Gymnemic acids inhibit rabbit glyceraldehyde-3-phosphate dehydrogenase and induce a smearing of its electrophoretic band and dephosphorylation

Yusuke Izutani[a], Takuya Murai[a], Toshiaki Imoto[b], Masatake Ohnishi[a], Masayuki Oda[a], Sumio Ishijima[a,*]

[a] *Graduate School of Agriculture, Kyoto Prefectural University, Kyoto 606-8522, Japan*
[b] *Department of Physiology, Faculty of Medicine, Tottori University, Yonago 683-8503, Japan*

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Abstract Gymnemic acids (GA) inhibited rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. Binding of GA to GAPDH was observed by surface plasmon resonance measurement. Incubation of GAPDH with GA induced a smearing of the GAPDH band in SDS–PAGE. The GA-induced smearing was diminished by prior incubation of GA with γ-cyclodextrin or by GA treatment with NAD. GA treatment did not affect the electrophoretic mobility of glucose-6-phosphate isomerase and dehydrogenase. GA treatment diminished the GAPDH band detected by an antibody to phosphoserine, but did not affect the phosphoserine bands of glucose-6-phosphate isomerase and dehydrogenase. These results indicated that GA specifically induced dephosphorylation of GAPDH.

Keywords: Gymnemic acids; Glyceraldehyde-3-phosphate dehydrogenase; Dephosphorylation; γ-Cyclodextrin; Rabbit

1. Introduction

Gymnemic acids (GA) are the saponins with a triterpenoid structure contained in *Gymnema sylvestre* leaves. GA have various physiological effects. They suppress taste sensitivity to sweetness [1,2], inhibit intestinal glucose absorption [3], and lower plasma glucose and insulin levels [4]. There are, however, few reports about molecular interaction of GA with proteins.

We previously found that a 37-kDa protein from fungiform papillae of bovine tongue is bound to GA-coupling gel [5]. The N-terminal sequence of nine amino acids of the 37-kDa protein is identical to the N-terminal amino acid sequence of bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC: 1.2.1.12). GAPDH is an NAD-linked dehydrogenase that catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate. Although this abundant protein (10–20% of total cellular protein) is commonly known as a key enzyme in glycolysis, a number of intriguing intracellular roles have been reported for mammalian GAPDH (reviewed in [6]). Early experiments described the autophosphorylation of GAPDH [7]. Dephosphorylation of GAPDH was also observed by incubation with NAD, NADH and glyceraldehyde 3-phosphate. Other laboratories confirmed this finding [8,9]. GAPDH exhibits kinase activity to phosphorylate other proteins [9,10]. GAPDH is phosphorylated by a number of cellular protein kinases, including protein kinase C [11], epidermal-growth-factor-receptor kinase [12] and Ca^{2+}/calmodulin-dependent protein kinase II [13]. These cumulative results demonstrate that mammalian GAPDH displays a number of diverse activities unrelated to its glycolytic function. We now examined interaction between GA and GAPDH and effect of GA on the electrophoretic mobility and dephosphorylation of GAPDH.

2. Materials and methods

2.1. Materials

Rabbit muscle GAPDH, yeast glucose-6-phosphate isomerase and dehydrogenase, and monoclonal antibody to phosphoserine (clone PSR-45) were obtained from Sigma, and rabbit muscle L-lactate dehydrogenase was obtained from Roche. *G. sylvestre* extract was donated by Dai-Nippon Meiji Sugar Co. Ltd. (Tokyo, Japan). Gymnemagenin was obtained from Maruzen Pharmaceuticals Co. Ltd. (Onomichi, Japan). Diaion HP20 was obtained from Mitsubishi Chemical Co. Ltd. (Tokyo, Japan).

2.2. Purification of GA

GA are the saponins with a triterpenoid structure and more than 10 kinds of GA and related compounds were isolated [14]. Because of the difficulty and tediousness of the isolation of each GA, we used here the GA preparation obtained as described below without isolation of each GA.

