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Isolation and Characterization of a Low-Molecular-Weight Immunoglobulin-Binding Protein from *Yersinia pseudotuberculosis*

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Abstract—A low-molecular-weight immunoglobulin-binding protein (IBP) bound with the cell envelope has been isolated from *Yersinia pseudotuberculosis* cells and partially characterized. This IBP is a hydrophilic protein with a high polarity index of 55.3%. The molecular weight of the protein has been determined by MALDI-TOF mass spectrometry as 14.3 kD. CD spectroscopy showed that the IBP has high contents of the β-structure and random coil structure. The IBP contains glycine as the N-terminal amino acid. The protein can be stored for a long time at acidic pH values but aggregates and loses activity at alkaline and neutral pH. The IBP binds rabbit IgG with optimum at pH of 6.0-7.5. The IBP interacts with IgG molecule in the Fc-fragment region. The protein retains activity after heating at 100°C in the presence of SDS.

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Key words: immunoglobulin G, Fc-fragment of IgG, immunoglobulin-binding protein

Immunoglobulin-binding proteins (IBPs) are important virulence factors of various bacterial pathogens. The nonimmune binding of immunoglobulins with cells through the IBPs is thought to protect bacteria against the action of complement [1, 2], decrease their opsonization and phagocytosis, which as a result allows the microorganisms to escape the influence of the host's immune system [1-4].

IBPs include a large group of proteins different in location in microorganisms, molecular structure, and binding abilities. These proteins have been found on the surface of bacterial cells, in the capsule, and in the culture medium [1, 2, 5]. Molecular weights of IBPs widely vary in the range from 20 to 350 kD. They include both monomeric and oligomeric proteins [4-15]. IBPs also vary in affinity for human and animal immunoglobulins of different classes and subclasses; they can also bind with different sites of immunoglobulin molecules [6-9]. A bacterial cell can concurrently contain a number of structurally and/or functionally different IBPs as tools influencing the host's immune system [10-12].

IBPs of gram-positive bacteria, such as staphylococci, streptococci, and peptococci, are best studied [3, 5-7]. Proteins capable of nonimmune binding of immunoglobulins have also been identified in gram-negative bacteria, including *Yersinia pseudotuberculosis* [13-15]. A high-molecular-weight protein was earlier isolated by us from the outer membrane proteins of *Y. pseudotuberculosis* and characterized [16].

This work presents results of isolation and purification of a low-molecular-weight IBP from the cell envelope of *Y. pseudotuberculosis*. Some physicochemical characteristics and biological activity of this protein have been determined.

MATERIALS AND METHODS

Materials used include: acrylamide (Serva, Germany); Source 15S (Pharmacia Biotech, Sweden) and HiTrap Desalting (Amersham Biosciences, Sweden) columns; Ripor membranes for ultrafiltration (Olaine, Latvia); SDS (Bio-Rad, USA); Fc-fragment of rabbit IgG and rabbit IgG conjugate with horseradish peroxidase (ICN Biomedicals, USA). Other reagents (Reakhim, Russia) of chemical purity qualification were

Abbreviations: IBP) immunoglobulin-binding protein; EIA) enzyme immunoassay.

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used without additional purification. Solutions containing 50 mM Tris-HCl (pH 8.0) and 50 mM CH_3COONa/CH_3COOH (pH 4.0) were used as buffer A and buffer B, respectively.

Microorganisms and conditions of bacterial cultivation. The *Y. pseudotuberculosis* strain 598 serovar IB was used. Conditions of the bacterial cultivation are described in [17].

