Microbiology (1997), 143, 349–356 Printed in Great Britain

Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes

G. Smolenski, P. A. Sullivan,† S. M. Cutfield and J. F. Cutfield

Author for correspondence: J. F. Cutfield. Tel: +64 3 479 7836. Fax: +64 3 479 7866.
e-mail: johnfc@sanger.otago.ac.nz

Biochemistry Department, University of Otago, PO Box 56, Dunedin, New Zealand

The recently discovered secreted aspartic proteinase multi-gene (*SAP*) family in *Candida albicans* has complicated assessment of proteolytic activity as a factor in the onset and development of *Candida* infections. Differential expression of the *SAP* genes under various conditions, as well as possible variation in the properties of the individual isoenzymes, have consequences for immunological detection, for targeted drug design and possibly for pathogenicity. It is therefore important to be able to monitor Sap isoenzyme profiles in different strains of *C. albicans* cultures, and to know the biochemical properties of each isoenzyme. We have employed a simple purification protocol based on strong anion exchange chromatography for the direct analysis of *C. albicans* Sap isoenzymes from culture filtrates, as well as recovery of individual Sap1, Sap2 and Sap3 products. In the case of Sap1, this involved development of an overexpression system using the pEMBLyex4 vector transformed into *Saccharomyces cerevisiae*. The *C. albicans* strains ATCC 10231 and 10261 were shown to produce different ratios of Sap2 and Sap3 under the same conditions. Analysis of all three purified proteins by gel electrophoresis, immunoblotting and proteinase assays which were designed to evaluate pH dependence, thermal stability and substrate specificity revealed similar but distinct properties for each isoenzyme. Although Sap3 was shown to be antigenically more similar to Sap2 than was Sap1, it was less similar in terms of thermal stability and activity at low pH, being more stable and more active.

Keywords: aspartic proteinases, *Candida albicans*, virulence factors, recombinant protein, isoenzymes

INTRODUCTION

An ongoing interest in the secreted aspartic proteinase (Sap) activity of *Candida* spp., and *Candida albicans* in particular, and its possible role as a virulence factor, has been heightened by the discovery of a multi-gene *SAP* family (references include Staib, 1969; Cutler, 1991; Ray *et al.*, 1991; Rüchel *et al.*, 1992; Magee *et al.*, 1993; Monod *et al.*, 1994; White & Agabian, 1995). Amongst the eight *SAP* genes identified so far, the protein products of four of these (Saps 1, 2, 3 and a putative Sap8) have been characterized to various extents. Indeed, the isoenzyme now known as Sap2, which represents the major product secreted *in vitro* by the yeast form of many strains of *C. albicans*, was first isolated and identified as an acidic proteinase by Remold *et al.* (1968). It was subsequently characterized further by Rüchel (1981) and since then a number of reports on the purification, activity and substrate specificity of this enzyme have appeared (Negi *et al.*, 1984; Kaminishi *et al.*, 1986; Ray & Payne, 1990; Bannerjee *et al.*, 1991; Yamamoto *et al.*, 1992; White *et al.*, 1993; Morrison *et al.*, 1993a, b; Fusek *et al.*, 1994; Tsushima *et al.*, 1994). The *SAP2* gene itelf was cloned and sequenced by Wright *et al.* (1992), being the second gene in the family to be identified. Most recently, the crystal structure of Sap2 from two different strains of *C. albicans* was determined (Cutfield *et al.*, 1995; Abad-Zapatero *et al.*, 1996), revealing details of the binding site for peptide-based

†**Present address:** Biochemistry Department, Massey University, Private Bag 11222, Palmerston North, New Zealand.

inhibitors. These structural studies showed that the binding site contained some amino acids that varied amongst the Sap family members, which might affect individual specificities.

Identification of the *SAP1* gene (Hube *et al.*, 1991) preceded characterization of its protein product by White *et al.* (1993), who also identified the *SAP3* gene and its protein. They further showed that the switching strain WO-1 secreted the Sap2 isoenzyme from white cells whereas the opaque cells expressed Saps 1, 2 and 3 (White & Agabian, 1995). Hube *et al.* (1994) observed the expression (transcription) of Saps 4, 5 and 6 in several strains only during serum-induced yeast-to-hyphal transition. It is now clear that the pattern of Sap isoenzyme production in *C. albicans* depends on strain, cell type and environmental factors. More speculative at this stage is the suggestion that the differential transcription of the various *SAP* genes is associated with adherence and colonization of host tissue.

Originally motivated by the need to produce milligram quantities of individual Sap isoenzymes for crystallization studies, we have now established a heterologous expression system for these proteins, as well as modifying existing purification protocols to produce a simple procedure for monitoring Saps 1, 2 and 3 in culture filtrates and for recovering pure protein. This has enabled us to compare some biochemical properties of these three isoenzymes from one strain and, where applicable, to compare them with other published results.

METHODS

Strains and plasmids. *Candida albicans* strains used were ATCC 10231 and 10261. *Saccharomyces cerevisiae* AH22 (Leu2$^-$ His2$^-$) (Hinnen *et al.*, 1978) was the host for recombinant Sap, while *Escherichia coli* DH5α was the host for cloning the *SAP1* gene. The plasmid pSap1 (referred to as CaPrA in Magee *et al.*, 1993) was provided by B. Hube (Universität Hamburg, Germany) and contains 5·4 kb *C. albicans* ATCC 10231 DNA including the *SAP1* open reading frame. The plasmids used for cloning and expression were pBluescript II KS(+/−) (Stratagene) and pEMBLyex4 (Cesareni & Murray, 1987), respectively.

