Biochemistry (Moscow), Vol. 67, No. 9, 2002, pp. 1062-1067. Translated from Biokhimiya, Vol. 67, No. 9, 2002, pp. 1282-1288.

Isolation and Characterization of the Immunoglobulin-Binding Protein from *Yersinia pseudotuberculosis*

G. A. Naberezhnykh*, E. V. Sidorin, P. S. Dmitrenok, N. Yu. Kim, and T. F. Solov'eva

Pacific Institute of Bioorganic Chemistry, Far East Division of the Russian Academy of Sciences, pr. 100-letiya Vladivostoka 159, Vladivostok, 690022 Russia; fax: (4232) 314-050; E-mail: naber@piboc.dvo.ru

Received August 15, 2001
Revision received October 17, 2001

Abstract—A high molecular weight immunoglobulin-binding protein localized on the surface of bacterial cells has been isolated from the protein fraction of the outer membrane of *Yersinia pseudotuberculosis*, and its properties are described. The immunoglobulin-binding protein is a trypsin-resistant and temperature-sensitive β-structured protein. As shown by MALDI-TOF mass spectrometry, after heating at 100°C the molecular weight of the protein constituted 37.5 kD. The native protein is capable of interacting with human and rabbit IgG but looses the ability to bind the immunoglobulins after the temperature denaturation. The immunoglobulin-binding protein binds to the Fc-fragments of the immunoglobulins and binding depends on the presence of calcium ions.

Key words: immunoglobulins, membrane proteins, immunoglobulin-binding proteins

Immunoglobulin-binding proteins (IBPs) constitute a heterogeneous family of proteins differing in their localizations in bacterial cells, binding properties, and molecular structure. IBPs from cell walls of the gram-positive bacteria (staphylococci of the A group, streptococci of the C and G groups, and peptococci) have been studied rather well [1, 2]. However, much less is known about the proteins from gram-negative bacteria that are capable of non-immune binding to immunoglobulins (Ig).

It is found that in most cases IBPs are revealed on the surface of bacterial cells. Using immunoblotting, IBPs were identified in the cell membrane of *Escherichia coli* as multiple bands in the region of 130-195 kD [3]. These proteins retained their activity even after heating to 100°C, binding immunoglobulins of different animals, but lost this ability after treatment with trypsin. However, IBP isolated from the fimbria of *Yersinia pestis* was capable of interacting only with human IgG. This protein has a high percentage of β-structured sites and exhibits a rather high resistance towards proteinases, high temperatures, and denaturing agents [4]. IBP from *Prevotela intermedia* interacts with human, monkey, porcine, and bovine IgG and does not bind rabbit, mouse, rat, and ovine IgG in spite of the great similarity of the structures of immunoglobulins from different sources [5]. A protein of 30 kD capable of binding to the Fc-fragments of different subclasses of IgG with high constants was isolated from *Pseudomonas maltophilia* [6].

It should be noted that a bacterium can possess several IBPs varying in their molecular weights. Immunoglobulin-binding proteins of 41 and 270 kD were found in *Haemophilus somnus*, the first of which being the main protein of the outer membrane of this microorganism [7]. The immunoglobulin-binding activity of *Fusobacterium nucleatum*, a bacterium that causes human pyorrhea, is associated with the polypeptides of 40 and 42 kD. These proteins are homologous to the terminal amino acid sequence of the Fom A porin [8].

In spite of the significant differences in the properties of IBPs from different organisms, they have a common feature—the ability to bind to the Fc-fragments of immunoglobulins, this being an important biological phenomenon. It is considered that the non-immune binding of the bacterial surface proteins to immunoglobulins prevents binding of the complement and decrease opsonization and phagocytosis of bacteria, this allowing them to avoid the effect of the host immune system [1, 9]. Thus, IBPs are of importance as factors of bacterial pathogenicity.

