UDP-sugar Pyrophosphorylase with Broad Substrate Specificity Toward Various Monosaccharide 1-Phosphates from Pea Sprouts*

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Toshihisa Kotake‡§, Daisuke Yamaguchi‡, Hiroshi Ohzono‡, Sachiko Hojo‡, Satoshi Kaneko‡, Hide-ki Ishida#, and Yoichi Tsumuraya‡‡

From the ‡Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan, ¶Biological Function Division, National Food Research Institute, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan, and the †Noguchi Institute, 1-8-1 Kaga, Itabashi-ku, Tokyo 173-0003, Japan

UDP-sugars, activated forms of monosaccharides, are synthesized through de novo and salvage pathways and serve as substrates for the synthesis of polysaccharides, glycolipids, and glycoproteins in higher plants. A UDP-sugar pyrophosphorylase, designated PsUSP, was purified about 1,200-fold from pea (Pisum sativum L.) sprouts by conventional chromatography. The apparent molecular mass of the purified PsUSP was 67,000 Da. The enzyme catalyzed the formation of UDP-Glc, UDP-Gal, UDP-glucuronic acid, UDP-L-arabinose, and UDP-xylose from respective monosaccharide 1-phosphates in the presence of UTP as a co-substrate, indicating that the enzyme has broad substrate specificity toward monosaccharide 1-phosphates. Maximum activity of the enzyme occurred at pH 6.5–7.5, and at 45 °C in the presence of 2 mM Mg2⁺. The apparent Kₘ values for Glc 1-phosphate and L-arabinose 1-phosphate were 0.34 and 0.96 mM, respectively. PsUSP cDNA was cloned by reverse transcriptase-PCR. PsUSP appears to encode a protein with a molecular mass of 66,040 Da (600 amino acids) and possesses a uridine-binding site, which has also been found in a human UDP-acetylhexosamine pyrophosphorylase. Phylogenetic analysis revealed that PsUSP can be categorized in a group together with homologues from Arabidopsis and rice, which is distinct from the UDP-Glc and UDP-N-acetylhexosamine pyrophosphorylase groups. Recombinant PsUSP expressed in Escherichia coli catalyzed the formation of UDP-sugars from monosaccharide 1-phosphates and UTP with efficiency similar to that of the native enzyme. These results indicate that the enzyme is a novel type of UDP-sugar pyrophosphorylase, which catalyzes the formation of various UDP-sugars at the end of salvage pathways in higher plants.

It is highly probable that the relative amounts and the architecture of cell wall polysaccharides and of the sugar moieties of glycolipids and glycoproteins in higher plants are regulated by the level of nucleotide sugars available, as well as by the levels of glycosyltransferases that incorporate monosaccharide units from respective nucleotide sugars into the polymers. Activated nucleotide sugars that serve as glycosyl donors for these polymers in higher plants are generated through de novo pathways, in which various UDP- and GDP-sugars are produced through sequential interconversions from UDP-Glc and GDP-Man as the starting substrates (1). In the salvage pathways, alternative routes to synthesize nucleotide sugars, free monosaccharides released during the degradation of polysaccharides and glycoconjugates are first phosphorylated by the action of monosaccharide kinases, then converted into nucleotide sugars by the action of pyrophosphorylases in the presence of the respective nucleotide triphosphates as co-substrates. Pyrophosphorylases principally catalyze both the following forward (synthesis of NDP-sugars) and reverse (pyrophospholytic) reactions (1): monosaccharides 1-phosphates + NTP ⇌ NDP-sugars + PP₃, where NTP and NDP are nucleoside triphosphates and diphosphates, respectively. Various kinases and pyrophosphorylases are involved in the salvage pathways and seem to regulate carbon flux, together with enzymes in de novo pathways, leading to modification of the architecture of polysaccharides and glycoconjugates (1). Indeed, an L-arabinose-sensitive ara mutant of Arabidopsis is known to be deficient in L-arabinokinase (ARA1, EC 2.7.1.46) and cannot reutilize free L-arabinose (L-Ara)₁² because of the block in the L-arabinose salvage pathway. Administration of exogenous L-Ara to the mutant causes severe growth inhibition, providing evidence of the importance of the salvage pathways for normal growth and development of plants (2). Further evidence came from the case of the mur1 mutant of Arabidopsis with dwarf phenotype, which shows a decrease in L-fucose (L-Fuc) content in rhamnogalacturonan II because of a defect in the conversion of GDP-Man to GDP-L-Fuc in the de novo pathway (3). Since the phenotype is rescued by addition of exogenous L-Fuc to the mur1 mutant, a normal level of GDP-L-Fuc seems to be supplied by the salvage pathway via L-Fuc 1-phosphate synthesis (1). These observations indicate that the network of the synthesis of nucleotide sugars is regulated through concerted actions of various enzymes involved in both de novo and the salvage pathways.

In cell wall polysaccharides, L-Ara and xylose (Xyl) are the predominant pentose constituents found in pectins, arabinogalactan proteins, xylolignans, and arabinoxylans, etc. They are...
incorporated into these polymers from UDP-β-L-arabinopyranose (UDP-L-Ara) and UDP-α-D-xylose (UDP-Xyl) by the action of L-arabinofuranosyltransferases and xylosyltransferases, respectively (4). UDP-Xyl is generated from UDP-glucuronic acid (GlcA) as the immediate substrate in the de novo pathway mediated by UDP-glucuronic decarboxylase (UDP-glucuronate carboxylase; EC 4.1.1.35) (5), that is then interconverted to UDP-β-L-Ara by the action of UDP-β-L-arabinose 4-epimerase (EC 5.1.3.5) (6). In the salvage pathway, UDP-L-Ara is generated by sequential conversions via L-Ara 1-phosphate catalyzed by the action of L-arabinokinase mentioned above, followed by UDP-L-Ara pyrophosphorylase (UTP: β-L-arabinofuranosylpyrophosphorylase, no EC number). In a similar manner, UDP-Xyl is produced by xylokinase and UDP-Xyl pyrophosphorylase (UTP: α-D-xylose-1-phosphate uridylyltransferase, EC 2.7.7.11). The latter enzyme has been detected in a crude enzyme preparation from mung bean seedlings (7), but it has been neither purified nor cloned so far. Furthermore, it is unclear whether distinct pyrophosphorylases specific to either of the pentose 1-phosphates are involved in the synthesis of each UDP-pentose or whether a single pyrophosphorylase with broad specificity toward pentose 1-phosphates is responsible for both UDP-pentoses (8).

