PROCESSING AND SECRETION OF BARLEY (1-3,1-4)- β -GLUCANASE IN YEAST

by

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DNA segments encoding signal peptides from mouse α -amylase, yeast acid phosphatase, and yeast invertase were fused in frame to a barley (1-3,1-4)- β -glucanase cDNA gene and expressed in yeast cells under the control of the phosphoglycerate kinase gene promoter. Pure β -glucanase is obtained by gel filtration of concentrated yeast cell supernatant. It was shown that the glucanase pre-protein was specifically processed and the mature protein efficiently secreted when the yeast invertase signal sequence directed secretion.

1. INTRODUCTION

Cloning and expression of genes in heterologous hosts have proven to be important techniques for the production and characterization of enzymes that are of interest to the bioindustry (9, 25, 34). Secretion of a heterologous protein product into the culture fluid can be the initial biological step in its purification and simplify downstream processing of the bioengineered product. Secretion is also an obligatory process if the heterologous gene confers the ability to utilize an otherwise inaccessible high molecular weight substrate to the host organism as is exemplified by amylolytic yeast (35).

The yeast *Saccharomyces cerevisiae* can be used as a host for heterologous gene expression by placing the foreign gene under the control of

a yeast promoter region, e.g. (2). Several factors have been identified which influence the efficiency of heterologous gene expression in yeast cells. Among these are the length and nucleotide sequence of the 5' end of the mRNA (24). The translation initiation region of the mRNA is of special importance and expression efficiency can be increased by incorporating a homologous initiation region (31).

The mechanisms for protein secretion in the small eukaryote are very similar to those first elucidated in animal cells. The signal sequence of pre-proteins apparently also interacts in yeast cells with a signal recognition particle and effects translocation of secretory proteins across the membrane of the rER (30, 36). Subsequently, the signal sequence is removed by a membrane-associated endopeptidase, the yeast signal pepti-

Abbreviations: ADHI = yeast alcohol dehydrogenase I gene; (r)ER = (rough) endoplasmic reticulum; kD = kilo Dalton; PGK = yeast 3'-phosphoglycerate kinase gene; SC = synthetic complete medium; SDS-PAGE = SDS-polyacrylamide gel electrophoresis; SRP = signal recognition particle.

dase, and the mature protein is generated (38). Typically, signal sequences are cleaved from the protein during or immediately after translocation to the rER lumen. The cleavage of the signal sequence plays an important role in the secretion of yeast invertase (17). In general, only large deletions or substitutions lead to severe defects in invertase secretion (27).

In the present report we describe the construction of yeast expression plasmids carrying a barley (1-3,1-4)- β -glucanase cDNA gene fused in frame with synthetic oligonucleotides encoding different signal peptides. These chimeric genes were cloned behind the yeast ADHI and PGK promoters. When yeast cells were transformed with such plasmids, active barley β -glucanase was synthesized and released to the culture medium. The enzyme was purified and the amino-terminal amino acid sequence analyzed as well as the specific activity of the enzyme.

2. MATERIALS AND METHODS

2.1. Microbial strains and culture media

Escherichia coli strain DH5 α (12) grown in standard LB medium was used as plasmid host. Ampicillin was used in a concentration of 100 μ g/ml. *Saccharomyces cerevisiae* cells of strain DBY746 (16) were cultured in either YPD or synthetic complete (SC) media (26). Barley β -glucan in a concentration of 0.1% (w/v) added to SC medium permitted the selection of yeast cells secreting β -glucanase by utilizing Congo red as indicator for the presence or absence of β -glucan around the colony (16).

2.2. Genetic transformations

E. coli DH5 α cells were transformed according to the published procedure (11). Saccha-

Saccharomyces cerevisiae DBY746 cells were transformed by the method of ITO et al. (15) using lithium acetate to induce competence.

