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Appearance and distribution of regioisomers in metallo- and serine-protease-catalysed acylation of sucrose in *N*,*N*-dimethylformamide

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ABSTRACT

The appearance and distribution of monoester regioisomers were investigated in the virtually irreversible acylation of sucrose with the enol ester, vinyl laurate, as acyl donor catalysed by serine proteases and a metalloprotease in the hydrophilic, aprotic solvent *N*,*N*-dimethylformamide. Sucrose laurate was obtained in yields from 12 to 53% after 48 h under different catalytic conditions. The serine protease ALP-901, derived from a *Streptomyces* sp., produced the highest yield at this reaction time, while reaction with the zinc-protease thermolysin achieved the overall highest yield (63%) after 6 h, with only monoesters synthesised. The total conversion of sucrose after 48 h ranged from 19 to 96%. The highest degree of conversion was observed in the reaction with thermolysin, while the reactions without protein and with ALP-901 resulted in 82% and 66% sucrose conversion, respectively. 2-*O*-Lauroyl sucrose was the most abundant monoester regioisomer synthesised and the highest concentration observed was 23.7 mM after 24 h in the thermolysin-catalysed reaction. The highest concentration of 2-*O*-lauroyl sucrose detected in the reaction catalysed by ALP-901 was 19.0 mM, while it was 17.0 mM the reaction without protein, both after 48 h. The detected appearance of the sucrose laurate regioisomers largely corresponded to the apparent rates of formation, and 2-*O*-lauroyl sucrose was among the first regioisomers to appear in all reactions. The observed sucrose laurate regioisomeric distribution after 48 h (2:3:4:6:1′:3′) was 72:5:2:1:7:14 in the reaction catalysed by ALP-901, and 74:5:2:1:7:13 in the reaction without protein. In the reaction catalysed by thermolysin the distribution was 71:5:2:–:9:13 after 6 h and 86:8:–:–:4:3 after 48 h of reaction. The esterification of sucrose with vinyl laurate without protein in the reaction mixture appeared to be catalysed in the presence of aluminosilicate molecular sieves. Non-catalytic protein in the reaction medium seemed to lower the catalytic activity of the molecular sieves.

1. Introduction

Sucrose fatty acid esters are widely used in the food, cosmetics, pharmaceutical and detergent industries, as they can be produced with a wide range of emulsifying, dispersing and solubilising properties [1,2]. These non-ionic surfactants are non-toxic and biodegradable, and some of them have shown antimicrobial effects [1,3,4]. Currently sucrose alkanoates are synthesised by conventional chemical processes at high temperatures, resulting in low regiospecificity and side reactions leading to alterations of product properties [2,5,6]. Biocatalysis has been shown as a strong alternative to conventional chemical synthesis, offering improved activity and selectivity while not needing the protection and deprotection steps often required in chiral and regioselective organic synthesis. In particular, lipases and proteases have been applied to the synthesis of sucrose alkanoates in organic solvents [7]. Lipases have been shown to catalyse esterification by reversed hydrolysis reactions and exhibit selectivity towards the primary hydroxyl groups of sucrose (6, 1′ and 6′) [8,9], while proteases catalyse transesterification reactions and exhibit selectivity towards primary or secondary hydroxyl groups in sucrose [10,11]. Hydrophilic, aprotic solvents, including *N*,*N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) are excellent solvents for carbohydrates but act as protein denaturants affecting enzyme activity and stability. Proteases have shown improved activity and selectivity with

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DMSO as co-solvent [12,13], while lipases have shown sensitivity to hydrophilic solvents, losing activity and exhibiting complete inactivation at DMSO concentrations of 20–30% and above [14,15]. Subtilisin has shown selectivity towards 1′-OH and 6-OH of sucrose in anhydrous DMF as well as in mixtures of buffer and DMSO [16–18], while the metalloprotease thermolysin catalysed the acylation of sucrose primarily at the 2-OH [19] and the alkaline protease AL-89 catalysed the esterification of 2-OH and promoted acyl migration to the 3-regioisomer of sucrose alkanoates [13]. The recent developments in methods for quantitative analysis of sucrose laurate using RP-HPLC with charged aerosol detection (CAD) [20] made more accurate studies of the esterification processes possible, and in particular offered increased sensitivity in the analysis of the distribution of regioisomers. In this work, we have investigated catalytic activity and appearance and distribution of monoester regioisomers in the irreversible acylation of sucrose with the enol ester vinyl laurate as acyl donor catalysed by serine proteases and a metalloprotease in the hydrophilic, aprotic solvent DMF.

