Development of monoclonal antibody against isoquinoline alkaloid coptisine and its application for the screening of medicinal plants

Jun-Sik Kim¹, Hiroyuki Tanaka¹, Chun-Su Yuan² and Yukihiro Shoyama^{1,*}

¹Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; ²Tang Center for Herbal Medicine Research & Department of Anesthesia and Critical Care, The Pritzker School of Medicine, The University of Chicago, 5841 S. Maryland Avenue, MC 4028 Chicago, Illinois 60637, USA; (*Author for correspondence; E-mail: shoyama@phar.kyushu-u.ac.jp; phone/fax: +81-92-642-6580)

Received 24 June 2004; accepted in revised form 7 July 2004

Key words: Coptisine, ELISA, Isoquinoline alkaloid, Monoclonal antibody, Small hapten

Abstract

In the development of immunoassay technique, the design of hapten containing a functional group suitable for protein conjugate is the key step for the preparation of antibodies against small molecules. Coptisine (MW 320), a bioactive constituent of *Berberis* and *Coptis* species, is small as an immunogen. In addition, coptisine has no reactive group in molecule for conjugating with a protein. To overcome this problem, 9-*O*-carboxymethyl-berberrubine was designed and conjugated with carrier protein. In order to confirm its immunogenicity, the ratio of hapten in the conjugate was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). After immunization, hybridomas secreting antibodies against coptisine were produced by fusing splenocytes with mouse myeloma cell line, P3-X63-Ag8-653. Among hybridomas, the clone 2A1 secreting anti-coptisine monoclonal antibody (MAb) 2A1-9E-1 was obtained through the limited dilution method. The MAb-based enzyme-linked immunosorbent assay (ELISA) against coptisine was developed and characterized. The linear range of the assay in this ELISA method was extended from 1.56 to 25 $\mu\text{g ml}^{-1}$ possessing the detection limit of 1.56 $\mu\text{g ml}^{-1}$. The established ELISA using MAb 2A1-9E-1 was applied for the survey of isoquinoline alkaloids in various medicinal plants.

Introduction

The enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (MAb) have become an important methodology for the qualitative and/or quantitative analysis of pesticides (Hammock et al. 1980), preservatives (Yoshida et al. 1995), environmental chemicals (Marco et al. 1995) and phytochemical compounds (Shoyama et al. 1999) having small molecular weight. In phytochemical studies, chromatographic methodologies such as HPLC and GC are sufficiently accurate and sensitive as analytical methods. However, there are

some limitations such as the number of samples, pretreatment of samples, time and labor consuming besides interference by other compounds included in herbal medicines. On the other hand, an ELISA method can be applied effectively for an analytical tool for determining the levels of phytochemical compound in commercial products, either as a single crude drug or in combination with other crude drugs resulting in establishment of primary criteria for the standardization of herbal medicines.

Coptis rhizome (*Coptis japonica* M., Ranunculaceae) containing coptisine, a bioactive

isoquinoline alkaloid has been used in traditional medicines as a remedy for gastroenteric disease. According to previous pharmacological studies, coptisine has gastric-mucous membrane protective effect (Hirano et al. 2001) and significant cancer cell growth inhibitory activities (Colombo et al. 2001). Therefore, the survey of coptisine in plant samples becomes important for the finding of new resources of coptisine as a candidate of new drug.

Immunization of coptisine may be quite difficult in the common way because coptisine is not only a small molecular weight, but also no reactive group in a molecule. However, a heterogeneous hapten, 9-*O*-carboxymethyl-berberrubine we designed as an intermediate for conjugation with carrier protein, shares a part of coptisine molecule and it may cross-react with coptisine due to similarity of structure.

