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Inhibitory effect of chlorogenic acid on digestion of potato starch

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Highlights:

- Chlorogenic acid inhibits the digestion of isolated potato starch both competitively and non-competitively.
- Five commercial potato varieties were analysed for phenolic content and starch digestibility.
- Digestibility is affected by multiple factors including phenolic, dry matter and starch content.

Abstract:

The effect of the chlorogenic acid isomer 5-O-caffeoylquinic acid (5-CQA) on digestion of potato starch by porcine pancreatic alpha amylase (PPAA) was investigated using isolated starch and cooked potato tuber as substrates. In vitro digestion was performed on five varieties of potato with varying phenolic content. Co- and pre-incubation of PPAA with 5-CQA significantly reduced PPAA activity in a dose dependent manner with an IC50 value of about 2 mg mL^{-1}. Lineweaver-Burk plots indicated that 5-CQA exerts a mixed type inhibition as k_m increased and V_{max} decreased. The total polyphenol content (TPC) of peeled tuber tissue ranged from 320.59 to 528.94 mg $100g^{-1}$ dry weight (DW) in raw tubers and 282.03 to 543.96 mg $100g^{-1}$ DW in cooked tubers. With the exception of Désirée, TPC and 5-CQA levels decreased after cooking. Principle component analysis indicated that digestibility is affected by multiple factors including phenolic, dry matter and starch content.

Keywords

Alpha amylase; starch; chlorogenic acid; enzyme kinetics; polyphenol; digestion; potato; *Solanum tuberosum*.

1. Introduction

Potato is the third most consumed food crop providing around 5% to 15% of dietary energy, primarily from starch, to various populations around the world. Potato has been labelled with a high glycaemic index (GI) because consumption of some potato products can cause a sharp increase of postprandial blood glucose concentration. However there is significant variation in the GI of various potato products (Weichselbaum, 2010). Many investigators have reported the effect of food processing (industrial or domestic) on the GI of potato (Ek, Brand-Miller and Copeland, 2012). For example, it has been reported that freshly boiled potato (GI 78) and instant mashed potato (GI 87) have a higher GI than French fries (GI 63) (Atkinson, Foster-Powell and Brand-Miller, 2008). Furthermore, the incremental area under curve (AUC) of freshly cooked potato decreased by 18% and 30% upon storage in the refrigerator for 1 and 5 days respectively. This observation is attributed to the retrogradation of amylose which reduces digestibility (Fernandes, Velangi, and Wolever, 2005). Henry, Lightowler, Strik and Storey (2005) determined the GI of commercially available potatoes in Great Britain and demonstrated that varieties prepared by boiling for 15 min showed a wide variation in GI values, ranging from 56 for Marfona to 94 for Maris Peer. Henry postulated that the difference was related to their texture, with waxy potatoes having a medium GI and floury potatoes having a high GI. We postulate that some of these differences may be attributed to the presence of endogenous polyphenolic substances acting as α-amylase inhibitors in the digestive tract.

Pancreatic alpha amylase is an endoglycosidase enzyme that has a significant role in carbohydrate digestion. It has been shown that inhibitors of α-amylase reduce bioavailability of glucose (Bozzetto et al., 2015). Controlling blood glucose level by α-amylase inhibitors may play a role in preventing hyperglycaemia in patients with diabetes mellitus. Some α-amylase inhibitors are naturally present in foods. Potato tubers contain many plant secondary metabolites including phenolics, carotenoids and polyamines. Their content and composition vary according to the variety of potato, conditions of cultivation, cooking and processing methods (Ezekiel, Singh, Sharma and Kaur, 2013, Ryes and Cisneros-Zevallos, 2003). Phenolics present in potatoes include phenolic acids, tannins, lignin, flavonoids, coumarins and anthocyanins (Ryes and Cisneros-Zevallos, 2003). 5-O-caffeoylquinic acid (5-CQA) is an isomer of chlorogenic acid (CGA) which makes up 90% of the phenolic content of potato (Malenberg and Theander, 1985). The concentration of 5-CQA is higher in the skin than in the medulla. Analysis has shown that cooked unpeeled potato contains between 9.1 to 12 mg 5-CQA per 100 g fresh weight compared to 0.86 to 6.6 mg for equivalent peeled samples (Mattila and Hellstrom, 2007). The variation in 5-CQA content suggests that different potatoes may inhibit pancreatic amylase to different extents.

Evidence from a number of in *vitro* and in *vivo* studies indicates inhibitory effects of polyphenols on enzymes involved in carbohydrate digestion. It was reported that 5-CQA, quinic acid (QA) and caffeic acid (CA) have mixed-type inhibitory effect against pure porcine pancreatic alpha amylase (PPAA) isomers I and II using *p*-nitrophenyl-α-D-maltoside as a substrate (Narita and Inouye, 2009). The most potent inhibitor was 5-CQA followed by CA and QA. In an *in vivo* animal study, oral intake of a 5-CQA solution (3.5 mg kg^{-1} body weight) during a glucose tolerance test lowered the height of glycaemia peaks at 10 and 15 min by 22 and 17% respectively compared to control (only glucose) (Bassoli et al., 2008). Rohn, Rawel and Kroll (2002) derivatized PPAA with a number of phenolic compounds and showed that 5-CQA reacted covalently with the enzyme and decreased its activity by about 50%. However, the mode of inhibition and potential effect on digestion of native potato starch has not been shown. The aim of the present study was to characterise the effect of 5-CQA on PPAA activity *in vitro* using potato starch as a substrate and to determine the *in vitro* digestibility of steam cooked potatoes from varieties which vary in their phenolic content.

