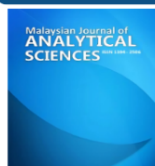

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Research Article

Preparation and characterisation of an anti-benzylacetone monoclonal antibody and its use for histochemical analysis of *trans*-cinnamic acid in *Cinnamomum cassia* branches using the dot blot method

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Abstract
The pharmacological effects of various inhaled aroma compounds have been investigated, such as benzylacetone used as a positive control in behavioural tests. To develop a monoclonal antibody (MAb) for detecting and quantifying the aroma compound benzylacetone in sera obtained from experimental animals, immunogenic conjugates were synthesised using 3-phenylpropanoic acid (3PPa), a benzylacetone-like compound coupled to carrier proteins. Mice were immunised with 3PPa-keyhole limpet haemocyanin, and a MAb-secreting hybridoma was obtained. Cross-reactivities and detection limits were determined for 36 compounds by enzyme-linked immunosorbent assay (ELISA). Although the MAb cross-reacted with several phenylpropanoids, alkaloids, and flavonoids, its detection limits (> 0.1 μg/mL) were unsuitable for ELISA. However, the MAb successfully visualised *trans*-cinnamic acid and 3PP using the dot blot method. The MAb strongly immunostained *trans*-cinnamic acid localisation in cinnamon bark. Therefore, this anti-benzylacetone MAb may be a powerful tool for visualising the biosynthesis and distribution of these phenylpropanoids in biological samples.

Keywords: *trans*-cinnamic acid, monoclonal antibody, immunostaining

Introduction

The inhalation of aroma compounds can have sedative, anti-depressant, anti-anxiety, and appetite-enhancing effects in mice [1-3]. In particular, the aroma compound benzylacetone is often used as a positive control compound in studies on sedative and appetite-enhancing effects [1,3]. Benzylacetone is also an aroma compound in the smoke of agarwood, which is a fragrant wood, because the chromones in agarwood are converted to benzylacetone and its analogues by thermal decomposition [4]. We have examined the behaviours of mice after inhalation of various aroma compounds, such as benzylacetone. In these studies, small amounts of aroma compounds showed effects; however, the quantities were too small to measure, even using solid-phase microextraction-gas chromatography-mass spectrometry (GC-MS) analysis. Therefore, another method is needed for analysing small amounts of vaporised compounds in air. Enzyme-linked immunosorbent assay (ELISA) is a suitable method for analysing the amounts of aroma compounds in liquid, and monoclonal antibodies (MAbs) have been used to quantify compounds at picogram per millilitre concentrations in solution [5]. MAbs against some aroma compounds have been prepared [6]; thus, we expected that we could prepare an anti-benzylacetone MAb to detect small amounts of vaporised volatile compounds in air. Furthermore, most phenylpropanoids are not conventionally stained by immunochemical methods. The successful use of this MAb technique, therefore, makes it possible to confirm the presence of small molecular compounds on blotting sheets. Benzylacetone, which has a molecular weight of 148.20 g/mol, is a hapten, which has no or weak immunogenicity [7], and thus must be combined with large-molecular-weight carrier proteins, such as human serum albumin (HSA), keyhole limpet haemocyanin (KLH), and ovalbumin (OVA), to generate an immune response. A sugar moiety or carboxylic group is a desirable feature for haptens that allows them to be coupled easily with carrier proteins. However, benzylacetone does not have either of these. 3-Phenylpropanoic acid (3PPa), which is structurally similar to benzylacetone, is a suitable analogue because it has a carboxylic acid group instead of an α-methyl ketone group. The carboxylic group of 3PPa can form amide or ester bonds with amino or hydroxyl groups on carrier

proteins to form a conjugate bearing the structure of benzylacetone, except the α-methyl moiety. In this study, we report in detail on the preparation of the anti-benzylacetone MAb and its characterisation, as well as on the use of this MAb in the development of an immunostaining method for histochemical localisation of *trans*-cinnamic acid in some parts of *Cinnamomum cassia*.

Materials and Methods

Chemicals and immunochemicals

3PPa, acetophenone, *trans*-anethole, benzylacetone, benzalacetone, berberine chloride, butylbenzene, butyrophenone, *trans*-cinnamyl alcohol, cortico sterone, *p*-cymene, eugenol, eugenol acetate, 1-phenyl-2-butanone, 2-phenylethylamine, 3-phenylpropanal, quercetin, 1-(3-dimethyl aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). *trans*-Cinnamic acid, methanol, and cholesterol were purchased from Kishida Chemical (Osaka, Japan). Daidzein, L-phenylalanine, L-tyrosine, tyramine, *o*- and *p*-coumaric acids, HSA, OVA, mouse serum albumin (MSA), polyethylene glycol, aminopterin, and HT Media Supplement (50×) Hybri-Max (hypoxanthine and thymidine) were purchased from Sigma-Aldrich (St. Louis, MO). *γ*-Amino-*n*-butyric acid (GABA), benzyl acetate, *β*-caryophyllene, coumarin, *l*-menthol, hesperidin, hesperetin, quinidine sulfate, KLH, bovine serum albumin (BSA), skim milk powder, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 4-chloro-1-naphthol, and glycerol were obtained from Fujifilm Wako Pure Chemical Corporation (Tokyo, Japan). *trans*-Cinnamaldehyde, benzaldehyde, glycyrrhizinic acid, and dimethyl sulfoxide (DMSO) were obtained from Nacalai Tesque (Kyoto, Japan). Thymol, *N*-benzylacetamide and 2-methoxycinnamaldehyde were purchased from Hayashi Pure Chemical (Osaka, Japan), CHEM-IMPEX (Wood Dale, IL) and WuXi LabNetwork (Wuhan) Chemical Technology Co., Ltd. (Wuhan, China), respectively.

