Enzymatic Formation of Ether Linkage Producing Shoyuflavones from Genistein and (±)-*trans*-Epoxysuccinic Acid

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The production mechanism of shoyuflavones, conjugated ethers of isoflavones with tartaric acid and isolated from fermented soy sauce, was studied. In the high molecular weight fraction of the culture extract of *Aspergillus oryzae*, genistein was transformed into shoyuflavone B in the presence of (±)-*trans*-epoxysuccinic acid but not in the low molecular one. *Asp. sojae* and *Asp. tamarii* showed high activity similar to *Asp. oryzae* but none of *Asp. niger*, *Rhizopus oligosporus*, and *Mucor praini* did. The contents of epoxysuccinic acids in the starting materials of soy sauce and the cultures of various *Asp.* fungi were determined as dimethyl 2-chloro-3-hydroxysuccinate derivatives by GC-MS. Although epoxysuccinic acids were contained in *Asp. oryzae*, *Asp. sojae*, and *Asp. tamarii* cultures, they were not found in soybeans and wheat. A possible producing mechanism for shoyuflavones by enzymatically conjugating isoflavones to (±)-*trans*-epoxysuccinic acid with ether linkage was suggested.

Keywords: *Isoflavone; trans-epoxysuccinic acid; Aspergillus oryzae; GC-MS*

INTRODUCTION

Three shoyuflavones, tartaric acid isoflavone derivatives, isolated from Japanese soy sauce (Kinoshita et al., 1997) have unique structures because of their ether linkages between the position 7 in isoflavones (i.e., daidzein, genistein, and 8-hydroxygenistein) and the position 3″ in tartaric acid (Figure 1). Although several naturally occurring tartaric acid esters such as caffeoyl esters (Snook et al., 1994) have been reported, shoyuflavones are the first series of tartaric acid ether compounds.

Recently, flavonoids including isoflavones have been paid great attention because of their various biological activities. Shoyuflavones have more intense inhibitory effects on the activities of histidine decarboxylase (HDC) from both mouse mastocytoma P-815 cells and *Clostridium perfringens* than those of daidzein and genistein (Kinoshita and Saito, 1998). Some isoflavone 7-*O*-substituted analogues, such as 7-benzoyloxy-4′-hydroxyisoflavone and 7-*O*-(ω-carboxyalkyl) derivatives of daidzein, were chemically synthesized (Nakayama et al., 1978). Keung et al. (1977) reported that 7-hydroxyl group blocked isoflavones were better inhibitors of human mitochondria aldehyde dehydrogenase than daidzein. Among them, 7-*O*-(ω-carboxyalkyl) derivatives of daidzein showed the most potent inhibitory effect. These results suggest that shoyuflavones may have other biological activities besides inhibition of HDC.

Although the mechanism of formation of shoyuflavones is not known, two possible processes can be assumed, enzymatic by *Asp.* fungi, yeast, and/or lactobacillus or chemical nonenzymatic synthesis. Shoyuflavones were originally found to play a significant role in differentiating Japanese soy sauces from various manufacturers by chemometric pattern recognition analysis of their HPLC profiles (Kinoshita et al., 1997). Generally, individual manufactures of soy sauce in Japan have been using their own strains of *Asp.* fungi for making their dry mash or koji. Therefore, using different strains of *Asp.* fungi must be one of the key factors for differentiating chemical compositions in their soy sauce products.

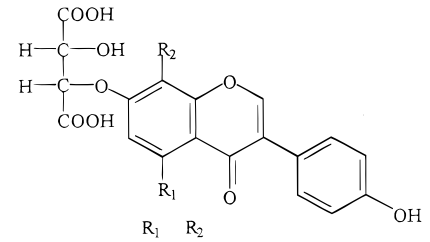

Figure 1. Structures of three shoyuflavones.