2. Materials and methods:

2.1 Potato samples

Tubers from five commercial varieties that differ in skin colour were purchased from food markets in Leeds, UK. Maris piper has creamy flesh and golden yellow skin; Maris peer has creamy flesh and skin; Désirée, Rooster and Mozart have reddish pink skin and light yellow flesh. Three potatoes from each variety were rinsed with water and dried with a paper towel. Then the potatoes were peeled to remove skin and cortex, cut into cubes (1 cm^3) and separated into 100 g batches. Potatoes cubes were placed into a steam pan and cooked for 30 min at boiling temperature. Cooked potatoes were mashed by using a fork and used for enzymatic digestion. The remaining raw and steam cooked mashed potato was immediately frozen at -80 °C, freeze dried and stored at -80°C for analysis of TPC and phenolic acid composition. Analyses were repeated at least three times with three batches of potato.

2.2 In vitro digestion of potato starch in presence of chlorogenic acid

The activity of PPAA (16 U/mg, Sigma Aldrich) enzyme on hydrolysis potato starch in the presence and absence of 5-CQA (Sigma Aldrich; PubChem CID 12310830) was examined by the method of Brenfeld (1955) and Kazeem, Adamson and Ogunwande (2013) with some modifications. One percent (w/v) soluble potato starch (Sigma Aldrich) was suspended in 20mM sodium phosphate buffer pH 6.9 buffer containing 6.7mM NaCl and gelatinized for 15 min at 90 °C then allowed to cool to 37°C before addition of PPAA at a concentration of 0.33 U ml^{-1}. The reaction was followed at 37°C for up to 20 minutes. 5-CQA (final concentration 1.5 mg mL^{-1}) was either added to the enzyme-substrate mixture at the start of the reaction or was pre-incubated for 10 minutes with the enzyme prior to addition of the substrate. All reactions were carried out in four replicates. Reducing sugar released was measured at two reaction times (5 and 20 min) using the 3, 5-dinitrosalicylic acid (DNS; Sigma Aldrich) colorimetric assay (Miller, 1959, Fei et al., 2014). An enzymatic kit was not used due the inhibition of enzymes in the kit by chlorogenic acid. Brayer has shown that maltose is the preferred leaving group for PPAA (Brayer et al., 2000), and therefore maltose was used for generating standard curve to quantify the reducing sugar released. The enzymatic activity of PPAA was determined in the presence of various concentrations of 5-CQA (0.08 - 2 mg mL^{-1}). IC_{50} was calculated as the concentration of 5-CQA required to inhibit 50% of enzyme activity.

2.3 Enzymatic kinetics and mode of inhibition

Michaelis-Menten kinetic parameters and mode of inhibition of PPAA by 5-CQA was determined from a Lineweaver-Burk plot. One mL of 5-CQA at concentrations ranging from 0 to 2 mg mL^{-1} was added to a mixture containing 1 mL of starch solution at concentrations from 0 to 6.6 mg ml^{-1} in the same buffer solution as described in the previous section. The reaction was initiated by addition of a fixed concentration of PPAA (0.33 unit mL^{-1}). The solution mixture was incubated for 5 min at 37 °C. The reducing sugar produced was determined by the DNS colorimetric method as described previously.

2.4 Determination of total starch content

Total starch (TS) was determined enzymatically according to the method of Goñi, Garcia-Alonso and Saura –Calixto (1997) with some modifications. Raw potato (50 mg) was homogenized in 6 mL of 2M KOH and then agitated using a shaking vortex at room temperature for 30 min. The agitation step was very important to ensure complete solubility of the starch. 3 mL of 0.4M sodium acetate buffer (pH=4.75) was added to the suspension and pH was adjusted to 4.75 by using 3M acetic acid. 60 µl of amyloglucosidase (AMG) from *Aspergillus niger* (70 U/mg, Sigma Aldrich) was added to the solubilized starch and hydrolysed for 45 min at 60 ˚C in a shaking water bath. The digestion mixture was centrifuged for 5 min and pH was neutralised with 0.2M NaOH. Glucose in the supernatant was measured using the DNS method. Glucose amount was converted to starch by multiplying by a factor 0.9.

2.5 Determination of free sugar content

Free sugar content was determined in order to correct the TS value in potato samples. Potato samples, 200 mg of raw or cooked tuber, were homogenized in 6 mL sodium acetate buffer (pH=4.75) and then centrifuged for 10 min. Soluble sugars were determined using the DNS method and high performance anion exchange chromatography with pulsed amperometric detection.

2.6 High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Sugar solutions (glucose, fructose, sucrose, maltose and maltotriose, all from Sigma Aldrich) were used as standards at concentrations of 0-0.2 μg mL^{-1}. Samples and standards were spiked with internal standard (fucose, final concentration of 0.05 μg mL^{-1}). Samples were filtered through PTFE membrane filters (0.2 μm pore size, Chromacol Ltd) and analyzed by HPAEC-PAD (Thermo Fisher DX500 instrument equipped with a GP40 gradient pump, ED40 electrochemical detector including gold working and silver reference electrodes and a LC20 column oven set at 30^{o}C). The analytical column used was CarboPac PA20 (3×150mm) with guard (3×30mm) with anion exchange capacities of 65μeq/column. The mobile phase was 200 mM NaOH and the flow rate was 0.4 mL/min. Injections (10 μL) were made by an AS500 autosampler. The elution programme was as follows: isocratic elution with 60mM NaOH from 0 to 8 min, followed by increasing gradient up to 140 mM NaOH to 17 min. The concentration was reduced back to 60mM and equilibration was carried out for 6 min.

