J. Biochem. 2011;150(4):431–438 doi:10.1093/jb/mvr073

JB THE JOURNAL OF BIOCHEMISTRY

Comprehensive characterization of secreted aspartic proteases encoded by a virulence gene family in *Candida albicans*

Received March 12, 2011; accepted May 22, 2011; published online June 5, 2011

Wataru Aoki[1,2], Nao Kitahara[2], Natsuko Miura[1,2], Hironobu Morisaka[2], Yoshihiro Yamamoto[3], Kouichi Kuroda[2] and Mitsuyoshi Ueda[2,*]

[1]Research Fellow of the Japan Society for the Promotion of Science, Sakyo-ku, Kyoto 606-8502; [2]Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502; and [3]Industrial Technology Center, Kyoto Municipal Industrial Research Institute, Simogyo-ku, Kyoto 606-8813, Japan

*Mitsuyoshi Ueda, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan. Tel: +81-(0)75-753-6110, Fax: +81-(0)75-753-6112, email: miueda@kais.kyoto-u.ac.jp

***Candida albicans* is a commensal organism, but causes life-threatening infections in immunocompromised patients. Certain factors such as yeast-hyphae transition and hydrolytic enzymes are suggested as virulence attributes of *C. albicans*. Among them, 10 types of secreted aspartic protease (*SAP*) genes have received particular attention as a major virulence gene family. However, their full functional repertoire, including its biochemical properties, remains to be elucidated. Hence, we purified all Sap isozymes using *Pichia pastoris* and comprehensively determined and compared their biochemical properties. While optimum pH of Sap7 was 6.5 and that of Sap8 was 2.5, presence of other Sap isozymes functioning within a broad range of optimum pH could allow *C. albicans* to survive and cause infections in various tissues. The substrate specificities of Sap isozymes were analysed by using FRETS-25Xaa libraries. Sap7 and Sap10 showed high substrate specificity, while other Sap isozymes had broad substrate specificities. Principal component analysis revealed that the 10 Sap isozymes were clustered into 3 distinct groups in terms of their substrate specificities. Interestingly, Sap4–6, which are coproduced in the hyphal form, were clustered as the same group, indicating that they may target similar host proteins. These results will lead to further understanding of *C. albicans* pathogenicity.**

Keywords: *Candida albicans*/secreted aspartic proteases (SAP)/substrate specificity/virulence factor/yeast-hyphae transition.

Abbreviations: A2pr, 2,3-diaminopropionic acid; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; Dnp, 2,4-dinitrophenyl; GPI, glycosylphosphatidylinositol; Nma, 2-(*N*-methylamino)benzoyl; PBS, phosphate-buffered saline; PCA, principal component analysis; SAP, secreted aspartic protease.

Candida albicans usually exists as a commensal organism and can be detected in ~50% of the human population (*1*). However, if the host immunity is compromised by AIDS or cancer, or if the balance of the normal microflora is disrupted, *C. albicans* opportunistically causes recurrent superficial or systemic candidiasis. Superficial candidiasis is widely spread and three-quarters of healthy women experience a vaginal infection (*2*, *3*). Notably, the mortality rate of systemic candidiasis is very high because of the lack of effective diagnoses and treatment. Hence, there is a growing need for a clear elucidation of the virulence mechanisms of *C. albicans* and for the development of novel pharmaceutical agents.

One of the major virulence attributes of *C. albicans* is constituted by secreted aspartic proteases (SAPs), encoded by a family of 10 *SAP* genes (*1*, *4–6*). *SAP* gene products are known to contribute to virulence processes such as adhesion, invasion and immune evasion. Differential regulation of certain *SAP* genes has been reported (*7*), thus, they might play individual roles at certain stages of the infection. Of the 10 *SAP* genes, expression of *SAP1* to *SAP3* has been observed in the yeast form. Among these, *SAP2* has been found to play a major role and is necessary for *in vitro* growth when BSA is the sole carbon source (*8*). *SAP4* to *SAP6* have been reported to be exclusively expressed in the hyphal form and have been related to systemic infections and the evasion from the host immune system (*9*). *SAP9*- and *SAP10*-encoding proteins, with a glycosylphosphatidylinositol-anchoring (GPI-anchoring) domain, are considered to maintain the cell wall integrity through the post-translational processing of cell wall proteins. *C. albicans* mutants lacking *SAP9* or *SAP10* have a reduced ability to adhere to epithelial cells and show attenuated pathogenicity during interaction with oral epithelial tissues (*10*).

It is known that Sap isozymes contribute to the pathogenicity of *C. albicans* (*1*), but the fundamental mechanisms contributing to their pathogenicity, such as the multiplicity of *SAP* genes and the physiological targets of Sap isozymes, remain to be elucidated. Clarifying the biochemical properties, especially substrate specificity of Sap isozymes, will lead to the answers of these questions. A few articles have reported substrate specificities of Sap isozymes. By using defined mixtures of peptide substrates, Koelsch *et al.* (*11*) reported that Sap1, 2, 3 and 6 cleaved peptide bonds between larger hydrophobic amino acids. Albrecht *et al.* (*10*) reported that Sap9 and Sap10 could cleave some synthetic peptides that contain basic amino acid motifs. However, more comprehensive analyses of the substrate specificities and physiological roles of all 10

Sap isozymes are indispensable for a clear understanding of the pathogenicity of *C. albicans*.

In this study, we succeeded in purifying all 10 Sap isozymes and elucidating comprehensive substrate specificities of the pathogenic proteins. The results will provide an insight into the fundamental pathogenic processes of *C. albicans*.

