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Trends & Developments in BioProcess Technology

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Development of Pregnancy-Associated Malaria Vaccine Using the ExpreS² Insect Cell Expression System

By CHARLOTTE DYRING

There are an estimated 225 million cases of malaria each year leading to nearly a million deaths, mostly in children under five years of age.[1] Of the five malaria parasite species that can infect humans *Plasmodium falciparum* is by far the most virulent, causing 89% of all malaria related deaths in 2008.[1] The *P. falciparum* parasite escapes the immune system by inserting antigens, functioning as adhesions, into the membrane of the infected erythrocyte. This allows it to sequester in the host microvasculature, thereby avoiding clearance in the spleen (Figure 1).

The binding is mediated by a family of antigens called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by var genes of which there are about 60 in each genome. Protective antibodies block the binding between the infected red blood cells and receptors on vascular endothelium. People living in endemic regions develop partial immunity to *P. falciparum* infection as a function of age.[2]

Women, who during childhood have developed immunity against malaria, are susceptible to malaria during first pregnancies. Parasites accumulate in the placenta, where a combination of altered blood flow and expression of chondroitin sulphate A (CSA) provides a new niche for parasites to sequester.[3] Constituting a major health problem in areas south of the Sahara, malaria in pregnant women manifests as death, anemia in the mother, impaired fetal development, low birth weight or spontaneous abortion. Fortunately, women can acquire immunity against pregnancy-associated malaria (PAM).[4] The average birth weight is significantly higher among second and third time childbirth than at first childbirth. This relatively fast development of immunity suggests that a vaccine to protect against pregnancy malaria can be developed.

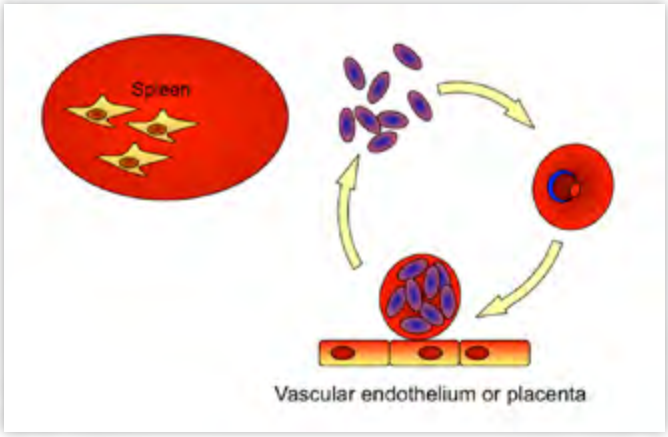

FIGURE 1. The multiple different disease forms are influenced by the expression of antigens on the surface of the infected red blood cells that enable the parasite to sequester to the vascular lining. Infected red blood cells are morphologically different from uninfected red blood cells and would, under normal circumstances, be filtered through the spleen where they would be cleared from the bloodstream.

In 2003, Centre for Medical Parasitology (CMP) at University of Copenhagen, Denmark, identified the malaria protein, which is responsible for parasite binding to CSA in the placenta. It was initially shown that only one gene, named var2csa, was highly expressed in only CSA-selected parasites.[5] Expression studies have since shown that parasites isolated from infected placenta express VAR2CSA in high quantities, and that this gene family is uniquely conserved.[6]

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VAR2CSA interacts with an unusually low-sulfated form of CSA, attached to proteoglycans in the intervillous spaces of the placenta.[7-10] Immunity to placental malaria is acquired over multiple pregnancies.[11] Evidence suggests that VAR2CSA is the main target of this protective immunity.[4] High levels of anti-VAR2CSA antibodies are correlated with better pregnancy outcomes, such as higher birth weight and protection against maternal anemia (Figure 2).[4] This, and the fact that VAR2CSA is unusually well-conserved (75–83% amino acid identity between variants[12]), suggests that it should be possible to obtain a VAR2CSA-based vaccine against PAM. The aim of the vaccine is to induce antibodies that can hinder parasite adhesion in the placenta and mediate circulation followed by destruction of infected red blood cells in the spleen (Figure 3).

