Preparation of anti-2,4-dichlorophenol and 2,4-dichlorophenoxyacetic acid monoclonal antibodies

Hiroyuki Tanaka¹, Shayu Yan¹, Norio Miura² and Yukihiro Shoyama^{1,*}

¹Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; ²Advanced Science and Technology Center for Cooperative Research, Kasuga, Fukuoka 816-8580, Japan; *Author for correspondence (e-mail: shoyama@phar.kyushu-u.ac.jp; phone: +81 92 642 6580; fax: +81 92 642 6580)

Received 4 March 2003; accepted in revised form 9 August 2003

Key words: Assay system, Biomedical application, 2,4-Dichlorophenol, 2,4-Dichlorophenoxyacetic acid, ELISA, Monoclonal antibody

Abstract

The ratios of hapten and bovine serum albumin (BSA) in an antigen conjugate were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Hybridomas secreting monoclonal antibodies against 2,4-dichlorophenoxyacetic acid (2,4-D) were produced by fusing 2,4-D-BSA conjugate-immunized splenocytes with a HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653. A substantial cross-reaction was observed for 2,4-dichlorophenol (2,4-DP) when compared with that observed for 2,4-D. The full measurement range for this assay is 0.2–3 $\mu\text{g ml}^{-1}$ for 2,4-DP. On the other hand, the range for 2,4-D is between 1 and 20 $\mu\text{g ml}^{-1}$.

Abbreviations: ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BSA – bovine serum albumin; 2,4-D – 2,4-dichlorophenoxyacetic acid; 2,4-DP – 2,4-dichlorophenol; ELISA – enzyme-linked immunosorbent assay; MAbs – monoclonal antibody; MALDI – matrix-assisted laser desorption/ionization; PBS – phosphate-buffered saline; TPBS – PBS containing 0.05% of Tween 20; SPBS – PBS containing 5% of skim milk

Introduction

The widespread use of various herbicides, insecticides, and fungicides in agriculture can lead to contamination of soil as well as ground and surface water. The negative consequences of such ecological contamination have increased the demand for monitoring systems to ensure water quality. The systemic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Figure 1) is widely used in a variety of agricultural and non-agricultural applications, and is typically monitored in environmental samples using HPLC methods, which require clean-up, although,

a number of potential field analytical methods have been reported (Garimella et al. 2000; Loper et al. 2002). Currently, 2,4-D contamination of water is ruled out by HPLC analysis using standard methods (Anastassiades and Schwack 1998; Wells et al. 2002). Although conventional qualitative and quantitative methods are available, alternative immunochemical methods may be more practical. These methods include enzyme immunoassay, immunoagglutination, fluorescence polarization, and enzyme assays as well as antibody-based biosensor methods (Miura et al. 1997; Horacek and Skladal 2000; Green et al. 2002; Taitt et al. 2002).

Figure 1. Structures of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DP), and related compounds.

Metabolite studies of 2,4-D have been conducted and have revealed that, in mice, a glycine conjugate and a 2,4-D-glucuronide are major metabolites, while 2,4-dichlorophenol (2,4-DP) is a minor metabolite. On the other hand, microbial degradation is considered to be the major route of 2,4-D breakdown in soil and water (Figure 1). Recently, Zipper et al. (1999) found that under anaerobic conditions, the concentration of 2,4-D decreased below the detection limit after 17 days and the most important mechanism of microbial degradation of 2,4-D involves the removal of the acetic acid side chain to yield 2,4-DP. Therefore, 2,4-DP appears to be the most important marker metabolite of environmental 2,4-D pollution.

In our ongoing investigations into monoclonal antibody (MAb) formation against naturally occurring bioactive compounds, we have established MAbs against forskolin (Sakata et al. 1994; Yanagihara et al. 1996), solamargine (Ishiyama et al. 1995), opium alkaloid (Shoyama et al. 1996), marijuana compounds (Tanaka et al. 1996), crocin (Xuan et al. 1999), ginsenoside Rb1 (Tanaka et al. 1999) and Rg1 (Fukuda et al. 2000a), and have developed potential applications (Fukuda et al. 2000b, c, 2001). In fact, we recently developed a new immunostaining method, known as Eastern blotting, against several glycosides (Tanaka et al. 1997; Fukuda et al. 2000c; Shan et al. 2001). More

recently, we reported the highly sensitive detection of benzopyrene using a surface plasmon resonance immunosensor (Miura et al. 2002). Here, we report the preparation of MAbs against 2,4-DP and 2,4-D.

