PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/62, C12N 1/19 // 15/81

A1

(11) International Publication Number: **WO 98/01473**

(43) International Publication Date: 15 January 1998 (15.01.98)

(21) International Application Number: PCT/DK97/00297

(22) International Filing Date: 4 July 1997 (04.07.97)

(30) Priority Data:
0748/96 5 July 1996 (05.07.96) DK

(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: EGEL-MITANI, Michi [DE/DK]; Gøngesletten 31, DK-2950 Vedbæk (DK). BRANDT, Jakob [DK/DK]; Tjørnevangen 1, st., DK-2700 Brønshøj (DK). VAD, Knud [DK/DK]; Duevej 112, 2. Tv., DK-2000 Frederiksberg (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR THE PRODUCTION OF PRECURSORS OF INSULIN, PRECURSORS OF INSULIN ANALOGUES, AND INSULIN LIKE PEPTIDES

(57) Abstract

The present invention relates to a novel method for the production of precursors of insulin, precursors of insulin analogues, and insulin like peptides in genetically modified yeast cells having reduced activity of YAP3 protease. The invention further comprises said genetically modified yeast cells, and a method for the preparation of said yeast cells.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHOD FOR THE PRODUCTION OF PRECURSORS OF INSULIN, PRECURSORS OF INSULIN ANALOGUES, AND INSULIN LIKE PEPTIDES

FIELD OF THIS INVENTION

The present invention relates to a novel method for the production of precursors of insulin, precursors of insulin analogues, and insulin like peptides in genetically modified yeast cells, said genetically modified yeast cells, and a method for the preparation of said yeast cells.

BACKGROUND OF THIS INVENTION

Expression of heterologous proteins in yeast after transformation of yeast cells with suitable expression vectors comprising DNA sequences coding for said proteins has been successful for precursors of insulin, precursors of insulin analogues, and insulin like peptides. Yeasts, and especially Saccharomyces cerevisiae, are preferred host microorganisms for the production of pharmaceutically valuable polypeptides due to the stable yield and safety.

A number of proteases, activated by the PEP4 gene product are responible for yeast protein degradation. Mutation in the PEP4 gene such as the pep4-3 mutation is often used to reduce cellular proteolysis whereby the quality and yields of heterologous proteins of interest can be improved. EP 341215 describes the use of a yeast strain that lacks carboxypeptidase yscα activity for the expression of the heterologous protein hirudin. Wild-type yeast strains produce a mixture of desulphatohirudin species differing in the C-terminal sequence due to the post-translational action of endogeneous yeast proteases on the primary expression product. It is shown that yeast mutant strains lacking carboxypeptidase yscα activity are unable to remove amino acids from the C-terminus of heterologous proteins and therefore give rise to integral proteins. The use of yeast strains defective in ysc A, B, Y, and/or S activity can only partially reduce random proteolysis of foreign gene products.

Another problem encountered in production of heterologous proteins in yeast is low yield, presumably due to proteolytic processing both in intracellular compartments and at the plasma membrane caused by aberrant processing at internal sites in the protein, e.g. secretion of human parathyroid hormone (Gabrielsen et al. Gene 90: 255-262, 1990; Rokkones et al. J. Biotechnol. 33: 293-306, 1994), and secretion of β-endorphine by S. cerevisiae (Bitter et al. Proc. Natl. Acad. Sci. USA 81: 5330-5334, 1984).

WO 95/23857 discloses production of recombinant human albumin (rHA) in yeast cells having a reduced level of yeast aspartyl protease 3 (Yap3p) proteolytic activity resulting in a reduction of undesired 45 kD rHA fragment, and in a 30 to 50% increased yield of recovered rHA produced by the haploid Δyap3 strain compared to the rHA produced by the corresponding haploid YAP3 wild-type strain.

Previously, Bourbonnais et al. (Biochimie 76: 226-233, 1994), have shown that the YAP3 protease gene product has in vitro substrate specificity which is distinct though overlapping with the Kex2p substrate specificity, and shown that Yap3p cleaves exclusively C-terminal to arginine residues present in the prosomatostatin's putative processing sites. Moreover, Cawley et al. (J. Biol. Chem. 271: 4168-4176, 1996) have determined the in vitro specificity and relative efficiency of cleavage of mono- and paired-basic residue processing sites by Yap3p for a number of prohormone substrates, such as bovine proinsulin.

Often it is advantageous to produce heterologous polypeptides in a diploid yeast culture, because possible genetical defects may become phenotypically expressed in a haploid yeast culture, e.g. during continuous fermentation in production scale, and because the yield may be higher (Fu et al. Biotechnol. Prog. 12: 145-148, 1996; Mead et al. Biotechnol. Letters 8: 391-396, 1986).

The purpose of the present invention is to provide an improved method for the production of secreted precursors of insulin, precursors of insulin analogues, and

insulin related peptides in a yeast expression system. Preferably, the production of precursors of insulin, precursors of insulin analogues, and insulin related peptides by the method of the invention is increased considerably, e. g. from about 60 to about 350%, more preferably from about 100 to about 300%, compared to the production of precursors of insulin, precursors of insulin analogues, and insulin like peptides in conventional yeast expression systems, and/or, preferably, the level of proteolysis of the secreted product resulting in an inhomogeneous product is decreased considerably.

SUMMARY OF THE INVENTION

The above identified purpose is achieved with the method according to the present invention which comprises culturing a yeast which has reduced activity of Yap3p or a homologue thereof and has been transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for a precursor of insulin, a precursor of an insulin analogue, or an insulin related peptide, and isolating said precursor of insulin, precursor an insulin analogue, or an insulin related peptide. Preferably, the yeast cells lack Yap3p activity through disruption of the YAP3 gene.

It has been found that using a YAP3 disrupted yeast strain for the production of heterologous polypeptides belonging to the insulin family including proinsulin, precursors of insulin, precursors of insulin analogues, and insulin like peptides, such as IGF-1, and SC hybrid result in a remarkably improved yield of up to about 200% compared to the yield from the corresponding YAP3 wild-type yeast strain. Moreover, the homogeneity in the sense of polypeptide chain length of said heterologous polypeptides is considerably improved.

