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Enzymatic rearrangement of chitine hydrolysates with β-*N*-acetylhexosaminidase from *Aspergillus oryzae*

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Abstract

The role of higher chitooligomers in medical applications is increasing due to their interesting biological activities. Transglycosylation activity of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* was employed to produce higher chitooligosaccharides (chitohexaose–chitooctaose) from a mixture of lower chitooligomers prepared by acid hydrolysis of chitin. Enzymatic rearrangement of the chitooligomer mixture was optimized in respect of substrate concentration, presence of inorganic salts, enzyme activity, and reaction time to achieve the highest production of longer chitooligomers.

Keywords: Chitooligosaccharides; β-*N*-Acetylhexosaminidase; *Aspergillus oryzae*; Enzymatic transglycosylation

1. Introduction

Chitooligomers (e.g., chitobiose (**2**), chitotriose (**3**), chitotetraose (**4**), chitopentaose (**5**), chitohexaose (**6**), chitoheptaose (**7**)) (Scheme 1) are increasingly employed in medical applications due to their interesting biological activities, e.g., antibacterial [1], lysozyme inducing [2], and immunostimulating [3] ones. Higher chitooligomers — chitohexaose and chitoheptaose — show antitumor activity against mice sarcoma 180 [4], and they also elicit chitinase activity in plants, thus triggering defense mechanisms against fungal pathogens [5]. Recently, chitotetraose was found to have strong stimulating activity towards natural killer cells [6]. Chitopentaose is an important building block for NOD factor synthesis [7].

Chitooligosaccharides (Scheme 1) can be obtained by chemical or enzymatic methods from chitin or chitosan. Fluorohydrolysis of chitin in anhydrous hydrogen fluoride led to a mixture of chitooligosaccharides **2**–**5** including GlcNAc (**1**) [8]. In an alternative method, peracetylated chitooligomers are obtained by acetolysis of chitin with a mixture of acetic anhydride and concentrated sulfuric acid [9,10]. A controlled hydrolysis of chitin using hydrochloric acid [11–16], subsequently improved [17,18] and recently optimized [19], is the most popular method for production of the chitin oligomers. Efficiency of

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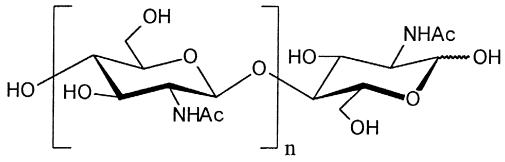

GlcNAc (n = 0) **1**
Chitobiose (n = 1) **2**
Chitotriose (n = 2) **3**
Chitotetraose (n = 3) **4**
Chitopentaose (n = 4) **5**
Chitohexaose (n = 5) **6**
Chitoheptaose (n = 6) **7**
Chitooctaose (n = 7) **8**

Chitin (n = 5000 - 10000)

Scheme 1.

this method is largely dependent both on mechanical (chitin grinding, ultrasound treatment) and chemical parameters (temperature, reaction time) and also on the reaction mixture processing (neutralisation, electrodialysis, gel filtration). The recently optimized method affords mostly chitooligomers **2**–**5** in a similar proportion as the fluorohydrolysis.

Enzymatic cleavage of chitin employing lysozyme or chitinase [20], hydrolysis of chitosan with chitinase followed by chemical *N*-acetylation of the hydrolysates [21], as well as lysozymic hydrolysis of partially *N*-acetylated chitosans [22], were shown to be useful in preparation of various *N*-acylchitooligosaccharides.

However, all these degradation methods mostly afford mixtures of lower chitooligomers (2–5), whereas the yield of higher chitooligomers (6–8) is very low, or they are not produced at all.

A reverse approach — using hydrolytic enzymes for the synthesis — was applied aiming to obtain higher chitooligomers. Transferase activity of chitinase was employed in the preparative synthesis of **6** and **7** from **4** and **5**, respectively. In this case, disaccharide elongation of the chitooligomer chain was achieved [23]. Also, the oxazoline derivative of (**1**) was used as a novel glycosyl donor for chitinase in enzymatic synthesis of **2** [24]. Higher chitooligosaccharides were synthesized from **2** as the initial substrate by lysozyme catalysis [25]. Fungal β-*N*-acetylhexosaminidase catalyzed formation of **2** from *p*-nitrophenyl β-*N*-acetylglucosamine and **1**. Consequently, also **3** and **4** were obtained from **2** used as a starting material [26]. The same strategy was employed for enzymatic synthesis of **5** and **6** starting from **3** or **4** [27].

