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Running title: Chitin deacetylase of *Flammulina velutipes*

Isolation and characterization of a gene coding for chitin deacetylase specifically expressed during fruiting body development in the basidiomycete *Flammulina velutipes* and its expression in the yeast *Pichia pastoris*

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Abstract

Fv-pda, a gene coding for chitin deacetylase, was isolated from the basidiomycete *Flammulina velutipes* by differential display targeted for genes specifically expressed during fruiting body development. The *fv-pda* open reading frame comprises 250 amino acid residues and is interrupted by 10 introns. The *fv-pda* cDNA was expressed in the yeast *Pichia pastoris*, and the resulting recombinant FV-PDA was used for enzymatic characterisation. The recombinant FV-PDA catalyses deacetylation of *N*-acetyl-chitooligomers, from dimer to pentamer, glycol chitin and colloidal chitin. The *fv-pda* was specifically expressed through the entire stage of fruiting body development, and the transcript was abundant in stipes of mature fruiting bodies. These results suggest that chitin deacetylase plays an important role in the process of fruiting of *F. velutipes*.

Introduction

Edible mushrooms (fruiting bodies of the basidiomycetes) are commercially cultivated worldwide. In commercial production of fruiting bodies, fruiting is usually induced by controlling various environmental factors that promote expression of regulatory gene(s), which in turn trigger a set of genes required for the initiation of fruiting. However, the precise mechanism of fruiting at the molecular level is still unclear; this explains why fruiting bodies can be artificially cultivated in only a limited number of basidiomycete species.

The basidiomycete *Flammulina velutipes* (called *Enokitake* in Japan) is commercially cultivated and fruited to produce foods with high nutritional value. Fruiting bodies of *F. velutipes* are induced and developed in a well-synchronized manner and are, therefore, ideal for studying temporal changes in gene expression during fruiting body development. Using this fungus, we isolated genes specifically expressed at the primordial (pin mushroom) stage by differential display (Yamada *et al.*, 2006). Analysis of these genes will help us to clarify the process of initiation and development of fruiting bodies at a molecular level and further improve the efficiency of artificial cultivation of fruiting bodies in *F. velutipes* as well as other mushrooms.

In this study, we focused on a polysaccharide deacetylase (PDA)-like gene (named 36b) (Yamada *et al.*, 2006) that was specifically expressed at the primordial stage but not in vegetative mycelia. The PDA family contains two major groups, peptidoglycan deacetylases and chitin deacetylases (Tsigos *et al.*, 2000), and their substrates; peptidoglycan and chitin are the major constituents of the cell walls of bacteria and fungi, respectively. Peptidoglycan deacetylase is associated with the formation of spore walls in *Bacillus subtilis* by deacetylation of *N*-acetyl-D-glucosamine (GlcNAc) moiety and a subsequent lactamisation (Fukushima *et al.*, 2002). In pathogenic strains of *Streptococcus pneumoniae*, peptidoglycan deacetylase is involved in the acquisition of a lysozyme-resistant phenotype that circumvents the defence system of host cells

(Vollmer & Tomasz, 2000).

In contrast to the bacterial peptidoglycan deacetylases, the physiological roles of fungal chitin deacetylases are still unclear. Chitin is a major component of fungal cell walls and is synthesized from a precursor, uridine-5-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc), by the action of chitin synthase. In a limited number of fungi (such as *Mucor* or *Absidia*), chitin synthesized in cell walls is immediately deacetylated to chitosan by chitin deacetylase (CDA, EC3.5.1.41). Although enzymatic characters of fungal CDA were reported from *Mucor rouxii* (Araki & Ito, 1974; Laurel & Bartnicki-Garcia, 1984; Kafetzopoulos *et al*., 1993), *Colletotrichum lindemuthianum* (Tsigos & Bouriotis, 1995; Tokuyasu *et al.*, 1996), *Absidia coerulea* (Gao *et al*., 1995) and *Cryptococcus neoformans* (Baker *et al.*, 2007), their physiological roles remain unclear. Therefore, it is of much interest to study *F. velutipes* CDA from the perspective of fruiting body formation, partly because the CDA related to fruiting body development has not been reported previously and thus might provide a new role for CDA. In this study, we describe an enzymatic character of the recombinant *F. velutipes* CDA expressed in the yeast *Pichia pastoris* and discuss its physiological role with regard to fruiting body formation.

Materials and Methods

Strains and vector

Escherichia coli JM109 and the plasmid vector pUC119 were used for construction of recombinant plasmids. Recombinant FV-PDA was expressed in host cells of *P. pastoris* X-33 using an expression vector pPICZαA (Invitrogen). A commercially cultivated *F. velutipes* dikaryotic strain, MH 092086, was used throughout this study. Culture conditions for fruiting were described in our previous article (Yamada *et al.*, 2005). Total RNAs were prepared from cells during fruiting body development or mature fruiting body tissues (pileus, stipe and root) using TRI reagents (Molecular

Research Center) according to the manufacturer's protocol.

Analysis of fv-pda gene

General manipulations of gene cloning and analysis were performed according to standard protocols (Sambrook & Russell, 2001). The original 36b cDNA fragment (250 bp in size) isolated by differential display (Yamada *et al.*, 2006) was used as a probe to obtain an entire genomic *fv-pda* fragment from the *F. velutipes* genomic library, constructed using the lambda DASH II phage vector. Nucleotide sequence was determined by a gene-walking method using an ABI PRISM 310 genetic analyzer (Applied Biosystems). The cDNA encompassing an entire open reading frame (ORF) was obtained by RT-PCR using total RNAs prepared from cells at a primordial stage. The primer set 36b CDAUP (5′-CAAACATTCGGCAACGGTCA-3′) was used, which was designed based on the sequence of 5′-untranscribed region (UTR) and oligo(dT)$_{15}$. By comparing genomic and cDNA sequences of *fv-pda*, the number and position of the introns were determined.

