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Cell-free production of the bifunctional glycoside hydrolase GH78 from *Xylaria polymorpha*

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ABSTRACT

The ability to catalyze diverse reactions with relevance for chemical and pharmaceutical research and industry has led to an increasing interest in fungal enzymes. There is still an enormous potential considering the sheer amount of new enzymes from the huge diversity of fungi. Most of these fungal enzymes have not been characterized yet due to the lack of high throughput synthesis and analysis methods. This bottleneck could be overcome by means of cell-free protein synthesis. In this study, cell-free protein synthesis based on eukaryotic cell lysates was utilized to produce a functional glycoside hydrolase (GH78) from the soft-rot fungus *Xylaria polymorpha* (Ascomycota). The enzyme was successfully synthesized under different reaction conditions. We characterized its enzymatic activities and immobilized the protein via FLAG-Tag interaction. Alteration of several conditions including reaction temperature, template design and lysate supplementation had an influence on the activity of cell-free synthesized GH78. Consequently this led to a production of purified GH78 with a specific activity of 15.4 U mg^{-1}. The results of this study may be foundational for future high throughput fungal enzyme screenings, including substrate spectra analysis and mutant screenings.

1. Introduction

Industry has taken advantage of the versatility of reactions that are catalyzed by fungal enzymes. About half of all commercial enzymes currently in use are of fungal origin [1]. Most of them are hydrolases primarily used in wine and dairy industry and in food industry in general [2]. Thus, amylases and rhamnosidases usually support fermentation processes, but are also applied in a medical context, especially in the preparation of certain metabolites. Amongst others, some of these metabolites display anti-cancer activities (ginsenoside-Rh1), antibiotic properties (chlorosporin C), anti-inflammatory activities (quercetin, prunin) and diverse modes of action against DNA and RNA viruses (prunin) (reviewed in [3]). Furthermore, the production of steroid drugs, like progesterone are performed with the help of rhamnosidases [3]. The range of putative fungal enzymes with industrial relevance is long and new ones are constantly being discovered and synthesized *de novo* [4]. This was the case for a glycoside hydrolase of *Xylaria polymorpha* an enzyme with unique properties [5].

Xylaria polymorpha (Xpo) – a fungus belonging to the ascomycetous class Sodariomycetes – lacks high-redox potential class-II peroxidases for lignin attack (as found in basidiomycetous fungi causing white-rot, i.e. manganese, lignin and versatile peroxidases), but harbors instead laccases and several extracellular hydrolases that enable the fungus to follow an alternative pathway of lignocellulose conversion, including a special glycoside hydrolase 78 (GH78). GH78 is a hybrid enzyme with four domains that exhibits α-L-rhamnosidase and feruloyl esterase activities. It was first described by Nghi et al. who produced the enzyme with the wild-type fungus, characterized it with respect to its substrate spectrum and determined enzyme kinetic data [5]. The enzyme turned out to hydrolyze ester-linkages between lignin and polysaccharide

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moieties in the plant cell wall and beyond that, it accomplishes an even stronger α-L-rhamnosidase activity, which is relevant for the degradation of hemicellulose and pectin chains.

Although GH78 is a secretory protein – since its polymeric substrates must be digested in the extracellular microenvironment of fungal hyphae – it does not have an N-terminal signal sequence for translocation. Despite the lacking signal sequence, glycosylation of the protein suggests that it is translocated into the endoplasmic reticulum (ER) via an alternative pathway [5]. A straightforward approach to monitor GH78's rhamnosidase activity makes use of the *p*-nitrophenyl α-L-rhamnopyranoside (*p*-NPRP) assay that follows the formation of the colored fission product *p*-nitrophenol (appendix Figure A. 2) [6].

The cultivation of *Xylaria polymorpha* and subsequent purification of the GH78 protein are time-consuming processes and can take up to eight weeks (Nghi et al., 2012). Therefore, this preparation method has limited suitability for applications, in which the enzyme needs to be modified multiple times, for example during enzyme engineering. Enzyme synthesis in a cell-free protein synthesis (CFPS) platform can facilitate these kinds of applications. CFPS is a sophisticated method to produce modified proteins within a few hours for enzymatic characterization. GH78 offers the ideal entry point for cell-free synthesis of fungal enzymes due to its intrinsic stability and lacking prosthetic groups. Once the methods for cell-free synthesis of active fungal enzymes are established, they represent an ideal platform for high throughput screenings. In this process, templates can be tested that may lead to an improvement or completely change the functionality of the individual enzyme (enzyme engineering) [7].

Eukaryotic CFPS systems have been developed to improve the production of "difficult-to-express proteins" [8–10]. Notable advantages of cell-free systems are the significant reduction of production time [11] and the possibility to perform high throughput screenings using various templates and synthesis conditions in a cost and time efficient way [12]. Enzymatic activity assays can be performed immediately after protein production or even during protein synthesis, for example, in a 96-well plate format. This makes CFPS a valuable tool for enzymology.

The basis for cell-free synthesis is provided by translationally active cell-lysates CFPS enables the user to address a broad spectrum of different applications for protein characterization. However, choosing the right cell-free system and attaining the appropriate reaction conditions for each individual application is mandatory. To date translationally active lysates from *Escherichia coli*, *Saccharomyces cerevisiae*, *Leishmania tarentolae*, wheat germ (WG) from *Triticum*, *Spodoptera frugiperda* (*Sf*21), chinese hamster ovary (CHO) from *Cricetulus griseus*, rabbit reticulocytes from *Oryctolagus cuniculus* and cultured human (*Homo sapiens*) cells are available [10,13]. In the present study, a clear influence of the lysate on the activity of GH78 was observed.

