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Steady state kinetics and regioselectivity for variants of StEH1 hydrolyzing different epoxide substrates

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Abstract

Solanum tuberosum epoxide hydrolase 1 (StEH1) originates from potato. It catalyzes the hydrolysis of epoxides into corresponding vicinal diols. In a previous study, directed evolution was used to gradually mutate the residues in the active site of StEH1. The mutants were designated RC1, RC1B1, RC1B1D33 and RC1B1D33E6. In this project, RC1, RC1B1 and RC1B1D33 were purified. Steady state kinetics for wild type StEH1 and the mutants RC1, RC1B1, RC1B1D33 and RC1B1D33E6 was studied spectrophotometrically together with the substrates R,R-TSO; S,S-TSO; *cis*-SO; R-4-NSO and S-4-NSO. All enzyme variants had a higher k_{cat}/K_m when hydrolyzing S,S-TSO and S-4-NSO compared to when they hydrolyzed R,R-TSO and R-4-NSO respectively. All mutants showed a decreased k_{cat}/K_m with S,S-TSO and R,R-TSO compared to the wild type. None of the mutants showed any drastic improvements in enzyme activity with *cis*-SO compared to the wild type. RC1B1D33 showed an 8-fold increase in k_{cat}/K_m in the hydrolysis of S-4-NSO compared to the other enzyme variants. Chiral HPLC was used to measure regioselectivity for StEH1 wt with *cis*-SO. It showed that the nucleophilic attack occurs exclusively at one specific carbon atom, giving R,R-hydrobenzoin as only product. The conclusion from these results could facilitate the development of a more enantio- and regiospecific enzyme and a greener line of chemical production.

Abbreviations

CASTing Combinatorial Active-site Saturation Testing

***cis*-SO** *cis*-stilbene oxide

[E] concentration of free enzyme

EH epoxide hydrolase

[EP] concentration of enzyme-product complex

[ES] concentration of enzyme-substrate complex

HPLC High Performance Liquid Chromatography

IPTG Isopropyl β-D-1-thiogalactopyranoside

[P] concentration of free product.

PCR Polymerase Chain Reaction

R-4-NSO R-4-nitro styrene oxide

Rac-NSO racemic nitro styrene oxide

R,R-TSO R,R-*trans*-stilbene oxide

[S] concentration of free substrate

S-4-NSO S-4-nitro styrene oxide

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

S,S-TSO S,S-*trans*-stilbene oxide

StEH1 *Solanum tuberosum* epoxide hydrolase 1

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1. Introduction

1.1 Enzyme catalysis

Enzymes catalyze chemical reactions by stabilizing the transition state, thereby lowering the activation energy so that equilibrium is reached faster. Enzymes are very selective and many of them can only catalyze one specific reaction. This specificity is due to the structure of the active site which allows only the right substrate to fit.

Enzymes can be utilized in the chemical industry when it is necessary to produce only one of the enantiomers of a chiral molecule. For instance, they can be used in drug manufacturing where the drug needs to function together with chiral biomolecules. One enantiomer can then act as a medicine while the other in worst case is toxic. If substrates are allowed to react in a non-chiral environment the product, if chiral, is most likely to be racemic. It might then be hard to separate the desired isomer from the other. In order to manufacture enantiopure products, enzymes might be of use. One advantage of using enzymes to catalyze chemical reactions is their ability to function in aqueous solutions without requiring excessive amounts of heat. They are therefore safer and more environmentally friendly than many techniques used today, important factors in green chemistry. The enzyme should also be able to function in moderate concentrations of organic solvents if it is to be used in the chemical industry. That is since many steps in organic synthesis can only be performed in organic solvents.

1.2 Epoxides and epoxide hydrolases

Epoxides, also known as oxiranes, consist of two carbon atoms and one oxygen atom forming a triangle. Due to their electrophilic nature and highly strained ring structure, epoxides are reactive and can interfere with DNA, thereby causing cancer (Morisseau and Hammock 2005). That is why epoxide hydrolases, EHs, play an important role in the metabolism of toxic compounds. They do so by catalyzing the hydrolysis of epoxides into corresponding vicinal diols in an S_N2-type reaction with water as the only co-substrate (Morisseau and Hammock 2005). EHs can also regulate signal molecules containing epoxide groups (Thomaeus *et al.* 2007).

In reactions catalyzed by EHs, one of the product enantiomers is often produced in a greater quantity then the other. Moreover, EHs don´t require cofactors which make them good candidates for development of efficient biocatalysts (Morisseau and Hammock 2005).

1.3 StEH1

The enzyme studied in this project is *Solanum tuberosum* epoxide hydrolase 1 (StEH1) which originates from potato. The crystal structure of StEH1 has been solved by molecular replacement with a resolution of 1,9 Å (Mowbray et al. 2006). StEH1 belongs to the α/β-hydrolase fold enzymes. Enzymes in this family consist of a β-sheet as a core surrounded by several α-helixes and also a helical lid domain (Figure 1). A catalytic triad in the core together with two tyrosine residues in the lid domain constitutes the active site.

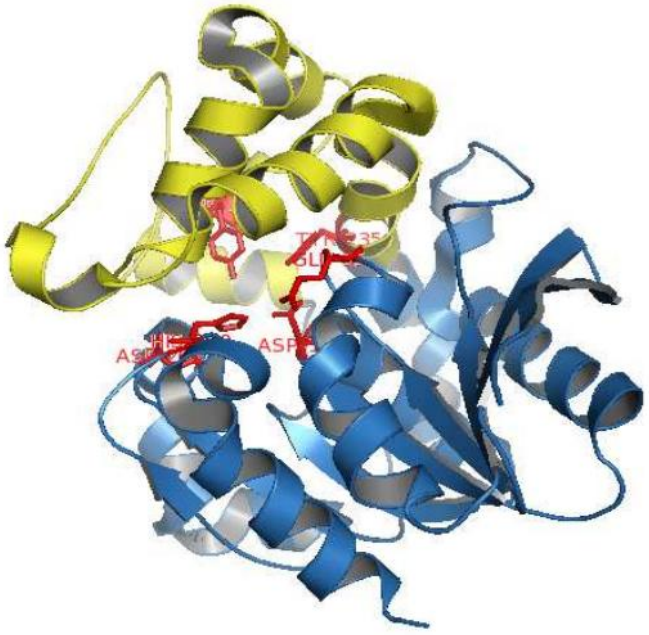


Figure 1. Overall structure of StEH1. Blue color indicates the β-sheet core flanked by α-helixes and the yellow indicates the lid domain. Red color shows the catalytic residues Asp105, Glu35, His300, Tyr154 and Tyr235. Image is from Huan Ma's Master thesis 2011.

Figure 2 shows a catalytic mechanism of StEH1 proposed by Thomaeus et al. 2007. The catalytic triad consists of Asp105, Asp265 (Asp265 not shown in Figure 3) and His300 (Mowbray et al. 2006). The nucleophilic Asp105 in the active site attacks one of the electrophilic epoxide carbons, thereby forming an alkylenzyme intermediate. Tyr154 and Tyr 235 facilitate the ring opening by stabilizing the leaving group with hydrogen bonds. Asp265 is located near His300 and together they form a base-charge relay pair. The acidic Asp265 interacts with His300 which become more polarized and basic, making it easier to activate the water molecule held in place by His300, Glu35 and Phe33. The activated water molecule hydrolyses the alkylenzyme intermediate by a nucleophilic attack to yield the diol product.

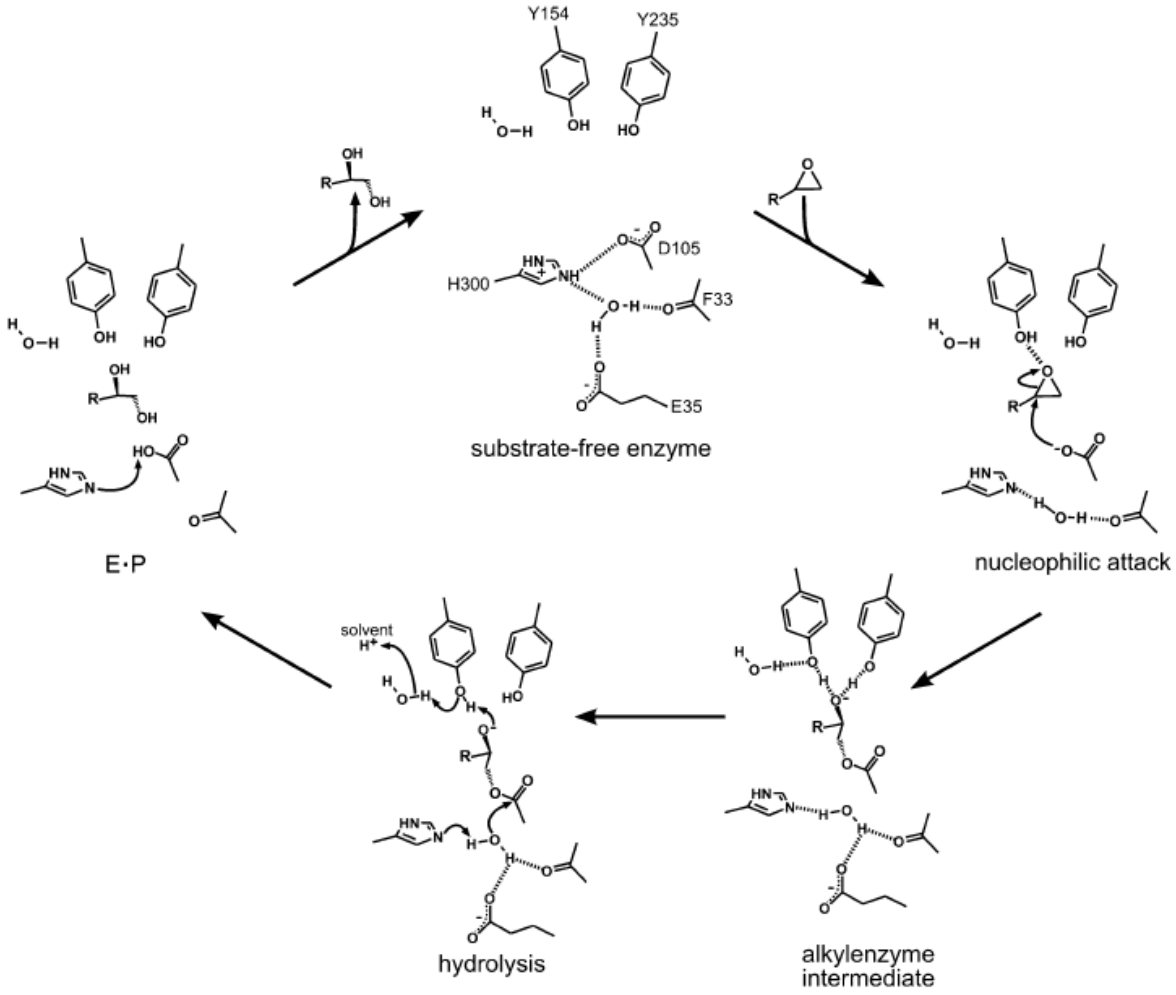


Figure 2. Catalytic mechanism of hydrolysis of epoxides by StEH1. Image is from Carlsson et al. 2012.

