J. Biol. Macromol., 22(2), 37-46 (2022)

Article

Binding of gymnemic acid II to mammalian glycerol-3-phosphate dehydrogenase

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Gymnemic acid II (GA II) is a saponin of triterpene glycoside isolated from the plant *Gymnema sylvestre*. It suppresses taste sensitivity to sweetness and inhibits intestinal glucose absorption. We found GA II inhibited glycerol-3-phosphate dehydrogenase (G3PDH) activity noncompetitively with a substrate, dihydroxyacetone phosphate, and induced band smearing of G3PDH in SDS-PAGE in a time-dependent manner; band smearing was observed but decreased when G3PDH was incubated with GA II for 1.5-2 h compared with smearing after incubation with GA II for 4 h. NADH was more effective than NAD for decreasing the smearing of the G3PDH band, suggesting that GA II distinguishes between NADH- and NAD-bound forms of G3PDH. The smearing of the G3PDH band was diminished by prior incubation of GA II with γ-cyclodextrin, but the suppressing effect of γ-cyclodextrin was decreased when G3PDH was incubated with GA II for 0.5 h before adding γ-cyclodextrin. These results suggest that the induction of the band smearing of G3PDH by GA II proceeded in two steps. GA II bound G3PDH and inhibited G3PDH activity but did not induce band smearing in SDS-PAGE during the first stage. Then, GA II induced a change in G3PDH that caused band smearing during the second stage, and the suppressing effect of γ-cyclodextrin on band smearing was decreased at this stage. Analysis using in-gel digestion coupled with mass spectrometry suggested that proteolysis at the amino acid A238-F251 region of G3PDH was affected by treatment with GA II.

Received July 1, 2022; Accepted August 3, 2022

Keywords: gymnemic acid II, glycerol-3-phosphate dehydrogenase, γ-cyclodextrin, in-gel digestion coupled with mass spectrometry

Introduction

Gymnemic acid (GA) is a saponin of triterpene glycoside isolated from the plant *Gymnema sylvestre*. *Gymnema sylvestre* is used to manage diabetes mellitus in several parts of India. *Gymnema sylvestre* extract is known to have antimicrobial and antihypercholesteolemic effects, hepatoprotective properties, and especially effects on obesity and diabetes mellitus [1]. These pharmacological activities

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are mainly due to triterpenoid saponins present in the leaf extracts, and GA is the major active compound [2]. GA has various physiological effects. GA suppresses taste sensitivity to sweetness [3,4], inhibits intestinal glucose absorption [5], and elicits antihyperglycemic effects through its ability to decrease blood glucose levels and enhance insulin sensitivity in patients with type 2 diabetes [6,7]. GA binds to several proteins, including glucose transporter [8], taste receptors T1R2/T1R3 [9], and liver X-receptor which regulates lipid metabolism in the liver [10].

GA is not a pure entity but is comprised of several types of homologs [11]. The aglycone part of GA is gymnemagenin, which is linked with glycone glucuronic acid and diversified esters (tigloyl, methylbutyroyl, etc.). Eighteen different types of gymnemic acids (GAs) were found in the leaves of *G. sylvestre* [2]. Aqueous extracts of the leaves of *G. sylvestre* were used for the isolation of GAs I-VI, the saponin fraction, which yields GAs XV-XVIII, and gymnemagenin, fractioned as GAs VIII-XII. *G. sylvestre* extracts contain triterpene saponins belonging to the oleanane and dammarane classes [1]. GAs, a complex mixture of oleanane saponins, are generally considered the active constituents, and the quality of the extracts and their formulations is assessed by the content of GAs [12]. Although several studies have reported the pharmacological effects of crude GA extracts, few studies on the effects of a single molecule have been reported. The sweet-suppressing effect of GAs differs among derivatives (GA I, GA II>GA III>GA IV) [13]. GAs I, II, XI, XII, and XV- XVIII are, among natural sweetness inhibitors, the most potent antisweet compounds known [14]. Because of the difficulty and tediousness of the isolation of each GA, some studies have used a GA preparation without isolation of each GA.

We previously used a GA preparation without isolation of each GA and found an interaction of GA with mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC: 1.2.1.12) and glycerol-3-phosphate dehydrogenase (G3PDH, EC: 1.1.1.8) [15-17]. GA inhibits GAPDH and G3PDH activity and induces a smearing of their bands in SDS-PAGE. GA does not induce a smearing of other enzymes involved in glycolysis, such as phosphoglucose isomerase, 6-phosphofructokinase, triosephosphate isomerase, 3-phosphoglycerate kinase, pyruvate kinase, and lactate dehydrogenase. GAPDH is a key enzyme in glycolysis, and G3PDH catalyzes the reversible biological reduction of glycerone phosphate using NADH as a reducing equivalent to form glycerol 3-phosphate, and it is involved in the hepatic metabolism of glycerol. These studies suggest that GA may have some pharmacological activities, including antidiabetic activity and lipid-lowering effects, via their interactions with GAPDH and G3PDH.

