Glycoprotein biosynthesis in *Saccharomyces cerevisiae*: *ngd29*, an *N*-glycosylation mutant allelic to *och1* having a defect in the initiation of outer chain formation

Ludwig Lehle[a,*], Antje Eiden[a], Klaus Lehnert[b], Anton Haselbeck[b], Erhard Kopetzki[b]

[a]*Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, 93040 Regensburg, Germany*
[b]*Boehringer Mannheim GmbH, Department of Genetics, 82377 Penzberg, Germany*

Received 20 June 1995

Abstract ***Outer chain*** **glycosylation in** ***Saccharomyces cerevisiae*** **leads to heterogeneous and immunogenic asparagine-linked saccharide chains containing more than 50 mannose residues on secreted glycoproteins. Using a [^{3}H]mannose suicide selection procedure a collection of *N*-glycosylation defective mutants (designated *ngd*) was isolated. One mutant, *ngd29*, was found to have a defect in the initiation of the *outer chain* and displayed a temperature growth sensitivity at 37°C allowing the isolation of the corresponding gene by complementation. Cloning, sequencing and disruption of *NGD29* showed that it is a non lethal gene and identical to *OCH1*. It complemented both the glycosylation and growth defect. Membranes isolated from an *ngd29* disruptant or an *ngd29mnn1* double mutant were no longer able, in contrast to membranes from wild type cells, to transfer mannose from GDPmannose to $Man_8GlcNAc_2$, the in vivo acceptor for building up the *outer chain*. Heterologous expression of glucose oxidase from *Aspergillus niger* in an *ngd29mnn1* double mutant produced a secreted uniform glycoprotein with exclusively $Man_8GlcNAc_2$ structure that in wild type yeast is heavily hyperglycosylated. The data indicate that this mutant strain is a suitable host for the expression of recombinant glycoproteins from different origin in *S. cerevisiae* to obtain mammalian oligomannosidic type *N*-linked carbohydrate chains.**

Key words: Protein glycosylation; Glycoprotein; Golgi glycosyltransferase; *Saccharomyces cerevisiae*

1. Introduction

Asparagine-linked glycosylation is a highly conserved and essential protein modification occurring in all eukaryotes [1–4]. The initial step of the pathway is the assembly of the core oligosaccharide $Glc_3Man_9GlcNAc_2$ on the lipid carrier dolichyl phosphate. After its en bloc transfer to nascent polypetide chains in the endoplasmic reticulum the carbohydrate part is further processed. Three glucose residues and one mannose are removed in the endoplasmic reticulum to give the $Man_8GlcNAc_2$ core. Further processing occurs in the Golgi and is different in mammalian cells and yeast. In mammals further removal of mannoses and selective addition of a limited number of different monosaccharides (GlcNAc, Gal, Fuc, sialic acid) occur to yield high mannose, hybrid or complex type structures. In *Saccharomyces cerevisiae*, processing predominantly involves addition of only mannose residues to give rise to a family of $Man_{9-15}GlcNAc_2$ species, the so-called *inner core*. On secreted glycoproteins some of the *inner core* saccharides are further, though not necessarily, processed by addition of an *outer chain* composed of an α1,6-linked polymannose backbone and highly branched α1,2- and α1,3-side chains [5]. The *outer chain* greatly varies in size (50–150 mannose residues) and contributes to the heterogeneity of most secreted yeast glycoproteins, as well as making these proteins highly immunogenic. Mainly inferred from known saccharide structures of various *outer chain* defective glycosylation mutants, it appears that formation of the *outer chain* occurs in a structurally and topologically controlled and coordinated elongation process rather than in a random fashion [6–8]. At least 7 steps require distinct mannosyltransferases. Here we report the isolation and characterization of a mutant, termed *ngd29*, with a defect in initiation of *outer chain* synthesis. It will be shown that membranes from a *ngd29* disruptant lacks the enzymatic activity to mannosylate $Man_8GlcNAc_2$, the in vivo acceptor for *outer chain* formation. Heterologous expression of glucose oxidase (GOD) from *Aspergillus niger* in the double mutant *ngd29mnn1* gives rise to a secreted product with a uniform saccharide chain of the composition $Man_8GlcNAc_2$, whereas GOD, when expressed in wild type yeast strains, is hyperglycosylated.