Donated *G. sylvestre* extract was the ethanol/water extract of *G. sylvestre* leaves. Extract (3 g) was suspended in 10 ml methanol and the supernatant was treated with 2.5 M H_2SO_4 to carry out acid precipitation at pH 2.5. The precipitate was dissolved in 30 ml of 25% methanol and applied to Diaion HP20 column. The column was washed with 25% and then 50% methanol, and GA preparation was eluted with 85% methanol.

GA were analyzed as gymnemagenin, the aglycone of GA. The obtained GA was redissolved in 50% ethanol, and alkaline and acid hydrolyses were performed [15]. Gymnemagenin content was determined by HPLC on a C_{18} reversed-phase column with UV detection at 213 nm using a linear gradient of acetonitrile (25–50%) in 0.1% phosphoric acid.

*Corresponding author. Fax: +81 75 703 5674.
E-mail address: ishijima@kpu.ac.jp (S. Ishijima).

Abbreviations: GA, gymnemic acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SPR, surface plasmon resonance; RU, response unit; γ-CD, γ-cyclodextrin

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2.3. Determination of GAPDH activity

The activity of GAPDH was measured spectrophotometrically utilizing modifications of the methods of Steck and Kant [16]. Briefly, sample was added to a cuvette containing 30 mM sodium pyrophosphate (pH 8.4), 13 mM sodium arsenate, 4 mM cysteine and NAD. The reaction was initiated by the addition of glyceraldehyde 3-phosphate and absorbance at 340 nm was measured for 2 min at 25 °C.

2.4. Surface plasmon resonance (SPR) measurement

The Biacore biosensor system, Biacore 2000, was used to measure real-time interaction between GAPDH and GA. GAPDH was covalently immobilized onto a CM5 sensor chip surface by the amine coupling method, which resulted in capture of about 20 000 response unit (RU) of GAPDH. Various concentrations of GA in the absence or presence of γ-cyclodextrin (γ-CD) in HEPES-buffered saline (pH 7.4) were applied over the sensor chip at a rate of 20 μl/min for 3 min.

2.5. Binding reaction, electrophoresis and Western blotting

GAPDH (125 μg/ml) was incubated with GA at 20 °C for 10 min to overnight, and SDS–PAGE sample buffer was added. The proteins (0.3–1.25 μg) were separated by SDS–PAGE. Gels were subsequently either stained for protein or electroblotted. For Western blotting, PVDF membranes were treated with a 1:500 diluted monoclonal antibody to phosphoserine (clone PSR-45, ascites fluid), and the antigen–antibody complex was visualized with horseradish peroxidase-conjugated anti-mouse IgG followed by incubation with 50 mM Tris–HCl (pH 7.4) containing 0.02% 3,3′-diaminobenzidine and 0.05% H_2O_2.

3. Results

3.1. Inhibition of GAPDH activity by GA

We here examined interaction of GA with GAPDH, using commercially obtained rabbit muscle GAPDH. Rabbit muscle GAPDH was bound to GA-coupling gel, as was the 37-kDa protein of bovine tongue [5]. Then, the effects of GA on GAPDH activity were examined. GAPDH activity was inhibited by GA (Fig. 1). GA inhibition type and inhibition constants were determined by initial velocity measurements at a series of glyceraldehyde 3-phosphate concentrations (Fig. 1A) and NAD concentrations (Fig. 1B). The results were plotted by Dixon [17]. The common intercept on the abscissa indicated that the GA inhibition was non-competitive with respect to glyceraldehyde 3-phosphate and NAD. The K_i values obtained from the linear plots of Figs. 1A and B were 0.21 and 0.20 mM, respectively.

