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The Pathway of Dephosphorylation of *myo*-Inositol Hexakisphosphate by Phytases from Wheat Bran of *Triticum aestivum* L. cv. Nourin #61

Tadao NAKANO,[1] Toshio JOH,[2] Kazumasa NARITA,[2] and Toshiro HAYAKAWA[2]

[1]*Graduate School of Science and Technology, Niigata University, Ikarashi 2-8050 Niigata, Niigata 950-2181, Japan*
[2]*Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Ikarashi 2-8050 Niigata, Niigata 950-2181, Japan*

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Phytases are the primary enzymes responsible for the hydrolysis of phytic acid, *myo*-inositol-1, 2, 3, 4, 5, 6-hexakisphosphate ($InsP_6$). The pathway of hydrolysis of $InsP_6$ by phytase from wheat bran of *Triticum aestivum* L. cv. Nourin #61 is proved in this study. Structures of the intermediates were established by a variety of nuclear magnetic resonance techniques (^1H-, two-dimensional ^1H-^1H coupling-correlation spectra and two-dimensional ^{31}P-^1H correlation spectra), gas chromatography, and bioassay. On the basis of the structures identified, initial hydrolysis of the phosphate ester occurs at the D/L-4 position of $InsP_6$ to yield D/L-Ins (1, 2, 3, 5, 6) P_5. After the dephosphorylation, the pathway of dephosphorylation is divided into two routes. The main route proceeds via D/L-Ins (1, 2, 5, 6) P_4, D/L-Ins (1, 2, 6) P_3 and D/L-Ins (1, 2) P_2, while the minor route proceeds via D/L-Ins (1, 2, 3, 6) P_4, Ins (1, 2, 3) P_3 and D/L-Ins (1, 2) P_2. D/L-Ins (1, 2) P_2 is hydrolyzed at the D/L-1 or 2-position, and finally *myo*-inositol is produced.

Key words: phytase; inositol phosphates; NMR; gas chromatography; dephosphorylation pathway

myo-Inositol hexakisphosphate ($InsP_6$, phytic acid) is known as the most abundant organic form of phosphate in cereal seeds. It has potential in the prevention and treatment of a range of cancers, however, its biological role remains uncertain. $InsP_6$ may simply be a phosphate or inositol store, being metabolized to other inositol phosphates or pyrophosphates. It may be a neurotransmitter, though the effects may be caused by its chelation with calcium. Another possible function arises from its antioxidative and metal chelating properties. $InsP_6$ is of interest to nutritionists, as it can prevent absorption of trace metals. Graf *et al.*[1] showed that chelation of Fe^{3+} to $InsP_6$ prevented the formation of the highly reactive hydroxyl radical ($HO^{\cdot}$) from the superoxide radical anion (O^{2-}) by the iron-catalyzed Haber-Weiss redox cycle.

On the other hand, bioavailability of *myo*-inositol phosphates, the hydrolysis products of $InsP_6$, has been recently reported. Ins (1, 2, 3) P_3, D/L-Ins (1, 2) P_2, and all isomeric $InsP_4$ were evaluated for their ability to depress $HO^{\cdot}$ production by the iron-catalyzed Haber-Weiss reaction. The results demonstrated that a 1, 2, 3-grouping of phosphates in *myo*-inositol was necessary for the depression, and also that D/L-Ins (1, 2) P_2 potentiated $HO^{\cdot}$ production.[2] Tarnow *et al.*[3] described how D-Ins (1, 2, 6) P_3 has previously been shown to reduce burn-induced oedema formation and the inflammation involved in the pathophysiology of progressive ischaemia.

Phytase (*myo*-inositol-hexakisphosphate phosphohydrolase) which catalyzes the hydrolysis of $InsP_6$ into inositol mono-, di-, tri-, tetra-, and penta- phosphates ($InsP_{1-5}$) and inorganic phosphates (Pi) has been investigated in various plants and microorganisms, and also the hydrolysis pathway of a few phytases has been investigated. By an alkaline phytase from lily pollen, initial dephosphorylation occurs at the D-5 position of $InsP_6$ to yield the symmetrical Ins (1, 2, 3, 4, 6) P_5, and finally to yield Ins (1, 2, 3) P_3.[4] Paramecium phytase degrades $InsP_6$ by stepwise dephosphorylation via D/L-Ins (1, 2, 3, 4, 5) P_5, D/L-Ins (1, 2, 3, 4) P_4 and Ins (1, 2, 3) P_3 finally to D/L-Ins (1, 2).[5]

Dephosphorylation of $InsP_6$ by phytase from wheat bran was investigated by Tomlinson & Ballou[6] and Lim & Tate.[7] But they could not identify all intermediate *myo*-inositol phosphates, and the pathway of dephosphorylation of $InsP_6$ has not been described completely. Their imcomplete results are probably attributable to the use of unpurified enzyme.

In a previous study, we purified two phytases (PHY1 and PHY2) homogeneously from wheat bran of *Triticum aestivum* L. cv. Nourin #61.[8] PHY1

To whom Correspondencse should be addressed: Toshio JOH (Tel: 81-25-262-6640; Fax: 81-25-262-6854; E-mail: joh@agr.niigata-u.ac.jp)

and PHY2 have high affinity for $InsP_6$ and show low sensitivity for inhibition by Pi. Therefore, we tried to identify the hydrolysis pathway of $InsP_6$ by use of the enzymes.