Materials and Methods

Strains and media

Escherichia coli strain DH5α [F^-, *ΔlacU169 (φ80lacZΔM15)*, *hsdR17* (r_K^-, m_K^+), *recA1*, *endA1*, *deoR*, *thi-1*, *supE44*, *gyrA96*, *relA1*, λ^-] (TOYOBO, Osaka, Japan) was used as a host for DNA manipulation. *C. albicans* strain SC5314 (American Type Culture Collection) was used for isolation of the *Candida* genome. *Pichia pastoris* strain GS115 (*his4*) (Invitrogen, CA, USA) was used as a host for protein production. *E. coli* transformants were grown in Luria-Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride] containing 50 μg/ml ampicillin. For protein production, *P. pastoris* transformants were pre-cultivated in buffered glycerol-complex medium (BMGY) [1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base w/o amino acids, 4×10^{-5}% (w/v) biotin and 1% (v/v) glycerol, 100 mM potassium phosphate (pH 6.0)]. Pre-cultivated transformants were grown in buffered methanol-complex medium (BMMY) [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base w/o amino acids, 4×10^{-5}% (w/v) biotin and 0.5% (v/v) methanol] for transcriptional induction.

Construction of expression plasmids

All the primers used in this study are presented in Supplementary Table SI. All reverse primers used for cloning *SAP* genes had a FLAG tag-encoding sequence (DYKDDDDK) at their 3′-terminal. DNA fragments that encoded the *SAP* genes were cloned from the genomic DNA extracted from *C. albicans* SC5314 by using the GenTLE High Recovery Kit (TAKARA Bio, Shiga, Japan) and were inserted into the pHIL-S1 plasmid (Invitrogen). The resulting recombinant genes were composed of the *PHO1* secretion signal sequence and each *SAP* gene. The sequences of the *SAP* genes were optimized for *P. pastoris* translation by using the QuikChange multi site-directed mutagenesis kit (Stratagene, CA, USA). Because *C. albicans* displays alternative CUG codon usage (Ser for Leu) (*12*), CUG codons in the 10 *SAP* genes were replaced by UCG codons, which encode Ser in *P. pastoris*. The resulting plasmids were named pHIL-Sap1 to pHIL-Sap10.

Production and purification of FLAG-tagged Sap isozymes

pHIL-Sap1 to pHIL-Sap10, and pHIL-S1, a control plasmid, were digested with the restriction enzyme *Sac*I. *P. pastoris* GS115 cells were transformed with the linear plasmids by using the Frozen-EZ Yeast Transformation II kit (Zymo Research, CA, USA). The *P. pastoris* transformant, which showed a Mut^+ phenotype, was grown in BMGY medium for 48 h at 30°C. The culture medium was centrifuged at 3000*g* for 5 min, and the cells were resuspended in BMMY medium for transcriptional induction, and were then grown for 24 h at 30°C. The supernatant of the culture medium was concentrated by using YM-10 (Millipore, MA, USA). Then, the supernatant was mixed with an anti-FLAG M2 affinity gel (Sigma-Aldrich, MO, USA) and rotated for 1 h at 4°C. The gel was washed with phosphate-buffered saline (PBS) (pH 7.4) to remove non-specific proteins. FLAG-tagged Sap isozyme was eluted from the gel using a 3× FLAG peptide (Sigma-Aldrich). The protein concentration was quantified by Protein Assay Bicinchoninate Kit (Nacalai tesque, Kyoto, Japan). The purified Sap isozyme was separated by SDS–PAGE with or without EndoH treatment. The gel was stained with Coomassie brilliant blue. The proteins in the bands were identified by a Voyager RP MALDI-TOF/MS (Applied Biosystems, CA, USA) and the homogeneity of Sap isozymes was confirmed. Amino acid sequencing of purified Sap isozymes was carried out by the Edman degradation method on a protein sequence system PPSQ-33A (Shimadzu, Kyoto, Japan), using a Hybond-P membrane (GE Healthcare, Buckinghamshire, UK).

Measurement of proteolytic activity and determination of optimum pH

The proteolytic activity of Sap isozymes was measured by using resorufin-labelled casein (Roche, Basel, Switzerland). Sap isozymes (final concentration, 5 nM) were mixed with 125 μl sodium citrate buffer (50 mM; pH 2.0–7.5) and the solutions were incubated at 37°C. pH values of reaction samples were measured and ensured of maintaining pH at designated values. For inhibitory assays, pepstatin A was mixed with the reaction solution (final concentration, 1 μM). After the reaction was complete, undigested resorufin-labelled casein was precipitated with 10% trichloroacetic acid and the fluorescence of the supernatant was measured at $\lambda_{ex} = 540$ nm and $\lambda_{em} = 612$ nm after neutralization with 25 μl of 4 M NaOH. For all types of Sap isozyme including Sap7 and Sap10, the FRETS-25Ala library (Peptide Institute, Osaka, Japan) was used as a substrate. Because we could not determine the specific activities of Sap7 and Sap10 using resorufin-labelled casein. The peptide library (final concentration, 10 μM) was mixed with each Sap isozyme (final concentration, 5 nM) in 200 μl sodium citrate buffer (50 mM; pH 2.0–7.5) at 37°C. The increase in fluorescence was kinetically measured at $\lambda_{ex} = 355$ nm and $\lambda_{em} = 460$ nm. The pH sensitivity of the fluorescence was calibrated by using standard curves at each pH. One unit of proteolytic activity was defined as that increasing the fluorescence by 1 min^{-1} at optimum pH.