It should be emphasized that, in most cases, the properties of gram-negative bacteria were investigated using total extracts of the outer membrane proteins. There were only a few cases when the individual proteins were isolated from bacterial species and their properties were studied.

Abbreviations: IBP) immunoglobulin-binding protein; FITC-IgG) rabbit immunoglobulins labeled with fluorescein.

* To whom correspondence should be addressed.

0006-2979/02/6709-1062$27.00 ©2002 MAIK "Nauka/Interperiodica"

The goal of this work was to isolate and to characterize the immunoglobulin-binding protein from *Y. pseudotuberculosis*.

MATERIALS AND METHODS

Bacterial cultures. *Y. pseudotuberculosis* (IB serotype) taken from a patient was grown at 25°C as described earlier [10]. In the present work we used acrylamide and divinyl sulfone from Serva (Germany), Sepharose CL 4B and Sephacryl S-300 from Pharmacia (Sweden), and detergent Brij 35 from Calbiochem (USA). Other chemicals were of analytical grade (Reakhim, Russia) and were used without additional purification.

Physical and chemical methods. CD spectra were recorded on a Jasco J-500A spectropolarimeter (Japan) in 0.1-cm quartz cuvettes in the peptide region of the spectrum (190-230 nm). Ellipticity [θ] was calculated as the mean residue ellipticity, taking the molecular weight of the mean residue as 110 daltons and the protein content as 100% according to the equation:

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

where S is the sensitivity of the scale, C is the protein concentration (mg/ml), d is the width of the cuvette (cm). The contents of the elements of the secondary structure of the protein were calculated according to Provencher [11].

Fluorescence spectra were measured using a Hitachi 850 spectrofluorimeter (Japan) at 25°C in a 1-cm quartz cuvette. Fluorescence of IgG labeled with FITC (FITC-IgG) was excited at 487 nm. The fluorescence spectra corrected by Rhodamine B (Wako Chemical Industries, Japan) were recorded, subtracting the Raman band of the buffer. The excitation and emission optical bandwidths were 5 nm.

MALDI mass-spectrometry was performed on a MALDI-TOF Biflex III mass spectrometer (Bruker, USA) in the reflex regime with recording of positive ions. α-Cyano-4-hydroxycinnamic acid (25 mg/ml) was used as matrix in the mixture with acetonitrile containing 0.1% trifluoroacetic acid. Samples were prepared by the drop drying method.

Composition of buffer solutions was the following: 0.12% Brij 35, 16.7 mM Tris-HCl, pH 7.5 (buffer A); 10 mM EDTA, 0.12% Brij 35, 16.7 mM Tris-HCl, pH 7.5 (buffer B); 10 mM EDTA, 0.12% Brij 35, 100 mM CH_3COONa/CH_3COOH, pH 4 (buffer C).

The fraction of outer membrane proteins was isolated according to Nurminen [12]. Bacterial cells were treated with lysozyme, and the resulting extract was gel filtered through a Sephacryl S-300 column (65 × 2 cm) equilibrated with buffer B. The protein fractions were analyzed by SDS-PAGE according to Laemmli [13]. A kit of proteins of molecular weights 20.1, 30.0, 43.0, 67.0, 94.0 kD (Sigma, USA) was used as the markers. The proteins separated in the gel were stained with the solution containing Coomassie R-250, 10% acetic acid, and 45% ethanol. Protein content in samples was determined spectrophotometrically by the Bradford's procedure, using lysozyme as the standard protein [14].

Preparation of the affinity sorbent. The affinity sorbent was prepared using IgG isolated from rabbit serum by ammonium sulfate precipitation with subsequent chromatography on DEAE-cellulose [15]. Sepharose CL-4B was activated by two methods using sodium periodate and divinyl sulfone according to the standard procedures [16]. Binding of the IgG to the activated Sepharose was performed as described in [16].

