ABSTRACT

MALONEY, KATHERINE PATTERSON. Sweetpotato Peel Proteins: Extraction Optimization and Susceptibility to Digestive Enzymes. (Under the direction of Dr. Jonathan Allen.)

North Carolina is the leading producer of sweetpotatoes in the United States. In 2009, 21% of the 679,780,240 pounds of sweetpotatoes that were produced in North Carolina were processed before entering the market. Generally, one of the first steps in processing is removal of the peel. The peel is considered a waste product, but contains potentially valuable compounds, such as proteins. The first objective of this research was to optimize a process for extracting proteins from industrial peel waste. Material from the primary peeling of raw roots and from a secondary peeling after blanching was obtained from a local sweetpotato processing facility. Peel was mixed with saline solvent to dissolve proteins, and then the mixture was centrifuged to separate non-soluble material. After the proteins were precipitated from the supernatant with $CaCl_2$, centrifugation was used to obtain a protein pellet. Linear segmented and quadratic models were used to optimize peel to solvent ratio, NaCl concentration, and $CaCl_2$ concentration. More proteins could be extracted from secondary peelings than primary peelings. The highest recovery, 32.0%, was obtained by mixing 1 g of secondary peelings with 59.7 mL of 0.025 mM NaCl and then precipitating with 6.8 mM $CaCl_2$. The protein banding pattern and glycosylation characteristics of the extract were similar to Caiapo, a commercial anti-diabetic supplement containing sweetpotato proteins.

In order to exhibit systemic effects, a protein must survive gastric and duodenal digestion, and then be absorbed. Sweetpotato proteins have been reported to possess anti-diabetic,

antioxidant, and anti-proliferative properties, but the mechanism by which the proteins evade digestion is unknown. The second objective of this research was to determine the susceptibility of sweetpotato proteins to digestive enzymes. Caiapo, an extract from primary peelings, and an extract from secondary peelings were incubated with pepsin, trypsin, and chymotrypsin. Samples were removed throughout the digestion procedure and protein breakdown was visualized with SDS-PAGE. Samples were also assayed for amylase activity and amylase inhibitory activity after incubation with pepsin. Proteins were present in all of the samples that were resistant to digestion by pepsin, trypsin, and chymotrypsin. The extract from secondary peelings exhibited lower resistance to pepsin than Caiapo and the extract from primary peelings. Compact structure is most likely responsible for the noted resistance to digestion, since the amino acid sequence of the major storage protein in sweetpotatoes, sporamin, showed numerous potential cleavage sites. In addition, heat treatment, which would cause denaturation, increased susceptibility of the protein to digestion. Trypsin inhibitors remained active after simulated gastric digestion, with the Caiapo and extract from primary peelings exhibiting higher inhibitory activity compared to the extract from secondary peelings. Active amylase and chymotrypsin inhibitors were not found in any of the samples after digestion. Modified glucose tolerance tests in rats confirmed the lack of digestion-resistant amylase inhibitors, and showed that sweetpotato proteins do not alter maltose digestion or glucose absorption.

Sweetpotato Peel Protein: Extraction Optimization and Susceptibility to Digestive Enzymes

by
Katherine Patterson Maloney

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APPROVED BY:

_____	_____
Dr. Jonathan Allen Committee Chair	Dr. Van-Den Truong
_____	_____
Dr. Gisele Passador-Gurgel	Dr. Sophia Kathariou

DEDICATION

To my parents and my husband.

BIOGRAPHY

Katherine Patterson Maloney was born and raised in Raleigh, NC. She received her B.S. in Biological Sciences and M.S. in Nutrition and Food Science from NCSU. Upon completion of her Ph.D. in Food Science, she will begin work at Novozymes.

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CHAPTER 1

Literature Review

1.1 Sweetpotato production in North Carolina

In 2008, North Carolina harvested more acres of sweetpotatoes than any other state (figure 1.1). During this year, North Carolina produced 8,740,000 cwt of sweetpotatoes and the US per capita consumption was reported at 5.1 sweetpotatoes. The counties that produced the most sweetpotatoes in North Carolina in 2008 were Nash, Sampson, Johnston, Wilson, Edgecombe, Wayne, Columbus, Greene, Cumberland, and Duplin (figure 1.2). In 2009, 87% of the sweetpotatoes planted in North Carolina were the Covington cultivar and 10% were the Beauregard cultivar, both of which are orange-fleshed varieties. The remaining 3% consisted of other orange-fleshed cultivars, and some white-fleshed and purple-fleshed cultivars (figure 1.3) *(1)*.

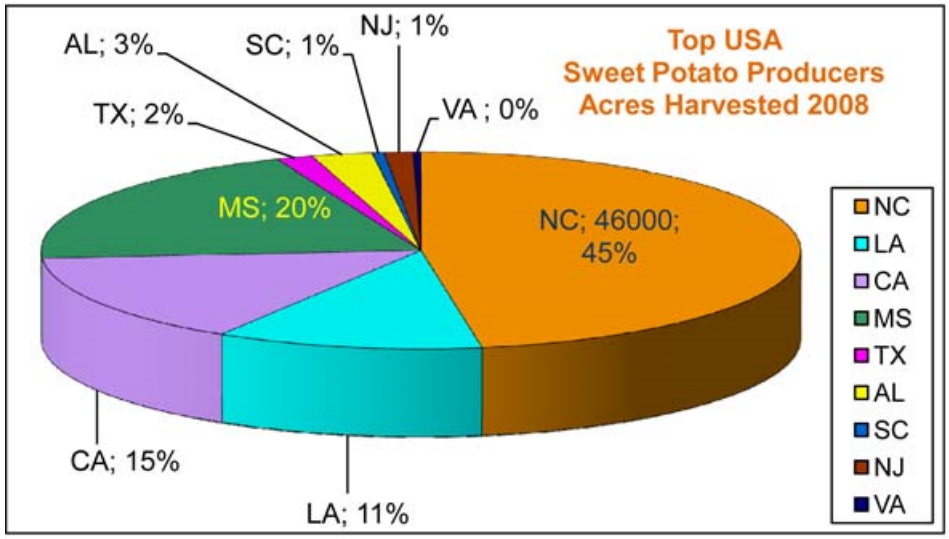

Figure 1.1. Percentage harvested by top 9 states of total sweetpotato acres in the US in 2008 *(1)*.

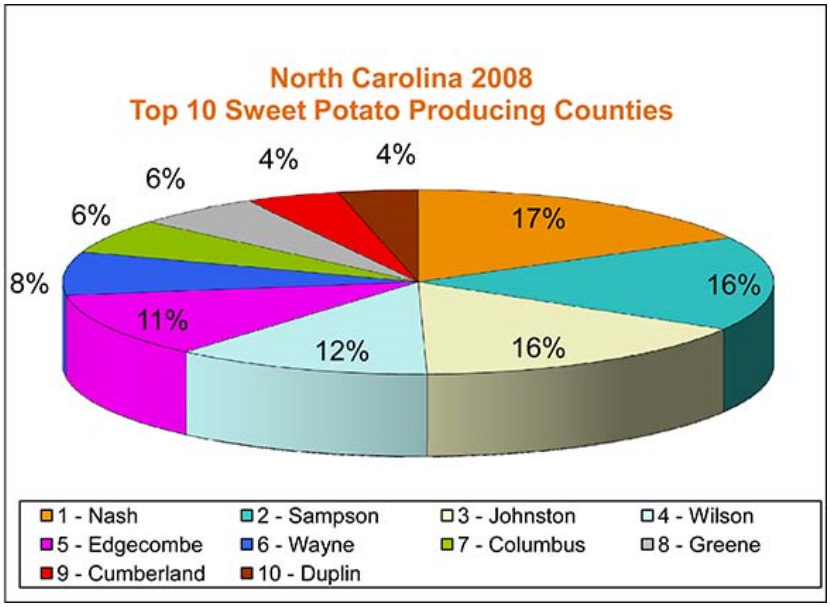

Figure 1.2. Percentage produced by top 10 counties of total sweetpotatoes produced in NC *(1)*.

Figure 1.3. Sweetpotato cultivars grown in North Carolina *(1)*.

In 2009, a total of 537,821,840 pounds of North Carolina sweetpotatoes were distributed to retail establishments (70%), food service establishments (18%), and the international market (5%). Another 141,958,400 pounds of sweetpotatoes were processed into frozen product (23%), canned product (21%), chips (14%), baby food (14%), or other processed product (28%), before entering the market *(2)*.

1.2 Processing of sweetpotatoes

Sweetpotatoes are traditionally viewed as holiday cuisine, with much higher sales in November and December than any other time of year *(3)*. In recent years, however, there has been a focus on developing processing techniques that maintain flavor and texture to increase year-round consumption of sweetpotatoes. Restructured sweetpotato fries have been improved by adding alginate-calcium to increase textural consistency *(4)*, as well as, adding sodium tripolyphosphate during blanching and sweetpotato flour during mixing to increase product firmness and dry matter content *(5)*. New methods for preserving sweetpotato puree and new uses for sweetpotato puree have also been investigated. Coronel and others *(6)* used continuous flow microwave processing on sweetpotato puree from orange-fleshed varieties to obtain a shelf-stable product. This technology was licensed to, and is currently being used by Yamco, LLC in Snow Hill, NC to create orange-fleshed sweetpotato purees of superior flavor and texture, with significant storage energy savings, compared to those previously available. Continuous flow microwave processing has also been applied to sweetpotato puree from purple-fleshed varieties *(7)*. Grabowski and others *(8)* optimized the process for

spray drying sweetpotato puree to create a powder that can, compared to traditional drying methods, be more easily incorporated into other foods, further expanding the sweetpotato market. These technologies have been reviewed by Truong and Ramesh *(9)*. US per capita sweetpotato consumption data is, currently, only available up to 2008, and shows a relatively stable market *(1)*. However, recent events, such as the opening of a new plant in Delhi, LA focused on producing various processed sweetpotato products, point to consumption being on the rise *(10)*.

1.3 Sweetpotato protein quantity

The protein content of sweetpotatoes can vary substantially depending on both cultivar and growing conditions. Analysis of various cultivars grown in North Carolina showed protein content to vary from 1.73% to 9.14% on a dry weight basis *(11)*. Similar results were obtained by a study conducted in Nigeria on 49 different cultivars of sweetpotato. Protein content was found to range from 1.39% to 9.4% on a dry weight basis *(12)*. Purcell and others *(13)* found that even within a cultivar, significant differences in protein content could exist. Sweetpotatoes of the cultivar Jewel were found to vary in protein content from 3.99% to 8.81% on a dry weight basis and sweetpotatoes of the cultivar Centennial were found to vary in protein content from 5.27% to 7.24% on a dry weight basis.

Bradbury and others *(14)* found that the peel of the sweetpotato was 50-80% higher in crude protein than the flesh. Makki and others *(15)* compared the peel and pulp of two varieties of

Egyptian sweetpotatoes and found that the peel was also higher in protein than the pulp, but the difference between the two values was smaller than that found by Bradbury and others *(14)*. The peel of the cultivar Abees contained 6.7% crude protein compared to 5.0% in the pulp, and the peel of the cultivar Giza 69 contained 7.4% protein compared to 4.9% in the pulp. Purcell and others *(16)*, when comparing the distribution of protein within the sweetpotato, also noted that the peel was higher in protein than the flesh.

1.4 Sweetpotato protein quality

Tryptophan, sulfur amino acids, and lysine have been identified as limiting amino acids in different sweetpotato cultivars. Purcell and others *(11)* compared the amino acid composition of six sweetpotato cultivars grown in North Carolina to the FAO reference protein and found that tryptophan and total sulfur amino acids were limiting. An excess of other essential amino acids was present, however. Bradford and others *(14)* found sulfur amino acids to be limiting in 65% of the cultivars tested from the Highlands of Papua New Guinea. Mu and others *(17)* found lysine and tryptophan to be limiting compared to the FAO reference in the Chinese cultivar 55-2 while many of the other essential amino acids were present in excess. Walter and others *(18)* compared protein extracts from the cultivars Jewel and Centennial to casein and found that the extracts were higher in total sulfur amino acids than casein, however, both casein and the extracts were still below the FAO reference. The protein efficiency ratio of the extracts was equal to that of casein. Ravindran and others *(19)* compared the in vitro digestibility of sweetpotato protein from 16 cultivars and found an

average digestibility of 75.8% with a range of 71.4% to 79.5%. Apparent metabolizable energy for poultry was then determined and found to be similar to values for corn, 14.54 MJ/kg versus 14.45 MJ/kg.

1.5 Effect of agricultural practices on sweetpotato protein content

The effect of nitrogen fertilization on protein content of sweetpotato roots has been extensively studied. Constantin and others *(20)* found that as nitrogen levels increased, protein content increased. The study, conducted in Louisiana, used nitrogen application levels of 0 kg/ha, 33.64 kg/ha, 67.28 kg/ha, and 100.92 kg/ha. Purcell and others *(21)*, in a study conducted in North Carolina, also found that nitrogen fertilization increased protein content of roots. Application levels were similar with 0 kg/ha, 56 kg/ha, and 112 kg/ha being applied during the 1977 trial and 0 kg/ha, 51 kg/ha, and 101 kg/ha being applied during the 1978 trial. Nitrogen fertilization did not change the ratio of non-protein nitrogen to total nitrogen. A more recent study *(22)*, conducted in Nigeria, also reported an increase in protein content with nitrogen fertilization up to 80 kg/ha. Application levels tested included 0 kg/ha, 40 kg/ha, 80 kg/ha, and 120 kg/ha. Ukom and others *(22)* found that 40-80 kg/ha was sufficient for maximum protein yield in most cultivars. Application above these levels did not further increase protein content. The highest protein yield obtained was 9.84% on a dry weight basis with a nitrogen application level of 80 kg/ha, a protein content value on the higher end of the ranges reported by other groups *(11, 12)*.

The effect of irrigation and fertilization with other minerals on root protein yield has also been studied. Constantin and others *(20)* found that irrigation negatively impacted protein content. Sweetpotatoes from plots maintained at 25% and 50% soil moisture had reduced protein content (6.5% and 6.7% on a dry weight basis, respectively) compared to those from the control plot (7.9% on a dry weight basis) in which only rainfall provided moisture to the soil. Purcell and others *(21)* found that potassium and sulfur fertilization did not alter the protein content of individual roots, however, potassium application did increase total root yield and thus total protein yield.

1.6 Changes during storage of sweetpotato roots

Purcell and others *(23)* found that protein content of roots decreases with storage; however, percent protein increases because dry matter is lost at a rate twice that of protein. Zhang and others *(24)* found that dry matter and starch content of sweetpotatoes were correlated ($r = 0.92$) and that starch content decreased during storage with rates of decrease differing among cultivar. Alpha-amylase activity was found to increase during the first 2 months of storage and then decrease with continued storage back to levels observed at harvest with cultivar again affecting rate. Trypsin inhibitor activity decreased slightly in some cultivars with extended storage (> 60 days).

1.7 Characterization of sweetpotato proteins

Sporamin A and sporamin B account for 80% of the total protein content of sweetpotato roots. Both proteins migrate to a position corresponding to a molecular weight of 25 kDa under denaturing SDS-PAGE. Under non-denaturing SDS-PAGE, however, sporamin A migrates to a position corresponding to a molecular weight of 31 kDa and sporamin B migrates to a position corresponding to a molecular weight of 22 kDa. The amino acid composition and immunological properties of the sporamins are very similar but not identical. Antibodies raised against sporamin A also recognized sporamin B. Neither protein was adsorbed on a concanavalin A-Sepharose column or stained by periodic acid-Schiff staining of a gel, thus it was concluded that neither was a glycoprotein *(25)*.

1.8 Sweetpotato trypsin inhibitors

Trypsin inhibitors in sweetpotatoes have been extensively studied. Bradbury and others *(14)* found a 67-fold range in trypsin inhibitor activity, from 0.33 to 22.1 trypsin inhibitory units (TIU), among sweetpotato cultivars from the Highlands of Papua New Guinea. Results of this study failed to find a significant correlation between crude protein content and trypsin inhibitory activity ($r = 0.057$). Later work *(26)* confirmed the lack of a significant correlation between crude protein content and trypsin inhibitory activity across cultivars; however, Bradbury and others found that within cultivar, crude protein content and trypsin inhibitory activity were correlated.

