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Doctoral Dissertation

Production of recombinant human acid sphingomyelinase
in *Nicotiana benthamiana*

PANYAWECHAMONTRI KULACHATR

January 2026

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DECLARATION

“I hereby declare that this dissertation is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which has been used in it. This dissertation has also not been submitted for any degree in university/institution previously.”

.....

PANYAWECHAMONTRI KULACHATR

2026

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List of abbreviations

°C	Degree Celsius
μM	Micromolar
2xYT	2x Yeast extract tryptone medium
4-MU	4-Methylumbelliferone
4-MUG	4-methylumbelliferyl-β-D-glucuronide
5'-UTR	5' Untranslated region
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
ASM	Acid sphingomyelinase
ASMSp	Original signal peptide from human ASM
AtADH	<i>Arabidopsis thaliana</i> alcohol dehydrogenase
AWF	Apoplastic wash fluid
BSA	Bovine serum albumin
BY2	Bright yellow 2
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CNS	Central nervous system
CTD	C-terminal domain
DEAE	Diethylaminoethyl Sepharose CL-6B
DNA	Deoxyribonucleic acid
dpi	Days post infiltration
<i>E. coli</i>	<i>Escherichia coli</i>
ERT	Enzyme replacement therapy
ExtASM	Full-length ASM fused to extensin signal peptide
ExtSp	Extensin signal peptide from <i>N. benthamiana</i>
F	Forward primer
FDA	The U.S. Food and Drug Administration
g	Gram
<i>g</i>	The acceleration due to gravity
GFP	Green fluorescent protein

GUS	β-glucuronidase
GMP	Good manufacturing practice
h	Hour
HIC	Hydrophobic interaction chromatography
HRP	Horseradish peroxidase
HSP	Heat shock protein
IEX	Ion exchange chromatography
kDa	Kilodalton
kV	Kilovolt
L	Liters
LSDs	Lysosomal storage disorders
MES	2-(<i>N</i> -morpholino) ethanesulfonic acid
mF	Millifarad
mg	Milligrams
min	Minute
mM	Millimolar
mRNA	Messenger RNA
<i>N. benthamiana</i>	<i>Nicotiana benthamiana</i>
nanoLC-MS/MS	Nano liquid chromatography-tandem mass spectrometry
NOS	Nopaline synthase
<i>NPC1</i>	NPC intracellular cholesterol transporter 1
<i>NPC2</i>	NPC intracellular cholesterol transporter 2
NPD	Niemann-Pick disease
NPT II	Neomycin phosphotransferase gene
ntExtASM	Truncated ASM fused to extensin signal peptide
OD ₆₀₀	The optical density at a wavelength of 600 nm
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PL	Proline-rich linker
pLDDT	Predicted local distance difference test
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride

R	Reverse primer
RB and LB	Right and left borders of T-DNA
RNA	Ribonucleic acid
RT-qPCR	Reverse-transcription quantitative PCR
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf21	<i>Spodoptera frugiperda</i> 21
Sf9	<i>Spodoptera frugiperda</i> 9
SMPD1	Sphingomyelin phosphodiesterase 1
Sp	Signal peptide
SP	Sulfopropyl-550C
SRT	Substrate reduction therapy
<i>tetR</i>	Tetracycline resistance
TSP	Total soluble protein
U	Unit

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Chapter 1

General Introduction

1.1 Lysosomal storage disorders

Lysosomal storage disorders (LSDs) are a complex group of over 70 rare inherited genetic conditions that occur when enzymes or proteins essential for proper lysosomal function are deficient or defective. Lysosomes, which are responsible for breaking down various substances within cells, become overwhelmed as substrates accumulate due to these enzyme deficiencies. This buildup leads to cellular dysfunction, causing a range of issues like tissue damage, organ failure, and disruption of normal metabolic processes. Although each disorder is individually rare, collectively, LSDs affect approximately 1 in 5,000 live births. LSDs usually appear in infancy and childhood, though some forms can have an onset in adulthood. The severity of LSDs can vary widely, with symptoms affecting different organs and tissues, often leading to significant neurological and physical impairments (Platt et al., 2018). The severity and symptoms of each disorder depend on which enzyme is deficient, what substances accumulate, and which organs or tissues are involved. The progression of LSDs can be understood in three stages (Figure 1): starting with genetic mutations that lead to changes at the cellular level, and then leading to symptoms in organs, ultimately affecting the body's overall function. Several studies have reported on the prevalence of LSDs (Chen et al., 2016; Parenti et al., 2021; Sheth et al., 2014). These disorders are recognized as a major public health burden in developing countries.

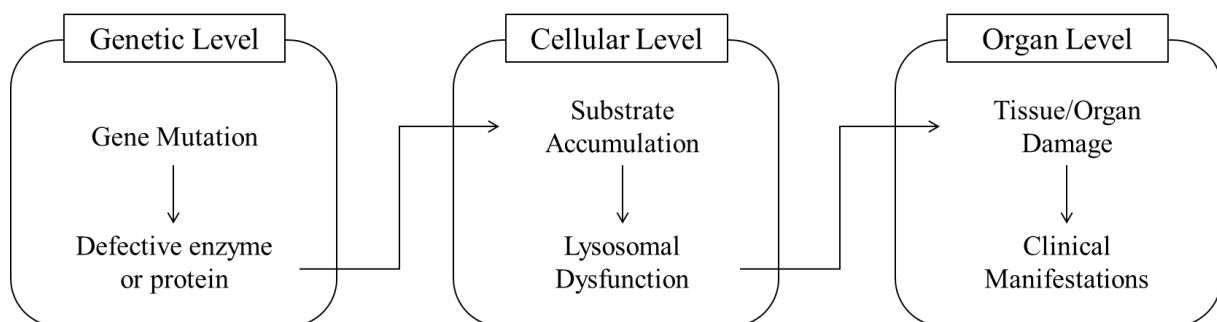


Figure 1 The diagram illustrating the progression of LSDs across three biological levels: genetic, cellular, and organ levels.

1.2 Niemann-Pick Disease

Niemann-Pick disease (NPD) is a rare and progressive LSDs caused by mutations affecting lysosomal function, leading to lipid accumulation and multi-organ involvement. NPD is classified into three main types based on the presence or absence of neurological problems: type A, type B, and type C. NPD types A and B result from mutations in the *SMPD1* genes, which encodes acid sphingomyelinase (ASM, EC 3.1.4.12), also known as sphingomyelin phosphodiesterase 1. ASM is a lysosomal enzyme that catalyzes the hydrolysis of sphingomyelin into phosphocholine and ceramide (Figure 2). Deficiency of ASM leads to the accumulation of sphingomyelin and other lipids in various organs, including the spleen, liver, lungs, and brain, which underlies the clinical manifestations of NPD types A and B (Gaudioso et al., 2023; Pinto et al., 2021). NPD type A is the most severe form, with symptoms appearing in infancy and complete loss of ASM activity, resulting in a life expectancy of less than three years (Schuchman & Desnick, 2017). NPD type B is generally milder, caused by partial loss of ASM activity, with symptom severity ranging from mild to severe depending on the individual. In contrast, NPD type C is caused by mutations in *NPC1* or *NPC2*, genes unrelated to *SMPD1*. These mutations disrupt intracellular cholesterol trafficking rather than sphingomyelin metabolism, leading to the accumulation of cholesterol and other lipids within lysosomes. Clinically, type C presents with progressive neurological symptoms such as ataxia, dystonia, and cognitive decline, as well as organ enlargement and neurodegeneration (Santos-Lozano et al., 2015). The key features of NPD are summarized in Table 1.

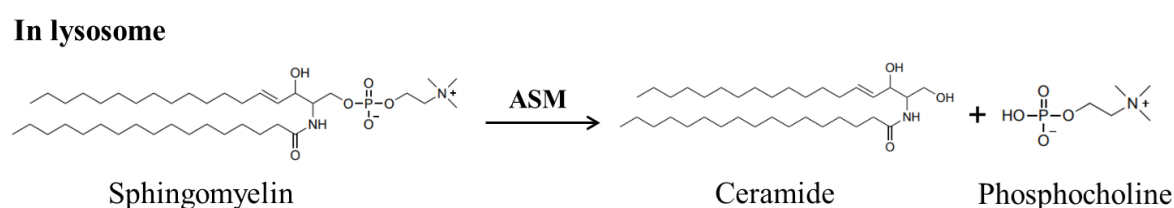


Figure 2 The enzyme reaction of ASM in lysosome of macrophage cells. (Adapted from Hofmann & Dixit, 1998)

Table 1 The characteristics of Niemann-Pick disease in different type.
(Summarized from MedlinePlus, 2020)

	NPD type A	NPD type B	NPD type C
Disease onset	Infancy	Childhood/Adulthood	Childhood/Adulthood
Major symptoms	Hepatomegaly, Splenomegaly, dysphagia, Enlarged bone marrow cavities		
Other symptoms	Brain and nervous system, Cherry red spots	Non-neurological involvement, ocular signs	Brain damage
Mutation in gene	<i>SMPD1</i>	<i>SMPD1</i>	<i>NPC1</i> and <i>NPC2</i>
Chromosome location	11p15.4	11p15.4	18q11.2 and 14q24.3
Frequency	1:250,000	1:250,000	1:150,000
Diagnosis	- Clinical examination and history - Blood tests (Measure of ASM amount in white blood cell or bone marrow sample) - Genetic testing		- Skin biopsy how the skin cells grow, Keep track of how they move and store cholesterol
Treatment	No effective	- Bone marrow transplants - Gene therapy - Blood transfusions - Enzyme replacement therapy (ERT)	- Enzyme inhibitor - Substrate reduction therapy (SRT)

1.3 Structure and biological activity of acid sphingomyelinase

The human ASM enzyme is a glycoprotein composed of a single polypeptide chain with a molecular weight of approximately 50-60 kDa due to the different glycosylation. The enzyme is synthesized as a precursor and undergoes essential post-translational modifications, including glycosylation, for proper folding and activity. The active enzyme is localized in the lysosome, and its optimal pH for enzymatic activity is acidic (around pH 4.5 – 5), which aligns with the acidic environment of the lysosome (Smith & Schuchman, 2008). The enzyme contains a catalytic domain that is responsible for the hydrolysis of sphingomyelin and is characterized by the presence of conserved residues required for substrate binding and catalysis. Recent researches have revealed that the role of ASM extends beyond simple sphingomyelin hydrolysis (Insausti-Urkia et al., 2020; Mir & Thirunavukkarasu, 2023). The biological activity of ASM is critical for the degradation of sphingomyelin in the lysosome. Sphingomyelin is a major component of cellular membranes, particularly in the plasma membrane and myelin sheaths. When ASM hydrolyzes sphingomyelin, it produces ceramide, which is a bioactive lipid that plays a role in various cellular functions, including apoptosis (programmed cell death), signal transduction, and the regulation of membrane fluidity (Choi et al., 2024). The production of ceramide also regulates the sphingolipid metabolic pathway by influencing the balance between sphingolipids and other lipid species. In addition to its role in lipid metabolism, ASM is involved in the regulation of intracellular signaling pathways. For example, ceramide generation can trigger cellular stress responses and activate pathways related to inflammation and cell death (Breiden & Sandhoff, 2021). Furthermore, ASM activity is important for the proper functioning of the immune system, as it influences the response to pathogens and the resolution of inflammation. There are six predicted *N*-glycosylation sites (N88, N177, N337, N397, N505, and N522) in the ASM polypeptide chain with eight intrachain disulfide bonds (Scrima et al., 2023). ASM consists of two main distinct domains; the N-terminal saposin domain, which facilitates membrane binding and activation of the enzyme by mediating interactions with lipid substrates, and a catalytic metallophosphatase domain, which features a special α -helical section at the C-terminus (CTD). These domains are connected by a proline-rich linker region (Xiong et al., 2016) (Figure 3). Similar to other lysosomal enzymes, ASM needs saposin to help it function properly in the body. The saposin domain helps bind and extract lipids from membranes, enabling their breakdown by ASM. It plays a critical role in lipid metabolism, facilitating the interaction of enzyme with membrane lipids (Olmeda et al., 2013; Popovic et al., 2012).

Meanwhile, the catalytic domain of ASM contains the active site responsible for hydrolyzing sphingomyelin into ceramide and phosphocholine. This process is essential for the degradation of sphingolipids, which are key components of cellular membranes. The cooperation between the catalytic and regulatory domains is crucial for the function of ASM, especially in the lysosome, where it helps regulate cellular processes and maintain lipid balance. By controlling sphingolipid metabolism, ASM plays an essential role in maintaining lipid homeostasis, which is vital for overall cell function and health (Breslow & Weissman, 2010).

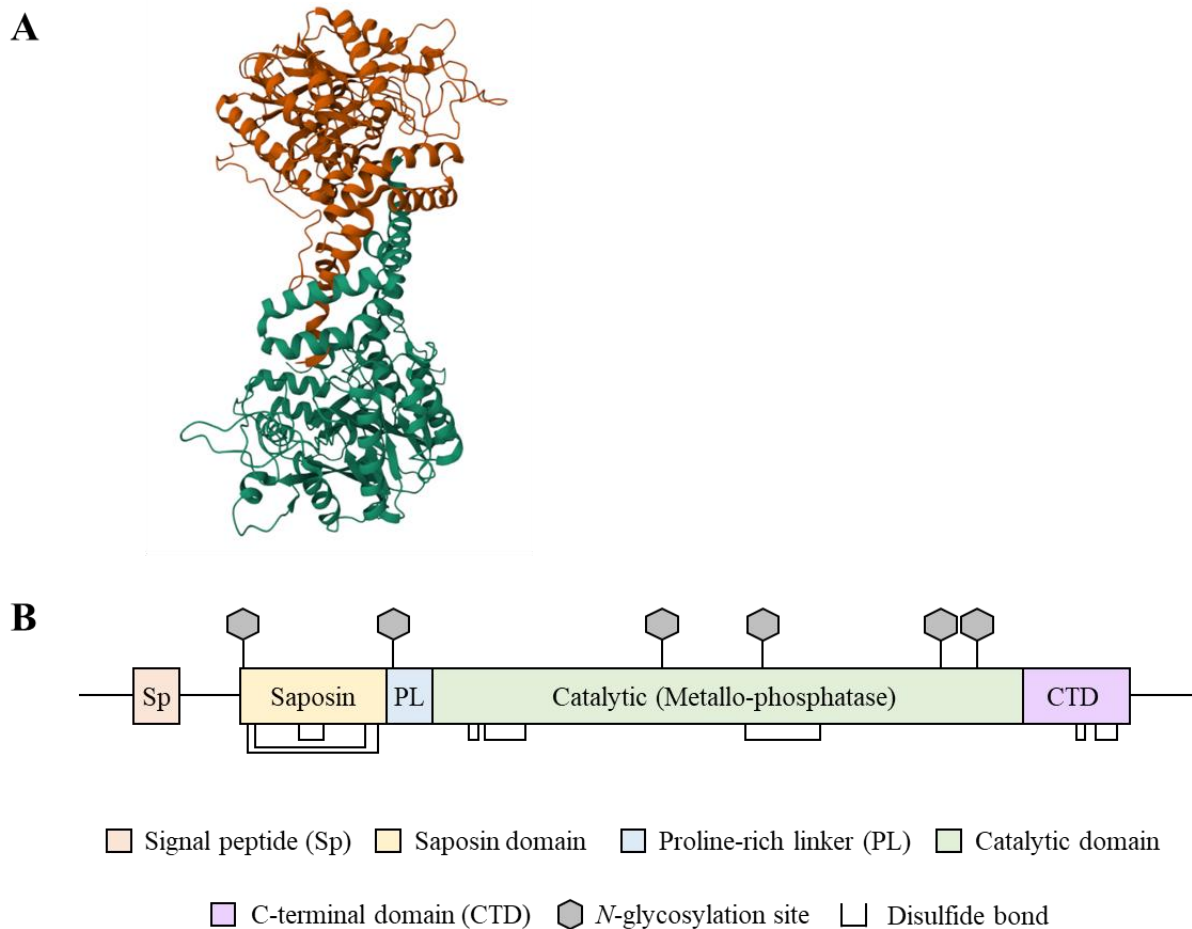


Figure 3 The structure of human acid sphingomyelinase (ASM). (A) Crystal monomeric structure of ASM (protein data bank: 5JG8, PubMed ID: 27349982). (B) Schematic diagram of ASM amino acids sequence. Six *N*-glycosylation sites and eight disulfide bonds were indicated. (Adapted from Gorelik et al., 2016 and Zhou et al., 2016).

1.4 Enzyme replacement therapy of people with Niemann-Pick disease type B

Enzyme replacement therapy (ERT) is being explored as a potential treatment option for NPD type B. This treatment consists of delivering a recombinant form of ASM to patients who are deficient in this crucial enzyme. The recombinant ASM is injected into the body to decrease sphingomyelin accumulation in tissues, which helps to reduce symptoms like liver and spleen enlargement (Kumar et al., 2024). Recombinant ASM has been produced in several expression systems, including Chinese hamster ovary (CHO) cells (He et al., 1999), *Spodoptera frugiperda* 21 (Sf21) cells (Lansmann et al., 2000), Sf9 cells (Gorelik et al., 2016).

Xenpozyme[®] (olipudase alfa; Sanofi, Paris, France) is a recombinant ERT developed to treat NPD type B, specifically for managing non-central nervous system (non-CNS) symptoms. Xenpozyme[®], manufactured by Sanofi, produced in CHO cells. This helps to ease symptoms, including organ enlargement and dysfunction. In 2022, Xenpozyme[®] became the first ERT approved to treat this rare and progressive lysosomal storage disorder, NPD type B, in both pediatric and adult patients. Its approval by major regulatory agencies, including the European Commission, Japan's SAKIGAKE designation, and the U.S. Food and Drug Administration (FDA), marked a major milestone in treating ASM deficiency (Keam, 2022). Xenpozyme[®] not only offers the first approved treatment for NPD type B but also serves as a potential model for treating other LSDs, demonstrating the effectiveness of the ERT approach and paving the way for possible combination therapies. Despite its efficacy, the high cost of Xenpozyme[®] – around \$7,142 per vial in the U.S. and an annual treatment cost of approximately \$780,000 – creates a significant financial barrier, limiting access for many patients who could benefit from this life-saving treatment (Beninger, 2023). Although ERT for NPD type B shows strong potential, high treatment costs continue to limit patient access.

1.5 Production of pharmaceutical proteins

The production of recombinant protein is one of the most powerful techniques used in life sciences, especially in the therapeutic market where human proteins produced through genetic engineering are essential. *Escherichia coli* was the first successfully expression system used for therapeutic protein (Itakura et al., 1977), with insulin being the first U.S. FDA-approved therapeutic protein produced in this system (Pillai & Panchagnula, 2001). Recently, there has been a growing focus on animal-free systems for the production of biopharmaceutical proteins. This shift is driven by concerns over the risk of contamination from human pathogens and the high production costs of proteins derived from animal cells (Butler & Spearman, 2014; Shanmugaraj et al., 2020). Recombinant therapeutic proteins are vital for the treatment and

prevention of numerous diseases. These proteins, including enzymes, antibodies, growth factors, and hormones are produced using different expression systems, such as bacteria, yeast, insect cells, mammalian cells and plants (Burnett & Burnett, 2019; Puetz & Wurm, 2019). However, each expression system has its own set of advantages and limitations, as summarized in Table 2, highlighting the need to choose the most appropriate system based on the specific requirements of the therapeutic protein being produced.

Table 2 Comparison of available expression hosts for the protein production.
Modified from Costa & Sautto, 2025 and Moussavou et al., 2015.

Expression systems	Bacteria	Yeast	Insect cells	Mammalian cells	Plants
Time required	Short	Medium	Medium	Long	Medium
Scale-up capacity	High	High	Medium	Low	High
Contamination risk	Medium	Low	High	High	Low
Protein yield	High	High	Medium	Medium	High
Glycosylation	No	High mannose	Mannose terminal	Correct	Plant specific
Production cost	Cheap	Moderate	Expensive	Expensive	Cheap
Storage cost	Cheap	Moderate	Expensive	Expensive	Cheap

Bacteria are simple to handle with and grow rapidly, but they are unable to perform key post-translational modifications and can accumulate endotoxins, which reduces their effectiveness. Yeast systems are impacted by hyper-mannosylation, which can decrease the effectiveness and half-life of therapeutic glycoproteins (Nielsen, 2013). Insect cell systems, while useful, are costly to cultivate and require expensive media (Giglio et al., 2024). Although most therapeutic proteins (89%) are produced using mammalian cells, specifically CHO cells, this method comes with high production costs and a risk of contamination by human pathogens (Walsh & Walsh, 2022).

1.6 Recombinant protein expression in plants for biopharmaceutical production

Recombinant protein production in plants has emerged as a promising and innovative methods for producing biopharmaceuticals and industrial proteins. This approach utilizes genetically modified plants to express foreign proteins, offering a cost-effective and scalable alternative to traditional expression systems like bacterial, yeast, or mammalian

cell cultures (Boehm, 2007). In recent decades, significant advancements in both academic research (Kim et al., 2024; Kopertekh, 2024; Schillberg & Finnern, 2021) and industry (Nosaki et al., 2021) has highlighted the increasing relevance of animal-free approaches in the production of therapeutic proteins. Plant-based expression systems are gaining attention for heterologous protein expression due to their unique benefits, including low cost, scalability, the ability to perform post-translational modifications, such as glycosylation, which is often necessary for the correct folding and function of therapeutic proteins (Debler et al., 2021; Gomord & Faye, 2004; Kim et al., 2020; Lee & Ko, 2017; Soni et al., 2022). Additionally, plant-based systems offer the advantage of being free from human pathogens, reducing the risk of contamination, a concern often associated with animal cell-based production systems (Giddings et al., 2000; Shin et al., 2019). This makes plant systems particularly attractive for producing biopharmaceuticals, such as vaccines, antibodies, and enzymes.

The journey of plant-based protein production began in 1986 with the successful expression of human growth hormone. This achievement was the first pharmaceutically relevant protein produced in a transgenic tobacco plant (Barta et al., 1986). This breakthrough opened the door for using plants as platforms to produce various therapeutic proteins. In 2012, the first commercial plant-based biopharmaceutical, Taliglucerase alfa (Elelyso®), was approved by the U.S. FDA, marking a significant milestone in the field of plant-based protein production (Fox, 2012). A variety of important pharmaceutical proteins have been successfully produced in plants like rice, maize, soybean, potato, and sunflower (Gerszberg & Hnatuszko-Konka, 2022; Ma et al., 2003).

1.7 *Nicotiana benthamiana* as a promising platform for biopharmaceutical productions

Nicotiana benthamiana has emerged as one of the most promising platforms for biopharmaceutical productions. This plant species, a member of the tobacco family, is increasingly being utilized for the expression of recombinant proteins due to its many advantages in both research and industrial-scale production. *N. benthamiana* is particularly valued for producing therapeutic proteins, such as vaccines, monoclonal antibodies, enzymes, and other biologically active molecules, making it a valuable asset in the biotechnology and pharmaceutical industries. Compared to other plant platforms, *N. benthamiana* stands out as an efficient and effective platform for recombinant protein production since its rapid growth, high biomass, and ease of genetic modification. This plant can quickly express large quantities of recombinant proteins, especially through transient expression, where genes are introduced for a short period, resulting in high yields in less time (Goodin et al., 2008; Li & Wang, 2019).

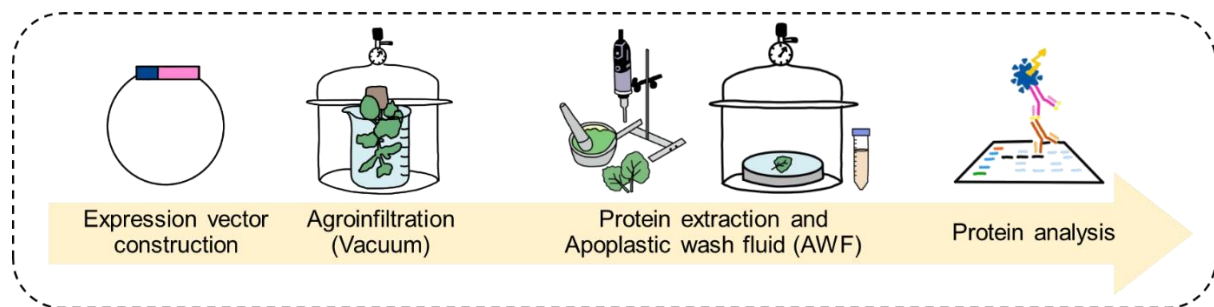
Furthermore, *N. benthamiana* is not used as a food or feed crop, which minimizes the risk of recombinant proteins contaminating the food supply (Tremblay et al., 2010). The suitability of *N. benthamiana* as a biopharmaceutical host has been strongly demonstrated in recent real-world applications. Notably, Medicago successfully developed a COVID-19 vaccine using *N. benthamiana* as the expression platform. The vaccine reached Phase III clinical trials and subsequently received regulatory approval from the Canadian government, marking a significant milestone for plant-based vaccines and illustrating the feasibility of large-scale, clinically relevant protein production in this species (Hager et al., 2022). These achievements underscore *N. benthamiana*'s potential as a rapid, scalable, and safe system for producing vaccines and other biopharmaceuticals. Although plant-based recombinant protein production offers many advantages, using *N. benthamiana* for biopharmaceutical production also presents several challenges. These challenges involve optimizing protein yield and quality, ensuring proper protein folding, and scaling up production from laboratory to industrial levels. Achieving this requires careful control over growth conditions, plant management, and harvesting techniques. Additionally, preserving the functionality and structure of the proteins produced in plants is still an area under active investigation. (Jiang et al., 2019; Lindström Battle et al., 2025).

1.8 Objectives of this study

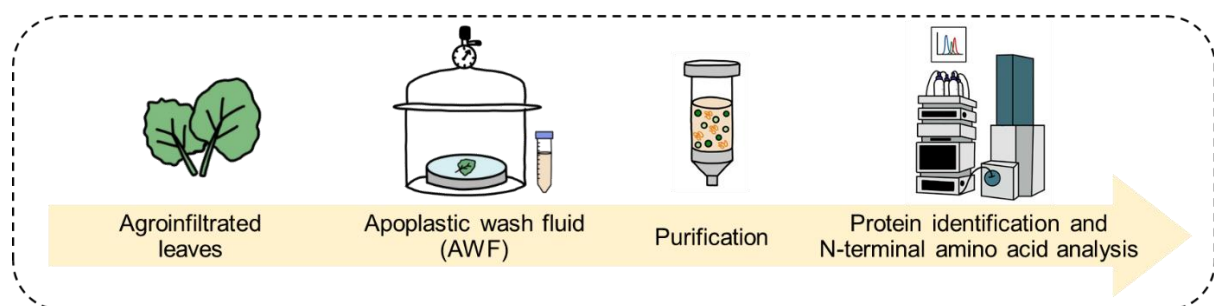
This study aims to explore the production of soluble recombinant human ASM using *N. benthamiana* as an expression system. The research seeks to overcome the challenges associated with the production of functional recombinant proteins in plant-based platforms and proposes *N. benthamiana* as a promising alternative for the expression and purification of therapeutic proteins. Specifically, the research evaluates the potential of *N. benthamiana* as an effective platform for recombinant enzyme production. The original full-length ASM and engineered truncated ASM with a plant-derived secretion signal are compared to optimize ASM expression, localization, and functionality (Chapter 2). Since ASM has not been previously produced in plant systems, this chapter primarily focuses on investigating the production and extraction of plant-produced ASM. Following the production of ASM in plants, the second part (Chapter 3) of this study aims to establish the purification method of ASM within the plant-based expression system. This research explores innovative strategies to improve the purification of ASM protein, including N-terminal amino acid analysis to confirm protein identity and processing. These efforts contribute to the advancing plant-based platforms for large-scale therapeutic protein production. In the third part (Chapter 4), the focus shifts to

increasing ASM expression levels by modifying the expression vector. Specifically, this chapter aims to enhance contribution by minimizing the size of the plant expression vector, thereby improving the efficiency of ASM production in *N. benthamiana*.

