

Identification of fibroblast growth factor 1 (FGF-1) in a black market product

Katja Walpurgis,^a Andreas Thomas,^a Tim Laussmann,^b Luis Horta,^c Sabine Metzger,^d Wilhelm Schänzer^a and Mario Thevis^{a*}

The use of growth factors for accelerated healing of sports injuries is restricted under the terms of the World Anti-Doping Agency (WADA) anti-doping code. Cheating athletes have used the black market as a source of performance-enhancing substances. Drugs that currently undergo clinical trials are frequently offered – despite the unknown health risks associated with the administration of unapproved pharmaceuticals. Recently, a new growth factor (referred to as fibroblast growth factor 1/FGF-1) with known effects on the repair and regeneration of damaged tissue was detected in an unlabelled black market product confiscated by the German customs. The identification of the protein was achieved by one- and two-dimensional polyacrylamide gel electrophoresis (SDS-PAGE and 2D-PAGE), different proteolytic digestions, immunological methods and nano-liquid chromatography high-resolution/high-accuracy Orbitrap mass spectrometry. The SDS-PAGE analysis revealed slight differences concerning the molecular weight of recombinant human and black market FGF-1. Using in-gel proteolysis, a truncation or modification located at the N-terminus of the protein was suggested. These findings demonstrate that drug candidates without clinical approval can be readily obtained from the black market, regardless of potential dangerous consequences for the consumer, which corroborates the necessity of proactive and preventive doping control approaches. In that regard, physiological concentrations of blood and urine specimens collected from healthy individuals were analyzed and were found to range below 28 pg/ml in urine, while there was no detectable FGF-1 in plasma. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: sport; doping; mass spectrometry; growth factors

Introduction

The protein family of fibroblast growth factors consists of 22 human members with a molecular weight between 17 and 34 kDa.^[1–3] Most of these share a common internal 120 amino acid core domain, which contains 28 highly conserved and six identical amino acids. FGFs are widely expressed in cells and tissues and involved in many different physiological processes among which FGF-1 is a single, non-glycosylated polypeptide consisting of 154 amino acids with a molecular weight of 17 kDa³. The literature also describes two N-terminally truncated forms of FGF-1, comprising residues 15–154 and 21–154 respectively (Figure 1). FGF-1 has a characteristic β -trefoil structure that contains 12 antiparallel β -strands, a heparin binding region and different domains for the interaction with FGF receptors.^[2,3]

In general, fibroblast growth factors fulfil many different biological functions. On the one hand, they are key regulators of multiple developmental processes and therefore strictly spatially and temporarily regulated in their expression during embryonic differentiation and development of organs.^[3,4] For example, they play a crucial role during bone formation, neural development, formation of the circulatory system, and the development of the lung. On the other hand, FGFs stimulate cell proliferation, migration and differentiation in adult organisms, where they additionally act as cell survival factors.^[1–3] Especially FGF-1 affects many cells like preadipocytes, endothelial cells, fibroblasts or neuroepithelial cells.^[1,3,5] Thus, FGF-1 is involved in angiogenesis, tissue repair, wound healing, adipogenesis, and homeostatic regulation.

Due to their mitogenic and angiogenic activities, FGFs are of great interest for the pharmaceutical industry. Potential therapeutic implications are FGF-mediated angiogenesis, wound and bone

fracture healing and neuroprotection.^[1,3,6,7] The therapeutic use of FGF-1 is however limited by its short protein half-life *in vivo*, resulting from a denaturation temperature around 40 °C and increased susceptibility to protease action. For this reason, it is essential to design stable mutants and modified forms of FGF-1 as well as specific delivery systems.^[1,3,6,7] Alternatively, the intramuscular injection of an expression plasmid encoding FGF-1 was investigated in case of chronic critical limb ischemia.^[8] The non-viral plasmid NV1FGF failed in Phase III TAMARIS trial in the end of 2010 and no drug has received full clinical approval yet.^[9]

Despite missing drug approval, the use of FGFs is restricted under the terms of the World Anti-Doping Agency (WADA) anti-doping code.^[10,11] Major reason is the positive effect of FGFs on muscle healing and regeneration, tendon and ligament repair, as well as wound healing and bone regeneration.^[1,12] Cheating athletes have been shown to use the black market as a source of performance enhancing substances such as the growth-hormone-releasing

* Correspondence to: Mario Thevis, Centre for Preventive Doping Research/Institute of Biochemistry, German Sport University Cologne, Germany. E-mail: thevis@dshs-koeln.de

a Centre for Preventive Doping Research/Institute of Biochemistry, German Sport University Cologne, Germany

b Centre for Education and Science of the Federal Revenue Administration, Cologne, Germany

c Autoridade Antidopagem de Portugal, Av. Prof. Egas Moniz (Estádio Universitário), 1600-190 Lisboa, Portugal

d Biological Medical Research Center, Heinrich-Heine-University of Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, 1600-190, Germany