In this paper, we described the specificity of hydrolysis of $InsP_6$ by purified phytases from wheat bran. The structure of the intermediate *myo*-inositol phoshates was analyzed by 1D- and 2D-NMR techniques and gas chromatography and the final product was identified by 2D-NMR techniques and bioassay method using *Schizosaccharomyces pombe*.[9]

Materials and methods

Purification of phytases from *Triticum aestivum* L. cv. Nourin #61. Wheat bran was obtained from Maruei Seifun Co. Ltd. (Niigata, Japan) and stored at 1–4°C. The cultivated variety was *Triticum aestivum* L. cv. Nourin #61 produced in Japan. Two phytases (PHY1 and PHY2) were purified homogeneously from the bran by the method of Nakano *et al.*[8]

Stability of phytases. The stability of phytases at 37°C was investigated. After 4 ml of 0.25 M acetate buffer (pH 5.5) containing 2.0 U of phytases was incubated at 37°C for 0, 24, 48, and 72 h, samples of the solution were withdrawn and the residual activity was examined as follows. The reaction mixture containing the enzyme solution and 15 mM $InsP_6$ was incubated at 37°C for 15 min, and the reaction was stopped by adding 10% (w/v) trichloroacetic acid in amounts equal to reaction mixture, and assayed for inorganic phosphate by the method of Fiske & Subbarow.[10]

One unit of enzyme activity was defined as the amount of enzyme that liberates 1 mmol of Pi from the substrate per minute under the assay conditions described in our previous paper.[8]

Hydrolysis of $InsP_6$ by phytase. $InsP_6$ was hydrolyzed by the purified enzyme in the following manner. The reaction mixture contained 250 mM acetate buffer (pH 5.5), 15 mM Na-$InsP_6$ (Sigma, U. S. A.), and 0.5 U/ml enzyme. Since the enzymes was inactivated about 50% by incubating at 37°C for 24 h, the enzyme solution corresponding to the lost activity was added to reaction mixture after 24 and 48 h. The reaction mixture was incubated at 37°C for 1, 6 and 72 h, and the reaction was stopped by adding 10% (w/v) trichloroacetic acid in amounts equal to the reaction mixture. The solution was immediately lyophilized, and stored in desiccator with silica gel at room temperature.

Separation of inositol phosphates. Inositol phosphates were separated by a modification of the procedure described by Radenberg *et al.*[11] All steps were done at 4°C. The lyophilized sample was dissolved in double distilled water and the pH adjusted to 7. The solution was put in at a flow rate of 2 ml/min to a column of Q-Sepharose (chloride form, ϕ1.5 cm × 115 cm). The column was thoroughly washed with 400 ml of double distilled water, and then inositol phosphates were eluted from the column with a linear gradient of 1 l of double distilled water in the mixing chamber and 1 l of 0.4 M HCl in the reservoir, and finally with 200 ml of 0.5 M HCl. The eluate was collected in 10-ml fractions. Samples of each fraction were assayed for inorganic phosphate by the method of Fiske & Subbarow[11] rapidly or after autoclaving 121°C, 30 min for the measurement of inorganic or organic phosphate compounds, respectively. Fractions containing organic phosphate compounds were immediately lyophilized to remove the HCl and analyzed by NMR and gas chromatography as described below.

NMR Spectroscopy. Spectra were recorded on a 400-MHz Bruker DPX-400 spectrometer operating at 400.13 MHz for ^1H, and at 161.96 MHz for ^{31}P. Samples were prepared by the method of Barrientos *et al.*,[4] and finally dissolved in 0.7 ml of D_2O. All spectra were recorded at 25°C. Chemical shifts of ^1H were referenced to the residual proton absorption of the solvent, D_2O (4.67 ppm). Typically, 64 scans with a recycle delay of 11 s between acquisitions were collected. The residual H_2O resonance was suppressed by a 2-s selective presaturation pulse. Two-dimensional ^1H-^1H coupling-correlation spectra were taken by using the COSY technique method of Bax & Freeman[12] and two-dimensional ^{31}P-^1H correlation spectra was done by using the method of Laussmann *et al.*[13]

Sample preparation for gas chromatography. To a lyophilized sample, 2 ml of dry diethyl ether was added and mixed well, and then about 1 g of dried molecular sieve was added. After gentle stirring for 10 min, the molecular sieve was removed and the precipitate of inositol phosphates was collected by centrifugation at 3,000 × *g* for 10 min, dried by N_2 gas, and then dissolved in 150 μl of TMS-PZ. Insoluble matter was collected by centrifugation at 3,000 × *g* for 10 min and discarded. The supernatant was displaced to a new glass centrifuge tube and left in the dark in a desiccator with silica gel overnight at room temperature. After drying by N_2 gas, the dried matter was dissolved in 1 ml of dry diethyl ether containing 10% methanol at 0°C, and then diazotized-methane solution was added to this solution. The diazotized matter was dried by N_2 gas, dissolved in 100 μl of TMS-PZ, and left in the dark in a desiccator with silica gel overnight at room temperature. After drying by N_2 gas, the dried matter was dissolved in 100 μl of ethyl acetate.

Analysis by gas chromatography. Standard *myo*-inositol-2-monophosphate (95%) and D/L-*myo*-inositol-1-monophosphate (75%) were obtained from Sigma (U. S. A.).

The TMS-derived InsP was analyzed with a Hitachi gas chromatograph (model G-5000A) equipped with a flame-ionization detector and a Chirasil-Val capillary column (25 m, 0.25 mm). The operation conditions were as follows: injector, 250°C; detector, 250°C; nitrogen, 30 ml/min; air, 400 ml/min; hydrogen, 40 ml/min; temperature program: 140°C for 30 min and then 4°C per min to 190°C and 190°C for 10 min.

