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Protease-catalyzed synthesis of disaccharide amino acid esters in organic media

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Abstract

An enzymatic synthesis of sugar amino acid esters has been developed in pyridine by using a commercial protease, Optimase M-440, from *Bacillus licheniformis*. Optimase M-440 showed broad substrate specificity toward amino acid esters as acyl donors and disaccharides as nucleophiles. Analysis of acylation sites indicated sucrose was acylated only at three primary hydroxyls. Trehalose, consisting of two glucose units, was acylated at two primary (6-OH and 6′-OH) and one secondary (3-OH). With sucrose and trehalose, diesters along with monoesters were enzymatically synthesized. In both cases, no triester was formed. Molecular size of nucleophiles, glucose and sucrose, have effects on the extent of acylation with D-amino acids. N-blocked phenylalanine, leucine, and methionine exhibited higher activity toward sucrose than lysine, aspartic acid, and tyrosine. Various leaving groups of tBoc-L-phenylalanine were used and cyanomethyl ester gave the highest rate of reaction. Optimization of initial water activity of Optimase M-440 (A_w = 0.2–0.3) was necessary to maintain catalytic activity and to prevent undesirable hydrolysis of activated esters.

Keywords: Optimase M-440; Amino acid esters; Acylation; Subtilisin; Organic solvents

1. Introduction

Hydrolases (lipases, esterases, and proteases) are well known to catalyze reverse reactions (esterification, transesterification, and lactonization) in organic media, which are impossible in the aqueous solution [1]. The properties have been utilized to synthesize pharmaceutical intermediates [2,3] and food ingredients [4]. Drawbacks of nonaqueous enzymology, such as low activity of enzymes in organic solvents, could be overcome by various strategies [5]. Many examples of using lipases and proteases in organic synthesis have been reported in the literature.

Regioselective synthesis and modification of carbohydrate conjugates is a problematical and arduous task due to the presence of multiple hydroxyl groups [6]. Despite the attractive properties of sugar esters, their industrial uses remain limited as they are difficult to synthesize chemically. Selective acylation of a given primary or secondary hydroxyl, however, requires even more significant synthetic control via blocking and deblocking methodologies.

Nowhere is regioselectivity of enzymes potentially more important than in the synthesis of sugar esters and their derivatives [7,8]. In addition to its use as a sweetener, sucrose is a renewable resource of great commercial importance [9]. Dordick's group reported regioselective acylation of sucrose with enzymes in the synthesis of biodegradable polymers containing sucrose [10–12]. Although various studies concerning the enzymatic synthesis of vinyl sugar esters and sucrose fatty acid esters were published [13–17], there are only a few reports on the synthesis of sugar amino acid esters especially with disaccharides to the best of our knowledge [18,19]. Sugars and amino acids, along with fatty acids, are major basic components of life and are cheap renewable raw materials. Various functionalities of amino acids could be utilized to modify carbohydrates and to introduce new functional groups, which could be used as moieties for chemoenzymatic biodegradable polymer synthesis [20,21].

In a related work, Fernandez et al. were able to synthesize peptides with unnatural amino acids by using various

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proteases in the presence of organic solvents, which could be used as polymerizable monomers in free-radical polymerizations [22]. Proteases are geared in the body to catalyze the hydrolysis of peptides and proteins. Compared to other proteases subtilisin has a broad substrate specificity including aliphatic-, aromatic acids and bulky substrates like glycopeptides [23,24]. In addition, proteases are known to show relaxed stereospecificity in organic solvents enabling D-amino acids to be incorporated into peptide backbone [25]. Due to a broad substrate specificity of subtilisin, sugars, unnatural substrates for proteases, also could act as nucleophiles in transesterification with amino acid esters. In an effort to broaden the applicability of the protease-catalyzed sugar amino acid ester synthesis [26], Optimase M-440, a commercial bulk enzyme screened for synthesis of monosaccharide amino acid esters, was examined for their ability to synthesize various disaccharide amino acid esters in pyridine. Disaccharide amino acid diesters could be employed as functional monomers containing two amine groups in subsequent polycondensation reactions.

2. Materials and methods

2.1. Enzymes

Optimase M-440 (Solvay Enzyme, Elkhardt, IN, USA) was used as a catalyst without pretreatment unless stated otherwise. The enzymes are dust-free granules and contain crude preparation of subtilisin Carlsberg from *Bacillus licheniformis* (EC 3.4.21.14). When initial water activity of enzymes was a reaction variable, enzymes were adjusted to a desired water activity before reaction. Normally this was done by equilibrating at room temperature for 3 days through the vapor phase in sealed containers over P_2O_5 ($A_w = 0.00$) and saturated salt solutions. Salts used were: LiCl ($A_w = 0.11$), CH_3COOK^+ ($A_w = 0.22$), $MgCl_2$-$6H_20$ ($A_w = 0.33$), $Mg(NO_3)_2$ ($A_w = 0.52$), NaCl ($A_w = 0.75$), KNO_3 ($A_w = 0.93$). When initial water activity was not a reaction variable, Optimase M-440 was used without pretreatment.

