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Development of an Immunosensor Based on Surface Plasmon Resonance for Simultaneous Residue Analysis of Three Pesticides —Boscalid, Clothianidin, and Nitenpyram— in Vegetables

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A simultaneous immunosensor based on surface plasmon resonance (SPR) was developed for determination of 3 pesticides —boscalid, clothianidin and nitenpyram— instead of the direct competitive enzyme-linked immunosorbent assays (dcELISAs) widely used as individual determination methods. Carboxy groups that introduced compounds to their pesticides were designed, and conjugates of them and bovine serum albumin were immobilized onto separate channels of the same sensor chip. When a mixture of 3 monoclonal antibodies reacted to each pesticide, and 3 pesticides were injected into the SPR immunosensor, each channel showed specific reactivity at 15 – 93 ng mL^{-1} for boscalid, 6.7 – 27 ng mL^{-1} for clothianidin, and 7.3 – 62 ng mL^{-1} for nitenpyram. Recovery tests using vegetables spiked with a mixture of 3 pesticides showed good results: 75 – 90%, 88 – 104%, and 72 – 105%, respectively, with a high correlation to results of the dcELISAs. The SPR immunosensor would be useful for the determination of pesticide residues in vegetables.

Keywords Insecticide, fungicide, sensor, antibody, immunoassay

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Introduction

Boscalid, 2-chloro-*N*-(4′-chlorobiphenyl-2-yl) nicotinamide, is a carboxamide fungicide that was introduced in 2002.[1] Both clothianidin [(*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine] and nitenpyram [(*E*)-*N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N*′-methyl-2-nitrovinylidenediamine] are neonicotinoid insecticides introduced in 2002 and 1995, respectively.[2,3] Clothianidin is a modified derivative of nitenpyram, although they have different ring structures; clothianidin has a chlorothiazol ring, whereas nitenpyram has a chloropyridine ring, as shown in Fig. 1.[4] Fungicides and insecticides are often applied simultaneously to agricultural fields when the plants are put to risks of fungal disease and insect damage, especially in hot and humid seasons. The combination of boscalid and clothianidin or nitenpyram is also applied widely to prevent fungal diseases and insect pests. It is important to monitor their residues simultaneously in agricultural products. The maximum residue limits (MRLs) in vegetables have been set to 1 – 40 mg kg^{-1} for boscalid, 0.2 – 40 mg kg^{-1} for clothianidin, and 0.5 – 5 mg kg^{-1} for nitenpyram in Japan, including some exceptional vegetables for which the ranges are lower.

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Generally, boscalid is determined by gas or liquid chromatography by mass spectrometry (LC-MS).[5-8] Clothianidin and/or nitenpyram are determined by high-performance liquid chromatography (HPLC) with diode-array detection or by LC-MS.[9-14] Boscalid and clothianidin can be determined simultaneously by multi-residue analysis using LC-MS.[15-17] These instrumental analyses are sensitive and accurate; however, these technologies are sophisticated, labor-intensive, and time-consuming.

Direct competitive enzyme-linked immunosorbent assays (dcELISAs) were developed for the monitoring of pesticide residues in vegetables as more simple and rapid methods compared to the above chromatography techniques, but of the single pesticide although simultaneous analysis is required in the field.[18-20] The development of simultaneous dcELISA for three pesticides was described previously.[21] Its reactivity was specific with each pesticide, but was difficult to put into practical use because optimization of the assay condition was complicated.

Immunosensors based on electrochemistry have been developed to determine pesticides in foods.[22-24] Immunosensors based on surface plasmon resonance (SPR immunosensors) have also been developed to determine pesticides.[20,25-27] Since SPR immunosensors especially enable real-time monitoring of the antigen-antibody interaction in contrast to the above dcELISAs and electrochemical immunosensors, they are expected to be applicable to pesticide residue analysis in fresh vegetables that will be quickly distributed. However, there have been no reports of any SPR immunosensor for the simultaneous