Media and growth conditions. YPD medium contained (g l^{-1}) yeast extract (2), peptone (2) and glucose (20); YBD medium contained (g l^{-1}) yeast extract (2), BSA (2) and glucose (20). Minimal medium contained salts, vitamins and trace elements (Wickerham, 1946) plus 20 g glucose l^{-1}. The expression medium contained salts, vitamins and trace elements as above plus glycerol (3%, v/v) and sodium lactate (2%, w/v). Sap1 production was induced with the addition of galactose (2%, w/v). Protein and vitamins were filter-sterilized as 20-fold and 200-fold concentrated stocks, respectively. Media for plates contained agar (30 g l^{-1}). Cultures were stored in YPD at 4 °C and subcultured at 2 monthly intervals. Plates were incubated at 28 °C. Liquid cultures were grown on a gyratory shaker (200 r.p.m.) at 28 °C. The initial volume was 20% of the nominal flask volume and the cultures were inoculated from overnight cultures.

DNA manipulations and transformations. The 5·4 kb *Bam*HI fragment was excised from pSap1 and subcloned into pBluescript II KS(+/−) to give pGS1. The plasmid pGS1 was digested with *Bam*HI and *Ksp*6321 to give two distinguishable fragments, each of approximately 1800 bp. These were isolated and digested with *Bgl*I to produce 1800 bp of genomic DNA and two fragments (1100 and 700 bp) of vector DNA. The 1800 bp product was gel-purified, end-filled using DNA polymerase I (Klenow fragment) and T4 DNA polymerase, and ligated into the *Sma*I site of pBluescript II KS(+/−) (pGS2). The DNA containing the *SAP1* gene was excised from pGS2 as a 1400 bp fragment using *Bam*HI and *Xba*I and directionally subcloned into the yeast expression vector pEMBLyex4 (pGS3). This fragment contained 14 bp of 5′ sequence, the 1173 bp *SAP* open reading frame and about 210 bp of 3′ sequence. The correct orientation of the subcloned fragment was confirmed by restriction mapping and sequencing. Strain AH22 was then transformed with pGS3 (Burgess & Percival, 1987) with selection for Leu$^+$ colonies on minimal medium plus histidine (100 mg l^{-1}).

Purification of secreted aspartic proteinases

(a) Sap2 and Sap3. *C. albicans* ATCC 10231 (or 10261) was grown as a starter culture overnight in 200 ml YPD medium in a 1 l flask at 30 °C, then added to 10 l YBD medium and incubated for 18 h at 30 °C with an aeration rate of 10 l min^{-1}. After cell harvesting, the medium was adjusted from pH 3·2 to pH 6·5 with 6 M NaOH and concentrated to 500 ml by ultrafiltration (10000 Da molecular mass cut-off). Following a buffer exchange with 10 mM sodium citrate (pH 6·8), the concentrate was applied to a pre-equilibrated DEAE-Sephacel column (24 cm × 2·5 cm) and eluted at a flow rate of 60 ml h^{-1} over a 1 l linear gradient from 10 to 300 mM sodium citrate (pH 6·3). Fractions containing the enzyme activity were pooled, concentrated to 10 ml by ultrafiltration, buffer-exchanged with 20 mM bis-Tris/HCl (pH 6·0) and filtered through a 0·45 μm membrane to remove any particulate matter. Aliquots (1 ml) of this enzyme concentrate were applied to a pre-equilibrated Pharmacia Mono Q HR 5/5 column and eluted with a linear gradient from 20 mM bis-Tris/HCl (pH 6·0) to the same buffer plus 300 mM KCl at a flow rate of 1·0 ml min^{-1} under the control of a Waters Millipore 650 Advanced Protein Purification System. Individual peak fractions containing Sap activity were concentrated, buffer-exchanged, and then subjected to another Mono Q chromatography step in order to achieve maximum purity. Purified enzyme samples were routinely stored frozen at −20 °C in 100 mM Tris/HCl (pH 6·7). Protein concentration was determined by the modified Lowry procedure of Peterson (1977), using BSA as a standard.

(b) Recombinant Sap1. Recombinant Sap1 was induced in 1 l cultures by the addition of 2·0% (w/v) galactose using the conditions described previously for heterologous expression in *S. cerevisiae* (Chambers *et al.*, 1993). The medium was harvested 12–24 h post-induction, concentrated 20–50-fold and buffer-exchanged into 20 mM bis-Tris/HCl (pH 6·0) prior to Mono Q ion exchange chromatography as described above for Saps 2 and 3.

Assays for enzyme activity

(a) BSA. Based on the assay of MacDonald & Odds (1980), enzyme activity was measured spectrophotometrically (Pharmacia-LKB Ultrospec II spectrophotometer) following the digestion of BSA as substrate. A typical reaction mix containing 500 μl 2·0% (w/v) BSA in 50 mM sodium citrate (pH 3·2), 100 μl 50 mM sodium citrate (pH 3·2) and 200 μl of culture supernatant was incubated for 30 min at 37 °C. The reaction was stopped by adding 200 μl 2 M perchloric acid and incubated on ice for 15 min. Precipitated protein was removed

by centrifugation at 14000 *g* for 5 min and the enzyme activity was measured by determining the increase in A_{280} of the supernatant. One enzyme unit for this system is a change in absorbance of 1·0 min^{-1}.

