Production of Human Compatible High Mannose-type (Man₅GlcNAc₂) Sugar Chains in Saccharomyces cerevisiae*

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Yasunori Chiba‡, Misa Suzuki‡, Satoshi Yoshida‡, Aruto Yoshida‡, Hiroshi Ikenaga‡, Makoto Takeuchi‡§, Yoshifumi Jigami‡, and Eiji Ichishima

From the ‡Central Laboratories for Key Technology, KIRIN Brewery Co., Ltd., Yokohama, Kanagawa 236-0004, Japan, and §National Institute of Bioscience and Human Technology, Tsukuba, Ibaraki 305-0046, Japan, and the Department of Bioengineering, Faculty of Engineering, Sohka University, Hachioji, Tokyo 192-0060, Japan

A yeast mutant capable of producing Man₅GlcNAc₂ human compatible sugar chains on glycoproteins was constructed. An vector for α-1,2-mannosidase with the “HDEL” endoplasmic reticulum retention/retention tag was designed and expressed in Saccharomyces cerevisiae. An in vitro α-1,2-mannosidase assay and Western blot analysis showed that it was successfully localized in the endoplasmic reticulum. A triple mutant yeast lacking three glycosyltransferase activities was then transformed with an α-1,2-mannosidase expression vector. The oligosaccharide structures of carboxypeptidase Y as well as cell surface glycoproteins were analyzed, and the recombinant yeast was shown to produce a series of high mannose-type sugar chains including Man₅GlcNAc₂. This is the first report of a recombinant S. cerevisiae able to produce Man₅GlcNAc₂-oligosaccharides, the intermediate for hybrid-type and complex-type sugar chains.

Saccharomyces cerevisiae is useful for the production of recombinant proteins of biological interest because of the established expression system, and it can be easily grown in large quantities. Moreover, yeast share the early steps of the mammalian Asn-linked glycosylation pathway. However, the mature Asn-linked oligosaccharides of yeast are mannan glycan chains and are highly antigenic against mammals. Thus, it would be necessary to eliminate the antigenicity of the sugar chains when recombinant therapeutic glycoproteins are produced in yeast.

Several genes concerned with the biosynthesis of yeast sugar chains have been cloned, and the glycosylation pathway of yeast has been clarified. The OCH1 gene encodes an α,1,6-mannosyltransferase that initiates α,1,6-polymannose outer chain formation on the Asn-linked inner oligosaccharide Man₅GlcNAc₂ in S. cerevisiae (1). MNN1 has been proposed as the structural gene for the α,1,3-mannosyltransferase that elongates the outer chain and the inner core oligosaccharide (2, 3). The och1 mnn1 double mutant accumulated a single oligosaccharide moiety, Man₅GlcNAc₂, a high mannose-type structure (1). This mutant may be useful to produce recombinant therapeutic glycoproteins without any antigenicity toward humans.

On the other hand, some glycoproteins of therapeutic value require complex-type sugar chains for their efficacy. Erythropoietin (EPO), 1 a hematopoietic glycoprotein factor produced in the kidney, has three complex-type Asn-linked sugar chains and one mucin-type sugar chain. It is reported that the composition and structure of each sugar chain affected the biological activity, the efficiency of secretion, and had profound effects on the half-life of EPO in the blood circulation (4). It seems that the most active form of the EPO molecule requires tetra-antennary Asn-linked sugar chains (5) with full sialylation, to prevent serum clearance by the action of the hepatic asialoglycoprotein binding protein (6, 7). When EPO was expressed in the och1 mnn1 mutant yeast, the recombinant EPO should have high mannose-type oligosaccharides, which are trapped by the mannan-binding proteins of serum, liver, and macrophages, or excreted in the urine through the kidney because of their small size.

