Original paper

Characterization of Acid Phosphatase (AphC) from the *Miso Koji* Mold, *Aspergillus oryzae* KBN630: AphC is Mainly Responsible for Both Acid Phosphatase Activity and 5'-IMP Dephosphorylation Activity in Soybean-*Koji* Culture

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Single disruption of seven acid phosphatase genes (*aphB-H*) demonstrated that the *aphC* gene is mainly responsible for acid phosphatase production in the soybean-*koji* culture of the *miso koji* mold, *A. oryzae* KBN630. None of the single disruptions of *aphB-H* affected growth in the culture. Both the acid phosphatase activity and 5'-IMP dephosphorylation activity of the *aphC* disruptant decreased by 95% compared to the wild-type strain. Utilizing the promoter of the *A. oryzae* *taaG2* gene, AphC was successfully overproduced in *A. oryzae* and secreted into liquid culture medium. The purified AphC had a molecular mass of 69.0 kDa, a pH optimum of 4.5, and a temperature optimum of 50°C. AphC exhibited high dephosphorylation activity towards the *umami* flavor enhancers, 5'-IMP and 5'-GMP, supporting the idea that AphC is involved in degradation of disodium 5'-ribonucleotides supplemented in *miso* products.

Keywords: acid phosphatase gene, *Aspergillus oryzae*, 5'-IMP dephosphorylation, *miso*, soybean-*koji* culture

Introduction

Miso, fermented soybean paste, is a Japanese traditional seasoning which is indispensable for Japanese cuisine, as is soy sauce (*shoyu*). The taste, aroma and color are important parameters of *miso* quality. In order to improve the taste, *miso* products are often supplemented with disodium 5'-ribonucleotides such as disodium

5'-inosinate (5'-IMP) and disodium 5'-guanylate (5'-GMP). These *umami* flavor enhancers work synergistically with glutamic acid naturally contained in *miso*.

The filamentous fungus *Aspergillus oryzae* is used for *miso* brewing. During preparation of its *koji* culture (i.e., solid starter culture), *A. oryzae* secretes a large variety of enzymes such as am-

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ylases and proteases when grown on steamed rice (for rice-*miso*), soybean (for soybean-*miso*), or barley (for barley-*miso*). These enzymes are essential for efficient maceration and degradation of the ingredients. Among the enzymes secreted by *A. oryzae*, acid phosphatases appear to catalyze the hydrolysis of disodium 5'-ribonucleotides, yielding insipid ribonucleosides and phosphoric acid. Therefore, in order to prevent dephosphorylation of disodium 5'-ribonucleotides, acid phosphatases must be inactivated by heating *miso* at 85°C for 15 min (Oike *et al.*, 1984). This high-temperature heating process not only requires large equipment and a large amount of energy, but also reduces *miso* quality, causing browning and a burnt smell. Thus, an *A. oryzae* strain that produces acid phosphatases at a very low level is highly desirable as a *miso koji* mold for the production of seasoned *miso*.

A. oryzae has been reported to produce several acid phosphatases (Fujishima *et al.*, 1964, Wang *et al.*, 1980, Oike *et al.*, 1984, Shimizu, 1993, Fujita *et al.*, 2003a, 2003b). Eight *aph* genes encoding acid phosphatase with similarity to the well-characterized *A. niger phyA* gene were identified in the *A. oryzae* genome database by BLAST searches (Yoshino-Yasuda *et al.*, 2012, Marui *et al.*, 2012). In our previous report, we directly showed that acid phosphatase A encoded by the *aphA* gene (GenBank accession number AB042805 / AP007157) in *A. oryzae* possesses some level of 5'-IMP and 5'-GMP dephosphorylation (Yoshino-Yasuda *et al.*, 2012). Disruption of the *aphA* gene in *A. oryzae* KBN630 resulted in only a 20% reduction in acid phosphatase activity in soybean-*koji* culture, a starter culture for soybean-*miso* brewing unique to the Chukyo area of Japan (Yoshino-Yasuda *et al.*, 2012).