Determination of substrate specificity of Sap isozymes

FRETS-25Xaa libraries (Peptide Institute) (Fig. 1) were used to determine substrate specificity of Sap isozymes, as described in a previous report (*13*). In brief, the library contains 475 peptides, separated into 19 sublibraries. The peptides in each sublibrary have a specific amino acid at the Xaa position as shown in Fig. 1. The 19 natural amino acids, except for Cys, were incorporated at the Xaa position because Cys promotes the aggregation of peptides. FRETS-25Xaa libraries were dissolved in DMSO at 2 mM concentration and prepared by 200-fold dilution with 50 mM sodium citrate buffer at optimum pH for determination of substrate specificity. Purified Sap isozymes (final concentration, 5 nM) were mixed with each FRETS-25Xaa sublibrary (final concentration, 10 μM) in a total volume of 200 μl sodium citrate buffer and the fluorescence increase at $\lambda_{ex} = 355$ and $\lambda_{em} = 460$ nm was kinetically measured under substrate-saturation conditions. Relative degradation rate was calculated by the following equation: Relative degradation rate = [(Degradation rate of each sublibrary)/(Average degradation rate of sublibraries)] − 1.

Principal component analysis of substrate specificity of Sap isozymes

To study the similarity between substrate specificities of Sap isozymes, the data were entered into a matrix, with each row representing the relative degradation rate and each column representing an isozyme. The data set was then imported into SIMCA P^+ (Umetrics Inc., Umeå, Sweden), which is generally used for a clustering analysis and the first three principal components were used for a score plot and a dendrogram representation (*14*).

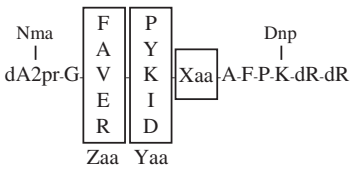

Fig. 1 Diagrammatic representation of the FRETS-25Xaa library. The library contains 475 peptides, separated into 19 sublibraries. Each sublibrary has a specific amino acid at Xaa position, which represents the 19 natural amino acids, except Cys. A mixture of 5 amino acids each (P, Y, K, I, D or F, A, V, E, R) was incorporated at the Yaa and Zaa position, respectively. dA2pr: D-2,3-diaminopropionic acid, dR: D-isoform of arginine.

Results

Expression of Sap-encoding genes and purification of the 10 Sap isozymes

DNA fragments encoding the *SAP* genes were cloned from the *C. albicans* SC5314 genome. Specifically, the DNA fragments encoding *SAP9* and *SAP10* were amplified as C-terminally truncated forms for the efficient secretion, because these genes encode GPI-anchored proteins (*10*). The deleted signal sequences for GPI-anchoring are very short and might not influence proper folding of Sap9 and Sap10. These DNA fragments were inserted into pHIL-S1, a vector for the secretory protein production by *P. pastoris*. *P. pastoris* was transformed with the plasmids, and cultivated in BMMY medium for transcriptional induction. The *P. pastoris* cells successfully produced all types of FLAG-tagged Sap isozyme, and the recombinant Sap isozymes were purified using an anti-FLAG affinity gel. As judged by SDS–PAGE, single (Sap1, 3, 5, 6, 10) or multiple (Sap2, 4, 7, 8, 9) distinct protein bands were observed as purified proteins, while no band was detected in the control (pHIL-S1) extract (Fig. 2). The result that purified Sap2 and Sap9 were separated into two bands on SDS–PAGE, was consistent with those from previous reports (*9*, *10*). MALDI-TOF/MS analysis revealed that the protein bands in each lane were derived from individual Sap isozymes (data not shown). Taken together, these results showed that Sap isozymes were purified without any contamination. Furthermore, the N-terminal sequence analysis of the Sap isozymes (Supplementary Table SII) (*8*, *15*, *16*) indicated that the partial processing of the Sap2 and Sap4 translation products had occurred, and that the Sap7 and Sap9 mature products were separated into two distinct subunits (*15*). In addition, previous observations showed that some Sap isozymes could be *N*-glycosylated (*1*, *4*, *17*, *18*). Consistent with these reports, treatment with EndoH, which cleaves oligosaccharides from *N*-linked glycoproteins, showed that Sap4, 6, 7, 8, 9 and 10 were *N*-glycosylated (Fig. 2). This result suggests for the first time that 6 out of 10 isozymes may be *N*-glycosylated proteins in *C. albicans*. Sap7, in particular, is a highly heterogeneous *N*-glycosylated protease, as revealed by the EndoH treatment, consisted of two subunits, shifting the smear band of Sap7 to a single 15-kDa band. The sum of apparent molecular weights of the 2 Sap7 subunits (67 kDa) was considerably larger than the theoretical molecular weight of Sap7 (47 kDa) (Table I). This result suggests that Sap7 occurs in glycosylated forms other than *N*-glycosylation.

Measurement of proteolytic activity

The proteolytic activity of purified Sap isozyme was quantified by using resorufin-labelled casein (for Sap1–6, 8, 9) or FRETS-25Ala library (for all types of Sap isozyme including Sap7 and Sap10) as substrates. All of the purified Sap isozymes exhibited proteolytic activities, while the control extract showed no proteolytic activity. This result indicates that the

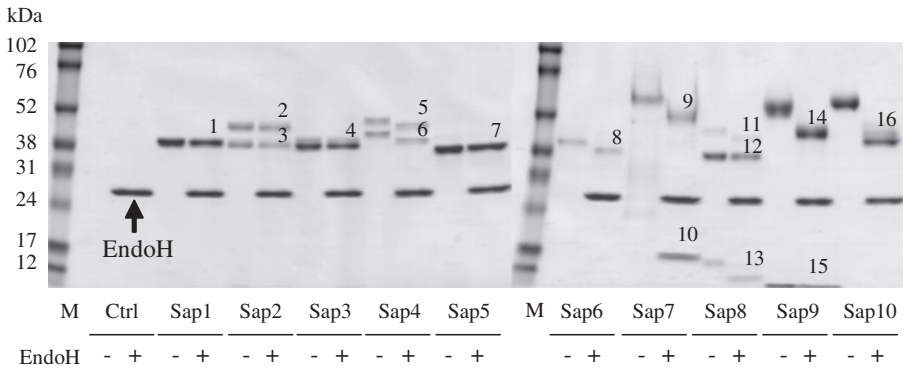

Fig. 2 SDS–PAGE analysis of Sap isozymes. In the lanes of Sap2, 4, 7, 8 and 9, we observed 2 or 3 distinct bands, which indicated partial processings or internal cleavage of the translation products. EndoH treatment (+) caused a band shift for Sap 4, 6, 7, 8, 9 and 10, suggesting that these Sap isozymes are *N*-glycosylated. The protein band of EndoH is marked with an arrow. All the bands, numbered from 1 to 16, were analysed by N-terminal sequencing. M: marker lane, Ctrl: control lane.

