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Physiological correlation between glycyrrhizin, glycyrrhizin-binding lipoxygenase and casein kinase II

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Abstract By means of glycyrrhizin (GL)-affinity column chromatography, a GL-binding lipoxygenase (gbLOX) was selectively purified from the partially purified soybean LOX-1 fraction. Polypeptide analysis of the purified gbLOX by SDS-PAGE detected two distinct polypeptides (p96 and p94), which were identical to LOX-3 as determined by their partial N-terminal amino acid sequences. Moreover, it was found that (i) phosphorylation of gpLOX by casein kinase II (CK-II) is significantly stimulated by 3 μM GL, but inhibited by 30 μM GL or 10 μM oGA; and (ii) gbLOX activity is enhanced when the enzyme is phosphorylated by CK-II in the presence of 3 μM GL. These results suggest that (i) CK-II is a kinase responsible for the activation of gbLOX through its specific phosphorylation; and (ii) GL is one of the regulatory substances for specific phosphorylation of gbLOX (LOX-3) by CK-II in plant cells.

Key words: Casein kinase II; Glycyrrhizin; Glycyrrhizin-binding lipoxygenase; Phosphorylation; Lipoxygenase activity, regulation; Soybean

1. Introduction

Glycyrrhizin (GL) is present in large quantities in the roots and rhizomes of liquorice, *Glycyrrhiza glabra* L., and is composed of a molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid. GL is an effective anti-inflammatory agent used in Chinese medicine [1,2] and several of its biological activities have been identified in the human body, i.e. hypolipidemic [3], anti-oxidative [3], anti-viral [4] and interferon-inducing activities [5]. However, the biochemical mechanisms involved in these GL-induced biological effects remain to be elucidated.

Recently, we (i) selectively purified a 96-kDa GL-binding protein (gb96) from an aqueous extract of soybeans, by means of GL-affinity column chromatography (HPLC); (ii) identified it as lipoxygenase 3 (LOX-3); (iii) demonstrated that it is an effective phosphate acceptor for CK-II in vitro; and (iv) showed that low levels (1–10 μM) of GL significantly stimulate the CK-II catalyzed phosphorylation of gbLOX in vitro [6]. These observations suggest that there is a tight physiological correlation between the activities of GL-binding enzymes (gbLOX and CK-II) and GL in plant cells. Therefore, the present study was carried out to determine (i) the effects of GL and a glycyrrhetinic acid derivative (oGA) on phosphorylation of gbLOX by CK-II in vitro, and (ii) the physiological significance of GL in the regulatory mechanism of gbLOX activity in vitro. Here, we show direct evidence for the physiological significance of GL on the regulation of gbLOX activity through its specific phosphorylation by CK-II. This is the first report concerning the physiological correlation between GL, gbLOX (LOX-3) and CK-II in vitro.

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Abbreviations: CK-II, casein kinase II; DTT, dithiothreitol; GA, glycyrrhetinic acid; gbLOX, glycyrrhizin-binding lipoxygenase; GL, glycyrrhizin; HPLC, high-performance liquid chromatography; LOX-1, lipoxygenase-1; poly-Arg, poly-L-arginine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham (Arlington Heights, USA); DTT, poly-Arg (5–10 kDa), α-casein (bovine milk) and trypsin from Sigma Chemicals (St. Louis, USA); and the Mono Q HR5/5 column from Pharmacia (Uppsala, Sweden). Partially purified soybean LOX-1 fraction (specific activity as LOX-1: 137200 U/mg) was obtained from Biozyme Laboratories Ltd. (UK). Goat anti-soybean LOX-1 was kindly provided by Dr. B. Axelred (Purdue University, USA).

2.2. GL and oGA

GL (20β-carboxyl-11-oxo-30-norolean-12-en-3β-yl-2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid, $C_{42}H_{62}H_{16} = 822.92$) and oGA (olean-11,13(18)-diene-3β,30-diol-3β,30-di-*O*-hemiphthalate disodium salt) were kindly supplied by Minophagen Pharmaceutical Co., Ltd. (Tokyo). These compounds were prepared as a 10 mM stock solution in 5% dimethyl sulfoxide and used after dilution with distilled water.

2.3. GL-affinity column

A GL-affinity column was prepared as originally described by Nakamura et al. [7], using Tresyl-5PW (packing gel for HPLC; Tosoh Mfg, Co., Ltd., Tokyo) and *N*-(glycyrrhizin)-30-α-lysine, as previously reported [6].

