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Screening of microorganisms producing a novel protein-asparaginase and characterization of the enzyme derived from *Luteimicrobium album*

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A screening system using enrichment culture has been established with the aim of obtaining a novel enzyme for protein modification that has not been previously reported. This enzyme catalyzes deamidation of the side-chain amide group of asparagine in proteins. Enrichment culture of 390 soil samples was carried out with Z-Asn-Gly as the sole source of nitrogen, and the reaction product, Z-Asp-Gly, was detected in the culture supernatant of 102 strains. Strains with particularly high activity were *Leifsonia* sp., *Luteimicrobium* sp., *Microbacterium* sp., and *Agromyces* sp., all belonging to the class Actinobacteria. Of these, a protein-asparaginase (PA) was obtained from the culture supernatant of *Luteimicrobium album* 333B-h1, and its reactivity with different substrates and its basic enzymatic characteristics were investigated. Addition of the enzyme solution resulted in specific deamidation of only the asparagine residue in insulin chain B. The enzyme showed no reactivity with free asparagine or asparagine in low molecular weight peptides. It was demonstrated that the enzyme reacts with various protein substrates. In particular, proteins that have open structures, such as casein or gelatin, were good substrates. The activity and stability of PA at different temperatures and pH values were investigated. It was found that a temperature of 37°C and a roughly neutral pH are optimal conditions for the enzyme.

[**Key words:** Soil bacteria; Protein-asparaginase; Deamidation enzyme; *Luteimicrobium album* 333B-h1; Enrichment culture]

Deamidation of proteins can greatly improve various physical and functional properties, and it is therefore expected to expand their uses. Of the amino acids that make up proteins, only glutamine and asparagine have amide groups, and these amino acids are converted by deamidation into glutamic acid and aspartic acid, respectively. The negative electric charge of a protein increases with deamidation, so that its isoelectric point is lowered, and the solubility of the protein increases. The electrostatic repulsion also increases, as a result of which there is reduced interaction or associativity. In addition, deamidation of proteins causes the three-dimensional structure to unravel, so that hydrophobic regions that were buried inside the molecule are exposed on its surface. The protein then becomes amphiphilic, and its emulsifying capacity, emulsion stability, foaming properties, and foam stability improve (1).

Protein deamidation can be achieved by chemical or enzymatic methods. Among the chemical methods, many approaches involving processing with mildly acidic or alkaline solutions have been reported. However, there are some problems, which include the non-specificity of all of these reactions, the severing of peptide bonds under acidic or alkaline conditions, and the production of unexpected byproducts. Enzymatic methods for protein deamidation that have been reported include the use of protein-glutaminase (PG; EC 3.5.1.44) (2,3), proteases (4,5), transglutaminase (6), and peptide-glutaminase (7,8). PG in particular can catalyze protein deamidation reactions without any side reactions. PG has already been found to increase the functions of wheat proteins, milk proteins (casein and whey protein), and soybean proteins (9–12). However, it has been reported that the substrate of PG is the glutamine residue present in proteins, and PG does not act on the asparagine residue (2). The amino acids that make up plant and animal proteins contain abundant glutamine and asparagine, and deamidation not just of glutamine, but of asparagine as well, may be expected to be effective in further modifying protein functions.

Asparaginase (EC 3.5.1.1) is widely known as an enzyme that catalyzes the hydrolysis of asparagine to aspartic acid, but it has no reactivity with respect to proteins or peptides (13). There have been some reports on enzymes that catalyze deamidation reactions of N-terminal asparagine residues with a free alpha-amino group (14), enzymes that catalyze deamidation of asparagine residues with a peptide C-terminal (15), and peptidoglutaminase-asparaginase from *Aspergillus sojae* that catalyzes the deamidation of glutamine residues and asparagine residues in low molecular weight peptides (16). However, to date, no enzymes are known to catalyze the deamidation of asparagine present in the interior of the primary structure of proteins. The objective of this study was to screen and isolate bacteria from soil to obtain a novel protein-asparaginase (PA), a deamidation enzyme that acts directly on the amide groups in the side chains of asparagine (Asn) in proteins. The present paper describes the screening of PA-producing bacteria by

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enrichment culture and the results of the investigation of the properties of PA obtained from the culture supernatant of *Luteimicrobium album* 333B-h1.

MATERIALS AND METHODS

Screening for PA-producing strains Soil samples collected from natural environments were inoculated into an enrichment medium consisting of 1.17% Yeast Carbon Base (YCB) (BD Difco, Tokyo, Japan) and 0.3% carboxybenzoxy (Z)-Asn-Gly (Peptide Institute, Osaka, Japan) (pH 7.2, adjusted with NaOH). The cultures were incubated with shaking at 120 rpm for 5 days at different temperature conditions. The culture liquid thus obtained was inoculated into an isolation medium consisting of 3% Tryptic Soy Broth (TSB) (BD Difco, Tokyo, Japan) and 1.5% agar (INA AGAR; Ina Food Industry, Nagano, Japan) (pH 7.2, adjusted with NaOH) and cultured at 30°C. The colonies that appeared following the culture were seeded onto the primary screening assessment medium consisting of 1.17% YCB, 1.5% agar, with or without 0.3% Z-Asn-Gly (pH 7.2, adjusted with NaOH), and cultured at 30°C for 2 days. Following this, the strains with greater growth on the plate containing Z-Asn-Gly were selected as primary candidate strains.

