Identification of peptides from camel milk that inhibit starch digestion

Rami M. Althnaibat, Heather L. Bruce, Michael G. Gänzle*)

University of Alberta, Dept. of Agricultural, Food and Nutritional Science, Edmonton, AB, Canada

*) Corresponding author footnote

Michael Gänzle,

University of Alberta, Dept. of Agricultural, Food and Nutritional Science

4-10 Ag/For Centre

Edmonton, AB

Canada, T6G 2P5

mail; mgaenzle@ualberta.ca

Abstract

Digestion of starch especially the rapidly digestible starch (RDS) leads to hyperglycemia. There is a clear relationship between postprandial hyperglycemia and diet-related health problems like diabetes and obesity, and the most useful therapy for these problems is to moderate postprandial blood glucose levels. This study aimed to determine the inhibition of starch digestion activities by peptides derived from camel milk proteins and to assess the effect of their amino acids charge and/or hydrophobicity. Starch digestion by pancreatic and brush border enzymes was assessed *in vitro* with a peptide to starch ratio of 1:3 (w/w). Hydrolysed whey proteins were more inhibitory than hydrolysed casein. Peptides were fractionated by cation exchange chromatography (CEX) and hydrophobic interaction chromatography (HIC). The successive chromatographic separation by CEX and HIC enriched positively charged peptides with hydrophobic amino acids. Whey protein hydrolysate inhibited starch hydrolysis by 16%; peptide fractions recovered after CEX and HIC inhibited starch hydrolysis by 33 – 36%. Peptides in the active fractions were identified by LC-MS/MS and the inhibitory activity of 6 synthetic peptides was evaluated. Two of these six peptides, LALDIEIATYR and VLDELTLAR, inhibited starch hydrolysis by 34 – 37%. In conclusion, specific peptides that are produced before or during *in vitro* digestion can inhibit starch digestion and may moderate postprandial blood glucose levels *in vivo*.

KEYWORDS: Camel milk, whey protein, bioactive peptides, starch digestion inhibition, charge, hydrophobicity.

1 Introduction

Starch is the storage polysaccharide in seeds of many plant crops including legumes and cereals (Perin & Murano, 2017). Starch is the only polysaccharide that is hydrolyzed by human intestinal enzymes and provides 45-65% of the daily dietary energy for humans worldwide (Augustin et al., 2015). Depending on the botanical origin, starch consists of 74%-82% amylopectin and 18%-26% amylose (Englyst et al., 1992; Miao & Hamaker, 2021). Starch has been classified as non-glycemic or glycemic (Augustin et al., 2015; Hasek et al., 2018). Glycemic starch includes rapidly digestible starch (RDS) and slowly digestible starch (SDS). Non-glycemic starch or resistant starch is not hydrolysed in the small intestine but fermented in the large intestine (Englyst et al., 1992).

Starch is digested in two stages: the intraluminal stage, which involves hydrolysis by salivary and pancreatic α-amylases; the brush border stage, which involve maltase/glucoamylase (MGAM, EC 3.2.1.20/3.2.1.3) and sucrase/isomaltase (SIM, EC 3.2.1.48/3.2.1.10) as the main enzymes with activity on oligosaccharides derived from starch, followed by absorption of glucose (Zhang et al., 2015). Digestion of glycemic starch, especially RDS, leads to fast rise of blood glucose levels. Postprandial hyperglycemia is related to diet-related health problems like diabetes and obesity while slowly digestible starch (SDS) liberates glucose more slowly and is considered more beneficial than RDS (Hanefeld & Schaper, 2007).

The ratio of amylopectin to amylose, crystallinity, porosity, surface area, and integrity all affect starch digestibility (Miao & Hamaker, 2021). The food matrix, e.g. the presence of proteins, dietary fibre, lipids and phenolic compounds, also leads to changes in starch digestion either through inhibition of starch digestive enzyme, or by modulation of the kinetics of digesta transit (Lopez-Rubio et al., 2008; Metzler-Zebeli et al., 2010; Wolever, 2017). Helical complexes like V-type crystalline starch are produced when free fatty acids or/and monoglycerides interact with

amylose, resulting in crystalline amylose that is resistant to digestion (Luo et al., 2020). Interactions between starch and phenolic compounds decrease starch digestibility by several mechanisms including inhibition of pancreatic α-amylase and brush border enzymes, enhancing amylose crystallinity, or by physical complexation (Li et al., 2020; Simsek et al., 2017; Sun et al., 2018). Proteins of wheat and other grains physically surround the granules of starch, limiting access of digestive enzymes (Bhattarai et al., 2018; Zhou et al., 2018). In addition, dietary fibre and peptides derived from protein hydrolysis inhibit starch digestion (Augustin et al., 2015; Layman et al., 2003).

