University of Nevada, Reno

Bio-fortification of Rice by Constructing a Starch-Binding and Lysine-Rich Protein to Supplement Diets of Lysine Deficient Populations

A thesis submitted in partial fulfillment of the
requirements for the degrees of

BACHELOR OF SCIENCE, BIOCHEMISTRY & MOLECULAR BIOLOGY
BACHELOR OF SCIENCE, NUTRITION

by

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May, 2013

UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

MICHELLE LINH NGO

entitled

Bio-fortification of Rice by Constructing a Starch-Binding and Lysine-Rich Protein to Supplement Diets of Lysine Deficient Populations

be accepted in partial fulfillment of the
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Christie Howard, Ph.D., Thesis Advisor

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May, 2013

Abstract

Lysine is an essential amino acid in the human diet and when consumed at insufficient levels, can lead to osteoporosis, reproductive disorders, and heart diseases. In an approach to increase the nutritional content of lysine in rice, a modular protein composed of a starch-binding protein, *CBM 21*,was fused to a lysine-rich protein, *ABY716351*. This alternative approach was geared towards genetically engineering a lysine-rich protein that can be added to white rice after milling. This approach is much different than the more classic molecular approach of genetically modifying the rice itself. This was done in order to gain a more acceptable perception of genetic engineering approach to fortifying rice with lysine for lysine deficient populations worldwide.

Acknowledgement

I would like to thank Dr. Christie Howard, Dr. Chong Tang, and Dr. David Shintani for all of their hard work, guidance, and encouragement. Without them, I would not have been able to accomplish what I have thus far. They have encouraged me to go far beyond what I had thought could be possible in my undergraduate career by allowing me to pursue my own research. I am thankful for this opportunity and I am grateful to have such amazing advisors. I would also like to thank all my colleagues in iGEM for all the good times and memories we shared in lab and at the iGEM 2012 Regional Jamboree: Americas West at Stanford and the 2012 World Championship Jamboree at MIT.

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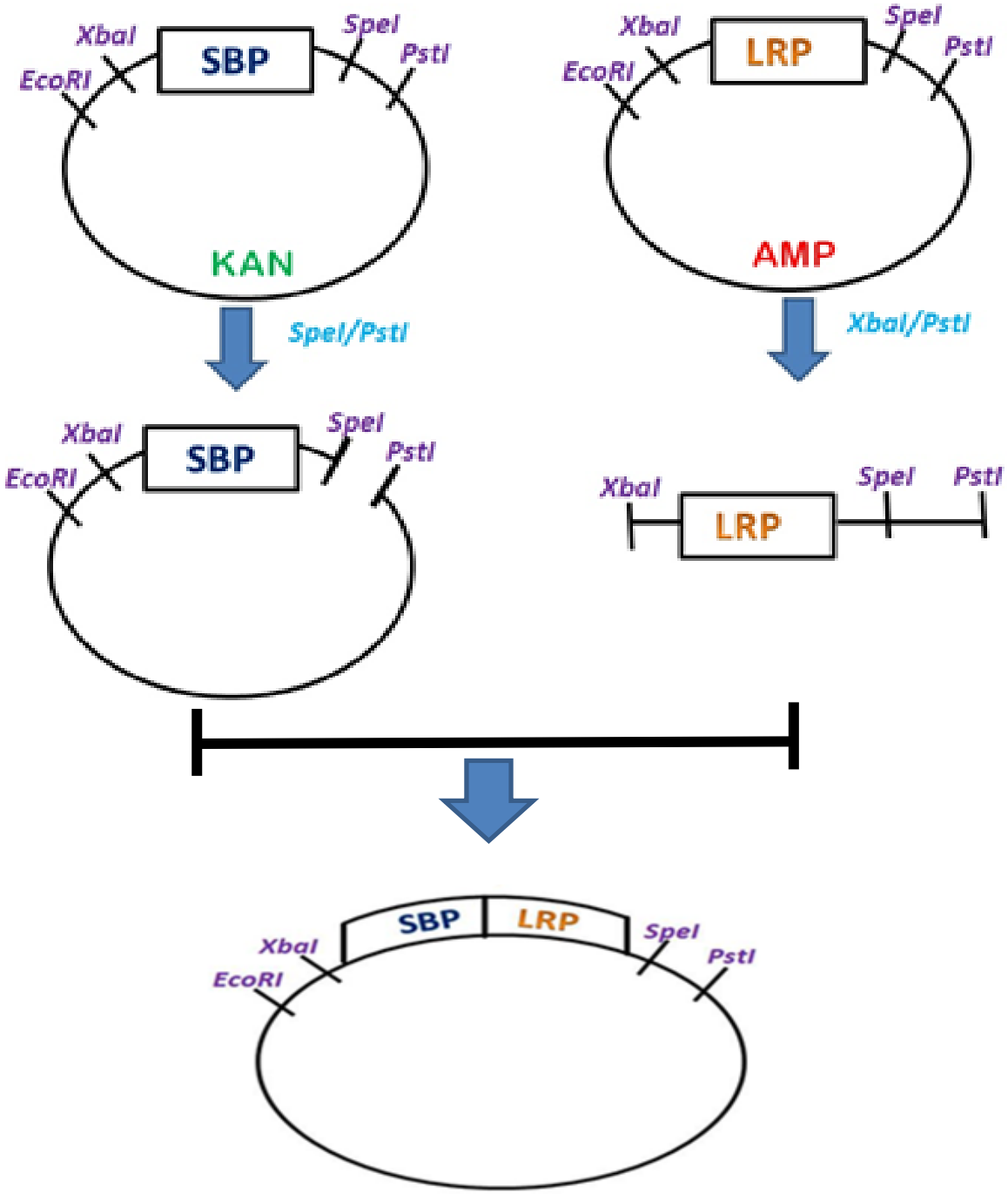

