Real Vegan Cheese Progress Report (Draft)

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Introduction:

The Real Vegan Cheese Project (RVC) is a grassroots, non-profit research project working to produce real cheese using cellular agriculture. The project also fosters public engagement with synthetic biology. We are dedicated to Open Science and making sure the results of our research are available to the global community to enable a sustainable animal-free dairy industry.

This progress report focuses on our work to express casein proteins using *Escherichia coli*. This report does not cover work using other expression systems such as yeast.

We are engineering *Escherichia coli* to synthesize bovine casein proteins, enabling cow-less cheese production. Animal agriculture contributes to animal suffering and a variety of environmental problems, including global warming, water pollution, and deforestation. The availability of a vegan cheese that has exactly the taste and texture of cow cheeses could help reduce cow milk and cheese consumption when more people are willing to switch to a plant-based diet. Animal dairy also contains lactose, a sugar that the majority of adults globally cannot consume. It is our hope that we can synthetically produce cheeses that are more ethical, sustainable, healthy, and inclusive.

The four main proteins in cow's milk are alpha-s1, alpha-s2, beta, and kappa casein. The hydrophobic caseins aggregate into casein micelles, with the hydrophilic tail of kappa-casein pointing outwards. The assembly of the casein micelles auto-assemble naturally,
During cheese making, this hydrophilic kappa-casein tail is cleaved off by chymosin (rennet), making the micelles coagulate into cheese curds. We are in the process of purifying all four caseins and performing experiments to create a milk without using animal caseins.

Methods:

Genetic engineering and expression of caseins:

To introduce each of the four casein genes (alpha S1, alpha S2, beta and kappa casein) into *E.coli* BL21 expression strain, we used the pET vector system (pET24a+). Using this vector, the expression of the casein proteins is inducible under the control of the lac operon. The inducible lac promoter can be switched on and off and is active in the presence of the sugar IPTG. To express the caseins, the bacterial cells were grown to a start optical density (OD_{600}) of 0.6 - 0.8. To induce casein expression, we add IPTG to 1mM concentration and proteins are produced for

2 hours at 37°C. With this strategy, we were able to produce all four bovine casein proteins recombinantly in *E.coli*.

Purification of caseins:

Alpha S1, alpha S2, and kappa casein were purified via ammonium sulfate precipitation (ASP). Beta casein was insoluble, so purification from inclusion bodies and a refolding protocol were necessary. Both methods do not require a protein tag, which is why we are able to use the exact same amino acid sequences as caseins from cows. Therefore, all four caseins are suitable for further investigations in milk reconstitution experiments and cheese curd formation. Because we found higher expression in *E.coli* than in the budding yeast *Pichia pastoris* (our original production host), we have decided to move forward with *E. coli*. Consequently, our caseins are not post-translationally modified as they would be in eukaryotic expression systems like yeast. We initially expected that phosphorylation of kappa casein has an impact on micelle formation, but in our preliminary experiments, the unphosphorylated kappa casein seemed to be fully capable of supporting micelle formation..

Results:

Expression of recombinant bovine caseins:

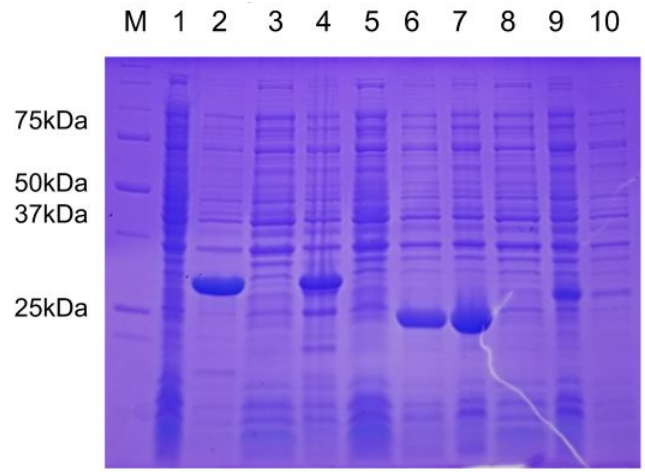

Fig. 1: M: marker; lanes 1-2: alpha S1 casein expression; lanes 3-4: alpha S2 casein expression; lane 5-7: kappa casein expression; lane 8-10: beta casein expression

Figure 1 shows protein expression of all four bovine caseins in *E. coli* before induction with IPTG and after 2 hours of induction for alpha S1 and alpha S2, and 2 as well as 4 hours for kappa and beta casein expression at 37°C. The calculated molecular weight of each of the caseins is: alpha S1 24 kDa, alpha S2 25 kDa, beta casein 24 kDa, and kappa casein 19 kDa. We noticed that all caseins run higher than expected in the SDS gel, but are in due proportion to each other.

