Efficient transesterification of sucrose catalysed by the metalloprotease thermolysin in dimethylsulfoxide

Ninfa Rangel Pedersen[a], Peter J. Halling[b], Lars Haastrup Pedersen[a,*], Reinhard Wimmer[a], Rune Matthiesen[a], Oene Robert Veltman[a,c]

[a] *Institute of Life Sciences, Aalborg University, Sohngårdholmsvej 49, 9000 Aalborg, Denmark*
[b] *Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, UK*
[c] *Danisco Cultor, Edwin Rahrs Vej 38, 8220 Brabrand, Denmark*

Received 18 March 2002; revised 19 April 2002; accepted 21 April 2002

First published online 26 April 2002

Edited by Judit Ovádi

Abstract Thermolysin catalyses the formation of sucrose esters from sucrose and vinyl laurate in dimethylsulfoxide, with a specific activity of 53 nmol/min/mg and 2-*O*-lauroyl-sucrose as the main product. Such transesterification reactions are normally observed only when the mechanism involves an acyl enzyme intermediate, as with lipases or serine proteases, and not with metalloproteases like thermolysin. A possible reason is the affinity of the active site of thermolysin for sugar moieties, as for the potent inhibitor phosphoramidon. The reaction is not catalysed by other proteins under the same conditions, and is inhibited by removal of the active site zinc.

Key words: Transesterification; Thermolysin; Carbohydrate fatty acids ester; Dimethylsulfoxide; Reaction mechanism

1. Introduction

Carbohydrate fatty acid esters with varying degrees of esterification are used in, e.g. the food, cosmetic and pharmaceutical industries [1]. These esters can be synthesised chemically or enzymatically. Chemical esterification methods are non-specific, often resulting in a broad range of carbohydrate esters. Chemical acylation of sucrose takes place with preference at the primary alcohol groups, 6-OH $\geq$ 6′-OH $>$ 1′-OH [2], however, under suitable conditions acylation have been shown to take place preferentially at the 2-OH position [3]. Enzymatically catalysed carbohydrate fatty acid esterification reactions are generally much more specific. Thus members of the *subtilisin* family (E.C.3.4.21.14) specifically catalyse the acylation of sucrose on the 1′-OH, i.e. on the fructose ring [4]. Lipases from *Humicola lanuginosa* and *Pseudomonas* sp. catalyse the acylation of sucrose mainly at the C-6 position [5]. The lipases and serine proteases used in these processes have in common the formation of a covalent acyl enzyme intermediate, which is believed to be essential for catalysis of transesterification reactions, as commonly used in such syntheses [6].

Thermolysin, (E.C.3.4.24.27) a 316 amino acid thermostable neutral metallo-endopeptidase produced by *Bacillus thermoproteolyticus*, is used widely in organic media for the synthesis of peptides [7,8] and is used in industrial scale for the synthesis of aspartame precursor [9]. Unlike serine proteases, it is not normally used to synthesise or hydrolyse esters, however, it has been used in a transesterification reaction of the diterpenoid paclitaxel [10]. A high-affinity transition state analogue inhibitor of thermolysin is phosphoramidon (*N*-α-L-rhamnopyranosyl-oxy-(hydroxyphosphinyl)-L-leucyl-L-tryptophan, $K_i = 21$ nM) [11]. As the sugar group of phosphoramidon binds to the S1 pocket in the active site of thermolysin, we anticipated that sugar moieties like sucrose in highly polar organic solvents might be able to bind into this pocket too. The S1′ pocket of thermolysin favours medium to large sized hydrophobic amino acid side chain residues, suggesting that the similar sized vinyl moiety of vinyl laurate would bind into it. Therefore we explored the possibility of using thermolysin to catalyse synthesis of sucrose esters by a transesterification reaction.

2. Materials and methods

2.1. Immobilisation of proteins/enzymes on celite

Thermolysin was obtained from Sigma (Denmark), Calbiochem (USA) and Roche (Germany). Bovine serum albumin, hen egg white lysozyme and carboxypeptidase A were purchased from (Sigma, Denmark). Celite (Sigma) was used as the support matrix for protein and enzyme immobilisation. Of each of these proteins, 30 mg was dissolved in 1 ml 50 mM Na–MOPS (3-morpholinopropanesulfonic acid) buffer at pH 7.5, mixed thoroughly with 1 g of acid washed celite and subsequently vacuum dried for 8 h [7]. The catalytic properties of the immobilised enzyme and proteins were investigated in synthesis of sucrose ester using celite without immobilised protein as a control.