Table I. Biochemical properties of Sap isozymes.

Isozyme	Sap1	Sap2	Sap3	Sap4	Sap5	Sap6	Sap7	Sap8	Sap9	Sap10
Theoretical kDa	36	36	37	37	37	37	47	35	53	45
Apparent kDa	39	38	37	39	36	38	52, 15	36	44, 11	42
N-linked glycosylation	−	−	−	+	−	+	+	+	+	+
Optimum pH	5.0	4.0	3.0	5.0	5.0	5.0	6.5	2.5	5.5	6.0
Specific activity (Casein, U/μg)	260	82	180	120	140	1100	ND	240	21	ND
Specific activity (FRETS-25Ala, U/μg)	6.0	5.1	3.9	1.3	0.66	3.6	0.048	0.69	1.3	0.071
Pepstatin A sensitivity	+	+	+	+	+	+	−	+	+	+

Theoretical kDa of mature Sap isozymes was calculated by the ExPASy Compute pI/Mw tool. Apparent kDa of mature Sap isozymes was calculated by the mobility of the bands with the EndoH treatment in SDS–PAGE. One unit of proteolytic activity was defined as that increasing the fluorescence by 1 min^{-1} at optimum pH using casein-resorufin labelled or the FRETS-25Ala library. ND, not detected.

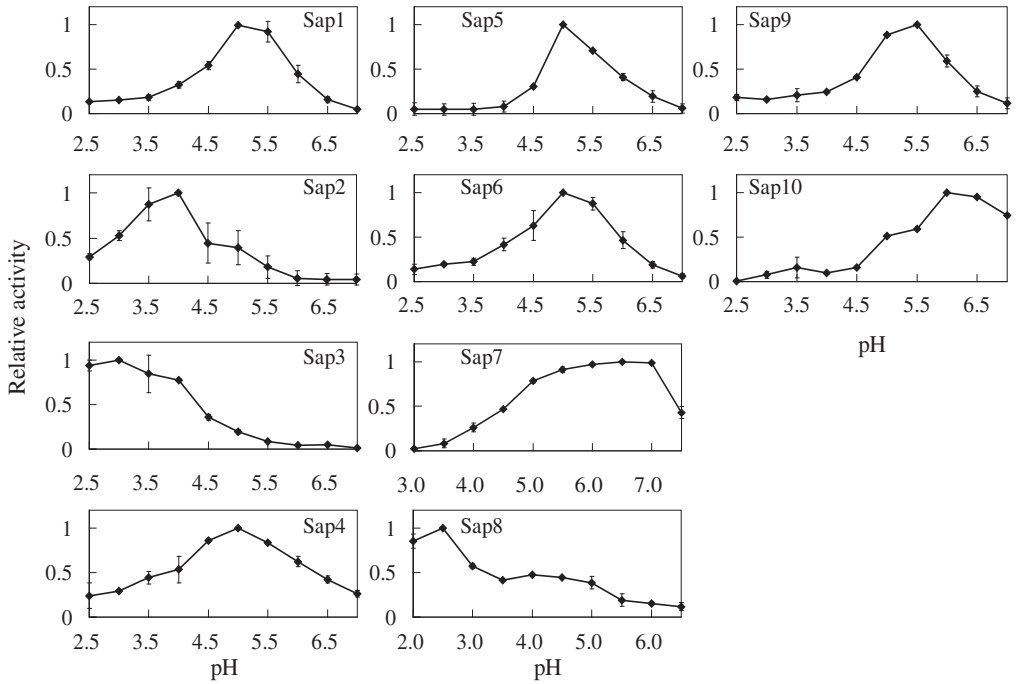

Fig. 3 pH dependence of proteolytic activity of Sap isozymes. Relative activities are presented as ratios of the activity at the optimum pH. The values represent the average of the data obtained from at least three independent experiments. The error bars indicate standard deviation.

proteolytic activities were not derived from contamination of *P. pastoris* proteases. A majority of the Sap isozymes were inhibited by pepstatin A, a classical aspartic protease inhibitor, as reported in previous studies (*9*, *11*). However, Sap7 was not inhibited by pepstatin A and any other protease inhibitors, such as leupeptin, phenylmethylsulfonyl fluoride and EDTA (data not shown). Sap7 is the most divergent isozyme (*5*); hence, it may have catalytic center different from those in other Saps. Together with a previous report showing a low inhibition potential of pepstatin A against Sap9 and Sap10 (*19*), pepstatin A might not be a good reagent to address the relationship between Saps and pathogenesis. The optimum pH of the proteolytic activity of Sap isozymes was determined by using 50 mM citrate buffer between pH 2.0 and 7.5 (Fig. 3). Most of the Sap isozymes displayed optimum proteolytic activities at pH 3.0–5.0 (*9*, *11*, *20*). Interestingly, the proteolytic activities of Sap7 and Sap8 were optimum at an aberrant pH of 6.5 and 2.5, respectively. The biochemical characteristics of Sap isozymes are summarized in Table I.