From the chemical structures of shoyuflavones (Figure 1), they must be formed from isoflavones (i.e., daidzein, genistein, and 8-hydroxygenistein). Daidzein and genistein are derived from their glycosides in soybeans (Wang and Murphy, 1994), and 8-hydroxygenistein might be produced from genistein during the fermentation process (Umezawa et al., 1975; Esaki et al., 1998). The first problem to be solved was to identify the source of another moiety consisting of four carbon atoms. As the possible precursors for shoyuflavones, several candidate compounds consisting of four carbon atoms, such as tartaric acid, unsaturated dicarboxylic acids (maleic or fumaric acid), succinic acid, epoxysuccinic acid, malic acids, or their isomers, among components in soy sauce or metabolites from *Asp.* fungi could be considered (Shibata et al., 1964). Among them, unsaturated carboxylic acids and epoxysuccinic acids seemed to be highly reactive. Considering chemical structures in shoyuflavones, however, unsaturated carboxylic acids may not be appropriate precursors. On the

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other hand, epoxysuccinic acids with a distorted bond angle inherent in the three-membered ring structure should be highly reactive.

Carboxylic acids and dicarboxylic acids, e.g., isomers of tartaric acid and epoxysuccinic acid, are often analyzed by high-performance liquid chromatography (HPLC) detected with UV absorbance or conductivity directly or after making their derivatives. However, these methods are neither sensitive nor selective enough for detecting carboxylic and/or dicarboxylic acids in the crude extract of fungi culture. However, gas chromatography−mass spectrometry (GC-MS) is highly sensitive and selective for detecting volatile derivatives of carboxylic and/or dicarboxylic acids. In this research, we intended to find out the formation mechanism of shoyuflavones.

MATERIALS AND METHODS

Materials. (±)-*trans*-Epoxysuccinic acid and *cis*-epoxysuccinic acid were obtained from Tokyo Kasei Industries (Tokyo, Japan). L-(+)-Tartaric acid, D-(−)-tartaric acid, and *meso*-tartaric acid were obtained from Nacalai Tesque (Kyoto, Japan). Genistein was obtained from Funakoshi (Tokyo, Japan). Shoyuflavone B was isolated according to the method by Kinoshita et al. (1997). A PD-10 column was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Preparation of fungi cultures. All fungus' strains were obtained from Noda Institute for Scientific Research (Noda, Chiba, Japan). Seventeen grams of wheat bran was mixed with distilled water (13 g) and the mixture in each Ferunbach flask was sterilized at 121 °C for 50 min. After cooling, spores of each fungus strain were inoculated and then incubated at 30 °C for 6 days.

Identifying Precursors for Shoyuflavones. To identify the precursor for shoyuflavones besides genistein, five compounds consisting of four carbon atoms, such as *cis*-epoxysuccinic acid, (±)-*trans*-epoxysuccinic acid, D-tartaric acid, L-tartaric acid, and *meso*-tartaric acid, were examined. One gram of *Asp. oryzae* IFO4206 culture was extracted with 5 mL of 50 mM sodium acetate−acetic acid buffer at pH 6.0. One hundred liters of 50 mM sodium acetate−acetic acid buffer at pH 6.0 containing 600 mM of each acid and 200 L of the culture extract were mixed and preincubated at 37 °C for 10 min. The reaction was started by adding 25 L of 3 mM genistein dissolved in dimethyl sulfoxide. After incubating at 37 C for 15 min, the reaction was stopped by adding 10 L of concentrated HCl. The amounts of shoyuflavone B produced in the cultures were analyzed by HPLC.

Assay for the Ability of Transformation from Genistein into Shoyuflavone B. The assay for the transformation ability of each fungus from genistein into shoyuflavones was performed in the presence of (±)-*trans*-epoxysuccinic acid. One gram of each fungus culture was extracted with 5 mL of 50 mM sodium acetate−acetic acid buffer at pH 6.0. After filtering, the activity of each culture extract was assayed as follows. One hundred liters of 50 mM sodium acetate−acetic acid buffer at pH 6.0 containing 600 mM (±)-*trans*-epoxysuccinic acid and 200 L of the extract of fungus culture were mixed and preincubated at 37 °C for 10 min. The reaction was started by adding 25 L of 3 mM genistein dissolved in dimethyl sulfoxide. After incubating at 37 °C for 15 min, the reaction was stopped by adding 10 L of concentrated HCl. The amounts of shoyuflavone B produced in the culture extract of each fungus were analyzed by HPLC.

