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Enzymatic digestion of EPO

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Introduction

The glycoprotein erythropoietin (EPO) has been on WADA's Prohibited List since 1990. Detection of EPO-abuse is based on the different isoelectric profiles of endogenous and recombinant EPO. Recently, it has been rumoured that athletes add chemicals containing proteolytic enzymes to their urine samples in order to remove traces of recombinant EPO (rEPO). Additionally, the number of analyzed urines void of EPO-signal has allegedly increased. In our own material from a major championship the IEF-profile for EPO was undetectable in 25 % of the samples.

Proteases, enzymes that break down proteins, can have a variety of sources as they are found naturally in our body, in plants and in fruits (i.e. bromelain in pineapple and papain in papaya), and they are added to laundry detergents as spot removal aid. In an attempt to resolve possible routes of cheating with enzymatic digestion of EPO, we wanted to determine the amount of laundry detergent needed to break down detectable EPO in urine and to see whether drinking or adding the juice from a protease-rich fruit like pineapple could have a similar EPO-degradable effect.

Methods

Powdered laundry detergent, pure protease (Subtilisin A) or pineapple juice was added to urines spiked with recombinant EPO, followed by incubation conditions trying to mimic the handling of athlete urine samples. In addition, pineapple juice and the bromelain-containing nutritional supplement MyoRepair was administered orally to a volunteer and urine collected in the following 12 hours. In order to test the efficiency of protease inhibitors in preventing protein breakdown in manipulated urine samples, the protease inhibitor cocktail Complete was added to urine before the addition of the proteases. After addition of protease (and inhibitor when stated), the urine samples (37 °C at t=0) were incubated at RT for 2 hours, followed by over night storage at 4 °C, unless otherwise stated. This setup was meant to mimic the handling of athlete urine samples. The samples were analysed by the standard

EPO-protocol including IEF-double blotting and chemiluminescent detection (Lasne et al., 2002). The effect of different protease inhibitors on Subtilisin A was tested using a protease assay kit (EnzChek from Molecular Probes), where a fluorescence-labelled casein derivative becomes highly fluorescent by protease-catalyzed hydrolysis. The resulting fluorescent signal was measured in a LAS 5000 scanner.

Results

Drinking 1 L of pineapple juice or swallowing a single dose of MyoRepair capsules equivalent to 800 mg bromelain had no apparent effect on endogenous EPO in urine in the following 12 hours (not shown). Adding juice directly to the urine, on the other hand, totally changed the IEF-profile, shifting the bands from the endogenous to the basic region of the gel (Figure 1C). This suggested that the pineapple proteases (bromelain) most likely are inactivated by metabolism in the gastrointestinal tract.