Sweetpotatoes contain multiple proteins that exhibit trypsin inhibitor activity. Sugiura and others *(27)* identified trypsin inhibitors with molecular weights of 23 and 24 kDa. Obidairo and Akpochafo *(28)* isolated 10 different trypsin inhibitors from sweetpotatoes, with the most active inhibitors having molecular weights of 12, 10, and 9.3 kDa. Hou and others *(29)* identified proteins with molecular weights of 73, 38, and 22 kDa as trypsin inhibitors. Jaw and others *(30)* purified a trypsin inhibitor with a molecular weight of 23 kDa. Trypsin inhibitors had no effect on chymotrypsin and pepsin activity *(27)*. Obidairo and others *(28)* showed that maximum inhibitor activity was attained with pH values between 7.5 and 8.5, and Sugiura and others *(27)* found that sweetpotato trypsin inhibitors are stable over a wide pH range. Incubation at pH values between 2 and 11 for 12 hours before assaying for inhibitor activity at pH=8 did not result in significant loss of activity. Hou and others *(31)* identified a native 64 kDa aspartic protease that was capable of degrading trypsin inhibitors.

Processing of sweetpotatoes can greatly affect the activity of trypsin inhibitors. Obidairo and Akpochafo *(28)* found that boiling sweetpotatoes for 40 minutes resulted in complete inactivation of trypsin inhibitors. Kiran and others *(32)* found microwave baking to be the most effective method for inactivating trypsin inhibitors in sweetpotatoes followed by boiling and then oven drying. Microwave baking for 180 seconds resulted in complete inactivation of trypsin inhibitors while boiling for 30 minutes resulted in 17-31% residual activity. Trypsin inhibitors in oven dried sweetpotato chips were relatively stable for 2 hours at 70°C, with 80-90% residual activity. After 2 hours, however, inactivation progressed at a more

rapid rate, with less than 20% activity remaining after 24 hours. Higher temperatures also led to more rapid inactivation, with inactivation complete after 4 hours at 100°C. Minor variations in thermostability were seen for different cultivars. The researchers also compared the effect of different flour preparation methods on residual trypsin inhibitor activity. Flour was prepared by drying either sweetpotato chips, grated sweetpotato pieces, or ground sweetpotato pieces at 70°C for 24 hours and then powdering. All methods resulted in 5-12% residual activity with no statistically significant differences between methods being noted. Zhang and Corke *(33)* found that moist heat treatment provided better inactivation of trypsin inhibitors than dry heat treatment. Dry heat treatment at 60, 80, and 100°C for 15 minutes resulted in average residual activities of 92, 84, and 71%, respectively, where as moist heat treatment at 60, 80, and 100°C for 15 minutes resulted in average residual activities of 71, 26, and 5%, respectively. Similarly to Kiran and others, Zhang and Corke found that the trypsin inhibitors of some cultivars were more heat resistant than others.

1.9 Sweetpotato amylase inhibitors

Shivaraj and others *(34)* found that sweetpotato protein exhibited high amylase activity with an average of 480 units/mg protein and a range of 274-758 units/mg protein. After heating the extract for 10 minutes at 80°C to destroy native amylase activity, amylase inhibitory activity was assayed via the dinitrosalycylic acid method. No amylase inhibitors were found in the sweetpotato extract. Rekha and others *(35)* found that native amylase activity remained after heating for 10 minutes at 80°C and thus used trichloroacetic acid to

selectively precipitate the amylases before assaying the extract for amylase inhibitory activity via the iodine binding method. Of the 100 cultivars studied, amylase inhibitors were found in 79.

Rekha and others *(36)* later studied the effect of processing on native amylase inhibitors in sweetpotatoes and found that cultivar played a significant role in the stability of the inhibitors. Boiling sweetpotato pieces in water for 30 minutes resulted in residual amylase inhibitor activities of 29.3 ± 1.1% (cultivar RS III), 29.1 ± 1.1% (cultivar S 62), 44.6 ± 1.9% (cultivar S 56-2), and 58.9 ± 0.7% (cultivar S 1195). Microwave baking resulted in complete amylase inhibitor inactivation in the S 62 cultivar after 120 seconds and the S 1195 cultivar after 180 seconds. Residual amylase inhibitor activities of 29.1 ± 1.1% and 19.2 ± 0.6% remained after 180 seconds in the cultivars RS III and S 56-2, respectively. Grating or blending, oven drying at 70°C for 24 hours, and then powdering to obtain flour resulted in complete inactivation of amylase inhibitors in all cultivars tested.

1.10 Antioxidant activity of sweetpotato proteins

Various antioxidant functions have been reported for sweetpotato proteins. Trypsin inhibitors isolated from sweetpotatoes were shown to possess DHA reductase and MDA reductase activities *(37)*. A 33 kDa trypsin inhibitor was isolated with preparative SDS-PAGE and found to possess scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical *(38)*. This 33 kDa trypsin inhibitor was later shown to possess

glutathione peroxidase-like activity *(39)*. Further studies showed that the trypsin inhibitors were able to prevent Cu^{2+}-induced human LDL peroxidation and hydroxyl radical-induced DNA damage of calf thymus in vitro. Degree of protection was dependent on the cultivar from which the trypsin inhibitors were isolated. Trypsin inhibitors isolated from the cultivar Tainong 65 had 3-fold greater activity against LDL peroxidation but trypsin inhibitors isolated from the cultivar Tainong 57 showed 10-fold greater hydroxyl radical scavenging ability. Hydrolysis with pepsin and chymotrypsin increased the scavenging activity of the trypsin inhibitors against DPPH, indicating that digestion would not eliminate the antioxidant properties *(40)*.

1.11 Anti-diabetic properties of sweetpotatoes

Proteins isolated from a white-skinned sweetpotato cultivar have been extensively studied for their anti-diabetic properties. Kusano and others *(41)* compared the anti-diabetic efficacy of white-skinned sweetpotato (WSSP) extract to troglitazone using obese Zucker fatty rats. The WSSP was administered at 100 mg/kg/day for 8 weeks. After 8 weeks, blood insulin levels were significantly lower in the WSSP group (384 ± 97 μU/mL) and troglitazone group (394 ± 94 μU/mL) compared to the control group (753 ± 214 μU/mL). Blood triglycerides and free fatty acid (FFA) levels were reduced in both the WSSP and troglitazone groups compared to the control group. Cholesterol was not significantly different in any of the groups, however. Blood glucose and blood insulin levels after glucose loading were also

reduced in the WSSP and troglitazone groups compared to the control group. Body weight was significantly higher in the troglitazone group compared to the WSSP and control groups.

In another animal study, Kusano and others *(42)* found that oral administration of 400 mg/kg/day of WSSP to KK-Ay mice significantly reduced blood glucose levels and increased glucose tolerance compared to the control. No significant differences in body weight were found between the group receiving WSSP and the control group. After 3 weeks of treatment, the group receiving WSSP had significantly higher expression of ACRP30, indicating that the anti-diabetic mechanism of action of WSSP may be increasing secretion of adiponectin, a hormone associated with insulin sensitivity.

Ludvik and others have performed several human trials with the WSSP extract, Caiapo *(43-46)*. In their first trial *(43)*, Ludvik and others randomly divided 18 male type II diabetic patients into 3 groups, control, 2 g Caiapo per day, and 4 g Caiapo per day. The double-blind, placebo-controlled study lasted 6 weeks. A significant decrease in fasting blood glucose (from 8.3 ± 0.6 to 7.2 ± 0.4 mmol/L), total cholesterol (from 4.97 ± 0.21 to 4.45 ± 0.18 mmol/L), and LDL cholesterol (3.12 ± 0.16 to 2.72 ± 0.16 mmol/L) was noted in the 4 g treatment group after 6 weeks. No significant changes were seen in body weight or blood pressure in any of the groups. Insulin sensitivity, measured by frequently sampled intravenous glucose tolerance test (FSIGT), increased in both treatment groups. A 37%

increase was noted in the 2 g treatment group and a 42% increase was noted in the 4 g treatment group. In a second trial, Ludvik and others *(44)* again randomly divided 18 male type II diabetic patients into 3 groups, control, 2 g Caiapo per day, or 4 g Caiapo per day. In this trial, oral glucose tolerance test (OGTT) was performed in addition to FSIGT. After 6 weeks of treatment, insulin sensitivity, measured by both FSIGT and OGTT, increased significantly in the 4 g treatment group. In the 2 g treatment group, only insulin sensitivity measured by FSIGT increased significantly. Ludvik and others *(45)* then performed a larger study with 61 type II diabetic patients randomly assigned to receive either placebo or 4 g per day of Caiapo. The study lasted 12 weeks. Significant decreases were seen in fasting blood glucose (from 143.7 ± 1.9 to 128.5 ± 1.7 mg/dl), HbA_{1c} (from 7.21 ± 0.15 to 6.68 ± 0.14%), and blood glucose 2 hours after OGTT (from 193.3 ± 10.4 to 162.8 ± 8.2 mg/dl) in the treatment group while no significant changes in these parameters were seen in the control group.

In a later trial, Ludvik and others *(46)* found similar results to Kusano and others *(42)* relating to the mechanism of action of WSSP on increasing insulin sensitivity. Previous results concerning Caiapo's ability to increase insulin sensitivity, lower fasting blood glucose, and lower HbA_{1c} were also confirmed by this 5 month study involving 61 type II diabetic patients randomized to receive either 4 g Caiapo per day or placebo. After 5 months, oral glucose insulin sensitivity, measured by OGTT, increased from 293 ± 15 to 321 ± 12 $mL/m^2/min$, fasting blood glucose decreased from 138 ± 4 to 128 ± 5 mg/dl, and HbA_{1c}

decreased from 6.46 ± 0.12 to 6.25 ± 0.11%. Additional parameters that were measured included plasma adiponectin and fibrinogen. Significant increases in plasma adiponectin (from 5.97 ± 0.65 to 6.63 ± 0.70 μg/mL) and significant decreases in fibrinogen (from 3.83 ± 0.16 to 3.64 ± 0.18 mg/mL) were seen in the treatment group. Thus, increasing adiponectin secretion appears to be the mechanism by which Caiapo exerts its anti-diabetic effects. The observed decrease in fibrinogen may indicate that Caiapo possesses other nutraceutical properties and may be useful as an anti-atherosclerotic supplement, as well.

Kusano and others *(47)* attempted to isolate the component responsible for the anti-diabetic effect of WSSP. Figure 1.4 shows the steps of the isolation procedure. The anti-diabetic component remained in the inner solution during dialysis, exhibited solubility in 85% ethanol, remained in solution upon the addition of 15% trichloroacetic acid, and passed through a filter with a 30 kDa molecular weight cut-off during ultrafiltration. The molecular weight of the active component was estimated at 22 kDa with gel filtration chromatography. The component was found to be acidic with ion exchange chromatography and contained both protein and sugar, so was assumed to be a glycoprotein. Oral administration of the isolated component to db/db mice for 2 weeks resulted in decreased blood glucose and blood insulin levels compared to the control.

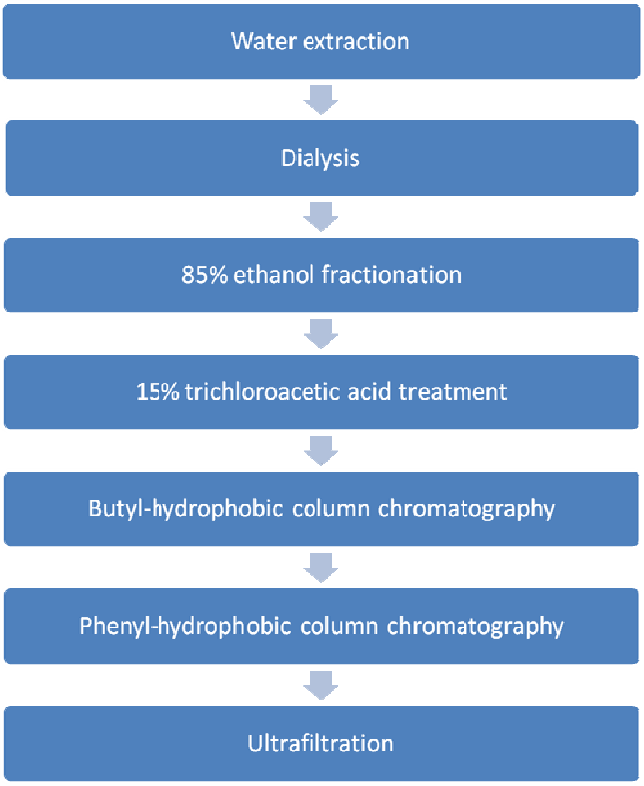

Figure 1.4. Isolation of WSSP anti-diabetic component by Kusano and others *(47)*.

A different anti-diabetic component than that isolated by Kusano and others from WSSP was later isolated by Ozaki and others *(48)*. Figure 1.5 shows the steps in the isolation procedure. The anti-diabetic component was not precipitated by ammonium sulfate. The isolated component was found to be an arabinogalactan-protein with a molecular weight of 126.8 kDa consisting of 95% (w/w) carbohydrate and 5% (w/w) protein. Oral administration of the isolated component to KK-Ay mice resulted in significantly lower fasting plasma glucose levels compared to the control group.

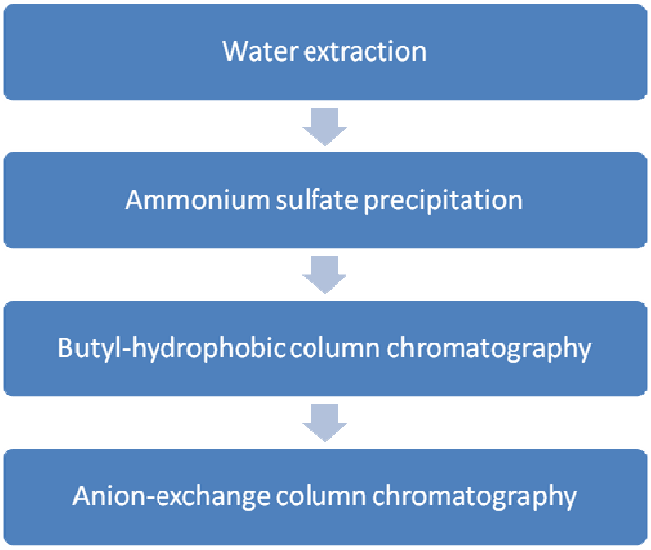

Figure 1.5. Isolation of WSSP anti-diabetic component by Ozaki and others *(48)*.

Zakir and others *(49)* compared the protein banding pattern of Caiapo to a sweetpotato cultivar commonly grown in Pakistan, White Star, and a sweetpotato cultivar commonly grown in the United States, Beauregard. The protein banding patterns of all samples were similar. The researchers also conducted a feeding trial with type II diabetics to compare the blood glucose lowering efficacy of Caiapo, White Star, and Beauregard. Blood glucose levels 2 hours after feeding were lower in the White Star and Beauregard groups compared to the glucose control. Blood glucose levels in the glucose control group were 296 ± 111.34 mg/dl, in the White Star group were 246 ± 87.07 mg/dl, in the White Star (skin) group were 216.29 ± 93.16 mg/dl, and in the Beauregard group were 257 ± 98.90 mg/dl. The addition of

Caiapo to glucose resulted in 2 hour blood glucose levels similar to the control, 301 ± 113.91 mg/dl. Corbitt *(50)* compared the glycemic index of Beauregard sweetpotatoes prepared by various methods. Steamed, baked and microwaved sweetpotato flesh were found to be medium glycemic index foods, with glycemic indices of 63 ± 8.4, 64 ± 10.0 and 66 ± 13.3, respectively. Dehydrated and raw sweet potato flesh were found to be low glycemic index foods, with glycemic indices of 40 ± 8.2 and 28 ± 7.3, respectively.

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CHAPTER 2

Chemical Optimization of Protein Extraction from Sweetpotato Peel

2.1 ABSTRACT

Proteins isolated from sweetpotato have been shown to possess anti-diabetic, antioxidant, and anti-proliferative properties. Sweetpotato peel generated during the processing of sweetpotatoes for puree currently has little market value and is a good source for protein extraction. The objective of this study was to chemically optimize the process for extracting protein from sweetpotato peel. The extraction procedure involved mixing peel with saline solvent to dissolve protein and then precipitating with $CaCl_2$. Quadratic and segmented models were used to determine the optimum NaCl concentration and peel to solvent ratio to maximize protein solubility while minimizing solvent usage. A segmented model was also used to optimize the concentration of $CaCl_2$ used for precipitation. The highest yield was obtained by mixing secondary peelings with 59.7 mL of 0.025 mM NaCl per g peel and then precipitating with 6.8 mM $CaCl_2$. SDS-PAGE comparison of the peel extract to Caiapo, a sweetpotato protein extract currently being marketed as an anti-diabetic supplement, revealed only minor differences. The results of this study show that potentially valuable protein can be extracted from peel generated during processing of sweetpotatoes and industrial costs can be minimized by using these optimum conditions.

