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Cloning, Sequencing of Glucoamylase cDNA from *Aspergillus niger* and It's Expression in *E.coli*

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Abstract A cDNA library was constructed using poly(A)$^+$mRNA of *Aspergillus niger* 3758 as template and phage λgt 10 DNA as vector. The glucoamylase gene was screened using a 456bp DNA fragment coding for 152 amino acid residues of *A.niger* glucoamylase as probe. 11 positive plaques were identified by hybridization in situ with ^{32}P-labelled probe. DNA from 6 of the 11 positive clones were isolated and restriction analysis and Southern hybridization were done. All of these phages DNA carry inserts with different sizes and hybridize with the DNA probe, which indicate the cloning of glucoamylase gene of *A. niger*. The physical map of the 2.1 kb cDNA fragment was constructed and its nucleotide sequence determined. From the sequence analysis, the cloned fragment consists of the 5′-noncoding sequence, the intact structural gene coding for glucoamylase and the 3′-nontranslated region. The cloned cDNAs were transferred to λgt 11, the resultant phages were used to infect *E. coli*. The plaques formed were transferred to nitrocellulose filter and probed with *A. niger* glucoamylase antibody. Positive result was obtained from the plaque carrying the 2.1kb fragment. This fragment was also ligated to expression plasmid pPL2 which was then transformed to *E.coli* JF1125, a new protein band was found by SDS-PAGE. These results show that the cloned glucoamylase cDNA was expressed in *E.coli*.

keywords cloning, sequencing, expression, glucoamylase cDNA, *Aspergillus niger*, *E. coli*

1 Introduction

Glucoamylase (EC 3. 2. 1. 3.) is a class of extracellular enzymes secreted by a variety of organisms, which hydrolyzed starch and related malto-oligosaccharides to yield glucose[1]. *Aspergillus niger* secretes two glycosylated forms of glucoamylase designated GAI and GAII[2~4]. Both forms are encoded by a single gene and have the same amino terminal sequence[4~7] and differ in the length of their carboxyl terminal seque-

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nce as a consequence of differential RNA splicing[8]. Glucoamylases have been widely used in commercial processes requiring the saccharification of starch, like in the food and the fermentation industry. The glucose produced is used as a substrate for the enzymatic production of fructose syrup and as a feed source for fermentations of various organisms. The glucoamylases used in industry are in the main produced by *A. niger*, *A. awamori* and *Rhizopus oryzae*[9], *Saccharomyces diastaticus*[10] and *Saccharomycopsis fibuligera*[11] also secrete glucoamylases. The cloning of glucoamylase gene would be of great value for the improvement of the industrial strain, for the construction of starch termentable *Saccharomyces cerevisiae* strain by introducing the cloned gene into it and for the construction of fungal expression and secretion system. In this paper, we report the molecular cloning and sequencing of the glucoamylase cDNA and its expression in *E. coli*.

2 Materials and Methods

2.1 Strains, phages and plasmids

Aspergillus niger 3758, the glucoamylase producing strain as a DNA donor, is provided by Food Fermentation Institute of Guangdong.

Phages λgt 10, λgt 11 and their hosts *E. coli* BNN93, BNN102, Y1088, Y1090 is provided by Shanghai Institute of Biochemistry.

E. coli HB101 containing the plasmid p4EX is a gift of Dr. Amsden from Infergene Company, USA.

Plasmid pPL2 and *E. coli* JF1125 is provided by Dr. Liu Sin Yen of Institute of Biochemistry.

2.2 Main reagents and media

Zeiss medium is used for the cultivation of *A. niger* and L Broth for bacteria growth.

The reagents used for mRNA isolation and purification, cDNA synthesis and cloning are as described in reference[12] and those for nucleic acid hybridization as in [13]. Restriction enzymes, T_4 DNA ligase and EcoRI linker are purchased from Promega or Sino-American Biotechnology Company

M_{13} Cloning and Sequencing Kits are from GIBCO-BRL.

Nitrocellulose filter is from Millipore.

2.3 Isolation and purification of poly(A)$^+$mRNA and synthesis and cloning of cDNA are as described in reference[12]

2.4 *In situ* plaque hybridization

The probe is a 456 bp DNA fragment in plasmid p4EX coding for the

329～481 amino acid sequence of *A. niger* glucoamylase which was excised from the plasmid by EcoRI-HindIII double digestion and isolated by 5%～15% sucrose density gradient centrifugation. The isolated fragment was labelled with ^{32}P-dCTP by nick translation and used as probe for *in situ* plaque hybridization and for Southern blot assay.