From these facts, we postulated that other Aphs are responsible for the remaining acid phosphatase activity. In order to clarify the major Aph with 5'-IMP dephosphorylation activity in *A. oryzae*, seven *aph* gene disruptants were constructed and their enzyme production in soybean-*koji* culture was examined. In addition, the *aphC* gene, demonstrated to be mainly responsible for both acid phosphatase activity and 5'-IMP dephosphorylation activity, was overexpressed under control of the *A. oryzae taaG2* gene promoter (Tsukagoshi *et al.*, 1989). Then AphC protein was purified and its enzymatic properties were characterized.

Materials and Methods

Fungal strains, culture media and transformation *A. oryzae* KBN630, obtained from Bio'c (Toyohashi, Japan), was used for DNA isolation. Two strains were used for transformation. One was the *pyrG*, *ku70* double deletion strain, *A. oryzae* KBN630-17K3 (Yoshino-Yasuda *et al.*, 2011), derived from *A. oryzae* KBN630. The second was the *alp*, *pyrG* double deletion strain, *A. oryzae* PDE1, derived from the industrial *shoyu koji* mold *A. oryzae* KBN616. In *A. oryzae* PDE1, the alkaline protease gene (*alp* gene) (Murakami *et al.*, 1991) was disrupted to produce recombinant proteins efficiently. The methods used to construct *A. oryzae* PDE1 will be published elsewhere. *A. oryzae* Δ aphA was used as an

aphA gene disrupted strain (Yoshino-Yasuda *et al.*, 2012). Rice starch (RS) 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 pellets as follows. Soybeans were soaked in water for 2 h, held overnight at 4°C, and then steamed for 30 min followed by autoclaving at 121°C for 5 min. Autoclaved soybeans were mashed, pelletized and coated with roasted barley flour before use. Protoplast transformation of *A. oryzae* was carried out as described previously (Kitamoto *et al.*, 1995).

DNA techniques and PCR methods Standard DNA techniques were used (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).

Disruption of *aph* genes An *aphB* gene disruption vector, pDisAphB, was constructed as follows. A 1.0-kb 5'- and a 1.0-kb 3'-flanking region of the *aphB* gene were amplified from *A. oryzae* genomic DNA using the primer pairs phyB1/phyB2 and phyB3/phyB4. A 1.8-kb fragment of the *pyrG* gene was amplified from *A. oryzae* genomic DNA with the primer pair pyrGN2/ pyrGC2 (Yoshino-Yasuda *et al.*, 2011). The three PCR-products and the *Bam*HI/*Sal*I-digested pUC18 were joined in a four-piece In-Fusion reaction using an In-Fusion Advantage PCR Cloning Kit (Takara Bio). The resultant plasmid was designated as pDisAphB, in which the *pyrG* gene was located between the 5'- and 3'-flanking regions of the *aphB* gene. The gene disruption fragment for *aphB* was amplified with the primer pair phyB1/phyB4 and pDisAphB as the template, and introduced into *A. oryzae* KBN630-17K. Genomic DNAs from transformants were used as the template for PCR analysis using the primer pair phyB1/phyB4 for verification of the *aphB* disruption. Using the primers listed in Table 1, the other six *aph* gene disruption vectors, pDisAphC to pDisAphH, were constructed by the same procedure as for pDisAphB. The gene disruption fragments for the six *aph* genes were also amplified by the same procedure as for the *aphB* gene, and introduced into *A. oryzae* KBN630-17K. Genomic DNAs from the transformants were analyzed by PCR using specific primer pairs for *aph* genes (phyC1/phyC4 for *aphC* gene, phyD1/phyD4 for *aphD* gene, phyE1/phyE4 for *aphE* gene, phyF1/phyF4 for *aphF* gene, phyG1/phyG4 for *aphG* gene and phyH1/phyH4 for *aphH* gene, respectively) for verification of gene disruption.