Bioassay for inositol. *myo*-inositol was assayed by a bioassay method using *Schizosaccharomyces pombe*.[9]

Results

We examined the pathway of dephosphorylation of $InsP_6$ by two phytase isozymes, PHY1 and PHY2. The results showed that the hydrolysis pathways of the isozymes are not different, therefore, only the pathway of PHY1 is reported here.

Hydrolysis and isolation of inositol phosphates

When the stability of PHY1 was examined at 37°C, the activity after 24, 48, and 72 h was about 50%, 25%, and 10% of 0-h, respectively (Fig. 1).

The inactivation of enzyme lowers the hydrolysis rate and prevents the identification of the final products. Consequently, the enzyme solution was added to reaction mixture after 24 and 48 h.

Inositol phosphates, hydrolysis products by PHY1, were separated by Q-Sepharose FF column chromatography. As shown in Fig. 2 (a), five peaks containing phosphate were detected for 1-h hydrolysis product and only one of them, pool G, contained inorganic phosphate. The elution patterns of 6-h and 72-h hydrolysis products are shown in Fig. 2 (b) and (c), respectively. As the hydrolysis of $InsP_6$ progressed, the fructions containing inositol phosphates were eluted early. This shows that a decrease of the number of phosphate ester bonds to inositol lowered adsorption to Q-Sepharose FF.

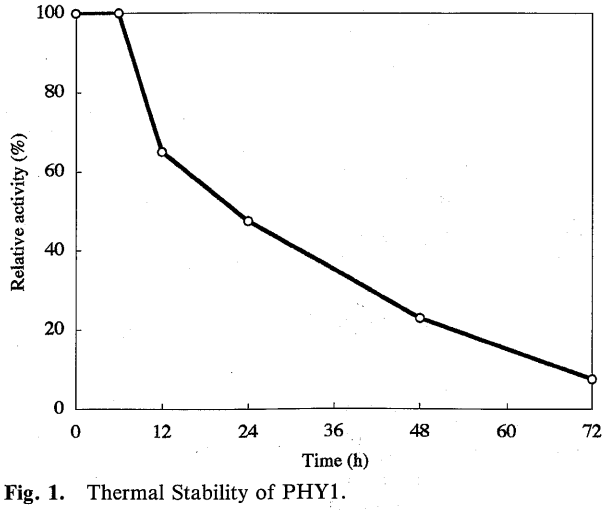

Fig. 1. Thermal Stability of PHY1.
The enzyme was incubated at 37°C and after given periods, the activity was assayed.

NMR identification of inositol phosphate isomers

Pools A, B, C, D, E, and F in Fig. 2 were analyzed by ^1H-NMR spectrum, two-dimensional ^{31}P-^1H correlation spectrum and ^1H-^1H COSY spectra.

The ^1H-NMR spectrum of pool A consists of three sets of protons (α, β, γ) in the ratio 1:2:3 (Fig. 3). Consistent with observations that equatorial proton

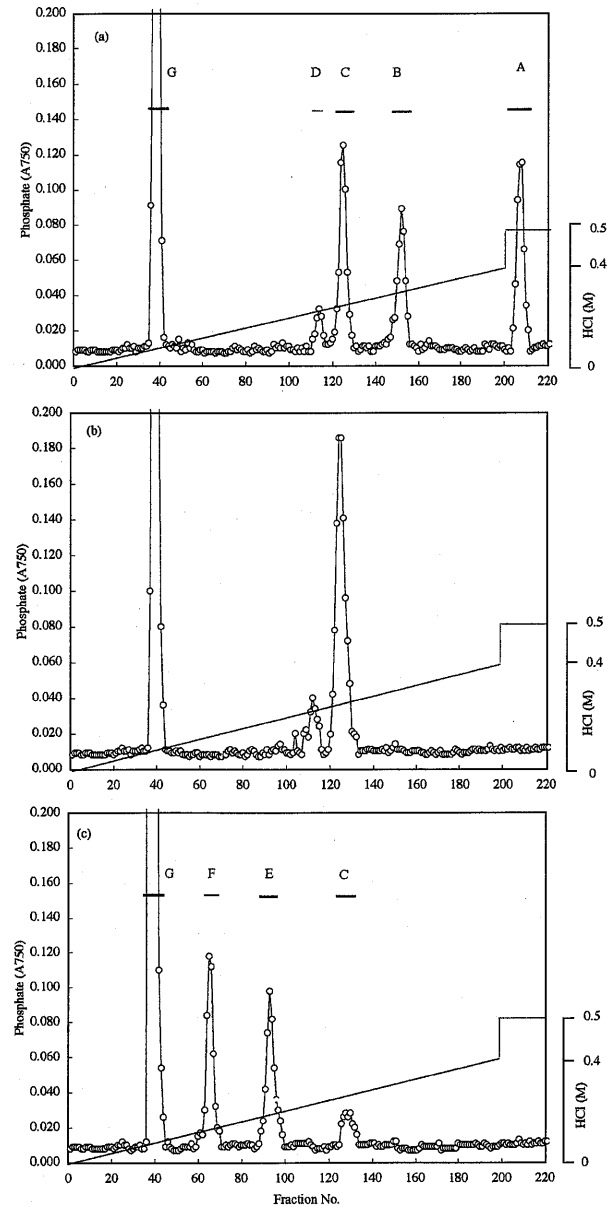

Fig. 2. Separation of InsP by Q-Sepharose FF.
After $InsP_6$ was hydrolyzed for 1 h (a), 6 h (b) and 72 h (c) by PHY1, the products were separated by the column. Bars (A-G) show the recovered and analyzed fractions.

