A Novel Protein-Deamidating Enzyme from Chryseobacterium proteolyticum sp. nov., a Newly Isolated Bacterium from Soil

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A novel protein-deamidating enzyme, which has potential for industrial applications, was purified from the culture supernatant of Chryseobacterium proteolyticum strain 9670T isolated from rice field soil in Tsukuba, Japan. The deamidating activities on carboxybenzoyloxy (Cbz)-Gln-Gly and caseins and protease activity were produced synchronously by the isolate. Both deamidating activities were eluted as identical peaks separated from several proteases by phenyl-Septarose chromatography of the culture supernatant. The enzyme catalyzed the deamidation of native caseins with no protease and transglutaminase activities. Phenotypic characterization and DNA analyses of the isolate were performed to determine its taxonomy. Physiological and biochemical characteristics, 16S rRNA gene sequence analysis, and DNA-DNA relatedness data indicated that the isolate should be placed as a new species belonging to the genus Chryseobacterium. The isolate showed no growth on MacConkey agar and produced acid from sucrose. The levels of DNA-DNA relatedness between the isolate and other related strains were less than 17%. The name Chryseobacterium proteolyticum is proposed for the new species; strain 9670 is the type strain (=FERM P-17664).

An enzyme catalyzing deamidation of proteins has a great potential for industrial applications. Deamidation of proteins can improve protein functionalities such as solubility, emulsification, and foaming and gelation properties, which are desired properties in some food proteins. Most plant proteins have poor solubility and functionality under mild acidic conditions, which are the pH ranges of most food systems, resulting in their limited use in foods. Because the contents of glutamine residue in plant proteins are generally high, deamidation of such proteins is one of the most promising ways to expand their uses and to improve their functionalities. Many studies of the chemical (mild acid or alkaline treatment) or physical (dry heat treatment) deamidation of food proteins had reported and demonstrated the effectiveness of deamidation for improvement of protein functionalities (see reference 25 for review). To avoid unfavorable side effects brought about by nonenzymatic treatments—for example, concomitant peptide bond cleavage, off-flavor formation, and amino acid racemization—enzymatic deamidations of proteins have been examined (see reference 10 for review). Protease (16), transglutaminase (23), and peptidoglutaminase (9) were used for this purpose. None of their primary reactions were deamidations, or the enzymatic substrates were peptides rather than proteins. Besides the improvement in protein functionalities, protein-deamidation enzymes could be used for many applications, including protein structure analysis.

In 1971, Kikuchi et al. (17) found an enzyme, peptidoglutaminase, from Bacillus circulans that deamidates the peptide-bound glutamines. This enzyme was not active on high-molecular-weight peptides, i.e., proteins such as caseins, unless the proteins were hydrolyzed to short peptides (17). In plants, the possible presence of protein deamidase in germinating wheat grains was reported, but the enzyme has not yet been fully purified and characterized (31).

To obtain a protein-deamidating enzyme of microbial origin, we have screened microorganisms from soils and successfully isolated a bacterium that produces the target enzyme. The enzyme deamidated native caseins without protease and transglutaminase activity. In the present study, we report the discovery of a novel protein-deamidating enzyme from a bacterium and the taxonomic determination of the isolate. The latter led to the proposal of a new species within the genus Chryseobacterium.

MATERIALS AND METHODS

Bacterial strains. Two strains, 9670T and 9671, isolated as described below were used. They were maintained on nutrient agar at 4°C. Type strains of Chryseobacterium gleum JCM 23415T (ATCC 35910T), Chryseobacterium indolgenes IFO 14944T (ATCC 29897T), Chryseobacterium balustinum IFO15053T (ATCC 33487T), Chryseobacterium meningosepticum IFO 12535T (ATCC 13253T), Empedobacter brevis IFO 14945T (NCTC 11099T), and Myroides odoratus IFO 14945T (ATCC 4653T) were used as reference strains for DNA-DNA hybridization studies.

