Purification and optimization of sequential biosynthetic enzymes for co-localization onto a nanocarrier

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Purification and optimization of sequential biosynthetic enzymes for co-localization onto a nanocarrier

by

Mollie Sue Tiernan

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:
Reuben Peters, Major Professor
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Iowa State University
Ames, Iowa
2013

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ABSTRACT

Flavonoids are polyphenolic plant secondary metabolites with many purposes, including providing pigmentation in plants and anti-oxidant and anti-cancerous activities in humans. There are over 10,000 different flavonoids known, making them one of the largest groups of natural products. One major sub-class of flavonoids is the flavan-3-ols, which are known for their health benefits and the ability to form proanthocyanidins, or condensed tannins. Many of the intermediates in this pathway are unstable and have not been isolated in planta. Another natural product of interest is sclareol, a diterpene alcohol, which is important in the fragrance and flavor industry as a precursor to Ambrox, and ambergris analog that can be used as a fixative in high end perfumes.

In this study we are aiming to create a synthetic complex using a polymer nanocarrier to co-localize two sequential enzymes in a pathway. The first system tried included the final two enzymes in flavan-3-ol biosynthesis, anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR). Each of these enzymes, along with the preceding enzyme, dihydroflavonol 4-reductase (DFR) has been recombinantly expressed in E. coli with a specific tag for binding to the nanocarrier. However, due to difficulty achieving ANS activity a backup system involved in sclareol synthesis was developed. Two enzymes, NgCPS and sSsSS, were used to convert geranylgeranyl-pyrophosphate (GGPP) to sclareol in a coupled assay. Each enzyme was purified with either a 6xHis tag or monomeric DM3 streptavidin tag for attachment to the nanocarrier. Assays to measure each enzymes activity have been designed, and our goal now is to test each enzyme’s activity before and after co-localization onto the nanocarrier.
CHAPTER 1: INTRODUCTION

Flavonoid Pathway

Flavonoids are one of the largest groups of plant natural products with over 10,000 known today (Tahara 2007). They have many functions in plants and benefits to human health. Flavonoids are known to function in plant defense as well as aiding in UV protection and attracting pollinators (Harborne and Williams 2000; Beecher 2004). One type of flavonoid, anthocyanins, a glycosylated version of anthocyanidin, are the largest group of water-soluble pigments known (Yan, Chemler et al. 2005). These compounds are responsible for the bright reds, oranges, and blues found in many flowers and fruits. Anthocyanins can be used as a natural alternative to current dyes that may be harmful. In humans and animals flavonoids, and more specifically, flavan-3-ols, have anti-oxidant and anti-cancerous properties. It has also been shown that flavonoids may have anti-obesity and anti-microbial properties (Chung, Wong et al. 1998; Cos, De Bruyne et al. 2004; Tsuda, Ueno et al. 2006). The upstream pathway has been extensively studied, but the later steps need further elucidation (Burbulis and Winkel-Shirley 1999; Pelletier, Burbulis et al. 1999). The natural occurrence, applications, and health benefits of flavonoids have also been reviewed (Gu, Kelm et al. 2003; Aron and Kennedy 2008). However, research involving the flavonoid pathway and possible applications makes this field of continued interest to researchers.

The flavonoid pathway stems from the phenylpropanoid pathway (Figure 1) and culminates in the production of flavonols, anthocyanidins, flavan-3-ols, and proanthocyanidins, which have a variety of functions as secondary metabolites.
As you can see in Figure 1 there are many possible product outcomes of the flavonoid pathway. One such outcome is the formation of proanthocyanidins, or condensed tannins, which are polymers of flavan-3-ols. Proanthocyanidins are responsible for the astringent taste in grapes and wine (Bate-Smith 1973). The branching point for this outcome is dihydroflavonol reductase, or DFR, which catalyzes the conversion of dihydroquercetin to leucoanthocyanidin (Figure 2). The intermediates in the last steps of this pathway are highly unstable. Leucoanthocyanidins polymerize readily upon a very
slight change in environmental conditions. These flavonoids are then converted to anthocyanidin, which contains a flavylanium cation by anthocyanidin synthase (ANS), then reduced by anthocyanidin reductase, or ANR, to form the final product in this branch of the flavonoid pathway--- epicatechin. Epicatechin, a flavan-3-ol can then polymerize to form proanthocyanidins. Although the flavonoid pathway has been studied for more than fifty years the last step in proanthocyanidin synthesis is unknown.

Figure 2. Late flavonoid pathway in *Arabidopsis thaliana.*
Flavonoid accumulation first begins in the seed coat of plants when it is exposed to UV light (Pelletier, Murrell et al. 1997). Flavonoid production can increase at any time in the plant’s life when exposed to external stress (Winkel-Shirley 2002). Several manipulations of plants have been used to study flavonoids. For instance, mutations that cause a clear to yellow phenotype due to disruption of the production of pigmentation in plants, called transparent testa, have been used to identify genes involved in flavonoid biosynthesis and map their loci by disrupting the production of plant pigmentation from flavonoids (Koornneef 1990; Shirley, Kubasek et al. 1995). There have also been 24 mutant knockout studies to analyze anthocyanidin presence during various stages of plant growth (Pelletier, Burbulis et al. 1999; Abrahams, Tanner et al. 2002; Abrahams, Lee et al. 2003).

*Arabidopsis thaliana* has been used by many researchers as a model system to study flavan-3-ol biosynthesis. There are several reasons for this: First, *Arabidopsis* has a fully sequenced genome, which simplifies genetic studies and manipulations. Also, all but one of the enzymes involved in flavan-3-ol biosynthesis are encoded by a single gene (Winkel-Shirley 2001). FLS, the exception, has six naturally occurring gene copies, but only one is responsible for activity in wild type plants (Pelletier, Murrell et al. 1997). Finally, *Arabidopsis thaliana* does not appear to encode an equivalent enzyme to leucoanthocyanidin reductase (LAR), which helps to further simplify the study of flavan-3-ol biosynthesis by limiting the product stereochemistry to that of (-)-epicatechin (Abrahams, Lee et al. 2003).