Herein, we examined the effects of purified gymnemic acid II (GA II) on G3PDH. Gymnemic acids I-IV were the components considered to be of prime importance and were first reported from the leaves of *G. sylvestre* [18]. GA II has been found to completely suppress the sweet sensation [19] and exhibit inhibitory activity against glucose uptake into rat small intestinal fragments [5].

Materials and methods

Materials

Rabbit muscle G3PDH and hexokinase were obtained from Roche. *G. sylvestre* extract was donated by Dai-Nippon Meiji Sugar Co., Ltd. (Tokyo, Japan). Gymnemagenin was obtained from Maruzen Pharmaceutical Co., Ltd. (Onomichi, Japan). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Purification

G. sylvestre extract is an ethanol/water extract of *G. sylvestre* leaves. Crude GAs

containing several acids in the mixture were obtained from the *G. sylvestre* extract as described previously [16]. GA II was purified by HPLC on a reversed-phase COSMOSIL $5C_{18}$-AR-II column eluting in isocratic mode with 0.1 M ammonium acetate/methanol (35:65) at a flow rate of 1.0 mL/min [20].

GA II was analyzed as gymnemagenin, the aglycone of GA II. The obtained GA II was dissolved in 50% methanol, and alkaline and acid hydrolyses were performed [16]. The gymnemagenin content was determined by HPLC on a C18 reversed-phase column.

Determination of G3PDH activity

The activity of G3PDH was measured spectrophotometrically as previously described [17] with modifications. Briefly, 0.1 mM NADH and the indicated concentrations of dihydroxyacetone phosphate (DHAP) and GA II were incubated in 0.1 M Tris-HCl buffer (pH 7.4) at 25°C for 5 min. The reaction was initiated by the addition of 8.1 nM G3PDH, and absorbance at 340 nm was measured for 2 min at 25 °C.

Binding reaction and SDS–PAGE

G3PDH and other proteins were incubated with GA II in 0.1 M Tris-HCl buffer (pH 7.4) at 20°C for 8 h, unless otherwise specified, and then SDS–PAGE sample buffer was added. The proteins were heated and separated by SDS–PAGE. The gel was stained with silver.

In-gel digestion and analysis by MALDI-TOF MS

For analysis by MALDI-TOF MS, 0.89 μM G3PDH was incubated with and without 0.8 mM GA II at 20°C for 8 h and was subjected to SDS–PAGE. The gel was stained with silver. In-gel digestions were performed according to a standard protocol with reduction and alkylation steps [21]. Proteins were digested with lysyl endopeptidase and chymotrypsin in 50 mM NH_4HCO_3 (pH 7.8) at 37°C for 6 h. The supernatants were transferred to tubes, and the residual proteolytic peptides were extracted twice from the gels using 50% CH_3CN and 5% TFA. All extractions were combined and vacuum dried. The resulting peptides were analyzed by matrix-associated laser desorption/ionization mass spectrometry (MALDI-MS). MS analysis was performed using an AXIMA Performance MALDI-TOF mass spectrometer (Shimadzu Co., Kyoto, Japan), and alpha-cyano-4-hydroxycinnamic acid solution was used as the MALDI matrix.

Results

Binding of GAs to G3PDH

We previously reported the binding of GAs to G3PDH [17]. We fractionated crude GAs by HPLC on a C18 reversed-phase column (Fig. 1A) and analyzed the binding of fractionated GA to G3PDH by its ability to induce a smearing of its band on SDS–PAGE (Fig. 1B). Fractions eluting at 10-13, 14-17, and 40-46 min induced a smearing of the G3PDH band. GA II was eluted at ~15 min [20]. GA II was purified by repeated HPLC on a C18 reversed-phase column. The purity of GA II was verified by HPLC and MS analyses. Based on the MS data, the purified GA II was identified.

Inhibition of G3PDH activity by GA II

G3PDH activity was inhibited by GA II (Fig. 2A). A linear plot of Dixon indicated that GA II inhibition was noncompetitive with respect to DHAP, and the *K*i value obtained was 0.3 mM (Fig. 2B).

Binding of GA II to G3PDH

Incubation of G3PDH with GA II induced smearing of the G3PDH band on SDS–PAGE (Fig. 3). GA II treatment did not induce smearing of the bovine serum albumin and hexokinase bands. Smearing of the G3PDH band was observed in a time-dependent manner (Fig. 4A); band smearing was observed but it decreased

