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δ^1-Pyrroline-5-carboxylic Acid Formed by Proline Dehydrogenase from the *Bacillus subtilis* ssp. natto Expressed in *Escherichia coli* as a Precursor for 2-Acetyl-1-pyrroline

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Proline dehydrogenase (PRODH) catalyzes the biosynthesis of Δ^1-pyrroline-5-carboxylic acid (P5C). The *Bacillus subtilis* subsp. natto gene for the proline dehydrogenase (BnPRODH) was cloned and expressed in *Escherichia coli*. Nucleotide sequence analysis of the clone revealed an open-reading frame that encodes 302 amino acid polypeptide with a calculated molecular mass of 34.5 kDa. The deduced amino acid sequence showed sequence similarity to bacterial PRODH and PutA of *E. coli*. The BnPRODH gene was cloned into pET21b and was expressed at a high level in *E. coli* BL21-(DE3). The expressed protein was purified by using nickel ion affinity column chromatography to homogeneity before characterization. The purified recombinant BnPRODH was used to produce P5C. Model system composed of P5C and methylglyoxal was set up to study the formation of 2-acetyl-1-pyrroline. Our data showed that P5C, derived from the conversion of L-proline by the purified recombinant PRODH, might react directly with methylglyoxal to form 2-AP. P5C/methylglyoxal pathway represents the first report of a biological mechanism by which 2-AP may be synthesized in vitro by PRODH.

KEYWORDS: Proline dehydrogenase; *B. subtilis* subsp. natto; 2-acetyl-1-pyrroline; δ^1-pyrroline-5-carboxylic acid

INTRODUCTION

2-Acetyl-1-pyrroline (2-AP) had been proposed as a character impact odorant causing the roasty note of bread crust and aromatic rice (*1*) at an extremely low odor threshold of 0.02 ng/L. It has been identified in a great variety of processed and cooked food products. 2-AP is reported to be formed by the nonenzymatic browning reaction between reducing sugars and free amino acids, such as proline and ornithine (*2*).

Various syntheses of 2-AP have been described in the literature (*3*). The first synthesis (*4*) of 2-AP was achieved by an oxidation of 2-(1-hydroxyethyl)pyrrolidine with a large excess of silver carbonate on Celite in benzene. Methods entailing the cyclization of 6-amino- or 6-azidohexane-2,3-dione to form 2-AP have been published. A short synthetic strategy was developed depending on an amino-protected functionalized vicinal diimine as a key intermediate (*5*). Recently, another synthetic procedure for the preparation of 2-acetyl-1-pyrroline from 2-pyrrolidinone was published. The described process provided access to 2-AP in gram quantity and 22% overall yield in four steps (*6*).

However, 2-AP has also been found in biological systems. Several strains of *Bacillius cereus* strains were reported to produce 2-AP at about 0.030−0.075 mg/kg after 2 day incubation on a standard plate-count agar (PCA) (*7*). Recently, some wine lactic acid bacteria were characterized as producing 2-AP at about 0.001−0.005 mg/L. The biosynthesis of 2-AP was studied by the wine lactic acid bacterium, *Lactobacillus hilgardii* DSM 20176 (*8*). Recently, 2-AP was identified by gas chromatography−mass spectrophotometry **(**GC-MS) from the cultures of the fungal strains *Acremonium nigricans*, *Aspergillus awamori*, and *Aspergillus oryzae* and a yeast strain, *Kluveromyces maxianus* var. *marxianus* (Hansen) (*9*). In plants, 2-AP has been detected in leaves from pandan (*Pandanus amaryllifolius*) (*10*), taro corms (*11*), uncooked Khao Dawk Mali 105 brown rice (*12*), and fresh bread flowers (*Vallaris glabra* Ktze) (*13*).

