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Preparation of Anti–fragrant Monoclonal Antibodies by the Rat Lymph Node Method and Their Characterization Using Enzyme–linked Immunosorbent Assay

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In the food and beverage industries there is a great demand for a sensitive and easy detection system of fragrant compounds in order to ensure product quality control and to avoid contamination. Benzaldehyde (BZ) and furfural (FF) are typical fragrant compounds for peach–flavored beverages, and methyl anthranilate (MA) is a typical flavor of grape beverages. For sensitive detection of these flavors by immunoreaction, we prepared monoclonal antibodies against BZ, FF and MA by using the rat lymph node method. The antibody for BZ had high selectivity toward BZ compared with other fragrant compounds and high affinity with an affinity constant of 1.9×10^{6} M^{-1}. The limit of detection (LOD) of BZ by indirect competitive ELISA (ic–ELISA) was 0.6 ng mL^{-1}. For FF, anti–FF mAb had high selectivity toward FF and high affinity constant of 2.5×10^{4} M^{-1}. The LOD of FF by ic–ELISA was 100 ng mL^{-1}. For MA, anti–MA mAb had high selectivity toward MA and high affinity constant of 1.3×10^{5} M^{-1}. The LOD of MA by ic–ELISA was 200 ng mL^{-1}. These monoclonal antibodies were suitable for detection of very small amounts of fragrant compounds in rinsed water of beverage industries.

INTRODUCTION

In beverage industries, automated bottle–filling production lines are employed at a high–speed filling–capacity, and consequently several tons of beverages are filled in a single stretch into bottles, cans or tetrapacks. The same bottle–filling production line is used routinely for various beverages of widely different aromas and flavors ranging from mineral water to fruit juices, coffee, etc. The production line must be free from contaminants before changing between different beverages. In particular, the line must be ultra–free from characteristic fragrant molecules. Excessive cleaning and/or rinsing cycles with the use of medium–hot water are applied to avoid failures. Close monitoring of the presence of trace amounts of fragrant compounds in cleaning discharges will simplify the cleaning process and will ultimately cut the production cost and time. Usually human odor–specialists are employed to detect the presence of trace amounts of fragrant compounds in cleaning process, but this occasionally involves failure. Thus, novel analytical systems allowing rapid and automated routine detection of trace amounts of low molecular weight fragrant are indispensable to the food and beverage industry in order to ensure high quality of bottled beverages and avoid contamination of different flavors. Benzaldehyde and furfural are the most popular peach flavors and methyl anthranilate is one of the grape flavors used in the beverage industries. These flavors have relatively low threshold levels and sometimes cause problem after changing between different beverages.

Conventional chromatographic and spectroscopic methods coupled to selective detectors (mass spectrometer, electrochemical and flame photometric detectors) were established for detection and quantification of such small molecular organic compounds with high sensitivity. However, these methods involve laboratory–base bulky instruments and labor–intensive sample pre–concentrations by orders of magnitude to reach detectable amounts of analytes (Kazemifard *et al.*, 2002; Albala–Hurtado *et al.*, 1997; Vinas *et al.*, 1993). Immunological methods are proven analytical tools for highly sensitive and selective determination of target analytes and involve no preliminary purification procedure (Luppa *et al.*, 2001; Mallat *et al.*, 2000; Kurita *et al.*, 2006; Ngundi *et al.*, 2005). A number of immunoassay methods, such as enzyme–linked immunosorbent assay (ELISA), fluorescence, chemiluminescence, and amperometric immunoassay methods, have been developed to transform the specific immunoaffinity interaction into a measurable physical response. However, most of these methods are highly disadvantageous, because they imply labeling of either antigen or antibody, long analysis times for *ca.* 2–6 h, extensive sample handling, and/or bulky and expensive instrumentation. In order to overcome the requirements of continuous real–time monitoring of target analyte, we have focused on the development of highly sensitive surface plasmon resonance (SPR) sensing

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methods for the detection of low molecular weight (LMW) compounds using an antigen–antibody reaction (Matsumoto *et al.*, 2005; Shankaran *et al.*, 2006; Gobi *et al.*, 2008, Nagatomo *et al.*, 2009a). For highly sensitive detection of LMW compounds by SPR immunosensor, antibodies with high selectivity and affinity are exclusively required. For this purpose, we have engaged in producing polyclonal and monoclonal antibodies (Sakai *et al.*, 2003; Matsumoto *et al.*, 2005; Nagatomo *et al.*, 2009b). Recently, a novel method was developed for preparation of rat monoclonal antibodies by using rat medial iliac lymph node cells (Kishoro *et al.*, 1995; Sado *et al.*, 1995; Sado *et al.*, 2006).