Affinity chromatography. $CaCl_2$ (final concentration, 30 mM) was added to a solution of the outer membrane proteins, and the resulting solution was allowed to circulate through a column for 16 h with the immobilized IgG. Then the column was washed with buffer A from the nonbound proteins, until the absorption value of the eluate reached 0.05 at 280 nm. The adsorbed proteins were eluted with buffer B containing 0.5 M NaCl. To elute completely the proteins bound to IgG, the column was washed with the eluting buffer overnight.

Ion-exchange chromatography. Ion-exchange chromatography of the proteins obtained during the affinity chromatography was performed sequentially on DEAE- and CM-Sepharose CL-4B columns (30 × 1.5 cm). The ion-exchangers were prepared as described in [15] and then equilibrated with one volume of buffer A or B. The proteins were eluted with a 0-2 M NaCl gradient in buffer A or B. The fractions were assayed for the immunoglobulin-binding activity and pooled.

Methods for assaying IgG-binding activity. Immunoenzymatic analysis, dot blot analysis, and electroblotting were performed according to standard procedures [17]. The activity of the IBP was assayed using animal immunoglobulins labeled by horseradish peroxidase and commercial conjugates of the anti-species antibodies.

Amino acid analysis. Protein samples were hydrolyzed in 5.7 M HCl for 24 h at 110°C in ampoules filled with nitrogen. The samples were analyzed using an Alpha-Plus 4151 amino acid analyzer (LKB, Sweden). N-Terminal amino acids were assayed as described in [18].

Localization of the IBP in *Y. pseudotuberculosis* cells. The bacterial cells were washed three times with buffer A, centrifuging the bacteria. Then 100 μl (50 μg) of rabbit FITC-IgG in the presence or in the absence of $CaCl_2$ were added to 8 μl of the cell suspension and the mixture was incubated for 2 h at 37°C. To inhibit binding, 40 μg of the IBP or 40 μg of rabbit IgG Fc-fragments were added to the mixture. The cells were washed three times with buffer A and suspended in 100 μl of the same buffer. The suspension (10 μl) was applied to a plate, dried in air, fixed with varnish, and assayed using a light microscope (Carl Zeiss, Germany). To assay the total fluorescence of the cells (50 μl), FITC-IgG were suspended in 1 ml of buffer A.

RESULTS AND DISCUSSION

Isolation and purification of the immunoglobulin-binding protein. We were first to reveal the immunoglobulin-binding activity in the fraction of the outer membrane proteins of *Y. pseudotuberculosis* using immunoenzymatic and dot-blot analysis.

The outer membrane proteins were isolated from the bacteria according to Nurminen [12] and purified by gel filtration on Sephacryl S-300. The isolated fraction contained the active immunoglobulin-binding protein as a minor component in the mixture with high molecular weight integral membrane proteins that could be dissolved in aqueous solutions only in the presence of a detergent, this complicating the separation of the individual protein.

In the first step, the IBP was separated from the outer membrane proteins using affinity chromatography on immobilized rabbit IgG. Two methods were used for immobilization of IgG: Sepharose CL-4B was activated with sodium periodate and divinyl sulfone. The second method resulted in lower content of the immobilized IgG, but in spite of this fact, the yield of the IBP was 25% more than that on the activation of the support with sodium periodate. This difference in the yield of the protein can be accounted for as the facilitation of binding of the ligand due to an increase in the distance between the support and IgG and, consequently, decrease in steric hindrances for the interaction [15]. The steric hindrances and, presumably, the association of the IBP with the detergent (nonionic detergent Brij 35) that was present in the solution in the micellar concentration determine the long time of interaction (18-20 h) between the original membrane proteins and the affinity sorbent required for maximal binding of IgG.

In the chromatography on the affinity sorbent, the immunoglobulin-binding activity was revealed in the fraction eluted with 0.5 M NaCl in the presence of 0.01 M EDTA. It should be noted that the immunoaffinity chromatography did not yield the individual IBP, presumably due to its complexing with other membrane proteins.