Fractionation of *Asp. oryzae* IFO4206 Culture Extract. One milliliter of the crude extract of the culture of *Aspergillus oryzae* IFO4206 was applied to a PD-10 gel filtration column, equilibrated 50 mM sodium acetate−acetic acid buffer (pH 6.0), then fractionated 0.5 mL per each fraction, being eluted with the same buffer.

HPLC Analysis. HPLC analyses were carried out on a Shimadzu liquid chromatograph system with an SIL-10A autoinjector, LC-10AD and SPD-10A detector (Shimadzu Corp., Kyoto, Japan). HPLC analyses were performed on a Wakosil-II 5C18HG column (4.6-mm i.d. 100 mm + 4.6-mm i.d. × 300 mm, Wako Pure Chemicals Industries, Tokyo, Japan) at room temperature, with a solvent system of a mixture of 0.05% trifluoroacetic acid and 33% acetonitrile in water. The flow rate was 1.0 mL/min and monitored at 260 nm.

Table I. Comparison of Effect of the Four-Carbon Compounds Consisted of Shoyuflavone B Formation by Addition to the Extract *Aspergillus oryzae* IFO4206

compound[a]	relative activity,[b] %
none	100
trans-epoxysuccinic acid	190
cis-epoxysuccinic acid	102
L-tartaric acid	104
D-tartaric acid	95
meso-tartaric acid	109

[a] Final concentration was 50 mM. [b] Activity in none was taken as 100%.

Determination of Epoxysuccinic Acid Isomers by GC-MS. Epoxysucinic acids were analyzed as dimethyl 2-chloro-3-hydroxysuccinate derivatives. Five grams of each material or the solid culture was extracted 3 times with 20 mL of methanol at 5 °C for 4 h. The extract was collected and concentrated to 5 mL by rotary evaporator. Then, the concentrated solution was refluxed with 6% HCl at 70 °C overnight. The refluxed solution was analyzed by a GC-MS system, HP5980 gas chromatograph equipped with HP5971 mass spectrometer (Hewlett-Packard, Palo Alto, CA). GC analyses were performed on a DB-Wax capillary column (60 m × 0.25-mm i.d., 0.25 μm df, J&W Scientific, Folsom, CA) using helium as carrier gas and flow rate at 0.9 mL/min. The oven temperature was elevated from 50 to 200 °C at 3 °C/min. The ion source temperature in MS analysis was at 180 C and ionization voltage was 70 eV. The amount of epoxysuccinic acids in each culture extract was calculated from the ratio of sum of two fragments, 108 and 137, in a dimethyl 2-chloro-3-hydroxysuccinate spectrum to that of 56 and 85 fragments in the spectrum of γ-valerolactone used as the internal standard.

RESULTS

Precursor of Shoyuflavones. The transformation of genistein into shoyuflavone B was increased about 190% from the reference by adding 50 mM of (±)-*trans*-epoxysuccinic acid, while the other four compounds showed no increase in the transformation ratio (Table 1). Thus, (±)-*trans*-epoxysuccinic acid was identified as one of essential precursors for producing shoyuflavones. Each fraction of the extract of *Asp. oryzae* IFO04206 culture fractionated by a PD 10 column was examined the transformation ability of shoyuflavone B from genistein when (±)-*trans*-epoxysuccinic acid was added. In the high molecular weight fractions, genistein was transformed into shoyuflavone B. On the other hand, the low molecular fractions did not produced shoyuflavone B regardless of adding (±)-*trans*-epoxysuccinic acid. This transformation ability completely disappeared after boiling the extract of the fungus culture at 100 °C for 10 min. These results suggested that this transformation should be caused by enzymatic reaction.