Suitably, the yeast is S. cerevisiae which lacks a functional YAP3 gene. However, other yeast genera may have equivalent proteases, i.e. homologues of Yap3p, e. g. the genera Pichia and Kluyveromyces as shown in WO 95/23857 and Clerc et al. (J. Chromat. B. 662: 245-259, 1994). A gene is regarded as a homologue, in general, if

the sequence of the translation product has greater than 50% sequence identity to Yap3p. Komano and Fuller (Proc. Natl. Acad. Sci, USA 92: 10752-10756, 1995) has identified the Mkc7 aspartyl protease from S. cerevisiae which is closely related to Yap3p (53% identity). Other aspartyl proteases of Saccharomyces include the gene products of PEP4, BAR1, and of open reading frames, the sequences of which are partially homologous with the YAP3 open reading frame, such as YAP3-link (coded by GenBank acc. No. X89514: pos. 25352-26878), YIV9 (Swiss Prot acc. No. P40583), and aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116). According to recently accepted yeast genome nomenclature the yeast gene names YAP3, YAP3 link, YIV 9, NO 4, and MKC 7 used herein correspond to the yeast open reading frame YLR120C, YLR121C, YIR039C, YDR349C, and YDR144C, respectively. Furthermore, the gene product of open reading frame YGL259W is included among the yeast aspartyl proteases.

Examples of yeasts include Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri, Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans.

A suitable means of eliminating the activity of a protease is to disrupt the host gene encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease including regulatory and/or coding regions, or, alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations. Other methods which may be suitable for down regulation of the protease include the introduction of antisense and/or ribozyme constructs in the yeast, e.g. Atkins et al. (Antisense and Development 5: 295-305, 1995) and Nasr et al. (Mol. Gen Genet 249: 51-57, 1995). One preferred method of disrupting the YAP3 gene in the yeast strain used in the method of the present invention are described by Rothstein (Method in Enzymol, 194: 281-301, 1991).

The precursors of insulin, precursors of insulin analogues, and insulin related peptides may be of human origin or from other animals and recombinant or semisynthetic sources. The cDNA used for expression of precursors of insulin, precursors of insulin analogues, or insulin related peptides in the method of the invention include codon optimised forms for expression in yeast.

By "precursors of insulin or precursors of insulin analogues" we include all precursors of human insulin, preferably precursors of des(B30) human insulin, porcine insulin, and insulin analogues wherein at least one Lys or Arg is present. Examples of preferred insulin analogues among those in which a Lys or Arg is present are insulin analogues in which Phe^{B1} has been deleted, insulin analogues in which the A-chain and/or the B-chain have an N-terminal extension and insulin analogues in which the A-chain and/or the B-chain have a C-terminal extension. Other precursors of insulin analogues which can be produced according to the present invention are such in which one or more of the amino acid residues, preferably one, two, or three of them, have been substituted by another codable amino acid residue. Thus in position A21 a parent insulin may instead of Asn have an amino acid residue selected from the group comprising Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Ser, Thr, Trp, Tyr or Val, in particular an amino acid residue selected from the group comprising Gly, Ala, Ser, and Thr. Precursors of insulin analogues produced according to the method of the present invention may also be modified by a combination of changes outlined above. Likewise, in position B28 a parent insulin precursor may instead of Pro have an amino acid residue selected from the group comprising Asp or Lys and in position B29 a parent insulin precursor may instead of Lys have the amino acid Pro.

Futhermore, by "precursors of insulin analogue" as used herein is meant a peptide having a molecular structure similar to that of human insulin precursor including the disulphide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulphide bridge between Cys^{A6} and Cys^{A11}, and which can be processed to a polypeptide having insulin activity. When the amino acid at position B1 is deleted, the position of the remaining amino acids of the B-chain are not renumbered. The expres-

sion "a codable amino acid residue" as used herein designates an amino acid residue which can be coded for by the genetic code, i. e. a triplet ("codon") of nucleotides.

Throughout the description and claims is used one and three letter codes for amino acids in accordance with the rules approved (1974) by the IUPAC-IUB Commission on Biochemical Nomenclature, vide Collected Tentative Rules & Recommendations of the Commission on Biochemical Nomenclature IUPAC-IUB, 2nd ed., Maryland, 1975.

The insulin related polypeptides are IGF-1 (insulin like growth factor-1) and insulin single-chain hybrids, such as the SC hybrid, which designates a polypeptide consisting of the insulin B- and A-chains connected by the IGF-I C-peptide, cf. Kristensen et al. (Biochem J. 305: 981-986, 1995), and WO95/16708, and the insulin single-chain hybrids described in EP 741188.

A second aspect of the invention provides a culture of yeast cells containing a polynucleotide sequence, preferably a first DNA sequence, encoding a precursor of insulin, a precursor of insulin analogues, or insulin related peptides, and a second polynucleotide sequence, preferably a second DNA sequence, encoding a secretion signal causing said precursor of insulin, precursor of insulin analogues, or insulin like peptides to be secreted from the yeast, characterized in that the yeast cells have reduced Yap3 protease activity. Preferably, the yeast cells are transformed with a hybrid vector comprising said first DNA sequence and said second DNA sequence, and, preferably, the yeast cells lack Yap3p activity, and this may conveniently be obtained through disruption of the YAP3 gene. The culture of yeast cells according to the invention is haploid or polyploid, preferably diploid.

The DNA encoding the precursor of insulin, precursor of insulin analogue, or insulin related peptide may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration on host chromosome is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.

If integration is desired, the DNA is inserted into an yeast integration plasmid vector, such as pJJ215, pJJ250, pJJ236, pJJ248, pJJ242 (Jones & Prakash, Yeast 6: 363,1990) or pDP6 (Fleig et al. Gene 46:237, 1986), in proper orientation and correct reading frame for expression, which is flanked with homologous sequences of any non-essential yeast genes, transposon sequence or ribosomal genes. Preferably the flanking sequences are yeast protease genes or genes used as a selective marker. The DNA is then integrated on host chromosome(s) by homologous recombination occured in the flanking sequences, by using standard techniques shown in Rothstein (Method in Enzymol, 194: 281-301, 1991) and Cregg et al. (Bio/Technol. 11:905-910, 1993).

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the precursor of insulin, precursor of insulin analogues, or insulin like peptides, which can then be recovered, as is known.

Useful yeast plasmid vectors include the POT (Kjeldsen et al. Gene 170: 107-112, 1996) and YEp13, YEp24 (Rose and Broach, Methods in Enzymol. 185: 234-279, 1990), and pG plasmids (Schena et al. Methods in Enzymol. 194: 289-398, 1991).

Methods for the transformation of S. cerevisiae include the spheroplast transformation, lithium acetate transformation, and electroporation, cf. Methods in Enzymol. Vol. 194 (1991). Pereferably the transformation is as described in the examples herein.