From the viewpoint of glycoengineering, we are trying to construct the mammalian-type glycosylation system in S. cerevisiae as a host to produce glycoprotein therapeutics (Fig. 1). The first aim of this research was to convert the mannose-type sugar chain of S. cerevisiae to a Man₅GlcNAc₂ sugar chain, because it is an intermediate for hybrid- and complex-type sugar chains. However the och1 mnn1 mutant can only produce the Man₅GlcNAc₂ structure (1). Further trimming of the mannose residues by α,1,2-mannosidase requires α-mannosidase I. Several α,1,2-mannosidases have been isolated from mammals, yeast, and fungi (8), and some mammalian α,1,2-mannosidase genes have been cloned (9, 10). During preparation of the manuscript, it was reported that a truncated soluble form of the human α,1,2-mannosidase IB was expressed as a secreted protein in Pichia pastoris (11). The S. cerevisiae α,1,2-mannosidase gene (MNS1) has been cloned (12) and expressed in S. cerevisiae. However, this enzyme only removes a specific single mannose residue from Man₅GlcNAc₂ and produces Man₄GlcNAc₂. The Aspergillus α,1,2-mannosidase gene (msdS1) has also been cloned and has been expressed successfully in yeast cells as a chimeric gene with the signal sequence of the aspergillopepsin I gene from Aspergillus saitoi (13, 14). The recombinant α,1,2-mannosidase activity was secreted into

1 The abbreviations used are: EPO, erythropoietin; ER, endoplasmic reticulum; CPY, carboxypeptidase Y; endo-H, endo-β-N-acetylgalactosaminidase; PA, 2-aminopyridine; PMSF, phenylmethylsulfonyl fluoride; GnT, N-acetylgalactosaminyltransferase; SD, synthetic minimal dextrose; CL, crude lysate; LSP, low speed pellet; HSP, high speed pellet; SSL, supernatant fraction; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MES, 2-morpholinoethanesulfonic acid.
the culture medium, indicating that the products of the msdS gene had passed through the yeast secretion pathway. Therefore, α-1,2-mannosidase could be used as a tool to produce the mammalian-type sugar chains in the yeast if this enzyme was retained in the endoplasmic reticulum (ER) or Golgi apparatus.

In yeast cells, the His-Asp-Glu-Leu (HDEL) C-terminal sequence of proteins acts as a retention/retrieval signal for the endoplasmic reticulum (ER) (15). Proteins with an HDEL sequence are bound by a membrane-bound receptor (Erd2p) (16, 17) and then enter a retrograde transport pathway for return to the ER from the Golgi apparatus. In this study, the expression of the A. saitoi α-1,2-mannosidase in the ER was demonstrated by adding “HDEL” to the C terminus of the α-1,2-mannosidase open reading frame. The introduced α-1,2-mannosidase was also shown to convert Asn-linked oligosaccharides into Man5GlcNAc2, the intermediate form for hybrid- and complex-type sugar chains, in mutant yeast cells with disruptions in three of the original mannosyltransferase genes (OCH1, MNN1, and MNN4).

EXPERIMENTAL PROCEDURES

Yeast Strain and Culture Conditions—The enzyme activity and the localization of the HDEL-tagged MsdSp was determined in S. cerevisiae pep4 disrupted YPH500 cells (MATa ura3–52 lys2–801 ade2–101 his3–Δ63 pep1–Δ63 his3–Δ200 leu2–Δ1 pep7–ADE2) (18). YS132–8B (MATa och1::LEU2 mnn1::URA3 mnn4::LYS2 leu2–Δ1 ura3–52 trp1–Δ1 lys2–801 his3–Δ200 ade2–101Δ53), which had been constructed by standard genetic methods (19), were used to analyze the Asn-linked sugar chains of carboxypeptidase Y (CPY) or mannoproteins. All strains were transformed by the method of Ito et al. (20). Transformants were selected on synthetic minimal dextrose (SD) medium with auxotrophic supplements.

DNA Constructs—For preparation of the HDEL-tagged MsdSp, the tag sequence was introduced by amplifying the 0.6-kilobase region between the HindIII site and the stop codon of the msdS gene with the following mutation primers: 5’-TCGCGCCGGAAGCTTACGACTCACC--3’ and 5’-TCTAGAATTCTGTCGTCGGTAAGA-3’. The
polymerase chain reaction product was subcloned into pCR-Script Amp SK (+) (Stratagene) and digested with HindIII and NotI. The coding region of the C-terminal domain of pGAM1, an expression plasmid for A. saitoi α-1,2-mannosidase (13), was substituted with the recovered 0.6-kilobase HindIII-NotI fragment to create the pGAM1H plasmid. The α-1,2-mannosidase sequence was confirmed by DNA sequencing.