For *in situ* hybridization, the dry nitrocellulose circle was placed neatly onto the surface of the top agarose so that it comes into direct contact with the plaques and let the DNA transferred onto the nitrocellulose filter. After 1～2 minutes, the filter was peeled off and immersed, with DNA side up, in 1.5 mol/L NaCl and 0.5 mol/L NaOH for denaturation. The filter was then transferred into neutralizing solution(1.5 mol/L NaCl, 0.5 mol/L Tris-HCl pH8.0) for 5 minutes. After rinsed with 2× SSPE, the DNA was fixed to the filter by baking for 2 hours at 80℃ in a vacuum oven. The filter was hybridized with ^{32}P-labelled denatured probe at 42℃ overnight after prehybridization for 4 hours at 42℃.

The hybridized filter was rinsed, dried and autoradiographed. Phages were picked up from the positive plaques and infected host *E. coli* BNN-102 which were plated on the top agarose and the second hybridization was done.

2.5 Southern blot assay

DNA extracted from positive plaques of *in situ* hybridization was digested with EcoRI. After agarose gel electrophoresis, the DNA was transferred to nitrocellulose filter by Southern transfer[14].

The filter with transferred DNA was prehybridized at 68℃ for 3 hours and hybridized to ^{32}P-labelled probe at 68℃ overnight. The hybridized filter was then autoradiographed after rinsing and drying.

2.6 Physical mapping and DNA sequencing

The cloned cDNA was removed from λgt 10 DNA with EcoRI digestion and isolated by low melting temperature agarose (LMTA). The purified DNA fragment was then digested with several restriction endonucleases. Each enzyme was used according to manufacturer's specification, physical map was constructed according to the data of single and double digestions of the used enzymes.

The DNA sequence was analysed in bacteriophage M_{13} by the dideoxy chain-termination method[15,16]. The Sequencing kit of BRL was used and the protocol was according to the instruction manual.

2.7 Preparation of glucoamylase antisera from *A. niger*[17,18]

Six guinea pigs were used as immuned animals (two of them as control),

after dilution, the antigen was mixed with adjuvent and ground into emulsion. 0.5ml of antigen emulsion was injected subcutaneously, 4 injections were done for every 2 weeks interval. Blood was taken from the immunized guinea pigs after the last digestion and serum isolated. The immuno-double extension method[17] was used to determine the titer. The antiserum was distributed into vials after adding sodium azide and storaged at -20℃.

2.8 Expression analysis of the cloned cDNA

The protocol of reference[19] was used. The cloned glucoamylase cDNA was excised from recombinant λgt 10 DNA with EcoRI and isolated with low melting temperature agarose and inserted into the EcoRI site of λgt 11 DNA, in vitro packaged and infected *E. coli* Y1088. The recombinant phages were picked up and used to infect *E. colic* Y1090. After IPTG induction, the plaques were then transferred to nitrocellulose filter and reacted with antiglucoamylase antibody and then with ^{125}I labelled protein A from *Staphylococcus aureus*. The filter was rinsed, dried and autoradiographed.

3 Results and Discussion

3.1 Constrution of cDNA library

The synthetic linker method was used to construct the cDNA library of *Aspergillus niger* 3758. The ds cDNA[12] were first methylated with EcoRI methylase, ligated with phosphorylated EcoRI linker, digested with EcoRI and then ligated with EcoRI digested λgt 10 DNA. The ligated product was packaged *in vitro* and the packaged phages were used to infect *E. coli* BNN102. 100ng of cDNA was used for cloning and 3.6×10^4 recombinant plaques obtained, the cloning efficiency is 3.6×10^5 pfu/μg cDNA.

3.2 Screening of glucoamylase cDNA from cDNA library

1.5×10^4 recombinant plaques were used for *in situ* hybridization to the ^{32}P-labelled probe. After screening and rescreening, 11 positive plaques were obtained(Fig.1). DNAs were extracted from 6 clones of these phages by rapid isolation method, cleaved with EcoRI and electrophoresed on agarose gel. The gel pattern shows that these recombinant phages contain insert fragments of 0.8, 1.7, 2.1, 2.5, 3.5 and 4.3kb respectively (Fig.2). Southern blot assay showed that all of these inserts were positively hybridized to the DNA probe of *A. niger* glucoamylase(Fig.3). These results indicats the cloning of glucoamylase cDNA of *A. niger* 3758.

