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Jesse Horne et al.

Citation of published version:
Horne, J., Beddingfield, E., Knapp, M., Mitchell, S., Crawford, L., Mills, S. B., Wrist, A., Zhang, S., & Summers, R. M. (2020). Caffeine and Theophylline Inhibit β-Galactosidase Activity and Reduce Expression in Escherichia coli. In ACS Omega (Vol. 5, Issue 50, pp. 32250–32255). American Chemical Society (ACS). https://doi.org/10.1021/acsomega.0c03909

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Article

Caffeine and Theophylline Inhibit β-Galactosidase Activity and Reduce Expression in *Escherichia coli*

Jesse Horne, Elizabeth Beddingfield, Madison Knapp, Stephanie Mitchell, Logan Crawford, Shelby Brooks Mills, Alexandra Wrist, Shuyuan Zhang, and Ryan M. Summers*

Cite This: *ACS Omega* 2020, 5, 32250−32255

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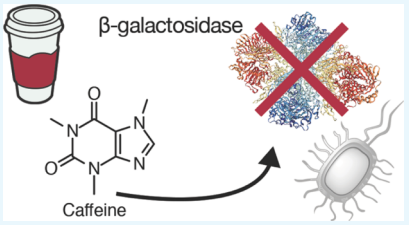
ABSTRACT: The β-galactosidase enzyme is a common reporter enzyme that has been used extensively in microbiological and synthetic biology research. Here, we demonstrate that caffeine and theophylline, common natural methylxanthine products found in many foods and pharmaceuticals, negatively impact both the expression and activity of β-galactosidase in *Escherichia coli*. The β-galactosidase activity in *E. coli* grown with increasing concentrations of caffeine and theophylline was reduced over sixfold in a dose-dependent manner. We also observed decreasing *lacZ* mRNA transcript levels with increasing methylxanthine concentrations in the growth media. Similarly, caffeine and theophylline inhibit the activity of the purified β-galactosidase enzyme, with an approximately 1.7-fold increase in K_M toward *o*-nitrophenyl-β-galactoside and a concomitant decrease in v_{max}. The authors recommend the use of alternative reporter systems when such methylxanthines are expected to be present.



INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a natural product used widely in foods and pharmaceuticals for its stimulatory and diuretic properties. Unfortunately, its wide use has led to the leaching of high amounts of caffeine into the environment,[1,2] presenting toxicity toward both macro-organisms and micro-organisms in several different ways. In plants, caffeine can exhibit autotoxic effects and inhibit seed germination.[3] Even at naturally occurring concentrations, caffeine can have adverse effects on aquatic species and has been shown to induce oxidative stress in bivalves and reduce lysosomal membrane stability in both bivalves and crustaceans.[4−6] Caffeine is also toxic to most bacteria and insects[7,8] and has been shown to cause mutation and cell death in yeast.[9] In spite of this, several synthetic biology studies with bacteria and yeast cells use theophylline (1,3-dimethylxanthine) or caffeine to control gene expression, especially when using theophylline-responsive riboswitches.[10−13]

Not surprisingly, because of the abundance of caffeine in the environment, some bacteria have evolved the ability to metabolize the compound.[14−16] We have isolated several bacterial strains capable of growth on caffeine as the sole carbon and nitrogen source. During our studies on the genes and enzymes responsible for caffeine degradation, we noticed a significant decrease in activity of the commonly used reporter enzyme β-galactosidase toward 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) when in the presence of caffeine. β-Galactosidase is a 465 kDa homotrimeric enzyme encoded by the *lacZ* gene in *Escherichia coli*.[17] The reduction of activity toward X-gal in the presence of caffeine was unexpected and led us to hypothesize that the caffeine could be affecting expression of the *lacZ* gene and/or activity of the β-galactosidase enzyme itself. Here, we demonstrate that caffeine and the related compound theophylline affect both of these parameters.