The primary candidate strains thus obtained were cultivated in the enrichment medium at 30°C for 24 h, and the deamidation product, Z-Asp-Gly of the supernatant, was analyzed using a high-performance liquid chromatography (HPLC) system (Agilent 1200 Series) equipped with a Microsorb 100-3 C18 column (100 × 4.6 mm) (Agilent Technologies, Inc., Santa Clara, CA, USA). The elution was carried out under isocratic conditions with 18% (v/v) acetonitrile containing 0.1% TFA at 25°C with UV detection at 215 nm.

Identification of candidate PA-producing strains The candidate strains obtained by screening were colonized on an agar medium consisting of 3% TSB and 1.5% agar and purified by single colony isolation, and chromosomal DNA was then extracted from the colonies using PrepMan (Applied Biosystems, Inc., MA, USA). With this DNA as the template, the target sequences were amplified using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplified DNA fragments were base sequenced with an Applied Biosystems 3130xl Genetic Analyzer using the primers 27F, 920R (5′-GTCAATTCCTTTGAGTTT-3′), and 1100R (5′-AGGGTTGCGCTCGTTG-3′). The nucleotide sequences thus determined were analyzed using the Basic Local Alignment Search Tool (BLAST) software of the DNA Data Bank of Japan (DDBJ) to search for bacterial strains with high homology.

Investigation of cultures of candidate PA-producing strains by test tube culture Candidate PA-producing strains that showed high deamidation activity were selected, and test tube culture was carried out to select the appropriate medium for enzyme production.

The different media were prepared with YCB containing no nitrogen (N) source to which was added 0.3% Z-Asn-Gly and each of 13 types of medium ingredient added to 1% as an N source: sodium caseinate, NZ Amine Type A, N-Z-Case (Wako Pure Chemical Corp., Osaka, Japan), Polypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), Casitone, Trypton Peptone, BACTO Peptone, BACTO Proteose Peptone No. 3, liver, beef extract, Soytone, malt extract, and yeast extract (BD Difco). With a medium using sodium caseinate, calcium carbonate (Junsei Chemical, Tokyo, Japan) was added to 1% to control the pH. The various bacterial strains were cultured in each of the N source media at 30°C for 24 h with shaking at 120 rpm. A sample of culture supernatant was analyzed by HPLC to determine the amount of Z-Asp-Gly. With samples showing deamidation of all Z-Asn-Gly after 24 h of culture, the supernatant was reacted with Z-Asn-Gly and assayed using an F-kit Ammonia (Roche Diagnostics, Basel, Switzerland) according to the amount of released NH_3.

Flask culture of *Luteimicrobium album* 333B-h1 and enzyme purification As a seed culture, 50 mL of a growth medium consisting of 3% TSB was inoculated to 1% from a glycerol stock and cultured at 37°C for 19 h. Following this, 50 mL of a culture medium consisting of 1.17% YCB and 1% Polypepton (pH 7.2) were added to a shaking flask, and the seed culture was inoculated to 1% and cultured at 37°C for 24 h with reciprocal shaking at 120 rpm. The culture solution was centrifuged at 6982 ×*g* at 4°C for 15 min, and 3.5 L of supernatant were concentrated approximately 25 times using an ultrafilter (Sartorius; molecular weight cutoff 10,000) and filtered using a Stericup 0.22 μm (Millipore, Germany). Sodium sulfate was dissolved in the filtrate to 1.0 M to give the purified raw material sample, and this was passed to a Hiprep Octyl FF 10/16 hydrophobic chromatography column (GE Healthcare, NJ, USA) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 1.0 M sodium sulfate. The adsorbed proteins were eluted by a linear concentration gradient from 1.0 M to 0 M sodium sulfate, the active fraction was collected, and the buffer was exchanged for 20 mM sodium phosphate buffer (pH 7.0). This was passed to a Hiprep DEAE FF 10/16 anion exchange chromatography column (GE Healthcare) equilibrated with the same buffer, and the adsorbed proteins were eluted by a linear concentration gradient from 0 M to 0.5 M sodium chloride. The active fraction was again collected, and, as before, the buffer was exchanged for 20 mM sodium phosphate buffer (pH 6.0), after which the Hiprep DEAE FF 10/16 anion exchange chromatography column was equilibrated, and the adsorbed proteins were eluted by a linear concentration gradient from 0 M to 0.5 M sodium chloride. The active fraction was concentrated using an ultrafilter and passed to a Superdex 200 10/300 gel filtration chromatography column (GE Healthcare) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride and eluted with the same buffer. The active fraction was mixed with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing a reducing agent and heat treated, after which electrophoresis was carried out with a 7.5% polyacrylamide gel (e-PAGEL E-T7.5L; Atto Corporation, Tokyo, Japan) or a 5–20% gradient gel (PAGEL SPG-520L; Atto Corporation). The gel was stained with Coomassie Brilliant Blue (Bio-Rad, Richmond, CA, USA).

