Drug Testing and Analysis

Published online in Wiley Interscience:

(www.drugtestinganalysis.com) DOI 10.1002/dta.29

Detection of the protease Bacillolysin in doping-control urine samples†

Introduction

Industrial, pharmaceutical and biochemical applications for proteases have a long history and proteolytic processes have been examined and understood for decades.[1] In contrast, the adulteration of urine samples in sports drug testing may represent a more urgent and recent phenomenon in the detection of protein-based prohibited substances.[2–5] Nowadays, enormous amounts of various peptidases are produced in bacteria in order to customize every proteolytic problem in industrial or biochemical implementation. Universal mixtures of proteases for optimal digestion under different conditions (such as pH, temperature and time of activity) are commercially available allowing proteolysis under all kinds of external conditions. Cheating sportsmen thus have a large variety of effective proteases to choose from, which needs to be taken into account when conducting doping analysis of protein-based performance-enhancing agents. This case report presents the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-mass spectrometry (LC-MS) approaches for the detection of manipulation using a protease in doping-control urine samples.

Case Report

Two athletes' urine samples arrived in the laboratory to be analysed for proteases. Suspicion was aroused after routine erythropoietin analyses were unsuccessful and the isoelectric focusing method used for EPO detection[6] showed a 'trace of burning'.[2] Analysis of the total protein in the urine specimens was performed by SDS-PAGE and, after suspicious results were obtained, solid phase extraction (SPE) and liquid chromatography-(tandem) mass spectrometry (LC-MS(/MS)) were performed, followed by a Swissprot database search for confirmation and identification of the protease as recently described.[3]

Four confiscated powders with unknown content were also to be analysed for potential protease content. Aqueous solutions (~10 mg/mL) were applied to SDS-PAGE and LC-MS(/(MS)) analysis as described also for urine samples.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of the urine samples and the powders was carried out as a screening procedure and this proved the presence of protease activity in the urine samples. This is shown in Figure 1. All urine samples on the gel represent a concentrate of 2 mL of urine. Lane 1 shows a blank urine sample with a typical protein pattern. Lanes 2 and 3 illustrate the doping control samples, which do not contain a normal protein pattern when compared with the blank sample but show only small amounts of proteins with low molecular weight, indicating degradation products of urinary proteins. Lanes 5 and 6 show the doping control samples after addition of two proteins (myoglobin and growth hormone) and incubation for 30 min at 37 °C to prove the proteolytic activity of the samples. Lane 4 is an aqueous solution of the added proteins in the same amounts that were added to the urine specimen. Lane 4 shows two clear bands for the two proteins but these are not visible in the urine samples, proving the degradation of proteins by proteases in the samples.

Lanes 7–10 represent the four powders (10 µg of powder per lane) and do not show signs of intact proteases or any medium to high molecular protein. Lanes 11 and 12 show two blank urine samples that were fortified with two proteases, Subtilisin or Bacillolysin. While addition of Subtilisin results in complete digestion of the proteins in the sample, Bacillolysin is less effective and some proteins as well as the protease band (< the 37 kDa molecular weight maker) are visible on the gel.

For LC-MS analysis, 1 mL of urine was extracted by SPE, the eluate was dried under reduced pressure and reconstituted in 2% acetic acid. Proteolysis and autolysis peptide products were separated by capillary- or nano-LC and analysed by mass spectrometry. The powder solutions were subjected to mass spectrometry without further preparation. Mass spectrometry files, including a full scan with accurate masses of precursor ions and automatic, data dependent MS/MS experiments of multiply charged ions, were subjected to a database search using Bioworks 3.3 with Swissprot/Uniprot database (2006, Thermo, Bremen). The peptide criteria for the identification of a protein were adapted from an earlier proposal.[3,7] For identification of a protein, at least two peptides (mass error <10 ppm) with a sequence coverage >10% must be detected and the retention time should not deviate more than 2% from a reference standard. The results of the database search are shown in Figure 2 for one of the powders (powder 2 from Figure 1) and one of the urine samples (sample 2 from Figure 1). All four powders contained Subtilisin and Bacillolysin from *Bacillus amyloliquefaciens,* confirmed by high-sequence coverages for Bacillolysin (P06832, 41% to 78%) and Subtilisin (P00782, 30% to 65%). Moreover, in three out of the four powders (powders 2–4) Aspergillopepsin A from *Aspergillus awamori* (P17946) was detected and another Subtilisin produced in *Bacillus subtilis* (P35835) was identified and fulfilled the identification criteria (sequence coverage 12% to 26%, 4–11 peptides). Interestingly, the combination of the three proteases covers the whole pH range: an alkaline protease (Subtilisin), a neutral protease (Bacillolysin) and an acidic enzyme (Aspergillopepsin), giving the appearance of a mixture to cover the degradation of all kinds of urinary proteins. The two urine samples contained Bacillolysin, as confirmed by sufficient sequence coverage of 17% and 33% and the detection of 5 and 14 peptides, respectively. A Bacillolysin standard (2 µL/mL of