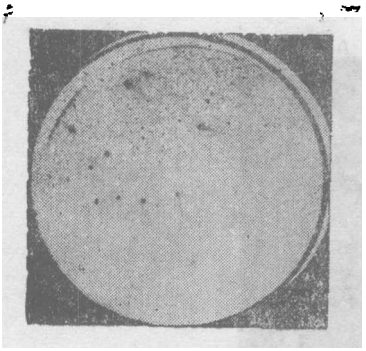

Fig.1 Autoradiograph of *in situ* hybridization of recombinant plaques with ladelled DNA probe of glucoamylase Nitrocellulose filter was blotted from a 120mm plate seeded with about 1×10^4 plques of the amplification stock. Phage DNA was transferred and immobilised in situ on nitrocellulose filter. The filter was exposed to ^{32}P-labelled DNA probe

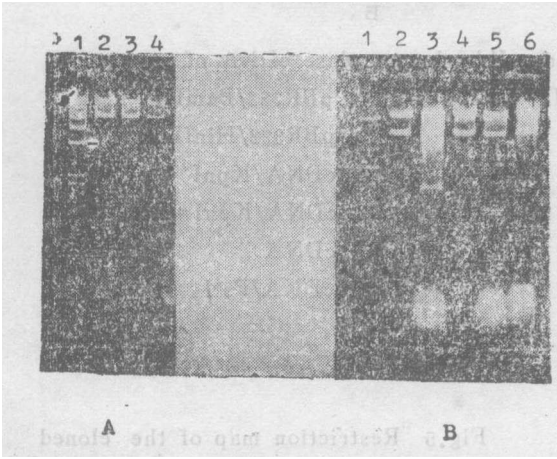

Fig.2 Restriction analysis of DNA from positive plaques. Lane(1) of A and B are λcI857S7DNA digested with HindIII, lane(4) of A and B are λgt10 DNA digested with EcoRI, the others are the positive phage DNAs cleaved with EcoRI

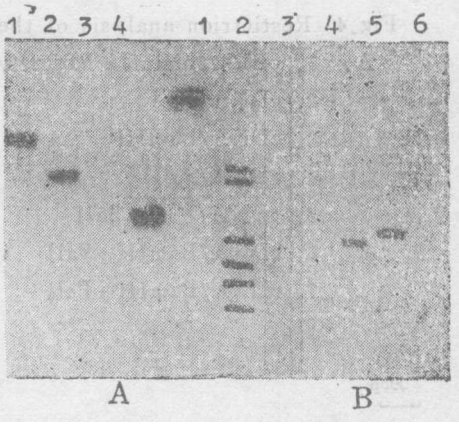

Fig.3 Autoradiograph of Southern blotting of DNAs from *in situ* hybridization positive phages. The phage DNAs in Fig. 2 were transferred to the filter and hybridized to ^{32}P-labelled DNA probe. Lane(1) of Fig. B is λcI 857S7 DNA digested with HindII

3.3 Physical mapping and sequencing of the cloned cDNA

One of the 2.1kb cloned cDNA fragment was used for further characterization. This fragment, after isolation by LMTA, was digested with BamHI, BglII, ClaI, HindIII, KpnI, PstI, SalI and XbaI respectively. The electrophoresis pattern(Fig.4) shows that there is one site for BamHI, ClaI, PstI and SalI, 2 sites for KpnI and nothing for BglII, HindIII and XbaI on the cDNA fragment. Double digestions were also done with BamHI, ClaI, KpnI, PstI and SalI. The physical map was thus constructed according to the data from single and double reciprocal digestions and is shown in Fig. 5.

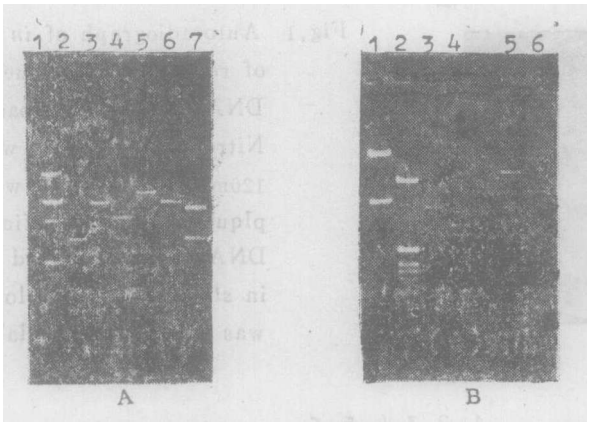

Fig.4 Restictrion analysis of the cloned 2.1kb glucoamylase cDNA of *A. niger*

A. 1. pBR322/HinfI + PBR322/BamHI + PstI
2. cDNA/PtsI
3. cDNA/BamHI
4. cDNA/SalI
5. cDNA/PstI + SalI
6. cDNA/BamHI + SalI
7. cDNA/BamHI + PstI