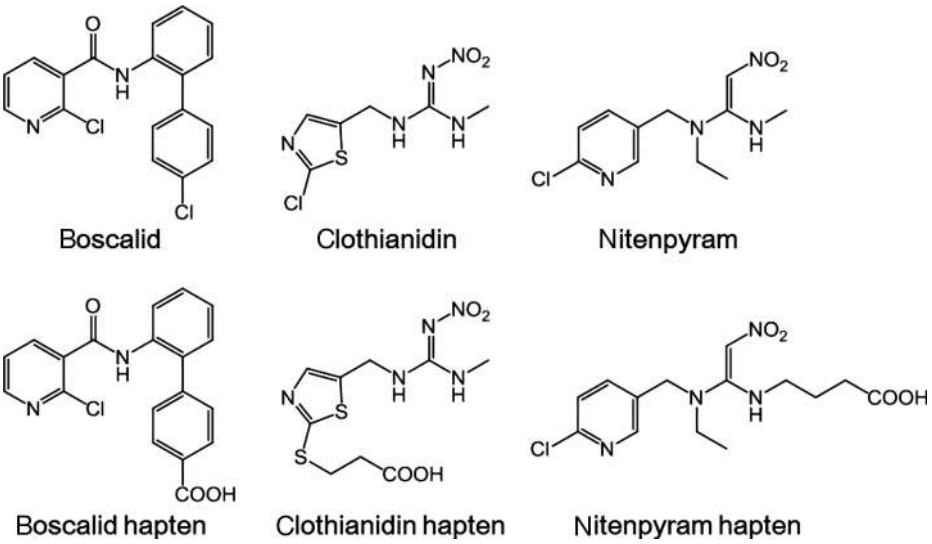

Fig. 1 Structure of boscalid, clothianidin, nitenpyram, and their haptens.

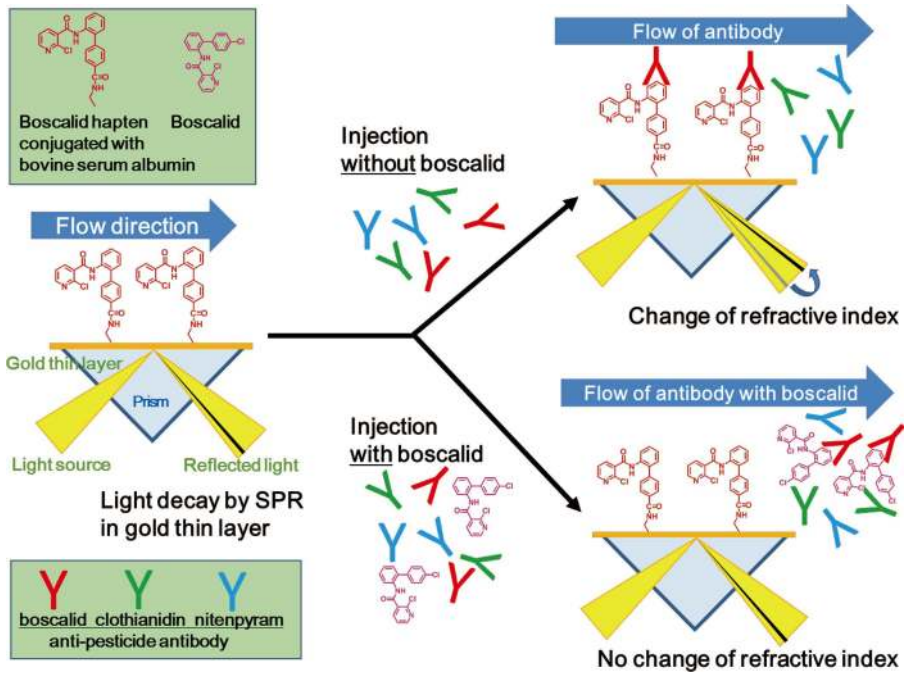

Fig. 2 Schematic illustration of boscalid determination on boscalid channel in the SPR immunosensor.

and rapid analysis of pesticides, although it involves required techniques.

We hypothesized that a simultaneous SPR immunosensor would be developed if each of the antigen-antibody interactions showed no cross-reaction to the other target pesticides. In this study, we have described the successful development of a simultaneous SPR immunosensor to determine boscalid, clothianidin, and nitenpyram, with no cross-reaction among the pesticides, as their boscalid part is shown in Fig. 2. The design of the haptens has also been discussed in the context of developing simultaneous immunosensors.