Isolation of strains. Water suspensions of 320 soil samples, collected from natural environments, such as grasslands, gardens, crop fields, livestock farms, riversides, forests, and dumping grounds in Tsukuba City, Japan, were inoculated into A medium consisting of 0.1% carboxybenzoxy (Cbz)-Gln-Gly (Peptide Laboratory, Osaka, Japan), 0.5% glucose, 0.02% KH2PO4, 0.02% MgSO4·7H2O, 0.0005% NaWO4·2H2O, 0.01% NaCl, 0.002% CuSO4·5H2O, 0.0005% NaMoO4·4H2O, 0.0005% MnSO4·H2O, and 0.001% CuSO4·5H2O (pH 8.0, adjusted with 6N NaOH). The cultures were incubated aerobically at 30°C for 6 days. Fresh A medium was inoculated with a portion of the above cultures and incubated at 30°C for 3 days. Bacterial and fungal strains were isolated by plating or streaking a portion of the culture onto Luria-Bertani agar (Oxoid, Basingstoke, United Kingdom) for bacteria or potato-dextrose agar (Difco Laboratories, Detroit, Mich.) for fungi. Isolated strains were inoculated into B medium containing 1.5% agar. Strains grown on the plates were collected and then inoculated into B medium consisting of 0.5% lactose, 1.0% peptone, 0.17% Na2HPO4·7H2O, 0.025% KH2PO4, 0.025% MgSO4·7H2O, and 0.005% FeSO4·7H2O (pH 7.2, adjusted with 6N NaOH). The cultures were incubated aerobiologically at 30°C for a period of from 2 to 7 days. Culture supernatants were subjected to enzyme assays. Two strains showing higher protein-deamidating activity were selected and purified by repeated streaking on the nutrient agar medium.

Enzyme assays. For deamidating activity, 100 μl of substrate solution containing 10 mM Cbz-Gln-Gly or 1.0% caseins (Hammersten, Merk, Poole, United Kingdom), 175.6 mM sodium phosphate buffer (pH 6.5), and 10 μM of enzyme solution were mixed and then incubated at 37°C for 60 min. The reaction was stopped by the addition of 100 μl of 12% trichloroacetic acid. For blank assays, enzyme solution was added after addition of trichloroacetic acid. After centrifugation at 18,000 × g for 5 min, released ammonia in the supernatant was determined by an NADH-glutamate dehydrogenase method (21) with an am-
monia determination kit according to the manufacturer’s instructions (Boehringer-Mannheim/Roche Diagnostics, Lewes, United Kingdom). One unit of enzy-
me was defined as the amount that released 1 μmol of ammonia per min under
the above conditions. Ammonia was also determined by a phenol method
for screening study. In this case, 10 μl of the supernatant was mixed with 100 μl of
0.1% phenol–0.005% sodium nitroprusside, and then 100 μl of 0.5% NaOH–
0.6% NaOAc was added. After 60 min, the A450 of the mixture was measured. For
proteinase activity, the A450 was measured in the supernatant from the above
decamidating activity assay when casein was used as a substrate. One unit of
protease activity was defined as the amount that caused an increase of 1 optical
density unit at 280 nm (OD280) per 60 min under the above conditions. Trans-
glutaminase activity was assayed by a horseradish method according to Folk and
Chung (5).

