SENSITIVITY IMPROVEMENT OF A DIRECT COMPETITIVE ELISA FOR ATRAZINE BY EXPLOITING LOW CROSS-REACTIVITY OF AN ATRAZINE-SPECIFIC RECOMBINANT ANTIBODY FAB-FRAGMENT

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ABSTRACT

The hapten-binding site of the antibody Fab-fragment K411B specific towards the herbicide atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) was modified by means of structural modeling and site-directed mutagenesis. A triple mutant (GlnL89Glu/ValH37Ile/GluL3Val) of the Fab-fragment showed an increased affinity towards the hapten H/Cl/C6 (4-amino-6-chloro-1,3,5-triazine-2-(6-aminohexanecarboxylic acid)) compared to the affinity of the wild-type Fab-fragment towards the same hapten. However, the mutant exhibited substantially lower affinity towards the hapten H/Cl/C6 than towards atrazine and the hapten iPr/Cl/C6 (4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid)), which is usually used in the synthesis of enzyme tracers in ELISA for atrazine. Advantage was taken of the low cross-reactivity and increased affinity of the mutant Fab fragment towards H/Cl/C6 to improve the sensitivity of a direct-competitive ELISA for atrazine. H/Cl/C6 was covalently conjugated with horseradish peroxidase (HRP), and the conjugate H/Cl/C6-HRP was used as enzyme tracer in the ELISA for atrazine. An eight-fold improvement in sensitivity of a direct-competitive ELISA for atrazine could be achieved using the tracer H/Cl/C6-HRP compared to the sensitivity of ELISA using the tracer iPr/Cl/C6-HRP. The detection limit for atrazine was as low as 0.01ì g/l.

Keywords: atrazine, direct-competitive ELISA, cross-reactivity, recombinant Fab-fragment, structural modeling

ABSTRAK

Situs pengikatan hapten dari fragmen antibodi Fab K411B yang spesifik terhadap herbisida atrazin (2-(etilamino)-4-(isopropilamino)-6-kloro-1,3,5-triazin) telah dimodifikasi dengan modeling struktur dan mutagenesis. Mutan dari fragmen Fab (GlnL89Glu/ValH37Ile/GluL3Val) menunjukkan peningkatan affinitas terhadap hapten H/Cl/C6 (4-amino-6-kloro-1,3,5-triazin-2-(asam 6-amino-heksankarbonat)) dibandingkan dengan affinitas Fab tipe liar K411B terhadap hapten yang sama. Namun demikian, mutan tersebut menunjukkan affinitas yang lebih rendah terhadap hapten H/Cl/C6 dibandingkan dengan affinitasnya terhadap atrazin dan hapten iPr/Cl/C6 (4-(isopropilamino)-6-kloro-1,3,5-triazin-2-(asam 6-amino-heksankarbonat)) yang biasa digunakan dalam sintesis enzim konjugat penanda (*tracer*) pada ELISA untuk mendeteksi atrazin. Rendahnya reaktivitas silang dan peningkatan affinitas dari mutan tersebut terhadap H/Cl/C6 dimanfaatkan untuk meningkatkan tingkat kepekaan dari ELISA kompetitif langsung dalam mendeteksi atrazin. H/Cl/C6 dikonjugasikan secara kovalen dengan enzim *horseradish peroxidase* (HRP), dan konjugat H/Cl/C6-HRP digunakan sebagai enzim konjugat penanda dalam ELISA untuk mendeteksi atrazin. Peningkatan sebesar delapan kali terhadap kepekaan ELISA untuk mendeteksi atrazin dapat diperoleh dengan menggunakan *tracer* H/Cl/C6-HRP dibandingkan dengan kepekaan ELISA yang menggunakan iPr/Cl/C6-HRP sebagai *tracer*. Konsentrasi minimum atrazin yang dapat dideteksi adalah 0,01 ì g/l.

