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ORIGINAL ARTICLE

Solubility enhancement of isoflavonoids by complexation with acyclic hexadecasaccharides, succinoglycan dimers isolated from *Sinorhizobium meliloti*

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Abstract *Sinorhizobium meliloti* produces succinoglycan, an acidic exopolysaccharide composed of a monomeric octasaccharide repeating unit with acetyl, succinyl, and pyruvyl groups, in both low- and high-molecular-weight forms. Among the low-molecular-weight succinoglycans, dimers were isolated from *S. meliloti* and purified using various chromatographic techniques. The dimers were classified as four types (D1, D2, D3, and D4) based on the number of succinyl moieties in their structure. The effect of succinoglycan dimers on the aqueous solubility of isoflavonoids, daidzein and genistein was investigated. The solubility of isoflavonoids increased in the presence of succinoglycan dimers, and the complexation between isoflavonoids and succinoglycan dimers was analyzed by UV–Vis (ultraviolet–visible) and NMR (nuclear magnetic resonance) spectroscopy. In the phase solubility study, succinoglycan dimer D3 was shown to have the highest stability constants (4951 M^{-1} for daidzein, and 4452 M^{-1} for genistein) among the four succinoglycan dimers. The morphological structures of daidzein and genistein with D3 were studied using scanning electron microscopy, and X-ray powder diffractometry. The results showed the natures of the complexes significantly different from the free isoflavonoids. Herein, we suggest that the succinoglycan dimers are able to act as effective complexing agents for the isoflavonoids.

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Introduction

Shinorhizobium meliloti is a nitrogen-fixing soil bacterium that has a symbiotic relationship with legume plants (*Medicago sativa*) through the formation of root hair nodules. Succinoglycan, which is an exopolysaccharide produced by *S. meliloti*, has been shown to play an important role during the symbiotic process [1, 2]. Succinoglycan is a polymer that consists of linear octasaccharide subunits containing one galactose and seven glucoses substituted by acetyl, pyruvyl, and succinyl groups [3]. The produced forms consist of both high molecular weight (HMW) and low molecular weight (LMW) fractions, but some reports has shown that a LMW form of succinoglycan can promote nodule invasion in legume [4, 5].

The LMW succinoglycan can be purified further as a monomer, dimer, and trimer, according to the number of subunits [6, 7]. In addition, the purified monomer has been investigated as chiral additives for the separation of chiral flavonoids [8, 9]. The successful enantioseparation of flavonoids using the succinoglycan monomer is based on the different interactions between the monomer and the *R* or *S* configuration of chiral flavonoids. Also, we previously examined the structural property of the succinoglycan dimers [10]. In this study, the hydrophobicity of succinoglycan dimers was evaluated based on interactions with hydrophobic fluorescent probes. Herein, we investigated the interaction between flavonoids exudated from the host plant and the succinoglycan dimers purified from *S. meliloti*, a symbiotic bacterium for the host plant.

Isoflavonoids found in legume are a class of flavonoid polyphenolic compounds. They are viewed as host-produced antibiotics, phytoalexin, and *S. meliloti* results in the accumulation of isoflavonoid phytoalexins in host-plant cells [11, 12]. Daidzein (7,4′-dihydroxyisoflavone) and genistein (5,7,4′-trihydroxyisoflavone) are common examples of isoflavonoids (Fig. 1a). The backbone of these compounds is structurally similar to estrogen and they have been shown to bind to the estrogen receptor (ERα and ERβ) [13]. Hence, they play an important role in preventing hormone dependent diseases, such as menopausal symptoms, osteoporosis, breast cancer, and prostate cancer [14, 15]. However, clinical use of these compounds is limited because of their low solubility in water; thus, there has been growing interest in improving the solubility of isoflavonoids. The aqueous solubility of isoflavonoids has been enhanced by complexation with cyclic oligosaccharides such as cyclodextrin (CD) [16–18]. Complexation of the hydrophobic guest by CD may have originated from the nonpolar cavity of the host molecule. For the synthesis of CD-guest complexes, co-crystallization is a widely used method, which can defined as forming crystalline complexes of two or more components through noncovalent interactions (primarily hydrogen bonding) [19, 20]. In the present study, the solubility of daidzein and genistein was shown to be enhanced by complexation with acyclic hexadecasaccharides, succinoglycan dimers, which have characteristic hydrophobic properties.

Experimental

Chemicals

Daidzein and genistein were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, Mo, USA).

