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Screening of Sterol Biosynthesis Inhibitors from Natural Products Using Recombinant Yeast Carrying Human Lanosterol Synthase

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Abstract – For the screening of inhibitors of sterol biosynthesis from natural products, a simple and rapid assay method was developed using recombinant yeast carrying human lanosterol synthase, main target of this assay method. Sterol biosynthesis inhibition activity was monitored only by the inhibition of growth of the recombinant yeast. By changing the substrate, this assay method can figure out which step is inhibited in the sterol biosynthesis by the test material. With this assay method total 102 plant samples were screened for their inhibitory activity of sterol biosynthesis. Among plant water extracts screened, 11 plant samples showed inhibitory activity on sterol biosynthesis in ergosterol (–) medium. For selection of the specific inhibitory materials, 11 plant samples were re-assayed in ergosterol (+) medium. After all 5 plant samples, *Abutilon avicennae* Gaertn. (stem), *Alnus japonica* Steud. (stem), *Amaranthus mangostanus* L. (aerial part), *Philadelphus schrenckii* Pupr. (leaf) and *Pimpinelia brachycarpa* Nakai (aerial part), showed specific inhibitory activity.
Key words – Sterol biosynthesis, human lanosterol synthase, recombinant yeast, assay method, screening of natural products

Introduction

Sterols play important roles in living organisms including mammal, plant and fungus by acting as membrane constituents or as hormones. For these reasons, reduction or deletion of sterols from living organisms gives rise to inhibition of growth or even death. As a consequence, from their reduction or deletion, we can expect to get anti-hypercholesterolemic drug or even anti-cancer drug in mammal, herbicide in plant and anti-fungal drug to fungus. The main purpose of this study lies in development of anti-hypercholesterolemic drug from natural product.

For mammal, hypercholesterolemia is regarded as the main reason of atherosclerosis. Several approaches have been used to lower blood cholesterol level. These are (1) inhibition of cholesterol biosynthesis (Biller *et al.*, 1991; Horie *et al.*, 1991; Taton *et al.*, 1992; Sonoda *et al.*, 1988), (2) inhibition of its absorption (Hashim and Van Itallie, 1965; Shepherd *et al.*, 1980), (3) increase of its catabolism (Mittinen, 1972; Yamamoto *et al.*, 1983) and (4) increase of its excretion to bile acid (Steward *et al.*, 1982; Paul *et al.*, 1980). Because more than 70% of the total input of body cholesterol are derived from *de novo* synthesis in the body (Dietschy and Wilson, 1970), blood cholesterol level has been expected to be effectively reduced by inhibition of cholesterol biosynthesis. Cholesterol in mammal is biosynthesized from acetate in the liver via more than 20 reaction steps (Scheme 1). So far the most frequent target of the biosynthetic inhibitor has been 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase) that is involved in the early main step, which is the rate-limiting step in the whole cholesterol biosynthesis (Rodwell *et al.*, 1976). And several HMG-CoA reductase inhibitors have been developed as cholesterol lowering drugs. However, the inhibition of this enzyme can cause the inhibition of synthesis of physiologically important materials like dolichol, ubiquinone and prenylated proteins, from this reason some side effects can be expected and have been reported. These include inhibition of cellular proliferation (Habenicht *et al.*, 1980), modulation of changes in the cell cycle (Jakobisiak *et al.*, 1991) and cellular morphology (Maltese *et al.*, 1985). Thus, recently many investigations have been concentrated on searching for the inhibitors of some other enzymatic steps that are unique to the steroid biosynthetic pathway. Reaction steps comprising last stage are demethylase and several oxido-reductases. From broard spectrum of P_{450} type reactions, there also can be expected of side effects and desmosterol accumulated by the reaction of desmosterol reductase can cause severe side effects. On the other hand,

