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Identification of Zinc-alpha-2-glycoprotein binding to clone AE7A5 anti-human Epo antibody by means of nano-HPLC and high-resolution accurate mass ESI-MS/MS

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Introduction

Nonspecific binding of the monoclonal anti-human Epo antibody (clone AE7A5; R&D Systems, Inc.) used for the detection of doping with recombinant erythropoietin by the worldwide practiced isoelectric focusing (IEF) and Western double blotting method [1] has been in discussion for several years. Publications in peer-reviewed journals addressed this subject in 2005 and 2006 and led to some scientific argumentation on the specificity of the employed detection antibody [2, 3, 4, 5, 6]. However, experienced users of the Epo-method have observed several additional bands (usually two to four) in the basic region of the pH 2-6 IEF-gel that was only occasionally detected in urine samples. Their relationship to Epo remained unclear for years. Nevertheless, it was obvious that these bands did not interfere with the endogenous urinary and recombinant Epo-IEF profiles and thus the evaluation of the profiles was not disturbed or questioned at all [2]. We were able to identify the protein causing these basic IEF-bands by means of carrier ampholyte IEF-PAGE, SDS-PAGE, Western blotting, and nano-ESI high resolution mass spectrometry.

Experimental

Materials

Carrier ampholytes (Servalytes 2-4, 3-4, 4-5, 4-6, and 6-8) were from Serva (Heidelberg, Germany). Acrylamide/bisacrylamide solution for isoelectric focusing (PlusOne ReadySol IEF, 40 % T, 3 % C), urea, ammonium peroxidisulfate (APS), and N,N,N′,N′-tetramethylethylenediamine (TEMED), Tris, and glycine were from GE Healthcare (Uppsala, Sweden). For SDS-PAGE separations BisTris-gels (NuPAGE, 10 % T) together with LDS

sample buffer and MOPS electrophoresis running buffer were used (Invitrogen, Carlsbad, CA). Devices for microfiltration (Steriflip) and ultrafiltration (Centricon Plus-20, Centricon YM-30, Microcon YM-30), PVDF-membranes (Durapore, Immobilon-P) were from Millipore (Billerica, MA). Methanol (HPLC grade), acetonitrile (ACN) (HPLC hypergrade), glacial acetic acid, formic acid (FA), and water for LC-MS (HPLC grade) were from Merck (Darmstadt, Germany). Reversed phase columns for peptide trapping (C18 PepMap 100, 300 µm x 5 mm, 5 µm particle size, 100 Å pore size) and nano-HPLC (C18 PepMap100, 75 µm x 15 cm, 3 µm particle size, 100 Å pore size) were bought from Dionex (Sunnyvale, CA). Glass emitters for online nano-ESI (360 µm OD, 20 µm ID, 10 µm tip ID) were from New Objective (Woburn, MA). Monoclonal mouse antibodies for the detection of erythropoietin (clone AE7A5) and zinc-alpha-2-glycoprotein (ZAG; clone 35) were acquired from R&D Systems (Minneapolis, MN) and BD Biosciences (San Jose, CA), respectively. The standard for human urinary erythropoietin (uhEpo; second international reference preparation) was from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK), standards for recombinant erythropoietin (rhEpo) from the European Directorate for the Quality of Medicines (BRP-Epo; Strasbourg, France) and Amgen (NESP; Thousand Oaks, CA). Recombinant zinc-alpha-2-glycoprotein (rhZAG) was obtained from BioVendor (Modrice, Czech Republic). Trifluoroacetic acid (TFA), dithiothreitol (DTT), iodoacetamide (IAA), trifluoroethanol (TFE), and ammonium bicarbonate were from Sigma-Aldrich (St. Louis, MO). Urine samples were either official samples taken from athletes for Epo-doping control or samples received from healthy volunteers.