In all mentioned enzymatic syntheses [23,25–27] rather expensive substrates such as *p*NP-β-GlcNAc and pure chitooligomers **2**, **3**, or **4** were used as starting materials.

We describe here a method for upgrading of a crude unseparated mixture of chitooligomers resulting from the acid hydrolysis of chitin according to Bredehorst et al. [19], which increases the yield of the higher chitooligosaccharides **6**–**8**. This method employs the transglycosylation activity of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* without any addition of synthetic glycosyl-donors.

2. Materials and methods

2.1. Enzyme

β-*N*-Acetylhexosaminidase (EC 3.2.1.52) from *Aspergillus oryzae* CCF 1066 originating from the library of fungal glycosidases of the Laboratory of Biotransformation, Institute of Microbiology, Prague [28] was prepared by induction as described previously [29].

2.2. Chitooligomer mixture

The crude mixture of chitooligomers was prepared by the procedure described by Bredehorst et al. [19] involving hydrochloric acid catalyzed cleavage of chitin and desalination of neutralized chitin hydrolysate by electrodialysis.

2.3. Transglycosylation reactions

Reaction conditions in analytical experiments: A crude mixture of chitooligomers (for typical HPLC analysis see Fig. 1) consisting of GlcNAc (**1**) (0.6%), $(GlcNAc)_2$ (**2**) (12.9%), $(GlcNAc)_3$ (**3**) (25.2%), $(GlcNAc)_4$ (**4**) (31.2%), $(GlcNAc)_5$ (**5**) (22.4 %), $(GlcNAc)_6$ (**6**) (6.3%), and $(GlcNAc)_7$ (**7**) (1.4%) (15 mg) was dissolved under ultrasound treatment in McIlvaine buffer (50 μl, 50 mM citric acid and 100 mM Na_2HPO_4, pH 4.5) and β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 (10 μl, 4.6 U) was added. The reaction mixture was incubated at 37°C. At respective times samples (5 μl) were taken and diluted with methanol (10 μl), heated for 5 min in a

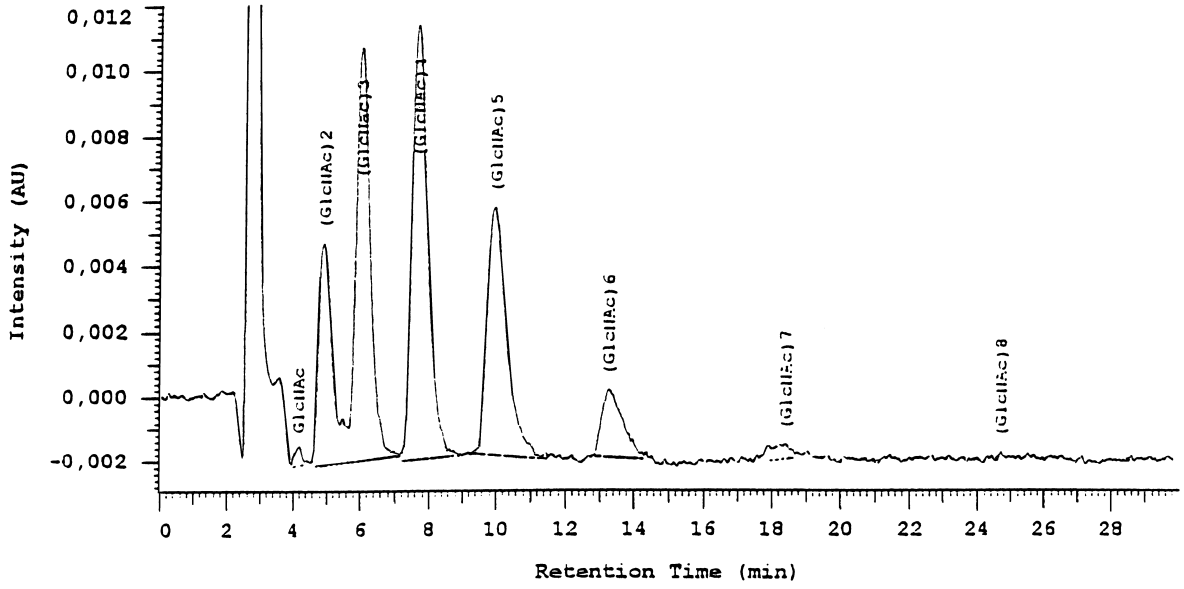

Fig. 1. HPLC analysis of the starting chitooligomer mixture.

boiling water bath to denaturate the enzyme and then analyzed by HPLC and TLC.