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On the basis of the evidence above, we hypothesized that methylglyoxal may react with Δ^1-pyrroline-5-carboxylic acid (P5C) to form 2-AP. P5C can be synthesized from the oxidation of L-proline by proline dehydrogenase (BnPRODH) in *Bacillus subtilis* subsp. natto. In this study, BnPRODH gene was expressed in *Escherichia coli* BL21 and was purified to homogeneity. A model system composed of methylglyoxal and P5C produced by BnPRODH was set up to study the formation of 2-AP. The present results provide the direct evidence that BnPRODH is responsible for the biosynthesis of 2-AP in *B. subtilis* subsp. natto.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Cultivation Conditions. *E. coli* JM109 and XL1-Blue (Stratagene, La Jolla, CA) were used as hosts for cloning, and *E. coli* BL21(DE3):pLysS (Novagen, Madison, WI) was used as the host for protein production. All *E. coli* strains were grown in Luria−Bertani (LB) medium at 37 °C. When necessary, the medium was supplemented with ampicillin (50 μg mL^{-1}). *B. subtilis* subsp. natto was cultivated at 30 °C in a medium containing 0.3% beef extract and 0.5% peptone. All *E. coli* strains were grown in LB medium at 37 °C. Plasmids pGEM-7Zf (+) (Promega, Madison, WI), pGEM-T easy vector (Promega, Madison, WI), and pET21b (Novagen, Madison, WI) were used as a cloning and expression vector.

Cloning of *BnPRODH* Gene. On the basis of the similarity of the nucleotide sequence of proline dehydrogenase (PRODH) gene among bacteria, the forward primer (5′-AGG GAG TCA TAT GTT GAG ACA TGT GTT TTT-3′) and reverse primer (5′-CTG CTC GAG CTT TTT CAA AAT TCC TTT TAA-3′) were designed. PCR was performed with a model PE2400 automatic thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR products were ligated to pGEM7Zf (+) vector, generating pGEM-BnPRODH. The nucleotide sequence was confirmed by sequencing with an automated laser fluorescence sequencer (Model 377, ABI PRISM, Foster, CA).

Expression and Purification of BnPRODH in *E. coli*. The coding sequence of BnPRODH was amplified using the forward primers (5′-A GGG AGT CATATG TTG AGA CAT GTG TTT TT-3′; the *Nde*I is underlined) and reverse primers (5′-CTG CTCGAG CTT TTT CAA AAT TCC TTT TAA-3′; the *Xho*I site is underlined). The PCR products were digested with *Nde*I and *Xho*I and were fused in frame to the His_6 tag of pET21b. *E. coli* BL21 (DE3) containing pET21-BnPRODH was grown at 37 °C. When the optical density at 595 nm reached 0.6−0.8, the culture was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. The cells were harvested and resuspended in 20 mM Tris (pH 8.0) and were disrupted by sonication. After centrifugation, the crude extracts were applied to a 5 mL HiTrap metal chelating column (GE Healthcare, Milwaukee, WI) charged with Ni^{2+}. The protein was eluted with a linear gradient of 0−600 mM imidazole containing 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9). The eluted protein fractions were dialyzed against 10 mM ammonium bicarbonate buffer (pH 7.9) to remove salts, and the purified proteins were lyophilized and stored at −70 °C. The purified proteins were separated by sodium dodecyl sulfate−10% polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was measured according to the Bradford method (*14*).

Assay of BnPRODH Activity. BnPRODH activity was determined with the spectrophotometric method described by Scarpulla and Soffer (*15*). The reaction was carried out at 25 °C in 50 mM Tris-HCl (pH 7.5) containing 100 mM L-proline, 10 mM KCN, 10 μM FAD, 0.2 mM phenazine methosulfate, 0.2 mM *p*-iodonitrotetrazolium (Sigma-Aldrich, St. Louis, MO), 0.75% Triton X-100, and enzyme. One unit of PRODH activity was defined as the amount of activity yielding reduction of 1.0 μmol *p*-iodonitrotetrazolium per min under the assay condition using the extinction coefficient 11.5 mM^{-1} cm^{-1}.

Synthesis and Characterization of P5C. For synthesis of P5C, purified BnPRODH (289 units) was incubated with 1−5 mmole L-proline, 5 mmole α-ketoglutarate, and 15 mmole pyridoxal-5′-phosphate in 100 mL potassium phosphate buffer (pH 7.2). The mixture was kept under constant agitation at 25 °C for 1 h. P5C concentration was determined following the method of Mezl and Knox (*16*). To 1.35 mL of reaction mixture and 1.5 mL of 10% trichloroacetic acid was added 0.15 mL of 0.1 M *o*-aminobenzaldehyde in 40% (v/v) ethanol. After 25 min at room temperature, the sample was cleared by centrifugation, and the absorbance in a 1 cm cuvette at 440 nm was determined. The extinction coefficient of P5C was 2.58 mM^{-1} cm^{-1}. The characterization of P5C was determined by using a Hitachi model 6000A HPLC equipped with a photodiode array detector (Model L-4500, Hitachi, Japan. 220−550 nm) following the method of O'Donnell et al. (*17*) with a slight modification.