2.2. Reagents

Sucrose, maltose monohydrate (mixed anomers α; 5%, β; 93%), trehalose dihydrate, standards of trimethylsilyl derivatives of sugars, N-protected amino acid derivatives, and 1,1,1,3,3,3-hexamethyldisilazane (Sil-A) were purchased from Sigma (St. Louis, MO, USA). D-(+)-Cellobiose and chloroacetonitrile were obtained from Tokyo Chemical Industry (Tokyo, Japan). Lactose and raffinose were from Difco Laboratories (Detroit, MI, USA). Silica gel 60 analytical plates and silica gel for column chromatography (230–400 mesh) were from Merck (Darmstadt, Germany). *N,N′*-dicyclocarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), acetone oxime and Amberlyst 15 ion-exchange resin were purchased from Aldrich (Milwaukee, WI, USA). Trifluoroacetic acid and molecular sieve (3Å, 4–8 mesh) were from Janssen Chimica (Geel, Belgium). All other chemicals and solvents used in this work were of the highest quality commercially available. Organic solvents were dried over molecular sieve and the water content of anhydrous pyridine was less than 0.01% (v/v) by Karl Fischer titration (Model 447, Coulomatic™ K-F Titrimeter®, Fischer Scientific, Bucks, AL, USA).

2.3. Analytical methods

2.3.1. Chromatography analysis

Periodically samples were withdrawn and centrifuged to remove enzymes. The solvent was evaporated, and the samples were diluted with a solvent mixture (acetonitrile:water = 60:40). Waters high-performance liquid chromatography (HPLC) system, equipped with Waters ultraviolet detector (254 nm) and μ-Bondapak C18 reverse phase column (mobile phase; acetonitrile:water = 50:50, pH 2.5 with trifluoroacetic acid), was used to monitor the disappearance of *tert*-butyloxycarbonyl-L-phenylalanine trifluoroethyl ester (tBoc-L-Phe-OTFE) and the products. Unreacted carbohydrates were analyzed on a IB-Sil NH_2 column (Phenomenex, Belmont, CA, USA) with a mobile phase consisting of a mixture of acetonitrile and water (70:30) with a refractive detector (Hitachi, Japan). The flow rate was 1.0 ml/min. Conversion was calculated from consumption of tBoc-L-Phe-OTFE or carbohydrates. In some cases, sugars also were analyzed by gas chromatography (Varian model 3300 with a 10-m Alltech AT-1 capillary column packed with polydimethylsiloxane; helium as a carrier gas, 15 ml/min; injector and detector port temperature were 300°C; the temperature increase of the column was from 250°C to 300°C at a rate of 5°C/min). Samples were derivatized with Sil-A according to a general methodology [27]. Thin-layer chromatography (TLC) was performed on Merck 60 F_{254} silica-gel-coated glass sheets with a appropriate mixture of solvents. The spots were visualized with a ultraviolet lamp and were also developed by spraying 8% phosphomolybdic acid in ethanol followed by heating at 110°C.

2.4. Product characterization

2.4.1. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) analysis

Amino acid esters chemically synthesized were dissolved in $CDCl_3$ (trimethylsilane was used as an internal standard) or dimethyl sulfoxide (DMSO)-d6 and ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Brüker AMX 200 or 300. Acylated products were dissolved in DMSO-d6 and the positions of acylation in all enzymatically prepared compounds were established by ^{13}C-NMR (Brüker AMX 500). In some cases, acylated products were also analyzed on MALDI-TOF mass spectrometry (Kratos KOMPACT MALDI-II, Manchester, UK). 2,5-dihydroxybenzoic acid

and a-cyano-4-hydroxycinnamic aid were used as the matrices.

2.5. Chemical synthesis of N-protected amino acid esters

Trifluoroethyl (TFE) esters of N-blocked amino acids were prepared by using DCC and DMAP as a coupling agent and a catalyst, respectively, according to the methods of Dhaon et al. [26,28]. Methyl and ethyl ester of tBoc-L-Phe were synthesized with Amerlyst 15 as a catalyst as reported in the literature [29].

2.5.1. tBoc-L-MetOTFE

^{13}C-NMR (DMSO-d6): δ 14.3 (s, 1C, -CH_2-CH_2-S-*C*H_3), 28.0 (s, 3C, (*C*H_3)$_3$-C), 29.5 (s, 1C, -*C*H_2-CH_2-S-CH_3), 29.9 (s, 1C, -CH_2-*C*H_2-S-CH_3), 52.3 (s, 1C, -NH-*C*H-), 59.0, 59.7, 60.4, 61.1 (q, 1C, -COO*C*H_2CF_3), 78.5 (s, 1C, (*C*H_3)$_3$-*C*-), 115.6, 120.6, 126.1, 131.6 (q, 1C,-CH_2*C*F_3), 155.6 (s, 1C, (*C*H_3)$_3$-C-OO*C*-), 171.2 (s, 1C, -*C*OOCH_2CF_3) (R_f = 0.77 with hexane:ethyl acetate = 7:4, solvent A).