Preparative scale experiments: The chitooligomer mixture (200 mg) was dissolved in McIlvaine buffer (664 μl, pH 4.5), β-*N*-acetylhexosaminidase (133 μl, 62 U) from *A. oryzae* 1066 was added and the mixture was incubated under vigorous shaking at 37°C for 9 h. Methanol (1.5 ml) was added to the reaction mixture and it was heated in a boiling water bath for 15 min to deactivate the enzyme. The mixture was then analyzed by HPLC and MS, and fractionated by gel chromatography on Bio-Gel P4 (2.5×90 cm column, 50°C, water as eluent).

2.4. Identification of products

Analysis of the chitooligomer mixture was carried out by HPLC (Waters 660 pump) with UV detection at 210 nm (Waters 996 PDA), using a Lichrospher NH_2 column (5 μm, 250×4 mm) at 40°C with mobile phase acetonitrile-water (70:30; 1 ml/min) in isocratic mode. Identification was performed by co-chromatography with authentic compounds.

Final structure confirmation of the oligomers was performed using MALDI-TOF (Bruker Biflex III) with 2,5-di-hydroxy-benzoic acid (15 μg/ml) as a matrix.

2.5. Isolation of products

Individual chitooligomers were isolated from the mixture by gel chromatography on Bio-Gel P-4 (2.5×90 cm column, temperature 50°C), with water as eluent (45 ml min^{-1}). The eluted chitooligomers were detected by TLC on silica gel plates (Merck 60 GF_{254}) using *n*-propanol/water/concentrated ammonia (7:2:1, v/v) as the solvent system. Spots were visualized by charring with 1 M H_2SO_4 in ethanol. Purity was checked with HPLC and identity of individual chitooligomers was verified by MALDI-TOF MS.

3. Results and discussion

3.1. Effect of substrate concentration

Initially, it was necessary to establish the optimal substrate concentration for the transglycosylation reaction with β-*N*-acetylhexosaminidase. Different fi-

nal concentrations (15, 100, 150, 225, and 250 mg/ml) of the chitooligomer mixture in McIlvaine buffer (pH 4.5) were tested on the analytical scale under the conditions described above. Almost complete hydrolysis of substrates occurred at concentrations of 15 up to 100 mg/ml. Formation of the higher chitooligomers (**6** and **7**) was observed at substrate concentrations over 150 mg/ml. The highest yields of the most desired compounds **6**–**8** were obtained at the substrate concentration 250 mg/ml. At higher concentrations, part of the mixture — presumably higher oligomers — remained insoluble despite sonication and heating to 40°C. A typical course of reaction performed with chitooligosaccharide concentration of 250 mg/ml is shown in Fig. 2. With increasing incubation time the reaction mixture became opaque due to increasing concentration of the higher chitooligomers **6**–**8**. Chitooligomer solubility decreases with the extension of its chain. The maximum yields of **6**–**8** were achieved within 9 h. Subsequently, the transglycosylation products were hydrolyzed followed by decrease of viscosity. After

