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Spectrophotometric Investigation of Plant Based Pancrelipase for Pancreatic Insufficiency

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Cystic Fibrosis and Pancreatic Insufficiency

Pancreatic insufficiency is generally identified as pancreatic function that will not allow the individual to grow, thrive, or maintain their health without pancreatic supplementation (Singh & Schwarzenberg, 2017). Pancreatic insufficiency often begins at birth or infancy in subjects with cystic fibrosis, most commonly in subjects presenting with homozygous recessive or heterozygous in which at least one of the two mutated genes are 'severe' mutations such as Δf508 (Singh & Schwarzenberg, 2017). Cystic fibrosis (CF) creates thick secretions that cause significant injury to the acinar tissue in the exocrine pancreatic tissue as early as fetal development, causing pancreatic insufficiency. Pancreatic insufficiency can lead to fatal malnourishment and failure to thrive beginning as a neonate (Singh & Schwazenburg, 2017). Even with the support of prescribed pancreatic enzymes, CF patients often still need supplemental vitamins due to the malabsorption of fat-soluble vitamins including vitamins A, E, K, and D (Bertolaso et al., 2014).

Pancreatic insufficiency and the malnourishment associated with CF often causes significant health complications throughout the rest of the body as well, including reduced bone density and

development of osteoporosis in CF patients of significantly younger ages than their non-CF osteoporotic peers (King et al., 2005). Malnourishment has also been found to be directly correlated with declining lung function trends in CF patients (Singh & Schwarzenberg, 2017). It is essential that patients with CF who have pancreatic insufficiency, approximately 85-90% of the patients, are treated with effective pancreatic enzyme replacement therapy (PERT) to achieve the best prognosis. PERT is an oral supplement that contains a combination of protease, lipase, and amylase typically known as pancrelipase in place of the digestive enzymes that are not functional in pancreatic insufficient CF patients. Protease is an enzyme that digests proteins, amylase is an enzyme that digests sugars and starches, and lipase is an enzyme that digests fats. Pancrelipase is dosed by lipase units based on the fat content in the meal.

Spectrophotometric Investigation of Plant-Based Pancrelipase for Pancreatic Insufficiency

In patients with CF and other conditions with pancreatic insufficiencies, the pancreas is unable to supply the enzymes required for digestion and absorption of food in the small intestines. Pancreatic inadequacies lead to malnourishment in patients that may be decreased by prescribed supplemental pancreatic enzymes (Singh & Schwarzenberg, 2017), but there may be additional enzymes with better digestive efficiencies. Currently, most CF-prescribed enzymes are sourced from porcine pancreatic lining. Although many CF patients can tolerate this therapy well, this can place the recipient of the enzymes at risk for several dangerous pathogens that can be carried by pigs, especially for the immunocompromised, which applies to most CF patients (Venkatesh & Kasi, 2022). There are patients with CF who also present with Alpha-gal syndrome (AGS); AGS can cause life threatening allergic reactions to red meat products. Just as patients with AGS have been found to have reactions to other medications that are derived from swine, these patients have been found to have serious reactions to porcine-derived pancrelipase (Swiontek et al., 2019).

A viable plant-based pancrelipase may present an alternative solution that has the potential to be more sustainably sourced. Bromelain is an enzyme concentrated in the stems and leaves of pineapple plants, *Ananas comosus*, and aids in digestion of proteins (Roxas 2008; Rathnavelu et al., 2016). The lipase found in ground oats, *Avena sativa*, aids in digestion of fats, specifically in patients with CF (Barnes et al., 1994). Additionally, extract of mango peels, *Mangifera indica*, contains amylase to aid in digestion of carbohydrates. (Mehrnoush & Yazid 2013). The hypothesis is the plant-based enzymes (bromelain, oat lipase, oat amylase, and mango amylase) will have significantly higher or comparable amounts of digested protein, fat, and carbohydrates compared to CF-prescribed pancreatic enzymes.

Potential Benefits of Plant-Based Enzymes

There are several potential benefits of a plant-based alternative form of pancrelipase for pancreatic insufficiencies. From an ethical standpoint, patients who observe religious or personal beliefs that prohibit the consumption of porcine derivatives currently have little other option than to consume porcine derived pancrelipase. While most religious leaders recognize that it is permissible as a lifesaving medication, offering a plant-based alternative could potentially alleviate ethical concerns for patients and prescribing care providers. Pancrelipase derived from any animal source has the potential to

introduce dangerous pathogens to the patient consuming the enzymes. Although there are a series of safety measures in place during the production and manufacturing of animal-based enzymes, using a plant-based alternative has the potential to eliminate the risk of animal derived pathogens being contracted by the immunocompromised CF patient (Venkatesh & Kasi, 2022).