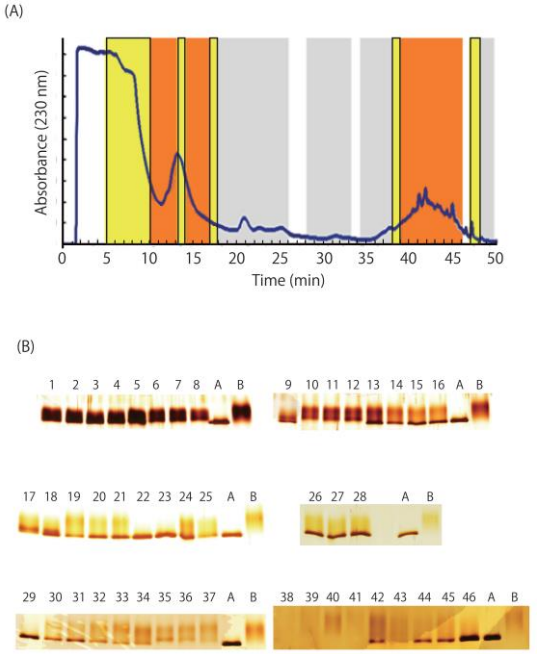

Fig. 1. HPLC chromatogram of crude GAs and HPLC fractions inducing G3PDH band smearing. (A) Crude GAs obtained from the *G. sylvestre* extract were fractionated on HPLC; column, COSMOSIL 5C_{18}-AR-II; eluents: A=0.1 M ammonium acetate-methanol (35:65, v/v), B=0.1 M ammonium acetate-methanol (10:90, v/v). Gradient elution was begun after 30 min, from 100% A to 100% B in 10 min. The flow rate was 1.0 ml/min. (B) Individual 1ml fractions eluted from 5-6 min (Lane 1) to 50-51 min (Lane 46) were collected, vacuum dried, and dissolved in 0.1 M Tris-HCl buffer (pH 7.4). G3PDH (1.65 μM) was incubated with an aliquot of each fraction and without (Lane A) and with (Lane B) 2 mM crude GAs at 20°C for 8 h and subjected to SDS–PAGE. Fractions inducing marked (orange), moderate (boxed yellow), mild (gray), and no (white) smearing of the G3PDH band are shown in (A). When measuring the distance of the upward movement of the G3PDH smearing band and comparing it with the distance of the movement induced by 2 mM crude GAs (Lane B), the smearing was divided into three categories: marked (>75%), moderate (50-75%), and mild (<50%).

when G3PDH was incubated with 0.5 mM GA II for 1.5-2 h compared with smearing after incubation with GA II for 4 h. Smearing of the G3PDH band was observed at concentrations greater than 0.15 mM GA II (Fig. 4B). The

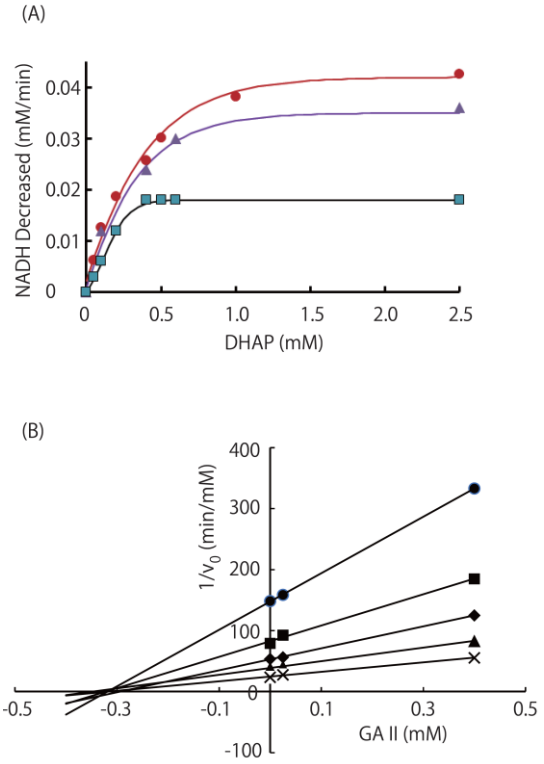

Fig. 2. Inhibition of G3PDH activity by GA II. (A) The initial velocity measurements were carried out at GA II concentrations of 0 (●), 25 (▲), and 400 (■) μM. All experiments were performed in triplicate. SEs were <3% and are covered by the symbols. (B) The G3PDH activity is plotted according to Dixon as a function of GA II concentration at dihydroxyacetone phosphate (DHAP) concentrations of 0.05 (●), 0.1 (■), 0.2 (◆), 0.4 (▲), and 2.5 (×) mM.

smearing of the G3PDH band was not observed when G3PDH was denatured in SDS-containing sample buffer and then incubated with GA II (Fig. 4C).

