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High-Yield Synthesis of *N*-Acetyllactosamine by Regioselective Transglycosylation

Amedeo Vetere,*,[1] and Sergio Paoletti*,†,[2]

**Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università degli Studi di Trieste, Via L. Giorgieri 1, 1-34127 Trieste, Italy; and †Istituto di Chimica Biologica, Facoltà di Medicina e Chirurgia, Università degli Studi di Sassari, Viale S. Pietro 43/B, 1-07100 Sassari, Italy*

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The synthesis of *N*-acetyllactosamine (D-Gal*p* β1-4D-Glc*p*NAc) with very low contamination of its isomer *N*-acetylallolactosamine (D-Gal*p* β1-6D-Glc*p*NAc) was obtained by use of regioselective transglycosylation activity of β-galactosidase from *Bacillus circulans* using lactose as the donor of D-Gal*p* and D-Glc*p*NAc as the acceptor. The reaction was conducted at 15°C and at pH 5.0. The incubation time was considerably reduced and the yield improved 100% with respect to the best results so far described in the literature.

N-Acetyllactosamine is one of the most representative core structure in oligosaccharide components of glycoproteins (1). It is the main component of the oligosaccharides obtained from human milk where it plays an important biological role (2). This disaccharide has been found to occur in sialyl-Lewis^{x}, identified as a functional ligand for E-, P-and L-selectin (3–6), which mediate trafficking and recruitment of blood-borne leukocytes to endothelial cells during normal (7) and pathological (8) inflammatory response to injury and infection. In addition, there is evidence that metastasis formation in some tumors seems to be linked to the expression of sialyl-Lewis^{x} (9–11). Chemical approaches has been developed for the synthesis of *N*-acetyllactosamine (12, 13) and other biological oligosaccharides (see 14 for a review). Enzymatic synthesis by use of glycosyltransferases has been reported (see 15, 16 for reviews; 17). Such enzymes are very attracting for their efficiency and high regio- and stereoselectivity but do not look to be very promising for large-scale preparative applications since they are not readily available and require cofactors for inducing the reaction. From a practical viewpoint the use of transglycosylation reactions of glycosidases is more interesting (see 18–20 for reviews). Glycosidases are much less expensive than glycosyltransferases. The first glycosidase-assisted synthesis of *N*-acetyllactosamine was described by Zilliken *et al.* (21) using β-galactosidase from *Lactobacillus bifidus,* but the yield was low (less than 5%). Other β-galactosidases have been used but the transfer of D-Gal*p* from donor occurred mainly on primary hydroxyl group (OH-6) or on OH-3 secondary group (22–25). More interesting results have been reported by use of β-galactosidase

[1] Corresponding author; Dipartimento di Biochimica, Biofisica e Chimica delle Macromelecole, Universtà degli Studi di Trieste, Via L. Giorgieri 1, 1-34127 Trieste (Italy). Fax: +39-40-6763691; e-mail: vetere@univ.trieste.it.

[2] Present permanent address: Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università degli Studi di Trieste, Via L. Giorgieri 1, I-34127 Trieste (Italy). Fax: +39-40-6763691; e-mail: vetere@univ.trieste. it.

Abbreviations: LacNAc, *N*-acetyllactosamine; AlloLacNAc, *N*-acetylallolactosamine; D-Gal*p* β1-4D-Glc*p*NAc, 4-O-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucopyranose; D-Galp β1-6D-Glc*p*NAc, 6-O-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucopyranose; D-Gal*p,* D-galactopyranose; D-GlcNAc*p,* 2-acetamido-2-deoxy-D-glucopyranose; D-Glc*p,* D-glucopyranose.

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from *Bacillus circulans* using lactose and D-Glc*p*NAc as substrates (25–27). These authors described a regioselective synthesis of *N*-acetyllactosamine with a yield of about 23% (with respect to D-Glc*p*NAc) with a contamination of 10% by its isomer *N*-acetylallolactosamine. In the present work we report a high-yield transglycosylation reaction with a high regioselectivity toward the formation of β1–4 linkage between D-Gal*p* and D-Glc*p*NAc using β-galactosidase from *B. circulans.*