Experimental

Reagents and chemicals

Boscalid, benalaxyl, fenhexamid, tecloftalam, clothianidin, nitenpyram, imidacloprid, dinotefuran, thiacloprid, thiamethoxam were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetamiprid was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Bovine serum albumin (BSA: Prod. No. A7888) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Horseradish peroxidase (HRP) was purchased from Toyobo Co., Ltd. (Osaka, Japan). The anti-boscalid monoclonal antibody (MoAb) was prepared as described previously.[20] The anti-clothianidin MoAb

and anti-nitenpyram MoAb were provided from Horiba Ltd. (Kyoto, Japan).[18] All other chemicals and reagents used were of analytical grade, and purchased from Wako Pure Chemical Industries, Ltd. or Nacalai Tesque, Inc. (Kyoto, Japan).

Hapten design for boscalid, clothianidin, and nitenpyram

The haptens for boscalid and clothianidin were synthesized as described previously.[18,20] On the other hand, the synthesis method for nitenpyram is described in detail in Supporting Information 1: "Synthesis of nitenpyram hapten" with the scheme in Fig. S1. Their structures were summarized in Fig. 1.

Preparation of hapten and protein conjugate

Boscalid, clothianidin, and nitenpyram haptens were, respectively, conjugated to BSA and HRP, as described previously.[20]

Constitution of SPR immunosensor

The SPR immunosensor comprised a commercially available microflow-type instrument (Biacore T200; GE Healthcare Europe, Munich, Germany), and its sensor chip had four channels coated with carboxymethyl dextran (CM5; GE Healthcare Europe), as described previously.[20,27] The details are described in Supporting Information 2: "Constitution of SPR immunosensor", except for the sample preparation method and for the reaction method to establish simultaneous pesticide residue analysis.

The pesticide standard solutions were prepared with 10% methanol to the following concentrations: boscalid and nitenpyram (3.1 – 200 ng mL^{-1}), clothianidin (1.6 – 100 ng mL^{-1}), dinotefuran (1.6 ng mL^{-1} – 10 μg mL^{-1}), thiacloprid (160 ng mL^{-1} – 100 μg mL^{-1}), and the other pesticides (10 μg mL^{-1}). The pesticides were mixed at the same final concentrations for simultaneous analysis. By contrast, the anti-boscalid, anti-clothianidin, and anti-nitenpyram MoAbs were diluted to 15 μg mL^{-1} with high ion strength phosphate buffered saline (modified PBS: 100 mmol L^{-1} phosphate, 150 mmol L^{-1} NaCl; pH 7.0) containing 0.2% BSA. A mixture of the MoAbs was also prepared at the same final concentration. The pesticide standard solution or the diluent prepared from the vegetables (75 μL) was mixed with an equal volume of the MoAb solution (75 μL), and used as measurement samples.

Measurement samples were allowed to flow serially through the first boscalid channel, the second clothianidin channel, and the last nitenpyram channel immobilized with each of the corresponding haptens and BSA conjugate for 180 s at 20 μL min^{-1}. The solution was continuously changed to the running buffer, and it was allowed to flow further for 180 s at 20 μL min^{-1} to obtain the *Kd* values.

dcELISA

dcELISA was performed as described previously.[20] The specific conditions for boscalid, clothianidin, and nitenpyram are described below. The anti-pesticide MoAbs were diluted with PBS (10 mmol L^{-1} phosphate, 150 mmol L^{-1} NaCl; pH 7.0) to the following concentrations: anti-boscalid MoAb (500 ng mL^{-1}), anti-clothianidin MoAb (250 ng mL^{-1}), and anti-nitenpyram MoAb (125 ng mL^{-1}). The hapten and HRP conjugates were diluted with modified PBS containing 0.2% BSA to the following concentrations: 250 ng mL^{-1} for boscalid, 50 ng mL^{-1} for clothianidin, and 1000 ng mL^{-1} for nitenpyram. Pesticide standard solutions were prepared in 10% methanol to the following concentrations: boscalid (0.038 – 156 ng mL^{-1}), clothianidin (0.024 – 100 ng mL^{-1}), and nitenpyram (0.24 – 1000 ng mL^{-1}).

