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PRODUCTION OF TERMINALLY N-DEACETYLATED OLIGOMERS OF CHITOSANE USING RECOMBINANT CHITOOLIGOSACHARIDE DEACETYLASE NodB OF BACTERIA *Mesorhizobium loti* EXPRESSED IN *Escherichia coli*

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Abstract

Chitin and chitosan oligomers affect the growth and development of plants and are able to induce plant resistance to infection with phytopathogens, which determines the interest in the preparation and use of these compounds. The influence of chitosan oligomers on the plant directly depends on the degree of deacetylation, but it is difficultly to obtain compounds with necessary structure using hydrolysis of the polymer or chemical synthesis. Such problems can be solved in the process of biosynthesis of chitooligosaccharides, when enzymes with specific activity are used. The selectivity of the chitooligosaccharide deacetylase (EC 3.5.1.-) of rhizobia to carry out the monodeacetylation of the chitooligosaccharides at the terminal position of the molecule causes interest in studying the possibility to use this enzyme for the synthesis of such compounds. In current work we have developed approaches for the synthesis of mono-deacetylated chitopentaose (tetra-N-acetylchitopentaose) using *Mesorhizobium loti* CIAM1026 enzyme chitooligosaccharide deacetylase. Heterologous expression of the *nodB* gene encoding the *M. loti* chitooligosaccharide deacetylase in *Escherichia coli* XL1-Blue MRF' and SHuffle express strains using the modified pOPE101mod-*nodB* vector with deleted *pelB* sequence resulted in soluble enzyme preparation. The amount of soluble enzyme was higher in SHuffle express strain, which was specially developed for correct formation of disulfide bonds in synthesized proteins. Studying the properties of the enzyme purified on Ni-NTA agarose showed its ability to deacetylate penta-N-acetylchitopentaose at the terminal position. Mass spectrometric analysis confirmed the use of practically the entire substrate for the preparation of deacetylated tetra-N-acetylchitopentaose. Methods for the separation and purification of deacetylated chitooligosaccharides by ion exchange chromatography followed by desalination have been developed. Synthesis of terminally N-deacetylated chitosan oligomers may be a necessary step in the preparation of their conjugates with biologically active compounds.

Keywords: chitin and chitosan oligomers, *Mesorhizobium loti* chitooligosaccharide deacetylase, pOPE101-215(Yol) and pRSETb vectors, biosynthesis, *Escherichia coli* SHuffle express and XL1-Blue MRF'

Chitin and chitosan oligomers (chitooligosaccharides) are widely used in medicine and agrotechnologies. In agriculture, interest in these compounds is associated with their ability to induce nonspecific resistance of plants to infection by phytopathogens due to the activation of the natural protective potential (elicitor activity) [1, 2], as well as to stimulate the growth and development of plants. The specific feature of chitooligosaccharides when used as regulators is non-toxicity even in significant quantities and ease of disposal, which makes them environmentally safe [3-5].

For the elicitor activity to manifest itself, the degree of deacetylation of chitooligosaccharides is important, because in case of some plant species (arabidopsis, rice, wheat), eliciting properties are shown by fully acetylated chitin oligomers, in case of others (peas, coffee, parsley), by partially or completely deacetylated chitosan oligomers [6-9]. In addition, deacetylation of chitooligosaccharides in certain positions may allow various chemical groups to covalently attach to them, which will significantly expand the range of such compounds.

In chemical synthesis, it is difficult to obtain compounds with the necessary degree of deacetylation. In particular, it is almost impossible to synthesize partially deacetylated chitin oligomers in this way due to the identical chemical activity of the amino groups in sugar residues, which does not allow the deacetylation reaction to be controlled due to harsh conditions. Similar problems can be solved during the biosynthesis of chitooligosaccharides, when enzymes with selective specificity are used and, as a result, products with a strictly defined structure are formed. In this connection, the search and study of enzymes involved in the synthesis of chitin oligomers and their deacetylated derivatives are of great interest.

In *Rhizobium* spp. bacteria, the *nodABC* genes [10-13], which are common for all rhizobial species, encode enzymes necessary for synthesizing the core structure of Nod factors, the signal molecules that control the development of legume-rhizobial symbiosis. NodC is an N-acetylglucosaminyltransferase catalyzing the synthesis of chitin oligomers. Chitooligosaccharide deacetylase NodB is required for deacetylation of chitooligosaccharide at the non-reducing end (terminal N-acetylglucosamine) [14], which allows fatty acid to be attached to it using NodA acyl transferase. The selective ability of rhizobial chitooligosaccharide deacetylase to carry out monodeacetylation at the terminal position determines the interest in studying the possibility of using this enzyme to synthesize deacetylated chitosan oligomers in vitro.