Suitable promoters for S. cerevisiae include the MFα1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CYCI promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoter. A suitable promoter in the genus Pichia is the AOXI (methanol utilisation) promoter.

The transcription terminal signal is preferably the 3' flanking sequence of a eucaryotic gene which contains proper signal for transcription termination and polyadenylation. Suitable 3' flanking sequences may, e.g. be those of the gene naturally linked to the expression control sequence used, i.e. corresponding to the promoter.

The DNA constructs that are used for providing secretory expression of precursors of insulin, precursors of insulin analogues, or insulin related peptides according to the invention comprising a DNA sequence that includes a leader sequence linked to the polypeptide by a yeast processing signal. The leader sequence contains a signal peptide ("pre-sequence") for protein translocation across the endoplasmic reticulum and optionally contains an additional sequence ("pro-sequence"), which may or may not be cleaved within yeast cells before the polypeptide is released into the surrounding medium. Useful leaders are the signal peptide of mouse α-amylase , S cerevisiae MFα1, YAP3, BAR1, HSP150 and S. kluyveri MFα signal peptides and prepro-sequences of S. cerevisiae MFα1, YAP3, PRC, HSP150, and S. kluyveri MFα and synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666. Furthermore, the precursor of insulin, precursor of insulin analogues, or insulin related peptides to be produced according to the the method of invention may be provided with an N-terminal extension as described in WO 95/35384.

The invention also relates to a method of preparing a yeast having reduced Yap3p activity comprising the steps of a) providing a hybrid plasmid containing a part of the YAP3 gene and suitable for transformation into a yeast cell, b) disrupting the YAP3 gene by deleting the fragment of YAP3 and inserting the URA3 gene instead to obtain a Δyap3::URA3 gene disruption plasmid, c) providing a yeast Δura3 deletion mutant, d)

transforming said mutant with said plasmid, and e) selecting the Δyap3::URA3 deletion mutants on a medium without uracil. Further the invention relates to a method of preparing a yeast having reduced Yap3p activity using antisense technology.

Moreover, the precursors of insulin, precursors of insulin analogues, or insulin related peptides to be produced according to the method of the invention may conveniently be expressed coupled to an N- or C-terminal tag or as a precursor or fusion protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the construction of the pS194 plasmid.

Fig. 2 shows the construction of plasmids pME834 and pME1389.

Fig. 3 is a restriction map of a general expression plasmid used herein.

Fig. 4 is a restriction map of the pME973 plasmid, containing the genes encoding the HO (homothallism) endonuclease and Ura3p inserted into the YEp13 plasmid.

DETAILED DESCRIPTION OF THIS INVENTION

Preferred embodiments of this invention are described in Table 1 below. Having knowledge of the art, it will be obvious to a skilled person to produce precursors of insulin, precursors of insulin analogues, or insulin related peptides by the method of the present invention and using the following constructs:

Table 1

Presequence (signal)	Prosequence	Heterologous protein
YAP3(1-21)	LA19[1]-KR	EEAEPK-insulin B chain(1-29)-AAK-insulin A chain(1-21)
YAP3(1-21)	LA19[1]-KR	EEPK-insulin B chain(1-29)-AAK-Achain(1-21)
MF α1(1-19)	MF α1(20-85)	Proinsulin
MF α1(1-19)	MF α1(20-85)	Insulin B chain(1-27)-DKAAK-insulin A chain
MF α1(1-19)	MF α1(20-85)	$E(EA)_3K$-insulin B chain(1-29)-AAK-insulin A chain(1-21)

MF α1(1-19)	MF α1(20-81)MAKR	DDDDK-IGF-1
MF α1(1-19)	MF α1(20-81) MAKR	SC hybrid
HSP150(1-18)	HSP150(19-67)-WIIAENTTLANVAMAKR	$E(EA)_3$PK-insulinB chain(1-29)-AAK-A chain(1-21)
MF α1(1-19)	MF α1(20-85)	$E(EA)_3$PK-insulin B chain(1-27)-DK-insulin A chain(1-21)
MF α1(1-19)	LA19[1]-KR	Insulin B chain(1-29)-AAK-insulin A chain(1-21)
spx3[2]	LaC212	Insulin B chain(1-29)-AAK-insulin A chain(1-21)
YAP3(1-21)	MF α1(20-81)MAKR	IGF-2
MF α1 (1-19)	MF α1(20-81)MAKR	IGF-2
MF α1 (1-19)	MF α1(20-81)MAXKR X=peptide bond or S or K or E or ARS	IGF-1
YAP3(1-21)	MF α1 (20-81)MAKR	IGF-1

[1] LA19, cf. SEQ ID NO:1 and WO 95/34666. [2] spx3-LaC212, cf. WO89/02463 and WO 90/10075.

The Genetic background of S. cerevisiae strains used herein is as follows:

E11-3C MATα YAP3 pep4-3 Δtpi::LEU2 leu2 URA3

SY107 MATα YAP3 pep4-3 Δtpi::LEU2 leu2 Δura3

ME1487 MATα Δyap3::URA3 pep4-3 Δtpi::LEU2 leu2 Δura3

ME1656 MATα Δyap3::ura3 pep4-3 Δtpi::LEU2 leu2 Δura3

ME1684 MAT**a** Δyap3::URA3::Δylr121c pep4-3 Δtpi::LEU2 leu2 Δura3

ME1695 MATα Δyap3::ura3 pep4-3 Δtpi::LEU2 leu2 Δura3

ME1719 MAT**a**/α Δyap3::URA3/Δyap3::ura3 pep4-3/pep4-3 Δtpi::LEU2/Δtpi::LEU2 leu2/leu2 Δura3/Δura3

MT663 MAT**a**/α YAP3/YAP3 pep4-3/pep4-3 Δtpi::LEU2/Δtpi::LEU2 leu2/leu2 URA3/URA3 HIS4/his4

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in

the fore-going description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

Example 1

Δyap3::URA3 gene disruption

The Δura3 deletion mutation was constructed as follows:
pJJ244 (pUC18 containing a 1.2 kb HindIII fragment of the URA3 gene) was digested with StyI and filled in with Klenow polymerase and self ligated. The resulting plasmid designated pS194 contains a 84bp of StyI-StyI fragment deletion of the URA3 gene, cf. Fig.1.