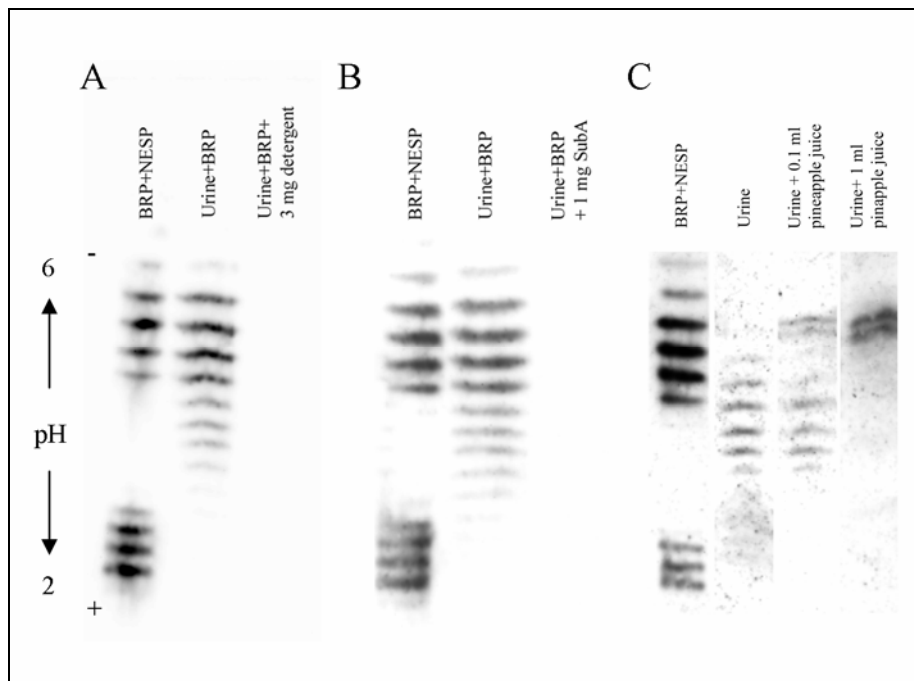


Figure 1: Direct manipulation of urines with proteases

A) Laundry detergent (3 mg) or B) pure protease (Subtilisin A, 1 mg) was added to urine samples (20 ml, 37 °C) spiked with 0.45 ng BRP and incubated for 2 h at RT. C) Pineapple juice (0,1 or 1 ml) was added to 20 ml urine and incubated for 2 h at RT. All samples were stored at 4 °C over night prior to ultrafiltration, IEF-double blotting and chemiluminescence detection according to the standard EPO-protocol.

Further, small amounts of protease were sufficient to remove both the endogenous and the added recombinant EPO-signal, from urine (Figure 1A and B).

We were interested to see whether we could detect proteolytic activity in manipulated urine samples after freezing. Powdered laundry detergent or pure protease was added to urines spiked with recombinant EPO (to obtain a stronger reference signal) and frozen. After storage at -20°C for several days, darbepoetin α was added to the urines, and the urine samples were analysed with the standard protocol as before. Enzymes added in about 2 to 3 times the amount needed to remove endogenous and added recombinant EPO on day 1 (i.e. 5 mg laundry detergent and 3 μg Subtilisin A, not shown), is sufficient to digest additionally added protein (darbepoetin α) after storage for several days at -20°C , implying that about 40-70 %, but not all, of the initial protease activity was lost during freezing (Figure 2A and B).

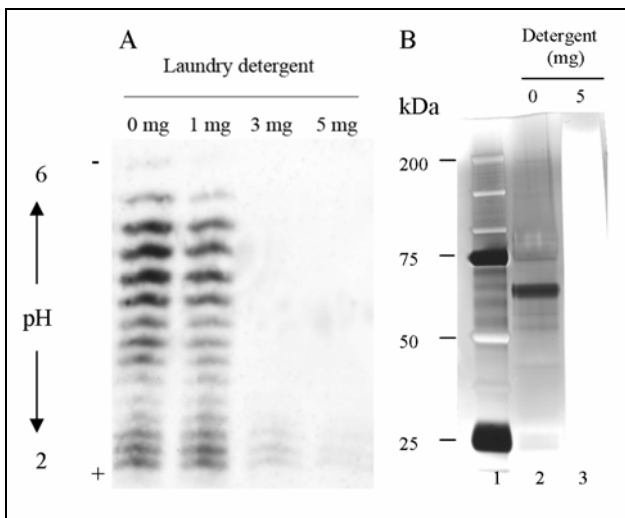


Figure 2: Protease stability

A) 20 ml urine was spiked with 0.45 ng BRP and 0, 1, 3 or 5 mg laundry detergent was added prior to 2 h incubation at RT. The samples were frozen (-20°C , 4 days), thawed and spiked with NESP (0.25 ng). After 2 h incubation in RT, the samples were stored overnight at 4°C , before ultrafiltration and IEF-double blotting according to standard EPO-protocol. B) Spiked samples treated as in A with no (lane 2) or 5 mg (lane 3)

laundry detergent, were run on SDS-PAGE (4-12%) and silver stained for total protein. 5 mg detergent removed all traces of protein (lane 3). Lane 1: Molecular weight standard.

We wanted to see if we could prevent the protein degrading effect of proteases by adding inhibitors to the urine prior to the proteases. The protease inhibitor cocktail Complete did not visibly inhibit the proteolytic activity of 3 mg laundry detergent (Figure 3A) or 10 μg Subtilisin A (Figure 3B) when added to 20 ml urine. This was also the case when the samples were not frozen (not shown). We then tested different serine and cysteine protease inhibitors using a protease assay kit. Very high inhibitor concentrations were needed to cause an effect, and none of the tested inhibitors were able to fully inactivate 0.15 $\mu\text{g}/\text{ml}$ Subtilisin A (Figure 4).