Bacterial cultures and the purification of succinoglycan dimers

Shinorhizobium meliloti Rm 1021 was grown in a rotary shaker at 30 °C in a GMS medium for 5 days [7]. Cells were removed by centrifugation, and the supernatant was concentrated. After adding 3 vol of ice-cold ethanol, the precipitate containing HMW succinoglycan was removed. The LMW succinoglycan in the supernatant was concentrated again, and another 7 vol of ice-cold ethanol were added. The supernatant was collected by centrifugation, and the putative LMW succinoglycan samples were applied to Bio-gel P6 with 0.5 % acetic acid. The monomers, dimers, and trimers of the succinoglycan subunit were separated. The dimers were further fractionated into D1, D2, D3, and D4 on a DEAE Sephadex A-25 using a linear gradient from 5 to 400 mM KCl in 10 mM MOPS buffer. Each dimer (D1, D2, D3, and D4) was collected and desalted using a Bio-gel P4 column.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS)

The mass spectra of the succinoglycan dimers were obtained with a MALDI-TOF mass spectrometer (Voyager-DE^{TM} STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA, USA) in the negative-ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

Phase-solubility study

Genistein and daidzein were dissolved in methanol, and the methanolic solution was added to aqueous solutions of D1 to D4 (0.0–4.0 mM). The mixtures were magnetically stirred for 24 h at 25 °C, shielded from light to prevent degradation of the molecules. After equilibration, the suspensions were lyophilized and the powder was added to 0.4 mL distilled water. After filtering through a 0.2 μm syringe filter (PTFE syringe filter, Whatman), the sample was diluted two fold prior to detection. The amount of dissolved genistein and daidzein was analyzed using a UV–Vis spectrophotometry (UV 2450, Shimadzu Corporation). The spectra were obtained from 220 to 400 nm, and the apparent stability constants (K_c) of isoflavonoids/D1 to D4 complex were calculated based on the phase-solubility diagrams using the following equation:

$$K_c\left(M^{-1}\right) = \text{slope}/S_0(1 - \text{slope})$$

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Preparation of complexes of isoflavonoids with succinoglycan dimers

Five millimolar isoflavonoids (daidzein and genistein) dissolved in 1 mL MeOH and 5 mM succinoglycan dimers dissolved in 1 mL water were mixed, and stirred at 25 °C for 24 h. After equilibration, methanol was evaporated using N_2 gas, and the mixture was lyophilized.

Nuclear magnetic resonance (NMR) spectroscopy

For the NMR spectroscopic analysis, we used a Bruker Avance 500 spectrometer to record ^{1}H-NMR spectra and the Nuclear Overhauser Effect Spectroscopy (NOESY) spectra. The chemical shift displacements were calculated according to the formula: $\Delta\delta = \delta_{(\text{complex})} - \delta_{(\text{free})}$, where $\delta_{(\text{free})}$ is the chemical shift of isoflavonoids without succinoglycan dimers, and $\delta_{(\text{complex})}$ is the chemical shift of isoflavonoids with succinoglycan dimers. The NOESY

