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Expression, Purification and Initial Characterization of Fungal Hydratases

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Abstract

Hydratases, belonging to the lyase enzyme class, form a broad class of enzymes that catalyze the addition of water to carbon-carbon double bonds in a variety of substrates. Due to the fact that the addition of water to C=C double bonds shows poor selectivity using chemical catalysts, the application of a regio- and stereoselective biocatalyst holds advantages in the production of secondary and tertiary alcohols.

In this thesis, recombinant hydratases of fungal origin were functionally expressed in two different hosts, *Escherichia coli* and *Pichia pastoris*, from codon-harmonized sequences. As well, biochemical protein analyses and an assay for reliable *in vitro* quantification of hydratase activity were developed. In order to obtain homogeneous hydratases for protein crystallization, potential N-glycosylation sites were removed by site-directed mutagenesis in all consensus sequences. Additionally, enzyme variants with amino acid exchanges in the native signal sequence were expressed and analysed. Functional hydratases were obtained, characterized and subjected to protein crystallization.

Zusammenfassung

Hydratasen, welche zur Enzymklasse der Lyasen gehören, bilden eine breite Klasse von Enzymen, die die Addition von Wasser an Kohlenstoff-Kohlenstoff-Doppelbindungen in einer Vielzahl von Substraten katalysieren. Da die Addition von Wasser zu C=C-Doppelbindungen unter Verwendung von chemischen Katalysatoren schlechte Selektivität zeigt, hat die Anwendung eines regio- und stereoselektiven Biokatalysators mehrere Vorteile bei der Herstellung von sekundären und tertiären Alkoholen.

In dieser Arbeit wurden Hydratasen pilzlichen Ursprungs codon-harmonisiert und in den zwei Expressionssystemen, *Escherichia coli* und *Pichia Pastoris,* produziert und biochemisch analysiert. Weiters wurde ein Assay für die zuverlässige *in vitro* Quantifizierung der Hydratasen-Aktivität entwickelt. Um homogene Hydratasen für die Proteinkristallisation zu erhalten wurden potentielle N-Glykosylierungsstellen durch ortsgerichtete Mutagenese in allen Konsensus-Sequenzen entfernt. Zusätzlich wurden Enzymvarianten mit Mutationen in der nativen Signalsequenz exprimiert und analysiert. Funktionelle Hydratasen wurden erhalten, charakterisiert und zur Proteinkristallisation eingesetzt.

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1 Introduction

Hydratases or hydro-lyases, belonging to the lyase enzyme class (EC) 4.2.1, catalyse the addition of water to isolated or conjugated carbon-carbon double bonds in a variety of substrates.[1] Although enzymatic hydration activity has been known for several decades and more than 100 hydratases have been discovered to date, only a few have been used in industrially relevant applications. The most prominent example is a fumarase from *Brevibacterium flavum*, used for the production of L-malate from fumarate on a multi-ton scale.[2] Due to the fact that the addition of water to C=C double bonds shows poor selectivity using chemical catalysts, the application of a selective biocatalyst holds advantages in the production of secondary and tertiary alcohols. Advantages include mild transformation conditions, reduced manufacturing costs as well as high stereo- and/or regioselectivity of the reaction.[1]

The glycoenzyme kievitone hydratase (KHS), secreted by the bean pathogen *Fusarium solani* f. sp. *phaseoli*, catalyses the conversion of the prenylated isoflavonoid kievitone, generating the less toxic metabolite kievitone-hydrate (

Figure 1).

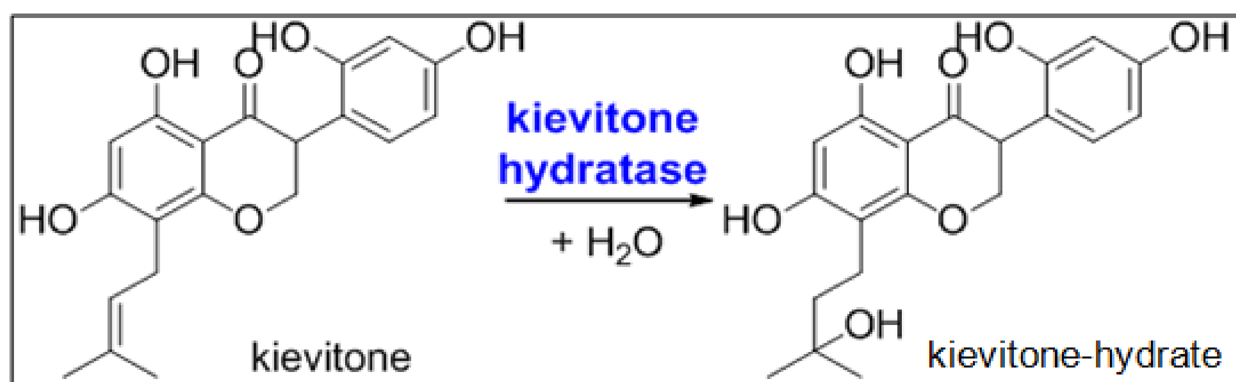

Figure 1: Conversion of kievitone to kievitone-hydrate by kievitone hydratase.

Kievtione is an antimicrobial phytoalexin produced by the French bean *Phaseolus vulgaris* upon microbial infection. Detoxification of kievitone is mediated by kievitone hydratase, adding water to the carbon-carbon double bond of the prenyl group and resulting in a tertiary alcohol.[3] Hence, KHSs are interesting enzymes due to their ability to selectively catalyse the addition reaction leading to the production of a tertiary alcohol, which is still challenging to produce via chemical synthesis.

However, detailed analyses of the enzyme were concerning activity and stability as well as substrate range are missing.

The aim of this thesis was the heterologous expression of KHSs at suitable amounts for biochemical characterization as well as crystallization experiments to characterize their three-dimensional structure. Similar work had been performed on recombinant oleate hydratase originating from *Elizabethkingia meningoseptica*[4]. Further goals were the engineering of KHSs regarding stereo- and/or regioselectivity as well as stability and activity on non-physiological substrates. Depending on the availability of structural information, rational design and directed evolution approaches were to be undertaken in the engineering of hydratases. Hence, recombinant expression of three KHSs, originating from different organisms, called *Aspergillus terreus* (*Ate*)-, *Fusarium solani* (*Fso*)- and *Naectria haematococca* (*Nha*)- KHS, was performed in two different expression systems, *E. coli* and *P. pastoris*.

First, KHSs were overexpressed in *E. coli*, since it is one of the most widely used hosts for recombinant protein expression, due to its ability of fast growth to high cell densities on economical substrates, its detailed characterization as well as access to a large number of vectors and strains.[5] Expression of the enzymes was followed by purification trials and development of a suitable assay for reliable quantification of KHS activity using the natural substrate kievitone as well as the potential alternative substrate cholesta-5,7,24-trien-3beta-ol (Figure 2).

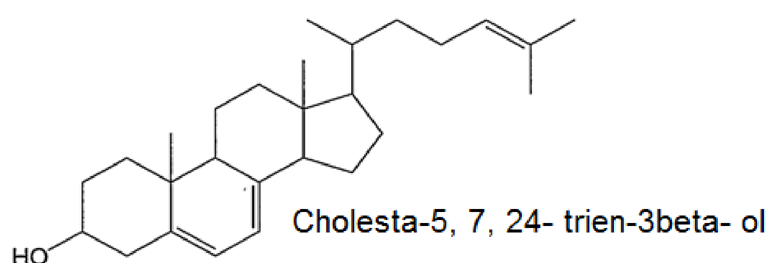

Figure 2: Potential alternative substrate cholesta-5, 7, 24- trien-3beta-ol tested for kievitone hydratase activity.

Since expression and purification in *E. coli* was not successful, *P. pastoris* was used as expression host. Expression in yeast might be an appropriate alternative to the expression in *E. coli*, because the KHS genes are derived from fungi and, therefore, could benefit from a eukaryotic expression host. *P. pastoris* provides many advantages as protein expression host, including growth to high cell densities,

posttranslational modifications, strong and tightly regulated promoters as well as expression of the recombinant proteins either intracellularly or through highly efficient secretion using suitable signal sequences. As a methylotrophic yeast, *P. pastoris* is able to metabolize methanol as sole carbon source for energy production.[6] Isolation of the yeasts' microsomal fraction indicated that KHSs are membrane fraction associated and definitely not cytosolic enzymes. Due to intracellular translocation, caused by an N-terminal signal sequence[7], N-glycosylation[8] of the proteins was observed. However, for crystallization experiments to elucidate the enzymes' three-dimensional structure and possibly the reaction mechanism, removal of N-linked glycans is often favored. Hence, N-glycosylation site muteins as well as enzymes with mutations concerning the native signal sequence were expressed in *P. pastoris* and analysed.

2 Materials and methods

All instruments and reagents, bacterial and yeast strains, expression plasmids and genes, as well as tools for molecular cloning are described in the Appendix section.

2.1 General methods

2.1.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed with 1% agarose gels containing 0.25 µg/mL of ethidium bromide in TAE buffer. Preparative gels were run at 90 V for 90 min and analytical gels were run at 130 V for 30 min. Sizes of separated DNA fragments were identified by comparison to GeneRuler™ DNA Ladder Mix.

2.1.2 DNA gel and PCR purification

Purification of separated DNA from preparative 1 % agarose gels and DNA amplified through PCR was achieved using the Wizard® SV Gel and PCR Clean Up System. DNA was eluted using 30 to 60 µL of ddH_2O (preheated to 65 °C).

2.1.3 Preparative DNA restriction

The total volume of purified plasmid or cleaned-up PCR was mixed with respective restriction enzyme(s) and recommended buffer for preparative DNA restriction. The mixtures were incubated at 37°C for 4 h when working with FastDigest™ restriction enzymes or at 37°C over night when working with regular restriction enzymes.

2.1.4 Control restriction

One µL of purified plasmid was mixed with 1 µL of FastDigest™ restriction enzyme(s) and 1 µL of appropriate buffer (10x) as well as 6 µL of ddH_2O. The reaction was performed at 37°C for 2 h.

2.1.5 Determination of DNA concentration

DNA concentration in 1 µL of purified DNA sample was determined by absorbance measurement at 260 nm using the NanoDrop 2000 UV-Vis spectrophotometer.

2.1.6 DNA ligation

For ligation, vector backbone and insert were mixed at a molar ratio of 1:3. One µL of T4-ligase and 1.5 µL of ligase buffer (10x) were added. A total volume of 15 µL was obtained by adding ddH$_2$O. The ligation reaction was incubated at 37°C for 30 min followed by enzyme inactivation at 72°C for 10 min. The mixtures were either instantly used for electrotransformation or stored at -20°C.

2.1.7 Transformation of electrocompetent *E. coli* cells

The ligated expression vector was transformed into electrocompetent *E. coli* cells using electroporation. Eighty µL of electrocompetent cells were mixed with 2 µL of plasmid and incubated on ice. Immediately after electroporation (program EC2: electric pulse of 5-6 ms at 2.5 kV) cells were regenerated in 1 mL of 2xTY or SOC medium, followed by incubation in a thermo mixer at 37°C and 650 rpm for 30 min. Defined amounts of regeneration culture were plated onto LB-plates provided with the respective antibiotic. Plates were incubated at 37°C over night.

2.1.8 Plasmid isolation

Several transformants were streaked onto fresh LB-plates supplied with the respective antibiotic. After 24 h of incubation at 37°C, plasmid DNA was isolated using the GeneJET™ Plasmid Miniprep Kit. Purified plasmid DNA was eluted using 60 µL of nuclease free water (preheated to 65°C). Control restriction of purified plasmid DNA was performed using FastDigest™ restriction enzymes. Restriction products were analysed on a 1 % agarose gel to confirm correct cloning, followed by storage at -20°C until further use.

2.2 Expression of kievitone hydratases in different *E. coli* strains

E. coli Top10F’ was used for cloning while *E. coli* BL21 Star™ (DE3) was used for expression. Additionally, seven other *E. coli* strains (see Appendix) were tested for improved protein expression.

2.2.1 Recombinant protein expression in LB-medium

The overnight culture (ONC) was prepared by inoculation of 5-10 mL of LB-medium, supplied with the respective antibiotics, of the respective *E. coli* strain and was incubated over night at 28°C and 120 rpm. The main culture was prepared by inoculation of 100 mL LB-medium containing the respective antibiotics in a 300 mL baffled flask to an OD_{600} of 0.1 and incubated at 28°C and 120 rpm. After 3 -4 h of growth, the cell cultures reached an OD_{600} of 0.8 to 1.0 and were induced with 20 µM isopropyl-β-D-thiogalactopyranosid (IPTG). Recombinant protein expression was performed at 28°C and 120 rpm for 6 h. Cells were harvested via centrifugation (4°C, 5000 rpm, 20 min) using an Avanti™ centrifuge equipped with a JA-10 rotor. Prior to further treatment, the cell pellet was stored at -20°C over night.

2.2.2 Preparation of CFE

The cell pellet was resuspended either in 25 mL of 50 mM sodium citrate, pH 5 for the initial preparation of cell free extract (CFE)[9], or 25 mL of 50 mM buffer with different pH values, including sodium citrate at pH 4, 5 or 6; potassium phosphate at pH 7 or 8 and Tris-HCl with a pH value of 9. Also, 25 µL of 1 M phenylmethylsulfonyl fluoride (PMSF) were added to the suspension. Then, the cell suspension was disrupted via sonication for 2x4 min at 80 % duty cycle and output control level 8 under cooling. Samples of total cell extract (TCE) were taken prior to centrifugation at 4°C, and 22,000 rpm for 40 min using an Avanti™ centrifuge and a JA-25.50 rotor. After centrifugation, CFE samples were collected.

2.2.3 Protein expression analysis

<u>Bio-Rad protein assay</u>

Protein concentrations in TCE and CFE were determined by Bio-Rad protein assay according to a 96 well plate protocol established by A. El- Heliebi[10]. In brief, 10 µL of (diluted) samples were mixed with 200 µL of BioRad reagent, which was diluted in a 1:5 ratio with ddH_2O, incubated for 5 minutes and measured in a microplate reader at 595 nm. A calibration curve was generated by applying different concentrations of Bovine serum albumin (BSA) in a range from 0.05 to 1.0 mg/mL. As a blank, 10 µL of ddH_2O were used.

SDS-PAGE (Sodiumdodecylsulphate-polyacrylamide gel electrophoresis)

Five 1 mm thick SDS-gels were prepared in two steps as follows. First, the separation gel (12.5%) was made using 9.43 mL of ddH_2O, 7.03 mL of Tris-HCl (1.5 M, pH 8.8), 11.25 mL of Acrylamid/Bis (30%), 281.25 µL of SDS (10% w/v), 140.63 µL of ammonium persulfate (APS, 10% w/v) and 28.13 µL of TEMED. The gels were poured and covered with n-butanol. After solidification, n-butanol was removed using filter paper. Then, the stacking gel (4%) was prepared using 11.45 mL of ddH_2O, 4.70 mL of Tris-HCl (0.5 M, pH 6.8), 2.45 mL of Acrylamid/Bis (30%), 187.50 µL of SDS (10% w/v), 93.75 µL of APS (10% w/v), 18.75 µL of TEMED and 10 µL of phenol-blue, followed by application of 15-well combs. Gels were stored at 4°C in Tris-HCl.

Prior to loading the gels, protein samples were mixed with SDS–loading dye consisting of NuPage® LDS Sample Buffer (4x) and NuPage® Sample Reducing Agent (10x) and were denatured at 37°C for 10 to 20 min. Ten or 15 µL of the protein samples as well as 5 µL of Novex® Sharp Pre-Stained Protein Standard were applied and gels were run in SDS–Running Buffer at 125 V and 30 mA for approximately 60 to 90 min.

For Coomassie Blue staining, gels were put in a plastic box and covered with Coomassie Blue staining solution for 10 min under moderate shaking at room temperature. Destaining of the gels was performed over night with 10 % acetic acid.

Immunoblot – Western blot[11]

Target proteins were detected in TCEs and CFEs by Western blotting. First, proteins were separated using SDS-PAGE, followed by blotting of macromolecules onto a nitrocellulose membrane. Therefore, a blotting sandwich between two electrodes drenched in transfer buffer was prepared. Starting from the cathode, 3 blotting pads and 2 filter papers soaked in transfer buffer were layered with the SDS-gel and nitrocellulose membrane. To complete the sandwich, 2 filter papers and 3 blotting pads soaked in transfer buffer were placed on top (anode). It was important, to avoid air bubbles in the pads and filter paper. The sandwich was placed into the blotting unit. The inner chamber was filled up with buffer, overlaying the sandwich, and the outer chamber was filled with a mixture of water and ice, used as cooling solution. Electrophoretic transfer from gel to membrane was conducted by applying an electric

field with a potential of 500 V, power of 50 W and current of 125 mA. Blotting was performed for 1 h.

After blotting and before proceeding with the protocol, the proteins on the membrane were stained with PonceauS (0.1 % w/v) in acetic acid (5 %) to test for transfer efficiency. Therefore, the membrane was put in a plastic box and covered with staining solution for 1 min under moderate shaking. Afterwards, the membrane was destained with ddH_2O. To avoid non specific binding of antibodies to the membrane, blocking was performed by covering the membrane with 50 mL of TBST-milk blocking solution for 1 h under moderate shaking at room temperature.

All Western blots were treated with the indirect detection method. First, an unlabeled primary antibody (H1029; anti-His antibody from mouse) binds to the antigen (His_{10}-tag of the target protein) and a horse radish peroxidase-labeled secondary antibody (A4416; Anti-mouse IgG) binds to the primary antibody. Then, the secondary antibody is detected by chemiluminenscence using the SuperSignal® WestPico Chemiluminescent Substrate kit.

Immediately after blocking, the membrane was covered with 50 mL of primary antibody solution and was incubated over night under moderate shaking at 4°C. The next day, the membrane was washed for 5x10 min with TBST, followed by application of the secondary antibody solution for 1 h under moderate shaking at room temperature. Thereafter, the membrane was rinsed for 3x5 min with TBST and transferred to the Bioimager G:Box HR16 for detection. Two components (peroxide and enhancer solution) were mixed in a 1:1 ratio for substrate reaction. The solution was pipetted onto the membrane, incubated for 2 min and pictures were taken after 30 s, 4 min and 12 min to monitor the progress using GeneSnap program.

2.2.4 Purification of recombinant protein

Purification of His_{10}-tagged[12] protein was performed with self-packed Ni-NTA affinity chromatography columns and gravity-flow protocol using the buffers listed in Table 1. Purification was always conducted with permanent cooling of all aliquots on ice.

Table 1: List of buffers used for purification of recombinant protein.

Buffer	Composition
Binding Buffer	50 mM phosphate, pH 8, with 10 mM imidazole
Washing Buffer	50 mM phosphate, pH 8, with 50 mM imidazole
Elution Buffer	50 mM phosphate, pH 8, with 250 mM imidazole
Desalting Buffer	50 mM potassium phosphate, pH 7
Stripping Buffer	20 mM sodium phosphate, 0.5 M NaCl, 50 mM EDTA, pH 7.4

Gravity flow purification

Ni-NTA affinity chromatography columns were equilibrated with 5 column volumes (CV) of Binding Buffer. Prior to purification, the prepared CFE was filtered through 0.22 µL syringe filter. Thirty mL of filtered CFE were loaded. The column was washed with 10 CV of Binding Buffer and 10 CV of Washing Buffer. Protein was eluted from the column with 5 CV of Elution Buffer. During purification, aliquots of the flow through and washing fractions were collected for assessment with SDS-PAGE. Regeneration of the column was performed prior to each purification in order to re-charge Ni Sepharose™ 6 Fast Flow. Residual Ni^{2+} was removed with 5 CV of Stripping Buffer. Leftover EDTA was removed by application of 5 CV of Binding Buffer followed by 5 CV of ddH_2O. The column was recharged by application of 1 CV of 0.1 M $NiSO_4$. Recharged beads were washed with 5 CV of ddH_2O followed by 5 CV of Binding Buffer and storage in 20% ethanol.

Desalting and concentration of purified protein

Desalting of purified protein was conducted with PD-10 columns for buffer exchange and imidazole removal. PD-10 columns were previously equilibrated with Desalting Buffer and protein was eluted with the same buffer. Elution fractions harboring protein were collected and concentrated with Amicon® Ultra-15 Centrifugal filter devices at 4°C and 5000 rpm for 5 -10 min to approximately 1 mL.

