Glycoside hydrolases of marine bacteria are promising tools in haemotherapy

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Abstract— **Universal donor blood of group O is widely used on an emergency basis when it is impossible to define for some reasons a blood type of the recipient, for pediatric transfusions, and, especially, in cases when the blood of unusual or rare phenotypes. For today in transfusion medicine, still there is a problem of production of a qualitative donor blood in necessary quantity. Despite occurrence of techniques of enzymatic production of a donor blood, it cannot widely be applied in clinical practice because the enzymes being used for blood groups conversion are isolated from pathogenic microbial strains or/and produce up to 70 % injuring erythrocytes. Herein, alpha-N-acetylgalactosaminidase and α-galactosidase of marine bacteria, operating at neutral values of pH, have been suggested for conversion of erythrocytes of blood group A, B and AB to O in biotechnological production of universal donor blood. Alpha-N-acetylgalactosaminidase isolated from marine bacterium *Arenibacter latericius* has found to be uncommon enzyme. It shows the maximum activity at pH 7.0-8.0 and remains stable up to 50°C. The enzyme does not cause nonspecific agglutination of erythrocytes and their hemolysis and unlike known analogs modifies erythrocytes of blood subgroup A2 and A1 into erythrocytes of blood group O. Characterization of the sequences encoding glycoside hydrolases of marine bacteria revealed that they shared about 20-40% overall amino acid identity with their terrestrial counterparts. For further comparison of *A. latericius* alpha-*N*-acetylgalactosaminidase gene family, the gene sequence was isolated and characterized. It has a single open-reading frame consisting of 1287 base pairs, and the deduced amino acid sequence revealed that the mature enzyme consisted of 428 amino acid residues. The enzyme was estimated to be a homodimer with a molecular mass of subunit 48.2 kDa. The *Arenibacter* enzyme has a conserved common gene structure with a new glycoside hydrolases family 109. The structure of the putative binding sites of *A. latericius* alpha-N-acetylgalactosaminidase with NAD+ and blood group A antigen was predicted by molecular docking.**

Keywords- *marine bacteria, glycosidase, alpha-N-acetylgalactosaminidase, homology model, blood group conversion, erythrocytes*

I. INTRODUCTION

Unlike known analogs, the enzymes from marine bacteria seem to be more suitable for use in biotechnology of production of a universal blood: a comfortable interval of pH for keeping the vital activity of erythrocytes, high specific activity and demanded specificity that does a transfusion as much as possible effective, safe and economic. Recent characterization of alpha-galactosidase isolated from marine bacterium *Pseudoalteromonas* sp. KMM701 showed that the enzyme has a maximum activity at pH 6.7-7.7 at 22ºC [1]. Alpha-galactosidase splits off galactose from melibiose, disaccharide Gala-l,3Gal and trisaccharide Galαl, 3(Fucαl,2)Gal that is a terminal structural element of the B antigen on erythrocytes of a blood type B. The results of immunological investigation of erythrocytes have shown that B antigens are completely transformed to H antigens in absence of aggregation of erythrocytes and their hemolysis. Enzyme does not show activity towards antigens A and H that is very important.

Alpha-N-acetylgalactosaminidases (EC 3.2.49), that is capable of removing alpha-1,3-bound residues of N-acetylgalactosamine from glycoproteins of blood group substances and A-erythrocytes at neutral pH, converting them into O group substances are of more greater practical interest. For example, alpha-N-acetylgalactosaminidase from chicken liver carries out conversion only blood subgroup A2 that is not a sufficient condition for complete conversion of erythrocytes of type A, and the enzyme works at value of acidic pH, that in its turn produces 70 % injuring erythrocytes [2]. The enzymes from *Clostridium perfringens* and *Elisabethkingia meningosepticum* recently characterized are unsafe for recipients because of their pathogenic microbial sources [3, 4].

