Preparation of monoclonal antibody against crocin and its characterization

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Abstract

Three crocin-carrier protein conjugates were synthesized and their hapten numbers were determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Three monoclonal antibodies against crocin were produced by hybridomas fused with the splenocytes immunized with crocin hemisuccinate-bovine serum albumin conjugate and HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653. They were identified as IgG_{2a} and IgG_{2b} possessing λ light chain, respectively. Their wide reactivities against crocetin glycosides were discussed.

Introduction

Crocin is the main carotenoid pigment isolated from the pistils of *Crocus sativus* L., which is famous for saffron in the folk (Pfander et al., 1975). Besides its popular utility as a coloring and flavoring agent owing to its unique physical properties, saffron was widely used as a folk medicine, which can not only remove blood stasis, promote blood circulation, but also allay fear, cure trance, ameliorate convulsion (Suzhou New Medical College, 1977). Recently its antitumor activity was also reported (Abdullaev, 1994; Escibano et al., 1996). Furthermore, our pervious investigations showed that crocin and other crocetin glycosides can influence the process of learning and memory (Sugiura et al., 1995a; Sugiura et al., 1996), ameliorate the ethanol-induced inhibition of long-term potentiation (LTP) in the hippocampus (Sugiura et al., 1994; Sugiura, 1995b), and crocin is the most effective constituents in *Crocus sativus* L. (Zhang et al., 1994; Sugiura et al., 1996).

In recent rapid development of molecular biosciences and their biotechnological applications, im-

munoassay systems using monoclonal antibodies (MAbs) against drugs and biologically active small molecular weight compounds have become an important tool (Weiler, 1990) for the studies on receptor binding analysis, enzyme assay, and quantitative and/or qualitative analytical techniques in animals or plants, owing to their specific affinity. In previous papers we reported the methods of direct determination for antigen conjugate by matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (Shoyama et al., 1993a; 1993b; Goto et al., 1994), and the preparation of the monoclonal antibodies (MAb) and enzyme-linked immunosorbant assay (ELISA) for forskolin (Sakata et al., 1994), solamargine (Ishiyama et al., 1996; Tanaka et al., 1997), marihuana compound (Tanaka et al., 1996) and opium alkaloid (Shoyama et al., 1996). In continuing studies on MAbs against naturally occurring bioactive compounds, we present here the preparation of MAb for crocin, its characteristics and an ELISA system.

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Material and methods

Chemicals and immunochemicals

Crocus sativus L. was donated by Taketa Saffron Association (Taketa city, Japan). Succinic anhydride, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (HOSu), bovine serum albumin (BSA) and human serum albumin (HSA) were provided by Nacalai Tesque (Kyoto, Japan). Complete and incomplete Freund adjuvant (CFA and IFA) were obtained from Difco (Detroit, USA). CHP 20P MCI gel was the product of Mitsubishi Chemical Corporation (Tokyo, Japan). All other chemicals were standard commercial products of analytical grade.

Isolation of crocin

Crocin (4 g) was isolated from 50% EtOH extract (40 g) of *Crocus sativus* L. by MCI gel chromatography using MeOH-H₂O as gradient eluent. It is identified with authentic sample of crocin by TLC and HPLC as previously reported (Morimoto et al., 1994).

Synthesis of crocin hemisuccinate (CHS)

A solution of crocin (50 mg) and succinic anhydride (50 mg) in anhydrous pyridine (1 ml) was kept at 4 °C overnight. After adding H₂O (100 μ l) for termination of the reaction, the solution was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1 N NaHCO₃ solution and applied to MCI gel Chromatography with MeOH-H₂O as gradient eluent. Crocin hemisuccinate (CHS, 22 mg) was yielded as a sodium salt, then transformed into acid by cation exchange gel.

Synthesis of activated crocin hemisuccinate

CHS (15 mg), EDC (3 mg) and HOSu (4.5 mg) were dissolved in anhydrous DMF (500 μ l) and stirred at 4 °C overnight. The reaction was terminated by H₂O (100 μ l), and the solution was applied immediately in next conjugating step.