2.2 INTRODUCTION

Proteins isolated from sweetpotato have been shown to possess many properties beneficial to human health. Caiapo, a protein extract from a white-skinned sweetpotato cultivar, has been shown to lower fasting blood glucose levels and increase insulin sensitivity in type II diabetics *(1-4)*. A 22 kDa acidic glycoprotein *(5)* and a 126.8 kDa arabinogalactan-protein *(6)* have been proposed as the active components of Caiapo. Sweetpotato trypsin inhibitor proteins have been shown to possess antioxidant properties with scavenging abilities against 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical *(7)*, and reactive nitrogen species *(8)* in vitro. Feeding sweetpotato trypsin inhibitor proteins to mice increased serum superoxide dismutase, catalase, and glutathione peroxidase activity *(9)*. Sweetpotato trypsin inhibitor proteins have also been shown to possess anti-proliferative properties, inhibiting the growth of NB4 promyeolcytic leukemia cells in vitro *(10)*. In addition to these nutritional benefits, sweetpotato proteins have been shown to possess the desirable functional properties of high water solubility and ability to stabilize emulsions over a wide pH range *(11)*.

Various solvents have been used to extract proteins from sweetpotatoes. Rekha and others *(12)* found that of the 5 buffers tested, homogenization with 0.02 M sodium phosphate buffer containing 0.3 M NaCl maximized extraction of alpha-amylase inhibitory proteins from taro. These results were then transferred to extraction of alpha-amylase inhibitory proteins from sweetpotatoes *(13)*. Shivaraj and others *(14)*, however, used distilled water to extract alpha-amylase inhibitory proteins from sweetpotatoes, and Purcell and others *(15)* and Walter and

Catignani *(16)* started their protein extractions by blending with water. Kusano and others *(5)* and Ozaki and others *(6)* also began their process to isolate the protein responsible for the anti-diabetic properties of sweetpotatoes by mixing with distilled water. Thus, the most efficient solvent for extraction of proteins from sweetpotatoes is unclear.

Various techniques for precipitating sweetpotato protein from solution once extracted have also been used. Purcell and others *(15)* employed both heat treatment and 0.5% calcium chloride while Walter and Catignani *(16)* used heat treatment and 0.1% calcium chloride to precipitate sweetpotato proteins. Peters *(17)* compared the efficiency of precipitation of sweetpotato proteins from solution with calcium chloride and ammonium sulfate and found that both methods worked equally well. The optimum concentration of calcium chloride to precipitate the maximum amount of protein while minimizing chemical usage, however, appears to be unknown.

Sweetpotato peel generated during the processing of sweetpotatoes for puree currently has little market value, but is a good source for protein extraction. The objective of this research was to chemically optimize the process for extracting proteins from sweetpotato peel. Proteins were dissolved by mixing peel with saline solvent and then precipitated with $CaCl_2$. Response surface methodology was investigated as an optimization technique; however, initial trials indicated that a linear segmented model fit the solvent to peel ratio data better than a second order polynomial model, and would thus give a better estimate for the optimum. A quadratic model was used to determine the optimum NaCl concentration to

maximize protein solubility. A linear segmented model was also used to optimize the concentration of $CaCl_2$ used for precipitation. After the optimum conditions for extraction were determined, SDS-PAGE was used to visualize the proteins present in the extract and compare the extract to Caiapo.

2.3 MATERIALS AND METHODS

2.3.1 Pilot Plant Trial

2.3.1.1 Chemicals and raw material

Calcium chloride was obtained from Sigma-Aldrich (St. Louis, MO). Sweetpotato peel was obtained from Yamco, LLC (Snow Hill, NC). Peel was from a mixture of orange-flesh cultivars including Beauregard, Jewel, and Covington. Peel was kept at -20°C until five days before use, at which time it was thawed at 4°C. Caiapo was obtained from Fuji-Sangyo Company (Japan).

2.3.1.2 Protein extraction process

Proteins were extracted by mixing 24 kg peel (wet weight) with 120 L deionized water in a pilot plant scale mixer in Schaub Hall (NCSU, Raleigh, NC). Insoluble material was removed from the mixture by pumping through a 1 mm mesh screen. The solution was

transferred to the Biomanufacturing Training and Education Center (BTEC) (NCSU, Raleigh, NC), where it was pumped into a pressurized holding tank and run through a Westfalia production-scale disc stack centrifuge (GEA Mechanical Equipment USA, Inc., Northvale, NJ) at a flow rate of 1 L per minute. The supernatant and insoluble material were retained for further analysis.

2.3.1.3 Protein precipitation process

Proteins were precipitated from the supernatant by adding 0.4% (w/v) calcium chloride under slight agitation and then centrifuging in a bench-top scale disc stack centrifuge (Alfa Laval United States, Richmond, VA) at a flow rate of 0.5 L per minute. The supernatant and precipitate were retained for further analysis.

2.3.1.4 Fraction analysis

Protein in liquid samples was determined using BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) and protein in solid samples was determined using the Kjeldahl method with 6.25 as the conversion factor. Atomic absorption was used to measure calcium content of the precipitate and the iodine-binding method was used to determine if starch was present in the precipitate.

2.3.2 Bench-top Process Optimization

2.3.2.1 Chemicals and raw material

Sodium chloride and calcium chloride were obtained from Sigma-Aldrich (St. Louis, MO). Solvents were made by adding the appropriate amount of NaCl to deionized water. Sweetpotato peel was obtained from a local processing plant (Yamco, LLC, Snow Hill, NC). Peel was from a mixture of orange flesh cultivars including Beauregard, Jewel, and Covington. Peel was obtained from two different points along the processing line, the initial peeling of the sweetpotatoes before any further processing, from here after referred to as "peel," and a secondary peeling after blanching of the sweetpotatoes, from here after referred to as "blanched peel." Upon receipt, the peel and blanched peel were freeze dried and stored at -20°C.

2.3.2.2 Protein extraction process

Proteins were extracted by mixing peel or blanched peel with saline solvent, centrifuging the mixture at 1,000 g for 5 minutes, and then vacuum filtering the supernatant through Whattman 4 filter paper. Insoluble material was discarded and protein in solution was determined using Bradford reagent (Thermo Fisher Scientific, Rockford, IL).

2.3.2.3 Protein precipitation process

Proteins were precipitated by adding calcium chloride, vortexing for 10 seconds, incubating for 15 minutes at either 25, 65, or 95°C, and then centrifuging at 1,000 g for 10 minutes to obtain a protein pellet. The supernatants were retained and protein in solution was determined using Bradford reagent (Thermo Fisher Scientific, Rockford, IL). The quantity of protein precipitated was calculated by subtracting protein in the supernatant after precipitation from protein in the supernatant before precipitation. After protein in solution was determined, the supernatants were discarded. The protein pellet was retained for further testing.

2.3.2.4 Determining optimum conditions for protein extraction

The results from each concentration of NaCl were fit to a linear segmented model using SAS (SAS, Cary, NC). The equation (1) was,

$$y = m_1 * x + b_1 \qquad x < \theta$$

$$y = m_2 * \theta + b_2 \qquad x > \theta$$

where y = protein extracted (mg), m = slope, x = solvent (mL), b = y-intercept, and θ = join point. The values obtained for protein extracted and join point were then fit to a quadratic model using SAS (SAS, Cary, NC). The equation (2) was,

$$y = \beta_1 * x^2 + \beta_2 * x + \beta_3$$

where y= protein extracted (mg) per g peel (dry weight) or join point and x = log NaCl (M). The NaCl concentration for maximum protein extraction was determined from the model with y = protein extracted (mg) per g peel (wet weight) and then join point at this NaCl concentration was determined from the model with y = join point.

2.3.2.5 Determining optimum conditions for protein precipitation

The percentage protein precipitated based on the initial protein in solution at each level of $CaCl_2$ addition was fit to a linear segmented model with SAS (SAS, Cary, NC). The equation (3) was,

$$y = m_1 * x + b_1 \qquad x < \theta$$

$$y = m_2 * \theta + b_2 \qquad x > \theta$$

where y = protein precipitated (%), m = slope, x = $CaCl_2$ (mM), b = y-intercept, and θ = join point. Optimum level of $CaCl_2$ addition was the join point since further addition beyond the join point would not increase % protein precipitated.

2.3.2.6 Comparison of Caiapo and peel extract with gel electrophoresis

Reducing SDS-PAGE was performed to compare the peel protein extract to Caiapo. Laemmli sample buffer and 10x Tris/Glycine/SDS running buffer were obtained from Bio-Rad (Hercules, CA), β-mercaptoethanol was obtained from Sigma-Aldrich (St. Louis, MO), and BenchMark pre-stained protein ladder was obtained from Invitrogen (Carlsbad, CA). Samples were run on a 15% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 200 V. One gel was stained with Imperial Protein Stain (Thermo Fisher Scientific, Rockford, IL) to view the total protein banding pattern and one gel was stained with Glycoprotein Staining Kit (Thermo Fisher Scientific, Rockford, IL) to view the glycoprotein banding pattern.

2.4 RESULTS AND DISCUSSION

2.4.1 Pilot plant trial

The mass balance for the pilot plant trial can be seen in figure 2.1. The starting material contained approximately 6.6% protein on a dry weight basis. This value is consistent with Makki and others *(18)* who found that the peel of the cultivar Abees contained 6.7% crude protein and the peel of the cultivar Giza 69 contained 7.4% crude protein. The total dry weight was 1479 ± 46 g and 98 ± 3 g of this was protein. Slightly more protein (66 ± 1 g) was lost in the insoluble material after mixing and centrifuging than was retained in the

supernatant (45 ± 4 g). Precipitation of proteins with calcium chloride from this solution followed by centrifugation resulted in a dry weight recovery of 324 ± 64 g containing 10 ± 0.4 g protein. The supernatant after precipitation contained more total protein (31 ± 3 g) than the precipitate; however, it was not in a concentrated form since the leftover liquid constituted 130 ± 2 L. The peel extract contained 3.3 ± 0.75% protein. This was lower than Caiapo, which contained 6.4% protein (table 2.1).

The method of Purcell and others *(15)* for extracting protein from whole sweetpotatoes was applied to protein extraction from sweetpotato peel by Makki and others *(18)* on the bench-top scale. Purity of the protein extract was lower for the peel than the pulp. One cultivar, Abees, yielded a product containing 33.5% protein when extraction was performed on the peel compared to a product containing 76.5% protein when extraction was performed on the pulp. Another cultivar, Giza 69, yielded a product containing 53.3% protein when extraction was performed on the peel compared to a product containing 80.9% protein when extraction was performed on the pulp. The percentage protein of the extracts produced in our pilot plant trial was much lower (3.27 ± 0.75%) than that of Makki and others *(18)*. Differences in the amount of pulp included in the peel samples could account for this discrepancy. Prior to partnering with an industrial sweetpotato processor, bench-top trials were performed with sweetpotatoes peeled by hand in our lab. Using this method, results similar to Makki and others *(18)* were obtained; extracts contained 40-60% protein (data not shown). The layer of pulp that remained attached to the peel during hand peeling was not present in the peelings obtained from the industrial peeler. Since extract protein purity has been shown to be higher

in pulp samples than peel samples *(18)*, the decrease in purity could be related to the lower amount of pulp in the industrial peelings.

Makki and others *(18)* found that the majority of the non-protein material in their peel extracts was carbohydrate, calculated by difference after determining crude fat and ash. Since iodine binding assay did not detect any starch in our extract, we assume that the majority of the non-protein material was non-starch polysaccharides. Due to the disappointing yield and product purity obtained from the pilot plant trial, bench-top work was undertaken to chemically optimize the process.

2.4.2 Bench-top optimization of the extraction process

The method chosen to optimize the extraction process involved fitting data to linear segmented and quadratic models despite the recent popularity of the response surface method to optimize protein extraction processes *(19-25)*. The response surface method is often chosen for optimization experiments because of the ability to obtain a vast amount of information from a small number of experiments and the ability to determine how the interaction of variables affects the response. However, a major limitation of the response surface method is that the data must be fit to a second order polynomial *(26)*. In this research, the fit limitation outweighed the advantages of using the response surface method. A second order polynomial fit for NaCl concentration versus protein extracted was able to be obtained by using a log transformation, however, solvent to solute ratio could not be

adequately fit to a second order polynomial. At a certain point, protein extracted remained constant despite continued addition of solvent, and determination of this point was important because it represented the most efficient solvent to solute ratio, i.e. protein extraction was maximized while solvent usage, and thus cost, was minimized. While the trend of protein recovery from sweetpotatoes at different ratios of solvent to solute could not be well explained by a second order polynomial model, the effect of solvent to solute ratio on protein recovery in other processes may be explained by a second order polynomial. For example, some protein extraction raw materials contain compounds that dissolve as solvent is increased, bind with protein, and lower total protein recovery. In this case, solvent to solute ratio versus protein recovery may fit well to a second order polynomial because as solvent is first increased, protein recovery will increase, a maximum will be reached, and then protein recovery will start to decrease as additional solvent is added and other compounds are extracted that bind with the protein and make it unavailable *(20, 27)*.

The results for proteins extracted over different solvent to peel and blanched peel ratios were fit to a linear segmented model for each of the NaCl concentrations tested. Figures 2.2 and 2.3 show the models fit to the data obtained for raw peel mixed with 0.001, 0.01, 0.1, and 1 mM NaCl and blanched peel mixed with 0.001, 0.01, 0.1, and 1 mM NaCl, respectively. A point was seen for each solvent in which increasing solvent volume no longer increased protein extracted. This point was termed the join point because it is the point where the two lines of the segmented model cross. The join point was important in this research because it

represents the optimum ratio of solvent to peel since addition of solvent beyond this point does not increase yield but adds to cost.

In order to determine the true optimum NaCl concentration rather than just the optimum of the NaCl concentrations tested, a quadratic model was fit to protein extracted versus log [NaCl]. A salting in and salting out effect was observed for both peel and blanched peel mixed with saline solvent. Figure 2.4 shows the quadratic model fit to log [NaCl] versus protein extracted for peel and figure 2.5 shows the quadratic model fit to log [NaCl] versus protein for blanched peel. The optimum NaCl concentration was log [NaCl] = -4.7 for peel and log [NaCl] = -4.6 for blanched peel. A quadratic model was also fit to join point versus log [NaCl] in order to find the minimum solvent required to extract maximum protein. Figures 2.4 and 2.5 show the quadratic models fit to log [NaCl] versus join point for peel and blanched peel, respectively. Once the optimum NaCl concentration had been determined, this value was substituted into the join point quadratic equation. The predicted join point for peel at log [NaCl] = -4.7 was 63.0 mL solvent per g peel (dry weight) and the predicted join point for blanched peel at log [NaCl] = -4.6 was 59.7 mL solvent per g blanched peel (dry weight). A trial was run using the optimum conditions predicted by the models, 63.0 mL of 0.02 mM NaCl per g peel and 59.7 mL of 0.025 mM NaCl per g blanched peel. The predicted protein extracted from peel was 2.30 mg per g peel (dry weight) and the experimental protein extracted was 2.36 ± 0.26 mg per g peel (dry weight). The predicted protein extracted from blanched peel was 4.37 mg per g blanched peel (dry weight) and the experimental protein extracted was 4.35 ± 0.06 mg per g blanched peel (dry weight).

Samples were freeze dried prior to the start of the optimization trial to increase weighing accuracy; however, this step would not be practical in an industrial setting, so the dry weight to wet weight conversion becomes important. A 1 g portion of dried material was equal to about 17.5 g wet material. Since previous researchers used wet weight to report starting material, this conversion also allows for comparison of our results to previous work. Purcell and others *(15)* extracted protein by combining 500 g sweetpotatoes with 1500 mL water. We found a slightly higher solvent to solute ratio to be necessary for maximum extraction of protein. It is important to note, however, that the starting materials were different. Purcell and others *(15)* used whole sweetpotatoes while we used only sweetpotato peel.