The flavonoid pathway must be carefully regulated due to interactions between enzymes and intermediates. For example, in order for proanthocyanidins to be formed
leucoanthocyanidin, the extension unit, must be present in higher concentrations than flavan-3-ols, the initiator unit (Abrahams, Lee et al. 2003). Many of the intermediates in this pathway are highly reactive and possibly toxic so they must be rapidly transported between enzymes in order to accumulate high levels of end product while keeping intermediate concentrations low (Mathews 1993; Ovadi and Srere 1996).
Flavonoids

Structure

Flavonols are polyphenolic compounds that are derived from phenylalanine and acetyl-CoA using the phenylpropanoid pathway. There are 9 sub-classes of flavonols (Figure 3), which include anthocyanidins and flavanols. The variance in these units depends on the placement of the hydroxyl group on the rings.

Leucoanthocyanidins

Leucoanthocyanidins are highly reactive intermediates in the flavonoid biosynthetic pathway. These 3, 4-diols are the extension units for proanthocyanidin formation. They have not yet been isolated in nature (Forkmann and Martens 2001), which may be because of their tendency to polymerize readily. The inability to isolate these compounds in nature may also be due to highly efficient exchange between the enzymes in the possible existence of a flavonoid biosynthetic enzyme complex. Leucoanthocyanidins may also convert back to their precursors, dihydroflavonols, under storage conditions (Pang, Peel et al. 2007). Because of their unstable nature,
leucoanthocyanidins are difficult to measure and generate and must be lyophilized or acidified immediately upon synthesis to avoid degradation.

**Anthocyanidins**

Anthocyanidins are the precursor to flavan-3-ols. They can exist as a flavylium cation, but that is uncommon to see in nature. They usually exist as salts or anthocyanins, glycosylated versions of anthocyaninins that are more stable. The small amounts of anthocyanidins found in nature are typically considered to be degradation products of anthocyanins (He, Mu et al. 2010). Anthocyanidins have a pH dependant color that varies from blue to red. Anthocyanins are best known for producing pigmentation in plants, which aids in defense against UV light and in the recruitment of plant pollinators (Harborne and Williams 2000). It has also been shown that the administration of blackberry extracts that are rich in anthocyanins to rats will halt tumor growth and may even shrink the tumor size (Desai, Olsen et al., 2010).

Anthocyanidin marks the committed step in the downstream flavonoid pathway because it is the substrate for two different enzymes. UDPglucose:flavonoid glucosyltransferase (UGFT) will glycosolate anthocyanidin to form an anthocyanin, a stable product. Anthocyanidin reductase (ANR) reduces anthocyanidin to a flavan-3-ol.

**Flavan-3-ols**

Flavan-3-ols are a class of flavonoids that contain a 3-hydroxyl group on the central ring (Figure 4). They possess two chiral carbons, leading to different possible stereochemistries. Flavan-3-ols can be found as single unit monomers, or they can condense to form dimers, oligomers, or polymers (Marles, Ray et al. 2003). These flavan-3-ol polymers are often called proanthocyanidins, or condensed tannins.
Flavan-3-ols are the most commonly found flavonoid in our diet (Scalbert and Williamson 2000). They can be found in many foods and beverages including vegetables, fruits, tea, cocoa and legumes. Food sources rich in flavan-3-ols have become referred to as “superfoods” for their high antioxidant activity. This has led to extensive research on flavan-3-ols and sources rich in these beneficial compounds.

It has been demonstrated that flavan-3-ols have anti-microbial/viral activity (Chung, Wong et al. 1998). They may also suppress Staphylococcus aureus resistance to antibiotics (Vinson, Mandarana et al. 2002). It is also known that flavan-3-ol monomers from plants rich in these flavonoids are capable of chelating transition metals (i.e. iron or lead), which leads to an increased resistance to high concentrations of these metals in the environment. In addition, invasive neighboring plant grown is slowed by (-)-epicatechin and (-)-catechin, flavan-3-ol monomers (Ahmad, Gupta et al. 2000; Bais, Vepachedu et al. 2003) which are also used in defense against invasive microorganisms (Scalbert, 1991).

Another interesting property of flavan-3-ols can be seen in extracts from flavonoid rich plants, which show anti-cancerous and cancer preventative effects (Grotewold, Lin et al. 2006; Ahmad, Cheng et al. 2000). The extracts have also been shown to induce apoptosis and reduce tumor formation (Cos, De Bruyne et al. 2004; Bagchi, Bagchi et al. 2000). Because of these implications in plant defense and human

Figure 4. The general structure of a flavan-3-ol.
health, flavan-3-ols have been widely studied. Future work on flavan-3-ols including potential sources and uses of these compounds remains important.

**Proanthocyanidins**

Proanthocyanidins, or condensed tannins (CTs) are the condensation products of flavan-3-ols. They are well known because their presence in grapes and wine leads to astringency. The astringency occurs because proanthocyanidins react with proline-rich proteins in saliva (Kall thraka, Bakker et al. 2001). It has been speculated that this astringency works as a defense mechanism for the grapes because it is unpalatable to animals and microbes (Bate-Smith, 1973).

There are three different proanthocyanidin types. A-type proanthocyanidins have two bonds between the flavan-3-ol units, whereas B-type proanthocyanidins have only one bond. Because of this, A-type proanthocyanidins confer more rigidity than B-type. Both structures can be seen in Figure 5. The third type of proanthocyanidins is C-type, which has a minimum of three units---one initiating and at least two extension units (Hemingway, Karchesy et al. 1989).