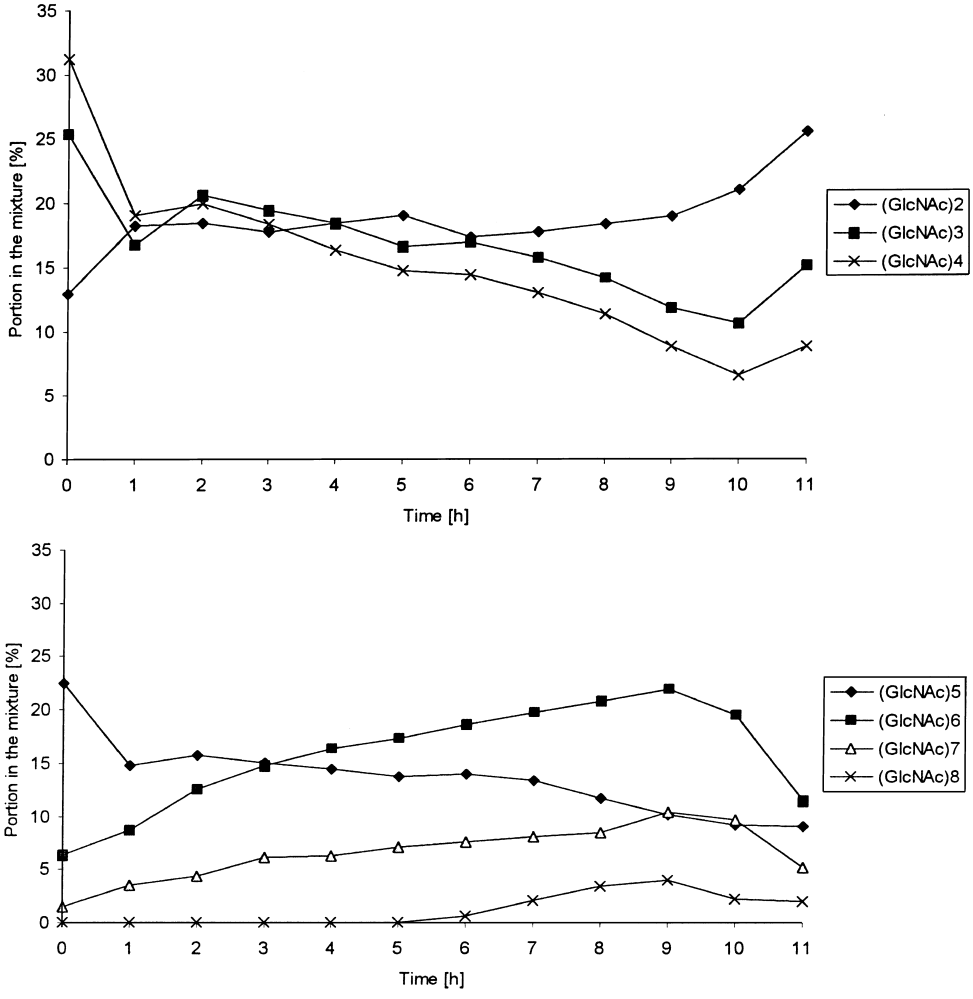

Fig. 2. Time profile of chitooligomer mixture transglycosylation with β-*N*-acetyl-hexosaminidase from *A. oryzae* CCF 1066 (37°C, pH 4.5, concentration of chitooligomer mixture 250 mg/ml). The reaction was monitored by HPLC.

24 h, all chitooligomers in the mixture were completely hydrolyzed to GlcNAc.

Kinetic of this reaction is very complex. All compounds (1–7) present in the reaction mixture can serve as potential donors as well as acceptors for the hexosaminidase. Water acts as another potential glycosyl acceptor and, therefore, not only transglycosylation products but also products of the hydrolysis are present in the reaction milieu. According to the time course of the reaction shown in Fig. 2, compounds **3**, **4** and **5** seem to be hydrolyzed preferably, whereas **2** is rather resistant. A different situation takes place during the transglycosylation of **2**, which yields a mixture of the compounds **1**–**4**. In this case, **2** is very readily cleaved, but **3** and **4** seem to be resistant to hydrolysis [26]. However, neither **3** nor **4** were present in the reaction medium before incubation and their concentrations increased in the course of the reaction. In our case, the concentrations of **6** and **7** (being very low in the original mixture) increase during the first 9 h to reach a maximum. Generation of **8** seems to start after accumulation of a critical amount of **7** — the glycosyl acceptor, in this case — in the reaction mixture. Concentration of **6**, **7**, and **8** commenced to decrease after 9 h of the reaction, when higher oligomers reached maximal concentration. As already mentioned, concentrations of the chitooligosaccharides in the mixture should be considered as a result of both transfer and hydrolytic reactions [26], and the rate of hydrolytic attack depends not only on the affinity of the enzyme to the individual chitooligosaccharides, but also on their concentrations. In addition, **1** as the end hydrolytic product, which accumulates in the reaction medium, noncompetively inhibits β-*N*-acetylhexosaminidase ($(K_i)_{GlcNAc} = 1.61$ mM [30]) and, thus, influences the kinetics of the transglycosylation reactions as well. Measurement and calculation of kinetic constants in such a complex system is very difficult and requires many approximations. Therefore, to predict any exact termination point of the reaction at maximum concentration of the desired compounds is difficult and the time course of the reaction must be carefully monitored by HPLC. Preparatory enzymatic chitooligomer rearrangements were reproducible in our hands under identical conditions. However, thorough mixing is absolutely crucial for the reproducibility.