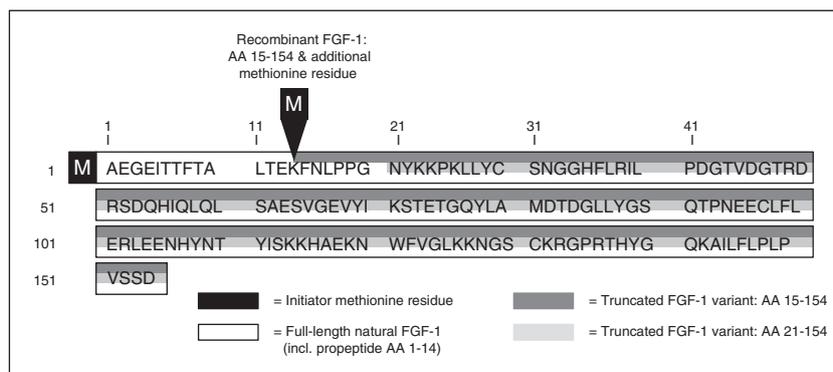


Figure 1. Amino acid sequences of full-length natural FGF-1 (white) and two N-terminally truncated variants comprising the amino acids 15–154 (dark grey) and 21–154 (light grey). Recombinant human FGF-1 consists of the amino acids 15–154 of natural FGF-1 and has an additional methionine-residue attached to the N-terminus.

peptides (GHRPs) or the insulin-like growth factor 1 (IGF-1).^[13–16] Drugs still undergoing clinical trials are frequently offered.

In this communication, the detection of FGF-1 in an unlabelled injection vial provided by the German customs is reported. The identification of the protein was accomplished by one- and two-dimensional polyacrylamide gel electrophoresis (SDS-PAGE and 2D-PAGE), OFFGEL fractionation, different proteolytic digestions, immunological methods and nano-liquid chromatography high-resolution/high-accuracy Orbitrap mass spectrometry.

Materials and methods

IPG strips (pH 3-10NL, 7 cm) were obtained from Bio-Rad (München, Germany) and polyacrylamide gels (1 and 10 wells, 1 mm, 12% Bis-Tris) as well as MOPS (3-(N-morpholino)propane-sulfonic acid) running buffer and lithium dodecyl sulfate sample buffer (LDS sample buffer) were from Invitrogen (Karlsruhe, Germany). Carrier ampholytes for pH 3–10 were purchased from Serva (Heidelberg, Germany) and Coomassie Blue (R-250) staining solution came from Fermentas (St Leon-Rot, Germany). A human FGF-1 enzyme-linked immunosorbent assay (ELISA) was obtained from R&D Systems (Wiesbaden, Germany). The proteases Lys-C and Asp-N (both sequencing grade) were bought from Roche (Mannheim, Germany) and Trypsin (sequencing grade) was purchased from Promega (Mannheim, Germany). All buffer ingredients as well as dithiothreitol (DTT), acrylamide, trifluoroacetic acid (TFA) and a silver staining kit were obtained from Sigma Aldrich (Deisendorf, Germany). All buffers were prepared using MilliQ water (arium pro UV-T, Sartorius Stedim, Aubagne Cedex, France). Acetonitrile (ACN) and acetic acid (HAC) were bought from Merck (Darmstadt, Germany) and solvents for nano-flow liquid chromatography were from Biosolve (Valkenswaard, Netherlands). For OFFGEL fractionation, a 12 well frame set was obtained from Agilent (Waldbronn, Germany) and IPG strips (pH 3-10NL, 13 cm) were purchased from GE Healthcare (München, Germany).

Unknown injection solution

A set of unlabelled, sealed injection vials with unknown content was obtained by the German customs. The vials contained a colourless lyophilisate which was dissolved in 1 ml of MilliQ water and subjected to different analytical approaches targeting the identification of ingredients potentially relevant for doping controls or being subject to the German drug law.

Reference material

Recombinant human FGF-1 was purchased from Sigma Aldrich as reference material. It has a molecular weight of 16 kDa and comprises the amino acids 15–154 of natural FGF-1 (Figure 1). Additionally, it contains a methionine residue attached to the N-terminus.