Bioactive peptides are generated by hydrolysis of proteins *in vivo* and/or *ex vivo* through digestive enzymes, microbial enzymes, and microbial fermentation (Park, 2009). Bioactive peptides in milk protein hydrolysates were suggested to inhibit starch digestion by inhibition of intestinal α-glucosidases (Lacroix & Li-Chan, 2013; Park, 2009). *In vivo* human trials demonstrated effects of milk protein hydrolysates (Sartorius et al., 2019), casein hydrolysates (Geerts et al., 2011), and whey protein hydrolysates (Chen et al., 2020) on the postprandial glucose levels but did not identify individual peptides with inhibitory activity on starch digestion.

Most studies with milk-derived peptides were conducted with bovine milk and only a few studies have investigated the bioactive peptides from camel milk (El-Agamy, 2009). Camel milk whey proteins consist of a high proportion of hydrophobic amino acids and contain a high content of Phe, Val, Leu, Lys, Glu and Pro (El-Agamy, 2009; Rafiq et al., 2015). These intrinsic characteristics of whey proteins from camel milk make these promising candidates for generation of bioactive peptides that inhibit starch digestion. The aims of this study therefore were: (i) to assess the effect of enzymatic hydrolysis of proteins from camel milk on *in vitro* starch digestion,

(ii) to enrich active peptides by fractionation with cation exchange chromatography and hydrophobic interaction chromatography, and (iii) to determine the sequences of active peptides.

2 Materials and methods

2.1 Isolation of cheese whey.

Unpasteurized and lyophilized skim milk from Bactrian camels was obtained from the Inner Mongolia Agricultural University, Hohot, China. Skim camel milk was reconstituted by dissolving the skim milk powder in water at a solid to water ratio of 1:10 (w/v) with vigorous stirring (1000 rpm, 23˚C) for 2h. The reconstituted milk was heated to 37˚C and 1mL camel chymosin (Chr. Hansen, Bayswater, Australia) was added to 1L milk, followed by incubation at 37℃ for 60min. Precipitated proteins were removed from the supernatant containing whey proteins and the caseinmacropeptide by centrifugation at 5,000×g for 60min at 4˚C. The latter step was repeated 3 times, and the supernatant was lyophilized and stored at -20˚C until further analysis.

2.2 Hydrolysis of casein and whey protein.

Whey proteins and casein were hydrolyzed by addition of proteases from *Aspergillus oryzae* (Flavourzyme, Sigma, Canada, EC: 232-752-2). A 10% (w/v) whey or casein solution was prepared, and the pH value for solution was adjusted to 6.0 using 0.1M HCL. Flavourzyme was added at 0.05% (v/v) and the mixture was agitated with glass beads at 50˚C for 24h to hydrolyze proteins. The reaction was stopped by heating to 95ºC for 5min, then the hydrolysates were lyophilized and stored at −20°C for further analysis. The hydrolysis was conducted in triplicate.

2.3 Hydrophobic interaction chromatography (HIC)

Camel milk whey hydrolysates were fractionated by HIC on an Octyl Sepharose CL-4B column (1.5cm × 15cm, Octyl Sepharose CL-4B, GE Healthcare, Chicago, IL) that was linked to a UV

detector (220nm). Freeze-dried camel milk whey hydrolysates were dissolved in 0.1% trifluoroacetic acid (TFA) and the pH was adjusted to 6.0. Of this solution, 250mL were loaded on the column. The column was washed with 250mL distilled water (pH 6) and eluted with 250mL 5% isopropanol in water with 0.1% TFA. The fractions were pooled based on the 220 and 280 nm absorbance, freeze-dried, and analyzed by starch digestibility assay as described above (Figure 1).