2.3. Enzymes and chemicals

Deoxy-nucleotides, restriction endonucleases and other enzymes used for DNA cloning were purchased from Boehringer Mannheim and conditions for their use were those recommended by the manufacturer. Plasmids pTZ19R and pGEMTM-7zf(+) were from Pharmacia and Promega, respectively. (α -³⁵S)-dATP with a specific activity higher than 1000 Ci/mmol was obtained from New England Nuclear. Modified T7 DNA polymerase (SequenaseTM) and SequenaseTM sequencing kit was from USB Corporation, while a GeneCleanTM kit from Bio101 Inc. was used for purification of DNA. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer Model 380A, desalted on Pharmacia NAPTM-10 columns containing SephadexTM G-50 and used without further purification. A β -glucanase assay kit was from Biocon, while barley β -glucan and Congo red were purchased from Sigma. SephacrylTM S-200HR was from Pharmacia.

2.4. Oligonucleotides

Oligonucleotides were designed as shown in Figure 1 based on the known coding regions for the yeast invertase signal peptide (5) and the yeast acid phosphatase signal peptide (33). The two sets of complementary oligonucleotides in TE buffer (10 mM Tris-Cl pH 7.8, 1 mM EDTA) were heated to 95 °C and slowly cooled down to room temperature and ligated (using T4 DNA ligase) onto the ApaI site of the gel-purified large fragment of BamHI and ApaI cut vector pAG

Figure 1. Oligonucleotides. The double-stranded oligonucleotides encoding the yeast invertase signal sequence (A) and the yeast phosphatase signal sequence (B). (US = upper strand, LS = lower strand).

Figure 2. Construction of yeast plasmids directing synthesis and secretion of barley (1-3,1-4)- β -glucanase with the aid of the yeast phosphatase 5 signal peptide (pMAG24) and the yeast invertase signal peptide (pMIG5). The transcription of the hybrid genes is under control of the yeast PGK promoter. Pr = Promoter, Ter = Terminator.

(Fig. 2). The oligonucleotides were not phosphorylated to avoid self ligation. The ligated complex was desalted using the GeneClean™ kit, then cut with BamHI and circularized. Both of the double-stranded oligonucleotides have PstI compatible 5' ends for cloning into vectors containing PstI cloning sites such as pG1 (23). The yeast alkaline phosphatase signal peptide code contains the codon AAT encoding Asn at position -2 with respect to the signal peptidase cleavage site. The corresponding codon of the oligonucleotide was changed to GCG encoding Ala facilitating its ligation to the ApaI digested end of plasmid pAG (Fig. 2). The construction of plasmids pMIG5 and pMPG24 (Fig. 2) were carried out by different strategies because the oligonucleotide encoding the yeast invertase signal peptide contains a HindIII restriction site.

2.5. Preparation and analysis of DNA

Plasmid DNA (miniprep DNA) was isolated from *E. coli* cells as described by HATTORI and SAKAKI (13). Standard techniques for DNA isolation and agarose gel electrophoresis have been described elsewhere (19). Plasmid DNA was digested with appropriate restriction endonucleases, and the resulting fragments were separated on 1% (w/v) agarose gels. The desired fragment was excised and the DNA recovered by using the GeneClean™ kit according to the manufacturer's recommendations. The DNA sequence around splice junctions was confirmed by restriction analysis, DNA sequence analysis, or both. Double-stranded DNA sequencing was performed according to the procedure by ZHANG et al. (39) except that miniprep DNA was used as template.

2.6. Plasmid constructions

The procedure is outlined schematically in Figure 2. In order to fuse the two double-stranded oligonucleotides encoding the signal peptides for yeast invertase and yeast acid phosphatase 5 onto the most distal 5' ApaI-cut end of the β -glucanase gene (16), the BamHI-PstI fragment containing a part of the barley β -glucanase gene from plasmid pYBAG17 (16) was cloned into the BamHI-PstI cut cloning vector pTZ19R

thereby creating the plasmid pAG which then was cut with BamHI and ApaI. The large fragment was purified and ligated with the double-stranded oligonucleotides with ApaI and BamHI compatible ends giving rise to plasmid pP5 (modified phosphatase signal code) and plasmid pI5 (invertase signal code). Hereafter, two different cloning procedures were followed because the double-stranded oligonucleotides contain different restriction sites.