Previously, we developed an approach to obtain chitin oligomers associated with the use of the N-acetylglucosaminyltransferase enzyme of two strains of rhizobial bacteria — *Rhizobium* sp. GRH2 and *Mesorhizobium loti* CIAM1803 [15]. In this study, we carried out heterologous expression of the *nodB* gene encoding *M. loti* CIAM1803 chitooligosaccharide deacetylase in two strains of *Escherichia coli* XL1-Blue-MRF' and SHuffle express. In this case, the plasmid pOPE101-215(Yol) was used, which allows the synthesized protein to transfer to the periplasmic space thanks to the inclusion in the pelB leader sequence, which ensures its safety and correctness of folding [16]. This plasmid was successfully used to synthesize the heavy and light chains of immunoglobulin G [16]. Earlier, an attempt was made to synthesize NodB of *Sinorhizobium meliloti* in *E. coli* BL21 (DE3) bacteria using the pET-3c vector, while the recombinant protein formed inclusion bodies, which required refolding (probably, therefore, the enzyme activity was low) [14]. An active soluble protein NodB of *Rhizobium* sp. GRH2 has been recently obtained by a group that synthesized it in BL21(DE3) cells transformed with the pET22b(+) vector [17], but the enzyme yield was not high enough.

In this work, using the pOPE101-215(Yol) vector and its modified version pOPE101mod with the *pelB* sequence removed, used to transform two strains of *E. coli* (SHuffle express and XL1-Blue MRF'), we managed to achieve a high yield of soluble protein, the NodB *Mesorhizobium loti* chitooligosaccharide deacetylase enzyme capable of deacetylating penta-N-acetylchitentaose in terminal position. Methods for selective extraction of the formed deacetylated chitooligosaccharides have been also developed.

The work aimed to study the possibility of obtaining terminally N-deacetylated chitosan oligomers using NodB chitooligosaccharide deacetylase, synthesized by heterologous expression of specially designed gene constructs.

Techniques. DNA was isolated from the *Mesorhizobum loti* CIAM 1803 strain (WDCM 966). The *E. coli* DH5α strain was used for standard cloning procedures. The synthesis of proteins was carried out in a mutant strain of *E. coli* C41 obtained on the basis of BL21(DE3) [18], as well as in strains XL1-Blue MRF' (Stratagene, USA) and SHuffle express (New England BioLabs, United Kingdom). *E. coli* strains were cultured in a liquid medium LB [19] or 2xYT (Difco, Netherlands) on a Heidolph Unimax 2010 orbital shaker (Heidolph Instruments GmbH, Germany) at 30 °C or 37 °C with the addition of ampicillin (100 μg/ml) in the presence of 0.04 mM or 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) as an inducer of expression. The strain of rhizobia was cultured in a liquid medium TY (trypton 5 g/l, yeast extract 3 g/l, $CaCl_2$ 0.5 g/l) at 28 °C.

When creating genetic constructs for the synthesis of NodB in *E. coli* bacteria, the full-size *nodB* gene was amplified on the DNA matrix of the *M. loti* CIAM1803 (WDCM 966) strain using primers into which the BamHI and EcoRI restriction sites were introduced for cloning in the pRSETb vector (Invitrogen, USA) or NcoI and NotI for cloning in the pOPE101-215(Yol) vector (Progen Biotechnik GmbH, Germany):

*nodB*_F_BamHI — GGGGATCCGATGAGACGTCTCGATGACAG,
*nodB*_R_EcoRI — GGGAATTCTCAGTGATGTTCTGGAAGCG,
*nodB*_F_NcoI — GGCCATGGCGATGAGACGTCTCGATGACAG,
*nodB*_R_NotI — GGGCGGCCGCGTGATGTTCTGGAAGCG.

Amplification (a Thermal Cycler C1000, Bio-Rad Laboratories, USA) was performed according to the following protocol: 5 min at 95 °C; 30 cycles — 30 s at 94 °C, 30 s at 51 °C and 30 s at 72 °C).

To obtain the modified vector pOPE101mod with the *pelB* sequence removed, the following primers were used:

*nodB*_F_NcoI — GGCCATGGCGATGAGACGTCTCGATGACAG,
pOPE101mod_R_NcoI — CCCCATGGCGGTTAATTTCTCCTCTT.

All vectors contained a His_6 sequence allowing the purification of recombinant proteins using metal chelate affinity chromatography.

The constructs were introduced into *E. coli* cells using electrical or chemical transformation methods [15, 20, 21]. Plasmid DNA was isolated from 3 ml culture using alkaline lysis [22].

To synthesize NodB protein, *E. coli* C41, XL1-Blue MRF' or SHuffle express cells were cultured in a liquid medium LB or 2xYT at 37 °C to a density of OD_{600} = 0.6-0.7, then IPTG was added to a final concentration of 0.04 or 0.4 mM, and culturing continued at 30 °C or 37 °C for 3 or 24 hrs. After completion of the synthesis, the cells were held on ice for 20 min, then precipitated by centrifugation at 3,500 g (J2-21, Beckman Coulter, Inc., USA) for 15 min at 4 °C. The cell sediment was gently resuspended in 50 mM Na-phosphate buffer (pH 7.4) containing a mixture of protease inhibitors (Sigma, USA), 1 mM dithiothreitol (DTT) and DNase (1 U/ml). The cells were sonicated 3 times for 30 s at an amplitude of 10 μm (Soniprep 150 Plus, MSE, United Kingdom), at 40-

sec intervals of holding on ice, then centrifuged at 100000 g (TL-100, Beckman Coulter, Inc., USA).

