Energimyndighetens titel på projektet – svenska Att konstruera förbättrad stabilitet och substratbindning i enzymer för effektiv hydrolys eller lignocellullosisk biomassa	
Energimyndighetens titel på projektet – engelska Engineering improved stability and substrate binding into enzymes for efficient hydrolysis of lignocellullosic biomass	
Universitet/högskola/företag Kungliga Tekniska Högskolan	Avdelning/institution Avdelning för glykovetenskap; Institution för kemi
Adress AlbaNova University Centre	
Namn på projektledare Lauren Sara McKee	
Namn på ev övriga projektdeltagare He Li: post-doctoral scientist Johan Larsbrink: collaborator Scott Mazurkewich: collaborator Francisco Vilaplana: advisor on some analytical techniques Erik Estreen, Rasmus Gustafsson, Vasiliki Makrygianni, Lova Sandin, Irem Ergenlioglu: Master's thesis project students	
Nyckelord: 5-7 st Biomass. Biorefinery. Enzymes. Non-catalytic domains. Thermal stability.	

Förord

This research was financed almost entirely by this award from Energimyndigheten. When Master's thesis students were involved in the work, there was some support from KTH in the form of partial salary compensation for the time spent on supervision. During the project, post-doctoral scientist Dr He Li received an award from ÅForsk foundation that enabled the purchase of an new ÄKTA-brand fat protein liquid chromatography (FPLC) machine that made much of our work in protein production more efficient and reduce dour reliance on external consultants for protein production.

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Sammanfattning

I denna slutliga projektrapport sammanfattar jag de framsteg som gjorts under 5 år i detta projekt finansierat av Energimyndigheten. I samband med försockring av biomassa för energigenerering är många av de flaskhalsar som påverkar tid, energi och resursförbrukning relaterad till de höga kostnaderna och låga hållbarheten för hydrolytiska enzymer. Vårt mål var att få sådana enzymer att fungera bättre och arbeta längre genom att öka deras värmestabilitet, vilket tenderar att ge en övergripande förbättring av den allmänna processstabiliteten på samma gång. Vi har gjort stora framsteg när det gäller att förstå en termostabiliseringseffekt som vi var de första att observera, genom att använda molekylärbiologi, enzymbiokemi och olika strukturbiologiska tekniker för att undersöka aktiviteten, effektiviteten, interaktionerna och värmetoleransen hos 20 nya enzymer. Vissa förseningar i projektgenomförandet på grund av Covid-19-pandemin, och en längre lista av enzymkandidater än vi från början förväntade oss att hitta, har gjort att vi inte nådde alla våra mål med att konstruera nya enzymer redo för användning i industrin. Ny finansiering har dock säkrats från Vetenskapsrådet som ska stödja rekryteringen av en doktorand för att genomföra detta arbete. Vi har publicerat flera artiklar, skriver just nu ännu fler och har handlet flera framgångsrika examensarbeten under denna tid, vilket visar att forskningen också bidragit till kunskapsutveckling och utbildning i Sverige.

Summary

In this final project report, I summarise the progress made over 5 years in this project funded by the Swedish Energy Agency. In the context of biomass saccharification for energy generation, many of the bottlenecks impacting time, energy, and resource consumption relate to the high cost and low durability of hydrolytic enzymes. Our goal was to make such enzymes work better and work for longer by increasing their heat stability, which tends to give an overall improvement in general process stability at the same time. We have made great progress in understanding a thermostabilisation effect that we were the first to observe, using molecular biology, enzyme biochemistry, and diverse structural biology techniques to probe the activity, efficacy, interactions, and heat-tolerance of 20 novel enzymes. Some delays in project implementation due to the Covid-19 pandemic, and a longer list of enzyme candidates than we initially expected to find, have meant that we did not reach all of our goals in engineering new enzymes ready for deployment in industry. However, new funding has been secured from the Swedish Research Council that will support the recruitment of a PhD student to complete this work. We have published several papers, are currently writing even more, and have supervised multiple successful Master's thesis projects during this time, showing that the research also contributed to knowledge development and education in Sweden.

Inledning/Bakgrund

The efficient production of biofuels from lignocellulosic biomass is dependent on the effective hydrolysis of plant biomass, to fully fractionate the plant cell wall.