Problems in vaccine development arise from the large size (350 kDa) and complex multi-domain structure of VAR2CSA (Figure 4) along with genetic variability among isolates. This complicates current large-scale production strategies. The focus in vaccine development is therefore to produce a small fragment of VAR2CSA that has the ability to induce functional cross-inhibitory antibodies.[13, 14]

In 2009, CMP demonstrated that the high CSA binding-affinity of VAR2CSA is retained in several shorter fragments of VAR2CSA and that DBL2X, including small regions from the flanking inter-domains, forms a

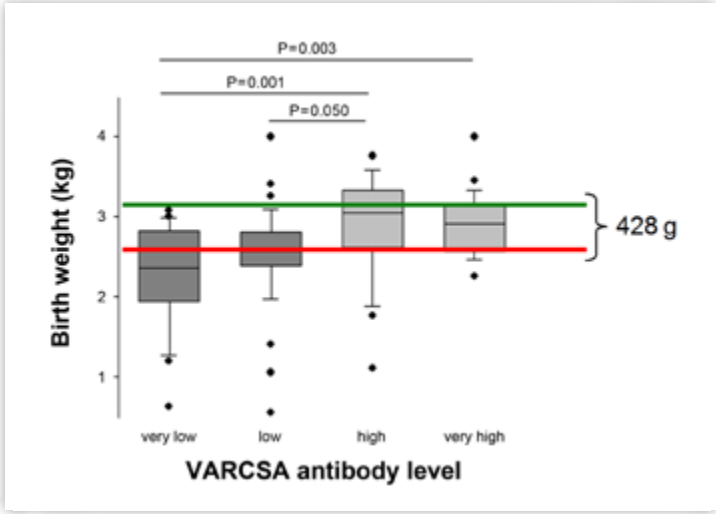

FIGURE 2. Birth weight as a function of VAR2CSA antibody levels in the women. Red indicates the median on "very low" and green indicates the median on "very high." High levels of anti-VAR2CSA antibodies are correlated with higher birth weight.[11]

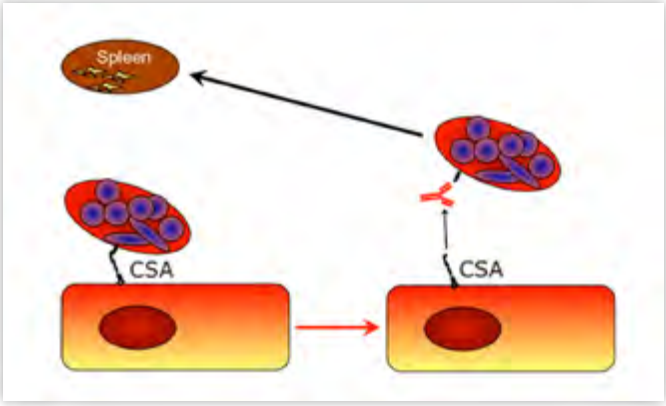

FIGURE 3. The aim of a vaccine is to induce antibodies that can hinder parasite adhesion in the placenta and mediate circulation followed by destruction of infected red blood cells in the spleen.

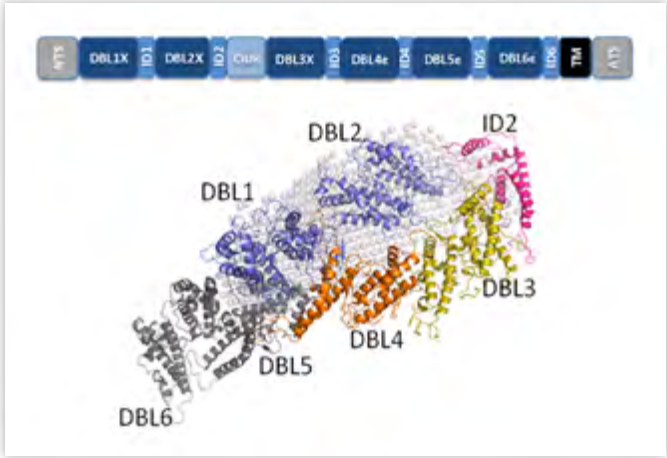

FIGURE 4. Schematic representation of VAR2CSA domains (top) and a var2csa structural model (bottom).[15]

compact core that contains the high-affinity CSA binding site (Figure 5).[15] CMP identified a subunit of VAR2CSA that induces the antibodies by inhibiting parasite binding to the placenta. This protein can form the active principle in a vaccine inducing antibodies blocking the binding of parasites in the placenta. VAR2CSA has, through a Gates foundation-funded project, been produced in a number of different expression systems, and production in baculovirus-infected insect cells (baculovirus expression vector system [BEVS]) clearly outperformed other expression systems evaluated at that time. Unfortunately, this production platform has significant cost disadvantages because of low VAR2CSA yields along with the added complexity of working with a virus (*e.g.*, heterogeneity of the product and inconsistency between batches).