Preparation of vegetable samples

A variety of vegetables belonging to different families, (broccoli (*Brassica oleracea* var. *italica*), cucumber (*Cucumis sativus* L.), lettuce (*Lactuca sativa* L.), spinach (*Spinacia oleracea* L.), tomato (*Solanum lycopersicum* L.), and Welsh onion (*Allium fistulosum* L.)) were purchased from a market in Kyoto city. dcELISAs, which are more sensitive than the SPR immunosensor, were used to confirm that they did not contain any boscalid, clothianidin, or nitenpyram. The vegetable samples (100 g) were homogenized in a blender. Pesticide mixtures dissolved in methanol (100 μL) were spiked into the homogenized samples (5.0 g) in 50 mL screw-cap tubes at the final concentrations of boscalid, clothianidin, and nitenpyram: A) 2, 0.75, and 1.5 μg g^{-1}; B) 4, 1.5, and 3 μg g^{-1}; C) 8, 3, and 6 μg g^{-1}, respectively. After standing for 30 min at room temperature, 25 mL of methanol was added to the homogenates. The tubes were shaken vigorously on a reciprocal shaker (Shaker SA320; Yamato Scientific Co., Ltd., Tokyo, Japan) for 30 min to extract the pesticides into the liquid phase. The extracts were centrifuged at 3000 rpm for 10 min at room temperature. The supernatants were diluted to 8.5-folds with distilled water to prepare 10% methanol equivalent solutions. They were further diluted with 10% methanol to adjust the concentrations to the working range of the SPR immunosensor or the dcELISA. The diluents were used to prepare measurement samples.

Results and Discussion

Hapten design

For simultaneous analysis, measurement samples containing the three MoAbs and the three pesticides were allowed to flow serially through the boscalid, clothianidin, and nitenpyram channels immobilized with each of haptens and BSA conjugate. The hapten on one channel is therefore necessary to react with the corresponding MoAb only without any cross reaction on the other channels.

As shown in Fig. 1, boscalid has a structure different from that of clothianidin, but has the same 2-chloropyridine ring structure as nitenpyram. It is present in the basic structure at the *ortho*-position in boscalid, but at the *para*-position in nitenpyram. Generally, it is easy to raise MoAbs that recognize such a difference in the angle. We presumed that the haptens for boscalid would not react with the anti-nitenpyram MoAb. By contrast, the 2-chlorobenzene ring in the basic structure of the boscalid at the *para*-position is similar to the structure of the 2-chloropyridine ring in nitenpyram, which might make it difficult for a MoAb to recognize the difference. Therefore, the linker of boscalid was extended from the chlorine position of the 2-chlorobenzene ring to inhibit any possible cross-reaction.

Clothianidin and nitenpyram belong to the same neonicotinoid insecticide group. Clothianidin has a nitroguanidine structure that is similar to the nitrovinylidenediamine structure in nitenpyram. Thus, their haptens might react with MoAb raised against another hapten, resulting in a failure to develop a useful simultaneous SPR immunosensor. In a previous study, two kinds of haptens of the insecticide etofenprox, whose linker sites were on opposite sides (at the ethoxy group and the phenoxybenzene group), induced different cross reactivity with the antibodies raised against the haptens.[30] Thus, we believed that the concept would be effective to develop a simultaneous SPR immunosensor. The clothianidin hapten was synthesized based on the published structure,[18] and then the linker for the nitenpyram hapten was introduced at the opposite side from

clothianidin (Fig. 1).

As shown in Table 1, each of the haptens actually functioned in the constituted SPR immunosensor without any cross-reaction with the other MoAbs, when each of the MoAb solutions flowed serially through the first boscalid channel, the second clothianidin channel, and the last nitenpyram channel.

Determination of pesticides by the SPR immunosensor

The anti-boscalid MoAb, the anti-clothianidin MoAb, and the anti-nitenpyram MoAb solutions were injected separately into the SPR immunosensor. The RU value of the MoAb solutions increased in a time-dependent manner, reaching 450 RU for boscalid, 1500 RU for clothianidin, and 310 RU for nitenpyram at 180 s from the reaction start point, as shown in Fig. 3A. The signals were returned to the baseline after washing with 3 kinds of regeneration buffers. The *Kd* values, calculated from the time course results, were determined as 2.9×10^{-12} mol L^{-1} for boscalid, 8.1×10^{-12} mol L^{-1} for clothianidin, and 7.7×10^{-12} mol L^{-1} for nitenpyram. All of the MoAbs showed high affinity to the corresponding hapten and BSA conjugate, as indicated by the *Kd* values. These high affinities are important to constitute an SPR immunosensor for the accurate determination of the pesticides.

