Pergamon

SCIENCE DIRECT®

Tetrahedron: *Asymmetry* 14 (2003) 667–673

TETRAHEDRON: *ASYMMETRY*

Synthesis of sucrose laurate using a new alkaline protease

Ninfa Rangel Pedersen, Reinhard Wimmer, Rune Matthiesen, Lars Haastrup Pedersen* and Amare Gessesse

Department of Life Sciences, Aalborg University, Sohngaardsholmsvej 49, 9000 Aalborg, Denmark

Received 17 October 2002; accepted 21 January 2003

Abstract—Sucrose laurate esters were synthesized from sucrose and vinyl laurate in organic solvents using an alkaline protease from a new alkalophilic strain, *Bacillus pseudofirmus* AL-89. Maximum synthetic activity was observed in the presence of 7.5% v/v water and in the pH range of 7–10. With protease AL-89 esterification occurred predominantly at the 2-*O*-position while subtilisin A-catalyzed monoester formation predominantly at the 1′-*O* position. In the absence of enzyme, buffer salts catalyzed non-specific reactions, resulting in the formation of a number of esters. Non-specific catalysis was also observed upon inhibition of the enzyme using a serine protease inhibitor or upon deactivation of the enzyme at pH above 10.

1. Introduction

Sugar esters find important applications in a variety of industrial processes including their use as biodegradable surfactants in the food and cosmetic industry, as antitumoral agents and plant growth inhibitors.[1,2] Currently sucrose esters are synthesized non-enzymatically at high temperatures, resulting in low specificity and side reactions with the formation of colored derivatives.[3] In recent years, hydrolytic enzymes have received increasing attention for specific and regioselective acylation of carbohydrates in organic solvents.[4–7] In addition to their selectivity, enzyme-catalyzed reactions are advantageous as they can be performed under milder conditions than non-enzymatic reactions, potentially lowering the cost of ester production.

The two main problems encountered with the enzymatic acylation of carbohydrates in organic solvents are the stability of the biocatalyst and the solubility of the sugar substrate.[6,7] While better sugar solubility is obtained in water miscible organic solvents, such as dimethylsulfoxide (DMSO) and dimethylformamide (DMF), most enzymes are denatured in the presence of these polar solvents.[7,8]

Lipases and serine proteases are commonly used for the synthesis of sugar fatty acid esters and the enzymes catalyze reactions by a similar mechanism.[9] To date only a handful of commercially available lipases and serine proteases have been used for the synthesis of sugar esters.[10] The serine protease, subtilisin is known to synthesize sucrose esters predominantly at the 1′-*O*-position in DMF and pyridine[11–13] and the metalloprotease, thermolysin has recently been shown to catalyze the acylation of sucrose at the 2-*O*-position in DMSO.[14] Thus, enzymes from different sources have different properties, including stability and selectivity in the presence of chemical denaturants. This shows the need to search for new and better biocatalysts that can withstand the reaction conditions required for efficient industrial productions. Herein, we report on the use of an alkaline protease from *Bacillus pseudofirmus* AL-89, recently isolated from an alkaline soda lake in Ethiopia[15] for sucrose ester synthesis in organic solvents.

2. Results

2.1. Effect of water

Protease AL-89 catalyzed the synthesis of sucrose esters using vinyl laurate as the acyl donor in DMF. No ester synthesis was detected in the absence of water showing that the enzyme needed to be hydrated to catalyze the reaction. Maximum synthetic activity was observed in the presence of 7.5% v/v water (Fig. 1). Following the

* Corresponding author. Tel.: +45 9635 8497; fax: +45 98141808; e-mail: lhp@bio.auc.dk

0957-4166/03/$ - see front matter
doi:10.1016/S0957-4166(03)00086-7

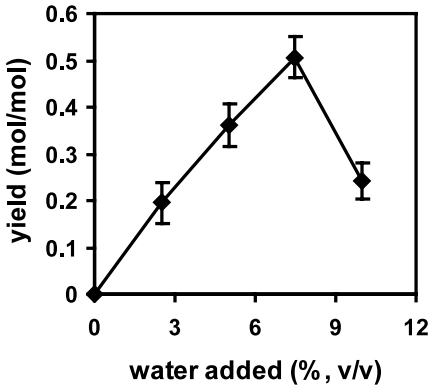

Figure 1. Effect of water on sucrose laurate ester synthesis catalyzed by protease AL-89 in DMF. The yield of ester was calculated on the basis of the carbohydrate substrate.

progress of the reaction in the presence of 10% v/v water by TLC, showed that ester formation reached a maximum at 12 h and decreased markedly at 24 h, resulting in a reduced yield of the ester.