B. 1. pBR322/BamHI + PstI
2. pBR322/HinfI
3. cDNA/KpnI
4. cDNA/KpnI + PstI
5. cDNA
6. cDNA/PstI

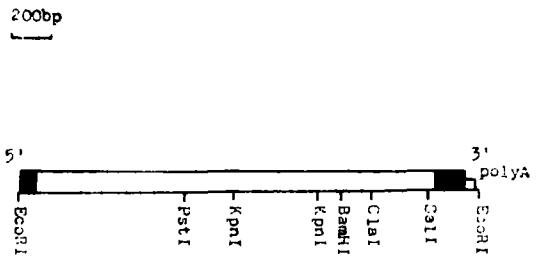

Fig.5 Restriction map of the cloned 2.1kb glucoamylase cDNA of *A. niger*. Black boxes are the 5- and 3-untranslated regions, poly A is shown by half thickness

The nucleotide sequence of the approximately 2.1kb cDNA was determined by the Sanger dideoxy chain termination method, the result of the nucleotide sequencing is shown in Fig.6 and Fig.7, from the sequence analysis, the cloned fragment is 2161bp long, and consists of 41bp 5'-noncoding sequence, 1920bp structural gene coding for the 640AA residues of glucoamylase GAI and 132bp 3'-noncoding sequence and poly A(56bp). The data show that the cloned 2.1kb cDNA is an intact gene for glucoamylase GAI. The sequence is in total agreement with the results of Boel[8] at 5' untranslated region and the structural gene but different at 3' noncoding region. The terminal codon is TAG instead of TGA, followed by four different bases and an extra AGCCAGCGG sequence is found in our case.

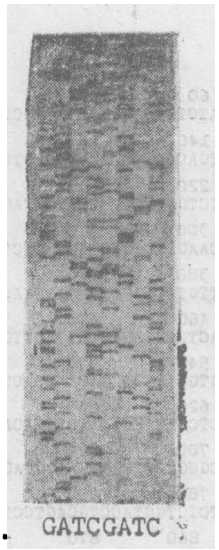

Fig.6 Autoradiograph of a 8% acrylamide/8M urea gel electrophoresis of sequencing products. Reactions were performed using single strand M_{13} *mp18* and *mp19* as the templates. ^{32}P-labelled DNA fragments were synthesized by DNA polymerase I(large fragment) and terminated by dideoxy

3.4 Expression of the cloned glucoamylase cDNA in *E. coli*

To analyse the expression of cloned glucoamylase cDNA in *E. coli*, the fragment of 2.5, 2.1 and 1.7kb were removed from the recombinant phage λgt 10 DNA by EcoRI digestion and inserted into the EcoRI site of λgt 11 DNA which is an expression vector. The packaged phages were used to infect E. coli. Plaques formed were immobilized on nitrocellulose filter and probed with *A. niger* glucoamylase antibody. The results is shown in Fig. 8. It shows the specific immunoreaction between the cell lysate infected by the recombinant phage λgt 11 containing the 2.1kb cDNA and the antiglucoamylase antibody labelled with ^{125}I. The result indicates that the cloned 2.1kb glucoamylase cDNA can expressed in *E. coli*.

The 2.1kb cDNA was also ligated to expression plasmid pPL2 which was then transformed to *E. coli* JF1125. The new protein band was found on the SDS-polyacrylamide gel electrophresis of soluble proteins from transformed *E. coli* JF1125, which indicates the exsistence of expression product in transformed cell(data not shown).

The 1.7kb fragment is too short to encode the 640 amino acid sequence of glucoamylase(the sequencing data indicate its deletion at 5′ end) or is not in frame, while the 2.5kb cDNA may have too long 5′ end and 3′ end noncoding sequences[20], they are not expressed in *E. coli*.