Ion-exchange chromatography. The IBP was isolated from the *Y. pseudotuberculosis* cell envelopes prepared by the method of Nurminen [18]. The cell envelopes were treated successively with buffers A and B for 30 min at room temperature and after each extraction centrifuged at 10,000*g* for 20 min at 20°C. The extract in buffer B (20 ml) was filtered though a 0.22-μm filter and placed onto a column as a specimen for ion-exchange chromatography. Ion-exchange chromatography was performed using an FPLC chromatograph (Amersham Pharmacia Biotech, Sweden) on a Source 15S column equilibrated with buffer B. After the specimen had been loaded, the column was washed with 10 ml of buffer B to remove the unbound and weakly bound proteins. The proteins were eluted from the sorbent using 0-1 and 1-2 M NaCl gradients (8.5 and 3 ml, respectively) in buffer B. The elution rate was 0.5 ml/min, and fractions of 0.5 ml volume were collected. The fractions were tested for the presence of immunoglobulin-binding activity by the dot-method. The active fractions were combined and concentrated by ultrafiltration on membranes with exclusion limit of 1000 daltons (Ripor, Latvia). After concentrating, gel filtration was additionally performed on a HiTrap Desalting column in buffer B. The fractions were combined by the activity monitored by the dot-method.

Determination of amino acid composition of the proteins. To determine the amino acid composition, the IBP specimens (2.5-3.0 nmol) were hydrolyzed at 110°C in 50 μl of 5.7 N HCl for 24, 48, and 72 h in ampules filled with nitrogen. The analysis was performed using an Alpha-plus 4151 amino acid analyzer (LKB, Sweden). The N-terminal amino acid analysis was performed as described in [19].

SDS-PAGE. Polyacrylamide gel electrophoresis in the presence of SDS was performed by the Laemmli method [20]. All specimens for electrophoresis were prepared without heating at 100°C, except for the cases mentioned specially. Proteins with molecular weights of 14.4, 20.1, 30.0, 43.0, 67.0, and 94.0 kD (Sigma, USA) were used as markers. The proteins separated in the gel were stained with Coomassie R-250 in 10% acetic acid and 45% ethanol.

Methods of chemical analysis. The total protein content was determined by Bradford's method [21] with lysozyme as the calibration protein. The total content of neutral monosaccharides was determined using phenol–sulfuric acid [22] with D-glucose as the standard.

CD spectra were recorded using a J-500A spectropolarimeter (Jasco, Japan) in quartz cuvettes with 0.1-cm pathlength for the peptide region of the spectrum. In the peptide region of the CD spectrum (190-240 nm) the ellipticity [θ] was taken equal to the ellipticity of the average residue (110 daltons) by the formula:

$$[\theta] = [\theta]_{obs} \cdot S \cdot 110/10 \cdot C \cdot d \ (\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}),$$

where S is the sensitivity of the device, C is the protein concentration (mg/ml), d is the cuvette thickness (cm). In the aromatic region of the CD spectrum (240-320 nm) the ellipticity was considered to be molar ($[\theta]_M$) and molecular weight of the protein was taken to be 14.3 kD. The scale of the spectropolarimeter was calibrated with 0.06% solution of 10-sulfonate-D-camphoric acid ammonium salt (Katayama Chemical, Japan). The ratio of the band ellipticities at 192 and 290 nm was 2.09. Contents of secondary structure elements of the protein were calculated using Provencher's method [23].

MALDI-TOF mass spectrometry. Mass spectrometry was performed using a Biflex III MALDI-TOF spectrometer (Bruker, USA) in a linear regimen, with recording of positive ions. 3,5-Dimetoxy-4-hydroxysinapinic acid (10 mg/ml) in acetonitrile containing 0.1% trifluoroacetic acid was used as a matrix. Specimens were placed onto a matrix-covered target by the drop drying method.

Methods of determination of the IgG-binding activity. Enzyme immunoassay (EIA), dot-analysis, and western-blotting were performed as described in [24, 25]. The IBP activity was determined with a commercial conjugate of normal rabbit IgG with horseradish peroxidase. The results of EIA were recorded with a μQuant spectrophotometer (BIO-TEK Instruments, USA) at 492 nm, with 0.04% solution of *o*-phenylenediamine as a chromogen.