2.2. Effect of buffer salts and immobilization matrices

With lauric acid as the acyl donor neither subtilisin A nor protease AL-89 catalyzed the synthesis of sucrose ester. However, both enzymes catalyzed the formation of sucrose esters when using vinyl laurate as the acyl donor. In all cases a reaction mixture without any enzyme added was used as a control to determine the extent of non-enzymatic catalysis. Sucrose ester formation was repeatedly observed in the control reaction mixtures. However, when vinyl laurate and sucrose in the appropriate solvent were mixed in the absence of buffer salts or Celite no ester formation was observed even after prolonged incubation. This showed that buffer salts in combination with the immobilization matrix catalyze the reaction between vinyl laurate and sucrose. To investigate the role of immobilization matrices on ester synthesis, in addition to Celite, three other matrices (silica gel, Amberlite XAD-7 and Accurel) were treated with 10 mM sodium carbonate buffer and used in the reaction. Sucrose laurate ester formation was observed with all the matrices tested. Given the wide variation in chemical composition of these matrices, the similarity in the rate of ester synthesis indicates that the buffer salts play a major role in catalyzing the reaction. The rate of ester synthesis increased on increasing the buffer salt concentration from 10 mM to 1 M (Fig. 2) further indicating that the buffer salts play an important role in the non-enzymatic formation of these esters. Similarly, when immobilized subtilisin A adjusted to pH 7.8 with 10 mM sodium phosphate buffer was used instead of protease AL-89, only monoesters were formed in the presence of enzyme, while a mixture of esters varying in the degree of substitution (DS) was formed in the absence of enzyme (Fig. 3B).

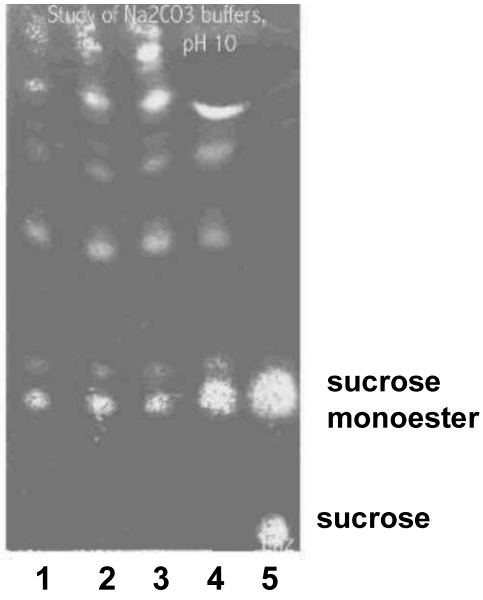

Figure 2. Effect of sodium carbonate concentration (pH 10) on the non-enzymatic synthesis of sucrose laurate esters in DMF. Na_2CO_3 was deposited on Celite. TLC analysis of samples incubated with 1.0 M (lane 1), 0.5 M (lane 2) and 0.1 M (lane 3) solutions of sodium carbonate for 6 h. Lane 4 shows a sample incubated with 10 mM sodium carbonate for 29 h, showing complete conversion. 100 mg Celite containing Na_2CO_3 buffer was incubated with 150 mM sucrose and 0.5 M vinyl laurate at 45°C Lane 5 shows a sample incubated with protease AL-89, adjusted to pH 10 with 10 mM sodium carbonate, and 7.5%, v/v water for 24 h at 45°C. The mobility of sucrose and sucrose laurate monoesters are indicated to the right.