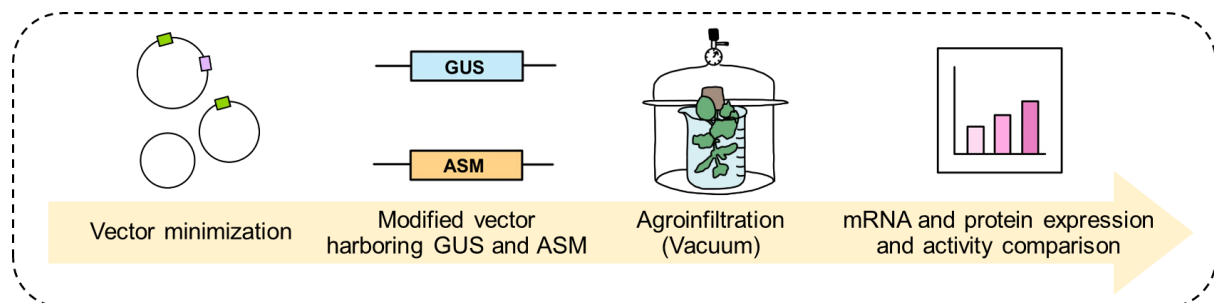
Objective 1: Production of recombinant human ASM as the soluble form in *N. benthamiana* (Chapter 2)



Objective 2: Establishment of ASM purification method in plant, along with protein identification and N-terminal amino acid analysis (Chapter 3)



Objective 3: Enhancement of ASM expression levels through the use of a modified expression vector (Chapter 4)



Chapter 2

Production of human acid sphingomyelinase in *Nicotiana benthamiana* whole plants

2.1 Introduction

Plants offer a promising system for the production of therapeutic proteins and non-pharmaceutical products due to several key advantages. These include: (1) low upstream processing costs, as *N. benthamiana* plants require simple cultivation conditions, providing cost efficiency; (2) a reduced risk of contamination from mammalian pathogens and bacterial endotoxins, ensuring greater safety; (3) the capacity of plants to perform post-translational modifications, particularly *N*-glycosylation, which are similar to mammals, facilitating the proper folding and assembly of recombinant proteins; and (4) the ability to maintain contained, controlled, and sterile conditions that comply with Good Manufacturing Practice (GMP) standards for biopharmaceutical proteins (Debler et al., 2021; Kashima et al., 2016; Kim et al., 2020; Shin et al., 2019). Notably, *N. benthamiana* plants have demonstrated high effectiveness as a platform for the production of recombinant lysosomal enzyme used in ERT. This is supported by many research studies reporting their successful application in producing therapeutic enzymes for diseases such as Gaucher disease (Uthailak et al., 2021), Fabry disease (Kytidou et al., 2017), Hunter syndrome (Sinha et al., 2024). As a result, it is no surprise that plant-based systems have gained growing attention as a next-generation platform for the production of biopharmaceuticals.

Niemann-Pick disease caused by mutations in the *SMPD1* genes, resulting in a deficiency of the lysosomal enzyme acid sphingomyelinase (ASM). This enzyme is responsible for catalyzing the hydrolysis of sphingomyelin into phosphocholine and ceramide. ERT has emerged as a promising treatment for NPD type B, offering a therapeutic approach by replacing the deficient enzyme in patients. Recombinant ASM is administered through injections to help reduce sphingomyelin accumulation, leading to a decrease in many organs.

Agroinfiltration is a method of transient expression in plants that allows for the production of large quantities of recombinant proteins. This technique not only enables rapid production but also ensures high protein expression levels, cost-effectiveness, and scalability. It is particularly valuable for producing vaccines or pharmaceuticals during pandemics due to its fast gene expression capabilities (Spiegel et al., 2022).

The use of expression cassettes to express a foreign gene in plants had a major influence on protein production. The 5'-UTR plays an essential role in the downstream gene expression of promoter activity. The 5'-UTR of the alcohol dehydrogenase gene from *Arabidopsis* (AtADH) was shown to effectively enhance the translational efficiency in tobacco BY2 cells (Sugio et al., 2008). Furthermore, another vital parameter of gene expression is the efficiency of the terminator. It regulates the level of expression by controlling the transcriptional termination and 3' end processing of RNA. *Arabidopsis* heat shock protein (HSP) terminator has been reported that can increased mRNA level about 2-fold over the yield more than nopaline synthase (NOS) terminator (Nagaya et al., 2010). Therefore, the combination of the translational enhancer and terminator was utilized in highly enhanced the levels of gene expression in transgenic plants.

The expression levels of native full-length ASM and an engineered truncated ASM in a plant-based system were compared in this chapter. The full-length ASM corresponds to full-length ASM individually fused to original and extensin signal peptide (ExtSp), while the truncated ASM has been modified to potentially enhance production and functional properties. This comparison aimed to evaluate expression efficiency, protein extraction, and enzymatic activity, laying the foundation for subsequent experimental steps in recombinant protein production.

2.2 Materials and Methods

2.2.1 Plant expression Vector construction

A gene encoding the full-length of human ASM (1,896 bp) was synthesized by GENEWIZ (Tokyo, Japan), using sequence information obtained from the KEGG database (Uniprot ID: P17405, NCBI Reference Sequence: NP_000534.3). The sequence was optimized for codons to enhance expression in *N. benthamiana* and adjusted to remove undesirable motifs, especially GGGXX, as noted by Bazzini et al. (2016). The optimized and original sequences are provided in the supplementary materials. The cDNA encoding ASM was amplified by PCR using the primers ASM-*Nde*I-F and ASM-*Sac*I-R, which are listed in Table 3. After amplification, the product was digested with *Nde*I and *Sac*I and ligated to the pRI201-AN plant expression vector (TaKaRa Bio, Shiga, Japan), which was also cut with *Nde*I and *Sac*I, obtaining the pRI201-ASM construct. To eliminate the native signal peptide, the full-length ASM gene was amplified using the nASM-F and n*Sal*I-ASM-R primers. A 26-amino acid signal peptide from *N. benthamiana* (ExtSp; MGKMASLFATLLVVLVSLSLASESSA), as described by Jiang et al. (2020), was amplified using the n*Nde*I-ExtSp-F and nExtSp-ASM-R primers. This ExtSp sequence was fused to the ASM gene through overlapping PCR, resulting in a full-length ASM construct containing the ExtSp. The resulting product was then digested with *Nde*I and *Sal*I restriction enzymes and inserted into the digested pRI201-AN vector, forming the pRI201-ExtASM construct. To make a truncated ASM, the pRI201-ExtASM plasmid served as the template for two separate PCR reactions using the primer n*Nde*I-ExtSp-F/nExt-ASM-R and truncated ASM-F/n*Sal*I-ASM-R. Both two resulting fragments were fused through overlapping PCR to create the truncated ASM that preserving the ExtSp sequence. Finally, the PCR product was subcloned into the pRI201-AN vector using *Nde*I and *Sal*I sites to yield the pRI201-ntExtASM. The schematic representation of ASM expression constructs are shown in Figure 4A.

Table 3 Primers used for plasmid construction.

Primer ^a	Sequences ^b (5' → 3')
ASM- <i>Nde</i> I-F	ATT <u>CATATG</u> CCAAGGTATGGAGCATC
ASM- <i>Sac</i> I-R	TAAGAGCTCTCAACAGAACAATGGCC
nASM-F	CTTGCTCTTTCTGACTCAAGGGTG
n <i>Sal</i> I-ASM-R	ATTGTCGACTCAACAGAACAATGGCC
n <i>Nde</i> I-ExtSp-F	ATT <u>CATATG</u> GGGAAAATGGCTTCTC
nExtsp-ASM-R	AGTCAGAAAGAGCAAGTGCTGAGCTTTCAGAAGC
nExt-ASM-R	ATAAAGGTTACCCCATCCTGCTGAGCTTTCAGAAGC
Truncated ASM-F	GGATGGGGTAACCTTACATG
35Sp-F	TGAGACTTTTCAACAAAGGG
HSpt-R	CTTATCTTTAATCATATTCCATAGTCCA

^a F and R represent forward and reverse primers, respectively.

^b Underlined sequences indicate the restriction enzyme recognition sites.

2.2.2 Plasmid transformation (Sambrook & Russell, 2001)

The pRI201-ASM, pRI21-ExtASM, pRI201-ntExtASM, and the negative control vector (Figure 4) were introduced into *E. coli* DH5α competent cells by the heat-shock method, as described by Sambrook & Russell (2011). Bacterial culture was spread on 2xYT agar plate containing 50 µg/mL of kanamycin. The positive transformants harboring ASM fragment were selected and isolated after screening by PCR with 35Sp-F and HSpt-R primers (Table 3). To confirm the results, plasmids were analyzed through restriction enzyme digestion and nucleotide sequencing. Subsequently, all plasmid constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 via electroporation using a MicroPulser electroporator (Bio-Rad, Hercules, CA), with the following settings: 2.4 kV voltage, 25 mF capacitance, and 200 Ω resistance. Transformants were selected by growing on media containing kanamycin (50 µg/mL), streptomycin (50 µg/mL), and rifampicin (50 µg/mL).

2.2.3 *Agrobacterium*-mediated transient expression

Wild-type *N. benthamiana* plants were grown in a controlled plant room under a 16 h light and 8 h of dark photoperiod at 25°C and 50% relative humidity. Five-week-old plants were utilized for agroinfiltration. Plants were co-agroinfiltrated with *A. tumefaciens*

containing the vector construct harboring target ASM and p19, a suppressor of RNA silencing from the Tomato bushy stunt virus (TBSV), kindly provided by Professor Atsushi Takeda (Ritsumeikan University), using vacuum infiltration in an infiltration buffer (10 mM MES, 10 mM MgSO₄, 100 µM acetosyringone, pH 5.5) (Wang et al., 2018; Zhang et al., 2023). *A. tumefaciens* harboring ASM and p19 were inoculated in 5 mL of 2xYT media containing kanamycin (50 µg/mL), rifampicin (50 µg/mL), and streptomycin (50 µg/mL). Then, incubated at 30°C with shaking for 2 days. Bacterial cultures were transferred to 200 mL of 2xYT broth in Sakaguchi flask with the same antibiotics and condition. Cultures (200 mL) were harvested and re-suspend in infiltration buffer to obtain each OD₆₀₀ at 0.5. Equal volumes of the ASM- and p19- containing plasmids were mixed in a 1:1 ratio for infiltration. *N. benthamiana* plants were then infiltrated using vacuum infiltration same as previously reported (Nguyen et al., 2023). Agroinfiltrated leaves were harvested to analyze the transient expression level of ASM. In this study, *A. tumefaciens* harboring pRI201-AN was used as a negative control. The experiment was performed in triplicate.

2.2.4 Protein extraction from agroinfiltrated leaves

Infiltrated leaves were ground into a fine powder in liquid nitrogen using a mortar and pestle. Crude protein powders from each collection days were suspended in ASM extraction buffer (25 mM citrate/phosphate, 0.01% Triton X-100, 0.1 mM ZnCl₂, 10 µM Leupeptin, 1 mM PMSF, and cComplete™ EDTA-free protease inhibitor cocktail tablet (Roche, Mannheim, Germany), pH 5.0) that modified from Pentchev et al., (1977) and Yoshida et al., (2017). The 0.5 g of crude protein was suspended in 1 mL of extraction buffer. After centrifugation at 12,000 x *g* for 20 min at 4°C, the supernatant was collected to new microcentrifuge tube. The protein concentration was calculated by the Bradford method (Bradford, 1976). The Protein Assay CBB Solution (5×) (Nacalai Tesque, Kyoto, Japan) was prepared according to the manufacturer's instructions. The absorbance of the protein samples was then measured at 595 nm using an iMark™ Microplate Absorbance Reader. A standard curve constructed using known concentrations of Bovine Serum Albumin (BSA, F-V, pH 5.2; Nacalai Tesque, Kyoto, Japan), was utilized to determine the protein concentrations in the samples. Then, the supernatant was analyzed by SDS-PAGE, Western blot analysis and ASM enzymatic assay as outlined below.

2.2.5 Collection of apoplastic wash fluid via vacuum infiltration

The apoplastic wash fluid (AWF) was prepared following the previous studies (Alkanaimsh et al., 2016; Jiang et al., 2020). Leaves were placed in a steel dish and submerged in AWF buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.02% Silwet L-77 (GE Toshiba Silicones, Tokyo, Japan)). The steel dish was then placed in a vacuum chamber for 1 min to allow infiltration. The buffer-infiltrated leaves were dried and then placed into a syringe, which was positioned inside a 50 mL Falcon tube. The leaves in the falcon tube were centrifuged at $1,000 \times g$ for 10 min at room temperature to separate the liquid from the solid material. The liquid collected was labeled as the AWF sample. The intracellular fraction was then extracted from the remaining leaf tissue using the same method applied to the agroinfiltrated leaves.

2.2.6 Protein analysis by SDS-PAGE and Western blot

Protein samples were mixed with one-fourth volume of 4x SDS-PAGE sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% β -mercaptoethanol and 0.2% bromophenol blue). To determine the optimal heating conditions, the mixture samples were heated at temperature ranging from 100°C to 50°C, with heating times of 5 and 10 min compared for each temperature. Denatured protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using either a SuperSep™ Ace 5-20% gradient gel (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) or self-prepared 10% acrylamide gel. Proteins in the polyacrylamide gel were stained with Coomassie Brilliant Blue (CBB) (CBB Stain One; Nacalai Tesque, Kyoto, Japan) following the manufacturer's instructions. For Western blot analysis, protein separated on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore-Sigma, Burlington, MA, USA). The membrane was blocked with 5% fat-free skim milk (Nacalai Tesque) in phosphate buffered saline with Tween-20 (PBST) buffer (1.47 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 2.7 mM KCl, 137 mM NaCl, and 0.05% Tween-20, pH 7.4) for 1 h at room temperature. It was then incubated with the rabbit polyclonal anti-ASM antibody (Cat# GTX114782; GeneTex, CA, USA) diluted 1:2,000 in Can Get Signal™ solution I (TOYOBO, Osaka, Japan) for 1 h at room temperature or overnight at 4°C. After washing the membrane three times with PBST for 10 min each, it was incubated with ECL™ anti-rabbit IgG conjugated to HRP (Cat# NA934V; Cytiva, Marlborough, MA, USA) at a 1:10,000 dilution in Can Get Signal™ solution II (TOYOBO) for 45 min at room temperature. Following a final wash with PBST (three times for 10 min

each), protein signals were developed using Immobilon Forte Western HRP substrate (Millipore-Sigma) and visualized using the Invitrogen iBright Imaging System 1500. Recombinant human ASM derived from CHO cells (Commercial rhASM; R&D System, Inc., Minneapolis, MN, USA) was used as a positive control, while wild-type and agroinfiltrated empty vector (pRI201-AN) served as negative controls. A prestained protein ladder (PageRuler™; Cat#26616, Thermo Scientific, Waltham, MA, USA) was utilized as a marker to estimate the molecular weights of proteins.

2.2.7 ASM activity measurement

The ASM activity was evaluated using the Acid Sphingomyelinase Assay Kit (Cat#ab252889; Abcam, Cambridge, UK). This colorimetric assay utilizes a probe to quantify phosphocholine produced from the sphingomyelin hydrolysis by ASM at pH 5.0, with absorbance read at 570 nm (OD). The ASM activity in the samples was determined according to the manufacturer's guidelines. One unit (U) of ASM activity was defined as the amount of enzyme that generates 1 μ mol of choline per minute at pH 5.0 and 37°C.

2.2.8 Statistical analysis

This study included three independent biological replicates. The results are presented as the mean $\pm$ standard deviation (SD). To ensure the reliability and reproducibility of the findings, each experiment was performed in triplicates. Statistical analyzes were conducted using IBM SPSS Statistics software (version 30). Analysis of variance (ANOVA) was applied, followed by Duncan's multiple range test to assess differences between groups. Results with a p -value ≤ 0.05 were regarded as statistically significant.

2.3 Results

2.3.1 Construction of plant vector and transient expression of ASM in *Nicotiana benthamiana*

The ASM protein, composed of 631 amino acids, includes a signal peptide at the N-terminus (residue 1-46) and the full-length ASM sequence (residue 47-631). The fragment of ASM was subcloned into the pRI201-AN plant expression vector to create the pRI201-ASM construct (Figure 4A). The plasmid was then introduced into *A. tumefaciens* LBA4404 by electroporation. To assess ASM expression in *N. benthamiana* plants, the pRI201-ASM construct was utilized for transient expression through agroinfiltration in 5-week-old *N. benthamiana* plants. Expression levels were compared between plants co-agroinfiltrated with p19 and those without. The expression of ASM extracted using protein extraction method was confirmed by Bradford assay, CBB staining, Western blot analysis, and ASM activity assay. The Western blot result (Figure 5A) showed ASM bands were detected a target band around 65 kDa using anti-ASM antibody in both conditions and cannot detected any bands in both negative controls; wild-type and empty vector plants. ASM expression was successfully achieved in the infiltrated plants. Co-expression with p19 led to increased ASM production in plants, as evidenced by a 1.5-fold (3.02 ± 0.11 mU/mg TSP) higher ASM activity (Figure 5B). However, despite this increase, the overall expression level of ASM was still relatively low compared to other proteins, as indicated by the CBB results (Figure 5A). This made the purification of ASM from the cell extract both difficult and challenging.

To improve expression and simplify purification, a shorter version of ASM was designed. This decision was based on the 3D structure of ASM from the AlphaFold database (AF-P17405-F1), which showed that the N-terminal region of the protein exhibited low predicted confidence scores (predicted Local Distance Difference Test; pLDDT) (Figure 4B). This indicated reduced reliability in the structural prediction for that region (Jumper et al., 2021). The removal of these amino acids does not affect the function of ASM. Therefore, residues 1 – 84 were excluded, resulting in a truncated ASM (residues 85-631). The original signal peptide was replaced with ExtSp (78 bp) to accelerate the extraction process and target the recombinant protein to the apoplast. Both the full-length ASM and the truncated ASM were fused with the ExtSp and inserted into pRI201-AN to generate two new constructs: pRI201-ExtASM and pRI201-ntExtASM, as illustrated in Figure 4A. According to the Western blot results, the intensity of ASM band showed a gradual increase, starting with the original signal peptide with ASM (pRI201-ASM: 65 kDa), followed by the extensin signal peptide with ASM

(pRI201-ExtASM: 65 kDa), and finally the extensin signal peptide with truncated ASM (pRI201-ntExtASM: 61 kDa) (Figure 6A). A similar pattern was observed in the enzymatic activity of ASM (Figure 6B), with the highest amount of crude ASM (4.01 ± 0.11 mU/mg) produced by pRI201-ntExtASM. Given these findings, the pRI201-ntExtASM construct, which demonstrated the highest ASM expression, was selected for the subsequent experiment.

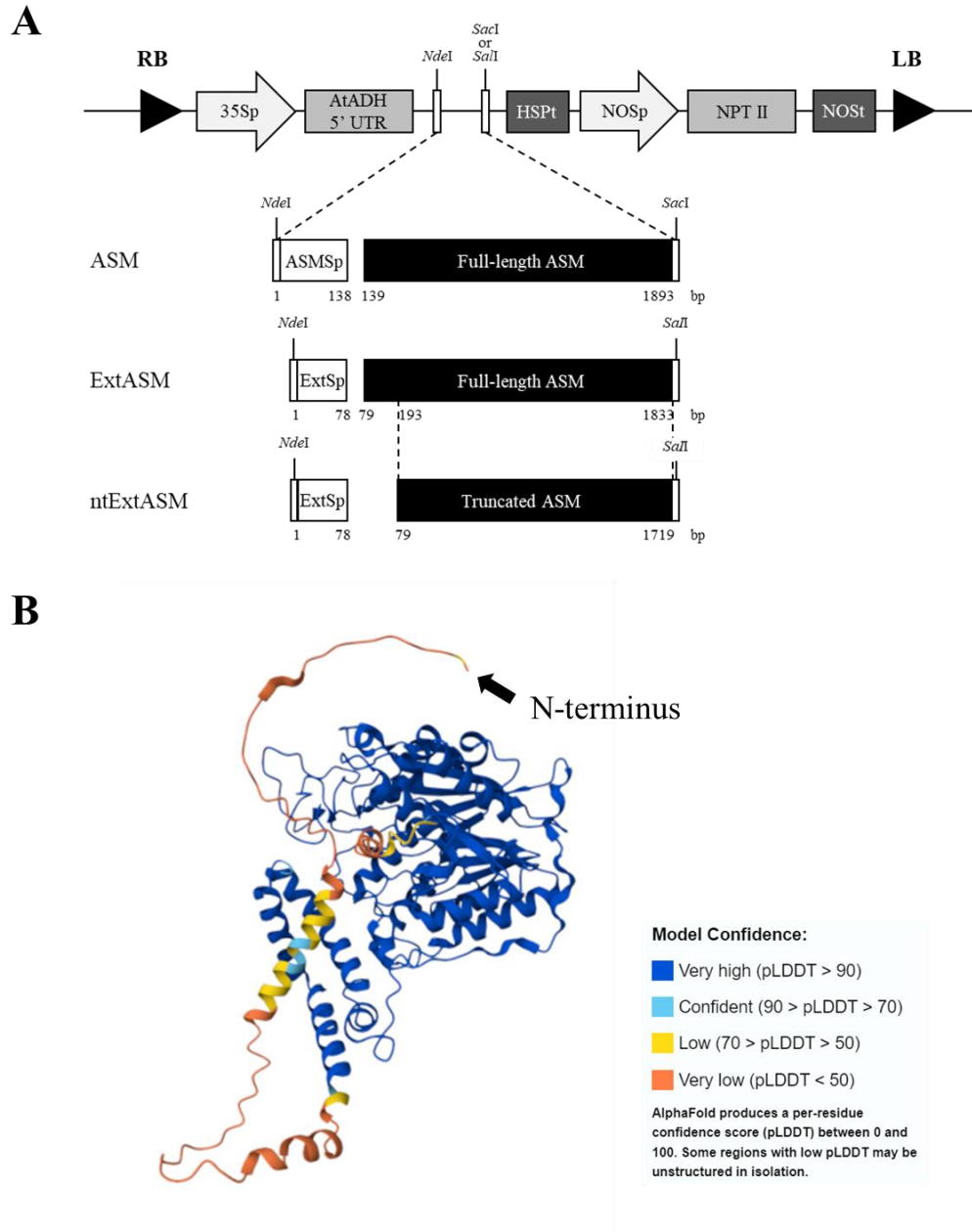


Figure 4 The schematic representation of ASM expression construct. (A) Schematic representation of ASM, ExtASM, and ntExtASM expression constructs in the pRI201-AN plant expression vector. ASMSp, Original signal peptide from human ASM; ExtSp, Extensin signal peptide from *N. benthamiana*; Truncated ASM; Amino acid residues 85 to 631 from the full-length ASM. (B) The predicted 3D structure of ASM from AlphaFold database (AF-P17405-F1). pLDDT: Predicted Local Distance Difference Test.

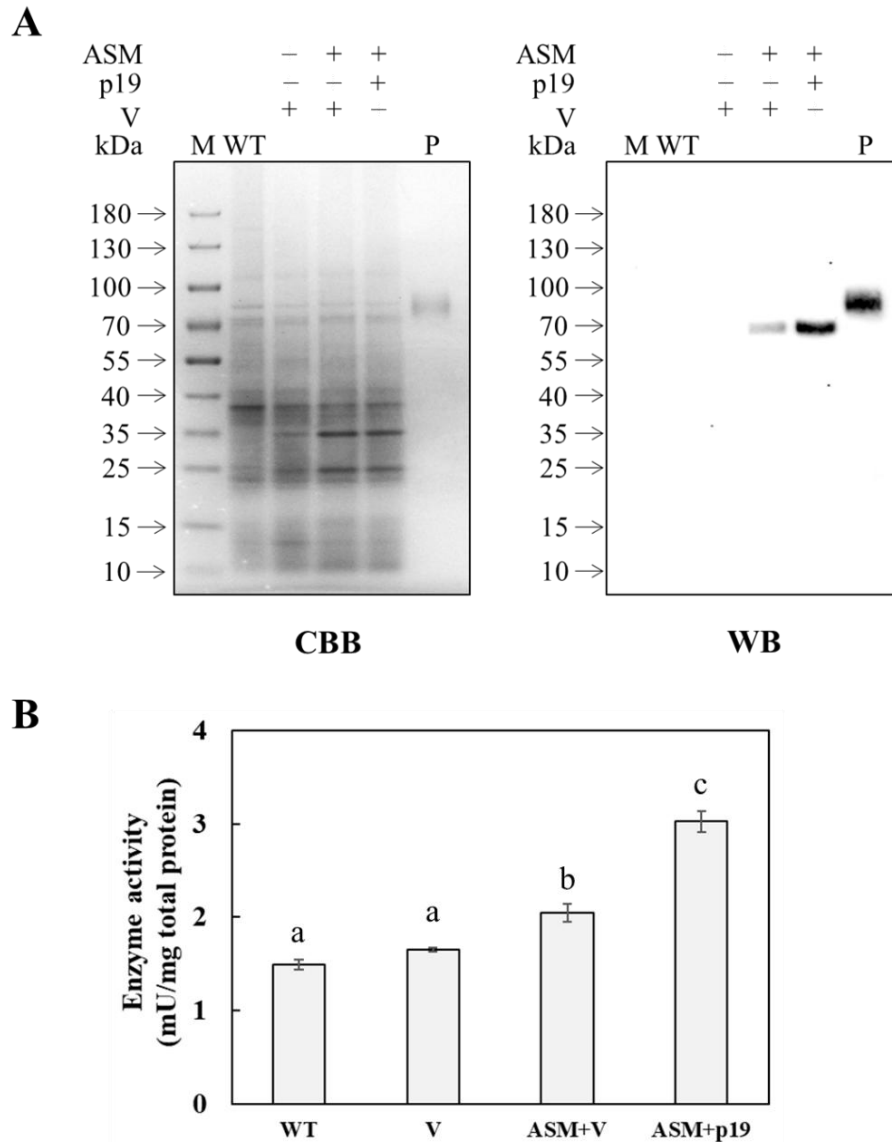


Figure 5 The confirmation of ASM expression in *N. benthamiana* plants. (A) Sodium dodecyl sulfate-polyacrylamide (5-20%) gel electrophoresis (SDS-PAGE) in CBB staining and Western blot analysis of ASM expression with and without p19 using the pRI201-ASM construct. M: Prestained protein ladder marker; WT: Wild-type plant; V: Empty vector; P: CHO-derived human ASM (5 ng) as a positive control. (B) ASM enzyme activity comparison between wild-type, empty vector, ASM with empty vector, and ASM with p19. Each data point reflects the average value $\pm$ standard deviation (SD), calculated from three biologically independent infiltration experiments ($n=3$). In the graph, bars labeled with different letters represent significant differences, all of which are highly significant ($p < 0.001$).