(b) Haemoglobin. A modification of the procedure given by Jones (1991) was used. The reaction mixture contained 750 µl 2·0% apo-haemoglobin (pH 3·2), 650 µl 200 mM glycine (pH 3·2) and 100 µl of sample solution. After 30 min incubation at 37 °C, samples (300 µl) were removed and added to 150 µl 1 M perchloric acid on ice, and briefly shaken. After centrifugation at 14000 *g* for 5 min, 75 µl supernatant was removed and added to 75 µl 0·5 M NaOH. Tyrosine-containing peptides in the neutralized sample were determined with Folin's reagent according to the Lowry method. To each 150 µl sample, 750 µl reagent consisting of 2·0% (w/v) sodium carbonate in 1·0 M NaOH, 1·0% (w/v) copper sulphate and 2·0% (w/v) sodium tartrate was added and incubated at room temperature for 10 min before addition of 75 µl 50% diluted Folin's reagent. After 30 min incubation at room temperature, the change in A_{750} was determined. One enzyme unit for this system corresponds to the production of 1·0 µg tyrosine min^{-1} measured as an A_{750} of 0·058.

(c) pH and thermal stability activity profiles. To examine pH/activity profiles using either substrate, a range of buffers was employed, each at 50 mM concentration. These were glycine, citrate, acetate and Tris/HCl. For measurements of thermal stability, samples (0·1 ml) of enzyme were heated for 15 min at a specified temperature and then assayed at 37 °C as above.

PAGE and immunoblotting. Proteinase samples containing 25 mM Tris/HCl (pH 7·0), 1·0% (v/v) SDS, 5·0% (v/v) glycerol, 2·5% (v/v) β-mercaptoethanol and 0·05% bromphenol blue were heated at 100 °C for 5 min prior to SDS-PAGE. Electrophoresis (Mini-Protean II; Bio-Rad) was for 2–3 h at 150 V (constant voltage) using the discontinuous buffer system described by Schägger & van Jagow (1987). Alternatively, native PAGE was performed without the addition of any of the denaturing components in the above system. Following electrophoresis, samples were transferred to nitrocellulose, incubated with a 1:3000 dilution of rabbit anti-proteinase antiserum (Ross *et al.*, 1990) and visualized using the detection system of Blake *et al.* (1984). The antigen was a highly purified preparation of Sap2 from strain ATCC 10261.

N-terminal sequencing. Enzyme and peptide samples (~ 100 pmol) were applied to Immobilon PVDF membrane or Polybrene-coated glass-fibre disks, respectively. Automated N-terminal sequencing was performed with a gas-phase instrument (Applied Biosystems model 470A-R/120A/610A). Fragments of Sap proteins for internal sequencing were obtained by treatment with V8 protease from *Staphylococcus aureus* at an enzyme to substrate ratio of 1:30 (w/w), in 100 mM ammonium bicarbonate for 50 min at 37 °C.

Reverse-phase HPLC. For analysis of substrate specificity, separations were carried out using a Waters Chromatography binary gradient system consisting of a U6K injector, two M6000A pumps, a 680/440 systems controller and detector with extended wavelength module. A wide-pore C8 silica-based Bakerbond column (4·6 mm × 250 mm) was employed in conjunction with a linear gradient, from 0·1% trifluoro-acetic acid (TFA) to 80% acetonitrile/0·02% TFA. Insulin B-chain (15 µg) in 50 mM citrate buffer (pH 3·2) was digested with 100 ng Sap, total volume 100 µl, for a specified time. The reaction was stopped by addition of 100 µl 0·1% TFA/20% acetonitrile, and injected immediately onto the column. Peptide products were analysed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Finnigan MAT) and N-terminal sequencing.

RESULTS

Purification of Sap2 and Sap3

An outline of the purification scheme used for obtaining homogeneous preparations of Saps 2 and 3 from the culture medium of *C. albicans* strain ATCC 10231, or indeed from other strains, is shown in Fig. 1. It is based on the well-known ability of aspartic proteinases, with their generally low isoelectric points, to bind to anion-exchange resins, and was first used by Remold *et al.* (1968) for *Candida* Sap. Two such steps have been employed in this study: the 'traditional' DEAE chromatography (in this case as per Rüchel, 1981 and Wright *et al.*, 1992) followed by a much faster and higher resolution separation with Mono Q (strong anion exchanger). However, it was not necessary to include the DEAE step as can be seen in Fig. 2, where culture filtrates, after concentration and buffer exchange, were applied directly to the Mono Q column. In the interests of column preservation and for higher protein loadings, the preliminary DEAE step is, however, recommended for removal of some extraneous protein. Sap activity was located in two sharp peaks identified by N-terminal sequencing as Sap2 (the larger) and Sap3, respectively. Further confirmation was obtained by sequencing internal fragments generated by treatment with V8 protease. A second round of Mono Q chromatography was considered important for the high degree of purity required for crystallization trials and for comparative studies of individual isoenzymes.

The one-step Mono Q procedure allowed rapid analysis

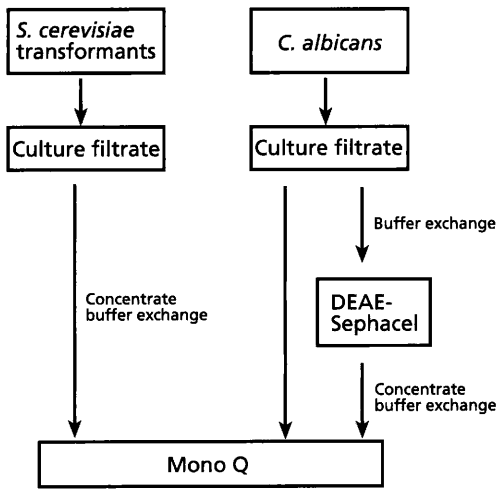

Fig. 1. Flow diagram of the purification scheme for either recombinant Sap or native Sap isoenzymes. The DEAE-Sephacel step is recommended for recovery of milligram quantities of protein but may be bypassed if rapid analysis of Sap isoenzyme profiles is required.