Table 1 Typical signal of anti-pesticide MoAbs onto each pesticide channel by the constituted SPR immunosensor

MoAb	Channel immobilized with hapten and BSA conjugate (delta RU)		
	Boscalid	Clothianidin	Nitenpyram
Anti-boscalid	450	0	0
Anti-clothianidin	2	1500	8
Anti-nitenpyram	0	3	310

The determination of each pesticide was initially examined using the SPR immunosensor. The signal produced between the hapten and BSA conjugate and the MoAb was inhibited by the corresponding pesticide in a concentration-dependent manner for all three pesticides, as shown in Figs. 3B – 3D. The inhibition curves were drawn using the signal data at 180 s from the reaction start point, as shown in Fig. 4. The 20, 50, and 80% inhibitory concentrations (IC_{20}, IC_{50}, and IC_{80}) were 15, 41, and 93 ng mL^{-1} for boscalid; 6.7, 15, and 27 ng mL^{-1} for clothianidin; and 7.3, 24, and 62 ng mL^{-1} for nitenpyram. The quantitative working ranges, defined as between the IC_{20} value and IC_{80} value, suggested that the SPR immunosensor was sufficiently sensitive to be applied to residue analysis of the target pesticides around the MRLs in the majority of vegetables.

The constituted SPR immunosensors were combined for the simultaneous analysis of their pesticides. A mixture of the three MoAbs was added to each pesticide, but also a mixture of their pesticides, and this mixture was injected into the SPR immunosensor. As shown in Fig. 4, the inhibition curves were almost identical to the above determination results for the individual MoAbs. The simultaneous SPR immunosensor could determine boscalid in the range of 15 – 93 ng mL^{-1}, clothianidin in the range of 6.7 – 27 ng mL^{-1}, and nitenpyram in the range of 7.3 – 62 ng mL^{-1}. This successful result could be attributed by the design of highly specific haptens that reacted only with the corresponding MoAb and by the use of high affinity MoAbs.

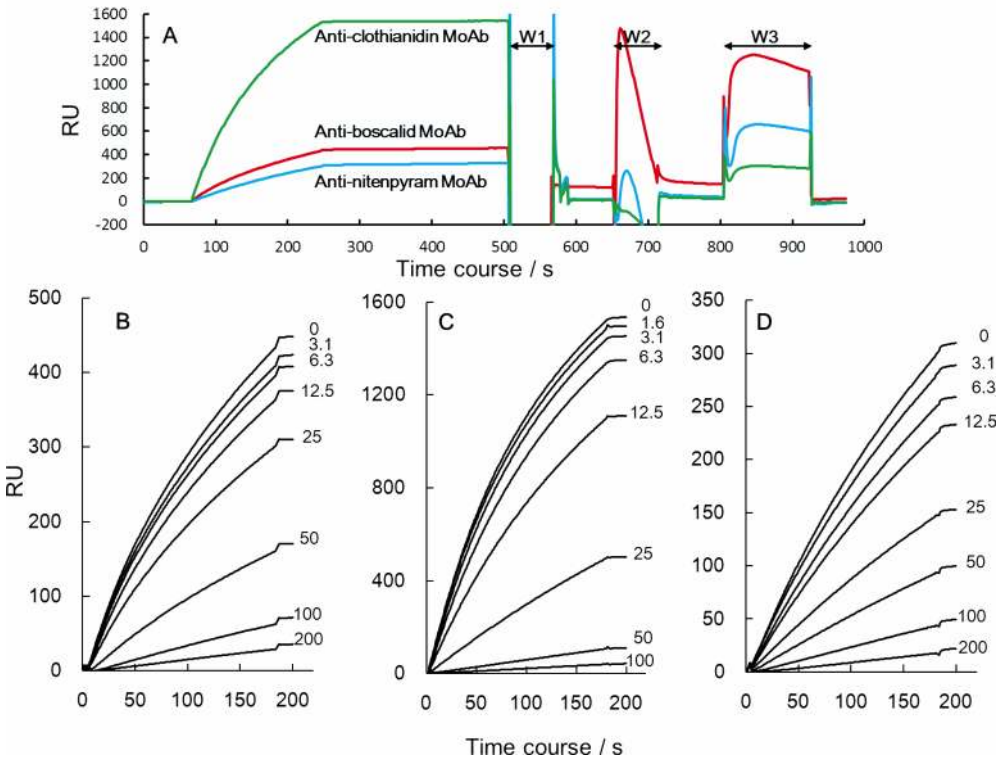

Fig. 3 Time course of anti-pesticide MoAbs reaction without the corresponding pesticides on each channel in SPR-immunosensor (A), and their signal reduction by the corresponding pesticides (ng mL^{-1}): (B) boscalid, (C) clothianidin, (D) nitenpyram. W1, W2, and W3 show regeneration steps by GdnHCl in acetic acid (pH 1.9), distilled water, and 0.2% SDS, respectively.

