New oligosaccharyltransferase assay method

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We developed a new in vitro assay for oligosaccharyltransferase (OST), which catalyzes the transfer of preassembled oligosaccharides on lipid carriers onto asparagine residues in polypeptide chains. The asparagine residues reside in the sequon, Asn-X-Thr/Ser, where X can be any amino acid residue except Pro. We demonstrate the potency of our assay using the OST from yeast. In our method, polyacrylamide gel electrophoresis is used to separate the glycopeptide products from the peptide substrates. The substrate peptide is fluorescently labeled and the formation of glycopeptides is analyzed by fluorescent gel imaging. Two in vitro OST assay methods are now widely used, but both the methods depend on previous knowledge of the oligosaccharide moiety: One method uses lectin binding as the separation mechanism and the other method uses biosynthetically or chemoenzymatically synthesized lipid-linked oligosaccharides as donors. N-linked protein glycosylation is found in all three domains of life, but little is known about the N-glycosylation in Archaea. Thus, our new assay, which does not require a priori knowledge of the oligosaccharides, will be useful in such cases. Indeed, we have detected the OST activity in the membrane fraction from a hyperthermophilic archaeon, Pyrococcus furiosus.

Keywords: enzyme assay method/fluorescence detection/N-linked protein glycosylation/oligosaccharyltransferase

Introduction

Oligosaccharyltransferase (OST) en bloc transfers preassembled oligosaccharides onto asparagine residues in nascent polypeptide chains (for recent reviews, see Knauer and Lehle 1999; Yan and Lennarz 2005; Kelleher and Gilmore 2006). Asparagine-linked glycosylation (N-glycosylation) is the most ubiquitous modification of secretory and membrane proteins. In this sense, it is not surprising that N-glycosylation is widespread not only in eukaryotic organisms, but also in archaeal and some eubacterial organisms (for recent reviews, see Szymanski et al. 2003; Upreti et al. 2003; Szymanski and Wren 2005; Kelleher and Gilmore 2006). The OST enzyme is a multisubunit protein complex residing in the endoplasmic reticulum (ER) membrane in eukaryotic cells. In Saccharomyces cerevisiae (yeast), the OST complex consists of nine subunits, all of which contain one or more predicted transmembrane segments (Knauer and Lehle 1999) and adopts a dimeric structure when solubilized in digitonin (Chavan et al. 2006). Among them, staurosporine and temperature sensitivity-3 (STT3) is the only conserved subunit in the three domains of life (Zufferery et al. 1995). In particular, a five-residue sequence, Trp-Trp-Asp-Tyr-Gly (WWDYG motif), is highly conserved. Accordingly, STT3 has been shown to be the catalytic subunit of the OST complex from yeast (Yan and Lennarz 2002; Nilsson et al. 2003; Karamyshev et al. 2005) and a Gram-negative eubacterium, Campylobacter jejuni (Feldman et al. 2005; Glover et al. 2005). The inactivation of the stt3 gene (alias, aglB) in an archaeon, Methanococcus voltae, resulted in the underglycosylation of flagellin and S-layer proteins (Chaban et al. 2006). Although the effects of the stt3 gene disruption on glycosylation were somewhat obscured due to the occurrence of O-glycosylation, this is the first experimental evidence for the involvement of the STT3 protein in the archaeal glycosylation process.

The oligosaccharide donor for N-glycosylation is the dolichol pyrophosphate-linked oligosaccharide (OS-PP-Dol) in eukaryotes: Glc3Man9GlcNAc2-PP-Dol for most eukaryotes, but similar sugar lipids with smaller oligosaccharides in the lower eukaryote, protista (Samuelson et al. 2005). In particular, GlcNAc2-PP-Dol is the in vivo donor in Giardia lambia. The oligosaccharide donor in the eubacterium, C. jejuni, is GalNAc2GalNAc2Bac-PP-Und, where Bac and Und represent bacillosamine (2,4-diacetoamido-2,4,6-trideoxyglucopyranose) and undecaprenol, respectively (Wacker et al. 2002; Young et al. 2002). In archaea, both dolichol-phosphate- and dolichol-pyrophosphate-linked oligosaccharides were detected in extracts of such cells (Lechner et al. 1985; Lechner and Wieland 1989; Kuntz et al. 1997; Burda and Aebi 1999). The glycan structure of the oligosaccharide donors is highly diverse in archaea, considering the huge variety of the N-glycan structures on asparagine residues (Schäffer and Messner 2004; Eichler and Adams 2005). Therefore, the chemical structure of the oligosaccharide donor is highly variable among the three domains of life.