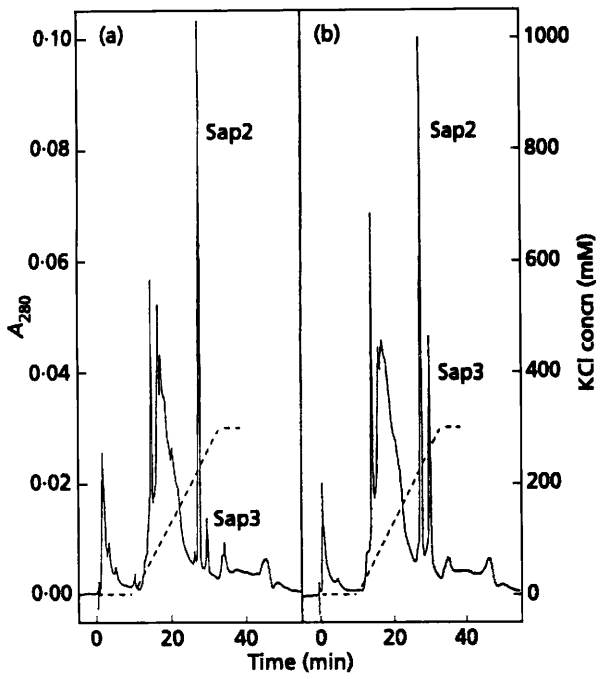

Fig. 2. Mono Q ion exchange chromatography of concentrated culture filtrate from *C. albicans* strains 10261 (a) and 10231 (b). Sap activity was eluted when a linear gradient of 0–300 mM KCl/20 mM bis-Tris/HCl (pH 6·0) was applied; flow rate was 1 ml min^{-1}.

of Sap isoenzyme profiles as shown in Fig. 2 for strains 10231 and 10261. Under the same growth conditions, these two strains produce Saps 2 and 3 in quite different ratios. Comparison of the A_{280} peak heights shows that 10231 produces considerably more Sap3 relative to Sap2 than does strain 10261 after 18 h growth, even allowing for the higher aromatic content of Sap3 (an extra tryptophan and two extra tyrosine residues), which would raise its extinction coefficient at 280 nm by a factor of 1·3, as calculated using the rules of Gill & von Hippel (1989). Thus for strain 10231, the predicted Sap2:Sap3 ratio is about 70:30 whereas for 10261 it is about 95:5. This was borne out by the enzyme recovery data given in Table 1 for 10231, with 40 mg pure Sap2 and 18 mg Sap3 being obtained from a 10 l culture. These data also show that, using the BSA assay, the specific activities of Saps 2 and 3 were very similar (5 U mg^{-1}) and that a 16-fold purification of the culture filtrate activity had been achieved. By comparison, 41 mg Sap2 and only 1·8 mg Sap3 was obtained from strain 10261 grown under the same conditions. The specific activity for Sap2 was the same as in 10231; however, for Sap3 it was 20% lower (4 U mg^{-1}). Similar relative activities were seen when haemoglobin was used as substrate.

Expression of Sap1 in *S. cerevisiae*

Heterologous expression of recombinant Sap1 was based on a fragment of DNA containing the full-length open reading frame identified originally from a genomic library of ATCC 10231 DNA (Hube *et al.*, 1991). The fragment was inserted into the multicopy yeast expression vector pEMBLyex4, allowing expression in *S. cerevisiae* strain AH22. The purification of Sap1, the main protein species secreted into the medium, was simple, involving just the one chromatography step with Mono Q (Fig. 1), to give a twofold purification of enzyme activity from the filtrate and a 69% total recovery. The purified enzyme had a specific activity of 3·2 U mg^{-1} using BSA as a substrate, which is less than for Saps 2 and 3 (5 U mg^{-1}) prepared from *C. albicans* as above. Although the final yield of protein was low [1·1 mg (l culture)$^{-1}$], the ease of purification largely compensated for this. Proof of the correct identity of the secreted protein was secured by N-terminal sequencing, mobility on SDS-PAGE, Western blotting and pH dependence of proteinase activity. Yeast cells transformed with vector only showed no Sap activity and no immunoreactivity. The partial sequence obtained (QAIPVTLNNE....) was indicative that *S. cerevisiae* had correctly processed the precursor form of the enzyme. Using the same heterologous expression system (A. D. Scadden & P. A. Sullivan, unpublished), recombinant Sap2 isoenzyme has also been produced and this was found to possess the same specific activity as the native protein. Therefore it can be reasonably expected that the recombinant Sap1 so produced will have similar activity to native Sap1.

Table 1. Purification of Saps 2 and 3 from *C. albicans* ATCC 10231

Purification step	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (-fold)
Culture medium	9780	1213	3917	0·3	100	1·0
Concentrate	506	873	874	1·0	73	3·2
DEAE-Sephacel	170	424	178	3·9	35	12·6
Concentrate	10·1	351	79	4·4	29	14·2
Mono Q Sap2	13·1	197·7	39·5	5·0	16·3	16·2
Sap3	6·2	93·2	18·6	5·0	7·7	16·2

