J. Microbiol. Biotechnol. (2001), **11**(3), 529–533

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

A Gene Encoding β-Amylase from *Saprolegnia parasitica* and Its Expression in *Saccharomyces cerevisiae*

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Received: January 29, 2001
Accepted: May 17, 2001

Abstract The β-Amylase cDNA fragment from the oomycete *Saprolegnia parasitica* was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using degenerate oligonucleotide primers derived from conserved β-amylase sequences. The 5' and 3' regions of the β-amylase gene were amplified using the rapid amplification of cDNA ends (RACE) system. It consisted of an open reading frame of 1,350 bp for a protein of 450 amino acids. Comparison between the genomic and cDNA sequences revealed that the intron was not present in the coding region. The deduced amino acid sequence of the β-amylase gene had a 97% similarity to the β-amylase of *Saprolegnia ferax*, followed by 41% similarity to those of *Arabidopsis thaliana*, *Hordeum vulgare*, and *Zea mays*. The β-amylase gene was also expressed in *Saccharomyces cerevisiae* by placing it under the control of the alcohol dehydrogenase gene (*ADC1*) promoter.

Key words: β-Amylase gene, cloning and expression, rapid amplification of cDNA ends, *Saprolegnia parasitica*

Amylases and debranching enzymes play a very significant role in starch-processing industries. Among these enzymes, β-amylase (1,4-α-glucan maltohydrolase, EC 3.2.1.2) is an exo-enzyme that hydrolyzes starch by removing maltose stepwise from the nonreducing ends of the substrate. β-Amylases are widely distributed in plants as well as in Gram-positive spore-forming bacteria. Many bacterial and plant β-amylase genes have been cloned and well characterized [12, 13]. Only a few β-amylases of amylolytic filamentous fungi and yeasts have been isolated and characterized [1, 3, 6]. However, the sequences of β-amylase genes from fungi and yeasts have not yet been reported. Species of *Saprolegnia* belonging to oomycetes are saprobic on plant and animal remains. Most species can utilize starch and glycogen as the sole source of carbon [5]. Recently, the extracellular β-amylase gene from *Saprolegnia ferax* has been cloned [1, 10]. In this report, we describe the cloning of the β-amylase gene from *Saprolegnia parasitica*, its nucleotide sequence and deduced amino acid sequence, along with the expression of the gene in *Saccharomyces cerevisiae*.

Spl. parasitica CBS540.67 was used as a source of the β-amylase gene, and *Escherichia coli* JM83 was used for transformation and propagation of plasmids. pGEM-T Easy vector (Promega, Madison, U.S.A.) was used as a cloning vector and also as a subcloning vector for DNA sequencing. *S. cerevisiae* SHY3 [2], *S. cerevisiae* INVSC1 (Invitrogen, San Diego, U.S.A.) and *S. cerevisiae* W303-1A [10] were used as hosts for yeast transformation. In addition, pYES2 (Invitrogen, San Diego, U.S.A.) was used for the construction of yeast recombinant plasmids. GY medium (0.25% Bacto-yeast extract and 1% glucose) was used for the culture of *Spl. parasitica*. All procedures for the plasmid manipulation, preparation, and transformation of *E. coli* were performed by the methods described by Sambrook *et al.* [15]. Yeast cells were transformed according to the lithium acetate/DMSO method of Hill *et al.* [7]. Genomic DNA of 4-day-old cultures of *Spl. parasitica* grown in a GY medium was isolated following the procedure of Zhu *et al.* [20] using benzyl chloride (Sigma, St. Louis, U.S.A.). Total RNA was extracted from mycelia of 2-day-old cultures in the β-amylase induction medium containing 0.25% Bacto-yeast extract and 1% soluble starch using a TRI REAGENT (Molecular Research Center, Cincinnati, U.S.A.). Polyadenylated RNA was isolated using Poly AT tract mRNA isolation systems (Promega, Madison, U.S.A.) and cDNA was synthesized with a cDNA synthesis kit (TaKaRa, Japan). Two degenerate primers, which correspond to highly conserved regions present in β-amylase genes of different species, were designed to amplify β-amylase specific cDNA fragments: Bamy-F (5'-