Proanthocyanidins have anti-microbial and anti-fungal effects in plants (Beecher 2004) but
they are best known for their health benefits in humans, which includes anti-oxidant activity as well as cardio and neuro-protective benefits (Aron and Kennedy 2008). The bioavailability of proanthocyanidins has been studied and they are the least absorbed class of flavonoids through the digestive track in humans (Tsang, Auger et al. 2005). It is much easier to metabolize flavan-3-ol monomers than oligomers or polymers (Scalbert, Deprez et al. 2000; Manach and Donovan 2004). It is even thought that flavanol monomers are not absorbed into the body until they are broken down into smaller metabolites (Scalbert 1991; Gonthier et al. 2003).
**Arabidopsis thaliana flavonoid biosynthetic enzymes**

Dihydroflavonol Reductase

Dihydroflavonol reductase (DFR) catalyzes the stereo-specific C-4 reduction of a dihydroflavonol to leucoanthocyanidin, a 3,4-cis diol as seen in figure 6 (Stafford and Lester 1985). This reaction is the beginning of a series of steps in flavan-3-ol biosynthesis with highly unstable intermediates. DFR is classified as a short-chain dehydrogenase/reductase and required NADPH as a cofactor (Petit, Granier et al. 2007). The crystal structure obtained from grape showed that the substrate is wrapped up by both the N- and C- terminals of the enzyme with the NADPH binding site located in the N-terminal, which forms a characteristic Rossmann fold (Petit, Granier et al. 2007). DFR from *Arabidopsis thaliana* has previously been recombinantly expressed and characterized in *E. coli* (Leonard, Yan et al. 2008).

**Anthocyanidin Synthase**

The enzyme that follows DFR in the flavonoid biosynthetic pathway in *Arabidopsis* is anthocyanidin synthase, or ANS. This enzyme is also referred to as leucoanthocyanidin dioxygenase (LDOX) and corresponds to the TDS4 gene (Abrahams, Lee et al. 2003). ANS is a 2-oxoglutarate dependent dioxygenase that requires the cofactors 2-oxoglutarate and Fe(II) for activity. ANS requires the presence of oxygen and it has been shown that an unusually high concentration of ascorbate is needed to
increase the activity of this enzyme to its maximum catalytic rate. AtANS has been purified and crystallized and the structure can be seen in figure 7 (Turnbull, Prescott et al. 2001).

ANS catalyzes the oxidation of flavan-3,4-diols to anthocyanidins, the step after DFR in the flavonoid biosynthetic pathway. Both the main substrate and product of ANS are relatively unstable. Also, there have been studies into the stereospecificity of this enzyme and the products formed. In vitro assays have shown that leucoanthocyanidin stereochemistry affects the product outcome with ANS catalyzing either the formation of dihydroquercetin or cyanidin. ANS also catalyzes the conversion of dihydroquercetin to quercetin after a second catalytic cycle (Turnbull, Nagle et al. 2003). The various reactions ANS catalyzes can be seen in figure 8. In further studies ANS was found to form a cyanidin in the direct flavan-3-ol pathway (Turnbull, Sobey et al. 2000). It should be noted that there are complications with ANS characterization due to substrate and product instability, the compounds forming complexes with the Fe(II) and further levels of oxidation. This leads to indirect evidence for the true mechanism and products of ANS. Using the natural 2,3 cis form of leucoanthocyanidin it was observed that quercetin was the most prevalent product of ANS (85% of the product profile) whereas cyanidin was the least prevalent product, accounting for only 2% (Turnbull, Sobey et al. 2000).
Anthocyanidin Reductase

Anthocyanidin reductase, or ANR is the last step in flavan-3-ol biosynthesis. This enzyme converts anthocyanidin to a flavan-3-ol (Figure 9), and is also dependent on NADPH. ANR resides in the BANYULS gene in arabidopsis. It has been shown that when this gene is mutated there is an accumulation of red anthocyanin pigments and loss of proanthocyanidins (Xie, Sharma et al. 2003). In *Arabidopsis thaliana* ANR catalyzes the formation of (-)-epicatechin from cyanidin (Xie, Sharma et al. 2003). A full characterization of AtANR recombinantly expressed in *E. coli* has been performed alongside ANR from *Medicago trucatula* (Xie, Sharma et al. 2004).
Backup platform: sclareol synthesis

In the event that the flavonoid biosynthesis system would not work with the experimental design, a backup platform with similar applications to the flavor and fragrance industry was chosen. Ambergris, waxy substance secreted by the sperm whale, has long been used as a fixative in high-end perfumes. It is well known for its musky and sweet earthy scent, but its use has become controversial due to the fact that the only natural source is an endangered and protected animal species (Caniard and Zerbe 2012). Ambergris is in short supply and has undergone price inflation, so a number of ambergris substitutes have been developed (Schalk and Pastore 2012). One such substitute is Ambrox (Figure 10), which is industrially produced by semisynthesis from sclareol, a naturally occurring diterpene alcohol (Gunnewich and Higashi, 2012).

Sclareol has been reported in four different plant species: *Salvia sclarea, Cistus creticus, Nicotiana glutinosa,* and *Cleome spinosa* (Caniard and Zerbe 2012). The main plant based source of sclareol is the flowers and leaves of *Salvia sclarea* (Clary sage), a biennial herb native of Southern Europe. However, despite successful cultivation of Clary sage, annual production and availability of sclareol varies substantially due to uncontrollable environmental factors (Caniard and Zerbe 2012). Thus, the development of an alternative, cost-effective and sustainable way to produce sclareol is desirable.

In plants diterpenes are derived from geranylgeranyl diphosphate (GGPP) through cyclization reactions catalyzed by diterpene synthases. Two types of cyclization
mechanisms occur in nature, which are separated into two classes of diterpene synthases: class I and class II, both of which are Mg$^{2+}$ dependent. Class II diterpene cyclases are catalyzed by the protonation dependent cyclization of GGPP, forming a bicyclic diphosphate intermediate, and Class I diterpene cyclases are initiated by ionization of the diphosphate ester (Peters 2010). Sclareol is produced via diterpene synthesis and can be synthesized by specific diterpene cyclases.

A class II diterpene synthase from *Nicotiana glutinosa* (NgCPS) is known to catalyze the reaction of GGPP to labda-13-en-8-ol diphosphate (LDPP) (Guo and Wagner 1995; Shepard 2002). Sclareol synthase has been successfully cloned and functionally characterized and the sclareol synthesis pathway had been reconstructed in recombinant *e. coli* (Schalk and Pastore 2012), so the LDPP produced by NgCPS will be converted to sclareol by sclareol synthase from *Sclarea sativa* (sSsSS) as seen in Figure 11.