Each plant-based enzyme investigated in this study has additional qualities of interest to the CF community. Bromelain, the protease found in pineapple plants, has anti-inflammatory properties that can help combat the chronic inflammation that plagues many CF patients (Rathnavelu et al., 2016; Munck, 2014). Bromelain has been found to have properties that help to fight off bacterial and fungal infections, including but not limited to *Escherichia coli* and *Vibrio cholerae* in the gut, and significant defense against several parasitic nematodes in the gastrointestinal system (Rathnavelu et al., 2016). Bromelain has unique properties that enhance antibiotic effectiveness against several strains of bacteria, including *Staphylococcus areus*, which often causes bouts of respiratory infections such as pneumonia and tuberculous in CF patients (Rathnavelu et al., 2016). Bromelain is significantly more effective when used in conjunction with other medications in treating sepsis when compared to antibiotics on their own (Rathnavelu et al., 2016). Due to the nature of cystic fibrosis, these infections are very common and often show resistance to treatment. Therefore, using a pancrelipase that also carries these defensive qualities may be an advantage as opposed to the currently prescribed pancrelipase. Finally, bromelain has been found to have an alkalizing effect when present in the digestive system. The release of bicarbonate that the duodenum in the digestive system is responsible for does not typically work as well with CF patients, often causing GERD and acid gastrointestinal environments (Kaur et al., 2004).

Oat lipase has been shown to be an effective lipase source for individuals with CF and qualities that aid in its ability to remain stable and not denature in acidic environments, potentially providing more efficient digestive qualities as a lipase, as well as an amylase (Barnes et al., 1994). Oats are also very sustainably sourced and easy to obtain, potentially lowering costs of production and distribution. Finally, the peel of mangoes contains an amylase that has been found to have antidiabetic effects, when introduced to the digestive system that are comparable to the same therapeutic levels of the commonly prescribed diabetic medication Metformin (Gondi & Rao, 2015). At least 50% of patients with CF develop cystic fibrosis related diabetes (CFRD) by the age of 18, and as patients age, the likelihood of developing CFRD increases. While CFRD demonstrated symptoms similar to those of Type 1 and Type 2 diabetes mellitus, it is not treatable by oral medication, and treatment relies on an insulin regime (Singh & Schwarzenberg, 2017.) The mango amylase may be able to provide support to the diabetic therapies used to impede the onset of CFRD or to help manage the symptoms and/or complications of CFRD.

Purpose

The hypothesis of this study is that the plant-based enzymes (bromelain, oat lipase, oat amylase, and mango amylase) will have significantly higher or comparable amounts of digested protein, fat, and carbohydrates compared to CF-prescribed pancreatic enzymes. As the enzymes increase in digestive efficiency, the quantity of monomers increases. Therefore, to test the efficiency of the plant-based enzymes, the quantity of monomers produced after introducing the respective digestive enzymes to the

food source was measured. Although previous studies have shown that bromelain is an effective protease for the treatment of multiple sources of pancreatic insufficiency (Rathnavelu et al., 2016), as well as oat lipase as an effective source of lipase for CF patients and oats as an amylase source (Smith & Bennett, 1974) and mango amylase as an effective amylase with antidiabetic properties (Gondi & Rao, 2015), the effectiveness of the enzymes as a complete pancrelipase source needs to be tested. Using all three plant-based sources as a pancrelipase source is the initial step to developing a complete plant-based alternative pancrelipase therapy.

Method

Sample Preparation

Samples were prepared using 100% cashew milk, *Anacardium occidentale,* as a nutritionally dense food source and a significant source of all three macromolecules being tested: with ~4g protein, ~10g fat and ~8g carbohydrate (Ahmad et al., 2017). A 1:3 ratio of cashew milk to distilled water was used to dilute the milk to adhere to the sample quality standard for accurate spectrophotometric results. Bromelain (raw pineapple leaves and stalk) was blended with distilled water with a 1:2 ratio. The mixture was then strained through a cheesecloth to remove solids. Oat lipase and amylase were prepared by creating a 1:3 ratio of finely ground oats diluted with distilled water. The mango amylase was prepared by blending the peel of a ripe mango, with distilled water creating a 1:2 ratio. The mango amylase mixture was then also strained through a cheesecloth to remove solids. CF-prescribed pancreatic enzymes (Rx), Pertzye 24,000 USP Supplemental Pancreatic Enzyme known as pancrelipase, was prepared by dissolving 1 capsule (1 dosage) in 60 mL distilled water.

