Short Communication

Cloning and Expression of the
Rhizopus Glucoamylase
Gene in YeastToshihiko ASHIKARI, Norihisa NAKAMURA,
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A variety of microorganisms produce glucoamylases (EC 3.2.1.3) which hydrolyze starch progressively from its non-reducing ends to yield glucose. These enzymes are useful in saccharifying starch in the commercial processes of glucose and alcohol production. Recently, the glucoamylase genes from *Aspergillus awamori*¹⁾ and *Asp. niger*²⁾ have been cloned and found to be homologous to each other. The glucoamylase produced by *Rhizopus* sp. is of great importance in that it

possesses greater hydrolyzing activity than glucoamylase produced by other species.

In this paper we report the molecular cloning of a glucoamylase gene from *Rhizopus* sp. and its expression in yeast. The details will be presented elsewhere.

The glucoamylase was purified from *Rhizopus* sp., and its N-terminal amino acid sequences and C-terminus were determined to be Ala-Ser-Ile-Pro- and -Ala, respectively. Further sequence studies revealed that the glucoamylase contains the sequence, -Thr-Trp-Ser-His-Ala. Oligonucleotides which have sequences, 5'-ACNTGGTCNCAQGC-3', corresponding to this amino acid sequence were synthesized (Q, T or C; N, any base) and used as probes. Chromosomal DNA isolated from spores of *Rhizopus* sp. was digested completely with *Hind*III and fractionated by a sucrose density gradient. DNA fragments in the fraction hybridized with synthetic oligomer probes were inserted into the *Hind*III site of pBR322. *E. coli* WA 802 cells were transformed with the resulting plasmids.

The transformants were screened for hybridization with the synthetic probes. Three clones harboring essentially the same plasmid, which contain the 4.3kb *Hind*III fragment

FIG. 1. Construction of the Glucoamylase Gene to be Expressed in Yeast.

Restriction maps are presented for the glucoamylase genes derived from cDNA (A) and chromosomal DNA (C), and constructed from A and C for expression in yeast (B). The entire glucoamylase gene without intervening sequences was constructed as follows. New *Sal*I sites were generated in A and B at the position indicated by a dashed line by *in vitro* mutagenesis. After recombination of A and C at the *Sal*I site, the sequence at the *Sal*I site was altered to the original sequence by *in vitro* mutagenesis. ATG and TAA indicate the initiation and termination codons of glucoamylase gene, respectively. Black boxes are the intervening sequences (these will be described elsewhere).

from *Rhizopus* DNA, were obtained and one of them was designated as pRGA39. A physical map of the DNA fragment inserted in pRGA39 is shown in Fig. 1C. The nucleotide sequence covering the glucoamylase coding region and both the 5'- and 3'-flanking regions was determined by the dideoxy-termination method.³⁾ Expression of the chromosomal glucoamylase gene in yeast was unsuccessful probably due to the existence of intervening sequences.

A cDNA library was constructed from *Rhizopus* total poly-(A) RNA according to the method of Okayama and Berg⁴⁾ and screened as to hybridization with the nick-translated DNA fragment containing the chromosomal glucoamylase gene as a probe. All clones harbored plasmids containing the cDNA glucoamylase gene which lacked the N-terminal regions in varying degrees and one of these plasmids was designated as pCGA239 (Fig. 1A). A recombinant plasmid, pCGA449 (Fig. 1B), which contained the 5'- and 3'-flanking regions and the entire coding sequence without intervening sequences was constructed from pRGA39 and pCGA239 through *in vitro* mutagenesis.⁵⁾ A large open reading frame encoding 604 amino acid residues is the best candidate for glucoamylase. Terminal amino acid sequence determined for the mature glucoamylase was found to be the 26th~29th residues of this amino acid sequence deduced from the nucleotide sequence.

A 2.3 kb *Hind*III-*Pvu*II fragment covering the glucoamylase gene from pCGA449 was introduced into the *Hind*III-*Pvu*II sites of yeast vector pYE227⁶⁾ which contained 2 μ m replication origin in yeast and a *LEU2*-selectable marker. The resulting plasmid, pYGA201, was introduced into the yeast strain, XS-30-2B (α , *trp1*, *leu2*, *his3*, *ura3*), which was constructed in our laboratory. One of the transformants was examined for growth on a medium containing starch as the sole carbon source (Fig. 2). The transformant grew at approximately the normal rate and glucoamylase was secreted into the medium in an amount of 0.005 u/ml. One glucoamylase unit

FIG. 2. Growth of the Transformants on Starch Medium.

Cells were cultivated aerobically at 30°C. The growth rate of the cells was determined from the absorbance at 660 nm. The yeast, XS-30-2B, harboring pYGA201 which contained the entire glucoamylase gene was grown on YPS (2% starch, 2% polypeptone and 1% yeast extract) medium (—▲—). The control yeast, XS-30-2B, without the plasmid was grown on YPS medium (—●—) or YPD (2% glucose, 2% polypeptone and 1% yeast extract) medium (—○—).

was defined as the amount of glucoamylase that released 1 μ mol of glucose per minute from soluble starch at 37°C. The production of the glucoamylase was confirmed immunologically using anti-glucoamylase antibody. These results indicate that the promoter and the putative signal sequence of the *Rhizopus* glucoamylase gene were functionally active in yeast.

High expression of the glucoamylase gene in yeast and efficient ethanol production from starch materials are now being utilized in our laboratory.

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