Tetrahedron Letters, Vol.31, No.15, pp 2131-2134, 1990
Printed in Great Britain

0040-4039/90 $3.00 + .00
Pergamon Press plc

ACCELERATED ESTERIFICATION OF AMINOACIDS WITH LIPOGLYCOSYLATED α-CHYMOTRYPSIN IN POLAR SOLVENTS.

Daniel Cabaret, Sophie Maillot and Michel Wakselman

CNRS-CERCOA, 2, rue Henri Dunant, F-94320 Thiais, France

Summary: A lipodisaccharide possessing a reactive aldopentose function, the 6-O-octyl-β-D-galactopyranosyl-(1→5)-L-arabinose (**5**), has been prepared. The reductive alkylation of four of the lysine residues of the bovine α-chymotrypsin led to a lipo-1-deoxyglycytolated enzyme. This modified protein efficiently catalyzed the synthesis of N-acetyl aminoacid ethyl esters in different solvents with 2.5-3 % water contents. Compared to the native enzyme, enhanced esterification rates were determined, particularly in tetrahydrofuran, ethyl acetate and acetonitrile.

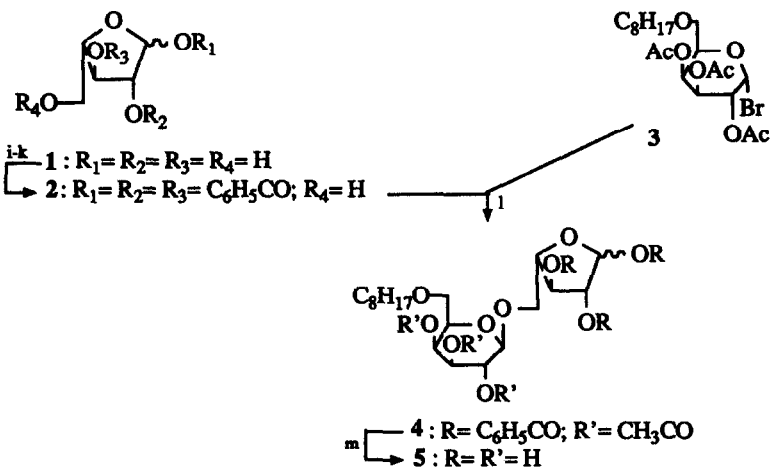
In the enzymic synthesis of esters or peptides, organic solvents are often used to decrease the activity of water; they shift the equilibrium of the reaction (thermodynamic control), or decrease the competitive hydrolysis of the substrate (kinetic control). In other enzyme-catalyzed conversions, solvents are sometimes needed to solubilize starting or end products. To reduce the denaturing effect of the solvent, several procedures have been proposed: polyphasic media [1], such as reverse micelles [2], suspended enzymes in hydrophobic solvents which do not strip off the essential layer of water around the catalyst [3] and high-molecular-weight polyethylene-glycol-modified enzymes soluble in low polar solvents [4]. Recently, Kise and Martinek have studied reactions of polar substrates in which an hydrophilic alcohol acts both as a reactant and as a solvent [5,6].

Some years ago, we suggested [7] the synthesis of amphiphilic reagents and the use, for organic synthesis, of the modified enzymes suspended in polar solvents with low water content [8]. In this system, the hydrophilic chains introduced can retain water molecules, whereas the hydrophobic substituents can insert into the solvent. *Altogether, a covalent model of a reverse micelle, in a polar solvent, can result.* Owing to the stabilizing effect of polyols and sugars on enzymes [10], lipoglycosylated reagents were designed. As the mild reductive alkylation of the ϵ-amino groups of external lysine residues preserves the positive charge, and the original locus for that charge on the protein surface [11], a latent aldehyde group was selected as the reactive function of the reagent.

On purpose, α-chymotrypsin, a globular protein which is not well formulated to operate in organic media [3], was chosen as the first enzyme to be studied. We report here the synthesis of a new lipodisaccharide reagent **5**, its reductive conjugation (1-deoxyglycitolation reaction) with α-chymotrypsin and the use of the new biocatalyst for the esterification of N-protected aminoacids in different organic solvents.

1. Synthesis of the 6-O-octyl-β-D-galactopyranosyl-(1→5)-L-arabinose 5;

Previously, we prepared several liposaccharides [12]. However, in the 1-deoxyglycitolation reaction, aldopentoses exhibit faster rates of alkylation than their more stable aldohexoses analogs [13]. As a too long condensation reaction time could have a detrimental effect on the catalytic activity of the new biocatalyst, the synthesis of a liposaccharide reagent having a furanose end has now been achieved in a few steps.



i: $ClC(C_6H_5)_3$/pyr; j: C_6H_5COCl/pyr; k: $ClSiMe_3$,NaI/CH_3CN; l: $Hg(CN)_2$, 72% yield; m: NaOMe/HOMe.