Aliquots of the soluble and insoluble protein fractions were separated in a 12-15% polyacrylamide gel (PAAG) in a Tris-glycine buffer in the presence of sodium dodecyl sulfate (SDS) (25 mM Tris-HCl pH 8.3; 192 mM glycine, 0.1% SDS) by the U.K. Laemmli method [23]. For the separation, the Mini-PROTEAN 3 system (Bio-Rad Laboratories, USA) was used, with the current intensity of 15-35 mA per gel. After completion of electrophoresis, the gels were washed with deionized water and either stained in Simple Blue solution (Invitrogen, USA) according to the manufacturer's protocol, or used for Western blot hybridization. The proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot semi-dry blotting device (Bio-Rad Laboratories, USA). To check the transfer efficiency, the membranes were stained with Ponce 4R, then washed 2 times for 10 min in TBS buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl), then 2 times for 15 min in TTBS buffer (50 mM Tris -HCl, pH 8.0; 150 mM NaCl, 0.05% Tween-20). Then they were incubated for 1 hr. in a blocking solution of TTBS containing 1% bovine serum albumin (BSA), then for 2 hrs with antibodies against a His_6 sequence (anti-His) conjugated with horseradish peroxidase, which were diluted in TTBS buffer with 0.5% BSA in the ratio of 1:2000. After that, the membranes were washed 2 times for 15 min in TTBS buffer, then 2 times for 10 min in TBS buffer. To demonstrate the reaction, the Clarity Western ECL Substrate, a chemiluminescent substrate (Bio-Rad Laboratories, USA) was used. The development of the reaction was analyzed on a G:BOX-CHEMI-XX9 system (Syngene, United Kingdom).

For the standard procedure for purification of the synthesized proteins containing a His_6 sequence, 100 μl of Ni-NTA (nickel-nitrilotriacetic acid) agarose (Thermo Fisher Scientific, USA) was used. Purification was carried out at 4 °C according to the protocol proposed by the manufacturer; the protein was washed from the column with 50 mM Na-phosphate buffer (pH 7.4) containing imidazole (300 mM).

In vitro deacetylation of penta-N-acetylchitentaentase using the obtained NodB protein was performed overnight at 28 °C in 100 μl of a solution containing 3-[N-morpholino] propanesulfonic acid (MOPS, 20 mM, pH 7.2), 5 μg of an enzyme purified on Ni-NTA agarose and 1 mg of penta-N-acetylchitentaose substrate. The reaction was stopped by boiling at 95 °C for 5 min, and the samples were centrifuged for 10 min at 14000 g (Mikro 22R, Hettich GmbH & Co. KG, Germany). The supernatant was dried in a vacuum evaporator (Concentrator Plus, Eppendorf, USA). Separation and purification of the obtained chitosan oligomer was performed using ion-exchange chromatography on Toyopearl-SP 650M (0.5×2 cm) (Sigma-Aldrich, USA); elution was performed with 0.2 M NaCl. A Sep-Pak C18 cartridge (Waters, USA) was used to remove salt from the eluate.

The products of the deacetylation reaction were analyzed on a Varian 902 FT/ICR MS ion cyclotron mass spectrometer (Agilent Technologies, USA) with a 9.4 T superconducting magnet. Desorption and ionization of the sample was performed using the third harmonic of the Nd:YAG laser (λ = 355 nm). The samples were dissolved in 2 μl of a 0.1% aqueous solution of trifluoroacetic acid (TFA), an aliquot (0.5 μl) was mixed on a target with an equal volume of the matrix (2.5-dihydroxybenzoic acid at a concentration of 20 mg/ml; the solvent for the matrix was a mixture of acetonitrile and 0.1% aqueous solution of TFA in the ratio of 30%:70%) and dried in air. Then the samples were subjected to laser irradiation (5 pulses per series). The molecular weight of the sample was determined by external calibration using standards.

Results. Construction of vectors that ensure the synthesis of

NodB protein in *E. coli* cells. For heterologous expression of the *nodB M. loti* CIAM1803 gene, two types of constructs were obtained — in the pRSETb vector under the control of the promoter of T7 bacteriophage and in the pOPE101-215(Yol) vector, in which the coding sequence of the gene was cloned under the synthetic promoter in the frame with the *pelB* sequence necessary for transfer of the synthesized protein to the periplasmic space.

Synthesis of chitooligosaccharide deacetylase (NodB) in the *E. coli* C41 strain using the pRSETb-*nodB* construct. A prerequisite for protein synthesis is the ability of the *E. coli* strain to stably maintain the expression vector, to be resistant to the foreign product and to ensure its stability. Mutant strains of *E. coli* C41(DE3) and C43(DE3), derived from BL21 (DE3) [18], have such properties. When the *E. coli* C41 cells transformed with the pRSETb-*nodB* construct were cultured in the presence of a 0.4 mM IPTG inducer, NodB protein synthesis with the expected molecular weight of about 25 kDa was observed (Fig. 1). NodB production was high, but at the same time, with its bulk accumulated in the insoluble fraction obtained at 3600 g, which indicated the formation of inclusion bodies (see Fig. 1). Variation of cultivation temperature and inducer concentration did not affect the increase in the yield of NodB protein in a soluble state. Similarly, earlier when using BL21(DE3) cells transformed with the pET-3c vector, in which the *nodB* gene was cloned under the T7 promoter, all the synthesized protein was in the insoluble fraction [14]. Thus, with vectors that ensure a very high level of NodB synthesis in *E. coli* cells, it cannot be obtained in a soluble state.