2.7 Determination of moisture content

Potato samples fresh and cooked (in triplicate) were weighed and frozen at -80°C then freeze dried for 48 hours. The moisture content was calculated as percentage of weight loss**.** Dry matter (DM) was calculated from the remaining dry yield.

2.8 In vitro starch hydrolysis of steam cooked potato

In order to test the hypothesis that the TPC affects the digestibility of starch in potato varieties, *in vitro* digestibility of different verities of potato were carried out. Steam cooked tubers from potato varieties (10 mg total starch mL^{-1}) with different TPC levels were digested using PPAA (0.66 unit mL^{-1}) for up to 180 min at 37ºC in a shaking water bath (75 rpm).

In addition, we also performed sequential digestion with AMG (60 µl of AMG (184 U) from *Aspergillus niger*, Sigma Aldrich) following PPAA digestion to degrade all digestion products to glucose. Before AMG digestion, the pH was adjusted to 4.75 and digestion performed for 30 min at 37ºC in a shaking water bath (75 rpm). Digestion products were detected using the DNS method and HPAEC-PAD.

2.9 Determination of total polyphenol content (TPC)

The method of extraction was adapted from Shakya and Navarre (2006). Phenolic compounds were extracted in four replicates taken from freeze dried raw and cooked potatoes. Freeze-dried powder (200 mg) was mixed with 1.5 mL of extraction buffer (50% MeOH, 2.5% metaphosphoric acid, 1 mM EDTA, chilled to 4ºC) and 500 mg of glass beads (1.0 mm in diameter; Fisher Scientific). Tubes were shaken with a vortex for 10 min at room temperature and then sonicated at 10 °C for 10 min. After sonication, tubes were shaken again with a vortex for 10 min. Tubes were centrifuged at 2500 g at 4ºC for 10 minutes and the supernatant was transferred to a clean tube. Extractions were repeated three times and supernatants combined. Samples were kept chilled at all times and not exposed to light. TPC of potato was measured by Folin-Ciocalteau method (Singleton and Rossi, 1965). Potato extract (50µL) was mixed with Folin-Ciocalteau reagent (50 µL, 1 N), sodium carbonate (150 µL, 20% w/v) and distilled water (750 µL). After shaking by vortex, the mixture was incubated in the dark at room temperature for 45 min. Absorbance was measured at 765 nm using a spectrophotometer (Cecil, CE 7200 Double Beam UV/VIS Spectrophotometer). Different concentrations of 5-CQA (10-300 µg mL^{-1}) were used to generate a standard curve.

2.10 Separation of phenolic acids using high performance liquid chromatography with diode array detector – mass spectrometry (HPLC-DAD-MS).

The HPLC-DAD-MS system consisted of a micro vacuum degasser (Prominence Degasser LC-20 A5, Shimadzu), a liquid chromatograph (Prominence Liquid Chromatograph LC-30 AD, Shimadzu), an auto sampler (Prominence Auto Sampler SIL-30 AC, Shimadzu), a diode array detector (Prominence Diode Array Detector system SPD-M20A, Shimadzu), a column oven (Prominence Column Oven CTO-20 AC, Shimadzu), a controller (Prominence Controller CBM-20 A, Shimadzu), and an MS detector with electrospray ion source and quadrupole analyser (Liquid Chromatograph Mass Spectrometer LCMS-2020, Shimadzu). The Labs solutions (Shimadzu) software was used to control the LC–MS system and for data processing. The column used for chromatographic separation was an Agilent Zorbax Eclipse plus C18 column 4.6 mm × 150 mm, 5 µm internal diameter. A gradient elution program of solvent A (0.1 % formic acid, 5% acetonitrile and 94% water) and solvent B (0.1 % formic acid, 5% water and 94% acetonitrile) was set as follows: 61-min; linear gradient from 0-51 min from 0% to 100% solvent B, isocratic elution from 51.1-56 min with 100% solvent B, linear gradient from 56-56.1 min to 0% solvent B and isocratic elution from 56.1-61 min with 0% solvent B. The column temperature was 35°C and flow rate of 0.5 mL min^{-1} and injection volume 10 µL. The diode array detection spectra was recorded at wavelengths of 280, 290, 315, 320 and 330 nm.

2.11 Statistical analysis

Statistical analysis was carried out using IBM SPSS statistics version 22 software program for window. An independent sample t-test was used to compare amylase activity in presence or absence of chlorogenic acid. One way ANOVA was used to analyse the differences between varieties in terms of compositon and digestibility, and also to test the effect of cooking on phenolic content. Differences were considered to be statistically significant when $p \leq 0.05$. Pearson correlations were performed between composition factors and AUC values.

Principle component analysis (PCA) was conducted to analyse the relationship between starch hydrolyzed and TS, DM, TPC and 5-CQA content of potatoes. PCA was performed using MATLAB software R2015a (MathWorks, Inc.).