We studied the distribution of microbial producers of alpha-N-acetylgalactosaminidases among 860 bacterial isolates from water, bottom sediment, algae, and animals collected in different regions of the Pacific, Indian, and Atlantic oceans (Collection of Marine Microorganisms of PIBOC FEB RAS). Only nineteen percent of the microorganisms tested synthesized the enzyme with the different level of activity and specificity towards A-erythrocytes [5]. In view of this application, we have selected N-acetylgalactosaminidase of marine bacterium *Arenibacter latericius* for future characterization [6]. In this paper, the molecular structure of *A. latericius* alpha-N-acetylgalactosaminidase and catalytic mechanism of blood group A antigen hydrolyzing are suggested.

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II. MATERIAL AND METHODS

A. Bacterium characterization and culture conditions

Arenibacter latericius KMM 426^T was isolated from sandy sediment sample collected from a depth of 20m (salinity, 32; temperature, 18 ºC) in the South China Sea, near the island of Ku-Lao-Re (lat152108N, long1090702E). The bacterial strain KMM 426^T was cultivated on medium containing 5.0 g/liter bactopeptone from Difco (USA), 1.0 g/liter glucose, 0.2 g/liter K2HPO4, 500 ml of distilled water, 500 ml of sea water, pH 7.8. Seeding material was grown on a shaker (150 rpm) in 250-ml shaker flasks (with medium of 50 ml) for 24-30 h at 25°C to the density 109 cells/ml. The resulting material was inoculated into 1000-ml flasks with 500 ml of the same fermentative medium.

B. Alpha-N-acetylgalactosaminidase purification

The bacterial cells were suspended in 0.01 M Na+-phosphate buffer, pH 7.2, and sonicated. Proteins were precipitated from the supernatant with 50-70% ammonium sulfate, and the resulting pellet was resolved and dialyzed in 0.01 M Na+-phosphate buffer, pH 7.2. The solution was loaded onto DEAE-Sepharose CL-6B (15.0×2.8 cm), and then on DEAE-Toyopearl 650 M (Toya Soda) (15.5×2.0 cm) in the same buffer, pH 7.2. Alpha-N-acetylgalactosaminidase activity was eluted with gradient of 0–1 M and 0-0.5 M NaCl, respectively. Active fractions were concentrated.

C. Effect of enzyme on erythrocytes blood group-specific glycoproteins

Erythrocytes washed with physiological solution were mixed with the enzyme solution
(2 U/ml) in isotonic phosphate buffer, pH 7.3. After 24 h incubation with stirring at 36°C, the erythrocytes were washed thrice with physiological solution (pH 7.3) and mixed with corresponding sera in a series of double dilutions on the plates. Agglutination titer was read after 1 h incubation at room temperature. Excision of N-acetylgalactosamine from BGG A +H was registered by an amino acid analyzer after acidic hydrolysis of low-molecular-weight fraction of the products of fermentolysis obtained by gel chromatography on Sephadex G-15.

D. Molecular cloning and sequence analysis

Recombinant DNA technique was performed using Ins T/Aclone^{tm} PCR Product Cloning Kit, restriction endonucleases, T4 DNA ligase, Long PCR mix (Fermentas), Smart Taq Polymerase (Topotili), automatic amplifier (Eppendorf). Two pair of primers (For1 - 5'-GG(G/A/T) GC(A/T) AA(A/G) TA(T/C) ATG GGN GGN TT (T/C) TC–3' and Rev1 - 5'-GG (A/G)TG (G/A)TC (G/A)TA (C/T)TT N TC–3; For2 - 5'-ATG ATG ATG GA(A/G) AA(T/C) GTN AA(T/C) TA–3' and Rev2 - 5'-GG (A/G)TG (G/A)TC (G/A)TA (C/T)TT N TC–3') were synthesized on the base of known alpha-N-acetylgalactosaminidase homologue sequences to amplify the active site region of alpha-N-acetylgalactosaminidase using *A. latericius* chromosomal DNA as a template. The C-termini region coding sequence was determined using only forward primers (F1 and F2). PCR products were cloned and sequenced using the automated PE/ABI 310 DNA sequencer and PE/ABI-ABI PRISM BigDye Terminator cycle sequencing Ready Reaction Kit (PE Applied Biosystems). *Escherichia coli* strains TOP10 or XL1 (Evrogen) were used for standard cloning procedures.
Nucleotide and amino acid sequences analysis were performed with Chromas, GenRunner. Nucleotide and amino acid sequences homology and similarity searches and alignments were carried out by using the BLAST and ClustalW, MUSCLE facilities. The Molecular Operating Environment version 2010.10 software [7] was used for 3D-structure modeling and visualization. The theoretical model of *A. latericius* alpha-N-acetylgalactosaminidase 3D-structure was constructed with the use of Homology module of MOE package on the based of X-ray structure of *E. meningosepticum* alpha-N-acetylgalactosaminidase (PDB ID: 2IXB) as template. Molecular docking alpha-N-acetylgalactosaminidase with blood group A antigen was performed with the use of Docking module of MOE.