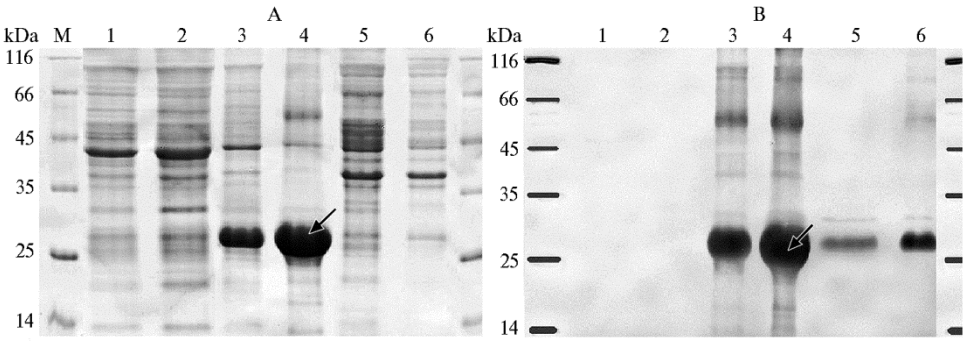

Fig. 1. Synthesis of chitooligosaccharide deacetylase (NodB) *Mesorhizobum loti* CIAM1803 in *Escherichia coli* C41 cells transformed with the pRSETb-*nodB* vector, after 20 h of culture: A — staining of Simple Blue gel, B — Western blot hybridization with anti-His antibodies. Transformation with the pRSETb vector without insert (control): 1 and 2 — cultivation without the addition of an isopropyl-β-D-thiogalactoside (IPTG) inducer and with 0.4 mM IPTG. Transformation with the pRSETb-*nodB* vector: 3 and 4 — cultivation without the addition of IPTG and with 0.4 mM IPTG (insoluble fractions obtained by centrifugation at 3600 g); 5 and 6 — culture without the addition of IPTG and with 0.4 mM IPTG (insoluble fractions obtained by centrifugation at 14000 g). M — molecular weight marker. The arrow indicates the synthesized protein.

Synthesis of chitooligosaccharide deacetylase in *E. coli* XL1-Blue MRF' and SHuffle express using the pOPE101-215(Yol) vector. To increase the yield of soluble *NodB* protein, we used the construct in the pOPE101-215(Yol) vector (Progen Biotechnik GmbH, Germany), which ensures the accumulation of protein in the periplasmic cell space. When pOPE101-215(Yol)-*nodB* was introduced into *E. coli* XL1-Blue MRF' and SHuffle express cells we observed protein synthesis with the expected molecular weight (about 25 kDa) in the presence of 0.04 and 0.4 mM IPTG. We judged this by the emergence of a product similar in motility to that synthesized in *E. coli* C41 cells when using the pRSETb-*nodB* construct. In both strains, NodB expression was

maximal when cultured overnight, at a temperature of 37 °C and an IPTG concentration of 0.4 mM (Fig. 2), but in the case of the XL1-Blue MRF' strain, NodB production was significantly higher (see Fig. 2). The protein obtained in *E. coli* XL1-Blue MRF' cells accumulated mainly in the insoluble fraction, and only a small amount of it was present in the soluble fraction (Fig. 3). In order to exclude the possibility of ineffective cell lysis when using a special buffer [16], we applied ultrasonic treatment. However, probably, due to the peculiarities of the structure of the synthesized NodB protein, we failed to detect its effective transfer to the periplasmic space. To assess the possible effect of *pelB* on the level of protein synthesis, we also obtained the pOPE101mod-*nodB* construct, from which we removed the fragment encoding this sequence.

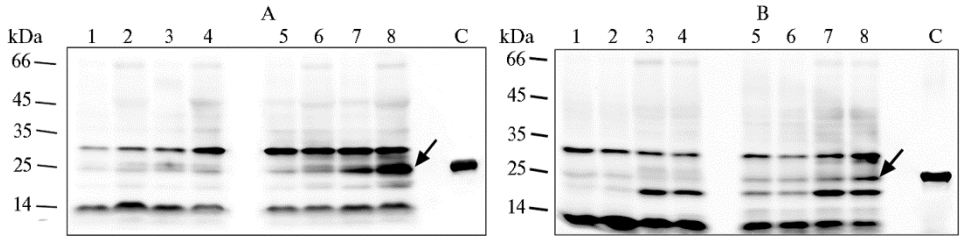

Fig. 2. Synthesis of chitooligosaccharide deacetylase (NodB) of *Mesorhizobum loti* CIAM1803 in *Escherichia coli* XL1-Blue MRF' (A) **and SHuffle express** (B) **cells transformed with the pOPE101-215(Yol)-*nodB* vector:** 1 and 2 — culture at 30 °C in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture at 30 °C with 0.4 mM IPTG for 3 and 20 hrs; 5 and 6 — culture at 37 °C with 0.04 mM IPTG for 3 and 20 hrs, 7 and 8 — culture at 37 °C with 0.4 mM IPTG for 3 and 20 hrs. C (control) — synthesis of NodB protein in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs at 37 °C in the presence of 0.4 mM IPTG (insoluble fraction). Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