A Venier UV-VIS spectrophotometer in combination with chemical reagents was used to measure the quantity of monomers produced by the enzymatic reactions between the enzymes and the food source. A spectrophotometer uses a beam of light through a small transparent curvet containing the sample to measure the absorbency at predetermined wavelengths in nanometers. The chemical reagents bond to the monomers and a chemical reaction causes a color change of the sample. Each reagent has a peak(s) where the most activity is expected to be found. Bradford reagent binds to amino acids produced when protein digestion occurs to measure protease efficiency at a peak of 594 nm. Rhodamine reagent binds to fatty acids produced when fat digestion occurs to measure lipase efficiency at a peak of 532 nm. Lugol's Iodine measures simple starches produced when starch digestion occurs to measure the efficiency of starch amylase at a peak of 620 nm.

Twenty-four tests were prepared containing 60 µL cashew milk as the source of the respective macromolecule (see Table 1), as well as 60 µL of the enzyme being tested, and 3 mL of each sample's respective reagent (see Figure 1). There were also samples used of each reagent by itself, the cashew milk by itself, the Rx pancrelipase by itself and each individual enzyme by itself, to serve as control.

Data Collection

A blank curvet was used to calibrate the spectrophotometer using distilled water. Once the machine was properly calibrated, 60µL of each solution was added to the curvet and measured on the spectrophotometer. Data at the peak wavelength for the reagent used was recorded. Each sample category trial was conducted three times to ensure precision and accuracy. The absorbance of the samples containing protein, Rx protease, and bromelain protease combinations were recorded using Bradford reagent at 594 nm light wavelength. The absorbance of the samples containing fat, Rx lipase, and oat lipase combinations were recorded using Rhodamine 6g reagent at 532 nm light wavelength. The absorbance of the samples containing carbohydrate, Rx amylase, and mango amylase combinations were recorded using iodine reagent at 620 nm light wavelength. The absorbance of the samples containing carbohydrate, Rx amylase, and oat amylase combinations were recorded using iodine reagent at 620 nm light wavelength.

Results

The absorbance rate was significantly different between fat, lipase Rx, and oat lipase using rhodamine reagent at light wavelengths of 488 nm (1-way ANOVA, $F(6,14) = 139.62$, $p < .001$). The absorbance was significantly higher in Rhodamine + fat, Rhodamine + fat + lipase Rx, Rhodamine + fat + oat lipase, and Rhodamine + fat + lipase Rx + oat lipase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$) (see Figure 3). The absorbance rate was significantly different between fat, lipase Rx, and oat lipase using rhodamine reagent at light wavelengths of 510-514.5 nm (1-way ANOVA, $F(6,35) = 493.05$, $p < .001$). The absorbance was significantly higher in Rhodamine + fat, Rhodamine + fat + lipase Rx, Rhodamine + fat + oat lipase, and Rhodamine + fat + lipase Rx + oat lipase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$) (see Figure 4). The absorbance rate was significantly different between fat, lipase Rx, and oat lipase using rhodamine reagent at light wavelengths of 532 nm (1-way ANOVA, $F(6,14) = 81.46$, $p < .001$). The absorbance was significantly higher in Rhodamine + fat, Rhodamine + fat + lipase Rx, Rhodamine + fat + oat lipase, and Rhodamine + fat + lipase Rx + oat lipase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$) (see Figure 5). The absorbance rate was significantly different between carbohydrate, amylase Rx, and mango amylase using Iodine reagent at light wavelengths of 620 nm (1-way ANOVA, $F(6,14) = 221.51$, $p < .001$). The absorbance was significantly higher in iodine + carbohydrate, iodine + carbohydrate + mango amylase, iodine + carbohydrate + amylase Rx, and iodine + carbohydrate + amylase Rx + mango amylase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$) (see Figure 6). The absorbance rate was significantly different between carbohydrate, amylase Rx, and oat amylase using Iodine reagent at light wavelengths of 620 nm (1-way ANOVA, $F(6,14) = 224.81$, $p < .001$). The absorbance was significantly higher in iodine + carbohydrate, iodine + carbohydrate + oats amylase, iodine + carbohydrate + amylase Rx, and iodine + carbohydrate + amylase Rx + oats amylase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$) (see Figure 7). The absorbance was significantly different between protein, protease, and bromelain combinations using Bradford reagent at light wavelengths of 594 nm (1-way ANOVA, $F(6,14) = 90.83$, $p < .001$). The absorbance was significantly higher in Bradford + protein, Bradford + protein + protease Rx, Bradford +

protein + bromelain, and Bradford + protein + protease Rx + bromelain than the other treatments; Bradford + protein was significantly lower than Bradford + protein + protease Rx + bromelain (Tukey-Kramer post hoc test, k = 7, df = 14, α = .05) (see Figure 8). The absorbance was significantly different between protein, protease, and bromelain combinations using Bradford reagent at light wavelengths of 593-597 nm (1-way ANOVA, $F(9,60) = 7061.08$, $p < .001$). The absorbance was significantly different between all treatments except protein + protease. Protein + bromelain, and protein + 1/2 bromelain; protein + protease + bromelain had significantly higher absorption than any of the other treatments (Tukey-Kramer post hoc test, k = 10, df = 60, α = .05) (see Figure 9).

