BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 17–22 (1998)
ARTICLE NO. RC989091

A Mushroom Fruiting Body-Inducing Substance Inhibits Activities of Replicative DNA Polymerases

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Received July 3, 1998

We found and isolated two natural products in the extract from a basidiomycete, *Ganoderma lucidum*, as eukaryotic DNA polymerase inhibitors. The compounds were identified as cerebrosides, (4*E*,8*E*)-N-D-2′-hydroxypalmitoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and (4*E*,8*E*)-N-D-2′-hydroxystearoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine, and were found to be identical to the mushroom fruiting body-inducing substances (FIS) reported. These cerebrosides selectively inhibited the activities of replicative DNA polymerases, especially the α-type, from phylogenetically broad eukaryotic species, whereas they hardly influenced the activities of DNA polymerase β, prokaryotic DNA polymerases, terminal deoxynucleotidyl transferase, HIV reverse transcriptase, RNA polymerase, deoxyribonuclease I, and ATPase. The inhibition of another replicative polymerase, the δ-type, was moderate. The inhibitions of the replicative polymerases were dose-dependent, and the IC_{50} for animal or mushroom DNA polymerase α was achieved at approximately 12 μg/ml (16.2 μM) and for animal DNA polymerase δ at 57 μg/ml (77.2 μM). FIS is possibly a DNA polymerase inhibitor specific to the replicative enzyme group, and the fruiting body formation may be required for the suppression of the DNA replication or the vegetative growth of the mycelium.

Eukaryotic DNA polymerases are designated as α, β, γ, δ, ϵ and ζ, each responsible for different DNA syntheses (1, 2). DNA polymerase α and δ are replicative enzymes, DNA polymerase β, δ and ϵ are repair-related enzymes, and DNA polymerase γ acts on mitochondrial replication; the function of DNA polymerase ζ is unknown. The current intense interest to define the precise *in vivo* roles of these polymerases has prompted us to undertake a major search for inhibitors of these enzymes (3-12). Subsequently, several fungi and mushrooms were found to produce such inhibitors, and one of the strongest inhibitors was found in a basidiomycete, *Ganoderma lucidum*. We have isolated the compound from the fruiting bodies of *Ganoderma lucidum*, which is known as a medicinal mushroom used in traditional Chinese medicine, as the so-called "*reishi*" or "*mannentake*" mushroom. The powder of the dried "*reishi* " was used as a cancer chemotherapy agent in the Imperial Court of ancient China. The compound was identified as a mixture of two cerebrosides by spectroscopic analyses as described in the later part of this report. Interestingly, these cerebrosides coincided with the substances which were able to induce the fruiting bodies of a mushroom, *Schizophyllum commune* reported in 1983 (13) and in 1985 (14), which were called the fruiting body-inducing substances (FIS). We therefore investigated the properties of the cerebrosides or FIS in their relation to the inhibitory effect of DNA polymerases.

EXPERIMENTAL PROCEDURE

Materials. Nucleotides and chemically synthesized template-primers such as poly(dA) and oligo$(dT)_{12\text{-}18}$ were purchased from Pharmacia (Uppsala, Sweden). [^{3}H]-dTTP (thymidine 5′-triphosphate, 43 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA). The fruiting caps of the "*reishi*" or "*mannentake*" mushroom were purchased from Japan Microbe Chemical Co. (Tokyo, Japan). All other reagents including enzymes were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan). DNA polymerase α was purified from the calf thymus by immuno-affinity column chromatography as described previously (15). Recombinant rat DNA polymerase β was purified from *E. coli* JMpβ5 as described by Date *et al.* (16). DNA polymerase δ was puri-

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0006-291X/98 $25.00

fied from developing cherry salmon (*Oncorhynchus masou*) testis (17). DNA polymerase I (α-like) and II (β-like) from a higher plant, cauliflower inflorescence, was purified according to the methods outlined by Sakaguchi *et al.* (3). Human immunodeficiency virus type-1 (HIV-1) reverse transcriptase and the Klenow Fragment of DNA polymerase I were purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). T4 DNA polymerase, Taq DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan). Calf thymus terminal deoxynucleotidyl transferase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA, USA). The *Coprinus* enzymes such as DNA polymerase α and β, RNA polymerase I and II, polynucleotide kinase, DNA-dependent ATPase and deoxyribonuclease were purified according to the methods in our previous reports (4, 8, 12, 18-21).