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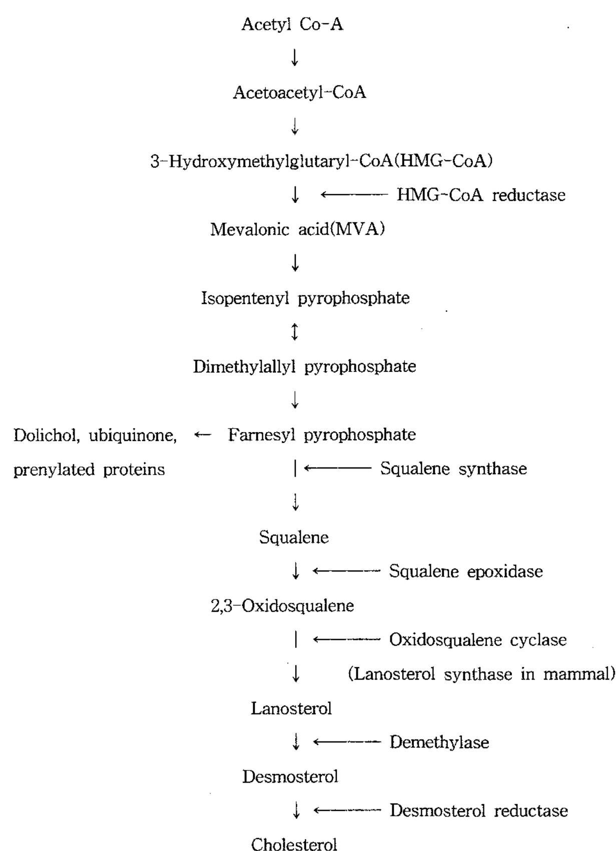

Scheme 1. Cholesterol biosynthetic pathway.

three kinds of enzymes, squalene synthase, squalene epoxidase and oxidosqualene cyclase located in the middle stage of cholesterol biosynthesis should be a new target for inhibition of cholesterol biosynthesis. Especially, oxidosqualene cyclase (OSC), lanosterol synthase (LS) of mammals (Kusano *et al.*, 1995; Sung *et al.*, 1995) can be a good target from its unique catalyzing properties of formation of cyclic lanosterol from acyclic oxidosqualene, inhibition of which can differ from other reactions in the body.

For the development of assay method for the screening of inhibitors of sterol biosynthesis from natural products, yeast transformant carrying human lanosterol synthase (hLS) (Sung *et al.*, 1995), main target for the development of cholesterol lowering material from natural products, was developed. With this recombinant yeast, a simple and rapid assay method was developed. With this assay method total 102 plant meterials are screened for their inhibitory activity.

Materials and Methods

Materials – Recombinant yeast having *GALI* promotor carrying human oxidosqualene cyclase (hOSC), pYES2h/GIL77, was used (Sung *et al.*, 1995). Positive control, lauryl dimethylamine oxide (LDAO) was purchased from Sigma Co. (USA).

Construction of recombinant yeast carrying human lanosterol synthase – Originally recombinant yeast having *GALI* promotor was cloned for the expression of hOSC using yeast expression vector, pYES2. For the construction of transformed yeast having *GPD* promotor, *Spe 1* site in cloning site of pYES2 vector was disrupted (pYES2ΔS) and *GPD* promotor was amplified by PCR from yeast genomic DNA. These two plasmids were digested with *Spe* I and *Hin*d III, ligated and propagated to get yeast expression vector having *GPD* promotor, pYES2G1. hOSC gene was transfered from pYES2h (*GALI* promotor) to pYES2G1 (*GPD* promotor) to get expression vector having *GPD* promotor and hOSC ORF, pYES2G1h. Transformed yeast, pYES2G1h/GIL77, was cloned with pYES2G1h and mutant yeast strain, GIL77, lacking LS.

Preparation of ergosterol (–)/(+) medium – Ergosterol (–) medium (synthetic complete medium-uracil + Tween 80 + hemin) and ergosterol (+) medium (synthetic complete medium - uracil + ergosterol + Tween 80 + hemin) were prepared. Formulation of the ergosterol (–)/(+) medium is shown in Table 1. YNB 10X stock solution was sterilized with membrane filtration. Hemin stock meant that 7.83 mg of hemin was dissolved in 6 ml of ethanol and 6 ml of sterilized deionized water with 60 μl of 2N NaOH. All of the stock solutions were kept at refrigerator. For ergosterol stock solution (kept at –20°C), 10 mg of ergosterol was