Electrophoretic separation and Western blotting

Isoelectric focusing (IEF) on carrier ampholyte (CA) slab gels was used for separating endogenous urinary and recombinant human erythropoietin isoforms (standards, urine samples). Two different pH-gradients, namely pH 2 to 6 and pH 3 to 5, and two different gels sizes (24 x 10 cm, 24 x 20 cm) were employed. The method (gel casting, sample preparation, IEF) was essentially performed as described by Lasne [7]. For large sized gels (pH 3-5 XL, pH 2-6 XL) electrophoresis conditions were adapted accordingly (Table 1). Interelectrode distances were 10 cm and 17 cm, respectively. The temperature of the cooling device was set to 8 °C. After focusing, gels were blotted on PVDF-membranes using modified Towbin buffer (25 mM Tris, 192 mM glycine, no methanol) as described by Lasne [7, 8]. For chemiluminescent detection of the specific and nonspecific binding of clone AE7A5 anti-Epo antibody single and double blots were used. Incubations with the anti-ZAG antibody (clone

35) were performed on first blots only and after the anti-Epo antibody was stripped by semidry blotting under acidic conditions (0.7 % acetic acid, 0.8 mA/cm^2, 10 min).

Table 1: Focusing conditions for low and high resolution IEF slab gels.

Step	pH 2 to 6 IEF gel (24 x 10 cm)	pH 3 to 5 XL, pH 2 to 6 XL IEF gels (24 x 17 cm)
Prefocusing	250 V / 150 mA / 70 W / 30 min	3500 V / 40 mA / 34 W / 60 min
Focusing	2000 V / 131 mA / 25 W / 4000 Vh	3500 V / 25 mA / 25 W / 1000 Vh 3500 V / 25 mA / 50 W / 9000 Vh

After incubation with a biotinylated secondary antibody (ImmunoPure goat anti-mouse IgG; Pierce, Rockford, IL) followed by several washing steps (0.5 % low fat milk in PBS) and a streptavidin-HRP incubation step (ImmunoPure; Pierce, Rockford, IL) the membranes were washed with PBS. Chemiluminescence was achieved by incubation of the blots in a luminol based substrate (West Pico; Pierce, Rockford, IL). All images were acquired with a CCD camera (epoCAM, ARC Seibersdorf research, Austria) and at variable exposure times depending on the initial signal intensity (e.g. 1 min, 2 min, 5 min, and 10 min). **SDS-PAGE** separations were performed on BisTris-gels (10 % T, 1.5 mm) at constant voltage (200 V) and for 50 min (MOPS running buffer). Samples and standards were heated at 70 °C for 10 min in LDS-sample buffer and under reducing conditions. Between 20 and 35 µL of the denatured protein solutions were applied on the gels via GELoader tips (Eppendorf; Hamburg, Germany). After electrophoresis gels and membranes (Durapore, Immobilon-P) were equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine, 20 % methanol) for 15 min and blotted at constant current (1 mA/cm^2) for 60 min (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell; BioRad, Hercules, CA). All subsequent steps were done exactly as described for the IEF-gels.

On-membrane protein elution

Bands showing nonspecific binding of clone AE7A5 anti-Epo antibody after IEF and double blotting were excised from the first membrane after stripping the antibody and drying of the membrane. The PVDF-membrane pieces were transferred to 2 mL Eppendorf tubes and incubated with 40 µL one fold LDS sample buffer at 95 °C for 5 min and under reducing conditions (Thermomixer, 700 rpm). After cooling on ice and centrifugation at 16,100 rcf for 5 min the eluates were directly applied on BisTris gels (10 % T, 1.5 mm). Electrophoretic

conditions were as described above. In order to show whether these bands resembled isoforms with approximately the same molecular mass, double blots of the SDS-PAGE gels were performed. In addition, the first membranes were also incubated with the clone 35 anti-ZAG antibody.

On-membrane tryptic digestion of nonspecifically bound proteins

For mass spectrometric identification of nonspecifically bound proteins bands on the 1st membranes of both the IEF and SDS-PAGE separations were excised and transferred to 2 mL Eppendorf tubes. Subsequently, 25 µL of a solution of 0.2 µg/µL trypsin (sequencing grade modified; Promega, Madison, WI) in 25 mM Tris-HCl buffer pH 8.5 containing 4 M urea were added and the tubes were incubated at 37° C overnight (Thermomixer, 700 rpm). For mass spectrometric analysis the tubes were sonicated for 2 minutes and 10 µL of the digest were mixed with 90 µL of 1 % acetonitrile (ACN) containing 0.05 % TFA.

