CONSTRUCTION OF A YEAST VECTOR DIRECTING THE SYNTHESIS AND RELEASE OF BARLEY (1→3,1→4)-β-GLUCANASE

by

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Two cloned cDNA sequences from barley aleurone, representing complementary parts of a barley (1→3,1→4)-β-glucanase gene were joined together and fused in frame to a DNA fragment encoding the mouse α-amylase signal peptide. The triplet determining the amino terminal amino acid residue of native β-glucanase was changed from ATC (isoleucine) to CTA (leucine) in order to accommodate the α-amylase signal sequence in the correct reading frame. This chimaeric cDNA was inserted in the yeast expression vector pMA56 behind the promoter from the yeast alcohol dehydrogenase I gene. Yeast cells harbouring such plasmids synthesize β-glucanase and secrete the enzyme to the culture medium.

1. INTRODUCTION

The enzyme (1→3,1→4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73) confers to an organism the enzymatic activity for the degradation of (1→3,1→4)-β-glucans. It is an endo-β-glucanase and it hydrolyses (1→4)-β-glycosidic bonds provided they follow a (1→3)-β-glycosidic bond. The substrate for this enzyme, (1→3,1→4)-β-glucan, is a major component of the endosperm cell walls of many cereals e.g. barley and oats (1). In barley approximately 75% of the endosperm cell wall material is contributed by (1→3,1→4)-β-glucans (14). During germination as well as in malting β-glucanase isoenzymes are secreted from the aleurone and scutellum, and released into the endosperm (26, 37, 45). Following degradation of the β-glucans in

the endosperm cell walls amylolytic and proteolytic enzymes can reach and degrade the endosperm starch and protein storage components.

Insufficient β-glucanase activity during malting gives rise to high levels of residual high molecular weight β-glucans in the wort (3), which can create problems during filtration of wort (33) and beer (23, 29). High β-glucan content may also lead to formation of precipitates in the finished beer (38). The practice of using unmalted barley as an adjunct in the brewhouse increases these problems (7). The barley β-glucanase is almost completely inactivated during kilning of the malt and mashing. However, (1→3,1→4)-β-glucanases from microbial sources (e.g. *Bacillus subtilis*, *Aspergillus*

niger and *Trichoderma reesii*) are less heat labile than barley β -glucanase. Such preparations may be added during mashing (2, 36) or at a later stage during fermentation (23), to reduce the content of high molecular weight β -glucan. A lager yeast which synthesizes and secretes (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase during fermentation and maturation of beer might alleviate the problems of beer filtration and formation of precipitates in the finished beer. In this paper we report the construction of a plasmid encompassing the coding information for the mature (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase isoenzyme II from barley fused in frame to a stretch of DNA encoding a mouse α -amylase signal peptide needed for translocation across the membrane of the endoplasmic reticulum. This chimaeric gene, with the potential of directing synthesis and secretion of barley β -glucanase, was inserted into the expression vector pMA56 behind the promoter of the alcohol dehydrogenase I gene from *Saccharomyces cerevisiae*. Upon transformation of a laboratory strain of yeast, β -glucan degrading activity was detected in the culture medium.

2. MATERIALS AND METHODS

2.1. Strains and media

E. coli strain JM83 (*ara*, Δ *lac-pro*, *str* A, *thi*, ϕ 80d *lacZ* Δ M15) (42) was used for all bacterial cloning experiments. The yeast strain used in this study was DBY746 (*Mat* α *his3 Δ 1 leu2-3 leu2-111 ura3-52 trp1 289*). Bacteria were grown on LB medium (1% Difco Bacto tryptone, 0.5% Difco Bacto yeast extract, 1% NaCl) supplied with ampicillin (50 μ g $\cdot$ ml⁻¹) and when appropriate for screening, with 50 μ g $\cdot$ ml⁻¹ of 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). YPD and synthetic complete (SC) media for yeast have been described previously (30).

