Characterization of a monoclonal antibody specific to a flavonol 2′-*O*-glucosyltransferase

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A monoclonal antibody to a partially purified preparation of 2′-*O*-glucosyltransferase was produced by *in vitro* immunization of spleen cells from BALB/c mice, followed by fusion with mouse myeloma cells. Hybridoma culture supernatants were screened by enzyme-linked immunosorbent assay for (*i*) their ability to produce immunoglobulins and (*ii*) their immunoreactivity with a partially purified enzyme preparation. The majority of the immunoglobulin-producing hybridomas were IgM secretors. Two highly immunoreactive IgM-secreting clones were chosen for further characterization. The supernatant fraction from a culture of one of these clones displayed 50% inhibition of the 2′-*O*-glucosyltransferase activity. The native form of the 2′-*O*-glucosyltransferase was essential for recognition, suggesting that the epitope recognized by the antibody is a conformational discontiguous one.

Key words: monoclonal antibody, *in vitro* immunization, flavonoid, *O*-glucosyltransferase.

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Nous avons produit un anticorps monoclonal contre une préparation partiellement purifiée de 2′-*O*-glucosyltransférase par immunisation *in vitro* des cellules spléniques de souris BALB/c, suivie d'une fusion avec des cellules de myélome de souris. Les surnageants des cultures d'hybridomes sont examinés par ELISA pour (*i*) leur pouvoir de produire des immunoglobulines et (*ii*) leur immonoréactivité avec une préparation enzymatique partiellement purifiée. La majorité des hybridomes produisant des immunoglobulines sont des sécréteurs d'IgM. Nous avons choisi deux clones IgM-sécréteurs fortement immunoréactifs pour plus ample caractérisation. La fraction surnageante issue d'une culture d'un de ces clones manifeste 50% d'inhibition de l'activité 2′-*O*-glucosyltransférasique. La forme native de la 2′-*O*-glucosyltransférase est essentielle à sa reconnaissance, preuve que l'épitope reconnu par l'anticorps est un épitope conformationnel morcelé.

Mots clés : anticorps monoclonal, immunisation *in vitro*, flavonoïdes, *O*-glucosyltransférase.

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Introduction

Flavonoid compounds are widely distributed in plants and are commonly *O*-glycosylated at positions 3, 7, and (or) 4′ (Harborne and Williams 1975). A number of partially methylated flavonols were identified as the 2′- and 5′-*O*-glucosides in *Chrysosplenium americanum* (Collins *et al.* 1981). A novel flavonol ring β-*O*-glucosyltransferase which catalyzes the transfer of the glucosyl moiety from UDP-glc to positions 2′- or 5′- of partially methylated flavonols (Fig. 1) was isolated from this plant (Bajaj *et al.* 1983). Previous work on this enzyme indicated the close similarity, in both chromatographic (Bajaj *et al.* 1983; Latchinian *et al.* 1987) and kinetic (Khouri and Ibrahim 1984) properties, of the 2′- and 5′-*O*-glucosylating activities. These results suggested that *O*-glucosylation of these two, para-oriented positions may be catalyzed by one enzyme. However, these two *O*-glucosylating activities were recently resolved by fast protein affinity chromatography (Latchinian *et al.* 1987).

In contrast with the enzymes of primary metabolism, those involved in the biosynthesis of secondary plant metabolites (alkaloids, flavonoids, etc.) are usually present in minute quantities. The low abundance of such enzymes renders their isolation and purification to homogeneity an extremely difficult task. Hybridoma technology allows the production of highly specific antibodies using partially purified protein antigen (Luben and Mohler 1980), as long as a specific method of screening for the desired antibodies is available. Furthermore, the use of a recently reported *in vitro* immunization technique (Reading 1982; Boss 1986) for the production of mAbs offers several advantages over the classical *in vivo* technique. The former requires only nanogram quantities of the protein antigen for sensitization (Luben and Mohler 1980) and a short immunization period (ca. 5 to 7 days).

In this paper we describe the combined application of hybridoma technology and *in vitro* sensitization technique for the production and characterization of a mAb specific to *Chrysosplenium* flavonol 2′-*O*-glucosyltransferase.

Materials and methods

Plant material

Chrysosplenium americanum Schwein ex Hooker (Saxifragaceae) was collected from St. Anicet (Que.) and was maintained in the greenhouse under controlled conditions of light, temperature, and humidity.