Expression of *aphC* under control of the *A. oryzae taaG2* pro-

Table 1. Oligonucleotide primers used in this study

Name	Sequence (5' to 3')	Direction
Primers for gene disruption		
The <i>aphB</i> gene		
phyB1	CGGTACCCGGGGATCCCAACCGTACTCCTAGGAGTGG	Forward
phyB2	TTCCAGCAGGCCTTGAAAGCATGGCGCACTATCGCGG	Reverse
phyB3	ATTGATCAGGCCTTTCGACCAGCTAACCAAGACTAGT	Forward
phyB4	ATGCCTGCAGGTCGACATGATCAACGAATTCCTTCAACG	Reverse
The <i>aphC</i> gene		
phyC1	CGGTACCCGGGGATCCTATGCCGGGATACTAACACAAT	Forward
phyC2	TTCCAGCAGGCCTTGCTATTAGTGGGTAATGCATAGTG	Reverse
phyC3	ATTGATCAGGCCTTTGAATGAGTGGGAGTATGTGCTC	Forward
phyC4	ATGCCTGCAGGTCGACAAGTCCAGATCTCGGAAATCAG	Reverse
The <i>aphD</i> gene		
phyD1	CGGTACCCGGGGATCCATCCTTAACAGTAGACTACTTGG	Forward
phyD2	TTCCAGCAGGCCTTGACAGCTAACACTTCTAGACTAAG	Reverse
phyD3	ATTGATCAGGCCTTTAACTTCCGAACCCGCTATGCTAG	Forward
phyD4	ATGCCTGCAGGTCGACAACCTTGACTCTCGTTCTTCGGTG	Reverse
The <i>aphE</i> gene		
phyE1	CGGTACCCGGGGATCCCTGTGATCACTTTGAATAACACC	Forward
phyE2	TTCCAGCAGGCCTTGCAACATAACTTACTTGAAGACCAG	Reverse
phyE3	ATTGATCAGGCCTTTGGTCGTGATATTTTGCTTGCCAC	Forward
phyE4	ATGCCTGCAGGTCGACAGGTCCAGATTTTCGGAGATTAG	Reverse
The <i>aphF</i> gene		
phyF1	CGGTACCCGGGGATCCAGAATACAACATGCTGACTATGG	Forward
phyF2	TTCCAGCAGGCCTTGATGCGATACTAAGCAAAGCAATC	Reverse
phyF3	ATTGATCAGGCCTTTGCTCAAAGGGTCACTTGATCGCC	Forward
phyF4	ATGCCTGCAGGTCGACATACTAGATGGAACCGGAAAGG	Reverse
The <i>aphG</i> gene		
phyG1	CGGTACCCGGGGATCCTTTAGACAATTCAGTCCCCTC	Forward
phyG2	TTCCAGCAGGCCTTGAAATGCAGCAGGTACGTTCTC	Reverse
phyG3	ATTGATCAGGCCTTTGGATCAAATACGGCAGGCTG	Forward
phyG4	ATGCCTGCAGGTCGACATAGAGGATCGGATCCAAAGAG	Reverse
The <i>aphH</i> gene		
phyH1	CGGTACCCGGGGATCCACACAGAAACAAACAAAAGATCTG	Forward
phyH2	TTCCAGCAGGCCTTGGTGAAGTTCTCTAGACTCTAGG	Reverse
phyH3	ATTGATCAGGCCTTTCCTTAATGACTGGAGCTATGGGC	Forward
phyH4	ATGCCTGCAGGTCGACCTTCATGAACGTGTCAAGCTCAC	Reverse
Primers for <i>aphC</i> expression vector		
fupyrGN	CGGTACCCGGGGATCCAAGCCGCTGCTGGAATTGACA	Forward
pyrGC3	TCAGAAGAAAAGGATGATCAATACC	Reverse
pyrGtaa	ATCCTTTTCTTCTGAATTCATGGTGTGTTTGATCATTTT	Forward
taaPrev	CATAAATGCCTTCTGTGGGGTTTATTGTT	Reverse
taaaphC	CAGAAGGCATTTATGCAGCAATTATTGCAATCAACGG	Forward
aphCSal	ATGCCTGCAGGTCGACGGGTTGATAGAGCTTGTTCTGGTGATC	Reverse