Synthesis of crocin hemisuccinate BSA conjugate (CHS-BSA)

The activated reaction mixture above was added to a solution of BSA (15.6 mg) in H₂O (500 μ l) and Et₃N (5 μ l), and it was stirred at 4 °C for 24 h. The reaction solution was dialyzed against water overnight, with the

water changed for several times. After lyophilizing, crocin hemisuccinate BSA conjugate (CHS-BSA, 22 mg) was obtained. Crocin hemisuccinate HSA conjugate (CHS-HSA) was synthesized in the same way.

Synthesis of HSA conjugate by NaIO₄ (CI-HSA)

Crocin (5 mg) dissolved in H₂O (1 ml) was added to a solution of NaIO₄ (2 mg) in H₂O (500 μ l) dropwisely, then stirred at room temperature for 1 h. A solution of HSA (6 mg) in 50 mM carbonate buffer (pH 9.6, 1 ml) was added and stirred at room temperature for 5 h. The reaction mixture was dialyzed against H₂O overnight, with the water changed for several times. After lyophilizing crocin-HSA conjugate (CI-HSA, 5 mg) was afforded.

Determination of hapten numbers by MALDI-MS

The hapten numbers in these three conjugates were determined by MALDI-TOF-MS. A small amount antigen conjugate (1–10 pmol) was mixed with a 10³-fold molar excess of sinapinic acid in an aqueous solution containing 10% trifluoroacetic acid. The mixture was subjected to a JMS-ELITE MALDI-TOF mass monitor and irradiated with a N₂ laser (337 nm, 3ns pulse). The ion formed by each pulse were accelerated by 25 kV potential into a 1.7 m evacuated tube and detected using an IBM PC compatible computer.

Immunization and hybridization

A solution of CHS-BSA (50 μ g) in 50 mM carbonate buffer (pH 9.6, 250 μ l) was emulsified with equal volume CFA, then injected intraperitoneally to a 7-week-old BALB/c female mouse. One week later the second injection was held with IFA instead of CFA. After the third injection in fourth week, the last boosting injection was carried on intravenously with a solution of CHS-BSA (50 μ g) in 50 mM carbonate buffer (pH 9.6, 100 μ l) two weeks later. Three days later, the splenocyte was isolated and fused with a HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-653, by the polyethylene glycol (PEG) method (Galfre and Milstein, 1981). Hybridomas producing MAb against crocin were cloned by the limited dilution method (Goding, 1980). Established hybridomas were cultured in 5% FCS eRDF medium.

Large-scale preparation of MAb against crocin in vitro and in vivo

Two methods were used for the large scale preparation of MAbs. 1: After incubation in 10% FCS-eRDF media for a week, the hybridoma was transferred to culture RD-1/eRDF media for another one-week incubation as previously reported (Murakami et al., 1982). About 300 ml supernatant was afforded by centrifugation. 2: After four times injections of pristine in 10 days, 7-week-old ICR nude mice were planted with hybridoma by intraperitoneal injection. One week later, the ascite was collected as more times as possible. About 30 ml ascite was obtained from 3 mice.

Concentrated with ultrafiltration, the supernatant or the ascite was developed on Protein A affinity column (Protein A sepharose 4 Fast Flow, Pharmacia Biotech AB, Uppsala, Sweden) using 20 mM sodium phosphate solution (pH 7.0) as binding buffer and 0.1M glycine solution (pH 3.0) as eluting buffer. The MAb effluent was dialyzed against PBS buffer and lyophilized. The yielding of MAb was analyzed by sandwich ELISA by comparison of authentic mouse IgG. The class and subclass of the MAbs were determined by Zymed's mouse monoAb ID kit (HRP, Zymed laboratories, Inc., So. San Francisco, USA).

