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Selectivity and stability of alkaline protease AL-89 in hydrophilic solvents

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ABSTRACT

The alkaline protease from *Bacillus pseudofirmus* strain AL-89 used vinyl fatty acid esters of increasing chain length from C10 to C18 equally well as substrates for esterification of sucrose in a reaction mixture of DMF and DMSO (1:1, v/v). The synthesized esters were purified and characterized by NMR and nano-electron spray MS. As evaluated by the initial reaction rates, the primary site of substitution of sucrose was at the C-2 position with the C-3 and C-3′ as secondary substitution sites. The enzyme catalysed the formation of 3-*O*-acyl sucrose from 2-*O*-acyl sucrose. The investigation did not reveal if the 3′-*O*-acyl sucrose was formed the same way. The synthesis of the 2-*O*-esters showed the characteristics of kinetically controlled reactions, whereas the formation of the 3-*O*- and 3′-*O*-esters showed the characteristics of equilibrium controlled reactions. The enzyme catalysed process was effected by initial water content, substrate molar ratio and reaction temperature. Under the reaction conditions of 0% initial water content, a molar ratio of sucrose to vinyl stearate of 1:1.5 and 70 °C an initial formation rate of 13.5, 2.9 and 2.1 μmol min^{-1} was achieved for 2-*O*-, 3-*O*- and 3′-*O*-stearoyl sucrose respectively with a specific initial synthesis rate of 2-*O*-stearoyl sucrose of 0.27 μmol min^{-1} mg^{-1} biocatalyst. In the absence of substrates the enzyme proved to be more stable in DMF than in water and DMSO at 50 °C. Mixing DMF with DMSO 1:1 (v/v) increased the stability and the half-life was found equal to that in water. In the presence of substrates a residual activity of 40% was observed after 24 h of incubation in the 1:1 (v/v) mixture of DMF and DMSO at 70 °C.

1. Introduction

Sugar fatty acid esters synthesized by enzymatic methods in organic solvents have been extensively investigated for the production of non-toxic, biodegradable surface active agents, some of which have shown antimicrobial properties [1]. In particular, lipases and proteases have successfully been applied as biocatalysts for these processes [2–4] where the solubility of the substrates, the activity and stability of the enzyme is influenced by the reaction medium. Aprotic, hydrophilic solvents such as DMSO are excellent solvents for carbohydrates but are at the same time protein denaturants affecting enzyme activity and stability significantly. Particularly lipases are sensitive to increased concentrations of hydrophilic solvents, loosing activity and exhibiting complete inactivation at DMSO concentrations of 20–30% [5–7]. Alkaline proteases, however, have shown improved catalytic properties both with regard to activity and enantio-selectivity at DMSO concentrations of 54–56% (v/v) [8]. Subtilisin is one of the best studied alkaline proteases as biocatalyst in hydrophilic solvents. Almarsson and Klibanov [9] demonstrated that DMSO in concentrations of 7.5–35% (v/v) improved activity of this protease increasing the esterification rate of 1-propanol with *N*-acetyl-L-phenylalanine ethyl ester up to 98-fold depending on the co-solvent. Regarding synthesis of sugar fatty acid esters, subtilisin has shown specificity for short chained acyl donors and regio-selectivity for the C-1′ and C-6 position of sucrose in anhydrous DMF as well as in a mixture of buffer and DMSO [10–12]. A completely different regio-selectivity was discovered with the alkaline protease AL-89 which catalysed the acylation of the C-2 position of sucrose using a medium chained acyl donor in a mixture of DMF and DMSO [13]. In the present paper, the stability and catalytic properties of the alkaline protease AL-89 in hydrophilic solvents is investigated further. In particular, the substrate specificity for vinyl fatty acid esters of increasing chain length and the regio-selectivity regarding acylation of sucrose was studied.

2. Materials and methods

2.1. Materials

Alkaline protease produced from *Bacillus pseudofirmus* strain AL-89 was used as a source of biocatalyst [14]. Celite (diatomaceous earth, 30–80 mesh), molecular sieves (3 Å, 8–12 mesh) and trichloroacetic acid (TCA) were purchased from Merck. Vinyl

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fatty acid esters of capric-, myristic- and palmitic acid were kindly donated by Japan Vam and Poval Co. (Osaka, Japan), vinyl laurate was purchased from Fluka. Vinyl stearate, sucrose, *n*-dodecyl-β-D-maltoside, azocasein, DMF and DMSO was supplied by Sigma–Aldrich. Acetronitrile (HPLC grade) was from Fisher Scientific. All other solvents and reagents were of analytical grade. The solvents and reactants used in synthetic reactions were stored over molecular sieves, minimum for 24 h prior to use.

2.2. Enzyme preparation

B. pseudofirmus strain AL-89 producing alkaline protease was cultivated as previously described [14]. The biomass was removed by centrifugation at 3000 × *g* for 20 min. The protease was precipitated from the cell free culture supernatant with 60% (w/v) ammonium sulphate and centrifuged at 6000 × *g*. The precipitate was then dissolved in 10 mM Tris–HCl buffer pH 8.0 and dialyzed against 10 mM sodium carbonate buffer pH 10.0 overnight. After dialysis, the frozen enzyme solution was lyophilized.

2.3. Proteolytic activity

Proteolytic activity was measured in aqueous medium at timed intervals of incubation at 60 °C with 0.5% azocasein in 20 mM Glycine-NaOH buffer pH 10.0 as a substrate according to Gessesse et al. [15]. One unit of activity was defined as the amount of enzyme causing an increase in absorbance at 440 nm against a reagent blank, $\Delta A_{440} = 0.01$.

The enzyme preparation used in the present investigation had a specific proteolytic activity of 1600.8 units mg^{-1}.