Partial purification of protein-deamidating enzyme. A preculture of strain
9670T in B medium grown at 25°C overnight was inoculated into the same
medium at a 1.0% concentration of preculture. The culture was incubated at
25°C with reciprocal shaking at 145 rpm for 48 h for a culture profile study or 24 h
for enzyme purification. The culture broth was centrifuged at 22,200 × g for 15
min at 4°C. After addition of 2 mM EDTA, the supernatant was concentrated
eightfold by ultrafiltration with a hollow-fiber-type membrane (AIP1010, MW
6000 cut; Asahi Chemical Industry, Tokyo, Japan). After dialysis of the concen-
trate against 2.0 M NaCl in 10 mM sodium phosphate buffer (pH 6.5), the dialysate
was centrifuged at 2.0 × g for 10 min at 4°C and filtered through a 0.45-μm-pore-
diameter membrane in order to remove the insoluble materials. The resultant
filtrate was applied to a phenyl-Sepharose High Performance Hiload 16/10 col-
umn (Amersham Pharmacia Biotech, Uppsala, Sweden) prequilibrium with 2.0
M NaCl in 10 mM sodium phosphate buffer (pH 6.5). The column was washed
to wash with two column volumes of 2.0 M NaCl in 10 mM sodium phosphate buffer (pH 6.5), and the deamidating activity was eluted by a linear concentration from 2.0 to 0 M NaCl
of 10 mM sodium phosphate buffer (pH 6.5). The elution was followed with 10
mM sodium phosphate buffer (pH 6.5). The chromatography was carried out
with a fast-performance liquid chromatography (FPLC) system (Amersham
Pharmacia Biotech) with all flow rates at 1.0 ml/min.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE) was performed by the method of Laemmli (20) using a 10 to 20% polyacrylamide gradient gel (Multigel; Daiichi Pure Chemicals, Tokyo, Japan).

Proteins were silver stained with a kit from Wako Pure Chemicals, Osaka, Japan.

Molecular weight markers were obtained from Daiichi Pure Chemicals.

Determination of phenotypic characteristics. Morphological and cultural
characteristics were observed on nutrient agar (Eiken, Tokyo, Japan) and
Trypto-Soy agar (Eiken). The pH range for growth was determined on broth
(Difco) filtered through a 0.45-μm-pore-diameter membrane after adjust-
ment to various pHs with HCl or NaOH. The cells grown on Trypto-Soy agar at
30°C were recorded by scanning electron microscopy. Physiological and bio-
chemical characteristics were determined according to reference 1 and Yabuuchi
et al. (33). DNA was extracted from the cells using a GenElute Bacterial DNA
Test Kit (Sigma, St. Louis, MO). DNA preparations were examined by a DNA
test agar (Difco), heart infusion broth (Difco) containing 12% gelatin, and a medium consisting of 1% Bacto Peptone (Difco), 0.5% NaCl, 0.05% ferric citrate, and 0.1% esculin, respectively. Indole production was tested by using 2% tryptophan in Difco broth and Kovac’s reagent (7). Urase activities, nitrate reduc-
tion, and malonate utilization were examined by using Christsen urease test
agar (Eiken), nutrient broth (Eiken) containing 1% potassium nitrate, and
malonate-phenylalanine medium (Kyokato, Tokyo, Japan), respectively. Mac-
Conkey agar was used for the detection of acid and gas formations. Flexirubin-
type pigment was detected according to the method of Yabuuchi et al. (35).

16S rRNA gene sequencing and analysis. Isolation of genomic DNA, PCR-
mediated amplification of 16S rRNA gene, and purification and sequencing of the
PCR product were performed according to the method of Shida et al. (27). Oligonucleotide primers 5'-CTGGGATCCATTTACTCGAGAGTTTGATCCTG
GGTCAG-3' (5' end of the 16S rRNA gene) and 5'-GGTTCCTCCATTAGCT
GATTCGAC-3' (3' end of the 16S rRNA gene) were used for PCR amplification of the 16S rRNA gene as described by Shida et al. (27). The amplified 16S gene was purified with a QiAquick spin PCR purification kit (Qiagen GmbH, Hilden, Germany) and then used as a sequencing template. Seven sequencing primers were used as described by Fox et al. (6). The sequence determination was compared with 16S rRNA gene sequences obtained from the
EMBL, GenBank, and DDBI databases. Multiple alignment of sequences, cal-
culation of nucleotide substitution rates (Kms values) (18), construction of a
neighbor-joining phylogenetic tree (26), and a bootstrap analysis with 1,000
replicates for evaluation of phylogenetic tree topology (4) were performed with
the CLUSTAL W version 1.5 program (30).

