2604 *Biochemistry* **2010,** *49,* 2604–2614
DOI: 10.1021/bi100094g

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Highly Oriented Recombinant Glycosyltransferases: Site-Specific Immobilization of Unstable Membrane Proteins by Using *Staphylococcus aureus* Sortase A[†]

Takaomi Ito,[‡,§] Reiko Sadamoto,[∥] Kentaro Naruchi,[‡] Hiroko Togame,[§] Hiroshi Takemoto,[§] Hirosato Kondo,[⊥] and Shin-Ichiro Nishimura*[,‡]

[‡]*Graduate School of Life Science and Frontier Research Center for Post-Genomic Science and Technology, Hokkaido University, N21, W11, Kita-ku, Sapporo 001-0021, Japan,* [§]*Discovery Research Laboratories, Pharmaceutical Research Division, Shionogi and Company, Ltd., N21, W11, Kita-ku, Sapporo 001-0021, Japan,* [∥]*Ochanomizu University, 2-1-1, Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan, and* [⊥]*Discovery Research Laboratories, Shionogi and Company, Ltd., 12-4, Sagisu 5-chome, Fukushima-ku, Osaka 541-0045, Japan*

Received January 20, 2010; Revised Manuscript Received February 22, 2010

ABSTRACT: Recombinant glycosyltransferases are potential biocatalysts for the construction of a compound library of oligosaccharides, glycosphingolipids, glycopeptides, and various artificial glycoconjugates on the basis of combined chemical and enzymatic synthetic procedures. The structurally defined glycan-related compound library is a key resource both in the basic studies of their functional roles in various biological processes and in the discovery research of new diagnostic biomarkers and therapeutic reagents. Therefore, it is clear that the immobilization of extremely unstable membrane-bound glycosyltransferases on some suitable supporting materials should enhance the operational stability and activity of recombinant enzymes and makes facile separation of products and recycling use of enzymes possible. Until now, however, it seems that no standardized protocol preventing a significant loss of enzyme activity is available due to the lack of a general method of site-selective anchoring between glycosyltransferases and scaffold materials through a stable covalent bond. Here we communicate a versatile and efficient method for the immobilization of recombinant glycosyltransferases onto commercially available solid supports by means of transpeptidase reaction by *Staphylococcus aureus* sortase A. This protocol allowed for the first time highly specific conjugation at the designated C-terminal signal peptide moiety of recombinant human β1,4-galactosyltranseferase or recombinant *Helicobacter pylori* α1,3-fucosyltransferase with simple aliphatic amino groups displayed on the surface of solid materials. Site-specifically immobilized enzymes exhibited the desired sugar transfer activity, an improved stability, and a practical reusability required for rapid and large-scale synthesis of glycoconjugates. Considering that most mammalian enzymes responsible for the posttranslational modifications, including the protein kinase family, as well as glycosyltransferases are unstable and highly oriented membrane proteins, the merit of our strategy based on "site-specific" transpeptidation is evident because the reaction proceeds only at an engineered C-terminus without any conformational influence around the active sites of both enzymes as well as heptad repeats of rHFucT required to maintain native secondary and quaternary structures during the dimerization on cell surfaces.

Glycosylation is one of the most important posttranslational modifications, and more than 50% of all proteins are presumed to be glycosylated in eukaryotes (*1*). The glycan chains in glycoconjugates, such as glycoproteins, glycosphingolipids, and proteoglycans, play crucial roles in a variety of physiological and pathological events, including cell differentiation and cell−cell interactions (*2*), cell adhesion (*3*, *4*), immunological responses (*5*, *6*), inflammation (*7*), viral and parasitic infections (*8*), and tumorigenesis (*7*, *9*). It has been well documented that the posttranslational glycosylation of proteins is not template-driven and glycan chains are synthesized by some complicated biosynthetic pathways in ER/Golgi with various glycosyltransferases and glycosidases (*10*).

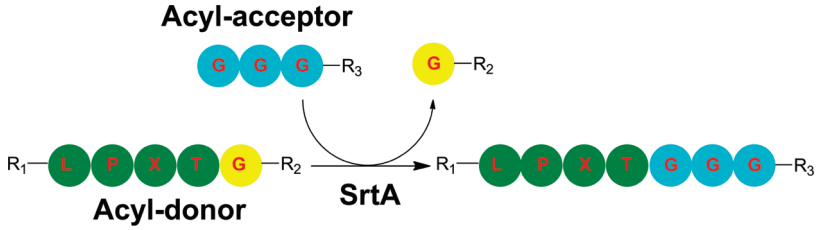
Basic studies on the relationship between the structure and function of the glycan chains in various glycoconjugates have greatly contributed to the application of characteristic features of carbohydrates in the development of modern biopharmaceuticals. For example, the genetic installation of multivalent sialylated *N*-glycan chains at some additional sites of the human hormone erythropoietin, which has three original *N*-glycan chains at Asn24, Asn38, and Asn83 residues in the native form, resulted in a considerably longer half-life of the hormone in blood compared with that of the native protein (*11*). Researchers also demonstrated that glycosylation of naked protein/peptide (nonglycosylated) drugs such as insulin and GLP-1 (glucagon-like peptide 1) improves their therapeutic activity in disease model mice (*12*−*14*). It is generally recognized that glycosyltransferase-catalyzed glycosylation is one of the most practical approaches for in vitro enzymatic synthesis of various glycoconjugates (*15*, *16*). Glycosyltransferases make up a family of membrane-bound enzymes that catalyze the transfer reaction of a specific sugar from its sugar−nucleotide donor to various

[†]This work is supported partly by a program grant for the National Project "Innovation COE program for future drug discovery and medical care" from the Ministry of Education, Culture, Science, and Technology, Japan.

*To whom correspondence should be addressed. E-mail: shin@glyco.sci.hokudai.ac.jp. Telephone: +81-(0)11-706-9043. Fax: +81-(0)11-706-9042.

 Published on Web 02/23/2010

Scheme 1: Transpeptidation Catalyzed by SrtA and General Acyl Donors and Acceptors



acceptor substrates with high chemo-, regio-, and stereoselectivity under mild reaction conditions. Recently, although a variety of recombinant glycosyltransferases may be available because of advances in cloning studies, commercialization is often hampered by the lack of operational stability of these unstable enzymes, coupled with their relatively high prices.

One way to overcome this obstacle is immobilization of the enzymes, affording improved operational stability and providing for its facile separation and reuse of costly biocatalysts. The most robust approach is immobilization of these enzymes on the solid supports through a covalent linkage. However, in most of the early methods, enzymes have been attached to the supporting materials through random amide bond or Schiff base formation by using potential amino acids such as lysine and arginine residues, resulting in significant loss of enzyme activity after immobilization (*17*−*19*). The site-specific reaction of existing or engineered cysteine residues with activated solid or gold surfaces can provide selective and oriented attachment (*20*, *21*) but is limited to situations in which cysteine residues can be optimally positioned and/or removed from the native protein sequence. Noncovalent bond-based immobilization of enzymes with some affinity interaction, such as biotin−avidin (*22*, *23*), anti-GST antibody (*24*), His tag−Ni (*25*), or maltose binding protein−amylose (*26*−*28*) interaction, has also been used to immobilize enzymes on agarose beads, magnetic nanoparticles, and the surface plasmon resonance (SPR)[1] plate, but the potential dissociation of the enzyme from the solid surface may interfere with the long-term storage and reusability of the immobilized enzyme.

In recent years, a number of selective ligation chemistries have been developed. These include intein-based systems (*29*), "click" chemistry, Staudinger ligation, or native chemical ligation (*30*−*33*). While promising, these chemoselective approaches involve multiple steps and require the introduction of special linkers to proteins and/or chemical modifications of the surface of solid supports. Therefore, there remains a significant need for a robust and much more versatile methodology for enzyme/protein immobilization that can be applied to a wide range of protein-based biotechnology. Enzymatic approaches for the conjugation or immobilization of proteins have attracted much attention because the substrate specificity of enzymes enables site-specific protein modification. For instance, we developed a highly efficient method for site-specific introduction of ω-aminoalkyl glycosides of oligosaccharides into mutant insulins based on the transpeptidase activity of bacterial or mammalian transglutaminase (TGase) when the suited position could be replaced genetically with a designated glutamine residue (*12*, *13*). It was also reported that use of phosphopantetheine transferase makes a site-selective protein immobilization possible (*34*). This method, however, requires incorporation of coenzyme A as a probe on a solid surface in addition to a natural or engineered phosphopantetheinylation site on the protein of interest. Recent studies have shown that *Staphylococcus aureus* sortase A (SrtA) provides a robust and gentle approach for the selective conjugation of proteins to solid surfaces (*35*−*37*). The bacterial transpeptidase SrtA is an extensively studied stapler enzyme with known substrate specificity (*38*). SrtA is used by Gram-positive bacteria to anchor surface proteins to the cell wall through a condensation reaction between a C-terminal LPXTG tag on the former and a polyglycine bridge in the latter (*39*). The enzyme cleaves the LPXTG sequence at the amide bond between the threonine and the glycine to form an acyl−enzyme complex. Nucleophilic attack by the amino group of the tris-glycine on the intermediate results in the formation of an LPXT−GGG bond and the liberation of the free enzyme (Scheme 1). Because of the low tolerance of SrtA for the deviation in the LPXTG recognition motif, this enzymatic ligation is highly selective. In particular, the very limited occurrence of this motif in native proteins seems to make the SrtA-mediated reaction attractive for protein modification (*40*−*42*). Recently, we also demonstrated the feasibility of this specific ligation reaction by SrtA for the construction and characterization of highly complicated glycoprotein models related to tumor-relevant MUC1 (*43*). Here, we report the successful use of SrtA for the covalent and site-specific immobilization of glycosyltransferases, recombinant human β1,4-galactosyltransferase (rhGalT) and recombinant *Helicobacter pylori* α1,3-fucosyltransferase (rHFucT), onto common solid supporting materials without a loss of activity or stability.