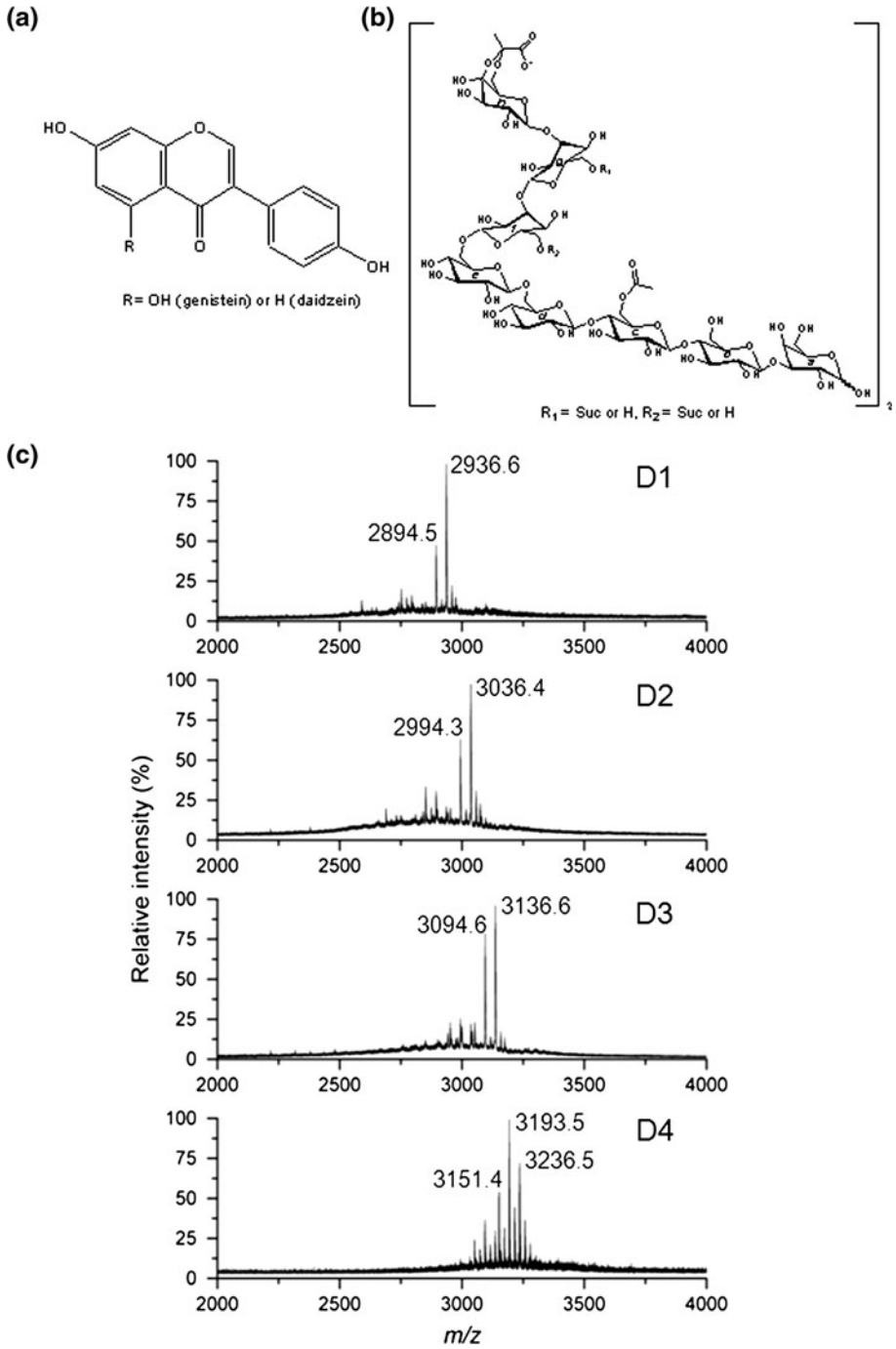

Fig. 1 Chemical structures of isoflavonoids (**a**) and succinoglycan dimers (**b**) which were called D1, D2, D3 and D4 according to the number of succinyl groups in the molecule, and (**c**) MALDI TOF mass spectra of the purified D1, D2, D3, and D4

spectra were recorded with 256/2048 complex data points using a pulse train to achieve a spin-lock field with a mixing time of 500 ms for the complex. The NMR spectroscopic analyses were carried out in 60 % d_6-DMSO/D_2O at room temperature.

Scanning electron microscopy (SEM)

The samples were mounted onto stubs using double sided adhesive tape and then made electrically conductive by coating with a thin layer of gold. The surface morphologies of the materials were examined under a scanning electron microscope (Jeol, JSM 6380, Tokyo, Japan).

X-ray powder diffractometry (XRPD)

Powder X-ray diffraction patterns were recorded with D8 FOCUS (Bruker Corp., Germany) X-ray diffractometer by using a Ni-filtered CuKα radiation ($\lambda = 1.5418$ Å), in the $5° \leq 2\theta \geq 45°$ range. The measurement conditions were