2. Materials and methods

2.1. Materials

All solvents were of HPLC grade, unless otherwise indicated. Methanol (MeOH), trifluoroacetic acid (TFA, reagent grade) and vinyl laurate (GC grade) were from Sigma–Aldrich, acetonitrile (ACN) was from Panreac Quimica, while *N*,*N*-dimethylformamide (DMF, GC grade) was from Fluka. Water (H_2O) of Type I purity (ELGA PURELAB flex) was degassed by sonication for 20 min (Bransonic 2510E-MT, water bath). Sucrose was from Nordic Sugar A/S, while molecular sieves (0.3 nm, aluminosilicate beads ~2 nm) were from Merck. Enzyme and protein formulations used were ALP-901 (crude formulation of ALP-101 from *Streptomyces* sp., Toyobo), subtilisin A (*Bacillus licheniformis*, lyophilised, Novozymes A/S), Alcalase 3.0 T (granulate of subtilisin A, Novozymes A/S), thermolysin (*Bacillus thermoproteolyticus*, lyophilised, Fluka) and casein (technical grade, Sigma–Aldrich).

2.2. Sucrose laurate synthesis reactions

Sucrose was powdered with mortar and pestle and dried at 120 °C for 24 h, while DMF and vinyl laurate were dried over molecular sieves (40 g/L) for at least 24 h. Reactions were performed by dissolving sucrose (50 mM) in DMF (5 mL) containing molecular sieves (40 g/L) in serum flasks (25 mL) with stirring magnets for ca. 10 min in a stirring block thermostat (50 °C, 600 rpm, VARIOMAG 15.5 with TELEMODUL 40 CT). Enzyme or protein formulation (10 g/L) was added where appropriate, vinyl laurate (400 mM) was added and the flasks stoppered with rubber caps and sealed with parafilm. Reactions with ALP-901, alcalase, and subtilisin were run in three parallels, and the reaction with thermolysin was run in two parallels. Control reactions were performed with non-catalytic protein (casein) and without protein; the reaction with casein was run in three parallels, as was the reaction without protein to 6 h, while a single reaction without protein was run to 48 h. The chemical background reaction was accounted for by a reaction with neither protein nor molecular sieves, performed in duplicate.

After 2, 4, 6, 24 and 48 h reaction time, samples (100 μL) were withdrawn from the reaction mixture using a syringe (1 mL) and transferred to centrifuge tubes (1.2 mL). After centrifugation (14,000 rpm, 3+ min) supernatant aliquots (50 μL) were diluted with methanol (ratio 1:9, 1:2 for the reaction without protein) for analysis by RP-HPLC.

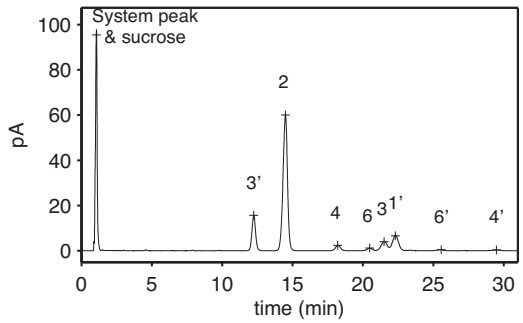

Fig. 1. Chromatogram of reaction mixture from reaction catalysed by thermolysin after 6 h. Peaks annotated with regioisomer substitution position. Retention times: sucrose, 1.1; sucrose laurate regioisomers, 12.2, 14.5, 18.2, 20.5, 21.5, 22.3, 25.6, 29.5.

2.3. RP-HPLC analysis

Samples (10 μL injections) were analysed using a Dionex UltiMate 3000 system (Thermo Scientific) with Corona Veo CAD charged aerosol detector (Thermo Scientific, N_2 at 61.5 psi, nebuliser at 50 °C). Stationary phase was a Symmetry C_{18} column (250 mm × 4.6 mm, 5 μm particle size, Waters) maintained at 45 °C, and eluents consisted of mixtures of water and acetonitrile, both with 0.05% (v/v) TFA, with flow rate 2.0 mL/min. The chromatographic data were collected using Chromeleon software (version 7.2, Thermo Scientific).