III. RESULTS AND DISCUSSION

The distribution of alpha-N-acetylgalactosaminidase activity in marine bacteria showed that the enzyme is rare and a highest number of its producers occurred as a free-living or marine animal-associated bacteria in tropical water of Pacific Ocean [5]. The extracellular glycosidase activity suggests that the bacterium is able to hydrolyze organic materials in the marine environment [8]. The enzyme exhibiting a high alpha-N-acetylgalactosaminidase activity isolated from marine bacterium *A. latericius* KMM 426^T, new genus of the family *Flavobacteriaceae*, was found to be unusual alpha-N-acetylgalactosaminidase. After three purification stages, the enzyme was homogenous and not contaminated with other glycosidase activities. It shows the maximum activity at pH 7.0-8.0 and stability up to 50°C during 30 min. The enzyme has exogenous mode of action. The enzyme does not cause nonspecific agglutination of erythrocytes and their hemolysis and unlike known analogs completely modifies erythrocytes of both blood subgroup A2 and A1 into erythrocytes of blood group O (Fig. 1, Table 1).

The enzyme properties provide a significant advantage of the enzyme over the alpha-N-acetylgalactosaminidase from liver that have optimum activity at pH less than 5.5 [2]. Characterization of the *A. latericius* alpha-N-acetylgalactosaminidase gene sequence (accession n.HQ108058) revealed that the enzymes shared about 84%

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and 70% overall amino acid homology with uncharacterized proteins of *Flavobacteria bacterium* и *Akkermansia muciniphila*, respectively, and 37% identity and 50% homology with its terrestrial prototype from *E. meningosepticum.* It has a single open-reading frame consisting of 1287 base pairs, and the deduced amino acid sequence revealed that the mature enzyme consisted of 428 amino acid residues. The enzyme was estimated to be a homodimer with a molecular mass of subunits 48.2 kDa and pI 7.99. *A. latericius* alpha-N-acetylgalactosaminidase is characterized by a high content of Gly (10.51%) Glu (7.24%) Leu (7.01%) Ala (6.78%) Lys (6.54%). Polar amino acid residues are predominant. A high content of glycine residues in the structure can be explained by psychrophility of most marine bacteria [8]. Despite the low level of identity and homology with the known alpha-N-acetylgalactosaminidases the tertiary structure analysis of the *Arenibacter* enzyme showed that it has a common molecular fold with a new glycosyl hydrolases family 109 (Fig. 2). It has been found that exogenous NAD^+ does not influence on the *A. latericius* alpha-N-acetylgalactosaminidase activity. The structure superposition showed cofactor binding specificity and strength similarity to *E. meningosepticum* alpha-N-acetylgalactosaminidase, belonging to family 109 (Fig. 2). The structure of the putative binding site of blood group A antigen trisaccharide with *A. latericius* alpha-N-acetylgalactosaminidase was predicted by molecular docking (Fig. 3). The trisaccharide moiety was found to be close to NAD^+ and residues Tyr 178, His 180, Tyr 209 and Tyr 292, which were invariant within the GH109 family.