The α-1,2-Mannosidase Assay—Man₆GlCN₄P₆ oligosaccharide was obtained from Seikagaku Co. (Tokyo, Japan) and labeled with 2-amino-2-deoxyribose (21). Pyridylaminated oligosaccharide (Man₆GlCN₄P₆-PA) was purified by gel filtration (TOYOPEARL HW-40, 1.6 × 73 cm, Tosoh Corp., Japan) and the purity confirmed by reversed-phase HPLC using an ODS-80TM column (4.6 × 15 cm, Tosoh Corp., Japan). Yeast cell extracts were cultured on SD medium lacking tryptophan. The cell density was determined at 600 nm using a 10-mm cuvette. The pellets were washed with deionized water, resuspended in extraction buffer (0.1 M sodium acetate buffer (pH 5.0) containing 1 mM phenylmethylsulfonyl fluoride (FMSF), and vortexed with acid-washed glass beads (425–600 μm diameter). Soluble cell extract was separated from cell debris by centrifugation and assayed for activity. Samples containing 10–100 μg of protein were incubated for 30 min with 150 pmol of Man₆GlCN₄P₆-PA in 0.1 M sodium acetate buffer (pH 5.0) at 37 °C. The assay was stopped by boiling, and the sample was filtered using an Ultrafree-MC centrifugal filter unit (0.22 μm pore size, Millipore, Milw., Milw.). The filters were analyzed by HPLC with a Hitachi P-1050 fluorescence spectrophotometer, using an ODS-80TM column (4.6 × 150 mm). The solvent and elution conditions used are as described by Kondo et al. (21). One unit of the enzyme was defined as the amount of enzyme that was required to liberate 1 μmol of mannose from Man₆GlCN₄P₆-PA per min at 30 °C and pH 5.0.

Marker Enzyme Assay—NADH cytochrome P-450 reductase, a marker enzyme for the ER, guanosine diphosphatase, and glucose-6-phosphate dehydrogenase, a cytosol marker, were assayed as described (22–24), respectively.

Western Blot Analysis—Rabbit anti-MasSp and rabbit anti-glucose-6-phosphate dehydrogenase antisera were obtained from Sawaday Technology (Tokyo, Japan). Mouse anti-CPY monoclonal antibody 10A5-B5, mouse anti-alkaline phosphatase monoclonal antibody 1D3-A10, and mouse anti-dolichol phosphate mannosyl synthase monoclonal antibody S55-A7 were purchased from Molecular Probes, Inc. (Eugene, OR).

Samples containing 1 μg of protein were subjected to SDS-PAGE. SDS-PAGE was carried out using the buffer system of Laemmli (25) in 10% gel. Electroblotting of the fractionated proteins onto polyvinylidene difluoride membrane (Millipore Corp.) was carried out by the method of Towbin et al. (26), and detection was performed essentially according to the method of Hsu et al. (27).

Subcellular Fractionation—Cells were grown in SD medium and were converted to spheroplasts by the method of Vita et al. (28). The following procedures were also performed at 4 °C. The spheroplasts were harvested by centrifugation. The spheroplasts were resuspended in a 0.15 M NaCl, 1 mM MnCl₂, and 1 mM CaCl₂ buffer (pH 7.2) containing 0.15% NaCl, 1 mM MnCl₂, and 1 mM CaCl₂ (pH 7.2). Liberated Asn-linked oligosaccharides were separated from the salts and peptides using an AG 501-X8 mixed bed resin (Bio-Rad), and from Nonidet P-40 using Bio-Beads S-X8 (Bio-Rad). Reductive pyridylation and structural analyses of the purified oligosaccharides were performed essentially as described by the method of Kondo et al. (21). Pyridylaminated (PA−) oligosaccharides were analyzed by HPLC using a size-fractionation column (TSKgel Amide-80, 4.6 × 250 mm, Tosoh Corp.) and a reverse-phased column (TSKgel ODS-80TM, 4.6 × 150 mm). Authentic PA-oligosaccharides and PA-glucose oligomers were purchased from Takara Shuzo Co. (Kyoto, Japan).