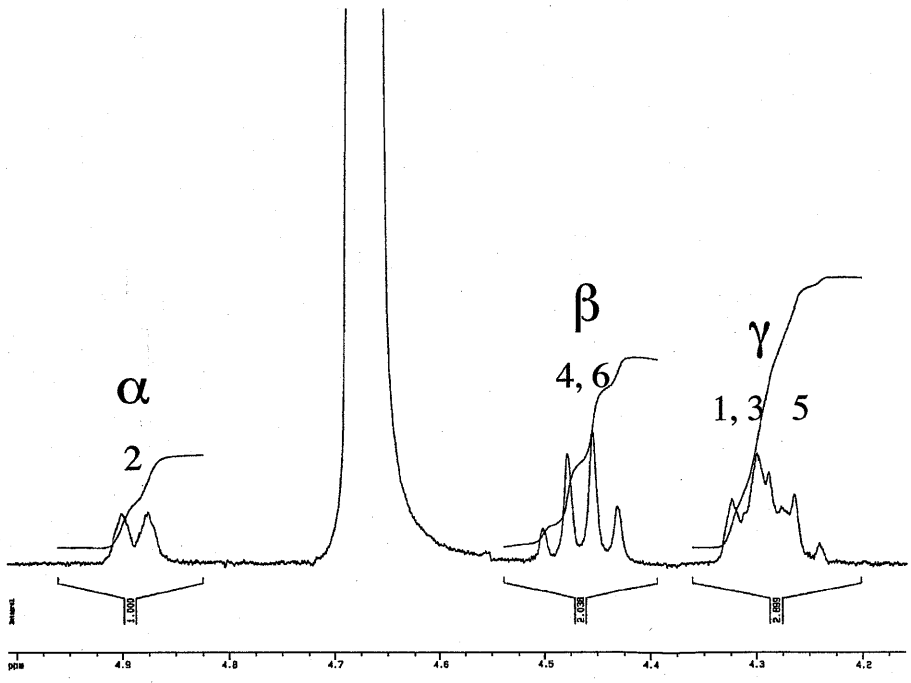

Fig. 3. ^{1}H-NMR Spectrum of Pool A.
Inositol phosphates were derived from the action of PHY1 on $InsP_6$, and separated by Q-Sepharose FF column chromatograpy.

resonances are generally downfield of axial protons, the one equatorial proton H (2) at 4.88 ppm, is split into a triplet of doublets, which appears as broad doublets because of one large $J_{H(2)-P}$ coupling and two small J_{ax-eq} vicinal coupling to H (1) and H (3). The enantiotopic H (4) and H (6) protons at 4.47 ppm are split into quartets due to two J_{ax-ax} vicinal couplings with protons on either side and one J_{H-P} coupling, all of similar magnitude. The enantiotopic H (1) and H (3) protons overlap with the H (5) proton at approximately 4.27 ppm. The H (5) proton is split into a quartet because of two vicinal J_{ax-ax} coupling and J_{H-P} coupling, all of similar magnitude. The H (1) and H (3) protons are split into broad triplets because of one J_{ax-ax} coupling and a J_{H-P} coupling of similar magnitude and a small J_{ax-eq} coupling with the H (2) proton. The ^{1}H-NMR spectrum of pool A was as same as $InsP_6$, therefore, pool A is identified as $InsP_6$.

Two-dimensional ^{31}P-^{1}H correlation spectrum of material from pool B is shown in Fig. 4. A two-dimensional ^{31}P-^{1}H correlation spectrum would provide information on H-P connectivities and thereby provide additional proof of structural assignment. Figure 4 shows H-P connectivities at a, b, c, and d protons and none for the e proton. Dephosphorylation would lead to upfield shift of the proton by approximately 0.5 to 1.0 ppm,[14] and significantly affect the splitting pattern of the inositol ring proton because of loss of the proton-phosphorous coupling. Compared with Fig. 3, proton β (4.47 ppm), proton b (4.42 ppm), which coupled phosphate A is estimated H (4) or H (6), and phosphate A is one phosphate, therefore, proton e which decoupled phosphate is estimated H (4) or H (6). H (1) or H (3) proton overlap with the H (5) proton at 4.11 ppm (proton d). These suggest that the pool B is D/L-Ins (1, 2, 3, 5, 6) P_5.

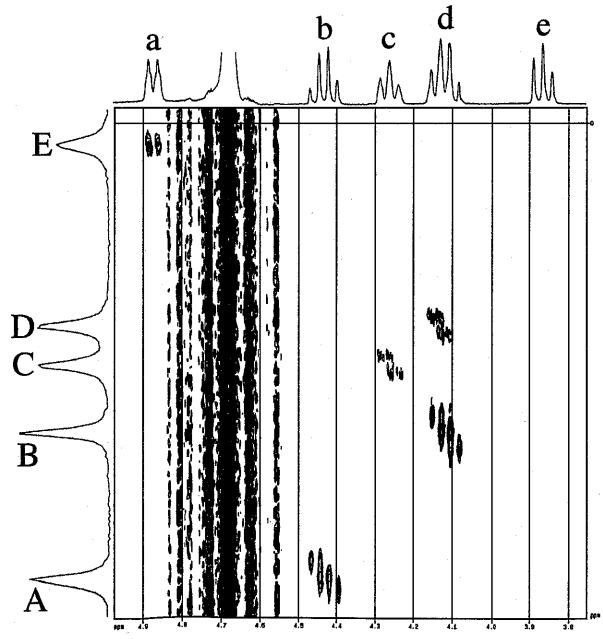

Fig. 4. Two-dimensional ^{31}P-Decoupled HMBC of Pool B with Attached ^{1}H and Proton-decoupled ^{31}P Spectrum.
Inositol phosphates were obtained as described in Fig. 3. Spectrum was recorded on a 400-MHz Burker DPX-400 spectrometer operating at 400.13 MHz for ^{1}H, and at 161.96 MHz for ^{31}P.

