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An acid phosphatase gene (*aphA* gene) from an industrial *miso koji* mold strain, *A. oryzae* KBN630, was disrupted by using the recently developed homologous gene replacement system for this strain. The *aphA* gene disruption did not affect growth on steamed soybean. Acid phosphatase production decreased by approximately 20% in the *aphA* gene disruptants compared with that of the wild-type strain. Utilizing the promoter of the *A. oryzae* TEF1 gene, AphA expressed in *A. oryzae* was successfully secreted into the culture medium. AphA had a molecular mass of 58.0 to 65.0 kDa, a pH optimum of 4.0, and a temperature optimum of 40°C. AphA had the ability to release inorganic phosphate from GMP and IMP. This is the first report to show directly that an *A. oryzae* acid phosphatase has the ability to hydrolyze GMP and IMP.

Keywords: *miso koji* mold, *Aspergillus oryzae*, acid phosphatase gene, gene disruption, overexpression

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Introduction

*Miso*, fermented soybean paste, is a Japanese traditional fermented soybean food. *Miso*, as with *shoyu* (soy sauce), is an indispensable seasoning in Japanese cuisine. The taste, aroma and color are important parameters of *miso* quality. In order to improve taste quality, disodium 5'-ribonucleotides such as disodium inosinate (IMP) and disodium guanylate (GMP) are added to *miso*. Disodium 5’-ribonucleotides are flavor enhancers of the *umami* taste, in synergy with the glutamic acid naturally contained in *miso*.

The filamentous fungus *Aspergillus oryzae* is used in the manufacture of *miso*. During preparation of the starter culture, *miso koji*, *A. oryzae* secretes a large variety of enzymes such as amylases and proteases when grown on steamed soybean; these enzymes are essential for efficient maceration and degradation of the soybean. Among the enzymes secreted by *A. oryzae*, acid phosphatases catalyze the hydrolysis of disodium 5'-ribonucleotides, yielding ribonucleoside, which is not a flavor enhancer. Therefore, to prevent the dephosphorylation of disodium 5'-ribonucleotides, acid phosphatases must be inactivated by heating of *miso* at 85°C for 15 min (Oike et al., 1984). However, the heating process leads to browning and a burnt smell, resulting in lowered *miso* quality. In order to overcome these problems, an *A. oryzae* strain that produces acid phosphatases at a very low level is highly desired as a *miso koji* mold.

Although several studies on acid phosphatases and phytases from *A. oryzae* have been reported (Fujishima et al., 1966; Wang et al., 1980; Oike et al., 1984; Shimizu, 1993; Fujita et al., 2003a, 2003b), little is yet known about their dephosphorylation activity on disodium 5'-ribonucleotides. Phytases belong to the family of histidine acid phosphatases, a subclass of phosphatases. Phytases efficiently cleave phos-
medium containing 3% rice starch, 1% polypeptone, 1% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, and 0.05% MgSO₄·7H₂O was used for liquid cultivation of *A. oryzae*. Soybean koji culture was carried out at 30°C for 43 h on steamed soybean. Soybean was soaked in water for 2 h and then steamed for 35 min. Steamed soybean was mashed and pelletized before use. Protoplast transformation of *A. oryzae* was carried out according to the method described previously (Kitamoto *et al.*, 1995).

**DNA techniques and PCR methods** Standard DNA techniques were used in this study (Sambrook and Russell, 2001). Genomic DNA of *A. oryzae* was prepared using a previously described method (Kitamoto *et al.*, 1993). PCR amplification was carried out with a GeneAmp9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). TaKaRa Ex Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan) and PfuUltra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA, USA) were used in PCRs. Oligonucleotide primers used in this study are shown in Table 1. Essential cloning steps were confirmed by sequencing on a model 4000LS DNA sequencer (LI-COR, Lincoln, NE, USA) and GenomeLab GeXP (Beckman Coulter, Brea, CA, USA).