The consensus motif of N-glycosylation (referred to as the sequon) is represented by Asn-X-Thr/Ser, where X can be any residue except Pro, throughout all three domains of life (Gavel and von Heijne 1990). Statistical sequence analyses of comprehensive sets of eukaryotic and archaeal glycoproteins revealed no clear rules regarding the flanking residues around the sequons (Ben-Dor et al. 2004; Petrescu et al. 2004; Abu-Qarn and Eichler 2006), except for the rare occurrence of proline at the position immediately after the sequons. This observation is consistent with the fact that tripeptides, such as Asn-Tyr-Thr, can...
serve as an acceptor substrate in the OST assay, provided that the N-terminal and C-terminal groups of the peptide are blocked. By contrast, in the eubacterium C. jejuni, the consensus sequon is N-terminally extended to Asp/Glu-X-Asn-X-Thr/Ser for efficient glycosylation in vivo (Kowarik et al. 2006) and in vitro (Chen et al. 2007). A simple N- and C-terminal capped tripeptide is, however, usable in the in vitro assay as an acceptor substrate, despite its low efficiency (Glover et al. 2005). Not all of the N-glycosylation sequons are modified. In fact, statistical analyses revealed that just 60–65% of the potential sequons are occupied (Petrescu et al. 2004). It is likely that conformational factors have a decisive role in determining whether a sequon will be modified (Knauer and Lehle 1999), but the reasons and the molecular mechanism for this are not fully understood. Thus, extensive experimental analyses of the OST reaction are necessary to make the prediction of glycosylation possible from amino acid sequences. The enzymatic properties of the OST complex can be investigated by in vivo assays, if the mutated proteins can be expressed in cells, but a serious problem arises because the N-glycosylation is essential for cell viability in most eukaryotic cells. In the case of yeast, a special method, called the plasmid shuffle was used to show that the mutations in the WWDYG motif abolished or substantially reduced the OST activity in yeast cells (Yan and Lennarz 2002). By contrast, the C. jejuni OST consists of the STT3 protein only, and the heterologous expression of the C. jejuni stt3 gene (alias, pgIB) was successful in Escherichia coli cells, and showed the essential role of the WWDYG motif in the OST activity (Wacker et al. 2002; Feldman et al. 2005; Nita-Lazar et al. 2005). Although these in vivo experiments are very useful, they have some limitations. Alteration of the OST activity may not be a direct consequence of the mutations. Many factors other than the OST activity itself may influence the effects of mutations, and make the interpretation obscure. For example, the OST complex interacts with the Sec61p pore complex, which is involved in protein import into the ER (Chavan et al. 2005; Chavan and Lennarz 2006).

In vitro assays using (partially) purified OST enzymes cover the shortcomings of the in vivo assays. In addition, enzyme kinetics studies provide valuable information on the enzymatic mechanism. The suggestion of the presence of a regulatory site of OS-PP-Dol, in addition to the catalytic site, in the eukaryotic OST complex provides an excellent example (Karaoglu et al. 2001; Kelleher et al. 2007). This interesting conclusion was derived from a substantial deviation from simple Michaelis–Menten enzyme kinetics. In one in vitro OST assay (Roos et al. 1994; Kelleher et al. 2001), radiolabeled oligopeptides are used, from which radioactive glycopeptide products are separated by binding to a lectin. In another in vitro assay (Bause and Hettkamp 1979; Sharma et al. 1981; Imperiali and Shannon 1991; Zufferey et al. 1995; Glover et al. 2005), radiolabeled lipid-linked oligosaccharides (LLO) are used as the donor, and radioactive glycopeptide products are recovered from the upper water phase, while the unreacted LLO remains in the lower organic phase.