RESULTS AND DISCUSSION

We first set out to determine if caffeine inhibits the expression of the *lacZ* gene in *E. coli* BL21(DE3). We chose this strain because it contains a native, inducible *lacZ* gene, which has been knocked out of many common laboratory strains. We began by growing *E. coli* BL21(DE3) at various concentrations of IPTG to determine the optimal inducer concentration for *lacZ* expression, which was measured by determining β-galactosidase activity using the Miller Assay.[18] As seen in Figure 1A, β-galactosidase activity toward *o*-nitrophenyl-β-galactoside (ONPG) increased greatly when IPTG was increased from 10 to 100 μM. Thus, we selected 100 μM IPTG for future studies because it gave sufficient expression to measure β-galactosidase activity but was still sensitive enough for us to measure enzyme activity accurately. When cells were grown in LB medium containing 100 μM IPTG and various concentrations of caffeine (Figure 1B) or theophylline, we observed decreasing β-galactosidase activity with increasing methylxanthine concentration in the growth media (Figure

Received: August 13, 2020
Accepted: December 3, 2020
Published: December 14, 2020

https://dx.doi.org/10.1021/acsomega.0c03909
ACS Omega 2020, 5, 32250−32255

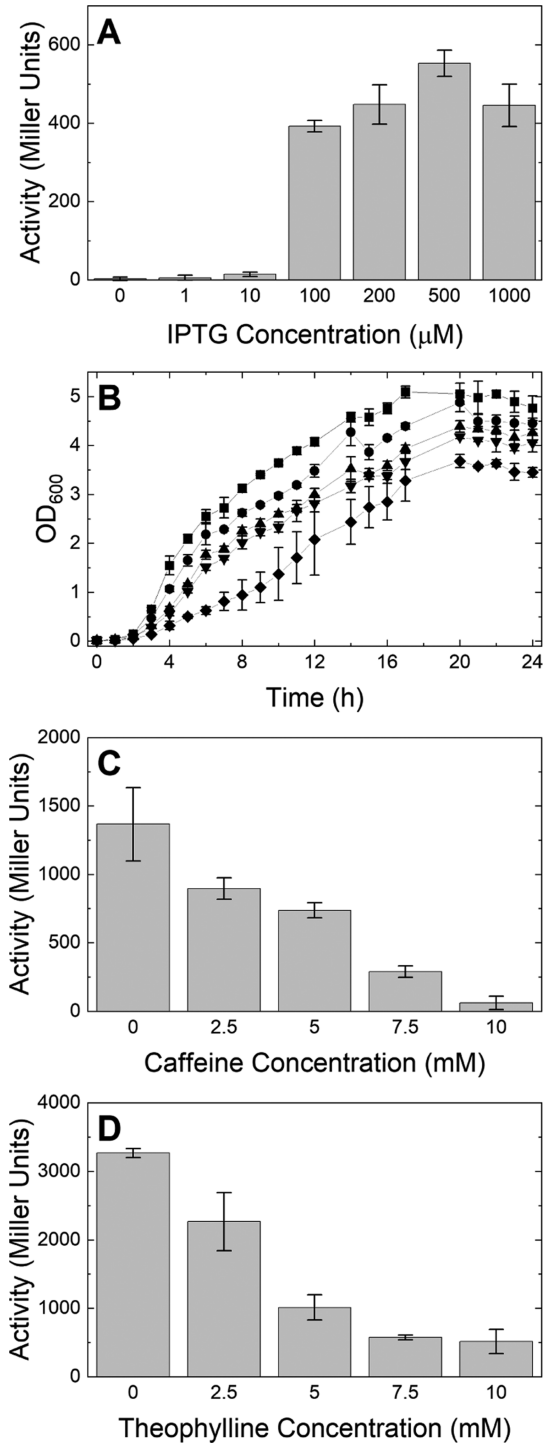


Figure 1. Effect of caffeine on cell growth and β-galactosidase activity in *E. coli* BL21(DE3). (A) β-Galactosidase activity at various IPTG concentrations. (B) Growth curves of BL21(DE3) in LB containing 0 (■), 2.5 (●), 5 (▲), 7.5 (▼), or 10 (♦) g/L caffeine. (C,D) β-Galactosidase activity of cells grown in LB containing various concentrations of caffeine (C) or theophylline (D). Error bars in all graphs represent standard deviations of triplicate results.

1C,D). There was no caffeine in the Miller assay; hence, these data suggest that caffeine and theophylline may inhibit the expression of *lacZ* in *E. coli*, as indicated by the reduced enzyme activity without inhibitors present. Cell growth was reduced by caffeine (Figure 1B) and theophylline (data not shown), and we suspect that the added stress from the presence of the methylxanthines may be the leading cause of reduced *lacZ* expression.

