HPLC AND MASS SPECTROSCOPIC CHARACTERIZATION OF MANGO (*Mangifera Indica* L.) GALLOTANNINS FOLLOWING ENZYMATIC HYDROLYSIS

A Senior Scholars Thesis

by

KIMBERLY ANN KRENEK

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

HPLC and Mass Spectroscopic Characterization of Mango (*Mangifera indica* L.) Gallotannins Following Enzymatic Hydrolysis. (April 2009)

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Mangos contain numerous compounds that have been shown to exhibit antioxidant properties. These compounds, most of which are polyphenolics, are linked to anti-cancer and anti-inflammatory activities in the body. Mangos more specifically boast a large number of high molecular weight compounds called gallotannins, composed of gallic acid units attached to glucose via a glycosidic linkage. It is unknown if these compounds are broken down into smaller molecules through the normal course of human digestion, or if food processing operations, such as the addition of a gallotannin-active hydrolyases, could be more effective in lowering the size of these molecules to increase the absorption and potential bioactivity. This research focused on understanding the chemical changes that occur to gallotannins derived from mangos following enzymatic hydrolysis and attempted to draw inferences relating to overall human health. Polyphenolics in mangos, cv. Ataulfo were extracted using a 1:1:1 acetone:ethanol:methanol mixture and further concentrated and clarified using a reverse-

phase C18 Sep-Pak cartridge. Mango extracts were treated with 20,000 U/ml and 13,000U/ml β-glucosidase with a time course of 2, 4, 6, and 8 hours in an optimal pH 5.0 citric acid buffer, and at a constant temperature of 35° C. Changes in mango polyphenolics following enzyme hydrolysis were monitored using a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source. B-glucosidase proved to be effective in the hydrolysis of some gallotannins but was incapable of hydrolyzing all gallotannins into free gallic acid. This was illustrated by the observance of an increase in penta, hexa, hepta-*O*- and a subsequent decrease in higher molecular weight compounds. The limitations for complete hydrolysis explains by the inability of β-glucosidase to cleave the glycosidic linkage due to steric hindrance created from having up to five gallic acid moieties attached to glucose, or from the inability of the enzyme to break *m*-dipside linkages between two or more galloyl groups. Incubating mango extract with both 20,000 U/mL and 13,000 U/mL resulted in an equivalent eight-fold increase in free gallic acid. Enzyme concentration was not the limiting factor in the hydrolytic reaction. Additionally, reaction time did not have a significant role in the hydrolytic rate, as the amount of free gallic remained relatively constant from 2 to 8 hours. These findings indicated that it was possible to increase low molecular weight gallotannin species following enzyme hydrolysis and will aid in future studies to understand the digestion and bioavailability mango phenolics.

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Lastly, but more importantly, I want to thank my family for being my biggest fans. I could not have asked for more love and encouragement from anyone. Thank you for supporting my dreams and being so interested in my work. Amanda, Hannah, and Colleen, thank you for being amazing sisters and serving as inspiration to me.

NOMENCLATURE

CV	Cultivar
L.M.W	Low Molecular Weight
H.M.W	High Molecular Weight
amu	Atomic Mass Unit

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CHAPTER I

INTRODUCTION: INCREASING MANGO BIOAVAILABILITY

Mango fruit overview

Mango (*Mangifera indica* L.) is a popular tropical fruit because of its unique taste, affordability and nutritional qualities. Mangoes are members of the family Anacardiaceae and are predominately grown in tropical and warm sub-tropical climates in places such as Asia, Africa, and the Americas. There are over one thousand different cultivars of mangos growing world-wide and mangos are considered to be one of the most widely eaten fruits. The mango is known to be an excellent source of many vitamins such as ascorbic acid, thiamine, riboflavin, and niacin, and ß-carotene. Not only are mangos rich in these nutrients, mangos are also high in non-nutrient phytochemical compounds. Recently much attention has been given to phytochemicals and the distinctive roles they play in anti-inflammatory and anti-cancer properties related to the consumption of fruits and vegetables. A need exists to chemically identify hydrolytic by-products from mango polyphenolics in an effort to eventually relate these compounds to the absorption of mango polyphenolics following human consumption.

This thesis follows the style of Journal of Food Science.

