Journal of
Emerging Investigators

The Effects of L-glutamate, L-glutamine, and L-aspartic Acid on the Amylase Production of *E. coli* Transformed with pAmylase

Kevin S. Liu and Brittany Franckowiak

Wilde Lake High School, Howard County, MD

Summary

Amylase catalyzes the hydrolysis of dietary starch into oligosaccharides. In humans, amylase is produced by the pancreas and salivary glands and is important in food digestion. Human amylase also has broad applications for therapeutic use in patients with pancreatic insufficiency, necessitating an efficient process for amylase production. *E. coli* transfected with human amylase cDNA can be used to produce amylase to fulfill these pharmaceutical production needs. It was reported that the production of *Thermus* maltogenic amylase in *E. coli* is enhanced by adding L-glutamate and L-glutamine. The purpose of this experiment was to determine whether the amino acids L-glutamate, L-glutamine, and L-aspartic acid could increase human amylase expression in *E. coli*. Based on their effect on the production of *Thermus* maltogenic amylase in *E. coli*, we hypothesized that L-glutamate and L-glutamine would increase human amylase production in *E. coli*, while L-aspartic acid would not. Bacteria with added L-glutamate produced 16-fold more amylase, according to Lugol's iodine staining, and 52-fold more amylase according to Benedict's solution. Bacteria with L-glutamine produced 6-fold more amylase, according to Lugol's iodine staining, and 26-fold more amylase according to Benedict's solution. These findings demonstrate L-glutamate and L-glutamine's ability to increase amylase production by increasing the bacterial cell population. This may result from the effect of L-glutamate and L-glutamine's nutritional properties, as well as their roles as precursors to amino acid synthesis. Our study presents preliminary results for the further development of a more productive way to produce amylase for the pharmaceutical industries.

Received: June 15, 2015; **Accepted:** January 13, 2016; **Published:** May 10, 2016

Introduction

Amylase is an enzyme that catalyzes the hydrolysis of amylose, or starch, into oligosaccharides. Amylase can be further classified as alpha, beta, or gamma-amylase (1). Alpha-amylase, made by the pancreas and salivary glands, hydrolyzes dietary starch into disaccharides and trisaccharides. These saccharides are further converted by other enzymes to glucose and used as a source of cellular energy (1). Consequently, amylase has an important role in digestion, where the secretion of amylase is vital to proper nutrient digestion and absorption (2, 3). Abnormal or unhealthy enzyme secretion by the pancreas can result in nutrient malabsorption and other symptoms that lead to malnutrition (4).

In addition to their role in digestion, amylases are also important ingredients in the food industry. Specifically, alpha and beta amylases are used in brewing beers and liquors made from starch-derived sugars. Amylases, along with yeast, are also used in bread making (5). Since amylase has a potential application in a wide number of industrial processes, research has been conducted to develop a more efficient method of amylase production.

Amino acids are monomers of proteins. The presence of amino acids has a noted effect on the production of *Thermus* maltogenic amylase in *E. coli* (7). Out of all the amino acids tested, L-glutamate most increased the production of *Thermus* maltogenic amylase in *E. coli* (7). This was attributed to L-glutamate's ability to counteract *E. coli*'s stringent response, which represses protein production in response to low levels of amino acids and induces proteins involved in synthesis and protection against damage (7). Specifically, the stringent response that L-glutamate counteracts is characterized as a stress response, induced when amino acid levels in nutrition are low, forcing the cell to construct proteins from available amino acids.

Since L-glutamate increases *Thermus* maltogenic amylase production in *E. coli*, we sought to determine whether L-glutamate and similar amino acids could also increase human alpha-amylase production in *E. coli*. In general, alpha-amylases have important commercial applications, such as in processing starch, brewing, and alcohol production (1, 5). *Thermus* maltogenic amylase is more suited for this application, because most commercial applications of amylase require high temperatures (5). However, human amylase has therapeutic applications; amylase is used in enzyme replacement therapy for those who suffer from pancreatic exocrine insufficiency (2, 3). Human amylase is more suited for therapeutic use because human body temperatures are lower than the optimal temperature required for *Thermus* maltogenic amylase to function. Thus, it is important to develop

techniques to increase alpha-amylase production for the aforementioned purposes. It is also necessary to conduct studies on both enzymes, as *Thermus* maltogenic amylase and human amylase are dissimilar: their protein sequence is only 28% identical. They may be regulated by different mechanisms and their production may respond differently to various amino acids. Therefore, we investigated whether the addition L-glutamate and L-glutamine caused an observable increase in amylase production by increasing the bacterial population size or by up-regulating amylase expression. It was hypothesized that L-glutamate would increase human alpha-amylase production in *E. coli*.

