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Identification of proteolytic enzymes in human urine

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Introduction

Beside compounds with low molecular weight protein drugs like erythropoietin (EPO) and insulin have been used by athletes to increase the chance of success. Recently, there was discussion about the possible abuse of proteases, because they might efficiently degrade these substances if added to the urine doping control sample. This and the fact, that proteases are easily available (over-the-counter drugs, dishwasher detergent, laundry powder etc.) are reasons why they may be of interest to athletes.

In this study more than 100 urine samples of athletes as well as standards of proteases and many blank urine samples have been analysed. The proteolytic activity was determined using fluorescence-based detection kits and 1D-SDS-PAGE [1]. Characteristic proteins were identified with mass spectrometry. Furthermore, blank urine samples were spiked with proteases. As a result endogenous and recombinant erythropoietin was eliminated completely. Beyond, it became evident that the urinary proteome depends on the pH value as well as on both the temperature and the period of storage. Resulting proteome pattern may misleadingly imply the abuse of proteases.

Our results indicate that the addition of proteases can be discovered anyway. Many proteases (or their products of cleavage in the case of autolysis) may be identified in low concentrations using mass spectrometry (up to 5µg/mL). After in-gel-digestion with trypsin we focus on the detection of unambiguous nonhuman peptides.

Methods

Sample preparation for EPO analysis

Three blank urine samples (25mL each) were spiked with EPO (30µL; 1200 U/L). An aliquot of each sample was spiked additionally with proteases (20µg/mL). After incubation (60min, 37°C) all samples were prepared according to the protocol for EPO analysis.

Sample preparation for the determination of the proteolytic activity

A Fluorescent Detection Kit (Sigma-Aldrich, PF0100) was used to determine protease activity of urine samples. Following the protocol 10µL of sample were mixed with 20µL of incubation buffer and 20µL of FITC-Casein substrate in an 1.5mL tube. After incubation at 37°C in the dark for 60min 150µL of 0.6M trichloroacetic acid were added. Incubation at 37°C for another 30min was followed by centrifugation (10min, 10000 x *g*) to precipitate the intact insoluble Casein substrate. Centrifugation was repeated if some casein-conglomerates were still found to float at the surface. Ten µL of the supernatant were added to 1mL of assay buffer (If necessary the tip of the pipette was mechanically cleaned with a small peace of tissue to remove residues). A volume of 200µL of this solution was transferred to a multiwell plate. Fluorescence intensity was recorded with excitation at 485nm monitoring the emission wavelength of 535nm.

Sample preparation for 1D-SDS-PAGE

Four to eight mL of urine sample were deep frozen (-80°C) and thawed (water bath) to increase precipitation. When performing experiments with spiked samples the protease was added prior to the first freezing step. After centrifugation (5min, 4000 x *g*) the supernatant was transferred to a filter device (AMICON Ultra-4, 10,000 MWCO). Another 25min of centrifugation (4000 x *g*) yielded 50-80µL concentrate of which up to 10µL were used for gel electrophoresis.

If more than 5mL of sample had to be used the centrifugate of a first 5mL-centrifugation step was transferred to another tube and the remaining sample volume (e.g. 3mL) was filtered through the same filter device. Centrifugation time was adjusted accordingly.

1D-SDS-PAGE

Electrophoresis was performed using a mixture of 10 (to 20)µL sample concentrate with 5µL (to 7.5µL) sample buffer and 2µL (to 3µL) 1M DTT. After incubation at 70°C (10min) the solutions were transferred to the wells of a NuPAGE Novex Bis-Tris Gel (Invitrogen). MOPS buffer was used as elution buffer (25mL + 475mL deion. H_2O). Antioxidant (NuPAGE, 250µL/100mL) was added to the Upper Buffer Chamber.

one gel: 200V, 40mA, ~110min

two gels: 200V, 80mA, ~110min

After electrophoresis the gels were washed with deion. H_2O (3 times à 5min), fixed with 80mL MeOH / 7% HOAc (15min) and washed again with deion. H_2O (10min) before staining with Coomassie Blue (5-10mL per gel).