Abilities of Transformation from Genistein into Shoyuflavone B in Cultures of Various Fungi. By adding (±)-*trans*-epoxysuccinic, some strains in *Asp.* fungi showed drastic increase in amounts of shoyuflavone B but some other strains and fungi did not show any significant change (Figure 2). Especially, some strains in *Asp. oryzae*, *Asp. sojae*, and *Asp. tamarii*, which are widely used for making koji in the soy sauce

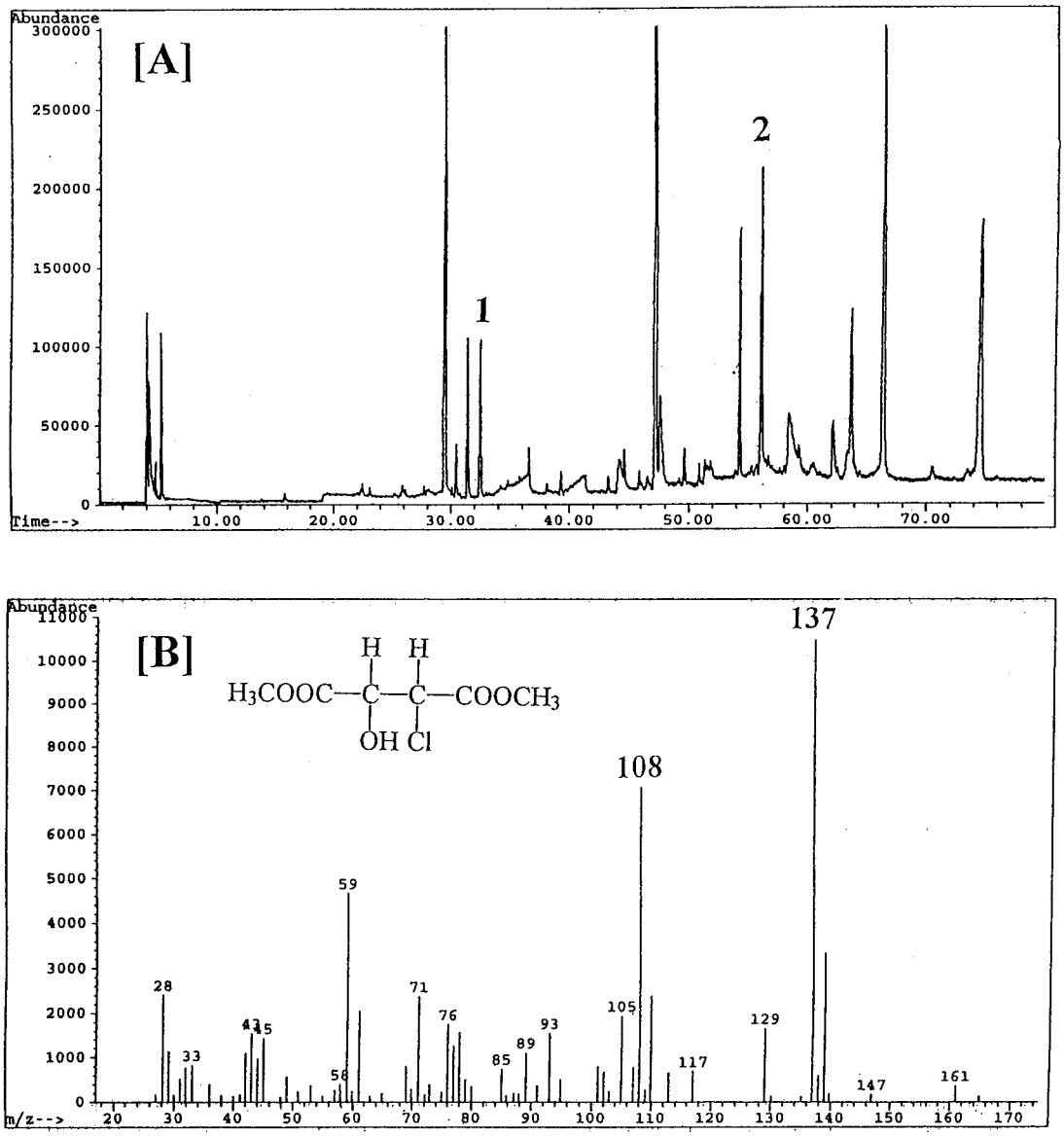

Figure 2. GC-MS data of the ethanol extract of *Asp. sojae* ATCC20245 culture. [A] Total ion chromatogram. 1, γ-valerolactone; 2, dimethyl 2-chloro-3-hydroxysuccinate. [B] Mass spectrum of the 2 in [A].