Fig.I: Synthetic scheme for the preparation of the reagent **5**.

The synthetic scheme is shown on Fig. 1. To avoid a possible intramolecular acyl transfer during the cleavage of a trityl protecting group, benzoylation was prefered to acetylation [14] and mild conditions [15] were used for the detritylation of the benzoylated arabinose. Königs-Knorr condensation with the previously described glycosyl donor **3**[12a] led to the protected disaccharide **4**. Removal of the esters groups easily gave the liposaccharide **5**.

2. Chemical modification of bovine α-chymotrypsin:

Using different ratios of reagent over enzyme, several experiments were performed. As an excess of reagent **5** led to insoluble inactive products, near stoichiometric conditions were selected. Thus, to a solution of 4 μmol of the enzyme in 10 ml of 0.1 M borate buffer pH 8.0, at 4°C, were added 56 μmol of **5** and after 15 min 0.56 mmol of $NaBH_3CN$. After 24 h, dialysis against 10^{-4} M HCl and water, and then lyophilization, gave the modified α-chymotrypsin as a fluffy white solid.

3. Properties of the modified enzyme:

Free amino groups were determined using the 2,4,6-trinitrobenzenesulfonic acid method [16]. On average,

four amino groups were modified per molecule of enzyme. The protein concentration was estimated by measuring the absorbance at 280 nm ($\epsilon = 5.10^4\ M^{-1}$). With *p*-nitrophenyl acetate as substrate, active site titrations were done, following the *p*-nitrophenolate anion burst at 402 nm [17]. The activities of the starting (Fluka) and modified α-chymotrypsin were found to be 76.5 and 50% respectively.

4. Aminoacid esterifications:[1a,5,18]

General procedure: To a solution of 0.1 μmol of the modified chymotrypsin in 0.125 ml of 0.1 M phosphate buffer pH 6.8, were added 4.6 ml of the organic solvent, 0.297 ml (5 mmol) of ethanol and 55 μmol of the N-acetyl aminoacid, at 30°C.

Particular cases: N-Ac-L-Phe: For this aminoacid, 0.15 instead of 0.125 ml of the buffer were used, which raised the water content from 2.5 to 3% [5b]; *$CHCl_3$:* The general conditions led to an inefficient two-phase system. Therefore 1.45 ml of EtOH and only 3.42 ml of the solvent were used; *EtOH:* 4.88 ml.

In all cases, good *pseudo*-first-order rate constants were obtained from the HPLC data: Nucleosil C_{18}-column; eluent: 35-65 (v/v) acetonitrile-water. The results are collected in Tables I and II, and compared with that of the native enzyme in the same experimental conditions. In ethanol (Kise's conditions [5]), there is no advantage of using the modified enzyme: the rate constants for the modified (k_m) and the native (k_n) enzymes, and therefore the reaction half-times (t_m and t_n) and the percentages of the formed aminoacid esters at the end of the reaction (e %) are about the same. In the other five solvents, the rates of the lipoglycosylated chymotrypsin-catalyzed esterifications are higher than those catalyzed by the native enzyme: $r = k_m/k_n \geq 1$ and $t_m \leq t_n$ [19]. The rate enhancement is particularly striking in THF, acetonitrile and ethyl acetate. There is no evident correlation of the rate acceleration factor *r* with the log *P* of the solvent [20]. At equilibria, the ester yields (e %) are however about the same using both biocatalysts.

	$CHCl_3$ (log *P*= 2.0)			THF (log *P*= 0.49)			acetone (log *P*= -0.23)		
	r[a)]	t_m[b)](e)	t_n(e)[c)]	*r*	t_m(e)	t_n(e)	*r*	t_m(e)	t_n(e)
Ac-Tyr	1.74	0.31(90)	0.55(90)	3.70	1.20(50)	4.42(50)	1.48	1.25(70)	1.58(70)
Ac-Trp	1.11	1.41(90)	1.56(92)	2.30	5.30(53)	12.2(53)	1.26	4.50(69)	5.60(69)
Ac-Phe	1.47	8.50(80)	12.5(87)	2.20	8.75(40)	11.2(40)	1.31	22.5(60)	29.5(60)

Table I: Esterification of N-acetyl aminoacids in chloroform, tetrahydrofuran and acetone.

a) $r = k_m/k_n$, ratio of the *pseudo*-first-order constants for esterifications of the N-acetyl-L-aminoacids catalyzed with the modified and the native enzymes.

b) t_m, t_n: half time, in hours, of the reaction using the modified or the native enzyme.

c) e: percentage of the aminoacid ethyl ester product at the end of the reaction.