For a better understanding of the regulation of the synthesis of the various nucleotide sugars in the salvage pathways, it is necessary to get information on individual monosaccharide kinases and pyrophosphorylases leading to the generation of particular nucleotide sugars. In this article, we report the purification, characterization, and cloning of a pyrophosphorylase involved in the synthesis of UDP-L-Ara from pea (Pisum sativum L.) sprouts. The enzyme appears to be a novel type of pyrophosphorylase generating various UDP-sugars from respective monosaccharide 1-phosphates. In this context, we also discuss the characteristics of the enzyme based on the comparison of amino acid sequences with other pyrophosphorylases as well as its possible function in vivo.

EXPERIMENTAL PROCEDURES

Materials—Pea (P. sativum L.) sprouts were purchased from a local market and incubated under illumination in a growth chamber at 25 °C for 1 day prior to preparation of the enzyme. Pea (cv. Dun) seeds were a gift from Murakami Seed (Yokohama, Japan). For RNA preparation, pea seedlings (1 kg) containing epicotyls and leaves were chopped with razor blades and homogenized with two times their weight in homogenization buffer containing 100 mM potassium phosphate, pH 6.9, 1 mM dithiothreitol, and 1 mM EDTA in a homogenizer. The homogenate was filtered through three layers of nylon mesh, then centrifuged at 1,500 × g for 15 min to remove cell debris. The crude enzyme fraction was further fractionated with crystalline ammonium sulfate. Proteins precipitated between 40 and 70% saturation with ammonium sulfate were collected by centrifugation for 30 min, dissolved in 120 ml of a column buffer (20 mM potassium phosphate buffer, pH 6.9, containing 0.5 mM dithiothreitol; this buffer was used throughout for all chromatography), and dialyzed overnight against the same buffer. The dialyzed sample was adsorbed onto a 2.8 × 73-cm DEAE-Sepharose FF (Amersham Biosciences) column, which had been equilibrated with the column buffer. The column was eluted with a linear gradient of 0–300 mM NaCl in the column buffer (total volume, 1.5 liters). The active fractions were collected, brought to 30% saturation with ammonium sulfate, and applied onto a 2 × 19.5-cm Butyl-Toyopearl 650M (Tosoh, Tokyo, Japan) column equilibrated with the buffer containing 30% saturated ammonium sulfate. The column was eluted with a linear gradient of 30–0% saturation with ammonium sulfate (total volume, 1.5 liters). The active fractions were combined, brought to 80% saturation with crystalline ammonium sulfate, and proteins were recovered by centrifugation at 18,000 × g for 40 min. The proteins were dissolved in 2 ml of the buffer and applied onto a 2 × 112-cm Sephacryl S-200 (Amersham Biosciences) column equilibrated with the buffer containing 100 mM NaCl. The column was eluted with the same buffer, and the active fractions were collected and dialyzed overnight against the buffer. The dialyzed sample was then applied onto a 0.7 × 12-cm DEAE-Sepharose FF column equilibrated with the column buffer. The column was eluted with a linear gradient of 0–300 mM NaCl in the buffer (total volume, 200 ml), and the active fractions were collected. The enzyme specimen was stored at 4 °C and used in the following experiments.

Peptide Sequencing—The purified enzyme (10 μg) was digested with 3 μg of V8 protease from Staphylococcus aureus was from Wako (Osaka, Japan).

Enzyme Assay—The activity of pea pyrophosphorylase (also called UDP-sugar pyrophosphorylase in this paper, because of its broad substrate specificity) was determined by monitoring the formation (forward direction of the enzyme action) of either UDP-Glc or UDP-L-Ara from respective Glc 1-phosphate or L-Ara 1-phosphate in the presence of UTP. The reaction mixture usually contained 50 mM MOPS-KOH buffer, pH 7.0, 2 mM MgCl₂, 0.01% (w/v) BSA, 1 mM monosaccharide 1-phosphate, 1 mM UTP, and enzyme in a final volume of 0.1 ml (this is referred to as the standard reaction mixture). The enzyme was preincubated at 35 °C for 5 min in buffer without substrates, and then the reaction was started by addition of monosaccharide 1-phosphate and UTP. After incubation at 35 °C for 10 min, the reaction was terminated by dialyzing the mixture in a boiling water bath for 2 min. The reaction products were detected according to the method described by Pauly et al. (14). The mixture was centrifuged at 10,000 × g for 5 min, and then 40-ml aliquots were analyzed on a high performance liquid chromatography (HPLC) system with a Shimadzu LC-10AD equipped with a CarboPac PA-1 column (4 × 250 mm, Dionex Japan). The column was eluted with 50 mM ammonium formate for the initial 2 min, followed by a linear gradient (50–650 mM, 2–26 min) and an isocratic elution with the eluent (1x, 26–32 min) at a flow rate of 1 ml per min and at 35 °C. The reaction products were monitored by absorbance at 262 nm, and the amount of UDP-sugar produced was estimated from the peak area based on the curve of UDP-Glc as the calibration standard. One unit of enzyme activity is capable of producing 1 μmol of UDP-Glc or UDP-L-Ara from respective monosaccharide 1-phosphates per min. When various monosaccharide 1-phosphates were examined for their efficiency as substrate for the enzyme, each UDP-sugar produced was confirmed by comparison of its elution time with respective standard UDP-sugars on HPLC. Elution times of standard nucleotide sugars as well as uridine nucleotides are shown in parentheses as follows: UDP-L-Ara (14.89 min), UDP-Gal (14.99), UDP-Xyl (15.16), UDP-Glc (15.24), UDP-GlcA (15.24), UDP-GlcA (24.53), UMP (14.04), UDP (18.39), UTP (23.72). When crude enzyme preparations were assayed for activity during purification of the enzyme, UMP was also detected in reaction mixtures possibly because of the presence of phosphatase(s) and/or pyrophosphatase(s) that degrade substrates and/or products.

