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Effects of Structural Changes in Bile Salt Hydrolase Enzyme on Biocatalytic Efficiency and Activation Energy at Working pH and Temperature Conditions

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Abstract

Microbial bile salt hydrolases (BSHs) catalyse the hydrolysis of glycine and taurine-linked bile salts in the small intestine of humans. Achieving the effects of structural changes in BSH molecules on biocatalytic efficiency (k_{cat}/K_m) and activation energy (E_a) is necessary to determine biocatalytic performances of the enzymes. Amino acids responsible for biocatalytic activity or substrate specificity in BSH molecules were modified to determine the effects of structural changes on k_{cat}/K_m values and E_a values of the bioconversion reactions. Purified wild type positive control enzyme (pCON2) and mutant recombinant target enzymes (F18L and Y24L) reacted with six conjugated pure bile salt substrates at working temperature and pH conditions. The results of the hydrolysis conversion analysis conducted at various pH conditions were used to estimate k_{cat}/K_m, and the assays conducted at various temperature conditions were used to approximate E_a of the biocatalytic reactions. The quantified k_{cat}/K_m value was found remarkably highest with mutant recombinant enzymes (Y24L), while the efficiency value with wild type (pCON2) was determined as lowest, indicating that the structural modifications in BSH molecules showed higher values. The alterations with the mutant-type enzymes F18L and Y24L resulted in decreasing k_{cat}/K_m and increasing E_a estimations of the hydrolysis conversion reactions.

Keywords

Bile salt, wild hydrolase, recombinant hydrolase, biocatalytic efficiency, activation energy

1 Introduction

BSH enzyme, a member of cholylglycine hydrolase (CGH) family, is produced by the intestinal tract microbiota bacteria, such as *Lactobacillus*, *Clostridium*, *Bacteroides*, and *Bifidobacterium*, and catalyses the hydrolysis bioreaction of glycine or taurine-conjugated bile acids to produce taurine or glycinamino acids and bile salts.[1,2] Through bile salt deconjugation, BSH enzymes function as specific signalling and regulating molecules in multiple physiological processes, and contribute to the treatment effect of human diseases such as hypercholesterolemia, colon cancer, diabetes, inflammation, antimetabolic functionality, anti-immune homeostasis, giardial activity, and cardiovascular diseases.[3,4] However, various studies have stated that extra deconjugation of tauro-conjugated bile salts and secondary bile acid production during the hydrolysis can cause formation of deoxicholic and lithocholic acids in the gastrointestinal tract, which may result in undefined toxic effects resulting in colon cancer.[5] Determination of the function of BSH amino acids that are responsible for the substrate specificity or biocatalytic activity can supply information on the role of formation of secondary toxic metabolites.[6] Therefore, the molecular structure of BSH was altered by modifying the amino acids that are hypothetically responsible for the specificity or the activity following site-directed mutagenesis procedures.

BSHs differ in subunit size and composition, parameters of pH and temperature optimum, kinetic properties, substrate specificity, gene organisation and regulation. To understand the characteristic features of BSH, the *bsh* gene is cloned into the expression vector, and production of the active pure BSH protein is required. Future structure analysis of BSH allows to discover key residues of the active site and substrate-binding pocket, and provide information on the substrate selectivity of BSHs. Two BSHs (BSH1 and BSH2) from *Lb. salivarius* were expressed and purified in a heterologous manner, and BSH1 showed a wide optimum pH range of 5.5 to 7.0, while a narrower pH range of 5.5 to 6.0 was determined for BSH2. The kinetic curves of BSH1 and BSH2 were found to be similar to the hyperbolic forms of Michaelis-Menten kinetics in the presence of dithiothreitol (DTT). The results of the obtained Michaelis-Menten kinetics constant (K_m) were found to be significant at pH (6.0) and temperature (37 °C).[1]

The modifications in the amino acid residues can affect the biocatalytic performances, and consequently k_{cat}/K_m and E_a of the enzymes in the substrate hydrolysis reactions. The evaluation of the biocatalytic efficiency can be calculated at working pH and constant temperature, and E_a can be estimated at working temperature and constant pH.