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CAYCAKTGYGGNGGNAAYGTNGGNGAY-3') and Bamy-R (5'-GTCGTTCATYTCCATRCANGTAAAGCA-3'); K=G or T, R=A or G, Y=T or C, N=A or C or G or T. PCR conditions were 94°C for 0.5 min (denaturation), 50°C for 1 min (annealing), and 72°C for 1 min (extension). These conditions were repeated for 32 cycles. The amplified fragments were purified using a QIA quick Gel extraction kit (Qiagen, Chatsworth, U.S.A.), and directly cloned in a pGEM-T Easy vector. The 5' and 3' cDNA ends were amplified with a Marathon cDNA Amplification kit (Clontech, Palo Alto, U.S.A.) according to the manufacturer's instructions. Both 5'- and 3'-rapid amplification of cDNA ends (RACE) reactions were performed with internal gene-specific primers (pB1 and pB2) and the adaptor primer, AP1, that was supplied in the kit. The internal gene-specific primers, pB1 and pB2, were derived from the PCR-amplified partial β-amylase cDNA sequence. The primer sequences were as follows: pB1 (5'-CGAGTCGTAGCTCGTAAAC-TCGCC-3'); pB2 (5'-GGCGAGTTTACGAGCTACGAC-TCG-3'); and AP1 (5'-CCATCCTAATACGACTCACTA-TAGGGC-3'). The pB1 and AP1 primers, and the pB2 and AP1 primers, were used to obtain 5'-RACE product and 3'-RACE product, respectively. Two gene-specific primers (PE5' and PE3'), based on the sequences from 5'- and 3'-RACE products, were designed to clone the cDNA encoding β-amylase. The primer sequences were as follows: PE5' (containing a *Eco*RI site: 5'-TTGGAATTCAGCATGGT-GCGTCTGCTTCCTTCG-3') and PE3' (containing a *Eco*RI site: 5'-CGGGAATTCTTCTTATACAGGATCGGATAC-AAA-3'). PCR conditions were initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 0.5 min, annealing at 62°C for 0.5 min and extension at 72°C for 1 min, followed by extension at 72°C for 5 min. With these primers and a genomic DNA template, PCR was performed as described above to obtain the genomic DNA sequence encoding β-amylase. The nucleotide sequence of the β-amylase gene was determined by using an ABI PRISM 377 DNA sequencer. The DNASIS and PC/Gene sequence analysis software systems were employed to analyze the DNA sequences. The nucleotide sequence and deduced amino acid sequence were analyzed with databases using the BLAST programs. The amplified DNA fragments of the whole open reading frame (containing their own signal sequences) from cDNA templates, after being digested with *Eco*RI, were inserted into the same site of plasmid pYES2 containing the *ADC1* promoter in replacement of the *GAL1* promoter [16]. Yeast transformants were incubated and selected on YPD1S3 plates containing 1% Bacto-yeast extract, 2% Bacto-peptone, 1% glucose, 3% soluble starch, and 2% Bacto-agar for 24–72 h at 30°C. Amylolytic clones were detected by halos around colonies. Buffered yeast extract-peptone starch or glucose medium containing 0.1 M sodium phosphate buffer (pH 6.0), 1% Bacto-yeast extract, 2% Bacto-peptone and 2% soluble starch, or 2% glucose (BYPS or BYPD) was used to assay the amylase activity that was secreted by *Spl. parasitica* and *S. cerevisiae* transformants. β-Amylase activity was determined using the method of Bai *et al.* [1]. The pH level of the enzyme reaction mixture and the temperature employed were 6.0 and 50°C, respectively. One unit of β-amylase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per ml per min. To identify reaction products, a thin-layer chromatography (TLC) was carrried out by the method of Bai *et al.* [1].