The assay for pyrophosphorylase of UDP-Glc (reverse direction of the enzyme action) was performed in a 0.1 ml reaction mixture containing 30 mM MOPS-KOH, pH 7.0, 2 mM MgCl₂, 0.01% (w/v) BSA, 1 mM monosaccharide 1-phosphate, and enzyme. The reaction was preincubated at 35 °C for 5 min, started by addition of monosaccharide 1-phosphate and UTP. After incubation at 35 °C for 10 min, the reaction was terminated by dialyzing the mixture against the reaction buffer. The reaction products were detected according to the method described by Pauly et al. (12) modified to prepare the crude enzyme fraction. All operations were carried out at 0–4 °C. Pea seedlings (1 kg) containing epicotyls and leaves were chopped with razor blades and homogenized with two times their weight in homogenization buffer containing 100 mM potassium phosphate, pH 6.9, 1 mM dithiothreitol, and 1 mM EDTA in a homogenizer. The homogenate was filtered through three layers of nylon mesh, then centrifuged at 1,500 × g for 15 min to remove cell debris. The crude enzyme fraction was further fractionated with crystalline ammonium sulfate. Proteins precipitated between 40 and 70% saturation with ammonium sulfate were collected by centrifugation for 30 min, dissolved in 120 ml of a column buffer (20 mM potassium phosphate buffer, pH 6.9, containing 0.5 mM dithiothreitol; this buffer was used throughout for all chromatography), and dialyzed overnight against the same buffer. The dialyzed sample was adsorbed onto a 2.8 × 73-cm DEAE-Sepharose FF (Amersham Biosciences) column, which had been equilibrated with the column buffer. The column was eluted with a linear gradient of 0–300 mM NaCl in the column buffer (total volume, 1.5 liters). The active fractions were collected, brought to 30% saturation with ammonium sulfate, and applied onto a 2 × 19.5-cm Butyl-Toyopearl 650M (Tosoh, Tokyo, Japan) column equilibrated with the buffer containing 30% saturated ammonium sulfate. The column was eluted with a linear gradient of 30–0% saturation with ammonium sulfate (total volume, 1.5 liters). The active fractions were combined, brought to 80% saturation with crystalline ammonium sulfate, and proteins were recovered by centrifugation at 18,000 × g for 40 min. The proteins were dissolved in 2 ml of the buffer and applied onto a 2 × 112-cm Sephacryl S-200 (Amersham Biosciences) column equilibrated with the buffer containing 100 mM NaCl. The column was eluted with the same buffer, and the active fractions were collected and dialyzed overnight against the buffer. The dialyzed sample was then applied onto a 0.7 × 12-cm DEAE-Sepharose FF column equilibrated with the column buffer. The column was eluted with a linear gradient of 0–300 mM NaCl in the buffer (total volume, 200 ml), and the active fractions were collected. The enzyme specimen was stored at 4 °C and used in the following experiments.

Enzyme Purification—A procedure described by Kobayashi et al. (12) for cloning of the UDP-sugar pyrophosphorylase gene, two degenerate primers (5'-GGCAATCAATGAACTCATGATGTTTTTTTTTTTTTTT-3'). For cloning of the UDP-sugar pyrophosphorylase gene, two degenerate primers (5'-GGCAATCAATGAACTCATGATGTTTTTTTTTTTTTTT-3').
pyrophosphorylase was monitored for subsequent chromatography. The enzyme fraction recovered from DEAE-Sepharose FF was brought to 30% saturation with ammonium sulfate and applied to a Butyl-Toyopearl column. Bound proteins were eluted with a linear gradient of 0–30% saturated ammonium sulfate. The 3-region was amplified with an internal specific primer (5'-GGCTCAACCAGCTTGTGAGG-3'), corresponding to RLTHSDG of the cloned sequence) and an adaptor primer (5'-GGCAGATCCAGATTCGAGATG-3'), using the single strand cDNA as a template under the following conditions: 0.5 min denaturing at 94 °C, 0.5 min annealing at 55 °C, and 2 min amplification at 72 °C, 30 cycles. The amplified DNA fragment was subcloned into a pGEM T-Easy vector (Promega, Madison, WI), and the nucleotide sequence was determined. Expression and Purification of Recombinant Enzyme—The coding region of the cloned cDNA of UDP-sugar pyrophosphorylase was amplified with a set of specific primers, S-1 (5'-GGATCCATGGCTTCCTCGTCGCCG-3') and A-1 (5'-GAGGCTCTATATATCTGGCGGCAC-3'), which contain restriction sites for BamHI and SacI, respectively. The cDNA fragment was subcloned into a pGEM5zf+ vector (Promega), and its nucleotide sequence was confirmed, then inserted between the BamHI and SacI sites of the pET32a expression vector (Novagen, Madison, WI). The construct was designed to express the recombinant enzyme fused to thioredoxin and His6-tags at the N terminus. The plasmid construct was transfected into a BL21 gold strain of E. coli (Novagen). The E. coli cells were grown at 10 °C, and the recombinant protein was induced by treatment with 0.5 mM isopropyl β-D-thiogalactopyranoside for 24 h. The E. coli cells were harvested and lysed in a buffer containing 50 mM potassium phosphate buffer, pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2% lysozyme from chicken egg (Wako). The lysate was put on a 1.5 × 6.0-cm chelating Sepharose FF column. The column was washed with 50 mM sodium-phosphate buffer, pH 7.2, containing 50 mM imidazole, and the bound protein was then eluted with the same buffer containing 250 mM imidazole. Approximately 5 mg of purified recombinant enzyme was obtained from a 500-ml culture of E. coli. The purified recombinant enzyme (1 mg) was digested with 0.1 unit of thrombin (Novagen) at 20 °C for 24 h in order to split off the fused thioredoxin and His6-tags, then dialyzed overnight against the column buffer mentioned under “Enzyme Purification.” The dialyzed sample was applied onto a 1.5 × 1.0-cm DEAE-Sepharose FF column equilibrated with the buffer. The column was eluted with a linear gradient of 0–300 mM NaCl in the buffer (total volume, 50 ml) and the active fractions were collected. The purified recombinant enzyme was examined for its purity, specific activity, and substrate specificity. Analyses of Product—To identify UDP-sugar formed by the action of the recombinant enzyme, a large scale reaction was employed. A reaction mixture consisting of 50 mM MOPS-KOH buffer, pH 7.0, 2 mM MgCl2, 1 mM L-Ara 1-phosphate, 1 mM UTP, 0.1 unit/ml inorganic pyrophosphatase from yeast, and 0.4 unit/ml recombinant enzyme in a final volume of 50 ml was incubated at 35 °C for 24 h: the rate of conversion to UDP-L-Ara was 84% based on the initial L-Ara 1-phosphate according to analysis on HPLC done as described above. After the reaction was terminated by heating in a boiling water bath, the mixture with Arachis hypogaea L. seed void volume and V1, indicated total bed volume of the column, respectively. D, the fraction from Sephacryl S-200 was subjected to a second DEAE-Sepharose FF chromatography. Bound proteins were eluted with a linear gradient of 0–300 mM NaCl.

