Food Control 26 (2012) 162–168

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Development of monoclonal antibody-based competitive immunoassays for the detection of picoxystrobin in cereal and oilseed flours

Josep V. Mercader [a,1], Javier Parra [a,1], Francesc A. Esteve-Turrillas [a], Consuelo Agulló [b], Antonio Abad-Somovilla [b], Antonio Abad-Fuentes [a,*]

[a] Department of Biotechnology, Institute of Agrochemistry and Food Technology (IATA–CSIC), Agustí Escardino 7, 46980 Paterna, València, Spain
[b] Department of Organic Chemistry, Universitat de València, Doctor Moliner 50, 46100 Burjassot, València, Spain

ARTICLE INFO

Article history:
Received 6 September 2011
Received in revised form
4 January 2012
Accepted 6 January 2012

Keywords:
ELISA
Hapten
Spacer arm
Strobilurins
Pesticide residues
Food safety

ABSTRACT

Picoxystrobin is a new generation fungicide primarily developed to be used in cereal crops. In the present study a novel collection of specific monoclonal antibodies has been produced using different immunizing haptens based on a carboxy-functionalized identical spacer arm attached to alternative positions of the target pesticide molecule. Two competitive enzyme-linked immunosorbent assays have been developed employing hapten heterology, one using the antibody-coated direct format and the other in the conjugate-coated indirect format. Both immunoassays have been characterized in terms of selectivity, solvent tolerance, and buffer conditions, affording similar limits of detection at or below 0.1 μg/L. Finally, the optimized assays were applied to the analysis of picoxystrobin in wheat, corn, oat, barley, and soybean flours. Average recovery values from spiked samples were between 84 and 115%.

1. Introduction

The intensive and occasionally abusive use of agrochemicals for crop yield improvement often leads to the presence of residual amounts of pesticides in cereals, possibly affecting the quality and safety of the final food product (Dornez et al., 2008; Ruske, Gooding, & Dobraszczyk, 2004). Picoxystrobin (PC) is a new broad-spectrum fungicide that belongs to the strobilurin family of pesticides and whose biological activity derives from the same β-methoxyacrylatetoxophore group found in the natural active principle (strobilurin A) produced by the fungus *Strobilurus tenacellus* (Clough, 1993). The mechanism of action of PC is the inhibition of mitochondrial respiration by binding to the Q_0 site of cytochrome b, thus blocking electron transport between cytochrome b and cytochrome c_1 which eventually leads to disruption of the energy cycle (Bartlett et al., 2002). PC is effective against highly destructive pests in cereal crops such as *Septoria tritici*, *Leptosphaeria nodorum*, yellow rust, brown rust, ear diseases, and eyespot in wheat crops; net blotch, brown rust, powdery mildew, and *Rhychosporium* in barley crops; crown rust and powdery mildew in oat crops; and sclerotinia in oilseed rape (DuPont Global Website, 2011). This strobilurin fungicide shows preventive and curative properties, and it is currently formulated and commercialized under different trademarks by DuPont and Syngenta for cereal and oilseed crop protection (Balba, 2007; Bartlett et al., 2001).

According to EC regulation 396/2005, the maximum residue limit (MRL) for PC in most cereals and oilseeds is 50 μg/kg, whereas a specific MRL of 200 μg/kg has been established for barley and oat (European Commission, 2005). At present, diverse analytical methodologies are available for the analysis of PC residues in foodstuffs, most of which are based on liquid or gas chromatography coupled to mass spectrometry detectors. Pesticide extraction is usually carried out with different organic solvents such as acetone (Hiemstra & de Kok, 2007), ethyl acetate (Schurek et al., 2008; Taylor, Keenan, Reid, & Uría-Fernández, 2008), or acetonitrile (Walorczyk & Gnusowski, 2009). In some cases, extraction was achieved by ultrasonic treatment combined with different clean-up approaches (Bo, Wang, Guo, Qin, & Lu, 2008; Campillo, Viñas, Aguinaga, Férez, & Hernández-Córdoba, 2010; Viñas, Martínez-Castillo, Campillo, & Hernández-Córdoba, 2010), whereas the