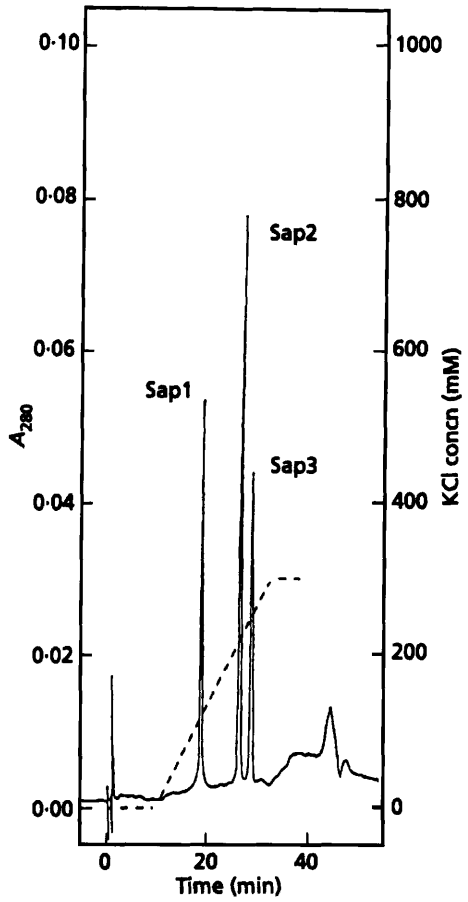

Fig. 3. Mono Q profile of Saps 1, 2 and 3 (previously purified) loaded as a mixture of arbitrary composition. Conditions are the same as in Fig. 2.

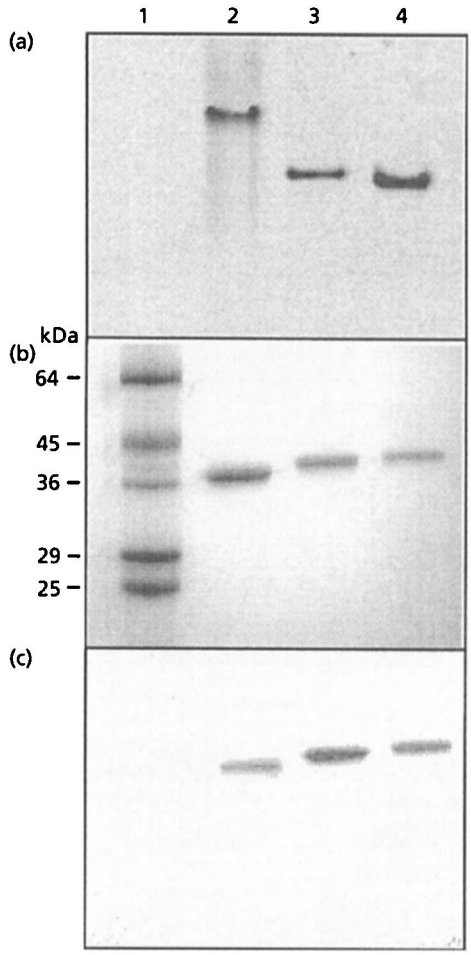

Fig. 4. (a) Native PAGE, (b) SDS-PAGE and (c) corresponding Western blot of Saps 1, 2 and 3 (lanes 2, 3 and 4, respectively) from 10231. Loadings were 3 μg Saps in (a) and (b), and 100 ng for (c). Lane 1 in (b) contains molecular mass marker proteins. Gels were stained with Coomassie blue and scanned by an Eagle Eye II (Stratagene) imaging system.

Comparative properties of Sap1, Sap2 and Sap3

The three Sap isoenzymes from strain 10231, purified as above, are readily distinguishable by anion exchange chromatography (Fig. 3) and also by native gel electrophoresis (Fig. 4a). Both of these methods exploit the overall charge on the molecule and it can be seen that the order of increasing negative charge was Sap3 > Sap2 > Sap1. Fig. 3 shows that if all three Saps were secreted at the same time, they could be easily identified by their elution times and quantified relative to each other from the A_{280} profile, remembering that the Sap3 peak should be reduced by a factor of 1·3 with respect to both Sap1 and Sap2. Analysis by SDS-PAGE (Fig. 4b) shows a running order Sap1 > Sap2 > Sap3, corresponding to apparent molecular masses of 38, 41 and 42 kDa, respectively, which are 3–6 kDa higher than the molecular masses deduced from their sequences. It is interesting to note that if the gel recipe was altered to increase the bis:acrylamide ratio, then Saps 2 and 3 ran parallel. There was no evidence (from carbohydrate analysis, amino acid sequencing and crystallography of Sap2) for any significant glycosylation which would explain the slower than expected migration that nevertheless is consistent with other gel analyses of Saps.

Western analysis of the three isoenzymes (Fig. 4c) using a polyclonal antibody preparation directed against purified Sap2 (Ross *et al.*, 1990) clearly showed that there were common epitopes amongst them, the order of reactivity being Sap2 > Sap3 > Sap1. Quantification of the Western blots by soft laser densitometry indicated that Sap3 was 90% and Sap1 65% immunoreactive in comparison to Sap2.

The pH dependence of the isoenzymes was determined using a range of buffers between pH 2·0 and 7·0. Similar profiles were observed in all three cases (Fig. 5a), although Sap3 showed significantly higher activity below pH 2·5 with both BSA and haemoglobin substrates. Above pH 7, all three Saps were essentially inactive. The pH optima using either substrate were all in the range 3·2–3·5.