2.2. Enzymatic ester synthesis

In general, enzymatic reactions were started by adding 150 mg (support plus protein) of immobilised enzyme or 4.5 mg free enzyme to either 0.03 or 0.3 M sucrose and 0.1 or 1.0 M vinyl laurate in dimethylsulfoxide (DMSO), total volume 1 ml. As a control, buffer-treated celite was incubated with sucrose and vinyl laurate in DMSO for 24 and 48 h. The reaction of 1.2 M vinyl laurate with 0.3 M sugars in the presence of celite–thermolysin and the celite–buffer control was investigated in the following solvents: DMSO, DMF, acetonitrile, *t*-butanol, *t*-amyl alcohol, ethyl acetate and pyridine. Reactions were carried out in 25 ml Schott bottles in a heating block with magnetic stirring at 250 rpm (Telemodule 40TC, Germany) and at 45°C for 24 h. For initial reaction rates, aliquots of reaction media were sampled at 15 min timed intervals until 120 min. All solvents were dehydrated and stored over 0.3 nm molecular sieves (Merck, Germany). Transesterification products were analysed using thin layer

*Corresponding author. Fax: +45-98-141808.
E-mail address: lhp@bio.auc.dk (L.H. Pedersen).

PII: S0014-5793(02)02753-9

chromatography (TLC), high-pressure liquid chromatography (HPLC) and mass spectroscopy.

2.3. Aspartame precursor synthesis

The method of Xing et al. [12] with modifications was used for the synthesis of CBZ-aspartame as described below with the immobilised thermolysin preparation. L-Phenylalanine methyl ester hydrochloride (50 mM; Sigma, The Netherlands) and N-CBZ-L-aspartic acid (50 mM; Sigma, The Netherlands) were dissolved in 1.79 ml *t*-amyl alcohol, with 88 μl tri-ethylamine and 120 μl MOPS buffer (50 mM, pH 7.5), resulting in an end concentration of 6% water in the reaction mixture. For the reactions, 100 mg of the immobilised enzyme (3 mg thermolysin) or 100 mg of buffer-treated celite as blank was used. Besides *t*-amyl alcohol, DMSO was also used as a solvent in the synthesis reaction. Reactions were carried out at 45°C for 24 h in closed Schott bottles (25 ml) on a Vario magnetic heating block stirred at 250 rpm. For initial reaction rates, aliquots of reaction media were sampled at 15 min timed intervals until 120 min. Synthesis was monitored using TLC and HPLC.

2.4. Protease inhibition studies

The effect of inhibitors was tested by direct addition to the reaction mixture (0.03 M sucrose and 0.1 M vinyl laurate in DMSO). Inhibitors and final concentrations were EDTA (50 or 100 mM), 1,10-phenanthroline (10 or 50 mM), phosphoramidon (10, 30 or 94 μM). Preincubation experiments were also performed. Thermolysin (15 mg) was allowed to react with 1,10-phenanthroline (15 mg) for 30 min at room temperature in MOPS buffer (50 mM, pH 7.5, containing 1 mM $CaCl_2$). The mixture was dialysed overnight (12 h, 4°C) against the same buffer and then deposited on celite and used in a reaction with 0.03 M sucrose and 0.1 M vinyl laurate in DMSO. Phosphoramidon (10, 33, 88, and 400 μM final concentration) was added to 15 mg thermolysin in 1 ml Na–MOPS buffer (50 mM, pH 7.5), and allowed to bind for 45 min. The solution was subsequently mixed with 500 mg celite, dried under vacuum for 8 h and used in the usual DMSO reaction mixture.

2.5. TLC analysis

Analytical TLC was performed using silica gel 60 and silica gel 60 F254 plates (Merck, Germany) with chloroform/methanol/acetic acid/water (3.5:1:0.4:0.1 v/v) as the mobile phase for carbohydrate fatty acid esters [13] Usually 2 μl samples were applied. Sucrose and sucrose fatty acid ester spots were visualised using methanol/sulphuric acid (50:50 v/v) and subsequent heating at 140°C for 10 min.

2.6. HPLC analysis

Samples of the reaction mixture were centrifuged for 15 min at 14 000 rpm in an Eppendorf microcentrifuge, to remove the immobilised enzyme. The sucrose fatty acid esters in the supernatant were analysed using reversed phase HPLC (Waters C18, 5 μm column, 4.6×250 mm HPLC cartridge). Substrates and products could be detected using a Knauer refractive index detector (Germany). Methanol 70 and 85% (v/v) in water was used as eluant, at a flow rate of 1 ml/min. Aspartame precursor in the supernatant was detected using acetonitrile and TFA (50 and 0.1% v/v respectively) in water as eluant using a flow rate of 1 ml/min.

