Two Proteins, Mn^{2+}, and Low Molecular Cofactor Are Required for *C*-Glucosyl-Cleavage of Mangiferin

Kanjana SANUGUL,[a] Teruaki AKAO,[b] Norio NAKAMURA,[a] and Masao HATTORI*,[a]

[a] *Institute of Natural Medicine, Toyama Medical and Pharmaceutical University; and* [b] *Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930–0194, Japan.*
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***C*-Glucosides, in which sugars are attached to the aglycone by carbon–carbon bonds, are generally resistant to acid and enzyme hydrolysis. The *C*-glucosyl bond of mangiferin, a xanthone *C*-glucoside, was cleaved by anaerobic incubation with a human intestinal bacterium, *Bacteroides* sp. MANG, to give norathyriol. A cell-free extract obtained by sonication of *B*. sp. MANG demonstrated cleaving activity for mangiferin to norathyriol by adding NADH, diaphorase, and dithiothreitol. Both high molecular weight (>10 k) and low molecular weight (<10 k) fractions obtained from the cell-free extract were required for the activity. $MnCl_2$ was necessary for the activity, but other metal ions were not. By purification of the high molecular weight fraction using DEAE-cellulose and Phenyl Sepharose column chromatography, two fractions, designated as proteins A and B, were separated and required for the activity. Neither protein A nor protein B alone showed any activity. This is the first report describing a *C*-glucosyl-cleaving enzyme from human intestinal bacterium that seems to involve a novel enzyme mechanism.**

Key words *C*-glucoside; mangiferin; human intestinal bacterium; *Bacteroides*; *C*-glucosyl-cleaving enzyme

C-Glucosides, in which the anomeric oxygen atom of glucose is replaced by a carbon atom, have been extensively studied during the last three decades.[1] It has been shown that the conformation of *C*-glucosides is significantly different from that of *O*-glucosides.[2] *C*-Glucosides are stable to enzymatic as well as acidic degradation, which is why they have potential as hydrolytically stable *O*-glucoside analogs for enzyme inhibitors.[3,4] The microflora in the human intestinal tract plays important roles in the metabolism of many substances. *C*-Glucosyl bonds of various *C*-glucosides including mangiferin, abrusin 2″-*O*-β-D-apioside, aloeresin A, aloesin, barbaloin, bergenin, homoorientin, puerarin, and safflor yellow B are also transformed to the corresponding aglycone by human intestinal bacteria.[5—11]

Most research has been focused on the synthesis of *C*-glucosides.[12] Naturally-occurring *C*-glucosides, however, have advantages and pharmacological activities different from their aglycones. However, to date, there is little information concerning the cleavage mechanism of *C*-glucosides by enzymes. Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), a xanthone *C*-glucoside from *Mangifera indica* L. (Anacardiaceae), can be used for the study of *C*-glucosyl-cleaving. In studies *in vivo*, mangiferin has been reported to have many biological activities, including antitumor,[13] antidiabetic,[14] antioxidant,[15] hepatoprotective,[16] and immunomodulative[17] activities, whereas its aglycone shows anti-inflammatory,[18,19] vasorelaxation,[20] and antiplatelet[21] activities. The specific bacterium involved in the *C*-glucosyl-cleaving of mangiferin was isolated and classified as *Bacteroides* sp. MANG.[22] Our previous study has shown that the mangiferin *C*-glucosyl-cleaving enzyme is an inducible enzyme produced by *Bacteroides* sp. MANG. The enzyme can break the C–C linkage in mangiferin to give norathyriol as an aglycone and seems to be different from *O*-glucosidases.[22]

The main objective of the present research was to study the *C*-glucosyl-cleaving activity at the enzymatic level. This paper describes the factors associated with the *C*-glucosyl-cleaving activity and purification of *C*-glucosyl-cleaving enzyme.