BSH and the other enzymes contain biocatalytically active group residues as binding sites or active sites that are directly dependent on physiological conditions such as pH and temperature. The dependency dominates the activity of enzymes and affects the amino acid chains of biocatalytic

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side residues. The other active groups of the enzyme sites coordinate formation of the enzyme-substrate complex and product. Except for ideal conditions, the extremely lower and higher pH and temperature values reduce the biocatalytic activity and damage the molecular structures of the enzymes. BSH enzymes have specific working pH and temperature conditions, and if the pH and temperature gradients are out of the ideal limits, then apparent activity loss and denaturation occur.

Amino acid residues in the active site chains of enzymes can be charged positively or negatively or protonated or deprotonated by increasing or decreasing the pH values, adjusting the activity of the enzymes. Low pH gives protonated negative charge value to the enzyme solution and to the binding sites, and high pH positively charges the sites. The negative and positive charges affect the strength of the bond between the amino acids in the sites of the enzymes and substrate that indicate the biocatalytic activity.[7]

The Michaelis-Menten equation, used to model the reaction rate V (1/time), is based on the reaction constant (K_m) of the substrates (S) and the maximum reaction rate (V_{max}). The Michaelis-Menten equation was linearised to obtain K_m and V_{max} constants using the Eadie-Hofstee linearisation approach, one of the most used linearisation methods.[8] According to the Eadie-Hofstee approximation equation, the linear equation of the Michaelis-Menten function was obtained by plotting the graph according to V *vs* V/S for estimation of V_m from the intersection of y axis, and V_m/K_m from the intersection of x axis.[9,10]

The model of an enzyme-catalysed reaction mechanism consists of the binding of the total amount of enzyme (e_T) to substrate, and then the catalysis of the reaction and release of the product. The binding of the substrate to the enzyme is determined by the affinity constant $K_A = k_1/k_{-1}$ and the Michaelis-Menten reaction constants (K_m), which are $K_S = (k_2 + k_{-1})/k_1$ for the substrate, and $K_P = (k_2 + k_{-1})/k_{-2}$ for the product. The maximum velocity (V_m) for the forward enzyme reaction equals to the multiplication of the biocatalytic rate constant (k_2) and the total amount of enzyme (e_T). Overall kinetic constants can be arranged in various combinations, and Michaelis-Menten velocity of enzyme kinetics was approached for the substrate concentration.[11]

The BSH enzyme hydrolysis is strictly temperature-dependent and actual temperature conditions are needed for ideal biocatalytic activity. The temperature had a noticeable direct effect on the kinetic energy of the molecular structure. Therefore, there is occurrence possibility of clever inactivation at low temperatures and severe denaturation at high temperatures in enzyme molecules. The temperature impacts the value of kinetic constants, which affect the E_a that is the function of the kinetic constants and reaction temperature, defined as the critical energy of the conversion reactions.[12] Enzymes decrease the E_a of the specific reactions, resulting in the increase in the reaction rates by specifically binding more strongly at the transition state than at the reactant state.[13] The Arrhenius equation was used to determine the E_a of enzymatic reactions, that can also be written in linear equation form, which was applied for plotting ln$k$ *vs* $1/T$. The value of $-E_a/R$ was calculated from the slope, and ln$A$ from the intercept of the plot. The k_{cat} and reaction temperature values were used in Arrhenius equation for determination of the E_a to obtain maximum conversion of the substrates and high product formation in enzyme-catalysed reactions.[14] The equations of Michaelis-Menten (V) and the maximum velocity (V_m), and the Arrhenius equation (k) and their linearised forms are given in Table 1.

