Abstract

In recent years there has been a dynamic development of protein engineering methods to obtain enzymes with new properties. Directed evolution methods allow the creation of enzymes with improved properties by random changes in the nucleotide sequence and the effects are tested by selection and screening procedures. On the other hand development of advanced bioinformatics tools causes the increasing use of molecular modeling methods and rational protein design.

Consumption of *trans* fatty acids (TFA) has been considered an important factor in the formation of cancer, coronary heart disease and insulin resistance. Most of these TFAs form as undesired by-products in the industrial hydrogenation of polyunsaturated vegetable oils. Enzymatic processes catalyzed by substrate selective lipases could replace chemical processes to develop methods for the production of edible fats without harmful *trans* fatty acids.

Lipase A from *Candida antarctica* exhibits favorable selectivity for *trans* unsaturated fatty acids. The selectivity results from the unique, straight construction of the acyl-binding tunnel to provide excellent fit *trans* fatty acids, which are characterized by a linear structure.

The aim of the experiments was to develop and evaluate the functionality of directed molecular evolution and rational protein design in improving the catalytic properties of *Candida antarctica* lipase A.

In the first stage of experiments the creation of recombinants libraries was developed and optimized. Conditions of amplification, cloning and expression of native lipase were developed. Correct construct was selected and then used in Error-prone PCR and Site-directed mutagenesis reactions.

Random mutations were induced with the use of a GeneMorph® II Random Mutagenesis Kit (Stratagene). Low (0-4.5 mutations/kb) and average (4.5-9 mutations/kb) mutation rate was used. It has been found that a high frequency of changes in the nucleotide sequence inactivates enzyme protein. Therefore, this type of mutation was not applied.

In a second step the selection and screening of obtained libraries in terms of lipolytic activities, in particular towards *cis*- and *trans*- isomers of fatty acids, was conducted.

A qualitative assessment of transformants was performed by the agar diffusion method in a medium with 3% (v:v) emulsion of tributyrin or triolein. Colonies that were able to hydrolyze at least one of the substrates were used in further steps of experiments,

ie. cell culture in 96-well microtiter plates followed by the determination of selectivity towards *cis*/*trans* dienes of linoleic acid (CLA) by gas chromatography.

The use of epPCR enabled the development of libraries of varied frequency of mutations and modified selectivity towards CLA isomers.

Analysis of the library after the first cycle of epPCR of *Candida antarctica* lipase A gene with average mutation frequency allowed to select the MA39 clone which exhibited selectivity towards *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers. Constant selectivity towards both isomers was α=0.13. The degree of CLA-TAG hydrolysis was 8.8%. Constant selectivity to *cis*-9, *trans*-11 and *trans*-10, *cis*-12 for native lipase A was α=0.01 and the degree of CLA-TAG hydrolysis 1.1%. After three cycles of epPCR selected transformant MA39 displayed 12- and 14-times higher selectivity towards *trans*-9, *trans*-11 and *trans*-10, *cis*-12 isomers compared to native lipase, respectively. The mutant was characterized by 11-fold increase of the ability of substrate hydrolysis (from 1.1% to 17.1%).

Based on the results of molecular modeling modification of *Candida antarctica* lipase A by site directed mutagenesis method using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene) was conducted. F149D and F222S variants characterized by distinct catalytic properties and increased selectivity to *trans* isomers compared to native lipase were obtained.

Obtained variants of *Candida antarctica* lipase A were examinated for the ability to hydrolyze substrates containing *trans*- and *cis*- isomers of fatty acids. For the determination of substrate preference the rate of hydrolytic activity value of *p*-NP esters of *trans*- elaidic acid (A_e) or *trans*- vaccenic acid (A_w) and activity of *cis*- oleic acid (A_o) was determined. The measurements confirmed higher preference for the hydrolysis of *p*-NP esters of vaccenic acid (C18:1 Δ11 *trans*) than for elaidic acid (C18:1 Δ9 *trans*).

The most favorable ratio of A_e/A_o and A_w/A_o was obtained after expression of modified lipases in Tuner (DE3)p*Lac*I cells at 30°C which was 3.17 and 4.41 (lipase F149D); 2.15 and 3.14 (lipase F222S), respectively. Native lipase was characterized by selectivity coefficient towards foregoing isomers 1.07 and 1.24, respectively. It has been shown that modified lipases were characterized by approximately 3- (variant F149D) and 2- fold (variant F222S) increase in substrate preference towards *trans* fatty acids compared to native lipase.

Variants of *Candida antarctica* lipase A and native lipase were tested for their ability to hydrolysis of oil containing 60% *trans* isomer. The obtained results were used to estimate the effect of the mutation on the increase of substrate selectivity. It has been

demonstrated that F149D and F222S mutants were characterized by 14- and 4.5-times higher ability to hydrolysis of oil containing 60% *trans* isomers compared to native lipase.