2. Materials and methods

2.1. Template generation

Templates for the synthesis of GH78 (amino acid sequence in the appendix) in cell-free systems were manufactured by Biocat GmbH (Heidelberg, Germany). The protein encoding sequence was integrated in a pUC57–1.8 k-vector backbone comprising further regulatory factors for CAP-independent protein synthesis using a CrPV-IRES [14]. Templates for protein synthesis in WG lysates were generated through Gibson assembly. For this purpose, the protein coding sequence was extracted from the pUC57 vector and cloned in a pIVEX1.3 vector. Codon optimization was performed using the "geneius" online tool (https://www.geneius.de/GENEius). Based on the original DNA sequence (XPO), sequence optimization was performed for *Spodoptera frugiperda* (*Sf*21), *Cricetulus griseus* (CHO) and *Saccharomyces cervisiae* (SCE). Codon optimizations consider the availability of translation components in the respective organism, like tRNA and aminoacyl-tRNA synthetases. While endogenous synthethases from the lysates (*Sf*21 and CHO) are used during cell-free protein synthesis tRNAs originating from S. cervisiae are supplemented to the lysates. Since the limiting factor is unknown, codon optimizations to all three organisms were performed.

2.2. Lysate preparation and cell-free protein synthesis

Lysate preparation of *Sf*21 and CHO lysates was performed as described earlier [15,16]. CHO cells were grown exponentially in suspension cultures in well-controlled fermenters at 37 °C using the ProCHO 5 serum-free medium (Lonza, Cologne, Germany). *Sf*21 cells were grown in fermenters at 27 °C in an animal component-free insect cell medium.Cells were harvested at a density of approximately 1–4 × 10^6 cells/mL. Collected cells were centrifuged at 200 x g for 10 min and washed once with a buffer consisting of 40 mM HEPES-KOH (pH 7.5), 100 mM NaOAc (CHO)/100 mM KOAc (*Sf*21), and 4 mM DTT. The cell pellet was resuspended to achieve a density of approximately 1–5 × 10^8 cells/mL. Cell disruption was accomplished by syringing the harvested cell pellet through a 20-gauge needle, followed by centrifugation at 10.000xg for 10 min to remove the nuclei and cell debris. The supernatant was applied to a size-exclusion chromatography column (Sephadex G-25, GE Healthcare, Freiburg, Germany) and the elution fractions (each 1 mL) with an RNA content above an absorbance of 100 at 260 nm were pooled. Residual mRNA was digested by micrococcal nuclease (S7) treatment. In this respect, 10 U/mL S7 nuclease (Roche, Mannheim, Germany) and 1 mM $CaCl_2$ (final concentration) were added to the eluate and the reaction mixture was incubated for 2 min (CHO)/ 10 min (*Sf*21) at room temperature. The reaction was inactivated by the addition of 6.7 mM EGTA (f.c.). Finally, translationally active lysates were immediately shock-frozen in liquid nitrogen and stored at − 80 °C in order to preserve maximum activity.

Production of proteins in translationally active lysates derived from cultured insect cells (*Sf*21) and Chinese hamster ovary (CHO) cells was performed as previously described [16,17] in batch based and continuous exchange cell-free (CECF) systems. A standard batch reaction mixture was composed of 40% (v/v) processed cell lysate. Further standard supplements comprise HEPES-KOH (pH 7.6, f.c. 30 mM; BioMol GmbH, Hamburg, Germany), KOAc (f.c. 135 mM, Merck, Darmstadt, Germany), $Mg(OAc)_2$ (f.c. 3.9 mM, Merck) and amino acids (complete 100 μM, Merck), spermidine (f.c. 0.25 mM, Sigma-Aldrich, St. Louis, USA). Creatine phosphate (20 mM; Roche, Grenzach, Germany), ATP (f.c. 1.75 mM, biotechrabbit GmbH, Berlin, Germany), CTP (f.c. 0.3 mM, biotechrabbit GmbH), GTP (f.c. 0.3 mM, biotechrabbit GmbH), UTP (f.c. 0.3 mM, biotechrabbit GmbH) and 0.1 mM (f.c.) $m^7G(ppp)G$ (Prof. Edward Darzynkiewicz, Warsaw University, Poland) were added as energy resources to the reaction. PolyG (IBA, Göttingen, Germany) primer was supplemented to the reaction at a final concentration of 4.5 μM in the batch mode. Plasmid DNA (f.c. 60 ng/μL) and 1 U/μL T7 RNA polymerase (f.c.) (Agilent technologies, Waldbronn, Germany) were added to start the reaction. For the conformation of successful cell-free protein synthesis, ^{14}C-labeled leucine (f.c. 30 μM, specific radioactivity 46.15 dpm/pmol; Perkin Elmer, Baesweiler, Germany) was added to the reaction. In the course of the work KOAc and $Mg(OAc)_2$ were replaced by KGlu (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) and $Mg(Glu)_2$ (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) in a f.c. of 135 mM and 3.9 mM respectively, which has been observed to increase the enzymatic activity of GH78 in batch reactions. Batch reactions were incubated for 3 h and CECF reactions were incubated for 24 h at 600 rpm unless otherwise noted. A standard CECF reaction consisted of a reaction mix (50 μL) and a feeding mix (1000 μL). Reaction mix was composed like a batch reaction without $m^7G(ppp)G$ cap analogue. Sodium azide was supplemented to the reaction to prevent microbial growth during cell-free synthesis. The feeding mix contained HEPES-KOH, salts, amino acids and nucleotides in the same concentration as in the reaction mixture and 0.02% of sodium azide. CECF synthesis was performed in a commercially available two chamber dialysis device (biotechrabbit GmbH, Berlin, Germany) for 24 h and 600 rpm in a thermomixer

(Eppendorf). Concentrations of 11 μM ^{14}C leucine (specific radioactivity 9.9 dpm/pmol) supplemented to the CECF reaction was to enable further qualitative and quantitative analysis of cell-free synthesized proteins.

