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Rene Lametsch/Fergal P. Rattray:

Plasmin-fri mejeri: Kromatografisk teknologi til fjernelse af plasminaktivitet fra mejeriprodukter

Plasmin-free dairy; chromatographic technology for the removal of plasmin activity from dairy products.

Mejeribrugets
ForskningsFond

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Final report

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1. Title of the project

Dansk: Plasmin-fri mejeri: Kromatografisk teknologi til fjernelse af plasminaktivitet fra mejeriprodukter
English: Plasmin-free dairy; chromatographic technology for the removal of plasmin activity from dairy products.

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4. Sources of funding

Milk Levy Fund and Arla Foods Ingredients

5. Project period

Project period with DDRF funding: January 2018-August 2022.

6. Project summary

Dansk:

Plasminogen, den zymogene form af plasmin, er en serinprotease, der overføres fra blodet til mælken og er ansvarlig for hydrolysen af kaseiner i mejeriprodukter. Plasminaktivitet kan være skadelig eller gavnlig for det enkelte mejeriprodukt. Effektiv kontrol af plasminaktivitet er derfor nødvendigt for at forbedre mejeriprodukternes funktionalitet og kvalitet. Det oprindelige formål med dette projekt var at udvikle en affinitetskromatografisk oprensningsmetode, der kunne bruges til at fjerne plasminaktivitet i mejeriprodukter. Det var intentionen, at metoden skulle bruges til at opnå fuld kontrol over plasminaktivitet i mælk og mejeriprodukter. Under arbejdet med den kromatografiske oprensning af plasminaktivitet fra sur valle, opdagede vi, at plasminaktiviteten blev opdelt i en bunden fraktion, en ikke-bunden fraktion og en tabt-aktivitets fraktion, hvilket svarede til hhv. 25, 50 og 25 % af den totale plasminaktivitet. Den ikke-bundne fraktion indeholdt de lateralt producerede plasminfragmenter: midi-, mini og mikro-plasmin. Derudover indeholdt den bundne, koncentrerede fraktion et protein med høj molekylvægt, der var større end fuld-længde plasmin. Proteinet med høj molekylvægt udviste plasminlignende aktivitet og så ud til at være et kompleks eller en forbindelse dannet mellem plasmin og immunoglobulin. Dette indikerer, at den observerede plasminaktivitet var meget kompleks. På grund af den observerede kompleksitet besluttede vi at studere disse forskellige plasminvarianter i yderligere detaljer og deres hydrolyse af kaseiner. Vi udviklede derfor *P. pastoris* produktionsprotokoller til rekombinant produktion af de forskellige plasminvarianter, fuld længde-, midi-, mini- og mikroplasmin. Vi oprensede disse for at analysere for forskelle i kaseinhydrolyse udført af plasminvarianterne ved brug af LC-MS/MS. Udviklingen af forskellige markørpeptider og deres peptidfragmenter fra hydrolysen af forskellige kaseiner (αS1-, αS2-, og β-kasein), afslørede, at der var en forskel i specificitet, men også i hastigheden af hydrolysen udført af de forskellige plasminvarianter.

Et lagringsforsøg med micellar kasein isolater (MCI) samt spraytørret MCI viste, at der ikke var nogen plasminaktivitet i det spraytørrede MCI-pulver, og der var meget lidt nedbrydning af kasein, hvilket indikerer, at plasmin og plasminogen bliver inaktiveret under spraytørringen. I MCI blev der fundet stor aktivitet af plasmin og høj aktivering af plasminogen under lagring, hvilket medfører en signifikant nedbrydning af kasein. Midi-plasmin var den mest dominerende form af plasmin, og den var mere stabil end den native plasminform ved stigende temperatur. Resultaterne indikerer at plasmininhibitorerne bliver fjernet under mikrofiltreringen sammen med β-lactoglobulin og derved øges aktiviteten af plasmin i MCI.