2.4. Residual activity

The residual proteolytic activity in aqueous medium of the alkaline protease AL-89 was investigated as function of incubation time in hydrophilic solvents in the absence and the presence of substrates, respectively at an enzyme concentration of 10 g L^{-1}. For the experiments 25 mL serum flasks were placed in a stirring block thermostat with magnetic stirring (Variomag 15.5, thermomodule 40) at 250 rpm (for description of the system, see Section 2.5). All experiments were performed in duplicates.

In the absence of substrates the lyophilized protease AL-89 was dissolved in 2 mL solvent respectively: water (MilliQ), DMF, DMSO and a mixture of DMF and DMSO (1:1, v/v) and incubated at 50 °C for 1 h. Samples were periodically withdrawn and the proteolytic activity in aqueous buffer was determined as described in Section 2.3.

In the presence of substrates incubation of the enzyme was performed in a mixture of DMF and DMSO (1:1) with sucrose (0.2 M) and vinyl stearate (0.3 M) for 24 h at 60 °C and 70 °C, respectively. Prior to incubation all substrates were dissolved in DMF:DMSO (1:1, v/v) containing molecular sieves and equilibrated for at least 24 h. 5 mL of this mixture was transferred to a 25 mL serum flask and lyophilized protease AL-89 (10 g L^{-1}) and molecular sieves (10 g L^{-1}) were then added. Aliquots were periodically withdrawn and immediately analyzed for proteolytic activity as described in Section 2.3.

2.5. Transesterification reaction

The esterification of sucrose using vinyl fatty acids as acyl donors was carried out in a reaction medium of DMF and DMSO (1:1, v/v) in the presence of 10 g L^{-1} molecular sieves. The reactions were carried out in 25 mL serum flasks containing teflon coated magnetic stirring rods and sealed with teflon coated rubber stoppers equipped with an injection needle and syringe for sample withdrawal. The serum flasks were placed in a stirring block thermostat with magnetic stirring (Variomag 15.5, thermomodule 40) at 250 rpm. The reaction volume was 5 mL and reaction mixture was composed of 0.2 M sucrose, 10 g L^{-1} alkaline protease AL-89 lyophilized preparation while the concentration of the acyl donor was varied as described below in this section. A control experiment replacing the enzyme with 10 g L^{-1} preparation of Celite equilibrated in 10 mM sodium carbonate buffer pH 10 and vacuum dried, and 10 g L^{-1} preparation of casein, dissolved in the same buffer and lyophilized, was performed respectively, in parallel with enzymatic reaction. All experiments were performed in duplicates and controls consisting of reaction mixture without enzyme were included. To monitor the progress of the reactions, 200 μL aliquots were periodically withdrawn, centrifuged at 3100 × *g* and the supernatant was analyzed by TLC and HPLC.

The effect of initial water content on initial reaction rates was investigated under the reaction conditions described above using vinyl stearate as acyl donor (0.2 M) at 45 °C. The water content of the reaction mixture was regulated by the addition of water from 0% to 10% (v/v).

The effect of substrate molar ratio was investigated by varying the initial vinyl stearate concentration from 0.2 M to 1.0 M maintaining the initial sucrose concentration at 0.2 M. The reactions were performed without water added at 45 °C.

The effect of temperature was investigated at a vinyl stearate concentration of 0.3 M and without water addition, varying the temperature from 40 °C to 80 °C.

The substrate specificity for vinyl fatty acid esters of increasing chain length was investigated at 60 °C and an acyl donor concentration of 0.2 M with vinyl fatty acid esters varying in chain length from C10 to C18.

2.6. Formation of 3-O-caproyl sucrose

Purified 2-*O*-caproyl sucrose was dissolved in a DMF:DMSO (1:1, v/v) to a final concentration of 0.96 mM and incubated at 60 °C and 250 rpm for 24 h in the absence and the presence of enzyme, respectively. The experiment was run in 1.5 mL Eppendorf tubes in volumes of 0.5 mL using a thermomixer (Eppendorf 5436). To the tube with enzyme, 10 g L^{-1} lyophilized alkaline protease AL-89 was added. Samples were periodically withdrawn at timed intervals and analyzed by RP-HPLC as described in Section 3.2.

3. Analytical procedures

3.1. TLC analysis

The reaction mixture (5 μL) was qualitatively analyzed by thin layer chromatography (TLC) using silica gel Plate 60 F_{245} (Merck) with chloroform:methanol (5:1, v/v) as mobile phase. The spots were visualized by applying vaporised sulphuric acid:methanol (1:1, v/v) to the plate followed by heating (150 °C, 5 min).

3.2. HPLC analysis

Substrate and product concentrations of the reaction medium were determined by HPLC (HP 1100 system) equipped with a Chromolith Performance RP-18e column (100 mm length, 4.6 mm diameter, Merck) and Evaporative Light Scattering Detector (ELSD, Scantec 900). The column temperature was 45 °C and the detector was operated at 3.0 bar of N_2 gas and a drift tube temperature of 50 °C. The samples were appropriately diluted with methanol containing 1 g L^{-1} *n*-dodecyl-β-D-maltoside as internal standard.

Table 1
RP HPLC mobile phase gradient for analyses of sucrose fatty acid esters.

Time (min)	% acetonitrile
(a) Used for esters of fatty acid chain lengths C10 and C12	
0	20
1	20
7	55
8.5	100
12	100
(b) Used for esters of fatty acid chain lengths C14–C18	
0	50
1	50
8	80
8.5	100
12	100

Sucrose monoester products were quantified based on internal standard concentration. The injection volume was 10 μL and the mobile phase was acetronitrile and water, the flow rate was 2 mL min^{-1}. Depending on the fatty acid chain length of the acyl donor two different the gradient programmes were used, see Table 1.

