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Extracellular Chitinases of Mutant Superproducing Strain *Serratia marcescens* M-1

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Abstract—Four extracellular proteins with chitinase activity capable of binding chitin substrates have been revealed in the culture liquid of chitinase superproducing mutant strain M-1 of *Serratia marcescens*. Proteins were analyzed by SDS-PAGE and MALDI-TOF mass spectrometry. Based on the data obtained, the proteins were identified as typical chitinases of *S. marcescens*: ChiA, ChiB, ChiC, and CBP21.

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Chitin is a water-insoluble linear polymer of N-acetyl-D-glucosamine, one of the most widespread polysaccharides in nature. It is contained in the investing tissues of arthropods and some other invertebrates and in the cell walls of fungi. According to available estimates, hundreds of millions of tons of chitin are formed in the biosphere annually [1]. Nevertheless, chitin is not accumulated in the environment. The main contribution to its natural processing and utilization is made by various microorganisms. They produce chitinases (poly[1,4-β(2-acetamido-2-deoxy-D-glucoside)] glycan hydrolases, EC 3.2.1.14) that catalyze the cleavage of high-polymer chitin molecules to products assimilated by cells.

The soil bacterium *Serratia marcescens* is among the most effective microorganisms capable of chitin utilization [2]. The chitinase system of *S. marcescens* contains at least one intracellular and four extracellular proteins. These proteins are encoded by different genes and have different physicochemical and enzymatic properties. Appearance of chitin-containing substrates in the environment induces the biosynthesis of extracellular chitinases. These chitinases designated as ChiA (58.8 kDa), ChiB (55.4 kDa), ChiC (51.7 kDa), and CBP21 (21.8 kDa) [3-9] are secreted into the extracellular space, adsorbed on chitin particles, and depolymerize them. The endochitinase ChiC catalyzes the hydrolysis of chitin polymers in internal regions [10], while exochitinases ChiA and ChiB successively depolymerize chitin molecules from the reducing and non-reducing ends, respectively [10, 11]. Protein CBP21 has no hydrolase activity and is supposed, through peculiar binding to chitin particles, to make them more susceptible to hydrolysis by chitinases ChiA, ChiB, and ChiC [6, 12, 13]. As a result, on joint action proteins ChiA, ChiB, ChiC, and CBP21 supplement each other with the effect of synergism [9]. The main product of such complex of enzymatic reactions is a dimer of N-acetyl-D-glucosamine, which is further cleaved to monomers by the intracellular enzyme N-acetyl-β-glucosaminidase (EC 3.2.1.30). Obviously, such arrangement of the chitinolytic system allows *S. marcescens* bacteria to assimilate more effectively various chitin substrates occurring in nature. The study of this system is of particular interest both in the scientific respect and in the context of the prospects of using chitinases in medicine, industry, and agriculture [14].

Previously, we constructed stable mutant strain M-1, a superproducer of chitinases, from the native strain *S. marcescens* B-10 [15-17]. On cultivation on media with various chitin substrates, the chitinase activity (CA) in the culture liquid (CL) of the mutant strain was hundreds of times higher than the respective CA values in the precursor CL [18]. At the same time, in contrast to strain B-10,

Abbreviations: CA, chitinase activity; CC, colloidal chitin; CL, culture liquid.

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rather high CA levels in the CL of strain M-1 were shown even in the absence of chitin inducers. Several extracellular proteins were revealed, the quantity of which correlated with the change of CA in CL. These proteins specifically bound chitin, and their molecular weights (62, 54-52, 43, 38, and 21 kDa) corresponded to the values reported for chitinases ChiA, ChiB, ChiC, and CBP21 of *S. marcescens* [18]. However, more strict evidence is needed for verification of these proteins as actual chitinases.

The specific goal of this work was identification of extracellular chitinases of the mutant stain *S. marcescens* M-1. For solution of this problem, proteins that can specifically bind chitin substrates and possess chitinase activity were isolated from the CL of strain M-1. These proteins were analyzed by the methods of SDS-PAGE and time-of-flight (MALDI-TOF) mass spectrometry. The data analysis proved that the revealed proteins were chitinases ChiA, ChiB, ChiC, and CBP21.