Figure 1. SBP-LRP Coding Region Construct. The starch-binding protein (SBP) was digested with SpeI and PstI and lysine-rich protein (LRP) was digested with XbaI and PstI prior to ligating the LRP insert into the 3' end of the SBP coding region to form the SBP-LRP coding region construct as illustrated above.

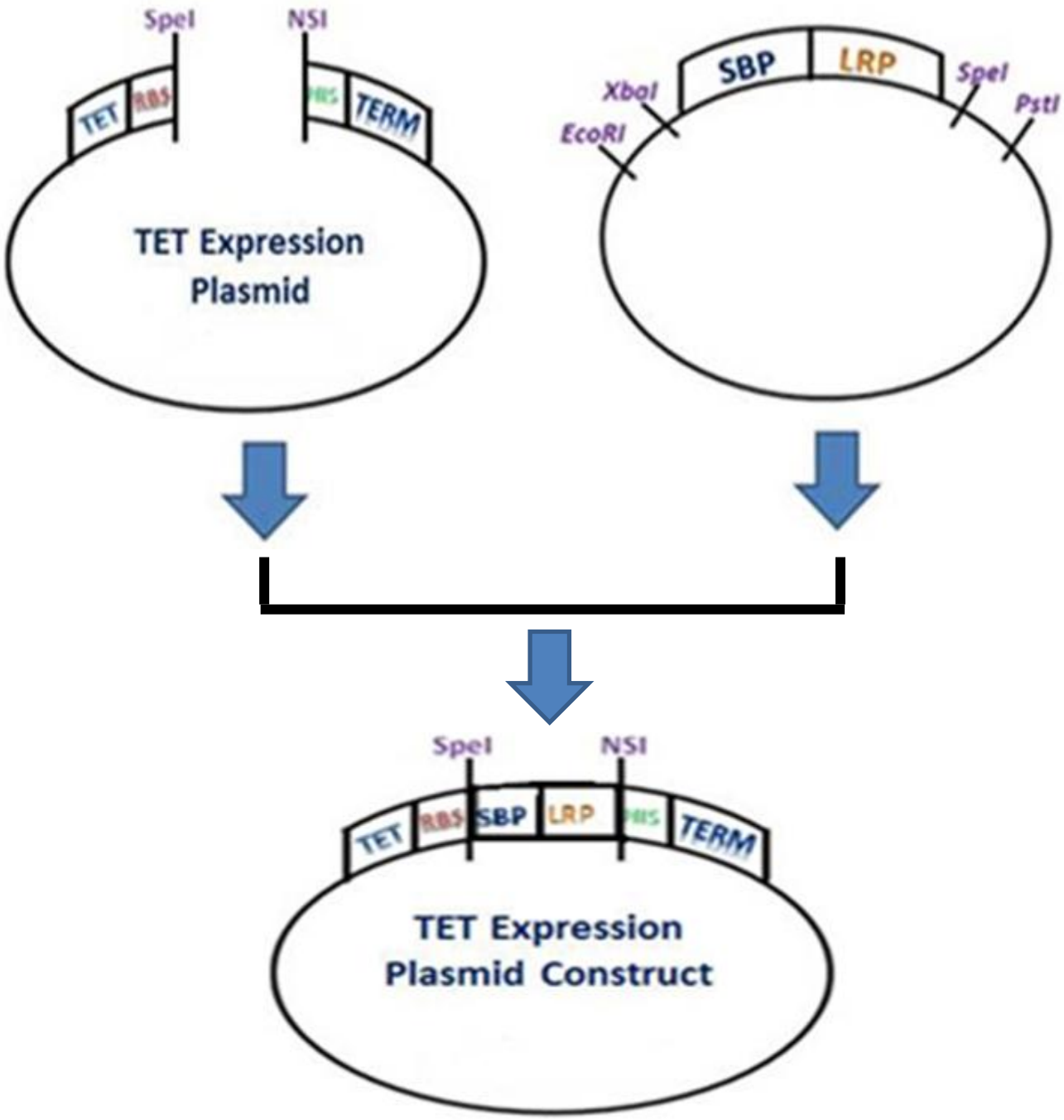

Figure 2. TET Promoter Expression Plasmid Construct. The starch-binding protein (SBP)-lysine-rich protein (LRP) coding region construct was digested with XbaI and PstI and the TET Promoter Expression Plasmid (124.9 ng/ul) was digested with SpeI and NSI and dephosphorylated before ligating to form the TET Promoter Expression Plasmid construct. The