Sodium chloride, potassium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate dodecahydrate, polyoxyethylene (20) sorbitan monolaurate (Tween 20), citric acid monohydrate, trisodium citrate dehydrate, hydrogen carbonate, and sodium carbonate were purchased from Fujifilm Wako Pure Chemical Corporation. Hydrogen peroxide (30%) was purchased from Tokyo Chemical Industry. BupH MES-Buffered Saline Packs were purchased from Thermo Fisher Scientific (Waltham, MA). Sodium hydroxide was purchased from Fujifilm Wako Pure Chemical Corporation and tris(hydroxymethyl) aminomethane (Tris) was purchased from Kishida Chemicals. Hydrochloric acid (35%) was purchased from, Junsei Chemical (Tokyo, Japan). These chemicals were used to prepare 20 mM phosphate buffer (PBS; pH 7.4), 0.05% Tween 20 containing PBS (T-PBS), 0.0–6% H_2O_2 containing 0.2 M citrate buffer (pH 4.0), 50 mM carbonate buffer, and 2-(*N*-morpholino)ethanesulfonic acid (MES)-buffered saline for immunisation and ELISA. In addition, these chemicals were used to prepare 20 mM phosphate buffer (pH 7.0), 100 mM citrate buffer (pH 2.7), and 1 M Tris-HCl buffer (pH 9.0) for purification of MAb.

Synthesis of 3PPa-carrier protein conjugates

3PPa-carrier protein conjugates were synthesised as follows. 3PPa (5 mg) was dissolved in methanol (0.5 mL) and mixed with 0.1 M MES buffer (0.5 mL). The mixture was mixed with KLH dissolved in MES buffer (1:1 ratio) and allowed to react at room temperature for 15 h. The mixtures were dialysed using dialysis membranes (Spectra/Por dialysis membrane, Spectrum Chemical Manufacturing, New Brunswick, NJ; molecular weight cut-off: 6–8 kD) at 4 °C for 2 days, and then lyophilised to obtain 2.8 mg of purified 3PPa-KLH conjugate. The 3PPa-HSA and 3PPa-OVA conjugates were synthesised in the same manner and yields of 4.0 and 5.5 mg, respectively, were obtained.

Determination of hapten number in 3PPa-carrier protein conjugates by matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry

This section was followed by our previous study [8] with some improvements. 3PPa-HSA was diluted in water and serially diluted into 1–10 pM conjugate solution. This solution was mixed with a matrix solution, which contained a 10^3-fold molar excess of sinapinic acid (Bruker Daltonics, Billerica, MA) dissolved in water containing acetonitrile (Fujifilm Wako Pure Chemical Corporation) at 30% (v/v) and trifluoroacetic acid (Nacalai Tesque) at 0.1% (v/v). The solution was spotted on a ground steel target plate (MTP 384, Bruker Daltonics), air dried, and analysed with a matrix-assisted laser desorption-ionisation time-of-flight (MALDI-TOF) mass spectrometer (Bruker Autoflex III, Bruker Daltonics) by using nitrogen laser irradiation (337 nm, 200 Hz maximum firing frequency). The spectra were analysed using flexControl software (Bruker Daltonics) and the hapten number of 3PPa-HSA was determined.

Animals

The animal studies were designed according to the recommendation of the Committee of Animal Experiments at Daiichi University, Fukuoka, Japan (authorisation number: 18007-23). Six-week-old female Balb/c mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were housed in colony cages (two mice per cage) at an ambient temperature of 25 ± 2 °C under a 12-h light–dark cycle. Water and pellet chow were administered *ad libitum*.

Immunisation and hybridisation

Female Balb/c mice were immunised by

intraperitoneal administration of the 3PPa-KLH conjugate eight times. In the first immunisation, the mice were administered a 1:1 emulsified mixture of 50 μg of 3PPa-KLH suspended in PBS and Freund's complete adjuvant (Sigma-Aldrich). Two weeks later, the second immunisation was conducted in the same manner except with Freund's incomplete adjuvants instead of the complete adjuvant. Then, starting 2 weeks later, the third to eighth immunisations were given at 2-week intervals by administering 100 μg of 3PPa-KLH suspended in PBS to the mice. Three days after the final immunisation, the mice were killed by cervical dislocation, the spleen was removed, and then the immune splenocytes were exenterated and fused to a hypoxanthine-aminopterin-thymidine (HAT)-sensitive myeloma cell line (SP2/0) using the polyethylene glycol method [9]. After HAT selection, the IXF1-A7Bf12 hybridoma cell line secreting MAb IXF1-A7Bf12 was cloned using two limiting dilution protocols. The IXF1-A7Bf12 hybridoma cell line was cultured at 37 °C under a 5% CO_2 atmosphere in eRDF medium containing 10% foetal bovine serum (FBS; HyClone, Thermo Fisher Scientific; lot: AXJ47554). The hybridoma was cultured in FBS-eRDF, the amount of FBS was decreased, and then the hybridoma was cultured in eRDF containing RD-1 for 2 weeks. Finally, serum-free culturing medium containing MAb IXF1-A7Bf12 was obtained [8].