The Δyap3::URA3 gene disruption plasmid pME1389 was constructed as follows:
The 2.6kb SacI-PstI fragment which contains the YAP3 gene in pME768 (Egel-Mitani et al. Yeast 6: 127-137, 1990) was inserted in 2.6 kb of the SacI-PstI fragment of pIC19R (Marsh et al. Gene 32: 481-485, 1984). The resulting plasmid is pME834. pME834 was digested with HindIII to form a deletion of the 0.7 kb YAP3 fragment and the 1.2 kb HindIII fragment of the URA3 gene (Rose et al. Gene 29: 113-124, 1984) was inserted instead. The resulting plasmid is pME1389. The construction of plasmids pME834 and pME1389 is shown in Fig. 2 in diagrammatic form.

S. cerevisiae strain E11-3C (MATα YAP3 pep4-3 Δtpi::LEU2 leu2 URA3), cf. ATCC 20727, US pat. 4766073, was transformed with linialized pS194 (BsgI digested) to make Δura deletion mutation. By selection on 5-FOA (5-fluoro-orotic acid) containing minimal plates, the Δura3 mutant designated SY107 was obtained.

The strain SY107 (MATα YAP3 pep4-3 Δtpi::LEU2 leu2 Δura3), was then transformed with pME1389 previously being cut by SalI and SacI, and 3kb fragment of Δyap3::URA3 was isolated for the transformation. Δyap3::URA3 deletion mutants

were selected on minimal plates without uracil. URA3 transformants were characterized by PCR and Southern hybridisation to confirm the correct integration of the Δyap3::URA3 fragment in the YAP3 locus. ME1487 was isolated as a Δyap3::URA3 deletion mutant (MATα Δyap3::URA3 pep4-3 Δtpi::LEU2 leu2 Δura3).

Example 2

Construction of a diploid Δyap3/Δyap3 strain

ME1487 was mutagenized by using EMS (methane-sulfonic acid ethylester) and ura3 mutants were selected on plates containing 5-FOA. One of the selected isolates, ME1656 was then subjected to mating type switch (Herskowitz and Jensen, Methods in Enzymol. 194: 132-146,1991) by transient transformation with pME973 shown in Fig. 4. pME973 contains the genes encoding the HO (homothallism) endonuclease and URA3 inserted into the YEp13 plasmid (Rose and Broach, Methods in Enzymol. 185: 234-279, 1990). From transient transformants, ME1695 was selected as the haploid strain, which had switched from MATα to MAT**a**, and have the following genetic background: MAT**a** Δyap3::ura3 pep4-3 Δtpi::LEU2 leu2 Δura3.

ME1695 was then crossed with ME1487 by micromanipulation (Sherman and Hicks, Methods in Enzymol. 194: 21-37, 1991) in order to get Δyap3/Δyap3 diploids. From the resulting diploids, ME1719 was selected as the strain with the following genetic background:

MAT**a**/α Δyap3::ura3/Δyap3::URA3 pep4-3/pep4-3 Δtpi::LEU2/Δtpi::LEU2 leu2/leu2 Δura3/Δura.

Example 3

Construction of a Δyap3::URA3::Δylr121c double disruption strain

In order to make a one-step gene disruption strain of the two closely linked genes encoding YAP3 and YLR121C, the following two oligonucleotide primers were synthesized:

P1 Length 57bp: YLR121C/URA3 primer

5′-GAT CGA ACG GCC ATG AAA AAT TTG TAC TAG CTA ACG AGC AAA GCT TTT CAA TTC AAT-3′

P2 Length 57bp: YAP3/URA3 primer

5′-CCA GAA TTT TTC AAT ACA ATG GGG AAG TTG TCG TAT TTA TAA GCT TTT TCT TTC CAA-3′

P1 and P2 each contains 40 nucleotides corresponding to sequences within the coding region of YLR121C and YAP3, respectively, as well as a HindIII site (AAGCTT) and 12 nucleotides corresponding to sequences flanking the URA3 gene (YEL021W). P1 and P2 were used for PCR using the URA3 gene as template. The resulting 1248bp PCR fragment contains the URA3 selective marker flanked with 40 nucleotides derived from the YAP3 or YLR121C encoding regions. The PCR fragment was then transformed into ME1655, and Δyap3::URA3::Δylr121c deletion mutants were selected and characterized as described in Example 1. ME1684 was isolated as a Δyap3::URA3::Δylr121c mutant with the following genetic background: MATα Δyap3::URA3::Δylr121c pep4-3 Δtpi::LEU2 leu2 Δura3.

Example 4

Transformation into yeast

In order to make yeast competent cells, yeast haploid strains SY107 and ME1487 or the diploid ME1719 strain were cultivated in 100ml YPGGE medium (1% yeast extract, 2% peptone, 2% glycerol, 2% galactose, 1% ethanol) to OD_{600} = 0.2. Cells

were harvested by centrifugation at 2000rpm for 5 min. and washed once by 10ml H_2O. Cells were resuspended in 10ml SED (1M sorbitol, 25mM Na_2EDTA pH8, 6.7mg/ml dithiothreitol) and incubated at 30°C for 15min. Cells were harvested by centrifugation and resuspended in 10ml SCE (1M sorbitol, 0.1M Na-citrate, 10mM Na_2EDTA, pH5.8) + 2mg Novozyme SP234 and incubated at 30°C for 30 min. After cells were harvested by centrifugation and washed once by 10ml 1.2M sorbitol and subsequently by 10ml CAS (1M sorbitol 10mM $CaCl_2$, 10mM Tris-HCl pH7.5), cells were harvested by centrifugation and resuspended finally in 2ml CAS. Competent cells were frozen in portion of 100µl per Eppendorf tube at -80°C.

Transformation was made as follows: Frozen competent cells (100µl) were warmed up quickly and 1µg plasmid DNA were added. Cells were incubated at room temp. for 15 min. and 1ml PEG solution (20% polyethyleneglycol 4000, 10mM $CaCl_2$, 10mM Tris-HCl pH7.5) was added. After 30min. at room temperature, cells were harvested by centrifugation at 2000rpm for 15min. and resuspended in 100µl SOS (1M sorbitol, 1/2 vol. YPGGE, 0.01% uracil, 7mM $CaCl_2$). After incubating at 30°C for 2 hours, cells were centrifuged and resuspended in 0.5ml 1M sorbitol. Cells were then spread on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) together with 6ml of top agar (YPD containing 2.5% agar). Plates were incubated at 30°C for 3 to 5 days until transformants appear.

