**Note**

Yolk of the Century Egg (Pidan) Contains a Readily Digestible Form of Free Vitamin B₁₂

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**Summary** In this study, we determined the vitamin B₁₂ content of commercially available century eggs (pidan) and characterized their vitamin B₁₂ compositions in detail. The egg yolk and white of century eggs (each 100 g wet weight) contained 1.9 ± 0.6 and 0.8 ± 0.3 μg of vitamin B₁₂, respectively. The vitamin B₁₂ compounds purified from the egg yolk and white were identified as vitamin B₁₂ using liquid chromatography-electrospray ionization/tandem mass spectrometry. The vitamin B₁₂ present in the yolk or white of century eggs was recovered completely in macromolecular fractions, but not in free vitamin B₁₂ fractions by Sephadex G-50 gel filtration. However, with respect to the vitamin B₁₂ bound to protein in the century egg yolk, approximately 52% of the free vitamin B₁₂ was formed during in vitro gastric digestion and no free vitamin B₁₂ was detected in the egg white.

**Key Words** century egg, egg yolk, In vitro gastric digestion, pidan, vitamin B₁₂

Vitamin B₁₂ (B₁₂) is synthesized only by certain bacteria (1) and it is concentrated primarily in the bodies of predators found higher in the food chain. Thus, foods derived from animals (meat, milk, eggs, fish, and shellfish) are considered to be the major dietary sources of B₁₂ (2). Raw and boiled whole chicken eggs contain 0.9 μg of B₁₂ per 100 g wet weight of the edible portion (3) and most of the B₁₂ is located in the egg yolk (4). The reported average bioavailable levels of B₁₂ in scrambled egg yolks, scrambled whole eggs, boiled eggs, and fried eggs are 8.2%, 3.7%, 8.9%, and 9.2%, respectively (3). In addition, the B₁₂ in eggs is absorbed poorly compared with that in other animal food products (5, 6).

A century egg ("pidan" in Chinese) is an alkaline-fermented ethnic food in China. Century eggs are traditionally made by preserving chicken or duck eggs in a mixture of salt, ash, and lime (CaO as a strong base), before wrapping them in rice husks for several weeks. During this treatment, the pH of the egg increases and the components of the egg change significantly. During this process, the egg yolk becomes dark green with a creamy consistency, while the egg white becomes an amber jelly. B₁₂ is unstable under alkaline conditions (2), so it is possible that this alkali treatment results in a significant loss of B₁₂ in eggs. However, little information is available about the B₁₂ content of the yolk and white of century eggs.

In this study, we determined the B₁₂ contents of various century eggs and characterized their B₁₂ composition to evaluate whether they are good sources of B₁₂.

**Materials and Methods**

Materials. B₁₂ was obtained from Sigma (St Louis, MO). A B₁₂ assay medium based on *Lactobacillus delbrueckii* (formerly *L. leichmannii*) ATCC 7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). Various century eggs prepared from duck eggs were purchased from local markets in Japan.

B₁₂ extraction and assay. B₁₂ was assayed using a microbiological technique based on *L. delbrueckii* ATCC 7830, according to the method described in the Standard Tables of Food Composition in Japan (7). After removing the shells from the duck century eggs, the egg white and yolk were separated. Each sample was homogenized using a mortar and pestle. An aliquot (4.0 g) of each homogenate was used as the test sample. The total B₁₂ compounds were extracted from each sample by boiling in acetic acid buffer (pH 4.5) containing 4.0 × 10⁻⁴% KCN in order to convert various B₁₂ compounds with different upper-ligands (e.g., B₁₂ coenzymes) into cyanocobalamin (B₁₂). The pH of the total B₁₂ extract aliquot was adjusted to 11.0 and autoclaved (MC-23; ALP Co., Ltd., Tokyo, Japan) at 121°C for 30 min to decompose the B₁₂ in the extract. The treated extract contained certain compounds (including deoxyribosides and deoxyribonucleotides) that are known to...
be alkali-resistant factors. *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (alkali-resistant factors) as well as B12, so the amount of B12 was calculated by subtracting the levels of the alkali-resistant factors from the total B12. The B12 assay was performed in triplicate.