Liquid Chromatography−Mass Spectrometry (LC-MS). All analyses (using 5 μL samples) were carried out in duplicate on a Thermo Finnigan LCQ DECA XP MAX single quadrupole instrument (Waltham, MA) with an ESI interface. A C18 column (150 × 2.0 mm, Phenomenex Co., Torrance, CA) with a Finnigan Surveyor HPLC pump was used throughout the analyses. Separation was achieved with an isocratic elution using 33:67 methanol/water solution for 30 min. The mass spectrometer was configured for positive ion chemical ionization with coronal discharge at ~4.5 kV. Nitrogen at 35 psi was used as the sheath gas. Mass parameters comprised a full scan mode *m*/*z* 100−220 in 1.5 s and selected ion monitoring *m*/*z* (±0.15 amu) with a dwell time of 0.25 s/window.

Synthesis and Characterization of 2-AP. The solution containing P5C (5 mmole) was mixed with methylglyoxal (5 mmole) in 100 mL phosphate buffer (0.1 M pH 7.2). After stirring for 30 min at room temperature (25 °C), the mixture was extracted eight times with dichloromethane (total volume = 250 mL). After drying with anhydrous sodium sulfate, the extract was further concentrated to a volume of 2 mL for GC-MS analysis. L-Proline, α-ketoglutarate, pyridoxal-5′-phosphate, and methylglyoxal were purchased from Sigma-Aldrich (St. Louis, MO).

Gas Chromatography−Mass Spectrophotometry (GC-MS). An aliquot (0.5 μL) of the extracted sample was injected into a gas chromatograph−mass spectrometer (Agilent 6890 and HP 5973 mass-selective detector, Agilent Technologies, Palo Alto, CA) equipped with a fused silica capillary column, HP-5MS, with (5%-phenyl)-methylpolysiloxane as nonpolar stationary phase (30 m × 0.25 mm i.d. × 0.25 μm). The sample was injected with spilt ratio of 20:1 and the flow rate of helium was 1 mL/min. The injection port temperature was 200 °C. The temperature of the HP-5MS column was programmed starting at 50 °C after injection. After 1 min, the temperature was increased at a rate of 5 °C/min from 50 to 250 °C and was held for 15 min. The mass spectrometer was operated in electron impact (EI) mode with an electron energy of 70 eV; ion source temperature, 250 °C; mass range *m*/*z* 40−250; scan rate, 0.68 s/scan; and EM voltage, 1832 V. The mass transfer line was set to 280 °C. Linear retention indices (RTs) were calculated using *n*-alkanes as reference.

Nucleotide Sequence Accession Number. The nucleotide sequence of *BnPRODH* has been deposited at the NCBI database under accession no. AF497244.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of the *BnPRODH* Gene. The gene encoding BnPRODH has been cloned from *B. subtilis* subsp. natto by PCR. The complete 909 bp sequence was determined, and it was found that the open-reading frame (ORF) encodes a polypeptide of 302 amino acids with an estimated molecular mass of 34.5 kDa. A search through the available protein sequence databases revealed that BnPRODH shared significant amino acid sequence similarity with bacterial PRODH, such as *Geobacillus kaustophilus* HTA426 (YP_148863, 63% identity), *Exiguobacterium sibiricum* (EAM86525, 55% identity), and *Thermus thermophilus* HB27 (YP_005183, 48% identity) (**Figure 1**). BnPRODH shows less similarity to PRODH domain of PutA (proline utilization A) from *E. coli* (*18*), but the degree of identity (approximately 30% identical at the amino acid sequence) still suggests that these two proteins were structurally related. PutA is a 1320 amino acid modular

Figure 1. Optimal alignment of *Bacillus subtilis* subsp. natto proline dehydrogenase (bn-pdh) with those of other proteins. The sequences listed are proline dehydrogenase (PRODH) from *Geobacillus kaustophilus* HTA426 (gk-pdh), *Exiguobacterium sibiricum* (es-pdh), *Thermus thermophilus* HB27 (tt-pdh), and PutA from *Escherichia coli* (ec-puta). The amino acid numbers are listed on the right, and they are numbered from Met-1 of the proteins. The black background regions indicate highly conserved amino acid residues. Dashes represent gaps introduced during the alignment process.