Materials and methods

Chemicals and immunochemicals

Coptisine chloride and structurally related compounds used in cross-reaction studies were of analytical grade and were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) and Nacalai tesque, Inc. (Kyoto, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Complete and incomplete Freund's adjuvants were obtained from Difco Labs. (MI, USA). Enriched RPMI1640-Dulbecco's-Ham's F12 medium (eRDF) and RD-1 additives were obtained from Kyokuto Pharmaceutical Ind., Ltd. (Tokyo, Japan). Goat peroxidase-labeled anti-mouse IgG was provided by Organon Teknika Cappel Products (West Chester, PA, USA). Fetal calf serum (FCS) was provided by Cambrex Co. (Wakersville, MA, USA). All other chemicals were standard commercial products of analytical grade.

Synthesis of hapten for conjugates

Synthesis of the hapten was carried out as outlined in Figure 1: 9-*O*-Carboxymethyl-berberrubine was synthesized following Iwasa's method (Iwasa et al. 1996) and Sadykov's method (Sadykov et al. 1967)

with modification as indicated below. Berberine (3 g) was heated at 190 °C for 15 min. The resulting mixture was dissolved in H₂O, and extracted with chloroform three times. Chloroform was evaporated, and the residue was purified by column chromatography using Sephadex LH20 and ODS gel to give berberrubine (2 g). Subsequently, chloroacetic acid (300 mg, 3.17 mol) was added to a stirred solution of berberrubine (100 mg, 0.31 mol) in chloroform at room temperature. And the reaction mixture was stirred for 3 h. After the solvent was evaporated, the residue was chromatographed to give 9-*O*-carboxymethyl-berberrubine. 9-*O*-Carboxymethyl-berberrubine was identified with authentic sample comparing the data of ¹H NMR.

Preparation of immunogen

9-*O*-Carboxymethyl-berberrubine (8 mg, 0.021 mmol) and 1-ethyl-3-(3'-dimethyl aminopropyl)-carbodiimide hydrochloride (EDC, 8 mg, 0.042 mmol) was added to pyridine solution (1 ml). The reaction mixture was added dropwise to H₂O solution (1 ml) containing BSA or HSA (8 mg), and then stirred for 3 h at room temperature. Subsequently, the mixture was dialyzed 5 changes of H₂O for 2 days at 4 °C, and lyophilized.

Direct determination of hapten ratio in conjugates by MALDI-TOF mass spectrometry (MS)

The hapten ratio in the hapten-carrier protein conjugates were determined by MALDI-TOF MS as previously described (Shoyama et al. 1993). A small amount (1–10 pmol) of hapten-conjugate was mixed with a 10³-fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid. The mixture (0.5–1.0 µl) was deposited on sample plate and air-dried. The mass spectra were acquired using a delayed extraction MALDI-TOF MS (Voyager Elite, PerSeptive Biosystems, Inc., Framingham, MA, USA) operated in the linear model. The instrument was generally operated at an accelerating voltage of 20 kV and a grid voltage of 90% of the accelerating voltage. The time of flight was measured by a 500 MHz transient digitizer board in the computer, and the data were analyzed using GRAMS/

Figure 1. Synthetic pathway of the hapten for conjugation with protein.

386 software (Galactis Industries Corp., Salem, NH, USA).

insulin, 20 $\mu\text{g ml}^{-1}$ of transferrin, 20 μM of ethanolamine and 25 nM of sodium selenite.

Immunization

Female, 6 weeks old BALB/c mice (Kyudo, Kumamoto, Japan) were immunized intraperitoneally with hapten-BSA conjugate solution four times biweekly. The first immunization (50 μg of immunogen) was injected as a 1:1 (v/v) emulsion in Freund's complete adjuvant to a total volume of 0.5 ml. The second immunization (50 μg of immunogen) was injected as a 1:1 emulsion in Freund's incomplete adjuvant. The third and final immunization (100 μg of immunogen) were given without any adjuvant 3 days prior to cell fusion.

Purification of MAb

MAb 2A1-9E-1 was purified using a protein G FF column (0.46 $\times$ 11 cm, Amersham Pharmacia Biotech, Uppsala, Sweden). The media containing the IgG was adjusted to pH 7 with 1 M Tris solution (pH 9) and loaded onto the column. After binding, the column was washed with 20 mM phosphate buffer (pH 7) and the adsorbed IgG was eluted with 100 mM of citrate buffer (pH 3). The eluted IgG was neutralized with 1 M Tris solution (pH 9), then dialyzed against 5 changes of H_2O for 3 days at 4 $^\circ\text{C}$, and finally lyophilized.

