



SLE: Unusual Recombinant Monoclonal Light Chain NGTA3-Pro-DNase Possessing Three Different Activities Trypsin-like, Metalloprotease and DNase

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Abstract

Objective: DNA- and myelin basic protein (MBP)-hydrolyzing antibodies can play an important role in the pathogenesis of systemic lupus erythematosus (SLE), therefore their analysis seems to be important.

Results: In mammals there are serine proteases, metalloproteases, and DNases. Each of these and many other enzymes catalyze only one chemical reaction. Very unusual and unpredictable situation was revealed by us in the case of monoclonal abzymes corresponding to the sera of patients with SLE. The small pools of phage particles displaying light chains with different affinity for myelin basic protein (MBP) were isolated by affinity chromatography on MBP-Sepharose. In contrast to canonical enzymes, one of twenty five MLChs demonstrated three different enzymatic activities; it efficiently hydrolyzed MBP (but not other proteins) and DNA. Other twenty four MLChs hydrolyzed only MBP. The proteolytic activity of NGTA3-pro-DNase was efficiently inhibited by specific inhibitors of serine-like (PMSF) and metalloproteases (EDTA). Protease and DNase properties of NGTA3-pro-DNase differ significantly from those for the corresponding canonical enzymes.

Conclusion: This is the first example of monoclonal antibodies with three different catalytic activities. The principal possibility of the existence of monoclonal antibodies with several different enzymatic activities is unexpected but very important for the further understanding of unknown biological functions of human immunoglobulins.

Keywords: Systemic lupus erythematosus; Recombinant monoclonal light chain; Hydrolysis of myelin basic protein and DNA

Abbreviations: Ab: Antibody; EDTA: Ethylenediamine Tetraacetic Acid; HAS: Human Serum Albumin; IPTG: Isopropyl β -D-1-Thiogalactopyranoside; MBP: Myelin Basic Protein; OP: Oligo Peptide; PFU: Plaque-Forming Unit; SDS-PAGE: SDS-Polyacrylamide Gel Electrophoresis; MLCh: Monoclonal Light Chain; MS: Multiple Sclerosis; SLE: Systemic Lupus Erythematosus; RA: Relative Activity.

Introduction

Abzymes against transition chemical states of different reactions were studied intensively reviewed in [1-3]. Similarly to artificial abzymes against analogs of transition states of catalytic reactions [1-3], naturally occurring abzymes may be antibodies (Abs) raised directly against enzyme substrates acting as haptens and mimicking transition states of catalytic reactions [3-6] or may be antiidiotypic Abs against active centers of various enzymes [7,8].

A special feature of autoimmune diseases including systemic lupus erythematosus (SLE) and multiple sclerosis (MS) is high concentrations of auto-Abs to many different endogenous antigens including DNA and myelin basic protein (MBP) [3-6]. Polyclonal natural IgG and/or IgA and IgM abzymes hydrolyzing DNA, RNA, polysaccharides, nucleotides, oligopeptides, and proteins from the sera of patients with several autoimmune and viral diseases were revealed

(reviewed in [3-6]). Healthy humans and patients with many diseases with insignificant autoimmune reactions usually lack abzymes or develop Abs with very low catalytic activities, often on a borderline of the sensitivity of detection methods.

It was shown that electrophoretically homogeneous polyclonal Abs from the sera of MS [9-13] and SLE [14-17] patients contains small fractions with DNase and MBP-hydrolyzing activities, which are intrinsic properties of IgGs and/or IgMs and IgAs. SLE and MS patients can generate polyclonal abzymes attacking MBP in the myelin-proteolipid sheath of axons and DNA and can play an important role in these diseases pathogenesis [6]. There are examples of monoclonal human [18,19] and mouse IgGs hydrolyzing DNA [20,21]. Bence-Jones proteins from patients with multiple myeloma should be considered as the first found natural human monoclonal abzymes [22]. Monoclonal light chain (MLCh) of anti-VIP abzyme was expressed in bacteria, purified, and found to possess an intrinsic VIP-hydrolyzing activity [23,24]. Two MLChs from patients with multiple myeloma with prothrombinase activity were identified [25]. Two MLChs from multiple myeloma patients specifically hydrolyzed viral gp120 protein [26,27]. Recently MLChs capable to degrade the active site of the urease of *Helicobacter pylori* and eradicate the bacterial infection in a mouse stomach was obtained [28]. Catalytic MLCh was obtained by immunizing with a peptide possessing a part of a sequence of a chemokine receptor, CCR-5, which is present as a membrane protein on the macrophage surface [29].

It should be mentioned, that all described above monoclonal abzymes catalyzed only one chemical reaction. At the same time, the origin of classical enzymes and catalytic antibodies has a completely different nature. To each canonical enzyme corresponds one gene that encodes its protein structure. The formation of genes encoding structure of Abs occurs due to V(D)J recombination, which is the unique mechanism of genetic recombination that occurs only in developing lymphocytes during the early stages of T and B cell maturation [30]. The process results in the highly diverse repertoire of immunoglobulins and it is a defining feature of the adaptive immune system and its development was a key event in the evolution of jawed vertebrates. The process ultimately results in novel amino acid sequences in the antigen-binding regions of immunoglobulins that allow for the recognition of antigens from nearly all pathogens; the recognition can be also "autoreactive" and leading to autoimmunity [30]. It should be mentioned, that immunization of autoimmune mice results in a dramatically higher incidence of abzymes with a higher activity than in conventionally used normal mouse strains [31,32], therefore the formation of abzymes in autoimmune diseases may be much more profuse. In addition, theoretically the immune system can produce 106 different Ab variants in response to a single antigen [33]. Moreover, an increased level of apoptosis in autoimmune patients may trigger production of abzymes directly to DNA and its complexes with various proteins. Therefore, situation with catalytic antibodies may be very complicated and somewhat unpredictable.

For example, DNases hydrolyze only DNA and are inactive in the hydrolysis of RNA, while RNases cannot hydrolyze DNA. Several monoclonal mouse SLE IgGs against B-DNA of different sequences efficiently hydrolyze single- and double-stranded DNA and RNA in a sequence-independent manner, with the hydrolysis of RNA being 30–100-fold faster than of DNA [20]. In addition, immunization of rabbits with DNA, RNA, DNase I, DNase II, and pancreatic RNase A produced IgGs with intrinsic DNase and RNase activities ([34] and references therein). It was shown that small fractions of the preparations of total polyclonal IgGs against DNase I, DNase II, and RNase demonstrate high DNase and RNase activities belong to antiidiotypic Abs to these enzymes, but they also contain small fractions interacting no with Sepharose bearing Abs against these enzymes, but interact with nucleic acids bound to these enzymes [34]. It means that these Abs are against nucleic acids bound with the enzymes used.

An immunoglobulin light chain phagemid library derived from peripheral blood lymphocytes of patients with SLE was recently used [35]. Small pools of phage particles displaying light chains with different affinity for MBP were isolated by affinity chromatography on MBP-Sepharose, and the fraction eluted with 0.5 M NaCl was used for preparation of individual monoclonal light chains (MLChs, 27–28 kDa). Seventy five of 440 individual colonies were randomly chosen, expressed in *E. coli* in a soluble form, and MLChs were purified by metal chelating chromatography. Twenty-five of 72 MLChs efficiently hydrolyzed MBP. Four of twenty-two MLChs analyzed in this article [35] demonstrated serine protease-like and three, thiol protease-like activities, while eleven MLChs were metalloproteases. The activity of three chimeric MLChs was inhibited by both PMSF and EDTA, two other by EDTA and iodoacetamide and one by PMSF, EDTA, and iodoacetamide [35]. These observations suggest an extreme diversity of anti-MBP abzymes in SLE patients.

Two of 25 MLChs were later analyzed in details [36,37]. Uninspected results were obtained. The activity of one MLCh-23

(NGTA1-Me-pro) was inhibited only by EDTA and it efficiently hydrolyzed MBP (but not other control proteins) [36]. NGTA1-Me-pro was shown to be abzyme with two independent metalloprotease active centers demonstrating two pH optima, two optimal concentrations of Me^{2+} ions, and two Km values for MBP [36].

MLCh-24 (NGTA2-Me-pro-ChTr) also efficiently hydrolyzed only MBP [37]. Its proteolytic activity was efficiently inhibited only by specific inhibitors of serine-like (PMSF) and metalloproteases (EDTA). It was shown, that MLCh-24 possesses independent serine-like and metal-dependent activities [37].