**Bioautography of B12 compounds using B12-dependent* Escherichia coli* 215.** Bioautography of corrinoid compounds was performed as described previously (8). The B12 extract (50 mL) prepared as described above was partially purified and concentrated using a Sep-Pak Plus® C18 cartridge (Waters Corp., Milford, MA) that had been washed with 5 mL of 75% (v/v) ethanol and equilibrated with 5 mL of distilled water. The C18 cartridge was then washed with 5 mL of distilled water and the B12 compounds were eluted through the cartridge using 2 mL of 75% (v/v) ethanol. The eluate was then evaporated in a centrifugal concentrator (Integrated SpeedVac® System ISS110; Savant Instruments Inc., Holbrook, NY) and the residual fraction was dissolved in 2.0 mL of distilled water. The concentrated B12 extracts (1 mL) that contained authentic B12 (50 μg/L), and pseudo-B12 (50 μg/L) were spotted onto a silica gel 60 TLC sheet and developed in the dark using 2-propanol/NH4OH (28%)/water (7:1:2 v/v) at 25°C. After drying, the TLC sheet was overlaid with agar containing a basal medium preincubated with *E. coli* 215 and incubated at 37°C for 20 h. The gel plate was then sprayed with a methanol solution containing 2,3,5-triphenyltetrazolium salt where the red coloration obtained indicated the growth of *E. coli* and thus the presence of B12 compounds in the sample.

**Identification of B12 compounds in century egg by liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS).** The B12 compounds in each sample were purified using a C18 cartridge under the same conditions described above. The purified extract was loaded onto an immunoaffinity column (EASI-EXTRACT® B12 Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany), and the corrinoids were purified according to the manufacturer’s protocol. The corrinoids from each sample and authentic B12 were dissolved in 0.1% (v/v) acetic acid and filtered with a Nanosep MF centrifuge device (0.4 μm, Pall Corp., Tokyo, Japan) to remove small particles. A 2-μL aliquot of the filtrate was analyzed using a LCMSIT-TOF coupled to an Ultra-Fast LC system (Shimadzu, Kyoto, Japan). Each purified sample was injected into an InertSustain column (3 μm, 2.0×100 mm; GL Science, Tokyo, Japan) and equilibrated with 85% solvent A [0.1% (v/v) acetic acid] and 15% solvent B (100% methanol) at 40°C. The B12 compounds were eluted using a linear gradient of methanol (15% solvent B for 0–5 min, 15–90% solvent B for 5–11 min, and 90–15% solvent B for 11–15 min). The flow rate was 0.2 mL/min. The electrospray ion (ESI) parameters for detecting the parent and daughter ions of the B12 compound were optimized after injecting authentic B12 into the MS detector. ESI-MS was operated in the positive ion mode using argon as the collision gas. The identification of B12 (m/z 678.2914) representing [M+2H]2+ was confirmed by comparing the observed molecular ions and their retention times.

**pH determination for the yolk and white of century eggs.** The pH values of the yolk and white of century eggs were determined at multiple points using a pH meter equipped with a flat electrode (ISFET 0040-10D).

**Sephadex G-50 gel filtration experiments.** The free B12 was separated from the yolk and white of century eggs using a column (1.4×10 cm, econo-pack column, BioRad Laboratories, Hercules, CA) of Sephadex G-50 fine (GE Healthcare UK Ltd., Little Chalfont, England) and then assayed. A portion (2.5 g) of egg white or egg yolk was added to 100 mmol/L potassium phosphate buffer (10 mL) and then homogenized using a mortar and pestle. Each homogenate was centrifuged at 10,000 ×g for 10 min at 25°C to remove insoluble materials. An aliquot (1.0 mL) of the supernatant was applied to the Sephadex G-50 column, which had been equilibrated with 100 mmol/L potassium phosphate buffer (pH 7.0). The column was eluted with the same buffer at a flow rate of 1.0 mL/min. The eluate obtained from the column was analyzed using a HPLC system equipped with a flat detector (SQV-2000, Zetasil 250 mm × 4.6 mm, Zorich, Japan)

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Table 1. B12 contents and pH values of yolk and white samples from various century eggs.