Figure 5 (A) shows the ^{1}H-^{1}H COSY spectrum of material from pool C. Proton c and f are coupled proton a, proton a is H (2), therefore, c, and f are H

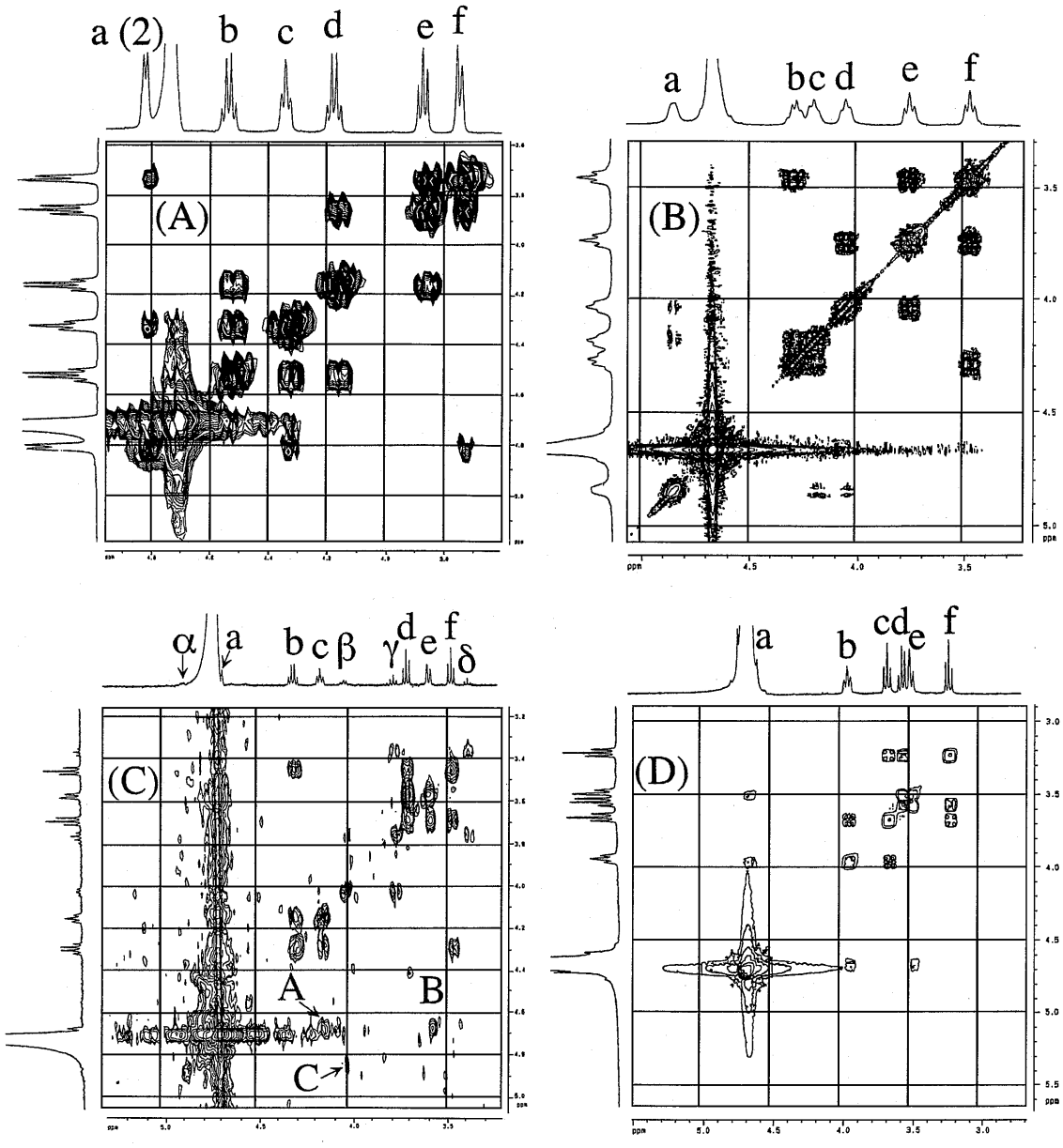

Fig. 5. Two-dimensional ^1H-^1H COSY Spectra of Inositol Phosphates Derived from the Action of PHY1 on $InsP_6$ with Attached ^1H Spectra.

Spectra were recorded on a 400-MHz Burker DPX-400 spectrometer operating at 400.13 MHz for ^1H. (A), pool C; (B), pool D; (C), pool E; (D), pool F.

(1) and H (3). Proton b is coupled proton c and d, and proton d is coupled proton e, therefore, proton d is H (5), and proton b and e are H (4) and H (6). H (1) or H (3), proton f, is shifted 0.36 ppm, and the change in splitting pattern from a triplet to sharp doublet, therefore, dephosphorylation is occurred. These suggest that the pool C is D/L-Ins (1, 2, 5, 6) P_4.

