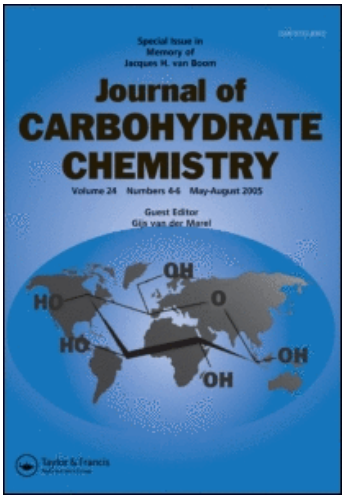

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Becker, K. -C. and Kuhl, P.(1999) 'Synthesis of *O*-β-Galactopyranosyl-L-Serine Derivatives Using β-Galactosidase in Aqueous-Organic Reaction Systems', Journal of Carbohydrate Chemistry, 18: 2, 121 — 129
To link to this Article: DOI: 10.1080/07328309908543986
URL: http://dx.doi.org/10.1080/07328309908543986

J. CARBOHYDRATE CHEMISTRY, 18(2), 121-129 (1999)

SYNTHESIS OF *O*-β-GALACTOPYRANOSYL-L-SERINE DERIVATIVES USING β-GALACTOSIDASE IN AQUEOUS-ORGANIC REACTION SYSTEMS

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Received April 3, 1998 - Final Form November 4, 1998

ABSTRACT

The galactosylation of amino protected serine methyl ester derivatives by β–galactosidases from *E. coli* and *A. oryzae* in aqueous-organic solvents was investigated. A comparison of enzyme activity in aqueous buffer and in several aqueous-organic mixtures revealed that the presence of an organic solvent normally causes a loss in enzyme activity. When the enzyme is used in suspensions with mainly undissolved lactose, the detrimental influence of an organic solvent is less marked if it does not exceed 25 % of the added mixture with water. Employing organic cosolvents, such as acetonitrile, 2-butanone, acetone or ethyl acetate, we obtained yields of the desired galactosylation products higher than those with the enzyme in purely aqueous solution. The amino protecting group shows a significant influence on the transglycosylation reaction in terms of yield, the best up to 28 % being achieved in the synthesis of Aloc-(Galβ1-)Ser-OMe with β–galactosidase from *E. coli* in a reaction mixture containing 8 to 15 % organic solvent.

INTRODUCTION

Glycoproteins play a central role in cell-cell recognition, bacterial and viral infection and oncogenesis.[1] Attachment of a sugar molecule to a biologically active

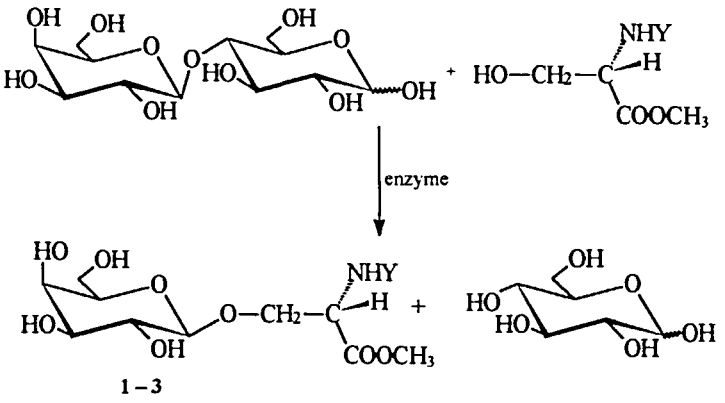
peptide sequence offers protection against proteolytic enzymes and improves receptor affinity. In glycoproteins oligosaccharide chains are bound N- or O-glycosidically to asparagine and serine/threonine, respectively.[2]

Glycosidases in particular are of great interest for the stereospecific synthesis of partial structures of naturally occuring glycoproteins. As reported previously, the O-glycosidic attachment of carbohydrates to a hydroxyaminoacid derivative by β-galactosidase was successfully performed.[3,4,5,6,7] However, the use of naturally occuring disaccharides as glycosyl donors mostly results in low yields of the desired product, and the outcome is very dependent on the origin of the enzyme.[8] Therefore, methodical improvements in the enzyme catalyzed synthesis of glycoconjugates are of significant importance.