Enzyme assays. The activities of DNA polymerases and bovine pancreas deoxyribonuclease I were measured by the methods described in previous reports (22-24). The activities of RNA polymerase, polynucleotide kinase and DNA-dependent ATPase were measured in standard assays according to the manufacturer's specifications, as described by Nakayama *et al.* (25), Soltis *et al.* (26) and Kornberg *et al.* (27), respectively.

RESULTS AND DISCUSSION

Extraction and purification of the cerebrosides from the fruiting caps of the basidiomycete, Ganoderma lucidum. As noted above, we have sought mammalian DNA polymerase inhibitors among microbially-derived products and have attempted to screen them systematically. We subsequently found a potent activity in the fruiting caps of the *Ganoderma* mushroom that inhibits calf thymus DNA polymerase α activity, and we then tried to extract the effective substance. After homogenization in a Waring blender, the fruiting caps (150 g) were extracted with 5 L acetone for 3 days. The evaporated extract (5.5 g) was partitioned between ethyl acetate and water and adjusted to pH 7. The residue (2.2 g) after evaporating the organic layer was subjected to silica gel column chromatography and then eluted with chloroform/methanol (v/v 8:1). The active fractions were collected (200 mg) and then loaded on a silica gel column chromatography and eluted with ethyl acetate/methanol/water (v/v/v 15:1:0.1). The active fractions were pooled and evaporated to dryness, and gave a white powder (16 mg).

Negative fast atom bombardment mass spectra (FAB MS) and high resolution fast atom bombardment mass spectra (HR FAB MS) were run on a glycerol matrix, and were recorded on a JEOL (Tokyo, Japan) JMS HX110 mass spectrometer. Major peak (compound **1** in Fig. 1): $[M-H]^+$ obsd. *m/z* 726.5529; calcd. *m/z* 726.5520 for $C_{41}H_{76}O_9N$. Minor peak (compound **2** in Fig. 1): $[M-H]^+$ obsd. *m/z* 754.5825; calcd. *m/z* 754.5833 for $C_{43}H_{80}O_9N$. Nuclear magnetic resonance (NMR) measurements were performed on a spectrometer (Bruker (Silberstreifen, Germany) AC-300 Plus). ^1H and ^{13}C spectra were recorded in CD_3OD solution at 300 and 75 MHz, and the chemical shifts given relative to TMS and CD_3OD solvent peaks were δ 0.00 and 77.0 ppm, respectively. ^{13}C (ppm); δ 175.7, 135.3, 133.2, 129.6, 123.3, 103.2, 76.5, 76.4, 73.5, 71.6, 71.4, 70.1, 68.2, 53.1, 39.3, 34.4, 32.3, 31.6, 29.3-28.9, 27.6, 27.2, 24.7, 22.3, 14.6, 13.0. ^1H (ppm); δ 5.72 (1H, dt, J=5.87, 15.27 Hz, H-5), 5.48 (1H, dd, J= 7.27, 15.27 Hz, H-4), 5.14, (1H, m, H-8), 4.26 (1H, d, J= 7.76 Hz, H-1″), 4.13 (2H, m, H-3 & 1b), 4.10 (2H, m, H-2, 2′), 3.86 (1H, d, J= 11.72 Hz, H-6″), 3.71, (2H, m, H-1a & 6″a), 3.32-3.16 (4H, m, H-2″, 3″, 4″ & 5″), 2.06 (2H, m, H-6 & 7), 1.97 (1H, t, J= 7.24 Hz, H-10), 1.59 (3H, s, H-19), 1.36-1.28 (20H, m, overlapping CH_2), 0.90 (6H, overlapping t, J= 6.35 & 6.98 Hz, H-18 & 16′). The molecular formula $C_{41}H_{77}O_9N$ for compound **1** was determined by negative HR FAB MS along with a compound **2** of $C_{43}H_{81}O_9N$. In the ^{13}C NMR, four olefinic carbon signals at δ 135.3, 133.2, 129.6 and 123.3 ppm showed the presence of two olefins in its structure. The simple doublet olefin protons at δ 5.72 and 6.25 was trans olefin, determined by the large coupling constant (J= 15.27 Hz). The β-D-glucopyranoside was assigned by the coupling constant of the anomeric proton at δ 4.26 (J= 7.76 Hz) and the chemical shift at δ 103.2 ppm. According to the NMR spectra and molecular formula, the inhibitor fraction was a mixture of two structurally-related cerebrosides (Fig. 1). Compounds **1** and **2** were (4*E*,8*E*)-*N*-D-2′-hydroxypalmitoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and (4*E*,8*E*)-*N*-D-2′-hydroxystearoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine, respectively (Fig. 1). They are each composed of a 1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and 2-hydroxypalmitoyl or 2-hydroxystearoyl as a fatty acid moiety, respectively (Fig. 1). The relative peak intensities to the compounds **1** and **2** were 71.3 % and 28.7 %, respectively, in the negative FAB MS. The structures of the cerebroside compounds **1** and **2** were identified as Sch II (13) and Sch IV (14), respectively, coinciding with the fruiting body-inducing substances in *Schizophyllum commune.* The cerebrosides, the mixture of compounds **1** and **2,** could induce the dikaryotic fruiting bodies in the tested mushroom strain, *Coprinus cinereus* (Fig. 2B). Namely, the fruiting body-inducing substance could be a DNA polymerase inhibitor. We therefore analyzed the biochemical properties of the cerebrosides in the following experiments. The mixture of both cerebrosides was used as the inhibitor fraction in the experiments, because of the difficulty of separating them from each other.