3.3. Purification and characterization of sucrose esters

2 mL of reaction mixture was subjected to preparative thin layer chromatography (TLC) using 0.5 mm silica gel plate (Analtech) with chloroform:methanol (5:1, v/v) as a mobile phase. 5 cm of the TLC plate was cut off and used for product detection. Silica gel with sucrose ester products was scraped off the TLC plate and ground into fine particles. The product was extracted with chloroform:methanol (1:1, v/v) three times at 60 °C under stirring. The extraction process and product purity were monitored by TLC and HPLC. After extraction, solvents were then evaporated under vacuum. The molecular weight and structure of the purified products were elucidated by nano-electron spray mass spectrometry and ^{1}H and ^{13}C NMR, respectively.

3.4. *Nano-electron spray mass spectrometry analysis*

The purified sucrose fatty acid esters were analyzed by nano-electron spray mass spectrometry. The samples were diluted in 50% methanol and 1% formic acid and analyzed using a hybrid QTOF mass spectrometer (MicroTOFq, Bruker Daltronics, Bremen, DE) using offline nanoelectrospray emitters (Proxeon Biosystems, Odense, Denmark). The mass spectra were recorded in positive ion mode.

3.5. NMR analysis

The position of acylation of the purified sucrose esters was determined by ^{1}H NMR and ^{13}C NMR. All NMR spectra were recorded at 298 K on a BRUKER DRX600 NMR spectrometer equipped with a triple-gradient TXI (H/C/N) probe. Spectra were recorded with TopSpin 1.3b16 and interpreted with CARA/NEASY. Assignments were based on DQF-COSY and multiplicity-edited HSQC spectra.

4. Results and discussion

4.1. Protease stability

The residual proteolytic activity in aqueous medium of alkaline protease AL-89 was investigated as function of incubation time in hydrophilic solvents as well as in water at 50 °C in the absence of substrates: during the first 10–20 min of incubation the loss of activity followed exponential decay at $R^2 = 0.9$ in all solvents (see Fig. 1). The protease proved to be more stable in DMF than in pure water with half-lives of $t_{d(1/2)\ DMF}$: 10 min and $t_{d(1/2)\ water}$: 4 min. However, the fastest deactivation was observed in DMSO ($t_{d(1/2)\ DMSO}$: 1 min). Mixing DMSO with DMF 1:1 (v/v) stabilised the activity compared to pure DMSO and the half-life was found equal to that in water.

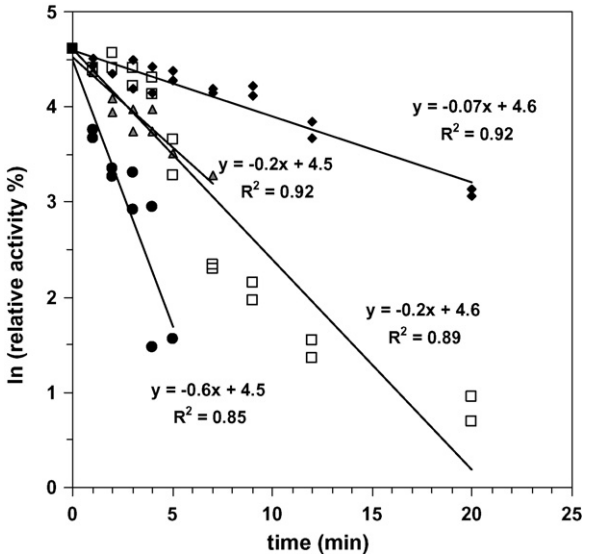

Fig. 1. Residual proteolytic activity of alkaline protease AL-89 after incubation in hydrophilic solvents at 50 °C. DMF(♦), water (□), DMF/DMSO mixture (▲), DMSO (●) Half-life: $t_{d(1/2)\ DMF}$: 10 min, $t_{d(1/2)\ water}$: 4 min, $t_{d(1/2)\ DMF/DMSO}$: 4 min, $t_{d(1/2)\ DMSO}$: 1 min. The lyophilized enzyme powder (10 gL^{-1}) was dissolved in the solvent and incubated at 50 °C under agitation 250 rpm.

In the presence of substrates the enzyme maintained 40% of its original activity even after 24 h of incubation in the 1:1 (v/v) mixture of DMSO and DMF at 70 °C (data not shown).

The conformation and dynamics of secondary structural elements of the alkaline serine protease, subtilisin Carlsberg in 100% DMSO was studied by Fourier-transform infrared (FTIR) spectroscopy and suggested on one hand random coil formation at room temperature due to complete unfolding [16]. On the other hand, formation of aggregation or intermolecular β-sheets at 30 °C resulting in very low catalytic activity has been reported [17]. At DMSO concentrations of 54–56% only a partial destruction of the tertiary structure of subtilisin was observed by FTIR and circular dichroism (CD). This structural change induced an enhanced conformational flexibility of the enzyme resulting in an increase in both hydrolytic activity and enantioselectivity towards (S)-ethyl-2-(4-substituted phenoxy) propanoates.

DMSO (25%, v/v) had a stabilising effect on α-chymotrypsin and two alkaline proteases, subtilisin Carlsberg and *Pseudomonas aeruginosa* protease PST-01. In fact the latter proved to be extremely stable at 30 °C in DMSO and a range of alcohols showing activity half-lives longer than 50 days. DMF destabilised both α-chymotrypsin and thermolysin but had a stabilising effect on the alkaline proteases, subtilisin Carlsberg in particular [8]. Ghorbel et al. [18] found that the *Bacillus cereus* BG1 protease was more stable at 30 °C in DMSO (25%, v/v) than in buffer (100%) and DMF (25%, v/v) showing half-lives of 50, 40 and 35 days, respectively.

Our results showed that the alkaline protease AL-89 was more stable in DMF (100%) than in water at 50 °C while DMSO (100%) de-activated the protease significantly at this temperature.