Further purification of the IBP was performed using ion-exchange chromatography. The chromatography on DEAE-Sepharose in the presence of the detergent resulted in separation of some impurity proteins capable of binding to the anion-exchanger. The IBP did not interact with the ion-exchanger and could be eluted with the starting buffer A. The obtained IBP-containing fraction was revealed by SDS-PAGE as a triple band of 120-140 kD (Fig. 1a, lane *3*). As shown by electroblotting (Fig. 1b, lane *3*), all three bands corresponded to the proteins exhibiting the immunoglobulin-binding activity.

During further purification on CM-Sepharose, the active protein was eluted with a solution of high ionic strength (1 M NaCl) as a single peak and was revealed on the electrophoregram and on the blot as the single band of 160 kD (Fig. 1, a and b, lane *4*).

Characteristics of the immunoglobulin-binding protein. N-Terminal amino acid analysis revealed a single amino acid, alanine, this confirming the purity of the isolated protein. The same N-terminal amino acid was revealed in the IBP fraction that was obtained by the ion-exchange chromatography on DEAE-Sepharose and yielded several bands on SDS-PAGE (Fig. 1, a and b, lane *3*).

The hydrolyzate of the active protein eluted from DEAE-Sepharose, unlike that eluted from CM-Sepharose, contains a substance that is eluted in the region of basic amino acids, but it does not correspond to any of the known amino acids. Its content constitutes about 20% of the total content of the amino acids (calculated from the areas of the peaks). This component is likely to be a compound containing the amino group, because it forms an intensively fluorescing derivative with dansyl chloride. However, two-dimensional thin layer chromatography in silica plates could not identify this derivative as one of the known dansyl-amino acids. This suggests that the IBP forms complexes with an unknown component that is destroyed on the ion-exchange chromatography. These complexes can differ in their composition resulting in the multiple bands on the electrophoregram. Separation of the protein-associated component results in transformation of the multiple bands into the single protein band. It can be assumed that the decrease in the electrophoretic mobility of the IBP observed after the removal of the component is due to conformational changes in the protein molecule and formation of molecules of less compact structure. The work on isolation and purification of the unknown component associated with the IBP is being continued to

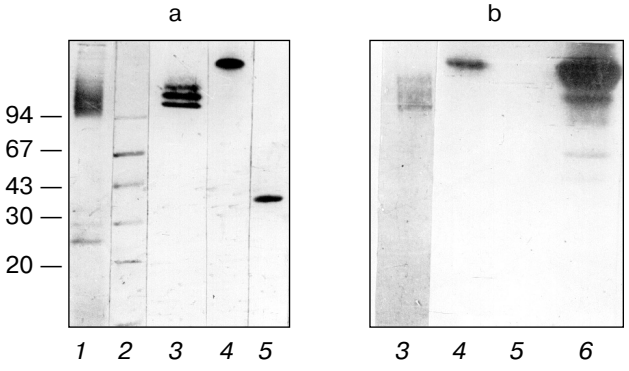

Fig. 1. Electrophoresis in a 9-20% gradient SDS polyacrylamide (a) and blotting of the same samples revealed by rabbit IgG and the second antibodies conjugated with peroxidase (b): *1*) original membrane proteins; *2*) protein markers; *3*) IBP eluted from DEAE-Sepharose; *4*) IBP eluted from CM-Sepharose; *5*) the same as *4* but preheated at 100°C; *6*) rabbit IgG (molecular weights of the protein markers in kD are given on the left).

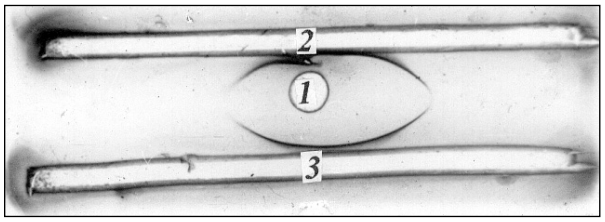

Fig. 2. Electrophoresis of the IBP in agarose (*1*). The IBP was revealed using rabbit IgG (*2*) and human IgG (*3*).