Sucrose laurate regioisomer analysis was conducted using isocratic elution with 37% acetonitrile in water for 31 min, as previously optimised for these analytes [20] (see Fig. 1). Oligoester analysis was conducted by elution with 50% ACN held to 5 min, followed by a gradient to 100% ACN at 10 min, and 100% ACN held to 15 min.

Standard curves for sucrose and 6-*O*-lauroyl sucrose were constructed from the analysis of sample series over the respective ranges of 0.05–60 and 0.06–90 μg injected analyte. CAD exhibits characteristic non-linear mass–response relations expressed by the equation:

$$A = aM^b \tag{1}$$

where A is the area response of the detector, M is the injected mass of analyte, and a and b are values specific to analytes and chromatographic conditions. Linear regression was applied to logarithmically transformed peak areas, and from the linearised mass–response relation:

$$\log A = b \log M + \log a \tag{2}$$

the coefficient b and the term $\log a$ were determined for each analyte [21]. By rearranging Eq. (1) and inserting the determined values, the injected mass of analyte (M, ng) were determined from peak area (A, pA s) according to the equations:

$$M \pm 2.1\% = \frac{A^{1.89}}{1.565} \tag{3}$$

for sucrose, and

$$M \pm 3.5\% = 4.20A^{1.09} \tag{4}$$

for sucrose laurate.

2.4. Mass spectrometry

Sucrose laurate product (lyophilised, 3.3 mg) in methanol (100 μL) was analysed by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry, as

Table 1
Initial rates of formation for 2-*O*-lauroyl sucrose.

Enzyme formulations (w/mol sieves)				Controls (w/mol sieves)		Control (w/o mol sieves)
Thermolysin	ALP-901	Alcalase	Subtilisin	Casein	No protein	Background
108 ± 6	22.2 ± 1.9	1.78 ± 0.17	0.765 ± 0.234	0.981 ± 0.020	3.24 ± 0.63	0.238 ± 0.019

Initial rates (μM/min) were calculated from 2-h samples, except for the casein reaction where it was calculated from the 4-h sample.

previously described [19], using DHB (2,5-dihydroxybenzoic acid) as matrix.

3. Results and discussion

All protease-catalysed reactions, as well as the control reactions with casein and without protein, resulted in the synthesis of sucrose laurate. Mass spectrometry of the sucrose laurate product gave characteristic signals from the molecular ion adducts with Na and K (*m*/*z* 547 and 563, respectively), corresponding to the observations of Wang et al. [22] for glucose, fructose and sucrose. 2-*O*-Lauroyl sucrose was the most abundant monoester synthesised, and its concentration increased over the first 6 h of reaction time in all reactions (see Fig. 2). The initial rates of formation for this regioisomer ranged from 0.238 to 108 μM/min (see Table 1), with the control reaction with neither protein nor molecular sieves showing the lowest rate. This reaction was considered as the chemical background reaction. The three conditions exhibiting highest initial rates were thermolysin, ALP-901 and no protein, and the rates with thermolysin and ALP-901 were, respectively, more than 450 and 90 times the rate of the chemical background reaction, and about 33 and 7 times the rate of the reaction without protein (but with molecular sieves).

Compared to the remaining reactions where molecular sieves were included, the initial rate of the reaction without protein was about 2 times that with alcalase, about 3.5 times the rate with casein and about 4 times the rate with subtilisin, indicating the presence of catalytic functionality other than enzyme under these reaction conditions. Ritthitham et al. [13] showed Celite to have catalytic activity in the reaction between sucrose and vinyl stearate in DMF/DMSO (1:1, v/v). The initial rate of formation for 2-*O*-stearoyl sucrose was reported to be 0.17 μM/min in medium without Celite, 1.7 μM/min with 10 g/L Celite in the medium, while it increased to 11.7 μM/min with 100 g/L. The molecular sieves employed in the present investigation were made of sodium aluminosilicate, a material similar to Celite, and the initial rate observed in the reaction without protein and the concentration of molecular sieves (40 g/L) corresponded well with their observations. In conjunction with these results, the lower initial rate with the non-catalytic protein casein added to the reaction, seemed to be due to the presence of protein and may be caused by an interaction between protein and the molecular sieves reducing the catalytic effect.