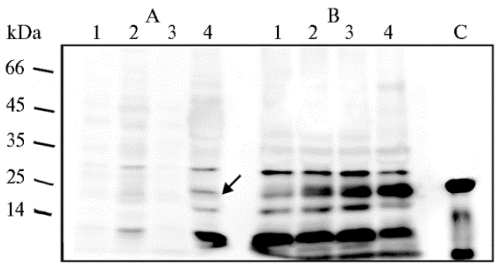

Fig. 3. Analysis of the content of NodB *Mesorhizobum loti* CIAM1803 protein in the soluble (A) **and insoluble** (B) **fractions of *Escherichia coli* XL1-Blue MRF' cells in transformation with the pOPE101-215(Yol)-*nodB* construct:** 1 and 2 — culture at 37 °C in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture at 37 °C with 0.4 mM IPTG for 3 and 20 hrs. C (control) — synthesis of NodB protein in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs at 37 °C in the presence of 0.4 mM IPTG (insoluble fraction). Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

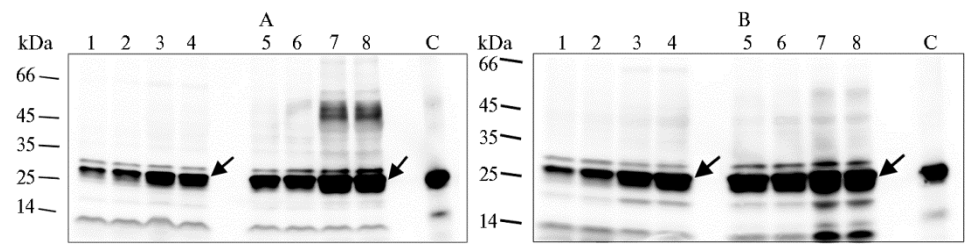

Fig. 4. Synthesis of chitooligosaccharide deacetylase NodB of *Mesorhizobum loti* CIAM1803 in transformation of *Escherichia coli* XL1-Blue MRF' (A) **and SHuffle express** (B) **with the pOPE101mod-*nodB* vector:** 1 and 2 — culture at 30 °C in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture at 30 °C with 0.4 mM IPTG for 3 and 20 hrs; 5 and 6 — culture at 37 °C with 0.04 mM IPTG for 3 and 20 hrs, 7 and 8 — culture at 37 °C with 0.4 mM IPTG for 3 and 20 hrs. C (control) — NodB protein synthesized in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs at 37 °C in the presence of 0.4 mM IPTG. Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

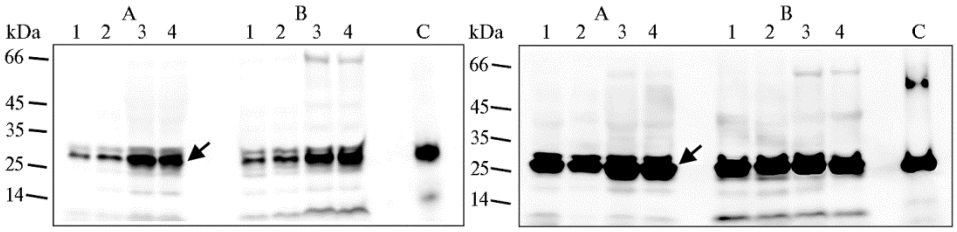

Fig. 5. The content of NodB of *Mesorhizobum loti* CIAM1803 protein in the soluble (A) **and insoluble** (B) **fractions in transformation of *Escherichia coli* XL1-Blue MRF'** (left) **and SHuffle express** (right) **with the pOPE101mod-*nodB* construct (culture at 30 °C):** 1 and 2 — culture in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture with 0.4 mM IPTG for 3 and 20 hrs. C (control) — synthesis of NodB protein in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs in the presence of 0.4 mM IPTG (insoluble fraction). Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

Synthesis of chitooligosaccharide deacetylase (NodB) in *E. coli* XL1-Blue MRF' and SPuffle express cells using the pOPE101mod-*nodB* vector. When using the pOPE101mod-*nodB* vector, from which the *pelB* leader sequence was removed, a higher level of NodB production was observed in two types of *E. coli* cells (XL1-Blue MRF' and Shuffle express) compared to that in the variant of transformation with the initial vector pOPE101-215(Yol)-*nodB* (Fig. 4, 5). When culture in the presence of 0.4 mM IPTG and at temperatures of 30 and 37 °C, a high level of NodB synthesis was observed already after 3 hrs in the cells of two *E. coli* strains, the XL1-Blue MRF' and SHuffle express (see Fig. 4, 5).