Abbreviations: BSA, bovine serum albumin; cELISA, competitive enzyme-linked immunosorbent assay; CR, cross-reactivity; d-cELISA, direct cELISA; DMF, *N*,*N*-dimethylformamide; HRP, horseradish peroxidase; i-cELISA, indirect cELISA; LOD, limit of detection; mAb, monoclonal antibody; MRL, maximum residue limit; OVA, ovalbumin; PC, picoxystrobin; RAM–HRP, polyclonal rabbit anti-mouse immunoglobulins conjugated to peroxidase.

* Corresponding author. Tel.: +34 963900022; fax: +34 963636301.
E-mail address: aabad@iata.csic.es (A. Abad-Fuentes).

[1] Both authors contributed equally to this article.

0956-7135/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodcont.2012.01.021

application of solid-phase microextraction resulted in a substantial improvement in sensitivity for the determination of PC in baby foods (Viñas, Campillo, Martínez-Castillo, & Hernández-Córdoba, 2009).

As complementary analytical tools to chromatographic methods, a large number of studies based on enzyme-linked immunosorbent assay (ELISA) technology have been published for the determination of a wide variety of agrochemical residues in diverse food commodities (Morozova, Levashova, & Eremin, 2005; Van Emon, Chuang, Dill, & Xiong, 2008). Immunochemical methods are simple, rapid, and sensitive. In addition, intricate sample treatments are often not required, so direct dilution in an aqueous solution usually suffices for the analysis of liquid samples or for extracts of solid samples. Thus, ELISAs constitute not only a very useful routine laboratory approach where a large number of repetitive analyses need to be performed, but also a means for screening for the presence of specific compounds in a considerable assortment of samples. Moreover, since immunoassays can be implemented in many different formats, they can be adapted to a great variety of analytical circumstances. Nevertheless, the technique requires high-quality immunoreagents together with extensive and thorough characterization and validation studies.

Whilst ELISA is a well-established methodology for the detection and analysis of certain chemical contaminants in cereals such as mycotoxins (Goryacheva, Rusanova, Burmistrova, & De Saeger, 2009; Skerritt, 1998), few studies have been published for the determination of fungicide residues by enzyme immunoassay in those complex food matrices (Danks, Chaudhry, Parker, Barker, & Banks, 2001; Jiang, Shi, Wu, & Wang, 2011). In previous publications, we described the syntheses of three PC derivatives functionalized with the same linker at rationally-selected sites of the target molecule (Parra et al., 2011) and the application of selected immunoassays to the analysis of PC residues in beer (Esteve-Turrillas et al., 2010). In the present study, novel monoclonal antibodies (mAb) to PC have been raised using regioisomeric PC haptens as immunogens. The affinity-purified antibodies were characterized in two competitive ELISA (cELISA) formats, the antibody-coated direct (d-cELISA) and the conjugate-coated indirect (i-cELISA) assays. In order to improve the limit of detection (LOD) of the selected immunoassays, the three available PC haptens were also evaluated as antigens in heterologous assays; i.e., assays employing conjugates carrying a different hapten to that employed for the generation of the antibody. A direct and an indirect cELISA were characterized and optimized for solvent tolerance and buffer conditions. Finally, both immunoassays were validated for the determination of PC residues present in different cereal and oilseed flours at concentration levels in accordance with the European MRLs for those foodstuffs.