Kata kunci: atrazin, ELISA kompetitif langsung, reaktivitas silang, fragment Fab rekombinan, modeling struktur

INTRODUCTION

Atrazine (Fig. 1) has been used extensively as herbicide and is a well known pollutant of water and soils due to its persistence and wide-spread application. Conventional methods for the determination of atrazin and its derivatives are GC and HPLC, which require liquid-liquid extraction and purification for sample preparation. The procedure is time-consuming, limited by the high expenditure, and involves toxic solvents and reagents (Dunbar *et al.*, 1990). In contrast to the chromatographic methods, enzyme immunoassays are quick and inexpensive methods to detect pesticide residues such as atrazine and its analogs (Weller *et al.*, 1992; Giersch, 1993). Sensitive atrazine immunoassays have been developed using either polyclonal or monoclonal antisera against atrazine with detection limits ranged from 0.01 µg/l to 0.1 µg/l (Schlaeppi, *et al.*, 1989; Witmann and Hock, 1989; Dunbar *et al.*, 1990; Giersch, 1993).

by this approach. Low cross-reactivity of an antibody to analytes could also be exploited to increase the sensitivity of immunoassays (Matuszczyk *et al.*, 1996; Gerdes *et al.*, 1997).

The recombinant technology of immunoglobulin production permits the expression of antibody fragments such as single-chain variable (scFv)-fragments and Fab-fragments in bacteria or eukaryotic systems (Saviranta *et al.*, 1998; Lange *et al.*, 2001; Kusharyoto *et al.*, 2002). Knowledge of the DNA sequence and the three-dimensional structure of an antibody provides information about the interactions between the antigen and antibody and leads close to a precise understanding of the recognition mechanism involved. The information also gives the possibility to alter the binding properties of the antibody by manipulation of the corresponding DNA sequence (Lamminmäki *et al.*, 1997; Miyazaki, *et al.*, 1999; Kusharyoto *et al.*, 2002).

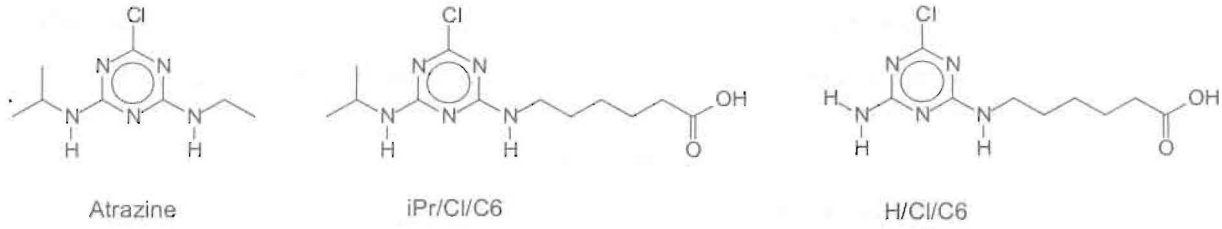

Figure 1. The structures of atrazine and the haptens used in the synthesis of enzyme tracers. iPr/Cl/C6: 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid), H/Cl/C6: 4-amino-6-chloro-1,3,5-triazine-2-(6-aminohexanecarboxylic acid)

Attempts to increase the sensitivity of immunoassays for small molecules or haptens have been described in several studies. Many of the improvements in sensitivity have been achieved by altering the structure of the hapten used to synthesize the hapten-protein conjugates as coating antigens or enzyme tracers (Tiefenauer and Andres, 1990, Colbert *et al.*, 1991). Particularly the so-called spacer recognition could be affected

In the previous study, an atrazine-specific antibody Fab-fragment K411B and a triple mutant of the Fab-fragment (GlnL89Glu/ValH37Ile/GluL3Val) were generated using recombinant technology and site-directed mutagenesis (Kusharyoto *et al.*, 2002). The mutant showed a four-fold increase in relative affinity towards the hapten H/Cl/C6. However, the affinity of the mutant towards H/Cl/C6 is significantly lower than

towards iPr/Cl/C6. In the present investigations, advantage was taken of the increased affinity and the low cross-reactivity of the mutant Fab fragment towards the hapten H/Cl/C6 to improve the sensitivity of a direct-competitive ELISA for the detection of atrazine.

MATERIALS AND METHODS

Chemicals

Horse radish peroxidase (HRP) and rabbit anti-mouse antibody (RAM) were obtained from Sigma (Deisenhofen, Germany). Di-(N-succinimidyl)-carbonat (DSC), N-(dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Tween 20, and all buffer salts and solvents were purchased from Fluka (Buchs, Switzerland). Atrazine and 3,3′,5,5′-tetramethylbenzidine (TMB) were available from Riedel de Häen (Seelze, Germany).

