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IDENTIFICATION OF EPO-Fc FUSION PROTEIN BY MEANS OF POLYACRYLAMIDE GEL-ELECTROPHORESIS WITH ISOELECTROFOCUSING (IEF-PAGE) AND IN PRESENCE OF SODIUM DODECYLSULPHATE (SDS–PAGE)/ LAUROYLSARCOSINATE FOR THE PURPOSE OF ANTI-DOPING CONTROL*

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The article is devoted to develop of an approach for the identification of new stimulator of hematopoiesis, EPO-Fc fusion protein, which is banned by the World Anti-doping Agency (WADA) to use by athletes since it has become doping. Existing methods of qualitative determination of this substances in routine practice of antidoping laboratories such as polyacrylamide gel-electrophoresis in presence of sodium dodecylsulphate (SDS-PAGE) or lauroylsarcosinate (SAR-PAGE) are insufficiently specific. The article shows the principal possibility of identification of EPO-Fc fusion protein by means of IEF-PAGE in carrier ampholyte-based gels with a pH range 2–6 after Fc-fragment removal via fermentative hydrolysis.It has been shown that the removing of the crystallizable fragment leads to decrease of molecular weight of whole hybrid molecule and to increase its electrophoretic mobility that allows to detect this banned substances with high specificity by existing methods. During the study the enzyme for hydrolytic cleavage and optimum conditions of hydrolysis of EPO-Fc in serum samples were selected.

Keywords: *EPO-Fc fusion protein, fermentative hydrolysis, electrophoresis, EPO-hinge fragment, antidoping control.*

Introduction

Recently the interest in studying fusion proteins EPO-Fc, new stimulators of blood formation, as potential means for treating people suffering from chronic renal failure and various forms of anemias rises more and more. These compounds have a number of improved pharmacokinetic and pharmacodynamic properties as compared to other famous erythropoiesis-stimulating agents (ESA). Now they are produced by a number of biotechnological companies only for research purposes. However, they are available in the "black" market. So, these preparations can be used by athletes along with other types of erythropoetins (EPO) in hardy sports for increasing erythrocytes production and, as a result, increasing the oxygen capacity of blood.

Fusion proteins EPO-Fc consist of one or two molecules of EPO hormone conjugated with the dimeric Fc-part of human IgG1, IgG2 or IgG4 including the hinged area, CH2 and CH3 domains [1–3]. Their molecular weight is from 110 to 130 kDa [2], which exceeds the limit of kidney filtration. Therefore, blood serum is a preferable matrix for their detection. The EPO and

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Fc parts can be interlinked directly or by means of a flexible peptide linker [4]. The full amino-acid sequence of such proteins is the property of biotechnological manufacturing firms and is not completely known.

The use of EPO-Fc proteins in sport is strictly regulated by the Forbidden List of WADA, in which they were included in 2012 according to article S2 "Peptide Hormones, Growth Factors, Substances Similar to Them and Mimetics" [5]. In order to determine them in blood serum samples, modern drug tests use qualitative analysis methods, such as polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and of sodium lauroyl sarcosinate (SAR-PAGE) [6–9]. However, these methods are insufficiently specific, because serum is a complex matrix and contains a large amount of proteins with molecular weights similar to that of EPO-Fc. This can result in the emergence of additional protein bands in the region of fusion protein detection due to nonspecific interaction with antibodies used in the analysis. In order to determine some blood formation stimulators (Darbepoetin or Mircera) the IEF-PAGE method with a gradient pH 2–6 [6, 9] is used, because these medicines have unique isoelectric profiles other than those of endogenous EPO. However, this method is inapplicable for detecting EPO-Fc because of bad separation of the later into separate isoforms [10].

This work is devoted to estimating the influence of enzymatic elimination of the Fc part on EPO-Fc electrophoretic characteristics and continues previous studies [11]. Among enzymes most often used for antibodies fragmentation we chose IdeS protease specific to human IgG. IdeS cleaves the amino-acid sequence after the Gly236 residue of the IgG hinged area of all subclasses. It is shown that removing the Fc part can improve EPO-Fc separation into discrete isoforms by the IEF-PAGE method with pH 2–6 gradient and increase the specificity of detection by the existing SDS/SAR-PAGE methods.

Experimental

Materials

All solutions and buffers were prepared with the use of deionized water. A 30% solution of acrylamide/bis, methyl red, morpholine-4-propanesulfonic acid (MPSA), phenol red, tris(hydroxymethyl)aminomethane hydrochloride (Tris hydrochloride), EDTA (acid), D,L-dithiothreitol (DTT), saccharose, 85% orthophosphoric acid, kerosene, bovine serum albumin (BSA), *N*- sodium lauroyl sarcosinate (sarcosyl) and human zero serum were purchased from Sigma-Aldrich (USA).

Urea, GelBond substrates for polyacrylamide gel, clips, frames and glasses for the gel (125×260×1.0 mm), tris(hydroxymethyl)aminomethane (Tris base), glycine, electrode strips, blotting paper (IEF), sodium dodecyl sulfate (SDS), ammonium persulphate, electrode paper, system for electrophoresis (Multiphor II) and a current source (EPS 3500 XL) for the system were purchased from GE Healthcare (Sweden).

N,N,N',N'-Tetramethylethylenediamine (TEMED), 12% Bis-Tris precast gels (18 holes), Criterion® cell for electrophoresis, extra blotting paper (Criterion format, 86×135 mm), CoomassieBio-SafeR-250 reagent for proteins coloring, XT buffer, XT reducing agent, XT MOPS buffer for electrophoresis, a device for semidry transfer of proteins (TransBlot SD), a current source (PowerPac HC) for all Western blots, fat-free powdered milk, Precision Plus Protein™ Dual Color precolored standards of molecular weights and ChemiDoc MP imaging system were ordered from Bio-Rad (USA).