Animals and myeloma cell line

Male BALB/c mice, 6 to 8 weeks old, were obtained from Charles River Canada Inc., and the mouse Ig-nonproducing myeloma cell line P3X63 Ag8.653 was kindly provided by J.M. Leclerc, St. Justine Hospital, Montreal.

Extraction and partial purification of enzyme protein

Protein extracts were prepared as previously described (Bajaj *et al.* 1983) and were fractionated with solid ammonium sulfate. The protein fraction that precipitated between 35 and 70% saturation was collected by centrifugation. Partial purification (ca. 200-fold) of the protein pellet was performed using the Phar-

ABBREVIATIONS: FPLC, fast protein liquid chromatography; HAT, hypoxanthine, aminopterin, and thymidine; Ig, immunoglobulin; IMDM, Iscove's modified Dulbecco's medium; mAb, monoclonal antibody.

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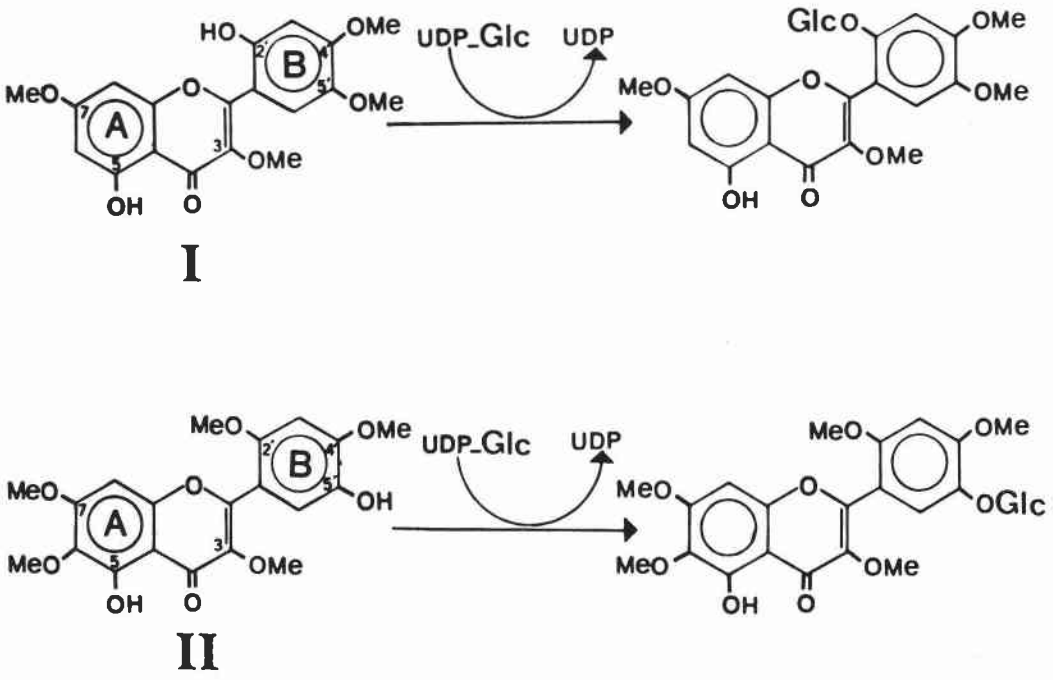

FIG. 1. *O*-Glucosylation of the 2′- and 5′-positions of partially methylated (Me = methyl group) flavonols of *Chrysosplenium americanum*. I, 5,2′-dihydroxy-3,7,4′,5′-tetramethoxyflavone; II, 5,5′-dihydroxy-3,6,7,2′,4′-pentamethoxyflavone.

macia FPLC system, by gel filtration on a column of Superose 12 (prep grade) HR 16/50, followed by ion-exchange chromatography on a column of Mono Q HR 5/5 (Latchinian *et al.* 1987). The protein fractions which contained both 2′- and 5′-*O*-glucosylating activity were pooled and used as the source of antigen in this study.

Isolation and stimulation of spleen cells

Spleen cell harvest and *in vitro* immunization were performed according to a reported procedure (Boss 1986) with some modification. The lymphocyte pellet, from three unimmunized male BALB/c mice, was suspended at 7.5×10^6 cells/mL in IMDM containing 10% fetal bovine serum (Flow Labs), 50 μM 2-mercaptoethanol, 2 mM glutamine (Gibco), 1 mM sodium pyruvate (Gibco), and 1% antibiotic–antimycotic solution (100×, Gibco). Muramyl dipeptide (Sigma) and antigen were added to the above suspension at final concentrations of 10 μg/mL and 2.5 μg/mL, respectively. The suspension was then transferred into tissue culture flasks and incubated at 37°C in a humidified atmosphere containing 8% carbon dioxide for 5 and 7 days prior to fusion.