In the presence of substrates a residual activity of 40% was observed after 24 h of incubation in a mixture of DMF and DMSO (1:1, v/v).

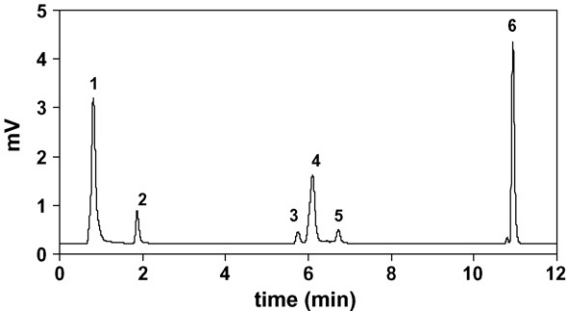

Fig. 2. HPLC-chromatogram of reaction mixture of the protease AL-89 catalysed process. RP C18 chromolith column (45 °C), ELSD (50 °C, N_2 3.0 bar), mobile phase: water and acetonitrile, flow: 2 mL min^{-1}. Peak no 1: sucrose, 2: *n*-dodecyl-β-D-maltoside (internal standard), 3: 3′-*O*-stearoyl-sucrose, 4: 2-*O*-stearoyl-sucrose, 5: 3-*O*-stearoyl-sucrose, 6: vinyl stearate.

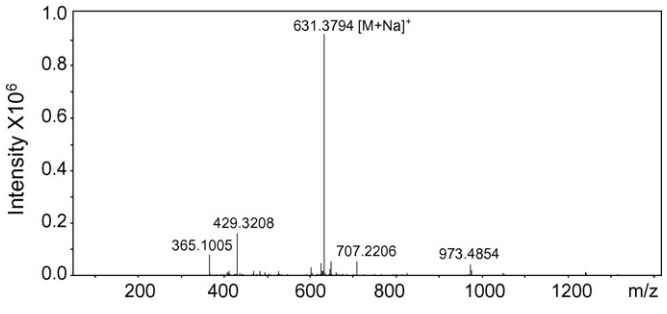

Fig. 3. Mass spectrum of purified 2-*O*-stearoyl sucrose. Masses below 500 *m*/*z* are solvent related.

4.2. Synthesis of sucrose stearate

Transesterification of sucrose with vinyl stearate in DMF:DMSO (1:1, v/v) was catalysed by the alkaline protease AL-89 and the catalytic properties were compared to a non-enzymatic catalyst, Celite and a non-catalytic protein, casein. With Celite and alkaline protease AL-89 respectively, similar product profiles were obtained after 24 h of reaction as analyzed by TLC and HPLC, showing that also Celite equilibrated at alkaline pH catalyzes sucrose stearate formation. No product formation was observed in the presence of casein. The alkaline protease AL-89 catalysed reaction was very specific towards formation of monoesters as three monoesters were detected in the reaction mixture. The main product was 2-*O*-stearoyl sucrose with 3-*O*-stearoyl sucrose and 3′-*O*-stearoyl sucrose present in lower amounts as identified by NMR and HPLC analyses. A chromatogram of the reaction mixture is shown in Fig. 2.

The monoesters were purified by preparative TLC and analyzed by ^{1}H and ^{13}C NMR (Table 2). The ^{1}H–^{13}C HSQC spectrum of each fatty acid ester was compared to the ^{1}H–^{13}C HSQC spectrum of sucrose. A significant difference was observed in the chemical shift at $\delta(^1\text{H};\ ^{13}\text{C}) = (4.65;\ 72.3)$ stemming from the H2/C2 atoms due to the presence of the acyl substitution at the C-2 position. In the 3-*O*-ester the substitution effected the shift at $\delta(^1\text{H};\ ^{13}\text{C}) = (5.20;\ 75.9)$ stemming from the H3/C3 atoms. On the inverse-detected carbon spectra measured, the C2′ carbon atom was not showing up because it is not bearing a hydrogen substituent.

Table 2
Chemical shifts (δ, ppm) of sucrose and its esters in chloroform/methanol 1:1 (v/v). On the inverse-detected carbon spectra measured, the C2′ carbon atom was not showing up because it is not bearing a hydrogen substituent.

Atom	Sucrose	2-*O*-ester	3-*O*-ester	3′-*O*-ester
C1	92.8	90.1	92.9	92.8
C2	72.3	73.5	70.7	72.3
C3	73.9	71.2	75.9	74.0
C4	70.7	71.0	68.9	70.4
C5	73.9	73.7	73.8	73.9
C6	61.8	61.9	61.6	61.8
H1	5.41	5.55	5.45	5.41
H2	3.48	4.65	3.62	3.48
H3	3.72	3.89	5.20	3.65
H4	3.36	3.43	3.51	3.39
H5	3.84	3.89	3.92	3.84
H6a	3.73	3.73	3.74	3.73
H6b	3.83	3.86	3.84	3.83
C1′	64.1	63.1	64.0	65.0
C3′	79.6	77.3	79.4	79.5
C4′	74.7	74.2	74.8	72.7
C5′	83.0	82.6	83.0	83.3
C6′	62.0	62.1	62.3	62.0
H1′a	3.68	3.54	3.69	3.66
H1′b	3.61	3.41	3.63	3.59
H3′	4.07	4.18	4.10	5.29
H4′	4.08	4.05	4.08	4.30
H5′	3.80	3.76	3.81	3.92
H6′a	3.79	3.80	3.79	3.79
H6′b	3.73	3.73	3.75	3.73

The mass of the sodium adducts of the purified sucrose stearate monoesters were determined by mass spectrometry as 631.3794 Da (see Fig. 3 and Table 4) which subtracted by the mass of sodium corresponded to the theoretical mass of sucrose stearate.