Ultrafiltration equipment (Amicon Ultra-0.5, maximum molecular weight 30 kDa), HPF Millex-HV filter elements, polypropylene syringes (20 ml), polivinylidenedifluoride (PVDF) membranes for Western blotting, Immobilon-P and Durapore, Immobilon™ Western Chemiluminescent HRP substrate, plastic syringes (50 ml) and a vacuum pump were purchased from Millipore corporation (USA).

QIAvac Plus 24 System was purchased from Qiagen (Germany).

Monoclonal antibodies were purchased from R&DSystems (anti-EPO, AE7A5 clone; USA). Biotinylated goat anti-mouse antibodies (H+L), streptavidin conjugated with horseradish peroxidase, peroxidase conjugate stabilizer, Tween-80 10% solution, Super Signal®West Femto Maximum sensitivity chemiluminescent substrate and NanoDrop 2000cUV-Vis spectrophotometer were received from ThermoScientific/Pierce company (USA).

IdeS-protease (5000 units) was purchased from Promega corporation (USA). Papain was received from Ferak company (Germany). Ampholytes for isoelectric focusing in polyacrylamide gel (Servalyt 2–4, 4–6, and 6–8) and pepsin (30 units/mg) is acquired from Serva company (Germany).

Glacial acetic acid and methanol were purchased from Merck (Germany). Sodium metabisulphite was acquired from Acros Organics (Belgium). Anhydrous glycerin, from Appli Chem GmbH (Germany). Immunoaffine purification set based on anti-EPO antibodies was purchased from MAIIA Diagnostics (Sweden). Tablets for preparing phosphatic buffer (PB) solution were ordered from Amresco (USA).

Human endogenous erythropoietin standard (international reference medicine) was received from National Institute of biological standards and controls (NIBSC, Great Britain). Recombinant human erythropoietin (BRP-EPO, batch 3.3) was acquired from Council of Europe (France). Preparations of recombinant EPO, darbepoetin-alpha (NESP, Aranesp™) and Mirtsera™ (CERA) produced by pharmaceutical companies Amgen (USA) and Hoffmann-la Roche (Switzerland), respectively, were used.

Recombinant human EPO-alfa/Fc fusion proteins were acquired from the pharmaceutical companies Gen Script (USA) and Cell Science Technology (USA).

Methods

Polyamide gel formation for IEF-PAGE

In order to prepare the gel, Servalyt ampholyte 2–4 (1.9 ml), Servalyt 4–6 ampholyte (1.9 ml), a 30% solution of acrylamide/bis (6.32 ml) and deionized water (14.17 ml) were added to urea (14.71 g) and saccharose (1.75 g) samples. The mixture was stirred in a beaker in vacuum for 15 min. TEMED (31 μl) and a 10% of solution of ammonium persulphate (310 μl) were added, and the mixture was immediately poured into a preliminarily prepared frame as described above [12].

IEF-PAGE EPO-Fc before and after enzymatic hydrolysis

0.5 M orthophosphoric acid and a 2% solution (w/v) of Servalyt 6–8 ampholyte, respectively, were used as an anolyte and a catholyte for the application on electrode strips. Kerosene was used for contact between the gel and the cooling platform of Multiphor II cell. In order to compare the isoelectric profile before and after EPO-Fc hydrolysis with IdeS protease with profiles of other preparations of recombinant EPO, 1 mkg of fusion protein preparation was incubated with IdeS in 50 μl of a 0.1% BSA/PB solution for 1 h (37°C, 450 rpm) in Eppendorf

Comfort thermomixer (Germany). The protease/protein ratio was as recommended by the producer [13]. Then the sample was diluted with the same solution to a concentration of 0.1 ng/μl (1 mkg of non-hydrolyzed preparation was also diluted to a concentration of 0.1 ng/μl). After this 12 μl samples were transferred into 0.5 ml test tubes. A 10% solution of Tween-80 detergent (1.2 μl) was added and applied into the polyacrylamide gel holes near samples of other rEPO preparations used as positive controls (0.2 ng per track in 0.1% of BSA/PB) and endogenous EPO. After prefocusing at constant tension (250 V, 30 min, 8°C, distance between electrodes: 10 cm) the gel was subjected to isoelectric focusing at 8°C and a constant electric current power of 25 W to attain in total 3600 volt-hours (3–3.5 h). Besides, a 10% solution of Tween-80 (1:10) was added to all rEPO samples. A 0.1% BSA/PB solution was used to dilute all the preparations. After isoelectric focusing the proteins were transferred to the Immobilon-P PVDF membrane.

SDS-PAGE and SAR-PAGE EPO-Fc before and after fermentative hydrolysis

Electrophoresis in the presence of sodium dodecylsulfate and lauryl sarcosinate was carried out as described in [14, 15] with little changes. In order to determine the electrophoretic mobility and molecular weight of EPO-Fc hydrolyzed by IdeS protease, 1 mkg of the preparation was placed in 50 μl of the PB solution in the ratio recommended by the producer [13] and incubated for 1 h (37°C, 450 rpm). Then the sample was diluted with deionized water to a concentration of 0.1 ng/μl and applied on the gel (0.4 ng per hole) together with other rEPO preparations (0.2 ng per hole, prepared with the use of deionized water). A 4-fold buffer solution of the sample [14] was added to the samples to obtain a 1-fold (in case of SAR-PAGE analysis method) or ready XT buffer solution of the sample (in case of SDS-PAGE). The samples were heated in reducing conditions (95°C, 5 min, 450 rpm, 100 mmol of DTT in case of SAR-PAGE and XT-reducing agent in case of SDS-PAGE) in 0.5 ml Eppendorf test tubes [14, 15]. Buffer solutions for electrophoresis were prepared on the basis of MOPS (SAR-PAGE: 50 mmol of MOPS, 50 mmol of Tris base, 0.1% sarcosyl and 1 mmol of EDTA; SDS-PAGE: ready 20-fold commercial XT MOPS buffer solution). A solution of sodium metabisulphite in water was added to the catholyte as an antioxidant to attain a final concentration of 5 mM (SAR-PAGE method). Gel electrophoresis was carried out with the use of an electrophoretic Criterion™ cell and PowerPac current source (Bio-Rad, USA) at a constant tension (200 V) for 50 and 55 min for the analysis by SAR-PAGE and SDS-PAGE, respectively. In all the experiments 12% Bis–Tris precast polyacrylamide gels were used.