3. Results and discussion

3.1 In vitro hydrolysis of potato starch in presence of chlorogenic acid

We evaluated the inhibitory effect of the 5-CQA on PPAA activity using potato starch as substrate at two incubation time periods of 5 and 20 min (figure 1A). The results indicate that 5-CQA significantly ($p \leq 0.05$) inhibited PPAA at both incubation times, the inhibition was more pronounced at 5 min (25.5% inhibition) compared to 20 min (1.5% inhibition). The difference in inhibition at the two reaction times indicates that 5-CQA is most efficient during early stages of hydrolysis, most probably in a competitive way. Pre-incubation of enzyme with 5-CQA for 10 min inhibited the enzyme by 53.8 and 28.3 % at 5 and 20 min respectively. In order to calculate the IC_{50}, PPAA activity was measured in the presence of increasing concentrations of 5-CQA (figure 1B). The IC_{50} was found to be around 2 mg mL^{-1} 5-CQA and this in agreement with results of Sun et al. (2016) who reported IC_{50} of 1.96 mg mL^{-1} for chlorogenic acid against PPAA hydrolysis of maize starch. The enzymatic kinetic properties were examined by applying Michaelis-Menten assumptions. The initial velocity of the reaction (*V*) was determined at various concentrations [S] of gelatinized potato starch in the absence and presence of various concentrations of the 5-CQA and a fixed concentration of PPAA. A Lineweaver-Burk plot produced linear relationships for $1/V$ against 1/[S] at various 5-CQA various concentrations (figure 1C). Increasing inhibitor concentrations led to an increase in slope and y-intercept on the vertical axis. The intersection of lines in the second quadrant suggests a mixed type of inhibition of 5-CQA against PPAA. Kinetic parameter V_{max} and k_m were obtained from Lineweaver-Burk plots (table 1); k_m values increased from 1.66 to 2.08 mg mL^{-1} starch with increasing inhibitor concentrations while maximum velocity (V_{max}) values decreased from 0.35 to 0.29 mg mL^{-1} min^{-1} maltose. These results also suggest that 5-CQA has a mixed-type inhibition (competitive and non-competitive) against PPAA when potato starch is used as a substrate. This mixed type inhibition behaviour was previously reported for 5-CQA using p-nitrophenyl-α-D-maltoside as a substrate (Narita and Inouye, 2009). Recently, Sun et al (2016) reported that chlorogenic and caffeic acid have a mixed type inhibitory effect on PPAA hydrolysis of maize starch. These two inhibitors decrease the V_{max}, by interacting more strongly with the enzyme-substrate complex than with the free enzyme (Sun et al 2016, Narita and Inouye, 2011). Rohn, Rawel and Kroll (2002) reported that the incubation of digestive enzymes (PPAA, trypsin, lysozyme) with simple phenolic compounds (CQA, caffeic acid, gallic acid and ferulic acid) for 24 hours resulted in covalent attachment of the phenolic compounds to the free amino groups of the enzymes and in consequence, decreased their activity irreversibly and non-competitively. The non-competitive inhibition of PPAA was reported for millet seed coat extract using potato starch as substrate (Shobana, Sreerama and Malleshi, 2009).

The inhibitory activity of phenolic acids is enhanced with increasing the number of phenolic sub-structures. The inhibitory effect of caffeic acid was enhanced 5-fold by combining with quinic acid to form chlorogenic acids (Narita & Inouye, 2011). We show that 5-CQA is able to inhibit PPAA activity on potato starch in both competitive and non-competitive manners.

3.2 Total phenolic content

The TPC of raw and cooked peeled potato samples is shown in table 2. TPC in fresh peeled potatoes varied from 320.59 mg 100 g^{-1} dry weight (DW; 5-CQA equivalent) in Rooster to 528.94 mg 100 g^{-1} DW in Maris Peer. TPC content was in the order Maris Peer > Mozart > Désirée > Maris Piper > Rooster from highest to lowest content. Variety affected TPC content significantly ($p \leq 0.05$). We did not measure TPC content in the skin of the potatoes, but observe that skin colour is not a good indicator of TPC content in the flesh.

In general, steam cooking affected the TPC in all potato samples. Cooking reduced the TPC in Maris Peer (-35%) and Mozart (-19%) significantly ($p \leq 0.05$). Smaller non-significant decreases were observed in Maris Piper (-7%) and Rooster (-12%) (p = 0.26 and 0.20 respectively). However, the TPC content increased in Désirée potatoes by 22% (p=0.04). This variation in the response to cooking has been reported in the literature (Tian, Chen, Ye, Chen, 2016).

There are many reports of TPC in different genotypes of potato in peeled, unpeeled, fresh and cooked forms. The TPC varies according to genotype, agricultural practices and method of extraction. Ah-Hen, Fuenzalida, Contreras, Vega-Galvez and Lemus-Mondaca (2012) examined the TPC in some native coloured potatoes from Chiloe Island (Southern Chile) and found that TPC in peeled potato samples varied from 192 to 1864 mg 100g^{-1} DW (ferulic acid equivalent). Lachman, Hamouz, Sulc, Orsak and Dvorak (2008) reported TPC in red and yellow-fleshed potato to range from 296 mg 100 g^{-1} DW in yellow-fleshed potato to 468 mg 100 g^{-1} DW (gallic acid equivalent) in purple-fleshed potato. These levels are consistent with the results obtained in this study. Recently, Tierno, Hornero-Mendez, Gallardo-Guerrero, Lopez-Pardo and Ruiz de Galarreta (2015) reported that boiling peeled potato for 30 min reduced the TPC of tubers by about 50% and similar results also were obtained by Lemos, Aliyu and Hungerford (2015). Meanwhile, Blessington, Nzaramba, Scheuring, Hale, Reddivari and Miller (2010) reported an increase in TPC after baking, frying and microwaving. Bembem and Sadan (2013) and Burgos et al. (2013) observed an increase in TPC after boiling, steam cooking, microwaving and pressure cooking. Therefore, an increase in level of TPC of Désirée potato in our study after steam cooking is in accordance with some published reports. The increased levels could be due to the release by cooking of phenolic or other compounds (Tian, Chen, Ye, Chen, 2016).