3.2. Effect of reaction temperature

Influence of temperature (25°C and 37°C) upon the course and the yield of transglycosylation was tested on the analytical scale under the conditions described above.

At 25°C, the transglycosylation proceeded slowly compared to 37°C; however, in both cases, yields of higher chitooligomers were almost identical. Maximum concentrations of **6** and **7** were achieved within 16 h, **8** reached its peak concentration after 18 h at 25°C (data not shown), while at 37°C, maximum yields of **6**, **7**, and **8** were detected already after 9 h. Slower transglycosylation reaction (25°C) is easier to control and it can be terminated at the optimum time before hydrolysis of the products occurs.

3.3. Effect of ammonium sulfate addition

Ammonium sulfate is known to lower water activity and, in some cases, to increase the yields of enzymatic transglycosylations [25,31,32]. Therefore, we investigated its effect on the chitooligosaccharide formation. The reaction was performed on the analytical scale, at 25°C, pH 4.5, with substrate concentration of 225 mg/ml in the presence of 10% of ammonium sulfate. No significant influence of $(NH_4)_2SO_4$ on the yield of **6** and **7** was observed (**8** was not detected in the presence of $(NH_4)_2SO_4$ at

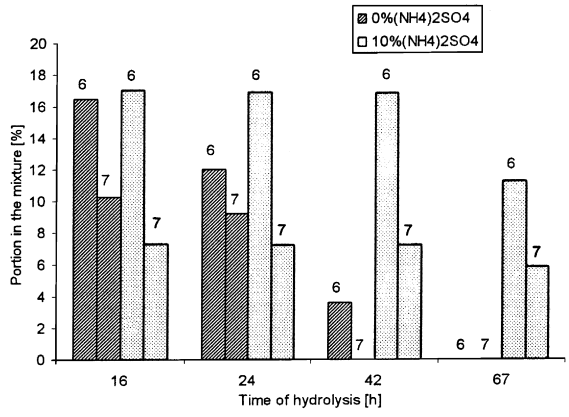

Fig. 3. Effect of $(NH_4)_2SO_4$ on the hydrolysis of transglycosylation products **6** and **7**. The enzyme reaction was performed on the analytical scale at 25°C, pH 4.5 with a concentration of chitooligomer mixture of 225 mg/ml.

Table 1
Composition of the starting and the enzymatically rearranged chitooligomer mixtures — preparatory reaction catalyzed by β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066

Compound	MALDI TOF MS, m/z [M + Na^+]	Starting mixture (%)	Rearranged mixture (%)	Change (%)
GlcNAc (**1**)	–	1	25	+24
$(GlcNAc)_2$ (**2**)	447	13	20	+7
$(GlcNAc)_3$ (**3**)	650	25	10	−15
$(GlcNAc)_4$ (**4**)	853	31	6	−25
$(GlcNAc)_5$ (**5**)	1056	22	8	−14
$(GlcNAc)_6$ (**6**)	1259	6	19	+13
$(GlcNAc)_7$ (**7**)	1462	1	8	+7
$(GlcNAc)_8$ (**8**)	1665	0	3	+3

all); however, degradation of the transglycosylation products — **6** and **7** — was much slower (Fig. 3). 75% of **6** and all **7** were hydrolyzed in the medium without ammonium sulfate after 42 h, and complete hydrolysis of all chitooligosaccharides occurred within 67 h. In the presence of $(NH_4)_2SO_4$, concentrations of **6** and **7** were not changed within 42 h, and only 30% and 25%, respectively, were cleaved within 67 h. Ammonium sulfate may act — besides lowering water activity — by "salting out" the higher chitooligomers from solution preventing their hydrolysis .

