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Enzymatic synthesis of the Tn antigen

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Abstract

The enzymatic synthesis of the Tn antigen (GalNAc-α-*O*-Ser), a glyco-aminoacid of great biological importance, is reported. The reaction was promoted by commercial α-*N*-acetylgalactosaminidase from *Acremonium* sp., using *p*-nitrophenyl-α-*N*-acetylgalactosamine as the donor. The kinetics were monitored by capillary electrophoresis and LC–UV-MS. For unprotected serine, the role of pH and temperature was investigated, finding that pH 5 and $T = 18\,^{\circ}C$ gave the best yield. Under these conditions a significant increase of the reaction rate was observed in comparison with previous literature data, using unprotected serine. The role of the bulkiness of the serine protecting groups on the yield was additionally considered, as well as the kinetic profiles generated by the use of two differently protected aminoacids. By proper choice of the protecting group, the reaction yield then increased from 5% (with unprotected serine) to about 50% (with *N*-Boc and *N*-methoxycarbonyl serine).

Keywords: Tn antigen; Enzymatic synthesis; α-*N*-Acetylgalactosaminidase; Capillary electrophoresis of oligosaccharides

1. Introduction

Researchers show an ever-increasing interest in the role played by oligosaccharides in countless biological processes [1–5]. Glycosylation alterations can be a significant marker of a modification of the patient conditions [6], and in particular some of them are strictly related with the occurrence of neoplastic processes [1,4,7–10]. Preparation in laboratory of biologically active saccharides provides sufficient amount of the compounds for the investigation of their role in the biological processes in which they are involved and for developing of vaccines and targeted drugs.

Tn antigen (GalNAc-α-*O*-Ser), a tumor-associated oligosaccharide typically expressed by breast cancer cells [11] is the simplest of these altered structures, but it is extremely important for its predictive value and for the possibility to use it as a building block for the synthesis of more complex oligosaccharides.

Among the possible preparative routes for the synthesis of oligosaccharides and their derivatives, enzymatic synthesis is increasingly being used because of the high regioselectivity, the possibility to use environmentally friendly aqueous solvents, and the relatively simple composition of reaction mixtures, which implies simpler purification routes. For the enzymatic synthesis of *O*-glycosides, glycosyltransferases are probably the most powerful tools, but their extreme specificity and the high cost of the required substrates (NDP-sugars) make them less applicable for large-scale preparative applications.

From a practical point of view, the use of glycosidases for transglycosylation reactions is more interesting. These enzymes usually act as hydrolases; however, they are versatile inasmuch as they can be forced to transfer a sugar donor to a suitable acceptor, upon choosing optimal experimental parameters like pH and temperature [12,13]. It is to be remarked that the mechanism underlying the enzymatic reactivity does not change. After the breaking of the glycosidic bond, the addition to a nucleophile takes place: in the hydrolysis case to a molecule of water, in the transglycolytic case to an acceptor with a free hydroxyl group.

Despite the increasing interest for the enzymatic syntheses promoted by glycosidases, only few works describe the

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enzymatic synthesis of glyco-aminoacids, in contrast to the wide literature concerning chemical strategies for the preparation of these relevant compounds [14,15]. This discrepancy can be attributed to the low reactivity of the hydroxyl group of serine and threonine with respect to the enzymatically induced transfer of glycosyl donors. Such drawback is counterbalanced by the excellent stereoselectivity of the enzymatic synthesis, which gives rise only to the desired anomer. A few examples have been reported in literature regarding the enzymatic synthesis of Tn antigen [14,16]. Johannson et al. [17] reported the use of *N*-acetyl-galactosamine as the donor, serine as the acceptor and α-*N*-acetylgalactosaminidase from bovine liver as the promoter; the best yield, which was reached after 6 days, turned out to be equal to 4%. More recently, it was shown [18] that the use of activated donors like 4-nitrophenyl *N*-acetyl-α-galactosaminide as well as the use of *N*-alkyloxycarbonyl serine ester derivatives as the acceptors significantly improved the performances of Tn synthesis promoted by α-*N*-acetylgalactosaminidase from *A. oryzae*. In particular, the reaction was noticeably faster and more productive (yield up to 40%) upon decreasing the size or bulkiness of the serine alkyloxy group.