One of the disadvantages of the TPC assay using Folin-Ciocalteau reagent is the low level of specificity. For instance, the reagent reacts with ascorbic acid and tyrosine, both abundant in potatoes. It has been reported that the ascorbic acid response to the Folin-Ciocalteau reagent is two times higher than gallic acid (Lachman, Hamouz, Sulc, Orsak and Dvorak, 2008). Therefore, results using TPC values need to be interpreted with caution. To address the non-specificity, the individual phenolic acids in potato extracts were identified using HPLC-DAD-MS (typical chromatogram in supplementary material 1). 5-CQA was the predominant phenolic acid in the flesh of raw and cooked potatoes. Other minor peaks were also observed but not quantified. The content of 5-CQA in raw potato ranged from the lowest in Désirée (10.36 mg 100 g^{-1} DW) to highest in Maris Piper (29.46 mg 100 g^{-1} DW) (table 2). In cooked tuber, the 5-CQA content was lowest in Rooster (6.51 mg 100 g^{-1} DW) and highest in Maris Piper (21.24 mg 100 g-1 DW). Similar to the effects of cooking on TPC, steam cooking significantly decreased the 5-CQA level of all potatoes tested ($p \leq 0.05$), except Désirée which saw an increase. 5-CQA in cooked Désirée was 1.7 time higher than in raw potato ($p \leq 0.05$). The level of chlorogenic acids in 50 unpeeled potato genotypes was reported by Navarre, Pillai, Shakya and Holden (2011) to be in the range 21.9 to 473.0 mg 100g^{-1} DW. Mattila and Hellstrom (2007) reported that cooked and peeled potato contain 4.13 to 31.2 mg 100 g^{-1} DW, in agreement with the current study. The colour of the skin has been suggested to be associated with TPC content in the flesh (Tierno, Hornero-Mendez, Gallardo-Guerrero, Lopez-Pardo and Ruiz de Galarreta, 2015). However, in this study, this was not found to be the case. Observed colour was not a good indicator of TPC and 5-CQA content in flesh. Rooster has red skin but its flesh showed one of the lowest TPC and 5-CQA contents amongst the varieties investigated. Conversely, Maris Piper has yellow skin and had the highest 5-CQA in cooked flesh. The HPLC results indicate that TPC content overestimates potato phenolic content but is still used in many studies.

3.3 In vitro starch hydrolysis of steam cooked potato

Hydrolysis of potato starch by PPAA was monitored by measuring the reducing sugar produced at different times using the DNS colorimetric method and the maltose content by HPAEC-PAD (typical chromatogram in supplementary material). The amount of sugar detected after PPAA hydrolysis was around 1.5 times higher using the DNS method than using HPAEC-PAD. This can be explained by DNS reacting with oligosaccharides produced by amylase digestion that can react with DNS (Van der Maarel, Van der Veen, Uitdehaag, Leemhuis and Dijkhuizen, 2002, Nigam and Singh, 1995, Robyt and Whelan, 1972). AMG was used to digest PPAA digestion products to glucose (figure 2) and in this case DNS and HPAEC-PAD gave similar results (data not shown). Using HPAEC-PAD, free soluble sugars including glucose, fructose and sucrose were detected at low levels at time 0 (supplementary material and table 2). While after hydrolysis by PPAA, maltose and maltotriose were detected as products of digestion (supplementary material) and after AMG digestion, glucose was the only product (not shown). The HPAEC-PAD also showed that the PPAA preparation contains sucrose, most probably as a stabiliser and this needs to be corrected for. Sucrose does react with DNS and therefore it is important to undertake a blank correction of the enzyme preparation. Enzymatic kits were not used because polyphenols inhibit the kit enzymes, underestimating the carbohydrate content (data not shown).

The area under the curve (AUC) for the *in vitro* digestion reactions with PPAA and PPAA+AMG were calculated for each potato type (table 3). There were significant ($p \leq 0.05$) differences in the AUC_{PPAA} and $AUC_{PPAA+AMG}$ between the varieties. Rooster showed consistently the highest extent of digestion. Meanwhile, Mozart and Maris Peer showed lower levels of digestibility with PPAA than Désirée and Maris Piper, but upon digestion with PPAA+AMG, the opposite trend was observed with Désirée and Maris Piper showing lower levels of digestibility compared to Mozart and Maris Peer. While there was no significant correlation between AUC_{PPAA} and TPC or 5-CQA content there was a significant negative correlation between $AUC_{PPAA+AMG}$ and 5-CQA and between $AUC_{PPAA+AMG}$ and TPC (with Pearson correlation coefficients $r = -0.49$, $p < 0.0001$ and $r = -0.30$, $p = 0.0001$ respectively).

In this study, the amount of steam cooked potato used as substrate contained 10 mg mL^{-1} of starch (similar to the first experiments) and around 0.08 mg mL^{-1} 5-CQA. According to figure 1B, this is a low concentration of 5-CQA, enough to inhibit up to 5.8 % of the alpha amylase activity. This amount of 5-CQA does appear to be enough to inhibit AMG. To better understand the relation between composition factors and starch digestibility, principle component analysis (PCA) was performed.