We report in this article first example of even more unusual recombinant MLCh-25, which binds and hydrolyzes DNA, MBP, and three oligopeptides corresponding to its four specific sequences (immunodominant sequences containing cleavage sites) demonstrating three different alternative activities: two proteolytic (serine-like and metal-dependent) and DNase activity.

Methods

Materials and chemicals

Most chemicals, proteins and the Superdex 200 HR 10/30 column were from Sigma, while chelating Sepharose from GE Healthcare. Human MBP was from the Department of Biotechnology, Research Center of Molecular Diagnostics and Therapy (Moscow). MBP-Sepharose was obtained by immobilizing of MBP on BrCN-activated Sepharose according to the standard manufacturer's protocol.

Amplification of phage library

We have used human lupus kappa light chains library (from three patients; 106 variants of different light chains); cDNA was cloned into the phagemid pCANTAB5His6 vector after a leader sequence of the phage coat protein gene pIII of a filamentous *E. coli* bacteriophage M13 between *Sma*I and *Not*I restriction sites using standard methods [24,38,39]. This library was a gift from S. Paul and S. Plaque (University of Texas Houston Medical School, USA); all details of this library preparation were described earlier [24]. Amplification of the VCSM13 helper phage and determination of its titer was carried out according to [24]. The amplification of the phage library was carried out as in [24,38,39].

Chromatography of phage particles on MBP-Sepharose

Preparations prepared using *E. coli* TG1 were exhausted from the main part of phage particles having affinity to DNA by chromatography on DNA-cellulose according to [18,19]. Then preparations of phage particles eluted from DNA-cellulose at loading (4 ml containing 2.5×10^{12} phage particles) were applied onto MBP-Sepharose column (3 ml) equilibrated with 20 mM Tris-HCl (pH 7.5) and the column was washed with the same buffer to zero optical density (A_{280}). For the control similar solution of phage particles corresponding to pCANTAB plasmid containing no library of light chains was used. The phage particles were eluted with the same buffer containing different concentrations of NaCl (0.01–3 M), and then with 50 mM glycine-HCl (pH 2.6) similarly to purification of polyclonal Abs [16,17]. Phage particles were collected, concentrated, and each fraction was precipitated using PEG/NaCl as in [24]. The titers of phage particles were determined (see below) and each fraction was assayed for MBP-hydrolyzing activity using intact MBP and four

oligopeptides (OPs) corresponding to the protein specific immunodominant sequences containing cleavage sites (see below).

Preparation of monoclonal phage particles

For preparation of soluble MLChs we have used *E. coli* HB2151 as in [35-37]. An overnight culture of *E. coli* HB2151 (200 µl) was placed in a flask containing 80 ml of 2YTL medium and this mixture was incubated with shaking at 37°C to $A_{600}=1.0$. The cells were centrifuged for 10 min (4000 rpm), and the pellet was re-suspended in 80 ml of 10 mM $MgSO_4$ for receiving a solution containing cells in initial concentration. The preparation of phage particles eluted from MBP-Sepharose with 0.5 M NaCl was diluted 100-fold in 2YTL, and 10 µl of this solution was added to 90 µl of the *E. coli* cells. The mixture was incubated for 30 min at 37°C and uniformly distributed over a Petri dish with agarized 2YTL containing 40 µg/ml ampicillin; the dish was incubated overnight at 37°C. For further analysis, 72 of 440 individual colonies from two dishes were randomly chosen.

To propagate individual colonies of phages, the material from each colony were grown overnight at 37°C in a Petri dish as described above. The cells were scraped to a vial containing 1.5 ml 2YTL medium supplemented with ampicillin (50 µg/ml), and the mixture was shaken at 37°C to $A_{600}=0.6$. Then 1.5 ml of 2YTL and isopropyl β-D-1-thiogalactopyranoside (IPTG) to the final concentration 2 mM were added, and the mixture was shaken at 37°C overnight. The suspension was centrifuged (1 min, 12000 rpm); the supernatant containing phage particles was collected and used to obtain monoclonal phages.

Purification of monoclonal light chains

Supernatant (50 ml) containing MLCh was dialyzed twice for 3 h at 4°C against 1 ml of H_2O and then overnight against Buffer A consisting of 50 mM Tris-HCl (pH 7.2), 0.5 M NaCl, and 1 mM DTT. The solution obtained was first applied on column with Sephadex G-75 for removal of different hydrophobic compounds and then on a HiTrap chelating Sepharose column (1 ml) charged with Ni^{2+} and equilibrated in buffer A for affinity chromatography [35-37]. After loading MLCh, the column was washed with the same buffer to zero optical density of the eluate. The bound MLChs were eluted with a gradient of imidazole (0-1 M) in 50 mM Tris-HCl (pH 7.2). Optical density was measured in all fractions. The fractions containing MLChs were dialyzed against 20 mM Tris-HCl (pH 7.5) and then concentrated. According of SDS-PAGE analysis these preparations contain small admixtures of several proteins (probably from medium, *E. coli* cells or phage particles) interacting with HiTrap chelating Sepharose. For isolation of homogeneous preparations of MLCh we used gel filtration. FPLC gel filtration of this preparation was performed on a Superdex 200 HR 10/30 column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl as in [16,17,35-37]. Before gel filtration, the MLCh samples were incubated in TBS containing 2.0 M $MgCl_2$ for 30 min at 20°C. The fractions containing [26-27] kDa MLCh were collected. About 0.3-0.8 mg of MLChs was obtained from 1 liter of the medium, depending on the preparation. Only fraction corresponding to the MLChs (containing no proteins admixtures) demonstrated MBP-hydrolyzing activity.

In order to protect the MLChs from bacterial contamination they were sterilized by filtration through a Millex filter (pore size 0.2 µm) and then concentrated in sterile condition and used for analysis. Incubation of standard bacterial medium with MLChs preparations did not lead to a formation of colonies. In this article we have analyzed

in detail only one MLCh (NGTA3-pro-DNase) demonstrating high MBP- and DNA-hydrolyzing activities (see above).

Western blotting and ELISA

The NGTA3-pro-DNase was analyzed by Western blotting onto a nitrocellulose membrane using horseradish peroxidase conjugated with mouse Abs against light chains of human Abs as in [16,17,35-37]. The interaction of the MLChs with MBP was also analyzed using standard ELISA plates with immobilized MBP [16,17,35-37], or double-stranded DNA [18,19]. After adsorption of the MLChs and a consecutive treatment of samples with horseradish peroxidase conjugated with mouse antibodies against light chains of human Abs the reaction mixtures were incubated with tetraethyl benzidine and H_2O_2 . The reaction was stopped with H_2SO_4 and optical density (A_{450}) was determined. The relative content of anti-MBP and anti-DNA MLChs in the samples was expressed as a difference in the relative absorption at 450 nm between experimental and control samples; controls with MBP (or DNA) but without MLChs were used. Controls with DNA but without MLChs as well as with MLChs not interacting with DNA gave the same results.

Proteolytic activity assay

The reaction mixture (10-40 µl) for analysis of MBP- or oligopeptide-hydrolyzing activity of MLCh, containing 20 mM Tris-HCl (pH 7.5), 0.5-1.0 mg/ml (28-56 µM) MBP or 0.33-1.0 mM one of four different oligopeptides, and 0.001-0.01 mg/ml (37-370 nM) of MLCh, was incubated for 0.1-24 h at 30°C. OP-17 (X-ENPVVHFFKNIVTPRTP), OP-19 (X-LSRFSWGAEGQLPGFGYGG), OP-21 (X-YLASASTMDHARHGFLPRRHR), and OP-25 (X-AQGTLKIFKLGGRDSRSGSPMARR) oligopeptides corresponding to four known IgG-dependent specific cleavage sites of MBP [13,16,17] and containing fluorescent residue 6-O-(Carboxymethyl) fluorescein ethyl ester (R) on its N-terminus were used.

The MBP cleavage products were analyzed by SDS-PAGE in 12% or 4-15% gradient gels with Coomassie R250 staining. The gels were imaged by scanning and quantified using GelPro v3.1 software. Finally, the activities of the MLCh preparations were determined as a decrease in the percentage of MBP converted from the initial to hydrolyzed forms taking into account incubation of MBP in the absence of Abs.

