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Production and Characterization of Islet Amyloid Polypeptide Using Recombinant Technology

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Production and Characterization of Islet Amyloid Polypeptide Using Recombinant Technology

Production and Characterization of Islet Amyloid Polypeptide Using Recombinant Technology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

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July 2015

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ABSTRACT

Islet amyloid polypeptide (IAPP) also called amylin is an amyloid-forming protein; IAPP is a proteinaceous hormone that comprises 37 amino acid residues. It is secreted along with insulin from the pancreatic β -cells to help it regulate the uptake and removal of glucose in the bloodstream. IAPP has been observed in the amyloid deposits found in pancreatic β -cells of most patients suffering from type II diabetes mellitus. This research project aims at producing recombinant amylin peptide. To achieve this goal, we used the pBAD plasmid vector which we introduced into *Escherichia coli* to express the peptide. Although the vector was successfully introduced into *E. coli*, production of the amylin protein was not detected under a variety of different expression conditions. Examination of the RNA produced from the *E. coli* showed expression of the amylin RNA which indicates that the protein is most likely degraded. The degradation may have resulted from the fact that the peptide in question is a small exogenous toxic peptide and as a result may have been degraded by cytoplasmic proteases to protect the cell. One way that this degradation could be overcome is to attach the protein to an endogenous fusion peptide, such as the maltose-binding protein, which could provide protection against proteases.

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DEDICATION

I dedicate my thesis to my family and many friends. A special sentiment of gratitude goes to my father Etienne DUVERNA who passed away and my mother Adrienne Romain Duverna who always encouraged me. I also dedicate this work to professors who have assisted me throughout the research project. I will always appreciate all they have done, especially Dr. Christa Hestekin as the thesis director for her many hours of proofreading and Dr. Tammy M. Lutz-Rechtin for helping me develop my lab skills.

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CHAPTER 1: INTRODUCING AMYLIN

INTRODUCTION

Many aged-related diseases are caused by protein aggregation. These include Parkinson disease, Huntington disease, Creutzfeldt–Jakob disease, type II diabetes mellitus (T2DM), and Alzheimer's disease (AD) [1]. Each of these diseases is typified by a buildup of amyloid deposits sprung from a variety of other proteins [1]. Parkinson disease is typified by a buildup of alpha-synuclein, Huntington disease by polyglutamine-containing aggregates, Creutzfeldt-Jakob disease by massive misfolded prion protein aggregates [1], AD by β -amyloid deposits, and T2DM by amylin aggregates [2]. These diseases are commonly characterized by amyloid deposits. These amyloid deposits are made up of misfolded and self-associated amyloid-forming peptides which are composed of cross- β -sheets [2]. Amylin, which is co-secreted with insulin, has been found in amyloid deposits in the pancreatic β -cells of most patients suffering from T2DM [2, 3]. Amylin was identified in 1987 [4]. It is mainly produced in the pancreatic β -cells, yet it is also synthesized in small amounts in various other organs such as the gastrointestinal tract, dorsal root ganglia, and in the kidney in the midst of development [4]. Studies revealed that huge aggregates of amylin have been identified in blood vessels and brain parenchyma [5]. Also, in the temporal lobe gray matter of patients suffering from diabetes, the presence of oligomeric amylin and plaques have been detected [5]. Interestingly, amylin has been found to deposit in blood vessels and brain parenchyma of patients who have AD without displaying any clinical symptoms associated with T2DM [5].

1.2. PHYSIOLOGICAL AND PATHOLOGICAL EFFECTS OF AMYLIN ON THE PANCREAS AND OTHER ORGANS

The role of amylin is not completely comprehended. Part of the problem is understanding the contrast between physiological (normal) and pathological (high) amounts of amylin in the body [6]. At physiological amount, amylin behaves like a growth factor which contributes to bone calcification [6], thereby preventing the resorptive activities of osteoclasts [7]. Amylin regulates nutrient uptake and metabolism. It does so by diminishing the amount of food absorbed, gastric acid released, and glucagon produced by pancreatic α -cells; amylin also accomplishes this function by repressing gastric unloading [8]. Overall, amylin is reported to accomplish a great deal of physiological functions; however their mechanisms are yet to be understood [6]. Amylin tends to aggregate when present in pathological (high) quantities, which has negative effects on cells [6]. Similar to other amyloid-forming proteins, soluble oligomers of amylin are believed to bring about cell death [9-11]. These soluble toxic oligomers interact with constituents of the membranes, phospholipids principally, and end up perforating the cell membrane (Figure 1) [9, 10]. This perforation alters the calcium ion equilibrium between the intracellular and extracellular contents as well as the cell lifespan [11-14]. Moreover, in its oligomeric form, amylin negatively impacts the cardiovascular system by provoking lipid degradation, augmenting the level of free fatty acid present in the plasma, turning on the renin-angiotensin-aldosterone system, and producing inflammatory and oxidative stress [11, 15, 16]. Data gathered recently indicate that too much amylin results in toxicity in other organs such as heart [16] and kidney of patients who are obese and diabetic [13]. It has been reported that aggregates of amylin found in the failing heart of diabetics may bring about heart failure [12].

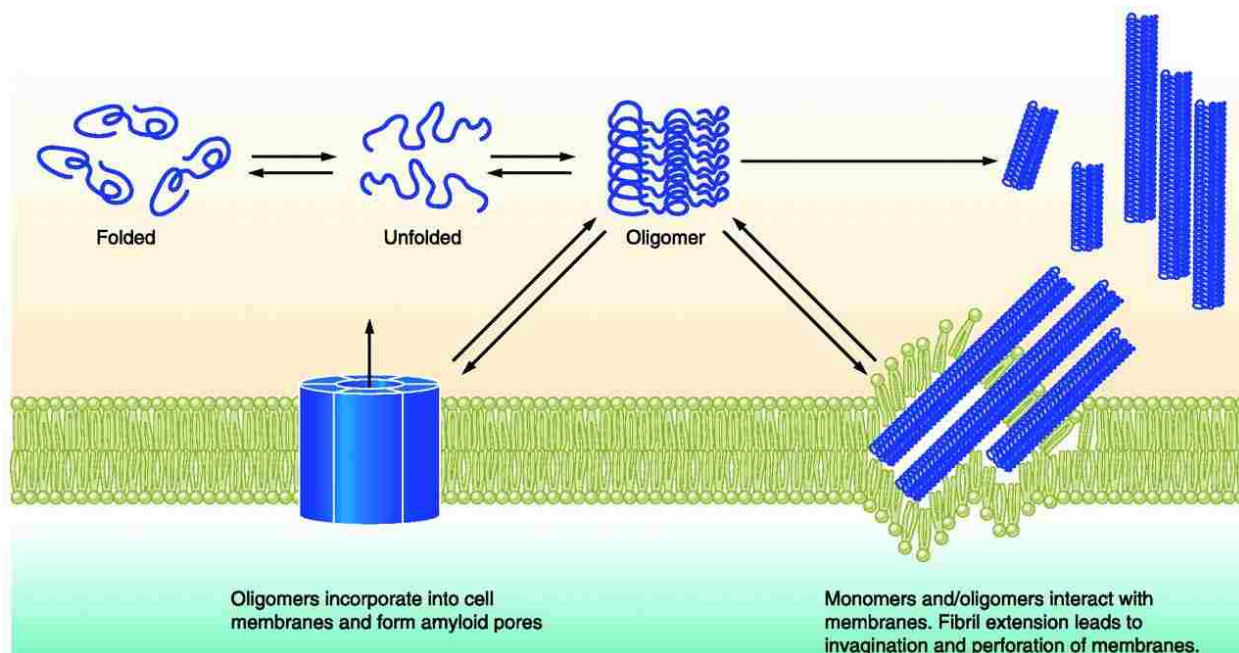


Figure 1. The completely folded protein, for some unknown reason, becomes unfolded and shows hydrophobic patches on its surface thereby starting aggregating into soluble toxic oligomers. The oligomers react with constituents of the membrane and end up creating holes in it [6]. Used with permission of the publisher, The American Physiological Society; April 2, 2015.

