Kiwifruit Effects on Starch Digestion by Salivary Amylase under Simulated Gastric Conditions

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Abstract

Background Postprandial hyperglycaemia is a risk factor for diabetes and its complications, and it is influenced by the rate of carbohydrate digestion and absorption. Human salivary amylase (HSA) initiates starch digestion in the mouth and plays an important role in starch digestion as it may continue starch digestion in the stomach during gradual gastric acidification. Previous research has shown that Hayward kiwifruit lowers the glycaemic response to co-ingested cereal foods. However, the underlying mechanisms have not been identified; many factors, including acidity combined with the buffering capacity of fruit organic acids, and the action of the kiwifruit protease, actinidin, may play a part, and interact with one another during digestion.

Aim To investigate effects of individual factors (fruit buffering capacity, acidity, pepsin and actinidin) and their interactions on HSA activity during 30 minutes of simulated gastric digestion and so to elucidate their role in the glycaemic response-lowering capacity of kiwifruit co-ingested with cereal foods.

Methods A semi-dynamic gastric model with automated titration was developed to mimic gastric digestion. This project had 6 stages: Stage 1: Determining the buffering capacity of various fruits. Stage 2: Development of a $CaCl_2$ fortified potassium-citrate buffer to approximate kiwifruit organic acid (referred as Ca-KF buffer), and testing pH effects on HSA activity during gastric acidification to pH 2.0. Stage 3: determining effect of Hayward kiwifruit, SunGold kiwifruit and Granny Smith apple on HSA activity during acidification. Stage 4: Testing effect of pepsin on HSA activity during acidification. Stage 5: Determine interaction of pepsin and actinidin from Hayward kiwifruit on HSA activity during acidification. Stage 6: Measure effect of pepsin with the three fruits in stage 3 on HSA activity in a Weet-Bix™ meal. HSA activity was measured as

sugar released in 15 and 30 minutes using a colorimetric assay. Protein analysis by SDS-PAGE and a kinetic assay were used to examine effects of pepsin-actinidin interaction on HSA survival in stage 5.

Results Buffering capacity differed between fruits. HSA activity decreased as pH dropped from 7.0 to 2.0 with little decrease in activity above pH 4.0. The Ca-KF buffer significantly inhibited HSA activity at a pH range of 3.0 to 6.0 ($p<0.01$) compared to $CaCl_2$ fortified saline solution. Pepsin inhibited HSA activity at pH 4.0 and below. Hayward kiwifruit significantly inhibited HSA activity at pH 4.0 and below ($p<0.01$) with the strongest inhibition at pH 4.0. A significant but small inhibition was contributed by SunGold kiwifruit ($p<0.01$) as well as Granny Smith apple ($p<0.05$) at pH 3.0 and below. HSA activity was inhibited more by pepsin alone than by pepsin and actinidin combined at pH 3.0 and below. Actinidin inhibited HSA activity more than pepsin alone at pH 4.0.

Conclusion The low pH and strong buffering capacity of kiwifruit lead to immediate inhibition of HSA activity in the stomach as well as promoting early activation of pepsin, which also inactivates HSA. Actinidin in Hayward kiwifruit actively degraded HSA at its optimal pH around 4.0 but pepsin degraded both actinidin and HSA when pH fell below 3.0. Overall, Hayward kiwifruit had a potential in retarding HSA in the stomach and in turn might lower glycaemic response to co-ingested starch.

Preface

This study was conducted under supervisor of Dr Bernard Venn from the Department of Human Nutrition at the University of Otago and in collaboration with Dr John Monro, Dr Suman Mishra and Dr Harry Martin from the Plant and Food Research Institute.

The candidate was responsible for

- Input into the study design and experiment protocol
- Preparing experiment materials, including purchasing test foods, blending fruits into pulps, weighing test foods and starch, making up chemical solutions, etc.
- Purchasing and setting up an overhead stirrer needed for low-speed stirring
- Setting up an automated titrator and optimizing titration protocol
- Undertaking laboratory health and safety trainings and equipment trainings including use of centrifuge, -80 °C freezer, titrator, spectrophotometer, microplate reader, etc.
- Learning and conducting starch digestion analysis, SDS-PAGE, actinidin colorimetric assay as well as Western blotting and HSA colorimetric assay that were not included in this thesis
- Inputting all experiment data for calculation and statistical analysis and interpreting results
- Writing this thesis

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List of Abbreviations and Nomenclature

<	Less than
>	More than
µl	Microlitre
µM	Micromolar
CHO	Carbohydrate
CN	Copy number
DI	Distilled
DNS	3,5-Dinitrosalicylic acid
EP	End point
FW	Fresh weight
g	Gram
GI	Glycaemic index
GL	Glycaemic load
HSA	Human salivary α-amylase
IDF	International Diabetes Federation
KF	Kiwifruit
L	Litre
M	Mole per litre
mEq	Milliequivalent
mg	Milligram
min	Minute
ml	Millilitre
mol	Mole
MW	Molecular weight

nm	Nanometer
OD	Optical density
ppm	Parts per million
RCT	Random control trial
rpm	Revolutions per minute
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SET	Set end-point titration
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

1. Introduction

In 2017, the International Diabetes Federation (IDF) reported a high prevalence of diabetes with 425 million adults (1 in 11 adults) worldwide diagnosed with the condition, while 50% of cases remain undiagnosed (1). The majority (90%) of cases are type 2 diabetes mellitus (T2DM) (2). Diabetes is estimated to contribute to 4 million early deaths annually and to cost 12% of the global health expenditure (727 billion USD) (1) as a result of ill health caused by direct and indirect effects of elevated blood sugar, which is a defining feature of diabetes. Given its enormous burden on mortality, morbidity and health economics (3), it is important to manage diabetes by controlling blood glucose, dietary and lifestyle factors which are important elements of blood glucose management especially for those who suffer from T2DM (4).

T2DM is a chronic metabolic disorder characterised by persistent hyperglycaemia due to a combination of pancreatic β-cell dysfunction and insulin resistance (5). Major risk factors for T2DM are obesity (6), sedentary lifestyle (7) and ageing (8). Additionally, diets with high glycaemic index (GI) and load (GL) have been found to be associated with risk (9). Studies on GI and GL have revealed a positive relationship between the *in vivo* rate of carbohydrate digestion and the glycaemic response to ingesting carbohydrate-containing foods (10-12). Starch is the main digestible carbohydrate in the human diet and abundant in staple foods such as potatoes, beans and cereals such as maize and rice, etc. (13). Understanding parameters governing the rate of starch digestion and associated glycaemic response is key to moderating the impact of diet on blood glucose in T2DM management.

Human salivary α-amylase (HSA) initiates starch breakdown in the mouth and its action continues in the stomach before the breakdown products and remaining starch enter the small

intestine for more complete hydrolysis by pancreatic α-amylase and brush border enzymes (13) before absorption. HSA is a major digestive enzyme in human saliva that breaks down amylose and amylopectin by cleaving α-1,4-glycosidic bonds (14). The enzyme has optimal activity at a pH around 5.0 at a temperature of 37 °C (15). Given the limited contact time that saliva has with foods in the mouth and its inactivation in an acidic stomach environment, the significance of HSA in starch hydrolysis was often thought to be negligible compared to pancreatic amylase (16-18). However, studies back in the 1900s found HSA activity in human duodenal juice suggesting the intact passage of HSA through the stomach and even reaching the duodenum (19-21). More evidence has led to the speculation that HSA is able to pass through the gut intact under protection of starch oligosaccharides and other dietary components, while hydrolysing a substantial amount of starch (20, 22, 23).

In the stomach, masticated food contents, so called boluses, are chemically and physically disintegrated by gastric secretions and gastric peristalsis to form chyme which is periodically emptied into the small intestinal for neutralization and further digestion at the pH optimum of the pancreatic amylases. (24). Although gastric acid has a pH close to 2.0, acidification generally starts at a higher pH and gradually declines to below 4.0, which inactivates HSA in up to 45 min depending on food contents (25). Therefore, food compositions and buffering capacity are important determinants of gastric acidification, continued HSA activity, and gastric emptying (GE) (26), and subsequent digestion and absorption in the small intestine (27).

Incorporating organic acid into foods (e.g., breads enriched with lactic acid) or co-consuming it with other foods (e.g., vinegar dressing on starchy foods) has been found to lower postprandial glycaemic response and attributed to delayed GE (28, 29). With a high content of vitamin C and a range of other organic acids, as well as dietary fibre, kiwifruit has been found to have healthful qualities (30). The non-sugar components of kiwifruit have been found to exhibit a glycaemic

response-lowering effect on co-ingested carbohydrates during *in vivo* gastrointestinal digestion (31). This suppression of glycaemic response by kiwifruit is possibly due to the acidity of the fruit combined with the buffering capacity of its organic acids (31), with a possible contribution from dietary fibre and various phytochemicals. In addition, the natural protease actinidin may play a role by degrading HSA.

Green kiwifruit (*Actinidia deliciosa* cv. Hayward) in particular is abundant in actinidin, which digests a broad range of proteins including HSA at its optimum pH around 4.0 (32). Limited but clear evidence has demonstrated that actinidin from green kiwifruit extracts is able to enhance gastric digestion and GE of a number of large dietary proteins (Molecular Weight>30 kDa) *in vitro* (33) and in rat and pig models (34, 35). However, little is known about the role of pH in gastric digestion involving kiwifruit except one recent study reported kiwifruit actinidin exerted little influence on gastric protein digestion when the pH was below 3.1 (36).

Several gaps in the literature can be identified. Firstly, evidence of the effect of pH on prolonged salivary digestion of starch that occurs under postprandial conditions in the stomach is scarce, with little attention given to the effect of gradual pH change on enzymes. Only Freitas' (24) gastric model has accounted for the physiological fact that stomach digestion starts at a near neutral pH and gradually acidifies. Secondly, little evidence is available to help to define and quantify the buffering capacity of kiwifruit that may be responsible for delaying GE (31). Moreover, little is known about the degradation of HSA by kiwifruit actinidin and pepsin, respectively, or how the interaction of actinidin with pepsin affects HSA survival under gastric conditions.

This masters project aimed to explore the role of kiwifruit components on starch digestion in the stomach using a semi dynamic model to monitor effect of changing gastric pH on HSA activity.

The research is novel in several respects: (1) measuring the effects of kiwifruit on gastric pH and buffering capacity, and comparing its buffering capacity with that of other fruits; (2) monitoring gastric activity of HSA at changing pH; (3) measuring gastric activity of HSA in the presence of the kiwifruit protease actinidin; (4) investigating the interaction of pepsin with actinidin and effect of the interaction on gastric survival of HSA. The project consisted of 6 stages starting from looking at individual factors (i.e. fruit buffering capacity, pH, pepsin, actinidin) on HSA activity under gastric conditions to the combination of multiple factors so that the mechanism can be understood step by step.

The hypotheses guiding the research were: (1) Organic acids in fruit lead to postprandial gastric acidification while conferring a buffering capacity that is different among fruits (i.e. green kiwifruit, gold kiwifruit, berries, apples and orange); (2) Starch hydrolysis by HSA in the stomach changes as a function of gastric pH; (3) HSA activity in the stomach is sufficient to extensively degrade starch before inactivation by changing pH; (4) HSA is degraded by pepsin as a function of pH; (5) HSA is degraded by kiwifruit actinidin as a function of pH; (6) the interaction of pepsin with actinidin under gastric conditions affects their joint degradation of HSA and is pH dependent. By addressing the foregoing hypotheses, the proposed research will help to clarify the possible role of organic acids, pH and actinidin activity in retarding the postprandial gastric degradation of ingested starch by HSA. By so doing it may help to identify their contribution to the ability of kiwifruit to reduce the glycaemic response to co-consumed starchy cereal in an equal carbohydrate exchange format. That is, it may support a role for kiwifruit in a healthy diet for managing T2DM.

2. Literature Review

This literature review discusses the effect of pH, and some kiwifruit components, on HSA activity under gastric conditions. It identifies gaps in our knowledge by providing an overview and analysis of the following topics:

- Importance of glycaemic response in diabetes
- Role of starch digestion in the glycaemic response
- Significance of HSA in starch digestion and survival in the gut
- Impact of kiwifruit organic acids and actinidin on gastric HSA activity
- Effect of kiwifruit in gastric digestion of starch with regards to GE

2.1. Search strategy

The literature search was carried out on Web of Science and Google Scholar using key words “glycaemic response”, “diabetes”, “salivary amylase”, “starch digestion”, “gastric digestion”, “gastric acidification”, “buffering capacity”, “gastric emptying”, “organic acids”, “citric acid”, “actinidin” and “pepsin”. Additional literature was obtained from reference lists and cross-references in published articles.

2.2. Glycaemic response and diabetes

The main burden of ill health associated with diabetes is well established as being the direct and indirect result of elevated blood glucose concentrations, both postprandial and chronic (2). Elevated postprandial blood glucose is thought to contribute to the syndromic character of

diabetes and its complications through combinations of several mechanisms, including exhaustion of β-cell function in the pancreas due to excessive insulin demand, non-specific glycation of biomolecules throughout the body, and indirect widespread damage and loss of immune function due to hyperglycaemia-induced oxidative stress (37).

In addition to its role in the health consequences of diabetes, hyperglycaemia may also play a role as an antecedent to diabetes through its contribution to obesity, which is one of the major risk factors for diabetes (6). Rapid digestion of starchy foods may contribute to obesity through a coupling of acute insulin response with postprandial glycaemia favouring lipogenesis and lack of satiety due to rapid food digestion (38). Therefore, the rate of starch digestion is a critical factor in dietary management of both the risk and consequences of diabetes.

Unlike type 1 diabetes mellitus (T1DM), T2DM is a non-insulin-dependent disease that is developed later in life and attributed to risk factors such as ageing (8), overweight and obesity (6), sedentary lifestyle (39) and unhealthy diets (9). The latest report by the International Diabetes Federation (IDF) on the global prevalence of diabetes claimed that in 2017, 425 million adults aged 20 to 79 years old around the world suffered from diabetes, with a mortality of 4 million lives and a cost of 727 billion USD of health expenditure because of hyperglycaemia and related complications, such as hypertension and cardiovascular disease (1). Almost half of the diabetic population was unaware of their condition (1) and 90% of them suffered from T2DM (2). Improving lifestyle and dietary factors to maintain a normal blood glucose level are important approaches to T2DM management and prevention, especially in developing countries where access to medical resources is limited (4).

2.3. Starch digestion

Starch comprises a large proportion of dietary carbohydrate, consumed in the form of potatoes, corn, rice, legumes and other cereals(13). Starch is made up of glucose residues linked by α-1,4-glycosidic bonds in linear segments and α-1,6-glycosidic bonds at branch points (15), and generally consists of 20-30% amylose (linear) and 70-80% amylopectin (branched). The compact linear structure of amylose makes it less accessible to α-amylase than amylopectin so it is more slowly hydrolysed than amylopectin (40). HSA produced by the salivary glands initiates starch digestion in the oral cavity by hydrolysing starch into smaller oligosaccharides while food is transformed into a bolus through mastication (24). Within a minute of ingestion (depending on variables such as food quantity, texture and flavour), boluses are swallowed and enter the stomach, where they are physically and chemically broken down, weakened by enzyme activities in gastric secretions at low pH and disintegrated by peristaltic contractions of the stomach wall, before being periodically emptied to the duodenum as chyme (24, 41). In the small intestine, pancreatic amylase and brush border enzymes complete the hydrolysis of starch and oligosaccharides to glucose for absorption into the bloodstream (15). Elevated blood glucose level triggers the release of insulin by pancreatic β-cells which facilitates glucose uptake by cells and lower blood glucose (5).

2.3.1. Rate of starch digestion and glycaemic control

As sugar release from dietary carbohydrates is the direct cause of rise in blood glucose, a knowledge of how carbohydrates, in this instance starch, are digested is key to glycaemic control. Several lines of evidence have indicated that the rate of digestion is an important factor of glycaemic response to foods (9-12). It has been found that the intrinsic digestibility of starch

in foods, measured by *in vitro* digestive analysis with pancreatic amylase, predicts glycaemic response to carbohydrate in foods when the foods are consumed by humans (42). In an randomised crossover trial, Ells et al (10) found that slowly digestible starch (~90% glucose released in 70 min *in vitro*) evoked a slower and more sustained rise in blood glucose compared to rapidly digestible starch (~90% glucose released in 10 min *in vitro*) which resulted in a sharp rise and fall in blood glucose.

Glycaemic index (GI) and glycaemic load (GL) provide measurements of the relative blood glucose raising ability of a fixed amount of carbohydrate in foods (GI) and when whole foods are eaten in variable amounts (GL). Both GI and GL are reported to be positively associated with risk of T2DM in a meta-analysis of 21 cohort studies (9). However, the data are not consistent as in three prospective studies, no significant associations were found between these dietary factors and risk of T2DM (43-45) while in two other studies, a positive association was found with GI but not GL (12, 46). Given the nature of observational studies, these results should be interpreted with caution. A meta-analysis of 10 randomised control trials (RCTs) showed low GI diets had marginal benefit on glycaemic control in diabetic patients by reducing medium-term glycated haemoglobin (HbA1c) levels (47). This was agreed by another meta-analysis of 6 recent RCTs which demonstrated significant difference ($p<0.001$) between low and high GI diets in HbA1c levels in patients with T2DM (48). However, a recent meta-analysis involving 185 prospective studies and 58 clinical trials revealed a weak association between dietary GI and GL with health (49). More large-scale RTCs are needed to verify the long-term effect of low GI and GL diets on glycaemic control in T2DM management.

2.4. Human salivary α-amylase (HSA)

HSA (α-1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1) is a major digestive enzyme in human saliva and accounts for 40-50% of salivary protein (50). This enzyme has a molecular weight of 56 kDa in its non-glycosylated form and 62 kDa in its glycosylated form (51). HSA consists of a single polypeptide chain of 496 amino acids folded into three domains (A, B and C) with the active site located in a $(\beta/\alpha)_8$-barrel in Domain A (52). In catalytic action, HSA cleaves α-1,4-glycosidic bonds in amylose and amylopectin yielding maltose, maltotriose and α-dextrin (14).

2.4.1. AMY1 gene copy number variation

HSA production and enzymatic activity levels vary among and within individuals and are subjected to regulation by environmental factors such as circadian rhythms (53) and physical and psychological stress (54). Genetic factors also account for between-person variability as copy number (CN) of the HSA coding gene *AMY1* varies from 1 to 15 among individuals (55). A positive correlation between *AMY1* gene CN and its expression and activity has been demonstrated in previous studies (55-57) although evidence on determinants of variation in *AMY1* CN is mixed (55, 57-59). Perry et al (57) reported a significantly larger CN of *AMY1* gene in populations historically fed on a high-starch diet (e.g., European Americans and Japanese) than that of populations on a low-starch diet (e.g., rainforest hunter-gatherers and Yakutians) possibly due to natural selection favouring more efficient starch digestion.

2.4.2. HSA survival in the gut

Evidence for an impact of HSA on starch digestion is mixed, which is understandable given the short duration of the oral phase of digestion, and deactivation of HSA by gastric acidity. HSA activity has an optimum pH range of 5.0 to 7.0 (15, 60). Fried et al (19) reported that inactivation of HSA was observed by a gastric pH between 3.8 and 3.3 *in vitro* and pH 3.0 and below *in vivo*. However, HSA is unlikely to become inactivated immediately upon entering the stomach, as adjustment of the gastric pH after consuming a solid-liquid meal (pH~6.7) to the fasted state (pH~1.7) took approximately 2 hours (25). Gastric acidification is therefore a gradual process, taking 45 to 60 minutes to reduce the pH to below pH 4.0, depending on the meal composition and its initial pH (61).