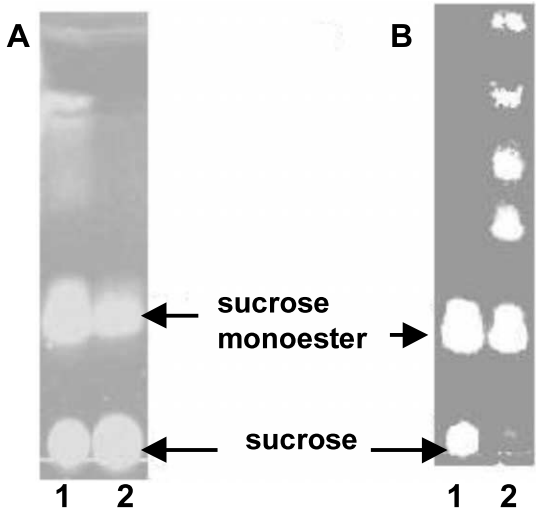

Figure 3. Enzymatic and non-enzymatic catalyzed sucrose ester synthesis in DMF–pyridine 1:1 v/v. Enzymes pH-adjusted and immobilized on Celite. TLC analysis of reactions: (A) 100 mg immobilized enzyme or Celite containing buffer was incubated with 150 mM sucrose and 0.5 M vinyl laurate at 45°C for 24 h Ester formation with PMSF inhibited (lane 1) or uninhibited (lane 2) protease AL-89, pH 10. (B) Ester formation in the presence (lane 1) or absence (lane 2) of subtilisin A, pH 7.8.

It is interesting to note that while buffers deposited on Celite or other matrices catalyzed the synthesis of a mixture of sucrose laurate esters (Fig. 2 and Fig. 3B), only monoesters were synthesized in the presence of protease AL-89 and subtilisin A (Fig. 3A and 3B). When PMSF (a serine protease inhibitor) inactivated enzyme was used in the reaction a mixture of esters similar to that obtained from the non-enzymatic reaction was formed (Fig. 3A). In addition, when the enzyme was replaced with BSA a similar product mixture was obtained.

2.3. Effect of solvents

With AL-89 adjusted at pH 10 and a water content of 7.5% v/v ester synthesis was observed in pyridine, DMF, and a 1:1 v/v mixture of DMF–pyridine and DMF–DMSO. No ester formation was detected in THF, acetone, dioxane, acetonitrile, or a 1:1 v/v mixture of the above solvents with DMF (Table 1).

2.4. The effect of pH

Using non-immobilized enzyme, protease AL-89 adjusted to pH 6 prior to lyophilization failed to catalyze ester synthesis while raising the pH from 7 to 10 in 1:1 v/v DMF:pyridine, ester formation was observed. Mass spectrometry analyses of the reaction products showed one peak corresponding to the calculated mass of sucrose laurate monoester as the sodium adduct (Fig. 4A). The reaction with enzyme adjusted at pH 11 and 12 gave four peaks corresponding to the calculated mass of the sodium adducts of the mono-, di-, tri and tetrasubstituted esters (Fig. 4B). At pH 11 and 12 no proteolytic activity in aqueous solution was detected, indicating that the enzyme was deactivated at these high pH values. The substitution pattern was identical to that observed with buffer salt deposited on immobilization matrix suggesting that the ester synthesis was catalyzed non-enzymatically.

The monoesters synthesized by protease AL-89 were analyzed by NMR (Table 2). In the range of pH 7–10, between 50 and 60% of the esters was at the 2-*O*-position and 5–25% were substituted at the 1′-*O*-position. Small amounts of other esters were also detected. A similar reaction carried out using subtilisin A adjusted in the pH range of 6–9 gave 60–75% of the 1′-monoester. HPLC analysis revealed no hydrolysis of the acyl donor occurred with either protease.

2.5. Hydrolysis of esters by protease AL-89

To investigate if sucrose laurate esters of DS 2-4 were hydrolyzed selectively by protease AL-89, resulting in the accumulation of sucrose monoesters, a mixture of non-enzymatically synthesized esters (Fig. 2) was incubated with AL-89 in increasing amounts of water.

Table 1. Effect of solvents on protease AL-89 catalyzed sucrose laurate ester formation

Solvent	Sucrose laurate (mM)	Solvent	Sucrose laurate (mM)	Solvent	Sucrose laurate (mM)
Pyridine	15	Acetone	0	DMSO	0
DMF	72	Acetonitrile	0	DMF–acetone	0
DMF–pyridine	80	dioxane	0	DMF–acetonitrile	0
DMF–DMSO	85	THF	0	DMF–dioxane	0

The reaction mixture (total volume 1 ml) in the respective solvents or a 1:1 mixture of solvents was composed of 7.5% v/v water, 10 mg of pH 10 adjusted lyophilized enzyme, 150 mM sucrose, and 0.5 M vinyl laurate. The total amount of ester formed after 24 h incubation was quantified by measuring the amount of unreacted vinyl laurate.