![Figure 11. Sclareol synthesis from geranylgeranyl diphosphate (GGPP).](image)

In this proposed backup platform NgCPS and sSsSS could be expressed and purified from *e. coli* with several different tags to be used for co-localization onto a nanoparticle to increase yields of sclareol, an important compound in the flavor and fragrance industry.
Enzyme Attachment

Enzyme attachment to a platform has many potential benefits to a biological process. The process can be made more efficient for commercial or industrial scale, and can be manipulated as such (Sheldon 2007). Immobilization may increase the stability of the enzyme being used, which can increase performance and percent recovery of the enzyme (Cabral and Kennedy 1993). Also, this stability may include higher tolerance to extreme pH or temperature ranges, as well as other conditions.

Lactose dehydrogenase and glucose dehydrogenase have been tethered to silica glass platforms (El-Zahab, Jia et al. 2004) and there were differences in the generation and use of the cofactor, NADH, between the two enzymes with variations in the chain length. Liu, Zhang, et al. 2009 duplicated this experiment using glutamate dehydrogenase in the place of glucose dehydrogenase, and both showed high turnover and regeneration of NADH.

There has been little related research concerning the biosynthesis of flavan-3-ols or sclareol. Leucoanthycyanidins have been immobilized and stabilized (Deffieux, Gaudrel-Grosay et al. 2009) but no instance of enzyme stabilization has been reported. However, similar benefits should be expected by using enzyme attachment with these systems as long as no negative interactions occur.

The aim is to co-localize two enzymes using a Nickel-6xHistidine binding and Biotin-Streptavidin binding to provide control over the placement and ratios of the enzymes in question to determine if any of the expected benefits to the system are present (Figure 12). The enzymes to be attached include Anthocyanidin synthase and
*Anthocyanidin reductase*, or *Nicotiana glutinosa* ent-copalol diphosphate synthase and *Sclareol synthase* in the event that active flavonoid biosynthesis protein cannot be obtained.

![Enzyme attachment scheme](image)

Figure 12. Enzyme attachment scheme for ANS and ANR. The same scheme would apply for NgCPS and sSsSS.
**Hypothesis**

The objective of this project is to create a more efficient or stable synthetic flavan-3-ol biosynthetic complex that will mimic the proposed flavonoid multi-enzyme complex. The aim of this research is to attach several enzymes in the late flavonoid biosynthetic pathway to a nanocarrier via site-specific attachments. The enzymes that will be attached to this nanocarrier catalyze the last steps in the biosynthesis of flavan-3-ols, which are compounds that have many benefits to human health as well as a plethora of other purposes as plant secondary metabolites.

The enzymes of interest will be purified and their activity assayed both before and after site-specific attachment to a nanocarrier. The attachment will occur by expressing each enzyme with a fusion tag that has specific binding for sites on the surface of the nanocarrier. The platforms that will be used are a 6xHistidine tag that will covalently bind to nickel on the surface of the nanocarrier, and a monomeric streptavidin tag that will specifically bind to biotin on the nanocarrier. These platforms can be attached to the nanocarrier concurrently or sequentially to control for the ratio of one enzyme to the other.

Co-localizing these enzymes onto a nanocarrier will be beneficial to the pathway in several ways. First, the attachment could stabilize the protein and allow it to withstand environments that it would not be able to in solution. This could help the protein remain active in harsh environments and avoid denaturation. Also, attaching sequential proteins could lead to intermediate shuttling between the enzymes allowing for a more efficient system of two or more enzymes. This intermediate shuffling would also avoid loss of substrate due to diffusion and unwanted side reactions.
CHAPTER 2: MATERIALS AND METHODS

Materials

Geranylgeranyl-pyrophosphate (GGPP) standard was purchased from Isoprenoids, LC (Tampa, FL). Standards for HPLC analysis (dihydroquercetin, quercetin, cyanidin chloride, and (-)-epicatechin) and all other reagents, substrates, and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). C41(DE3) chemically competent cells were purchased from Lucigen (Middleton, WI) for protein expression. Top10 competent cells were purchased from Invitrogen (Carlsbad, CA). All DNA primers were synthesized by Invitrogen or the DNA facility of Iowa State University’s Office of Biotechnology. Accuprime Pfx polymerase was ordered from Invitrogen. Takara Primestar HS Polymerase was ordered from Fischer Scientific (Waltham, MA).

DNA Cloning and Recombination

Native AtANR was obtained from the TAIR database. Amino acid sequences for AtDFR and AtANS were taken from the TAIR database and were codon optimized for growth in E. coli and synthesized into pUC57 (Genscript USA Inc.). A monomeric streptavidin tag was placed on the N-terminus of AtANS with a (glycine3-serine1)4 linker in between the amino acid sequences of the tag and AtANS.

Accuprime Pfx polymerase (Invitrogen) was used for PCR of AtANR with an initial melting temperature of 95°C for 5 minutes, then 30 seconds at 94°C, 62°C for 30 seconds and an elongation time of 2 minutes at 72°C for 34 cycles, followed by 7 minutes at 72°C and 10 minutes at 4°C. AtANS and AtDFR were cloned using Takara Primestar HS polymerase (Fischer Scientific). Both genes were clones using the Primestar 2-step method with an elongation time of 2.5 minutes for AtANS and 1.75 minutes for AtDFR.
The primers used were ordered from Invitrogen and diluted to 5 nmol concentrations. They can be seen in Table 1. DNA gel electrophoresis was used to determine and isolate the correct gene using a 1.5% agarose gel. The DNA band with the appropriate size was purified by a Millipore DNA gel purification kit and confirmed with full genetic sequencing.