Determination of substrate specificities

FRETS-25Xaa libraries were used to determine the substrate specificities of Sap isozymes, and to analyze the similarities and the differences among them. ESI-TOF/MS analysis showed that almost all peptides were cleaved between Xaa and Ala. The relative degradation rate for each library is shown in Fig. 4. As described in the experimental procedures, the substrate library with an average degradation rate is scored as zero. In addition, a higher relative degradation rate indicated an easier cleavage of the substrate library by Sap isozymes than other sublibraries. As a result, Sap1–6 and Sap8 cleaved almost all the libraries close to the average degradation rate, indicating their broad substrate specificities. However, they relatively preferred positively charged or large hydrophobic amino acids such as Arg, Lys, Leu, Phe and Tyr at the Xaa position, as partially shown in a previous report for Sap1–3 and Sap6 (*11*). In contrast, Sap7, 9 and 10 had very high substrate specificities and preferred Met, Arg and His, respectively. Consistent with previous report, these Sap isozymes also preferred Arg and Lys at the Xaa position (*19*).

Principal component analysis

Principal component analysis (PCA) was used to compare the similarities and the differences between Sap isozymes and to categorize Sap isozymes according to their substrate specificities (Fig. 5A and B). In the score plot and the dendrogram representation of PCA, Sap isozymes that have similar substrate specificities are clustered in close proximity. In contrast, Sap isozymes with different substrate specificities are clustered at a considerable distance. As a result, Sap7 and Sap10 were plotted far from other Sap isozymes and designated as Group 1. This view supports the result that Sap7 and 10 had high substrate specificities, which were quite different from other Sap isozymes. In addition, Sap4–6 were categorized as the same group (Group 2), and Sap1–3 and Sap8–9 were together categorized as Group3.

Discussion

Secreted aspartic proteases (Sap1–10) of *C. albicans* are important virulence attributes and many studies have proven the relationship between *SAP* gene

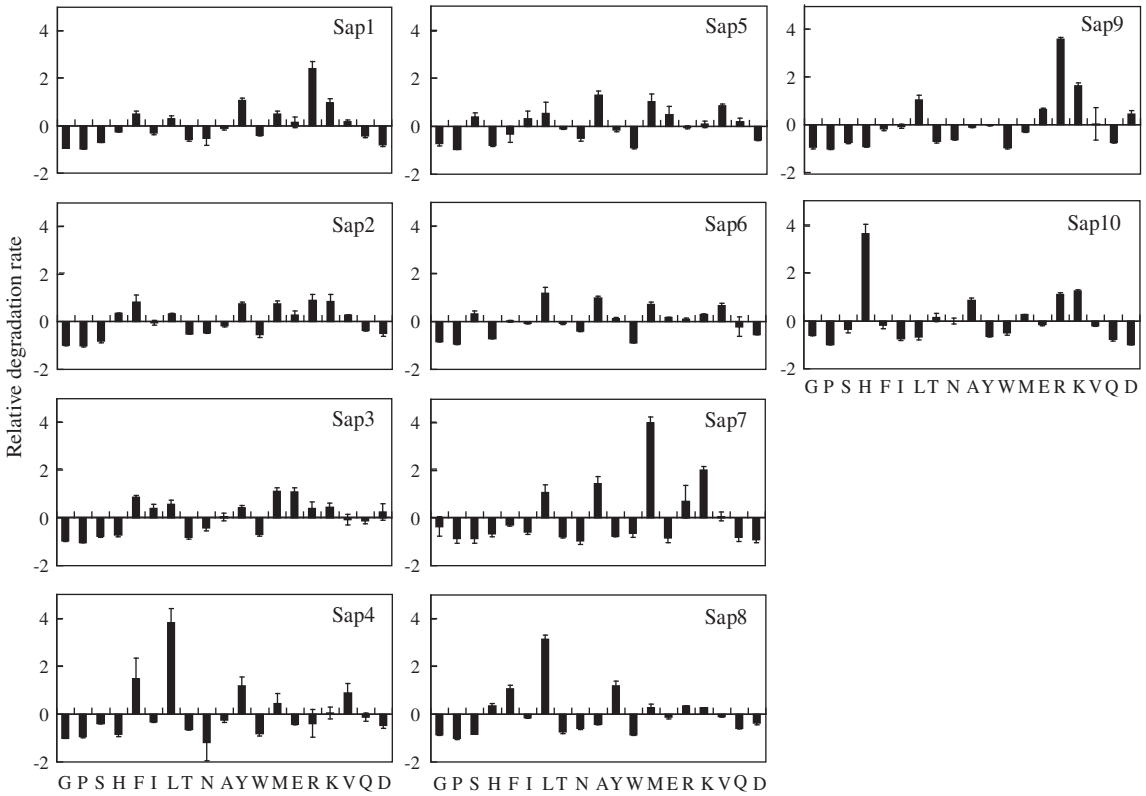

Fig. 4 Substrate specificities of Sap isozymes. The horizontal axis indicates individual FRETS sublibraries and the vertical axis indicates relative degradation rate calculated by the equation provided in the experimental procedures. The average degradation rate was scored as zero. In addition, sublibraries that exhibited a negative degradation rate were more difficult to be cleaved; those with positive values were cleaved more easily. The values represent the average of the data obtained from at least three independent experiments. The error bars indicate standard deviation.

expression and the pathogenicity of *C. albicans*. Only Sap1 to Sap3, but not other Sap isozymes, were efficiently produced by *C. albicans in vitro* (*8*, *9*, *20*); hence, it is difficult to study the characteristics of Sap isozymes using *C. albicans* cells because sufficient amounts of Sap isozymes cannot be obtained for enzymatic experiments. In this study, we used the *P. pastoris* expression system and FLAG-tag affinity purification for the rapid and efficient production of recombinant Sap isozymes. We used *P. pastoris* to produce Sap proteins due to several reasons. *P. pastoris* has a strong, inducible promoter and we can obtain large amount of heterologous proteins for determining various enzymatic parameters. *P. pastoris* is a eukaryote and has chaperone proteins required for proper folding. This is the first successful approach using the system to produce all the 10 types of Sap isozyme.