MATERIALS AND METHODS

Reagents. The work was performed with 3,5-dinitrosalicylic acid (Sigma, USA), N-acetyl-D-glucosamine (Koch-Light Laboratories, England), reagents for SDS-PAGE (Serva, Germany), and crab shell chitin (Fluka, Switzerland). The kit of marker proteins for molecular weight determination containing phosphorylase B (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carboanhydrase (30 kDa), trypsin inhibitor from soybean (20.1 kDa), and α-lactalbumin (14.4 kDa) was produced by Pharmacia (Sweden). Other reagents were produced domestically with especially pure, chemically pure, and analytically pure grades.

Object of research. The object of research was a mutant bacterial strain *S. marcescens* M-1 obtained due to the effect of nitrosomethylurea on a synchronized culture of the native strain *S. marcescens* B-10 VKM [15, 16]. Strain *S. marcescens* M-1 was deposited as B-3273 at the Central Museum of Industrial Microorganisms of the All-Russia Research Institute of Genetics and Selection of Microorganisms (VNII Genetika).

Chitin preparations. Powdery chitin with the particles less than 60 μm was obtained by grinding the pieces of crab shell chitin in a ball mill and sieving (screen opening, 60 μm). Possible admixtures of mineral salts, proteins, and lipids were removed from the chitin powder by sequential treatment with 1 M HCl, 1 M NaOH, and ethanol, followed by drying.

Colloidal chitin (CC) was obtained by dissolution of powdery chitin in concentrated hydrochloric acid followed by precipitation in distilled water [19]. The resulting CC was used to prepare chitin suspension (10 mg/ml as converted per dry weight) in deionized water. The CC suspension was used as a substrate for CA determination and as an adsorbent for isolation of chitinases.

Preparation of *S. marcescens* M-1 extracellular proteins. The culture of strain *S. marcescens* M-1 was grown in medium containing (g/liter): powdery chitin (20.0), yeast extract (5.0), $(NH_4)_2SO_4$ (1.0), $MgSO_4 \cdot 7H_2O$ (0.3), KH_2PO_4 (1.36), pH 8.2-8.4. The culture was incubated for 6 days at 30°C on a shaker at 100 rpm. On completion of the cultivation, the *S. marcescens* cells were precipitated by centrifugation at 30,000*g* for 30 min at 4-8°C. Proteins from the supernatant were precipitated by ammonium sulfate at 80% of the saturating concentration. The precipitate was dissolved and dialyzed against deionized water. The dialyzate was filtered through a sterilizing filter (pore diameter 0.22 μm). Protein concentration and CA were determined in the resulting filtrate.

Study of pH effect on binding of *S. marcescens* M-1 extracellular proteins with CC. Colloidal chitin suspension (1.2 ml) was centrifuged (10,000*g*, 10 min, 4-8°C) and the supernatant discarded. The residual of the CC was suspended in 500 μl of double universal buffer [20] containing 50 mM Na_2HPO_4, 50 mM H_3BO_3, 36 mM citric acid, and 0.24 M NaOH. The pH value in the universal buffer was preliminarily brought to 4.0, 5.0, 6.0, 7.0, and 8.0 using hydrochloric acid solution. Aliquots of the preparation of *S. marcescens* M-1 extracellular proteins, each containing 0.65 mg protein (300 mU CA), and deionized water were added to the resulting suspensions to 1 ml and thoroughly mixed. The mixtures were incubated in an ice bath for 30 min under periodic stirring. The CC with adsorbed proteins was separated by centrifugation at 10,000*g* for 10 min at 4°C. The CC residuals were twice washed in the universal buffer with respective pH. Chitinase activity and protein concentration were determined in the supernatants containing protein not bound to CC. Total quantity and CA of the protein adsorbed on CC was estimated by the difference between the protein quantity and CA in the initial aliquots and the total protein content and CA in the supernatants with unbound material.

The proteins bound to CC were analyzed as follows. The CC precipitate was supplemented with 50 μl of 10% SDS (to final concentration of 2%), thoroughly mixed, and kept at −20°C for 10-20 h. Then the suspension was thawed and incubated in a water bath for 10 min. The precipitate of CC that had lost its amorphous structure was separated by centrifugation. Desorbed proteins in the supernatant were analyzed by SDS-PAGE in 10% gel.