Purification of MAb IXF1-A7Bf12

The hybridoma culture medium supernatant was purified using a Protein G Sepharose 4 Fast Flow column (GE Healthcare, Chicago, IL; length 5 cm, diameter 1 cm). The medium-free culturing medium containing immunoglobulin (IgG) was centrifuged at 3000 rpm for 15 min to remove the hybridoma, and then the supernatant was filtered using a glass filter (Merck Millipore, Burlington, MA; 2 μm pores). The supernatant was loaded into the column using a peristaltic pump (SJ-1211II-H Perista, ATTO, Tokyo, Japan) at 4 °C overnight, and the column was washed with 20 mM phosphate buffer (pH 7.0). The MAb was eluted with 100 nM citrate buffer (pH 2.7) and neutralised using Tris-HCl buffer (pH 9.0). Fractions containing the MAb were dialysed against water at 4 °C for 2 days. After lyophilisation, MAb (72.2 mg) was obtained. The isotype of the MAb was investigated using a Mouse Antibody Isotyping Kit (Immunology Consultants Laboratory Inc., Portland, OR) [8]. The purity of MAb IXF1-A7Bf12 was calculated by sandwich ELISA. Anti-mouse IgG antibody (H+L, from goat, MP Biochemicals, Santa Ana, CA) was adsorbed to the wells of a 96-well immunoplate (Nunc, Roskilde, Denmark), and then it was treated with PBS containing 5% skimmed milk (300 μL) for 1 h to reduce non-specific adsorption. Standard protein mouse IgG (Sigma-Aldrich) was dissolved in T-PBS to prepare a 100 ng/mL solution, which was then diluted stepwise to plot a standard curve. The MAb dissolved in 10% T-PBS (100 μg/mL) was also diluted stepwise. The plate was washed with T-PBS and reacted with the standards and MAb solutions for 1 h. The plate was washed with T-PBS, and then the standard mouse IgG and MAb were combined with 100 μL of a 1:1000 dilution of peroxidase-labelled anti-mouse IgG (Fc specific, from goat, Sigma-Aldrich) solution for 1 h. After the plate was washed three times with T-PBS, 100 μL of 0.003% H_2O_2 and 0.3 mg/mL ABTS containing 0.1 M citrate buffer (pH 4.0) were added to each well and incubated for 15 min. Absorbance was measured with a microplate reader (CHROMATE 4300, Awareness Technology Inc., Palm City, FL) at 405 and 492 nm. The MAb solutions were quantified using their absorbance in the quantitative range of the IgG standard. The concentrations of the solutions were multiplied by the dilution ratio to obtain the concentrations of the MAb in 5 mg/mL MAb solution, and then the concentrations of the MAb were multiplied 100 and divided by 5 mg to yield the purity of the MAb [8]. All reactions were conducted at room temperature.

Characterisation of MAb against proteins by direct ELISA

The reactivities of anti-3PPa MAb to 3PPa-HSA, 3PPa-OVA, and other proteins, such as HSA, BSA, MSA, KLH, and OVA, were determined by direct ELISA. The conjugate and proteins (100 μL, 1 μg/mL) were adsorbed to the wells of a 96-well immunoplate that was treated with PBS containing 5% skimmed milk (300 μL) for 1 h. The plate was washed with T-PBS, and MAb was dissolved in 10% DMSO-PBS to prepare 18 μg/mL solutions. The solution was diluted stepwise, added to the plate, and incubated for 1 h. The plate was washed with T-PBS, MAb was combined with peroxidase-labelled anti-mouse IgG (Fc specific) solution diluted 1:5000 in Can Get Signal Solution 2 (Toyobo, Osaka, Japan) for 1 h. After washing the plate three times with T-PBS, 100 μL of 3,3′,5,5′-tetramethylbenzidine solution (SureBlue, SeraCare Life sciences, Milford, MA) were added to each well and incubated for 5 min, and then reaction was stopped by adding 0.5 M sulfuric acid solution (100 μL). Absorbance was measured by a microplate reader at 450 and 630 nm [8].

Cross-reactivity of MAb by competitive ELISA

Competitive ELISA was conducted as follows. 3PPa-OVA was adsorbed (100 μL, 1 μg/mL) to a 96-well immunoplate and treated with PBS containing 5% skimmed milk (300 μL) for 1 h. The plate was washed with T-PBS, and benzylacetone and several compounds were dissolved in 10% DMSO-PBS or MES to prepare 10 mM solutions, which were diluted stepwise to 9.8×10^{-3} mM with 10% DMSO-PBS or MES buffer. Those solutions were added to the plate, and 50 μL of 10% DMSO-PBS or MES was added to the rest of the wells as a control. The MAb (50 μL, 0.5 μg/mL dissolved in 10% DMSO-PBS or T-PBS)

solution was reacted at 4 °C overnight. The following methods were the same as for direct ELISA.

The solutions of benzylacetone related compounds and other compounds were prepared from stock solutions in DMSO. The cross-reactivities of MAb against benzylacetone and various compounds were determined according to Weiler and Zenk's equation [10].

Immunostaining and immunohistochemical analysis using the dot blot method

Standard compounds were dissolved in methanol to prepare 10 mM solutions, except for L-tyrosine, which was dissolved in 0.1% $NaHCO_3$ aqueous solution. The standard solutions (2 µL) were spotted on a positively charged polyethersulfone (PES) membrane (Pall Life Sciences, Port Washington, NY; 0.2 µm pores) using the tip of a micropipette, and then the membrane was air-dried. The membrane was put in a tray with lid, and soaked in 0.1 M MES buffer solution containing 2% EDC and 1% NHS at room temperature for 1 h. The solution was removed and the membrane was soaked in 1% BSA-PBS solution for 3 h with shaking. After removing the 1% BSA-PBS solution, the membrane was washed using PBS, and then it was soaked in 5% skimmed milk-PBS solution overnight at room temperature with shaking. The skimmed milk solution was removed, the membrane was washed with PBS, and then it was reacted with 2.3 µg/mL MAb dissolved in T-PBS or PBS for 3 h with shaking. The membrane was washed with T-PBS twice and reacted with 500-fold diluted secondary antibody (anti-mouse anti-mouse IgG (H+L), from goat) for 1 h with shaking. The membrane was washed with T-PBS twice, rinsed with PBS, and then the membrane was reacted with the substrate solution, which was a 1:10 mixture of 10 mg/mL 4-chloro-1-naphthol methanol solution and PBS containing 0.03% hydrogen peroxide, for 20 min at room temperature. The reaction was stopped by washing with distilled water, and the immunostained PES membrane was allowed to dry and was photographed or scanned immediately [11]. Vertically and horizonally cut branches of *Cinnamomum cassia* (L.) J. Presl (Lauraceae) were blotted on the PES membrane and were immunostained as well.