We used fluorescent substrates to determine optimum pH of the proteolytic activity of Sap isozymes. Majority of the Sap isozymes (Sap1–6) showed highest proteolytic activity at pH 3.0–5.0, consistent with previous reports (*8*, *9*, *19–21*), which are typical for aspartic proteases. In contrast, Sap7 and Sap8 showed characteristic properties. Surprisingly, Sap7 showed optimal activity at neutral pH, which is an unusual pH for aspartic protease activity, and the inhibition of proteolytic activity by pepstatin A was extremely low. These properties are exceptional for aspartic proteases, although some examples are known as following. Renin, an aspartic protease, also known as angiotensinogenase, exhibits optimal activity at neutral pH (*22*). Aspergillopepsin II from *Aspergillus niger* has unconventional catalytic residues (Glu and Asp) and is not inhibited by pepstatin A (*23*). Further investigations are required to clarify the catalytic mechanism of Sap7. In addition, Sap8 showed the highest proteolytic activity at pH 2.5, which was the lowest among all Sap isozymes. Although the virulence mechanism of Sap8 is unknown, it may be contributing to the viability of *C. albicans* at very low pH. We clarified that Sap isozymes display a broad range of optimum pH, as depicted in Table I. This property could allow *C. albicans* to survive and cause infections in various tissues.

Substrate specificities of all 10 Sap isozymes were comprehensively determined for the first time by using FRETS-25Xaa libraries. For comprehensive profiling of substrate specificity, these libraries are superior to conventional methods that utilize separate peptide substrates. As a result, Sap1–6, and Sap8 showed relatively broad substrate specificities against the FRETS sublibraries. These broad substrate specificities provide not only essential nitrogen source, but also a barrier against the host immune defense, which supports previous observations that Sap2 can degrade many host proteins *in vitro*, such as mucin (*24*), IgG (*25*), IgA (*26*), complements (C3b, C4b and C5) (*27*) and albumin (*1*). It is particularly noteworthy that Sap4–6, which are hyphae-specific proteases, were

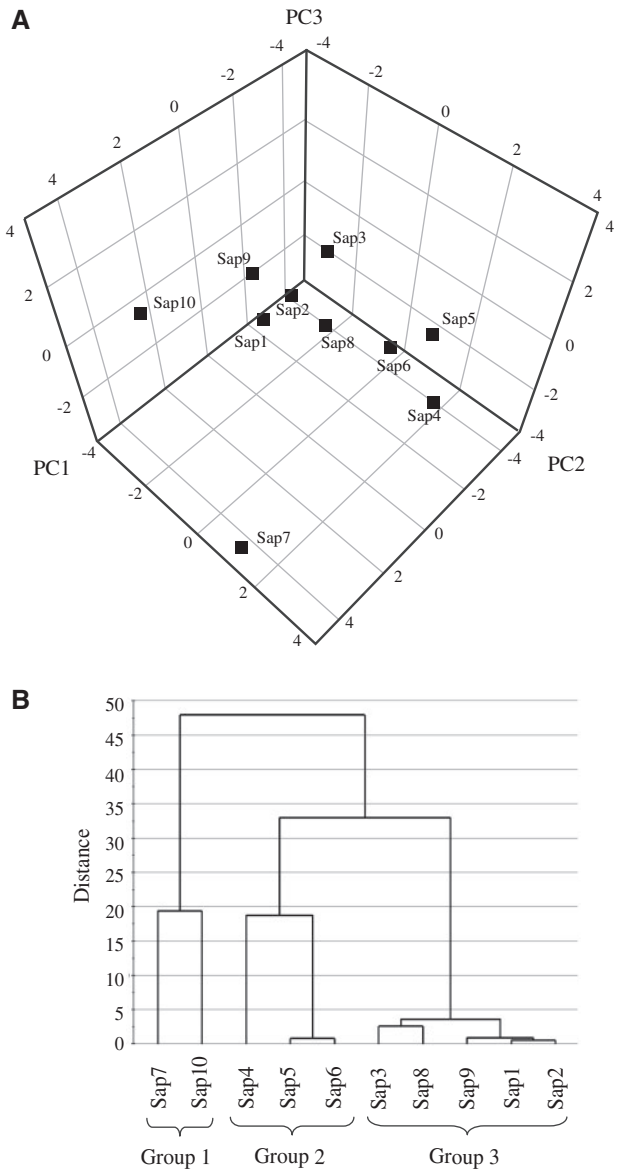

Fig. 5 Principal component analysis of substrate specificities of Sap isozymes. (A) Score plot representation of the PCA analysis. SIMCA P^+ was used to study the similarity between substrate specificities of Sap isozymes. Three axes of the score plots (PC1–PC3) represent the first, second and third principal component, respectively. Each symbol represents one of the Sap isozymes. Sap isozymes with similar substrate specificities are clustered in close proximity. (B) Dendrogram representation of the PCA analysis. The height is proportional to the distance between the clusters in the score plot. The distance was calculated by using SIMCA P^+ software. Sap7 and Sap10 were designated as Group 1 far from other Sap isozymes. This view supports the result that Sap7 and 10 had high substrate specificities, which were quite different from other Sap isozymes.

clustered in the same group by PCA. We do not know the reason why *C. albicans* produces the proteases with highly similar substrate specificities and similar optimum pH ranges at the same time. They may have a similar series of target proteins. Sap4–6 appeared to be involved in systemic infections and in resistance against phagocytic attacks (*9*, *28*, *29*). Thus, identification of the physiological targets of Sap4–6 in phagocytic cells will be important to clarify the roles of Sap4–6 in pathogenesis.