MATERIALS AND METHODS

Materials. Lactose, *N*-acetylallolactosamine, D-Glc*p*NAc, D-Glc*p*, D-Gal*p*, *o*-nitrophenyl β-D-galactopyranoside and nicotinamide-adenine dinucleotide were purchased from Sigma (St Louis, MS, USA), β-galactose dehydrogenase (E.C. 1.1.1.48) was from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.), *N*-acetyllactosamine was from Dextra Laboratories Ltd (Reading, U.K.), β-galactosidase (E.C. 3.2.1.23) from *B. circulans* was from Daiwa Kasei Co. (Osaka, Japan), Bio-Gel P2 was from Bio-Rad (Richmond, CA, USA), colorimetric kit Sera-Pak for glucose determination was from Bayer Diagnostics (Saint Denis, France), LiChrosorb-NH_2 HPLC column was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Purification and analytical methods. Products of transglycosylation were purified by gel permeation chromatography on two serial columns (2.0 × 100 cm each) of Bio-Gel P2 equilibrated in water. The elution was followed by use of R-403 Waters differential refractometer. The analysis of transglycosylation reaction involved the use of an Beckman HPLC system (GOLD Model) equipped with a UV-visible detector, monitoring at 210 nm (N-acetyl group), and a LiChrosorb-NH_2 column (5 μm, 250 × 4.00 mm I.D.). The column was eluted under isocratic conditions using acetonitrile:water (84.5:15.5) as mobile phase. Free galactose was determined enzymatically by use of galactose dehydrogenase as described by Beutler (28). Yield of transglycosylation products was determined according to calibration curves determined by HPLC for *N*-acetyllactosamine and *N*-acetylallolactosamine. Free glucose was determined by use of a colorimetric kit. Free D-Glc*p*NAc was determined by a modification of the Morgan-Elson assay for hexosamine (29). Lactose was determined measuring the glucose content, as described above, after enzymatic hydrolysis with β-galactosidase.

Enzyme assays. β-galactosidase activity was assayed as follows. To 450 μl of 4 mM *o*-nitrophenyl β-D-galactopyranoside in 50 mM potassium phosphate (pH 6.4) 50 μl of enzyme solution were added and the mixture incubated at 37°C for 4 min. The enzymatic reaction was stopped by adding 1 ml of 0.1 M Na_2CO_3, and then the liberated *o*-nitrophenol was determined spectrophotometrically at 410 nm. One unit of activity was defined as the amount of the enzyme releasing 1 μmole of *o*-nitrophenol per min.

Structural identification methods. ^{13}C- and ^{1}H.N.M.R. decoupled spectra were measured on a Bruker AC 200 MHz spectrometer using a multinuclear 5 mm probe. 500 μl of deutered water were used for all the measurements with a typical sample concentration of 5 mg/ml for ^{1}H measurements and 30 mg/ml for ^{13}C measurements. The spectra were obtained at 297K.

Ionspray Mass Spectrometry data were recorded on a API-I PE-SCIEX quadrupole mass spectrometer connected to a syringe pump for the injection of the samples.

The sample was dissolved in 50% aqueous acetonitrile 5 mM ammonium acetate and injected at a flow rate of 7 μl/min. Spectra were recorded in the positive ion mode using a step size of 0.1 amu, an orifice potential of 50 V and an ionspray voltage of 5000 V

Kinetics of the synthesis of N-acetyllactosamine. 4.5 g of lactose and 900 mg of D-Glc*p*NAc were dissolved in 8.1 ml of water. This solution was divided into three groups of three aliquots (i.e. 900 μl each). To each aliquot of each group 1.0 ml of buffer solution was added, with the following features: 100 mM sodium acetate for experiments at pH 5.0, 100 mM potassium phosphate for pH 6.4 and 100 mM Tris-HCl for pH 8.0. A solution (100 μl, 6 units) of β-galactosidase from *B. circulans* was added to each aliquot. The three aliquots of each pH group were incubated at 15°C, 24°C and 55°C for 6 hours, respectively. During incubation, on every hour, 100 μl of each sample were collected, added to 900 μl of distilled water and heated in a boiling water bath for 10 min and then immediately cooled in ice. After centrifugation at 11,000 rpm for 5 min, the clear supernatants were analyzed by HPLC as described before.

Synthesis and purification of N-acetyllactosamine. 500 mg of lactose and 100 mg of D-Glc*p*NAc were dissolved in 1.9 ml of 50 mM sodium acetate (pH 5.0). 6 units (100 μl) of β-galactosidase from *B. circulans* were added. After incubation at 15°C for 3 h, the mixture was heated in a boiling water bath for 10 min and immediately cooled in ice. It was then centrifuged at 11,000 rpm for 5 min and the clear supernatant was loaded on a Bio-Gel P2 column setup and eluted as described hereabove. 20 μl of each peak emerging from the columns were analyzed by HPLC. Fractions containing *N*-acetyllactosamine were pooled and concentrated under reduced pressure and freeze-dried. On an aliquot of this lyophilized product contamining carbohydrates were determined. The remainig part was re-dissolved and re-chromatographed as described before. Fractions corresponding to the main peak, containing *N*-acetyllactosamine, were pooled and the volume reduced to about 2 ml under reduced pressure and freeze-dried.