**Construction of the disruption vector for the aphA gene** An *aphA* gene disruption vector, pDisAphA, was constructed as follows. A 1.0-kb 5'- and a 1.1-kb 3'-flanking regions of the *aphA* gene were amplified from *A. oryzae* genomic DNA with primer pairs phyA1/phyA2 and phyA5/phyA6. A 1.8-kb fragment of the *pyrG* gene was amplified from *A. oryzae* genomic DNA with primer pair phyA3/phyA4. The three PCR-products and the SmaI-digested pUC18 were joined in a four-piece In-Fusion reaction using an In-Fusion Advantage PCR Cloning Kit (Takara Bio). The resultant plasmid

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>phyA1</td>
<td>5'-TCGAGCTCGTATACCCCTTTCAATTGGAAGAA-3'</td>
</tr>
<tr>
<td>phyA2</td>
<td>5'-CAGCGGCTTTGACACTCGTACAGCCGAC-3'</td>
</tr>
<tr>
<td>phyA3</td>
<td>5'-TTGATCAAGCCTGCTGGAATTGACATTA-3'</td>
</tr>
<tr>
<td>phyA4</td>
<td>5'-GTGACGGGAGATTGTACGAACAGATGGCCC-3'</td>
</tr>
<tr>
<td>phyA5</td>
<td>5'-ACAATCTCCCCGGTGACCGTGATCAA-3'</td>
</tr>
<tr>
<td>phyA6</td>
<td>5'-CTCTAGAGGATCCCTCCCAGGATTCCACACAG-3'</td>
</tr>
<tr>
<td>fupyrGN</td>
<td>5'-CGGTAACCCGGGATCCAAGCGCTGATGACTGACAC-3'</td>
</tr>
<tr>
<td>pyrGC2</td>
<td>5'-AAAAGCTCGTATGTCACTCGGAGGATTT-3'</td>
</tr>
<tr>
<td>pyrGtef</td>
<td>5'-ATTGATCAAGCCTTCTACCTGGAACCAGACAGGC-3'</td>
</tr>
<tr>
<td>tefPrev</td>
<td>5'-ATTCCATTGGAAGGTTGTCGAGCATTGACAC-3'</td>
</tr>
<tr>
<td>tefaphA</td>
<td>5'-ATGCCTGCAGGGGAGGATCCTGACAGAAGGATGGG-3'</td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Fungal strains, culture media and transformation** An industrial *miso koji* mold, *A. oryzae* KBN630, was used for isolation of DNA. The *pyrG*, *ku70* double deleted strain, *A. oryzae* KBN630-17K3 (Yoshino-Yasuda *et al.*, 2011), derived from *A. oryzae* KBN630, and the *alp*, *pyrG* double disrupted strain, *A. oryzae* KO2, derived from the industrial *shoyu koji* mold *A. oryzae* KBN616, were used for transformation. In *A. oryzae* KO2, the alkaline protease gene (*alp* gene) (Murakami *et al.*, 1991) was disrupted to produce recombinant proteins efficiently. The construction methods of *A. oryzae* KO2 will be published elsewhere. Rice starch (RS)
Acid phosphatase gene from *Aspergillus oryzae*

was designated as pDisAphA, in which the *pyrG* gene was located between the 5' and 3'-flanking regions of the *aphA* gene.

**Construction of the expression vector for the *aphA* gene**
To express the *aphA* gene under the control of the *A. oryzae* *TEF1* gene promoter, an expression plasmid, pTFAphA, was constructed as follows. A 1.8-kb fragment of the *pyrG* gene was amplified from *A. oryzae* genomic DNA with primer pair fupyrGN/pyrGC2. The 0.8-kb fragment of the *A. oryzae* *TEF1* gene promoter was amplified from *A. oryzae* genomic DNA with primer pair pyrGtef/tefPrev. A 2.0-kb fragment of the *pyrG* gene was amplified from *A. oryzae* genomic DNA with primer pair tefaphA/aphASal. The three PCR-products were digested with *HindIII* and *SalI* and joined in a four-piece In-Fusion reaction using an In-Fusion Advantage PCR Cloning Kit. The resultant plasmid was designated as pTFA-aphA, in which the *TEF1* gene promoter was positioned precisely next to the *aphA* coding region.