There are chemical and metabolic connections between the three amino acids chosen for this investigation. Chemically, both aspartic acid and glutamate are classified as acidic amino acids (12). Metabolically, glutamine can be converted to glutamate through glutaminase, releasing ammonia in the process (13). Glutamine can also be synthesized from glutamate through glutamine synthetase (10). Both aspartic acid and glutamate are classified as some of the most metabolically active amino acids (13). Since these three amino acids share close metabolic and chemical relationships, we decided to study them together to explore their biologic effects on human alpha-amylase production in transformed *E. coli*. Here, we found that the addition of L-glutamate or L-glutamine enhanced the amount of amylase production by individual bacterial cells. We also demonstrated that both L-glutamate and L-glutamine increased the amylase produced and the total population of *E. coli*.

Results

To semi-quantify the amount of amylase produced by *E. coli* transformed with pAmylase, we set up two color scales for Lugol's iodine staining and for Benedict's solution coloration, respectively (**Figures 1A** and **B**). These two scales were based on serial dilutions of a 1 mL aqueous solution with 10 mg amylase. Amylase was added to each amylose solution so as to generate maltose. In the presence of amylose, Lugol's iodine creates a black solution. When amylase catalyzes amylose, the addition of Lugol's iodine results in a solution of a lighter shade (**Figure 1A**). This can be attributed to the reduced presence of amylose. Benedict's solution is green-blue in the presence of amylose. However, in the presence of maltose, Benedict's solution becomes brown-orange (**Figure 1B**). Next, we used the color scale of the dilution series shown in **Figure 1** to semi-quantify the amount of amylase in different experimental groups.

Since we had three amino acids to test, we set up four experimental groups. The control group did not receive any added amino acids. The other three groups received 20 mM of L-glutamate, L-glutamine, or L-aspartic acid. All groups received the same amount of the bacteria initially and were shaken at the same temperature for the same amount of time. For each of the three trials in this experiment, each group had nine replicates. As shown in **Figure 2**, adding 20 mM glutamate or 20 mM glutamine in culture broth significantly enhanced the production of amylase, as determined by both Lugol's iodine assay (**Figure 2A**) and by Benedict's solution assay (**Figure 2B**). The addition of 20 mM L-aspartic acid in culture broth did not affect the production of amylase.

To further test whether both amino acids increase amylase production by enhancing the numbers of bacteria or by enhancing the amylase production of each individual bacteria cell, we investigated the effects of L-glutamate, L-glutamine, and L-aspartic acid on the yield of bacteria numbers by counting colony forming units (CFU). The CFU is proportional to the number of viable bacteria produced. Adding 20 mM L-glutamate or 20 mM L-glutamine to the culture media significantly increased the number of CFUs in both groups (**Figure 3**), suggesting that both L-glutamate and L-glutamine can increase the production of *E. coli*. Including 20 mM L-aspartic acid did not affect the number of CFUs of

Figure 1

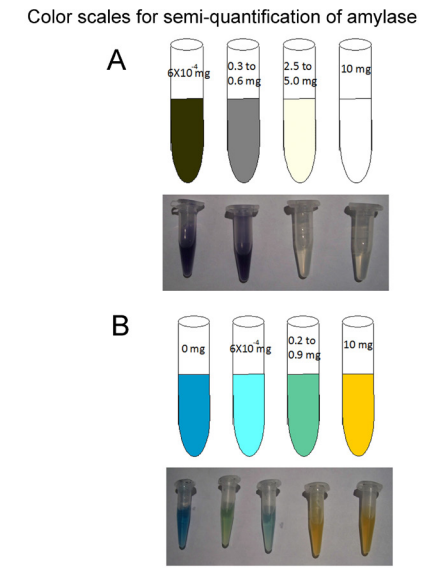

Figure 1: Color chart used to determine amylase concentration. (A) Top row is the color scale used for semi-quantification of amylase with Lugol's iodine method. Lower row is a typical measurement of amylase, showing different levels of darkness after the reaction was stopped. **(B)** Top row is the color scale used for semi-quantification of amylase with Benedict's solution method. Lower row is a typical measurement of amylase, which shows different colors reflecting different amounts of amylase.