Measurement of asparagine deamidation activity Enzyme solution (10 μL) was added to 190 μL of 0.2 M phosphate buffer (pH 6.5) containing 30 mM Z-Asn-Gly and reacted at 37°C for 10 min, after which the reaction was halted by the addition of 200 μL 12% trichloroacetic acid (TCA). The reaction solution was centrifuged (13,000 ×*g*, 4°C, 5 min), and the ammonia in the supernatant thus obtained was measured using an F-kit Ammonia. For the unit of enzyme activity, the enzyme activity that generated 1 μmol of ammonia in 1 min under the reaction conditions detailed above was defined as 1 unit (U).

N-terminal sequencing analysis The N-terminal amino acid sequence of the enzyme or substrate sample was analyzed using a protein sequencer (PPSQ-21A; Shimadzu Corporation, Kyoto, Japan).

Substrate reactivity of the enzyme Insulin chain B (Sigma, MO, USA) was dissolved to 2.5 mg/mL in 0.1 M sodium phosphate buffer (pH 6.8). To 45 μL of this substrate solution, 45 μL of either enzyme solution or water as a control were added, and this was reacted for 1 h at 37°C. The reaction was then halted with 10 μL 1 M hydrochloric acid. After centrifuging, the supernatant was filtered and analyzed by HPLC. The HPLC analysis device was a UHPLC Agilent 1290 Infinity LC, and the column was a ZORBAX 300SB-C18 2.1 × 50 mm, 1.8 μm. Elution buffer A was 0.1% TFA, and elution buffer B was 0.1% TFA/80% acetonitrile. The elution conditions were buffer A to buffer B: 5%–50%, 0 min to 5 min.

The reaction solution was dissolved in 0.2 M phosphate buffer (pH 6.5) containing 10 mM synthetic peptides, Z-Asn-Gly, Z-Gln-Gly (Peptide Institute, Osaka, Japan), Z-Asn (Kokusan Kagaku, Tokyo, Japan), Asn-Gly, pGlu-Asn-Gly, Gly-Asn, Gly-Asn-Gly, Pro-Leu-Gly-Asn, Leu-Ala-Asn, Leu-Gly-Asn (Eurofins, Tokyo, Japan) or D or L-Asn (Nacalai Tesque, Kyoto, Japan). To 100 μL of this substrate solution, 10 μL (3 U/mL) of enzyme were added, and this was reacted for 10 min at 37°C. The reaction was halted by the addition of 100 μL 12% TCA, and the ammonia in the reaction solution was measured using an F-kit Ammonia.

To examine the reactivity with various different proteins, a sodium phosphate buffer solution (0.2 M, pH 6.5) was prepared containing 2% (w/v) of α-casein (Sigma), α-lactalbumin (Sigma), sodium caseinate (Miprodan, Nippon Shinyaku Co., Ltd., Tokyo, Japan), whey protein (Bipro, Davisco, Eden Prairie, MN, USA), porcine type A gelatin (Sigma), bovine type B gelatin (Sigma), fish gelatin (Nippi Inc., Tokyo, Japan), egg albumin (Sigma), and bovine serum albumin (Sigma). Powdered skimmed milk (low heat type, Yotsuba Milk Products Co., Ltd., Hokkaido, Japan) was dissolved in the buffer to 6% w/v. The PA solution (10 μL, approximately 5 U/mL) was added to 100 μL of each of these substrate solutions and reacted for 1 h at 37°C, after which the ammonia in the supernatant was measured.

Temperature characteristics of the enzyme Enzyme solution (10 μL) was added to 190 μL 0.2 M Tris-HCl buffer solution (pH 7.5) containing 30 mM Z-Asn-Gly to give a final concentration of 2–2.5 μg/mL. The resulting solution was reacted for 30 min at 30°C, 37°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C, and the amount of ammonia generated by the reaction was measured. To evaluate the thermal stability, 1 mg/mL enzyme solution were dissolved in 0.2 M of buffer solution at each pH to a final concentration of 2.5 μg/mL, and incubated for 30 min at 30°C, 37°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C. After this, the enzyme solution was quickly moved to an ice bath, and the enzyme activity was measured.

pH characteristics of the enzyme Enzyme solution (10 μL) was added to 190 μL of 0.2 M buffer solution at different pH values containing 30 mM Z-Asn-Gly to give a final concentration of 2–2.5 μg/mL. The buffer solutions were: acetic acid buffer solution at pH 3.5, 4.0, 4.5, 5.0, and 5.5; citric acid buffer solution at pH 5.5 and 6.0; phosphate buffer solution at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5; and Tris-HCl buffer solution at pH 8.0, 8.5, and 9.0. The reaction was carried out for 30 min at 37°C, and the ammonia generated by the reaction was measured. To evaluate the enzyme stability, an enzyme solution was prepared by diluting 1 mg/mL of enzyme solution in a 0.2 M buffer solution at each pH and leaving it for 3 days at 4°C, after which the enzyme activity was measured.

