Characterization of the Prolyl Dipeptidyl Peptidase Gene (dppIV) from the Koji Mold Aspergillus oryzae

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The koji mold Aspergillus oryzae secretes a prolyl dipeptidyl peptidase (DPPIV) when the fungus is cultivated in a medium containing wheat gluten as the sole nitrogen and carbon source (MMWG). We cloned and sequenced the DPPIV gene from an A. oryzae library by using the A. fumigatus dppIV gene as a probe. Reverse transcriptase PCR experiments showed that the A. oryzae dppIV gene consists of two exons, the first of which is only 6 bp long. The gene encodes an 87.2-kDa polypeptide chain which is secreted into the medium as a 95-kDa glycoprotein. Introduction of this gene into A. oryzae leads to overexpression of prolyl dipeptidyl peptidase activity, while disruption of the gene abolishes all prolyl dipeptidyl peptidase activity in MMWG. The dppIV null mutants did not exhibit any change in phenotype other than the absence of prolyl dipeptidyl peptidase activity, suggesting that this activity is not essential. This loss of activity diminished the number of dipeptides and increased the number of larger peptides present in the MMWG culture broth. These effects were reversed by the addition of purified, recombinant DPPIV from the methyloptrophic yeast expression vector Pichia pastoris. Our results suggest that the DPPIV enzyme may be of importance in industrial hydrolysis of wheat gluten-based substrates, which are rich in Pro residues.

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Aspergillus oryzae and the closely related Aspergillus sojae are used in industrial and traditional koji fermentations. During such fermentation, these molds secrete a large variety of carbohydrates and proteases that are essential for efficient solubilization and hydrolysis of the soybean or wheat raw materials. Protein degradation is a complex multistep process involving endopeptidases and exopeptidases (14). To date, two neutral endopeptidases (NPI and NPII) (28, 29), an alkaline endopeptidase (ALP) (24, 30) and an aspartic protease (PEPO) (49, 50) have been identified and purified from koji molds. The genes encoding NPII (48), ALP (10, 47), and PEPO (5) from A. oryzae have been cloned and characterized. The exopeptidases characterized in A. oryzae are aminopeptidases (31–33, 39), carboxypeptidases (25–27, 34), and a prolyl dipeptidyl peptidase (46). Since wheat gluten contains a large number of Pro residues (1), this last enzyme could be of particular importance in the degradation of wheat gluten during koji fermentation.

Recently, two dipeptidyl peptidase genes (dppIV and dppV) from Aspergillus fumigatus have been cloned and characterized (3, 4). The dppIV gene encodes a prolyl dipeptidyl peptidase (DPPIV) which releases N-terminal X-Pro residues, while the dppV gene encodes a dipeptidyl peptidase (DPPIV) which releases N-terminal X-Ala, His-Ser, and Ser-Tyr dipeptides. DPPIV activity previously detected in A. oryzae (46) was probably due to an enzyme homologous to the A. fumigatus DPPIV. To date, the role of prolyl dipeptidyl peptidase activity has not been analyzed in the context of fermentation and hydrolysis of wheat gluten (WG) by A. oryzae. Our objectives were to clone the A. oryzae gene encoding DPPIV and to compare the peptide profiles of a WG-containing medium hydrolyzed by wildtype or dppIV disruptant strains.

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MATERIALS AND METHODS

Strains and plasmids. Escherichia coli LE392 was used as a host strain for propagation of bacteriophages. Plasmid pMTL21-H6,6, containing the A. fumigatus dppIV gene that was used as a probe, was kindly provided by A. Beauvais, Institut Pasteur, Paris, France. Plasmids pMTL20 (9), pNEB 193 (New England Biolabs, Beverly, Mass.), pBluescript SK– (Stratagene, La Jolla, Calif.), and pCL1920b (6) were used in subcloning procedures. E. coli DH5α was transformed by using competent cells and standard protocols (43).

Both A. oryzae 44 and TK3 from our strain collection were used as the wild type. A. oryzae NF1 is a uridine auxotroph derived from TK3 by targeted disruption (51) and was used as the recipient for transformations. Plasmid pNPF28 containing the A. oryzae TK3 ppyG gene (P. van den Broek; GenBank accession no. Y13811) was used as a selection marker in the cotransformation experiment to overexpress DPPIV in A. oryzae. Aspergillus nidulans 033 (hisA4 argF4) obtained from A. J. Clutterbuck, Fungal Genetic Stock Centre, Glasgow, Scotland, A. fumigatus CBS 144.89 and Aspergillus niger CBS 126.49 were used to test secreted prolyl dipeptidyl peptidase activity.