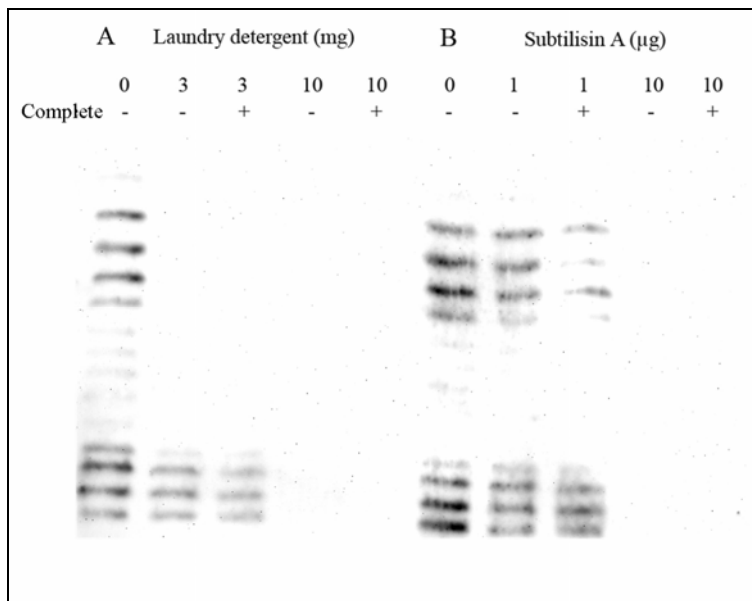


Figure 3: Effect of Complete inhibitor
 Samples were treated as described in Fig. 2. In addition, some of the samples were treated with 800 µl of the protease inhibitor Complete prior to adding the protease. The presence of the inhibiting cocktail had no visible effect on the proteolytic effect of neither laundry detergent (A) nor Subtilisin A (B). The protease activity was reduced by freezing the samples.

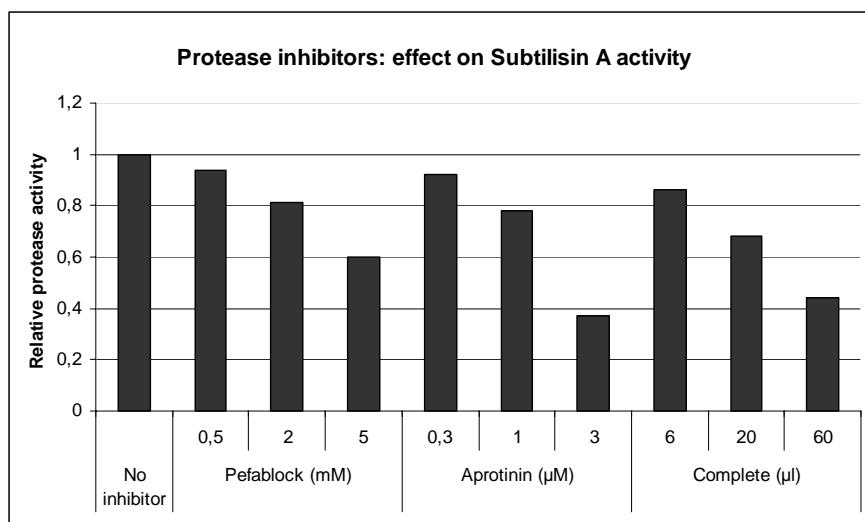


Figure 4: Protease inhibitors' effect on Subtilisin A

Different serine protease inhibitors were added to 0.15 µg/ml Subtilisin A (equals 3 µg in 20 ml) in 10 mM PBS pH 7.3, prior to a fluorescently labelled casein derivative (EnzChek Protease Assay Kit). Both Pefablock and Aprotinin were tested at concentrations 3x higher

than the upper range given by the manufacturer (Sigma), and 60 µl Complete in this assay equals 30x the concentration used in the EPO-procedure. Despite the high concentrations, none of the tested inhibitors were able to fully inhibit the catalytic activity of Subtilisin A.

Summary

Minute amounts of protease have the proteolytic capacity of degrading all proteins present in urine. Adding protease inhibitors in relatively high concentrations is not sufficient to quench the proteolytic activity of laundry detergent or Subtilisin A. The presence of proteolytic activity in doping control samples is possible to detect using a protease detection kit.

References: Lasne, F., Martin, L., Crepin, N. and de Ceaurriz, J. (2002) Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant EPO. *Anal Biochem* **311**, 119-126.