The cleavage products of different OPs were separated by TLC on Kieselgel F60 plates using acetic acid-n-butanol- H_2O (1:4:5) system. The plates were dried and photographed. To quantify the intensities of the fluorescent spots after TLC, OPs incubated without MLCh were used as controls. Photographs of the plates were imaged by scanning and quantified using GelPro v3.1 software. All quantitative measurements (initial rates) were taken under the conditions of the pseudo-first order of the reaction within the linear regions of the time courses (15-40% of MBP or hydrolysis of OPs) and dependence of the rate on MLCh concentration.

pH dependencies were analyzed using different buffers (50 mM): MES-NaOH (pH 5.4-6.6), Tris-HCl (pH 6.0-8.6) and glycine-NaOH (pH 9.0-10.0). In some cases, $MgCl_2$, $MnCl_2$, $CuCl_2$, $CoCl_2$, $NiCl_2$, $ZnCl_2$, or $CaCl_2$ at different concentrations were used. For analysis of a possible type of proteolytic activity, MLCh (0.3-0.5 mg/ml) was pre-incubated for 30 min at 25°C with one of specific inhibitors of different proteases: iodoacetamide (10 mM), PMSF (1 mM), or EDTA (10-100 mM), and aliquots of these mixtures were then added to the standard reaction mixture. Substrate specificity of NGTA3- pro-DNase was

analyzed using MBP, human serum albumin, human milk lactoferrin and four different OPs.

DNase activity assay

DNase activity was analyzed using supercoiled (sc) DNA as described earlier for DNase I, DNase II, and human serum catalytic antibodies [9,10]. Initially, the reaction mixtures (20 μ l) contained 18 μ g/ml supercoiled pBluescript DNA, 5 mM MgCl₂, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5), 2 μ l of the phage preparation after chromatography on DNA-cellulose or purified light chain NGTA3-pro-DNase (1-10 nM), and were incubated for 0.5-2 h at 37°C.

Later, the specific optimal conditions were used. Dependencies of the relative DNase activity on the concentration of metal ions were analyzed using KCl, NaCl, MnCl₂, MgCl₂, CaCl₂, CuCl₂, CoCl₂, NiCl₂, or ZnCl₂ (each at 0.005-20 mM).

After incubation 3 μ l of buffer containing 1% SDS, 20 mM EDTA, pH 8.0, 30% glycerol and 0.005% bromophenol blue was added to reaction mixture. Electrophoresis was performed in 0.8% agarose gel until the bromophenol blue migration to 2/3 of the way. DNA gel stained with ethidium bromide (0.5 μ g/ml, 2-4 h). The images of ethidium bromide-stained gels were captured on a Sony DSC-F717 camera and a relative amount of DNA in different bands was analyzed using ImageQuant v5.2 (Molecular Dynamics, GE Healthcare, UK).

Similarly to DNase I and polyclonal abzymes from autoimmune patients [9,10,40,41], depending on the incubation time and type of metal ions, MLCh was capable to hydrolyze plasmid DNA forming single breaks in one strand of supercoiled DNA (relaxed DNA) and then multiple breaks yielding linear DNA. Finally, it hydrolyzed DNA into short and medium-length oligonucleotides.

However, after such a deep hydrolysis of DNA, it was very difficult to estimate relative activity of MLCh. Therefore, to estimate the DNase activity quantitatively, we have found the concentration of MLCh preparation and the time of incubation sufficient to convert scDNA into the relaxed form without further noticeable fragmentation after 0.2-2 h of incubation. Finally, the activities of the MLCh preparations were determined as a decrease in the percentage of DNA converted from the initial supercoiled form to the relaxed form (and sometimes additionally linear form), corrected for the distribution of DNA between these bands in the control (incubation of pBluescript in the absence of Abs).

All measurements (initial rates) were taken within the linear regions of the time courses (15-40% of DNA hydrolysis) and a complete transition of the supercoiled plasmid (18 μ g/ml=6.0 nM) to the nicked form was taken for 100% activity. This approach allows normalization of the relative activity of MLChs and calculation of k_{cat} value.

In some experiments, the MLChs were extensively dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.1 M EDTA and then three times against 1 mM Tris-HCl (pH 7.5); before the experiments, metal ions were removed from all solutions using a 5 ml Chelex column. The RAs of these preparations were assayed as described above using a standard reaction mixture.

Dependency of the activity on pH was analyzed using different buffer systems as described above for analysis of protease activity.

In gel assay of protease activity

Analysis of MBP- and DNA-hydrolyzing activity of NGTA3-pro-DNase after SDS-PAGE was performed similarly to [16-19,35-37]. MLCh (10-15 μ g) was pre-incubated at 30°C for 30 min under nonreducing condition (50 mM Tris-HCl, pH 7.5, 1% SDS, and 10% glycerol). After standard SDS-PAGE electrophoresis of MLCh to restore its MBP-hydrolyzing activity, SDS was removed by incubation of the gel for 1 h at 30°C with 4 M urea and washed 10 times (7-10 min) with H₂O.

Then 2-3-mm cross sections of longitudinal slices of the gel were cut up and incubated with 50 μ l of 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl for 4-6 days at 4°C to allow protein refolding and eluting from the gel. The solutions were removed from the gels by centrifugation and used for assay of MBP and DNA hydrolysis as described above. Parallel control longitudinal lanes were used for detecting the position of MLCh on the gel by Coomassie R250 staining.

In situ analysis of DNase activity

DNase activity of NGTA3-pro-DNase was determined *in situ* after separation of proteins in 4-15% gradient SDS-PAGE gels containing 3 μ g/ml calf thymus DNA as in [18,19,42].

For the analysis of DNase activity (and to remove SDS), the gels were washed at 25°C for 4 h in 40 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100, 4 mM MgCl₂, and 0.2 mM CaCl₂ and then incubated in the same buffer containing 0.1 mM CaCl₂ but without Triton X-100 for 2 h followed with a fresh portion of the same buffer for 1-2 days at 30°C. To visualize the regions of DNA hydrolysis, the gels were stained with ethidium bromide. The parallel longitudinal slices were used to detect the position of the MLCh in the gel by Coomassie R250 staining.