newly synthesized TET Promoter Expression Plasmid construct contained the TET promoter →R0040 (iGEM), ribosome binding domain (RBS) →B0034 (iGEM), His tag, and the double terminator (TERM) → B0010 and B0012 (iGEM) as illustrated above.

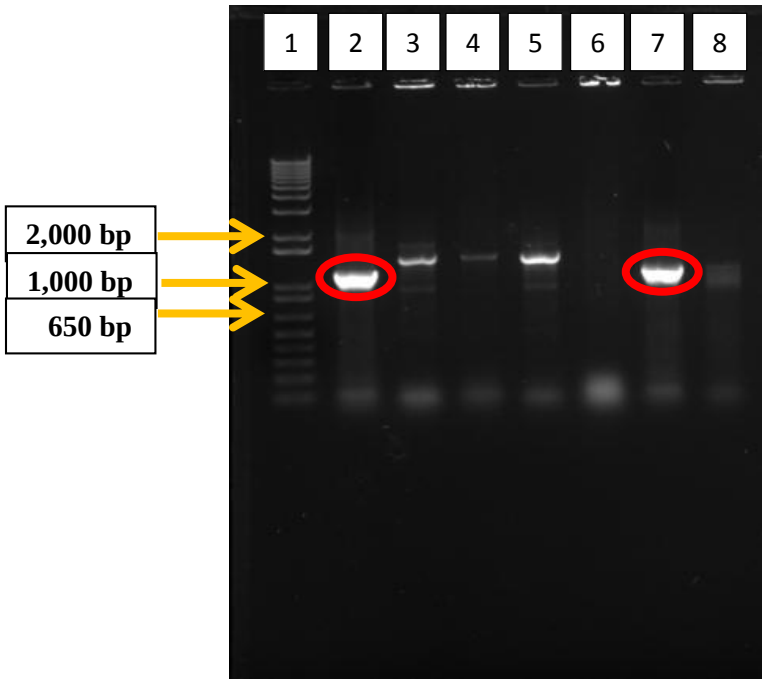

Figure 3. Colony PCR Gel Analysis of TET Promoter Expression Plasmid Construct. Results obtained from colony PCR on the TET Promoter Expression Plasmid Construct showed the construct at approximately 1,200 base pairs (bp). The following samples were loaded into each lane: 1 kilobase (kb) ladder (Lane 1), TET Promoter Expression Plasmid Construct (Lanes 2 and 7), and samples obtained from colleagues (Lanes 3-6 and 8).