MATERIALS AND METHODS

Chemicals and Bacterial Strain *Bacteroides* sp. MANG was isolated from human feces as previously described.[22] Mangiferin was purchased from Sigma-Aldrich Co. (U.S.A.). Diaphorase derived from *Clostridium kluyveri* was obtained from Oriental Yeast Co. (Osaka, Japan) and NADH from Wako Pure Chemical Industries Ltd. (Osaka, Japan). General anaerobic medium (GAM) broth was purchased from Nissui Seiyaku Co. (Tokyo, Japan). All other chemicals were of analytical reagent grade.

Preparation of Cell-Free Extract The *B*. sp. MANG was cultured under anaerobic conditions at 37 °C for 12 h in 2 l of GAM medium containing 0.2 mM mangiferin, harvested, and then suspended in 250 ml of 50 mM phosphate buffer (pH 7.3). The bacterial cells were disrupted by 10 sonic bursts of 30 s each (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT, U.S.A.) on ice in a cold room, and then centrifuged at 100000 ***g*** for 60 min (Ultracentrifuge Beckman Optima XL-70, Beckman Instruments, Fullerton, CA, U.S.A.) at 4 °C to obtain the supernatant as a cell-free extract. The cell-free extract was used as a starting material for the enzyme purification.

Preparation of High Molecular Weight (HMW) and Low Molecular Weight (LMW) Fractions A cell-free extract was first separated to HMW and LMW fractions by centrifugation through Centriplus (Millipore, U.S.A.) with a 10 k molecular weight cut-off at centrifugal force 3000 ***g*** at 4 °C for 2—3 h. Substances whose molecular weights were less than 10 k (lower part of the filter) were collected as an LMW fraction. The upper part was further separated by Sephadex G-25 gel filtration column chromatography (1.3×42 cm). The column was equilibrated and eluted with 50 mM phosphate buffer (pH 7.3). Five milliliters fractions were collected

* To whom correspondence should be addressed. e-mail: saibo421@ms.toyama-mpu.ac.jp

and monitored at 280 nm. High molecular weight proteins eluted in the earlier fractions were combined and concentrated again by a centrifugal filter and called the HMW fraction.

***C*-Glucosyl-Cleaving Activity Assay for Mangiferin** Assay of Cell-Free Extract: The reaction mixture containing 20 mM NADH, 20 units of diaphorase, 20 mM dithiothreitol, 1 mM mangiferin, 30 μl of a cell-free extract, and 10 mM phosphate buffer (pH 7.3) in a final volume of 100 μl was anaerobically incubated at 37 °C for 1 or 2 h. The reaction was stopped by adding 100 μl of acidified 1-butanol (saturated with distilled water and acidified with 0.1% acetic acid). A portion of the butanol extract was dried using a Speed Vac SC 110 dryer (Savant Instruments, NY, U.S.A.) and then dissolved in 50% methanol. The metabolite, norathyriol, from the reaction mixture was analyzed by HPLC as described below. The air-flushed sample of the cell-free extract was prepared by air bubbling under aerobic conditions for 2 min. A vacuum pump was used to eliminate air from one portion of the sample. The effects of various metals and a chelating agent on the *C*-glucosyl-cleaving activity of the cell-free extract were tested by adding the metal chloride and EDTA·2Na to the reaction mixture as described above.

Assay of HMW Fraction: The reaction mixture contained 20 mM NADH, 20 units of diaphorase, 20 mM dithiothreitol, 1 mM mangiferin, 30 μl of the HMW fraction, and 10 mM phosphate buffer (pH 7.3) (with/without 30 μl of the LMW fraction and 1 mM metal chloride) in a final volume of 100 μl. The reaction mixture was anaerobically incubated at 37 °C for 1 or 2 h. The amount of norathyriol was determined as below.

Assay of Partially Purified Enzyme: During the enzyme purification process, the activity assay was markedly complicated by the addition of appropriate amounts of the other corresponding protein. The reaction mixture containing 20 mM NADH, 20 units of diaphorase, 20 mM dithiothreitol, 1 mM mangiferin, and 0.25 mM $MnCl_2$, together with an optimized amount of the LMW fraction and an enzyme solution in a final volume of 100 μl, was anaerobically incubated at 37 °C for 2 h. If partially purified protein A was determined, partially purified protein B (preliminary preparation on Phenyl Sepharose column chromatograph, see Enzyme Purification below) was added to the reaction mixture and *vice versa*. The reaction was analyzed as below.