3.4. A preparative-scale reaction

Preparatory reactions were carried out with 200 mg of chitooligomers (concentration of 250 mg/ml) under conditions described in Materials and methods. The yields after 9 h and MS data of chitooligomers are summarized in Table 1. HPLC analysis of the

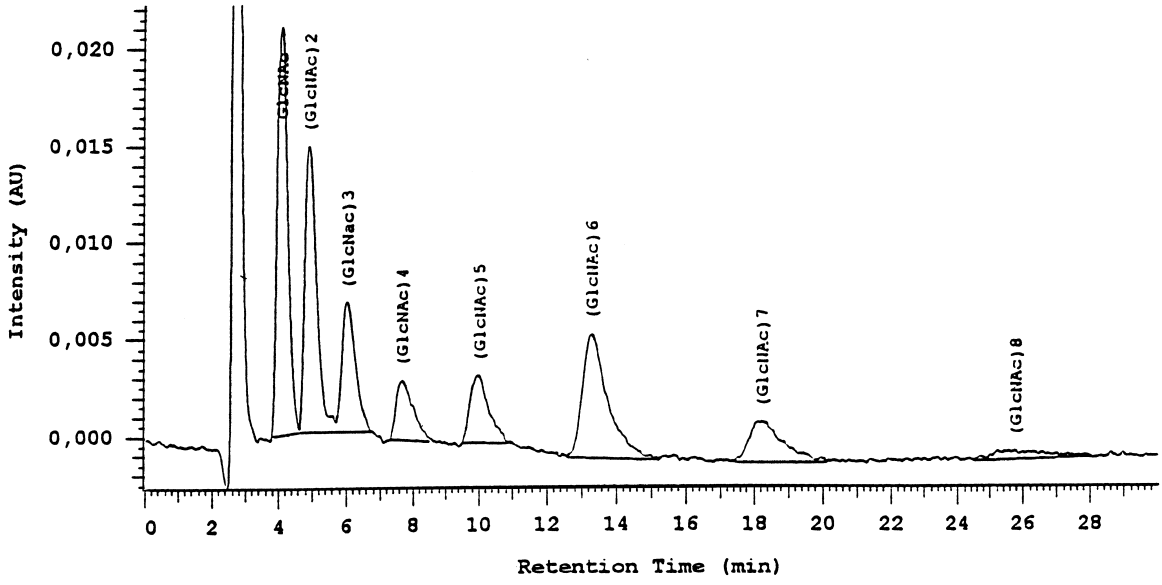

Fig. 4. HPLC analysis of the chitooligomer mixture after rearrangement with β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066-preparative-scale.

rearranged chitoligomer mixture is shown in Fig. 4. Individual chitooligosaccharides were easily separated using gel chromatography on Bio-Gel P4 to afford 5 mg of **8**, 16 mg of **7**, 35 mg of **6**, 14 mg of **5**, 10 mg of **4**, 26 mg of **3**, 46 mg of **2**, and 55 mg of **1**.

This novel approach to the preparation of higher oligosaccharides by enzymatic transglycosylation employing a mixture of unseparated chitooligomers as a starting material has numerous advantages. The crude mixture of chitooligosaccharides resulting from acid hydrolysis of chitin [19] is a much easier available substrate than synthetic glycosyl donors as, e.g., *p*NP-β-GlcNAc [26] or pure lower chitooligomers [23,25,27] used for the enzymatic synthesis of the higher oligosaccharides. Intermediate separation of the respective chitooligomers can be done after the enzymatic reforming reaction, eliminating thus one chromatographic step. The only one purification procedure of the crude HCl/acetolyzed hydrolysate of chitin is its desalting by electrodialysis.

Lower chitooligosaccharides present in the starting mixture in relatively high concentrations (mainly **3**, **4** and **5**) somehow reduce water activity and increase efficiency of the transglycosylation [33,34]; they also partly saturate the enzyme [25] and, therefore, decrease probability of enzymatic hydrolysis of the desired compounds **6**, **7** and **8**.

When higher chitooligosaccharides **4** [26], **6** and **7** [25] are prepared from **2** through enzymatic transglycosylation, first of all, sequential formation of glycosyl acceptors **3**, **4**, **5** and **6** is necessary. Some of these reaction steps can be rate-limiting for the whole transglycosylation process [25]. In the crude mixture used in our method, all the glycosyl acceptors are already present.

This is also first report of preparation of the compound **8** by the enzymatic transglycosylation.

4. Conclusions

Controlled enzymatic rearrangement of the chitooligosaccharides employing β-*N*-acetylhexosaminidase from *A. oryzae* is an elegant and easy method for increasing the yield of the higher chitooligomers in the crude chitin hydrolysate without the need of the synthetic glycosyl donors. Therefore, it is relatively cost effective method for the preparation of higher chitooligosaccharides applied in medicine.

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