**Purification, amino acid sequencing, and deglycosylation of APha from an overproducing *A. oryzae* transformant**
*A. oryzae* transformant APA4 was grown in RS medium for 5 days. After removal of mycelia through filtration, proteins in 150 mL of the culture filtrate were precipitated by the addition of ammonium sulfate at >80% saturation, and dissolved in and dialyzed against 10 mM Tris-HCl buffer (pH 7.0). The crude enzyme solution was loaded on a HR16/20 Fast Flow Q-Sepharose anion-exchange column (GE Healthcare, Buckinghamshire, UK) equilibrated in the same buffer and eluted with a linear gradient from 0 M to 0.3 M NaCl. The APhA-containing fractions were dialyzed against the same buffer and loaded again on a HR16/20 Fast Flow Q-Sepharose anion-exchange column. Then, adsorbed proteins were eluted with a linear gradient from 0 M to 0.3 M NaCl.

To analyze the N-terminal amino acid sequence, the purified enzyme was applied to PVDF using a ProSorb device (Applied Biosystems) and sequenced on an Applied Biosystems Procise 491 sequencer according to the manufacturer’s instructions (Applied Biosystems).

Deglycosylation of the purified enzyme was done with endoglycosidase H (Glyko, Novato, CA, USA) according to the procedure provided by the manufacturer, with denaturation of the protein before addition of endoglycosidase H.

**Enzyme assay**
Acid phosphatase activity was measured according to the slightly modified procedure described by Oike *et al.* (1984). An aliquot of the enzyme solution was incubated with 1 mM PNPP in 100 mM acetate buffer (pH 4.0) at 40°C for 10 min. The reaction was terminated by adding 10% trichloroacetic acid solution. After the addition of 2 M Na₂CO₃ solution, the amount of liberated *p*-nitrophenol (PNP) was measured by absorbance at 405 nm. One enzyme unit was defined as the amount of enzyme that liberated 1 μmol of PNP per min under the assay conditions. In the case of soybean *koji* culture, acid phosphatase activity was measured at 37°C for 20 min instead of 40°C for 10 min. The pH optimum of the enzyme was measured by incubating the enzyme for 10 min at 40°C in 100 mM sodium acetate buffers at various pHs (3.0 to 7.0). The temperature optimum was measured by incubation for 10 min at various temperatures (25°C to 65°C) in 100 mM sodium acetate buffer (pH 4.0). The thermal and pH stabilities were measured after incubation of the enzyme at various temperatures (25°C to 65°C) for 30 min and at various pH (3.0 to 7.0) for 1 h at 30°C, respectively. Substrate specificity was investigated as follows. The enzyme solution was incubated with 2 mM of each substrate for 30 min at 40°C in 100 mM acetate buffer (pH 4.0). The amount of released inorganic phosphate was measured using a Phosphor C Test Kit (Wako Pure Chemical, Osaka, Japan).

Alpha-amylase activity was measured according to the procedure of the officially approved method (Nishiya, 1993). Neutral protease activity was measured according to the slightly modified procedure of the officially approved method (Nishiya, 1993). McIlvaine buffer (pH 6.0) was used as a reaction buffer instead of McIlvaine buffer (pH 3.0).

**Results and Discussion**

*In silico cloning and disruption of the *aphA* gene*  
*A. oryzae* produces several acid phosphatases and phytases. Although some of them have been purified and their N-terminal amino acid sequences determined (Fujita *et al.*, 2003a, 2003b), the corresponding genes encoding them could not be found in the genome database of *A. oryzae* (i). Therefore, we surveyed putative acid phosphatase genes from the *A. oryzae* genome database by BLAST search. Five putative acid phosphatase genes and 8 phytase genes were found. It was considered that the expression of the putative acid phosphatase genes might be repressed by the phosphate from phytate in soybean because these genes are homologous to the phosphate repressible acid phosphatase gene from *Aspergillus niger* (MacRae *et al.*, 1988) and *Penicillium chrysogenum* (Hass *et al.*, 1992). Among the remaining candidate genes, the gene (NITE DOGAN ID: AO090023000692) with the highest similarity to the well-characterized *A. niger* *phyA* gene (van Hartingsveldt *et al.*, 1993) was chosen. This gene was identical to the previously reported phytase gene from *A. oryzae* RIB40 (GenBank/EMBL/DDBJ accession no. AB042805) (Uchida *et al.*, 2006) and was designated as *aphA* gene. Then, we attempted to assess whether APha exhibits hydrolytic activity against disodium 5'-ribonucleotides.