EXPERIMENTAL PROCEDURES

Materials and Methods. All commercially available solvents and reagents were used without further purification. Uridine 5′-diphospho-D-galactose, 2Na (UDP-Gal), and guanosine 5′-diphosphate-L-fucose (GDP-Fuc) were purchased from Yamasa Co. Matrix-associated laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) data were recorded with a Bruker REFLEX III or AUTOFLEX II instrument using 2,5-dihydroxybenzoic acid as a matrix. Kinetic analysis was performed with an HPLC system equipped with an Inertsil ODS-3 column (4.6 mm × 150 mm) with a flow rate of 1.0 mL/min at room temperature. The column was equilibrated with 5% acetonitrile and eluted with the following acetonitrile gradient: 5% at time zero, 15% at 3 min, and 30% at 12 min. The chromatography was monitored by using UV absorption at 315 nm. Data were fitted by nonlinear regression to the Michaelis−Menten equation using Sigmaplot (Systat Software Inc.).

Preparation of S. aureus Sortase A. Recombinant *S. aureus* sortase A (SrtA) was prepared according to the

[1]Abbreviations: SrtA, *Staphylococcus aureus* sortase A; rhGalT, recombinant human β1,4-galactosyltransferase; rHFucT, recombinant *Helicobacter pylori* α1,3-fucosyltransferase; SPR, surface plasmon resonance; PCR, polymerase chain reaction; IPTG, isopropyl β-D-1-thiogalactopyranoside; TB, Terrific broth; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GDP, guanosine 5′-diphosphate; LacNAc, *N*-acetyl-D-lactosamine; DTT, dithiothreitol; UDP, uridine 5′-diphosphate.

procedure previously reported (*43*, *44*). A truncated form of SrtA lacking the first 59 N-terminal amino acids was cloned from *S. aureus* RN4220 genomic DNA by PCR using primers 5′-GATATACATATGCAAGCTAAACCTCAAATTCCG-3′ and 5′-GTGGTGCTCGAGTTTGACTTCTGTACAAAGAT-3′ and subcloned into the pET-23b vector for expression. In a typical procedure, *Escherichia coli* BL21(DE3) harboring the SrtA expression plasmid was precultured in LB broth containing 50 μg/mL carbenicillin at 37 °C overnight. The overnight culture was inoculated into a 1 L flask containing 250 mL of LB broth containing 50 μg/mL carbenicillin and 0.5% glucose. The cells were grown at 37 °C until the OD_{600} reached 0.7. Expression of recombinant protein was induced by the addition of IPTG to a final concentration of 0.3 mM for 18 h at 20 °C. The cells were harvested by centrifugation and resuspended in buffer [50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 5 mM imidazole]. After the sonication, supernatants were obtained by centrifugation and applied to the 5 mL HisTrapHP column (GE Healthcare Bio-Sciences). The column was eluted with a linear gradient of imidazole from 0 to 500 mM. The pooled fractions were dialyzed against 50 mM Tris-HCl (pH 7.5), concentrated, and stored at −80 °C.

Preparation of ghe rhGalT Fusion Protein. Human β1,4-galactosyltransferase (hGalT) was cloned from a human kidney cDNA library (Clontech) and expressed as a fusion protein with a maltose binding protein tag (*45*). To insert the sorting signal, "LPETG", and a His_6 tag at the C-terminus of hGalT, the expression plasmid was constructed in two steps. First, the hGalT gene was amplified by PCR using primers 5′-GGAGAATTCCGGACCGGAGGG-3′ and 5′-TAGCTCG-AGGCCGGTTTCCGGAAGGTCGACGCTCGGTGTCCC-GATGTC-3′ and cloned into the EcoRI−XhoI site of pET-23a. Next, the hGalT gene was amplified by PCR using primers 5′-GGAGAATTCCGGACCGGAGGG-3′ and 5′-ACGAAG-CTTTCAGTGGTGGTGGTGGTGGTGCTCGAGGCCG-GTTTCC-3′ and inserted into the EcoRI−HindIII-digested pMal-p2x vector, resulting in an MBP-hGalT-LPETG-His_6 expression construct (hGalT-Sor). In a typical procedure, the *E. coli* JM109 transformed by the rhGalT-Sor expression plasmid was precultured in LB broth containing 50 μg/mL carbenicillin at 37 °C overnight. The overnight culture was inoculated into a 2 L flask containing 500 mL of modified TB medium (40 g/L glycerol, 24 g/L yeast extract, 12 g/L peptone, 72 mM K_2HPO_4, 1.7 mM KH_2PO_4, and 50 μg/mL carbenicillin). The cells were grown at 28 °C until the OD_{600} reached 1.5. Expression of recombinant protein was induced by the addition of IPTG to a final concentration of 0.3 mM for 20 h at 28 °C. The cells were harvested by centrifugation and disrupted by sonication. Nucleic acids were precipitated by the addition of polyethyleneimine to a final concentration 0.7%. Ammonium sulfate was added to the supernatant of lysate up to 70% saturation. The precipitated protein was suspended in 50 mL of buffer [20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 5 mM imidazole] and applied to a 5 mL HisTrapHP column. The column was eluted with linear gradient of imidazole from 0 to 500 mM. The pooled fractions desalted by ultrafiltration were applied to a HiTrap DEAE FF column (GE Healthcare Bio-Sciences) equilibrated with 20 mM Tris-HCl (pH 8.0). The column was eluted with a linear gradient of NaCl from 0 to 150 mM. The pooled fractions were desalted, concentrated, and stored at −80 °C.

Preparation of the rHFucT Fusion Protein. *H. pylori* α1,3-fucosyltransferase (HFucT) with deletion of 42 C-terminal residues (*46*, *47*) was amplified from *H. pylori* J99 genomic DNA by PCR using primers 5′-GTTCTTGAATTCATGTTCC-AGCCCCTATTAGA-3′ and 5′-GTTCTTGTCGACGCCGC-CGCCATAATTAAC-3′ and inserted into the EcoRI−SalI site of the rhGalT-Sor expression plasmid mentioned above to attach LPETG and His_6 to the C-terminus. To remove the MBP tag, the HFucT gene was amplified by PCR using primers 5′-CACCA-TGTTCCAGCCCTATTAGA-3′ and 5′-CCGCTCCCGGCG-GATTTGTCCTAC-3′ and ligated pET101/D-TOPO (Invitrogen), resulting in an HFucT-LPETG-His_6 expression construct (HFucT-Sor). In a typical procedure, the *E. coli* JM109(DE3) transformed by the HFucT expression plasmid was precultured in LB broth containing 50 μg/mL carbenicillin at 37 °C overnight. The overnight culture was inoculated into a 1 L flask containing 250 mL modified TB medium. The cells were grown at 37 °C until the OD_{600} reached 1.5. Expression of recombinant protein was induced by the addition of IPTG to a final concentration of 1 mM for 23 h at 20 °C. The cells were harvested by centrifugation and disrupted by sonication. The supernatant of the lysate was applied to a 25 mL DEAE-Sepharose FF column (GE Healthcare Bio-Sciences) equilibrated with 50 mM Tris-HCl (pH 8.0). The column was eluted with a linear gradient of NaCl from 0 to 1 M. The pooled fractions were applied to a 5 mL HisTrapHP column. The column was washed with 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl and eluted with a linear imidazole gradient from 0 to 350 mM. The pooled fractions were buffer-exchanged and reapplied to a 5 mL HisTrapHP column. The pooled fractions were buffer-exchanged into 100 mM HEPES-NaOH (pH 8.0), 100 mM NaCl, and 0.01% Triton X-100. Glycerol (20%, v/v) was added before the sample was aliquoted and stored at −80 °C.

SrtA-Based Ligation of a Model Nonapeptide with Various Acyl Acceptors. An acyl donor nonapeptide (AHLPKTGLR-NH_2) was prepared via the general protocol for Fmoc amino acid-based solid phase peptide synthesis (SPPS). Triglycine (**1**) and 5-amino-1-pentanol (**2**) were purchased from Wako chemicals (Tokyo, Japan). Biotin derivatives (**4** and **5**) were purchased from Biotium, Inc. ω-Aminoalkyl sugar (**3**) was synthesized as previously reported (*12*). The reaction mixture contained 0.2 mM nonapeptide, 5 mM acyl acceptor (1 mM in the case of biotin derivatives), and 280 μM SrtA in sortase reaction buffer [50 mM Tris-HCl (pH 7.5), 0.05% Triton X-100, 5 mM $CaCl_2$, 100 mM NaCl, and 1 mM 2-mercaptoethanol]. The mixture was incubated at 37 °C for 9 h and subjected to MALDI-TOF MS. For the conjugation of rhGalT-sor with biotinylated compound **4** or **5**, the reaction mixture was composed of 5 μM glycosyltransferase as the acyl donor, 1 mM **4** or **5**, and 280 μM SrtA in sortase reaction buffer. The mixture was incubated at 37 °C overnight. After SDS−PAGE of the reaction solution, the gel was analyzed via Coomassie brilliant blue staining and by immunoblotting using streptavidin-bound HRP.