Quantitative Analysis of Mangiferin and Its Product, Norathyriol, by HPLC[22] The conditions used for reversed phase HPLC (Shimadzu Co., Japan) were as follows: recorder, C-R6A Chromatopac; pump, LC-6A; system controller, SCL-6B; monitor, SPD-6A; injector, SIL-9A; column, YMC-Pack ODS-AP AP-302 (YMC Co., Kyoto, Japan); flow rate, 1 ml/min; detection, 260 nm; solvent system, 10—40% acetonitrile linear gradient in 0.1% trifluoroacetic acid.

Protein Measurement The protein content of the cell-free extracts was determined by the method of Lowry.[23] Sample (0.1 ml) containing protein was pretreated with 0.1 ml of 1 N NaOH. One milliliter of copper–tartrate–Na_2CO_3 solution was added, the mixture shaken well, and allowed to stand at 37°C for 10 min (copper–tartrate–Na_2CO_3 solution was freshly prepared by dissolving 100 ml of 2% Na_2CO_3 with 1 ml of 1% $CuSO_4 \cdot 5H_2O$ and 1 ml of 2% tartrate sodium potassium $\cdot 4H_2O$ in distilled water). Folin-Ciocalteu phenol (0.1 ml) was then rapidly added and mixed immediately. The mixture was incubated at 37 °C for 30 min and then monitored by measuring the absorbance at 650 nm. Bovine serum albumin was used as a standard for the protein assay.

Enzyme Purification From the preliminary study, we found that in addition to the LMW fraction, there were at least 2 protein fractions required for *C*-glucosyl-cleaving activity. A 150 ml of a cell-free extract was applied to a DEAE-cellulose (DE-52, Whatman Biosystems Ltd., England) column chromatograph (3×9 cm) equilibrated with 50 mM phosphate buffer (pH 7.3). The column was washed with 50 ml of the same buffer and 100 ml of 100 mM phosphate buffer (pH 7.3) and then eluted with 400 ml of NaCl concentration gradient (0—400 mM) in 100 mM phosphate buffer (pH 7.3). Three milliliters fractions each were collected and monitored by measuring the absorbance at 280 nm and *C*-glucosyl-cleaving activity for mangiferin. The active fraction pooled was further purified using a Phenyl Sepharose CL-4B (Amersham Biosciences, Sweden) column chromatograph (3×4 cm) equilibrated with 100 mM phosphate buffer (pH 7.3) containing 200 mM NaCl. The column was immediately eluted with 50 ml of 20 mM phosphate buffer (pH 7.3) before washing first with 50 ml of 10 mM and then 2.5 mM Tris–HCl (pH 6.8). The column was finally eluted with distilled water. Two milliliters fractions were collected and monitored as described above.

RESULTS

Factors Associated with *C*-Glucosyl-Cleaving Activity in a Cell-Free Extract from *B.* sp. MANG Disruption of the bacterial cells by sonication resulted in the loss of *C*-glucosyl-cleaving activity. The activity was restored by the addition of various reagents such as NADH, diaphorase, and dithiothreitol, which were all required for maximum activity (Table 1), to remove oxygen. The activity was decreased by flushing atmospheric air into a cell-free extract even in the presence of the above three factors and was recovered by eliminating air (data not shown), suggesting that reducing conditions are important for activity.

The activity of a cell-free extract was remarkably stimulated by the addition of $MnCl_2$ and moderately by $MgSO_4$ and $CaCl_2$, whereas other metals, such as $ZnCl_2$ and $CuSO_4$, had no effect (data not shown). On the other hand, a chelating agent, EDTA·2Na, markedly inhibited the *C*-glucosyl-cleaving activity in a concentration-dependent manner (up to 81% inhibition at 3 mM).