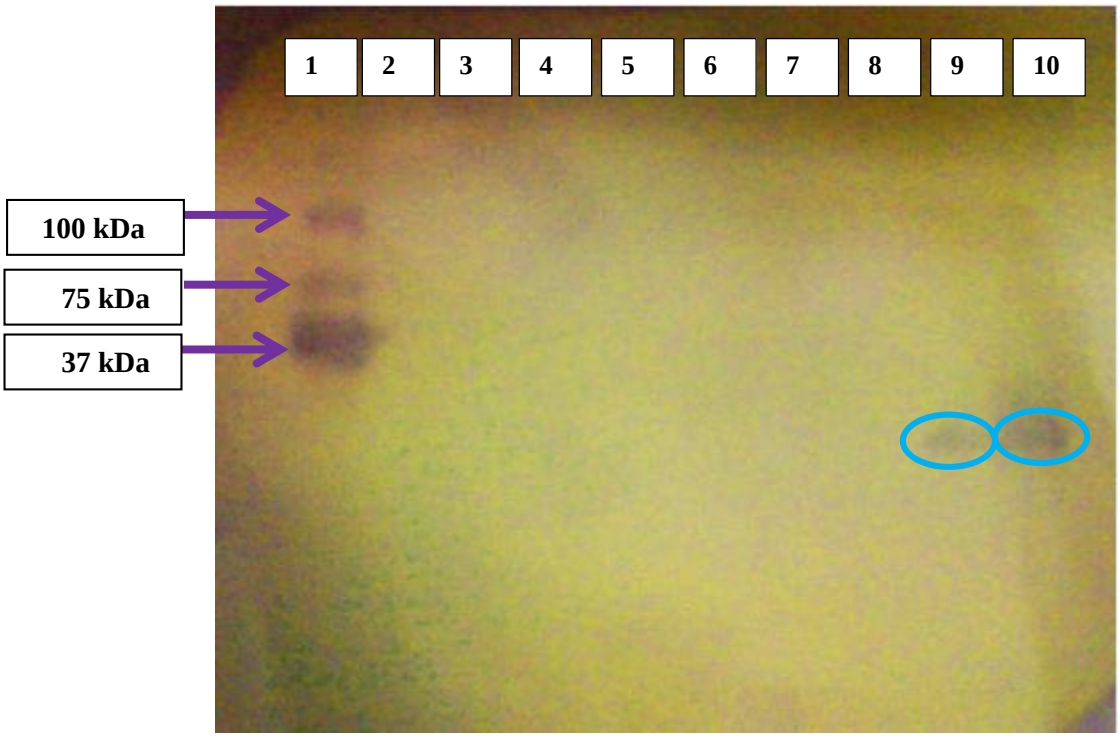

Figure 4. Western Transfer Blot Membrane of SBP-LRP fusion protein.
A colony from TET Promoter Expression Plasmid Construct transformed in BL21 cells as shown by the bands less than 37 (kilodaltons) kDa. The lanes were as followed accordingly: Bio-Rad Kaleidoscope Standards ladder (Lane 1), samples obtained from colleagues (Lanes 2-7), pellet from TET Promoter Expression Plasmid Construct (Lane 9), and supernatant from TET Promoter Expression Plasmid Construct (Lane 10).