1.4 Directed evolution

The diol formed by epoxide hydrolysis can often exist as two enantiomers. This could, as mentioned earlier, cause complications in the chemical industry when only one of them is desirable. If enzymes are used to solve this problem, they should generate one enantiomer of the product in a far greater yield than the other. The enzyme should also have a reduced specificity and be able to hydrolyze several different epoxide substrates, thereby being a versatile device in the chemist's "toolbox". In order to produce an enzyme like that, directed evolution can be used. Directed evolution can be described as an *in vitro* version of natural selection. An enzyme with promising properties is identified and the cDNA coding for the enzyme is mutated. A collection of different cDNA mutants is called a library. The mutated cDNA from the library are transformed into host cells and the different enzyme variants are produced, giving rise to an enzyme library. A screening method is used to identify the enzyme variants with improved properties. Their cDNA can then be further mutated and the cycle is repeated until a desired enzyme is identified.

A dilemma is that it is impossible to mutate all amino acids since the library would get way too big to handle. However, this can be solved by using Combinatorial Active-site Saturation Testing (CASTing) (Reetz, 2007). The structure of the enzyme is studied provided that the crystal structure is available. Amino acids which are believed to be important for, in this case, catalytic function are identified and divided into libraries. Each library contains 1-3 amino acids, or more. The size of the library can be drastically reduced and thereby easier to handle when only the important amino acids are mutated instead of all. The amino acid(s) in the first library is mutated by using PCR (Polymerase Chain Reaction) with special primers that can give rise to different amino acids on the desired position. A library of cDNA can then be constructed. Transformation, protein expression and screening are then used to identify hits. The cDNA from the hits serve as a template for mutation of amino acid(s) in the next library etc.

1.5 Enzyme mutants in this project

By using CASTing, several mutants of StEH1 has been created. These include RC1, RC1B1, RC1B1D33 and RC1B1D33E6 (Carlsson *et al.* 2012). The numbers and letters refer to the different libraries the mutants come from. Figure 3 shows the active site of wild type StEH1 where the residues constituting the different libraries are displayed. The actual residue in each library of the different enzyme variants are shown in Table 1. The change from large residues to smaller for the three last mutants should make the active site larger compared to the wild type, with the largest active site found in RC1B1D33E6. In general, enzymes get more versatile with big active sites since more different substrates can fit (Carlsson *et al.* 2012).

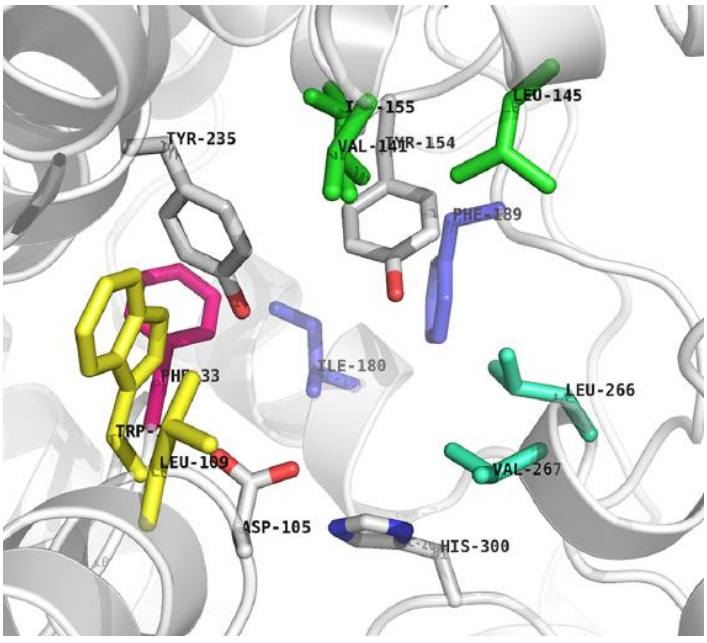


Figure 3. Active site of wild type StEH1. White sticks indicate catalytic residues while colored sticks indicate residues in the different libraries. Pink = library A (F33), yellow = library B (W106 and L109), green = library C (V141, L145 and I155), dark blue = library D (I180 and F189) and light blue = library E (L266 and V267). Image is from Carlsson et al. 2012.

Table 1. Residues in the positions for the libraries of the different enzyme variants (Carlsson *et al.* 2012).

Library	**A**	**B**		**C**			**D**		**E**	
Position	**33**	**106**	**109**	**141**	**145**	**155**	**180**	**189**	**266**	**267**
StEH1 wt	Phe	Trp	Leu	Val	Leu	Ile	Ile	Phe	Leu	Val
RC1	Phe	Trp	Leu	Lys	Leu	Val	Ile	Phe	Leu	Val
RC1B1	Phe	Leu	Tyr	Lys	Leu	Val	Ile	Phe	Leu	Val
RC1B1D33	Phe	Leu	Tyr	Lys	Leu	Val	Ile	Leu	Leu	Val
RC1B1D33E6	Phe	Leu	Tyr	Lys	Leu	Val	Ile	Leu	Gly	Val

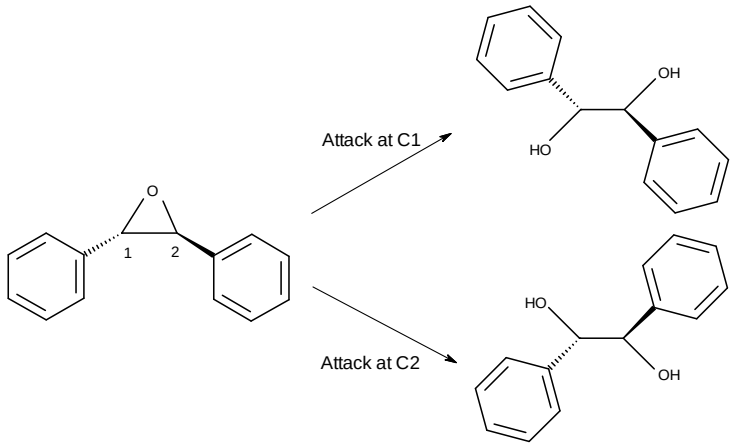
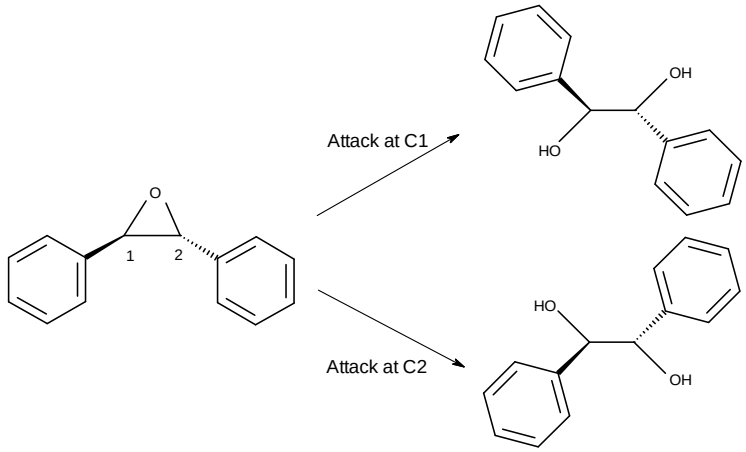
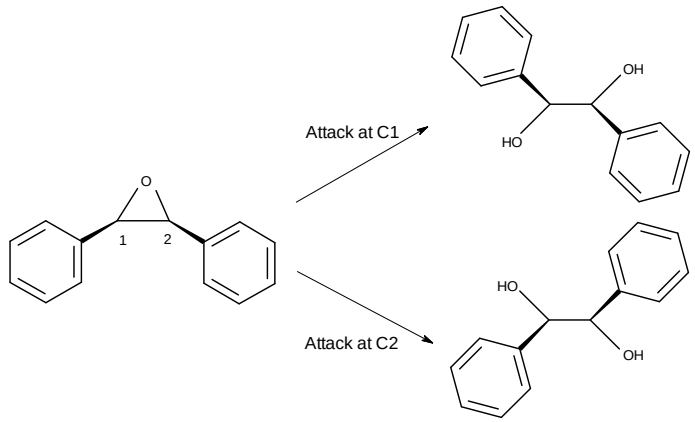
1.6 Enantio- and regioselectivity