RESULTS

Study of the effect of reaction parameters on the kinetics. In the first part of this study we evaluated the effect of some experimental conditions (i.e. pH and temperature) on the yield and regioselectivity of the synthesis of *N*-acetyllactosamine.

As shown in Figure 1, the effect of a change of pH in the range of values considered was found to depend upon the reaction temperature. The largest effect on the synthesis of *N*-acetyllactosamine was recorded at 15°C after 1 h (at this temperature, at pH 8.0 the yield was about 71% of that at pH 5.0), whereas this difference practically dropped to zero at 55° C (after 2 h). On the opposite, the effect of changing pH was almost negligible on the synthesis of *N*-acetylallolactosamine at 15°C at all times, whereas the synthesis of such allo-isomer reached a reduction to 70% on passing from pH 5.0 to pH 8.0 at 55°C. As expected, the experiments performed at the intermediate

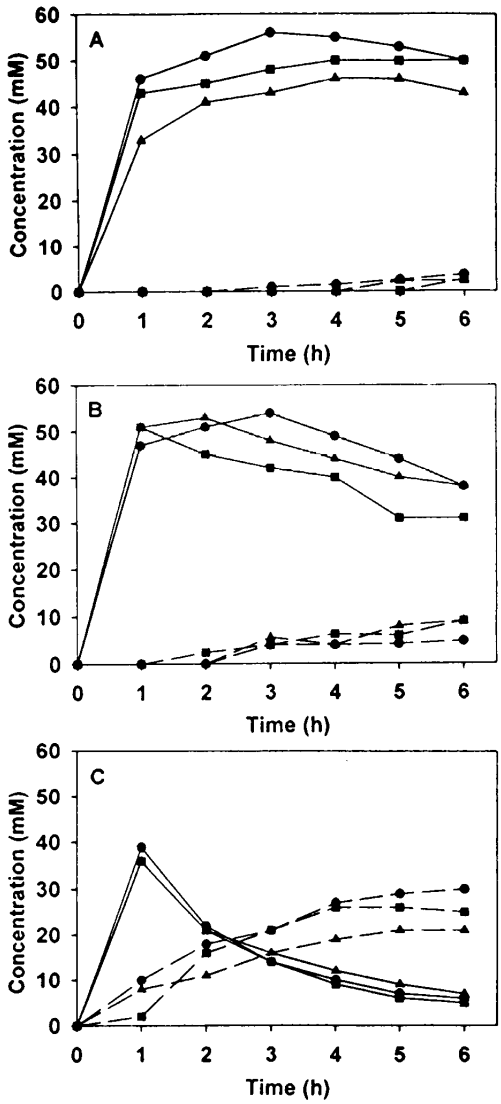

FIG. 1. Time-course of the production of *N*-acetyllactosamine (—) and *N*-acetylallolactosamine (—) at pH 5.0 (●), pH 6.4 (■) and pH 8.0 (▲) during 6 h of incubation at 15°C (A), 25°C (B) and 55°C (C).

temperature of 24°C gave an intermediate behaviour. The very strong effect of temperature emerges from a detailed inspection of the results of Figure 1. In fact, performing incubation at 15°C or at 24°C the maximum production of *N*-acetyllactosamine was reached after about 3 h. In this short period a low production of *N*-acetylallolactosamine, always lower than 10%, was observed, with the best result obtained with the incubation performed at 15°C (Fig. 1A and B). At this temperature the production of *N*-acetyllactosamine expressed as ratio LacNac/(LacNac + AlloLacNAc) remains close to its maximum level for almost all the period of incubation. At 24°C even since the second hour a decrease in the production of *N*-acetyllactosamine was observed, together with a corresponding increase in the appearance of its isomer which after 6 h reached 18–20% of total products. At 55°C the maximum production of *N*-acetyllactosamine was obtained after 1 h of incubation but the yield was significantly lower than those obtained in the other experimental conditions and it dropped off ever since the second hour of incubation (Fig. 1C). On the other side the production of *N*-acetylallolactosamine raised rapidly. Even after 1 h it corresponded to 20–25% of total reaction products (Fig. 1C). In all conditions considered, prolonging the incubation period up to 24 h was demonstrated to bring to a conspicuous decrease in the overall production of *N*-acetyllactosamine, in the ratio LacNac/(LacNac + AlloLacNAc) and in the sum of LacNac + AlloLacNAc (data not shown).