	EtOH (log *P*= -0.24)			CH_3CN (log *P*= -0.33)			EtOAc (log *P*= -0.68)		
	$r^{a)}$	$t_m{}^{b)}$(e)	t_n(e)$^{c)}$	*r*	t_m(e)	t_n(e)	*r*	t_m(e)	t_n(e)
Ac-Tyr	1.07	7.0 (87)	7.30(87)	3.13	4.6 (80)	14.4(78)	5.69	3.25(76)	18.5(74)
Ac-Trp	0.94	13 (89)	12.1(89)	2.18	44 (82)	96 (74)	5.18	5.33(74)	27.7(63)
Ac-Phe	0.95	45(65)	42(70)	1.35	63(59)	85.8(29)	2.61	10(60)	26(60)

Table II: Esterification of N-acetyl aminoacids in ethanol, acetonitrile and ethyl acetate.

References and Notes

[1] a) J. L. Vidaluc, M. Baboulene, V. Speziale, A. Lattes and P. Monsan, *Tetrahedron*, 1983, **39**, 269; b) D. K. Eggers, H. W. Blanch and J. H. Prausnitz, *Enzyme Microb. Technol.*,1989, **11**, 84.

[2] P. Walde, Q. Peng, H. Fadnavis, E. Battistel and P. L. Luisi, *Eur. J. Biochem.*, 1988, **173**, 401.

[3] a) A. M. Klibanov, *Trends Biochem. Sci.*, 1989, **14**, 141; b) J. S. Dordick, *Enzyme Microb. Technol.*, 1989, **11**, 194.

[4] a) K. Takahashi, A. Ajima, T. Yoshimoto, M. Okada, A. Matsushima, Y. Tamaura and Y. Inada, *J. org. Chem.*, 1985, **50**, 3414; b) Y. Inada, K. Takahashi, T. Yoshimoto, A. Ajima, A. Matsushima, and Y. Saito, *Trends Biotechnol.*, 1986, **4**, 190; c) H. Gaertner and A. Puigserver, *Eur. J. Biochem.*, 1989, **181**, 207; d) M. T. Babonneau, R.Jacquier, R. Lazaro and P. Viallefont, *Tetrahedron Lett.*, 1989, **30**, 2787.

[5] a) H. Kise and H. Shirato, *Tetrahedron Lett.*, 1985, **26**, 6081. b) H. Kise, H. Shirato and H. Noritomi, *Bull. Chem. Soc. Jpn.*, 1987, **60**, 3613; c) H. Kise and H. Shirato, *Enzyme Microbiol. Technol.*, 1988, **10**, 582.

[6] V. Cerovsky and K. Martinek, *Coll. Czech. Chem. Comm.*, 1989, **54**, 266.

[7] M. Wakselman and D. Cabaret, *Biocatalysis in organic media*, C. Laane, J. Tramper and M. D. Lilly eds, Elsevier 1987, 253.

[8] subsequently, a soluble amphiphile-coated lipase (uncovalent complex) has been used in low polar solvents[9].

[9] a) Y. Okahata, Y. Fujimoto and K. Ijiro, *Tetrahedron Lett.*, 1988, 5133; b) Y. Okahata and K. Ijiro, *J. Chem. Soc. Chem. Comm.*, 1988, 1392.

[10] a) D. I. Metelitsa and A. N. Eremin, *Russ. Chem. Rev.*, 1987, **56**, 1096; b) J. F. Carpenter and J. H. Crowe, *Biochemistry*, 1989, **28**,3916.

[11] G. M. Means, *J. Protein Chem.*, 1984, **3**, 121

[12] a) D. Cabaret, R. Kazandjan and M. Wakselman, *Carbohydr. Res.*, 1986, **146**,464; b) D. Cabaret and M. Wakselman, *ib*, 1989, **189**,341

[13] W. S. D. Wong M. M. Kristjansson, D. T. Osuga and R. E. Feeney, *Int. J. Peptide Protein Res.*, 1985, **26**, 55.

[14] C. du Mortier, O. Varela and R. M. de Lederkremer, *Carbohydr. Res.*, 1989, **189**, 79.

[15] A. Klemer, M. Bieber and H. Wibers, *Liebig's Ann. Chem.*, 1983, 1416.

[16] R. Fields, *Methods Enzymol.*, 1972, **25B**,464.

[17] a) B. S. Hartley and B. A. Kilby, *J. Biol. Chem.*, 1954, **56**, 1954; b) M. L. Bender, F. J. Kezdy and F. C. Wedler, *J. Chem. Ed.*, 1967, **44**, 84.

[18] a) M. H. Remy, P. Bacou, C. Bourdillon and D. Thomas, *Biochim. Biophys. Acta*, 1987, **911**, 252; b) M. Reslow, P. Adlercreutz and B. Mattiasson, *Eur. J. Biochem.*,1988, **172**, 573

[19] In the esterification of Ac-Tyr in CH_3CN or EtOAc, α-chymotrypsin modified with the 6'-O-octyl melibiose is more efficient than the biocatalyst formed from the melibiose (a reducing disaccharide).

[20] C. Laane, S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng.*, 1987, **30**, 81.

(Received in France 20 December 1989)