2.5.2. tBoc-L-Leu-OTFE

^{1}H-NMR ($CDCl_3$): δ 0.91, 0.94 (d, 6H, -CH_2-CH-(CH_3)$_2$), 1.41 (s, 9H, tBoc), 1.43–1.53 (m, 2H, -CH_2-CH-(CH_3)$_2$), 1.60–1.73 (m, 1H, -CH_2-CH-(CH_3)$_2$), 4.20–4.44 (m, 1H, -CH-NH-), 4.50–4.63 (m, 2H, -COO-CH_2CF_3), 4.96 (d, 1H, -CH-NH-) (R_f = 0.82 with solvent A).

2.5.3. Cbz-L-Asp-OTFE (OTFE)

^{13}C-NMR (DMSO-d6): δ 35.0 (s, 1C, -CH-*C*H_2-$COOCH_2CF_3$), 50.0 (s, 1C, -*C*H-NH-), 59.8, 60.3, 60.8, 61.3 (q, 1C, -COO*C*H_2CF_3), 65.8 (s, 1C, -CH-NH-COO*C*H_2-Ar-), 127.7–136.6 (m, 5C, CH-NH-$COOCH_2$-Ar), 155.8 (s, 1C, Ar-CH_2OO*C*-NH-), 168.4 (s, 1C, -CH_2-*C*OOCH_2CF_3), 169.4 (s, 1C, -CH-*C*OOCH_2CF_3) (yield 89.3%, R_f = 0.63 with solvent A).

2.5.4. Nα, Nϵ-di-tBoc-L-lysine-2,2,2-trifluoroethyl ester (di-tBoc-L-LysOTFE)

The finely powdered dicyclohexyl ammonium salt of N-protected lysine, Nα, Nϵ-di-tBoc-L-Lys-COO dicyclohexyl ammonium (2.64 g), was added to a two-phase system of ethyl acetate (40 ml) and a solution of $KHSO_4$ (2 g in 40 ml of water). The mixture was shaken until the dicyclohexyl ammonium salt was completely dissolved. The aqueous phase was extracted with ethyl acetate (20 ml × 2). The combined organic layer was washed with water until they are free of sulfate ions, dried over anhydrous sodium sulfate, and evaporated to dryness in vacuo. The residue (free acid, 1.71 g) was dissolved in dichloromethane. DMAP (61.3 mg) and 2,2,2-trifluoroethanol (0.80 ml) were added, and the solution was cooled in an ice bath. DCC (1.016 g) was added with stirring. The purification was performed as in the synthesis of tBoc-L-PheOTFE [26].

^{1}H-NMR (CD_3OD): δ 1.32 (s, 9H, tBoc), 1.59–1.63 (m, 2H, -*C*H_2-CH_2-CH_2-CH_2-NH-), 1.66–1.70 (m, 2H, -CH_2-CH_2-*C*H_2-CH_2-NH-), 2.95–2.99 (m, 2H, -CH-*C*H_2-CH_2-CH_2-CH_2-NH-), 4.20–4.44 (m, 1H, -*CH*-NH-), 4.48–4.53 (m, 2H, -COO-CH_2CF_3), 4.77 (d, 1H, -CH_2-CH_2-CH_2-CH_2-NH-), 5.32 (d, 1H, -*CH*-NH-) (1.21 g, yield 70%, R_f = 0.84 with solvent A).

2.5.5. N-tBoc-L-phenylalanine cyanomethyl ester (tBoc-L-PheOCM)

Cyanomethyl esters of N-protected amino acids were prepared following the procedure of Schwyzer et al. [30]. A solution of tBoc-L-Phe (4.0 g) and chloroacetonitrile (2.52 ml) was cooled in an ice bath. Triethylamine (3.15 ml) was added to the solution for 30 min and maintained at room temperature overnight. White precipitates were formed and filtered. Water (10 ml) and ethyl ether (60 ml) were added, and organic phase was washed with 0.5 N HCl solution (10 ml), 10% saturated aqueous $NaHCO_3$ (10 ml) and water. The solvent was evaporated at room temperature. The purification was performed as in the synthesis of tBoc-L-PheOTFE.

^{1}H-NMR (DMSO-d6): δ 1.33 (s, 9H, tBoc), 2.97 (m, 2H, -CH_2-Ar), 4.23 (m, 1H, -CH-CH_2-Ar), 4.98 (s, 2H, -CH_2-CN), 7.25 (m, 5H, -CH_2-Ar), 7.44 (d, 1H, -NH-CH-) (4.13 g, yield 90.4%, R_f = 0.92 with solvent A).