In-solution tryptic digestion of recombinant zinc-alpha-2-glycoprotein

A stock solution (1 µg/µL) of recombinant zinc-alpha-2-glycoprotein (rhZAG) was prepared by reconstituting the lyophilized protein formulation with Milli-Q water. In order to remove interfering buffer substances the protein was precipitated by adding cold acetone (-20 °C, 150 µL) to 30 µL of the stock solution. After overnight incubation at –20 °C, centrifugation at 16100 rcf for 5 min, and subsequent acetone removal, the pellet was dried for 10 min at ambient temperature and resolubilized in 25 µL of 100 mM ammonium bicarbonate stock solution. Protein denaturation and reduction of disulfide bridges was done by adding TFE (25 µL) and DTT (1 µL, 200 mM) (60 °C, 60 min, Thermomixer, 400 rpm). The resulting free thiol-groups were carbamidomethylated by adding 4 µL of a freshly prepared solution of iodoacetamide (200 mM) and incubating in the dark at 25 °C for 60 min (Thermomixer, 400 rpm). Excess IAA was removed by adding 1 µL of the 200 mM DTT-solution (25 °C, 60 min, Thermomixer, 400 rpm). Subsequently, 300 µL of Milli-Q water, 100 µL ammonium bicarbonate stock solution (100 mM), and 0.6 µL trypsin solution (dissolved in 25 mM ammonium bicarbonate solution at a concentration of 1 µg/µL) were added. The mixture was incubated at 37 °C (Thermomixer, 400 rpm) overnight. For mass spectrometric analysis aliquots of the digest were mixed 1:1 with 1 % ACN containing 0.05 % TFA.

LC-MS/MS

Tryptic digests were separated on a Dionex Ultimate 3000 dual gradient nano-HPLC system (Sunnyvale, CA) in online preconcentration mode. For peptide trapping a C18 PepMap 100

column (300 µm x 5 mm, 5 µm particle size, 100 Å pore size) was used and was operated at a flow rate of 20 µL/min. After loading the peptides on the trap column salts were removed by washing the column with loading solvent (2 % ACN with 0.05 % TFA). After 3 min the trap column was switched in line with the analytical column (C18 PepMap100, 75 µm x 15 cm, 3 µm particle size, 100 Å pore size), which was operated at a flow rate of 300 nL/min. Solvents A (2 % ACN with 0.1 % FA) and B (80 % ACN with 0.1 % FA) were used as mobile phases. A linear gradient starting at 0 % B and ending at 80 % B after 120 min was used. Then both columns were washed with 100 % B for 25 min and reequilibrated after switching the trap column off line with 0 % B and loading solvent, respectively, for a further 25 min. Injection volumes were 1 µL. The autosampler was operated at 4 °C. UV absorption was recorded at 214 nm (3 nL in line flow cell). The column oven temperature was 25 °C.
Eluting peptides were sequenced with low energy CID and an LTQ-Orbitrap FT mass spectrometer (Thermo Electron, Bremen). A Proxeon nano-ESI source (Odense, Denmark) equipped with a New Objective glass emitter (360 µm OD, 20 µm ID, 10 µm tip ID) for online nano-ESI (Woburn, MA) was used. The mass spectrometer was operated in positive ion mode with a spray voltage of 2 kV and a capillary temperature of 160 °C. Data were acquired in data-dependent mode (one full MS scan (m/z 300-2000) at a resolving power of R = 60,000 at m/z 400 followed by MS/MS scans of the three or five most intense ions in the linear ion trap) with a cycle time of approximately 1 s for the top three experiment (one full MS scan experiment at R = 60,000 in the Orbitrap took ca. 1 s and thus enabled three parallel MS/MS experiments in the linear ion trap). The lock-mass option of the orbitrap analyzer was used for all measurements and enabled online recalibration of the instrument. The polydimethylcyclosiloxane background ion at m/z 445.120025 was used as lock-mass. Mass accuracy was usually better than 5 ppm. Dynamic exclusion was enabled with 30 s exclusion duration. Automated gain control (AGC) target settings were 2 x 10^5 for full MS scans in the FT analyzer and 1 x 10^4 for MS/MS scans in the linear ion trap. The high-resolution instrument was chosen in order to enable online charge state determination of precursor ions and to additionally use monoisotopic precursor mass information for subsequent bioinformatic analyses [9].