UV-Vis spectroscopy

UV-Vis absorbance spectra of purified and concentrated protein were measured from 250 to 1000 nm in semi micro quartz cuvettes using a Specord 205/BU spectrophotometer. Protein concentrations were calculated using the absorbance at 280 nm.

2.2.5 Determination of kievitone hydratase activity

In vitro activity assay with kievitone and HPLC-UV analysis

The method used for the HPLC-UV measurements was adapted from the protocol provided by DSM. It was developed for reliable quantification of substrate and product after enzymatic reactions. Sample aliquots of 5 µL were injected. The eluents used for this method were nuclease free water and acetonitrile. A gradient profile in reversed phase chromatography was applied. The method started with 5% acetonitrile and progressed to 98% acetonitrile at a constant flow rate of 0.6 mL/min with a total run time of 30 min for each measurement. The column, having a pressure limit of 130 bar, was heated to 40°C. HPLC-UV results were evaluated with the HPLC-UV software of Agilent Technologies.

Prior to the assays, a 100 mM kievitone stock in ethanol was prepared. In order to establish a calibration curve for kievitone, dilutions of 0.004, 0.02, 0.1, 2, 5 and 10 mM of kievitone in 50 mM sodium citrate, pH 5, were prepared and quantified via HPLC-UV measurement at 220 and 291 nm.

For the assay, *E. coli* BL21 Star™ (DE3) was cultivated for preparation of CFEs as described above. To record an activity profile of KHS at different pH values, buffers of pH 4 to pH 9 were used. The standard activity assay was performed in a total volume of 100 µL in tightly screwed PYREX® reaction tubes aligned at a 45° tilt. Hence, 98 µL of CFEs were mixed with 2 µL of kievitone in ethanol stock. The samples were shaken over night at 28°C and 150 rpm. Afterwards, protein was precipitated by the addition of 3 assay volumes of acetonitrile, 15 min of incubation at room temperature and 300 rpm, followed by 5 min of centrifugation. Additionally, a chemical standard (98 µL of 50 mM citrate buffer, pH 5, combined with 2 µL of kievitone stock) and a biological standard (98 µL of CFE of an empty vector control and 2 µL of the kievitone stock) were prepared. Finally, the solution was transferred into HPLC vials and samples were analysed by HPLC-UV.

In vitro activity assay with alternative substrate and HPLC-UV/MS analysis

Sample aliquots of 10 µL were injected into the device. The composition of the mobile phase was kept constant. Hence, the method worked with an isocratic elution mode, pumping a mixture of 80% ethanol, 20% methanol and 0.1% trifluoro acetic acid at a constant flow rate of 0.6 mL/min. The column, having a pressure limit of 230 bar, was kept at 20°C. Total run time of each measurement was 20 min. The UV-Vis-detector was adjusted to measure at a wavelength of 210 nm. The MS was adapted to measure four separate channels. The “SCAN-mode” detected all masses between 200 and 800 m/z-ratio. Each of the three “SIM-modes” was adjusted to detect the mass of either positive ionized substrate or the masses of positive or negative ionized product. HPLC-UV/MS results were evaluated with the HPLC-UV/MS software of Agilent Technologies.

Prior to the assays, 100 mM substrate and product stocks in ethanol were prepared. For the assay, *E. coli* BL21 Star™ (DE3) was cultivated for preparation of CFEs as described above. 50 mM potassium phosphate, pH 7, was used for pellet resuspension. The standard activity assay was performed in a total volume of 100 µL in tightly screwed PYREX® reaction tubes aligned at a 45° tilt. Hence, 98 µL of CFEs were mixed with 2 µL of substrate or product in ethanol stock. Additionally, 5 µL of detergent Tween® 20 were added. The samples were shaken over night at 28°C and 150 rpm. Afterwards, lipids were extracted according to the protocol established by Folch et al.[13]. Additionally, a chemical standard (98 µL of 50 mM potassium phosphate, pH 7 with 2 µL of substrate or product stock) and biological standards (98 µL of CFE of an empty vector control with or without 2 µL of the substrate stock) were prepared. Finally, the solution was transferred into HPLC vials and samples were analysed by HPLC-UV/MS.

2.3 Expression of kievitone hydratases in *P. pastoris*

2.3.1 Transformation of expression vector into *P. pastoris* cells

For linearization of the expression vector, 30 µL of purified plasmid DNA, 2 µL of restriction enzyme *Pst*I, 7 µL of 10x Orange Buffer and 31 µL of ddH_2O were mixed and incubated at 37°C over night. The linearized plasmids were separated on a preparative 1% agarose gel. Bands of the linearized expression vectors were cut and purified. Purified vectors were eluted using 30 µL of nuclease free water (preheated to 65°C).

In parallel, electrocompetent *P. pastoris* CBS7435 Δ*his4* cells were generated. One µg of linearised plasmid was transformed into 80 µL of electrocompetent cells and incubated for 2 h at 28°C[14]. Regenerated cultures of 100 µL of resuspended pellet were plated onto YPD-Zeocin^{100} plates and incubated at 30°C for at least 3 days.

2.3.2 Cultivation of *P. pastoris* transformants and expression analysis

Complex media containing 1% of glycerol (BMGY) or 1 % of methanol (BMMY) were prepared. Five mL of BMGY were inoculated with several colonies of each strain, respectively. ONCs were incubated over night at 28°C, 180 rpm and 80% humidity. In parallel, the colonies were streaked out onto YPD-Zeocin^{100} plates. For main cultures 25 mL of BMGY media were filled into 300 mL baffled flasks and inoculated to an OD_{600} of 0.1. Cell broths were incubated at 28°C, 120 rpm and 80% humidity for 24 h. Then, 25 mL of BMMY were added and additionally, every 12 h, 250 µL of methanol were added for 48 h (*Pichia* Expression Kit, Invitrogen[A]).

After 48 h of induction, 6 OD_{600} units of cell cultures were harvested and treated according to Riezman cell disruption protocol[15]. The samples were prepared to be loaded onto an SDS–gel, followed by either Coomassie Blue staining or immunoblot analysis.

[A] https://tools.thermofisher.com/content/sfs/manuals/easyselect_man.pdf

2.3.3 Isolation of *P. pastoris* microsomes

Total microsomes were isolated from *P. pastoris* upon cultivation as described before in 2 L baffled Erlenmeyer flasks, using 400 mL cell culture. Cells were harvested using Avanti™ centrifuge equipped with a JA-10 rotor at 5000 rpm and 4°C for 5 min and were washed twice with dH_2O. Cell pellets were weighed, resuspended in 1 mL/g CWW TE buffer and 2 µL of 1 M PMSF per g CWW. Cells were disrupted with glass beads by shaking for 3 min in a Merckenschlager Cell Homogenizer under CO_2 cooling. Cell lysate was centrifuged using Avanti™ centrifuge equipped with a JA-10 rotor at 5000 rpm and 4°C for 10 min to remove glass beads and unbroken cells. The resulting supernatant, i.e. the homogenate, was centrifuged using Avanti™ centrifuge and a JA-25.50 rotor at 10000 rpm and 4°C for 15 min. The pellet representing the mitochondrial fraction was resuspended in TE buffer. The mitochondrial supernatant was centrifuged again using Avanti™ centrifuge and a JA-25.50 rotor at 10000 rpm and 4°C for 15 min, followed by ultracentrifugation at 45000 rpm and 4°C for 45 min using Optima™ LE-80K Ultracentrifuge. The supernatant after ultracentrifugation was the cytosolic fraction. The microsomal pellet was resuspended in TE buffer with Dounce homogenizer using a loose fitting pestle. Five hundred µL of each fraction were collected. Protein concentration in the fractions was determined by Bio-Rad assay and 50 µg of total proteins were precipitated with trichloroacetic acid on ice for 1 h. Protein analyses were performed according to SDS-PAGE and immunoblot protocols.

2.3.4 Native signal sequence

Analysis of the amino acid sequences of KHSs (see Appendix section) displayed an N-terminal signal sequence as predicted by the proteomics tools Phobius and SignalP on the ExPASy web page[16]. To elucidate the function of the putative signal peptide, constructs without the signal sequence (nosig) and with a masked signal sequence using an N-terminal FLAG[17]-tag were designed.

2.3.5 Amino acid exchange: Site-directed mutagenesis

Site-directed mutagenesis

Potential N-glycosylation sites were removed using PCR-based site-directed mutagenesis of Asparagine to Alanine residues in all N-glycosylation consensus sequences as predicted by the post-translational modifications (PTMs)-analysis tool on the UniProt web page[8]. PCR reactions were prepared using 0.5 µL of 15-30 ng/µL template DNA in a 2-step protocol. Two mixtures were prepared for each gene containing 17.45 µL of ddH_2O, 2.5 µL of HF buffer (5x), 2.5 µL of 2 mM dNTPs, 0.8 µL of DMSO, 0.5 µL of Phusion DNA polymerase and 1.25 µL of either forward or reverse primer (Table 16). After initial incubation at 98°C for 30 s, 18 cycles were carried out at 98°C for 10 s, 65°C for 30 s and 72°C for 9 min, followed by 72°C for 7 min. The two half reactions were combined at the end of cycle 5. Removal of methylated plasmid template was achieved with *Dpn*I digestion at 37°C for 2 h. Restriction was stopped by heating at 70°C for 30 min, followed by transformation into *E. coli* Top10F'.

Plasmid sequencing

Twelve µL of isolated plasmid and 3 µL of 10 µM primer (Table 17) were sent to Microsynth Austria GmbH (Vienna, Austria). Sequencing results were analysed on CLC Main Workbench program. Correct variants were transformed into the expression strain.

3 Results

The three kievitone hydratase genes (see Table 2 and Appendix section) sequence-optimized for expression in *E. coli*, were provided by DSM Innovative Synthesis GV (Geleen, the Netherlands). Genes in codon-optimized form for expression in *P. pastoris* were designed by Anita Emmerstorfer-Augustin and provided by DSM Innovative Synthesis GV (purchased from DNA2.0).

Table 2: Kievitone hydratase genes, sequence-optimized for expression in *P. pastoris* or *E. coli*.

Gene	Organism	Gene length [bp]	Protein size [kDa]
Ate-KHS-His_{10}	*Aspergillus terreus*	1152	41.2
Fso-KHS-His_{10}	*Fusarium solani*	1104	40.3
Nha-KHS-His_{10}	*Naectria haematococca*	1098	39.9

3.1 Expression of kievitone hydratases in different *E. coli* strains

The genes, sequence optimized for expression in *E. coli*, were amplified by PCR, using primers containing *Hind*III and *Nde*I restriction sites as well as a C-terminal His_{10}-tag (Table 15) for cloning into the pET26b(+) expression vector (Figure 3). Codon-optimized KHS genes for expression in *P. pastoris* were amplified by PCR, using primers containing *Not*I and *Nde*I restriction sites (Table 15) for cloning into the same *E. coli* expression vector. Basic cloning procedures were performed in *E. coli* Top10F'. Plasmids from several clones of each construct were isolated and control cuts with respective restriction enzymes were conducted. After confirmation of successful ligation, 2 µL of each expression plasmid were transformed into eight different *E. coli* strains (see Table 3) to create the strains for heterologous protein expression.

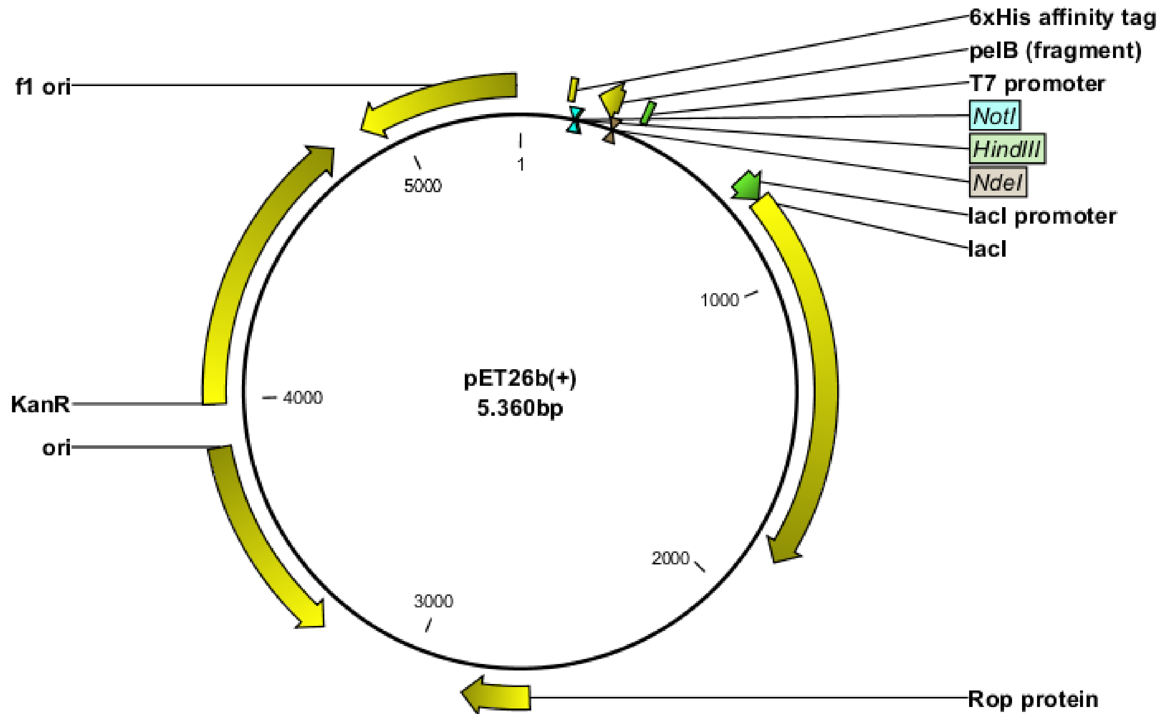

Figure 3: Map of *E. coli* expression vector pET26b(+).

Expression of the C-terminally His_{10}-tagged KHSs from pET26b(+) vector, which was induced with IPTG, was tested using eight different *E. coli* strains. Selection was driven by kanamycin and the respective antibiotics listed in Table 3.

Table 3: *E. coli* strains used for KHS expression.

Strain	Antibiotics and inducers for cultivation in LB medium
E. coli BL21 Star™ (DE3)	
E. coli BL21 (DE3) CodonPlus-RIL	20 mg/L Chloramphenicol
E. coli BL21 (DE3) TaKaRa5	20 mg/L Chloramphenicol, 0.5 mg/mL L-arabinose
E. coli BL21 (DE3) TaKaRa1	20 mg/L Chloramphenicol, 0.5 mg/mL L-arabinose, 5 ng/mL Tetracycline
E. coli Rosetta (DE3) pLysS	20 mg/L Chloramphenicol
E. coli K12 ER2508	
E. coli TUNER	
E. coli Arctic Express	15 mg/L Gentamycin

3.1.1 Protein expression analysis

Initially protein expression analysis was conducted using B-Per™ Bacterial Protein Extraction Reagent for cell disruption. Therefore, 5 OD_{600} units of *E. coli* cell culture, cultivated at 28°C and 120 rpm, were harvested 6 and 20 h after induction with IPTG. Cells were harvested at 10,000 rpm for 5 min. Hundred µL of B-Per™ were added followed by pellet resuspension by vortexing and centrifugation at 14,000 rpm for 3 min. Protein concentration in the supernatant, representing the soluble fraction, was determined using Bio-Rad assay. Then, SDS-PAGE and Western blotting were performed. However, cell disruption and separation of insoluble as well as soluble fraction was not successfully accomplished using B-Per™ reagent and the above described procedure, most likely due to low sample volumes. Hence, the signals that were obtained always represented the total cell lysate and never the soluble fraction. Additionally, there were never any signals obtained for the *Ate*-KHS. Therefore, all further work focused on expression analysis of *Nha*- and *Fso*-KHS.

Subsequent cultivation of seven different *E. coli* strains expressing *Nha*-KHS-His_{10} in sequence optimized form for *E. coli* or *P. pastoris* as well as initial preparation of CFEs was performed as described in the Materials and methods section, followed by SDS-PAGE and Western blot analysis. Therefore, 2 µL of TCE and 10 µL of CFE were mixed with SDS–loading dye to a total volume of 15 µL, denatured at 37°C for 10 min and loaded on the gel.

Analysis of protein expression in *E. coli* BL21 star™ (DE3) and *E. coli* Rosetta (DE3) (Figure 4 and Figure 5), *E. coli* BL21 (DE3) CodonPlus-RIL (Figure 6 and Figure 7), *E. coli* BL21 (DE3) TaKaRa5 and TaKaRa1 (Figure 8 and Figure 9) as well as *E. coli* TUNER and *E. coli* K12 ER2508 (Figure 10 and Figure 11) expressing C-terminally His_{10}-tagged *Nha*-KHS showed similar results. In most strains, KHSs were only found in TCE fractions but never in soluble fractions, indicating formation of inclusion bodies.

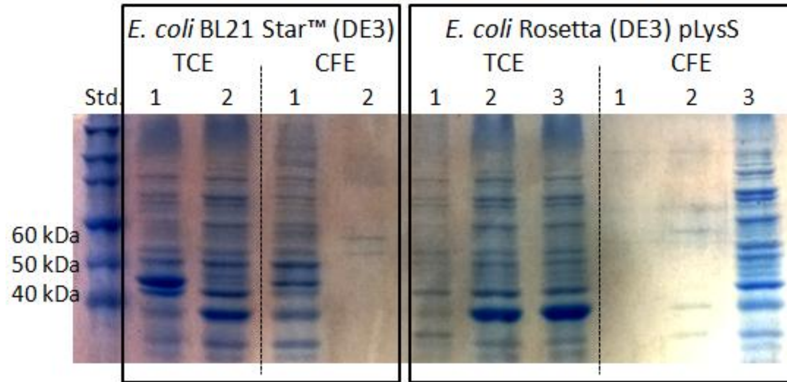

Figure 4: SDS-PAGE analysis of *E. coli* BL21 star™ (DE3) and *E. coli* Rosetta (DE3) pLysS expressing C-terminally His_{10} -tagged *Nha*-KHS (39.9 kDa). Proteins were expressed at 28°C for 6 h upon induction with IPTG. Cell pellets were resuspended in 50 mM sodium citrate, pH 5, and disrupted via sonication. Total cell extracts (TCE) and soluble fractions (CFE) were loaded. 1: pET26b(+) empty vector control strain. 2: *Nha*-KHS-His_{10} codon optimized for expression in *P. pastoris*. 3: *Nha*-KHS-His_{10} codon optimized for expression in *E. coli*. Standard: Novex®Sharp Prestained Protein Standard.

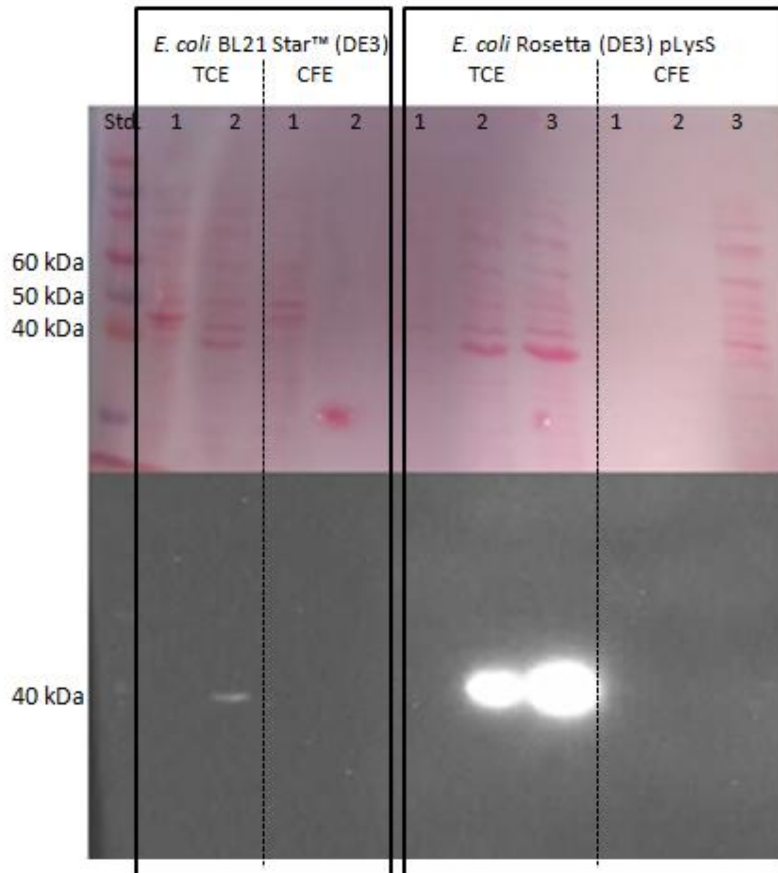

Figure 5: Western blot analysis of *E. coli* BL21 star™ (DE3) and *E. coli* Rosetta (DE3) pLysS expressing C-terminally His_{10} -tagged *Nha*-KHS (39.9 kDa). Legend see Figure 4.