Table 1 – Michaelis-Menten equations and maximum velocity (V_m), and Arrhenius equation and linearised form

Michaelis-Menten	$V = \frac{V_m S}{K_m + S}$
Linearised Michaelis-Menten (Eadie-Hofstee)	$V = V_m - K_m \frac{V}{S}$
Maximum velocity	$V_m = k_{cat} e_T$
Arrhenius	$k = Ae^{-E_a/RT}$
Linearised Arrhenius	$\ln k = \ln A + \left(-E_a / RT\right)$

The molecular structure of the amino acid residues of BSH enzymes was engineered according to the amino acid modification applications.[2] Ninhydrin reagent test was conducted for detection of the presence of amino acids in the sample, having the reaction taking place between the amino group and the ninhydrin reagent.[21]

This research aimed to investigate the effects of the structural changes in the BSH molecules on k_{cat}/K_m and E_a at ideal pH and temperature ranges.

2 Materials and methods

The assays were performed according to the standard experimental procedures using six primary and secondary bile acids (glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), and taurochenodeoxycholic acid (TCDCA)) as the substrates. The bile acid standards and other chemicals were obtained from Sigma-Aldrich. The BSH enzymes were obtained through transferring the amplified bile salt hydrolase (BSH) gene from *Lactobacillus plantarum* to *Escherichia coli*. Partially purified wild type enzyme (pCON2) and mutant recombinant target enzymes (F18L and Y24L) were used in the duplicated hydrolysis conversion assays carried out at a) pH values of 3.0, 4.0, 5.0, and 7.0 at a constant temperature of 37 °C, and at b) 25, 37, 45, 55, and 65 °C at a constant pH value of 6.0. The standard Ninhydrin assay method was applied to detect the activity of wild-type recombinant and mutant BSH enzymes.[21]

The conversion data of the substrates observed at different pH values at constant temperature were used to determine the biocatalytic efficiency, and the data collected at differ-

ent temperature and constant pH conditions were applied to assess E_a, consistent with previously accomplished studies.

3 Results

The data plots of Eadie-Hofstee have given the most satisfactory linear lines among the three equations. Effects of structural changes on k_{cat}/K_m and E_a of human-sourced purified wild type and mutant BSHs were investigated at ideal pH and temperature ranges. The molecular structure of recombinant BSHs were altered by modifying the amino acids evidently responsible for biocatalytic activity following the site-directed mutation procedures.

The six bile salts as pure substrates were applied for the hydrolysis reactions with the purified enzymes at ideal pH and temperature conditions. The biocatalytic activities and the reaction mechanisms of the BSH enzymes were evaluated by estimating the overall Michaelis-Menten kinetic constants for the converted substrate concentrations. The reaction kinetic constants, the rate of binding of substrates to the enzymes (K_m), and the maximum reaction rate (V_m) values were determined using the hydrolysis conversions data for the experimented pH and temperature values. The bioprocess of an enzyme-catalysed reaction mechanism consists of the binding of the total amount of enzyme (e_T) to substrate, and then the catalysis of the reaction and the release of the product. The biocatalytic rate constant (k_{cat}), ratio of maximum velocity (V_m), and bioreaction constant (K_m) were determined to approximate the biocatalytic efficiency values. The kinetic constants were used to model the reaction rates (V) for the determination of biocatalytic efficiency, and E_a for the enzymes and substrates. The biocatalytic efficiency and E_a values of purified wild type positive control enzyme (pCON2) were compared to the purified mutant recombinant enzymes (F18L and Y24L) at the investigated pH and temperature values.

3.1 Effect of structural changes on biocatalytic efficiency

In this part of the research, the purified BSH enzymes were applied to hydrolyse the six pure conjugated bile salts as substrates at varied pH and constant temperature conditions to approximate the biocatalytic efficiency values of the BSHs. The hydrolysis conversion data of the six substrates measured at the experimented pH values were applied to determine K_m and V_m, which were used to estimate V and k_{cat}/K_m of the BSHs.

The hydrolysis conversions of the six pure bile salt substrates were tested with purified wild and mutant enzymes (F18L and Y24L) at different pH and fixed temperature conditions as presented in Fig. 1.