2.4.3 Bench-top optimization of the precipitation process

The optimum concentration of $CaCl_2$ for precipitation of the extracted proteins was determined using a linear segmented model, as well. The join point was again important because it represented the concentration above which additional $CaCl_2$ would no longer increase yield but would increase cost. The effect of temperature was also investigated. Incubation of peel solution at 95°C greatly reduced the concentration of $CaCl_2$ required for maximum precipitation of protein. The join point at 95°C was 9.3 mM $CaCl_2$ where as the join point at 65°C was 27.6 mM $CaCl_2$ and at 25°C was 32.7 mM $CaCl_2$ (figure 2.6). Incubation temperature of blanched peel solution, however, did not significantly affect

concentration of $CaCl_2$ required for maximum precipitation of protein. The join point when the model was fit to points from all temperatures was 6.8 mM $CaCl_2$ (figure 2.7).

Purcell and others *(15)* heated sweetpotato protein solution to 65°C and then added 0.5% $CaCl_2$ to precipitate the chromoplast proteins. After removing this fraction, the researchers heated the solution to 95°C to coagulate and precipitate the remaining proteins. In contrast to these findings, we did not observe an increase in total proteins precipitated when the protein solution was incubated at 95°C. We did, however, observe a drastic decrease in the amount of $CaCl_2$ required for maximum precipitation of proteins from the peel solution when temperature was increased to 95°C. Makki and others *(18)* applied the procedure of Purcell and others *(15)* to sweetpotato peel and found that protein concentrates from sweetpotato peel were lower in purity than protein concentrates from sweetpotato pulp. We observed that $CaCl_2$ addition beyond the point of maximum protein precipitation led to dilution of the protein by precipitation of non-protein material, presumably soluble fiber, which could explain the low protein concentration in peel extracts compared to flesh extracts. Sweetpotato peel is higher in fiber than sweetpotato flesh *(18)*, so the dilution would presumably be greater in samples prepared with peel. Walter and Catignani *(16)* modified the procedure of Purcell and others *(15)* for their studies on the nutritional quality of sweetpotato protein extracts. One of the changes was a reduction in the amount of $CaCl_2$ used for protein precipitation. Instead of using 34 mM (0.5%), the authors used 6.8 mM (0.1%). This $CaCl_2$ concentration is lower than the amount we found to be required for maximum precipitation of proteins from sweetpotato peel solutions. The optimum $CaCl_2$

concentration for precipitation of proteins from solutions made from whole sweetpotatoes was not investigated in this study. It may be that the optimum for peel proteins is different than the optimum for flesh proteins.

The lack of reduction in the amount of $CaCl_2$ required for maximum precipitation from blanched peel solution with incubation at 95°C was most likely due to heat sensitive proteins having already been denatured during blanching and being able to more readily interact with the added ions even at low temperatures. When peel solution was heated to 95°C, the amount of $CaCl_2$ required for maximum precipitation (9.3 mM) began to approach the amount required for maximum precipitation from blanched peel solution at any temperature (6.8 mM), indicating that denaturation of proteins was likely responsible for the change.

For peel, the optimum conditions for protein precipitation resulted in 14.4% of the protein in the starting material being recovered in the peel extract, which consisted of 4.8% protein, calculated from % nitrogen with a conversion factor of 6.25. In the case of blanched peel, the optimum conditions for protein precipitation resulted in 32.0% of the protein in the starting material being recovered in the blanched peel extract, which consisted of 41.3% protein, calculated from % nitrogen with a conversion factor of 6.25. The higher purity of the blanched peel extract may be due to either the lower amount of $CaCl_2$ added for protein precipitation or the difference in composition between the blanched peel and the peel. It was noted that $CaCl_2$ also precipitated non-protein material, presumably soluble fiber. The rate of precipitation of non-protein material increased more quickly compared to the rate of

precipitation of additional protein, as the concentration of $CaCl_2$ was increased. In regards to the differences in composition between the blanched peel and peel, the blanched peel contained more pulp than the peel because the blanched peel was obtained from a secondary peeling step. As was previously mentioned, extract protein purity from pulp samples has been shown to be higher than extract protein purity from peel samples when prepared by $CaCl_2$ precipitation *(18)*. The higher fiber content of the peel compared to the pulp *(18)* is likely a major contributing factor to this difference in extract protein purity.

2.4.4 Comparison of protein extraction from peel and blanched peel

Blanched peel appears to be a better starting material for protein extraction and concentration. More protein was present in blanched peel than the peel (8.2% versus 6.4% protein), more protein could be recovered from blanched peel than peel (32.0% versus 14.4%), and the extract from blanched peel was more concentrated than the extract from peel (41.3% versus 4.8% protein). The proteins extracted from blanched peel may be of less use, however, due to possible loss of the desirable nutraceutical properties from heat treatment. Kusano and others *(28)* reported that boiling in water inactivated the anti-diabetic component of white-skinned sweetpotato extract. Other activities of sweetpotato proteins have also been reported to be heat labile. Trypsin inhibitors and amylase inhibitors in sweetpotatoes can be inactivated by heat treatment, with the degree of inactivation varying based on time, temperature, and type of heat treatment *(29-32)*. For the same reason, even though heating

the peel solution decreased the amount of $CaCl_2$ required for maximum protein precipitation, doing this may be undesirable because nutraceutical activities may be lost.

2.4.5 Comparison of protein quantification methods

Protein in the starting material and the final product was estimated by nitrogen analysis with a conversion factor of 6.25. It is likely that protein was overestimated in the starting material due to the presence of non-protein nitrogen. Up to 30% of the nitrogen in some cultivars of sweetpotatoes may be classified as non-protein nitrogen, depending on storage time *(33)*. At 107 days of storage, Purcell and others *(34)* found that the non-protein nitrogen fraction of the Jewel cultivar was composed mostly of amino acids, asparagine made up 61%, aspartic acid made up 11%, glutamic acid made up 4%, serine made up 4%, and threonine made up 3%. It is unlikely that these small peptides or free amino acids would be precipitated with $CaCl_2$ if they were not precipitated with trichloroacetic acid, and thus protein recovery may be underestimated due to overestimation of protein in the starting material when based on nitrogen.

BCA assay was used to quantify protein in solution during the pilot plant trial while Bradford assay was used to quantify protein in solution during the bench-top optimization. Bovine serum albumin was used to prepare a standard curve for each assay. It was discovered that Bradford assay and BCA assay yield very different protein concentrations for the same solution. Performing BCA assay with a representative peel solution gave 0.90 ± 0.02 mg/mL

protein while Bradford assay on the same solution gave 0.04 ± 0.01 mg/mL. A similar trend was seen with a blanched peel solution. BCA assay gave 1.91 ± 0.03 mg/mL while Bradford assay gave 0.07 ± 0.01 mg/mL. Similar results for the optimum NaCl concentration and peel to solvent ratio were obtained during a trial using BCA assay for protein quantification instead of Bradford assay, so the proposed optimum conditions do not appear to be affected by the lack of inter-assay consistency. Bradford assay most likely underestimated protein in solution. BCA assay is largely based on the peptide bond while the presence of specific amino acid residues, namely arginine, lysine and histidine, is important in Bradford assay. Mu and others *(11)* found that sweetpotato protein contained 5.30% arginine, 5.20% lysine, and 1.55% histidine. Similarly, Walter and Catignani *(16)* found that one fraction of sweetpotato protein from the Jewel cultivar contained 5.90%, 5.16%, and 2.70% and another fraction contained 6.33%, 7.03%, and 3.21% arginine, lysine, and histidine, respectively. Another cultivar, Cenntenial, contained 5.91%, 5.21%, and 2.88% in one fraction and 6.34%, 6.43%, and 2.96% in another fraction of arginine, lysine, and histidine, respectively. The standard curve that was used to determine sweetpotato protein in solution was prepared using BSA, which is much higher in these amino acids than sweetpotato protein. BSA contains 11.8% arginine, 15.3% lysine, and 6.75% histidine *(35)*.

2.4.6 Comparison of Caiapo and peel extract with gel electrophoresis

SDS-PAGE showed major protein bands present in Caiapo at 22 kDa and 58 kDa. These bands were also present in the peel extract; however, the intensity of the 58 kDa band was

reduced in the peel extract compared to Caiapo (figure 2.18). Despite the absence of any apparent protein bands above 58 kDa with total protein staining, a high molecular weight glycoprotein was found in both the extract and Caiapo after glycoprotein staining. A 22 kDa glycoprotein was also present in both the peel extract and Caiapo (figure 2.19).

The proteins extracted appear to be similar, at least in molecular weight, to proteins known to have unique nutritional benefits. Glycoprotein staining (figure 2.19) revealed that the 22 kDa protein of both the extract and Caiapo contained sugar, indicating that the active anti-diabetic component of Caiapo isolated by Kusano and others *(5)* may also be present in the extract. A higher molecular weight glycoprotein was also seen in both the extract and Caiapo, which may correspond to the active anti-diabetic component isolated by Ozaki and others *(6)*. The protein was not detected by the Imperial protein stain; however, a very intense colored band was seen with glycoprotein staining, which would fit with a product containing 95% sugar and 5% protein, the composition reported by Ozaki and others *(6)*.

2.5 CONCLUSIONS

Primary industrial peeling of sweetpotatoes yields a material in which concentration of the protein is difficult, due to co-precipitation of non-protein material. Much better protein concentration results can be attained using material from the secondary peeling after blanching. The optimum conditions for extracting and precipitating protein from the sweetpotato peel were presented. Using the optimum conditions, 32.0% of the protein

present in blanched peel was recovered in the extract, which consisted of 41.3% protein. Preliminary work with gel electrophoresis and glycoprotein staining suggests that the anti-diabetic proteins in Caiapo, an extract of a white-skinned sweetpotato cultivar, may also be present in extracts from orange-fleshed sweetpotatoes.

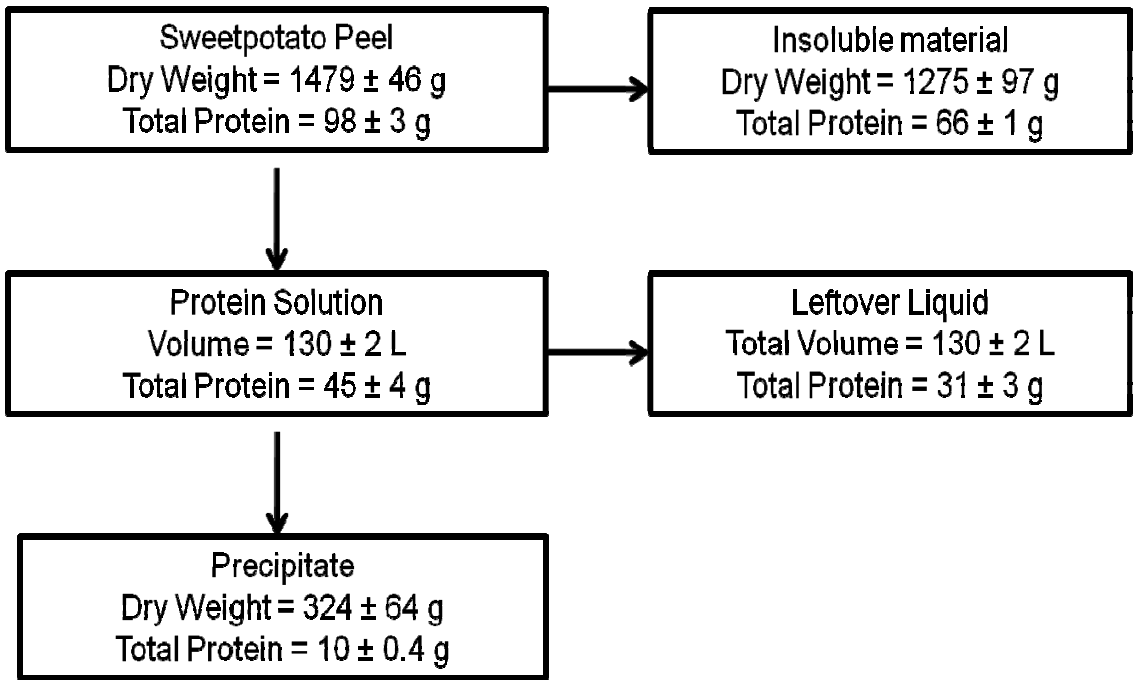

Figure 2.1. Mass balance during pilot plant extraction of protein from sweetpotato peel.

Table 2.1. Compositional comparison of Caiapo and pilot plant trial peel extract. Protein was determined by Kjeldahl nitrogen analysis with a conversion factor of 6.25, calcium was determined by atomic absorption, and starch was determined by iodine binding.

Component	**Caiapo**	**Peel Extract**
Protein	6.38%	3.27 ± 0.75%
Calcium	None detected.	0.14%
Starch	None detected.	None detected.

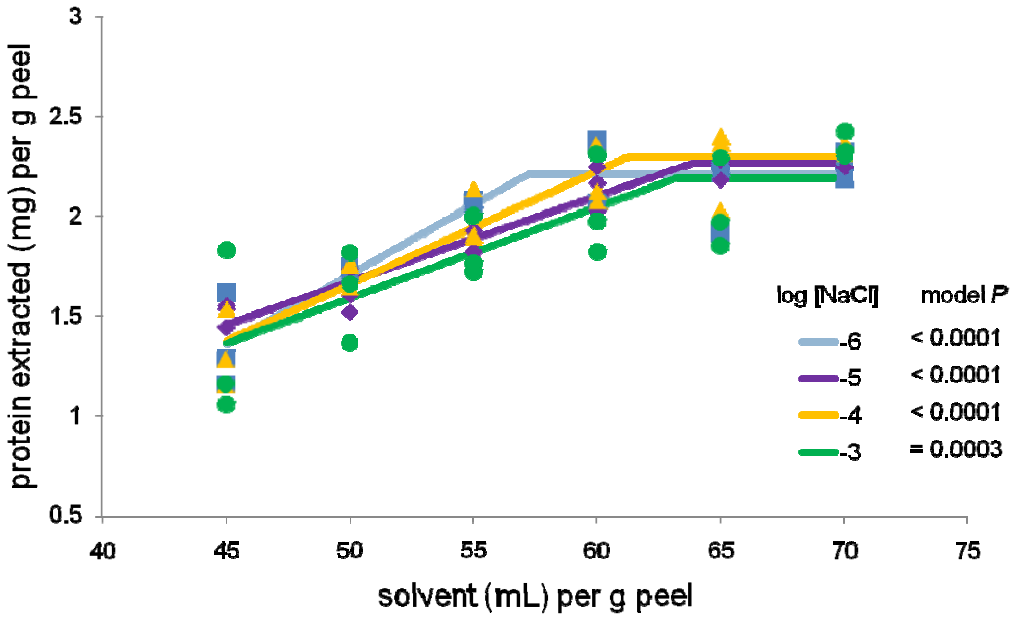

Figure 2.2. Linear segmented models fit to extraction data for peel.

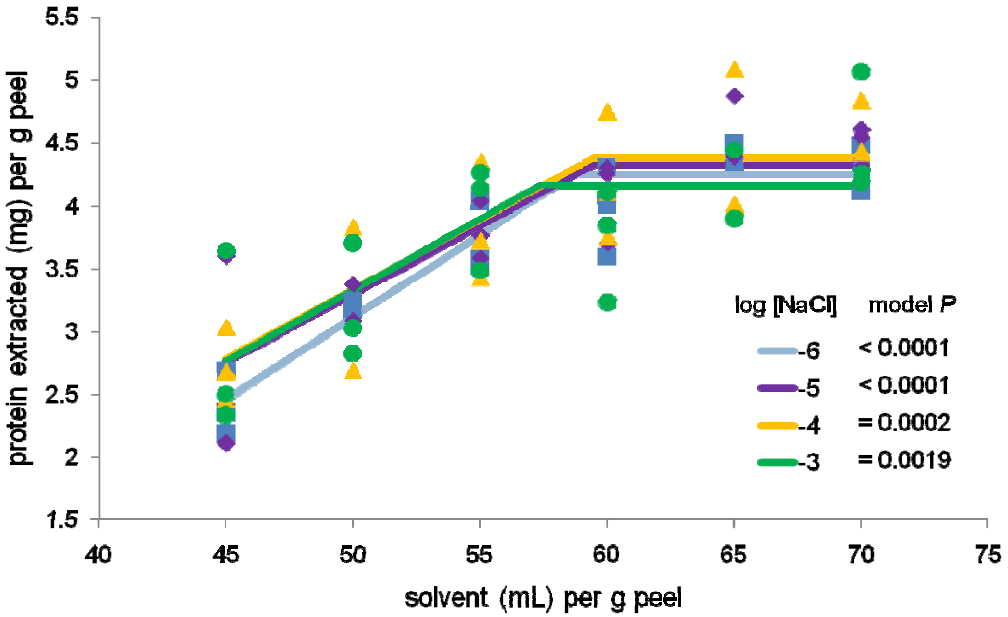

Figure 2.3. Linear segmented models fit to extraction data for blanched peel.