RESULTS

Isolation of bacterial strains. Repeated liquid cultures and subsequent plate culture using Cbz-Gln-Gly as the sole nitrogen source were used to enrich for protein-deamidating enzyme producers from soils. From 320 soil samples, 150 bacteria and 294 fungi were isolated and examined for protein-deami-
dating enzyme productivity in their culture supernatants. Among positive isolates, two bacteria showed significantly higher activities of deamidation of both Z-Gln-Gly and caseins. These isolates, designated as strains 9670T and 9671, origi-
nated from soils of a rice field and the bank of a brook, respectively. They were used for the following studies.

Culture profile of the isolates. Figure 1 shows the culture profile of strain 9670T. A similar profile was obtained for strain 9671. At the late exponential growth phase (16-h culture), deamidating activities on both Cbz-Gln-Gly and caseins began to be produced significantly and simultaneously. Protease activity was also produced, accompanied by the deamidating activities. The pH of the culture broth began to rise at the same
time with increasing ammonia produced, which might be re-
leased from peptone contained in the medium by the deami-
dating activities. Maximum deamidating activities were ob-
erved at 24 h of culture with 0.258 U/ml on Cbz-Gln-Gly and 0.228 U/ml on caseins. The enzyme productivities of strain 9671 were lower than those of strain 9670T by ca. 30%.

Partial purification of the protein-deamidating enzyme. Although ammonia-releasing activity from caseins was observed in the culture supernatants of the isolate, it was necessary to confirm whether the enzyme deamidated high-molecular-
weight peptides, i.e., proteins, or merely deamidated short peptides produced by the protease activity. For this purpose, we tried to purify the deamidating activity from the culture supernatant of strain 9670T. After trials of several kinds of chromatography and conditions, including ion-exchange chroma-
tagrapy, gel filtration, and chromatofocusing, it was found that hydrophobic chromatography on a phenyl-Sepharose column successfully resulted in the separation of deamidating
activities from protease activities. The ultrafiltration and dialysis procedure described in Materials and Methods had also contributed to the removal of most of the protease activity (ca. 98%). At around 0.2 M NaCl in its gradient (elution volume of 113 ml), both deamidating activities on caseins and Cbz-Gln-Gly were eluted as identical peaks separated from several protease peaks (Fig. 2). A minor peak of deamidating activities was observed in the unabsorbed fraction (elution volume of 25 ml) with crossover by protease peaks. The main fraction for the deamidating enzyme at an elution volume of 113 ml was used in the following study. Protease activity in this fraction was less than the level of the minimal detection limit (<0.003 U/ml).

Analysis by SDS-PAGE indicated this fraction contained a main protein band of 20 kDa with several minor protein bands. Evidence of protein-deamidating enzyme. To confirm that the enzyme can deamidate high-molecular-weight proteins, caseins were incubated with the above enzyme fraction for 16.5 h at 37°C, and the products were subjected to SDS-PAGE. As shown in Fig. 3, casein treated with the deamidating enzyme fraction was scarcely degraded (lane 4), compared to the control casein treated without enzyme (lane 2), whereas casein was completely hydrolyzed by the culture supernatant which contains proteolytic activities besides the deamidating activity (lane 3). Ammonia released in these reaction mixtures was also determined and found to be at concentrations of 7.12, 0.02, and 8.04 mM in the reaction mixture with the deamidating enzyme, control casein mixture, and the reaction mixture with the culture supernatant, respectively. Provided an average molar content of amido-containing amino acid residues, Gln and Asn, in caseins of 26.8 mol/mol of protein and assuming an average molecular weight of casein of 23,261, which were calculated based on the numbers of both amino acids in four casein components (αs1-, αs2-, β-, and γ-caseins) and the relative content of each component in the casein preparation (34), the deamidation degree (millimolar ammonia released/millimolar total amido content in the substrate casein) × 100 was estimated as 62.0% for the reaction product by the deamidating enzyme fraction. These results imply that the casein treated by the deamidating enzyme fraction was deamidated in a high-molecular-weight state, not in a small-peptide state (i.e., not after degradation). It can be concluded, therefore, that the deamidating enzyme fractionated from the culture supernatant of strain 9670T can deamidate high-molecular-weight proteins (caseins).