Cell fusion and production of MAb

Splenocytes were isolated from the immunized mouse, and fused with a hypoxanthine-aminopterin-thymidine (HAT) sensitive mouse myeloma cell line, P3-X63-Ag8-653, by the polyethylene glycol (PEG) method (Galfre et al. 1981). And hybridoma producing MAb against coptisine were cloned twice by the limited dilution method (Goding et al. 1980). Subsequently, established hybridomas were scaled up and cultured in serum-free media (SFM), enriched RPMI1640-Dulbecco's-Ham's F12 medium (eRDF) supplemented with RD-1 additives containing 9 $\mu\text{g ml}^{-1}$ of

Direct ELISA procedures

A 100 μl of hapten-HSA (1 $\mu\text{g ml}^{-1}$ in 50 mM carbonate buffer, pH 9.6) was allowed to adsorb onto the wells of a 96-well flat-bottomed polystyrene immunoplate (NUNC, Roskilde, Denmark) for 1 h, and washed three times with washing buffer, PBS containing 0.05% Tween-20 (TPBS). The plate was treated with 300 μl of PBS containing 5% skimmed milk (SPBS) for 1 h to minimize nonspecific adsorption, and washed three times with TPBS and reacted with 100 μl of MAb for 1 h. Subsequently, the plate was washed three times with TPBS and MAb was combined with

100 μ l of 1:1000 dilution of peroxidase-labeled anti-mouse IgG as secondary antibody for 1 h. After washing the plate three times with TPBS, 100 μ l of 100 mM citrate buffer (pH 4.0) containing 0.003% H_2O_2 , and 0.3 mg ml^{-1} of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ABTS (Wako Pure Chemical Ind. Ltd., Osaka, Japan) was added to each well. And then the plate was incubated for 20 min. After the incubation, the reaction was stopped with 0.001% NaN_3 . Absorbance was measured using an immunoplate reader (ImmunoMini NJ-2300, NUNC, Roskilde, Denmark) at 405 and 490 nm. All reactions were carried out at 37 °C.

Competitive ELISA procedures

A 100 μ l of hapten-HSA (1 μ g ml^{-1} in 50 mM carbonate buffer, pH 9.6) was allowed to adsorb onto the wells of an immunoplate for 1 h, and washed three times with TPBS. The plate was treated with 300 μ l of SPBS for 1 h. After washing three times with TPBS, 50 μ l of various concentrations of coptisine or its structurally related compound solutions dissolved in 10% of methanol were introduced into the wells of plate with coated hapten-HSA. And an equal volume of MAb solution was added to the wells, and incubated for 1 h. Subsequently, the plate was washed three times and MAb was combined with 100 μ l of 1:1000 dilution of peroxidase-labeled anti-mouse IgG as secondary antibody for 1 h. After washing the plate three times with TPBS, 100 μ l of 100 mM citrate buffer (pH 4.0) containing 0.003% H_2O_2 , and 0.3 mg ml^{-1} of ABTS was added to each well, and the plate was incubated for 20 min. After the incubation, the reaction was stopped with 0.001% NaN_3 . Absorbance was measured using an immunoplate reader at 405 and 490 nm. All reactions were carried out at 37 °C.

For data analysis, absorbance values from standards were mathematically fitted to a four-parameter logistic equation:

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where y is the absorbance value; A , the maximum absorbance at no analyte present, B , the curve slope at the inflexion point, C , the x value at the inflexion point (corresponding to the analyte concentration that reduces absorbance to 50% of

the maximum, IC_{50}), and D is the minimum absorbance.