2.7. Mass spectroscopy analysis

After 24 h the enzyme reaction mixture was centrifuged for 15 min at 14 000 rpm in an Eppendorf microcentrifuge to remove the immobilised enzyme. From the supernatant, 20 μl was subsequently diluted in a total of 1.5 ml methanol. After repeating the centrifugation procedure, compounds present in the supernatant were analysed either by electrospray mass spectroscopy (ESMS), on an Esquire Mass Spectrometer (Brucker, Germany) or by matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) using dried droplet and CCA (α-cyano-4-hydroxycinnamic acid) as matrix.

2.8. Nuclear magnetic resonance (NMR) analysis of the reaction products

NMR studies were carried out on a Bruker DRX600 spectrometer equipped with a TXI (H/C/N) xyz-gradient probe. Samples were dissolved in pyridine-d_5 and analysed at 303K by means of 2QF-COSY, TOCSY (120 ms mixing time) and ^{13}C–^{1}H HSQC spectra.

3. Results and discussion

The immobilised thermolysin preparation (150 mg) catalysed ester synthesis from sucrose (0.3 M) in both DMSO and DMF with yields of 44 and 52% respectively after 24 h of reaction. TLC analysis showed less intense ester spots with maltose, maltotetraose or maltoheptaose as compared to sucrose in the same solvents. Smaller amounts of ester were also formed from sucrose or maltose in pyridine. No synthesis of sucrose esters was detected in acetonitrile, *t*-amylalcohol or ethyl acetate, solvents generally used with thermolysin in peptide synthesis [7,8]. However, significant ester spots were observed when using 50% (v/v) mixtures of these same solvents with DMSO. The key factor here is the solubility of disaccharides in DMSO. No ester synthesis was observed in the celite control experiment as determined by TLC, indicating that the synthesis reaction was catalysed by thermolysin.

DMSO is a powerful enzyme denaturant and hence generally considered to be incompatible with enzymatic activity. However, hydrogen bond forming solvents are known to function as water mimics introducing new catalytic properties to the enzyme significantly different from those in water [14]. Thus, DMSO has been used successfully as solvent in a proteinase N-catalysed synthesis of sucrose esters [15]. To investigate if the observed reaction represented a weak general catalysis by protein functional group, the experiments were repeated with hen egg white lysozyme, bovine serum albumin and carboxypeptidase A immobilised the same way as thermolysin. No sucrose ester products were detected in any of the solvents used. The fact that no reaction was detected with the zinc metalloprotease carboxypeptidase A indicates that the reaction was enzymatically catalysed by thermolysin.

The reaction in DMSO was studied in more detail: sucrose esters were synthesised in similar amounts when using unimmobilised thermolysin. With reduced substrate concentrations (0.03 M sucrose and 0.1 M vinyl laurate), the progress of the reaction was monitored by TLC and HPLC. With 100 mg celite–enzyme (3 mg/ml thermolysin), there is a linear initial synthesis of ester with a rate of 53 nmol/min/mg (Fig. 1), which was 2.7 times lower than the initial reaction rate of aspartame precursor synthesis (141 nmol/min/mg). The com-

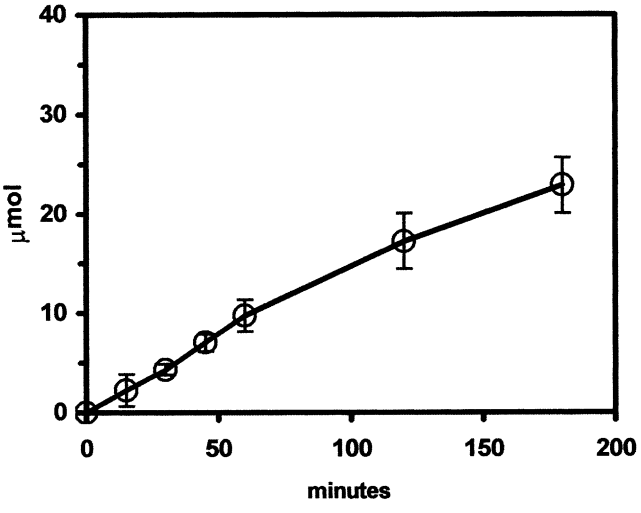

Fig. 1. Initial reaction rate of thermolysin-catalysed sucrose ester synthesis (100 mg enzyme preparation, 0.03 M sucrose and 0.1 M vinyl laurate).