Example 5

Heterologous protein expression plasmid

Yeast-E.coli shuttle vector (Fig. 3) used in the following examples contains a heterologous protein expression cassette, which includes a DNA sequence encoding a leader sequence followed by the heterologous polypeptide in question operably placed in between the TPI promoter and TPI terminator of S. cerevisiae in a POT plasmid (Kjeldsen et al. 1996, *op. cit.*). Examples are shown as follows:

Table 2

Presequence (signal)	Prosequence	Heterologous protein	plasmid (Fig. 3)
YAP3(1-21)	LA19[1]-KR	EEAEPK-insulin B chain(1-29)-AAK-insulin A chain(1-21)	pAK729
MFα1(1-19)	MFα1(20-81)MAKR	EEAEAEEAK-insulin B chain(1-29)-AAK-insulin A chain(1-21)	pJB152

[1] LA19, cf. SEQ ID NO:1 and WO 95/34666.

Example 6

Expression of insulin precursor EEAEPK-B chain(1-29)-AAK-A chain(1-21)

Insulin precursor EEAEPK-B chain(1-29)-AAK-A chain(1-21) (referred to as "EEAEPK-MI3" below) expression plasmid pAK729 equivalent to the plasmid shown in Fig. 3, in which the leader sequence-polypeptide is YAP3(1-21)-LA19KR-EEAEPK-MI3), was transformed into ME1487 (Δyap3), ME1719 (Δyap3/Δyap3) and SY107 (YAP3 WT). Transformants were selected by glucose utilization as a carbon source on YPD plates (1% w/v yeast extract, 2% w/v peptone, 2% glucose, 2% agar). ME1541 and YES1729 are pAK729 transformants obtained from ME1487 (Δyap3) and ME1719 (Δyap3/Δyap3), respectively, whereas ME1540 is the pAK729 transformant obtained from SY107 (Δyap3). Transformants were inoculated in 5ml YPD + 5mM $CaCl_2$ liquid medium and incubated at 30°C for 3 days with shaking at 200rpm. Culture supernatants were obtained after centrifugation at 2500rpm for 5 min. and 1ml supernatants were analyzed by reverse phase HPLC. Production levels shown in Table 3 were average value of cultures from 2 independently isolated transformants (Exp. 1) or values from a mixculture of 3 transformants, and were normalized so that YAP3 wild type level was taken as 100%. HPLC analyses showed that ME1541 and YES 1729 produced 1.7 to 2.5 times more insulin precursor than ME1540.

HPLC settings for analysis of precursors of insulin

HPLC-Column: 4 x 250 mm Novo Nordisk YMC-OdDMeSi C18 5 um

Column temp.: 50°C

Flowrate: 1 ml/min

HPLC solvents:

A: 10 % (v/v) acetonitrile in 0.2 M Na2SO4, 0.04 M H3PO4 pH adjusted to 2.3 with ethanolamine

B: 50 % (v/v) acetonitrile in water

Inj. vol:: 150 µl

Insulin precursor is eluated from the HPLC columns with the following acetonitrile gradient:

Ins.Pre.: 23.6 % acetonitrile to 34.0 % acetonitrile in 40 min.

Table 3

TRANSFORMANT	HOST	PLASMID	EEAEPK-MI3	
			Exp. 1	Exp. 2
ME1540	SY107	pAK729	100%	100%
ME1541	ME1487	pAK729	247%	177%
YES1729	ME1719	pAK729	ND	167%

Example 7

Expression of EEAEAEAK-B chain (1-29)-AAK-A chain (1-21) insulin precursor

Insulin precursor EEAEAEAK-B chain(1-29)-AAK-A chain(1-21) (refered to as "EEAEAEAK-MI3" below) expression plasmid pJB152 equivalent to the plasmid shown in Fig. 3, in which the leader sequence-polypeptide is MFα1(1-81)MAKR-EEAEAEAK-MI3 (Kjeldsen et al. 1996, *op. Cit*) was transformed into ME1487 (Δyap3), ME1719 (Δyap3/Δyap3) and SY107 (YAP3 WT) and transformants were selected and analysed as desribed in Example 4. ME1600 is the pJB152 transfor-

mant obtained from ME1487(Δyap3), whereas ME1599 is the pJB152 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 4, were an average value from 2 independently isolated transformants, and were normalised so that the haploid YAP3 wild-type level of EEAEAEAK-MI3 insulin precursor was taken as 100%. HPLC analyses showed that ME1600 produced 3.7-fold more EEAEAEAK-MI3 insulin precursor than ME1599. Moreover, the insulin precursor produced from ME1600 was homogeneous compared to that from ME1599, which produced 32% N-terminal trunkated insulin precursor in form of B chain(1-29)-AAK-A chain(1-21) (designated "MI3" in Tabel 4)

Table 4

TRANSEORMANT	HOST	PLASMID	MI3	EEAEAEAK-MI3
ME1599	SY107	pJB152	32%	100%
ME1600	ME1487	pJB152	0%	371%

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gln Pro Ile Asp Asp Thr Glu Ser Asn Thr Thr Ser Val Asn Leu Met
1 5 10 15

Ala Asp Asp Thr Glu Ser Arg Phe Ala Thr Asn Thr Thr Leu Ala Leu
20 25 30

Asp Val Val Asn Leu Ile Ser Met Ala
35 40

CLAIMS

1. A method for the production of a precursor of insulin, a precursor of an insulin analogue, or an insulin related peptide in yeast, comprising culturing a yeast having reduced Yap3p activity or a homologue thereof, said yeast being transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for said precursor of insulin, precursor of an insulin analogue, or an insulin related peptide and isolating said precursor of insulin, precursor of an insulin analogue, or an insulin related peptide.

2. A method according to claim 1, wherein the yeast lacks Yap3 protease activity.

3. A method according to claim 1, wherein the yeast is a diploid yeast.

4. A method according to any one of claims 1, 2 or 3, wherein the yeast is selected from the group consisting of Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri, Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans.

5. A method according to claim 3 wherein the yeast is S. cerevisiae.

6. A method according to any one of the preceding claims wherein the yeast additionally has reduced protease activity selected from the group of proteases coded for by the BAR1, STE13, PRA, PRB, KEX1, PRC, CPS, and the YAP3 homologues MKC7, YAP3-link (coded by GenBank acc. No. X89514:25352-26878), YIV9 (Swiss Prot acc. No. P40583), and aspartyl protease (IV) (coded by GenBank acc. No. U28372:326-2116) genes.

7. A method according to any one of the preceding claims wherein the yeast additionally has reduced protease activity selected from the group of proteases coded for by the STE13, PRA, PRB, KEX1, and PRC genes.

8. A method according to any one of claims 1 to 5, wherein the yeast additionally has reduced protease activity of the aparatyl protease encoded by the yeast open reading frame YGL259W or selected from the group of serine proteases coded for by the KEX2 gene and the yeast open reading frame YCR045C and YOR003W.