We decided to explore the activity of α-*N*-acetylgalactosaminidase from *Acremonium spiriferum*, a commercially available enzyme, so-far never used for the synthesis of such kind of molecules. The enzymatic activity was studied in detail by monitoring the kinetics with different analytical approaches. The enzyme was tested with unprotected serine as the acceptor using either α-*N*-acetylgalactosamine ("reverse hydrolysis") or its *p*-nitrophenyl derivative ("transglycosylation") as donor; a significant increase of the reaction rate in comparison with previous literature data was obtained in the latter case. On the contrary, the yield practically did not increase, remaining lower than 5% for both reactions.

Since previous works have demonstrated that the yield of the enzymatic synthesis of glyco-aminoacids can be improved by suitably protecting carboxyl- and amino-group of the acceptor [19,20] we have carried out a systematic study of reaction yield as function of different protecting groups. In comparison with unprotected serine, the use of protecting groups produced a yield increase up to 12-fold.

2. Materials and methods

2.1. Materials

N-Acetyl-α-D-galactosamine (Gal*N*Ac), 4-nitrophenyl-*N*-acetyl-α-D-galactosamine (Gal*N*Ac-*p*NP), L-serine, 9-fluorenylmethyl-chloroformate chloride (Fmoc-Cl), sodium tetraborate (borax) and methanol were purchased from Sigma (St. Louis, MO, USA); α-*N*-acetylgalactosaminidase from *A. spiriferum* was purchased from Seikagaku (Tokyo, Japan); standard GalNAc-α-*O*-Ser was purchased from Calbiochem-VWR, ammonium acetate, acetonitrile, sodium bicarbonate, sodium dodecyl sulphate (SDS), sodium hydroxide and acetic acid were from Merck (Darmstadt, Germany); *N*-Boc-L-serine methyl ester, L-serine methyl ester hydrochloride, methyl-chloroformate, potassium carbonate anhydrous and chloroform were from Fluka (Buchs, Switzerland); dioxane was from Aldrich (St. Louis, MO, USA).

2.2. Capillary electrophoresis

Capillary electrophoresis experiments were carried out using a Hewlett-Packard HP ^{3D}Capillary electrophoresis system (diode array UV detector). The experimental conditions for *M*icellar *E*lectro *K*inetic *C*apillary chromatography (MEKC) were the following: buffer, 25 mM borax + 100 mM SDS; temperature, 25 °C; voltage, 15 kV; UV detection at 195 nm and 260 nm; fused silica capillaries were from Agilent Technologies ($L = 80.5$ cm; $l = 72$ cm; i.d. = 50 μm; extended light path). Before sample injection (50 mbar × 6 s), a 4-min wash with the running buffer was necessary, which had followed a 2-min wash with 0.1 M sodium hydroxide (pressure: 950 mbar). Quantification was carried out by using peak areas divided by migration time [21].

2.3. Mass spectrometry

Electrospray mass spectrometry (ESI-MS) was performed using an ion trap mass spectrometer (model DecaXP, ThermoFinnigan, San Jose, CA, USA), operating in positive ionization mode (+4.5 kV). The sheath gas (N_2) flow was typically equal to 0.23 L/min. The temperature of the heated capillary was set at 240 °C. Flow-injection studies were carried out with a flow equal to 3 μL/min. High performance liquid chromatography coupled with UV and mass spectrometry detectors (HPLC–UV-MS) was carried out upon connecting the mass spectrometer to a Surveyor ThermoFinnigan HPLC system, equipped with a MS pump, an autosampler and a PDA detector. XCalibur (ThermoFinnigan) data system software (version 1.3) allowed the instruments control as well as data acquisition and processing. Kinetic studies using protected serine as the acceptor were carried out by HPLC–MS, with a Zorbax SB-Phenyl column (3.0 × 2.50 mm, 5.0 μm) (Agilent). All the separations were performed at ambient temperature (Flow: 200 μL/min, injected volume: 10 μL). When using *N*-Boc-L-serine methyl ester, the gradient depicted in Table 1 was applied; electrospray mass spectrometry was carried out using both *t*otal *i*on *c*urrent (TIC) monitoring and *s*ingle *i*on *m*onitoring (SIM), set at m/z equal to 423 ($[M+H]^+$). Kinetic studies using *N*-methyloxycarbonyl-L-serine methyl ester were carried out in isocratic conditions (70% A, 30% B, A: 5 mM CH_3COONH_4/CH_3COOH, pH 3.5, B: CH_3OH); ESI-MS was carried out using both TIC and SIM, set at m/z equal to 381

Table 1
HPLC Gradient for kinetic studies using *N*-Boc-L-serine methyl ester as the acceptor

T (min)	0	8	20	28	30	36
%A	97	97	10	10	97	97
%B	3	3	90	90	3	3

Mobile phase—Eluent A: 5 mM CH_3COONH_4/CH_3COOH, pH 3.5; Eluent B: CH_3OH.