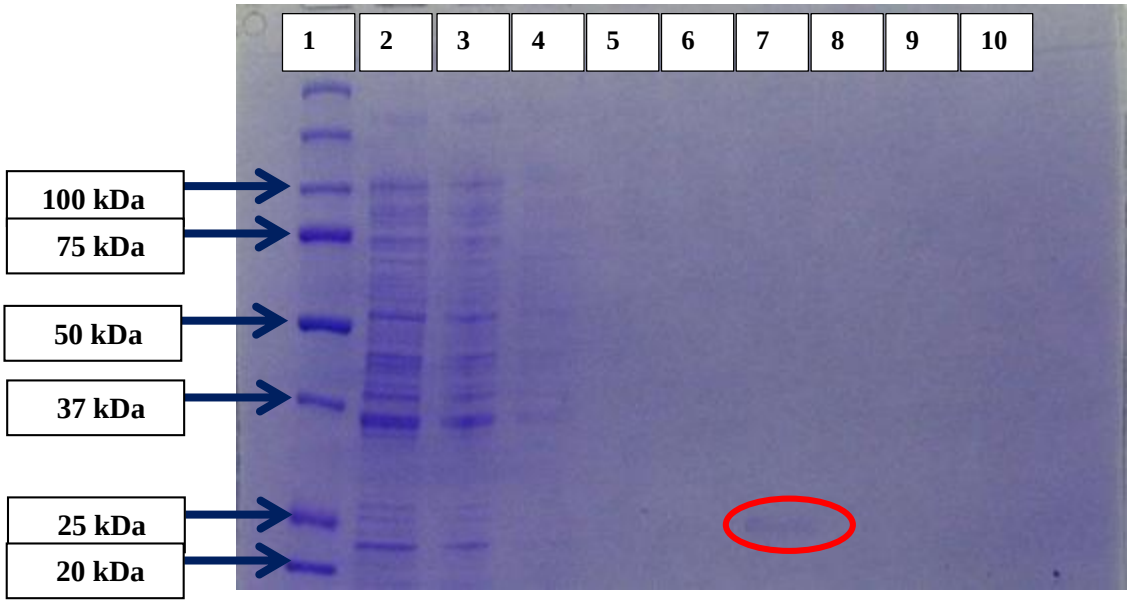

Figure 5. Ni-NTX Nickel Column Purification and SDS-PAGE Coomassie Stained Gel Analysis of SBP-LRP fusion protein. A colony from TET Promoter Expression Plasmid Construct transformed in BL21 cells resulting in a band below 25 (kilodaltons) kDa. The lanes were loaded as followed: Bio-Rad Kaleidoscope Standards ladder (Lane 1), protein supernatant (Lane 2), protein run-through (Lane 3), wash 1 (Lane 4), wash 2 (Lane 5), wash 3 (Lane 6), elution 1 (Lane 7), and empty lanes (Lanes 8-10).

Appendix

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   1 taaaatcgcg gccgcttcta gaatggcatc gatcccgagc agcgcgtccg ttcagctgga
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Appendix 1. SBP-LRP Coding Region Construct Sequence.
The sequence of the starch-binding protein (SBP)-lysine-rich protein (LRP) coding region construct was given to confirm the expected sequence of the SBP-LRP coding region construct. Colonies used for sequencing were obtained from the transformation of the SBP-LRP construct in BL21 cells (Invitrogen).

Introduction

Lysine is an essential amino acid consumed through meats, beans, and legumes in the diet (Van de Poll et al., 2005). Lysine is required for growth, bone development, tissue repair, and producing antibodies, hormones, enzymes, and collagen. Lysine has been linked to reducing symptoms of herpes infections, stress-induced anxiety, and aiding in the production of carnitine to decrease harmful LDL cholesterol levels (Shaw, 2011). This essential amino acid is limited in the diets of vegans and people who are on a strict wheat-based diet (Emery, 2005). Lysine deficiency can lead to osteoporosis, fatigue, anemia, hair loss, reproductive disorders, and heart diseases for those who cannot obtain the daily recommended intake of lysine through their diet (Shaw, 2011). The recommended daily allowance for lysine is 12 mg per kg of body weight (Torun, 2005).