2.4 Cation exchange chromatography (CEX)

Camel milk whey hydrolysates (Table 1) were fractionated by CEX on a 1.5cm × 15cm, SP-Sepharose fast flow column (GE Healthcare, Chicago, IL). Freeze-dried camel milk whey hydrolysates were dissolved in distilled water to a concentration of 1g/L and the pH was adjusted to 7.0. Of this solution, 250mL were loaded on the column and the column was washed with distilled water (pH 7). The column was eluted with a linear gradient of 0 to 2M NaCl in water and the fractionation was monitored by measuring the absorption at 220nm. The fractions were pooled based on peaks, then part of each pooled fraction was freeze-dried, and analyzed for the starch digestibility assay as described above.

Fraction (F1) from the CEX was further purified and sub-fractionated by HIC as described above. The column was washed with 0.1% TFA and eluted with 5% isopropanol in 0.1% TFA. The fractions were pooled based on peaks, freeze-dried, and analyzed with the starch digestibility assay as described in section 2.7. (Figure 1).

2.5 Peptide sequencing

Fraction F1 after HIC and the pooled fractions F1* and F2* after CEX-HIC were selected for peptide sequencing by LC-MS/MS. Peptide sequencing was carried out by Alberta Proteomics and Mass Spectrometry Facility in the Department of Biochemistry of the University of Alberta.

Briefly, 50µg of sample was dissolved in 100mM ammonium bicarbonate to a concentration of 1.0g/L, reduced with dithiothreitol and alkylated with iodoacetamide. Samples were then digested overnight with trypsin (2µg, Promega sequencing grade) at 37˚C. After digestion, the pH of the samples was adjusted to 3-4 with formic acid, dried, dissolved in water + 0.2% formic acid, and desalted (Pierce C18 tips). The sample was additionally analysed without trypsin hydrolysis.

The peptides were resolved and ionized by using nano-flow HPLC (Easy-nLC 1000, Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) with an EASY-Spray capillary HPLC column (ES800A, Thermo Scientific). The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey orbitrap spectra using external mass calibration, with a resolution of 35,000 and m/z range of 300–1700. The 12 most intense multiply charged ions were sequentially fragmented by using high energy collision induced dissociation and spectra of their fragments were recorded in the orbitrap at a resolution of 17,500. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and the database was searched using SEQUEST (Thermo Scientific). Search parameters included a strict false discovery rate (FDR) of 0.01, a relaxed FDR of 0.05, a precursor mass tolerance of 10ppm and a fragment mass tolerance of 0.01Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

2.6 Peptide synthesis

Six short peptides identified in the most potent HIC fractions and CEX-HIC fractions were chosen for peptide synthesis. The selected peptide sequences were synthesized by Canada Peptide (Pointe-Claire, Quebec, Canada) with 92.5 – 97.9 % purity. The effect of the peptides on starch digestibility was assayed as described in section 2.7.

2.7 *In vitro* digestibility of starch and starch-peptide mixtures

Starch digestion *in vivo* simultaneously hydrolyses dietary proteins and peptides through the activity of pepsin, pancreatic enzymes, and brush border peptidases. To mimic the *in vivo* situation, the assay for starch digestibility used pancreatic enzymes and brush border enzymes that also have protease or peptidase activity. Starch (7.5mg; potato starch, Sigma, Canada) or peptide-starch mixtures (2.5mg peptide: 7.5mg starch) were suspended in 1mL of water, heated for 10min at 85℃ to gelatinize the starch, and incubated at 37℃ for 16h. Protein or peptide samples that were analysed are shown in Table 1. Digestion of starch and proteins or peptides was carried out by adding 0.5mg of pepsin (250U/mg, Sigma, Canada) and incubation at pH 2.0 and 37℃ with agitation at 200rpm for 30min. The pH of the digesta was adjusted to pH 6.0 with 2M NaOH prior to addition of porcine pancreatic enzymes and brush border enzymes from the rat intestinal mucosa (Tsunehiro et al., 1999). In brief, 1ml of 50mM sodium maleate buffer pH 6.0 containing 0.07g porcine pancreatin (Sigma, USA; 45U/mg lipase, 42U/mg amylase, and 3.0U/mg protease) and 10g/L rat small intestinal enzyme (Sigma, USA) was added to 1ml of digesta. After adding ~ 5 glass beads (5mm diameter), the reaction mixture was incubated at 37℃ and pH 6 for 4h with agitation at 200rpm. The digestion process was stopped by heating to 95℃ for 4min. The samples were cooled on ice and centrifuged at 5, 000 ×g for 5min at 4˚C. The glucose concentration for samples and controls was measured with the D-glucose (GOPOD-format) kit (Megazyme, Bary, Ireland) (Figure 1).