English:

Plasminogen, the zymogen form of plasmin, is a serine protease that is transferred from blood to the milk and is responsible for the hydrolysis of casein in dairy products. Activity from plasmin can be detrimental or beneficial to an individual dairy product. Efficient control of plasmin activity is therefore required for the improvement of the functionality and quality of dairy products. The initial aim of this project was to develop an affinity chromatographic removal approach that could be used to remove plasmin activity from milk. It was believed that this could be used to obtain full control of plasmin activity in dairy products. However, during the work with the chromatographic removal of plasmin activity from acid whey it was discovered that plasmin activity was divided into a bound-fraction, non-bound fraction, and lost activity fraction, which corresponded to 25, 50, and 25 % of the total plasmin activity, respectively. The non-bound fraction contained the lateral generated plasmin-fragments: midi-, mini-, and micro-plasmin. Furthermore, the bound concentrated fraction contained a high molecular weight protein, that was larger than that of full-length plasmin. This high molecular weight protein exhibited plasmin-like activity and were indicated to be a complex or an association formed between plasmin and immunoglobulin. This indicates that the observed plasmin activity was more complex than expected. Due to this observed complexity, we decided to study the different sized plasmin variants and their hydrolysis of casein in detail. Therefore, novel *P. pastoris* production protocols for the recombinant production of the different plasmin variants, full-length-, midi-, mini-, and micro-plasmin were developed. Difference

in casein hydrolysis by the plasmin variants was studied using LC-MS/MS. Different marker peptides and their fragments from the hydrolysis of casein (αS1-, αS2-, and β-caseins), revealed that there was a difference in specificity, but also in the rate of the hydrolysis by the different plasmin variants.

A storage experiment of casein with micellar casein and spray dried MCI showed that there was no plasmin activity in the spray dried MCI powder and limited degradation of casein. In MCI, large activity of plasmin was found leading to a significant degradation of casein. Midi-plasmin was the dominating form of plasmin and was more stable than the native form of plasmin. The results indicate that the plasmin inhibitors are removed during micro filtration together with β-lactoglobulin resulting in a high activity of plasmin in MCI.

7. Project aim

Dansk:

Formålet med projektet er som følger:

(i) at udvikle en ny mejerienhedsoperation baseret på affinitetskromatografi til fremstilling af "plasmin-fri" mejeriprodukter.

(ii) at anvende den nye mejerienheds drift til produktion af mælk med forlænget holdbarhed.

(iii) at anvende den nye mejerienheds drift til produktion af kaseinkoncentrater/-isolater med forbedret funktionalitet.

(iv) at anvende den nye mejerienhedsoperation til produktion af valleproteinkoncentrater/-isolater med forbedret funktionalitet.

English:

The objectives of the project are as follows:

(i) to develop a new dairy unit operation based on affinity chromatography to produce “plasmin-free” dairy products.

(ii) to apply the new dairy unit operation for the production of liquid milk with extended shelf-life.

(iii) to apply the new dairy unit operation for the production of casein concentrates/isolates with improved functionality.

(iv) to apply the new dairy unit operation for the production of whey concentrates/isolates with improved functionality.

8. Background for the project

The dairy industry produces among other milk, whey protein concentrates and isolates that have a high protein level and are further processed into other products. The inherent enzyme, plasmin, is known to degrade casein in dairy products, resulting in undesired effects such as reduced shelf life, quality deterioration, gelling and development of off-flavors.

The project is centered around removal of plasmin from the dairy products using affinity chromatography and other chromatographic methods. Focus is on developing a process that is scalable and may be applied in industrial settings. The process has two main benefits: It will reduce the plasminogen/plasmin activity in the whey or milk, and it enables recovery of plasminogen/plasmin in high concentrations, which may subsequently be used in the production of other dairy products, where degradation of caseins is desirable.

Affinity chromatography is a relatively well-known technology in the bio-pharmaceutical industry, which may be easily transferred to an increasingly sophisticated dairy industry. Affinity chromatography separates proteins by means of a reversable interaction between a protein (in this case plasmin) and a specific biding molecule. The technique is extremely selective resulting in high purification levels. A purification process that would be tedious and time consuming – even impossible – using other techniques, may be obtained using affinity chromatography.

The focus of the project is to screen, design, and develop affinity chromatography for removal of plasmin (and its components) from milk.

Pilot plant and subsequent full-scale experiments (using long shelf-life dairy products and whey protein concentrates/isolates) will be carried out to transform and adjust the lab scale results to actual production settings at the dairies.