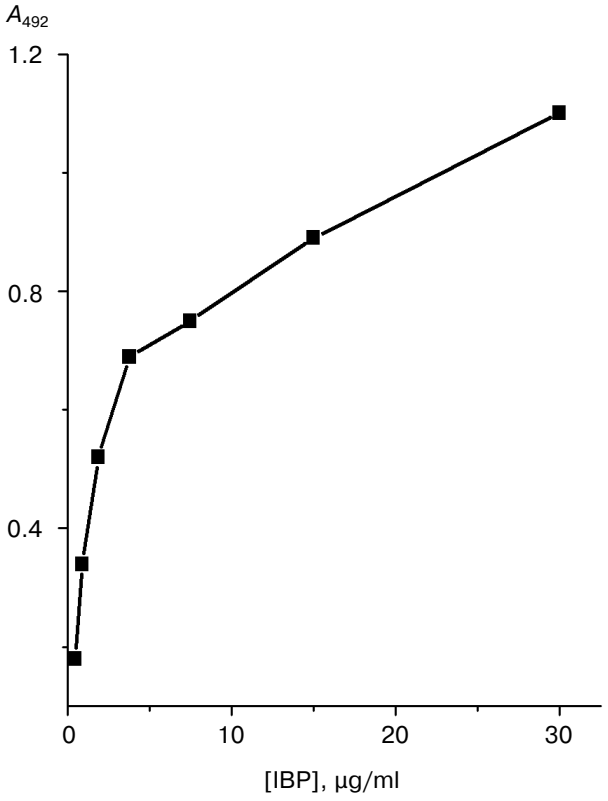

Fig. 3. Dependence of immunoglobulin-binding activity of IBP on IBP concentration. Polystyrene plates were coated with increasing concentrations of IBP, and then rabbit IgG were added. The interaction between IgG and the IBP were detected using anti-IgG antibodies conjugated with peroxidase.

determine the nature of its effect on the protein conformation.

On immunoelectrophoresis in agarose, the IBP moves to the cathode yielding a single precipitation arc (Fig. 2). Thus, under the conditions employed (barbital buffer, pH 8.6), the IBP has a positive charge and can be revealed with rabbit or human IgG as a homogenous protein.

Heating of the IBP under denaturing conditions (2% SDS, 100°C) results in a significant increase in its mobility during SDS-PAGE and it can be revealed as the single band corresponding to 37 kD (Fig. 1a, lane *5*). The molecular weight of the polypeptide was measured more exactly using MALDI-TOF mass spectrometry. A single peak corresponding to 37.5 kD was revealed in the mass-spectrum of the IBP preheated at 100°C. However, we failed to obtain mass spectra for the native protein form. Presumably, it is connected with its high molecular weight, weak ionization, or dissociation into monomers under the conditions of MALDI-TOF mass-spectrometry.

The isolated protein has rather hydrophobic properties because it can be dissolved in aqueous solutions only in the presence of detergents; the removal of the detergents results in aggregation of the protein.

Circular dichroism analysis was used to characterize the secondary structure of the IBP. A negative band with maximum at 213 nm and a shoulder at 222 nm, and also an intense positive band at 192 nm were observed in the peptide region of the CD spectrum of the IBP. The shape of the spectrum indicated a high content of β-structure in the protein. The results of calculation of the secondary structure elements according to Provencher [11] presented in the table confirm the conclusion made based on qualitative analysis of the shape of the CD spectrum. As seen from the table, the IBP is a β-structured protein.

The comparatively low molecular weight of the denatured IBP together with the low electrophoretic mobility of the native protein suggests that the IBP has oligomeric structure.

Binding of the IBP to immunoglobulins. To investigate the ability of the IBP to bind to rabbit IgG, a polystyrene plate was coated with increasing IBP concentrations prepared by sequential two-fold dilutions of the IBP, then treated with rabbit IgG, and the complex formed was revealed using the anti-IgG antibodies labeled with peroxidase. Figure 3 illustrates the dependence of the immunoglobulin-binding activity of the IBP on its concentration.