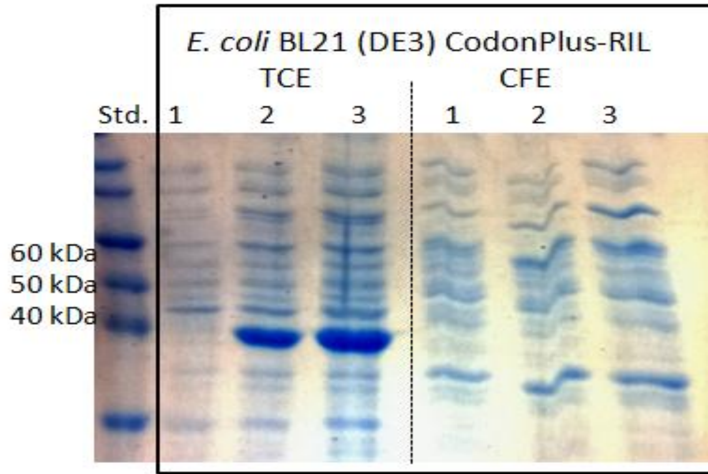

Figure 6: SDS-PAGE analysis of *E. coli* BL21 (DE3) CodonPlus-RIL expressing C-terminally His_{10} -tagged *Nha*-KHS (39.9 kDa). Proteins were expressed at 28°C for 6 h upon induction with IPTG. Cell pellets were resuspended in 50 mM sodium citrate, pH 5, and disrupted via sonication. Total cell extracts (TCE) and soluble fractions (CFE) were loaded. 1: pET26b(+) empty vector control strain. 2: *Nha*-KHS-His_{10} codon optimized for expression in *P. pastoris*. 3: *Nha*-KHS-His_{10} codon optimized for expression in *E. coli*. Standard: Novex®Sharp Prestained Protein Standard.

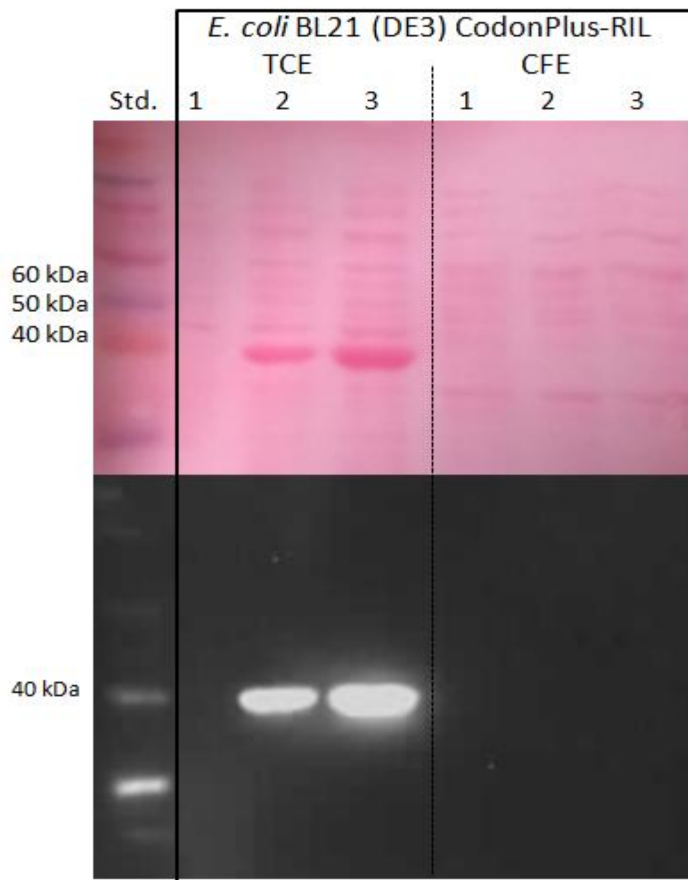

Figure 7: Western blot analysis of *E. coli* BL21 (DE3) CodonPlus-RIL expressing C-terminally His_{10} -tagged *Nha*-KHS (39.9 kDa). Legend see Figure 6.

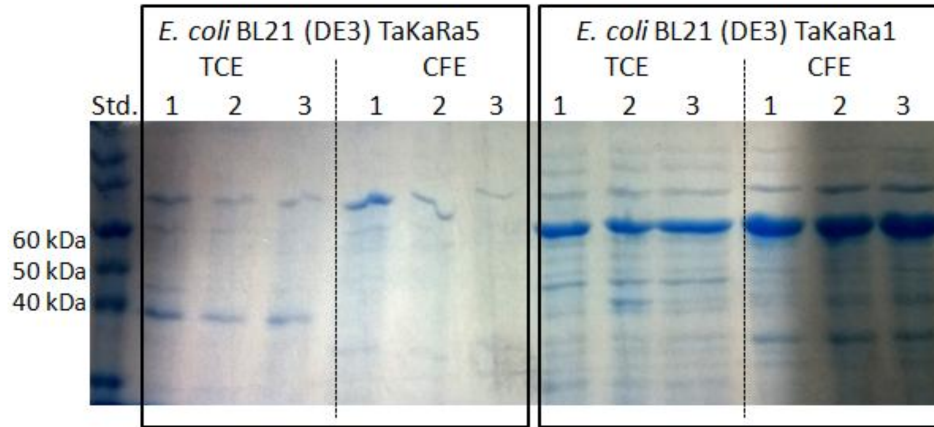

Figure 8: SDS-PAGE analysis followed by Coomassie staining of *E. coli* BL21 (DE3) TaKaRa5 and TaKaRa1 expressing C-terminally His_{10}-tagged *Nha*-KHS (39.9 kDa). Proteins were expressed at 28°C for 6 h upon induction with IPTG. Cell pellets were resuspended in 50 mM sodium citrate, pH 5, and disrupted via sonication. Total cell extracts (TCE) and soluble fractions (CFE) were loaded. 1: pET26b(+) empty vector control strain. 2: *Nha*-KHS-His_{10} codon optimized for expression in *P. pastoris*. 3: *Nha*-KHS-His_{10} codon optimized for expression in *E. coli*. Standard: Novex®Sharp Prestained Protein Standard.

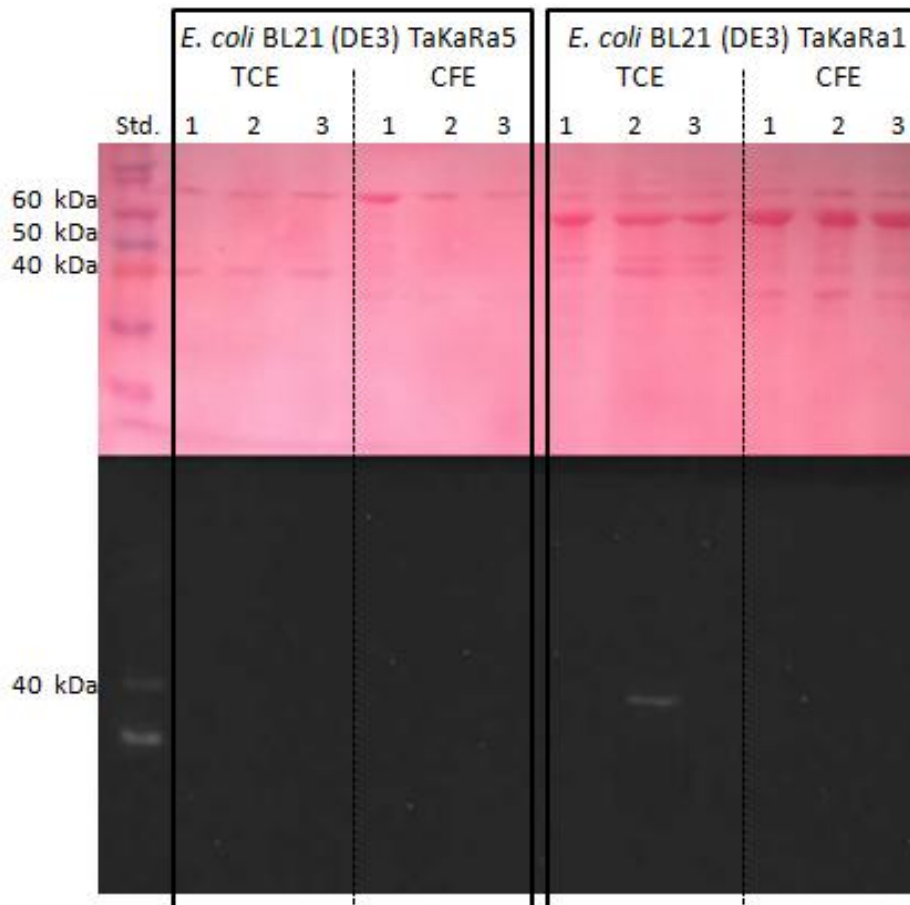

Figure 9: Western blot analysis of *E. coli* BL21 (DE3) TaKaRa5 and TaKaRa1 expressing C-terminally His_{10} -tagged *Nha*-KHS (39.9 kDa). Legend see Figure 8.

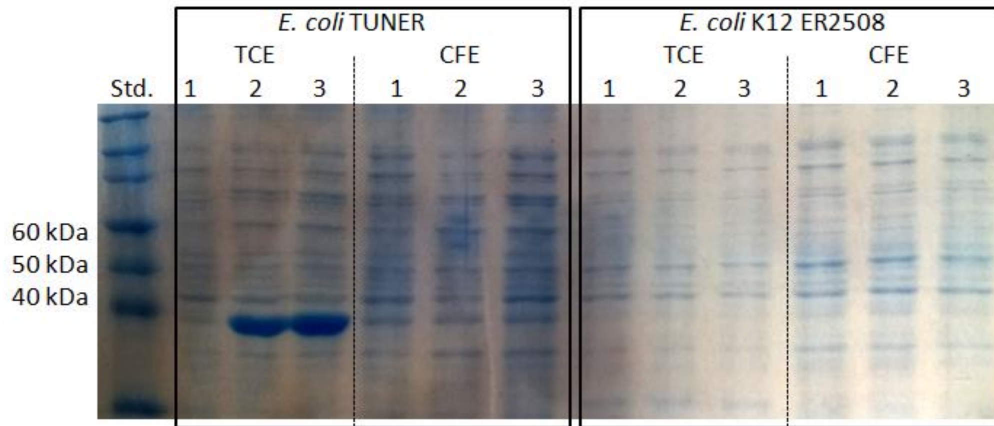

Figure 10: SDS-PAGE analysis followed by Coomassie staining of *E. coli* TUNER and *E. coli* K12 ER2508 expressing C-terminally His_{10} -tagged *Nha*-KHS (39.9 kDa). Proteins were expressed at 28°C for 6 h upon induction with IPTG. Cell pellets were resuspended in 50 mM sodium citrate, pH 5, and disrupted via sonication. Total cell extracts (TCE) and soluble fractions (CFE) were loaded. 1: pET26b(+) empty vector control strain. 2: *Nha*-KHS-His_{10} codon optimized for expression in *P. pastoris*. 3: *Nha*-KHS-His_{10} codon optimized for expression in *E. coli*. Standard: Novex®Sharp Prestained Protein Standard.

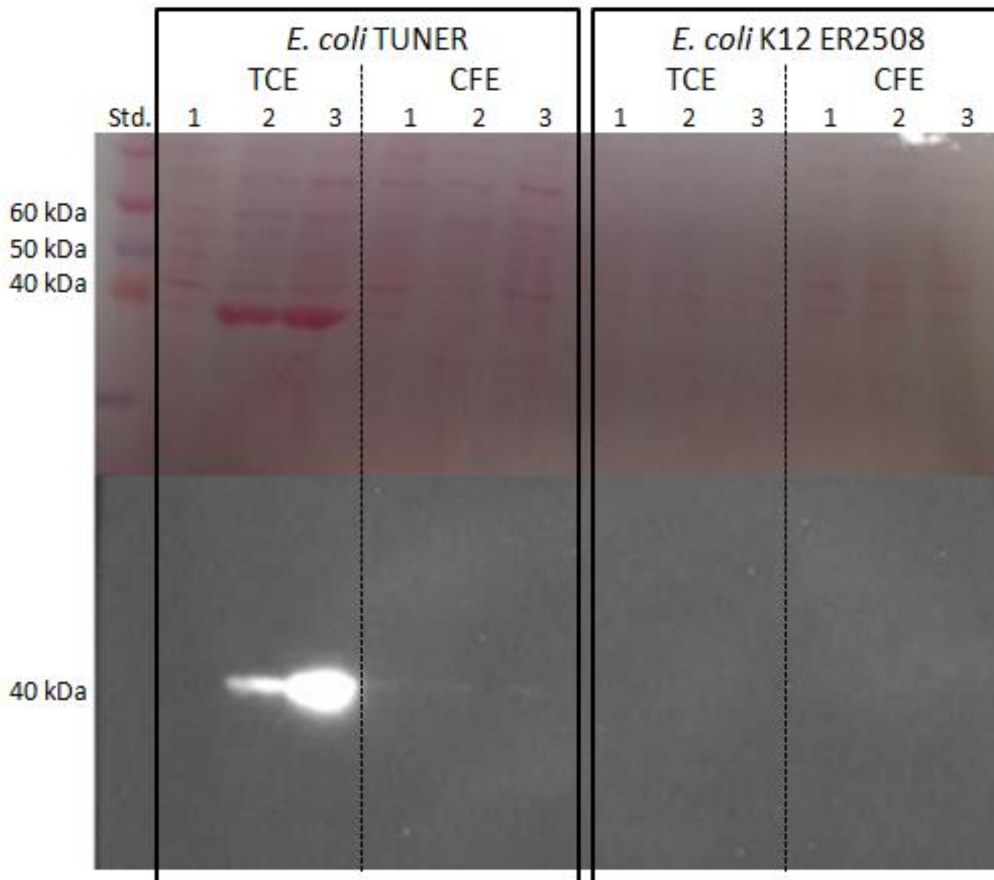

Figure 11: Western blot analysis of *E. coli* TUNER and *E. coli* K12 ER2508 expressing C-terminally His_{10} -tagged *Nha*-KHS (39.9 kDa). Legend see Figure 10.

All *E. coli* strains used for expression of KHSs were chosen due to their improved characteristics concerning the expression of eukaryotic proteins in a prokaryotic host.

The *E. coli* TUNER strain is a *lacZY* deletion mutant of BL21. The *lacY* mutation enables uniform entry of IPTG into all cells in the culture, which leads to adjustable levels of protein expression. The *E. coli* Rosetta (DE3) strain is a BL21 derivative as well, designed to improve expression of eukaryotic proteins containing codons rarely used in *E. coli* (Novagen® User Protocol TB009 Rev. F 0104[B]). The *E. coli* BL21 (DE3) TaKaRa5 and TaKaRa1 strains harbor different types of plasmids expressing molecular chaperones for a proper protein folding process (Chaperone Competent Cell BL21 Series #9120 – 9125 TaKaRa[C]).

However, the expression of KHSs in the different strains showed similar results. In the majority of the strains, KHSs were only found in insoluble, but never in soluble fractions. However, there were small differences in the expression levels when examining different strains, e.g. expression of *Nha*-KHS-His_{10} codon-optimized for *P. pastoris* was higher in the *E. coli* Rosetta (DE3) and *E. coli* TUNER strains (Figure 5 and Figure 11).

Next, CFE samples of *Fso*-KHS-His_{10} and *Nha*-KHS-His_{10}, optimized for *E. coli* and cloned into pET26b(+) vector were analyzed via Western blotting. The best results were obtained with *E. coli* BL21 star™ (DE3), since both KHSs could be detected in the CFE via Western blot analysis (Figure 12). Preparation of CFEs was conducted as described in the Materials and methods section, using buffers with different pH values ranging from pH 4 to pH 9. Samples of the insoluble fraction were taken and 2 µL thereof were mixed with 13 µL of SDS–loading dye. The protein concentration in CFEs was determined by Bio-Rad assay and prepared to obtain 1 mg/mL of protein in 50 µL of SDS–loading dye. Samples were denatured at 37°C for 10 min and 15 µL were loaded onto the SDS gel.

A freshly prepared primary antibody solution was used for immunoblotting of the CFE samples whereas for TCEs, a much older aliquot was applied. Therefore, the intensities of the signals of TCEs and CFEs are not comparable.

[B] https://www.med.unc.edu/pharm/sondeklab/files/resource-files/manuels/novagen_competent_cells2

[C] http://www.takara.co.kr/file/manual/pdf/9120-25_e.v0707.pdf

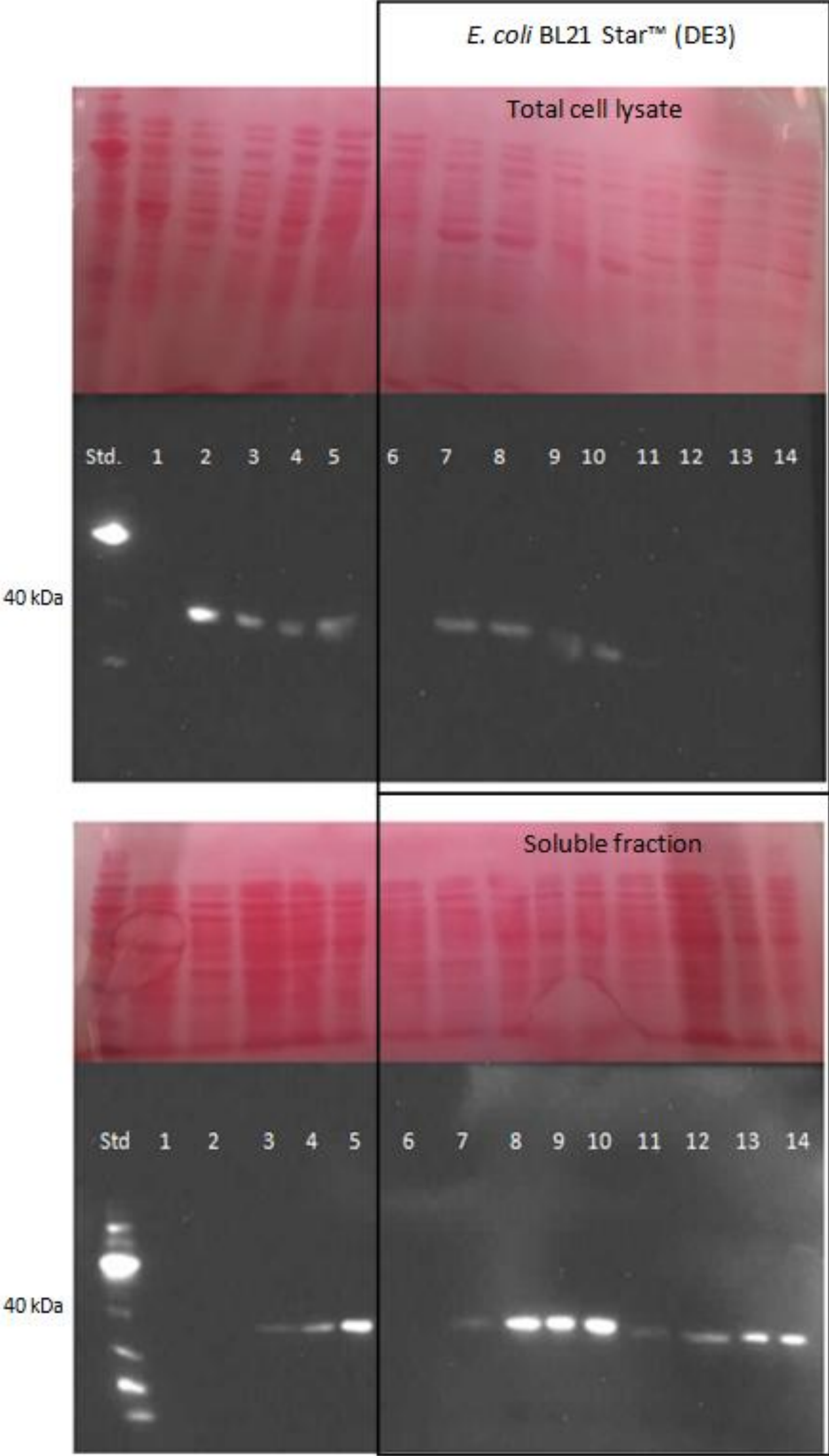

Figure 12: Western blot analysis of *E. coli* BL21 star™ (DE3) expressing C-terminally His_{10} -tagged *Fso*- (40.3 kDa) and *Nha*-KHS (39.9 kDa). Proteins were expressed at 28°C for 6 h upon induction with IPTG. Cell pellets were resuspended in buffers with different pH values and disrupted via sonication. Total cell extracts (TCE) and soluble fractions (CFE) were loaded. Lanes 1-5: pMS470_ *Nha*-KHS-His_{10}, pH 5 (Positive control). Lane 6: pET26b(+) empty vector control. Lanes 7–10: pET26b(+)_*Nha*-KHS-His_{10}, pH 5, 6, 7 or 8. Lanes 11–14: pET26b(+)_*Fso*-KHS-His_{10}, pH 5, 6, 7 or 8. Standard: Novex®Sharp Prestained Protein Standard.