Table 1. Formulation of synthetic complete medium without uracil

Constituents	Concentration in medium
Drop-out mixture	2.0 mg/ml
YNB	6.7 mg/ml
Hemin	13.0 μg/ml
Ergosterol (ergosterol (+) medium only)	20.0 μg/ml
Tween 80	10.0 μg/ml

Table 2. Composition of drop-out mixture

Compound	Quantitiy	Compound	Quantity
Adenine	0.5 g	Leucine	4.0 g
Alanine	2.0 g	Lysine	2.0 g
Arginine	2.0 g	Methionine	2.0 g
Asparagine	2.0 g	*p*-Aminobenzoic acid	0.2 g
Aspartic acid	2.0 g	Phenylalanine	2.0 g
Cysteine	2.0 g	Proline	2.0 g
Glutamine	2.0 g	Serine	2.0 g
Glutamic acid	2.0 g	Threonine	2.0 g
Glycine	2.0 g	Tryptophan	2.0 g
Histidine	2.0 g	Tyrosine	2.0 g
Inositol	2.0 g	Valine	2.0 g
Isoleucine	2.0 g		

dissolved in 5 ml of ethanol and 5 ml of Tween 80.

At first, for preparation of ergosterol (–) medium, drop-out mixture (Table 2) was dissolved in deionized water. After autoclaving in a condition at 121°C, 15 psi for 15 minutes, the calculated volume of YNB, hemin stock solution and Tween 80 were added to the autoclaved solution. In case of ergosterol (+) medium, ergosterol stock solution was substituted for Tween 80.

Assay method – With the transformed yeast above, the assay method for inhibition of sterol biosynthesis was established by only measuring of the yeast growth (Scheme 2). After mixing the sample or control solution (100 μl) and medium (800 μl), yeast suspension (OD_{600nm} ≒ 2, 100 μl) was added and incubated at 30°C for 6 hours with shaking (200 rpm). After incubation OD of the incubation solution was detected at 600 nm and the inhibition rate was calculated.

Plant samples – Plant samples and parts used in the experiments, collected in the yards around Korea, are as follows: *Abutilon avicennae* Gaertn. (stem), *Aconitum triphyllum* Nakai (aerial part), *Aconogonum polymorphum* T. Lee (aerial part), *Actinidia polygama* Max. (aerial part), *Adenocaulon himalaicum* Edgew. (aerial part), *Alangium platanifolium* var. *macrophylum* Wanger. (leaf), *Alnus japonica* Steud. (leaf), *Alnus japonica* Steud. (stem), *Amaranthus mangostanus* L. (aerial part), *Arabis glabra* Bernh. (aerial part), *Aralia elata* Seem. (fresh leaf), *Arisaema amurense* var. *serratum* Nakai (aerial part), *Artemisia capillaris* Thunb. (aerial part), *Artemisia iwayomogi* Kitamura (aerial part), *Artemisia japonica* Thunb. (aerial part), *Artemisia montana* Pampan. (aerial part), *Asparagus schoberioides* Kunth. (aerial part), *Aster incisus* Fisch. (aerial part), *Astilbe chinensis* var. *davidii* Fr. (aerial part), *Astragalus membranaceus* Bunge (root), *Astragalus membranaceus* Bunge (adventitious root), *Astragalus mongholicus* Bunge (adventitious root), *Broussonetia papyrifera* Vent. (leaf), *Carduus crispus* L. (aerial part), *Carduus crispus* L. (whole plant), *Carpesium abrotanoides* L. (aerial part), *Cephalonoplos segetum* Kitamura (leaf and stem), *Chamaecyparis obtusa* Endl. (leaf and stem), *Chamaecyparis obtusa* Endl. (fruit), *Chamaecyparis pisifera* Endl. (leaf), *Chloranthus japonicus* Sieb. (aerial part), *Cimicifuga heracleifolia* Kom. (leaf), *Circaea cordata* Royle (aerial part), *Cirsium pendulum* Fisch. (aerial part), *Clematis ochotensis* Poir. (aerial part), *Clematis trichotoma* Nakai (aerial part), *Corydalis pallida* Pers. (aerial part), *Cudrania tricuspidata* Bureau (leaf and stem), *Desmodium oxyphyllum* DC. (aerial part), *Dianthus superbus* var. *longicalycinus* Williams (aerial part), *Dioscorea quinqueloba* Thunb. (aerial part), *Dioscorea septemloba* Thunb. (aerial part), *Equisetum arbense* L. (aerial part), *Erigeron annuus* Pers. (aerial part), *Erysimum aurantiacum* Max. (aerial part), *Euonymus alatus* Sieb. (leaf), *Fraxinus rhynchophylla* Hance (leaf), *Galium trachyspermum* A. Gray (aerial part), *Gypsophila oldhamiana* Miq. (aerial part), *Houttuynia cordata* Thunb. (aerial part), *Hylomecon vernale* Max. (aerial part), *Hypericum ascyron* L. (aerial part), *Lagerstroemia speciosa* Pers. (leaf), *Lepidium apetalum* Willd. (aerial part), *Lilium concolor* var. *partheneion* Bak. (aerial part), *Lindera obtusiloba* Bl. (leaf), *Lithospermum erythrorhizon* S. et Z. (root), *Lysimachia barystachys* Bunge (aerial part), *Lysimachia clethroides* Buby (aerial part), *Mallotus japonicus* Muell.-Aro. (fruit and leaf), *Morus bombycis* for. *kase* Uyeki (leaf), *Morus bombycis* Koidz. (leaf), *Oenothera odorata* Jacq. (aerial part), *Orobanche coerulescens* Steph.(aerial part), *Paederia scandens* Merr.(aerial part), *Panax ginseng* C. A. Meyer (steamed root), *Persicaria thunbergii* H. Gross (aerial part), *Philadelphus schrenckii* Pupr. (leaf), *Phryma leptostachya* var. *asiatica* Hara (aerial part), *Pimpinelia brachycarpa* Nakai (aerial part), *Pinus strobus* L. (leaf