2.4. Assay for LOX activity

LOX activity was measured polarographically by the method described originally by Grossman and Zakut [8], using a Clark oxygen electrode. The LOX fractions were added to reaction mixtures containing 3 mM linolenic acid and 0.1 M sodium phosphate (pH 6.5 or 9.0). Oxygen consumption (ratio of oxygen taken up by the sample to total oxygen dissolved in the initial reaction mixture) was recorded for the indicated periods at 25°C. The linear part of the curve was used for calculation of the reaction rate, as previously reported [6]. The activity (1 unit) is defined as the quantity of enzyme catalyzing the consumption of 1 nmol O_2/min per μg at 25°C.

2.5. Purification of CK-II

CK-II was highly purified from a 1.0 M NaCl extract of mouse brain by means of successively phospho-cellulose column chromatography, gel filtration on a Superdex 200pg column (HPLC) and ion-exchange chromatography on a Mono Q column (HPLC), as previously reported [9].

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2.6. Determination of gbLOX activity after its phosphorylation by CK-II in vitro

To determine the stimulatory and inhibitory effects of GL on gbLOX activity, phosphorylation of gbLOX by CK-II was carried out by incubation of purified gbLOX (approx. 1 μg) with CK-II (approx. 50 ng), 2 mM DTT, 2 μg of poly-Arg (CK-II activator), 5 mM Mg^{2+} and 50 μM ATP in the presence or absence of GL. After incubation (35 min at 30°C), 6 mM linolenic acid (a substrate for LOX, final 3 mM) in 0.1 M MES buffer (pH 6.5) was added to the reaction mixtures (50 μl), and then further incubated for 5 min at 30°C. The enzyme reaction was arrested by the addition of 25 μl of 4 N HCl. Aliquots (10 μl) of the reaction mixtures were spotted on a thin-layer plate (TLC aluminum sheet silica gel 60, Merck), and then developed in the solvent mixed with 70% ether and 30% hexane. Oxidized products (linoleate hydroperoxides) formed from linolenic acid by LOX-3 on the TLC plate were specifically detected using 1% *N,N*-dimethyl-*p*-phenylenediamine (DMPD) in a mixture of methanol-water-acetic acid (128:25:1) [10].

2.7. Phosphorylation of gbLOX by CK-II in vitro

To phosphorylate gbLOX by CK-II in vitro, the purified gbLOX fraction (Mono S fraction, approx. 1 μg) was incubated for 20 min at 30°C with CK-II (approx. 50 ng, purified from mouse brain) in reaction mixtures (50 μl) comprising 40 mM Tris-HCl (pH 7.6), 2 mM DTT, poly-Arg (2 μg), 5 mM Mg^{2+} and 30 μM [γ-^{32}P]ATP (1500 cpm/pmol). Phosphorylation was arrested by the addition of SDS-sample buffer (50 μl). The ^{32}P-labeled gbLOX in aliquots (20 μl) of the reaction mixtures was directly detected by autoradiography after SDS-PAGE, as previously reported [6].