STRUCTURE OF AMYLIN

Amylin, also known as islet or insulinoma amyloid polypeptide (IAPP), comprises 37 amino acid residues. It is part of the calcitonin superfamily; other members of this family include calcitonin (CT), calcitonin gene-related peptides (CGRP), and adrenomedullin [17]. Amylin has in common with the other members of the CT family a disulfide bond between cysteine residues 2 and 7 and an amide group at the C-terminus (Figure 2); the amidated C-terminus and the disulfide bond are added posttranslationally and are crucial for biological functions [18, 19]. Amylin has been reported to have a random-coil conformation [20]. CD and NMR studies have demonstrated that amylin has a transitory amphipathic helix at the N-terminus region [21-24]. When placed in solution, the helix goes from residues 5 to 28 [22].

The C-terminus of amylin lacks a defined structure [20]. It is believed that the helix-shaped region is crucial for receptor binding and may contribute a great deal to amylin aggregation. The secondary structure of human amylin has been determined using high-resolution NMR; its N-terminal helix spans from cysteine 7 and to valine 17. There is another helix from asparagine 21 to serine 28; in between these 2 helices there is a turn which spans from histidine 18 to serine 20. Near the C-terminus exists a short helical segment from glycine 33 to asparagine 35 (Figure 4) [21]. Human and rat amylin have basically the same quaternary structure. However, some differences between these two stem from the fact that rat amylin has three proline residues which the human version lacks (Figure 3); the differences result in a human amylin which is amyloid-forming and a rat amylin which is not [6]. This amphipathic helix does not reach the very end of the N-terminus. Instead this terminal region has a rigid ring.

Peptide	1	10	20	30	40	50					
h-ADM	-	YRQSMN	NFQGLRS	FGCRFG	TCTVQK	LAHQIY	QPTDKD	KDNVAP	RSKIS	SPQGY-NH ₂	
p-ADM	-	YRQSMN	NFQGLRS	FGCRFG	TCTVQK	LAHQIY	QPTDKD	KDGVAP	RSKIS	SPQGY-NH ₂	
r-ADM	-	YRQSMN	- -	QGSRS	TGCRFG	TCTM	QKLAHQ	IYQPTD	KDKDGM	APRNKIS	SPQGY-NH ₂
h-CGRP- α	-										
h-CGRP- β	-										
h-amylin	-										
h-CT	-										
e-CT	-										
s-CT	-										

Figure 2. Comparison of primary structures between adrenomedullin, CGRP, amylin, and calcitonin. Notice that, being members of the same family, all these peptides have a disulfide bond between cysteine residues 2 and 7 and share a common amide group at the C-terminus [18]. Used with permission of the publisher, The Begell House; April 10, 2015.

	1	5	10	15	20	25	30	35																													
Human-CGRP	A	C	D	T	A	T	C	V	T	H	R	L	A	G	L	L	S	R	S	G	G	V	V	K	N	N	F	V	P	T	N	V	G	S	K	A	F
Human-Amylin	K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	V	H	S	S	N	N	F	G	A	I	L	S	S	T	N	V	G	S	N	T	Y
Monkey-Amy	K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	V	R	S	S	N	N	F	G	T	I	L	S	S	T	N	V	G	S	D	T	Y
Cat-Amylin	K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	I	R	S	S	N	N	L	G	A	I	L	S	P	T	N	V	G	S	N	T	Y
Dog-Amylin	K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	V	R	T	S	N	N	L	G	A	I	L	S	P	T	N	V	G	S	N	T	Y
Rat-Amylin	K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	V	R	S	S	N	N	L	G	P	V	L	P	P	T	N	V	G	S	N	T	Y

Figure 3. Amino acid sequence similarity between human CGRP and some amylin homologs. Notice the presence of proline residues in rat amylin. This small difference results in a human amylin which aggregates and a rat amylin which does not [18]. Used with permission of the publisher, The Begell House; April 10, 2015.

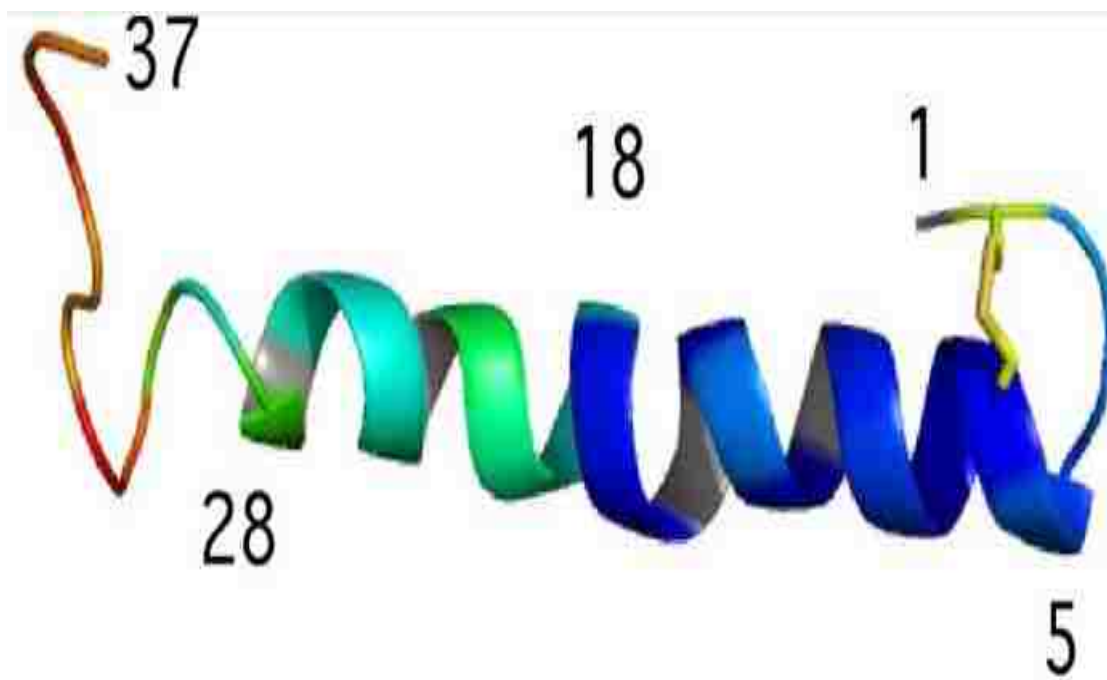


Figure 4. Ribbon diagram of the NMR structure of human amylin determined in SDS micelles. The first four residues in the structure form a hairpin loop by the disulfide bond. The last nine residues near the C-terminus are unfolded. The structure center is occupied by an α -helix spanning approximately from residues 5 to 28. The N-terminal helix spans from cysteine 7 and valine 17; another helix from asparagine 21 to serine 28; in between these 2 helices, a turn from histidine 18 to serine 20. Near the C-terminus, a short helical segment from glycine 33 to asparagine 35 [22]. Used with permission of the publisher, The American Society for Biochemistry and Molecular Biology; August 19, 2011.

EXPRESSION, LOCATION, PROCESSING, AND RELEASE OF AMYLIN

Similar to most peptide hormones, amylin is synthesized as a prehormone in the β -cells of the pancreas. This preproamylin, which comprises 89 amino acid residues, is made up of a signal sequence of 22 amino acid residues, two peptide segments at each extremity and the proamylin in between (Figure 5) [25, 26]. This 22-amino acid signal sequence targets amylin to the endoplasmic reticulum, a principal route of nearly all secretory proteins. There, the signal sequence gets cleaved off to yield proamylin which, in the late Golgi and the secretory vesicles, is converted into amylin before being released into the bloodstream to accomplish its functions as a completely active hormone [25, 27].

The amylin gene is localized on the short arm of the chromosome 12 where it is expressed from a single allele of a gene [18]. Among the three exons which the preproamylin is translated from, only the last two code for the complete molecule [28]. It is important to note that it is a requirement that this C-terminal glycine stay for amylin to be completely active [6]. Both amylin and insulin genes are under the control of the transcription factor PDX-1 and they both have promoters that resemble each other [29]. The transcription factor PDX-1 controls the effects of glucose on both genes [30-33]. Although the promoters have a certain resemblance, researchers have found that amylin and insulin genes are not always expressed at the same time. This finding proves that the co-secretion of these proteins can be altered in certain circumstances [6].



Figure 5. Primary sequence of preproamylin with its 3 internal endoprotease sites. Remark how crucial the specificity of CPE is by leaving this glycine at the C-terminus of the peptide. This glycine is not part of the primary structure of amylin, yet contributes a great deal to its biological functions [6]. Used with permission of the publisher, The American Physiological Society; April 2, 2015.