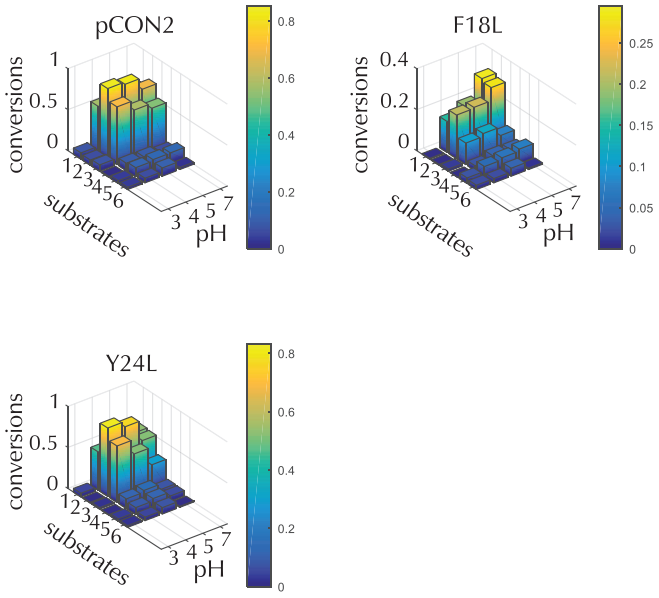

Fig. 1 – Conversions of six pure bile salts reacted with purified wild type enzyme (pCON2) and mutant recombinant enzymes (F18L and Y24L) at various pH values and constant temperature of 37 °C. (Substrates: 1 – GCA, 2 – GDCA, 3 – GCDCA, 4 – TCA, 5 – TDCA, 6 – TCDCA)

The reaction rate (V) was predicted upon quantifying approximation of K_m and V_m, and the graphs of the reaction rate (V) *vs* hydrolysed amount of the six substrates and experimented pH values are shown in Fig. 2.

The biocatalytic rate constant determinations were achieved using V_m estimations and e_T values, which were determined by activity of the purified mutant recombinant

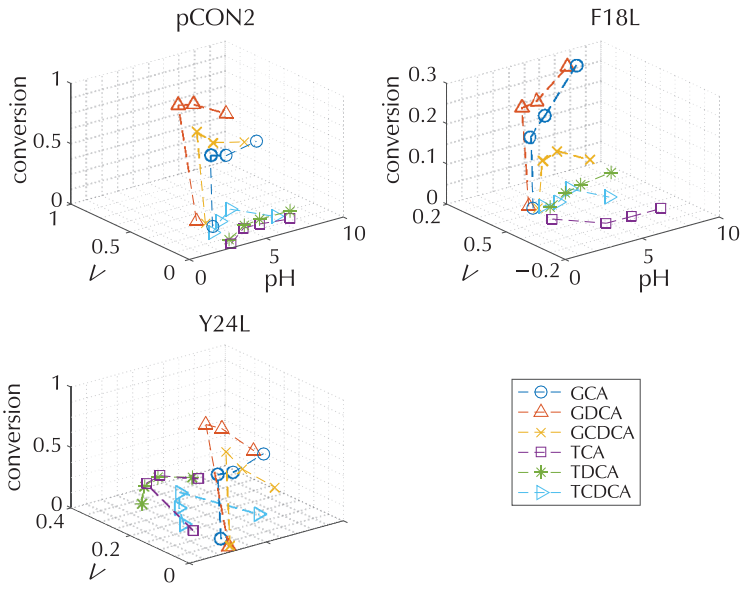

Fig. 2 – The estimated reaction rates (V) *vs* hydrolysis conversion of the six pure bile salt substrates tested on the purified wild type positive control and purified mutant recombinant enzymes at various pH values and constant temperature of 37 °C

enzymes. The ratio of K_m over k_{cat} was used to attain k_{cat}/K_m assessments of the BSHs bioprocess. The biocatalytic efficiency graphs for the six bile salt substrates hydrolysed by the BSH enzymes are presented in Fig. 3.