Double Western-blotting and visualization

After electrophoresis in the presence of sodium dodecyl sulfate and sodium lauroyl sarcosinate the gel was treated with Bjerrum buffer solution (incubated for 3×10 min) on a Promax 1020 shaker with a heated Incubator 1000 block (Heidolph, Germany) [16]. After the analysis by IEF-PAGE the modified Towbin buffer solution (50 mM Tris-glycine buffer solution, pH 8.3, without methanol) was used for this purpose. The gel was kept in this solution for at least 3 min. Membranes cut off according to the gel size and blotting paper were soaked in a Tris-glycine buffer solution (25 mM, pH 8.3, without methanol). Then the proteins were transferred onto an Immobilon-P PVDF membrane at constant current (1 mA/cm^2, 1 h in case of SDS/SAR-PAGE and 30 min in case of IEF-PAGE). One block of Criterion blotting paper and 4 layers of thin electrode paper were placed at each side of the Immobilon-P/Durapore/gel "sandwich". After electrotransfer the Immobilon-P membrane (blot-I) was incubated in a 5 mM DTT solution in PB (37°C, 60 min.). Then the blot was blocked for 1 h in a 5% OM/PB solution, incubated in 1% OM/FB solution containing anti-EPO mouse antibodies (R&D Systems, AE7A5 clone, 1:1000 titer v/v, 37°C, 1 h), washed in PB (3×1 min, 3×10 min), and bound anti-EPO mouse antibodies were electrotransferred onto another PVDF membrane (blot-II) in acidic conditions (0.8 mA/cm^2, 10 min) as described by Lasne et al. [17]. Then blot-II was washed in PB (3×1 min), blocked for 1 h in a 5% OM/PB solution, incubated in a 1% OM/PB solution

containing biotinylated goat anti-mouse antibodies (1:12500 titer v/v, 2–8°C, overnight), washed PB (3×1 min, 3×10 min) and incubated in a 1% OM/PB solution containing a streptavidin conjugate of horseradish peroxidase (1:256 titer v/v, 1 h, 37°C). Finally, the membrane was washed in PB (3×1 min, 3×10 min) and incubated for 5 min in a chemiluminescent substrate (Super Signal® West Femto Thermo Scientific and Immobilon™ Western Chemiluminescent HRP substrate for SDS/SAR-PAGE and IEF-PAGE methods, respectively). ChemiDoc MP gel documenting (Bio-Rad, the USA) system was used for the chemiluminescent signal visualization and registration.

Choosing the optimal enzyme for Fc part elimination

In order to choose the most specific enzyme for the fusion protein hydrolysis we carried out SDS-PAGE followed by coloring with Coomassie R-250 solution. To prepare 4 samples into 7 μl of 50 mM Tris-HCl buffer solution, a solution of EPO-Fc (3 μl, 3 mkg) was added. IdeS protease (in the ratio recommended by the producer), papain (5 units) and pepsin (5 units) were added to three of the solutions. Glacial acetic acid (1 μl) was added to the sample containing pepsin, because the enzyme is active only in acidic media. Samples of IdeS protease, papain and pepsin were prepared separately. For this purpose, 20 units of IdeS, 10 units of papain and 10 units of pepsin were added to 9 μl of 50 mM Tris-HCl buffer solution. A 4-fold XT buffer solution of the sample was added to each of the samples until obtaining a 1-fold solution. The mixtures were heated under reducing conditions (95°C, 5 min, 450 rpm, the XT-reducing agent to obtain a 1-fold solution). All the samples were transferred into holes of precast 12% Bis-Tris gel with the samples of preliminarily colored molecular weight standards (7 μl per gel hole). A ready 20-fold XT MOPS buffer solution (diluted to 1-fold) was used as an electrode buffer solution for SDS-PAGE. Electrophoresis was carried out at a constant tension (200 V) for 55 min. The gel was washed with deionized water (3×5 min) and incubated in Coomassie R-250 solution for 45 min. The gel was washed with deionized water (5×10 min), and the colored protein strips were visualized with the use of gel-documenting ChemiDoc MP system (Bio-Rad, the USA).

Choosing the optimal conditions of EPO-Fc hydrolysis in blood serum samples

Blood serum contains endogenous immunoglobulins, the object of IdeS protease hydrolysis. They can compete with EPO-Fc and reduce the reaction efficiency. So, it is necessary to add a surplus of the protease and to measure the general protein concentration in the sample after immunopurification. In order to determine the optimal IdeS/general protein ratio EPO-Fc (0.3 ng) was added into 500 μl of blood serum samples (9 samples), and immunopurification was carried out with the use of EPO Purification Detection kits produced by MAIIA Diagnostics (Sweden) and vacuum system QIAvac 24 (Germany) [18, 19]. The buffer from the kit was used for elution. Then the samples were neutralized with buffer A, concentrated with the use of Amicon 0.5 Ultra devices (20 min, 20000 g), and the total aquantity of protein in the eluate was measured by means of a microvolume NanoDrop 2000 spectrophotometer in the Protein A280 mode (light absorption at wavelength 280 nm, UV region). After this IdeS protease was added (1, 2.5 and 5 units per 1 mkg of general protein). The mixture was incubated in a thermomixer (37°C, 450 rpm) for 30, 60 and 120 min. Eluates were analyzed by SAR-PAGE followed by double western blot and chemiluminescent detection as described above.

Results and Discussion

Choosing an enzyme for EPO-Fc hydrolysis

Now scientists most often use streptococcal pyrogenic endotoxin B (SpeB proteinase) [20–22], papain [23], pepsin [24] and IdeS protease [25–28] for the fragmentation of antibodies and preparation of stable $F(ab`)_2$ and Fc fragments. We will discuss further in detail abilities of each of the above enzymes.