Synthesis of enzyme tracers

The carboxylated hapten derivatives H/Cl/C6 and iPr/Cl/C6 (Fig. 1) were synthesized according to the methods described elsewhere (Weller *et al.*, 1992). The hapten derivatives (100 µmol), NHS (130 µmol) and DSC (40 µmol) were mixed in 1 ml dried dimethoxyethane. After 1 h stirring EDC (130 µmol) was added and stirred for 4 h to obtain the NHS-ester of the hapten derivatives. To a solution of HRP (0.15 µmol) in 0.5 ml of 0.1 M sodium carbonate buffer (pH 8.5) NHS-ester solution was added dropwise within 15 min. The mixture was then stirred for 2 h at room temperature. After centrifugation the soluble hapten-HRP conjugates were purified by means of gel-filtration on Sephadex G-25 in a PD-10 column (Amersham-biotech, Uppsala, Sweden).

Expression and purification

E. coli JM101 containing either the expression vector pASK85-K411B of the wild-type or the mutant Fab fragment was grown on Luria Broth (LB) agar plates containing 100 µg/ml ampicillin. Single colonies were picked and grown overnight in 10 ml LB containing 100 µg/ml ampicillin at 30°C. This culture was then diluted 1:40 with LB-medium, grown at 30°C in 2 l shaking flask to an OD_{550} of 0.8 - 1.0, after which anhydrotetracycline (IBA, Göttingen, Germany) was added to a final concentration of 0.2 µg/ml, and growth was continued for 4 h. The cells were harvested by centrifugation (5000 g, 15 min, 4°C) and then resuspended in 50 mM PBS containing 300 mM NaCl. After addition of lysozyme to a final concentration of 0.5 mg/ml and incubation at room temperature for 20 min, the suspension was sonicated to isolate the Fab fragments expressed in the periplasmic space of *E. coli*.

Purification of the Fab fragments was performed by immobilized metal-ion affinity chromatography (IMAC) on TALON™ chromatography matrix (Clontech, Heidelberg, Germany). Four ml of the matrix were loaded onto a PD-10 column and equilibrated with 50 mM PBS pH 7.2 containing 300 mM NaCl. Ten milliliters periplasmic extract from a 0.4 l culture were applied to the column. After washing with 40 ml of the same buffer the Fab fragments were eluted from the column with 100 mM imidazole in 50 mM PBS pH 7.2 containing 300 mM NaCl. Removal of the imidazole and NaCl was performed by gel-filtration on Sephadex G-25 in PD-10 column using 50 mM PBS pH 7.2 as eluent.

ELISA protocol

The IC_{50} values (concentrations at 50% relative absorbance (B/B_0) of the calibration

curves) were determined by a direct competitive ELISA. In all steps a volume of 200 µl/well was employed. After each incubation the microtiter plate (MaxiSorp, NUNC, Roskilde, Denmark) was washed five times with 8 mM PBS pH 7.2 supplemented with 0.1% Tween 20. The microtiter plate wells were first coated overnight at 4°C with rabbit anti-mouse antibody (RAM; 1µg/ml in 50 mM carbonate buffer pH 9.6). The Fab fragments (0.2 µg/ml in 80 mM PBS pH 7.2) were then bound to the RAM by incubation for 2 h at room temperature. The enzyme tracer was mixed with a serial dilution of the hapten and incubated in the wells at room temperature for 1 h. After incubation of 200 µl of the substrate solution (400 µl TMB-solution (6 mg TMB in 1 ml DMSO) and 100 µl of 1% H_2O_2 dissolved in 25 ml of 50 mM Na-acetate buffer pH 5.5) for 15 min and stopping the reaction with 100 µl 1 M H_2SO_4 the absorbance in each well was read at 450 nm, with 620 nm as reference. Cross-reactivity was calculated as the ratio of the IC_{50} value for H/Cl/C6 to the IC_{50} value of the cross-reacting iPr/Cl/C6 or atrazine. The limit of detection was determined by substracting the threefold standard deviation (error probability: 3%) from the maximum relative absorbance (Matuszczyk *et al.*, 1996).