In order to develop highly sensitive SPR immunosensors, we firstly prepared anti–benzaldehyde, anti–furfural and anti–methyl anthranilate monoclonal antibodies by using medal iliac node cells and characterized their properties by using solid–phase enzyme–linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Materials and Apparatus

Bovine serum albumin (BSA), *N*–hydroxysuccinimide (NHS), *o*–phenylenediamine (*o*–PD), 1–ethyl–3–(3–dimethyl aminopropyl) carbodiimide (EDC), disodium *p*–nitrophenylphosphate (*p*–NPP), 2–furan carboxylic acid and gelatin were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Ovalbumin (OVA, Grade VI), guanidine hydrochloride and 4–carboxybenzaldehyde (CBZ, >98%), benzaldehyde (BZ, >99%), 1–methyl 2–aminoterephthalate (MAT), alkaline phosphatase labeled anti–rabbit immunogloblin G (ALP labeled anti–rabbit IgG), ALP labeled anti–rat IgG, 5–fromyl 2–furancarboxylic acid (FFC) were obtained from Sigma (St. Louis, MO, USA). 2,4–Dinitrophenylhydrazine (DNPH) and furfural (FF) were from Kanto Chemical Co., Inc. (Tokyo, Japan). Mariculture keyhole limpet hemocyanin (KLH) and *Concholepas concholepas* hemocyanin (CCH) were from Pierce (Rodkford, IL, USA). GIT medium and methyl anthranilate (MA) were obtained from Wako Pure Chemicals Ind., Ltd. (Osaka, Japan). Freund's complete adjuvant was obtained from Difco (Detroit, MI, USA). Sevofrane was from Maruishi Pharmaceutical Co. Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and BM–condimed H1 were from Gibco (OK, USA) and Roche (Basel, Switzerland), respectively. Horse radish peroxidase labeled polyclonal rabbit anti–rat immunoglobulin G (HRP labeled anti–rat IgG) was from Daco Japan Co. (Tokyo, Japan). The HiTrap Protein G column and PD–10 column were purchased from GE healthcare UK Ltd. (Amersham Place, Little Chalfont, England). Isotyping test kit (RMT1) was from AbD serotec (Cosmo Bio, Tokyo, Japan). All other reagents were of analytical–reagent grade. All buffer solutions were prepared using water purified with a Milli–Q filter (Millipore, Bedford, MA, USA).

ELISA measurements were performed using 96–well immunoplates (NUNC, No. 446612, Roskilde, Denmark) and a microplate reader (Wallac 1420, Perkin Elmer Life Science Japan, Tokyo, Japan). Spectrophotometric measurements were performed using a Shimadzu Multi Spec 1500 (Kyoto, Japan).

Preparation of fragrant analog–protein conjugate

CBZ–KLH conjugate: An ice–cooled solution of 1.8 mg (12 μmol) of CBZ in 1 mL dioxane was stirred with 2.3 mg (20 μmol) NHS, 50 mg Na_2SO_4 and later 34 mg (177 μmol) EDC over night at room temperature (RT). The resulting mixture was centrifuged and the supernatant solution was slowly added to a solution of 5 mg KLH in 1 mL borate buffer (12.5 mM, pH 8.0). The addition of the supernatant solution was divided in three portions (180 μL each) with 30 min interval. After incubating it at RT for 3 h, the reaction mixture was dialyzed against borate buffer (12.5 mM, pH 8.0) for one days (three times change) at 5 °C and was then lyophilized. Condensing a chromophoric compound, DNPH, with aldehydes was used to estimate the number of BZ haptens per CBZ–KLH conjugate. Control experiments using the initial KLH protein had been carried out to corroborate the number of haptens determined. The hydrazone adduct has an absorbance maximum around 380 nm with a molar extinction coefficient of 22,000 M^{-1} cm^{-1} (Dalle–Donne *et al.*, 2003; Levine *et al.*, 1990), and the average number of BZ haptens per CBZ–KLH unit was determined to be 24.2. The CBZ–OVA conjugates were also prepared by almost the same way with CBZ–KLH conjugates, and the average number of BZ haptens per CBZ–OVA unit was 1.8.