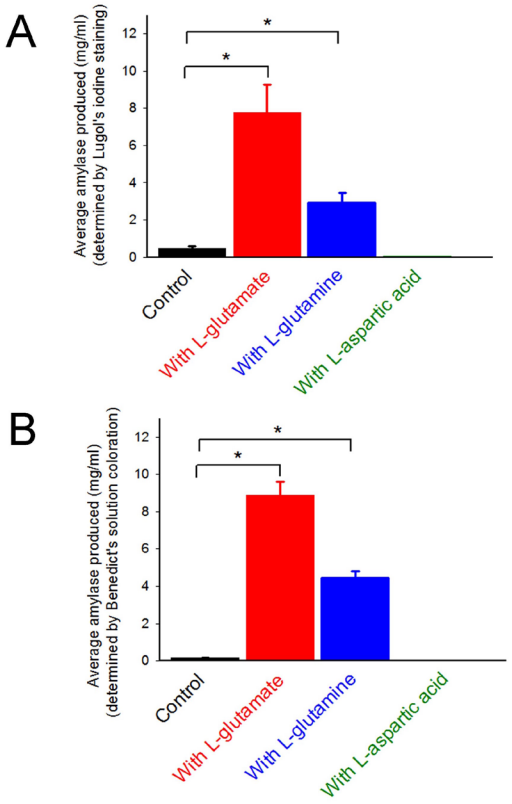

Figure 2: L-glutamate and L-glutamine enhance the production of amylase. Addition of 20 mM L-glutamate or L-glutamine significantly enhanced the production of amylase, as measured with **(A)** Lugol's iodine method or with **(B)** Benedict's solution method. An asterisk (*) denotes significance ($p < 0.05$) as determined by ANOVA test, compared to the control culture without extra amino acids added. Results represent triplicate measurements from each of three independent experiments. Error bars represent standard error.

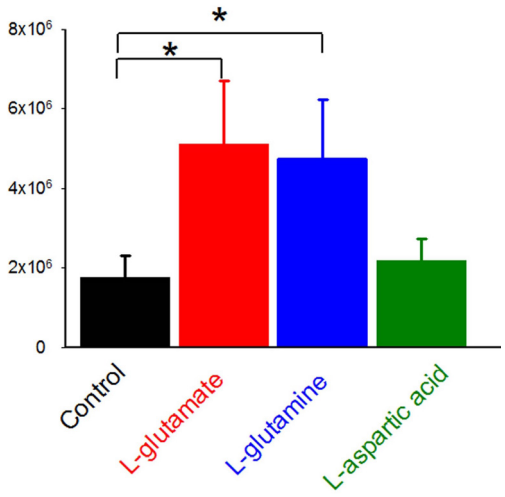

Figure 3: L-glutamate and L-glutamine increase the CFU of *E. coli*. Both L-glutamate and L-glutamine increased the CFU of bacterial cultures, indicating that both amino acids enhanced the population growth of *E. coli*. An asterisk (*) denotes significance ($p < 0.05$) as determined by ANOVA test, compared to the control culture without extra amino acids added. Results represent triplicate measurements from each of three independent experiments. Error bars represent standard error.

bacteria culture. These effects on the CFU demonstrate that both L-glutamate and L-glutamine can increase the numbers of viable bacteria in the culture.

We further calculated a ratio of the amount of amylase produced to the number of CFUs from the same experimental group. As shown in **Figure 4A** and **4B**, both L-glutamate and L-glutamine increased this ratio, indicating that L-glutamate and L-glutamine can increase the production of amylase by each bacterial cell.

Taken together, our results show that L-glutamate and L-glutamine can enhance the overall production of amylase, increase the population growth of *E. coli*, and increase the production of amylase by individual bacterial cells.