Immobilization of rhGalT and rHFucT on EAH-Sepharose. Typical SrtA-mediated immobilization of glycosyltransferases was conducted via the following procedure. The reaction mixture containing 12.5 μM rhGalT-Sor and 840 μM SrtA in sortase reaction buffer and 2 volumes of 7 μmol of amino reactive group/mL of EAH-Sepharose (GE Healthcare Bio-Sciences) in equilibrium by sortase reaction buffer were mixed and incubated with shaking at 37 °C overnight. After the immobilization, the reaction mixture was filtered and the solid support was washed with 25 mM HEPES buffer (pH 7.5) (0.01% Triton X-100) containing 0.5 M NaCl. The recovered solid material was suspended and stored in 25 mM HEPES buffer. In case for the

preparation of immobilized rHFucT, the ligation by SrtA was performed at 25 °C overnight. After the incubation, the reaction mixture was filtered and the solid support was washed with 50 mM Tris-HCl buffer (pH 7.5) (1 M NaCl and 0.01% Triton X-100). The recovered supporting material was suspended and stored in 50 mM Tris-HCl buffer.

The conventional chemical and random immobilization of rhGalT onto commercially available EAH-Sepharose was conducted by reacting enzyme with preactivated supporting material treated with 2.5% glutaraldehyde, and the collected solid support was washed with water and 0.1 M sodium phosphate buffer (pH 7.0) as follows. A half-volume of 25 μM rhGalT-Sor was added to glutaraldehyde-activated Sepharose, and the mixture was incubated at 4 °C for 13 h. Subsequently, 0.1 M Tris-HCl (pH 7.5) was added for quenching the resulting activated functional group. After the immobilization reaction, the solid support was washed and stored with 25 mM HEPES buffer. We also used NHS-activated Sepharose (GE Healthcare Bio-Sciences) as an alternative common supporting material for chemical immobilization as follows (*19*). A half-volume of 25 μM rhGalT-Sor was added to NHS-activated Sepharose, and the mixture was incubated at 4 °C for 13 h. Subsequently, 0.1 M Tris-HCl (pH 7.5) was added for quenching, and the supporting material was washed and stored with 25 mM HEPES buffer.

Evaluation of the Relative Activity, Specificity, and Recyclability of the Immobilized Glycosyltransferases. 4-Methylumbelliferyl *N*-acetyl-β-D-glucosamine (Mu-GlcNAc) was used as a glycosyl acceptor substrate for the measurement of the D-galactose transfer activity of immobilized rhGalT. Reaction was initiated by addition of 0.5 mM UDP-Gal to the solution [50 mM HEPES (pH 7.5), 10 mM $MnCl_2$, 0.01% Triton X-100, 1 mM 2-mercaptoethanol, and 0.1 mM Mu-GlcNAc]. After the mixture had been incubated at 25 °C for 15 min, an equivalent volume of 8 M GuHCl was added to stop the reaction. Kinetics were performed by HPLC-based protocols described in Materials and Methods. Similarly, 4-methylumbelliferyl *N*-acetyllactosamine (Mu-LacNAc) was used as glycosyl acceptor substrate for the measurement of the extent of fucosylation reaction by immobilized rHFucT. Reaction was initiated by addition of 1 mM GDP-Fuc to the solution [50 mM Tris-HCl (pH 8.0), 1 M NaCl, 50 mM $MgCl_2$, 0.01% Triton X-100, and 0.3 mM Mu-LacNAc]. After the solution had been incubated at 30 °C for 30 min, an equivalent volume of 8 M GuHCl was added to stop the reaction and the mixture was subjected to HPLC-based kinetic analysis. To determine the net activity of immobilized glycosyltranaferases, the activities of rhGalT-Sor or rHFucT-Sor protein of supernatant were quantified both in the presence and in the absence of SrtA, that is, the amount of immobilized proteins corresponding to those estimated by the image analysis (LAS-4000, Fujifilm) after SDS−PAGE stained with CBB.

Repeated use of the immobilized enzymes was conducted as follows. Reaction was started by addition of 2.5 mM UDP-Gal and 2.5 mM GDP-Fuc to the solution [50 mM Tris-HCl (pH 7.5), 10 mM $MnCl_2$, 50 mM $MgCl_2$, 0.01% Triton X-100, 1 M NaCl, 0.25 mM Mu-GlcNAc (95 μg), immobilized rhGalT (52 μL, 1.8 μg of protein), and immobilized rHFucT (68 μL, 6.0 μg of protein), in 1 mL of reaction buffer]. After incubation at 25 °C with shaking, immobilized glycosyltransferases were separated with a spin filter column and washed with 50 mM Tris-HCl (pH 8.0), 1 M NaCl, and 0.01% Triton X-100. Recovered immobilized glycosyltransferases were employed for the next reaction.

RESULTS

Substrate Specificity of SrtA toward Various Acyl Acceptors. In the previous reports about substrate specificity of SrtA, the LPXTG motifs were favored as acyl donors over all positional variants in vitro (*37*) and were necessary and sufficient for directing proper sorting of proteins in vivo (*48*). However, we considered that tolerance against acyl acceptors in SrtA-based transpeptidation would be much more beneficial than the tight specificity for the acyl donor substrates in terms of the versatility of SrtA-based potential biotechnology, as shown in hydroxylaminolysis of *S. aureus* surface proteins (*49*), conjugation of protein harboring the LPXTG motif on the polystyrene beads modified with alkylamine (*35*), and ligation of an LPXTG motif-containing peptide and sugar derivative (*50*), despite the ligation of LPXTG motif-containing *O*-glycopeptide and highly steric hindered *N*-glycopeptide (*43*). Our interest was focused on the tolerance and acceptability of acyl acceptor substrates because this might be a key to defining the feasibility of the SrtA-based protein engineering. We first tested the potentials of SrtA to conjugate a plausible model acyl donor, nonapeptide containing the "LPXTG" sequence, with general aliphatic amines (**2**−**5**) listed in Figure 1. After incubation of nonapeptide and the alkylamine derivatives with SrtA under a condition described in Experimental Procedures, MALDI-TOF MS of the reaction mixture indicated the formation of the expected products through their transpeptidase reactions (Figure 2). The results suggest that non-natural alkylamine derivatives **2**−**5** are potent acyl acceptor substrates as well as the natural acyl acceptor, triglycine (**1**). It seems likely that SrtA recognizes at least the terminal aminoethyl ($H_2NCH_2CH_2$-) moiety of these compounds bearing an aliphatic amine and can use them as convenient acyl acceptors.

Recombinant Human β1,4-Galactosyltransferase Having LPETG-HHHHHH at the C-Terminus (*rhGalT-Sor*). A cloned gene encoding hGalT with a C-terminal sorting signal peptide "LPETG" followed by His_6 was inserted into the pMal-p2X vector and overexpressed in *E. coli* JM109, producing the MBP-rhGalT-LPETG-His_6 fusion protein (rhGalT-Sor) (Figure 3a). rhGalT-Sor was purified by Ni affinity chromatography and DEAE-Sepharose chromatography, appearing as a single band on SDS−PAGE analysis (Figure 3b). We then examined the SrtA-mediated conjugation of rhGalT-Sor with a synthetic acyl acceptor, aminoalkyl-biotin derivative (**4**). Purified rhGalT-Sor and compound **4** were incubated overnight with or without SrtA, and the reaction mixtures were analyzed via SDS−PAGE. As anticipated, CBB staining and immunoblotting with streptavidin-HRP indicated that rhGalT-Sor can be coupled with aminoalkyl-biotin derivative **4** in a SrtA-dependent manner, confirming that the LPETG sequence in the C-terminal region of rhGalT-Sor worked as an acyl donor for SrtA and was transferred to the terminal amino group of compound **4** (Figure 3c). Recombinant hGalT-Sor exhibited a reactivity similar to those of all other synthetic acyl acceptors used in this study (data not shown).

Site-Specific Immobilization of rhGalT to Alkylamine-Displaying Sepharose. Successful site-specific biotinylation of rhGalT-Sor described above encouraged us to challenge the site-specific immobilization of rhGalT onto a solid support through SrtA-based transpeptidation. Instead of biotin derivative **4**, EAH-Sepharose, a commercially available supporting soft material which displays multiple amino alkyl functional

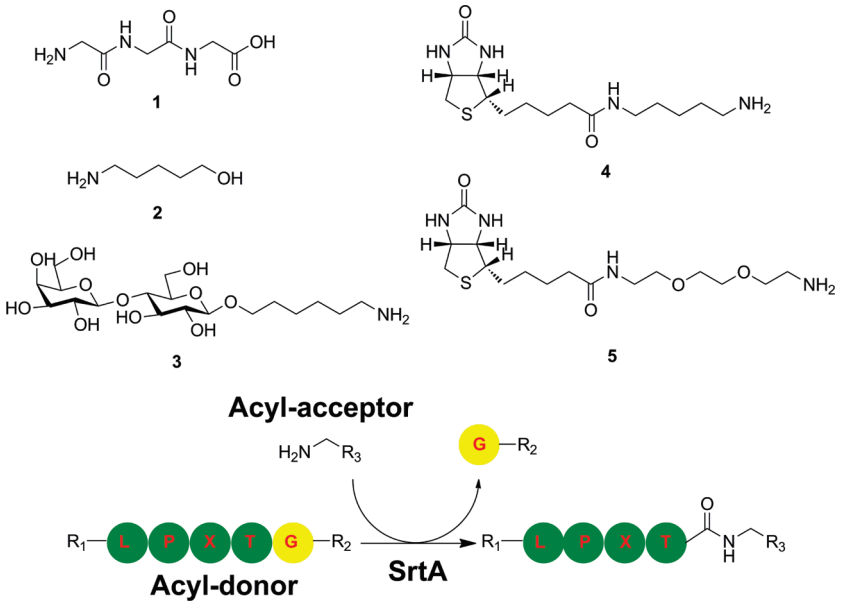


FIGURE 1: Synthetic acyl acceptors tested in this study and newly formed anchor region.