Discussion

When comparing the data coupled with the statistical analysis, this study found the plant-based enzymes all had significantly higher amounts of fats, proteins, and carbohydrates digested when compared to the prescribed pancreatic enzymes (Rx). The plant-based enzymes from pineapple bromelain, oats, and mango have equal or higher amounts of the proteins, fats, and carbohydrates digested in cashew milk than the prescription pancreatic enzymes (see Figures 3-9). Plant-based enzymes may be an alternative to the current enzyme supplementation for patients with pancreatic insufficiencies, including CF patients. In previous studies, bromelain was found to have anti-inflammatory properties and anti-microbial properties in the intestinal track, resulting in lower amounts of fat in stool, as well as a reduction in pain (Rathnavelu et al. 2016). Bromelain is also shown to aid in the effectiveness of antibiotic and other medical treatment of common serious cystic fibrosis complications including but not limited to pneumonia, bronchitis, tuberculosis, *Staphylococcus aureus* infections, sepsis, *Escherichia coli*, and intestinal parasites (Rathnavelu et al., 2016). Ongoing pancrelipase using this source of bromelain may potentially help to prevent or lessen the onset of these complications with the addition of medication. Bromelain was also reported to have an alkalinizing effect when introduced to the digestive track (Rathnavelu et al. 2016); this may be advantageous because of the lack of bicarbonate released by the duodenum in CF patients.

Oats as an affective source of lipase and amylase are shown to be stable in acidic environments such as that of the highly acidic cystic fibrosis digestive tract. This may result in more effective digestion and absorption of fats and carbohydrates, resulting in less malnourishment and overall better prognosis of related complications (Gondi & Rao, 2015). The peel of mangos has antidiabetic properties that have been found to be comparable to metformin in lowering blood glucose levels (Gondi & Rao, 2015). This is useful because many CF patients develop cystic fibrosis-related diabetes (CFRD). The results of this study indicate that these plant-based enzymes may be a viable alternative to supplemental protease, lipase, and amylase enzymes currently prescribed to CF patients. The plant-based enzymes may be a more desirable option for religious or dietary practices as opposed to most prescribed pancreatic supplemental enzymes that are derived from swine origins. The nature of plant-based enzymes as opposed to animal-based enzymes likely eliminates the risk of alpha-gal syndrome reactions as well as the risk of contracting an animal derived pathogen. Studies have shown that malnourishment has a direct correlation to lung function and overall health in CF patients (Singh & Schwarzenberg, 2017). Having alternative options for supplemental enzymes along with the added benefits of the plant-based

enzymes investigated in this study may enable more patients with CF to thrive. A previous trial conducted by the authors found the efficiency of protein digestion increased as the quantity of bromelain increased (Lowe & Spring, 2021). Further research will investigate appropriate dosage of plant-based enzymes and their efficiencies with additional food sources.

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Table 1

Prepared Solutions with Their Respective Reagents

Bradford Reagent	Rhodamine 6G Reagent	Lugol's Iodine	Lugol's Iodine
protein (cashew milk)	fat (cashew milk)	carbohydrate (cashew milk)	carbohydrate (cashew milk)
Rx (protease)	Rx (lipase)	Rx (amylase)	Rx (amylase)
bromelain (protease)	oats (lipase)	oats (amylase)	mango (amylase)
protein + Rx	fat + Rx	carbohydrate + Rx	carbohydrate + Rx
protein + bromelain	fat + oats	carbohydrate + oats	carbohydrate + mango amylase
protein + Rx + bromelain	fat + Rx + oats	carbohydrate + Rx + oats	carbohydrate + Rx + mango amylase

Figure 1

Test Tubes Containing Prepared Solutions with Their Respective Reagents

Figure 2

Absorbance of Protein with Bromelain Protease Using Bradford Reagent at 594 nm Light Wavelength