($[M+H]^+$). Quantification was achieved by evaluating peak areas from SIM data.

2.4. Nuclear magnetic resonance

Nuclear magnetic resonance was carried out on a JEOL ECX (400 MHz) spectrometer. The solvent was deuterated water.

Selected ^{1}H-NMR data for Tn antigen obtained with unprotected serine: δ 4.88 (d, 1H, J = 10.0 Hz, H-1); δ 4.12 (d, 1H, J = 6.5 Hz, Ser H-α); δ 4.08 (d, 1H, J = 10.8 Hz, H-2); δ 4.00 (dd, 1H, J = 10.9 Hz, H-4); δ 3.99 (dd, 1H, J = 3.0 Hz, H-3); δ 1.98 (s, 3H, NHAc). Assignments were confirmed by comparison with literature data [14].

2.5. Synthesis of *N*-methyloxycarbonyl-L-serine methyl ester

N-methyloxycarbonyl-L-serine methyl ester was prepared upon reaction of serine methyl ester (0.84 mmol, 3 mL acetone) with methyl chloroformate (4 equiv.) and potassium carbonate anhydrous (6 equiv.) for 12 h under reflux; after solvent evaporation, methanol containing sodium hydroxide (4%) was added and the mixture was stirred at ambient temperature for 2 h; finally, methanol was removed and the product was recovered in water and then extracted with chloroform [22] (yield 50%).

2.6. Kinetics of the enzymatic synthesis of Tn antigen

Each kinetic study was repeated three times; therefore, the reported kinetic studies correspond to average molar yields versus reaction time.

(a) Kinetic studies using GalNAc as the donor (reverse hydrolysis) and unprotected serine as the acceptor

0.023 mmol of GalNAc and 0.046 mmol of serine were dissolved in 200 μL of 50 mM ammonium acetate buffer pH 5.0. 0.6 U of enzyme were added and the solution was incubated at 37 °C. During incubation, 25 μL of reaction mixture were collected at defined time intervals, diluted with 225 μL of water, heated in a boiling water bath for 10 min and then immediately cooled in ice. The aliquots were then freeze-dried. Every aliquot was derivatized with 9-fluorenylmethyl-chloroformate (Fmoc): the freeze-dried sample was recovered with 40 μL of a saturated sodium bicarbonate aqueous solution and 40 μL of Fmoc-Cl (0.4 mmol) in dioxane were added. The mixture was stirred overnight at room temperature and centrifuged at 10,000 rpm (microcentrifuge 4214 ALC International, Milan, Italy). To draw the kinetic profile of the enzymatic synthesis, the supernatant was then analyzed by MEKC-UV.

(b) Kinetic studies using GalNAc-pNP as the donor (transglycosylation) and unprotected serine as the acceptor

0.028 mmol of GalNAc-*p*NP and 0.048 mmol of serine were dissolved in 1.0 mL of 50 mM ammonium acetate buffer (pH 4.0, 5.0 and 6.0). After complete solubilization 0.6 U of enzyme were added and portions of the solution were incubated at 18, 37 or 55 °C. The aliquots were analyzed as described in the above paragraph.

(c) Kinetic studies using *N*-protected serine methyl ester as the acceptor

N-Boc serine methyl ester or N-methyloxycarbonyl serine methyl ester. 0.028 mmol of GalNAc-pNP and 0.045 mmol of protected serine were dissolved in 1.0 mL of 50 mM ammonium acetate buffer (pH 5.0). After complete solubilization 0.6 U of enzyme were added and portions of the solution were incubated at 18, 37 or 55 °C, respectively. During incubation, 25 μL of reaction mixture were collected at defined time intervals (see results), diluted with 225 μL of water, heated in a boiling water bath for 10 min and then immediately cooled in ice. The aliquots were then freeze-dried and recovered in water (265 μL). To draw the kinetic profile of the reaction, the aliquots were monitored without pre-treatment by HPLC–MS.