In order to test whether caffeine and theophylline specifically inhibited the *lacZ* expression or if the decrease in activity was due to a global suppression of gene expression, we used qPCR to calculate transcript levels of *lacZ* and glucokinase (*glk*) relative to the constitutively expressed malate dehydrogenase (*mdh*) when cells were grown in the presence of 0, 5, and 10 mM methylxanthine. The *glk* gene expression remained fairly constant, irrespective of methylxanthine concentration (Figure 2B). However, we observed a significant decrease in *lacZ* expression when cells were grown with both 5 and 10 mM caffeine or theophylline (Figure 2A), indicating that the transcription of *lacZ* is reduced in the presence of methylxanthines.

We also tested expression of the yellow fluorescent protein amilGFP gene in *E. coli* BL21(DE3) at different concentrations of caffeine and theophylline. As can be seen in Figure 2C, the fluorescence remained relatively unchanged when cells were grown with increasing levels of methylxanthines, indicating that gene expression was not significantly affected. Thus, the *lacZ* expression appears to be inhibited by caffeine and theophylline at a greater level than expression of other genes. However, whether the expression of *lacZ* is specifically inhibited by methylxanthines is beyond our ability to determine, given the data presented in this study. A full RNAseq analysis of the cells grown at different concentrations of caffeine and theophylline would provide greater insights into which genes are affected by methylxanthines.

We also sought to determine if caffeine inhibits the activity of β-galactosidase once it has been expressed. In this case, we assayed purified β-galactosidase in the presence of 10 mM caffeine or theophylline to determine kinetics of inhibition. We first cloned the *lacZ* gene into the pET28a(+) vector with an N-terminal hexahistidine tag, expressed the gene in *E. coli* BL21(DE3), and purified the enzyme to near homogeneity with a Ni-NTA column (Figure 3A). Because of a wide range of reported molar absorbance coefficients for *o*-nitrophenol (ONP),[19−22] we determined the absorbance coefficients for ONP alone and with 10 mM caffeine or theophylline in 50 mM potassium phosphate buffer (pH 7.0) at 420 nm (Table 1, Figure S1). These values are on the lower end of the reported molar absorptivity[23] and do not vary much in the presence of caffeine or theophylline.

The purified β-galactosidase activity toward ONPG was significantly reduced in the presence of both caffeine and theophylline (Figure 3B, Table 1). The K_M value for ONPG increases by approximately 1.7-fold with caffeine and theophylline, while v_{max} is reduced by 27% and 13%, respectively. From these data, we have calculated inhibition constants of 15.0 ± 1.36 and 13.7 ± 1.97 mM for caffeine and theophylline, respectively. Although these parameters do not neatly fit into the traditional inhibitor mechanisms, a Lineweaver−Burk plot of the data for ONPG concentrations at or above 0.25 mM shows all three curves intersecting near the *x*-axis (Figure 3C), indicating that both caffeine and theophylline most closely approximate competitive inhibitors for β-galactosidase. These results are intriguing as the structures of caffeine and theophylline greatly differ from those of lactose and ONPG. We also note that two other compounds with slightly similar ring structures, pyridindolol and imidazole, have been shown to inhibit the activity of β-galactosidase.[24,25]

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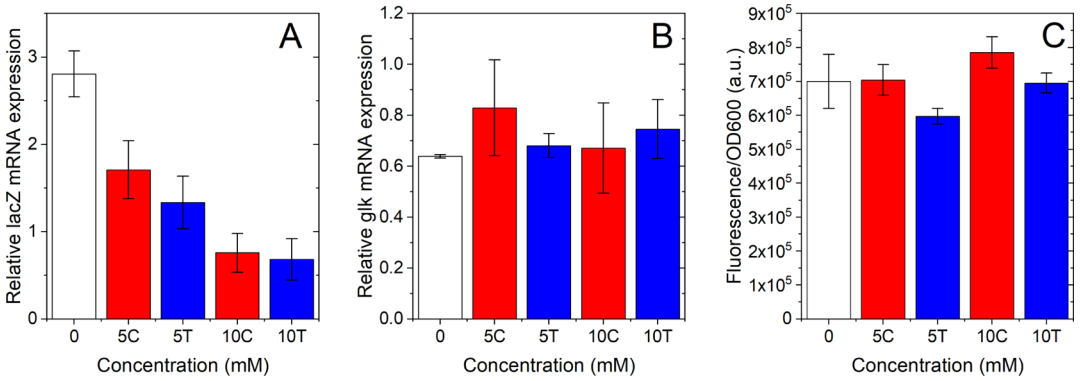

Figure 2. Effect of caffeine and theophylline on gene expression in *E. coli* BL21(DE3). Expression of *lacZ* (A) and *glk* (B) mRNA relative to the *mdh* gene. (C) Expression of amilGFP in *E. coli* BL21(DE3). White bar, no methylxanthines; red bars, caffeine; blue bars, theophylline. Error bars in all graphs represent standard deviations of triplicate results.