* *Correspondence to: Mario Thevis, Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany. E-mail: thevis@dshs-koeln.de*

† *Articles in this section are subject to screening for scientific accuracy but do not undergo the traditional model of peer review.*

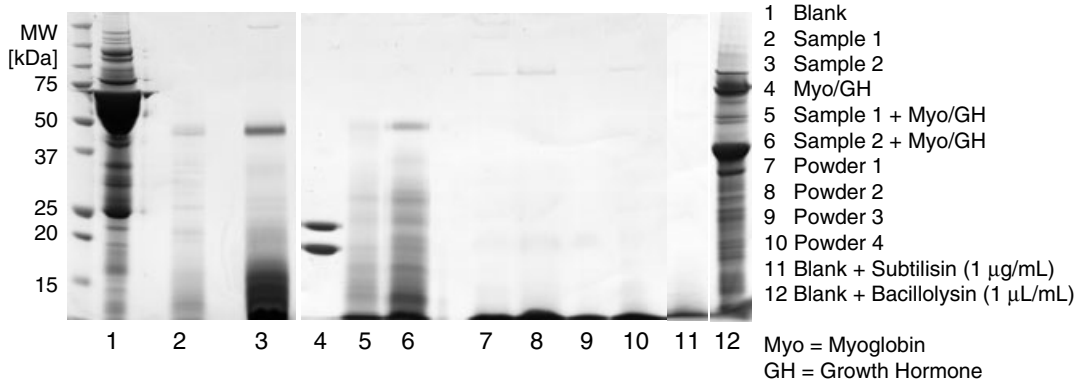

Figure 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of doping control samples, samples fortified with myoglobin/growth hormone, confiscated powders and blank urine samples fortified with proteases.