Bioinformatics

BioWorks 3.3 (Thermo Electron, San Jose, CA) was used for searching the experimental tandem mass spectra (MS/MS data) against the UniProt protein database. SEQUEST search parameters were set as follows: trypsin (fully enzymatic, cleaving at both ends), two missed

cleavage sites, 5 ppm peptide mass tolerance, monoisotopic precursor mass, and 1 amu fragment ions tolerance. Oxidized methionine was set as variable modification and in cases where the protein mixture was treated with IAA before tryptic cleavage carbamidomethylcysteine was set as fixed modification. The top SEQUEST hit was manually confirmed according to the criteria proposed by Yates et al. [10].

Results

Electrophoretic separation and Western blotting

Isoelectric focusing (IEF) of the same urine samples (pH 2-6, pH 2-6 XL, and pH 3-5 XL) showed that the nonspecific binding behaviour of clone AE7A5 anti-Epo antibody was not influenced by the gel size or pH-gradient used and thus was reproducible. Within these pH-ranges usually three to four extra bands were detected in the extreme basic region of the gels after double blotting (Fig. 1a). However, it must be emphasized that not all samples showed these extra bands. Due to the better separation of the bands on the pH 3-5 XL gels these gels were used for all subsequent SDS-PAGE and MS experiments.

SDS-PAGE and double blotting was performed with urinary ultrafiltration retentates which showed extra bands during IEF and resulted in electropherograms with one additional band. Its apparent molecular mass was higher than the apparent mass of uhEpo but slightly lower than the mass of NESP (Fig. 1d).

On-membrane protein elution

Elution of the three to four extra bands from one and the same IEF-lane (Fig. 1b) followed by SDS-PAGE and double blotting showed identical apparent molecular masses for these bands (Fig. 1c). Hence, it was most likely that the bands represented isoforms of one single protein.

LC-MS/MS

A shotgun proteomics approach was used in order to identify the protein causing the nonspecific binding of the clone AE7A5 antibody. After cutting the four bands out of the 1st PVDF-membrane the proteins were on-membrane tryptically digested. Subsequent nano-LC-MS/MS runs and processing of the MS/MS spectra with Bioworks 3.3 software revealed a series of possible proteins. Among them ZAG was present in all four fractions and showed highest sequence coverages (ca. 52-62 %) and identification scores (cross correlation values between ca. 298-336; Fig. 2 represents the results of band number one).

