DETERMINATION OF THE RATE LIMITING STEP DURING ZEARALENONE HYDROLYSIS BY ZENA

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Key Words: pre-steady state kinetics, mycotoxin degradation, enzyme engineering, zearalenone

Zearalenone (ZEN) is a mycotoxin produced by different *Fusarium* species, including *Fusarium graminearum* and *Fusarium culmorum* which infect major crop plants worldwide. Biological decontamination is a favorable method to reduce ZEN concentration in contaminated feed. Previously we identified, cloned and produced a ZEN hydrolyzing alpha/beta hydrolase from a gram-positive soil bacterium, taxonomically assigned to the species *Rhodococcus erythropolis*. This enzyme was named $ZenA_{Re}$. $ZenA_{Re}$ hydrolyzes zearalenone to hydrolyzed zearalenone (HZEN) which then decarboxylates further to decarboxylated hydrolyzed zearalenone (DHZEN). Due to low heat stability and susceptibility to low pH it was considered unfit for industrial application as feed additive active in the animal gastro intestinal tract. The homologous enzyme from *Streptomyces coelicoflavus* ($ZenA_{Scfl}$) showed higher thermostability, but with higher K_m and lower k_{cat}. It was used as starting point for enzyme engineering, resulting in a variant with lower K_m and increased catalytic efficiency ($ZenA_{Scfl041}$). All three enzymes were subjected to pre-steady state enzyme kinetics to elucidate their reaction mechanism and identify the rate limiting step as a basis for further targeted engineering.

It was shown that substrate binding and affinity of all variants is significantly driven by conformational change (enzyme closure). $ZenA_{Re}$ revealed the highest affinity to the substrate. This is partially related to increased binding affinity but also to the fast kinetics of the consecutive enzyme isomerization (closure) which brings the complex to the reactive mode. A six-step kinetics mechanism, including the enzyme isomerization steps, was proposed for all three variants. The rate-limiting step differs between $ZenA_{Scfl}$ variants and $ZenA_{Re}$. Both, $ZenA_{Scfl}$ and $ZenA_{Scfl041}$, are limited by the first chemical step forming the intermediate form. $ZenA_{Re}$ is limited by the second chemical step (hydrolysis of the intermediate) at a temperature above 298 K. The rate-limiting step is shifted to the enzyme opening at the temperature below this temperature threshold. This shift of the rate-limitation is also visible by non-linearity of the Arrhenius plot at a similar temperature range. The results showed that different ZenA variants may need different approaches for improvement of kinetics by enzyme engineering.