2.2. Plasmids

The plasmid pUC13 (42) was used for the construction of a cDNA library and in all other cloning experiments involved in vector constructions. The plasmid pA5 (15) was prepared by Dr. G.B. FINCHER, La Trobe University, Mel-

bourne, Australia. In this plasmid a barley β -glucanase cDNA has been cloned in the PstI site of pUC9 (42). The cDNA encodes 291 amino acid residues and the first six codons are believed to represent part of a transient signal peptide. They are followed by 285 codons determining the amino acid sequence from the amino terminal end of the mature enzyme. The plasmid pMS12 (39) contains a mouse amylase cDNA gene (16) where part of the oligo(dG:dC) tails used for cloning has been removed by treatment with Bal31 exonuclease, leaving only three dG:dC pairs at the 5' end. The yeast expression vector pMA56 (41) was from Dr. B.D. HALL, University of Washington, Seattle, USA. It encodes most of the *E. coli* plasmid pBR322 including both the ampicillin resistance gene and the origin of replication. It also contains the yeast alcohol dehydrogenase I gene (ADCI) promoter, a yeast *TRP1* gene for selection and the origin of replication from the yeast 2 μ circular plasmid. A unique EcoRI cloning site is situated immediately downstream from the ADCI promoter.

2.3. Enzymes and chemicals

Reverse transcriptase was from Life Science Inc., St. Petersburg, Florida, USA, and terminal deoxynucleotidyl transferase was from Pharmacia-PL Biochemicals. Restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerases, T4 polynucleotide kinase, alkaline phosphatase from calf intestine and deoxy-nucleoside triphosphates as well as their di-deoxy derivatives were from Boehringer Mannheim. Low melting point agarose was from Litex and GIBCO-BRL. Barley β -glucan was from Sigma and chemicals for oligonucleotide synthesis were from Applied Biosystems. Tetramethylammonium chloride was from Aldrich and radioactive nucleotides were from New England Nuclear.

2.4. Construction of a cDNA library

Messenger RNA isolated from the aleurone layer of barley half-grains treated with 10 μ M gibberellic acid (27) was supplied by J. MUNDY, Department of Biotechnology, Carlsberg Research Laboratory. The cDNA was synthesized

by the method of WICKENS et al. (43) using oligo(dT) to prime first strand synthesis. The double stranded cDNA was tailed with oligo(dC) (37) and annealed with pUC13 linearised with SmaI and tailed with oligo(dG). Annealing was at 65 °C for 15 minutes followed by slow cooling to room temperature achieved by turning off the waterbath and leaving the mixture over night. Without further processing it was then used to transform *E. coli* strain JM83.

2.5. Transformation

E. coli cells were grown and prepared for transformation as described by LEDERBERG and COHEN (22), and the competent cells were stored as previously described (39). Yeast cells were transformed according to the method of ITO et al. (20) using lithium nitrate to induce competence.

2.6. Oligonucleotide synthesis and purification

Oligonucleotides were synthesized from *o*-methyl phosphoramidites using an Applied Biosystems Model 380A synthesiser. The products were separated from failure sequences, due to premature termination, on 14% polyacrylamide gels containing 7 M-urea and then eluted in three washes of deionised water at 37 °C for 30 minutes each, followed by drying in vacuum. The redissolved oligonucleotides were extracted with an equal volume of phenol/chloroform (1:1) and precipitated from 70% alcohol. The mixed oligonucleotide probe used for screening was purified as described by SMITH (35).

2.7. Screening the cDNA library

Individual cDNA clones were streaked on sheets of nitrocellulose (BA85, Schleicher & Schuell) placed on LB solid medium with ampicillin. The cells were initially grown for 16 hours at 37 °C and then transferred to new medium supplied with chloramphenicol (250 $\mu\text{g} \cdot \text{ml}^{-1}$) for plasmid amplification. Incubation on this medium was for 20 hours at 37 °C. The cells were lysed and the DNA fixed on the nitrocellulose filters as described by MANIATIS et al. (24). The library was screened with a 12-fold redundant oligonucleotide

representing the amino acid sequence Tyr-Ile-Phe-Ala-Met-Phe, residues 273-278 in barley β -glucanase (15). The probe was labelled using T4 polynucleotide kinase in the presence of γ - ^{32}P -ATP ($1.0 \cdot 10^{14} \text{ Bq} \cdot \text{mmol}^{-1}$). Before incubation with the radioactive probe the filters were prehybridized at 22 °C for 4 hours in $6\times\text{SSC}$ ($1\times\text{SSC}$ is 0.15 M-NaCl, 0.015 M-sodium citrate) 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone and 50 $\mu\text{g} \cdot \text{ml}^{-1}$ of sheared heat denatured herring sperm DNA. Hybridisation of three 20 $\times$ 20 cm filters was in 30 ml of this solution with 10 pmole of labelled 17-mer ($5\times 10^4 \text{ Bq} \cdot \text{pmol}^{-1}$) for 18 hours at 22 °C. The filters were washed with multiple changes of $6\times\text{SSC}$ at 22 °C to remove the unbound probe before washing two times, each for one hour at 40 °C, with 250 ml of a tetramethylammonium chloride wash solution (3 M-tetramethylammonium chloride, 50 mM-Tris-HCl, pH 8.0, 2 mM-EDTA, 1 $\text{mg} \cdot \text{ml}^{-1}$ SDS) described by WOOD et al. (44). The filters were then exposed on Kodak XAR-5 films with Kodak X-Omatic Regular intensifying screens at -80 °C for 20 hours.