Cell fusion

Fusion partners, namely mouse myeloma cells and *in vitro* sensitized spleen cells, were hybridized following a recently described method (Lane *et al.* 1986) at a ratio of 1:2, lymphocytes to myeloma cells. Cells were suspended in selective HAT (Gibco) medium containing 15% fetal bovine serum, and distributed at 2.1×10^5 total cells per well in 96-well tissue culture plates. Two fusions were performed, one on day 5 and the other on day 7 after *in vitro* immunization. Screening for Ig production was done 10 days after each fusion by ELISA (see below). ELISA was also used to screen for hybridomas which specifically react with the partially purified *O*-glucosyltransferase preparation. Highly immunoreactive hybridomas were cloned twice by limiting dilution at 1 cell per well, retested by ELISA for reactivity, and if found positive were expanded *in vitro*.

ELISA

Immulon-2 plates (Dynatech Labs) were coated with 1 μg/mL partially purified enzyme protein or affinity purified goat antibodies to mouse IgG or IgM (Kirkegaard & Perry Labs) in carbonate buffer, pH 9.6. The plates were incubated overnight at 4°C and assayed as previously described (Campbell 1984), with some modification. BSA (2.5%) in phosphate-buffered saline was used to block uncoated sites, and Tween 20 (0.05%) in phosphate-buffered saline was used to wash the plates between incubations. Goat anti-mouse IgG or IgM coupled with peroxidase (Kirkegaard & Perry Labs) was used as the secondary antibody. The reaction was revealed with a freshly prepared solution of *o*-phenylenediamine (0.04 mg/mL, Sigma) in phosphate–citrate buffer, pH 5.0, in presence of 0.01% hydrogen peroxide. After stopping the reaction with sulfuric acid, the OD was measured at 492 nm using an EL308 microplate reader (Bio-Tek). Mouse preimmune serum, as well as myeloma cell and feeder cell culture media, were used as controls in order to confirm the specificity of mAb binding to the partially purified preparation of *O*-glucosyltransferases.

Immunodetection following nondenaturing and SDS–PAGE (Western blotting)

The partially purified preparation of *O*-glucosyltransferases was resolved either by 12% SDS–PAGE (Laemmli 1970) or by 7.5% nondenaturing PAGE (Davis 1964), which were run using the Bio-Rad Mini-PROTEAN II dual slab cell. In order to localize *O*-glucosyltransferase activity, nondenaturing gels were run at 4°C, sliced horizontally into 1.5-mm pieces, crushed in 20 mM Tris-HCl, pH 7.8, containing 14 mM 2-mercaptoethanol, incubated overnight at 4°C, and assayed for 2′- and 5′-*O*-glucosylating activity.

Electrophoretic transfer of protein bands from the gels onto nitrocellulose was carried out (Towbin *et al.* 1979) using the Bio-Rad Mini Trans-Blot cell. Following transfer, nitrocellulose strips were blocked with BSA (2.5%) in Tris-buffered saline, and Tween 20 (0.05%) in Tris-buffered saline was used to wash the strips between incubations. The strips were incubated with either test (mAb) or control (myeloma or feeder cells) supernatants. Mouse preimmune serum was also used as a control. Goat antibodies to mouse IgM (or IgG) labeled with peroxidase were used as secondary antibodies. Immunoreactive bands were visualized by incubating the strips in Tris-buffered saline containing 0.5 mg/mL 4-chloro-1-naphthol, 0.015% hydrogen peroxide, and 16.6%

TABLE 1. Specific fusion efficiency

Fusion	Ig-producing wells (% of total) IgM	IgG	Specific immunoreactive wells (% of IgM-secretors)
Day 5	50.5	3.6	22.2
Day 7	45.9	0.5	18.6

NOTE: ELISA was applied to determine the class and immunoreactivity of the antibodies produced by the hybridomas; goat anti-mouse IgG or IgM and a partially purified *O*-glucosyltransferase preparation were used, respectively. The reaction was revealed by peroxidase-labeled goat anti-mouse IgG or IgM.

aqueous methanol until color development. Neither control supernatants nor preimmune serum revealed any immunoreactive bands.