RESULTS AND DISCUSSION

By means of a structural model and site-directed mutagenesis a triple mutant (GlnL89Glu/ValH37Ile/GluL3Val) of the Fab antibody fragment K411B was generated, previously. The mutant showed a four-fold increase in relative affinity towards the hapten H/Cl/C6 compared to affinity of the wild type (Kusharyoto *et al.*, 2002). Direct competitive ELISA using the tracer iPr/Cl/C6-HRP was performed in order to determine the affinity and cross-reactivity of the mutant GlnL89Glu/ValH37Ile/GluL3Val towards the hapten iPr/Cl/C6, H/Cl/C6 and atrazine. The normalized calibration curves are sigmoidal (Fig. 2). Using the mutant, IC_{50} values of 2.5 nM for iPr/Cl/C6, 13.2 nM for atrazine and 36.4 nM for H/Cl/C6 were determined. Thus, the affinity of the mutant towards H/Cl/C6 was significantly lower than the affinity towards iPr/Cl/C6, and even towards atrazine. The cross-reactivity of the mutant towards H/Cl/C6 compared to iPr/Cl/C6 was 7%, and compared to atrazine was 37%.

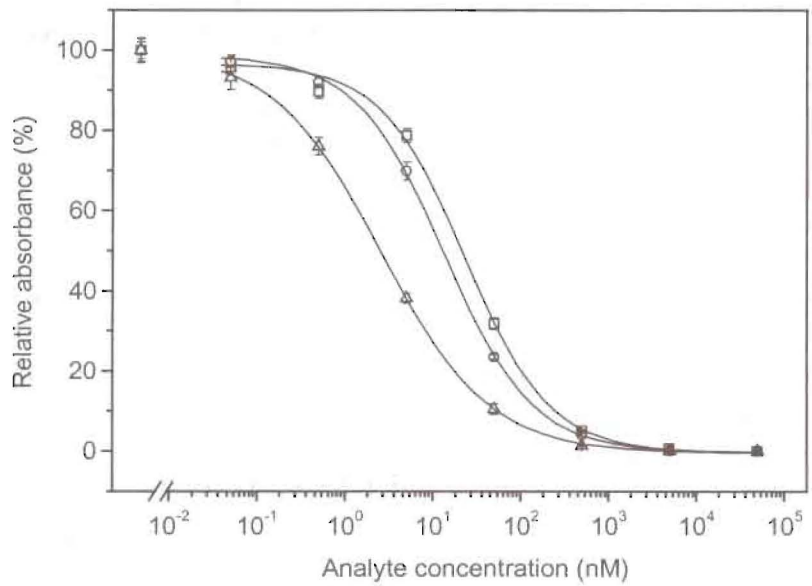

Figure 2. Comparison of normalized calibration curves of direct-competitive ELISA with the mutant GlnL89Glu/ValH37Ile/GluL3Val for atrazine (○), iPr/Cl/C6 (Δ) and H/Cl/C6 (□) using the tracer iPr/Cl/C6-HRP. (Dilution of the enzyme tracer: 1 : 5000; error bars: range of triple determination)

Direct competitive ELISA for atrazine using the wild-type Fab fragment K411B and the tracer H/Cl/C6-HRP could actually be possible, however, the ratio between the maximum and minimum absorbance was too low to perform reproducible ELISA measurements (Fig. 3). The absorbance obtained in ELISA with the tracer H/Cl/C6-HRP was six times higher using the mutant GlnL89Glu/ValH37Ile/GluL3Val than using the wild-type Fab fragment. The higher signal could be solely due to the higher affinity of the mutant towards H/Cl/C6 compared to the affinity of the wild-type to the same hapten. Thus, advantage was taken of the lower affinity of the mutant Fab fragment towards H/Cl/C6 compared to atrazine and the increased relative affinity of the mutant towards H/Cl/C6 to improve the sensitivity of a direct competitive ELISA for the detection of atrazine.