2.8 Statistical Analysis.

Starch digestibility assay was performed in triplicate biological repeats with three technical repeats, and the results are presented as means ± standard error. To determine the statistical differences between the samples, p-values were calculated using Tukey Pairwise Comparisons at

95% Confidence in Minitab 19 (The differences between the conditions are considered significant if p-value < 0.05).

3 Results

3.1 Starch digestibility inhibition of camel whey and whey hydrolysates.

The starch digestibility assay was applied to starch alone or mixtures of peptides / proteins and starch in a ratio of 1:3 (w/w peptide: starch). The digestibility assay included addition of pepsin, pancreatic enzymes, and brush border enzymes to mimic the enzymes involved in starch and protein digestion in the digestive tract. It was previously shown that the activity of brush border glycosyl hydrolases from rat intestinal mucosa corresponds the activity of human brush border enzymes (Oku et al., 2011).

Whey and casein inhibited starch hydrolysis by about 10 and 7%, respectively (Table 1). Enzymatic hydrolysis of whey and casein with flavourzyme increased the inhibition of starch hydrolysis by whey and casein hydrolysates to about 17 and 11%, respectively (Table 1). Hydrolyzed whey consists of 13.6% proteins or peptides while the protein or peptide content in the casein hydrolysate is more than 85%, therefore, any peptides in the whey fraction presumably are more active and subsequent analyses focused on whey hydrolysates.

3.2 Purification of Bactrian camel whey hydrolysate.

Peptides obtained by Flavourzyme hydrolysis of camel whey proteins were first fractionated by HIC. Fractions were characterized with respect to the inhibition of starch hydrolysis. Fractionation of by HIC resulted in five fractions (Figure 2). Among these fractions, fraction 1 (F1) was most inhibitory to starch hydrolysis (Figure 3).

In addition, peptides in the whey hydrolysate were fractionated by CEX. Whey hydrolysate purified by CEX was collected in four fractions (Figure 4A). The chromatogram and the inhibition of starch hydrolysis by the fractions are shown in Figure 4B and 5B, respectively. Fraction F1 from CEX exhibited the highest inhibitory activity on starch hydrolysis (Fig. 5B) and was subsequently fractionated by HIC (CEX-HIC). Of the fractions eluting from the HIC column, fractions F1* and F2* were most inhibitory to starch hydrolysis. These two fractions were pooled and selected for peptide sequencing by LC-MS/MS. Peptides in fraction F1 eluting from HIC was also sequenced for comparison.

3.3 Peptide sequences in fractions inhibiting starch hydrolysis.

The fractionated peptides were sequenced by LC/MS/MS after trypsin digestion. A total of 22 peptides were identified in fraction F1 after HIC separation. Peptide sequences consisted of 7-24 amino acids with molecular weights (Mw) ranging from 994 to 2933Da (Table 2). In the pooled fractions F1* and F2* obtained after fractionation on CEX and HIC, 13 peptides were identified after trypsin digestion. In addition, 2 peptides were identified in a sample that was analyzed without a trypsin digestion step. The molecular weights of the peptide sequences ranged from 956 to 1708Da, and the peptides contained 8-19 amino acids (Table 3).

3.4 Inhibition of starch hydrolysis by synthesized peptides

The starch digestibility assay as described above was applied to the selected synthesized peptides sequences to determine their inhibitory activity on starch hydrolysis. All sequences matched *Camelus bactrianus* proteins with 100% identity and coverage (Table 4). Table 4 illustrates that two sequences that were identified after fractionation on HIC and CEX and HIC; LALDIEIATYR (LR11) and VLDELTLAR (VR9) were as active as the entire fraction. LR11 and VR9 inhibited

starch hydrolysis by about 37 and 33%, respectively. The remaining peptides were inactive or much less active (Table 4). The two active peptides carried a single negative charge at neutral pH and included 4 – 6 hydrophobic amino acids. Peptides with lower activity differed in their net charge at neutral pH (uncharged or positively charged) and/or included only 1 – 3 hydrophobic amino acids.