Plant materials and preparation of test samples

Fourteen kinds of medicinal plants were selected randomly in the herbal museum, Graduate School of Pharmaceutical Science, Kyushu University. Each powdered sample (30 mg) was sonicated in 3 ml of methanol for 30 min. The mixture was centrifuged at 380 g for 5 min, then the supernatant was collected. This procedure was repeated three times, and the combined supernatant was adjusted to volume of 9 ml with methanol. The sample solution was filtered with 0.45 μ m membrane and diluted with H_2O for analysis individually.

Results and discussion

Synthesis of hapten for conjugates

The specificity and selectivity of immunoassay are mainly determined by antibody. Since small molecule compound has no immunogenicity, in order to elicit an immune response in the host animal, a small molecule compound must be conjugated to a carrier protein that could be functioned as a primary immunogen. This hapten should preserve as much as possible the chemical structure, electronic distribution and spatial conformation of the target compound. Therefore, the design of hapten for the carrier protein conjugation is the key step in the immunoassay technique for small molecules.

In the case of coptisine (MW 320), it is too small to be immunized and should be conjugated to a carrier protein that will function as the primary immunogen as already discussed above. In addition, the structure of coptisine has no reactive group such as NH_2 , COOH , OH , SH , CO or CHO for linkage to a protein. To overcome this problem, 9-*O*-carboxymethyl-berberrubine having a partial structure of coptisine was selected as a candidate of conjugation, since berberrubine has a hydroxyl group conjugated to a carrier protein resulting in production of antibody which may cross-react with coptisine. The designed hapten and carrier protein were conjugated with a zero-length cross-linking procedure mediated by the

water-soluble carbodiimide EDC to prevent the production of antibodies against the cross-linker bridge (Tedder et al. 1972). The first reaction was started from heating of berberine at 190°C resulting in berberrubine in a high yield as indicated in Figure 1. Berberrubine was treated with monochloroacetic acid to give 9-*O*-carboxymethyl-berberrubine. And then after the carbodiimide reacts with carboxylic groups on 9-*O*-carboxymethyl-berberrubine to form a highly active *O*-acylisourea intermediate. The activated carboxylic group then reacts with a primary amine of lysine group in carrier protein to form an amide bond releasing in the EDC mediator as a soluble isourea derivative. As results, MAb 2A1-9E-1 has recognized the methylene group of target compound particularly. Coptisine has two methylene groups in structure but berberine and berberrubine have one. So the binding capacity of MAb 2A1-9E-1 for coptisine is stronger than that for berberine and berberrubine.

Direct determination of hapten ratio in conjugates by MALDI-TOF MS

In order to confirm immunogenicity of its conjugate, the hapten ratio in the hapten-carrier protein conjugate was determined by MALDI-TOF MS. Using experimental results and a molecular weight of 66,433 for BSA, the calculated value of the

Figure 2. Direct determination of the hapten ratio in hapten-protein conjugates by MALDI-TOF mass spectrometry. $[M + H]^+$ and $[M + 2H]^{2+}$ are single- and double-protonated molecules of hapten-BSA.

hapten component (MW 380) was m/z 1022, indicating at least three molecules of hapten conjugated with BSA. This hapten number was estimated to be enough for immunization. The number of hapten moieties contained in the hapten-HSA conjugate was determined, by its spectrum, to be approximately four molecules (Figure 2).

Preparation of monoclonal antibody

After the injection of the hapten conjugate to mice, the general format for the preparation of hybridoma has been investigated previously developed in our laboratory (Ishiyama et al. 1996). The splenocytes from the mouse immunized with hapten-BSA were hybridized with myeloma cells, P3-X63-Ag8-653. The titer of all supernatants in culture plate was investigated by direct ELISA and 17 strains were selected. After scaled up for a week, seven strains had high titers to hapten-BSA, whereas low titers to BSA. From the strains produced antibodies of high titer and grown more rapidly, three strains were selected and cloned twice by the limited dilution method. Finally, the clone 2A1-9E-1 was chosen due to its reactivity and specificity for coptisine. The clone 2A1-9E-1 was grown in eRDF media giving anti-coptisine MAb.