production, showed the higher activities. On the contrary, activities in all of *Asp. niger*, *Asp. awamori*, *Asp. usami* ut. shirousami, *Rhizopus oligosporus*, *Monascus anka*, and *Mucor praini* were negligible regardless of added (±)-*trans*-epoxysuccinic acid.

Contents of Epoxysuccinic Acids in the Starting Materials and Culture Extracts of Various Fungi. Some results obtained from GC-MS analysis of the ethanol extract of *Asp. oryzae* ATCC20245 culture are shown in Figure 3. (±)-*trans*-Epoxysuccinic acid and its isomers were detected as dimethyl 2-chloro-3-hydroxysuccinates around at 57 min (Figure 3A) and discriminated from coeluting components by selecting fragments 108 and 137 (Figure 3B). The fragment ion at *m*/*z* 137 was produced by losing [−$COOCH_3$] from the molecular ion and the ions at *m*/*z* 108 were derived from further loss of [−CHO]. Table 2 shows contents of epoxysuccinic acid in culture extracts, soybeans, wheat, and wheat bran. Epoxysuccinic acid was only found in the culture extracts of *Asp. oryzae*, *Asp. sojae*, and *Asp. tamarii* by widely ranging their contents from 9 to 78 g/g. However, epoxysuccinic acid was not detected in the starting materials of soy sauce, i.e., soybeans, wheat, and wheat bran. This result strongly suggested that (±)-*trans*-epoxysuccinic acid was produced by some strains of *Asp.* fungi and those strains are widely used in manufacturing soy sauce. Thus, we propose a scheme for the enzymatic formation of shoyuflavones by conjugating genistein and (±)-*trans*-epoxysuccinic with an ether linkage as shown in Figure 4.

DISCUSSION

This paper describes that the ether linkages in shoyuflavones are enzymatically produced by some strains of *Asp.* fungi. Two precursors needed for this transformation have been identified as isoflavones and (±)-*trans*-epoxysuccinic acid. Considering the soy sauce manufacturing process, major isoflavones, i.e., daidzein and genistein, are derived from their glycosides in soybeans, while (±)-*trans*-epoxysuccinic acid is produced by *Asp.* fungi during the koji period.

The biosynthesis of ether linkages in shoyuflavones is particularly interesting. Although enzymatic syntheses of ether linkages are not fully understood yet, some enzymes cleaving ether linkages have been found (Koga