FFC–KLH conjugate: FFC–KLH conjugates were prepared by almost the same way with CBZ–KLH conjugates except the starting amount of FFC (3.36 mg; 24 μmol). FFC–OVA conjugates were also prepared as above. The average number of FF haptens per FFC–KLH unit was estimated to be *ca.* 50.

MAT–CCH conjugate: MAT (2.3 mg; 12 μmol) was dissolved in 1 mL of DMF and 50 mg Na_2SO_4, 23 mg NHS and 34 mg EDC were added at 4 °C under stirring for 2 h and then the mixture was stirred overnight at RT. The resulting mixture was centrifuged and the supernatant solution was slowly added to a solution of 50 mg CCH in 4 mL borate buffer (12.5 mM, pH 8.0) containing 50 mg Na_2CO_3. After stirring overnight at RT, the reaction mixture was dialyzed against borate buffer (12.5 mM, pH 8.0) for one day (three times change) at RT and was then lyophilized. The conjugation of MAT to CCH was confirmed by UV spectrum. The MAT–OVA conjugates were prepared by using the Fmoc method (Louis *et al.*, 1972; Louis, 1987), and the average number of MA haptens per MAT–OVA unit was 8.2.

Immunization of rats

Rats, anesthetized with sevofrane, were immunized with fragrant analog–protein conjugates according to the following procedures. Conjugates dissolved in phosphate buffered saline (PBS, 1 mg mL^{-1}) were thoroughly emulsified with an equal volume of Freund's complete adjuvant in two 2–mL glass syringes with a three–way stopcock. After emulsification was completed, the emulsion

was sucked in one ml of plastic syringe attached with 25 G×1" needle.

Nine–week–old female WKY rats (Charles River Japan, Inc., Yokohama) were injected intramuscularly at the right and left tail base with the prepared emulsion (0.2 mL in total). Rats were kept in plastic cages containing wood shavings for bedding. All animal experiments were conducted under the control of the guideline for Animal Experiment in Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

The rats were bled every week from tail veins after the immunization, and the antisera were collected by centrifugation of the blood samples. The antisera were tested by direct–ELISA. Ninety–six–well immunoplates were coated with 100 μL of fragrant analog–protein conjugates (immunogen, 10 μg mL^{-1} in PBS) overnight at RT. The plates were then treated with 150 μL of 1% gelatin for 1 h at RT, and reacted with antisera at nine different dilutions (1/100–1/25,600 in PBS), which were added to the wells (100 μL to each well) and incubated for 1.5 h at RT. For antisera taken from the immunized rats with CBZ–KLH or FFC–KLH, the solution of HRP labeled anti–rat IgG (1/1000 dilution in PBS) was added (100 μL to each well) and incubated for 1 h at RT. Then, 150 μL of the substrate solution (0.4 mg mL^{-1} of *o*–PD and 0.4 μL mL^{-1} of 30% H_2O_2 in PBS) was added to each well and incubated for 30 min at room temperature. After the reaction, 50 μL of 3 M sulfuric acid was added to terminate the enzyme reaction, and then the absorbance at 490 nm was measured. For antisera from MAT–CCH, the solution of ALP labeled anti–rat IgG (1/2000 dilition in PBS) was added (100 μL to each well) and incubated for 45 min at RT. After the incubation, 100 μL of the substrate solution (2 mg mL^{-1} of *p*–NPP in 50 mM carbonate buffer, pH 7.4, containing 0.1 mM Zn^{2+} and 1 mM Mg^{2+}) was added to each well and incubated for 30 min at RT, and absorbance at 405 nm was measured. The plates were rinsed three times with PBS containing 0.05% Tween 20 (PBST) at each step. The rats were bled without boost injection for 2–3 weeks for obtaining both iliac lymph cells and polyclonal antibodies.

Cell fusion

A mouse myeloma cell line, Sp2/0–Ag14 (Dainihon Sumitomo Pharmacy), was used for cell fusion. The procedures were almost the same as our previous paper (Nagatomo *et al.*, 2009b).