Mango phytochemicals

Extensive research has been conducted to quantify and characterize compounds from kernels, peels, and leaves of the mango (Barreto and others 2008; Berardini and others 2004). Mango pulp has proven to be more difficult however, and studies have been on-going to identify unambiguously compounds located in the pulp. Reports suggest that the phytochemical content of mango pulp consists of gallic acid, mangiferin, quercetin glycosides and many identified and uncharacterized hydrolyzable tannins (Schieber and others 2000). Other compounds in smaller concentrations include *p*-OH-benzoic acid, *m*-coumaric acid, *p*-coumaric acid, and ferulic acid (Kim and others 2007). In most mango varieties, free gallic acid, 3,4,5-trihydroxybenzoic acid, is the predominant compound present and has been shown to possess a high antioxidant capacity with numerous implications to overall human health (Shanrzad and Bitsch 1998). Gallic acid units possess three hydroxyl groups and an acid group which allow the compound to link with another gallic acid to form an ester, digallic acid (Masibo and He 2008). Gallic acid is an essential component to a group of compounds present in mango pulp called gallotannins.

Tannins

Tannins, in general, are water soluble phenolic secondary metabolites that range in size from 300 to 3000 Daltons (D) and are classified into four different groups based on similar structural characteristics. Such groups include gallotannins, ellagitannins, complex tannins and condensed tannins (Mingshu and others 2006). Tannins are known for their ability to bind proteins to form either soluble or insoluble complexes

(Hangerman and others 1992). Gallotannins are the predominant class of tannins identified from mango pulp and range in size from 787 – 1243 Daltons (Berardini and others 2004). Gallotannins consist of a sugar, primarily glucose, surrounded by several gallic acid units which can further be attached to other gallic acid units via a *m*-depside bond (Mueller-Harvey 2001). Research has suggested that the larger a polyphenolic compound is in size, the less the likelihood for intestinal absorption and subsequent bioavailability. Therefore, an important area of research is to understand the hydrolytic products of gallotannins as a means of understanding their absorption and bioactivity of the mango as a whole.

Enzyme treatment

Only by first evaluating enzyme hydrolysis techniques and understanding how these processes relate to the size and chemical composition of the resultant molecule can a later assessment of reduced molecular weight be made on potential health benefits of mangos. It is known that certain bacteria, yeasts, and molds produce enzymes that are capable of breaking down these gallotannins (Mingshu and others 2006). β-glucosidases can also be found in the epithelial cells of the small intestine where it is thought that sugar-linked molecules are broken into smaller units that are subsequently absorbed by these cells. (Nemeth and others 2003). Both β-glucosidase and tannase have been used for hydrolysis of tannins, but further studies need to be completed in this area to determine the most effective enzyme to use, or if a combination of the two enzymes could be more effective (Kikuzaki and others 2000). It is therefore hypothesized that

hydrolysis of gallotannins via the addition of β-glucosidase will increase the concentration of free gallic acid, as well as lower molecular weight polyphenolics, and will potentially enhance the bioavailability of mango polyphenolics. Quantifying and characterizing the gallotannins and their hydrolytic by-products will aid in future research with a goal of understanding industrial food processing techniques that will aid in better or more efficient uses of nutritionally dense foods such as mango.

CHAPTER II

METHODOLOGY

Mango extraction

Whole mango fruits, *Mangifera indica* L., cv. Ataulfo, were received in May and allowed to fully ripen. Upon ripening, peels and kernels were removed. Pulp was blended and stored at -20°C until needed.

Polyphenolic extraction

To extract polyphenolic compounds from mango pulp, 200 g of mango pulp was placed in a beaker with 600 mL of 1:1:1 acetone, methanol, and ethanol (v/v/v). The extract was initially filtered through cheesecloth to remove large particles and subsequently filtered through diatomaceous earth. This process was repeated twice, using the pulp remaining on the cheesecloth in the first filtrations and extracted in 400mL, 200mL, respectively. The extraction solvents were evaporated under reduced pressure at < 45°C using a rotary evaporator. Methanol was added to the extract and the extract was centrifuged to precipitate pectin. The supernant was collected, precipitate re-extracted, and methanol was again evaporated under reduced pressure at <45°C. The mango extract was washed through a Waters Sep-Pak® Vac 35cc 10g C18 cartridge to remove sugars, residual pectin, and to concentrate phenolic compounds. Bound phenolic compounds were fractioned using 25% methanol and 100% methanol to separate (LMW) low molecular weight compounds from high molecular weight compounds based on their

affinity to C18. Each extract was brought to a total volume of 100 mL in a pH 5.0 buffer solution.