Semi-quantitative RT-PCR analysis

The amount of *fv-pda* transcript was determined by semi-quantitative RT-PCR for total RNAs prepared from cells at different stages. The first strand cDNA was synthesized from 200 ng of total RNA by an RNA PCR kit (Takara Bio) using an oligo(dT)$_{15}$ primer. PCR amplification was performed using a primer set of 36b CDAUP and 36b CDADW (5′-CTCTGCTTATCCCATCCACT-3′). The reaction mixture contained dNTP mix (0.2 mM each), primers (0.2 μM each), Ex-Taq polymerase (2.5 U) (Takara Bio) and the first strand cDNA. The reaction was performed for 40 cycles of denaturation at 95 °C for 60 s, annealing at 65 °C for 60 s and extension at 72 °C for 60 s. The cDNA fragment (450 bp) of glyceraldehyde-3-phosphate dehydrogenase (*gpd*) was also amplified as internal control for quantitative standardization since the

transcription level of this gene remains constant at any stage. The primers used for amplification of *F. velutipes gpd* were GPD-fwd (5′-GCNACNCARAARACIGTIGA-3′) and GPD-rev (5′-CCCCAYTCRTTRTCRTACCA-3′) designed based on the conserved sequences among fungal *gpd* genes.

Expression of *fv-pda* cDNA in *P. pastoris*

Fv-pda cDNA fragments corresponding to the whole ORF, but excluding a putative signal sequence, were amplified by PCR using a primer set of 36b DWPP (5′-GAATTCGCCCCGACCAATGAGAAA-3′) with an *Eco*RI recognition site (underlined) and fvpdaUP-PET (5′-TCTAGACATCTCCAGTCAGAAGTGGG-3′) with *Xba*I recognition site (underlined). The amplified fragment was double-digested with *Eco*RI and *Xba*I and then inserted into an expression vector pPICZαA previously digested with the same restriction endonucleases. The resulting recombinant plasmid pPICZα–*fv-pda* was linearized at a single *Sac*I site located in the vector and introduced into competent cells of *P. pastries* X-33 by electroporation using the GenePulser (Bio-rad) (field strength of 7.60 kV/cm, time constant of 8.44 ms). Zeocin-resistant transformant colonies were selected according to the protocol of the EasySelect Pichia Expression kit (Invitrogen).

Cells of *P. pastoris* M33 possessing pPICZα–*fv-pda* were pre-cultured in YPD medium (1% yeast extract, 2% polypepton, 2% glucose) at 30 °C for 16 h with shaking. The pre-culture was transferred into a BMMY medium (1% yeast extract, 2% polypepton, 1.34% yeast nitrogen base, 100 mM potassium phosphate buffer (pH 6.0), 0.00004% biotin and 0.5% methanol (v/v)) after adjusting the cell density of the initial culture (OD_{600} =1.0). The culture was incubated at 30 °C for 7 days with shaking at 200 rpm, and methanol was added at a final concentration of 0.5% at every 24 h to induce an expression of recombinant FV-PDA. After the cultivation, cells were removed by

centrifugation and ammonium sulfate was dissolved into the supernatant to yield 85% saturation. The resulting precipitate was collected by centrifugation (5,500 rpm, 15 min, 4 °C), dissolved in a small volume of 20 mM potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer. Recombinant FV-PDA was purified by affinity chromatography targeted on poly-histidine tag using a His-Trap kit (GE Healthcare).

Enzyme assay

Polysaccharide deacetylase activity was assayed by determining the amount of acetate liberated from substrate during the reaction using an F-kit (Roche). The reaction mixture (0.5 ml) contained 20 mM Tris-HCl buffer (pH 7.0), 5 mM substrates (adjusted by a concentration of *N*-acetyl groups of each substrate) and an appropriate amount of the enzyme. The mixture was incubated at 30 °C for 60 min and then immersed in boiling water for 5 min to arrest the reaction. A part of the mixture was used for identifying the acetate. The substrates used were GlcNAc and its oligomers, from dimer to pentamer (Seikagakukougyo). Colloidal chitin (Nacalaitesque) and glycol chitin were also used. Glycol chitin was prepared by chemical acetylation of glycol chitosan (Wako Pure Chemical Industries) according to a method by Shimahara & Takiguchi (1988). One unit (U) of the activity was defined as an amount of enzyme releasing 1 μmol of acetate per minute. To evaluate product inhibition of the enzyme, the reaction was performed using glycol chitin as the substrate with addition of various concentrations of acetate. In this case, the initial velocity of reaction was determined by measuring the amount of amino groups of glycol chitin according to the method of Kauss & Bauch (1988).

Results

Gene analysis of fv-pda

The 350-bp cDNA fragment (named 36b) whose nucleotide sequence showed a

significant homology to those of polysaccharide deacetylase was isolated as one of the representative genes specifically expressed at the primordial stage but not in vegetative mycelia (Yamada *et al.*, 2006). Using the 36b cDNA as a probe, we located the full-length *fv-pda* gene within the 2.9-kbp *Kpn*I–*Sal*I fragment in the genome of *F. velutipes* (Fig. 1(a)). Nucleotide sequence was determined for the 1.9-kbp DNA fragment that encompasses a putative *fv-pda* ORF. The cDNA corresponding to the whole ORF was also obtained by RT-PCR to determine the number and position of introns. The determined sequence has been deposited in DDBL/GenBank/EMBL database (accession number, AB250372).