3.2. Binding measurement of GA to immobilized GAPDH

Binding of GA to immobilized GAPDH on the sensor chip was analyzed using SPR. An increase in the RU value was observed for the binding of GA alone, while that was significantly reduced in the presence of γ-CD, which can bind to GA by the inclusion complexation (Fig. 2). The dissociation constant of GA binding to GAPDH was estimated to be 8.9 mM. The binding of GA to other immobilized proteins, such as hen egg lysozyme and bovine serum albumin, used as negative control, resulted in only background changes reflected in the bulk refractive index. These results indicated interaction between rabbit muscle GAPDH and GA in a specific manner.

3.3. Effect of GA on electrophoretic mobility of GAPDH

Incubation of GAPDH with GA induced a significant smearing of the GAPDH protein band in SDS–PAGE. The GA treatment-induced smearing was observed in a time- and dose-dependent manner as shown in Figs. 3A and B. Treatment with 1.3 mM GA for 0 min did not induce the smearing of GAPDH band. The smearing of GAPDH protein band was not observed with gymnemagenin, the aglycone of GA (Fig. 3C). Prior incubation of GA with γ-CD abolished the

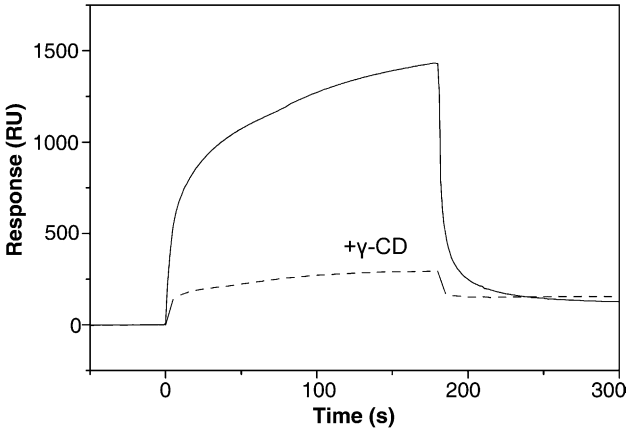

Fig. 2. Typical sensorgrams of interaction between GAPDH and GA. A 250 μM solution of GA was passed over the immobilized GAPDH (solid line) and in the presence of a 250 μM solution of γ-CD (dashed line) at 25 °C.

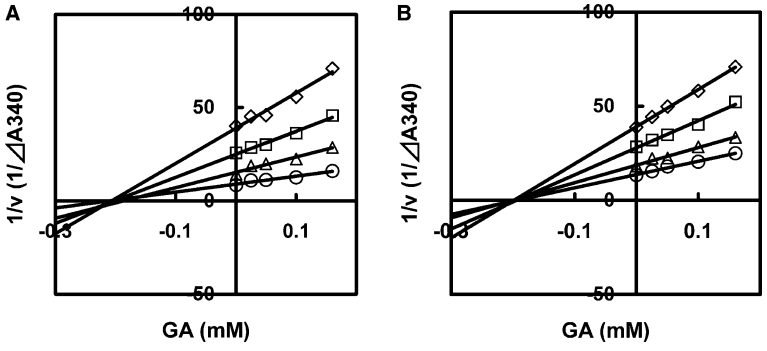

Fig. 1. Inhibition of GAPDH activity by GA. The GAPDH activity is plotted according to Dixon as a function of GA concentration (A) at glyceraldehyde 3-phosphate concentrations of 30 μM (◇), 45 μM (□), 75 μM (△), 190 μM (○) and 100 μM NAD, and (B) at NAD concentration of 12.5 μM (◇), 25 μM (□), 50 μM (△), 100 μM (○) and 250 μ M glyceraldehyde 3-phosphate. The initial velocity measurements were carried out in duplicate.