The pH optimum of the binding reaction between the IBP and rabbit IgG was determined in 10 mM sodium acetate buffer (pH 4.5, 5.0, 6.0), 10 mM Tris-HCl buffer (pH 7.0, 7.5, 8.0), and 10 mM carbonate buffer (pH 9.0, 10.0). The interaction of IBP with rabbit immunoglobulin was inhibited by supplementing the conjugate with the rabbit IgG Fc-fragment to the concentration of 0.5 mg/ml at pH 7.5 during the binding reaction in EIA.

RESULTS AND DISCUSSION

Isolation and purification of IBP. Proteins capable of binding rabbit IgG were detected by EIA and western-blotting in lysates of *Y. pseudotuberculosis* cells. In the blot, the IBPs were displayed as several diffuse bands in the region from 7 to 20 kD (Fig. 1, lanes *1*).

Figure 1 shows that the cell envelopes mainly contain the same active proteins as those detected in the cell lysates of the whole cells (lanes *2*). Thus, the IBPs are

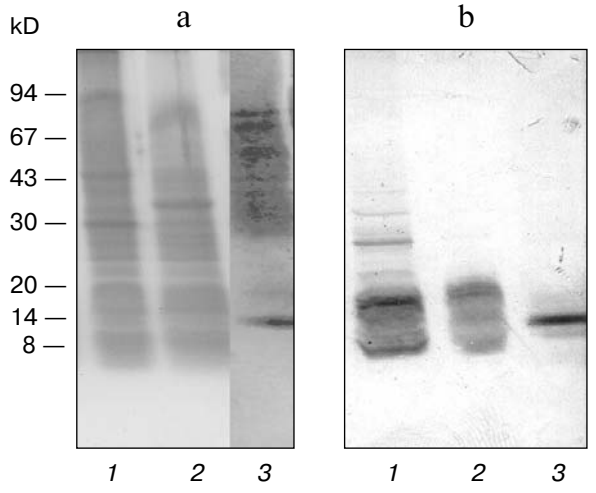

Fig. 1. Electrophoregram of *Y. pseudotuberculosis* proteins in 10-25% gradient SDS-polyacrylamide gel (a) and western-blot of the same specimens revealed with rabbit IgG conjugated with horseradish peroxidase (b). *1*) Lysate of the whole cells in 2% SDS; *2*) cell envelopes of the bacteria prepared by Nurminen's method and dissolved in 2% SDS; *3*) the IBP eluted from a Source 15S cation-exchange column after storage for seven days in 50 mM Tris-HCl buffer (pH 8.0) (molecular weights of the marker proteins are shown on the left).

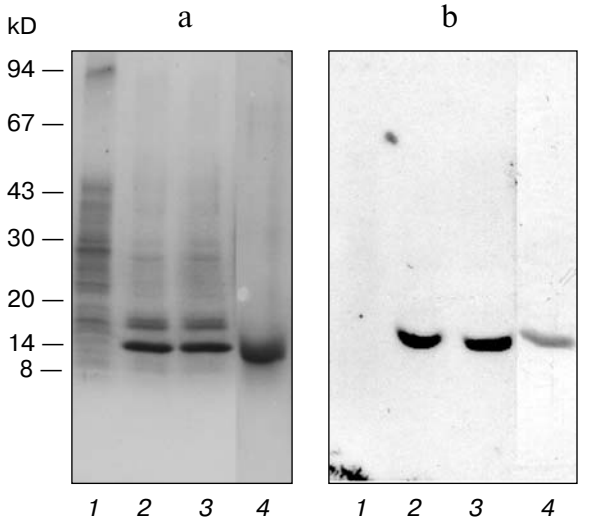

Fig. 2. Electrophoregram in 10-25% gradient SDS-polyacrylamide gel (a) and western-blot of the same specimens revealed with rabbit IgG conjugated with horseradish peroxidase (b). *1*) Extract of the cell envelopes in 50 mM Tris-HCl buffer (pH 8.0); *2*) extract of the cell envelopes in 50 mM sodium acetate buffer (pH 4.0); *3*) extract of the cell envelopes in 50 mM sodium acetate buffer (pH 4.0) after heating for 7 min at 100°C in the sample buffer; *4*) the IBP eluted from a Source 15S cation-exchange column (molecular weights of the marker proteins are shown to the left).

concluded to be mainly located in the cell envelopes of *Y. pseudotuberculosis*.