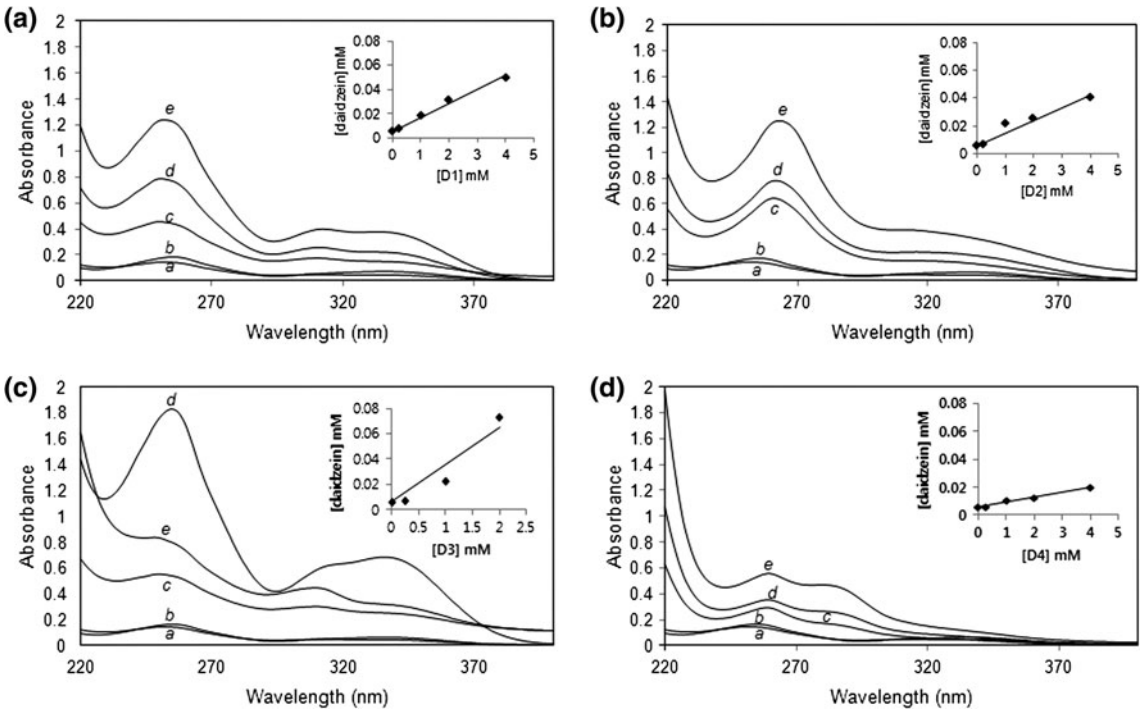

Fig. 2 UV–Vis absorbance of daidzein (125 μM) in various concentrations of D1 (**a**), D2 (**b**), D3 (**c**), and D4 (**d**) [succinoglycan dimers] = 0, 0.25, 1, 2, 4 mM (*a*–*e*). *Insets* show the phase solubility diagrams of daidzein–succinoglycan dimers complexes in water at 25 °C

as follows: voltage of 40 kV, current of 40 mA, increment of 0.02°, scan rate of 0.8 s/step.

Results and discussion

Succinoglycan dimers isolated from *S. meliloti* Rm 1021 were purified using size exclusion and anion exchange chromatographic techniques. The chemical structure of succinoglycan dimers is shown in Fig. 1b, and the linkage sequence is [Glcβ-1,3-Glcβ-1,3-Glcβ-1,6- Glcβ-1,6-Glcβ-1,4-Glcβ-1,4-Glcβ-1,3-Gal$\beta(\alpha)$-1]$_2$. Also, the hexa-deca-saccharide was modified with two acetyl at *c* glucoses, two pyruvyl groups at *h* glucoses, and one to four succinyl groups at the *g* and *f* glucoses (Fig. 1b). Accordingly, the dimers were classified as four types (D1, D2, D3, and D4) based on the number of succinyl moieties [6]. The structure of the respective D1, D2, D3, and D4 was confirmed with MALDI-TOF MS (Fig. 1c) [7]. The molecular ions in negative-ion mode ([D1-H]$^-$, [D2-H]$^-$, [D3-H]$^-$, and [D4-H]$^-$) were observed at *m/z* 2936.6, 3036.4, 3136.6, and 3236.5, and the additional sodium adducts were shown with small intensity. This result confirms that the mass intervals of D1, D2, D3, and D4 are 100 corresponding to *O*-ester linked succinyl residue. Also, the loss of acetyl group was detected with 42 mass difference from original molecular ions, and the acetyl release in D4 increased compared with D1, D2, and D3.

The effect of succinoglycan dimers on the solubility of isoflavonoids was examined using the phase solubility methods. The phase solubility diagram is a widely accepted method to assess the effect of CD complexation on guest solubility [16–18]. Figure 2a, d shows the UV absorption spectra of daidzein alone in water and those in the presence of succinoglycan dimers (D1 to D4). As the amount of succinoglycan dimers increased, the absorbance of daidzein was also enhanced. Phase solubility diagrams of D1 to D4 (see insets of Fig. 2) were obtained using the UV spectra of different concentrations of the samples. Daidzein solubility after the addition of D3, D1, D2, and D4 was improved by up to 12.4-, 8.6-, 6.8-, and 3.4-fold, respectively. Since the absorbance value of daidzein reached a plateau at a D3 concentration of 2 mM of D3 (Fig. 2c), the slope of curve was evaluated at concentrations ranging from 0 to 2 mM. In addition, the slope of the curve from D1, D2 and D4 was evaluated between 0 and 4 mM.

Table 1 Stability constants for daidzein/succinoglycan dimers and genistein/succinoglycan dimers

Succinoglycan dimers	Daidzein K_c (M^{-1})	Genistein K_c (M^{-1})
D1	1949	2292
D2	1576	–
D3	4951	4452
D4	620	–