The ELISA for atrazine was performed using the tracer H/Cl/C6-HRP, obtained by covalent conjugation *via* the carboxyl group of the hapten H/Cl/C6 with HRP, and the result was compared with the ELISA using the tracer iPr/Cl/C6-HRP, which is usually used in the detection of atrazine by ELISA. The fitted and normalized calibration curves of the ELISA for atrazine using each of the tracers showed the typical sigmoidal curves (Fig. 4). The calibration curve obtained by using the tracer H/Cl/C6-HRP was displaced to lower concentrations compared to that using the tracer iPr/Cl/C6-HRP. Using the tracer H/Cl/C6-HRP, the ELISA for atrazine with the mutant showed an IC_{50} value of 1.7 ± 0.2 nM (0.37 µg/l) and a detection limit of 0.013 ± 0.003 µg/l, which is comparable to the most sensitive ELISA for atrazine (Witmann and Hock, 1989). In comparison, an IC_{50} value of 13.4 ± 0.4 nM (2.89 µg/l) and a detection limit of $0.18 \pm 0,02$ µg/l were obtained, if the tracer iPr/Cl/

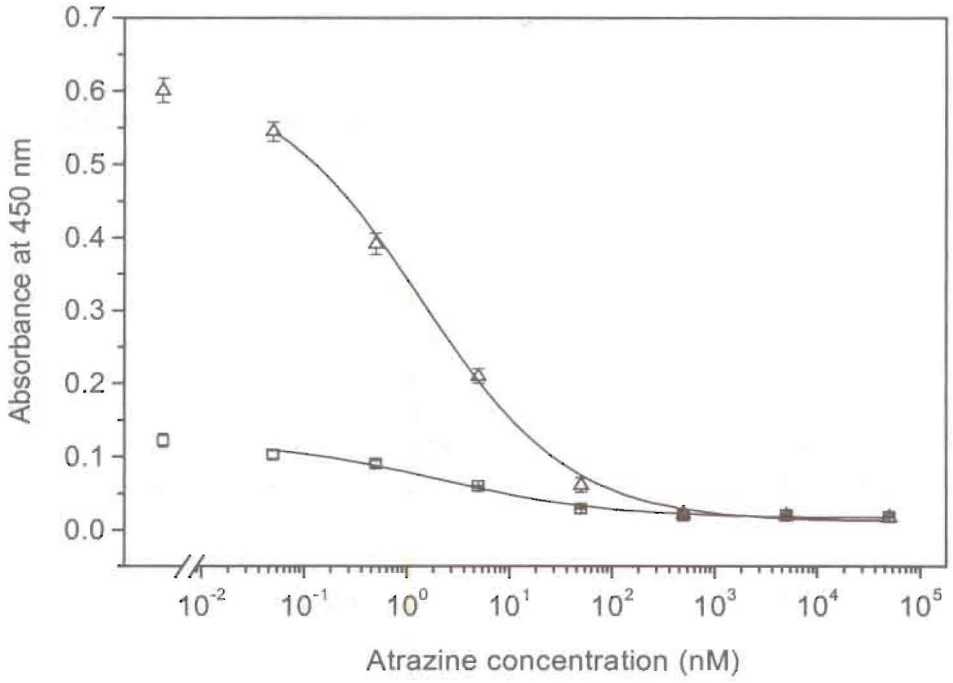

Figure 3. Comparison of the absorbance at 450 nm in direct–competitive ELISA for atrazine obtained with the wild-type K411B (□) and the mutant GlnL89Glu/ValH37Ile/GluL3Val (Δ) using the tracer iPr/Cl/C6-HRP. (Dilution of the enzyme tracer: 1 : 5000; error bars: range of triple determination)

C6-HRP was used. Thus, by taking advantage of the lower affinity of the mutant to the hapten H/Cl/C6 compared to iPr/Cl/C6 and atrazine, an eight-fold improvement in sensitivity of a direct competitive ELISA for atrazine could be achieved.

group. If H/Cl/C6-HRP was used in the competitive ELISA for atrazine, the preference of the binding site of the mutant Fab fragment to bind atrazine was higher than towards H/Cl/C6-HRP. Therefore, lower concentration of atrazine was required to obtain 50% inhibition (binding)