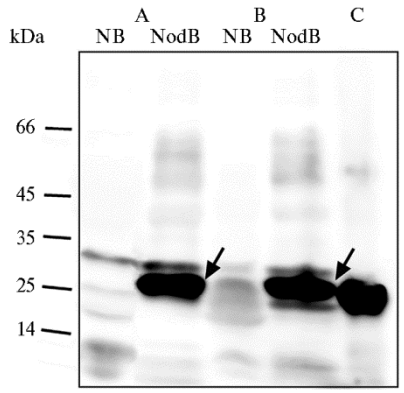

Fig. 6. Purification of recombinant protein NodB of *Mesorhizobum loti* CIAM1803 on Ni-NTA agarose: A and B — transformation of *Escherichia coli* XL1-Blue MRF' and SHuffle express strains with the pOPE101mod-*nodB* construct, C (control) — transformation of *E. coli* C41 with pRSETb-*nodB*; NB is a protein that did not bind to Ni-NTA agarose, NodB is a protein purified on Ni-NTA agarose. Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

In this case, the amount of soluble protein was higher at a temperature of 30 °C and with the use of the SHuffle express strain (see Fig. 5), specially designed to ensure the correct formation of disulfide bonds in proteins [24]. The increase in the yield of the recombinant product in the soluble state as the cultivation temperature decreases can probably be determined by the fact that under such conditions the synthesis of chaperones in the cell enhances [25]. Thus, we managed to obtain chitooligosaccharide deacetylase in a soluble state.

Analysis of the products of deacetylation of penta-N-acetyl chitopentaose by the *M. loti* CIAM1803 chitooligosaccharide deacetylase enzyme. To test the enzymatic activity of the obtained chitooligosaccharide deacetylase, we studied its ability to deacetylate penta-N-acetyl chitopentaose which is the main substrate of this enzyme in *M. loti* bacteria. For this purpose, we purified the recombinant protein obtained during the synthesis in *E. coli* XL1-Blue MRF' and SHuffle express cells with the use of metal chelate affinity chromatography on Ni-NTA agarose (we obtained about 200 μg of partially purified protein from 50 ml culture) (Fig. 6). The purified enzyme was incubated with the substrate (penta-N-acetyl chitopentaose). Mass spectrometry of the samples obtained after the deacetylation reaction catalyzed by NodB

chitooligosaccharide deacetylase (Fig. 7) revealed a compound with the mass-to-charge ratio (m/z) of 1014.390. The expected mass of chitopentaose deacetylated at one position is 991. However, an H^+ or Na^+ ion usually attaches to the test substance during a mass spectrometric analysis. If Na^+ attaches, the molecular weight of the analyzed substance will be 991 + 23 = 1014. Thus, the substance to be synthesized is tetra-N-acetyl chitopentaose (monodeacetylated chitopentaose). The analysis showed that chitooligosaccharide deacetylase obtained by synthesis in *E. coli* SHuffle express and XL1-Blue MRF' bacteria has the necessary enzymatic activity.

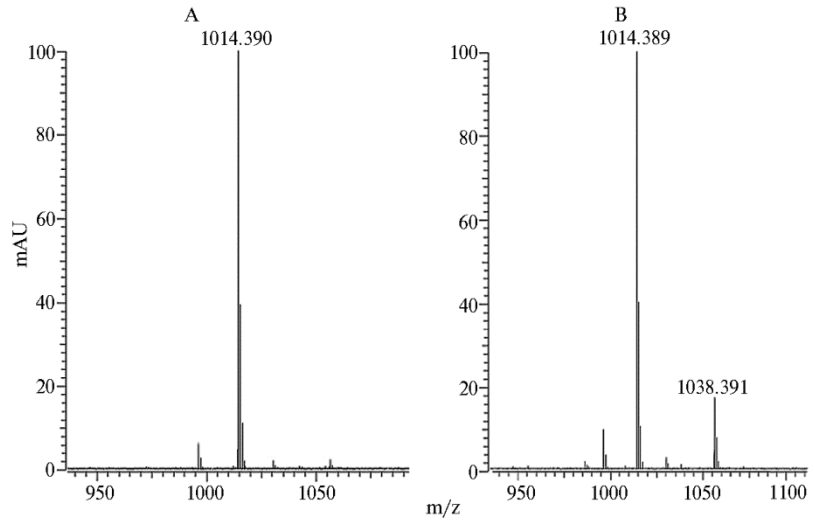

Fig. 7. Mass spectrometry analysis of the products of deacetylation of penta-N-acetyl chitopentaose by the *Mesorhizobum loti* CIAM1803 chitooligosaccharide deacetylase enzyme synthesized in *E. coli* SHuffle Express (A) **and XL1-Blue MRF'** (B) **strains with the pOPE101mod-*nodB* vector** (Varian 902 FT/ICR MS mass spectrometer, Agilent Technologies, USA).