The catalytic efficiency of Celite equilibrated at alkaline pH and alkaline protease AL-89 was compared. The esterification rate in the presence of protease was comparatively faster in all reactions investigated (see Table 3). Compared to the reaction with Celite (10 g L^{-1}), the initial rate of sucrose stearate synthesis in the presence of 10 g L^{-1} alkaline protease AL-89 was increased 162-fold for 2-*O*-stearoyl sucrose, 120-fold for 3-*O*-stearoyl sucrose and 110-fold for 3′-*O*-stearoyl sucrose, revealing a preference of the protease for the C-2 position. All three monoesters were synthesized from the start of the protease catalysed process but the initial rate of 2-*O*-ester formation was 4.5 and 7.4 times faster than the formation of the 3-*O*- and the 3′-*O*-ester, respectively.

With subtilisin as biocatalyst Polat et al. [19] reported the synthesis of sucrose stearate with acylation taking place at the primary hydroxyl group of the C-1 position with a 15% product yield in anhydrous pyridine. Adding trace amounts of DMSO 1′,6-di-*O*-stearoyl-sucrose was observed as a minor product, while no product formation was found using 100% DMSO as reaction medium. 3-*O*-acyl sucrose was synthesized, via the dibutylstannylene acetal intermediate by chemical reaction with di-*n*-butyline oxide and fatty acid (C12–C18) anhydrides as acyl donors in DMF, in yields of 2–5% after prolonged the reaction time for 3 days [20]. Using butyl lithium as catalyst in DMF 6-*O*-lauroyl sucrose was obtained as the primary product with cyanoethyl-, methylthioethyl- and methyl esters as acyl donors, respectively [21], whereas *N*-acylthiazolidinethiones afforded the formation of 2-*O*-acyl sucrose as the major product [22]. With triethylamine as a basic catalyst in DMF and 3-*N*-stearoyl-thiazolidinie-2-thione as acyl donor at 40 °C they obtained acylation of sucrose at the C-2 position and to a minor extent at the C-3 position in yields of 46% and 8% respectively in 18 h, demonstrating a pre-eminent reactivity of the hydroxyl group at the C-2 position. Plou et al. [23] previously reported that Celite and Eupergit C, respectively catalysed the formation of lauric acid mono- and di-esters of sucrose.

4.3. Effect of initial water content

Initial water contents higher than 5% (v/v) significantly reduced the rate of sucrose stearate synthesis. The decrease in synthetic rates could be explained by water acting as a competitive nucle-

Table 3
Initial reaction rates of transesterification reaction catalysed by Celite and alkaline protease AL-89 in DMF/DMSO (1:1, v/v). Experimental conditions: 0.2 M sucrose; 0.2 M vinyl stearate: 45 °C, 250 rpm.

Reaction with	Amount of catalyst ($g\,L^{-1}$)	Initial reaction rate ($mM\,h^{-1}$)		
		2-*O*-stearoyl sucrose	3-*O*-stearoyl sucrose	3′-*O*-stearoyl sucrose
Control[a]	–	0.01 ± 0.002	0.01 ± 0.006	0
Celite[b]	10	0.1 ± 0.03	0.03 ± 0.02	0.02 ± 0.01
Celite[b]	100	0.7 ± 0.03	0.08 ± 0.02	0.08 ± 0.01
Alkaline protease AL-89[c]	10	16.2 ± 1.0	3.6 ± 1.6	2.2 ± 1.5

[a] Sucrose and vinyl stearate at ratio 1:1 neither Celite nor enzyme was added.
[b] Acid washed Celite (500 mg) was mixed with 5 mL of 10 mM sodium carbonate buffer pH 10.0 and subsequently vacuum dried overnight.
[c] Concentrated alkaline protease AL-89 in 60% ammonium sulphate solution was dialyzed twice against 10 mM sodium carbonate buffer pH 10.0 and subsequently lyophilized.

ophile in the reaction. The initial reaction rate was considerably decreased in the reaction at 7.5% water with 87% for the formation of 2-*O*-stearoyl sucrose and 84% for 3-*O*-stearoyl sucrose. The highest initial reaction rates were observed in range of 0–2.5% water with a tendency towards an optimum at 2.5%. However no significant differences in initial reaction in this range were established (see Fig. 4).

Kitagawa et al. [24] reported that the water content necessary for catalysis of transesterification reactions in organic media depends on the enzyme. For 6-*O* vinyl adipoyl glucose synthesis catalysed by the alkaline protease from *Bacillus subtilis*, the reaction needed an initial water content of 20% (v/v) to achieve maximum conversion rate; however, with the alkaline protease from *Streptomyces* sp., the transesterification activity decreased with addition of water. For the *B. subtilis*, proteinase N catalysed synthesis of sucrose methacrylate yields of 93% were accomplished by adding 4.7% (v/v) water in DMF [25]. In the present study, the reaction proceeded efficiently at initial water contents between 0% and 2.5%.

4.4. Effect of initial vinyl stearate concentration

As shown in Fig. 5, the initial synthetic rate at 45 °C increased with increasing acyl donor concentration from 0.2 M to 0.3 M. The highest initial reaction rate was obtained at a molar ratio of sucrose to vinyl stearate of 1:1.5. The reaction reached steady state relatively slower at molar ratios higher than 1:2. Therefore higher vinyl stearate concentrations were found to be unfavorable for sucrose stearate synthesis. At a vinyl stearate concentration higher than 0.4 M, the reaction medium became increasingly viscous and an oily phase was formed, a phenomenon that may lead to a mass transfer limitation of the reaction.