Discussion

E. coli bacteria transformed with pAmylase can be significant sources of human amylase for therapeutic applications (2, 3) and for the food industry (5). This investigation sought to find an amino acid suitable for increasing the amount of amylase produced by transformed *E. coli*. The results show that amylase production in the group that received L-glutamate increased 16-fold, as detected by Lugol's iodine staining, and approximately 52-fold, as detected by Benedict's solution coloration, compared to the control group. Since both assays confirm the increase, it is conclusive that L-glutamate increases amylase production in *E. coli*. The results also show that the group that received L-glutamine increased its amylase production 6-fold, as evidenced by Lugol's iodine staining, and 26-fold, as evidenced by Benedict's solution coloration, compared to the control group. Both L-glutamate and L-glutamine also increased the numbers of CFU within each group, suggesting that both amino acids enhanced the population growth of *E. coli*.

L-glutamate is nutritionally beneficial since it is ranked with L-glutamine and ammonia as a major nitrogen source (7). The nutritional properties of L-glutamate and L-glutamine could, to some extent, explain the larger populations of *E. coli* in the groups that received L-glutamine and L-glutamate. Bacterial cultures that received L-glutamate and L-glutamine had significantly more CFUs than the control group. This can partially explain why bacteria with these amino acids produced more amylase, since a larger population could correlate to more amylase synthesis. Most cells use glutamine as a precursor to L-glutamate (8, 9). This could account for the similarity in results between the groups that received

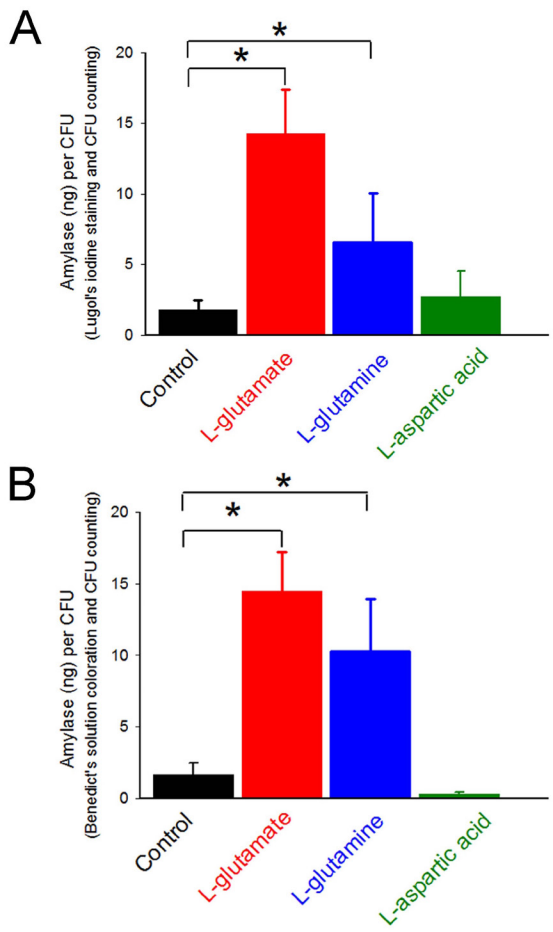

Figure 4: L-glutamate and L-glutamine increase the ratio of amylase produced per CFU compared to control cultures. Addition of L-glutamate or L-glutamine significantly ($p < 0.05$) increased the ratio, suggesting both amino acids can increase the yield of amylase by each individual bacterium, either determined by **(A)** Lugol's iodine method or **(B)** Benedict's solution method. L-aspartic acid has no effects on the ratio. An asterisk (*) denotes significance ($p < 0.05$) as determined by ANOVA test. Error bars represent standard error.

L-glutamine and L-glutamate.

We also demonstrate that each CFU that received L-glutamate or L-glutamine produced more amylase than a CFU of the control group. This is corroborated by both Lugol's iodine staining and Benedict's solution, which shows that a CFU that receives L-glutamate or L-glutamine produced more amylase than one of the control group. Van Heeswijk and colleagues have pointed out that glutamate to L-glutamine conversion is a precursor to the synthesis of many amino acids (10). Furthermore, glutamate can be converted to glutamine, and vice versa, via various chemical pathways. This could explain the increase of human alpha-amylase expression seen in this experiment, because the groups that received either L-glutamate or L-glutamine would have more biosynthesis of amino acids. This would also explain the similar effects on amylase production observed in bacteria with added L-glutamate or L-glutamine. As a result, this could correlate to more protein synthesis, and thus more amylase produced.