Plasmid pI5 was cut with BamHI-ClaI and the purified small fragment was ligated with the large fragment of BamHI-ClaI cut vector pTG1. This construct is called pIG. The small HincII fragment from pIG containing the invertase signal peptide code fused in frame with the structural gene of barley (1-3,1-4)- β -glucanase gene was then ligated into the SmaI site of the cloning vector pGEM™-7zf(+) making plasmid pGEMIG6. The small BamHI fragment from pGEMIG6 was purified and ligated into BglII cut and dephosphorylated vector pMA91 to give plasmid pMIG5.

Plasmid pP5 was cut with PstI and HindIII, the large fragment purified and ligated with the PstI-HindIII fragment containing most of the β -glucanase gene from plasmid pYBAG17 giving rise to plasmid pPG, which was then cut with BamHI and SacI. The small fragment was purified, the ends made blunt with T4-DNA polymerase and the fragment cloned into the BglII cut and blunt ended vector pMA91 to give plasmid pMPG24.

Plasmid pMAG17 (not shown in Fig. 2) was constructed by ligation of the small blunt ended EcoRI fragment (containing the mouse α -amylase signal peptide code fused in frame with the β -glucanase structural gene behind the PGK promoter) from pYBAG17 into the BglII digested and blunt ended pMA19.

Table 1. Yeast plasmids directing synthesis and secretion of β -glucanase.

Plasmid	Promoter	Secretion signal
pYBAG17	ADHI	mouse α -amylase
pMAG17	PGK	mouse α -amylase
pMPG24	PGK	yeast phosphatase
pMIG5	PGK	yeast invertase

Figure 3. Gel filtration column chromatography of β -glucanase from SC-medium of yeast cells transformed with plasmid pMIG5 and the vector pMA91 without the β -glucanase gene. Fraction 52 from yeast cells transformed with plasmid pMIG5 contains pure β -glucanase.

Plasmid pYBAG17 has been described (16).

Expression-secretion cassettes used in this study are listed in Table I.

2.7. Purification of β -glucanase

Yeast cell cultures were centrifuged at $13,000\times g$, the supernatants filtrated through a $1.2\ \mu\text{m}$ filter and concentrated by ultrafiltration using a MinitanTM equipment from Millipore followed by a final concentration step in an Amicon ultrafiltration cell. The filters used had a cut-off of 10 kD. Concentrated supernatants (2-3 ml) were applied onto a SephacrylTM S-200HR gel filtration column ($2.6\times 65.0\ \text{cm}$) equilibrated with 50 mM NaCl, 50 mM Na-acetate, pH 5.0. The proteins were eluted with the same buffer at a flow rate of 30 ml/h. By this procedure the β -glucanase was well separated from other proteins in the culture fluid (Fig. 3).

Barley (1-3,1-4)- β -glucanase isoenzyme II from germinating barley was purified as described by WOODWARD and FINCHER (37).

2.8. Determination of β -glucanase activity

The Biocon assay kit using Azo-barley β -glucan as substrate was used for determination of β -glucanase activity (20). The assay conditions

were as recommended by Biocon. Using (1-3,1-4)- β -glucanase isoenzyme II purified from germinating barley it was estimated that 1 mg of enzyme produces 200 OD₅₉₀ units in a standard assay. This conversion factor was used for determination of the yield of β -glucanase in crude supernatants from yeast cells transformed with β -glucanase expression plasmids.

2.9. SDS-polyacrylamide gel electrophoresis

The purity and molecular weight of the purified enzyme were analyzed by SDS-polyacrylamide gel electrophoresis in a 13% separating gel using the technique first described by LAEMMLI (18). Gels were fixed, washed, and stained as described by BALLANCE and SVENDSEN (4).

2.10. Amino-terminal sequencing of purified (1-3,1-4)- β -glucanase

Amino acid sequence analyses were performed on a gas-phase sequencer (Applied Biosystems model 470A) equipped with an on-line HPLC detection system (Applied Biosystems model 120A PTH analyzer) according to the manufacturer's instructions.