AMYLIN AND TYPE 2 DIABETES

The factors contributing to the pathogenesis of T2DM have been rigorously investigated [34-36]. At present, two main factors are clearly involved [37, 38]. The first is that insulin becomes less effective, resulting in a higher need for the insulin peptide. The second important factor is the inadequacy of pancreatic beta-cells. Among other manifestations of this insufficiency, a drop in the beta- cells quantity and a reduction in their roles have been reported [38, 39].

In previously conducted research, islets taken from T2DM patients have been found to secrete less insulin and could not normalize the level of glucose in diabetic animals into which these islets were transplanted; however, it is important to note that the response obtained from the glucagon hormone which exerts a countereffect on glycemia was quite normal [40]. Islets obtained from patients with T2DM were at lower quantity compared to those from patients who were free from T2DM. Also, there was islet amyloid, albeit at very low amount, in samples taken from diabetics whereas the samples from non-diabetic patients did not have any [41, 42]. This could indicate that amyloids play a role in β -cell degradation.

Aggregated amylin exists in non-diabetic patients although its impacts on the cells are less severe than they are in diabetic individuals [43, 44]. Through meticulous work, researchers found that, even though aggregated amylin is also present in patients with no T2DM, amylin aggregation is linked to a smaller islet volume because of the drop in the amount of cells [45, 47]. Nevertheless, only the reduction in the islet volume cannot explain why the response from the insulin hormone is ineffective. Some researchers found that fibrils in the vicinity of the β -cells perforate the cell membrane and reach the interior of the cell [48], therefore decreasing their viability. Recently, it has been shown that interactions between amyloid fibrils and the membrane

disturb the influx of Ca^{2+} which may seriously hamper the proper functions of the islets [49].

One important aspect concerning the link between islet amyloid and T2DM is the sites where the fibrils form. Amylin aggregates are typically formed outside the cell [48]. However, there is evidence of amylin deposits that start occurring inside the cell. Studies done on human islets transplanted into mice or transgenic mice expressing human amylin showed that amylin start aggregating inside the cell [50-56]. O'Brien et al found that in β -cell tumors amylin also deposited intracellularly [57]. Studies done on diabetic baboons showed that amylin aggregated both inside and outside the cell [1].

WHY IS IT OF INTEREST TO STUDY AMYLIN?

T2DM is one of the many age-associated diseases which include AD [1]. Up to 8.3% of the US inhabitants have diabetes and nearly 95% of adult American diabetics are diagnosed with T2DM [58]. Amylin in its pathological state evidently has its contribution whether in triggering the onset of T2DM or in worsening a pre-existing genetic predisposition to the disease. In either case, amylin deposits are considered to be a significant factor as they affect the proper function of the pancreatic β -cells. So, it is logical to think that any study related to T2DM should to a large extent consider the amylin peptide. Understanding amylin aggregation can aid in gaining better information about the onset of the disease and also in designing drugs to combat the disease. In this study, we aimed to express recombinant amylin in *Escherichia coli*. We hoped that by expressing and using our own recombinant amylin, we could have a peptide in its active state with a high purity. While chemically synthesized peptide also has high purity, the presence of solvent residues can affect the aggregation kinetic. Therefore, the recombinantly produced peptide should allow us to obtain more correct data about how amylin aggregates *in vitro* so that it can be used in further studies and to gain better insights about the early aggregation steps of amylin and its implications in T2DM.

CHAPTER 2: PREPARATION OF PLAMID VECTORS AND CONSTRUCTS

2.1. INTRODUCTION

At present, systems expressing amyloid-forming peptides in *E. coli* can do so in two manners: express the peptide directly or express the peptide with a solubilizing peptide or protein domain [59]. Fusion partners to express amyloid-forming peptides include maltose-binding protein [60, 61], glutathione S-transferase [62, 63], thioredoxin [64, 65], and poly (NANP) [66]. Vectors designed to produce amylin and amylin variants were previously described by Yonemoto and co-workers. In their work, they took advantage of the fact that amyloid-forming proteins tend to aggregate naturally and accordingly designed their vector by fusing it to the BCL-X1 $\frac{1}{2}$ fusion partner which directed the fusion peptide into inclusion bodies in *E. coli* (Figure 6). However, the amylin peptides obtained in their work were insoluble with no biological activity. This inactivity and insolubility stem from the fact that protein aggregates have the same biophysical properties as inclusion bodies [67, 68] which are made of misfolded proteins with hydrophobic patches exposed on their surface [69]. In our work, we were interested in producing our peptide in its biologically active form. Even though direct expression of peptide is not much reliable due to the fact such small peptides are susceptible to proteolysis, we decided to express our peptide with no solubilizing (fusion) peptide fragment. In order to enhance the formation of the disulfide bond between cysteines 2 and 7 of the amylin peptide which can be formed only in the periplasm in *E. coli*, the peptide was fused to the ompA leader sequence which directs proteins to the periplasm in bacteria [70].

In order to clone the amylin gene, pBAD/Myc-His A Vector, bought from Invitrogen, was used for tight regulation of the gene expression. The insert, which includes the amylin DNA

(Table 1), was designed by us and made by Invitrogen (Table 1). Designing the insert included the creation of a multiple cloning site containing some of the restriction sites from the pBAD/Myc-His A Vector including NcoI and XhoI sites and the insertion of the OmpA nucleotide sequence (Figure 7) (Table 1) right before the start codon for translocation of the peptide into the periplasm to favor and enhance disulfide bond formation. NcoI and XhoI restriction enzymes were used to digest the construct containing the amylin nucleotide sequence attached to the OmpA nucleotide sequence and the pBAD/Myc-His A plasmid vector at their respective cutting sites. T4 DNA ligase was used to ligate the OmpA-amylin nucleotide sequence to the pBAD/Myc-His A plasmid vector.

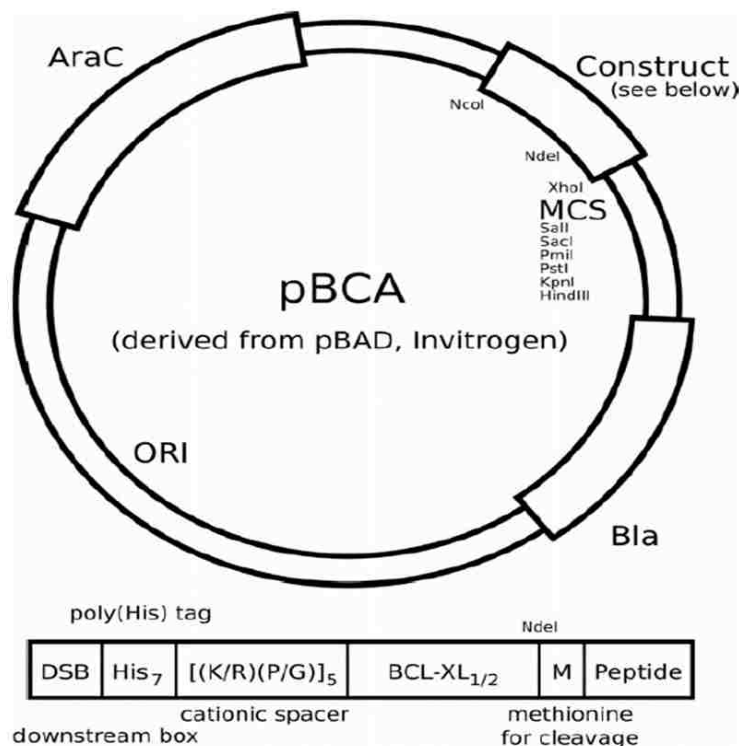


Figure 6. pBCA vector with the construct by Yonemoto which serves as a model for the construction of our construct [59]. Used with permission of the publisher, The Protein Science Society; April 2, 2015.