Qualitative and quantitative GC-MS analysis

Qualitative and quantitative analyses of the compounds immunostained in the dot blot method were performed by GC-MS. Samples were prepared as follows. Dried cinnamon bark which was obtained from the rest of the *C. cassia* branch that was used for immunohistochemical analysis by the dot blot method was powdered using a mill mixer. The powder (316.6 mg) was added to a mixture of pentane, dichloromethane and acetic acid (3166 µL, 1000:1000:1), the mixture was stirred for 13 h, and then centrifuged at 5000 rpm for 5 min. The supernatant was transferred to a new vial and dried using anhydrous sodium sulfate. The sample solution was prepared by dissolving 1 mg of *trans*-anethole (>98%, Tokyo Chemical Industry), which is not found in *C. cassia* bark, as an internal standard in 1 mL of the extract solution. The sample solution was analysed on a GC-MS system (JMS-Q1500GC, JEOL, Tokyo, Japan) equipped with an Inertcap WAX column (GL Sciences, Tokyo, Japan; 60 m × 0.25 mm, 0.25 µm film thickness). The following operating conditions were similar to those described previously [12], with some modifications: injector temperature, 250 °C; carrier gas, helium; column flow, 1.0 mL/min; injection volume, 1 µL (splitless); and mass range *m/z* 50–500. The oven program was hold at 50 °C for 4 min, increase by 4 °C/min to 250 °C, and hold at 250 °C for 40 min. *trans*-Cinnamic acid, 3PPa, and internal standard *trans*-anethole were identified by comparing their mass spectra with those in a data library (NIST11, National Institute of Standards and Technology) and by their retention indices. Tetracosane (Fujifilm Wako Pure Chemical Corporation), hexacosane (Tokyo Chemical Industry Co., Ltd.), octacosane, and triacontane (Sigma-Aldrich) were used as authentic compounds to obtain the GC-MS retention indices. Quantitative analysis of *trans*-cinnamic acid and 3PPa contained in the cinnamon bark was performed using the relative standard curves. Samples containing these two compounds at seven concentrations from 0.5 mg/mL to 3.2×10^{-5} mg/mL, that is, the 5-fold stepwise diluted concentrations, were used to prepare the standard curves. *trans*-Anethole was added to the solvent as an internal standard in every sample at a concentration of 1 mg/mL. The fragment ion peaks used in the quantification were *m/z* 148 for *trans*-anethole, *m/z* 147 for *trans*-cinnamic acid, and *m/z* 91 for 3PPa.

Results and Discussion

Conjugate synthesis, immunisation, and purification of MAb

In a previous report, a conjugate that was combined with 3 mol of crocin was used to immunise mice and obtain a MAb [13]. Therefore, we expected that it would be feasible to immunise mice and yield a new MAb against aroma compounds. HSA, KLH, and OVA were reacted with 3PPa (1:1, w/w) using EDC/NHS to yield immunogenic conjugates. The reactions yielded 4.0 mg of 3PPa-HSA conjugate, 2.8 mg of 3PPa-KLH conjugate, and 5.5 mg of 3PPa-OVA conjugate. The number of hapten molecules combined with the carrier protein was determined for the 3PPa-HSA conjugate with MALDI-TOF-MS. The molecular weight of 3PPa-HSA was 68211.9 *m/z* and that of HSA was 66625.1 *m/z*. Therefore, the increase in weight following the conjugation of the hapten was determined to be 1586.8 *m/z*. This value was then divided by the difference in molecular weight between 3PPa (150.18 g/mol) and water (18.02 g/mol), a net value of 132.16 g/mol, to determine the hapten number as 12.0. This result indicates that 1 mol of HSA was

successfully combined with 12 mol of 3PPa. Balb/c mice hyperimmunised with 3PPa-KLH yielded splenocytes producing an antibody reactive to 3PPa-HSA and the splenocytes were fused with SP2/0 myeloma cells using the polyethylene glycol method. After strain selection and cultivation, a hybridoma strain, IXF1-A7Bf12, that produced the MAb was established. The hybridoma was initially grown in 5% FBS-eRDF medium. Subsequently, it was cultivated in eRDF medium containing the RD-1 supplement, which is a serum-free formulation consisting of sodium selenite, insulin, transferrin, and 2-aminoethanol. After 2 weeks of cultivation, 400 mL of serum-free medium containing the MAb against aroma compounds was obtained. The MAb was extracted from the serum-free medium using Protein G affinity chromatography, and 72.2 mg of the MAb was obtained. The MAb had a κ light chain and was classified into the IgG1 subclass antibody group. The purity of the MAb was determined using sandwich ELISA against mouse IgG. The purified MAb contained 9.16 ± 0.28% IgG1 after dialysis and lyophilisation.

Reactivity of MAb against several conjugates and carrier proteins

The reactivity of MAb against several 3PPa conjugates and carrier proteins was analysed using direct ELISA by measuring the MAb concentration (**Figure 1**). The absorbance of the carrier proteins increased at MAb concentrations higher than 2.29 µg/mL in terms of IgG (25 µg/mL). The absorbance of MSA was about 10 times higher than those of other carrier proteins. The absorbance of 3PPa-HSA and 3PPa-OVA increased at MAb concentrations higher than 0.14 µg/mL in terms of IgG (1.56 µg/mL), and 3PPa-HSA had an absorbance approximately twice that of 3PPa-OVA at a lower MAb concentration. The results suggested that 3PPa-OVA was a suitable fixed antigen for indirect competitive ELISA. Absorbance was approximately 1.0 at a MAb concentration of 0.69 µg/mL in terms of IgG (7.50 µg/mL), but this was not an appropriate MAb concentration for indirect competitive ELISA assays to be performed well. An antibody concentration of 0.5 µg/mL in terms of IgG (5.5 µg/mL) with an absorbance of approximately 0.5 was selected for competitive ELISA.