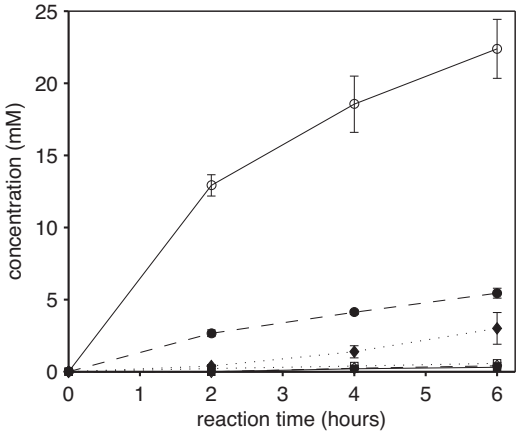

Fig. 2. Progress curves for 2-*O*-lauroyl sucrose. Reaction conditions: (–○) thermolysin, (–●) ALP-901, (···□) alcalase, (–■) subtilisin, (–◊) casein, (···♦) no protein. Error bars indicate standard deviation, determined from triplicate reactions, except for thermolysin (duplicates).

The lower initial rates of the subtilisin formulations compared to the reaction without protein, indicated decreased catalytic activity in these reactions, similar to what was observed in the presence of the non-catalytic protein, casein. Thus, catalytic activity specifically ascribable to subtilisin in either of the two formulations could not be distinguished from that of the non-catalytic protein, casein. Substrate inhibition, particularly with vinyl laurate, could decrease the activity of the enzymes, although subtilisin has been reported to catalyse the acyl-transfer between vinyl laurate and sucrose in DMF–pyridine (1:1, v/v) at higher acyl donor concentrations than employed in the present investigation [10]. Subtilisin and other alkaline proteases have also been reported to exhibit high stability in pure DMF, in fact, higher than in other commonly used hydrophilic, aprotic solvents, such as dimethyl sulfoxide (DMSO), and in some cases higher than in water [13,23], although the granulate of subtilisin A (Alcalase) was observed to dissolve in the reaction medium. In addition, the tautomerisation by-product of acyl transfer with vinyl esters, acetaldehyde, can act as an alkylating agent through formation of Schiff's bases with lysine residues in the protein, which destabilises some enzymes [24], although the presence of molecular sieves has been indicated as beneficial through trapping of the acetaldehyde [25].

The concentration of sucrose was observed to decrease progressively during the course of all the reactions, and the total conversion of sucrose after 48 h ranged from 19 to 96% (see Table 2). The highest degree of conversion was observed in the reaction with thermolysin, while the reactions with no protein and ALP-901 showed degrees of conversion above 50%. The sucrose laurate yield after 48 h ranged from 12 to 53%, and the reaction with ALP-901 resulted in the highest yield. To allow for the complete ester substitution of sucrose, 8 equiv. of acyl donor were used in the reactions. The yield of sucrose laurate compared to the substrate conversion in the reactions with thermolysin, ALP-901 and without protein indicated the formation of di- and oligoesters at the expense of monoesters. The presence of oligoesters was observed after 48 h in these reactions, and the detected amounts of mono- and oligoesters corresponded to the degree of sucrose conversion. Previous reports suggest that a lower ratio of acyl donor to acyl acceptor would increase the selectivity towards monoester product [10,26].

Table 2
Sucrose conversion and sucrose laurate yields after 48 h.

Reaction	Sucrose conversion (%)	Sucrose laurate yield (%)
Thermolysin	96 ± 3	29 ± 2
ALP-901	66 ± 5	53 ± 4
Alcalase	34 ± 5	44 ± 5
Subtilisin	19 ± 8	12 ± 3
Casein	21 ± 9	34 ± 9
No protein	82 ± 2	46 ± 10

Conversion expressed as ratio of concentration decrease to initial concentration, and yield expressed as ratio of detected concentration to theoretical maximum.