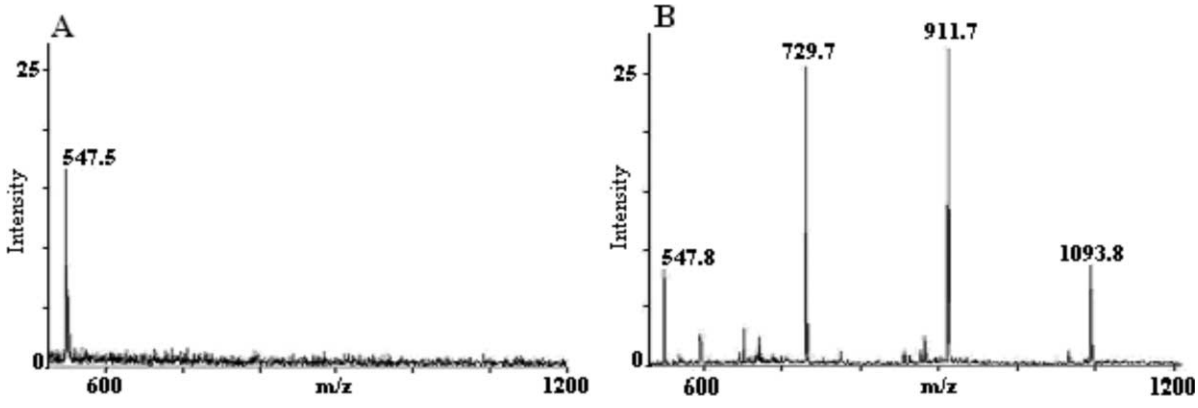

Figure 4. Mass spectra of sucrose laurate esters. Sucrose esters obtained with protease AL-89 adjusted to pH 10 (A) and pH 12 (B). Masses of sodium adducts of mono-, di-, tri- and tetralaurate esters shown on labelled peaks. The unlabelled peaks were also present in the calibration spectra.

Table 2. The effect of pH on the position of acylation of sucrose catalysed by protease AL-89 and subtilisin A

Protease	pH	1′-*O*-Lauroyl ester (%)	2-*O*-Lauroyl ester (%)	6-*O*-Lauroyl ester (%)	6′-*O*-Lauroyl ester (%)	Unidentifiable compounds
AL-89	7.0	N.D.	~60	N.D.	N.D.	D
AL-89	8.0	5–10	55–60	3–5	N.D.	D
AL-89	9.0	15–20	40–50	5–10	N.D.	D
AL-89	10.0	20–25	~50	~15	N.D.	N.D.
Subtilisin A	6.0	60	N.D.	~20	~20	N.D.
Subtilisin A	7.8	75	N.D.	10–15	10–15	N.D.
Subtilisin A	9.0	65	5	15	15	N.D.

Immobilized enzyme (100 mg) in DMF:pyridine, 1:1 v/v was incubated with 150 mM sucrose and 0.5 M vinyl laurate at 45°C for 24 h. The position of acylation was determined by NMR analysis. D: detected, N.D.: not detected.

After 24 h of incubation at 45°C the reaction mixture was analyzed by TLC. At water content of up to 7.5% v/v no hydrolysis of the sucrose esters was detected. At water contents of 10 and 20% v/v hydrolysis of sucrose esters was evident, with the concomitant production of sucrose. However, no accumulation of monoesters was observed.

3. Discussion

Protease AL-89 adjusted at pH 10 catalyzed the synthesis of sucrose laurate esters using vinyl laurate as the acyl donor in three hydrophilic solvents. Ester synthesis was observed only when water was present in the reaction medium. In organic solvents the enzyme conformation is believed to be very rigid, leading to significant reduction in activity.[16] Hydration is thought to reduce the rigidity of the enzyme by forming multiple hydrogen bonds with the amino acid residues, thus increasing the conformational flexibility of the enzyme.[16,17] However, increasing the concentration of water past the optimum resulted in a significant reduction in ester yield, indicating an increase in the rate of hydrolysis.

In contrast to lipases, proteases do not catalyze the esterification reaction between native fatty acids and sucrose, but both of these serine hydrolases readily catalyze a transesterification reaction with vinyl esters as acyl donors.[11,18] The use of such substrates for ester synthesis results in the liberation of enols, which rapidly tautomerize to the corresponding aldehyde or ketone,[19] driving the reaction forwards and avoiding the need for continuous removal of the water liberated, which would otherwise be necessary in an esterification reaction.