Powder 2

retention time (min)	Peptides	MH+	ΔM (ppm)	z
Bacillolysin(EC 3.4.24.28, P06832)				
1.39	N.YKNLPNTDAGDYGGVHTN.S	1935,88	0,92	3
13.30	P.NTDAGDYGGVHTN.S	1320,55	0,46	2
13.71	K.YGQPDNFKN.Y	1082,49	0,91	2
14.38	N.YKNLPNTDAGD.Y	1207,56	2,37	2
14.45	N.YKNLPNTDAGD.Y	1207,56	2,37	2
14.60	D.YGGVHTNSGIPNKAA.Y	1485,74	1,18	3
14.63	S.LSNPTKYG.Q	879,46	2,66	2
14.70	D.AGDYGGVHTNSGIPNK.A	1586,76	1,12	3
14.72	N.SGIPNKAAYNT.I	1135,57	1,98	2
15.03	H.TNSGIPNKAAYNT.I	1350,66	2,64	2
15.15	L.VSSTTNQFTTSSQ.R	1387,63	1,86	2
15.17	S.LSNPTKYGQPD.N	1219,60	2,54	2
15.19	L.VSSTTNQFTTSSQ.R	1387,63	1,86	2
15.24	Y.KNLPNTDAGDYGGVH.T	1557,73	1,69	3
15.28	D.AGDYGGVHTNSGIPNKAA.Y	1728,83	2,23	3
15.29	R.SLSNPTKYG.Q	966,49	1,68	2
15.31	D.AGDYGGVHTNSGIPNKAA.Y	1728,83	2,54	3
15.34	R.SLSNPTKYG.Q	966,49	2,12	2
15.37	N.TDAGDYGGVHTNSGIPNK.A	1802,83	2,25	3
15.41	Y.KNLPNTDAGDYGGVHTN.S	1772,82	2,36	3
15.52	S.LSNPTKYGQP.D	1104,57	1,89	2
15.55	R.SLSNPTKYGQPDN.F	1420,67	2,09	2
15.56	S.LSNPTKYGQP.D	1104,57	1,77	2
15.57	Y.KNLPNTDAGDYGGVHTN.S	1772,82	2,44	2
15.98	N.TDAGDYGGVHTNSGIPNKAA.Y	1944,90	1,74	3
16.25	S.LSNPTKYGQPDNFKN.Y	1722,84	2,27	3
16.26	R.SLSNPTKYGQP.D	1191,60	1,81	2
16.32	S.LSNPTKYGQPDNFKN.Y	1722,84	2,10	2
16.50	L.NYENQPGALNE.S	1248,55	1,56	2
16.70	N.YKNLPNTDAGDYGGVH.T	1720,79	2,12	2
16.72	N.YKNLPNTDAGDYGGVHTN.S	1935,88	2,25	2
16.73	R.SLSNPTKYGQPDNFKN.Y	1809,88	2,37	3
16.78	N.YKNLPNTDAGDYGGVHTN.S	1935,88	2,50	2
16.82	R.SLSNPTKYGQPDNFKN.Y	1809,88	2,18	2
16.86	N.LPNTDAGDYGGVHTN.S	1530,68	2,56	2
16.87	N.LPNTDAGDYGGVH.T	1315,59	1,14	2
16.89	R.SLSNPTKYGQPDNFKN.Y	1809,88	2,65	2
16.91	N.LPNTDAGDYGGVHTN.S	1530,68	2,48	2
16.93	D.AGDYGGVHTNSGIPNKAAY.N	1891,89	2,31	3
16.95	T.TGTGTTLKGKTVSL.N	1363,78	2,55	3
17.23	N.YKNLPNTDAGDY.G	1370,62	1,75	2
17.30	K.YGQPDNFKNY.K	1245,55	1,89	2
17.46	D.LYGSQDAASVE.A	1139,52	0,98	2
17.77	T.LVSSTTNQFTTSSQ.R	1500,72	1,83	2
18.12	T.LVSSTTNQFTTS.S	1285,63	1,97	2
18.21	R.AAVDAHYNL.G	973,47	1,54	2
18.27	L.SKPTGTQIITYD.L	1323,68	2,16	2
18.38	T.LVSSTTNQF.T	996,50	1,01	2
18.39	N.LNYENQPGALNES.F	1448,67	1,85	2
18.56	R.SLSNPTKYGQPDNFKNY.K	1972,94	1,79	3
18.60	N.LNYENQPGALNE.S	1361,63	2,00	2
....	....			

Sequence coverage: 60 %

retention time (min)	Peptides	MH+	ΔM (ppm)	z
Powder 2 Subtilisin (EC 3.4.21.62. P00782)				
14.71	A.AAGNEGTSGSSSTVGY.P	1444.62	2.56	2
15.14	N.PFQDNNSHGTHVAGTVA.A	1751.81	1.86	3
15.84	S.TLPGNKYGAYN.G	1197.59	2.06	2
16.08	Q.STLPGNKYGAYN.G	1284.62	2.27	2
16.48	D.SSNQRASFSSVGPE.L	1452.67	2.07	2
16.61	Q.STLPGNKYGAYNGT.S	1442.69	3.17	2
17.29	Y.AQSVPYGVSQ.I	1035.51	1.17	2
17.42	T.TTKLGDSF.Y	868.44	1.28	2
17.74	A.AAGNEGTSGSSSTVGYPGKYP.S	1986.90	2.54	2
17.87	E.NTTTKLGDSF.Y	1083.53	1.45	2
18.08	Y.PGKYPSVIA.V	931.52	1.20	2
18.43	D.KAVASGVVVVA.A	999.62	0.89	2
18.48	L.ENTTTKLGDSF.Y	1212.57	1.30	2
19.53	N.MSLGGPSGSAALKAA.V	1317.68	2.63	2
19.93	D.SSNQRASFSSVGPELD.V	1680.78	3.07	2
20.54	Y.GAYNGTSMASPHVAGAAAL.I	1745.83	3.23	2
20.87	D.SSNQRASFSSVGPEL.D	1565.76	2.72	2
21.23	R.SSLENTTTKLGDSF.Y	1499.72	2.97	2
21.27	R.SSLENTTTKLGDSF.Y	1499.72	2.24	2
23.38	K.VLGADGSGQYSW.I	1239.56	2.83	2
23.54	K.VAGGASMVPSETNPFQD.N	1706.77	3.33	2
25.42	K.VLGADGSGQYSW.I	1239.56	0.76	2
26.05	Y.GAYNGTSMASPHVAGAAALIL.S	1972.00	3.21	2
28.70	A.GTVAALNNSIGVLGVAPSASL.Y	1911.05	3.35	2