Characteristics of MAb 2A1-9E-1 against coptisine

The reactivity of MAb 2A1-9E-1 against coptisine was determined by direct ELISA. The isotype of secreted antibody was determined by using a mouse MAb isotyping kit. MAb 2A1-9E-1 was classified as IgG₁ that had κ light chain. The reactivity of MAb was tested by direct ELISA using various concentrations (Figure 3).

Sensitivity and specificity of assay

The calibration curve was prepared by the competitive ELISA as indicated in Figure 4. The linear range of the assay was extended from 1.56 to 25 $\mu\text{g ml}^{-1}$ with the correlation coefficient (R^2) of 0.991, as indicated in Figure 4. The detection limit for coptisine was also estimated to be 1.56 $\mu\text{g ml}^{-1}$ by

Figure 3. Reactivity of MAb 2A1-9E-1 against hapten-HSA. To examine the reactivity of the antibody, various concentrations of antibody were added to each well of an immunoplate coated with hapten-HSA ($1 \mu\text{g ml}^{-1}$). Error bars were $\pm$ SD.

Figure 4. Standard curve for the determination of coptisine by competitive ELISA. The insert shows the linearized calibration curve by the same experimental data. Various concentrations of hapten were incubated with MAb 2A1-9E-1 ($1 \mu\text{g ml}^{-1}$) in an immunoplate adsorbed with hapten-HSA ($1 \mu\text{g ml}^{-1}$). A/A_0 , A_0 is the absorbance with no coptisine present, and A is the absorbance with coptisine present. The data represents the means of triplicate wells. This sigmoidal curve was obtained by averaging 12 individual standard curves performed in the course of coptisine determination in samples. Error bars were $\pm$ standard deviation.

IC₉₀. The specificity of MAb 2A1-9E-1 was estimated by cross-reactivity measurement according to Weiler and Zenk's equation (Weiler et al. 1976). The ELISA was specific for coptisine having low

cross-reactivity with structurally related compounds, berberine, palmatine and jateorrhizine, but did not react with other alkaloids such as hydrastine, corydaline and papaverine (Table 1). Moreover, MAb 2A1-9E-1 did not react with various compounds of such as flavonoids, terpenes, saponins and anthraquinones (Table 2). These results show that the presence of charged residue in the coptisine skeleton may be necessary for the epitope for MAb 2A1-9E-1. Furthermore, the methylene group in a molecule is also needed.

Accuracy and variation of assay

Standard curves for the competitive ELISA of coptisine from four consecutive days were compared, and the variations were calculated. The variations of repeatability between triplicates from well-to-well (intra-assay), and reproducibility from plate-to-plate (inter-assay) were measured. Although there are potential interferences such as organic solvent effects, coating step, the position of sample in immunoplate, multi-channel pipetter, edge effects due to evaporation, uneven temperature during incubation, and day-to-day variation, the results of accuracy and variation were acceptable. The coefficient of variation in intra-assay and inter-assay were below 5% in most cases (Table 3).

Reactivity of MAb against various medicinal plants

As an application of the competitive ELISA, we attempted the survey of isoquinoline alkaloids in various medicinal plants. Table 4 shows the reactivity of MAb 2A1-9E-1 against various medicinal plants. From these results, it becomes evident that MAb 2A1-9E-1 reacted with the extract of samples contained isoquinoline alkaloids such as *Coptis japonica*, *Berberis thunbergii*, *Corydalis turtschaninovii*, *Hydrasis canadensis*, and *Phellodendron amurense*. The extract of *Cinchona succirubra* was, however, strongly reactive. To find out the reason that MAb 2A1-9E-1 reacts with this plant, we examined the reactivity against quinine as main constituent in this medicinal plant. But we reached a conclusion that there is no reactivity of major constituent, quinine (Table 2). The reason why the *Cinchona succirubra* plant was detectable is still

Table 1. Cross-reactivities of MAb 2A1-9E-1 against coptisine and its related compounds