A few early human studies in the 1900s provided evidence for researchers to speculate that HSA may still remain active while passing through the gastrointestinal tract (19, 21). Bergeim (20) reported that up to 76% and 59% of starch in mashed potatoes and bread respectively, was hydrolysed in the stomach. Little is known about the mechanism behind passage of intact HSA through the stomach. Skude et al (21) found HSA activity was present in over 75% of duodenal aspirate samples and accounted for 15% and 40% of total amylase activity in normal subjects and chronic pancreatitis patients, respectively (21). Similar observations were made by Fried et al (19) who estimated that HSA accounted for 11% of total amylase in duodenal samples of a normal subject compared to 27% of that in an achlorhydric subject after a hamburger meal. In addition, the hydrolytic products of dietary components might in part protect HSA from gastric inactivation (19). This hypothesis was supported by Rosenblum et al (22) who found in *in vitro* studies that 1% starch was able to preserve 56% of HSA activity after 60 min exposure to pH 3.0 at 37 ^{0}C and up to 5% maltose and maltotriose also conferred significant protection of HSA activity against low pH.

2.4.3. A dynamic gastric model investigating role of HSA in starch digestion

An extensive amount of work has been done investigating the kinetics of starch digestion *in vitro* with little attention given to the oral digestion or gastric digestion processes (11, 17, 60, 62-64). Only Freitas et al (60) has used a dynamic gastric model which not only accounted for the effect of the oral phase on starch hydrolysis but also mimicked postprandial gastric digestion during a change in pH from 6.0 to 2.0. The oral phase of Freitas et al. simulated mastication by mixing saliva (collected from a non-smoker) with artificially chewed wheat bread crumbs. *In vivo* oral digestion was performed in the same fashion but chewed by the same person who supplied saliva. Four types of gastric digestions were conducted testing two digestion circumstances (snack and lunch) with water and inactivated pepsin as controls. (1) snack-type digestion of a water-based bolus (SWB, 30 min digestion at pH 6.0 to 2.0 plus 30 min at constant pH 2.0); (2) snack-type digestion of a saliva-based bolus (SSB, same as SWB); (3) lunch-type digestion of a saliva-based bolus (LSB, 60 min digestion at pH 6.0 to 2.0 plus 30 min at constant pH 2.0); (4) constant pH digestion of a saliva-based bolus (CSB, 60 min digestion at constant pH 6.0). Gastric digestion was performed using DiDGI® with continuous HCl and pepsin influx and stirring at 37 ^{0}C.

Amylolytic activity of saliva as a function pH revealed that HSA exerted maximal activity at pH 6.0 to 7.0 but lost 50% activity at pH 4.0 and was completely inactivated at pH 3.5. These findings were in line with other observations (15, 19). Boluses produced *in vitro* and *in vivo* were highly comparable in their response to HSA digestion, indicating that *in vitro* HSA digestion could closely mimic that of oral digestion of starch. Compared to earlier gastric models processing at a stable acidic pH (11, 63, 64), Freitas's model presented a more realistic picture of starch digestion in oral and gastric phases. Similar trends in glucose release (80% starch

released in 20 min digestion) were observed in both snack and lunch type digestions, clearly showing the important role of HSA activity in the stomach in the overall digestion of starch.

2.5. Interaction of kiwifruit with HSA

Recent research by Mishra et al (31) has indicated that a lowering of glycaemic response when kiwifruit is partially exchanged for cereal, on an equal carbohydrate basis, is due to more than simple substitution of kiwifruit sugars for cereal starch. When cereal was consumed either with kiwifruit sugars alone, or with the whole kiwifruit containing the same amount of sugars, glycaemic response was lower with the whole kiwifruit (31). This showed that there were components other than sugars in the kiwifruit that could lower glycaemic response to starchy foods when co-ingested with kiwifruit.

The non-sugar factors in kiwifruit responsible for the lowering of glycaemic response could include retardation of digestion due to the effects of kiwifruit dietary fibre on the physical properties of the gastric chyme (65), and inhibition of glucose uptake by phenolics from kiwifruit (66). However, in light of recent reports on the possible importance of HSA in starch digestion and glycaemic response (23, 60), another possibility is that the kiwifruit could be inhibiting the digestion of starch by HSA in the stomach.

There are several ways in which kiwifruit might inhibit HSA, or reduce its effects in the stomach:

- Kiwifruit organic acids may rapidly reduce the pH of the gastric chyme to below the optimum pH range for HSA activity.
- The organic acids, citric acid in particular, may directly inhibit HSA activity.

- The kiwifruit protease actinidin may degrade HSA.
- The reduction in pH due to the kiwifruit organic acids may stimulate the activity of the gastric protease, pepsin, which may interact with the kiwifruit actinidin and degrade HSA.
- The effect of low pH and buffering due to the kiwifruit organic acids may cause a delay in GE, which delay the arrival of carbohydrates at the sites of glucose absorption in the small intestine.

Gastric digestion is governed by a range of food-related factors such as food composition and buffering as well as psychological factors such as gastric secretions and GE (24). The effect of kiwifruit on HSA activity in the stomach is complicated by the fact that the above factors will all interact. In particular, actinidin and pepsin, both being proteases, may degrade one another with surviving activity depending on the pH at which the interaction occurs.

2.5.1. Kiwifruit organic acids

Kiwifruit is a good source of ascorbic acid (Vitamin C), ranging from 50 to 430 mg per 100 g fresh weight (FW) depending on the cultivar, for instance, SunGold kiwifruit of Zespri contains nearly three times the concentration of Vitamin C than that in oranges (161 mg/100 g FW and 51 mg/100 g FW, respectively) (67). There are a range of other organic acids in kiwifruit including citric acid, quinic acid and malic acid. The compositions of most organic acids are similar across different kiwifruit cultivars analysed except that quinic acid is present in higher concentration in gold kiwifruit (*Actinidia deliciosa cv. 'Hort16A'*) than in green kiwifruit (*Actinidia chinensis cv. 'Hayward'*) (68).

2.5.2. Acidity

The presence of organic acids is the main cause of fruit acidity. There has been little research on the influence of organic acids on the activity of HSA in the stomach, presumably because the activity of HSA in gastric digestion has for many years been thought to be unimportant. However, Freitas et al (60) found that a gradual rate of change in gastric acidification provided the opportunity for HSA to rapidly hydrolyse a large proportion of starch until the pH had reduced to the point where the enzyme had been inactivated. Freitas et al (69) plotted the dependence of HSA on pH showing activity decreased as the pH was reduced. Thus, they proposed that reducing HSA activity through premature acidification could slow down amylolysis and reduce glycaemic response to starchy foods. This was demonstrated by inhibited HSA activity by lemon juice *in vitro*. Lemon juice, which lowered the pH of chyme to ~ 2.5 significantly reduced starch release in bread to 35% compared with 84% in water (pH ~ 6.0) at the end of *in vitro* gastric digestion (69). In addition, the protective effect of oligosaccharides on HSA at low pH proposed by Rosenblum et al (22) was not observed in this case, indicating acidification by co-ingestion of acidic foods could be an effective approach to reduce oro-gastric hydrolysis of starch and ultimately glycaemic response of starchy foods.

Abundant in organic acids, kiwifruit has a low pH of 3.0-3.5 depending on cultivar, orchard and time of harvest (70). Previous research by Mishra et al (31) demonstrated that green kiwifruit (200 g pulp, pH 3.3) lowered the pH of co-ingested breakfast cereal (47.3 g, pH 5.3) to 3.7, which would possibly inhibit HSA activity in the stomach.

2.5.3. Citric acid

It has been shown that citric acid can inhibit HSA activity (71), possibly because HSA requires Ca^{2+} ions as cofactors for enzymatic activity while citric acid is a chelating agent which reduces the availability of free and functional Ca^{2+} to HSA (52). A forensic saliva identification study showed a significant decrease ($p<0.05$) of 92.2% and 99.7% in HSA activity in saliva samples contaminated with citric acid-containing soft drinks (8.7 mg and 7.1 mg of citric acid/L, respectively) (71). Therefore, organic acids could inhibit HSA both through their binding of Ca^{2+} by citric acid as well as their effects on pH.

2.5.4. Kiwifruit actinidin

Actinidin (EC 3.4.22.14) is a cysteine protease comprising up to 40% of soluble proteins in green kiwifruit but less than 1% in gold cultivars (72, 73). Actinidin consists of a single polypeptide chain of 220 amino acid residues with a molecular weight (MW) of 23 kDa and appears as a band somewhere at 24-30 kDa on SDS-PAGE gel (74). Other major proteins in kiwifruit identified include kiwellin (28 kDa on PAGE), thaumatin-like protein (24 kDa on PAGE) and kirola (17 kDa on PAGE) (74, 75). Actinidin contains a free sulfhydryl bond in its structure that is essential for its activity but also makes it susceptible to oxidation (33).

Actinidin catalyses the hydrolysis of a broad range of proteins at a pH range of 3.0 to 6.0 with an optimum of 4.0 (76). The interaction between actinidin and HSA had not been explored until one recent study by Martin et al (32) in which actinidin was shown to be highly active against HSA. Five minutes of exposure to green kiwifruit containing actinidin at 37 °C resulted in 83% loss of

HSA activity as assessed a colorimetric assay and 64% loss using potato starch as substrate (32).

2.6. Pepsin

Pepsin is a principle digestive enzyme responsible for partial digestion of proteins into smaller peptides in the stomach. Pepsin consists of 330-350 amino acids residues and has a MW of 35 kDa (77). Its zymogen, pepsinogen, is secreted by chief cells on gastric mucosa and activated at low pH upon HCl release by parietal cells (77). Determinations of pH optimum of pepsin have varied slightly depending on substrate and buffer used, but most studies (78-81) have found that pepsin activity was maximal at pH 1.5 to 2.0 and minimal at pH 5.0 and above. Piper and Fenton (81) plotted a composite profile of pH activity and stability of pepsin (**Figure 1**) showing that human pepsin exerted maximal activity at pH 2.0 and started to lose activity as pH increased until it lost activity at pH 5.5 and became irreversibly inactivated at pH 7.5 and above. It was also revealed that pepsin was stable at pH 1.0 to 6.0, a range where peptic activity could be restored if pH lowered to pH 2.0 and below (81) which explained presence of peptic activity up to pH 5.0 observed elsewhere (78).

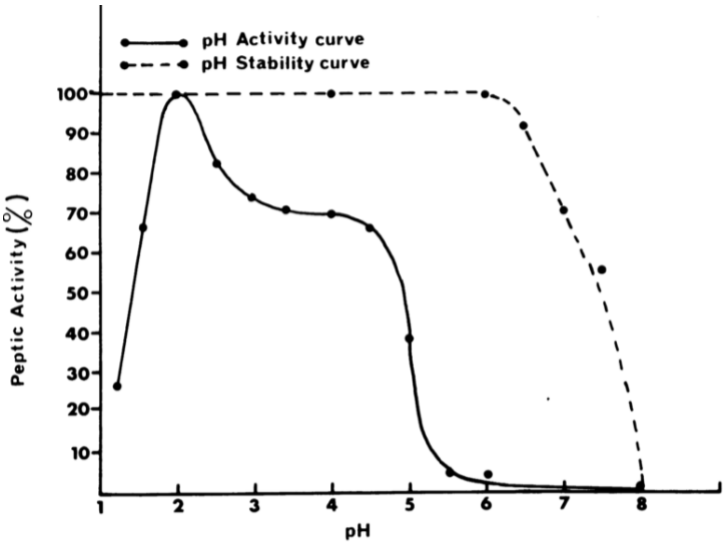

Figure 1 pH activity and stability curves of pepsin

Gastric acidity was thought to be the major limiting factor to HSA survival in the gut while little attention was paid to the influence of the gastric protease, pepsin on HSA activity. A study back in the early 1990's revealed that pepsin resulted in more complete inhibition of salivary isoamylase than HCl alone (82). Given the pH dependence of pepsin, co-ingestion of kiwifruit (pH 3.0-3.5) may lower the pH of the gastric chyme, promoting early activation of pepsin which would contribute to further degradation of HSA in the stomach.

2.6.1. Interaction of actinidin with pepsin

The interaction between actinidin and pepsin in the stomach has not yet been studied, but as they are both proteases they could degrade one another with the outcome depending on pH. There have been anecdotal claims that kiwifruit is able to assist in gastric digestion. Given the wider pH range and specificity of actinidin than pepsin, actinidin was proposed to hydrolyse a broad range of proteins when pepsin activation was impaired at high pH, or open up protein structures for peptic activity contributing to a more rapid and complete digestion of proteins in

the stomach (83). However, this line of thought is challenged as the gastric acidity (~pH 2.0 and lower) is outside the optimum pH range of actinidin and the influence of pepsin on actinidin is less known.

The role of kiwifruit containing actinidin in gastric digestion has been explored both *in vitro* (33, 36) and *in vivo* (34, 35) in relation to protein digestion. Kaur et al (33) investigated the effect of actinidin extracts from green kiwifruit on gastric digestion of a range of proteins by simulating gastric digestion at pH 1.9 with pepsin for 30 min by assessing protein degradation on SDS-PAGE. They found that the addition of actinidin enhanced digestibility of some relatively large proteins compared with pepsin alone, with digestion of α-, β-, k-caseins of sodium caseinate increased by 36%, 33% and 48%, respectively (33). However, a low pH of 1.9 might have impaired the catalytic activity of actinidin and solubility of some proteins leading to an underestimation, as digestive contents do not immediately encounter such a low pH in the stomach. A more profound effect of actinidin on protein breakdown was found by Donaldson et al who adopted an *in vitro* gastric model where freeze-dried beef was digested over a pH range of 1.3 to 6.2 by pepsin and actinidin of different concentrations for 60 min (36). Protein hydrolysis was increased by 27.5% with the addition of actinidin at a pH around 3.1 and this enhancing effect weakened as pepsin concentration increased. Despite the difference in enzyme concentrations between the two trials, the elevation of gastric pH from 1.9 to 3.1 contributed to nearly twice the amount of increase (27.5% in Donaldson et al's trial vs. 15% in Kaurl et al's trial) in hydrolysis of beef muscle protein attributed to addition of actinidin. *In vitro* results indicated that actinidin enhanced gastric digestion of proteins especially when pepsin and acid secretions were insufficient.

Kiwifruit actinidin was shown to increase gastric digestion and emptying of dietary proteins in pig (35) and rat (34) models by Montoya et al. Gastric chyme of growing rats fed 6 protein diets with

(freeze-dried green kiwifruit) and without (freeze-dried gold kiwifruit) actinidin treatment (equivalent to consuming 2 kiwifruit with an average-size meal for an adult) was obtained 60 min after gavage. Actinidin significantly increased the digestion of gluten by 3.2 fold and GE of beef muscle diets by 43% ($p<0.05$). These *in vivo* results were consistent with previous *in vitro* findings confirming that actinidin enhanced gastric digestion of some high-MW dietary proteins (MW>32 kDa). Although pH data were not available, higher physiological pH in the stomach could be accounted for by the enhanced effect of actinidin in digesting some protein. In addition, a significant negative correlation ($p<0.05$) between protein digestion and GE suggesting a possible role of kiwifruit actinidin in increasing the rate that dietary proteins are digested and emptied from the stomach for further degradation and absorption in the small intestine. Similar patterns were seen in growing pigs fed diets containing beef muscle and fresh kiwifruit pulp and assessed for protein breakdown in the gastric chyme over a 7 hour postprandial period (35). Significant increased digestion of beef muscle proteins ($p<0.05$), especially high-MW proteins (MW>34 kDa) was seen when actinidin was present either in the form of green kiwifruit pulp or gold kiwifruit supplemented with purified actinidin over 3 hours.

Since kiwifruit actinidin was found to not only actively digest a broad range of proteins including HSA (32, 76) but also assisted digestion of high-MW proteins (MW>32 kDa) in the stomach when concentration and pH environment is not optimal for pepsin activity (33-36), it is rational to speculate that kiwifruit actinidin may degrade HSA (MW~56 kDa) rapidly before food enters the stomach and works together with pepsin during gastric acidification.

2.7. Buffering capacity

Buffering capacity is the ability to resist a change in pH when acid or alkali is added. The buffering capacity of a food is mainly attributed to its composition such as organic acids and salts (84). A limited number of studies that have linked buffering capacity to organic acids in fruits have been related to alcoholic fermentation (85, 86) and dental erosion (87). Torjia et al (85) found that organic acids in grapes, tartaric and malic acids for example, were responsible for wine acidity and conferred different buffering capacity, which would prevent pH alterations in the process of fermentation. Li et al (86) investigated the effects of organic acids on buffering capacity of wort and observed a positive linear positive relationship between buffering capacity and level of organic acids. Among a range of organic acids, acetic acid and citric acid were found to contributed most substantially to the buffering capacity of wort (86).

There has been little research on the effect of buffering capacity of organic acids on digestion and it has been limited to animal models (88, 89). The animal research found that feedstuffs with high buffering capacity led to pH remaining high in the stomach and proximal digestive track, impairing the activation of pepsin and breakdown of proteins in the stomach and leading to toxicity from excessive protein fermentation in the jejunum and colon (88). Incorporating organic acids was able to lower the acidity and buffering capacity of the feed, promoting peptic digestion of dietary proteins and inhibiting bacterial growth in the stomach (89). Mishra et al (31) proposed that sustained reduction in meal pH to 3.7 combined with a further delay in gastrointestinal pH adjustment to pH 2.0 due to the high buffering capacity of kiwifruit demonstrated in an *in vitro* titration analysis, could perhaps delay GE and partly accounted for the glycaemic lowering effect observed *in vivo*.

2.8. Gastric emptying (GE)

Incorporating organic acids in the processing and consumption of starchy foods has been adopted as a dietary approach to glycaemic control (40). The effects of vinegar dressing (29, 90-97) and sourdough fermentation (28, 98-103) on postprandial glycaemia has been extensively studied *in vitro*, and *in vivo* with small sample sizes ($n<16$). A meta-analysis of 11 cross-over clinical trials including 204 subjects in good health or with glucose metabolism disorders reported that acetic acid, administrated as vinegar contributed to a small but significant decrease in postprandial blood glucose and insulin responses ($p<0.01$). However, two of the trials claimed this effect was only significant when vinegar was consumed with high GI meals but not with low GI meals (93, 96). The underlying mechanisms have not yet been fully understood. Two of the possible factors were delayed GE (29, 104) and inhibited amylase activity by acidity discussed earlier (69, 92).

A growing body of evidence has recognised the rate of transit of digesta from the stomach to the duodenum, that is, GE rate is a major determinant of postprandial glycaemic excursions in both healthy people (27, 105) and in those with diabetes (106, 107). Slowing GE is thought to decrease the rate that digestive contents enter the small intestine for predominant starch digestion and absorption and so reduce the postprandial glycaemic response and subsequent insulin demand. However, GE was found to determine the initial rise in blood glucose concentration as plasma glucose and insulin levels were positively related to GE at 30-60 min, but negatively related to GE at 120 min postprandially (27, 105, 107). This is due to a complex bidirectional relationship existing between GE and postprandial glycaemic response involving small intestinal inhibitory feedback, in which hypoglycaemia accelerates GE while hyperglycaemia slows GE (108).