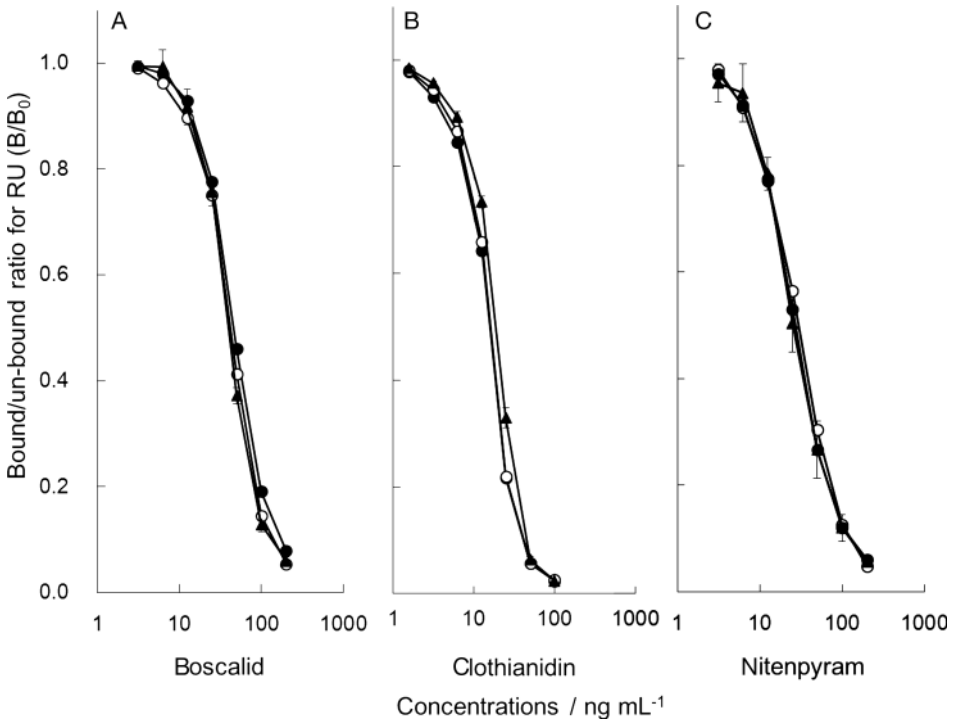

Fig. 4 Inhibition curves for each of the pesticides by SPR-immunosensor: (A) boscalid, (B) clothianidin, (C) nitenpyram. (●) shows inhibition curve for each of the MoAbs with the 1 pesticide, (○) shows inhibition curve for the mixture of 3 MoAbs with the 1 pesticide, and (▲) shows inhibition curve for the mixture of 3 MoAbs with 3 pesticides. Each data point is the mean of triplicate in independent examinations; error bars indicate ± SD.

Cross reactivity

The constituted SPR immunosensor was highly specific to boscalid, clothianidin, and nitenpyram. However, it was not clear whether other structurally related pesticides would cross-react in the SPR immunosensor. Therefore, the cross-reactivity of the anti-boscalid MoAb was examined using fenhexamid, tecloftalam, and benalaxyl, which belong to the same carboxamide fungicide group. The cross-reactivity of anti-clothianidin and anti-nitenpyram MoAbs was examined using acetamiprid, imidacloprid, dinotefuran, thiacloprid, and thiamethoxam, which are all neonicotinoid insecticides. The three MoAbs were mixed, and the mixture was further mixed with each of the pesticides. After injection into the SPR immunosensor, each of the IC_{50} values was obtained from inhibition curves drawn from the time course signal. The cross-reactivity (%) of the MoAbs was obtained from their ratio with the target pesticide. As described in Table 2, the sensor channel for boscalid was specific to boscalid. The sensor channel for nitenpyram was also specific to nitenpyram despite acetamiprid, imidacloprid, and thiacloprid having the 2-chloropyridine ring bound on the *para*-position like nitenpyram. It was speculated that the anti-nitenpyram MoAb used would recognize nitrovinylidenediamine *via* the 2-chloropyridine ring of nitenpyram, because the linker of the hapten was extended from the methyl amine, which exists on the opposite side from the 2-chloropyridine ring.