enzyme consisting of a DNA binding domain (residues 1−47), PRODH domain (residues 261−612), and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) domain (residues 650−1130) (*19*). According to the crystal structure of the PRODH domain of *E. coli* PutA (*20*), and the studies of site-directed mutagenesis (*19*), Glu289, Lys329, Asp370, Arg431, Leu432, Tyr437, Leu514, Tyr540, Arg555, Arg556, and Arg559 were likely to be involved in catalytic mechanism for PRODH activity. Sequence analysis revealed that all residues corresponding to these catalytic residues are clearly present in BnPRODH.

Purification and Characterization of the Recombinant BnPRODH. The coding region of the *BnPRODH* gene was cloned into pET21b vector under the control of the T7 promoter. The protein product contained a C-terminal His_6 tag, derived from pET 21b. After expression in *E. coli* BL21 (DE3), the protein product was purified from cell extract by nickel ion affinity column chromatography. The yield of the recombinant protein was 22 mg/L *E. coli* culture. The purified protein product showed a single band with a molecular mass of 35 KDa on SDS-PAGE (**Figure 2**). It is in good agreement with the value estimated from the deduced amino acid sequence of BnPRODH. The enzyme activity of recombinant BnPRODH was determined with *p*-iodonitrotetrazolium as the electron acceptor under saturating substrate concentration to show the formation of P5C. This indicated that BnPRODH underwent a dehydrogenation to remove two hydrogen atoms, leading to the transformation of L-proline to P5C. The final specific activity of the purified enzyme was 28.9 units/mg. The optimal pH for the purified BnPRODH was measured using a series of biological buffers

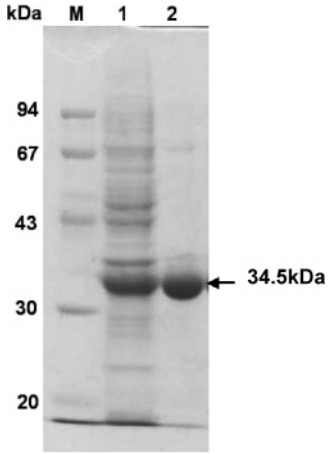

Figure 2. SDS-PAGE analysis of purified BnPRODH. Lane M, molecular mass standard; lane 1, crude cell extract from *E.coli* BL21/pET21-BnPRODH; lane 2, BnPRODH purified from *E.coli* BL21/pET21-BnPRODH by nickel ion affinity column chromatography. The proteins were analyzed by 10% SDS-PAGE, and the gel was stained with coomassie brilliant blue R-250.

with various pH's. The purified recombinant BnPRODH exhibited the maximum activity within a pH range of 7.5−8.0 and a temperature of 25 °C (**Figure 3A** and **3B**). The enzyme is temperature sensitive, losing 70% of enzymatic activity upon incubation at 45 °C for 15 min.

Synthesis of Δ^1-Pyrroline-5-carboxylic Acid. The formation of P5C was confirmed by reaction with *o*-aminobenzaldehyde