The immunoglobulin-binding activity of the IBP is strongly affected by the presence of calcium ions in the reaction mixture. The dependence of the immunoglobulin-binding activity of the IBP on the concentration of calcium ions in the solution (10-70 mM) was assayed using the immunoenzymatic analysis. Binding of IgG was

Content of the secondary structure elements in IBP isolated from the pseudotuberculosis bacillus

Sample in 20 mM Tris-HCl (pH 8.0)	α-Helix	β-Structure	β-Turn	Random coil
IBP	0.00	0.67	0.32	0.01

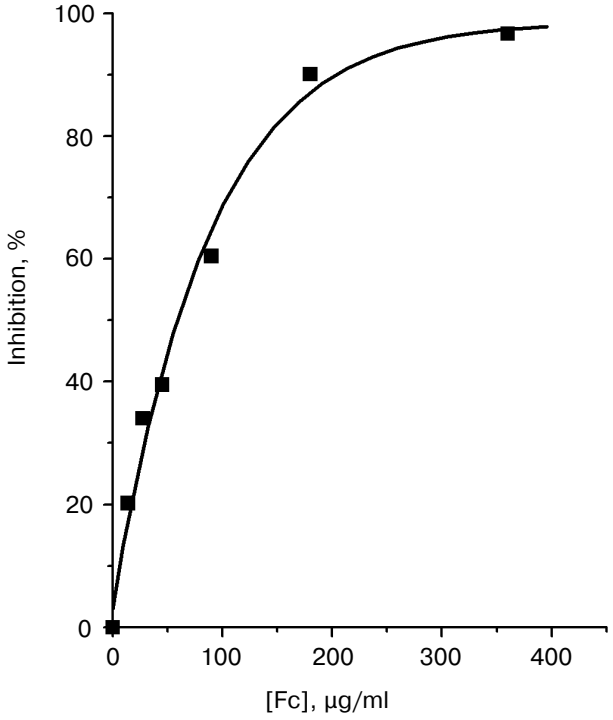

Fig. 4. Inhibition of binding of IBP to rabbit IgG by Fc-fragments. Increasing concentration of Fc-fragments of rabbit IgG were incubated with adsorbed IBP, and then rabbit IgG were added. The results were detected as described in the legend to Fig. 3.

maximal in the region of 20-30 mM $CaCl_2$ concentration. Due to this fact, the addition of $CaCl_2$ (final concentration, 30 mM) increases the yield of the active protein during the affinity chromatography by 75%. Only in the presence of calcium ions IBP gives a single precipitation line with rabbit and human IgG during electrophoresis in agarose. It should be noted that the immunoglobulin-binding activity of IBP from *Mycoplasma salivarium* was shown to depend on bivalent manganese ions [19].

The interaction between IBP and rabbit IgG was inhibited on the addition of the Fc-fragments of IgG into the reaction mixture (Fig. 4). This suggests that the Fc-site of the immunoglobulin molecule is involved in binding of IBP. These results are consistent with literature data concerning the localization of the binding sites of IBPs from other microorganisms on the immunoglobulin molecule [1, 4, 7].

The immunoenzymatic analysis and electroblotting showed that the IBP lost its ability to bind immunoglobulins after the thermal denaturation (Fig. 1, lane *5*). Previously, the decrease in the immunoglobulin-binding activity with the increase in the temperature of the medium to 40°C was observed for protein L from *Peptostreptococcus magnus*, this being accounted for by