Isolation of *S. marcescens* M-1 extracellular chitinases. Chitinases were isolated by the modified procedure of Roberts and Cabib [21]. Colloidal chitin suspension (10 ml) was centrifuged at 10,000*g* for 10 min at 4°C. The CC precipitate was suspended in 10 ml of double universal buffer (pH 8.0). A solution of extracellular proteins with total CA of 10 U and deionized water were added to the resultant suspension to achieve volume of 20 ml. The mixture was thoroughly mixed, incubated for 30 min in an ice bath under periodic stirring, and centrifuged at

10,000*g* for 15 min at 4°C. The CC precipitate was washed three times in 15 ml of universal buffer, pH 8.0. Then CC with the adsorbed chitinases was suspended in 5 ml of universal buffer, pH 5.5, and incubated for 8-10 h at 37°C until the CC was solubilized. The mixture was centrifuged at 10,000*g* for 15 min at 4°C. The supernatant containing chitinases was collected and dialyzed against deionized water until the absence of reducing sugars in the dialyzate. Protein concentration and CA were determined in the dialyzate.

Determination of chitinase activity. Chitinase activity was determined by the rate of formation of reducing sugars (as converted per N-acetyl-D-glucosamine) using CC (3 mg/ml) in the universal buffer (pH 5.5) as a substrate [19]. The concentration of reducing sugars was determined by the reaction with 3,5-dinitrosalicylic acid [22]. The amount of the enzyme catalyzing substrate cleavage with the formation of 1 μmol of N-acetyl-D-glucosamine in 1 min at 37°C was taken as a unit of activity. The CA value was calculated as the mean of three parallel analyses, with variation coefficient not exceeding 7%.

Protein assay. Protein concentration in preparations under study was determined by the binding of Coomassie G-250 [23], with human serum albumin as a calibrator.

SDS-PAGE in 10% polyacrylamide gel. Electrophoresis was performed in 10% polyacrylamide gel plates (0.8 × 120 × 120 mm) in the presence of 0.1% SDS followed by staining with Coomassie R-250 [24]. The molecular weights of proteins under study were determined by the calibration curve of dependence of marker protein mobility on the logarithms of their molecular weights, linearized by the least-squares method.

Identification of *S. marcescens* proteins by time-of-flight (MALDI-TOF) mass spectrometry. For mass spectrometry followed by protein identification, the polyacrylamide regions containing the respective proteins under study were cut out and treated by the modified method of Rosenfeld et al. [25]. With this purpose, the cut-out pieces of gel with the proteins were washed free from Coomassie R-250 and SDS in a solution of 50% acetonitrile and 0.1% trifluoroacetic acid. The proteins contained in the gel were reduced with 45 mM DTT in 0.2 M ammonium bicarbonate (60°C, 30 min) followed by alkylation with 100 mM iodoacetamide in 0.2 M ammonium bicarbonate at room temperature for 30 min. Then the gel pieces were dehydrated in 100% acetonitrile. For protein hydrolysis, 20 μl of 0.2 mM modified trypsin (Promega, USA) solution in 40 mM ammonium bicarbonate containing 5 μM $CaCl_2$ was added to each piece followed by incubation for 30 min at room temperature. Then 60 μl of the buffer for peptide extraction (40 mM ammonium bicarbonate, 5 μM $CaCl_2$, 10% acetonitrile, pH 8.1) was added to the gel pieces followed by incubation for 16-18 h at 37°C. Peptide fragments of the proteins extracted from the gel were concentrated and desalinated on C_{18} ZipTips microcolumns (Millipore, USA). The peptide mixture was eluted from the microcolumn to the target instrument plate by saturated matrix solution (10 mg/ml α-cyano-4 oxycinnamic acid in a mixture of 50% acetonitrile and 0.1% trifluoroacetic acid).

Mass spectra were obtained and recorded in a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Canada) in positive ion mode in the mass range of 700-3000 Da. Proteins were identified by searching for the appropriate candidates in the annotated NCBI and Swiss-Prot databases using Mascot software (Matrix Science Ltd., London; http://www.matrixscience.com/search_form_select.html). The following parameters were used for the search: acceptable deviation of charged peptide weight, 50 ppm; presence of oxidized methionine residues and carboxylated cysteine residues; identification reliability no less than 95%.

RESULTS AND DISCUSSION

The ability to bind chitin is one of the typical characters of extracellular chitinases of *S. marcescens* [8, 12, 26, 27]. Considering this, we have used the procedure of selective sorption on chitin to reveal the corresponding enzymes in the CL of mutant strain M-1 [21]. Colloidal chitin with highly dispersed amorphous structure of chitin particles, providing high efficiency and reproducibility of chitinase adsorption, was chosen as a chitin sorbent.

