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Identification of a putative active site residue in the exo-β-(1,3)-glucanase of *Candida albicans*

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Recombinant exo-β-(1-3)-glucanase from *Candida albicans* was expressed in *Saccharomyces cerevisiae* and purified. The enzyme contains a number of short blocks of sequence homology with several genes for cellulases of the family A glycanases including the conserved sequence motif NEP which has previously been shown to be important in the catalytic function of several cellulases. Site directed mutagenesis of this glutamic acid residue in the 1,3 glucanase (E230D, E230Q) decreased the enzymatic activity 15,000- and 400-fold, respectively. This suggests that the E of the NEP participates in catalysis of the exoglucanase and other related glycanases.

Site-directed mutagenesis; Exo-β-(1,3)-glucanase; *Candida albicans*

1. INTRODUCTION

β-Glucan hydrolases (β-glucanases) secreted to the cell surface of many yeasts and fungi participate in the metabolism of β 1,3/1,6 glucan, a major structural component of the cell wall. Genes for exo-β-(1,3)-glucanases cloned from *Saccharomyces cerevisiae* [1] and *Candida albicans* [2] have 58% identity in deduced amino acid sequences but no overall homology with other glycanases. The catalytic mechanisms and substrate binding sites of yeast β-glucanases however should have features in common with other glycanases. For example it is well established that acidic residues function as nucleophiles (or in stabilizing the transition state) and as proton donors in glycanases such as lysozyme [3] and cellulases [4–6]. Both exoglucanase genes contain the sequence motif NEP which is also highly conserved in members of the family A cellulases [7]. Point mutations of this glutamic acid to the isosteric glutamine in two bacterial β-(1,4)-endoglucanases resulted in a loss of enzymatic activity [6]. In this paper we report the effects of point mutations of residue E^{230} on the catalytic activity of the exoglucanase of *C. albicans*.

2. MATERIALS AND METHODS

2.1. *DNA manipulations*

Standard conditions were used for molecular cloning, hybridization, transformation, transfection and electrophoresis [8]. End-filling of 3′ recessed restriction fragments was with DNA polymerase I (Klenow fragment). Genomic DNA from *S. cerevisiae* was isolated as described by Cryer et al. [9]. DNA fragments were recovered from agarose gels using a Geneclean II kit (Bio 101). DNA fragments were labeled with [α-^{32}P]dATP (Amersham) as described by Feinberg et al. [10].

Oligonucleotides (22-mers) for mutagenesis were produced on an Applied Biosystems 380B synthesizer: E230D: TTG CTT AAT GAT CCA TTG GGT C; E230Q: TTG CTT AAT CAA CCA TTG GGT C; and D230E: TTG CTT AAT GAA CCA TTG GGT C. Mutagenesis was carried out using an oligonucleotide-directed in vitro mutagenesis kit, version 2.1 (Amersham). The region around each mutation was sequenced using a Sequenase kit (United States Biochemicals) with [α-^{35}S]dATP (Amersham) and the universal M13 forward primer. Yeast transformations were carried out according to Burgers et al. [11] using Zymolyase 20T from Seikagaku Kogo. All other reagents were of analytical grade.

2.2. *Organisms and plasmids*

Escherichia coli strains DH5α and TG1 [12] were hosts for plasmids and bacteriophage M13, respectively. The plasmid pUC9 [12] was used for subcloning experiments, pEMBLyex4 [13] for expression in *S. cerevisiae* and the bacteriophage M13mp19 [12] for sequencing and mutagenesis. The plasmids pXG29 [2] and pBG3 [14] contained the exoglucanase genes from *C. albicans* and *S. cerevisiae* (*EXG1*), respectively. An exoglucanase deficient mutant of *S. cerevisiae*, strain AWY-1, was produced by disruption of *EXG1* of strain DBY746 (α *his3 1 leu2-3 leu2-112 ura3-52 trp1-289 gal can1*) obtained from the Yeast Genetic Stock Center, Berkeley. A 3.5 kb *Hin*dIII fragment from pBG3 containing the *EXG1* gene was cut within the gene at the single *Xba*I site and partially end-filled with dTTP and dCTP using DNA polymerase I (Klenow fragment). A 1.1 kb *Hin*dIII fragment from pBG3 containing the *URA3* gene was partially end-filled with dATP and dGTP using DNA polymerase I (Klenow fragment) and ligated to the *Xba*I cut to generate the plasmid pAW5. The 4.6 kb *Hin*dIII fragment from pAW5 containing the *EXG1:URA3* construct was used to transform DBY746 to URA$^+$. Southern blots of restriction digests of the genomic DNA of one transformant, AWY-1, with both the *URA3* and *EXG1* genes as probes showed that the uracil prototrophy had arisen as a result of homologous recombination at the exg1 locus.