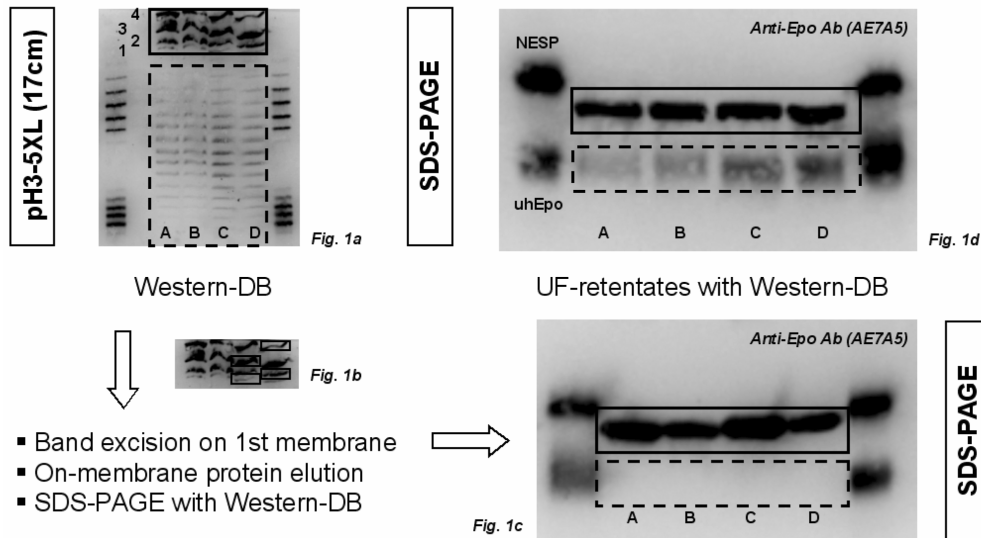
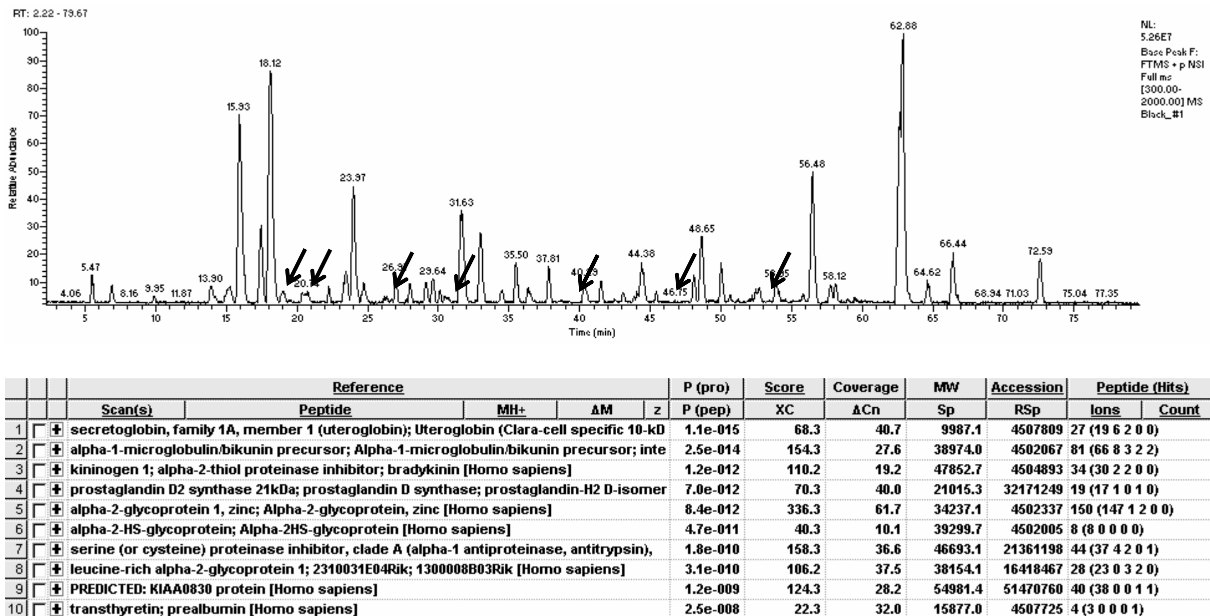


Figure 1: Isolation of the unknown protein. Isoelectric focusing on a pH 3-5 XL carrier ampholyte gel resulted in good separation of the four nonspecifically detected bands *(Fig. 1a)*. Excision of the bands from the 1st PVDF-membrane *(Fig. 1b)* combined with on-membrane elution and subsequent SDS-PAGE showed that all four bands had the same apparent molecular mass *(Fig. 1c)*. In comparison, direct SDS-PAGE of the same urinary retentates led to a similar result but with the uhEpo bands still present *(Fig. 1d)*.