There are many other factors besides acidity and buffering capacity conferred by the kiwifruit, including dietary fibre and various phytochemicals affecting gastric viscosity, particle breakdown and extent of digestion (65), that may all interact to affect the acidification and disintegration of digestive contents in the stomach and impose a delay on the rate of GE (40). Therefore, it is quite likely that kiwifruit could have a delaying effect, which would also retard glucose uptake from the small intestine and depress glycaemic response.

Since HSA plays a significant role in the oro-gastric digestion of starch and the enzyme is susceptible to acid, citric acid inhibition and kiwifruit actinidin and pepsin degradation, a dietary approach targeting HSA inhibition has the potential to reduce glycaemic responses to starchy foods. As a fruit carrying all factors mentioned above that contribute to HSA inhibition individually and interact collectively to delay GE, green kiwifruit may have the potential to reduce the rate of starch hydrolysis contributed by HSA during oro-gastro digestion and ultimately lower overall glycaemic response to co-ingested starchy foods. All above factors of kiwifruit should be reviewed and investigated thoroughly so that the mechanisms behind the glycaemic lowering effect of kiwifruit can be understood.

3. Methods

3.1. Stage 1 Buffering capacity of fruits

The aim of the Stage 1 experiments was to investigate the buffering capacity of various fruits during simulated gastric digestion both as individual fruits and when combined with a starchy food (Weet-Bix™ or oats). The buffering capacity was determined from the quantity of hydrochloric acid (HCl) of approximately physiological concentration required to reduce the pH of the gastric digestion medium to pH 2.0, and subsequently, the amount of sodium hydroxide (NaOH) required to raise the pH from 2.0 to 7.0. The addition of HCl (acid) and NaOH (base) was at approximately the *in vivo* rate, determined from literature values, so the titrations also gave an indication of the effect of buffering by fruit on the time taken for gastric acidification.

3.1.1. Automated titration

Stock solutions preparation

Hydrochloric acid (HCl; 0.5 M) and sodium hydroxide (NaOH; 0.5 M) solutions were used as titrants for carrying out the acid-alkali titration. To prepare 0.5 M HCl, 49.1 ml of HCl stock (concentration: 32%, density: 1.16 g/L) was added to distilled (DI) water and made up to 1 L in a glass beaker. Calculations for working out the volume of HCl stock required are shown below:

Moles of HCl in 1 L of solution = molarity (0.5 M) × volume of solution (1.0 L) = 0.5 mol

Mass of HCl = moles of HCl (0.5 mol) × molar weight of HCl (36.46 g/mol) = 18.23 g

Volume of HCl = mass of HCl (18.23 g) ÷ density of stock (1.16 g/ml) = 15.72 ml

Volume of stock solution = volume of HCl (15.72 ml) ÷ concentration of stock (32.0%) = 49.10 ml

To make 0.5 M NaOH, 20±0.01 g laboratory grade NaOH pellets were weighed on a calibrated weighing scale (Mettler Toledo, Columbus, OH, USA) and dissolved in DI water and made up to 1 L in a glass beaker. Calculations for working out the weight of NaOH required are shown below:

Moles of NaOH in 1 L of solution = molarity (0.5 M) × volume of solution (1 L) = 0.5 mol

Weight of NaOH = moles of NaOH (0.5 mol) × molar weight of NaOH (40.0 g/mol) = 20.00 g

Titrator setup

A Metrohm 902 Titrando (Metrohm AG, Herisau, Switzerland) was used to perform automated acid-alkali titration under the control of supporting software Tiamo (Metrohm AG, Herisau, Switzerland). To mimic gastric digestion, acid/alkali dosing rate was set at a constant 2 ml/min, which was calculated based on literature data (gastric acid secretion rate 30 mEq/hr, 30 min for 'snack-type' meal digestion) (60, 61). A semi-dynamic gastric digestion system was set up as shown in **Figure 2**. Digestion medium was constantly mixed at 37±2 °C in a 1 L beaker by a heating magnetic stirrer (Thermo Fisher Scientific, Waltham, MA, USA) beneath and an overhead stirrer (Xin Rui Instruments, Jiangsu, China) simultaneously operating at 10 rpm. Titration was performed using Set End-point Titration (SET) method with 2 endpoints (EP), pH 2.0 and 7.0, and customized parameters.

Figure 2 Set up of a semi-dynamic gastric digestion system

Test foods preparation

Fruits used in the titrations were fresh Pink Lady and Granny Smith apples (PAK'nSAVE), fresh Navel oranges (PAK'nSAVE), frozen black berries, strawberries, raspberries and blueberries (Woolworths), fresh Hayward kiwifruits (Zespri), frozen SunGold kiwifruits (Zespri), all in a ready-to-eat state of ripeness. Fresh fruits were stored at 4 ℃ until use, for no more than 2 days. Frozen fruits were stored at -20 ℃ and thawed at 4℃ overnight. All fruits except berries were peeled, diced and blended into pulps. To fully release organic acids, diced apples were weighed before being stewed on medium-low heat for 10 min and then reweighed to determine moisture loss during cooking. The stewed apples were blended with enough DI water added to compensate for moisture lost in cooking. Fruit pulps and artificial gastric salt solution (7 g/L gastric salt consisting of 50 g NaCl, 4 g $CaCl_2$, 1 g $NaHPO_4$ and 0.1 g $MgCl_2$) were prepared and heated to 37℃ in a water bath (Grant Instruments, Cambridge, UK) beforehand. Weet-Bix™

(Sanitarium, 67 g CHO per 100 g) and rolled oats (Harraways, 56.2 g CHO per 100 g) were the starchy foods. The Weet-Bix™ biscuits consisted of lightly compressed whole wheat flakes and were easily crushed dry by hand to simulate chewing.

3.1.2. Titration experiments

To test and compare the buffering capacity of test foods, experiments consisted of titration of a water blank and two starchy foods controls, the individual fruit, and the individual fruit and a starchy food combined (**Table 1**) and were conducted in triplicate.

Table 1 The compositions of test foods

Test foods	Fruit pulp (g)	Starchy food (g)	Water (ml)	Gastric medium (ml)
Water	-	-	100	200
Weet-Bix™	-	41.8	100	200
Rolled oats	-	51.6	100	200
Individual fruit	100	-	-	200
Fruit and Weet-Bix™	100	41.8	-	200
Fruit and rolled oats	100	51.6	-	200

Test foods were mixed as shown in **Table 1**. Fruit pulps (100±0.01 g) were weighed into a 1L beaker, or in treatments not containing fruit DI water (100±0.01 ml) substituted for fruit pulp to keep a constant volume. Crushed Weet-Bix™ (41.8±0.01 g) or rolled oats (51.6±0.01 g), each containing 28 g available CHO were added in all except water blank and individual fruit

experiments. Lastly gastric salt solution (200 ml), which was the least amount required to dissolve Weet-Bix™ biscuits and keep the pH electrode immersed, was added to all meals. The beaker was then moved onto the heating magnetic stirrer. Titration started once temperature reached 37 ℃, (within 5 min) and the pH reading had not changed for at least 5 seconds.

Addition of starch significantly increased the viscosity of the digestion medium. Therefore, constant manual mixing was needed during acidification and early stage of neutralisation, especially in trials where Weet-Bix™ was used, because the dosing rate up to the set maximum rate was highly dependent on pH readings. Frequent checking of the pH electrode was also necessary as Weet-Bix™ and oats were likely to get clustered onto electrode probe.

The following data were exported from Tiamo to Excel: (1) time (min); (2) pH; (3) HCl/NaOH volume dispensed (ml); (4) dosing rate (ml/min); (5) temperature (℃). The HCl/NaOH dispensing volume was multiplied by 0.5 to convert from ml to mEq as the molarity of titrant was 0.5 M. The pH (y axis) and mEq of HCl/NaOH (x axis) were used to draw a titration curve for each test food. Data were plotted to compare the rate of acidification and neutralisation between test foods and buffering capacity between fruits.

3.2. Stage 2 Effect of pH on HSA activity

The aim of Stage 2 experiments was to investigate the effect of pH on HSA activity over a pH range of 7.0 to 2.0. A buffer was developed so that the activity of HSA could be monitored at intervals of 1 pH unit.

3.2.1. Buffer development

A buffer solution was made by dissolving 0.5±0.01 g potassium hydroxide (KOH) and 1.0±0.01 g citric acid in DI water to form 100 ml solution. It was named 'kiwifruit buffer' because it had similar amount of potassium (K) and citric acid as Zespri SunGold and 'Hayward' green kiwifruit (315 mg K and 900 mg citric acid per 100 g SunGold; 300 mg K and 970 mg citric acid per 100 g Hayward). $CaCl_2$ (100 ppm) was added to stabilize the HSA and reduce its inhibition by citric acid. The solution was then diluted 1:2 with water, as in titration 100 g kiwifruit pulp is diluted by approximately 200 ml of gastric salt solution. The final solution had a pH around 4.5 and was adjusted to pH 7.0 with 1 M KOH. This diluted $CaCl_2$ fortified 'kiwifruit buffer' will be referred as 'Ca-KF buffer' in the following context. To test the inhibition of HSA by citric acid in the buffer the activity of HSA in Ca-KF buffer titrated with 0.5 M HCl and NaOH was compared with its activity in $CaCl_2$ fortified saline solution, referred as 'Ca-saline' (3% sodium chloride containing 100 ppm $CaCl_2$).

3.2.2. Starch digestion analysis

The activity of HSA over a pH scale of 7.0 to 2.0 was measured by dissolving 2.5 ± 0.01 g pre-gelatinised starch (BO11C, Davis Trading Company, Auckland, New Zealand) in 50 ml Ca-KF buffer, titrating the buffer from pH 7.0 to pH 2.0 with HCl (0.5 M), and measuring the activity of HSA against the dissolved starch at each pH. During acidification, 1 ml of sample solution was taken out, in duplicate, at pH 7.0, 6.0, 5.0, 4.0, 3.0 and 2.0 into 10 ml test tubes. Fifty µl of fresh human saliva was added into each tube and mixed by pipetting. The saliva was the student's own saliva collected after at least 6 hours of fasting. All tubes were incubated at 37±2 ℃ for 10 minutes before adding 4 ml absolute ethanol to stop the digestion and precipitate undigested starch. The ethanolic samples were centrifuged (1000 rpm, 5 min, at room temperature) and stored at 4℃ before analysis of starch digestion products in the supernatant.

Products of starch digestion (glucose, maltose and dextrins) in the ethanolic supernatants were measured as reducing sugars after a secondary digestion that converted the starch digestion products to free glucose. For the secondary digestion, supernatant (50 µl) of each sample was incubated with 250 µl of "Enzyme solution A" for 15 min at room temperature. The Enzyme solution A consisted of 100 µl invertase (Megazyme E-INVERT, Megazyme, Bray, Co. Wicklow, Ireland) and 100 µl amyloglucosidase (Megazyme E-AMGDF, Megazyme, Bray, Co. Wicklow, Ireland) per 10 ml acetate buffer (pH 5.2), Free glucose was then measured as reducing sugar by the 3,5-Dinitrosalicylic acid (DNS) method (**Figure 3**). DNS solution was made by mixing 0.5 mg/ml glucose, 4 M NaOH and DNS reagent (containing 10 g/L DNS, 16 g/L NaOH, 300 g/L potassium sodium tartrate) at a ratio of 1:1:5. For the reducing sugar analysis DNS solution (750 µl) was added into each tube and the tubes were heated in boiling water for 15 min and cooled down in an ice bath before adding 4 ml of DI water to dilute. The absorbance was read at 530

nm using an auto-calibrated GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The absorbance data was entered into an Excel table to calculate mg of glucose released per g of starch as shown below.

$$mg\ glucose\ in\ digestion\ medium\ =\ 10 \times 50 \times 5 \times \frac{OD\ (sample) - OD\ (blank)}{OD\ (10\ mg/ml\ glucose\ standard)} \quad (1)$$

$$\frac{mg\ (starch\ digested)}{g\ (sample)} = 0.9 \times \frac{(1)}{2.5\ g}$$

NB: Concentration of the glucose standard = 10 mg/ml; Volume of digestion medium= 50 ml; Correction factor = 0.9; Dilution factor = 5

	Test	Blank	5 mg/ml glucose standard	10 mg/ml glucose standard
Sample	50 µl	-	-	-
DI water	-	50 µl	-	-
Glucose	-	-	50 µl	50 µl
Enzyme A	250 µl	250 µl	250 µl	250 µl
Mix well and incubate at room temperature for 15 min				
DNS reagent	750 µl	750 µl	750 µl	750 µl
Mix well and incubate in boiling water for 15 min and then cool down in ice bath for 2 min				
DI water	4 ml	4 ml	4 ml	4 ml
mix well and read absorbance at 530 nm against blank				

Figure 3 Process of DNS assay and compositions of reagents

The amount of starch digested in 10 min by 50 µl of saliva in Ca-KF buffer and saline solution from pH 7.0 to 2.0 were compared in the same graph to illustrate the effect of pH and Ca-KF buffer on HSA activity. A student t-test was performed to test for any significant difference ($p<0.01$) between Ca-KF buffer and Ca-saline solution.

3.3. Stage 3 Effect of pepsin on HSA activity

The aim of Stage 3 experiments was to investigate the effect of pepsin on HSA activity over a pH range of 7.0 to 2.0. The previous experiments suggested that use of pre-dissolved pre-gelatinised starch solutions lead to pipetting errors due to the high viscosity of the starch solutions, producing errors in HSA analysis. Therefore, to improve accuracy a new approach to measuring HSA activity was adopted in which the pre-gelatinised starch was weighed into the analysis tube.

3.3.1. Pepsin reaction

Duplicate samples containing 5 ml of Ca-KF buffer were adjusted to pH 7.0, 6.0, 5.0, 4.0, 3.0 and 2.0 using 0.5 M HCl and KOH. One ml of saliva and 100 µl of pre-made 10% (w/v) pepsin (Sigma P7125, Sigma-Aldrich, St. Louis, MO, USA) were added into each tube before incubation at 37±1 ℃ in a water bath for 30 min. To eliminate the effect of pH and only look at the effect of pepsin on HSA activity after exposure to pepsin, samples were then neutralised to pH 7.0 (HSA active, pepsin inactive) using 0.5 M KOH and then made up to 10 ml with DI water. Neutralisation started from samples at lower pH to higher pH so that the samples with the highest pepsin activity could be inactivated as soon as possible.

3.3.2. Starch digestion analysis

One ml of each neutralised sample was added into a test tube containing 50±0.01 mg of pre-gelatinised starch. Digestion was initiated by incubating all tubes at 37±1 ℃. HSA activity was

determined by measuring the amount of starch digested at 0 min, 15 min and 30 min. Reaction was terminated by adding 4 ml of absolute ethanol at each time point. 0 min sample contained 1 ml of buffer (pH 7.0) without saliva or pepsin. The same procedure as described for Stage 2 was conducted to measure absorbance of all samples using DNS assay and calculate mean mg/g starch digested in Excel.

3.4. Stage 4 Effect of kiwifruit actinidin on HSA activity

The aim of the experiments in Stage 4 was to investigate the pH-dependence of the effect of kiwifruit actinidin on HSA activity. The protocol was developed step by step, with the following parameters set up and modified along the way: (1) concentration of kiwifruit sample; (2) pH of buffer; (3) control for fruit sugars; (4) pH adjustment method; (5) sampling method.

3.4.1. Sample preparation

Ready to eat green Hayward kiwifruit supplied by Zespri were skinned, blended into pulp and stored at -20 ℃. Frozen pulp was thawed and spun at 3000 rpm for 20 min at 4 ℃ to obtain actinidin extracts from the supernatant. Three different concentrations of kiwifruit samples (**Table 2**) were tested in duplicates. The control sample (0% kiwifruit, **Table 2**) contained only saliva and buffer.

Table 2 Kiwifruit samples consisting of kiwifruit extracts and Ca-KF buffer at target pH

Concentration of kiwifruit sample (%)	50%	10%	0%
Volume of kiwifruit extracts (ml)	2.5	0.5	0
Volume of pH adjusted Ca-KF buffer (ml)	2.5	4.5	5
Total volume (ml)	5	5	5

The experiment was carried out in duplicate. Pre-gelatinised maize starch (50±0.01 mg) was weighed into test tubes beforehand. The Ca-KF buffer was adjusted to pH 7.0, 6.0, 5.0, 4.0, 3.5,

3.0 and 2.0 using 0.5 M HCl and KOH and the Titrando titrator. A new pH point of 3.5 was of interest because 3.5 was found to be the initial pH of the digestion medium when kiwifruit was co-ingested with Weet-Bix™ or oats. At each pH, three concentrated samples were made by mixing kiwifruit extracts with buffer already adjusted to that pH (**Table 2**). Each sample was then immediately manually adjusted for pH again within 5 minutes due to the strong buffering capacity of kiwifruit.

3.4.2. Kiwifruit actinidin reaction

As soon as the pH was adjusted, 1 ml of saliva was added into the sample and incubated for 30 min at 37±1 ℃ after mixing. At the end of 30 min, all samples were taken out of the water bath and neutralised to pH 7.0 using KOH and made up to a final volume of 10 ml to standardise conditions for analysis of surviving HSA activity (**Figure 4**).

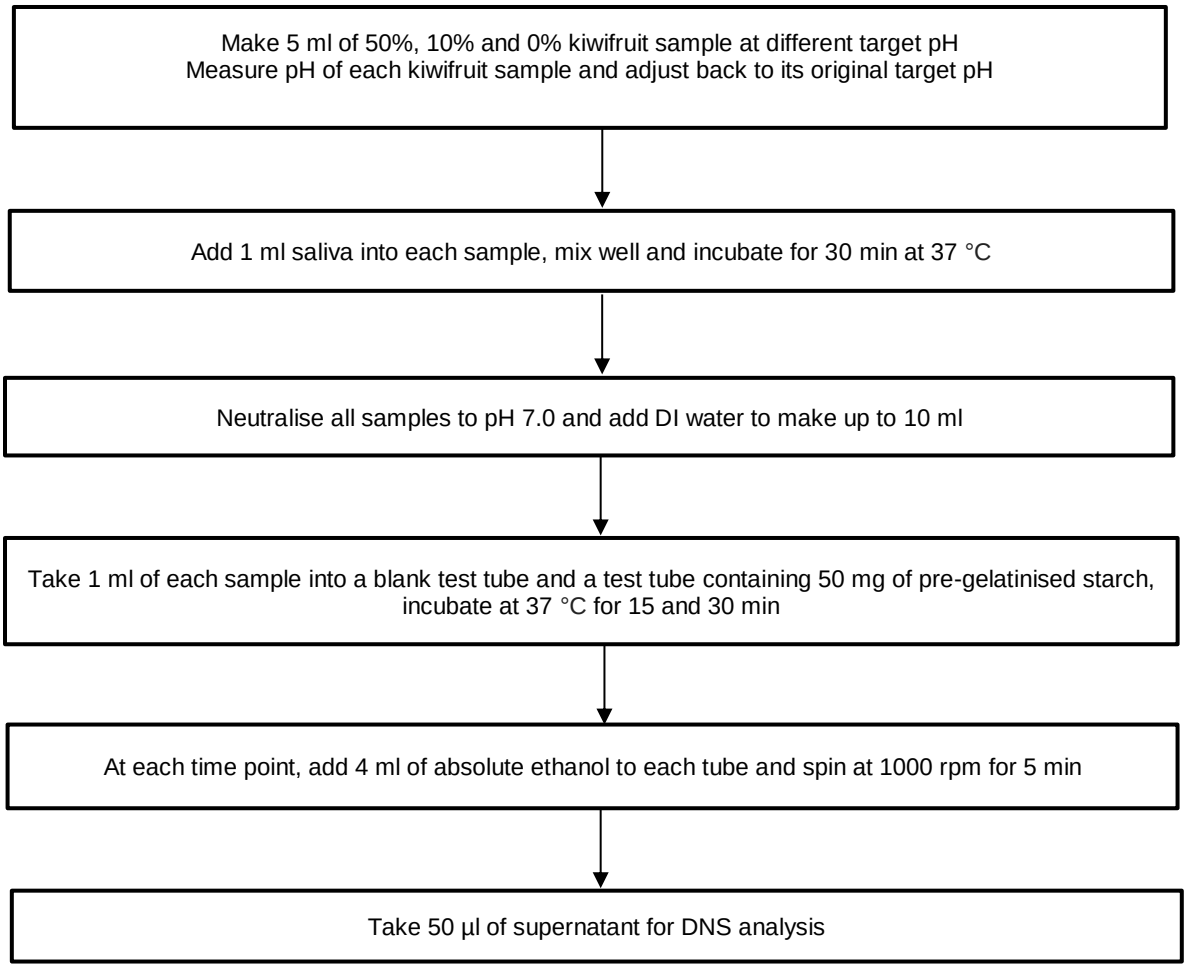

Figure 4 Flow chart of the initial experiment protocol

3.4.3. Starch digestion analysis

As shown in **Figure 4**, 1 ml of each neutralised sample was taken to digest 50±0.01 mg pre-gelatinised starch for 0, 15 and 30 min at 37±1 ℃. The sample solution was dispensed into the tube at an angle and at a speed which would quickly disperse and dissolve the starch to form a uniform solution. A clean Pasteur pipet was used as a stirring rod to aid dissolving if a lump was seen. Kiwifruit extracts contained fruit sugars that were included in total reducing sugars by DNS method. Therefore, another 1 ml was taken from all samples except control (0% kiwifruit)

into a blank test tube to determine kiwifruit sugars in the sample. The amount of fruit sugars was then subtracted from the total reducing sugars to determine the amount of starch digested by HSA in each sample. Starch digestion at time 0, 15 and 30 min was plotted to reflect the effect that kiwifruit actinidin had had on HSA activity due to exposure to kiwifruit at different pH levels. A student t-test was performed to test for any significant difference ($p<0.01$).