SDS-PAGE

Prior to SDS-PAGE, denaturation of proteins and reduction of disulfide bonds was achieved by adding 3 μmol of DTT and 7 μl of LDS sample buffer (4x) to 20 μl of each dissolved sample followed by heating for 10 min at 70 °C. SDS-PAGE was performed on 8 cm, 12% Bis-Tris gels (1.0 mm thick, 10-well) at 125 V constant voltage for 90 min (XCell SureLock Mini-Cell (Invitrogen), SE 260 (GE Healthcare, München, Germany)). Afterwards, gels were stained with Coomassie Blue and scanned on a light transmission scanner (GE Healthcare) using a red filter and a resolution of 300 dpi in transparent mode. As a more sensitive staining method for lower protein amounts, a silver staining kit was used according to the manufacturer's instructions.

In-gel proteolytic digestions

The selected gel bands and spots were excised and destained twice for 10 min with 100 μl of 100 mM $\text{NH}_4\text{HCO}_3/\text{ACN}$ (1:1). After dehydration of the washed bands for 10 min with 50 μl of ACN, they were dried in a vacuum centrifuge and rehydrated in 10 μl of a trypsin solution (20 $\mu\text{g}/\text{ml}$ in 50 mM NH_4HCO_3 , pH 8) for 45 min at 4 °C. After rehydration, 20 μl of 100 mM NH_4HCO_3 were added to each sample and the proteins digested 3–16 h at 37 °C. Extraction of the tryptic peptides was achieved by shaking the gel bands for 10 min at 1000 rpm in 100 μl of 50% ACN/1% TFA for a total of three times. Combined extracts were dried under reduced pressure and peptides resolved in 50 μl of 2% HAC.

For in-gel proteolytic digestion with Lys-C and Asp-N, protein bands were washed and dried following the same protocol. After rehydration in 18 μl of a solution containing 0.8 μg of Lys-C and 0.4 μg of Asp-N, 5 μl of ACN and 27 μl of 25 mM Tris-HCl (pH 8.5) were added to a total volume of 50 μl . Proteins were digested for 18 h at 37 °C and the obtained peptides were extracted with 100 μl of 50% ACN/1% TFA in three repetitive steps.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Liquid chromatography was performed on a Waters nano-Acquity UPLC™ system (Eschborn, Germany) equipped with a Symmetry C18 precolumn (5 µm, 180 µm x 20 mm, operated at a flow rate of 5 µl/min) and a BEH130C18 peptide column (1.7 µl, 100 µm x 100 mm, operated at a flow rate of 750 nl/min). A gradient program with 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B was used as follows: 3 min 97% A, 3–5 min 80% A, 5–45 min 40% A, 45–48 min 20% A, 48–50 min 3% A, 50.01 min 97% A, 15 min equilibration. The LTQ Orbitrap (Thermo, Bremen, Germany) was used in combination with a nanospray ion source in positive mode with 1.4 kV ionization voltage. Full scan spectra were recorded with a resolution of 30000 FWHM (at m/z 400). For MS/MS experiments, the collision energy was set to 35% (arbitrary unit, Xcalibur 2.0 SR 2, Thermo Fisher) using helium as collision gas. As a damping gas, nitrogen supplied by a CMC nitrogen generator (CMC Instruments, Eschborn, Germany) was used. The chosen acquisition method included an accurate mass full scan in the Orbitrap analyzer and data-dependent MS/MS experiments in the linear ion trap fragmenting the three most abundant ions with a charge state > 1 in each scan.

Identification of proteins in digested samples

The evaluation of the MS data was accomplished with Proteome Discoverer 1.0 containing the SwissProt and UniProt databases (2008, Thermo). To further include unspecific proteolytic peptides, the database search was performed without selecting a specific enzyme. The identification of a protein was considered successful if at least two peptides were detected or the sequence coverage was above 10% (according to WADA and literature recommendations^[17,18]). Moreover, the agreement of molecular weight and isoelectric point of detected proteins with the respective position of a selected band/spot on the gel was evaluated.

Quantification of FGF-1 by means of SDS-PAGE & ELISA

For quantification of the black market FGF-1, SDS-PAGE was performed with 20 µl of the dissolved sample and a calibration curve with 5, 10, 25, 50, 75, and 100 ng of recombinant human FGF-1 as reference compound. The band volumes were measured using a light transmission scanner (*vide supra*) and ImageQuant™ TL software (GE Healthcare).

A commercial FGF-1 ELISA kit was used to confirm the FGF-1 concentration estimated by means of SDS-PAGE and to determine physiological FGF-1 values in urine and plasma. A total of 15 urine and plasma samples was collected from healthy volunteers (10 male and 5 female, age between 20 and 40, sampling conducted at rest) and stored at -20°C until analysis. All participants provided written consent and the study was approved by the local ethics committee. The ELISA was performed as recommended by the supplier. All samples were determined in duplicates and the dissolved black market product was measured at dilutions of 1:2000 and 1:4000.