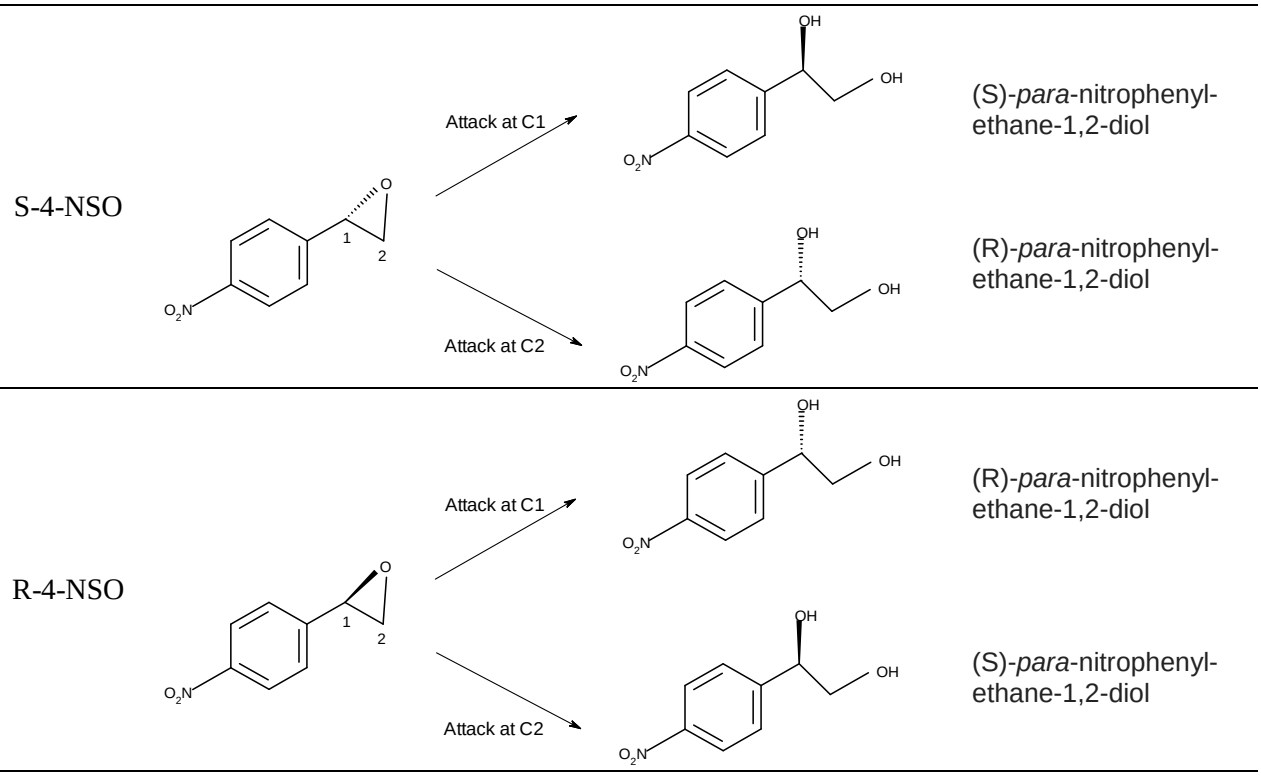
Two aspects are critical in order for an enzyme to favor one product enantiomer, namely enantioselectivity and regioselectivity. When the active site is asymmetric, the enzyme's specificity affects chiral substrate molecules by which one of the enantiomers will fit in the active site better than the other, a phenomenon referred to as enantioselectivity. Enantioselectivity results in a higher reaction rate for the preferred enantiomer in a racemic mixture, leading to a greater production of the corresponding product than that of the other enantiomer per unit of time.

The S_N2-reaction of epoxide hydrolysis can sometimes give rise to two different enantiomers of the product depending on which of the two carbons that are attacked to open the epoxide ring. This is affected by the regioselectivity of the enzyme. In a simple S_N2-reaction, the carbon is attacked by the nucleophile on the opposite side of the leaving group. The leaving group moves away and the three other substituents binding to the attacked carbon atom follow in the same direction. The bonds of the three moving substituents are in the same plane during the transition state and when the leaving group is gone they have inverted their configuration.

The epoxides used in this project were R,R-TSO (R,R-*trans*-stilbene oxide); S,S-TSO; *cis*-SO (*cis*-stilbene oxide); R-4-NSO (R-4-nitro styrene oxide) and S-4-NSO. Table 2 shows their structure and corresponding products depending on which carbon atom is attacked during hydrolysis with water. OH^- attacks one of the carbon atoms in the epoxide ring, causing the oxygen atom to leave and the other substituents to invert their configuration. The leaving oxygen atom binds a hydrogen atom from the surrounding water so that the vicinal diol is formed. The configurations of the substituents binding to the non-attacked carbon atom are unchanged.

Table 2. Structures for the different epoxide substrates used in this project and their corresponding products formed by hydrolysis with water. The ratio between the formed products is affected by regioselectivity.

Substrate	Possible reactions	Products
S,S-TSO		Meso-hydrobenzoin Meso-hydrobenzoin
R,R-TSO		Meso-hydrobenzoin Meso-hydrobenzoin
cis-SO		S,S-hydrobenzoin R,R-hydrobenzoin



The two products formed from S,S-TSO are identical so regioselectivity does not affect the product outcome. That is also the case with R,R-TSO. Regioselectivity can however generate different products when *cis*-SO, R-4-NSO and S-4-NSO are hydrolyzed.

An enantioselective enzyme lacking activity for the unfavored substrate enantiomer would only generate one enantiomer of the product from a racemic substrate solution. This providing that one enantiomer of the substrate could only give rise to one enantiomer of the product. A drawback in such case is that only half of the substrates can be turned into useful products. The optimal outcome from the directed evolution would instead be an enantioconvergent enzyme. That is an enzyme which due to regioselectivity can convert both enantiomers of the substrate into the same enantiomer of the product. The initial substrate solution would then be utilized more efficiently.

1.7 Chiral HPLC

The two enantiomers of a chiral molecule have the same physical properties and can only be separated in a chiral environment, such as chiral High Performance Liquid Chromatography (HPLC). When the stationary phase is chiral it will interact differently with the two enantiomers. The enantiomer interacting the most with the stationary phase will be the most retained and will thereby be eluted last.

1.8 Enzyme kinetics

An enzymatic reaction can be described as shown in Scheme 1. Regarding the kinetics it can be divided into pre-steady state, steady state and post-steady state. The pre-steady state occurs immediately when the enzyme and substrate have been mixed together and it normally only lasts for a few microseconds as [ES] increases. During the subsequent steady state, the concentration of intermediates is constant and [s] is decreasing in the same rate as [P] is increasing. After a while, normally seconds or minutes, the reaction rate slows down as [S] is decreased. The reaction has then entered the post-steady state and [E] is increasing.

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

Scheme 1. A simplified enzymatic reaction. E = free enzyme, S = free substrate, ES = enzyme-substrate complex, EP = enzyme-product complex and P = free product.

A saturation curve describes how the initial reaction rate, V_0, during steady state depends on [S] (Figure 5). The reaction rate increases linearly as [S] increases provided that [S] is quite low. However, at high [S] the enzymes get saturated and addition of extra substrates have little effect on the reaction rate. The maximum reaction rate is designated V_{max} and the [S] required to reach half of this velocity is designated K_m.

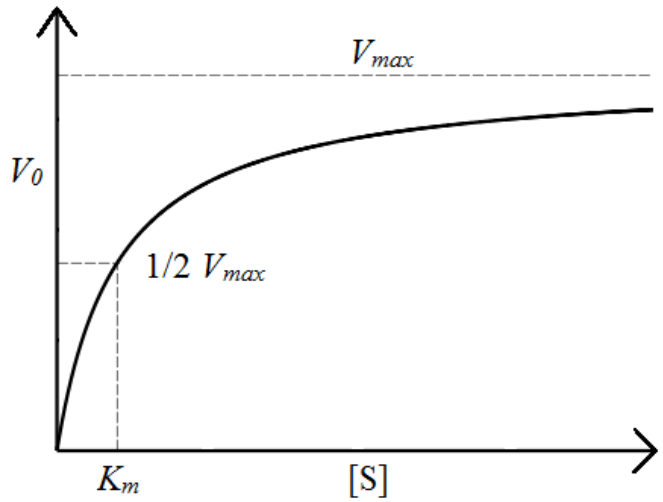


Figure 5. A saturation curve showing how V_0 depends on [S]. K_m corresponds to the [S] required to reach half V_{max}.

The relation between V_0, V_{max}, [S] and K_m is described by the Michaelis-Menten equation (Equation 1).

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{(Equation 1)}$$

Another constant which is essential in describing enzyme activity is the turn over number, k_{cat}. This shows the number of substrate molecules turned into product by each enzyme molecule per unit of time when V_{max} is reached.

The specificity constant, k_{cat}/K_m, is often used to compare the kinetics of different enzymes. A good enzyme can rapidly convert many substrate molecules into product and do so even in low substrate concentrations, leading to a high k_{cat}/K_m for the enzyme. This constant can be obtained even if the saturation curve has the shape of a straight line instead of a hyperbola. This would be the case for instance if the substrate is not soluble at concentrations needed to reach enzyme saturation. Since only the concentrations were the substrate is completely dissolved can be used, the deflection of the saturation curve would then be too vague to make precise predictions of k_{cat} and K_m. However, [S] in those cases is very small compared to K_m and can thereby be neglected in the denominator of the Michaelis-Menten equation to give Equation 2. The straight line generated when V_0 is plotted against [S] has the slope of V_{max}/K_m which, if the enzyme concentration has been taken into consideration, is equal to k_{cat}/K_m.

$$V_0 = \frac{V_{max}}{K_m}[S] \qquad \text{(Equation 2)}$$

1.9 This project

RC1, RC1B1 and RC1B1D33 were purified while the other enzyme variants were already available in sufficient amounts. Steady state kinetics for wild type StEH1 and the mutants RC1, RC1B1, RC1B1D33 and RC1B1D33E6 was studied spectrophotometrically together with the substrates R,R-TSO; S,S-TSO; *cis*-SO; R-4-NSO and S-4-NSO. Chiral HPLC was used to measure regioselectivity for StEH1 wt with *cis*-SO. Regioselectivity for R-4-NSO and S-4-NSO was not measured due to lack of time.

2. Material and methods

2.1 Chemicals and bacteria

The chemicals used in this project were of the highest purity commercially available. The bacteria strain used to produce all enzyme variants was *E. coli* XL-1-Blue carrying plasmids called pREP4 GroEL/ES. These plasmids contained genes for kanamycin resistance and formation of chaperonins for improved folding of produced proteins. The cDNA for the different enzyme variants were located on vectors containing genes for ampicillin resistance, IPTG (Isopropyl β-D-1-thiogalactopyranoside) inducer and a His-tag to facilitate protein purification. The bacteria had been transformed with the vectors before the project began.

