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Chemical permeabilization and *in situ* removal of daidzein from biologically viable soybean (*Glycine max*) seeds

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After 24 h of chemical permeabilization with 20% (v/v) methanol at 25 °C, the amount of daidzein released from soybean seeds is 15 to 20% of the amount (0.0423 ± 0.0045 mg/g seed dry wt) obtained by physical grinding. With this chemical permeabilization condition, 70% of the permeabilized seeds are still able to germinate. The release of daidzein is enhanced to 33% with the addition of XAD-4 to 20% (v/v) methanol without affecting seed viability.

Key words: chemical permeabilization, *in situ* product removal, daidzein, soybean

Introduction

The production of useful compounds by plants is of great interest in biotechnology and pharmacology. Because secondary plant metabolites are often stored within the vacuoles of plant cells, chemical permeabilization has been studied to release intracellular metabolites while maintaining the viability of the plant cells in order to preserve the plant biomass (Brodelius, 1988; Brodelius and Nilsson, 1983; Dörnenburge and Knorr, 1992; Felix, 1991; Park and Martinez, 1992; Parr *et al*., 1984; Sim *et al*., 1994). Some investigators report that plant cells can survive after certain permeabilizing conditions. Another strategy that has been used to improve productivity is *in situ* removal of metabolites using solid adsorbents which can provide a high concentration gradient of metabolites across the cellular membranes (Asada and Shuler, 1989; Cormier *et al*., 1992; Payne *et al*., 1988; Robin and Rhodes, 1986; Sim *et al*., 1994; Strobel *et al*., 1991). Other methods, such as substrate induction, elicitor addition, and genetic engineering, have been evaluated in order to improve metabolite synthesis. However, only a few processes have been commercialized due to low yields and high economic costs of cell culture systems (Chrispeels and Sadava, 1994).

The objective of the present study is the use of chemical permeabilization and *in situ* product removal to extract a high-value metabolite from plant tissue. The release of daidzein (Figure 1) , an isoflavonoid with potential anticancer properties, from soybean seeds is used as the model system for this investigation. Soybean seeds are selected as the plant tissue model because they have well-defined seed coats which may be a major barrier to the permeabilization process. Furthermore, tissues inside the seeds are relatively uniform as compared with those of leaves and stems. In addition, the viability of the seeds can be readily quantified by germination. Dimethylsulfoxide (DMSO), methanol and XAD-4 are used as the model solvents and solid adsorbent, respectively.

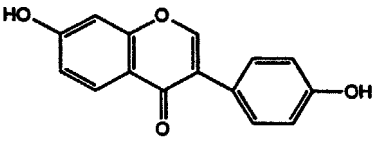

Figure 1 Chemical structure of daidzein.

Materials and methods

Permeabilization

Whole soybean (*Glycine max*) seeds, 2 g, were equilibrated with 20 ml distilled water for 6 h. The seeds were then added to 20 ml solvent solutions and shaken at 150 rpm at 25 °C for various times. After centrifuging the solution at 15,000 **g** at 20 °C for 30 min, 5 ml supernatant was diluted with distilled water to 25% (v/v) solvent and loaded on a C_{18} Sep-Pak disposable column. Daidzein was eluted from the column with 4 ml methanol (confirmed by the use of daidzein standard) at 1 ml/min and the solvent was evaporated with a stream of air. Methanol, 1 ml, was added to dissolve the dry extract. The sample was then kept at 4 °C until analysis by HPLC. To study the effect of *in situ* adsorption, 0.5 g (with 60% (w/w) water content) of the styrene-divinylbenzene solid adsorbent, XAD-4, was added to the solution. XAD-4 was separated by filtration

through a sieve with 0.1 mm pore size diameter. The metabolite was eluted from the XAD-4 by washing twice with methanol for 1 h for each washing. Prior to use, XAD-4 was immersed in methanol and then washed with several volumes of water and dried with paper towels.

The total amount of daidzein in the seeds was obtained by grinding extraction (adapted from methods cited in Kaufman *et al*., 1997). Whole seeds covered with liquid N_2 were ground into a fine powder. Ground seed, 2 g, were extracted twice with 20 ml 80% (v/v) methanol for 24 h at 25 °C. The sample was centrifuged at 15000 g at 20 °C for 30 min. The supernatant was then treated as the sample obtained from permeabilization described previously.