The putative *fv-pda* ORF encoded a polypeptide comprising 250 amino acid residues whose molecular mass was 28 kDa (Fig. 1(b)). The ORF was interrupted by 10 introns, all of which had a consensus sequence of fungal introns (GTPuNGPy–PyAG) on both splicing sites (Breathnach & Chambon, 1981). Upstream of the putative start codon, a TATA box-like sequence (TATAAAA; from –70 to −64), CAAT box-like sequence (CCAATC; from –145 to −139) and GC box-like sequence (GGGCGG; from –325 to −316) were found. Downstream of the stop codon, a sequence similar to a polyadenylation signal motif (AATTGA; from 1513 to 1519) was located. An analysis of the N-terminal amino acid sequence using SignalP software (http://www.cbs.dtu.dk/services/SignalP/) estimated that the signal sequence comprised 19 amino acids with a high fidelity. A BlastP homology search using the deduced amino acid sequence as a query revealed significant degrees of similarity with those of polysaccharide deacetylases (PDA) belonging to family 4 carbohydrate esterase (Fig. 2). The highest similarity was found with PDA from *C. neoformans* (47% identity), which is known as a pathogen of cryptococcal meningoencephalitis (Biondo *et al.*, 2002).

Expression analysis of fv-pda by RT-PCR

We examined the transcript level of *fv-pda* during fruiting body development. First,

northern blot analysis was performed using total RNAs prepared from various growth stages; however, no significant signal was detected on any of the stages (data not shown). This result implies that the amount of *fv-pda* transcript was fairly low. Next, the amount of transcript was evaluated by RT-PCR as described in Materials and Methods. DNA fragments of expected size (750 bp) were amplified from cells at the primordial stage (10 days after fruiting body induction) and at mature fruiting body (16 days after fruiting body induction), but not from vegetative mycelia (Fig. 3(a)). To determine the localization of *fv-pda* transcript in tissues of the mature fruiting body, RT-PCR analysis was performed on total RNAs prepared from pileus, stipes and roots (Fig. 3(b)). The transcript could be detected from all tissues tested, but the level of the transcript was significantly higher in stipes than in pileus and roots.

Characterisation of recombinant FV-PDA expressed in P. pastoris

The *fv-pda* cDNA corresponding to the ORF by removing the N-terminal putative signal sequence was inserted into the expression vector pPICZα and introduced into cells of *P. pastoris* X-33. When the resulting transformant cells were grown in an induction medium with the addition of methanol, a polyhistidine-tagged recombinant protein was detected in the culture fluid. Contrary to our expectation, the yield of the recombinant FV-PDA was extremely low (1 mg of protein per litre of culture fluid). The purified recombinant FV-PDA had a molecular mass of 31 kDa, calculated from the mobility on SDS-PAGE, which is consistent with the molecular mass calculated from its deduced amino acid sequence (31,520 Da), indicating the absence of glycosylation in the recombinant protein (Fig. S1).

The recombinant FV-PDA was tested for its deacetylation activity using various substrates (Table 1). The enzyme reacted with GlcNAc oligomers, from dimer to pentamer but not with monomeric GlcNAc. The initial velocity of deacetylation increased with the increase in the number of polymerization (n) in the substrates,

(GlcNAc)n. Polymeric substrates, colloidal chitin and glycol chitin were also deacetylated. It should be noted that the activity was significantly higher towards soluble chitosan (degree of deacetylation, 70%). On the contrary, the enzyme did not react with peptidoglycan (prepared from *B. subtilis* cell wall). These results indicate that FV-PDA catalyzes reactions that release acetyl groups from chitinous substrates and should be denoted as chitin deacetylase.

The recombinant FV-PDA exhibited the maximum activity at 60 °C and pH 7, and the activity remained almost constant at alkaline pHs (Fig. 4). Addition of bivalent cations, Co^{2+}, $Ca^{2+}$and Zn^{2+}, increased the reaction velocity to 163%, 138% and 129%, respectively, while Cu^{2+} and Ni^{2+} significantly inhibited the velocity (<35%) (Fig. 5). Addition of EDTA (at a final concentration of 10 mM) eliminated the activity, but a simultaneous addition of Co^{2+} (1 mM) completely restored the activity. This clearly indicates that FV-PDA requires bivalent cations such as Co^{2+}, Ca^{2+} or Zn^{2+} for catalysis. The enzyme exhibited deacetylation activity towards glycol chitin even in the presence of 100 mM acetate, indicating the absence of product inhibition.

Discussion

In this study, we characterized the *fv-pda* gene that is especially expressed during fruiting body development in the basdiomycete *F. velutipes*, and determined that it encodes a chitin deacetylase, using a recombinant FV-PDA expressed in *P. pastoris*.

The amino acid sequence of the *fv-pda* ORF exhibited significant similarities (28–47% identity) to those of fungal PDAs available in the database, which could be categorized into family 4 carbohydrate esterase. Among the enzymes of this family, a three-dimensional structure was recently reported for the peptidoglycan *N*-acetylglucosamine deacetylase from *Staphylococcus pneumoniae* (Blair *et al.*, 2005). This 3D structure reveals a relative configuration of five conserved motifs comprising a catalytic center as well as related amino acid residues for binding to metal ions, a

characteristic that is indispensable for catalysis (Caufrier *et al.*, 2003). The *fv-pda* ORF similarly conserves five motifs, including Asp46 and His195 for catalytic center and Asp47, His101 and His105 for binding to metal ion (Fig. 2).