Fig. 1. Purification of UDP-sugar pyrophosphorylase from pea sprouts. A, protein fraction precipitated at 40–70% saturated ammonium sulfate from a crude extract of pea sprouts was applied to a DEAE-Sepharose FF column. Bound proteins were eluted with a linear gradient of 0–500 mM NaCl. The fractions were assayed for UDP-Glc pyrophosphorylase activity (open circles) and UDP-β-D-arabinofuranosyl pyrophosphorylase activity (closed circles) and monitored for absorbance at 280 nm (dotted line). The solid line represents the concentration of NaCl. The horizontal bar indicates pooled fractions. Only the activity of UDP-Glc pyrophosphorylase was monitored for subsequent chromatography. B, the enzyme fraction recovered from DEAE-Sepharose FF was brought to 30% saturation with ammonium sulfate and applied to a Butyl-Toyopearl column. Bound proteins were eluted with a linear gradient of 30–0% saturated ammonium sulfate. C, the fraction from Butyl-Toyopearl was concentrated and applied to gel-permeation chromatography on Superose 6, and V0, void volume and V1, indicated total bed volume of the column, respectively. D, the fraction from Sephacryl S-200 was subjected to a second DEAE-Sepharose FF chromatography. Bound proteins were eluted with a linear gradient of 0–300 mM NaCl.
UDP-sugar Pyrophosphorylase with Broad Substrate Specificity

**RESULTS**

**Purification of a Pyrophosphorylase from Pea Sprouts**—We looked for activity of UDP-L-Ara pyrophosphorylase in plant sources and found the activity, together with UDP-Glc pyrophosphorylase activity, in a crude enzyme preparation of pea sprouts. The enzyme was purified from the crude preparation by ammonium sulfate fractionation and several subsequent chromatographic operations: during purification, both enzyme activities were monitored after each chromatographic step. The assay was done to estimate UDP-Glc pyrophosphorylase activity. UDP-L-Ara pyrophosphorylase activity is shown in parentheses.

| TABLE I Purification of UDP-sugar pyrophosphorylase from pea sprouts |
|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | Total protein    | Total activity   | Specific activity | Purification    | Yield            |
| Crude extracts   | 5790             | 1220             | (144)             | 0.211           | (0.025)          | 1.0              | (1.0)           | 100.0           | (100.0)         |
| 40–70% Ammonium  | 1150             | 218              | (80.8)            | 0.190           | (0.070)          | 0.9              | (2.8)           | 17.9            | (56.1)          |
| sulfate fraction |                 |                  |                   |                 |                  |                  |                  |                 |                 |
| 1st DEAE-Sepharose FF | 57.7 | 137              | (57.3)            | 2.37            | (0.99)           | 11.2             | (39.6)          | 11.2            | (39.8)          |
| Butyl-Toyopearl  | 1.45             | 52.2             | (15.7)            | 36.0            | (10.8)           | 171              | (432)           | 4.3             | (10.9)          |
| Sephacryl S-200  | 0.166            | 4.26             | (1.57)            | 25.7            | (9.46)           | 122              | (373)           | 0.35            | (1.09)          |
| 2nd DEAE-Sepharose FF | 0.018 | 1.15            | (0.54)            | 63.9            | (30.0)           | 303              | (1200)          | 0.09            | (0.37)          |

* Extracts prepared from 1 kg of pea sprouts.

**Properties of Enzyme**—The properties of the purified enzyme were examined for both forward (synthesis of UDP-sugars) and reverse (pyrophospholysis) directions. When the purified enzyme was incubated with G1c 1-phosphate and UTP, the production of UDP-Glc increased proportionally with incubation times and the amount of enzyme protein in reaction mixtures and there was a concomitant decrease in the content of UTP on HPLC analysis. The activity, however, ceased after prolonged incubation times (see Fig. 4) or with higher amounts of protein because of the reversible character of pyrophosphorylases. This was also the case for pyrophospholysis using UDP-Glc and PP1 as the substrates. Similar results were observed when G1c 1-phosphate was replaced by l-Ara 1-phosphate as the substrate. The enzyme showed the absolute requirement of divalent cations for activity (Table II) and gave maximal activity in the presence of Mg2+ or Mn2+ to produce UDP-Glc from G1c 1-phosphate and UTP as do UDP-Glc pyrophosphorylases from human, Acanthamoeba castellani, and potato (17–19). The result obtained is consistent with the previous studies (17–19), in which MgUTP and MgPP1 complexes are postulated to be the actual substrates for the UDP-Glc pyrophosphorylases in both forward and reverse directions. The effect of various concentrations (1–10 mM) of Mg2+ on enzyme activity was measured, and activity was found to be almost constant in the range from 1–5