2.8. DNA purification

Plasmid DNA was prepared from *E. coli* by the alkaline lysis method of BIRNBOIM and DOLY (5) which for large scale preparations was followed by CsCl equilibrium centrifugation. DNA fragments for subcloning were purified from low melting point agarose gels by heating with an equal volume of buffer (10 mM-Tris-HCl, pH 7.4, 1 mM-EDTA) at 65 °C for ten to fifteen minutes followed by extraction with phenol (twice) and chloroform. However, quite often the agarose slice containing DNA was simply melted and the liberated DNA was used without further purification (13). In such cases ligation reactions were performed at 25 °C.

2.9. DNA sequencing

Recombinant plasmids of pUC13 were

Figure 1. Construction of plasmid pBAG carrying the coding information for mature (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase from barley, fused in frame with a mouse α -amylase signal peptide. Firstly, the 310 bp EcoRI fragment from pMS12 was cloned into pUC13 (not shown) to give plasmid pMSig. The unwanted ApaI-SalI fragment was cut out and the remaining fragment of pMSig, containing the vector (unshaded) and the signal peptide code (black shading), was isolated and ligated to the synthetic adaptor (an ApaI-Sau3A fragment) (hatched shading) and the Sau3A-SalI fragment from pA5 encoding the major part of the barley (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase (grey shading). The resulting plasmid, pMSG, was then digested with EcoRI-HindIII. The 990 bp fragment containing the pA5 cDNA was partially digested with TaqI, and the EcoRI-TaqI fragment of 877 bp was isolated after electrophoresis in LMP agarose. This fragment and the TaqI-EcoRI fragment from pBA170 (vertical bars) were ligated together into EcoRI-cut pUC13 to give the plasmid pBAG.

sequenced directly (42) by the di-deoxy chain termination method of SANGER et al. (32) using

direct and reverse primers and when appropriate primers within the glucanase gene. Plasmid DNA was denatured in 0.2 M-NaOH (12) before annealing of a primer. The radioactive label was α - 35 S-dATP ($18.5 \cdot 10^{12}$ Bq $\cdot$ mmol $^{-1}$). The dried gels were exposed on Kodak XRP1 films for 1-2 days.

2.10. Construction of a plasmid directing synthesis and secretion of barley β -glucanase

The strategy for construction of a plasmid carrying the coding information for mature (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase from barley, fused in frame with a piece of DNA encoding a mouse α -amylase signal peptide, is diagrammed in Figure 1. The plasmid was constructed from the following components: the 5' end of an α -amylase cDNA gene, a synthetic adaptor fragment and the barley β -glucanase cDNA clones pA5 and pBA170.

Cloning of the α -amylase signal peptide coding region. The β -glucanase cDNA clone in pA5 does not encode a complete signal peptide and is thus devoid of an initiation codon. Therefore, a 310 bp fragment representing the 5' end of a mouse α -amylase mRNA was isolated from pMS12 and subcloned in pUC13 to give a plasmid called pMSig. This fragment specifies part of the non-coding region, the initiation codon, and the signal peptide. The signal peptide code ends in an ApaI restriction site which facilitates fusing of another protein code in frame to this signal peptide code.

Synthesis and cloning of an adaptor fragment. An adaptor fragment was synthesized to link the DNA fragment encoding the amylase signal peptide to the cDNA insert of pA5. This fragment should have an ApaI restriction site in one end for attachment to the signal peptide code, and a Sau3A site near the other end corresponding to a site in the β -glucanase cDNA (position 160 in Figure 3). Thus, the adaptor fragment should be about 100 bp or more. To accomplish that two oligonucleotides, a 78-mer and a 50-mer, were synthesized and purified as described in a previous section. These two oligonucleotides represented complementary strands of the 5' end of the β -glucanase cDNA (position

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      TaqI
C GGC ATC GAG ACC TAC ATC TTC GCC ATG TTC AAC GAG AAC
  Ala Ile Glu Thr Tyr Ile Phe Ala Met Phe Asn Glu Asn
      270                               280