4 Discussion

Protein and peptides can delay starch digestion by inhibition of the enzymes responsible for starch digestion, such as α-amylase, α-glucosidase (Augustin et al., 2015; Gangoiti et al., 2018; Layman et al., 2003; Yu et al., 2012). This study demonstrates that some of the peptides derived from camel milk whey protein also inhibit starch digestion. Purification and fractionation of camel milk whey protein peptides depending on the content of hydrophobic and positively charged amino acids strongly increased the inhibition of starch digestion. For two of the peptides, inhibition of starch digestion was confirmed by assays with chemically synthesized peptides.

Camel milk whey protein is not as well studied as bovine milk whey protein and the biological activities of bioactive peptides derived from camel milk whey protein are not fully explored (Jafar et al., 2018). The *in vitro* and *in vivo* studies reported that the antihyperglycemic activity of bovine whey protein hydrolysates are higher than that of the casein hydrolysates (Park, 2009; Sartorius et al., 2019). Pepsin-treated bovine α-lactalbumin exhibited the highest antihyperglycemic activity compared to other pepsin-treated whey proteins including bovine serum albumin, β-lactoglobulin, lactoferrin, and whey protein isolate, whereas the β-lactoglobulin showed the lowest antihyperglycemic activity (Lacroix & Li-Chan, 2013). We confirm a higher activity of whey proteins for casein and whey protein hydrolysates from camel milk whey. Bactrian camel milk whey hydrolysates with 13.6% protein content showed an inhibition of starch hydrolysis that was

236 higher than the inhibition by a casein hydrolysate. The fractionation of the camel milk whey protein
237 hydrolysate by HIC or by CEX-HIC further increased the activity more than two-fold (Table 1),
238 suggesting that charged and hydrophobic amino acids enhance activity.

239 Inhibition of starch digestive enzymes has been reported for protein hydrolysates and peptides
240 derived from albumin (Yu et al., 2012), legumes (Ngoh & Gan, 2016), cumin (Siow & Gan, 2016),
241 and milk (Lacroix & Li-Chan, 2013) but only few studies reported specific peptide sequences with
242 inhibitory activity. The cumin seed derived peptide FFRSKLLSDGAAAAKGALLPQYW
243 inhibited α-amylase by 25% (Siow & Gan, 2016). KLPGF derived from albumin inhibited
244 α-amylase and α-glucosidase activities with IC_{50} values of about 120 and 59 μM, respectively (Yu
245 et al., 2012). We identified LALDIEIATYR and VLDELTLAR as peptides with the highest
246 inhibitory activity on starch hydrolysis; the remaining synthesized peptides had only limited
247 inhibitory activity on starch hydrolysis (Table 4). Peptides from camel whey proteins with *in vitro*
248 inhibitory activity on digestive glucosidases thus contain one or more negatively charged residues
249 (E or D), one positively charged residue (K or R) with a single negative charge at physiological
250 pH. Moreover, aromatic or aliphatic hydrophobic residues (I, V, L, Y, F or W) account for about
251 45% (4/9 and 5/11) of the amino acids. α-Lactalbumin is the major component of camel milk whey
252 protein constituting about 47% (Laleye et al., 2008) but peptides that inhibited intestinal
253 α-glucosidases were derived from minor whey proteins (Table 4), which further supports that
254 inhibitory activity is specific for the peptide sequence.

255 Peptides that inhibit starch digestion act locally in the gastrointestinal tract (Xu et al., 2019), and
256 the bioavailability of these peptides is affected by digestive enzymes in the gastrointestinal tract,
257 metabolism, and absorption. Our assay differs from previous studies by including brush border
258 enzymes in the *in vitro* assay for starch digestion. This approach has the advantage of simulating