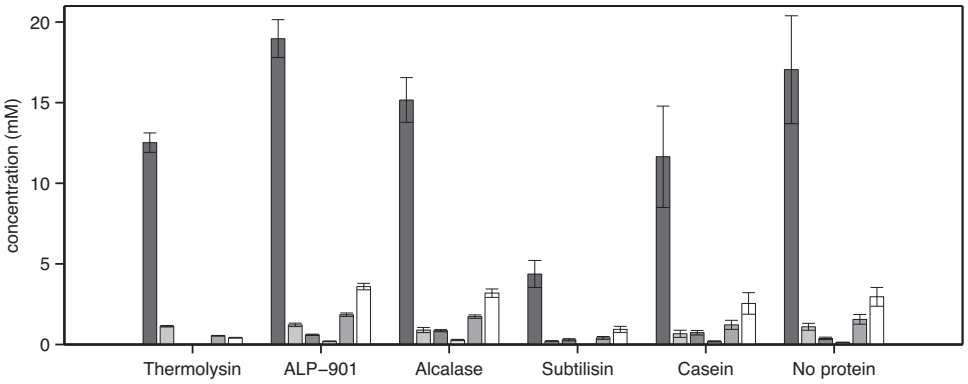

Fig. 3. Catalyst effect on sucrose laurate regioisomer concentration in reaction mixture after 48 h. Regioisomers of sucrose laurate: (■) 2, (■) 3, (■) 4, (■) 6, (■) 1′, □ 3′. The 4′- and 6′-regioisomers were not detected except in trace amounts in the reaction without protein. Error bars indicate standard deviations, determined from triplicate reactions, except for thermolysin (duplicates).

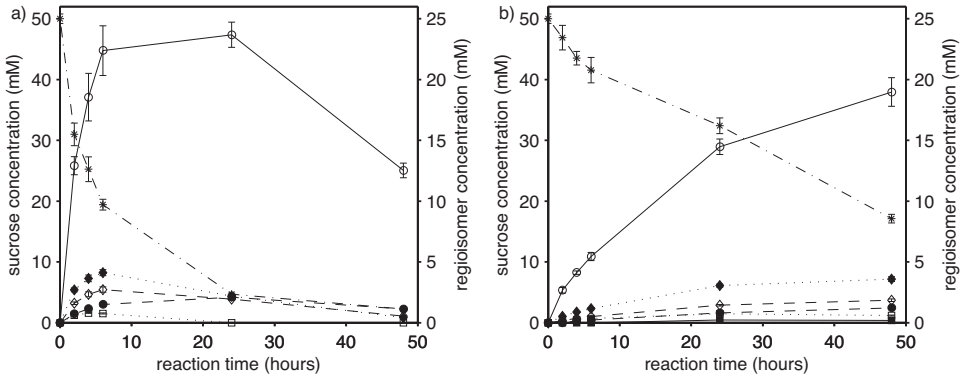

Fig. 4. Progress curves for the reactions catalysed by (a) thermolysin and (b) ALP-901. Sucrose: (–•✱); sucrose laurate regioisomers: (–○) 2, (–●) 3, (···□) 4, (–■) 6, (–◇) 1′, (···◆) 3′. Error bars indicate standard deviations determined from duplicates for thermolysin and triplicates for ALP-901.

The total concentration of sucrose laurate observed after 48 h differed between the reaction conditions, however, 2-*O*-lauroyl sucrose was the most abundant regioisomer in all reactions (see Fig. 3). The regioisomeric distribution of sucrose monoesters after 48 h was similar in the reactions with alcalase, subtilisin and casein, while it differed somewhat in the reactions with ALP-901 and without protein, whose distributions were similar to each other. In the background control, very low concentrations of only the 2- and 3′-regioisomer were detected during 6 h of reaction. The reaction with thermolysin exhibited a unique regioisomeric distribution with lower proportion of the 3′-regioisomer and the 4-regioisomer not detected (see Table 3). The detected appearance of the sucrose laurate regioisomers largely corresponded to the apparent rates of formation, based on regioisomeric distribution and concentration after 48 h.

The reaction progress over 48 h for the two enzyme-catalysed reactions with the highest initial rates was compared (see Fig. 4). In the thermolysin-catalysed reaction the highest concentration of 2-*O*-lauroyl sucrose (23.7 mM) was observed at 24 h (see Fig. 4a). Other regioisomers detected from 2 h, in descending order of concentration, were 3′, 1′, 3 and 4 (see Table 3). The 4-regioisomer concentration peaked at 4 h and could no longer be detected from 24 h. The concentrations of regioisomers 3′ and 1′ peaked at 6 h, while the concentration of the 3-regioisomer was highest at 24 h. The overall highest yield of sucrose laurate (63 ± 5%) was detected in the reaction with thermolysin already after 6 h (see Fig. 4a),

Table 3
Catalyst effect on appearance and distribution of sucrose laurate regioisomers.