HPLC analysis of daidzein

The amount of daidzein was analyzed by HPLC using VYDAC C_{18} (ODS) column, 4.6 × 250 mm. A linear gradient was run starting with 20% (v/v) methanol containing 0.1% (v/v) trifluoroacetic acid and ending with 86% methanol containing 0.1% (v/v) trifluoroacetic acid at 30 min. The flow rate and temperature was 1.3 ml/min and 40 °C, respectively. The eluate was mornitored at 260 nm. Identity of daidzein is verified 1) by direct comparison of retention time to that of pure daidzein standard and 2) by the comparison of the sample peak with and without the addition of internal daidzein standard. The amount of daidzein was calculated by the use of a standard curve that plots peak areas for known concentrations of standard.

Soybean viability test

After permeabilization, soybean seeds were washed with 20 ml well-stirred distilled water for 6 h. The viability of soybean seeds (n=20) was tested by germination in a mixture of Vermiculite : Perlite (1:1, w/w) at 25 °C in a greenhouse. The number of permeabilized seeds that can germinate was recorded.

Data analysis

The amount of daidzein (0.0423 mg/g seed dry wt) extracted by physical grinding and extraction with 80% (v/v) methanol was used as the basis for calculating the % daidzein released. For viability test, 20 seeds were used as the sample size. All treatments were made in triplicate. To study the effect of DMSO, methanol and time on permeabilization, the factorial design of experiment for the ANOVA was performed. The statistical software program, JMP (version 3.1.6) was used to fit the experimental data into a second-order model: $y = a + b_1x_1 + c_1x_2 + b_2x_1^2 + c_2x_2^2 + dx_1x_2$, where y is the % daidzein release or % soybean seed viability; a is the constant or intercept, b_1 and c_1 are the linear coefficients, b_2 and c_2 are the quadratic coefficients, d is the cross product coefficient, and x_1 and x_2 represent the concentrations of DMSO and methanol. These coefficients were then used to construct the contour plots of the % daidzein release and % soybean seed viability at different solvent concentrations and times.

Results and discussion

Effect of DMSO and methanol on the release of daidzein and viability of soybeans

Soybean seeds were hydrated for 6 h and permeabilized in various combinations of water, DMSO, and methanol for 16 h. The contour plots of the % daidzein release and the % soybean viability at different mixture concentrations are shown in Figure 2. The contour plots indicate that the % release of daidzein varies in proportion to the concentrations of the solvents. In addition, methanol solutions show a higher releasing-capability than do DMSO solutions. 20% (v/v) methanol can release up to 15% stored daidzein whereas higher than 50% (v/v) DMSO is required to release the same amount of daidzein. Furthermore, the addition of DMSO to the methanol solution does not increase the % release of daidzein as compared to the use of methanol solution alone at the same solvent concentration. In the case of % viability of soybean, the contour plots indicate that the viability of treated seeds varies inversely to the concentration of solvents. Furthermore, methanol solutions have a higher destructive effect on seed viability

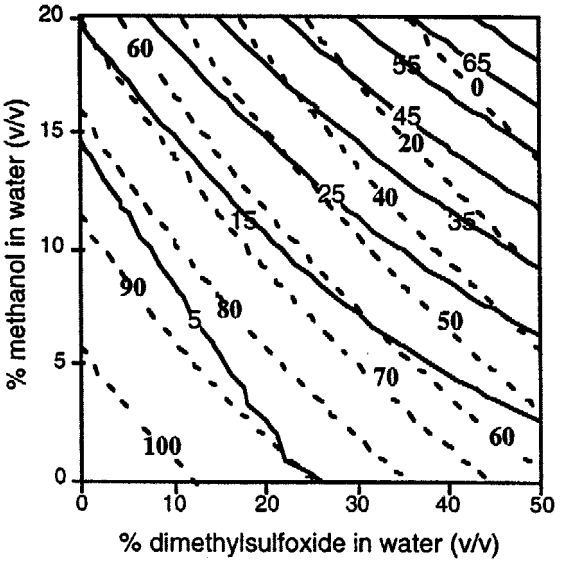

Figure 2 Contour plots of the % release of daidzein and the % viability of soybean after permeabilization of seeds with various concentrations of solvent mixtures for 16 h. The amount of daidzein (0.0423 mg/g seed dry wt) obtained by grinding extraction and the total number of seeds (n=20) grown were used as the basis for the percentage calculation: % daidzein release (——), % soybean seed viability (- - - - -).

than do DMSO solutions. More than 90% of seeds treated with 20% (v/v) DMSO are still viable whereas only 70% of seeds treated with 20% (v/v) methanol, germinate.