Serum antibody titers and screening assay

Anti–BZ antibody: The supernatants were screened for production of BZ–antibody by indirect ELISA. ELISA plates were coated with 100 μL of CBZ–OVA conjugate (10 μg mL^{-1}) overnight at RT. The plates were washed and blocked with 1% gelatin for 1 h at RT. The plates were washed and 50 μL of the supernatant of each cultured well were added to each well. One series of experiments was performed to check the association of supernatants (antibodies) to the coating conjugates. The other series of experiments was performed to check whether the supernatants (antibodies) will be inhibited with free BZ as follows: after the addition of supernatant, 50 μL of BZ solution was added at the final concentration of 50 μg mL^{-1}. The subsequent procedures for both experiments were almost the same as Section of immunization of rat. The cells, which were confirmed the production of anti–BZ antibody and the ability of association toward free BZ, were suspended in GIT medium containing 10% FBS (SGIT). The suspensions were subjected to the limit dilution methodology, and the screening and cloning were repeated for two times, and then monoclonal antibody producing hybridomas were established. The hybridomas were cultured in SGIT, and the supernatant was collected and purified with protein G column and PD–10 column according to the procedures recommended by the manufacturers. The isotype of the monoclonal antibody was IgG2a.

Anti–FF antibody: The supernatants were screened for production of anti–FF antibody by indirect ELISA. The procedures were almost the same as above except using FFC–OVA conjugate as a solid phase protein. The isotype of the monoclonal antibody was IgG1.

Anti–MA antibody: The screening procedures were almost the same as above except using MAT–OVA conjugate as a solid phase protein. The isotype of the monoclonal antibody was IgG2b.

Indirect competitive ELISA (ic–ELISA) for fragrant compounds

Ic–ELISAs for BZ (or FF): Ic–ELISAs for BZ (or FF) were performed as follows. Ninety–six–well immunoplates were coated with 100 μL of CBZ–OVA conjugate, 1 μg mL^{-1} in PBS (or FFC–OVA conjugate, 1 μg mL^{-1}), over night at RT. The following day, the plates were washed three times with PBST and treated with 150 μL of 1% gelatin for 1 h at RT. The plates were washed three times with PBST, reacted with 100 μL of the equivalent mixtures of anti–BZ monoclonal antibody (anti–BZ mAb), 1 μg mL^{-1} in PBS (or anti–FF mAb, 5 μg mL^{-1}), and serially diluted BZ (or FF) for 1.5 h at RT. The plates were washed again three times with PBST, and the solution of HRP labeled anti–rat IgG (1/1000 dilution in PBS) was added (100 μL to each well) and incubated for 1 h at RT. The plates were washed again, and 150 μL of the substrate solution (0.4 mg mL^{-1} of *o*–PD and 0.4 μL mL^{-1} of 30% H_2O_2 in PBS) was added to each well and incubated for 30 min at RT. After the reaction, 50 μL of 3 M sulfuric acid was added to terminate the enzyme reaction, and then the absorbance at 490 nm was measured.

Ic–ELISAs for MA: Ninety–six–well immunoplates were coated with 100 μL of MAT–OVA conjugate, 3 μg mL^{-1} in PBS, over night at RT. The following day, the plates were washed three times with PBST and treated with 150 μL of 1% gelatin for 1 h at RT. The plates were washed three times with PBST, reacted with 100 μL of the equivalent mixtures of anti–MA monoclonal antibody (anti–MA mAb), 0.4 μg mL^{-1} in PBS, and serially diluted MA for 1.5 h at RT. After washing with PBST the solution of ALP labeled anti–rat IgG (1/2000 dilution in PBS) was added (100 μL to each well) and incubated for

45 min at RT. After the incubation, 100 μL of the substrate solution (2 mg mL^{-1} of *p*–NPP in 50 mM carbonate buffer, pH 7.4, containing 0.1 mM Zn^{2+} and 1 mM Mg^{2+}) was added to each well and incubated for 30 min at RT, and absorbance at 405 nm was measured.