Reaction	Reaction time					Regioisomeric distribution after 48 h (2:3:4:6:1′:3′)[a]
	2 h	4 h	6 h	24 h	48 h	
Thermolysin	2, 3, 4, 1′, 3′					86:8:–:–:4:3
ALP-901	2, 4, 1′, 3′	3, 6				72:5:2:1:7:14
Alcalase	2	1′, 3′		3, 4, 6		69:4:4:1:8:14
Subtilisin	2		3′	4, 1′	3	71:3:5:–:7:15
Casein		2, 3′		3, 4, 1′	6	69:4:4:1:7:15
No protein	2, 3′	3, 4, 6, 1′		[b]	4′, 6′	74:5:2:1:7:13

Regioisomer identity is indicated under the reaction time of first detection for each reaction condition.
[a] The regioisomers 4′ and 6′ were only detected in trace amounts in the reaction without protein. Sums of ratios can differ from 100 due to rounding.
[b] The reaction without protein was not sampled after 24 h.

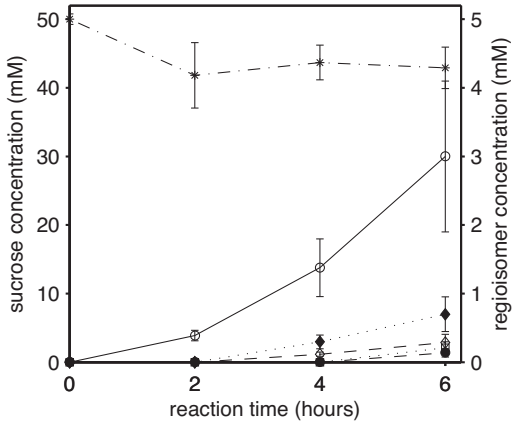

Fig. 5. Progress curves for the reaction without protein. Sucrose: (-·✱); sucrose laurate regioisomers: (–○) 2, (–●) 3, (···□) 4, (–■) 6, (–◇) 1′, (···◆) 3′. Error bars indicate standard deviations determined from triplicate reactions.

where the regioisomeric distribution was 71:5:2:9:13 (2:3:4:1′:3′, 6, 6′ and 4′ not detected) and the degree of sucrose conversion indicated only monoester product was present. The subsequent decline in sucrose laurate concentration was due to oligoester formation, as previously mentioned.

In the reaction catalysed by ALP-901 the concentration of 2-*O*-lauroyl sucrose increased progressively during the reaction to 19.0 mM at 48 h (see Fig. 4b). Other regioisomers detected at 2 h, in descending order of concentration, were 3′, 1′ and 4, while the 3- and 6-regioisomers were detected from 4 h (see Table 3). The concentrations of regioisomers 4 and 6 peaked at 24 h, while the other regioisomers detected were observed in highest concentration after 48 h of reaction.

The reaction progress over 6 h for the control reaction without protein was also considered (see Fig. 5). The 2-regioisomer was the most abundant at 3.00 mM after 6 h, and the regioisomeric distribution at this time, 69:3:5:1:7:16 (2:3:4:6:1′:3′), was very similar to the distributions observed in the reactions with alcalase, subtilisin and casein after 48 h (see Table 3). The 3′-regioisomer was detected from 2 h and the regioisomers 1′, 4, 6 and 3, in descending order of concentration, were detected from 4 h. The concentration of sucrose varied irregularly between the time points and the standard deviations were also large, possibly as a result of the samples analysed containing sucrose concentrations close to the upper limit of the detector range. After 48 h, all regioisomers were detected in the sample and the highest concentrations of all regioisomers were observed, with 17.0 mM of 2-*O*-lauroyl sucrose detected.