Inhibition of O-glucosyltransferase activity by mAb

Initially, a partially purified preparation of *O*-glucosyltransferases was stabilized with an optimum concentration of BSA (1.25 mg/mL). The preincubation mixtures which consisted of 40 μL of stabilized enzyme protein and 160 μL of different dilutions of control or test supernatants were incubated for 30 min at room temperature. Aliquots (80 μL) from the various preincubation mixtures were then assayed for 2′- and 5′-*O*-glucosyltransferase activity using a standard assay (see below). The controls used were mouse preimmune serum, feeder cell supernatant, and myeloma culture medium.

O-Glucosyltransferase assay and product identification

O-Glucosyltransferase assay and product identification were performed as previously described (Bajaj *et al.* 1983) using UDP-[U-^{14}C]Glc (Amersham) as the glucosyl donor. The substrates used were 5,2′-dihydroxy-3,7,4′,5′-tetramethoxyflavone (I, Fig. 1) and 5,5′-dihydroxy-3,6,7,2′,4′-pentamethoxyflavone (II, Fig. 1) for the 2′- and 5′-*O*-glucosylating activities, respectively (Fig. 1). The reaction products were identified by cochromatography with their corresponding 2′- and 5′-*O*-glucosides (Collins *et al.* 1981) on Polyamid-6 MN using benzene – methyl ethyl ketone – methanol (8:1:1, by volume) as the solvent system, and autoradiographed on x-ray film.

Protein determination

Protein concentration was determined using the Bio-Rad protein assay reagent (Bradford 1976) and BSA as standard.

Results and discussion

We have applied the novel technique of *in vitro* immunization (Reading 1982; Boss 1986) for the production of a mAb to *Chrysosplenium* flavonol 2′-*O*-glucosyltransferase, because it requires only minute quantities of soluble protein for sensitization. Furthermore, pure protein is not required as a source of antigen for the production of mAbs (Luben and Mohler 1980) since the hybridomas produced are subcloned more than once, giving rise to cell lines secreting monospecific, single-clone antibodies which are then tested and selected according to their specificity. Therefore, the difficulty of further purifying the *O*-glucosyltransferases to homogeneity has been circumvented by using a partially purified enzyme preparation as the antigen source and an *in vitro* immunization system.

Sensitization and selection

Since the *O*-glucosylating proteins are minor components of the partially purified protein preparation, 2.5 μg of the latter was added per millilitre of the *in vitro* immunization culture medium, although nanogram quantities were reported to be sufficient (Luben and Mohler 1980). However, the majority of Ig-producing hybridomas, as determined by ELISA, were IgM-secretors (Table 1). This result is in agreement with that of Erkman *et al.* (1987) who reported the production of antibodies only of the IgM class to a human plasma protein. This is not unexpected since *in vitro* immunization results in a primary immune response only. ELISA was also used for a second screening of the IgM-producing hybridomas, but this time using a partially purified preparation of *O*-glucosyltransferases with the aim of selecting hybridomas which specifically react with the enzyme protein (Table 1). The enzyme preparation did not show any immunoreactivity with preimmune serum as detected by ELISA. A few of the strongly immunoreactive IgM-producing hybridomas were cloned twice, thus generating single-clone cell lines. Two of the these cell lines, namely C3-2 and C7-1 from day 5 and day 7 fusions, respectively, were stable and, therefore, selected to be expanded *in vitro* for further characterization. Monoclonal antibodies C3-2 and C7-1 had similar ELISA reactivities on both goat anti-mouse IgM and on partially purified enzyme preparation (data not shown).

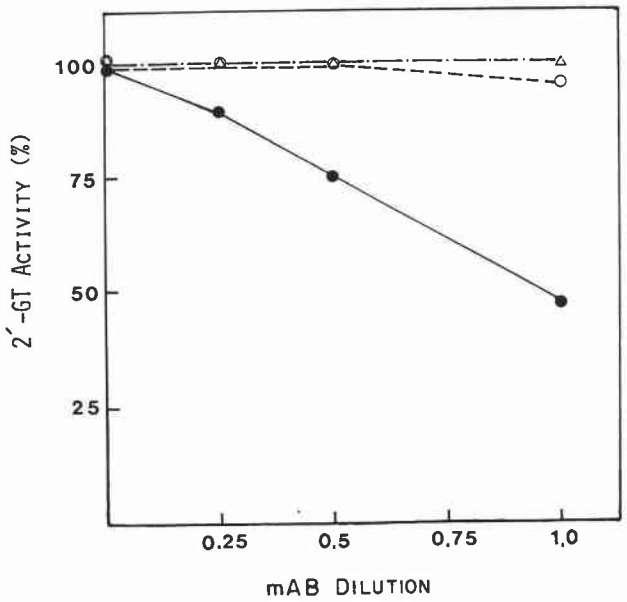

FIG. 2. Inhibition of 2′-*O*-glucosyltransferase activity by mAbs. The enzyme preparation was preincubated for 30 min at room temperature with different dilutions of mAbs. ●, C3-2; ○, C7-1; △, control supernatant. The remaining 2′-*O*-glucosyltransferase (2′-GT) activity was determined by the standard assay.