**TABLE II Effect of metal ions on the activity of UDP-sugar pyrophosphorylase**

| Metal ion | Relative activity |
|-----------|-------------------|
| None      | 0.0 ± 0.0         |
| Mg2+     | 100.0 ± 7.5       |
| Mn2+     | 75.6 ± 3.5        |
| Zn2+     | 13.7 ± 2.6        |
| Ba2+     | 0.0 ± 0.0         |
| Ca2+     | 0.0 ± 0.0         |
| Cu2+     | 0.0 ± 0.0         |
| Hg2+     | 0.0 ± 0.0         |
| Na+      | 0.0 ± 0.0         |
| K+       | 0.0 ± 0.0         |
| Al3+     | 0.0 ± 0.0         |
| Fe3+     | 0.0 ± 0.0         |

*The final concentration of metal ions was 2 mM.

b Activity is expressed as percent of that in the presence of 2 mM Mg2+.

with 7:4:2 (v/v/v) 1-butanol/ethanol/water as the solvent (to wash out salts from the mixture), followed by 5:2 (v/v) ethanol/0.1 M ammonium acetate, pH 6.8 (to migrate and separate UDP-L-Ara from other reaction components). A strip of paper corresponding to UDP-L-Ara was cut and developed again with the first solvent. A pure UDP-L-Ara specimen was extracted from the paper and lyophilized (yield, 8 mg).

Identification of the isolated UDP-L-Ara was performed by NMR spectroscopy. The sample was dissolved in D2O and NMR spectra were recorded at room temperature. 1H-, 13C-, and 31P-NMR spectra were recorded at 600, 150, and 243 MHz, respectively, with a JEOL JNM-ECA 600 spectrometer. Dioxane was used as the reference for 31P-NMR (δ = 0.0 ppm). PFG-COSY, PFG-HMBC, and PFG-HMBC were recorded for assignment of signals.
The enzyme appeared to be a novel type of pyrophosphorylase capable of using different kinds of monosaccharides 1-phosphates as glycosyl donors to form corresponding UDP-sugars.

The enzyme utilized various monosaccharide 1-phosphates in the forward reaction to form UDP-sugars. Among eight monosaccharide 1-phosphates examined in this study, the enzyme preferred Gal 1-phosphate and Glc 1-phosphate as glycosyl donors. Despite the significant structural similarity of the enzyme to a human UDP-N-acetylgalactosamine (UDP-HexNAc) pyrophosphorylase (AGX1, see below), GlcNAc 1-phosphate was a poor substrate for the enzyme. This is consistent with what is known about the pathways of nucleotide sugars in plants, in which these sugars are activated mainly through UDP derivatives (1). The enzyme did not utilize Glc 6-phosphate as a substrate either. It would be interesting to examine whether galacturonic acid 1-phosphate and l-rhamnose 1-phosphate act as substrates for the enzyme, because corresponding UDP-galacturonic acid and UDP-l-rhamnose are also main metabolites in the pathway (1). Because these two monosaccharide 1-phosphates are not commercially available, further study will be required to clarify their reactivity.

Kinetics of Enzyme—Effects of substrate concentration on activity and turnover numbers of the purified enzyme were examined for both forward and reverse reactions by measuring activities with varying concentrations of the respective substrates. The resulting $K_m$ and $V_{max}$ values are listed in Table V. The $K_m$ values of the enzyme for hexose 1-phosphates were lower than those for pentose 1-phosphates; in other words, the enzyme has higher affinity for hexose 1-phosphates than for pentose 1-phosphates.

cause of its broad substrate specificity. Among eight monosaccharide 1-phosphates examined in this study, the enzyme preferred Gal 1-phosphate and Glc 1-phosphate as glycosyl donors. Despite the significant structural similarity of the enzyme to a human UDP-N-acetylgalactosamine (UDP-HexNAc) pyrophosphorylase (AGX1, see below), GlcNAc 1-phosphate was a poor substrate (4% efficiency if compared with Glc 1-phosphate) for the enzyme. Man 1-phosphate and l-Fuc 1-phosphate did not serve as substrates for the enzyme. This is consistent with what is known about the pathways of nucleotide sugars in plants, in which these sugars are activated mainly through UDP derivatives (1). The enzyme did not utilize Glc 6-phosphate as a substrate either. It would be interesting to examine whether galacturonic acid 1-phosphate and l-rhamnose 1-phosphate act as substrates for the enzyme, because corresponding UDP-galacturonic acid and UDP-l-rhamnose are also main metabolites in the pathway (1). Because these two monosaccharide 1-phosphates are not commercially available, further study will be required to clarify their reactivity.

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V<sub>max</sub> values for hexose 1-phosphates are also higher. These kinetic characteristics are consistent with the preference of the enzyme for hexose 1-phosphates as substrate that is apparent if one compares activities toward various monosaccharide 1-phosphates (Table IV). The K<sub>m</sub> values (0.048 and 0.25 mM) for UTP and PP<sub>i</sub> are comparable to those (0.058–0.33 mM and 0.11–2.4 mM, respectively) obtained for UDP-Glc pyrophosphorylase from human, A. castellanii, and potato (17–19). However, the K<sub>m</sub> values (0.34 and 0.34 mM) for Glc 1-phosphate and UDP-Glc are roughly double or even higher than those (0.14–0.18 mM and 0.04–0.12 mM, respectively) of the three pyrophosphorylases. Sowokinos et al. (20) have reported biphasic kinetics for a UDP-Glc pyrophosphorylase from potato giving two different K<sub>m</sub> values for UTP and Glc 1-phosphate on Lineweaver-Burk plots. However, we did not observe such biphasic kinetics for pea UDP-sugar pyrophosphorylase. The V<sub>max</sub> values (81 and 166 μmol/min/mg protein) of the pea enzyme for respective UTP and Glc 1-phosphate are similar to those (94 and 64 μmol/min/mg, respectively, determined at 0.05–0.2 mM of substrate concentrations) of the potato enzyme (20). The V<sub>max</sub> values (145 and 164 μmol/min/mg) for UDP-Glc and PP<sub>i</sub> are, however, much lower than those (890 and 827 μmol/min/mg, respectively) of the potato enzyme.