Enzymatic hydrolysis is the most sustainable route to hydrolysis due to the low energy consumption and limited volumes of hazardous waste generated compared to chemical approaches. However, due to low catalytic efficiency, enzyme loading requirements are very high, such that the cost of hydrolytic enzymes can account for around 15% of the final price of bioethanol. Large numbers of complementary enzymes are required for effective biomass deconstruction, so even small improvements in enzyme stability or efficiency can lead to significant cost savings. The switch to a fully bio-based economy requires a fossil-free route to fuel production that is economically viable, so any cost savings in production can have a broad impact.
Most enzyme mixtures used for biomass deconstruction today are derived from industrial microbes that secrete cocktails of enzymes and other proteins. These enzyme mixtures tend to be poorly acting at the high temperatures that are often used in industry. In my research group, we discovered that a previously totally uncharacterised protein domain (Bacterial Fascin-like Domains, BFLDs) can bind polysaccharides to promote enzyme attachment to substrates and at the same time drastically enhance the thermostability of enzymes. Our goals with the project were to characterise a large number of enzymes that natural contain BFLDs in order to understand the thermal stabilisation phenomena, and then to use the knowledge to engineer biofuel-relevant enzymes with high heat tolerance.

The project leader Dr Lauren McKee was appointed to the position of Associate Professor (Lektor) in Cell Wall Biochemistry in late 2021, and worked on this project throughout its duration, identifying enzyme targets for characterisation, managing collaborations at KTH and Chalmers University, and recruiting new team members. She was joined in the biochemical research by senior post-doctoral fellow at KTH Dr He Li, who led most of the lab work, and whose salary was paid by this project for almost 3 years. Dr Li and Assoc Prof McKee co-supervised several Master's thesis students who each characterised 1-2 examples of relevant heat-stable enzymes. While some targets are still under characterisation, others have been fully studied, and some have now been published. In collaboration with Assoc Prof Johan Larsbrink and Dr Scott Mazurkewich at Chalmers, structural analysis by X-ray crystallography and small angle X-ray scattering (SAXS) have been performed, giving valuable initial insights into the inter-domain interactions that underpin thermostabilisation of enzymes by BFLDs. Moreover, thanks to our extensive research, the BFLD "domains of unknown function" have now been re-classified as family 92 carbohydrate binding modules (CBM92) on the CAZy database (https://www.cazy.org/CBM92.html).

Genomförande

Work package 1: discovery of BFLD-enzyme targets by bioinformatic screening; cloning and expression of select targets. Exceeding our targets, we discovered several hundred novel enzymes that contain BFLD/CBM92 domains, many of which were predicted to have activities with high relevance for the biofuel and biorefinery industries.

Work package 2: characterisation of enzyme activity and heat tolerance; comparison of efficacy and heat tolerance in enzymes produced with and without the BFLD. We first developed a consistent workflow to characterise the carbohydrate-degrading activity, thermostability, and more general process stability of enzymes in an efficient manner.

Work package 3: assessment of the polysaccharide-binding preferences of the BFLDs. We used pull-down assays to explore binding of BFLDs to diverse polysaccharides and biomass extracts.

Work package 4: assessment of enzyme-BFLD activity on complex biomass in industrial conditions. High substrate concentrations and high temperatures were used in assays testing enzyme-BFLD pairs for hydrolysis of biomass (sugarcane bagasse, spruce wood chips, agricultural mushroom residues).

Resultat

Work package 1: After narrowing down the list of candidates to ensure a representative assessment of their diversity, a total of 20 distinct BFLD-containing enzymes could be recombinantly produced and purified. Each was successfully produced in variant forms both with and without the BFLD, in order to assess its impact on enzyme activity.

Work package 2: We found that the BFLDs contributed a 10-30°C increase in enzyme thermostability. By collaborating with the start-up company EnginZyme, we were able to look more closely at the dynamics of enzyme behaviour at high temperatures, and found that the BFLDs unfold first when exposed to high temperature, delaying the denaturing of the enzyme domains by significant periods of time (manuscript in preparation). In many, but not all, cases, the removal of the BFLD led to a reduction in enzyme activity rate. This depended on the ligand binding preference of the BFLD.

Work package 3: In some cases, we found that the BFLDs bound to the polysaccharide that was the substrate of their enzyme partner – the removal of these domains led to a sharp drop in enzyme activity. However, in the majority of cases we examined, there was no loss of activity when the BFLD was removed, as the accessory domain interacted with a polysaccharide that was not the same as the enzyme's substrate. This striking observation led us to explore the role of BFLD's in attachment to complex intact biomass substrates, an unexpected avenue for the project. We are currently working with microscopists in France and the USA to explore this phenomenon, which might permit the rational design of enzyme cocktails with high activity on complex substrates. It was not possible within the project's timeframe and budget to design new engineered combinations of enzyme-BFLD pairs as was originally planned. This was partly due to the larger-than-expected number of enzyme candidates to explore but was also largely connected to Covid19-related delays in recruiting the post-doctoral fellow and difficulties during that time in getting regular safe access to the needed research facilities at KTH.