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Less than 1% exoglucanase activity was detected in the culture medium of AWY-1 and no exoglucanase could be detected by Western blot analysis.

2.3. *Media*

E. coli cultures carrying plasmids were grown in Luria broth supplemented with 100 $\mu g \cdot ml^{-1}$ ampicillin and strains carrying M13 were grown in 2 × YT broth [8]. *S. cerevisiae* cultures were grown either in YPD media containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose, or strains containing plasmids in the minimal medium of Wickerham [15] except that the carbon source was 3% (v/v) glycerol, 2% (v/v) sodium lactate and 0.05% glucose. Histidine, tryptophan, leucine and uracil were added as needed at 0.03 mg/ml^{-1}.

2.4. *Site-directed mutagenesis*

The *C. albicans* exoglucanase gene [2] was digested from pXG29 as a 1.34 kb *AseI–AhaII* fragment, blunted with DNA polymerase I (Klenow fragment) and cloned into the *SmaI* site of pUC9 (pXAG1). This fragment contained the entire open reading frame plus 16 bp upstream from the initiation codon and 8 bp downstream of the termination codon. The gene was recovered from pXAG1 as an *EcoRI–HindIII* fragment and cloned into M13mp19. Single-stranded DNA was prepared and the NEP motif was mutated to NDP and NQP using the oligonucleotide-directed in vitro mutagenesis kit. $E^{230}D$ and $E^{230}Q$ mutants were selected and the regions around the mutations were sequenced confirming the base changes.

2.5. *Expression of exoglucanase in* S. cerevisiae

Double-stranded DNA from each mutant and pXAG1 was digested with *EcoRI*, end-filled with DNA polymerase I (Klenow fragment) and digested with *HindIII*. The 1.34 kb fragments released were separated on 0.8% agarose gels, recovered using a Geneclean II kit and cloned into the *SmaI* and *HindIII* sites of the expression plasmid pEMBLyex4 yielding pNDP3, pNQP4 and pYOG1 (wild type).

2.6. *Enzyme purification*

Recombinant exoglucanase was purified from 1 l cultures grown in minimal medium in shake flasks at 200 rpm, 28°C. Cultures were inoculated to an $OD_{600} = 0.2$, grown to $OD_{600} = 0.4$ and 2% (w/v) galactose was added to induce expression. Growth was continued for a further 12 h, the cells were harvested by centrifugation (4,000 × *g*, 10 min) and the medium was concentrated to 5 ml by ultrafiltration (M_r = 10,000 cut-off filter). The concentrate was dialysed against 50 mM sodium phosphate pH 7.0, $(NH_4)_2SO_4$ was added to 0.6 M, the solution was passed through a 0.22 μm filter and loaded onto a FPLC Phenyl Superose HR 5/5 column (Pharmacia) pre-equilibrated with buffered 0.6 M $(NH_4)_2SO_4$. The column was eluted at a flow rate of 0.5 $ml \cdot min^{-1}$ with a reverse gradient of 0.6–0.0 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate, pH 7.0 over 30 min (Fig. 1). The fractions (0.5 ml) containing the enzyme were pooled and the solvent was changed to 5 mM sodium acetate, pH 5.6, using a Centricon-10 microconcentrator (Amicon). The enzyme preparations were finally concentrated to 0.5 ml and stored with 0.02% azide at 4°C.

2.7. *Protein analysis*

Exoglucanase activity was determined with laminaran, 7.8 $mg \cdot ml^{-1}$, in 100 mM sodium acetate, pH 5.6, and 0.08 $mg \cdot ml^{-1}$ bovine serum albumin. Assays (125 μl) were incubated for 30 min at 37°C and the reactions were stopped by heating to 100°C for 10 min. Glucose formation was measured by the glucose oxidase method [16] and one unit of enzyme activity produced 1 μmol of glucose per min. Alternatively, enzyme activity was determined with 4-methylumbelliferyl β-D-glucoside, 8 mM, in 20 mM sodium acetate, pH 5.6, and 0.08 $mg \cdot ml^{-1}$ bovine serum albumin. Assays (250 μl) were incubated for 30 min at 37°C and the reactions were stopped with 500 μl of 0.1 M glycine-NaOH, pH 10.3. The formation of the methylumbelliferone was measured by emission at 450 nm with excitation at 384 nm in a spectrophotofluorimeter. One unit of enzyme activity produced 1 μmol of methylumbelliferone per min. The assay with methylumbelliferyl-glucoside was approximately twenty times more sensitive than the laminaran assay. Protein was estimated by a modified Lowry method [17] using bovine serum albumin as a standard. SDS-PAGE was carried out with 10% (w/v) acrylamide gels [18] and Western blotting was as described previously [2].