Synthesis in optimized conditions and purification of N-acetyllactosamine. To confirm the yield calculated by HPLC analyses and to characterize the products we performed an incubation using the optimal experimental conditions so far determinated, and purified the transglycosylation products by gel permeation chromatography on Bio-Gel P2 (see 'Experimental'). After the first chromatographic purification a chromatographic profile as reported in Figure 2 was obtained. By HPLC analysis (see 'Experimental') we identified peak III as the one containing *N*-acetyllactosamine (Fig. 2) with the lowest content of Glc*p*NAc. Composition analysis of peak III indicated that it was composed for about 83% by the transglycosylation products, and for about 17% overall by contamining monosaccharides (0.1% D-glc*p*, 4 to 5% D-Glc*p*NAc), and disaccharides (10–15% lactose). It was then resorted to undergo the pool of fractions of peak III to a repeated gel permeation chromatography experiment. The chromatographic profile is reported in Figure 3, where IIIa stands for the peak corresponding to the pure reaction products. Composition analysis of the pool IIIa gave no detectable contaminant. After pooling, the volume of the fractions containing *N*-acetyllactosamine was reduced under reduced pressure and freeze-dried to give 40–42 mg of an amorphous solid.

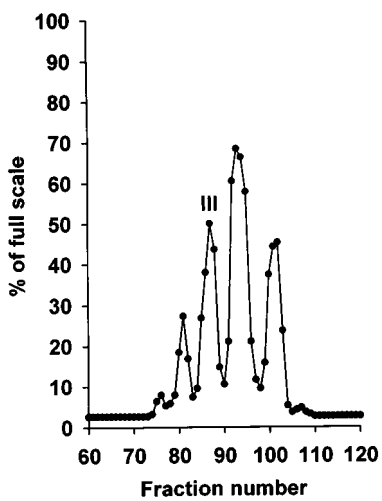

FIG. 2. Elution profile of gel permeation chromatography on Bio-Gel P2 of the incubation mixture.

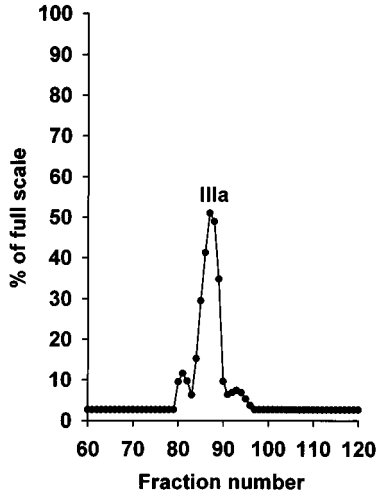

FIG. 3. Elution profile of gel permeation chromatography on Bio-Gel P2 of peak III.

Determination of the relative amounts of *N*-acetylallolactosamine and of *N*-acetylallolactosamine by HPLC analysis was performed both on the reaction mixture and after gel permeation chromatography separation on pooled fractions, in turn corresponding both to peak III and to IIIa. In all three cases the relative molar ratio [LacNAc/(LacNac + AlloLacNac)] was the same within 1%, thus indicating that the separation/purification procedures did not lead to any significant loss of either of the two *N*-acetyllactosamine isomers. The quantitative recovery of the reaction products and of the unreacted reagents was confirmed by running serial chromatographic separations with standards.

The nature of products obtained was confirmed by HPLC, ^{1}H- and ^{13}C-N.M.R. spectroscopy and by Ionspray Mass spectrometry. Comparison of HPLC elution profilles of our reaction product with standard *N*-acetyllactosamine and *N*-acetylallolactosamine confirms the high regioselectivity of transglycosylation reaction (Fig. 4). The ^{1}H- and ^{13}C-N.M.R. spectra of our transglycosylation product are superimposible on that of standard *N*-acetyllactosamine. The ionspray mass spectrum of the sample shows two ions at m/z 384.2 and 406.0 corresponding to $[M + H]^{+}$ and $[M + Na]^{+}$, respectively, corresponding to the molecular weight of the *N*-acetyllactosamine.

DISCUSSION AND CONCLUSIONS

The study of the effect of physico-chemical parameters of practical interest, like pH and temperature, on the kinetics of regioselective synthesis of *N*-acetyllactosamine by use of β-galactosidase from *B. circulans* has shown that in the pH range from 5.0 to 8.0 the value of pH 5.0 is the best to ensure the desired goal. This finding is in complete agreement with what already reported by Sakai *et al.* (26).