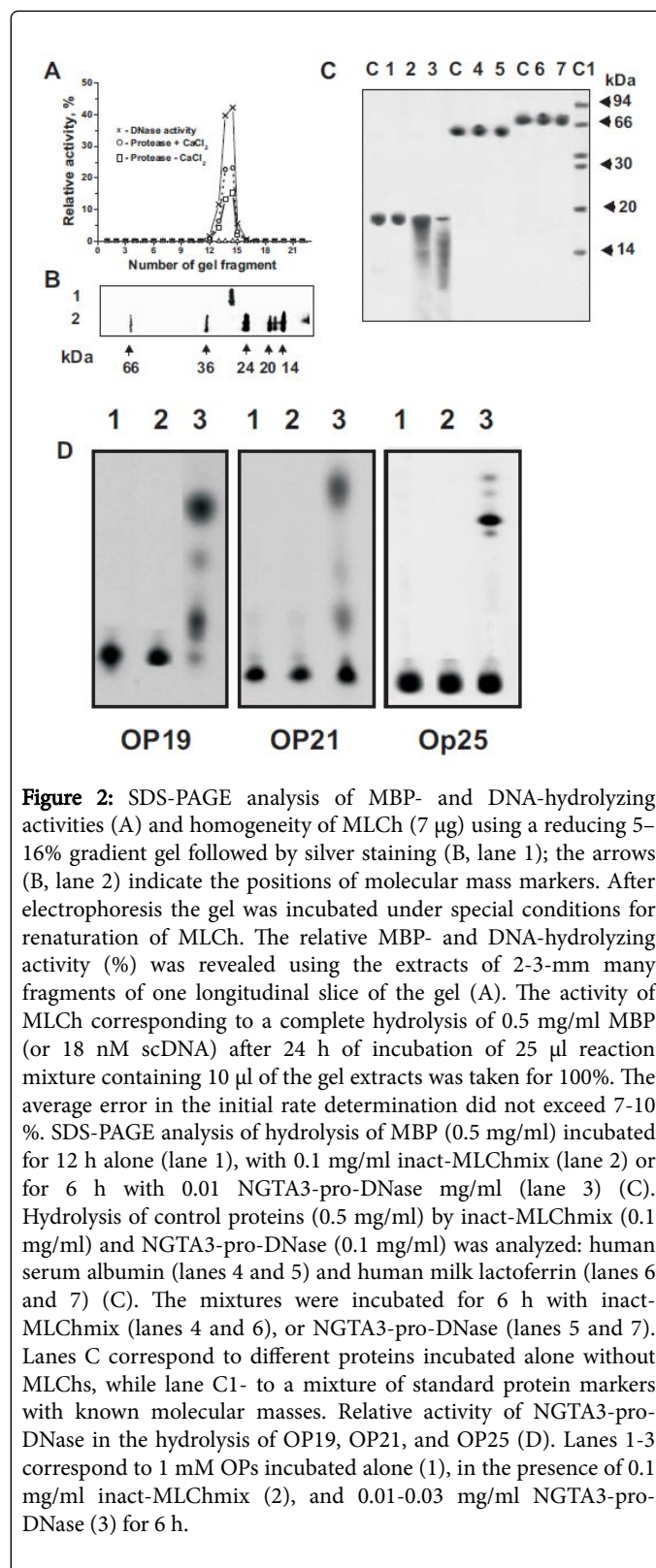
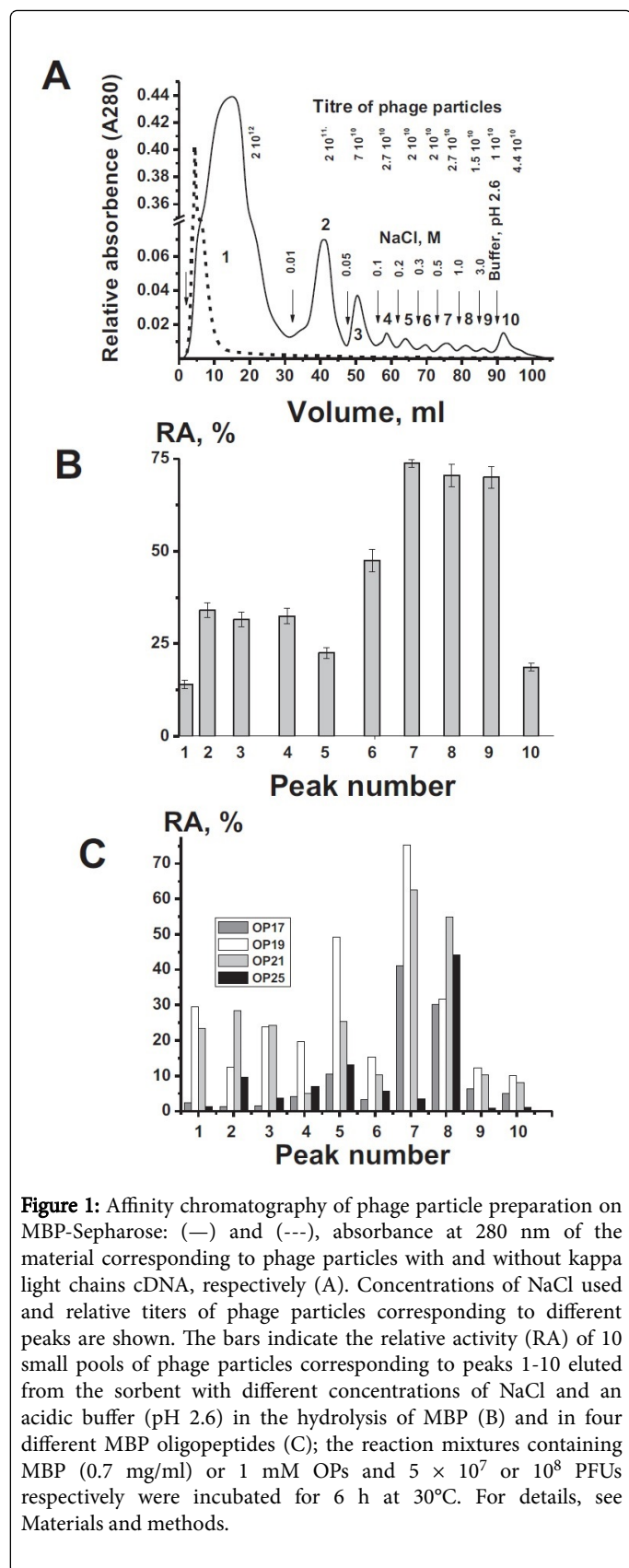
Fluorescence measurements

Fluorescence was measured in a thermostated (25°C) Hitachi MPF-2A spectrofluorimeter. Excitation was performed at 296 nm and fluorescence emission detected at 330 nm. The complex formation mixture contained 20 mM Tris-HCl (pH 7.5), and 0.3 mg/ml MBP. Aliquots (0.2-0.5 μ l) of d (TAGAAGATCAAAA) oligonucleotide were consecutively added to the mixture, and changes in MBP fluorescence spectrum (ΔF) were recorded, with correction for dilution.

The K_d values of MBP: oligonucleotide complexes were calculated from the Scatchard equation $\Delta F = \Delta F_{max} - K_d(\Delta F/[L])$, where [L] is the concentration of free oligonucleotide in the mixture [43]. The estimation error did not exceed 5-10%.

Determination of kinetic parameters

The K_m and V_{max} (k_{cat}) values were calculated from the dependencies of V versus [MBP] or [DNA] by least-squares non-linear fitting using Microcal Origin v5.0 software and presented as linear transformations using a Lineweaver-Burk plot [43]. The concentrations of MBP and DNA were varied. Errors in the values determination were within 8-12%. The results are reported as mean \pm S.E. of at least 2-3 independent experiments.



Results

Affinity chromatography of phage particles on MBP-Sepharose

Polyclonal anti-MBP Abs from the sera of SLE and MS patients, are usually very heterogeneous in their affinity for MBP and can be separated into many subfractions by chromatography on MBP-Sepharose [16,17]. Therefore, we first separated phage particles (*E.coli* TG1) containing a pool of various MLChs with different affinity for MBP by chromatography on MBP-Sepharose (Figure 1A). The complete pool of phage particles containing MLChs bound with MBP-Sepharose were distributed between ten peaks eluted during chromatography (Figure 1A) and all fractions corresponding to new small pools obtained were active in the hydrolysis of MBP (Figure 1B) and its four OPs (Figure 1C). However, we have not observed any detectable protein peaks having remarkable affinity for MBP-Sepharose after similar affinity chromatography of phage particles corresponding to pCANTAB plasmid containing no cDNA of light chains (Figure 1A). It means that the pools of MLChs of all 10 fractions of phage particles with different affinity to MBP contain not only inactive but also catalytically active light chains possessing MBP-hydrolyzing activity.

Preparation of individual homogeneous recombinant MLChs

Individual colonies corresponding to recombinant MLChs have been obtained using *E. coli* HB2151 and phage particles eluted from MBP-Sepharose with 0.5 M NaCl (peak 7, Figure 1A); this fraction demonstrated relatively high affinity and catalytic activity in hydrolysis of intact MBP (Figure 1B) and its four different OPs (Figure 1C). The phage particles of this fraction were growing on two Petri dishes with agar and separated colonies were used for preparation of individual MLChs.

On the end of recombinant MLChs there is a sequence of six histidine residues; this hexapeptide interacts efficiently with Ni²⁺ ions [24]. 72 preparations of monoclonal MLChs corresponding to randomly chosen colonies were purified by chromatography on HiTrapTM chelating Sepharose charged with Ni²⁺ ions and by following gel filtration. Twenty five of 72 recombinant MLChs efficiently hydrolyzed MBP. Twenty-four MLChs were described earlier [35-37]. One recombinant NGTA3-pro-DNase monoclonal light chain (below marked as MLCh-25) demonstrating relatively high MBP-hydrolyzing activity and MLChs of 5 single colonies without activity were used in this article for more detail analysis.

The electrophoretical homogeneity of ~26-27-kDa NGTA3-pro-DNase and control preparation of equimolar mixture of 5 MLChs without activity (inact-MLChmix) was confirmed by SDS-PAGE with silver staining (for example, Figure 2B, lane 1).

Characterization of individual homogeneous recombinant MLChs

It was shown, that MLCh-25 and inact-MLChmix demonstrated positive answer with mouse IgGs (conjugated with horseradish peroxidase) against human Abs light chains at Western blotting and positive ELISA answer using plates with immobilized MBP. NGTA3-pro-DNase was active in the hydrolysis of MBP (Figure 2C) in the absence of external metal ions (lane 2) and in the presence of CaCl₂ (lane 3), while inact-MLChmix preparation did not hydrolyze MBP

(Figure 2C, lane 1). To exclude possible artifacts due to hypothetical traces of contaminating proteases, the MLCh-25 was subjected to SDS-PAGE and its MBP-hydrolyzing activity was detected after extraction of proteins from the separated gel slices (Figure 2A).