Sap7–10 have received less experimental and clinical attention to date than Sap1–6. Through this study, we performed quantitative and comprehensive enzymatic studies of substrate specificities of Sap4–5 and Sap7–8 for the first time. Although Sap7 mRNA expression was detected in asymptomatic *Candida* carriers and vaginal and oral candidiasis patients, but not reproduced under laboratory growth conditions (*30–32*). Particularly in the intravenous model of systemic candidiasis, the *SAP7* null mutant exhibited an attenuated virulence, while the *SAP7* null mutant showed only a slight difference in organ fungal burdens (*33*). Together with the result that the proteolytic activity of Sap7 was optimal at neutral pH, and that it had high substrate specificity, it is possible that Sap7 degrade distinct host proteins in multiple organs before *C. albicans* cells acidify the ambient pH.

Sap9 and Sap10 have structural similarities to yapsins of *Saccharomyces cerevisiae* and may function to maintain the cell surface integrity by the processing of cell wall proteins (*6*, *34*). However, Sap9 and Sap10 may have other physiological roles besides the processing of cell wall proteins. For instance, Meiller *et al.* (*35*) showed that Sap9 was involved in the proteolysis of histatin-5, a salivary antimicrobial peptide. Antimicrobial peptides are found in many species and have important roles in the host innate immunity. Almost all antimicrobial peptides are cationic and hydrophobic in nature and exert their activity by disrupting negative-charged membranes (*36–38*). Using FRETS-25Xaa, we revealed that Sap9 preferred Arg predominantly, and Lys and Leu relatively (basic and hydrophobic amino acids). This substrate specificity of Sap9 could enable *C. albicans* cells to efficiently degrade antimicrobial peptides and resist host immunity. In contrast to Sap9, Sap10 had characteristic substrate specificity. We first demonstrated that His was the most preferred amino acid by Sap10, a rare digestive site for yapsin-like aspartic proteases. This result is consistent with the previous observation that Sap10 could cleave a peptide between His and Asn (*10*). However, we do not know about the physiological targets of Sap10. Further investigations are warranted to clarify the significance of the rare substrate specificity of Sap10.

In conclusion, we succeeded in performing a full-scale, side-by-side analysis of substrate specificities of Sap isozymes and their possible roles in the pathogenicity of *C. albicans*. These results will lead to further understanding of *C. albicans* pathogenicity and help in the design of specific inhibitors for individual Sap isozymes, which can be used as pharmaceuticals or research tools.

SUPPLEMENTARY DATA

Supplementary Data are available at *JB* Online.

Funding

A research fellowship from the Japan Society for the Promotion of Science for Young Scientists; Regional Innovation Creation R&D Programs.

Conflict of interest
None declared.