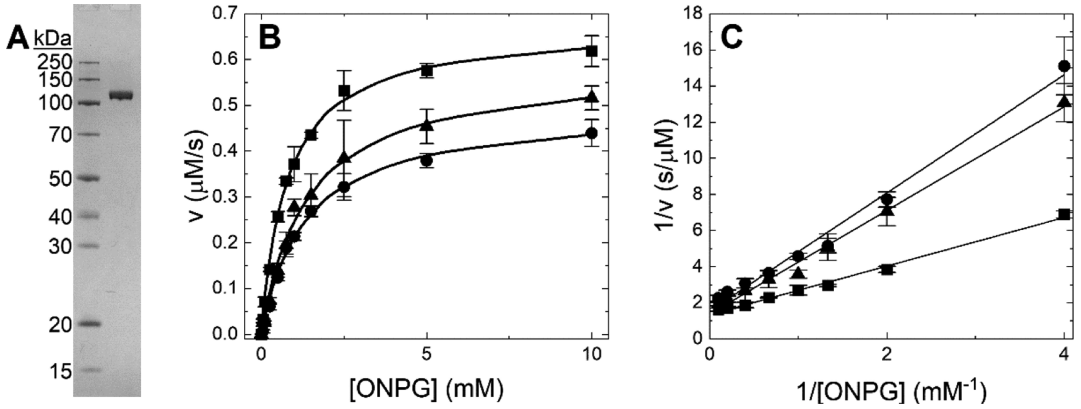

Figure 3. Enzymatic activity of purified *β*-galactosidase. (A) SDS-PAGE of 2 *μ*g of purified *β*-galactosidase. (B) Activity of 57.9 pg of purified *β*-galactosidase with no inhibitor (■), 10 mM caffeine (●), or 10 mM theophylline (▲). (C) Lineweaver–Burk plot of *β*-galactosidase activity; symbols are the same as in (B). Each point represents the average of three replicates, while error bars depict the standard devation.

Table 1. Kinetic Parameters of *β*-Galactosidase in the Presence of 10 mM Methylxanthines

inhibitor	ε (mM^{-1} cm^{-1})	v_{max} (μM/s)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M	K_I (mM)
no methylxanthine	3516	0.677 ± 0.011	0.816 ± 0.042	1088 ± 18	1333 ± 71	
10 mM caffeine	3620	0.495 ± 0.011	1.362 ± 0.086	796 ± 18	585 ± 39	15.0 ± 1.36
10 mM theophylline	3528	0.591 ± 0.022	1.412 ± 0.143	949 ± 36	672 ± 73	13.7 ± 1.97

To reaffirm that caffeine acts as a competitive inhibitor of the *β*-galactosidase enzyme, computer protein–ligand docking studies were conducted. Preliminary results demonstrate that caffeine binds within the same region of *β*-galactosidase as lactose, as shown in Figure 4. Caffeine interacting within the same binding pocket as the natural substrate, lactose, reaffirms the competitive inhibition of the methylxanthines. Further studies are necessary to demonstrate the cause of this competition and broader impacts of this inhibition.

To avoid the issue of reporter indicator competition, we suggest the use of alternative reporter systems when methylxanthines are expected. One such option is a luciferase reporter for monitoring gene expression.[26,27] Luciferases are enzymes from bioluminescent organisms that produce light. When fused to a luciferase reporter gene, the gene expression can be correlated to light emission. Fluorescent proteins are another commonly chosen reporter system, as specific wavelengths are used for transmission and detection, and proteins with nonoverlapping spectra may be used together.[28] Common choices include green, blue, and red fluorescent proteins. Ghim et al. provided greater coverage of reporter proteins commonly used in their review.[29] Of note, no publications were found that methylxanthines affected luciferase or fluorescent protein alternative reporter systems, but further studies may be necessary to rule out reporter inhibition in unique cases. We have demonstrated that the expression of amilGFP in *E. coli* from a strong constitutive promoter is not affected by caffeine and theophylline (Figure 2C), indicating that fluorescent proteins may not be as impacted as *β*-galactosidase in the presence of methylxanthines.