***C*-Glucosyl-Cleaving Activity of HMW and LMW**

Table 1. Effects of NADH, Diaphorase and Dithiothreitol on *C*-Glucosyl-Cleaving Activity of a Cell-Free Extract

Additives	Relative activity (%)
None	0
NADH and diaphorase	24.2
Dithiothreitol	3.8
NADH, diaphorase and dithiothreitol	100.0

The *C*-glucosyl-cleaving activity in a cell-free extract was determined in the presence of each additive before anaerobic incubation at 37 °C for 2 h.

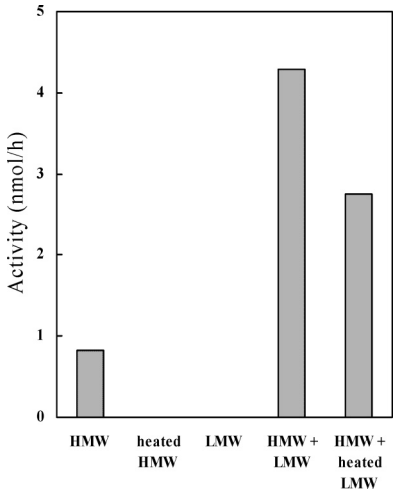

Fig. 1. Requirement for Both HMW and LMW Fractions for *C*-Glucosyl-Cleaving Activity

HMW (260 μg protein/ml) and LMW fractions were obtained from cell-free extract by ultrafiltration and gel filtration chromatography. *C*-Glucosyl-cleaving activity was determined by anaerobic incubation of NADH, diaphorase and dithiothreitol together with HMW (30 μl) and/or LMW (30 μl) fractions at 37 °C for 2 h. Heated samples were treated at 95 °C for 30 min before assaying for activity.

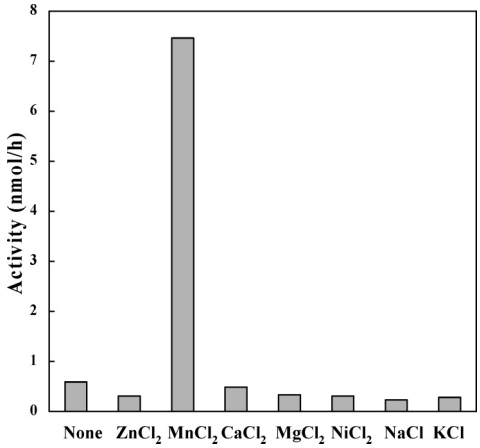

Fig. 2. Effects of Metal Ions on *C*-Glucosyl-Cleaving Activity of HMW Fraction

The effects of various metal ions listed below on *C*-glucosyl-cleaving activity were examined by adding 1 mM of each metal salt to the reaction mixture assay of HMW fraction before anaerobic incubation at 37 °C for 2 h.

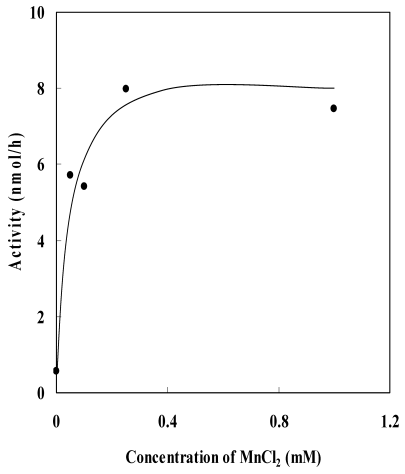

Fig. 3. Effects of $MnCl_2$ Concentration on *C*-Glucosyl-Cleaving Activity in HMW Fraction

To determine the Mn^{2+} ion concentration-dependence of *C*-glucosyl-cleaving activity, the HMW fraction was incubated under standard conditions in the presence of the indicated concentration of $MnCl_2$.