References

1. Naglik, J.R., Challacombe, S.J., and Hube, B. (2003) *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev.* **67**, 400–428
2. Sobel, J.D. (1992) Pathogenesis and treatment of recurrent vulvovaginal candidiasis. *Clin. Infect. Dis.* **14**, S148–S153
3. Sobel, J.D. (1988) Pathogenesis and epidemiology of vulvovaginal candidiasis. *Ann. NY Acad. Sci.* **544**, 547–557
4. Schaller, M., Borelli, C., Korting, H.C., and Hube, B. (2005) Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48**, 365–377
5. Calderone, R.A. and Fonzi, W.A. (2001) Virulence factors of *Candida albicans*. *Trends Microbiol.* **9**, 327–335
6. Naglik, J., Albrecht, A., Bader, O., and Hube, B. (2004) *Candida albicans* proteinases and host/pathogen interactions. *Cell Microbiol.* **6**, 915–926
7. Schaller, M., Januschke, E., Schackert, C., Woerle, B., and Korting, H.C. (2001) Different isoforms of secreted aspartyl proteinases (Sap) are expressed by *Candida albicans* during oral and cutaneous candidosis *in vivo*. *J. Med. Microbiol.* **50**, 743–747
8. White, T.C., Miyasaki, S.H., and Agabian, N. (1993) Three distinct secreted aspartyl proteinases in *Candida albicans*. *J. Bacteriol.* **175**, 6126–6133
9. Borg-von Zepelin, M., Beggah, S., Boggian, K., Sanglard, D., and Monod, M. (1998) The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Mol. Microbiol.* **28**, 543–554
10. Albrecht, A., Felk, A., Pichova, I., Naglik, J.R., Schaller, M., de Groot, P., Maccallum, D., Odds, F.C., Schafer, W., Klis, F., Monod, M., and Hube, B. (2006) Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. *J. Biol. Chem.* **281**, 688–694
11. Koelsch, G., Tang, J., Loy, J.A., Monod, M., Jackson, K., Foundling, S.I., and Lin, X. (2000) Enzymic characteristics of secreted aspartic proteases of *Candida albicans*. *Biochim. Biophys. Acta.* **1480**, 117–131
12. Berman, J. and Sudbery, P.E. (2002) *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat. Rev. Genet.* **3**, 918–930
13. Tanskul, S., Oda, K., Oyama, H., Noparatnaraporn, N., Tsunemi, M., and Takada, K. (2003) Substrate specificity of alkaline serine proteinase isolated from photosynthetic bacterium, *Rubrivivax gelatinosus* KDDS1. *Biochem. Biophys. Res. Commun.* **309**, 547–551
14. Chaloupkova, R., Sykorova, J., Prokop, Z., Jesenska, A., Monincova, M., Pavlova, M., Tsuda, M., Nagata, Y., and Damborsky, J. (2003) Modification of activity and specificity of haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26 by engineering of its entrance tunnel. *J. Biol. Chem.* **278**, 52622–52628
15. Cawley, N.X., Chino, M., Maldonado, A., Rodriguez, Y.M., Loh, Y.P., and Ellman, J.A. (2003) Synthesis and characterization of the first potent inhibitor of yapsin 1. Implications for the study of yapsin-like enzymes. *J. Biol. Chem.* **278**, 5523–5530
16. Morrison, C.J., Hurst, S.F., Bragg, S.L., Kuykendall, R.J., Diaz, H., Pohl, J., and Reiss, E. (1993) Heterogeneity of the purified extracellular aspartyl proteinase from *Candida albicans*: characterization with monoclonal antibodies and N-terminal amino acid sequence analysis. *Infect. Immun.* **61**, 2030–2036
17. Hornbach, A., Heyken, A., Schild, L., Hube, B., Loffler, J., and Kurzai, O. (2009) The glycosylphosphatidylinositol-anchored protease Sap9 modulates the interaction of *Candida albicans* with human neutrophils. *Infect. Immun.* **77**, 5216–5224
18. Chen, Y.C., Wu, C.C., Chung, W.L., and Lee, F.J. (2002) Differential secretion of Sap4-6 proteins in *Candida albicans* during hyphae formation. *Microbiology* **148**, 3743–3754
19. Schild, L., Heyken, A., de Groot, P.W., Hiller, E., Mock, M., de Koster, C., Horn, U., Rupp, S., and Hube, B. (2011) Proteolytic cleavage of covalently linked cell wall proteins by *Candida albicans* Sap9 and Sap10. *Eukaryot. Cell* **10**, 98–109
20. Smolenski, G., Sullivan, P.A., Cutfield, S.M., and Cutfield, J.F. (1997) Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes. *Microbiology* **143**, 349–356
21. Hube, B. and Naglik, J. (2001) *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology* **147**, 1997–2005
22. Mizuno, K., Ojima, M., Gotoh, M., Hashimoto, S., and Fukuchi, S. (1985) True renin in human pituitary tissue. *J. Neurochem.* **44**, 633–636
23. Huang, X.P., Kagami, N., Inoue, H., Kojima, M., Kimura, T., Makabe, O., Suzuki, K., and Takahashi, K. (2000) Identification of a glutamic acid and an aspartic acid residue essential for catalytic activity of aspergillopepsin II, a non-pepsin type acid proteinases. *J. Biol. Chem.* **275**, 26607–26614
24. Colina, A.R., Aumont, F., Deslauriers, N., Belhumeur, P., and de Repentigny, L. (1996) Evidence for degradation of gastrointestinal mucin by *Candida albicans* secretory aspartyl proteinases. *Infect. Immun.* **64**, 4514–4519
25. Kaminishi, H., Miyaguchi, H., Tamaki, T., Suenaga, N., Hisamatsu, M., Mihashi, I., Matsumoto, H., Maeda, H., and Hagihara, Y. (1995) Degradation of humoral host defense by *Candida albicans* proteinases. *Infect. Immun.* **63**, 984–988
26. Ruchel, R. (1986) Cleavage of immunoglobulins by pathogenic yeasts of the genus *Candida*. *Microbiol. Sci.* **3**, 316–319
27. Gropp, K., Schild, L., Schindler, S., Hube, B., Zipfel, P.F., and Skerka, C. (2009) The yeast *Candida albicans* evades human complement attack by secretion of aspartic proteases. *Mol. Immunol.* **47**, 465–475
28. Sanglard, D., Hube, B., Monod, M., Odds, F.C., and Gow, N.A. (1997) A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. *Infect. Immun.* **65**, 3539–3546
29. Naglik, J.R., Moyes, D., Makwana, J., Kanzaria, P., Tsichlaki, E., Weindl, G., Tappuni, A.R., Rodgers, C.A., Woodman, A.J., Challacombe, S.J., Schaller, M., and Hube, B. (2008) Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* **154**, 3266–3280
30. Lian, C.H. and Liu, W.D. (2007) Differential expression of *Candida albicans* secreted aspartyl proteinase in human vulvovaginal candidiasis. *Mycoses* **50**, 383–390
31. Naglik, J.R., Rodgers, C.A., Shirlaw, P.J., Dobbie, J.L., Fernandes-Naglik, L.L., Greenspan, D., Agabian, N.,

and Challacombe, S.J. (2003) Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. *J. Infect. Dis.* **188**, 469–479
32. Naglik, J.R., Newport, G., White, T.C., Fernandes-Naglik, L.L., Greenspan, J.S., Greenspan, D., Sweet, S.P., Challacombe, S.J., and Agabian, N. (1999) *In vivo* analysis of secreted aspartyl proteinase expression in human oral candidiasis. *Infect. Immun.* **67**, 2482–2490
33. Taylor, B.N., Hannemann, H., Sehnal, M., Biesemeier, A., Schweizer, A., Rollinghoff, M., and Schroppel, K. (2005) Induction of *SAP7* correlates with virulence in an intravenous infection model of candidiasis but not in a vaginal infection model in mice. *Infect. Immun.* **73**, 7061–7063
34. Chaffin, W.L. (2008) *Candida albicans* cell wall proteins. *Microbiol. Mol. Biol. Rev.* **72**, 495–544
35. Meiller, T.F., Hube, B., Schild, L., Shirtliff, M.E., Scheper, M.A., Winkler, R., Ton, A., and Jabra-Rizk, M.A. (2009) A novel immune evasion strategy of *Candida albicans*: proteolytic cleavage of a salivary antimicrobial peptide. *PLoS One* **4**, e5039
36. Sorensen, O.E., Borregaard, N., and Cole, A.M. (2008) Antimicrobial peptides in innate immune responses. *Contrib. Microbiol.* **15**, 61–77
37. Brown, K.L. and Hancock, R.E. (2006) Cationic host defense (antimicrobial) peptides. *Curr. Opin. Immunol.* **18**, 24–30
38. Brogden, K.A., Ackermann, M., McCray, P.B. Jr, and Tack, B.F. (2003) Antimicrobial peptides in animals and their role in host defences. *Int. J. Antimicrob. Agents* **22**, 465–478