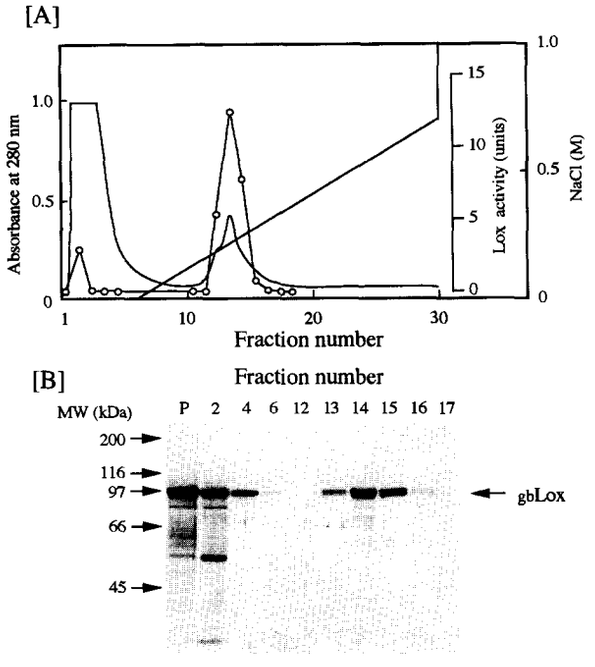

Fig. 1. (A) Purification of gbLOX from the partially purified soybean LOX-1 fraction by GL-affinity column chromatography (HPLC). The partially purified LOX-1 fraction (about 10 mg) was applied on a GL-affinity column (1.2×16 cm) equilibrated previously with 20 mM MES-NaOH buffer (pH 6.8). Elution was carried out with a linear gradient of 0.0 and 0.7 M NaCl in the same buffer at a flow rate of 1.0 ml/min, collecting 1.0 ml fractions. Aliquots (10 μl) of the indicated fractions were assayed for LOX activity at pH 6.5 using linolenic acid as a substrate (○). (B) Polypeptides in the indicated fractions were detected by staining with Coomassie Brilliant Blue R250 (CBB R250) after SDS-PAGE. P: Partially purified LOX-1 fraction.

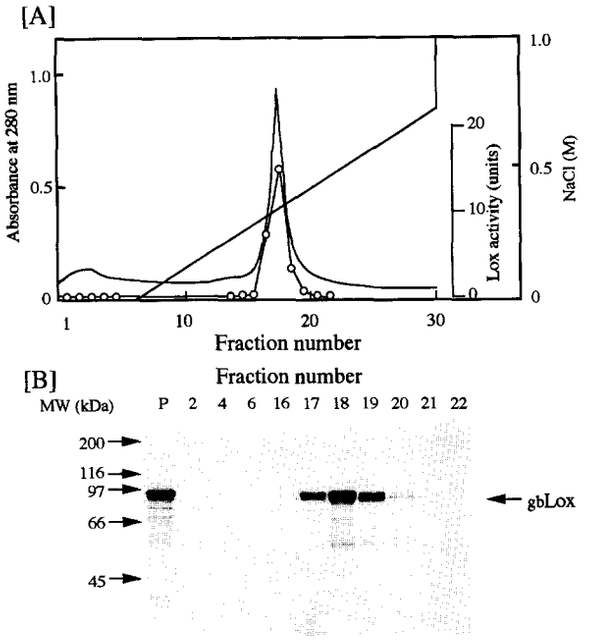

Fig. 2. (A) Final purification of gbLOX in the GL-affinity fraction by Mono S column chromatography (HPLC). The GL-affinity fraction (about 2.5 mg) was applied on a Mono S column, previously equilibrated with 20 mM MES-NaOH buffer (pH 6.8). Elution was carried out with a linear gradient of 0.0 and 0.7 M NaCl in the same buffer at a flow rate of 1.0 ml/min, collecting 1.0 ml fractions. Aliquots (10 μl) of the indicated fractions were assayed for LOX activity at pH 6.5 using linolenic acid as a substrate (○). (B) Polypeptides in the indicated fractions were detected by staining with Coomassie Brilliant Blue R250 after SDS-PAGE. P: Pre-Mono S fraction (GL-affinity fraction).

3. Results

3.1. Selective purification of gbLOX by GL-affinity column chromatography

By means of GL-affinity column chromatography, a GL-binding LOX (gbLOX) was selectively purified from the partially purified soybean LOX-1 fraction. The gbLOX was eluted from the GL-affinity column between 0.2 and 0.3 M NaCl, and the elution profile of gbLOX corresponded exactly to gbLOX activity at pH 6.5, using linolenic acid as a substrate (Fig. 1). It comprised approx. 16.5% of the total protein in the LOX-1 fraction. The gbLOX in the GL-affinity fraction was further purified by Mono S column chromatography (HPLC) and was eluted between 0.4 and 0.5 M NaCl as a single protein peak exactly corresponding to gbLOX activity (Fig. 2A). Polypeptide analysis by SDS-PAGE detected two distinct polypeptides (96 kDa (p96) and 94 kDa (p94) polypeptides) in the purified gbLOX Mono S fraction (Fig. 2B).

The partial N-terminal amino acid sequences of three fragments (p68, p50 and p46) generated from p96 and two fragments (p56 and p38) from p94, respectively, after separate digestions of these two polypeptides with trypsin were identical to the corresponding sequences of LOX-3, as previously reported [6]. Polypeptide mapping confirmed no difference in the polypeptide fragments generated from p96 and p94 after limited digestion with modified trypsin (data not shown). In addition, Western blotting analysis of p96 and p94 showed that these two polypeptides cross-react with anti-soybean LOX-1 (Fig. 3). From these results, it is concluded that