In contrast, the anti-clothianidin MoAb cross-reacted with dinotefuran at the same level as clothianidin. The cross-reactivity was 119%. Dinotefuran and clothianidin have a common structure: the 1,3-dimethyl-2-nitroguanidin group. The hapten linker of clothianidin was extended from the chlorine atom position of the thiazole ring, which exists on the opposite side of the nitroguanidin group, as shown in Fig. 1. We speculated that the anti-clothianidin MoAb must recognize this common structure.

Table 2 Cross-reactivity of the MoAbs with the structurally related pesticides by the SPR immunosensor

Pesticides examined	Cross-reactivity (%) of MoAbs		
	Anti-boscalid	Anti-clothianidin	Anti-nitenpyram
Boscalid	100[a]	<0.18	<0.22
Clothianidin	<0.18	100	<0.22
Nitenpyram	<0.18	<0.18	100
Fenhexamid	<0.18	NT[b]	NT
Tecloftalam	<0.18	NT	NT
Benalaxyl	<0.18	NT	NT
Acetamiprid	NT	<0.18	<0.22
Imidacloprid	NT	<0.18	<0.22
Dinotefuran	NT	119	<0.22
Thiacloprid	NT	0.21	<0.22
Thiamethoxam	NT	<0.18	<0.22

a. Each data is the mean of duplicates in independent examinations.
b. NT means not tested.

Thus, the cross-reactivity examination suggested that the SPR immunosensor could determine dinotefuran in addition to boscalid, clothianidin, and nitenpyram. Dinotefuran, clothianidin, and nitenpyram, which belong to the same insecticide group, are not usually applied to an agricultural field at the same time. Thus, it was suggested that the constituted SPR immunosensor can determine boscalid and dinotefuran simultaneously, in addition to boscalid and clothianidin, or boscalid and nitenpyram.

Table 3 Recovery examination of pesticide mixtures spiked in vegetables by the SPR immunosensor

Spiked pesticide conc./μg g^{-1}			Welsh onion		Lettuce		Cucumber	
			Rec[a]	RSD[a]	Rec	RSD	Rec	RSD
A	Boscalid	2	79.7[b]	2.49	76.4	1.44	77.2	0.00
	Clothinidin	0.75	92.3	1.15	100	0.77	99.8	0.38
	Nitenpyram	1.5	78.0	8.63	80.8	5.07	84.1	6.91
B	Boscalid	4	87.2	2.49	78.9	1.44	89.6	2.49
	Clothinidin	1.5	96.3	3.32	98.5	1.01	96.9	2.39
	Nitenpyram	3	84.1	18.3	98.5	5.07	95.2	6.91
C	Boscalid	8	83.8	3.80	85.5	1.44	88.8	1.44
	Clothinidin	3	98.7	1.01	87.9	0.38	99.8	1.38
	Nitenpyram	6	90.7	1.91	94.1	1.92	101	5.07

Spiked pesticide conc./μg g^{-1}			Tomato		Broccoli		Spinach	
			Rec	RSD	Rec	RSD	Rec	RSD
A	Boscalid	2	75.5	3.80	78.9	1.44	74.7	2.49
	Clothinidin	0.75	96.7	3.66	97.4	1.92	95.6	2.39
	Nitenpyram	1.5	85.2	5.07	74.1	1.92	71.9	5.07
B	Boscalid	4	82.2	4.98	82.2	2.49	81.3	7.61
	Clothinidin	1.5	94.5	2.99	99.6	1.33	98.7	4.32
	Nitenpyram	3	89.6	3.32	94.1	8.36	94.1	15.0
C	Boscalid	8	78.0	10.1	83.0	3.80	77.2	2.49
	Clothinidin	3	100	2.39	104	1.15	95.8	1.53
	Nitenpyram	6	98.5	8.36	101	8.36	105	18.9

a. Rec shows recovery (%) and RSD shows relative standard deviation (%). b. Each Rec. is the mean of triplicate in independent examinations.