Under physiological conditions and provided that specificity requirements are met, the usual result of a glycosidase action on a disaccharide is the hydrolysis of the glycosidic bond. In order to achieve mainly the formation of glycosidic linkages, manipulations of the reaction system are necessary, e.g., by adding organic cosolvents and lowering the water activity this way.[9] β-Galactosidase-mediated transglycosylation procedures in the presence of water-miscible organic solvents were also applied to the synthesis of β-D-galactosyl disaccharide derivatives and *n*-alkyl β-D-galactopyranosides.[6,10,11] Initially, we have studied the activity of two commercially available β-galactosidases in the presence of different organic solvents. The most suitable of these solvents have then been applied in moderate amounts in order to improve the synthesis of galactosyl-serine derivatives according to Scheme 1.

RESULTS AND DISCUSSION

At the beginning of our studies, the influence of aqueous-organic solvent mixtures on the activity of *E. coli* and *A. oryzae* β-galactosidase was investigated. Depending on the kind of organic solvent as well as its concentration, diminished enzyme activities were observed. Employing dimethylformamide, dimethylsulfoxide or pyridine, even in small amounts, gives a dramatic loss in enzyme activity and the β-galactosidase is damaged irreversibly. However, in experiments using ethyl acetate, cyclohexane, isopropyl ether or toluene, a sufficient residual enzyme activity after 24 h was found.



(Aloc = allyloxycarbonyl, Boc = *tert*-butyloxycarbonyl, Z = benzyloxycarbonyl)

Scheme 1

Selected results are given in Table 1 and 2. No correlation was found between enzyme stability and physico-chemical properties of the solvent. Based on the results from the activity tests, we have chosen acetonitrile, acetone, ethyl acetate and 2-butanone for galactosylation reactions.

On many occasions we observed that the lactose content is very important in terms of suppressing the detrimental influence of an organic solvent on the enzyme activity. Increasing the amount of lactose from 0.2 to 1.5 mmol in mixtures of aqueous acetone or acetonitrile and β-galactosidase from *E. coli* resulted in a considerable stabilization of the biocatalyst in these media. This is demonstrated in Table 3. Preliminary experiments showed that this seems to be valid also for β-galactosidase from *A. oryzae*.

The positive effect of sugar additives in stabilizing β-galactosidase from *A. oryzae* against deactivation by organic solvents was studied by Sundaram and Shubhada.[12] Based on their results, it seems possible that high lactose concentration, followed by high concentrations of glucose and galactose during hydrolysis, shows a similar effect in terms of protecting the enzyme against the detrimental influence of the organic solvent.

TABLE 1. Effect of organic solvent on the activity of β-galactosidase from *E. coli* after 24 h at 37 °C [a]

Cosolvent [%]	Residual enzyme activity [%]							
	DMF	DMSO	ethyl acetate	acetone	aceto-nitrile	pyridine	2-butanone	dichloro methane
5	70	83	100	92	91	–	98	104
10	64	–	92	90	88	48	86	97
15	52	39	91	92	90	32	90	80
20	19	15	–	80	56	–	84	–
30	5	–	65	–	–	–	–	25
50	0	0	40	38	11	0	25	14

a. Mixtures of aqueous-organic solvents and enzyme (10 U) with a total volume of 2 mL were agitated by a magnetic stirrer.

TABLE 2. Effect of organic solvent on the activity of β-galactosidase from *A. oryzae* after 24 h at 37 °C [a]

Cosolvent [%]	Residual enzyme activity [%]							
	DMF	DMSO	ethyl acetate	acetone	aceto-nitrile	pyridine	2-butanone	dichloro methane
5	90	89	98	95	94	–	95	101
10	84	84	102	92	93	72	91	95
15	78	64	101	90	90	63	92	90
20	52	–	95	90	–	–	–	84
30	39	–	92	–	–	–	65	67
50	10	5	85	70	38	0	44	42

a. For experimental details see Table 1.

The enzymatic synthesis of serine galactosides strongly depends on the protecting groups of the serine derivative and the type of glycosyl donor.[3,4,13] Applying β-galactosidases from different sources (e.g. also from *A. niger*, *B. circulans* and bovine liver) we obtained best results with the enzyme from *E. coli*. As glycosyl donors we obtained good results with low-priced lactose as well as the more active ONPG (*ortho*-nitrophenyl-β-D-galactopyranoside).[13] Although the latter gives higher yields than lactose as glycosyl

TABLE 3. Residual activity at 37 °C after 24 h of β-galactosidase from *E. coli* in aqueous-organic mixtures with lactose [a]

	residual enzyme activity [%]		
	lactose content [mmol]		
30 % (v/v) cosolvent	**0.2**	**1**	**1.5**
acetone	49	93	96
acetonitrile	22	72	89

a. Mixtures of Na-phosphate buffer (0.03 M; pH 7.3), cosolvent and enzyme (50 U) with a total volume of 300 µL and lactose were shaken on a thermo-mixer.

donor, we observed formation of undesired side products, such as ONP-digalactosides with various linkages.[14,15] At the kinetically-controlled maximum of product formation a considerable amount of unreacted ONPG was still present, which we were not able to separate from the desired product without significant losses.