2. Materials and methods

2.1. Yeast strains and media

The following strains were used: *X2180-1A* (*MATa SUC2 mal mel gal2 CUP1*), W303 (*MATa/MATα leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 ade2-1/ade2-1 trp1-1/trp1-1 can1-100/can1-100*), BMY 3-9A (*MATα ngd29 ura3-52 leu2-1,112 his3-Δ200*), YG0091 (*Δalg5::HIS3 ade2-101 his3Δ200 ura3-52*), LB2303-3A (*MATa mnn1 trp1 ura3-52*), LLY1 (*ngd29::URA3 mnn1 ura3-52 trp1*). Standard yeast media and yeast DNA technology methods have been used as described [9].

2.2. Isolation and disruption of NGD29 locus

Yeast *N*-glycosylation defective mutants (*ngd*) were isolated by a [^{3}H]mannose suicide selection procedure according to [10]. The *ngd29* mutant was transformed by a genomic library containing 10–20 kb *Sau*3A fragments in YCp50 [11] and uracil prototrophic transformants were selected at the restrictive temperature at 37°C. Five positive clones were obtained out of 7000 transformants. For further analysis and sequencing the threee *Eco*RI restriction fragments of the 9 kb insert (see Fig. 2A) were subcloned into YEp352. For the *NGD29* disruption the part between the *Hpa*I-*Bam*HI sites (600 bp) within the ORF was replaced by *URA3* in a subclone (pAE8) containing the non-complementing 1600 bp *Eco*RI-*Eco*RI fragment. From the resulting plasmid pRE/L1 the *Eco*RI fragment was excised and transformed into the wild type diploid W303 strain and the haploid *mnn1* mutant LB 2303-3A. Gene disruption was confirmed by Southern analysis.

2.3. Mannosyltransferase assay

Yeast membranes were isolated from mid-log cells of *mnn1* mutant,

*Corresponding author. Fax: (49) (941) 943-3352.

SSDI 0014-5793(95)00789-X

ngd29 mnn1 double mutant or *X2180-1A* wild type cells grown in YEPD as described earlier [12]. Oligosaccharide acceptors $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ were isolated from the lipid-linked oligosaccharide fraction after metabolic labeling of yeast cells with [^{3}H]mannose [10]. Upon mild acid hydrolysis free oligosaccharides were recovered by Bio Gel-P4 column chromatography [10]. The standard mannosyltransferase incubation mixture contained: 50 mM Tris-HCl (pH 7.4), 10 mM $MnCl_2$, 0.06% Triton X-100, 0.5 mM 1-deoxymannojirimycin, ^{3}H-labeled $Man_xGlcNAc_2$ (20000 cpm) acceptor and membranes (1 mg protein) in a total volume of 0.1 ml. After incubation for 60 min at 25°C the reaction was stopped with 0.2 ml methanol. The precipitate was washed with 0.2 ml water and the combined supernatants concentrated and analyzed by HPLC on a Supelcosil LC-NH_2 column (25 cm × 4.6 mm) using a gradient from 70 to 50% acetonitrile/water within 75 min and a flow rate of 1 ml.

2.4. Analysis of invertase

During the mutant screening invertase was analyzed by activity staining using native polyacrylamide gel electrophoresis (5% gel) according to [13]. Cells were derepressed for invertase in YEP medium containing 0.1% glucose for 90 min, lysed with glass beads in 50 mM Tris-HCl (pH 7.5), 5 mM mercaptoethanol and an aliquot of the supernatant obtained after centrifugation, equivalent to 5 OD_{578}, was loaded onto the gel. For metabolically labeling of invertase with [^{35}S]methionine 20 OD_{578} of log phase cells were derepressed and pulsed for 30 min with 115 μCi in 5 ml minimal medium containing 0.1% glucose. Then 0.2 mg/ml unlabeled methionine was added and incubated for another 30 min. Glass bead lysis and immunoprecipitation have been described [14]. Labeled invertase was analyzed by 7.5% SDS-PAGE.