Equilibrium ratios of UDP-Glc in the reaction catalyzed by UDP-sugar pyrophosphorylase were determined for both forward and reverse directions by monitoring the amount of UDP-Glc produced or consumed in reaction mixtures (Fig. 4A). In both directions a final molar ratio of 0.30 mol% UDP-Glc was obtained. This value is comparable to that (0.34) observed for UDP-Glc pyrophosphorylase from human erythrocytes (17). Given that 0.70 mol% Glc 1-phosphate (which cannot be detected by the HPLC method employed) remained at the equilibrium state, the apparent equilibrium constant (K) was therefore 0.18 for the forward direction and 5.4 for the reverse direction.

PP<sub>i</sub> is a product in the reaction directed toward production of UDP-Glc. It appears that accumulation of this product in reaction mixtures caused product inhibition (Table III). To confirm this effect of PP<sub>i</sub>, inorganic pyrophosphatase from yeast was added to reaction mixtures to deplete this compound by converting it into P<sub>i</sub>, during the progress of the reaction (Fig. 4B). As a result, the rate of UDP-Glc production dramatically increased, giving rise to complete conversion (99.7% of Glc 1-phosphate into UDP-Glc. We therefore used pyrophosphatase to produce effectively a large amount of UDP-1-Ara by recombinant UDP-sugar pyrophosphorylase (see below).

cDNA Sequence of Enzyme—Since the N terminus of the purified enzyme was blocked, the enzyme was subjected to partial digestion with V8 proteinase. The peptide sequence of an internal fragment released from the purified enzyme with an apparent molecular mass of ~31,000 Da on SDS-PAGE was YNQDLPXRASGYPD, where the 8th residue could not be determined because of its low signal intensity. The sequence was very similar to partial sequences of an Arabidopsis open reading frame (At5g52560, YNQLDPLRASGFPD, 86.7% identity) and a rice cDNA sequence (AK064009, YNQLPDLLRASGHPD, 86.7% identity); the function of these genes has not yet been clarified so far.

A partial fragment of cDNA for the purified UDP-sugar pyrophosphorylase was amplified by reverse transcriptase-PCR (RT-PCR) using cDNA from pea sprouts as the template and a set of degenerate primers designed based on the conserved sequences found in both At5g52560 and AK064009. The 506-bp PCR product contained the sequence determined by the peptide sequence analysis mentioned above (Fig. 5A). A full-length cDNA encoding the purified enzyme was isolated by the following 3’-RACE and 5’-RACE procedures.

The cloned cDNA, PsUSP (Pisum sativum UDP-sugar pyrophosphorylase) appeared to encode a polypeptide of 600 amino acids (molecular mass 66,040 Da) (Fig. 5A). The peptide sequence deduced from PsUSP cDNA contained neither apparent secretory signal, mitochondrial targeting, nor chloroplast transit sequence, and analysis by iPSORT (21) predicted the product of PsUSP was localized in the cytoplasm. The calculated

TABLE V

Kinetics of UDP-sugar pyrophosphorylase

To examine the effects of UTP on enzyme activity, activity in the forward direction (UDP-sugar formation) was measured under standard assay conditions by incubation with varying concentrations of UTP in a range of 0.02–1.8 mM in the presence of 1 mM Glc 1-phosphate. To examine the effects of monosaccharide 1-phosphates on the activity, reactions were carried out with varying concentrations (0.2–2.8 mM) of various monosaccharide 1-phosphates in the presence of 1 mM UTP. The K<sub>m</sub> and V<sub>max</sub> values were calculated from a Hanes-Woolf plot using the obtained activities. For the reverse reaction of UDP-sugar degradation (pyrophospholysis), kinetic parameters for UDP-Glc and PP<sub>i</sub> were calculated in a similar manner with varying concentrations (0.04–0.55 mM) of either UDP-Glc or PP<sub>i</sub>, in the presence of 1 mM of the respective counter substrates.

| Substrate     | K<sub>m</sub> (mM) | V<sub>max</sub> (μmol/min/mg protein) |
|---------------|------------------|-----------------------------------|
| UDP-sugar formation |                  |                                   |
| UTP           | 0.048            | 81                                |
| Glc 1-phosphate | 0.34             | 106                               |
| Gal 1-phosphate | 0.58             | 161                               |
| GlcA 1-phosphate | 0.48             | 66                                |
| Ara 1-phosphate | 0.96             | 71                                |
| Xyl 1-phosphate | 1.98             | 49                                |
| UDP-sugar degradation |            |                                   |
| UDP-Glc       | 0.34             | 145                               |
| PP<sub>i</sub> | 0.25             | 64                                |
isolectric point for the enzyme was 5.82. The amino acid sequence deduced from \textit{PsUSP} was 77 and 72\% identical with those from \textit{Arabidopsis} At5g52560 and rice AK064009, respectively. The sequence of \textit{PsUSP} shared low but significant similarities with UDP-Glc pyrophosphorylase from potato (15\%) (22) and a human UDP-HexNAc pyrophosphorylase (AGX1, 15\%) (23) at the amino acid level. \textit{PsUSP} contains putative pyrophosphorylase consensus and uridine-binding motifs in the regions between Val123 and Lys137, and Val233 and Tyr257, respectively (Fig. 5A). Based on photolabeling analyses of AGX1 with N3-UDP-[32P]HexNAc, these motifs have been shown to comprise amino acid residues essential for the pyrophosphorylase activity (24). Phylogenetic analysis revealed that \textit{PsUSP} can be categorized into a group together with At5g52560 and AK064009, which is apparently independent from those of the UDP-Glc pyrophosphorylase and UDP-HexNAc pyrophosphorylase genes (Fig. 5B). Since pyrophosphorylase(s) participating in the salvage of pentoses (\textit{t}-Ara and Xyl) in higher plants have not yet been identified so far (7, 8), this phylogenetic relationship suggests that the products of At5g52560 and AK064009 along with \textit{PsUSP} act as key enzymes salvaging, after phosphorylation, free pentoses as well as other sugars (at least Glc, Gal, and GlcA) released during the degradation of polysaccharides and glycoconjugates and have physiological functions distinct from those of UDP-Glc pyrophosphorylase and UDP-HexNAc pyrophosphorylase.