2. Materials and methods

2.1. Reagents and instrumentation

Analytical-grade PC (methyl (E)-3-methoxy-2-{2-[6-(trifluoromethyl)-2-pyridyloxy methyl]phenyl}acrylate, CAS Registry No. 117428-22-5, MW 367.32 g/mol) and other employed pesticides were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). All pesticide standards were prepared as concentrated solutions in *N*,*N*-dimethylformamide (DMF) and were kept at −20 °C in amber glass vials. Sephadex G-25 HiTrap Desalting columns and HiTrap Protein G HP columns from GE Healthcare (Uppsala, Sweden) were used for conjugate and antibody purification, respectively. Polyclonal rabbit anti-mouse immunoglobulins conjugated to peroxidase (RAM–HRP) was from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) fraction V and Hybridoma Fusion and Cloning Supplement were purchased from Roche Applied Science (Mannheim, Germany). HT (hypoxanthine–thymidine) and HAT (hypoxanthine–aminopterin–thymidine) supplements and gentamicine solution were obtained from Gibco BRL (Paisley, Scotland). Horseradish peroxidase (HRP), ovalbumin (OVA), *o*-phenylenediamine, cell culture media, fetal bovine serum, polyethylene glycol, amino acid solutions, Red Blood Cell Lysing Buffer Hybri-Max, and Freund's adjuvants were from Sigma–Aldrich (Madrid, Spain). P3-X63-Ag-8.653 mouse plasmacytoma cell line was from the European Collection of Cell Cultures (Wiltshire, UK). Culture plastic ware and Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY). ELISA plates were washed with an ELx405 microplate washer from BioTek Instruments (Winooski, VT) and the absorbance values were read in dual wavelength mode (492–650 nm) with a PowerWave HT device, also from BioTek Instruments.

Composition, concentration, and pH of the employed buffers were: (i) PB, 100 mM sodium phosphate buffer, pH 7.4; (ii) PBS, 10 mM sodium phosphate buffer, pH 7.4, with 140 mM NaCl; (iii) PBST, PBS containing 0.05% (v/v) Tween 20; (iv) 2× PBST, 20 mM sodium phosphate, pH 7.4, with 280 mM NaCl and 0.05% (v/v) Tween 20; (v) CB, 50 mM sodium carbonate–bicarbonate buffer, pH 9.6; (vi) Washing solution, 150 mM NaCl and 0.05% (v/v) Tween 20; (vii) Enzyme substrate buffer, 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4; and (viii) CitBT, 100 mM citrate buffer, pH 6.0, with 32 mM NaCl and 0.05% (v/v) Tween 20.

2.2. Protein–hapten conjugates

Three PC haptens (PCa6, PCb6, and PCo6) with the same linker at three alternative tethering sites were employed in the present study (Fig. 1). Those regioisomeric haptens contained a functional carboxylate group which was activated for coupling to the free amine groups of the carrier proteins. The synthesis of the PC derivatives and the preparation of the different conjugates were previously described (Esteve-Turrillas et al., 2010; Parra et al., 2011). BSA was used for immunogen preparation, OVA for conjugate-coated indirect assays, and HRP as enzyme tracer in d-cELISAs.

2.3. Monoclonal antibody production

Animal manipulation was performed according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were immunized with BSA–PCb6 and BSA–PCo6 conjugates following equivalent procedures to those used in a previous study where the immunogen was BSA–PCa6 (Esteve-Turrillas et al., 2010). Briefly, sets of four mice each received three 200 μL intraperitoneal injections at three week intervals. Injections consisted of a 1:1 emulsion of a 1 mg/mL conjugate solution in PBS and Freund's adjuvant (complete for the first dose and incomplete for subsequent ones). After a resting period of at least 3 weeks from the last injection with adjuvant and four days before cell fusion, a booster injection in PBS was administered. For hybridoma production, murine myeloma cells and spleenocytes from immunized mice were fused using polyethylene glycol 1500. Next, hybrid cells were discriminated by employing aminopterin-containing supplement. Then, they were cloned by limiting dilution and cultured following published protocols (Mercader & Abad-Fuentes, 2009).

After cell fusion, a sequential double-screening strategy was applied in order to identify hybridomas producing high-quality