moter To express the *aphC* gene under control of the *A. oryzae* *taaG2* gene promoter, an expression plasmid, pTAAphC, was constructed as follows. A 1.8-kb fragment of the *pyrG* gene was amplified from *A. oryzae* genomic DNA using the primer pair fupyrGN/pyrGC3. A 0.6-kb fragment of the *A. oryzae* *taaG2* gene promoter was amplified from *A. oryzae* genomic DNA using the primer pair pyrGtaa/taaPrev. A 2.0-kb fragment of the *aphC* gene was amplified from *A. oryzae* genomic DNA using the primer pair

taaaphC/aphCSal. The three PCR-products and the *Bam*HI/*Sal*I-digested pUC18 were joined in a four-piece In-Fusion reaction using an In-Fusion Advantage PCR Cloning Kit. The resultant plasmid was designated as pTAAphC, in which the *A. oryzae* *taaG2* gene promoter was positioned precisely next to the *aphC* gene coding region. Then, plasmid pTAAphC was introduced into *A. oryzae* PDE1.

To confirm the introns of the *aphC* gene, RT-PCR was per-

formed using a High Fidelity RNA PCR Kit (Takara Bio) with total RNA from the high-AphC-producing strain described below. Double stranded cDNA synthesis was performed using the primer pair Adaptor Primer FB and taaaphC. The amplified fragment was cloned into *Hinc*II-digested pUC118 for sequencing.

Purification, amino acid sequencing, and deglycosylation of AphC from the overproducing *A. oryzae* transformant *A. oryzae* transformant APC15 was grown in RS medium for 5 days. After removing the mycelia by filtration, 50 mL of the culture filtrate was dialyzed against 10 mM Tris-HCl buffer (pH 7.0). The crude enzyme solution was loaded on an HR16/20 Fast Flow Q-Sepharose anion-exchange column (GE Healthcare, Buckinghamshire, UK) equilibrated in the same buffer and eluted with a linear gradient of 0 M to 0.25 M NaCl. The AphC-containing fractions were pooled, dialyzed against the same buffer, and rechromatographed on an HR16/20 Fast Flow Q-Sepharose anion-exchange column under the same conditions. The AphC-containing fractions were dialyzed against 10 mM Tris-HCl buffer (pH 7.0) and brought to 40% saturated $(\text{NH}_4)_2\text{SO}_4$, followed by loading on a HiLoad 26/10 Phenyl Sepharose HP (GE Healthcare) equilibrated in the same buffer containing 40% saturated $(\text{NH}_4)_2\text{SO}_4$. The column was washed with the equilibration buffer, and bound protein was eluted with a linear gradient of 40% to 0% saturated $(\text{NH}_4)_2\text{SO}_4$. The AphC-containing fractions were dialyzed against 10 mM Tris-HCl buffer (pH 7.0) and were determined to be pure by SDS-PAGE.

In order to analyze the N-terminal amino acid sequence, purified enzyme was applied to PVDF using a ProSorb device (Applied Biosystems) and sequenced on an Applied Biosystems Procise 491 sequencer (Applied Biosystems).

Purified enzyme was deglycosylated with endoglycosidase H (Glyko, Novato, CA, USA) according to the procedure provided by the manufacturer. The protein was denatured before addition of endoglycosidase H.

Enzyme assay Acid phosphatase activity was measured using a slight modification of the procedure described by Oike *et al.* (Oike *et al.*, 1984). An aliquot of the enzyme solution was incubated with 1 mM *p*-nitrophenylphosphate (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 addition of 2 M Na_2CO_3 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 pHs (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 the officially approved method (Nishiya, 1993). Neutral protease activity was measured according a slight modification 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). IMP dephosphorylation activity was measured as follows: 1 mL of 20 mM IMP was mixed with 0.2 mL of 200 mM acetate buffer (pH 4.0) and incubated with 0.1 mL of dialyzed culture extract at 37°C for 20 min. The reaction was terminated by adding 0.1 mL of 1 N NaOH, followed by neutralization with 0.1 mL of 1 N HCl. The liberated phosphate was measured using Pi ColorLock ALS (Innova Biosciences Ltd., Cambridge, UK). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of phosphate per min under the assay conditions.