**Properties of Recombinant Enzyme**—A recombinant \textit{PsUSP} expressed in \textit{E. coli} was obtained as a protein fused to thioredoxin and His tags. The recombinant \textit{PsUSP} was purified by chelating chromatography and digested by incubation with thrombin. The protein was then purified on a DEAE-Sepharose FF column to remove the fused thioredoxin and His tags at the N terminus. The recombinant \textit{PsUSP} appeared as a single band with molecular mass of 70,000 Da (Fig. 6). The slightly higher molecular mass of the recombinant enzyme compared with that (67,000 Da) of the native enzyme purified from pea sprouts possibly resulted from an additional sequence between the thrombin cleavage site and \textit{PsUSP} inserted into the pET32a. The specific activity of the recombinant \textit{PsUSP} (95.0 unit/mg protein) was comparable to that (63.9 unit/mg protein) of the native enzyme purified from pea sprouts. The recombinant enzyme catalyzed the formation of various UDP-sugars from respective monosaccharide 1-phosphates and UTP, exhibiting broad substrate specificity toward monosaccharide 1-phosphates similar to the native enzyme (Table IV). These results confirm that the cloned \textit{PsUSP} encodes UDP-sugar pyrophosphorylase with characteristics identical to those of the native enzyme purified from pea sprouts.

**Analysis of Product**—We characterized a product generated from \textit{t}-Ara 1-phosphate by the action of \textit{PsUSP}, because this is the first identification at the molecular level of a plant pyrophosphorylase capable of producing UDP-pentoses. UDP-\textit{t}-Ara was produced in a high yield (more than 80\% of initial \textit{t}-Ara 1-phosphate) when inorganic pyrophosphatase was added to
the reaction mixture. The configuration of the UDP-1-Ara produced was determined by NMR analysis (Table VI). The significant signal of a one-proton doublet of doublets at $\delta$ 5.53 ppm ($J_{1,2}$ 3.4 Hz, $J_{1,P}$ 6.9 Hz, H-1 of l-Ara) in the $^1$H-NMR spectrum of the synthesized UDP-1-Ara and the cross-peak from H-1 to C-5 (64.74 ppm) in HMBC spectrum (not shown) indicated that the configuration of the product l-arabinopyranose was $\beta$. The signals at $\delta$ -7.28 ppm and -8.78 ppm (2d, $J$ 20.4 Hz) in the $^{31}$P-NMR spectrum indicate a diphospho linkage. All other signals in the $^1$H- and $^{13}$C-NMR spectra were in accordance with those of UDP-1-Ara reported by Pauly et al. (14), and Ernst and Klaffke (25). These results establish unambiguously that the product is UDP-$\beta$-l-arabinopyranose, a metabolite in the pathway of nucleotide sugars in higher plants (1).

**DISCUSSION**

UDP-sugar pyrophosphorylase (PsUSP) purified from pea sprouts as well as the corresponding recombinant protein expressed in E. coli exhibited broad substrate specificity generating not only UDP-Glc from Glc 1-phosphate and UTP, but also other UDP-sugars including UDP-Gal, -GlcA, -Xyl, -l-Ara, and -GlcNAc from the respective monosaccharide 1-phosphates, though the last compound (UDP-GlcNAc) was not produced efficiently (Table IV). We demonstrated clearly that the enzyme possesses the ability to convert 1-phosphates of Xyl and l-Ara, whose metabolism has not yet been clarified so far (8), to respective UDP-pentoses in the salvage pathway of higher plants. The properties of the enzyme differ from those of other plant UDP-Glc pyrophosphorylases in optimal pH. The relatively low optimal pH (pH 6.5–7.5) for the pea enzyme compares with pH 8.5 (for potato enzyme) (20) and pH 8.0–9.0 (Sorghum enzyme) (26), suggesting different intercellular and/or tissue localization of the pea enzyme. Kinetics of the enzyme also differ: biphasic substrate saturation kinetics with two different $K_m$ values for UTP and Glc 1-phosphate, which resulted from the oligomeric nature of the enzyme protein, were observed for the potato enzyme (20), while the pea enzyme gave single kinetic parameters (Table V). Moreover, phylogenetic analysis revealed that pea enzyme falls into a novel class of pyrophosphorylase with broad substrate specificity similar to the pea enzyme. This class of enzyme may play multiple physiological roles distinct from other types of pyrophosphorylase in the salvage pathway of higher plants. Low but discernible similarity to euakaryotic UDP-Glc pyrophosphorylase and UDP-HexNAc pyrophosphorylase suggests that this class of enzyme has a common ancestor with these other classes of pyrophosphorylases. The name, UDP-sugar pyrophosphorylase, has already been applied to a pyrophosphorylase found in a thermotolerant bacterium, *Thermus caldophilus*, which catalyzes the production of various UDP-sugars from respective 1-phosphates of Glc, GlcNAc, and Xyl in this order of efficiency, but with low efficiency from 1-phosphates of Gal and Man (27). However, the enzyme has not been purified as a homogenous protein, therefore it is not clear whether this is a single enzyme with broad substrate specificity toward monosaccharide 1-phosphates. In addition, the preference of pea enzyme to Gal 1-phosphate but not to GlcNAc 1-phosphate (Table IV) make pea enzyme markedly different from the bacterial enzyme with respect to substrate specificity. Broad substrate specificity of pea UDP-sugar pyrophosphorylase toward various monosaccharide 1-phosphates (Table IV) as well as high efficiency of production of UDP-sugars in the

**FIG. 6. SDS-PAGE of recombinant PsUSP at different purification steps.** The recombinant PsUSP was expressed in E. coli. The protein (~ 1 mg) obtained after different purification steps was analyzed by SDS-PAGE. Protein in the gel was stained with Coomassie Brilliant Blue R-250. Lanes S, molecular mass markers; lane 1, lysate of E. coli; lane 2, recombinant PsUSP purified on a chelating column; lane 3, thrombin-digested recombinant PsUSP. The arrow indicates the purified recombinant PsUSP.