Experimental

Chemicals and immunochemicals

We purchased 2,4-D and 2,4-DP from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). BSA and HSA were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was obtained from Organon Teknika Cappel Products (West Chester, PA, USA). All other chemicals were standard commercial products of analytical grade.

Synthesis of antigen conjugates

2,4-D-carrier protein conjugates were synthesized as previously reported (Erlanger et al. 1957), with some modification. Briefly, isobutylchloroformate (10 μ l) and *N*-triethylamine (10 μ l) were added to a dioxane solution (1 ml) of 2,4-D (5.0 mg) dropwise, and stirred at room temperature for 2 h. To this, H₂O (2 ml) containing BSA (5.0 mg) was added, and stirred at room temperature for 16 h. The reaction mixture was dialyzed against H₂O five

times, and then lyophilized to give 2,4-D conjugate (2,4-D-BSA) (5.2 mg).

The hapten number in the 2,4-D-BSA conjugate was determined by MALDI-TOF mass spectrometry, as previously described (Goto et al. 1994). A small amount (1–10 pmol) of antigen conjugate was mixed with a 10^3 -fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid. The mixture was subjected to a JMS time-of-flight (TOF) mass monitor and irradiated with a N_2 laser (337 nm, 150 ns pulse). The ions formed by each pulse were accelerated by a 20-kV potential into a 2.0-m evacuated tube and were detected using a detector linked to a computer.

Immunization, hybridization and purification of MAb

BALB/c female mice were immunized with the 2,4-D-BSA conjugate, and MAbs were generated as described in previous protocols (Sakata et al. 1994) with some modification. The first immunization (50 μ g protein) was administered as a 1:1 emulsion in Freund's complete adjuvant. The second immunization (50 μ g protein in each injection) was administered as a 1:1 emulsion in Freund's incomplete adjuvant. On the third day after the final immunization (100 μ g protein), splenocytes were isolated and fused with a HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653, using the polyethylene glycol (PEG) method. Hybridomas producing MAb reactive to 2,4-D were cloned using the limited dilution method. Established hybridomas were cultured in eRDF medium supplemented with 10 μ g ml^{-1} insulin, 35 μ g ml^{-1} of transferrin, 20 μ M ethanolamine and 25 nM selenium (ITES).

MAbs were purified using a Protein G FF column (0.46 $\times$ 11 cm, Pharmacia Biotech, Uppsala, Sweden). The cultured medium (200 ml) containing the IgG was adjusted to pH 7 with 1 M Tris solution (pH 9) and applied to the column, and the column was then washed with 10 mM phosphate buffer (pH 7). Adsorbed IgG was eluted with 100 mM citrate buffer (pH 3). The eluted IgG was neutralized with 1 M Tris solution (pH 9), dialyzed against H_2O three times, and finally lyophilized.

Direct ELISA using 2,4-D-BSA

The reactivity of MAbs to 2,4-D-BSA (5 molecules of 2,4-D per molecule of BSA) was determined by ELISA. A 96-well immunoplate (NUNC Roskilde, Denmark) to which 100 μ l of 2,4-D-BSA had been adsorbed (1 μ g ml^{-1}) was treated with 300 μ l phosphate-buffered saline containing of 5% skim milk (SPBS) for 1 h in order to reduce non-specific adsorption. The plate was washed three times with PBS containing 0.05% Tween 20 (TPBS) and reacted with 50 μ l of MAb for 1 h. The plate was washed three times with TPBS, and then the MAb combined with 100 μ l of a 1000-fold diluted solution of peroxidase-labeled anti-mouse IgG (Organon Teknica Cappel Products) for 1 h. After washing the plate three times with TPBS, 100 μ l of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.006% H_2O_2 and 0.3 mg ml^{-1} 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Wako, Osaka, Japan), was added to each well and incubated for 15 min. Absorbance at 405 nm was measured with a micro plate reader (Model 450 Microplate Reader Bio-Rad Laboratories, CA, USA). All reactions were carried out at 37 $^{\circ}C$.