The E. coli-Pichia pastoris shuttle vector pPIC9 was obtained by using the P. pastoris expression system from Invitrogen (San Diego, Calif.). The P. pastoris expression vector pKJ115 was constructed by cloning the expression cassette of pPIC9, flanked by two BglII sites, into the BamHI site of pCL1920b. In pKJ115, the expression cassette of pPIC9 was flanked by two 5′ smd sites for linearization of the DNA, before transformation of P. pastoris GS115 (Fig. 1). The His+ Mut+ P. pastoris strain GS115, used as a host for transformation, was obtained from Invitrogen.

A. oryzae growth media. Aspergillus minimal medium (MM) was prepared as described by Pontecorvo et al. (40). Wheat gluten minimal medium (MMWG) contained MM and 1% (wt/vol) WG (Sigma, St. Louis, Mo.). Wheat gluten-wheat gluten hydrolysate minimal medium (MMWGHH) contained MM, 0.1% (wt/vol) WG (Sigma), and 0.1% (wt/vol) WG hydrolysate (WGHH). WGHH was prepared by hydrolyzing nonvital WG powder (Roquette, Freres, Lestrem, France) with alcalase (Novo Nordisk, Bagsvaerd, Denmark). This preparation was used 20% (wt/wt) substrate concentration and an enzyme-to-substrate ratio of 1:50 (weight of protein) for 6 h at 60°C and a constant pH of 7.5. Alcalase was then heat inactivated at 90°C for 10 min. After centrifugation of the hydrolysate, the supernatant was lyophilized to give WGH and stored at room temperature. It contained mainly peptides that were shown by size-exclusion chromatography on a Sephadex peptide HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) (data not shown) to be in the range of 200 to 10,000 Da. Only minimal amounts of free amino acids were detected.

Genomic DNA extraction of A. oryzae. Genomic DNA from lyophilized mycelium was isolated as described by Raiser and Brola (41) and purified by using Genomic-tips (Qiagen, Hilden, Germany).

Cloning and sequencing of the A. oryzae dppIV gene. The A. oryzae genomic library was constructed in λEMBL3 as previously described (25). Recombinant plaques of the A. oryzae 44 genomic library were immobilized on nylon membranes (Genescreen; Biotechnology Systems, Boston, Mass.). These filters were
to 2 cycles of 1 min at 98°C, 2 min at 56°C, and 2 min at 72°C, followed by 27 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C, and 1 cycle of 1 min at 94°C, 1 min at 56°C, and 10 min at 72°C. The gel-purified PCR products were recovered with Quia II (Qiagen) and directly ligated into the pGEM-T vector (Promega, Madison, Wis.) to generate plasmid pNF113.

Southern blotting. Agarose gel electrophoresis of total A. oryzae genomic DNA was blotted onto nylon membranes (Hybond-N+; Amersham Pharmacia Biotech) in accordance with the standard protocol (43). The membranes were prehybridized at 65°C for 45 min in Rapid-blot buffer (Amersham Pharmacia Biotech) before hybridization for 5 h in the same solution containing randomly primed 32P-labeled probes. The membranes were exposed to X-ray film after three washes in 0.1× SSC-0.1% SDS at 65°C. Denstometric scans of autoradiographs were obtained by using the Herolab GmbH (Wiesloch, Germany) Enhanced Analysis System.

Standard PCs. Two hundred nanograms of DNA, 1 μl of oligonucleotides each at 50 mM, and 6 μl of deoxyribonuclease trifluorophosphates each at 2 mM were dissolved in 50 μl of PCR buffer number 2 [20 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 10 mM (NH4)2SO4, 0.1% Triton X-100, 100 μl of nuclease-free bovine serum albumin per ml] to which a drop of Dynawax (Finnzymes, Espoo, Finland) was added. To each reaction mixture, 2.5 μl of cloned Pfu DNA polymerase (Stratagene) in 50 μl of 1× PCR buffer was added. These reaction mixtures were subjected to 30 cycles of 1 min at 95°C, 1 min at 52°C, and 3 min at 72°C with a Perkin-Elmer DNA thermal cycler.