(d) Scale-up

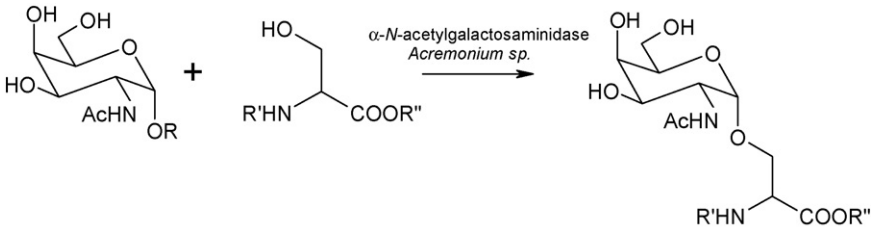
After the optimization of the reaction conditions, a scale-up of the syntheses at points (b) and (c) was carried out, by simply using 10× volumes and amounts of the reactants. At the time corresponding to the maximum yield (5 h for the reaction with *N*-methyloxycarbonyl serine methyl ester and 10 h for *N*-Boc serine methyl ester), the enzyme was inactivated by heating in a boiling water bath for 10 min, then the reaction mixture was purified by gel filtration chromatography on two serial columns (2.0 cm × 100 cm each) of Bio Gel P2 (Bio-Rad, Hercules, CA, USA) equilibrated in water. The flow was set at 4 mL/h. The fractions containing the pure product were identified by electrospray mass spectrometry and proton nuclear magnetic resonance.

3. Results and discussion

3.1. Unprotected serine

Notwithstanding the relatively low yield expected with the use of native serine as the acceptor [17], still the synthesis of Tn antigen using unprotected serine (Scheme 1) was initially explored for two main reasons. First, the product achieved by this approach would not have to be subjected to deprotection reactions, which are necessary to get the native Tn when using protected serine; this implies that no product loss associated with these processes would occur. Secondly, no previous use of α-*N*-acetylgalactosaminidase from *Acremonium* sp. for promoting Tn synthesis has ever been reported.

Kinetics of the reaction were studied by analyzing aliquots at different reaction times by MEKC-UV. This technique is ideal for the analysis of low quantities of product (the injected amounts were in the order of nL), and a method has been settled to obtain a good resolution of peaks. Since the product is not effectively detected by UV lamp, the derivatization with Fmoc was necessary. The identity of the product was then confirmed by co-injection of purified standard (a commercial sample of GalNAc-α-*O*-Ser derivatized with Fmoc following the procedure described above) and by flow-injection ESI-MS, which evidenced the presence of a peak at *m*/*z* equal to 519, corresponding



Scheme 1. Enzymatic synthesis of Tn antigen using *GalNAc* (R=H) and *GalNAc-pNP* (R=*p*-nitro phenyl) as the donor and serine (R′=R″=H) and protected serine as the acceptor (R′=Boc, R″=Me; R′=methyloxycarbonyl, R″=Me).

to $[M+H]^+$. The activated donor, i.e. GalNAc-*p*NP, was tested first in view of its well-known efficiency as a leaving group. After the assessment of the conditions for the monitoring of kinetic profiles, an accurate analysis of the best experimental conditions, namely pH and temperature, was carried out. In particular, a study of the reaction yield as a function of pH was performed at 18 °C (the enzyme is reported to be more stable at this temperature), in the known range of enzyme stability (pH 4.0–6.0). The best results were found at pH 5.0. At pH 6.0 no formation of the product could be noticed, while at pH 4.0 (Fig. 1) the product was obtained in small amount and electropherograms show a rich pattern of peaks, due to the presence of many side-products.

Three different temperatures have been investigated (18, 37 and 55 °C, respectively).

The kinetic curves (Fig. 2) show an increase of the yield as a consequence of the decrease of the temperature of incubation. Upon increasing temperature, the peak moves toward shorter times of incubation: it is likely that at high temperature the enzyme reacts too fast and its hydrolytic activity induces the degradation of the product before it can accumulate.