In this report, we have developed a third in vitro assay, which uses polyacrylamide gel electrophoresis (PAGE) as the separation mechanism. The peptide substrate is labeled with a fluorescent dye, and the glycopeptide products are detected and quantified by fluorescence gel imaging. We show the potency of our in vitro assay using membrane fractions from yeast and a hyperthermophilic archaeon, Pyrococcus furiosus. We expect that this method will be widely accepted, because of its efficient and user-friendly PAGE format.

**Results and discussion**

**Analysis of the OST reaction mixture by sodium dodecyl sulfate (SDS)-PAGE**

The OST enzyme requires two substrates, an LLO as the donor and an oligopeptide as the acceptor (Figure 1). The products are glycopeptide and the corresponding lipid-pyrophosphate. We prepared a digitonin-extracted membrane fraction that contains OST from yeast cells, and separately, a crude LLO fraction derived from a hyperthermophile (Chavan et al. 2005). Although these in vivo experiments are very useful, they have some limitations. Alteration of the OST activity may not be a direct consequence of the mutations. Many factors other than the OST activity itself may influence the effects of mutations, and make the interpretation obscure. For example, the OST complex interacts with the Sec61p pore complex, which is involved in protein import into the ER (Chavan et al. 2005; Chavan and Lennarz 2006).

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**Fig. 1.** Schematic representation of the reaction catalyzed by the oligosaccharyltransferase (OST). A hexapeptide containing the N-glycosylation sequon, Asn-X-Thr, with a fluorescent dye, TAMRA, at the N-terminus is the acceptor substrate. The lipid-linked oligosaccharide (LLO) is the donor substrate. The in vivo LLO donor in yeast is Glc3Man9GlcNAc2-PP-Dol. The products are a glycopeptide and a lipid-pyrophosphate. Hexagons, oligosaccharide chain; circle, phosphate group; rectangle, dolichol.
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Fig. 2. SDS-PAGE analysis of the reaction mixtures of the in vitro yeast OST assay. The membrane fraction containing the OST from yeast, the LLO fraction from yeast, and the TAMRA-peptide were mixed, incubated at 30°C, and subjected to SDS-PAGE. The fluorescence image was photographed with an image analyzer. The gel images were cropped to show the region in the Rf (mobility relative to bromophenol blue dye) range of 0.5–0.8. The main product band (open triangles) migrated to the position of Rf 0.65, whereas the TAMRA-peptide (open squares) migrated to Rf 0.80. The minor products are indicated by filled triangles. (A) The necessity of each component for the reaction, and the effect of 5 mM EDTA. The reaction time was 24 h. (B) A calibration curve. The pixel intensity of the SDS-PAGE bands was integrated (AU) and corrected by subtracting the background (BG). The exposure time was 180 s (filled circles) or 10 s (open circles, the AU-BG values were multiplied by 18). (C) Metal ion dependence of the reaction and plot of the main product formation as a function of the final concentration of ions. The reaction time was 3 h. (D) Time-course of the reaction and plot of the main product formation as a function of time. Mn²⁺ was added at 5 mM.

fraction, the LLO fraction, and the TAMRA-peptide were included in the reaction mixture (Figure 2A). The addition of 5 mM ethylenediaminetetraacetic acid (EDTA) in the reaction mixture completely inhibited the formation of the band, suggesting that an endogenous metal ion was necessary for the reaction (Figure 2A). The quantity of the product was estimated using a TAMRA-labeled 22-residue peptide as an external standard (Figure 2B). Excellent linearity (R² = 0.999) was obtained over three orders of magnitude for the concentration of the TAMRA dye. The addition of exogenous manganese ions (Mn²⁺) stimulated the formation of the product band (Figure 2C). In addition to the major band, minor product bands with a higher rate of migration were also visible. Magnesium ions (Mg²⁺) also had the same effect, but were less efficient. The stimulatory effects of the divalent metal cations are consistent with the previous studies of the yeast OST (Sharma et al. 1981), the hen oviduct OST (Welplly et al. 1983), and porcine liver OST (Hendrickson and Imperiali 1995). Manganese ions were added at the final concentration of 5 mM in all of the following experiments.