CAG AAG GAC AGC GGC GTG GAG CAG AAC TGG GGA CTC TTC
  Gln Lys Asp Ser Gly Val Glu Gln Asn Trp Gly Leu Phe
      290

TAC CCC AAC ATG CAG CAC GTC TAC CCC ATC AAC TTC TGA
  Tyr Pro Asn Met Gln His Val Tyr Pro Ile Asn Phe *
      300

      SacI
CGGAGCTCGTCTGCTGTTAAGTCCTACTTGTCTTGTTAACGAGCTAGTAA

AAAGTCATGTTACGGAACTTGACGAGCTACTCGTTGGAGAGCCTCTTAA

TTACCTCCTCTTTTTCACATGAGGGATAAGAAGCTATGAGTTAATAACCAGA

      EcoRI
CCCATTTACTGTGAATT(C)n

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Figure 2. The nucleotide sequence of cDNA pBA170. This cDNA was selected from a library made from barley aleurone mRNA, using a redundant 17-mer oligonucleotide representing amino acid residues 273-278 of barley β -glucanase (underlined). The cDNA encodes the 38 C-terminal amino acid residues of the (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase enzyme. Restriction sites used for construction of pBAG plasmids and analysis are labelled. The stop codon is marked by an asterisk and a putative polyadenylation signal with a bar above.

76-180 in Figure 3) and had a 23 nucleotide overlap at their 3' ends. Approximately 500 ng of each of the purified oligonucleotides were annealed in 50 μ l of DNA polymerase buffer (10 mM-Tris-HCl, pH 7.4, 10 mM-MgCl₂, 50 mM-NaCl) by incubation at 95 °C for 10 minutes, 60 °C for 16 hours and then cooling on ice followed by incubation with DNA polymerase I (Klenow fragment) and dCTP, dGTP, dTTP and dATP, each at a final concentration of 0.080 mM. α -³²P-dATP was added to a final specific activity of 3.7 · 10¹¹ Bq · mmol⁻¹. Incubation was at 14 °C for 2 hours. The double stranded products were treated with T4 polynucleotide kinase in the presence of ATP and cloned in the SmaI site of pUC13 to give a series of plasmids called pSP.

Splicing the β -glucanase cDNA gene onto the signal peptide coding region. The cloned adaptor fragment from pSP71 was recovered from low melting point agarose as a 240 bp Sau3A frag-

ment which was then digested with ApaI. The plasmid pMSig, carrying the 5' end of an amylase cDNA gene was cut with ApaI and SalI and the large fragment – vector plus signal peptide code – was isolated. The β -glucanase cDNA in pA5 has a Sau3A site that corresponds to the one present in the adaptor fragment (position 160 in Figure 3). There are no other Sau3A sites in pA5 downstream from this position. Therefore, the cDNA insert from pA5 was isolated as a Sau3A-SalI fragment where the SalI site is in the plasmid polylinker as in pMSig. These three fragments were ligated and the ligation mixture used to transform *E. coli*. A plasmid with the components fused correctly was selected and will be referred to as pMSG.

Construction of a complete β -glucanase cDNA gene. The cDNA clone pBA170, described in this paper, carries an insert representing the 3' end of the β -glucanase gene. Near the 5' end of the cDNA is a TaqI site which corresponds to the TaqI site near the 3' end of the cDNA in pA5. Also an EcoRI site is present in the cDNA of pBA170, immediately prior to the poly(dG:dC)-tail used for cloning. Thus, the cDNA fragment needed to complement the coding information of pA5 could be isolated as a TaqI-EcoRI fragment. However, the TaqI site suitable for joining the cDNAs together is not the only one present in the pMSG insert. Two more are located further upstream in the glucanase cDNA. Therefore, this plasmid was first digested with EcoRI and HindIII to yield a 990 bp fragment with the chimaeric cDNA. This digest was treated with TaqI for 2, 4, 8, 16 and 32 minutes before the enzymatic reaction was stopped by addition of EDTA. The DNA fragments were separated on a low melting point agarose gel and an 877 bp fragment was isolated. The two fragments carrying complementary parts of a β -glucanase cDNA gene and having compatible TaqI ends were ligated into the EcoRI site of pUC13. To reduce self ligation the vector had been purified from low melting point agarose and treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli* strain JM83 and transformed cells were selected on LB medium with ampicillin and X-gal. Plasmid DNA from white colonies was analysed with restriction enzymes and a clone