2D-PAGE

For 2D-PAGE, 50 ng of black market and recombinant human FGF-1 were mixed with multichatotropic sample solution (7.7 M urea, 2.2 M thiourea, 44 mM Tris, 4.4% CHAPS) to a total volume

of 100 µl. Moreover, an ampholyte solution was added in a concentration of 0.5% to optimize the focusing of the proteins. Reduction of disulfide bonds was accomplished with 5 µmol of DTT for 45 min at room temperature (RT). Afterwards, cysteine residues were derivatized with 15 µmol of acrylamide for 45 min at RT. Excess acrylamide was eliminated with further 10 µmol of DTT for 10 min at RT prior to rehydration loading of the samples to 7 cm, non-linear IPG strips with a pH gradient of 3–10. Isoelectric focusing (IEF) was performed overnight in an Ettan IPGphor 2 (GE Healthcare) with the following voltage gradient: 300 V until 200 Vhs, 300–1000 V within 300 Vhs, 1000–5000 V within 4500 Vhs and the voltage was halted at 5000 V until the focusing was stopped the next morning. The maximum current per strip was set to 50 µA. Following IEF, strips were equilibrated twice for 10 min in 1.5 ml of LDS sample buffer. For SDS-PAGE, 8 cm, 12% Bis-Tris gels were used again (1.0 mm thick, 1-well, 125 V, 90 min). After staining with Coomassie Blue, gels were digitalized using the light transmission scanner. The protein composition of the different spots was determined by means of in-gel tryptic digestion and LC-MS/MS experiments as described above.

OFFGEL fractionation

The proteinogenic ingredients of the black market product were fractionated based on their pI and in liquid phase using the Agilent 3100 OFFGEL Fractionator System (Agilent, Waldbronn, Germany) in combination with a 12-well frame set and 13 cm IPG strips ranging from pH 3 to 10. For this purpose, 24 mg of lyophilisate were dissolved in 500 µl of MilliQ water and 360 µl of this solution were mixed with 1.44 ml of 1.25 x protein OFFGEL stock solution (8.4 M urea, 2.4 M thiourea, 80 mM DTT, 6% Glycerol, 1.2% ampholytes pH 3–10) according to the manufacturer's instructions. After rehydration of the IPG strip, 150 µl of the sample were applied to each well and fractionation was performed over night using an OFFGEL default method for proteins. Afterwards, the different fractions were analyzed by means of SDS-PAGE, silver staining, in-gel tryptic digestion, and LC-MS/MS (*vide supra*).

Results and discussion

Identification of FGF-1 in a black market product

The lyophilized contents of the unlabelled injection vials provided by the German customs were initially analyzed for proteinogenic components by means of SDS-PAGE. Figure 2 shows the SDS-PAGE gel separating aliquots of the ingredients of two injection vials. Several bands with molecular weights between 10 and 150 kDa are visible. In order to identify these proteins, gel bands were subjected to in-gel tryptic digestion and analyzed by LC-MS/MS. Most of the bands contained several characteristic plasma proteins or fragments such as albumin (Band 1–8, 10, and 11), hemopexin (Band 4) and haptoglobin (Band 4 and 11). By contrast, Band 9 with a molecular weight of approximately 16 kDa comprised FGF-1. Although FGF-1 was at that time a rather unknown growth factor in conjunction with black market products, its application in the treatment of sports injuries is restricted under the terms of the World Anti-Doping Agency (WADA) anti-doping code since 2010.^[12] For this reason, a further characterization and quantification of the black market FGF-1 were conducted.

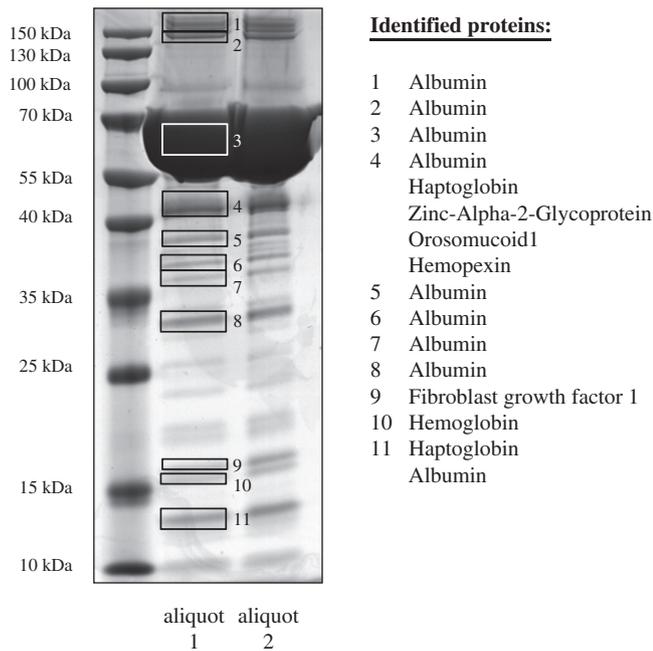


Figure 2. SDS-PAGE gel (Coomassie staining) of the black market product. The encircled and numbered bands were subjected to in-gel tryptic digestion and mass spectrometric identification.