The cysteine proteinase produced by streptococci of serogroup B (SpeB) – causative agents of various skin infections, soft tissues and mucous membranes in humans – was the first proteinase used for the hydrolysis of monoclonal antibodies [29]. It is applied for proteolytic cleavage of heavy IgG chains into F(ab`)$_2$ and Fc fragments [20, 21]. Previously, it was considered that SpeB cleaves IgG between the amino-acid residues Gly236–Gly237 of the hinge region distinguishing 237GPSVFLFP244 [20, 21]. However, later works [22] prove that SpeB hydrolyzes the bond between the amino-acid residues Thr225–Cys226 and only of IgG1 subclass (Figure 1, blue frame), which results in the formation of two Fab and Fc fragments. In addition, the IgG2 and IgG3 cleavage site is not known, and IgG4 is not hydrolyzed at all. SpeB cleaves immunoglobulins of all other classes IgM, IgA, IgD, IgE [20, 25] and some proteins: vitronectin, fibrin, fibrinogen etc. [20]. Thus, SpeB has no strict specificity to IgG and cannot be used for cleaving EPO-Fc, the structure of which includes an Fc part of IgG2 or IgG4.

Other proteolytic enzymes widely used for the structural analysis of antibodies are pepsin and papain [23, 30]. Pepsin effectively hydrolyzes peptide bonds between hydrophobic and aromatic amino acids Phe, Trp, Leu, more rare Met, Glu, and it has wide specificity.

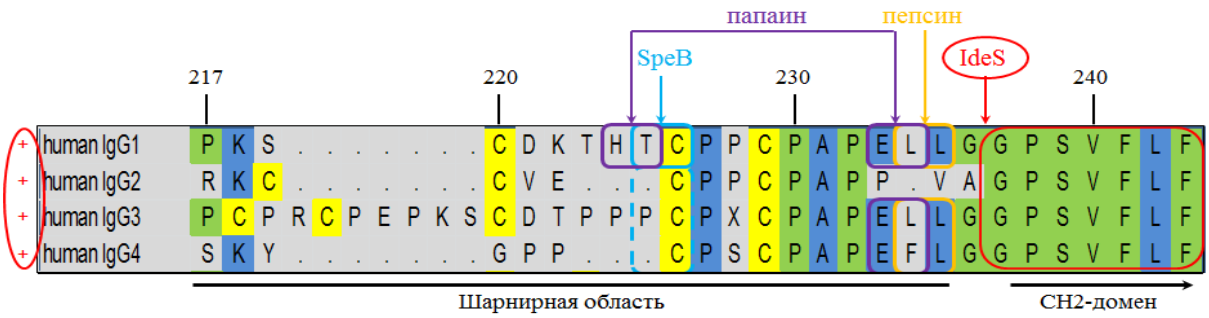

Figure 1. The amino-acid structure of hinged regions of human IgG of various subclasses and the sites of SpeB, pepsin, papain and IdeS cleavage. The red frame shows the IdeS protease recognition site that is present in all subclasses of human IgG. The blue frame indicates the cleavage of the peptide bond between the amino-acid residues upon SpeB hydrolysis; the yellow frame, upon hydrolysis by pepsin; the violet one, by papain.
[папаин means papain; пепсин means pepsin;
Шарнирная область means Hinge region; домен means domain]

Pepsin cleaves the Phe234–Leu235 and Leu234–Leu235 peptide bonds of IgG1 and IgG4 molecules [30] (but not of IgG2) replacing the amino acids in the same sites Val234 and Ala235 (Figure 1, yellow frame). Pepsin is active in acidic conditions and is sensitive to pH change. Papain affects the peptide bonds between Gly233 and Leu234, and also between the amino acids showing basic properties. According to several works [23, 30] the enzyme cleaves IgG at two sites: first His224–Thr225 resulting in the formation of two Fab and one Fc fragment, then Glu233–Leu234 (Figure 1, violet frame). Only studies concerning mouse antibodies [31] and human IgG1 [23, 30] were carried out. Besides, these are not the only sites of hydrolysis. As the time of hydrolytic influence increases, cleavage at other sites can occur. It was established that IgG1 is cleaved at two sites, IgG3 and IgG4 – only at Glu233–Leu234 site, and IgG2 hydrolysis proceeds extremely inefficiently [32]. Besides, it is necessary to control and optimize the hydrolysis conditions for each subclass of IgG separately to provide a high yield of reaction products. Pepsin and papain give various reaction products depending on the duration of hydrolytic influence, and they can cleave EPO-Fc into low molecular weight peptides. Despite some positive aspects of using these enzymes, such as availability and low price, they do not

have the required selectivity and specificity: they cleave many other blood serum proteins. Because the structure of the fusion protein may contain Fc parts of different IgG subclasses, these enzymes cannot be used for EPO-Fc hydrolysis.

The cysteine IdeS protease isolated from *Streptococcus pyogenes* has strict specificity to human IgG [25, 27]. It selectively cleaves the molecules of all IgG subclasses into stable $F(ab`)_2$ and Fc fragments distinguishing 237GPSVFLFP244 in the structure of the fusion protein of the hinged region (Figure 1, red frame). The hydrolysis process is schematically shown in Figure 2.