Cell-free synthesis based on WG lysates were performed according to the RTS 100 Wheat Germ CECF Kit Manual (biotechrabbit GmbH, Berlin, Germany). Reaction mixtures were incubated at 21 °C for 24 h at 900 rpm.

To validate successful protein synthesis, radioactive labeling of the proteins with ^{14}C-leucine was performed which allows qualitative characterization by autoradiography and quantitative analysis through scintillation counting as described earlier [16]. The received values from scintillation counting are measured in counts per minutes (cpm) which are subsequently converted to disintegrations per minute (dpm). Protein yields were calculated using the measured counts, the molecular weight of the synthesized protein, the specific radioactivity A_{spec} (Eq. 1) and the total number of leucines were present in the target protein (Eq. 2).

$$A_{spec} = \frac{c_{14C-Leu} \bullet A_{14C-Leu\ \ Stock}}{c_{total\ \ Leu}} \quad (1)$$

$$concentration\left[\frac{\mu g}{ml}\right] = \frac{measured\ \ counts\ \left[\frac{dpm}{ml}\right] \bullet molecular\ \ weight\ \left[\frac{\mu g}{pmol}\right]}{A_{spec}\left[\frac{dpm}{pmol}\right] \bullet \#_{leucines\ \ in\ \ the\ \ protein}} \quad (2)$$

2.3. GH78 activity measurement

The α-L-rhamnosidase activity of cell-free synthesized GH78 was assayed photometrically using *p*-nitrophenyl α-L-rhamnopyranoside (*p*-NPRP; Sigma-Aldrich) as substrate [6,18]. Each well of a 96-well microtiter plate contained 2.5 mM *p*-NPRP in 50 mM sodium acetate buffer (pH 5.0) in an overall reaction volume of 100 μL. Dependent on the enzyme synthesis yield, different volumes of the supernatant fraction were added to the substrate to start the reaction. The supernatant fraction contains the active cell-free synthesized GH78 enzymes. The reaction proceeded for 4 h at room temperature (unless otherwise noted) and was stopped by adding 40 μL sodium carbonate solution (1 M) to each well (resulting in a pH around 11). Absorbance at 405 nm was determined using a microplate reader (Mithras^2 LB 943, Berthold). The amount of synthesized *p*-nitrophenol was calculated by using a calibration curve.

Enzymatic activity over time was determined using the above described assay (with 100 μL *p*-NPRP) with stepwise stopping of the reaction after different time points. For kinetic measurements, different concentrations of *p*-nitrophenyl α-L-rhamnopyranoside (0.1–2.5 mM) were applied. After adding the enzyme, mixtures were incubated at 37 °C and the reactions were stopped after 30–120 min by adding 40 μL of 1 M sodium carbonate solution. Kinetic data (K_m, k_{cat}) were calculated by plotting inverse substrate concentrations against invers reaction velocity according to Lineweaver and Burk.

2.4. GH78 FLAG-Tag purification

Purification of GH78 was performed by FLAG-Tag protein capture using Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich). 20 μL magnetic bead suspension (50%) was loaded onto a small column and washed twice with 200 μL TBS. A cell-free translation mixture containing the synthesized enzyme was centrifuged at 4 °C and 16.000g for 10 min 500 μL of the supernatant were added to the agarose and incubated over night at 4 °C on a rotator. The binding supernatant (BSN) was removed and the agarose was washed five times with 500 μL TBS (W1–5). Subsequently, the agarose was incubated with 50 μL elution buffer (3 x FLAG-peptide, 100 ng/μL in TBS) two times (E1–2). Each fraction was collected individually and analyzed by autoradiography and scintillation counting. GH78 rhamnosidase activity was determined as described above.

2.4.1. Substrate specificity screening

Substrate specificity of cell-free produced GH78 was studied with five substrates and compared with the wild-type enzyme expressed in *X. polymorpha* as described earlier [5]. Specific activities were determined by the hydrolysis of the following substrates: *p*-nitrophenyl α-L-rhamnopyranoside, *p*-nitrophenyl α-L-arabinofuranoside, naringine, *p*-nitrophenyl β-D-glucopyranoside and *p*-nitrophenyl β-D-cellobioside.

2.5. GH78 FLAG-Tag immobilization and activity determination

The immobilization of GH78 was performed similar to the purification. 20 μL magnetic bead suspension (50%) was loaded onto a small column and washed twice with 200 μL TBS. A cell-free translation mixture containing the synthesized enzyme was centrifuged at 4 °C and 16.000g for 10 min 200 μL of the supernatant were added to the agarose and incubated over night at 4 °C on a rotator. The binding supernatant (BSN) was removed and the agarose was washed three times with 500 μL TBS (W1–3). The TBS was depleted and the beads were incubated repeatedly with 100 μL TBS buffer containing 2.5 mM *p*-NPRP. 40 μL of a 1 M $NaCO_3$ solution was added to the obtained 100 μL fractions, which were then analyzed by measuring the absorbance at 405 nm with a microplate reader. The principle of the immobilization procedure is depicted below (appendix Figure A. 1).

3. Results

3.1. Increasing the amount of active GH78 by synthesis optimization in batch-format cell-free protein synthesis

Reaction condition adaptations were performed in a batch mode reaction. The choice of the counter anion in cell-free synthesis was relevant during enzyme production and activity. GH78 was synthesized in CHO and *Sf*21 lysate-based batch reactions using either acetate or glutamate as counter anion (Fig. 1). In batch mode, CHO based CFPS led to higher total target protein yields, while *Sf*21-based CFPS led to higher total GH78 enzyme activity. Regarding the protein yield, glutamate had a negative effect on the reaction in the CHO-lysate based CFPS system and a positive effect in the *Sf*21-lysate based CECF system. Regarding the absolute activity, a significant increase of absolute enzyme activity was observed between both counter anions for the enzymes produced in the CHO-lysate based CFPS system. An almost twofold increase in absolute activity was observed for GH78 produced in the insect-lysate based CFPS system (Fig. 1b). The relative activity of the enzyme was therefore higher in both CFPS systems when glutamate instead of acetate was supplied as counter ion (Fig. 1c).