Fruiting body-inducing activity of the cerebrosides. Bioassays for the fruiting body-inducing activity (FIS activity) were performed as described by Uno *et al.* (28). The test strain was *Coprinus cinereus* (5026 + 5132). The strain's mycelium can vegetatively grow in a malt-yeast agar medium, but under such conditions, the fruiting bodies are hardly formed (see Fig. 2A). When the cerebrosides were added to the agar medium, fruiting bodies were efficiently induced (Fig. 2B). These cerebrosides must be the FIS.

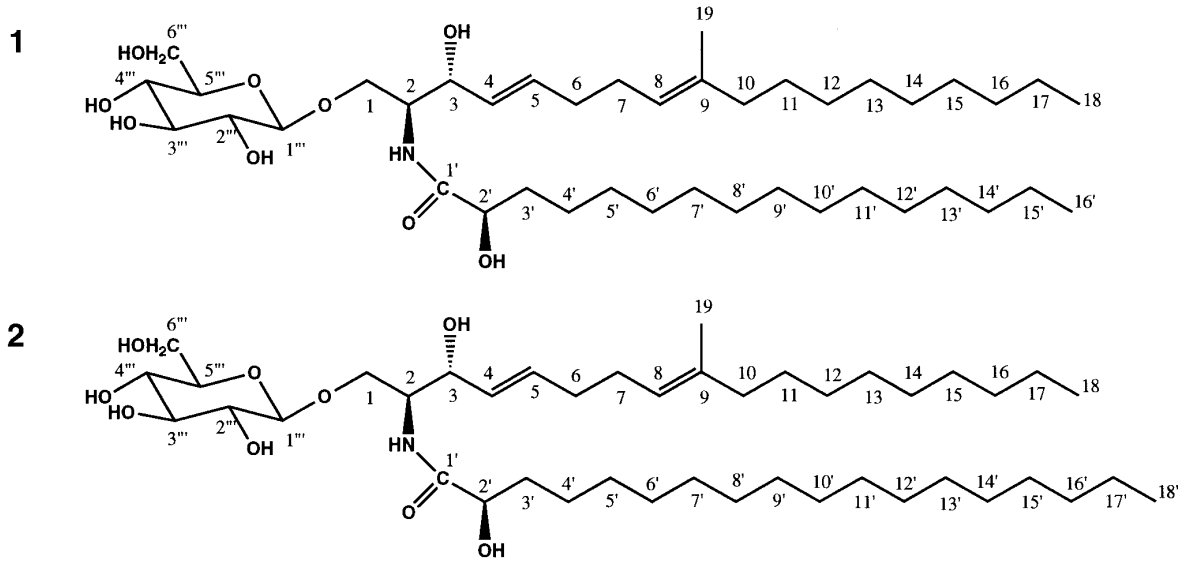

FIG. 1. Structures of cerebroside compounds **1** and **2** from the fruiting caps of a basidiomycete, *Ganoderma lucidum*.