The time-course study showed that the product formation was reached a plateau after 24 h (Figure 2D).

The new SDS-PAGE bands are glycopeptides

We confirmed that the product bands are glycopeptides. After the 20-h reaction, PNGase F, which cleaves between the innermost GlcNAc and asparagine residues of N-linked glycopeptides, was added to the reaction mixture. The bands disappeared, as expected (Figure 3A). The concanavalin A (ConA) lectin, immobilized to agarose, was mixed with the reaction mixture. More than half of the bands were recovered with the resin, suggesting that the bands contained α-linked mannose and terminal glucose residues (Figure 3B). A TAMRA-peptide library based on the known glycosylation sequon was synthesized, and was assayed in the presence and absence of yeast LLO (Figure 3C). The product bands were observed when the peptide substrate contained the Asn-X-Thr or Asn-X-Ser sequences, in the presence of LLO. The replacement of the first
The products of the yeast OST reaction are glycopeptides. (A) Effect of PNGase F treatment. After the 20-h OST reaction, PNGase F (glycopeptidase F, 0.25 mU, Takara BIO, Shiga, Japan) was added, and the incubation was continued for one more hour. (B) ConA-agarose binding. ConA-agarose (Wako, Tokyo, Japan), preequilibrated with 50 mM Tris–HCl, pH 7.5, containing 0.1 M NaCl, was mixed with the reaction mixture, washed twice, and heated with 5× SDS sample buffer. (C) Effect of the amino acid substitutions of the N-glycosylation sequon in the peptide substrate. The reaction mixtures were incubated for 43 h in the presence and absence of yeast LLO.

![Fig. 3.](image)

**Fig. 3.** The products of the yeast OST reaction are glycopeptides. (A) Effect of PNGase F treatment. After the 20-h OST reaction, PNGase F (glycopeptidase F, 0.25 mU, Takara BIO, Shiga, Japan) was added, and the incubation was continued for one more hour. (B) ConA-agarose binding. ConA-agarose (Wako, Tokyo, Japan), preequilibrated with 50 mM Tris–HCl, pH 7.5, containing 0.1 M NaCl, was mixed with the reaction mixture, washed twice, and heated with 5× SDS sample buffer. (C) Effect of the amino acid substitutions of the N-glycosylation sequon in the peptide substrate. The reaction mixtures were incubated for 43 h in the presence and absence of yeast LLO.

The simple mass difference between the glycopeptide products and the peptide substrates cannot explain the excellent separation on SDS-PAGE. The unmodified TAMRA-peptide migrated near the electrophoretic front, as expected from its small molecular mass, 1 kDa. A branched structure with a bulky oligosaccharide chain in the glycopeptides decreases the rate of migration substantially, probably due to the sieving action of the polyacrylamide gel. The appropriate setting of the electric

![Fig. 4.](image)

**Fig. 4.** MALDI-TOF-MS spectrum of the ConA-captured materials in the reaction mixture of the yeast OST assay. A ladder of ions at 162-Da intervals was observed. The inset shows the MS/MS analysis of the ion of m/z 3004.15. The cross-ring cleavage of the innermost GlcNAc residue is shown schematically. The peptide substrate used for the MS/MS analysis was an eight residue peptide, TAMRA-Arg-Gly-Asn-Ser-Thr-Val-Thr-Arg-NH₂, instead of the six residue peptide substrate.
Detection of the OST activity in a peptide substrate useful for the separation of the glycopeptide products and the reaction solution. Regardless of the mechanism, SDS-PAGE is age by its denaturating effects on miscellaneous proteins in the current value is essential for the good separation. The role of SDS is unclear, but it is expected to clarify the migration image by its denaturating effects on miscellaneous proteins in the reaction solution. Regardless of the mechanism, SDS-PAGE is useful for the separation of the glycopeptide products and the peptide substrates.