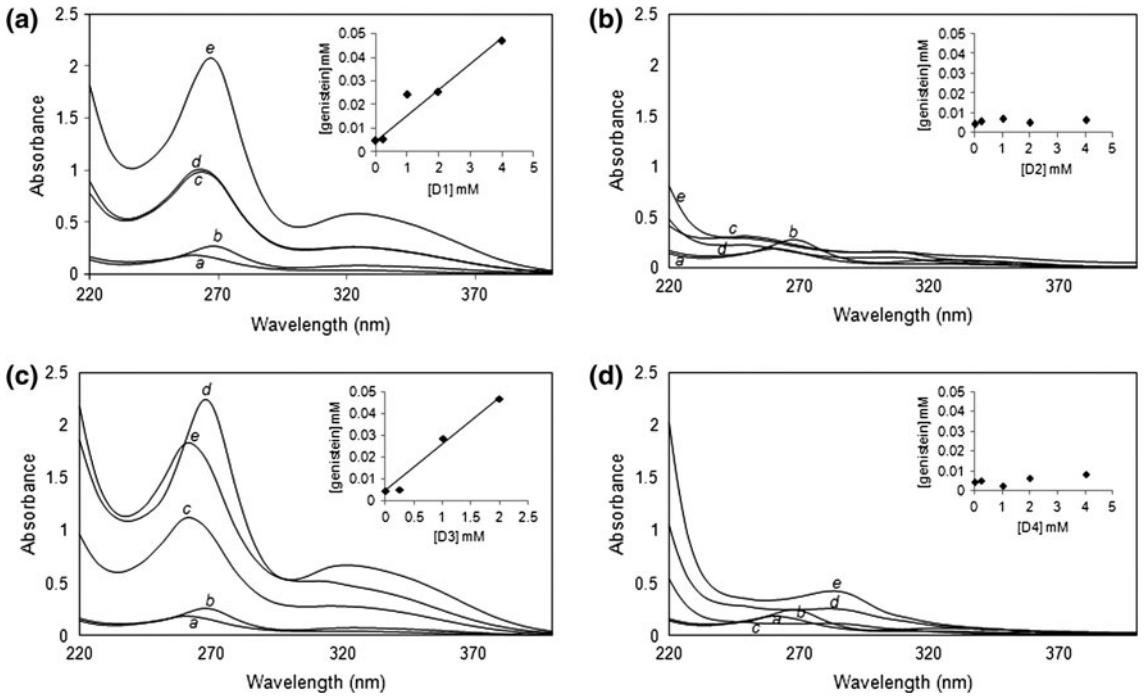

Fig. 3 UV–Vis absorbance of genistein (125 μM) in various concentrations of D1 (**a**), D2 (**b**), D3 (**c**), and D4 (**d**). [succinoglycan dimers] = 0, 0.25, 1, 2, 4 mM (*a–e*). *Insets* show the phase solubility diagram of genistein–succinoglycan dimers complexes in water at 25 °C

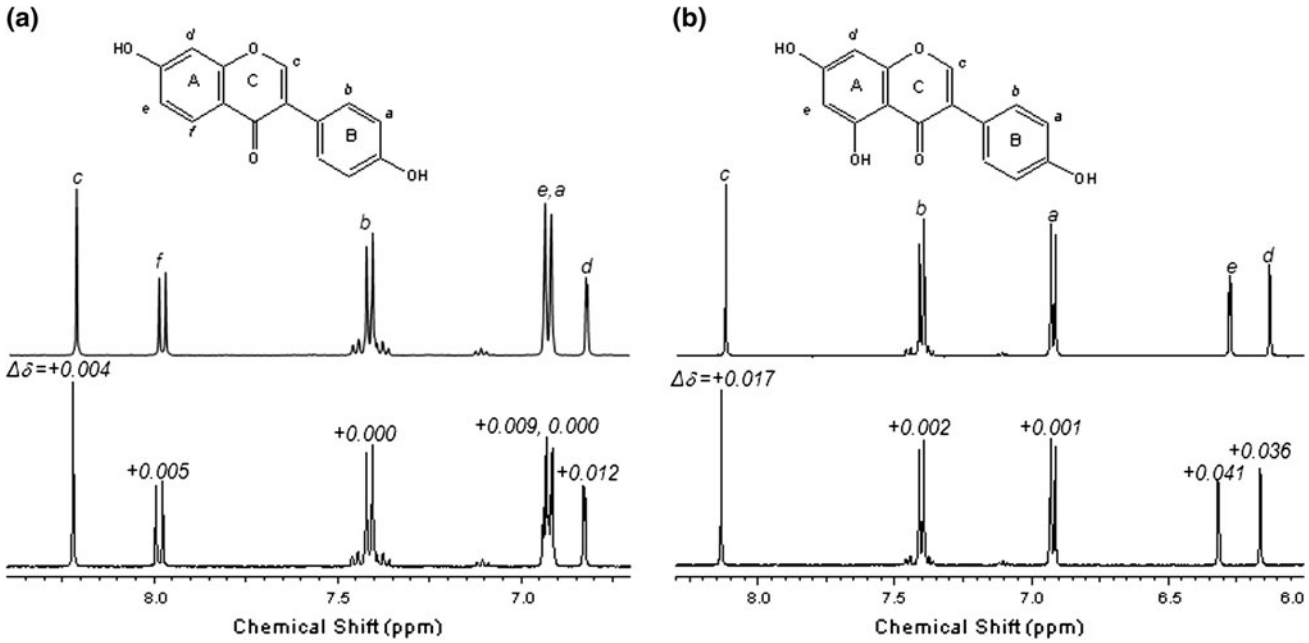

Fig. 4 Partial ^{1}H NMR spectra of daidzein (**a**) and genistein (**b**) in the absence (*top*) and presence (*bottom*) of D3