In the absence of external metal ions and in the presence of CaCl₂ MBP-hydrolyzing activity was detected only in the band corresponding to the light chains (Figure 2A). Since SDS dissociates any protein complexes, the detection of proteolytic activity in the gel region corresponding only to the MLCh-25, together with the absence of any other bands of the activity or protein (Figure 2), as well as the absence these activities in the case of inact-MLChmix (Figures 2C and 2D) provides direct evidence that recombinant MLCh-25 possess MBP-hydrolyzing activity.

It was shown previously, that polyclonal SLE and MS IgGs purified on MBP-Sepharose hydrolyze only MBP, but not many other tested proteins [11,12,16,17]. Similar situation was observed in the case of abzymes against other proteins and peptides: HIV-1 reverse transcriptase and integrase, human serum albumin, casein, thyroglobulin, and intestinal vasoactive peptide [44-47]. It has been shown, that NGTA3-pro-DNase efficiently hydrolyzes MBP in the absence of external metal ions (Figure 2C, lane 2) and in the presence of 2 mM CaCl₂ (Figure 2C, lane 3).

In the same conditions (in contrast to canonical proteases hydrolyzing all proteins) there was no observed detectable hydrolysis of control non-specific proteins, (Figure 2C, lanes 4-7). In contrast to inact-MLChmix, NGTA3-pro-DNase efficiently hydrolyzed three OPs corresponding to MBP antigenic determinants (OP19, OP21 and OP25) (Figure 2D). At the same time, in contrast to canonical proteases hydrolyzing all peptides, NGTA3-pro-DNase did not efficiently hydrolyze OP17.

Type of proteolytic activity of recombinant MLCh

It was previously shown that, in contrast to polyclonal MS IgGs, MBP-hydrolyzing abzymes from SLE patients are more sensitive to EDTA and less sensitive to PMSF, which is a specific inhibitor of serine-like proteases [16]. Figure 3A demonstrated that NGTA3-pro-DNase is not sensitive to specific inhibitors of thiol-like (iodoacetamide) and acidic-like (pepstatin A) proteases. Preincubation of MLCh-25 containing intrinsic metal ions with specific inhibitor of serine-like proteases (PMSF) leads to decrease in its activity for 67 ± 5%. Human and mammalian intact polyclonal Abs are known to interact with different metal ions and they do not completely lose intrinsically bound metal ions during the standard procedure of their purification [48,49]. The dialysis of MLCh-25 against EDTA or addition of EDTA to MLCh containing only intrinsically bound Me²⁺-ions led to a decrease in its activity for 33 ± 3% (Figure 3A). And average Me²⁺-dependent proteolytic activity of MLCh-25 containing only intrinsically bound Me²⁺-ions was approximately 2.0-fold lower (Figure 2A), but after addition of external Ca²⁺ ions became to be 2.2-fold higher than its serine like activity (Figure 3B).

Dependence of MLCh protease activity upon different metal ions

We have compared the effect of different metal ions on the proteolytic activity of MLCh-25 in the hydrolysis of MBP (Figure 3B). It was shown that in contrast to canonical metal-independent serine-, thiol-, and acidic-like proteases, seven different external metal ions activate NGTA3-pro-DNase in the following order: Ca²⁺ ≥ Ni²⁺ > Co²⁺

$\approx \text{Mn}^{2+} \geq \text{Cu}^{2+} \approx \text{Zn}^{2+} \geq \text{Mg}^{2+}$ (Figure 3B). An optimal concentration of CaCl_2 , which is the best activator of MLCh-25, was 3 mM (Figure 3C). The use of the combinations of Ca^{2+} with other metal ions led to a significant decrease in the metal-dependent proteolytic activity of MLCh as compared with CaCl_2 taken separately (Figure 3D).

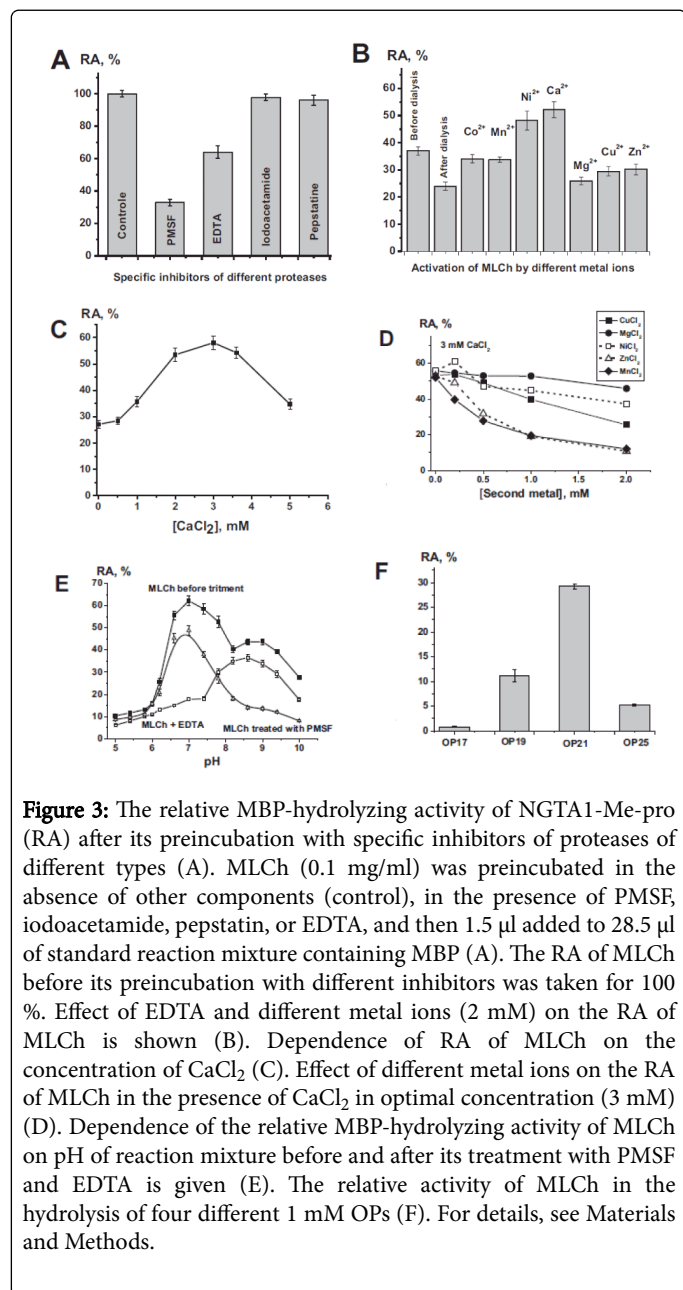


Figure 3: The relative MBP-hydrolyzing activity of NGTA1-Me-pro (RA) after its preincubation with specific inhibitors of proteases of different types (A). MLCh (0.1 mg/ml) was preincubated in the absence of other components (control), in the presence of PMSF, iodoacetamide, pepstatin, or EDTA, and then 1.5 μl added to 28.5 μl of standard reaction mixture containing MBP (A). The RA of MLCh before its preincubation with different inhibitors was taken for 100 %. Effect of EDTA and different metal ions (2 mM) on the RA of MLCh is shown (B). Dependence of RA of MLCh on the concentration of CaCl_2 (C). Effect of different metal ions on the RA of MLCh in the presence of CaCl_2 in optimal concentration (3 mM) (D). Dependence of the relative MBP-hydrolyzing activity of MLCh on pH of reaction mixture before and after its treatment with PMSF and EDTA is given (E). The relative activity of MLCh in the hydrolysis of four different 1 mM OPs (F). For details, see Materials and Methods.

Many known canonical human metalloproteases are calcium-dependent cysteine proteases [50,51] or Zn^{2+} -dependent enzymes [52], while their activity in the presence of other metal ions is absent or significantly lower. MLCh-25 is not cysteine protease demonstrating maximal activity in the presence of Ni^{2+} and Ca^{2+} , but effects of five other metal ions on its activity are to some extent comparable (Figure 3B). In addition, serine-like activity of MLCh-25 is approximately 2.2-fold lower than its Me-dependent activity in the presence of metal ions.

pH optimum MLCh proteolytic activity

In contrast to all human canonical proteases having one pronounced pH optimum, polyclonal catalytic IgGs from the sera of individual MS and SLE patients demonstrate in the MBP hydrolysis quite distinct pH dependencies (from one to four-five pH optima) within a wide range of pH values (5-10) [11,12,16]. Taking into account inhibition of MLCh-25 by PMSF and EDTA it was reasonable to expect a possibility of the existence of two optimal pH values. MLCh demonstrates two optimal pHs (Figure 3E). After treatment of MLCh-25 with PMSF, its metalloprotease activity was maximal at pH 8.6 (Figure 3E). In the presence of EDTA, serine-like protease activity demonstrated pH optimum at 7.0 (Figure 3E). The existence of two optimal pHs might be a consequence of accidental agglomeration of two different clones on Petri dishes. To obtain repeatedly chosen single colonies of phages, the phage material corresponding to this clone was re-grown in Petri dishes and five new single clones were randomly chosen. Purified MLCh preparations corresponding to five new single mono-colonies demonstrated the same two values of pH optima. It means that NGTA3-pro-DNase was single after the first step of its selection and that this recombinant MLCh-25 is characterized by two different pH optima in the hydrolysis of MBP and possess serine-like and metalloprotease activities.