The IBPs were isolated from the bacteria by successive extraction of the cell envelopes with buffer A and buffer B (Fig. 2, lanes *1* and *2*). Figure 2 shows that the treatment of the cell envelopes with buffer A results in extraction of various proteins lacking the immunoglobulin-binding activity. The active protein was mainly extracted with buffer B, which appeared in the blot as a single band in the region of 14 kD. The blots of the cell envelope lysate and extract of them in buffer B were significantly different (Figs. 1 and 2): the cell envelope contained more active proteins than the extract. These differences could be caused by the inability of the acidic buffer to extract all active proteins from the cell envelopes. However, no active proteins were detectable in the cell envelopes by dot- and blot-analysis after several successive extractions with acetate buffer. Based on these data, it was suggested that the acidic buffer could not extract completely the active proteins from the cell envelopes, but they were denatured during the extraction and lost activity, and this prevented their detection in the blot. Possibly, the active proteins were totally extracted with the acidic buffer, but underwent a structural modification which did not affect the activity but was accompanied by changes in the electrophoretic mobility of the proteins. In the last case, all active proteins under the influence of the acidic buffer were likely to acquire virtually the same apparent molecular weight (because they appeared in the blot as a single band). Therefore, these proteins were suggested to be different molecular forms of the same protein. To answer these questions, further studies are needed.

IBPs were isolated from the cell envelope extract of the bacteria with the acidic buffer and subjected to chromatography under medium pressure on a Source 15S cation-exchange column. Under the fractionation conditions, the active protein was eluted from the cation exchanger with a solution of a high ionic strength (0.6 M NaCl) as a single asymmetric peak (Fig. 3) and appeared in the blot as a single band with the mobility in the region of 14 kD (Fig. 2b, lane *4*). By polyacrylamide gel electrophoresis, the active protein fraction was shown to contain small amounts of peptides with apparent molecular weights lower than 8 kD. To further purify the IBP from low-molecular-weight compounds (<5 kD), gel chromatography on a HiTrap Desalting column in buffer B was used (Fig. 4). By SDS-PAGE and N-terminal amino acid analysis, the isolated IBP was shown to be homogenous. The resulting IBP contained glycine as the N-terminal amino acid and 6% of monosaccharides.

Chemical and physicochemical characteristics of the IBP. By data of MALDI-TOF spectrometry, the molecular weight of the IBP in 50 mM sodium acetate buffer (pH 4.0) is 14.3 kD (Fig. 5). Along with the peak of the IBP molecular ion, in the IBP mass spectrum an addi-

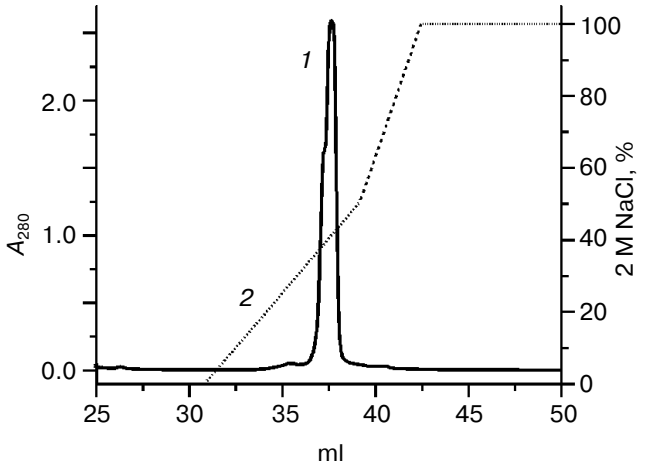

Fig. 3. Profile of IBP elution from a Source 15S cation-exchange column in 50 mM sodium acetate buffer (pH 4.0). *1*) Protein (A_{280}); *2*) gradient of 50 mM sodium acetate buffer (pH 4.0) containing 2 M NaCl.