3.4.4. Further modifications

A few more modifications were made regarding kiwifruit concentrations, pH adjustment, and sampling method. The final protocol for this part is shown in **Figure 5** with modifications highlighted in bold. Firstly, 0% and 50% were selected to be the two concentrations to be tested. Secondly, 5 ml of kiwifruit extracts was adjusted to each target pH before mixing with pH-adjusted buffer to make up a 10 ml 50% kiwifruit sample. No further pH adjustment was needed. 2-Mercaptoethanol (0.2%) was added into extracts as an antioxidant to stabilise actinidin during pH adjustment and incubation. In addition, the experiment scale was increased with the ratio between reagents kept the same so that 15 and 30 min samples could be taken from the sample tube during starch digestion analysis. Furthermore, SunGold kiwifruit and Granny Smith apple were tested in the same fashion for comparison, as they do not contain actinidin.

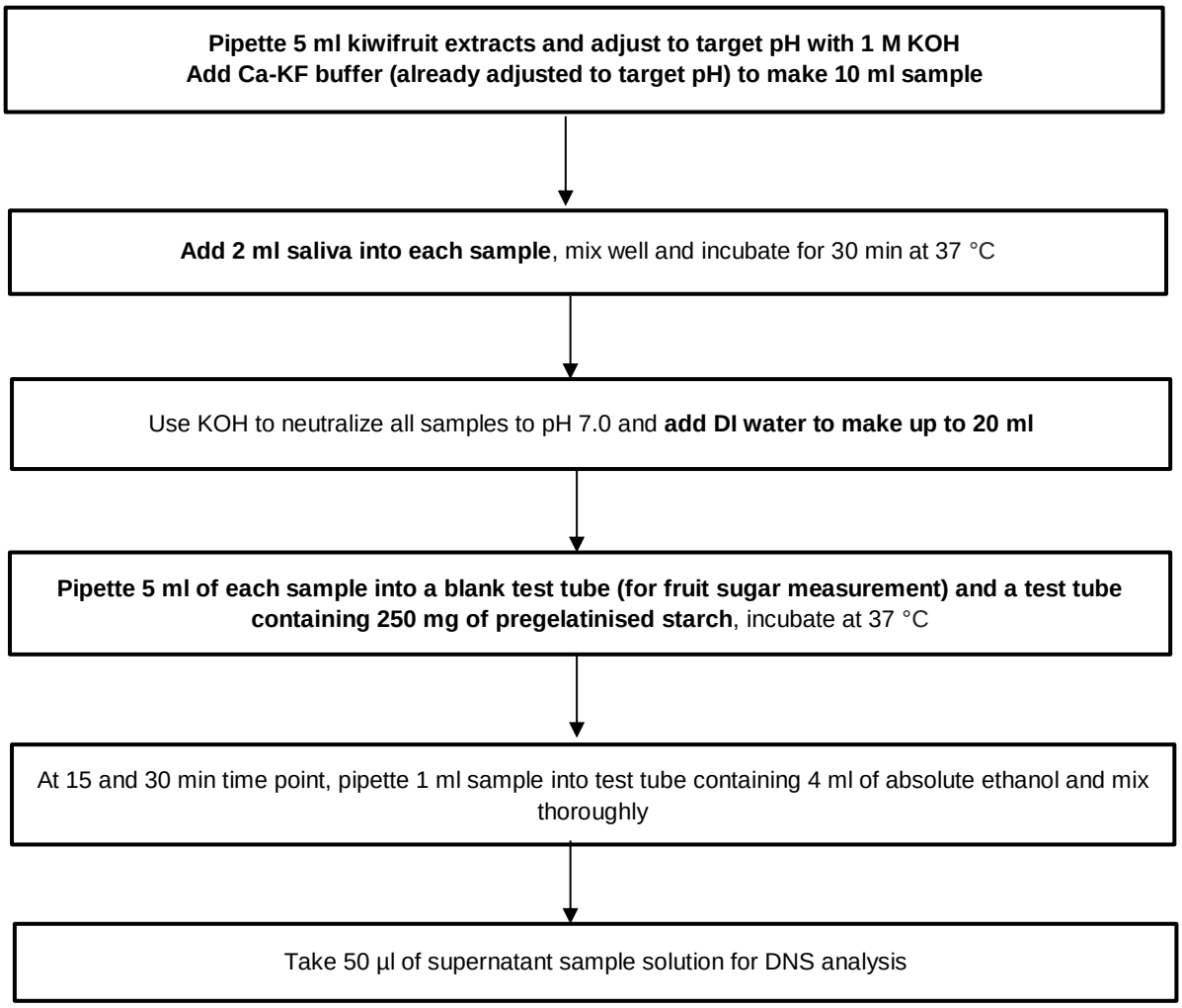

Figure 5 Flow chart of the modified experiment protocol with modifications in bold

3.5. Stage 5 Effect of kiwifruit actinidin-pepsin interaction on HSA activity

The aim of Stage 5 experiments was to investigate how the interaction between pepsin and kiwifruit actinidin affected their joint effect on HSA activity at pH 4.0, 3.5, 3.0 and 2.0. This pH range was selected because Stage 1 results suggested co-ingestion of kiwifruit would lower the pH of a food bolus to around pH 3.5-4.0 due to the acidity and buffering capacity of kiwifruit.

3.5.1. Gastric digestion and starch digestion analysis

A similar digestion procedure was followed as in Stage 4 (Figure 4) except that 0.24 ml of 10% (w/v) pepsin (Sigma P7125, Sigma-Aldrich, St. Louis, MO, USA) was added along with saliva (**Figure 6**). In addition, an extra tube containing the same reagents except kiwifruit extracts was used as a pepsin control for each sample.

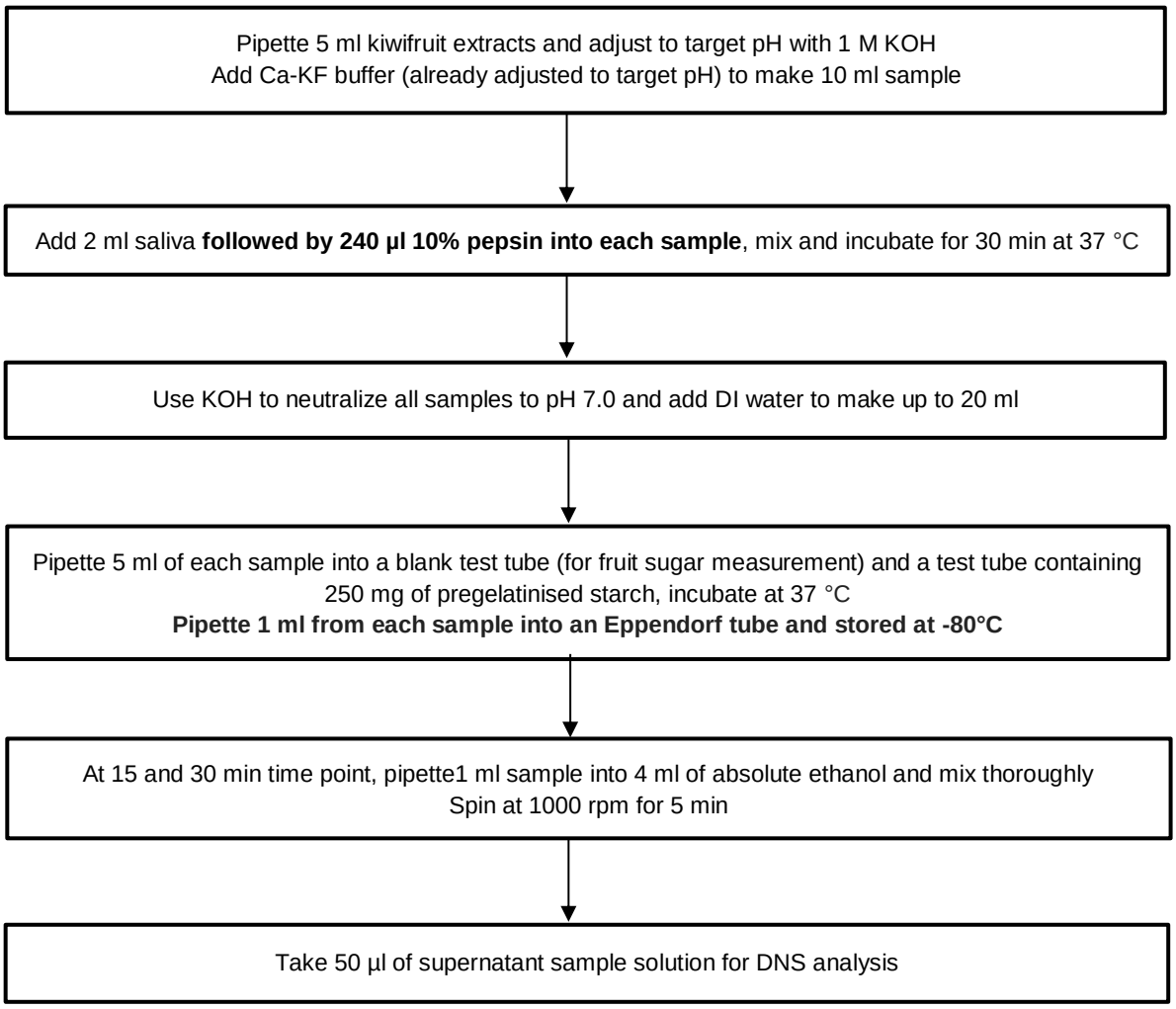

Figure 6 Flow chart of the experiment protocol with new procedures in bold

Starch digestion analysis was used to determine the amount of starch digested by HSA remaining in each tube, to reveal how HSA activity was affected by the interaction between actinidin and pepsin at pH 4.0, 3.5, 3.0 and 2.0 during 30 min digestion. A one millilitre sample was also removed from the digestion medium for gel-electrophoresis, and immediately stored at -80 °C until analysis.

3.5.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gastric digestion samples stored at -80 ℃ were used for protein analysis in duplicate on step gradient polyacrylamide gels (10%, 15% and 20% acrylamide) using a Bio-Rad Mini-Protean® II electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) following the method of Laemmli (109). Step-gradient polyacrylamide gels were cast in pre-made gel chambers before well-forming combs were inserted (**Table 3** shows the gel composition). After polymerisation the combs were removed and the wells were filled with SDS/Tris/Glycine running buffer.

Table 3 The compositions of 10%, 15% and 20% acrylamide gels and stacking gel

	10%	15%	20%	Stacking
H_2O	1.7 ml	1 ml	0.3 ml	2.25 ml
Acrylamide/Bis-acrylamide, 30% solution	1 ml	2 ml	2.7 ml	0.68 ml
1.5 M Tris pH 8.8	1 ml	1 ml	1 ml	-
0.5 M Tris pH 6.8	-	-	-	1 ml
10% SDS	40 µl	40 µl	40 µl	40 µl
2,2,2-Trichloroethanol	20 µl	20 µl	20 µl	-
10% Ammonium persulfate	20 µl	20 µl	20 µl	20 µl
N,N,N',N'- Tetramethylethylenediamine	4 µl	4 µl	4 µl	4 µl

Thawed samples were made to the same concentration with sample loading buffer (**Table 4**) and treated with E64 (Sigma E3132, Sigma-Aldrich, St. Louis, MO, USA) and Pepstatin A (Sigma P5318, Sigma-Aldrich, St. Louis, MO, USA) which are two selective inhibitors of actinidin and pepsin, respectively. Samples were then boiled at 95 ℃ for 5 min and centrifuged

at 14,680 rpm for 1 min before being loaded onto the gels along with a molecular weight marker. Electrophoresis was performed at 0.05A for approximately 70 min at room temperature. Protein bands in the gels were visualised with Trichloroethanol (ReagentPlus®, ≥99%, Sigma T54801, Sigma-Aldrich, St. Louis, MO, USA) which allows fluorescent detection of tryptophan-containing proteins without staining. The gels were scanned under ultraviolet light by the Gel Doc XR+ Imager (Bio-Rad Laboratories, Hercules, CA, USA) and the protein bands were analysed by Quantity One® 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Table 4 The compositions of sample loading buffer (2X loading buffer for 10 ml)

	ml (for 10 ml)	Concentration
0.5 M Tris pH 6.8	2 ml	0.1 M
Glycerol	2.5 ml	25%
10% SDS	4 ml	4%
H_2O	1.5 ml	/
Bromophenol blue	2 mg	0.02%
2-Mercaptoethanol	0.4 ml	4%

3.5.3. Actinidin kinetic assay

The same batch of frozen gastric digestion samples as used for gel electrophoresis were used for kinetic analysis of actinidin, in duplicate, on white 384-shallow well microplates (ProxiPlate-384 Plus, PerkinElmer, Waltham, MA, USA) in 20 µl volumes. Thawed samples were put through a series of gradient dilutions using 0.1 M sodium acetate buffer pH 4.5 containing 0.2% 2-Mercaptoethanol and 0.2% Igepal. Kiwifruit was homogenised in a glass Dounce hand-held

homogeniser and assayed at a final dilution of x20 in the microplate. Actinidin activity was determined from coumarin fluorescence read at excitation and emission wavelengths of 351 and 430 nm (bandwidth 20 nm) by a Tecan Spark® 20 M multimode microplate reader (Tecan Group, Männedorf, Switzerland) controlled by SparkControl software immediately after addition of the coumarin-yielding substrate Z-Phe-Arg-7-amido-4-methylcoumarin Hydrochloride (Sigma C9521, Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 200 µM. Data were plotted in Excel with fitted curves to compare actinidin activity at pH 4.0, 3.5, 3.0 and 2.0 during 30 min of interaction with pepsin and HSA.

3.6. Stage 6 Meal study

The aim of the Stage 6 experiments was to investigate the effect of green kiwifruit pulps on HSA activity co-ingested with Weet-Bix™ during simulated gastric acidification.

3.6.1. Saliva secretion

To get an estimation of how much saliva is secreted in chewing of 41.8$\pm$0.01 g Weet-Bix™ until ready to be swallowed, hand-crushed dry biscuits were chewed by the student and expectorated into a re-sealable plastic bag at onset of the urge to swallow. The wet boluses were mixed by massaging the sealed bag. Four sub-samples were taken and weighed individually (recorded as Wy) immediately before transferred to a vacuum oven (Lab-Line Instruments, Inc., Melrose Park, IL, USA) for drying at 100 °C overnight under vacuum. Each dried sub-sample was reweighed (weight recorded as Wx). Calculations for working out the theoretical amount of saliva to add to 41.8 g of Weet-Bix™ in the whole bolus were carried out as follows:

Saliva as a % of dry weight (S) = (Wy - Wx) $\times$ 100 / Wy %
Therefore, weight of saliva required for 41.8 g of Weet Bix is S/100 $\times$ 41.8 g

As this was an estimation, the moisture content of the dry Weet-Bix™ and the dry matter content of saliva were not allowed for, as they are both minor proportions of the materials.

3.6.2. Gastric digestion

To investigate the effect of pepsin-actinidin interaction on HSA activity during simulated gastric digestion of Hayward kiwifruit and Weet-Bix™ combined, experiments consisted of titration of a Weet-Bix™ control and three treatments (Kiwifruit, pepsin, Kiwifruit + pepsin; **Table 5**) were conducted in duplicate.

Table 5 Compositions of test foods

Test product	Enzyme	Starch
Weet-Bix™ (control)	28 ml saliva[1]	41.8 g Weet-Bix™
	(100 g water)	
Actinidin	28 ml saliva	41.8 g Weet-Bix™
	100 g Hayward pulp	
Pepsin	28 ml saliva	41.8 g Weet-Bix™
	(100 g water)	
	7 ml 10% pepsin[2]	
Actinidin & pepsin	28 ml saliva	41.8 g Weet-Bix™
	100 g Hayward pulp	
	7 ml 10% pepsin	

[1] 28 ml saliva was calculated according to data obtained from saliva secretion experiment which found saliva secreted to chew Weet-Bix™ was 66% of the dry weight of Weet-Bix™ biscuits so theoretically 28 ml saliva would be secreted to chew 41.8 g Weet-Bix™.
[2] Previously gastric digestion study (unpublished) used 1 ml 10% pepsin in 50 ml digestion medium so 7 ml of the same pepsin was used in 340 ml digestion medium.

The compositions of control and treatments were shown in **Table 5** and the experiment protocol was shown in **Figure 6**. Thawed green kiwifruit pulp, fresh saliva, 7 g/L gastric salt solution, 10% w/v pepsin were pre-warmed to 37 °C in a water bath beforehand. Crushed Weet-Bix™ (41.8±0.01 g) were weighed into a 1 L beaker and mixed with 100±0.01 g of kiwifruit pulps for 30 seconds. Pre-warmed saliva (28 ml, based on preliminary data on saliva secretion) was added and mixed for 1 min using a spatula before adding 7 ml of 10% pepsin and 200 ml of gastric salt solution. The above procedure was completed in 4 min. The first sample was taken at 5 min and added into a tube containing 4 ml of absolute ethanol. Samples were taken at each 0.5 pH unit. Due to high viscosity of the sample, pipette tips were cut to prevent blocking. Tips were inserted into ethanol for dispensing and ejected into the tube for mixing. The mixture was poured into a 500 ml measuring cylinder to measure final volume after completion. Volume of digesta for each sample was calculated from the final volume and amount of acid dispensed at each sampling as recorded by the titrator's program Tiamo. The calculated volumes were used for calculating starch digestion in Excel from results of DNS analysis of digestion products as before. Data were plotted to compare between Weet-Bix™ control and three treatments, to reveal the effect of pepsin-actinidin interaction on HSA activity.

