"Engineering bacterial arylsulfatases for high activity towards alpha-configured steroid sulfates: new mutant enzymes to improve doping control."

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Project Overview

When athletes dope the drugs are changed by the body and excreted in the urine. These drug metabolites must be processed by anti-doping laboratories to enable detection using a range of sophisticated techniques. An enzyme called beta-glucuronidase, isolated from Escherichia coli bacteria, is routinely used by anti-doping labs to process samples prior to analysis. It has become an essential tool used by analysts in the fight against doping. Unfortunately, this beta-glucuronidase enzyme only works on some drug metabolites called glucuronides leaving others called sulfates unprocessed, and so doping may go undetected. Creating a mild and universal enzyme to process sulfate metabolites would significantly improve anti-doping analysis.

In earlier WADA-funded research we engineered an enzyme from the bacterium Pseudomonas aeruginosa called an arylsulfatase that is able to process the sulfate metabolites that E. coli beta-glucuronidase cannot. Our work improved enzyme activity for testosterone sulfate hydrolysis by over 270-fold and increased the substrate scope. However, the activity for some drug metabolites remained low leading to inefficient hydrolysis. In this project will employ laboratory-based methods of rapid evolution to enhance the substrate scope of the P. aeruginosa arylsulfatase enzyme for anti-doping applications.

The project outcome will be mild and universal arylsulfatase enzymes for processing drug metabolites that will complement E. coli beta-glucuronidase. The new enzyme will be rigorously evaluated by the WADA-accredited Australian Sports Drug Testing Laboratory. Including the improved enzyme in the methods used to process drug metabolites will increase the sensitivity of analysis and allow doping to be detected for a longer period after an athlete takes a banned drug. We expect this improved P. aeruginosa arylsulfatase will join E. coli beta-glucuronidase and also become an indispensable tool used by anti-doping laboratories in the fight against doping.

Results and Conclusions:

Steroid abuse still makes up a large proportion of the incidences of doping in world sport. This abuse leaves tell-tale metabolites in the urine. To date, anti-doping labs have focussed on one class of steroid metabolites: those with glucuronide conjugates. There is now a wealth of evidence suggesting that another class, steroids with sulfate conjugates can in some cases provide longer lasting markers of doping. However, steroid sulfates are difficult to detect by gas chromatography-mass spectrometry (GC-MS) methods that are essential for producing evidence in suspected cases of doping.

Before GC-MS analysis, steroid metabolites must be prepared by first hydrolysing the glucuronide or sulfate conjugates. Most glucuronides can be efficiently hydrolysed by a bacterial enzyme, but no general sulfatase enzyme is available to hydrolyse the sulfate esters. The aim of this WADA-funded project was to engineer a sulfatase enzyme to meet this need.

WADA's first grant for sulfatase engineering (WADA 13A13MM) allowed us to find and optimise mutations in *Pseudomonas aeruginosa* sulfatase (PaS). The best combinations of mutation allowed PaS to hydrolyse testosterone sulfate (TS) 150 times faster than the original bacterial enzyme. However, this version of PaS, like its predecessors was biased towards steroids with a beta configured hydroxyl group, such as TS, or dehydroepiandrosterone sulfate (DHEAS). The alpha configured steroid sulfates such as epitestosterone sulfate (ETS), androsterone sulfate (AS) or etiocholanolone sulfate (ECS) were hydrolysed thousands of times slower, if at all.

This one year WADA follow-up grant (WADA 16A06MM) has allowed us to take one PaS variant (named PVFV-PaS) that had significant ECS activity and engineer it towards the alpha configured steroid sulfates. We used genetic engineering to prepare thousands of PaS genes with mutations scattered in regions that we had discovered to be important for binding steroid sulfates. Our assays examined thousands of these variants in microlitre-scale reactions to find the best mutations for ECS hydrolysis. The work resulted in the identification of several new beneficial mutations: the best combination resulted in 15 times more activity towards ECS compared with PVFV-PaS. Over two WADA-funded projects, we have taken an enzyme with no detectable activity for alpha configured steroid sulfates and have prepared a variant with enough activity to be applied in anti-doping laboratories. Our work has also developed the know-how to prepare gram quantities of this purified enzyme from two litres of bacterial culture: enough to process more than a litre of urine samples for GC-based analysis.

The project also tested several PaS variants with pooled urine samples to evaluate which endogenous steroids could be detected when compared with no treatment or a typical beta-glucuronidase treatment. The GC-mass spectrometry method detected 38 steroids, 14 of which were enhanced by PaS treatment. That is, without PaS treatment, 14 steroid sulfates did not contribute to the GC-MS steroid profile. Further analysis revealed that eight steroid signals were enhanced by PaS treatment compared with the industry standard beta-glucuronidase and five were enhanced by using an engineered PaS variant compared with the original PaS enzyme.

In conclusion, we have developed sulfatases that can hydrolyse many of the steroid sulfates important for anti-doping analysis under similar conditions as already used for steroid glucuronide hydrolysis. The studies have revealed fundamental knowledge about the sulfatase enzymes (*ACS Catal.* **2018**, *8*, 8902−8914) and have found application in sample preparation prior to anti-doping analysis (*Drug Test. Analysis* **2017**, *9*, 1695-1703; *Analytica Chimica Acta* **2018**, *1030*, 105-114).