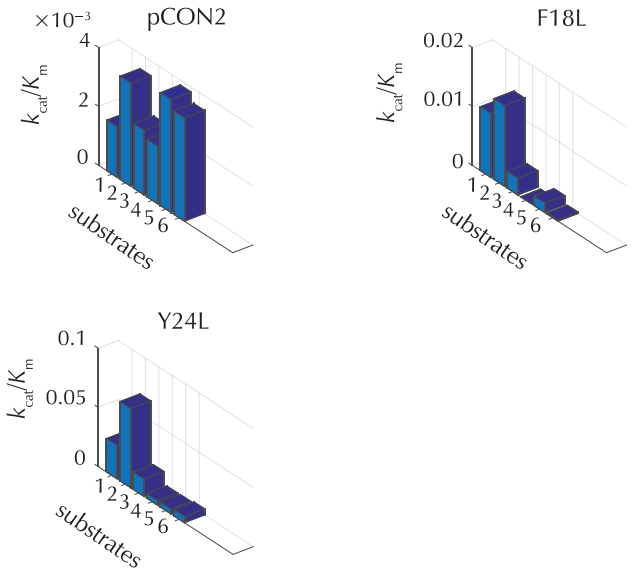

Fig. 3 – Estimated biocatalytic efficiency (k_{cat}/K_m) for six bile salt substrates hydrolysed by the BSH enzymes at pH values and constant 37 °C temperature conditions. (Substrates: 1 – GCA, 2 – GDCA, 3 – GCDCA, 4 – TCA, 5 – TDCA, 6 – TCDCA)

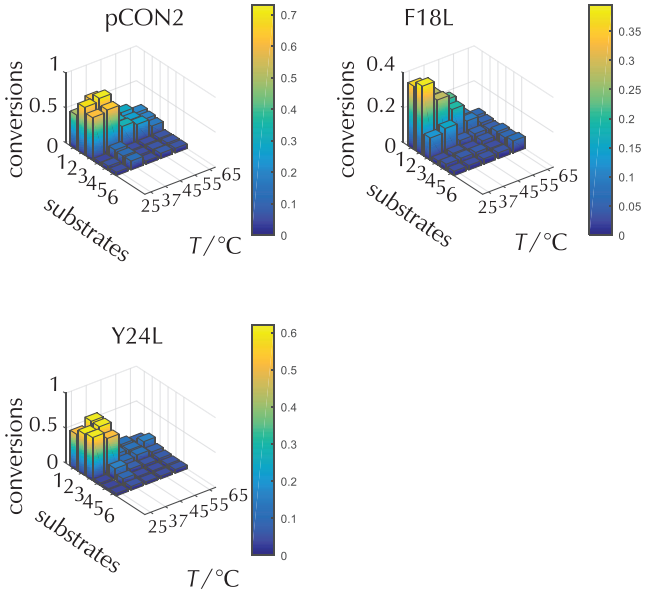

Fig. 4 – The hydrolysis conversions of six pure bile salts reacted with the purified wild type enzyme positive control and mutant recombinant enzymes at various temperatures and constant pH value of 6.0. (Substrates: 1 – GCA, 2 – GDCA, 3 – GCDCA, 4 – TCA, 5 – TDCA, 6 – TCDCA)

3.2 Effect of structural changes on E_a

Effect of structural alterations in the molecular structures of the BSH enzymes on E_a was evaluated in this section. The amino acids responsible for biocatalytic activity of BSHs molecules were modified to determine the effects of structural changes on E_a at ideal temperature gradients. The six pure conjugated bile salt substrates were reacted with purified BSH enzymes at different temperatures and fixed pH conditions. The hydrolysis conversions data were used to determine K_m and V_m values, which were used to estimate the reaction rates (V).