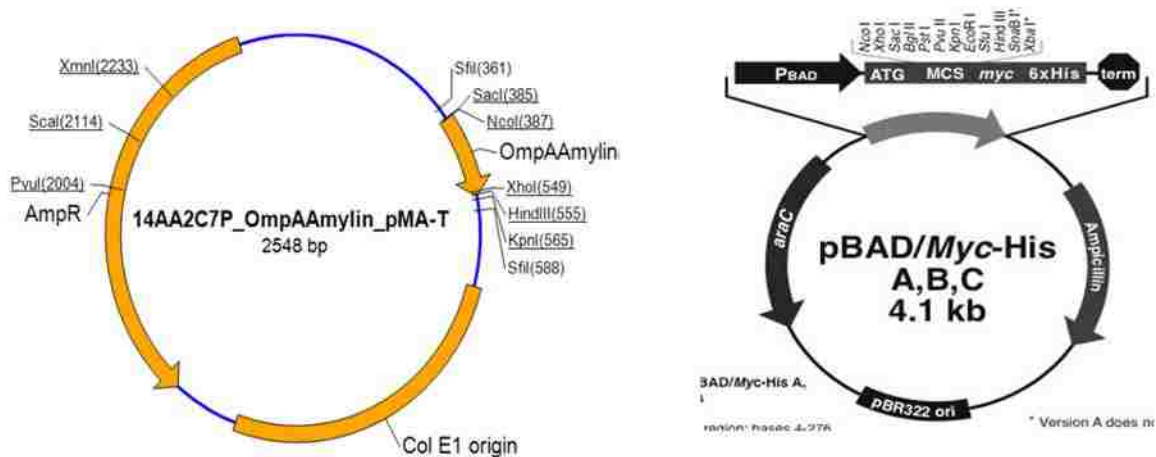


Figure 7. The construct on the left with AmpR, OmpA-Amylin, and restriction enzyme sites such as NcoI and XhoI which were used for digestion. On the right, pBAD/*Myc*-His A Vector in which we introduced our insert.

http://tools.lifetechnologies.com/content/sfs/manuals/pbad_man.pdf

Table 1. (A) Nucleotide sequence of *ompA* (39bp) (codon-optimized for *E.coli*); (B) nucleotide sequence of the human amylin (110bp) (codon-optimized for *E.coli*)

A
5' ATGAAAAAAAAACCGCAATTGCCATTGCAGTTGCACTGGCA 3'
3' TACTTTTTTTGGCGTTAACGGTAAACGTCAACGTGACCGT 5'
B
5' AAATGTAATACCGCAACCTGTGCAACCCAGCGTCTGGCAAATTTCTGGTTCATAGCAGCAATAACTTTGGTGCA
3' TTTACATTATGGCGTTGGACACGTTGGGTCGCAGACCGTTTAAAAGACCAAGTATCGTCGTTATTGAAACCACGT
ATTCTGAGCAGCACCAATGTTGGTAGCAATACCTAT 3'
TAAGACTCGTCGTGGTTACAACCATCGTTATGGATA 5'

2.2 MATERIALS AND METHODS

2.2.1 PEPTIDE PRODUCTION IN LB

The plasmid-containing OmpA-amylin DNA was introduced into TOP10 electrocompetent cells. One fresh cell colony grew overnight in 5 mL of LB media with 0.15 mg/mL of ampicillin. One mL of the cell sample was transferred into 100 mL of LB media with 0.15 mg/mL of ampicillin. The cells were allowed to grow until they had OD600 values of 0.57 and 0.75 at which points 2% arabinose was added. The incubation was performed at 37°C prior and after induction. After induction, samples were collected at 2h, 6h, 8h, and overnight. At each time point, samples were centrifuged, supernatant was disposed of, and pellet was frozen at -20°C. The frozen cell samples were treated with buffer (50 mM Sodium Phosphate, 1 mM EDTA, 150 mM NaCl, pH 7.4) and were sonicated 4 times (30 seconds each time) before loading them onto a Tris-tricine-SDS-15% PAGE. Fifteen μ L of protein sample and 10 μ L of protein standard were applied to the gel. Gels were run at 200 Volts for 85 minutes. After running, the gels were silver stained.

2.2.2 PEPTIDE PRODUCTION USING DIFFERENT AMOUNTS OF ARABINOSE IN M9

Since arabinose induces the vector to produce the protein, its amount was varied to study its effect. One fresh cell colony grew overnight in 5 mL of LB media with 0.15 mg/mL of ampicillin. Two mL of cell sample were then incubated in 200 mL of minimal M9 media (1 mL of cells for 100 mL of media separately) with 0.15 mg/mL of ampicillin added to each medium. The cells grew until an OD600 of 0.75 was reached before the addition of 1% arabinose in one sample and 2% in the other. The incubation was performed at 37°C prior to and after induction.

After induction, samples were collected at 2h, 6h, 8h, and overnight. At each time point, samples were centrifuged, supernatant was disposed of, and pellet was frozen at -20°C . The frozen cell samples were treated with buffer (50 mM Sodium Phosphate, 1 mM EDTA, 150 mM NaCl, pH 7.4) and were sonicated 4 times (30 seconds each time) before loading them onto a Tris-tricine-SDS-15% PAGE. Fifteen μL of sample and 5 μL of loading dye were applied to the gel; 10 μL of protein standard was also loaded. Gels were run at 200 Volts for 85 minutes. Upon running, the gels were stained overnight with Coomassie brilliant blue and destained for approximately 3 hours.

2.2.3. PEPTIDE PRODUCTION AT 25°C , 16°C , AND 4°C IN LB

Next we examined the influence of decreasing the temperature at which the cells were growing since this can among other decrease the proteolytic activities in the cytosol. In that endeavor, one fresh cell colony grew overnight in 5 mL of LB media 0.15 mg/mL of ampicillin. One mL of cell sample was incubated in 100 mL of LB media at room temperature with 0.15 mg/mL of ampicillin added to the media. The cells grew until an OD600 value of 0.80 was reached before the addition of 2% arabinose. The incubation was performed at room temperature ($\sim 25^{\circ}\text{C}$) prior to and after induction. After induction, samples were collected at 4h and overnight. Another 1 mL of cell sample was incubated in 100 mL of LB at 37°C before induction and was switched to 16°C after induction with 2% arabinose. Samples were taken 6 h after induction and overnight. At each time point, samples were centrifuged, supernatant was disposed of, and pellet was frozen at -20°C . The frozen cell samples were treated with buffer (50 mM Sodium Phosphate, 1 mM EDTA, 150 mM NaCl, pH 7.4) and were sonicated 4 times (30 seconds each time) before loading them onto a Tris-tricine-SDS-15% PAGE. Fifteen μL of sample and 5 μL of

loading dye were applied to the gel; 10 μL of protein standard were also loaded. The gels were run at 200 Volts for 85 minutes. After running, the gels were stained overnight with Coomassie brilliant blue and destained for approximately 3 hours. In the same intention of determining the effects of the temperature on the protein expression, one fresh cell colony grew overnight at 37°C in 5 mL of LB media with 0.15 mg/mL of ampicillin. One mL of the cell sample was incubated in 100 mL of LB media at 37°C for 4 h with the same concentration of ampicillin used in the previous experiments. The cells grew until an OD600 of 0.57 was reached before the addition of 2% arabinose. After induction, the cells grew at 4°C . Samples were collected for analysis after 5h, 15h, and 24h. Another 1 mL of cell sample was incubated in 100 mL of minimal M9 media at 37°C for 9 h before induction with 2% arabinose and cells were left to continue growing at 37°C after induction for 48 h. Samples were taken for analysis after 24h and overnight. At each time point, samples were centrifuged, supernatant was disposed of, and pellet was frozen at -20°C . The frozen cell samples were treated with buffer (50 mM Sodium Phosphate, 1 mM EDTA, 150 mM NaCl, pH 7.4) and were sonicated 4 times (30 seconds each time) before loading them onto a Tris-tricine-SDS-15% PAGE. Five μL of sample diluted in 12.5 μL of buffer and 15.7 μL of loading dye were applied to the gel; 7 μL of protein standard were also loaded. Gels were run at 100 Volts for 2 hours. After running, gels were silver stained according to a protocol provided by the silver staining kit supplier.

2.2.4. PEPTIDE PRODUCTION AT 4°C IN LB

One mL of cell sample was incubated in 100 mL of LB media at 37°C for 4 h with 0.15 mg/mL of ampicillin added to the media. Cells grew until an OD600 value of 0.57 was reached before the addition of 2% arabinose. After induction, the cells were incubated at 4°C . Samples

were collected for analysis after 5 h, 10 h, and 19 h. The analysis was performed on a Tris-tricine-SDS-15% PAGE. Fifteen μl of sample and 15 μl of loading dye were applied onto the gel; 15 μl and 10 μl (left and right respectively) of protein standard were also loaded. Gels were run at 100 Volts for 2 hours. After running, gels were stained overnight with Coomassie brilliant blue and destained for approximately 3 hours for a better visualization of the protein of interest.