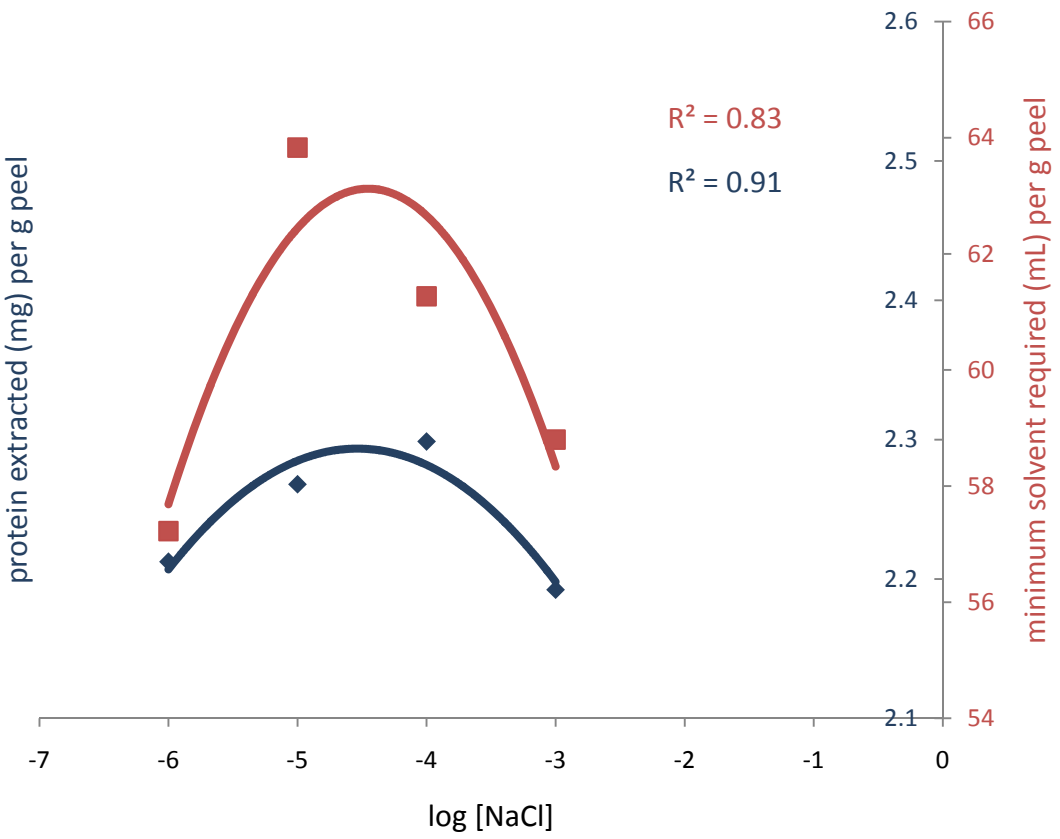

Figure 2.4. Optimizing NaCl concentration and solvent to peel ratio for protein extraction from peel using quadratic models.

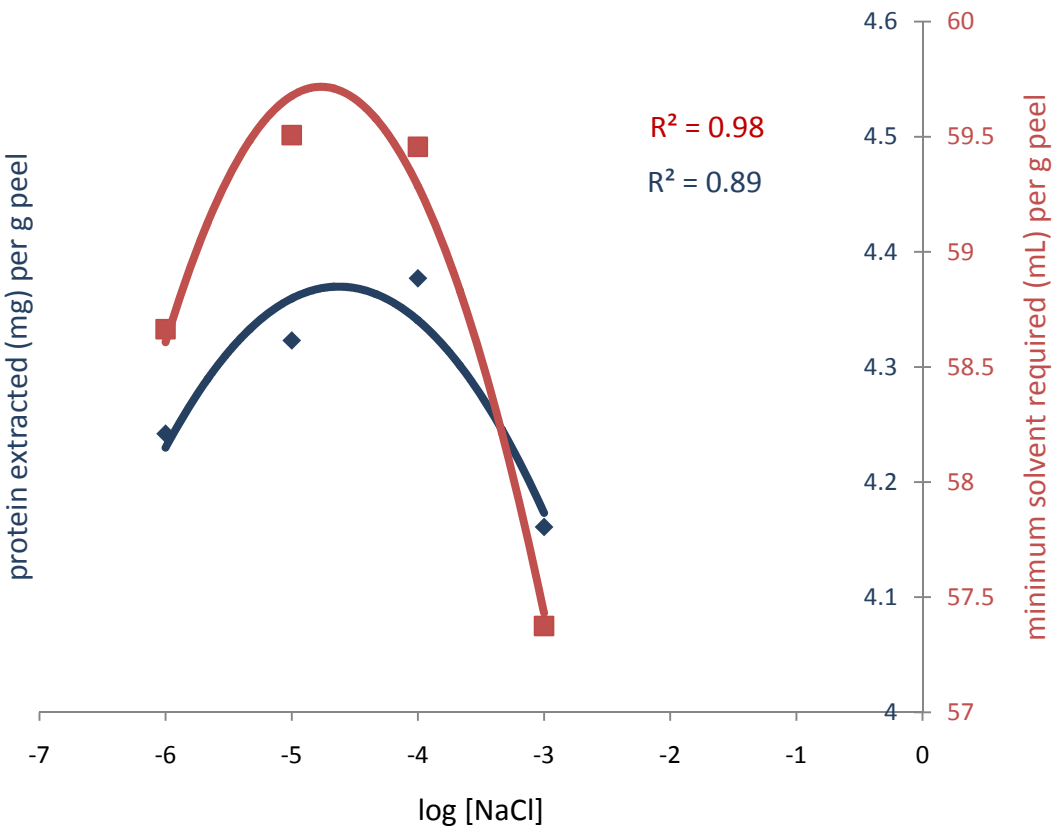

Figure 2.5. Optimizing NaCl concentration and solvent to peel ratio for protein extraction from blanched peel using quadratic models.

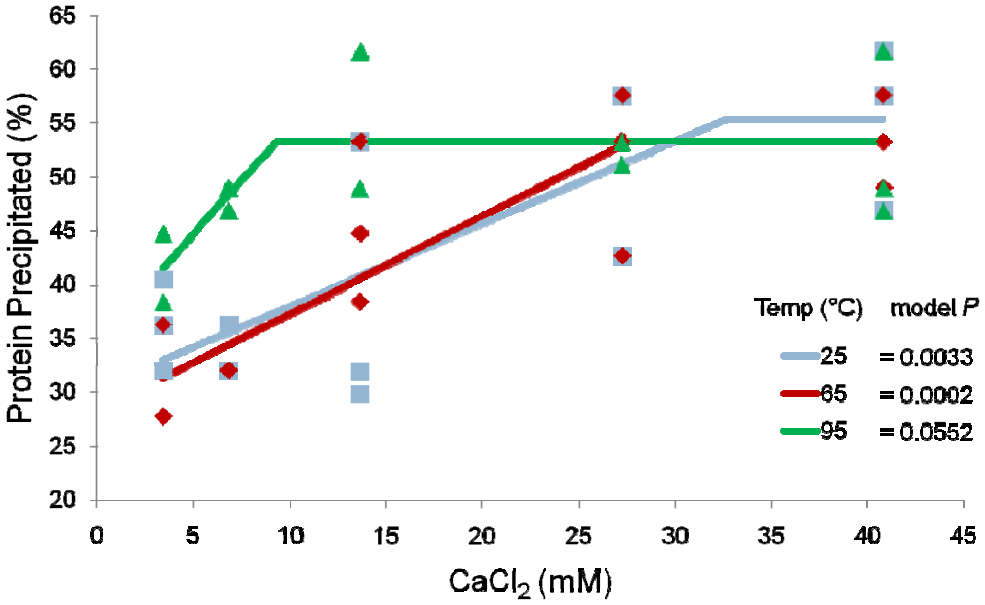

Figure 2.6. Linear segmented models fit to peel precipitation data.

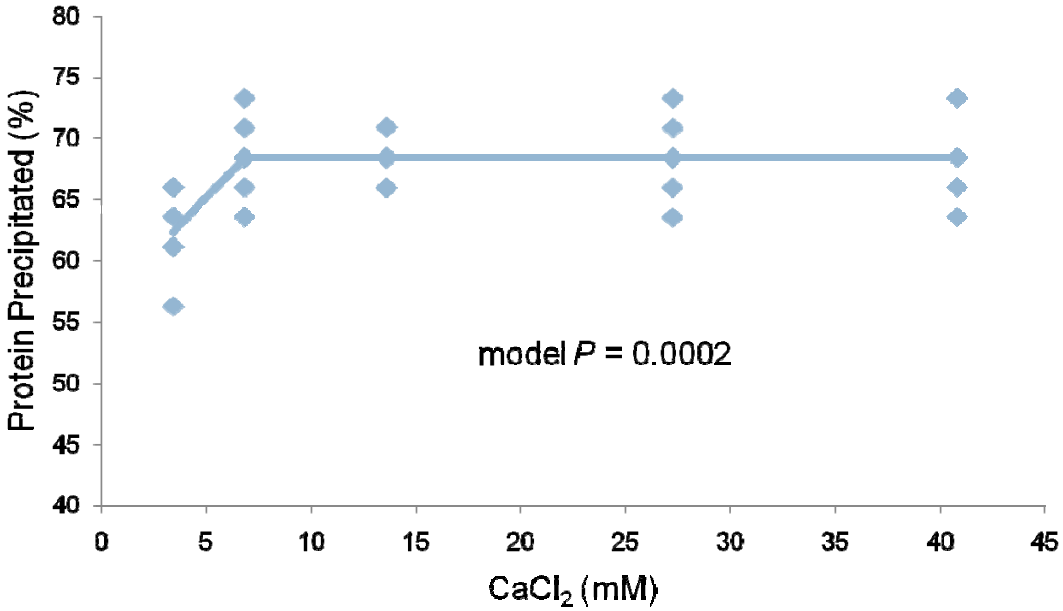

Figure 2.7. Linear segmented model fit to blanched peel precipitation data all temperatures combined.

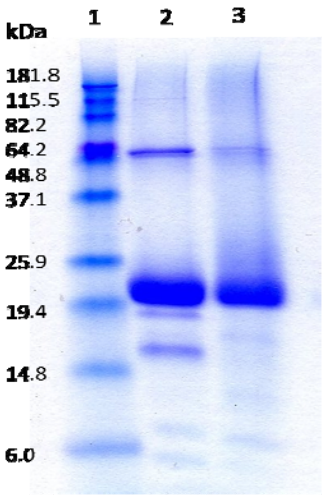

Figure 2.8. Comparison of Caiapo and peel extract protein banding patterns. SDS-PAGE of protein ladder (lane 1), Caiapo (lane 2), and peel extract (lane 3). Gel was stained with Imperial protein stain.

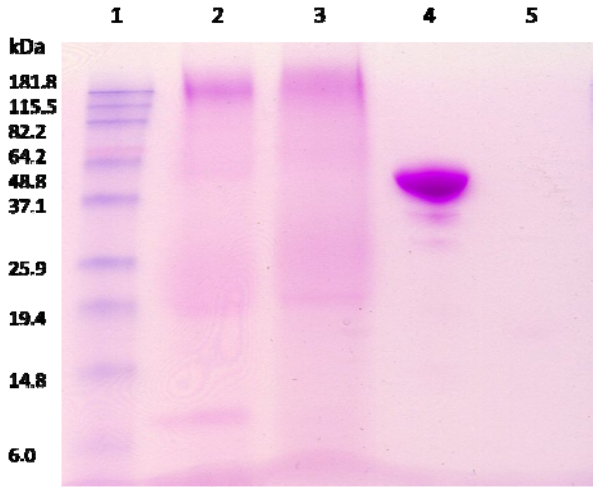

Figure 2.9. Comparison of Caiapo and peel extract glycoprotein banding patterns. SDS-PAGE of protein ladder (lane 1), Caiapo (lane 2), peel extract (lane 3), horseradish peroxidase positive control (lane 4), and soybean trypsin inhibitor negative control (lane 5). Gel was stained with glycoprotein staining kit.

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CHAPTER 3

Susceptibility of sweetpotato proteins to digestive enzymes

3.1 ABSTRACT

Sweetpotato proteins have been shown to possess antioxidant properties in vitro and in animals, anti-proliferative properties in vitro, and anti-diabetic properties in animals and humans. The ability of a protein to exhibit systemic effects is somewhat unusual since proteins are typically susceptible to digestive enzymes. This study was undertaken to better understand how digestive enzymes affect sweetpotato proteins. Sweetpotato proteins from 3 different sources, Caiapo, an extract from a white-skinned sweetpotato cultivar currently sold as an anti-diabetic supplement, an extract from the peel of orange-fleshed sweetpotatoes, and an extract from blanched peel of orange-flesh sweetpotatoes, was incubated with pepsin, trypsin, and chymotrypsin. Aliquots were removed and proteins were visualized with SDS-PAGE as the reaction progressed. Aliquots removed after pepsin incubation was complete were also assayed for amylase inhibitory activity. Sporamin, the major storage protein in sweetpotatoes, which functions as a trypsin inhibitor as well, exhibited resistance to pepsin, trypsin, and chymotrypsin. Sporamin from blanched peel was less resistant to pepsin digestion than sporamin from Caiapo and peel. Trypsin inhibitory activity remained after simulated gastric digestion, with the Caiapo and peel samples exhibiting higher inhibitory activity compared to the blanched peel sample. Amylase and chymotrypsin inhibitory activity was not present in any of the samples after digestion. Lack of amylase inhibitory activity was confirmed by modified glucose tolerance test in rats.

3.2 INTRODUCTION

Sweetpotato proteins possess numerous nutraceutical properties. Caiapo, a protein extract from a white-skinned sweetpotato cultivar, is currently being marketed as an anti-diabetic supplement and has been shown to lower fasting blood glucose levels and increase insulin sensitivity in type II diabetics *(1-4)*. In vitro, sweetpotato trypsin inhibitor proteins have been shown to possess antioxidant properties with scavenging abilities against 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical *(5, 6)*, and reactive nitrogen species *(7)* and in mice, have been shown to increase serum superoxide dismutase, catalase, and glutathione peroxidase activity *(8)*. Anti-proliferative properties (NB4 promyeolcytic leukemia cells in vitro) have also been reported *(9)*.

Multiple proteins isolated from sweetpotatoes have been shown to possess trypsin inhibitory activity *(10-13)*; however, the protein found in the greatest quantity that possesses trypsin inhibitory activity is the 25 kDa storage protein, sporamin. Sporamin makes up over 80% of the total protein found in sweetpotatoes *(14)*. Trypsin inhibitory activity has been shown to vary by cultivar. Bradford and others *(15)* found a 67-fold range in trypsin inhibitory activity, from 0.33 TIU to 22.1 TIU, among sweetpotato cultivars from the Highlands of Papua New Guinea. Nutraceutical properties of the trypsin inhibitors have also been shown to vary by cultivar. Hou and others *(6)* found that trypsin inhibitors isolated from the cultivar Tainong 65 exhibited a higher degree (3-fold) of protection against Cu^{2+}-induced human LDL peroxidation than trypsin inhibitors isolated from the cultivar Tainong 57. Trypsin

inhibitors from Tainong 57, however, exhibited a higher degree (10-fold) of protection against hydroxyl radical-induced DNA damage of calf thymus compared to trypsin inhibitors isolated from Tainong 65. Trypsin inhibitors are stable over a wide pH range *(11)*, but their activity can be reduced by processing *(10, 16, 17)*.

While less studied, amylase inhibitors have also been identified in sweetpotatoes with amylase inhibitory activity varying by cultivar *(18)*. The stability of the amylase inhibitors to processing has been found to vary by cultivar *(19)*. Amylase inhibitors, in general, have been investigated for their potentially positive impact on diabetes management and weight control, due to their ability to slow and/or reduce starch digestion, thus slowing and/or reducing glucose absorption, lowering glycemic response and possibly lowering caloric absorption. In order to function as an inhibitor of intestinal digestive enzymes in vivo, a protein must survive gastric digestion. Several studies found high in vitro inhibitory activities, but then failed to find the same inhibitory activities in vivo *(20, 21)*, possibly due to susceptibility of the inhibitor to digestion. Therefore, in this study, extracts were subjected to in vitro gastric digestion prior to assaying for amylase inhibitory activity.