9. A method according to any one of the preceding claims wherein the yeast additionally has the pep4-3 mutation.

10. A method according to any one of the preceding claims, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a pre-sequence (signal) or a prepro-sequence linked in the proper reading frame to a second DNA sequence coding for a precursor of insulin, precursor of an insulin analogue, or an insulin related peptide, and a DNA sequence containing yeast transcription termination signals.

11. A method according to claim 10, wherein the yeast promoter is selected from the group consisting of the MFα1 promoter, CYC1 promoter, galactose inducible promoters such as GAL1, GAL7, and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoters.

12. A method according to claim 10, wherein the hybrid vector comprises a first DNA sequence encoding a signal peptide of mouse α-amylase, S. cerevisiae MFα1, BAR1, YAP3 and HSP150 and S. kluyveri MFα signal peptides or prepro-sequences of S. cerevisiae MFα1, YAP3, PRC, HSP150 and S. kluyveri MFα and/or synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666.

13. A method according to any one of the preceding claims wherein the DNA sequence encoding the precursor of insulin, precursor of an insulin analogue, or an insulin related peptide is selected from the group consisting of DNA sequences encoding precursor of proinsulin, IGF-1, precursor of insulin and insulin analogues such as EEAEPK-insulin B chain(1-29)-AAK-insulin A chain(1-21) and insulin B chain(1-27)-DKAAK-A chain(1-21).

14. A culture of yeast cells containing a DNA sequence encoding a precursor of insulin, precursor of an insulin analogue, or an insulin related peptide and a second DNA sequence encoding a secretion signal causing said precursor of insulin, precursor of an insulin analogue, or an insulin related peptide analogue to be secreted from the yeast, characterized in that the yeast cells have reduced Yap3p activity.

15. A culture of yeast cells transformed with a hybrid vector comprising said DNA sequence and said second DNA sequence.

16. A culture according to any one of claims 14 to 17, wherein the yeast cells lack Yap3p activity.

17. A culture according to any one of claims 14 to 16, wherein the yeast cells are diploid yeast cells.

18. A culture according to any one of claims 14 to 17, wherein the yeast is selected from the group consisting of Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri, Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans.

19. A culture according to claim 18, wherein the yeast is S. cerevisiae.

20. A culture according to any one of the claims 14 to 19, wherein the yeast cells additionally have reduced protease activity selected from the group of proteases coded for by the BAR1, STE13, PRA, PRB, KEX1, PRC, CPS, and the YAP3 homologues MKC7, YAP3-link (coded by GenBank acc. No.X89514: pos. 25352-26878), YIV9 (Swiss Prot acc. No. P40583), and aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116) genes.

21. A culture according to any one of the claims 14 to 19, wherein the yeast cells additionally have reduced protease activity selected from the group of proteases coded for by the STE13, PRA, PRB, KEX1, and PRC genes.

22. A culture according to any one of the claims 14 to 19, wherein the yeast cells additionally have reduced protease activity of the aparatyl protease encoded by the yeast open reading frame YGL259W or selected from the group of serine proteases coded for by the KEX2 gene and the yeast open reading frame YCR045C and YOR003W.

23. A culture according to any one of claims 14 to 22, wherein the yeast cells additionally have the pep4-3 mutation.

24. A culture according to any one of claims 14 to 23, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a pre-sequence (signal) or a prepro-sequence linked in the proper reading frame to a second DNA sequence coding for a precursor of insulin, precursor of an insulin analogue, or an insulin related peptide, and a DNA sequence containing yeast transcription termination signals.

25. A culture according to claim 24, wherein the yeast promoter is selected from the group consisting of the MFα1 promoter, CYC1 promoter, galactose inducible promoters such as GAL1, GAL7, and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoters.

26. A culture according to claim 24, wherein said first DNA sequence encodes a signal peptide of mouse α-amylase, S cerevisiae MFα1, BAR1, YAP3 and HSP150 and S. kluyveri MFα signal peptides or prepro-sequences of S. cervisiae MFα1, YAP3, PRC, HSP150 and S. kluyveri MFα and/or synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666.

27. A culture according to any one of claims 14 to 26, wherein the DNA sequence encoding the precursor of insulin, precursor of insulin analogue, or insulin related peptide is selected from the group consisting of DNA sequences encoding precursor of proinsulin, IGF-1, precursor of insulin and insulin analogues such as EEAEPK-insulin B chain(1-29)-AAK-insulin A chain(1-21) and insulin B chain(1-27)-DKAAK-A chain(1-21).

28. A method of preparing a yeast having reduced Yap3p activity comprising the steps of a) providing a hybrid plasmid containing the a part of the YAP3 gene and suitable for transformation into a yeast cell, b) disrupting the YAP3 gene by deleting the fragment of YAP3 and inserting a URA3 gene instead to obtain a Δyap3::URA3 gene disruption plasmid, c) providing a yeast Δura3 deletion mutant, d) transforming said mutant with said plasmid, and e) selecting Δyap3::URA3 deletion mutants on a medium without uracil.