1
GAATTCGGGAAGAATACTGCCAACAGCATAGCAAA ATG AAA TTC TTC CTG CTG CTT TCC CTC ATT GGA TTC TGC
EcoRI

100
TGG GCC CTA GGG GTG TGC TAC GGC ATG AGC GCC AAC AAC CTG CCG GCG GCG AGC ACC GTC GTG AGC
ApaI Leu

150
ATG TTC AAG TCC AAC GGG ATC AAA TCG ATG CGG CTG TAC GCT CCC AAC CAG GCG GCG CAG GCG
Sau3A TaqI

200
GTC GGC GGC ACG GGC ATC AAC GTC GTC GTC GGG GCT CCT AAC GAC GTC CTC TCC AAC CTC GCC GCC

250
AGC CCG GCA GCG GCC GCC TCG TGG GTC AAG AGC AAC ATC CAG GCG TAC CCC AAG GTT TCC TTC CGG

300
TAC GTC TGC GTC GGA AAC GAG GTC GCC GGC GGC GCC ACC CGG AAC CTC GTC CCG GCA ATG AAG AAC

350
GTG CAT GGC GCG CTC GTC GCC GCT GGG CTG GGC CAC ATC AAG GTG ACC ACG TCG GTG TCG CAG GCC

400
ATC CTC GGC GTG TTC AGC CCG CCC TCC GCC GGG TCC TTC ACC GGG GAG GCG GCC GCG TTC ATG GCG
ApaI

450
CCC GTG GTG CAG TTC CTT GCC CGC ACC AAC GCG CCG CTC ATG GCC AAC ATT TAC CCG TAC CTG GCC

500
TGG GCT TAC AAC CCG AGC GCC ATG GAC ATG GGC TAC GCT CTC TTC AAC GCG TCC GGC ACC GTG GTC

550
AGG GAC GGC GCC TAC GGG TAC CAG AAC CTG TTC GAC ACC ACC GTG GAC GCC TTC TAC ACG GCC ATG

600
GGC AAG CAC GGC GGC TCC AGC GTG AAG CTG GTG GTG TCG GAG AGC GGG TGG CCG TCG GGC GGC GGC

650
ACG GCG GCG ACT CCG GCC AAC GCT AGG TTC TAC AAC CAG CAC CTC ATC AAC CAC GTC GGC CGC GGC

700
ACC CCA CGC CAC CCG GGC GCC ATC GAG ACC TAC ATC TTC GCC ATG TTC AAC GAG AAC CAG AAG GAC

750
AGC GGC GTG GAG CAG AAC TGG GGA CTC TTC TAC CCC AAC ATG CAG CAC GTC TAC CCC ATC AAC TTC

800
TGA CGGAGCTCGTGTGCTCGTTAAGTCCCTACTTGTCTTGTTAACGAGCTAGTAAAAAGTCATGTTACGCGAACTTGACGAGCTACT
SacI

850
CGTTTGGAGAGCCTCTTAATTACCTCCTCTTTTCACATGAGGGATAAGAACGTATGAGTTAATAACCAGACCCATTACTGTGAATTC
EcoRI

900
1000 1050 1150

Figure 3. Sequence of complete β -glucanase coding region attached to the signal peptide code from a mouse α -amylase gene. The codon for the amino terminal amino acid residue of the mature β -glucanase polypeptide has been changed from ATC (isoleucine) to CTA (leucine) in order to accommodate the signal peptide code. Restriction sites referred to in the text are underlined and arrows mark oligonucleotides used as primers for sequencing.

having the fragments correctly spliced together (pBAG) was subjected to DNA sequencing across the splice junctions to confirm the construction.

Cloning of a β -glucanase cDNA gene in a yeast expression vector. The complete β -glucanase cDNA gene behind a mouse α -amylase signal

peptide code was isolated as an EcoRI fragment from pBAG and ligated into the EcoRI cloning site of the yeast expression vector pMA56 to give pYBAG plasmids. The orientation of the cDNA gene relative to the ADCl promoter was determined from double digests with the restriction endonucleases SacI and BamHI since there is a unique SacI site in the β -glucanase cDNA gene near the 3' end and a unique BamHI site in the vector. The *Saccharomyces cerevisiae* strain DBY746 was transformed with plasmids carrying the cDNA gene in each orientation.