2.5. Expression of glucose oxidase (GOD) from Aspergillus niger in yeast

GOD from *Aspergillus niger* [15] was fused at the C-terminus with 4 additional His-residues to aid metal chelate affinity purification of GOD-$(His)_4$. The attachment of this His-tail had no effect on expression, secretion or enzymatic properties of GOD. The GOD-$(His)_4$ structural gene including its own signal sequence was expressed under the control of the maltose inducible and glucose repressible α-glucosidase expression cassette using a derivative of the episomal replicating vector YEp24 [16]. Wild type and *ngd29mnn1* mutant were transformed. Secreted GOD was isolated from the medium by affinitychromatography on commercially available Ni-chelate resin [17,18]. For carbohydrate analysis 0.31 mg protein was digested with 4 U *N*-glycosidase F (Boehringer) according to the manufacturer's protocol for 16 h at 37°C. Released carbohydrate was freed from protein by ultrafiltration (Ultrafree, Millipore) and analyzed by HPEAC equipped with a PAD detector (CarboPac PA-100 column, linear gradient of 0–10% 0.5 M sodium acetate in 0.1 M NaOH within 60 min, flow rate 1 ml/min).

3. Results

3.1. Isolation and characterization of ngd29 mutant

A [^{3}H]mannose suicide selection [10] has been used to isolate yeast mutants with a temperature sensitive *N*-linked glycosylation defect, termed *ngd* mutants. Briefly it is assumed that mutants with a deficiency in *N*-glycosylation will incorporate less [^{3}H]mannose as compared to wild type cells and will survive long-term storage in the presence of the isotope. Possible candidates were further screened for altered glycosylation of invertase and carboxypeptidase. Among 350 mutants with a reduced mannose incorporation 70 isolates revealed an altered glycosylation phenotype. One mutant, termed *ngd29*, showed a distinct defect in *outer chain* formation of invertase and was further investigated. As can be seen from Fig. 1A, upon native gel electrophoresis wild type invertase migrates in this system as a diffuse band, whereas that from *ngd29* and also from *mnn9* migrates faster and with a typical pattern of 4 bands. These distinct bands reflect oligomeric forms (di-, tetra-, hexa- and octamer), which in wild type invertase are not visible due to the

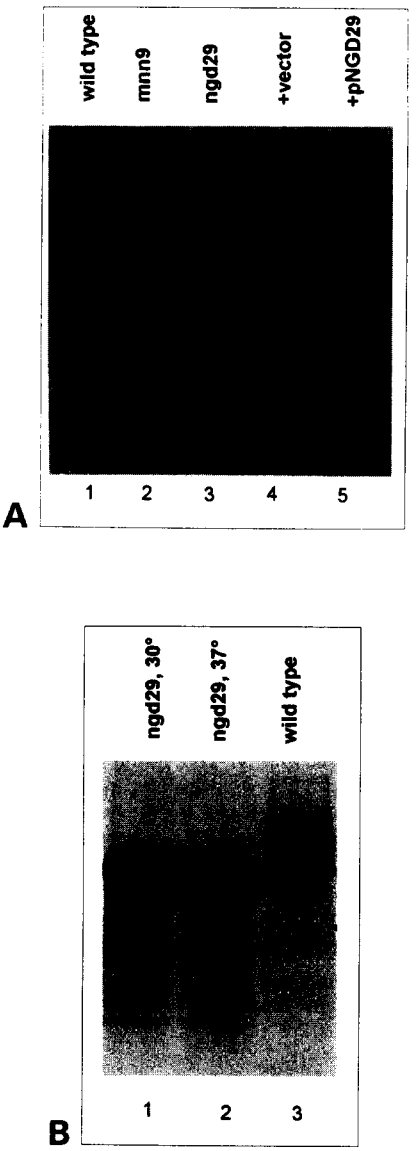

Fig. 1. (A) Native polyacrylamide gel electrophoresis of invertase. Cell extracts from wild type cells (lane 1), *mnn9* (lane 2), *ngd29* (lane 3), *ngd29* complemented with vector YCp50 (lane4) and *ngd29* complemented with YCp50*(NGD29)* (lane 5) were analyzed as described in section 2. (B) SDS-PAGE of metabolically labeled invertase. Cells were labeled with [^{35}S]methionine and processed as described in section 2. *ngd29* at 30°C (lane 1), *ngd29* at 37°C (lane 2), X2180 wild type (lane 3).