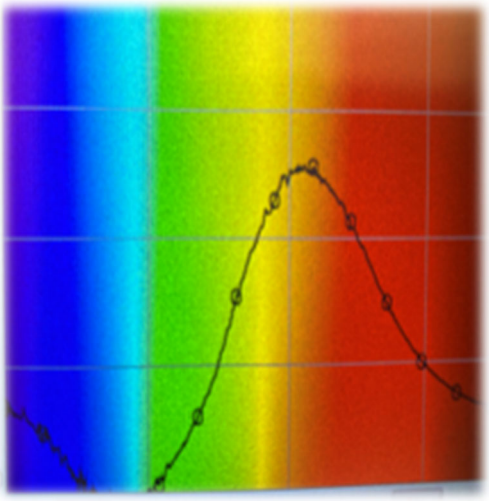
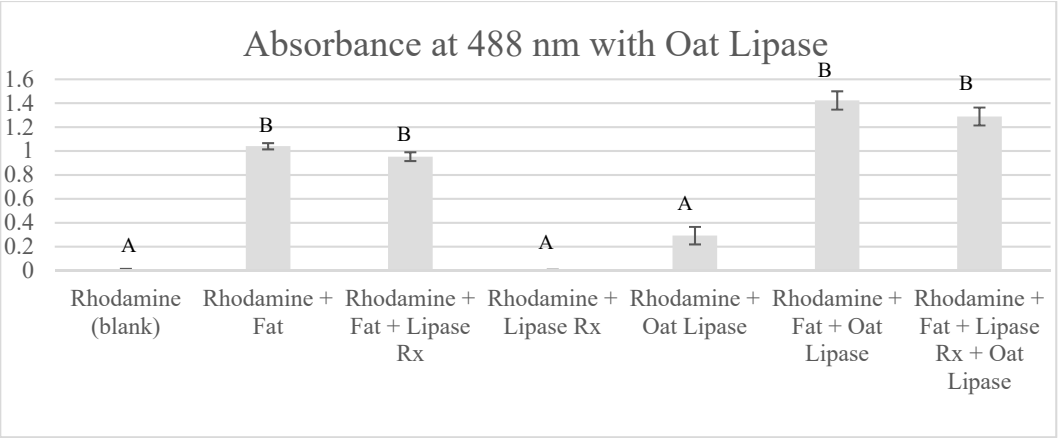


Figure 3

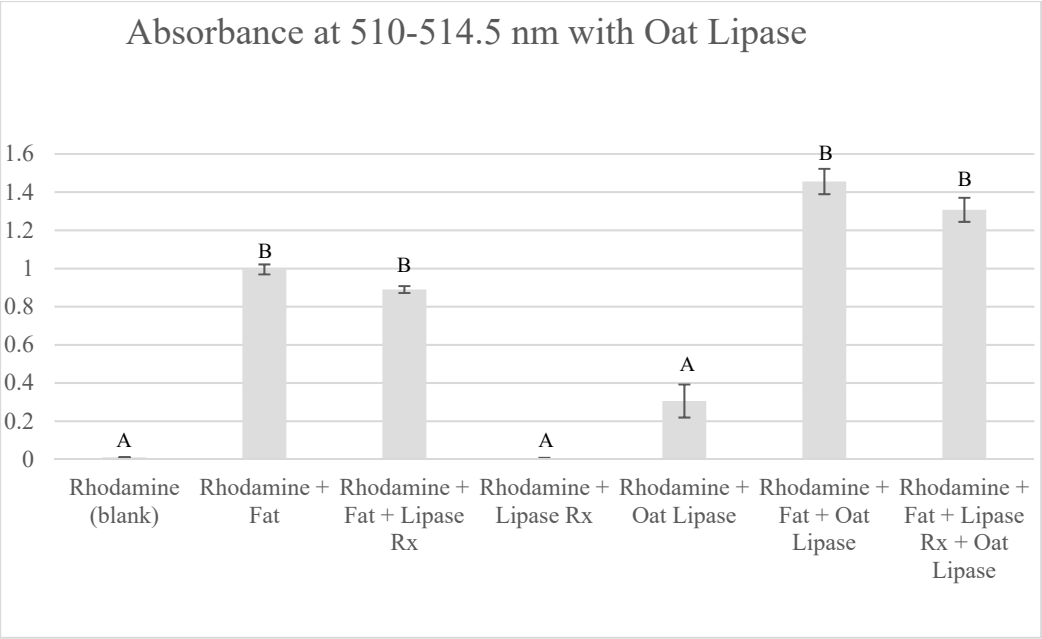
Absorbance of Fat, Lipase Rx, and Oat Lipase Combinations Using Rhodamine Reagent at Light Wavelengths of 488 nm (n = 3, mean ± SD)



The absorbance rate was significantly different between fat, lipase Rx and oat lipase using rhodamine reagent at light wavelengths of 488 nm (1-way ANOVA, $F(6,14) = 139.62$, $p < .001$). The absorbance was significantly higher in Rhodamine + fat, Rhodamine + fat + lipase Rx, Rhodamine + fat + oat lipase, and Rhodamine + fat + lipase Rx + oat lipase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$).