It is interesting that at 37 °C the kinetic profile shows two distinct peaks. An explanation for this behaviour could be that at this temperature there is the formation of one or more intermediate products (like for instance, Gal*N*Ac–Gal*N*Ac-*p*NP, the presence of which was detected in the reaction mixture by HPLC–MS) that can act as the donor when Gal*N*Ac-*p*NP is completely hydrolyzed. This point, however, deserves a further investigation to better assess the origin of the phenomenon.

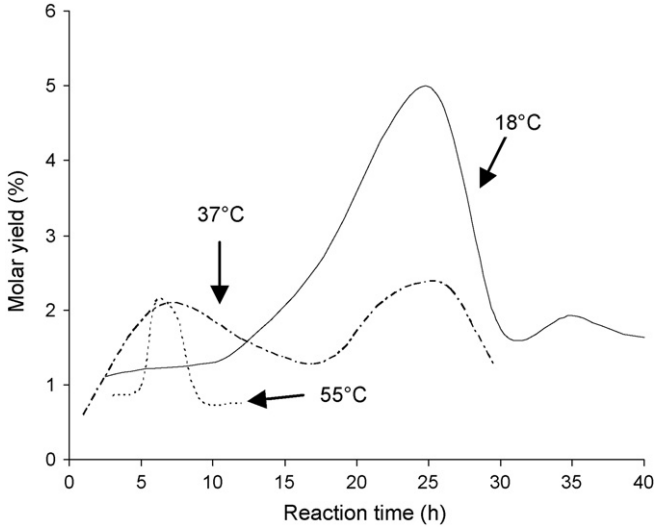


Fig. 2. Dependence on the reaction time of the molar yield of Tn antigen synthesis at pH 5.0. Unprotected serine as the acceptor at 18, 37 and 55 °C.

Even under optimal experimental conditions (18 °C, pH 5.0), the yield of the synthesis (5%) was not practically improved with respect to that previously reported by Johansson et al. (4%), who used α-*N*-acetylgalactosaminidase from beef [17]. Anyway, there are some relevant differences. The most evident is the reaction time, which with α-*N*-acetylgalactosaminidase from *Acremonium* sp. was only 25 h, in comparison with the 6 days needed to obtain the same result with the enzyme

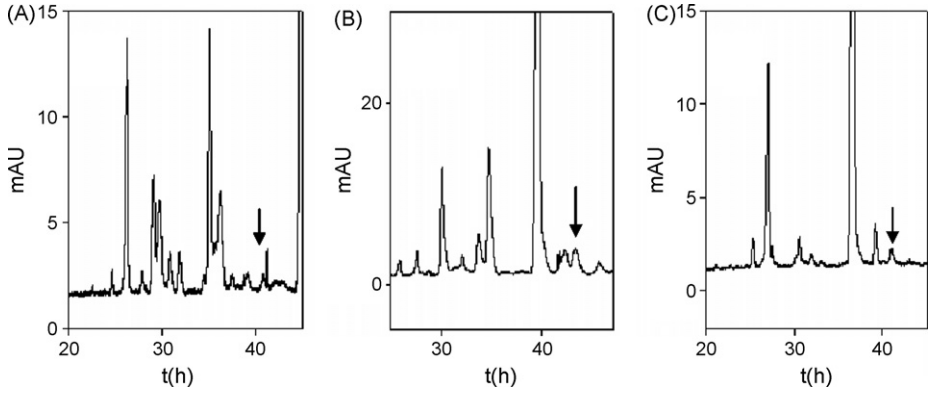


Fig. 1. Electropherograms of Tn antigen synthesis mixture using unprotected serine as the acceptor after 15 h of reaction at: (A) pH 4.0, (B) pH 5.0 and (C) pH 6.0. Experimental conditions are reported in Section 2. Arrows indicate the product.

from beef liver. Moreover, the temperature needed for α-*N*-acetylgalactosaminidase from beef liver to get some production in a reasonable time is 50 °C, while the reaction here reported does not need such a high temperature value. Besides the different origin of the enzyme, a possible reason for the present good results is represented by the use of an activated donor, i.e., GalNAc-*p*NP, which, owing to the *p*-nitrophenyl leaving group, is well-known to improve the efficiency of a saccharide moiety transfer on the acceptor molecules [1,18,23]. This hypothesis is confirmed by the results presently obtained with the non-activated donor (i.e., *N*-acetylgalactosamine) at pH 5.0 and $T = 37$ °C. In this case, the product of the reaction could very hardly be detected, due to its low concentration. Indeed, from a MEKC-UV monitoring of the Fmoc-derivatized synthesis mixture, Tn antigen was shown to be obtained with a yield lower than 1% even after 72 h incubation. This result is not surprising in view of the known difficulty in obtaining a satisfactory yield in reverse hydrolysis reaction: it was decided to perform the experiment anyway for comparative reasons.