Figure 5 (B) shows the ^1H-^1H COSY spectrum of material from pool D. Proton c and d are coupled proton a, proton a is H (2), therefore, c and d are H (1) and H (3). Proton e is coupled to proton d and f, and proton f coupled to proton b, as a result proton b and e are H (4) and H (6), and proton f is H (5). H (5), proton f, is shifted 0.66 ppm, and the change in splitting pattern from a quartet to triplet, therefore, dephosphorylation is occurred. These suggest that the pool D is D/L-Ins (1, 2, 3, 6) P_4.

Figure 5 (C) shows the ^1H-^1H COSY spectrum of material from pool E. Two type of inositol phosphates exist in this pool, because the protons of a, b, c, d, e, and f (InsP-A), as well as the protons of α, β, γ, and δ (InsP-B) are coupled to each other, but InsP-A and InsP-B are not coupled to each other. Proton a (H (2) resonance of InsP-A) overlap with water resonances. Proton c and e are coupled to H (2) (Fig. 5 (C), A and B), therefore, c and e are H (1) and H (3). Proton is coupled to proton b, and proton b is

coupled to proton f, as a result proton b and d are H (4) and H (6), and proton f is H (5). Compared with Fig. 5 (A), H (5) is shifted 0.62 ppm, and the change in splitting pattern from a quartet to sharp triplet, therefore, dephosphorylation has occurred. These suggest that InsP-A is D/L-Ins (1, 2, 6) P_3. Proton β is coupled to H (2) (proton α) (Fig. 5 (C), C) and proton γ, and proton γ coupled to proton δ, as a result proton β is H (1) and H (3), proton γ is H (4) and H (6), and proton δ is H (5). Compared with Fig. 5 (B), proton b, H (4) or H (6) is shifted 0.53 ppm, and the change in splitting pattern from a quartet to triplet, therefore, dephosphorylation has occurred. These suggest that InsP-B is Ins (1, 2, 3) P_3.

Figure 5 (D) shows the ^1H-^1H COSY spectrum of material from pool F. Proton a, H (2), overlap with water resonances. Proton b and e are coupled to H (2), therefore, b and e are H (1) and H (3). Proton c is coupled to proton b and f, proton f is coupled to proton c and d, as a result proton c and d are H (4) and H (6), and proton f is H (5). Proton c and d were dephosphorylated, becouse proton c and d are eiter a triplet, so these suggest that the pool F is D/L-Ins (1, 2) P_2.

Identification of inositol monophosphate by gas chromatography

Figure 6 (A) is a gas chromatogram of standard Ins (2) P_1. One large peak and two small peaks appeared. Judging from the peak size, the large peak (*Rt* 46.18 min) was considered to be Ins (2) P_1. Small peaks (*Rt* 47.12 and 47.75 min) are probably other inositol phosphates. Figure 6 (B) is a gas chromatogram of standard D/L-Ins (1) P_1. Three peaks were detected. Compared with the gas chromatogram of standard Ins (2) P_1, the peak of *Rt* 46.17 min was considered to be Ins (2) P_1. Other two peaks, compared with the analytical report of enantiomers of inositol monophosphate by Leavitt & Sherman,[15] peaks of *Rt* 47.15 min and 47.8 min were considered to be L-Ins (1) P_1 and D-Ins (1) P_1, respectively. This results show that the small peaks (*Rt* 47.12 and 47.75 min) of Fig. 6 (A) are L-Ins (1) P_1 and D-Ins (1) P_1, respectively. When the material from pool G was analyzed, the chromatogram showed three peaks; *Rt* 46.25 min, 47.23 min and 47.90 min (Fig.6 (C)). This result suggests that pool G contains Ins (2) P_1, L-Ins (1) P_1 and D-Ins (1) P_1, respectively. The rate of peak areas was 3: 1: 1, respectively.

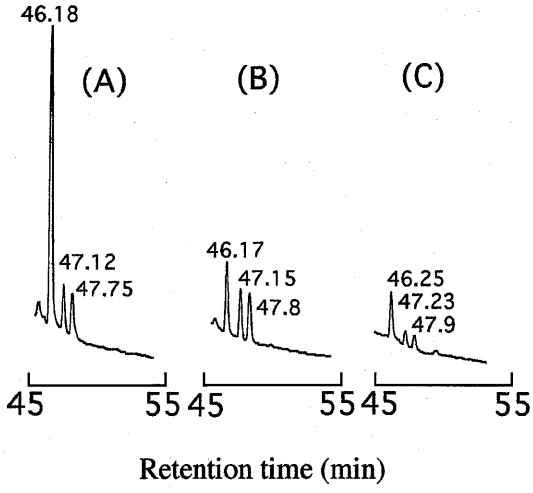

Fig. 6. Gas Chromatogram of Inositol Monophosphate on a Chirasil-Val Column.

The methylated and trimethylsilylated inositol monophosphate was analyzed as described in materials and methods.

(A), Standard as Ins (2) P_1; (B), Standard as D/L-Ins (1) P_1; (C), pool G.

Analysis of non-adsorbed fractions

Non-adsorbed fractions from Q-Sepharose of 72-h hydrolysis products were collected in 10-ml fractions. Each fraction was rapidly assayed for inorganic phosphate and *myo*-inositol by the bioassay method. Inorganic and organic phosphate was not detected in any fractions, however, *myo*-inositol was detected in fractions 15 to 22 (Fig. 7). When the fractions from 20 to 21 were lyophilized, and analyzed by ^1H-^1H coupling-correlation spectra, the fractions were confirmed to contain *myo*-inositol (data not shown).

Discussion

In this study, we discovered the hydrolysis pathway of $InsP_6$ by phytases purified homogeneously from wheat bran of *T. aestivum* L. cv. Nourin #61.