**TABLE VI**

NMR data of UDP-l-arabinose

| 1-Arabinose | Ribose | Uracil |
|-------------|--------|--------|
| ppm         | ppm    | ppm    |
| Hz          | Hz     | Hz     |
| $^1$H signals |        |        |        |
| H-1         | 5.53 (d) | 4.26–4.33 (m) | 5.91 (d) |
| H-2         | 3.74 (dt) | 4.21 (m) | 5.51 (d) |
| H-3         | 3.85 (dd) | 4.12 (ddd) | 5.11 (d) |
| H-4         | 3.85 (broad s) | 4.17 (ddd) | 4.21 (m) |
| H-5a        | 3.64 (dd) | 4.12 (ddd) | 5.11 (d) |
| H-5b        | 4.04 (d) | 4.17 (ddd) | 4.21 (m) |
| $^{13}$C signals |        |        |        |
| C-1         | 96.98 (d) | 84.03 (d) | 89.14 |
| C-2         | 69.18 (d) | 74.54 | 167.61 |
| C-3         | 69.50 | 70.44 | 152.61 |
| C-4         | 69.64 | 84.03 (d) | 103.44 |
| C-5         | 64.74 | 65.71 | 142.40 |
| C-6         |        |        |        |
| $^{31}$P signals |        |        |        |
| $-7.28$ (d) | $J$ 20.4 | $-8.78$ (d) |
presence of pyrophosphatase in the forward reaction (Fig. 4B) would seem to make the enzyme a useful tool for synthesizing various UDP-sugars.

Pea UDP-sugar pyrophosphorylase catalyzes pyrophosphorylation of UDP-sugars in the reverse reaction as do other nucleotide sugar pyrophosphorylases. The equilibrium ratios of UDP-Glc (Fig. 4A) in reaction mixtures for both directions indicate that the enzyme in fact prefers pyrophosphorylation, rather than the formation of UDP-sugars from monosaccharide 1-phosphates and UTP. However, pyrophosphorylation of UDP-Glc was considerably reduced by the addition of inorganic pyrophosphatase to the reaction mixtures (Fig. 4B). Higher plants contain high levels of pyrophosphatase and the intracellular level of inorganic PPi is unlikely to reach levels to trigger pyrophospholytic cleavage of UDP-sugars (8). Therefore, it is very likely that pea pyrophosphorylase plays a role in the formation of UDP-sugars in the salvage pathway rather than in the degradation of UDP-sugars.

It is highly probable that various enzymes involved in the salvage and de novo pathways regulate the synthesis and flow of nucleotide sugars in a concerted manner. It has been demonstrated that exogenous application of Gal inhibits the auxin-induced elongation growth of oat coleoptile segments (28). This may be caused by the accumulation of Gal 1-phosphate through the action of galactokinase, which in turn interferes with the synthesis of UDP-Glc as an analogue of G1c 1-phosphate and/or by depleting cytoplasmic phosphate as well as uridine phosphate pools (1, 28). A decrease in UDP-Glc likely results in inhibition of cell wall synthesis. However, the effect of Gal 1-phosphate on UDP-Glc pyrophosphorylase has not yet been verified in vitro, and enzymes that catalyze the formation of UDP-Gal from Gal 1-phosphate and UTP remain to be identified. Since the UDP-sugar pyrophosphorylase reported in this study possesses relatively high activity toward Gal 1-phosphate compared with other monosaccharide 1-phosphates, the enzyme appears to favor the synthesis of UDP-Gal by utilizing selectively Gal 1-phosphate among various monosaccharide 1-phosphates available in vivo. As a result, the formation of UDP-sugars (at least UDP-Glc, -GlcNAc, -Xyl, and -Ara) (Table IV) other than UDP-Gal was suppressed when free Gal was exogenously applied to plants. This aspect of the behavior of the enzyme may explain the toxicity of Gal in higher plants.

The sequence of PsUSP shared low but significant similarity (15%) with UDP-HexNAc pyrophosphorylase (AGX1) (23). Teritary structure prediction with the three-dimensional-PSSM program showed that the structure of PsUSP is closely related to AGX1 (E-value, 1.44e-62) (29), although GlcNAc 1-phosphate served as a poor substrate for PsUSP. Interestingly, the amino acid residues encompassing the sugar N-acetyl arm of UDP-GlcNAc in AGX1 (Asn223 and Ala228) are substituted with more bulky residues in PsUSP (His240 and Gln257), as are the corresponding residues in a human UDP-Glc pyrophosphorylase (UGPase1, GenBank access no. AAH74004, His223 and Trp233) (30). These bulky residues probably impede the access of GlcNAc to the catalytic site of PsUSP, thereby altering the substrate specificity of the enzyme. Another feature that distinguishes PsUSP from AGX1 is insertions of amino acid residues near the uridine binding (from Gln228 to Arg239) and the pyrophosphorylase consensus motifs (from Gly111 to Arg117), which may alter the conformation required for substrate recognition. However, to address the conformational features that result in the broad substrate specificity unique to PsUSP, stereochemical analysis of the three-dimensional structure of PsUSP would be required.

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