The upper temperature for thermal stability using either substrate was observed to be 45 °C for each of the three isoenzymes (Fig. 5b). Both Saps 1 and 2 lost approxi-

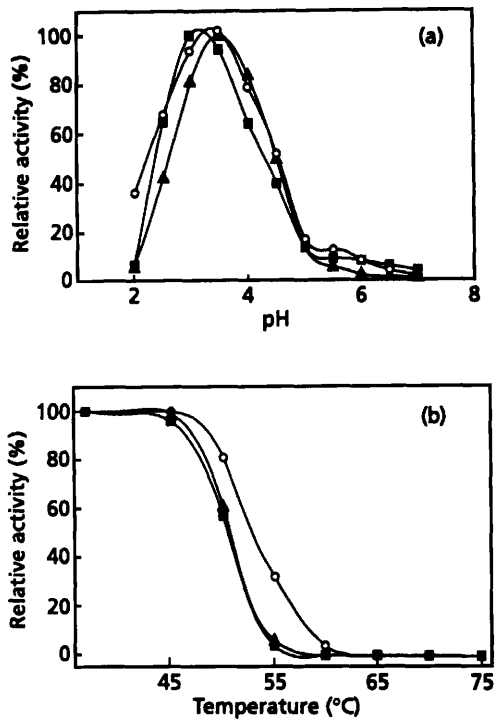

Fig. 5. (a) pH profiles and (b) thermal stability profiles for Saps 1 (■), 2 (▲) and 3 (○) from strain 10231. Activity assays used BSA as substrate. Similar results were obtained with haemoglobin as substrate. Points shown are the mean of triplicates.

mately 40% of their activity after incubation at 50 °C, while by 55 °C activity was almost abolished. In contrast, Sap3 was 80% active following incubation at 50 °C and it still retained 30% activity at 55 °C, indicating greater resistance to thermal denaturation than the others. Bannerjee *et al.* (1991) reported similar thermal stability data to those obtained with Sap2 for the (presumed) Sap2 from strain SC5314.

There have been many studies on the substrate specificity of what is now known as Sap2, including a variety of different Sap-producing strains and involving both natural and synthetic substrates. These experiments have demonstrated that the enzyme can cut at many sites with no particular sequence discrimination. We wished simply to compare the pattern of cleavage for just one substrate, insulin B-chain, by each of the Sap isoenzymes. The results as analysed by reverse-phase HPLC indicated that the A_{214} profiles are quite complex, with most of the peptide peaks being common to all three enzymes but varying in height (Fig. 6). Analysis of some of these peaks showed that a favoured cleavage site lies between B24 Phe and B25 Phe (peak * is the B25–30 peptide), but also indicated several other sites close together in the region B12–B15 (Val-Glu-Ala-Leu), similar to the original finding by Remold *et al.* (1968). With this particular substrate, therefore, there are no major differences in specificity amongst the three isoenzymes.

DISCUSSION

This paper presents (a) the first report of recombinant Sap production, (b) the use of a straightforward protocol for recognizing and purifying Sap isoenzymes and (c) a comparison of some fundamental properties of Saps 1, 2 and 3 from a non-switching strain, ATCC 10231.

The *SAP1* gene from *C. albicans* strain 10231 was expressed as a correctly processed (secreted) recombinant protein in *S. cerevisiae*, albeit at a modest level, although no special effort was made to optimize the construct. There was very little other protein secreted into the medium, thus simplifying the purification process. As measured, the specific activity of the pure Sap1 was lower than that of Saps 2 and 3 obtained from cultures of *C. albicans* 10231. Purification of Saps 2 and 3 was almost as simple as for Sap1, the key step being the rapid, high-resolution ion exchange step with Mono Q. Analysis of fractions following Mono Q chromatography showed that under proteinase-inducing conditions (YBD medium), the Sap activity secreted by 10231 (or 10261) was associated with Saps 2 and 3 only. Sap1 expression has so far only been observed in the opaque phase of the switching strain WO-1 and it is not yet clear under what conditions, if any, 10231 could express the *SAP1* gene product. Using oligonucleotide probes specific for Saps 1, 2 and 3, Northern analyses of both 10231 and 10261, grown under the same conditions as for Sap protein production, were consistent with the protein results obtained in this study (R. J. Wright & P. A. Sullivan, unpublished). An earlier report by Wright *et al.* (1992) had indicated some expression of Sap1 in 10261 but the full-length probe used in that experiment would have allowed cross-hybridization to occur. It is clear then that evidence from both mRNA and secreted protein levels shows that Sap2 is the major isoenzyme expressed and that strain 10231 is a significantly greater producer of Sap3 than is 10261 under the conditions used.

Reasonably pure preparations of Sap2 (known previously under a variety of names, including *CAP*, *PRA* and *PEP* gene products) have long been attainable. Most of these protocols have incorporated a DEAE chromatography step following the original work of Remold *et al.* (1968), together with at least one other step. This group combined gel filtration with DEAE ion exchange as did Rüchel (1981), who subsequently introduced pepstatin-linked affinity chromatography (Rüchel *et al.*, 1982). A pseudo-affinity step followed by gel filtration was employed by Ray & Payne (1990), while most other preparations have incorporated variations on the above themes (summarized by Morrison *et al.*, 1993b). Analysis of purity of these Sap preparations has been carried out by various methods, including SDS-PAGE, immunoblotting and isoelectric focusing with confirmation by N-terminal sequencing in some cases. Given that Saps 1, 2 and 3 are of similar molecular mass (approximately 36 kDa), they migrate close together in denaturing gels and are not easy to discriminate in mini-gels, especially so Saps 2 and 3 (see also White *et al.*, 1993). We have