Sample preparation for MSMS

A relevant band of an SDS-PAGE experiment was transferred to a 1.5mL-tube to perform in-gel-digestion with modified trypsin according to a general procedure:

100µL of 100mM NH_4HCO_3 and CH_3CN (1:1) were added to the gel spots. After 10min the supernatant was removed and the spots were dehydrated with 35µL CH_3CN for another 10 minutes before the spots were dried in a vacuum centrifuge (~5min).

The samples were reduced adding 10-20µL of 10mM (1,54mg/mL) 1,4-Dithiothreitol (DTT) in 50mM NH_4HCO_3 and heating for 60min at 60°C. After cooling to room temperature 10-20µL of freshly prepared iodoacetic acid in 50mM NH_4HCO_3 were added followed by incubation at 45°C (45min).

Prior to the digestion the samples were treated with 50µL of 100mM NH_4HCO_3 (10min). Again, after 10min the supernatant was removed and the spots were dehydrated with 25µL of CH_3CN (10min). After removal of any remaining supernatant the last two steps (washing and dehydrating) were repeated. Finally, the samples were dried in a vacuum centrifuge for 5min.

The gel spots were then treated with 5-10µL of 50mM containing 10µg/µL modified trypsin storing the samples for 45min at 4°C. Another 10-20µL of 100mM NH_4HCO_3 were added before incubation/digestion overnight at 37°C.

To recover the peptides the gel spots were first washed with 10-20µL deion. H_2O discarding any supernatant and then extracted 3 times with 50% CH_3CN containing 1% triflouroacetic acid (100µL/10min each). The combined extracts were dried in a vacuum centrifuge (~1h) and resuspended in 20µL of 0.1% HAc/0.005% TFA (97 parts) and ACN (3 parts).

MS: Orbitrap, Thermo Fisher Scientific

LC: Agilent 1100 Series (A: 0.1% HAc/0.01% TFA; B: ACN+0.1%/0.01% TFA)

Results and Discussion

Effect of proteases on urinary proteins and their identification

The addition of 20µg/mL protease to urine samples results in a complete lack of Erythropoietin (EPO) as can be seen in figure 1. Since incubation time is very short an abuse of proteases is conceivable.

In routine analysis there are samples with little or no EPO. To determine if the lack of EPO in these samples is due to protease activity a commercially available fluorescent detection kit purchased from Sigma-Aldrich (PF0100) has been used. The proteolytic activity of blank and spiked urine samples (20µg/mL protease) can be easily differentiated (see figure 2).