Cross-reactivities of MAb in competitive ELISA

Benzylacetone, which has ketone and phenyl groups, has many analogues with similar functional groups, such as *trans*-cinnamaldehyde and benzalacetone. The MAb may also have affinities with these analogues. Therefore, the cross-reactivity of the MAb against various analogues was investigated following a method from our previous studies [14,15]. The cross-reactivities of the MAb were investigated by indirect competitive ELISA and calculated using Weiler and Zenk's method (**Table 1**) [10]. We set the reactivity of the MAb with benzylacetone as 100%. The cross-reactivities of benzalacetone, *trans*-cinnamaldehyde, *trans*-cinnamyl alcohol, and *N*-benzylacetamide were 9.91-, 1.16-, 31.72-, and 5.44-fold higher, respectively, than the reactivity of benzylacetone. Compounds exhibiting a more planar conformation generally demonstrated stronger reactivity compared to their more flexible counterparts. This trend was observed when comparing compound pairs such as benzylacetone and benzalacetone, 3-phenylpropanal and *trans*-cinnamaldehyde, and 3PPa and *trans*-cinnamic acid (see video files in the supporting information). In addition, recognition by the MAb was observed to be weakened by shorter aliphatic chains, as in benzaldehyde and acetophenone, as well as by the variation in the position of the carbonyl group when comparing benzylacetone, 1-phenyl-2-butanone, and butyrophenone. The compounds without carbonyl or hydroxyl groups, such as *trans*-anethole, eugenol, eugenol acetate and butylbenzene, were not recognised by the MAb. These findings suggest that the distances between the key amino acid residues in the binding site and the hapten's carbonyl or hydroxyl groups and phenyl group are spatially comparable to the corresponding distances in planar phenylpropanoids. These results suggest that the presence of a carbonyl group at the 3-position relative to the phenyl group, combined with a double bond between them, leads to stronger MAb recognition. Some compounds, such as *trans*-cinnamic acid, and 2-phenylethylamine, showed cross-reactivity similar to but not exceeding that of benzylacetone.

From the perspective of amines and amino acids, compounds such as L-tyrosine and L-phenylalanine exhibited comparatively low cross-reactivities. This low reactivity is likely attributable to the ionisation of the carboxylic acid group under the physiological pH of 7.4. The same observation was made for 3PPa. This observation underscores that the overall reactivity is substantially influenced by differences in chemical structure. Specifically, the molecular structures of compounds such as *trans*-cinnamic acid and 3PPa are hypothesised to exhibit higher reactivity than their ionic counterparts. Furthermore, other compounds that are difficult to vaporise, such as the flavonoid quercetin, showed 2.8-fold higher reactivity than that of benzylacetone. The MAb exhibited cross-reactivities with some compounds bearing a benzene ring, such as flavonoids, berberine, and thymol. The recognition of flavonoids and berberine by the MAb was likely due to the specific spatial arrangement of the aromatic rings and the presence of key substituents that can participate in hydrogen bonding. This is particularly relevant for the oxygen- or nitrogen-containing moieties found at positions corresponding to the 3-position of the hapten structure. This suggests that hydrogen bonding may be involved in MAb-hapten binding. The weak recognition observed for hesperidin and hesperetin is likely due to the flexible rotation of the B ring linked to the C-position of the

chromanone structure. Based on these results, MAb IXF1-A7Bf12 was identified as an anti-benzylacetone MAb showing cross-reactivities against benzalacetone and other compounds, including several phenylpropanoids, in ELISA. Furthermore, other factors affecting MAb-hapten binding estimated by molecular simulations were a planar conformation and substituents forming hydrogen bonds with the MAb, which acts as both a hydrogen donor and receptor, and the distance between the aromatic ring and carbonyl group.

Standard curves for benzylacetone and *trans*-cinnamaldehyde analogues

The standard curves for volatile compounds with cross-reactivities over 50% (benzylacetone, 3-phenylpropanal, benzalacetone, *trans*-cinnam aldehyde, *trans*-cinnamyl alcohol, and *trans*-cinnamic acid) were calculated by competitive ELISA using the anti-benzylacetone MAb. The calibration ranges were 3.9×10^{-2} to 10, 9.8×10^{-3} to 10, 9.8×10^{-4} to 0.5, 9.8×10^{-3} to 5, 9.8×10^{-4} to 0.5, and 3.9×10^{-2} to 10 mM, respectively (supporting information; Fig. S1). The lowest limit of detection was 9.8×10^{-4} mM (0.13 µg/mL), for *trans*-cinnamyl alcohol. In general, ELISA can detect picogramme samples[5]; however, the ELISA using anti-benzylacetone MAb in this study would not be sufficiently sensitive for analysing small amounts of volatile compounds contained in mouse serum after administration via inhalation. Furthermore, limit of detection for *trans*-cinnamaldehyde by GC-MS was 0.1 µg/mL in our previous study [15]; thus, the limit of detection for *trans*-cinnamaldehyde by ELISA using this MAb of 9.8×10^{-3} mM (1.3 µg/mL) indicated that ELISA was an order of magnitude less sensitive.

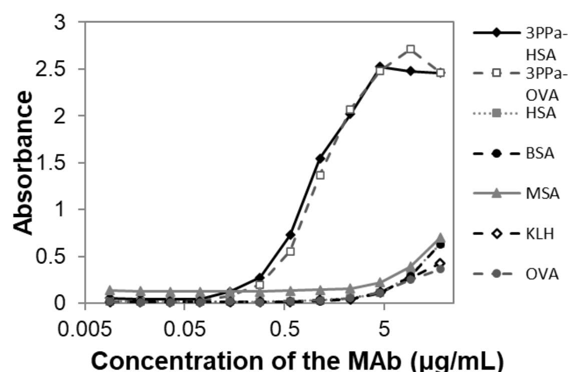

Figure 1. Reactivity of MAb against several 3PPa conjugates and carrier proteins

Table 1. Cross-reactivities of anti-benzylacetone MAb against various compounds

Compound	Cross Reactivity (%)	Compound	Cross Reactivity (%)
Phenylpropanoids		Terpenoids	
Benzylacetone	100.00	*l*-Menthol	<3.93
3-Phenylpropanal	72.78	Thymol	4.95
3-Phenylpropanoic acid	21.08	*p*-Cymene	<3.93
Benzalacetone	990.52	*β*-Caryophyllene	<3.93
trans-Cinnamaldehyde	115.93	Glycyrrhizinic acid	<3.93
trans-Cinnamyl alcohol	3171.86	Flavonoids	
trans-Cinnamic acid	61.60	Hesperidin	40.25
1-Phenyl-2-butanone	9.56	Hesperetin	27.02
Butyrophenone	9.87	Quercetin	279.66
Benzaldehyde	30.00	Daidzein	18.48
Acetophenone	34.17	Alkaloids	
Benzyl acetate	4.29	Berberine chloride	34.15
Coumarin	9.67	Quinidine sulfate	<3.39
trans-Anethole	<3.93	Serum compounds	
Eugenol	<3.93	Cholesterol	<3.39
Eugenol acetate	<3.93	Corticosterone	<3.39
Butylbenzene	<3.93		
N-Benzylacetamide	543.57		
Amino acids and amines			
L-Tyrosine	7.07		
Tyramine	13.99		
L-Phenylalanine	3.19		
2-Phenylethylamine	72.78		
GABA	<3.93		