heterogeneity of the *outer chain*. The *mnn9* mutant was isolated earlier [19] and shown to have a small portion of α1,6-linked backbone left. Since crossing of *ngd29* and *mnn9* did complement each other, it was concluded that the *ngd29* defect is caused by a different gene. The glycosylation deficiency was further substantiated by analyzing metabolically labeled invertase (Fig. 1B). The apparent molecular mass of *ngd29* invertase was 83 kDa and identical to core glycosylated invertase of *sec18* at the restricted temperature (data not shown). *sec18* is a secretion defective mutant with a *ts*-block between ER and Golgi [20]. The *ngd29* glycosylation defect of invertase occurs at all growth temperatures; at 37°C in addition a growth phenotype is displayed that allowed to clone the relevant gene. The temperature-dependent growth sensitivity is not a lesion in a pro-

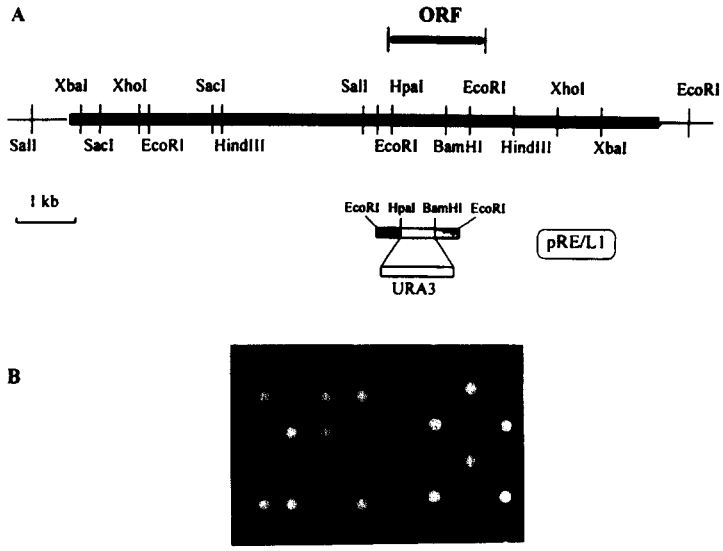

Fig. 2. (A) Partial restriction map of *NGD29*. For disruption the *Hpa*I-*Bam*HI part within the 1.6 kb *Eco*RI-*Eco*RI fragment was replaced by *URA3* to give plasmid pRE/L1. (B) Tetrad analysis of *ngd29* disrupted diploid W303. Most of the tetrads gave two large (wild type) and two small (*ngd29*) colonies.

tein activity rather is an indirect effect related to glycosylation at the elevated temperature. The wild type *NGD29* gene was isolated by complementation of the *ts* growth phenotype with a yeast genomic library in the centromer vector YCp50. Among 7×10^3 transformants 5 clones were able to complement both the *ts* growth phenotype and invertase underglycosylation (Fig. 1A). One plasmid, designated pAE0, was further analyzed. The restriction map is shown in Fig. 2A. A *Sal*I-*Xho*I subclone was able to complement the glycosylation and growth phenotype. Subsequent sequencing revealed that *NGD29* is identical to *OCH1* [21]. Disruption of wild type diploid cells followed by tetrad analysis gave two large (wild type) and two small (*ngd29* mutant) colonies (Fig. 2B). Thus *NGD29/OCH1* is not essential for cell growth but is required for tolerance at higher temperature.

3.2. *NGD29/OCH1 encodes the mannosyltransferase initiating outer chain biosynthesis*

On account of biochemical data it was postulated that *OCH1* encodes the elongation rather the initiation α1,6-mannosyltransferase [21]. Subsequent analysis of the structure of *N*-linked oligosaccharide chains in the *och1* mutant questioned this earlier finding [22]. In order to clarify this open point, the mannosyltransferase activity of the *outer chain* initation was directly measured. Microsomal membranes from the *mnn1* single mutant (control) and *ngd29mnn1* double mutant were isolated and used to assay mannosyl transfer from GDPmannose to $Man_8GlcNAc_2$ acceptor substrate. The *ngd29* disruption in the *mnn1* background was chosen in order to obtain unambiguous interpretation of the results. The *MNN1* gene causes minor *core*-modifications by adding α1,3-linked mannose residues, what may cover in the assay the true α1,6-initiation reaction. As shown in Fig. 3, membranes from *mnn1* cells as enzyme source (as well as from wild type; data not shown) are able to elongate $Man_8GlcNAc_2$ to $Man_9GlcNAc_2$ with a small amount of $Man_{10}GlcNAc_2$, the next higher homolog (trace B). However, no transfer occured with membranes from the Δngd29 disruptant (trace A). We also investigated the acceptor specificity of the reaction. Besides $Man_8GlcNAc_2$ also $Man_9GlcNAc_2$ (trace C), but not $Man_5GlcNAc_2$ (data not shown) acts as substrate.