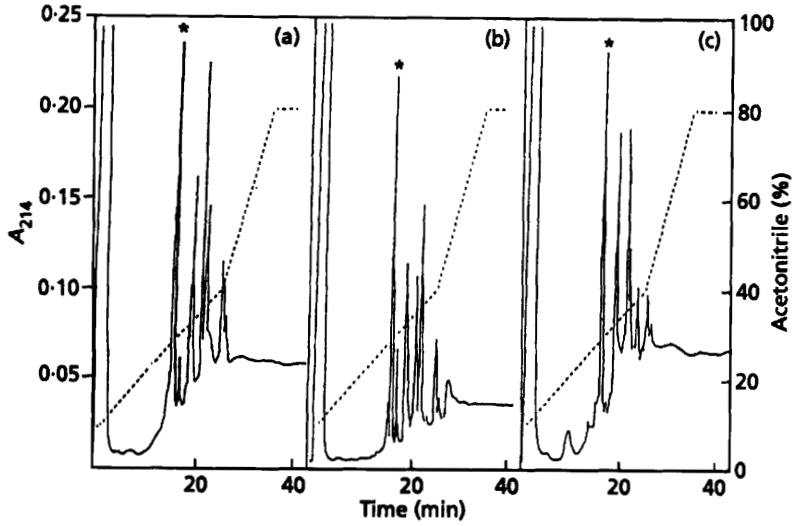

Fig. 6. Reverse-phase HPLC profiles of insulin B-chain digested with (a) Sap1, (b) Sap2 and (c) Sap3. Conditions are as described in Methods. Peak ★ is the B25–30 peptide.

shown that non-denaturing gels offer better discrimination and high-resolution fast anion exchange chromatography even more so.

It was recently pointed out that despite the many and varied studies on the biochemical properties of Sap, there were inconsistencies in the results, in part due to lack of identification of the various isoenzymes (White & Agabian, 1995). Bannerjee *et al.* (1991) characterized two activities in cultures of SC5314 which presumably corresponded to Saps 2 and 3, and some earlier studies had also suggested more than one activity, including those of Remold *et al.* (1968). The first clear demonstration of the presence of three distinct isoenzymes was by White *et al.* (1993), who showed that a single strain, WO-1, is able to secrete Saps 1, 2 and 3 and that the levels of these isoenzymes were dependent on environmental factors. We have also isolated the three isoenzymes (Sap1 as a recombinant), from a non-switching strain in this case, and compared several of their basic functional properties. In terms of pH profile, there was little difference, though Sap3 appears to be considerably more active at pH 2. Of greater interest perhaps is the higher thermal stability exhibited by Sap3, but more stringent denaturation tests would need to be carried out in order to confirm that this isoenzyme possesses greater structural stability. As far as the specificity of substrate cleavage is concerned, the results found with insulin B-chain reiterate earlier findings of broad specificity, regardless of isoenzyme type. Some preliminary results using synthetic peptides indicate that there may be some better defined specificity preferences among the Saps but even so this would seem to be of little importance *in vivo*.

Although phylogenetic analysis of Sap proteins by Monod *et al.* (1994) has shown that Sap1 and Sap3 display similar sequence similarity to Sap2, the immunoblotting results indicated that the anti-Sap2 antibodies recognized Sap3 better than they did Sap1. The possibility that the Sap2 preparation used as antigen (Ross *et al.*, 1990) may have contained a small amount of Sap3 is unlikely as it had been extensively purified to give a clean amino acid sequence and a single band on SDS-PAGE. Examination of the known three-dimensional structure of Sap2 (Cutfield *et al.*, 1995) offers a possible explanation for the different responses. There are several exposed regions on the surface of the molecule that are possibly antigenic and that vary in sequence among the isoenzymes, with Sap1 showing more radical substitutions than Sap3, relative to Sap2. For example, the loop region 49–53 in Sap1 has two basic amino acids not seen in the others; the loop region 241–244 also has a different charge distribution and there are a number of exposed single-site substitutions involving change in charge or in polarity.

ACKNOWLEDGEMENTS

We acknowledge the support of the Health Research Council of New Zealand for grants awarded to J. F. C. and P. A. S., and for a postgraduate scholarship to G. S. D. Carne assisted with amino acid sequencing. We are grateful to Dr B. Hube for the *SAP1* clone.

REFERENCES

Abad-Zapatero, C., Goldman, R., Muchmore, S. W., Hutchins, C., Stewart, K., Navaza, J., Payne, C. D. & Ray, T. L. (1996). Structure of a secreted aspartic protease from *C. albicans* complexed with a potent inhibitor: implications for the design of antifungal agents. *Protein Sci* **5**, 640–652.

Bannerjee, A., Ganesan, K. & Datta, A. (1991). Rapid purification of secretory acid proteinase from *Candida albicans* and its characterization. *Indian J Biochem Biophys* **28**, 444–448.

Blake, M. S., Johnston, K. H., Russell-Jones, G. J. & Gotschlich, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal Biochem* **136**, 175–179.

Burgess, P. M. J. & Percival, K. J. (1987). Transformation of yeast spheroplasts without cell fusion. *Anal Biochem* **163**, 391–397.

Cesareni, G. & Murray, J. (1987). Plasmid vectors carrying the replication origin of filamentous single-stranded phages. *Genet Eng* **9**, 135–154.

Chambers, R. S., Walden, A. R., Brooke, G. S., Cutfield, J. F. & Sullivan, P. A. (1993). Identification of a putative active site residue in the exo-β-(1,3)-glucanase of *Candida albicans*. *FEBS Lett* **3**, 366–369.

Cutfield, S. M., Dodson, E. J., Anderson, B. F., Moody, P. C. E., Marshall, C. J., Sullivan, P. A. & Cutfield, J. F. (1995). The crystal structure of a major secreted aspartic proteinase from *Candida albicans* in complexes with two inhibitors. *Structure* **3**, 1261–1271.

