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## An inhibitory substance of glyceraldehyde 3-phosphate dehydrogenase in urine of diabetic patients

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# An inhibitory substance of glyceraldehyde 3-phosphate dehydrogenase in urine of diabetic patients\*

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## Abstract

In urine of diabetics, a significantly great inhibitory activity of glyceraldehyde 3-phosphate dehydrogenase (GAP-Dehyd) was observed compared with that of normal subjects. The 0.2 ml of urine from 41 patients with diabetes mellitus inhibited 0.5 units of GAP-Dehyd by 27.2 +/- 3.0% (mean +/- S.E.M.), while that from 17 normal volunteers inhibited only by 9.0 +/- 1.0% (P less than 0.05). This inhibitory substance was extracted by 90% ethanol from diabetic urine and partially purified by anion exchange chromatography using Dowex-1 (HCOO type). The molecular weight of this substance was confirmed to be 100–300 daltons by an analysis on Biogel P-4 gel filtration chromatography. And analysis by thin layer chromatography using silicagel plate showed that this inhibitor was a ninhydrin reactive substance which has not been reported previously. From the above facts, it was assumed that the inhibitory substance of GAP-Dehyd in urine of diabetics was a new acidic compound of low molecular weight containing an amino residue in the molecule.

**KEYWORDS:** glyceraldehyde 3-phosphate dehydrogenase, inhibitor, diabetes mellitus, urine

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## AN INHIBITORY SUBSTANCE OF GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE IN URINE OF DIABETIC PATIENTS

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*Abstract.* In urine of diabetics, a significantly great inhibitory activity of glyceraldehyde 3-phosphate dehydrogenase (GAP-Dehyd) was observed compared with that of normal subjects. The 0.2 ml of urine from 41 patients with diabetes mellitus inhibited 0.5 units of GAP-Dehyd by  $27.2 \pm 3.0\%$  (mean  $\pm$  S. E. M.), while that from 17 normal volunteers inhibited only by  $9.0 \pm 1.0\%$  ( $P < 0.05$ ). This inhibitory substance was extracted by 90% ethanol from diabetic urine and partially purified by anion exchange chromatography using Dowex-1 (HCOO<sup>-</sup> type). The molecular weight of this substance was confirmed to be 100-300 daltons by an analysis on Biogel P-4 gel filtration chromatography. And analysis by thin layer chromatography using silicagel plate showed that this inhibitor was a ninhydrin reactive substance which has not been reported previously. From the above facts, it was assumed that the inhibitory substance of GAP-Dehyd in urine of diabetics was a new acidic compound of low molecular weight containing an amino residue in the molecule.

*Key words:* glyceraldehyde 3-phosphate dehydrogenase, inhibitor, diabetes mellitus, urine

It was reported that an inhibitory substance(s) of glyceraldehyde 3-phosphate dehydrogenase (GAP-Dehyd) was present in plasma of diabetics (1, 2). But the chemical identification of this substance has not been performed yet, and its originating organ and role in abnormal carbohydrate metabolism in diabetes mellitus are not fully understood at present.

Bornstein and his co-workers reported that this inhibitory substance was a polypeptide derived from the pituitary gland (3, 4), *i.e.* a peptide fragment of growth hormone (5). However, Schwartz and Turfus (2) have recently showed that a nonpeptide inhibitory substance of GAP-Dehyd with lower molecular weight was present in plasma of diabetics, and the peptide of growth hormone was not detected.

In an attempt to verify the presence of an inhibitory substance of GAP-

Dehyd in diabetes mellitus, the inhibitory activity of urine of diabetics was assayed.

#### MATERIALS AND METHODS

*Urine samples.* Urine samples were collected at 9–12 a.m. from 41 patients (aged 20–67) with diabetes mellitus who visited and were hospitalized at Okayama University Hospital. The samples were stored at  $-20^{\circ}\text{C}$  until use. Control urine samples were collected at 10–11 a.m. from 17 normal volunteers (aged 20–47). Most of the subjects were not fasted.