The hydrolysis conversions of the six bile salt substrates tested with purified wild and mutant enzymes (F18L and Y24L) at different pH and fixed temperature conditions are presented in Fig. 4.

Fig. 5 shows reaction rates of the conjugated six bile salt substrates assayed with wild type enzyme positive control (pCON2) and mutant enzymes at 25, 37, 45, 55, 65 (°C) temperatures and fixed pH 6.0 conditions.

The assessed k_{cat} values determined at the experimented temperature conditions were used to estimate E_a for experimented enzymes and substrates. The E_a of hydrolysis reactions of the BSHs, wild type positive control enzyme (pCON2), and mutant recombinant enzymes (F18L and Y24L) that catalysed the six bile acids at 25, 37, 45, 55, 65 (°C) temperatures and constant pH 6.0 conditions was evaluated to investigate the modification effect on the biocatalytic activity of the wild

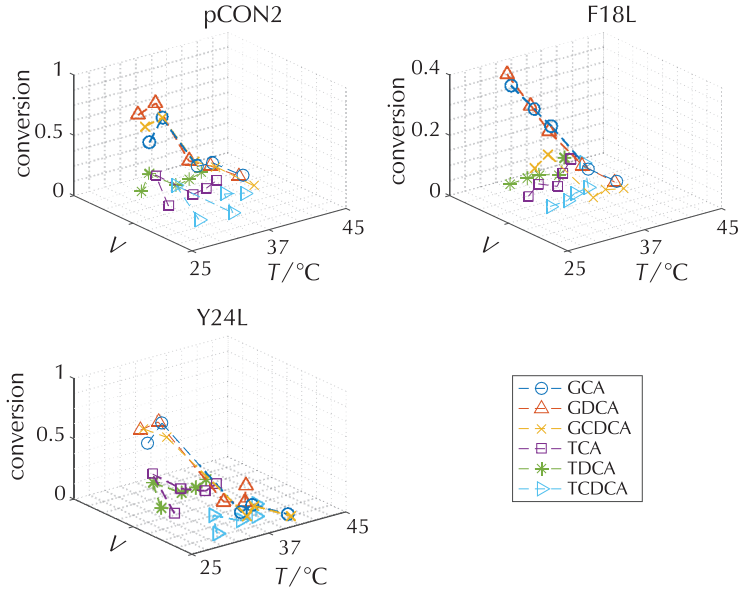

Fig. 5 – Graphs of the reaction rates, hydrolysis of the six pure bile salt substrates, and tested temperatures. The substrates were assayed with purified wild type enzyme positive control and purified mutant enzymes at various temperatures and constant pH value of 6.0.

and recombinant enzymes. The estimated k_{cat} and experimented temperature values were used to evaluate the E_a as illustrated in Fig. 6.

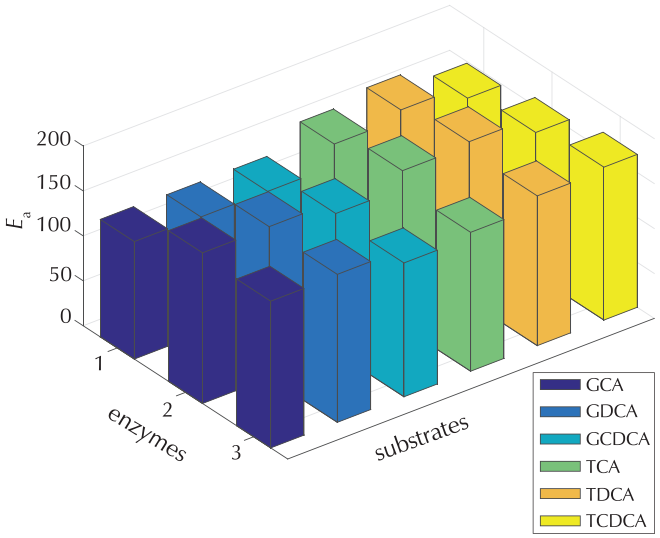

Fig. 6 – Estimated E_a values of the reactions of the purified wild type positive control enzyme and mutant recombinant enzymes. (Enzymes: 1 – pCON2, 2 – F18L, 3 – Y24L)

4 Discussion

Modifications in molecular structure of the recombinant BSHs are presumed to affect the biocatalytic performances of BSHs due to the biocatalytically active group residues of the enzymes that control the hydrolysis reactions at ideal surrounding pH and temperature conditions.