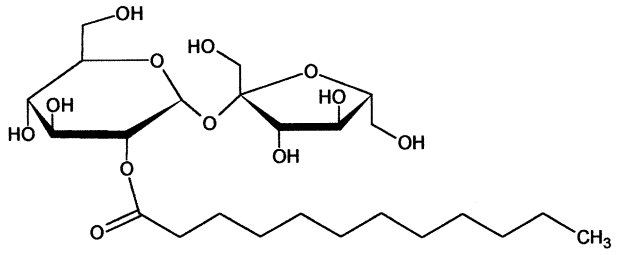

Fig. 2. Haworth projection of 2-*O*-lauroyl-sucrose, the main product of the thermolysin catalysed transesterification reaction between sucrose and vinyl laurate.

parable catalysis rate of aspartame and sucrose ester synthesis suggests that the latter reaction is enzymatic. In agreement with Nagasayu et al., aspartame synthesis could not be observed using DMSO as the solvent [16]. Polyols are known to stabilise proteins [17] and studies of thermolysin have shown that small carbohydrates contribute to the hydration and activation of the enzyme [18]. This effect could explain that addition of water is not necessary for sucrose ester synthesis in DMSO. In the aspartame precursor synthesis reaction where carbohydrates are not present, water seems to play an essential role in hydration of the enzyme as the reaction did not proceed in absence of water.

Using 0.03 M sucrose and 0.1 M vinyl laurate there was complete conversion to monolauroyl-sucrose esters after 12 h. Their composition was confirmed by molecular weights from mass spectrometry (ESMS and MALDI-TOF). From the amount of cross peaks in the ^{13}C–^{1}H HSQC NMR spectrum of the product it could be concluded that there were at least four different sucrose esters present. The main product (90%) was purified and could be determined as 2-*O*-lauroyl-sucrose (Table 1, Fig. 2). When the concentration of the substrates was increased to 0.3 M sucrose and 1.0 M vinyl laurate, using the same amount of enzyme, mono-, di-, tri- and tetra-substituted esters were detected by mass spectrometry and TLC. The observed regioselectivity is different from that reported for other enzymes. Molecular electrostatic potential profiles of sucrose has shown that the 2-OH is the most readily deprotonated hydroxyl group in aprotic solvents such as DMF and DMSO [3]. However, using high amounts of subtilisin (30 mg/ml), in a reaction mixture consisting of 10 mM sucrose in DMF and a 20% excess of trichloroethylbutyrate as acyl donor Riva et al. [4] obtained 1′-*O*-butyroyl-sucrose as the major product (90% of total). The acylation of sucrose using subtilisin from *Bacillus subtilis* consistently resulted in the 1′-*O* ester as the major product with various acyl donors in different solvents including DMF [4,18]. *Candida antarctica* lipase catalyses the acylation of sucrose monoesters at the 6 and 6′ positions in refluxing *t*-butanol [19] and at the 6 position in *t*-amyl alcohol:DMSO 4:1 (v/v) using *H. lanuginosa* lipase [5]. Therefore, in the particular solvent the regioselectivity of the enzyme is the determining factor for the products obtained.

Table 1
NMR signal assignment of 2-*O*-lauroyl-sucrose in pyridine-d_5

Carbon atom	δ (^{1}H, ppm)	δ (^{13}C, ppm)
1	6.40	90.6
2	5.43	74.6
3	4.84	72.1
4	4.25	72.2
5	4.84	74.8
6	4.4/4.29	62.4
1′	4.31/4.19	63.7
2′	–	not found
3′	5.27	77.8
4′	5.07	75.0
5′	4.52	84.2
6′	4.37/4.33	63.0

To further characterise the nature of catalysis of this reaction by thermolysin, the effects of inhibitors were studied. Thermolysin has a catalytic zinc ion bound to the active site [20] and four calcium ions that contribute to its structural integrity and high stability [21]. In aqueous medium the enzyme is inhibited by removal of the zinc ion using chelators as 1,10-phenanthroline and EDTA. The former specifically removes the zinc ion leaving thermolysin structurally intact, whereas the aspecific chelator EDTA removes both the zinc and calcium ions. Addition of 50 mM EDTA to the DMSO reaction mixture completely prevented reaction. In contrast, addition of 50 mM 1,10-phenanthroline to the reaction mixture had no effect. However, it might be that the inhibitor is ineffective at removing zinc ions in DMSO medium. When thermolysin was incubated with aqueous 1,10-phenanthroline, then dialysed, deposited on celite as before, and added to DMSO containing sucrose and vinyl laurate, no reaction was observed. Thus we conclude that the zinc ion is indeed essential for the ester synthesis reaction observed.