Characterization

Characterization of the mAbs produced was performed by inhibition studies using the standard enzyme assay. Thus, in order to demonstrate the specificity of the produced mAbs for 2′- and 5′-*O*-glucosyltransferases, we studied the ability of C3-2 and C7-1 culture supernatants to inhibit enzyme activity. Precautions taken were as follows: (*i*) to avoid a protein stabilization effect by the added mAb or control supernatants on the 2′- and 5′-*O*-glucosylating activities, the enzyme protein was previously stabilized with an optimum concentration of BSA (1.25 mg/mL); (*ii*) the protein concentrations of the test and control supernatants were made equal; and (*iii*) the enzyme preparation was used at concentrations low enough to avoid masking of the inhibition produced by the mAbs. Immunoreactive mAb C3-2 inhibited 2′-*O*-glucosyltransferase activity (Fig. 2), whereas immunoreactive C7-1 did not reveal any significant inhibi-

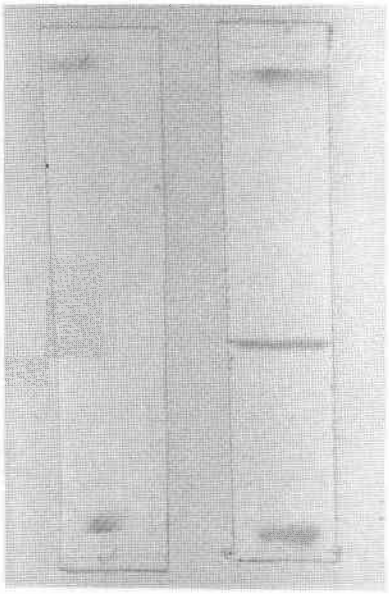

FIG. 3. Western blot analysis of mAb C3-2. Partially purified *O*-glucosyltransferase preparation was subjected to 7.5% nondenaturing PAGE and transferred to nitrocellulose, followed by immunodetection using control supernatant (A) or mAb C3-2 (B).

tion. Furthermore, undiluted C3-2 culture supernatant displayed 50% inhibition of the 2′-*O*-glucosyltransferase activity. The percent inhibition decreased upon dilution of the supernatant (Fig. 2). Neither C3-2 nor C7-1 inhibited the 5′-*O*-glucosyltransferase activity. These results demonstrate that mAb C3-2 specifically inhibited the 2′-*O*-glucosyltransferase.

Inhibition of enzyme activity by an antibody indicates that the latter recognizes an epitope which is part of the native structure of the enzyme protein. This seems to be the case with mAb C3-2 which inhibited 2′-*O*-glucosyltransferase activity. Monoclonal antibody C3-2, but not C7-1, displayed a significant immunoreactive band after electrophoretic transfer of protein from nondenaturing gels onto nitrocellulose (Fig. 3). This immunoreactive band corresponded with the region of 2′-*O*-glucosyltransferase activity on nondenaturing polyacrylamide gels. Immunodetection after SDS–PAGE of the enzyme preparation, using C3-2 and C7-1 culture supernatants, did not reveal immunoreactive bands. This is further evidence for the recognition by mAb C3-2 of a determinant in the native spatial conformation of the 2′-*O*-glucosyltransferase, thus implying recognition of an assembled topographic epitope rather than a segmental contiguous one (Berzofsky 1985). It should be pointed out that the inability of the mAb C3-2 to inhibit the 5′-*O*-glucosyltransferase does not exclude the possibility that it may bind to this enzyme species. However, the fact that the highly purified 5′-*O*-glucosyltransferase (Latchinian *et al.* 1987) did not reveal any immunoreactive bands on Western blots indicates that mAb C3-2 neither inhibits nor binds to this form of the enzyme.

To our knowledge, this is the first reported instance in which the combined application of *in vitro* immunization and hybridoma technology has been successfully used to produce an antibody to a flavonol *O*-glucosyltransferase. The protocol used seems to provide a very efficient tool for the production of antibodies to enzymes of low abundance in plant tissues.

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