9. Sub-activities in the entire project period

The main objective with the project is to investigate following three questions:

(i) Is it possible to fully control plasmin activity in UHT liquid milk, casein concentrates/isolates and whey protein casein concentrates/isolates?

(ii) Are there any new emerging technologies that can be applied to remove plasmin activity from milk?

(iii) To what extent can product quality be improved, and shelf-life extended beyond what is currently possible?

This project is proposing to answer these questions by applying an *affinity chromatography* approach to selectively remove plasmin and its components (plasminogen, plasminogen activator inhibitor, plasmin activator and plasmin inhibitor) from milk, and thus create plasmin-free milk.

The removal of plasmin from milk is illustrated in Figure 1. The process consists of pumping the milk (or whey) through a separation column which is packed with the highly selective affinity media. The affinity media captures and removes the plasmin its components (plasminogen, plasminogen activator inhibitor, plasmin activator and plasmin inhibitor) from the milk. The technology is gentle and does not affect the milk proteins in any negative way.

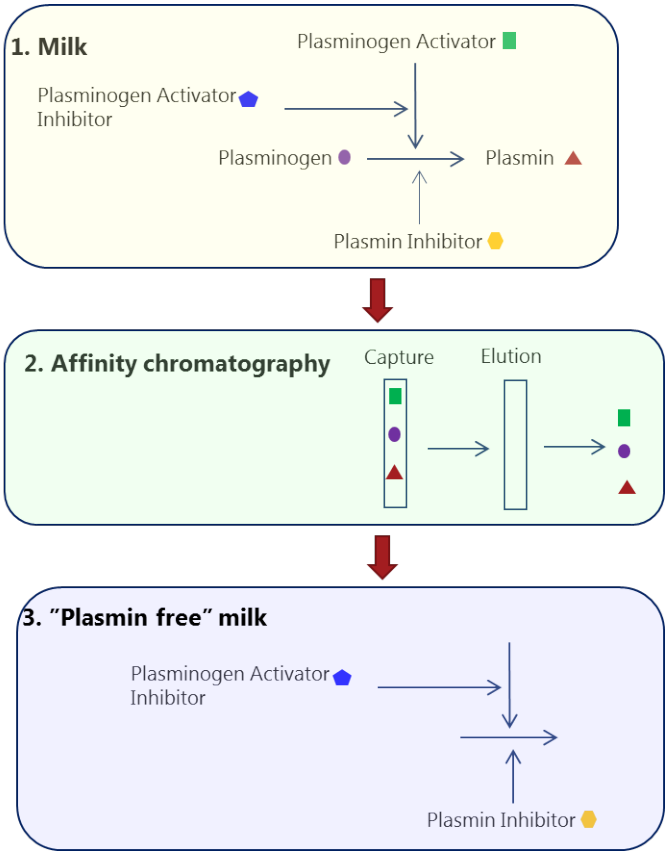

Figure 1. Use of affinity chromatography to produce **plasmin-free** milk. **1.** Milk or whey feed. **2.** Affinity chromatography for capture of Plasmin ▲, Plasminogen ● and Plasminogen activator ■**3.** Milk or whey with plasmin activity removed.

The main activities are divided into work packages (WP) from and listed below:

<u>WP1. Screening commercial affinity chromatography media for plasmin/plasminogen capture.</u>

Responsible: FOOD-KU.

This work package comprises screening commercial affinity chromatography media for plasmin/plasminogen ▲/● capture (Figure 2). Several commercial sources based on the capture molecule, trans-4-(aminomethyl) cyclohexanecarboxylic acid (a lysine analogue) are available (for example Plasminogen Removal Gel from GE Health Care Life Sciences). Capture from milk is believed to be the most challenging due to the strong association of plasmin/plasminogen with the casein, while in contrast capture from whey should be relatively easy. It is expected that modification of pH and/or temperature of the milk and whey may be necessary to achieve maximum capture efficiency. Initial experiments will be performed as a batch process to quickly establish optimal conditions.