To obtain the *aphA* disrupted *A. oryzae* strain, *A. oryzae* KBN630-17K3 was transformed using a DNA fragment am-
plified by PCR with the primer pair phyA1/phyA6, and using plasmid pDisAphA as a template (Fig. 1A). Transformants were examined by detection of the DNA fragments amplified by PCR from the transformants using the primer pair phyA1/phyA6. For 6 out of the 8 transformants examined, only a 3.9-kb fragment was detected (Fig. 1B, lanes 2 to 7), whereas for the other 2 transformants, a 2.6-kb fragment, in addition to the 3.9-kb fragment, was detected (Fig. 1B, lanes 1 and 8). These results indicated that the aphA gene was disrupted in the former 6 transformants. Three of the aphA gene disruptants were used for further experiments.

The enzyme productivity of the aphA gene disruptant was compared with that of the wild-type strain to investigate the effect of the aphA gene disruption on acid phosphatase production. Three aphA gene disruptants grew normally on steamed soybean, indicating that the aphA gene disruption did not affect the growth when steamed soybean was used as the source of nutrients. As shown in Table 2, acid phosphatase production decreased by approximately 20% in the aphA gene disruptants compared with that of the wild-type strain. In contrast, amylase and neutral protease production in the aphA gene disruptants increased by approximately 10% compared with those of the wild-type strain. These results suggest that disruption of the aphA gene might have some positive effect on the production of the other enzymes, such as amylases, in the wild-type strain. Therefore, the effect of aphA gene disruption may be underestimated, since it is possible that the other acid phosphatases are also increased in the aphA gene disruptant. To examine the contribution of the aphA gene product to overall acid phosphatase activity, disruptants of other acid phosphatase genes must be obtained.

Overexpression and purification of AphA In order to characterize the enzymatic properties of the aphA gene product, the aphA gene was efficiently expressed under the

<table>
<thead>
<tr>
<th>Strain</th>
<th>α-Amylase (% of control)</th>
<th>Neutral protease (% of control)</th>
<th>Acid phosphatase (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔaphA-1</td>
<td>778 ± 54.4 (122)</td>
<td>31,883 ± 1460.2 (110)</td>
<td>324 ± 30.5 (84)</td>
</tr>
<tr>
<td>ΔaphA-2</td>
<td>721 ± 43.1 (113)</td>
<td>30,061 ± 1803.8 (104)</td>
<td>304 ± 32.7 (79)</td>
</tr>
<tr>
<td>ΔaphA-3</td>
<td>695 ± 68.6 (109)</td>
<td>30,547 ± 601.0 (105)</td>
<td>309 ± 13.1 (80)</td>
</tr>
<tr>
<td>A. oryzae</td>
<td>636 ± 14.8 (100)</td>
<td>29,028 ± 338.0 (100)</td>
<td>387 ± 22.3 (100)</td>
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<tr>
<td>KBN630-17K3</td>
<td>636 ± 14.8 (100)</td>
<td>29,028 ± 338.0 (100)</td>
<td>387 ± 22.3 (100)</td>
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</tr>
</tbody>
</table>

The enzyme activity is shown as U/g koji.
The activity of two independent experiments is presented as the average ± standard deviation.
control of the *A. oryzae TEF1* gene promoter, as the *TEF1* gene promoter is one of the strongest promoters in *A. oryzae*. An expression vector, pTFAphA, carrying the *aphA* gene under the control of the *A. oryzae TEF1* gene promoter, was constructed as described in the Materials and methods. The nucleotide sequence of the *aphA* gene was identical to that of the *A. oryzae* RIB40 phytase gene and consisted of 1,469 bp, including one intron of 68 bp. After pTFAphA was introduced into *A. oryzae* KO2, transformants grown in RS medium were assayed for extracellular acid phosphatase activity to select a high-AphA-producing strain for further study. The enzyme levels ranged from 2.3 to 12.1 U/mL (21 to 112 mg/L) for five individual transformants examined, whereas *A. oryzae* KO2, carrying the *pyrG* gene, alone showed no acid phosphatase activity.

