectively, following well-established conjugation chemistry protocols (Avrameas and Ternynck, 1969; Mattson et al., 1993; Singh et al., 2004).

2.2. Immunization of rabbits

New Zealand white rabbits (two 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 (Papasarantos et al., 2010) and then administered subcutaneously to the host-animals, according to the method of Vaitukaitis (1981). The animals were boosted initially six weeks after first exposure and subsequently every four weeks. Incomplete Freund's Adjuvant was used in boosting immunizations (Harlow and Lane, 1988). Blood was collected two weeks after each booster injection. Antisera were obtained with low speed centrifugation of whole blood.

Care of animals was provided in accordance to the corresponding European legislation.

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

Isolation of the antibody (whole IgG-antibodies fraction) from the antisera was accomplished through sequential precipitation with caprylic acid and ammonium sulfate, as previously described (Perosa et al., 1990) with slight modifications. Briefly, 1 mL of antiserum 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×*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×*g*, 30 min, 4 °C) and the precipitated IgG-antibodies were resuspended in 0.01 M PBS, pH 7.4. The IgG-antibodies were finally dialysed for 72 h against 0.01 M PBS, pH 7.4. IgG purity was tested with SDS–PAGE (Chevalier, 2010). Protein concentration was measured using the BCA method (Sorensen and Brodbeck, 1986).

2.4. ELISA evaluation

2.4.1. ELISA coating

2-Mercaptobenzimidazole coupled to a suitable lysine-dendrimer, which had been pre-functionalized with 3-maleimidopropionic acid (Ogawa and Taneda, 1979; Bayer et al., 1985), was employed as the immobilized hapten in the in-house developed ELISA system. The lysine-dendrimer was prepared with a solid phase peptide synthesis protocol (Amblard et al., 2006), appropriately modified as previously described by our team (Papasarantos et al., 2010).

2.4.2. ELISA buffers

Coating buffer: 0.01 M 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) 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^{-1} in coating buffer, 100 μL per well, overnight, 37 °C). The following day, the liquid was discarded and the wells were washed once with 0.01 M PB, pH 7.4 (250 μL per well). Blocking was performed with a 2% BSA solution in PBS-T (200 μL per well, 1 h, room temperature). After blocking, the liquid was discarded, the wells were washed three times with PBS-T and then incubated (100 μL per well, 2 h, 37 °C) with serial dilutions (1:1000–1:50,000) of the antiserum under evaluation or with increasing concentrations of the corresponding antibody solution (0.01–20 μg mL^{-1}) in diluting buffer 1. After incubation, the liquid was discarded, the wells were washed three times with PBS-T and then incubated (100 μL per well, 2 h, 37 °C) with a commercially available anti-rabbit IgG coupled to horseradish peroxidase (anti-rabbit IgG/HRP, product of Sigma–Aldrich), at 1:3000 dilution in diluting buffer 2. Afterwards, the liquid was discarded, the wells were washed three times with PBS-T and finally incubated (100 μL per well, 30 min, 37 °C) with an ABTS (product of Sigma–Aldrich, 1 mg mL^{-1})/H_2O_2 (0.003%) solution in 0.1 M citrate/phosphate buffer, pH 4.5. After color development, the OD was measured (405 nm) in a microtiter plate reader (Sirio S, SEAK) and the corresponding titer curves were plotted.

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