3.1.2 Purification of recombinant protein

On the basis of Western blot analyses (Figure 12), the most promising expression strain *E. coli* BL21 Star™ (DE3) [pET26b(+)_*Nha/Fso*-KHS-His_{10}] was chosen for larger-scale cultivations in 2 L baffled Erlenmeyer flasks containing 200 mL cell culture as described in the Materials and methods section. For preparation of the CFE, 50 mM phosphate buffer, pH 8, was used.

Protein purification was performed via Ni-NTA columns. The purification profiles are shown in Figure 13 and Figure 15.

UV-VIS absorbance spectra were recorded (Figure 14 and Figure 16) to calculate the concentration of purified protein using the molar extinction coefficient ε_{280} calculated via the ProtParam tool on the ExPASy[16] web portal. For *Fso*-KHS, an ε_{280} of 92820 M^{-1} cm^{-1}, and for *Nha*-KHS, an ε_{280} of 83310 M^{-1} cm^{-1} were calculated.

Figure 13 represents the purification profile of the *Fso*-KHS-$His_{10.}$ The CFE as well as the elution fraction did not show a distinct band in the mass range of 40 kDa, corresponding to the expected size of the hydratase of 40.3 kDa. However, the TCE showed a strong band in the expected range. This implies to the formation of inclusion bodies.

Figure 13: Purification of *Fso*-KHS-His_{10} (40.3 kDa) from *E. coli* BL21 star™ (DE3) cultivation. Standard: Novex®Sharp Prestained Protein Standard. EV: CFE of empty vector control. TCE: Total cell extract. CFE: Cell free extract. FT: Flow through. WF: Washing fraction (1-10 using10 mM imidazole, 11-17 using 50 mM imidazole). EF: pooled, desalted (50 mM potassium phosphate, pH 7) and concentrated purified protein.

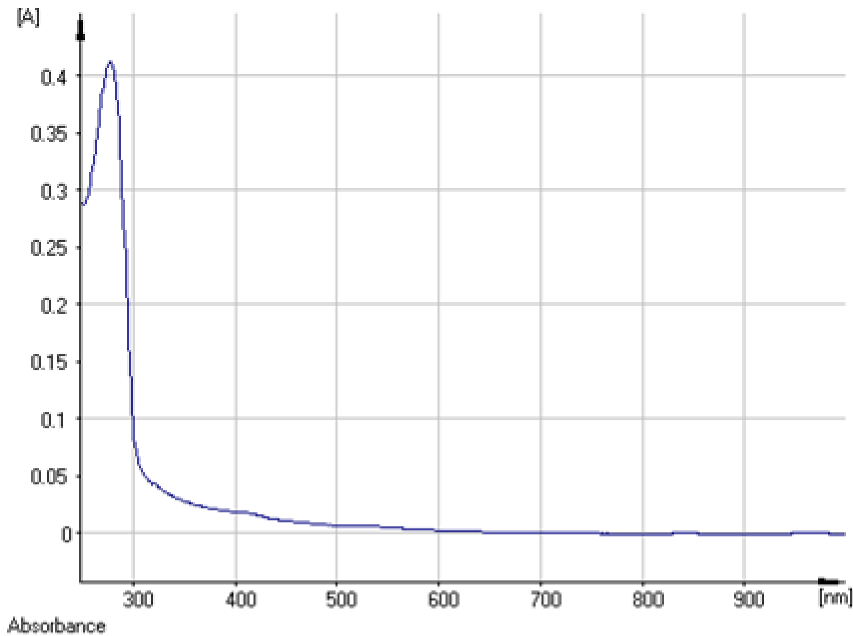

Figure 14: UV-VIS absorption spectrum of *Fso*-KHS-His$_{10}$ in the EF: pooled, desalted (50 mM potassium phosphate, pH 7) and concentrated purified protein. Protein concentration: 4.4*10^{-6} M or 0.18 mg/mL.

The purification profile of *Nha*-KHS-His$_{10}$ showed a massive band in the range of around 40 kDa in the TCE, which matched the expected size of *Nha*-KHS-His$_{10}$ (39.9 kDa). The lack of any distinct band at the same size in the CFE indicated extensive formation of inclusion bodies. The elution fraction showed faint double bands at about 40 kDa, potentially representing *Nha*-KHS-His$_{10}$.

Figure 15: Purification of *Nha*-KHS-His$_{10}$ (39.9 kDa) from *E. coli* BL21 star™ (DE3) cultivation. Standard: Novex®Sharp Prestained Protein Standard. EV: CFE of empty vector. TCE: Total cell extract. CFE: Cell free extract. FT: Flow through. WF: Washing fraction (1-13 using10 mM imidazole, 14-25 using 50 mM imidazole). EF: pooled, desalted (50 mM potassium phosphate, pH 7) and concentrated purified protein. EF2: last elution fraction.

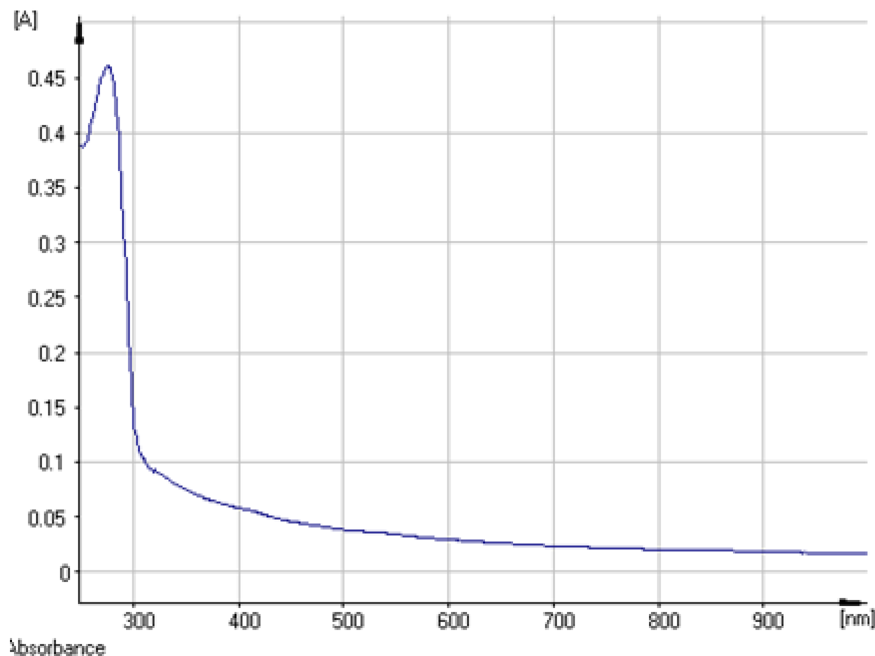

Figure 16: UV-VIS absorption spectrum of *Nha*-KHS-His_{10} in the EF: pooled, desalted (50 mM potassium phosphate, pH 7) and concentrated purified protein. Protein concentration: $5.4*10^{-6}$M or 0.21 mg/mL.

Considering the low yield of protein in the elution fractions, as well as the lack of enzyme purity, these samples were not suitable for protein crystallization attempts.

3.1.3 Determination of kievitone hydratase activity

In vitro activity assay with kievitone and HPLC-UV analysis

All assays were performed as described in the Materials and methods section, followed by HPLC-UV measurement at wavelengths of 220 nm, which was adapted from the protocol by DSM, and 291 nm[18]. Owing to the very limited availability of kievitone, determination of KHS activity was performed only in single measurements.

Quantitation of kievitione was initially attempted by plotting peak areas against concentrations of kievitone dilutions in a range from 10 mM to 4 µM, which were prepared from a 100 mM kievitone stock in ethanol (Figure 17).

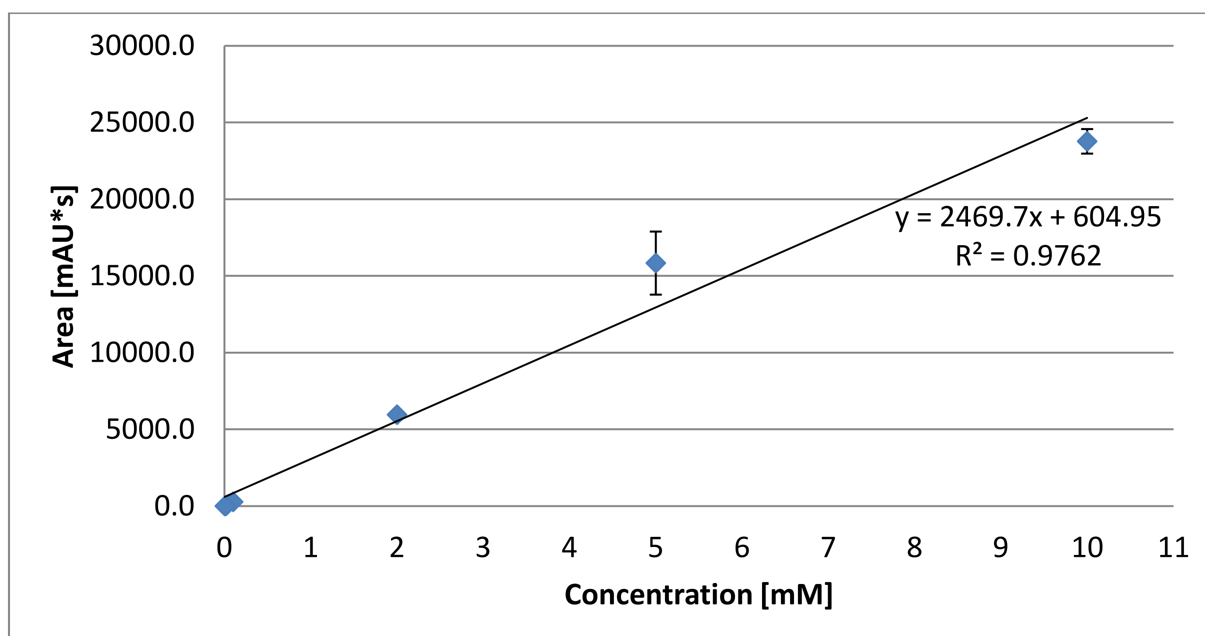

Figure 17: Calibration curve for kievitone dissolved in ethanol and quantified with HPLC-UV at 291 nm.

For the assay, *E. coli* BL21 Star™ (DE3) [pET26b(+)_*Nha/Fso*-KHS-His10] was cultivated for preparation of CFEs as described above. To record an activity profile of KHS at different pH values, buffers of pH 4 to pH 9 were used. A biological standard and a chemical standard were measured as well. HPLC-UV obtained chromatograms at 291 nm and 220 nm of all the assays performed with CFE harboring either *Fso*-KHS-His_{10} (Figure 18 and Figure 19) or *Nha*-KHS-His_{10} (Figure 20 and Figure 21) are shown. The results are consistent, showing conversion of kievitone to kievitone-hydrate from pH 5 to pH 8 for both KHSs. Kievitone eluted at a retention time of approximately 21 min and the product kievitone-hydrate at a retention time of approximately 17 min. At pH 4 and pH 9 the substrate was not transformed into product. However, absolute quantitation was not attempted, due to the limited access to kievitone.

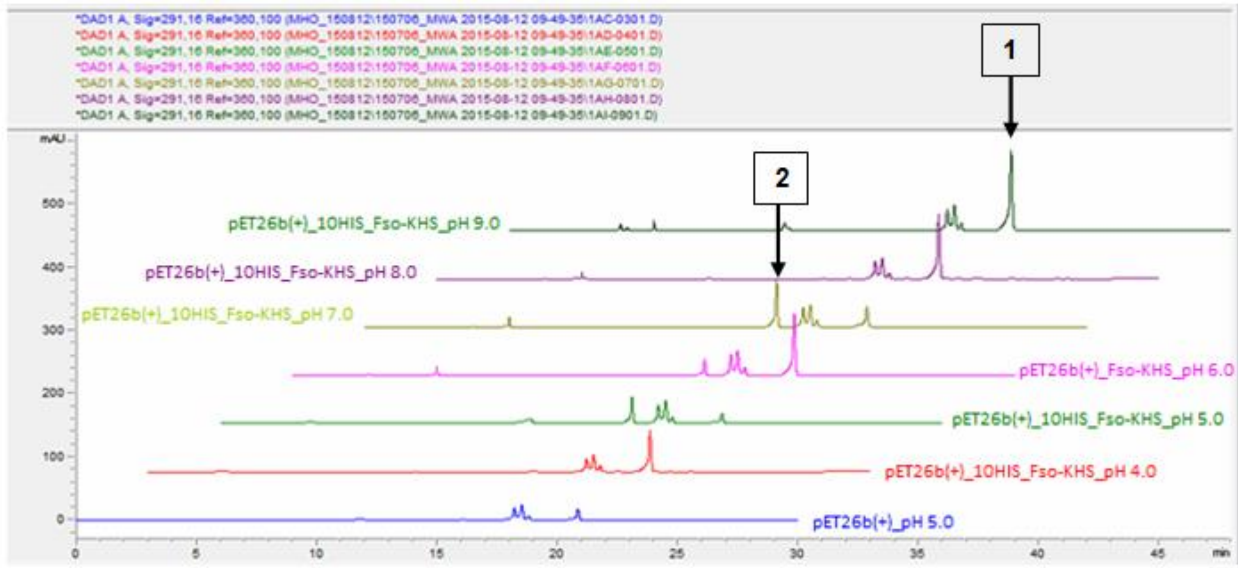

Figure 18: Chromatograms of HPLC-UV measurement at 291 nm of KHS assay performed with CFEs harboring *Fso*-KHS-His$_{10}$. Assays were carried out at pH values from 4 to 9. Chromatogram of HPLC-UV measurement at 291 nm of the biological standard (empty vector control) at pH 5 is shown in blue. 1: Substrate kievitone with a retention time of 21 min. 2: Product kievitone-hydrate with a retention time of 17 min.

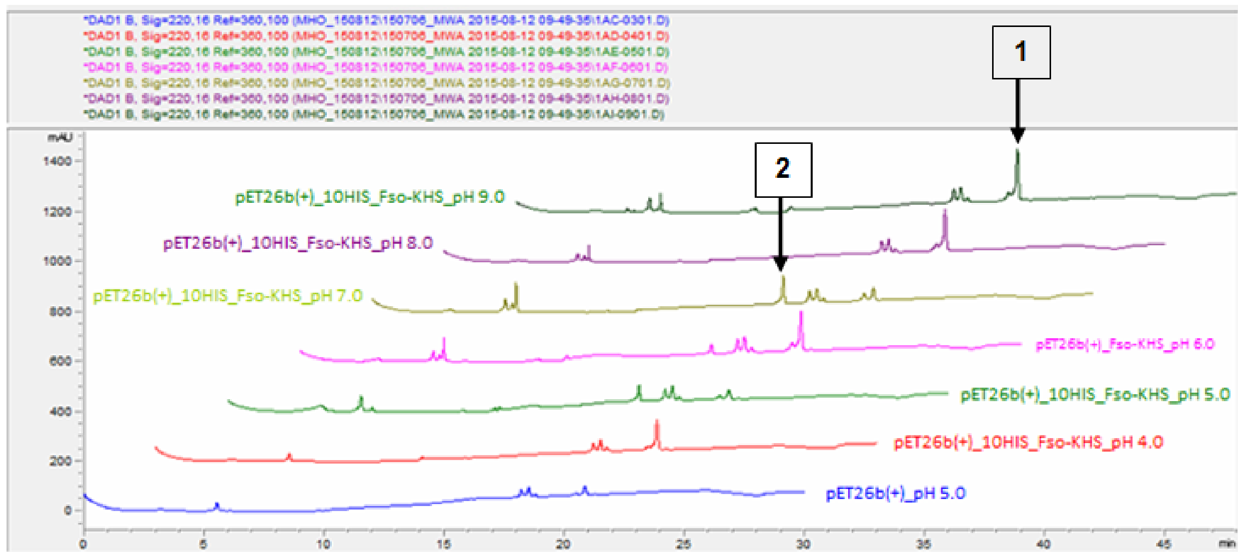

Figure 19: Chromatograms of HPLC-UV measurement at 220 nm of KHS assay performed with CFEs harboring *Fso*-KHS-His$_{10}$. Legend see Figure 18.

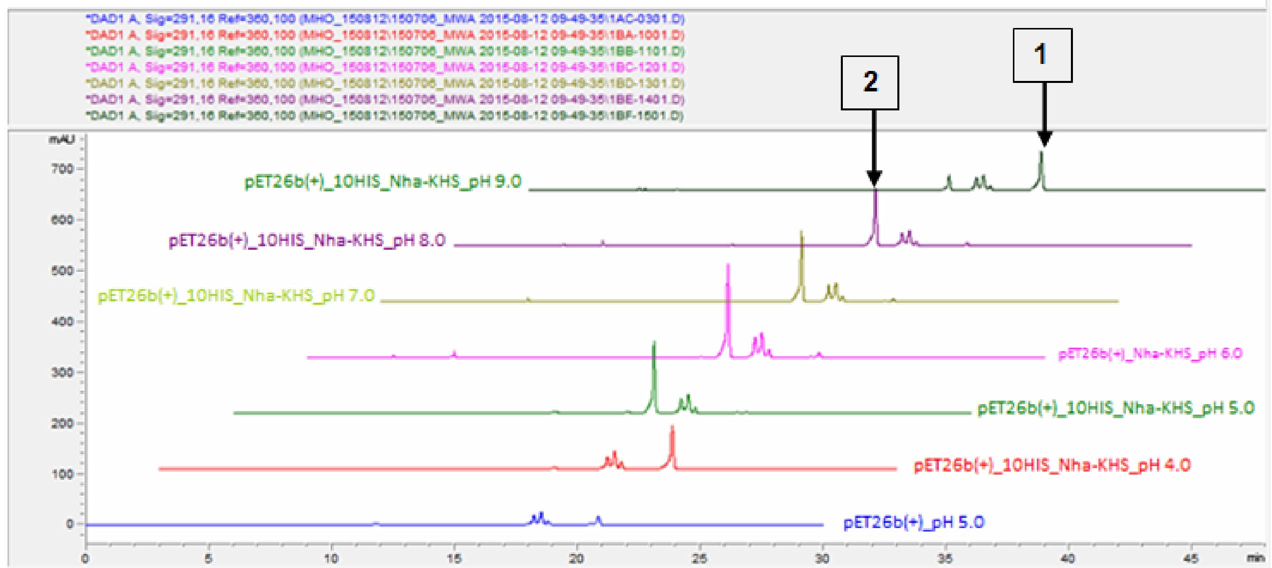

Figure 20: Chromatograms of HPLC-UV measurement at 291 nm of KHS assay performed with CFEs harboring *Nha*-KHS-His$_{10}$. Assays were carried out at pH values from 4 to 9. Chromatogram of HPLC-UV measurement at 291 nm of the biological standard (empty vector control) at pH 5 is shown in blue. 1: Substrate kievitone with a retention time of 21 min. 2: Product kievitone-hydrate with a retention time of 17 min.