Effect of NADH, NAD, and dihydroxyacetone phosphate on GA II-induced smearing of the G3PDH band

G3PDH is an NAD-linked dehydrogenase and it catalyzes the reaction reversibly. The reported apparent *K*m values of rabbit muscle G3PDH for NADH and NAD are both 0.01 mM, and that for DHAP is 0.15 mM [22]. We examined the effect of NADH, NAD, and

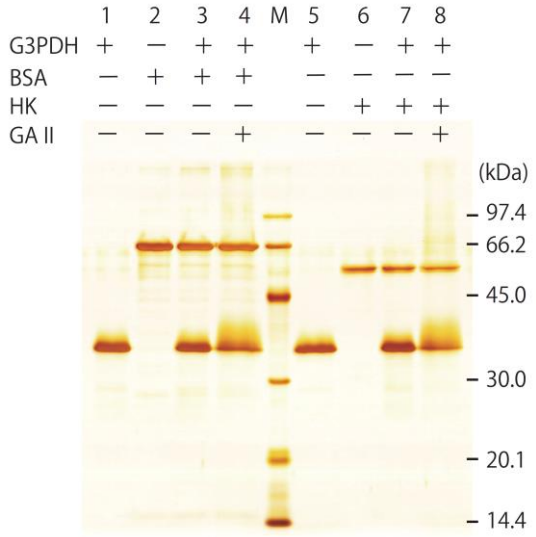

Fig. 3. The effect of GA II on the electrophoretic mobility of G3PDH, bovine serum albumin (BSA), and hexokinase (HK). G3PDH (0.6 μM), BSA (0.6 μM), and HK (0.31 μM) were incubated without (Lanes 1-3 and 5-7) and with (Lanes 4 and 8) 0.54 mM GA II for 8 h and then subjected to SDS–PAGE. The gel was stained with silver. Lanes 1 and 5: G3PDH, Lanes 2: BSA, Lanes 3 and 4: G3PDH and BSA, Lane 6: HK, Lanes 7 and 8: G3PDH and HK, Lane M: protein markers.

DHAP on GA II-induced smearing of the G3PDH band. NADH and NAD had different effects on the GA II-induced smearing of the G3PDH band (Fig. 5A); NADH was more effective than NAD for decreasing the smearing of the G3PDH band. NADH diminished the smearing of the G3PDH band at a concentration of 0.5 mM. NAD had little effect on smearing at a concentration of 0.5 mM, and a concentration-dependent decrease in smearing was observed from 3.5 mM to 30 mM NAD. DHAP, a cosubstrate with NADH, had little effect on the GA II-induced smearing of the G3PDH band at a concentration of 5 mM (Fig. 5B).

Effect of γ-cyclodextrin on GA II-induced smearing of the G3PDH band

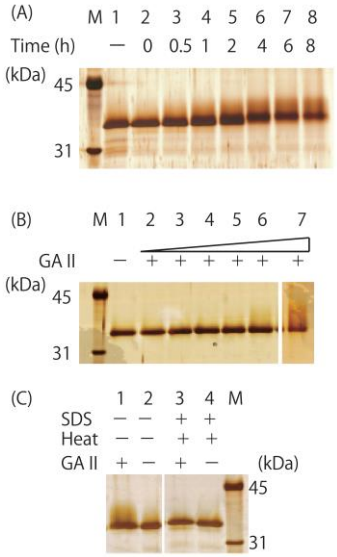

Fig. 4. The effect of GA II on the electrophoretic mobility of G3PDH. G3PDH (0.89 μM) was incubated with GA II and subjected to SDS–PAGE. The gel was stained with silver. (A) G3PDH was incubated without (Lane 1) and with 0.5 mM GA II for 0 h (Lane 2), 0.5 h (Lane 3), 1 h (Lane 4), 2 h (Lane 5), 4 h (Lane 6), 6 h (Lane 7), and 8 h (Lane 8). (B) G3PDH was incubated without (Lane 1) and with 8 μM (Lane 2), 13 μM (Lane 3), 21 μM (Lane 4), 42 μM (Lane 5), 0.15 mM (Lane 6), and 0.42 mM (Lane 7) GA II for 8 h. (C) G3PDH was heated at 95 °C for 5 min with SDS-containing sample buffer (Lanes 3 and 4). Heat-treated (Lanes 3 and 4) and untreated (Lanes 1 and 2) G3PDH was incubated with (Lanes 1 and 3) and without (Lanes 2 and 4) 0.7 mM GA II for 8 h. Lane M: protein markers.

The binding of GA to GAPDH was significantly reduced in the presence of γ-cyclodextrin [16]. The smearing of the G3PDH band was diminished when G3PDH was incubated with GA II that had been pre-treated with γ-cyclodextrin (Fig. 6A). This effect of γ-cyclodextrin was not observed when G3PDH was incubated with GA II first, followed by the addition of γ-cyclodextrin. The suppressive effect of γ-cyclodextrin on the GA II-induced smearing of the G3PDH band was observed after GA II was incubated with γ-cyclodextrin for 0-