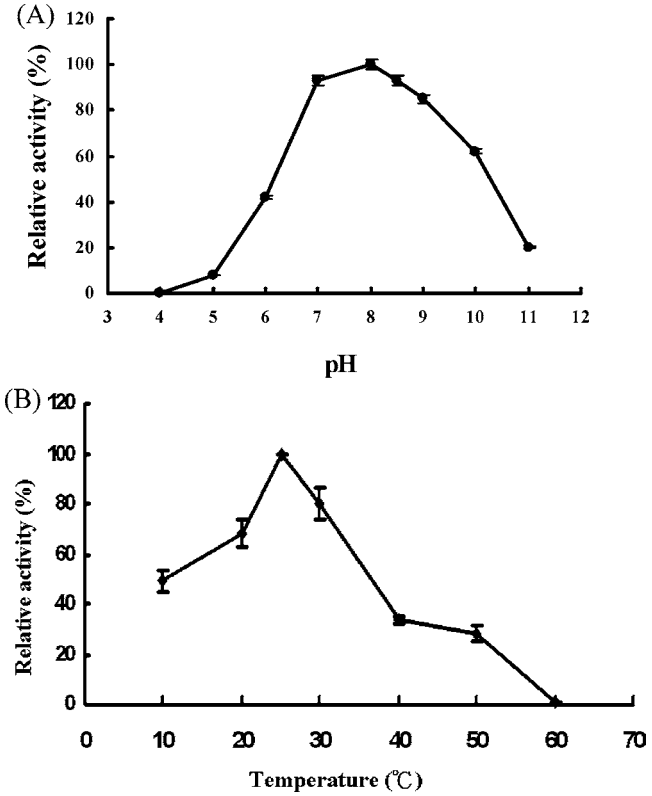

Figure 3. Effect of pH (**A**) and temperature (**B**) on the activity of recombinant BnPRODH. Recominant BnPRODH was incubated with L-proline in 1 mL buffers at various pH's and temperatures. Vertical bars indicate standard deviation ($n = 3$).

using chemically synthesized P5C (*16*) as a standard. The Schiff base product between P5C and *o*-aminobenzaldehyde was characterized as a dihydroquinazolium compound with molecular weight of 217. Absorption spectrum of the product by the reaction between P5C and *o*-aminobenzaldehyde was recorded by an HPLC coupled with a photodiode array detector, and the molecular weight was characterized by LC-MS. The spectrum is shown in **Figure 4** with absorption maxima at 230, 294, and 436 nm, which are all in good agreement with the data reported by O'Donnel et al. (*17*). The MS spectrum is shown in **Figure 5** with M^{+1} ion 218. The formation of P5C in the model system is dose-dependent on proline concentration as shown in **Figure 5**. A 100 mL reaction mixture containing 5 mmol of L-proline and purified BnPRODH produced 2.2 mmol of P5C, which presented 44% conversion to P5C. It has been suggested that L-proline might be converted to P5C in large quantities by proline oxidase (*21*), but successful application of this approach has not been reported. Enzymatic method for synthesizing gram quantities of P5C using purified ornithine aminotransferase has been reported (*22*). L-Ornithine is converted to P5C with the simultaneous conversion of α-ketoglutarate to L-glutamic acid. It has previously been synthesized chemically by the hydrolysis of γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde with hydrochloric acid (*16*). This produces a relatively pure compound which is a mixture of the D- and L-stereoisomers. Another chemical method employing oxidation of hydroxylysine to P5C has been reported, but it most probably produces a similar mixture of the D- and L-stereoisomers. The periodate oxidation of hydroxylysine offers a simple and efficient route to form Δ^1-pyrroline-5-carboxylate (*23*).

Characterization of 2-Acetyl-1-pyrroline in the Reaction Mixtures. In the model system of P5C and methylglyoxal, volatile compounds were extracted with methylene chloride and were analyzed by GC-MS. The mass spectrum for 2-AP (Kovats' RT index, HP-5ms, 918) from the reaction mixture shows some major ions at molecular ion at *m*/*z* 111 (12) and other major ions at 43 (100), 41 (61), 83 (51), 42 (23), 68 (14), 69 (8), 55 (3.6), 52 (2.2), 67 (2.9), and 54 (1.5). The breakdown of the methylene moiety $CH_2{=}CH_2$ andacetyl moiety $CH_3{-}CO$ leads to the formation of the *m*/*z* 83 fragment and the *m*/*z* 54 pyrroline ring. It was essentially identical to 2-AP reported from pandan leaf extract (*10*).

By using the internal standard 2,4,6-trimethylpyridine, concentration of 2-AP in the model system of P5C/methylgyoxal was estimated to be 0.27 ± 0.06 mmole which accounted for the 6.3% conversion rate of P5C to 2-AP.

