Licorice-derived compounds inhibit linoleic acid (C:18:2ω6) desaturation in soybean chloroplasts

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Abstract Although glycyrrhizic acid, a major constituent of licorice root, has important pharmacological effects in humans, the biological activity of glycyrrhizic acid and its aglycone glycyrrhetinic acid in plants is unknown. Here we report that these licorice-derived compounds and the analog carbenoxolone inhibit desaturation of linoleic acid (C18:2ω6) in soybean chloroplasts using monogalactosyldiacylglycerol and phosphatidylcholine substrates in an in vitro assay for desaturase activity. At 10 nM glycyrrhetinic acid, there is significant inhibition of desaturation of linoleic acid suggesting that licorice-derived compounds could prove useful in investigating biochemical pathways of linoleic acid desaturation in plant chloroplasts and plant desaturase regulation, which has application in modification of plant response to environmental stress, as well as optimization of oil seed composition.

Key words: Linoleic acid desaturase; Licorice; Soybean chloroplast

1. Introduction

Licorice was used during the time of Hammurabi [1]. Indeed, Hippocrates, Theophrastus, Pliney the Elder and Galen have cited the extract of the root of *Glycyrrhiza glabra* as having important medicinal properties, including healing of ulcers and wounds and quenching thirst [1–3]. It is now known that glycyrrhizic acid and its aglycone glycyrrhetinic acid (Fig. 1) present in the root extract are responsible for these biological activities [1–3]; these compounds exert their biological effects in humans by inhibiting 15-hydroxyprostaglandin dehydrogenase [4–6] and 11β-hydroxysteroid dehydrogenase [7–10], enzymes that catalyze the conversion of prostaglandins and glucocorticoids, respectively, into inactivate metabolites. This ancient medicinal lore has been applied in modern medicine to raise the levels of prostaglandins E_2 and $F_{2\alpha}$ and cortisol for treatment of conditions where increased levels of prostaglandins and glucocorticoids have therapeutic benefits. Thus, carbenoxolone (Fig. 1), an analog of glycyrrhizic acid, is used to increase the levels of prostaglandins E_2 and $F_{2\alpha}$, which promotes healing of peptic ulcers [4–6]. Licorice is also used to increase glucocorticoid levels [9], which has been useful in treating some cases of Addison's and Simmons's disease [11,12].

However, little is known of the role of either glycyrrhizic acid or glycyrrhetinic acid in plants, although glycyrrhizic acid constitutes 1–3% of *G. glabra* roots [13]. The high concentrations of such a complex molecule in *G. glabra* suggests that either glycyrrhizic acid or one of its metabolites has a biological role either in *G. glabra* or in an organism that interacts with this plant [14,15]. Because glycyrrhetinic acid inhibits oxidoreductases that metabolize hydrophobic substrates, we reasoned that this compound may inhibit one or more enzymes of fatty acid metabolism in plants. To test this hypothesis, we have investigated the effect of licorice-derived compounds on desaturation of fatty acids in chloroplasts, using a recently developed procedure [16] that stabilizes desaturase enzyme activity in vitro, permitting characterization of the 18:2 desaturase(s) that is located in chloroplast envelopes in plant leaf tissue [16–18]. Here we report that licorice-derived compounds inhibit enzymatic conversion of linoleic acid (C18:2ω6) to α-linolenic acid (C18:3ω3) in soybean leaf chloroplasts. C18:3ω3 is a dietary essential fatty acid [17,19], as well as a precursor of other fatty acids that have important industrial applications. Moreover, regulation of fatty acid desaturation is important in tolerance of plants to extremes of cold and heat [17,20]. Our findings suggest that licorice-derived compounds, coupled with the ability to stabilize chloroplast n-3 desaturase activity in vitro [16,21], should provide a unique and valuable tool to study desaturation of C18:2 to C18:3, which could have important applications in agriculture.

2. Materials and methods

Glycyrrhizic acid, 18α- and 18β-glycyrrhetinic acid, and carbenoxolone were purchased from Sigma Chemical Co. and used as received.