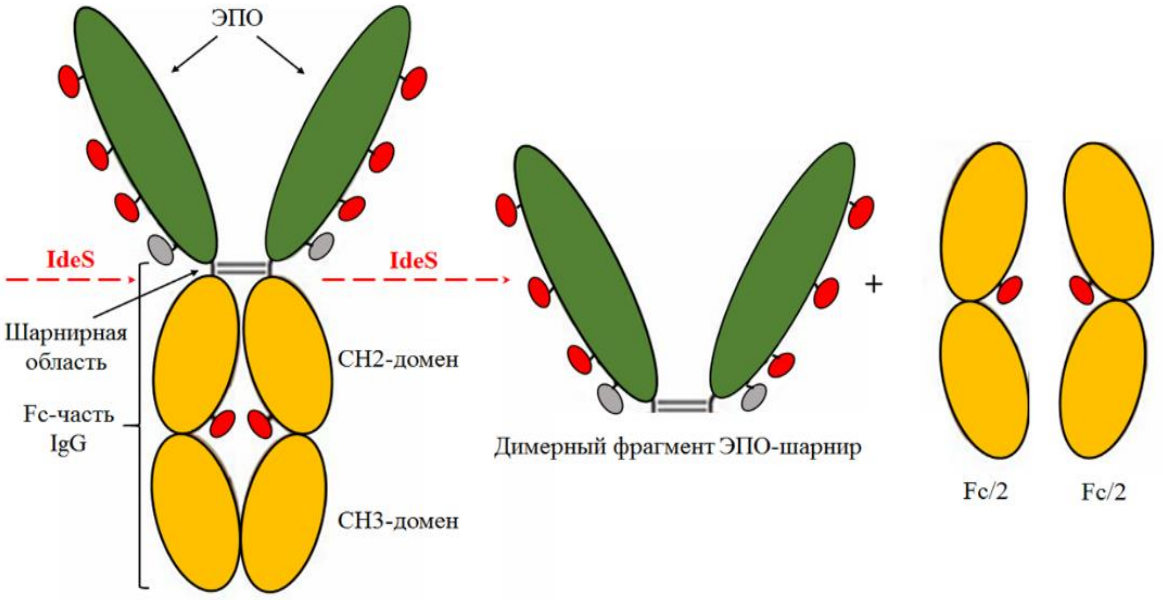

Figure 2. Scheme of EPO-Fc hydrolysis with IdeS protease.
[ЭПО means EPO; Шарнирная область means Hinge region; Fc-часть means Fc part;
CH2-домен means CH2 domain; CH3-домен means CH3 domain;
Димерный фрагмент means dimeric fragment; ЭПО-шарнир means EPO hinge]

Unlike the above enzymes, IdeS-protease has several undoubted advantages. It does not show activity with respect to immunoglobulins of other classes (IgM, IgA, IgD, IgE). As SpeB, it provides high yields of reaction products ($\geq$ 95%) in a short time period. Its activity peak is in neutral media, and it has a simple protocol of hydrolysis. In addition, IdeS protease has been successfully used for several years for the fragmentation of both IgG [28, 33, 34] and Fc-fusion proteins [35, 36].

In order to choose the optimal enzyme we carried out SDS-PAGE of EPO-Fc samples hydrolyzed by IdeS-protease, papain and pepsin followed by coloring with R-250 Coomassie reagent. The results are shown in Figure 3.

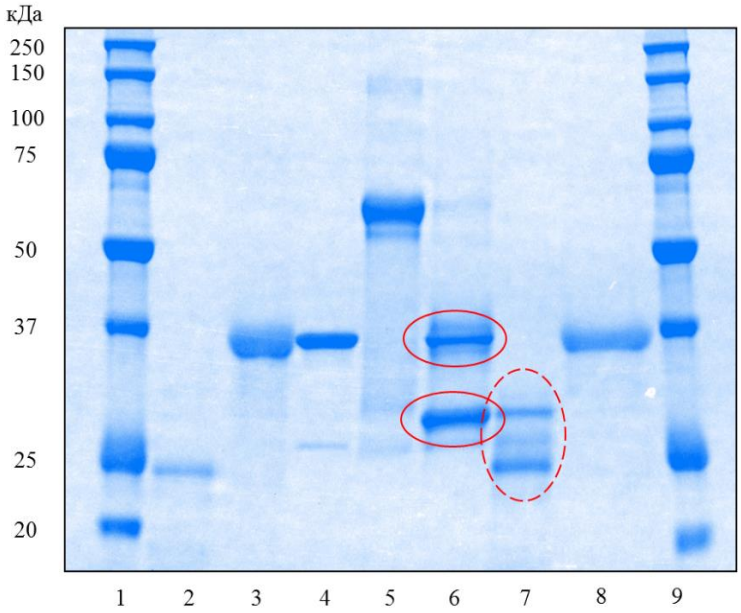

Figure 3. Electrophoregram of gel SDS-PAGE with samples of EPO-Fc hydrolyzed by IdeS, papain and pepsin, colored by R-250 Coomassie solution (in denaturating conditions):
1, 9 – preliminarily colored standards of molecular weights; 2 – papain; 3 – pepsin; 4 – IdeS-protease;
5 – pure EPO-Fc; 6 – EPO-Fc hydrolyzed by IdeS protease; 7 – EPO-Fc hydrolyzed by papain;
8 – EPO-Fc hydrolyzed by pepsin.
[кДа means kDa]

When hydrolyzing the fusion protein with IdeS protease, EPO-Fc is specifically cleaved into two monomeric Fc parts with molecular weights about 27 kDa (Figure 3, line 6, the lower strip is marked with an oval) and an EPO fragment integrated with the IgG hinged region with a molecular weight of 36–37 kDa (Figure 3, line 6, the upper strip is marked with an oval).

Papain cleaves EPO-Fc less specifically (line 7). One can see a protein strip of lower intensity corresponding to the Fc part of IgG and a protein strip with a molecular weight about 25 kDa (dotted line) obviously inappropriate to the fragment containing EPO and a part of IgG. As compared to two above enzymes, pepsin has no evident specificity at all. Most likely, it cleaves EPO-Fc into low molecular weight peptides. No protein strips were detected in the sample containing the mixture of the fusion protein and enzyme except for the strip of pepsin itself.

On the basis of above arguments and conclusions IdeS protease was chosen for EPO-Fc hydrolysis.

Characterization of EPO-Fc electrophoretic properties before and after hydrolysis by SDS/SAR/IEF-PAGE

According to the producer instruction [13] EPO-Fc hydrolysis was carried out as follows. Protease was added to a solution containing the fusion protein in 1:1 ratio, and the mixture was incubated in the phosphatic buffer (1 h, 37°C). After isoelectric focusing of the hydrolysis products in polyacrylamide gel at pH 2–6 followed by double Western blotting and chemiluminescent detection the unique isoelectric profile of the dimeric fragment $(\text{EPO hinge})_2$ differing from all known EPO analogs was visualized (Figure 4). The isoprofile of each individual rEPO is determined by the total charge of its molecule and, as a rule, is unique. This is used in the modern drug test for analyzing unknown samples of urine and blood serum for the presence of erythropoetins of recombinant nature.