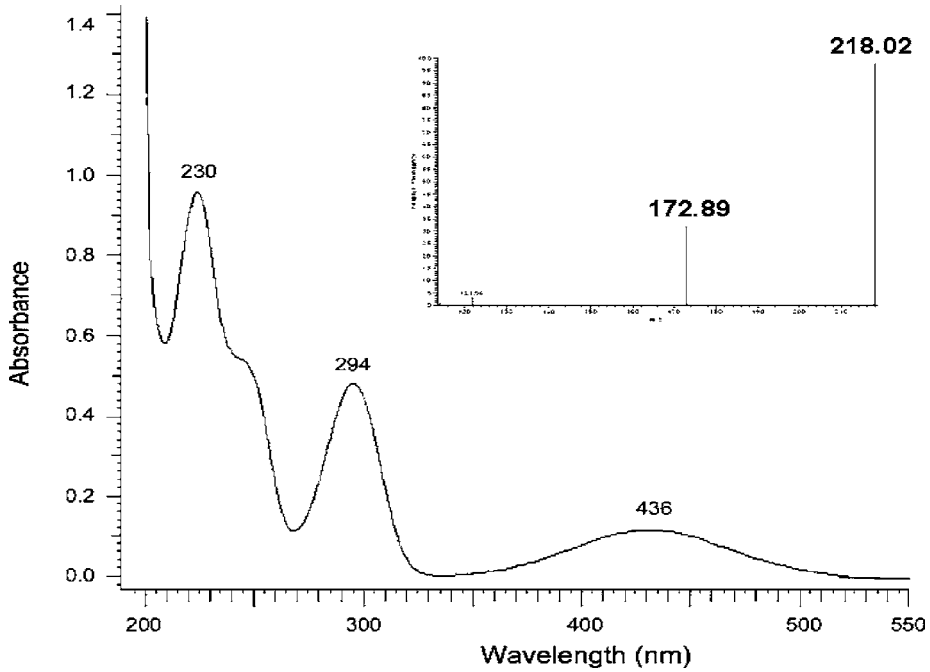

Figure 4. UV–vis spectrum and LC mass spectra (M^+ 218.02) of the product formed by the reaction between Δ^1-pyrroline-5-carboxylic acid and *o*-aminobenzaldehyde.

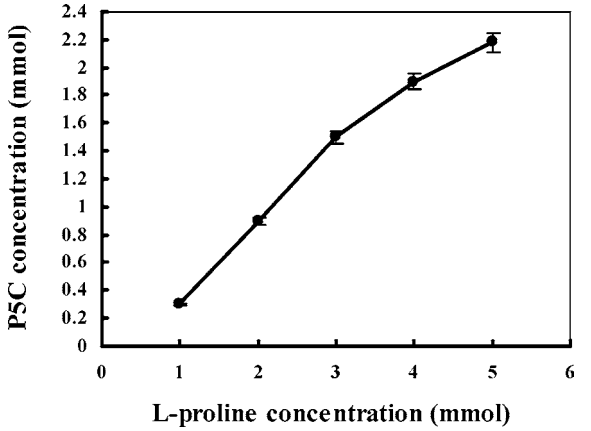

Figure 5. Synthesis of Δ^1-pyrroline-5-carboxylic acid as a function of L-proline concentration.

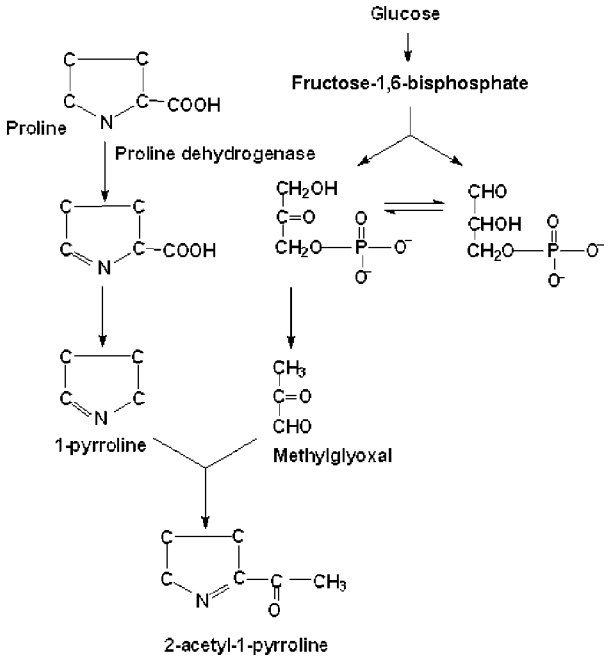

Figure 6. Proposed mechanism for 2-acetyl-1-pyrroline production by proline dehydrogenase (BnPRODH) from the *Bacillus natto* gene expressed in *Escherichia coli.*