Figure 4

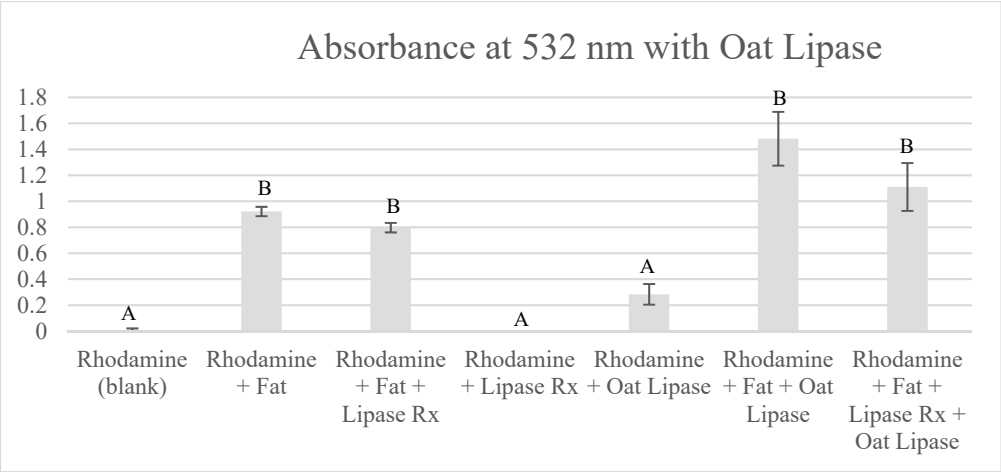
Absorbance of Fat, Lipase Rx, and Oat lLipase Combinations Using Rhodamine Reagent at Light Wavelengths of 510-514.5 nm (n = 6, mean ± SD)



The absorbance rate was significantly different between fat, lipase Rx and oat lipase using rhodamine reagent at light wavelengths of 510-514.5 nm (1-way ANOVA, $F(6,35) = 493.05$, $p < .001$). The absorbance was significantly higher in Rhodamine + fat, Rhodamine + fat + lipase Rx, Rhodamine + fat + oat lipase, and Rhodamine + fat + lipase Rx + oat lipase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$).

Figure 5

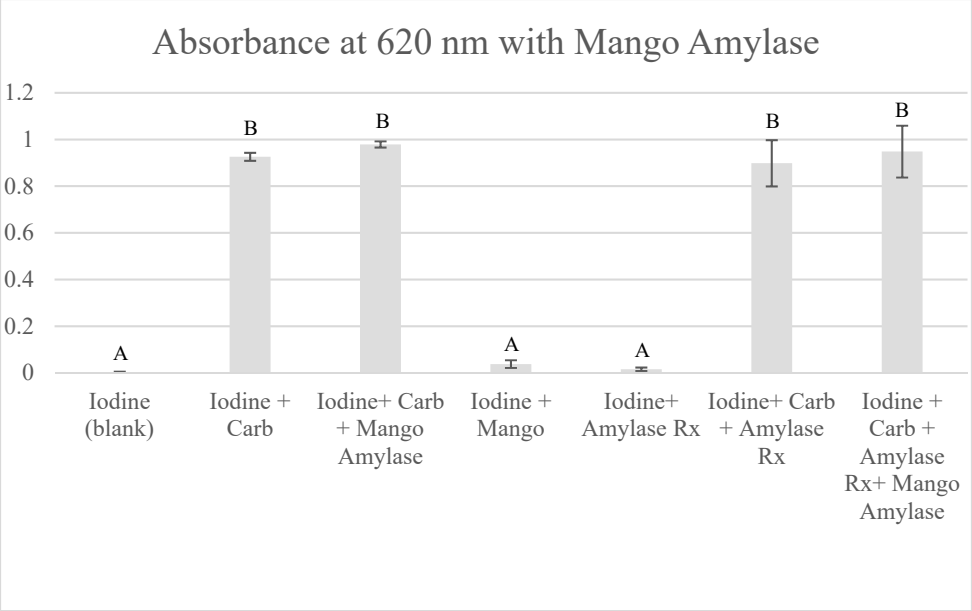
Absorbance of Fat, Lipase Rx, and Oat Lipase Combinations Using Rhodamine Reagent at Light Wavelengths of 532 nm (n = 3, mean ± SD)



The absorbance rate was significantly different between fat, lipase Rx, and oat lipase using rhodamine reagent at light wavelengths of 532 nm (1-way ANOVA, $F(6,14) = 81.46$, $p < .001$). The absorbance was significantly higher in Rhodamine + fat, Rhodamine + fat + lipase Rx, Rhodamine + fat + oat lipase, and Rhodamine + fat + lipase Rx + oat lipase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$).