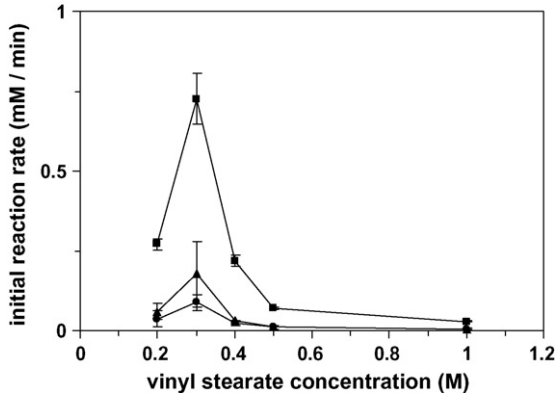

Fig. 5. Effect of initial vinyl stearate concentration on initial rate of 2-*O*-stearoyl sucrose (■), 3-*O*-stearoyl sucrose (▲), 3′-*O*-stearoyl sucrose (●) synthesis. Reaction media consisted of 0.2 M sucrose, different amounts of vinyl stearate, 0% initial water content and 10 $g\,L^{-1}$ lyophilized alkaline protease AL-89. Reaction was carried out in DMF/DMSO (1:1, v/v) at 45 °C.

4.5. Effect of temperature

The effect of temperature on the initial rate of sucrose stearate synthesis is shown in Fig. 6 and the optimum temperature was found at 70 °C. The initial rate for synthesis of 2-*O*-stearoyl sucrose was increased 45-fold as compared to 40 °C. The increase in temperature from 40 °C to 70 °C increased the initial formation

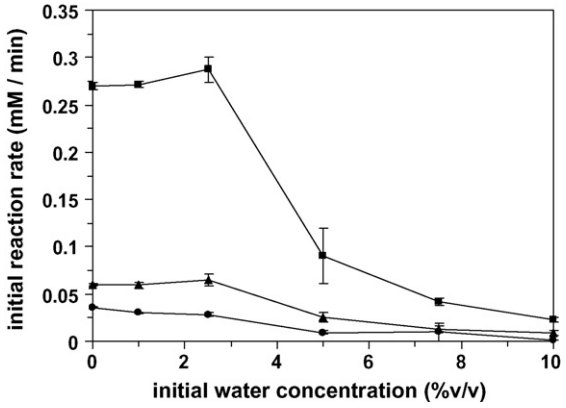

Fig. 4. Effect of water content on the initial reaction rate of 2-*O*-stearoyl sucrose (■), 3-*O*-stearoyl sucrose (▲), 3′-*O*-stearoyl sucrose (●) synthesis. *Reaction conditions*: the reaction was performed in 5.0 mL DMF:DMSO (1:1, v/v) containing 0.2 M sucrose and 0.2 M vinyl stearate in the presence of 10 $g\,L^{-1}$ lyophilyzed alkaline protease AL-89 at 250 rpm, 45 °C for 6 h.

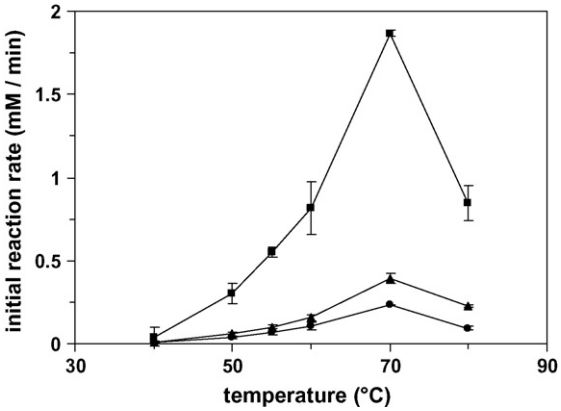

Fig. 6. Effect of reaction temperature on initial rate of 2-*O*-stearoyl sucrose (■), 3-*O*-stearoyl sucrose (▲), 3′-*O*-stearoyl sucrose (●) synthesis. Reaction media consisted of 0.2 M sucrose, 0.3 M vinyl stearate, 0% initial water content and 10 $g\,L^{-1}$ lyophilized alkaline protease AL-89. Reaction was performed in DMF:DMSO (1:1, v/v).

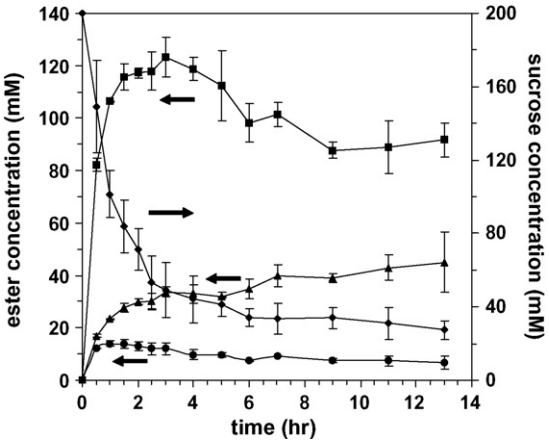

Fig. 7. Progression curves of sucrose stearate formation. 2-*O*-stearoyl sucrose (■), 3-*O*-stearoyl sucrose (▲), 3′-*O*-stearoyl sucrose (●), sucrose (♦). The reaction mixture consisted 0.2 M sucrose, 0.3 M vinyl stearate and 10 g L^{-1} lyophilized alkaline protease AL-89. The reaction was carried out in 5 mL DMF:DMSO (1:1, v/v) total volume at 70 °C.

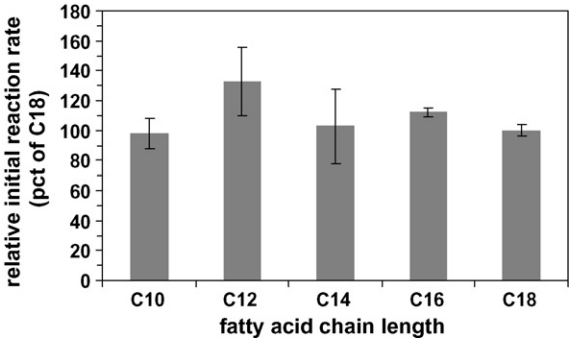

Fig. 8. Relative rates of formation of 2-*O*-acyl sucrose as effected by fatty acid chain length of the acyl donor.

rate of 3-*O*-ester 76 times and the formation of 3′-*O*-ester 63 times.