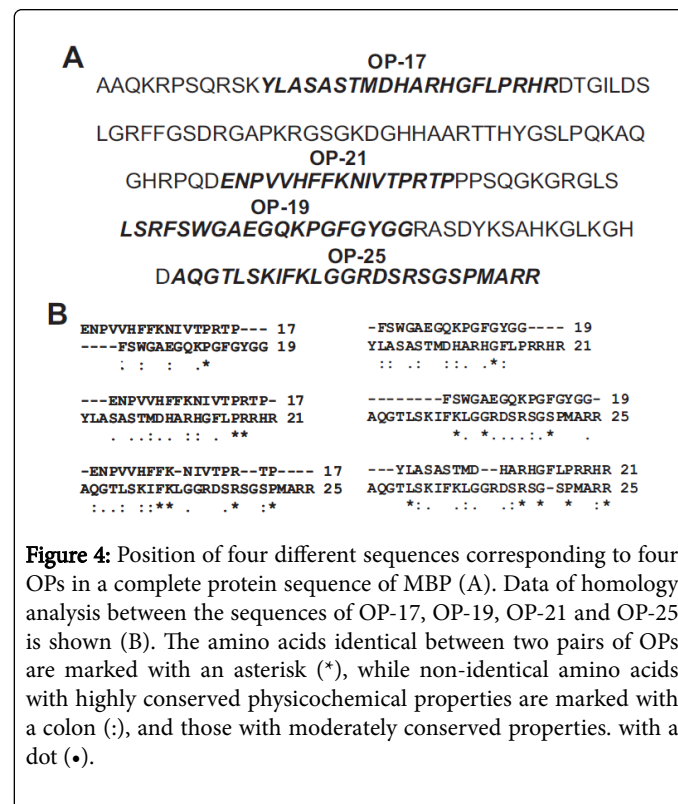


Figure 4: Position of four different sequences corresponding to four OPs in a complete protein sequence of MBP (A). Data of homology analysis between the sequences of OP-17, OP-19, OP-21 and OP-25 is shown (B). The amino acids identical between two pairs of OPs are marked with an asterisk (*), while non-identical amino acids with highly conserved physicochemical properties are marked with a colon (:), and those with moderately conserved properties, with a dot (•).

Protein sequence site specificity of MLCh

Several abzyme-dependent specific sites of intact MBP cleavage are clustered within four known immunodominant regions of MBP [16,17]. It was obviously, that NGTA3-pro-DNase corresponds to Abs against one of four known immunodominant regions containing cleavage sites of MBP. Therefore, it is reasonable to assume, that MLCh-25 will bind and hydrolyze only one of four OPs. However, an unexpected result was obtained. MLCh-25 was to some extent

unspecific and efficiently hydrolyzed three of four MBP oligopeptides; the activity decreased in the following order: OP21>OP19>OP25, while hydrolysis of OP17 was very insignificant (Figure 3F).

Taking into account the absence of an exceptional specificity of MLCh in the hydrolysis of OPs, it was interesting to analyze a possible homology of four OPs corresponding to four different cleavage sites disposed in different parts of MBP (Figure 4A). Figure 4B demonstrates the sequence alignment of four OPs. The similarity between four sequences is not absolute and only some positions fully coincide (marked with an asterisk), while several positions show good (marked with a colon) or moderate (marked with a dot) conservation of physicochemical and structural properties. Thus, MLCh-dependent hydrolysis of free MBP oligopeptides can to some extent be a consequence of their partial homology and possible to similarity of spatial properties.

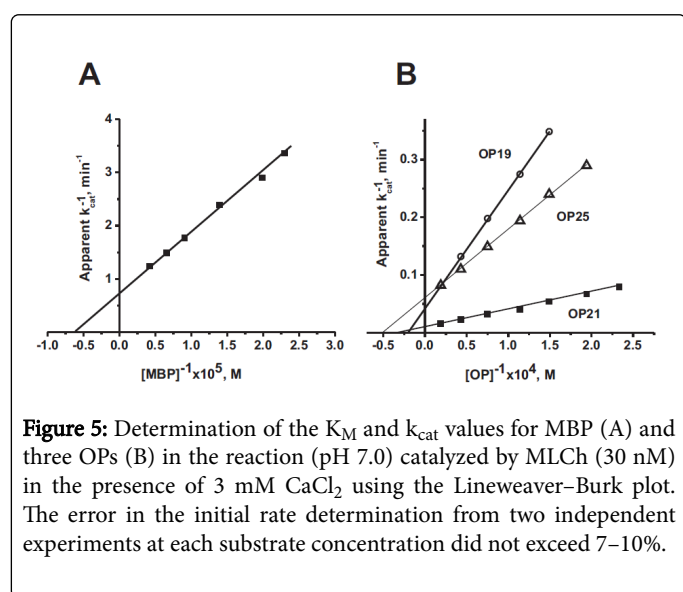


Figure 5: Determination of the K_M and k_{cat} values for MBP (A) and three OPs (B) in the reaction (pH 7.0) catalyzed by MLCh (30 nM) in the presence of 3 mM $CaCl_2$ using the Lineweaver–Burk plot. The error in the initial rate determination from two independent experiments at each substrate concentration did not exceed 7–10%.

Affinity of MLCh for substrates

The MLCh in the presence of 3 mM $CaCl_2$ (pH 7.0) demonstrated K_m for intact MBP ($15 \pm 1.1 \mu M$) and k_{cat} value $0.4 \pm 0.03 \text{ min}^{-1}$ (Figure 5A). The K_m and k_{cat} values for three different OPs were also determined: OP19 ($0.5 \pm 0.02 \text{ mM}$, $25.2 \pm 1.2 \text{ min}^{-1}$), OP21 ($0.3 \pm 0.02 \text{ mM}$, $95.2 \pm 6.2 \text{ min}^{-1}$), and OP25 ($0.2 \pm 0.02 \text{ mM}$, $16.6 \pm 1.2 \text{ min}^{-1}$) (Figure 5B).

DNase activity of MLCh

Twenty four of 72 MLChs efficiently hydrolyze only MBP. Only one of twenty five MLChs (NGTA3-pro-DNase) efficiently hydrolyzed not only MBP, but also DNA (for example, Figure 6A). It was shown, that only NGTA3-pro-DNase of twenty five MLChs demonstrated positive ELISA answer using plates with immobilized DNA with mouse IgGs (conjugated with horseradish peroxidase) against human Abs light chains.

To exclude possible artefacts due to hypothetical traces of contaminating canonical DNases, the purified preparation of NGTA3-pro-DNase was subjected to SDS-PAGE with following analysis of DNase activity of eluates of the gel cross sections similarly to analysis of MBP-hydrolyzing activity. DNase activity was detected only in the band corresponding to the MLCh-25 (Figure 2A). In addition, DNase

activity of NGTA3-pro-DNase was revealed using *in situ* analysis after separation of proteins in gradient SDS-PAGE gels co-polymerized with calf thymus DNA (Figure 6B).