Table 1. Primers used in cloning PCR, 5’ to 3’

<table>
<thead>
<tr>
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<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>AtDFR</td>
<td>CACCATGGTGTCACCAAAAGAAAACGGTG</td>
<td>TCAAGCACAACATCTGTGTGCACCGAGCATC</td>
</tr>
<tr>
<td>AtANS</td>
<td>GGGACAAGTTTTGATACAAAAAGGAGCTTGGGAGGA</td>
<td>GGGGACCCTTTGTACAAAGAAAGGCAAGGACAGGAAG</td>
</tr>
<tr>
<td></td>
<td>GATAGAACCAGGAGCTTGGGAGGAAGGAAGGACAG</td>
<td>TGGGATTTTAATCATTTTTTTCAGACTTTCACAA</td>
</tr>
<tr>
<td>AtANR</td>
<td>CACCATGGACAGACTTTACACACACCCGGATCG</td>
<td>CACCTCATTTAGCTTTGATCAAATCCTTTGACTC</td>
</tr>
</tbody>
</table>

Using the SD/D TOPO enzyme from Invitrogen’s Gateway® recombination system AtDFR and AtANR DNA fragments were inserted into pENTR by following protocol. The BP clonase enzyme from the Gateway® system was used to insert AtANS into pDONR221 by following protocol. All three were transformed using Top10 cells (Invitrogen) and propagated onto 1.5% agarose NZY medium plates with 50 µg/mL kanamycin for antibiotic resistance selection. AtANR was inserted into the destination vector pDest17, and both AtANS and AtDFR were inserted into pDest15 and AtANS was also cloned into pDest14 using the Gateway® LR clonase enzyme following protocol. They were then transformed into Top10 cells for DNA propagation. pDest14 contains no tag, pDest15 contains an N-terminal GST tag, and pDest 17 contains an N-terminal Ni²⁺ tag.
NgCPS in the entry vector pENTR had been previously made by the Peters Lab and was obtained and cloned into pDest17. sSsSS in pDest17 had been previously made by the Peters Lab and was also obtained. Accuprime Pfx polymerase (Invitrogen) was used to clone out NgCPS, sSsSS, and DM3 (from DM3-sAtANS) with 19 bp overhangs to add the DM3 tag to the insert for future attachment to the nanoparticle. Primers used for cloning can be seen in Table 2. After addition of the DM3 tag, NgCPS-DM3 and sSsSS-DM3 were sequence verified and cloned into pDest15 for expression.

Table 2. Primers used to add DM3 tag to NgCPS and sSsSS

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM3-F</td>
<td>CACCATGGACCCGAGCAAAG</td>
</tr>
<tr>
<td>DM3-R</td>
<td>TCAACCGCCACCACTGCC</td>
</tr>
<tr>
<td>DM3-NgCPS-R</td>
<td>GCTTACCTGAATATCCATCGGAACCGGCCACCACGGCC</td>
</tr>
<tr>
<td>DM3-NgCPS-F</td>
<td>GCAGTGGTGGCGGTTCCGATGGATATTCAGGTAAAG</td>
</tr>
<tr>
<td>DM3-sSsSS-F</td>
<td>GTGGCAGTGGTGCCGTCGATGGATATTCAGGTAAAG</td>
</tr>
<tr>
<td>DM3-sSsSS-R</td>
<td>TTCTTTTCATTTTTCGTCGTAACCCACGTGAC</td>
</tr>
</tbody>
</table>

**Protein Expression**

For protein expression of DFR, ANS, ANR, NgCPS, and sSsSS the genes were transformed to C41 (DE3) cells. 50 µg/mL carbenicillin was included for antibiotic selection during each step of the expression process. One colony from the transformation was selected to inoculate 5 mL NZY media starter cultures and shaken at 200 rpm at 37°C overnight. They were enlarged to 50 mL NZY media at 37°C for two hours then
transferred to 1L NZY media and allowed to grow to an optical density of 0.6-0.8 $A_{600}$.
The cells were then allowed to equilibrate at 16°C for 1 hour before they were induced by
the addition of 1 mM isopropyl-β-thiogalactopyranoside (IPTG). The cells were left to
shake overnight for a total induction time of 16 hours.

Cells were harvested by centrifuging the media at 5,000 g for 20 minutes at 4°C.
The supernatant was discarded and the cell pellet was re-suspended in lysis buffer (50
mM Bis-Tris, pH 6.8, 150 mM KCl, 10 mM MgCl$_2$, 10 % glycerol for 6xHis tagged
protein or 20 mM Tris-HCl pH 7.0, 200 mM NaCl, 1 mM EDTA, 1 mM β-
mercaptoethanol for other applications). The re-suspended cells in lysis buffer were
lysed by sonication (3 x 8 s continuous output at 30 % amplitude). The lysed cells were
clarified by centrifugation at 15,000 g for 20 minutes at 4°C.

**Protein Purification**

AtDFR (42800.6 Da) was purified using glutathione-agarose resin (Sigma-
Aldrich). 1 mM Triton®X-100 was added to the clarified cell lysate containing
pDest15/AtDFR (70,155 kDa) before incubation with 3 mL resin (50% v/v in PBS-T) on
a rotisserie at 4°C for 1-2 hours. The glutathione-agarose resin was prepared by swelling
the lyophilized powder in sterile water overnight at 200 mL/g on a rotisserie at 4°C. The
agarose beads were then washed thoroughly with 10 volumes of equilibration buffer
(PBS-T) before incubation with the clarified cell lysate. 1 mM dithiothreitol (DTT), 0.1
mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM phenylmethylsulfonyl fluoride
(PMSF) were added during incubation and to each following step.

After the incubation period, resin was collected by spinning at 1,000 rpm for 5
minutes in a clinical centrifuge at 4°C. The supernatant and each following wash were
collected for SDS-PAGE analysis. The resin was re-suspended in 5 mL PBS-T and collected as described previously. This wash step was repeated 3 times. The resin was then re-suspended in a final wash and transferred to a mini column under gravity flow and the wash was allowed to flow through and collected. 10 mL of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5) was added to the column and the eluate was collected and analyzed by SDS-PAGE. The purified protein was quantified by $A_{280}$ using the GST-tagged AtDFR extinction coefficient of 85,930 M$^{-1}$cm$^{-1}$.