Inhibition by the cerebrosides of activities of DNA polymerases and the other DNA metabolic enzymes. As shown in Fig. 3A, the cerebrosides at 60 μg/ml (81.3 μM) were found to significantly inhibit the activity of a typical replicative polymerase, α-type, such as calf thymus DNA polymerase α and DNA polymerase I (α-like polymerase) from a higher plant, cauliflower. Calf thymus DNA polymerase α inhibition was non-competitive with both the template-primer DNA and the substrate (the Km was unchanged at concentration of 6.2 μM of poly(dA)/oligo(dT)12-18 and of 2.2 μM dTTP). The inhibition constant (Ki) values, obtained from Dixon plots, were found to be 6.5 μg/ml (8.8 μM) and 16 μg/ml (21.7 μM) for the template-primer DNA and the substrate, respectively. Aphidicolin, which is a representative of the polymerase α inhibitors, was also investigated. The Ki values were 21 μg/ml (62.0 μM) for the template DNA and 5.2 μg/ml (15.3 μM) for the substrate. The cerebrosides have stronger affinity to the DNA polymerase α than aphidicolin. The cerebrosides weakly or hardly influenced the activities of rat DNA polymerase β and cauliflower DNA polymerase II (β-like polymerase). Interestingly, the cerebrosides moderately inhibited another replicative polymerase, fish DNA polymerase δ (Fig. 3A). We recently found that the partial amino acid sequences of a 110 kDa polypeptide of a new DNA polymerase species from the testis of a fish, cherry salmon (*Oncorhynchus masou*), are almost homologous to that of human DNA polymerase δ (ref. 29, Yamaguchi *et al.* in preparation). We used this fish DNA polymerase δ in the inhibition test, because it is too difficult to obtain mammalian DNA polymerase δ routinely, and because our purpose of this report is to screen which class of polymerase is affected with the agent. DNA polymerase ϵ and ζ were harder obtainable. Since the enzymes inhibited are correlated mostly to the DNA replication, the cerebrosides may be an inhibitor of the DNA replication, and subsequently, of the cell cycle progression or the simple cell reproduction. Under the conditions used here, HIV-1 reverse transcriptase, the prokaryotic DNA polymerases (i.e., the Klenow Fragment of DNA polymerase I, T4 DNA polymerase and Taq polymerase) and the DNA-metabolic enzymes (i.e., T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I) were not inhibited at all (Fig. 3A). Calf thymus terminal deoxynucleotidyl transferase was weakly inhibited by the cerebrosides (Fig. 3A). The inhibition seemed to occur only for the replicative polymerases, a result leading us to speculate that the fruiting body formation may be required for the prevention of the DNA replication.

Whether the polymerase inhibitory effect also occurs on the mushroom polymerases is critical in relation to the FIS activity. The DNA polymerases and the other DNA metabolic enzymes from *Coprinus cinereus*, the basidiomycete tested (Fig. 2), were also tested (Fig. 3B). The cerebrosides at 60 μg/ml (81.3 μM) potently influenced the activity of DNA polymerase α, but had no inhibitory effect on DNA polymerase β or the other DNA metabolic enzymes (Fig. 3B). Mushroom DNA polymerase δ, ϵ and ζ could not be tested because it has not been identified in *Coprinus* or any basidiomycete yet. However, the inhibition data for the α-type, one of the replicative polymerases, suggests that even in the basidiomycetes the replicative polymerases must be selectively affected by the cerebroside.

Figure 3C shows the dose-response curves of the cerebrosides to calf thymus DNA polymerase α, rat DNA polymerase β, fish DNA polymerase δ and *Coprinus*