Quantification of black market FGF-1

For the determination of the FGF-1 content in the black market product, at first a quantification by means of SDS-PAGE was used. Figure 3 shows the SDS-PAGE gel with the calibration curve from 5 to 100 ng of recombinant human FGF-1. From the dissolved black market product containing FGF-1, 20 µl were applied to the gel. By measuring the band volumes of the calibration curve and the black market product with ImageQuant™ TL, an amount of 36.9 ng FGF-1 per 20 µl solution and therefore 1.9 µg FGF-1 per injection vial was estimated.

Moreover, an ELISA test specific for FGF-1 was used to confirm the FGF-1 concentration determined by SDS-PAGE. It yielded an

amount of 1.6 µg FGF-1 per injection vial and therefore lies in the same range as the previously measured value.

Determination of physiological FGF-1 values in plasma and urine

Additionally, the ELISA was used to determine physiological FGF-1 values in urine and plasma. A total of 15 plasma and 15 urine samples was collected from healthy volunteers and stored at -20 °C until analysis. Only 8 out of 15 urine samples and none of the plasma samples contained detectable amounts of FGF-1. All measured values were below the lowest standard with 31.2 pg/ml (data not shown). As free FGFs are rapidly degraded by proteases, they increase their stability *in vivo* through binding to heparin and heparan sulfate proteoglycans present at the cell surface and in the extracellular matrix.^[2,3,19] Thus, FGFs are not only protected against proteases but also regulated in their interaction with FGF receptors. Moreover, the extracellular matrix acts as a storage depot for FGFs, releasing them only in case of cell death or damage. Therefore, only very low amounts of FGF-1 in plasma and urine were to be expected.

Further characterization of black market FGF-1

Comparing the electrophoretic behavior of black market and recombinant human FGF-1, SDS-PAGE revealed slight differences concerning their molecular weight. As depicted in Figure 4, the black market FGF-1 appears slightly smaller than the recombinant human FGF-1. For this reason, both forms of FGF-1 were analyzed by means of different proteolytic digestions and MS/MS experiments. Figure 5 shows the sequence coverage of recombinant and black market FGF-1 as accomplished by proteolytic digestions with trypsin, Lys-C and Asp-N. For both forms of FGF-1, a high sequence coverage of 82% and 72%, respectively was accomplished. But while for recombinant FGF-1 both the characteristic N-terminal peptide containing the attached methionine-residue (Figure 6) and the C-terminal peptide were detected, the N-terminal peptide of the black market FGF-1 remained undetected. Therefore, it can be assumed that an N-terminally

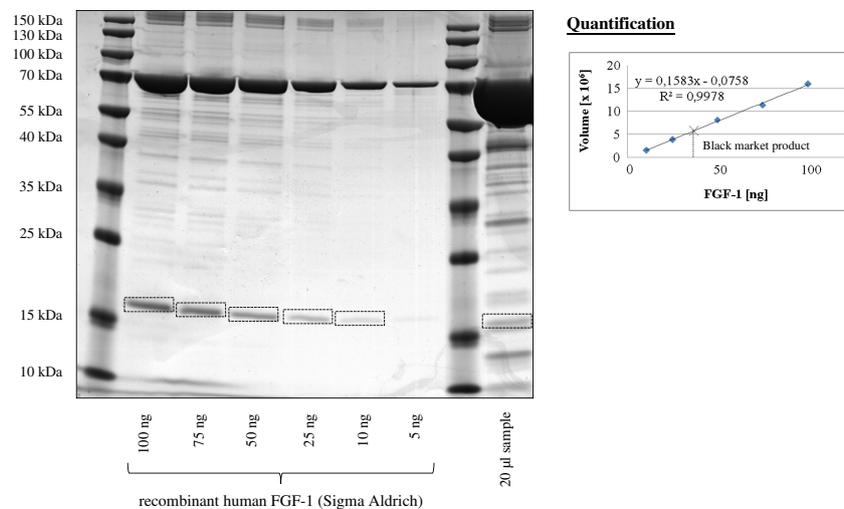


Figure 3. Quantification of FGF-1 by means of SDS-PAGE (Coomassie staining). Using ImageQuant™ TL for the determination of the band volumes, an amount of approximately 1.9 µg FGF-1 per injection vial was estimated.



Figure 4. Mass difference between recombinant human and black market FGF-1 (Coomassie-stained SDS-PAGE gel).