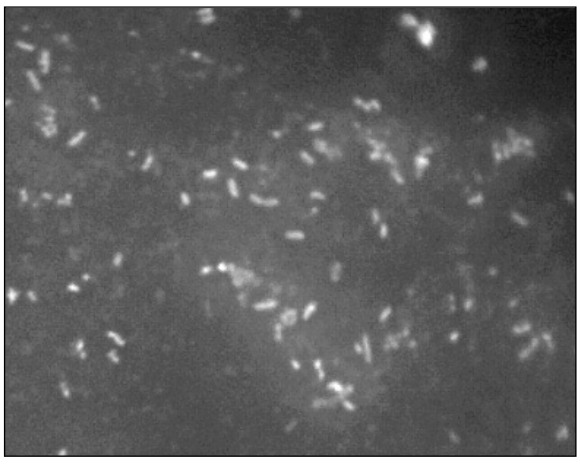

Fig. 5. Binding of rabbit IgG labeled with FITC to non-fixed cells of *Y. pseudotuberculosis*.

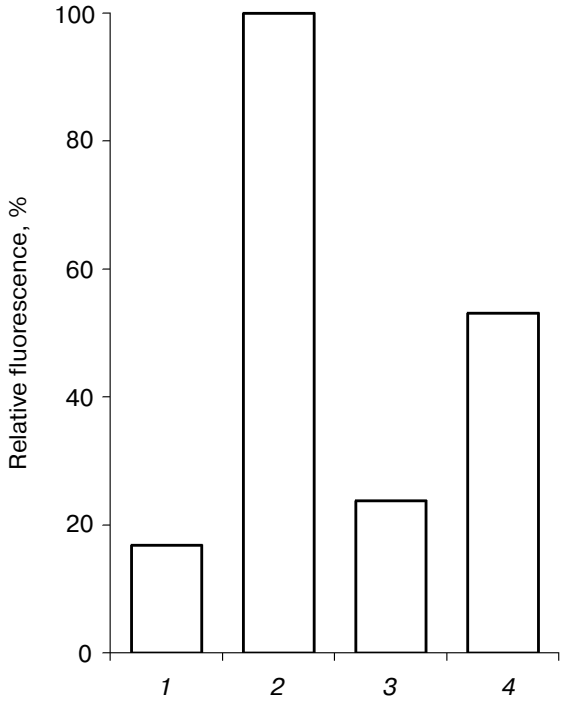

Fig. 6. Total fluorescence of the pseudotuberculosis bacteria after interaction with rabbit IgG labeled with FITC: *1*) in the absence of calcium ions; *2*) in the presence of calcium ions; *3*) in the presence of IBP; *4*) in the presence of Fc-fragments of IgG. Inhibition by the IBP and Fc-fragments was performed in the presence of calcium ions.

changes in the conformation of the protein molecule and the decrease in the affinity of the interaction [20].

Localization of the immunoglobulin-binding protein from *Y. pseudotuberculosis*. Fluorescent microscopy showed that the treatment of the cells of the pseudotuberculosis bacteria by the non-immune rabbit IgG labeled by fluorescein resulted in binding of the IgG to the surface of the cells (Fig. 5). These results indicate that IBP is exposed to the surface of the bacterial cell. The method of isolation of IBP and its hydrophobic properties also suggest that IBP is localized in the outer membrane of the bacteria.

The fluorescence intensity of the bacteria labeled with FITC-IgG significantly increased in the presence of calcium ions and decreased in the presence of the purified IBP and the Fc-fragments of rabbit IgG (Fig. 6). The fact that the interaction of the bacteria with FITC-IgG is only in part inhibited by IBP and Fc-fragments of rabbit IgG suggests the presence of some additional factors on the cell surface providing direct binding of IgG without participation of Fc-fragments.

Thus, a high molecular weight immunoglobulin-binding protein localized on the surface of bacterial cells has been isolated from *Y. pseudotuberculosis*, and its properties have been described. This protein is resistant to trypsin, sensitive to temperature, and completely β-structured. The IBP interacts with Fc-fragments of immunoglobulins and its binding depends on calcium ions.