3.3. *Heterologous expression and secretion of glucose oxidase from* Aspergillus niger *in* ngd29mnn1 *double mutant*

In order to demonstrate the utility of a *ngd29mnn1* strain as a host to synthesize secreted glycoproteins with uniform *core*-saccharide chains, we expressed glucose oxidase (GOD) from *Aspergillus niger* in such a strain. In wild type yeast cells, GOD migrates as a broad band ranging from 80–140 kDa (Fig. 4) with a carbohydrate content of 65%, whereas in *ngd29mnn1* it is a very distinct band of 80 kDa with a carbohydrate content of 11%. The specific activity of 230 U/mg protein measured in wild type yeast and 228 U/mg in the mutant is not influenced by the carbohydrate content and is the same as for the native *Aspergillus niger* enzyme. HPLC analysis of the oligosaccharide fraction of GOD expressed in the *ngd29mnn1* double mutant reveals that it is uniform and exclusively $Man_8GlcNAc_2$ (Fig. 5).

4. Discussion

Outer chain attachment is a typical *N*-glycosylation modification of *S. cerevisiae* that leads to hyperglycosylated proteins with a heterogeneous and highly immunogenic carbohydrate moiety. This modification can be considered as a way of carbohydrate processing analogous to the formation of complex/hybrid type structures of higher eukaryotes. Thus it has been shown that large polymannose chains of yeast invertase are processed to complex type chains, when this glycoprotein is expressed in frog oocytes [23]. A functional explanation for building up the *outer chain* is not possible at the moment. As shown here (Fig. 2B) and elsewhere [21], disruption of *outer chain* synthesis is not lethal for yeast. However, defects in *outer chain* synthesis may lead to reduced growth, a clumpy morphol-

ogy and distortion of the cell wall [19]. In the present study it is shown that a null mutation of *NGD29* leads both to a loss of the enzymatic activity transferring mannose from GDP-Man to $Man_8GlcNAc_2$ in vitro and to a loss of *outer chain* formation in vivo as revealed by analyzing the glycan chain of glucose oxidase. As compared to the heterogeneous carbohydrate size of GOD expressed in a wild type strain a distinct oligosaccharide of the size $Man_8GlcNAc_2$ is synthesized in the *ngd29mnn1* double mutant.

We have not determined yet the α1,6-linkage of the newly synthesized $Man_9GlcNAc_2$ product due to limited amounts. Since the addition of α1,3-linked mannose, the only minor modification of the core that can take place [5], was ruled out with the help of the *mnn1* mutation and on account of the properties of Ngd29p/Och1p as an integral type II Golgi membrane protein [21] it can be concluded that it is the initiation-α1,6-mannosyltransferase reaction that is affected.