Further reaction condition adaptations comprising Poly G and template concentration variation did not increase enzyme yield or the enzymatic activity of the cell-free produced GH78 to a relevant extent (appendix Figure A. 3).

3.2. Enhancing synthesis efficiency by altering the template sequence in CHO-based CFPS

Modifications of the template DNA sequence in the coding and non-coding regions can lead to increased protein yield and activity in CFPS. Different template modifications were designed to increase GH78 yield in the eukaryotic lysate-based CFPS. These modifications were codon optimization, addition of an N-terminal tag, and modification of the 5`UTR. The modified templates were applied in the reaction for CFPS. Target protein yield and enzyme activity were determined for each modification in a *Sf*21 and in a CHO cell-lysate based CFPS. The original sequence was codon-optimized to an *Sf*21 system (GH78 Sf21 opt.). Using alternative sequences, the *Cricetulus griseus* optimized template (GH78 C. griseus opt.), the *Xylaria polymorpha* optimized template

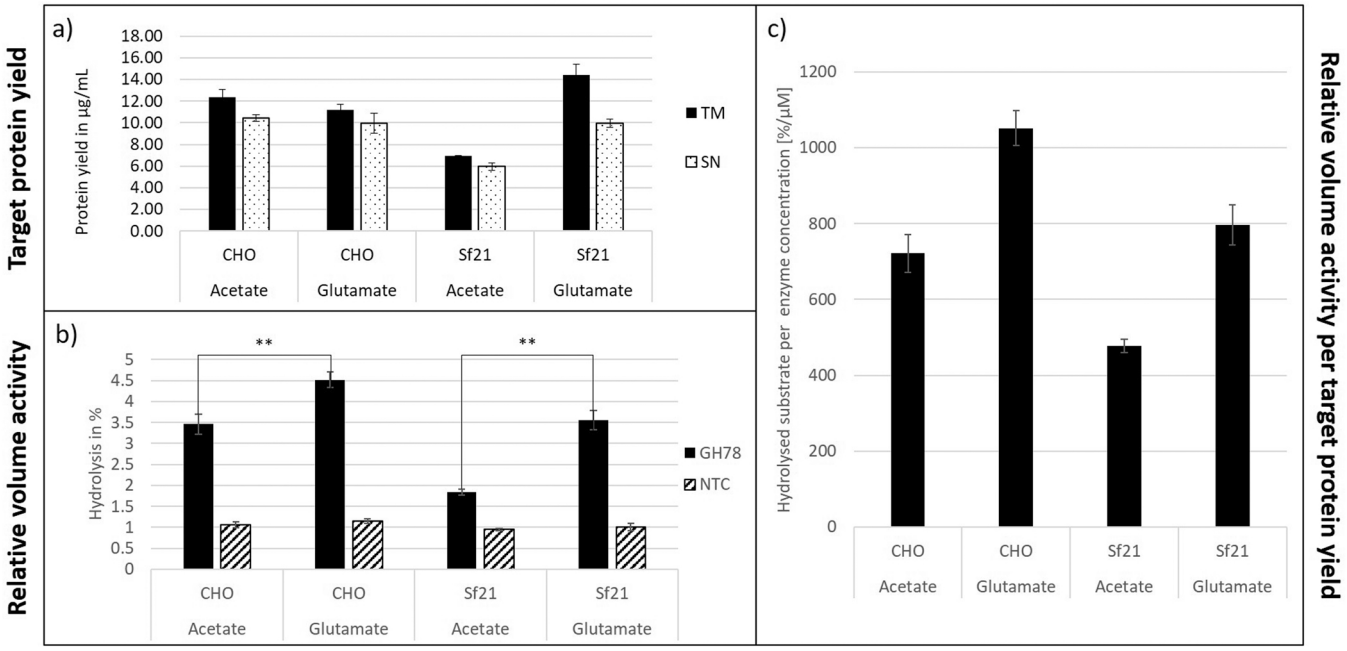

Fig. 1. Comparison of FLAG-GH78 yield and activity in CHO and Sf21 lysate batch based cell-free protein synthesis systems using two different counter anions at 24 °C (acetate and glutamate both in a final concentration of 143 mM added to the lysate). a) Total protein yield was determined by scintillation counting in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000g. b) p-NPRP assay of cell-free synthesized FLAG-GH78 using 3 µL of the supernatant fraction compared to a no template control. The bars represent the percentage of hydrolyzed substrate after an incubation time of 2 h at 37 °C. c) p-NPRP assay values in relation to the incubated enzyme molarity. The standard deviation was calculated from triplicates. Statistical significance was determined with t-tests for two independent samples (** $p < 0.01$).

(GH78 *X. polymorpha* opt.), and the respective N-terminal His (His-GH78) and Flag-tagged (Flag-GH78) templates resulted in both higher target protein yield and higher concentration of active enzyme during synthesis (Fig. 2).