In order to describe the complete hydrolysis pathway, it is necessary to take into consideration an inhibition of the activity by liberated Pi or an inactivation of the enzyme during the reaction. Therefore, the stability of the enzymes was examined under the

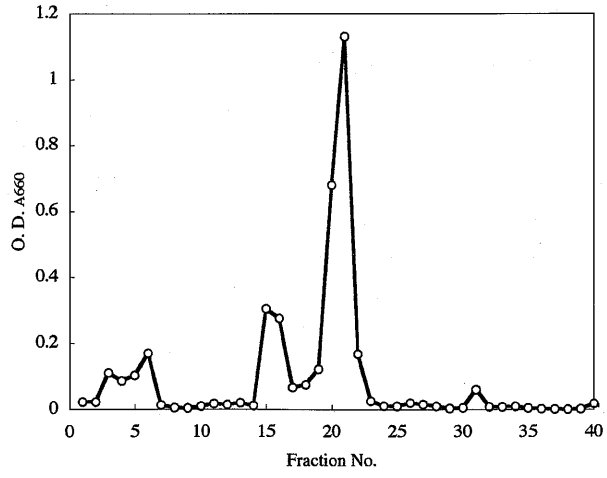

Fig. 7. Detection of Inositol in Non-adsorbed Fractions of Q-Sepharose FF Column Chromatography.

The 72-h hydrolyzed product was separated by the column and *myo*-inositol was assayed for the non-adsorbed fractions (No. 1–40) by bioassay.

reaction conditions. Since the results showed that the activity is reduced to half after 24 h, we added the enzyme corresponding to inactivated amounts to the reaction mixture after 24 h and 48 h. The rate of hydrolysis of $InsP_6$ reached 85% after 72 h by this procedure (data not shown). PHY1 and PHY2, which were used in this study, show low sensitivity for inhibition by Pi compared to other phytases.[8] Consequently, the high hydrolysis rate may reflect the low sensitivity. The properties of the enzymes were very helpful in analyzing the complete hydrolysis pathway.

NMR techniques allowed us to establish unambiguously the structures of *myo*-inositol and *myo*-inositol phosphates. However, NMR cannot distinguish between enantiotopic protons, for example, at this time we cannot say which enantiomer of D/L-Ins (1, 2, 5, 6) P_4 is produced. We achieved the direct separation of enantiomers of L-Ins (1) P_1 and D-Ins (1) P_1 using Chirasil-Val capillary column gas chromatography. The result showed that Ins (2) P_1, L-Ins (1) P_1 and D-Ins (1) P_1 were produced as Ins P_1, and quantities of L-Ins (1) P_1 and D-Ins (1) P_1 were almost equality. Therefore, each inositol phosphate contains both the D and L enantiomers.

On the basis of the structures of the hydrolysis products identified, we found that the pathway of dephosphorylation of $InsP_6$ by phytases from wheat bran (PHY1 and PHY2) (Fig. 8). Because the elution patterns in Q-Sepharose FF of PHY2 was completely identical with that of PHY1, the pathway of dephosphorylation by PHY2 was considered to be the same as that of PHY1. The enzyme nomenclature recommended by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB) recognizes two phytases, 3-phytase (EC 3. 1. 3. 8) which initiates the dephosphorylation of $InsP_6$ at D-3 position and 6-phytase (EC 3. 1. 3. 26) which initiates the dephosphorylation of $InsP_6$ at L-6 position.[16] PHY1 and PHY2 initially hydrolyzed the phosphate at the D/L-4 (D/L-6) position of $InsP_6$. PHY1 and PHY2 belong to a group of 6-phytase (EC 3. 1. 3. 26). After dephosphorylation at the D/L-4 (D/L-6) position of $InsP_6$, the pathway of dephosphorylation is divided into two routes. Since the quantity of D/L-Ins (1, 2, 5, 6) P_4 (pool C in Fig. 2) was larger than that of D/L-Ins (1, 2, 3, 6) P_4 (pool D in Fig. 2), the main route proceeds via D/L-Ins (1, 2, 5, 6) P_4, D/L-Ins (1, 2, 6) P_3 and D/L-Ins (1, 2) P_2, while the minor route proceeds via D/L-Ins (1, 2, 3, 6) P_4, Ins (1, 2, 3) P_3 and D/L-Ins (1, 2) P_2. At the D/L-1 or 2-position of D/L-Ins (1, 2) P_2 was hydrolyzed, and finally produced *myo*-inositol.

Dephosphorylation of $InsP_6$ by phytase from wheat bran was investigated by Tomlinson & Ballou[6] and Lim & Tate.[7] Lim & Tate[7] described about the pathways of dephosphorylation of $InsP_6$ by phytase F_2. Main route proceeded via D-Ins (1, 2, 3, 5, 6) P_5, D-Ins (1, 2, 3, 6) P_4, Ins (1, 2, 3) P_3 and D/L-Ins (1, 2) P_2. At the D/L-1-position, D/L-Ins (1, 2) P_2 was hydrolyzed, and finally produced *myo*-inositol. This route was similar to the minor route of PHY1 and PHY2. On the other hand, Tomlinson & Ballou[6] identified L-Ins (1, 2, 5, 6) P_4, L-Ins (1, 2, 6) P_3, Ins (1, 2, 3) P_3, L-Ins (1, 2) P_2, L-Ins (1) P_1, Ins (2) P_1 and