In order to have systemic effects, such as the effects seen in Caiapo administration, proteins must survive both gastric and intestinal digestion, so that they will have the chance to be absorbed. A first step in determining if protein extracts from orange-flesh cultivars of sweetpotato may be useful as an anti-diabetic supplement, like the extract from white-skinned sweetpotatoes, Caiapo, is to ensure proteins would be able to be absorbed intact by

the body, i.e. are resistant to digestion. A limitation to the use of proteins for therapeutic treatment is their susceptibility to digestive enzymes *(22)*; however, if the protein can remain intact in the presence of digestive enzymes, absorption may occur. Several proteins have been shown to pass through the intestinal barrier intact *(23-25)*. The objectives of this study were to determine if proteins were present in sweetpotato extracts that were resistant to digestive enzymes and if activities were retained after in vitro digestion, in order to better understand how sweetpotato proteins could exhibit systemic effects in the body. Caiapo, a sweetpotato protein supplement already on the market, was compared to extracts from two other potential sources for sweetpotato protein supplements.

3.3 MATERIALS AND METHODS

3.3.1 Enzymes and chemicals

Enzymes were obtained from Sigma-Aldrich (St. Louis, MO). The reported activity for pepsin from porcine gastric mucosa was 3802 units/mg protein, α-chymotrypsin from bovine pancreas was 59.3 units/mg protein, and trypsin from porcine pancreas was 14476 BAEE units/mg protein. Soluble starch was obtained from Sigma-Aldrich (St. Louis, MO). Caiapo was obtained from Fuji-Sangyo Company (Japan).

3.3.2 Raw material

Sweetpotato peel was obtained from a local processing plant (Yamco, LLC, Snow Hill, NC). Peel was from a mixture of orange-flesh cultivars including Beauregard, Jewel, and Covington. Peel was obtained from two different points along the processing line. Material was obtained from the initial peeling of the sweetpotatoes before any further processing, from here after referred as "peel," and material was obtained from a secondary peeling after blanching of the sweetpotatoes, from here after referred to as "blanched peel." Upon receipt, the peel and blanched peel were freeze dried and stored at -20°C.

3.3.3 In vitro gastric digestion

For in vitro gastric digestion, the low-protease digestion assay of Mandalari and others *(26)* was followed with some modifications. Saturated solutions of peel, blanched peel, and Caiapo in 150 mM NaCl, pH=2 were incubated at 37°C for 10 minutes. A 100 µL portion of pepsin solution (5 mg/mL, pH=2) was then added to 10 mL of each protein solution. Solutions were incubated at 37°C for 1 hour with 100 µL portions removed at 0, 1, 2, 5, 10, 30, and 60 minutes. The reactions were stopped by addition of 20 µL 0.5 M NaOH.

3.3.4 In vitro duodenal digestion

For in vitro duodenal digestion, the low-protease digestion assay of Mandalari and others *(26)* was again followed with some modifications. After simulated gastric digestion for 60 minutes, the pH of the protein solutions was adjusted to 7 using 0.5 M NaOH. A 100 µL portion of either trypsin solution (25 µg/mL, pH=7) or chymotrypsin solution (0.5 mg/mL, pH=7) was then added to 5 mL of each protein solution. Solutions were incubated at 37°C for 1 hour with 100 µL portions removed at 0, 1, 2, 5, 10, 30, and 60 minutes. The reactions were stopped by addition of 20 µL SigmaFAST protease inhibitor tablet solution (1 tablet dissolved in 10 mL DI water).

3.3.5 Amylase activity assay

The method of Bernfeld *(27)* with some modifications was used to determine the amylase activity of the peel, blanched peel, and Caiapo before and after digestion, and to determine if amylase inhibitors were present in the extracts after digestion. A 1% soluble starch solution was prepared by dissolving 1 g soluble starch in 100 mL warm 20 mM sodium phosphate buffer containing 150 mM NaCl, pH=7. For the assay of amylase activity, a 250 µL portion of each protein solution before and after in vitro gastric digestion for 60 minutes was combined with 250 µL sodium phosphate buffer, so that the final reaction buffer consisted of 20 mM sodium phosphate buffer containing 150 mM NaCl, pH=7. For the assay of amylase inhibitory activity after gastric digestion, a 250 µL portion of each protein solution after in

vitro gastric digestion for 60 minutes was combined with 250 µL sodium phosphate buffer containing porcine pancreatic amylase, so that the final reaction buffer consisted of 20 mM sodium phosphate buffer containing 150 mM NaCl, pH=7. Amylase activity of the samples containing digested peel, blanched peel, or Caiapo in combination with porcine pancreatic amylase were compared to a sample containing only porcine pancreatic amylase. All solutions were incubated at 37°C for 15 minutes and then 1 mL of soluble starch solution was added to the protein solutions to start the reaction. Samples were incubated at 37°C for 3 minutes and then the reaction was stopped by the addition of 2 mL of 3,5-dinitrosalycylic acid reagent. Absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount required to liberate 1 µmol maltose per minute under the conditions of the assay.

3.3.6 Gel electrophoresis

Changes in proteins over time during incubation with pepsin, trypsin, and chymotrypsin were visualized with reducing SDS-PAGE. Laemmli sample buffer and 10x Tris/Glycine/SDS running buffer were obtained from Bio-Rad (Hercules, CA), β-mercaptoethanol was obtained from Sigma-Aldrich (St. Louis, MO), and SeeBlue Plus2 protein standard was obtained from Invitrogen (Carlsbad, CA). Samples were run on a 15% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) at constant voltage (200 V). Gels were stained with Imperial Protein Stain (Thermo Fisher Scientific, Rockford, IL). Band density was quantified with UN-SCAN-IT gel analysis software (Silk Scientific, Inc., Orem, UT).

3.3.7 Modified glucose tolerance tests in rats

Male Wistar rats (n=12) were fasted for 6 hours prior to treatment, but were given free access to water. Treatments, consisting of 300 mg carbohydrate (glucose, maltose, or starch) and 16 mg protein (Caiapo, peel extract, or flesh extract) dissolved in saline solution, were administered via gastric intubation. Controls containing only carbohydrate were also administered. Blood was collected from the tail vein immediately before intubation, and at 1 and 2 hours after intubation. Blood glucose was determined with a glucometer. Each rat received every treatment in random order, with at least 48 hours between treatments.

3.3.8 Statistical analysis

Data were analyzed by ANOVA in JMP (SAS, Inc., Cary, NC). Mean differences were determined with Tukey-Kramer test ($P < 0.05$).

3.4 RESULTS AND DISCUSSION

3.4.1 Susceptibility of sporamin to digestive enzymes

Visualization of proteins with SDS-PAGE at various points during digestion of Caiapo, peel extract, and blanched peel extract with pepsin, trypsin, and chymotrypsin revealed that sporamin exhibits resistance to cleavage by these enzymes. The density of the sporamin

band in Caiapo and peel extract remained unchanged despite incubation with pepsin, trypsin, and chymotrypsin (figures 3.1-3.2). The density of the sporamin band in blanched peel extract decreased as incubation time increased, however, resistance to digestive enzymes was still noted (figure 3.3). After 1 minute of incubation with pepsin, sporamin band density in the blanched peel sample decreased to 55% of the original band density. After 60 minutes, the band density decreased to 23% of the original density. The sporamin that remained after 60 minutes of incubation with pepsin was resistant to digestion by trypsin and chymotrypsin, indicated by the lack of difference between the sporamin band density at the start of incubation and after 60 minutes of incubation. Generally, protein resistance to digestion is due to either a unique amino acid sequence, causing digestive enzymes to not recognize cleavage sites, or compact structure, that hinders the ability of the digestive enzymes to reach the cleavage sites. ExPASy PeptideCutter was used to predict potential cleavage sites on sporamin for pepsin, trypsin, and chymotrypsin, in order to determine if resistance was due to a unique amino acid sequence. The sequence used for sporamin was GenBank accession AAB52550, determined by Yeh and others *(28)*. Sporamin contained numerous potential cleavage sites for pepsin, trypsin, and chymotrypsin (figures 3.4-3.6), indicating that a unique amino acid sequence is not likely the mechanism for the resistance of sporamin to digestion. The compact structure of the protein is more likely responsible for the lack of cleavage by digestive enzymes. Sugiura and others *(11)* found that sweetpotato trypsin inhibitors with molecular weights of 23 and 24 kDa (probably sporamin) were stable at pH=2 and 37°C. Since this is within the pH range and temperature of the stomach, it is likely that potential cleavage sites were inaccessible to pepsin due to the structural stability of sporamin.

Compact structure has been found to play a role in reducing the digestibility of several proteins, including chickpea albumin *(29)*, lupin γ-conglutin *(30)*, and many allergenic albumins *(31)*.

In contrast to our findings, Hou and others *(6)* reported that free amino ends increased during incubation of the major root storage protein isolated from the sweetpotato cultivars Tainong 57 and Tainong 65 with pepsin and chymotrypsin. The digestion procedure used by Hou and others was more exhaustive than our procedure (incubation with pepsin for 8 hours and then chymotrypsin for 4, 8, or 12 hours), however, which could account for the difference in results. Hou and others noted that the susceptibility to digestion of the protein did not hinder its antioxidant properties. In fact, scavenging ability against DPPH radical increased upon hydrolysis.

An additional band around 60 kDa was seen in the Caiapo sample that was also resistant to pepsin, trypsin, and chymotrypsin digestion. This may be the same protein that Chen and others *(32)* found exhibited glutathione reductase activity and was resistant to trypsin and chymotrypsin digestion. A 60 kDa protein was not seen in the peel and blanched peel samples, but this was likely due to the concentration being below the staining threshold, not the absence of the protein. Caiapo exhibited higher solubility than the peel and blanched peel samples at pH=2, and thus more proteins were able to be visualized.

3.4.2 Trypsin and chymotrypsin inhibitory activity

Trypsin and chymotrypsin inhibitory activity was determined by monitoring the degradation of the pepsin band during incubation with trypsin and chymotrypsin. Trypsin inhibitors were present in the Caiapo, peel extract, and blanched peel extract after digestion with pepsin. Pepsin band density remained unchanged in the Caiapo and peel samples during incubation with trypsin (figures 3.1-3.2). Trypsin inhibitory activity was lower in the blanched peel sample than the Caiapo and peel samples. After 1 minute of incubation with trypsin, pepsin band density was reduced to 90% of the original band density, and after 60 minutes, pepsin band density was reduced to 18% of the original density (figure 3.3). These results are consistent with previous studies that found heat treatment reduced the activity of sweetpotato trypsin inhibitors *(10, 16, 17)*. Trypsin inhibitors isolated from other sources have also been shown to exhibit resistance to pepsin digestion that is reduced by heat treatment. Liao and others *(33)* found that a trypsin inhibitor isolated from *Cassia obtusifolia* seeds, completely resistant to pepsin digestion before heating, became susceptible to pepsin digestion upon heating. Band density did not change in the non-heated sample after 60 minutes of incubation with pepsin; however, band density was reduced by 40% after 20 minutes of incubation with pepsin in the heated sample.

Chymotrypsin inhibitory activity did not appear to be present in any of the samples. The pepsin band was completely eliminated after 1 minute of incubation with chymotrypsin in all samples and degradation products were visible (figures 3.1-3.3). While dual trypsin-

chymotrypsin inhibitors have been identified in some plants *(34-38)*, the sweetpotato trypsin inhibitors do not appear to fall into this category, since trypsin inhibitory activity was present and chymotrypsin inhibitory activity was not present in the same samples. These results are consistent with Sugiura and others *(11)* who found that sweetpotato trypsin inhibitors did not affect chymotrypsin activity.

3.4.3 Amylase activity and amylase inhibitory activity

Caiapo and peel extract exhibited amylase activity; however, blanched peel extract did not exhibit amylase activity. These findings are in agreement with Hagenimana and others *(39)* who showed that sweetpotato α-amylase and β-amylase were rapidly inactivated at high temperatures, such as those that would be used for blanching. The amylase activity of Caiapo and peel extract was eliminated by pepsin digestion (figure 3.7). The activity of porcine pancreatic amylase was not significantly changed by the presence of pepsin digested Caiapo, peel extract, or blanched peel extract, indicating that amylase inhibitors were not present after pepsin digestion (figure 3.8).

Amylase inhibitors have been isolated from numerous plants, including legumes *(40)*, grains *(41, 42)*, and tubers *(18, 37, 43)*, as well as several types of seeds *(44-46)*, but their effects in vivo have been inconsistent. Carlson *(21)* found that despite high in vitro activity of a bean amylase inhibitor, in humans, glycemic response was not reduced by addition of the amylase inhibitor supplement to a high starch meal. Similarly, Bo-Linn and others *(20)* found that

calories absorbed were not reduced by amylase inhibitor supplements given in conjunction with a high starch meal. Lack of stability to digestion was cited as a possible reason for the ineffectiveness of these supplements in vivo. Gibbs and Alli *(40)* later found that an amylase inhibitor isolated from white kidney bean was resistant to pepsin digestion, but was readily hydrolyzed by trypsin and chymotrypsin. If amylase inhibitors were present in the sweetpotato samples, they are likely susceptible to pepsin digestion, and thus would result in the same type of situation described above when consumed as a dietary supplement, i.e. would not exhibited amylase inhibitory activity in vivo.

It is possible, however, that amylase inhibitors were not present in the sweetpotato samples prior to digestion. Shivaraj and others *(47)* did not find amylase inhibitors in the sweetpotatoes they tested and Rekha and others *(18)* only found amylase inhibitors in 79 of the 100 accessions they tested. The presence of native amylase activity in sweetpotato protein complicates assays for amylase inhibitors. Shivaraj and others *(47)* overcame this issue by heating the extract for 10 minutes at 80°C to eliminate native amylase activity while Rekha and others *(18)* selectively precipitated the amylases with trichloroacetic acid prior to assaying for amylase inhibitory activity. Since both of these methods could lead to inactivation or removal of amylase inhibitors as well as amylases, we attempted to take into account the native amylase activity in our calculations of amylase inhibitory activity by assaying for native amylase activity and porcine pancreatic amylase activity separately and then together, instead of inactivating or removing the native amylases. Expected activity was calculated by adding the native amylase activity and the porcine pancreatic amylase activity

assayed separately, and then amylase inhibitory activity was determined by subtracting experimental amylase activity of the sweetpotato protein and the porcine pancreatic amylase assayed together from the expected amylase activity. No amylase inhibitory activity was found in any of the extracts (data not shown) using this method. We decided to proceed with the digestion and then assay for amylase inhibition again after amylase activity was removed by digestion, since it has been suggested that the activities of α-amylase (like the porcine pancreatic amylase we used in the assay) and β-amylase (found in sweetpotatoes) may not be additive *(48)*, which could have masked the presence of amylase inhibitors. This digestion method for eliminating native amylase activity was well-suited for our study because only the presence of digestion-resistant amylase inhibitors was important, since amylase inhibitors susceptible to digestion would be unlikely to exhibit nutraceutical effects in the body.

3.4.4 Modified glucose tolerance tests in rats

Lack of amylase inhibitory activity was confirmed in vivo by a modified glucose tolerance test with starch as the carbohydrate source. No statistically significant differences in blood glucose at 1 hour and 2 hours after intubation were seen between the control (starch) and the treatment (starch + Caiapo, starch + peel extract, and starch + flesh extract) groups. In addition, tests were performed with glucose and maltose as the carbohydrate source to determine if sweetpotato proteins altered glucose absorption or possessed maltase inhibitory activity, respectively. No statistically significant differences in blood glucose at 1 hour and 2 hours after intubation were seen between the control and the treatment groups with these

carbohydrate sources either (table 3.1). Short-term blood glucose lowering effects have been reported for sweetpotatoes *(49)*, but based on the results of this study, the effect does not appear to be due to the protein fraction. Short-term blood glucose reduction could be due to other sweetpotato components, such as fiber.

3.5 CONCLUSIONS

Sporamin, in Caiapo and extracts from orange-fleshed sweetpotato peel, was resistant to pepsin digestion in vitro. This resistance was most likely due to the compact structure of the protein because the amino acid sequence of the protein suggested that numerous pepsin cleavage sites were present, and blanching, which would cause denaturation of the protein, increased susceptibility to pepsin. Sporamin that was not digested by pepsin was also resistant to trypsin and chymotrypsin digestion. Again, this was most likely due to the compact structure of the protein because the amino acid sequence showed that cleavage sites for trypsin and chymotrypsin were present. Trypsin inhibitors persisted through in vitro gastric and duodenal digestion; however, chymotrypsin and amylase inhibitors were not present after digestion. This work is a first step in understanding how proteins isolated from sweetpotatoes can exhibit systemic effects.