RESULTS AND DISCUSSION

Enrichment culture from soil samples A total of 390 soil samples were enrichment cultured, from which 3494 strain colonies were extracted. Of these, 2001 strains were selected as primary candidate strains on the basis of greater growth on a YCB agar culture medium containing Z-Asn-Gly, and evaluation was carried out by liquid culture. The supernatant of 24-h culture liquids was

analyzed by HPLC, and conversion of Z-Asn-Gly to Z-Asp-Gly was found with 124 strains. The majority of strains with which Z-Asn-Gly was found in the culture liquid were enrichment cultured at 30°C, although the culture was also carried out at 20°C, 40°C, 42°C, and 50°C to obtain different bacterial strains. Among these different strains, 102 strains with Z-Asp-Gly generation of greater than 0.2 mM were taken as candidate PA-producing strains.

Activity evaluation and identification of candidate PA-producing strains The genus and species of candidate PA-producing strains were identified by means of 16S rDNA analysis. As the results of identification and the amounts of Z-Asp-Gly generation for each strain showed, the majority of strains for which Z-Asp-Gly generation was found were identified as *Leifsonia xyli*, with multiple *Microbacterium* sp., *Agromyces* sp., *Paenibacillus* sp., and *Citerobacter* sp. also obtained. *Leifsonia xyli* and other species of genus *Leifsonia* showed a wide distribution of Z-Asp-Gly generation from 0.2 mM to 7.0 mM, and exhibited a normal distribution with a peak at 3.0−4.0 mM. *Microbacterium* sp. also showed a wide range of Z-Asp-Gly generation, from 1.0 mM to 5.0 mM. On the other hand, *Paenibacillus* sp. showed Z-Asp-Gly generation of $\leq$ 2.0 mM. For the other genus and species, three strains showed Z-Asp-Gly generation of $\geq$ 1.0 mM, these being two *Agromyces* sp. and one species of genus *Luteimicrobium*. The other strains showed Z-Asp-Gly generation of $<$ 1.0 mM.

A total of 102 candidate PA-producing strains were identified, comprising 71 *Leifsonia* sp., 11 *Microbacterium* sp., 2 *Agromyces* sp., 1 *Luteimicrobium* sp., 10 *Paenibacillus* sp., 1 *Rhizobium* sp., 1 *Enterobacter* sp., 2 *Citerobacter* sp., 1 *Arthrobacter* sp., 1 *Rahnella* sp., and 1 *Raoultella* sp. Of these, 10 strains that were selected by screening as strains with high deamidation activity were 1 *Leifsonia* sp., 2 *Microbacterium* sp., 1 *Agromyces* sp., 1 *Luteimicrobium* sp., 2 *Paenibacillus* sp., 1 *Rhizobium* sp., and 1 species each of *Enterobacter* and *Citerobacter* from the class γ-Proteobacteria. The greatest numbers of candidate PA-producing strains were *Leifsonia* sp., followed by *Microbacterium* sp., *Agromyces* sp., and *Luteimicrobium* sp. These are all members of the class Actinobacteria, suggesting that many species of this lineage have the ability to produce deamidation enzymes.

Investigation of culture of candidate PA-producing strains by test tube culture Ten strains that were selected as candidate PA-producing strains were test tube cultured in a YCB culture medium containing different N sources with Z-Asn-Gly for 24 h, and the culture supernatant was analyzed by HPLC. Table 1 shows the amount of Z-Asp-Gly generated in the representative media. Among the 10 strains, enzyme production was particularly high in the two species *Luteimicrobium album* 333B-h1 and *Agromyces* sp. 225A-i. These two species showed high Z-Asp-Gly generation (7−9 mM) with most N source media (except only Z-Asn-Gly). *Leifsonia xyli* 338A-d2 showed relatively high PA activity (Z-Asp-Gly generation $>$ 6.0 mM) with 7 types of culture media, including liver and malt extract. With the other strains, high deamidation activity was found with a few types of culture media, and differences were seen between strains in the N source that showed high activity. *Microbacterium testaceum* 170A-e showed the highest activity with liver as the N source, while 210A-j, identified as the same genus *Microbacterium*, showed optimal activity with BACTO Peptone. The five strains described above, 333B-h1, 225A-i, 338A-d2, 170A-e, and 210A-j, belong to the class Actinobacteria. It was thus shown once again that many of the PA-producing microorganisms are in the class Actinobacteria.

Among the microorganisms from different classifications, while *Paenibacillus* sp. (355A-b and 212B-a1) from the class Firmicutes, the γ-Proteobacteria *Enterobacter asburiae* 144A-b and *Citerobacter freundii* 181A-a1, and the α-Proteobacteria *Rhizobium radiobacter* 388A-b1 showed relatively high activity in only a few culture media, the overall trend was for low activity with any N source medium or else no activity at all.