RESULTS

Expression of the α-1,2-Mannosidase—Measurement of α-1,2-mannosidase activity by the Nelson-Somogyi method was attempted but was not sensitive enough to detect the amount of enzyme present. We have developed a new assay for α-1,2-mannosidase using a fluorescent oligosaccharide. PA-oligosaccharide made it possible to assay picomole per minute-ordered enzyme activity. For the assay of the α-1,2-mannosidase activity in vitro, we used Man₆GlCN₄P₆-PA oligosaccharide as a substrate. The optimal assay conditions, such as enzyme concentration, reaction time, and substrate concentration were determined as described under “Experimental Procedures.” A soluble form of A. saitoi α-1,2-mannosidase was constructed with the HDEL ER retention/retrieval signal sequence at the N-terminus. This construct was subcloned into the multicopy plasmid pG3, termed pGAM1H, and was used to transform S. cerevisiae YPH500 cells. α-1,2-Mannosidase activity that converted Man₆GlCN₄P₆-PA substrate into Man₆GlCN₄P₆-PA was observed in the cell extracts of the recombinant yeast with the pGAM1H vector (Fig. 2B), whereas there was no such activity in the extract of the recombinant yeast transfected...
with the pG3 vector only (Fig. 2A). The activity of ER α-1,2-mannosidase and vacuole α-mannosidase in yeast were not detected under these assay conditions. Four milliunits of the enzyme activity was recovered from a 500-ml yeast culture.

**Localization of the α-1,2-Mannosidase—**To determine the localization of the expressed α-1,2-mannosidase in yeast, we investigated the subcellular distribution of the enzyme. Fig. 3A illustrates the protocol for the fractionation of the yeast cells.

The α-1,2-mannosidase activity was localized primarily in the LSP fraction (77%) (Table I). The LSP fraction also contained 69% of NADPH cytochrome P-450 reductase (ER marker) (31, 32). In contrast, most of the guanosine diphosphatase (70%), a Golgi marker (23), was found in the HSP fraction. Kex2p (33), a late Golgi marker, was split into the HSP and HSS fractions. The cytosol marker, glucose-6-phosphate dehydrogenase, was detected mainly in the HSS fraction (76%).

The Western blot pattern also showed that the ER marker protein (dolichol phosphate mannose synthase) and the vacuolar membrane protein (alkaline phosphatase) were localized in the LSP fraction, whereas both CPY, which is a soluble protein in the vacuole, and cytosolic glucose-6-phosphate dehydrogenase were fractionated in HSS fraction (Fig. 3B). The introduced α-1,2-mannosidase gene products were detected in the LSP fraction. Because it is known that the LSP fraction contained the vacuole in addition to the ER (34), discontinuous sucrose density centrifugation was performed to determine whether the expressed α-1,2-mannosidase was localized in the ER or the vacuole (Fig. 4). Alkaline phosphatase, a vacuolar marker enzyme, was distributed to fractions 1–3, the most light density fraction. In contrast, dolichol phosphate mannose synthase, an ER marker, was distributed to fractions 4–6. This result indi-
cated that the vacuole and ER are well separated from each other in this system. The signals of the α-1,2-mannosidase appeared around fraction 5. The results strongly suggested that the α-1,2-mannosidase with the HDEL-tag is mainly localized in the ER.

Oligosaccharide Structures of the Recombinant Triple Mutant Yeast—The apparent molecular mass of the CPY produced in the recombinant yeasts was analyzed on SDS-PAGE followed by Western blot analysis. YS132–8B, which has disrupted OCH1, MNN1, and MNN4 genes, will not have any outer mannosyl chains on its glycoproteins. As shown in Fig. 5, the CPY from YS132–8B carrying the null vector gave a single signal with an apparent molecular mass of 62 kDa on SDS-PAGE. However the CPY from YS132–8B harboring the pGAMH1 plasmid gave an additional signal below the original one, indicating that the sugar chains of the CPY have been trimmed by the introduced α-1,2-mannosidase. Treatment of each cell lysate with endo-H gave a single signal of an N-deglycosylated CPY (Fig. 5, third and fourth lanes).

