Note

Occurrence in Soybeans of a Novel Vitamin B₆ Conjugate that Liberates Pyridoxine by β-Glucosidase Action after Alkali Treatment

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A type of vitamin B₆ conjugates (B₆X), which liberates free vitamin B₆ by alkaline and successive β-glucosidase hydrolyses, is known to occur in rice bran and wheat bran. Conflicting experimental results, however, have been reported about the occurrence of B₆X in soybeans. This study afforded evidence for B₆X occurring in soybeans: certainly a highly purified B₆X preparation from whole soybeans liberated pyridoxine when it was treated with alkali followed by β-glucosidase hydrolysis, and 5′-O-(β-D-glucopyranosyl)pyridoxine by alkali treatment alone. The B₆X content varied with cultivars, of which a certain kind contained no B₆X.

Key words: vitamin B₆ conjugate; pyridoxine conjugate; derivative of pyridoxine glucoside; soybean; B₆X

Grains and legumes are rich in vitamin B₆, and regarded as good sources of vitamin B₆ for the Japanese. Vitamin B₆ in plant foods, however, is incompletely available to animals, because it occurs largely in conjugated forms. For the accurate assessment of the bioavailable vitamin B₆ from foods, studies on structural elucidation, bioavailability, and distribution of vitamin B₆ conjugates are essential.

A predominant form of vitamin B₆ conjugates is 5′-O-(β-D-glucopyranosyl)pyridoxine (PNG) distributed widely in plant foods but not in animal foods. The bioavailability of PNG is very low; it is estimated to be less than 40% in rats and about 58% in humans. An antagonistic effect of PNG on pyridoxine (PN) metabolism occurs in humans, although the effect is less pronounced than that shown in rats. In addition to PNG, another type of vitamin B₆ conjugate (B₆X), which liberates free vitamin B₆ by alkaline and successive β-glucosidase hydrolyses, occurs in rice bran and wheat bran. From rice bran, B₆X was isolated, and identified as 5′-O-(6-O-(4′-acetoxy-4′H-dioxinodec-3′-acetyl)-β-D-glucosyl)pyridoxine. The chemical structure of B₆X in wheat bran has not been identified yet. We measured the contents of various forms of vitamin B₆ in 8 plant foods by a differential microbiological assay, in which B₆X was calculated from the values of free vitamin B₆ released by β-glucosidase hydrolysis of food samples with and without treatment with 0.7 M potassium hydroxide. The B₆X content in foods was as follows (% of the total B₆): rice bran, 38; wheat bran, 19; powdered peas, 21; defatted soybeans, 27; cauliflower, spinach, pumpkin, and immature broad beans, 0. Later, Gregory and Ink analyzed B₆X vitamins, PNG, and B₆X in several plant foods by HPLC. The B₆X content (% of the total B₆) was 43 for rice bran, 11 for wheat bran, zero for oat bran, 12 for raw carrots, and zero for soy flour. The analytical values for rice and wheat brans were compatible, but that for soybeans was inconsistent with our data taken by bioassay. The discrepancy may be due to a difference in analytical method, raising a question about the occurrence of B₆X in soybeans. On this account, this study was done to discover whether B₆X is really present in soybeans.

A vitamin B₆-depleted basal medium for microbiological assay was purchased from Nissui Pharmaceutical Co. Sweet almond β-glucosidase (E.C.3.2.1.21) was a product of Sigma Chemical Co., and other reagents were obtained from Nacalai Tesque Inc. PNG was prepared as described previously. The microbiological assay was done using Saccharomyces uvarum (ATCC 9080) as a test organism. B₆X was detected at all the steps of isolation from soybeans as described previously. Before the microbiological assay, food samples were treated as described previously. A highly purified B₆X preparation from soybeans was analyzed before and after the treatment by a modification of HPLC, in which the mobile phase consisting of 50 mM potassium phosphate buffer (pH 3.50), 100 mM sodium perchlorate, and 5.0% methanol was used. B₆X was eluted at about 42 min by raising the methanol concentration from 1.0% to 5.0%.

The contents of various forms of vitamin B₆ in whole soybeans (unidentified cultivar I) were measured microbiologically, and the analytical values were expressed in μg PN equivalent/100 g as the means ± SD of 6 measurements: total vitamin B₆, 361 ± 29; free forms, 100 ± 11; glycosylated and phosphorylated forms, 222 ± 33; B₆X, 50 ± 20 (14% of the total B₆). This assay also showed the occurrence of B₆X in soybeans, although the B₆X content was lower than our previous result. An attempt was, therefore, made to confirm its occurrence by HPLC. Soybeans (900 g) were powdered and extracted with 2.71 of boiling 70% ethanol. The