2.11. Detection of β -glucanase activity

Yeast cells harbouring pYBAG plasmids were grown at 30 °C on SC medium without tryptophan containing 0.1 g · l⁻¹ barley β -glucan and, in some cases, 0.3% Congo Red. Plates without Congo Red were incubated for 48 hours. The cells were then washed off and the plates stained with a 0.1% solution of Congo Red (10). Plates containing Congo Red were incubated for 7 days and then photographed. To analyse if β -glucanase was released to the culture medium in a free form yeast cells were grown for 18 hours in 10 ml cultures (SC without tryptophan), harvested, resuspended in fresh medium and further incubated for 24 hours. The cleared cell supernatant was concentrated 20 fold in a Speed Vac Concentrator (Savant Instruments, Inc.) and 100 μ l samples were pipetted into 6.0 mm diameter wells in a 1% agar plate with 0.1 g · l⁻¹ of barley β -glucan and 0.1% Congo Red in 10 mM-sodium acetate-HCl, pH 5.0 (25). The plates were incubated for 48 hours and photographed.

2.12. Containment

All experiments involving recombinant DNA were carried out under BL1 laboratory conditions and waste containing biological material was autoclaved.

3. RESULTS

3.1. Isolation and identification of a cDNA clone representing the 3' end of a barley β -glucanase gene

A cDNA library of approximately 1000 clones generated from barley aleurone mRNA was screened with a 17-mer oligonucleotide of 12 fold redundancy. The probe represents amino acid residues 273-278 of barley (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucanase (15). The oligonucleotide hybridised specifically to clone pBA170 and the sequence of this clone was determined using denatured plasmid DNA as template (Figure 2). The cDNA encodes 38 amino acid residues followed by a TGA stop codon. The deduced amino acid sequence shows a perfect match to the previously determined carboxy terminal sequence of barley β -glucanase isoenzyme II. Following the stop codon is a noncoding region of 169 nucleotides. The clone does not extend into a poly(A)-tail, but a putative polyadenylation signal (AATAA) is present near the 3' end.

3.2. Construction of pMSig

An EcoRI fragment of 310 bp from pMS12 was subcloned in pUC13. This fragment encodes the 5' end of mouse α -amylase mRNA including the initiation codon and the signal peptide needed for translocation across the membrane of the endoplasmic reticulum, an initial step in secretion of proteins. A recombinant plasmid with this fragment inserted in the EcoRI site of the pUC13 polylinker in the orientation suitable for later constructions was identified by digestion with both Apal and PstI (not shown).

3.3. Synthesis and cloning of the adaptor fragment

A synthetic adaptor fragment was used to splice a DNA fragment encoding the mouse signal peptide together with the β -glucanase cDNA from pA5. The adaptor fragment was synthesized as two partially complementary oligomers which were annealed and made double stranded using DNA polymerase I (Klenow fragment). The products were then cloned in the SmaI site of pUC13. The double stranded prod-

ucts were analysed on a sequencing gel before cloning, and it was evident that only a fraction of the molecules had the desired length. Therefore, clones were selected on the basis of length of a *Sau3A* restriction fragment containing the synthetic piece. Another criterium for selection was the presence of both an *ApaI* and a *Sau3A* site. Clones fulfilling both criteria were analysed by sequencing, and the clone pSP71 was found to have the desired structure. During this analysis it became clear that the DNA polymerase had often terminated prematurely and occasionally had introduced failures such as misincorporations and missing bases in the DNA synthesized.

3.4 Construction of pMSG

The β -glucanase cDNA from pA5 was connected with the mouse α -amylase signal peptide code via the synthetic adaptor and the structure was corroborated by sequencing across the junctions. To fulfill the requirement for an *ApaI* site in the synthetic adaptor it was necessary to introduce a different codon for the amino terminal amino acid residue. It was therefore changed from ATC (isoleucine) to CTA (leucine). This is a neutral amino acid substitution and we do not believe that it has any effect on the activity of the mature enzyme.