2.5.6. tBoc-L-LeuOCM

^{1}H- NMR (DMSO-d6): H 0.83, 0.89 (d, 6H, -CH_2-CH-(CH_3)$_2$), 1.37 (s, 9H, tBoc), 1.41–1.50 (m, 2H, -CH_2-CH-(CH_3)$_2$), 1.51–1.58 (m, 1H, -CH_2-CH-(CH_3)$_2$), 3.95–4.05 (m, 1H, -*CH*-NH-), 4.98 (s, 2H, -$COOCH_2CN$), 7.36 (d, 1H, -CH-*NH*-) (R_f = 0.95 with solvent A).

2.5.7. tBoc-L-TyrOCM

^{1}H- NMR (DMSO-d6): δ 1.33 (s, 9H, tBoc), 2.83 (m, 2H, -CH_2-Ar-OH); 4.23 (m, 1H, -CH-CH_2-Ar-), 4.96 (s, 2H, -CH_2-CN), 6.64, 6.68, 7.35, 7.39 (dd, 5H, -CH_2-Ar-OH), 7.37 (d, 1H, -*NH*-CH-), 9.22(s, 1H, -CH_2-Ar-*OH*) (R_f = 0.80 with ethyl acetate).

2.5.8. N-tBoc-L-phenylalanine oxime ester (tBoc-L-PheOxime)

tBoc-L-Phe (0.796 g), DMAP (0.037 g), and acetone oxime (0.44 g) were dissolved in CHCl3 (10 ml) and the solution was cooled with stirring in an ice bath at 0°C. To the solution was added DCC (0.62 g). The white precipitate was formed after addition. The reaction was monitored by TLC. The precipitate [dicyclohexylurea (DCU)] was filtered and the solution was washed with water and saturated $NaHCO_3$ (10 ml). The solvent was evaporated at room temperature. The purification was performed with a elution solvent mixture (hexane:acetone = 9:1).

^{1}H-NMR (DMSO-d6): δ 1.33 (s, 9H, tBoc), 1.80 (s, 3H, -N=C-*cis*-CH_3), 1.93 (s, 3H, -N=C-*trans*-CH_3), 2.97 (m, 2H, -CH_2-Ar), 4.28 (m, 1H, -CH-CH_2-Ar), 7.25 (m, 5H,

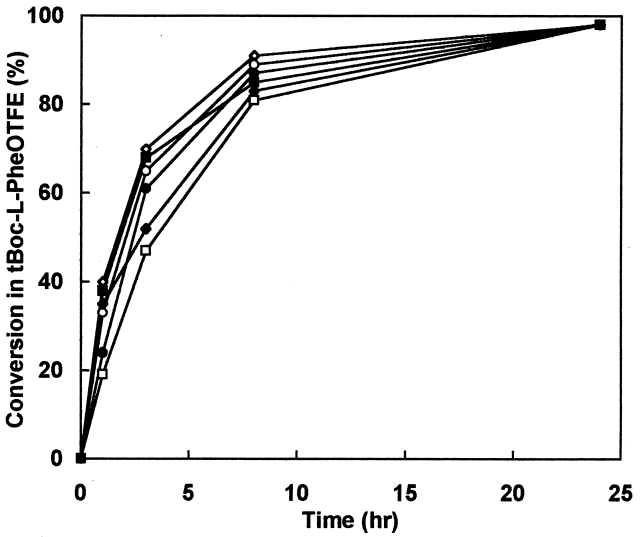

Fig. 1. Effect of the sugars on Optimase M-440 catalyzed transesterification between tBoc-L-PheOTFE and (○) sucrose, (■) lactose, (◇) cellobiose, (●) raffinose, (□) trehalose, and [24] maltose in pyridine. The concentrations of sugars and tBoc-L- PheOTFE were 0.1 M and concentration of Optimase M-440 was 100 mg/ml.

-CH_2-Ar), 7.39 (d, 1H, -*NH*-CH-) (0.514 g, yield 53%, R_f = 0.45 with solvent A).

2.6. Enzymatic synthesis of sugar amino acid esters

2.6.1. Small-scale reactions

The initial concentrations of amino acid esters and sugars were 0.1 M. The reactions were initiated upon addition of enzyme (Optimase M-440 25 mg/ml, except for Fig. 1, 100 mg/ml) to pyridine (1 ml) containing the substrates in 5-ml sealed glass vials placed in a shaking incubator with a stirring speed of 250 rev./min at 45°C. The reaction progress was monitored by TLC, HPLC, and gas chromatography analysis.