The recombinant FV-PDA catalysed deacetylation of chitin-related substrates and should be denoted as chitin deacetylase (CDA) (Table 1). Fungal CDAs have been characterized from *M. rouxii* (Araki & Ito, 1974; Laurel & Bartnicki-Garcia, 1984; Kafetzopoulos *et al.*, 1993), *Aspergillus nidulans* (Alfonso *et al.*, 1995) and *C. lindemuthianum* (Tsigos & Bouriotis, 1995; Tokuyasu *et al.*, 1996). In the case of *C. lindemuthanum* CDA, the protein is highly glycosylated such that two-thirds of its molecular weight (150 kDa) is occupied by carbohydrates (Tsigos & Bouriotis, 1995). *S. cerevisiae* CDA (Cda2p) is also a glycosylated protein, and the removal of a polysaccharide chain completely eliminates its activity (Martinou *et al.*, 2002). On the contrary, FV-PDA does not contain any consensus sequences for *N*-glycosylation in its ORF. The molecular mass of the recombinant FV-PDA suggests that it was not glycosylated, although the host *P. pastoris* often glycosylates recombinant proteins. While FV-PDA is likely to be a non-glycosylated protein, we could not rule out the possibility that it is *O*-glycosylated in *F. velutipes*. In most cases, PDAs require Co^{2+} ions for their catalytic function, as is the case with the recombinant FV-PDA (Fig. 5) (Martinou *et al.*, 2002; Shrestha *et al.*, 2004; Blair *et al.*, 2005). Fungal CDAs could be divided into two groups according to the criteria of product inhibition caused by acetate. *M. rouxii* and *A. coerulea* CDAs are significantly inhibited by acetate, while those from *A. nidulans* and *C. lindemuthianum* are not inhibited. The activity of FV-PDA was not inhibited by acetate at a concentration of 100 mM and can thus be grouped into the latter group. A variation of enzymatic characters among fungal CDAs may be ascribed to a difference in their physiological roles.

Two types of fungal CDAs have been reported from the perspective of cellular localisation. One is a periplasmic (cell wall-bound) CDA that was reported from *M.*

rouxii (Kafetzopoulos *et al.*, 1993), *A. coerulea* (Gao *et al.*, 1995) and *S. cerevisiae* (Christodoulidou *et al.*, 1996, 1999). The other is an extracellular CDA reported from *C. lindemuthianum* (Tsigos & Bouriotis, 1995) and *A. nidulans* (Alfonso *et al.*, 1995). Periplasmic CDAs from *M. rouxii* and *A. coerulea* are tightly coupled with a chitin synthase within cell walls and deacetylates newly synthesized chitin chains while they are premature and non-crystallized. A physiological significance of the conversion of chitin into its deacetylated derivative chitosan has not yet been clarified in these fungi. Another periplasmic CDA of *S. cerevisiae* deacetylates chitin in spore walls and is associated with integrity and stress resistance of spores (Mishra *et al.*, 1997). On the other hand, a plant pathogenic fungus, *C. lindemuthianum*, secretes CDA at the time of infection against the host plant. This secreted enzyme helps fungal cells acquire resistance to chitinases produced by plant host cells; it is one of the defense systems that result in the conversion of chitin to chitosan on the surface of fungal cell walls (Tsigos & Bouriotis, 1995). *A. nidulans* secretes CDA at the late stationary phase of culture, and this CDA was reported to correlate with autolysis (Alfonso *et al.*, 1995). In fungal cell walls, the removal of acetyl groups from chitin liberates free amino groups that are positively charged at physiological pH. These amino groups can be potentially modified by acylation (Lerouge *et al.*, 1990), sulfonation (Thunburg *et al.*, 1982) or formation of intramolecular lactam (Mitsuoka *et al.*, 1999; Warth & Strominger, 1972). These modifications are likely to alter physicochemical properties of cell walls. Further examination is required to clarify the physiological role of the deacetylation of chitin in the fungal cell walls.

Semi-quantitative RT-PCR analysis revealed that the *fv-pda* gene was specifically expressed during development of the fruiting body (Fig. 3(a)). This result suggests that FV-PDA would play an important role in the formation of cell walls of fruiting bodies through conversion of chitin to partially deacetylated chitosan. FV-PDA may be associated with the integrity of basidiospores (as in the case of ascospores in *S.*

cerevisae) (Mishra *et al.*, 1997), although this notion is contrary to our observation that *fv-pda* was expressed in all fruiting body tissues, including the stipe and root (Fig. 3(b)). Nonetheless, FV-PDA would alter the structure of the cell wall during the process of fruiting body formation. This alteration may be required for morphological changes during the process of fruiting such as agglutination of mycelia, flexibility of stipes or defense against microbial infection. We are currently trying to construct a gene knockdown mutant of *F. velutipes* in which *fv-pda* is suppressed by RNA interference. This should clarify the role of FV-PDA during the process of fruiting in *F. velutipes.*

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Figure legends

Fig. 1 (A) Restriction map of the genomic DNA region containing the *fv-pda* gene.