The observed selectivity of both thermolysin and ALP-901 was predominantly towards formation of the 2-regioisomer. This corresponded to previous reports on thermolysin-catalysed acyl transfer reactions in hydrophilic, aprotic solvents [19,27], and reports on catalysis by another alkaline protease, AL-89, in vinyl fatty acid acyl transfers to sucrose in DMF-DMSO (1:1, v/v) [13]. Previously reported subtilisin-catalysed reactions with short-chained acyl donors in various DMF–co-solvent mixtures, have shown selectivity for acylation at the 1′-position of sucrose [11,16,28], however, as outlined above, the catalytic activity of the subtilisin preparations employed in the present investigation appeared to be negligible and the regioisomeric distribution. At 48 h the regioisomeric distribution of the thermolysin catalysed reaction differed significantly from the remaining reactions, while the proportion of the 4-regioisomer of the reactions with ALP-901 and without protein was half that of the three slower reactions. Nuclear magnetic resonance studies of sucrose in DMSO solution have showed intramolecular hydrogen bonds between 2-OH and both 1′-OH and 3′-OH [29], which have conformation-stabilising effects. Computer modelling of molecular electrostatic potential profiles showed the 2-OH substituent to be the most electropositive hydroxyl group, and experimental confirmation by benzylation in DMF resulted in the order of reactivity 2-OH > 1′-OH > 3′-OH [30]. Acid chloride acylation in water have also confirmed the 2-OH as the most acidic and reactive substituent [31,32]. Molinier et al. [33] showed that acyl migration in sucrose monoesters is catalysed by aqueous, hydrophilic organic media, resulting in rapid conversion from the 2- to the 3-regioisomer, which has also been shown to be catalysed by the protease AL-89 [13], and slower further conversion to the 6-regioisomer. Even slower migrations favour the primary hydroxyl groups 6, 1′ and 6′. The resulting regioisomeric distribution of sucrose acylation does not necessarily express the relative reactivity of the hydroxyl groups, but the intricate competition between esterification and migration [32]. In the current reactions, the regioisomeric distribution conformed largely to the reactivity predicted by the electrostatic potential with the major products being the 2-, 3′- and 1′-regioisomers, indicating that acyl migration was not favoured.

The distinctly different product compositions after 48 h in the reaction with thermolysin seemed to be a result of the overall higher reaction rate and the reaction thus progressing further over the reaction time studied. The reaction progress over 48 h for ALP-901 resembled that over 6 h for thermolysin, as supported by the similarity of the sucrose laurate regioisomeric distribution and degree of sucrose conversion at these respective reaction times, and by the decline in the concentration of some regioisomers from after 4 h with thermolysin and after 24 h with ALP-901.

4. Conclusion

The acylation of sucrose with vinyl laurate acyl donor in the hydrophilic, aprotic solvent *N*,*N*-dimethylformamide resulted in the formation of sucrose laurate in yields from 12 to 53% after 48 h under different catalytic conditions. The serine protease ALP-901 produced the highest yield at this reaction time, while the reaction with thermolysin achieved the overall highest yield (63%) after 6 h, with only monoesters synthesised. 2-*O*-Lauroyl sucrose was the most abundant monoester regioisomer synthesised, it was among the first regioisomers to appear and its concentration increased over the first 6 h in all reactions. The highest concentration of 2-*O*-lauroyl sucrose observed was 23.7 mM after 24 h in the thermolysin-catalysed reaction, while in the reaction catalysed by ALP-901 and in the reaction without protein, it was 19.0 mM and 17.0 mM after 48 h, respectively. The initial rates of formation of the reactions catalysed by thermolysin and ALP-901 were more than 450 and 90 times the rate of the chemical background reaction, respectively.

The detected appearance of the sucrose laurate regioisomers largely corresponded to the apparent rates of formation, based on initial rates of formation for the 2-regioisomer and monoester concentration and regioisomeric distribution after 48 h. The observed sucrose laurate regioisomeric distribution after 48 h (2:3:4:6:1′:3′) was 72:5:2:1:7:14 in the reaction catalysed by ALP-901, and 74:5:2:1:7:13 in the reaction without protein. In the reaction catalysed by thermolysin, the distribution was 71:5:2:–:9:13 after 6 h, while it was 86:8:–:–:4:3 after 48 h. The concentration of sucrose laurate was observed to decline with reaction times above 6 h in the reaction with thermolysin due to oligoester formation.

The reaction between sucrose and vinyl laurate with no protein in the reaction mixture appeared to be catalysed by aluminosilicate molecular sieves present in the reaction medium, with an initial rate of formation 13 times higher than that of the chemical background reaction. Non-catalytic protein in the reaction medium seemed to lower the catalytic activity of the molecular sieves.

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