Inadequate dietary intake of lysine is a major concern in areas where rice is the main food supply. This leads to a widespread problem of protein-energy malnutrition (PEM) and nutritional deficiencies that can eventually lead to disease in first world and developing countries. Rice is a major staple and energy food source to over half of the world's growing population (FAO, 1998). Rice provides over 70% of dietary energy for Asian countries such as, Cambodia, Myanmar, Bangladesh, and Vietnam. These countries depend on rice for their caloric intake (Kennedy et al., 2003). Through traditional methods of rice cooking, it has been shown that parboiling decreases the content of lysine compared to the lysine intake of raw rice (Eggum et al., 1984). Studies have shown that genetic engineering is a successful strategy that does not alter the genetic composition of the rice grain (Beyer, 2010). One study showed that lysine-rich rice was as safe as non-transgenic rice (Zhou et al., 2012). Genetically engineering rice to increase

lysine content has been shown to increase the lysine content by 2.5 fold when compared to polished rice that an average adult consumes (Bhullar, 2012). However, most people in first world and developing countries may not accept genetically engineered rice because of the stigma associated with genetically modified foods, such as Golden Rice. Cultural and individual perceptions and environmental problems such as cross-breeding of crops are factors that contribute to consumer acceptance of genetically engineered rice.

In this research, an alternative approach is taken to increase lysine levels in rice by fusing the C-terminal end of a starch-binding protein, *CBM 21,* derived from *Rhizopus oryzae,* a fungus thriving on dead organic starch species, to the lysine-rich protein, *ABY71635.1,* a clone derived from a chili pepper, *Capsicum frutescens* to construct a modular fusion protein (Lin 2009). The goal of this research is to produce a modular fusion protein with a starch-binding protein and a lysine-rich protein in *E. coli* and purify this protein, much like you would a pharmaceutical product. This protein can then be commercially bound to both rice and lysine during post-harvest production.

Materials and Methods

Part I. Coding Region Construct

The starch-binding protein (SBP) construct was digested with SpeI and PstI and lysine-rich protein (LRP) was digested with XbaI and PstI. PCR purification (Qiaquick) followed. The fragments were ligated together to form the coding region construct of SBP-LRP (Figure 1). This ligation product was transformed into BL21 cells (Invitrogen) followed by plating onto Kanamycin (KAN) Lauria Broth (LB) agar plates. Colony PCR was conducted using Forward Primer LRP 9 SBP Sense 1214 (5' AAGAAAGTGACGGTCGTTTATGC 3') and Reverse Primer Anti467 10X (5' ATGGTTTCTTCCGCCTTATCCTG 3') to check for the correct SBP-LRP colonies (Appendix 1).

Part II. TET Promoter Expression Plasmid Construct

The SBP-LRP construct was digested with XbaI and PstI. Results of the digestion were analyzed using 1.2% agarose gels stained with ethidium bromide. The successful colony was cultured with Terrific Broth-Kanamyacin (TB-KAN). PCR and PCR purification using glass milk (Mobio UltraClean 15 DNA Purification) was performed on the digest before it was ligated with TET Promoter Expression Plasmid (124.9 ng/ul) that consisted of a TET promoter →R0040 (iGEM), ribosome binding domain (RBS) → B0034 (iGEM), His tag, and a double terminator (TERM) →B0010 and B0012 (iGEM) as shown in Figure 2. The TET Promoter Expression Plasmid was constructed, digested with SpeI and NSI, and dephosphorylated by colleagues, Justin Emlen and Dafne Ordonez, prior to ligation of the PCR product and expression plasmid. Transformation

into BL21 (DE3) cells was plated onto 1.2% LB agar plates with Ampicillin (AMP) and Chloramphenicol (CM) followed by colony PCR (Figure 3).