Previously it was shown that *Y. pseudotuberculosis* could inhibit phagocytosis of bacteria by macrophages mediated by the Fc-receptors [21]. However, the protein responsible for this activity of the pseudotuberculosis bacteria has not been described. We assume that the protein localized on the surface of the cell and capable of binding the Fc-fragments of IgG can be responsible for the antiphagocytic activity.

The authors are grateful to T. A. Zykova (Pacific Institute of Bioorganic Chemistry, Far East Division of the Russian Academy of Sciences) for help in the analysis of N-terminal amino acids.

This work was supported by the Russian Foundation for Basic Research (grant 01-04-49331).

REFERENCES

1. Widders, P. R. (1991) in *Bacterial Immunoglobulin-Binding Proteins: Microbiology*, *Chemistry*, *and Biology* (Boyle, M. D. P., ed.) Academic Press Inc, San Diego, pp. 375-396.
2. Ezepchuk, Yu. V. (1977) in *Biomolecular Background of Bacterial Pathogenicity* [in Russian], Nauka, Moscow, pp. 56-63.
3. Sandt, C. H., Wang, Y.-D., Wilson, R. A., and Hill, C. W. (1997) *Infect. Immun.*, **65**, 4572-4579.
4. Zavyalov, V. P., Abramov, V. M., Cherepanov, P. G., Spirina, G. V., Chernovskaya, T. V., Vasiliev, N. P., and Zavyalova, G. A. (1996) *FEMS Immunol. Med. Microb.*, **14**, 53-57.
5. Labbe, S., and Grenier, D. (1995) *Infect. Immun.*, **63**, 2785-2789.
6. Grover, S., McGee, Z. A., and Odell, W. D. J. (1991) *Immun. Meth.*, **141**, 187-197.
7. Yarnall, M., Gogolewski, R. P., and Corbeil, L. B. (1989) *J. Gen. Microbiol.*, **135**, 1993-1999.
8. Guo, M., Han, Y. W., and Sharma, A. (2000) *Oral Microbiol. Immunol.*, **15**, 119-123.
9. Widders, P. R., Dorrance, L. A., Yarnall, M., and Corbeil, L. B. (1989) *Infect. Immun.*, **57**, 639-642.
10. Ovodov, Yu. S., Gorshkova, R. P., and Tomshich, S. V. (1971) *Immunochemistry*, **8**, 1071-1079.
11. Provencher, C. W., and Glocker, J. (1981) *Biochemistry*, **20**, 33-37.
12. Nurminen, M., Lounatmaa, K., Sarvas, M., Makela, P. H., and Nakae, T. (1976) *J. Bacteriol.*, **127**, 941-995.
13. Laemmli, U. K. (1970) *Nature*, **2**, 680-685.
14. Gasparov, V. S., and Degtyar', V. G. (1994) *Biochemistry* (Moscow), **59**, 563-572.
15. Osterman, L. A. (1985) in *Chromatography of Proteins and Nucleic Acids* [in Russian], Nauka, Moscow, pp. 277-281.
16. Ber, E., and Mani, J.-C. (1989) in *Affinity Chromatography* (Din, P., Johnson, W., and Middle, F., eds.) [Russian translation], Mir, Moscow, pp. 48-83.
17. Mikhailov, A. T., and Simirskii, V. N. (1991) in *Methods of Immunochemical Analysis in Developmental Biology* [in Russian], Nauka, Moscow, pp. 155-192.
18. Gray, R. (1972) *Meth. Enzymol.*, **25**, 121-138.
19. Shibata, K., Sawa, Y., Inoe, S., Noda, M., and Watanabe, T. (1994) *FEMS Microbiol. Lett.*, **123**, 305-310.
20. Beckingham, A., Bottomley, S. P., Hinton, R., Sutton, B. J., and Gore, M. G. (1999) *Biochem. J.*, **340**, 193-199.
21. Fallman, M., Andersson, K., Hakansson, S., Magnusson, K.-E., Stendahl, O., and Wolf-Watz, H. (1995) *Infec. Immun.*, **63**, 3117-3124.