Slow migrations of protein bands in the casein treated with the deamidating enzyme fraction were observed (Fig. 3, lane 4 as compared to lane 2). Such slow migration on SDS-PAGE was reported for chemically deamidated gluten (2). This phenomenon might be considered to be caused by the increased negative charge in the deamidated protein, which should decrease the affinity between the protein and the negatively charged SDS molecule due to an electrostatic repulsion and
then decrease the total negative charge of the protein-SDS complex.

The deamidating enzyme fraction had no transglutaminase activity, as measured by hydroxamate formation between Cbz-Gln-Gly and hydroxyalamine, which is a common characteristic of transglutaminase. Furthermore, no formation of higher-molecular-weight, cross-linking products was observed in the reaction products of caseins by the deamidating enzyme fraction, as judged by SDS-PAGE (Fig. 3, lane 4 compared to lane 2). Casein is one of the best substrates for transglutaminase-catalyzed protein cross-linking, and the cross-linked products of caseins can be easily detected by SDS-PAGE. The deamidating enzyme from strain 9670T therefore could be distinguished from transglutaminase.

**Phenotypic characteristics.** Strains 9670T and 9671 showed the same morphological and physiological characteristics. They were rod-shaped, nonmotile, and nonsporing. Gram staining was negative. The cells were 0.4 to 0.5 μm wide and 0.8 to 2.0 μm long (Fig. 4). They were aerobic and positive for oxidase and catalase, producing an insoluble yellow or orange pigment, which turned red with 3% KOH and returned to orange by neutralization, indicating a flexirubin type of pigment.

Phenotypic characterization of the isolates indicated that they were included in the genus *Chryseobacterium*, which belongs to the family *Flavobacteriaceae*. Differential characteristics were reported among seven genera of *Flavobacteriaceae*, including the genera *Chryseobacterium*, *Flavobacterium*, *Emperor*, *Weeksella*, *Bergeyella*, *Riemerella* (33), and *Myroides* (32). Except for the acid-forming property from sucrose, all other properties of the isolates matched those of *Chryseobacterium*. In the genus *Chryseobacterium*, six species (*C. gleum*, *C. indologenes*, *C. balustinum*, *C. indoltheticum*, *C. meningosepticum*, and *C. scophthalmum*) are recognized at present. Besides acid formation from sucrose, the new isolates were distinguished from these six existing species: acid formation from mannitol and growth on MacConkey agar for *C. gleum*; malonate utilization for *C. indologenes*, G+C content, acid formation from mannitol, growth at 36 to 37°C, and growth on MacConkey agar for *C. balustinum*; G+C content, acid formation from mannitol, and growth on MacConkey agar for *C. indoltheticum*; growth on MacConkey agar for *C. meningosepticum*; and acid formation from glucose and mannitol, growth at 36 to 37°C, urease activity, and indole production for *C. scophthalmum* (Table 1). These results indicated that the new isolates should be placed as a new species in the genus *Chryseobacterium*.

**16S rRNA gene sequence and phylogenetic analysis.** The determined 16S rRNA sequence of strain 9670T showed higher similarities to those of a group consisting of several *Chryseobacterium* strains with 96.0, 95.9, 95.1, and 94.9% similarity to *C. gleum*, *C. indologenes*, *C. balustinum*, and *C. indoltheticum*, respectively. A recent published sequence of the 16S rRNA gene from *Chryseobacterium* sp. (22) showed 95.1% similarity to that of the strain 9670T. A second group consisted