*Reagents.* Crystalline rabbit muscle GAP-Dehyd (EC. 1. 2. 1. 12),  $\beta$ -NAD and D-glyceraldehyde 3-phosphate (GAP) were purchased from Sigma Chemical Co., Ltd., U.S.A. . Ninhydrin solution was purchased from Tokyo Kasei Co., Ltd., and petroleum pitch charcoal with a spherical granule (BAC-LQ) was supplied by Kureha Kagaku Co., Ltd., Tokyo.

*Assay of inhibitory activity of GAP-Dehyd.* The assay of inhibitory activity of GAP-Dehyd in urine was performed according to the method of Schwartz and Turfus (2). The reaction mixture contained: 2.50 ml of 0.1 M pyrophosphate buffer containing 0.02 M EDTA, pH 8.45; 0.10 ml of  $1.02 \times 10^{-2}$  M  $\beta$ -NAD; 0.10 ml of GAP-Dehyd solution (5 units/ml); and 0.20 ml of urine from the patients. The reaction was started by adding 0.10 ml of  $1.02 \times 10^{-2}$  M GAP and the optical density at 340 nm was measured at  $25 \pm 3^{\circ}\text{C}$  for 5 min using a Hitachi Spectrophotometer Model 124. The inhibitory activity (%) was calculated as follows,  $[(\text{O. D. cont.} - \text{O. D. test}) / \text{O. D. cont.}] \times 100$ .

*Determination of the molecular weight.* The molecular weight of the inhibitory substance of GAP-Dehyd was determined by gel filtration chromatography on Biogel P-4 (column  $1.5 \times 82$  cm) using vitamin B<sub>12</sub> (mol. wt. 1350) and CoCl<sub>2</sub> (mol. wt. 130) as standard. 0.05 M acetic acid was used as an effluent at a flow rate of 10 ml/h, and 3 ml fractions were collected.

*Purification of the GAP-Dehyd inhibitory substance.* The GAP-Dehyd inhibitory substance was extracted with ethanol (final 90%) from urine (0.5–1 liter) of diabetics, and dried in vacuo at below  $50^{\circ}\text{C}$ . The dried residue was dissolved in 200 ml of distilled water, and then 50 g of Dowex-50 (H<sup>+</sup> type) and 50 g of activated charcoal was added to it and stirred for 30 min at  $4^{\circ}\text{C}$ . After removing the resin and charcoal by filtration on a glass filter (No. 3), the solution was adjusted to pH 8 by adding triethylamine and applied to a column of Dowex-1 (HCOO<sup>-</sup> type) ( $3 \times 35$  cm). Elution was carried out at first with 300 ml of 10% ethanol and then with a linear gradient of 0–3 N formic acid containing 10% ethanol.

*Thin layer chromatography.* The fraction containing the inhibitory substance obtained from the Dowex-1 column was analysed by thin layer chromatography using Merck precoated Kiesel Gel 60. After developing with n-butanol-acetic acid-water (5:3:5) or ethanol-acetic acid-water (90:5:5) as a mobile phase, it was sprayed with ninhydrin solution for the detection of peptides.

## RESULTS AND DISCUSSION

Inhibitory effects of GAP-Dehyd in urine samples from a normal subject and three diabetics are shown in Fig. 1. Inhibition of GAP-Dehyd was not

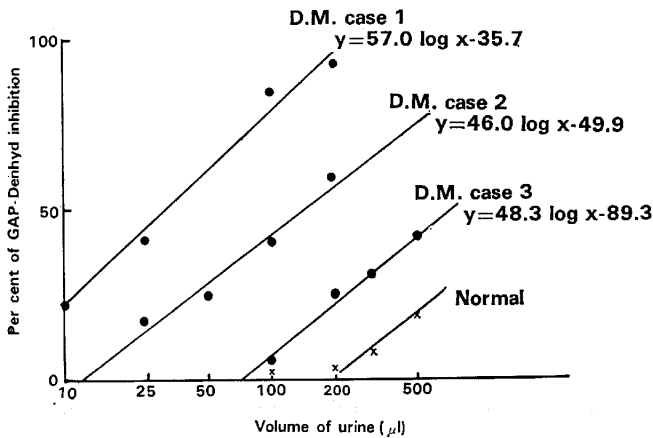


Fig. 1. Dose response of urinary inhibitory activity of GAP-Dehyd. Urine samples (10-500  $\mu$ l) from a normal and three patients with diabetes mellitus were added to the medium for assay of GAP-Dehyd activity and the GAP-Dehyd inhibitory activity was determined as described in the text,