In the absence of enzyme, but in the presence of the buffer salts or bases added for pH adjustment, the formation of esters also occurred and within 24 h complete conversion of sucrose to esters of DS 1-4 was observed. The non-regioselective synthesis of sucrose esters of varying DS catalyzed by 0.1 M phosphate buffer has been reported previously.[20]

In the presence of active enzyme (protease AL-89 or subtilisin A) only monoesters were synthesized. However, not all sucrose was converted during 24 h. With protease AL-89 sucrose esters predominantly substituted at the 2-*O*-position were obtained. In contrast, with subtilisin the monoesters substituted primarily at the 1′-*O*-position were obtained. Incubation of the non-enzymatically synthesized heterogeneous mixture of esters with protease AL-89 at a water content of 0%–7.5% v/v did not generate any breakdown products, demonstrating that appearance of only monoesters in the enzyme catalyzed reaction was not due to hydrolysis of sucrose esters of DS 2-4. Ester hydrolysis occurred at water contents of 10% and 20% v/v, but no selectivity towards hydrolysis of esters of DS 2-4 was observed. Furthermore, at a water content of 7.5% and below, no hydrolysis of the acyl donor was observed. With an initial molar ratio of 3.3 equivalents of acyl donor to acyl acceptor, the absence of higher esters in the protease-catalyzed process was not due to limited availability of the acyl donor.

When the enzyme was deactivated using an active site specific serine protease inhibitor (PMSF) and used in the reaction, a mixture of esters similar to that formed in the non-enzymatic reaction was obtained. Unreacted sucrose and a lower yield of higher esters than the yield of monoesters was observed after 24 h incubation, showing that the non-enzymatically catalyzed formation of higher esters occurred more slowly than the enzyme catalyzed formation of monoesters. In the presence of pH adjusted, active enzyme both enzymatic and non-enzymatic catalysis would be expected to occur simultaneously, resulting in a mixture of mono- and higher esters at a thermodynamically controlled stage of the process. Therefore we concluded that the regioselective synthesis of monoester in the presence of active enzyme was enzymatically catalyzed and that at reaction time of 24 h the ester synthesis seems to be kinetic controlled.

Previous studies showed that the solvent composition in the reaction medium could significantly affect the rate of enzyme-catalyzed reactions in organic media.[6–8] In our study, of the 13 different solvents and solvent mixtures tested, sucrose ester synthesis was detected in three of the solvents. The solvent plays two important

roles, determining the solubility of the sugar and altering the stability of the enzyme. Thus, it is essential to select the best solvent for high sugar solubility whilst maintaining enzyme activity so as to increase the yield of esters synthesized in organic solvents.

The pH at which the enzymes are treated prior to lyophilization is also very important for synthetic activity in organic solvents.[16] Protease AL-89 adjusted in the pH range of 7-10 catalyzed the synthesis of only monoesters of sucrose. When the pH was raised to 11–12 the enzyme was deactivated and a mixture of esters similar to that obtained from the buffer-catalyzed reaction was formed. Protease AL-89 is an alkaline serine protease with maximum stability around pH 10 in aqueous medium. It becomes unstable at pH values above 10.5.[15] Thus, the esters synthesized at pH 11 and 12 must result from non-enzymatic catalysis as the enzyme is inactive at this high pH.

The monoester formed in the pH range of 7–10 by protease AL-89 was predominantly (50–60%) 2-*O*-lauroyl sucrose, showing that the enzyme has selectivity towards reaction at the 2-*O*-position. In contrast, 60–75% of the monoesters obtained using subtilisin A in the pH range 6–9 were at the 1′-*O* position. The identity of the ester products was confirmed by NMR analysis and were shown to be identical to those reported by Potier et al.[11]

Subtilisin has been shown to synthesize esters predominantly at the 1′-*O*-position in DMF and pyridine.[11–13] Thus, protease AL-89 differed from subtilisin with respect to regioselectivity. A recent investigation has shown that another serine protease (Proleather) also catalyses acylation of sucrose at the 2-*O*-position in DMF using divinyl adipate as the acyl donor.[5] These results show that the regioselectivity of different serine proteases is not the same.

In some cases the regioselectivity of an enzyme has been shown to be affected by the solvent. For example, the alkaline protease from *Streptomyces* spp. catalyzed the synthesis of the 6-*O*-galactose ester in DMF, but in DMF:DMSO 4:1 v/v the 2-*O*-galactose ester was formed.[21]

In conclusion, protease AL-89 adjusted at pH between 7 and 10 catalyzed the acylation of sucrose in a transesterification reaction with vinyl laurate. The enzyme differed from subtilisin in that it shows specificity for reaction at the 2-*O* position of sucrose in DMF:pyridine (1:1, v/v).