According to Higuchi and Connors [21], these curves can be classified as A_L type, which is consistent with a 1:1 molecular association. From the slope of the linear fit, the stability constants (K_c) were determined and summarized in Table 1. The order of the stability constant with daidzein was D3 > D1 > D2 > D4. The solubility of genistein by adding D1 to D4 was also investigated (Fig. 3). D2 and D4 showed no or minimal effects on the genistein aqueous solubility, but both D3 and D1 enhanced the solubility by up to 10.0-fold. D3 was more efficient than D1 in enhancing the solubility of genistein, and this effect plateaued at 2 mM D3, which was similar to that of

Table 2 Changes in the chemical shifts of daidzein and genistein by succinoglycan dimers

Succinoglycan dimers	$\Delta\delta$ of daidzein (ppm)						$\Delta\delta$ of genistein (ppm)				
	a	b	c	d	e	f	a	b	c	d	e
D1	0.000	0.000	0.000	0.004	0.003	0.002	0.000	0.000	0.000	0.002	0.004
D2	0.000	0.001	0.007	0.025	0.017	0.011	–	–	–	–	–
D3	0.000	0.000	0.004	0.012	0.009	0.005	0.001	0.002	0.017	0.036	0.041
D4	0.002	0.003	0.028	0.091	0.061	0.039	–	–	–	–	–

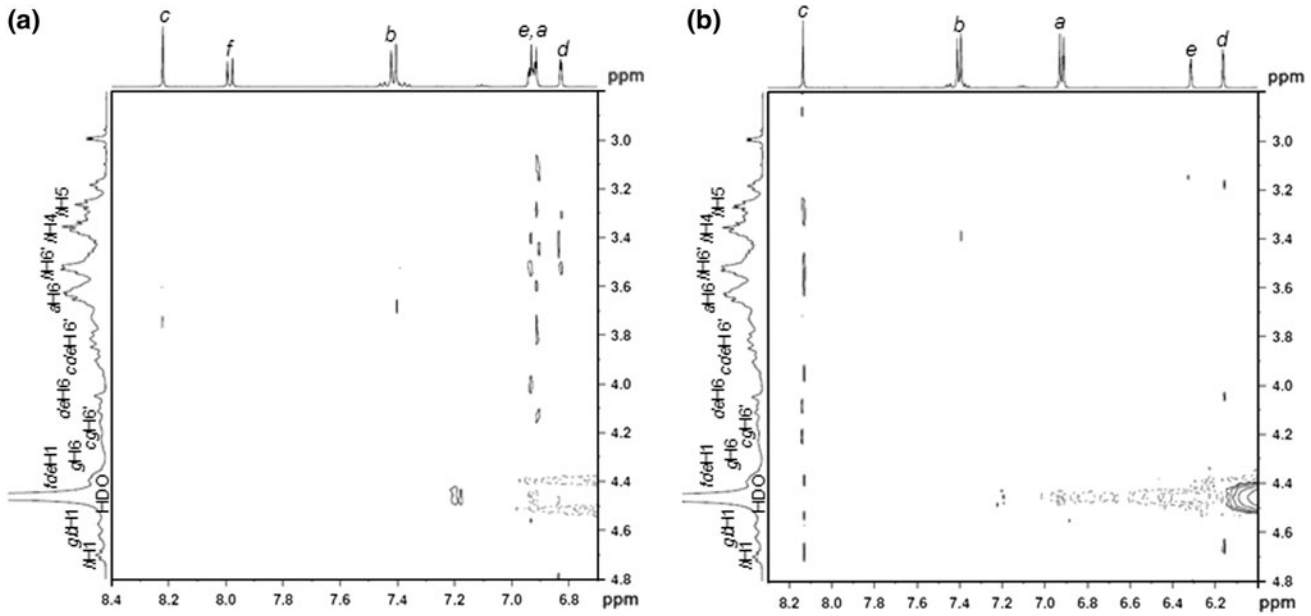

Fig. 5 Partial NOESY spectra of daidzein/D3 (**a**) and genistein/D3 (**b**)

daidzein. As a result, D3 showed the highest stability constants (4951 M^{-1} for daidzein, and 4452 M^{-1} for genistein) among the four succinoglycan dimers tested. In the case of cyclic oligosaccharides, β-CD, Methyl β-CD, and HP β-CD have been used as complexation agents of daidzein and genistein [16–18]. The order of the stability constants for these compounds was HP β-CD > Methyl β-CD > β-CD. However, in the present study, we found that the linear hexadecasaccharides as the complexation agent could enhance the aqueous solubility of isoflavonoids due to the hydrophobicity of the succinoglycan dimers [10].