The oligosaccharide structures of glycoproteins produced in these yeasts were analyzed using CPY as a model glycoprotein. While the sugar chains of CPY produced in the null vector were eluted at the Man8GlcNAc2-PA position on the amide column (Fig. 6A, graph a), those produced in the yeast with the pGAMH1 plasmid showed four peaks at positions corresponding to Man5GlcNAc2-PA, Man6GlcNAc2-PA, Man7GlcNAc2-PA, and Man8GlcNAc2-PA, respectively (Fig. 6A, graph b). The molar ratio of each glycoform was Man5GlcNAc2-PA:Man6GlcNAc2-PA:Man7GlcNAc2-PA:Man8GlcNAc2-PA = 27:22:22:29. The fraction eluted at the position corresponding to Man5GlcNAc2-PA (indicated with an open arrow in Fig. 6A) was pooled and subjected to reversed-phase chromatography. Only one peak was observed at the same position as authentic Man5GlcNAc2-PA, Man6GlcNAc2-PA, Man7GlcNAc2-PA, and Man8GlcNAc2-PA, respectively (Fig. 6B); this is the smallest structure of mammalian-type high-mannose sugar chains.

Besides CPY, we also investigated the oligosaccharide structures of cell wall mannoproteins. As shown in Fig. 6C, the mannoproteins produced in the yeast with the pGAMH1 plasmid contained Man5GlcNAc2. The molar ratio of each glycoform in mannoproteins was Man5GlcNAc2-PA:Man6GlcNAc2-PA:Man7GlcNAc2-PA:Man8GlcNAc2-PA = 10:13:16:61.

**DISCUSSION**

α-Mannosidase I digests α-1,2-mannosidic linkages and converts Man5GlcNAc2 oligosaccharide into Man6GlcNAc2. This is the first step in the biosynthesis of hybrid-type and complex-type sugar chains from high mannose-type sugar chains. There are several successive enzymatic reactions necessary to complete complex-type structures. N-Acetylglucosaminyltransferase (GnT)-I, α-mannosidase II, GnT-II, β-1,4-galactosyltransferase, etc. work in succession in mammalian cells. Since the α-1,2-mannosidase acts upstream in the biosynthetic pathway of oligosaccharides, it must be located either in the ER or the early Golgi apparatus to reconstruct this system in yeast. We have already succeeded in expressing the A. saitoi α-1,2-mannosidase as a chimeric protein with a transmembrane domain of Och1p (data not shown). Although Och1p resides in the early Golgi apparatus of yeast, the expressed chimeric enzyme was localized not only in the Golgi apparatus, but also in the ER and the cytosol fractions. We could not detect any Man5GlcNAc2 sugar chain structure in the recombinant yeast (data not shown). Evidence suggested that the mislocalization of the chimeric α-1,2-mannosidase prevented the trimming of sugar chains in the yeast. In this study, we attempted to localize the α-1,2-mannosidase to the yeast ER using a retention/retrieval signal.

There has been several retention/retrieval systems proposed to date. Some of these systems require a transmembrane domain and/or a cytoplasmic tail. Xaa-Xaa-Arg-Arg (XXRX, X is any amino acid) in the N-terminal cytoplasmic domain and Lys-Lys-Xaa-Xaa (KKXX), in the C-terminal cytoplasmic domain of membrane proteins are known as retrieval signals for the ER. It has been demonstrated that the N-terminal 16 amino acids of the alkaline phosphatase in the cytoplasmic tail contain a vacuolar sorting signal in S. cerevisiae (36). Lussier et al. reported that an N-terminal cytoplasmic domain was necessary for Kre2p to correctly localize in the Golgi apparatus and that the entire Kre2p cytoplasmic tail plus the transmembrane domain and 36 amino acids in the luminal stem region were required to localize a Pho8p reporter protein in the yeast Golgi apparatus (35). These results suggested that there is no accurate signal for the retention of exogenous membrane proteins in the ER or Golgi apparatus of yeast. Therefore, we constructed an expression vector with an HDEL signal for the transfer of soluble α-1,2-mannosidase proteins from the Golgi apparatus to the ER.