Gessesse et al. [14] reported that the alkaline protease from *B. pseudofirmus* strain AL-89 has an optimum pH for proteolytic activity in aqueous reaction medium at pH 11.0 and an optimum temperature at 70 °C in the presence of Ca^{2+} and at 60 °C in the absence of Ca^{2+}. Thus, the optimum temperature of alkaline protease for synthetic activity was in the same range as for the hydrolytic activity.

4.6. Progression of the reaction under optimized conditions

The progression of sucrose stearate synthesis catalysed by alkaline protease AL-89 under optimized conditions is shown in Fig. 7. The reaction was conducted at 70 °C with substrate molar ratio 1:1.5 without addition of water. Formation rates of 13.5 μmol min^{-1}, 2.9 μmol min^{-1} and 2.1 μmol min^{-1} were achieved for 2-*O*-, 3-*O*- and 3′-*O*-stearoyl sucrose, respectively with a specific synthesis rate of 2-*O*-stearoyl sucrose of 0.27 μmol min^{-1} mg^{-1} biocatalyst. A maximum concentration of 123 mM of the 2-*O*-ester was obtained after 3 h, where after it decreased to a final concentration of 92 mM at the termination of the reaction, showing a progression profile of a kinetically controlled reaction [26]. After 30 min of reaction no significant differences in the concentration of the 3′-*O*-ester was observed. After 6–7 h a constant sucrose concentration was observed at which point the 3-*O*-ester had reached a concentration of 40 mM at the expense of the 2-*O*-ester concentration. The final degree of sucrose conversion was 86.5% and the final yields of 2-*O*-, 3-*O*- and 3′-*O*- were 53.2, 26.0 and 4.0%, respectively. The progression curves showed two different control mechanisms of reaction. The synthesis of the 2-*O*-ester showed the characteristics of kinetically controlled reaction – whereas the 3-*O* and 3′-*O* showed the characteristics of an equilibrium controlled reaction, where product concentration increases until the point of equilibrium is reached [26].

4.7. Effect of fatty acid vinyl ester chain length

The protease was able to use fatty acid vinyl esters of chain lengths from C10 to C18 as substrates. The NMR spectra obtained for the individual sucrose fatty acid ester were similar to the results presented in Table 2. The masses of the corresponding sodium adducts were determined by nano-electron spray MS (see Table 4). The initial synthesis rates of 2-*O*-acyl sucrose were not effected by the chain length of the acyl donor (see Fig. 8).

Alkaline proteases have previously been shown to use activated short chained fatty acids as substrates for esterification of a range of mono- and disaccharides [3,7,10,11,13,25]. In most investigations substitution of primary alcohol groups of the carbohydrate were obtained. Changing the solvent from DMF to a mixture of DMF and DMSO (4:1, v/v) changed the position of substitution from the primary alcohol at the C-6 position to the secondary alcohol at the C-2 position of galactose using *Streptomyces* sp. protease as biocatalyst [7]. Esterification of sucrose using activated esters of longer chained fatty acids as substrates has been obtained with Proteinase N and Alcalase as biocatalysts in DMF and pyridine, respectively. In both investigations 1′-*O*-acyl sucrose was obtained as the primary product with triethyl stearate (C18) and vinyl laurate (C12) as acyl donors, respectively [27,28]. The present investigation showed that the protease AL-89 preferentially catalysed the synthesis of 2-*O*-acyl sucrose in DMF and DMSO (1:1) using vinyl fatty acid esters of chains length C10–C18 equally well as substrates (Fig. 8). Occurrence of 3 and 3′-*O*-acyl sucrose were detected in reactions with the respective acyl donors in similar ratios to the corresponding 2-*O*-acyl sucrose as observed with the sucrose stearate isomers. Thus the control mechanisms of the reaction were not effected by the chain length of the acyl donor and again the equilibrium mechanism controlled the formation of the 3 and 3′-*O*-acyl sucrose isomers. RP

Table 4
Molecular mass of sucrose monoester with different acyl chain length analyzed by nano-electron spray mass spectrometry.

Compound	Molecular formula	Molecular mass		Molecular mass (FAB-MS)[a]
		With Na	Without Na	
Sucrose caprate	$C_{22}H_{39}O_{12}$	514.2785	491.2785	495.2424
Sucrose laurate	$C_{24}H_{43}O_{12}$	547.2661	524.2661	523.2754
Sucrose myristate	$C_{26}H_{47}O_{12}$	575.2946	552.2946	551.3014
Sucrose palmitate	$C_{28}H_{51}O_{12}$	603.3303	580.3370	579.3370
Sucrose stearate	$C_{30}H_{55}O_{12}$	631.3794	608.3794	607.3652

[a] Fast atom bombardment-mass spectrometry [35].

Table 5
RP HPLC retention times of sucrose fatty acid esters.