2.11. Computer analysis of DNA and protein sequences

The prediction of protein secondary structure was performed using the algorithm of GARNIER et al. (10), whereas the algorithm of HOPP and WOODS (14) was used to predict protein hydrophobic and hydrophilic regions. All sequence analysis procedures were performed using the MicroGenieTM analysis program (28).

2.12. Containment

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

3. RESULTS AND DISCUSSION

3.1. Promoters

The plasmids pYBAG17 and pMAG17 both contain a hybrid gene of the mouse α -amylase

signal peptide code fused in frame to the barley cDNA gene encoding mature (1-3,1-4)- β -glucanase. Both plasmids have a 2 μ origin for replication in yeast and pYBAG17 has a TRP1 gene for selection while pMAG17, derived from pMA91 (21), has a LEU2 gene as the selectable marker. The β -glucanase expression in yeast cells transformed with pYBAG17 is directed by the ADHI gene promoter while expression of β -glucanase in cells transformed with pMAG17 is controlled by the PGK gene promoter. The amount of β -glucanase measured in supernatants from yeast cells transformed with pYBAG17 was estimated to be 1-5 μ g per litre and about 100 μ g/l when pMAG17 was directing β -glucanase expression. The amount of internal active β -glucanase in yeast transformants containing either of the two plasmids was approximately 10% of the quantity secreted. We have not determined whether this intracellular β -glucanase is present in the secretory apparatus, in the cytosol, or as a combination of both. SØGAARD and SVENSSON (32) compared secretion levels of barley α -amylase from yeast cells and found that the PGK promoter directed a 200 fold higher secretion level of the product as compared to the secretion level of the heterologous gene under ADHI promoter control. That we only observe a 20 fold increase in the amount of β -glucanase secreted to the medium by exchanging the promoter region may be a reflection of either suboptimal translation initiation regions of the messages or incorrect processing of the pre-protein (see later) resulting in scrambling of the translocation or secretion apparatus of the yeast cells. A secretion level of 100 μ g/l of β -glucanase may be the uppermost level that can be achieved with this message.

3.2. Signal peptide structure

The signal peptide per se influences the secretion efficiency. The pMA91 plasmid derivatives pMPG24 and pMIG5 (Fig. 2) differ only with respect to the signal peptide code which is fused to the structural gene of barley (1-3,1-4)- β -glucanase. The modified yeast acid phosphatase signal peptide (encoding Ala instead of Asn in position -2 with respect to the signal peptidase cleavage site) is encoded by plasmid pMPG24

and the yeast invertase signal peptide is encoded by plasmid pMIG5, and the untranslated 5' end of the message transcribed from these two constructs is approximately 40 nucleotides long (22). The two plasmids direct synthesis and secretion of substantially more β -glucanase than the construct pMAG17 (mouse α -amylase signal peptide code), and yeast cells containing pMIG5 secrete consistently higher levels of β -glucanase than transformants containing pMPG24. The amounts and activities of secreted β -glucanase from different transformants after 72 hours of growth is shown in Table I. Based on the elution profile of concentrated pMIG5 supernatant applied onto a SephacrylTM S-200HR column (Fig. 3), it was estimated that approximately 20% of the total amount of secreted protein is β -glucanase. Transformants harbouring pMIG5 secrete approximately 5 mg of β -glucanase per litre medium, pMPG24 0.5 mg/l, and pMAG17 0.1 mg/l. There is a significant difference in the specific activities of the purified enzymes. When compared to β -glucanase activity from the pMIG5-transformants, the relative specific activity from pMPG24 is 30% and from pMAG17 is 20%. The lower secretion level of β -glucanase directed by pMPG24 (as compared to pMIG5) might be due to the substitution of Asn to Ala in the signal peptide at position -2 with respect to the signal peptidase cleavage site generating a less efficient signal for secretion of protein.