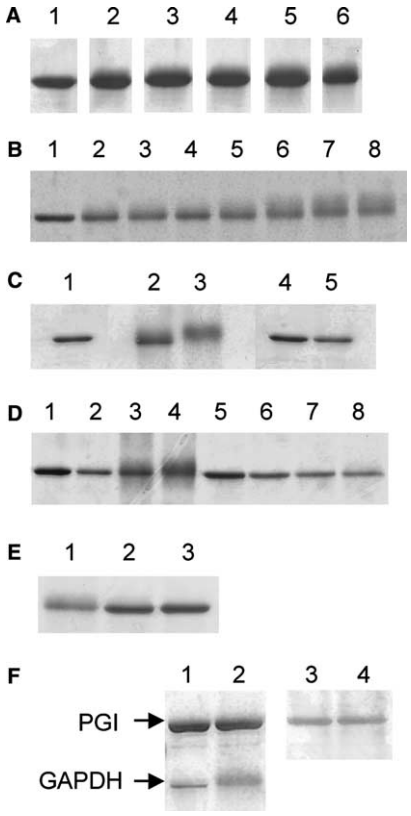

Fig. 3. The effect of GA on electrophoretic mobility of GAPDH. GAPDH (125 μg/ml) was incubated with GA, and 0.3–1.25 μg of protein was subjected to SDS–PAGE. (A) GAPDH was incubated with 1.3 mM GA for 0 min (lane 1), 10 min (lane 2), 1 h (lane 3), 3 h (lane 4), 18 h (lane 5), and 40 h (lane 6). (B) GAPDH was incubated with 0 mM (lane 1), 0.13 mM (lane 2), 0.25 mM (lane 3), 0.38 mM (lane 4), 0.5 mM (lane 5), 1.3 mM (lane 6), 2.5 mM (lane 7) and 3.8 mM (lane 8) GA for 8 h. (C) GAPDH was incubated without (lane 1) and with 0.15 mM (lane 2) and 1.5 mM (lane 3) GA, and 0.15 mM (lane 4) and 1.5 mM (lane 5) gymnemagenin. (D) GAPDH was incubated with 0.05 mM (lanes 1 and 5), 0.15 mM (lanes 2 and 6), 0.5 mM (lanes 3 and 7), 1.5 mM (lanes 4 and 8) GA. In lanes 5–8, GA was previously mixed with 7.5 mM γ-cyclodextrin. (E) GAPDH was incubated with 3 mM GA in the absence (lane 1) and presence of 0.15 mM (lane 2) and 1.5 mM (lane 3) NAD. (F) GAPDH and glucose-6-phosphate isomerase (PGI) (lanes 1 and 2) and glucose-6-phosphate dehydrogenase (lanes 3 and 4) were incubated without (lanes 1 and 3) and with (lanes 2 and 4) 1.5 mM GA for 8 h. The gel was stained with silver.

GA-induced effect on the GAPDH band in SDS–PAGE (Fig. 3D). In contrast, when GAPDH was incubated with GA, and then γ-CD was added, the addition of γ-CD did not diminish the GA-induced smearing of the GAPDH band (data not shown). GAPDH is an NAD-linked dehydrogenase. When GAPDH was incubated with GA in the presence of NAD, the GA-induced smearing of GAPDH was diminished (Fig. 3E). In contrast, glyceraldehyde 3-phosphate, a substrate of GAPDH, and glycerol had no significant effect on the GA-induced smearing of GAPDH (data not shown). Treatment of GA did not induce a smearing of protein bands of other enzymes in glycolysis, such as glucose-6-phosphate isomerase and dehydrogenase (Fig. 3F) and lactate dehydrogenase (data not shown). These results indicated that the GA-induced smearing of GAPDH band in SDS–PAGE was not due to general detergent effects of GA.

Fig. 4. The effect of GA on phosphorylation at serine residues of GAPDH (Western blotting). (A) GAPDH was incubated without (lane 1) and with 0.13 mM (lane 2), 0.25 mM (lane 3), 0.38 mM (lane 4) and 0.5 mM (lane 5) GA. (B) Glucose-6-phosphate isomerase was incubated without (lane 1) and with (lane 2) 3 mM GA.