Fractions from a Cell-Free Extract The cell-free extract was separated into HMW and LMW fractions using an ultrafiltration membrane and gel filtration chromatography. The proteins in the HMW fraction were obtained with a yield of 65% of the original protein in the cell-free extract. It was difficult to calculate the yield of the LMW fraction because the identity of the LMW fraction was unknown. (However, we estimated it to be a 7.8% yield based on protein content.) As shown in Fig. 1, the HMW fraction even in the presence of NADH, diaphorase and dithiothreitol was slowly catalyzed to produce norathyriol, whereas none of the *C*-glucosyl-cleavage activity was found in the LMW fraction. However, a combination of both fractions increased the activity by about 5-fold compared to that obtained with the HMW fraction alone. Furthermore, the HMW fraction resulted in loss of the activity after heating at 95 °C for 30 min, whereas the heated LMW fraction still promoted the activity when it was added to the HMW fraction. The enzyme promoting activity of the LMW fraction was not affected by treatment with alkaline protease and trypsin. Protein elimination using a cold methanol method did not influence the cofactor. It showed a molecular weight less than 2000 using Sephadex G-25 gel filtration chromatography.

The effects of various metal ions (1 mM) on activity in the HMW fraction were tested (Fig. 2). Incubation of the HMW fraction with $MnCl_2$ markedly stimulated the activity, whereas other metal ions such as $ZnCl_2$, $CaCl_2$, $MgCl_2$, $NiCl_2$, NaCl, and KCl had no or only a slight effect on the activity. $MnCl_2$ showed a concentration-dependent activation of *C*-glucosyl-cleaving activity, with near maximum activity at a low concentration of 0.25 mM (Fig. 3). The *C*-glucosyl-cleaving activity in the HMW fraction together with $MnCl_2$ was also inhibited by addition of EDTA·2Na, but the activity was restored by increasing the amount of $MnCl_2$ in the reaction mixture (data not shown). In fact, the HMW fraction was essential for the *C*-glucosyl-cleaving reaction and still required the LMW fraction even in the presence of $MnCl_2$ for the highest activity.

Partial Purification of *C*-Glucosyl-Cleaving Enzyme A preliminary study showed that the *C*-glucosyl-cleaving activity was not detectable after first purification with hydrophobic chromatography on Phenyl Sepharose. The activity could only be detected if fractions eluting with 20 mM phosphate buffer (pH 7.3) were combined with fractions eluting with distilled water. We named these two protein fractions protein A and B, respectively. To follow the purification of each protein fraction, preliminary preparation of proteins A and B was necessary for detecting activity.

The *C*-glucosyl-cleaving enzyme was first separated from cell-free extract by ion-exchange chromatography using a DEAE-cellulose column (Fig. 4A). The *C*-glucosyl-cleaving activity was measured by adding NADH, diaphorase, dithiothreitol, $MnCl_2$, and the LMW fraction to the assay system. At least 2 proteins, designated proteins A and B, which were eluted with a linear gradient of NaCl, were separated for *C*-glucosyl cleaving activity. Each protein profile could be suc-