Enzyme hydrolysis

The enzyme β-glucosidase was purchased from MP Biomedicals and stored at -20°C. An enzyme solution was prepared immediately prior to incubation in a pH 5.0 buffer with a concentration of 13,000 U/mL and 20,000 U/ml. One unit (U) of β-glucosidase will liberate 1.0µmol of glucose per minute at 35°C. Ataulfo mango fractions (stock, L.M.W., H.M.W) at a volume of 2.0 mL were treated with 200 µL of the prepared beta-glucosidase enzyme solution. The samples were incubated following a time course at 35°C, with samples pulled at 30 minutes, 1, 2, and 4 hrs. Following incubation, each sample was acidified to pH 3.0 to inactivate β-glucosidase.

Chemical analysis

Changes in mango polyphenolics following enzyme hydrolysis were monitored using a Thermo Finnigan LCQ Deca XP Max MS*n* ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). Separation of compounds was completed with a Dionex (Sunnydale, CA) Acclaim® 120 A, (4.6 X 250 mm; 5µM). Mobile phases used included 0.5% formic acid in water (solvent A), and 0.5% formic acid in acetonitrile (solvent B) at 0.4 mL/min. The mobile phases were run in a gradient program and began with 100% A for 5 minutes. Solvent B was changed from 0-30% in

25 minutes, to 50% in 15 minutes, to 100% in 25 minutes, back to 0% in 2 minutes. The gradient run was finished with 100% A for 8 minutes.

CHAPTER III

RESULTS AND DISCUSSIONS

Polyphenolic characterization

Compound 1 was determined to be a mono-galloyl glucoside. (fig. 1). This compound showed a predominate ion of *m/z* 331 and a loss of 162 amu in the MS^2 scan event. The loss of 162 corresponds to the loss of a glucose molecule minus a water molecule. The predominate ion in MS^2 was 169, the molecular weight of gallic acid.

Compound 2 was identified as gallic acid based on molecular weight, fragmentation pattern, and wavelength. An ion of *m/z* 169 was most abundant and MS^2 showed a fragmentation pattern of 125 *m/z*. This pattern is typical of benzoic acid derivatives. (Barreto and others 2008). A wavelength of 271 was observed, characteristic of gallic acid.

Compound 3 was determined to be mangiferin. A predominate ion of *m/z* 421 was found followed by *m/z* 301, 331, and 403 at MS^2. This identification is supported by (Shieber and others 2003).

Compound 4 showed a *m/z of 938,* which corresponds to the molecular weight of a penta-galloyl glucoside. This determination was further supported by the fragmentation

pattern observed at MS^2. The predominate ion was 768 followed by 787 *m/z.* A molecular weight of 787 is the known molecular weight of a tetra-galloyl glucoside.

Compound 5 was determined to be a hepta-galloyl glucoside. This compound showed a *m/z* of 1242. In MS^2, a fragmentation pattern of 1089, 939, and 787 *m/z* was observed. The predominate ion; *m/z* of 1089, can be identified as a hexa-galloyl glucoside (Berardini and others 2004).

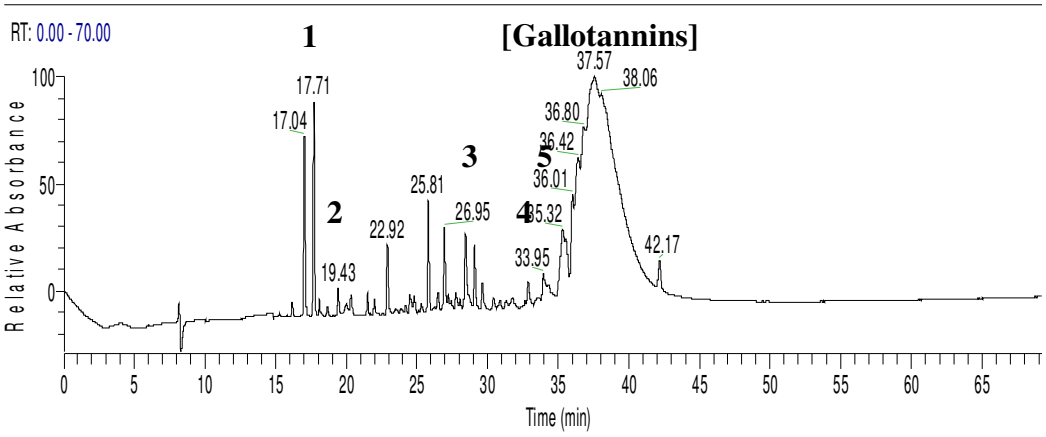

Figure 1 – Chromatograph of Mango polyphenolics cv. Ataulfo before enzymatic hydrolysis. Compounds 1-5 were identified using and HPLC-MS^n –ESI.