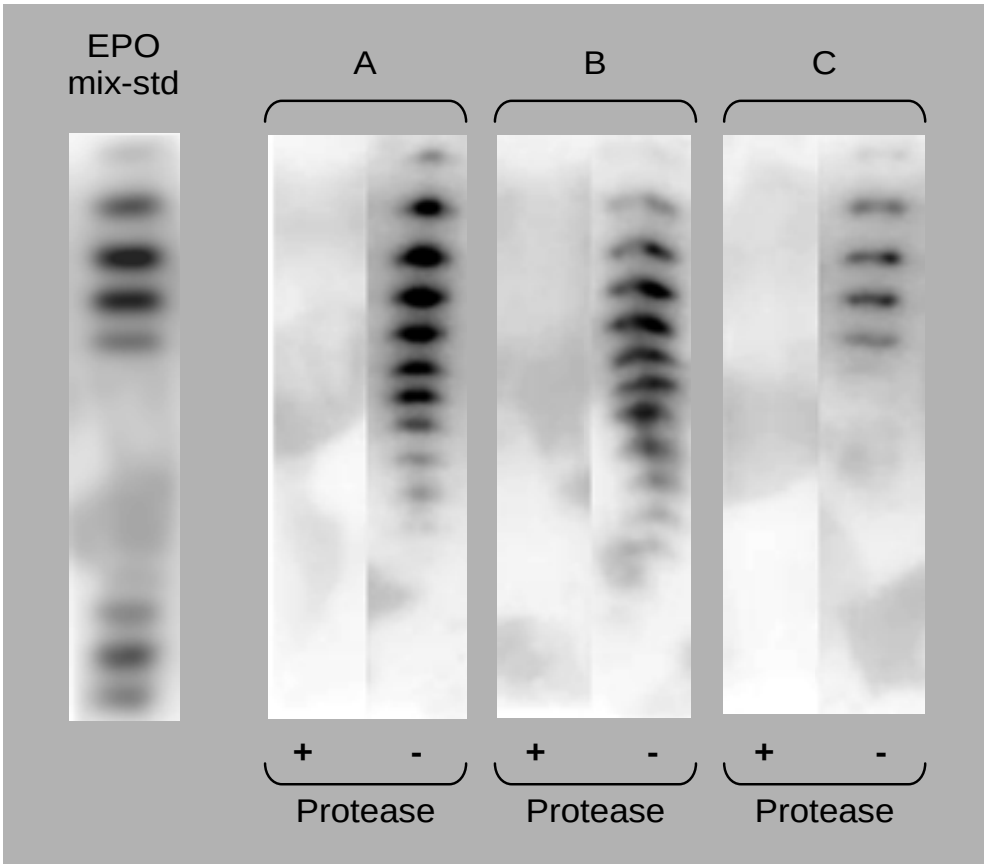

Fig.1: Results of EPO analysis after IEF and immuno-blotting of protease-spiked and blank urine samples.

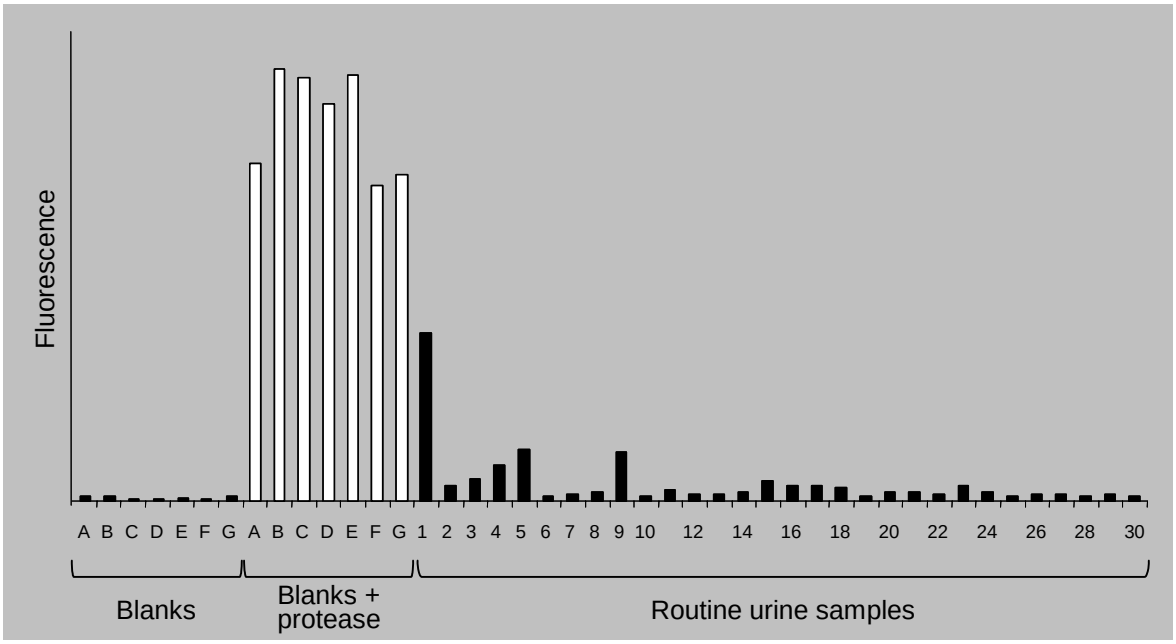

Fig.2: Indirect determination of protease activity of blank and spiked urine samples and routine doping control samples.

Additionally, randomly chosen routine doping control samples were analysed. Most of the corresponding fluorescence values are in the range of blank samples. Only samples 1, 4, 5 and 9 show an increased protease activity (see figure 2). These samples do not necessarily represent positive doping cases because some diseases may lead to a higher excretion of endogenous proteases. As a consequence, the determination of proteolytic activity may only be applied as a relatively economic screening method. Further analysis of the urinary proteome by SDS-PAGE is suggested.