Figure 4 shows the result of the IEF-PAGE analysis of ESA preparations used as positive standards and of endogenous human erythropoietin (eEPO), *from left to right*: isoprofiles of

EPO-Fc, Mircera, endogenous EPO, biological reference preparation α-, β-rEPO (BRP, Biological Reference Preparation, equimolar mixture of epoetin-α and -β, track 4, the upper 6 strips) and Aranesp (NESP, track 4, 4 strips at the bottom) and EPO-Fc after hydrolysis by IdeS. The isoprofiles of α-, β-rEPO, Mircera and the product of EPO-Fc hydrolysis of the dimeric fragment $(EPO\text{-}hinge)_2$ by IdeS are determined in the main region of the polyacrylamide gel. The isoelectric profile of rEPO preparation Aranesp upon isoelectric focusing is determined in the acidic region of the gel. The main and acidic regions of the gel are divided according to the positions of the first intensive protein strip of α-, β-rEPO and the most intensive protein strip of Aranesp. The endogenous region is between these regions.

The isoprofile of the dimeric fragment of EPO conjugated with the hinged region of IgG significantly differs both in the nature of glycoforms distribution and in their quantity with respect to other ESA. It consists of 10–12 discrete glycoforms, the most intensive of which are detected in the region between the isoprofiles of α-, β-rEPO and Mircera. It was established that the distance between the glycoforms of the isoprofile of the dimeric fragment $(EPO\ hinge)_2$ is less than between those of Mircera.

Besides, EPO-Fc samples before and after IdeS hydrolysis were analyzed by SDS/SAR-PAGE. These methods do not have sufficient specificity for determining EPO-Fc. The case is that blood serum contains a large amount of proteins having molecular weights similar to that of EPO-Fc monomer. These proteins can reduce the determination selectivity (Figure 5). This occurs, for example, due to nonspecific binding of secondary antibodies with the albumins or heavy chains of antibodies and is shown as additional protein strips visualized in the region of EPO-Fc detection. Repeated analysis of such "suspicious" samples does not confirm the presence of fusion protein in them.

After IdeS hydrolysis of the Fc hybrid followed by SAR-PAGE (Figure 6a) and SDS-PAGE (Figure 6b) analysis, decrease in the molecular weight of the whole molecule was noted. The protein strip of the monomeric EPO hinge fragment rather than that of dimeric $(EPO\ hinge)_2$ was registered, because the electrophoresis was carried out in denaturating conditions [11]. After removal of the Fc fragment the electrophoretic mobility of the fusion protein molecule increased, and the protein strip of the EPO-Fc monomer was found in the region between the strips of α-, β-rEPO and NESP (Figure 6, below, red frame) with a lower content of serum proteins. This will allow differentiating specifically the fusion protein from other proteins with similar molecular weights that can interact with secondary antibodies in the course of the analysis. So, we showed that IdeS hydrolysis can be used for detecting EPO-Fc by the existing SDS/SAR-PAGE methods recommended by WADA. The change of the protein strip position due to hydrolysis will allow distinguishing specifically the fusion protein strip from the strips of other proteins of human biological liquids.

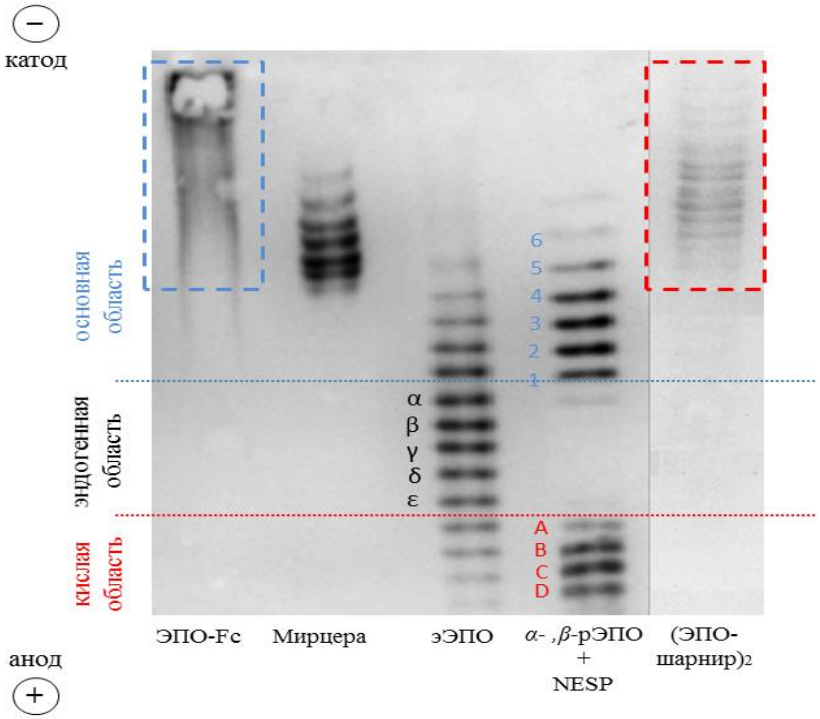

Figure 4. Isoelectric profiles of various ESA *(from left to right)*: EPO-Fc (the left top corner is marked with a blue dotted line), Mircera, endogenous EPO, a mixture of α-, *β*-rEPO + Aranesp and EPO-Fc treated with IdeS protease (dimeric fragment (EPO hinge)$_2$, the profile of glycoforms is marked with a red dotted line, the right top corner). *According to the computer program for annotation and assessment of isoelectric GASepo profiles [37] used for processing results glycoforms detected in the main region of the gel should be designated by Arabic numerals, and those detected in the acidic region, by capital letters of the Latin alphabet. Isoforms of EPO profile in the endogenous region are designated by letters of the Greek alphabet.*
[катод means cathode; основная область means basic region; эндогенная область means endogenous region; кислая область means acidic region; анод means anode; ЭПО-Fc means EPO-Fc; Мирцера means Mircera; эЭПО means eEPO; α-, β-рЭПО means α-, β-rEPO; (ЭПО-шарнир)$_2$ means (EPO hinge)$_2$]