Effect of permeabilization time on the release of daidzein and the viability of soybeans

Besides the concentration of solvent, the time of permeabilization is another important factor. Hydrated soybeans were permeabilized in different concentrations of methanol (10, 20 and 30%, v/v, in water) for various times (0, 2, 4, 8, 12, 16, 24 and 36 h). The contour plots of the % daidzein release and the % soybean viability at different periods and methanol concentrations are shown in Figure 3. The contour plots clearly show that there is an overlap region that can be used to release a moderate amount of daidzein, and at the same time, to maintain a high level of soybean viability. If 70% viability is set as a criterion, up to 20% (v/v) methanol solution can be used to release 20–25% stored daidzein after permeabilization for 24 h.

The release profiles of daidzein and the viability profiles of treated soybeans permeabilized with 20% (v/v) methanol and 30% (v/v) DMSO as well as water are illustrated in Figure 4. The results clearly show that methanol and DMSO solution can be used to significantly improve the release of daidzein as compared to water alone. The release

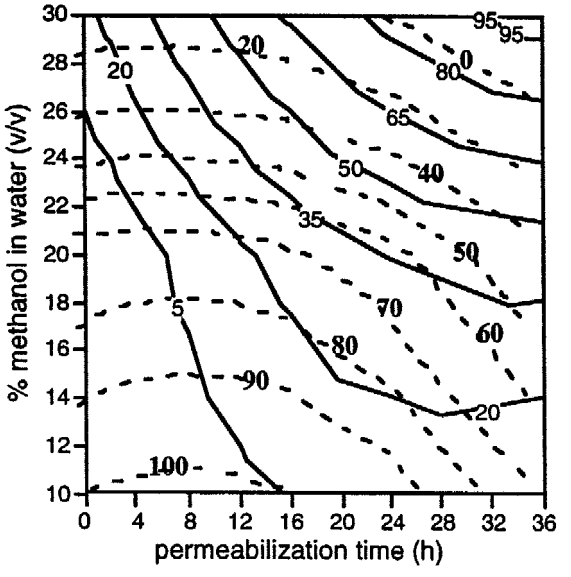

Figure 3 Contour plots of the % release of daidzein and the % viability of soybean after permeabilization of seeds with various methanol concentrations and times. The amount of daidzein (0.0423 mg/g seed dry wt) obtained by ginding extraction and the total number of seeds (n=20) grown were used as the basis for the percentage calculation: % daidzein release (——), % soybean seed viability (- - - -).

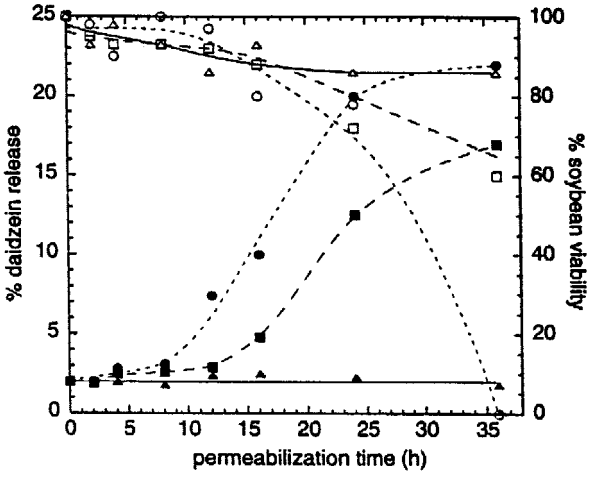

Figure 4 The release profile of daidzein and the viability profile of the soybean seeds after permeabilization for different periods. The amount of daidzein (0.0423 mg/g seed dry wt) obtained by ginding extraction, and the total number of seeds (n=20) grown were used as the basis for the percentage calculation: the % release of daidzein permeabilized with water (—▲—), 20% (v/v) methanol (- -●- -), 30% (v/v) DMSO (—■—), and the % soybean viability treated with water (—△—), 20% (v/v) methanol (- -○- -), 30% (v/v) DMSO (—□—). The values are the average of 3 replicates. The standardard deviations of the % daidzein release and soybean viability are <4 % and <10%, respectively (not shown).