2.6.2. Large-scale reactions

To obtain diesters of disaccharides and activated amino acid esters, preparative scale synthesis of sucrose esters and trehalose esters were performed in anhydrous pyridine. Sucrose (1.03 g) and tBoc-L-PheOTFE (4.16 g) were dissolved in 50 ml of pyridine at 45°C in a 250-ml round bottom flask. The reaction was initiated upon addition of Optimase M-440 (5 g) to the reaction mixture. The reaction mixture was terminated after 8 days when more than 90% of tBoc-L-PheOTFE was consumed. The enzyme was removed by centrifugation, and the solvent was evaporated under vacuum at 60°C. The acylated products were isolated by silica gel chromatography (3 × 13 cm, 230–400 mesh) by using eluents consisting of ethyl acetate:methanol:water (18:2:1) and (24:1.0:0.5) in the stepwise manner. For trehalose esters, trehalose dihydrate (2.26 g) was more easily dissolved in 50 ml of pyridine than sucrose. Optimase M-440 (5 g) and tBoc-L-Phe-OTFE (4.17 g) was added to the trehalose solution. Silica gel chromatography (4.5 × 18 cm) was performed with an eluent consisting of ethyl acetate:methanol:water (24:1:0.5) for purification of monoesters and diesters. Pure products were pooled and checked with TLC and HPLC. Pure isolated products were dissolved in DMSO-d6 and analyzed by ^{13}C-NMR for acylation site determination.

3. Results and discussion

3.1. Reactivities of disaccharides and trisaccharide with tBoc-L-PheOTFE

In the previous study, Optimase M-440 showed a broad substrate specificity toward various monosaccharides in pyridine [26]. With glucose as a nucleophile it gave only 6-O-aminoacyl monoester. For the use of sugar esters of amino acids as monomers for polymer synthesis, it is necessary to obtain sugar amino acid diesters. Disaccharides were tested for the formation of diesters. Initially we examined the suitability of pyridine as a reaction medium for the enzymatic transesterification of disaccharides. tBoc-L-PheOTFE was chosen as acyl donor because this amino acid ester can be simply monitored by TLC and HPLC and the blocking group can be easily removed by established chemical methods (trifluoroacetic acid treatment). Small scale reactions of the disaccharides with tBoc-L-PheOTFE were performed shaking a solution of the substrates in pyridine. Lactose and cellobiose have very low solubility in pyridine. Maltose and trehalose are readily soluble. However, tBoc-L-PheOTFE reacted with disaccharides very rapidly irrespective of solubility of disaccharides. In 7 h, 80% of tBoc-L-PheOTFE reacted. TLC analysis showed several spots after 25 h for disaccharides. The phenomena probably resulted from multi-acylation on disaccharides. Among the disaccharides, maltose gave highest initial reaction rate (Fig. 1). No significant differences in initial reaction rate were observed between disaccharides and raffinose.

3.2. Regioselectivity of sucrose and trehalose acylation

Encouraged by the broad substrate specificity of Optimase M-440 toward disaccharides and the ability to acylate at multiple hydroxyls, we investigated the regiospecificity of Optimase M-440 in more detail. It is desired to establish whether subtilisin could regioselectively acylate carbohydrates larger than monosaccharides with amino acids to obtain sugar amino acid diesters. Sucrose (three primary hydroxyls and five secondary hydroxyls) and trehalose (two primary and six secondary hydroxyls) were selected as model compounds. Sucrose is an ideal model substrate for this study, both because it is highly complex and it is a commercially useful sugar. Trehalose is currently used as protective materials during freeze-drying of biological entities such as cells and pharmaceutical proteins [31,32].

Table 1
^{13}C-NMR chemical shifts (ppm)[a] of sucrose and tBoc-L-phenylalanyl sucrose esters (DMSO-d6)

Carbon	Sucrose	1′-O-ester	6-O-ester	6-O-ester	1′,6-O-ester	1′,6′-O-ester
C2′	104.0	102.2	104.4	104.1	102.1	102.4
C1	91.7	92.3	91.7	91.8	92.0	92.0
C5′	82.6	82.7	79.2	82.7	82.7	77.1
C3′	77.0	77.1	76.9	77.1	76.4	76.2
C4′	74.3	74.5	74.9	74.6	74.6	74.8
C3	72.9	72.8	72.9	72.8	73.4	73.0
C5	72.8	72.8	72.8	70.2	70.2	72.8
C2	71.6	71.6	71.7	71.6	71.2	71.4
C4	69.9	70.0	70.2	70.0	69.8	70.1
C6′	62.1	62.4	66.5	62.4	63.0	63.8
C1′	62.0	65.4	61.9	61.9	64.3	63.8
C6	60.5	60.6	60.9	62.6	62.1	60.9

[a] Assignments of acylation site according to Yoshimoto et al. [35].