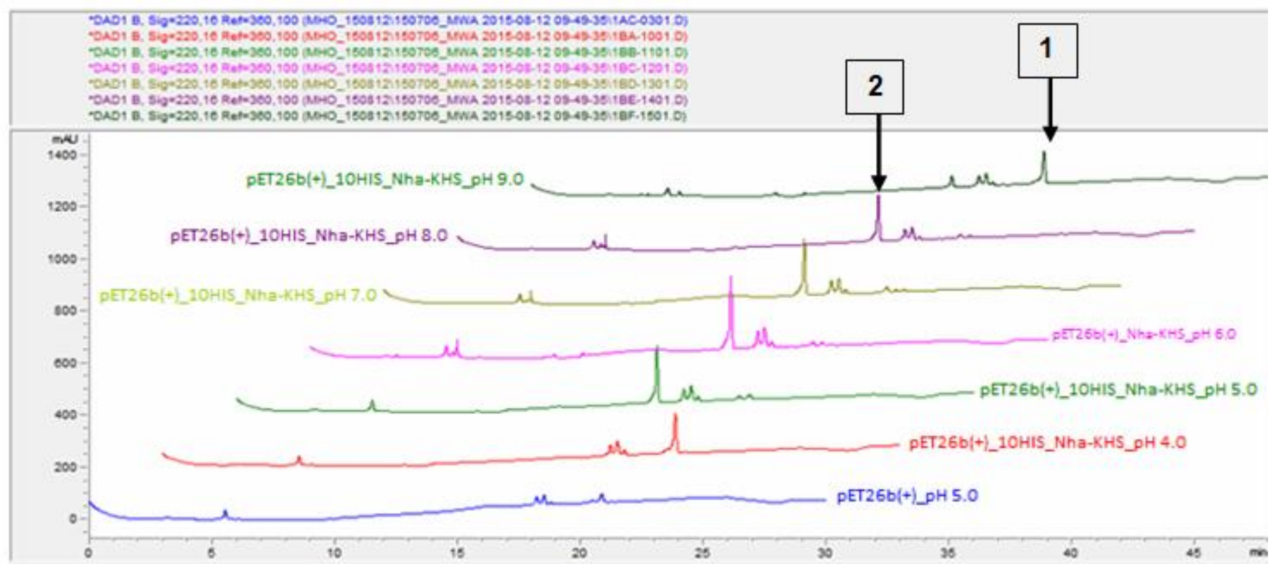

Figure 21: Chromatograms of HPLC-UV measurement at 220 nm of KHS assay performed with CFEs harboring *Nha*-KHS-His$_{10}$. Legend see Figure 20.

The graphs shown in Figure 22 and Figure 23 represent the area-values obtained from HPLC-UV measurement at 220 nm of chemical and biological standards as well as *Fso*- and *Nha*-KHS assays performed at different pH values. The *Nha*-KHS showed the highest activity, with almost complete conversion at several pH values and a maximum activity at pH 6. The *Fso*-KHS displayed highest conversion at a pH of 7. Due to the low area value, the biological standard was considered to be an outlier.

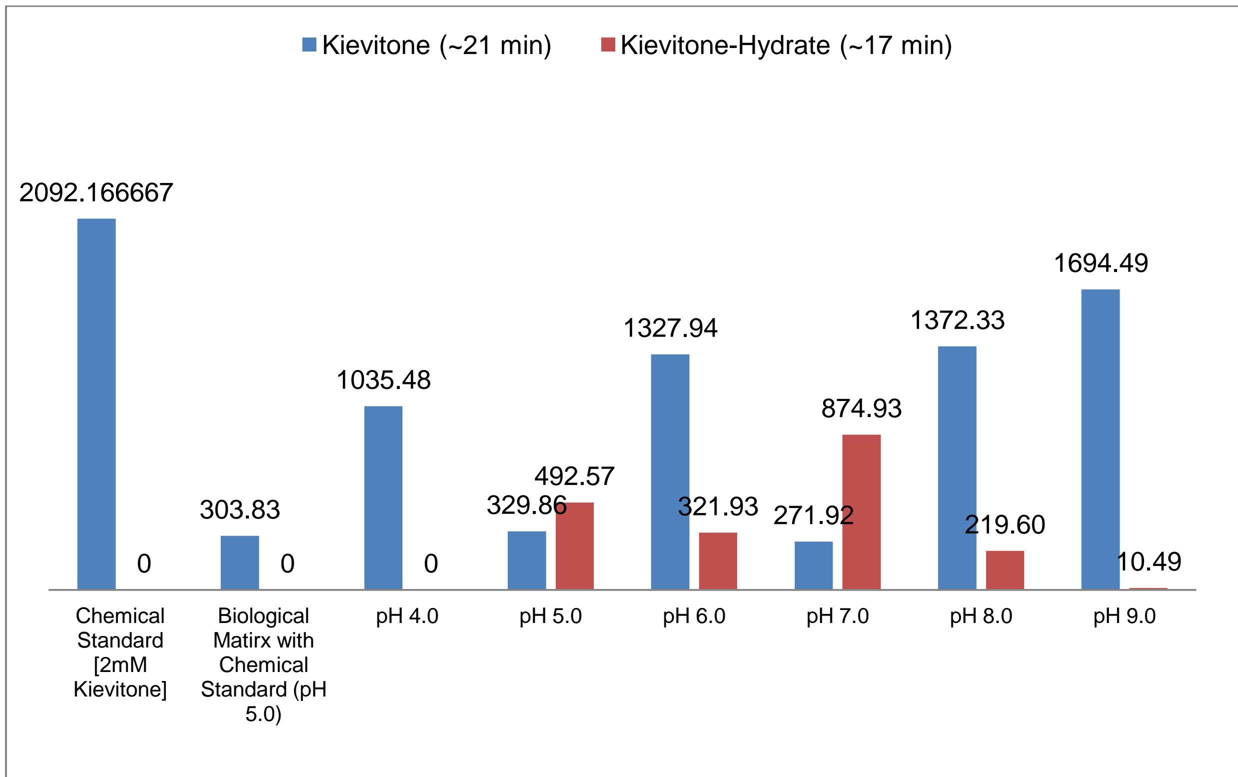

Figure 22: Area-values [mAU*s] obtained from HPLC-UV measurement at 220 nm of chemical standard and biological matrix with chemical standard as well as *Fso*-KHS assays performed at different pH values

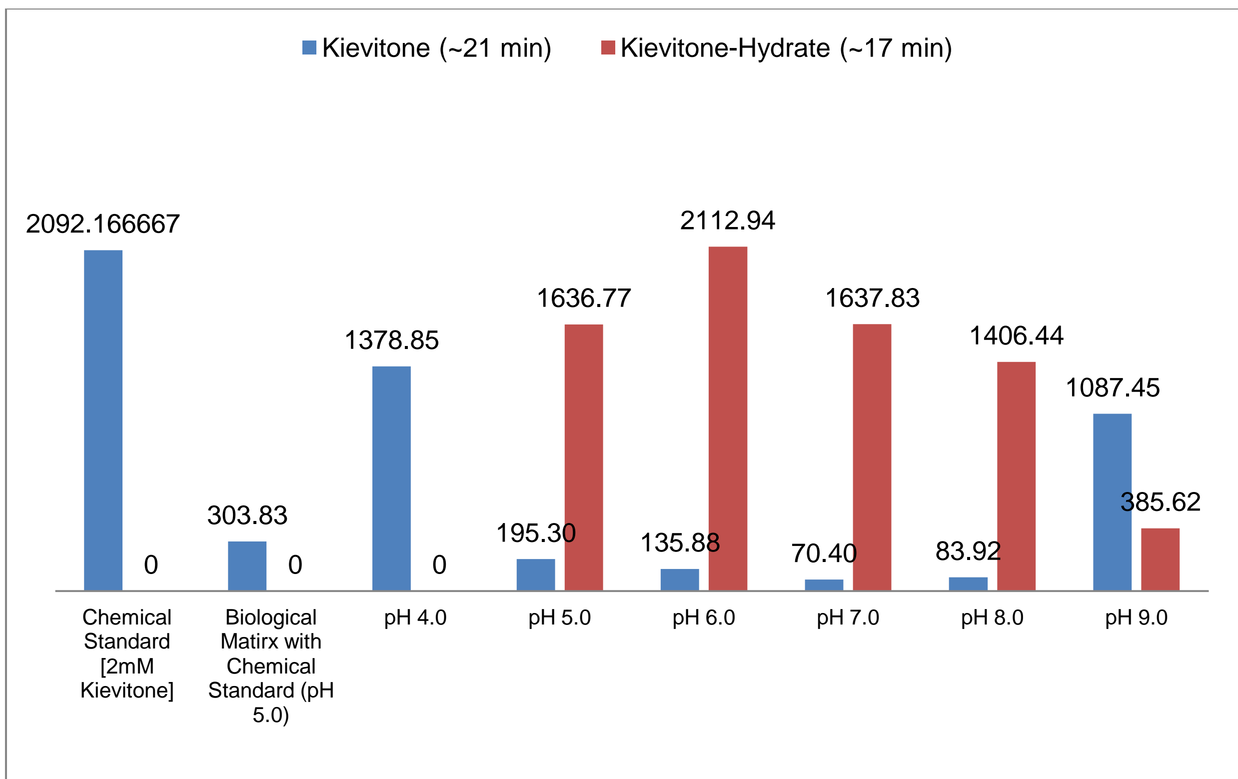

Figure 23: Area-values [mAU*s] obtained from HPLC-UV measurement at 220 nm of chemical standard and biological matrix with chemical standard as well as *Nha*-KHS assays performed at different pH values.

In vitro activity assay with alternative substrate and HPLC-MS analysis

KHS activity assays with cholesta-5,7,24-trien-3beta-ol as a potential alternative substrate were performed in triplicates as described in the Materials and methods section, followed by HPLC-UV/MS measurement.

The MS was adjusted to measure four separate channels. The SCAN-mode detected all masses between 200 and 800. Each of the three SIM-modes was modified to detect the mass of either positive ionized substrate (365) or the masses of positive or negative ionized product (383 or 381). Every sample was analysed using the appropriate mode.

Chromatograms of substrate and product standard are shown in Figure 24 A and B, respectively. Retention times of substrate and product were determined to be 9 min and 5 min, respectively. Biological standards without and with substrate are shown as well (Figure 24 C and D). Figure 24 E represents the obtained HPLC-UV/MS chromatograms after cholesta-5,7,24-trien-3-beta-ol conversion using CFEs harboring Nha-KHS-His_{10}.

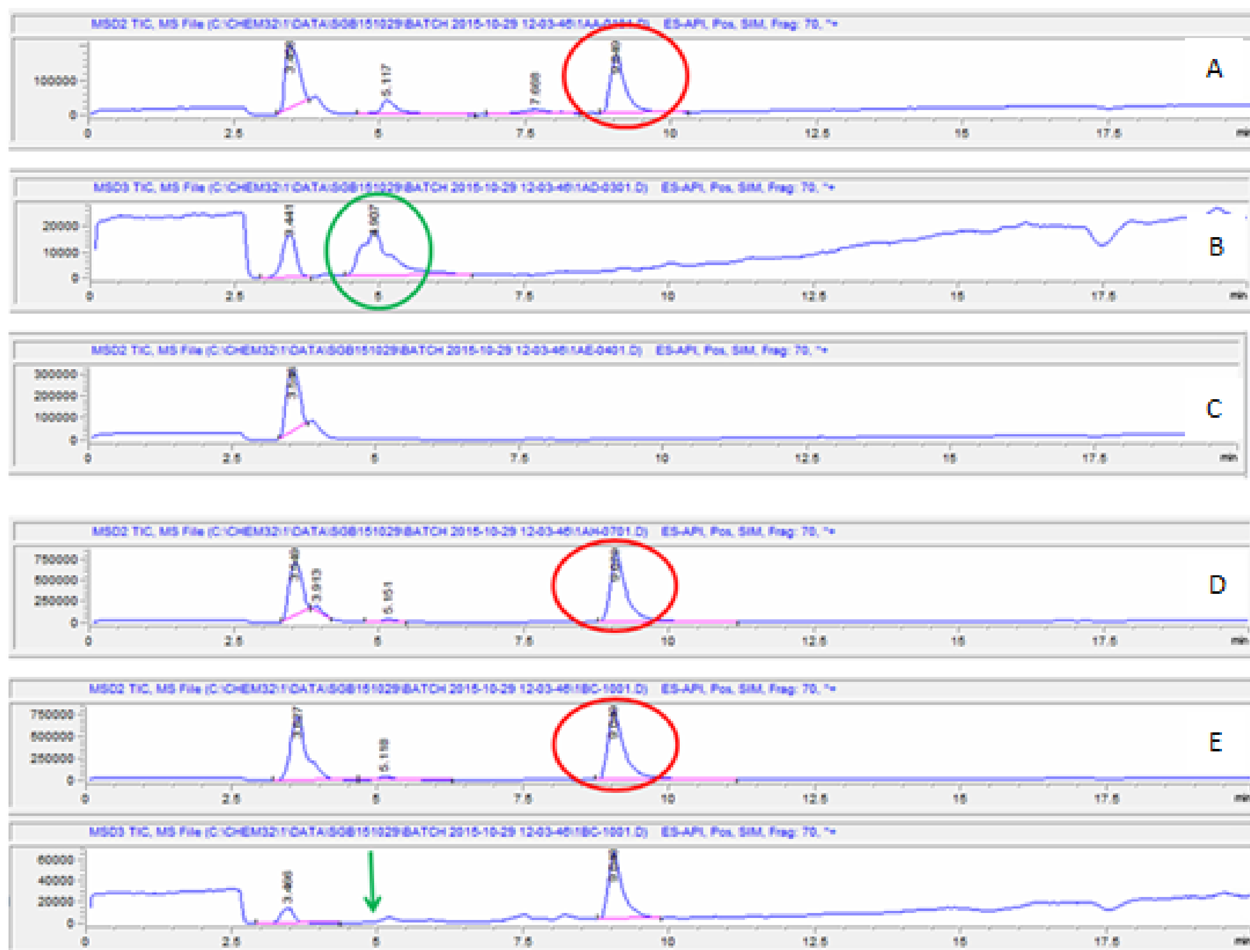

Figure 24: Chromatograms of the particular SIM-mode of HPLC-UV/MS measurement of KHS assays performed with the potential alternative substrate cholesta-5,7,24-trien-3-beta-ol and CFEs harboring *Nha*-KHS-His$_{10}$. Assays were carried out at pH 7. **A**: SIM mode positive ionized substrate (365) of substrate standard. **B**: SIM mode positive ionized product (383) of product standard. **C**: SIM mode positive ionized substrate (365) of biological standard without substrate. **D**: SIM mode positive ionized substrate (365) of biological standard with substrate. **E**: SIM mode positive ionized substrate of conversion with the CFE (upper figure, 365) and positive ionized product (lower figure, 383) conversion with the CFE. Red: cholesta-5,7,24- trien-3-beta-ol. Green: 25-hydroxy-cholesta-5,7-dienol.

The lack of a distinct product peak at the expected retention time of 5 min in the according SIM-mode as well as the presence of a substrate peak suggested that *Nha*-KHS did not convert the cholesta compound.

The area-values obtained from the HPLC-UV/MS measurement are displayed in Figure 25. Substrate as well as product standard showed rather low area values in comparison to the values obtained for the biological standard as well as KHS activity assay.

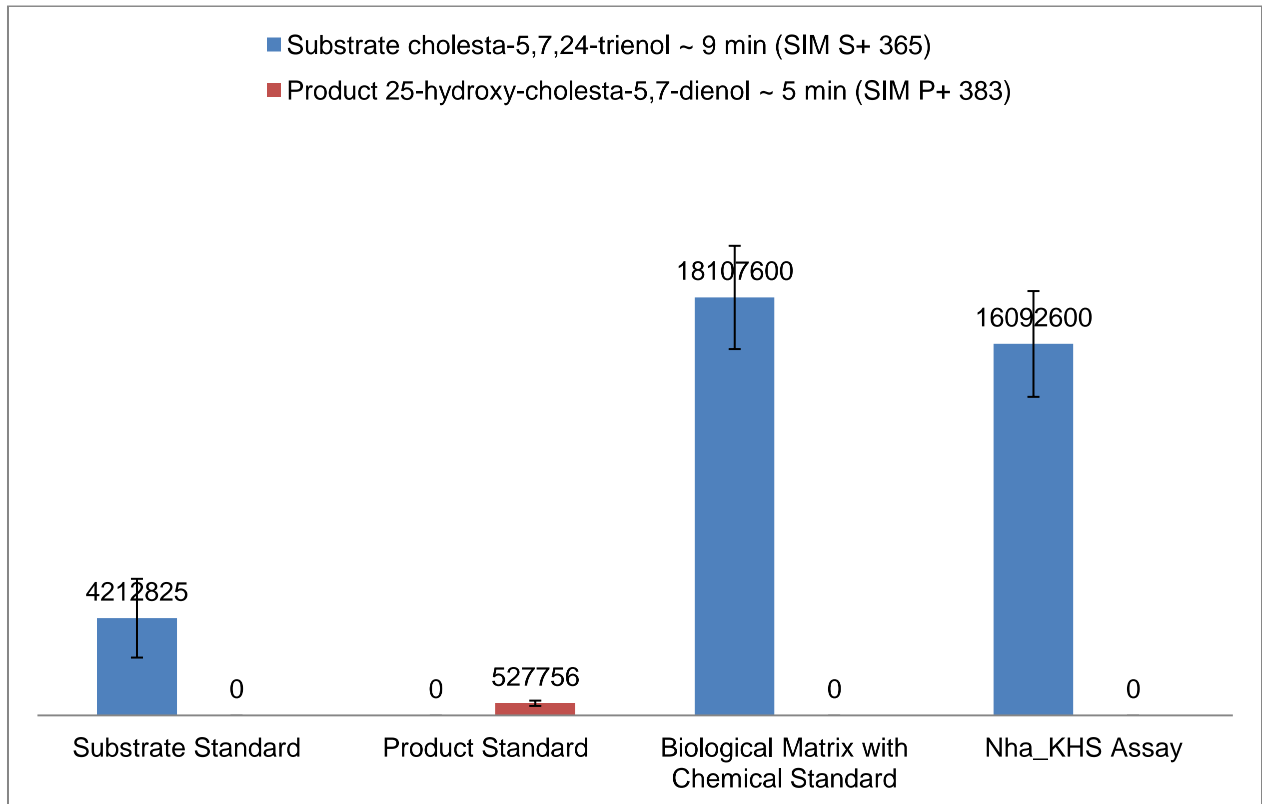

Figure 25: Area-values [mAU*s] of the particular SIM-mode obtained with HPLC-UV/MS measurement of chemical substrate or product standards and biological matrix with chemical standard as well as *Nha*-KHS assays at pH 7.

3.2 Expression of kievitone hydratases in *P. pastoris*

Cloning of *Fso*- and *Nha*-KHS-His_{10} sequence-optimized for expression in *P. pastoris* into the *E. coli* - *P. pastoris* shuttle vector pPpB1_S[19] (Figure 26) was performed by Anita Emmerstorfer-Augustin. Therefore, kievitone hydratase genes were amplified with PCR, using primers containing *Not*I and *EcoR*I restriction sites (Table 15). These two constructs were used in all experiments.

Transformation of the vector as well as cultivation and expression of the C-terminally His_{10}-tagged *Fso*- and *Nha*-KHSs was performed as described in the Materials and methods section.

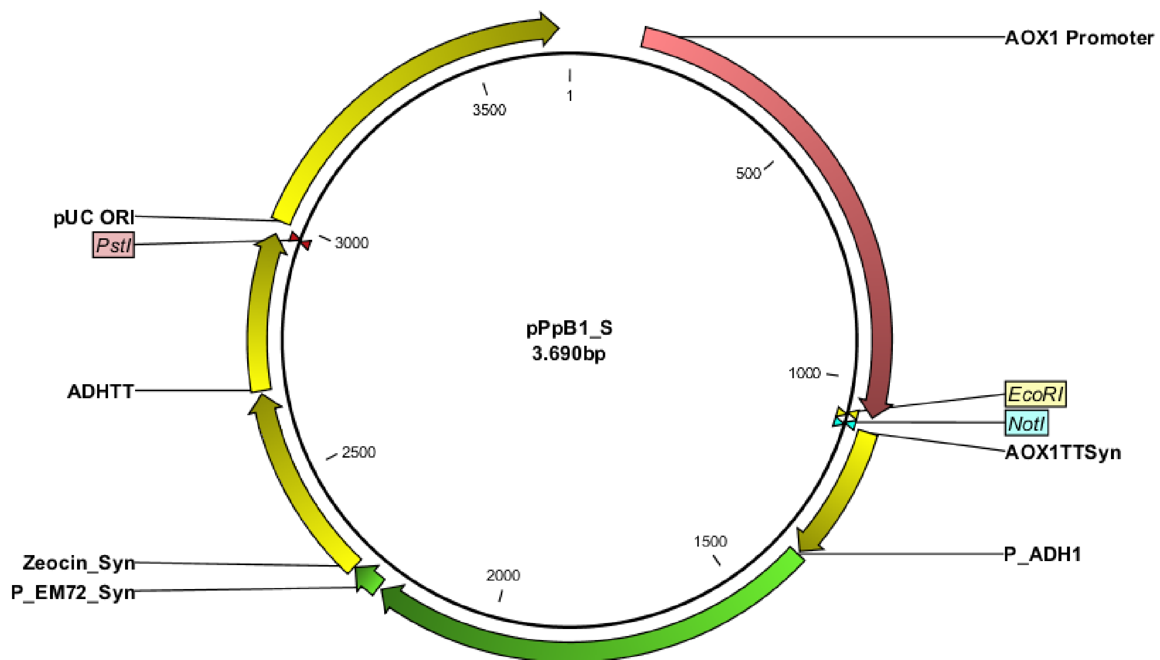

Figure 26: Map of *P. pastoris* expression vector pPpB1_S.