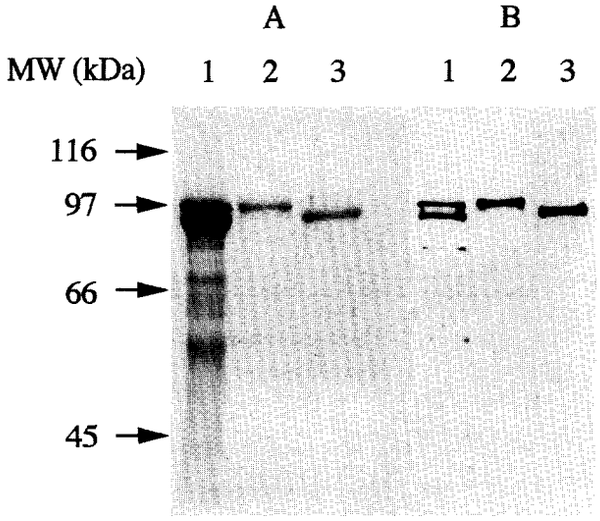

Fig. 3. Western blotting of p96 and p94. Polypeptides in fraction 14 (Fig. 1) were analyzed by SDS-PAGE. After SDS-PAGE, p96 and p94 on the gel were separately extracted with 20 mM phosphate buffer (pH. 6.5) containing 0.5 M NaCl and 0.1% Triton X-100. Extracted p96 and p94 were separately analyzed again by SDS-PAGE. The polypeptides were detected by staining with Amido Black 10B (A) and also subjected to Western blotting using anti-soybean LOX-1 (B), as reported previously [6]. Lanes: 1, polypeptides in GL-affinity fraction 14; 2, p96; 3, p94.

(i) the partially purified soybean LOX-1 fraction contains gbLOX, which could be selectively separated from LOX-1 by GL-affinity column chromatography (HPLC); and (ii) gbLOX is identical to LOX-3.

3.2. *Phosphorylation of gbLOX by CK-II in vitro*

As reported previously [6], it was confirmed that the purified gbLOX fraction (Mono S fraction) was phosphorylated by CK-II in vitro (Fig. 4A, lanes 3–6). This phosphorylation was significantly stimulated by GL at low doses between 1 and 10 μM, whereas it was completely inhibited by 30 μM GL (Fig. 4A, lane 7) or 10 μM oGA (lane 9). Maximum stimulation (about 3.2-fold) of the CK-II catalyzed phosphorylation of gbLOX was observed when 3 μM GL were added to the reaction mixtures (Fig. 4B). Similar stimulation was also observed with oGA at lower doses (0.3–3.0 μM), as compared with those determined with GL.

3.3. *Effect of GL on gbLOX activity after its phosphorylation by CK-II*

To determine the effect of GL on gbLOX activity after its phosphorylation by CK-II in vitro, a minimum assay system for the determination of LOX activity was established. In this assay, a minimum amount (approx. 1 μg) of the purified gbLOX was added to the reaction mixtures (pH 7.6) in order to achieve complete phosphorylation by CK-II. After incubation (35 min at 30°C) with CK-II in the presence of poly-Arg (2 μg), 50 μM ATP and 5 mM Mg^{2+}, an effective substrate (3 mM linolenic acid) for LOXs in 0.1 M MES buffer (pH 6.5) was added to the reaction mixtures, and then further incubated for 5 min at 30°C. The peroxidized products formed from linolenic acid by the enzyme action were specifically detected by 1% *N,N*-dimethyl-*p*-phenylenediamine (DMPD) after development on a TLC plate. As shown in Fig. 5, it was found that (i) phosphorylation of gbLOX by CK-II resulted in a significant increase in its activity (lane 2); (ii) gbLOX activity at pH 6.5 was significantly stimulated when gbLOX was phosphorylated by CK-II in the presence of 3 μM GL (lane 3), but was significantly inhibited at 30 μM (lane 4). In this experimental system, it was confirmed that (i) increased gbLOX activity was exactly dependent upon the incubation time with CK-II and ATP up to 35 min at 30°C; (ii) this increase was reproduced when GTP instead of ATP was used as a phosphate donor for CK-II, whereas it was prevented by CK-II inhibitors (heparin and dextran sulfate); and (iii) LOX substrates (linoleic acid and arachidonic acid) and their oxidized products (linoleic acid hydroperoxide, 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 12-HETE at 1 mM reduced slightly the CK-II catalyzed phosphorylation of gbLOX. Taken together, all these results suggest that (i) CK-II is a kinase responsible for the activation of gbLOX activity; and (ii) GL regulates directly phosphorylation of gbLOX by CK-II in vitro.