Flask culture and partial enzyme purification of *Luteimicrobium album* 333B-h1 Since it was suggested that 333B-h1, 225A-i, and 338A-d2, three strains that belong to the class Actinobacteria, have high enzyme productivity, enzyme purification was conducted to verify the existence of an enzyme that deamidates asparagine in polypeptides or proteins. Purification was carried out not only to exclude protease activity, but also to estimate a protein with PA function. If protease is contained in the culture solution, it is possible that the deamidation activity for asparagine by a peptide or amino acid is being evaluated, rather than the asparagine in the protein. Attempts were made to purify PA from cultures of the three strains. However, in 225A-i, it was difficult to separate the supernatant from the culture broth solution by centrifugation due to the high viscosity of the solution. There is a report showing that a strain of the genus Agromyces is related to biofilm formation, and a microbiological analysis is further necessary (17). In terms of 338A-d2, during the preparation of the PA fraction, the activity was decreased and purification could not be completed until the protease activity was removed (data not shown). In this study, we successfully purified PA from 333B-h1 culture broth to almost a single band and investigated the basic characteristics of PA.

A large quantity of 333B-h1 culture broth containing Polypepton as the N source was concentrated, and this was purified by successive hydrophobic chromatography, ion-exchange chromatography, and gel filtration chromatography, and the results are shown in Table 2. From approximately 3 L of culture broth, a purified sample with a total PA activity of 13.6 U was obtained. The chromatogram from the gel filtration chromatography, which was the

TABLE 1. Investigation of medium for candidate PA-producing strains.

Strain	Amount of Z-Asp-Gly generated in different N sources (mM)							
	Z-Asn-Gly only	Z-Asn-Gly+Casitone	Z-Asn-Gly+Polypepton	Z-Asn-Gly+BACTO Peptone	Z-Asn-Gly+Liver	Z-Asn-Gly+Beef extract	Z-Asn-Gly+Soytone	Z-Asn-Gly+Malt extract
333B-h1	1.63	7.77	7.67	7.49	7.88	7.88	7.81	7.5
225A-i	3.28	8.23	7.88	8.29	7.04	2.06	7.98	2.34
338A-d2	6.79	5.28	1.81	5.43	7.55	6.19	5.76	6.59
170A-e	3.79	ND	ND	ND	6.64	0.45	5.31	1.42
210A-j	1.37	0.44	0.14	6.04	0.82	0.06	0.13	1.89
212B-a1	1.67	0.83	0.98	0.68	0.41	0.93	0.37	1.51
355A-b	1.51	ND	ND	0.11	1.92	2.58	ND	2.56
144A-b	0.53	ND	0.06	ND	ND	0.09	ND	1.85
181A-a1	0.52	ND	0.2	ND	0.35	0.32	ND	1.28
388A-b1	0.59	ND	ND	ND	4.51	ND	ND	ND

ND, not detected. 333B-h1, *Luteimicrobium album*; 225A-i, *Agromyces* sp.; 338A-d2, *Leifsonia xyli*; 170A-e, *Microbacterium testaceum*; 210A-j, *Microbacterium* sp.; 212B-a1, *Paenibacillus lactis*; 355A-b, *Paenibacillus* sp.; 144A-b, *Enterobacter asburiae*; 181A-a1, *Citerobacter freundii*; 388A-b1, *Rhizobium radiobacter*.

TABLE 2. *Luteimicrobium album* 333B-h1 culture broth purification table.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery rate (%)
Ultrafilter (molecular weight cutoff: 10000)	145.52	219	1.5	100
Hiprep Octyl FF 16/10	53.5	85.5	1.6	39.1
Hiprep DEAE FF 16/10 (pH 7.0)	2.59	49.2	19	22.5
Hiprep DEAE FF 16/10 (pH 6.0)	1.86	27.9	15	12.8
Superdex 200 10/300	0.53	13.6	25.9	6.2

final purification process, is shown in Fig. 1A. The highest PA activity was detected in fraction 1B4. SDS—PAGE revealed a protein band estimated to be PA at about 110 kDa (Fig. 1B). To more accurately confirm the molecular mass, fraction 1B4 was concentrated and SDS—PAGE was carried out on a gel for separating proteins with higher molecular mass. As shown in Fig. 1C, two bands were observed. N-terminal analysis of these bands revealed a common five N-terminal sequence (Ala-Val-Thr-Ala-Asp-). Therefore, it was inferred that due to contaminant protease in the original culture medium, PA was subjected to the post-translational modification, resulting in the formation of PA with different molecular masses. In the future, it will be necessary to determine the gene sequence and primary structure of PA and to analyze its homology with a protein of known structure.