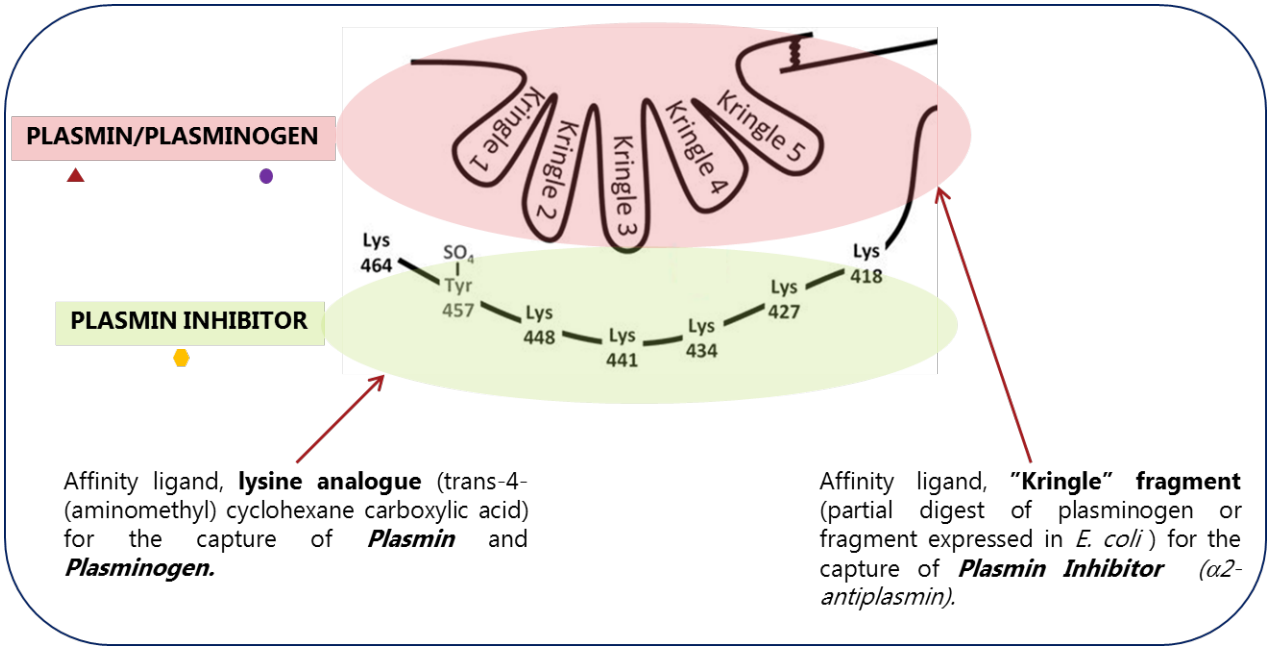

Figure 2. Principle of affinity chromatography capture. Exploiting the "Kringle-Lysine" interaction between plasmin/plasminogen ▲/● and plasmin inhibitor (αs2-antiplasmin) ⬣.

WP2. Design and development of tailored affinity chromatography media for plasmin inhibitor, plasminogen activator inhibitor and plasminogen activator capture.

Responsible: FOOD-KU

This work package is the most complex as no commercially available affinity chromatography media exists for the capture of plasmin inhibitor ⬣, plasminogen activator inhibitor ⬟ and plasminogen activator ■. However, there are detailed reports in the literature describing the experimental production of these affinity media (Figure 3). Purification of plasmin inhibitor requires the coupling of the "Kringle fragment" onto a support molecule (such as CNBr-activated Sepharose). Two possible approaches exist for the purification of the "Kringle fragment", namely (i) partial digestion of plasminogen and subsequent purification of the fragment using lysine-Sepharose and gel filtration (Wiman, 1980), and (ii) cloning of the DNA fragment encoding the "Kringle fragment" in a suitable host organism (*E.coli*) (Bian *et al*., 2013).

The capture molecule, anhydrourokinase has been described for the capture of human plasminogen activator inhibitor (Figure 3A). For the capture of plasminogen activator, the capture molecule, tripeptide GLU-GLY-ARG has been used for human plasminogen activator (Patel *et al*., 1990) (Figure 3B). It is believed that this same principle can be readily applied to a bovine dairy system. An experienced post-doctoral protein chemist will be employed for the chromatography experiments. Once all the prototype affinity chromatography methods have been established, they will be applied to milk (and whey) to capture the various plasmin components. Similar to WP1, experiments in this WP will be performed as a batch process in order to establish optimal pH and temperature conditions. Furthermore, a full cost-benefit analysis of the new technology will be performed.