Cross-reactivity (%)
coptisine 100	berberine 20.6
palmatine 20.4	berberrubine 29.8
jateorrhizine 14.0	hydrastine n.i. ^a
corydaline n.i. ^a	papaverine n.i. ^a

Cross-reactivity (%) = [(quantity of coptisine that inhibits antibody binding by 50%)/(quantity of cross-reactive compound that inhibits antibody binding by 50%)] × 100. The values are averages from triplicate determinations.^an.i. is no inhibition at the highest concentration (100 µg ml⁻¹).

obscure and under further investigation. Previous reports demonstrated that in sample nos. 1–6 plants contained isoquinoline alkaloids as listed in Table 4. Therefore these plants are suggested to be candidates of coptisine resources except sample no. 3 because the value is low (16.1%). We also

determined the reactivities against the crude extracts of various alkaloid containing and non-alkaloid containing medicinal plants as indicated in Table 4. From this survey it has been clear that tropane alkaloids contained in *Atropa* and diterpene type alkaloid contained in *Aconitum* spp. did

Table 2. Cross-reactivities of MAb 2A1-9E-1 against various compounds

Compounds	Types	Cross-reactivity (%)
Sennoside A	Anthraquinone	n.i. ^a
Sennoside B	Anthraquinone	n.i.
(-)-Epicatechin	Catechin	n.i.
Baicalin	Flavonoid	n.i.
Baicalin	Flavonoid	n.i.
Arbutin	Hydroquinone	n.i.
Swertiamarin	Secoiridoid	n.i.
Artemisin	Sesquiterpenoid	n.i.
Ginsenoside Rb1	Saponin	n.i.
Saikosaponin a	Saponin	n.i.
Saikosaponin d	Saponin	n.i.
Glycyrrhizin	Triterpenoid	n.i.
Paeoniflorin	Monoterpenoid	n.i.
Albiflorin	Monoterpenoid	n.i.
Quinine	Alkaloid	n.i.

^an.i. is no inhibition at the highest concentration (200 µg ml⁻¹).

Table 3. Variations among ELISA runs for the analysis

Coptisine (µg ml ⁻¹)	Coefficient of variation (%)	
	Inter-assay	Intra-assay
1.56	4.23	2.76
3.13	3.09	3.30
6.25	2.90	2.44
12.5	2.56	2.38
25	2.81	0.66

The measured values were mean ± SD for four plates and triplicate wells for each concentration within one plate from four consecutive days. The variations on replicates from well to well and plate to plate are defined as intra-assay and inter-assay variation, respectively.

Table 4. Reactivities of MAb 2A1-9E-1 against various medicinal plants

Sample No.	Family	Plants	Main constituents	Inhibition (%)
1	Rutaceae	<i>Phellodendron amurense</i>	Alkaloids	63.2
2	Papaveraceae	<i>Corydalis turtshianovii</i>	Alkaloids	50.6
3	Menispermaceae	<i>Jateorhiza columba</i>	Alkaloids	16.1
4	Berberidaceae	<i>Berberis thunbergii</i>	Alkaloids	43.1
5	Ranunculaceae	<i>Coptis japonica</i>	Alkaloids	79.8
6		<i>Hydrastis canadensis</i>	Alkaloids	68.7
7		<i>Aconitum japonicum</i>	Alkaloids	0
8	Solanaceae	<i>Atropa belladonna</i>	Alkaloids	9.3
9	Rubiaceae	<i>Cephaelis ipecacuanha</i>	Alkaloids	0
10		<i>Cinchona succirubra</i>	Alkaloids	96.1
11	Campanulaceae	<i>Platycodon grandiflorum</i>	Saponins	6.5
12	Araliaceae	<i>Panax ginseng</i>	Saponins	0
13	Leguminosae	<i>Cassia angustifolia</i>	Anthraquinones	0
14	Labiatae	<i>Scutellaria baicalensis</i>	Flavonoids	15.5

Inhibition (%) = $[(A_0 - A_S)/A_0] \times 100$. The values are averages from triplicate determinations at the highest concentration (1 mg ml⁻¹). A_0 is the absorbance in the absence of the test sample and A_S is that in the presence of the test sample.

not react to the MAb resulting in only limited to the isoquinoline type alkaloids.