Evidence of enzymatic deamidation of protein-bound asparagine The insulin B chain is a polypeptide consisting of 30 amino acids. The third and fourth from the N terminus are Asn and Gln, respectively, which are convenient substrates for judging the deamidation reaction of asparagine and glutamine. The PA fraction from 2nd ion exchange chromatography was reacted with insulin chain B for 1 h at 37°C and analyzed by HPLC. A slight difference was observed in the elution time. The retention time of insulin B chain before and after reaction was 4.88 min and 4.96 min, respectively (Fig. 2A). The solution after reaction was analyzed by a protein sequencer, and the N-terminal sequence was found to be Phe-Val-Asp-Gln-. Since the unreacted N-terminal sequence was Phe-Val-Asn-Gln-, this indicates that asparagine, which is the third base from the N-terminal of the unreacted sequence, was converted to aspartic acid as a result of deamidation by the enzyme. There was no change to glutamine, which is the fourth base from the N-terminal of the unreacted sequence, suggesting that the present enzyme is specific to the asparagine residue.

When the reaction mixture containing 2% sodium casein incubated with PA sample (9 U/mL) at 37°C for 3 h was subjected to SDS—PAGE (Fig. 2B), no significant change was produced in the molecular mass of casein. This means that the enzyme sample had no hydrolytic activity after purification. Also, after the enzymatic reaction, it was recognized that the α-casein band shifted slightly to the upper side. The same phenomenon was confirmed with protein-glutaminase-treated casein (3), and it is inferred that the PA-induced deamidation also influenced the mobility of the band by changing the interaction of negatively charged casein to SDS.

Substrate specificity of PA from *Luteimicrobium album* 333B-h1 Deamidation activity was investigated using asparagine and peptides containing asparagine or glutamine in place of Z-Asn-Gly as the substrate (Table 3). In total, 1.09 mM of released ammonia was generated with pGlu-Asn-Gly, indicating activity near the level of that with Z-Asn-Gly (1.47 mM). There was virtually no reaction with Leu-Ala-Asn, Z-Asn, Asn-Gly, L-Asn, D-Asn. Pro-Leu-Gly-Asn, Leu-Gly-Asn, or Gly-Asn. Furthermore, Gly-Asn-Gly showed little PA activity, approximately one-tenth that of Z-Asn-Gly. Judging from the difference in the reactivity between Gly-Asn-Gly and pGlu-Asn-Gly (Z-Asn-Gly), it was conjectured that the molecular weight or structure of the amino acids on the N-terminal side may have influenced the reactivity. In addition, there was almost no reaction with Leu-Ala-Asn, Pro-Leu-Gly-Asn or Z-Asn, suggesting that reaction does not readily occur with Asn on the C-terminal. It was also confirmed that there was no reactivity toward Z-Gln-Gly. Based on these results, it may be conjectured that PA derived from the 333B-h1 strain is an enzyme that does not act on Gln, but acts readily on Asn in polypeptide chains (Asn not located at the N-terminal or the C-terminal). Specifically, this enzyme is completely distinguished from the previously reported enzymes such as peptide-asparaginase (15) and protein NH_2-terminal asparagine deamidase (14). In the future, it will be necessary to conduct a detailed enzymatic reaction rate analysis to obtain the K_m value and V_{max} for each substrate.

The reactivity of PA with different proteins was also investigated (Table 3). To investigate the possibility of application to foods and

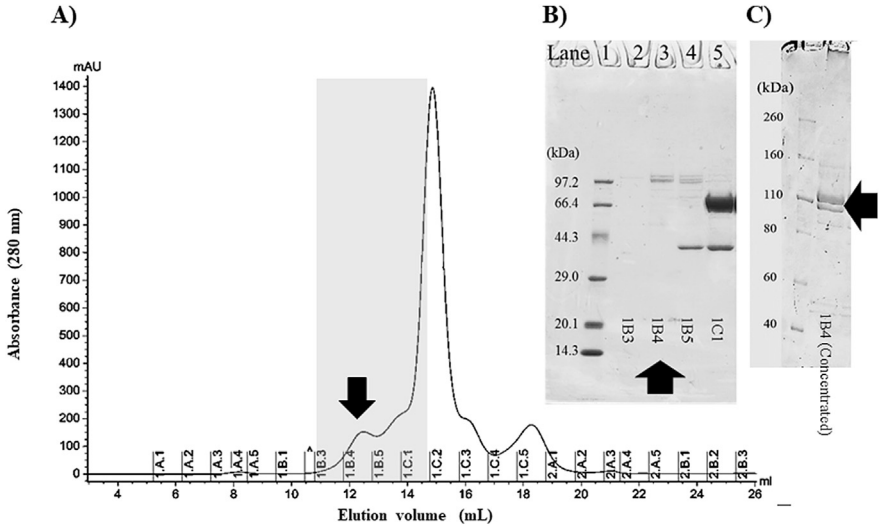

FIG. 1. Final purification using gel filtration chromatography of PA derived from *Luteimicrobium album* and electrophoresis of the active fraction. (A) Gel filtration chromatogram. Mobile phase; 0.1 M phosphate buffer (pH 7.0) + 0.1 M NaCl. (B) SDS—PAGE of the fraction shown by the shaded area in panel A (1B3—1C1). (C) SDS—PAGE of a concentrated sample of 1B4 (the fraction with the highest activity).