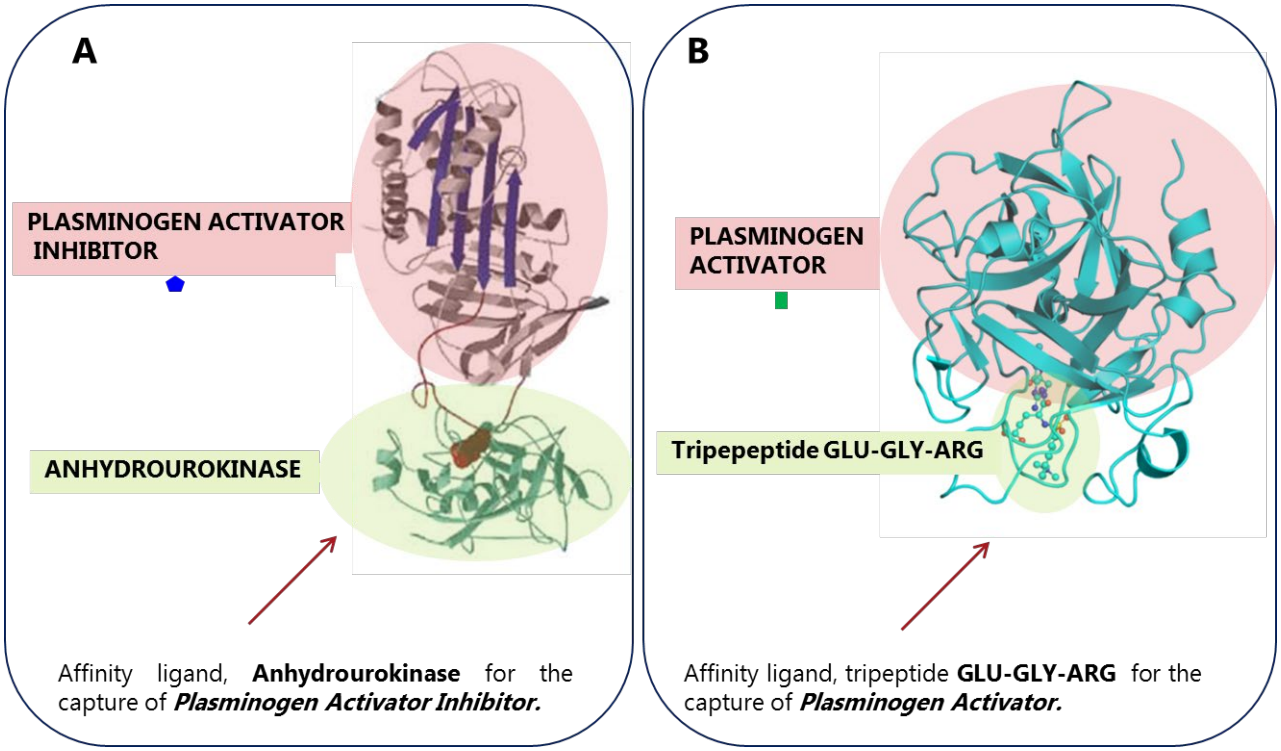

Figure 3. Principle of affinity chromatography capture. **(A)** Exploiting the "Serpin–protease" interaction between Plasminogen Activator Inhibitor ⬟ and Anhydrourokinase. **(B)** Exploiting the "arginine–enzyme active site" interaction between Plasminogen Activator ■ and tripeptide GLU-GLY-ARG (human Plasmin Activator depicted).

WP3. Development of a new dairy unit operation based on affinity chromatography.

Responsible: FOOD-KU

The affinity chromatography media developed in WP1 and WP2 will be used to produce plasmin-free milk (Figures 2 and 3). In essence, a new dairy unit operation will be developed in this WP. In view of the colloidal nature of milk, Expanded Bed Adsorption (EDA) affinity chromatography will be used to ensure that the separation column does not block and that high flow rates are possible thus ensuring an economically viable process. While for the treatment of whey, Fixed Bed (FB) affinity chromatography will be the more suitable in view of the fact that the proteins are in solution. Treatment of casein products is envisaged to be similar to that of milk.