4. Discussion

In the present study, gbLOX in the partially purified soy-

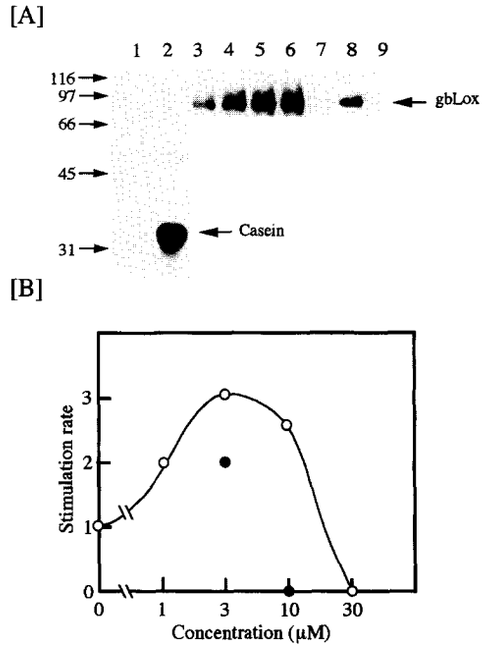

Fig. 4. Phosphorylation of gbLOX by CK-II and the effect of GL on its phosphorylation in vitro. Under the CK-II assay conditions, the purified LOX-3 (Mono S fraction, approx. 1 μg) was incubated for 20 min at 30°C with CK-II (approx. 20 ng, purified from mouse brain) at 30°C in the presence of various doses of GL. (A) The ^{32}P-labeled gbLOX in the reaction mixtures was directly detected by autoradiography after SDS-PAGE. Lanes: 1, gbLOX alone; 2, CK-II incubated with α-casein (1 μg) in the presence of poly-Arg (2 μg); 3, LOX-3 incubated with CK-II and poly-Arg in the absence of GL; 4, as lane 3 in the presence of 1 μM GL; 5, as lane 3+3 μM GL; 6, as lane 3+10 μM GL; 7, as lane 3+30 μM GL; 8, as lane 3+3 μM oGA; 9, as lane 3+10 μM oGA. (B) [^{32}P]Phosphorylated gbLOX on the autoradiogram was determined densitometrically. Stimulation rate '1' represents the enzyme activity of gbLOX phosphorylated by CK-II in the absence of GL. Phosphorylation of gbLOX by CK-II was determined in the presence of the indicated doses of GL (○) or oGA (●).

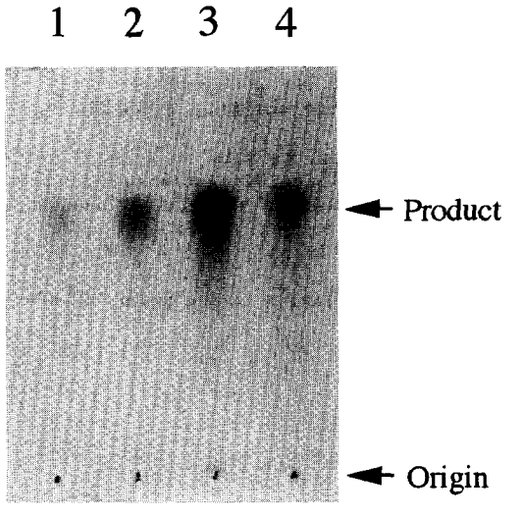

Fig. 5. Stimulation of LOX-3 activity after phosphorylation of gbLOX by CK-II in the presence of GL in vitro. Purified gbLOX (approx. 1 μg) was fully phosphorylated by CK-II under the standard assay conditions (incubation for 35 min at 30°C). After phosphorylation, the enzyme activity of phosphorylated and unphosphorylated gbLOXs was determined using a thin-layer plate as described in Section 2. Lanes: 1, gbLOX alone; 2, phosphorylation of gbLOX by CK-II in the absence of GL (control); 3, 3 μM GL; 4, 30 μM GL.

bean LOX-1 fraction was selectively purified by means of successive GL-affinity column chromatography (Fig. 1) and Mono S column chromatography (Fig. 2). It was found that (i) phosphorylation of gbLOX by CK-II resulted in a significant stimulation of its activity (Fig. 5); and (ii) gbLOX activity was further stimulated when the enzyme was phosphorylated by CK-II in the presence of low doses (1–10 μM) of GL, but this activity was significantly inhibited by 30 μM GL or 10 μM oGA (Figs. 4 and 5). The stimulatory effect of its phosphorylation by CK-II in the presence of low doses of GL was significant since 3 μM GL inhibited approx. 10% of gbLOX activity [6]. The data suggest that GL functions as a regulatory substance for specific phosphorylation of gbLOX by CK-II in plant cells and oGA (a GA-derivative), at one-third the concentration of native GL, inhibits CK-II activity (phosphorylation of gbLOX) in vitro.