Jung and colleagues (7) have determined that the gene expression of *Thermus* maltogenic amylase in *E. coli* increased due to L-glutamate because L-glutamate counteracted the cell's stringent response. Jung's investigation dealt with *Thermus* maltogenic amylase expression. However, this present study deals with human alpha-amylase. Despite the differences between the two types of amylase, Jung's explanation could possibly help to interpret the increased amylase production seen in this study.

Human error was unavoidable when making observations. This especially holds true when determining the darkness of the Lugol's iodine staining or the coloration of the Benedict's solution when assaying amylase concentration. Human error was also possible when creating the semi-quantitative scale (**Figure 1**) to assay amylase concentration, since not all possibilities of coloration or staining could have been accounted for. Although we used two color scales instead of one for semi-quantification, human errors were unavoidable. The ideal method of color measurement would be to use a transmittance meter, which would dramatically reduce human errors.

In summary, we found that addition of L-glutamate or L-glutamine can significantly increase the production of amylase by *E. coli* transformed with a plasmid encoding human amylase. L-aspartic acid has no effect on the production of amylase. Our study presents preliminary results for a possible method of scaling amylase production for therapeutic use, especially for those with pancreatic exocrine insufficiency (14, 15). The results of this investigation could be of note to those in the pharmaceutical industry. It is recommended that for future studies, a more reliable method of measuring amylase and bacterial concentration could be used. The enzymatic activity of human alpha-amylase should be measured using the same methods utilized in other studies as in Jung et al (7) using beta-cyclodextrin as a substrate.

Methods

Agar plates with 100 µg/mL ampicillin were used for the selection of positive colonies, storage of stock colonies, and for plate counts (11). Competent *E. coli* were transformed with pAmylase (11); this human alpha-pAmylase plasmid was created by inserting the amylase gene into the pUC18 plasmid vector.

Bacteria were transformed first by thawing the competent bacteria on ice. We added 5 µL of the

plasmids to the thawed bacterial solution and mixed by gently swirling the pipette tip. The solution of bacteria and plasmids was left to incubate for thirty minutes on ice. The solution was then heated at 42°C for 45–60 seconds and placed on ice for 2 minutes. We added 3 mL of LB broth to the transformed tube, and the solution of bacteria was later incubated in a shaker at 37°C at 150 rpm for 45 minutes. A dilution of the transformed bacteria consisting of 10 µL bacteria and 90 µL LB broth was plated on agar plates with 100 µg/mL ampicillin for the selection of positive colonies. This plate served as the stock from which bacteria was continually drawn.

To prepare cultures for the experimental group, a sterile pipette tip was used to remove a colony from the stock of bacteria; this was placed in 7.5 mL LB broth and shaken at 37°C for most of a day. This resulted in a cloudy broth. We added 0.4 mL of this bacterial suspension to every culture tube in the experimental groups. This ensured an equal initial amount of bacteria per culture. We added 4.1 mL LB to each culture tube. The control group received 0.5 mL water. The experimental groups each had a final concentration of 20 mM L-glutamate, L-glutamine, or L-aspartic acid.

The cultures were shaken overnight at 37°C. After shaking, a small sample of culture was saved from at least one culture in each group. Each culture was poured into a 15 mL centrifuge tube and centrifuged at 4000 rpm for 10 minutes. The supernatant was then separated from the cell pellet that accumulated at the bottom. Each tube of supernatant was divided into two tubes with 300 µL supernatant in each. We added 1 mL of a 3% amylose solution to each tube. Half of the tubes received 20 µL of Lugol's iodine. All protein assays were temporarily left for 24 hours; afterwards, the darkness of the staining of Lugol's iodine was recorded. The other half of the protein assay received 300 µL of Benedict's solution. This half of the protein assay was heated at 100°C for two minutes on a heating-block or hot-bath. The coloration of the protein assay was then recorded. The staining of iodine and coloration of Benedict's solution corresponded to a scale based on serial dilutions of 10 mg amylase/mL.