2.2 Protein expression and purification

One single colony of the bacteria coding for RC1, RC1B1 or RC1B1D33 was inoculated over night at 30°C and 200 rpm in 10 ml 2TY-medium. The 2TY-medium contained 100 μg/ml ampicillin and 30 μg/ml kanamycin. The overnight culture was diluted in 750 ml 2TY media with 50 μg/ml ampicillin and 15 μg/ml kanamycin. It was incubated at 30°C and 200 rpm until the OD_{600} reached 0.4. IPTG was added to a final concentration of 1 mM so that the production of StEH1 variants and chaperonins were induced. The cell cultures were incubated overnight at 30°C and 200 rpm. After 12 min of centrifugation at 5000 rpm, a cell pellet was obtained and stored at -80°C until the subsequent protein purification.

The cell pellet was thawed and resuspended in 25 ml binding buffer [10 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole and 0.02 % sodium azide (pH 7.0)] with 25μl DNase and ½ EDTA-free protease inhibitor cocktail tablet. The cells were lysed by ultrasonication and the lysate was centrifuged for 1 h at 4°C and 15000 rpm (Beckman Coulter JA-25.50). 2 μl of the supernatant was stored for future analysis with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and the rest was mixed with 1 ml IMAC gel to which the His-tagged enzymes could bind. The gel was incubated for 1 hour under moderate agitation. The IMAC gel and its content were loaded in a column which was plugged until the gel had settled. The liquid was allowed to run through the gel and 2 μl of flow through was stored for SDS-PAGE. Approximately 25 ml washing buffer [10 mM sodium phosphate, 0.5 M NaCl, 100 mM imidazole and 0.02 % sodium azide (pH 7.0)] was added to the column and 2 μl of flow through was stored for SDS-PAGE. Elution buffer [10 mM sodium phosphate, 0.5 M NaCl, 300 mM imidazole and 0.02 % sodium azide (pH 7.0)] was added to the column to elute the His-tagged enzyme and 2 μl of flow through was stored for SDS-PAGE. The eluted flow through was concentrated by centrifugation in a vivaspin 20 until only 2.5 ml of the enzyme solution remained. The protein solution was desalted in a PD10 column by using sodium phosphate buffer [0.1 M sodium phosphate (pH 7.4)] according to the protocol from GE Healthcare. 2 μl of the desalted enzyme solution was stored for SDS-PAGE.

SDS-PAGE was performed to confirm that the majority of the enzyme was found in the eluted and desalted fraction and that the sample did not contain unwanted proteins. The protein concentrations were determined by measuring the absorbance at 280 nm.

2.3 Enzyme kinetics

Steady state kinetics was measured with a Shimadzu UV-1800 spectrophotometer at 30°C. Sodium phosphate buffer (0.1 M, pH 6.8 and preheated to 30°C) and pure enantiomers of each substrate (R,R-TSO; S,S-TSO; *cis*-SO; R-4-NSO or S-4-NSO) was added to a cuvette to the final substrate concentrations shown in Table 3. The substrates had initially been dissolved in acetonitrile, leading to a final concentration of 1% (v/v) of acetonitrile in the reaction mixture. The enzyme was added with the concentrations shown in Table 3 to start the reaction. The initial decrease in absorbance was measured at 229 nm ($\Delta\varepsilon$ = -15 $mM^{-1}\cdot cm^{-1}$) (Wixtrom and Hammock 1988) for R,R-TSO; S,S-TSO and *cis*-SO while 310 nm ($\Delta\varepsilon$ = -1

$mM^{-1} \cdot cm^{-1}$) (Westkaemper and Hanzlik 1981) was used for R-4-NSO and S-4-NSO. The decrease in absorbance over the course of time with non preheated reaction mixtures of S- and R-4-NSO appeared to have a lagging phase in the beginning. In those cases, the mixture of substrate and buffer in the cuvette was heated to 30°C for 2 minutes in the spectrophotometer before addition of enzyme. All measurements were performed in triplicates in a cuvette with the path length 1 or 0.5 cm (Table 3) depending on the initial absorbance.

In order to consider an activity too small to detect, the measurements were made for at least 3 minutes with an enzyme concentration over 2 μM at first and then a concentration under 0.05 μM. The measurements were made on the highest substrate concentration, the lowest and two concentrations in between.

Table 3. Conditions in the reaction mixture during measurements of steady state kinetics. Substrate concentration, enzyme concentration and cuvette length depending on substrate and enzyme used are shown.

Substrate	Enzyme	[S] (μM)	[E] (μM)	Cuvette length (cm)
S,S-TSO	StEH1 wt	2-50	0.02	1
	RC1	2-50	0.03	1
	RC1B1	2-50	0.06	1
	RC1B1D33	13-50	0.04	1
	RC1B1D33E6	6-50	2.63	1
R,R-TSO	StEH1 wt	3-50	0.04	1
	RC1	3-50	0.03	1
	RC1B1	3-50	3.16	1
	RC1B1D33	3-50	3.85	1
	RC1B1D33E6	3-50	0,03-2,63[a]	1
Cis-SO	StEH1 wt	13-50	4.00	1
	RC1	13-50	2.99	1
	RC1B1	25-50	3.16	1
	RC1B1D33	25-50	0.04-3.85	1
	RC1B1D33E6	25-50	0.03-2.63	1
S-4-NSO	StEH1 wt	8-500	1.00	0.5
	RC1	16-500	0.15	0.5
	RC1B1	16-625	1.34	0.5
	RC1B1D33	16-417	0.04	0.5
	RC1B1D33E6	31-750	0.66	0.5
R-4-NSO	StEH1 wt	125-750	4.00	0.5
	RC1	125-750	2.99	0.5
	RC1B1	125-750	0.03-2.69	0.5
	RC1B1D33	125-750	3.85	0.5
	RC1B1D33E6	125-750	0.03-2.63	0.5

[a] Enzyme concentrations given in intervals show the concentrations used before the activity was considered too small to detect.

The activity measured as a concentration change was calculated by using Lambert-Beers law and after dividing the activity with the enzyme concentration, the desired kinetic parameters were obtained from the program Simfit. The Simfit package contains RFFIT which calculates k_{cat}/K_m and MMFIT which calculates k_{cat} and K_m respectively.

2.4 Chiral HPLC

Sodium phosphate buffer (0.1 M, pH 6.8), *cis*-SO (final concentration 3 mM) and StEH1 wt (final concentration 2 μM) were added to an eppendorf tube. A control sample with the same buffer and the same concentration of *cis*-SO but without enzyme was also made to check the spontaneous hydrolysis. The samples were incubated over night at 30°C and 200 rpm. Methanol was added to stop the reaction. The samples were centrifuged under vacuum for 5 h at 800 rpm to evaporate the buffer and methanol. Each sample was dissolved in 150 μl mobile phase [90% hexane and 10% isopropanol] and centrifuged at 13000 rpm for 5 min to get rid of unwanted particles. In order to be able to identify the peaks from the HPLC, pure solutions of *cis*-SO and the two diols S,S-hydrobenzoin and R,R-hydrobenzoin (all with the final concentration 5 mM) were made with mobile phase as solvent. 110 μl of each sample were transferred to glass vials compatible with the HPLC machine. HPLC was performed with a Daicel Chiralpak AS-H (250 × 4.6 mm) and a Shimadzu SPDM20A photometric unit where the mobile phase had the flow rate 0.5 ml/min and the injection volume was 60 μl of each sample. The diol products were detected at 229 nm.

3. Results

3.1 Protein expression and purification

SDS-page indicated negligible amounts of unwanted proteins in the purified samples. The protein concentrations were determined by measuring the absorbance at 280 nm and using Lambert-Beers law (Table 4). Because of the lack of unwanted proteins, the protein concentration was considered equal to the enzyme concentration. The extinction coefficient for the different enzyme variants was calculated by equation 3:

$$\varepsilon = \varepsilon_{WT} + \Delta Trp \times 5500 + \Delta Tyr \times 1490 + \Delta Cys \times 125 \qquad \text{(Equation 3)}$$

Where $\varepsilon_{WT} = 59030\ M^{-1} \cdot cm^{-1}$ (Elfström & Widersten 2005).

Table 4. Protein concentrations in the different solutions of purified enzymes.

Enzyme variant	Protein concentration (μM)
RC1	66,5
RC1B1	134,4
RC1B1D33	163,4

3.2 Enzyme kinetics

The saturation curves generated by Simfit are shown in Appendix 9.1. Figure 6A shows an example of a hyperbolic shaped saturation curve which enables the extraction of k_{cat}, K_m and k_{cat}/K_m. Figure 6B shows an example of a saturation curve obtained when enzyme saturation was not reached and only k_{cat}/K_m could be extracted.

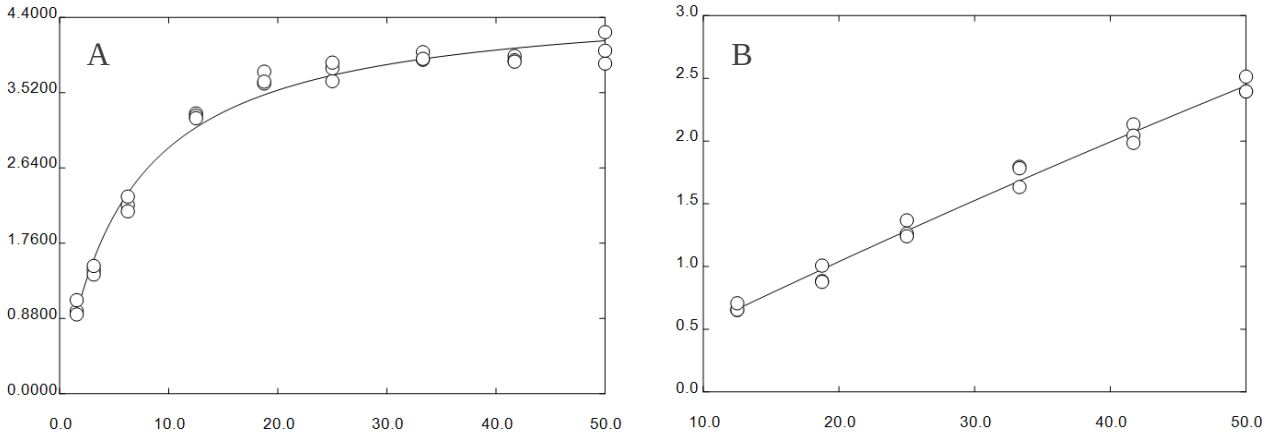


Figure 6. Saturation curves obtained from hydrolysis of S,S-TSO with (**A**) StEH1 wt. (**B**) RC1B1D33.