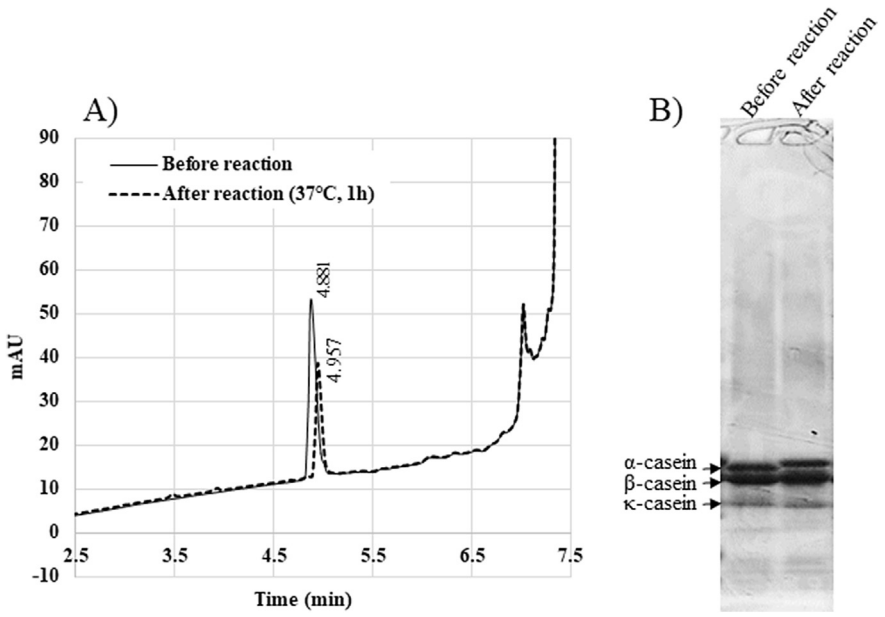

FIG. 2. (A) Reactivity of PA from 333B-h1 with insulin B chain (solid line: before reaction, broken line: after reaction). (B) Sodium casein incubated with PA (9 U/mL) at 37°C for 3 h.

medicines, the reactivity of PA was evaluated mainly on representative food proteins as previously reported (2). The amounts of released ammonia when PA was added to 2% substrate solutions of different proteins and reacted for 1 h at 37°C were recorded. There were reactions with various proteins, and α-casein in particular, one of the main proteins found in milk, generated a large amount of ammonia. It was also confirmed that the deamidation of α-casein was found to be dependent on the amount of enzyme and the time (data not shown). Similarly, sodium caseinate is a relatively good substrate although it produced a slightly weaker reaction than α-casein, probably due to the effect of β-casein and κ-casein. β-Lactoglobulin (β-Lg) and α-lactalbumin (α-La) are major whey proteins and the number of Asn residues is 5 and 8, respectively (18,19). Although both are globular proteins, PA reacted well with α-La, but showed no reaction with β-Lg. This is presumably because β-Lg has fewer Asn residues than α-La. Furthermore, it is possible that all Asn residues are buried in folded molecules; therefore, it can be inferred that the protein in the whey protein isolate reacted only with α-La. Much less ammonia was released from skim milk than α-casein. This can be explained by the casein micelle structure and poor reactivity for β-Lg. The egg albumin and bovine serum albumin showed no reaction for the same reason as for β-Lg. Soy protein isolate which comprises a mixture of globular proteins showed ammonia generation by the deamidation, probably because conglycinin and other soy proteins have a high asparagine content (20). Acid-treated gelatin also reacted comparatively well. Since this protein has a random structure as well as caseins, it can be considered that the asparagine present in these proteins is in a location where it can react readily. Alkali-treated gelatin (type B) showed lower reactivity than acid-treated gelatin, and this was probably because alkali treatment causes chemical deamidation, thus lowering the asparagine content (21). Thus, the number of asparagine residues in the higher-order and the primary protein structure affects the reactivity of the present enzyme.

TABLE 3. Substrate specificity of PA from *Luteimicrobium album* 333B-h1.

Substrate	Released NH_3 (mM)
Peptide substrate	
Z-Asn-Gly (30 mM)	1.47[a]
pGlu-Asn-Gly	1.09[a]
Pro-Leu-Gly-Asn	0.154[a]
Leu-Gly-Asn	0.148[a]
Gly-Asn	0.166[a]
Gly-Asn-Gly	0.142[a]
Leu-Ala-Asn	N.D.[a]
Z-Asn	N.D.[a]
Asn-Gly	N.D.[a]
L-Asn	N.D.[a]
D-Asn	N.D.[a]
Z-Gln-Gly	N.D.[a]
Protein	
α-Casein	4.69[b]
Sodium caseinate	2.98[b]
Skim milk	1.22[b]
α-Lactalbumin	1.84[b]
β-Lactoglobulin	N.D.[b]
Whey protein isolate	1.17[b]
Soy protein isolate	0.98[b]
Egg albumin	N.D.[b]
Bovine serum albumin	N.D.[b]
Type A bovine gelatin	2.23[b]
Type B porcine gelatin	0.73[b]
Fish gelatin	1.27[b]

[a] Ten microliters (3 U/mL) of enzyme were added to 100 μL of 30 mM substrate solution and incubated for 10 min at 37°C.
[b] The enzyme solution (0.025U of PA) was added to each protein solution (2%) and incubated at 37°C for 1 h.