Structural assignments of acylation sites were performed by ^{13}C-NMR for acylated products as described by Yoshimoto et al. [33]. The positions of acylation of sucrose mono- and diesters were assigned by comparing the chemical shifts of sucrose esters and those of the parent sucrose (Table 1). Peaks having 60–10 ppm of chemical shift are sucrose moiety, and peaks newly appearing downfield in 126–138 ppm are amino acid moiety (phenyl). Reaction of sucrose and tBoc-L-PheOTFE catalyzed by Optimase M-440 resulted in the formation of a mixture of three monoesters and two diesters. No secondary hydroxyl participated in the enzymatic acylation. In the absence of enzyme, no reaction was observed. Under the conditions of high conversion, the reaction gave rise to both monoesters and diesters. No sucrose triesters were formed. Sucrose esters were separated on TLC plates in the ascending order (6′-O-, 1′-O, 6-O-, 1′, 6′-O-, and 1′,6-O-aminoacyl sucroses). With sucrose as an acyl acceptor, all three primary hydroxyls were acylated by carboxylic acid of tBoc-L-PheOTFE (two primary hydroxyls on fructose moiety (1′OH and 6′-OH) and one primary 6-OH on glucose). These three monoesters (**3a**-) were further acylated into two diesters (1′,6-O-diester, **4a** and 1′,6′-O-diester, **4b**) (Fig. 2). The amounts of sucrose esters could not be accurately calculated because of repeated purification steps.

On the contrary, in case of trehalose, composed of two glucose moieties, both primary hydroxyl (6-OH, 6′-OH) and secondary hydroxyl (3-OH, 3′-OH) were acylated. Subsequently monoesters were then converted into their diesters, 6,6′-O-diester and 3,6′-O-diester (Table 2). As with sucrose, no triester was detected on TLC. Because of symmetry of trehalose, 3,6′-O-diester could not be distinguished from 3,6-O-diester. The ratio of diesters to monoesters was 49:51 (wt%). Both diesters were obtained in almost equal amounts (3′,6-O-diester:6,6′-O-diester = 48:52). The ratio of monoesters obtained after purification was 87:13 (6-monoester:3-monoester). MALDI-TOF mass spectrometry confirmed that the molecular weight of diesters (sucrose 1,6′-O-diester: found 836.9, calculated 836.9; trehalose 6,6′-O-diester: found 836.3, calculated 836.9). In a previous study, glucose, a monosaccharide, as a nucleophile afforded only 6-O-monoester by the same enzyme [26].

In case of carboxylic acids, sucrose was acylated at the C1′ position followed by acylation at the C6 with subtilisin BPN′ and Carlsberg [34]. Oosterom et al. reported that a lipase from *Candida antarctica* showed acylation at C6 and C6′ of sucrose and at C6 of trehalose with short-chain and long-chain fatty acids [15]. However, in the present study, when an activated amino acid ester (tBoc-L-PheOTFE), a natural substrate for a protease, was used as an acyl donor, acylation at C1′, C6, and C6′, of sucrose and at C6 and C3′ of trehalose can take place simultaneously.

3.3. Effect of initial water activity

Water activity of the reaction mixture plays an important role in the rate of catalysis as well as maintenance of the enzyme conformation in organic solvents [35]. The effect of water activity (A_w) on the reaction progress was studied. Optimase M-440 was observed to possess optimal activity in terms of conversion of sucrose in the range of A_w = 0.2–0.3 (Fig. 3). At lower A_w, the reaction rate was slow probably due to enzyme inactivation caused by water stripping by phosphorous pentoxide (P_2O_5). At higher A_w, one of the substrate, tBoc-L-Phe-OTFE, was converted into the unreactive free amino acid, tBoc-L-Phe. At A_w = 0.93, the sucrose conversion was 65% that of A_w = 0.32. These results demonstrate that initial water activity of Optimase M-440 has to be controlled to maintain catalytic activity and to prevent hydrolysis of activated esters of amino acids.

3.3.1. Comparison of reactivity of tBoc-L-, D-Phe-OTFE with sucrose and glucose

From the previous study [26] and above results, Optimase M-440 could accept mono-, di-, and trisaccharides as nucleophiles. Raffinose (trisaccharide) was acylated with L-amino acids as effectively as sucrose. Sugars could be