The kinetic parameters k_{cat}, K_m and k_{cat}/K_m obtained by Simfit are shown in Table 5. The low activity of *cis*-SO resulted in a very irregular decrease in absorbance over the course of time regardless of enzyme used. The validity of the kinetic parameters for *cis*-SO are therefore dubious. R-4-NSO together with RC1B1D33E6 did sometimes show a low activity uncorrelated with the substrate concentration. Since the absorption lines were impossible to replicate and the vast majority of the measurements indicated no activity, the activity for R-4-NSO and RC1B1D33E6 was considered too small to detect.

Table 5. k_{cat}, K_m and k_{cat}/K_m for the different combinations of substrate and enzyme.

Substrate	Enzyme	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
S,S-TSO	StEH1 wt	4.6 ± 0.07	6.1 ± 0.4	760 ± 40
	RC1	3.7 ± 0.06	8.6 ± 0.5	430 ± 20
	RC1B1	ND[a]	ND	71 ± 1
	RC1B1D33	ND	ND	54 ± 2
	RC1B1D33E6	ND	ND	1.5± 0.06
R,R-TSO	StEH1 wt	ND	ND	175 ± 9
	RC1	ND	ND	97 ± 6
	RC1B1	ND	ND	0.9 ± 0.06
	RC1B1D33	ND	ND	0.5 ± 0.04
	RC1B1D33E6	NA[b]	NA	NA
Cis-SO	StEH1 wt	ND	ND	0.07 ± 0.009
	RC1	ND	ND	0.09 ± 0.01
	RC1B1	ND	ND	0.02 ± 0.003
	RC1B1D33	NA	NA	NA
	RC1B1D33E6	NA	NA	NA
S-4-NSO	StEH1 wt	0.3 ± 0.007	25 ± 3	9.1 ± 1
	RC1	1.0 ± 0.03	70 ± 8	15 ± 1
	RC1B1	0.4 ± 0.009	41 ± 4	10 ± 1
	RC1B1D33	5.0 ± 0.2	59 ± 7	85 ± 8
	RC1B1D33E6	ND	ND	5.9± 0.3
R-4-NSO	StEH1 wt	0.06 ± 0.006	460 ± 90	0.14 ± 0.01
	RC1	ND	ND	0.050 ± 0.004
	RC1B1	NA	NA	NA
	RC1B1D33	ND	ND	0.11 ± 0.002
	RC1B1D33E6	NA	NA	NA

[a] ND = no data available since enzyme saturation was not reached.

[b] NA = no activity found.

3.3 Chiral HPLC

The chiral HPLC displayed no signs of spontaneous hydrolysis for the sample with *cis*-SO incubated without enzyme. The sample with StEH1 wt added showed that R,R-hydrobenzoin was the only product formed.

4. Discussion

The k_{cat} for StEH1 wt with S,S-TSO obtained in this project is slightly higher than previous data (Elfström & Widersten 2005) while the obtained K_m is about nine times higher. This could be explained with the steep slope of the saturation curve where small measurement errors make a big difference in K_m. The difference in K_m leads to a difference in k_{cat}/K_m which is five times higher in the literature. The difference in k_{cat}/K_m for StEH1 wt with R,R-TSO

obtained in this project compared to the literature (Elfström & Widersten 2005) is even higher but the conclusion that k_{cat}/K_m is higher for S,S-TSO than R,R-TSO is the same.

k_{cat}/K_m decreased drastically with both S,S-TSO and R,R-TSO when RC1 was mutated to generate RC1B1. The difference is a Trp in RC1being substituted with a Leu in RC1B1. This change could make more room in the active site, possibly causing the transition state to fit worse. K_m would then be increased with a subsequent decrease in k_{cat}/K_m. The increase in K_m was observed for S,S-TSO but could not be detected experimentally for R,R-TSO. k_{cat}/K_m decreased even more for both S,S-TSO and R,R-TSO when RC1B1D33 was mutated to generate RC1B1D33E6. The reason could again be due to a larger active site since Leu was replaced with a smaller Gly.

The low activities and irregular absorption lines obtained from measurements on *cis*-SO leads to difficulties in making reliable comparisons between the different enzyme variants. It can at least be stated that none of the mutants showed any drastic improvements in enzyme activity compared to the wild type.

k_{cat} for StEH1 was significantly higher with S-4-NSO compared with R-4-NSO even though a previous study (Elfström & Widersten 2005) has shown a k_{cat} ten times higher with Rac-NSO (racemic NSO) than what was now obtained with S-4-NSO. This difference can so far only be explained with measurement errors. The enzyme variants showing apparent activity together with R-4-NSO all had a higher K_m compared to when they hydrolyzed S-4-NSO. This contributed to the k_{cat}/K_m which was shown to be higher for S-4-NSO than R-4-NSO with all mutants and the wild type.

The ratio in kinetic parameters between the different enzyme variants hydrolyzing S-4-NSO in this project corresponds well with the ratios found when the same mutants were hydrolyzing S-styrene oxide (Carlsson et al. 2012), that is, NSO without the nitro group. Both studies show that RC1B1D33E6 has the highest K_m followed by RC1. This project and Carlsson et al. 2012 also show that RC1B1D33 has the highest k_{cat}/ K_m, mainly because of the high k_{cat}. k_{cat}/K_m for RC1B1D33 with S-4-NSO was about eight times higher compared to the other enzyme variants hydrolyzing the same substrate. Both S-4-NSO and R-4-NSO got increased k_{cat}/K_m when the aromatic Phe in RC1B1 was substituted with a smaller Leu in RC1B1D33 in this project as well as in the literature (Carlsson et al. 2012). This change in k_{cat}/K_m could be due to the larger active site.

The kinetic parameters and their ratios between the different enzyme variants hydrolyzing R-4-NSO in this project varied quite a lot from the data previously obtained with the same mutants together with R-styrene oxide (Carlsson et al. 2012). It is unclear whether these differences are due to measurement errors or if the electron withdrawing nitro group in NSO has a more significant affect on the kinetics of R-4-NSO than on S-4-NSO.

The chiral HPLC with *cis*-SO and StEH1 wt showed that R,R-hydrobenzoin was the only product formed. This implies that the nucleophilic attack only occurs at carbon 2 in Table 2 were *cis*-SO is the substrate. A possible application of the exhibited regioselectivity could be in the production of the R,R-diol from a racemic *cis*-SO solution.

5. Conclusion

Several of the mutants had a larger active site than the wild type and should thereby have an improved ability to hydrolyze different epoxide substrates. However, all mutants in the kinetic studies showed a decreased k_{cat}/K_m with S,S-TSO and R,R-TSO compared to the wild type. The kinetic parameters obtained for *cis*-TSO were quite unreliable due to low activities, but none of the mutants showed any drastic improvements in enzyme activity compared to the wild type. All enzyme variants had a higher k_{cat}/K_m when hydrolyzing S-4-NSO compared to when they hydrolyzed R-4-NSO. A contributing factor was the lower K_m together with S-4-NSO. Some residues proved to have a great impact on the kinetic properties when mutated. For instance, RC1B1D33 showed an 8-fold increase in k_{cat}/K_m in the hydrolysis of S-4-NSO compared to the other enzyme variants. The chiral HPLC with *cis*-SO and StEH1 wt showed that the nucleophilic attack occurs exclusively at one specific carbon atom, giving R,R-hydrobenzoin as only product. This behavior in regioselectivity could possibly be used in the production of the R,R-diol from a racemic *cis*-SO solution.

The next step in the research should be to measure regioselectivity for S-4-NSO and R-4-NSO together with the enzyme variants that showed apparent activity in the kinetic studies. The data provided in this rapport can hopefully increase the understanding of the structure-function relationship for StEH1 and facilitate the development of a more enantio- and regiospecific enzyme. This could make the chemical industry nicer to Mother Nature. Mother Nature who gives us so many nice things, such as enzyme-containing potatoes.

6. Svensk populärvetenskaplig sammanfattning

Olika mutanter av ett enzym från potatis undersöktes för att se hur de påverkade diverse epoxider. Mutanterna kunde i olika grad påverka vilken spegelbild av slutprodukten som bildades samt i vilken hastighet. Resultatet kan göra den kemiska industrin miljövänligare.

Enzymer är proteiner som kan skynda på kemiska reaktioner utan att själva förbrukas. Eftersom de kan arbeta utan att kräva giftiga lösningsmedel eller höga temperaturer är användandet av enzymer väldigt miljövänligt. Enzymer har en så kallad aktiv yta där de molekyler som ska reagera, substraten, kan fastna och omvandlas till produkt. Beroende på vilka aminosyror, proteinernas byggstenar, som bygger upp aktiva ytan så kan olika substrat få plats och de kan reagera olika snabbt.

Många molekyler kan förekomma i två olika former som är spegelbilder av varandra. I den kemiska industrin vill man ofta ha endast den ena spegelbilden eftersom den till exempel kan fungera som ett läkemedel medan den andra spegelbilden kan vara giftig. Om en sådan molekyl som kan förekomma i två former framställs utan hjälp av enzymer kommer den erhållna blandningen antagligen innehålla båda spegelbilderna i lika stor mängd. Det kan sedan vara svårt att separera dem. Denna blandning med två spegelbilder av produkten kan ha uppkommit på två sätt. Den kan ha bildats genom att ursprungslösningen innehöll båda spegelbilderna av substratet och att dessa gav upphov till varsin spegelbild av produkten.

Alternativt fanns bara en spegelbild av substratet i början men beroende på hur det reagerade kunde båda spegelbilderna av produkten bildas.