Avidity of anti–fragrant mAb to various kinds of fragrant compounds

The avidity of anti–fragrant mAb to fragrant compounds was investigated by ic–ELISAs using each fragrant analog–OVA conjugate as a coating antigen–protein conjugate. The ELISA procedures were the same as described in Section of ic–ELISA for fragrant compounds. The IC_{50} was defined as the concentration of added fragrant compounds that yield 50% inhibition compared with no inhibition (100%). Molar cross–reactivities were related to each target fragrant compound (100%); namely, all molar cross–reactivities were determined in relation to each fragrant standard inhibition curve. The molar cross–reactivity of each derivative was calculated according to Weiler's equation (Weiler and Zenk, 1976):

$$CR = (IC_{50}^{*}/IC_{50}) \times 100$$

where CR is molar cross–reactivity (%), IC_{50}* is the IC_{50} of each standard (M), and IC_{50} is the IC_{50} of fragrant compounds (M).

Data analysis

Experimental values obtained from indirect competitive assays were converted into inhibition values (%B/B_0) by using following equation:

$$\%B/B_0 = 100 \times B/B_0$$

In ic–ELISA, B is the absorbance value for each standard and B_0 is the absorbance value resulting from a zero dose standard (blank value). Curve fitting of standard curves was performed by use of following equation (Holthues *et al.*, 2005):

$$Y = 100/[1+(x/c)^b]$$

where c is the midpoint, b is the slope of the curve, and × is the standard concentration. The limit of detection (LOD) was calculated as three times the standard deviation of a blank value, following IUPAC rules.

RESULTS AND DISCUSSION

Establishment of monoclonal antibody hybridoma

Anti–BZ hybridoma: The establishment of the hybridoma cells which produce monoclonal anti–BZ antibody (anti–BZ mAb) was one cell of 84 fusion positive cells. The inhibition profile of anti–BZ mAb by the ic–ELISA is shown in Fig. 1 along with that of rat anti–BZ–KLH polyclonal antibody (anti–BZ pAb). As shown in Fig. 1, the anti–BZ mAb showed the LOD of 0.6 ng mL^{-1} (ppb) toward BZ. This sensitivity is much higher than that using polyclonal antibody. The affinity constants between free BZ and anti–BZ antibodies (monoclonal and polyclonal) were also estimated by using Seligman's method (Seligman, 1994) using the data of Fig. 1. The evaluated values were K_A=1.9×10^6 M^{-1} and K_A=1.3×10^3 M^{-1} for anti–BZ mAb and anti–BZ pAb, respectively.

Anti–FF hybridoma: The establishment of the hybridoma cells which produce monoclonal anti–FF antibody (anti–FF mAb) was two cells of 90 fusion positive cells. We denoted the hybridomas as FF–mAb1 cell and FF–mAb2 cell. We selected the FF–mAb1 hybridoma as a best one in consideration of relatively strong affinity to solid phase FFC–OVA and strong affinity to free FF. The inhibition profile of anti–FF mAb1 by the ic–ELISA is shown in Fig. 2 along with that of rat anti–FF–KLH polyclonal antibody (anti–FF pAb). As shown in Fig. 2, the anti–FF mAb1 showed the LOD of 0.1 μg mL^{-1} (0.1 ppm) toward FF. Although the LOD of anti–FF mAb toward FF was a little worse than that of anti–FF pAb, the affinity constant between free FF and anti–FF mAb1 (K_A= 2.5×10^4 M^{-1}) was better than that between free FF and anti–FF pAb (K_A=2.1×10^3 M^{-1}). These results (LODs and affinity constants) seemed to reflect that the percent inhibition in low concentration region (0.1 ppb–0.1 ppm) with polyclonal antibody changed moderately whereas that of monoclonal antibody in 0.1 ppm level changed sharply.

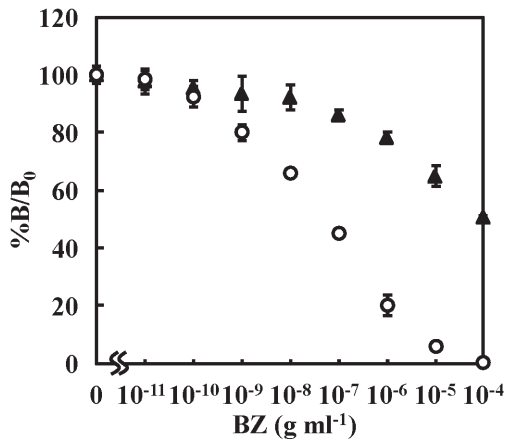

Fig. 1. Standard curve of BZ in indirect competitive ELISA. The results are shown as mean values. Symbols are as follows: ▲, polyclonal antibody (n=3); ○, monoclonal antibody (n=3)