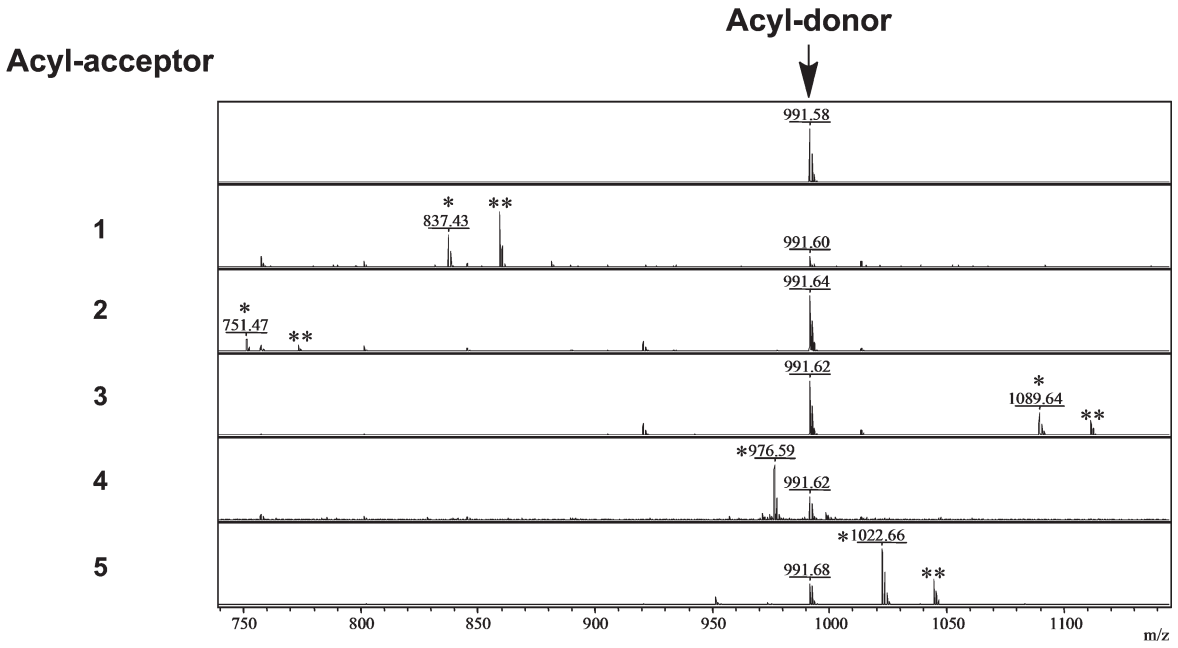


FIGURE 2: SrtA-mediated conjugation of the model peptide with compounds **1**−**5** monitored by MALDI-TOF MS. The acyl donor (AHLPKTGLR-NH_2, $[M + H]^+$ calcd, *m*/*z* 991.61) and each acyl acceptor were incubated in the presence of SrtA. Asterisks and double asterisks denote the respective products conjugated with acyl acceptors and $[M + H]^+$ and $[M + Na]^+$, respectively. Calculated $[M + H]^+$ *m*/*z* values of the expected products are as follows: *m*/*z* 837.45 for **1**, *m*/*z* 751.48 for **2**, *m*/*z* 1089.60 for **3**, *m*/*z* 976.57 for **4**, and *m*/*z* 1022.57 for **5**.

groups [-$OCH_2CH(OH)CH_2NH(CH_2)_6NH_2$], was employed as an acyl acceptor for site-specific immobilization. Recombinant hGalT-Sor and EAH-Sepharose were vigorously mixed in the presence of SrtA at 37 °C overnight. Then, the reaction mixture was separated by spin filtration. The resulting solid support was washed with 25 mM HEPES buffer containing 0.5 M NaCl to remove nonspecifically adsorbed rhGalT-Sor. Image analysis following SDS−PAGE of the separated solution phase (Figure 4) showed that the band of rhGalT-sor was reduced in magnitude by 33% in the presence of SrtA (lane 1) compared with that in the absence of SrtA (lane 2), indicating that the reduced portion of rhGalT-Sor corresponds to the amount of enzyme immobilized on the supporting material by SrtA. Interestingly, SDS−PAGE analysis of the reaction mixture incubated in the absence of EAH-Sepharose (lane 3) showed the generation of an extra band with a molecular mass of 99.3 kDa, suggesting that ligation between rhGalT-Sor (80.6 kDa) and SrtA (17.8 kDa) may occur at the ε-amino group of lysine residues of SrtA itself as the acyl acceptor substrate. However, this side reaction should become negligible as indicated when a large excess of acyl acceptor groups [-$OCH_2CH(OH)CH_2NH(CH_2)_6NH_2$] due to EAH-Sepharose is supplied (lane 1).

To demonstrate the merit of this strategy, we tested the galactose transferring activity in comparison with those of rhGalT immobilized by two conventional protocols as summarized in Table 1. It was suggested that two chemical anchoring protocols based on the reaction of aldehyde groups or active ester groups on Sepharose showed relatively high protein immobilizing

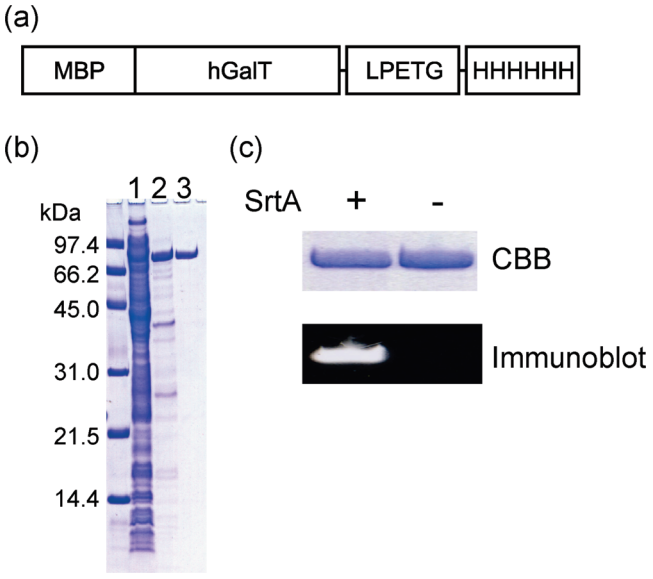


FIGURE 3: Recombinant hGalTSor as an acyl donor for SrtA-mediated conjugation. (a) Structure of recombinant MBP-fused hGalT with the LPETG sorting signal inserted and a His tag at the C-terminus (rhGalT-Sor). (b) Purification of rhGalT-sor: lane 1, cell lysate; lane 2, Ni affinity chromatography; lane 3, DEAE-Sepharose chromatography. The arrow shows a band due to rhGalT-Sor. (c) SDS−PAGE and immunoblot analysis of biotinylated rhGalT during the SrtA-mediated reaction between rhGalTSor and compound **4**. Immunoblotting analysis by means of streptavidin-HRP indicates that the desired biotinylation occurs only in the presence of SrtA.

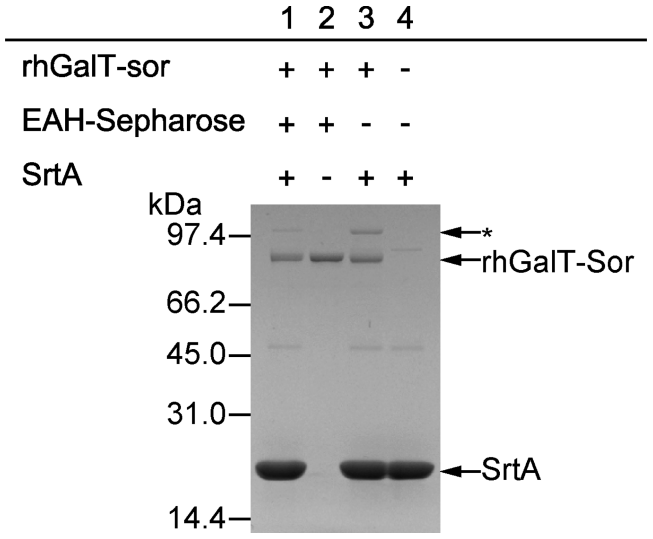


FIGURE 4: Immobilization of rhGalTSor by SrtA-mediated transpeptidation with EAH-Sepharose: lane 1, immobilization of rhGalT-Sor with EAH-Sepharose in the presence of SrtA; lane 2, without SrtA; lane 3, without EAH-Sepharose; lane 4, only SrtA. The asterisk indicates a newly generated band.

yields such as 56 and 100% due to random cross-linking with lysine and arginine residues, while the yield of the SrtA-mediated site-specific immobilization was found to be 33%. However, it is clear that the relative specific activity (net activity per protein immobilized) of the rhGalT immobilized by SrtA proved to be the highest (90%), and others were only 25 and 23%. Moreover, a kinetic study using Mu-GlcNAc as a glycosyl acceptor substrate showed that the apparent K_m value of the immobilized rhGalT (rhGalT-Sepharose) (54.7 ± 6.0 μM) was quite similar to that of soluble hGalT-Sor (25.4 ± 8.4 μM) and the value from the previous report (35.9 μM) (*51*). These results demonstrate that rhGalT was immobilized on EAH-Sepharose by SrtA-mediated specific transpeptidation of the C-terminal Sortase signal peptide without having a significant influence on the intact conformation of active glycosyltransferases. Indeed, it was reported that the immobilization of GalTs by chemical random cross-linking to activated solid supports, such as CNBr (*19*), glutaraldehyde, and *N*-hydroxysuccinimide (NHS)-activated Sepharose (*52*) led to low levels of retention of enzyme activity and low immobilization efficiencies because of random and multipoint cross-linking.