The effect of temperature on the enzymatic reaction turned out to be much less trivial. As it can be seen by the cumulative graph of Figure 5A summarizing the results at pH 5.0, a decrease of temperature from 55°C to 15°C brings about an unexpected dramatic increase in the formation of the desired isomer *N*-acetyllactosamine and a parallel decrease of the undesired alloform. In all cases maxima in the *N*-acetyllactosamine production are reached, but analysis of the time course of the overall production of the two isomers (i.e. LacNAc + AlloLacNAc) (see Figure 5B) clearly indicates that, besides regioselectivity, also the overall transglycosylation efficiency tends to decrease with time, for all temperatures considered.

A quantitative analysis of the many kinetic pathways of β-galactosidase catalyzed reactions needs a deeper investigation however: on the basis of the present results one can already state that

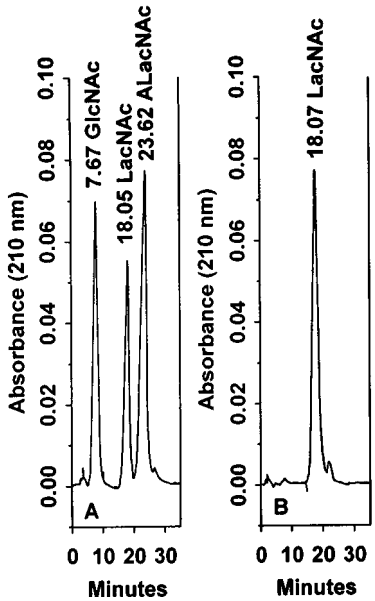

FIG. 4. HPLC profiles of standards (A) and transglycosylation product (B).

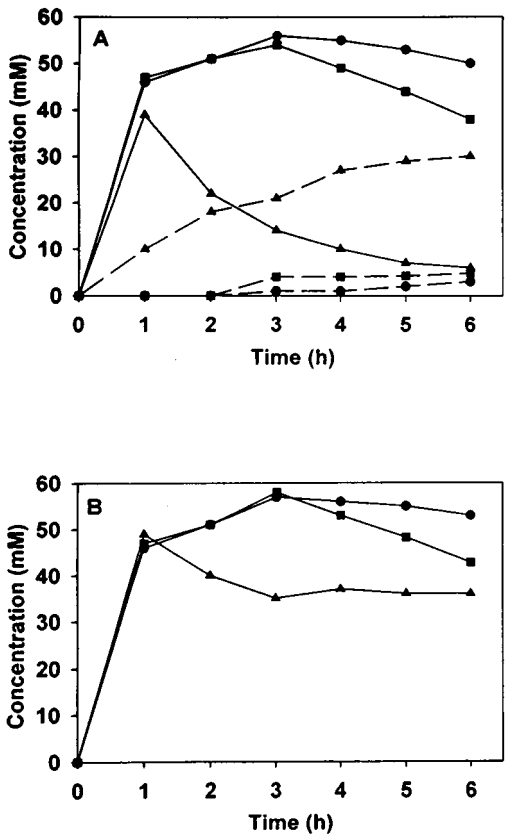

FIG. 5. Time-course of the production of *N*-acetyllactosamine (—) and *N*-acetyllallolactosamine (—) (A) and of (LacNAc + AlloLacNAc) (B) at pH 5.0. (●) Incubation at 15°C; (■) incubation at 24°C and (▲) incubation at 55°C.

at pH 5.0:1) lower temperature favours both the transglycosylation in general and the formation of *N*-acetyllactosamine in particular; 2) the competing glycohydrolase activity of the enzyme tends to grow significantly only after a few hours (3 h ca.), being generally favored by an increase of temperature; 3) a tendency to levelling off of the overall amount of transglycosylation products at 55°C can be easily explained by a partial thermal inactivation of the enzyme.

On purely comparative grounds, the present reaction conditions seem to be able to produce a dramatic increase in the efficiency with respect to the already reported ones (26): 1) the yield (expressed as the percentage of moles of D-Glc*p*NAc which have undergone transglycosylation) has practically doubled, on passing from 23% to 42%; 2) the regioselectivity (expressed as the ratio of LacNAc over LacNAc + AlloLacNAc) has increased from 90% to 98%; 3) the optimum incubation time has shortened from 24 h to 3 h; 4) the temperature (which can be looked upon as an index of energetic cost in any industrial application) has reduced from 30°C to 15°C; 5) the amount of enzyme required for the above performance was reduced from 15 U to 6 U.

In conclusion, the present study demonstrated the possibility to significantly improve the regioselectivity of β-galactosidase from *B. circulans* and its yield in transglycosylation reactions thus allowing a practical application of the method, starting from cheap sugars (lactose and D-Glc*p*NAc) and commercially available enzyme. Work is in progress to clarify the role of the various isoforms of the enzymes contained in the commercial preparation (30).

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