**Detection of the OST activity in a P. furiosus membrane fraction**

The Triton X-100-solubilized membrane fraction from the cells of a hyperthermophilic archaeon, *P. furiosus*, was prepared and used for the OST assay. The incubation of the membrane fraction with the TAMRA-peptide at 65 °C produced a new band that migrated at a different rate from those of the glycopeptides derived from yeast OST (Figure 5A). The addition of the *P. furiosus* LLO fraction was unnecessary, indicating that the membrane fraction contained endogenous LLO. The discrepancy between the yeast and *P. furiosus* membrane fractions with respect to the presence of endogenous LLO might be explained by the use of the different detergents, digitonin, and Triton X-100, respectively. The same TAMRA-peptide library as with the yeast OST demonstrated that the product formation was fully consistent with the N-glycosylation sequon (Figure 5B). The addition of EDTA did not inhibit the reaction up to 10 mM. This is probably due to endogenous metal ions. OST assay using purified *P. furiosus* OST is necessary for future work. To our knowledge, this is the first biochemical demonstration of an in vitro OST reaction of archaea.

**Comparison of the present assay method with the existing methods**

There are two conventional in vitro assay systems established for the mammalian, yeast, and bacterial OSTs. In one method, the peptide acceptor is the 125I-radiolabeled tripeptide, Nα-Ac-Asn-[125I]Tyr-Thr-NH₂ (Roos et al. 1994; Kelleher et al. 2001). The 125I-glycopeptide product is separated from the peptide substrate by binding to the ConA lectin, and then is subjected to scintillation counting. In the other method, 14C- or 3H-labeled LLOs, such as GlcNAc₂-P-Dol and GalNAc-Bac-PP-Und, are prepared biosynthetically (Bause and Hettkamp 1979; Sharma et al. 1981) or chemoenzymatically (Imperiali and Zimmerman 1990; Glover et al. 2005). An aqueous/organic phase separation partitions the radiolabeled glycopeptide product into the aqueous phase, while the LLO remains in the organic phase. The aqueous phase is subjected to scintillation counting.

Although these two methods work well, there is one limitation: we must know the LLO structure or at least the N-glycan structure to design the assay properly. By contrast, the assay developed in this study does not require a priori knowledge of the oligosaccharide structure, since the labeled substrate is the peptide, and the separation mechanism relies on the different mobility in SDS-PAGE. Indeed, we successfully detected the OST activity in the Triton X-100-solubilized membrane fraction from an archaeon, *P. furiosus* (Figure 5). An extra advantage of the PAGE method is the separation of the glycopeptide products from each other. The presence of the minor bands in the yeast OST assay demonstrates the heterogeneity of the oligosaccharide structure of the glycopeptides (Figures 2 and 3). Note that thin layer chromatography (TLC) was previously used for the separation of glycopeptides produced by yeast OST (Wieland et al. 1987). The minor bands are not due to the degradation of the peptide moiety of the glycopeptides by endogenous proteases during the assay. We found that the glycosylation effectively protected the peptide moiety from proteolysis (data not shown). We added glycosidase inhibitors, 1-deoxynojirimycin and/or 1-deoxymannojirimycin, in the reaction mixture, but the amount of the minor products was not reduced. Thus, the minor bands are ascribed to the variety of the oligosaccharide structures in the LLO preparation, which is thought to arise by exposure to cellular glucosidases and mannosidases during the LLO isolation. The ladder of ions in the MS spectrum supports the notion of the N-glycan heterogeneity (Figure 4). The different mobility of the product of *P. furiosus* OST from those of yeast OST suggests the presence of a new N-glycan structure in *P. furiosus* (Figure 5). In fact, the *P. furiosus* product did not bind to ConA lectin, which suggests that the oligosaccharide structure does not contain α-linked mannose or terminal glucose residues. Its higher electrophoretic mobility suggests that there are fewer monosaccharide residues in the *P. furiosus* N-glycan.