3.2. Protected serine

Among the numerous works reporting the advantageous increase of yield by using protecting groups on the aminoacidic moiety, some report the effect of *N*-protecting groups on serine methyl ester for the synthesis of Tn antigen promoted by α-*N*-acetylgalactosaminidase from *A. oryzae* [18]. A decrease of the bulkiness of the *N*-protecting moieties was shown to increase the yield, up to 40% (on molar basis). In these works, however, there is not a direct comparison with the

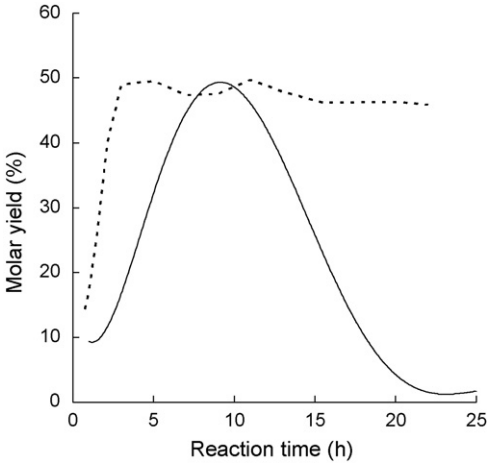


Fig. 4. *N*-Boc serine methyl ester (full curve) and *N*-methyloxycarbonyl serine methyl ester (dotted curve) as the acceptor, respectively (both at 37 °C).

results obtained with unprotected acceptor and no details on the kinetics are provided. In order to clarify the behaviour of α-*N*-acetylgalactosaminidase from *Acremonium* sp. and, hopefully, to further improve the synthesis yield, kinetic studies were performed using *N*-Boc serine methyl ester and *N*-methyloxycarbonyl serine methyl ester as the acceptor. Despite its apparently disadvantageous steric hindrance, *N*-Boc group can be more easily and effectively removed with respect to less bulky moieties, like the *N*-methyloxycarbonyl analogue [22]; moreover, *N*-Boc serine methyl ester is commercially available. The use of *N*-methyloxycarbonyl serine methyl ester as the acceptor was also explored for comparison, in order to check