Sequence coverage: 60 %

Sample 2

Bacillolysin(EC 3.4.24.28, P06832)				
12.97	H.AATTGTGTTLKGK.T	1206,67	2,55	2
13.25	A.TTGTGTTLKGK.T	1064,59	1,07	2
13.37	T.KIGVNKAEQ.I	986,56	1,73	2
13.41	S.STFKDAKAA.L	938,49	2,23	2
13.91	K.FNRNSYDNKGGK.I	1399,67	0,83	3
14.64	S.SQRAAVDAHYN.L	1231,58	1,29	3
15.66	N.SYDNKGGKIVS.S	1167,60	0,70	2
16.24	T.ITKIGVNK.A	872,56	1,75	2
16.70	M.THGVTQETANLN.Y	1284,62	1,98	2
16.81	T.ITKIGVNKAEQ.I	1200,69	1,39	2
18.59	Y.LTPSSTFKDAKAA.L	1336,71	2,31	2
18.76	K.IVSSVHYGSRY.N	1267,64	2,05	2
21.40	L.SKPTGTQIITY.D	1208,65	1,02	2
21.76	R.AAVDAHYNLGKVY.D	1420,72	2,43	2

Sequence coverage: 33 %

Figure 2. Results of the Swissprot database search exemplarily for powder 2 and sample 2.

urine, Sigma P1236) added to a blank urine sample was measured to confirm the database search. The retention time criteria were met and peptides of Bacillolysin from *Bacillus amyloliquefaciens* with a sequence coverage of 41% were identified. Obviously, Bacillolysin belongs to the class of proteases that is autolysed and proteolysed slowly and therefore it was the only protease detected in the doping-control specimens. Due to the production of Bacillolysin in the same bacteria as Subtilisin, it may also occur as contamination from the production process and may enable or facilitate the detection of proteases if Subtilisin was used. For both doping-control samples, adverse analytical findings with regard to tampering were reported.

Discussion

Mass spectrometry-based analytics is the most common tool for the unique and specific identification of banned substances in doping control. This procedure is commonly used to determine small target analytes with low ng/mL concentrations and well defined criteria for identification. Recently, this technique was also adopted for the unambiguous determination of peptides or proteins with performance-enhancing potential by means of liquid chromatography coupled with tandem mass spectrometry.[8,9] These procedures share a common approach of searching for known and well defined target analytes with elucidated metabolic fate. In the present case report, doping control was advanced by a technique that sought out unstable and dynamic targets in the respective specimens. The proteolytic process provides a complete heterogeneous spectrum of peptides, which is not dirigible. Thus, manual elucidation of the acquired raw data is not possible and computer-assisted database searches are necessary to identify the characteristic peptides occurring in urine.

This case report provides an example of the identification of proteases in urine using a state-of-the-art analytical approach.

Yours,

MAXIE KOHLER,[a] ANDREAS THOMAS,[a] HANS GEYER,[a] LUIS HORTA,[b] WILHELM SCHÄNZER,[a] MARIO THEVIS[b]*

[a] Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Germany

[b] Laboratorio de Analises de Dopagem, Instituto do Desporto de Portugal, Lisboa, Portugal

References

[1] N. Vasantha, L. D. Thompson, C. Rhodes, C. Banner, J. Nagle, D. Filpula, *J. Bacteriol.* **1984**, *159*, 811.
[2] M. Thevis, J. Maurer, M. Kohler, H. Geyer, W. Schänzer, *Int. J. Sports Med.* **2007**, *28*, 545.
[3] A. Thomas, M. Kohler, K. Walpurgis, W. Schänzer, M. Thevis, *Drug Test. Anal.* **2009**, *1*, 81.
[4] M. Thevis, W. Schänzer, *Curr Proteomics.* **2005**, *2*, 191.
[5] M. Thevis, M. Kohler, W. Schänzer, *Drug Discov. Today* **2008**, *13*, 59.
[6] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, *Anal. Biochem.* **2002**, *311*, 119.
[7] M. Thevis, J. A. Loo, R. R. Loo, W. Schänzer, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 297.
[8] M. Thevis, W. Schänzer, *Anal. Bioanal. Chem.* **2007**, *388*, 1351.
[9] M. Thevis, W. Schänzer, *Analyst* **2007**, *132*, 287.