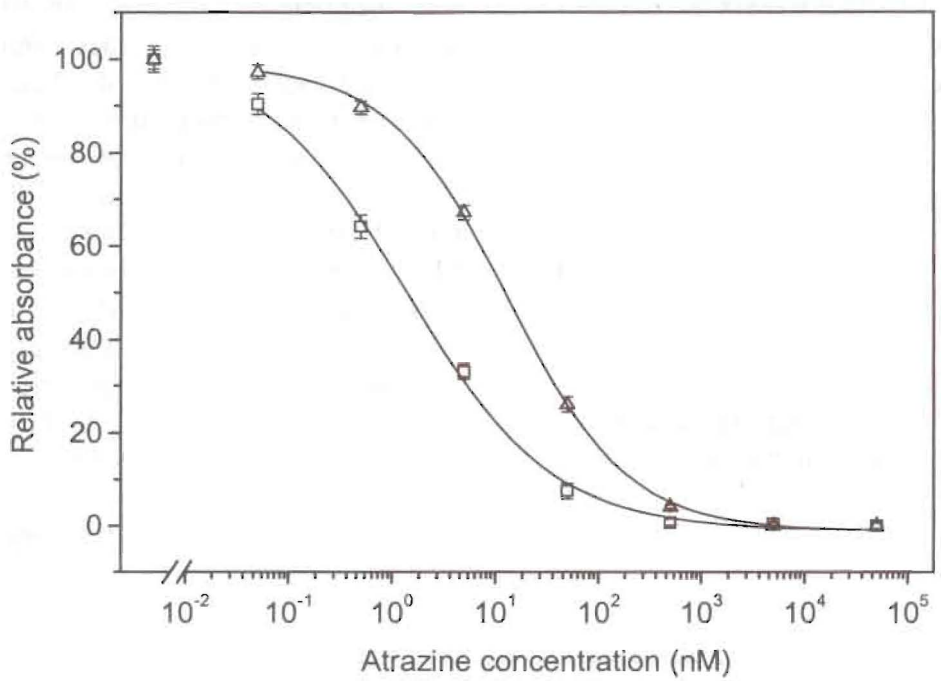

Figure 4. Comparison of normalized calibration curves of direct-competitive ELISA for atrazine with the mutant GlnL89Glu/ValH37Ile/GluL3Val using the tracer H/Cl/C6-HRP (□) and iPr/Cl/C6-HRP(Δ). (Dilution of all enzyme tracers: 1 : 5000; error bars: range of triple determination)

Discussion

The results of this investigation showed that structural differences of the haptens used in the synthesis of the enzyme tracer could be exploited to improve the sensitivity of a direct competitive ELISA. Compared to iPr/Cl/C6 and atrazine, H/Cl/C6 lacks the isopropyl group, which is replaced by a significantly smaller hydrogen atom. In the previous study, we proposed a specific binding pocket for the isopropyl group of iPr/Cl/C6 or atrazine at the bottom of the hapten-binding site of the Fab fragment K411B, which accomodates an isopropyl group much better than any other alkyl group (Kusharyoto *et al.*, 2002). The triple mutation in the mutant Fab fragment did not alter the preference of the binding site to isopropyl of the hapten-binding sites in ELISA using the tracer H/Cl/C6-HRP than using iPr/Cl/C6-HRP, resulting in increased sensitivity of ELISA. Compared to atrazine the tracer iPr/Cl/C6-HRP was bound with higher affinity by the mutant due to the recognition of the C6-alkyl group of the tracer by the binding site. If such a spacer recognition was reduced or eliminated, i.e. by replacing residues of the binding site responsible for spacer recognition, the sensitivity of ELISA could also be improved (Kusharyoto, unpublished data).

In other studies, advantage was taken of the low cross-reactivity of antibodies towards particular haptens to develop sensitive

immunoassays. For instance, the use of 2,4-dichlorobutyric acid (2,4-DB)-HRP tracer and exploiting the low affinity of the 2,4-D-specific antiserum 7/89 towards 2,4-DB offered the possibility of determining the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) more sensitively in a direct competitive ELISA than using the tracer 2,4-D-HRP or 2,4-D-aminohexanoic acid-HRP (Matuszczyk *et al.*, 1996). The low cross-reactivity of a 2,4-D-specific antibody E2/E5 towards 2-methyl-4-chlorophenoxyacetic acid (MCPA) was employed to develop a displacement immunoassay for the determination of 2,4-D (Gerdes *et al.*, 1997). MCPA conjugated with BSA was used as coating antigen. When MCPA-BSA was replaced by 2,4-D-BSA in the chosen assay format, a displacement of bound antibodies was only observed at the very high concentration of the analyte, thus, reducing the sensitivity. In the study presented here, not only the structural difference between the haptens was exploited, but the hapten-binding site of the antibody was manipulated as well to enable ELISA for the detection of atrazine with improved sensitivity.