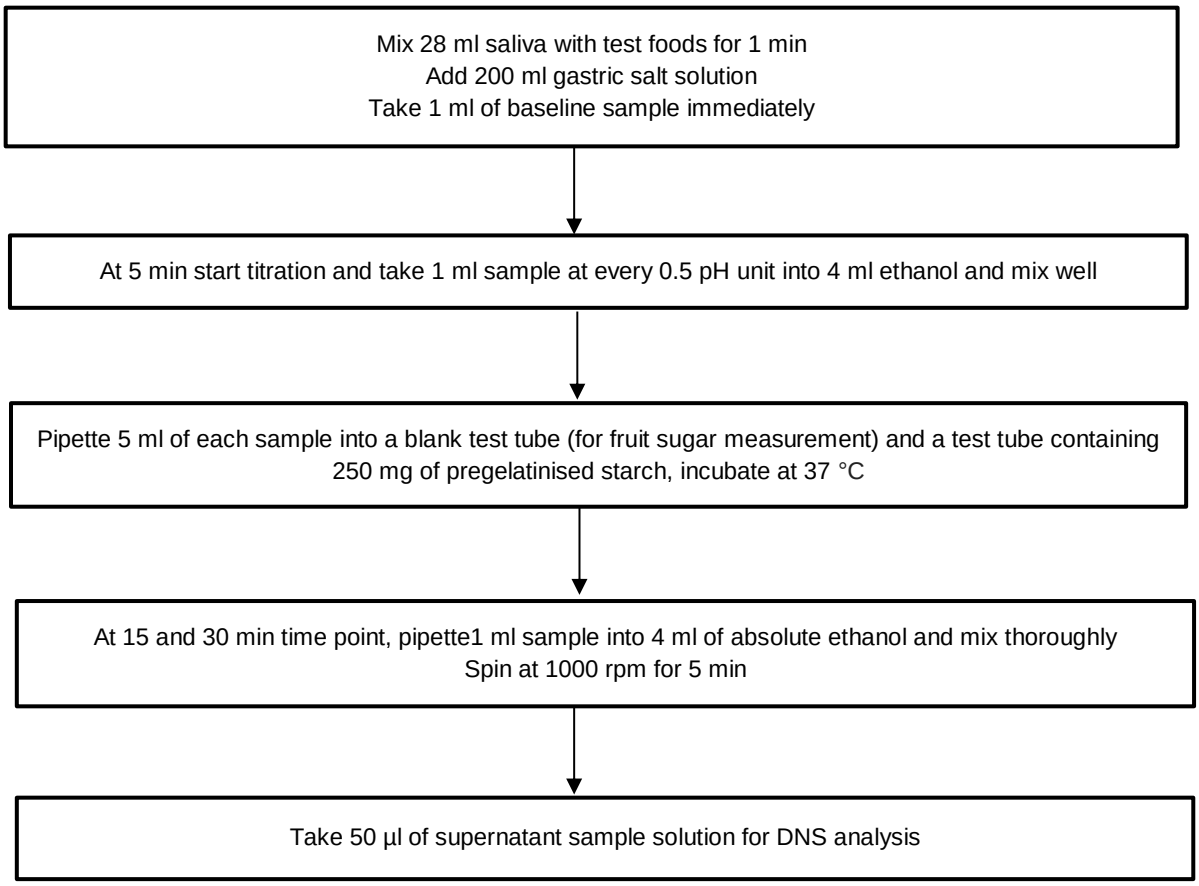

Figure 7 Flow chart of the experiment protocol

4. Results

4.1. Buffering capacity of fruits

The buffering capacity of test foods measured as mEq of 0.5 M HCl required to titrate from their initial pH down to gastric pH 2.0, and in the amount of 0.5 M NaOH consumed to titrating back towards intestinal pH 7.0 is shown in **Table 6**. Addition of 200 ml of gastric salt solution did not alter the pH of test foods. Titration of water blank showed that the gastric salt solution had no buffering effect. All of the fruits tested were able to substantially reduce the pH of the gastric medium from near neutral to within a pH range of 2.9-3.6. However, despite the similarity of the initial pH of the gastric media immediately after adding the fruit, titration revealed that they differed considerably in buffering capacity. The test food with the greatest buffering capacity was SunGold kiwifruit that consumed an average of 13.4 mEq HCl compared with 4.66 mEq consumed by the test food with the least buffering capacity, the Pink Lady apple.

Table 6 Acid (0.5 M HCl) and alkali (0.5 M NaOH) required (mean±SD) to titrate test foods[1] from their initial pH down to pH 2.0 and subsequently up to pH 7.0 at room temperature

Test foods	Initial pH	Acid (mEq)	Alkali (mEq)
Water blank	7.7	2.8±0.3	3.1±0.3
Pink lady apple	3.2	4.7±0.1	11.5±0.2
Blueberry	3.0	5.4±1.1	16.7±1.4
Granny smith apple	3.3	5.8±0.3	14.5±0.8
Strawberry	3.4	6.8±0.1	15.8±0.5
Raspberry	2.9	6.9±0.0	31.7±0.2
Blackberry	3.3	8.8±0.1	21.6±0.2
Hayward kiwifruit	3.3	10.5±0.8	30.8±0.3
Orange	3.6	11.0±0.2	21.7±0.5
SunGold kiwifruit	3.4	13.4±0.2	35.0±1.0

The buffering capacity of a control and three fruits (Granny smith apple, Hayward and SunGold kiwifruits) when co-ingested with Weet-Bix™ is shown in **Table 7**.

[1] Test foods consisted of 100 g fruit pulp and 200 ml gastric salt solution, while water blank had 100 g water substituted for fruit. Titration was performed at a constant dosing rate with acid and alkali dispensed at 2 ml/min.

Table 7 Acid (0.5 M HCl) and alkali (0.5 M NaOH) (mean±SD) and mean time required (min) required to titrate test foods[1] from their initial pH down to pH 2.0 and subsequently up to pH 7.0 at room temperature

Test foods	Initial pH	Mixture pH	HCl (mEq)	Time (min)	NaOH (mEq)	Time (min)
Weet-Bix™ control	-	5.5	11.4±0.1	12	13.8±0.0	14
Granny smith apple + Weet-Bix™	3.3	4.0	12.5±0.7	14	24.6±0.9	26
Hayward kiwifruit + Weet-Bix™	3.3	3.6	17.7±2.5	20	43.2±1.6	45
SunGold kiwifruit + Weet-Bix™	3.4	3.7	22.3±0.2	23	46.5±0.6	48

The titration curves of the control and the three fruits (Granny smith apple, Hayward and SunGold kiwifruits) when co-ingested with Weet-Bix™ in a simulated gastric acidification and a subsequent intestinal neutralization is shown in **Figure 8**.

[1] Test foods consist of 41.8 g crushed Weet-Bix™, 100 g fruit pulp and 200 ml gastric salt solution, while control had 100 g water substituted for fruit. Titration was performed at a constant dosing rate with acid and alkali dispensed at 2 ml/min.

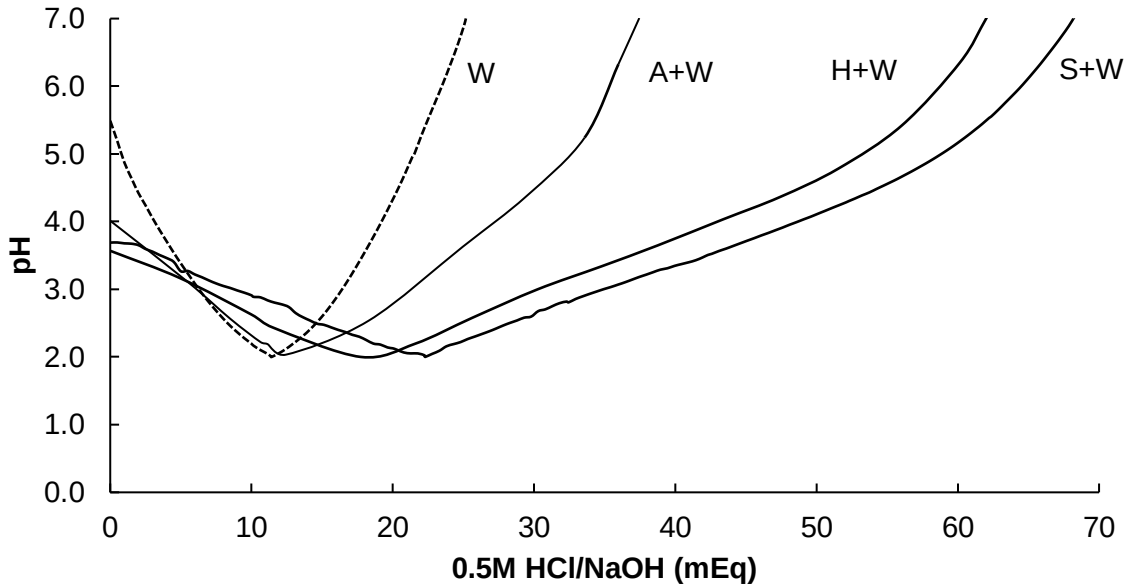

Figure 8 Mean acid and alkali (mEq) required to titrate Weet-Bix™ control (W), Granny smith apple + Weet-Bix™ (A+W), Hayward kiwifruit + Weet-Bix™ (H+W) and SunGold kiwifruit + Weet-Bix™ (S+W) down to pH 2.0 and subsequently up to pH 7.0 at room temperature

The buffering capacity of a control and three fruits (Granny smith apple, Hayward and SunGold kiwifruits) when rolled oats is shown in **Table 8**.

Table 8 Acid (0.5 M HCl) and alkali (0.5 M NaOH) (mean±SD) and mean time required (min) to titrate test foods[1] from their initial pH down to pH 2.0 and subsequently up to pH 7.0 at room temperature.

Test foods	Initial pH	Mixture pH	HCl (mEq)	Time (min)	NaOH (mEq)	Time (min)
Oats control	-	5.8	15.4±0.1	18	15.9±0.3	17
Granny smith apple + oats	3.3	4.0	17.4±0.1	18	29.0±0.3	31
Hayward kiwifruit + oats	3.3	3.5	23.6±0.2	25	47.1±0.5	49
SunGold kiwifruit + oats	3.4	3.8	24.3±0.1	26	47.3±0.4	49

The titration curves of the control and the three fruits (Granny smith apple, Hayward and SunGold kiwifruits) when co-ingested with rolled oats in a simulated gastric acidification followed by a subsequent intestinal neutralization is shown in **Figure 9**.

[1] Test foods consist of 51.6 g rolled oats, 100 g fruit pulp and 200 ml gastric salt solution, while control had 100 g water substituted for fruit. Titration was performed at a constant dosing rate with acid and alkali dispensed at 2 ml/min.

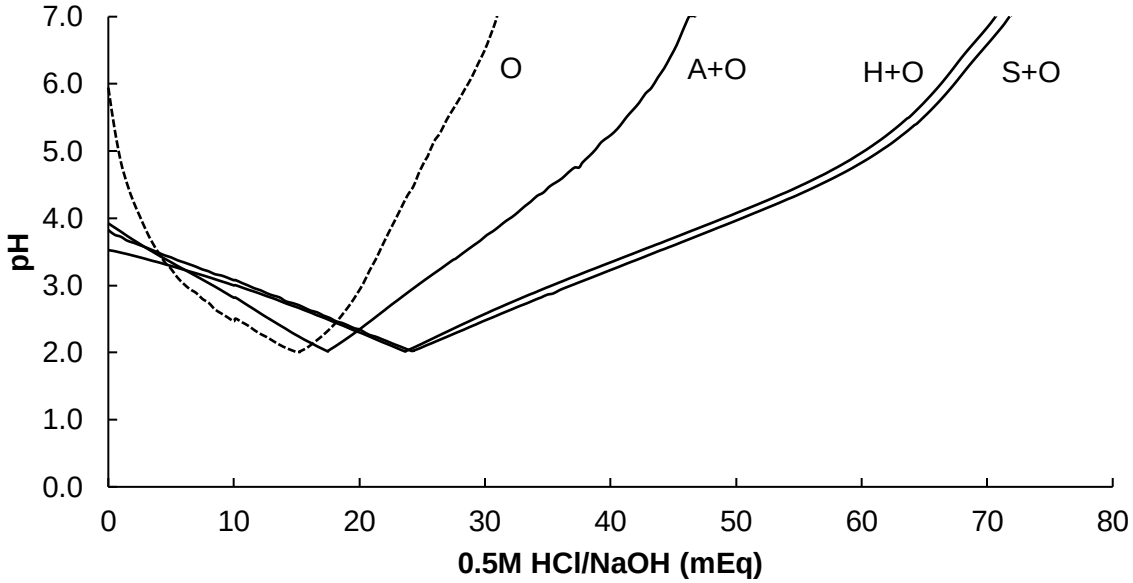

Figure 9 Mean acid and alkali (mEq) required to titrate oats control (O), Granny smith apple + oats (A+O), Hayward kiwifruit + oats (H+O) and SunGold kiwifruit + oats (S+O) down to pH 2.0 and subsequently up to pH 7.0 at room temperature

The buffering capacity of the fruits is clearly to be seen in the titration curves of the fruit-cereal combinations (**Figures 8 and 9**). Although the fruit-cereal combinations were similar in initial pH the acid consumed in acidification to pH 2.0 was almost twice as much for the kiwifruit-cereal combination as for the cereal alone. Addition of each of the three fruits reduced the pH while increased the buffering capacity in combination with both cereal foods in the order of SunGold> Hayward> Apple.

4.2. Effect of pH and Ca-KF buffer on HSA

The activity of HSA in Ca-KF buffer that was developed for the experiments against Ca-saline solution at a pH range of 7.0 to 2.0 was studied by measuring mg/g starch digested in 10 minutes at 37°C (**Table 9**). Starch digestion by saliva in Ca-saline solution and Ca-KF buffer was significantly different at pH 3.0 to 6.0 ($p<0.001$).

Table 9 Starch digested (mean±SD) in 10 minutes in by 50 µl of saliva in Ca-saline solution and Ca-KF buffer at a pH range of 7.0 to 2.0 at 37°C

pH	Starch digested (mg/g)		
	Ca-saline solution	Ca-KF buffer	P value
7.0	366.4±89.7	318.8± 56.0	0.203
6.0	373.2±75.3	264.2± 60.3	p<0.001
5.0	309.2± 38.7	152.6± 53.5	p<0.001
4.0	201.9± 27.5	19.6± 10.0	p<0.001
3.0	43.8± 13.5	15.2± 8.8	p<0.001
2.0	19.3± 5.3	15.9± 8.3	0.154

A scatterplot of starch digestion in 10 minutes by 50 µl saliva in Ca-saline solution and Ca-KF buffer a pH range of 7.0 to 2.0 is shown in **Figure 10**. HSA in saliva used for this experiment exhibited maximal activity at pH 7.0 and minimal activity at pH 2.0 and a decreasing trend in starch digestion as pH decreased was observed in both solutions. Little starch was digested by

HSA in Ca-KF buffer at pH 4.0 and 3.0 compared to a significantly larger amount digested in Ca-saline buffer.

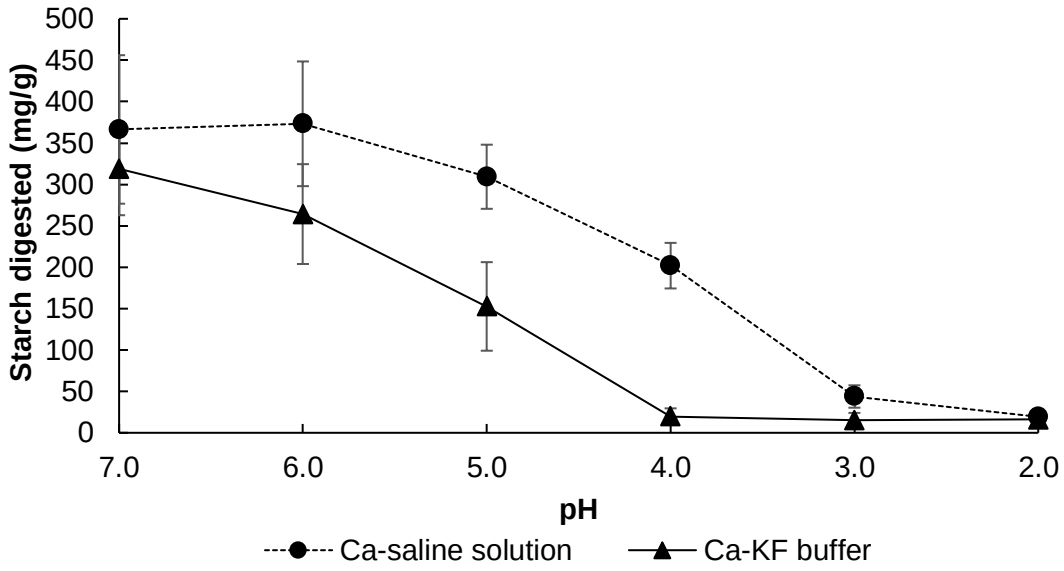

Figure 10 Mean starch digested (mg/g) in 10 minutes in by 50 µl of saliva in Ca-saline solution and Ca-KF buffer at a pH range of 7.0 to 2.0 at 37°C

Given the little starch digestion by 50 µl saliva in 10 min in Ca-KF buffer at low pH, the maximal activity of HSA in a larger amount of saliva (50 µl and 100 µl) over a longer period of time (0-60 min) was investigated (**Table 10**).

Table 10 Starch digested (mean±SD) over 60 minutes by 50 µl and 100 µl fresh saliva in neutralized Ca-KF buffer (pH 7.0) at 37°C

Time (min)	Starch digested (mg/g)	
	50 µl saliva	100 µl saliva
0	6.7±0.9	21.9±2.7
5	136.7±24.1	216.9±19.0
10	189.4±23.6	313.4±27.8
20	377.3±50.2	425.4±22.4
30	377.3±59.1	422.8±17.6
40	388.4±54.9	460.9.4±32.9
60	465.8±122.1	488.7±46.4

A scatterplot of starch digestion by HSA in 50 and 100 µl of saliva at its optimal pH 7.0 over 60 minutes is shown in **Figure 11**. Although 100 µl saliva digested more starch than 50 µl saliva, a rapid increase in starch digestion in 20 minutes followed by a plateau was observed in both cases.