As can be seen in figure 3 the relative concentrations of proteins in urine samples may vary. Some protein pattern of the blank urine samples do even resemble those of spiked urine samples (fig 4).

Apparently, the lack of proteins in a sample cannot be regarded as a sufficient criterion for positive doping cases. Consequently, it is important to identify proteases in a sample which is possible using mass spectrometry.

In order to find out if exogenous proteases may be distinguished from endogenous ones a 25kDa bovine chymotrypsin peptide was prepared (see methods). Analysis and calculation of one of the obtained MSMS spectra (see figure 5) yielded the amino acid sequence YNSLTINNDITLLK. The sequence shows only slight but significant differences to the corresponding human chymotrypsin one (FSILTVNNDITLLK) making distinction possible.

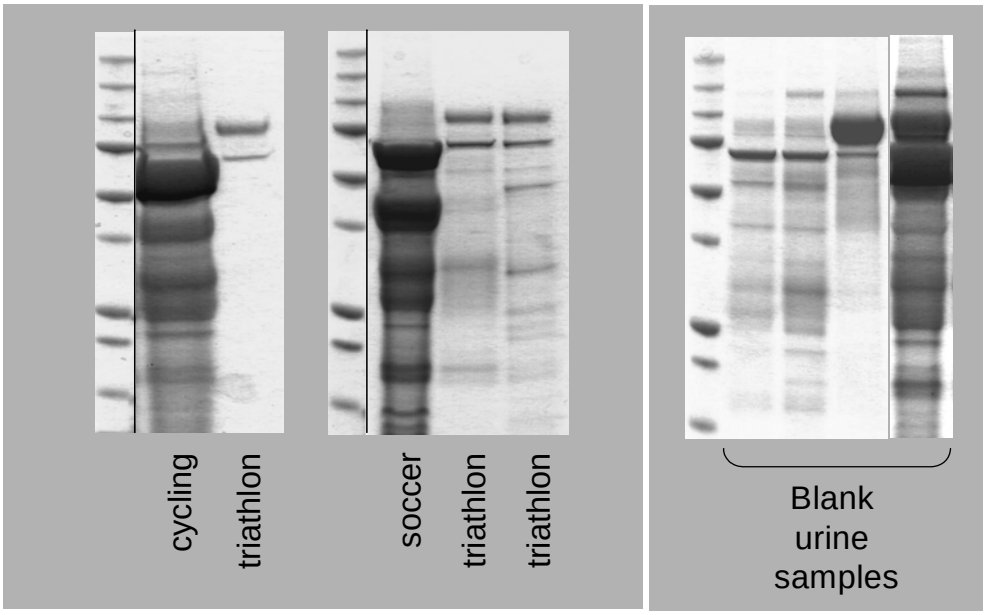

Fig.3: Urinary proteome pattern of routine doping control samples (left) and blank urine samples (right);