Direct ELISA using CHS-BSA

The reactivity of samples or MAbs to CHS-HSA was determined by direct ELISA. A 96-well immunoplate (NUNC, Roskilde, Denmark) absorbed by 100 μ l of 1 μ g/ml CHS-HSA in 50 mM carbonate buffer (pH 9.6) was treated with 300 μ l PBS containing 0.2% gelatin (G-PBS) for 1 h to reduce nonspecific adsorption. The plate was washed with PBS containing 0.05% Tween 20 (T-PBS) and reacted with 100 μ l samples or MAbs for 1 h. The plate was washed with T-PBS for three times, and then the plate was incubated with 100 μ l 2000-time-diluted peroxidase-labeled goat antimouse IgG (Organon Teknika Cappel Products, Westchester, USA) for 1 h. With the plate washed by T-PBS for three times, 100 μ l substrate solution, 0.3 mg/ml 2, 2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) (Wako, Tokyo, Japan) in 0.1 M citrate buffer (pH 4.0) containing 0.006% H_2O_2 , were added to each wells and incubated for 20 min. Absorbance at 405 nm was measured with a FAR400 electrophotometer (SLT-LABINSTRUMENTS, Salzburg, Austria) using 490 nm as reference. All the reactions were proceeded at 37 $^{\circ}$ C.

Figure 1. Synthetic pathway of antigen conjugate.

Table 1. Hapten numbers of crocin-carrier protein conjugates

Conjugate	Succinate method		NaIO ₄ method
	CHS-BSA	CHS-HSA	CI-HSA
Protein used (μ mol)	0.23	0.23	0.09
Hapten used (μ mol)	13.9	29.3	5.1
[M] ⁺	75646.6	98342.2	69505.2
Hapten number	8.6	29.6	3.1

Competitive ELISA

A 96-well immunoplate absorbed by CHS-HSA (100 μ l) was treated with G-PBS (300 μ l) for blocking. After washing with T-PBS, 50 μ l crocin or other related compounds were incubated with 50 μ l MAb for 1 h. After incubation with secondary antibody and substrate as same as direct ELISA, the absorbance at 405 nm was measured. All the reactions were proceeded at 37 $^{\circ}$ C.

Figure 2. Immunization result of CHS-BSA. All the sera were collected 3 days after injection. Serum collected after secondary injection was diluted for 600 times, and the others for 3200 times.

Results and discussion

Conjugation with carrier protein and hapten number determination by MALDI-TOF-MS

As described in Figure 1, two conjugate pathways were used for the conjugation with carrier protein, and the hapten numbers of three conjugates were determined by MALDI-TOF-MS (Table 1). About 3 mol of crocin were conjugated with HSA in the case of CI-HSA. With the diol bond of glucose moiety cleaved by NaIO_4 , the hapten was conjugated with the protein via Schiff's base bond. However, since crocin has many functional groups reactive to NaIO_4 the exact structure of CI-HSA is obscure. On the other hand, only a single hydroxyl group on C-6 of terminal glucose was conjugated with HSA or BSA through hemisuccinate linker under mild condition. The hapten number of CHS-BSA was determined as 8.6, while that of CHS-HSA as 29.6 according to the most intensive molecular ion peaks. Difference of the hapten numbers between these two conjugates may occur depending on the different ratio of hapten and proteins used.

Production and characteristics of the MAbs against crocin

After four times injections with CHS-BSA every 2 weeks, the BALB/c mice used to derive the cell clone described in this study were hyperimmunized as indicated in Figure 2. About 2×10^8 spleno-

Figure 3a. Reactivities of MAbs against crocin. 100 ng/ml MAb was added to a 96-well immunoplate precoated with various concentrations of CHS-HSA.