The α1,6-mannosyltransferase has been partially purified

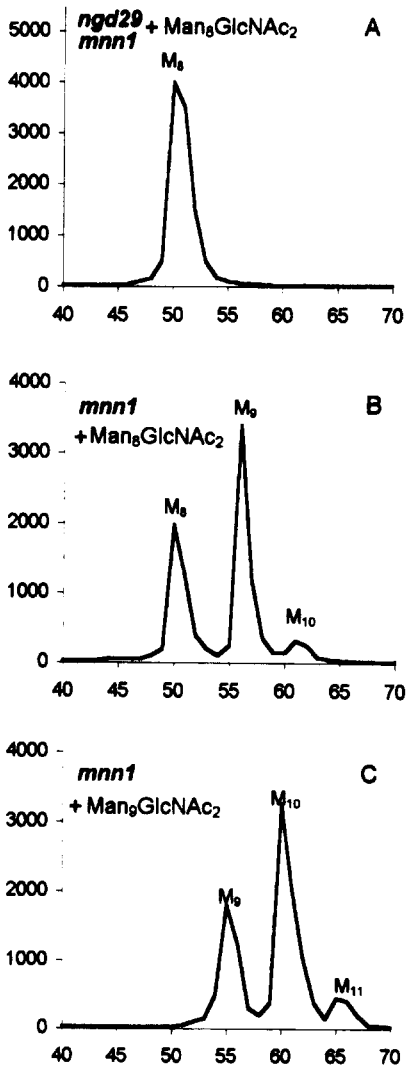

Fig. 3. Analysis of mannosyltransferase activity of *outer chain* initiation. Membranes from *ngd29mnn1* (trace A) and *mnn1* (trace B and C) were isolated and mannosyltransfer from GDPMan to $Man_8GlcNAc_2$ (trace A and B) or $Man_9GlcNAc_2$, (trace C) was measured as described in section 2. Products were analyzed by HPLC. M_{8-11} stands for $Man_{8-11}GlcNAc_2$ and indicates the elution position of authentic standards. Ordinate: radioactivity in counts/min; abscissa: fraction number.

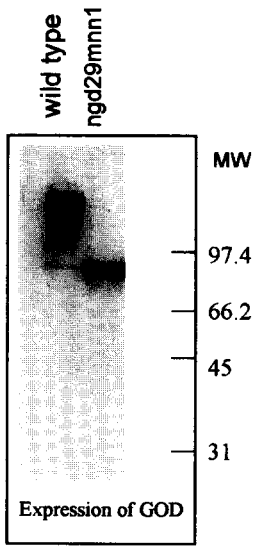

Fig. 4. Heterologous expression of GOD from *Aspergillus* in yeast. GOD was expressed in wild type yeast or *ngd29mnn1;* the material secreted into the medium was purified by metal affinity chromatography and analyzed by PAGE.

[24,25]. It was shown that both $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ act as substrates. This is also in agreement with the observation that *outer chain* formation is not prevented in a null mutant of the ER α-mannosidase that is not able to process $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ [26]. We have confirmed the substrate specificity with regard to $Man_{9/8}GlcNAc_2$ and extended by investigating also $Man_5GlcNAc_2$ as possible acceptor. In the *alg3* mutant protein glycosylation occurs from the $Man_5GlcNAc_2$-PP-Dol donor, nevertheless *outer chains* are formed on this truncated oligosaccharide [27–29]. However, in vitro we have not been able to demonstrate such a transfer. This is not necessarily in contradiction to the in vivo results. It could mean that $Man_5GlcNAc_2$ is a very poor substrate and under the selective pressure in *alg3* the level of the initiating α1,6-mannosyltransferase is increased. Also *alg3* reveals some leakiness [27–29] so that the *outer chain* made in this strain may result also in part from elongation of $Man_8GlcNAc_2$ rather from $Man_5GlcNAc_2$.

Yeast has become a favourite organism for heterologous production of proteins from human or other origin. So far the addition of the large, heterogeneous and immunogenic polymannose part is a serious hinderance for the utility of this expression system. The use of the *Δngd29Δmnn1* strain as an host that produces secretory glycoproteins with a defined and uniform *N*-linked oligosaccharide component identical to the mammalian oligomannosidic type structure may overcome these problems.

Acknowledgements: This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB43) and Fonds der Chemie.

References

[1] Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–634.
[2] Tanner, W. and Lehle, L. (1987) Biochim. Biophys. Acta 906, 81–99.
[3] Herscovics, A. and Orlean, P. (1993) FASEB J. 7, 540–550.