Purification of N-terminal deacetylated chitooligosaccharides. We also investigated the possibility to separate deacetylated chitooligosaccharides (due to the presence of a free amino group in the molecule) by ion-exchange chromatography. It was necessary to solve the problem of removing the aqueous-saline eluent after chromatography. To isolate the N-terminal deacetylated chitooligosaccharide, we used ion-exchange chromatography on Toyopearl-SP 650M; elution was performed with 0.2 M NaCl. A Sep-Pak C18 cartridge was used to remove salt from the eluate. This is due to the fact that oligomers of both chitin and chitosan are able to reversibly interact in the aqueous eluent with reversed-phase sorbents, and the time of their yield from the column is several times longer than the yield of inorganic salts [26]. The chitooligosaccharide solution after ion-exchange chromatography (no more than one volume of the cartridge) was passed through a sorbent, which was then washed with water. NaCl molecules pass through a hydrophobic medium with practically no dilution effect; therefore, to achieve complete desalting, it is enough to flush the column with 3 cartridge volumes. After this, the chitooligosaccharide was eluted with water; the desalted solution was lyophilized and analyzed. Thus, we have developed methods for the selective isolation of deacetylated chitooligosaccharides.

In our work, we studied the possibility of synthesizing the chitooligosaccharide deacetylase enzyme (NodB) of *M. loti* bacteria in *E. coli* cells in a soluble state and using it to obtain terminally N-deacetylated chitosan oligomers. In soil rhizobial bacteria, chitooligosaccharide deacetylase is a soluble cytoplasmic

protein that participates in the initial stages of the synthesis of signal molecules of Nod-factors, in particular, deacetylates the chitin oligomers (n = 4-6) obtained at the first stage of synthesis at the non-reducing end [27]. The possibility of obtaining terminally N-deacetylated chitin oligomers in vitro using the chitooligosaccharide deacetylase enzyme is of practical interest, since such compounds are very difficult to obtain during chemical synthesis, but it is convenient to use them for covalent attachment of biologically active substances.

For the heterologous expression of the *nodB M. loti* gene in *E. coli* bacteria, several genetic constructs were used, including those enabling the accumulation of the synthesized protein in the periplasmic space. However, in our experiments during the synthesis in *E. coli* C41 cells with the pRSETb-*nodB* construct, as well as in XL1-Blue MRF' and SHuffle express with the pOPE101-215(Yol)-*nodB* construct, we initially failed to obtain a significant amount of NodB protein in a soluble state. Most of the synthesized protein in the cells was contained in the insoluble fraction of cells. The observed ineffective removal of NodB into the periplasmic space was probably associated with the conformational features of this protein.

However, by removing the *pelB* sequence from the expression vector, we were able to increase the production of soluble protein in the SHuffle express and XL1-Blue MRF' cells. At the same time, the quantitative yield of NodB protein after purification on Ni-NTA agarose was quite high. Testing of the activity of purified soluble NodB protein confirmed its ability to deacetylate penta-N-acetylchitentaose at its non-reducing end. Mass spectrometry analysis showed that practically the entire substrate was used to obtain deacetylated tetra-N-acetyl-chitopentaose. A similar activity was detected for another rhizobium enzyme, NodB of *Rhizobium* sp. GRH2, synthesized in a soluble state in *E. coli* BL21(DE3) cells using the construct in the pET-22b(+) vector [17].

Thus, for the first time, the proposed approach using the modified vector pOPE101mod-*nodB* allows the synthesis of *Mesorhizobum loti* active chitooligosaccharide deacetylase in a new type of *Escherichia coli* SHuffle express and XL1-Blue MRF' cells with high quantitative yield. For the purpose of separation and purification of the resulting deacetylated chitooligosaccharides, we have developed methods based on ion-exchange chromatography followed by desalting.

REFERENCES

1. Abu-Muriefah S.S. Effect of chitosan on common bean (*Phaseolus vulgaris* L.) plants grown under water stress conditions. *Int. Res. J. Agric. Sci. Soil Sci.*, 2013, 3: 192-199.
2. Chatelain P.G., Pintado M.E., Vasconcelos M.W. Evaluation of chitooligosaccharide application on mineral accumulation and plant growth in *Phaseolus vulgaris*. *Plant Sci.*, 2014, 215-216: 134-140 (doi: 10.1016/j.plantsci.2013.11.009).
3. Khan W., Prithiviraj B., Smith D.L. Chitosan and chitin oligomers increase phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities in soybean leaves. *J. Plant Physiol.*, 2003, 160: 859-863 (doi: 10.1078/0176-1617-00905).
4. Aziz A., Trotel-Aziz P., Dhuicq L., Jeandet P., Couderchet M., Vernet G. Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. *Disease Control and Pest Management*, 2006, 96(11): 1188-1194 (doi: 10.1094/PHYTO-96-1188).
5. Gayoso G., Pomar F., Novo-Uzal E., Merino M., de Ilarduya O.M. The Ve-mediated resistance response of the tomato to *Verticillium dahliae* involves H_2O_2, peroxidase and lignins and drives PAL gene expression. *Plant Biology*, 2010, 10(232): 1471-2229 (doi: 10.1186/1471-2229-10-232).
6. Kaku H., Nishizawa Y., Ishii-Minami N., Akimoto-Tomiyama C., Dohmae N., Takio K., Minami E., Shibuya N. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *PNAS USA*, 2006, 103: 11086-11091 (doi: 10.1073/pnas.0508882103).
7. Cabrera J.C., Messiaen J., Cambier P., Van Cutsem P. Size, acetylation and concentration of chitooligosaccharide elicitors determine the switch from defense involving PAL activation to cell death and water peroxide production in Arabidopsis cell suspensions. *Physiologia Plantarum*,