Computational sequence analysis A homology search was performed using the BLAST search of DOGAN, the Database of the Genomes Analyzed at NITE (<http://www.bio.nite.go.jp/dogan/top>). The search for signal peptides was performed using SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>). The search for glycosylation sites was performed using two online glycosylation site prediction servers, NetOGlyc 3.1 (<http://www.cbs.dtu.dk/services/NetOGlyc/>) for prediction of O-linked glycosylation sites and NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) for prediction of N-linked glycosylation sites.

Results and Discussion

Construction and characterization of acid phosphatase gene disruptants In our previous report, we identified eight *aph* genes encoding acid phosphatase (Aph) with similarity to the well-characterized *A. niger phyA* by searching the *A. oryzae* genome database using BLAST (Table 2). Characterization results for the *ahpA* disruptant suggested that another Aph (or Aphs) is responsible for the majority of acid phosphatase activity. In order to identify the major Aph (or Aphs) possessing IMP dephosphorylation activity, we constructed seven *aph* disruptants in addition to the *aphA* disruptant. As an example, a construction scheme for the *aphB* gene disruption is shown in Fig. 1A. Genomic DNAs from eight randomly selected transformants were analyzed by PCR using the primer pair phyB1/phyB4. Only a 3.8-kb band was detected for five of the transformants, indicating successful *aphB* gene disruption (data not shown). Disruption of the other genes was performed using the same procedure as for *aphB*. The efficiencies of the eight *aph* gene disruptions were: 62.5% for *aphB*, 100% for *aphC*, 87.5% for *aphD*, 100% for *aphE*, 62.5% for *aphF*, 87.5% for *aphG* and 100% for *aphH*. As shown in Fig. 1B, gene disruption of the

targeted *aph* gene did not affect the other *aph* genes.

In order to investigate the effects of the single *aphA-H* gene disruption on acid phosphatase production, the enzyme productivity of the disruptants was compared to the wild-type strain, *A. oryzae* KBN630. The single disruptants of the *aphA-H* gene grew normally in soybean-*koji* culture, comparable to *A. oryzae* KBN630, indicating that *aphA-H* gene disruption did not affect growth when steamed soybeans were used as the source of nutrients. As shown in Table 3, *aphA*, *aphC* and *aphE* gene disruptants had lower acid phosphatase activity and 5'-IMP dephosphorylation activity than *A. oryzae* KBN630, whereas the enzyme activities of the other disruptants did not decrease. The acid phosphatase activity and 5'-

IMP dephosphorylation activity of the *aphC* gene disruptant were severely decreased, to 3% and 6%, respectively, compared to *A. oryzae* KBN630. These results strongly indicate that AphC is mainly responsible for both acid phosphatase activity and 5'-IMP dephosphorylation activity in soybean-*koji* culture.

On the basis of transcriptional expression analysis of the *aph* genes in solid-state rice and soybean culture, the *aph* genes in *A. oryzae* were classified into type R and type S due to their higher expression in solid-state Rice and Soybean culture, respectively (Marui *et al.* 2012). Disruption of the *aphC* gene, which was categorized as type S, resulted in a marked decrease in both acid phosphatase activity and 5'-IMP dephosphorylation activity in soybean-*koji* culture. However, disruption of the *aphB* and *aphG* genes, which were categorized as type S, reduced neither of the enzyme activities. In contrast, disruption of the *aphA* gene categorized as type R reduced both of the enzyme activities. These results indicate that transcriptional expression of the *aph* genes are regulated in a complicated manner by the PHO regulatory pathway, pH regulation, and other unknown mechanisms. Moreover, amylase activities in the *aphA-H* gene disruptants increased by 12 – 30% when compared to the wild-type strain, and the neutral protease activities increased by 2 – 9% except in the *aphE* disruptant. Although the reason is still unclear, these results suggest that disrupt-

Table 2. Acid phosphatase genes in *A. oryzae* studied during this work

Gene	Gene ID	Genbank accession number
<i>aphA</i>	AO090023000692	AP007157 / AB042805
<i>aphB</i>	AO090120000167	AP007166
<i>aphC</i>	AO090010000202	AP007175 / AB775132
<i>aphD</i>	AO090011000174	AP007171
<i>aphE</i>	AO090023000448	AP007157
<i>aphF</i>	AO090023000481	AP007157
<i>aphG</i>	AO090124000063	AP007165
<i>aphH</i>	AO090005000912	AP007151

Fig. 1. Construction scheme for *aphB* disruption (A) and confirmation of *aphA-H* single disruptants (B).