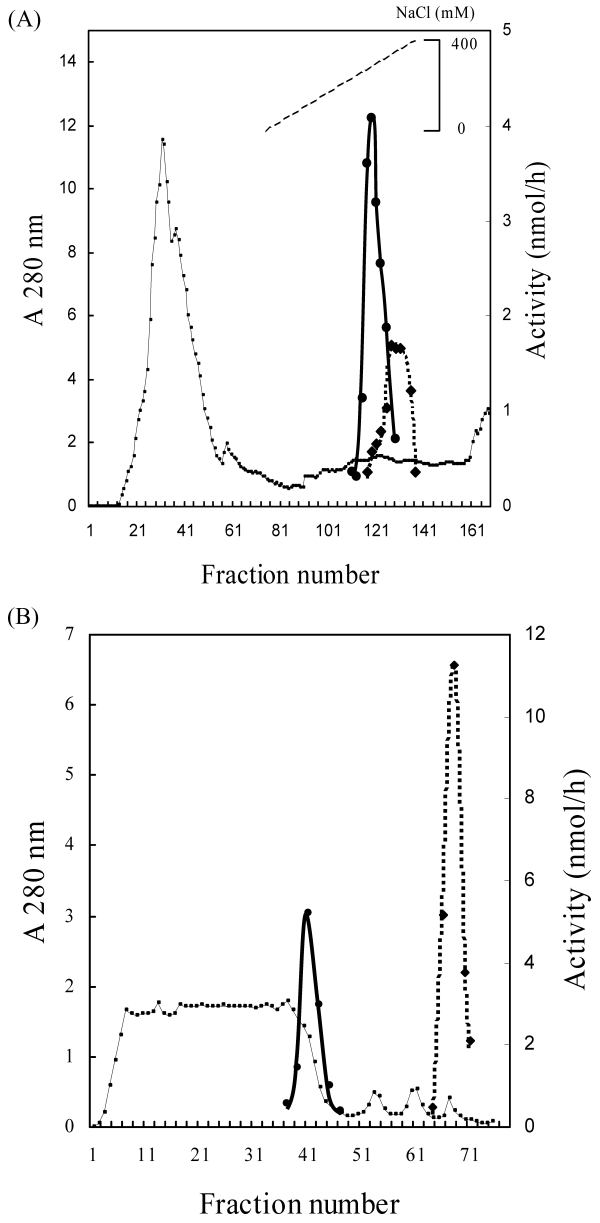

Fig. 4. Purification of *C*-Glucosyl-Cleaving Enzyme by (A) DEAE-Cellulose and (B) Phenyl Sepharose Column Chromatography

(A) The DEAE-cellulose column was eluted with a linear gradient of NaCl (0—400 mM) in 100 mM phosphate buffer (pH 7.3). The active fractions (protein A together with protein B) were pooled and then applied to a Phenyl Sepharose column (B). The column was eluted with 20 mM phosphate buffer (pH 7.3) and then H_2O. Fractions were collected and monitored by measuring the absorbance at 280 nm (■) and *C*-glucosyl-cleaving activity in mangiferin. The *C*-glucosyl-cleaving activity was measured in the presence of NADH, diaphorase, dithiothreitol, $MnCl_2$, optimized LMW fraction, and mangiferin, together with either protein A (11 mg/ml) or protein B (6.6 mg/ml) (prepare beforehand) to determine the other. Both proteins were required for *C*-glucosyl-cleaving activity; proteins A (●) and B (◆) are shown.

cessfully obtained by adding the other partially purified protein to the reaction mixture. For example, partially purified protein A (or B), which was obtained from the preliminary Phenyl Sepharose chromatography, was added to the reaction mixture to determine protein B (or A). Protein A eluted first. Their peaks were near each other, and there was some overlap. If protein A or B was not added to the assay system, a single peak was obtained (data not shown). Since the two proteins did not completely separate from each other, the active fractions (protein A as well as protein B) were pooled together and further purified.

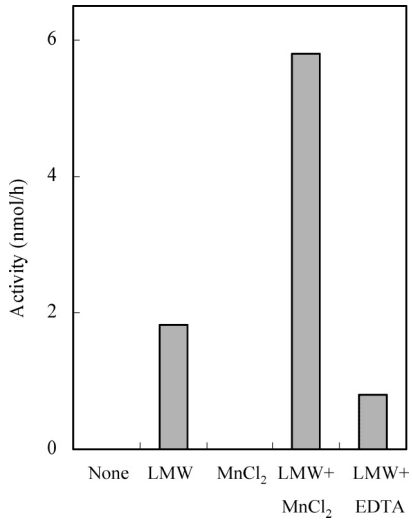

Fig. 5. Effects of Various Factors on the *C*-Glucosyl-Cleaving Activity of Partially Purified Enzymes

The effects of LMW fraction (5 mg of dry weight/ml) and $MnCl_2$ (0.25 mM) on the activity of partially purified enzyme were tested. The reaction mixture consisted of partially purified proteins A (5.4 mg/ml) and B (3.2 mg/ml), NADH, diaphorase, dithiothreitol, and mangiferin. The final concentration of EDTA·2Na used in this experiment was 0.25 mM. The LMW fraction was separated from the cell-free extract with an initial volume of 250 ml by ultrafiltration, freeze-dried to obtain 480 mg residues, and then dissolved in distilled water before using.