Table 1- Change in identified mango polyphenolic compounds after enzyme hydrolysis

peak #	compound name	Hydoylsis A	Hydrolysis B
1	mono-gallyol glucoside	(-) 90%	(-) 94%
2	gallic acid	(+) 88%	(+) 88%
3	mangiferin	(+)40%	(+) 6%
4	penta-galloyl-glucoside	(+)50%	(+) 35%
5	hepta-galloyl glucoside	(+)42%	(+) 32%

Hydrolysis A: 13,000 U/mL β-glucosidase, Hydrolysis B: 20,000 U/mL β-glucosidase. (+), (-) indicates increase or decrease

Enzymatic hydrolysis

It was hypothesized that β-glucosidase would be effective in hydrolyzing gallic acid units attached to glucose via a glycosidic linkage, and thus an increase in gallic acid would be observed. Incubation of the polyphenolic mango extract with 13,000 U/mL β-glucosidase resulted in a decrease in the amount of compound 1 present in the extract by 90%, and an increase in compound 2,3,4, and 5 by 88%, 40%, 50%, and 42%, respectively (Table 1). The same extract was treated with 20,000 U/mL β-glucosidase presented similar results. Compound 1 decreased by 94%, and compounds 2,3,4, and five increased by 88%, 6%, 35%, and 32% respectively. Figure 2 and Figure 3 show the increase and decrease in peak areas of the previously mentioned compounds based on the amount of β-glucosidase used in incubation.

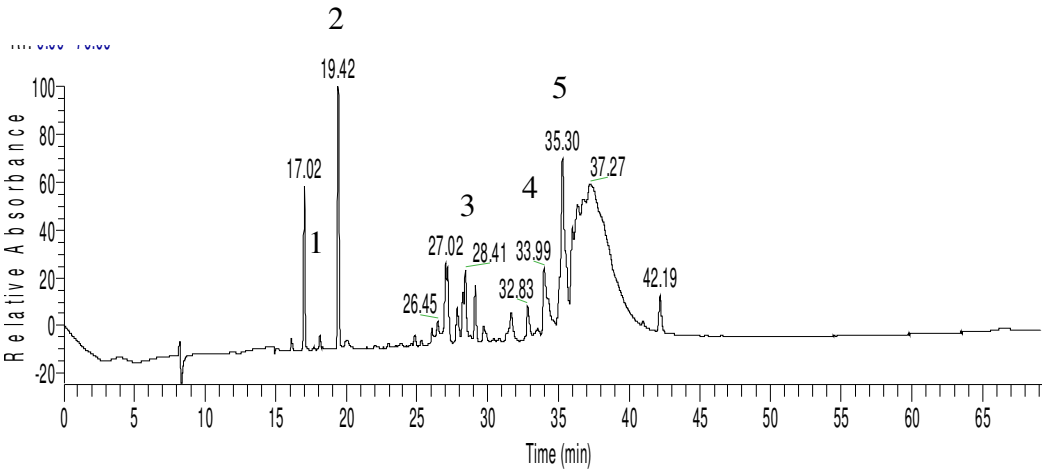

Figure 2- Chromatograph of mango polyphenolics cv. Ataulfo after enzymatic hydrolysis with 13,000 U/mL β-glucosidase.