A. Scheme for *aphB* gene disruption is shown. PCR-amplified DNA fragments from the *aphB* gene disruption vector, pDisAphB, were transformed into *A. oryzae* KBN630-17K3. Gray and black boxes indicate the 5'-flanking region and a part of the coding region of the *aphB* gene, respectively, and closed and open arrows indicate the *aphB* gene and *pyrG* gene regions, respectively. The direction of the arrow indicates the orientation of the *aphB* and *pyrG* genes. Small arrows indicate the positions of the oligonucleotide primers used.

B. Agarose gel electrophoresis of amplified DNA fragments in the *aphA-H* gene regions of the eight *aph* disruption strains using primer pairs phyB1/phyB4 to phyH1/phyH4 listed in Table 1, and phyA1/phyA6 from our previous report (Yoshino-Yasuda *et al.*, 2012).

Table 3. Effects of *aphA-H* gene disruption on enzyme production in soybean-*koji* culture

Strain	Enzyme activity (units/g <i>koji</i> culture) (% of control)			
	Acid phosphatase	5'-IMP dephosphorylation	α -Amylase	Neutral protease
$\Delta aphA$	394 $\pm$ 39.4 (80)	547 $\pm$ 57.9 (89)	959 $\pm$ 50.0 (130)	20,800 $\pm$ 461 (106)
$\Delta aphB$	482 $\pm$ 52.3 (98)	692 $\pm$ 18.7 (112)	894 $\pm$ 7.8 (121)	20,200 $\pm$ 385 (102)
$\Delta aphC$	13 $\pm$ 0.6 (3)	36 $\pm$ 10.3 (6)	899 $\pm$ 15.6 (122)	20,800 $\pm$ 307 (106)
$\Delta aphD$	478 $\pm$ 27.2 (97)	632 $\pm$ 16.5 (103)	853 $\pm$ 4.2 (116)	20,400 $\pm$ 461 (104)
$\Delta aphE$	378 $\pm$ 14.1 (76)	481 $\pm$ 1.0 (78)	869 $\pm$ 2.8 (118)	18,500 $\pm$ 461 (94)
$\Delta aphF$	528 $\pm$ 4.4 (107)	667 $\pm$ 39.2 (108)	881 $\pm$ 7.1 (120)	22,000 $\pm$ 537 (112)
$\Delta aphG$	496 $\pm$ 64.2 (100)	661 $\pm$ 75.7 (107)	924 $\pm$ 40.3 (125)	21,400 $\pm$ 615 (109)
$\Delta aphH$	452 $\pm$ 20.7 (91)	590 $\pm$ 30.0 (96)	822 $\pm$ 24.0 (112)	21,000 $\pm$ 93 (107)
KBN630	494 $\pm$ 28.3 (100)	615 $\pm$ 21.2 (100)	736 $\pm$ 42.4 (100)	19,700 $\pm$ 707 (100)

Activity of two independent experiments is presented as average $\pm$ standard deviation.

Fig. 2. SDS-polyacrylamide gel electrophoresis of AphC purified from *A. oryzae* transformant APC15.

AphC was purified as described in the Materials and Methods. The gel was stained with Coomassie Brilliant Blue. Lane 1, molecular-mass markers [*E. coli* β -galactosidase (116.0 kDa), rabbit muscle phosphor-ylase b (97.4 kDa), bovine serum albumin (69.0 kDa), glutamate dehy-drogenase (55.0 kDa), porcine muscle lactate dehydrogenase (36.5 kDa), bovine liver carbonate anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa), and egg white lysozyme (14.4 kDa)]; lane 2, purified AphC; lane 3, endoglycosidase H treated AphC.

tion of the *aphA-H* genes have a positive effect on the production of other enzymes such as amylase. Therefore, further studies on the *aphA-H* gene disruptants will be valuable for elucidating the *aph* genes regulatory mechanisms in *koji* culture and be helpful for in-creasing the amylase and protease productivities of *A. oryzae*.