To clone a part of the β-amylase cDNA sequence from *Spl. parasitica*, the published amino acid sequences for β-amylase from soybean, barley, sweet potato, *Arabidopsis thaliana*, and *Spl. ferax* [9, 10, 13] were compared. Based on the analysis, two degenerate primers designed from highly homologous regions of β-amylase sequences were synthesized, and PCR reactions were performed. The reaction yielded a cDNA fragment of 777 bp in length, which was expected to be a part of the DNA sequence encoding β-amylase from the deduced amino acid sequence analysis with BLAST programs. 5' and 3' RACE reactions were performed to clone the full-length β-amylase cDNA. RACE for the 3' end of the cDNA resulted in a 772 bp cDNA fragment. It was confirmed that, according to the sequence analysis, the 3' clone was identical to the overlapping region of the downstream sequence of the partial β-amylase cDNA fragment that also contained a part of a poly(A) tail. The 5' clone (666 bp) was also identical to the overlapping region of an upstream sequence of the partial β-amylase cDNA fragment. From these three cDNA fragments, a 1,415 bp cDNA sequence including the β-amylase coding region was finally confirmed by the sequence analysis. PCR amplification by primers PE5' and PE3' successfully yielded the cDNA and genomic DNA sequences encoding β-amylase. The nucleotide sequence of the *Spl. parasitica* β-amylase gene is shown in Fig. 1. Comparison of the cDNA and genomic DNA sequences revealed that, unlike most filamentous fungal genes, no intron was present in the coding region [10]. The open reading frame between the start codon and the termination codon (TAA) consisted of 1,350 bp, encoding a 450-amino acid protein with a molecular weight of 49,279 Da. The deduced 450-amino acid protein showed a hydrophobic region near the N-terminus and this region possibly represented a signal sequence with a good conformity of the typical signal sequence structure [19]. The putative signal sequence of pre-β-amylase consisted of 18 amino acids and its cleavage site appeared to be between Ala (position 18) and Glu (position 19). The sequence coincided well with the -3, -1 rule of von Heijne [18]. The deduced amino acid sequence of *Spl. parasitica* β-amylase gene, which differed from that of the *Spl. ferax* β-amylase gene (accession No. AF257459) at 14 residues, possessed a 97% similarity to the β-amylase of *Spl. ferax*. However, no marked