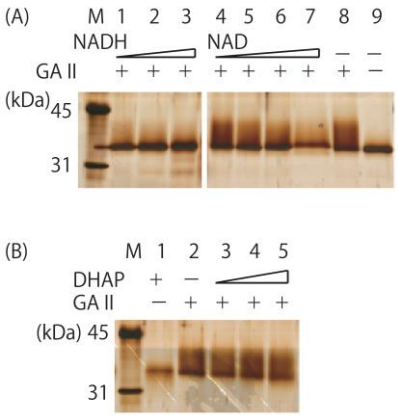

Fig. 5. The effect of NADH, NAD and dihydroxyacetone phosphate (DHAP) on GA II-induced smearing of G3PDH. G3PDH (0.5 μM) was incubated with 0.5 mM GA II for 8 h and subjected to SDS–PAGE. The gel was stained with silver. (A) G3PDH was incubated with (Lanes 1-8) and without (Lane 9) GA II in the absence (Lane 8) and presence of 0.5 (Lane 1), 3.5 (Lane 2), and 5.0 (Lane 3) mM NADH and 0.5 (Lane 4), 3.5 (Lane 5), 5.0 (Lane 6), and 30 (Lane 7) mM NAD. (B) G3PDH was incubated without (Lane 1) and with (Lanes 2-5) GA II in the presence of 0.5 (Lane 3), 2.5 (Lane 4), and 5.0 (Lanes 1 and 5) mM dihydroxyacetone phosphate. Lane M: protein markers.

0.5 h (Fig. 6B). The suppressing effect was observed when γ-cyclodextrin was added immediately after mixing GA II with G3PDH, but this effect was decreased when γ-cyclodextrin was added after incubation of GA II with G3PDH for 0.5 h (Fig. 6C). These results indicated that the interaction of G3PDH and GA II was inhibited by forming an inclusion complex between GA II and γ-cyclodextrin, but the GA II-induced change in G3PDH was stable even after the addition of γ-cyclodextrin.

GA II-binding sites on G3PDH

We previously observed that the proteins eluted from the upper and lower parts of the smearing G3PDH band again migrated to the positions corresponding to the upper and lower parts of the smearing G3PDH band in SDS–PAGE, respectively [17]. These results

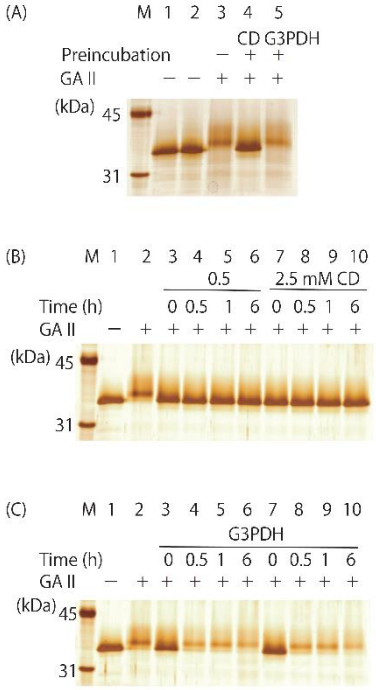

Fig. 6. The effect of γ-cyclodextrin on GA II-induced smearing of G3PDH. G3PDH (0.89 μM) was incubated with GA II and γ-cyclodextrin (CD), and subjected to SDS–PAGE. The gel was stained with silver. (A) G3PDH was incubated for 8 h without (Lane 2) and with 0.36 mM γ-cyclodextrin (Lane 1), 0.18 mM GA II (Lane 3), and 0.18 mM GA II that had been incubated with 0.36 mM γ-cyclodextrin for 8 h (Lane 4). Lane 5: G3PDH was incubated with 0.18 mM GA II for 8 h and then incubated with 0.36 mM γ-cyclodextrin for 8 h, Lane M: protein markers. (B) GA II (0.5 mM) was incubated without (Lane 2) and with 0.5 (Lanes 3-6) and 2.5 (Lanes 7-10) mM γ-cyclodextrin for 0 (Lanes 3 and 7), 0.5 (Lanes 4 and 8), 1 (Lanes 5 and 9), and 6 (Lanes 6 and 10) h, and then G3PDH was added and incubated for 8 h. (C) G3PDH was incubated without (Lane 1) and with GA II for 0 (Lanes 2, 3 and 7), 0.5 (Lanes 4 and 8), 1 (Lanes 5 and 9), and 6 (Lanes 6 and 10) h and then incubated without (Lane 2) and with 0.5 (Lanes 3-6) and 2.5 (Lanes 7-10) mM γ-cyclodextrin for 8 h. Lane M: protein markers.

suggested that G3PDH migrated as a GA-bound form in electrophoresis and that GA-bound G3PDH showed smearing of its band. G3PDH was treated with and without GA II and was