Table 1: Comparison of Relative Specific Activities of Immobilized rhGalTs

method for immobilization	immobilization yield: activity (%)	immobilization yield: protein (%)	relative specific activity (%)
SrtA-mediated	30	33	90
glutaraldehyde	14	56	25
N-hydroxysuccinimide	23	100	23
soluble rhGalT			100

Site-Specific Immobilization of rHFucT. H. pylori α1,3-fucosyltransferase (HFucT) has a heptad repeat region at its C-terminus that consists of a leucine zipper motif potentially mediating dimer formation (*46*, *47*). The following positive and hydrophobic region seems to act as a cell membrane anchor (*47*). In addition, truncation of the C-terminal region of HFucT demonstrated that tandem haptad repeats are essential for the formation of a dimeric structure as well as the stability of the enzyme (*46*, *47*). Thus, our attention was directed to the feasibility of SrtA-mediated site-specific immobilization of such dimeric and highly organized membrane-bound proteins at the engineered C-terminus containing the Sortase signal peptide. The HFucT gene was truncated at the positive and hydrophobic region and introduced into pET101 with a C-terminal LPETG sorting signal followed by His_6 (rHFucT-Sor) as well as above construction for expression of rhGalT-sor (Figure 5a). rHFucT-Sor was overexpressed in *E. coli* and homogeneously purified by DEAE-Sepharose chromatography and Ni affinity chromatography (Figure 5b). Purified rHFucT-Sor and alkylamino-biotin derivative **4** were incubated with or without SrtA under a similar condition for the conjugation with rhGalT-sor. The immunoblotting analysis by streptavidin-HRP showed that rHFucT-Sor was also SrtA-dependently coupled with the biotin derivative, confirming that the LPETG sequence in the C-terminal region of rHFucT-Sor worked as an acyl donor for SrtA and was transferred to the terminal amino group of compound **4** (Figure 5c). As anticipated, the immobilization reaction of rHFucT with EAH-Sepharose in the presence of SrtA proceeded smoothly; the yield was estimated to be 57% on the basis of the difference in the image analysis of SDS−PAGE of the soluble fractions between lane 1 (100%) and lane 3 (43%) (Figure 5d), and the activity of immobilized rHFucT (rHFucT-Sepharose) was determined to be 54% using GDP-Fuc and Mu-LacNAc, indicating that the relative specific activity was 94% as well as the result in the case of the immobilization of rhGalT (90%). Kinetic parameters (K_m and V_{max}) of rHFucT-Sepharose were found to be quite similar to those of soluble rHFucT (*53*) (Table 2). As shown in Figure 6, there was an optimal concentration (0.2−0.3 mM) of glycosyl acceptor substrate Mu-LacNAc and its inhibitory effect was noticeable under higher concentrations

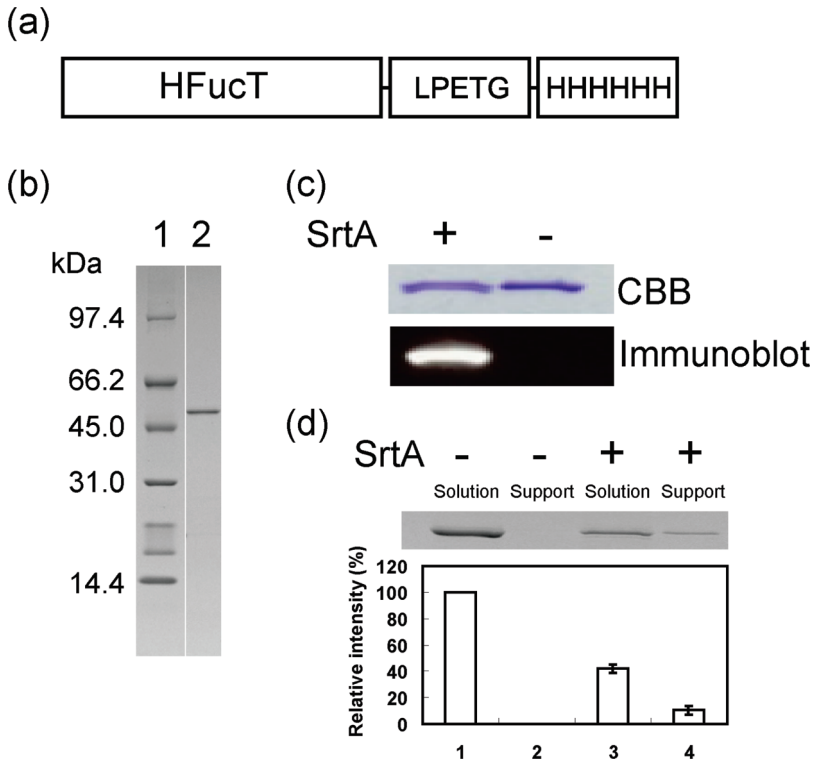


FIGURE 5: Recombinant HFucT-Sor as an acyl donor for SrtA-mediated reactions. (a) Structure of recombinant HFucT-Sor with the LPETG sorting signal inserted and a His tag at the C-terminus (rHFucT-Sor). (b) Purified rHFucT-Sor indicated by an arrow. (c) SDS−PAGE and immunoblot analysis of biotinylated rHFucT. (d) SDS−PAGE and image analysis of SrtA-mediated immobilization of rHFucT-Sor. After the immobilization, the reaction mixtures were subjected to separation as the solution phase and supports by spin filtration: lanes 1 and 2, without SrtA; lanes 3 and 4, with SrtA.

Table 2: Kinetic Parameters of Soluble and Immobilized rHFucTs

substrate	soluble rHFucT	immobilized rHFucT
GDP-fucose		
V_{max} (mmol mg^{-1} min^{-1})	1.25 ± 0.02	1.49 ± 0.06
K_m (mM)	0.108 ± 0.004	0.079 ± 0.010
Mu-LacNAc		
V_{max} (mmol mg^{-1} min^{-1})	7.7 ± 1.5	9.5 ± 3.0
K_m (mM)	0.75 ± 0.17	0.61 ± 0.22
K_i (mM)	0.16 ± 0.04	0.099 ± 0.038

in both cases. Since dimeric rHFucT composed of two subunits has two C-terminal signal peptides (-LPETG-HHHHHH), we hypothesized that the individual subunit (monomer) should still exist on the solid support through the covalent cross-linking even when the noncovalent interaction by tandem heptad repeats between two subunits is dissociated by being exposed to some severe buffer solution containing 1% SDS and 0.1 M DTT. Judging from the result shown in Figure 5d (lane 4), approximately 35% of the immobilized "dimeric" rHFucT seemed to be immobilized by using only single covalent bonding and 65% of the dimer employed at the C-terminal end two signal peptides for the conjugation by SrtA-based transpeptidation.

Recycleability of rhGalT-Sepharose and rHFucT-Sepharose Evaluated by One-Pot Synthesis of the Lewis X Antigenic Derivative. One of the most important characteristics in the immobilization of the catalytic enzyme is its stability during repeated use. We demonstrated high potentials of the two immobilized recombinant glycosyltransferases, rhGalT-Sepharose and rHFucT-Sepharose, by one-pot production of a Lewis X antigenic trisaccharide derivative from Mu-GlcNAc as a starting material (Figure 7a). One-pot reaction was initiated by addition of glycosyl donor substrates (2.5 mM UDP-Gal and 2.5 mM GDP-Fuc) to the reaction mixture [50 mM Tris-HCl (pH 7.5)] containing rhGalT-Sepharose (52 μL in 1 mL of the final reaction mixture), rHFucT-Sepharose (68 μL in 1 mL of the final reaction mixture), and 0.25 mM Mu-GlcNAc (95 μg). The reaction was monitored by quantification of Mu-GlcNAc, Mu-LacNAc, and Mu-Lewis X (Figure 7b), and after 7 h, the reaction mixture was separated simply with a spin filtration column. Recovered rhGalT-Sepharose and rHFucT-Sepharose were subjected to the next trial of Lewis X synthesis under the same condition described above. During the repetitive use of these immobilized enzymes, it seems that the relative activity of rHFucT-Sepharose was reduced to be 50% within three trials, while rhGalT-Sepharose did not lose its initial activity. However, no significant loss of activity for this HFucT-Sepharose was detected during 3−10 trials, suggesting that this result might be caused by dissociation of the noncovalently bound monomer from the dimeric rHFucT immobilized by the single covalent cross-linking on EAH-Sepharose due to some mechanical stress in a buffered solution containing a high concentration of substrates and salts (Figure 7c). In this repeated trial, it was demonstrated that "milligram scale synthesis" of Mu-Lewis X trisaccharide can be performed by a total of 10 repeated uses because the theoretical yield by one cycle of reactions was estimated to be 173 μg. It should be emphasized that our strategy using immobilized glycosyltransferases is really suited to the scale-up preparation if requested for pharmaceutical or industrial purposes because many mammalian and bacterial glycosyltransferases have now been proven to be produced as desirable fusion proteins using various expression systems.

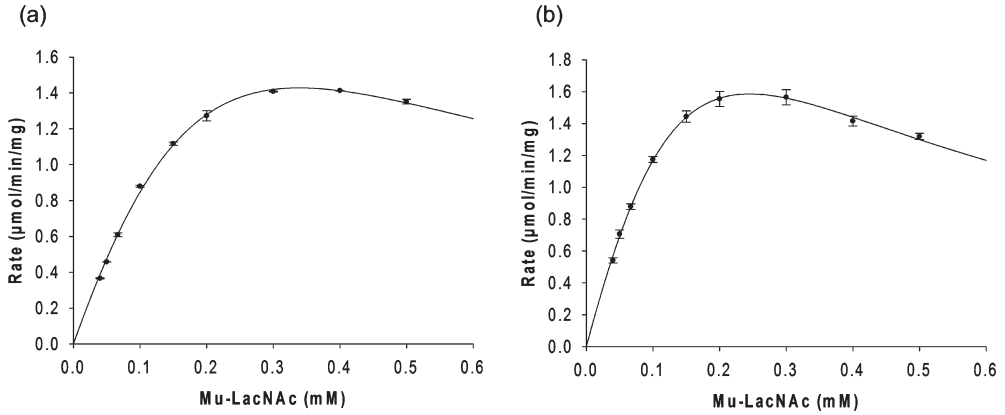


FIGURE 6: Dependence of the concentration of the glycosyl acceptor substrate (Mu-LacNAc) during the glycosylation reaction by rHFucTs: (a) soluble rHFucT and (b) immobilized rHFucT.