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**TABLE 1. Characteristics differentiating strains 9670T and 9671 from existing *Chryseobacterium* species**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>9670T and 9671&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C. gleum</th>
<th>C. indologenes</th>
<th>C. balustinum</th>
<th>C. indoltheticum</th>
<th>C. meningosepticum</th>
<th>C. scophthalmum</th>
</tr>
</thead>
<tbody>
<tr>
<td>G + C content (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.1 (n = 2)</td>
<td>37.6 ± 1.0 (n = 6)</td>
<td>37.7 ± 0.3 (n = 3)</td>
<td>33.1 (n = 1)</td>
<td>33.8 (n = 1)</td>
<td>37.0 ± 0.5 (n = 8)</td>
<td>34.2 ± 0.4 (n = 7)</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth at 36–37°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth on MacConkey agar</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>7/12</td>
<td>V</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Urease activity</td>
<td>–</td>
<td>7/12</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>18/49</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>24/49</td>
</tr>
<tr>
<td>Malonate utilization</td>
<td>–</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> n is the number of strains tested. +, all strains tested positive; –, all strains tested negative.

<sup>b</sup> Results for strains 9670T and 9671 were from this study.

<sup>c</sup> Data from Holmes et al. (11).

<sup>d</sup> Data from Yabuuchi et al. (15).

<sup>e</sup> Data from Mudarris et al. (24).

<sup>f</sup> Mean ± standard deviation.

<sup>h</sup> Number of strains positive/number of strains tested.

<sup>i</sup> V, variable. The number of strains positive is not available.

<sup>j</sup> NA, not available.
of Riemerella anatipestifer, Bergerella zoohelicom, and C. meningosepticum, with 92.2 to 93.5% similarities. Other related strains, such as Weeksella virosa, Empedobacter brevis, Flavobacterium aquatile, and Myroides odoratus, had 83.6 to 87.1% similarities. A phylogenetic tree constructed by the neighbor-joining method showed that strain 9670T exists as an independent branch within the above-mentioned group having higher sequence similarities (Fig. 5). The bootstrap analysis resulted in relatively high values of more than 75% for all of the branches within this group.

DNA base composition and DNA-DNA hybridization. The G+C content of strains 9670T and 9671 was 37.1 mol%. The levels of DNA-DNA relatedness were estimated by using these two strains, four type strains from Chryseobacterium (C. balustinum, C. gleum, C. indologenes, and C. meningosepticum), and two other related type strains (Empedobacter brevis and Myroides odoratus) (Table 2). The DNA-DNA relatedness value between strains 9670T and 9671 was 94%. Low values (14 to 17%) of relatedness were observed between the new isolates and three strains, C. gleum, C. indologenes, and C. balustinum, which showed higher similarities in 16S rRNA gene sequences to strain 9760T. The values for strain 9670T to three other strains, C. meningosepticum, E. brevis, and F. odoratus, were only 8 or 7, 4 and 3%, respectively. A relatively higher value (31%) in this analysis was observed between C. gleum and C. indologenes. All of these results supported the phylogenetic tree illustrated from 16S rRNA gene sequence analysis (Fig. 5).

**DISCUSSION**

Protein-deamidating activity was found in the culture supernatant of a newly isolated bacterium, strain 9670T. Both Cbz-Gln-Gly- and casein-deamidating activities and protease activ-

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### TABLE 2. Levels of DNA-DNA relatedness between strains 9670T and 9671 and various test isolates

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IFO 12535&lt;sup&gt;T&lt;/sup&gt;</th>
<th>JCM 2410&lt;sup&gt;T&lt;/sup&gt;</th>
<th>IFO 15053&lt;sup&gt;T&lt;/sup&gt;</th>
<th>IFO 14944&lt;sup&gt;T&lt;/sup&gt;</th>
<th>IFO 14943&lt;sup&gt;T&lt;/sup&gt;</th>
<th>IFO 14945&lt;sup&gt;T&lt;/sup&gt;</th>
<th>9670&lt;sup&gt;T&lt;/sup&gt;</th>
<th>9671</th>
</tr>
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<tbody>
<tr>
<td>C. meningosepticum IFO 12535&lt;sup&gt;T&lt;/sup&gt;</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. gleum JCM 2410&lt;sup&gt;T&lt;/sup&gt;</td>
<td>10</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. balustinum IFO 15053&lt;sup&gt;T&lt;/sup&gt;</td>
<td>8</td>
<td>14</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. indologenes IFO 14944&lt;sup&gt;T&lt;/sup&gt;</td>
<td>9</td>
<td>31</td>
<td>14</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. brevis IFO 14943&lt;sup&gt;T&lt;/sup&gt;</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. odoratus IFO 14945&lt;sup&gt;T&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain 9670&lt;sup&gt;T&lt;/sup&gt;</td>
<td>8</td>
<td>17</td>
<td>14</td>
<td>17</td>
<td>4</td>
<td>3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Strain 9671</td>
<td>7</td>
<td>17</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>94</td>
</tr>
</tbody>
</table>