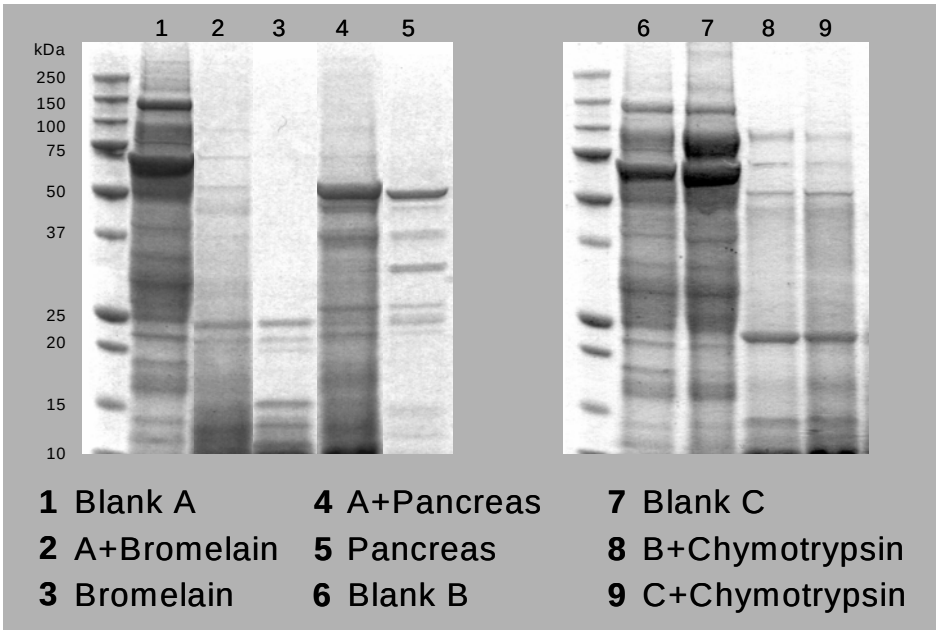

Fig.4: Urinary proteome pattern of blank urine samples (1, 6, 7), spiked urine samples (2, 4, 8, 9) and standard solutions of proteases (3, 5).

Stability of the urinary proteome and proteases

With respect to storage of doping control samples experiments were performed to investigate the stability of the urinary proteome and proteases added to urine samples. Duration of storage, temperature and the pH value were parameters of interest.

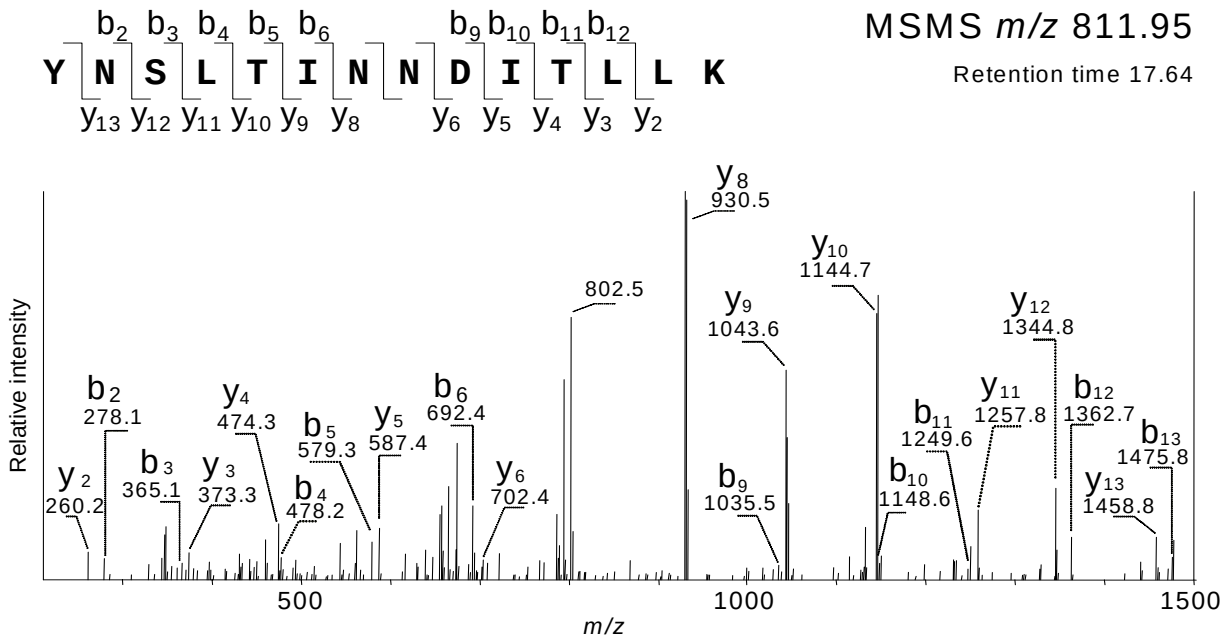

Fig.5: MSMS spectrum of a 25kDa bovine chymotrypsin peptide after in-gel-digestion.

Two blank urine samples were adjusted to the pH values 5, 6, 7 and 8, respectively and the samples were stored at -20°C, +4°C and room temperature. As can be seen in figure 6a) there is no obvious change in the proteome pattern at -20°C. Similar results were obtained for the samples stored at +4°C (not shown).