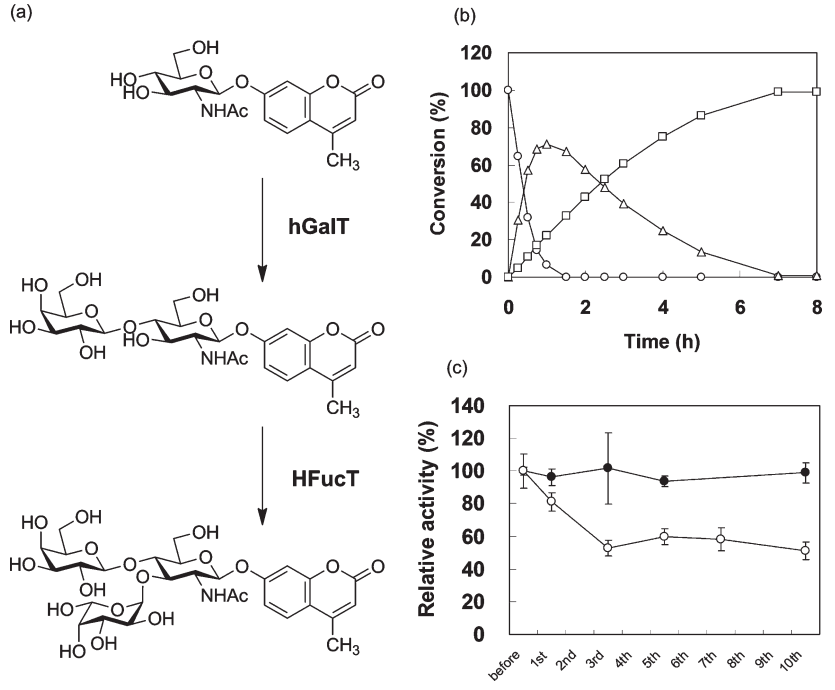


FIGURE 7: Reuseability of rhGalT and rHFucT immobilized on EAH-Sepharose by SrtA. (a) Synthesis of Lewis X by two glycosyltransferases. (b) Time course of the production of Lewis X: (○) Mu-GlcNAc, (△) Mu-LacNAc, and (□) Mu-Lewis X. (c) Enzymatic activity of immobilized glycosyltransferases during repetitive use: (●) rhGalT and (○) rHFucT.

DISCUSSION

It is not surprising that the artificial immobilization of membrane-bound glycosyltransferases is extremely difficult because various mammalian glycosyltransferases involving human β1,4GalT exhibit dynamic conformational changes during the catalytic reaction (*54*) and many bacterial glycosyltransferases such as HFucT require the formation of multimeric and highly oriented structures on the bacterial membrane (*46*, *47*, *53*). In this study, we established a general method for one-step covalent immobilization of unstable membrane-bound glycosyltransferases to solid surfaces in a site-specific manner by SrtA-based transpeptidation. Our approach can provide a highly efficient and site-specific coupling reaction of enzymes with EAH-Sepharose at their engineered C-termini without any loss of sugar transfer activity. The reuseability of the immobilized enzymes, rhGalT-Sepharose and rHFucT-Sepharose, was also demonstrated by repetitive syntheses of the Lewis X antigen (Figure 7), which is strongly expressed on the embryo stem cell surface and is also related to cellular recognition during fertilization and tumorigenesis. It is clear that the conventional chemical immobilization by using common supporting materials such as glutaraldehyde or NHS activated-Sepharose must accompany the significant loss of enzymatic activity due to nonspecific, random, and multiposition cross-linking at abundant lysine and arginine residues. Therefore, the merit of our strategy based on "site-specific" transpeptidation is evident because this reaction proceeds only at an engineered C-terminus without any conformational influence around the active site.

Reversible site-specific immobilization based on affinity interaction, such as His-Tag-Ni resin, biotin-avidin, and maltose binding protein-amylose resin (*26*−*28*, *55*, *56*), has the potential for dissociation of enzymes from supporting materials or denaturation of the affinity tag during repetitive use. Our results also indicated the disadvantage of noncovalent affinity-based conjugation in the case of the immobilized dimeric rHFucT in which one of the two C-terminal signal peptides was used for the

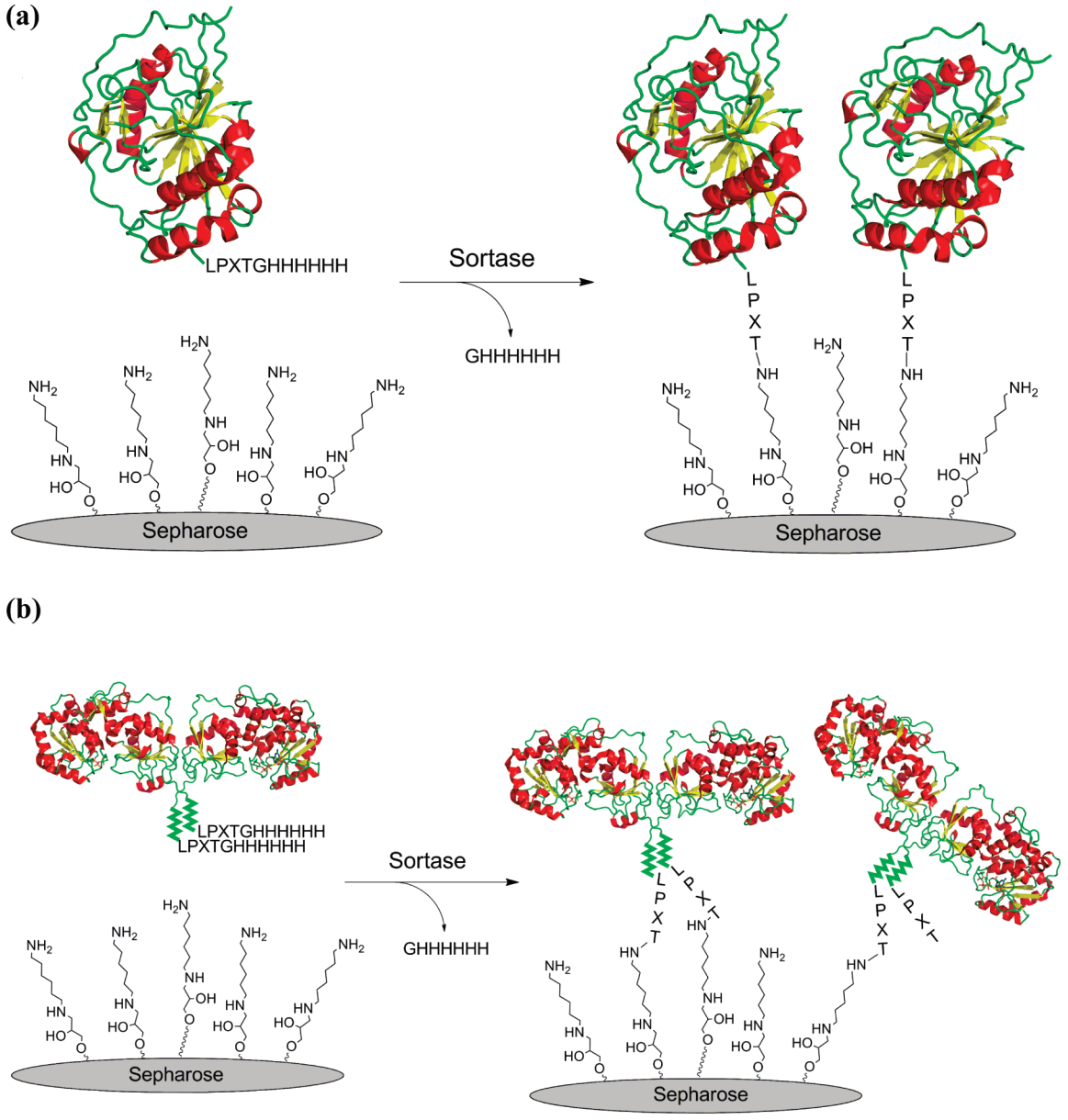


FIGURE 8: SrtA-based site-specific immobilization of rhGalT (a) and rHFucT (b).

covalent cross-linking with EAH-Sepharose (Figure 5). During the recycling experiments (n = 1−3), we may detect the significant loss of the activity due to the dissociation of the monomer under the fragile immobilization condition described above (Figure 7). Irreversible site-specific immobilization is therefore an ideal approach to the successful goal of practically recycleable immobilization of biologically important common proteins and enzymes. Actually, there are many approaches such as click chemistry and native chemical ligation, coupled with site-specific modification by intein-based methods, or the use of farnesyltransferase (*31*, *32*, *57*, *58*). Although such chemical ligation appears to show superior characteristics in terms of specificity and reactivity, these methods need the preparation of individual special linkers or tedious processes for the chemical modification of supporting materials. SrtA-mediated immobilization made possible site-specific irreversible reactions of recombinant glycosyltransferases at only the engineered C-terminus without any further modification. Moreover, we revealed that SrtA exhibited a wide range of acceptability toward simple aliphatic amines such as acyl acceptors, while acyl donors must carry strictly the LPXTG moiety in the C-terminal region (Figures 1 and 2). Although the crystal structure of *S. aureus* SrtA with the acyl acceptor has not been reported, the crystal structure of *S. aureus* SrtB (*59*), a very similar bacterial transpeptidase, with an inhibitor and acyl acceptor, triglycine, suggests tolerance in the interaction of various aliphatic amines with this acyl acceptor binding cavity (*34*, *35*). Interestingly, the ε-amino group of lysine, a native aliphatic amine structure distributed abundantly in general proteins and even SrtA itself, could become an acyl acceptor of SrtA-mediated transpeptidation (*60*−*63*). It should be noted that site-specific protein immobilization occurs predominantly in the solid surfaces when a large excess of aliphatic amino groups is displayed to prevent formation of an undesired complex between acyl donors and SrtA (Figure 4).