För att få fram den ena spegelbilden av produkten i ren form skulle enzymer kunna användas. Deras aktiva yta är nämligen inte symmetrisk. Detta gör att de kan snabba på reaktionen för den ena spegelbilden av substratet mer än för den andra. De kan också låta det rena substratet reagera på det sätt som bara ger den ena spegelbilden av produkten. Detta var tanken som ett forskarlag vid Uppsala universitet hade. De studerade epoxider, det vill säga molekyler som innehåller en ring av två kolatomer och en syreatom. Epoxider kan reagera med vatten och bilda dioler, alltså molekyler där två kolatomer binder varsin syreatom med tillhörande väteatom. Denna reaktion snabbades på med hjälp av enzymet StEH1 som kom från potatis. StEH1 oskadliggör i vanliga fall epoxider som är farliga för potatisen eftersom de kan förändra till exempel DNA. Forskarna hade framställt olika mutanter av StEH1 med olika aminosyror i aktiva ytan.

En projektarbetare fick sedan uppgiften att undersöka egenskaperna hos originalversionen av StEH1 samt fyra mutanter av denna. Detta gjordes tillsammans med epoxiderna R,R-TSO; S,S-TSO; *cis*-SO; R-4-NSO och S-4-NSO. De båda substraten R,R-TSO och S,S-TSO är spegelbilder av varandra men varje spegelbild kan bara ge upphov till en spegelbild av produkten. *cis*-SO kan bara förekomma i en form men kan bilda två spegelbilder av produkten. R-4-NSO och S-4-NSO är varandras spegelbilder och varje form kan ge upphov till två spegelbilder av produkten.

Med hjälp av en så kallad spektrofotometer undersöktes reaktionshastigheten för de olika substraten tillsammans med de fem olika enzymvarianterna. Eftersom substratet absorberar ljus annorlunda än produkten kan förändringen i absorbans visa hur snabbt reaktionen går. Mätningarna visade att alla enzymvarianter arbetade effektivare tillsammans med S,S-TSO än med R,R-TSO. Alla mutanter hade lägre effektivitet än originalversionen av StEH1 för dessa två substrat. Ingen av mutanterna visade heller några förbättringar i effektivitet tillsammans med *cis*-SO. Reaktionen med S-4-NSO snabbades på mer av samtliga enzymvarianter jämfört med R-4-NSO. En mutant som benämndes RC1B1D33 visade en klar ökning i effektivitet tillsammans med S-4-NSO jämfört med originalet av StEH1.

Sedan studerades i vilken proportion de båda spegelbilderna av produkten bildades utifrån *cis*-SO tillsammans med originalversionen av StEH1. Reaktionen fick ske fullständigt för att bilda så många diolmolekyler som möjligt. Produkterna som bildats analyserades med en så kallad HPLC där provet fick färdas igenom en kolonn där ena spegelbilden av produkten kom ut innan den andra. Därefter gick det att se hur stor mängd som bildats av de båda formerna. Det visade sig att endast ena spegelbilden, den som kallas R,R-hydrobenzoin, bildats.

Genom att se vilka förändringar som skett när olika aminosyror i aktiva ytan på StEH1 bytts ut hoppas nu forskarna få bättre förståelse för sambandet mellan struktur och funktion för enzymet. Tanken är att sedan gå vidare och göra enzymet ännu bättre på att bara bilda ena spegelbilden av produkten. Men nu är vi i alla fall ett steg närmre en grönare kemikalieproduktion.

7. Acknowledgements

Thank you Åsa Janfalk Carlsson and Mikael Widersten for this opportunity to get a brief but worthwhile insight into the real world of research. Despite your full schedules you have been able to give me all the essential information needed. I appreciate the great assistance I got from you Åsa during the enzyme purification and HPLC.

Emil Hamnevik and Huan Ma, thanks for the useful tips received when I was confused. I would also like to thank my parents and classmates for all support.

Now when the project is over, I´m looking forward to a good night of sleep without dreaming of the spectrophotometer.

8. References

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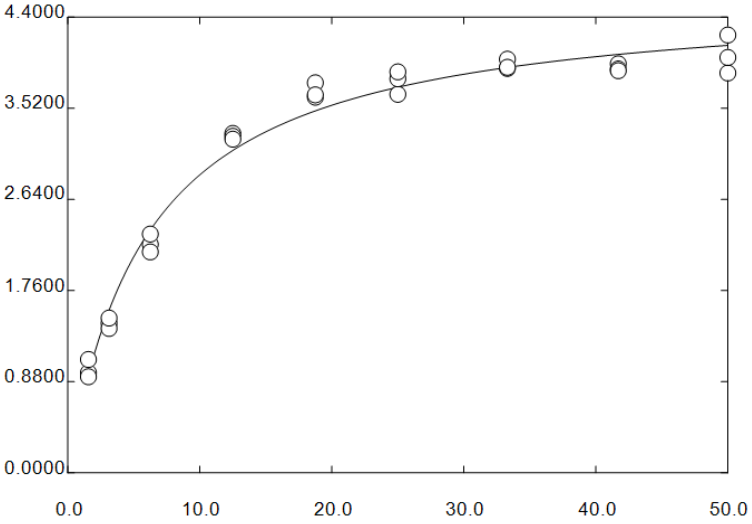
9. Appendix

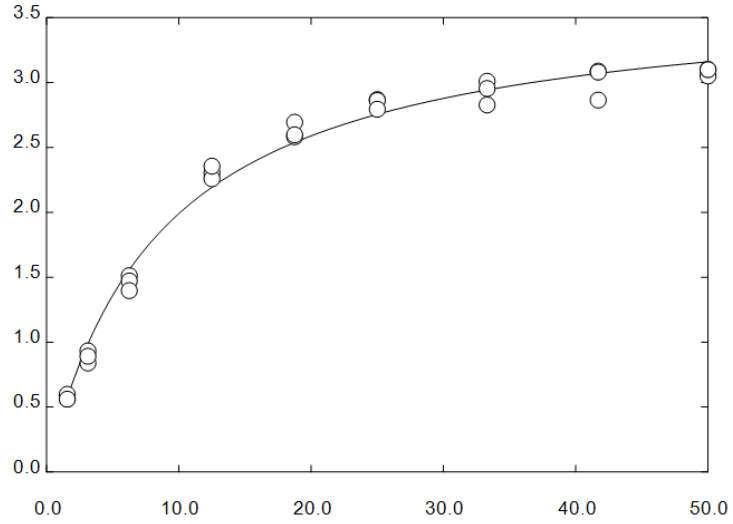
9.1 Saturation curves.

Saturation curves generated from the measurements of steady state kinetics. The y-axis indicates V_0 / [E] (s^{-1}) and the x-axis shows the [S] (μM).