The desaturase assay was done as described by Norman et al. [16]. Briefly, chloroplast homogenates (prepared by disruption in 0.5% CHAPS) were combined by ultrasonication with [^{14}C]C18:2/C18:3] MGDG or PC substrate and incubated in medium (30 mM HEPES-KOH, pH 8.0, containing 330 mM sorbitol, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% w/v BSA, 2.0 mM ATP, 2.7 mM NADPH, ferredoxin (10 μg/ml), catalase (2000 U/ml), and 0.5% CHAPS) with inhibitor concentrations as indicated. The assay contained from 200 μg to 250 μg of chloroplast. Glycyrrhetinic acid was dissolved in DMSO and equivalent volumes of the solvent were added to controls. After incubation (45 min at 25°C) reactions were terminated by the addition of 2 ml of methanol and lipids extracted and purified. Relative amounts of radioactivity in C_{18} fatty acids were determined by HPLC with in-line radioactivity detection. 100% activity is 28.5 and 15.2 nmol fatty acid desaturated/mg chlorophyll/hr for MGDG and PC substrates, respectively.

The radiolabelled MGDG substrate was prepared by silica Sep-Pak, TLC, and HPLC fractionations of lipid extracts from leaf tissue, which was preincubated with radiolabelled fatty acids according to Norman et al. [16]. In the initial experiment, aliquots (25–50 nmol, specific activity 0.54–1.20 μCi (20.16–44.4 kBq)/μmol) of the total MGDG

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preparations were used; in later experiments, molecular species were used.

3. Results and discussion

Previous studies indicate that monogalactosyldiacylglcyerol (MGDG) and phosphatidylcholine (PC) are substrates for 18:2 desaturases in plant chloroplasts [16,23]. In an initial study, we incubated MGDG containing ^{14}C-labelled C18 fatty acids with soybean chloroplast membranes and measured conversion of C18:1 to C18:2 and C18:3 in the presence of glycyrrhetinic acid at 10^{-8}M to 10^{-7}M. After 45 min at 25°C, the reaction was stopped and fatty acids in the MGDG fraction were isolated by HPLC with in-line radioactivity detection [16]. At 10^{-7}M, glycyrrhetinic acid decreased the desaturation of C18:2 by about 80% (data not shown). To more precisely examine 18:2 desaturation, we used another strategy in which MGDG and PC substrates with C18:2 in the sn-1 and C18:3 in the sn-2 positions, respectively (Fig. 2) were incubated with soybean chloroplast membranes in the presence of different concentrations of glycyrrhetinic acid and two related compounds: carbenoloxone and glycyrrhizic acid, which contain 18β-glycyrrhetinic acid with different C3 substituents (Fig. 1). We also tested 18α-glycyrrhetinic acid to investigate stereospecificity in the inhibition of C18:2 desaturase activity in soybean chloroplasts. As shown in Fig. 3, glycyrrhetinic acid, carbenoxolone and glycyrrhizic acid inhibited conversion of C18:2 to C18:3 at nM concentrations, whereas 18α-glycyrrhetinic acid was inactive. Substituents at C3 appear to modestly reduce the inhibition of the 18:2 desaturation for MGDG substrates: carbenoxolone is about as effective as glycyrrhizic acid. There is little effect on inhibition of C18:2 PC desaturation.

Examination of Fig. 3 reveals that even at a concentration of 10^{-4} M, licorice-derived compounds only partially inhibit desaturation of either C18:2 MGDG or C18:2 PC, and the degree of inhibition depends on the substituent at C3 of the

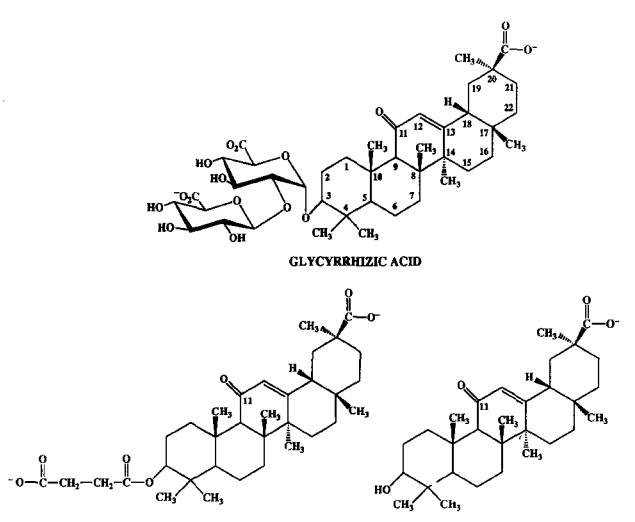
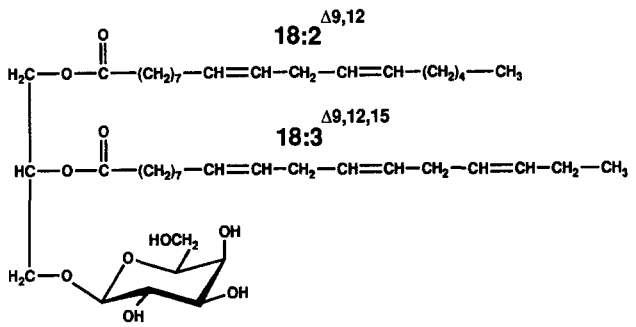