29. A method of preparing a yeast having reduced Yap3p activity using antisense technology.

30. A method according to claims 28 or 29 wherein the yeast is S. cerevisiae.

31. Any novel feature or combination of features described herein.

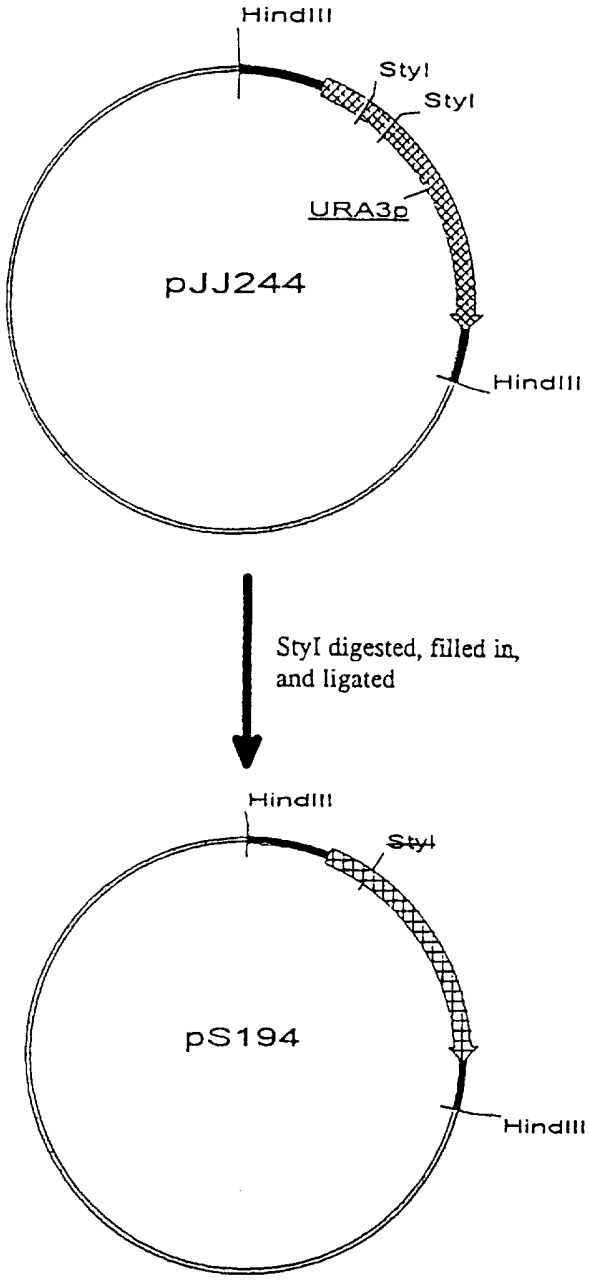
HindIII
StyI
StyI
URA3p
pJJ244
HindIII
StyI digested, filled in,
and ligated
HindIII
StyI
pS194
HindIII

Fig. 1

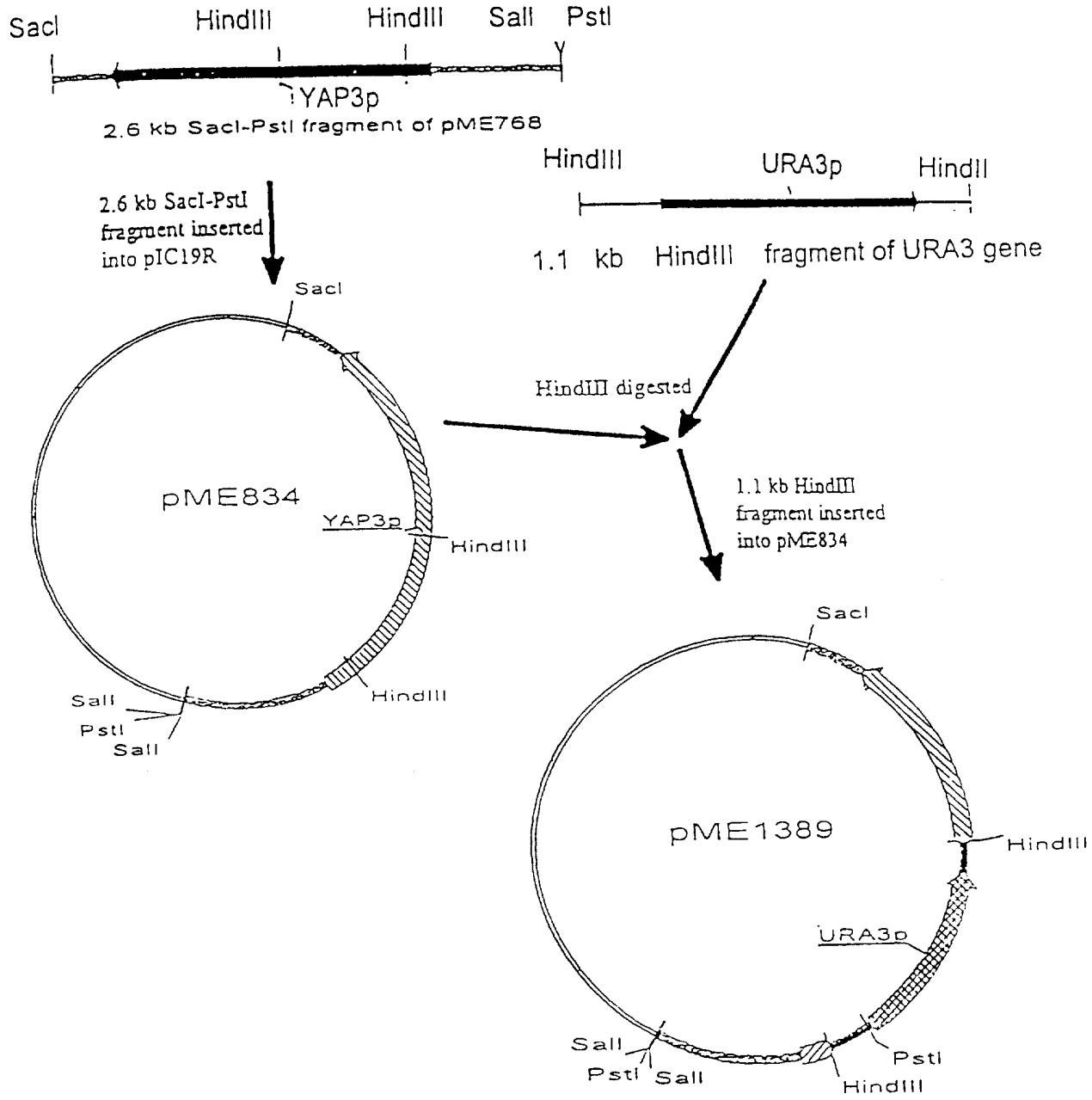
SacI
HindIII
HindIII
SalI
PstI
YAP3p
2.6 kb SacI-PstI fragment of pME768
2.6 kb SacI-PstI fragment inserted into pIC19R
HindIII
URA3p
HindII
1.1 kb HindIII fragment of URA3 gene
HindIII digested
1.1 kb HindIII fragment inserted into pME834
pME834
YAP3p
HindIII
SalI
PstI
SalI
pME1389
URA3p
HindIII
SalI
PstI
SalI
PstI
HindIII