Part III. Protein Expression and Detection

Expression was carried out by culturing one successful colony from the Bl21 (DE3) transformation in 10 ml LB-AMP-CM medium. By centrifuging at 13,000 rpm for 10 minutes, a pellet was obtained from the culture of the BL21 (DE3) transformation. The pellet was frozen in a solution of dry ice and 95% ethanol and heated at 90°C until it thawed completely. This freeze-thaw step was repeated 5-6 times. The lysed pellet was then centrifuged for 20 minutes at 13,000 rpm, separating the supernatant and the pellet. A total of 50 ul 3x Laemmli Loading Dye was added to the supernatant and 10 ul of the dye was added to the pellet. Both samples were put on a heating block for 10 minutes at 100°C and centrifuged for 1-5 seconds to remove the condensation before loading 30 ul of each sample into the SDS-PAGE gel (Bio-Rad). The samples were run in 1X SDS PAGE running buffer (Qiagen) at 200 volts for 30 minutes before transferring the protein from the SDS-PAGE gel (Bio-Rad) to the nitrocellulose membrane at 165 volts for 20 minutes. This was followed by the proceeding steps to the Western Transfer Blot protocol. The nitrocellulose membrane was washed in PBStween (PBST) for one hour and blocked with blocking buffer consisting of 0.2% PBST and 5% dry milk for another hour before discarding. Incubation of the nitrocellulose membrane with 0.5 ul of anti-His HRP antibody (Invitrogen) followed and was then left to incubate overnight. Following the overnight incubation, the nitrocellulose membrane was washed with PBST for 10 minutes twice before being washed by PBS for 10 minutes. SureBlue Reserve TMB

Microwell Peroxidase Substrate (1-Component) was used to develop the nitrocellulose membrane from the TOP 10 (Invitrogen) and BL21 (DE3) transformation (Figure 4).

Part IV. Protein Production and Purification

The successful expression and detection of the protein from the BL21 (DE3) transformation was used in large-scale production by culturing in 100 ml LB-AMP-CM medium. By centrifuging for 30 minutes at 13,000 rpm, a pellet was obtained from the 100 ml LB-AMP-CM culture of the BL21 (DE3) transformation. The Ni-NTX Nickel Column (Thermoscientific) protocol included equilibrium buffer (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, adjusting the pH to 8.0 using NaOH), washing buffer (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, adjusting pH to 6.3 using HCl), and elution buffer (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, adjusting pH to 4.5 using HCl) (Qiagen). A total of 2 ml of equilibrium buffer (Qiagen) was added prior to lysing the pellet using the sonic pulsification method. Sonic pulsification was required for 10 seconds and the pellet was allowed to sit in ice for 10 seconds; this step was repeated a total of 6-8 times. The lysed pellet was then divided into 10 tubes and centrifuged for 15 minutes at 13,000 rpm. The supernatant from the protein was combined for later use. A total of 500 ul of HisPur Ni-NTA Resin (Thermoscientific) was added to the Ni-NTX Nickel Column (Thermoscientific) and was allowed to pass through. One ml of equilibrium buffer (Qiagen) was added to the Nickel column and passed through; this was repeated two times. An amount of 5 ml of supernatant from the protein was drained through the Nickel column and collected as the protein run-through. A total of 1 ml of washing buffer (Qiagen) was added to the Nickel column and collected. This step was performed three times to collect the wash 1, wash 2, and wash 3 samples. Then 300 ul of elution buffer

(Qiagen) was added to the Nickel column and allowed to sit for 30 minutes before being drained and collected. This was repeated to obtain the elution 1 and 2 samples. A total of 20 ul 3x Laemmli Loading Dye was added to 40 ul of each sample. All samples were put on a heating block for 10 minutes at 100℃ and centrifuged for 1-5 seconds to remove the condensation before loading 30 ul of each sample into the SDS-PAGE gel (Bio-Rad). The samples were run in 1X SDS PAGE running buffer (Qiagen) at 165 volts for 30 minutes. Once completed, the SDS-PAGE Coomassie Staining protocol was performed. The SDS-PAGE gel (Bio-Rad) was rinsed with double deionized water before staining with Coomassie Brilliant Blue solution (Invitrogen) for one hour and soaking overnight with double deionized water (Figure 5).

The large-scale production and purification using the High Flow Amylose Resin Column (NEB) followed a similar protocol to the Ni-NTX Nickel Column (Thermoscientific) and Coomassie Brilliant Blue Staining protocols mentioned above. Modifications to the High Flow Amylose Resin Column protocol included column buffer made with 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, and 1mM EDTA (NEB). The column buffer was also used as the washing buffer and prepared at pH 10 to make the elution buffer (NEB). A total of 500 ul of High Flow Amylose Resin column buffer (NEB) was added and drained from the Amylose column in preparation for the collection of the 5 ml run-through of the protein supernatant. The washing buffer (20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 1mM EDTA) and elution buffer (20 mM Tris-HCl (pH 10), 0.2 M NaCl, and 1mM EDTA + 10 mM maltose) were used respectively following the protocol (NEB).