Overexpression and purification of AphC In order to charac-terize the enzymatic properties of the *aphC* gene product, the *aphC* gene from *A. oryzae* KBN630 (GenBank accession number AB775132) was efficiently expressed under control of the *A. ory-zae* *taaG2* gene promoter, which is one of the strongest promoters in *A. oryzae*. During the construction of the *aphC* gene expression plasmid, pTAAphC, nucleotide sequence analysis revealed that the

aphC gene from *A. oryzae* KBN630 consists of 1,678 bp, including two introns of 47 bp, and contains an open reading frame encoding 527 amino acids. The introns were subsequently confirmed by se-quencing the cDNA fragment of the *aphC* gene. The *aphC* gene from *A. oryzae* KBN630 differs from that of *A. oryzae* RIB40 (GenBank accession number AP007157) by 25 bp in the nucleotide sequence, which led to two amino acid substitutions: Asn-279 to Tyr, and Ala-344 to Ser. After pTAAphC was introduced into *A. oryzae* PDE1, transformants grown in RS medium were assayed for extracellular acid phosphatase activity in order to select a high-AphC-producing strain for further study. The enzyme levels ranged from 203 to 490 U/mL (0.14 to 0.34 mg/L) for the seven transformants examined, whereas the control strain, *A. oryzae* PDE1 carrying the *pyrG* gene, showed no acid phosphatase activi-ty.

AphC was purified to homogeneity from the culture superna-tant of the highest AphC producing strain, APC15, by two an-ion-exchange chromatography purifications followed by hydropho-bic interaction chromatography, as described in the Materials and Methods. AphC was purified 2.1-fold, with recovery of 49.4% of the initial activity. The specific activity of the purified AphC was 1,459 U/mg of protein, which was approximately 13 times higher than that of AphA. The purified AphC migrated as a band of ap-proximately 69.0 kDa on SDS-PAGE (Fig. 2, lane 2).

The N-terminal amino acid sequence of the purified AphC pro-tein was chemically determined as Ala-Pro-X-X-X-X-Ala-Ala-Ala (X denotes an unidentified residue). This sequence corresponds to the deduced amino acid sequence from Ala-22 to Ala-31, except for the five residues from Thr-24 to Ser-28. Thr-24 was one of the O-linked glycosylation sites predicted by NetOGlyc 3.1, and might be the reason why the residues from Thr-24 to Ser-28 could not be identified. Analysis using SignalP 4.0 predicted that the first 21 N-terminal amino acids of AphC function as a signal sequence, which is consistent with the results of N-terminal amino acid deter-mination. Therefore, the mature AphC consists of 506 amino acids with a calculated molecular mass of 55,105 Da. Since the calculat-

Table 4. Comparison of the properties of AphC with those of other acid phosphatases from *A. oryzae*

	AphC	AphA ^a	ACP-I ^b	ACP-II ^b	ACP-III ^b	AphK1 ^c
Molecular mass (kDa)	69.0	58.0 to 65.0	110	58	56	70
pH optimum	4.5	4.0	4.5	5.0	5.5	5.5
pH stability	3.5 to 6.5	3.0 to 7.0	4.3 to 5.5	4.8 to 6.5	5.0 to 5.5	2.0 to 7.0
Optimum temperature (°C)	50	40	60	40	45	60
Thermal stability (°C)	< 25	< 35	< 45	< 35	< 40	ND ^d

^aThe results of AphA were taken from our previous report (Yoshino-Yasuda *et al.* 2012).^bThe results of ACP-I, ACP-II and ACP-III were taken from the report by Fujita *et al.* (Fujita *et al.* 2003a).^cThe results of AphK1 were taken from the report by Shimizu (Shimizu 1993).^dND, not determined.**Table 5.** Comparison of AphC substrate specificity with those of other acid phosphatases from *A. oryzae*