To confirm the interaction between isoflavonoids and succinoglycan dimers, nuclear magnetic resonance (NMR) spectroscopic analyses were carried out. ^{1}H-NMR spectroscopy is a suitable method for the evaluation of non–covalent interactions at the molecular level [22]. Due to the extremely poor aqueous solubility of isoflavonoids, 60 % d_6-DMSO in D_2O was used as an NMR solvent to obtain optimum solubility of both species [23]. The assignments of the proton signals of daidzein and genistein are shown in Fig. 4, and the peaks were shifted by the addition of D3. A and C ring protons of daidzein and genistein were downshifted, which was different from B ring protons. Table 2 shows the variations in the chemical shifts of daidzein and genistein by succinoglycan dimers. There are some different aspects between the stability constant in the purely aqueous solvent system (Table 1) and the degree of chemical shift changes in 60 % d_6-DMSO in D_2O environments. This may be due to the presence of DMSO in the solvent. According to Zheng et al. [24] 20 % DMSO resulted in a 20-fold decrease in complexation between flavonoids and a cyclodextrin derivative via binding competition or flavonoids extraction. However, it will not significantly change the basic mode of interaction [23, 24]. The preferential change of *c* to *f* protons corresponding to A and C ring was evident as indicated in Table 2. Thus, the A and C ring protons of daidzein and genistein may be related to succinoglycan dimers, rather than the B ring protons of those compounds. In addition, two dimensional Nuclear Overhauser Effect (NOE) signals were detected in the nuclear overhauser enhancement spectroscopy

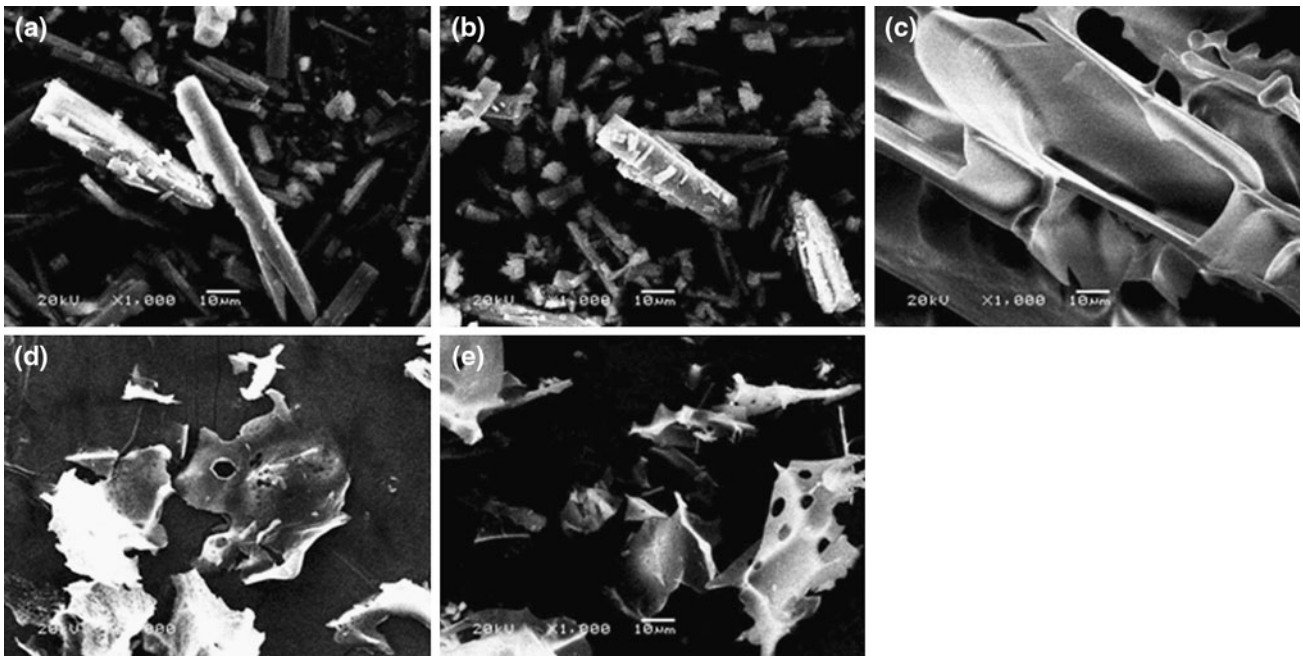

Fig. 6 SEM images of daidzein (**a**), genistein (**b**), D3 (**c**), daidzein/D3 (**d**), and genistein/D3 (**e**)

(NOESY) experiment. This approach has been frequently used to elucidate the intermolecular interaction of inclusion complexes, since two protons located close in space can induce an NOE cross-peak [25]. The assignment of D3 protons required two-dimensional NMR (HSQC Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC)), one–dimensional NMR (^{1}H, ^{13}C, and Distortionless Enhancement by Polarization Transfer (DEPT)-135) experiments (data not shown), and the use of previous data on ^{1}H and ^{13}C chemical shifts of the succinoglycan monomer [6, 24, 26]. D3 signals forming a broad hump containing H6 of *c*, *d*, *e*, and *h* glucoses were correlated with *c* to *e* protons in the A and C ring of daidzein (Figs. 1b, 5a). Also, D3 protons containing H1 of *d* to *f* glucoses and H6 of *c* to *e* glucoses were correlated with *c* to *e* protons in the A and C ring of genistein (Figs. 1b, 5b). These NMR spectroscopic results demonstrated the interaction between the protons of isoflavonoids and D3.