Work package 4: This work is ongoing in collaboration with researchers in Sweden, the USA, and France. In addition, follow-on project financing has been secured from Vetenskapsrådet to recruit a PhD student to work on this topic. Preliminary tests performed by Dr He Li during this funded project were promising but need to be repeated and scaled up before a publication can be prepared.

Diskussion

We have made tremendous progress in understanding the roles of BFLDs in controlling the specificity, efficacy, and thermostability of the enzymes to which they are appended. Through the papers and theses already completed, and those still in preparation, we have established that the now-named CBM92 domains coordinate enzyme attachment to complex substrates, which is key for developing processes for enzymes to act directly on biomass with minimal pre-treatment, thus reducing costs and process time for working with biomass in the energy context. Moreover, we have come a lot closer to understanding the thermostabilisation effect. Although it is not seen in every case of a BFLD/CBM92 pair, we are now better able to predict when the effect will be seen. In the coming years, with support for a PhD student from Vetenskapsrådet, we will complete our structural analysis of these proteins, finally understanding the inter-domain interactions that underpin thermostabilisation, and letting us design new enzymes with high temperature tolerance. We plan to develop cloning plasmids that carry BFLD/CBM92 domains so that other researchers in industry or academia can generate heat-tolerant variants of their own enzymes, while we continue to showcase examples of enzymes with surprisingly high innate heat-resistance. As stated in the original project proposal, these new technologies will permit savings in the biomass saccharification process and help promote the economic sustainability of biomass utilisation in Sweden and beyond.

Publikationslista

Published papers:

Family 92 carbohydrate-binding modules specific for β-1,6-glucans increase the thermostability of a bacterial chitinase. Li H, Hao M, Kvammen A, Inman AR, Srivastava V, Bulone V, **McKee LS*.** *Biochimie* 212 (2023) 153-160 *corresponding

Structural and biochemical analysis of family 92 carbohydrate-binding modules uncovers multivalent binding to β-glucans. Hao M-S, Mazurkewich S, Li H, Kvammen A, Saha S, Koskela S, Inman AR, Nakajima M, Tanaka N, Nakai H, Brändén G, Bulone V, Larsbrink J, **McKee LS***. *Nature Communications* 15 (2024) 3429 *corresponding

Multiple enzymatic approaches to hydrolysis of fungal β-glucans by the soil bacterium *Chitinophaga pinensis.* Lu Z, Rämgård C, Ergenlioglu I, Sandin L, Hammar H, Andersson H, King K, Inman AR, Hao M, Bulone V, **McKee LS***. *The FEBS Journal* 290 (2023) 2909-2922 *corresponding

Published Master's theses:

The role of inter-domain linkers in the stability of modular Glycoside Hydrolases. Erik Estreen, KTH, 2024.

Exploring the diversity of family GH16 enzymes appended to family CBM92 carbohydrate binding modules. Rasmus Gustafsson, KTH, 2023.

Exploring the impact Carbohydrate Binding Modules (CBMs) have on Glycoside Hydrolases (GHs) in regard to enzymatic properties and thermostability. Vasiliki Makrygianni, KTH, 2022.

Exploring the influence of a new CBM family on the thermostability of polysaccharide-degrading enzymes. Lova Sandin, KTH, 2022.

Discovery and Characterization of a Novel Bacterial β-1,6 Glucanase from the Soil Bacterium *Chitinophaga pinensis*. Irem Ergenlioglu, KTH, 2020.

Manuscripts in preparation:

Beyond saccharification: The importance of enzymes in a wood materials biorefinery. **McKee LS**, Lawoko M, Vilaplana F, Olsson L, Larsbrink J.

A diversity of carbohydrate-degrading activities rely on CBM92 accessory domains for stability to high temperatures. Li H, Sandin L, Makrygianni V, Gustafsson R, **McKee LS**.

Fungal Cell Walls: the rising importance of carbohydrate-active enzymes. Yao R, Berrin J-G, **McKee LS**, Bisarro B.

Carbohydrate binding modules with the beta-trefoil structure: Diverse roles in carbohydrate cross-linking and enzyme stabilisation. Mazurkewich S, Larsbrink J, **McKee LS**.

Referenser, källor

Bilagor

Administrative final report is also attached.