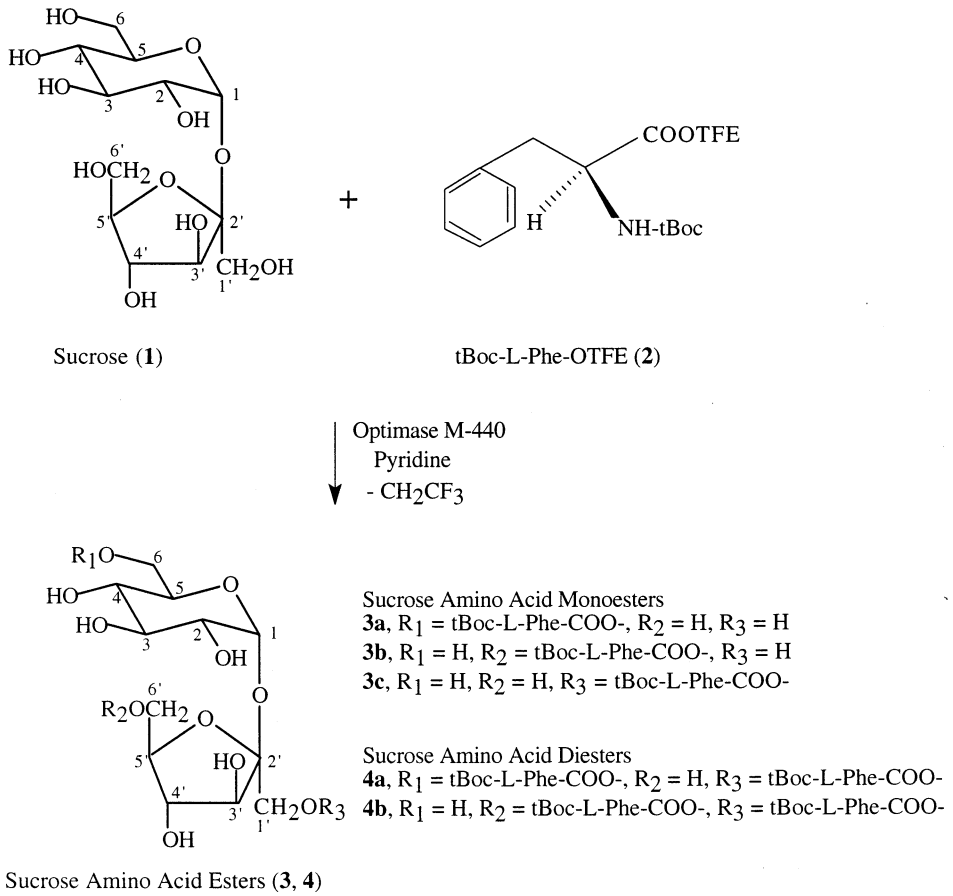

Fig. 2. Schematic diagram of Optimase M-440-catalyzed regioselective synthesis of tBoc-L-phenylalanyl sucrose esters in pyridine.

used as acyl acceptors for Optimase M-440 catalyzed transesterification with activated amino acid esters. Then, we undertook the study of effects of absolute configuration of acyl donors in the acylation of sucrose and glucose in pyridine. Glucose is a monosaccharide and smaller in molecular size and has fewer hydroxyls than sucrose. With both nucleophiles, tBoc-D-PheOTFE gave much lower conversion than tBoc-L-PheOTFE (data now shown). When tBoc-D-PheOTFE was employed as an acyl donor, glucose showed about 20% conversion in 10 h, whereas sucrose gave almost no significant conversion even after longer incubation. In order to rationalize these results, we assumed that the configuration of tBoc-D-Phe-COO-enzyme complex influenced the position of sucrose and glucose in the active site of Optimase M-440 [36]. Because sucrose is bulkier than glucose and less likely to be accommodated in the

Table 2
^{13}C-NMR chemical shifts (ppm)[a] of trehalose and tBoc-L-phenylalanyl trehalose esters(DMSO-d6)

Carbon	Trehalose	6-O-ester	3-O-ester	3,6′-O-ester	6,6′-O-ester
C1	93.1	93.5	93.4	93.5	93.7
C1′	93.1	93.4	93.1	93.3	93.7
C3	72.9	72.9	76.6	76.5	72.8
C3′	72.9	72.9	72.7	72.7	72.8
C5	72.4	69.7	72.2	72.5	69.7
C5′	72.4	72.6	72.2	69.5	69.7
C4	70.1	70.2	67.6	67.6	70.2
C4′	70.1	70.2	70.1	69.8	70.2
C2	71.6	71.6	69.5	69.3	70.2
C2′	71.6	71.4	72.2	71.4	71.4
C6	60.7	64.1	60.7	60.2	64.1
C6′	60.7	60.8	60.7	64.1	64.1

[a] Assignments of acylation site according to Yoshimoto et al. [35].

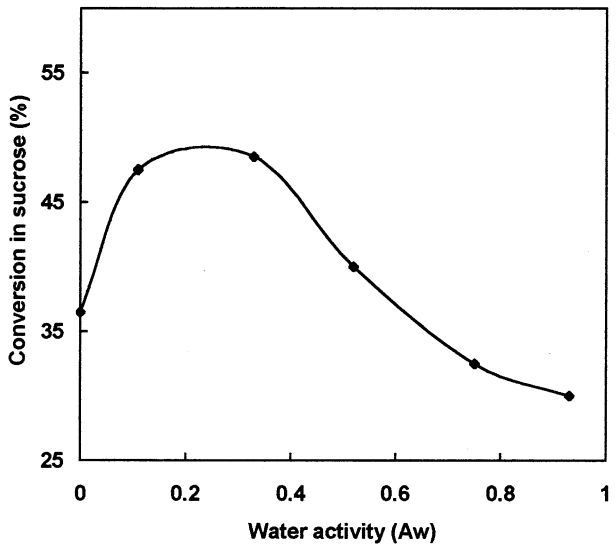

Fig. 3. Effect of initial water activity of Optimase M-440 on conversion of sucrose in Optimase M-440 catalyzed transesterification between sucrose and tBoc-L-PheOTFE in pyridine. The concentration of sucrose and tBoc-Phe-OTFE were 0.1 M and the reactions were performed over 24 h. The concentration of Optimase M-440 was 25 mg/ml.

active site, the conversion of tBoc-D-PheOTFE was much lower in case of sucrose than glucose.