Figure 6

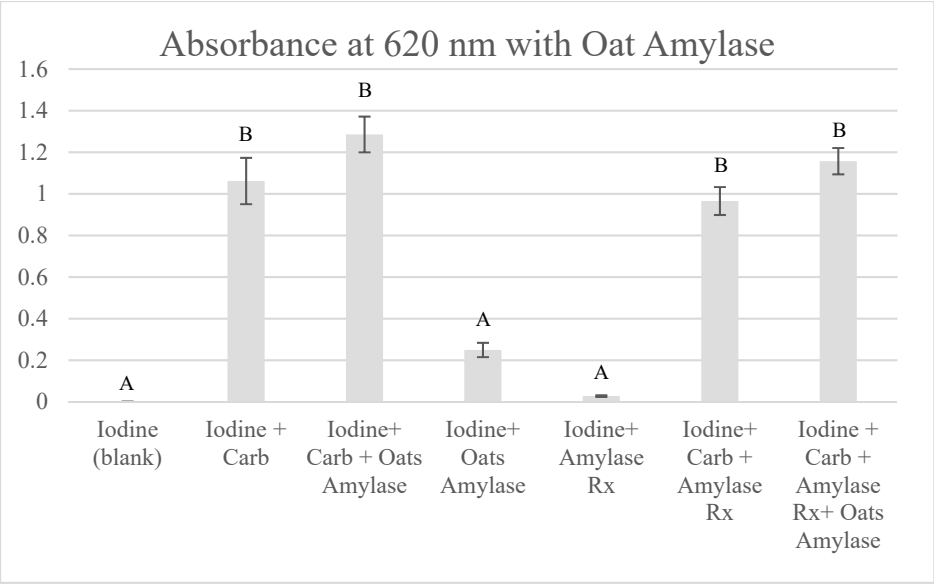
Absorbance of Carbohydrate, Rx Amylase, and Mango Amylase Combinations Using Iodine Reagent at Light Wavelengths of 620 nm (n = 3, mean ± SD)



The absorbance rate was significantly different between carbohydrate, amylase Rx and mango amylase using Iodine reagent at light wavelengths of 620 nm (1-way ANOVA, $F(6,14) = 221.51$, $p < .001$). The absorbance was significantly higher in iodine + carbohydrate, iodine + carbohydrate + mango amylase, iodine + carbohydrate + amylase Rx, and iodine + carbohydrate + amylase Rx + mango amylase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$).

Figure 7

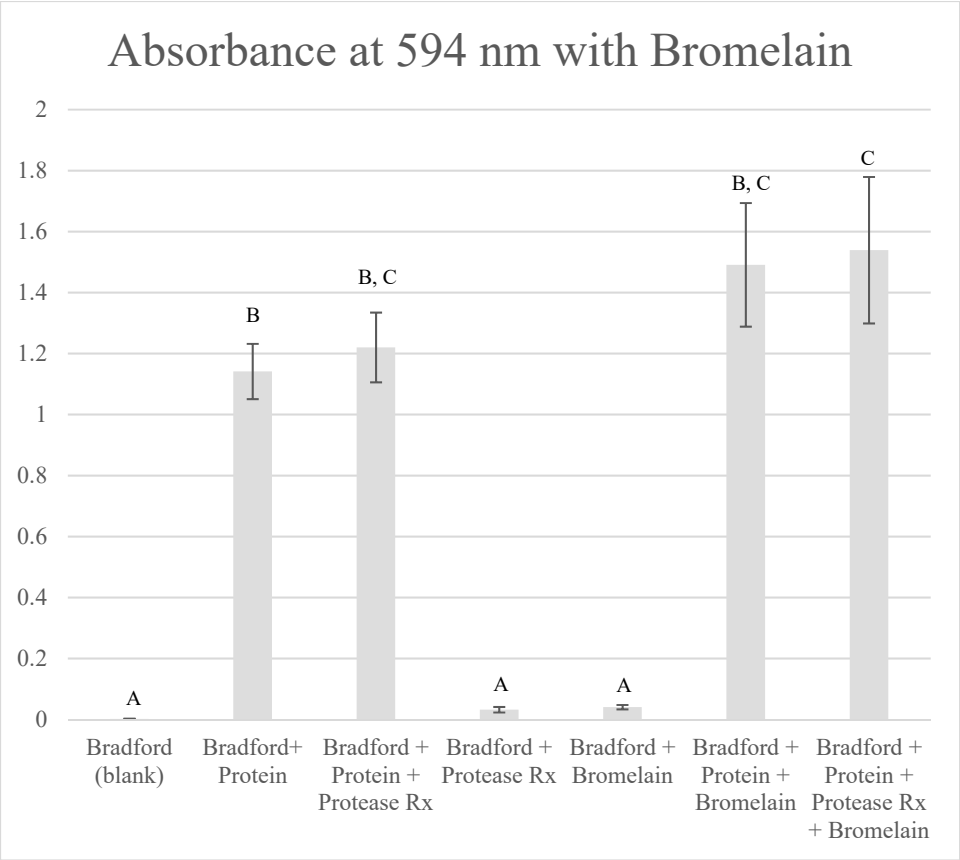
Absorbance of Carbohydrate, Rx Amylase, and Oat Amylase Combinations Using Iodine Reagent at Light Wavelengths of 620 nm (n = 3, mean ± SD)