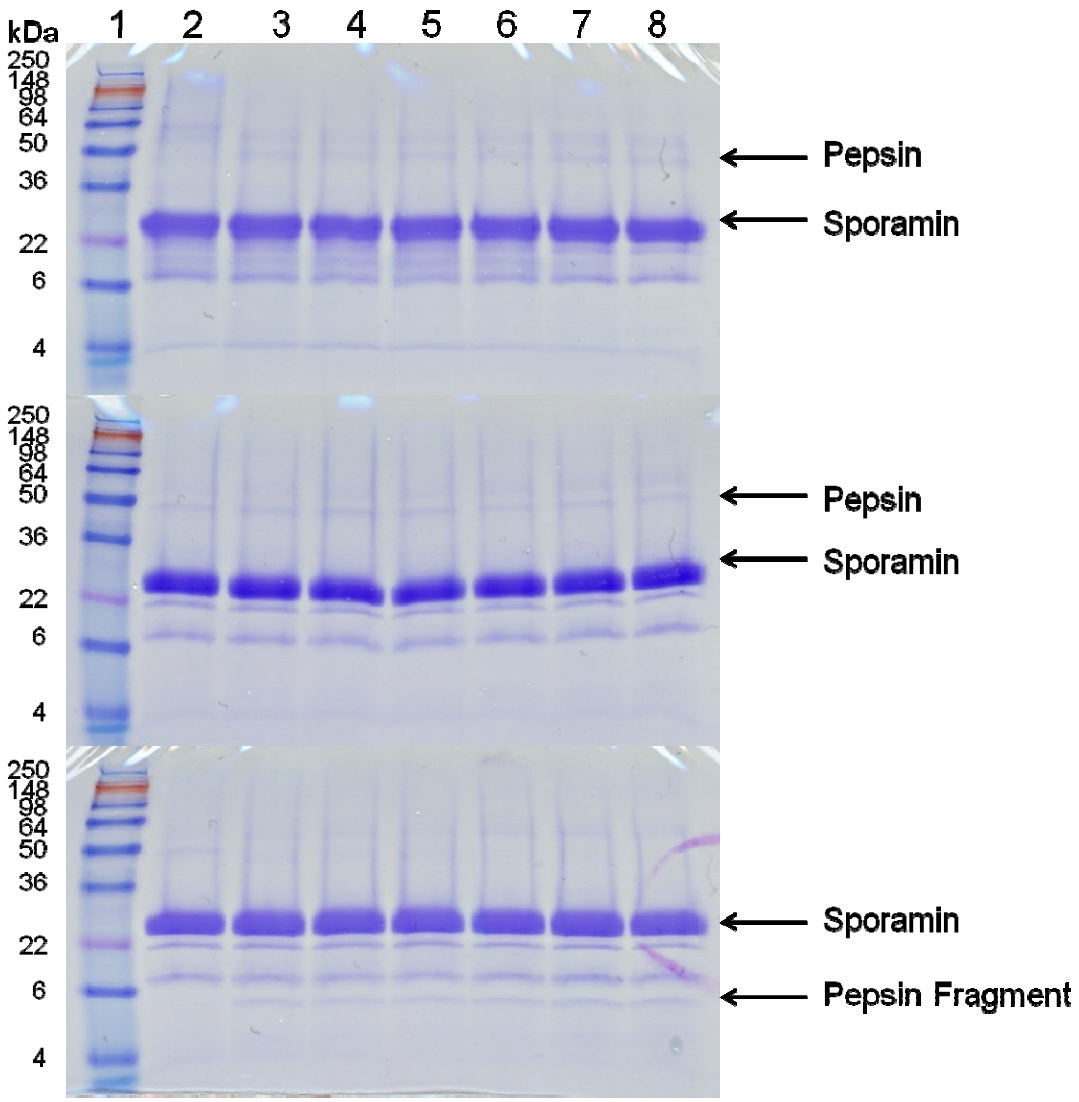

Figure 3.1. SDS-PAGE of Caiapo digested with pepsin (top), trypsin (middle), and chymotrypsin (bottom). Lane 1 contains SeeBlue Plus2 Pre-Stained Standard. Lanes 2-8 contain samples in which the reaction was stopped at 0, 1, 2, 5, 10, 30, and 60 minutes, respectively.

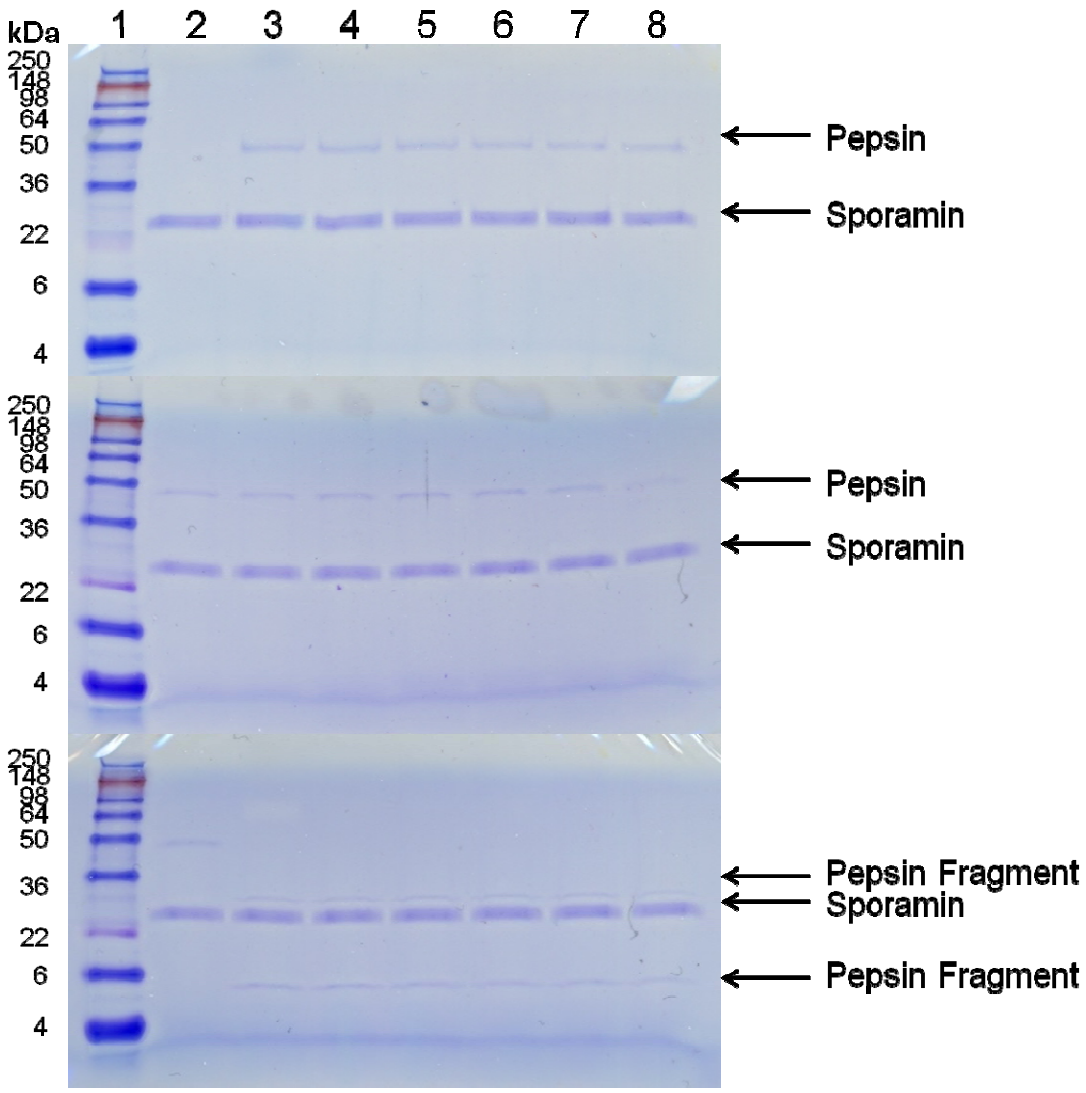

Figure 3.2. SDS-PAGE of sweetpotato peel digested with pepsin (top), trypsin (middle), and chymotrypsin (bottom). Lane 1 contains SeeBlue Plus2 Pre-Stained Standard. Lanes 2-8 contain samples in which the reaction was stopped at 0, 1, 2, 5, 10, 30, and 60 minutes, respectively.

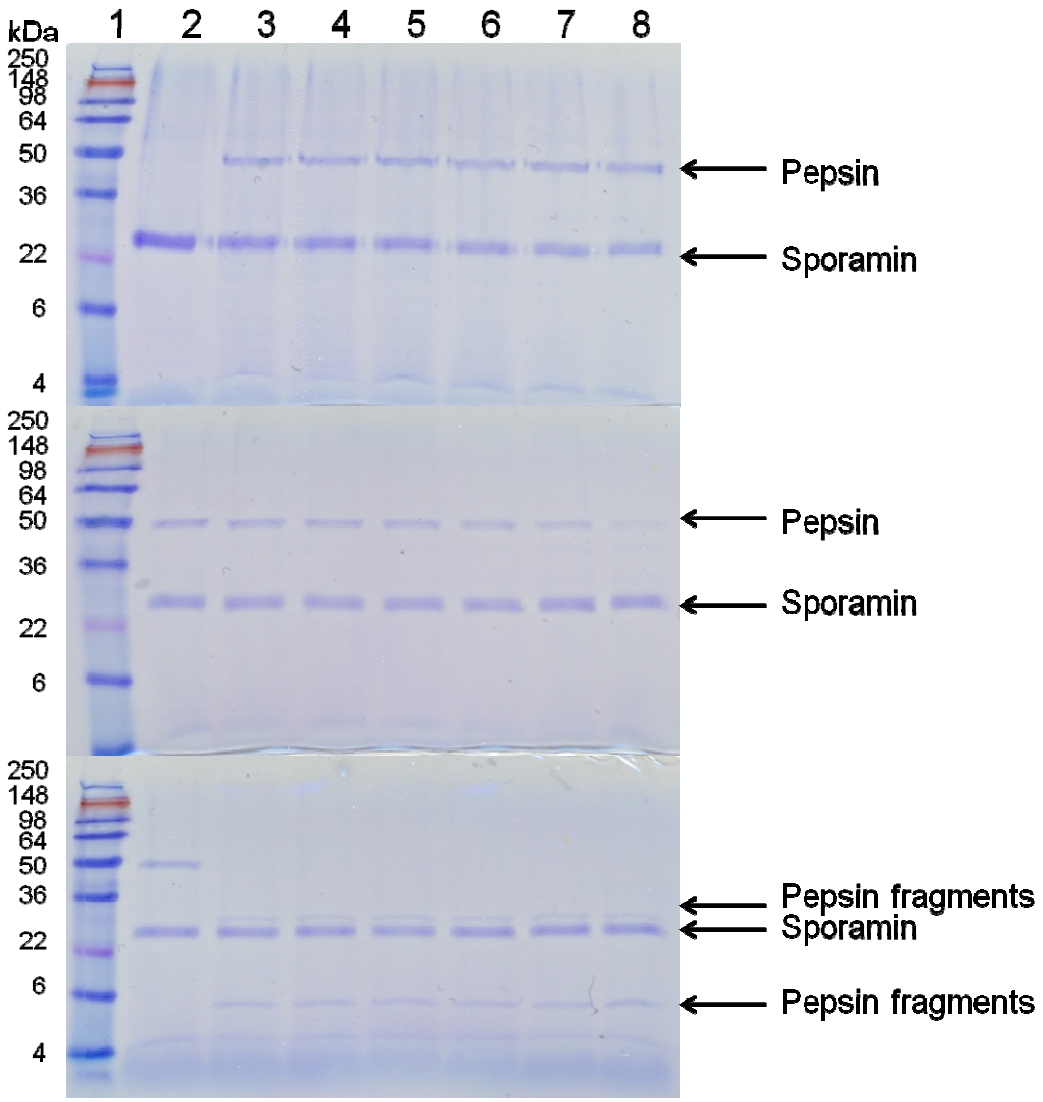

Figure 3.3. SDS-PAGE of blanched sweetpotato peel digested with pepsin (top), trypsin (middle), and chymotrypsin (bottom). Lane 1 contains SeeBlue Plus2 Pre-Stained Standard. Lanes 2-8 contain samples in which the reaction was stopped at 0, 1, 2, 5, 10, 30, and 60 minutes, respectively.

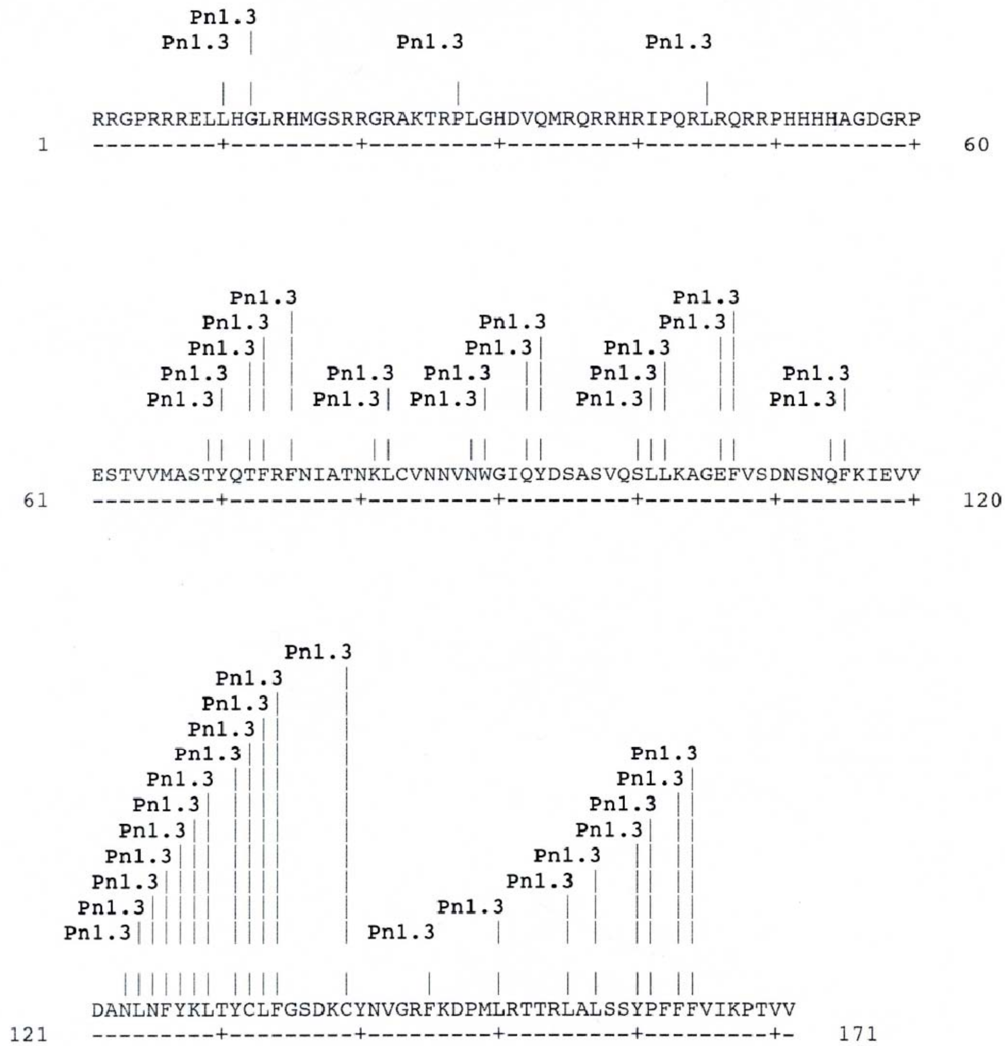

Figure 3.4. Theoretical pepsin cleavage sites of sporamin determined by inputting sporamin protein sequence from GenBank accession AAB52550 into ExPASy PeptideCutter.