Competitive ELISA

2,4-D-BSA (100 μ l, 1 μ g ml^{-1}) was adsorbed in the wells of a 96-well immunoplate, which was then treated with 300 μ l of 5% SPBS for 1 h in order to reduce non-specific adsorption. Fifty microliters of various concentrations of 2,4-D, 2,4-DP, and 2,4-D-related compounds dissolved in 10% of MeOH solution were incubated with 50 μ l of MAb solution for 1 h. The plate was washed three times with TPBS, and then the MAb was combined with 100 μ l of a 1000-fold diluted solution of peroxidase-labeled anti-mouse IgG for 1 h. After washing the plate three times with TPBS, 100 μ l of the substrate solution was added to each well and incubated for 15 min. Absorbance at 405 nm was measured using a micro plate reader. The cross-reactivities of 2,4-D, 2,4-DP and related compounds were determined according to Weiler and Zenk's equation (Weiler and Zenk 1976).

Figure 2. Direct determination of 2,4-D-BSA conjugate by matrix-assisted laser desorption/ionization-TOF mass spectrometry. $[M+H]^+$, $[M+2H]^{2+}$ are single and double protonated molecules of 2,4-D-BSA, respectively.

Results and discussion

Direct determination of hapten-carrier protein conjugate by MALDI-TOF mass spectrometry

It is well-known that the hapten number of an immunogen is important for immunization of small molecular compounds. Analysis of hapten-carrier protein conjugate by MALDI-TOF mass spectrometry directly determined the suitability of hapten number for immunization, as reported previously (Shoyama et al. 1993; Goto et al. 1994). Figure 2 shows the MALDI-TOF mass spectrum of the antigen, 2,4-D-BSA conjugate. A broad peak coinciding with the conjugate of 2,4-D and BSA appeared at around m/z 73 647. Using a molecular weight of 66 433 for BSA, the calculated value of 2,4-D component (MW 221) is 73 771 resulting in a hapten number of 33, which is considered sufficient for immunization when compared to previous results using solamargine (Ishiyama et al. 1995).

Production and characteristics of MAbs against 2,4-D

The hyperimmunized BALB/c mice, used to derive the cell clones described in this study, yielded splenocytes that were fused with P3-X63-Ag8-653 myeloma cells using the procedure established procedure by this laboratory (Sakata et al. 1994). One hybridoma producing MAb reactive to 2,4-D was obtained, and classified as IgG1 having κ light chains.

The reactivity of the IgG-type MAbs against 2,4-D were tested by direct ELISA using varying antigen concentrations. MAb 2C4 had higher reactivity, as shown in Figure 3.

Assay sensitivity and specificity

Following competition, free MAb is bound to polystyrene microtitre plates precoated with 2,4-D-BSA. Under these conditions, the full measuring

Figure 3. Reactivities of IgG type MAb against 2,4-D-BSA conjugate. To examine the reactivity of the antibody, varying concentrations of antibody were added to each well of a 96 well-immunoplate coated with 2,4-D-BSA ($1 \mu\text{g ml}^{-1}$).

Figure 4. Calibration curves of 2,4-D and 2,4-DP using Mab 9F in competitive ELISA. Various concentrations of 2,4-D and 2,4-DP were incubated with Mab 9F precoated with 2,4-D-BSA ($1 \mu\text{g ml}^{-1}$). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm. (—○—): 2,4-D (—): 2,4-DP.

range of the assay extends from 1 to $20 \mu\text{g ml}^{-1}$, as shown in Figure 4.

Table 1 shows the cross-reactivity profile for several structurally related compounds. Because

Table 1. Cross-reactivities (CR %) of anti-2,4-D Mab 2C4.

Compound	CR (%)
2,4-Dichlorophenoxyacetic acid (2,4-D)	100
2,4-Dichlorophenol (2,4-DP)	680
2,4,5-Trichlorophenoxyacetic acid	<3
<i>p</i> -Chlorophenoxyacetic acid	<3
2,4-Dichlorobenzoic acid	<3