The amino acid sequence of many secreted protein precursor segments has been determined, yet no apparent clear homologies have been detected at the primary sequence level, and the amino acids neighbouring the cleavage site also varies among the pre-proteins. Hence it is likely that certain general conformational features of the signals are functionally important for translocation. These may include the presence of charged amino acids in the amino-terminal region of the signal peptide followed by a predominantly hydrophobic central core and a less hydrophobic carboxyl-terminal segment which includes the signal peptidase cleavage site.

Figure 4 shows the calculated hydrophobicity/hydrophilicity (14) of the signal peptides (with neighbouring regions) used in this study. The figure illustrates that the central core of the

Figure 4. Structure analysis. (a) mouse α -amylase (b) yeast phosphatase 5 (c) yeast invertase signal peptides and neighbouring β -glucanase sequence. The arrow marks the peptide bond between the signal sequence and the β -glucanase protein. Prediction of secondary structure (A = α -helix, B = β -sheet, T = turn, blank = random coil) was done by using the rules of GARNIER et al. (10) and listed next to the amino acid number. Hydropobicity/hydrophilicity analysis of the sequences was performed according to the method of HOPP and WOODS (14).

invertase signal peptide displays the strongest hydrophobicity whereas the shorter mouse α -amylase signal peptide has a less pronounced hydrophobic structure. Perhaps the invertase signal peptide interacts most efficiently with the translocation apparatus of the yeast cell because of a more pronounced hydrophobic core.

Secondary protein structure predictions have demonstrated a high degree of conformational similarity among signal peptides. There is a high probability that the hydrophobic core of the signal peptide adopts an α -helix conformation and that this core segment is followed by a region capable of forming a β -turn structure required for cleavage. Through spectroscopic studies ROSENBLATT et al. (29) have shown that the signal sequence of the pre-parathyroid hormone assumes a highly structural α -helical conformation and they demonstrated that the structure of this sequence was as predicted by the CHOU and FASMAN rules (8). Figure 4 also shows the predicted secondary structures of the mouse α -amylase signal peptide, the yeast acid phos-

phatase signal peptide and the yeast invertase signal peptide when they are fused to the β -glucanase protein. The structures are based on the algorithm of CHOU and FASMAN (8) as modified by GARNIER et al. (10), and show that the invertase signal adopts an α -helical conformation from the amino-terminal end to the signal peptidase cleavage site whereas the modified phosphatase signal is less regularly α -helical. It also appears that the predicted secondary structure of the α -amylase signal peptide is very irregular. The conformational predictions, like that of others (3, 6, 29) indicate that an α -helix structure in front of a β -structure, as in the case of the invertase signal peptide, may delineate the COOH-terminus and cleavage site of the signal sequence. This structure may optimize the translocation process as indicated by the high secretion level directed by the pMIG5 construct (Table II). As discussed next, the pMIG5 construct codes for a pre-protein which is accurately processed by the yeast signal peptidase.

Table II. Secretion of β -glucanase from yeast (per litre medium).

Plasmid	Total protein in supernatant	Yield of β -glucanase ¹⁾	Amount of β -glucanase in 5 ml peak fraction ²⁾	Relative specific activity of β -glucanase in peak fraction ²⁾
	mg	μ g	μ g	
pYBAG17	–	< 5	–	–
pMAG17	20	100	70	20
pMPG24	20	500	80	30
pMIG5	20	5000	1000	100

¹⁾ Calculated by measuring the β -glucanase activity in concentrated crude supernatants, i.e. the amount of β -glucanase corresponds to the activity from an equal quantity of barley enzyme.

²⁾ The protein concentration was measured in pMIG5 using the BioRad assay. From the concentration obtained it was calculated that 1 OD₂₈₀ unit corresponds to approximately 0,55 mg/ml of protein. This conversion was used for calculation of relative specific activities. The protein concentration in supernatants from cells transformed with pMAG17 and pMPG24 was too low to be measured in the BioRad assay.