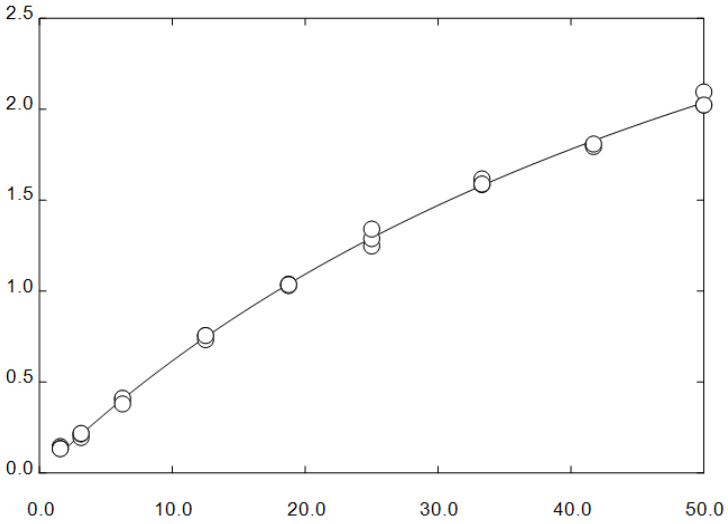
S,S-TSO with StEH1 wt



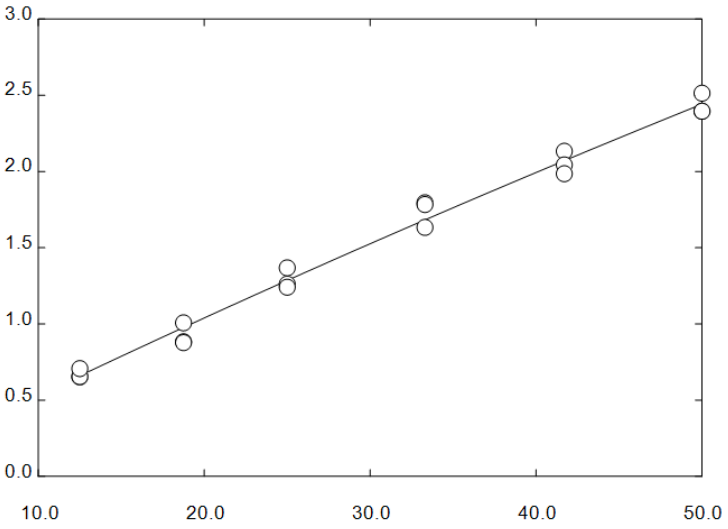
S,S-TSO with RC1



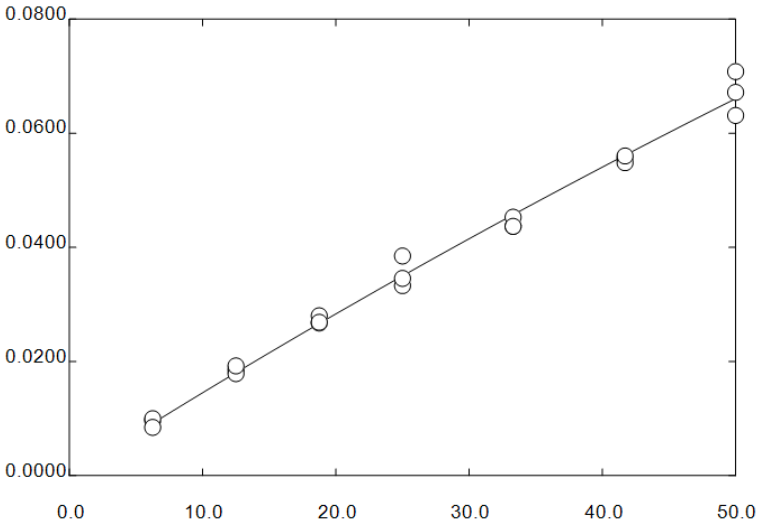
S,S-TSO with RC1B1



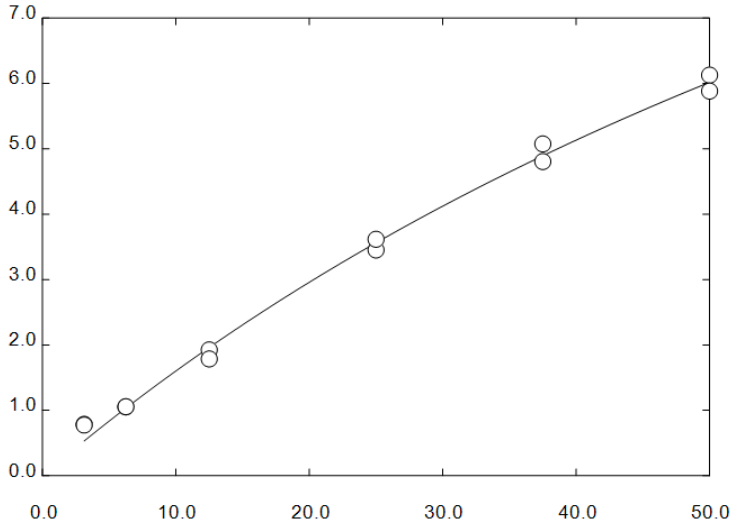
S,S-TSO with RC1B1D33



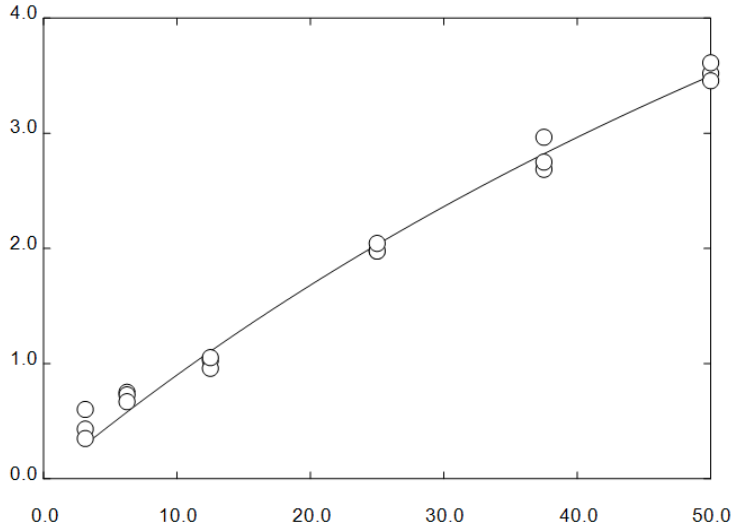
S,S-TSO with RC1B1D33E6



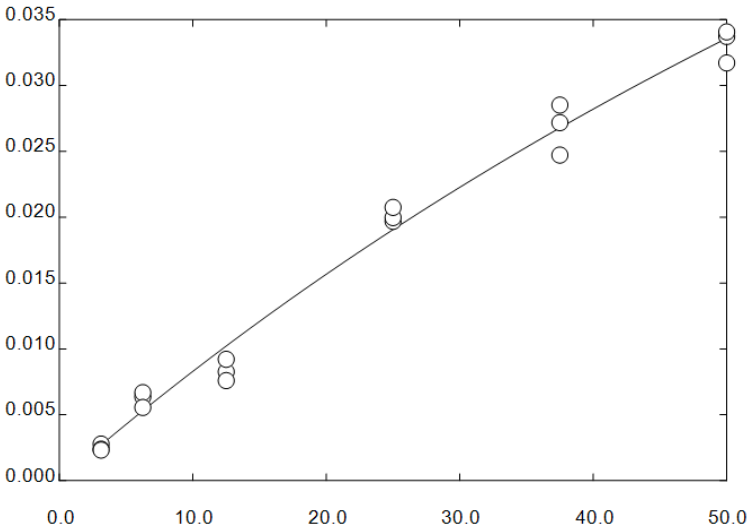
R,R-TSO with StEH1 wt



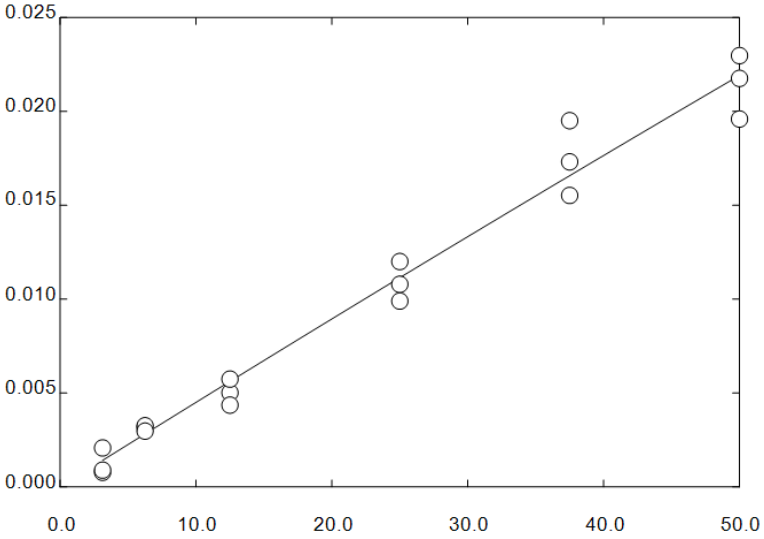
R,R-TSO with RC1



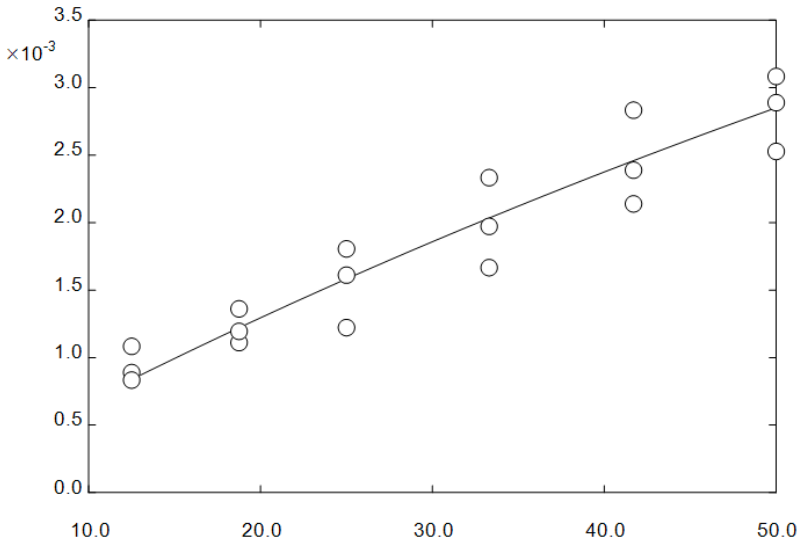
R,R-TSO with RC1B1



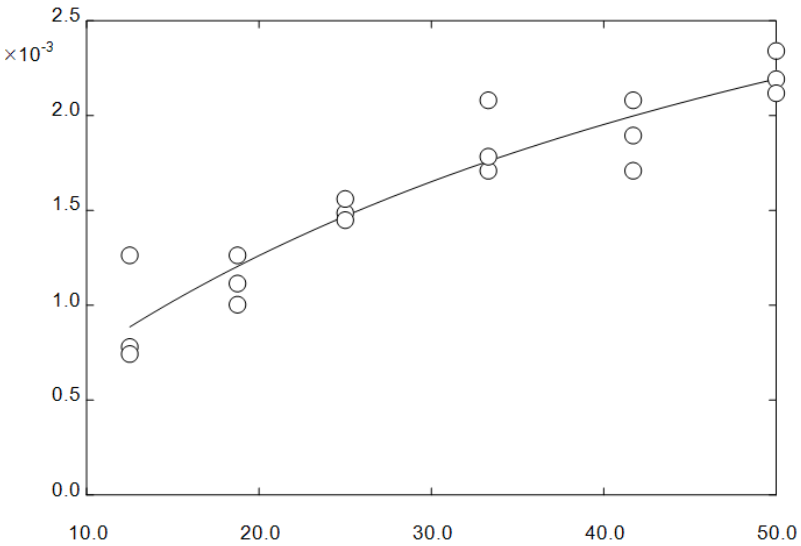
R,R-TSO with RC1B1D33