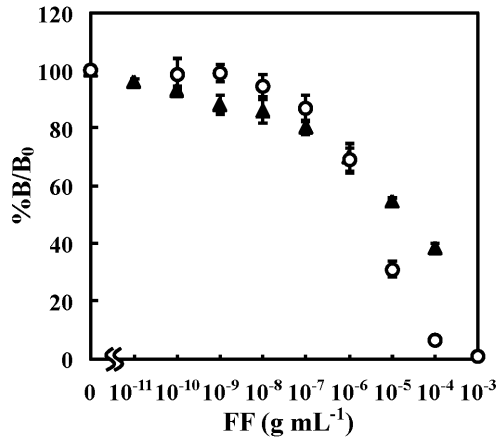

Fig. 2. Standard curve of FF in indirect competitive ELISA. The results are shown as mean values. Symbols are as follows: ▲, polyclonal antibody (n=3); ○, monoclonal antibody (n=6)

We used FF–mAb1 hybridoma hereafter.

Anti–MA hybridoma: The establishment of the hybridoma cells which produce monoclonal anti–MA antibody (anti–MA mAb) was three cells of 1370 fusion positive cells. We denoted the hybridomas as MA–mAb1 cell to MA–mAb3 cell. We selected the MA–mAb1 hybridoma as a best one in consideration of relatively strong affinity to solid phase MAT–OVA and strong affinity to free MA. The inhibition profile of anti–MA mAb1 by the ic–ELISA is shown in Fig. 3 along with that of rat anti–MAT–CCH polyclonal antibody (anti–MA pAb). As shown in Fig. 3, the anti–MA mAb1 showed the LOD of 0.2 μg mL–1 (0.2 ppm) toward MA. This sensitivity is much higher than that of polyclonal antibody. We used MA–mAb1 hybridoma hereafter. The affinity constants between free MA and anti–MA antibodies (monoclonal and polyclonal) were also estimated by using Seligman's method using the data of Fig. 3. The evaluated values were K_A=1.3×$10^5 M^{-1}$ and K_A=9.8×$10^2 M^{-1}$ for anti–MA mAb1 and anti–MA pAb, respectively.

Avidity of anti–fragrant mAbs to various kinds of fragrant compounds

The avidity of the prepared antibodies (anti–fragrant mAbs) to various compounds other than target fragran compound was evaluated by ic–ELISAs. The coating antigen–protein conjugate was each fragrant analog–OVA conjugate. Midpoints (IC_{50}) and molar cross–reactivity for BZ are listed in Table 1. As shown in Table 1, the anti–BZ mAb showed high selectivity toward relating compounds (or fragrant compounds) except vanillin (20.7%) and furfural (14.8%). CBZ, the starting compound of immunogen, showed the cross reactivity of *ca.* 58% and this compound is not contained in food beverage. Benzoic acid, a food preservative, did not show any affinity. Overall, the anti–BZ mAb seemed to be valuable for selective detection of target fragrant compound (BZ).

Midpoints (IC_{50}) and molar cross–reactivity for FF are listed in Table 2. As shown in Table 2, very small or almost no avidity were shown with BZ (13.9%), 2–furancarboxylic acid(<1%), vanillin (<1%), benzoic acid (<1%), and γ–undecalactone (none). FFC, the starting compound of immunogen, showed the cross reactivity of *ca.* 76% and this compound is not contained in food beverage. From these results, we judged the anti–FF mAb1 is valuable.

Midpoints (IC_{50}) and molar cross–reactivity for MA are also listed in Table 3. As shown in Table 3, the anti–MA mAb1 showed rather high selectivity toward relating

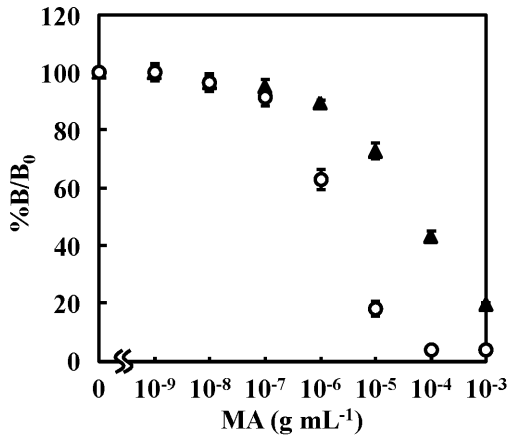

Fig. 3. Standard curve of MA in indirect competitive ELISA. The results are shown as mean values. Symbols are as follows: ▲, polyclonal antibody (n=3); ○, monoclonal antibody (n=6)