3.4 Principle component analysis (PCA)

Principle component analysis was conducted in order to determine the relationship between potato composition (TS, DM, TPC, 5-CQA) and percent of hydrolysed starch (as measured using $AUC_{PPAA+AMG}$). Figure 3 show the loading and scores of the characteristics of the five potato cultivars with the first two PCs explaining 72% of the total variance respectively. PC1 explained 47% and PC2 explained 24% of total variance. For PC1, TS, DM, 5-CQA and TPC had positive loading and starch hydrolysis had negative loading. TS and DM had positive loading for PC2 while 5-CQA and TPC had negative loading for PC2. According to the PCA scores, potato varieties segregated according to their components: Maris Piper showed high DM, high 5-CQA content and low digestibility. Rooster, Maris Peer and Mozart potato appeared in one cluster and showed the opposite characteristics. The digestibility of Désirée appeared to be heavily influenced by TPC, in contrast to the other varieties. It appears that the same single factor does not strongly determine digestibility in all varieties.

4. Conclusions:

This study is the first to examine the mechanism of inhibition of pancreatic alpha amylase by 5-CQA using potato starch as a substrate. Kinetic analyses showed a mixed-type inhibition, with stronger inhibition at earlier incubation times (5 min compared to 20 min). *In vitro* digestion of cooked potato tubers showed that the 5-CQA content in tubers is probably too low to affect PPAA digestion, but a significant effect was observed when AMG was also used. The inhibitory effect of 5-CQA on multiple carbohydrate digestive enzymes needs further investigation.

The results presented in this paper suggest that multiple factors affect potato digestibility, and the effects may be variety specific. Testing a higher number of varieties is required and ultimately testing the digestibility of the varieties *in vivo* will also confirm whether these observations have biological significance.

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Table and figure captions

Table 1. Kinetic parameters K_m and V_{max} of pancreatic alpha amylase enzyme with increasing concentrations of caffeoylquinic (5-CQA).

[5-CQA] mg mL^{-1}	k_m mg mL^{-1}	V_{max} (mg mL^{-1} min^{-1})
0	1.66	0.35
1	1.69	0.32
1.5	1.85	0.31
2	2.08	0.29

Table 2. Total phenolic content (TPC), caffeoylquinic (5-CQA) and dry matter (DM) content in raw and cooked potatoes.

Potato varieties	TPC (mg 100 g^{-1} DW) *		5-CQA (mg 100 g^{-1} DW)		DM (g 100 g^{-1} FW)	
	Raw	Cooked	Raw	Cooked	Raw	Cooked
Desiree (R)	445.14±32.89^{b}	543.96±20.68^{a}	10.36±0.16^{d}	17.83±0.40^{b}	21.04±0.10^{ac}	20.08±0.11^{abc}
Mozart (R)	524.64±27.10^{a}	425.53±17.36^{b}	16.84±0.59^{b}	11.12±0.31^{c}	16.12±0.81^{b}	19.10±1.13^{a}
Rooster (R)	320.59±15.34^{c}	282.03±5.07^{b}	13.07±0.70^{c}	6.51±0.23^{e}	22.12±3.13^{a}	19.52±0.10^{abc}
Maris Piper (Y)	375.69±29.06^{d}	349.18±13.58^{c}	29.46±1.19^{a}	21.24±0.42^{a}	20.67±0.10^{ac}	21.47±0.61^{b}
Maris Peer (Y)	528.94±15.72^{a}	343.83±11.57^{c}	11.12±1.86^{cd}	7.40±0.35^{d}	18.90±0.47^{acd}	19.66±0.22^{ac}

R= red skin, Y= yellow skin; DW = dry weight; FW = fresh weight

*Chlorogenic acid equivalent

Values are means of four determinations±SD

Mean value within a column with different subscript letter ^{a,b,c,d,e} indicate significant differences (one-way ANOVA, $P \leq 0.05$).

Table 3. Composition of peeled potatoes in terms of carbohydrate content (total starch (TS) and free sugars), and area under curve (AUC) of hydrolysed starch in cooked tubers. AUC$_{PPAA}$ is AUC for starch hydrolysed by porcine pancreatic alpha amylase (PPAA) alone, while AUC$_{PPAA+AMG}$ is for starch hydrolysed by PPAA followed by amyloglucosidase (AMG).

Potato varieties	**TS (g 100 g^{-1} FW)***	**Free sugars (g 100 g^{-1} FW)****	**AUC$_{PPAA}$ g maltose. min*****	**AUC$_{PPAA+AMG}$ g glucose. min*****
Desiree	13.4±1.44^{b}	2.64±0.06^{a}	517.35±12.82^{b}	752.59±29.19^{c}
Mozart	17.48±1.22^{a}	1.06±0.02^{b}	484.05±5.21^{c}	970.41±38.82^{b}
Rooster	13.61±0.80^{b}	1.15±0.01^{b}	599.25±1.31^{a}	1046.43±48.58^{a}
Maris Piper	16.97±3.62^{ab}	0.98±0.06^{b}	507.00±11.51^{b}	648.22±22^{d}
Maris Peer	15.76±2.44^{b}	1.09±0.02^{b}	486.27±5.30^{c}	1044.82±41.32^{a}

FW=Fresh weight

*(n=9), **(n=3),***(n=4)

Mean value within a column with different subscript letter ^{a,b,c,d} indicate significant differences (one-way ANOVA, $P \leq 0.05$).