2.2.5 PEPTIDE PRODUCTION FOR POSSIBLE SECRETION IN MINIMAL M9

In order to check for possible secretion of the protein in the media, we took one fresh cell colony which grew overnight at 37°C in 5 mL of LB media with 0.15 mg/mL of ampicillin. One mL of cell sample was incubated in 100 mL of minimal M9 media at 37°C for 9 h until an OD600 value of 0.57 was reached; the protein expression was induced with 2% arabinose and cells were left to continue growing at 37°C for 48 h. Samples were taken for analysis after 24 h and overnight. At each time point, samples were centrifuged, supernatant was disposed of, and pellet was frozen at -20°C. The frozen cell samples were treated with buffer (50 mM Sodium Phosphate, 1 mM EDTA, pH 7.4) and were sonicated 4 times (30 seconds each time) before loading them onto a Tris-tricine-SDS-15% PAGE. Five μL of sample diluted in 12.5 μL of buffer and 15.7 μL of loading dye were applied to the gel; 7 μL of protein standard were also loaded.

Gels were run at 100 Volts for 2 hours. After running, gels were silver stained according to a protocol provided by the silver staining kit supplier.

2.2.6 RESULTS

The construct containing the *ompA*-amylin DNA and the pBAD/*Myc*-His A plasmid vector were separately introduced to the TOP10 cells chemically. After this chemical transformation, the plasmid DNA were extracted from the cells and were subject to digestion and ligation. The construct and the pBAD/*Myc*-His A plasmid vector were digested by *Nco*I and *Xho*I and ligated together by T4 DNA ligase. The success of the digestion-ligation reaction was confirmed by double digestion of plasmid clones analyzed on agarose gel electrophoresis using the LONZA FLASHGEL system. The *ompA*-amylin-pBAD/*Myc*-His A was introduced into TOP10 cells by electroporation. The *ompA*-amylin-pBAD/*Myc*-His A was then extracted and was then sent for sequencing to confirm the transformation success. The protein expression was induced by L-arabinose and samples were subject to Tris-tricine SDS-15% PAGE analysis for detection of the expected 4.3 KDa-peptide. Samples were collected at various times post-induction and run on a Tris-tricine SDS-15% PAGE to confirm whether the protein was produced. The protein produced should have corresponded to a band with a molecular weight of 4.3 kDa. Although the gel is over-stained, it is clear in Figure 8 that there are no bands around the 4.3 kDa molecular weight for the initial set of conditions tested. Growth conditions such as media, temperature and amount of inducer may interfere with a successful protein production [60]. We started to probe the amount of arabinose assuming that too much of it may have resulted in overexpression of the peptide; this overexpression would likely have caused the peptide either to form inclusion bodies or to kill the cell because of its toxicity [61, 62].

Therefore, we decided to reduce the amount of the inducer (arabinose). So, the same protocol was used for another protein production with the intention of probing whether or not a reduction of the amount of arabinose used to induce the peptide resulted in better protein expression (Figure 9). As it can be seen in Figure 9, no 4.3-kDa band appeared on the gel leading to the conclusion that decreasing the amount of inducer did not aid in the production of the protein.

Since amylin is a small protein, it was possible that it was digested by proteases in the cytoplasm before reaching the periplasm. Therefore, the next step was to examine the use of lower temperatures which should diminish the speed of the metabolic reactions inside the cell and decrease the activity of the cytoplasmic proteases [63]. The lower speed of metabolism could lengthen the time for the cells to grow, which would allow a better formation of the disulfide bond in the peptide and give the protein more time for better folding. As a result, it could ultimately provide protection against proteases since high temperatures favor protein aggregation which in return can trigger proteolytic activities [63, 64]. Therefore, protein productions were undertaken and their inductions were performed at lower temperatures, specifically 25°C and 16°C (Figure 10), and 4°C (Figure 11). Still no bands were seen at 4.3 kDa under any of these conditions.

Another possibility for the problems with detecting the amylin protein was that it was secreted by the cell and therefore located in the growth media rather than the cell pellet itself. The secretion could have resulted from the fact that peptide was fused to a leader sequence and that bacteria ordinarily secrete a few proteins to the extracellular media [65, 66]. However, no amylin protein was detected in the growth media either.

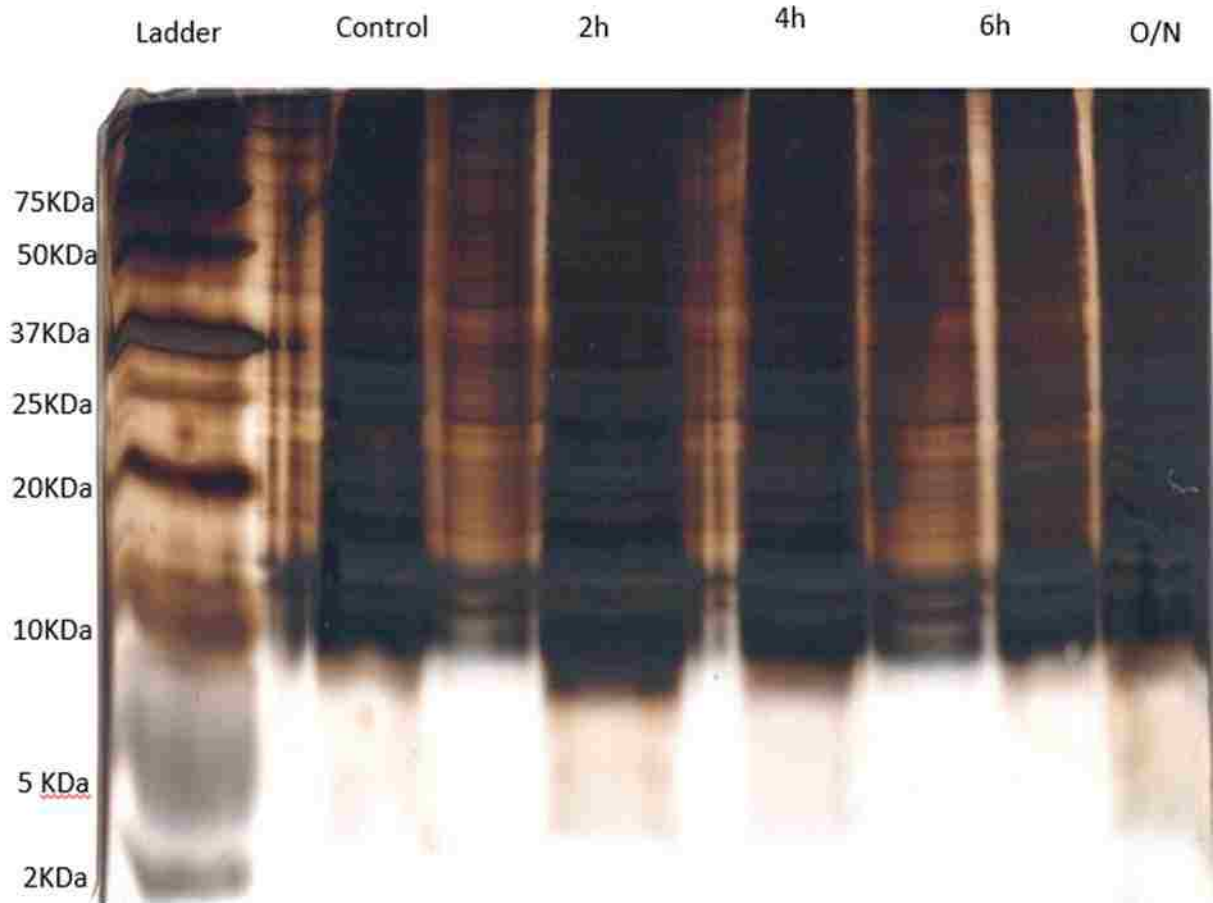


Figure 8. Peptide production performed in LB. h= hour; control= no arabinose. Samples collected 2h, 6h, 8h after induction and overnight. A 4.3-kDa band was expected to appear which does not seem to be present on the gel. Gel was silver stained.