cross-reactivity is the most important factor in determining the value of an antibody, the assay specificity was tested by determining the cross reactivities of the MABs with various related compounds. The cross-reactivities of the MABs obtained were examined by competitive ELISA and calculated by the method reported by Weiler and Zenk (1976) (Table 1). Although Mab 2C4 had very low cross-reactivity with 2,4,5-trichlorophenoxyacetic acid, *p*-chlorophenoxyacetic acid, 2,4-dichlorobenzoic acid and undetectable cross-reactivity with other phenols, with 2,4-DP, a cross-reactivity of 680% was observed. Judging from these findings, it is evident that the 2,4-dichlorophenoxy group was antigenic. This cross-reactivity is the major advantage of the antibody reagents used in this ELISA procedure, because when total concentrations of 2,4-D and 2,4-DP in water and/or soil samples are needed, this MAB can be used. similar to the results of anti-solamargine MAB (Ishiyama et al. 1995). Although 2,4-D is an important principle marker for water and soil pollution, 2,4-D is rapidly metabolized into 2,4-DP under anaerobic conditions and this compound accumulates in soil (Zipper et al. 1999). This suggests that the history of 2,4-D pollution is reflected in soil concentrations of 2,4-DP. Therefore, when analysis of underground soil pollution is required, ELISA using Mab 2C4 may be a suitable tool for practically monitoring environmental pollution.

In order to analyze the concentration of 2,4-D and 2,4-DP individually by ELISA using anti-2,4-D MABs, we performed a simple separation method using weak cation-exchange gels resulting in complete separation individually, which was confirmed by HPLC (data not shown). In this case the methanol fraction contained only 2,4-DP, while 2,4-D was adsorbed onto the ion-exchange gel. The concentration of 2,4-DP was then analyzed using the calibration curve shown in Figure 4. The sensitivity for this compound is

much higher than that for 2,4-D, thus reflecting the higher cross-reactivity. This suggests that the full measuring range of this assay extends from 200 ng ml⁻¹ to 3 µg ml⁻¹. This is the first example to establish the assay system of 2,4-DP, and moreover this system can be available for the determination of total concentration of 2,4-D and 2,4-DP which estimate the history of contamination with 2,4-D.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Uehara Memorial Foundation. Support for this work by New Energy and Industrial Technology Development Organization (NEDO) is greatly acknowledged.