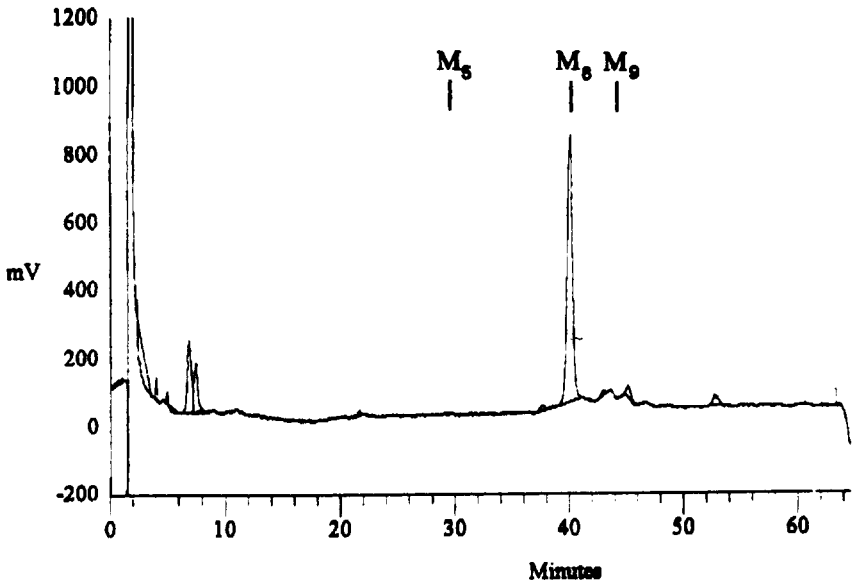

Fig. 5. HPLC analysis of *N*-glycosidase F released oligosaccharide fraction from GOD expressed in *ngd29mnn1* strain. M_5, M_8, M_9 indicate the position of Man_{5-}, Man_{8-} and $Man_9GlcNAc_2$, respectively.

[4] Schachter, H. (1991) Glycobiology 1, 453–461.
[5] Ballou, C.E. (1990) Methods Enzymol. 185, 440–470.
[6] Gopal, P.K. and Ballou, C.E. (1987) Proc. Natl. Acad. Sci. USA 84, 8824–8828.
[7] Ballou, L., Alvarado, E., Tsai, P., Dell, A. and Ballou, C.E. (1989) J. Biol. Chem. 264, 11857–11864.
[8] Flores-Carreón, A., Hixson, S.H., Gomez, A., Shao, M.C., Krudy, G., Rosevear, P.R. and Wold, F. (1990) J. Biol. Chem. 265, 754–759.
[9] Guthrie, C. and Fink, G.R. (1994) Methods Enzymol. 194.
[10] Huffaker, T.C. and Robbins, P.W. (1982) J. Biol. Chem. 257, 3203–3210.
[11] Rose, M.D., Novick, P., Thomas, J.H., Botstein, D. and Fink, G.R. (1987) Gene 60, 237–243.
[12] Knauer, R. and Lehle, L. (1995) FEBS Lett. 344, 83–86.
[13] Gabriel, O. and Wang, S.F. (1969) Anal. Chem. 27, 545–554.
[14] Franzusoff, A., Rothblatt, J. and Schekman, R. (1991) Methods Enzymol. 194, 1053–1060.
[15] Kriechbaum, M., Heilmann, H.J., Wientjes, F.J., Hahn, M., Jany, K.D., Gassen, H.G. (1989) FEBS Lett. 255, 63–66.
[16] Kopetzki, E., Buckel, P. and Schuhmacher, G. (1989) Yeast 5, 11–24.
[17] Porath, J., Carlsson, J., Olsen, I. and Belfrage, G. (1975) Nature 258, 598–599.
[18] Hochuli, E., Bannwarth, W., Döbeli H., Genz, R. and Stüber, D. (1988) Biotechnology 6, 1321–1325.
[19] Ballou, L., Cohen, R.E. and Ballou, C.E. (1980) J. Biol. Chem. 255, 5986–5991.
[20] Esmon, B., Esmon, P. and Schekman, R. (1984) J. Biol. Chem. 359, 10322–10327.
[21] Nakayama, K., Nagasu, T., Shimma, Y., Kuromitsu, J. and Jigami, Y. (1992) EMBO J. 11, 2511–2519.
[22] Nakanishi-Shindo, Y., Nakayama, K.-I., Tanaka, A., Toda, Y. and Jigami, Y. (1994) J. Biol. Chem. 268, 26338–26345.
[23] Roitsch, T. and Lehle, L. (1989) Eur. J. Biochem. 181, 733–739.
[24] Romero, P.A., Sleno, B. and Herscovics, A. (1994) Glycobiology 4, 135–140.
[25] Reason, A.J., Dell, A., Romero, P.A. and Herscovics, A. (1991) Glycobiology 1, 387–391.
[26] Puccia, R., Gromdin, B. and Herscovics, A. (1993) Biochem. J. 290, 21–26.
[27] Huffaker, T.C. and Robbins, P.W. (1983) Proc. Natl. Acad. Sci. USA 80, 7466–7470.
[28] Verostek, M.F., Atkinson, P.H. and Trimble, R.B. (1993) J. Biol. Chem. 268, 12095–12103.
[29] Verostek, M.F., Atkinson, P.H. and Trimble, R.B. (1993) J. Biol. Chem. 268, 12104–12115.