4. Experimental

4.1. Materials and methods

4.1.1. Materials. Celite, silica gel, Amberlite XAD 7, sucrose, molecular sieves (3 Å, 8–12 mesh), phenyl methylsulfonyl fluoride (PMSF) and organic solvents were from Sigma, St. Louis, MO, USA. Accurel EP100 was from Akzo Nobel, Obernburg, Germany. Silica gel 60 and thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). Lauric acid and vinyl laurate were from Fluka (Buchs, Switzerland). The organic solvents and vinyl laurate were stored over molecular sieves.

4.1.2. Enzyme sources. Subtilisin A was a kind gift from Novozymes, Denmark. Protease AL-89 was produced in the laboratory using *B. pseudofirmus* AL-89. The medium used for enzyme production was composed of (g/l) peptone, 5.0; yeast extract, 2.0; casein 5.0; K_2HPO_4, 1.0; magnesium sulfate, 0.2; calcium chloride, 0.1; glucose, 5.0 and Na_2CO_3, 10.0. Sodium carbonate was autoclaved separately and added to the rest of the medium after cooling. 500 ml of the medium in 2 l flasks was inoculated with 40 ml of pre-culture grown overnight and incubated 48 h at 37°C in a shaking incubator. The culture was centrifuged at 3000×*g* for 20 min and the cell free culture supernatant used as the enzyme source. To concentrate the enzyme ammonium sulfate was added to the cell free culture supernatant to 75% saturation followed by centrifugation at 6000×*g*. The precipitate was dissolved in water and dialyzed against three changes of Mili Q water and lyophilized.

4.1.3. Preparation of the enzyme catalyst. For sucrose ester synthesis, the enzyme was used as an immobilized protein or as a lyophilized protein powder directly into the reaction medium. Enzyme immobilization was done by adsorption or deposition. For deposition, 30 mg of protein was dissolved in 10 mM Na_2CO_3 buffer (pH 10) and mixed with 1 g of acid washed Celite followed by vacuum drying for 3 h. As a control the same amount of buffer without any protein added was mixed with Celite and dried under vacuum. In addition, 1 ml of different molarities (1.0, 0.5, 0.1 and 0.01 M) of Na_2CO_3 buffer, pH 10 was each deposited on 1 g Celite, dried under vacuum and used as control.

Alternatively the pH of the enzyme in aqueous solution was adjusted to 10 with either 0.1 M KOH or 0.1 M NaOH and then deposited on Celite and vacuum dried for 3 h. A corresponding control without enzyme was made with water containing the same volume of 0.1 M KOH or 0.1 M NaOH. The enzyme preparations were stored in a dessicator at 4°C.

Immobilization by adsorption was carried out using a modification of the method of Ulijin et al.[22] Enzyme (10 mg) dissolved in 2.5 ml of 10 mM sodium carbonate buffer, pH 10 was added to 100 mg Celite, Amberlite XAD-7 or Accurel EP100 and gently shaken at ambient temperature until no proteolytic activity detected in the liquid phase. The immobilized enzyme preparation was washed several times with DMF–pyridine (1:1 v/v). Celite and Accurel EP100 treated in a similar way without the addition of enzyme were used as controls. The immobilized enzyme and controls were used immediately in an ester synthesis reaction as described below.

For the preparation of lyophilized powders the enzyme (protease AL-89 and subtilisin A) was first dialyzed

against water, followed by adjustment of the pH to the desired values (from pH 6–12) and lyophilized. The pH-adjusted enzyme powder was directly used in the reaction medium.

4.1.4. Ester synthesis. The reaction mixture in total a volume of 1 ml, contained 150 mM sucrose, 0.5 M vinyl laurate, immobilized enzyme (100 mg) or lyophilized enzyme (10 mg) and appropriate amount of water or for control reactions, 100 mg of Celite buffer control with or without water. The different solvents tested were DMF, DMSO, pyridine, acetone, acetonitrile, dioxane or a 1:1 v/v mixture of these solvents with DMF. The reaction mixture was incubated at 45°C with constant stirring at 250 rpm. After 24 h the reaction was terminated by removing the immobilized enzyme by filtration through glass wool or by centrifuging to remove the lyophilized enzyme powder. Samples from the above reaction mixture were then analyzed qualitatively using TLC, or quantified using HPLC by measuring the unused vinyl laurate and/or sucrose. The esters were further characterized by mass spectrometry and NMR.