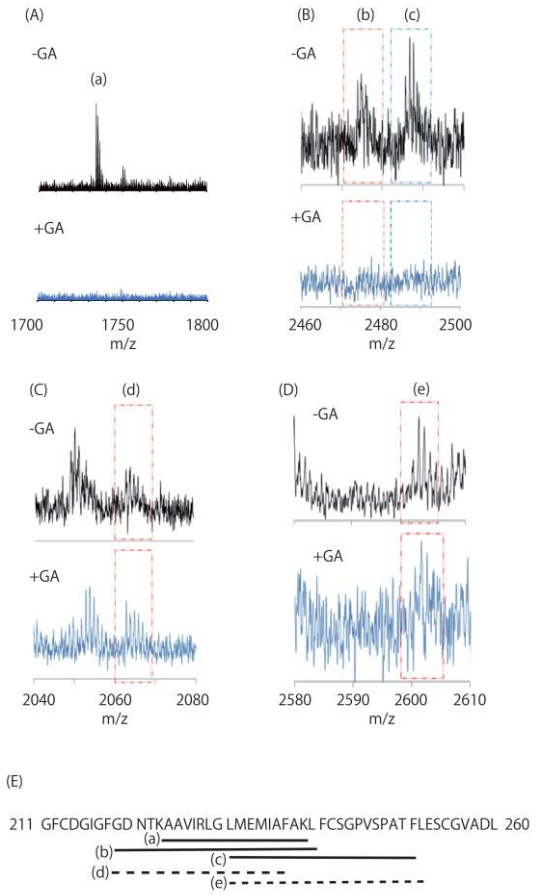

Fig. 7. Comparison of MS spectra of lysyl endopeptidase (A) and chymotrypsin (B-D)-digested peptides from G3PDH (-GA) and GA II-treated G3PDH (+GA). The relative ion abundance of peptides A224-K239 (a) in A and G219-L240 (b) and M232-F251 (c) in B from GA II-treated G3PDH were decreased relative to that of peptides from GA II-untreated G3PDH. The relative ion abundance of peptides G219-F237 (d) in C and M232-L252 (e) in D were the same as untreated and GA II-treated G3PDH. The amino acid sequence from G211 to L260 of G3PDH is shown in E. Peptides (a)-(c) are indicated by solid lines, and peptides (d) and (e) are indicated by dashed lines.

subjected to SDS–PAGE. The proteins in the gels of untreated G3PDH and in the upper parts of the smearing G3PDH band were digested with lysyl endopeptidase and chymotrypsin. The resulting peptides were subjected to matrix-associated laser desorption/ionization mass spectrometry (MALDI-MS). The peptide maps of G3PDH and GA II-treated G3PDH were similar, which is expected because of the small interaction site in the G3PDH-GA II complex. However, careful inspection of the MALDI mass spectra revealed significant differences in proteolysis at several amino acids (Fig. 7). In the proteolysis of GA II-treated G3PDH relative to untreated G3PDH, there was a reduction in the relative ion abundance of lysyl endopeptidase-digested peptide at *m*/*z* 1733 [amino acids (a.a.) A224-K239] (Figs. 7A and 7E(a)), indicating that digestion at K223-A224 and/or K239-L240 was protected by the binding of GA II. There was also a reduction in the relative ion abundance of chymotrypsin-digested peptides at *m*/*z* 2474 (a.a. G219-L240) and *m*/*z* 2486 (a.a. M232-F251) (Figs. 7B and 7E(b) and (c)), indicating that digestion at F218-G219 and/or L240-F241 and digestion at L231-M232 and/or F251-L252 were protected by the binding of GA II. However, there was no difference in the relative ion abundance of chymotrypsin-digested peptides at *m*/*z* 2065 (a.a. G219-F237) (Figs. 7C and 7E(d)) and *m*/*z* 2603 (a.a. M232-L252) (Figs. 7D and 7E(e)), indicating that digestion at F218-G219 and F237-A238 and digestion at L231-M232 and L252-E253 were not protected by the binding of GA II. These results suggested that proteolysis at amino acids A238-F251 was affected by the binding of GA II.

Discussion

GA comprises several types of glucuronides of gymnemagenin, which are differently acylated. We fractionated crude GAs by HPLC on a C18 reversed-phase column. Fractions eluting at 10-13, 14-17, and 40-46 min showed binding to G3PDH (Fig. 1). Fractions eluting at 40-46 min contained aglycones without a sugar moiety in the molecules [20]. These results suggested that the glucuronate

moiety was not important for the binding of GA to G3PDH and are consistent with our previous results that a smearing of the G3PDH band was observed with β-glucuronidase-treated GA [17]. We selected and purified GA II from the fraction eluting at 14-15 min for the analyses of binding to G3PDH.

G3PDH activity was inhibited by GA II (Fig. 2). An amphiphilic terpenoid fraction from *G. sylvestre* induces flip-flop of membrane lipids [23], and some triterpene compounds inhibit intracellular enzymes such as aldose reductase, protein tyrosine phosphatase 1B, glycogen phosphorylase and 11β-hydroxysteroid dehydrogenase type 1 [24] as well as G3PDH.