In yeast, two α-mannosidases have been found, and these have different substrate specificity and pH optima to the A.
saitoi α-1,2-mannosidase. S. cerevisiae ER α-mannosidase (Mns1p) cannot act on Man5GlcNAc2 oligosaccharide, and vacuolar α-mannosidase (Ams1p) cannot act at pH 5.0. Whereas A. saitoi α-1,2-mannosidase can remove the α-1,2-linked mannose of Man5GlcNAc2 oligosaccharide at pH 5.0. Based on these facts, we have developed an assay method that specifically detects A. saitoi α-1,2-mannosidase activity.

The subcellular fractionation experiments indicated that the product of the msdS gene was mainly localized in the LSP fraction (Fig. 3), which includes the ER, vacuole, and plasma membrane. However, it is unlikely that MsdSp was localized in the vacuole, because the signal distribution of MsdSp was quite different from that of CPY, which is the vacuolar marker (Fig. 3). Furthermore, MsdSp will never be anchored at the plasma membrane because it is a soluble protein. The fractionation in the sucrose discontinuous gradients also showed that the signal distribution of the product of the msdS gene did not match with that of the vacuole but with that of the ER.

CPY was chosen as one of the reporter glycoproteins to analyze the glycosylation phenotype of the genetically constructed yeast, because it has four Asn-linked oligosaccharides of known structure (37), and it has an established purification method (29). The triple mutant strain (YS132–8B) used in this study lacks three of the yeast mannosyltransferase activities, of known structure (37), and it has an established purification method (29).

In this study, S. cerevisiae was manipulated to produce Man5GlcNAc2 N-glycan. Increasing the efficiency of the α-1,2-mannosidase reaction remains to be done. Furthermore, the Man5GlcNAc2 N-glycan is a hybrid and a complex-type intermediate, the latter of which is better suited and more effective for human therapeutics. We have already succeeded in expressing GrnT-I, GrnT-II, and β-1,4-galactosyltransferase activities in yeast, but to make hybrid- and complex-type sugar chains in yeast cells, co-expression of GrnT-I and α-1,2-mannosidase is required and is an object of our future research.

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REFERENCES

1. Nakanishi-Shindo, Y., Nakayama, K. I., Tanaka, A., Toda, Y., and Jigami, Y. (1993) J. Biol. Chem. 268, 26338–26345
2. Graham, T. R., and Emr, S. D. (1991) J. Cell Biol. 114, 207–218
3. Yip, C. L., Welch, S. K., Kleib, F., Gilbert, T., Seidel, P., Grant, F. J., O'Hara, P. J., and MacKay, V. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2723–2727
4. Takeuchi, M., and Kobata, A. (1991) Glycobiology 1, 337–346
5. Takeuchi, M., Inoue, N., Strickland, T. W., Kubota, M., Wada, M., Shimizu, R., Hoshi, S., Konotsumi, H., Takasaki, S., and Kobata, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7819–7822
6. Fukuda, M. N., Sasaki, H., Lopez, L., and Fukuda, M. (1989) Blood 73, 84–89
7. Spivak, J. L., and Hegns, B. B. (1988) Blood 73, 90–99
8. Yoshida, T., and Ichiyama, E. (1995) Biochim. Biophys. Acta 1263, 159–162
9. Herscovics, A., Schneikert, J., Athanassiadis, A., and Moremen, K. W. (1994) J. Biol. Chem. 269, 9864–9871
10. Lal, A., Schiotz, I. F., Forsey, W. T., Neame, P. J., and Moremen, K. W. (1994) J. Biol. Chem. 269, 9872–9881
11. Tremblay, L. O., Campbell Dyke, N., and Herscovics A. (1998) Glycobiology 8, 595–595
12. Camirand, A., Heysen, A., Grondin, B., and Herscovics, A. (1991) J. Biol. Chem. 266, 15120–15127
13. Inoue, T., Yoshida, T., and Ichiyama, E. (1995) Biochim. Biophys. Acta 1253, 141–145
14. Fujita, A., Yoshida, T., and Ichiyama, E. (1997) Biochem. Biophys. Res. Commun. 238, 779–783
15. Pelham, H. R. (1988) EMBO J. 7, 913–918
16. Lewis, M. J., and Pelham, H. R. (1990) Nature 348, 162–163
17. Semenza, J. C., Hardwick, K. G., Dean, N., and Pelham, H. R. (1990) Cell 61, 1349–1357