The PAGE separation and the direct fluorescence imaging eliminate the sample handling on a sample-by-sample basis, making the method very convenient. Since the present method is a radioisotope-independent assay, it is free from inconvenience, such as, the short-half life of 125I-labeled tripeptides, and the low or uncertain specific activity of 3H- or 14C-labeled LLO. By contrast, TAMRA peptides are stable for long-term dark storage and permit easy quantification. The fluorescence detection is potentially sensitive. The minimal detectable quantity of the glycopeptide is 1 fmol, in our routine assay conditions. The use of two different fluorescent dyes will enable competitive experiments of two different peptide substrates. We expect that the new assay method presented here will be widely used, due to its user-friendly PAGE format, and will compliment the existing in vitro assay methods.
Materials and methods

Preparation of a membrane fraction and LLO from yeast cells

Yeast cells were grown at 30°C to a density of 2.0 (OD600) in YPD medium. The yeast cells (1 g) were harvested by centrifugation at 3000 x g for 10 min, suspended in 10 mL of 20 mM Tris–HCl, pH 7.5, containing 50 mM 2-mercaptoethanol, and incubated at 30°C for 10 min. After the centrifugation and washing in 20 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 1 M KCl, the cells were suspended in 10 mL of the same buffer containing Zymolyase-100T (10 mg, Cosmo Bio, Tokyo, Japan), and were incubated at 30°C for 30 min with gentle shaking. The resultant spheroplast cells were centrifuged and washed in the buffer twice, and then were suspended in 2 mL of 20 mM Tris–HCl, pH 7.5, 0.5 M sucrose, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). The washed spheroplast cells were disrupted in a bath-type sonicator (120 W) for 10 min. The suspension was centrifuged at 10,000 x g for 30 min to remove the cell debris. The supernatant was ultracentrifuged at 100,000 x g for 1 h, and the membrane pellet was resuspended in 1.5 mL of 20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 1 mM DTT, 0.1 mM PMSF, the protease inhibitor cocktail, and 1.5% (w/v) digitonin (Wako, Osaka, Japan). The mixture was then ultracentrifuged at 100,000 x g for 1 h again, and the clarified supernatant was collected, diluted to reduce the concentration of digitonin to 0.15%, and kept as the digitonin-solubilized membrane fraction for the OST assay. Typically, 15 mL of membrane fraction were obtained from 1-g wet cells.

The yeast cells were disrupted with glass beads for the preparation of LLO (dolichyl-phosphosphate-linked oligosaccharides), as described (Kelleher et al. 2001). Typically, 1 mL of LLO fraction was obtained from 1-g wet cells.

Preparation of the membrane fraction from P. furiosus cells

P. furiosus cells were stored at −80°C as a 50% glycerol stock. The growth medium consisted of 10 g tryptone, 5 g yeast extract, 10 g starch, 24 g NaCl, 10.8 g MgCl2·6H2O, and 4.0 g Na2SO4, per liter. The growth medium (1 L) was autoclaved, inoculated with 10 g of the glycerol stock, and then incubated in an oven, preheated at 98°C. The cells were cultured overnight without agitation. The caps of the bottles were kept loose because of the production of hydrogen gas. After the bottles cooled, the cells were harvested by centrifugation at 7000 x g for 10 min. One gram of wet cells was obtained per liter of culture. The cells were disrupted by an osmotic shock treatment: the frozen cells were suspended in 20 mM Tris–HCl, pH 7.5, containing 2 mM MgCl2, and 10 U mL−1 benzonase (Merck, Whitehouse Station, NJ), and were incubated at ambient temperature for 2 h. The suspension was centrifuged at 9000 x g for 10 min, and the clear supernatant was kept as the cytosol fraction. The pellet was resuspended in 20 mM Tris–HCl buffer, pH 7.5, homogenized by a probe-type sonicator at a low power setting, and centrifuged again. The pellet was kept as the cell debris fraction. The supernatant was ultracentrifuged at 100,000 x g for 1 h, and the membrane pellet was resuspended in 20 mM Tris–HCl, pH 7.5, containing 0.5 M NaCl, and 1% (v/v) Triton X-100, and was incubated at ambient temperature for 1 h. This suspension was ultracentrifuged again, and the supernatant was kept as the Triton X-100-solubilized membrane fraction. Typically, 2 mL of membrane fraction were obtained from 1-g wet cells.