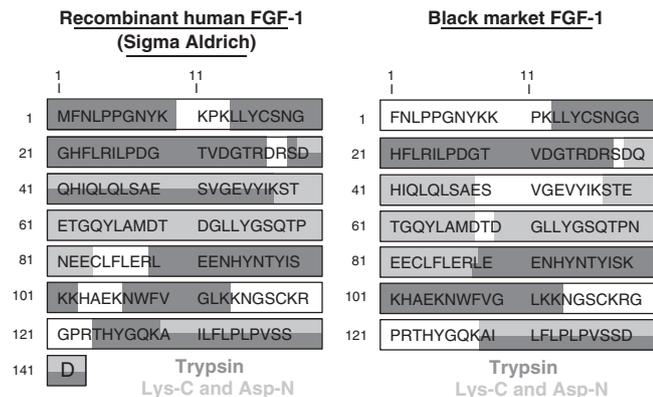


Figure 5. Sequence coverage of recombinant human and black market FGF-1 as accomplished by in-gel proteolytic digestions with Trypsin (dark grey) and a combination of Lys-C and Asp-N (light grey).

truncated or modified form of FGF-1 is present in the black market product, which needs to be further characterized.

2D-PAGE of FGF-1

In order to elucidate the assumed truncation or modification of the black market FGF-1, 2D-PAGE was performed revealing especially differences concerning the isoelectric point (pI). Figure 7

shows the 2D-PAGE gels of black market and recombinant human FGF-1. Both gels display two spots of FGF-1, which have isoelectric points of approximately pH 7.7 and 8.5. Recombinant human FGF-1 (A) has a theoretical pI of 7.73.^[20,21] The faint spot at pH 7.7 can be attributed to this protein. The unexpected second protein observed at pH 8.5, however, requires further characterization to specify the reason for the pH shift. For the black market FGF-1 (B), the 2D-gel shows the same spot pattern, which allows the assumption that the potential truncation has only little or no influence on the pI of the protein.

OFFGEL fractionation

In addition to the above reported experiments, a pI-based OFFGEL fractionation was performed to further purify and characterize the truncated or modified black market form of FGF-1. The obtained fractions were analyzed by means of SDS-PAGE, silver staining, in-gel tryptic digestion and LC-MS/MS. Figure 8 shows the SDS-PAGE gels of the 12 OFFGEL fractions, the unfractionated black market product and FGF-1 reference material. Fraction 10 clearly displays a band with a molecular weight of approximately 15 kDa, in which FGF-1 was identified according to the previously described bottom-up proteomic approaches. Therefore, the OFFGEL fractionation turns out to be an appropriate approach to fractionate complex samples like the FGF-1 black market product for further analysis steps like SDS-PAGE.

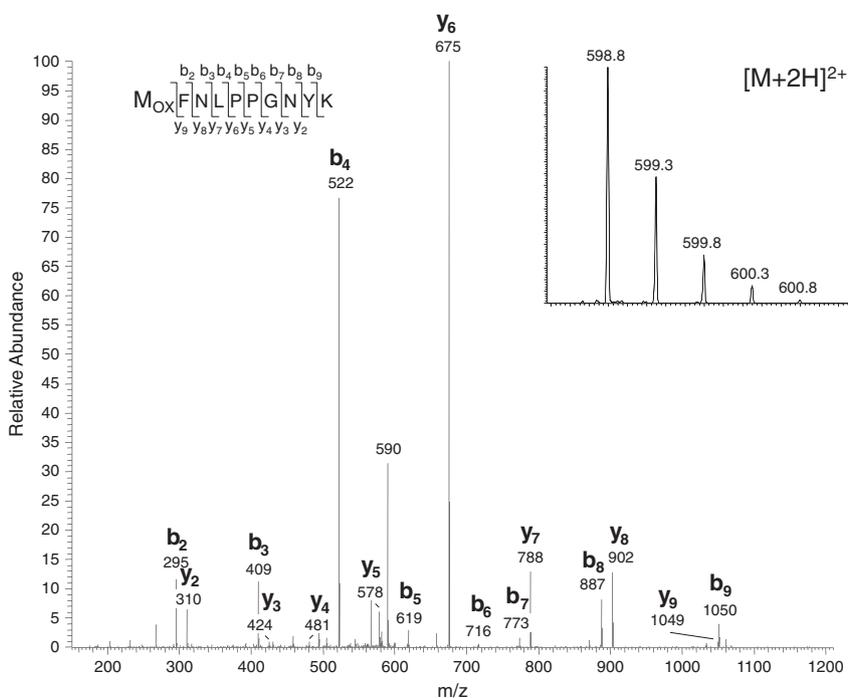


Figure 6. Nano-ESI product ion spectrum of the N-terminal tryptic peptide M_{ox} [FNLPPGNYK] (AA: 1–10; RT: 22.82 min, m/z : 598.8) of recombinant human FGF-1.