On the contrary, using lactose, the desired glycoamino-acid derivative can be easily separated from lactose and its hydrolysis products. The addition of 10 – 15 % (v/v) acetonitrile, acetone, ethyl acetate or 2-butanone to a highly concentrated reaction mixture of 1 mmol lactose and 2 – 3 mmol serine derivative promotes the formation of the transglycosylation product. As can be seen from Fig. 1, in the synthesis of **1** yields up to 28 % were obtained, depending on the type and the amount of solvent. One reason for this effect may be the improved solubility of the serine derivative in the presence of these organic solvents. As far as water activity is concerned, it cannot be expected that it is much reduced by the relatively small amounts of organic solvents used.[16,17] In reaction mixtures containing more than 50 % of organic solvent, no galactosylation of the serine derivatives took place. Under the conditions described above, the reaction optimum was reached between 15 and 20 h. The yields obtained are higher than those reported so far with β-galactosidase from *E. coli* in purely aqueous solutions.[3,4]

Protecting the amino group of the serine ester with the Boc or Z group gave lower yields in comparison to those achieved with Aloc-Ser-OMe. With the Fmoc (*N*-Fluorenylmethyloxycarbonyl)-protected serine methyl ester no galactosylation occurred

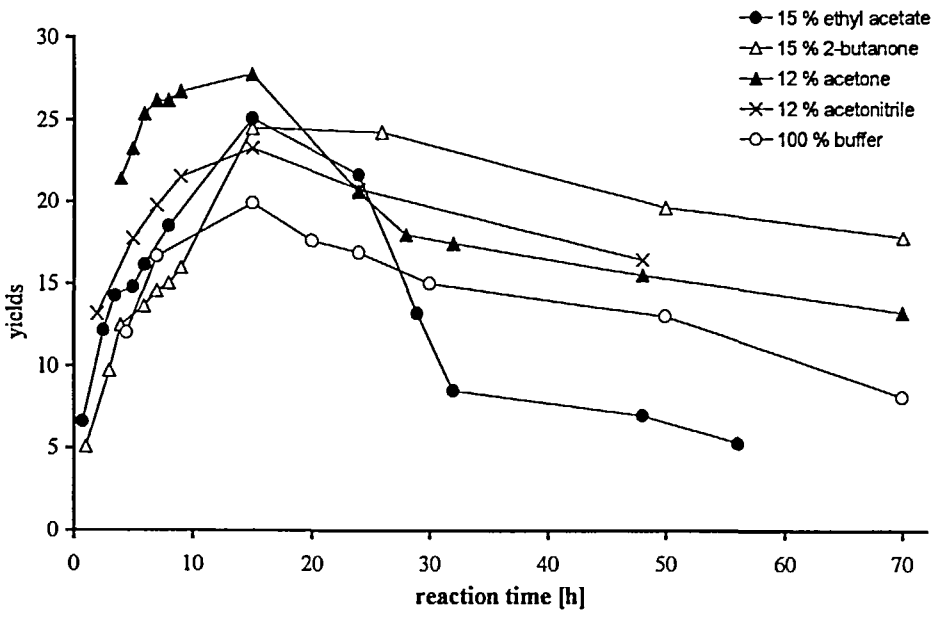


Figure 1. Effect of organic solvents on the formation of Aloc-(Galβ1-)Ser-OMe using β–galactosidase from *E. coli* and lactose as glycosyl donor.

at all. A correlation between structure of the N-protecting group and yield of the enzymatic galactosylation appears possible, but this phenomenon requires further investigation. Employing two different β-galactosidases with protected serine derivatives, we obtained the results summarized in Table 4. The product yields given here are about 20 to 80 % higher than those reported so far.[3,4] Although β-galactosidase from *A. oryzae* was more resistant towards the influence of organic solvents, considerably lower yields were obtained compared with the enzyme from *E. coli* under the same experimental conditions.