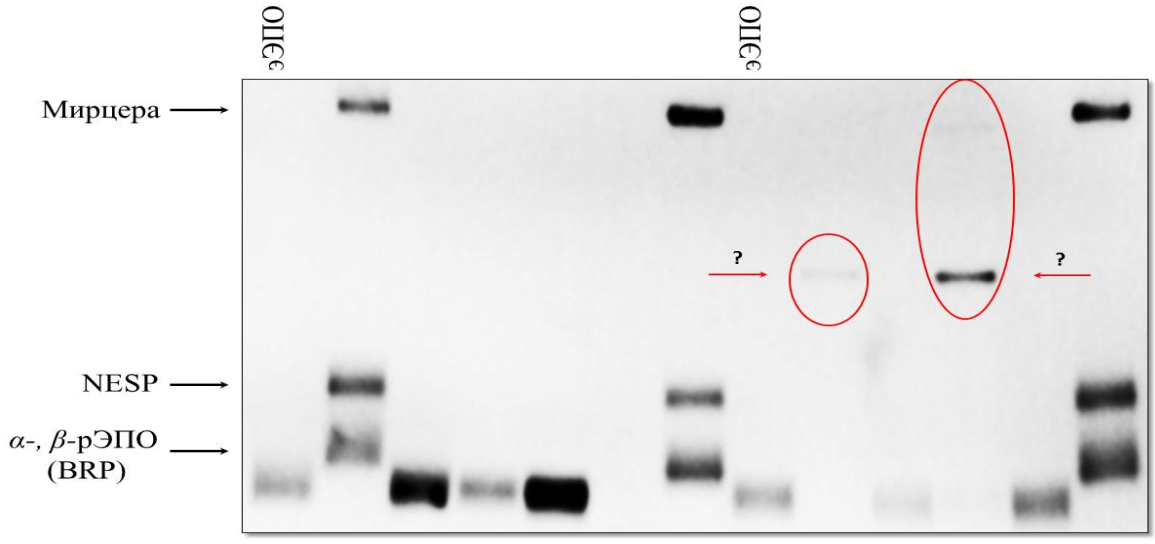

Figure 5. Results of SAR-PAGE of real blood serum samples of athletes. The strips of foreign proteins are marked with ovals.

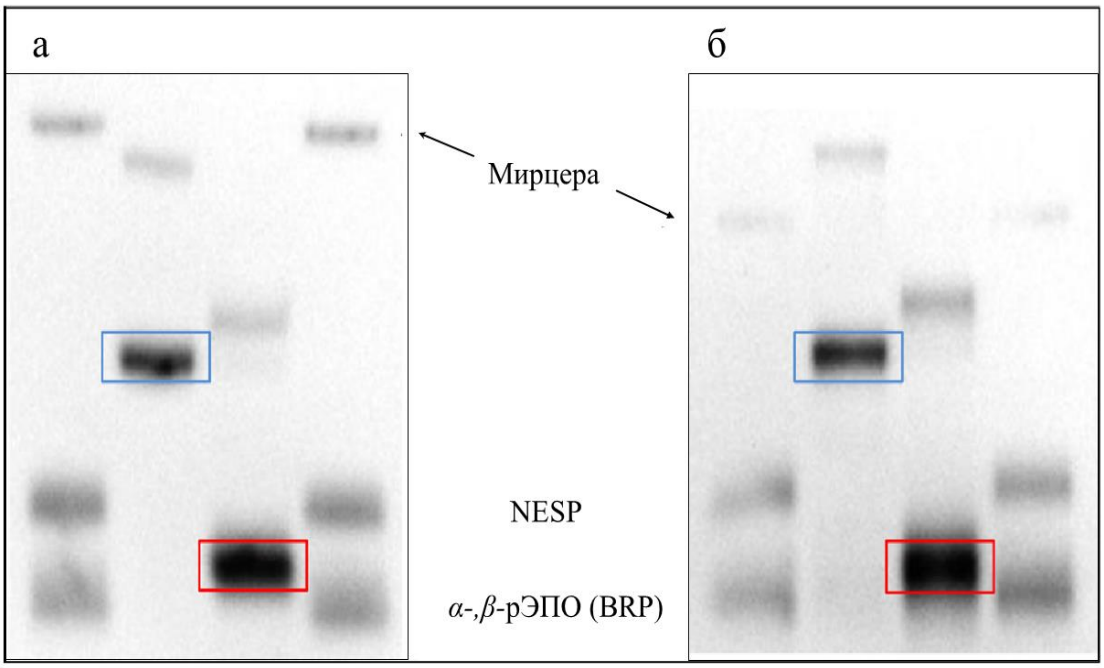

Figure 6. Study of the electrophoretic mobility of EPO-Fc monomer before (above, in the blue frame) and after (below, in the red frame, EPO-hinge fragment) IdeS hydrolysis of EPO-Fc with respect to the positions of protein strips of α-, β-REPO, NESP and Mircera [11]

[а means a; б means b; Мирцера means Mircera; α-, β-рЭПО means α-, β-rEPO]

Determination of optimal conditions for the hydrolysis of EPO-Fc isolated from blood serum samples

The possibility of using immunoaffine columns produced by MAIIA for isolating EPO-Fc has already been studied before [10]. Now it is the only approach widely used in routine practice of anti-doping laboratories of the world for isolating EPO analogs from blood serum samples. The columns have a high recovery degree, and sample preparation does not take much time [19].