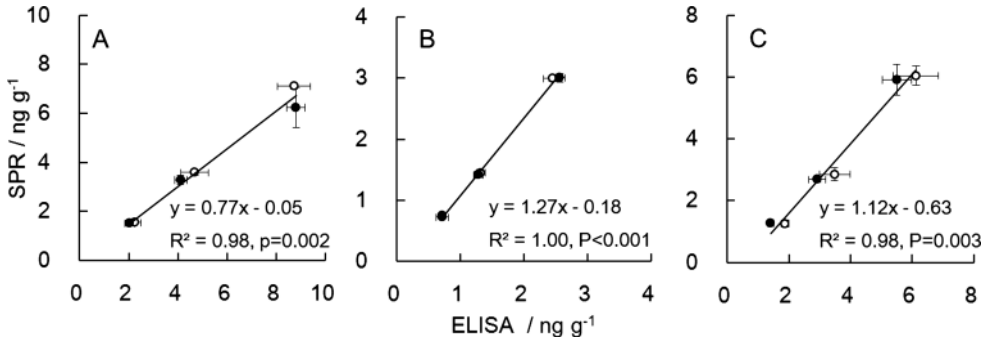

Fig. 5 Correlation of pesticide concentrations determined in cucumber (○) and tomato (●) samples spiked with mixture of 3 pesticides, between dcELISA and SPR-sensor: (A) boscalid, (B) clothianidin, and (C) nitenpyram. Each data point is the mean of triplicate in independent examinations; error bars indicate ± SD.

Recovery of pesticides spiked in vegetables

The recovery of the pesticides by the SPR immunosensor was examined using vegetable homogenates spiked with a mixture of boscalid, clothianidin, and nitenpyram. The lower quantitative limits of the three pesticides in vegetables were estimated to be 0.77 μg g^{-1} for boscalid, 0.34 μg g^{-1} for clothianidin, and 0.37 μg g^{-1} for nitenpyram from the preparation method of vegetable samples. The sensitivity was adequate to determine their concentrations around the MRLs for Welsh onion, lettuce, cucumber, tomato, broccoli, and spinach: 5 – 40 μg g^{-1} for boscalid, 1 – 40 μg g^{-1} for clothianidin, and 5 μg g^{-1} for nitenpyram. Mixtures of three pesticides were spiked at the following concentrations of boscalid, clothianidin, and nitenpyram: A) 2, 0.75, and 1.5 μg g^{-1}; B) 4, 1.5, and 3 μg g^{-1}; C) 8, 3, and 6 μg g^{-1}. Table 3 shows that the recovery values were 75 – 90% for boscalid, 88 – 104% for clothianidin, and 72 – 105% for nitenpyram. The results suggested that the SPR immunosensor could determine the pesticides simultaneously with satisfactory recovery. The relative standard deviation (RSD) values associated with the recovery tests also showed a high repeatability at 0.00 – 10.1%, except for nitenpyram, which showed RSD values of 18.3% in Welsh onion (3 μg g^{-1}), 15.0% in spinach (3 μg g^{-1}), and 18.9% in spinach (6 μg g^{-1}). The results suggested that the constituted sensor is applicable for quantitative residue analysis of boscalid and clothianidin, and for the semi-quantitative analysis of nitenpyram in vegetables.

Correlation results between the dcELISA and the SPR immunosensor

The applicability of the SPR immunosensor was confirmed by comparing the results obtained from the immunosensor with those of the individual dcELISAs, which, except for nitenpyram,

had been evaluated by previous studies.[18,20] As shown in Fig. 5, the SPR immunosensor results correlated highly with those of the dcELISAs: R^2 = 0.98 for boscalid, R^2 = 1.00 for clothianidin, and R^2 = 0.98 for nitenpyram, with a slight bias of their slope: 0.77 for boscalid, 1.27 for clothianidin, and 1.12 for nitenpyram. It was confirmed that the developed SPR immunosensor could determine the three kinds of pesticides residues simultaneously in vegetables.

Conclusions

Our study indicated that the developed SPR immunosensor could be applied to the simultaneous analysis of the three pesticides: boscalid, clothianidin, and nitenpyram. The individual sensitivities were adequate to determine the concentrations around the MRLs of the tested vegetables. The SPR immunosensor is applicable to a wide range of vegetables, such as Welsh onions, lettuce, cucumber, tomato, broccoli, and spinach, which belong to different families. The results also indicate that further development of simultaneous immunosensors is possible using the highly specific haptens and the high-affinity MoAbs. Such simultaneous SPR immunosensors would be useful for rapid, accurate, and simultaneous pesticide analyses.

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Supporting Information

The synthesis method for nitenpyram hapten, and preparation and regeneration methods of the sensor-chip in SPR immunosensor are described in detail in Supporting Information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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