Sample or control solution	100 μl
a. Negative control: SDW	
b. Positive control : LDAO	
c. Sample	
Buffer	800 μl

↓ Voltexing

Yeast suspension (OD_{600nm} ≒ 2)	100 μl

↓ Vortexing

↓ Shaking incubation(200 rpm)

↓ 30℃, 6 hrs

Detection of OD at 600 nm

$$\text{Inhibition}(\%) = 100 - \frac{T-\{(Co+So)+(S-So)\}}{C-Co} \times 100$$

$$= 100 - \frac{T-(Co+S)}{C-Co} \times 100$$

T : OD of sample treatment system after incubation

Co: OD of negative control system before incubation

C : OD of negative control system after incubation

So: OD of sample treatment system without yeast suspension before incubation

S : OD of sample treatment system without yeast suspension after incubation

Scheme 2. Assay system.

and stem), *Polygonatum lasianthum* var. *coreanum* Nakai (aerial part), *Polygonatum odoratum* var. *pluriflorum* Ohwi (aerial part), *Polystichum tripteron* Presl. (aerial part), *Potentilla chinensis* Ser. (aerial part), *Quercus salicina* Bl. (leaf), *Rehmannia glutinosa* Liboschitz aerial part), *Rhamnus davurica* Pall. (leaf), *Rhodiola sachalinensis* A. Bor. (root), *Rhus chinensis* L. (leaf), *Rubus crataegifolius* Bunge (aerial part), *Sambucus williamsii* var. *coreana* Nakai (leaf), *Saururus chinensis* Baill. (aerial part), *Schizopepon bryoniaefolius* Max. (aerial part), *Scopolia japonica* Max. (aerial part), *Scorzonera albicaulis* Bunge (aerial part), *Smilax sieboldii* Miq. (leaf), *Staphylea bumalda* DC. (leaf and stem), *Stephanandra incisa* Zabel (leaf), *Streptopus ajanensis* var. *japonica* Max. (aerial part), *Synurus deltoides* Nakai (aerial part), *Synurus excelsus* Kitamura (aerial part), *Thea sinensis* L. (leaf), *Torilis japonica* DC. (aerial part), *Torreya nucifera* S. et Z. (leaf), *Trichosanthes kirilowii* Max. (aerial part), *Ulmus laciniata* Mayr. (leaf), *Ulmus macrocarpa* Hance (leaf), *Zingiber mioga* Rosc. (aerial part)

Sample extraction – Two grams of each dried sample was powdered, weighed, and extracted twice with distilled water under reflux for 2 hours. The solution was filtered and lyophilized. One hundred μl of each sample (15 mg/ml) dissolved in sterilized deionized water was used for the determination of inhibitory activity.