| Sample | Egg yolk | Egg white | Whole egg | Edible portion | Egg yolk | Egg white |
|--------|----------|-----------|-----------|----------------|----------|-----------|
| a      | 2.3±0.2  | 0.6±0.3   | 1.6±0.2   | 54.6±0.8       | 8.8±0.1  | 9.0±0.1   |
| b      | 2.5±0.3  | 1.2±0.1   | 1.8±0.1   | 48.5±0.9       | 8.1±0.5  | 8.0±0.6   |
| c      | 1.5±0.2  | 0.8±0.2   | 1.2±0.1   | 56.4±0.6       | 9.0±0.2  | 9.2±0.3   |
| d      | 1.3±0.3  | 0.6±0.1   | 1.0±0.1   | 48.7±0.9       | 8.5±0.1  | 8.8±0.1   |
| All    | 1.9±0.6  | 0.8±0.3   | 1.4±0.4   | 51.2±4.1       | 8.6±0.4  | 8.7±0.5   |

The white and yolk were separated after removing the shells from century egg samples a–d (n=3 for each sample). Each sample was homogenized using a mortar and pestle. An aliquot (4.0 g) of each homogenate was used as the test sample. B12 was assayed using the microbiological technique based on *L. delbrueckii* ATCC 7830 as described in “Materials and Methods.” The pH values of yolk and white samples were determined at multiple points using a pH meter equipped with a flat electrode. All values represent mean ±SD.
fractionated at 0.5 mL. Next, 1,000 μL of 0.57 mol/L acetate buffer (pH 4.5) and 40 μL of 0.5% (w/v) KCN were added to each fraction, mixed vigorously, and left overnight at 4˚C in the dark. B12 was assayed using the microbiological method. The macromolecular and free B12 fractions were estimated with blue dextran 2000 (GE Healthcare) and authentic B12, respectively, by measuring the absorbance at 280 nm.

In vitro gastric digestion of century egg and boiled chicken eggs. Eggs were subjected to in vitro gastric digestion using a method that simulated the human digestion system (9). Century and boiled chicken eggs (each egg yolk and white) were sampled. An aliquot (5 g) of each sample was homogenized with distilled water (5 mL) using a mortar and pestle. The homogenate was mixed with 3 mL of 0.01% (w/v) pepsin solution and 29 mL of 0.06 mol/L HCl, and then incubated at 37˚C in the dark for 1.5 h in vitro (gastric digestion). The digestive reaction was stopped by adding 5 mL of 1 mol/L NaHCO3 and incubating on the ice for 5 min. The mixture was centrifuged at 10,000 × g for 10 min and the supernatant was used in the free B12 assay as described above.

Results and Discussion

B12 contents of century eggs

The B12 contents of various duck century eggs (samples a, b, c, and d; n = 3) were analyzed using the L. delbrueckii ATCC 7830 microbiological assay method (Table 1). The yolks and whites (each 100 g wet weight) of the century eggs contained approximately 1.9 ± 0.6 μg and 0.8 ± 0.3 μg of B12, respectively. However, previous reports indicate that B12 is located only in the egg yolk.
fraction of raw chicken egg and not in the egg white fraction (10). The B12 content of whole century eggs (per 100 g wet weight) was calculated as approximately 1.4 ± 0.4 μg and a similar value (1.1 μg) is reported in the Standard Tables of Food Composition in Japan 2010 (10).

Although the century eggs had been prepared by treating duck eggs with lime (CaO) as a strong base for several months, the pH values of the century egg yolk and white were 8.6 ± 0.4 and 8.7 ± 0.5, respectively. Our

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**Fig. 3.** LC/ESI-MS/MS chromatograms of the B12 compounds purified from the yolk and white of century egg sample c. Purified B12 compounds were analyzed using the LC/MS-IT-TOF system. Panels A and D show the total ion chromatograms and those (m/z 678.2914) of B12 compounds purified from the egg yolk and white, respectively. Mass spectra of purified B12 compounds at 7.5 min are shown in panels B and E, respectively (the magnified spectrum range from m/z 678.0 to m/z 680.0 is shown as an insert in each panel). The MS/MS spectra for the peaks of the purified B12 compounds at m/z 678.2902 and m/z 678.2881 are shown in panels C and F, respectively.
preliminary experiments indicated that approximately 13.5% of hydroxocobalamin was removed when it was solubilized with 100 mmol/L of sodium bicarbonate buffer (pH 8.7) and left in the dark for 6 mo at 25°C. However, an identical loss was also found under conditions at pH 7.0. Thus, it is unclear whether the B12 content decreased significantly during the processing of century eggs because we have no information about the B12 content of raw duck eggs. Our results suggest that the B12 located in the egg yolk is partly transferred to the egg white due to the denaturation of egg proteins during the CaO treatment of eggs.