For increased enzyme synthesis, a continuous exchange format cell-free (CECF) system was applied, which increased the protein yields ten-fold in the CHO lysate-based CFPS. CECF also resulted in an increased absolute enzyme activity, although the relative GH78 activity per produced protein was higher in the batch-format synthesis. In the CECF format, an almost four-fold increase in protein yield up to 85 µg/mL could be achieved by adding an N-terminal FLAG-tag to the enzyme produced in the CHO-based dialysis-format protein synthesis. The

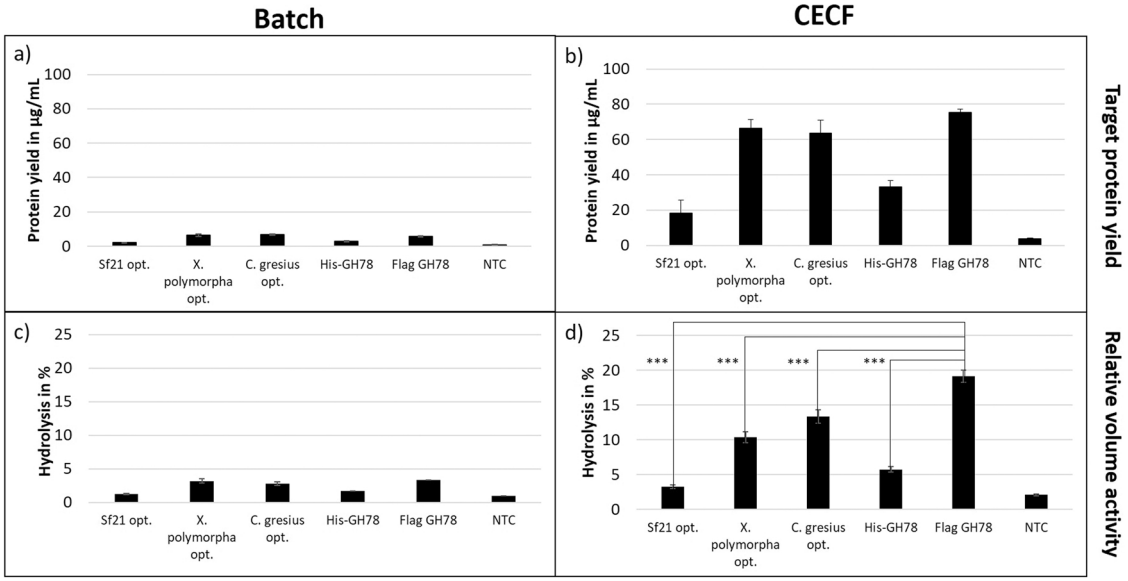

Fig. 2. Enhancing synthesis efficiency by modifying the template for cell-free protein synthesis in a CHO lysate-based cell-free protein synthesis system. GH78 yield determination by scintillation counting in the supernatant fraction (SN) after centrifugation at 16,000g in a batch based system (a) and in a CECF system (b). The standard deviation was calculated from triplicates. p-NPRP assay of cell-free synthesized GH78 using the supernatant fraction compared to a no template control of enzyme produced in a batch-based format (c) and in the CECF format (d), respectively. Columns represent the percentage of hydrolyzed substrate after an incubation time of 2 h at 37 °C. Standard deviation was calculated from triplicates. Statistical significance was determined with t-tests for two independent samples (*** $p < 0.001$).

correct size of the GH78 constructs could be verified by autoradiography showing the *de novo* synthesized proteins (Fig. 3).

Further attempts on sequence modifications were performed comprising *Saccharomyces cerevisiae* codon optimization, the use of species independent translation initiation sites, truncation of the sequence by the first two domains, the use of a melittin signal sequence, and the addition of alternative initiation sequences (appendix Fig. 4A). However, these modifications did not lead to increased activity of cell-free produced GH78. Similar attempts using *Sf*21 batch and dialysis-format CFPS resulted in similar trends for the different templates but to lower yields and volume activity.

3.2.1. Temperature adaptations

The synthesis temperature plays an important role with regard to the yield and the folding efficiency of cell-free produced proteins. GH78 was synthesized in a CHO lysate-based CECF-format system at temperatures between 15 °C and 33 °C, and the protein yields and enzyme activities were compared (Fig. 4). The highest protein amount in the translation mix was obtained at 30 °C with a yield of 190 μg/mL. In contrast, the optimal temperature for active enzyme synthesis was between 18 °C and 21 °C. The maximum relative activity (active enzyme in relation to the amount of total protein synthesized) was calculated for 18 °C and below.

Batch synthesis of GH78 in the CHO CFPS system has previously shown a synthesis temperature optimum at 24 °C for the active enzyme (appendix Figure A. 5).

3.3. Enzyme kinetic analysis

By using continuous exchange-format CFPS it was possible to prolong the reaction time and further increase the enzyme yield. The adapted synthesis conditions were applied to receive a maximum amount of active enzyme for kinetic analysis (appendix). Three different eukaryotic cell-free systems (CHO, *Sf*21 and WG) were compared directly regarding absolute yield during protein synthesis and their kinetic constants (Table 1). GH78 from CHO CFPS had the lowest K_m values with 0.46 mM, followed by GH78 from WG CFPS with 0.5 μM and *Sf*21 CFPS with 0.7 mM. The k_{cat} of the enzyme produced in the CHO system was with 4.4 s^{-1} and 6.0 s^{-1} in the WG system lower in comparison to GH78 produced in the *Sf*21 system with 9.9 s^{-1} resulting in a higher enzymatic efficiency of 15 mM/s for the GH78 generated in *Sf*21 lysate.

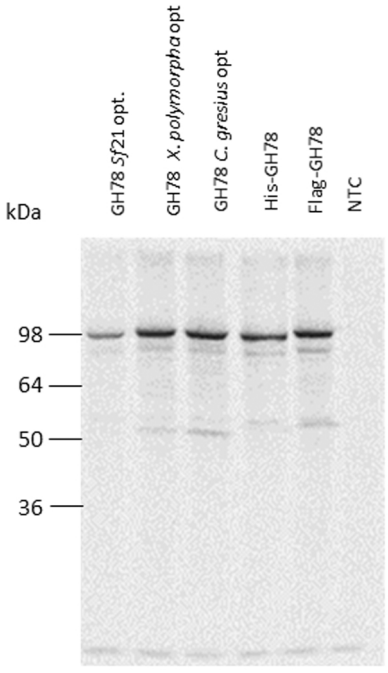

Fig. 3. Qualitative analysis of GH78-construct variants synthesized in a CHO lysate-based cell-free reaction. An autoradiography based on a 10% SDS-PAGE visualizes the size of the radioactive labeled proteins in the cell-free reaction.