AtANS (with GST tag: 83902.28 Da; without: 57902.28 Da) with the GST tag was purified following the same protocol as AtDFR. ANS lysate with no tag was purified on an AKTA FPLC system using a Mono Q HR 5/5 (GE Healthcare) column. Buffers used are as follows: buffer A was 50 mM Bis-Tris buffer and buffer B was 50 mM Bis-Tris and 3 M NaCl, both pH 6.8. The method is as follows: 0-4 minutes at 2% buffer B, 4-14 minutes gradient to 10% buffer B, 14-17 minutes at 100% buffer B. Flow rate was 1 mL/ min with 0.5 mL fractions collected, all at 4°C. Final protein was quantified by $A_{280}$ using the GST-tagged AtANS extinction coefficient of 126 110 M$^{-1}$ cm$^{-1}$ and 84 950 M$^{-1}$ cm$^{-1}$ with no GST tag.

AtANR (38,745 Da) was purified using Ni-NTA resin (Sigma-Aldrich). 3 mL resin (50% v/v in bind buffer: 50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole) was incubated with the clarified lysate containing pDest17/AtANR for 1-2 hours at 4°C on a rotisserie. 1 mM DTT was added during the incubation period and to each additional step. The resin was collected as described previously and washed 3 times with wash buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 20 mM imidazole). The resin was then re-suspended in a final wash and transferred to a mini column under
gravity flow and the wash was allowed to flow through. 10 mL of elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 250 mM imidazole) was added to the column and the eluate was collected and analyzed by SDS-PAGE. The purified protein was quantified by A_{280} using the extinction coefficient of 31,750 M^{-1} cm^{-1} for pDest17/AtANR.

NgCPS and sSsSS in pDEST17 followed the same purification procedure as 17/ANR. NgCPS and sSsSS with the DM3 tag were cloned into pDest15, and followed the same purification procedure as for DFR and ANS. Constructs made along with their molecular weights and extinction coefficients used to verify the correct size via SDS-PAGE and concentration of constructs can be seen in Table 3.

<table>
<thead>
<tr>
<th>Protein construct</th>
<th>Molecular weight</th>
<th>Extinction coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-NgCPS-DM3</td>
<td>128251.53</td>
<td>216670 M^{-1} cm^{-1}</td>
</tr>
<tr>
<td>6xHis-NgCPS</td>
<td>83637.72</td>
<td>126720 M^{-1} cm^{-1}</td>
</tr>
<tr>
<td>GST-DM3-sSsSS</td>
<td>106049.15</td>
<td>164710 M^{-1} cm^{-1}</td>
</tr>
<tr>
<td>6xHis-sSsSS</td>
<td>61304.15</td>
<td>74760 M^{-1} cm^{-1}</td>
</tr>
</tbody>
</table>

**Activity Assays**

AtDFR assays consisted of 100 mM Tris-HCl pH 7.0, 2 mM NADPH, and 100 µM dihydroquercetin (DHQ) in 1 mL final volume. 100 µg purified protein was added to the reaction, which was incubated at 37°C for 30 minutes and then stopped with 1 mL ethyl acetate extraction. The extraction was repeated two times for a final volume of 3 mL. Each extraction was mixed by vortexing for 1 minute, and any emulsions were broken by centrifugation at 3,000 rpm in a clinical centrifuge. The final extract was dried.
under nitrogen gas and the residual was dissolved in an n-butanol/HCl (95%/5% v/v) solution and incubated at 95°C for 30 minutes as described (Stafford and Lester 1980) to convert the unstable leucocyanidin to cyanidin. The cyanidin concentration was then analyzed by UV absorption at 550 nm to determine enzymatic activity.

AtANS assays were designed to measure the conversion of dihydrorquercetin (DHQ) to quercetin. Each contained 100 mM MOPS, pH 6.2, 20 mM ascorbate, 160 μM 2-oxoglutarate, 40 μM FeSO₄, 0.1 mg/mL bovine serum albumin, 10 μL catalase, 800 μM DHQ and varying amounts of enzyme in a total volume of 1 mL. Assays were incubated at 37°C for 10 minutes to 1 hour and stopped with the addition of 25 μL formic acid. Any precipitate was cleared by centrifugation at 3,000 rpm in a clinical centrifuge. 100 μL of the acidified assay was analyzed on an Agilent 1200 series HPLC with an Eclipse C18-reverse phase column (150 mm X 4.6 mm; bead size, 5 μm, Agilent). The method is as follows: 0-10 min 10%-40% acetonitrile, 10-15 min 40%-10% acetonitrile with 1% formic acid throughout.

To test the activity of AtANR an assay was designed to measure the conversion of cyanidin chloride to epicatechin. The assay contained 100 mM Tris-HCl, pH 7.0, 2 mM NADPH, 100 μM cyanidin chloride, and 100 μg purified protein in a total volume of 1 mL. Each assay was incubated at 37°C for 30 minutes and stopped with ethyl acetate. The assay was extracted with 3 x 1 mL ethyl acetate as described previously and the extracts were dried under nitrogen. They were then re-dissolved in 30 μL methanol for HPLC analysis using the same procedure as previously described with a 25 μL injection.

Sclareol synthase assays used a coupled assay procedure to test for the conversion of GGPP to sclareol. 50 μM GGPP was dried under nitrogen, then the following assay
constituents were added to the final concentration indicated: 50 mM HEPES pH 7.4, 5 mM MgCl$_2$, 10 % glycerol, 100 mM KCl, 5 mM DTT, and H$_2$O to 1 mL. The reaction mixture was incubated at 30°C for 10 minutes before addition of NgCPS and sSsSS. Upon enzyme addition the assay was briefly vortexed and incubated for 1 hour at 30°C. The reaction was stopped with 3 x 1 mL hexane extraction. The assay extract was dried under nitrogen and re-suspended in 50 µL hexane before running on the GC-FID to quantify the amount of sclareol produced and GC-MS to verify sclareol as the product formed.
CHAPTER 3: RESULTS AND DISCUSSION

Arabidopsis thaliana Flavonoid biosynthetic enzymes

DFR was expressed because of the stability and availability of its substrate. Leucoanthocyanidin, the substrate for ANS, is unstable and cannot be purchased. By including DFR in the system, leucoanthocyanidin could be produced, lyophilized, and stored at -80°C for future assays. Alternatively, DFR could be included in the assay mixture bound to glutathione resin along with dihydroquercetin to make leucoanthocyanidin for the immobilized ANS then removed via centrifugation.