3.3.2. Transesterification with different acyl donors

Five esters of N-protected phenylalanine—methyl (Me), ethyl (Et), oxime, cyanomethyl (CM), and trifluoroethyl (TFE) ester—were chemically synthesized and employed as acyl donors along with free amino acid (tBoc-L-Phe) for transesterification of sucrose. Cyanomethyl-, oxime-, and TFE esters exhibited higher initial reaction rates and conversion of sucrose (12 h) than methyl-, ethyl ester, and free amino acid (Table 3). Activated amino acid esters such as TFE, CM, and oxime esters seem to be required as acyl donors in acylation of carbohydrates, whereas in enzymatic peptide synthesis, methyl and ethyl esters are commonly used.

Table 3
Effects of different leaving groups of N-protected phenylalanine on Optimase M-440 catalyzed transesterification reactions between sucrose and amino acid esters[a]

N-tBoc-L-Phe-OX	Initial reaction rate (μmol/h per ml)	Conversion (12 h) (%)
tBoc-L-Phe	0.3	2.0
tBoc-L-PheOMe	1.0	5.5
tBoc-L-PheOEt	4.5	11.0
tBoc-L-PheOxime	13.3	40.2
tBoc-L-PheOCM	21.6	42.8
tBoc-L-PheOTFE	19.0	43.9

[a] The concentrations of sucrose and amino acid esters were 0.1 M and enzyme concentration was 25 mg/ml. The initial reaction rates and conversion were calculated from the consumption of amino acid esters. Experiments were performed as described in Section 2.

Table 4
Effects of different amino acid residues on the enzymatic synthesis of sucrose esters by transesterification between sucrose and amino acid esters[a]

N-protected amino acid esters	Initial reaction rate (μmol/h per ml)	Conversion (10 h) (%)	Conversion (24 h) (%)
tBoc-L-LeuOCM	14.0	41.6	56.3
tBoc-L-LeuOTFE	11.6	42.1	54.6
tBoc-L-PheOTFE	12.6	42.9	54.2
tBoc-L-TyrOCM	5.6	9.0	31.3
tBoc-L-MetOTFE	19.0	58.3	59.6
Cbz-L-AspOTFE	5.6	12.0	31.2
di-tBoc-L-LysOTFE	5.0	12.1	22.9

[a] The concentrations of sucrose and activated amino acid esters were 0.1 M and enzyme concentration was 25 mg/ml. The initial reaction rates and conversion were calculated from the consumption of sucrose. Experiments were performed as described in Section 2.

3.3.3. Effect of kinds of amino acid esters

Conventionally, fatty acids were employed for the enzymatic synthesis of biosurfactants such as sucrose esters. However, fatty acids cannot give additional functionality. With amino acids, there are several additional functionalities in the side chain. The synthesis of polyesters containing sucrose and amino acids requires a minimum of two hydroxyl groups in the sucrose to be linked with two amino acids for use as monomer in polycondensation reactions. This prompted us to investigate whether binding of amino acids at the active site of Optimase M-440 and reactivity of acylation would also be dependent on the structure of amino acid side chain. Initially we prepared various activated esters of amino acids chemically. Among the amino acids, methionine, leucine, and phenylalanine of hydrophobic nature was favored over basic (lysine) and acidic (aspartic acid) amino acids (Table 4). Interestingly, tyrosine, a good substrate in enzymatic peptide synthesis, exhibited lower reaction rate than amino acids of similar structure like phenylalanine. Hydroxyl at aromatic ring could give steric hindrance in the transesterification.

4. Conclusions

In summary, various disaccharides could be acylated with various amino acid esters in pyridine by a commercial enzyme, Optimase M-440. Analysis of acylation site indicated sucrose was acylated only at primary hydroxyls. Trehalose of two glucose units was acylated both at one primary hydroxyl (6-OH) and one secondary hydroxyl (3-OH). 6-OH was preferentially acylated than 3-OH. Acylation of disaccharides (sucrose and trehalose) with activated amino acid ester, tBoc-L-PheOTFE resulted in the formation of diesters, which are appropriate monomers for polymerization. No triesters of sucrose and trehalose were formed under the reaction conditions. Optimization of synthesis of sucrose amino acid esters was performed in terms of water

activity, leaving groups of amino acid esters, and different amino acid residues. The study on applications of sugar amino acid diesters as monomers in polycondensation reaction is in progress.

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