WP4. Pilot-scale production of plasmin-free milk, casein, and whey products.

Responsible: FOOD-KU and Arla Foods

Long-life plasmin-free milk will be manufactured at pilot scale (100 liter-scale). Milk will be treated using the affinity chromatography media developed in WP1 and WP2, and with the conditions established in WP3. Long-life plasmin-free milk will be compared to that of regular UHT treated milk (control). In particular, shelf-life will be monitored using extensive biochemical and sensory analysis. Advanced LC-MS/MS analysis of the milks during storage will be conducted to establish if as expected that the level of proteolysis in the plasmin-free milk is lower than that of the regular UHT treated milk (control). It is envisaged that for the production of plasmin-free milk, that microfiltration will be used in order to remove bacterial cells and spores. Whey and casein products will also be treated to produce plasmin-free whey, and subsequently used to produce various protein powders. Plasmin-free powders will be compared against the regular powders and analyzed for benefits in terms of improved sensory properties, shelf-life and functionality.

WP5. Industrial implementation of affinity chromatography technology for production of plasmin-free milk, casein and whey products.

Responsible; FOOD-KU and Arla Foods

In the final work package, the most promising plasmin-free product(s) developed in WP 4 (milk, casein or whey products) will be performed at an industrial scale (10,000-liters batch). Scale-up of the affinity chromatography technology will require the participation of specialist suppliers of industrial scale chromatography equipment. Identified suppliers include Upfront Chromatography A/S (Denmark) and NovaSep Process SAS (France). As in the pilot scale trials in WP4, the plasmin-free product(s) will be subjected to extensive stability, biochemical and sensory analysis during storage. It is keenly anticipated that at least some of the plasmin-free products will demonstrate extended shelf-life and superior functionality compared to that of the control products.

10. Deviations

During the work with the chromatographic removal of plasmin activity from acid whey it was discovered that plasmin activity was divided into a bound-fraction, non-bound fraction, and lost activity fraction, which corresponded to 25, 50, and 25 % of the total plasmin activity, respectively. The non-bound fraction contained the lateral generated plasmin-fragments: midi-, mini-, and micro-plasmin. Furthermore, the bound concentrated fraction contained a high molecular weight protein that was larger than that of full-length plasmin. This high molecular weight protein exhibited plasmin-like activity and indicated to be a complex or an association formed between plasmin and immunoglobulin. This indicates that the observed plasmin activity was more complex than expected. Due to this observed complexity, we decided to study the different sized plasmin variants and their hydrolysis of casein in detail. These results revealed that it was not possible to control the plasmin activity in the milk by removing the plasmin with the use of affinity chromatography, which resulted in changes in WP4/WP5. The work in WP4 was investigating the development of plasmin activity during the production and storage of micellar casein isolates.

11. Project results

WP1. Screening commercial affinity chromatography ligands for plasmin/plasminogen capture.

Focus has been on development and characterization of the purification procedure for plasminogen/plasmin in acid whey. We have developed a robust system that consistently removes plasminogen/plasmin from acid whey.

The system is based on affinity chromatography and is a relatively simple system that may easily be implemented in industry. It is a novel approach when it comes to studying the plasminogen/plasmin system in dairy products. We have focused on the scalability of the affinity chromatography system in the range of 100 mL-700 mL. This looks promising and further scaling of the process is ongoing.

We have further developed a robust fluorescence-based assay that may be used to determine the plasminogen/plasmin activity for different media. The assay is applicable for measurements on milk, whey products and concentrates. The assay may also be used to determine the ratio between plasminogen and plasmin. The system is also scalable for larger volumes.

WP2. Design and development of tailored affinity chromatography ligands for plasmin inhibitor, plasminogen activator inhibitor and plasminogen activator capture.

WP2 revealed that the plasmin system is more complex than expected. We have identified proteolytic active fragments from plasmin in the acid whey. The fragments most likely result from autolytic self-cleavage of plasmin. Additional studies are required to further characterize the proteolytic fragments, called midi, mini, and micro-plasmin.

Recombinant expression and purification of the fragments has been applied to study the fragments one by one using a model organism. The results may help improve the quality of whey products and milk. Midi, mini, and micro plasmin will be available, and the effect of the enzymes will be tested on dairy products.