DFR was successfully purified from E. coli using glutathione resin. Correct protein size was confirmed via SDS-PAGE, and the amount of purified protein was quantified using the extinction coefficient of 85,930 M⁻¹cm⁻¹. Activity was confirmed using the previously stated assay procedure to determine cyaniding concentration at 550 nm. Note the pink color observed in Figure 13, which shows a successful DFR assay. DFR activity was retained after 4+ months storage at -80°C and can be used as a way to produce ANS substrate in future assays.

ANR was designed to be able to be immobilized with a Ni-6xHis attachment, which is why it was cloned into and expressed in the pDest17 vector. ANR was
expressed, purified, and quantified using the extinction coefficient 31,750 M\(^{-1}\) cm\(^{-1}\). Purified protein size was verified via SDS-PAGE. To test the activity of ANR the turnover from cyanidin to epicatechin was measured. Cyanidin has a maximum absorbance of 550 nm, whereas epicatechin has an absorbance at 280 nm. ANR assays were performed as previously described and run on the HPLC to verify activity by epicatechin production (Figure 14). ANR was shown to retain activity after storage at -80°C for several months, and should be able to be used in future assays with ANS and DFR included.

When *e. coli* optimized ANS was ordered it was designed with an N-terminal monomeric streptavidin tag, DM3, that was separated from the enzyme with a (glycine\(_3\)-

![Figure 14](image_url)

Figure 14. HPLC chromatograms of ANR assay including substrate and product standards at 280 nm: A. cyanidin chloride standard. B. epicatechin standard. C. ANR assay extract showing the assay product, epicatechin, formation.
serine$_4$ linker to provide flexibility between the nanoparticle and the enzyme. ANS was first purified using the GST tagged pDest15 vector. Purified protein was quantified using the extinction coefficient of $126\,110\, M^{-1}\, cm^{-1}$, and protein size was verified by SDS-PAGE. The ANS activity assay was extremely difficult to replicate and ANS showed little or no activity in most purifications. To optimize the assay and consistently achieve ANS activity a range of different assay conditions was attempted. pH ranges from 6.0-8.0 were tested using several different buffer systems (HEPES, MOPS, MES, Tris-HCl) and the best pH was found to be 6.5. However, even at this pH ANS activity (measured by turnover of DHQ to quercetin on the HPLC) was rarely seen and not at all consistent. The ANS sequence was double checked and found to be correct, and the protein that was purified was consistent with the expected GST-DM3-ANS size. To determine if either the GST-tag or addition of DM3 was causing the lack of ANS activity each was removed individually and both were removed altogether leaving only the ANS sequence which is known to be active (Turnbull et al., 2000). Following the previously described protocol the enzyme was cloned into pDest14 both with and without the DM3 tag and purified via FPLC (Figure 15), but no activity could be seen in either case. After several months of attempting to purify an active form of ANS with no
success it was decided that work should be started on the backup sclareol synthase system.

**Backup system: sclareol synthase**

NgCPS and sSsSS were expressed with several combinations of the DM3 and 6xHis tag to provide flexibility in attachment to the nanoparticle. NgCPS was expressed and purified with both an N-terminal 6xHis tag and a C-terminal DM3 tag. sSsSS was also expressed and purified with an N-terminal 6xHis tag and a C-terminal DM3 tag as well as an N-terminal DM3 tag. Activity of each construct was verified by setting up coupled assays and running the assay extracts on the GC-FID and GC-MS to detect sclareol.

Figure 16 shows a sclareol standard run on the GC-FID as well as a successful coupled assay extract. First, the N-terminal 6xHis tagged enzymes were coupled to test activity of the native enzymes with no manipulations. After activity was shown with this 6xHis tag the DM3 tagged constructs were introduced. NgCPS showed the highest activity with the N-terminal 6xHis tag, although the C-terminal DM3
tag showed limited activity as well. To keep future work simplified only 6xHis tagged NgCPS was used to test sSsSS constructs. sSsSS showed efficient sclareol production with the 6xHis tag as well as both the N- and C-terminal DM3 tags.

The GC-FID peak areas for several preparations of sSsSS can be seen in Table 4. Each sample was also run on the GC-MS to verify that sclareol was produced. These results indicate that the NgCPS and sSsSS sclareol synthase system can be used while moving forward with the enzyme co-localization.

Table 4. GC-FID data of coupled NgCPS and sSsSS assays with various

<table>
<thead>
<tr>
<th>NgCPS tag</th>
<th>sSsSS tag</th>
<th>ug NgCPS / sSsSS</th>
<th>uM GGPP</th>
<th>Peak Area</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>His</td>
<td>His</td>
<td>50/50</td>
<td>50</td>
<td>1112.28</td>
<td>14.612</td>
</tr>
<tr>
<td>His</td>
<td>C-term DM3</td>
<td>50/50</td>
<td>50</td>
<td>220.889</td>
<td>14.599</td>
</tr>
<tr>
<td>His</td>
<td>C-term DM3</td>
<td>50/50</td>
<td>50</td>
<td>112.485</td>
<td>14.593</td>
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<tr>
<td>His</td>
<td>His</td>
<td>50/50</td>
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<td>1183.53</td>
<td>14.608</td>
</tr>
<tr>
<td>His</td>
<td>N-term DM3</td>
<td>50/50</td>
<td>50</td>
<td>154.613</td>
<td>14.597</td>
</tr>
<tr>
<td>His</td>
<td>His</td>
<td>50/50</td>
<td>50</td>
<td>137.745</td>
<td>14.594</td>
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<tr>
<td>His</td>
<td>His</td>
<td>50/50</td>
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<td>1028.22</td>
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<tr>
<td>His</td>
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<td>50/50</td>
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<td>887.617</td>
<td>14.605</td>
</tr>
<tr>
<td>His</td>
<td>N-term DM3</td>
<td>50/50</td>
<td>50</td>
<td>139.725</td>
<td>14.597</td>
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<tr>
<td>No enzyme control</td>
<td>0/0</td>
<td>50</td>
<td>41.891</td>
<td>14.602</td>
<td></td>
</tr>
</tbody>
</table>