Casein purifications:

Alpha S1 casein:

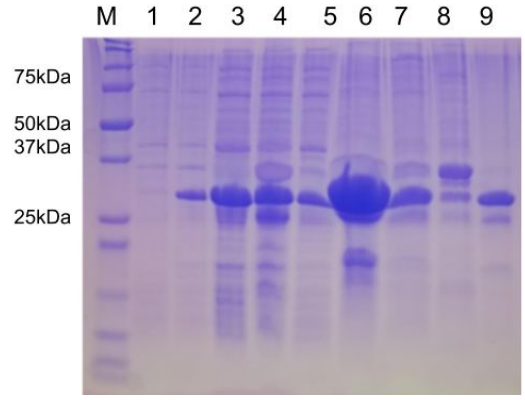

Fig. 2 : M= marker ;lanes 1-2: expression of alphaS1; lanes 3-5: lysate preparation; lanes 6-7: ASP 20-40%, lanes 8-9: concentration of alpha S1

After expression of alpha S1 casein in bacteria, we used sonication to lyse the bacterial cells and purified the protein by ammonium sulfate precipitation (ASP), as shown in Figure 2. At a concentration of 20% ammonium sulfate, alpha S1 casein precipitated, whereas most other proteins remained in solution (lane 6). This method eliminates most other proteins, but some few contaminants remained in the precipitated fraction. Alpha S1 solubilizes after stirring the pellet in salt buffer. We found that alpha S2 and kappa casein also could be purified by ASP. Kappa casein showed similar contaminants in the ASP fraction and was soluble in salt buffer after the purification procedure, whereas alpha S2 was found to be difficult to solubilize and more research needs to be done to find proper buffer conditions for this casein.

Beta casein:

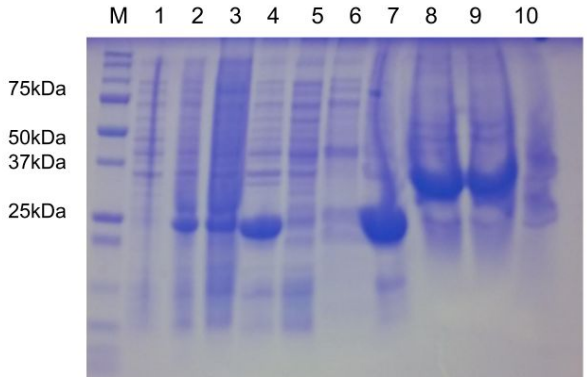

Fig. 3: M= marker; lane 1: Expression t=0; lane 2: expression t=1; 3: lysate after sonication; 4: pellet after sonication; 5: supernatant after sonication; 6: wash with triton; 7: inclusion bodies; 8-10: incubation in Gua-HCl

Since beta casein was found to be insoluble after the cell lysis and was located in the cell pellet (Figure 3, lane 4) we developed a method to purify this casein from inclusion bodies. Luckily, beta casein seems to be the most prominent protein in this fraction, as shown in Figure 3, lane 7. Lanes 8-10 show, that beta casein could be solubilized with 6M Guanidinium HCl (Gua-HCl). We were able to refold the casein after this step in a large volume of high salt buffer (1M NaCl) and concentrated the protein in low salt buffer.

Initial kappa casein milk reconstitution experiments:

After expressing our recombinant kappa casein in bacteria, we purified the protein via ammonium sulfate precipitation. At a concentration of 20% ammonium sulfate, kappa casein precipitated whereas most other proteins remained in solution. We then resuspended 32mg of this kappa casein (see Fig.4) in salt buffer and combined it with the other three caseins (alpha S1, alpha S2 and beta, purified from milk-derived casein powder from Sigma Aldrich) to reconstitute milk. All other typical milk ingredients like salts and fats (used here in form of heavy cream) were added to create a milk as close to cow milk as possible. By adding the enzyme chymosin (rennet), the milk curdled and formed a tiny mozzarella cheese (see Fig. 5). The taste was mild and not distinguishable from conventional available mozzarella cheeses. In a negative control without kappa casein, the cheese didn't curdle perfectly and was crumbly.

Fig. 4: This is how 32mg of purified and dried kappa casein look like.

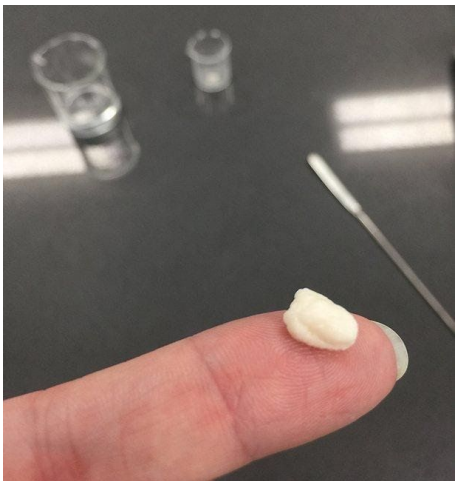

Fig. 5: First mini mozzarella made from purified kappa casein and other caseins from milk in salt buffer.

Future:

Since we were able to purify beta and alpha S1 casein successfully and in adequate amounts, we are going to test milk formation and cheese curdling with these caseins. We are going to optimize and finalize the purifications of alpha S2 and repeat the cheese forming experiments with kappa casein. In the future, we seek to replace all four caseins with our vegan protein variants and create a mozzarella cheese that we can refine and age using a set of lactic acid bacteria.