Proposed Biosynthesis Pathway for 2-Acetyl-1-pyrroline. Although a significant amount of 2-AP (3.3% and conversion from 1-pyrroline) in 1-pyrroline/methylglyoxal model system under boiling condition has been detected (*1*), we demonstrated here for the first time that P5C which is the metabolite from L-proline is the key precursor for 2-AP synthesis. Romanczyk et al. (*7*) reported that supplement with L-proline in plate count agar (PCA) medium inoculated with *B. cereus* induced a dose-dependent production of 2-AP. Suprasanna et al. (*24*) found that L-proline is an effective precursor for 2-AP synthesis in cell and callus cultures of rice. Among the three amino acids, L-glutamic acid, L-ornithine, and L-proline, L-proline can best produce 2-AP in both rice callus and seeding in a dose-dependent manner (*25*). Recently, L-proline was found to enhance the in vitro synthesis of 2-AP in semidifferentiated callus culture of *Pandanus amaryllifolius* (*26*). Iyer and Caplan (*27*) reported the induction of stress-related genes in rice by P5C but did not clarify whether this induction was due to P5C being toxic. Cyclic P5C is in equilibrium with glutamate-γ-semialdehyde and is unstable in aqueous solution (*16*). Since aldehydes are highly reactive, P5C might react with various cellular compounds and thereby develop toxicity. It was found that even moderate concentrations of L-proline are toxic for *Arabidopsis* in axenic culture. They hypothesized that toxicity is not due to the existence of L-proline, but rather P5C is the effector of proline-induced cell death. P5C might also act as a single molecule activating processes related to apoptosis (*28*).

Results of stable isotope tracer experiments using ^{15}N-proline indicated that the nitrogen source of 2-AP was L-proline in *B. cereus* (*7*) and rice callus (*25*). We cloned the gene encoding *B. subtilis* subsp. natto BnPRODH, which can convert L-proline into P5C. 2-AP was found in the mixture of P5C and methylglyoxal kept at room temperature (25 °C) for 30 min. The production of 2-AP by *Bacillus* PRODH at ambient temperature supported the possibility of a nonthermal formation pathway for 2-AP by other microorganisms, reported previously, such as *B. cereus* (*7*), *Saccharomyces cerevisiae* (*1*), and several lactic acid bacteria, such as *Lactobacillus hilgardii* and *Lactobacillus brevis* (*8*).

Methylglyoxal (MG) has been used as one of the major intermediates for 2-AP synthesis in model system. Ahmed et al. (*29*) pointed out that acetone, threonine, and glucose may generate methylglyoxal. Among them, formation of methylglyoxal from glucose through triosephosphate pathway deserves special interest. Methylglyoxal is predominantly generated by the nonenzymatic degradation of triose phosphate intermediate through fragmentation and removal of phosphate from 1,3-dihydroxyacetone phosphate. Phillips and Thornalley (*30*) ascribed the formation of methylglyoxal to the excess glucose metabolized through Embden-Meyerhof pathway.

In *B. cereus*, increase of glucose in PCA medium from 0.2 to 1% leads to a 3-fold increase of 2-AP (145 to 458 μg/kg). We postulated that a high level of glucose resulted in the accumulation of MG following a similar pathway described by Phillips and Thornalley (*30*). By adding 1% of $^{13}C_6$-glucose, Romanczyk et al. (*7*) demonstrated that two carbons from glucose were incorporated within 2-AP. The α-dicarbonyls, such as methylglyoxal, are extremely reactive as glycating agents for collagen, enzymes, and other important cellular components, and they are toxic to cultured cells. They are elevated in the plasma of diabetic individuals and are found in increased levels in patients showing evidence of early diabetic nephropathy and retinopathy (*31*). We hypothesized that the added glucose was metabolized by *B. cereus* via glycolysis pathway and reacted with the P5C synthesized from L-proline to form 2-AP.

Costello and Henschke (*8*) proposed that acetylation at the C-2 position of the 1-pyrroline intermediate by the accumulated acetyl CoA may lead to the formation of the 2-AP. Hypothetical pathway leading from 1-pyrroline and methylglyoxal was proposed by Hofmann and Schieberle (*32*). Our data showed that P5C, derived from L-proline by the purified recombinant BnPRODH, might react directly with methylglyoxal and lead to the formation of 2-AP. On the other hand, P5C may also degrade to 1-pyrroline and condense with methylglyoxal to form 2-AP (**Figure 6**). P5C/methylglyoxal pathway represents the first report of a biological mechanism by which 2-AP may be synthesized in vitro by BnPRODH.

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