References

- Anastassiades M. and Schwack W. 1998. Analysis of carbendazim, benomyl, thiophanate methyl and 2,4-dichlorophenoxyacetic acid in fruits and vegetables after supercritical fluid extraction. *J. Chromatogr. A* 825(1): 45–54.
- Erlanger B.F., Borek F., Beiser S.M. and Lieberman S. 1957. Steroid-protein conjugates I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. *J. Biol. Chem.* 228: 713–727.
- Fukuda N., Tanaka H. and Shoyama Y. 2000a. Formation of monoclonal antibody against a major ginseng component, ginsenoside Rg1 and its characterization. *Cytotechnology* 34(3): 197–204.
- Fukuda N., Tanaka H. and Shoyama Y. 2000b. Applications of ELISA, Western blotting and immunoaffinity concentration for survey of ginsenosides in crude drugs of *Panax* species and traditional Chinese herbal medicines. *Analyst* 125(8): 1425–1429.
- Fukuda N., Tanaka H. and Shoyama Y. 2000c. Isolation of the pharmacologically active saponin ginsenoside Rb1 from ginseng by immunoaffinity column chromatography. *J. Nat. Prod.* 63(2): 283–285.
- Fukuda N., Tanaka H. and Shoyama Y. 2001. Double staining of ginsenosides by western blotting using anti-ginsenoside Rb1 and Rg1 monoclonal antibodies. *Biol. Pharm. Bull.* 24(10): 1157–1160.
- Garimella U.I., Stearman G.K. and Wells M.J. 2000. Comparison among soil Series and extraction methods for the analysis of trifluralin. *J. agric. Food Chem.* 48(12): 5874–5880.
- Green T.M., Charles P.T. and Anderson G.P. 2002. Detection of 2,4,6-trinitrotoluene in seawater using a reversed-displacement immunosensor. *Anal. Biochem.* 310(1): 36–41.
- Goto Y., Shima Y., Morimoto S., Shoyama Y., Murakami H., Kusai A. and Nojima K. 1994. Determination of tetrahydrocannabinolic acid-carrier protein conjugate by matrix-assisted laser-desorption ionization mass-spectrometry and antibody-formation. *Org. Mass Spectrom.* 29(11): 668–671.
- Horacek J. and Skladal P. 2000. Effect of organic solvents on immunoassays of environmental pollutants studied using a piezoelectric biosensor. *Anal. Chim. Acta* 412(1–2): 37–45.
- Ishiyama M., Shoyama Y., Murakami H. and Shinohara H. 1995. Production of monoclonal antibodies and development of an ELISA for solamargine. *Cytotechnology* 18(3): 153–158.
- Loper B.R., Cobb W.T. and Anderson K.A. 2002. Chemical marker for ALS-inhibitor herbicides: 2-aminobutyric acid proportional in sub-lethal applications. *J. Agric. Food Chem.* 50(9): 2601–2606.
- Miura N., Ogata K., Sakai G., Uda T. and Yamazoe N. 1997. Detection of morphine in ppb range by using SPR (surface-plasmon-resonance) immunosensor. *Chem. Lett.* 8: 713–714.
- Miura N., Sasaki M., Sakai G. and Gobi K.V. 2002. Regenerable surface plasmon resonance (SPR)-based immunosensor for highly sensitive measurement of sub-ppb levels of benzo(a)pyrene. *Chem. Lett.* 3: 342–343.
- Taitt C.R., Anderson G.P., Lingerfelt B.M., Feldstein M.J. and Ligler F.S. 2002. Nine-analyte detection using an array-based biosensor. *Anal. Chem.* 74(23): 6114–6120.
- Tanaka H., Goto Y. and Shoyama Y. 1996. Monoclonal antibody based enzyme immunoassay for marihuana (cannabinoid) compounds. *J. Immunoassay* 17(4): 321–342.
- Tanaka H., Fukuda N. and Shoyama Y. 1999. Formation of monoclonal antibody against a major ginseng component, ginsenoside Rb1 and its characterization. *Cytotechnology* 29: 115–120.
- Tanaka H., Putalun W., Tsuzaki C. and Shoyama Y. 1997. A simple determination of steroidal alkaloid glycosides by thin-layer chromatography immunostaining using monoclonal antibody against solamargine. *FEBS Lett.* 404(2–3): 279–282.
- Sakata R., Shoyama Y. and Murakami H. 1994. Production of monoclonal antibodies and enzyme immunoassay for typical adenylate cyclase activator, forskolin. *Cytotechnology* 16: 101–108.
- Shan S.J., Tanaka H. and Shoyama Y. 2001. Enzyme-linked immunosorbent assay for glycyrrhizin using anti-glycyrrhizin monoclonal antibody and an eastern blotting technique for glucuronides of glycyrrhetic acid. *Anal. Chem.* 73(24): 5784–5790.
- Shoyama Y., Fukada T. and Murakami H. 1996. Production of monoclonal antibodies and ELISA for thebaine and codeine. *Cytotechnology* 19(1): 55–61.
- Shoyama Y., Sakata R., Isobe R. and Murakami H. 1993. Direct determination of forskolin bovine serum albumin conjugate by matrix-assisted laser-desorption ionization mass-spectrometry. *Org. Mass Spectrom.* 228(9): 987–988.
- Xuan L.J., Tanaka H., Xu Y.M. and Shoyama Y. 1999. Preparation of monoclonal antibody against crocin and its characterization. *Cytotechnology* 29(1): 65–70.
- Yanagihara H., Sakata R., Minami H., Tanaka H., Shoyama Y. and Murakami H. 1996. Immunoaffinity column

- chromatography against forskolin using an anti-forskolin monoclonal antibody and its application. *Anal. Chim. Acta* 335(1–2): 63–70.
- Weiler E.W. and Zenk M.H. 1976. Radioimmunoassay for the determination of digoxin and related compounds in *Digitalis lanata*. *Phytochemistry* 15: 1537–1545.
- Wells R.J., Hook J.M., Al-Deen T.S. and Hibbert D.B. 2002. Quantitative nuclear magnetic resonance (QNMR) spectroscopy for assessing the purity of technical grade agrochemicals: 2,4-dichlorophenoxyacetic acid (2,4-D) and sodium 2,2-dichloropropionate (Dalapon sodium). *J. Agric. Food Chem.* 50(12): 3366–3374.
- Zipper C., Bolliger C., Fleischmann T., Suter M.J.F., Angst W., Müller M. and Kohler H.P.E. 1999. Fate of the herbicides mecoprop, dichlorprop, and 2,4-D in aerobic and anaerobic sewage sludge as determined by laboratory batch studies and enantiomer – specific analysis. *Biodegradation* 10: 271–278.