A. oryzae transformation. A. oryzae NFI was transformed as previously described (12). The A. nidulans pygG gene and the A. oryzae ppyG gene were used as the selection markers in A. oryzae dppIV gene disruption and overexpression, respectively. Transformants were selected on MM.

Enzyme assays. For in situ detection of DPPIV activity, spots of the test strains were resuspended in 200 μl of SP2 buffer (20 mM KH2PO4 adjusted to pH 2.0 with HCl and 0.9% NaCl) in microtiter plates and replica plated onto petri dishes containing MMWG covered by a filter (Chrl; Whatman, Maidstone, England). The plates were then incubated for 2 days at 30°C. DPPIV activity was detected on the filter by the methods of Lojda (20) and Aratake et al. (2). The filters were reacted with a solution containing 3.0 mg of gleyol proline (4-N-p-nitroanilide in 0.25 ml of N2,N-dimethylformamide and 5.0 mg of o-dianisidine, tetrazotized in 4.6 ml of 0.1 M sodium phosphate buffer (pH 7.2) for 10 min at room temperature.

DPPIV activity was determined in culture broth by UV spectrometry with the synthetic substrate alanine-proline-p-nitroanilide (Bahcem, Bubendorf, Switzerland) as described by Sarah et al. (45). Fifty microliters of culture broth was added, and the reaction was continued for up to 60 min at 57°C. A control experiment with blank substrate and blank culture broth was carried out in parallel. Release of the chromophor group p-nitroanilide was measured at 400 nm, and activities were expressed as milliunits per milliliter (nanomoles per minute per milliliter). For practical purposes, 1 U of DPPIV activity was defined as the activity producing an absorbance of 0.001 min−1 in a proteolytic assay at optimum pH.

Expression of the A. oryzae dppIV gene in P. pastoris. The plasmid used to express the A. oryzae dppIV gene in P. pastoris was constructed by cloning a PCR product of the dppIV gene in the multiple-cloning sites of the E. coli-P. pastoris shuttle vector pKJ115. In detail, the A. oryzae dppIV coding region was amplified by standard PCR with primers 3261 and 3260 (Table 1). The PCR product was purified with the High Pure PCR product purification kit (Boehringer) and then digested by EcoRV and NotI restriction enzymes for which a site was previously designed at the 5′ extremity of the primers. By using standard protocols (43), the digested PCR products were then cloned into the SmalI and NotI sites of the pPic9 cassette multiple-cloning site of plasmid pKJ115.

Spheroplasts of P. pastoris were transformed with 10 μg of DNA linearized by SsrnI. Transformants were selected on histidine-deficient medium (1 M sorbitol, 1% [wt/vol] dextrose, 1.34% [wt/vol] yeast nitrogen base without amino acids, 0.00004% [wt/vol] biotin, 0.005% amino acids [i.e., 0.005% [wt/vol] of each l-glutamic acid, l-methionine, l-lysine, l-leucine, l-isoleucine]) and screened for

![FIG. 1. Map of plasmid pKJ115. REP, pCL1920 replicon (19); SPC, spectinomycin resistance gene; LACPO, LACZ promoter; 3′ AOX1; P. pastoris alcohol oxidase gene (AOX1) promoter; SS, signal peptide sequence of α-factor; 3′ AOX1 (TT), AOX1 terminator; HIS4, P. pastoris histidinol dehydrogenase gene; 3′ AOX1, 3′ AOX1 downstream sequence; LACZ, pUC19 LACZ fragment.](image-url)
insertion of the construct at the AOX1 site on methanol-minimal plates (1.34% [wt/vol] yeast nitrogen base without amino acids, 0.00004% [wt/vol] biotin, 0.5% [vol/vol] methanol).

Transformants unable to grow on media containing only methanol as a carbon source were assumed to contain the correct yeast genomic location and to have resulted from integration events in the AOX1 locus displacing the AOX1 coding region. The selected transformants were grown to near saturation at 30°C in 10 ml of glycerol-based yeast medium (BMGY; 0.1 M potassium phosphate buffer [pH 6.0], containing 1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 1.34% [wt/vol] YNB without amino acids, 1% [vol/vol] glycerol, and 0.00004% [wt/vol] biotin). Cells were harvested and resuspended in 2 ml of the same medium with 0.5% (vol/vol) methanol instead of glycerol (BMMM) and incubated for 2 days, after which time the supernatant was harvested and 10 µl was loaded on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels to identify clones producing DPPIV.