3.4. Substrate conversion of cell-free recombinant produced and wild-type expressed GH78

A direct comparison between purified, recombinant r*Xpo*GH78 produced in a CHO lysate-based CFPS and in a commercially available WG lysate based CECF and wild-type *Xpo*GH78 secreted by the fungus was performed regarding substrate conversion and specific activities. A purification of the cell-free produced enzymes was necessary in this case to avoid potential unspecific enzymatic activities originating from the lysate. GH78 produced in *Sf*21 based CFPS could not be purified successfully. Different substrates were tested and the specific activities here calculated (Table 2). For the conversion of *p*-NPRP and Naringin, GH78 produced by CHO CFPS achieved specific activities of 15.4 U mg^{-1} and 5.6 U mg^{-1}, respectively, which are considerably higher compared to the wild-type enzyme and the enzyme produced in the wheat-germ lysate. No esterase activity on the substrate methylferulate was measured for the cell-free produced enzymes.

Regarding the conversion of rhamnose educts, the recombinant cell-free synthesized GH78 enzymes are performing with a similar (WG) or with a higher specific activity (CHO) in comparison to wild-type GH78 from *Xylaria Polymorpha*. A multifunctionality, that was observed for the wild-type GH78 was not observed for recombinant cell-free synthesized GH78. Dependent on the individual application, different cell-free systems are beneficial for the enzyme production.

3.5. FLAG-Tag immobilization of GH78

After successful immobilization and purification of cell-free synthesized GH78 on Anti-FLAG M2 magnetic beads, we focused on monitoring the activity of GH78 bound to Anti-FLAG M2 magnetic beads. The scintillation results indicated that about 75% of the protein amount that was incubated with the beads was immobilized (appendix Figure A. 6Error! Reference source not found.a). No significant amount of GH78 was detected in the washing fractions. p-NPRP hydrolysis activity of the bound enzyme was measured ten times consecutively for 15 min each (appendix Figure A. 6Error! Reference source not found.b). A decrease in substrate turnover was observed after consecutive substrate incubation. The measured enzyme activity in the last 15 min incubation step was about 30% compared to the first step.

4. Discussion

CFPS is yet an underrepresented tool for the large domain of enzymology, even though the potentials are well known and published [19,20]. Especially enzyme discovery as well as advanced protein engineering could become more straight forward in cell-free systems in comparison to cell-based methods [20]. This does not only include the discovery of natural enzymes by high throughput experiments, but also the generation of new artificial enzymes through directed enzyme evolution and enzyme engineering [21]. Furthermore, CFPS allows the characterization of a protein in a particularly fast and efficient manner, which is shown in this publication by the example of an enzyme that has relevance for basic and ecological research as well as for industrial applications [3]. GH78 from *X. Polymorpha* is suitable for this purpose, as it exhibits high intrinsic stability and thus provides a perfect entry point for cell-free synthesis of fungal enzymes [5]. The knowledge gained can be used as a basis for further types of enzymes that can be produced in the future using cell-free systems.

Cell-free protein production usually requires optimization and adaptations to the individual enzyme of interest, especially for *de-novo* synthesis of a certain protein type in a cell-free system. The reaction

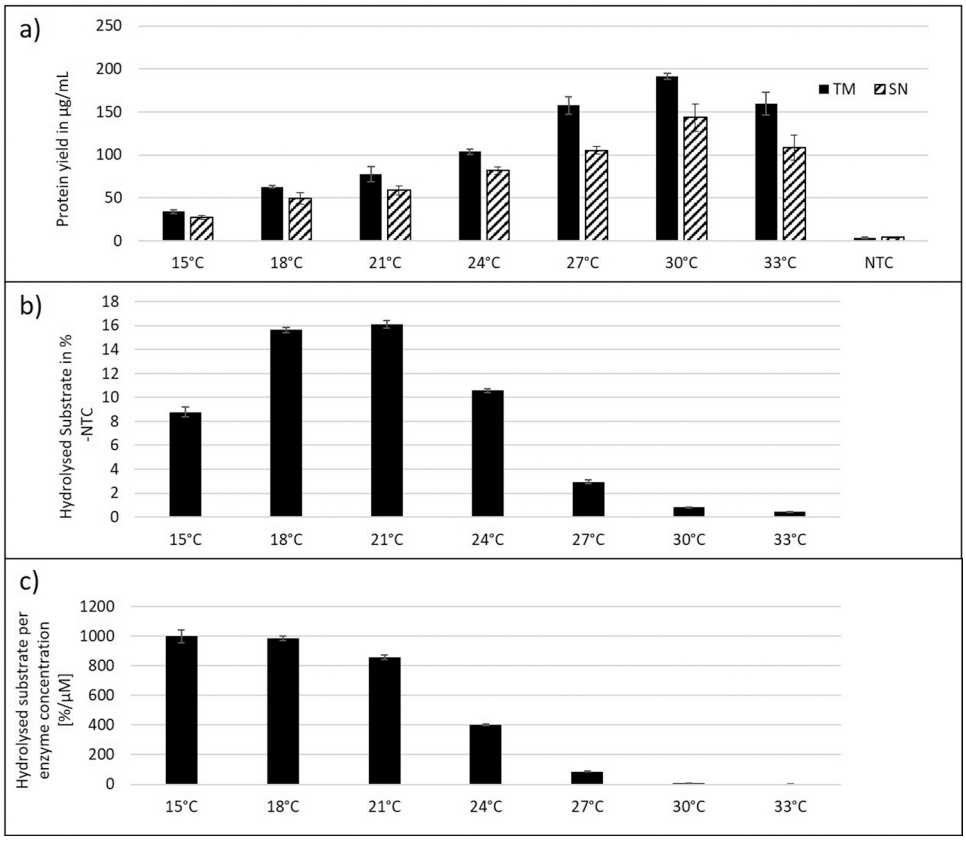

Fig. 4. Comparison of GH78 yield and activity in CHO-lysate CECF protein synthesis systems at different synthesis temperatures. a) Total protein yield determined by scintillation counting of GH78 produced under varying temperatures in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000g. Standard deviation was calculated from triplicates. The no template control was performed at 21 °C. b) p-NPRP assay of cell-free synthesized GH78 using 3 µL of the supernatant fraction. Bars represent the percentage of hydrolyzed substrate after an incubation time of 2 h. The standard deviation was calculated from triplicates. c) p-NPRP assay values in relation to the incubated enzyme molarity. The standard deviation was calculated from triplicates.