2006, 127: 44-56 (doi: 10.1111/j.1399-3054.2006.00677.x).
8. Conrath U., Domard A., Kauss H. Chitosan-elicited synthesis of callose and of coumarin derivatives in parsley cell suspension cultures. *Plant Cell Rep.*, 1989, 8(3): 152-155 (doi: 10.1007/BF00716829).
9. Dzung N.A., Khanh V.T.P., Dzung T.T. Research on impact of chitosan oligomers on biophysical characteristics, growth, development and drought resistance of coffee. *Carbohyd. Polym.*, 2011, 84: 751-755 (doi: 10.1016/j.carbpol.2010.07.066).
10. Spaink H., Sheeley D. M., van Brussel A.A.N., Glushka J., York W.S., Tak T., Geiger O., Kennedy E., Reinhold N., Lugtenberg B.J.J. A novel highly unsaturated fatty acid moiety of lipooligosaccharide signals determines host specificity of *Rhizobium*. *Nature*, 1991, 354: 125-130 (doi: 10.1038/354125a0).
11. Mergaert P., D'Haeze W., Geelen D., Promé D., Van Montagu M., Geremia R., Promé J.C., Holsters M. Biosynthesis of *Azorhizobium caulinodans* nod factors. *J. Biol. Chem.*, 1995, 270(49): 29217-29223 (doi: 10.1074/jbc.270.49.29217).
12. Roche P., Maillet F., Plazanet C., Debelle F., Ferro M., Truchet G., Prome J.-C., Denarie J. The common *nodABC* genes of *Rhizobium meliloti* are host-range determinants. *PNAS USA*, 1996, 93: 15305-15310 (doi: 10.1073/pnas.93.26.15305).
13. Southwick A.M., Wang L.X., Long S.R., Lee Y.C. Activity of *Sinorhizobium meliloti* NodAB and NodH enzymes on thiochitooligosaccharides. *J. Bacteriol.*, 2002, 184(14): 4039-4043 (doi: 10.1128/JB.184.14.4039-4043.2002).
14. John M., Rohrig H., Schmidt J., Wieneke U., Schell J. Rhizobium NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. *PNAS USA*, 1993, 90: 625-629 (doi: 10.1073/pnas.90.2.625).
15. Leppyanen I.V., Artamonova T.O., Lopatin S.A., Varlamov V.P., Tikhonovich I.A., Dolgikh E.A. Biosynthesis of hexa and pentameric chitooligosaccharides using N-acetyl-glucoseaminyl transferase from rhizobial bacteria. *Russian Journal of Genetics: Applied Research*, 2014, 4(5): 368-381 (doi: 10.1134/S2079059714050098).
16. Schmiedl A., Breitling F., Winter C., Queitsch I. Dübel S. Effect of unpaired cysteines on yield, solubility and activity in various recombinant antibody formats expressed in *E. coli*. *J. Immunol. Methods*, 2000, 242(1-2): 101-114 (doi: 10.1016/S0022-1759(00)00243-X).
17. Hamer S.N., Cord-Landwehr S., Biarnes X., Planas A., Waegeman H., Moerschbacher B.M., Kolkenbrock S. Enzymatic production of defined chitosan oligomers with a specific pattern of acetylation using a combination of chitin oligosaccharide deacetylases. *Sci. Rep.*, 2015, 5: 8716 (doi: 10.1038/srep08716).
18. Miroux B., Walker J.E. Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.*, 1996, 260: 289-298 (doi: 10.1006/jmbi.1996.0399).
19. Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.*, 1951, 62: 293-300.
20. Inoue H., Nojima H., Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, 1990, 96: 23-28 (doi: 10.1016/0378-1119(90)90336-P).
21. Dower W.J., Miller J.F., Ragsdale C.W. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.*, 1988, 16: 6127-6145.
22. Birnboim H.C., Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, 1979, 7: 1513-1523.
23. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, 227: 680-685.
24. Lobstein J., Emrich C.A., Jeans C., Faulkner M., Riggs P., Berkmen M. Shuffle, a novel *Escherichia coli* protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. *Microb. Cell Fact.*, 2012, 11: 753 (doi: 10.1186/1475-2859-11-56).
25. Caspeta L., Flores N., Pérez N.O., Bolívar F., Ramírez O.T. The effect of heating rate on *Escherichia coli* metabolism, physiological stress, transcriptional response, and production of temperature induced recombinant protein: a scale-down study. *Biotechnol. Bioeng.*, 2009, 102(2): 468-482 (doi: 10.1002/bit.22084).
26. Lopatin S.A., Ilyin M.M., Pustobaev V.N., Bezchetnikova Zh.A., Varlamov V.P., Davankov V.A. Mass-spectrometric analysis of N-acetylchitooligosaccharides prepared through enzymatic hydrolysis of chitosan. *Anal. Biochem.*, 1995, 227, 285-288 (doi: 10.1006/abio.1995.1282).
27. Schmidt J., Wingender R., John M., Wieneke U., Schell J. *Rhizobium meliloti* nodA and nodB genes are involved in generating compounds that stimulate mitosis of plant cells. *PNAS USA*, 1988, 85(22): 8578-8582.