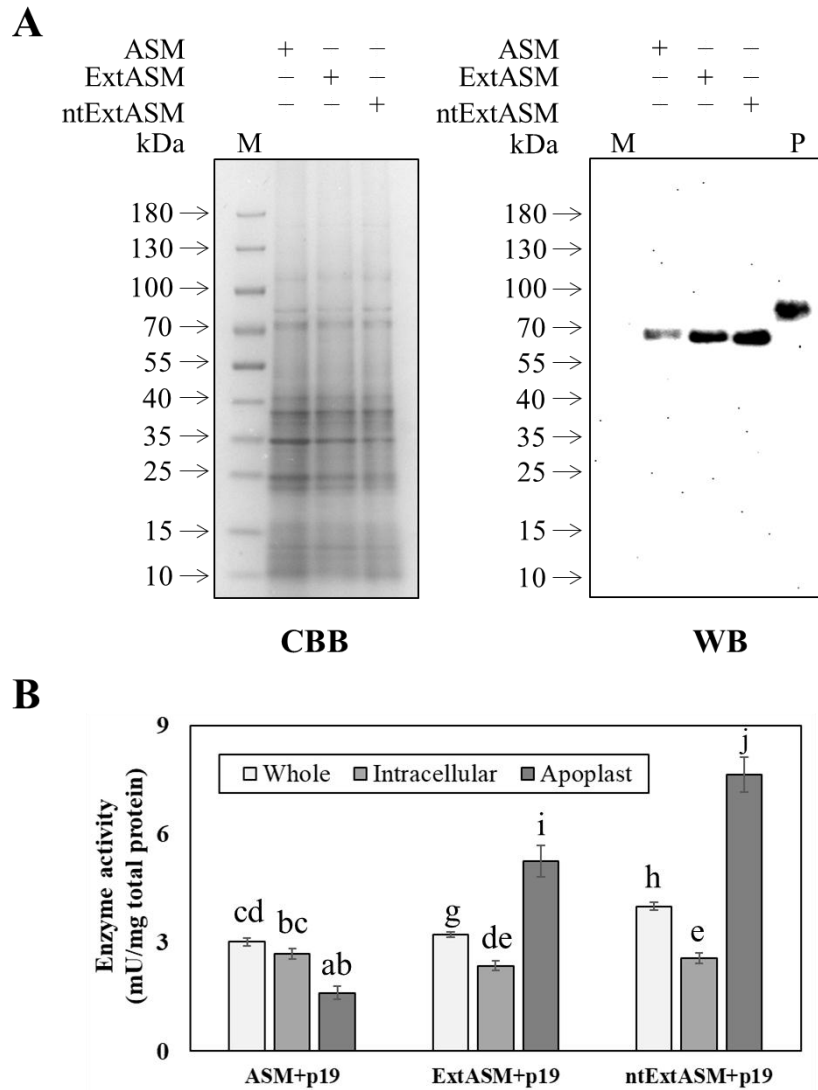


Figure 6 The transient production of ASM from different constructs in *N. benthamiana* plants. (A) Sodium dodecyl sulfate-polyacrylamide (5-20%) gel electrophoresis (SDS-PAGE) in CBB staining and Western blot analysis of ASM expression in combination with p19 co-expression, using different constructs (pRI201-ASM, pRI201-ExtASM, and pRI201-ntExtASM). M: Prestained protein ladder marker; P: CHO-derived human ASM (5 ng). (B) Enzyme activity of ASM crude extracted obtained from different constructs (pRI201-ASM, pRI201-ExtASM, and pRI201-ntExtASM). The data indicates the mean $\pm$ SD of three independent infiltration trials (n=3).

2.3.2 Optimization of boiling temperature and duration for sample preparation

Target ASM may be prone to loss or degradation due to heat, highlighting the need for a systematic investigation into the effects of temperature and duration on its stability. In this study, samples were subjected to boiling at temperatures ranging from 100°C to 50°C, with exposure durations of 5 and 10 min at each temperature. Initially, when comparing the results to the control condition (boiling at 100°C for 5 min), the Western blot analysis presented in Figure 7 indicated that ASM levels were either similar to or exhibited significant differences after 5 min at the various temperatures tested. However, a more pronounced effect was observed at the 10 min mark, where the ASM band intensity steadily increased as the temperature decreased, suggesting a protective effect at lower temperatures.

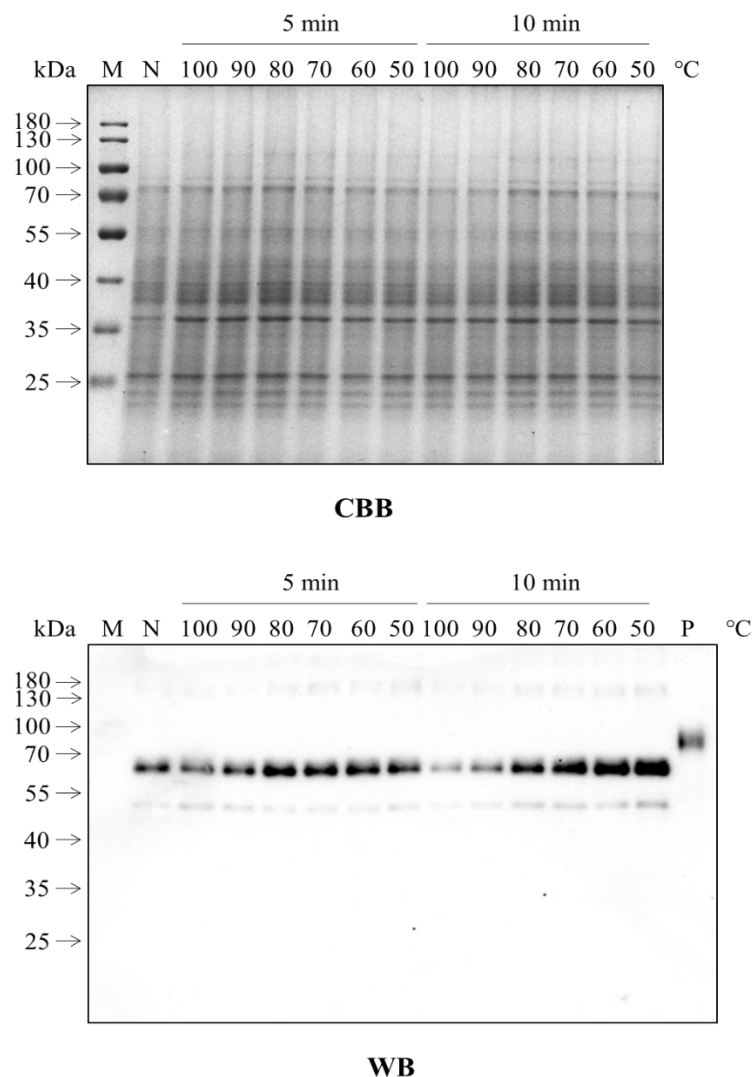


Figure 7 SDS-PAGE (10%) in CBB and Western blot analysis were performed to examine the impact of boiling temperature (from 100°C to 50°C) and time (5 and 10 min) on the samples. M: Prestained protein ladder marker; N: Crude extract without heat treatment; P: CHO-derived human ASM (5 ng).

2.3.3 Apoplastic wash fluid recovery

Apoplastic wash fluid is an effective method for protein production, as it enables the recovery of apoplast-localized proteins while minimizing contamination from other cellular components. In this study, recombinant ExtASM and ntExtASM were guided to the apoplast using the secretion signal, ExtSp. By utilizing this signal peptide, the total protein content in the leaf tissue is reduced because a portion of the expressed protein is secreted into the apoplastic space rather than accumulated inside the cells. This secretion limits the buildup of intracellular proteins and decreases the overall complexity of the total leaf extract. As a result, the subsequent purification step is simplified, facilitating the separation of ASM from other cellular proteins. To confirm the isolation of apoplast-targeted ASM from agroinfiltrated leaves of *N. benthamiana*, the infiltrated leaves were harvested and divided into two groups for analysis. To achieve the AWF sample, the leaves in the first group were immersed in AWF buffer, centrifuged, and the intracellular samples were extracted from the residual tissue using ASM extraction buffer. The whole leaves in the second group were directly extracted using the same extraction buffer as the intracellular samples. Results from SDS-PAGE and Western blot analysis (Figure 8) verified that ASM could be successfully recognized and recovered in the AWF as an apoplast-localized protein. This was further supported by the distinctive presence of ASM bands in the AWF fraction, indicating that the protein had indeed been targeted and isolated from the apoplastic space. In contrast, extracting ASM from the cell extract was less efficient and more challenging due to the presence of multiple cellular components and the difficulty of separating ASM from other intracellular proteins.

Furthermore, other ASM expression constructs, including variants with different signal peptides or truncations, were tested for AWF collection, and these experiments exhibited a similar trend to those observed in Figure 9. These results suggest that the AWF collection method is reproducible and can be applied to various constructs with consistent outcomes. AWF simplifies the purification process by specifically targeting ASM to the apoplast. The enzyme activity data presented in Table 4 further supports the effectiveness of this approach, as it shows a marked increase in ASM activity in the AWF compared to other fractions, emphasizing the successful enrichment of the apoplast-localized enzyme.

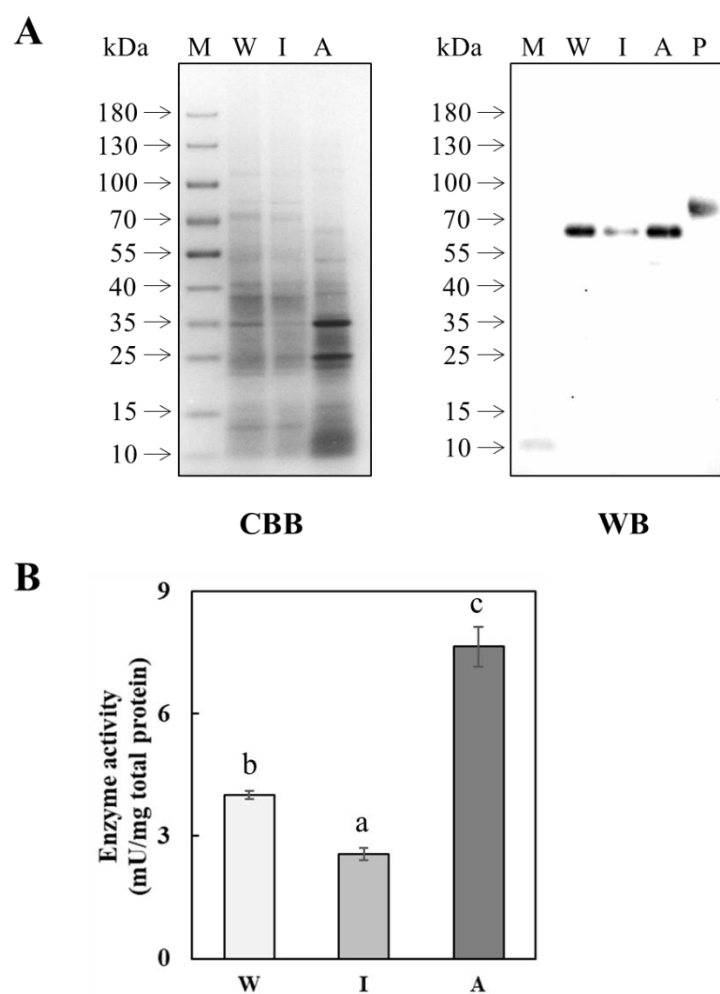


Figure 8 The recovery of ASM from the apoplastic wash fluid (AWF) sample. (A) SDS-PAGE (5-20%) in CBB and Western blot analysis. M: Prestained protein ladder marker; W: Whole-plant crude extract; I: Intracellular crude extract; A: AWF; P: CHO-derived human ASM (5 ng). (B) Enzyme activity compared between whole-plant, intracellular, and AWF samples. The data represent the average $\pm$ SD from three independent infiltration experiments (n=3).

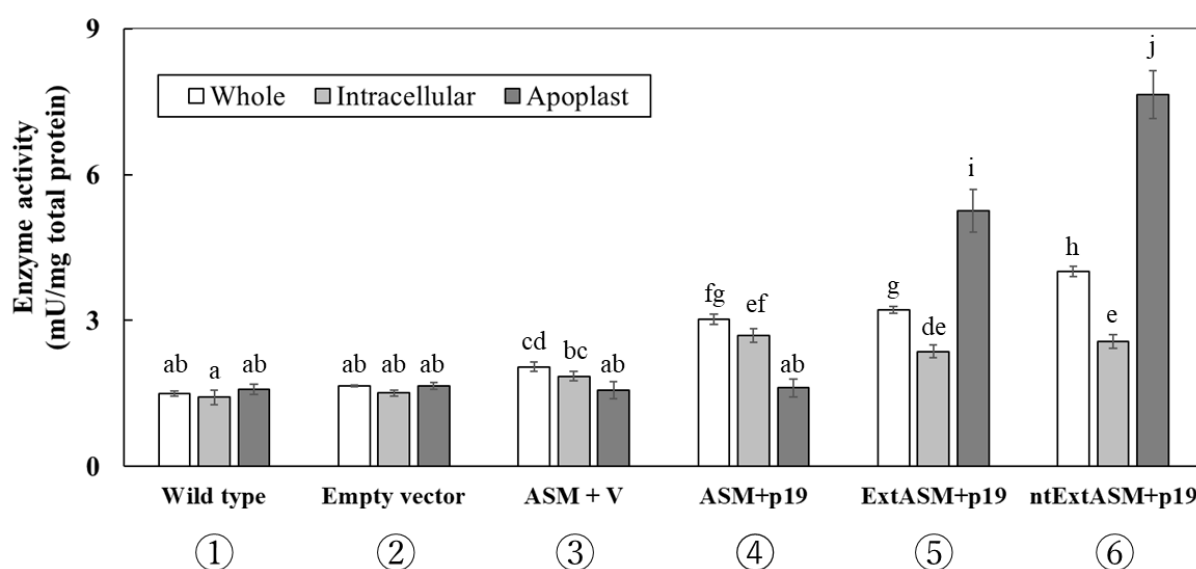


Figure 9 The enzyme activity of ASM extracted from leaves at 6 dpi was compared between whole-plant, intracellular, and apoplast samples, V: Empty vector. This data indicates the mean $\pm$ standard deviation (SD) of three separate infiltration trials (n=3).

Table 4 Summarize enzyme activity of ASM from the crude extract using different constructs (n=3).

Crude extract	Enzyme activity (mU/mg total soluble protein)		
	Whole	Intracellular	Apoplast
① Wild type	1.49 $\pm$ 0.05	1.43 $\pm$ 0.15	1.59 $\pm$ 0.11
② Empty vector	1.65 $\pm$ 0.02	1.51 $\pm$ 0.06	1.66 $\pm$ 0.07
③ ASM + Empty vector	2.05 $\pm$ 0.09	1.86 $\pm$ 0.10	1.57 $\pm$ 0.17
④ ASM + p19	3.02 $\pm$ 0.11	2.69 $\pm$ 0.14	1.61 $\pm$ 0.19
⑤ ExtASM + 19	3.22 $\pm$ 0.07	2.37 $\pm$ 0.13	5.26 $\pm$ 0.44
⑥ ntExtASM + p19	4.01 $\pm$ 0.11	2.56 $\pm$ 0.14	7.64 $\pm$ 0.49

2.4 Discussion

A hereditary illness known as NPD type B is brought on by a lack of the enzyme ASM and appears as neurological disorder, respiratory problems, and organ expansion. The primary treatment for this disease is ERT, which is considered to be disease-modifying. Recombinant human ASM is therefore essential to ERT for NPD type B. According to reports, the first and only approved ERT for the long-term therapy of NPD is still Xenpozyme[®] (olipudase alfa), a commercially manufactured recombinant ASM in CHO cells (Hon et al., 2024; Syed, 2023). Although efficient, there are a number of disadvantages to using animal cell-based expression systems to produce therapeutic proteins, including the expensive manufacturing and the possibility of virus contamination. A notable incident occurred in 2009 when Genzyme Corporation discovered viral contamination (vesivirus 2117) in one of its bioreactors at the Allston facility. This contamination impaired the growth of CHO cells used for biologics production, resulting in a 6 – 8 weeks shutdown for decontamination and several months of production delays for their flagship product, Cerezyme[®] (Hollak et al., 2010). This highlights the risks and operational challenges associated with animal-based production systems.

In contrast, plant-based expression systems provide a promising alternative for recombinant protein production. They offer substantial economic advantages by lowering production costs and reducing the risks of viral contamination. Plant expression systems are free from the human pathogens that can affect animal (Giddings et al., 2000). For example, Taliglucerase alfa (Elelyso[®]), a plant-produced recombinant enzyme for treating Gaucher's disease, became the first plant-made therapeutic product approved by the FDA in May 2012 (Fox, 2012). Plant-based drugs have also proven to be more cost-effective than animal-derived products, with plant-made therapies being approximately 48% cheaper to produce. This is particularly evident in the comparison of prices for recombinant GCase produced in CHO cells (Cerezyme[®]) versus GCase produced in genetically engineered carrot suspension cells (Elelyso[®]). The former costs \$2,460 per 400 U/vial, while the latter costs only \$1,300 per 400 U/vial (Canadian Agency for Drugs and Technologies in Health, 2017). For developing of therapeutic proteins, plant-based expression systems offer an attractive alternative to get around these obstacles.

In this study, recombinant ASM was produced in *N. benthamiana* using agroinfiltration for transient expression. To boost the amount of ASM produced in plants, the target construct was co-expressed with a p19 suppressor. Two versions of ASM were tested:

one fused with its original signal peptide and the other with a plant-derived signal peptide called ExtSp. Both forms were successfully produced as functional proteins. However, signal peptides are specific to the organisms they come from and may not work equally well in different species. The original ASM signal peptide did not perform as efficiently in the plant system, resulting in lower enzyme activity compared to the ExtSp-fused ASM. This reduced performance is partly due to features of the ASM signal peptide, such as its hydrophobic region and repeating sequence (LALALALALALA), which can affect how well the protein is processed and functions in plants (Rhein et al., 2014). As a result, the ASM signal peptide is less effective in non-native systems. In contrast, the ExtSp signal peptide is better recognized by the plant's own secretion system and is known to work reliably in plants. Its N-terminal region helps direct the protein to be secreted properly in plant cells, making it a better option for plant-based protein production (De Loose et al., 1991; Petersen et al., 2021).

Recombinant ASM produced in whole *N. benthamiana* plant exhibited the expected ASM activity, while the low enzyme activity observed in both wild-type and empty vector agroinfiltrated plants was due to endogenous plant sphingomyelinase (Liu et al., 2021). Notably, the crude enzyme activity of recombinant ASM in this study (4.01 ± 0.11 mU/mg TSP and 7.64 ± 0.49 mU/mg TSP in whole and apoplast samples, respectively) was much higher than that of the wild-type plants (1.49 ± 0.05 mU/mg TSP and 1.59 ± 0.11 mU/mg TSP in whole and apoplast samples, respectively) and the empty vector (1.65 ± 0.02 mU/mg TSP and 1.66 ± 0.07 mU/mg TSP in whole and apoplast samples, respectively)

To sum up, the goal of this chapter is to produce recombinant human ASM as the soluble form using agroinfiltration in *N. benthamiana*. The target construct was utilized for the transient expression of ASM using agroinfiltration in the whole plant, with co-expression of p19 to enhance the yield of ASM. This study reported and developed a transient expression system of ASM production, offering a rapid method for producing therapeutic proteins in *N. benthamiana*.

2.5 Summary

Human ASM plays a crucial role in the hydrolysis of sphingomyelin into ceramide and phosphocholine, a process essential for various cellular functions such as membrane dynamics, cell signaling, and apoptosis regulation. In this study, the first successful functional recombinant production of ASM in *N. benthamiana* is reported. The findings demonstrate that ASM can be transiently expressed as a functional protein in *N. benthamiana*, offering a promising platform for recombinant protein production. The successful expression and extraction of functional, apoplast-localized ASM in *N. benthamiana* marks the beginning of its potential for research and therapeutic use, particularly in treating LSDs like NPD type B, where ASM deficiency is a key contributing factor. Additionally, this study highlights the potential of *N. benthamiana* as an effective host for producing complex human proteins, which can be scaled up for research and therapeutic application in the future.

Chapter 3

Tag-free human acid sphingomyelinase purification in *Nicotiana benthamiana* whole plants

3.1 Introduction

Although plant-based systems have several benefits, the downstream processing – especially the purification step, presents significant challenges and difficulties. The presence of various proteins, metabolites, and secondary substances in plant matrices makes this process complex and critical, as these components can hinder the effective isolation of target proteins (Schillberg et al., 2019). This is further complicated by the tendency of recombinant proteins to aggregate or form inclusion bodies in plants, when expression level is more than 10% of the total soluble protein (Venkataraman et al., 2023). Despite employing different purification strategies to overcome issues such as aggregation, post-translational modifications, and instability in protein production, the purification process still struggles to effectively isolate recombinant human proteins from plant sources.

The purification of recombinant ASM from two primary host platforms – mammalian and insect cells – has been the subject of extensive research (Gorelik et al., 2016; Miranda et al., 2000; Qiu et al., 2003). Typical ASM purification protocols employ techniques such as affinity chromatography, hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEX), and size exclusion chromatography (SEC), all of which are effective for isolating the target protein with high precision. Many studies have reported using multi-step purification strategies, often involving at least three or more distinct steps, to achieve the high purity levels required for downstream applications (He et al., 1999; Lansmann et al., 2000). In some cases, specialized tags are incorporated into the recombinant ASM, facilitating the purification process and enhancing both the yield and efficiency of isolation. However, while these multi-step purification processes can be effective, they often present challenges related to protein degradation, aggregation, and the potential loss of biological activity. Each additional step in the purification process increases the risk of these issues, ultimately leading to reduce protein yield and a loss of ASM activity (Yamanaka et al., 1981).

Tag-free recombinant protein purification is an emerging and increasingly important approach in protein engineering and biotechnology. While affinity tags are often useful and convenient, they can sometimes interfere with the functionality, stability, or activity of recombinant proteins (Islam et al., 2019). Moreover, most affinity tags are expensive, which drives up the overall cost of protein production (Fong et al., 2010). The removal of these affinity tags after purification also presents a challenge, adding complexity and additional costs to the process (Tubb et al., 2009). To address these issues, tag-free purification methods have been developed to enable the direct isolation of native, functional proteins without the need for additional tags. In plant-based systems, tag-free purification offers several advantages. It helps produce proteins that are more likely to maintain their native structure and biological activity. Tag-free methods also can achieve high levels of protein purity (Nakagawa et al., 2024). Additionally, plants are ideal hosts for recombinant protein production due to their ability to produce complex proteins with post-translational modifications, which are essential for the functionality of many therapeutic proteins. However, isolating tag-free proteins from plant systems presents its own challenges, primarily due to the complex plant matrix that can complicate the purification process.

In this study, I aimed to establish an efficient and practical ASM purification method in plants by developing a tag-free purification strategy. This approach reduces the process to only two steps, minimizing the risk of degradation and aggregation while ensuring high-purity ASM with preserved activity and yield. The objective was to streamline the purification process, offering a more efficient solution for large-scale production of functional ASM in plant-based systems. Recent advances in tag-free purification methods, such as optimized chromatography, precipitation, and affinity capture techniques, have made this approach increasingly viable and desirable in plant-based biotechnology, further supporting the feasibility of this study.

3.2 Materials and Methods

3.2.1 Vector construction of histidine-tagged ASM expression

To facilitate protein purification and site-specific cleavage, a recombinant vector was designed to incorporate a C-terminal 6×histidine (His) tag and a Tobacco Etch Virus (TEV) protease cleavage site. The starting construct pRI201-ASM was modified through a two-step PCR process. In the first step, primers *nNdeI-ASMSp-F* and *nTEV-R* were used to amplify the initial fragment. This product then served as the template for a second PCR using *nNdeI-ASMSp-F* and *nSalI-HT-R*. The final fragment was digested with *NdeI* and *SalI*, and ligated into the binary vector pRI201-AN (TaKaRa Bio), generating the construct pRI201-ASMHT, which encodes a C-terminal His-tagged protein with a TEV site. Further modifications were made to the pRI201-ExtASM construct, which expresses ASM fused with the ExtSp signal peptide, to introduce both N- and C-terminal His and TEV tags. For N-terminal tagging, two separate DNA fragments were amplified using pRI201-ExtASM as the template. The first PCR used primers *nNdeI-Extsp-F/nExtsp-HT-R*, and the second used *nTEV-ASM-F/nSalI-ASM-R*. These two fragments were then fused via overlap extension PCR to produce the final N-terminally tagged product. This fragment was cloned into pRI201-AN, resulting in the pRI201-ExtHTASM construct. For C-terminal tagging, a similar two-step PCR approach was followed using the primer pairs *nNdeI-Extsp-F/nTEV-R* and then *nNdeI-Extsp-F/nSalI-HT-R*. The final PCR product was cloned into pRI201-AN, yielding the pRI201-ExtASMHT construct. All primers used in this study were summarized in Table 5. All recombinant vectors were first transformed into *E. coli* DH5 α and then into *A. tumefaciens* LBA4404, as described in Chapter 2, Section 2.2.2.

Table 5 Primers used for 6xHis and TEV tag insertion.

Primer ^a	Sequences ^b (5' → 3')
nNdeI-ASMSp-F	ATT <u>CATATG</u> CCAAGGTATGGAGC
nTEV-R	GCCCTGAAAATACAGGTTTTTCACAGAACAATGGCCTAG
nSall-HT-R	ATTGTCGACTCAATGGTGATGGTGATGATGGCCCTGAA AATACAG
nNdeI-ExtSp-F	ATT <u>CATATG</u> GGGAAAATGGCTTCTC
nExtsp-HT-R	AAAATACAGGTTTTTCATGGTGATGGTGATGATGTGCTG AGCTTTCAGAAG
nTEV-ASM-F	GAAAACCTGTATTTTCAGGGCCTTGCTCTTTCTGACTCA AGG
nSall-ASM-R	ATTGTCGACTCAACAGAACAATGGCC

^a F and R represent forward and reverse primers, respectively.

^b Underlined sequences indicate the restriction enzyme recognition sites.

3.2.2 Transient expression in *N. benthamiana* and apoplastic wash fluid extraction

All constructs containing the 6x His tag in *A. tumefaciens* LBA4404 strains were co-agroinfiltrated with the p19 silencing suppressor plasmid to confirm ASM expression. In addition, *A. tumefaciens* harboring the selected vector expressing the truncated ASM (pRI201-ntExtASM) were also co-agroinfiltrated with the p19. This construct was further used for large-scale protein expression and purification. To determine the optimal harvest time, agroinfiltrated leaves from multiple plants were collected at various time points, ranging from 2 to 10 days post-agroinfiltration (dpi). The transient protein expression in the leaves of *N. benthamiana* was conducted according to the protocol outlined in Chapter 2, Section 2.2.3. After harvesting, the leaves were separated into whole, intracellular, and apoplast fractions, as described in Chapter 2, Sections 2.2.4 and 2.2.5. The leaves collected at each time point were subsequently extracted and analyzed for target protein expression using Bradford assay, ASM enzymatic activity, SDS-PAGE, and Western blot analysis. For large-scale production, the agroinfiltration procedure was upscaled to include 12 five-week-old *N. benthamiana* plants, cultivated under controlled conditions. The harvested tissue from these 12 plants was used to recover the apoplast fraction, which was then processed for further purification.