Structurally functional researches of glycosidases of marine bacteria will create a basis for optimal genetic construction of recombinant protein for working out of a new highly effective and hi-tech method of universal donor blood production for a safe and easy accessible transfusion.

TABLE I.

Polyclone-A	Agglutination titer of donor A erythrocytes before and after *A. latericius* enzyme treatment								
	2	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*
Anty-A+ transformed erythrocytes	-	-	-	-	-	-	-	-	-
Anty-A+ non transform eruthrocytes	+	+	+	+	+	+	+	+	-

Galβ1-R 2 1 Fucα	GalNAcα1-3Galβ1-R 2 1 Fucα
H antigen	**A antigen**

Figure 1. H and A antigen structures.

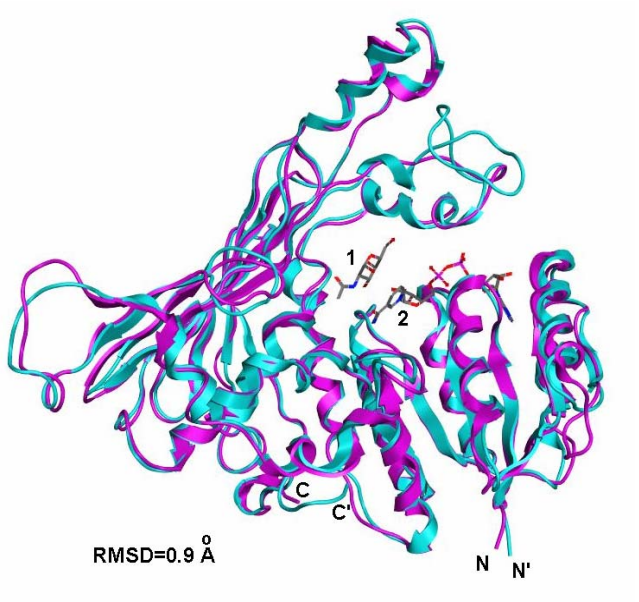

Figure 2. The ribbon diagram of superposition of the homology model of alpha-N-acetylgalactosaminidase from *Arenibacter latericius* KMM 426^T (magenta) and crystal structure of the complex of alpha-N-acetylgalactosaminidase from *Flavobacterium meningosepticum* (cyan) with N-acetyl-2-deoxy-2-amino-galactose (1) and NAD^+ (2).

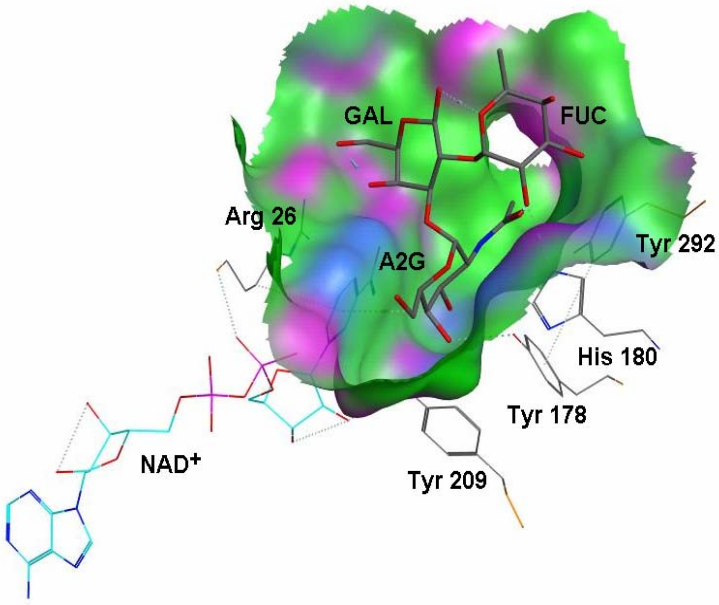

Figure 3. The putative binding site of alpha-N-acetylgalactosaminidase *Arenibacter latericius* KMM 426^T with blood group A trisaccharide. Molecular surface near trisaccharide is shown in magenta for H-binding, blue for mild polar and green for hydrofobic.

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