Table 1
Yields and kinetic constants based on Michalis-Manton-Kinetic of GH78 produced in different cell-free systems. Kinetic constants were determined with "Origin 2019" via non-linear regression.

Cell-free system	Template	Maximal Protein yield in the TM [µg/mL]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [mM/s]
WG CECF	GH78-His pIVEX1.3	550	0.5 ± 0.1	6.0 ± 0.6	11 ± 4
*Sf*21 CECF	GH78 pUC57–1.8k	30	0.7 ± 0.1	9.9 ± 0.8	15 ± 4
CHO CECF	FLAG-GH78 pUC57–1.8k	190	0.46 ± 0.07	4.4 ± 0.2	10 ± 2

Table 2
Specific activities of purified wild-type GH78 from X. polymorpha (wXpoGH78) and the purified recombinant enzyme (rXpoGH78) synthesized by CHO and wg lysate-based CFPS.

Substrates	Specific activity (U mg^{-1})		
	rFLAG-*Xpo*GH78 (CHO)	r*Xpo*GH78-His (WG)	w*Xpo*GH78
p-Nitrophenyl α-L-rhamnopyranoside	15.4	1.5	2.5
p-Nitrophenyl α-L-arabinofuranoside	n.d.	0.1	1.1
Naringine	5.6	2.0	1.0
p-Nitrophenyl β-D-glucopyranoside	n.d.	0.1	1.6
p-Nitrophenyl β-D-cellobioside	n.d.	0.1	0

temperature is one of the factors displaying a significant impact on the target enzyme activity [22], since it not only affects the total protein yield but is a crucial factor for protein folding as well [23]. Temperature screening of GH78 synthesis showed that the default temperature of 27 °C, which is considered to be optimal for *Sf*21 lysate-based CFPS [9], resulted in fact in the highest protein yield. In the CHO cell-free system a temperature of 30 °C resulted in maximal protein yield, which is indeed considered the default temperature for these lysates. However, this does not necessarily imply that the synthesized protein is completely soluble, correctly folded and active. Seemingly, the lower synthesis rate has led to a more consistent protein folding process, which promoted correct folding and less aggregation and hence higher relative activity [24]. It should also be taken into consideration that GH78 folding is optimal at lower synthesis temperatures, since the protein is of fungal origin and therefore needs to be active and correctly folded at a moderate temperature range [25]. In contrast to eukaryotic CFPS, synthesis at low temperatures is difficult to achieve for recombinant protein synthesis in *E. coli* systems usually operated at 37 °C [26]. Considering fungal enzymes are becoming more and more relevant for research and industrial applications [27], this information will be advantageous for future approaches addressing similar enzymes.

In addition to optimizing the synthesis temperature, the supplementation of additives and the substitution of components in the cell-free reaction mixtures typically influence enzyme activity. The substitution of acetate by glutamate was previously shown to enhance luciferase activity in a yeast lysate-based CFPS system [22]. This also applies to the synthesis of GH78 in CHO and *Sf*21 lysate-based CFPS systems. Glutamate is a predominant anion found in cells, which suggests that it may also stabilize protein synthesis and especially the protein folding processes in cell-free systems, thereby enhancing the relative enzyme activity [28]. For other fungal enzymes, co-factor introduction may play an even larger role and is still the bottleneck of successful synthesis of active fungal oxidoreductases such as unspecific peroxygenases (UPOs)

[29].

Similar to initiation trans-acting factors that are known as ITAFs, it is likely that the N-terminal sequence context ("cis-acting factors") may have an influence on IRES mediated translation initiation as well [30,31]. Thus, adaptations of the template sequence in proximity to the translation initiation site were performed to potentially increase the target protein yield and thereby the concentration of produced active enzyme. One effective template adaptation is the use of other codons [32]. This does not change the protein sequence but leads to an altered mRNA sequence and thus also to a different mRNA fold, which affects translation. By default, *Sf*21 optimized coded sequence is utilized for CFPS, which lead to reduced synthesis efficiency in contrast to *Cricetulus gresius* and *X. polymorpha* optimized sequences. Along with the codon optimizations, the addition of the N-terminal tags in particular led to an improvement in the cell-free synthesis of GH78. Similar effects on the activity of enzymes [33] and on the stability of enzymes [34] have been shown before. This indicates a strong influence of the mRNA sequence context immediately after CrPV-IRES at least for the applied template. Furthermore, increased target protein synthesis were observed with the N-terminal modification with MEL-EGFR-START (appendix Figure A. 4). The epidermal growth factor receptor (EGFR) has previously been successfully synthesized in a CHO-based CECF system with high yields using the melittin signal sequence (MEL) [8]. This has led to the hypothesis that the MEL-EGFR template inherits a favorable sequence for CFPS in close proximity to the N-terminus. However, the lack of enzyme activity in this variant as well as in the MEL- and MEL-EGFR-START modifications rather indicate a misfolding or substrate channel blocking of the enzyme resulting from the N-terminal sequence modification. Further template modification did not result in increased GH78 activity either (appendix Figure A. 4). This study shows how even small changes in the structure of the template can have a massive impact on the yield and functionality of a target protein. Therefore, it makes sense to consider modifications of the template design when synthesizing proteins that are difficult to produce in a cell free production system.