The Danish biotech company, ExpreS^{2}ion Biotechnologies, has developed and optimized a virus-free insect cell expression system, ExpreS^2, based on *Drosophila* Schneider-2 (S2) cells.[16] The ExpreS^2 system replaces the BEVS system: (a) to express truncation variants of VAR2CSA for development of the first pregnancy malaria vaccine candidate; and (b) in performing the first clinical studies in humans in a collaboration under the Danish National Advanced Technology Foundation together with CMP and CMC Biologics, Denmark. S2 cells have been in use for several decades to generate stable cell lines for recombinant protein production. They have proved to be very effective for the production of a broad variety of protein classes such as viral proteins, toxins, membrane proteins, and enzymes (Table 1).[17, 18] They express heterologous proteins at high levels—these proteins are authentically processed and are biologically active. Recombinant proteins made in S2 cells have been evaluated in clinical trials, and the S2 expression system has been approved by the FDA and European authorities for production of material for human clinical trials Phase 1 and 2, so far. The strengths of the S2 cell-based expression platform reside in the relative speed of production which allows fast access to the required proteins, excellent expression capability, scalability, applicability to high cell density perfusion cultivation, and regulatory friendliness.

For applications such as vaccines, where cost of manufacturing can weigh more than in other medical uses of recombinant proteins, a possible low-cost system such as S2 becomes very relevant. Selection of the appropriate expression system for recombinant or subunit vaccines is currently considered one of the most significant technical challenges for the vaccine industry. A significant obstacle to

FIGURE 5. Schematic representation of various VAR2CSA fragments and the corresponding K_D values. The figure shows tested fragments along with K_D values determined in the kinetic analysis. The kinetic analysis was performed on an Attana A100 quartz crystal microbalance (Attana AB). K_D values of the binding between the recombinant protein and immobilized CSA are indicated at the right column, N/A means that it was not possible to calculate the K_D value due to very low binding affinity. The minimal high affinity binding region is ID1-ID2a 72 kDa.[15]

TABLE 1. Examples of the many protein families that have been expressed using S2 cells.

Target Families	S2-Produced Examples
Anti-angiogenic	Endostatin and canstatin
Cytokines and growth factors	Vascular endothelial growth factor (VEGF) , interleukin 5 (IL5), IL12, erythropoietin (EPO), RANKL, tumor necrosis factor (TNF), lymphotactin
Enzymes	Sea Raven type II anti-freeze protein
Plasma components	Human transferrin (hTf)
Monoclonal antibodies	Immunoglobulin G (IgG), IgM, single-chain variable fragment (scFv) fusion protein, Fc chimeras, antibody-binding fragments (Fab)
Viral proteins	Japanese encephalitis virus (JEV), Rabies virus, West Nile virus, Influenza virus, Dengue fever virus
Virus like particles	Japanese encephalitis virus (JEV), glycoprotein (E)
Receptors (soluble versions)	CD23 and ADAM33 genes, epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2)
Integrins and related receptors	Platelet integrin IIbβ3, soluble urokinase-type plasminogen activator receptor (suPAR)
G protein coupled receptors (GPCRs)	Mammalian D1 and D2 dopamine receptors, human μ-opioid receptor (MOR)

the development of these promising and sorely-needed vaccines is the development costs involved. The geographic distribution of malaria, affecting mainly populations in poor countries, has meant that the development of a vaccine to prevent PAM, like for other tropical diseases, suffers from lack of economic incentives or political motivation. Public-private global health partnerships have been created to try to improve the development path for preventing and treating neglected diseases, but cost of development remains a critical parameter, and low-cost manufacturing platforms will play a vital role in enabling the progress of the more modern recombinant protein-based products necessary to address these diseases. An efficient manufacturing platform such as the *Drosophila* S2 cell-based system, which is robust and easily scalable, may become critical in enabling the development of these types of vaccines.