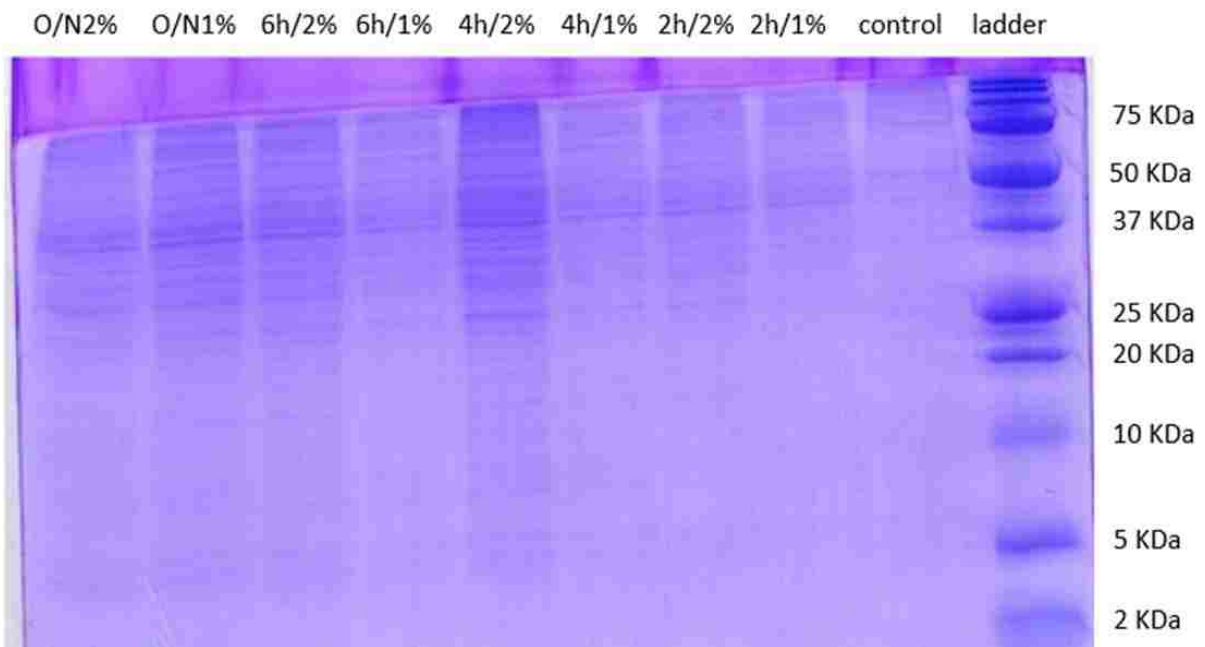


Figure 9. Peptide production using different amounts of arabinose in M9; O/N = overnight, h=hour, control=no arabinose, %= % of arabinose added. Cells grew in M9; Samples were collected 2h, 6h, 8h after induction and overnight. Gel was stained with Coomassie brilliant blue.

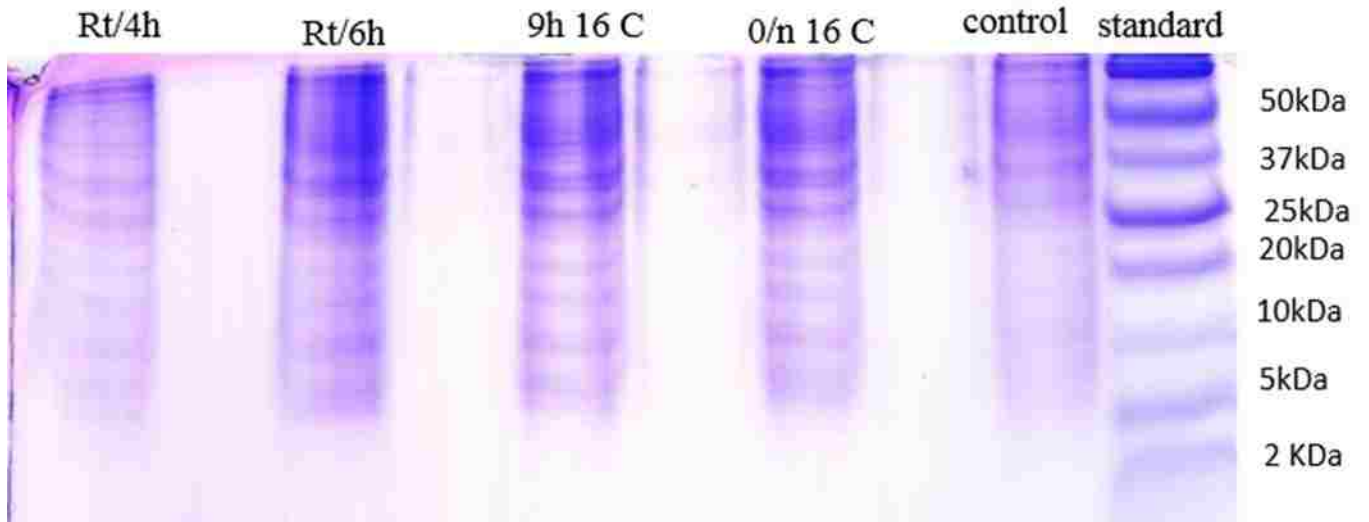


Figure 10. Peptide production at room temperature and 16 degree C in LB; RT= room temperature, o/n= overnight, control= no arabinose. Cells grew in LB; growth at room temperature and 16 degree C.

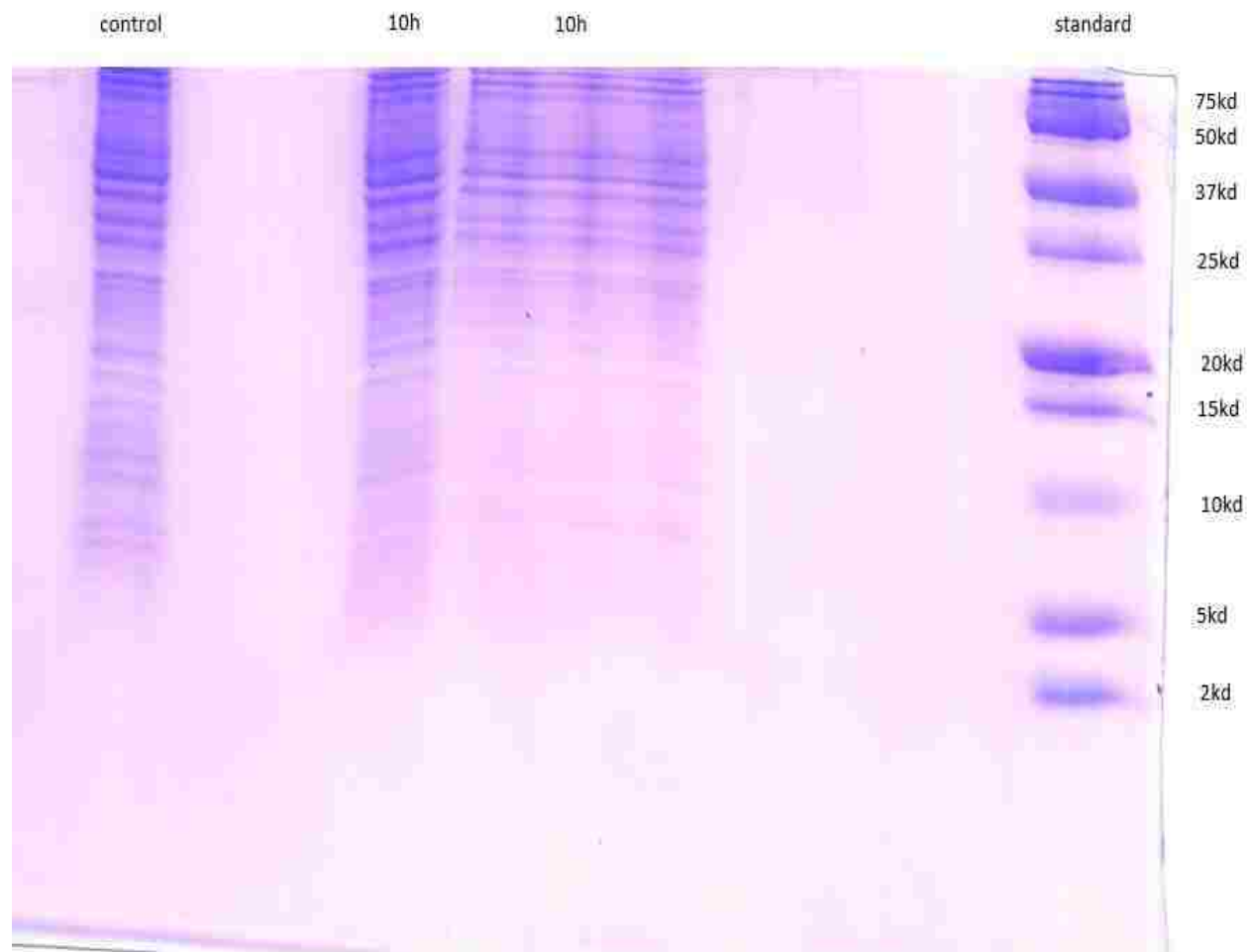


Figure 11. Peptide production at 4 degree C in LB; control= no arabinose. Cells grew in LB.