By contrast, there is a change in the pattern after three days when storing the samples at room temperature (figure 6b). The samples with a pH value greater than 6 are characterized by sever degradation. Measurement of nitrite implies bacterial activity in these samples. It is noteworthy, that any bacterial activity can apparently be reduced to a minimum by adjusting the pH of a sample. Although it is unlikely in these days that doping control samples are exposed to room temperature for such a long time the results provide evidence that the lack of proteins in a urine sample does not necessarily indicate an abuse of proteases.

To examine the stability of proteases in the urine matrix some blank urine samples were spiked with chymotrypsin and trypsin and stored at -20°C, +4°C and room temperature, respectively. No obvious autolysis was observed after 6 days even at room temperature (figure 7). The intensities of the characteristic bands at 25 to 20kDa representing chymotrypsin and trypsin do not change. Thus, the handling and storage of doping control samples as performed in these days does not seem to interfere with a potential identification of proteases investigated in this study.

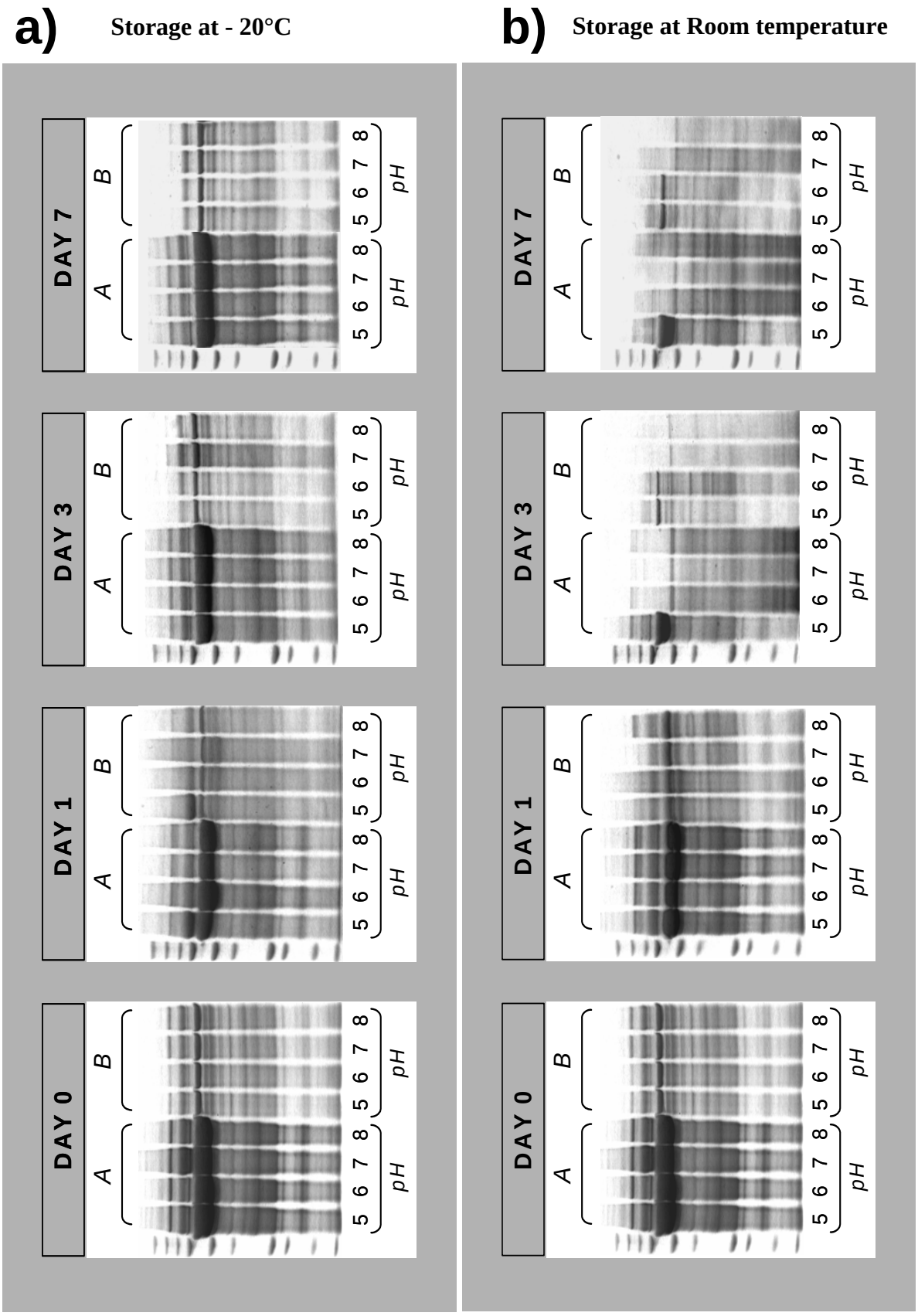

Fig.6: Urinary proteome of blank urine samples versus different pH values and the period of storage: Samples were stored at a) -20°C and b) at room temperature prior to workup