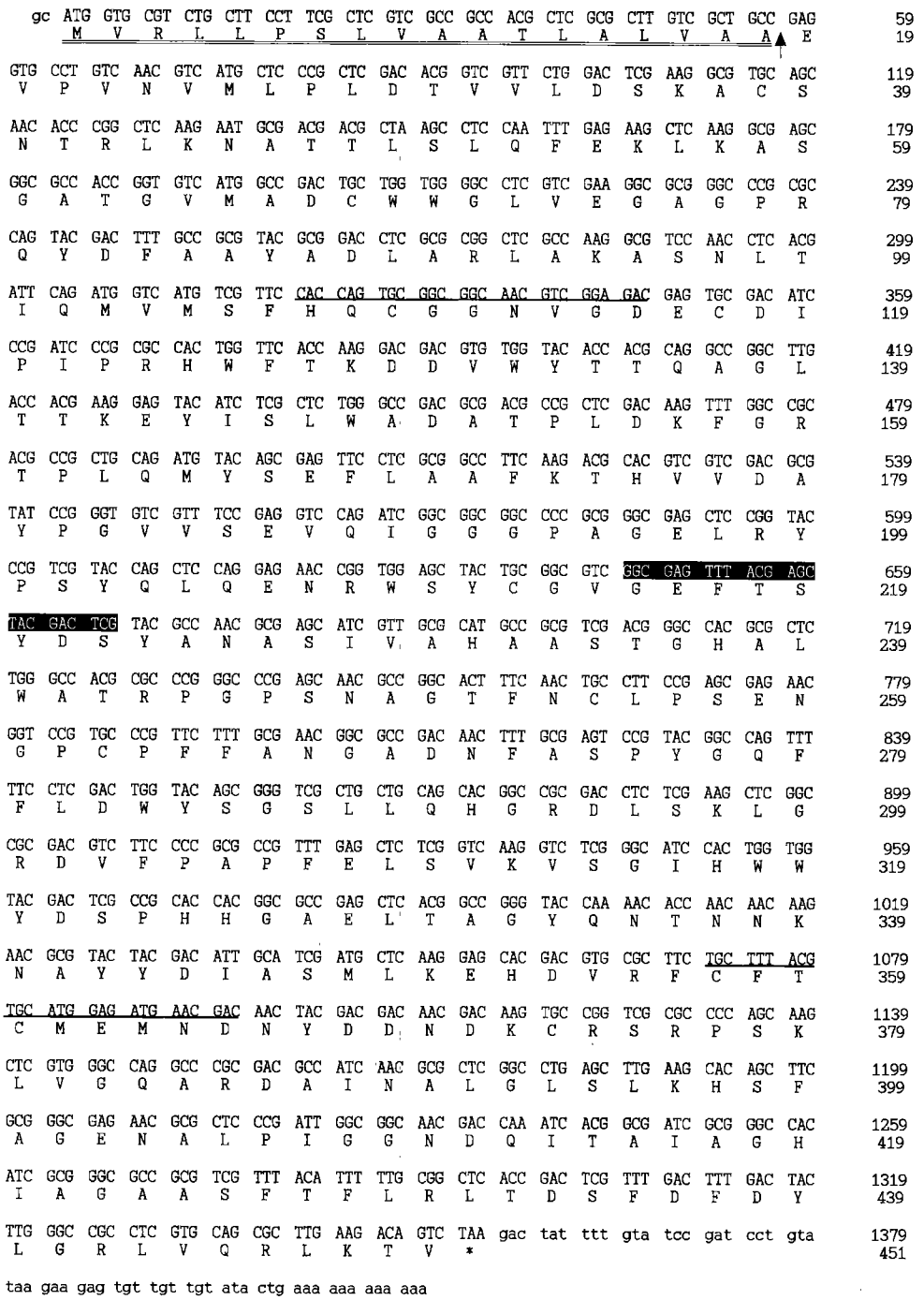

Fig. 1. Nucleotide sequence of the *Spl. parasitica* β-amylase gene and its deduced amino acid sequence.
The nucleotide sequence is not interrupted by any introns. The cleavage site of the 18-amino acid putative signal sequence (double underlined) is indicated by an upward arrow. The areas of degenerate primers, Bamy-F and Bamy-R, are marked by underlines. The gene specific primers, pB1 and pB2, which were used for RACE, are marked by a shadowed box. The nucleotide sequence reported in this paper was deposited in the GenBank database under an accession number AF336116.

differences in enzymatic properties were observed [1]. A comparison of the amino acid sequence of the *Spl. parasitica* β-amylase with those of other plant and bacterial β-amylases revealed the following degrees of identity: 41% with *Arabidopsis thaliana* (accession No. AJ250341), *Hordeum vulgare* (D21349), and *Zea mays* (AF068119), followed by 40% with *Oryza sativa* (L10345), and 31% with *Bacillus megaterium* (AJ250858). Seven regions of

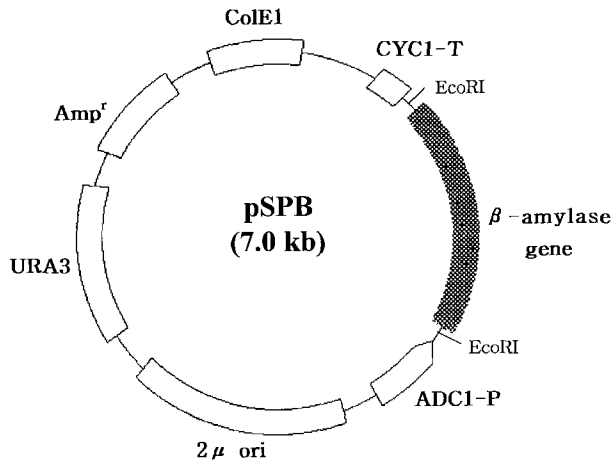

Fig. 2. Genetic map of plasmid pSPB containing the β-amylase gene.
CYC1-T, iso-1-cytochrome c gene terminator; ColE1, replicative origin of ColE1 (pUC); Amp^r, ampicillin resistance gene; 2 μ ori, yeast 2 μ origin; ADC1-P, alcohol dehydrogenase isoenzyme 1 gene promoter.