The entire nucleotide sequence of *fv-pda* was obtained by combining the determined sequences shown by arrows. Box represents the ORF with start and stop codons. Ten closed boxes indicate introns.

(B) Nucleotide and deduced amino acid sequence of *fv-pda*.

Numbers show the nucleotide number from the start codon (+1). Asterisk indicates a stop codon. Exons and introns are shown by upper case and lower case letters, respectively. An open triangle shows a cleavage site of a putative signal sequence. Putative TATA box (−64 to −70), CAAT box (−139 to −145) and GC box (−316 to −325) upstream of the start codon are *underlined*. A polyadenylation signal is *double-underlined*. The sequence has been deposited in the DDBJ database (accession number, AB250372).

Fig. 2 Comparison of deduced amino acid sequences among fungal polysaccharide deacetylases belonging to family 4 carbohydrate esterase.

Numbers show the amino acid residue number starting from the initial codon. The amino acids shared by four or more of the six aligned sequences are shown as black boxes. Five highly conserved motifs among family 4 carbohydrate esterases are underlined. Open triangles indicate putative catalytic residues and closed triangles indicate putative metal ion ligand residues. The sequences aligned here are from *F. velutipes* FV-PDA (this work, AB250372), *Cryptococcus neoformans* PDA (AJ414580), *Schizophyllum commune* CDA (AF271216), *Mucor rouxii* CDA (Z19109), *Saccharomyces cerevisiae* CDA (AY557948) and *Colletotrichum lindemuthianum* CDA (AY633657).

Fig. 3 Semi-quantitative RT-PCR analysis of *fv-pda* transcripts during fruiting body

development.

(A) RT-PCR products were amplified for 40 cycles from total RNAs prepared from cells of vegetative mycelium just before induction (M), 4 h (4H), 2 days (D2), 6 days (D6), 10 days (primordial, Pr) and 16 days (fruiting body, F) after induction. (B) Mature fruiting bodies were divided into three tissues: pileus (Pi), stipes (S) and root (R). RT-PCR products were amplified at 20–40 cycles from total RNAs prepared from each tissue. The transcripts of glyceraldehyde-3-phosphate dehydrogenase (*gpd*) were also amplified to confirm that an equal amount of total RNAs was used for each sample as a starting material.

Fig. 4 Effects of temperature (A) and pH (B) on enzyme activity of the recombinant FV-PDA.

The initial reaction velocity was measured at each condition using $(GlcNAc)_4$ (A) and glycol chitin (B) as substrates. Citrate buffer (●), Tris-HCl buffer (▲) and Na_2CO_3-NaOH buffer (■) were used at a final concentration of 20 mM.

Fig. 5 Effect of metal ions and chelator on enzyme activity of recombinant FV-PDA.

The relative activity was measured using $(GlcNAc)_4$ as a substrate with addition of various metal ions (1 mM) and EDTA (10 mM).

Supplementary figure legends

Fig. S1 Expression of *fv-pda* cDNA in the methylotrophic yeast *Pichia pastoris*.

SDS-PAGE (S) and Western blotting using an anti histidine-tag antibody (W) analysis of the recombinant FV-PDA purified by Ni affinity chromatography targeted for 6× histidine tag fused to the C-terminus of the recombinant protein. Prestained protein molecular weight size marker (M).

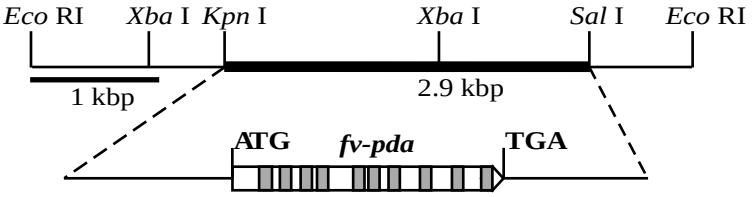
Table 1 Deacetylation activity of FV-PDA towards various substrates

Substrate	Released acetic acid (nmol)	Relative activity* (%)
GlcNAc	0	0
$(GlcNAc)_2$	4.95	6.6
$(GlcNAc)_3$	18.07	24.1
$(GlcNAc)_4$	38.88	51.7
$(GlcNAc)_5$	75.00	100.0
Glycol chitin	11.70	15.6
Colloidal chitin	13.39	17.9
Chitosan (DD† 70)	151.23	202.0
Peptidoglycan	0	0

*The amount of acetate liberated after a 60-min reaction compared with the reaction with $(GlcNAc)_5$.

†degree of deacetylation.

(A)



(B)