<sup>a</sup> IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan.

<sup>b</sup> NT, not tested.
ity were produced synchronously during the course of culture. The deamidating enzyme was separated from proteases by phenyl-Sepharose chromatography. Native caseins were not degraded by the enzyme, while more than 60% of amido groups in caseins were estimated to be deamidated, indicating that the enzyme can deamidate high-molecular-weight peptides (i.e., proteins) in the native state. The enzyme from strain 9670T is the first protein-deamidating enzyme of microbial origin. From the industrial point of view, the microbial protein-deamidating enzyme has great significance, because it opens the way to mass production of such an enzyme, which will find many applications.

Two peptidoglutaminases that catalyze the deamidation of peptide-bound glutamine residues have been found in Bacillus circulans: peptidoglutaminase I (EC 3.5.1.43), which deamidates the γ-carboxyamido group of C-terminal glutamine residue; and peptidoglutaminase II (EC 3.5.1.44), which deamidates the γ-carboxyamido groups of N-terminal and internal glutaminyl residues. Both enzymes, however, cannot deamidate caseins unless the caseins are prehydrolyzed (17). Gill et al. (8) reported that peptidoglutaminases are not active against caseins and whey proteins even after denaturation and are only active against glutaminyl residues in peptides with a molecular weight below 5,000. Hamada (9) reported slight enhancements of peptidoglutaminase-catalyzed protein deamidation using heat- and/or alkaline-treated proteins, but the degrees of deamidation were very low (0.8 to 3.0% for caseins) compared to those for the preparations treated by the combinations with proteolysis (ca. 38%).

Transglutaminase (EC 2.3.2.13) is an enzyme with a wide distribution ranging from mammals to microorganisms. The enzyme catalyzes the acyl transfer reaction in which the γ-carboxyamido groups of glutaminyl residue in proteins or peptides are the acyl donor. A variety of amines, including lysyl residues of proteins, can act as acyl acceptors. When lysyl residues of protein act as acyl acceptors, cross-linked products with higher molecular weights are formed through intermolecular isopeptide bonding. In the absence of amines in the reaction system, water can act as an acyl acceptor, resulting in the deamidation of glutaminyl residues in proteins. The protein-deamidating enzyme from strain 9670T was distinguished from transglutaminase, because no transglutaminase activities were detected based on the lack of hydroxamate formation and lack of cross-linked product formation from caseins.

The physiological role of the protein-deamidating enzyme produced by the microorganism is unknown. In germinating seeds in plants, deamidations of storage proteins preceding their proteolytic degradation were observed, and the possible involvement of a protein-deamidating enzyme in this process has been pointed out (31). Observed simultaneous expressions of the protein-deamidating enzyme and proteases into a culture medium by strain 9670T (Fig. 1) may suggest the involvement of a protein-deamidating enzyme in the degradation process of proteins to be utilized as energy or nutritional sources in cooperation with proteases. It was reported that proteins isolated from germinated seed, which were deamidated and conformationally changed, had an increased susceptibility to proteolytic digestion (19).