The versatility of the engineered enzymes immobilized on the solid surface in the modification of larger glycoproteins is under investigation, and the results will be communicated in the near future. Since the effect of "upside down" surface glycosyltransferases on the accessibility of glycoproteins might be closely related to their orientation in the Golgi apparatus, our artificial

synthetic system should provide us with insight into the mechanism of biosynthetic pathways and functional roles of membrane glycosyltransferases.

CONCLUSION

As illustrated in Figure 8, we developed a general method for the site-specific immobilization of recombinant glycosyltransferases, rhGalT and rHFucT, at an engineered C-terminal moiety. This approach allowed for highly efficient covalent immobilization of typically unstable membrane-bound enzymes without any conformational influence on its dynamic catalytic site bearing the flexible loop. It was demonstrated that a bacterial dimeric rHFucT composed of two subunits can also be immobilized successfully on the surface of solid supports in a well-oriented manner. This result indicates clearly the wide applicability of our strategy in site-directed conjugation of unstable membrane-associated proteins under a standardized protocol. These practically promising immobilized glycosyltransferases will greatly contribute to the construction of a glycan compound library (*15*, *16*, *43*) and accelerate the discovery research of potential disease-relevant biomarkers and diagnostic/therapeutic monoclonal antibodies (*64*).

ACKNOWLEDGMENT

We thank Dr. H. Hinou of Hokkaido University for critical reading the manuscript and helpful suggestions and comments.

SUPPORTING INFORMATION AVAILABLE

Lineweaver−Burk plots to determine kinetic parameters (Figures S1−S3). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Apweiler, R., Hermjakob, H., and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta 1473*, 4–8.
2. Crocker, P. R., and Feizi, T. (1996) Carbohydrate recognition systems: Functional triads in cell-cell interactions. *Curr. Opin. Struct. Biol. 6*, 679–691.
3. Kannagi, R., Izawa, M., Koike, T., Miyazaki, K., and Kimura, N. (2004) Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. *Cancer Sci. 95*, 377–384.
4. Akama, T. O., Nakagawa, H., Sugihara, K., Narisawa, S., Ohyama, C., Nishimura, S., O'Brien, D. A., Moremen, K. W., Millan, J. L., and Fukuda, M. N. (2002) Germ cell survival through carbohydrate-mediated interaction with Sertoli cells. *Science 295*, 124–127.
5. van Kooyk, Y., and Rabinovich, G. A. (2008) Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat. Immunol. 9*, 593–601.
6. Rudd, P. M., Elliott, T., Cresswell, P., Wilson, I. A., and Dwek, R. A. (2001) Glycosylation and the immune system. *Science 291*, 2370–2376.
7. Dube, D. H., and Bertozzi, C. R. (2005) Glycans in cancer and inflammation: Potential for therapeutics and diagnostics. *Nat. Rev. Drug Discovery 4*, 477–488.
8. Acosta-Serrano, A., Almeida, I. C., Freitas-Junior, L. H., Yoshida, N., and Schenkman, S. (2001) The mucin-like glycoprotein superfamily of *Trypanosoma cruzi*: Structure and biological roles. *Mol. Biochem. Parasitol. 114*, 143–150.
9. Ono, M., and Hakomori, S. (2004) Glycosylation defining cancer cell motility and invasiveness. *Glycoconjugate J. 20*, 71–78.
10. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The carbohydrate-active EnZymes database (CAZy): An expert resource for glycogenomics. *Nucleic Acids Res. 37*, D233–D238.
11. Elliott, S., Lorenzini, T., Asher, S., Aoki, K., Brankow, D., Buck, L., Busse, L., Chang, D., Fuller, J., Grant, J., Hernday, N., Hokum, M., Hu, S., Knudten, A., Levin, N., Komorowski, R., Martin, F., Navarro, R., Osslund, T., Rogers, G., Rogers, N., Trail, G., and Egrie, J. (2003) Enhancement of therapeutic protein in vivo activities through glycoengineering. *Nat. Biotechnol. 21*, 414–421.
12. Sato, M., Sadamoto, R., Niikura, K., Monde, K., Kondo, H., and Nishimura, S.-I. (2004) Site-specific introduction of sialic acid into insulin. *Angew. Chem., Int. Ed. 43*, 1516–1520.
13. Sato, M., Furuike, T., Sadamoto, R., Fujitani, N., Nakahara, T., Niikura, K., Monde, K., Kondo, H., and Nishimura, S.-I. (2004) Glycoinsulins: Dentritic sialyloligosaccharide-displaying insulins showing a prolonged blood-sugar-lowering activity. *J. Am. Chem. Soc. 126*, 14013–14022.
14. Ueda, T., Tomita, K., Notsu, Y., Ito, T., Fumoto, M., Takakura, T., Nagatome, H., Takimoto, A., Mihara, S.-I., Togame, H., Kawamoto, K., Iwasaki, T., Asakura, K., Oshima, T., Hanasaki, K., Nishimura, S.-I., and Kondo, H. (2009) Chemoenzymatic Synthesis of Glycosylated Glucagon-like Peptide 1: Effect of Glycosylation on Proteolytic Resistance and in vivo Blood Glucose-lowering Activity. *J. Am. Chem. Soc. 131*, 6237–6245.
15. Fumoto, M., Hinou, H., Ohta, T., Ito, T., Yamada, K., Takimoto, A., Kondo, H., Shimizu, H., Inazu, T., Nakahara, Y., and Nishimura, S.-I. (2005) Combinatorial synthesis of MUC1 glycopeptides: Polymer blotting facilitates chemical and enzymatic synthesis of highly complicated mucin glycopeptides. *J. Am. Chem. Soc. 127*, 11804–11818.
16. Naruchi, K., Hamamoto, T., Kurogochi, M., Hinou, H., Shimizu, H., Matsushita, T., Fujitani, N., Kondo, H., and Nishimura, S.-I. (2006) Construction and structural characterization of versatile lactosaminoglycan-related compound library for the synthesis of complex glycopeptides and glycosphingolipids. *J. Org. Chem. 71*, 9609–9621.
17. Dyal, A., Loos, K., Noto, M., Chang, S. W., Spagnoli, C., Shafi, K. V. P. M., Ulman, A., Cowman, M., and Gross, R. A. (2003) Activity of *Candida rugosa* lipase immobilized on Fe_2O_3 magnetic nanoparticles. *J. Am. Chem. Soc. 125*, 1684–1685.
18. MacBeath, G., and Schreiber, S. L. (2000) Printing proteins as microarrays for high-throughput function determination. *Science 289*, 1760–1763.
19. Nishiguchi, S., Yamada, K., Fuji, Y., Shibatani, S., Toda, A., and Nishimura, S.-I. (2001) Highly efficient oligosaccharide synthesis on water-soluble polymeric primers by recombinant glycosyltransferases immobilised on solid supports. *Chem. Commun.,* 1944–1945.
20. Ichihara, T., Akada, J. K., Kamei, S., Ohshiro, S., Sato, D., Fujimoto, M., Kuramitsu, Y., and Nakamura, K. (2006) A novel approach of protein immobilization for protein chips using an oligo-cysteine tag. *J. Proteome Res. 5*, 2144–2151.
21. Terrettaz, S., Ulrich, W. P., Vogel, H., Hong, Q., Dover, L. G., and Lakey, J. H. (2002) Stable self-assembly of a protein engineering scaffold on gold surfaces. *Protein Sci. 11*, 1917–1925.
22. Yin, J., Liu, F., Li, X., and Walsh, C. T. (2004) Labeling proteins with small molecules by site-specific posttranslational modification. *J. Am. Chem. Soc. 126*, 7754–7755.
23. Chattopadhaya, S., Tan, L. P., and Yao, S. Q. (2006) Strategies for site-specific protein biotinylation using *in vitro*, *in vivo* and cell-free systems: Toward functional protein arrays. *Nat. Protoc. 1*, 2386.
24. Ramachandran, N., Hainsworth, E., Bhullar, B., Eisenstein, S., Rosen, B., Lau, A. Y., Walter, J. C., and LaBaer, J. (2004) Self-assembling protein microarrays. *Science 305*, 86–90.
25. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Global analysis of protein activities using proteome chips. *Science 293*, 2101–2105.
26. Fujiyama, K., Ido, Y., Misaki, R., Moran, D. G., Yanagihara, I., Honda, T., Nishimura, S.-I., Yoshida, T., and Seki, T. (2001) Human N-acetylglucosaminyltransferase I. Expression in *Escherichia coli* as a soluble enzyme, and application as an immobilized enzyme for the chemoenzymatic synthesis of N-linked oligosaccharides. *J. Biosci. Bioeng. 92*, 569–574.
27. Toda, A., Yamada, K., and Nishimura, S.-I. (2002) An engineered biocatalyst for the synthesis of glycoconjugates: Utilization of β1,3-N-acetyl-D-glucosaminyltransferase from *Streptococcus agalactiae* type Ia expressed in *Escherichia coli* as a fusion with maltose-binding protein. *Adv. Synth. Catal. 344*, 61–69.
28. Nagahori, N., Niikura, K., Sadamoto, R., Taniguchi, M., Yamagishi, A., Monde, K., and Nishimura, S.-I. (2003) Glycosyltransferase microarray displayed on the glycolipid LB membrane. *Adv. Synth. Catal. 345*, 729–734.
29. Youngeun Kwon, M. A. C. J. A. C. (2006) Selective immobilization of proteins onto solid supports through split-intein-mediated protein trans-splicing13. *Angew. Chem., Int. Ed. 45*, 1726–1729.