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 -70  tataaaaagacgcgatgcttacccgctcaatttcccagctcacgctctgcttatcccatccactattacc
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      M  L  F  S  A  I  A  L  S  V  L  A  C  V  A  T  V  E  A△A  P  T  N  E
  71  AGAAACGTCAACAGGCGCAGGTCATCTATGGCTGCACTCAACCCAACACCGTCGCCCTCACTTTCgtaag
        K  R  Q  Q  A  Q  V  I  Y  G  C  T  Q  P  N  T  V  A  L  T  F
 141  tggtcaacgtctatatcatgtcgtttgtttccggctgacatcacctccttcgtcattctttagGACGATG
                                                                  D  D  G
 211  GCCCATATTGGTATATgcaagtctttcccttgcgttcagctgtcgtcttgctgaaggtctgcgcacagAT
        P  Y  W  Y  I                                                    Y
 281  ACGACATTAGTAACGCCTTGACGTCCAGGGGTGCAAAGGGGACCTTCTTTTTCAgtgagtctttttaatc
        D  I  S  N  A  L  T  S  R  G  A  K  G  T  F  F  F  S
 351  ccgacttttggtcctccttgctaacgcagagcctgatttcgaaagGCGGAAACAACTgtatgagcgaccc
                                                    G  N  N  Y
 421  tcgacatgattcactccaggcaaactaacttccttccagATCAACGCATTTATGACCAGGGCGCCATTGA
                                                 Q  R  I  Y  D  Q  G  A  I  D
 491  CCAGATAAAATACGCGTACAACGCAGGACACCAAGTTGCGTCGCACACATGGGGGCATAAAGACCTTACA
       Q  I  K  Y  A  Y  N  A  G  H  Q  V  A  S  H  T  W  G  H  K  D  L  T
 561  TCCTTAACGTGGGATCAAAgtgggtggcatctcatttcagcgaaatccttgcgatgttcacatgtatgct
      S  L  T  W  D  Q  I
 631  gcagTTCACGATGAAATGTGGCGTGTGGAATgtatgtaccttctttctattgccactctattttgacgta
            H  D  E  M  W  R  V  E  L
 701  tgtcaacagTGGCTCTACAACGAATTGTCGGTGTTCAACCTGCGTTCATGCGCCCACgtaagctcatcca
                  A  L  Q  R  I  V  G  V  Q  P  A  F  M  R  P  P
 771  ttcatggtatccgcgaaacgtccatgctgaccacaatctatattagCTTACGGCAACTACGATGACGAAG
                                                       Y  G  N  Y  D  D  E  V
 841  TCCTTTACGCATCTTATATTCGCGGCCAGAAGGTCGTCATTTGGGACTTCGAgtaagccccttcctccc
       L  Y  A  S  Y  I  R  G  Q  K  V  V  I  W  D  F  D
 911  ctctttctccgctcctcaactgaccaattctacccccttagCTCTGGCGATTCCGTGGGAGCCTCCGCG
                                                    S  G  D  S  V  G  A  S  A
 981  GCTGAGAGCAAAGAACGCTACCGCACCACCTCCCTCAACCGCCCTTCAACACTCCTCGCTTTGAACCACG
      A  E  S  K  E  R  Y  R  T  T  S  L  N  R  P  S  T  L  L  A  L  N  H  E
1051  AAACCATCGgtaggctatttgatctcttgaggctcataggctgatgatatacgtgtacccatctagAATC
       T  I  E                                                          S
1121  TACCGCACATGATGTTATCCCGTACGCTATCGACCGCCTGCAGAGCGCTGGATACCGTCTGGTGACCGTT
       T  A  H  D  V  I  P  Y  A  I  D  R  L  Q  S  A  G  Y  R  L  V  T  V
1191  GCCGAATGTTTGGGCGAACAGCCCTACCAATGGACTGGGGCGCCACAGACTCCCACTgtatgtgttcggt
      A  E  C  L  G  E  Q  P  Y  Q  W  T  G  A  P  Q  T  P  T
1261  tctgcccccatcttatgcttttgacgagctaacctgatttgacagTCTGACTGGAGATGCTGAaatcttc
                                                    S  D  W  R  C  *
1331  acgccgaaagttctttcttcttcatggacgcattggatttccttcatctccgggacatttcacgaactct
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Fig. 1 Yamada *et al*.