	Reference Scan(s) / Peptide / MH+ / ΔM / z	P (pro) P (pep)	Score XC	Coverage ΔCn	MW Sp	Accession RSp	Peptide (Hits) Ions / Count
1	secretoglobin, family 1A, member 1 (uteroglobin); Uteroglobin (Clara-cell specific 10-kD	1.1e-015	68.3	40.7	9987.1	4507809	27 (19 6 2 0 0)
2	alpha-1-microglobulin/bikunin precursor; Alpha-1-microglobulin/bikunin precursor; inte	2.5e-014	154.3	27.6	38974.0	4502067	81 (66 8 3 2 2)
3	kininogen 1; alpha-2-thiol proteinase inhibitor; bradykinin [Homo sapiens]	1.2e-012	110.2	19.2	47852.7	4504893	34 (30 2 2 0 0)
4	prostaglandin D2 synthase 21kDa; prostaglandin D synthase; prostaglandin-H2 D-isomer	7.0e-012	70.3	40.0	21015.3	32171249	19 (17 1 0 1 0)
5	alpha-2-glycoprotein 1, zinc; Alpha-2-glycoprotein, zinc [Homo sapiens]	8.4e-012	336.3	61.7	34237.1	4502337	150 (147 1 2 0 0)
6	alpha-2-HS-glycoprotein; Alpha-2HS-glycoprotein [Homo sapiens]	4.7e-011	40.3	10.1	39299.7	4502005	8 (8 0 0 0 0)
7	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin),	1.8e-010	158.3	36.6	46693.1	21361198	44 (37 4 2 0 1)
8	leucine-rich alpha-2-glycoprotein 1; 2310031E04Rik; 1300008B03Rik [Homo sapiens]	3.1e-010	106.2	37.5	38154.1	16418467	28 (23 0 3 2 0)
9	PREDICTED: KIAA0830 protein [Homo sapiens]	1.2e-009	124.3	28.2	54981.4	51470760	40 (38 0 0 1 1)
10	transthyretin; prealbumin [Homo sapiens]	2.5e-008	22.3	32.0	15877.0	4507725	4 (3 0 0 0 1)

Figure 2: Nano-LC separation of one of the nonspecifically detected bands on the pH 3-5 XL IEF-gel. Bands were cut out of the 1st PVDF-membrane and digested on-membrane with trypsin. MS/MS spectra were searched against UniProt using Bioworks 3.3. Zinc-alpha-2-glycoprotein was the protein with the highest identification score and sequence coverage (arrows indicate the position of the identified ZAG-peptides in the chromatogram).

In order to confirm the identity of the protein, MS/MS spectra of a tryptic digest of recombinant zinc-alpha-2-glycoprotein were also acquired and manually compared with the peptide spectra of the presumptive protein (Fig. 3). Detailed analysis of those peptides within the ZAG sequence which were not automatically identified by SEQUEST revealed that they resembled peptides bearing N-glycosylation sites. Naturally, these peptides were not part of the UniProt database.

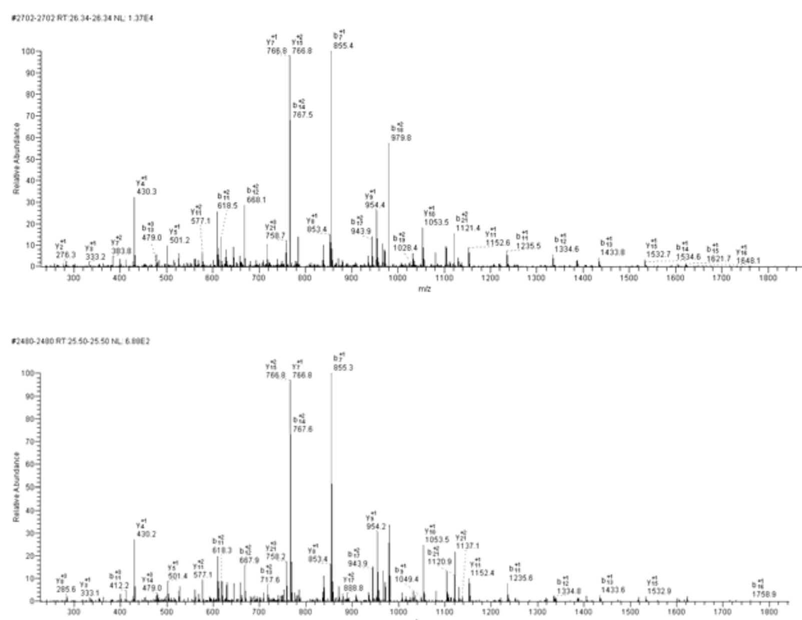


Figure 3: Manual confirmation of the SEQUEST search result according to [10] and by comparative sequence analysis of the MS/MS spectra of human urinary ZAG and human recombinant ZAG. The example above shows the MS/MS spectra of precursor mass 796.4 (3+) of uhZAG (above) and rhZAG (below). The sequence of the peptide was identified as NILDRQDPPSVVVTSHQAPGEK.