profiles show sigmoidal kinetics which can be divided into three phases: lag phase, release phase and saturation phase. During lag phase, only a small amount of daidzein is released. The amount of daidzein released then increases significantly in the release phase. In the saturation phase, daidzein is still released, but in lower amounts as compared to those during release phase. Like the release profile, the viability profile can be divided into three regions: high tolerance region, intermediate tolerance region, and intolerance region. In the high tolerance region, the solvents do not have much impact on soybean seed viability. More than 80% of treated seeds are viable. The viability then starts to decrease to levels lower than 80% in the intermediate tolerance region. This region is designated as such due to the fact that some treated seeds can survive the treatment and remain viable. In the intolerant region, the viability of permeabilized seeds is completely lost.

The release and viability profiles suggest that a good solvent system should provide a release profile with short lag phase and release phases as well as high levels in the amount of metabolite in the saturation phase. At the same time, it should provide a viability profile with long high tolerance and intermediate tolerance phases. These condi-

tions will allow one to obtain an overlap region that can be used to permeabilize high levels of metabolites, and at the same time, to maintain a high level of seed viability.

Effect of XAD-4 on the release of daidzein and viability of soybeans

Hydrated soybeans were permeabilized in 20% (v/v) methanol in the presence and absence of XAD-4 for different periods. Profiles for the release of daidzein and the viability of soybeans are illustrated in Figure 5. The results clearly show that XAD-4 significantly increases the amount of daidzein released, namely, from 20% to 33% after permeabilization for 24 h. This indicates a synergistic effect between permeabilization and *in situ* compound removal on the release of daidzein. The soybean seed viability profiles with and without XAD-4 are relatively similar, indicating that XAD-4 does not have any effect on seed viability.

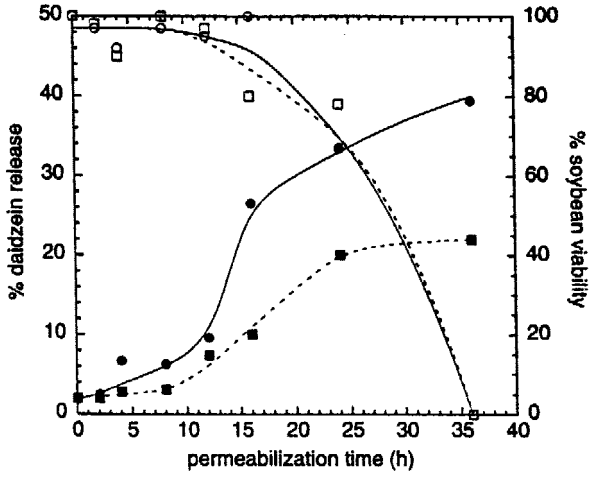

Figure 5 The release profile of daidzein and the viability profile of the soybean seeds after permeabilization for different times. The amount of daidzein (0.0423 mg/g seed dry wt) obtained by ginding extraction, and the total number of seeds (n=20) grown were used as the basis for the percentage calculation: the % release of daidzein permeabilized with 20% (v/v) methanol (- -■- -), 20% (v/v) methanol with XAD-4 (—●—), and soybean seed viability treated with 20% (v/v) methanol (- -□- -), 20% (v/v) methanol with XAD-4 (—○—). The values are the average of 3 replicates. The standardard deviations of the % daidzein release and soybean viability are <6% and <10%, respectively (not shown).

Conclusions

The concept of a combination of permeabilization and *in situ* product removal can be used to directly release intracellular metabolites from viable plant tissues such as found in seeds. We realize that the specific conditions achieved in this study may not be valid for all other systems. However, the information derived from this study will be of fundamental importance for the development and improvement of permeabilization conditions for other metabolites and tissues such as leaves and stems of mature plants.

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