■ CONCLUSIONS

In summary, we have determined that caffeine and theophylline reduce expression of the *lacZ* gene and inhibit the activity of its product, *β*-galactosidase. A preliminary molecular docking analysis indicated a similarity in binding location of both lactose and caffeine within the enzyme. These findings demonstrate another interesting property of natural methylxanthines and their effects on microbes. Because many

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ACS Omega 2020, 5, 32250–32255

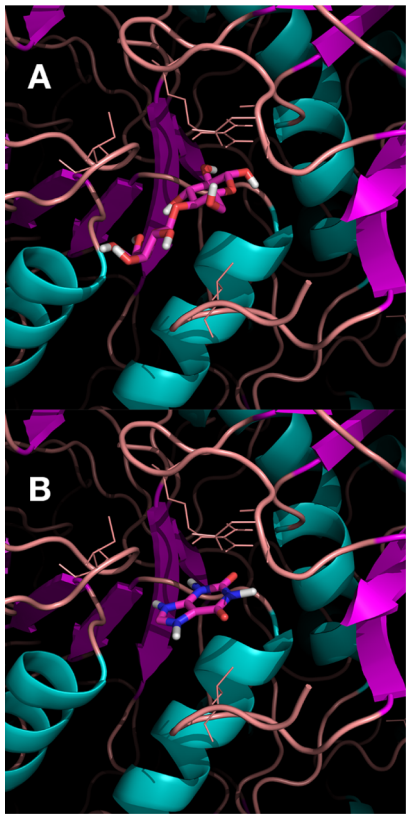

Figure 4. Virtual molecular screening of lactose (A) and caffeine (B) in the β-galactosidase enzyme. Both ligands have a similar binding location, demonstrating likely competitive inhibition.

synthetic biology studies use *lacZ* as the reporter gene, we advise that studies involving caffeine or theophylline should use a different reporter, such as a fluorescent protein.

EXPERIMENTAL SECTION

Chemicals. All chemicals used in this study were purchased from VWR (Radnor, PA). Enzymes and their buffers were obtained from New England Biolabs (Ipswich, MA).

Cell Growth. *E. coli* BL21 (DE3) cells were used in this study. Unless otherwise stated, cells were grown in Luria Bertani broth at 37 °C, with orbital shaking at 200 rpm. To determine optimum IPTG concentrations, cells were grown with increasing concentrations of IPTG to an OD_{600} of approximately 0.6.

Activity Assays. The expression of *lacZ* was measured through a Miller Assay.[18] Briefly, approximately 10^6 BL21-(DE3) cells (100 μL of a culture with OD_{600} of 1.0) grown in LB with 100 μM IPTG and increasing concentrations of caffeine or theophylline (0−10 mM) were resuspended in 1 mL Z-buffer-containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 38 mM 2-mercaptoethanol (pH 7.0). Cells were lysed by adding 30 μL chloroform and 20 μL of 0.1% SDS. The reaction was started by adding 200 μL of freshly prepared 4 mg/mL ONPG in water and stopped with 500 μL of 1 M Na_2CO_3. The amount of ONP produced was determined by measuring absorbance at 420 nm. Activity in Miller Units was determined with the following equation

$$\text{specific activity} = \frac{1000 \times \Delta A_{420}}{\text{mL of cells} \times A_{600} \times \text{time (min)}}$$

Purified enzyme activity was determined by incubating 57.9 pg of purified β-galactosidase in 200 μL containing 1 mM MgCl, 50 mM 2-mercaptoethanol, and increasing concentrations of ONPG (0 to 10 mM) in 50 mM potassium phosphate buffer (pH 7.0). Reaction progress was monitored by measuring absorbance at 420 nm in a Molecular Devices iD3 plate reader, and activity was calculated by taking the initial rate (linear portion) with the following equation:

$$v = \frac{\Delta A_{420}}{\varepsilon \times l \times \text{time(s)}}$$

where ε is the molar absorbance coefficient and l is the path length (depth of reaction in the microplate well). Michaelis Menten kinetic parameters were calculated by nonlinear regression.