3.3. Signal peptide processing

SDS-PAGE of purified β -glucanase from the yeast culture medium showed a single Coomassie blue-staining band (Fig. 5). The 2-3

kD difference in apparent molecular weight between the native barley β -glucanase and the enzyme secreted from yeast cells is most likely due to different glycosylation patterns in the two

Figure 5. SDS-PAGE. Coomassie stained protein profiles. (C) Total secreted protein from yeast cells transformed with pMIG5. (Y) Purified barley β -glucanase from yeast cells transformed with pMIG5. (B) Purified barley (1-3,1-4)- β -glucanase isoenzyme II. (M) Molecular weight marker. The relative mobilities of the standards (in kD) are indicated.

Figure 6. Amino acid sequence flanking the presumed signal peptidase cleavage site (marked with an arrow) of the β -glucanase pre-proteins synthesized in yeast cells transformed with plasmid pMAG17 (a) and pMPG24 (b). The result of amino-terminal amino acid sequencing is shown below the pre-protein sequence. The detected amino acids are shown in descending order with the most abundant amino acid at the top.

Incorrect protein folding of the secreted proteins may enhance proteolytic break-down of the enzymes and give rise to a heterogenous population of amino-termini.

organisms. When purified β -glucanase from yeast cells was subjected to consecutive Edman degradation, the analysis of β -glucanase from pMAG17 revealed a heterogenous population of amino-terminal amino acids and we were not able to detect a major signal peptidase cleavage site (Fig. 6a). Only a minor proportion of the β -glucanase produced from pMPG24 (modified phosphatase signal peptide) was correctly processed (Fig. 6b), suggesting that the yeast signal peptidase(s) cannot process the modified phosphatase signal peptide with complete fidelity when the β -glucanase protein acts as the passenger protein. However, the amino-terminal sequence determination of purified β -glucanase directed by the pMIG5 construction (containing the invertase signal sequence code) exclusively gave the sequence Leu-Gly-Val-Cys-Tyr-Gly-Met-Ser-Ala-Asn. A precise cleavage had oc-

curred between the Ala (position no. 19) and Leu (position no. 20) residues of the β -glucanase pre-protein. The invertase signal sequence was more efficiently processed and the enzyme more efficiently secreted than those pre-proteins synthesized with either the mouse α -amylase signal sequence or the modified yeast phosphatase signal sequence (Table II). Altered protein folding may provide an explanation for the differences observed in processing of pre-proteins and these processing alterations correlate with the secretion efficiencies. This may be due to a combined effect of suboptimal signal peptide interactions with various components involved in the translocation apparatus. Incorrect processing of the pre-protein may also generate a substrate less easily transported through the secretory system of the yeast cells. This interpretation is supported by the results of ANDREWS et al. (1) who showed that deletions in pre-prolactin beyond the signal peptidase cleavage site alter folding of the pre-protein thereby reducing the translocation efficiency of the polypeptide molecules. They also found that a small sequence deletion in the mature protein, close to the signal peptidase cleavage site, reduces the signal peptidase accessibility by more than 70%. ANDREWS et al. (1) speculate that over the course of evolution a particular signal sequence is tailored to achieve optimal translocation of the appropriate passenger domain.

Correct processing of the pre-proteins lead to a secreted protein with a high specific activity (Table I). The specific activities of the incorrectly processed β -glucanase proteins secreted with the aid of either the mouse α -amylase signal peptide or the yeast phosphatase signal peptide were only 20-30% of that obtained by using the invertase signal peptide. These data emphasize the importance of signal peptidase processing to ensure maximum foreign protein secretion in yeast.

The invertase signal peptide has also been used to transport heterologous human interferon from yeast cells (7). The protein was secreted and correctly processed by the putative yeast signal peptidase. Perhaps the invertase signal peptide code contains an optimal translation initiation region which, in combination with an optimal signal peptidase recognition sequence

of the nascent polypeptide chain, leads to a highly efficient secretion process.