**Identification of corrinoid compounds by the E. coli 215 bioautography**

The B12 compounds found in century eggs were analyzed using an E. coli 215 bioautogram after separation by silica gel 60 TLC (Fig. 1). Each yolk sample from century eggs produced a single clear spot with an Rf value identical to that of authentic B12 (Fig. 1A). The same results were also obtained using the egg white samples (Fig. 1B). These results indicate that the yolks and whites of century eggs contain B12 but not pseudovitamin B12, which is biologically inactive in humans.

**LC/ESI-MS/MS analysis**

To precisely identify the corrinoid compounds present in century eggs, corrinoids were purified from the egg yolk and white samples and identified using LC/ESI-MS/MS. Authentic B12 was eluted as a peak with a retention time of 7.5 min (Fig. 2A). In the mass spectrum of authentic B12, a doubly-charged ion with an m/z of 678.2943 [M+2H]2+ was prominent (Fig. 2B). The exact mass calculated from its formula (C63H88CoN14O14P) was 1354.5674 g/mol and the isotope distribution data showed that B12 was the major doubly-charged ion under the LC/ESI-MS conditions used in our analyses. The MS/MS spectrum of authentic B12 indicated that its dominant ion at m/z 359.0984 was attributable to the nucleotide moiety (α-ribazole-5′-phosphate, C14H19N2O7P, exact mass = 358.0929 g/mol) (Fig. 2C). The corrinoid purified from the yolk of century egg sample c was eluted as one ion peak. The mass spectrum of the main peak with a retention time of 7.5 min indicated the presence of a doubly-charged ion with m/z 687.2902 in the purified sample (Fig. 3A and B). The MS/MS spectrum of the purified compound with a monovalent ion with m/z 359.0984 was identical to that of authentic B12 (Fig. 3C). The egg white from sample c yielded identical results (Fig. 3D, E, and F). The remaining century egg samples also yielded identical results to sample c (data not shown) by LC/ESI-MS/MS. These results indicate that the yolk and white of century eggs contained authentic B12.

**Occurrence of free B12 in the yolk and white of century eggs**

The homogenates of selected samples were analyzed using Sephadex G-50 gel filtration to evaluate whether the yolk and white of the century eggs contained “free B12” or “protein-bound B12.” As shown in Fig. 4, the B12 found in the egg yolk and white was completely recovered only in the macromolecular fractions, probably the protein-bound B12 fractions. These results indicate that both the yolk and white of century eggs contained the protein-bound-B12 but not free B12 despite the denaturation of the egg proteins.

**In vitro gastric digestion of century and boiled chicken eggs**

The most important step in the gastrointestinal absorption of B12 from ingested food is the release of free B12 from the food protein-bound B12 complex during gastric digestion (6). To evaluate whether free B12 is formed from the protein-bound B12 complex during gastric digestion of century eggs, the yolk and white fractions were treated by in vitro gastric digestion and the free B12 formed was separated by Sephadex G-50 gel filtration. Boiled chicken eggs were treated under identical conditions and used as controls. Approximately 52.7±9.1% of the B12 found in the yolk of century eggs was recovered in the free B12 fractions, whereas free B12 was not detected in any of the egg white fractions (Fig. 5A and B). In the boiled chicken egg yolk, approximately 10.3±2.1% of the B12 was recovered in the free...
B12 fractions, whereas no B12 was detected in any of the egg white fractions (Fig. 5C and D). Katsura et al. (6) reported that approximately 16% of the B12 found in boiled whole chicken eggs was recovered in the free B12 fractions under identical gastric digestion conditions. Thus, these results confirmed the low bioavailability (8.9%) of B12 in boiled chicken egg (3). These results suggest that century egg yolks yield a readily digestible form of free B12 during gastric digestion. Considering the in vitro digestion rate (52%), the consumption of five yolks from century eggs could satisfy the recommended dietary allowance for B12 in adults (2.4 µg/d) (11, 12). Thus, our results indicate that century eggs are a better source of B12 compared with typical cooked chicken eggs or egg products.

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