An example of a *Drosophila* S2-based protein expression system is the ExpreS^2 platform developed by ExpreS^{2}ion Biotechnologies which consists of: (a) highly efficient vectors; (b) a proprietary and optimized expression host derived from *Drosophila* S2; (c) a very effective transfection reagent to introduce the vector DNA into cells at high efficiency; and (d) a cell culture medium developed to sustain increased growth and high-titer production of recombinant proteins in *Drosophila* S2 cells. The platform allows for high success rates of recombinant protein expression, high yields, reproducibility, ease of scalability, time savings, and compliance with regulatory requirements. The team that developed the platform has extensive experience in the use of stable transfected S2 cells for production of a broad range of recombinant proteins for research use and for clinical development. The team received approval and performed production of Pharmexa's HER-2 AutoVac™ and RANKL AutoVac™, used in human clinical trials Phase 1 and 2, and non-human primate studies, respectively. The effort of optimizing each component of the production platform resulted in the ExpreS^2 system (Figure 6) being easy to use with a high success rate for expression of a broad range of protein classes, at consistently high yields, with excellent reproducibility, ease of scale-up, time/cost savings, and cGMP and regulatory compliance.

All together, the ExpreS^2 platform offers several important practical advantages to production of recombinant VAR2CSA truncation variants and upstream process development: transient expression can be attained in 3–4 days allowing for fast screening and initial selection of multiple VAR2CSA variants for further development. Generation of stable, high-yielding cell lines requires only three weeks after transfection allowing for larger scale production of VAR2CSA variants for purification and immunizations. Thus, by using the S2 expression system, time from DNA to VAR2CSA variants produced in stable polyclonal pools of transformed cells can be reduced from months to just weeks. The stability of the polyclonal pools, and the natural gene amplification that occurs, removes the time-consuming step of clonal isolation from the critical path in the discovery phase. This characteristic feature of the S2-based system is particularly useful in screening many different proteins and/or variants thereof for evaluation and characterization of candidates, both in discovery and in R&D, in a short timeframe and is a huge advantage for development of a VAR2CSA-based vaccine. Process development, including upstream and downstream, and the establishment of analytical tools can also benefit from the use of stable pools of S2 transfectants for protein production as these steps can be optimized while screening and the selection of a monoclonal-producing cell line takes place. This ability to have both activities in parallel dramatically speeds up the process allowing for best use of resources.

A VAR2CSA-based vaccine against PAM must be able to induce a strong protective immune response. In this, the most important aspect is the generation of IgG antibodies capable of inhibiting placental-specific parasite adhesion. More than 30 different variants of truncated VAR2CSA were

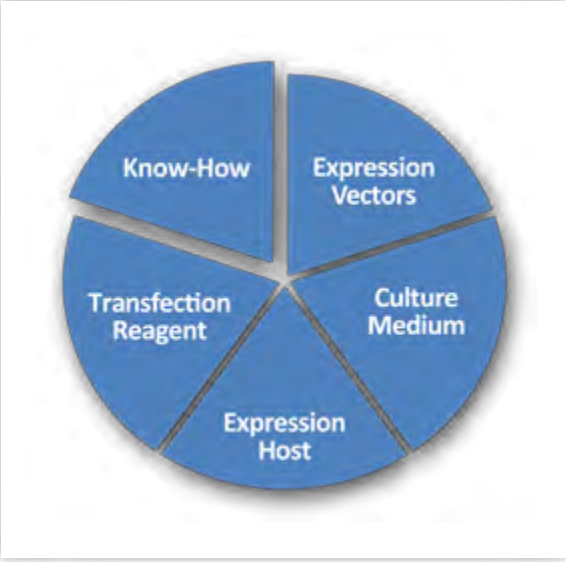

FIGURE 6. The ExpreS^2 protein expression package.