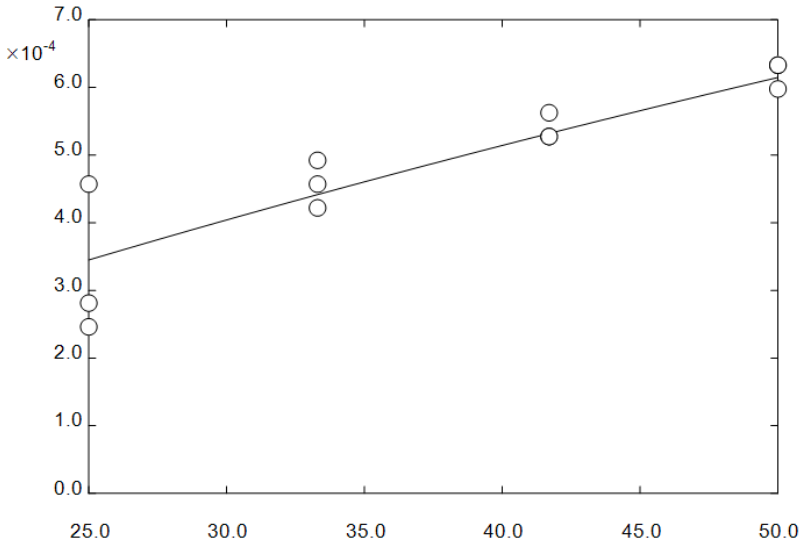
cis-TSO with StEH1 wt



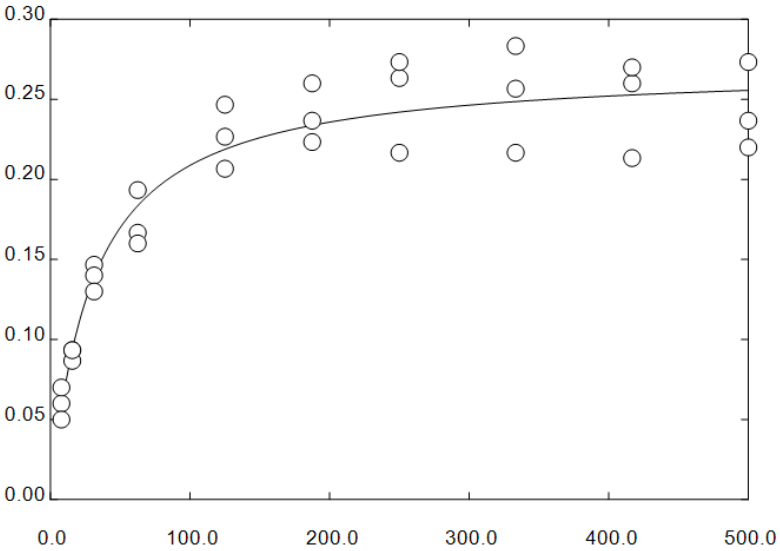
cis-TSO with RC1



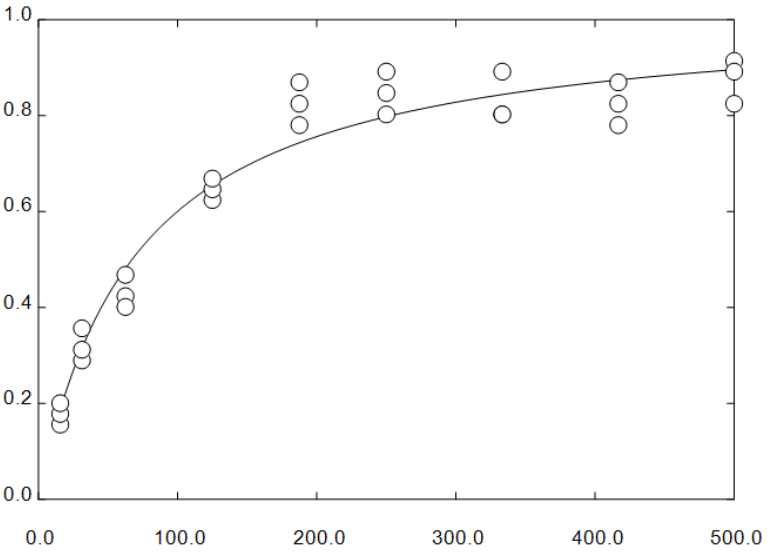
cis-TSO with RC1B1



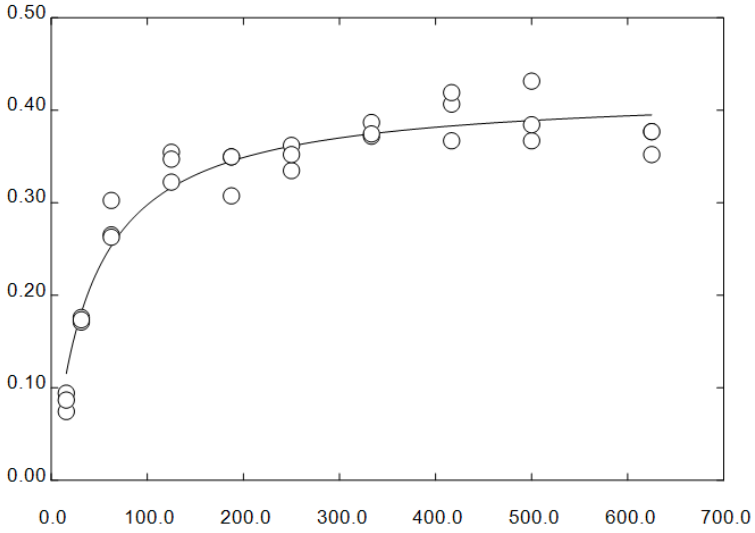
S-4-NSO with StEH1 wt



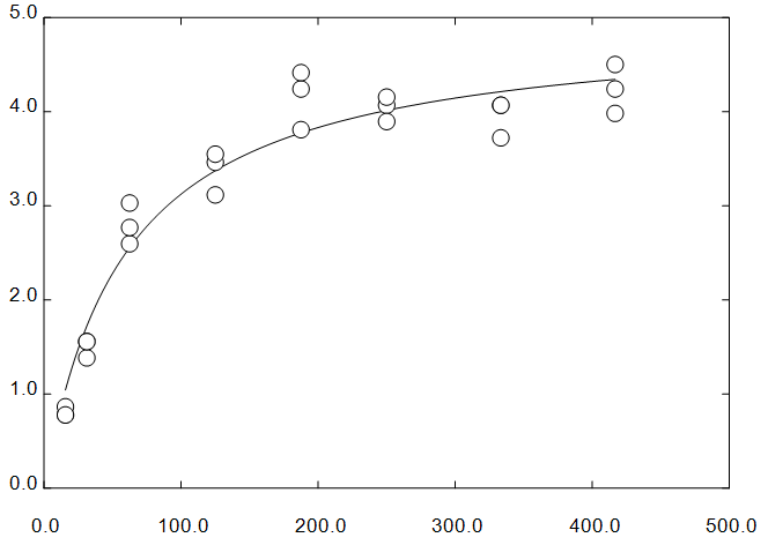
S-4-NSO with RC1



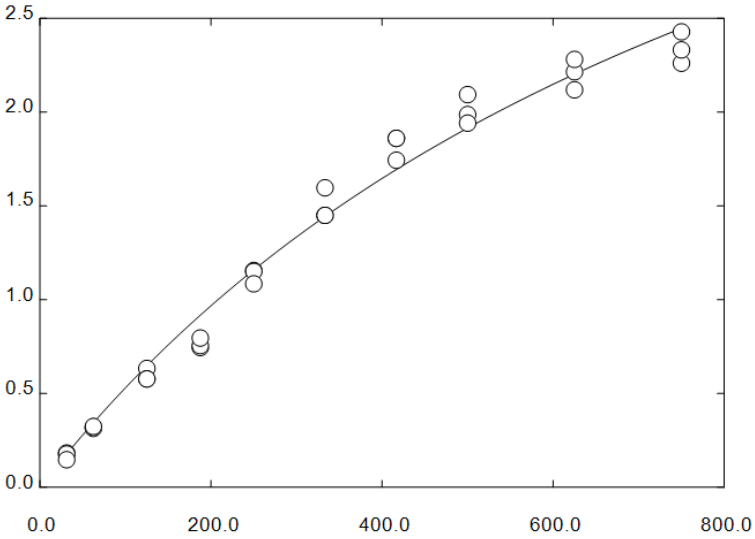
S-4-NSO with RC1B1



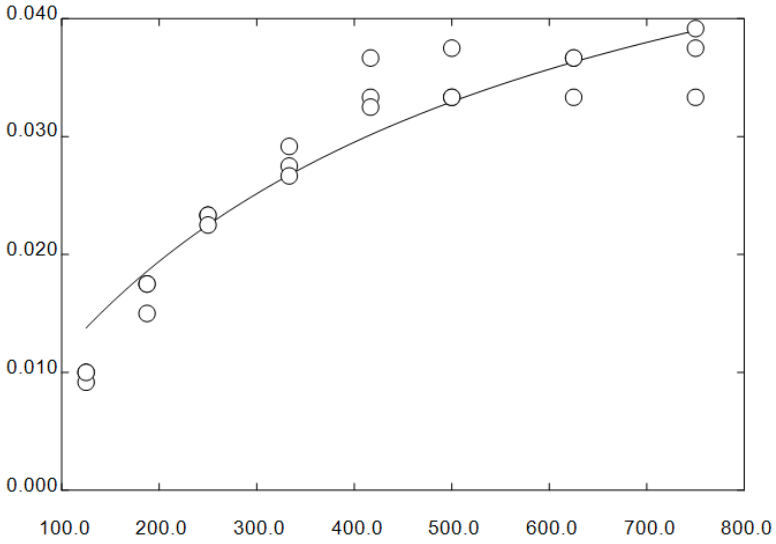
S-4-NSO with RC1B1D33



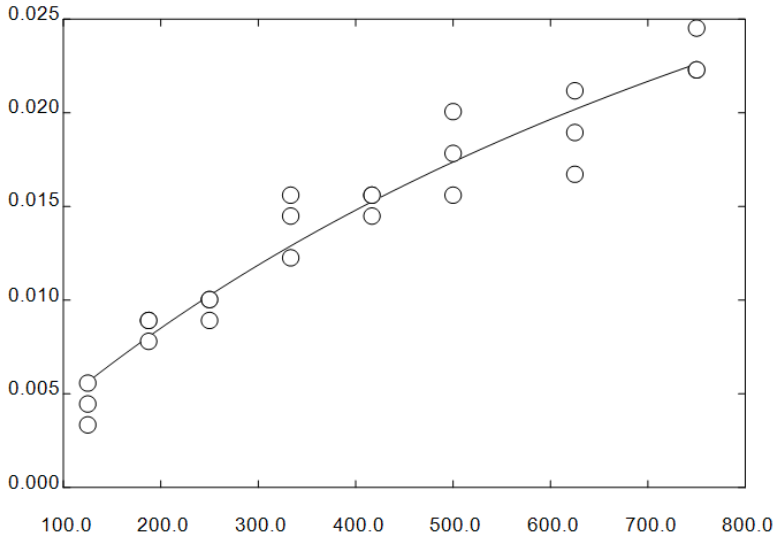
S-4-NSO with RC1B1D33E6



R-4-NSO with StEH1 wt



R-4-NSO with RC1



R-4-NSO with RC1B1D33