Fig. 1. Structures of glycyrrhizic acid, glycyrrhetinic acid and carbenoxolone. Stereochemistry of the hydrogen at C18 affects the orientation of the E ring. With an 18β-hydrogen, rings D and E are *cis*-fused. With an 18α-hydrogen, rings D and E are in *trans*-configuration Only compounds with the 18β-orientation inhibit human 11β-hydroxysteroid dehydrogenase.



18:2/18:3 Monogalactosyldiacylglycerol

Fig. 2. Structure of C18:2/C18:3 MGDG. Desaturation of C18:2 takes place on the sn-1 position. The PC substrate has phosphorylcholine at sn-3 instead of the monogalactosyl group.

inhibitor. Thus, about 15–20% of the C18:2 MGDG desaturase activity is resistant to either glycyrrhetinic acid or carbenoxolone and about 50% is resistant to glycyrrhizic acid. About 22%, 43% and 60% of C18:2 PC desaturase activity is resistant to glycyrrhetinic acid, carbenoxolone, and glycyrrhizic acid, respectively. We also find that combinations of licorice-derived compounds at 10^{-6} M did not completely eliminate desaturase activity towards MGDG or PC substrates. Incubation of chloroplast membranes with 10^{-6} M glycyrrhetinic acid and carbenoxolone or glycyrrhizic acid and carbenoxolone or glycyrrhetinic acid and glycyrrhizic acid inhibited only 85 to 90% of the conversion of C18:2 to C18:3. This suggests there are differences in the affinity of desaturase isozymes [23,24] for licorice-derived compounds, depending on the substituent at C3 of glycyrrhetinic acid, with at least one desaturase not being inhibited by the compounds that we used.

Our experiments indicate that there is a specific interaction between licorice-derived compounds and enzyme(s) that catalyze desaturation of C18:2 MGDG and C18:2 PC desaturation because nM concentrations of either glycyrrhetinic acid or carbenoxolone significantly inhibit enzyme activity. Glycyrrhizic acid and carbenoxolone are water soluble which makes it unlikely that these compounds are partitioning into a hydrophobic membrane and disrupting desaturation by non-specific chaotropic effects. Moreover, other experiments show that either glycyrrhetinic acid or carbenoxolone at 10^{-6} M inhibit conversion of C18:1 to C18:2 by 36% and 32%, respectively (data not shown), which is very different from the effects of these compounds on conversion of C18:2 to C18:3. Lastly, the inactivity of the 18α isomer of glycyrrhetinic acid indicates a stereospecific requirement for inhibition of C18:2 desaturase activity by glycyrrhetinic acid.

Our findings have application in medicine and agriculture that relate to regulation of C18:3ω3 levels in plants. This fatty acid is essential to human health [17,19] and is not synthesized by humans, who must obtain α-linolenic acids from dietary sources. C18:3ω3 is a precursor of eicosapentaenoic acid (C20:5ω3) and docosahexaenoic acid (C22:6ω3), which protect humans against cardiovascular disease [19]. These two fatty acids are synthesized mainly in brown and red algae, which are foods for fish, the usual source of ω3 fatty acids in human diet and in food supplements.