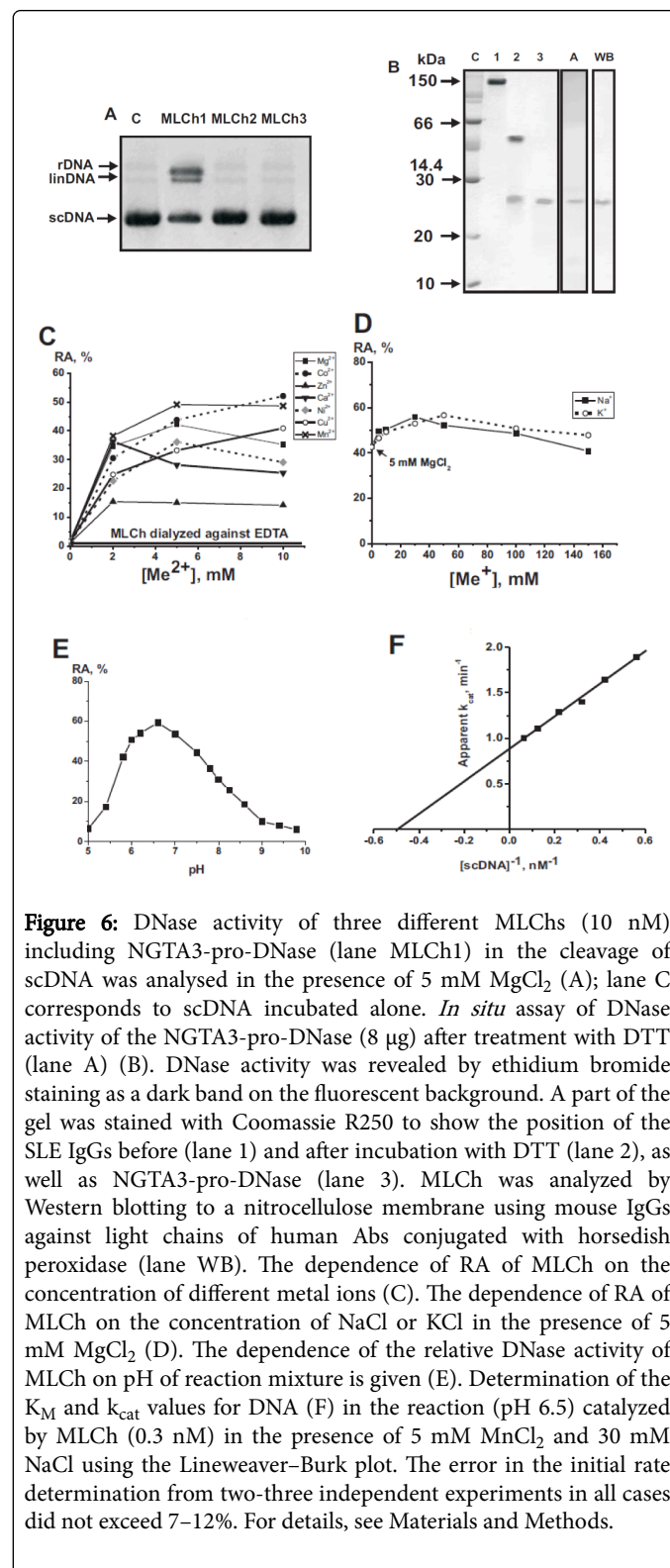


Figure 6: DNase activity of three different MLChs (10 nM) including NGTA3-pro-DNase (lane MLCh1) in the cleavage of scDNA was analysed in the presence of 5 mM $MgCl_2$ (A); lane C corresponds to scDNA incubated alone. *In situ* assay of DNase activity of the NGTA3-pro-DNase (8 μg) after treatment with DTT (lane A) (B). DNase activity was revealed by ethidium bromide staining as a dark band on the fluorescent background. A part of the gel was stained with Coomassie R250 to show the position of the SLE IgGs before (lane 1) and after incubation with DTT (lane 2), as well as NGTA3-pro-DNase (lane 3). MLCh was analyzed by Western blotting to a nitrocellulose membrane using mouse IgGs against light chains of human Abs conjugated with horseradish peroxidase (lane WB). The dependence of RA of MLCh on the concentration of different metal ions (C). The dependence of RA of MLCh on the concentration of NaCl or KCl in the presence of 5 mM $MgCl_2$ (D). The dependence of the relative DNase activity of MLCh on pH of reaction mixture is given (E). Determination of the K_M and k_{cat} values for DNA (F) in the reaction (pH 6.5) catalyzed by MLCh (0.3 nM) in the presence of 5 mM $MnCl_2$ and 30 mM NaCl using the Lineweaver–Burk plot. The error in the initial rate determination from two–three independent experiments in all cases did not exceed 7–12%. For details, see Materials and Methods.

Ethidium bromide staining of the gels after the electrophoresis of the MLCh revealed sharp dark bands against a fluorescent background

of DNA in the gel zone corresponding only to the MLCh-25 and there were no other peaks of proteins or DNase activity (Figure 6B). MLCh demonstrated positive answer at Western blotting to a nitrocellulose membrane using mouse IgGs against light chains of human Abs conjugated with horsedish peroxidase (Figure 6B).

Dependence of MLCh DNase activity on different metal ions

Many pro- and eukaryotic DNases including human DNase I (h-DNase I) [40,41] as well as DNase abzymes from the sera of patients with different pathologies [3-6,34] are Me^{2+} -dependent, while other DNases including DNase II and some subfractions of DNase sIgAs from human milk are Me^{2+} -independent. Therefore, we have analyzed the relative activities (RAs) of the MLCh before and after its dialysis against EDTA; after dialysis the MLCh completely lost the activity, but recovered DNase activity after addition of different external metal ions (Figure 6C). It is known that optimal Me^{2+} -cofactor of DNase I is Mg^{2+} and its optimal concentration is 10 mM [40,41]. Other metal ions very weakly activate DNase I. At the same time, the maximal and nearly the same activity at 2 mM MeCl_2 was observed for Mn^{2+} , Ca^{2+} , and Mg^{2+} (Figure 6C). Optimal concentration for Mn^{2+} , Mg^{2+} , and Ni^{2+} was observed at their 5 mM concentration, while for Ca^{2+} and Zn^{2+} at 2 mM. In addition, the increase in Ca^{2+} concentration higher 2 mM led to the inhibition of MLCh-25 DNase activity (Figure 6C). Interestingly, the DNase activity of MLCh was increased in the presence of Co^{2+} and Cu^{2+} up to 10 mM concentration. In contrast to DNase I, Mn^{2+} and Co^{2+} are the best activators of MLCh-25. MLCh-25 demonstrate a very individual dependence of DNase activity on different metal ions ($\text{Mn}^{2+} \approx \text{Co}^{2+} > \text{Mg}^{2+} > \text{Cu}^{2+} \approx \text{Ni}^{2+} \geq \text{Ca}^{2+} > \text{Zn}^{2+}$), which is completely different in comparison with that for DNases I.

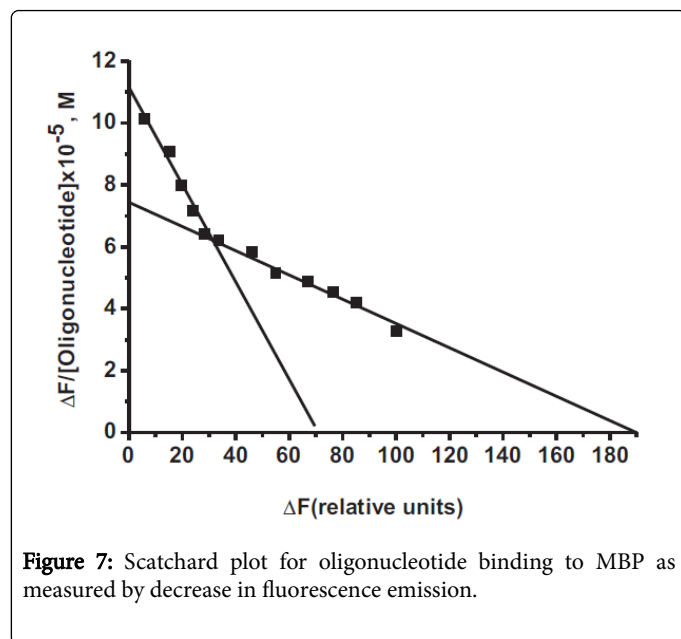


Figure 7: Scatchard plot for oligonucleotide binding to MBP as measured by decrease in fluorescence emission.

It was shown recently, that the optimal concentrations of NaCl or KCl in the case of various MLChs having only DNase activity may be very different and varied in range 10-100 mM [18,19]. Therefore, we have compared the dependences of DNase activity of NGTA3-pro-DNase on concentrations of NaCl or KCl in the presence of Mg^{2+} and Mn^{2+} at fixed concentration (5 mM). One can see that at 30-40 mM of NaCl or KCl the activity of NGTA3-pro-DNase is higher for only 10-15% (Figure 6D). Activation of DNases in the presence of Na^+ and

K^+ is usually associated with optimization of the structures of DNA and enzymes for effective catalysis.

pH optimum of DNase activity of MLCh

The pH optima for various canonical DNases are very different, but each of them usually demonstrates only one pH optimum [40,41]. In contrast to all canonical DNases, pool of polyclonal DNase Abs from the sera patients with different diseases can contain from one to many monoclonal abzymes; some preparations can demonstrate from one to 2-8 pronounced optima at pH range from 5 to 10 [3-6,34]. We have analyzed pH optimum for the MLCh-25 in the presence of 5 mM MnCl_2 and 30 mM NaCl. MLCh-25 demonstrated well expressed pH optima at pH 6.5-6.6 (Figure 6E). Finally using optimal conditions the K_m (2 ± 0.2 nM) for scDNA (and $k_{cat} = (1.1 \pm 0.1) \times 10^{-3} \text{ min}^{-1}$) in the reaction catalyzed by MLCh-25 was determined (Figure 6F).