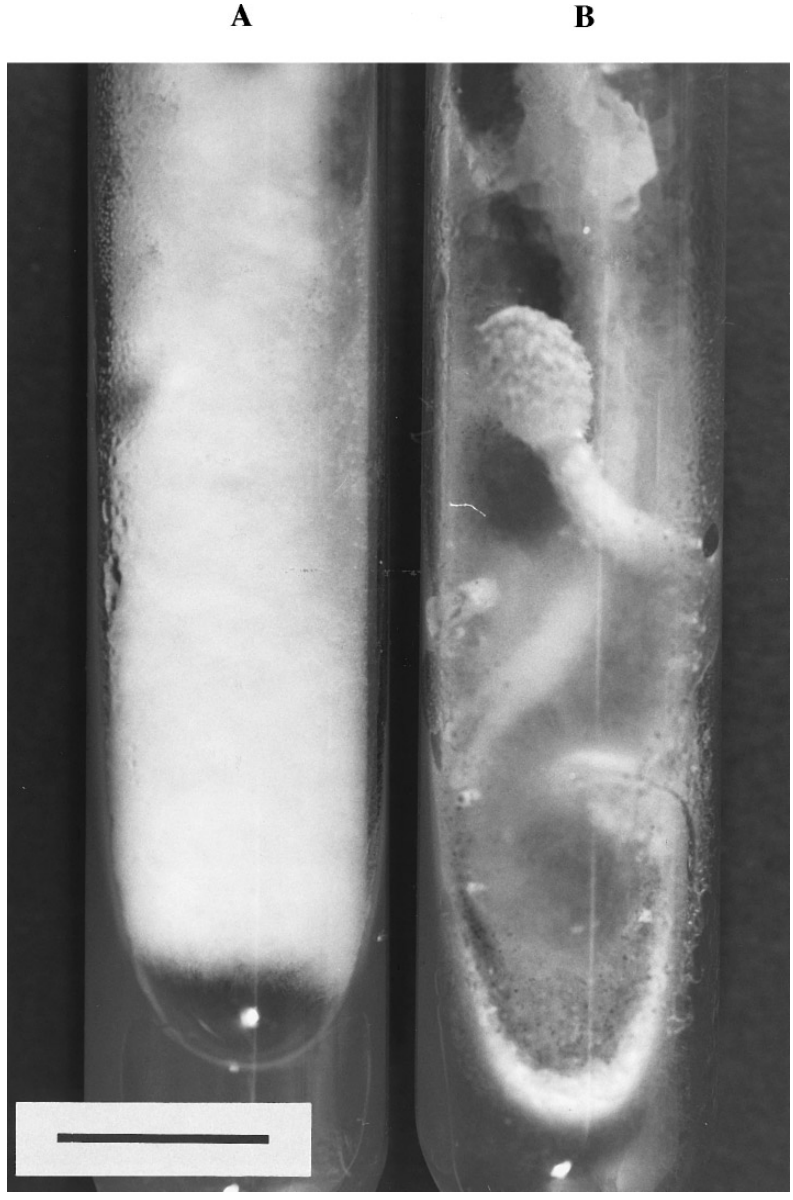

FIG. 2. Fruiting body-inducing activity of the cerebrosides isolated from the basidiomycete, *Ganoderma lucidum*. Dikaryotic cultures of strain 5026 + 5132 of *Coprinus cinereus* 14 days after inoculation with or without cerebrosides. (A) without cerebrosides; (B) with 200 μg cerebrosides. The bar indicates 1 cm.

DNA polymerase α. The inhibition by the cerebrosides of the α-type polymerase was very strong and dose-dependent, with 50 % inhibition for both DNA polymerase α observed at the dose of 12 μg/ml (16.2 μM); almost complete inhibition (more than 80 %) was achieved at approximately 30 μg/ml. The inhibition of the δ-type polymerase was moderate and also dose-dependent, with 50 % inhibition observed at the dose of 57 μg/ml (77.2 μM), and almost complete inhibition (more than 80 %) was achieved at more than 100 μg/ml. In contrast, the cerebrosides had very weak effects on the rat DNA polymerase β activity. We therefore concluded that the cerebrosides as FIS could be an inhibitor specific to replicative DNA polymerases, especially the α-type, *in vitro* from a phylogenetically wide range of eukaryotic species, including the fungus enzyme.

As mentioned briefly above, the fruiting body formation is regarded as a model system for studies of differentiation and morphogenesis mechanisms of fungi. For the mushroom reproduction, the fruiting body formation is genetically essential in the long range, and for the morphogenesis, it is a critical process. It is conceivable that a certain regulatory substance produced during the mycelium growth plays an important role at the initiation stage of the fruiting body formation, as in the case of the sexual morphogenesis of basidiomycetes