AphA was purified to homogeneity from the culture supernatant of the highest AphA producing strain, APA4, by anion-exchange chromatography, as described in the Materials and Methods. AphA was purified 4.3-fold, with recovery of 21.4% of the initial activity. The specific activity of the purified AphA was 108.4 U/mg of protein. AphA migrated as a broad band between 58.0 to 65.0 kDa on SDS-PAGE (Fig. 2, lane 2), which was approximately 10.0 to 17.0 kDa larger than the calculated molecular mass of 47,581 Da, based on the amino acid sequence for the mature protein. We assumed that the observed differences between theoretical and apparent molecular masses might be caused by protein glycosylation. This idea is supported by the presence of 7 potential sites for N-linked glycosylation of the Asn-X-Ser/Thr type occurring at residues 104, 119, 206, 219, 338, 351, and 375 in the AphA amino acid sequence, as well as by the decrease of the apparent molecular mass to 48.0 kDa after treatment of the purified AphA with endoglycosidase H (Fig. 2, lane 3). The molecular mass of the purified AphA was different from that of the identical gene product overexpressed in another *A. oryzae* strain previously reported (Uchida *et al.*, 2006). The differences in molecular mass might be due to differences in modification of glycosylation, since different extents of glycosylation were observed even with different batches of *A. fumigatus* phytase expressed in the *A. niger* expression system (Wyss *et al.*, 1999b).

The N-terminal amino acid sequence of the purified AphA protein was Gln-Ser-Asn-Thr-Val-Asp-Glu-Gly-Tyr-Gln (X denotes an unidentified residue). This sequence was in complete agreement with the deduced amino acid sequence from Gln-28 to Gln-38, except Cys-30, which is not chemically determined. Analysis using the SignalP 3.0 program (ii) predicted that the first 19 N-terminal amino acids of AphA functioned as a signal sequence. Therefore, amino acids 20 to 27 of AphA were presumed to represent a propeptide similar to other fungal phytases (Wyss *et al.*, 1999a, Lassen *et al.*, 2001). The N-terminal amino acid sequence of AphA was quite different from those of two acid phosphatases and two phytases from *A. oryzae* RIB128. These results indicate that AphA might not be secreted in the case of *A. oryzae* RIB128.

**Enzymatic properties of AphA** The enzymatic features of AphA, including pH and temperature optima and pH and thermal stabilities, were measured for the purified protein. AphA had a pH optimum of 4.0 and a temperature optimum of 40°C. AphA was stable over a wide range of pH, from 3.0 to 7.0. AphA was stable up to 35°C and inactivated sharply at temperatures above 35°C. As shown in Table 3, the temperature optimum and thermal stability of AphA were similar to those of *A. oryzae* ACP-II, while the pH optimum and pH stability of AphA were different from those of *A. oryzae* ACP-II (Fujita *et al.*, 2003a). On the other hand, the pH and temperature optima for AphA were lower than reported for the identical gene products overexpressed in another *A. oryzae* strain previously reported (Uchida *et al.*, 2006). Differences in experimental conditions, such as the substrate, might have significant effects on the enzymatic features, since the different extents and patterns of glycosylation did not have a significant effect on the catalytic properties of the fungal phytases (Wyss *et al.*, 1999a).
The substrate specificity of the purified enzyme was examined. The substrates used in this study were as follows: PNPP, phytate, glycerophosphate, pyrophosphate, d-glucose-6-phosphate, GMP, and IMP. AphA displayed broad substrate specificity (Table 4). PNPP was hydrolyzed at the fastest rate; this rate was about 2-fold greater than those from phytate, glycerophosphate, and d-glucose-6-phosphate. AphA showed low but distinct activities with GMP and IMP. These results indicated that AphA could be involved in the dephosphorylation of disodium 5’-ribonucleotides added to miso. As shown in Table 4, AphA had a different substrate specificity that that of other A. oryzae acid phosphatases. On the basis of the differences in the N-terminal amino acid sequence, enzymatic features, and substrate specificity, AphA is distinct from the acid phosphates or phytases reported by Fujita et al. (2003a, 2003b) and Shimizu (1993).

To our knowledge, this is the first report to show that an A. oryzae acid phosphatase has the ability to release inorganic phosphate from GMP and IMP. We are now in the process of examining the hydrolytic activity on disodium 5’-ribonucleotides of an additional 7 phytases, encoding genes with high similarities to the aphA gene found in the genome database of A. oryzae (i).

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**URL cited**


ii) http://www.cbs.dtu.dk/services/SignalP/ (2011.8.2)