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REFERENCES

- ANDREWS, D.W., E. PERARA, C. LESSER & V.R. LINGAPPA: Sequences beyond the cleavage site influence signal peptide function. *J. Biol. Chem.* 263, 15791-15798 (1988)
- ASHIKARI, T., N. KIUCHI-GOTO, Y. TANAKA, Y. SHIBANA, T. AMACHI & H. YOSHIZUMI: High expression and efficient secretion of *Rhizopus oryzae* glucoamylase in the yeast *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 30, 515-520 (1989)
- AUSTEN, B.M.: Predicted secondary structures of amino-terminal extension sequences of secreted proteins. *FEBS Lett.* 103, 308-313 (1979)
- BALLANCE, G.M. & I. SVENDSEN: Purification and amino acid sequence determination of an endo-1,3- β -glucanase from barley. *Carlsberg Res. Commun.* 53, 411-419 (1988)
- CARLSON, M.R., R. TAUSSING, S. KUSTU & D. BOTSTEIN: The secreted form of invertase in *Saccharomyces cerevisiae* is synthesized from mRNA encoding a signal sequence. *Mol. Cell. Biol.* 3, 439-447 (1983)
- CHAN, S.J., C. PATZELT, J.R. DUGUID, P. QUINN, A. LABRECQUE, B. NOYES, P. KLEIN, R.L. HENRIKSON & D.F. STEINER: In: *From Gene to Protein: Information transfer in normal and abnormal cells*, eds. T.R. Russel, K. Brew, H. Gaver & J. Schultz. Academic Press, New York, pp. 361-378 (1979)
- CHANG, N.C., M. MATTEUCCI, L.J. PERRY, J.J. WULF, C.Y. CHEN & R.A. HITZEMAN: *Saccharomyces cerevisiae* secretes and correctly processes human interferon hybrid proteins containing yeast invertase signal peptides. *Mol. Cell. Biol.* 6, 1812-1819 (1986)
- CHOU, P.Y. & G.D. FASMAN: Predictions of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 47, 45-148 (1978)
- COLE, G.E., P.C. MCCABE, D. INLOW, D.H. GELFAND, A. BEN-BASSAT & M.A. INNIS: Stable expression of *Aspergillus awamori* glucoamylase in distiller's yeast. *Bio/Technology* 6, 417-421 (1988)
- GARNIER, J., D.J. OSGUTHORPE & B. ROBSON: Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120, 97-120 (1978)
- GOLUB, E.I.: "One minute" transformation of competent *E. coli* by plasmid DNA. *Nucleic Acids Res.* 16, 1641 (1988)
- HANAHAN, D.: In: *DNA cloning: A Practical Approach* (Glover, D., ed) Vol. 1, p. 109. IRL Press, Oxford, England (1985)
- HATTORI, M. & Y. SAKAKI: Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* 152, 232-238 (1986)
- HOPP, T.P. & K.R. WOODS: Predictions of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* 78, 3824-3828 (1981)
- ITO, H., Y. FUKUDA, K. MURATA & A. KIMURA: Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153, 163-168 (1983)
- JACKSON, E.A., G.M. BALLANCE & K.K. THOMSEN: Construction of a yeast vector directing the synthesis and release of barley (1-3,1-4)- β -glucanase. *Carlsberg Res. Commun.* 51, 445-458 (1986)
- KAISER, C.A. & D. BOTSTEIN: Secretion-defective mutations in the signal sequence for *Saccharomyces cerevisiae* invertase. *Mol. Cell. Biol.* 6, 2382-2391 (1986)
- LAEMMLI, U.K.: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680-685 (1985)
- MANIATIS, T., E.F. FRITSCH & J. SAMBROOK: In: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 1-540 (1982)
- MCCLEARY, B.V.: *Proceedings of the 19th Convention of the Institute of Brewing (Australia and New Zealand Section)*, Hobart (1986)
- MELLOR, J., M.J. DOBSON, N.A. ROBERTS, M.F. TUTT, J.S. EMTAGE, S. WHITE, P.A. LOWE, T. PATEL, A.J. KINGSMAN & S.M. KINGSMAN: Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. *Gene* 24, 1-14 (1983)
- MELLOR, J., M.J. DOBSON, N.A. ROBERTS, A.J. KINGSMAN & S.M. KINGSMAN: Factors affecting