CHAPTER 3: RT-PCR TO TROUBLESHOOT THE CAUSE OF THE LIKELY FAILURE OF THIS PROTEIN PRODUCTION

3.1. INTRODUCTION

Since the amylin protein was unable to be detected by either varying the growth conditions or the media, it was possible that the issue was upstream of the protein production. In order for a protein to be produced, the mRNA must be transcribed from the DNA. The cytoplasm hosts the ribosomes, sites of protein synthesis. Ribosomes are the ultimate destinations of messenger RNA molecules which, in our case, carry the information that will be translated into amylin. Therefore, the messenger RNA can be of paramount importance in the troubleshooting work. One method that can be used to find out whether the messenger RNA was synthesized is RT-PCR. RT-PCR uses reverse transcriptase to convert RNA into cDNA, which is more robust for analysis.

3.2 MATERIALS AND METHODS PRIMER DESIGN

The amino acid sequence of human amylin was taken from a paper by Sunil J. Wimalawansa [25]. The amino acid sequence was then reverse-translated into the human amylin DNA and was codon-optimized for *E. coli*. Despite the fact the plasmid vector was designed in such a manner that the protein expression includes the leader sequence, the primers were designed for the amplification of only the amylin DNA (Table 2). The amylin primers were designed in our lab and were made by Integrated DNA technology (IDT). The primers for the 16S rRNA [67, 68] were provided by Dr. Jernigan (University of Arkansas, department of chemical engineering).

Table 2 Primers used in the RT-PCR for the amylin and 16S DNA

DNA	Forward Primer	Reverse Primer
Amylin	5'AAATGTAATACCGCAACCTGTGCAAC'3	5'ATAGGTATTGCTACCAACATTGGTGC'3
16S rRNA	5'TTACCGCGGCTGCTGGCAC'3	5'AGGTCCAGACTCCTACGGG'3

CELL CULTURE AND INDUCTION

TOP10 competent cells (From Life Technology; California, USA) were incubated overnight in a shaker at 37°C in 5 mL of LB media with 0.15 mg/mL of ampicillin. Two 1 mL-cell samples were then transferred to 150 mL of LB media (75 mL each) to which were added ampicillin (same concentration previously used) (228 µL, 228 µL). Both samples, incubated in a shaker at 37°C, grew until an OD600 value of 0.45 was reached. At this point, the expression was induced with 2% arabinose; from this point, one sample grew until an OD600 value of 0.55 (15 minutes after induction) and the other sample to an OD600 value of 0.75 (30 minutes after induction). Both samples were harvested at their respective ODs and were ready for RNA extraction.

RNA EXTRACTION

The total cell RNA was extracted using UltraClean® Microbial RNA Isolation Kit from MO-BIO (cat #:15800-50). 1.8 ml of cell culture (TOP10 cells) were centrifuged at 11,000 rpm for 30 seconds. The supernatant was decanted and the tubes were spun one more time for 30 seconds and the supernatant was completely removed. The cell pellet was resuspended in 300 µl of Solution MR1 (provided in the kit) and gently vortexed to mix. Resuspended cells were transferred to a MicroRNA bead tube where 15 µl of Solution MR2 (provided in the kit) were added to the MicroRNA Bead Tube and vortexed briefly to mix. The tubes were heated at 65°C for 10 minutes. The tubes were secured horizontally on a flat-bed vortex pad with tape and vortexed at maximum speed for 10 minutes. The supernatant was transferred to a clean collection tube. Five hundred µl of solution MR3 (Lysis buffer provided in the kit) were added to the supernatant and vortexed for 5 seconds. Two hundred-fifty µl of Solution MR4 (Lysis buffer

provided in the kit) were then added to the mixture; the mixture was incubated at 4°C for 5 minutes. The tubes were centrifuged for 1 minute at 11,000 rpm. The entire volume of supernatant was transferred, while avoiding the pellet, to a collection tube. About 650 µl were loaded into a spin filter and centrifuged at 11,000 rpm for 30 seconds. The flow through was discarded and the remaining supernatant was added to the spin filter and centrifuged at 11,000 rpm for 30 seconds. Three hundred µl of Solution MR5 (provided in the kit) were added and centrifuged for 30 seconds at 11,000 rpm. The flow through was discarded. The collection tubes were centrifuged again for 1 minute at 11,000 rpm. The spin filter basket was placed in a new collection tube. Fifty µl of RNase-free water were added to the center of the white filter membrane. The collection tubes were centrifuged for 30 seconds. The spin filter was discarded and RNA extract was stored at -80°C.

REVERSE TRANSCRIPTION POLYMERASE (RT-PCR)

The RT-PCR was performed as indicated in the protocol provided with GoTaq® Probe 2-Step RT-qPCR System kit from Promega. RNA concentration was determined by analysis with a Nanodrop spectrometer. RNA template and primers were combined on ice. Two µL of RNA template (15 min post-induction, 175ng/µl; 30 min post-induction, 190.8 ng/µl) were combined with 2 µL of oligo (dT) primers (provided in the kit), 2 µL random primers (1 µL, 1 µL) (provided in the kit), and 8 µL of nuclease-free water (provided in the kit) to a final volume of 7 µL for each reaction. In addition, a control reaction was performed which contained no reverse transcriptase. Our RNA extract was assumed not to be contaminated with genomic DNA; therefore, no DNase treatment was performed. RNA and primers were denatured in a thermocycler at 70°C for 5 minutes and immediately cooled down on ice for 5 minutes then

centrifuged for 10 seconds. RNA and primers were kept on ice prior to adding the reverse transcription reaction mix. Three μl for each cDNA synthesis reaction to be performed were prepared and vortexed gently to mix. 4.9 μl of nuclease-free water GoScript™, 4 μl of 5X reaction buffer, 1.6 μl of MgCl_2 , 1 μl of PCR nucleotide mix, 0.5 μl of recombinant RNasin® ribonuclease inhibitor, and 1 μl of GoScript™ reverse transcriptase were combined to a total volume per reaction of 13 μl (all these reagents were provided in the kit). Five reactions were performed: 1 reaction for each of the cell growths (15 min post-induction and 30 min post-induction), 1 positive control reaction with 16S rRNA, 1 negative control reaction with no cDNA template, and 1 more reaction to compensate pipetting errors. Thirteen μl of the reverse transcription mix were added to each RNA + primer tubes for a final reaction volume of 20 μl . The tubes were placed and incubated in a thermocycler at 25°C for 5 minutes to anneal, 42°C for 45 minutes to extend, and at 70°C for 15 minutes to inactivate the reverse transcriptase. The cDNA samples were stored at -20°C.