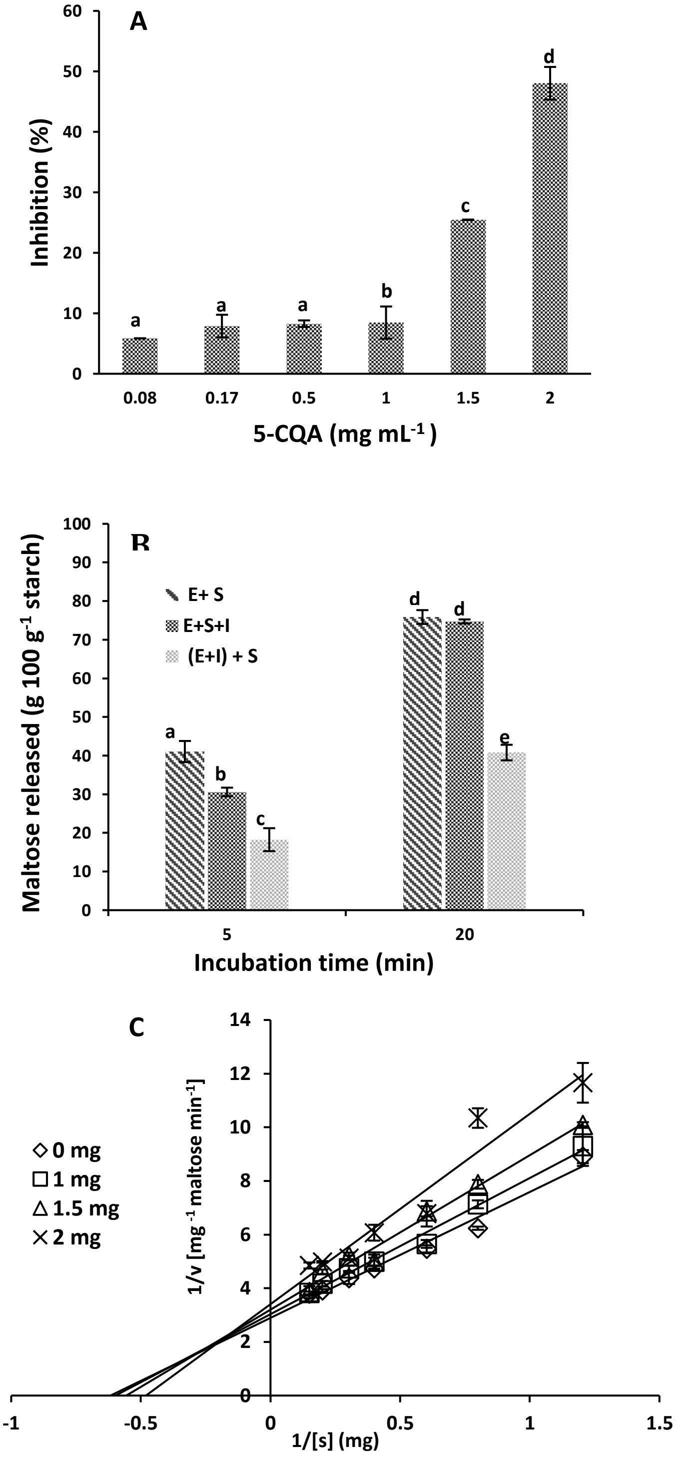


Figure 1. Effect of 5-caffeoylquinic acid (5-CQA) on the activity of porcine pancreatic alpha amylase (PPAA) activity using 1% potato starch as substrate. A) Effect of pre-incubation and co-incubation of 5-CQA (1.5 mg mL^{-1}) on the digestion of 1% starch by PPAA (0.33 unit mL^{-}

^{1}) at 5 and 20 minutes incubation. E = enzyme, S = substrate and I = inhibitor. B) Effect of increasing concentration of 5-CQA on inhibition of PPAA relative to control (no inhibitor) at 5 min incubation. Each bar represents mean of four measurements ± standard deviation. Bars with different letters have significant difference at $p<0.05$. C) Lineweaver-Burk plot for porcine pancreatic alpha amylase (PPAA) catalysed hydrolysis of increasing concentration of potato starch in the presence of 5-caffeoylquinic acid (5-CQA; 0, 1, 1.5 and 2 mg mL^{-1}). Each point represents mean of four measurements ± standard deviation.