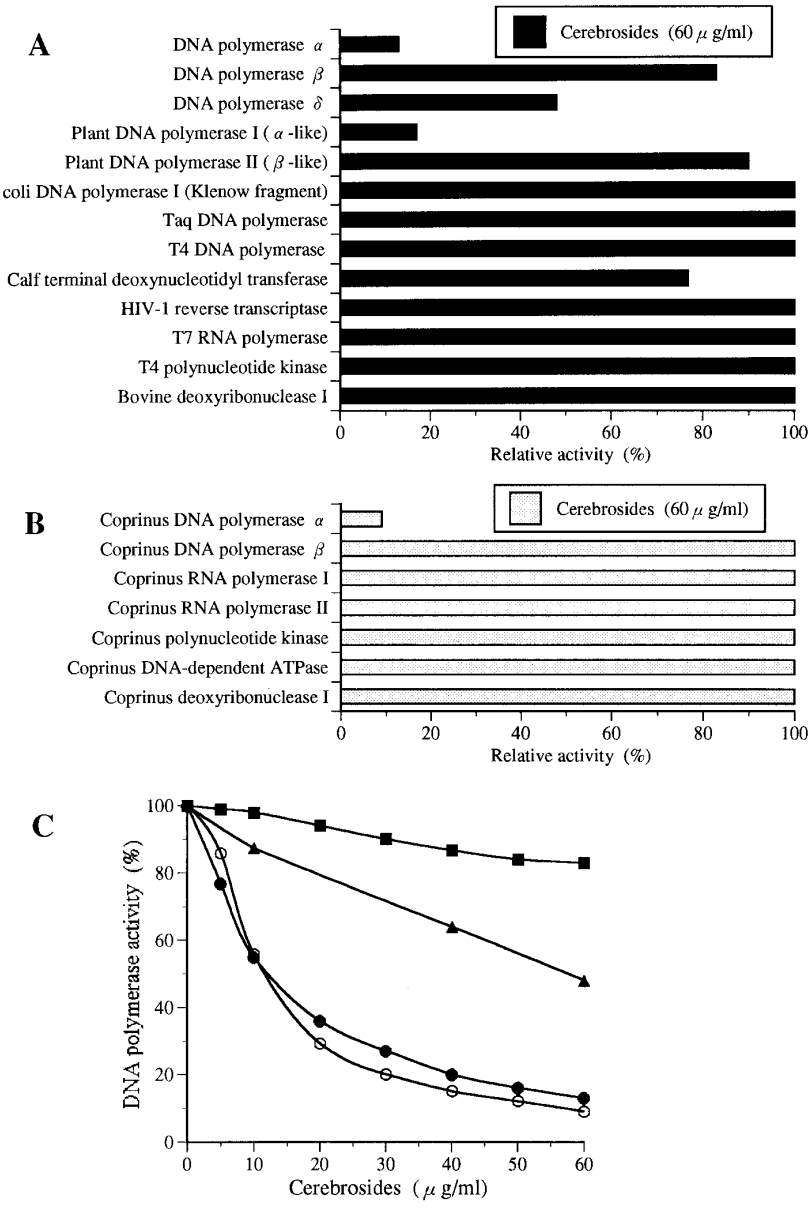

FIG. 3. (A, B) Effect of cerebrosides on the activities of various DNA polymerases and other enzymes (A) and *Coprinus* enzymes (B). DNA polymerase α, β, and δ were purified from calf thymus, rat recombinant and fish (cherry salmon, *Oncorhynchus masou*), respectively. The cerebrosides (60 μg/ml) were incubated with each enzyme (0.05 units). The enzymatic activity was measured as described in previous reports (22-24). Enzyme activity in the absence of cerebrosides was taken as 100%. (C) Inhibition of animal DNA polymerase α, β, δ and *Coprinus* DNA polymerase α activities by cerebrosides. Calf DNA polymerase α (·), rat DNA polymerase β (■) fish DNA polymerase δ (▲), and *Coprinus* DNA polymerase α (○) (0.05 units each) were pre-incubated with the indicated concentrations (0 to 60 μg/ml) of purified cerebrosides, and then assayed for these enzyme activities as described in the previous reports (22-24).

Rhodosporidium toruloides (30) and *Tremella mesenterica* (31). The substance is FIS, as often mentioned in this report. The biochemical mechanism of fruiting body formation has not yet been elucidated. The cerebrosides that were found as an inhibitor of eukaryotic replicative polymerases are the FIS, and the FIS did not affect the activities of the repair-related polymerase (DNA polymerase β) and the other DNA metabolic enzymes. If the cerebrosides can penetrate viable mycelium cells, they must affect the *in vivo* polymerase activities. It is possible that the fruiting body-inducement requires to suppress the activity of the replicative polymerase and to stop the mycelium cell proliferation.

ACKNOWLEDGMENTS

We thank Mr. J. Nishikawa and Dr. Y. Minegishi for providing the fruiting caps of a basidiomycete, *Ganoderma lucidum*, and Ms.

Y. Tsuya, Ms. A. Tosaka, Ms. M. Takenouchi, Ms. H. Sasaki, and Ms. S. Inagaki of our laboratory for their technical support.

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