Fig. 2

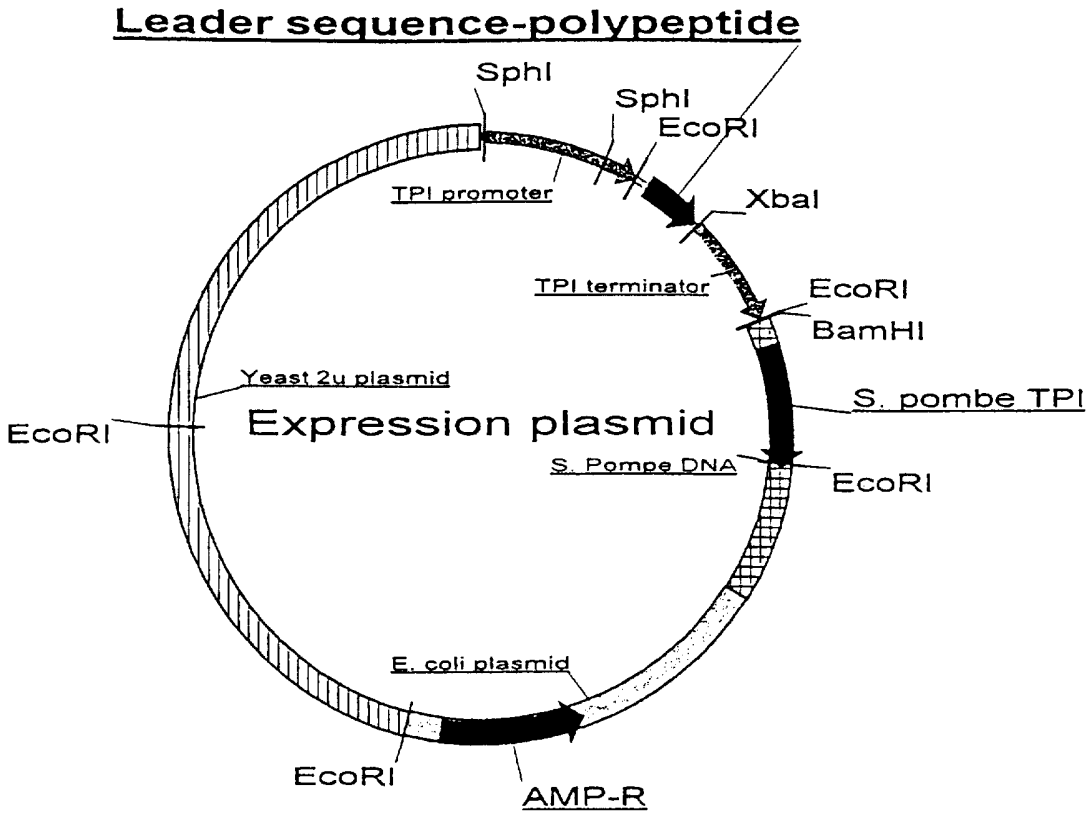
Leader sequence-polypeptide
SphI
SphI
EcoRI
TPI promoter
XbaI
TPI terminator
EcoRI
BamHI
Yeast 2u plasmid
S. pombe TPI
EcoRI
Expression plasmid
S. Pompe DNA
EcoRI
E. coli plasmid
EcoRI
AMP-R

Fig. 3

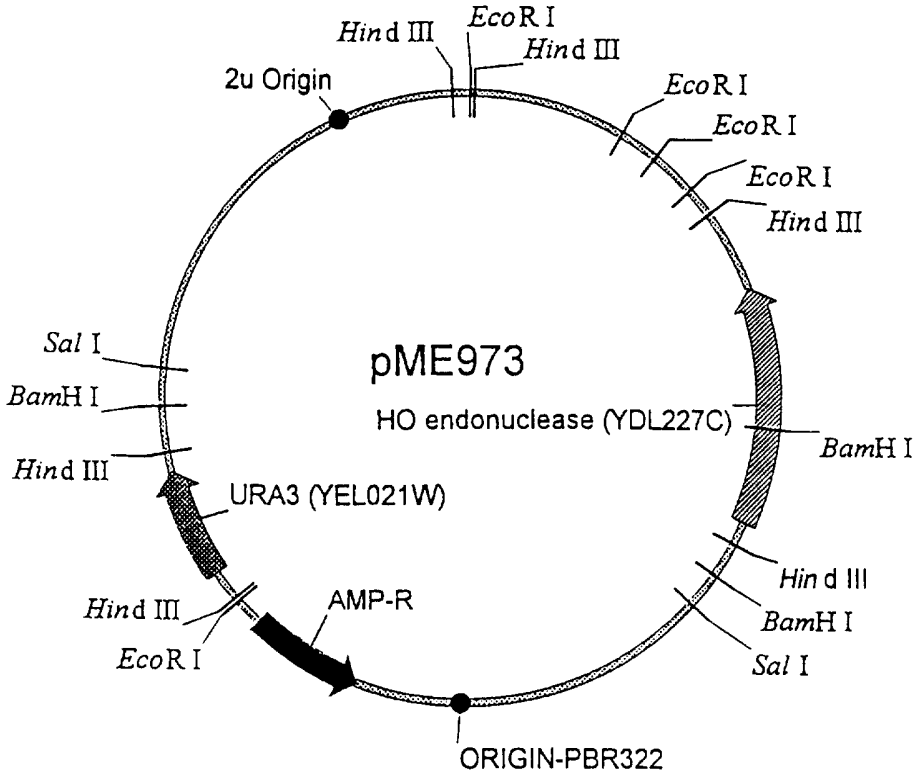
Hind III
EcoR I
Hind III
2u Origin
EcoR I
EcoR I
EcoR I
Hind III
Sal I
pME973
BamH I
HO endonuclease (YDL227C)
BamH I
Hind III
URA3 (YEL021W)
Hind III
AMP-R
BamH I
EcoR I
Sal I
ORIGIN-PBR322

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00297

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62, C12N 1/19 // C12N 15/81
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS, MEDLINE, DBA, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9523857 A1 (DELTA BIOTECHNOLOGY LIMITED), 8 Sept 1995 (08.09.95)	1-31
	--	
A	Dialog Information Services, File 154, Medline, Dialog accession no. 08582247, Medline accession no. 96200865, Kjeldsen T. et al: "A removable spacer peptide in an alpha-factor-leader/insulin precursor fusion protein improves processing and concomitant yield of the insulin precursor in Saccharomyces cerevisiae", Gene (NETHERLANDS) Apr 17 1996, 170 (1) p107-12	1-31
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 1997	30.10.1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00297

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 154, Medline, Dialog accession no. 06460032, Medline accession no. 90224362, Egel-Mitani M. et al: "A novel aspartyl protease allowing KEX2-independent MF alpha propheromone processing in yeast", Yeast (ENGLAND) Mar-Apr 1990, 6 (2) p127-37 -- --------	1-31

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/10/97

International application No.
PCT/DK 97/00297

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9523857 A1	08/09/95	AU 1818395 A	18/09/95
		CA 2183241 A	08/09/95
		EP 0749478 A	27/12/96
		GB 2301365 A,B	04/12/96
		GB 9404270 D	00/00/00
		GB 9616724 D	00/00/00