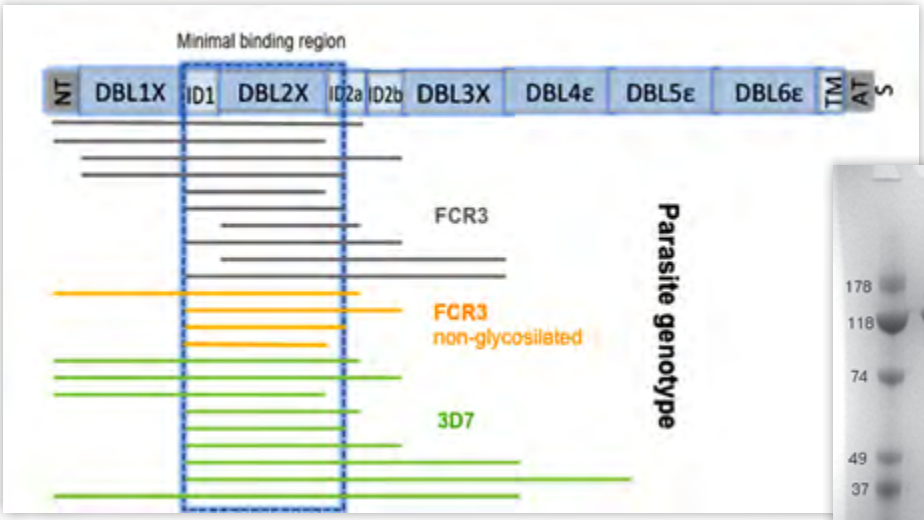

FIGURE 7 (left). Examples of VAR2CSA truncation variants cloned into ExpreS^2 vectors and transfected into S2 cells.

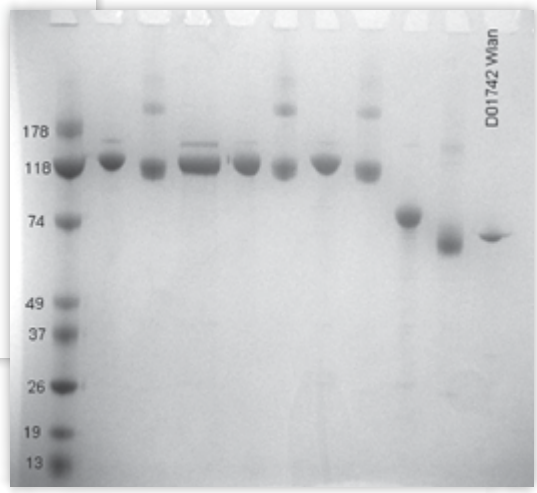

FIGURE 8 (right). Western blot showing expression of different truncated variants of VAR2CSA expressed in the ExpreS^2 system.

cloned into ExpreS^2 vectors (some examples are shown in Figure 7) and produced using the optimized ExpreS^2 system (Figure 8). The variants that were expressed were purified. To test the immunogenic characteristics of the produced fragments, they were used in the immunization of rats (Figure 9). Wistar rat anti-sera raised against the VAR2CSA variants were produced by injection of 30 µg of recombinant protein in Freund's complete adjuvant followed by three booster injections of 15 µg of protein in Freund's incomplete adjuvant at three week intervals. Anti-sera were collected one week after each injection. Sera raised against all fragments containing the CSA binding site inhibited parasite adhesion to CSA. Importantly, sera raised against ID1-ID2a genes resulted in almost complete inhibition. This suggests that the minimal CSA binding fragments retain the capacity for inducing a strong anti-adhesive immune response (Figure 10). Several of the produced VAR2CSA variants induced potent antibodies as shown in the binding inhibition assay.

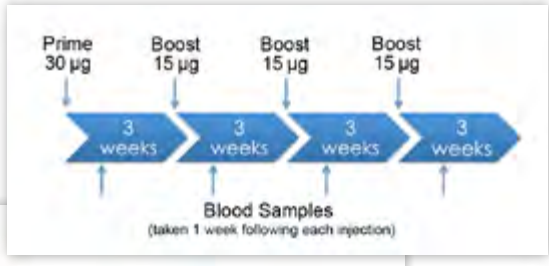

FIGURE 9 (right). Injection scheme of immunization of Wistar rats with protein in Freund's complete adjuvant.