3.2.3 Purification of recombinant ASM in *N. benthamiana*

Initial attempts to purify ASM were carried out using the full-length construct containing its native signal peptide. Two purification strategies were individually explored: hydrophobic interaction chromatography (HIC) and affinity chromatography. For HIC, NaCl was added to the crude extract sample to a final concentration of 1 M to enhance hydrophobic interactions. The sample was loaded onto a Phenyl-650M column (Tosoh Corporation, Tokyo, Japan) pre-equilibrated with high-salt buffer. Then, protein was eluted by gradually decreasing the NaCl concentration from 1 M to 0 M. In the affinity chromatography approach, ASM expressed from a construct containing a 6×His tag was purified using a TALON[®] metal affinity column, with elution carried out using imidazole, which competes with the His tag for binding to the resin.

Due to the limited efficiency and reproducibility of these initial methods, an alternative purification strategy was developed using a truncated ASM construct and a two-step ion exchange chromatography approach. A two-step purification strategy was employed to isolate ASM-producing recombinant proteins from *N. benthamiana* plants, utilizing both cation and anion exchange chromatography techniques. The process began by adjusting the AWF sample to a pH of 5.0, followed by centrifugation at 12,000 × *g* for 20 min at 4°C to remove any cellular debris. The clear supernatant was then carefully loaded onto a Toyopearl SP-550C strong cation exchange chromatography column (1 cm x 2.5 cm; Tosoh Corporation), which had been equilibrated with a buffer containing 25 mM citrate/phosphate, 0.01% Triton X-100, and 0.1 mM ZnCl₂, pH 5.0. The resin effectively bound only the desired proteins, allowing the unbound proteins to be washed away. This was accomplished by washing the column with 10 column volumes of wash buffer (20 mM Tris-HCl, 0.01% Triton X-100, and 0.1 mM ZnCl₂, pH 6.0). The bound proteins were then carefully eluted using 10 column volumes of elution buffer (20 mM Tris-HCl, 0.01% Triton X-100, 0.1 mM ZnCl₂, and cOmplete protease inhibitor cocktail, pH 8.0). The elution fractions were collected, providing a purified protein solution that underwent further refinement using a DEAE Sepharose[™] CL-6B weak anion exchange chromatography column (1 cm x 1.5 cm; Cytiva, Tokyo, Japan). The purified protein was then eluted from DEAE Sepharose[™] CL-6B using a similar elution buffer (10 column volumes) to the one used for the initial cation exchange column.

3.2.4 Methanol-chloroform protein precipitation

The eluted proteins were collected and concentrated via methanol-chloroform precipitation, following a modified protocol from Wessel & Flügge, 1984. In this process, the protein solution was mixed with methanol (4 parts), chloroform (1 part), and water (3 parts), resulting in a final ratio of protein:methanol:chloroform:water of 1:4:1:3. The mixture was vortexed thoroughly and centrifuged at 11,000 x *g* for 2 min at 4°C, during which the protein formed at the interface. The upper aqueous layer was carefully removed and discarded, and an additional three parts of methanol were added to the mixture. The solution was then vortexed thoroughly again and centrifuged at 13,000 x *g* for 5 min at 4°C, allowing the protein to precipitate as a pellet. After removing the supernatant, the protein pellet was dried in a SpeedVac. Finally, the pellet was resuspended in 1x SDS-PAGE sample buffer, which had been prepared by diluting the 4x SDS-PAGE sample buffer, as described in Chapter 2, Section 2.2.6.

3.2.5 CBB staining and Western blot analysis

The purified protein precipitate was analyzed by SDS-PAGE (5-20% or 10%), with the PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) serving as the protein marker. CBB staining and Western blotting were carried out according to the protocol described in Chapter 2, Section 2.2.6.

3.2.6 ASM activity assay

ASM activity was measured using the Acid Sphingomyelinase Assay Kit (Cat#ab252889; Abcam, Cambridge, UK), as described in Chapter 2, Section 2.2.7. An enzyme unit is defined as the amount of enzyme needed to release 1 μmol of choline/min. Enzyme activity and specific activity were reported as units/mg total soluble protein (TSP) and units/mg purified protein, respectively.

3.2.7 Analysis of N-terminal amino acid sequence by protein sequencer

DEAE Sepharose™ CL-6B purified protein was separated using a 10% SDS-PAGE gel and transferred to a PVDF membrane. After three 5-min washes with MilliQ water, the membrane was stained for 3 min with a dye solution (0.1 mg/mL CBB R-250, 10% methanol, and 7% acetic acid). The membrane was then destained by washing twice for 5 min and once for 15 min with a destaining solution (5% methanol and 7% acetic acid). After three additional

5-min washes with MilliQ water, the membrane was allowed to dry overnight. The target band on the membrane was carefully excised from the membrane, placed in a new microcentrifuge tube, and stored at -20°C before being sent to the Protein Research Institute at Osaka University for N-terminal peptide sequencing via Edman degradation using a Shimadzu PPSQ-53A gradient system.

3.2.8 Protein identification using nanoLC-MS/MS

The purified protein from the DEAE SepharoseTM CL-6B column was separated using a 10% SDS-PAGE gel and stained with CBB as described earlier. The target band (~61 kDa) was excised into small pieces and de-stained with 50 mM NH₄HCO₃ in 50% acetonitrile. The gel pieces were subsequently dehydrated twice with acetonitrile and completely dried using a SpeedVac. The excised ASM band underwent reduction and alkylation according to the protocol in Chen et al., 2016. In-gel digestion was carried out overnight at 37°C using pepsin (Promega, Madison, WI). The digested peptides were extracted from the gel, and the reaction was activated by heating at 95°C for 5 min. The digested peptides were analyzed by nanoLC-MS/MS using an ESI-Qq-TOF mass spectrometer (microTOF-Q II; Bruker Daltonics, Bremen, Germany) for protein identification (Nguyen et al., 2023). The obtained MS data were analyzed and compared against the SwissProt and NCBI protein databases using the MASCOT search engine to validate the identity of the purified plant-derived ASM protein.

3.2.9 Statistical analysis

Three identical biological replicates were used in this study. The mean ± standard deviation (SD) represents the reported findings. Each experiment was conducted in triplicate to ensure the precision and consistency of the results. Data were analyzed using IBM SPSS Statistics (v30) with ANOVA and Duncan's multiple range test, considering $p \leq 0.05$ as statistically significant.

3.3 Results

3.3.1 Transient production of 6x His-tagged ASM in infiltrated leaves

Several ASM constructs were generated with 6xHis tags fused to either the N- and C-terminal. These constructs were transiently expressed in *N. benthamiana* using plant expression vectors (Figure 10). Expression was carried out by vacuum infiltration, as described in Chapter 2, Section 2.2.3. Protein production was confirmed by SDS-PAGE and Western blot analysis (Figure 11A, 11B, and 11C). A Western blot probed with rabbit anti-ASM detected ASM at approximately 65 kDa, confirming successful expression of ASM from all constructs. However, the Western blot probed with mouse anti-6xHis did not show the expected bands in lanes 2, 4, and 5 of Figure 11C, which correspond to constructs containing the 6xHis tag. This lack of signal may be due to degradation by endogenous plant enzymes.

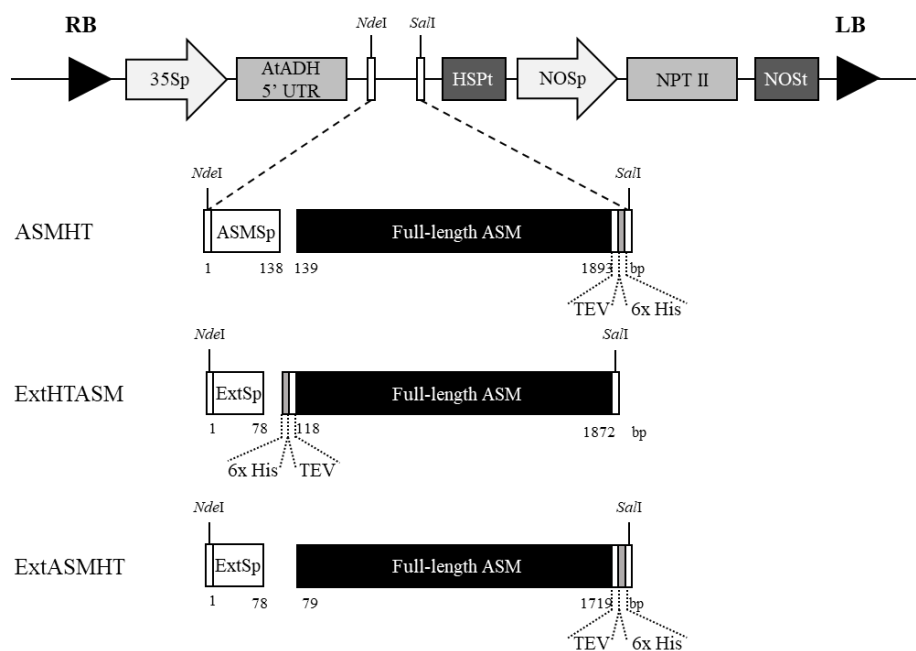


Figure 10 Schematic representation of 6xHis-tagged ASM (ASMHT, ExtHTASM, and ExtASMHT) expression constructs in the pRI201-AN plant expression vector. ASMSp, Original signal peptide from human ASM; ExtSp, Extensin signal peptide from *N. benthamiana*; 6x His, histidine tag (HT); TEV, Tobacco Etch Virus cleavage site.

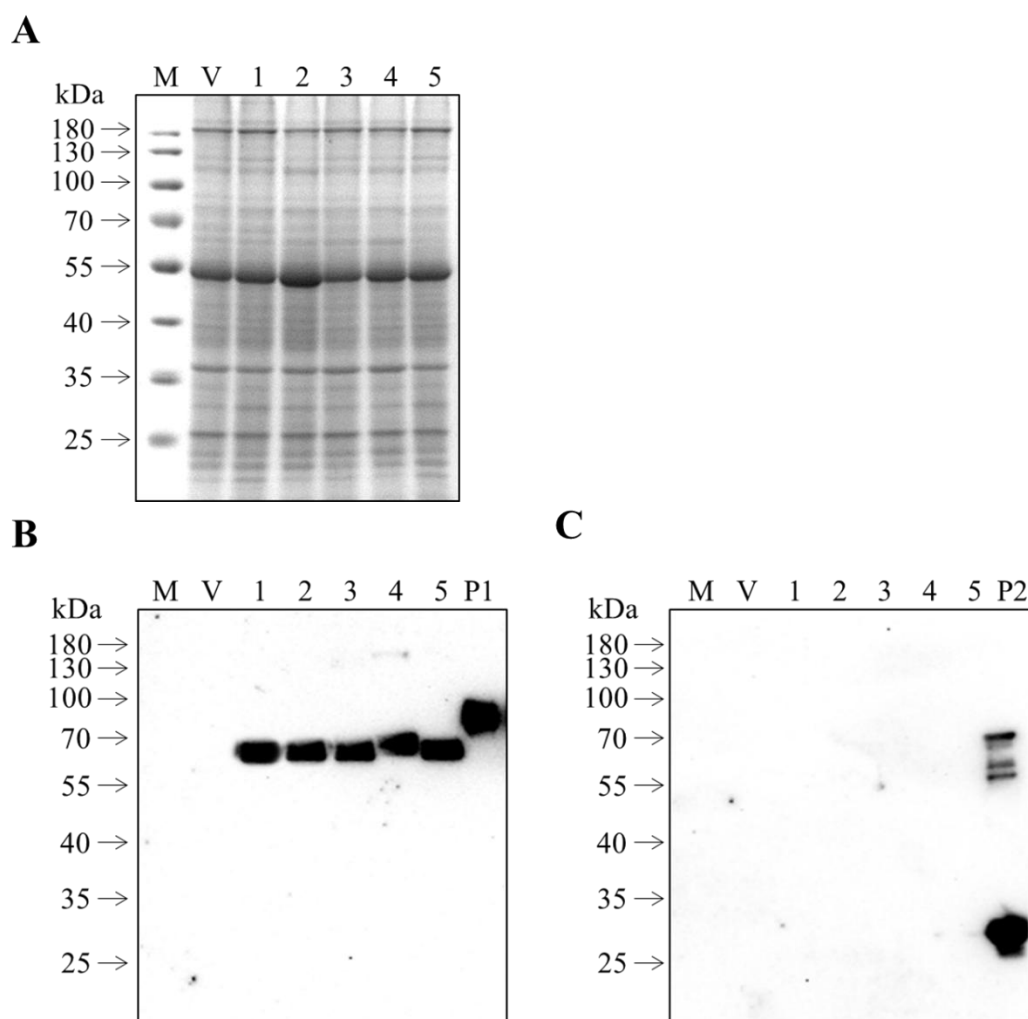


Figure 11 6xHis-tagged ASM expression using different constructs. (A) SDS-PAGE. (B) Western blot analysis was performed using rabbit anti-ASM antibody (1:2,000) as a probe. (C) Western blot analysis was performed using mouse anti-6x His antibody (1:2,000) as a probe. M: Prestained protein ladder marker; V: Empty vector; 1: pRI201-ASM construct; 2: C-terminal His-tagging of the pRI201-ASM construct (pRI201-ASMHT); 3: pRI201-ExtASM; 4: N-terminal His-tagging of the pRI201-ExtASM construct (pRI201-ExtHTASM); 5: C-terminal His-tagging of the pRI201-ExtASM construct (pRI201-ExtASMHT); P1: CHO-derived human ASM; P2: *E. coli*-derived 7x His-TEV.

3.3.2 Initial Trials for ASM Purification

Purifying ASM from plants proved to be technically challenging. The low expression level of ASM, along with the complex mixture of plant proteins and possible breakdown by plant-derived proteases, made purification particularly difficult. Initial purification attempts using hydrophobic interaction chromatography (Phenyl-650M column) were unsuccessful, resulting in low purity and significant protein loss throughout the procedure (Figure 12). To improve purification, several ASM constructs were designed to include a 6xHis tag to enable purification using TALON[®] affinity resin. A TEV protease recognition site was also inserted between ASM and the His-tag to allow tag removal after purification. The constructs tested included: (i) C-terminal His-tagged ASM (pRI201-ASMHT), (ii) N-terminal His-tagged ExtASM (pRI201-ExtHTASM), and (iii) C-terminal His-tagged ExtASM (pRI201-ExtASMHT). Western blot with an anti-ASM antibody can detect the target protein (Figure 13B). However, the anti-His antibody did not detect the His-tagged proteins, and purification using TALON[®] resin failed to recover the target protein (Figure 13C). These findings suggest that the His-tags were either degraded or hidden due to the structure of the protein in the plant system. Interestingly, both N- and C-terminal His-tagged versions showed similar results, indicating that either end of the ASM protein may be prone to degradation in *N. benthamiana*. The AlphaFold-predicted 3D structure of human ASM (AF-P17405-F1) shows that both the N- and C-terminals are located in regions with relatively low predicted confidence scores or low pLDDT values, suggesting that these terminal regions are flexible or disordered (Figure 4B). Van der Lee et al. (2014), discussed the functionality of disordered regions and their role in protein degradation which can only occur when the disordered terminal end is of a certain length and noted that degradation may be initiated by these regions either at the protein terminus or internally. Unstructured regions frequently have site recognized by proteases and are more easily cleaved than structured regions, which may increase susceptibility to proteolytic degradation by antibodies or binding to affinity resin. This observation could explain the apparent loss or inaccessibility of the His-tags in plant-expressed ASM. Since the 6xHis tag-based purification strategy was unsuccessful – likely due to tag degradation or inaccessibility – an alternative approach was pursued. A truncated version of ASM was fused to ExtSp to enhance secretion and simplify purification for potential scale-up. However, due to ongoing difficulties with tag detection and protein recovery, the final strategy focused on using a construct expressing tag-free ASM to improve protein integrity and yield during purification.

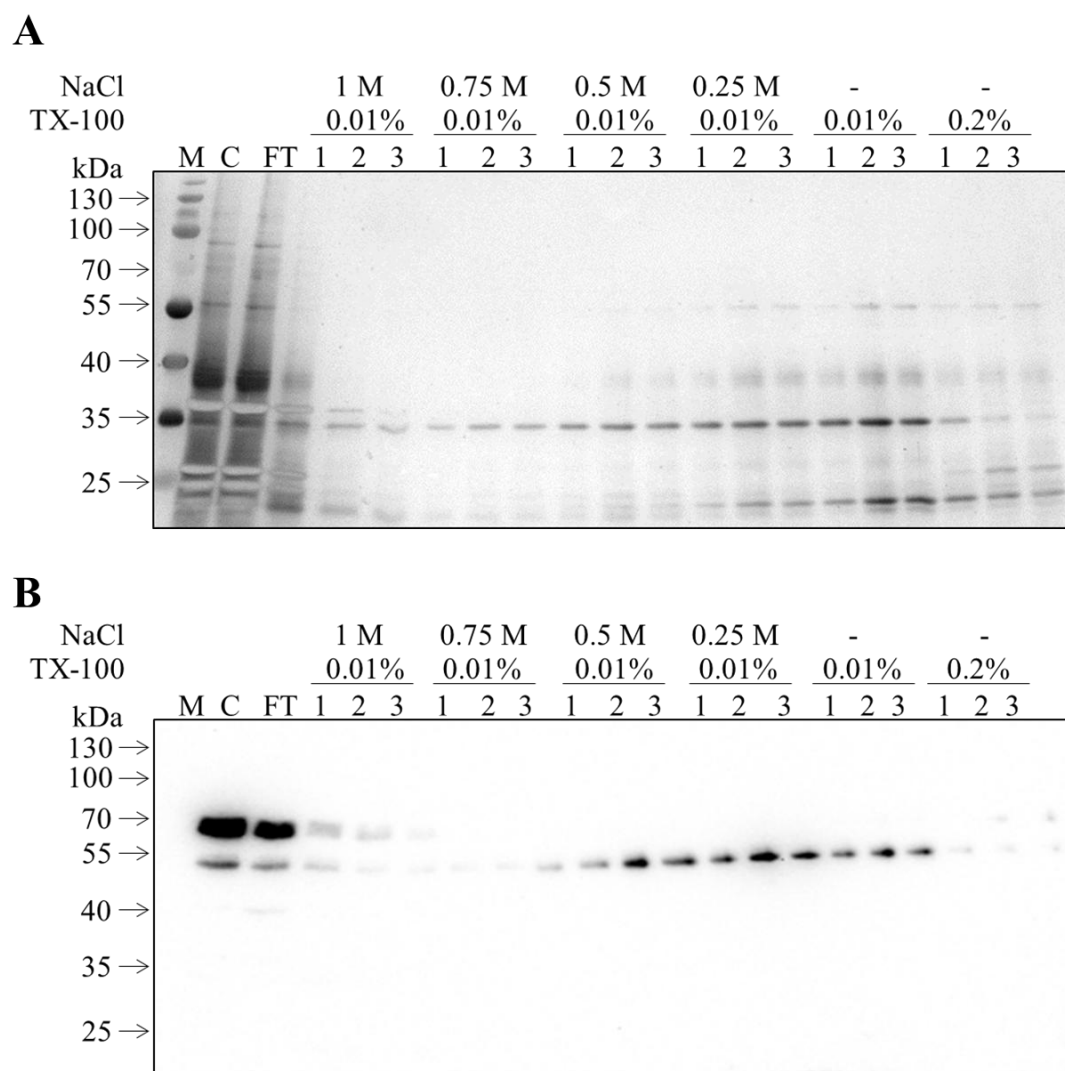


Figure 12 Purification of recombinant ASM using hydrophobic interaction chromatography (Phenyl-650M column). (A) SDS-PAGE and (B) Western blot analysis was carried out using rabbit anti-ASM antibody (1:2,000) as a primary antibody. M: Prestained protein ladder marker; C: Crude extract protein; FT: Flow-through sample; 1-3: Elution fractions 1 to 3; TX-100: Triton X-100.

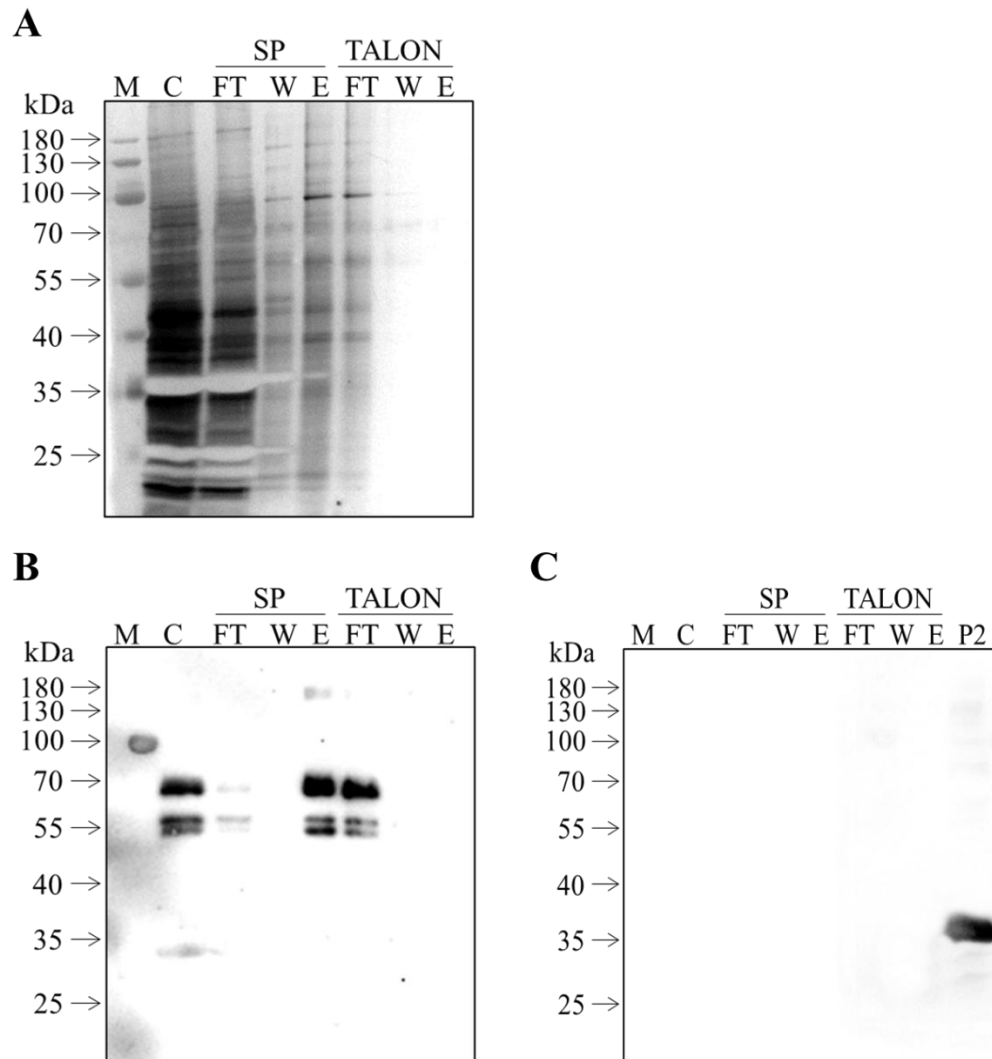


Figure 13 Purification of recombinant ASM was performed using N-terminal His-tagging of the pRI201-ntExtASM construct with SP-550C and TALON columns. (A) SDS-PAGE. (B) Western blot analysis was performed using rabbit anti-ASM antibody (1:2,000) as a probe. (C) Western blot analysis was performed using mouse anti-6x His antibody (1:2,000) as a probe. C: Crude protein; FT: Flow-through sample; W: Wash sample; E: Elution sample; P2: *E. coli*-derived 7x His-TEV.

3.3.3 Time-course analysis of the ASM production

This experiment was performed to identify the time point with the highest expression of ASM for optimizing its production. To determine the optimal expression levels of plant-derived ASM for large-scale production for using in the purification process, agroinfiltrated leaves were harvested at 2, 4, 6, 8, and 10 dpi. Total soluble protein (TSP) extracts from each sample were initially analyzed to quantify ASM expression. Crude leaf extracts were subjected to SDS-PAGE followed by Western blot analysis. The analysis revealed varying band intensities, with the expected 61 kDa ASM band detected as early as 2 dpi and persisting through 10 dpi. No ASM band was observed in extracts from wild-type and empty vector control leaves, as shown in Figure 14. However, ASM expression steadily increased after infiltration, peaking at 6 dpi before declining. Notably, a degraded form of ASM around 50 kDa appeared after 6 dpi. These results suggest that the highest level of ASM expression occurred at 6 dpi. To further investigate the localization of ASM, infiltrated leaves harvested from 2 to 10 dpi were also extracted to assess apoplast-localizing ASM level. Both intracellular samples and AWF samples were then analyzed using SDS-PAGE and Western blotting. These additional analyses yielded similar findings to those observed in whole leaf samples, confirming that ASM expression peaked at 6 dpi, without significant degradation visible at this point. The accumulation of ASM was similar between the whole leaf samples and AWF when equal amounts of TSP (5 µg) were used. In contrast, the intracellular samples showed lower levels of ASM accumulation, as indicated by the Western blot results. These findings demonstrate that ASM was recognized and recovered as a protein that is found in apoplasts. While the ASM accumulation in the AWF and whole leaf samples was comparable, there was a substantial difference in the TSP amounts in SDS-PAGE. This lower TSP concentration in AWF facilitated the purification of the target ASM from other plant proteins. Consequently, the AWF sample harvested at 6 dpi was selected for the subsequent protein purification steps.

