Determining the enantiomeric composition of the dipeptide Alanylglycine with Marfey's reagent

Noor Naji || Mentor: Dr. Gregory Barding

California State Polytechnic University, Pomona || Department of Chemistry and Biochemistry

Kellogg Honor's College Capstone Project

Introduction

Understanding genotypes that enhance plant survival under climate-stressing conditions is relevant to today's agricultural economy. Previous studies using submergence stress and recovery tests have shown that the SUB1A gene in rice plants does increase chance of survival during complete submergence. Those tests also showed AlaGly concentrations decreased after submergence, but did not recover upon returning to standard conditions like the other plant metabolites. This indicates a possibly unknown metabolic pathway and its implications on survival mechanisms. Additionally, other research has shown that the AlaGly is present only as D-Alanylglycine (D-AG) and in relatively high abundance. Since concentrations of analytes usually lead to an understanding of their respective functions, this project focuses on quantifying D-AG. And since both D/L enantiomers may be present, separating the two is important. Derivatizing with Marfey's reagent (MR) was chosen for chiral separation due to ease of derivatization and its ability to enhance the dipeptide's sensitivity in the UV region at 340 nm using HPLC-UV.

Materials and Methods

Sample Preparation For Rice Samples: Thirty mg of *Oryza sativa ssp. japonica* cv. M202(*Sub1*) rice tissue was weighed. Then, 1mL of 80:20 Methanol and H_2O mixture, and 300µL of chloroform were added. After centrifuging, the sample was centrivapped until dry. A 300µL aliquot was used for derivatization.

Derivatization Method: The derivatization procedure follows Bhushan and Brückner's method with some modifications. After adding either rice tissue or D/L-AG, Marfey's reagent in 1% acetone and 1M NaHCO3 were added. The samples were then heated for 1hr at 35 ºC. Samples were cooled and quenched with 2M HCl, then diluted with the 10% acetonitrile and 0.1% formic acid mixture.

HPLC Data Acquisition: After filtration, samples were analyzed via HPLC-UV at 340 nm. The aqueous phase was 10% acetonitrile and 0.1% acetic acid in water, and the organic phase was 99.9% acetonitrile and 0.1% acetic acid. Analysis was conducted with 10µL sample injections at a 0.5mL/min flow rate and 30 ºC oven temperature.

HPLC Chromatograms

D/L-AG Standards

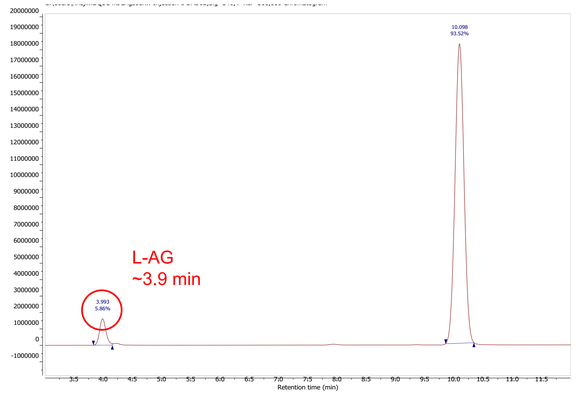


Figure 1 The 0.5mM L-Alanylglycine standard with a retention time of ~3.9 mins, eluted earlier than the D-enantiomer as predicted by literature. .

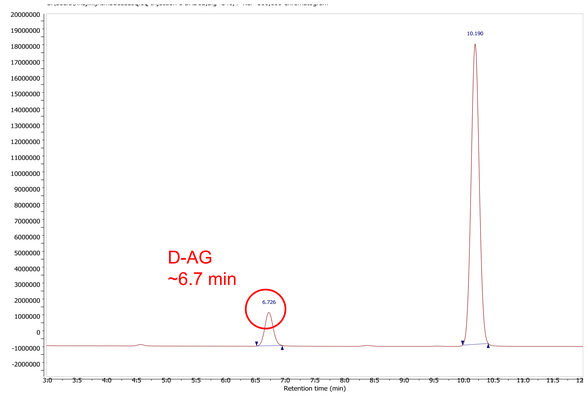


Figure 2 The 0.5mM D-Alanylglycine standard with a retention time of ~6.7 mins, eluted later than the L-enantiomer as predicted by literature.

Rice Samples

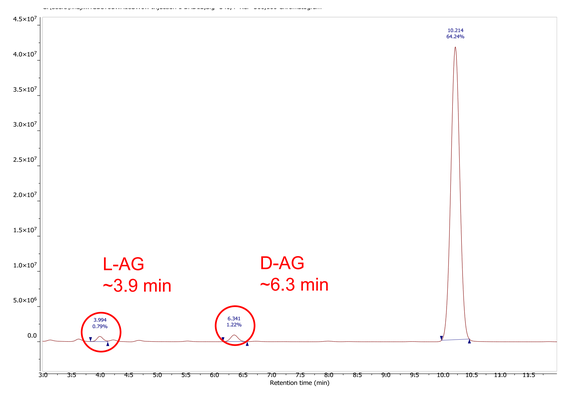


Figure 3 The spiked rice sample with D/L-AG standards at expected retention times to confirm presence and chirality of AG in raw samples.