3.2.1 Isolation of *P. pastoris* microsomes

Isolation of total microsomes was performed using *P. pastoris* CBS7435 strain expressing *Fso*-KHS-His_{10} or *Nha*-KHS-His_{10} from a construct for intracellular location. This expression construct was chosen to discover in which compartment of the cell KHSs are situated upon expression with their native signal sequence. Cultivation and protein expression as well as cell fractionation by differential centrifugation were carried out as described in the Materials and methods section.

Five hundred µL of each fraction were harvested for subsequent analysis. Additionally, the protein concentration of each fraction was determined via Bio-Rad assay. The samples were diluted to a protein concentration of 1 mg/mL in 50 µL of SDS–loading dye. Then, 15 µL of denatured samples were loaded onto an SDS gel, followed by Western blot analysis (Figure 27).

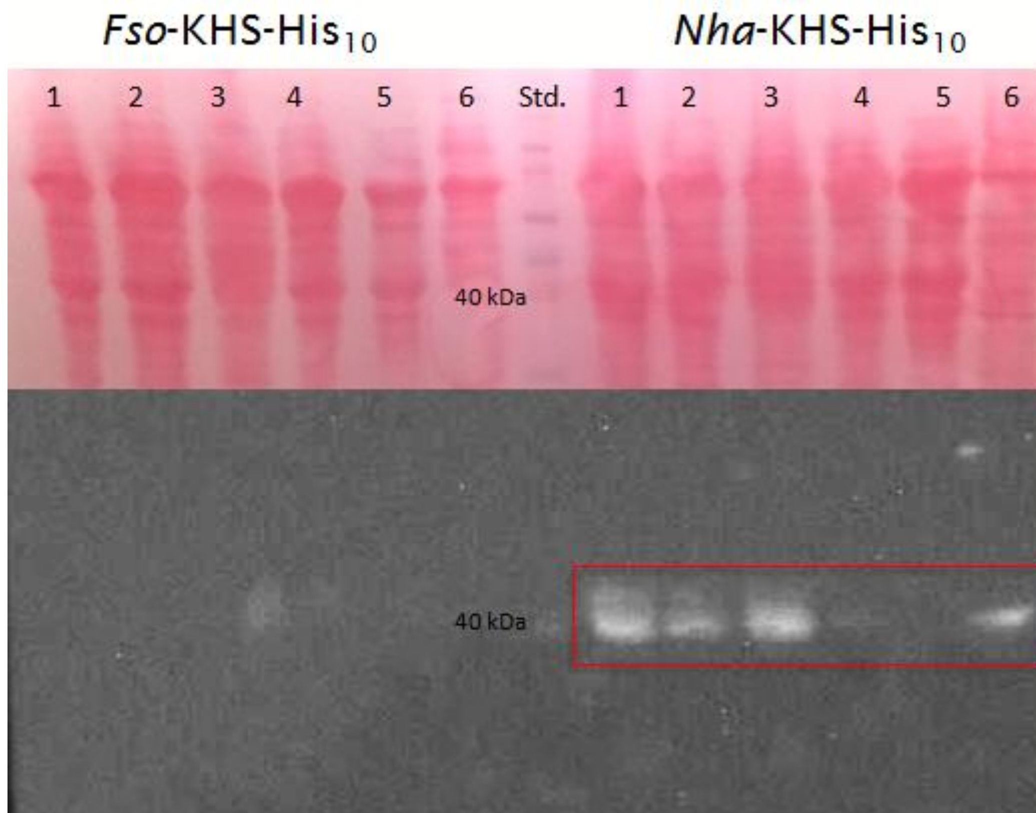

Figure 27: Western blot analysis of fractions from isolation of total microsomes of a *P. pastoris* CBS7435 strain intracellularly expressing *Fso*-(40.3 kDa) or *Nha*-KHS-His$_{10}$ (39.9 kDa). Strains were grown in BMGY medium for 24 h and induced with 0.5 % methanol every 12 h for 48 h. Fractions were obtained by differential centrifugation as described in the Materials and methods section. 1: Cell lysate. 2: Homogenate. 3: Mitochondrial fraction. 4: Post-mitochondrial supernatant. 5: Cytosolic fraction. 6: Total microsomes. Standard: Novex®Sharp Prestained Protein Standard.

Signals were obtained for *Nha*-KHS-His$_{10}$ in several of the membrane fractions, i.e. mitochondrial fraction (lane 3) and total microsomal fraction (lane 6). However, the hydratase could not be detected in the cytosolic fraction (lane 5). These results indicate that KHSs are likely membrane fraction associated proteins. No signals were obtained for the *Fso*-KHS-His$_{10}$ samples.

3.2.2 Native signal sequence

Fso- and *Nha*-KHS without the N-terminal signal sequence (nosig) or with an N-terminal FLAG-tag masking the signal sequence were amplified by PCR using primers listed in Table 15.

Transformation, cultivation and expression of the two KHS constructs was performed as described in Materials and methods part. Five transformants of each hydratase were cultivated. Following cultivation, 6 OD_{600} units of cell culture were harvested and treated according to the Riezman cell disruption protocol[15]. Protein pellets were mixed with 50 µL of SDS–loading dye, denatured for 5 min at 95°C and 15 µL were loaded onto an SDS gel, followed by immunoblot analysis (Figure 28 and Figure 29).

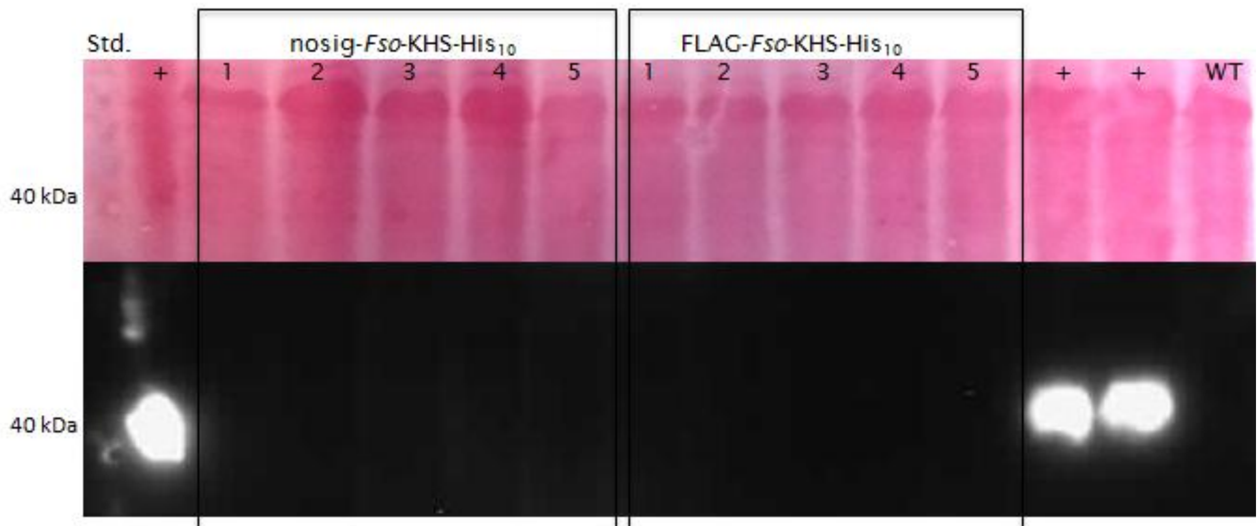

Figure 28: Western blot analysis of *P. pastoris* CBS7435 Δ*his4* strains expressing *Fso*-KHS-His_{10} (40.3 kDa) without N-terminal signal sequence (nosig) or masked signal sequence (FLAG-tag). Strains were grown in BMGY medium for 24 h and induced with 0.5 % methanol every 12 h for 48 h. Approx. 1.5 OD_{600} units were loaded on a SDS–gel after Riezman cell disruption. Standard: Novex®Sharp Prestained Protein Standard. WT: *P. pastoris* CBS7435 Δ*his4*. +: *P. pastoris* CBS7435 expressing wild type *Fso*-KHS-His_{10}.

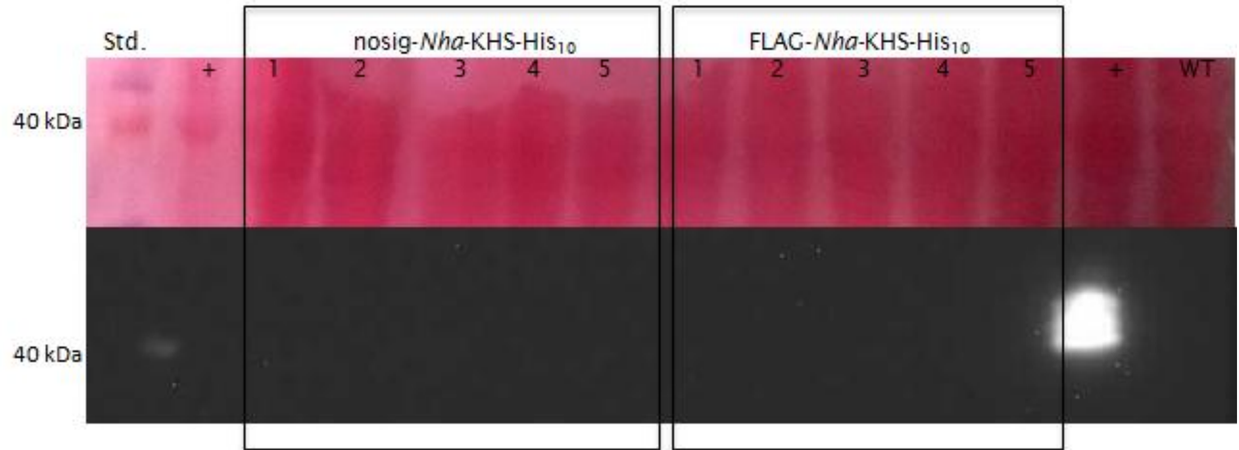

Figure 29: Western blot analysis of *P. pastoris* CBS7435 Δ*his4* strains expressing *Nha*-KHS-His$_{10}$ (39.9 kDa) without N-terminal signal sequence (nosig) or masked signal sequence (FLAG-tag). Strains were grown in BMGY medium for 24 h and induced with 0.5 % methanol every 12 h for 48 h. Approx. 1.5 OD_{600} units were loaded on a SDS–gel after Riezman cell disruption. Standard: Novex®Sharp Prestained Protein Standard. WT: *P. pastoris* CBS7435 Δ*his4*. +: *P. pastoris* CBS7435 expressing wild type *Nha*-KHS-His$_{10}$.

Neither for the nosig nor for the FLAG-tagged *Fso*- and *Nha*-KHS-His$_{10}$, signals were obtained through Western blotting (Figure 28 and Figure 29). Hence, after removing or masking the N-terminal, native signal sequence, neither of the tested KHSs were expressed intracellularly in *P. pastoris*.

3.2.3 Amino acid exchange: Site-directed mutagenesis

Potential N-glycosylation sites of *Fso*- and *Nha*-KHS-His$_{10}$ were removed by exchanging Asp for Ala residues via PCR-based site-directed mutagenesis. The amino acid sequences of both hydratases with emphasized potential N-glycosylation sites are shown in the Appendix section in Table 14.

According to sequence analysis, *Fso*-KHS and *Nha*-KHS contain 6 and 4 potential N-glycosylation sites, respectively. Five *Fso*-KHS-His$_{10}$ variants harboring the amino acid exchanges N108A, N132A, N144A, N194A and N326A, were generated. One of the variants with the replacement N219A could not be constructed. All four *Nha*-KHS-His$_{10}$ N-glycosylation site mutants, with the exchanges N112A, N132A, N144A and N219A were generated.

Transformation, cultivation and expression were performed as described in the Materials and methods section. Three transformants of each N-glycosylation site variant were analyzed by Western blotting after Riezman cell disruption.

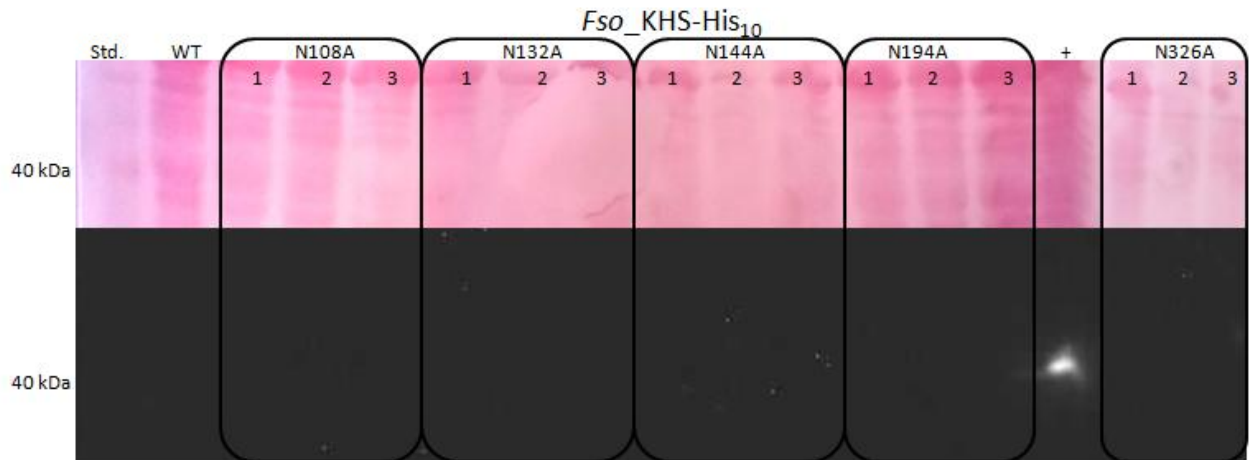

Figure 30: Western blot analysis of *P. pastoris* CBS7435 Δ*his4* strains expressing *Fso*- KHS-His$_{10}$ (40.3 kDa) harboring mutation of potential N-glycosylation sites at the indicated positions. Strains were grown in BMGY medium for 24 h and induced with 0.5 % methanol every 12 h for 48 h. Approx. 1.5 OD$_{600}$ units were harvested and prepared to be loaded on a SDS – gel. Standard: Novex®Sharp Prestained Protein Standard. WT: *P. pastoris* CBS7435 Δ*his4*. +: *P. pastoris* CBS7435 strain expressing wild type *Fso*- KHS-His$_{10}$.

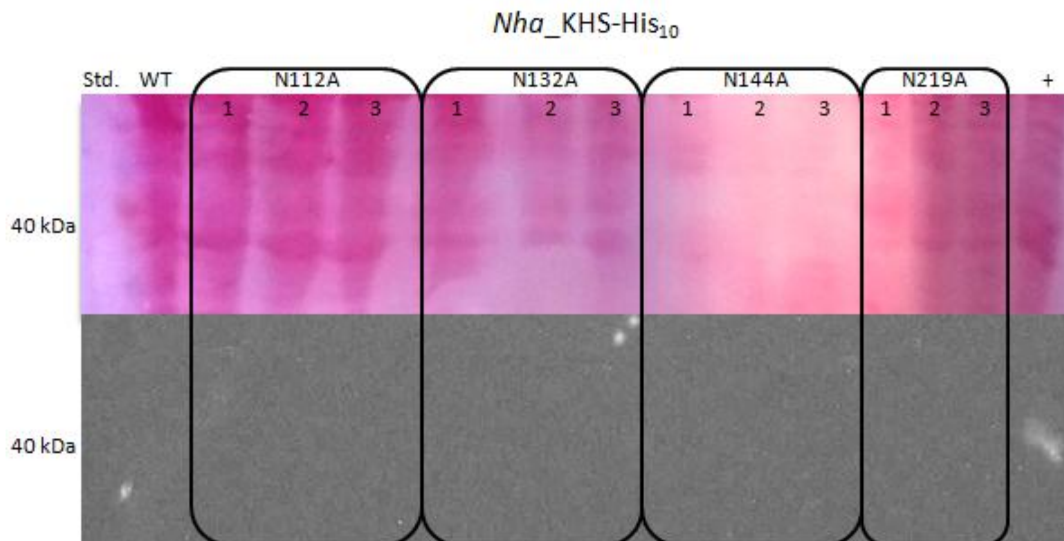

Figure 31: Western blot analysis of *P. pastoris* CBS7435 Δ*his4* strains expressing *Nha*-KHS-His$_{10}$ (39.9 kDa) harboring mutation of potential N-glycosylation sites at the indicated positions. Strains were grown in BMGY medium for 24 h and induced with 0.5 % methanol every 12 h for 48 h. Approx. 1.5 OD$_{600}$ units were harvested and prepared to be loaded on a SDS – gel. Standard: Novex®Sharp Prestained Protein Standard. WT: *P. pastoris* CBS7435 Δ*his4*. +: *P. pastoris* CBS7435 strain expressing wild type *Nha*-KHS-His$_{10}$.

Figure 30 and Figure 31 show, that no signals were obtained via Western blot analysis for any variant with potential N-glycosylation site Asp exchanged for Ala. Hence, exchange of single amino acids at different positions of the recombinant protein abrogated enzyme production in *P. pastoris*.

4 Discussion

4.1 Expression of kievitone hydratases in different *E. coli* strains

Recombinant expression of KHSs in *E. coli* strains mostly led to formation of inclusion bodies, hence insoluble and most likely inactive protein[20]. Western Blot analysis of several strain showed clearly visible signals only in the TCE. However, soluble expression of *Fso*- and *Nha*-KHS sequence optimized for *E. coli* and cloned into pET26b(+) vector was achieved in *E. coli* BL21 Star™ (DE3), as demonstrated by the bands at approximately 40 kDa in CFEs on the respective Western Blot (Figure 12). However, expression of hydratases sequence optimized for the expression in *P. pastoris* is ongoing and might be an appropriate alternative to the expression in *E. coli*. The KHS genes are derived from fungi and, therefore, could benefit from a eukaryotic expression host.

In the purification profile of *Nha*-KHS-His_{10} (Figure 15), the TCE showed a massive band in the mass range of around 40 kDa, which matched the expected size of *Nha*-KHS-His_{10} (39.9 kDa). The lack of any distinct band at the same size in the cell free extract indicated extensive formation of inclusion bodies. The elution fraction displayed two slight bands at approximately 40 kDa, matching the expected size of the hydratase, but also other bands, indicating contaminations. The purification profile of the *Fso*-KHS-His_{10} (Figure 13) showed similar results.

Consequently, expression of *Fso*- and *Nha*-KHS in *E. coli* was no suitable approach in yielding sufficient purified protein for characterization and crystallization.

Development of a KHS activity assay and analysis of enzymatic conversions via HPLC-UV/MS was accomplished. In order to avoid degradation of the substrate or product but enable quantitative precipitation of protein, acetonitrile was chosen as precipitation agent. In previous experiments, different precipitation methods, including the addition of methanol or ethanol as well as treatment at high temperature, had been tested, in order to see if the substrate was stable and to determine which method precipitated the largest amounts of protein. Hence, acetonitrile was the only agent fulfilling the requirements. Since purification of both KHSs was not successful, *in vitro* KHS activity assays with *E. coli* BL21 Star™ (DE3) cell free extracts harboring either of the enzymes were performed. Chromatograms of HPLC-UV measurements

at 220 nm and 291 nm of the KHS assays with CFEs harboring *Fso*- or *Nha*-KHS-His_{10} showed that substrate conversion was achieved for both enzymes. In order to determine the pH optimum of the enzymes, *Fso*- and *Nha*-KHS-His_{10} were incubated in buffers with pH values ranging from 4 to 9 during assays. The best conversions from kievitone to kievitone-hydrate were obtained at pH 7 for *Fso*-KHS and at pH 6 for *Nha*-KHS.