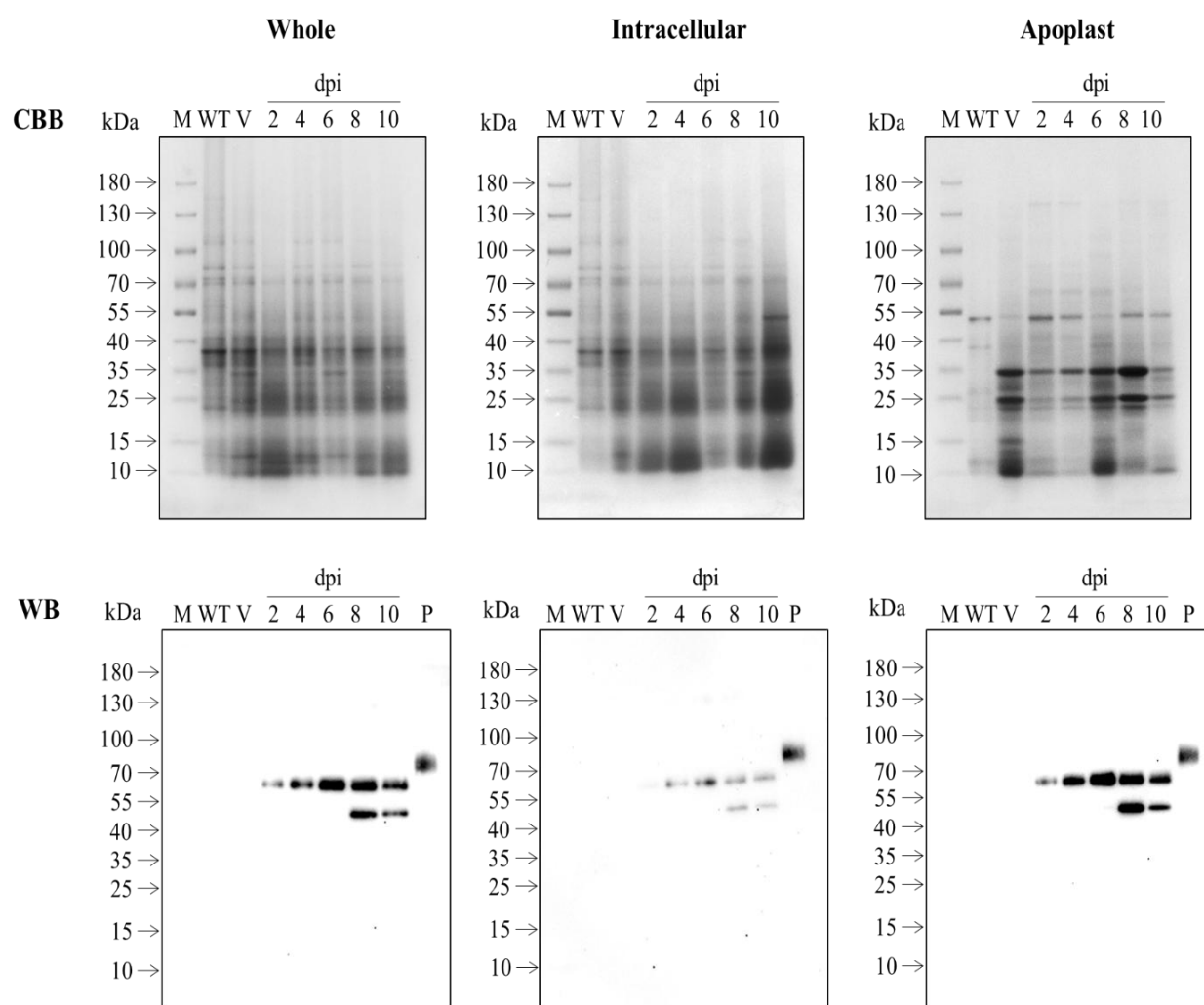


Figure 14 A time-course analysis of ASM production during 2 until 10 days post-infiltration (dpi) was conducted using SDS-PAGE and Western blot analysis. M: Prestained protein ladder marker (Thermo Scientific PageRuler Cat#26616); WT: Wild type plant; V: Empty vector (pRI201-AN); P: CHO-derived ASM (5 ng), probed by rabbit anti-human ASM (1:2,000).

3.3.4 ASM purification by two steps ion exchange chromatography

The AWF sample served as the crude protein source to purify tag-free recombinant human ASM from *N. benthamiana* plants. To effectively isolate ASM from other proteins according to their charge differences, a two-step ion exchange chromatography method was employed, utilizing SP-550C and DEAE Sepharose™ CL-6B columns in sequence. To optimize the protein charge system, the AWF sample was adjusted to pH 5.0 before being loaded onto the initial SP-550C column, ensuring proper protein binding. The elution from the SP-550C column at pH 8.0 was subsequently pooled and applied to the second column, DEAE Sepharose™ CL-6B, for further purification. The purity of ASM (61 kDa) was evaluated through SDS-PAGE and Western blot analysis (Figure 15A), which indicated minimal presence of other proteins in the purified fraction, confirming the success of the purification process. Each purification step eliminated loosely bound, non-specific proteins from the plant matrix, resulting in the successful isolation of tag-free recombinant human ASM. Figure 15B illustrates the specific enzyme activities of the crude protein from AWF, SP-550C-purified protein, and DEAE Sepharose™ CL-6B-purified protein. The enzyme activity of ASM progressively increased throughout the purification procedure. After purification, the ASM-specific activity in the purified sample (128.18 ± 4.18 mU/mg purified protein) increased by up to 21 times compared to the crude sample (6.04 ± 0.33 mU/mg TSP), with a yield of approximately 49%. The total amount of ASM produced was about 3.5 µg/g of fresh leaf weight. Table 6 summarizes the total activities, purification fold, and yield at each purification step, demonstrating the effectiveness of the two-step chromatography method in isolating highly purified ASM. This purification approach emphasizes the value of AWF as an essential starting material and showcases the successful application of ion exchange chromatography for purifying tag-free recombinant human ASM from *N. benthamiana*.

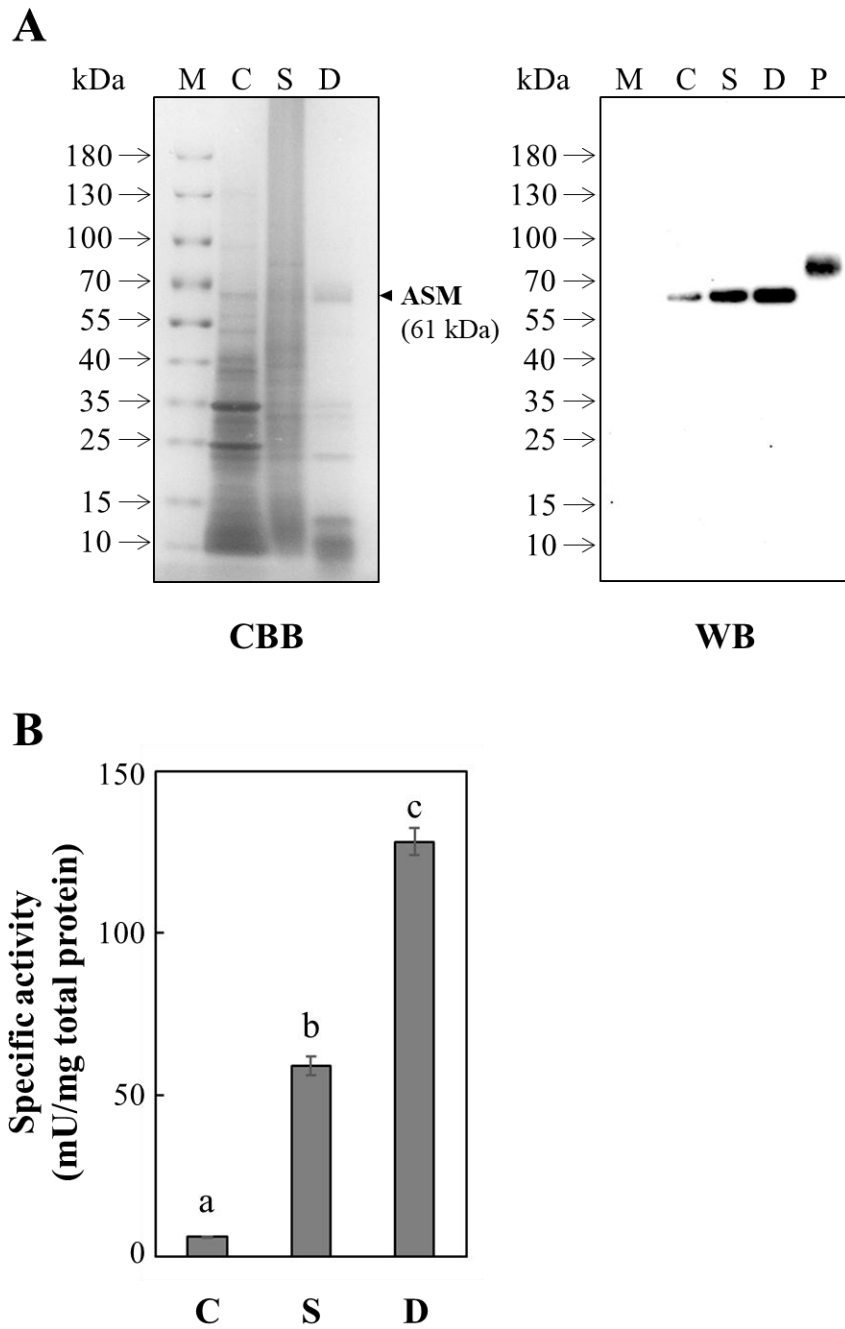


Figure 15 Tag-free recombinant ASM purification from *N. benthamiana* utilizing AWF samples at 6 dpi. (A) SDS-PAGE and Western blot analysis of purified ASM by two-step purification, M: Prestained protein ladder marker (Thermo Scientific PageRuler Cat#26616). C: Crude protein from AWF. S: SP-550C-purified. D: DEAE SepharoseTM CL-6B-purified. P: CHO-derived ASM (5 ng), probed by rabbit anti-human ASM (1:2,000). (B) Specific activity of purified ASM over the purification step.

Table 6 ASM purification from AWF sample (Crude). Purification was performed using two-step chromatography columns including SP-550C strong cation exchange chromatography (SP) and DEAE SepharoseTM CL-6B weak anion exchange chromatography (DEAE).

Purification step	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Specific activities (mU/mg)	Total activity (U)	Purification fold	Yield (%)
Crude	50	0.58	29	6.04 ± 0.33	0.175	1.0	100
SP	25	0.112	2.8	59.07 ± 2.95	0.165	9.78	94.29
DEAE	10	0.067	0.67	128.18 ± 4.18	0.086	21.22	49.14

3.3.5 Identification of proteins and N-terminal amino acid sequencing in ASM-producing plants

To identify the purified protein, the target band from the SDS-PAGE gel was excised and enzymatically digested with pepsin to break it into smaller glycopeptides. These peptides were then analyzed using nanoLC-MS/MS. The resulting mass spectrometry data were interpreted using the MASCOT search engine and compared against the SwissProt and NCBI protein databases. The top hit from the MASCOT analysis corresponded to sphingomyelin phosphodiesterase (acid sphingomyelinase) from *Homo sapiens*, confirming that the purified protein was human ASM. Furthermore, the nanoLC-MS/MS result was analyzed using the BioTools software (version 3.2, Bruker Daltonics) to confirm the sequence coverage, showing a 95.1% match with the reference protein sequence (Figure 16). Amino acid sequence alignment of the plant-derived ASM with the human ASM sequence is shown in the supplementary material. To gain deeper insight into the purified ASM structure, the N-terminal amino acid sequence of the purified protein was determined by Edman degradation. The sequence obtained, GWGNLTCPIC, matched the predicted N-terminus of mature ASM, confirming that the signal peptide had been correctly cleaved during processing. This cleavage site occurs at amino acid position 26, which was also accurately predicted by SignalP 6.0 (Teufel et al., 2022) with a high confidence score of 0.95 (Figure 17). So, the mature ASM protein starts at amino acid position 27. Interestingly, another N-terminal sequence, MGKM, was also detected, suggesting that some of the ASM protein still retained its signal peptide. This finding implies that signal peptide cleavage was not completely efficient, resulting in a mixed population of mature and uncleaved ASM.



Figure 17 SignalP6.0 prediction of secreted truncated ASM cleavage.

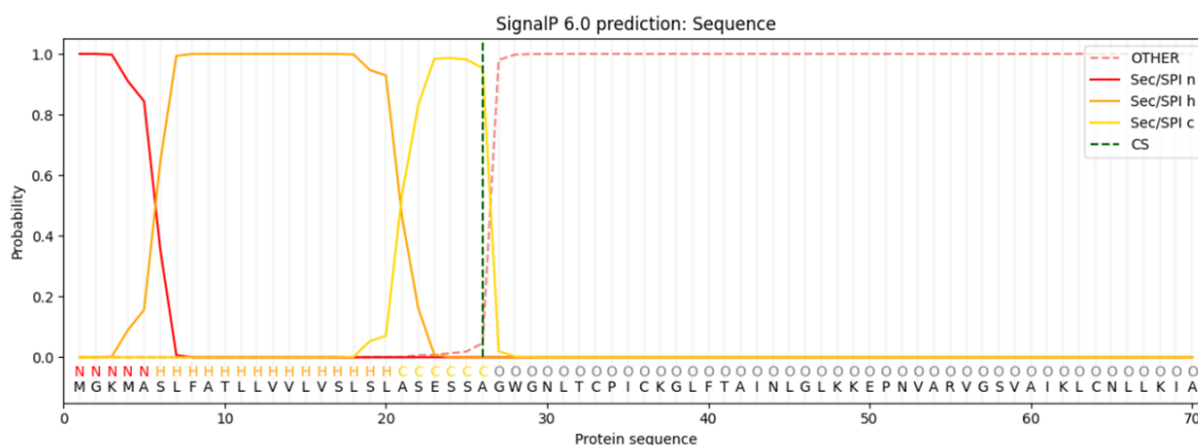


Figure 17 SignalP6.0 prediction of secreted truncated ASM cleavage.

3.4 Discussion

This study marks the first successful attempt to establish an effective purification process for recombinant human ASM expressed in plants using a two-step purification method. The AWF sample was utilized as the crude protein source to purify tag-free recombinant human ASM from *N. benthamiana* plants. This strategy was chosen due to the challenges associated with directly extracting ASM from cell extracts, which is ineffective and hindered by the complex mixture of cellular proteins. Additionally, more advanced purification techniques were required because the recombinant ASM used in this study lacked affinity tags. The approach employed, involving Sulfopropyl-550C (SP-550C) and DEAE Sepharose™ CL-6B ion exchange chromatography columns, has proven to be effective in isolating tag-free recombinant human ASM from *N. benthamiana* plants. This represents a significant advancement in the production and purification of ASM. The two-step ion exchange chromatography method efficiently separated ASM from other plant-derived proteins based on charge differences, resulting in a highly purified enzyme fraction, though not completely pure, with minimal contamination from other proteins.

Generally, ASM has been purified from human brain tissue using a complex seven-step process, involving several chromatographic techniques: Concavalanin A-Sepharose, Octyl-Sepharose, Hydroxylapatite, DEAE-cellulose, Red A-agarose, Sephadex G-200, and DEAE-cellulose with ampholyte elution (Yamanaka & Suzuki, 1982). This method, which results in a specific activity of up to 13.33 U/mg protein, is both time-consuming and labor-intensive. Previous publication reported the use of FLAG-tagged ASM, which was purified through a series of steps including anti-FLAG immunoaffinity chromatography, Superose-12 gel filtration chromatography, and a second round of anti-FLAG immunoaffinity chromatography (Schissel et al., 1998). This multi-step purification process offers a more efficient way of isolating high-purity ASM, although it still requires significant time and resources.

Compared to previous studies on recombinant human ASM purification, this approach demonstrates several advantages. For instance, in other studies, recombinant ASM produced in *E. coli* is often inactive and unglycosylated, limiting its biological activity. While bacterial systems can produce large quantities of recombinant protein, they often result in protein aggregation and improper folding. In contrast, insect and mammalian cell lines have shown more success in expressing full-length or mutant forms of ASM, but these systems tend to be more complex and expensive. Insect and mammalian cell systems such as Sf21 and CHO cells have been able to achieve specific activities of 7.33 U/mg and 8.73 U/mg, respectively,

with genetic engineering further improving enzyme activity (Bartelsen et al., 1998; He et al., 1999). For instance, a study involving CHO cells substituted the final cysteine residue of ASM with serine, boosting its specific activity to 91.1 U/mg – much higher than the wild-type enzyme (Qiu et al., 2003). Similarly, fusing ASM with a twin-strep-tag II and expressing it in HEK293S GNT1(-) cells led to a remarkable specific activity of 360 U/mg (Aldosari et al., 2019). Moreover, the use of affinity tags can sometimes introduce challenges during the purification process, including the need for tag removal and potential loss of protein activity (Amarasinghe & Jin, 2015; Guan & Chen, 2014). In contrast, the plant expression system used in this study not only allows for correct protein folding but also simplifies the purification process by eliminating the need for affinity tags. This is a significant advantage, as it reduces the risk of losing enzyme activity during the tag removal process (Pina et al., 2014). Recombinant proteins made in plant cells often show lower activity compared to those produced in mammalian or insect cells. This is likely due to several related reasons. First, plant cells have natural enzymes called proteases that can break down the protein during or after production, which reduces the overall yield and may affect how well the protein works (Pillay et al., 2014). Second, the protein-folding systems in plant cells – especially in the endoplasmic reticulum – may not work as efficiently, which can lead to protein folding incorrectly or forming aggregates (Wilbers et al., 2017). Third, plant cells handle post-translational modifications differently than human cells. For example, they may not add the same sugar chains (glycosylation) or form disulfide bonds properly, both of which are important for the stability and function of many therapeutic proteins (Strasser, 2016).

In this study, the buffer conditions used during the purification process were different compared to those in previous studies (Duan et al., 2003; Pentchev et al., 1977). Other studies employed a NaCl gradient for washing and elution, we utilized pH adjustments, which not only streamlined the ion exchange chromatography but also enhanced the overall efficiency of the purification process. This modification reduced protein aggregation and degradation, resulting in a more efficient and time-saving procedure. As a result, ASM production reached approximately 3.5 mg/kg of fresh leaf weight, and the specific enzyme activity increased 21-fold, from 6.04 ± 0.33 mU/mg in the crude sample to 128.18 ± 4.18 mU/mg in the purified fraction. This significant enhancement in enzyme activity demonstrates the effectiveness of the two-step ion exchange chromatography method and its potential for scaling up recombinant protein production in plants. With a yield of around 49%, this method shows promising potential for large-scale production, maintaining high enzyme activity and purity.

Recent advances in plant-based recombinant protein expression have been driven by the development of viral vectors that enable high protein yields, ranging from 0.5 to 5 g/kg of fresh leaf biomass or over 50% of total soluble proteins. For instance, protein-derived from *N. benthamiana*, such as model proteins like GFP, has been produced at levels of 3-5 g/kg using high-expression vectors (Chen et al., 2011), and human iduronate-2-sulfatase (IDS) expression has reached almost 2 g/kg (Sinha et al., 2024).

Overall, this chapter presents a novel and effective strategy for purifying recombinant human ASM from plants. The simplicity and effectiveness of the two-step ion exchange chromatography method make it a valuable tool for future recombinant protein production applications, offering a more scalable and cost-effective alternative to traditional expression systems. Further optimization of the process could potentially enhance yield and efficiency, paving the way for large-scale production of recombinant human ASM for therapeutic applications.

3.5 Summary

This study successfully establishes an effective two-step purification process for recombinant human ASM expressed in *N. benthamiana* plants, marking a significant advancement in the production and purification of this enzyme. By utilizing the AWF sample as a crude protein source and employing SP-550C and DEAE Sepharose™ CL-6B ion exchange chromatography, ASM was efficiently isolated, demonstrating high enzyme activity with minimal contamination. Unlike traditional methods, which require complex and time-consuming procedures such as the seven-step purification process used for brain-derived ASM, the two-step approach used in this study simplifies the process, resulting in a 21-fold increase in enzyme activity and a promising yield of 49%. This method holds great potential for scaling up recombinant protein production and offers a more cost-effective and efficient alternative to traditional systems. With further optimization, this process could significantly enhance yield and efficiency, paving the way for industrial-scale production of ASM.

Chapter 4

Engineering reduced-size expression vectors for improved protein yield in *N. benthamiana* whole plants

4.1 Introduction

The size and structure of binary vectors used in *A. tumefaciens* – mediated transient expression have a significant impact on transformation efficiency, gene delivery, and protein yield. Large binary vectors like pBI121 often contain non-essential bacterial sequences – such as the *lac* region, multiple antibiotic resistance genes and reporter cassettes – which increase plasmid size without contributing to protein expression in plants. These redundant elements can reduce cloning efficiency, hinder transformation, and place unnecessary metabolic burden on both bacterial and plant hosts. Therefore, minimizing vector backbone size by eliminating non-functional regions has become an approach to enhance recombinant protein production in plants (Lee et al., 2012; Ye et al., 2008).

Recent studies have demonstrated that smaller vector constructs not only replicate more efficiently in *Agrobacterium* but also promote higher gene expression in host plant cells (Peyret & Lomonosoff, 2013). This is particularly important in large-scale molecular farming, where maximizing protein yield while minimizing genetic load is crucial for both cost-effectiveness and biosafety. In addition, minimizing vector size facilitates easier manipulation, more efficient cloning, and reduced risk of unintended horizontal gene transfer of antibiotic resistance genes.

Agroinfiltration represents a revolutionary approach in plant biotechnology, offering an efficient and rapid approach for the transient expression of recombinant proteins in plants (Norkunas et al., 2018). The success of this technology is strongly relying on specific buffer components that play crucial roles in optimizing protein yield. Three key additives – p19, acetosyringone, and ascorbic acid – are considered critical for maximizing recombinant protein yield.

Each factor plays a distinct and essential role in enhancing the efficiency of transient protein expression:

- **p19 silencing suppressor:** this crucial additive prevents post-transcriptional gene silencing (PTGS), allowing sustained expression levels. By suppressing PTGS, p19 enables prolonged transgene expression and enhanced protein accumulation (Jay et al., 2023).
- **Acetosyringone:** this phenolic compound acts as a virulence inducer in *Agrobacterium*, triggering the expression of virulence genes and enhancing T-DNA transfer efficiency. Additionally, it promotes the accumulation of VirB proteins essential for DNA transfer and activates heat-shock proteins that facilitate the transformation process (Xi et al., 2018).
- **Ascorbic acid:** serving as an antioxidant, it counteracts reactive oxygen species (ROS) produced during the agroinfiltration process. By preventing oxidative damage and cell necrosis, it creates a more favorable cellular environment for protein synthesis and accumulation (Zhao et al., 2017).

A clear understanding of these buffer components is vital for refining agroinfiltration strategies and enhancing recombinant protein yields in plants. Their combined effects create an improve the conditions for T-DNA transfer, maintain stable transgene expression, and promote high levels of protein production – establishing them indispensable tools in the advancement of plant-based recombinant protein production.

In this study, we present a two-step approach to enhance recombinant protein production in *N. benthamiana* by minimizing vector backbone and optimizing agroinfiltration conditions. Using β -glucuronidase (GUS) and acid sphingomyelinase (ASM) as model proteins, we demonstrate that vector modification and buffer optimization significantly improve expression levels, offering practical strategies to advance transient expression systems in plant biotechnology.

4.2 Materials and Methods

4.2.1 Plasmid construction and vector minimization

Minimized vector constructs were generated by stepwise deletion of non-essential regions from the original pBI121 plasmid (14,758 bp). Specifically, the *lac* promoter/operator region (872 bp) and the tetracycline resistance (*tetR*) gene (1,626 bp) were removed to reduce plasmid size. In the first step of minimization, the *lac* promoter/operator region was deleted from pBI121. The plasmid backbone was amplified by PCR using primers *XhoI-lac-F* and *XhoI-lac-R*, which flank and exclude the *lac* region. The amplified product was digested with *XhoI* at both ends and ligated to produce a version of pBI121 lacking the *lac* promoter/operator region, referred to as pBI121 Δ *lac*. In the next step, the tetracycline resistance region was removed from pBI121 Δ *lac*. This was accomplished by amplifying the plasmid backbone using two sets of primers: *Sall-tetR1-F/HindIII-tetR1-R* and *HindIII-tetR2-F/Sall-tetR2-R*, generating two separate PCR fragments. These fragments were digested with *Sall* and *HindIII*, then ligated to form the final minimized vector, designated pBI121 Δ *lac* Δ *tetR* or mpBI121. The new mpBI121 plasmid construct was generated by replacing the β -glucuronidase (*GUS*) reporter gene with the green fluorescent protein (*GFP*) gene, which was used as a negative control in the experiment. The GFP fragment, derived from pRI201-GFP plasmid (Yamamoto, 2023), was digested with *HindIII* and *EcoRI*. It was then ligated into the mpBI121 vector, which had been digested with same restriction enzymes, resulting in the mpBI121-GFP construct. All constructed plasmids were first transformed into *E. coli* DH5 α for propagation and subsequently introduced into *A. tumefaciens* LBA4404, as detailed in Chapter 2, Section 2.2.2. The resulting constructs were verified by colony PCR, restriction enzyme digestion, and DNA sequencing. Primer sequences used in these steps are provided in Table 7.

Table 7 List of primers used in stepwise plasmid minimization.

Primer ^a	Sequences ^b (5' → 3')
<i>XhoI-lac-F</i>	ATA <u>CTCGAGAC</u> GACACACTTGTCTACTCC
<i>XhoI-lac-R</i>	TAA <u>CTCGAGC</u> AGTAGCGACAGAATCAAG
<i>Sall-tetR1-F</i>	AATG <u>TCGACAT</u> GCACATACAAATGGACG
<i>HindIII-tetR1-R</i>	TATA <u>AAGCTTC</u> GTCTCGAGCAGTAGCGAC
<i>HindIII-tetR2-F</i>	ATTA <u>AAGCTTC</u> ACACTTGTCTACTCCAA
<i>Sall-tetR2-R</i>	ATTG <u>TCGACTT</u> CGCACGATATACAGG

^a F and R represent forward and reverse primers, respectively.

^b Underlined sequences indicate the restriction enzyme recognition sites.

4.2.2 Antibiotics sensitivity test

Antibiotic sensitivity testing was conducted to evaluate the presence or absence of tetracycline resistance in *A. tumefaciens* strains carrying different plasmids; pBI121, pBI121 Δ lac, and mpBI121. Transformed strain were cultured on 2xYT agar plates containing the following antibiotics; 50 µg/mL each of kanamycin, streptomycin, and rifampicin. A parallel set of plates was prepared with the same antibiotics, with the addition of tetracycline at 10 µg/mL. All agar plates were incubated at 30°C for 2 days and observed for colony growth.

4.2.3 Generation of plasmid encoding acid sphingomyelinase

Based on the findings from Chapter 2, the pRI201-ntExtASM construct yielded the highest expression level. To further enhance and verify this expression using the minimized vector, the *GUS* gene in pBI121, pBI121 Δ lac, and mpBI121 constructs was substituted with ntExtASM. Additionally, the AtADH 5' UTR was introduced upstream of the coding sequence, and the NOS terminator was replaced with the HSP terminator to enhance transcription and translation efficiency. For the pBI121 Δ lac construct, the backbone was amplified by PCR using *EcoRI*-M13-F and *HindIII*-Splac-R primers (Table 8), and the PCR product was digested with *EcoRI* and *HindIII*. In parallel, the pBI121 and mpBI121 backbone were directly digested with the same restriction enzymes. The ntExtASM insert was excised from the pRI201-ntExtASM construct using *EcoRI* and *HindIII* to isolate the target fragment. This fragment was then ligated into the prepared backbone to generate pBI121-ntExtASM, pBI121 Δ lac-ntExtASM, and mpBI121-ntExtASM. All newly constructed plasmids were introduced into *E. coli* DH5 α and

A. tumefaciens LBA4404 (Chapter 2, Section 2.2.2). These constructs were then agroinfiltrated into *N. benthamiana* plants for further experiments.

Table 8 Primers utilized for amplification of the pBI121 Δ lac backbone.

Primer ^a	Sequences ^b (5' → 3')
<i>Eco</i> RI-M13-F	TATGAATTC <u>ACTGGCCGTCGT</u>
<i>Hind</i> III-Splac-R	TATAAGCTTCGCCGCCAGCATTGACA

^a F and R represent forward and reverse primers, respectively.

^b Underlined sequences indicate the restriction enzyme recognition sites.