**Purification of DPPIV produced by A. oryzae.** Ten milliliters of centrifuged culture broth was ultrafiltered on a Centricon-10 filter unit (Amicon, Beverly, Mass.) to a final volume of 2.5 ml. This aliquot was loaded onto a PD-10 column (Amersham Pharmacia Biotech) and eluted with 3.5 ml of distilled water. The eluate was loaded onto a second column containing 500 µg of hydroxyapatite (Bio-Rad, Hercules, Calif.), and the protein was eluted by a sodium phosphate buffer gradient (50 to 250 mM) at pH 7.0. In a typical experiment, approximately 500 µg of pure, active DPPIV was recovered; this corresponds to a yield of about 54%.

**Treatment with N-glycosidase F.** Four milliliters of culture broth containing secreted prolyl dipeptidyl peptide from an overproducing strain was ultrafiltered on a Centricon-50 filter unit (Amicon) to a final volume of 112 l. Ap-401 (10 µg) was added (final detergent concentrations, 0.1% SDS and 0.5% potassium phosphate buffer [pH 7.0], 25 mM EDTA, and 0.15% SDS) and then heated at 100°C for 5 min.

**N-terminal sequence determination of deglycosylated DPPIV from A. oryzae.** After SDS-PAGE, protein bands were directly blotted with a Mini Trans-Blot unit (Bio-Rad) onto an Immobilon P20 (Millipore, Bedford, Mass.) membrane and visualized by staining with Coomassie blue R-250. After the membrane was air dried, DPPIV-containing bands were excised with a razor blade. In situ automated Edman degradation for N-terminal sequence analysis was performed on the blot with a gas-phase sequencer (model 477A; PE Applied Biosystems), equipped for on-line detection of the amino acids released.

**Peptide profiling.** A. oryzae wild-type strain TK3 and A. oryzae disruptant D1 were grown in parallel in 200 ml of MMWG for 7 days at 37°C without shaking. The cultures were then filtered through two layers of cheesecloth. To 500-µl aliquots of culture medium was added either 3 µg of purified DPPIV (around 320 µU) or 20 µl of enzyme buffer without DPPIV. The aliquots were incubated for 24 h at 37°C, heated at 95°C for 10 min, centrifuged, and analyzed by size exclusion chromatography on a Superdex peptide HR 10/30 column. Separation of the amino acids and peptides from treated culture broth aliquots was based on molecular size (range, 100 to 7,000 Da). Chromatography was performed under isocratic conditions with 1% trifluoroacetic acid-20% acetonitrile in water at a flow rate of 0.5 ml/min. Amino acid and peptide peaks were detected at 215 nm. The molecular size (range, 100 to 7,000 Da). Chromatography was performed under isocratic conditions with 1% (wt/vol) triamterene sodium sulfate (SDS-PAGE) gels to identify clones producing DPPIV.
pNFF28 carrying the \textit{A. oryzae} \textit{pyrG} gene as the selection marker. Transformants were selected on MM. Ninety-five \textit{pyrG} transformants were incubated on MMWGH for 2 days at 30°C before screening for secreted DPPIV activity was performed. Sixteen transformants clearly exhibited increased staining compared to that of the wild type (Fig. 3B). In liquid MMWG medium, the maximum activity of DPPIV obtained in \textit{A. oryzae} DPPIV-overexpressing transformants was 17 times higher than that of the \textit{A. oryzae} \textit{NF1 pyrG} control strain. Four transformants, designated S1 to S4, were selected because of their elevated DPPIV activity.

\textbf{FIG. 2.} Construction of plasmid pNFF129 used for \textit{dppIV} gene disruption. The 1.5-kb \textit{NcoI} fragment of pNFF125 containing an internal part of the \textit{A. oryzae} \textit{dppIV} gene was replaced with the 1.8-kb \textit{NcoI} fragment from pNFF39 containing the functional \textit{A. nidulans pyrG} gene.

\textbf{FIG. 3.} Screening of \textit{A. oryzae} \textit{dppIV} null mutants (open arrows) (A) and DPPIV overproducers (solid arrows) (B).