3.5. Construction of a yeast vector directing synthesis of β -glucanase

The two cDNAs were spliced together via a common *TaqI* site and a complete β -glucanase coding region attached to a signal peptide code is contained within two *EcoRI* sites. The entire sequence is shown in Figure 3. This cassette was inserted behind the yeast *ADHI* promoter in pMA56 (Figure 4). The orientation of the insert was determined from *BamHI*-*SacI* double digests. If the cDNA gene is inserted in the orientation suitable for expression two fragments of about 6.7 kb and 2.7 kb are expected. With the opposite orientation the fragments would be 7.8 kb and 1.6 kb in size. In Figure 5 the result of an electrophoretic analysis of plasmids digested with *BamHI* and *SacI* is shown together with an identical digest of pMA56 (lane 9). The plasmid in lane 7 (pYBAG17) gives rise to the fragments expected when the cDNA is

Figure 4. The barley β -glucanase expression plasmid pYBAG17. This plasmid consists of the insert from pBAG inserted in the *EcoRI* site of pMA56, adjacent to the *ADHI* gene promoter and in the correct orientation for expression.

inserted in the correct orientation while the plasmid in lane 8 (pYBAG13) displays the pattern expected for the cDNA being inserted in the opposite orientation. Lanes 2 and 3 in this figure show the same two plasmids digested with *EcoRI* compared with an *EcoRI* digest of pUC13 carrying the complete cassette (lane 4), and of pMA56 (lane 1). As may be seen the excised fragments are of identical size. Lanes 5 and 6 show *SacI* digests pYBAG17 and pYBAG13. Digestion with *SacI* alone results in only one fragment showing that only one copy of the cDNA gene has been cloned.

3.6. Expression of a β -glucanase gene in yeast

The *Saccharomyces cerevisiae* strain DBY746 was transformed to tryptophan prototrophy using the plasmids pMA56, pYBAG13 and pYBAG17. Transformed yeast cells were selected on SC-medium without tryptophan, transferred to fresh selective plates supplied with 0.1 g $\cdot$ l⁻¹ of barley β -glucan and Congo Red and incubated for several days. A clearing zone around a colony indicates degradation of β -glucan to an extent where it no longer binds the dye. As can be seen in Figure 6A only the construction pYBAG17, having the β -glucanase gene

Figure 5. Orientation of the β -glucanase gene relative to the yeast promoter in pYBAG plasmids. DNA was digested with restriction enzymes as indicated at the bottom of the figure; pMA56 (lanes 1 and 9), pYBAG17 (lanes 2, 5 and 7), pYBAG13 (lanes 3, 6, and 8) and pBAG (lane 4). Size markers are in kbp.

inserted in the orientation suitable for expression, has a clearing zone – a halo – around the colony whereas the medium around pYBAG13 (reverse orientation) and pMA56 (parental plasmid) is well stained.

In other experiments the yeast was grown on plates without the dye. The cells were then washed off the plates before staining. In that case the medium underneath the cells transformed with pYBAG13 and pMA56 was stained slightly stronger than the surrounding medium whereas underneath pYBAG17 the medium remained unstained (Figure 6B).

To test further whether the heterologous gene product was actually released from the cell surface and present in the medium in a free form, yeast cells harbouring recombinant plasmids were grown in liquid medium (SC without tryptophan) for 24 hours. They were then har-

vested, transferred to fresh medium and incubated for another 24 hours. After this period the cleared supernatants were concentrated 20-fold and assayed for β -glucanase activity by placing aliquots in wells cut in agar plates containing barley β -glucan and Congo Red. Cell supernatants from pYBAG17 carrying yeast gave rise to haloes around the wells (Figure 6C).

4. DISCUSSION

In this report we describe the isolation and identification of a cDNA clone representing the 3' end of a barley (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase gene. This cDNA was spliced together with a previously isolated cDNA, pA5 (15) to constitute the entire protein coding region for the mature form of (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase isoenzyme II from barley aleurone. This β -glucanase gene was still

Figure 6. β -glucanase assays for yeast containing the plasmids 1: pYBAG17, 2: pYBAG13 or 3: pMA56. A. Yeast was grown 7 days on selective medium (SC without tryptophan) with $0.1 \text{ g} \cdot \text{l}^{-1}$ barley β -glucan and 0.3% Congo Red. B. Yeast was grown 2 days on selective medium containing $0.1 \text{ g} \cdot \text{l}^{-1}$ barley β -glucan. Colonies were washed off before staining with a 0.1% solution of Congo Red. C. Concentrated yeast cell supernatants, prepared as described in section 2.11 were pipetted into wells in agar plates containing 0.1% Congo Red, barley β -glucan $0.1 \text{ g} \cdot \text{l}^{-1}$ and 10 mM-sodium acetate-HCl pH 5.0.