4.2.4 Agroinfiltration for transient expression and protein isolation

All *A. tumefaciens* strains carrying the original and modified plasmid constructs were used for agroinfiltration to assess the expression of GUS. The transient expression protocol followed the method previously mentioned in Chapter 2, Section 2.2.3. Infiltrated leaves were harvested at 6 dpi and extracted using the extraction buffer (50 mM Sodium phosphate pH 7.0, 10 mM β -mercaptoethanol, 10 mM EDTA, 0.1% Triton X-100) (Sheshukova et al., 2022). Total soluble protein was quantified using the Bradford and assay, and GUS enzymatic activity was measured to evaluate reporter gene expression.

4.2.5. Blue light-based validation of GFP expression

GFP expression was evaluated by observing the infiltrated leaves under blue light to confirm the successful introduction and expression of the modified backbone vector. Leaves agroinfiltrated with the mpBI121-GFP and mpBI121 constructs were harvested at 6 dpi and exposed to 470 nm blue light (Digi Print Doc; Biotools, Gunma, Japan). Fluorescence images were captured in a dark condition using a Canon Kiss X5 camera with the following settings: exposure time 0.25 sec, aperture F4.5, and ISO 1600. The captured images were adjusted for brightness (+40%) and contrast (-20%), and the fluorescence patterns were compared with those of non-infiltrated control (wild-type) leaves.

4.2.6 Extraction of total RNA and cDNA synthesis

Using the RNeasy Plant Mini Kit (QIAGEN), total RNA was extracted from infiltrated leaf tissues following the provided protocol. The 0.2 g of leaf material was placed in a Simport screw cap tube (2 mL; T332-7, Ina Optica, Osaka, Japan) containing two metal beads

and rapidly frozen in liquid nitrogen. While frozen, the samples were ground into a fine powder by shaking twice at 1,200 rpm for 30 sec treated with DNase (Promega) according to the supplier's guidelines. Afterwards, complementary DNA (cDNA) was produced from the DNase-treated RNA using the SuperScript™ IV VILO™ Master Mix (Invitrogen) as instructed. The cDNA was then stored at -20°C until further use.

4.2.7 Reverse-transcription quantitative PCR (RT-qPCR) for mRNA expression analysis

RT-qPCR was carried out as described by Bennett et al. (2015). Each cDNA sample was tested three times, with each test coming from a different RNA extraction. The reaction mix (20 µL total) included 5 ng/µL of cDNA, 300 nM CXR reference dye (Promega), 10 pmol/µL of each forward and reverse primers, 2x GoTaq® qPCR Master Mix (Promega). The tests were run on a QuantStudio 3 system (Applied Biosystems) using a 96-well reaction plate. (0.1 mL, Applied Biosystems, Foster city, CA, USA). The primers used for RT-qPCR are listed in Table 9 and the PCR condition is shown in Table 10. Fluorescence was measured after each cycle. At the end, a melt curve test was done to check the specificity of the results. All samples for each reference gene were processed together to ensure accuracy. Gene expression was normalized using three housekeeping genes: 18S ribosomal RNA, elongation factor 1-alpha (EF1α), and actin. The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) with three biological replicates for each gene.

Table 9 Primers used for RT-qPCR.

Primer ^a	Sequences (5' → 3')
GUS F	ATCCATCGCAGCGTAATGC
GUS R	TCGCAGAACATTACATTGACG
18S F	GCAAGACCAAACTCAAAGG
18S R	TGTTTCATATGTCAAGGGCTGG
EF1α F	AGCTTTACCTCCCAAGTCATC
EF1α R	AGAACGCCTGTCAATCTTGG
Actin F	CTATTCTCCGCTTTGGACTTGGCA
Actin R	AGGACCTCAGGACAACGGAAACG

^a F and R represent forward and reverse primers, respectively.

Table 10 RT-qPCR cycling condition.

Temperature	Duration	Number of cycles
50°C	1 min	1 cycle
95°C	10 min	
95°C	15 sec	40 cycles
55°C	30 sec	
60°C	1 min	
95°C	25 sec	1 cycle
60°C	1 min	
95°C	15 sec	

4.2.8 GUS enzymatic activity assay

GUS activity was quantified using a fluorometric assay based on the enzymatic conversion of 4-methylumbelliferyl- β -D-glucuronide (4-MUG) (Tokyo Chemical Industry, Tokyo, Japan) into the fluorescent product 4-methylumbelliferone (4-MU) (Tokyo Chemical Industry). TSP supernatants were incubated with 4-MUG substrate at 37°C for 1 h. The reaction was stopped by adding sodium carbonate buffer, and fluorescence was measured using a fluorescent spectrophotometer (F-7100; Hitachi Ltd, Tokyo, Japan) with an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The fluorescence intensity of 4-MU was used to calculate GUS enzymatic activity. The mpBI121-GFP construct was included as negative control. One enzyme unit (U) is 1 μ mol of substrate converted per minute at 37°C.

4.2.9 Apoplastic wash fluid recovery

Infiltrated leaves were harvested at 6 dpi and fractionated into 3 different groups; whole, intracellular, and apoplast samples, following the procedures described in Chapter 2, Sections 2.2.4 and 2.2.5. Each fraction was subjected to a comparative analysis of ASM expression through total protein quantification (Bradford assay), ASM activity measurements, SDS-PAGE, and Western blot analysis.

4.2.10 Optimizing transient expression parameters for ASM

To optimize transient expression condition for ASM, eight different cases (Figure 18) were tested to assess their impact on protein yield. The tested variables included the co-expression or absence of the p19 silencing suppressor (at OD₆₀₀ = 0.5), as well as the additional of 100 µM acetosyringone, and 0.56 mM ascorbic acid (Zhao et al., 2017). Each buffer was prepared in infiltration medium, typically containing 10 mM MES and 10 mM MgSO₄. Bacterial cultures were adjusted to an OD₆₀₀ of 0.5, mixed with the respective infiltration buffers and incubated at room temperature for 2 h when the buffer included acetosyringone or ascorbic acid. For buffers containing ascorbic acid, the incubation was performed in the dark to preserve reagent stability.

	V	1	2	3	4	5	6	7	8
mpBI121-ntExtASM	-	+	+	+	+	+	+	+	+
p19	-	-	+	-	-	+	+	-	+
Acetosyringone (100 µM)	-	-	-	+	-	+	-	+	+
Ascorbic acid (0.56 mM)	-	-	-	-	+	-	+	+	+
mpBI121	+	+	-	+	+	-	-	+	-

Figure 18 Infiltration buffer components and experimental variables used for transient ASM expression in *N. benthamiana*, including co-expression with p19, and the addition of acetosyringone and ascorbic acid. V: Empty vector (mpBI121); 1-8: Cases 1-8.

4.2.11 Measurement of ASM enzyme activity

ASM activity was quantified using the Acid Sphingomyelinase Assay Kit (K-3200; Echelon Biosciences, Salt Lake City, UT, USA) according to the manufacturer's instructions. This fluorometric assay measures the hydrolysis of sphingomyelin by ASM, producing a fluorescent signal proportional to enzyme activity. Samples were incubated at 37°C for 3 h under shaking conditions. Fluorescence was measured using a fluorescent spectrophotometer (F-7100), and enzyme activity was expressed as µU/mg total protein.

4.2.12 Analysis of protein expression via SDS-PAGE and Western blot

A 10% SDS-PAGE gel was used to separate the protein samples, with the PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) included as a reference for molecular weight estimation. After SDS-PAGE, the gels were subjected to CBB staining and Western blot, as described in Chapter 2, Section 2.2.6.

4.2.13 Analysis of results

The study involved three independent biological replicates. Data are expressed as the mean $\pm$ SD. Each experiment was carried out in triplicate to guarantee the reliability and consistency of the results. Moreover, statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test to determine significant differences. Differences with a p -value ≤ 0.05 were considered statistically significant.

4.3 Results

4.3.1 Construction and validation of size-reduced plasmids

The *lac* promoter/operator region and tetracycline resistant (*tetR*) gene present in the original pBI121 plasmid are unnecessary for transient expression in plants. The *lac* promoter/operator region is primarily used for bacterial expression and regulation and does not contribute to protein production in plant cells. Similarly, the *tetR* gene serves only as a bacterial selectable marker and increases plasmid size without improving plant expression efficiency. Retaining these elements can impose unnecessary metabolic burden on both *Agrobacterium* and plant hosts, reduce cloning and transformation efficiency, and complicate vector handling. Therefore, these regions were specifically targeted for deletion to streamline the vector, reduce its size, and optimize it for high-yield recombinant protein expression in plants. To enhance the expression efficiency of recombinant proteins for high-yield production in plants, the original pBI121 plasmid was modified through stepwise deletions of non-essential elements, specifically the *lac* region and *tetR* gene. The resulting optimized vector is illustrated in Figure 19. To confirm successful modification of the vector, an antibiotic sensitivity test was conducted using constructs original pBI121, pBI121 Δ *lac*, and mpBI121. All constructs grew on plates without tetracycline, indicating viable transformation. However, only pBI121 and pBI121 Δ *lac* showed growth on tetracycline-containing plates, while mpBI121 did not, confirming the deletion of the tetracycline resistance gene. Consistent growth on kanamycin, streptomycin, and rifampicin plates further verified successful transformation. Overall, these results confirm the effective removal of both the *lac* and tetracycline resistance regions from the original vector.

The ntExtASM fragment, containing the CaMV 35S promoter, AtADH 5'UTR, and HSP terminator from the pRI201-ntExtASM construct (Chapter 2), was subcloned into three minimized vector – pBI121, pBI121 Δ *lac*, and mpBI121 – to generate the pBI121-ntExtASM, pBI121 Δ *lac*-ntExtASM, and mpBI121-ntExtASM constructs, respectively. (Figure 20).

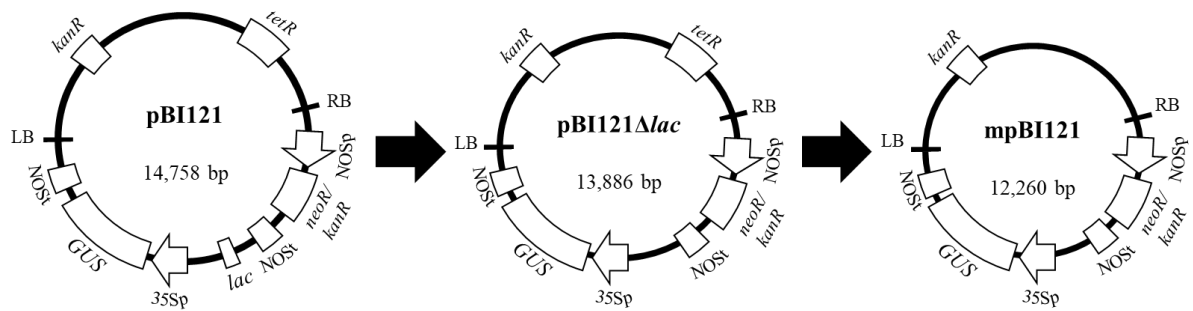


Figure 19 Schematic representation of minimized vector (pBI121, pBI121 Δ lac, and mpBI121). *tetR*: Tetracycline resistant gene; NOSp: Nopaline synthase promoter; neoR/kanR: Neomycin/ Kanamycin resistant gene; NOST: Napoline synthase terminator; *lac*: *lac* promoter/operator region; 35Sp: *Cauliflower mosaic virus* 35S RNA promoter; *GUS*: β -glucuronidase gene; RB and LB, Right and Left borders of T-DNA, respectively.

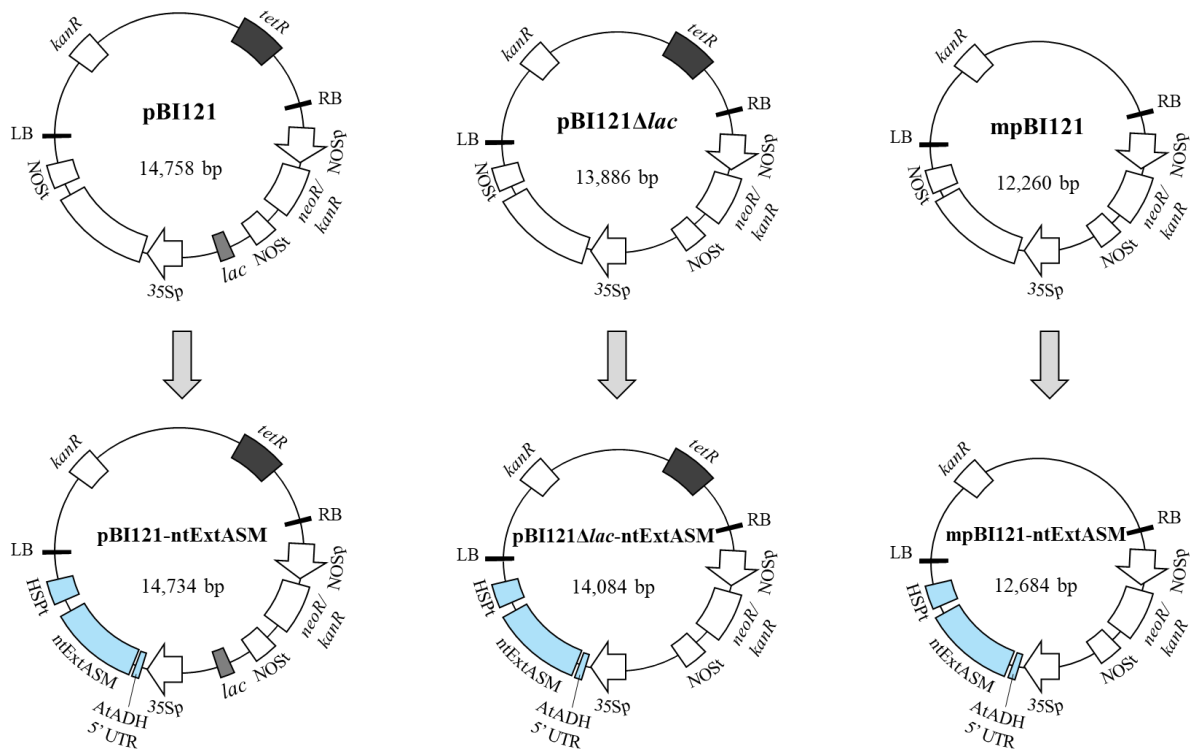


Figure 20 Schematic diagram of the engineered minimized vector containing ntExtASM. *tetR*: Tetracycline resistant gene; NOSp: Nopaline synthase promoter; neoR/kanR: Neomycin/ Kanamycin resistant gene; NOST: Napoline synthase terminator; *lac*: *lac* promoter/operator region; 35Sp: *Cauliflower mosaic virus* 35S RNA promoter; *GUS*: β -glucuronidase gene; RB and LB, Right and Left borders of T-DNA, respectively; AtADH 5' UTR: 5' untranslated region of *Arabidopsis thaliana* alcohol dehydrogenase gene; HSPt: Heat shock protein; ntExtASM: Truncated ASM fused to extensin signal peptide.

The mpBI121-GFP construct (Figure 21A) was generated by replacing the *GUS* reporter gene in the mpBI121 vector with the *GFP* gene. This construct was used as a negative control. All vectors were agroinfiltrated into *N. benthamiana* leaves. GFP expression from the mpBI121-GFP plasmid was confirmed by fluorescence detection under blue light condition. At 6 dpi, GFP fluorescence was observed in infiltrated leaves, while no fluorescence was detected in wild-type (non-infiltrated) and mpBI121 (empty vector) control leaves. The observed fluorescence in Figure 21B confirmed the functionality of the modified vector backbone in supporting recombinant protein expression in plant.

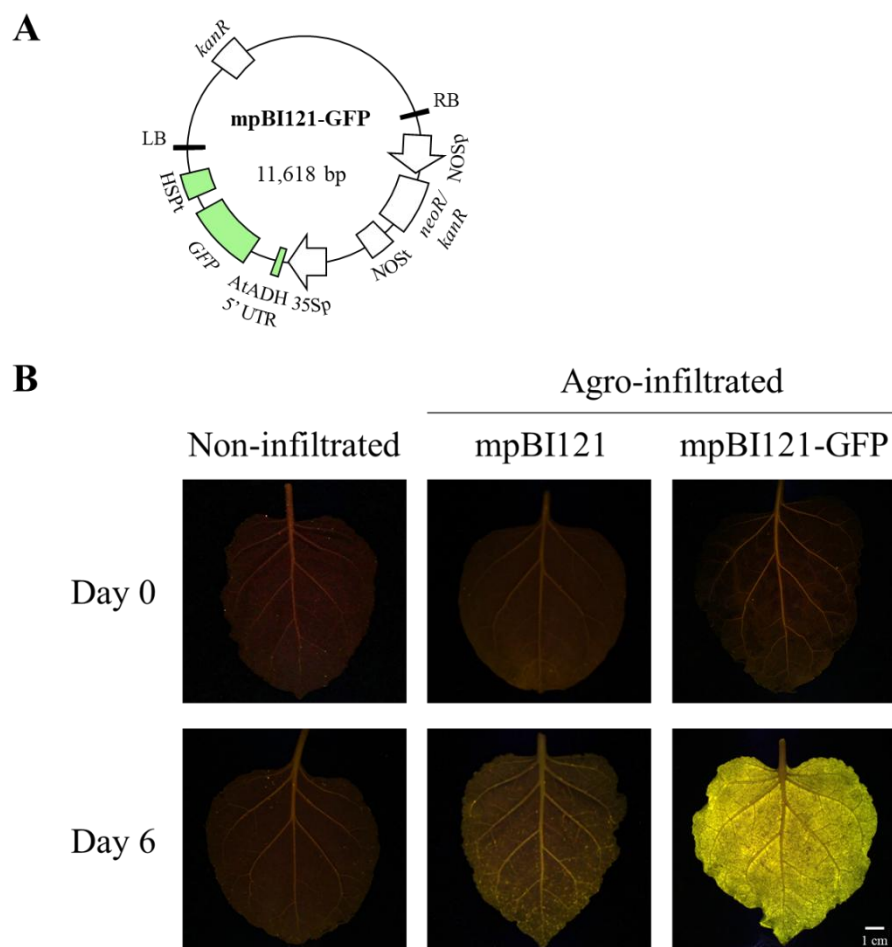


Figure 21 (A) Schematic representation of the mpBI121-GFP construct. (B) GFP expression in *N. benthamiana* leaves agroinfiltrated with mpBI121-GFP, visualized under blue light and compared to non-infiltrated and empty control (mpBI121) leaves.

4.3.2 Comparative analysis of mRNA expression in different expression vectors

To examine how vector size influences transgene expression, we measured *GUS* mRNA levels in *N. benthamiana* leaves after agroinfiltration using RT-qPCR. Three constructs – pBI121, pBI121 Δ *lac*, and mpBI121 – were compared, with mpBI121-GFP used as a negative control. Gene expression was normalized against three reference genes (18S, EF1 α , and actin), which showed consistent results across all samples. Compared with pBI121, *GUS* expression increased by about 1.30-fold with pBI121 Δ *lac* and 2.02-fold with mpBI121 (Figure 22A). No *GUS* transcripts were detected in the control. To further explore the reason for these differences, we compared the *GUS* transcript levels in infiltrated leaves with all constructs. RT-qPCR analysis showed slightly lower Δ Ct values in samples infiltrated with pBI121 Δ *lac* and mpBI121 compared to pBI121, indicating higher *GUS* transcript accumulation.

To confirm these findings with another gene, we examined the expression of *ASM* using the same set of constructs. RT-qPCR analysis showed that *ASM* transcript levels increased in a size-dependent manner: a 1.36-fold rise with pBI121 Δ *lac*-ntExtASM and 2.05-fold rise with mpBI121-ntExtASM compared to pBI121-ntExtASM (Figure 22B). No *ASM* expression was detected in the empty mpBI121 vector control. All fold change data from mRNA expression analysis of both *GUS* and *ASM* are summarized in Table 11.

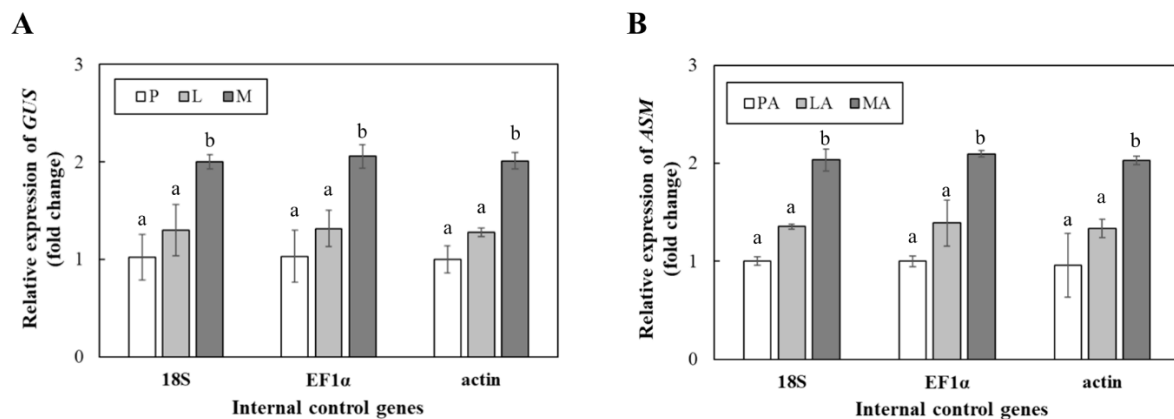


Figure 22 Analysis of mRNA expression levels. (A) Relative *GUS* mRNA levels were determined by RT-qPCR and normalized using plant housekeeping genes as internal control genes (18S, EF1 α , and actin), in plants carrying the following constructs: P: pBI121; L: pBI121 Δ *lac*; M: mpBI121. (B) Relative *ASM* mRNA expression, measured by RT-qPCR and normalized to the same housekeeping genes, with the constructs: PA: pBI121-ntExtASM; LA: pBI121 Δ *lac*-ntExtASM; MA: mpBI121-ntExtASM.

Table 11 Relative mRNA expression levels of *GUS* and *ASM*, presented as fold changes and normalized to plant internal control genes (n=3).

Target genes	Constructs	Fold change relative to control		
		18S	EF1 α	actin
<i>GUS</i>	pBI121	1.02 $\pm$ 0.23	1.03 $\pm$ 0.26	1.00 $\pm$ 0.07
	pBI121 Δ <i>lac</i>	1.30 $\pm$ 0.27	1.32 $\pm$ 0.19	1.28 $\pm$ 0.12
	mpBI121	2.00 $\pm$ 0.14	2.06 $\pm$ 0.04	2.01 $\pm$ 0.08
<i>ASM</i>	pBI121-ntExtASM	1.00 $\pm$ 0.04	1.00 $\pm$ 0.03	0.96 $\pm$ 0.11
	pBI121 Δ <i>lac</i> -ntExtASM	1.35 $\pm$ 0.05	1.39 $\pm$ 0.23	1.34 $\pm$ 0.04
	mpBI121-ntExtASM	2.03 $\pm$ 0.33	2.10 $\pm$ 0.09	2.03 $\pm$ 0.05

4.3.3 Transient expression of GUS and ASM using various minimized vector constructs

Transient expression of two proteins, GUS and ASM, was examined in *N. benthamiana* leaves using various minimized vector constructs. All vectors were introduced via vacuum agroinfiltration, and protein expression was analyzed at 6 dpi. As shown in Figure 23A, all minimized vectors retained GUS expression, with markedly higher activity observed in constructs of reduced size. Notably, some GUS activity was detected in control (mpBI121-GFP) leaves, likely due to endogenous GUS-like activity in the plant (Alwen et al., 1992). The pBI121 Δ *lac* and mpBI121 vectors exhibited GUS activities of 30.93 $\pm$ 2.16 nU/mg (1.33-fold) and 40.75 $\pm$ 1.43 nU/mg (1.76-fold), respectively, compared to the original pBI121 (23.20 $\pm$ 0.36 nU/mg), indicating enhanced expression efficiency through vector minimization.

ASM activity assays showed a parallel trend to GUS results, with 102.95 $\pm$ 1.66 μ U/mg (1.43-fold) and 125.32 $\pm$ 3.35 μ U/mg (1.75-fold) observed for the pBI121 Δ *lac*-ntExtASM and mpBI121-ntExtASM constructs, respectively, compared to pBI121-ntExtASM vector (71.81 $\pm$ 2.96 μ U/mg) (Figure 23B). Overall, both GUS and ASM displayed consistent expression patterns, where smaller, minimized vectors significantly improved protein expression and activity relative to the conventional pBI121 construct. That summarized in Table 12. Similarly, Western blot analysis of ASM-expressing constructs revealed a distinct band at approximately 61 kDa, confirming successful expression from each minimized vector (Figure 24).

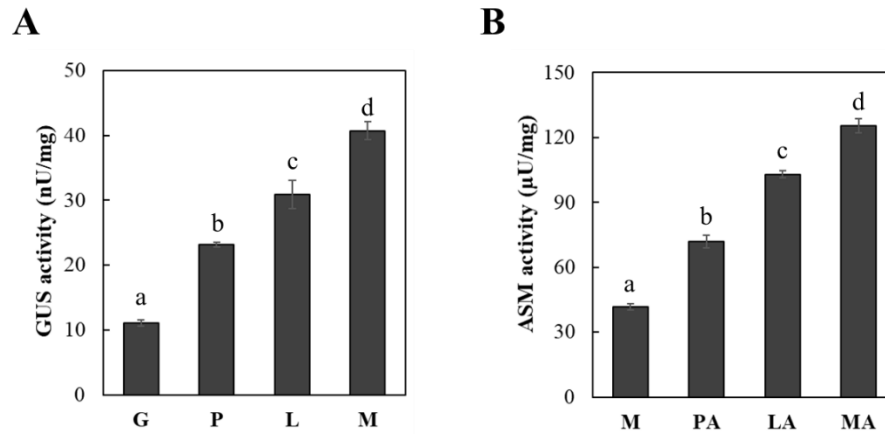


Figure 23 (A) GUS enzyme activity in leaf extracts at 6 dpi using different vector constructs. G: mpBI121-GFP; P: pBI121 (P); L: pBI121 Δ lac; M: mpBI121. (B) ASM enzyme activity in *N. benthamiana* leaves at 6 dpi following agroinfiltration with the indicated constructs. M: mpBI121; PA: pBI121-ntExtASM; LA: pBI121 Δ lac-ntExtASM; MA: mpBI121-ntExtASM. Each value represents the mean $\pm$ standard deviation (SD) from three independent biological infiltration experiments.

Table 12 Summary the enzyme activity of GUS and ASM in crude extract derived from the minimized vector constructs (n=3).