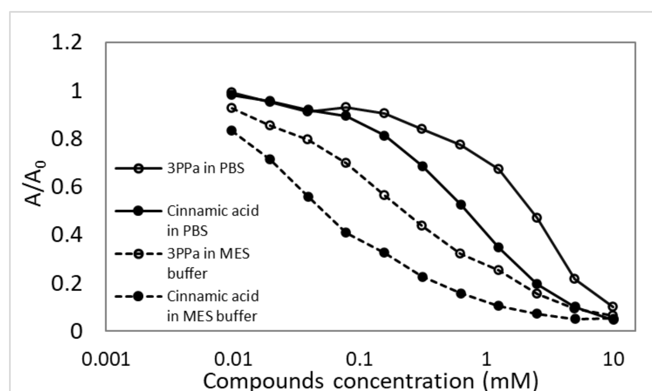

Figure 2. Effect of pH on the MAb's recognition of 3PPa and *trans*-cinnamic acid

Effects of pH, surfactant, and temperature on affinity of carboxylic compounds

3PPa and *trans*-cinnamic acid, which have a carboxyl group, were ionised in PBS (pH 7.4)/10% DMSO-PBS (1:1) solution. The effects of carboxyl group ionisation were evaluated by comparing cross-reactivities measured by competitive ELISA with the anti-benzylacetone MAb in solution containing MES buffer (pH 4.7) instead of PBS. The A/A_0 value of 3PPa was observed at an 8.75-fold lower concentration in MES buffer than in PBS (**Figure 2**; A/A_0 = 0.5: 2.18 mM in PBS and 0.25 mM in MES buffer), and that of *trans*-cinnamic acid was observed at a 13.25-fold lower concentration in MES buffer than in PBS (**Figure 2**; A/A_0 = 0.5: 6.85×10^{-1} mM in PBS and 5.17×10^{-2} mM in MES buffer). These results suggested that the reaction with the MAb was weaker when electrons were delocalised over the ionised carboxylic acid. On L-tyrosine and L-phenylalanine, an amino group and a carboxyl group would be ionised or become a zwitterion under neutral conditions. Therefore, tyramine and 2-phenylethylamine, the putrefactive amines of L-tyrosine and L-phenylalanine, would show stronger effects than the original amino acids, because they were less ionised at pH 7.4 than L-tyrosine and L-phenylalanine and did not form zwitterions. Therefore, the optimal pH for analysis would be the value at which the ionising compounds predominantly exist in their un-ionised molecular form.

Dot blot method and concentration-dependent staining

In the dot blot method using anti-benzylacetone MAb, 3PPa and *trans*-cinnamic acid were strongly immunostained purple using 4-chloro-1-naphthol, whereas compounds without a carboxylic group, including amino acids like L-tyrosine and L-phenylalanine, or with a short aliphatic chain, such as benzoic acid, were not stained (**Figure 3A**). In addition, 3PPa-HSA and 3PPa-OVA were strongly stained, whereas the carrier proteins were not stained at all. 3PPa and *trans*-cinnamic acid were immunostained strongly by the MAb diluted in PBS (**Figure 3A**) but weakly by the MAb diluted in T-PBS (**Figure 3B**). The dot blot reacted weakly when the anti-benzylacetone MAb was diluted using T-PBS. Similar cases were observed in competitive ELISA using the MAb (**Figure S2**). These cases suggested that surfactants such as Tween 20 could inhibit reaction of the MAb with target aroma compounds. These findings suggest that a surfactant-free solvent should be prepared to optimally enhance the antibody-antigen reaction for detecting and quantifying small compounds. In this study, the cross-reactivities of the MAb observed using the dot blot method and immunostaining were different from those observed using competitive ELISA. Not only aroma compounds such as benzylacetone and benzalacetone but also some aromatic compounds like quercetin and berberine were not immunostained in the dot blot method despite exhibiting reactivity in indirect competitive ELISA (**Figure 5**). It is considered that capability of compounds linked with carrier protein would be concerned with cross-reactivity of compounds in dot blot method. Because, hapten linked with carrier protein such as 3PPa-HSA was strongly immunostained using anti-benzylacetone MAb. Therefore, haptens like *trans*-cinnamic acid would be not only fixed stably on the membrane as conjugates, but enhance antigen-antibody reaction in dot blot method. Some compounds owning amino group can form a bond with carrier protein, however those compounds would be ionised in MES buffer (pH 4.5) and dissolved in the buffer. Therefore, those compounds may not have been immunostained. Additionally, some compounds owning carboxylic group were not immunostained in the cases that phenyl group has substituents like *o*- and *p*-coumaric acids or aliphatic chain is short like benzoic acid. According to some previous reports, the same cross-reactivity characteristics were observed for sennosides A and B in both the dot blot method and ELISA [8,11,16]. However, some other studies reported that cross-

activities for ginsenoside Re were different between the dot blot method and ELISA [17,18]. The cross-reactivities of these two methods using anti-benzylacetone MAb are expected to be similar to the case of anti-ginsenoside Re MAb, and the anti-benzylacetone MAb showed high specificity against *trans*-cinnamic acid and 3PPa in immunostaining, despite low specificity in ELISA. Stepwise diluted 3PPa and *trans*-cinnamic acid solutions were stained by the dot blot method, and the strengths of the spots depended on the 3PPa and *trans*-cinnamic acid concentrations (**Figure 3C**). Visible spots were observed for 6.25×10^{-1} to 20 mM 3PPa and 2.5 to 20 mM *trans*-cinnamic acid. The smallest compound that has been immunostained is D-Ala (molecular weight: 89.09 g/mol) with 3-amino-9-ethylcarbazole [19], and the smallest compound that has been immunostained using 4-chloro-1-naphthol is aristolochic acid II (molecular weight: 311.25 g/mol) [20]. The molecular weight of *trans*-cinnamic acid (148.16 g/mol) is half less than that of aristolochic acid II; therefore, *trans*-cinnamic acid is the smallest compound immunostained using 4-chloro-1-naphthol. Although D-Ala was not been blotted on PES or PVDF membranes, it was immunostained on a section of rat pancreas [19]. Therefore *trans*-cinnamic acid is the smallest compound visualised on a PES membrane by immunostaining. The strength of immunostaining of the stepwise-diluted standards was dependent on the concentration of the samples, with stronger immunostaining at higher concentrations. Our immunostaining method would be suitable for semi-quantitative analyses.