incomplete since at its 5' end it only encoded six amino acids of a signal peptide and thus lacked an initiation codon. Therefore, it was fused in frame with a DNA fragment encoding the 15 amino acid signal peptide of mouse salivary α -amylase. The amylase signal peptide was used because it was known to be compatible with the yeast secretory system (39). The β -glucanase cDNA was attached to the signal peptide encoding DNA via a 96 base pair synthetic adaptor fragment with an *Apa*I site corresponding to the 3' end of the gene fragment encoding the amylase signal peptide in one end, and a *Sau*3A site corresponding to position 160 in the glucanase gene in the other end. To take advantage of the *Apa*I site in the amylase gene it was necessary to change the codon for the amino terminal amino acid of mature β -glucanase from ATC (isoleucine) to CTA (leucine). It is unlikely that this amino acid substitution will affect signal peptide cleavage since the signal peptidase cleavage site appears to be determined by the sequences within the signal peptide itself and not by sequences beyond the cleavage site (17), and since it is a neutral amino acid substitution it is assumed that β -glucanase activity remains unchanged. The chimaeric cDNA gene was introduced in *Saccharomyces cerevisiae* via the expression vector pMA56 and it was shown that the barley gene is expressed and the gene product exported from the cells to the culture medium.

The only other (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase for which the primary structure is known is the *Bacillus subtilis* enzyme (28). This gene has also been cloned in yeast – *E. coli* shuttle vectors and introduced into yeast by two groups (9, 18, 19). Both groups have demonstrated β -glucanase activity in cell free extracts prepared from yeast carrying the β -glucanase gene and CANTWELL et al. (9) have observed low levels of extracellular β -glucanase activity.

Over the last few years several groups have obtained secretion of heterologous gene products synthesized by *Saccharomyces cerevisiae*. It seems that amylolytic enzymes of both fungal, plant and mammalian origin are effectively secreted and released to the culture medium via their natural signal sequence (40). Attempts to obtain secretion of other heterologous proteins have mainly focussed on fusion of the gene with

a signal peptide coding region from yeast, e.g. the yeast α -factor gene (6, 8, 34) and the gene fragment encoding the signal peptide code of the secreted form of yeast invertase (11). JIGAMI et al. (21) synthesized a DNA fragment encoding the signal peptide of chicken lysozyme and fused that to a synthetic human lysozyme gene. This synthetic gene was expressed in yeast under the control of the *GAL10* promoter, and about 60% of the total lysozyme activity was released to the medium. These results together with those reported in this paper demonstrate that a non-yeast signal peptide may be used to direct secretion of a different heterologous protein synthesized by yeast.

In some yeast genes it has been found that the codons for individual amino acids are used in a non-random fashion. BENNETZEN and HALL (4) have analysed the codon usage in some highly expressed yeast genes and found a strong codon usage bias. For example they found that the codons GGG, CCC, AAA, TTT, only G+C or only A+T were never used, except ATT which is one of two preferred lysine codons and GCC that is one of the two codons always found for alanine. The protein coding region of the barley (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucanase has a remarkably high G+C content (67%) and a strongly biased codon usage towards G or C in the wobble position (15). In 94% of the codons this position is occupied by G or C. In this gene there are about one hundred of the codons which are never found in highly expressed yeast genes. For example, all 33 glycine codons in this gene are GGG or GGC and 17 out of 18 proline codons are CCC or CCG. One might therefore fear that such a gene would not be expressed in yeast.

The most abundant amino acid in barley β -glucanase is alanine (43 residues) and 35 of the alanine codons are GCC or GCG, but the GCC alanine codon (22 of 43) happens to be one of the exceptions from the 100% rule mentioned above. The so-called preferred yeast codons for the amino acids phenylalanine, tyrosine, and asparagine are those with G or C in the third position. In the barley β -glucanase these amino acids are all entirely encoded by such codons, namely 14, 15 and 24 codons, respectively. Thus, the strong codon usage bias found in the barley β -glucanase gene has a dual effect, both

giving rise to codons preferred by yeast for some amino acids and to codons rarely found in highly expressed yeast genes.

The level of β -glucanase expression in terms of protein amount still remains to be established as well as how big a fraction of the enzyme synthesized is actually being exported. However, small scale fermentation experiments with yeast carrying the pYBAG17 plasmid have recently been performed in SC-medium supplied with high molecular weight barley β -glucan. The preliminary results indicate that the amount of β -glucanase released from the yeast cells is sufficient to degrade substantial amounts of the barley β -glucans normally present in wort. Future experiments will be directed towards the integration of the barley β -glucanase gene in the genome of brewing yeast strains and to testing the effect of this on wort and beer.

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