Conclusion

The sensitivity of direct competitive ELISA for atrazine could be improved by exploiting the lower affinity of a mutant Fab fragment towards the hapten H/Cl/C6 than towards the analyte atrazine. The mutant was obtained by rational design-based modification of the hapten-binding site. The increase in sensitivity was enabled using a hapten derivative lacking a key determinant for binding to the hapten-binding site of the antibody fragment. The use of the hapten with low cross-reactivity, H/Cl/C6, in ELISA for atrazine was also facilitated by the increased affinity of the mutant towards this hapten, so that sufficient signal for reproducible ELISA could be obtained.

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REFERENCES

Colbert, D.L., S.A. Eremin & J. Landon. 1991. The effect of fluorescein labels on the affinity of antisera to small haptens. *J. Immunol. Methods* 140: 227-233

Dunbar, B., B. Riggle & G. Niswender. 1990. Development of enzyme immunoassay for the detection of triazine herbicides. *J. Agric. Food Chem.* 38: 433-437

Gerdes, M., M. Meusel & F. Spener. 1997. Development of a displacement immunoassay by exploiting cross-reactivity of a monoclonal antibody. *Anal. Biochem.* 252: 198-204

Giersch, T. 1993. A new monoclonal antibody for the sensitive detection of atrazine with immunoassay in microtiter plate and dipstick format. *J. Agric. Food Chem.* 41: 1006-1011

Kusharyoto, W., J. Pleiss, T.T. Bachmann & R.D. Schmid. 2002. Mapping of a hapten-binding site: molecular modeling and site-directed mutagenesis study of an anti-atrazine antibody. *Prot. Eng.* 15: 233-241

Lamminmäki, U., B.O. Villoutreix, P. Jauria, P. Saviranta, M. Vihinen, L. Nilsson, O. Teleman & T. Lövgren. 1997. Structural analysis of an anti-estradiol antibody. *Mol. Immunol.* 34: 1215-1226

Lange, S., J. Schmitt & R.D. Schmid. 2001. High-yield expression of the recombinant, atrazine-specific Fab fragment K411B by

the methylotrophic yeast *Pichia pastoris*. *J. Immunol. Methods* 255: 103-114

Matuszczyk, G., D. Knopp & R. Nießner. 1996. Development of an ELISA for 2,4-D: characterization of two polyclonal antisera. *Fresenius J. Anal. Chem.* 354: 41-47

Miyazaki, C., Y. Ina, Y. Yamada, H. Takahashi, J. Sawada & Y. Kurosawa. 1999. Changes in the specificity of antibodies by site-specific mutagenesis followed by random mutagenesis. *Prot. Eng.* 12: 407-415

Saviranta, P., M. Pajunen, P. Jauria, M. Karp, K. Pettersson, P. Mäntsälä & T. Lövgren. 1998. Engineering the steroid-specificity of an anti-17-²À-estradiol Fab by random mutagenesis and competitive phage panning. *Prot. Eng.* 11: 143-152

Schlaeppi, J.M., W. Föry & K. Ramsteiner. 1989. Hydroxyatrazine and atrazine determination in soil and water by enzyme linked immuno sorbent assay using specific monoclonal antibodies. *J. Agric. Food Chem.* 37: 1532-1538

Tiefenauer, L.X. & R.Y. Andres. 1990. Biotinyl-estradiol derivatives in enzyme immunoassays: structural requirements for optimal antibody binding. *J. Steroid Biochem.* 35: 633-639

Weller, M.G., L. Weil & R. Niessner. 1992. Determination of triazine herbicides by ELISA-optimization of enzyme tracer synthesis. *Fresenius J. Anal. Chem.* 343: 51-52

Wittman, C. & B. Hock. 1989. Improved enzyme immunoassay for the analysis of s-triazines in water sample. *Food Agric. Immunol.* 1: 211-224