Substrate	Relative activity ^a (%)					
	AphC	AphA ^b	ACP-I ^c	ACP-II ^c	ACP-III ^c	AphK1 ^d
Sodium <i>p</i> -nitrophenylphosphate	100	100	100	100	100	100
Sodium phytate	0.04	54.0	14	255	23	3.3
Sodium glycerophosphate	12.1	49.2	10	0	81	93.3
Sodium pyrophosphate	16.2	33.3	ND ^e	ND	ND	183.3
D-Glucose-6-phosphate	6.5	43.4	52	0	45	ND
5'-GMP	19.2	8.1	ND	ND	ND	ND
5'-IMP	37.3	6.9	ND	ND	ND	ND

^aHydrolysis rate of *p*-nitrophenylphosphate was taken as 100%.^bThe results of AphA were taken from our previous report (Yoshino-Yasuda *et al.* 2012).^cThe results of ACP-I, ACP-II and ACP-III were taken from the report by Fujita *et al.* (Fujita *et al.* 2003a).^dThe results of AphK1 were taken from the report by Shimizu (Shimizu 1993).^eND, not determined.

ed molecular mass was smaller than that of the purified AphC by approximately 14.0 kDa on SDS-PAGE (Fig. 2, lane 2), we assumed that the differences between the theoretical and apparent molecular mass are caused by protein glycosylation. This idea is partly supported by the presence of six putative N-linked glycosylation sites (Asn-106, Asn-113, Asn-178, Asn-213, Asn-257, and Asn-275) and three O-linked glycosylation sites (Thr-24, Ser-32, and Thr-35) in AphC predicted by the programs NetNGlyc 1.0 and at NetOGlyc 3.1, respectively. Treatment of the purified AphC with endoglycosidase H, which cleaves N-linked mannose-rich oligosaccharides, decreased the apparent molecular mass to approximately 58.0 kDa (Fig. 2, lane 3). However, this value is still larger than the theoretical mass and may be due to O-linked glycosylation, as described above.

Enzymatic properties of AphC The enzymatic features of AphC, including pH and temperature optima and pH and thermal stabilities, were investigated with the purified protein. AphC has a pH optimum of 4.5 and a temperature optimum of 50°C. AphC is stable at a wide range of pHs, from 3.5 to 6.5. AphC was moderately inactivated at temperatures up to 30°C, but sharply inactivated at temperatures above 30°C. No enzyme activity remained at temperatures above 50°C. This finding means that AphC has a low intrinsic resistance to heat inactivation.

The substrate specificity of AphC was examined with a series

of phosphate compounds under the same conditions as AphA (Table 4, Yoshino-Yasuda *et al.*, 2012). AphC displayed rather broad substrate specificity but had very little activity toward phytate. AphC showed considerably higher activity toward 5'-GMP (19.2%) and 5'-IMP (37.3%). As shown in Tables 4 and 5, the enzymatic properties and substrate specificity of AphC clearly differ from those described for other *A. oryzae* acid phosphatases, AphA, ACP-I, ACP-II, ACP-III, and AphKI. Based on the specific activities of AphC (1,459 U/mg) and AphA (108 U/mg) toward PNP, AphC had about 30-fold and 70-fold higher specific activity toward 5'-GMP and 5'-IMP than AphA, respectively. This result suggests that AphC is largely involved in disodium 5'-ribonucleotide dephosphorylation activity in *A. oryzae* in soybean-*koji* culture. This unique substrate specificity appeared to correlate with the significant decrease in 5'-ribonucleotide dephosphorylation activity observed in the soybean-*koji* culture of the *aphC* disruptant (Table 3). From a practical point of view, it will be extremely interesting to investigate the expression levels of *aphC* in *A. oryzae* KBN630 and other industrial strains used in soybean-*koji*. We anticipate that the degradation of 5'-ribonucleotides, such as 5'-IMP and 5'-GMP that are supplemented in *miso* products, can be prevented by use of the *aphC* disruptant. Further studies are contemplated to verify the effectiveness of the *aphC* disruptant in laboratory scale *miso* brewing using soybean-*koji* culture. It is also necessary to consider the pos-

sible effect of lactic acid bacteria and yeasts involved in *miso* brewing. Moreover, we will construct the triple gene disruptant of *aphA*, *aphC*, and *aphE* in order to further decrease acid phosphatase activity.

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