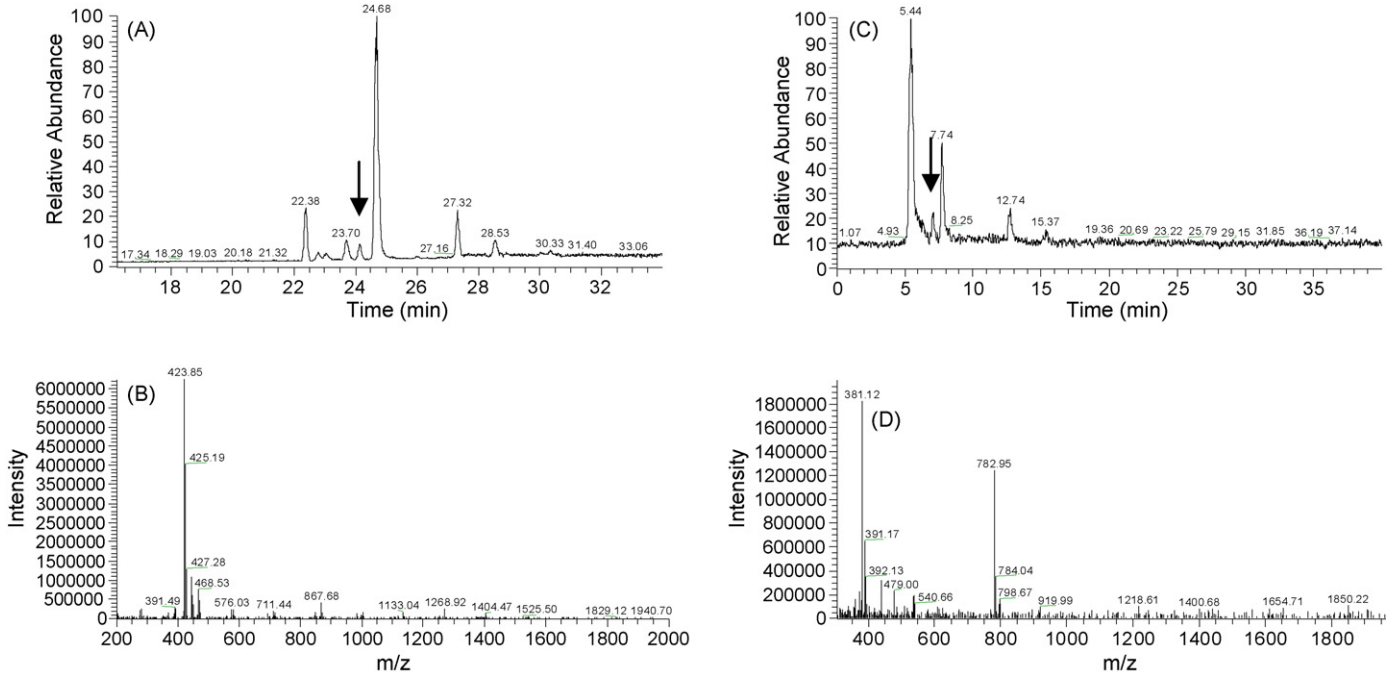


Fig. 3. HPLC–ESI-MS analysis of Tn antigen synthesis at the maximum of reaction at 37 °C at pH 5.0. The arrow indicates the peak associated with the antigen. Experimental conditions are reported in Section 2. Left side: *N*-Boc serine methyl ester as the acceptor (A) Chromatogram, TIC; (B) mass spectrum corresponding to the peak having a retention time equal to 24.11 min. Right side: *N*-methyloxycarbonyl serine methyl ester as the acceptor (C) chromatogram, TIC; (D) mass spectrum corresponding to the peak having a retention time equal to 7.17 min.

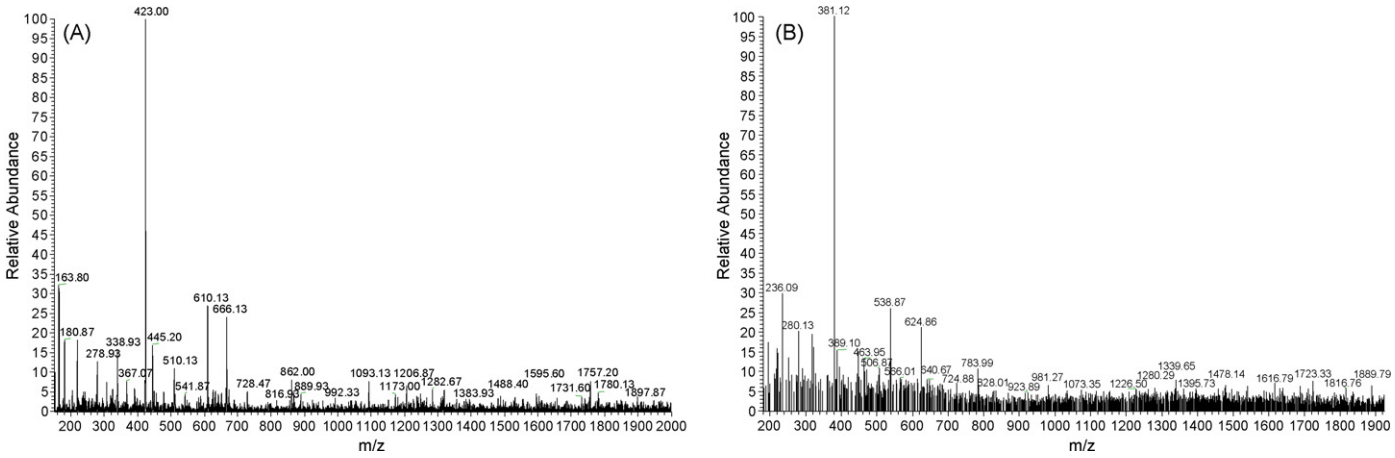


Fig. 5. Analysis ESI-MS of the products recovered after gel filtration chromatography of the reactions with (A) *N*-Boc serine methyl ester (MW 422 gmol^{-1}) and (B) *N*-methyloxycarbonyl serine methyl ester (MW 380 gmol^{-1}) in the mg-scale.

if the *N*-protecting group structure influenced the yield of Tn synthesis promoted by α-*N*-acetylgalactosaminidase from *Acremonium* sp. in the same way as the reported enzyme.