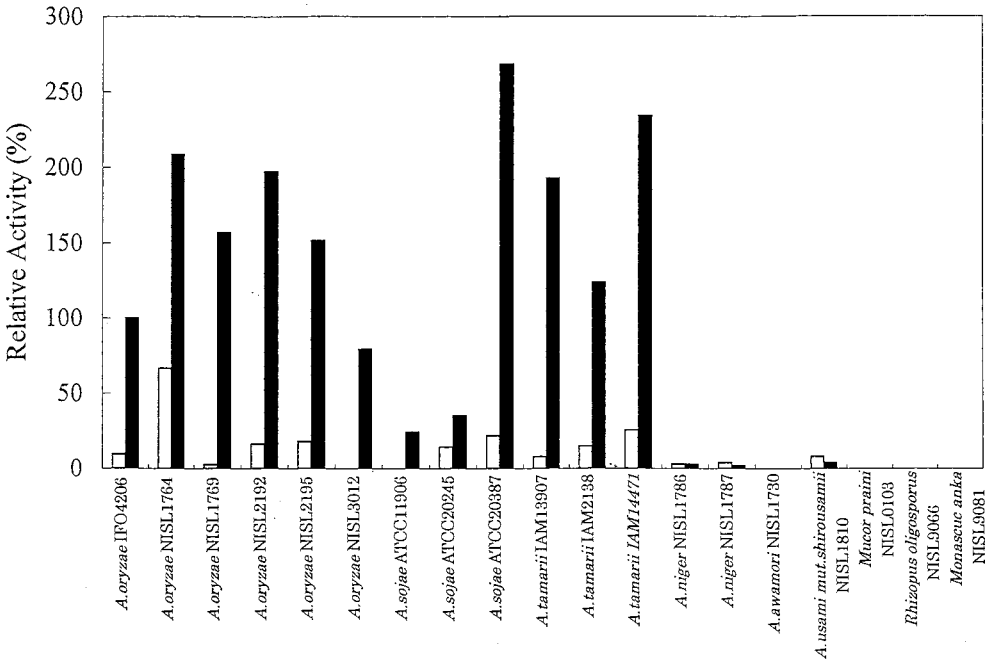

Figure 3. Ability of various fungi to transform genistein into shoyuflavone B. (□) (±)-*trans*-epoxysuccinic acid not added; (■) (±)-*trans*-epoxysuccinic acid added. The ability of *Asp. oryzae* IFO4206 in the presence of (±)-*trans*-epoxysuccinic acid was taken as 100%. NISLE, strain numbers being preserved in Noda Institute for Scientific Research.

Table 2. Contents of Epoxysuccinic Acids in the Extracts of Various Fungi Culture and Materials

source		epoxysuccinic acids,[a] μg/g
A. oryzae	IFO4206	48
A. oryzae	NISL1769	19
A. oryzae	NISL1764	78
A. sojae	ATCC11906	36
A. sojae	ATCC20245	53
A. sojae	ATCC20387	9
A. tamarii	IAM2138	11
A. tamarii	IAM14471	29
wheat		nd
soybean		nd
wheat bran		nd

[a] Wet weight. nd, not detect. NISL, strain numbers being preserved in Noda Institute for Scientific Research.

et al., 1993). Carboxymethyloxysuccinatelyase (EC 4.2.99.14) (Peterson and Llaneza, 1974) and hydrolases such as *trans*-epoxysuccinate hydrolase (EC 3.3.2.4) (Jacobs et al., 1991) are examples of these enzymes. Except for methoxylation for hydroxyl group by O-methyltransferases (Ebel et al., 1972; Wengenmyer et al., 1974; Poulton et al., 1977), only a few enzymatic syntheses of ether linkages have been reported. The divinyl ether synthase following lipoxygenase reaction to produce divinyl ether fatty acid is an example of such enzymes (Grechkin et al., 1995; Galliard and Phillips, 1972). However, until now, no ether synthase has been isolated in the pure form. Including ether lipids and ether phospholipids, the formation mechanism of ether linkages in natural compounds has not been fully understood yet (White et al., 1996). (±)-*trans*-Epoxysuccinic acid produced from *meso*-tartaric acid by *trans*-epoxysuccinate hydrolase (EC 4.2.1.37) in *Asp. fumigatus* (Wilkoff and Martin, 1963) and *Pseudomonas purida* (Martin and Foster, 1955) has been reported. Although epoxides such as (±)-*trans*-epoxysuccinic acid have a three-membered epoxide ring that makes them highly reactive, until now no natural compound having an ether linkage derived from (±)-*trans*-epoxysuccinic acid has been found. Naturally, no enzyme catalyzing the formation of an ether linkage from (±)-*trans*-epoxysuccinic acid has been reported. On the other hand, several enzymatic conversions of epoxide for detoxification by hydrolase (Jacobs et al., 1991), isomerase (Hartman et al., 1989; Weijers et al., 1995), and carboxylase (Allen and Ensign, 1997) have been reported. Some epoxysuccinyl derivatives, such as *trans*-epoxysuccinyl derivatives from *Myceliophthora thermophila* (Yaginuma et al., 1989) and from *Asp. oryzae* (Sato et al., 1996; Yamada et al., 1998), have been isolated as the inhibitors against cysteine protease. However, they are completely different from those of shoyuflavones because of keeping an epoxide ring in their structures. By cleaving the epoxy ring in (±)-*trans*-epoxysuccinic acid, isoflavones could be conjugated with ether linkage to form shoyuflavone. This reaction might prevent excess epoxide from having toxic effects, such as alkylating agents, due to accumulation of epoxide in the cultures of *Asp.* fungi.