After activity was confirmed in both NgCPS and sSsSS with separate tags, the next step was to purify large amounts of the protein constructs for future use in testing enzymatic activity before and after co-localization onto the nanoparticle. Table 5 shows the constructs prepared and concentrations after purification. Each purification was stored at -80°C for future testing. The constructs will be tested both before and after co-localization onto the nanocarrier to determine if co-localization increases the product yield, specifically by measuring the formation of sclareol.
Table 5. Final sclareol synthase constructs purified

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Tag</th>
<th>Date prepared</th>
<th>Final concentration (ug/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17/ssss</td>
<td>His</td>
<td>11/20/12</td>
<td>0.616</td>
</tr>
<tr>
<td>17/ngcps</td>
<td>His</td>
<td>11/26/12</td>
<td>0.681</td>
</tr>
<tr>
<td>17/ssss</td>
<td>His</td>
<td>12/2/12</td>
<td>0.863</td>
</tr>
<tr>
<td>17/ngcps</td>
<td>His</td>
<td>12/2/12</td>
<td>0.807</td>
</tr>
<tr>
<td>15/ngcps-dm3</td>
<td>C-terminal DM3</td>
<td>12/14/12</td>
<td>0.142</td>
</tr>
<tr>
<td>15/ssss-dm3</td>
<td>C-terminal DM3</td>
<td>12/14/12</td>
<td>0.239</td>
</tr>
<tr>
<td>17/ngcps</td>
<td>His</td>
<td>12/18/12</td>
<td>0.238</td>
</tr>
<tr>
<td>17/ssss</td>
<td>His</td>
<td>12/18/12</td>
<td>0.386</td>
</tr>
<tr>
<td>15/dm3-ssss</td>
<td>N-terminal DM3</td>
<td>1/7/13</td>
<td>0.599</td>
</tr>
<tr>
<td>15/ssss-dm3</td>
<td>C-terminal DM3</td>
<td>1/7/13</td>
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<tr>
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<td>His</td>
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<td>15/dm3-ssss</td>
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<td>1/9/12</td>
<td>0.274</td>
</tr>
<tr>
<td>17/ngcps (2)</td>
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<td>0.231</td>
</tr>
<tr>
<td>17/ngcps (6)</td>
<td>His</td>
<td>1/10/13</td>
<td>0.304</td>
</tr>
</tbody>
</table>
CHAPTER 4: CONCLUSION

The aim of this project was to develop a system of multiple sequential enzymes with tags for attachment onto a nanoparticle platform. It is thought that this co-localization might lead to increased output of the desired product by keeping the enzymes in close proximity to each other in the reaction mixture. This could occur by reduction of unstable intermediate side reactions and by intermediate shuttling that kinetically favors product formation by the enzymes. In order to test these effects, a system of two or more enzymes with several fusion tags to be developed, expressed and purified. Also, there needed to be a way to measure and compare product formation before versus after immobilization to quantify the results of co-localization.

Two biosynthetic pathways with applications towards human health and the flavor and fragrance industry were investigated. The first system, involved in flavonoid biosynthesis, was chosen because the pathway has been well characterized. The end products, flavan-3-ols, have been shown to have anti-cancerous and anti-oxidant properties in humans. Thus, creating an efficient system to express and purify the enzymes involved for co-localization onto a nanocarrier is desirable as a way to industrially synthesize large amounts of the beneficial compounds. The second system investigated involves the synthesis of sclareol, a compound that has applications in the flavor and fragrance industry as a precursor to ambrox, an analog of ambergris, which has long been used as a fixative in high-end perfumes but has become expensive and has limited availability.

The enzymes of interest for each pathway were obtained and manipulated to have one of two tags for attachment onto a nanocarrier: either an N-terminal 6xHis-tag or a
monomeric streptavidin tag, DM3. Once the correct constructs were obtained they were cloned into *E. coli*, expressed, and purified. The purified proteins were then tested in assays that were developed and optimized to measure the conversion of substrate to product.

The first system attempted was the flavonoid system, where the sequential enzymes *anthocyanidin synthase* (ANS) and *anthocyanidin reductase* (ANR) would be attached to the platform, and *dihydroflavonol reductase* would be included in the reaction mixture to convert dihydroquercetin to leucoanthocyanidin, the unstable substrate for the ANS/ANR system. ANS would be expressed with a DM3 tag for attachment, and ANR would include a 6xHis tag. Unfortunately, this flavonoid system did not work collectively due to difficulties achieving activity in ANS. DFR and ANR were purified with high activity even after several months storage at -80°C, but even after a year of work attempting to achieve consistent activity in ANS using several different constructs and purification methods, the lack of promising progress led to a project transition to work on the sclareol synthase backup system.

The backup system was much more straightforward to work with. Within a short time frame consistent activity in coupled assays was achieved with several different combinations of tags for enzyme attachment. NgCPS, the first enzyme in the sclareol synthesis system showed high activity with an N-terminal His-tag, and moderate activity with a C-terminal monomeric streptavidin tag, DM3. Sclareol synthase, or sSsSS, also showed high activity with an N-terminal His-tag and constructs with an N- and C-terminal DM3 tag showed good activity as well. This enables the enzymes to be co-
localized onto a nanoparticle platform with control over attachment method and placement, as well as the ratios of enzymes attached.

Several milligrams of each sclareol synthase construct have been prepared for future work on this project. The next step is to test the enzymes activity before and after attachment to a nanoparticle to compare the product yields. Different combinations of tagged enzymes can be used to compare the attachment methodologies and test for differences between the constructs that have been made and their relative activities. The data collected using the purified proteins that have been made should add to current information known about how enzymes interact when they are attached to a platform and any benefits this attachment may provide. In the larger scale this technique could be used for more efficient production of compounds of interest by increasing the product yield using the strategies that have been outlined. A working system which enables future work to study these possibilities has been developed and will be employed to gain valuable insight into the interactions and benefits of a multiple enzyme system co-localized onto a nanoparticle platform.
REFERENCES


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