Immunohistochemical analysis on a slice of *C. cassia* branch

The dot blot method was used for immunohistochemical analyses of C. cassia branches cultivated in the botanical garden in Daiichi University of Pharmacy. The branches were blotted on the PES membrane by applying even pressure, and then the membrane was immunostained with the anti-benzylacetone MAb. The regions of the membrane blotted with the medulla and xylem were gradually stained red-brown; therefore, the branch (**Figure 4A**), and the membrane before immunostaining (**Figure 4B**) and after immunostaining (**Figure 4C**) were compared to show which parts were immunostained.

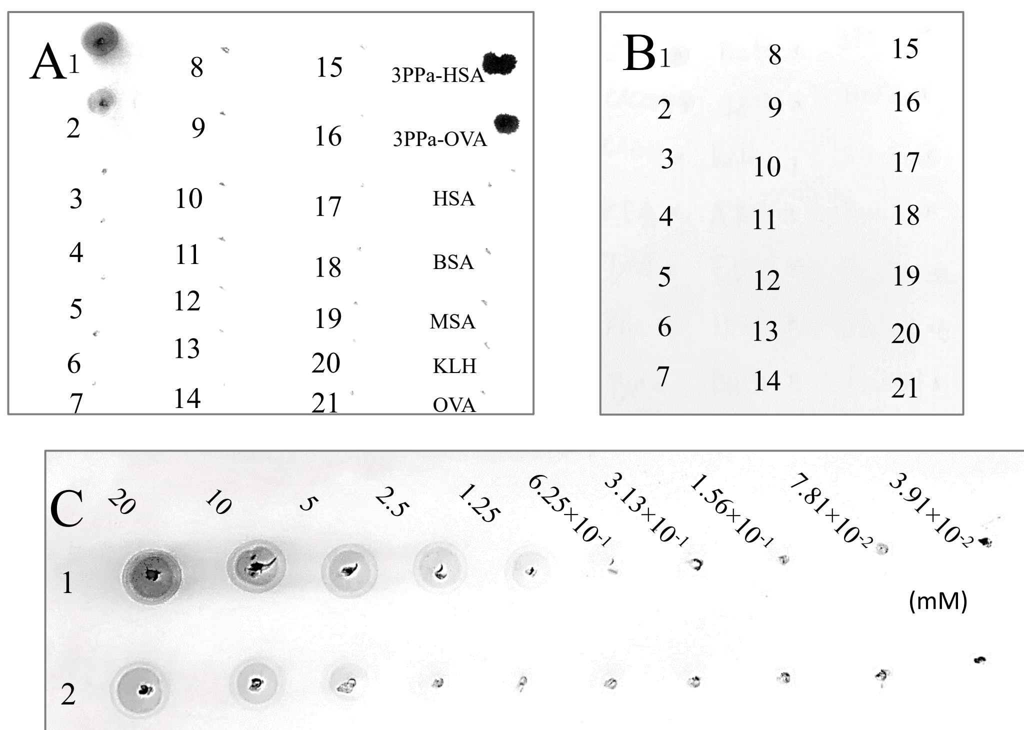

Figure 3. Dot blot analysis of aroma compounds using the anti-benzylacetone Mab. A: treated with the MAb diluted in PBS solution. B: treated with the MAb diluted in T-PBS solution. C: Immunostaining of stepwise diluted 3PPa and *trans*-cinnamic acid. In A and B, the spots were 2 μL of 10 mM compound solutions, and 1 μL of 1 mg/mL conjugate and carrier protein solutions. Compounds : 1. 3PPa, 2. *trans*-cinnamic acid, 3. *trans*-cinnamyl alcohol, 4. 2-phenylethylamine. 5. tyramine, 6. phenylalanine, 7. tyrosine, 8. benzylacetone, 9. *trans*-cinnamaldehyde, 10. benzalacetone, 11. *trans*-anethole, 12. eugenol, 13. 1-phenyl-2-butanone, 14. butyrophenone, 15. 3-phenylpropanal, 16. benzyl acetate, 17. *N*-benzylacetamide, 18. butylbenzene, 19. benzaldehyde, 20. acetophenone, 21. benzoic acid.