WP3. Development of a new dairy unit operation based on affinity chromatography.

We have characterized the removed component, which turned out to be plasminogen. The purified protein was characterized using SDS PAGE GEL, Zymogram Gels, and MALDI-MS/MS protein identification.

The results indicate that we can remove a specific protein from the acid whey without changing the composition of the remaining whey protein mix. This is important as it improves the functionality of the resulting whey products.

Scalability is extremely important for the industrial aspects of the project. We are in the process of testing the effect of increased flow rate. So far, this seems to be scalable – which is a promising result.

WP4. Development of plasmin activity during the production and storage of micellar casein isolates.

Samples from the production of micellar casein isolates (MCI) were analyzed for plasmin activity, plasminogen-related activity, autolysis of plasmin, and degradation of casein stored for 30 days at 5C, 21C, and 37C. Samples of skimmed milk, MCI (both pasteurized and non-pasteurized), and spray dried MCI powder were included in the study. No plasmin activity was found in the spray dried MCI powder. Furthermore, a limited degradation of casein was seen, indicating that plasmin and plasminogen was inactivated by the spray drying. The activity of plasmin and plasminogen was high in MCI stored at 21C and 37C, resulting in a significant degradation of casein. Midi-plasmin was the most dominant plasmin species, and it proved to be more stable than the native plasmin at elevated temperatures. The results indicate that the plasmin inhibitors are removed during the micro filtration process along with β-lactoglobulin – thereby increasing the plasmin activity in MCI.

12. The relevance of the results, including relevance for the dairy industry

While it is always difficult to predict the future direction with regard to the implementation of a new technology, it is believed that the transfer of a known technology from the biopharmaceutical industry to the dairy industry is realistically feasible. Furthermore, in the context of the Danish industry there is already a depth of knowledge available from companies such as Novozymes and Novo Nordisk where this type of technology is routinely used. The Danish dairy industry can benefit from this experience and apply it to dairy specific operations.

The project has extended our knowledge of the plasmin system in acid whey and demonstrated that the original hypothesis that plasmin could be removed from a dairy stream via lysine affinity chromatography was only partly acceptable due to a large non-binding fraction comprising autolyzed plasmin derivatives.

The hydrolysis of the (recombinantly produced) unbound fraction derivates of casein protein was also studied and gave new insights into the kinetics of casein hydrolysis by midi, micro and mini plasmin. This was very elegant basic research into the plasmin system and indeed a new higher mol weight plasmin-immunoglobulin complex in the bound fraction was also identified.

The original WPs pertaining to pilot scale chromatographic removal of plasmin from whey or milk became irrelevant as 50% of the PL activity remained unbound, hence the divergence of the project plan at this point. The project was redirected to study the plasmin system in MCI – an ingredient that is receiving increased attention these years – and where basic research is still required for the dairies to obtain a competitive edge.

13. Communication and knowledge sharing about the project

Papers in international journals:

Casper Normann Nurup, Tamás László Czárán, and Fergal P. Rattray. (2020). A chromatographic approach to understanding the plasmin-plasminogen system in acid whey International Dairy Journal, 106,104705. DOI:10.1016/j.idairyj.2020.104705

Easily read papers:

Fergal P. Rattray (2019) Plasminfri mejeriprodukter. Ny metode til fremstilling af plasminfri mejeriprodukter med forbedret holdbarhed, kvalitet og funktionalitet. Mælkeritidende 2019 (8): 10-11. https://maelkeritidende.dk/sites/default/files/udgivelser/mt_08_hoej_oploes.pdf

Student theses:

Casper Normann Nurup. 2021. New insights into the bovine Plasminogen system. PhD thesis. Department of Food Science, Faculty of Science, University of Copenhagen.

Oral presentations at scientific conferences, symposiums etc.:

Casper Normann Nurup, Science club presentations at Department of Food Science, University of Copenhagen, January 2020.

Casper Normann Nurup, Presentation at DDRF/MFF steering group meeting – Technology, 5 November 2018.

14. Contribution to master and PhD education

Casper Normann Nurup completed his PhD as part of the project.

15. New contacts/projects

None.