GA II treatment induced smearing of the G3PDH band in a time-dependent manner but did not induce smearing of the bovine serum albumin and hexokinase bands (Figs. 3 and 4). These results suggested that the band smearing of G3PDH was not due to the effects of GA II on the electrophoresis of proteins but was instead due to the specific binding of GA II to G3PDH. GA II-induced smearing of the G3PDH band was not observed when G3PDH was denatured by SDS and it was diminished by NAD and NADH. These results indicate that the band smearing of G3PDH was due to the interaction of GA II with native G3PDH. NADH was more effective than NAD for the decrease in the smearing of the G3PDH band (Fig. 5), while the apparent *K*m values of rabbit muscle G3PDH for NADH and NAD are reported to both be 0.01 mM. GA II may distinguish between NADH- and NAD-bound forms of G3PDH.

GAs suppress taste responses to various sweet substances in humans. This sweet-suppressing effect of GAs is diminished by rinsing the tongue with γ-cyclodextrin. GAs directly interact with taste receptors T1R2/T1R3, and this interaction is inhibited by forming an inclusion complex between GAs and γ-cyclodextrin [9]. Similarly, the binding of GA II to G3PDH was inhibited by forming an inclusion complex between GA II and γ-cyclodextrin. (Fig. 6). However, it should be noted that the suppressing effect of γ-cyclodextrin on the band smearing was decreased when G3PDH was incubated with GA II for 0.5 h, and only then γ-cyclodextrin was added. G3PDH activity was noncompetitively inhibited by GA II, and this inhibition was observed shortly after mixing GA II with G3PDH (Fig. 2). Band smearing was observed but decreased when G3PDH was incubated with GA II for 1.5-2 h compared with smearing after incubation with GA II for 4 h (Fig. 4). These results suggested that the induction of the band smearing of G3PDH by GA II proceeded in two steps. GA II bound G3PDH and inhibited G3PDH activity but did not induce band smearing in SDS–PAGE at the first stage. Then, GA II induced a change in G3PDH that showed band smearing at the second stage, and the suppressing effect of γ-cyclodextrin on band smearing was decreased at this stage. Thus, the effect of GA II on G3PDH was observed in two steps.

The peptide maps of GA II-treated and untreated G3PDH were compared, and the results suggested that proteolysis at the amino acid A238-F251 region was affected by GA II treatment (Fig. 7). Since monomeric and denatured G3PDH was digested in the SDS gel, the protection of proteolysis by GA II treatment was not due to the conformational change of G3PDH induced by the GA II-G3PDH complex formation but may be due to the binding of GA II to that region, and these results indicated that the binding between G3PDH and GA II was stable during SDS–PAGE. By analogy with the crystal structure of human G3PDH 1 (PDB code 1x0v), (1) this region is on the interface of the two subunits, (2) this region of each subunit faces each other, (3) the loop portion of this region protrudes outside the molecule, and (4) this region does not interact directly with the substrate. These results are consistent with the finding that GA II inhibited G3PDH activity and that the inhibition was noncompetitive with

respect to DHAP (Fig. 2).

Acknowledgments

The authors thank Marina Suzuki for HPLC analysis of the gymnemic acids and the Kyoto Integrated Science and Technology Bio-Analysis Center (KIST-BIC) for its assistance with the MALDI-MS analysis.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

[1] Khan F, Sarker MMR, Ming LC, Mohamed IN, Zhao C, Sheikh BY, Tsong HF and Rashid MA (2019) Comprehensive review on phytochemicals, pharmacological and clinical potentials of *Gymnema sylvestre*. Front. Pharmacol. 10: 1223. https://doi.org/10.3389/fphar.2019.01223

[2] Ye W, Liu X, Zhang Q, Che CT, Zhao S (2001) Antisweet saponins from *Gymnema sylvestre*. J Nat Prod. 64:232-235. https://doi.org/10.1021/np0004451

[3] Warren RM, Pfaffmann C (1959) Suppression of sweet sensitivity by potassium gymnemate. J Appl Physiol 14: 40-42. https://doi.org/10.1152/jappl.1959.14.1.40

[4] Kurihara Y (1969) Antisweet activity of gymnemic acid A1 and its derivatives. Life Sci 8: 537-543. https://doi.org/10.1016/0024-3205(69)90449-4

[5] Yoshikawa M, Murakami T, Matsuda H (1997) Medicinal foodstuffs. X. Structures of new triterpene glycosides, gymnemosides-c, -d, -e, and -f, from the leaves of *Gymnema sylvestre* R. Br.: influence of gymnema glycosides on glucose uptake in rat small intestinal fragments. Chem Pharm Bull 45: 2034-2038. https://doi.org/10.1248/cpb.45.2034

[6] Hirata S, Abe T, Imoto T (1992) Effects of crude gymnemic acid on the oral glucose tolerance test in human being. J Yonago Med Assoc 43: 392-396.