Morphological changes can frequently be employed as a tool to evaluate the interaction between drugs and host [27–29]. In the present study, SEM analysis was performed to investigate the morphologies of pure isoflavonoids, D3, and their combinations. Figure 6 shows SEM microphotographs of daidzein, genistein, daidzein/D3, genistein/D3, and D3. Daidzein and genistein were characterized by regular shaped crystals, while D3 was present as amorphous particles. After complexation, the original morphologies of isoflavonoids (daidzein and genistein) or D3 disappeared. The drastic change in particle shape of

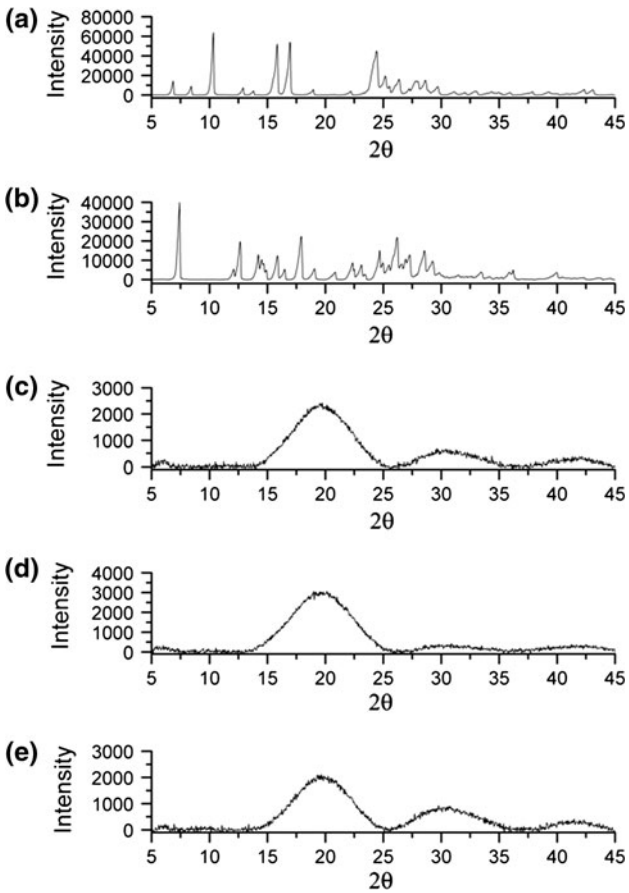

Fig. 7 X-ray diffraction patterns of daidzein (**a**), genistein (**b**), D3 (**c**), daidzein/D3 (**d**), and genistein/D3 (**e**)

daidzein/D3 and genistein/D3 corroborates the formation of a new solid phase. Further evidence for the nature of the daidzein/D3 and genistein/D3 is provided by XRPD experiments (Fig. 7). The powder diffraction patterns of daidzein and genistein displayed sharp peaks, which are the characteristic of an organic molecule with crystallinity. On the other hand, the daidzein/D3 and genistein/D3 have completely different patterns from crystalline isoflavonoids. This results indicate that the isoflavonoids are completely included in the D3 molecule, and the D3 solid complex exist in the amorphous state.

In conclusion, four different dimers (D1 to D4) of the succinoglycan subunit were isolated and purified from *S. meliloti* using various chromatographic methods. Complexation of the succinoglycan dimers with isoflavonoids was studied using UV–Vis and NMR spectroscopic analyses. The aqueous solubility of isoflavonoids (daidzein and genistein) was enhanced by the addition of succinoglycan dimers. The solubilizing efficiency of D3 was found to be the higher than those of the other succinoglycan dimers (D1, D2, and D4). Also, the interaction of isoflavonoids with succinoglycan dimer D3 was further analyzed by 2D NMR spectroscopy, and natures of the complexes were characterized by SEM and XRPD. Based on this analysis, the acyclic succinoglycan dimers hold great promise for use as an effective solubilizing agent through complexation with the isoflavonoids. In addition, the direct chemical interaction of succinoglycan dimers with isoflavonoids, phytoalexin exudated from the legume plant, may be closely related with the symbiotic relationship between the host and its symbiotic bacteria. More detailed studies on the biological function of succinoglycan dimers in the symbiosis with legume are in progress.

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