Table 1. Cross reactivities of anti–BZ mAb for BZ relevant compounds

Compound	IC_{50} (M)	Cross reactivity (%)
benzaldehyde (BZ)	2.50×10^{-6}	100
4–carboxy benzaldehyde	4.30×10^{-6}	58.1
vanilin	1.20×10^{-5}	20.7
furfural (FF)	1.70×10^{-5}	14.8
4–formypheoxy acetic acid	2.00×10^{-5}	12.0
4–methoxy benzaldehyde	8.40×10^{-5}	3.00
methyl anthranilate (MA)	No inhibition	–
5–formyl 2–furancaroxylic acid	No inhibition	–
γ–undecalactone	No inhibition	–
benzoic acid	No inhibition	–

Table 2. Cross reactivities of anti–FF mAb1 for FF relevant compounds

Compound	IC_{50} (M)	Cross reactivity (%)
furfural (FF)	4.53×10^{-5}	100
5–formyl–2–furancarboxylic acid	6.00×10^{-5}	75.5
benzaldehyde (BZ)	3.26×10^{-4}	13.9
2–furancarboxylic acid	4.70×10^{-3}	<1
vanilin	5.23×10^{-3}	<1
benzoic acid	6.06×10^{-3}	<1
γ–undecalactone	No inhibition	–

Table 3. Cross reactivities of anti–MA mAb1 for MA relevant compounds

Compound	IC_{50} (M)	Cross reactivity (%)
methyl anthranilate (MA)	1.91×10^{-5}	100
methyl 2–amino–3–carboxybenzoate	4.36×10^{-6}	438
1–methyl–2–aminoterephthalate	2.26×10^{-5}	84.5
N–methylanthranilic acid methyl ester	2.56×10^{-5}	74.6
o–toluic acid methyl ester	1.29×10^{-4}	14.8
2'–aminoacetophenone	1.30×10^{-4}	14.7
2–aminobenzaldehyde	1.09×10^{-3}	1.75
anthranilic acid	2.01×10^{-3}	<1
benzaldehyde (BZ)	2.01×10^{-3}	<1
furfural (FF)	No inhibition	–
o–phenylenediamine	No inhibition	–

compounds (or fragrant compounds) except methyl 2–amino–3–carboxybenzoate (438%), MAT (84.5%) and *N*–methylanthranilic acid methyl ester (74.6%). Methyl 2–amino–3–carboxybenzoate and MAT, which have the MA skeletal structure, are not contained in food beverage. *N*–methylanthranilic acid methyl ester is a flavor of grape and its smell is almost same as MA. This means acceptable to measure *N*–methylanthranilic acid methyl ester together with MA. From these results, we judged the anti–MA mAb1 is valuable.

CONCLUSIONS

Monoclonal antibodies against BZ, FF and MA were successfully prepared by using the rat lymph node method. One hybridoma cell which produce anti–BZ antibody were produced out of 84 fusion positive cells. The anti–BZ mAb has high selectivity toward BZ compared with other fragrant compounds and high affinity constant of $1.9\times10^5 M^{-1}$ to BZ. The LOD of BZ by ic–ELISA was 0.6 ng mL^{-1} (0.6 ppb). For FF, two hybridoma cells which produce anti–FF antibody were produced out of 90 fusion positive cells. The best antibody (anti–FF mAb1) has high selectivity toward FF compared with other fragrant compounds and high affinity constant of $2.5\times10^4 M^{-1}$ to FF. The LOD of FF by ic–ELISA was 100 ng mL^{-1} (100 ppb). For MA, three hybridoma cells which produce anti–MA antibody were produced out of 1370 fusion positive cells. The best antibody (anti–MA mAb1) has high selectivity toward MA compared with other fragrant compounds and high affinity constant of $1.3\times10^5 M^{-1}$ to MA. The LOD of MA by ic–ELISA was 200 ng mL^{-1} (200 ppb). These monoclonal antibodies were suitable for detection of very small amounts of fragrant compounds in rinsed water of beverage industries.

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