Target proteins	Crude extracts	Enzyme activity	Units
GUS	mpBI121-GFP	11.11 $\pm$ 0.49	nU/mg
	pBI121	23.20 $\pm$ 0.36	
	pBI121 Δ lac	30.93 $\pm$ 2.16	
	mpBI121	40.75 $\pm$ 1.43	
ASM	mpBI121	41.79 $\pm$ 1.49	μU/mg
	pBI121-ntExtASM	71.81 $\pm$ 2.96	
	pBI121 Δ lac-ntExtASM	102.95 $\pm$ 1.66	
	mpBI121-ntExtASM	125.32 $\pm$ 3.35	

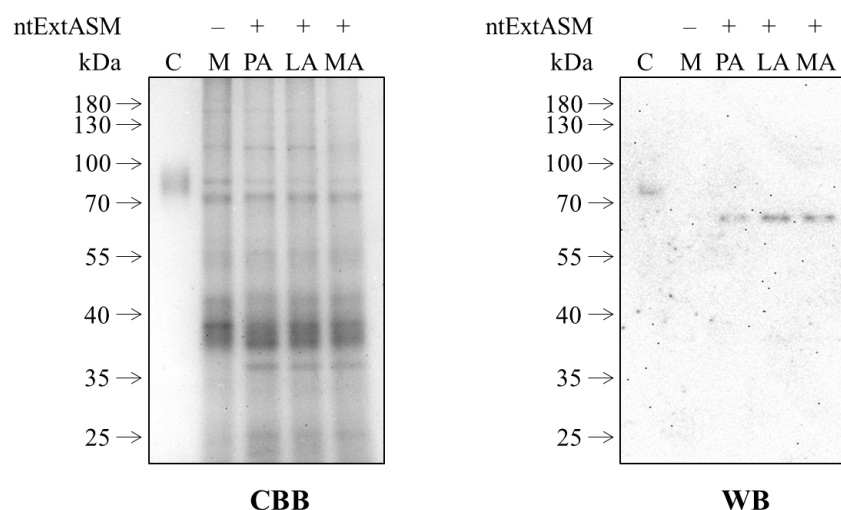


Figure 24 Assessment of ASM expression in *N. benthamiana* using minimized vector constructs. SDS-PAGE (10%) followed by CBB staining and Western blot analysis was performed to detect ASM expression. C: CHO-derived human ASM (5 ng) as a positive control; M: mpBI121 (empty vector); PA: pBI121-ntExtASM; LA: pBI121 Δ lac-ntExtASM; MA: mpBI121-ntExtASM, probed by rabbit anti-human ASM (1:2,000).

4.3.3 Extensin signal peptide facilitates apoplastic expression and recovery of ASM

To assess the localization and secretion of ASM to the apoplast, as intended by the inclusion of the extensin signal peptide in the construct, agroinfiltrated *N. benthamiana* leaves were analyzed for ASM distribution. SDS-PAGE analysis and ASM enzyme activity assays were performed on all three fractions – whole leaf extract, intracellular, and apoplast. The results clearly showed that ASM was predominantly present in the apoplastic wash fluid, with a strong band observed on Western blot (Figure 25A) and corresponding enzymatic activity detected (Figure 25B), indicating successful recovery of the protein in the apoplastic space. Minimal ASM activity was detected in the intracellular fraction, further supporting the effective targeting and accumulation of ASM in the apoplast. These findings confirm that the extensin signal peptide successfully directed ASM secretion to the apoplast, and that the recovery method was efficient for isolating apoplast-localized proteins. All ASM activities in apoplast recovery are separated and presented in Table 13.

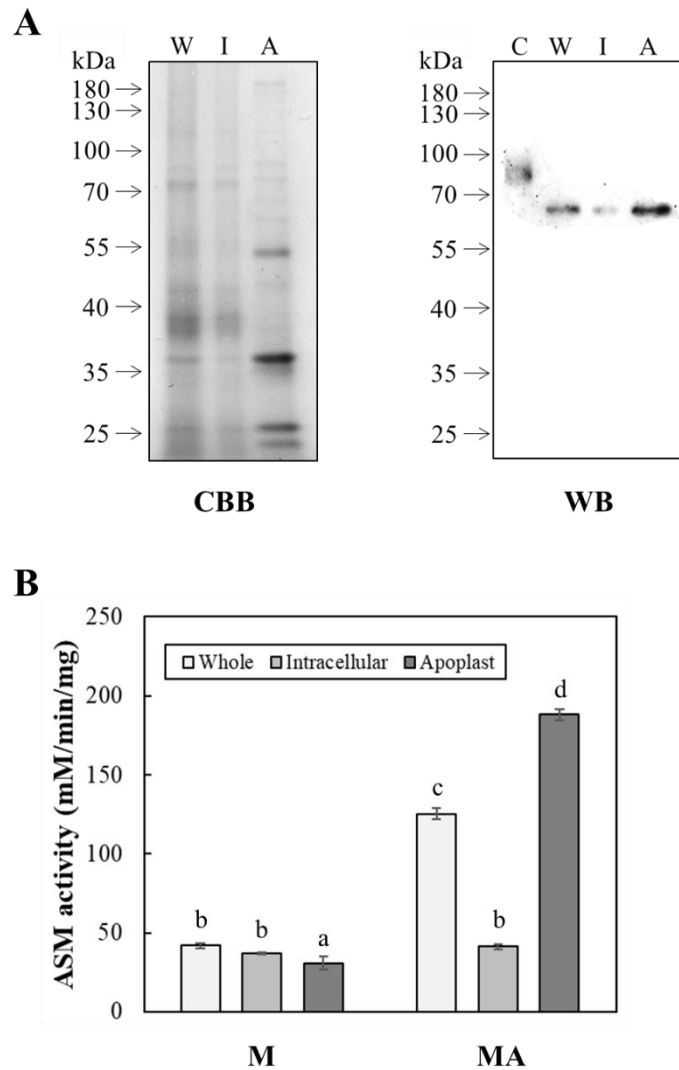


Figure 25 Isolation of ASM from the apoplastic wash fluid (AWF) fraction. (A) SDS-PAGE (10%) in CBB staining and Western blot analysis. C: CHO-derived human ASM (5 ng) as a positive control; W: Whole-plant crude extract; I: Intracellular crude extract; A: AWF, probed by rabbit anti-human ASM (1:2,000). (B) ASM enzyme activity was compared across whole, intracellular, and AWF samples from plants infiltrated with mpBI121 (M) and mpBI121-ntExtASM (MA) constructs (n=3).

Table 13 Comparison of ASM activity in apoplast recovery between mpBI121 and mpBI121-ntExtASM vectors (n=3).

Crude extracts	ASM activity (μ U/mg)		
	Whole	Intracellular	Apoplast
mpBI121	41.79 $\pm$ 1.49	37.05 $\pm$ 0.50	30.83 $\pm$ 4.29
mpBI121-ntExtASM	125.32 $\pm$ 3.35	41.24 $\pm$ 1.75	188.20 $\pm$ 3.63

4.3.4 Improving transient expression conditions for ASM production

Eight different cases (Figure 18) were evaluated to identify the most effective combination for maximizing ASM expression in *N. benthamiana*. These conditions differed in the inclusion of p19 co-expression, acetosyringone, and ascorbic acid, with an empty vector (mpBI121; V) serving as the negative control. Across all conditions, the highest ASM activity was consistently detected in the AWF, confirming successful secretion of the recombinant protein (Figure 26). Importantly, all four cases that produced the highest ASM activity (cases 5-8) co-expressed with p19, emphasizing its crucial role in enhancing transient expression by suppressing post-transcriptional gene silencing. Among them, case 8, which combined p19 co-expression with acetosyringone, and ascorbic acid, yielded the greatest ASM activity in the apoplast fraction (545.56 ± 4.28 $\mu\text{U}/\text{mg}$), representing a 2.9-fold increase compared with case 1 (190.11 ± 10.82 $\mu\text{U}/\text{mg}$), the traditional condition without enhancers) and a 16.6-fold increase compared to the vector control (32.93 ± 9.82 $\mu\text{U}/\text{mg}$) and approximately a 1.2-fold improvement over the next best condition (case 5, 522.80 ± 7.39 $\mu\text{U}/\text{mg}$), highlighting the additional benefit of acetosyringone and ascorbic acid supplementation (Table 14). The strong performance of case 8 can be attributed to the synergistic effects of its components p19 co-expression suppressed RNA silencing, acetosyringone induced *Agrobacterium* virulence gene expression to improve T-DNA delivery, and ascorbic acid reduced oxidative stress, stabilizing the recombinant protein. SDS-PAGE analysis of apoplast extracts (Figure 27) further confirmed these results, showing the most intense ~ 61 kDa band in case 8, consistent with the highest ASM enzyme activity observed. Collectively, these findings demonstrate that p19 co-expression is essential for high-level ASM expression, and that combining p19, acetosyringone, and ascorbic acid results in an optimized infiltration condition that maximizes recombinant protein yield and secretion efficiency in *N. benthamiana*.

Table 14 Summary of ASM activity using different infiltration buffers during agroinfiltration (n=3), corresponding to cases 1-8 described in Chapter 4, Section 4.2.10.

Conditions ^a	ASM activity (μU/mg)		
	Whole	Intracellular	Apoplast
V	38.95 ± 5.36	36.39 ± 8.77	32.93 ± 9.82
Case 1	120.41 ± 4.43	43.92 ± 5.43	190.11 ± 10.82
Case 2	255.41 ± 2.94	65.41 ± 10.67	456.15 ± 12.02
Case 3	182.83 ± 12.22	49.52 ± 5.29	373.49 ± 6.35
Case 4	147.93 ± 3.76	47.14 ± 8.34	250.15 ± 9.13
Case 5	284.36 ± 2.78	77.21 ± 10.49	522.80 ± 7.39
Case 6	269.97 ± 7.33	69.26 ± 6.64	495.71 ± 9.33
Case 7	237.17 ± 13.46	52.57 ± 10.54	412.77 ± 8.20
Case 8	314.36 ± 2.89	94.47 ± 6.11	545.56 ± 4.28

^a V: mpBI121 (empty vector) used as a negative control

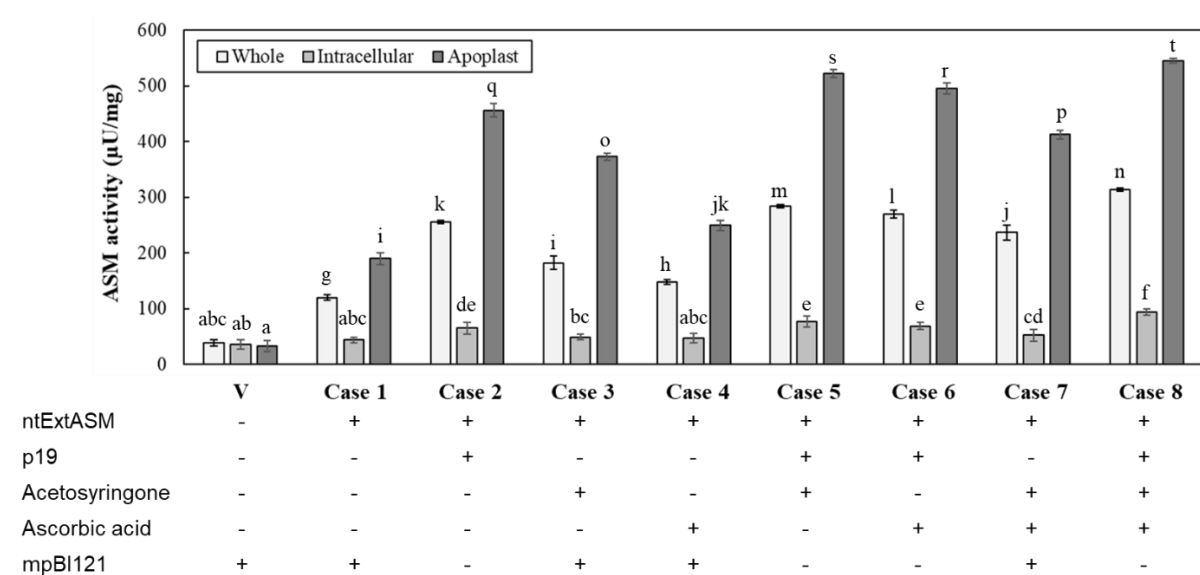


Figure 26 Comparison of ASM enzyme activity under different buffer conditions. V represent empty vector (mpBI121). Data represent the mean ± standard deviation (SD) from three independent infiltration experiments (n=3).

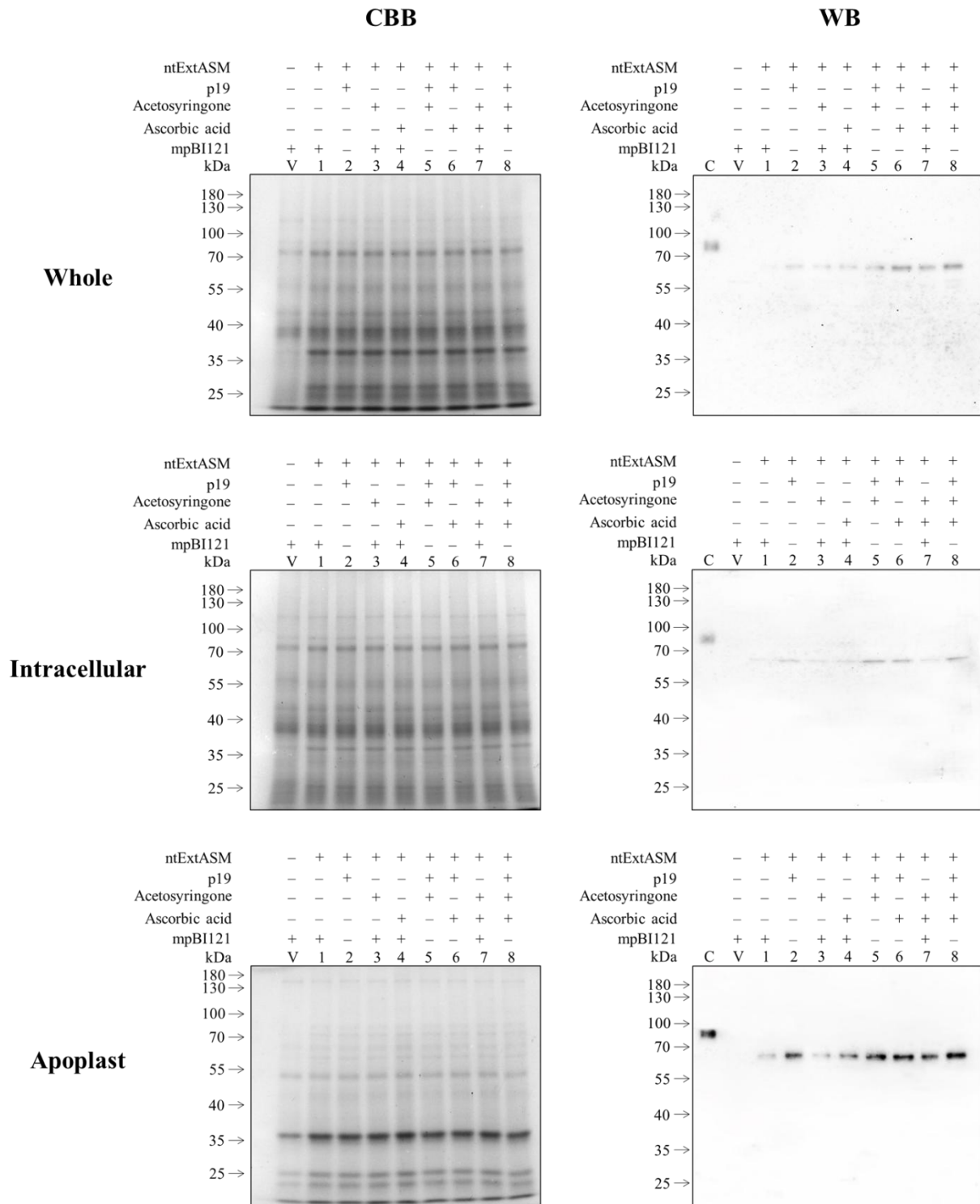


Figure 27 Analysis of ASM expression using various infiltration buffers for transient expression, assessed by SDS-PAGE followed by CBB staining and Western blot of whole, intracellular, and apoplast samples. C: CHO-derived human ASM (5 ng) used as a positive control and detected using rabbit anti-human ASM antibody (1:2,000).

4.4 Discussion

Reduction of non-essential sequences from binary vectors like pBI121 – such as the *lac* region and *tetR* – can significantly improve transient expression in *N. benthamiana*. These elements are primarily bacterial selection or cloning markers that serve no functional role in plant expression and may contribute unnecessary DNA length, which can reduce transformation efficiency and increase metabolic burden during *Agrobacterium*-mediated delivery. By removing these non-essential regions, the overall vector size is minimized, facilitating more efficient T-DNA transfer and potentially enhancing transcriptional and translational performance in plant cells. This streamlining strategy therefore improves the overall efficiency of transient protein expression without affecting essential components required for gene expression in plants. In this work, minimizing the size of the expression vector contributes to enhance protein expression in *N. benthamiana*. The minimized vector mpBI121 produced substantially higher mRNA levels of both *GUS* and *ASM* compared with the original pBI121 vector. Relative expression analysis confirmed that both *GUS* and *ASM* transcripts were significantly upregulated in plants transformed with mpBI121, indicating that vector minimization improves transcriptional efficiency. This increase in mRNA abundance corresponded closely with the elevated enzyme activities observed for both proteins, where *GUS* and *ASM* showed 1.76-fold and 1.75-fold higher activity, respectively, than those produced from pBI121. These findings demonstrate that reducing the binary vector size from pBI121 to the smaller mpBI121 markedly boosts recombinant protein production by enhancing both mRNA accumulation and protein yield. The consistent pattern across two distinct proteins suggests that vector minimization effectively improves transient expression in *N. benthamiana*. This outcome aligns with previous reports showing that smaller, simplified vectors enhance cloning efficiency, facilitate more effective T-DNA delivery, and ultimately lead to higher protein yield (Beritza et al., 2024). Recent advances in plant molecular farming further indicate that compact binary vectors, when paired with optimized constructs and delivery methods, can outperform larger systems, including those based on viral replicons, in terms of both consistency and scalability (Sainsbury et al., 2009; Szakasits et al., 2007). Mechanistically, larger vectors are more prone to transformation inefficiencies, as increased plasmid size hinders DNA transfer, replication, and maintenance in both bacterial and plant cells (Chan et al., 2002).

In *Agrobacterium*, smaller binary vectors promote greater T-strand accumulation and stronger *vir* gene activation, particularly following acetosyringone induction, which enhances T-DNA mobilization (Xi et al., 2018). In plant cells, excessive T-DNA length can reduce integration efficiency and expression stability by increasing the likelihood of incomplete transfer events, rearrangements, or transcriptional silencing of large transgenes (Frary & Hamilton, 2001). Overall, vector minimization represents practical strategy for boosting recombinant protein production in plant expression systems by enhancing both transcriptional and translational efficiency.

The composition of the infiltration buffer plays a critical role in the efficiency of transient expression. Among the eight buffer conditions tested, those containing p19 consistently resulted in higher ASM expression. This finding supports previous studies showing that p19, a viral suppressor of RNA silencing, prevents post-transcriptional gene silencing (PTGS), thereby allowing sustained transgene expression (Garabagi et al., 2012; Voinnet et al., 2003). The inclusion of acetosyringone – a compound that induces *Agrobacterium* virulence genes – also enhanced protein yield by promoting more efficient T-DNA transfer (Leuzinger et al., 2013; Xi et al., 2018). Additionally, ascorbic acid acted as an antioxidant, mitigating oxidative stress and cell death during infiltration, which contributed to a more favorable environment for protein accumulation (Akram et al., 2017; Ali et al., 2019; Wang et al., 2024). The highest levels of ASM expression were achieved when all three components were used together, highlighting the synergistic effect of an optimized infiltration buffer.

To further assess the performance of the minimized vector system, ASM activity was compared between constructs expressed from mpBI121-ntExtASM (Chapter 4) and the previously used pRI201-ntExtASM (Chapter 2). As shown in Table 15, the mpBI121-based construct consistently produced higher ASM activity across all sample types – whole, intracellular, and AWF. Among these, the AWF fraction exhibited the highest ASM activity in both expression systems, demonstrating that the majority of recombinant ASM is successfully secreted into the apoplast and that AWF is the optimal fraction for recovery and activity assessment. Specifically, mpBI121-ntExtASM yielded 570.43 ± 7.26 $\mu\text{U}/\text{mg}$ of ASM activity in the AWF, a 1.14-fold increase compared to 500.11 ± 8.96 $\mu\text{U}/\text{mg}$ from the pRI201-ntExtASM construct. This trend in enzymatic activity was further supported by CBB staining and Western blot analysis (Figure 28), which showed visibly stronger ASM bands in all fractions from the mpBI121 construct. The enhanced band intensity in the AWF fraction also

confirmed that the secreted ASM was abundant and intact. The consistency between the enzymatic and protein detection results confirms that the mpBI121 vector enhances both expression and secretion efficiency compared to the original pRI201-based system. Overall, these findings highlight the benefit of vector minimization for improving recombinant protein production and secretion in plant systems, establishing mpBI121 as a promising platform for future applications in molecular farming or gene expression.

Table 15 Comparative analysis of ASM enzymatic activity from constructs based on mpBI121 and pRI201 vector systems (n=3).

Crude extract	Enzyme activity ($\mu\text{U}/\text{mg}$)		
	Whole	Intracellular	Apoplast
mpBI121-ntExtASM	387.97 ± 15.58	105.29 ± 13.09	570.43 ± 7.26
pRI201-ntExtASM	287.51 ± 15.94	62.14 ± 8.96	500.11 ± 8.96

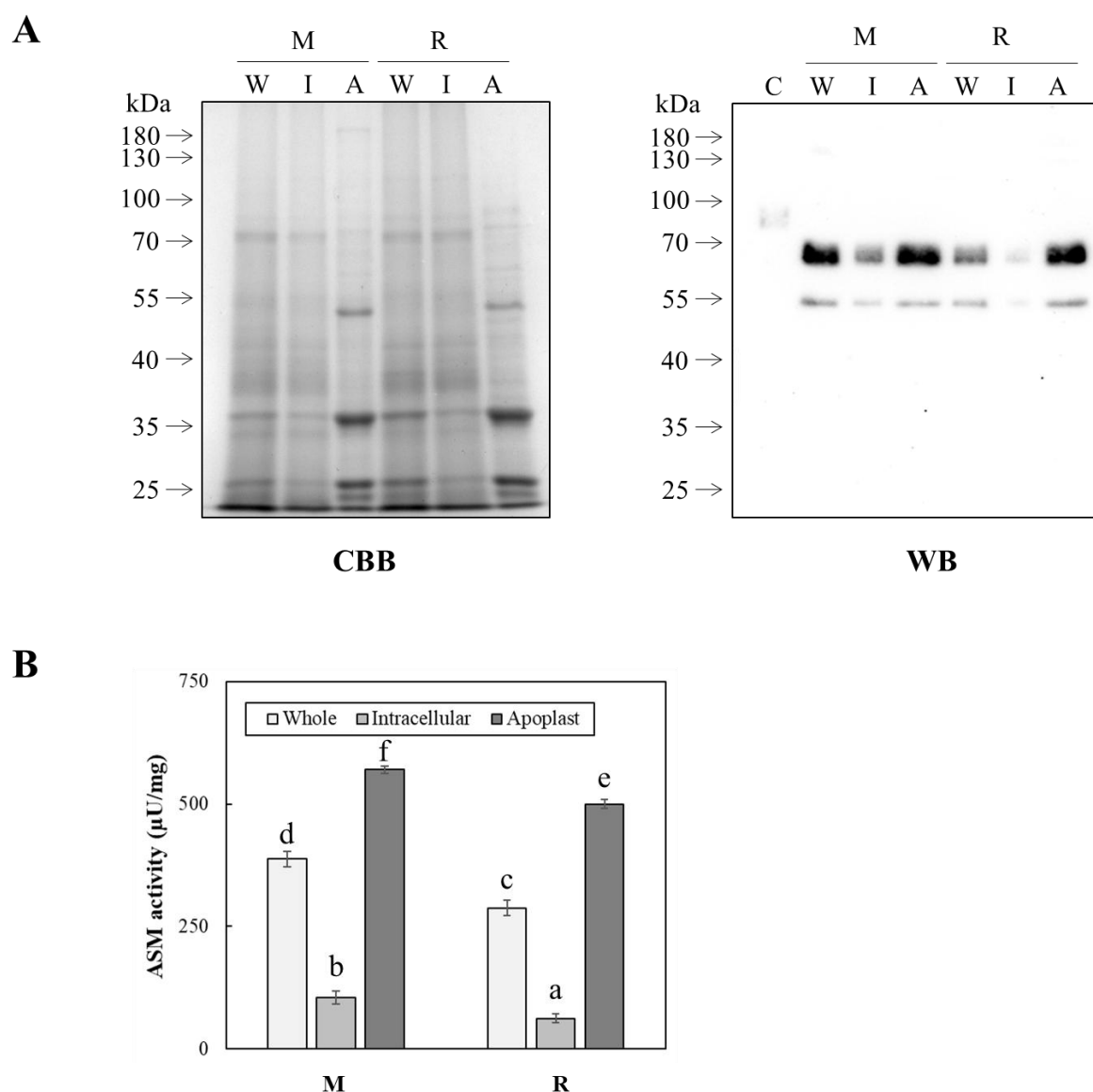


Figure 28 Comparison of ASM expression levels between mpBI121-ntExtASM (M) and pRI201-ntExtASM (R) constructs across whole, intracellular, and AWF samples. (A) SDS-PAGE (10%) followed by CBB and Western blot analysis. Sample types include; W: Whole-plant crude extract; I: Intracellular crude extract; A: AWF; C: CHO-derived human ASM. (B) Enzyme activity of ASM measured in whole, intracellular, and AWF samples from both constructs. Data represent the mean $\pm$ standard deviation (SD) from three independent agroinfiltration experiments.

4.5 Summary

This study focused on reducing the size of the commonly used plant expression vector pBI121 and improve the production of ASM in *N. benthamiana*. A minimized version of the vector, called mpBI121, was developed by removing unnecessary sequences such as the *lac* region and *tetR* gene. Experimental results showed that the minimized vector significantly improved gene expression, with *GUS* and *ASM* mRNA levels increasing by 2.02- and 2.05-fold, respectively, compared with pBI121. Similarly, *GUS* and *ASM* protein levels were enhanced by approximately 1.76- and 1.75-fold. In addition, the study optimized the infiltration buffer components used for *Agrobacterium*-mediated transient expression, further contributing to improved recombinant protein yield. Co-expression with the p19 silencing suppressor, together with adding acetosyringone and ascorbic acid in the infiltration buffer, resulted in the highest *ASM* expression, with most of the protein detected in the apoplast. When comparing the mpBI121 vector to another vector, pRI201, mpBI121 consistently gave higher *ASM* activity in all samples. The AWF had the highest enzyme activity, reaching $570.43 \pm 7.26 \mu\text{U}/\text{mg}$ – 1.14 times more than the pRI201 vector. In conclusion, minimizing the vector and optimizing the buffer significantly improved both the expression and secretion of *ASM* in plants, showing that this approach is effective for producing recombinant proteins in *N. benthamiana*.

Chapter 5

General conclusion and perspective

General conclusion

Plant-based expression systems are increasingly recognized as an attractive alternative for the production of therapeutic proteins. These systems offer numerous advantages, including lower production costs compared to traditional animal cell-based systems, the potential for large-scale production, the ability to produce complex proteins with proper post-translational modifications, and reduced risk of viral contamination especially human pathogen (Buyel, 2018; Kulshreshtha et al., 2022; Xu et al., 2017). Despite these benefits, the successful implementation of plant-based systems for producing therapeutic proteins has been hindered by several challenges, including issues with protein yield, stability, and the efficiency of purification methods. This study has made significant strides in overcoming these limitations, particularly in the context of recombinant human ASM production in *N. benthamiana* plants.