GFP Measurement. To measure GFP fluorescence in *E. coli*, plasmid pK1073024 (Biobrick BBa_K1073024) was transformed into strain BL21(DE3) by heat shock. This plasmid encodes a constitutively expressed amilGFP protein which is visible to the naked eye as a yellow color and also fluorescent. Cells containing pK1073024 were grown in LB with 34 μg/mL chloramphenicol. After 16 h of cultivation, fluorescence of cells was determined using a SpectraMax i3x plate reader (Molecular Devices, San Jose, CA) with 485 nm excitation and 535 nm emission. Measurements were taken in triplicate, and readings were standardized by dividing fluorescence by the OD_{600} of the cell cultures.

qPCR. The mRNA levels of the *lacZ*, *glk*, and *mdh* genes in *E. coli* BL21(DE3) were measured using qPCR. Cells were grown overnight in LB containing 0, 5, or 10 mM caffeine or theophylline and then inoculated into fresh media containing the same methylxanthine concentration and 100 μM IPTG and harvested when the OD_{600} was about 0.8−1.0. Total RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific) according to manufacturer instructions. The cDNA synthesis was carried out with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and random hexamer primer following kit instructions, with a thermocyler program of 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 5 min. Primer pairs for the qPCR reaction were LacZ-qPCR-F2/R2 (GTATCGCCAAAATCACCGCC/CGTTTCGTCAG-TATCCCCGT), Glk-qPCR-F1/R1 (TACAGCCGTCTTT-CACCTCG/AGTGGTGAAATCTCGCAGGC), and Mdh-qPCR-F1/R1 (TGTTTGCCTTTCAGTTCCGC/AGTTGC-GATTGCTGCTGAAG). Each qPCR reaction was carried out in triplicate with a time program of 50 °C for 2 min, 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 60 s.

Cloning and Heterologous Expression of lacZ. The *lacZ* gene was cloned into the NdeI and BamHI restriction sites of the pET28a(+) vector with an N-terminal hexahistidine (His_6) tag, resulting in pET28-His_6-lacZ. This plasmid was transformed into *E. coli* BL21 (DE3) cells. Cells harboring the plasmid were grown in LB broth with 30 μg/mL kanamycin at 37 °C with 200 rpm agitation. When the culture reached an optical density of 0.9, the IPTG was added to a concentration of 0.1 mM and the culture was shifted to 18 °C and grown overnight with shaking at 200 rpm. Cells were harvested by centrifugation at 10,000*g* for 10 min at 4 °C and stored at −80 °C prior to lysis.

About 10.6 g of frozen cells was resuspended in binding buffer (10 mM imidazole and 300 mM NaCl in 50 mM potassium phosphate buffer, pH 7.5) to a concentration of

https://dx.doi.org/10.1021/acsomega.0c03909
ACS Omega 2020, 5, 32250−32255

about 1 g/mL and lysed by sonication with 30% amplitude, 1 s on and 1 s off for 5 min total. The lysate was clarified by centrifugation at 20,000*g* for 10 min at 4 °C, and the supernatant was collected as the cell extract. Enzyme purification was carried out at 4 °C using an automated Bio-Rad NGC chromatography system. About 25 mL of the cell extract was loaded onto a 1 mL (bed volume) Ni-NTA column (GE Healthcare), washed with 10 column volume binding buffer, and eluted in 5 column volume elution buffer (250 mM imidazole and 250 mM NaCl in 50 mM potassium phosphate buffer, pH 7.5). The purified protein fractions were combined, concentrated, and dialyzed into KPGD buffer [5 mM potassium phosphate buffer, pH 7.5 containing 5% (vol/vol) glycerol and 1 mM dithiothreitol (DTT)] using a Macrosep Advance centrifugation tube (MWCO 10 kDa), and 100 μL aliquots were stored at −80 °C until ready for use. Protein concentrations were determined with the Bradford Assay.[30]

Protein−Ligand Molecular Docking Analysis. The structural model of the *E. coli* β-galactosidase enzyme in conformation with lactose from Juers et al. was obtained from the Protein Data Bank (accession code 1JYN).[31,32] The virtual molecular screening of caffeine and lactose was carried out using the software package PyRx for model preparation and setup.[33] AutoDock Vina[34] within PyRx was used for molecular docking. Protein and ligand visualization was performed in the open-source software PyMOL.[35]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03909.

Calibration curves to determine absorbance coefficients for ONP (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Ryan M. Summers − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States;* orcid.org/0000-0003-1358-6605; Phone: 205-348-3169; Email: rmsummers@eng.ua.edu

Authors

Jesse Horne − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Elizabeth Beddingfield − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Madison Knapp − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Stephanie Mitchell − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Logan Crawford − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Shelby Brooks Mills − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Alexandra Wrist − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Shuyuan Zhang − *Department of Chemical and Biological Engineering, The University of Alabama, Tuscaloosa, Alabama 35487, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c03909

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by University of Alabama research funds.

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https://dx.doi.org/10.1021/acsomega.0c03909
ACS Omega 2020, 5, 32250−32255