The saved culture sample was then diluted and spread out evenly on an agar plate with 100µg/mL ampicillin. The plate was then inverted and left to incubate at 37°C for a day. The number of discrete colonies was later recorded.

Lugol's solution contains iodine, which is a chemical indicator of starch molecules. When iodine in the Lugol's solution binds to the starch molecule, Lugol's solution changes its color. The changes in color are directly related to the amount of starch in the solution. This characteristic can be used to determine whether starch appears in the sample or the starch is being digested or removed from the sample.

Benedict's solution is another color indicator for glucose and maltose. Benedict's solution contains a copper compound that reacts with the aldehyde group on glucose and maltose. Different amount of these sugars appearing in the Benedict's solution will give a variety of colors during the assay. Since semi-quantifying method sensitivity can vary, we included both Lugol's iodine solution and Benedict's solution assays in our study to increase the experimental accuracy.

Since this is a semi-quantification experiment, human error was one of our concerns. We used two color scales instead of one in order to reduce human error. In this way, if similar results in amylase production were detected using two separate assays, both results would confirm and reinforce the presence of an increase or decrease in amylase production.

Following incubation and shaking of the cultures, 100 µL of broth was removed from one culture within each group. Each sample was spread evenly on a separate corresponding agar plate and then incubated for 16 hours in an inverted position. Data was collected by counting the number of bacterial colonies in each group. CFUs were calculated by multiplying the numbers of colonies formed on the agar plate by the dilution factor.

The results are expressed as means ± standard error. Statistical significance was tested with a one-way ANOVA (analysis of variance). The level of statistical significance was set at $p < 0.05$.

Acknowledgements

We would like to acknowledge Dr. Martin Schneider of the University of Maryland, Baltimore, for providing the instruments.

References

1. Van der Maarel MJ, et al. "Properties and applications of starch-converting enzymes of the alpha-amylase family." *J Biotechnol*. 94.2 (2002):137-155. Print.
2. Berry AJ. "Pancreatic enzyme replacement therapy during pancreatic insufficiency." *Nutr Clin Pract*. 29.3 (2014): 312-321. Print.
3. Ferrone M, Raimondo M, Scolapio JS. "Pancreatic enzyme pharmacotherapy." *Pharmacotherapy*. 27.6 (2007): 910-920. Print.
4. Pezzilli R, et al. "Quality of life in patients with chronic pancreatitis." *Dig Liver Dis*. 37.3 (2005): 181-189. Print.
5. de Souza PM, de Oliveira Magalhães P. "Application of microbial α-amylase in industry - A review." *Braz J Microbiol*. 41.4 (2010): 850-861. Print.
6. Reece, Jan, et al. *Campbell Biology: Concepts and Connections*. San Francisco: Pearson, 2012. Print
7. Jung HM, et al. "L-glutamate enhances the expression of Thermus maltogenic amylase in Escherichia coli."

Biotechnol Prog. 20.1 (2004): 26-31. Print
8. Forchhammer K. "Glutamine signalling in bacteria." *Front Biosci.* 12 (2007): 358-370. Print.
9. Feehily C, Karatzas KA. "Role of glutamate metabolism in bacterial responses towards acid and other stresses." *J Appl Microbiol.* 114.1 (2013): 11-24. Print.
10. van Heeswijk WC, Westerhoff HV, Boogerd FC. "Nitrogen assimilation in Escherichia coli: putting molecular data into a systems perspective." *Microbiol Mol Biol Rev.* 77.4 (2013): 628-695. Print.
11. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* 2nd ed. New York: Cold Spring Harbor Laboratory, 1989. Print
12. Lodish, Harvey, et al. *Molecular Cell Biology.* 3rd ed. New York: W. H. Freeman, 1995. Print
13. Ramakrishnan S, Prasannan KG, Rajan R. *Textbook of Medical Biochemistry.* 3rd ed. New Dehli: Orient Longman, 2001. Print
14. Bartel MJ, et al. (2015). "Pancreatic exocrine insufficiency in pancreatic cancer: A review of the literature." *Dig Liver Dis.* 47.12 (2015): 1013-1020. Print.
15. Fieker A, Philpott J, Armand M. "Enzyme replacement therapy for pancreatic insufficiency: present and future." *Clin Exp Gastroenterol.* 4 (2011): 55-73. Print.