Results

Part I. Construction of SBP-LRP Fusion Construct

In order to generate a SBP-LRP fusion construct, the plasmid containing the SBP (361 bp) was opened and LRP (709 bp) was inserted into the 3' end of the SBP coding region as illustrated in Figure 1. The coding region construct SBP-LRP was transformed into BL21 (DE3) cells followed by colony PCR and confirmed with sequencing. The size of the new construct was ~1000 kb (data not shown) and the sequence for the SBP-LRP coding region construct was given in Appendix 1.

Part II. TET Promoter Expression Plasmid Construct Transformation

A successful colony from the coding construct was cultured and underwent PCR and PCR purification before being ligated into the TET Promoter Expression Plasmid as depicted in Figure 2. This new TET Promoter Expression Plasmid construct included: the TET promoter →R0040 (iGEM); ribosome binding domain (RBS) →B0034 (iGEM); His tag; and the double terminator (TERM) → B0010 and B0012 (iGEM). Transformation into BL21 (DE3) cells was performed followed by colony PCR to confirm the successful transformation of the TET Promoter Expression Plasmid in BL21 (DE3) cells (Figure 3). The bright bands that appeared in lanes 2 and 7 at approximately 1,200 base pairs (bp) showed that the transformation into BL21 (DE3) cells were successful according to Figure 3. To prepare for protein expression, the working colonies were cultured with Terrific Broth (TB) – Kanamyacin (KAN).

Part III. Protein Expression and Detection in Western Transfer Blot and SDS-PAGE Coomassie Stained Gel

The protein was expressed and detected in the Western Transfer Blot membrane that was developed from the BL21 (DE3) expression presented in Figure 4. The supernatant and pellet from the protein were expressed in lanes 9 and 10 below 37 kilodaltons (kDa). The size band was smaller than the expected 37 kDa protein.

Part IV. Large-Scale Protein Production and Purification

The BL21 (DE3) cells described above were purified using a Ni-NTX Nickel Column (Thermoscientific) and a High Flow Amylose Resin Column (NEB). Only the Ni-NTX Nickel Column (Thermoscientific) purification method was successful. The SDS-PAGE Coomassie Stained gel from the Ni-NTX Nickel Column method indicated a purified band that was expressed in lane #7 as the first sample of the elution, referred to as elution 1, just below 25 kilodaltons (kDa) (Figure 5).

Discussion

The digestions and ligations that were prepared to design the SBP-LRP coding region and TET Promoter Expression Plasmid Constructs (Figures 1 and 2) were successfully transformed in BL21 (DE3) cells as depicted in the PCR colony of the TET Promoter Expression Plasmid Construct (Figure 3). The size of the protein detected in the Western Transfer Blot was below 37 kDa (Figure 4), which was followed by large-scale production and Ni-NTX Column purification of this protein. The SDS-PAGE Coomassie Stained gel detected a protein band slightly below 25 kDa (Figure 5). This probably resulted from choosing an incorrect colony obtained from the transformation of the TET Promoter Expression Plasmid Construct into BL21 (DE3) cells. Lack of binding to the High Flow Amylose Resin Column (NEB) and the aberrant size of the protein detected, indicates that the isolated protein was most likely only lysine-rich protein (LRP). This was further confirmed by the Ni-NTX Column used for the Western Transfer Blot (Figure 4).

Although, results for expression of the SBP-LRP protein were not obtained, this method of genetically engineering a lysine-rich protein (LRP) to a starch-binding protein (SBP) to produce a modular fusion protein can help increase lysine intake without altering genes in the rice plant itself. The starch-binding domain will bind to the starch in rice to prevent lysine from leeching away during the washing of the rice prior to cooking. Public distrust of genetically modified foods such as Golden Rice and unsuccessful trials with vitamin powders in dusting procedures show that a SBP-LRP fusion protein may be a better alternative to supplementing post-harvested rice to increase lysine intake in over half of the world's population who are dependent on rice.

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