- heterologous gene expression in *Saccharomyces cerevisiae*. *Gene* 33, 215-226 (1985)
23. MIYAJIMA, A., N. NAKAYAMA, I. MIYAJIMA, N. ARAI, H. OKAYAMA & K. ARAI: Analysis of full-length cDNA clones carrying GalI of *Saccharomyces cerevisiae*: Model system for cDNA expression. *Nucleic Acids Res.* 12, 6397 (1984)
 24. OLSEN, O.: Analysis of the effect of dG·dC homopolymer tails on expression of a mouse α -amylase cDNA gene in yeast. *Carlsberg Res. Commun.* 52, 91-97 (1987)
 25. PENTTILÄ, M.E., P. LEHTOVAARA, M. BAILY, T.T. TEERI & J.K.C. KNOWLES: Efficient secretion of two fungal cellobiohydrolases by *Saccharomyces cerevisiae*. *Gene* 63, 103-112 (1988)
 26. PETERSEN, J.G.L., M.C. KIELLAND-BRANDT, S. HOLMBERG & T. NILSSON-TILLGREN: Mutational analysis of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*. Mapping of *ilv2* and *ilv5*. *Carlsberg Res. Commun.* 48, 21-34 (1983)
 27. PREUSS, D. & D. BOTSTEIN: Secretion-defective mutations in the signal sequence for *Saccharomyces cerevisiae* invertase. *Mol. Cell Biol.* 6, 2382-2391 (1986)
 28. QUEEN, C. & L.J. KORN: A comprehensive sequence analysis program for the IBM personal computer. *Nucleic Acids Res.* 12, 581-599 (1984)
 29. ROSENBLATT, M., N.V. BEAUDETTE & G.D. FASSMAN: Conformational studies of the synthetic precursor-specific region of pre-proparathyroid hormone. *Proc. Natl. Acad. Sci. USA* 77, 3983-3987 (1980)
 30. ROTHBLATT, J.A. & D.I. MAYER: Secretion in yeast: Reconstitution of the translocation and glycosylation of α -factor and invertase in a homologous cell-free system. *Cell* 44, 619-628 (1986)
 31. SMITH, R.A., M.J. DUNCAN & D.T. MOIR: Heterologous protein secretion from yeast. *Science* 229, 1219-1224 (1985)
 32. SØGAARD, M. & B. SVENSSON: Expression of two barley α -amylase cDNAs in yeast and characterization of the secreted proteins. In preparation.
 33. THILL, B.B., R.A. KRAMER, K.J. TURNER & K.A. BOSTIAN: Comparative analysis of the 5'-end of two repressible acid phosphatase genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 3, 570-579 (1983)
 34. THIM, L., M.T. HANSEN, K. NORRIS, I. HOEGH, E. BOEL, J. FORSTROM, G. AMMERER & N.P. FIIL: Secretion and processing of insulin precursors in yeast. *Proc. Natl. Acad. Sci. USA* 83, 6766-6770 (1986)
 35. TUBB, R.S.: Amylolytic yeasts for commercial applications. *TIBTECH* 98-104 (April 1988)
 36. WATERS, M.G., W.J. CHIRICO & G. BLOBEL: Protein translocation across the yeast microsomal membrane is stimulated by a soluble factor. *J. Cell. Biol.* 103, 2629-2636 (1986)
 37. WOODWARD, J.R. & G.B. FINCHER: Purification and chemical properties of two 1-3;1-4- β -glucan endohydrolases from germinating barley. *Eur. J. Biochem.* 121, 663-669 (1982)
 38. YADEAN, J.T. & G. BLOBEL: Solubilization and characterization of yeast signal peptidase. *J. Biol. Chem.* 264, 2928-2834 (1989)
 39. ZHONG, H., R. SCHOLL, J. BROWSE & C. SOMMERVILLE: Double stranded sequencing as a choice for DNA sequencing. *Nucleic Acids Res.* 16, 1220 (1988)

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