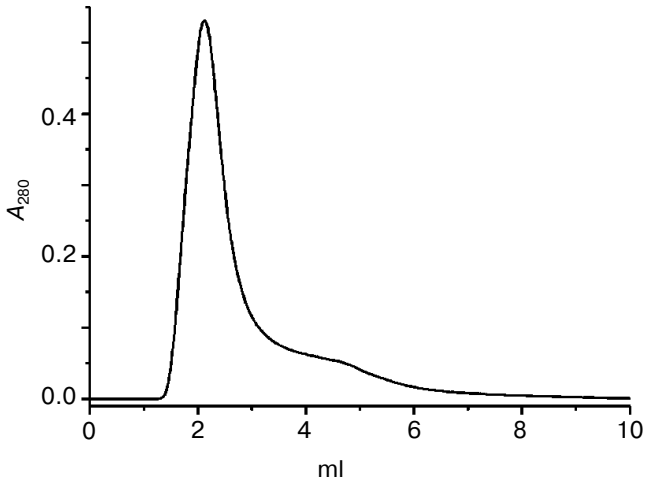

Fig. 4. Profile of IBP elution during gel chromatography on a HiTrap Desalting column in 50 mM sodium acetate buffer (pH 4.0).

tional peak at 7.1 kD is present which can belong to both the two-charged molecular ion of the 14.3-kD protein and the molecular ion of a 7.1-kD protein. We believe the first variant to be more likely because no protein with molecular weight of about 7 kD has been detected in the electrophoregrams and blots of the isolated IBP.

It was found that the IBP could be stored for a long time in sodium acetate buffer at pH 4.0 without losing activity or aggregating. In neutral or alkaline buffers, as well as in solutions with a low ionic strength, the isolated IBP tended to aggregate. In the mass spectrum of the IBP dissolved in deionized water, one can see IBP-produced oligomeric structures: dimer, trimer, and tetramer (Fig. 6). By data of SDS-PAGE and blotting, the aggregates produced during the IBP specimen storage for seven days in Tris-HCl buffer (pH 8.0) had no immunoglobulin-binding activity (lanes *3* and *4* in Figs. 1 and 2, respectively). The aggregation associated with the loss of the activity also occurred on placing IBP into a buffer containing the nonionic detergent Triton X-100 (data not presented). Heating of the IBP at 100°C for 7 min in the sample buffer used during the Laemmli method did not change the electrophoretic mobility of the IBP and its ability to bind immunoglobulins (Fig. 2, lanes *2* and *3*).

From the data of amino acid analysis, protein polarity index was calculated for IBP by Capaldi's method [26] as the sum of polar amino acids (42.1%) plus the half-sum of amino acids intermediate between polar and

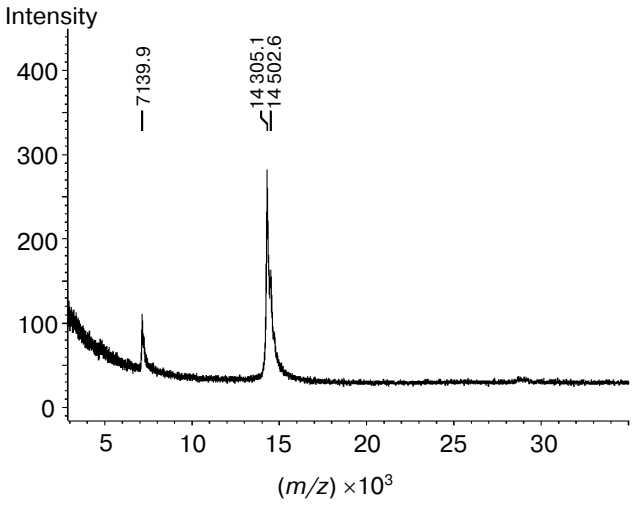

Fig. 5. MALDI-TOF mass spectrum of the IBP in 50 mM sodium acetate buffer (pH 4.0).