GoTaq® Probe qPCR master mix and nuclease-free water were thawed at 25°C. GoTaq® Probe qPCR Master Mix was vortexed for 5 seconds. Six reactions were prepared: 1 reaction for each of sample (one sample taken 15 minutes after induction, another one taken 30 minutes after induction), 1 negative control which contained no cDNA template, 1 positive control reaction (16S cDNA), 2 more reactions to compensate pipetting errors since the positive control was treated like a separate sample therefore could not be mixed with any other sample. Ten μl of GoTaq® Probe qPCR Master Mix (2X), 1 μl of forward primer (20X), 1 μl of reverse primer (20X) (primers for the amylin cDNA and the 16S cDNA were added in their respective tubes), and 4 μl of nuclease-free water were combined to a total volume of 17 μl . The reaction mix was made in one tube for 4 reactions including the negative control reaction and another reaction to

compensate pipetting errors. In another tube, 2 more reactions: one for the positive control and the other one for pipetting mistakes. After the combination of GoTaq® Probe qPCR Master Mix, PCR primers and nuclease-free water together, the 2 tubes were vortexed for complete mix. Seventeen μl of the reaction mix were added in 4 distinct tubes labeled according to their content (0.55cDNA, 0.75 cDNA, 16S cDNA, no-cDNA template control). Three μl of cDNA template or water (negative control) were added directly to the reaction mix. The tubes were briefly centrifuged and thermocycling was performed under standard conditions; the tubes were placed in a thermocycler and incubated at 95°C for 2 minutes to activate the GoTaq® polymerase (1 cycle), 95°C for 15 seconds to denature the cDNAs (40 cycles), and at 60°C for 1 minute to anneal and extend the amplified products.

AGAROSE GEL ELECTROPHORESIS

In order to ascertain that we obtained the expected product which was the human amylin cDNA, a 2.2 % agarose gel electrophoresis was performed. TBE buffer (10X) was made by mixing in 1 L of deionized water 54 g of Tris base, 27.5 g of boric acid, and 2.92 g of EDTA. To make the 2.2% agarose gel, 1.4 g of agarose was put in 60 mL of 1X TBE. The solution was heated in microwave for two segments of 40 seconds in order to minimize the amount of bubbles and then allowed to cool down for 1 minute during which time 3 μL of GelRed dye were added for visualization under UV light. The gel was poured in the tray and allowed to solidify for 30 minutes. The gel was run at 80 volts for 2h 30 minutes and was visualized under UV light.

3.3. RESULTS

As it can be seen in Figure 12, the well that has the negative control (no-cDNA template) has nothing in it except the primers which appear at the very bottom of the gel due to their low molecular weight (approximately 30 bp).

The two wells that contain the amylin amplicons (the 2 samples) show the expected product which is 111 bp. The well containing the positive control shows a product whose molecular weight is 204 bp which corresponds to the segment of the 16S rRNA that was amplified.

3.4. DISCUSSION

This project aimed at synthesizing human amylin using recombinant DNA techniques. We intended to do so by attaching the ompA amino acid sequence at the N-terminal of the human amylin amino acid sequence. The ompA peptide served solely as a signal sequence to favor translocation into the periplasm where it was more likely to form the disulfide bond; it was not designed to enhance the solubility of the peptide or to favor detection and purification of the peptide. Toward this goal, a Histag (6 histidine residues peptide) is part of the pBAD/Myc plasmid vector attached to the N-terminal segment of the signal sequence. We tried to express the peptide under many different experimental conditions. These changes consisted of dropping the temperature in order to decrease the rate at which metabolism occurs in the cell thereby creating time for the peptide to fold properly and protecting it against proteolysis, decreasing and increasing the inducing agent (L-arabinose) in order to avoid overexpression which would likely bring about inclusion bodies; we did not want our protein to form inclusion bodies due to the fact we wanted a product with full biological activity. However, as described in Ch.2 none of these changes produced the desired result. Therefore, we decided to probe the possible cause of the failure to see the peptide production. One possibility was that the protein was secreted in the media rather than the periplasm. However, analysis of the growing media determined this was not the case. Another possibility was that the problem may have occurred at the gene level instead of the protein level. Therefore, an RT-PCR was performed. Analysis of the 2.2% agarose

gel electrophoresis (Figure 12) indicates that the human amylin messenger RNA was synthesized. Since the mRNA was made, the problem seems to be more likely related to the lifetime of the small peptide in the cytosol. The most probable cause of our inability to detect the amylin protein seems to be proteolysis; the protein was most likely degraded by cytosolic proteases. One way to overcome this issue might be to consider fusing the peptide to other more efficient protein expression tags such as the maltose-binding protein (MBP). MBP has been known to enhance the solubility of many proteins to which it has been fused and also to facilitate the translocation of the protein into the periplasm for disulfide bond formation since MBP is synthesized in the cytoplasm and must be translocated into the periplasm where it plays its transport function.

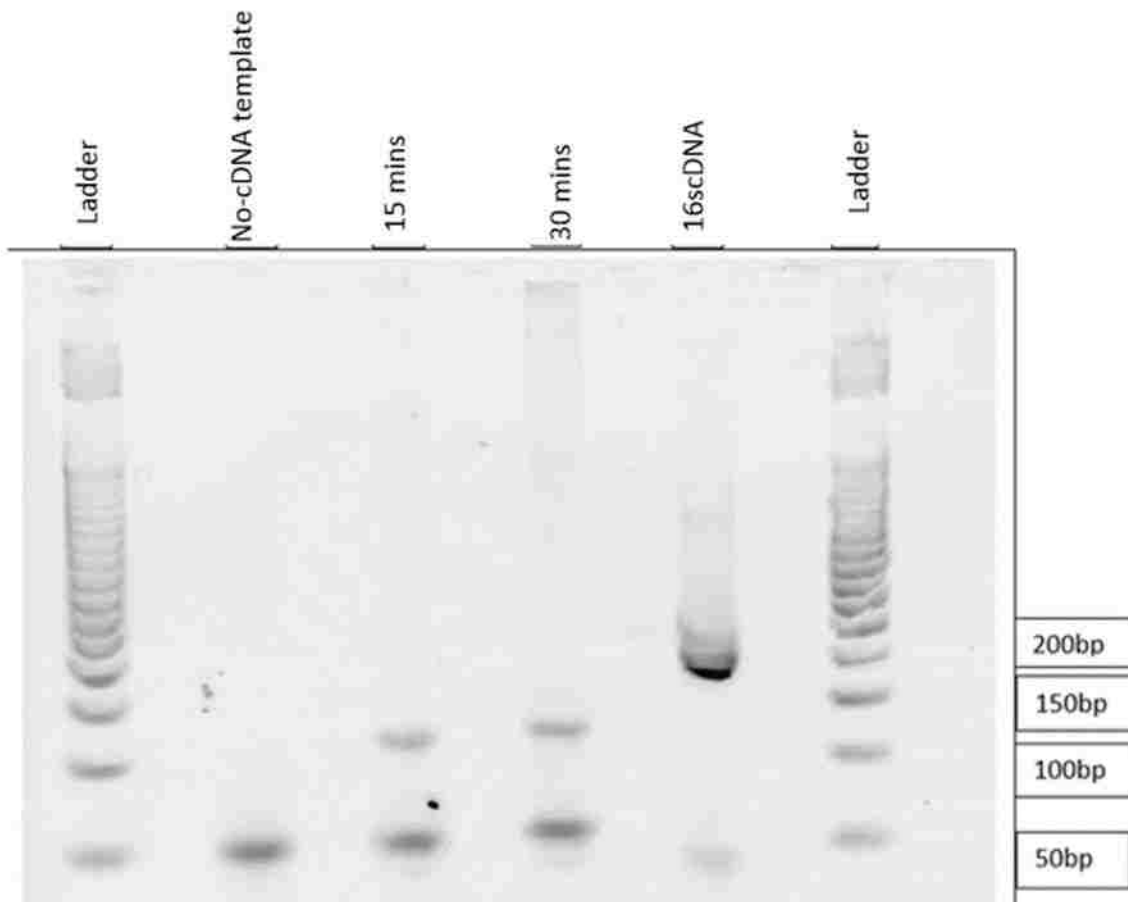


Figure 12. 2.2% agarose gel electrophoresis analysis of the RT-PCR products. The wells located at both extremities left and right contain DNA ladder. From right to left: the 16S rRNA (positive control) (204bp), human amylin cDNA (111bp), the no-cDNA template control (negative control). A no-reverse transcriptase control was not run on the gel; if need for reproduction of this experiment be, one may run a no-reverse transcriptase control as another negative control.

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Title: Islet Amyloid Polypeptide, Islet Amyloid, and Diabetes Mellitus

Author: Per Westermark ,Arne Andersson ,Gunilla T. Westermark

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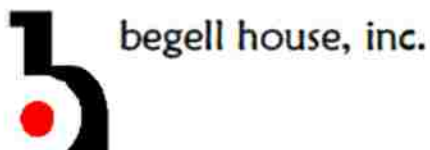
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