high homology in known β-amylases were found in the deduced amino acid sequence of the *Spl. parasitica* β-amylase [13]. However, a comparison of *Spl. parasitica* β-amylase with other fungal β-amylases could not be made, with an exception of *Spl. ferax* β-amylase [10], since the nucleotide sequence data of β-amylase genes from fungi have not yet been reported. The chimeric plasmid containing β-amylase-encoding cDNA was named pSPB (Fig. 2). Clear halos around the colonies of *S. cerevisiae* transformants harboring pSPB could be observed on YPD1S3 agar plates after 3 days of incubation at 30°C (Fig. 3). According to the analysis of the enzymatic reaction products by TLC, the amylase produced by the *S. cerevisiae* transformants

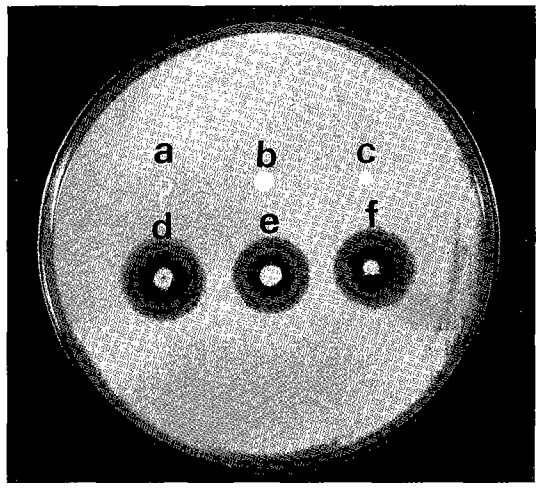

Fig. 3. Amylolytic activities of *S. cerevisiae* transformants harboring pSPB on YPD1S3 plates (YPD including 3% soluble starch).
Halos only developed around colonies secreting β-amylase. a, *S. cerevisiae* SHY3; b, *S. cerevisiae* W303-1A; c, *S. cerevisiae* INVSC1; d, SHY3/pSPB; e, W303-1A/pSPB; f, INVSC1/pSPB.

Table 1. β-Amylase activities of *Spl. parasitica* and *S. cerevisiae* transformants.

Strains	β-Amylase activity (U/ml)	
	BYPD[a]	BYPS[b]
Spl. parasitica	0.3[c]	3.0
S. cerevisiae SHY3/pSPB	13.0	8.5
S. cerevisiae W303-1A/pSPB	9.7	2.6
S. cerevisiae INVSC1/pSPB	9.5	1.3

[a]BYPD. Buffered yeast extract-peptone glucose medium containing 0.1 M sodium phosphate buffer (pH 6.0), 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose.
[b]BYPS. BYP supplemented with 2% soluble starch.
[c]Values are the means of results from triplicate experiments, and they are expressed in β-amylase activities present in the culture supernatants which were obtained from 4-day-old cultures in the above media.

harboring pSPB degraded starch to form maltose exclusively which could not hydrolyze maltotriose and either α- or β-cyclodextrins, along with the fact that it was also sensitive to 1 mM *p*-chloromercuribenzoate, similar to other β-amylases [1, 8]. These results confirmed that the product of the *S. cerevisiae* transformants was β-amylase [9]. Cell-free culture fluids from *Spl. parasitica* and various *S. cerevisiae* transformants were assayed for β-amylase activity (Table 1). β-Amylase production by *S. cerevisiae* transformants occurred in the media containing either starch or glucose, that was not diminished by the presence of glucose, unlike that by *Spl. parasitica*. This result was due to the modified *ADC1* promoter without the regulatory site responsible for the repression of *ADC1* gene expression in the presence of glucose [11]. *S. cerevisiae* SHY3 (maltose-assimilating strain) harboring pSPB grew on starch as the carbon source, since starch hydrolysis by β-amylase primarily liberated maltose. However, *S. cerevisiae* W303-1A and INVSC1, which were not able to assimilate maltose, utilized starch inefficiently, when transformed with pSPB. The relatively low β-amylase activity of these transformants observed in the starch containing media was possibly due to poor growth. The *Spl. parasitica* β-amylase gene could now be coexpressed with both the α-amylase gene and glucoamylase gene in industrial yeasts [4, 8, 14, 17], and used for the production of alcohol and single-cell protein from starch biomass.

Acknowledgment

This work was supported by the Brain Korea 21 Project.

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