4.1.5. Hydrolysis of esters using protease AL-89. Sucrose laurate esters of up to four substitutions were synthesized in DMF using sodium carbonate deposited onto Celite. After complete conversion, sodium carbonate Celite control was separated from the reaction medium by centrifugation and DMF was evaporated on a vacuum drier leaving behind the esters. These esters were dissolved in DMF:pyridine, 1:1 v/v and 10 mg of protease AL-89 was added with or without the addition of water and incubated at 45°C with constant stirring at 250 rpm. After 24 h the reaction mixture was analyzed by TLC.

4.1.6. Enzyme inhibition. To the enzyme (subtilisin A or AL-89, 10 mg) dissolved in MiliQ water adjusted to pH 7.5 or 10, with 0.1 M KOH, was added 35 μl of a 235 mM stock solution of phenyl methyl sulfonyl fluoride (PMSF) in methanol. After reacting for 30 min with occasional shaking, remaining proteolytic activity was determined and the mixture was freeze dried. Inhibited freeze-dried enzyme (subtilisin A or AL-89) was incubated with sucrose and vinyl laurate in DMF:pyridine 1:1 v/v in a similar manner to the uninhibited enzymes.

4.2. Analytical methods

4.2.1. Proteolytic and protein assays. Proteolytic activity was assayed using casein as substrate at pH 10 as described earlier.[23]

One unit (U) of protease activity was defined as the amount of enzyme, which resulted in the release of 1 μg of amino acid (equivalent to tyrosine) per minute. Protein concentration was determined by the Bio-Rad Bradford assay using bovine serum albumin as a standard. Protease activity of subtilisin A used was 71.84 U/mg protein. Protease activity of AL89 used was 19.12 U/mg protein.

4.2.2. Thin-layer chromatography (TLC). For TLC analysis, 2 μl of the reaction mixture was applied to silica gel 60 plates (0.1 mm) and chloroform/methanol/acetic acid/water 70:20:8:2 v/v was used as mobile phase.[7] Unused substrate and esters were detected by spraying with methanol/sulfuric acid (1:1 v/v) and heating at 140°C for 10 min.

4.2.3. Purification of sucrose esters. After removal of the enzyme by filtration or centrifugation, the reaction mixture was mixed with three volumes of hexane, resulting in the precipitation of the unreacted sucrose and sucrose laurate esters. After washing with *n*-hexane three times to remove residual vinyl laurate, the precipitate was dissolved in methanol and subjected to preparative TLC (0.5 mm TLC plates). The sucrose ester band was collected and eluted from a column with ethyl acetate/methanol/water (17:4:1 v/v/v).[24] Ester fractions were pooled, dried under vacuum and analyzed using MS and NMR.

4.2.4. High-pressure liquid chromatography (HPLC). Analysis was carried out by reversed-phase HPLC using a system equipped with a Waters-Physics pump, a Waters Nucleosil 100-C18 column (250×4.6 mm) and a refractive index detector (Knauer, Germany) For analysis, methanol/water 7:3 v/v or methanol/water 85:15 v/v[16] was used as the mobile phase at a flow rate of 1 ml/min at 40°C. The degree of conversion was calculated from the concentrations of the substrate (sucrose and/or vinyl laurate).

4.2.5. Mass spectrometry (MS). After 24 h reaction, the catalyst was removed from the reaction mixture as described earlier. To 20 μl of the reaction mixture 1.5 ml methanol was added. From this mixture 2 μl were analyzed by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS on a Bruker Reflex III, using dried droplet and CCA (α-cyano-4-hydroxycinnamic acid) as matrix. The spectra where calibrated using Angiotensin II (1046.5423 Da, Sigma), Bombesin (1619.8229 Da, Fluka), Adrenocorticotropic hormone fragments 18–39 (2465.1989 Da, Sigma), and Somatostatin 28 (3149.4783 Da, Sigma).

4.2.6. NMR. NMR studies were carried out on a BRUKER DRX600 spectrometer equipped with a TXI(H/C/N)xyz-grad probe. Solutions of the samples in pyridine-d_5 were prepared and analyzed by means of 2QF-COSY, TOCSY (120 ms mixing time) and ^{13}C–^{1}H–HSQC spectra.