Discussion

An extreme diversity of polyclonal IgG, IgA, and IgM abzymes in their affinity for MBP and DNA was shown previously using different methods [9-12,16,17,42]. Interestingly, when polyclonal IgGs were eluted from MBP-Sepharose or DNA-cellulose by a NaCl gradient (0-3 M), the Ab optical density and proteolytic activity were distributed all over the chromatography profiles. Several fractions were eluted from both sorbents only with 2-3 M MgCl_2 or with acidic buffer (pH 2.6) in the conditions destroying strong immunocomplexes. We have expected similar situation using chromatography on MBP-Sepharose in the case of separation of phage particles containing kappa light chains on their surfaces. Figure 1A shows the distribution of the phage particles (and their MBP-hydrolyzing activity) all over the profile of the chromatography on MBP-Sepharose. The data are indicative of the extreme diversity of SLE anti-MBP recombinant kappa light chains in their affinity for MBP. In previously published [35-37] and this article for a preparation of individual recombinant MLChs we have used phage particles eluted from MBP-Sepharose with 0.5 M NaCl (Figure 1A). One of 25 MLChs efficiently hydrolyzed not only MBP, but also DNA.

Many known canonical human metalloproteases are calcium-dependent cysteine proteases [50,51] or Zn^{2+} -dependent enzymes [52], while their activity in the presence of other metal ions is absent or significantly lower. MLCh-25 is not cysteine protease demonstrating maximal activity in the presence of Ca^{2+} and Ni^{2+} , but effects of five other metal ions on its activity are to some extent comparable (Figure 3B). In addition, serine-like activity of MLCh-25 is approximately 2.2-fold lower than its Me-dependent activity in the presence of Ca^{2+} Ni^{2+} ions.

All known canonical mammalian proteases have one pronounced pH optimum. It was shown, that after the treatment of MLCh-25 with PMSF pH optimum of metalloprotease activity corresponds to 8.5 (Figure 3E). After suppression of Me^{2+} -dependent activity by EDTA, maximum of serine-like protease activity was observed at 7.0 (Figure 3E) and both pHs were different in comparison with human trypsin and chymotrypsin (7.8-8.0).

It is known, that canonical proteases efficiently hydrolyze all proteins. After the separation on MBP-Sepharose polyclonal SLE and MS IgGs hydrolyze only MBP (see above). MLCh-25 hydrolyzes efficiently only MBP and three of four different OPs corresponding to four different immunodominant regions of MBP (Figure 3F). Since specific inhibitors of different proteases except PMSF and EDTA did

not decrease remarkably MBP-hydrolyzing activity of MLCh (Figure 3A), it may be considered as unusual protease specifically recognizing and hydrolyzing only MBP due to two alternative active centers with serine-like and metalloprotease activities.

The affinity of polyclonal SLE and MS IgGs to MBP varied from 0.1 to 1 μM [6,16,17], while affinity of MLCh-25 to MBP 10-100-fold lower (15 μM) (Figure 5A). In addition, affinity of three different OPs (0.2-0.5 mM) to MLCh is about 13-30-fold lower than that to MBP (Figure 5).

The catalysis mediated by artificial abzymes is usually characterized by relatively lower reaction rates than for canonical enzymes [3-6,34]. The known k_{cat} values for natural abzymes from autoimmune patients catalyzing many different chemical reactions vary in the range of 1×10^{-5} -40 min^{-1} [3-6,34,53] and refs therein). NGTA3-pro-DNase demonstrated relatively high k_{cat} value in the hydrolysis of OPs [16.6-95.2 min^{-1}] and lower in the hydrolysis of OBM (0.4 min^{-1}). Importantly, globular molecules of intact IgGs interact with variable parts of both heavy and light chains of abzymes [3-6,34]. At the same time, the catalytic centers of abzymes, including proteolytic ones, are usually located on the light chain, while the heavy chain is mainly responsible for the specific antigen recognition and the increased antigen affinity for abzymes. The separation of the light chains leads to decrease in the substrate affinity and in the lifetime of the existence of the complex and, as a consequence, to an increase in the turnover number and V_{max} (k_{cat}) of the reaction [3-6,34]. As it was shown previously, short oligopeptides interact mostly with the light chain of intact Abs, which possesses a 100-1000-fold lower affinity for intact proteins [17-53]; similar situation was observed for MLCh-25. Higher affinity and lower k_{cat} for MLCh-25 in the case of MBP may be due to some specific additional contact of light chain with MBP in comparison with OPs.

It is obviously, that MLCh-25 should correspond to Abs against only one antigenic determinant of MBP. At the same time, it efficiently hydrolyzes three of four OPs corresponding to MBP (Figure 3F). It was shown that the hydrolysis of nonspecific OPs corresponding to MBP by anti-integrase abzymes of HIV infected patients is caused by a partial homology between sequences of oligopeptides and several fragments of integrase [54]. Figure 4B demonstrates that four different OPs of MBP are to some extent homologous. Since OP21 is the best substrate one can propose that MLCh correspond to Abs against this sequence of MBP. At the same time, MLCh efficiently hydrolyzes only three of four OPs (Figure 3F). Probably, that not only partial homology, but also spatial structures of free OPs of the same oligopeptides within globular proteins can play important role in their cleavage by abzymes against MBP. One cannot, that in spite of comparable homology between four OPs (Figure 4B), spatial structure of free OP17 may be not optimal for cleavage in comparison with that for free OP21.

The most unexpected result is that in addition to the two types of proteolytic activity, MLCh-25 also possesses DNase activity. It was shown that DNase activity is intrinsic property of NGTA3-pro-DNase (Figure 6A). The K_{m} and k_{cat} values for polyclonal Abs against DNA are usually varied in the ranges 0.5-10 nM and 10^{-5} -40 min^{-1} , respectively [3-6,34,53]. The affinity of MLCh for scDNA turned out to be unexpectedly high, $K_{\text{m}}=2.0 \pm 0.2$ nM, and as a consequence the k_{cat} was relatively low (1.1 ± 0.1) $\times 10^{-3}$ min^{-1} . The affinity of scDNA for MLCh-25 (in terms of K_{M} values) is about 3.5 orders of magnitude higher than affinity of scDNA for DNase I ($K_{\text{M}}=46$ -58 μM) [53], while k_{cat} is significantly lower (k_{cat} for DNase I is approximately 1.0×10^5 min^{-1}). The question is why MLCh-25 against MBP can hydrolyze

DNA. It is believed that MBP and anti-MBP Abs cannot interact with DNA or RNA. It was recently shown that anti-MBP Abs efficiently interact with nucleic acids [55]. It should be noted, that MBP is a very hydrophobic protein. Therefore, we did not exclude, that MBP can also interact with different molecules including nucleic acid. Quenching of MBP tryptophan fluorescence emission decrease was used for the estimation of the MBP affinity for oligonucleotide. Figure 7 demonstrates Scatchard plot of the dependence of relative decrease in fluorescence on the concentration of oligonucleotide. Interaction of pure MBP with oligonucleotide is characterized by two Kd values: 65 ± 5 and 250 ± 20 μM . It means that MBP in principle can interact with DNA. In addition, one cannot exclude that the affinity of MBP as a component of axons myelin can be higher due to interaction of DNA with other components of myelin (different proteins, oligosaccharides, and lipids) contacting with MBP.

It is possible to suggest that 24 of 25 MLCh interacting only with MBP correspond to Abs directly against this protein [35-37], while NGTA3-pro-DNase may be against the complex of MBP with DNA. In the latter case, it is impossible to exclude possibility of a formation of the chimeric MLChs possessing affinity for MBP and for DNA, and also hydrolyzing these absolutely different substrates.

Three different activities of MLCh-25 were extremely unpredictable, but this phenomenon may be important for the further development in studies of the yet unknown possible biological functions of immunoglobulins.

Acknowledgments

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