Figure 3b. Reactivities of MAbs against crocin. MAb of series concentration was added to a 96-well immunoplate precoated with 25 ng/ml CHS-HSA.

cytes were yielded and fused with P3-X63-Ag8-653 HAT-sensitive mouse myeloma cell by routinely established procedure in this laboratory (Sakata et al., 1994; Ishiyama et al., 1996; Shoyama et al., 1996). After HAT-selection incubation, screening with direct and competitive ELISA and single cell cloning by limited dilution method (Goding, 1980), 3 hybridomas producing MAbs reactive to crocin were obtained. Their isotype and light chain were classified by indirect ELISA using rabbit IgG specially reactive to the class, subclass or light chain of mouse IgG. MAb 12a and 1d were identified as IgG_{2a}, while MAb 11h as

Table 2. Cross reactivities of the MABs against crocin

Compounds	Cross reactivities of different MABs (%)		
	12a	1d	11h
Crocin	100	100	100
Crocetin triglucoside	39.6	39.5	66.9
Crocetin diglucoside	26.8	25.9	61.6
Crocetin	2.6	4.4	1.5
Gentiobiose	—	—	—
Cellobiose	—	—	—
Maltose	—	—	—
β -Carotene	—	—	—
Nerol	—	—	—
Geraniol	—	—	—
Citrol	—	—	—
Citronellol	—	—	—
Cholesterol	—	—	—

— Cross reactivity < 0.05.

Figure 4. Reactivity of MAb 11 h. MAb 11 h of various concentration was added to a 96-well immunoplate precoated with CHS-HSA of different concentration.

IgG_{2b}. They all possess λ light chain. Their reactivities against crocin were tested by varying antigen or antibody concentration as described in Figure 3. MABs 12a and 1d had almost the same reactivity while MAB 11 h was more sensitive against crocin.

The cross reactivities of crocetin glycosides as indicated in Table 2 were calculated by the method reported by Weiler and Zenk (1976). From the data of Figure 4 which showed the reactivity of MAB 11 h in logarithmic plot, the optimal concentrations of antigen and antibody were determined as 25 ng/ml and 100 ng/ml respectively, based on two principles that

Figure 5. Calibration standard curve of MAB 11 h against crocetin glycosides. Various concentration of crocetin glycosides was incubated with 100 ng/ml MAB 11 h in a 96-well immunoplate precoated with 25 ng/ml CHS-HSA.

the concentration of MAB should be consistent with that of antigen and the absorbance should be among the most precise range (0.2–0.8). Figure 5 indicated standard calibration curves of crocetin glycosides obtained by competitive ELISA. Finally according to the concentration of free hapten or other relevant compounds, by which the binding between the antibody and antigen was halfly inhibited, the cross reactivities of crocin, crocetin triglucoside, crocetin diglucoside and crocetin were calculated as 100%, 66.9%, 61.6% and 1.5%, respectively. Besides, a series of compounds possessing analogical structure framework as crocin, such as carotenoids, monoterpenoids and sugars were screened, but no cross reactivities were found except crocetin glycosides. For MAB 12a and 1d, the cross reactivities were identified in the same way (data not shown). It is indicated that only part of the hapten molecule, especially the framework of carboxylic glycoside is important for the binding to antibody. One possible reason is that the molecule chain of crocin is too long to effect as a single epitope.

Competitive ELISA

The MAB following competition of free hapten is bound to the immunoplate precoated with CHS-HSA. Under this condition, the full measuring range of the assay extends from 10 ng/ml to 200 ng/ml of crocin as indicated in Figure 6. Due to its wide cross reactivities against crocetin glycosides, this ELISA system can only approximately reflect the total amount of

Figure 6. Calibration curve of crocin. Various concentration of crocin was incubated with 100 ng/ml MAb 11 h in a 96-well immunoplate precoated with 25 ng/ml CHS-HSA.

crocetin glycosides even though it is much more sensitive than TLC and HPLC analysis (Morimoto et al., 1994). Furthermore, no pretreatment of the sample is needed. The ELISA was not specific for crocin, however it did not react with monoterpenoids, carotenoids and sugars. This wide reactivity is the main advantage of the antibody used in this ELISA. It is better than a special antibody for the metabolic study on crocin and the study of pharmacologically active mechanism of crocin on LTP in the central nervous system. It is suggested this MAb should be helpful to the further study on the presumable receptor in the brain. Further application of these MAbs is in progress.

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