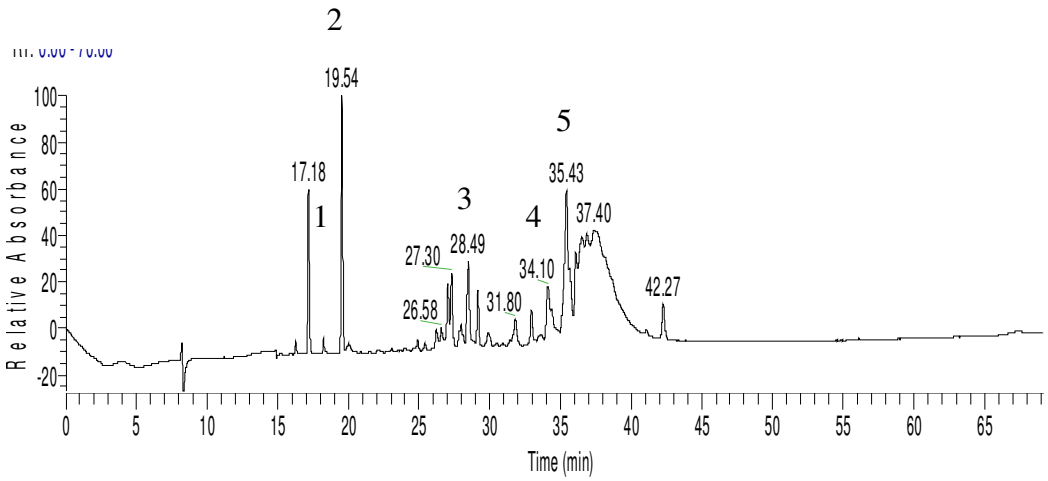

Figure 3- Chromatograph of mango polyphenolics cv. Ataulfo after enzymatic hydrolysis with 20,000 U/mL β-glucosidase.

Compound 1, a mono-galloyl glucoside, almost completely disappeared in the chromatograms produced from the extracts subjected to enzymatic hydrolysis. This can be explained by the ease in which β-glucosidase is able to cleave the bond between the glucose molecule and the gallic acid unit. The increase in gallic acid is attributed in most part to the hydrolysis of the mono-gallyol glucosides present in the extract.

Compound 2, gallic acid was the target compound for this study. After hydrolysis with β-glucosidase, an 8-fold increase in gallic acid was observed from incubation with both 13,000 U/mL and 20,000 U/mL β-glucosidase. If all gallotannins were hydrolyzed into their respective moieties, the amount of increase observed after hydrolysis would be expected to be higher than an 8-fold increase.

Compound 3, mangiferin, also increased after enzymatic hydrolysis. Mangos peels are known to contain mangiferin gallates. Mangiferin gallates have one gallic acid unit attached to the glucose molecule in the usual mangiferin structure via a glycosidic

linkage (Barreto and others 2008). This increase in mangiferin can be credited to the hydrolysis of the previously mentioned mangiferin gallates that are present in lower concentrations in the pulp of mangos.

Compounds 4 and 5 are both gallotannins that were identifiable. Both the hepta-galloyl glucosides and the penta-galloyl glucosides increased by 30-50%. This increase in the middle weight gallotannins can be explained by the ability for β-glucosidase to cleave only certain strategic bonds on the gallotannin structure, but cannot cleave every bond. A noticeable decrease in the gallotannin "hump" can be observed. This may be a false positive for HMW gallotannins hydrolysis because gallotannins have the ability to bind protein. (Feldman and others 1998). This binding can be seen as a precipitate formed during the incubation period and may have resulted in an inaccurate determination of gallotannin hydrolysis.

Figure 4 illustrates the effect that enzyme concentration had on the amount of hydrolytic products identified. Incubating the mango extract with 13,000 U/mL β-glucosidase versus 20,000 U/mL β-glucosidase did not have an effect of hydrolytic products. Figure 5 shows how reaction time affected enzyme hydrolysis. Maximum hydrolysis was reached between two to four hours of incubation time at 35°C in the pH 5.0 buffer.