30. Lue, R. Y., Chen, G. Y., Hu, Y., Zhu, Q., and Yao, S. Q. (2004) Versatile protein biotinylation strategies for potential high-throughput proteomics. *J. Am. Chem. Soc. 126*, 1055–1062.
31. Helms, B., van Baal, I., Merkx, M., and Meijer, E. W. (2007) Site-specific protein and peptide immobilization on a biosensor surface by pulsed native chemical ligation. *ChemBioChem 8*, 1790–1794.
32. Yu, C. C., Lin, P. C., and Lin, C. C. (2008) Site-specific immobilization of CMP-sialic acid synthetase on magnetic nanoparticles and its use in the synthesis of CMP-sialic acid. *Chem. Commun.,* 1308–1310.
33. Kalia, J., Abbott, N. L., and Raines, R. T. (2007) General method for site-specific protein immobilization by Staudinger ligation. *Bioconjugate Chem. 18*, 1064–1069.
34. Wong, L. S., Thirlway, J., and Micklefield, J. (2008) Direct site-selective covalent protein immobilization catalyzed by a phosphopantetheinyl transferase. *J. Am. Chem. Soc. 130*, 12456–12464.
35. Parthasarathy, R., Subramanian, S., and Boder, E. T. (2007) Sortase A as a novel molecular "stapler" for sequence-specific protein conjugation. *Bioconjugate Chem. 18*, 469–476.
36. Chan, L., Cross, H. F., She, J. K., Cavalli, G., Martins, H. F., and Neylon, C. (2007) Covalent attachment of proteins to solid supports and surfaces via Sortase-mediated ligation. *PLoS One 2*, e1164.
37. Clow, F., Fraser, J. D., and Proft, T. (2008) Immobilization of proteins to biacore sensor chips using *Staphylococcus aureus* sortase A. *Biotechnol. Lett. 30*, 1603–1607.
38. Kruger, R. G., Otvos, B., Frankel, B. A., Bentley, M., Dostal, P., and McCafferty, D. G. (2004) Analysis of the substrate specificity of the *Staphylococcus aureus* sortase transpeptidase SrtA. *Biochemistry 43*, 1541–1551.
39. Ton-That, H., Faull, K. F., and Schneewind, O. (1997) Anchor structure of *Staphylococcal* surface proteins. A branched peptide that links the carboxyl terminus of proteins to the cell wall. *J. Biol. Chem. 272*, 22285–22292.
40. Pritz, S., Wolf, Y., Kraetke, O., Klose, J., Bienert, M., and Beyermann, M. (2007) Synthesis of biologically active peptide nucleic acid-peptide conjugates by sortase-mediated ligation. *J. Org. Chem. 72*, 3909–3912.
41. Popp, M. W., Antos, J. M., Grotenbreg, G. M., Spooner, E., and Ploegh, H. L. (2007) Sortagging: A versatile method for protein labeling. *Nat. Chem. Biol. 3*, 707–708.
42. Antos, J. M., Miller, G. M., Grotenbreg, G. M., and Ploegh, H. L. (2008) Lipid modification of proteins through Sortase-catalyzed transpeptidation. *J. Am. Chem. Soc. 130*, 16338–16343.
43. Matsushita, T., Sadamoto, R., Ohyabu, N., Nakata, H., Fumoto, M., Fujitani, N., Takegawa, Y., Sakamoto, T., Kurogochi, M., Hinou, H., Shimizu, H., Ito, T., Naruchi, K., Togame, H., Takemoto, H., Kondo, H., and Nishimura, S.-I. (2009) Functional neoglycopeptides: Synthesis and characterization of new class MUC1 glycoprotein models having core 2-based *O*-glycan and complex-type *N*-glycan chains. *Biochemistry 48*, 11117–11133.
44. Mao, H., Hart, S. A., Schink, A., and Pollok, B. A. (2004) Sortase-mediated protein ligation: A new method for protein engineering. *J. Am. Chem. Soc. 126*, 2670–2671.
45. Shibatani, S., Fujiyama, K., Nishiguchi, S.-I., Seki, T., and Maekawa, Y. (2001) Production and characterization of active soluble human β1,4-galactosyltransferase in *Escherichia coli* as a useful catalyst in synthesis of the Gal β1→4 GlcNAc linkage. *J. Biosci. Bioeng. 91*, 85–87.
46. Sun, H. Y., Lin, S. W., Ko, T. P., Pan, J. F., Liu, C. L., Lin, C. N., Wang, A. H., and Lin, C. H. (2007) Structure and mechanism of *Helicobacter pylori* fucosyltransferase. A basis for lipopolysaccharide variation and inhibitor design. *J. Biol. Chem. 282*, 9973–9982.
47. Ma, B., Wang, G., Palcic, M. M., Hazes, B., and Taylor, D. E. (2003) C-terminal amino acids of *Helicobacter pylori* α1,3/4 fucosyltransferases determine type I and type II transfer. *J. Biol. Chem. 278*, 21893–21900.
48. Fischetti, V. A., Pancholi, V., and Schneewind, O. (1990) Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. *Mol. Microbiol. 4*, 1603–1605.
49. Ton-That, H., Mazmanian, S. K., Faull, K. F., and Schneewind, O. (2000) Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Sortase catalyzed in vitro transpeptidation reaction using LPXTG peptide and NH_2-Gly_3 substrates. *J. Biol. Chem. 275*, 9876–9881.
50. Samantaray, S., Marathe, U., Dasgupta, S., Nandicoori, V. K., and Roy, R. P. (2008) Peptide-sugar ligation catalyzed by transpeptidase sortase: A facile approach to neoglycoconjugate synthesis. *J. Am. Chem. Soc. 130*, 2132–2133.
51. Kanie, Y., Kirsch, A., Kanie, O., and Wong, C. H. (1998) Enzymatic assay of galactosyltransferase by capillary electrophoresis. *Anal. Biochem. 263*, 240–245.
52. Schneider, R., Hammel, M., Berger, E. G., Ghisalba, O., Nueesch, J., and Gygax, D. (1990) Immobilization of galactosyltransferase and continuous galactosylation of glycoproteins in a reactor. *Glycoconjugate J. 7*, 589–600.
53. Lin, S. W., Yuan, T. M., Li, J. R., and Lin, C. H. (2006) Carboxyl terminus of *Helicobacter pylori* α1,3-fucosyltransferase determines the structure and stability. *Biochemistry 45*, 8108–8116.
54. Ramakrishnan, B., Boeggeman, E., Ramasamy, V., and Qasba, P. K. (2004) Structure and catalytic cycle of β-1,4-galactosyltransferase. *Curr. Opin. Struct. Biol. 14*, 593–600.
55. Schmid, E. L., Keller, T. A., Dienes, Z., and Vogel, H. (1997) Reversible oriented surface immobilization of functional proteins on oxide surfaces. *Anal. Chem. 69*, 1979–1985.
56. Claudine Augé, A. M., Tahrat, H., Marc, A., Goergen, J.-L., Cerutti, M., Steelant, W. F. A., Steelant, Delannoy, P., and Lubineau, A. (2000) Outstanding stability of immobilized recombinant (1,3/4)-fucosyltransferases exploited in the synthesis of Lewis a and Lewis x trisaccharides. *Chem. Commun.,* 2017–2018.
57. Duckworth, B. P., Xu, J., Taton, T. A., Guo, A., and Distefano, M. D. (2006) Site-specific, covalent attachment of proteins to a solid surface. *Bioconjugate Chem. 17*, 967–974.
58. Lin, P. C., Ueng, S. H., Tseng, M. C., Ko, J. L., Huang, K. T., Yu, S. C., Adak, A. K., Chen, Y. J., and Lin, C. C. (2006) Site-specific protein modification through Cu(I)-catalyzed 1,2,3-triazole formation and its implementation in protein microarray fabrication. *Angew. Chem., Int. Ed. 45*, 4286–4290.
59. Zong, Y., Mazmanian, S. K., Schneewind, O., and Narayana, S. V. (2004) The structure of sortase B, a cysteine transpeptidase that tethers surface protein to the *Staphylococcus aureus* cell wall. *Structure 12*, 105–112.
60. Ton-That, H., and Schneewind, O. (2003) Assembly of pili on the surface of *Corynebacterium diphtheriae*. *Mol. Microbiol. 50*, 1429–1438.
61. Budzik, J. M., Marraffini, L. A., Souda, P., Whitelegge, J. P., Faull, K. F., and Schneewind, O. (2008) Amide bonds assemble pili on the surface of bacilli. *Proc. Natl. Acad. Sci. U.S.A. 105*, 10215–10220.
62. Kang, H. J., Coulibaly, F., Clow, F., Proft, T., and Baker, E. N. (2007) Stabilizing isopeptide bonds revealed in Gram-positive bacterial pilus structure. *Science 318*, 1625–1628.
63. Manzano, C., Contreras-Martel, C., El Mortaji, L., Izore, T., Fenel, D., Vernet, T., Schoehn, G., Di Guilmi, A. M., and Dessen, A. (2008) Sortase-mediated pilus fiber biogenesis in *Streptococcus pneumoniae*. *Structure 16*, 1838–1848.
64. Ohyabu, N., Hinou, H., Matsushita, T., Izumi, R., Shimizu, H., Kawamoto, K., Numata, Y., Togame, H., Takemoto, H., Kondo, H., and Nishimura, S.-I. (2009) An essential epitope of anti-MUC1 monoclonal antibody KL-6 revealed by focused glycopeptide library. *J. Am. Chem. Soc. 131*, 17102–17109.