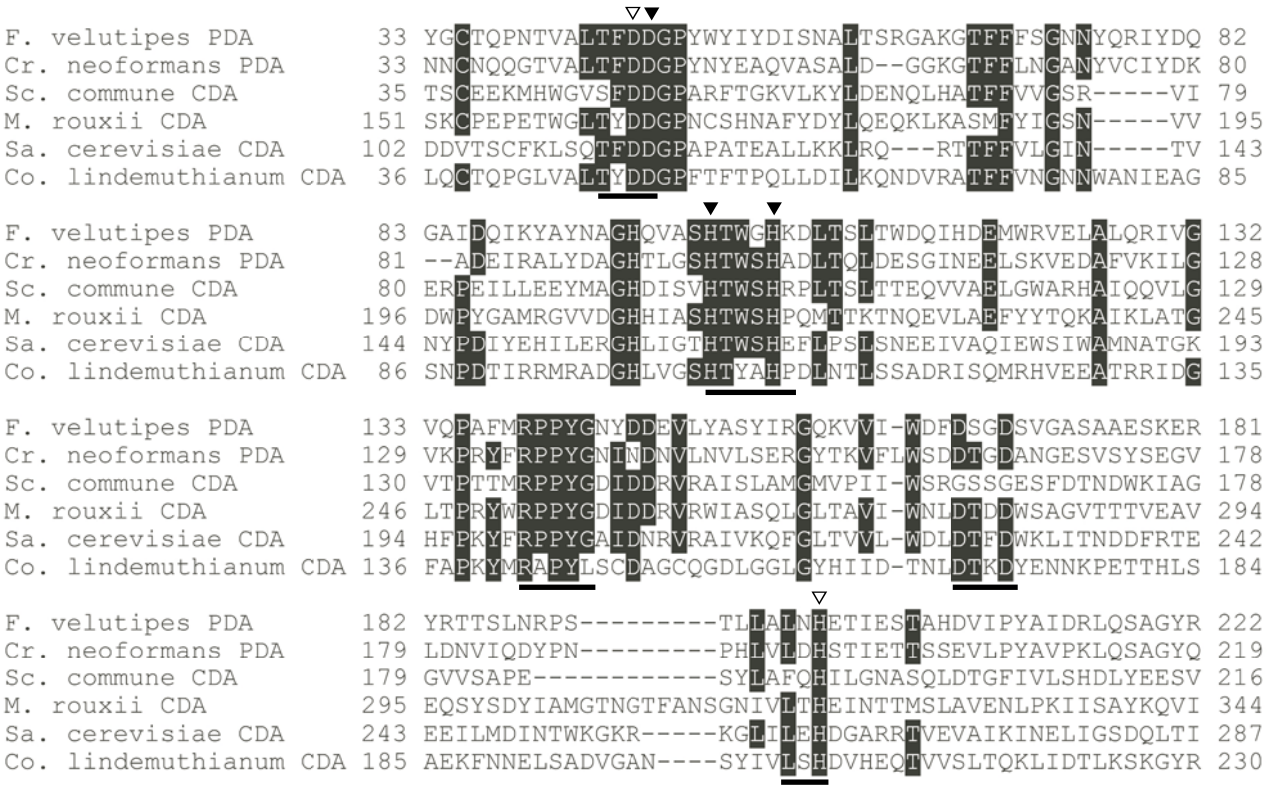

Fig. 2 Yamada *et al*.

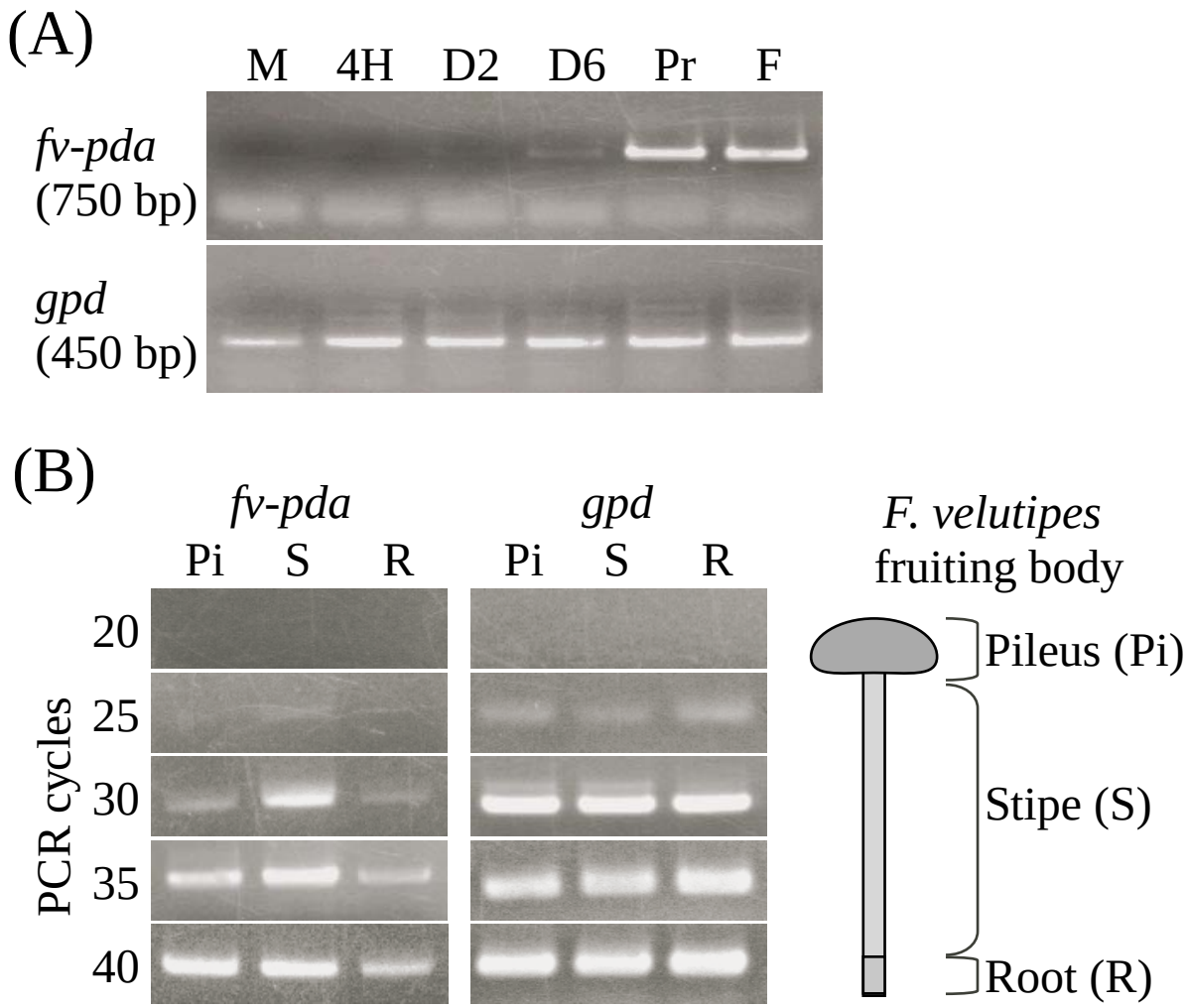

Fig. 3 Yamada *et al*.