Two strains, 9670T and 9671, were isolated from soils in various natural environments of the Tsukuba area, Japan, as producers of the protein-deamidating enzyme. Phenotypic characterization, 16S rRNA sequencing, and DNA-DNA hybridization studies indicated the isolates belonged to a new species in the genus Chrysobacterium. The genus Chrysobacterium is an emended one for some strains originally isolated as “Flavobacterium-like bacteria.” In 1994, Vandamme et al. (33) proposed that six strains (F. gleum, F. indologenes, F. balustinum, F. indoltheticum, F. meningosepticum, and F. scopophilum) should be given a new separate status based on the previously reported phenotypic and chemotaxonomic features as well as rRNA cluster analysis, and they coined a new name, Chrysobacterium, for these strains. The well-characterized species C. gleum (11) was selected as the type species of this genus. They pointed out that C. meningosepticum, well known as a pathogenic strain, had the most aberrant features within this genus. In this study, we recognized the newly isolated strains as the genus Chrysobacterium based on the results from both phenotypic and DNA analyses (DNA-DNA hybridization and 16S rRNA sequencing). DNA analyses also indicated that the isolates were closely related to a group of Chrysobacterium species, except for C. meningosepticum. The strains belonging to Chrysobacterium have been isolated from various ecosystems, such as water, soils, fish, marine environments, and clinical specimens. Many bacteria isolated from food environments, such as milk and butter (15), were recently recognized as members of the genus Chrysobacterium (12, 13). More recently, an isolate from fish was determined as a strain belonging to the genus by 16S rRNA analysis (22). The isolates we studied here were from soils of a rice field and the bank of a brook in Japan. These recent reports suggest a wide distribution of Chrysobacterium strains in various natural environments, although early studies of the taxonomy of “Fla-

vobacterium-like bacteria,” some of which are presently placed in Chrysobacterium, had been mainly focused on clinical strains. The new isolates here produced highly proteolytic activities. This characteristic was also mentioned for some Chrysobacterium strains (originally isolated as flavobacteria) from dairy foods (14).

We propose a new species with the name Chrysobacterium proteolyticum sp. nov. Strain 9670 was designated the type strain of Chrysobacterium proteolyticum. A description of the new species is given below.

**Description of Chrysobacterium proteolyticum sp. nov.**

*Chrysobacterium proteolyticum* (pro.te.o.lyt.ic. cum. Gr. n. pro-
teo; Gr. adj. lyticus, dissolving; M.L. neut. adj. proteolyticum, protein dissolving, proteolytic). Cells are gram-negative, non-
sporeforming, and nonmotile rods (0.4 to 0.5 by 0.8 to 2.0 μm). Circular, orange or light-pinkish cream colonies are formed on nutrient agar at 30°C for 2 days. Yellow or orange insoluble, flexirubin-type pigment is produced. The organism shows growth at 37°C but not at 42°C. The pH range for growth is from 5 to 9 and that for optimal growth is 6 to 8. Growth occurs aerobically, not anaerobically. No growth is observed on Mac-

Conkey agar. Catalase and cytochrome oxidase reactions are positive, but urease negative. The organism is positive for in-
dole production, weakly positive for H₂S formation, but negative for malonate utilization, nitrate reduction, denitrification, 3-ketolactose formation, and the Voges-Proskauer test. Lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, and phenylalanine deaminase are negative. Hydrolyses of esculin, Tween 80, starch, tyrosine, casein, gelatin, DNA, and o-nitrophenyl-β-D-galactopyranoside are positive. The organ-
ism produces acid from l-arabinose, D-glucose, malto-
ose, sucrose, trehalose, and soluble starch; produces acid weakly from glycerol and mannitol; and does not produce acid from adon-
itol, cellobiose, ethanol, inositol, inulin, lactose, raffinose, rhamnose, or salicin. The G + C content of the DNA is 37.1 mol% (determined by high-performance liquid chromatography).

Strains 9670T and 9671 were obtained from soil samples from Tsukuba, Ibaraki, Japan. The type strain, 9670, has been de-
posed in the Patent Microorganism Depository, National In-
stitute of Bioscience and Human Technology (Tsukuba, Ja-
pan), as strain FERM P-17664. The strain will be made available for research.

REFERENCES

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