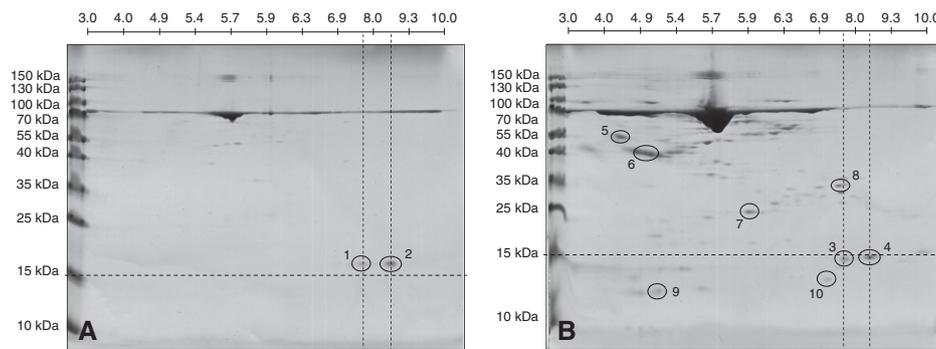


Figure 7. Coomassie-stained 2D-PAGE gels (pH 3-10NL) of recombinant human FGF-1 (A) and the black market product (B). The encircled spots 1–10 were applied to in-gel tryptic digestion and mass spectrometric identification. Thus, the following proteins were identified: Spots 1–4 – Fibroblast growth factor 1; Spot 5 – Alpha-2-HS-glycoprotein and Leucine-rich alpha-2-glycoprotein 1; Spot 6 – Zinc-alpha-2-glycoprotein, Spot 7 – Albumin, Spot 8 – Albumin and Keratin 1; Spot 9 – Haptoglobin, Spot 10 – Albumin.

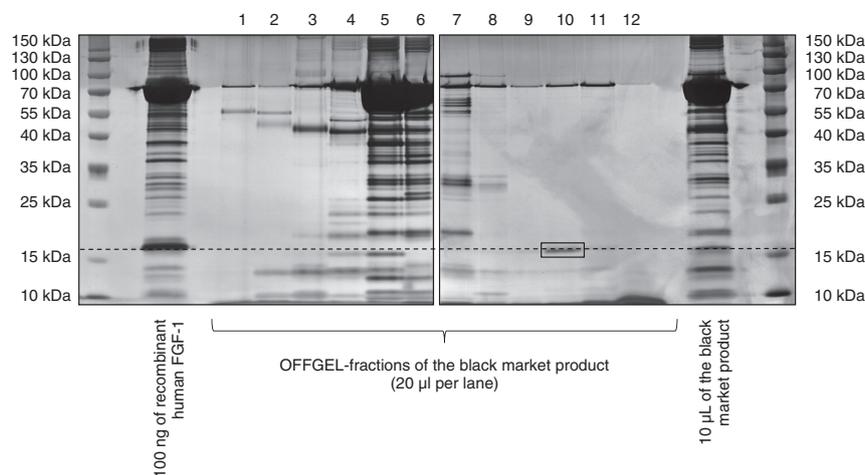


Figure 8. SDS-PAGE gels (silver staining) of 100 ng of recombinant human FGF-1, 10 µL of unfractionated black market product and 20 µL of each of the 12 OFFGEL fractions. The encircled band in fraction 10 contained FGF-1 at a sequence coverage of 28%.

Concluding remarks

In this report, the identification of an unknown truncated or modified form of FGF-1 was described, which was found in an injection vial confiscated by the German customs. Considering the facts, that the World Anti-Doping Agency has prohibited the use of FGFs in sports since 2010 and that FGF-1 was isolated from a lyophilisate probably intended for subcutaneous or intramuscular injection, it can be assumed that the identified product was intended as a performance-enhancing agent.

However, the treatment of sports injuries is not the only potential application for FGF-1. The positive effect of FGF-1 on the proliferation of fibroblasts has also been of interest to the cosmetics industry using FGF-1 as new ingredient of anti-aging skin care products targeting the reduction of wrinkles and the rejuvenation of the skin.^[22,23] But as these cosmetics are superficially applied to the skin and therefore distributed as creams or oily sera, it is rather unlikely, that the confiscated injection vial was intended as a beauty product.

Due to the fact that up to now no drug has achieved clinical approval, there are no data available concerning the appropriate dosage of FGF-1 administered topically or by injection. Therefore, it cannot be assessed if an amount of 2 µg FGF-1 per injection vial

as determined for the black market product is therapeutically relevant.