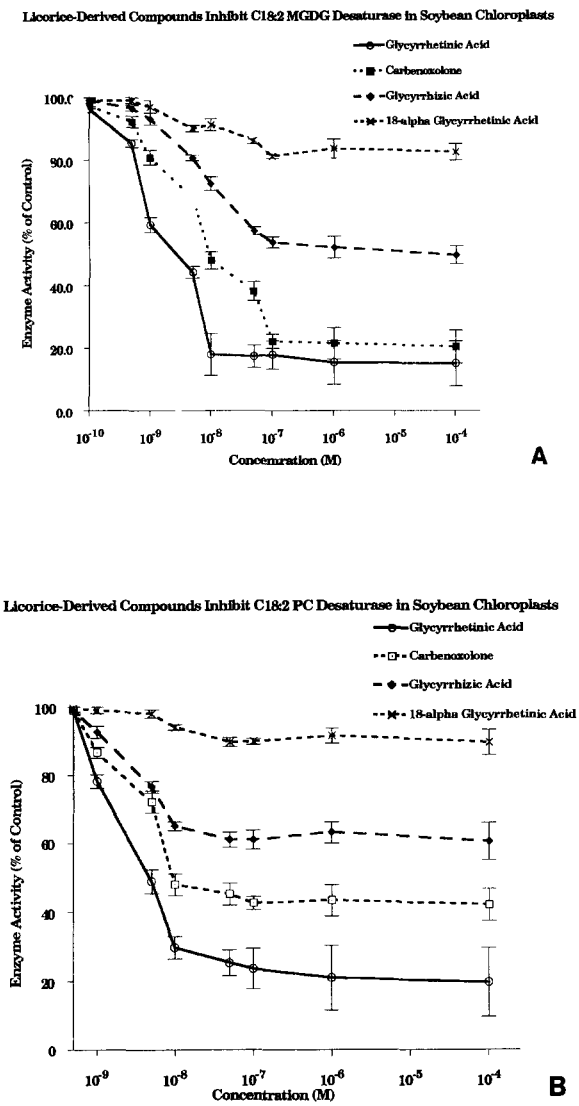


Fig. 3. Effect of glycyrrhetinic acid, carbenoxolone and glycyrrhizic acid on desaturation of 18:2/18:3 MGDG and 18:2/18:3 PC by soybean chloroplast membranes. (A) Inhibition of 18:2/18:3 MGDG desaturation. (B) Inhibition of 18:2/18:3 PC desaturation.

Understanding the regulation of C18:3 fatty acid synthesis is of interest for optimizing the fatty acid content of soybean oil and other vegetable oils for important industrial uses and to improve the tolerance of plants to extremes of cold and heat [17,20]. The high affinity of glycyrrhetinic acid, carbenoxolone and glycyrrhizic acid in inhibiting C18:2ω6 desaturation suggests that these compounds may be useful in studying plant enzymes involved in fatty acid desaturation.

Studies with radioactive glycyrrhizic acid indicate that this compound is not present in the green parts of *G. glabra* [13]. It appears that this plant excludes glycyrrhizic acid from its chloroplasts, where our findings suggest glycyrrhizic acid would be toxic to *G. glabra*. In fact, compounds that inhibit fatty acid synthesis in chloroplasts have been developed as herbicides [25]. Glycyrrhetinic acid and related compounds may be useful for this purpose because they inhibit desaturases at nM concentrations, in contrast to the substituted pyridazinone compound BASF 13–338, which inhibits plant C18:2 desaturase at 100-fold higher concentrations [16,25]. Indeed, we have preliminary evidence that spraying 100 μM glycyrrhetinic acid can inhibit the growth of lambsquarters, *Chenophodium album*, in the greenhouse. An herbicidal application for licorice-derived compounds is attractive because glycyrrhetinic acid, carbenoxolone and glycyrrhizic acid are known to have low toxicity towards humans, and in fact, are useful therapeutics in treating ulcers and glucocorticoid deficiency.

At this time, the function of glycyrrhizic acid in *G. glabra* is unknown. Glycyrrhizic acid or a metabolite may protect *G. glabra* against various organisms in the rhizosphere such as bacteria, fungi, insects and vertebrates. Activity of glycyrrhizic acid in soil bacteria is likely because glycyrrhizic acid inhibits 3α,20β-hydroxysteroid dehydrogenase in the soil bacterium *Streptomyces hydrogenans* [26]. This 3α,20β-hydroxysteroid dehydrogenase is homologous to proteins in other soil bacteria, including *rhizobia* that infect soybeans and alfalfa [14,15,27,28], as well as human 11β-hydroxysteroid dehydrogenase and 15-hydroxyprostaglandin dehydrogenase. Like soybeans and alfalfa, *G. glabra* is a legume. Glycyrrhizic acid or its metabolites may interact with an enzyme that regulates the concentration of small hydrophobic molecules involved in *G. glabra*-rhizobia communication [29–31], a process that has many similarities to steroid- and prostaglandin-mediated communication in mammals [2,14,15].

Thus, licorice, which is used by humans as a flavoring in candy and beverages and as an herbal medicine, may have new benefits to offer us: tools for studying fatty acid desaturation in plants to improve the production of nutritional and industrial fatty acids in plants and extend the climate tolerance of plants, as well as providing a safe herbicide for agriculture.

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