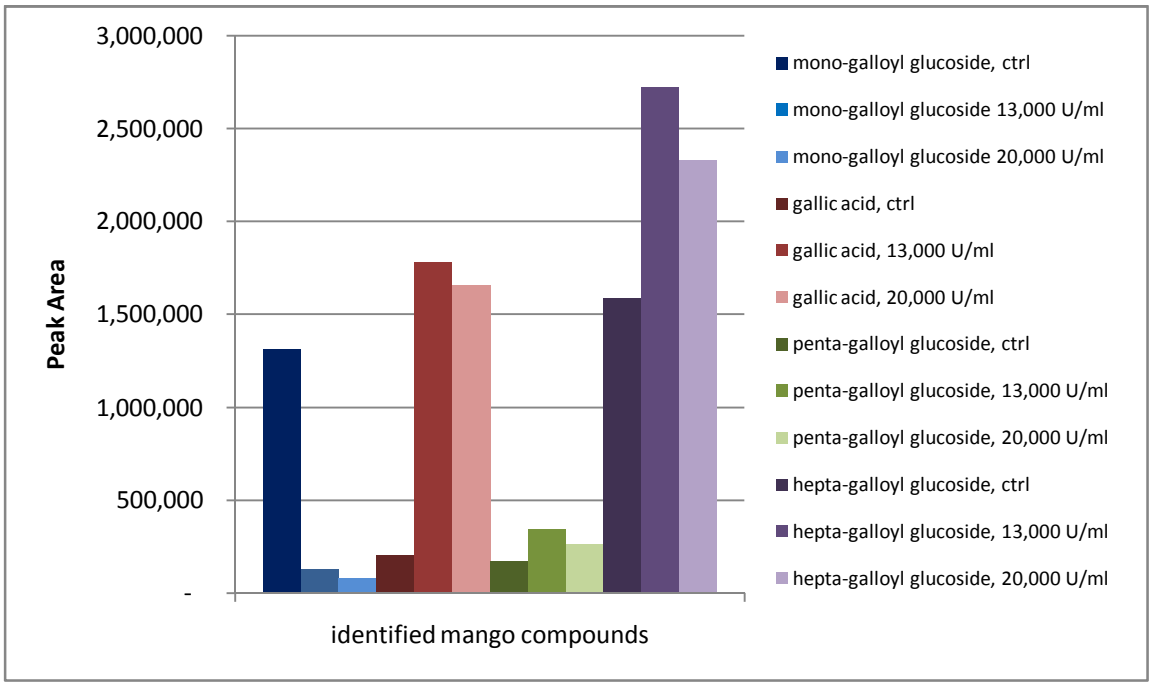

Figure 4 - The effect of β-glucosidase concentration on the hydrolysis of mango polyphenolics.

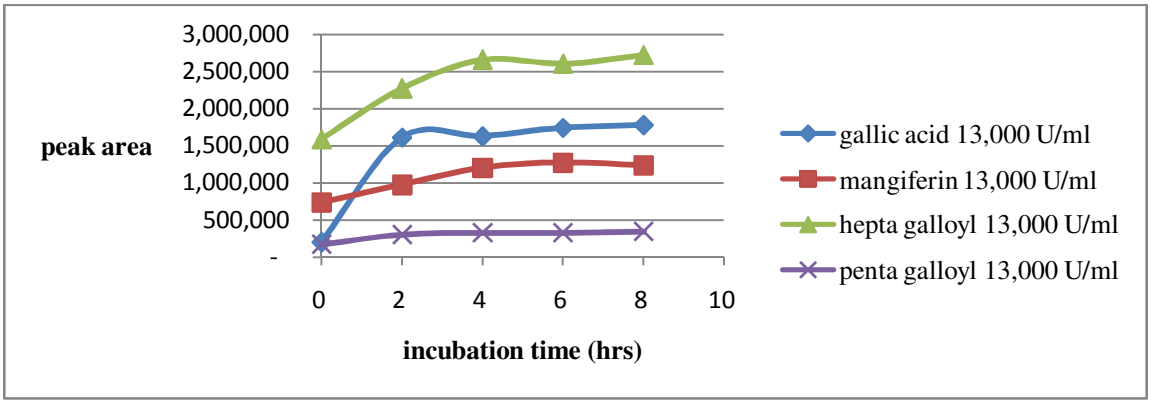

Fig. 5 - The effects incubation time had on mango polyphenolic hydrolysis.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The hydrolyase enzyme β-glucosidase proved to be effective in the hydrolysis of some gallotannins, but the hydrolytic reaction was never successfully run to completion. This was illustrated by the observance of an increase in middle-weight gallotannins, (penta, hexa, hepta-gallotannins) and a decrease in high molecular weight compounds. The limitations for complete hydrolysis could be explained by the inability of β-glucosidase to cleave the beta-linkage due to steric hindrance created from having five gallic acid moieties attached to glucose. Incubating mango extract with both 20,000 U/mL and 13,000 U/mL resulted in an eight-fold increase in free gallic acid. Therefore, enzyme concentration did not seem to affect gallotannin hydrolysis when the difference was not greater than around 10,000 U/mL. Time did not play a significant role in the hydrolysis of gallotannins, as the amount of gallic cleaved remained relatively stable from 2 to 8 hours. These findings showed an increase in lower molecular weight species following enzyme hydrolysis and will aid in future studies to understand the digestion and bioavailability mango phenolics.

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