However, these findings demonstrate that potential drugs without clinical approval can be obtained easily from the black market, regardless of potential dangerous consequences for the consumer.

Acknowledgements

The presented work was conducted with support of the Federal Ministry of the Interior of the Federal Republic of Germany, the Instituto do Desporto de Portugal, and the Manfred-Donike Institute for Doping Analysis (Cologne, Germany).

References

- [1] Y.R. Yun, J.E. Won, E. Jeon, S. Lee, W. Kang, H. Jo, J.H. Jang, U.S. Shin, H.W. Kim. Fibroblast growth factors: biology, function, and application for tissue regeneration. *J. Tissue Eng.* **2010**, *2010*, 218142.
- [2] D.M. Ornitz, N. Itoh. Fibroblast growth factors. *Genome Biol.* **2001**, *2*, Reviews 3005.
- [3] M. Zakrzewska, E. Marcinkowska, A. Wiedlocha. FGF-1: from biology through engineering to potential medical applications. *Crit. Rev. Clin. Lab. Sci.* **2008**, *45*, 91.

- [4] B. Thisse, C. Thisse. Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev. Biol.* **2005**, *287*, 390.
- [5] L. Hutley, W. Shurety, F. Newell, R. McGeary, N. Pelton, J. Grant, A. Herington, D. Cameron, J. Whitehead, J. Prins. Fibroblast growth factor 1: a key regulator of human adipogenesis. *Diabetes* **2004**, *53*, 3097.
- [6] M. Zakrzewska, D. Krowarsch, A. Wiedlocha, J. Otlewski. Design of fully active FGF-1 variants with increased stability. *Protein Eng. Des. Sel.* **2004**, *17*, 603.
- [7] J.F. Culajay, S.I. Blaber, A. Khurana, M. Blaber. Thermodynamic characterization of mutants of human fibroblast growth factor 1 with an increased physiological half-life. *Biochemistry* **2000**, *39*, 7153.
- [8] S. Nikol, I. Baumgartner, E. Van Belle, et al. Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. *Mol. Ther.* **2008**, *16*, 972.
- [9] American Heart Association. 2010 clinical trial/clinical science abstracts. *Circulation* **2010**, *122*, 2215.
- [10] WADA. Prohibited List 2011. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/To_be_effective/WADA_Prohibited_List_2011_EN.pdf [December 2010].
- [11] L. Creaney, B. Hamilton. Growth factor delivery methods in the management of sports injuries: the state of play. *Brit. J. Sports Med.* **2008**, *42*, 314.
- [12] 2010 Prohibited List - Summary of Major Modifications. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Summary_of_Modifications_2010_EN.pdf [April 2011].
- [13] A. Thomas, M. Kohler, J. Mester, H. Geyer, W. Schänzer, M. Petrou, M. Thevis. Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement. *Drug Test. Analysis* **2010**, *2*, 144.
- [14] M. Kohler, A. Thomas, K. Walpurgis, K. Terlouw, W. Schänzer, M. Thevis. Detection of His-tagged Long-R(3)-IGF-I in a black market product. *Growth Horm. IGF Res.* **2010**, *20*, 386.
- [15] M. Kohler, A. Thomas, H. Geyer, M. Petrou, W. Schänzer, M. Thevis. Confiscated black market products and nutritional supplements with non-approved ingredients analyzed in the cologne doping control laboratory. *Drug Test. Analysis* **2010**, *2*, 533–537.
- [16] M. Thevis, A. Thomas, M. Kohler, S. Beuck, W. Schänzer. Emerging drugs: mechanism of action, mass spectrometry and doping control analysis. *J. Mass Spectrom.* **2009**, *44*, 442.
- [17] WADA. Technical Document - TD2010IDCR. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20Assays_May%2008%202010_EN.doc.pdf [June 2011].
- [18] M. Thevis, *Mass Spectrometry in Sports Drug Testing - Characterization of Prohibited Substances and Doping Control Analytical Assays*. John Wiley & Sons, New Jersey, **2010**.
- [19] M. Zakrzewska, A. Wiedlocha, A. Szlachcic, D. Krowarsch, J. Otlewski, S. Olsnes. Increased protein stability of FGF1 can compensate for its reduced affinity for heparin. *J. Biol. Chem.* **2009**, *284*, 25388.
- [20] ExPASy Proteomics Server. Available at: <http://expasy.ch/> [March 2011].
- [21] Universal Protein Resource (UniProt). Available at: <http://www.uniprot.org> [March 2011].
- [22] Serum Cellular Repair - not just another anti-aging product. Available at: <http://www.esheesthetic.com/product.php?SKU=1234> [March 2011].
- [23] Dermatropin. Available at: <http://natropin.net/dermatropin.htm> [March 2011].