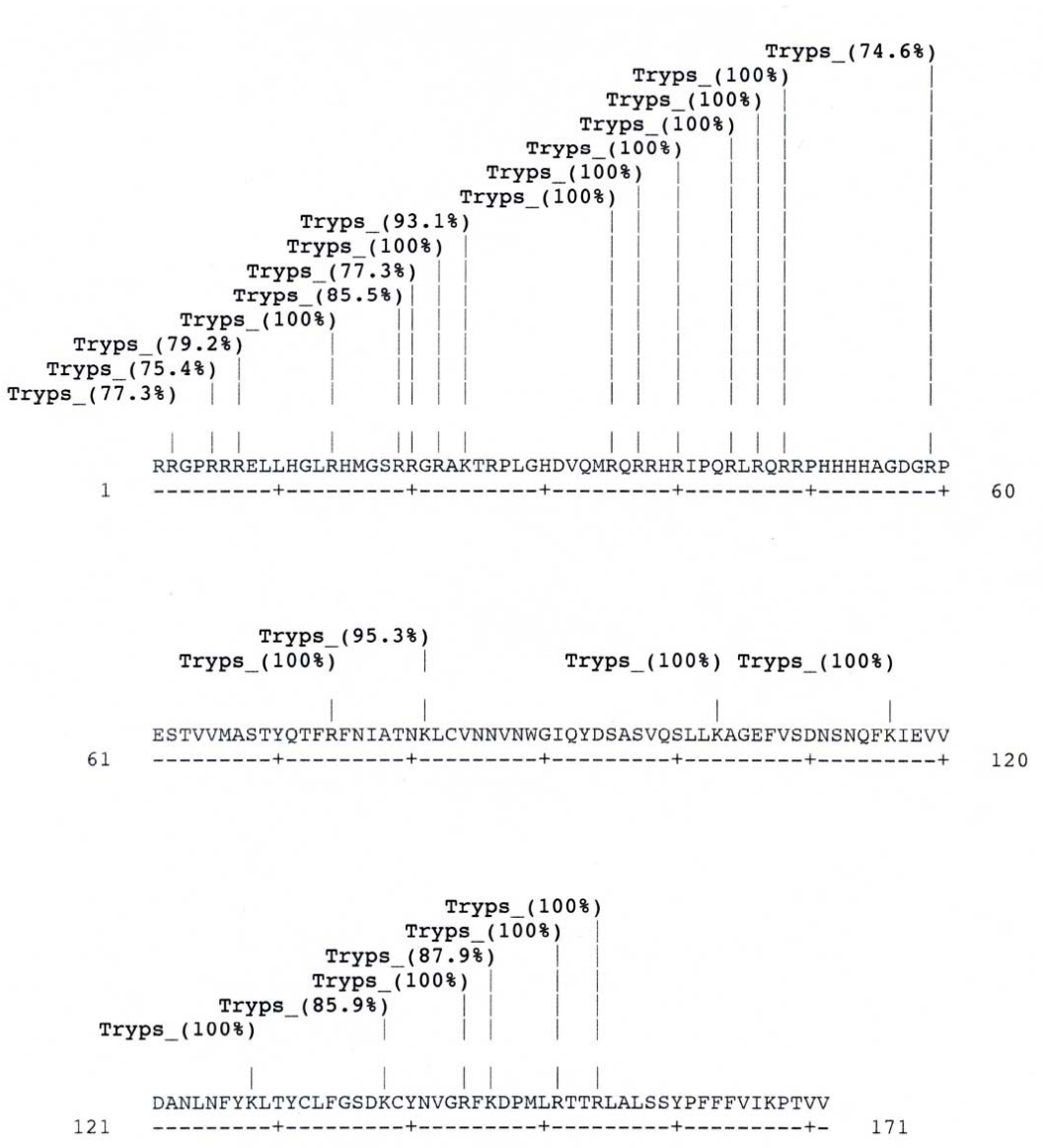

Figure 3.5. Theoretical trypsin cleavage sites of sporamin determined by inputting sporamin protein sequence from GenBank accession AAB52550 into ExPASy PeptideCutter. Likelihood of cleavage is shown in parenthesis. Only sites with a likelihood of cleavage greater than 50% are shown.

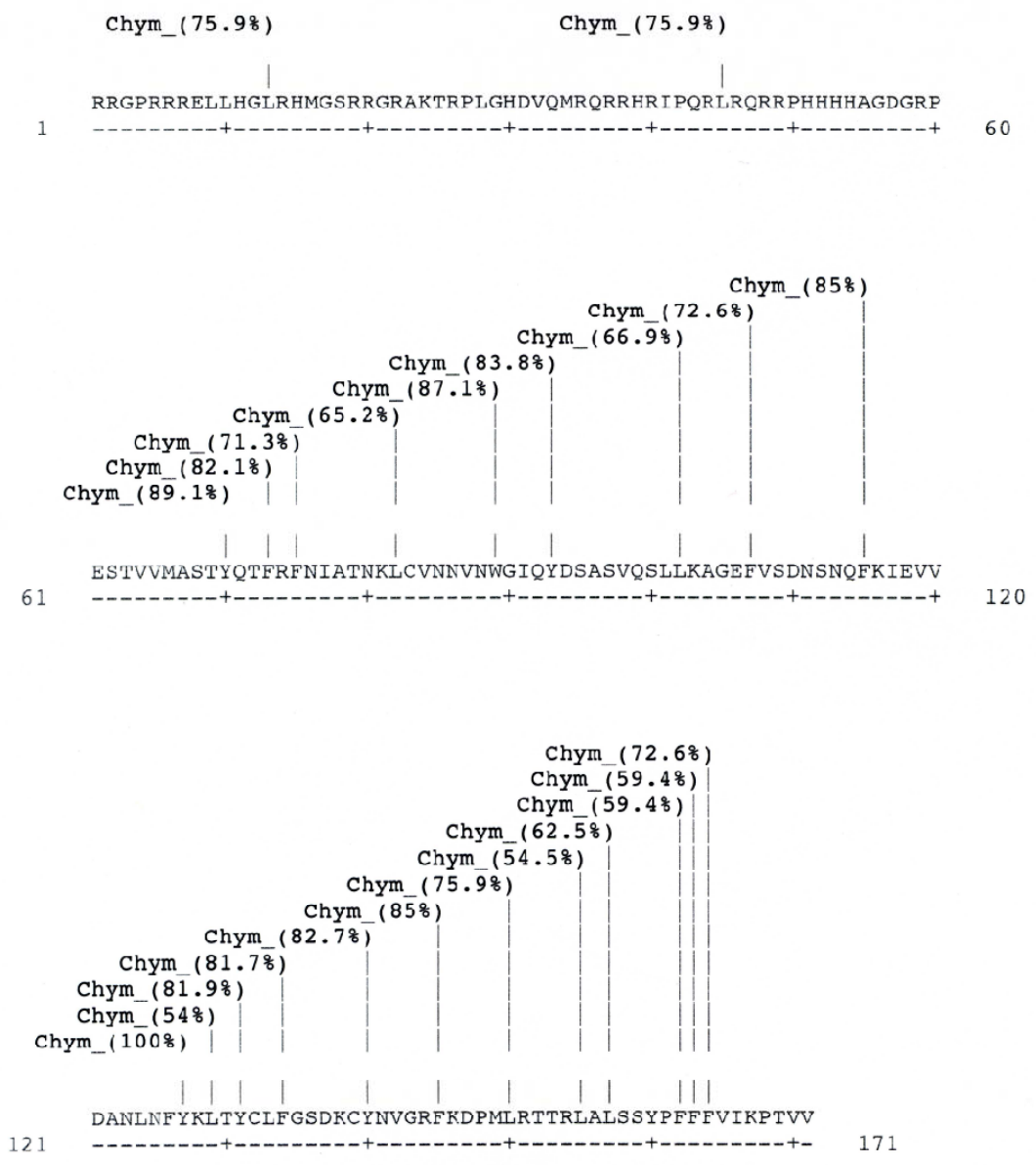

Figure 3.6. Theoretical chymotrypsin cleavage sites of sporamin determined by inputting sporamin protein sequence from GenBank accession AAB52550 into ExPASy PeptideCutter. Likelihood of cleavage is shown in parenthesis. Only sites with a likelihood of cleavage greater than 50% are shown.

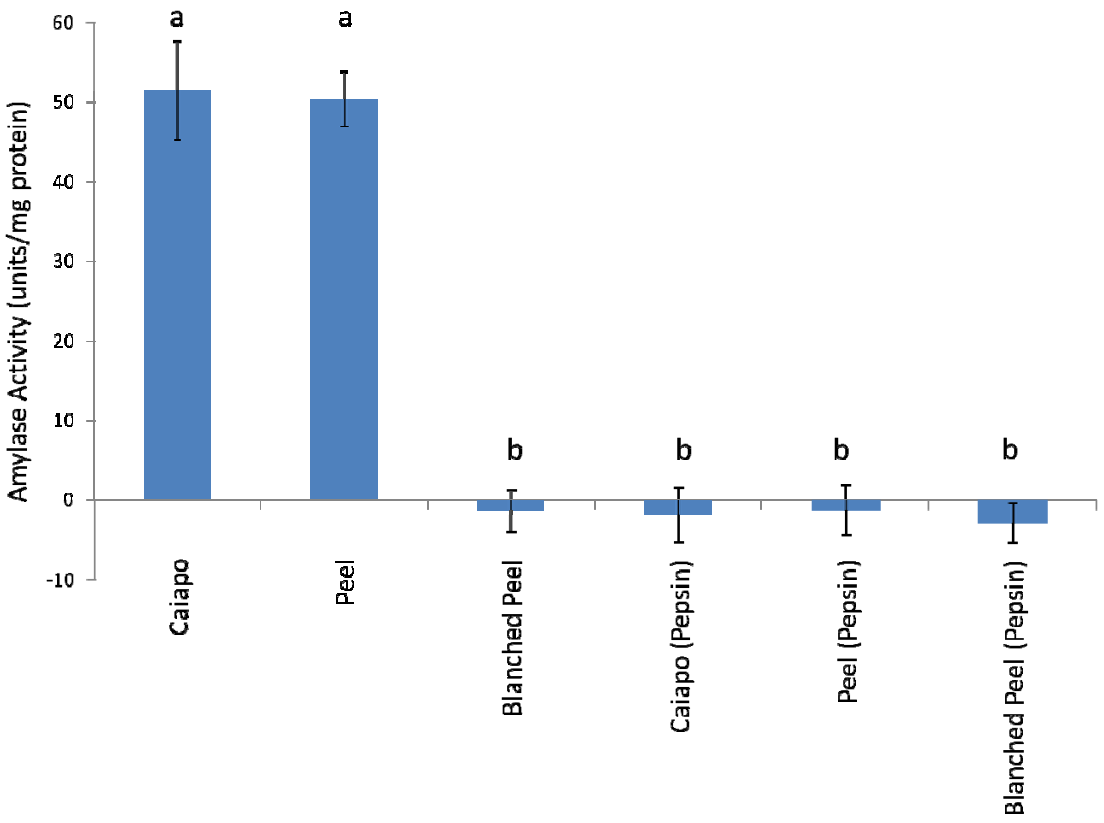

Figure 3.7. Amylase activity of Caiapo, peel, and blanched peel before and after incubation with pepsin for 1 hour at pH=2 and 37°C. Different letters represent statistically significant differences ($P < 0.05$).

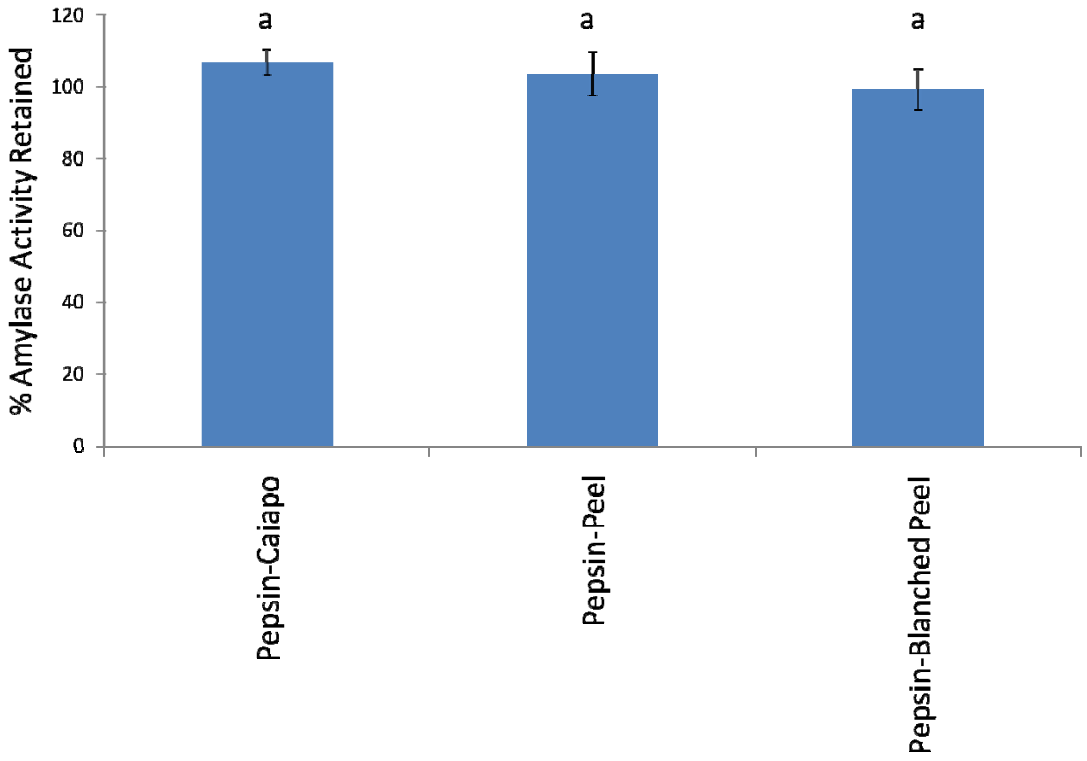

Figure 3.8. Activity of porcine pancreatic amylase (% retained) in the presence of Caiapo, peel, and blanched peel previously incubated with pepsin for 1 hour at pH=2 and 37°C. No statistically significant differences were observed ($P < 0.05$).

Table 3.1. Modified glucose tolerance tests in rats. Carbohydrate (300 mg) and protein (16 mg) were administered via gastric intubation. Average blood glucose values followed by standard deviation are given. No statistically significant differences in blood glucose were observed between treatments at 0, 1, or 2 hours ($P < 0.05$).

	Blood Glucose (mg/dl)		
Treatment	0 h	1 h	2 h
Starch + Casein	102 ± 26	128 ± 37	118 ± 24
Starch + Caiapo	96 ± 15	136 ± 29	121 ± 30
Starch + Peel Extract	110 ± 28	126 ± 20	123 ± 19
Starch + Flesh Extract	93 ± 12	122 ± 23	111 ± 22
Maltose + Casein	96 ± 34	120 ± 37	125 ± 32
Maltose + Caiapo	105 ± 36	127 ± 40	122 ± 22
Maltose + Peel Extract	111 ± 17	136 ± 18	130 ± 24
Maltose + Flesh Extract	96 ± 32	135 ± 34	106 ± 31
Glucose + Casein	101 ± 36	123 ± 36	124 ± 25
Glucose + Caiapo	101 ± 26	125 ± 21	128 ± 20
Glucose + Peel Extract	110 ± 23	125 ± 30	116 ± 22
Glucose + Flesh Extract	90 ± 18	118 ± 31	112 ± 36

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CHAPTER 4

Conclusions

Waste streams of the sweetpotato processing industry, such as peelings, contain valuable compounds, and could serve as an additional revenue stream provided these compounds could be efficiently extracted. This research, conducted in partnership with Yamco, LLC, a sweetpotato puree processing facility, showed that protein can be more efficiently extracted and concentrated from the secondary peeling after blanching of sweetpotatoes compared to the primary peeling before blanching. Using the determined optimum extraction (59.7 mL of 0.025 mM NaCl per g blanched peel) and precipitation (6.8 mM $CaCl_2$) conditions, 32.0% of the protein present in blanched peel was recovered in the extract, which consisted of 41.3% protein. Examination of activities during simulated gastric and duodenal digestions revealed that blanched peel retained some trypsin inhibitory activity after digestion, indicating that the protein responsible for the effect was not completely denatured by the blanching process and was resistant to digestive enzymes. Since sweetpotato trypsin inhibitors have been shown to possess antioxidant and anti-proliferative effects, their retained activity implies that a protein supplement made from blanched peel may provide nutraceutical benefits. In addition, proteins of the same molecular weight and glycosylation characteristics as those identified as anti-diabetic agents in white-skinned sweetpotatoes were seen in the extracts produced from orange-fleshed sweetpotatoes. Future research should focus on confirming the nutraceutical properties of the protein extracted from the peel of orange-flesh cultivars of sweetpotato.