Cutler, J. E. (1991). Putative virulence factors of *Candida albicans*. *Annu Rev Microbiol* **45**, 187–218.

Fusek, M., Smith, E. A., Monod, M., Dunn, B. M. & Foundling, S. I. (1994). Extracellular aspartic proteinases from *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* yeasts differ substantially in their specificities. *Biochemistry* **33**, 9791–9799.

Gill, S. C. & von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* **182**, 319–326.

Hinnen, A., Hicks, J. R. & Fink, G. R. (1978). Transformation of yeast. *Proc Natl Acad Sci USA* **75**, 1929–1933.

Hube, B., Turver, C. J., Odds, F. C., Eiffert, G. J., Boulnois, G. J., Kochel, H. & Rüchel, R. (1991). Sequence of the *Candida albicans* gene encoding the secretory aspartate proteinase. *J Med Vet Mycol* **29**, 129–132.

Hube, B., Monod, M., Schofield, D. A., Brown, A. J. P. & Gow, N. A. R. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol Microbiol* **14**, 87–99.

Jones, E. W. (1991). Tackling the protease problem in *Saccharomyces cerevisiae*. *Methods Enzymol* **194**, 444–446.

Kaminishi, H., Hagihara, Y., Hayashi, S. & Cho, T. (1986). Isolation and characteristics of collagenolytic enzyme produced by *Candida albicans*. *Infect Immun* **53**, 312–316.

MacDonald, F. & Odds, F. C. (1980). Inducible proteinase of *Candida albicans* in diagnostic serology and in the pathogenesis of systemic candidosis. *J Med Microbiol* **13**, 423–435.

Magee, B. B., Hube, B., Wright, R. J., Sullivan, P. A. & Magee, P. T. (1993). The genes encoding the secreted aspartyl proteinases of *Candida albicans* constitute a family with at least three members. *Infect Immun* **61**, 3240–3243.

Monod, M., Togni, G., Hube, B. & Sanglard, D. (1994). Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Mol Microbiol* **13**, 357–368.

Morrison, C. J., Hurst, S. F., Bragg, S. L., Kuykendall, R. J., Diaz, H., McLaughlin, D. W. & Reiss, E. (1993a). Purification and characterization of the extracellular aspartyl proteinase of *Candida albicans*: removal of extraneous proteins and cell wall mannoprotein and evidence for lack of glycosylation. *J Gen Microbiol* **139**, 1177–1186.

Morrison, C. J., Hurst, S. F., Bragg, S. L., Kuykendall, R. J., Diaz, H., Pohl, J. & Reiss, E. (1993b). 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.

Negi, M., Tsuboi, R., Matsui, T. & Ogawa, H. (1984). Isolation and characterization of proteinase from *Candida albicans*: substrate specificity. *J Invest Dermatol* **83**, 32–36.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* **83**, 346–356.

Ray, T. L. & Payne, C. D. (1990). Comparative production and rapid purification of *Candida* acid proteinase from protein-supplemented cultures. *Infect Immun* **58**, 508–514.

Ray, T. L., Payne, C. D. & Morrow, B. J. (1991). *Structure and Function of the Aspartic Proteinases*, pp. 173–183. Edited by B. M. Dunn. New York: Plenum Press.

Remold, H., Fasold, H. & Staib, F. (1968). Purification and characterisation of a proteolytic enzyme from *Candida albicans*. *Biochim Biophys Acta* **167**, 399–406.

Ross, I. K., de Bernardis, F., Emerson, G. W., Cassone, A. & Sullivan, P. A. (1990). The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant. *J Gen Microbiol* **136**, 687–694.

Rüchel, R. (1981). Properties of a purified proteinase from the yeast *Candida albicans*. *Biochim Biophys Acta* **659**, 99–113.

Rüchel, R., Tegeler, R. & Trost, M. (1982). A comparison of secretory proteinases from different strains of *Candida albicans*. *Sabouraudia* **20**, 233–244.

Rüchel, R., de Bernardis, F., Ray, T. L., Sullivan, P. A. & Cole, G. T. (1992). *Candida* acid proteinases. *J Med Vet Mycol* **1**, 123–132.

Schägger, H. & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**, 368–379.

Staib, F. (1969). Proteolysis and pathogenicity of *Candida albicans* strains. *Mycopathol Mycol Appl* **37**, 345–348.

Tsushima, H., Mine, H., Kawakami, Y., Hyodoh, F. & Ueki, A. (1994). *Candida albicans* aspartic proteinase cleaves and inactivates human epidermal cysteine proteinase inhibitor, cystatin A. *Microbiology* **140**, 167–171.

White, T. C. & Agabian, N. (1995). *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type and levels are determined by environmental factors. *J Bacteriol* **177**, 5215–5221.

White, T. C., Miyasaki, S. H. & Agabian, N. (1993). Three distinct secreted aspartyl proteinases in *Candida albicans*. *J Bacteriol* **175**, 2997–3005.

Wickerham, L. G. (1946). A critical review of the nitrogen assimilation tests commonly used in the classification of yeasts. *J Bacteriol* **52**, 293–301.

Wright, R. J., Carne, A., Hieber, A. D., Lamont, I. L., Emerson, G. E. & Sullivan, P. A. (1992). A second gene for a secreted aspartate proteinase in *Candida albicans*. *J Bacteriol* **174**, 7848–7853.

Yamamoto, T., Nohara, K., Uchida, K. & Yamaguchi, H. (1992). Purification and characterisation of the secretory proteinase of *Candida albicans*. *Microbiol Immunol* **36**, 637–664.

Received 9 July 1996; revised 4 October 1996; accepted 8 October 1996.