\textbf{FIG. 4.} Southern blot analysis of \textit{A. oryzae} \textit{dppIV} disruptants. \textit{NcoI}-digested genomic DNA from four pNFF129 transformants without prolyl dipeptidase activity (D1 to D4) and two transformants retaining enzyme activity (D5 and D6) were hybridized under high-stringency conditions with a PCR fragment encoding the whole DPPIV as a probe. \textit{A. oryzae NF1 pyrG} (NF1) DNA was used for comparison.

\textbf{FIG. 5.} Southern analysis of four prolyl dipeptidyl peptidase-overproducing transformants (S1 to S4). \textit{A. oryzae NF1 pyrG} DNA was used for comparison. \textit{EcoRV} (A)- and \textit{ApaI-EcoRV} (B)-digested genomic DNAs, hybridized under high-stringency conditions with a PCR fragment encoding the whole DPPIV as a probe, are shown.
these same transformants were determined to be 10, 3, 8, and 17 times higher than the activity of A. oryzae NF1 pyrG+ control strain after growth for 7 days at 30°C in 100 ml of liquid MMWG without shaking. These data confirm that an increase in the number of gene copies results in a higher production level but without linear correlation. This could be due to the fact that, besides the number of gene copies, the site of integration also may affect the expression of the introduced genes (52).

Identification of DPPIV protein in culture medium. Culture broth from the prolyl dipeptidyl peptidase-overproducing transformant S4 and from A. oryzae NF1 pyrG+ as a control strain were analyzed by SDS-PAGE to identify the DPPIV protein. A broad smear was visible in the region of 95 kDa for A. oryzae transformant S4 and from A. oryzae NF1 pyrG+ control strain as well as in the A. oryzae NF1 pyrG+ control (data not shown), suggesting that the DPPIV protein was glycosylated. After the samples were treated with N-glycosidase F, a band of about 85 kDa appeared in the NF1 pyrG+ control as well as in the prolyl dipeptidyl peptidase-overproducing strain (Fig. 6, lane 3) but not in the A. oryzae NF1 pyrG+ control (data not shown), indicating that the DPPIV protein was deglycosylated. The amount of deglycosylated DPPIV obtained from the overexpressing transformant culture broth was substantial enough to determine the initial amino acid residues of the N terminus of the protein by in situ automated Edman degradation. The N-terminal sequence of the mature protein was determined to be Leu-Asp-Val-Pro-Arg-, indicating that DPPIV was the major protein species in the excised band.

Expression of A. oryzae DPPIV in P. pastoris. P. pastoris transformants secreted A. oryzae DPPIV enzyme in the BMMY medium at the maximal rate of 100 μg/ml (8,000 mU/ml) after 48 h of expression. This rate is about 800 times that of A. oryzae TK3 in liquid MMWG (10 mU of DPPIV/ml). Under identical culture conditions, P. pastoris GS115 did not secrete any DPPIV (data not shown). Recombinant DPPIV was active between pH 4.0 and 9.0, with optimal activity at pH 7.0 (data not shown), and had a specific activity of 100 mU/μg when Ala-Pro-p-nitroanilide was used as a substrate.

SDS-PAGE analysis showed that the secreted, recombinant A. oryzae DPPIV was the major protein present in the culture medium of P. pastoris. This protein migrated as a slightly smearing band between 95 and 100 kDa (Fig. 6, lane 1). However, upon treatment with N-glycosidase F, this broad band gave rise to a sharp band at 85 kDa (Fig. 6, lane 2), similar to the one obtained after deglycosylation of the DPPIV secreted by A. oryzae dppIV cotransformants (Fig. 6, lane 4).

Role of DPPIV activity in WG hydrolysis. To test the function of DPPIV in WG hydrolysis, we compared the peptide profiles of hydrolysates from WG-containing medium after inoculation with both the DPPIV disruptant D1 and the wild-type TK3 strain (Fig. 7A). By size exclusion chromatography analysis, the Ala-Pro dipeptide control eluted after 35 min (Fig. 7C). In the culture broth of wild-type strain TK3, a large peak of dipeptides was obtained. However, the lack of DPPIV activity in the D1 strain gave only a residual peak, which perhaps resulted from the action of another dipeptidyl peptidase homologous to that characterized in A. fumigatus (3). The level of larger peptides (elution time, 25 to 35 min) was higher in the disruptant culture broth than in that of the wild-type strain.

However, when purified DPPIV produced in P. pastoris was added to the disruptant culture broth at the level of wild-type activity and incubated for 24 h, the pattern of the peptide profile was similar to that of the wild type (Fig. 7B), thus confirming the role of this enzyme in peptide degradation.