3.4. Effect of GA on GAPDH phosphorylation

GAPDH is autophosphorylated [7] and phosphorylated by a number of protein kinases [11–13]. Phosphoserine band was detected for commercially obtained rabbit muscle GAPDH (Fig. 4A). When GAPDH was incubated with GA, the phosphoserine band was diminished in a GA dose-dependent manner (0–0.5 mM). No phosphoserine band of GAPDH was detected after treatment of higher concentrations (1.3–3.8 mM) of GA. Phosphoserine bands were also detected for commercially obtained glucose-6-phosphate isomerase (Fig. 4B) and dehydrogenase (data not shown). Incubation of glucose-6-phosphate isomerase and dehydrogenase with 3 mM GA had no effect, and the phosphoserine bands were detected after treatment with GA.

4. Discussion

GA have a specific and profound effect on the sweetness of sugars and other sweet substances [18], and change glucose utilization patterns and enzyme activities in experimentally induced diabetic animals [19]. Although GAPDH is considered to be a classical glycolytic enzyme, recent evidence demonstrated that mammalian GAPDH displays a number of diverse activities unrelated to its glycolytic function [6,20]. To our knowledge, this is the first report that directly observed interaction of GA with a protein, GAPDH.

GA induced inhibition of GAPDH activity (Fig. 1) and a smearing of the GAPDH protein band in SDS–PAGE (Fig. 3). Gymnemagenin did not affect the electrophoretic mobility of GAPDH. Prior incubation of GA with γ-CD diminished the smearing of GAPDH band. Incubation of glucose-6-phosphate isomerase and dehydrogenase and lactate dehydrogenase with GA did not induce a smearing of their protein bands. Together with the results of SPR measurement (Fig. 2), these results indicated that GA specifically bound to GAPDH, and induced a smearing of its protein band. Similar behavior in SDS–PAGE is observed with tau proteins bound to phospholipids [21]. Interaction of tau proteins with phospholipids results in a smearing of the protein band on SDS–PAGE.

GAPDH is an NAD-linked dehydrogenase. The presence of NAD diminished the GA-induced smearing of GAPDH band. The results indicated the involvement of NAD-binding site of GAPDH in GA binding. Dixon plot analysis of GAPDH activity indicated that GA acted as a non-competitive type inhibitor for NAD (Fig. 1B). GA did not induce a smearing of NAD-linked lactate dehydrogenase and NADPH-linked glucose-6-phosphate dehydrogenase. These results suggested that GA did not directly bind to NAD-binding site of

GAPDH, but that NAD binding to GAPDH induced conformational change of the enzyme, and this diminished GA binding to GAPDH.

Phosphorylation of protein sometimes induces shift in the phosphorylated protein band to lower mobility in SDS–PAGE. In the present case, however, disappearance of the GAPDH band detected by an antibody to phosphoserine was accompanied by the smearing of GAPDH band in SDS–PAGE. Occurrence of other phosphorylation of GAPDH was impossible because GAPDH was incubated with GA in the absence of MgATP and any protein kinase added. The results presented in Fig. 4 together with other experiments suggested that incubation with GA induced dephosphorylation of GAPDH, and that the smearing of electrophoretic band of GAPDH is due to the dephosphorylation of the enzyme. GAPDH is reported to be dephosphorylated by incubation with NAD, NADH and glyceraldehyde 3-phosphate without any phosphatase added [7]. Although the possible existence of contaminant phosphatase in the GA preparation has not been excluded completely, the present data showing that dephosphorylation of glucose-6-phosphate isomerase and dehydrogenase was not observed with incubation with GA make this unlikely. GA did not affect the activity of calf intestine alkaline phosphatase and no phosphatase activity was detected in the GA preparation, assayed with *p*-nitrophenylphosphate (data not shown). The results further supported that GA-induced dephosphorylation of GAPDH was not due to activation of contaminated phosphatase.

We show here that GA bind to GAPDH. Because of the multiple functions of GAPDH, the binding of GA to GAPDH may be related to some of the physiological effects of GA.

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