Acknowledgements

The present work was financed by Aalborg University. The authors wish to thank Professor P. Halling (University of Strathclyde, Glasgow, UK) for valuable suggestions and Associate professor Dr. M. Wiebe (Aalborg University, Denmark) for valuable suggestions and critical reading of the manuscript.

References

1. Nishikawa, Y.; Yoshimoto, K.; Ohkawa, M. *Chem. Pharm. Bull.* **1979**, *27*, 2011–2015.
2. Okabe, S.; Saganuma, M.; Tada, Y.; Ochiaia, Y.; Sueoka, E.; Kohya, H.; Shibata, A.; Takahashi, M.; Mizutani, M.; Matsuzaki, T.; Fujiki, H. *Jpn. J. Cancer Res.* **1999**, *90*, 669–676.
3. Nakamura, S. *Oleochemicals* **1997**, *8*, 866–874.
4. Ferrer, M.; Cruces, M. A.; Bernabe, M.; Ballesteros, A.; Plou, F. J. *Biotechnol. Bioeng.* **1999**, *65*, 10–16.
5. Ferreira, L.; Ramos, M. A.; Gil, M. H.; Dordick *Biotech. Progress* **2002**, *18*, 986–993.
6. Plou, F.; Cruces, M. A.; Pastor, E.; Ferrer, M.; Fuentes, G.; Pastor, E.; Bernabé, M.; Christensen, M.; Comelles, F.; Parra, J. L.; Ballesteros, A. *J. Biotechnol.* **2002**, *96*, 55–66.
7. Pedersen, N. R.; Wimmer, R.; Emmersen, J.; Degn, P.; Pedersen, L. H. *Carbohydr. Res.* **2002**, *337*, 1179–1184.
8. Laane, C.; Vos, K.; Boeren, S.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 486–493.
9. Brady, L.; Brozowski, A. M.; Derewenda, Z. S.; Dodson, E.; Dodson, G.; Tolley, S.; Turkenburg, J. P.; Christiansen, L.; Jensen, B. H.; Norskoveif, L.; Thim, L.; Menge, U. *Nature* **1990**, *343*, 767–770.
10. Riva, S.; Roda, G. In *Methods in Non-aqueous Enzyme Enzymology*; Gupta, M. N., Ed.; 2000; pp. 146–159.
11. Potier, P.; Bouchu, A.; Gagnaire, J.; Queneau, Y. *Tetrahedron: Asymmetry* **2001**, *12*, 2409–2419.
12. Riva, S.; Chopineau, J.; Kieboom, A. P. G.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 584–589.
13. Rich, J. O.; Bedell, B. A.; Dordick, J. S. *Biotechnol. Bioeng.* **1995**, *45*, 424–432.
14. Pedersen, N. R.; Halling, P. J.; Pedersen, L. H.; Wimmer, R.; Matthiesen, R.; Veltman, O. R. *FEBS Lett.* **2002**, *519*, 181–184.
15. Gessesse, A.; Hatti-Kaul, R.; Gashe, B. A.; Mattiasson, B. *Enzym. Microb. Technol.* **2003**, *32*, in press.
16. Klibanov, A. M. *Nature* **2001**, *409*, 241–246.
17. Rupley, J. A.; Gratton, E.; Carreri, G. *TIBS* **1983**, *8*, 18–22.
18. Plou, F.; Cruces, M. A.; Pastor, E.; Ferrer, M.; Bernabé, M.; Ballesteros, A. *Biotechnol. Lett.* **1999**, *21*, 635–639.
19. Kvittingen, L. *Tetrahedron* **1994**, *50*, 8253–8274.
20. Potier, P.; Bouchu, A.; Gagnaire, J.; Queneau, Y. *Tetrahedron Lett.* **2000**, *41*, 3597–3600.
21. Kitagawa, M.; Fan, H.; Raku, T.; Shibatani, S.; Maekawa, Y.; Hiraguri, Y.; Kurane, R.; Tokiwa, Y. *Biotechnol. Lett.* **1999**, *21*, 355–359.
22. Ulijin, R. V.; Erbeldinger, M.; Halling, P. J. *Biotechnol. Bioeng.* **2000**, *69*, 633–638.
23. Gessesse, A.; Gashe, B. A. *Biotechnol. Lett.* **1997**, *19*, 479–481.
24. Degn, P.; Pedersen, L. H.; Duus, J. Ø.; Zimmermann, W. *Biotechnol. Lett.* **1999**, *21*, 275–280.