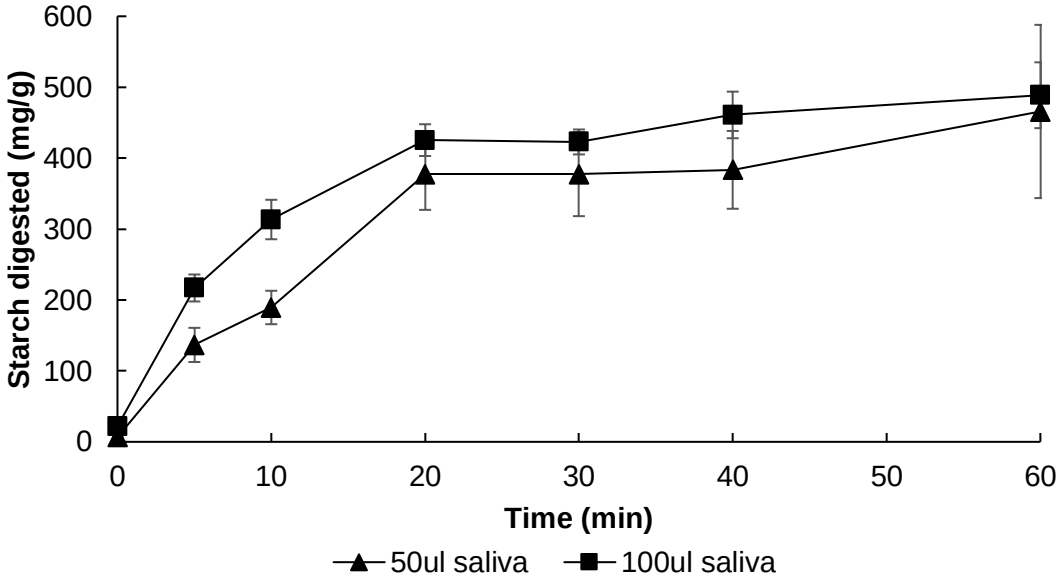

Figure 11 Mean starch digested (mg/g) over 60 minutes by 50 µl and 100 µl fresh saliva in neutralized Ca-KF buffer (pH 7.0) at 37°C

4.3. Effect of pepsin on HSA

Given the pH sensitivity of HSA to pH treatments, all samples were neutralized before conducting starch digestion analysis to eliminate the effect of pH on HSA activity from this part onwards. In addition, one ml of saliva would be used as starch digestion by 50 µl and 100 µl of saliva might not be sufficient for investigating effects of additional treatments on HSA activity.

The activity of HSA under the effect of pepsin at different pH was investigated by measuring mg/g starch digested by remaining HSA at pH 7.0 after 30 minutes of pre-incubation with 10% pepsin at a pH range of 7.0 to 2.0 (**Table 11**).

Table 11 Starch digested (mean±SD) in 15 and 30 minutes by saliva after 30 minutes pre-incubation with 10% pepsin in Ca-KF buffer at a pH range of 7.0 to 2.0 at 37°C

pH before neutralization	Starch digested (mg/g)	
	15 min starch digestion	30 min starch digestion
7.0	611.5±72.2	681.7±106.4
6.0	624.1±99.1	583.3±110.8
5.0	575.3±89.8	634.1±83.7
4.0	355.5±40.5	507.6±73.0
3.0	328.6±161.6	485.7±151.1
2.0	6.2±6.1	4.0±3.5

A scatterplot of remaining HSA activity after pepsin treatment as measured by starch digestion in 15 and 30 minutes is shown in **Figure 12**. HSA appeared to be able to resist 30 minutes of pre-incubation with 10% pepsin at about pH 5.0 and above but activity declined as pH dropped below 4.0. No HSA activity was found at pH 2.0.

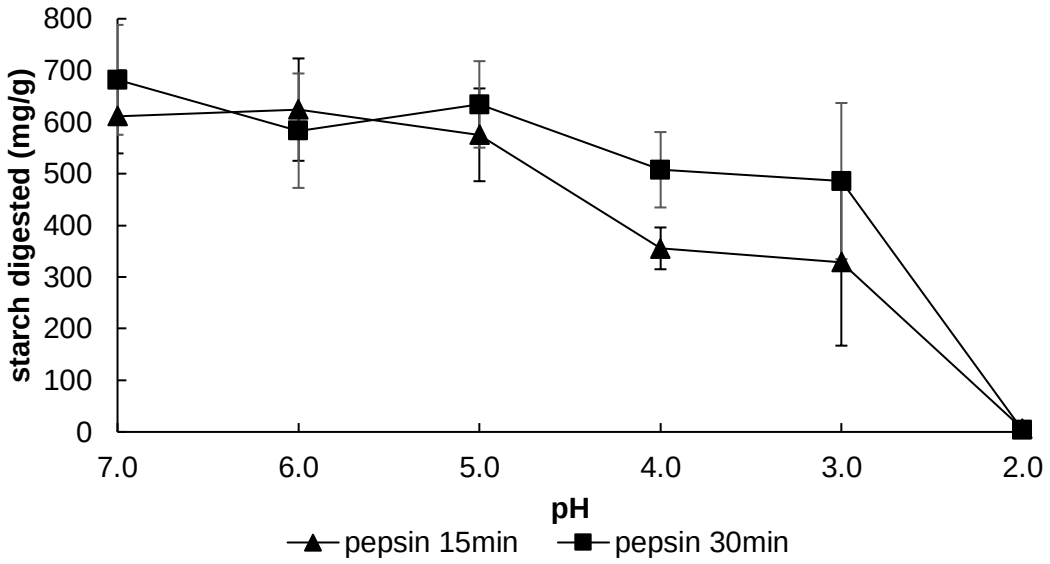

Figure 12 Mean starch digested (mg/g) in 15 and 30 minutes by saliva after 30 minutes pre-incubation with 10% pepsin in Ca-KF buffer at a pH range of 7.0 to 2.0 at 37°C

4.4. Effect of fruits on HSA

4.4.1. Hayward kiwifruit

The effect of 30 min Hayward kiwifruit treatment at different pH on HSA activity as measured by mg/g starch digested by remaining HSA is shown in **Figure 13**. Hayward kiwifruit treatment at pH 4.0 resulted in a drop in HSA activity.

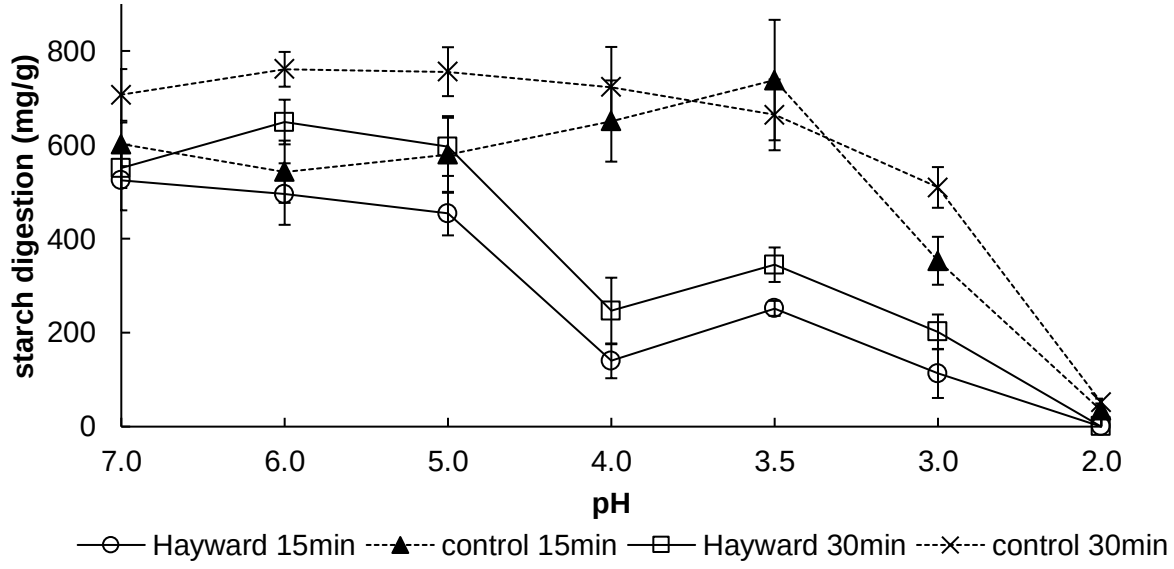

Figure 13 Mean starch digested (mg/g)[1] in 15 and 30 minutes by saliva after 30 minutes pre-incubation with in Ca-KF buffer at a pH range of 7.0 to 2.0 at 37°C with 50% Hayward kiwifruit and without kiwifruit (control)

[1] A background reading of 20.3 mg/g (blank containing 50 mg pre-gelatinized starch in pH 7.0 Ca-KF buffer and no HSA) was subtracted from all samples.

A student t-test was conducted to test the significance of difference between starch digestion by remaining HSA in saliva controls and Hayward treated samples and the p values are reported in **Table 12**. Pre-incubation with Hayward kiwifruit inhibited HSA activity as shown by a significant reduction ($p<0.01$) in both 15 and 30 minutes of starch digestion in the Hayward treated sample compared with controls at pH 4.0 and below.

Table 12 p values of a student t-test for Hayward treatment

	p value of t-test (Hayward treatment vs. control)						
	pH 2.0	pH 3.0	pH 3.5	pH 4.0	pH 5.0	pH 6.0	pH 7.0
15 min starch digestion	p<0.001	p<0.001	p<0.001	p<0.001	0.007	0.264	0.037
30 min starch digestion	p<0.001	p<0.001	p<0.001	p<0.001	0.013	0.003	0.003

4.4.2. SunGold kiwifruit

Pre-incubation with SunGold kiwifruit at pH 3.5 and below led to inhibited HSA activity as shown in **Figure 14.** The data point at pH 3.5 for control at 30 min may have included an outlier as indicated by the acute deviation in the graph and large SD. There was little HSA activity remained in SunGold samples treated at pH 3.0. In contrast to the Hayward kiwifruit the SunGold did not cause inhibition of HSA at pH 4.0.

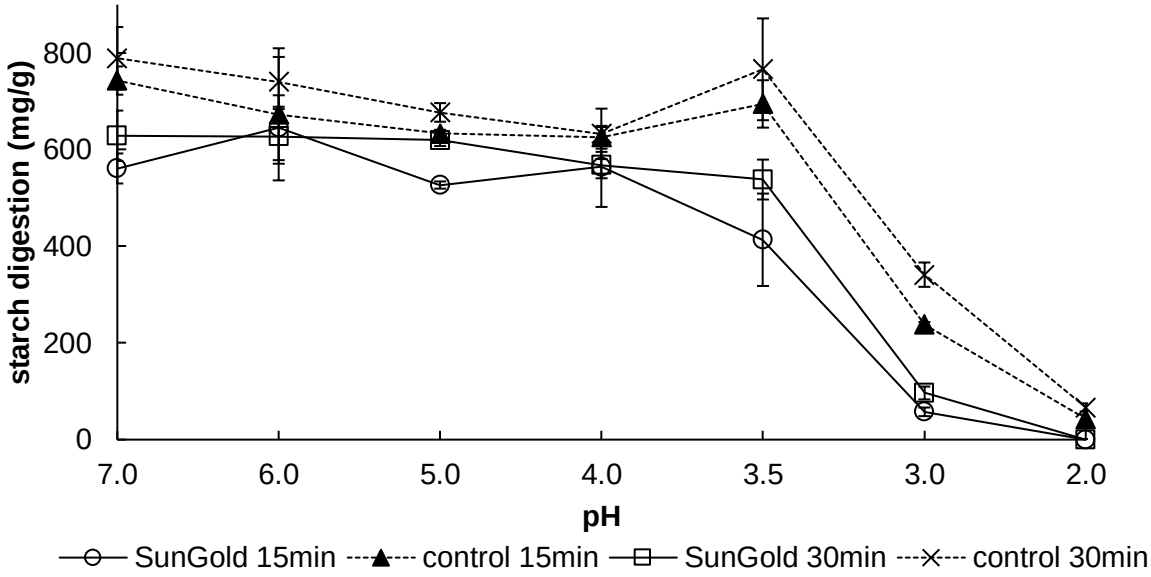

Figure 14 Mean starch digested (mg/g)[1] in 15 and 30 minutes by saliva after 30 minutes pre-incubation with 50% SunGold kiwifruit in Ca-KF buffer at a pH range of 7.0 to 2.0 at 37°C

P values of a student t-test are reported in **Table 13**. Pre-incubation with SunGold kiwifruit inhibited HSA activity as shown by a significant reduction ($p<0.01$) in both 15 and 30 minutes of starch digestion in the SunGold treated sample compared with controls at pH 3.0.

Table 13 p values of a student t-test for SunGold treatment

	p value of t-test (SunGold treatment vs. control)						
	pH 2.0	pH 3.0	pH 3.5	pH 4.0	pH 5.0	pH 6.0	pH 7.0
15 min starch digestion	0.002	p<0.001	0.027	0.380	0.066	0.674	0.147
30 min starch digestion	p<0.001	p<0.001	0.046	0.169	0.238	0.379	0.035

[1] A background reading of 12.7 mg/g (blank containing 50 mg pre-gelatinized starch in pH 7.0 Ca-KF buffer and no HSA) was subtracted from all samples.

4.4.3. Granny Smith apple

Figure 15 shows the starch digestion by remaining saliva in controls and Granny Smith apple treated samples pre-incubated at a pH range of 7.0 to 2.0. HSA activity were consistent throughout the pH range in both control and Granny Smith apple treated samples.

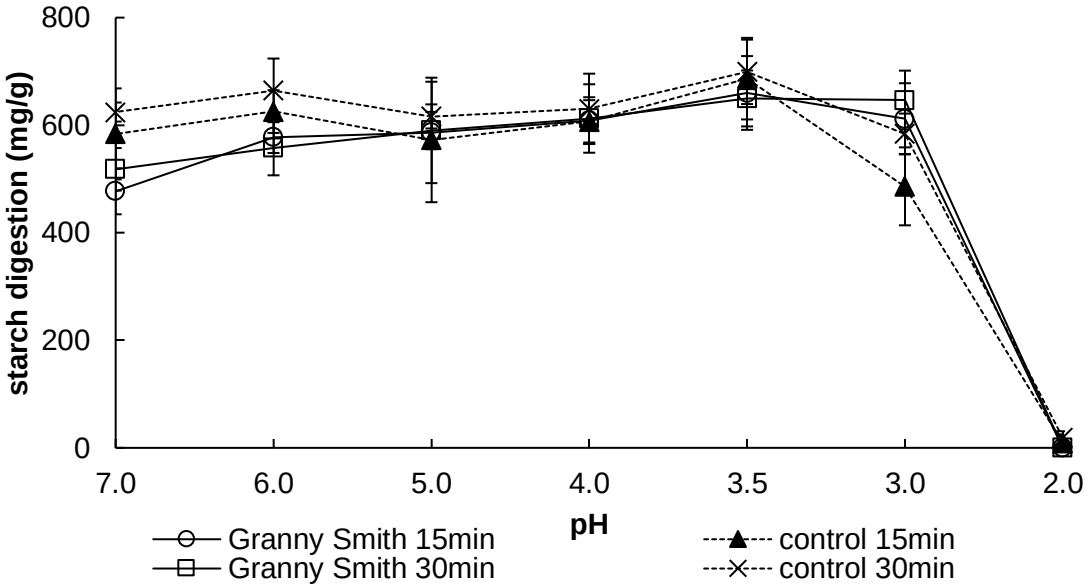

Figure 15 Mean starch digested (mg/g)[1] in 15 and 30 minutes by saliva after 30 minutes pre-incubation with 50% granny smith apple in Ca-KF buffer at a pH range of 7.0 to 2.0 at 37°C

A student t-test (p values reported in **Table 14**) revealed a significant but small difference ($p<0.05$) between HSA activity in controls and Granny Smith apple treated in 30 min digestion.

[1] A background reading of 14.9 mg/g (blank containing 50 mg pre-gelatinised starch in pH 7.0 Ca-KF buffer and no HSA) was subtracted from all samples.

Table 14 p values of a student t-test for Granny Smith apple treatment

	p value of t-test (apple treatment vs. control)						
	pH 2.0	pH 3.0	pH 3.5	pH 4.0	pH 5.0	pH 6.0	pH 7.0
15 min starch digestion	0.124	0.007	0.196	0.974	0.748	0.157	0.063
30 min starch digestion	0.012	0.021	0.035	0.783	0.230	0.005	0.014

4.5. Effect of pepsin and kiwifruit actinidin on HSA

4.5.1. Starch digestion

The effect of pepsin alone and pepsin combined with Hayward kiwifruit on HSA activity at pH 4.0 to 2.0 is presented in **Figure 16**. Pre-incubation with pepsin alone at pH 3.0 and 2.0 completely inactivated HSA activity as no starch digestion was seen at those low pH levels. Treatment with pepsin and Hayward kiwifruit combined lowered the HSA activity at pH 4.0 and 3.5, but more HSA activity survived at pH 3.0 compared to treatment with pepsin alone.

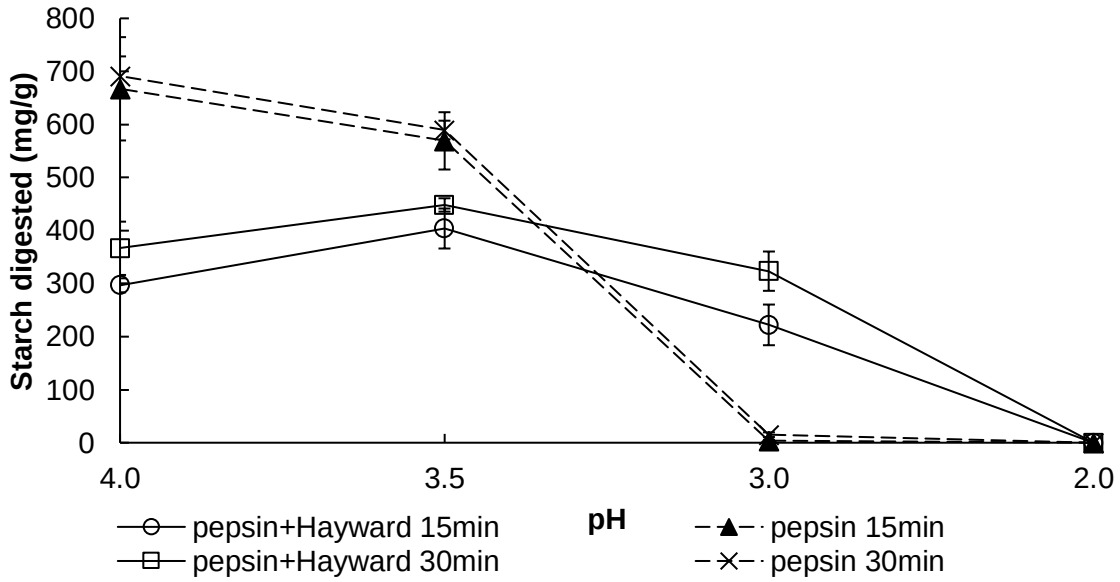

Figure 16 Mean starch digested (mg/g)[1] in 15 and 30 minutes by saliva after 30 minutes pre-incubation with 10% pepsin and 50% Hayward kiwifruit in Ca-KF buffer at a pH range of 4.0 to 2.0 at 37°C compared with incubation with 10% pepsin alone

[1] A background reading of 15.5 mg/g (blank containing 50 mg pre-gelatinised starch in pH 7.0 Ca-KF buffer and no HSA) was subtracted from all samples.

4.5.2. SDS-PAGE

SDS-PAGE analysis of pre-incubated samples (**A**) [1] and samples from a small scale experiment following the same protocol (**B, C, D**) [2] are presented in **Figure 17**. Both experiments produced similar results although the concentrations of pepsin and kiwifruit differed between them. HSA, pepsin and actinidin appeared on SDS as bands at approximately 56, 40 and 28 kDa, respectively. Pepsin actively degraded actinidin as well as HSA with and without the presence of actinidin at pH 2.0 and 3.0. Pepsin activity declined as the pH increased above 3.5. Actinidin was the most active at degrading HSA at pH 4.0 when pepsin was inhibited.