Since kievitone is not commercially available and had to be chemically synthesized by DSM in a costly, multi-step process specifically for the project, the access to the natural substrate is highly limited. Therefore, the access to an alternative substrate is essential for thorough protein characterization experiments. In this work, cholesta-5,7,24-trienol was tested, since it also contains a prenyl side chain with the to be hydrated double bond. Also, the sterol group is somewhat similar in size to the flavonoid structure of kievitone, although much more hydrophobic. The chromatograms obtained from the HPLC-MS measurements led to a substrate signal in all samples, but no product signal in the assays performed with enzyme. Hence, *Nha*-KHS-His_{10} did not show activity on the cholesta compound, most likely because the structure lacks several OH-groups and is bulkier in comparison to kievitone. In consequence, hydrophobicity of the substrate or interactions with the substrates' OH-groups, respectively as well as the size of the substrate could be important for substrate binding to the active site of the KHS.

4.2 Expression of kievitone hydratases in *P. pastoris*

As the overexpression of KHSs in *E. coli* did not yield satisfying amounts of soluble enzyme, expression of mutated KHSs in *P. pastoris* was performed.

First, to identify the localization of kievitone hydratases, isolation of total microsomes from *P. pastoris* cells expressing *Fso*- or *Nha*-KHS-His_{10} was performed.[21] Literature research suggested the enzymes are most likely not membrane associated, since no conserved sequence indicating a transmembrane domain or any other membrane-association was found. However, isolation of the microsomal fraction followed by Western blotting indicated that kievitone hydratases are membrane fraction associated and not cytosolic enzymes. Additionally, the signals displayed a haze towards higher molecular weights, indicating the possibility of N-glycosylation (Figure

27). Since only the cell lysates, but not the culture supernatants, were analyzed for expression of KHSs the possibility that part of the enzyme was secreted from *P. pastoris* remains due to the presence of the native signal peptide.

The amino acid sequence of the *Fso*- and *Nha*-KHSs revealed a predicted native signal sequence, which might direct the enzymes into the secretory pathway of *P. pastoris*. Through this signal peptide, it is possible that the KHSs are translocated into the endoplasmic reticulum lumen and are secreted via vesicles from the endoplasmic reticulum to the Golgi and plasma membrane, where they should be released into the extracellular space. However, the enzymes appear to get stuck somewhere in the vesicular transport route and are therefore localized in the lumen of one of the secretory organelles.

Targeting of the KHSs into the secretory pathway is also a likely reason for the observed N-glycosylation of the proteins. The potential N-glycosylation sites of *Nha*-KHS and *Fso*-KHS were mutated through site-directed mutagenesis to express the enzymes without *N*-glycans in *P. pastoris* for characterization and, especially, protein crystallization. The muteins obtained include *Nha*-KHS-His$_{10}$ N112A, N132A, N144A and N219A as well as *Fso*-KHS-His$_{10}$ N108A, N132A, N144A, N194A and N326A. After expression of the variants in *P. pastoris*, cell extracts were analyzed by Western Blot in order to find variants that do not exhibit any glycosylation-induced size-shift and test them for activity. If active, these non-glycosylated KHSs would have been purified and applied for crystallization studies. However, no functional expression was achieved, most likely due to misfolding of the protein caused by the mutations. Additionally, the lack or masking of the N-terminal native signal sequence of KHSs with a FLAG-tag prevented the enzymes from being actively expressed in *P. pastoris*. Expression only occured when the signal sequence was present, only demonstrated for the positive control. Hence, KHSs likely require a signal sequence for proper expression in *P. pastoris*. Deficiency or masking of the signal peptide possibly caused the enzymes to get stuck in a secretory organelle or improper protein folding and rapid degradation of the enzymes.

5 Conclusions and outlook

A reliable *in vitro* activity assay for determination of KHS activity was developed. *Fso*- and *Nha*-KHSs were successfully expressed in *E. coli*. In subsequently performed assays, active expression of both proteins was confirmed and a pH profile was obtained. However, purification from *E. coli* CFE did not yield in satisfying amounts of purified and soluble protein, preventing any further investigations. Since the overexpression of KHSs in *E. coli* was not achieved, expression of KHS variants in *P. pastoris* was performed. Site-directed mutagenesis targeting the potential N-glycosylation sites as well as mutagenesis of the N-terminal, native signal sequence of *Fso*- and *Nha*-KHSs was followed by expression in *P. pastoris*. However, functional enzyme expression was not achieved.

Based on the current project status, the focus is set on the establishment of a high-throughput kievitone hydratase activity assay that enables to screen for improved enzyme variants using alternative substrates, e.g. prenylated phenols or xanthohumol[22] (Figure 32).

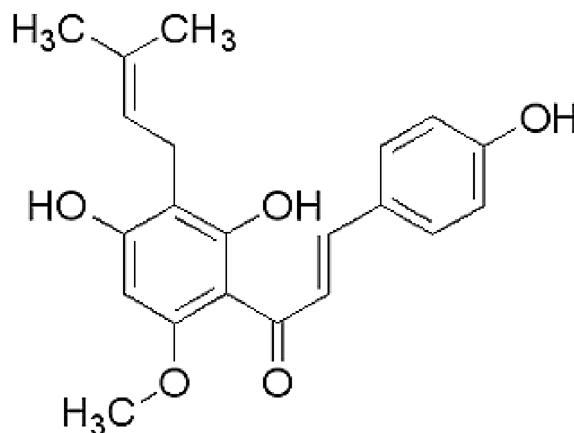

Figure 32: Potential alternative substrate xanthohumol tested for kievitone hydratase activity.

Xanthohumol has already been proven to be an alternative substrate accepted by *Nha*-KHS using *E. coli* CFE as well as the enzyme purified from *P. pastoris*. Furthermore, biochemical characterization, including enzyme kinetics and thorough

assessment of optimal conditions for stability and activity using xanthohumol as model substrate is ongoing. Moreover, crystallization of *Nha*-KHS-His_{10} is in progress. In case of successful 3D structure elucidation, rational engineering and/or directed evolution approaches will be used to improve the desired enzyme properties.

6 References

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7 List of abbreviations

Table 4: List of abbreviations.

Abbreviation	**Description**
Ate	*Aspergillus terreus*
BMGY/BMMY	Buffered Glycerol-/Methanol- complex Medium
bp	Base pair
CFE(s)	Cell free extract(s)
CV	Column volumes
CWW	Cell wet weight
dH_2O	Water, deionized
ddH_2O	Water, double distilled
DMSO	Dimethyl sulfoxide
EC	Enzyme class
EtBr	Ethidium bromide
EV	Empty vector control
Fso	*Fusarium solani*
His_{10}-tag	10 Histidine residues added to the protein for purification
HPLC-UV/MS	High performance liquid chromatography – ultra violet/mass spectrometry
IPTG	Isopropyl-β-D-thiogalactopyranosid
KHS(s)	Kievitone hydratase(s)
Nha	*Naectria haematococca*
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SIM	Selected ion monitoring
WT	Wild type
TCE(s)	Total cell extract(s) (lysate) with soluble and insoluble fraction
YPD	Yeast Peptone Dextrose Medium

8 Appendix

8.1 Instruments and devices

Table 5: Instruments and devices.

Task	Instrument/Device	Manufacturer
Absorbance measurement	FLUOstar Omega BioPhotometer Plus Specord 210/205 dual-beam spectrophotometer DU® 800 UV-Visible Spectrophotometer Microplate, 96 well, PS, U-bottom, MICROLON® Semi-Micro-Cuvettes, PS, 10 x 10 x 45 mm	BMG Labtech, Germany Eppendorf, Germany Analytik Jena, Germany Bio-Rad, USA Greiner bio-one AG, Germany Greiner bio-one AG, Germany
Agarose gel electrophoresis	PowerPac™ Basic + Sub-Cell GT	Bio-Rad, USA
Cell harvest/Centrifugation	Microcentrifuge 5415R Tabletop centrifuge 5810R Avanti™ centrifuge, JA-10 and JA-25.50 rotors Optima™ LE-80K Ultracentrifuge	Eppendorf, Germany Eppendorf, Germany Beckman Coulter™, USA Beckman Coulter, USA
Desalting/buffer exchange	PD-10 Desalting Columns	GE Healthcare Life Sciences, USA
Determination of DNA/protein concentration	NanoDrop 2000 UV-Vis Spectrophotometer	Thermo Scientific, USA
Electrotransformation	MicroPulser™ Electroporation Cuvettes (2 mm gap)	Bio-Rad, USA Life Technologies, USA
Extraction after activity assay	Vibrax VXR basic	IKA, Germany
HPLC-UV	Agilent 1100 series, G1379A Degasser Agilent 1200 series, G1311A QuatPump Agilent 1100 series, G1367A WPALS Agilent 1100 series, G1316A,	Agilent Technologies, USA and Canada

	COLCOM Agilent 1100 series, G1315B, DAD Agilent 1260 Infinity, G1321B, 1260 FLD Purospher® STAR RP-18 endcapped (5µm) LiChroCART® 250-4.6	 Merck Millipore, Ireland
HPLC-MS	Agilent 1200 series, G1312B, BinPumpSL Agilent 1100 series, G1367C, HiP-ALS SL Agilent 1200 series, G1314C, VWD SL Agielnt 1200 series, G1316B, TCC SC Agilent Technologies, LC/MSD SL YMC PAH, Classical Analytical HPLC column (4.6 mm i.d.), S-3 µm, 150x4.6 mm	Agilent Technologies, USA and Canada YMC GmbH Europe, UK
HPLC vials	1.5 ml Rollrandflasche, 32 x 11.6 mm, Klarglas, 1. hydrolytische Klasse, enge Öffnung 11 mm Verschluss: Aluminium Bördelkappe, farblos lackiert, mit Loch; PTFE rot/Silicon weiß/PTFE rot, 45° shore A, 1,0 mm	Markus Bruckner Analysentechnik, AUT
Immunoblotting (Western blotting)	PowerEase500 XCell SureLock™ Electrophoresis *SureLock*® cell and blot module Nitrocellulose Blotting Membrane, 0.45 µm Bioimager G:Box HR16	Life Technologies, USA Thermo Scientific, USA GE Healthcare Life Sciences, USA SynGene, UK
Incubator (28°C and 37°C)	BINDER Kühlbrutschränke	Binder GmbH, Germany
Magnetic stirrer	IKAMAG RCT	IKA, Germany

Mixing (small volumes)	Vortex – Genie 2	Scientific Industries Inc., USA
PCR reaction	GeneAmp® PCR System 2700	Applied Biosystems, USA
pH-value measurement	inoLab WTW 720 pH meter	WTW GmbH, Germany
Plate shaker	Heidolph Titramax 1000	Fisher Scientific, USA
Protein concentration	Ultrafiltration with Amicon® Ultra-15 Centrifugal filter devices, 15 mL, 30 kDa molecular weight cutoff	Merck Millipore, Ireland
Protein electrophoresis	NuPAGE® SDS-PAGE Gel System Custom made 4-12.5% SDS-Gels 1.0 mm, 15 wells	Life Technologies, USA
Protein purification	HisTrap FF Crude column, 5 mL HiTrap TALON® Crude column, 5 mL	GE Healthcare Life Sciences, USA GE Healthcare Life Sciences, USA
Reaction tubes for activity assay	PYREX® culture tubes	SciLabware, UK
Shaker (small volumes)	Thermomixer comfort	Eppendorf, Germany
Shaker for cell cultivation/activity assay	Multitron Standard	Infors AG, Swizerland
Sonication	Sonifier 2501	Branson, USA
Sterile work	UNIFLOW KR130 biowizard	UNIQUIP, USA
Weighing	Lab scale: SI-202 Precision scale: Kern Scale ABS 220-4	Denver Instrument, USA Kern &Sohn GmbH, Germany

8.2 Reagents

Table 6: Reagents and supplier.

Reagent	Supplier
Acetic acid	Roth GmbH, Germany
Acetonitrile	VWR, USA
Acrylamide/Bis	Sigma Aldrich, USA
Agar Agar	Roth GmbH, Germany
Agarose LE	Biozyme, Germany
Ampicillin (Amp)	Sigma-Aldrich, Germany
Ammonium persulfate (APS)	Roth GmbH, Germany
Anti-mouse IgG (A4416)	Sigma-Aldrich, Germany
Aqua bidest. (ddH_2O)	Fresenius Kabi GmbH, Austria
Bacto™ Agar	BD, USA
Biorad Protein Assay dye reagent (5x)	Bio-Rad, USA
Bovine Serum Albumine	Roth GmbH, Germany
B-Per™ Bacterial Protein Extraction Reagent	Thermo Scientific, USA
Chloramphenicol (Cm)	Roth GmbH, Germany
Cholesta-5,7,24-trienol	DSM, Geleen, Netherlands
Citric acid	Roth GmbH, Germany
Dimethylsulfoxide	Roth GmbH, Germany
Dithiotreitol (DTT)	Roth GmbH, Germany
Enzymes and adequate buffers, various	Thermo Scientific, USA
Ethanol	Roth GmbH, Germany
Ethidium bromide	Roth GmbH, Germany
Ethyl acetate	Roth GmbH, Germany
D-Glucose	Roth GmbH, Germany
Di-potassium hydrogen phosphate	Roth GmbH, Germany
DNA Loading Dye (6x)	Thermo Scientific, USA
GeneRuler™ DNA Ladder Mix	Thermo Scientific, USA
Glycerol	Roth GmbH, Germany
HEPES	Roth GmbH, Germany
Hydrochloric acid	Roth GmbH, Germany
Imidazole	Sigma-Aldrich, Germany
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Peqlab Biotechnologie GmbH, Germany
Kanamycin (Kan)	Sigma-Aldrich, Germany
Kievitone	DSM, Geleen, Netherlands
LB (Lennox)	Roth GmbH, Germany
MassRuler DNA Ladder Mix, Ready-to-Use	Thermo Scientific, USA
Methanol	Roth GmbH, Germany
Monoclonal Anti-polyHistidine antibody produced in mouse (H1029)	Sigma-Aldrich, Germany
N,O-Bis(trimethylsilyl)trifluoroacetamide	Sigma-Aldrich, Germany
Novex®Sharp Pre-Stained Protein Standard	Thermo Scientific, USA
NuPage® LDS Sample Buffer (4x)	Life Technologies, USA
NuPage® Sample Reducing Agent (10x)	Life Technologies, USA
Peptone	Roth GmbH, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Germany
PonceauS	Sigma-Aldrich, Germany

Potassium chloride	Roth GmbH, Germany
Potassium dihydrogen phosphate	Roth GmbH, Germany
SDS	Life Technologies, USA
Sodium chloride	Roth GmbH, Germany
Sodium citrate	Roth GmbH, Germany
Sodium hydroxide	Roth GmbH, Germany
SuperSignal® WestPico Chemiluminescent Substrate	Thermo Scientific, USA
TEMED	Roth GmbH, Germany
Tetracycline (Tet)	Sigma-Aldrich, Germany
Tris	Roth GmbH, Germany
Tween® 20	Roth GmbH, Germany
Yeast Extract	Bacto Laboratories Pty Ltd, Australia
Zeocin	InvivoGen, USA

8.3 Kits and enzymes

Table 7: Applied kits and enzymes.

Enzyme/Kit	**Supplier**
FastDigest™ restriction enzymes	Thermo Scientific, USA
Gene Jet™ Plasmid Miniprep Kit	Thermo Scientific, USA
Phusion High-Fidelity DNA Polymerase	Thermo Scientific, USA
Restriction enzymes	Thermo Scientific, USA
T4 DNA Ligase	Thermo Scientific, USA
Wizard® SV Gel and PCR Clean Up System	Promega Corporation, USA

8.4 Media and buffers

Table 8: Media and buffers.

Medium/Buffer	Composition
Ampicillin stock, 100 mg/mL	100 mg/mL dissolved in ddH_2O
BEDS solution	1 mL 0.1 M bicine NaOH (10x), pH 8.3, 300 µL ethylene glycol, 500 µL DMSO, 2 mL 5 M sorbit (5x), 6.2 mL dH_2O
Binding Buffer for protein purification	50 mM phosphate buffer, pH 8 with 10 mM imidazole
BMGY/BMMY-medium	1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6), 1.34 % YNB, $4x10^{-5}$ % biotin, 1 % glycerol or 0.5 % methanol
50 mM Citrate buffer, pH 4, 5 and 6	Mixture of 50 mM citric acid and 50 mM sodium citrate
Chloramphenicol stock, 20 mg/mL	20 mg/mL dissolved in ddH_2O
Coomassie Brilliant Blue G-250 staining solution	1 g of Coomassie Brilliant Blue in 1 liter of the following solution: Methanol (50% [v/v]), acetic acid (10% [v/v]), dH_2O (40%)
50 mM Di-potassium-hydrogen phosphate	8.71 g dissolved in 1 L ddH_2O
Desalting Buffer for protein purification	50 mM potassium phosphate buffer, pH 7
1 M DTT	1.54 g DTT / 10 mL ddH_2O
Elution Buffer for protein purification	50 mM phosphate buffer, pH 8 with 250 mM imidazole
IPTG stock (100 mM)	1.19 g IPTG dissolved in 50 mL ddH_2O
Kanamycin stock, 15 or 40 mg/mL	15 or 40 mg/mL dissolved in ddH_2O
LB-agar	LB-Medium + 20 g/L Agar Agar
LB-medium	10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl
50 mM Potassium dihydrogen phosphate	6.8 g dissolved in 1 L ddH_2O
50 mM Potassium phosphate buffer, pH 7 and 8	Mixture of 50 mM Potassium dihydrogen phosphate and 50 mM Di-potassium hydrogen phosphate
Primary antibody solution (H1029)	1:2000 dilution of H1029 in 1% BSA 50 mL TBST, 0.5 g BSA and 25 µL antibody
SDS-Running Buffer (10x)	28 g Tris, 144 g glycine, 10 g SDS. Fill to 1 L with dH_2O.
Secondary antibody solution (A4416)	50 mL TBST-milk and 10 µL antibody
SOC-Medium	20 g/L tryptone, 0.58 g/L NaCl, 5 g/L yeast extract, 2 g/L $MgCl_2$, 0.16 g/L KCl, 2.46 g/L $MgSO_4$, 3.46 g/L dextrose

TAE buffer, 50x	242 g L^{-1} Tris, 14.6 g L^{-1} EDTA, 57.1 mL L^{-1} acetic acid, adjusted to final volume with ddH_2O
TBS (10x) TBST (1 L) TBST-milk	30.3 g Tris, 87.6 g NaCl, pH 7.5 (HCl), fill to 1 L with dH_2O 100 mL 10x TBS, 300 µL Tween®20, fill to 1 L with dH_2O 50 mL TBST and 2.5 g skim milk powder
TE buffer	10 mM Tris, 1 mM EDTA, pH 7.5 adjusted with 1 M HCl
Transfer buffer (20x) Transfer buffer (1 L)	29 g 12 mM Tris, 144 g 96 mM Glycine, fill to 1 L with dH_2O 50 mL 20x Transfer buffer, 100 mL methanol, 850 mL dH_2O
50 mM to 1 M Tris-HCl, pH 7 to 9	Tris diluted in 1 L ddH_2O, adjusted with 1 M HCl to desired pH value
Washing Buffer for protein purification	50 mM phosphate buffer, pH 8 with 50 mM imidazole
YPD-medium	1 % yeast extract, 2 % peptone, 2 % glucose
Zeocin stock, 100 mg/mL	100 mg/mL dissolved in ddH_2O

8.5 Strains, plasmids and genes

Table 9: *E. coli* and *P. pastoris* strains.