TAMRA-peptide synthesis

The crude grade peptides were custom-synthesized with an N-terminal TAMRA (5/6-carboxytetramethylrhodamine, mixed isomers, absorption/emission max. 549 nm/566 nm) fluorophore, and a C-terminal amide group (OPERON Biotechnologies, Tokyo, Japan). The amino acid sequence was Gly-Asn-Ser-Thr-Val-Thr, where the underlined sequence was changed in the assay to test the N-glycosylation sequon. Each peptide was purified by reverse-phase high-performance liquid chromatography (HPLC). The peptides resolved into two peaks corresponding to the 5- and 6-isomers of the TAMRA fluorophore. We found that the slowly eluting peak of the TAMRA-peptide yielded a clearer SDS-PAGE background in the OST assay, for unknown reasons. The concentration of the peptides was determined by the absorbance at 558 nm, with an extinction coefficient of 90,000 M⁻¹ cm⁻¹ in aqueous solution.

OST assay

A 10 µL aliquot of LLO in CHCl₃·CH₃OH·H₂O (10:10:3) was transferred to a 1.5-mL plastic tube and dried in a SpeedVac concentrator (ThermoSavant, Holbrook, NY). Six microliter of LLO buffer (50 mM Tris–HCl, pH 7.5, containing 10 mM MnCl₂) was added to the assay tube, and the solution was sonicated in a bath-type sonicator (120 W) for 5 min. Three microliter of 0.01 mM TAMRA-peptide solution and 3 µL of the membrane fraction containing yeast OST were added, and the mixture was incubated at 30°C. The reaction was stopped by the addition of 2.5 µL of 5 × SDS sample buffer and was heated at 95°C for 5 min. The reaction mixtures were separated by SDS-PAGE (gradient gel 15–25%, size 90 × 90 × 0.9 mm³, Daiichi, Tokyo, Japan) under a constant current mode. The optimal electric current was about 23 mA per gel. Standard current, 30–40 mA, resulted in poor separation. The fluorescence images of the gels were recorded with a LAS-3000 multicolor image analyzer (Fuji Film, Tokyo, Japan), using the green LED (520 nm) illuminator and a 575DF20 filter. It was unnecessary to detach the glass plates from the gel. The typical exposure time was 180 s. The gel images were quantified using the Image Gauge software (Fuji Film). To obtain a calibration curve, a 22-residue peptide with an N-terminal TAMRA dye was used as a standard. Caution must be exercised in a serial dilution of the TAMRA-peptide to avoid adsorption on plastic tubes: mixing with the SDS sample buffer first, and then dilution. The brightness and contrast of the gel images were processed by Graphic converter, v4.0. The concurrent running of prestained molecular weight markers on the gel is not recommended because some of the staining dyes are fluorescent.

The assay of the membrane fraction containing P. furiosus OST was carried out in the same manner as that for yeast OST, except that the addition of LLO was omitted because the membrane fraction contains endogenous LLO. The reaction mixture was incubated overnight at 65°C.

MS/MS analysis of the yeast OST assay products

The matrix used was DHB (2,5-dihydroxybenzoic acid, Shimidzu GLC, Tokyo, Japan) dissolved at 10 mg/mL in 0.1% TFA: acetonitrile (6:4, v/v). Equal volumes (0.5 µL each) of
the sample and the matrix solution were mixed and dried on the target plate. MS and MS/MS spectra were acquired in the positive ion reflection mode, using a matrix assisted laser desorption/ionization quadrupole ion trap time of flight (MALDI-QIT-TOF) mass spectrometer (AXIMA-QIT, Shimadzu Biotech, Kyoto, Japan).

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Conflict of interest statement

None declared.

Abbreviations

ConA, concanavalin A; DTT, dithiothreitol; EDTA, ethylene-diaminetetraacetic acid; ER, endoplasmic reticulum; HPLC, high-performance liquid chromatography; LLO, lipid-linked oligosaccharide; MALDI-QIT-TOF, matrix assisted laser desorption/ionization quadrupole ion trap time of flight; MS, mass spectrometry; OS-PP-Dol, dolichol pyrophosphate-linked oligosaccharide; OST, oligosaccharyltransferase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-PAGE; STT3, starch- and temperature sensitivity-3; TAMRA, carbboxytetramethylrhodamine.

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