observed with less than 200  $\mu$ l of normal urine. However an appreciable inhibition of GAP-Dehyd was observed with smaller amounts of diabetic urine (10-100  $\mu$ l). The inhibition was proportional to the log dose of urine and parallel dose response curves were obtained. Many diabetic cases showed very great inhibitory activity of GAP-Dehyd in the urine. The mean inhibitory activity of 49 urine samples from 41 diabetic patients was  $27.2 \pm 3.0\%$  (mean  $\pm$  S. E. M.), while the inhibitory activity of urine from 17 normal volunteers was  $9.0 \pm 1.0\%$ . A significant difference of inhibitory activity was observed between normal and diabetic urine ( $p < 0.05$ ). But, there were no significant correlation between the inhibitory activity and the degrees of severity (mild, moderate and severe) of the disease. In order to clarify the differences of the urinary concentration of the inhibitory substance among the degrees of severity of the disease, further estimation of the other constitutions of urine, such as the concentration of creatinine and urea, will be required.

The molecular weight of this inhibitor was determined by gel filtration chromatography on Biogel P-4 and confirmed to be 100-300 daltons, which is similar to that of the nonpeptide inhibitory substance described by Schwartz and

Turfus (2). One peak of inhibitory activity was found at the same effluent position with  $\text{CoCl}_2$  (Fig. 2).

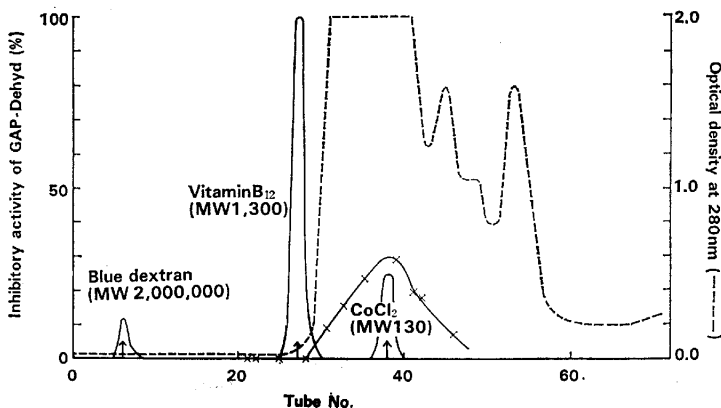


Fig. 2. Elution pattern of GAP-Dehyd inhibitory activity from Biogel P-4. Ninety percent ethanol extract of urine (10 ml) from a diabetics (case 3) was dried in vacuo and dissolved in 2 ml of 0.05 M acetic acid. The solution was applied on a column of Biogel P-4 (1.5×82 cm) and eluted with the same solvent at 4°C. The effluent fractions (3 ml each) were collected at a rate of 10 ml/h, and the GAP-Dehyd inhibitory activity was determined using 100  $\mu\text{l}$  of each effluent. The inhibitory activity was observed at tube No. 30-50 of the same effluent position with  $\text{CoCl}_2$ .

Furthermore, the physico-chemical property of this substance was similar to that of the nonpeptide inhibitory substance which is adsorbed on anion exchange resins, but different to that of the peptide inhibitor described by Bornstein *et al.* (1, 4). By the addition of 1 ml of Dowex-1 ( $\text{Cl}^-$ ) or DEAE-Sephadex to 1 ml of urine which had been adjusted the pH to 6-9, the inhibitory activity disappeared completely from the supernatant, while, by the addition of Dowex-50 ( $\text{H}^+$ ) or charcoal at pH 2-4, no decrease in the activity was observed.

On the other hand, GAP-Dehyd was not substantially inhibited by the addition of 0.2 ml of creatinine solution (1 mg/ml) to the reaction mixture, indicating that the inhibitory substance of GAP-Dehyd in urine is not creatinine. And it is also different from urea, because this inhibitory substance of GAP-Dehyd had negative charge since it adsorbs to anion exchange resins.