Acyl moiety	Retention time (min)		
	3′-*O*-ester	2-*O*-ester	3-*O*-ester
Caprate (C10)	4.82	5.023	5.49
Laurate (C12)	6.28	6.49	6.92
Myristate (C14)	2.13	2.37	2.89
Palmitate (C16)	3.82	4.15	4.74
Stearate (C18)	5.67	6.02	6.63

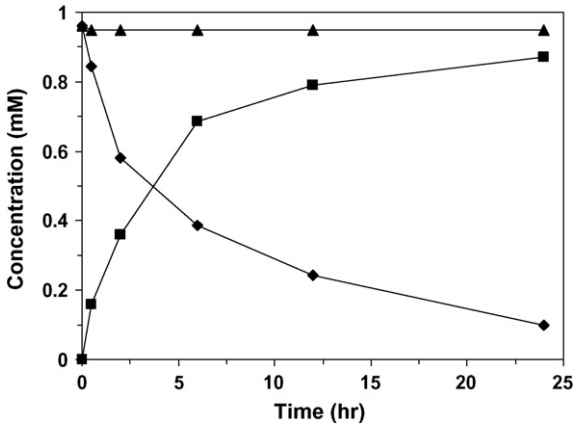

Fig. 9. Formation of 3-*O*-caproyl sucrose catalysed by alkaline protease AL-89 in DMF and DMSO (1:1, v/v). 2-*O*-caproyl sucrose in control without enzyme (▲), 2-*O*-caproyl sucrose in the reaction with enzyme (♦), and 3-*O*-caproyl sucrose in the reaction with enzyme (■). The reaction was carried out in 0.5 mL total volume of DMF:DMSO (1:1, v/v) at 250 rpm, 60 °C.

HPLC retention times of the individual sucrose fatty acid esters are listed in Table 5.

4.8. Formation of 3-O-caproyl sucrose

The purified 2-*O*-caproyl sucrose was incubated as the only compound in the presence and the absence of enzyme respectively, under conditions identical to those of the investigation of substrate specificity. In the absence of enzyme the concentration of 2-*O*-caproyl sucrose remained constant for the entire period of incubation (24 h). In the presence of enzyme the 3-*O*-caproyl sucrose was formed from the start at the expense of 2-*O*-caproyl sucrose. At the same time no free fatty acid and no formation of 3′-*O*-caproyl sucrose was observed as detected by RP-HPLC with ELSD (see Fig. 9). These results showed that the enzyme used 2-*O*-caproyl sucrose as substrate for the formation of 3-*O*-caproyl sucrose and that the latter was not formed as a result of non-enzymatic acyl migration. Acyl migration from the C-2 to the C-3 position of sucrose has previously been observed in the presence of inorganic catalysts in DMF by Molinier et al. [21], in DMSO by Cruces et al. [29] and even in aqueous reaction media by Thevenet et al. [30]. In DMSO no significant migration from the C-3 to the C-6 position was observed but the presence of water accelerated this process [21].

Acyl migration has also been studied intensively in relation to mono- and diacyl xylitol formation in lipase catalysed reactions, where it has been shown to occur later in the process, and at a rate of at least one order of magnitude slower than the enzyme catalysed ester synthesis [31]. In lipase catalysed hydrolysis of triacyl glycerides to mono- and diacyl glycerides the choice of reaction medium was shown to influence the migration significantly, and it occurred extensively in the presence of aliphatic hydrocarbons and water-miscible alcohols, respectively, whereas it was much slower in ethers than in alkanes [32,33]. Sjursnes and Anthonsen [34] showed that the rate of intramolecular acyl migration in 1,2 dibutyrin was dependent on the polarity of solvents, water activity and presence and type of salt in the reaction mixture with the highest migration rates found in non-polar solvents.

Our results showed that the protease catalysed the formation of the 2-*O*-acyl sucrose as a result of an esterification reaction using vinyl activated acyl donors and that the enzyme used 2-*O*-acyl sucrose as substrate to form the 3-*O*-acyl sucrose, while the formation of primary esters were not observed.

5. Conclusion

The alkaline protease from *B. pseudofirmus* strain AL-89 efficiently catalysed the synthesis of sucrose fatty acid monoesters using vinyl fatty acid esters as acyl donors in a reaction mixture of DMF and DMSO (1:1, v/v). The protease used acyl donors ranging in fatty acid chain length from C10 to C 18 equally well as substrates for esterification of sucrose as the initial reaction rates were not significantly different. As evaluated by the initial reaction rates, the primary site of substitution of sucrose was at the C-2 position with the C-3 and C-3′ as secondary substitution sites. The enzyme catalysed the formation of 3-*O*-acyl sucrose from 2-*O*-acyl sucrose. The investigation did not reveal if the 3′-*O*-acyl sucrose was formed the same way.

The non-enzymatic catalyst, Celite equilibrated at alkaline pH was shown to catalyze the synthesis of sucrose stearate, but the corresponding protease catalysed reaction was superior both in terms of initial reaction rates and product yield. The initial water content, substrate molar ratio and reaction temperature were shown to effect the protease catalysed synthesis of sucrose monoesters and the highest initial reaction rate was obtained with 0–2.5% initial water addition at a temperature of 70 °C and a molar ratio of sucrose to vinyl stearate of 1:1.5. At these conditions initial formation rates of 13.5 μmol min^{-1}, 2.9 μmol min^{-1} and 2.1 μmol min^{-1} were achieved for 2-*O*-, 3-*O*- and 3′-*O*-stearoyl sucrose, respectively with a specific initial synthesis rate of 2-*O*-stearoyl sucrose of 0.27 μmol min^{-1} mg^{-1} biocatalyst. The final degree of sucrose conversion was 86.5% and the final yields of 2-*O*-, 3-*O*- and 3′-*O*- were 53.2, 26.0 and 4.0%, respectively. The synthesis of the 2-*O*-ester showed the characteristics of kinetically controlled reaction – whereas the 3-*O* and 3′-*O* showed the characteristics of an equilibrium controlled reaction. In the absence of substrates the enzyme proved to be more stable in DMF than in water and DMSO at 50 °C, whereas in the presence of substrate, a residual activity of 40% was observed even after 24 h of incubation in a mixture of DMF and DMSO (1:1, v/v) at 70 °C.

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