Polypeptide analysis by SDS-PAGE detected at least two distinct polypeptides (p96 and p94) in the purified gbLOX fraction (Fig. 2). These two polypeptides were identical to LOX-3, because (i) the partial N-terminal amino acid sequences of at least five polypeptide fragments (p68, p56, p50, p48 and p38) generated from p96 and p94 by the limited proteolysis of each polypeptide with modified trypsin were identical to the corresponding sequences of LOX-3; and (ii) polypeptide mapping confirmed no difference in the polypeptide fragments generated from p96 and p94 after their limited digestion with modified trypsin (data not shown). p94 exhibited both GL-binding ability and LOX activity at pH 6.5, similar to those observed in p96. From these results, it is concluded that p94 is formed from p96 by limited proteolysis during LOX-1 purification.

Separation of gbLOX from GL-unbinding LOX (guLOX) in the partially purified soybean LOX-1 fraction by GL-affinity column chromatography (HPLC) suggests that there are two groups of LOXs in an aqueous extract of soybeans: LOX-1 and LOX-2 belong to the GL-unbinding LOX (guLOX) group, whereas LOX-3 belongs to the gbLOX group. This grouping is supported by the amino acid sequences among soybean LOXs, since LOX-1 (839 amino acids) and LOX-2 (865 amino acids) share more than 92% sequence identity at the amino acid level. In contrast, LOX-3 (857 amino acids) exhibits only 70% sequence identity with the predicted amino acid sequence of LOX-1 [11]. Since these three soybean LOXs (LOX-1–LOX-3) possess high homology in their amino acid sequences, p96 and p94 in the purified gbLOX fraction (Mono S fraction) cross-react with anti-soybean LOX-1 (Fig. 3). In addition, the 14 amino acid sequence (NSVTSVGGIIGOGL) at the N-terminal position (27–41) of LOX-3 are missing in the sequences of LOX-1 and LOX-2 [12]. Therefore, there is a possibility that the 14 amino acid sequence of LOX-3 may be related to one of the GL-binding sites, since the synthetic oligo-polypeptide composed of these 14 amino acid residues shows binding activity with a GL-affinity column (Shimoyama and Ohtsuki, unpublished observation). However, it will be necessary to determine the amino acid sequences of all GL-binding sites since there are at least four GL-binding sites per molecule on LOX-3, to which end work is now in progress.

The stimulatory effect of GL on the phosphorylation of LOX-3 by CK-II in vitro was analyzed by polypeptide mapping after complete phosphorylation in the presence of 3 μM GL. However, no differences were detected in the polypeptide fragments generated proteolytically from p96 or p94 after their full phosphorylation by CK-II in the presence or absence of 3 μM GL (data not shown). Therefore, it is concluded that GL at 3 μM significantly stimulates phosphorylation of gbLOX by CK-II, but has no effect on the CK-II phosphorylation sites of gbLOX.

Our observation that high doses (over 30 μM) of GL inhibit the activities of GL-binding enzymes (gbLOX and CK-II) in vitro (Fig. 5), suggests that saponins, including GL, generally may be responsible for suppression of the activities of GL-binding protein kinases (CK-II [13,14] and cAMP-dependent protein kinase [15]) in plant cells. In addition, our preliminary experiments showed that (i) the activities of GL-binding enzymes (gbLOX and CK-II) were highly stimulated in the presence of suitable concentrations (approx. 1–10 μM) of saponins; and (ii) the cellular levels of saponins and gbLOX (LOX-3) rapidly decreased during germination of soybeans (gbLOX level in stalk and seed leaf was about one-tenth, that of an aqueous extract of soybeans). The latter observation is basically consistent with the report by Kato et al. [16] in soybeans, because the physiological balance between saponins, including GL, and GL-binding enzymes is very important in the metabolic functions, such as the oxygenation of unsaturated fatty acids by gbLOX through its specific phosphorylation by CK-II and the protein kinase-mediated signal transduction, in growing plant cells.

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