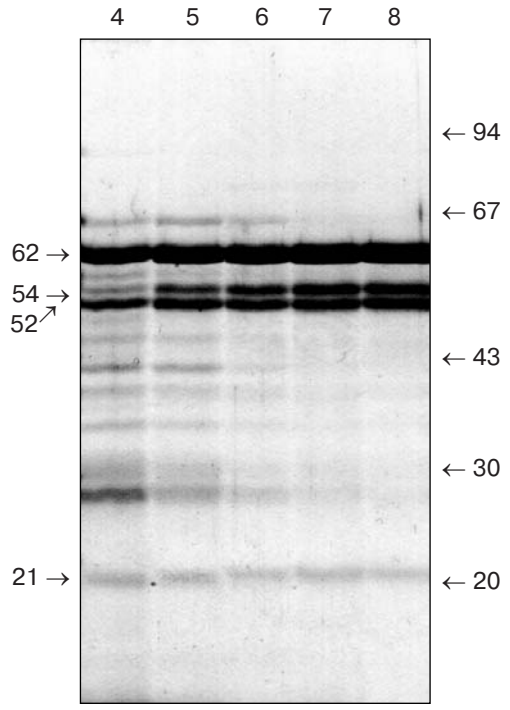
The analysis showed that the binding of *S. marcescens* M-1 extracellular proteins with CC could be affected by some sorption conditions. In particular, total protein bound to CC decreased more than twice on changing pH from 4.0 to 8.0. At the same time, the CA in the material not bound to CC was approximately equal with all of the tested pH values: about 10% of the initial CA.

More detailed study of CC-binding proteins by the method of SDS-PAGE showed that pH influenced not only the total amount of proteins but also protein composition (figure). A rather large group of proteins with electrophoretic mobility corresponding to the molecular weights of 62 to 14 kDa were strongly bound to chitin at pH 4.0. As pH was increased to 8.0, the number of adsorbed proteins significantly decreased. Only the CC affinity of proteins with 62, 52, and 21 kDa was unchanged, and the affinity of protein with 54 kDa slightly increased visually. Thus, only four proteins (62, 54, 52, and 21 kDa) maintained the efficiency of chitin binding over the whole tested pH range. Since the CA of CC-binding proteins practically did not change on varying pH, these results suggested that the proteins of 62, 54, 52, and 21 kDa were most likely the chitinases of *S. marcescens* M-1. To test this assumption, the enzyme activity of these proteins was determined as follows: the proteins binding CC at pH 8.0 were isolated in native

Results of identification of extracellular proteins of *S. marcescens* M-1

Tested protein	Identified protein	Peptides identified in the tested chitinases of *S. marcescens**		
		peptide position in the primary protein structure	monoisotopic mass of peptide (MH^+), m/z	amino acid sequence of identified peptide
62 kDa	ChiA *S. marcescens*	24-37	1411.8040	AAPGKPTIAWGNTK
	Accession number	38-55	1966.0207	**FAIVEVDQAATAYNNLVK**
	by NCBI:	85-101	1683.7536	**EAWSGPSTGSSGTANFK**
	P07254	159-172	1617.7988	**VVGSYFVEWGVYGR**
		206-216	1277.6411	EIEGSFQALQR
		226-237	1325.7139	**VSIHDPFAALQK**
		241-250	1151.5295	**GVTAWDDPYK**
		251-260	1078.5641	**GNFGQLMALK**
		251-260	1094.5590	**GNFGQLMALK oxidized (M)**
		268-287	2228.1024	**ILPSIGGWTLSDPFFFMGDK**
		321-340	2104.0306	**GANPNLGSPQDGETYVLLMK**
		321-340	2120.0256	**GANPNLGSPQDGETYVLLMK oxidized (M)**
		357-367	1139.5870	**YELTSAISAGK**
		474-480	836.4188	**NGIVDYR**
54 kDa	ChiB *S. marcescens*	163-174	1329.6840	**TLLNQQTIADGR**
	Accession number	223-244	2348.1379	**ITNHQAALFGDAAGPTFYNALR**
	by NCBI:	245-257	1590.7474	**EANLGWSWEELTR**
	P11797	285-294	1154.5916	IVMGVPFYGR oxidized (M)
		344-357	1743.7871	QLEQMLQGNYGYQR oxidized (M)
		394-410	2029.8595	QQQLGGVMFWHLGQDNR oxidized (M)
		411-420	1057.5438	**NGDLLAALDR**
		421-439	2167.9164	**YFNAADYDDSQLDMGTGLR oxidized (M)**
		479-494	1738.8117	WGYITSAPGSDSAWLK
52 kDa	ChiC *S. marcescens*	1-24	2444.2039	MSTNNIINAVAADDAAIMPSIANK
	Accession number	69-86	2040.0112	**GQGIPTFKPYNLSDAEFR**
	by NCBI:	88-97	1057.5676	QVGVLNSQGR
	Q8VQN1	98-113	1607.9090	AVLISLGGADAHIELK
		152-159	812.5167	TVLPAALK
		171-184	1713.8596	**NFIISMAPEFPYLR oxidized (M)**
		233-248	1906.9360	EDFLYYLTESLVTGTR
		258-277	2065.0528	FVIGLPSNNDAAATGYVIDK
		278-286	1039.5247	**QAVYNAFAR**
		296-308	1537.7031	**GLMTWSINWDNGK oxidized (M)**
		356-374	2011.0574	**LSWAAATGALPIASYTVYR**
21 kDa	CBP21	1-10	1102.5203	HGYVESPASR
	S. marcescens	37-53	1638.7798	**GFPQAGPADGHIASADK**
	Accession number	54-66	1569.7471	**STFFELDQQTPTR**
	by NCBI:	74-82	1037.4978	**TGPNSFTWK**
	O83009	87-93	874.4093	**HSTTSWR**