Effects of temperature and pH on PA activity of *Luteimicrobium album* 333B-h1 As shown in Fig. 3A, the optimum temperature for PA is 37°C, and reactivity drops off sharply above 40°C. In order to investigate the thermal stability, the enzyme solution was warmed in advance to the determined temperature and incubated for 30 min, after which the activity was calculated with the activity at 4°C taken to be 100%. The results showed that the residual activity decreased with increasing temperature, and above the optimal temperature of 37°C, the enzyme was almost completely inactivated as a result of 30-min pre-incubation (Fig. 3B). The optimal pH for PA was 6.5, and the relative reactivity at pH 5.5–6.5 was 90% (Fig. 3C). Outside this range, the activity dropped off with increasing alkalinity or acidity. The residual reactivity after storing for 3 days at 4°C in buffers at different pH values is shown in Fig. 3D. At pH 4.5–8.0, there was ≥ 80% residual activity, indicating relative stability across a wide pH range.

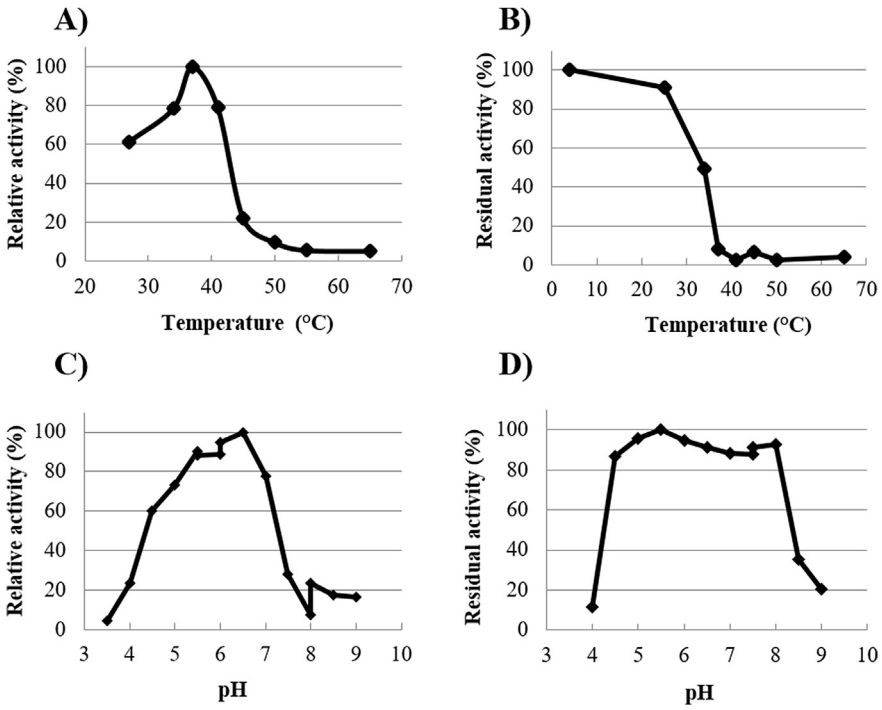

FIG. 3. Enzymatic properties of PA from *Luteimicrobium album* 333B-h1. (A) Optimal temperature: PA activity was determined in 0.1 M phosphate buffer, pH 6.8 at 25–65°C for 10 min. (B) Thermal stability: PA activity was determined after the incubation of purified PA in a 0.1 M phosphate buffer, pH 7.0 at 5–65°C for 30 min. (C) Optimal pH: PA activity was measured in buffers (0.1 M) with various pH values. The buffers used were acetate buffer, pH 3.5–5.5 (closed squares); phosphate buffer, pH 6.0–8.0 (open squares); Tris-HCl buffer, pH 7.0–9.0 (closed circles); and carbonate bicarbonate buffer, pH 9.0–11.0 (open circles). (D) pH stability: PA activity was measured after the incubation of purified PA in buffers (20 mM) with various pH values for 3 days at 4°C. (A–D) Assays were carried out in duplicate, and the average value is shown.

In conclusion, a novel enzyme (PA) that catalyzes the deamidation of asparagine residue in proteins was screened from soil bacteria using an enrichment culture. This study focused on PG derived from *Luteimicrobium album* 333B-h1, one of the PA-producing bacterial strains belonging to the class Actinobacteria. It was found that PA had reactivity toward various protein substrates, and the response of PA at different temperatures and pH values was characterized. In order to investigate the mechanism of deamidation catalysis of the novel PA, it will be necessary to determine the primary structure of the PA and carry out research into the deamidation reaction kinetics using different substrates and inhibitors. In future work, the PA from other bacterial strains should be investigated. Furthermore, future research must clarify the function of PA and investigate the possibility of application in foods and pharmaceutical fields.

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