A sensitive and simple GC-MS method for determining quantities of epoxysuccnic acid isomers (i.e., *trans*-, *cis*-, and *meso*-epoxysuccinic acid) as dimethyl 2-chloro-3-hydroxysuccinates was established in this research. Identification of organic acids, e.g., (±)-*trans*-epoxysuccinic acid, by HPLC analysis is sometimes difficult because organic acids generally lack suitable chromophores or fluorophores for UV detection. Further, organic acids cannot be reliably determined due to coelution of other compounds. Thus, these compounds should be derivatized for quantitative analysis of them. This method may be useful not only for analyzing epoxysuccinic acids but also for analyzing any other low molecular carboxylic acids present in liquid or solid matrixes.

The ability to form shoyuflavones was found in some strains of *Asp.* fungi used in soy sauce fermentation. Until now, shoyuflavones or related compounds have not been found in any other soy products. There were at least two possible compounds transformed from *trans*-

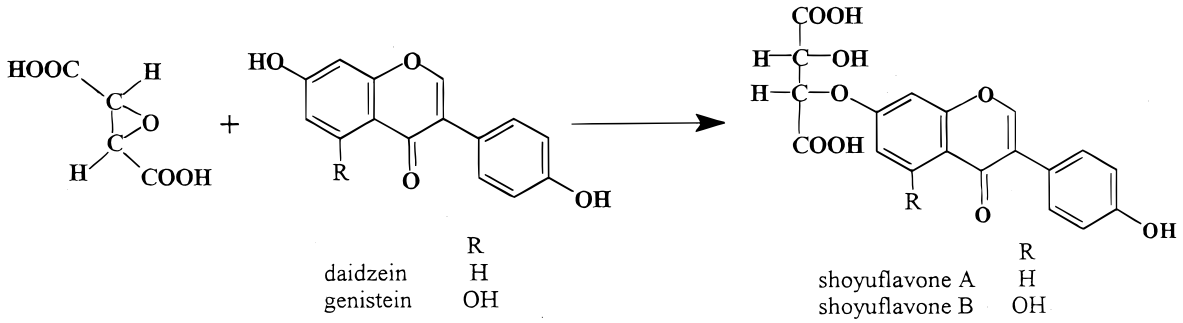

Figure 4. Possible scheme for the formation of shoyuflavones from isoflavones and (±)-*trans*-epoxysuccinic acid by *Asp. oryzae*.

epoxysuccinic acid by *Asp. oryzae*, tartaric acid ether derivatives such as shoyuflavones or *trans*-epoxysuccinyl derivatives (Yamaha et al., 1998). From recent studies, the former compounds were isolated from soy sauce and the latter from the liquid culture. Although *Asp. oryzae* is also widely used for producing miso, shoyuflavones have not been found in them yet. However, from a theoretical point of view, shoyuflavones might be found in miso or other fermented foods in the near future. According to the results obtained from soy sauce (Kinoshita et al., 1998), the amounts of shoyuflavone in soy sauce depends on the fermentation conditions.

In this study, we have suggested a possible scheme for producing shoyuflavones from epoxides and isoflavones catalyzed by a novel and unknown enzyme. Studies on the enzyme that produce shoyuflavones are in progress.

ABBREVIATION USED

HDC, histidine decarboxylase; NISLE, strain numbers being preserved in Noda Institute for Scientific Research.

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