3. RESULTS

3.1. *Homology among exoglucanases and cellulases*

A comparison of the two yeast exoglucanase sequences with those of bacterial endo-β-(1,4)-glucanases (cellulases) showed no overall homology but there are several short blocks of homology including residues 229–231, a highly conserved sequence NEP (Fig. 2). We therefore carried out site-directed mutagenesis studies to show that the E of NEP is a catalytic residue of an exo-β-(1,3)- glucanase.

3.2. *Analysis of the exoglucanase expressed in* S. cerevisiae

Strain AWY-1 was transformed with each plasmid, the recombinant exoglucanase was purified to homogeneity from each culture (see section 2) and checked for purity by SDS PAGE and Western blots (data not shown). Expression levels for the E230D, E230Q and wild type enzymes were comparable, and Table I shows a typical purification for the wild type enzyme.

K_m and V_{max} values were determined for the three preparations with both laminaran and methylumbelliferyl-glucoside as substrates (Table II). With laminaran the E230D and E230Q mutations resulted in 15,000-fold and 400-fold decreases in V_{max}, respectively, whereas the K_m values increased 1.8-fold and 1.2-fold, respectively. With methylumbelliferyl-glucoside the V_{max} values of E230D and E230Q mutations decreased 15,000-fold and 150-fold, respectively, but the K_m values were decreased to approximately one third that of wild type enzyme. The *C. albicans* exoglucanase also catalyses a transglucosylation reaction [19] and the E230D

Table I

Purification of recombinant exoglucanase

Purification step	Total activity (U)	Total protein (mg)	Specific activity	Yield (%)	Purification (-fold)
Spent medium concentrate	58	0.8	73	100	1
Phenyl Superose	14	0.065	215	24	3

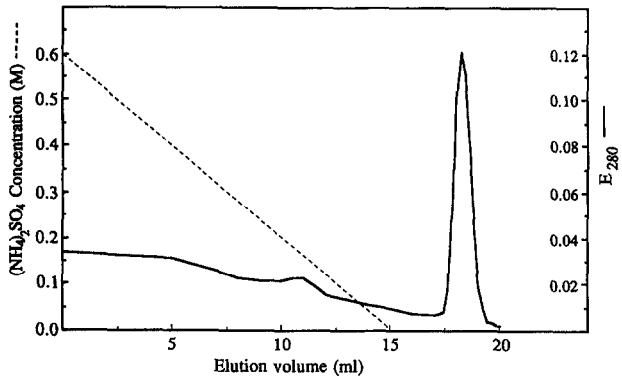

Fig. 1. Phenyl Superose column chromatography. Samples were applied to an FPLC phenyl Superose HR 5/5 column and eluted with a flow rate of 0.5 ml·min^{-1} and a gradient of 0.6–0 M $(NH_4)_2SO_4$ over 30 min. Fractions, 0.5 ml, were analysed for activity with laminaran and by a Western blot analysis.

mutant also showed a 15,000-fold decrease in this activity (data not shown). Mutagenesis of the E230D mutant back to wild type (D230E) resulted in an enzyme with V_{max} and K_m values of the wild type enzyme.

4. DISCUSSION

Gene disruption of the *EXG1* gene in *S. cerevisiae* produced no discernible difference in growth rate or morphology, consistent with previous studies [20,21] and the exoglucanase activity (< 1%) detected in the culture medium of the disruptant AWY-1 was probably due to other previously reported exoglucanases [22]. The use of strain AWY-1 as an expression host eliminated the possibility of contamination with exoglucanases of *S. cerevisiae* and facilitated the analysis of mutant forms of the *C. albicans* exoglucanase with very low activity. Overexpression of the *C. albicans* gene in strain AWY-1 had no noticeable effect on the growth and morphology and the enzyme was apparently expressed and processed as in *C. albicans*.

The recombinant exoglucanase was purified to homogeneity using a simple one-step procedure. As previously reported, the *C. albicans* exoglucanase has a high affinity for phenyl groups [2], and eluted at the end of the negative salt gradient on the phenyl Superose column. The wild type and mutant enzymes expressed in *S. cerevisiae* also eluted similarly indicating no gross changes in the structure.