This substance was purified by anion exchange chromatography using Dowex-1 ( $\text{HCOO}^-$  type) from urinary samples exhibiting strong inhibitory activity of GAP-Dehyd, which were obtained from a patient with diabetes mellitus and a patient of Werner's syndrome with glucose intolerance. The elution patterns from the Dowex-1 ( $\text{HCOO}^-$ ) column by gradient elution of formic acid (0-3 N) are shown in Fig. 3. To avoid the non-specific adsorption of the inhibitory substance to the resin, ethanol was added to the elution buffer to make a

## GAP-Dehyd Inhibitor in Diabetes Mellitus

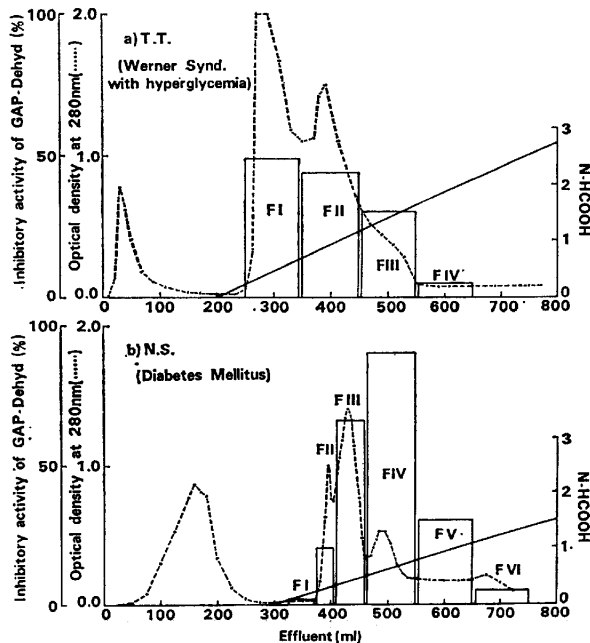


Fig. 3. Elution diagrams of inhibitory activity of GAP-Dehyd from Dowex-1 ( $\text{HCOO}^-$  type) by gradient elution with 0-3 N formic acid.

90% ethanol extracts of urine (500 ml) from a patient (T. T.) of Werner's syndrome with glucose intolerance (a) and urine (1,000 ml) from a patient (N. S.) with diabetes (b) were applied on column of Dowex-1 ( $\text{HCOO}^-$  type) ( $1.5 \times 25$  cm or  $3 \times 45$  cm) and were eluted at first with 200 ml (a) or 300 ml (b) of 10% ethanol and then eluted with 0-3 N formic acid gradient [600 ml (a) or 1,000 ml (b)] containing ethanol (10%).

final concentration of 10%. The inhibitory substance in urine from both the patients was similarly eluted at 0.3-0.7 N of formic acid.

The fractions I-II in Fig. 3-a and fractions III-V in Fig. 3-b were lyophilized and then dissolved in 10 ml of 0.05 M acetic acid, respectively. One ml of the each solution was applied to Merck precoated Kiesel Gel 60 for preparation. After developing with two different solvent systems for 1-2 h, the inhibitory substance was eluted from 1 cm silicagel bands with 0.05 M acetic acid. The inhibitory activity of GAP-Dehyd was recovered from the thin layer plates at  $R_f$  0.4 and 0.9 (n-butanol-acetic acid-water (5 : 3 : 5) and ethanol-acetic acid-water (90 : 5 : 5) solvent system) corresponding to a ninhydrin reacted spot. Therefore, the GAP-Dehyd inhibitor in urine was assumed to be a ninhydrin reactive substance of low molecular weight, which has similar physico-chemical properties to the non-peptide inhibitory substance found in plasma by Schwartz and Turfus (2). But, at present, it is not certain whether the inhibitory substance of GAP-

Dehyd in urine is an identical compound to the inhibitory substance in plasma, and further purification of both the substances is required for identification and also for biological studies on these substances.

In this communication, we reported the presence of an inhibitory substance of GAP-Dehyd in urine of diabetics, which may affect carbohydrate metabolism. The elucidation of the chemical and biological properties of this GAP-Dehyd inhibitory substance will aid in solving some important problems on the abnormal carbohydrate metabolism in diabetes mellitus.

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