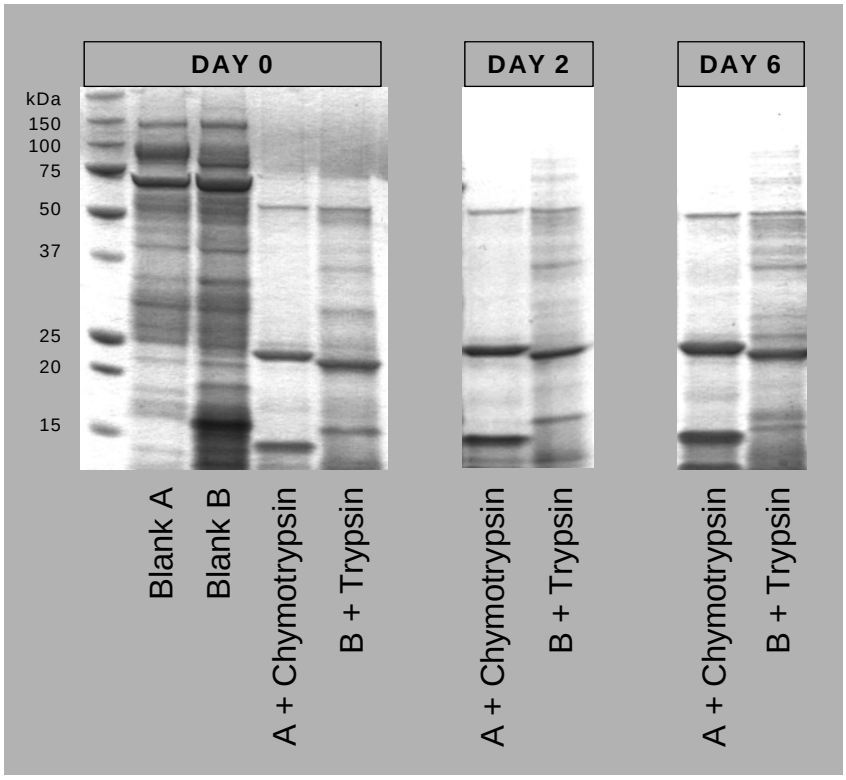

Fig.7: Urinary proteome of blank urine samples and samples spiked with chymotrypsin and trypsin with respect to the period of storage.

Conclusion

The urinary proteome may be degraded effectively by proteases. Many proteases are stable and no autolysis is observed even at room temperature. The stability is a requirement in order to identify proteases in a sample by means of mass spectrometry or – indirectly – the determination of the proteolytic activity. Commercially available fluorescence-based detection kits provide a relatively economic screening method for proteolytic activity although the information obtained with these kits is limited. When interpreting urinary proteome pattern it has to be kept in mind that the proteome may be altered depending on the pH, the temperature and the period of storage, resulting protein pattern that misleadingly might imply the abuse of proteases. This, and the fact, that there are proteases that are endogenous and exogenous points out the need of an unambiguous identification of proteases. The combination of 1D-SDS-PAGE and MSMS meets these requirements.

References

[1] Thevis, M., Maurer, J., Kohler, M., Geyer, H., Schänzer, W. (2007) Proteases in Doping Control Analysis. *International Journal of Sports Medicine* **28**, 545-549