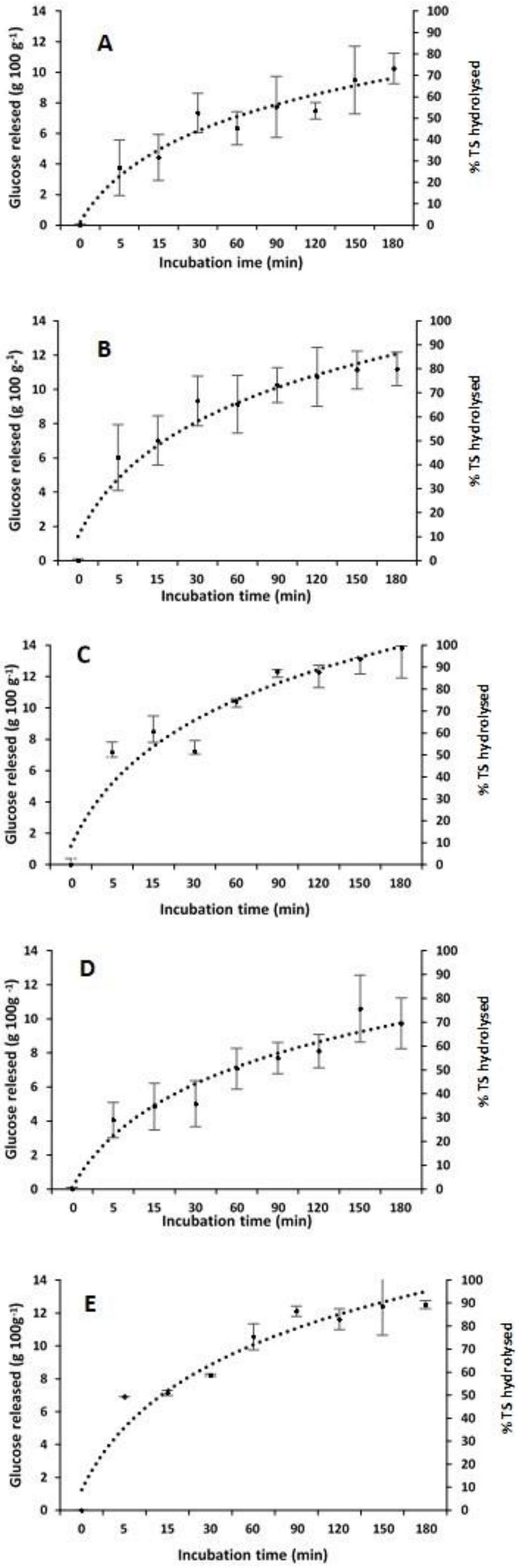


Figure 2. Starch hydrolysis curves of steam cooked potato samples digested with 0.66 units of porcine pancreatic alpha amylase (PPAA) followed by 180 units of amyloglucosidase (AMG).

A) Désirée B) Mozart. C) Rooster. D) Maris Piper. E) Maris Peer. Data expressed as amount of glucose released and as % of total starch (TS) at each time point as measured by DNS (n=9). Error bars represent standard deviation of mean.

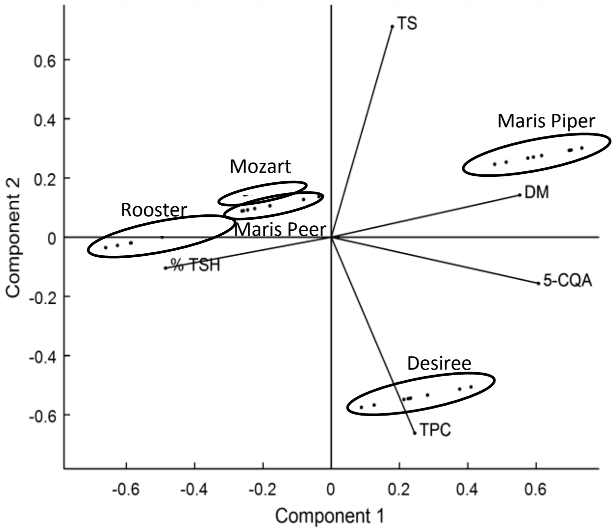
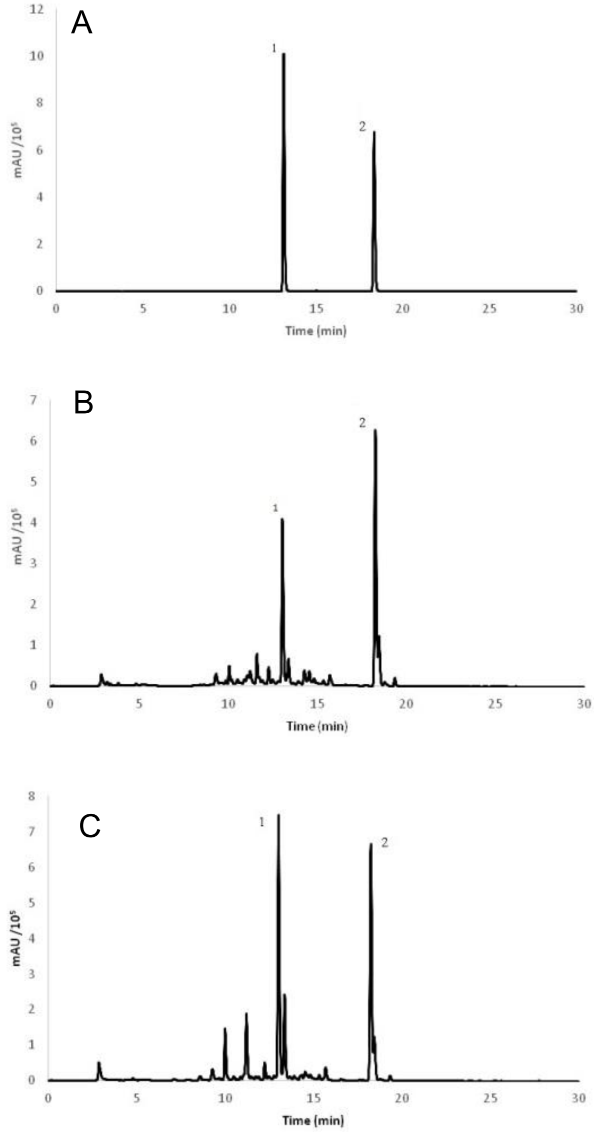


Figure 3. Principle component analysis (PCA) using composition and digestibility data from five potato varieties. Graph shows loading of potato components; total starch (TS), dry matter (DM), total polyphenol content (TPC), 5-caffeoylquinic acid (5-CQA) content and percentage total starch hydrolysed (%TSH) and scores of potato varieties according to PCA 1 and 2.

Supplementary material

Figure 1.

HPLC-DAD chromatogram of A) standard compounds, B) raw potato sample (Desiree) and C) cooked potato sample (Desiree). Peaks were detected at wavelength 330 nm. Peaks; (1) 5-caffeoylquinic (5-CQA) (2) sinapic acid (external standard).



Supplementary material

Figure 2.

HPAEC-PAD chromatogram of total sugars present after digestion of a potato sample (Maris Piper) for 120 min. Peaks; (1) fucose (external standard), (2) glucose, 3) fructose, 4) sucrose, 5) maltose, 6) maltotriose. Glucose, fructose and sucrose were already present at time 0 (not shown).