The purified wild type pCON2 was used as the positive control enzyme, and the purified mutant recombinant enzymes (F18L and Y24L) were used as the target enzymes in hydrolysis of the six pure conjugated bile salts (GCA, GDCA, GCDCA, TCA, TDCA and TCDCA) to estimate the biocatalytic efficiency and E_a at different pH and temperature conditions.

The biocatalytic performances were investigated by determination of biocatalytic efficiency estimated from hydrolysis reactions at ideal pH and constant temperature, and E_a approximated from hydrolysis reactions at ideal temperatures and constant pH, in accordance with the studies accomplished using natural and mutant enzymes.[2–23]

The BSHs were applied to the six substrates for hydrolysis conversions to assess the reaction rate (V) and biocatalytic efficiency constants of BSHs at ideal pH values of 3.0, 4.0, 5.0, 7.0 and constant 37 °C temperature conditions. The hydrolysis conversions of the six substrates at experimented pH ranges are given in Fig. 1, which indicates overall high conversions in glyco-conjugated bile salt substrates with wild type control enzyme (pCON2) at 4.0, 5.0, 7.0 pH points that were found ideal pH ranges for the whole conversion reactions. The lowest conversions were observed with the tauro-conjugated bile salt substrates, and mutant recombinant enzyme (F18L) performed low biocatalytic activity when compared to the other conversions. The reaction rate of hydrolysis conversions at experimented pH ranges is presented in Fig. 2, which indicates overall high reaction rate in glyco-conjugated bile salt substrates with wild type enzyme (pCON2) and the mutant recombinant enzyme (Y24L). Low reaction rate was recorded with mutant recombinant enzyme (F18L), which performed lower conversion reactions when compared to the other enzymes, indicating low enzyme-substrate interactions and low force of the bonds between the amino acids in active sites of the enzymes and binding sites of the substrates.

Fig. 3 shows significantly highest biocatalytic efficiency value for mutant recombinant enzymes (Y24L). Dramatically low reaction rate values were recorded at pH 3.0 due to the low pH, which reduced the biocatalytic activity of the enzymes. The pH parameter reflected the binding progress of the enzymes to the substrates; thus, the low pH reduced binding k_{cat}/K_m values, which resulted in lower yield in enzyme reactions.[16]

The biocatalytic efficiency value of mutant recombinant enzymes (Y24L) was found significantly highest, and the value of wild type (pCON2) was determined as lowest, suggesting high biocatalytic performance of the mutant recombinant enzymes over the wild type (pCON2) enzyme. The modifications in molecular structure of BSHs showed higher values. The biocatalytic efficiency of hydrolysis reactions of the glyco-conjugated bile salts was found slightly higher at ideal pH 5.5 and 41 °C temperature conditions.[12]

The data of hydrolysis conversions of the six conjugated GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA bile salt substrates were reacted with wild type enzyme positive control (pCON2), and mutant recombinant target enzymes (F18L and Y24L) at 25, 37, 45, 55, 65 (°C) temperatures and constant pH 6.0 conditions were used to quantify the reaction rate and E_a values. The ideal temperature range was observed at 25 and 37 °C temperatures, as shown in Fig. 4, in which the highest hydrolysis conversions were recorded with reaction of wild type enzyme (pCON2) and the mutant recombinant enzyme (Y24L) with the glyco-conjugated substrates. The highest conversion occurred at 37 °C representing the tested highest conversion. Evident low conversions were observed with the tauro-conjugated bile salt substrates, and mutant recombinant enzyme (F18L) performed with low biocatalytic activity compared to the other enzymes.