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Abbreviations: B₆X, a type of vitamin B₆ conjugates to liberate free vitamin B₆ by β-glucosidase action after alkali treatment; PNG, 5′-O-(β-D-glucopyranosyl)pyridoxine; PN, pyridoxine
residue obtained by filtration was again extracted with 1.8 l of the same solvent. The combined extract was concentrated below 40°C in vacuo in the dark. The precipitate formed during concentration was removed by centrifugation. To the supernatant was added 500 ml of Amberlite XAD-4, followed by stirring for 30 min. After the resin was washed with distilled water, B6X was eluted from the resin with 3 liters of 40% ethanol. The eluate was concentrated to 50 ml as described above, adjusted to pH 7.0 with dil. ammonia, and put on a column (2.5×25 cm) of Dowex 1×4 (acetate form). B6X was eluted with 600 ml of 0.1 M acetic acid. The fraction containing B6X was concentrated to 50 ml, and put on a column (1.8 × 15 cm) of P-cellulose (H⁺), followed by elution with water. The purified B6X fraction containing about 0.11 mg of PN equivalent (microbiological assay) was concentrated and analyzed by HPLC before and after the following treatments 1~4: (1) The B6X fraction (1 ml) was adjusted to pH 5.0 with 0.2 M sodium acetate, and incubated at 37°C with 60 units of β-glucosidase dissolved in 1 ml of 0.2 M acetate buffer. After incubation of 3 h, 1 ml of 3 N perchloric acid was added. The mixture was left at 2~4°C for 40 min in the dark, adjusted to pH 3.5 with 5 N potassium hydroxide, filled up to 5 ml with distilled water, and finally centrifuged. An appropriate volume of the supernatant was injected into the HPLC apparatus. (2) To the B6X fraction (1 ml) was added 163 μl of 5 M potassium hydroxide to give a potassium hydroxide concentration of 0.7 M, and left at room temperature in the dark. After 4 h, the reaction mixture was neutralized with 5 N hydrochloric acid, 3 N perchloric acid was added to the mixture to a final concentration of 1 M, and the mixture was treated in the manner above-mentioned. (3) The B6X fraction (1 ml) was treated with 0.7 M potassium hydroxide as described above, adjusted to pH 5.0 with 0.2 M sodium acetate, and digested with 60 units of β-glucosidase. Subsequent operations were the same as described in treatment 1. (4) The B6X fraction (1 ml) was digested with β-glucosidase and then treated with potassium hydroxide. Subsequent operations were the same as described in treatment 2.

As shown in Fig. 1, the B6X fraction (the untreated) gave a single peak of B6X on a HPLC chromatogram. On treatment 2, the B6X peak disappeared and a peak attributable to PNG appeared. This peak fraction was confirmed to be PNG, but not to be 4'-O-(β-D-glucopyranosyl)pyridoxine as follows: (1) the Rf on a paper chromatogram with a solvent of n-butanol/benzene/pyridine/water (5/1/3/3, v/v) upper phase was the same as that of PNG; (2) the peak fraction gave a positive Gibbs color reaction with 2,6-dichloroquinone chloroimide and a negative one in the presence of bovic acid. Treatment 3 decomposed B6X to release PN (0.1 mg), which was confirmed by paper chromatography. The quantities of PNG (0.11 mg PN equivalent) liberated by alkali treatment, and PN (0.1 mg) liberated by β-glucosidase hydrolysis after alkali treatment, which were measured by HPLC, were comparable to each other. Treatment 1 did not give PN or PNG, the chromatogram being the same as obtained for the B6X fraction (the untreated). Treatment 4 produced PNG, and gave the same chromatogram as treatment 2. These results indicate that B6X is surely present in soybeans and that B6X must be a derivative of PNG, probably with organic acid(s) attached to the glucose moiety or the glucose and PN moieties of PNG. Its chemical structure is undoubtedly different from that of rice bran B6X, because soybean B6X was adsorbed on the column of Dowex 1 × 4 (acetate form), while rice bran B6X passed through the column. On the basis of the chemical structure of B6X, it seems reasonable to assume that the bioavailability of B6X is similar to or less than that of PNG, depending upon a rate of its decylation before and/or after intestinal absorption.

Considering the results of Gregory and Ink, it is highly probable that there exist cultivars of soybeans which contain little or no B6X. On this account, the B6X content in a few cultivars was measured by HPLC, soybeans being treated as described previously. The means of 2 measurements (μg PN equivalent/100 g) were as follows: 172 for Toyoosuzu; 112 for Hatsuysuki; zero for Tohoku-110; 197 for Tohoku-106; 50 for unidentified cultivar 1, used in these experiments; and 97 for unidentified cultivar 2. A cultivar, Toyoosuzu, was cultivated at Hokkaido Prefecture, Hatsuysuki at

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**Fig. 1.** Changes in HPLC Chromatogram of Soybean B6X Preparation by Various Hydrolysis Treatments.

A: highly purified B6X preparation as such. B: the B6X preparation was treated with 0.7 M potassium hydroxide for 4 h at room temperature in the dark. C: the B6X fraction was treated with 0.7 M potassium hydroxide and then digested with β-glucosidase.
Province of Ontario, Canada, and two cultivars, Tohoku-110 and -106, at Kagoshima Prefecture. Thus, we consider the B<sub>6</sub>X content varies with cultivars of soybeans, though it is probably influenced by climatic and agricultural factors in an identical cultivar.

In summary, this study clarified that B<sub>6</sub>X really occurred in soybeans, that its chemical structure was different from that of rice bran B<sub>6</sub>X, and that the B<sub>6</sub>X content varied with different cultivars of soybeans, of which a certain kind contained no B<sub>6</sub>X.

References