Binding of clone AE7A5 anti-Epo antibody to zinc-alpha-2-glycoprotein

After successful identification of the unknown protein by mass spectrometry the binding behaviour of clone AE7A5 anti-Epo antibody was studied in greater detail. For that purpose both pH 2-6 XL and pH 3-5 XL IEF-gels were used. Recombinant human ZAG (2 µg) was separated together with uhEpo, BRP-Epo, and NESP (0.2 ng each). It could be clearly demonstrated that the anti-Epo antibody not only bound to the various erythropoietins but also to several rhZAG isoforms. Stripping and reprobing of the first blot with the clone 35 anti-ZAG antibody (Fig. 4) showed that (1) AE7A5 only bound to the most abundant rhZAG isoforms and that (2) uhZAG was also present in uhEpo, but at a lower concentration (the

NIBSC-standard for uhEpo contains 10 IU (80 ng) uhEpo and about 2 mg of other urinary proteins). The same experiment was repeated with samples of athletes. Only urines containing an elevated amount of uhZAG led to nonspecific binding of the clone AE7A5 antibody to uhZAG isoforms (Fig. 5). An investigation of a possible correlation between the uhZAG concentration and that of Epo was not part of this study.

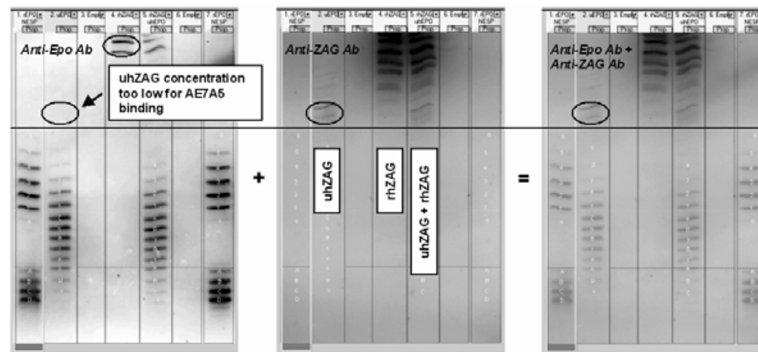


Figure 4: Separation of BRP-Epo and NESP, uhEpo, rhZAG, and a mixture of uhEpo and rhZAG (lanes 1, 2, 4, 5 from left to right) on a pH 2-6 XL IEF-gel (double blots). The 1st PVDF-membrane was incubated with clone AE7A5 anti-Epo antibody, stripped and then reincubated with clone 35 anti-ZAG antibody. The most intense uhZAG isoforms were observed in the region where the nonspecific binding usually occurred for the urinary samples.

Conclusion

It could be demonstrated that ZAG – which is an abundant protein in urine and which is present in many body fluids and tissues - was present in all of the investigated urinary samples screened for Epo-doping. It was also detectable in the reference standard used for human endogenous urinary erythropoietin (NIBSC). The binding of the monoclonal anti-human EPO antibody (clone AE7A5) to ZAG occured in a highly concentration dependant manner. Consequently, only samples rich in urinary ZAG caused detectable binding of the AE7A5 antibody to ZAG and thus led to the unexplained extra bands on the Epo-IEF gels.

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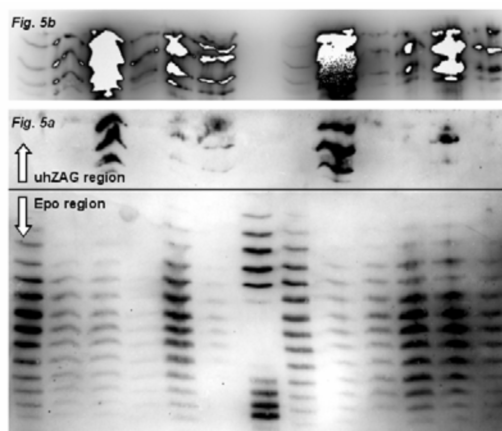

Figure 5: Samples of athletes were screened for Epo on a pH 3-5 XL gel. Nonspecific binding of the anti-Epo antibody (clone AE7A5) occured on few samples *(Fig. 5a)*. Stripping and reprobing of the first membrane with the anti-ZAG antibody (clone 35) revealed that uhZAG was present in all samples but AE7A5 bound only to those urines which had elevated amounts of uhZAG *(Fig. 5b)*.