Phosphoramidon is a specific inhibitor of thermolysin, but addition to the DMSO reaction mixture at up to 100 μM concentration had no effect on sucrose laurate synthesis. In this case, there was no effect even if the inhibitor and enzyme were pre-incubated in aqueous media before immobilisation. It may be that in DMSO the affinity of the enzyme for phosphoramidon is much weaker than in water. The hydrophobic side chains of the Leu and Trp residues will certainly be much better solvated in DMSO, and the hydrophobic driving force for binding to the enzyme will disappear.

4. Conclusions

This report describes a new thermolysin catalysed transesterification reaction between sugars and vinyl laurate using DMSO as a solvent. Although thermolysin does not normally synthesise carbohydrate esters, the reaction appears to be a true enzyme-catalysed process. This is supported by: (1) the absence of product formation by buffer-treated support matrix (celite); (2) the lack of product formation by other proteins; (3) an initial specific activity comparable with that of aspartame precursor synthesis; and (4) the dependence on the active site zinc. The major product was 2-*O*-monolauroyl-sucrose. This novel activity opens new possibilities for enzymatic synthesis of sugar fatty acid esters and related compounds.

Acknowledgements: We thank Dr. Marilyn Wiebe for valuable suggestions and Aalborg University for the PhD grant to N.R.P.

References

[1] Nishikawa, Y., Yoshimoto, K., Nishijima, M., Fukuoka, F. and Ikekawa, T. (1987) Chem. Pharm. Bull. 29, 505–513.
[2] Haines, A.H. (1976) Adv. Carbohydr. Chem. Biochem. 33, 11–109.

[3] Lichtenthaler, F.W., Immel, S. and Pokinskyj, P. (1995) Liebigs Ann. 11, 1939–1947.
[4] Riva, S., Chopineau, J., Kieboom, A.P. and Klibanov, A. (1988) J. Am. Chem. Soc. 110, 584–589.
[5] Ferrer, M., Cruces, M.A., Plou, F.J., Bernabé, M. and Ballesteros, A. (1999) Biotechnol. Bioeng. 65, 10–16.
[6] 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. and Menge, U. (1990) Nature 343, 767–770.
[7] Clapés, P., Torres-Lluis, J. and Adlercreutz, P. (1995) Bioinorg. Med. Chem. 3, 245–255.
[8] Ulijn, R.V., Erbeldinger, M. and Halling, P.J. (2000) Biotechnol. Bioeng. 69, 633–638.
[9] Cheetham, P.S.J. (2000) in: Applied Biocatalysis, 2nd edn. (Straathof, A.J.J. and Adlercreutz, P., Eds.), pp. 93–152, Harwood Academic Publishers, Amsterdam.
[10] Khmelnitsky, Y.L., Budde, C., Arnold, J.M., Clark, D.S. and Dordick, J.S. (1997) J. Am. Chem. Soc. 119, 11554–11555.
[11] De Kreij, A., Venema, G. and van den Burg, B. (2000) J. Biol. Chem. 275, 31115–31120.
[12] Xing, G.W., Li, X.W., Tian, G.L. and Ye, Y.H. (2000) Tetrahedron 3517–3522.
[13] Degn, P., Pedersen, L.H., Duus, J.O. and Zimmermann, W. (1999) Biotechnol. Lett. 21, 275–280.
[14] Kitaguchi, H. and Klibanov, A.M. (1989) J. Am. Chem. Soc. 111, 9272–9273.
[15] Potier, P., Bouchu, A., Descotes, G., Gagnaire, J. and Queneau, Y. (2001) Tetrahedron Asymmetry 12, 2409–2419.
[16] Nagasayu, T., Miyanaga, M., Tanaka, T., Sakiyama, T. and Nakanishi, K. (1994) Biotechnol. Bioeng. 43, 1118–1123.
[17] Kitaguchi, H. (1996) in: Enzymatic Reactions in Organic Media (Koskinen, A.M.P. and Klibanov, A.M., Eds.), pp. 224–243, Blackie Academic and Professional, London.
[18] Mejri, M. and Mathlouthi, M. (2001) Carbohydr. Polym. 45, 161–167.
[19] Woudenberg-van Oostrom, M., Rantwijk, F. and Sheldon, R. (1996) Biotechnol. Bioeng. 49, 328–333.
[20] Matthews, B.W., Colman, P.M., Jansonius, J.N., Titani, K., Walsh, K.A. and Neurath, H. (1972) Nature 238, 41–43.
[21] Dahlquist, F.W., Long, J.W. and Bigbee, W.L. (1976) Biochemistry 15, 1103–1111.