[1] Experiment A used 10% pepsin (>400 U/mg protein) and 50% kiwifruit.
[2] Experiment was a small scale experiment conducted in Eppendorf tubes following the same protocol as experiment A, but used 5 mg/ml pepsin (3200-4500 U/mg protein) and 40% kiwifruit.

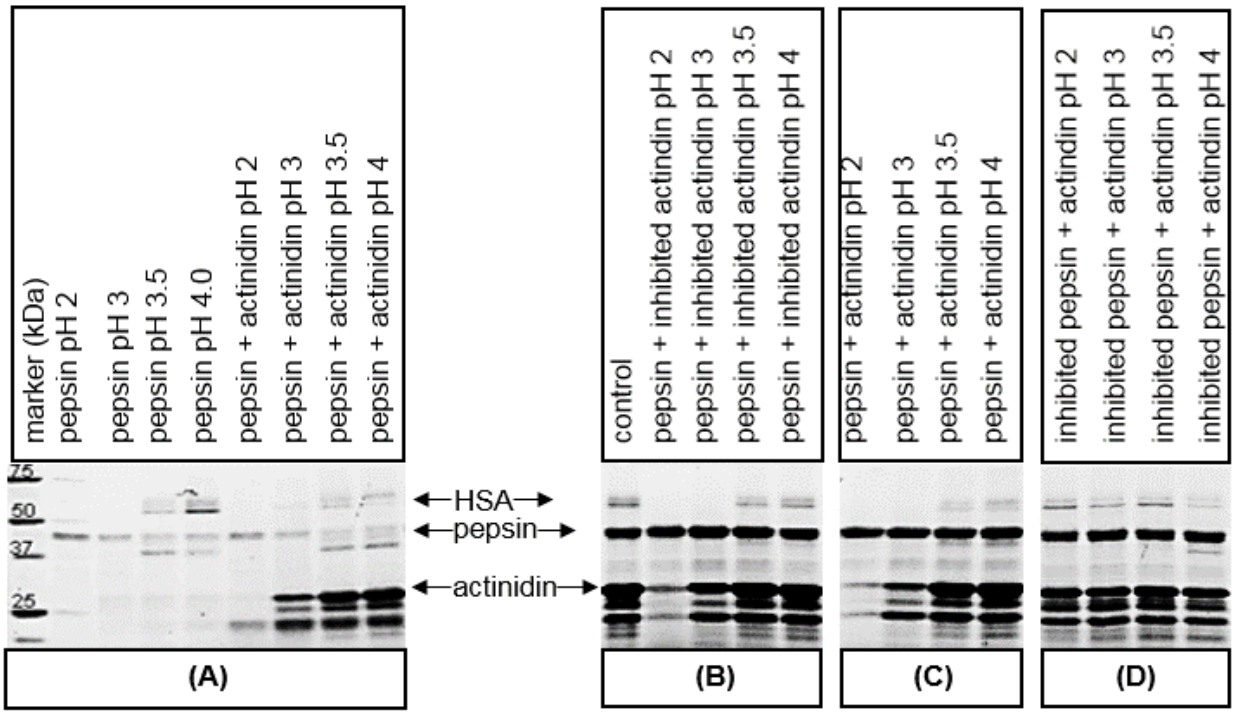

Figure 17 SDS-PAGE of HSA digested with pepsin and kiwifruit actinidin. (A) Neutralized pre-incubated samples (30 min, 37°C) in which saliva was treated with pepsin alone and pepsin + Hayward kiwifruit at pH 2.0, 3.0, 3.5 and 4.0. (B) The control sample contained HSA, inhibited pepsin (with its selective inhibitor pepstatin) and inhibited actinidin (with its selective inhibitor E-64). In the test samples, saliva was treated with pepsin + Hayward kiwifruit with inhibited actinidin; (C) The saliva samples contained pepsin + Hayward kiwifruit with active actinidin; (D) The saliva samples contained inhibited pepsin (with its selective inhibitor pepstatin) + Hayward kiwifruit with active actinidin

4.5.3. Actinidin activity

The kinetic activity of actinidin that remained in the samples after pre-incubation with pepsin is presented in **Figure 18**. The sample pre-incubated at pH 4.0 exerted the highest actinidin activity, while pre-incubation with pepsin at pH 2.0 and 3.0 led to complete loss of actinidin activity.

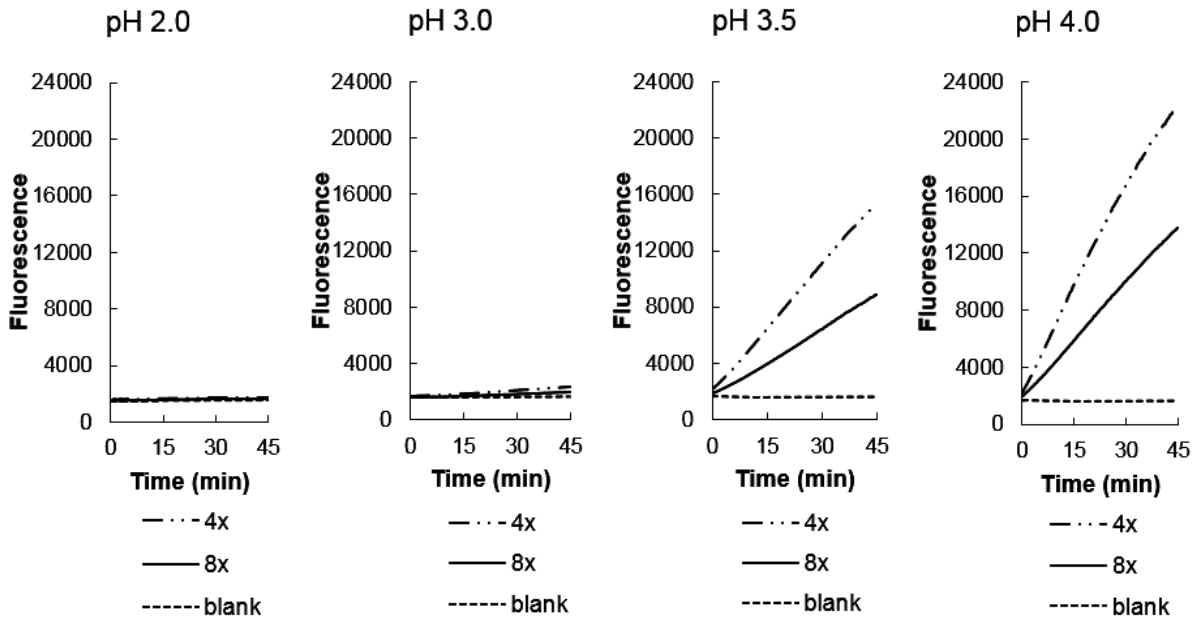

Figure 18 The kinetic activity of remaining actinidin after pre-incubation at pH 2.0, 3.0, 3.5 and 4.0 followed by neutralization was determined from coumarin fluorescence read at excitation and emission wavelengths of 351 and 430 nm. Samples were subjected to gradient dilutions using 0.1 M sodium acetate buffer (pH 4.5), the blank contained only acetate buffer

4.6. Effect of pepsin and kiwifruit actinidin on HSA in Weet-Bix™ meal

4.6.1. Saliva secretion

Approximately 28 g (28 ml) of saliva is required for chewing 41.8 g Weet-Bix™ according to an average of 66.1% saliva secretion of dry Weet-Bix™ (**Table 15**). This estimation will be used in the following experiment to mimic the oral and gastric phase of starch digestion.

Table 15 Estimation of saliva secretion determined from weight difference between chewed Weet-Bix™ (wet bolus) and freeze-dried bolus samples produced in a chewing test.

	Sample 1	Sample 2	Sample 3	Sample 4
Weight of wet bolus (g)	4.7	5.6	4.9	5.4
Weight of dried bolus (g)	2.8	3.4	3.0	3.2
Weight of saliva (g)[1]	1.9	2.2	1.9	2.2
% saliva of dry weight	67.9%	64.7%	63.3%	68.8%
Mean	66.2%			

4.6.2. Starch digestion

Samples in this part of the experiment were not neutralized after pre-incubation. The amount of starch digested (mg/g) (including fruit sugars where applicable) during gastric acidification in

[1] Difference between wet and dried bolus was assumed to be loss of saliva. The moisture content of the dry Weet-Bix™ and the dry matter content of saliva were not accounted for.

each test food[1] is presented in **Figure 19**. Granny Smith apple is compared with the other fruit treatments at the same sample pH although its digestion time was the shortest due to low buffering capacity. Starch digestion was consistent as pH decreased from 5.0 to 2.0 in both control and pepsin treated samples (**Figure 19 A**), while fruit treated samples started at a low pH resulting in low starch digestion (**Figure 19 B**).

Table 16 Time and pH of each sample during a simulated gastric acidification of Weet-Bix™ control (W), Hayward kiwifruit + Weet-Bix™ (H+W), SunGold kiwifruit + Weet-Bix™ (S+W), granny smith apple + Weet-Bix™ (A+W), pepsin + Weet-Bix™ (P+W) and pepsin + Hayward kiwifruit + Weet-Bix™ (P+H+W)

pH of sample	**Digestion time (min)**					
	W	**H+W**	**S+W**	**A+W**	**P+W**	**P+H+W**
Initial pH (at 5 min) [2]	5.4	3.7	3.7	4.1	5.3	3.6
5.0	5.5	-	-	-	5.3	-
4.5	6.5	-	-	-	6.5	-
4.0	8.0	-	-	-	8.0	-
3.5	9.5	6.7	7.0	8.8	9.7	6.5
3.0	11.3	13.7	13.0	12.2	11.7	13.2
2.5	13.3	20.0	19.0	16.0	13.8	19.8
2.0	16.9	26.0	24.3	19.9	17.6	25.4

[1] Test foods consisted of 41.8 g Weet-Bix™, 28 ml saliva, 100 g fruit pulp, 200 ml gastric salt solution and 7 ml 10% pepsin where applicable. Control had 100 g water substituted for fruit.
[2] Simulated gastric acidification commenced after 5 min of mixing. Samples were taken for DNS analysis at 5 min and at each 0.5 pH unit afterwards. Time at each sampling point was exported from the software Tiamo.

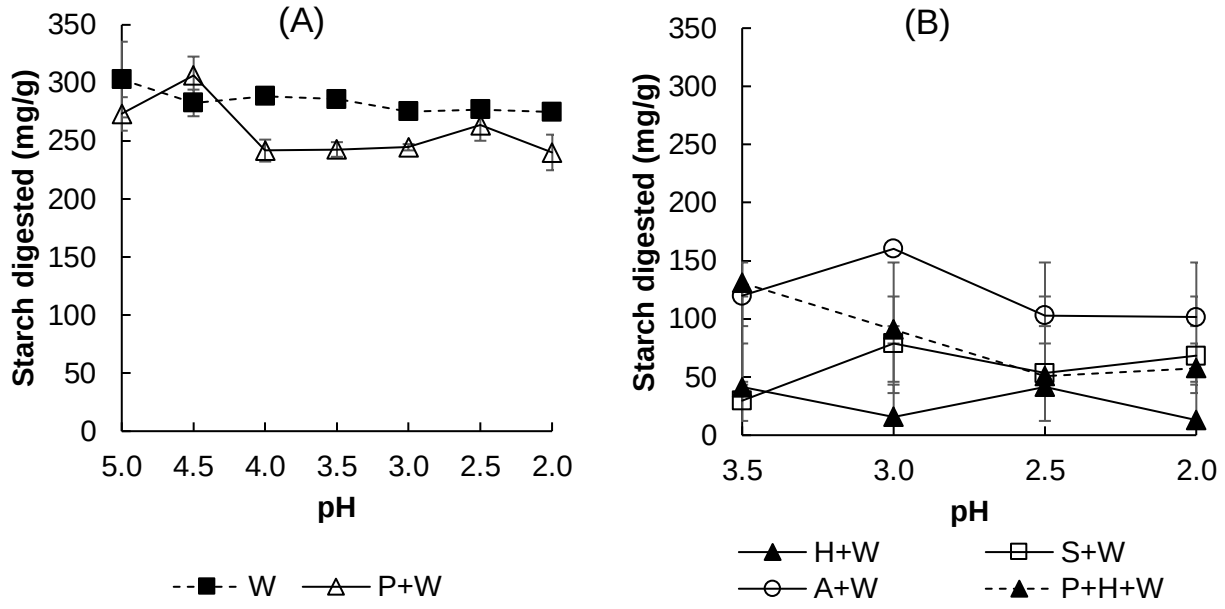

Figure 19 Mean starch digested (mg/g) (including fruit sugars where applicable) in test foods at different pH levels during a simulated gastric acidification. (A) Comparison between Weet-Bix™ control (W) and pepsin + Weet-Bix™ (P+W) that are similar in digestion digestion time at each sample pH. (B) Comparison between Hayward kiwifruit + Weet-Bix™ (H+W), SunGold kiwifruit + Weet-Bix™ (S+W), granny smith apple + Weet-Bix™ (A+W) and pepsin + Hayward kiwifruit + Weet-Bix™ (P+H+W) that are similar in digestion time

5. Discussion

This study is the first to investigate factors underlying the glycaemic lowering effect of green kiwifruit on co-ingested starchy foods. The impetus for this work originated from a report by Mishra et al who speculated on mechanisms for a glycaemic-dampening effect of kiwifruit on starch (31). The current work was undertaken to elucidate the possible fruit related factors in glycaemic response to co-ingested cereal foods in light of the significant role of HSA in gastric digestion of starch recognised by Freitas et al (23, 60).

A semi-dynamic gastric model was developed for this project, involving automated titration at a physiological rate, which enabled mimicking of gastric digestion and analysis of HSA at a changing pH. The experimental results revealed that firstly, the low pH and strong buffering capacity of kiwifruit contributed to instant acidification of a food bolus causing acid inhibition of HSA. Secondly, organic acids such as citric acids in kiwifruit may inhibit HSA directly. Thirdly, kiwifruit actinidin degraded HSA at its optimal pH of 3.5-4.0 which was the pH of the fruit. The strong buffering capacity of both kiwifruit varieties (Hayward and SunGold kiwifruit) enabled prolonged effects of acid inhibition on HSA by delaying gastric acidification. The protease actinidin in Hayward kiwifruit resulted in additional inhibition by degrading HSA at its optimal pH around 4.0. However, the low pH of kiwifruit also promoted the activation of pepsin which degraded HSA as well as actinidin at pH 3.0 and below. Therefore, our experiments suggest that kiwifruit is likely to promote retardation of gastric starch digestion by HSA through lowering gastric pH which enables acid inhibition and early activation of pepsin, additional partial suppression by citric acid as well as protease effect of actinidin (Hayward kiwifruit in particular). Moreover, it is plausible that a delay in gastrointestinal pH adjustment due to strong buffering of kiwifruit may impose a delay in GE which determines the rate that digestive contents enter the small intestine for glucose absorption. Overall, the above factors and their interactions may in

part explain the lowered glycaemic response to starchy foods when green kiwifruit is co-ingested in an equal carbohydrate exchange format.

5.1. Buffering capacity of various fruits

The buffering capacity of foods measured as titratable acidity has been claimed to have an impact on health conditions such as dental erosion in humans (87) and gut fermentation toxicity in livestock (88, 89). Our study partially filled a knowledge gap by investigating the buffering capacity of different fruits (i.e. kiwifruit, berries, apples and orange) during a simulated gastric digestion by titrating at a physiological rate.

Based on the results of titration as shown in **Table 6**, the test fruits could be ranked as follows according to their buffering capacity in a simulated gastric acidification: SunGold kiwifruit > orange > Hayward kiwifruit > blackberry > raspberry > strawberry > granny smith apple > blueberry > pink lady apple. SunGold kiwifruit (the most buffering fruit) consumed nearly three times as much acid as Pink Lady apple (the least buffering fruit) in titrating to pH 2.0 despite a small difference in initial pH (0.2). Hayward kiwifruit consumed nearly twice the amount of acid as Granny Smith apple while having the same initial pH of 3.3. Therefore, the buffering capacity of fruits can vary considerably in spite of fruit pH.

Granny Smith apple, Hayward and SunGold kiwifruit were further investigated as part of a cereal meal because of their similarity in fruit pH but disparity in buffering capacity. Granny Smith apple was chosen instead of pink lady apple because it is more commonly found in the market and stewed Granny Smith apple is a popular breakfast food. Mixing the three fruits with either Weet-Bix™ (**Figure 8**) or oats (**Figure 9**) reduced pH while increasing the buffering

capacity of both cereal foods in the order of SunGold kiwifruit > Hayward kiwifruit > Granny Smith apple, consistent with the buffering capacity of these fruits titrated alone. Mishra et al (31) demonstrated that nearly three times as much acid (0.5 M HCl) was used to titrate a meal that comprised of 200 g Hayward kiwifruit and 47.3 g Weet-Bix™ (pH 3.7) to pH 2.5 than Weet-Bix™ alone (pH 5.3). In our experiment, adding 100 g Hayward kiwifruit increased the acid consumption by 1.5 times as shown in **Table 7**.

The level of organic acids is likely to be responsible for the difference in buffering capacity of fruits, as organic acids have been found to determine the acidity and buffering capacity in fruits (85, 86). A positive linear relationship was observed between levels of organic acids and buffering capacity, especially with acetic acid and citric acid (86). Citric acid is a common organic acid in fruits and is present in SunGold and Hayward kiwifruit; and in Granny Smith apples at the approximate concentration of 900, 970 and 26.4 mg/g, respectively (72, 110). However, citric acid contents can vary depending on orchard and storage condition after harvest as Marsh et al (70) reported that citric acid levels in kiwifruit decline during storage at 4°C.

The effect of highly buffering kiwifruit on delaying gastric pH adjustment of cereal meals that was demonstrated in our study may slow down the rate that gastric chyme is emptied into the duodenum for further digestion and absorption as proposed by Mishra et al (31). Delayed GE has been claimed to be an underlying mechanism for the reduced postprandial glycaemic response to sourdough bread (28) and starchy foods with added vinegar (29), attributed to the presence of lactic acid and acetic acid, respectively. Therefore, it is plausible that citric acid in kiwifruit may delay GE and in turn reduce postprandial glycaemia as it is an organic acid.

The research on buffering capacity of fruits has so far only scratched the surface as the food and meal components used in the experiment were simple. More in depth analysis of the effect

of fruit organic acids in complex meals containing other buffering components, for instance, milk, which is a common component of cereal meal is needed. In addition, the importance of fruit ripeness, which affect the level of organic acids and fruit acidity and the possibility of using pH adjusted fruit organic acids in controlling gastric pH and related digestive function are worthy of further investigation.

5.2. Ca-KF buffer and pH inhibition of HSA

Note that in the experiments investigating the effects of pH on HSA activity incubation was carried out in individual test tubes at different pH levels instead of taking continuous samples at different pH in one titration experiment because the latter method could produce another variable, reaction time which would be different between solutions with different buffering capacities.

The Ca-KF buffer we developed contained similar concentrations of K and citric acid as in the kiwifruit and was diluted to reflect the change in concentration in titration (100 g pulp diluted with 200 ml gastric salt solution). Citric acid is one of the main organic acids in kiwifruit and has been found to inhibit HSA due to its chelating effect, which reduces the availability of free Ca^{2+} ions required for HSA's enzymatic function (52, 71). $CaCl_2$ has been used for stabilising HSA in forensic saliva identification (71). $CaCl_2$ (100 ppm) was found to enhance HSA activity and reduce peak starch apparent viscosity by 77% by Morris et al (111) and therefore was added to the buffer. Our result showed that HSA activity in the Ca-KF buffer was significantly lower than that in Ca-saline solution at a pH range of 6.0 to 3.0 ($p<0.001$), possibly due to the inhibition of citric acid.