Kinetic studies were carried out as a function of temperature (18 and 37 °C, respectively), while pH was kept constant at 5.0.

HPLC–ESI-MS was used to monitor the synthesis course, due to the good ESI-MS response of the protected product and the impossibility to derivatize it with a cromophore. Fig. 3A, shows the chromatographic profile (TIC) of the reaction mixture after 10 h at 37 °C, while panel B reports the ESI-MS spectrum of the peak at retention time equal to 24.11 min, which demonstrated the successful formation (see peak at *m*/*z* 423, $[M+H]^+$) of the product. The kinetics of the reaction at 18 °C was also studied, and an opposite behaviour with respect to that of the unprotected serine: in this case an increase of temperature causes a yield increment.

The use of *N*-methyloxycarbonyl serine methyl ester was also investigated (Scheme 1), in order to verify the effect of the size of serine *N*-protecting group on the Tn synthesis yield. Again, the good ESI-MS response of the protected product without the need of derivatization allowed the reliable monitoring of the synthesis course by HPLC–ESI-MS. Fig. 3C shows the chromatographic profile (TIC) of the reaction mixture after 3 h at 37 °C, which was found to be the optimal temperature also using this acceptor. Fig. 3D reports the ESI-MS spectrum of the peak at retention time equal to 7.17 min, which demonstrated the successful formation of the product (see peak at *m*/*z* 381, $[M+H]^+$ and *m*/*z* 783, $[2M+Na]^+$).

The best results were obtained for both reactions at 37 °C, pH 5.0. Fig. 4 reports the kinetic profiles of the reactions carried out with the two differently protected serine molecules in these conditions.

The values of analytical molar yield (50%) and reaction time (3 or 9.5 h for the *N*-methyloxycarbonyl and for the *N*-Boc serine derivatives, respectively) were particularly advantageous with respect to those previously reported [17,18]. It is noticeable that the kinetic curve of the synthesis carried out using *N*-methyloxycarbonyl serine methyl ester is characterized by the presence of a plateau; it is therefore possible to deduce a poor hydrolytic action of the enzyme on the produced Tn antigen derivative, at variance with the *N*-Boc case.

Given the very similar amount of achievable Tn antigen with the two derivatives, the different time-profiles of the yield demonstrate that, in the case of *N*-acetylgalactosaminidase from *Acremonium* sp., the bulkiness of the serine *N*-protecting group plays a much more important role as to the hydrolytic step than toward the transglycolytic one.

3.3. Scale-up

The scale-up to the mg-scale was performed for the optimized reactions.

When using the two protected serines, an incomplete solubilization of the aminoacid was observed in the first stages of the kinetics, which tends to disappear during the conversion into the product. Apparently this problem does not affect the reaction yield: this was evaluated gravimetrically after gel-filtration chromatography. Fig. 5 reports the ESI-MS spectra of the eluates and shows that the pure product is recovered. The gravimetric yields resulted to be approximately 10% lower than the analytical yields.

4. Conclusions

We have demonstrated by the use of various analytical techniques (MEKC-UV, HPLC–UV-MS) that an easily available enzyme, such as α-*N*-acetylgalactosaminidase from *Acremonium* sp., can be an efficient promoter of the synthesis of glycoaminoacids, by using an activated donor (e.g., Gal*N*Ac-*p*Np) and a conveniently protected aminoacid. As to the latter point, it was possible to obtain a high analytical yield (50%) with a 12-fold increase with respect to the use of unprotected serine; correspondingly, the yield of the reaction carried out without leaving-groups on the donor was lower than 1%. A yield as high as 50% is quite unusual for reactions involving *N*-acetylgalactosamine using low concentrations of enzyme and of the donor. The bulkiness of the protecting groups did not significantly affect the maximum reaction yield, whereas it was

shown to be critical to tune the ensuing hydrolysis of the serine derivative.

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