[7] Sugihara Y, Nojima H, Matsuda H, Murakami T, Yoshikawa M, Kimura I (2000) Antihyperglycemic effects of gymnemic acid IV, a compound derived from *Gymnema sylvestre* leaves in streptozotocin-diabetic mice. J Asian Nat Prod Res 2: 321-327. https://doi.org/10.1080/10286020008041372

[8] Wang Y, Dawid C, Kottra G, Daniel H, Hofmann T (2014) Gymnemic acids inhibit sodium-dependent glucose transporter 1. J Agric Food Chem 62:5925-5931. https://doi.org/10.1021/jf501766u

[9] Sanematsu K, Kusakabe Y, Shigemur N, Hirokawa T, Nakamura S, Imoto T, Ninomiya Y (2014) Molecular mechanisms for sweet-suppressing effect of gymnemic acids. J Biol Chem 289: 25711-25720. https://doi.org/10.1074/jbc.M114.560409

[10] Renga B, Festa C, De Marino S, Di Micco S, D'Auria MV, Bifulco G, Fiorucci S, Zampella A (2015) Molecular decodification of gymnemic acids from *Gymnema sylvestre*. Discovery of a new class of liver X receptor antagonists. Steroids 96: 121-131. https://doi.org/10.1016/j.steroids.2015.01.024

[11] Suttisri R, Lee I-S, Kinghorn, AD (1995) Plant-derived triterpenoid sweetness inhibitors. J Ethnopharmacol 47: 9-26. https://doi.org/10.1016/0378-8741(95)01248-c

[12] Patil AN, Nirmal SA, Chavan AK (2013) Development and validation of HPTLC method for estimation of gymnemic acid in microencapsulated antidiabetic polyherbal formulations. Acta Chromatogr 25: 601-625. https://doi.org/10.1556/AChrom.25.2013.4.1

[13] Kurihara, Y (1992) Characteristics of antisweet substances, sweet proteins, and

sweetness-inducing proteins. Crit. Rev. Food Sci. Nutr 32: 231-252. https://doi.org/10.1080/10408399209527598

[14] Kim N-C, Kinghorn, AD (2002) Sweet-tasting and sweetness modifying constituents of plants. In Studies in Natural Products Chemistry; Attaur, R, Ed.; Elsevier, Vol. 27 (Part H), pp 3−57. https://doi.org/10.1016/S1572-5995(02)80033-3

[15] Izutani Y, Hirai H, Takei T, Imoto T, Ishijima S, Ohnishi M (2002) Studies on the proteins from fungiform papillae of bovine tongue: Glyceraldehyde-3-phosphate dehydrogenase is bound to gymnemic acid-coupling gel. J Biol Macromol 2: 60-63

[16] Izutani Y, Murai T, Imoto T, Ohnishi M, Oda M, Ishijima S (2005) Gymnemic acids inhibit rabbit glyceraldehyde-3-phosphate dehydrogenase and induce a smearing of its electrophoretic band and dephosphorylation. FEBS Lett 579:4333-4336. https://doi.org/10.1016/j.febslet.2005.06.070

[17] Ishijima S, Takashima T, Ikemura T, Izutani Y (2008) Gymnemic acid interacts with mammalian glycerol-3-phosphate dehydrogenase. Mol Cell Biochem 310:203-208. https://doi.org/10.1007/s11010-007-9681-5

[18] Porchezhian E, Dobriyal RM (2003) An overview on the advances of *Gymnema sylvestre*: chemistry, pharmacology and patents. Pharmazie 58: 5-12

[19] Yoshikawa K, Amimoto K, Arihara S, Matsuura K (1989) Structure studies of new antisweet constituents from *Gymnema sylvestre*. Tetrahedron Lett 30: 1103-1106. https://doi.org/10.1016/S0040-4039(01)80371-3

[20] Imoto T, Yamamoto FM, Miyasaka A, Hatano H (1991) High-performance liquid chromatography-atmospheric pressure ionization mass spectrometry of gymnemic acids. J Chromatogr 557: 383-389. https://doi.org/10.1016/S0021-9673(01)87146-6

[21] Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 1: 2856–2860. https://doi.org/10.1038/nprot.2006.468

[22] Warkentin DL, Fondy TP (1973) Isolation and characterization of cytoplasmic L-glycerol-3-phosphate dehydrogenase from rabbit-renal-adipose tissue and its comparison with the skeletal-muscle enzyme. Eur J Biochem 36: 97-109. https://doi.org/10.1111/j.1432-1033.1973.tb02889.x

[23] Behuria HG, Sahu SK. (2020) An anti-microbial terpenoid fraction from *Gymnema sylvestre* induces flip-flop of fluorescent-phospholipid analogs in model membrane. Appl Biochem Biotechnol 192: 1331-1345. doi: 10.1007/s12010-020-03399-3

[24] Nazaruk J, Borzym-Kluczyk M. (2015) The role of triterpenes in the management of diabetes mellitus and its complications. Phytochem Rev 14: 675-690. doi: 10.1007/s11101-014-9369-x

Communicated by Emiko Okuda-Ashitaka