Results and Discussion

Assay method – A simple and rapid assay method was developed using recombinant yeast carrying human lanosterol synthase, main target in the sterol biosynthesis. Sterol inhibition activity was monitored by only inhibition of growth of the recombinant yeast. By using this assay method, we can screen, first of all, anti-hypercholesterolemic material only when inhibition site is proven to be oxidosqualene cyclase which is exchanged by human lanosterol synthase, anti-mycotic material, plant growth regulators or even anti-cancer drugs considering the homology of the genes participating in sterol biosynthesis among these organisms. This assay method is very simple and convenient to assay many plant sample in one time. Moreover, by changing the substrate, this assay method can figure out which step is inhibited in the sterol biosynthesis by the test material (Table 3).

Screening in ergosterol (-) medium – With this assay system, 102 plant samples were screened for sterol biosynthesis inhibition. Inhibitory activity (%) of each sample was compared with that of LDAO at the final concentration of 1.5 mg/ml. Considering the ingredients in plant sample, plant samples showing over 10% inhibition were thought to be active. Eleven out of the 102

Table 3. Site specificity of the assay method

Substrate	Site specific inhibitors				
	Squalene synthase	Squalene epoxidase	Oxidosqualene cyclase	Demethylase	Cytotoxic
Farnesyl pyrophosphate	–	–	–	–	–
Squalene	+	–	–	–	–
Oxidosqualene	+	+	–	–	–
Lanosterol	+	+	+	–	–
Ergosterol (+)	+	+	+	+	–
Ergosterol (–)	–	–	–	–	–

Table 4. Sterol biosynthesis inhibition of plant water extracts

Scientific name (part used)	Family name	Inhibition (%)[a]	
		ergosterol (–)	ergosterol (+)
Abutilon avicennae Gaertn. (stem)	Malvaceae	25.56	–11.66
Alnus japonica Steud. (stem)	Betulaceae	22.74	1.81
Amaranthus mangostanus L. (aerial part)	Amaranthaceae	16.41	–11.70
Astragalus membranaceus Bunge (root)	Leguminosae	32.21	37.10
Carduus crispus L. (aerial part)	Compositae	26.91	12.09
Cirsium pendulum Fisch. (aerial part)	Compositae	13.22	10.69
Lindera obtusiloba Bl. (leaf)	Lauraceae	14.28	13.74
Philadelphus schrenckii Pupr. (leaf)	Saxifragaceae	11.23	8.60
Pimpinelia brachycarpa Nakai (aerial part)	Umbelliferae	11.18	–5.14
Quercus salicina Bl. (leaf)	Fagaceae	19.62	14.63
Ulmus macrocarpa Hance (leaf)	Ulmaceae	19.20	23.07

[a]Inhibition of positive control, LDAO (3 mM), in ergosterol (–) medium was 100%.

samples showed inhibition in ergosterol (–) medium (Table 2). Inhibition in ergosterol (–) medium could be resulted from specific inhibition on an enzyme related to sterol biosynthesis or nonspecific inhibition to yeast growth, that is, cytotoxicity.

Screening in erg(+) medium – To confirm the specific inhibition, subsequent assay was carried out in ergosterol (+) medium. As a result of subsequent assay in ergosterol (+) medium (Table 4), five plant samples, *Abutilon avicennae* Gaertn. (stem), *Alnus japonica* Steud. (stem), *Amaranthus mangostanus* L. (aerial part), *Philadelphus schrenckii* Pupr. (leaf) and *Pimpinelia brachycarpa* Nakai (aerial part) showed specific inhibitory activity to yeast growth dependent of ergosterol biosynthesis.

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