Strain	Genotype	Source
E. coli Top10F'	F'{*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80 *lac*ZΔM 15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U galK *rps*L *end*A1 *nup*G	Thermo Scientific, USA
E. coli BL21 Star™ (DE3)	F^- *omp*T *hsd*$S_B(r_B^- m_B^-)$ *gal dcm rne*131 (DE3)	Thermo Scientific, USA
E. coli BL21 (DE3) CodonPlus-RIL	*E. coli* B F^- *ompT hsdS*$(r_B^-\ m_B^-)$ dcm^+ Tet^r *gal λ*(DE3) *endA Hte* [*argU ileY leuW* Cam^r]	Institute of Biochemistry, Graz University of Technology, Peter Augustin, Dipl.-Ing. BSc
E. coli BL21 (DE3) TaKaRa5	*E. coli* BL21: F^- , *ompT*, *hsdS*$_B(r_B^-\ m_B^-)$, *gal, dcm* Plasmid: pKJE7 Chaperone: dnaK-dnaJ-grpE Promoter: *araB* Resistant marker: Cm Inducer (final conc.): L-Arabinose (0.5 mg/ml)	
E. coli BL21 (DE3) TaKaRa1	*E. coli* BL21: F^- , *ompT*, *hsdS*$_B(r_B^-\ m_B^-)$, *gal, dcm* Plasmid: pG-KJE8 Chaperone: dnaK-dnaJ-grpE groES-groEL Promoter: *araB* *Pzt-1* Resistant marker: Cm Inducer (final conc.): L-Arabinose (0.5 mg/ml), Tetracycline (1 - 5 ng/ml)	
E. coli Rosetta (DE3) pLysS	F^- *ompT hsdS*$_B(r_B^-\ m_B^-)$ *gal* dcm *λ*(DE3 [lacI lacUV5-T7 gene 1 *ind1 sam7 nin5*]) pLysSRARE (Cam^R)	
E. coli K12 ER2508	F^- *ara-14 leuB6 fhuA2 Δ(argF-lac)U169 lacY1 lon::miniTn*10(Tet^R) *glnV44 galK2 rpsL20*(Str^R) *xyl-5 mtl-5 Δ(malB) zjc::Tn*5(Kan^R) *Δ(mcrC-mrr)*$_{HB101}$	
E. coli TUNER	F^- *ompT hsdS*$_B(r_B^-\ m_B^-)$ *gal* dcm *lacY1*	

E. coli Arctic Express	*E. coli* B F^- *ompT* *hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal endA* Hte [*cpn10 cpn60* $Gent^r$]	
P. pastoris CBS7435 Δ*his4*	Accession: PRJEA62483	ACIB c/o Institute of Molecular Biotechnology, Graz University of Technology, Anton Glieder, Ao.Univ.-Prof. Mag.rer.nat. Dr.rer.nat.

Table 10: Expression vectors.

Plasmid	Feature	Source
pET26b(+)	N-terminal His_6-tagging bacterial expression vector with T7 promoter, lac operon, signal sequence and kanamycin resistance marker for periplasmic protein expression via pelB leader sequence.	EMD Millipore, USA
pPpB1_S	*Escherichia coli-Pichia pastoris* shuttle vector harbouring replication origin (pUC ORI), promoters (P_ADH1 and P_EM72_Syn) and selection marker (Zeocin_Syn).	Institute of Molecular Biotechnology, Technical University of Graz.

Table 11: Kievitone hydratase genes, sequence-optimized for expression in *P. pastoris* or *E. coli*.

Gene	Organism	Gene length [bp]	Protein size [kDa]
Ate-KHS-His_{10}	*Aspergillus terreus*	1152	41.2
Fso-KHS-His_{10}	*Fusarium solani*	1104	40.3
Nha-KHS-His_{10}	*Naectria haematococca*	1098	39.9

Table 12: Nucleotide sequences of *Fso*- and *Nha*- KHSs. Sequences optimized for expression in *E. coli* (5' → 3').

Fso-KHS (1063 bp)
1 CATATGATGA TTAGCTCAGT ATTGGTGGCA GGGGTAGTGG CAGTTAGCGC AGCATTGGCA 61 AGCAAGCACC CGAAACAGTA TAGCTTTAAG CCGGAAGATG CCGAGACTAT CTGGAATGGT 121 GACATTCCGG TTCTGTATGA TTTCGGTGAC AGCCAATCTG CGAGCTATTC GGGTTCCTGG 181 TGGACCTCTA GCTACATCAC GGGTACGAAC GGCGAGCAGT ATCTGGTGAT CTCGCATTAC 241 CTGGATACCC CTGTGTTCAC GTATTTCCGT GCCAGCACGC TGAACCTGGA AACCTTGGAC 301 TACAATCAAT TCATTACCCT GGGCAACAAT ACCGCGAACA GCACCACGCT GGATGTAAAA 361 GTGGGCGATA ATGGTATTCA GAGCCTGACC GCAGACAACA TTAGCCAGCA ACGCGCGTAC 421 GCAAACGACG AGAATGTCAC TTTCGACATC ACGTTTGATG CTACCAGCCG TGTGATTAGC 481 AATGCCGGTG CGGGTGTTTT CCAGTTCGGT CCGAGCATTA CCTACGAATG GGGTCTGCCG 541 AACTGCCGTA CCCAGGGTTC TGTCACGGAC ACGGGTGGTA AAAACATTAC GGTTGACCCA 601 GCGAAGAGCT TTACCTGGTA TGATCGTCAG TGGGGCACCG CGGCTGTGAC CTCTGGCAAT 661 TGGACCTGGT TTCAAATGCA TATCCCGGAG ACTTCCTACA AACTGAGCGT CTGGATTATT 721 GATAACGACG TGACCAATCA ATTTAGCCGT TTTGCCACGA TCCGCGGTGA TAATGATGAG 781 TTCCAAGTCC TGCCGTTGGA ATGGAAACCG ATCTATGATC GCACCTACCA ATCCACCGCA 841 GCGGACATTC TGTACCCGCT GGACTGGGAG CTGGACATCA GCGGTTTTGG CGTGTTCCAG 901 TTGAGCAGCA TCCTGGATGA CCAAGAAATC GTCGGCACGA CGGCAATCCA GACCGCGTAC 961 GAGGGCTTCG TTACCTTTAA CGGCACCGTC CACAACAAGA AGGTGCAGGG TTACGGCCTG 1021 GTTGAAGTTG TTTATTCCAA TTGGGAGAGC CTGTAATAAG CTT
Nha-KHS (1057 bp)
1 CATATGCGTG CATCTTTTCT TTTGACGGCA GGTTTAGCAA CGGCGGCAGT AGGTAGAGCG 61 AAGTCCGTCC CAAAGAAGTT TCCGTTTAAG CCGGAGAACA GCAAAACGAC TGGCACCAAT 121 GCGATCCCTA TCGTCTATGG TCTGAGCGAA AGCCAACCGA ATAGCGTTGG TGGTTCTTGG 181 TGGTCTAGCA GCTACATTAC CACTACCAAC AACGAGCAAT ACGTCGTGCT GGCGCACTAT 241 CTGGACAACC CGGTTTATAC GTATTTTCGC GCGAGCACCC TGAACCTGGA GACTAACGAA 301 TACCACCAGT ACGTGACCGT GGGTTCCAGC ACGCCGAACA TCACCACCCT GGATGTCAGC 361 GTCGGCAACA ACGGTATCAA GAGCGAGAGC GAGGATAACC TGTCGAAATT GCGCAGCTAC 421 AGCAATCATG ACAATGTGAC CTTCGATATT ACTTACGACG CTACCACGGG TGCGGTGGCC 481 AATGGCGGTG CAGGCACCTT CCAGTTTGGT GAGGGCCTGA CCTGGGAGTT TGGCCTGCCA 541 AGCGCCAAAA CCGAGGGTAG CTTGACCGTT CACGGTGAAA AACTGGCGAT TGATCCGGCG 601 AAATCGCATA CGTGGTACGA CCGTCAATGG GGTAACACGG CAGCAATTCC GTCTAATTGG 661 ACGTGGTTCC AGCTGCACAT CCCGTCCACC GAATACAAGA TCTCCGCGTG GATTTTCAGC 721 GATCCGTTCC GTAATACCGA AACCCGTTTT GCGACGATTC GCGGTGCGAA TGACGAAACC 781 CTGGTTCTGC CGTTGGAATT TACGCCGATC TATAAACGTA CCTATGAGAG CGCGACGGGT 841 CGTGTAACGT ACCCGCTGGA CTGGAAACTG AAGATTTCTG GCTTCGGCGA TTTCAAACTG 901 AGCAGCTATA CCGAGGACCA GGAATTGGTT GGCGAGGACG CTCTGCAAAC CGCCTACGAG 961 GGTTTCATCA CGTTCAGCGG CAATGTCCAT AGCAAGCCGG TGCAGGGTTA TGGCCTGGTT 1021 GAAATTGTGT ACAGCACGTG GGATGTTTAA TAAGCTT

Table 13: Nucleotide sequences of Fso- and Nha- KHSs. Sequences optimized for expression *P. pastoris* (5' → 3').

Fso-KHS-His$_{10}$ (1104 bp)
1 GAATTCCGAA ACGATGATGA TTTCATCTGT CTTGGTCGCC GGTGTTGTCG CAGTCTCTGC
61 CGCTCTAGCT AGCAAGCATC CCAAACAATA CTCTTTCAAG CCAGAGGATG CTGAGACCAT
121 CTGGAATGGC GACATTCCTG TATTGTACGA TTTCGGTGAC TCTCAATCTG CTTCTTACTC
181 CGGTTCTTGG TGGACTAGTT CATACATTAC TGGTACTAAC GGTGAACAAT ATCTAGTGAT
241 TTCTCATTAT CTGGATACAC CAGTTTTTAC TTATTTCAGA GCATCAACTC TAAACCTGGA
301 AACTTTGGAT TATAATCAGT TCATTACTTT GGGTAATAAT ACTGCTAATT CTACTACCCT
361 TGATGTTAAG GTCGGAGACA ATGGAATCCA AAGTCTGACC GCCGATAATA TTTCTCAACA
421 GCGTGCTTAC GCAAACGATG AAAATGTCAC TTTCGATATC ACTTTTGATG CTACATCTCG
481 TGTTATCTCT AATGCCGGCG CTGGAGTGTT TCAGTTTGGC CCTTCTATTA CCTATGAGTG
541 GGGTCTTCCA AACTGTCGTA CCCAGGGTTC TGTTACTGAT ACAGGTGGAA AAAATATCAC
601 CGTGGACCCT GCTAAGTCAT TCACTTGGTA TGACAGACAA TGGGGCACAG CCGCAGTCAC
661 TTCTGGTAAC TGGACTTGGT TCCAAATGCA TATTCCAGAA ACTTCTTATA AGTTGAGTGT
721 TTGGATTATC GACAACGATG TTACCAACCA GTTCTCCCGT TTCGCCACCA TCAGAGGAGA
781 CAATGATGAG TTCCAAGTGC TTCCACTGGA GTGGAAGCCA ATTTATGACA GAACTTACCA
841 ATCTACTGCC GCTGATATCC TGTACCCATT GGATTGGGAA CTTGATATCT CTGGTTTCGG
901 AGTCTTCCAA CTTTCTTCCA TTCTTGATGA CCAAGAAATC GTCGGTACTA CCGCCATCCA
961 GACCGCATAC GAAGGATTTG TCACTTTTAA TGGTACTGTT CATAATAAAA AGGTTCAGGG
1021 TTACGGCCTT GTGGAAGTCG TGTATTCTAA TTGGGAGTCT CTTCATCACC ATCACCATCA
1081 CCATCACCAT CACTAAGCGG CCGC
Nha-KHS-His$_{10}$ (1098 bp)
1 GAATTCCGAA ACGATGAGAG CTTCCTTTCT TCTGACCGCC GGTTTGGCTA CCGCAGCCGT
61 GGGACGTGCT AAGTCTGTGC CTAAGAAATT TCCTTTCAAG CCAGAAAATT CTAAGACCAC
121 AGGAACAAAT GCAATTCCCA TAGTATATGG CCTGAGTGAA TCCCAACCAA ATTCTGTCGG
181 TGGATCTTGG TGGTCATCTT CCTATATCAC TACCACTAAT AACGAACAAT ACGTCGTTTT
241 GGCTCATTAT TTGGACAATC CTGTCTACAC TTACTTCCGT GCTTCCACTT TGAATCTTGA
301 GACTAATGAG TACCACCAGT ACGTTACAGT GGGTTCTTCC ACTCCTAATA TAACTACCTT
361 GGATGTTTCT GTGGGTAACA ATGGCATTAA AAGTGAGTCC GAAGATAATC TTTCTAAGCT
421 GAGATCATAC TCTAACCATG ATAATGTGAC CTTCGATATT ACTTACGACG CCACCACTGG
481 AGCTGTCGCT AATGGAGGCG CCGGAACTTT CCAATTCGGA GAAGGTTTGA CATGGGAGTT
541 CGGTTTGCCT TCTGCTAAAA CAGAGGGCTC CCTGACCGTT CATGGTGAAA AACTTGCTAT
601 CGACCCTGCT AAATCCCATA CATGGTATGA TCGTCAGTGG GGAAATACTG CCGCTATCCC
661 CTCTAACTGG ACTTGGTTCC AACTTCATAT TCCATCCACA GAATACAAAA TTAGTGCATG
721 GATTTTCTCC GACCCATTCA GAAACACAGA AACCCGTTTT GCAACTATTA GAGGTGCAAA
781 TGATGAAACA CTGGTACTAC CTTTGGAATT TACACCAATC TACAAAAGAA CCTACGAATC
841 TGCCACCGGA AGAGTTACAT ATCCTCTTGA TTGGAAATTG AAGATTTCCG GATTCGGTGA
901 TTTCAAACTT AGTTCTTATA CAGAAGATCA AGAGCTAGTT GGTGAAGATG CCCTTCAAAC
961 AGCATACGAG GGATTCATTA CCTTCAGTGG TAATGTGCAC TCTAAACCCG TGCAGGGGTA
1021 TGGCCTTGTG GAAATCGTTT ACAGTACTTG GGATGTCCAT CACCATCACC ATCACCATCA
1081 CCATCACTAA GCGGCCGC

Table 14: Amino acid sequences of *Fso*- and *Nha*-KHSs (N-terminus → C-terminus).

Fso-KHS-His_{10} (40,3 kDa), ACCESSION: AAA87627
M M I S S V L V A G V V A V S A A L A S K H P K Q Y S F K P E D A E T I W N G D I P V L Y D F G D S Q S A
S Y S G S W W T S S Y I T G T N G E Q Y L V I S H Y L D T P V F T Y F R A S T L N L E T L D Y N Q F I T L G
N N T A N S T T L D V K V G D N G I Q S L T A D N I S Q Q R A Y A N D E N V T F D I T F D A T S R V I S N A
G A G V F Q F G P S I T Y E W G L P N C R T Q G S V T D T G G K N I T V D P A K S F T W Y D R Q W G T A
A V T S G N W T W F Q **M** H I P E T S Y K L S V W I I D N D V T N Q F S R F A T I R G D N D E F Q V L P L E
W K P I Y D R T Y Q S T A A D I L Y P L D W E L D I S G F G V F Q L S S I L D D Q E I V G T T A I Q T A Y E
G F V T F N G T V H N K K V Q G Y G L V E V V Y S N W E S L H H H H H H H H H H
Nha-KHS-His_{10} (39,9)
M R A S F L L T A G L A T A A V G R A K S V P K K F P F K P E N S K T T G T N A I P I V Y G L S E S Q P N
S V G G S W W S S S Y I T T T N N E Q Y V V L A H Y L D N P V Y T Y F R A S T L N L E T N E Y H Q Y V T
V G S S T P N I T T L D V S V G N N G I K S E S E D N L S K L R S Y S N H D N V T F D I T Y D A T T G A V A
N G G A G T F Q F G E G L T W E F G L P S A K T E G S L T V H G E K L A I D P A K S H T W Y D R Q W G
N T A A I P S N W T W F Q L H I P S T E Y K I S A W I F S D P F R N T E T R F A T I R G A N D E T L V L P L
E F T P I Y K R T Y E S A T G R V T Y P L D W K L K I S G F G D F K L S S Y T E D Q E L V G E D A L Q T A
Y E G F I T F S G N V H S K P V Q G Y G L V E I V Y S T W D V H H H H H H H H H H
Signal sequence   Potential N-glycosylation sites His_{10} – Tag

8.6 Primers

All primers were purchased from Integrated DNA Technologies (IDT).

Table 15: List of primers used for amplification of target genes. Gray: *Nde*I restriction site. Green: *Hind*III restriction site. Blue: *Not*I restriction site. Red: *EcoR*I restriction site.

Primer	Sequence (5' → 3')
Fw(*Fso*_KHS_*Nde*I)	TATACATATGATGATTAGCTCAGTA
Rv(*Fso*_KHS_10His_*Hind*III)	GCCAAGCTTATTAGTGATGGTGATGGTGATGGTGATGGTGAT GCAGGCTCTCCCAATTG
Fw(*Nha*_KHS_*Nde*I)	TATACATATGCGTGCATCTTTTC
Rv(*Nha*_KHS_10His_*Hind*III)	GCCAAGCTTATTAGTGATGGTGATGGTGATGGTGATGGTGAT GAACATCCCACGTGCTG
Fw(*Ate*_KHS_*Nde*I)	TATACATATGGACATTGCACGC
Rv(*Nha*_KHS_10His_*Hind*III)	CCAAGCTTATTAGTGATGGTGATGGTGATGGTGATGGTGATG GCTCTCGCCGCTGAAAATG
Fw(*Ate*_KHS-His10, *Nde*I)	ATATACATATGGACATTGCCCGTG
Fw(*Fso*_KHS-His10, *Nde*I)	ATATACATATGATGATTTCATCTGTCTTG
Fw(*Nha*_KHS-His10, *Nde*I)	ATATACATATGAGAGCTTCCTTTCTTCTGAC
Rv(*Not*I_Pp_HisOHYs)	GAGCGGCCGCCCTTTAGTGATGGTGATGGTGATG
Fw(*Nha*_nosig_*EcoR*I)	GAAAGAATTCCGAAACGATGAAGTCTGTGCCTAAGAAATTTC
Fw(*Fso*_nosig_*EcoR*I)	GAAAGAATTCCGAAACGATGAGCAAGCATCCCAAACA
Fw(Flag_*Nha*_*EcoR*I)	GAAAGAATTCCGAAACGATGGATTACAAAGACGATGACGATAA GGAGCTTCCTTTCTTCTGAC
Fw(Flag_*Fso*_*EcoR*I)	GAAAGAATTCCGAAACGATGGATTACAAAGACGATGACGATAA GATGATTTCATCTGTCTTGGTC

Table 16: List of primers used for site-directed mutagenesis. Blue/Yellow: Codon/reverse complement codon alanine.

Primer	Sequence (5' → 3')
Fw_N112A_*Nha*	TTCTTCCACTCCTGCTATAACTACCTTGG
Rv_N112A_*Nha*	CCAAGGTAGTTATAGCAGGAGTGGAAGAA
Fw_N132A_*Nha*	GAGTCCGAAGATGCTCTTTCTAAGCTG
Rv_N132A_*Nha*	CAGCTTAGAAAGAGCATCTTCGGACTC
Fw_N144A_*Nha*	TCTAACCATGATGCTGTGACCTTCGAT
Rv_N144A_*Nha*	ATCGAAGGTCACAGCATCATGGTTAGA
Fw_N219A_*Nha*	CTATCCCCTCTGCTTGGACTTGGTT
Rv_N219A_*Nha*	AACCAAGTCCAAGCAGAGGGGATAG
Fw_N108A_*Fso*	GTTCATTACTTTGGGTGCTAATACTGCTAATTCTAC
Rv_N108A_*Fso*	GTAGAATTAGCAGTATTAGCACCCAAAGTAATGAAC

Fw_N132A_*Fso*	CAAAGTCTGACCGCCGATGCTATTTCTCAACAGCGTG
Rv_N132A_*Fso*	CACGCTGTTGAGAAATAGCATCGGCGGTCAGACTTTG
Fw_N144A_*Fso*	CTTACGCAAACGATGAAGCTGTCACTTTCGATATCAC
Rv_N144A_*Fso*	GTGATATCGAAAGTGACAGCTTCATCGTTTGCGTAAG
Fw_N194A_*Fso*	CTGATACAGGTGGAAAAGCTATCACCGTGGACCCTG
Rv_N194A_*Fso*	AGGGTCCACGGTGATAGCTTTTCCACCTGTATCAG
Fw_N219A_*Fso*	CGCAGTCACTTCTGGTGCTTGGACTTGGTTCCAAATG
Rv_N219A_*Fso*	CATTTGGAACCAAGTCCAAGCACCAGAAGTGACTGCG
Fw_N326A_*Fso*	GAAGGATTTGTCACTTTTGCTGGTACTGTTCATAATA
Rv_N326A_*Fso*	TATTATGAACAGTACCAGCAAAAGTGACAAATCCTTC

Table 17: Sequenicng primer for KHS constructs.

Primer	**Sequence (5' → 3')**
Rv(kep1 seq2)	CCAAACCCCTACCACAAGATATTC