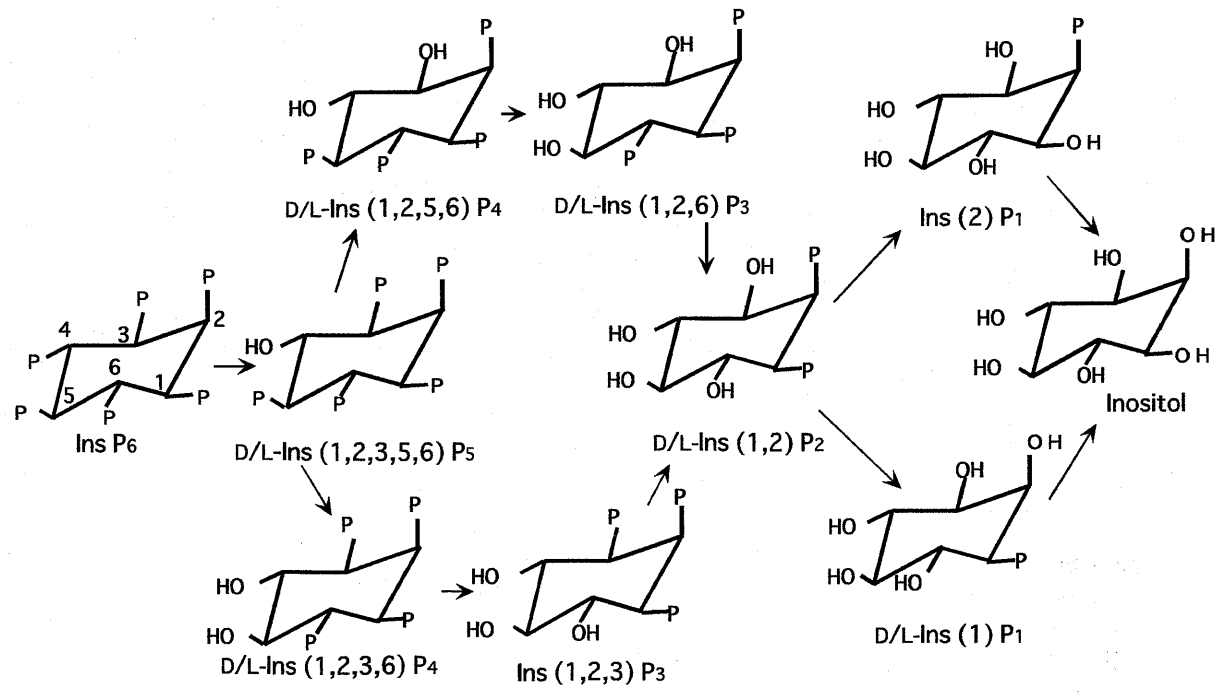

Fig. 8 The Pathway of Dephosphorylation of $InsP_6$ by Phytase from Wheat Bran of *T. aestivum* L. cv. Nourin #61.

myo-inositol as degradation products of $InsP_6$ by crude phytase. Though they could not identified precursors of L-Ins (1, 2, 5, 6) P_4 and Ins (1, 2, 3) P_3, it is presumed that L-Ins (1, 2, 3, 5, 6) P_5, and L-Ins (1, 2, 3, 6) P_4 or L-Ins (1, 2, 3, 5) P_4 are their precursors, respectively. The pathway of dephosphorylation of $InsP_6$ speculated by Tomlinson & Ballou[6] was similar to that of PHY1 and PHY2. But the D and L enantiomers of inositol phosphates coexist in the hydrolysis products by PHY1 and PHY2, and in this respect, both pathways are different.

The pathways of dephosphorylation of $InsP_6$ were investigated about several phytases besides wheat bran. Paramecium phytase was initially hydrolyzed L-6 (D-4) position, and finally to D/L-Ins (1, 2) P_2,[5] *E. coli* phytase P2 was also hydrolyzed at L-6 (D-4) or L-1 (D-3) position, and finally to D/L-Ins (2, 4, 5) P_3, Ins (1, 2, 3) P_3, D/L-Ins (1, 2, 4) P_3 or D/L-Ins (1, 2, 5) P_3,[17] maize root phytase (Phy1.1) was also hydrolyzed L-6 (D-4) position, and finally to D/L-Ins (1, 2, 5, 6) P_4,[18] and alkaline phytase from lily pollen was initially hydrolyzed in the D/L-5 position, finally to Ins (1, 2, 3) P_3.[4] In general, acid phytases initially hydrolyze $InsP_6$ at the L-6 (D-4) position, and the next hydrolysis occurs adjacent to the free hydroxyl group and, therefore, the initial position of hydrolysis is a major determinant of subsequent points of attack. Attack adjacent to a free hydroxyl group could be due to greater nucleophilicity of the hydroxyl oxygen compared to the ester bond oxygen.[19] Although the rate of removal of the second and third phosphates are significantly lower, possibly due to reduced specificity for $InsP_5$ and $InsP_4$ and inhibition by the phosphate released.[4] Actually, most of acidic phytases have been repoted to be able to hydrolyze only up to $InsP_3$ or $InsP_2$ *in vitro*. However, PHY1 and PHY2 finally produced *myo*-inositol. This result demonstrates that PHY1 and PHY2 are very useful enzymes for hydrolyzing $InsP_6$ due to the high affinity for $InsP_6$ and the low sensitivity for inhibition for Pi, which we described in previous paper.[8]

Recently, bioavailabilities of $InsP_{1-5}$ are expected for medical and food science. But, all bioavailabilities of $InsP_{1-5}$ have not been elucidated and further studies are still neeeded. PHY1 and PHY2 can be obtained easily from wheat bran, an industrial waste, and used for the preparation of $InsP_{1-5}$. Now, we are trying to find new bioavailabilities of the prepared $InsP_{1-5}$.

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