CONCLUSIONS

The galactosylation of N^{α}-protected serine methyl esters by the action of β-galactosidases from *E. coli* and *A. oryzae* can be improved by carrying out the reactions in the presence of moderate amounts of selected organic solvents. Especially useful are

TABLE 4. Synthesis of galactosylated N^{α}-protected serine methyl esters using β-galactosidases from *E. coli* and *A. oryzae* in aqueous-organic reaction systems [a]

Product	Cosolvent [% v/v]	Reaction time [h]	Yields [%] with β-Galactosidase from	
			E. coli	*A. oryzae*
Aloc-(Galβ1-)Ser-OMe	acetone (15)	18	28	11
Boc-(Galβ1-)Ser-OMe	2-butanone (10)	15	18	9
Z-(Galβ1-)Ser-OMe	2-butanone (10)	15	15	< 1

a. 37 °C; 1 mmol lactose; 2 mmol Aloc-Ser-OMe/Boc-Ser-OMe, 3 mmol Z-Ser-OMe; 200 U enzyme; 0.5mL total volume of solvents (buffer and cosolvent).

acetone, acetonitrile, 2-butanone and ethyl acetate in concentrations up to 15 % (v/v). Performing these reactions in highly concentrated suspensions using cheap lactose as both galactosyl donor and enzyme protectant presents an advantageous alternative method for the synthesis of *O*-β-galactopyranosyl-L-serine derivatives, even though the yields remain rather modest so far.

EXPERIMENTAL

Materials. β-galactosidases from *E. coli* (420 U/mg) and *A. oryzae* (6 U/mg) were obtained from Fluka and Armano, respectively. Lactose and analytical standards for HPLC of glucose and galactose were provided from Sigma. Serine methyl ester was purchased from Fluka. The N^{α}-protected serine methyl esters were synthesized according to published procedures.[18,19] Buffers were supplied by Fluka, for β-galactosidase from *E. coli* Na-phosphate buffer (0.03 M; pH 7.3) and for β-galactosidase from *A. oryzae* Na-citrate buffer (0.1 M; pH 5.0) were used. All organic solvents (p.A.) were supplied by Aldrich.

Enzyme activity measurements. The enzyme (10 U) was incubated at 37 °C in continuously stirred aqueous-organic mixtures containing various amounts of organic solvent. At different time intervals aliquots of 20 µL were withdrawn and the activity was determined by the ONPG method.[20] The results are expressed as percentage of enzyme activity in buffer solution (Table 1 and 2).

Transglycosylation reaction. Reactions were performed at 37 °C in 2-mL vials. Lactose-monohydrate (360 mg, 1 mmol) and Aloc-Ser-OMe (406 mg, 2 mmol), Boc-Ser-OMe (438 mg, 2 mmol) and Z-Ser-OMe (760 mg, 3 mmol), respectively, were suspended in buffer solution appropriate for the enzyme used. Then the enzyme (200 U, suspended in 20 µL buffer) and the organic solvents given in Table 4 were added. The total amount of buffer and organic cosolvent was 0.5 mL. All reactions were performed in a thermo-mixer and vigorously shaken at a frequency of 1000 oscillations per minute. The reaction was monitored by withdrawing aliquots at different times followed by HPLC analysis. After a reaction time of 15 – 20 h the mixture was extracted with dichloromethane. The aqueous phase was concentrated under vacuum and then subjected to preparative HPLC.

Purification and analytical methods. Purification of the serine galactosides was achieved by a GAT/Waters HPLC system on a LiChrospher NH_2 column (10 µm), 250 × 10 mm from Merck using an acetonitrile-water eluent (80/20) at a flow rate of 8 mL/min. Analytical HPLC was done with a LiChrospher NH_2 column (5µm), 250 × 4 mm from Merck using an acetonitrile-water gradient (0 – 40 % water) at a flow rate of 1 mL/min. Analyses and purifications were performed using a light scattering detector from S.E.D.E.X. (France). ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX 500 spectrometer (500 MHz). The samples were dissolved in d_6-dimethylsulfoxide. The signals obtained were in agreement with published data.[3,4]

ACKNOWLEDGEMENT

This work was partly funded by the Deutsche Forschungsgemeinschaft (DFG) and the CHEMTEC-LEUNA GmbH. The authors thank as well Dr G. Liebscher, Dr D. Scheller, Mrs H. Ihrke and Mrs R. Jauch for analytical and technical support.

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