Currently, Xenpozyme[®] (olipudase alfa) is the only ERT approved for the long-term treatment of NPD type B. It is a recombinant enzyme produced in CHO cells (Diaz et al., 2022; Lachmann et al., 2023). Although Xenpozyme[®] is the only FDA-approved therapeutic for NPD type B, it is associated with significant challenges, the most notable of which is its high cost. The production of recombinant enzymes in CHO cells is expensive due to the complex biotechnological processes and strict quality controls required. As a result, the cost of Xenpozyme[®] can reach several hundred thousand dollars annually, making it unaffordable for many patients. This situation highlights the urgent need for alternative production systems that can provide more affordable and scalable solutions for therapeutic enzyme like ASM (Brady & Love, 2021). A plant-based system is especially crucial for disease requiring ERT, like NPD type B, where high production cost of current treatments limit accessibility (Wraith, 2006).

This study successfully demonstrates the production and purification of recombinant human ASM using *N. benthamiana* as a host plant. Through agroinfiltration and co-expression of the p19 protein, we achieved efficient transient expression of ASM, resulting in significantly higher enzyme activity compared to wild-type and control plants. The truncated ASM fused to *N. benthamiana* extensin signal peptide, reached a peak expression reached at 4.01 ± 0.11 mU/mg TSP in the crude sample and exhibited ASM activity of 7.64 ± 0.49 mU/mg in the apoplast sample, confirming the successful recovery of apoplast-localized protein. This highlights the effectiveness of the plant-based expression system in producing functional recombinant ASM. Additionally, a two-step ion exchange chromatography method was developed to purify the recombinant ASM, which proved to be a simpler, more efficient alternative to multi-step processes previously reported (Yamanaka & Suzuki, 1982). This method resulted in a substantial increase in enzyme activity and demonstrated high purity, positioning plant-based expression systems as a promising alternative to conventional production systems. The ASM specific activity in the purified sample increased 21-fold after purification, to 128.18 ± 4.18 mU/mg, from 6.04 ± 0.33 mU/mg in the crude sample. ASM production was about 3.5 µg/g of fresh leaf weight, yielding a yield of about 49%.

Another key achievement of this study was the successful engineering of a minimized plant expression vector, mpBI121, by removing non-essential sequences such as the *lac* region and tetracycline resistance gene from the original pBI121. The mpBI121 vector demonstrated significantly improved transient expression of both the reporter protein (GUS) and the target protein (ASM), by 1.76-fold and 1.75-fold, respectively, compared to the original pBI121. Further enhancements were achieved by optimizing the infiltration buffer; the combination of p19, acetosyringone, and ascorbic acid, along with incubation at room temperature in the dark, resulted in the highest ASM expression. This demonstrates the synergistic effect of vector design and buffer composition in maximizing protein production. Additionally, compared to the previously used expression vector pRI201-ntExtASM, the mpBI121-ntExtASM construct yielded higher ASM levels across all fractions – whole, intracellular, and AWF – with the AWF fraction reaching 570.43 ± 7.26 µU/mg, a 1.14-fold increase over the 500.11 ± 8.96 µU/mg observed with pRI201.

In summary, this study not only confirms that functional, purified human ASM can be successfully produced in *N. benthamiana*, but also highlighting the benefits of using a minimized expression vector and optimized agroinfiltration conditions to enhance protein expression.

Future Perspective

Looking ahead, this research highlights the potential of plant-based systems for large-scale production of recombinant human proteins, particularly for therapeutic applications. The successful development of a simplified purification process offers a cost-effective and scalable solution for producing ASM and other therapeutic proteins. Further optimization of this method could lead to even higher yields and efficiencies, making it feasible for industrial-scale production. The ability to produce high-quality, therapeutic proteins in plants not only addresses the high costs and complexity of traditional expression systems but also opens the door for broader applications in the production of proteins for treating various diseases, including lysosomal storage disorders and beyond. Ultimately, this approach could contribute to a more sustainable and efficient global healthcare system by replacing animal and insect cell-based platforms with plant-based alternatives.

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Supplementary Materials

Nucleic acid sequences

Full-length ASM with original signal peptide (ASM)

ATGCCAAGGTATGGAGCATCTCTTAGACAGTCTTGCCCAAGGTCTGGTAGAGAG
CAAGGACAAGATGGTACTGCTGGTGCACCGGGTTTGCTTTGGATGGGTTTGGTGC
TTGCTCTTGCAATTGGCACTTGCAATTGGCTCTTGCTCTTTCTGACTCAAGGGTGCTT
TGGGCTCCAGCTGAAGCACATCCTCTTTACCTCAAGGTCACCCAGCAAGGCTTC
ATAGGATCGTGCCTAGGCTTAGGGACGTTTTTCGGATGGGGTAACCTTACATGTCC
TATTTGTAAGGGTCTTTTCACAGCTATCAATCTTGGTTTGAAAAAGGAGCCTAAC
GTGGCTAGGGTGGGTTCTGTTGCTATTAACTTTGTAACCTTTTGAAGATCGCAC
CTCCAGCTGTGTGCCAGTCAATCGTGCCTTGTTCGAGGATGACATGGTGGAAGT
GTGGAGGAGATCAGTGTTGTCTCCTTCAGAGGCTTGCGGATTGCTTCTTGGTTCT
ACTTGCGGACACTGGGACATCTTCTCATCATGGAATATCTCATTGCCTACAGTGC
CAAAGCCACCTCCTAAGCCTCCTTCTCCTCCAGCTCCGGGTGCTCCAGTTTCTAG
GATTCTTTTCTTGACAGATTTGCACTGGGACCACGACTACTTGGAGGGAAGTATGAT
CCAGATTGCGCTGACCCTTTGTGTTGCAGAAGGGGTTCTGGATTGCCTCCAGCAT
CTAGACCGGGTGCTGGTTACTGGGGTGAATACTCTAAGTGCGACCTTCCACTTAG
GACTTTGGAATCTCTTTTGTCTGGTCTTGGACCAGCTGGACCTTTTGACATGGTGT
ACTGGACTGGTGACATTCCAGCTCACGATGTTTGGCACCAGACTAGGCAAGATC
AACTTAGGGCATTGACTACAGTTACAGCATTGGTGAGGAAGTTCCTTGGTCCAGT
TCCAGTTTATCCAGCAGTTGGTAACCACGAGTCAACTCCAGTAACTCATTCCCA
CCACCTTTTATCGAGGGAAATCACTCTTCAAGGTGGTTGTACGAGGCTATGGCTA
AGGCTTGGGAACCATGGTTGCCAGCTGAGGCTCTTAGAACTTTGAGGATCGGTG
GTTTTTACGCACTTTCTCCATAACCGGGTTTGAGATTGATCTCTCTTAACATGAAT
TTCTGTTCTAGGGAAAACCTTTTGGCTTTTGATCAACTCTACAGACCCAGCTGGTC
AGCTTCAATGGCTTGTGGGTGAACTTCAAGCTGCAGAGGATAGAGGAGATAAAG
TTCATATTATCGGTCACATTCCTCCGGGTCACTGCTTGAAGTCTTGGTCTTGGAAT
TATTATAGGATTGTGGCTAGGTACGAGAACACTTTGGCAGCTCAATTCTTCGGAC
ACACACACGTGGACGAGTTCGAAGTTTTCTACGACGAGGAGACTCTTTCTAGGCC
TTTGGCAGTGGCTTTCTTGGCACCTTCTGCAACTACTTATATTGGACTTAATCCGG
GTTACAGAGTGTACCAGATCGACGGAAATTACTCTGGATCATCTCACGTGGTGCT
TGACCACGAGACTTATATCCTTAATCTTACACAAGCTAACATTCCGGGTGCAATC
CCACACTGGCAGTTGCTTTATAGAGCTAGGGAGACATACGGTTTGCCAAACACTC
TTCCAACAGCATGGCACAACCTTGTTTATAGGATGAGAGGAGATATGCAGCTTTT
TCAGACTTTTTTGGTTCCTTTACCACAAGGGTCACCCACCATCAGAGCCATGTGGA
ACACCTTGTAGATTGGCTACTCTTTGCGCTCAACTTTCAGCAAGGGCTGACTCTC
CAGCTCTTTGTAGACATTTGATGCCAGATGGTTCTCTTCCAGAAGCTCAGTCTTTG
TGGCCTAGGCCATTGTTCTGTTGA

Full-length ASM with extensin signal peptide (ExtASM)

ATGGGGAAAATGGCTTCTCTATTTGCCACTCTTCTAGTAGTTTTAGTGTCACCTTAG
CTTAGCTTCTGAAAGCTCAGCACTTGCTCTTTCTGACTCAAGGGTGCTTTGGGCTC
CAGCTGAAGCACATCCTCTTTACCTCAAGGTCACCCAGCAAGGCTTCATAGGAT
CGTGCCTAGGCTTAGGGACGTTTTCGGATGGGGTAACCTTACATGTCCTATTTGT
AAGGGTCTTTTACAGCTATCAATCTTGGTTTGAAAAAGGAGCCTAACGTGGCTA
GGGTGGGTCTGTTGCTATTAACTTTGTAACTTTTGAAGATCGCACCTCCAGCT
GTGTGCCAGTCAATCGTGCACCTTGTTTCGAGGATGACATGGTGGAAGTGTGGAGG
AGATCAGTGTTGTCTCCTTCAGAGGCTTGCGGATTGCTTCTTGGTTCTACTTGCGG
ACACTGGGACATCTTCTCATCATGGAATATCTCATTGCCTACAGTGCCAAAGCCA
CCTCCTAAGCCTCCTTCTCCTCCAGCTCCGGGTGCTCCAGTTTCTAGGATTCTTTT
CTTGACAGATTTGCACTGGGACCACGACTACTTGAGGGAACTGATCCAGATTGC
GCTGACCCTTTGTGTTGCAGAAGGGGTCTGGATTGCCTCCAGCATCTAGACCGG
GTGCTGGTTACTGGGGTGAATACTCTAAGTGCGACCTTCCACTTAGGACTTTGGA
ATCTCTTTTGTCTGGTCTTGGACCAGCTGGACCTTTTGACATGGTGTACTGGACTG
GTGACATTCCAGCTCACGATGTTTGGCACCAGACTAGGCAAGATCAACTTAGGG
CATTGACTACAGTTACAGCATTGGTGAGGAAGTTCCTTGGTCCAGTTCCAGTTTA
TCCAGCAGTTGGTAACCACGAGTCAACTCCAGTTAACTCATTCCCACCACCTTTT
ATCGAGGGAAATCACTCTTCAAGGTGGTTGTACGAGGCTATGGCTAAGGCTTGG
GAACCATGGTTGCCAGCTGAGGCTCTTAGAACTTTGAGGATCGGTGGTTTTTACG
CACTTTCTCCATAACCCGGGTTTGAGATTGATCTCTCTTAACATGAATTTCTGTTCT
AGGGAAAACCTTTTGGCTTTTGATCAACTCTACAGACCCAGCTGGTCAGCTTCAAT
GGCTTGTGGGTGAACTTCAAGCTGCAGAGGATAGAGGAGATAAAGTTCATATTA
TCGGTCACATTCTCCGGGTCACTGCTTGAAGTCTTGGTCTTGGAATTATTATAGG
ATTGTGGCTAGGTACGAGAACACTTTGGCAGCTCAATTCTTCGGACACACACACG
TGGACGAGTTCGAAGTTTTCTACGACGAGGAGACTCTTTCTAGGCCTTTGGCAGT
GGCTTTCTTGGCACCTTCTGCAACTACTTATATTGGACTTAATCCGGGTACAGA
GTGTACCAGATCGACGGAATTACTCTGGATCATCTCACGTGGTGCTTGACCACG
AGACTTATATCCTTAATCTTACACAAGCTAACATTCCGGGTGCAATCCCACACTG
GCAGTTGCTTTATAGAGCTAGGGAGACATACGGTTTGCCAAACACTCTTCCAACA
GCATGGCACAACCTTGTTTATAGGATGAGAGGAGATATGCAGCTTTTTTCAGACTT
TTTGGTTCCTTTACCACAAGGGTCACCCACCATCAGAGCCATGTGGAACACCTTG
TAGATTGGCTACTCTTTGCGCTCAACTTTCAGCAAGGGCTGACTCTCCAGCTCTTT
GTAGACATTTGATGCCAGATGGTTCTCTTCCAGAAGCTCAGTCTTTGTGGCCTAG
GCCATTGTTCTGTTGA

Truncated ASM with extensin signal peptide (ntExtASM)

ATGGGGAAAATGGCTTCTCTATTTGCCACTCTTCTAGTAGTTTTAGTGTCACCTTAG
CTTAGCTTCTGAAAGCTCAGCAGGATGGGGTAACCTTACATGTCCTATTTGTAAG
GGTCTTTTTCACAGCTATCAATCTTGTTTTGAAAAAGGAGCCTAACGTGGCTAGGG
TGGGTTCTGTTGCTATTAACTTTGTAACCTTTTGAAGATCGCACCTCCAGCTGTG
TGCCAGTCAATCGTGCACTTGTTTCGAGGATGACATGGTGGAAGTGTGGAGGAGA
TCAGTGTTGTCTCCTTCAGAGGCTTGCGGATTGCTTCTTGTTTCTACTTGCGGACA
CTGGGACATCTTCTCATCATGGAATATCTCATTGCCTACAGTGCCAAAGCCACCT
CCTAAGCCTCCTTCTCCTCCAGCTCCGGGTGCTCCAGTTTCTAGGATTCTTTTCTT
GACAGATTTGCACTGGGACCACGACTACTTGAGGGAACTGATCCAGATTGCGC
TGACCCTTTGTGTTGCAGAAGGGGTTCTGGATTGCCTCCAGCATCTAGACCGGGT
GCTGGTTACTGGGGTGAATACTCTAAGTGCGACCTTCCACTTAGGACTTTGGAAT
CTCTTTTGTCTGGTCTTGACCAGCTGGACCTTTTGACATGGTGTACTGGACTGGT
GACATTCCAGCTCACGATGTTTGGCACCAGACTAGGCAAGATCAACTTAGGGCA
TTGACTACAGTTACAGCATTGGTGAGGAAGTTCCTTGGTCCAGTTCCAGTTTATC
CAGCAGTTGGTAACCACGAGTCAACTCCAGTTAACTCATTCCCACCACCTTTTAT
CGAGGGAAATCACTCTTCAAGGTGGTTGTACGAGGCTATGGCTAAGGCTTGGA
ACCATGGTTGCCAGCTGAGGCTCTTAGAACTTTGAGGATCGGTGGTTTTTACGCA
CTTTCTCCATAACCCGGGTTTGAGATTGATCTCTCTTAACATGAATTTCTGTTCTAG
GGAAAACTTTTGGCTTTTGATCAACTCTACAGACCCAGCTGGTCAGCTTCAATGG
CTTGTGGGTGAACTTCAAGCTGCAGAGGATAGAGGAGATAAAGTTCATATTATC
GGTCACATTCCCTCCGGGTCAGTGCCTGAAGTCTTGCTTGGGAATTATTATAGGAT
TGTGGCTAGGTACGAGAACACTTTGGCAGCTCAATTCTTCGGACACACACACGTG
GACGAGTTCGAAGTTTTCTACGACGAGGAGACTCTTTCTAGGCCTTTGGCAGTGG
CTTTCTTGGCACCTTCTGCAACTACTTATATTGGACTTAATCCGGGTACAGAGTG
TACCAGATCGACGGAAATTACTCTGGATCATCTCACGTGGTGCTTGACCACGAGA
CTTATATCCTTAATCTTACACAAGCTAACATTCCGGGTGCAATCCCACACTGGCA
GTTGCTTTATAGAGCTAGGGAGACATACGGTTTGCCAAACACTCTTCCAACAGCA
TGGCACAACCTTGTTTATAGGATGAGAGGAGATATGCAGCTTTTTTCAGACTTTTT
GGTTCCTTTACCACAAGGGTCACCCACCATCAGAGCCATGTGGAACACCTTGTA
ATTGGCTACTCTTTGCGCTCAACTTTCAGCAAGGGCTGACTCTCCAGCTCTTTGTA
GACATTTGATGCCAGATGGTTCTTCCAGAAGCTCAGTCTTTGTGGCCTAGGCC
ATTGTTCTGTTGA

Nucleic sequences alignment between original and codon-optimized ASM

[illegible]

Original	CTCAGTTCTTTGGCCACACTCATGTGGATGAATTTGAGGTCTTCTATGATGAAGAGACTCTGAGCCGGCCGCTGGCTGTA
Codon-optimized	CTCAATTCTTCGGACACACACGTTGGACGAGTTCGAAGTTTCTACGACGAGGAGACTCTTTCTAGGCCTTTGGCAGTG *** **
Original	GCCTTCCTGGCAGCCAGTGCAACTACCTACATCGGCCTTAATCCTGGTTACCGTGTGTACCAAATAGATGGAACTACTC
Codon-optimized	GCTTCTTGGCAGCTTCTGCAACTACTTATATTGGACTTAATCCGGGTTACAGAGTGTACCAGATCGACGGAAATTACTC ** **
Original	CGGGAGCTCTCAGTGGTCCTGGACCATGAGACCTACATCCTGAATCTGACCCAGGCAAACATACCGGGAGCCATACCGC
Codon-optimized	TGGATCATCTCAGTGGTGCTTGACCACGAGACTTATATCCTTAATCTTACACAAGCTAACATTCGGGGTGAATCCAC **
Original	ACTGGCAGCTTCTCTACAGGGCTCGAGAAACCTATGGGCTGCCCCAACACACTGCCTACCGCCTGGCACAACCTGGTATAT
Codon-optimized	ACTGGCAGTTGCTTTATAGAGCTAGGGAGACATACGGTTTGCCAAACACTCTTCCAACAGCATGGCACAACCTTGTATAT *****
Original	CGCATGCGGGGCGACATGCAACTTTTCCAGACCTTCTGGTTTCTCTACCATAAGGGCCACCCACCCTCGGAGCCCTGTGG
Codon-optimized	AGGATGAGAGGAGATATGCAGCTTTTTCAGACTTTTGTGTTCTTACCACAAGGGTCACCCACCATCAGAGCCATGTGG * **
Original	CACGCCCTGCCGTCTGGCTACTCTTTGTGCCAGCTCTCTGCCCGTGCTGACAGCCCTGCTCTGTGCGCCACCTGATGC
Codon-optimized	AACACCTTGTAGATTGGCTACTCTTTGCGCTCAACTTTCAGCAAGGGCTGACTCTCCAGCTCTTGTAGACATTTGATGC ** **
Original	CAGATGGGAGCCTCCCAGAGGCCAGAGCCTGTGGCCAAGGCCACTGTTTGTCTAG
Codon-optimized	CAGATGGTCTCTTCCAGAAGCTCAGTCTTGTGGCCTAGGCCATTGTTCTGTTGA *****

Amino acid sequences

Full-length ASM with original signal peptide (ASM)

MPRYGASLRQSCPRSGREQGQDGTAGAPGLLWMGLVLALALALALALALSDSRVL
WAPAEAHPLSPQGHPARLHRIVPRLRDVFGWGNLTCPICKGLFTAINLGLKKEPNVA
RVGSVAIKLCNLLKIAPPAVCQSIVHLFEDDMVEVWRRSVLSPSEACGLLLGSTCGH
WDIFSSWNISLPTVPKPPPKPPSPPAPGAPVSRILFLTDLHWDHDYLEGTDPCADPLC
CRRGSGLPASRPGAGYWGEYSKCDLPLRTLESLLSGLGPAGPFDMVYWTGDIPAH
DVWHQTRQDQLRALTTVTALVRKFLGPVPVYPVAVGNHSTPVNSFPPPFIEGNHSSR
WLYEAMAKAWEPWLPAEALRTL RIGGFYALSPYPGLRLISLNMNFC SRENFWLLINS
TDPAGQLQWLVGELQAAEDRGDKVHIIGHIPPGHCLKSWSWNYYRIVARYENTLAA
QFFGHTHVDEFEVFYDEETLSRPLAVAFLAPSATTYIGLNPGYRVYQIDGNYSGSSHV
VLDHETYILNLTQANIPGAIPHWQLLYRARETYGLPNTLPTAWHNLVYRMRGDMQL
FQTFWFLYHKGHPPSEPCGTPCRLATLCAQLSARADSPALCRHLM PDGSLPEAQLSW
PRPLFC

Full-length ASM with extensin signal peptide (ExtASM)

MGKMASLFATLLVVLVSLSLASESSALALSDSRVLWAPAEAHPLSPQGHPARLHRIV
PRLRDVFGWGNLTCPICKGLFTAINLGLKKEPNVARVGSVAIKLCNLLKIAPPAVCQS
IVHLFEDDMVEVWRRSVLSPSEACGLLLGSTCGHWDIFSSWNISLPTVPKPPPKPPSPP
APGAPVSRILFLTDLHWDHDYLEGTDPCADPLCCRRGSGLPASRPGAGYWGEYS
KCDLPLRTLESLLSGLGPAGPFDMVYWTGDIPAHDVWHQTRQDQLRALTTVTALVR
KFLGPVPVYPVAVGNHSTPVNSFPPPFIEGNHSSRWLYEAMAKAWEPWLPAEALRTL
RIGGFYALSPYPGLRLISLNMNFC SRENFWLLINSTDPAGQLQWLVGELQAAEDRGD
KVHIIGHIPPGHCLKSWSWNYYRIVARYENTLAAQFFGHTHVDEFEVFYDEETLSRPL
AVAFLAPSATTYIGLNPGYRVYQIDGNYSGSSHVLDHETYILNLTQANIPGAIPHWQ
LLYRARETYGLPNTLPTAWHNLVYRMRGDMQLFQTFWFLYHKGHPPSEPCGTPCRL
ATLCAQLSARADSPALCRHLM PDGSLPEAQLSWPRPLFC

Truncated ASM with extensin signal peptide (ntExtASM)

MGKMASLFATLLVVLVSLSLASESSAGWGNLTCPICKGLFTAINLGLKKEPNVARVG
SVAIKLCNLLKIAPPAVCQSIVHLFEDDMVEVWRRSVLSPSEACGLLLGSTCGHWDIF
SSWNISLPTVPKPPPKPPSPPAPGAPVSRILFLTDLHWDHDYLEGTDPCADPLCCRR
GSGLPASRPGAGYWGEYSKCDLPLRTLESLLSGLGPAGPFDMVYWTGDIPAHDVW
HQTRQDQLRALTTVTALVRKFLGPVPVYPVAVGNHSTPVNSFPPPFIEGNHSSRWLY
EAMAKAWEPWLPAEALRTL RIGGFYALSPYPGLRLISLNMNFC SRENFWLLINSTDP
AGQLQWLVGELQAAEDRGDKVHIIGHIPPGHCLKSWSWNYYRIVARYENTLAAQFF
GHTHVDEFEVFYDEETLSRPLAVAFLAPSATTYIGLNPGYRVYQIDGNYSGSSHVVL
DHETYILNLTQANIPGAIPHWQLLYRARETYGLPNTLPTAWHNLVYRMRGDMQLFQ
TFWFLYHKGHPPSEPCGTPCRLATLCAQLSARADSPALCRHLM PDGSLPEAQLSWPR
PLFC

Amino acid sequences alignment between human and plant-derived ASM

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Human      MPRYGASLRQSCPRSGREQGQDGTAGAPGLLWMGLVLALALALALA LALSDSRVLWAPAEAHPLSPQGHPARLHRIVPRL
Plant-derived -----MGKMASLFFATLLVVLVSLSLAS

Human      RDVFGWGNLTCPICKGLFTAINLGLKKEPNVARVGSVAIKLCNLLKIAPPAVCQSIVHLFEDDMVEVWRRSVLSPSEACG
Plant-derived ESSAGWGNLTCPICKGLFTAINLGLKKEPNVARVGSVAIKLCNLLKIAPPAVCQSIVHLFEDDMVEVWRRSVLSPSEACG
          *****

Human      LLLGSTCGHWDIFSSWNISLPTVPKPPPKPPSPAPGAPVSRILFLTDLHWDHDYLEGTDPCADPLCCRRGSGLPSPASR
Plant-derived LLLGSTCGHWDIFSSWNISLPTVPKPPPKPPSPAPGAPVSRILFLTDLHWDHDYLEGTDPCADPLCCRRGSGLPSPASR
          *****

Human      PGAGYWGEYSKCDLPLRTLESLLSGLGPAGPFDMVYWTGDIPAHDVVHQTRQDQLRALTTVTALVRKFLGPPVPVYPAVGN
Plant-derived PGAGYWGEYSKCDLPLRTLESLLSGLGPAGPFDMVYWTGDIPAHDVVHQTRQDQLRALTTVTALVRKFLGPPVPVYPAVGN
          *****

Human      HESTPVNSFPPPFIEGNHSSRWLYEAMAKAWEPWLPAEALRTLRIIGGFYALSPYPGLRLISLNMNFCSRENFWLLINSTD
Plant-derived HESTPVNSFPPPFIEGNHSSRWLYEAMAKAWEPWLPAEALRTLRIIGGFYALSPYPGLRLISLNMNFCSRENFWLLINSTD
          *****

Human      PAGQLQWLVGELQAAEDRGDKVHIIGHIPPGHCLKSWSWNYYRIVARYENTLAAQFFGHTHVDEFEVFYDEETLSRPLAV
Plant-derived PAGQLQWLVGELQAAEDRGDKVHIIGHIPPGHCLKSWSWNYYRIVARYENTLAAQFFGHTHVDEFEVFYDEETLSRPLAV
          *****

Human      AFLAPSATTYIGLNPGRVYQIDGNYSGSSHVLDHETYIILNLTQANIPGAIPHWQLLYRARETYGLPNTLPTAWHNLVY
Plant-derived AFLAPSATTYIGLNPGRVYQIDGNYSGSSHVLDHETYIILNLTQANIPGAIPHWQLLYRARETYGLPNTLPTAWHNLVY
          *****

Human      RMRGDMQLFQTFWFLYHKGHPPEPCGTPCRLATLCAQLSARADSPALCRHLMPDGSLPEAQSLWPRPLFC
Plant-derived RMRGDMQLFQTFWFLYHKGHPPEPCGTPCRLATLCAQLSARADSPALCRHLMPDGSLPEAQSLWPRPLFC
          *****

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The yellow-highlighted region indicates the original signal peptide of full-length human ASM, while the green-highlighted region represents the extensin signal peptide of *N. benthamiana* used in the truncated, plant-derived ASM.

List of publication

Panyawechamontri, K., Kajiura, H., Misaki, R., Fujiyama, K. (2025). Production and purification of tag-free recombinant human acid sphingomyelinase in *Nicotiana benthamiana*. *Plant Cell Rep.*, 44, 247. <https://doi.org/10.1007/s00299-025-03618-3>

List of manuscript

Panyawechamontri, K., Kajiura, H., Misaki, R., Fujiyama, K. Vector minimization and infiltration optimization for enhanced protein expression in *Nicotiana benthamiana*. (In preparation)

Oral presentation

Panyawechamontri, K., Kajiura, H., Misaki, R., Fujiyama, K. (2022) “The transient expression in *Nicotiana benthamiana* of human acid sphingomyelinase for enzyme replacement therapy”, The 24th academic exchange seminar between Shanghai Jiao Tong University and Osaka University, online meeting, 29th November 2022.

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