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        10        20        30        40        50        60        70        80
AATTCCGAGAGCCTGAGCTTCATCCCCAGCATCATTACACGTCAGCAATGTCGTTCCGATCTCTACTGGCCCTGAGCGGC
        90       100       110       120       130       140       150       160
CTCGTCTGCACAGGGTTGGCAAATCTGATTTCCAAGCCCGCCACCTTGGATTCATGGTTGAGCAACGAAGCGACCGTGGC
       170       180       190       200       210       220       230       240
TCGTACTGCCATCCTGAATAACATCGGGGCGGACGGTGCTTGGGTGTCGGGCGCCGGACTCTGGCATTGTCGTTGCTAGTC
       250       260       270       280       290       300       310       320
CCAGCACGGATAACCCGGACTACTTCTACACCTGGACTCGCGACTCTGGTCTCGTCCTCAAGACCCTCGTCGATCTCTTC
       330       340       350       360       370       380       390       400
CGAAATGGAGATACCAGTCTCCTCTCCACCATTGAGAACTACATCTCCGCCCAGGCAATTGTCCAGGGTATCAGTAACCC
       410       420       430       440       450       460       470       480
CTCTGGTGATCTGTCCAGCGGCGCTGGTCTCGGTGAACCCAAGTTCAATGTCGATGAGACTGCCTACACTGGTTCTTGGG
       490       500       510       520       530       540       550       560
GACGGCCGCAGCGAGATGGTCCGGCTCTGAGAGCAACTGCTATGATCGGCTTCGGGCAGTGGCTGCTTGACAATGGCTAC
       570       580       590       600       610       620       630       640
ACCAGCACCGCAACGGCCATTGTTTGGCCCCTCGTTAGGAACGACCTGTCGTATGTGGCTCAATACTGGAACCAGACAGG
       650       660       670       680       690       700       710       720
ATATGATCTCTGGGAAGAAGTCAATGGCTCGTCTTTCTTTACGATTGCTGTGCAACACCGCGCCCTTGTCGAAGGTAGTG
       730       740       750       760       770       780       790       800
CCTTCGCGACGGCCGTCGGCTCGTCCTGCTCCTGGTGTGATTCTCAGGCACCCGAAATTCTCTGCTACCTGCAGTCCTTC
       810       820       830       840       850       860       870       880
TGGACCGGCAGCTTCATTCTGGCCAACTTCGATAGCAGCCGTTCCGGCAAGGACGCAAACACCCTCCTGGGAAGCATCCA
       890       900       910       920       930       940       950       960
CACCTTTGATCCTGAGGCCGCATGCGACGACTCCACCTTCCAGCCCTGCTCCCCGCCGCCGCTCGCCAACCACAAGGAGG
       970       980       990      1000      1010      1020      1030      1040
TTGTAGACTCTTTCCGCTCAATCTATACCCTCAACGATGGTCTCAGTGACAGCGAGGCTGTTGCGGTGGGTCGGTACCCT
      1050      1060      1070      1080      1090      1100      1110      1120
GAGGACACGTACTACAACGGCAACCCGTGGTTCCTGTGCACCTTGGCTGCCGCAGAGCAGTTGTACGATGCTCTATACCA
      1130      1140      1150      1160      1170      1180      1190      1200
GTGGGACAAGCAGGGGTCGTTGGAGGTCACAGATGTGTCGCTGGACTTCTTCAAGGCACTGTACAGCGATGCTGCTACTG
      1210      1220      1230      1240      1250      1260      1270      1280
GCACCTACTGTTCGTCCAGTTCGACTTATAGTAGCATTGTAGATGCCGTGAAGACTTTCGCCGATGGCTTCGTCTCTATT
      1290      1300      1310      1320      1330      1340      1350      1360
GTGGAAACTCACGCCGCAAGCAACGGCTCCATGTCCGAGCAATACGACAAGTCTGATGGCGAGCAGCTTTCCGCTCGCGA
      1370      1380      1390      1400      1410      1420      1430      1440
CCTGACCTGGTCTTATGCTGCTCTGCTGACCGCCAACAACCGTCGTAACTCCGTCGTGCCTGCTTCTTGGGGCGAGACCT
      1450      1460      1470      1480      1490      1500      1510      1520
CTGCCAGCAGCGTGCCCGGCACCTGTGCGGCCACATCTGCCATTGGTACCTACAGCAGTGTGACTGTCACCTCGTGGCCG
      1530      1540      1550      1560      1570      1580      1590      1600
AGTATCGTGGCTACTGGCGGCACCAGTACGACGGCTACCCCCACTGGATCGGGCAGCGTGACCTCGACCAGCAAGACCAC
      1610      1620      1630      1640      1650      1660      1670      1680
CGCGACTGCTAGCAAGACCAGCACCAGTACGTCATCAACCTCCTGTACCACTCCCACCGCCGTGGCTGTGACTTTCGATC
      1690      1700      1710      1720      1730      1740      1750      1760
TGACAGCTACCACCACCTACGGCGAGAACATCTACCTGGTCGGATCGATCTCTCAGCTGGGTGACTGGGAAACCAGCGAC
      1770      1780      1790      1800      1810      1820      1830      1840
CGCATAGCTCTGTAGTGCTGACAAGTACACTTCCAGCGACCCGCTCTGGTATGTCACTGTGACTCTGCCCGCTGGTGAGTC
      1850      1860      1870      1880      1890      1900      1910      1920
GTTTGAGTACAAGTTTATCCGCATTGAGAGCGATGACTCCGTGGAGTGGGAGAGTGATCCCAACCGAGAATACACCGTTC
      1930      1940      1950      1960      1970      1980      1990      2000
CTCAGGCGTGCGGAACGTCGACCCGCGACGGTGACTGACACCTGGCGGTAGAAACTCAATCCATTTCGCTATAGTTAAAGG
      2010      2020      2030      2040      2050      2060      2070      2080
ATGGGGATGAGGGCAATTGGTTATATGATCATGTATGTAGTGGGTGTGCATAATAGTAGTGAAATGGAAGGCCAAGTCAT
      2090      2100      2110      2120      2130      2140      2150      2160
GTGATTGTAATTCAGCCAGCGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGG

AATT
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Fig.7 The compete nucleotide sequence of the cloned 2.1kb glucoamylase cDNA of *A. niger*. Initiation and termination codon are underlined

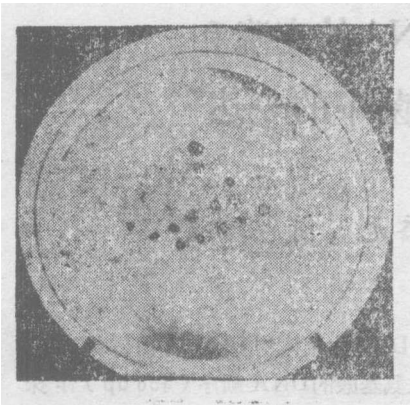

Fig. 8 Autoradiograph of immunoblotting assay of phage λgt 11 containing glucoamylase cDNA fragments. Phage DNA were transforred to nitrocellulose filter which was then probed with ^{125}I-labelled glucoamylase antibody. The positive plaques are those carrying the 2.1kb fragment

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黑曲霉葡萄糖淀粉酶cDNA的克隆和序列分析及其在大肠杆菌中的表达*

李文清** 罗进贤 张添元 何 玲

摘 要 以poly(A)⁺mRNA为模板，噬菌体λgt 10 DNA为载体构建了黑曲霉3758的cDNA的文库。用编码黑曲霉葡萄糖淀粉酶329—418位氨基酸的DNA顺序(456 bp)作探针从该文库中筛选葡萄糖淀粉酶cDNA。采用原位噬菌斑杂交法获得11个阳性噬菌斑。从其中6个噬菌体中提取DNA进行限制酶切分析和印迹法转移杂交试验，发现这些阳性噬菌体DNA含有不同大小的插入片段，它们都能与DNA探针杂交，表明已克隆了葡萄糖淀粉酶的cDNA。采用单酶切及交叉双酶切法制定了2.1 kb cDNA的物理图并对2.1 kb片段作了全序列分析。序列分析结果显示，克隆的2.1 kb片段含5′端非编码区，编码葡萄糖淀粉酶的结构基因及3′端非编码区。克隆的cDNA片段转移至表达载体λgt11，感染大肠杆菌后，产生的噬菌斑转移硝酸纤维素滤膜后与^{125}I标记的抗葡萄糖淀粉酶抗体进行免疫反应，结果证明含2.1 kb cDNA的λgt11表现葡萄糖淀粉酶阳性。2.1 kb片段也曾转至质粒pPL2，转化大肠杆菌JF 1125。细胞裂解物在SDS-PAGE电泳中可看到新的蛋白带。这些结果表明，克隆的黑曲霉葡萄糖淀粉酶cDNA可以在大肠杆菌中表达。

关键词 黑曲霉，葡萄糖淀粉酶，cDNA克隆，序列分析，基因表达，大肠杆菌

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