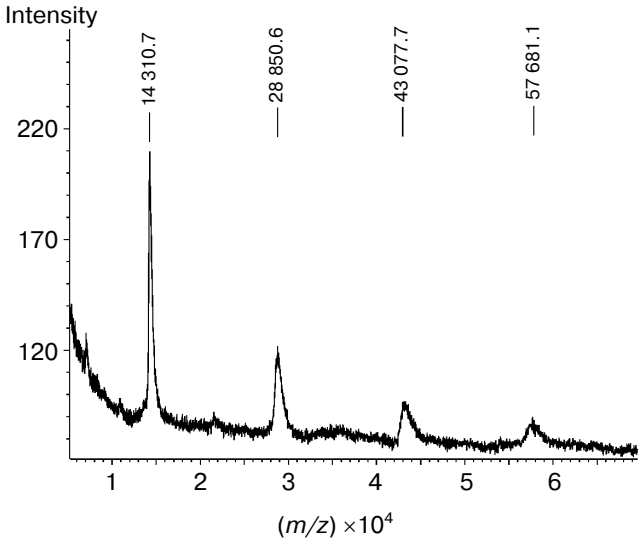

Fig. 6. MALDI-TOF spectrum of the IBP in deionized water.

Data of amino acid analysis of low-molecular-weight IBP from *Y. pseudotuberculosis* for calculation of the polarity index by Capaldi's method [26]

Polar amino acids	Molar %	Amino acids intermediate between hydrophilic and hydrophobic ones	Molar %
Lys	7.1	Thr	6.4
Arg	6.6	Ser	8.3
Asx	18.2	Tyr	4.9
Glx	10.2	His	2.7
		Gly	4.0
Total	42.1	Total	26.4
		Half-sum	13.2
		Index of protein polarity	55.3

hydrophobic amino acids (13.2%). The polarity index of IBP is 55.3% (table). Such high polarity is characteristic for most water-soluble proteins, 47 ± 6% [26].

The CD spectrum in the peptide region (Fig. 7a) was typical for the β-type proteins because it had a wide band at the wavelength of 220 nm. The presence of α-helical portions was confirmed by the maximum at 204 nm. The spectrum in the far-UV region of 190-200 nm indicated the presence of a large quantity of the random coil structure. Fractions of the secondary structure elements of the low-molecular-weight IBP from *Y. pseudotuberculosis* in 50 mM sodium acetate buffer (pH 4.0) calculated from the CD spectra by Provencher's method were 6, 29, 19, and 46% (α-helical, β-structure, β-turn, and random coil structure, respectively).

In the near-UV region (from 300 to 250 nm) of the CD spectrum (Fig. 7b) an intense positive band is observed (to 18 units) with maxima at 280 and 286 nm, which indicate the presence of tyrosine and tryptophan residues, respectively. Based on the CD spectrum in the aromatic region, it was concluded that the protein should have a rather rigid tertiary structure (because the band is intense), although aromatic amino acid residues were surrounded symmetrically, which was proved by a small number of bands and a symmetric appearance of the curve. Obviously, the protein globule structure was a little loosened. The CD spectra were recorded in 50 mM sodium acetate buffer at pH 4.0 (the conditions providing for the highest stability of the protein).