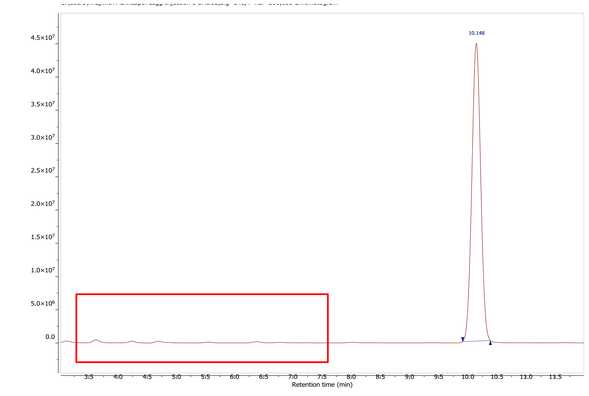


Figure 4 Raw unspiked rice sample with low intensity peaks in the 3-7 min range. Zoom in of boxed region can be seen in Figures 5 and 6.

Calibration Curve

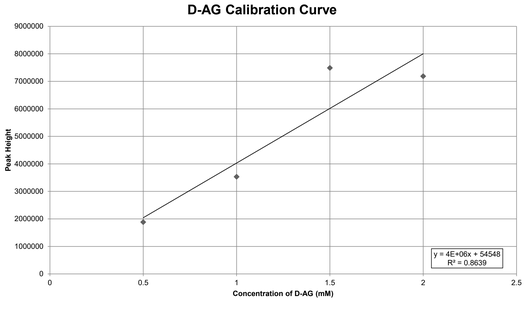


Figure 7 Calibration curve using D-AG standards from 0.5mM to 2.0mM. Extrapolation predicts a D-AG concentration of 0.03 mM for rice sample.

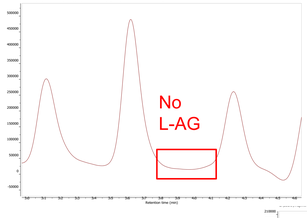


Figure 5 Figure is a scaled portion of Figure 4 at the 3.0-4.5 min region. Lack of peak at 3.9 mins confirms absence of L-AG.

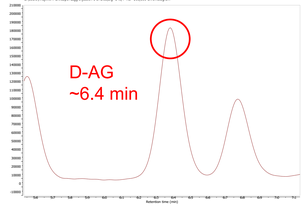
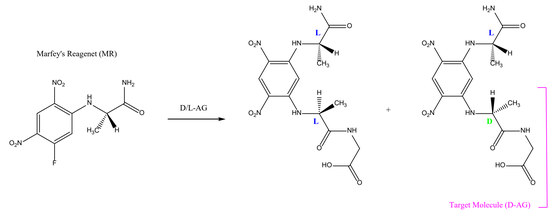


Figure 6 Figure is a scaled portion of Figure 4 at the 5.5-7.0 min region. Peak at 6.4 mins confirms presence of D-AG.

Results and Discussion

To confirm that the derivatization procedure and conditions yield a sufficient separation, D and L-AG standards were made. Figure 1 illustrates the L-AG standard eluting at 3.9 mins, and Figure 2 shows the D-AG standard eluting at 6.7 mins. This indicates (1) the separation process is well-resolved with retention times 2 mins apart and (2) the experiment matches previous studies that reported the D-enantiomer eluting later than L. The other large peak at 10 mins is unchanged for all samples, indicating it might be unreacted MR. For the rice samples, the presence of D-AG was confirmed by spiking a similarly treated sample with D and L. As seen in Figure 6, a scaled portion of Figure 4, there is a peak at exactly 6.4 mins, which matches the retention time of D-AG in the spiked sample. However, a scaled portion of the rice sample in Figure 5, indicates that there is no peak where L-AG is expected. Thus, the rice tissue contains D-AG, but not L. To quantify the D-AG in the rice tissue, a calibration curve of D-AG standards ranging from 0.5mM to 2mM was constructed (Figure 7.) Extrapolating the calibration yields 0.03mM of D-AG in the rice tissue. This concentration value is likely to be under the limit of quantitation due to possible extrapolation errors.



Conclusion

Experimental results illustrate that Marfey's reagent is a suitable chiral derivatizing agent for the target analyte, D-Alanylglycine. The rice tissue sample was confirmed to contain D-Alanylglcyine, but not the L-enantiomer, which is generally unusual for a multicellular organism. Since the analyte has been confirmed to be in the rice plant and has been quantified, the next step is to conduct submergence and recovery tests to understand D-alanine in the metabolic pathway and its relation to D-Alanylglycine's function.