The two proteins were clearly separated from each other by subsequent hydrophobic chromatography using a Phenyl Sepharose column (Fig. 4B). Proteins A and B were eluted as a single peak from the column with 20 mM phosphate buffer (pH 7.3) and then distilled water, respectively. The activities of both enzymatic peaks were not observed without addition of the other protein. Moreover, even in the presence of both proteins A and B, $MnCl_2$ and the LMW fraction were absolutely required for the activity (Fig. 5). The specific activities of proteins A and B showed 2-fold and 7-fold increases, respectively, with specific activity of 17 nmol/h/mg protein, and 54 nmol/h/mg protein compared to those obtained by DEAE-cellulose.

DISCUSSION

There are many factors that might affect the properties of the enzyme. A cell-free extract obtained by sonication exhibited cleaving activity by the addition of NADH, diaphorase, and dithiothreitol. This *C*-glucosyl-cleaving ability may be conducted under aerobic conditions, but for the enzymatic activity assay, it was necessary to perform it under strict anaerobic conditions. The *C*-glucosyl-cleavage activity was markedly increased by the addition of $MnCl_2$, not other metal chloride salts, whereas the addition of EDTA·2Na resulted in the loss of the activity, suggesting a requirement for Mn^{2+}.

The HMW fraction was thought to be a part of a protein or enzyme that was involved in the C–C bond cleaving because its activity disappeared after heating, and *vice versa* for the LMW fraction. This result indicates that both parts, the protein and heat-stable low molecular compound, were necessary for restoring activity.

To the best of our knowledge, at the beginning of purification of the *C*-glucosyl-cleaving enzyme by anionic exchange

chromatography using a DEAE-cellulose column, the active fractions were eluted as a single peak with a linear gradient of NaCl. However, after the highly active fractions were pooled and further purified by hydrophobic column chromatography with a Phenyl Sepharose column, the loss of *C*-glucosyl-cleaving activity was observed in individual fractions. It must be noted that the activity was restored by the mixing of two distinct protein fractions, designated as protein A and protein B, eluted from the column with 20 mM phosphate buffer (pH 7.3) and distilled water, respectively. At least two proteins were involved in the *C*-glucosyl-cleaving activity. For the subsequent purification of each protein fraction, preliminary preparation of proteins A and B from this step was necessary. Therefore, we attempted to measure proteins A and B again on a DEAE-cellulose column (Fig. 4A). The *C*-glucosyl-cleaving activity was measured not only by adding NADH, diaphorase, dithiothreitol, $MnCl_2$, and a cofactor in the LMW fraction to the assay system, but also by the addition of protein A for measuring protein B and *vice versa*. Their elution positions on the DEAE-cellulose column were close to each other and some parts even overlapped. Therefore, we previously mistakenly concluded that the enzyme was eluted as a single enzyme peak. The partially purified protein A and B did not transform mangiferin to norathyriol without the addition of an unknown low molecular cofactor in the LMW fraction. Neither protein A nor protein B alone showed any activity, even in the presence of all other factors. In fact, a low molecular weight cofactor passed through the DEAE-cellulose column during the purification of proteins A and B from a cell-free extract, indicating that the low molecular weight cofactor is different from proteins A and B.

In the present study, the activities of partially purified enzymes were decreased by the addition of H_2O_2 (data not shown), which is in contrast to the partially purified *C*-glucosyl-cleaving enzyme from dyer's saffron seedlings, which exhibited peroxidase properties.[24)]

The C–C bonds of *C*-glucosides are difficult to break by chemical and biological methods. Since the *C*-glucosyl-cleaving enzyme for mangiferin is different from *O*-glucosidases,[22)] the mechanism of the enzymatic cleavage of the *C*-glucosyl bond remains a mystery. The mechanism seems to be complex and may not be completed in one step based on the fact many factors are required for activity. This is the first report that examined several factors related to the *C*-glucosyl-cleavage of mangiferin.

Further studies will be needed to clarify many outstanding problems such as the purification and characterization of proteins A, B and a low molecular cofactor in the LMW fraction, as well as the glucose part of the reaction, which should help us understand the mechanism of the reaction more clearly.

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