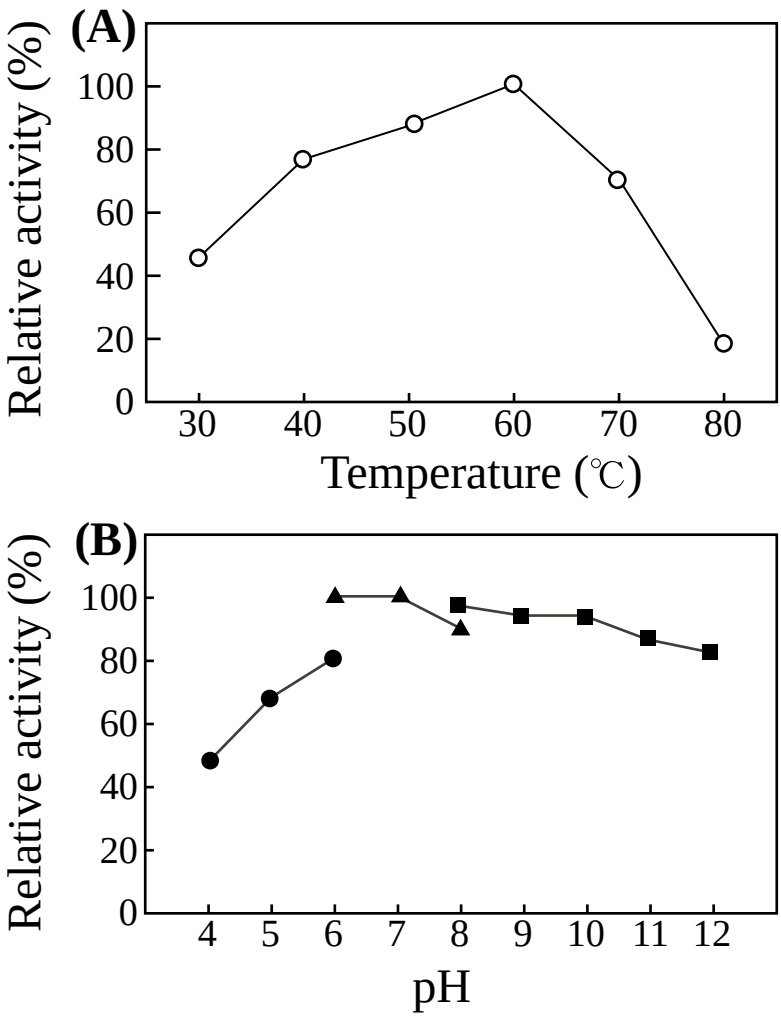

Fig. 4 Yamada *et al*.

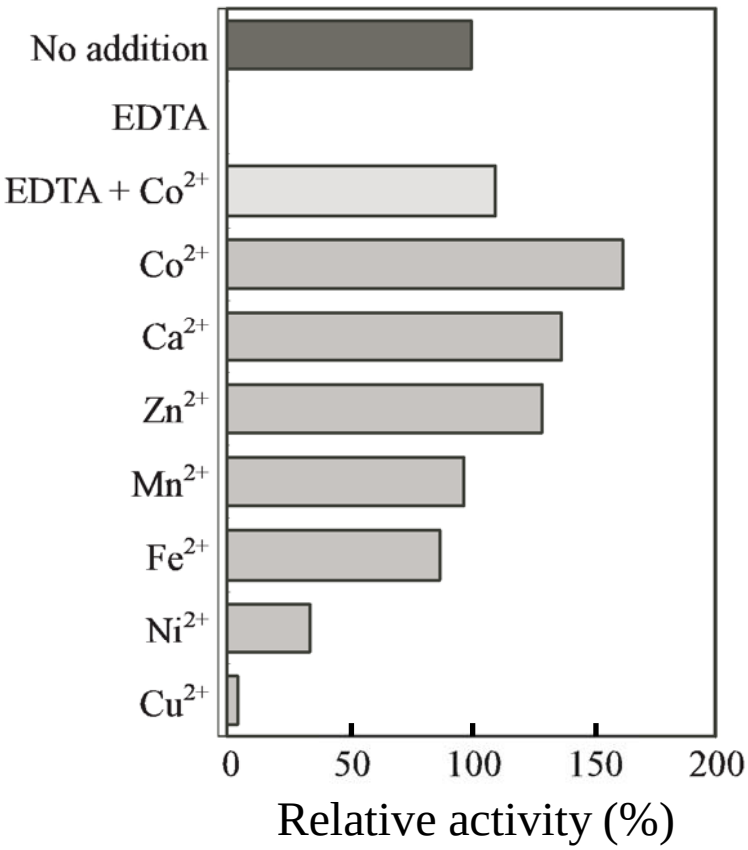

Fig. 5 Yamada *et al*.

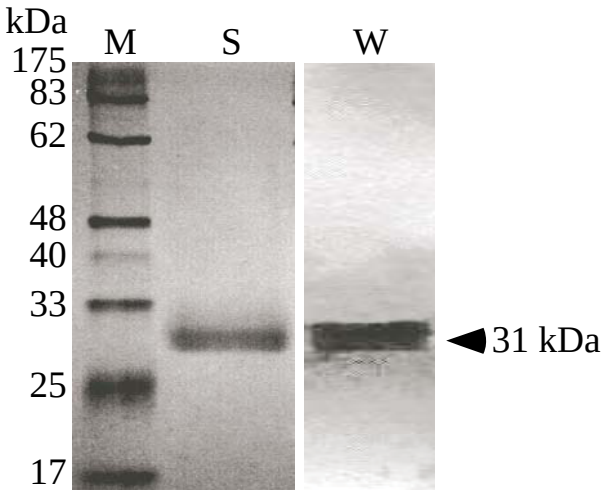

Fig. S1 Yamada *et al*.