The absorbance rate was significantly different between carbohydrate, amylase Rx and oat amylase using Iodine reagent at light wavelengths of 620 nm (1-way ANOVA, $F(6,14) = 224.81$, $p < .001$). The absorbance was significantly higher in iodine + carbohydrate, iodine + carbohydrate + oats amylase, iodine + carbohydrate + amylase Rx, and iodine + carbohydrate + amylase Rx + oats amylase than the other treatments (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$).

Figure 8

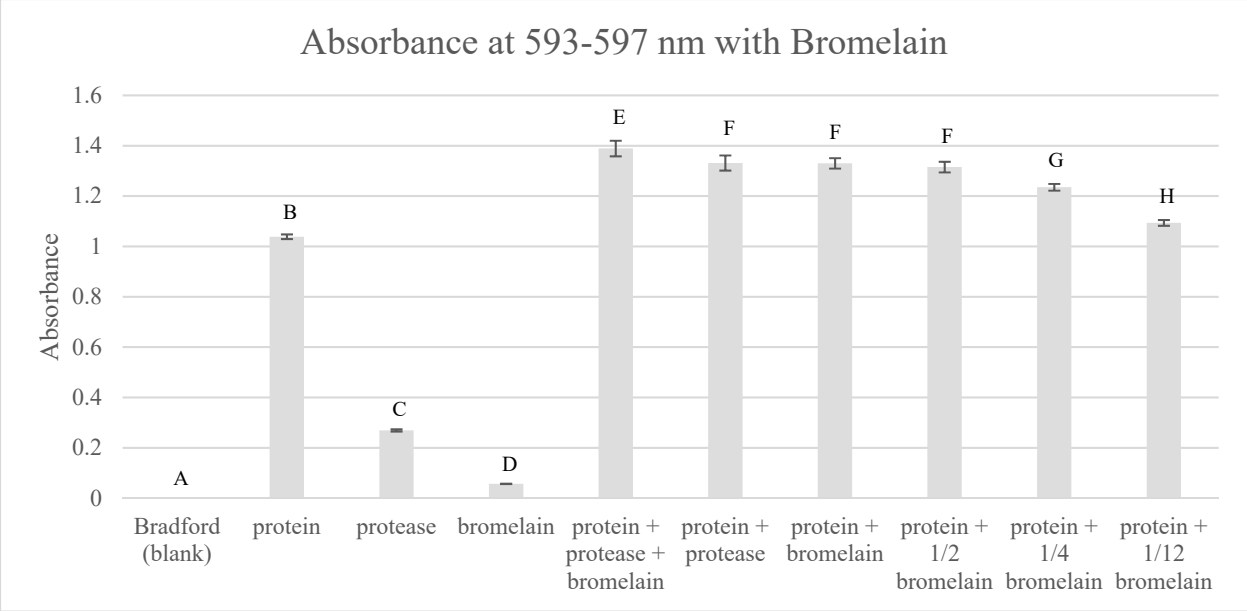
Absorbance of Protein, Rx Protease and Bromelain Protease Combinations Using Bradford Reagent at Light Wavelengths of 594 nm (n = 3, mean ± SD)



The absorbance was significantly different between protein, protease, and bromelain combinations using Bradford reagent at light wavelengths of 594 nm (1-way ANOVA, $F(6,14) = 90.83$, $p < .001$). The absorbance was significantly higher in Bradford + protein, Bradford + protein + protease Rx, Bradford + protein + bromelain, and Bradford + protein + protease Rx + bromelain than the other treatments; Bradford + protein was significantly lower than Bradford + protein + protease Rx + bromelain (Tukey-Kramer post hoc test, $k = 7$, $df = 14$, $\alpha = .05$).

Figure 9

Absorbance of Protein, Protease, and Bromelain Combinations Using Bradford Reagent at Light Wavelengths of 593-597 nm (n = 7, mean ± SD)



The absorbance was significantly different between protein, protease, and bromelain combinations using Bradford reagent at light wavelengths of 593-597 nm (1-way ANOVA, $F(9,60) = 7061.08$, $p < .001$). The absorbance was significantly different between all treatments, except protein + protease, protein + bromelain, and protein + 1/2 bromelain were not significantly different; protein + protease + bromelain had significantly higher absorption than any of the other treatments (Tukey-Kramer post hoc test, $k = 10$, $df = 60$, $\alpha = .05$).