IBP binding with rabbit immunoglobulins G. According to the literature, the interaction of IBP with immunoglobulins depends on pH of the medium, and these dependences vary for IBPs from different species of bacteria. Thus, protein A from *Staphylococcus aureus* and protein G from the group G streptococci effectively react with IgG over a wide range of pH values (4-10). The binding maxima for protein G and protein A are at pH 6.0 and 8.0, respectively [27]. The binding of the IBP under study with immunoglobulin G at different pH values is presented in Fig. 8. The diagram shows that the IBP interacts with the immunoglobulin in a narrower range of pH values (5-8) than proteins A and G, and the maximum binding occurs at pH 6.0.

Most of the IBPs described in the literature bind the IgG molecule in the region of the Fc-fragment [1-5, 7-16]. To determine on the immunoglobulin molecule the

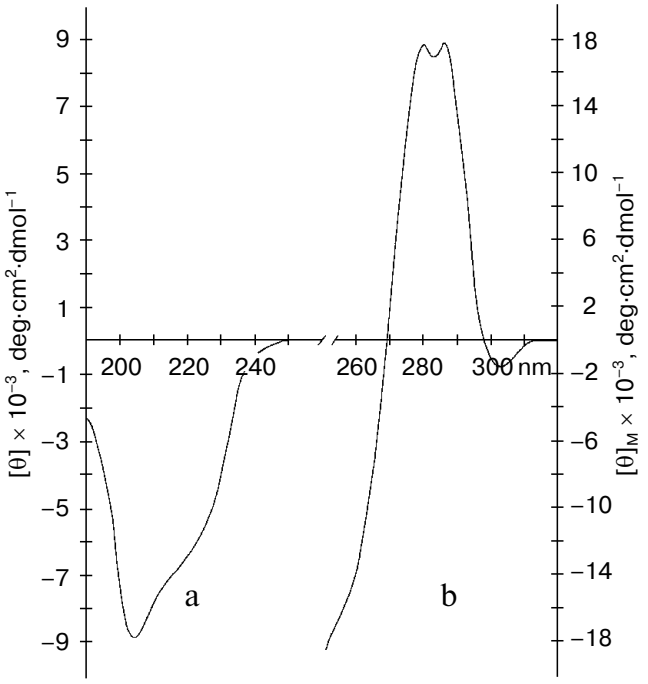

Fig. 7. The CD spectra of the IBP specimen in 50 mM sodium acetate buffer (pH 4.0) recorded in the far (a) and near (b) regions of the UV spectrum.

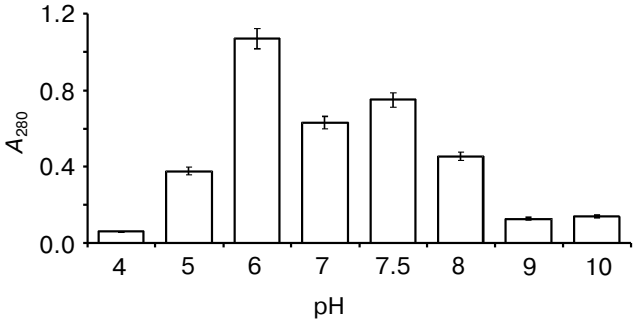

Fig. 8. The influence of pH on IBP binding with rabbit IgG.

binding site for the IBP under study, this interaction was inhibited by addition of the rabbit IgG Fc-fragment. By competitive EIA the binding reaction was shown to be inhibited by 92 ± 4%. Consequently, the IBP from *Y. pseudotuberculosis*, similarly to other proteins of this family, binds to the Fc-fragment of IgG.

Thus, from *Y. pseudotuberculosis* a low-molecular-weight protein was isolated which displayed nonimmune interaction with immunoglobulins through the Fc-fragment. Earlier we isolated from this microorganism a high-molecular-weight IBP, which also bound with the Fc-fragment of rabbit IgG [16]. Thus, we have found in pseudotuberculosis bacteria two proteins of the family of Fc-binding proteins, which are different in chemical and physicochemical properties. At present, we cannot determine whether they are products of conversion of the same protein or different proteins from *Y. pseudotuberculosis*. The work in this line will be continued.

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