**FIG. 6. Analysis of Asn-linked oligosaccharides in the triple mutant strain YS132–8B.** A, chromatogram of the sugar chains of CPY on HPLC using a TSKgel Amide-80 column. Graph a, from YS132–SB harboring null vector; graph b, from YS132–SB harboring the expression plasmid, pGAMH1. B, the peak indicated by the open arrow in panel A was pooled and subjected to HPLC using a TSKgel ODS-80TM column. Graph a, standard sugar chain of Man1–3[Man1–3Man1–6]Man1–6Man81–4GlcNAc81–4GlcNAc-PA; Graph b, the pooled fraction in panel A. C, chromatogram of the sugar chains of mannoproteins on HPLC using a TSKgel Amide-80 column. Graph a, from YS132–SB harboring null vector; graph b, from YS132–SB harboring the expression plasmid, pGAMH1. The elution times of authentic PA-sugar chains were indicated by arrows: M5, Man5GlcNAc2-PA; M6, Man6GlcNAc2-PA; M7, Man7GlcNAc2-PA; M8, Man8GlcNAc2-PA.
Mammalian-type High Mannose Sugar Chains Produced in Yeast

18. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
19. Sherman, F., and Hicks, J. (1991) Methods Enzymol. 194, 21–37
20. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
21. Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., and Ikenaka, T. (1990) Agric. Biol. Chem. 54, 2169–2170
22. Kubota, S., Yoshida, Y., Kumaoka, H., and Furumichi, A. (1977) J. Biochem. (Tokyo) 81, 197–205
23. Abeijon, C., Orlean, P., Robbins, P. W., and Hirschberg, C. B. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6935–6939
24. Wang, J. L., and Buhler, D. R. (1981) J. Toxicol. Environ. Health 8, 639–648
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
27. Hsu, S.-M., Raine, L., and Panger, H. (1981) J. Histochem. Cytochem. 29, 577–580
28. Vida, T. A., Graham, T. R., and Emr, S. D. (1990) J. Cell Biol. 111, 2871–2884
29. Johansen, J. T., Breddam, K., and Ottesen, M. (1976) Carlsberg Res. Commun. 41, 1–14
30. Peat, S., Whelan, W. J., and Edwards, T. E. (1961) J. Chem. Soc., 29–34
31. te Hessen, S., Rauhut, R., Aebersold, R., Abelson, J., Aebi, M., and Clark, M. W. (1991) Eur. J. Cell Biol. 56, 8–18
32. te Hessen, S., Janetzky, B., Leible, L., and Aebi, M. (1992) EMBO J. 11, 2071–2075
33. Redding, K., Holcomb, C., and Fuller, R. S. (1991) J. Cell Biol. 113, 527–538
34. Gaynor, E. C., te Hessen, S., Graham, T. R., Aebi, M., and Emr, S. D. (1994) J. Cell Biol. 127, 653–665
35. Lusser, M., Sdicu, A. M., Ketela, T., and Bussey, H. (1995) J. Cell Biol. 131, 913–927
36. Cowles, C. R., Snyder, W. B., Burd, C. G., and Emr, S. D. (1997) EMBO J. 16, 2769–2782
37. Trimble, R. B., Maley, F., and Chu, F. K. (1983) J. Biol. Chem. 258, 2562–2567