* Unique peptides found only in *S. marcescens* chitinases corresponding to the given accession number of NCBI database are shown in bold.



The effect of pH on the binding of *S. marcescens* M-1 extracellular proteins with CC. The composition of CC-bound proteins was analyzed by the method of SDS-PAGE in 10% polyacrylamide gel. Numerals above the lanes correspond to the pH values used. Arrows on the left indicate the proteins specifically binding with CC and their calculated molecular weights (kDa). Arrows on the right indicate electrophoretic mobilities and molecular weights (kDa) of marker proteins

form and their chitinolytic properties were analyzed. The resulting preparation contained a set of proteins with molecular weights of 62, 54, 52, and 21 kDa and had high CA (5 U/mg protein).

Thus, the study of the binding of extracellular proteins of *S. marcescens* M-1 with CC revealed a set of the most probable proteins of the chitinase system of this strain but left unsolved the problem of their structural correspondence to the typical chitinases of *S. marcescens*. Mass spectrometric analysis of these proteins was performed to answer this question. Regions of a polyacrylamide gel containing proteins with molecular weights of 62, 54, 52, and 21 kDa were cut out and treated with trypsin. The formed peptides were analyzed by MALDI-TOF mass spectrometry. The results of Mascot analysis of peptide maps are given in the table. The presented results of identification are statistically reliable. They demonstrate that the four proteins of 62, 54, 52, and 21 kDa are identified as *S. marcescens* chitinases ChiA, ChiB, ChiC, and CBP21, respectively. All of the proteins under study have been shown to possess unique peptides (marked bold in the table), which are not found by the Mascot software in any other proteins except for the corresponding chitinases of *S. marcescens*. The finding of such unique peptides substantially increased the reliability of identification of the extracellular chitinases of *S. marcescens* M-1.

Thus, the findings lead to the conclusion that the mutant strain *S. marcescens* M-1 produces a complete set of extracellular chitinases: ChiA, ChiB, ChiC, and CBP21. This may imply that pleiotropic mutation in the genome of this strain has not resulted in compositional changes of secreted chitinases. The molecular sizes of proteins comprising this set, as determined by their electrophoretic mobility, approximately correspond to the literature data [3-9], suggesting the absence of noticeable disturbances in their primary structure. Our preliminary data have also shown that the main enzymatic properties of the chitinase complex and individual chitinases of *S. marcescens* M-1 are similar to those reported for chitinase preparations of other *S. marcescens* strains [9, 21].

In conclusion, it should be noted that the chitinase preparations of this strain prove to be effective for plant protection from pests [28, 29] and diseases [30]. Hence, accumulation of information about the properties of these enzymes will provide more successful application of these preparations for solution of the specified (and probably some other) applied problems.

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