Enzyme kinetic analysis using unpurified GH78 revealed only minor differences between the GH78 proteins synthesized in *Sf*21, CHO and WG CFPS systems. Unsurprisingly, almost equal Michaelis-Menten constants for *p*-NPRP hydrolysis were obtained for enzyme proteins produced in all three systems. However, a higher k_{cat} was measured for GH78 produced in the *Sf*21 cell lysate-based CECF system, indicating that a higher ratio of active enzyme was synthesized. However, a reduced absolute amount of active GH78 was produced in *Sf*21-based CFPS compared to CHO and WG CFPS. Reduced enzyme production rates most likely promoted the generation of a higher percentage of correctly folded, active enzyme [35]. Additional factors that may have affected the kinetic measurement are the components in the lysates that could have an influence to *p*-NPRP conversion during the activity assay. Wild-type GH78 produced with *X. polymorpha* showed previously more than twice as high catalytic efficiency (k_{cat}/K_m =34 mM/s) [5] as cell-free produced GH78 but also required several days for production and was purified before kinetic experiments were performed. It should be noted, that cell-free produced GH78 may display different post-translational modifications, e.g. the lack of complete glycosylation in comparison to the wild-type protein and therefore could behave different in the *p*-NPRP assay. While glycosylation is not crucial for the catalytic properties of an enzyme, it can alter its conformation and therefore its function and specific activities [36,37]. The absence of sugars on the enzymes' surface can have both stimulating and inhibiting effects on the specific activities as described e.g. for proteases [38] and other fungal hydrolases such as esterases [39].

Non-glycosylated, purified cell-free produced GH78 (CHO CFPS) showed a higher rhamnosidase activity compared to its glycosylated wild-type counterpart. On the other hand, the esterase activity typically found in w*Xpo*GH78 was lost. Similar to the different migration of the protein in the native gel (appendix Figure A. 8), these altered substrate specificities may be explained by glycosylation-induced conformation changes [38] as well as protein dynamic changes [37,40], promoting or inhibiting substrate binding and product release. In this case, the lack of glycosylation seems to enable the purified cell-free produced enzyme to perform efficient conversion of rhamnosidase substrates, but the hydrolyzation of esters like methylferulate was not accomplished. Similar effects regarding altered functionality have been observed when expressing eukaryotic proteins in bacteria [41]. *Sf*21 and CHO CFPS would be able to perform core glycosylation in their inherent microsomes [10], however, the addition of a signal sequence for translocation has led to a significant reduced enzyme activity for GH78, further optimization to produce a functional protein containing a suitable signal-sequence is required.

High yield CECF based GH78 synthesis enabled immobilization and purification of the synthesized enzyme, besides screening tests, and kinetic and structural analyses. FLAG-Tag binding, which is one of the most straightforward immobilization methods [42,43], was not only used for purification but also for immobilization of the enzyme in order to monitor its activity in a bound state. This lays the foundation for future applications such as protein arrays, enzyme purification and the development of enzyme reactors. In general, enzyme immobilization is of increasing interest, e.g. for industrial and medical applications, especially if it can be performed directly after protein synthesis thereby avoiding cell-lysis procedures. Particular advantages include easy recovery of enzymes and products, reusability of enzymes and continuous operation of enzymatic processes [44]. By repeated substrate incubation of the immobilized GH78 in a small reactor, the enzyme was reusable after performing its catalytic function. An increased stability of immobilized enzymes has been repeatedly described and may generally improve their reaction performance (e.g. in [45]). The decrease of GH78 activity after the first repeats was probably caused by partial denaturation of the enzyme leading to diminished catalytic activity. Reaction conditions could be further adapted and improved to increase the stability of the immobilized enzyme. Furthermore, purified GH78 in higher amounts should be studied in structural studies (e.g. by X-ray crystallography).

The importance of rhamnosidases and other fungal enzymes for chemical and medical industry is remarkable [3]. The spectrum of potential applications might be even improved through site-directed mutagenesis and enzyme engineering. In this context CFPS can play a role in screening processes. In addition to the template changes shown here, the effects of modifications to the active site or substrate access sites can be analyzed on chosen enzymes. Rational design and enzyme evolution for the increase of specific catalytic efficiencies or extension of the enzymatic functionalities are generally imaginable using high throughput methods [21,46,47]. CFPS enables straightforward synthesis using various modified templates in parallel and thereby allowing for rapid enzymatic characterization of the produced mutated enzyme.

5. Conclusion

Our results show that CFPS based on eukaryotic cell-lysates is a highly efficient method for the synthesis of active fungal proteins thereby improving the performance of different test procedures. Using GH78 from *X. polymorpha,* various reaction conditions could be screened exemplarily in cell-free systems. This brings up a broad band of experimental opportunities that will not only lead to deeper insights into the nature of the GH78 and related hydrolase proteins as such but will also improve the application field in an industrial context. Furthermore, the gained knowledge may be adopted tor other (fungal) enzymes that are even more difficult to synthesize. Thus, adequately optimized reaction conditions may bear the fundament for the successful synthesis of active fungal oxidoreductases e.g. the industrially relevant fungal peroxidases and peroxygenases (UPOs). Immobilization methods as exemplarily pointed out here for GH78, may also be applicable to reaction conditions of other (fungal) proteins. In the near future, high throughput screenings of fungal enzyme mutants will be predestined as subject of eukaryotic

cell-free systems including approaches of targeted enzyme engineering.

CRediT authorship contribution statement

Jan Felix Knauer: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft. **Christiane Liers:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Stephanie Hahn:** Formal analysis, Investigation. **Doreen A. Wuestenhagen:** Resources. **Anne Zemella:** Supervision, Writing – review & editing. **Harald Kellner:** Conceptualization. **Lisa Haueis:** Methodology. **Martin Hofrichter:** Writing – review & editing, Resources. **Stefan Kubick:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.enzmictec.2022.110110.

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