Table II

Kinetic properties of exoglucanase mutants

	K_m (mM)		V_{max} (μmol·min^{-1}·mg^{-1})	
	K_m (mg·ml^{-1})			
	MUG	Laminaran	MUG	Laminaran
Wild type	2.0	2.2	60	300
E230D	0.6	3.9	0.004	0.018
E230Q	0.7	2.6	0.4	0.8

It is generally thought [23] that enzymatic hydrolysis of glycosidic linkages involves a carboxylate group, capable of forming a covalent glycosyl-enzyme intermediate (or an ion pair in the case of lysozyme). A proton donor which may also be a carboxylate is probably required to facilitate the reaction, however no detailed mechanistic studies have yet been carried out on a β-(1,3)-exoglucanase. Mutagenesis of the catalytic residues in the paradigmal lysozyme (Glu^{35}, Asp^{52}) resulted in a total loss of activity when measured on defined substrates, although the Asp^{52} mutant still retained 5% activity towards a complex substrate [3]. Other examples include the mutation of the putative proton donor in the endo-β-(1,4)-glucanase of *C. thermocellum* which resulted in a 4,000-fold decrease in activity [5], and decreases of 10,000-fold and 100–2,000-fold for the putative nucleophile and proton donor, respectively, of the β-glucosidase from *A. faecalis* [4].

Mutations of E^{230} in the *C. albicans* exoglucanase to Q and D resulted in decreases in activity of 150–400 and 15,000-fold, respectively. The difference in activity displayed by the two mutants (i.e., Q > D) however is not

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1  RGIQIILDRHRP    GSGGQSELWYTSQYP    ESRWISDWKMLADRYKNNP   TVIGADLH NEP H
2  LGAYCIVDIHNYARWNGGIIGQGGPT         NAQFTSLWSQLASKYASQS   RVW FGIM NEP H
3  VGIKVILDVHSPETDNQGHNYPLWYNTTIT     EEIFKKAWVWVAERYKNDD   TIIGFDLK NEP H
4  VGLKIMLDIHSIKTDAMGHIYPVWYDEKFT     PEDFYKACEWITNRYKNDD   TIIAFDLK NEP H
5  CGMYAIINLH    HDNTWIIPTYANEQRS      KEKLVKVWEQIATRFKDYD   DHLLFETM NEP R
6  LGIYVIIDWHILSDNDPNIY               KEEAKDFFDEMSELYGDYP   NVI YEIA NEP N
7  LGIYVIIDWHILNDGNPNQN               KEKAKEFFKEMSSLYGNTP   NVI YEIA NEP N
8  LNMYVIIDWHILSDNNPNTY               KEQAKSFFQEMAEEYGKYS   NVI YEIC NEP N
9  NDMYVIVDWHVHAPGDPRDP               YAGAEDFFRDIAALYPNNP   HII YELA NEP S
10 NDMYAIIGWHSHSAENN                  RSEAIRFFQEMARKYGNKP   NVI YEIY NEP
11 NNIRVWIDLHGAPGSQNGFDNSGLRDSYNFQNGDNTQVTLNVLNTIFKKYGGNEYSDVVIGIELL NEP L
12 NSLKVWVDLHGAAGSQNGFDNSGLRDSYKFLEDSNLAVTTNVLNYILKKYSAEEYLDTVIGIELI NEP L
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Fig. 2. Homology amongst yeast exoglucanases and cellulases. Data taken from [2] and [6]. (1) *Bacillus polymyxa* cel; (2) *Trichoderma reesei* EG3; (3) *Clostridium thermocellum* celB; (4) *Caldocellum saccharolyticum* celB; (5) *Clostridium thermocellum* celE; (6) *Bacillus subtilis* strain N_4-N_2; (7) *Bacillus subtilis* PAP115; (8) *Clostridium acetobutylicum* cel; (9) *Bacillus subtilis* strain 1139; (10) *Erwinia chrysanthemi* celZ; (11) *Candida albicans* EXG1; (12) *Saccharomyces cerevisiae* EXG1.

immediately explicable in terms of nucleophile function unless some deamidation of the E230Q mutant has occurred. Hence E^{230} might be more generally involved in transition-state stabilization, as a proton donor or in the maintenance of the required conformation. The active site of the *C. albicans* exoglucanase contains sub-sites for six glucose residues (H.J. Stubbs and P.A. Sullivan, unpublished results) and mutations in this region could increase the binding energy at one site but decrease the binding at others. This could account for the effects of the mutations on the K_m values; i.e. decreased values for 4-methylumbelliferyl glucoside and increased values for the polysaccharide laminaran (degree of polymerization = 28).

Baird et al. [6] first showed that the conserved NEP motif in two cellulases of the family A glycanases was essential for enzymatic activity and subsequently this motif has been noted in other members of the family [24]. A recent affinity labelling study of an endo-β-(1,4)-cellulase from *Trichoderma reesei* (a member of the family A glycanases) identified another residue, not the glutamic acid residue of the NEP motif, as the nucleophile and it was suggested that the E of the NEP is probably a proton donor [24]. Active site labelling studies as well as crystallographic studies which have been initiated [25] will be needed to establish the role of E^{230} in the exoglucanase. The demonstration of the importance of E^{230} further supports the hypothesis of the homology between yeast exoglucanases and cellulases.

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