There was a decreasing trend in HSA activity as pH decreased from 7.0 to 2.0 in both Ca-saline and Ca-KF buffer solutions (**Figure 10**) indicating a pH effect on HSA activity. A low pH of 3.0 and below inactivated HSA as shown by little starch digestion at pH 3.0 and 2.0 in both solutions. Freitas et al (60) found that HSA exerted maximal activity at 6.2 and no activity at 3.0 at 20 °C using potato starch. Fried et al (19) claimed that inactivation of HSA occurred at pH 3.3-3.8 *in vitro* using an inhibitor assay. In our experiments, HSA had an optimal pH at 7.0, retained most of its activity at pH 4.0 (in Ca-saline solution) and was inactivated at pH 2.0.

When incubated at the optimal pH of 7.0 over 60 minutes with either 50 µl or 100 µl saliva, starch digestion increased rapidly over 20 minutes followed by a plateau (**Figure 11**). A similar observation was made by Freitas et al (60) who found a plateau was reached by 15 min in an incubation at pH 6.0. Another increase in starch digestion was observed in our experiment at 60 min which was not seen by Freitas et al (60). Ca-KF buffer was the buffer used throughout the experiments so its inhibition of HSA should always be taken into account.

The gastric pH can be raised up to 5.5-6.5 by a solid-liquid meal and takes nearly 2 hours to return to the fasted state (pH 1.7-2.0) in healthy adults (25, 64). The pH of a normal meal is close to optimum for HSA activity so the enzyme can continue hydrolysing starch in the stomach until the pH drops to below 4.0 that may take up to 45 minutes (61). The gradual acidification process provides a window for HSA to hydrolyse about 59% and 76% of starch in mashed potatoes and bread, respectively, in the stomach (20). This prolonged starch digestion by HSA may be higher in people who have insufficient gastric acid secretion as Fried et al (19) found that HSA accounted for 27% of total amylase output in subjects with normal stomach function compared with 11% in achlorhydric subjects after a hamburger meal. Therefore, acidic fruits with strong buffering capacity like kiwifruit are likely to promote acid inhibition of HSA during oral and gastric phase of starch digestion by maintaining the low pH of a food bolus.

The focus on citric acid in this study was because citric acid was known to inhibit HSA activity through both acid and chelating effect. It was therefore important to establish that the citric acid in kiwifruit did not have such a substantial inhibitory effect on HSA activity that it would be impossible to measure the effects of other factors such as pH and pepsin on HSA activity. While our study demonstrated that HSA activity could be measured in the presence of potassium and citric acid, in future research the buffer could be made to include a full range of kiwifruit organic acids, such as malic and quinic acids to give a more valid representation of kiwifruit juice.

5.3. Pepsin inhibition of HSA

Note that from this part onwards all pre-incubated samples were neutralised to eliminate the pH effect on HSA before carrying out starch digestion analysis. Therefore, starch digestion in the following context reflects the survival of HSA after pre-incubation. In addition, one ml saliva was used to increase starch digestion so that the effect of additional treatment would not be masked due to the pH sensitivity of HSA.

As shown in **Figure 12**, the inhibition effect of pepsin on HSA was clear at pH 5.0 and below when compared to pH 6.0 and 7.0 where pepsin was inactive. No starch digestion was observed in samples pre-incubated at pH 2.0. One explanation could be that pepsin degraded HSA at its optimal pH. An alternative explanation could be that at pH 2.0, HSA could have been irreversibly inactivated. Previous studies as well as the SDS-PAGE results in this study revealed that pepsin could break down dietary proteins in the stomach once activated by HCl at low pH around 2.0 (77). Pepsin is most active at pH 1.5-2.0 and inactive at pH 5.0 and above (78, 79). However, 70% of peptic activity was found to be present at pH 4.0 *in vitro* by Pier and Fenton

(81). Little was known about the direct effect of pepsin on HSA since gastric acidity was recognised as the major factor responsible for HSA inactivation in the stomach (15). However, one early study revealed that pepsin could contribute to a more extensive inhibition of HSA than HCl alone (82).

Pepsin is unlikely to be active during the early stage of gastric digestion of a normal meal (pH 5.5-6.5). However, if a food bolus containing kiwifruit can enter the stomach at a low pH (~pH 3.5), it could possibly promote early activation of pepsin and further inhibit HSA activity in the stomach especially in people who are achlorhydric

The limitation in this part of the experiment was a lack of control to clarify the complete inhibition of HSA at pH 2.0. Although the activity of HSA and pepsin as a function of pH has been extensively studied individually, conclusion on the inhibitory effect of pepsin will benefit from a comparison between control and the pepsin treated sample.

5.4. Fruit inhibition of HSA

A fruit sugar control was incubated at the sample condition as each fruit treated sample and the amount of fruit sugar was subtracted from the total reducing sugar measured by DNS to determine the amount of sugar released by HSA. A new pH point 3.5 was of interest because it was the pH of food bolus when Weet-Bix™ and oats were mixed with Hayward and SunGold kiwifruit *in vitro*.

Pre-incubation with Hayward kiwifruit significantly inhibited HSA activity especially when the pH was below 5.0 ($p<0.001$) (**Table 12**). HSA activity was most inhibited by Hayward kiwifruit at pH

4.0 before complete acid inhibition at pH 2.0 (**Figure 13**). Actinidin, a natural protease abundant in Hayward kiwifruit might be responsible for HSA inhibition at this particular pH as it has been reported to actively degrade HSA at its optimal pH of 4.0 in one recent study by Martin et al (32).

Pre-incubation with SunGold kiwifruit significantly reduced the activity of HSA at pH 3.5 and below ($p<0.05$) (**Table 13**) and the inhibition was most profound at pH 3.0 before the enzyme was denature by pH 2.0 (**Figure 14**). This may be a result of additional citric acid inhibition since SunGold kiwifruit was found to be more buffering possibly due to a higher concentration of citric acid compared with Hayward kiwifruit and Granny Smith apple. Degradation of HSA at pH 4.0 was not observed in SunGold, which further suggests the effect of actinidin in Hayward kiwifruit as Hayward and SunGold kiwifruit are similar in nutritional composition, except that actinidin is abundant in Hayward but only found in trace amount in SunGold (74, 75). Gold kiwifruit has been used as a control for Hayward by Montoya et al to investigate the effect of actinidin on gastric protein digestion *in vivo* (34, 35).

As shown in **Figure 15**, pre-incubation with Granny Smith apple resulted in little inhibition on HSA activity at pH 3.0 and below. Neutralisation of samples pre-incubated at pH as low as 3.0 restored more HSA activity in apple treated sample than that in kiwifruit treated samples. This difference could be a result of lower citric acid level and absence of actinidin in the Granny Smith apple compared with the kiwifruit.

Among three fruit treatments, Hayward kiwifruit inhibited HSA activity significantly more at a pH range of 7.0 to 2.0 than SunGold kiwifruit and Granny Smith apple. Inhibition was greatest at pH 4.0 possibly due to the presence of the actinidin protease in Hayward. SunGold kiwifruit

contributed to a significant but smaller inhibition on HSA at pH 3.5 and below and Granny Smith apple had little inhibition on HSA along the pH range.

A limitation of the research on actinidin activity against HSA was that in the titration experiments whole kiwifruit pulp was used compared with kiwifruit extracts used in this part of the experiment. Therefore, there was no absolute certainty that the inhibitory effects observed were due to actinidin alone. However, actinidin is known to be highly active in homogenised kiwifruit and almost absent in SunGold kiwifruit which was in consistency with our results.

5.5. Pepsin and actinidin interaction in HSA inactivation

The effect of actinidin and pepsin together on HSA activity was further investigated at a pH range from 4.0 to 2.0 because the pH of a food bolus was around 3.5 when kiwifruit was co-ingested, because pepsin and actinidin are both proteases yet little is known about how they affected one another. Actinidin has been reported to have a broader specificity and pH range of activity compared with pepsin (83, 112). Hayward kiwifruit containing actinidin was found to enhance gastric digestion of large dietary proteins (MW>32 kDa) that were less digestible by pepsin (33-35) or when pH and pepsin concentration in the stomach are not optimal (36). The degradation of HSA by actinidin at pH 4.0 observed earlier was again seen in this experiment (**Figure 16**). Addition of Hayward kiwifruit further reduced HSA activity at 4.0 and 3.5 compared with pepsin treatment alone. Pre-incubation with pepsin alone resulted in complete inhibition on HSA activity at pH 3, while HSA pre-treated with pepsin and actinidin combined at this pH was able to digest around 300 mg/g (30%) starch once neutralised. It is possible that introducing an extra protein weakened the proteolytic capacity of pepsin.

As there were three proteins (HSA, pepsin and actinidin) interacting with each other, SDS-PAGE analysis was needed to provide direct insight into the observations described above. Analysis of pre-incubated samples by SDS-PAGE in **Figure 17 A** showed trends consistent with the results of starch analysis. In samples treated with pepsin alone, the HSA band disappeared at pH 2.0 and 3.0 and became clearer as pH increased from 3.5 to 4.0 in accordance with the results of starch analysis, in which no starch digestion occurred at pH 2.0 and 3.0 and more substantial starch digestion occurred at 3.5 and 4.0. In samples treated by pepsin and Hayward kiwifruit combined, both the actinidin and the HSA bands disappeared at pH 2.0, presumably as a result of active peptic activity at its optimal pH. At pH 3.0, although the HSA band was not visible, a thinner but clear actinidin band was present suggesting a weaker peptic activity at this pH. At pH 3.5 and 4.0, both HSA and actinidin bands were clear, indicating little peptic activity at higher pH. Overall, it is plausible that pepsin activity declines as pH increases and when there is more than one protein for it to digest.

The interactions between pepsin, actinidin and HSA at pH 2.0, 3.0, 3.5 and 4.0 after 30 minutes of incubation at 37 °C were verified by a small scale experiment in Eppendorf tubes (**Figure 17 C**). Pepsin bands were thicker in **Figure 17 B, C, D** because 5 mg/ml of more purified pepsin (3200-4500 U/mg protein) was used compared to 10% pepsin (>400 U/mg protein) in starch digestion. Pepsin in both assays had a final concentration of 800 U pepsin protein per ml. In addition, 40% kiwifruit was used instead of 50% kiwifruit to produce the same amount of protein as pepsin in order to be compared on SDS-PAGE. Despite this, the bands were almost identical between **Figure 17 A** and **C**.

To further investigate which protease is more powerful under gastric conditions, actinidin was inhibited by its selective inhibitor E-64 and pepsin was inhibited by its selective inhibitor

pepstatin**.** Pepsin degraded both HSA and E-64 inhibited actinidin at pH 2.0 and 3.0 as shown by thinned actinidin bands (**Figure 17 C**). Actinidin actively degraded HSA at pH 4.0, but it did not degrade either active pepsin or pepstatin-inhibited pepsin at any pH (**Figure 17 D**). A kinetic assay that directly measured actinidin activity remained in the pre-incubated samples, providing confirmation that actinidin resisted pepsin digestion at pH 3.5 and 4.0 but was susceptible to pepsin digestion at pH 3.0 and below (**Figure 18**). This was in line with the *in vitro* finding by Donaldson et al (36) who found that actinidin had little effect when pH was below 3.1 during a simulated gastric digestion at changing pH (1.3-6.2) followed by a duodenal digestion at a constant pH of 6.4, suggesting that kiwifruit actinidin is more likely to have a protease effect in the stomach rather than the duodenum.

On the contrary, the work of Rutherfurd and Montaya's research team have demonstrated more than once that kiwifruit actinidin might pass through the gastrointestinal digestive track intact promoting protein digestion (33-35, 83, 113) even though our *in vitro* results have clearly shown that actinidin was highly susceptible to degradation by pepsin once the pH has dropped to pH 2.0 and below (**Figure 17** &**18**). Grozdanovic et al (114) who found that actinidin resisted 2 hours of pepsin digestion even under ideal conditions for pepsin (pH 2.0) and therefore came to the conclusion that actinidin is capable of reaching the intestine mucosa intact. Grozdanovic et al (114) later demonstrated in mice that actinidin disrupted intestinal epithelium tight junctions and increased intestinal permeability causing food allergy. Bublin et al (115) also reported that 20% actinidin remained intact after 1 hour of peptic digestion at pH 2.0. This supports the role of Hayward kiwifruit in gastrointestinal digestion of dietary proteins as observed *in vitro* (113) and *in vivo* (83).

Our experiments revealed that pepsin and actinidin both could actively degrade HSA at their individual optimal pH. Pepsin degraded actinidin at its optimal pH but not vice versa. Thus, we

propose that during gastric digestion actinidin may degrade HSA at pH 4.0 and 3.5 where pepsin is not able to exert maximal activity. However, pepsin may degrade both HSA and actinidin when pH drops to 3.0 and below. However, further studies may be warranted to confirm our results due to the mixed findings in the literature illustrated above.

5.6. Weet-Bix™ meal

Starch digestion by HSA in a Weet-Bix™ meal under the effect of fruits (Hayward, SunGold kiwifruit and Granny Smith apple) and pepsin was analysed during a simulated gastric digestion. An average of 66% saliva per gram dry weight of Weet-Bix™ (28 ml saliva for 41.8 g Weet-Bix™) was determined in a chewing test (**Table 15**). Due to a difference in buffering capacity, samples taken at the same pH might have been digested for a different length of time. Therefore, samples that were close in digestion time were compared in one graph. A similar amount of starch was digested during gastric digestion in both the Weet-Bix™ control and pepsin treated samples as shown in **Figure 19 A**, suggesting starch digestion could have been completed before sampling. Five minutes was the least time required to get ready for titration (steps including adding reagents in the order of Weet-Bix™, fruit pulps, saliva, pepsin, gastric salt solution and mixing; achieving a stable pH reading and a temperature of 37 °C). No difference in starch digestion between pepsin treated sample and control at pH 2.0 indicated a failure in pepsin activation. It took 6 min for the pH to drop from 3.0 to 2.0, which might not be sufficient for pepsin to activate and act on HSA. Most *in vitro* methods for gastric digestion either dissolve pepsin in HCl before adding or add pepsin after digesta has been acidified to pH 2.0 (33, 60, 116). Moreover, peptic digestion at pH 2.0 was carried out for 30 minutes, while our experiment stopped once pH reached 2.0.

Fruit sugar controls were not included in the fruit-cereal digestion which were designed to show the increase in starch digestion rather than the total amount of sugar released. Therefore, trends rather than the amounts of reducing sugars can be compared between treatments (**Figure 19 B**). Although variation was large, a similar trend can be observed between SunGold kiwifruit and Granny Smith apple treated samples, while the Hayward kiwifruit treated sample showed a different trend, possibly due to the presence of actinidin. A decreasing trend as pH decreased was observed in pepsin and Hayward combined treatment. Acidification of samples treated with pepsin and Hayward to pH 2.0 took 25 minutes which could be sufficient to activate pepsin at low pH and degrade HSA. Addition of Hayward kiwifruit halved the amount of starch digested by HSA in pepsin treated samples despite inclusion of kiwifruit sugar in the former. This may support our speculation that the kiwifruit can promote early pepsin activation and more complete pepsin digestion owing to its high acidity and strong buffering capacity.

A major problem in this part of the experiment was the delay incurred during setting up the digestion of Weet-Bix™ in the presence of kiwifruit and pepsin. The current work has shown that starch digestion by HSA is rapid (< 5 min). In real life there are no delays between mastication, swallowing and emptying of the gastric chyme. Time did not permit, but priority should be given to streamlining the semi-dynamic digestion system for seamless operation.

5.7. Strengths and limitations

The strengths of our study include (1) development of a semi-dynamic gastric model consisting of automated titration which enabled acid/alkali dispensing at a physiological rate and analysis of the digestion medium at a changing pH; (2) the first to investigate the role of Hayward kiwifruit in glycaemic lowering in recognition of the significance of HSA in gastric starch digestion; (3)

step-wise investigation of the individual and combined effects of a number of factors (Ca-KF buffer – pH – fruits – pepsin – fruits and pepsin) on HSA activity in the stomach; (3) measurement of the effects of the above factors on HSA activity as a function of pH in recognition of the gradualness of the gastric acidification process; (4) filling a knowledge gap by investigating the buffering capacity of fruits in gastric digestion as well as interactions between pepsin, actinidin and HSA; (5) use of SDS-PAGE and actinidin kinetic assay as validation for the end-point starch analysis assay; (6) inclusion of an oral phase digestion using an estimated amount of saliva determined from a chewing test.

Overall, the limitations of our study are: (1) adoption of simple meal components in titration experiments generating results that may have limited application to real-life complex meal; (2) use of one person's saliva in experiments as HSA activity can vary among and within individuals; (3) lack of measurement of individual organic acid levels in fruits; (4) lack of a control for pepsin treatment in the study of the effect of pepsin on HSA activity; (5) uncertainty around the actinidin protease effect due to difference in fruit components used between experiments and meal study (kiwifruit pulp vs. kiwifruit extracts); (6) large standard deviations produced by natural lack of homogeneity in the fruit-cereal samples used in starch digestion; (7) lack of optimization to the meal study protocol due to insufficient time; (8) small sample size in most experiments due to insufficient time resulting in difficulty in conducting more vigorous statistical analysis.

5.8. Conclusions and further directions

To conclude, our project reveals that HSA is capable of rapidly digesting a considerable amount of starch at near-neutral pH which will be the pH of a starchy meal upon entering the stomach.

However, HSA is susceptible to low pH so that incorporation of acidic fruits, e.g., kiwifruit can result in immediate acid inhibition on HSA activity in the mouth and the strong buffering capacity of kiwifruit is likely to prolong the inhibition effect as food enters the stomach. Furthermore, HSA is susceptible to pepsin degradation at low pH (3.0 and below). Co-ingested kiwifruit is able to instantly acidify the food bolus to a pH level (~3.5) that promotes activation of pepsin. Hayward kiwifruit contains a natural protease actinidin that can actively degrade HSA at its optimal pH around 4.0 when pepsin cannot exhibit maximal activity. However, it is unclear whether inclusion of this extra protein actinidin has weaken the strength of pepsin when pH is not optimal for pepsin. When gastric pH falls below 2.0 HSA and actinidin is unlikely to resist pepsin degradation. In addition, the strong buffering capacity of kiwifruit, possibly owing to the high level of organic acids such as citric acid, is likely to delay GE and therefore further contribute to reduced glycaemic response to co-ingested starchy foods.

There are a number of factors that can be optimized and investigated in further research. Firstly, the buffering capacity of fruits in more complex meal should be investigated with measurement of organic acid compositions included. Secondly, a full range of organic acids can be included to make up a buffer that represents kiwifruit juice. Thirdly, pepsin can be activated in HCl before adding into the digestion model and a longer period of digestion at pH 2.0 is needed. In addition, the interaction between pepsin and actinidin should be investigated under different pH. Moreover, other factors in kiwifruit should be considered and tested for, for instance, dietary fibre, which may play a role in GE by affecting gastric viscosity and particle breakdown (40, 65). Last but not least, future research with large sample size are needed to confirm the statistical significance of the above observations made in this study.

Gastric digestion is an important link to intestinal digestion and absorption of starch. It is also a complex process where many factors come into play so their individual effect and interactions

need to be carefully examined to help understand the mechanisms underlying glycaemic response to starchy foods.

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