On the basis of information provided by the producer it is possible to conclude that 1 unit of protease is sufficient for the hydrolysis of 1 mkg of IgG [13]. Considering that blood serum contains endogenous immunoglobulins that can remain in the eluate after immunopurification and compete in the course of hydrolysis with EPO-Fc thus reducing its efficiency, it was decided to add more IdeS. In order to determine the optimal IdeS/general protein ratio we prepared 9 samples of serum (500 µl) containing 0.3 ng of EPO-Fc and subjected them to sample preparation with the use of immunoaffine columns from the EPO Purification kit produced by MAIIA Diagnostics (Sweden) according to the instruction. Then we measured the general concentration of protein in the eluates by means of a Nanodrop 2000 spectrophotometer (ThermoScientific, Germany), added 1, 2.5 and 5 units of IdeS protease per 1 mkg of general protein and incubated the mixtures within 30 min, 1 h and 2 h at 37°C as recommended by the producer [13]. Temperature effect on the hydrolysis was not studied. Figure 7 shows the results.

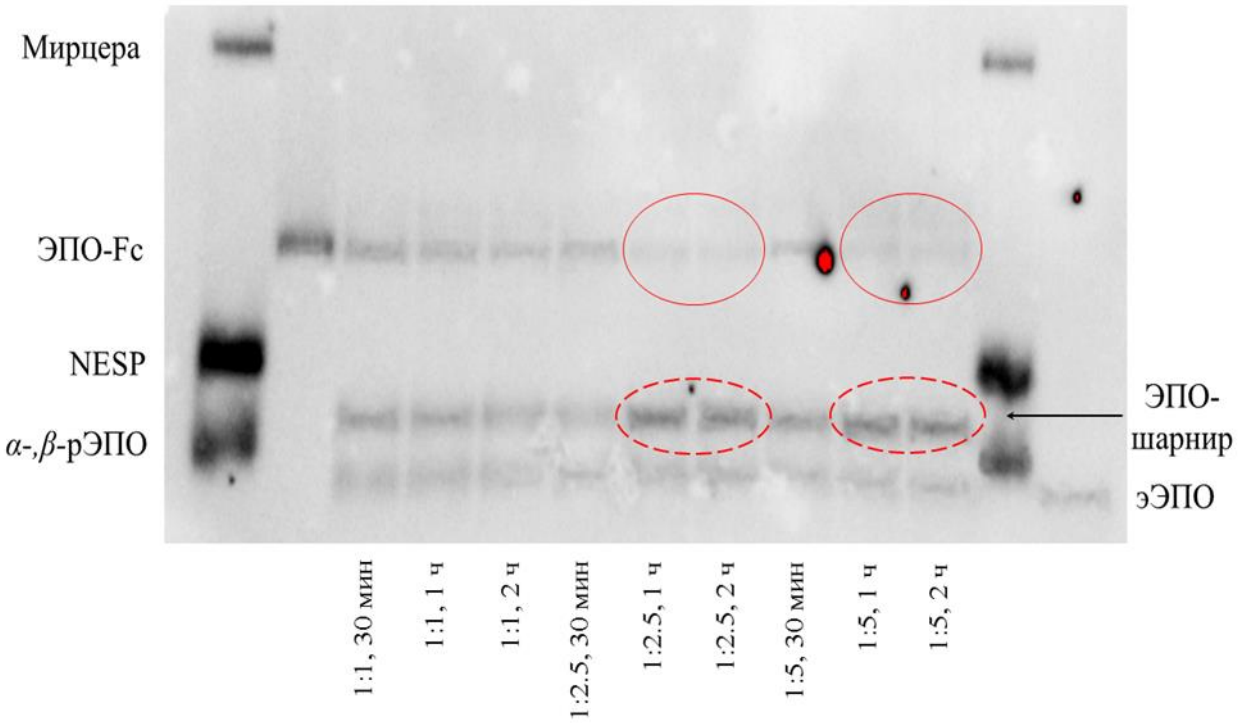

Figure 7. Choosing the optimal IdeS/general protein ratio for the hydrolysis of EPO-Fc isolated from serum samples.

[ЭПО-Fc means EPO-Fc; Мирцера means Mircera; эЭПО means eEPO; α-, β-рЭПО means α-, β-rEPO; (ЭПО-шарнир)$_2$ means (EPO hinge)$_2$; мин means min; ч means h]

It was found that the hydrolysis was complete in samples with general protein/IdeS ratio 1:2.5 and incubation time 1 h and more, as well as in samples with ratio 1:5 and the same incubation time: 1 h and more. The protein strip of EPO-Fc monomer was practically not detected in the samples with these ratios (Figure 7, marked with the red oval, above), and the yield of the hydrolysis product – EPO-hinge fragment – was higher (the protein strip of higher intensity is detected, marked with the red dotted line, below). In case of the sample with general protein / protease ratio 1:2.5 and incubation time 30 min this time was apparently insufficient for complete hydrolysis of the fusion protein. Hydrolysis of samples with ratio 1:1 requires more than 2 hours for complete elimination of the Fc part. As a result, the following conditions were chosen as optimal for the hydrolysis of EPO-Fc after immunopurification of serum samples: general protein / IdeS ratio 1:2.5 at incubation time 1 h.

Conclusions

As a result of the study the optimal enzyme allowing to specifically and effectively eliminate the Fc part of the fusion protein molecule was chosen.

It was established that the product of EPO-Fc hydrolysis by IdeS – the dimeric EPO fragment conjugated with the hinged region of human IgG – (EPO-hinge)$_2$ – shows a unique isoelectric profile when analyzed by IEF-PAGE. Its profile is not comparable to any of the isoprofiles of the known ESA and endogenous EPO. This can be used in modern drug tests for EPO-Fc identification.

The analysis of hydrolyzed EPO-Fc by the existing SDS/SAR-PAGE methods showed that the fusion protein molecule loses a part of its molecular weight. As a result, the protein strip of the hydrolysis product – EPO-hinge fragment – is shifted to the region between the protein strips of α-, β-REPO and Aranesp, and its position is also unique. This can be used as an

additional proof of the presence of Fc hybrids in blood serum samples.

Besides, the work enabled finding the optimal conditions for the hydrolysis of fusion protein in blood serum samples after immunopurification.

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