The BSHs were exerted to the six substrates for hydrolysis conversions to determine the reaction rate and biocatalytic efficiency coefficients of BSHs at experimented temperature conditions. The reaction rates of the conversions of the glycol-conjugated substrates with the reaction of wild type enzyme (pCON2) and the mutant recombinant enzymes (Y24L) were estimated as highest at ideal temperature points 25 and 37 °C, as shown in Fig. 5. Remarkably low reaction rates were detected at 45, 55, and 65 °C temperature conditions.

The E_a values for the biocatalytic conversion reactions were approximated according to the studies completed previ-

ously using the kinetic constants and experimented temperature values. The estimated E_a results are presented in Fig. 6, which reveals a high E_a value in mutant-type enzymes (F18L and Y24L) reactions, and lower E_a was recorded for the conversion reactions performed by wild type enzyme (pCON2). The alterations in molecular structure of BSHs led to a significant increase in E_a of the hydrolysis reactions.

5 Conclusion

Six conjugated pure bile salt substrates were used for the hydrolysis conversion analyses with purified wild type positive control enzyme (pCON2) and purified mutant recombinant enzymes (F18L and Y24L). The wild type (pCON2) enzyme demonstrated a low value, and the mutant (Y24L) enzyme showed a high k_{cat}/K_m value, demonstrating that structural modifications in BSH molecules result in higher k_{cat}/K_m values. The estimated E_a was found low with wild type enzyme pCON2, and the modified mutant-type enzymes (F18L and Y24L) resulted in higher E_a values, indicating that the modifications had increased the E_a of the enzyme reactions. This study shows that the modification of the amino acids responsible for the activity in the BSH enzyme molecular structure, lowered k_{cat}/K_m and increased E_a values of hydrolysis conversion reactions.

AUTHOR STATEMENT

Yakup Ermurat: Conceptualisation, Methodology, Data curation, Visualisation, Original draft preparation, Mehmet Öztürk: Conceptualisation, Methodology, Reviewing and Editing, Supervision, Cansu Önal: Conceptualisation, Methodology, Investigation, Zekiye Kılıçsaymaz: Methodology, Investigation.

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SAŽETAK

Učinci strukturnih promjena hidrolaze žučne soli na biokatalitičku učinkovitost i energiju aktivacije pri radnim uvjetima pH i temperature

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Mikrobne hidrolaze žučnih soli (BSH) kataliziraju hidrolizu soli glicina i taurina u tankom crijevu ljudi. Aminokiseline odgovorne za biokatalitičku aktivnost ili specifičnost supstrata u BSH molekulama modificirane su da bi se odredio utjecaj strukturnih promjena BSH molekula na biokatalitičku učinkovitost (k_{cat}/K_m) i energiju aktivacije (E_a) reakcija biokonverzije. Pročišćen prirodni enzim pCON2, upotrijebljen kao kontrola, a mutantni enzimi (F18L i Y24L) reagirali su sa šest čistih supstrata konjugiranih žučnih soli pri radnim uvjetima pH i temperature. Eksperimenti provedeni pri različitim pH vrijednostima primijenjeni su u procjeni k_{cat}/K_m vrijednosti biokatalitičkih reakcija, dok su eksperimenti provedeni pri različitim temperaturama primijenjeni u aproksimaciji njihovih E_a vrijednosti. Iznos k_{cat}/K_m bio je najveći kod mutantnih enzima (Y24L), a najniži kod kontrole (pCON2), što ukazuje da su strukturne modifikacije u BSH molekulama povezane s većom učinkovitosti. Preinake kod mutantnih enzima F18L i Y24L rezultirale su smanjenjem vrijednosti k_{cat}/K_m te povećanjem procijenjene E_a vrijednosti reakcija hidrolize.

Ključne riječi
Žučne soli, prirodna hidrolaza, preinačena hidrolaza, biokatalitička učinkovitost, energija aktivacije

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