To identify the compounds contained in the immunostained regions of the bark, the rest of the bark from cinnamon branch used in immunostaining was analysed by GC-MS. The sample solution contained $4.3 \times 10^{-3} \pm 2.9 \times 10^{-4}$ mg/mL of *trans*-cinnamic acid (retention index (RI): 2853; standard RI: 2852; reference RI [21]: 2844), whereas 3PPa (standard RI: 2611; reference RI [22]: 2603) was not detected. We extracted 316.6 mg of cinnamon bark with 3166 µL of solvent. Thus, the amount of *trans*-cinnamic acid contained in 1 mL of sample solution corresponded to the amount in 0.1 g of cinnamon bark. In other words, 1 g of cinnamon bark contained 43 ± 2.9 µg of *trans*-cinnamic acid.In the dot blot method, *p*- and *o*-coumaric acids contained in cinnamon bark [23] were not stained (Fig. 5) and other aromatic compounds without a carboxylic group such as quercetin [24], coumarin, and 2-methoxycinnamaldehyde contained in cinnamon bark and berberine were also not stained. Therefore, *trans*-cinnamic acid was the main compound immunostained in the branch slice of *C. cassia*. Histochemical and quantitative analyses of *trans*-cinnamic acid contained in several parts of *C. cassia* or related species have been reported [25,26]. The parts of the cinnamon plants that were analysed were mainly the bark, heartwood, and leaf. However, branches or twigs were considered as a single part, even though they consist of bark and a core part. The histochemical localisation of *trans*-cinnamic acid in smaller parts of cinnamon plants has not been reported. We found that *trans*-cinnamic acid was localised in the bark and was not present in the core of the branches; this is the first visualisation of the localisation of *trans*-cinnamic acid on a PES membrane by immunostaining. In a study on *C. osmophloeum*, which is congeneric with *C. cassia*, *trans*-cinnamic acid was identified as a biosynthetic precursor that was metabolised to *trans*-cinnamaldehyde [27]. Another study indicated that some genes for the enzymes correlated with *trans*-cinnamaldehyde biosynthesis in *C. osmophloeum* were expressed in the bark of *C. cassia* [28]. The *trans*-cinnamic acid that was immunostained in this study was derived from the precursors in the biosynthesis of *trans*-cinnamaldehyde, or from biosynthesised *trans*-cinnamaldehyde that was slowly oxidised by air [29]. The *trans*-cinnamic acid content in the *C. cassia* branch bark was too low to be coloured by immunostaining on the PES membrane. Thus, the *trans*-cinnamic acid adsorbed on the PES membrane was not only blotted from the surface of the *C. cassia* branch slice, but also from the upper tissues. The aqueous fluid in the tissue at the surface was absorbed by the PES membrane, and when the slice was pressed from the top, more fluid moved to the lower surface and was absorbed by the membrane. In addition, the tissue fluid dried on the PES membrane concentrating the *trans*-cinnamic acid, and therefore the colour was strong on the PES membrane. The vertical branch slice was weakly stained because of its shape. The vertical slice was pressed from the top, like the horizontal slice, but because the upper surface of the branch was semi-circular, the pressure was higher on the heartwood part of the lower flat surface than on the bark, which was further from the pressure source. In this study, anti-benzylacetone MAb IXF1-A7Bf12 was successfully prepared, but it was not suitable for ELISA methods because the detection limit of the MAb was too high to determine and quantify the amount of benzylacetone in the glass cage used in feeding and locomotor tests. A more sensitive MAb would be created by immunisation using KLH-*p*-(3-oxobutyl)benzoic acid conjugate, together with a hybridoma genetically engineered with IXF1-A7Bf12 cell genes. Furthermore, reactions in surfactant-free solution would be useful for achieving high reactivities of ELISA and immunostaining. Dot blot methods enabled visualisation and semi-quantitative analyses of *trans*-cinnamic acid and 3PPa. Further studies are needed for the anti-benzylacetone MAb to determine the small amount of benzylacetone present in cage when used in animal tests.

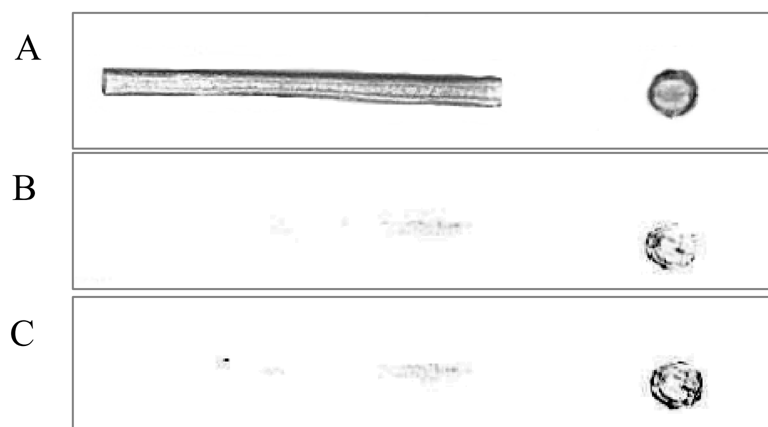

Figure 4. Immunohistochemical analyses of *trans*-cinnamic acid in *C. cassia*. A: scanned vertical (left) and horizontal (right) cut branches of *C. cassia*. B: blotted membrane before immunostaining. C: blotted membrane after immunostaining.

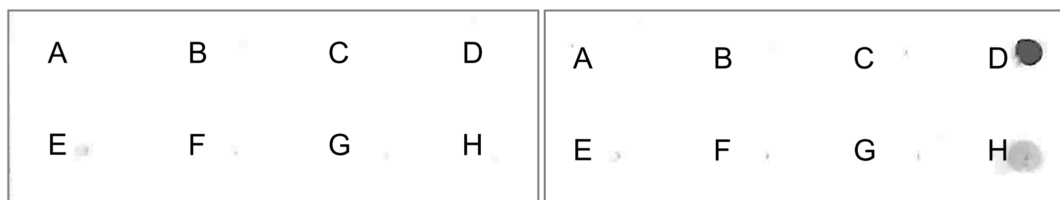

Figure 5. Immunostaining of aromatic compounds observed in *Cinnamomum cassia* and berberine. Left: Immediately before immunostaining, right: 20 min after immunostaining started. A: coumarin, B: *o*-coumaric acid, C: *p*-coumaric acid, D: 3PPa-HSA (positive control), E: quercetin, F: berberine chloride, G: 2-methoxycinnamaldehyde, H: *trans*-cinnamic acid (positively reacting compound)

Conclusion

In conclusion, we demonstrated immunostaining of compounds with molecular weights of less than 200 g/mol using anti-benzylacetone MAb on a PES membrane. These results can be applied to histochemical analyses in phytobiology and phytochemistry. The immunostaining method could be used to reveal the localisation of compounds in plant tissues more precisely without using expensive instruments such as a mass spectrometry imaging system. Moreover, the relative amount of the compound blotted on the PES membrane can be estimated using detection and imaging systems for western blotting with some colouring reagents for UV absorbance or chemiluminescence. Our immunostaining method using anti-benzylacetone MAb may be a powerful tool for revealing the localisation of these compounds in animal tissues after administration and for clarifying pharmacological mechanisms.

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