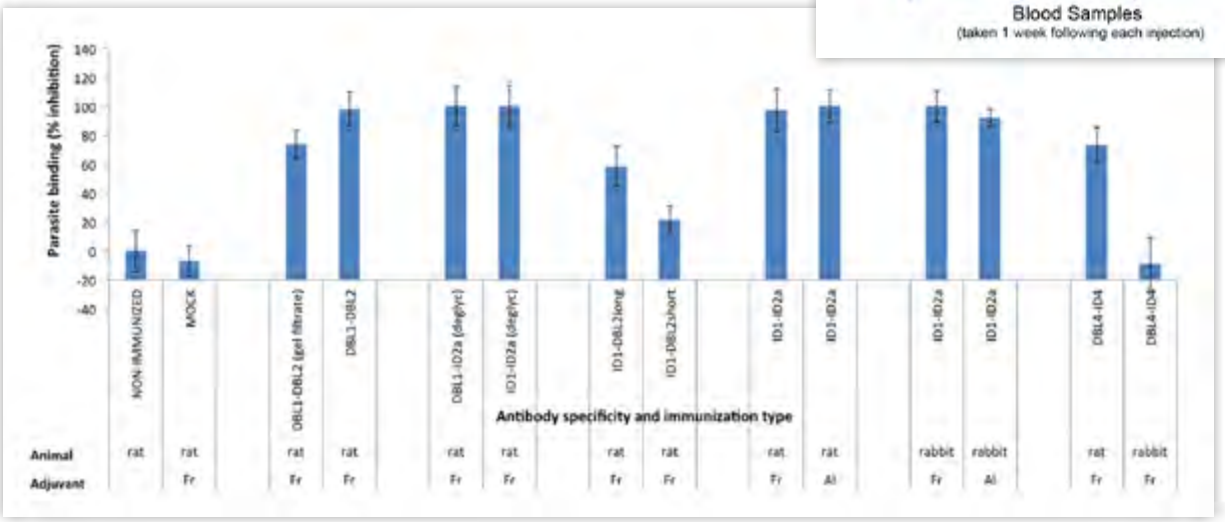

FIGURE 10. Inhibition of FCR3 parasites binding to CSA by anti-VAR2CSA sera generated by immunization of Wistar rats with VAR2CSA variants expressed in S2 cells.

It is important that a vaccine is capable of inhibiting placental adhesion regardless of parasite strain origin. A major concern in vaccine development is therefore the high interclonal diversity among parasite variants. While recombinant full-length VAR2CSA is very immunogenic, the antibodies produced are not cross-inhibitory.[19] A recent study shows that DNA vaccination with ID1-DBL2X from FCR3 induces antibodies that are cross-inhibitory, inhibiting CSA adhesion of other laboratory strains as well as parasites isolated in the field.[20] This supports the use of this small fragment in a PAM vaccine. We are currently testing the cross-reactivity of the lead S2 antigen-induced antibodies in our collaborator laboratories (in Benin and Tanzania) and freshly isolated placental malaria parasites. Preliminary data shows a promising level of cross-inhibition.

Variants of ExpreS^2-produced VAR2CSA protein are currently being screened for selection of the best vaccine candidate. A stable cell line system such as ExpreS^2 allows for a broader variety of upstream process options compared to BEVS. VAR2CSA variants have been produced in fed-batch and in perfusion with both production modes leading to high production yields. Perfusion allows for smaller footprint installations, a factor that may allow for more flexibility in early stages of production.

In looking for more flexible, low capital-cost investment alternatives to manufacturing, expression of VAR2CSA variants using the ExpreS^2 platform has been tested in disposable bioreactors. S2 cells expressing truncated variants of the VAR2CSA protein have been cultivated in a single-use system (CELL-tainer® by CELLution Biotech B.V.) at 4–15 L scale resulting in cell counts of 40–50 E6 cells/mL and viabilities above 94% in simple batch cultures without applying oxygen or pH control. The resulting protein was used to raise antibodies in animals and tested positively for opsonizing and parasite adhesion blocking responses.[21]

This ongoing work has demonstrated how the ExpreS^2 platform is feasible to use for expression of a large number of protein variants in larger scale for screening of expressability and production of functional antibodies that are also cross-reactive. We are currently working to screen further VAR2CSA variants and to optimize a fed-batch process that gives sufficient yield for the process to be economically viable. The tox batch will be produced by CMC Biologics, supported by the Danish National Advanced Technology Foundation.

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