DISCUSSION

Several facts support the conclusion that we have successively cloned the gene encoding the prolyl dipeptidyl peptidase and that this gene is responsible for the enzyme activity detected in A. oryzae culture broth. (i) Disruption of the dppIV gene abolished all detectable prolyl dipeptidyl peptidase activity. (ii) Prolyl dipeptidyl peptidase activity increased upon introduction of multiple copies of the 4.8-kb Apal-EcoRV fragment, which encompasses the dppIV gene, into A. oryzae. (iii) Insertion of the dppIV coding region into the P. pastoris expression system led to high-level secretion of prolyl dipeptidyl peptidase. Under identical culture conditions, wild-type P. pastoris does not produce any prolyl dipeptidyl peptidase activity.

As secreted protein, A. oryzae DPPIV is synthesized as a preprotein precursor. The N-terminal sequence of the mature protein, determined to be Leu-Asp-Val-Pro-Arg-, is preceded by a 16-amino-acid (16-aa) signal peptide with a hydrophobic core of 9 aa and a putative signal peptidase cleavage site in accordance with the −3,−1 von Heijne’s rule (54). The DPPIV protein molecules generated after signal peptide cleavage are 755 aa long. The polypeptidic chain of the mature protein has a calculated molecular mass of 85.5 kDa, which is in accordance with that estimated for the deglycosylated protein by SDS-PAGE. The amino acid sequence of A. oryzae DPPIV contains six potential N-linked glycosylation (Asn-X-Thr) sites which are conserved in A. fumigatus DPPIV, and the secreted enzyme contains approximately 10 kDa of N-linked carbohydrate (Fig. 6).

A. oryzae DPPIV is closely related to A. fumigatus DPPIV. These enzymes are homologous to human CD26 (11), Saccharomyces cerevisiae DPAP B (42), Xanthomonas maltophilia DP IV (17) and Flavobacterium meningosepticum DP IV (16), with around 35% identity, based on the algorithm of Needleman and Wunsch (36). The greatest homology is seen at the 200 aa of the C terminus of the proteins. In this area, we find the Gly-
X-Ser-X-Gly consensus sequence for the catalytic site of serine proteases (8). In the \textit{A. oryzae} DPPIV amino acid sequence, the Ser 618 residue in the Gly-X-Ser-X-Gly consensus sequence forms, with Asp 695 and His 730, which are conserved in the previously cited prolyl dipeptidyl peptidases, a catalytic triad described for serine proteases and lipases (7). However, unlike the \textit{Aspergillus} DPPIVs, which are secreted proteins, the human, rat (38), yeast, \textit{F. meningocepticum}, and \textit{X. maltophilia} prolyl dipeptidyl peptidases are membrane-bound proteins and contain an N-terminal noncleavable signal sequence which serves as a membrane anchor. By contrast, the deduced amino acid sequences of DPPIVs from \textit{Lactococcus lactis} (X-PDAP) (21, 35), \textit{Lactobacillus delbrueckii} (PepX) (22), and \textit{Lactoba}-

cillus helveticus} (PepX) (53) are located intracellularly (18) and do not exhibit significant sequence similarity with the aforementioned DPPIVs (data not shown).

The \textit{dppIV} null mutants did not exhibit any detectable change in phenotype other than the absence of prolyl dipeptidyl peptidase activity, suggesting that this activity is not essential for viability, which is consistent with the absence of prolyl dipeptidyl peptidase activity in wild-type \textit{A. niger} strains. However, the loss of prolyl dipeptidyl peptidase activity in the \textit{dppIV} null mutant grown on liquid MMWG led to a diminution in the number of dipeptides released and a simultaneous accumulation of larger peptides. These effects were reversed by the addition of purified recombinant DPPIV. Our results suggest an important role for this enzyme in koji fermentation processes, especially when a WG-based substrate rich in Pro residues is used. It is likely that another dipeptidyl peptidase is also involved during hydrolysis of WG. The residual peak observed in the culture broth of the \textit{A. oryzae} disruptant may be due to an alanyl dipeptidyl peptidase that is present in \textit{A. oryzae} (data not shown) and homologous to the DPPIV enzyme of \textit{A. fumigatus}. The role of this group of enzymes in koji fermentation is currently being investigated.

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**REFERENCES**