Conclusions

The linear range of the assay in this ELISA method was extended from 1.56 to 25 µg ml⁻¹ possessing the detection limit of 1.56 µg ml⁻¹. The sensitivity and selectivity of ELISA are sufficient to determine coptisine for the analysis. The ELISA method described in this study can be applied as an analytical tool for determining the levels of coptisine in biological samples such as plasma, urine and bile for *in vivo* pharmacokinetic studies.

As an application, we attempted to detect for isoquinoline alkaloids in various medicinal plants by using the established ELISA. This established method may be helpful for the screening medicinal plants containing the isoquinoline alkaloids. Also we will attempt to exploit MAb 2A1-9E-1 for the applications such as immunoblot procedure and immunochromatography for the identification of coptisine in plant. These results will be reported elsewhere.

Acknowledgments

The research in this paper was supported by the research fund of Kyushu University Foundation

and Japan Kampo Medicine Manufacturers Association.

References

- Colombo M.L., Bugatti C., Mossa A., Pescalli N., Piazzoni L., Pezzoni G., Menta E., Spinelli S., Johnson F., Gupta R.C. and Dasaradhi L. 2001. Cytotoxicity evaluation of natural coptisine and synthesis of coptisine from berberine. *Il Farmaco* 56: 403–409.
- Galfre G. and Milstein C. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 73: 3–46.
- Goding J.W. 1980. Antibody production by hybridomas. *J. Immunol. Meth.* 39: 285–308.
- Hammock B.D. and Mumma R.O. 1980. Potential of immunochemical technology for pesticide analysis. In: Harvey J. and Zweig G. (eds), *Recent Advances in Pesticide Analytical Methodology*. American Chemical Society, Washington, pp. 321–352.
- Hirano H., Osawa E., Yamaoka Y. and Yokoi T. 2001. Gastric-mucous membrane protection activity of coptisine derivatives. *Biol. Pharm. Bull.* 24: 1277–1281.
- Ishiyama M., Shoyama Y., Murakami H. and Shinohara H. 1996. Production of monoclonal antibodies and development of an ELISA for solamargine. *Cytotechnology* 18: 153–158.
- Iwasa K. and Kamigauchi M. 1996. 13-hydroxylation of tetrahydroberberine in cell suspension cultures of some *corydalis* species. *Phytochemistry* 41: 1511–1515.
- Marco M.P., Gee S. and Hammock B.D. 1995. Immunochemical techniques for environmental analysis II. Antibody production and immunoassay development. *Trends Anal. Chem.* 14: 415–425.
- Sadykov Y.D., Davidyants S.B., Poroshin K.T. and Grigina I.N. 1967. Synthesis of esters and ethers from the alkaloid berberine. *Dokl. Akad. Nauk Tadzh SSR* 10: 18–21.
- Shoyama Y., Sakata R., Isobe R. and Murakami H. 1993. Direct determination of forskolin–bovine serum albumin conjugate by matrix assisted laser desorption ionization. *Org. Mass. Spectr.* 28: 987–988.
- Shoyama Y., Tanaka H. and Fukuda N. 1999. Monoclonal antibodies against naturally occurring bioactive compounds. *Cytotechnology* 31: 9–27.
- Tedder J.M., Nechvatal A. and Murray A.W. 1972. Amino acids and proteins. In: *Basic Organic Chemistry*. John Wiley & Sons, Chapter 6, pp. 305–342.
- Weiler E.W. and Zenk M.H. 1976. Radioimmunoassay for the determination of digoxin and related compounds in *Digitalis lanata*. *Phytochemistry* 15: 1537–1545.
- Yoshida A. and Takagaki Y. 1995. Detection of food additives by enzyme immunoassay. *Nippon Rinsho* 53: 2316–2321.