the *in vivo* digestion more closely, however, because the commercial preparation of brush border enzymes also includes brush border peptidases, the peptides used may have been further modified during the digestion assay. Pepsin hydrolysis in the stomach is the first step in food protein digestion and proteins are then further hydrolysed by the pancreatic proteases trypsin and chymotrypsin, and by brush border peptidases (Hooton et al., 2015). Brush border enzymes that contribute to peptide hydrolysis include the peptidyl dipeptidase, aminopeptidase N, dipeptidyl aminopeptidase IV, γ-glutamyltranspeptidase, aminopeptidase A, and carboxypeptidase (Hooton et al., 2015; Mentlein, 2004; Yoshioka et al., 1988). The biological activity of peptides depends on the degree of hydrolysis by these digestive enzymes (Xu et al., 2019; Yoshioka et al., 1988; Zambrowicz et al., 2015). Proteins with high content of proline are resistant to gastric and pancreatic peptidases, and proline-rich peptides are thus most likely to escape the digestion and to reach the intestinal membrane in relatively intact sequence to face the brush border enzymes (Mentlein, 2004; Yoshioka et al., 1988). Peptides with multiple proline residues including IPP (Nongonierma & FitzGerald, 2016), VPP (Ten Have et al., 2015) and HLPLP (Sánchez-Rivera et al., 2014) have been detected in the plasma of human and animals. Only few of the peptides in the active fractions and none of the synthetic peptides with high inhibitory activity included proline in their sequence (Tables 2 – 4), however, because peptides that inhibit starch digestion may be active in the luminal stage of starch digestion by pancreatic amylases (Zhang et al., 2015), resistance to hydrolysis by brush border enzymes is not a prerequisite for activity. Whey-derived peptides were shown to inhibit brush border peptidases (Lacroix & Li-Chan, 2014), which may have increased the time needed for hydrolysis of peptides or peptide fractions that were assessed in the present study with respect to *in vitro* activity.

Several *in vivo* studies demonstrated that dietary peptides or proteins may reduce the relative glycemic response. VAGTWY from trypsin-treated bovine whey proteins showed significant decrease in postprandial glucose level in mice with an IC_{50} value about 174 μM (Uchida et al., 2011). Bovine whey proteins were more effective in reducing postprandial blood glucose levels in humans than other proteins (Wolever, 2017) but the effect was also observed with glucose rather than starch as carbohydrate source (Gunnerud et al., 2013; Lan-Pidhainy & Wolever, 2010) and thus relates to mechanisms that are independent of the rate of starch hydrolysis. Human intervention studies with bovine casein hydrolysates or whey protein hydrolysates also consistently reported reduced postprandial blood glucose level (Geerts et al., 2011; Manders et al., 2006; Sartorius et al., 2019). Because the available clinical studies all used bovine milk but did not compare different peptides or peptide fractions, and did not relate the *in vivo* effect on the relative glycemic response to the rate of starch hydrolysis in the small intestine, it is not possible to relate the *in vitro* inhibitory effect on starch digestion that was observed in the present study to these clinical data. Moreover, while a protein to starch ratio of 1:3 (w/w) may match the dietary intake of protein (hydrolysates) and starch, the same ratio by far exceeds the intake of peptide fractions or synthetic peptides as dietary supplement. The contribution of the inhibition of luminal or brush border glucosyl hydrolases by whey protein-derived peptides on the glycemic response thus remains subject to future investigations.

Conclusion

The prevalence of food-related chronic diseases including diabetes mellitus has increased worldwide. Rapid digestion of dietary starch leads to hyperglycemia that may lead to the development of insulin resistance and diabetes mellitus. Delaying carbohydrate digestion is an accepted approach as a treatment of type-2 diabetes. The present study identified specific whey

304 peptides from camel milk with inhibitory activity on *in vitro* starch hydrolysis. This finding may
305 inform future studies to improve the use of (camel) milk proteins or protein hydrolysates in dietary
306 intervention strategies to reduce the risk of insulin resistance and diabetes.

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311 **References**

312 Augustin, L. S. A., Kendall, C. W. C., Jenkins, D. J. A., Willett, W. C., Astrup, A., Barclay, A.
313 W., Björck, I., Brand-Miller, J. C., Brighenti, F., Buyken, A. E., Ceriello, A., La Vecchia, C.,
314 Livesey, G., Liu, S., Riccardi, G., Rizkalla, S. W., Sievenpiper, J. L., Trichopoulou, A.,
315 Wolever, T. M. S., … Poli, A. (2015). Glycemic index, glycemic load and glycemic response:
316 An International Scientific Consensus Summit from the International Carbohydrate Quality
317 Consortium (ICQC). *Nutrition, Metabolism and Cardiovascular Diseases*, *25*(9), 795–815.
318 https://doi.org/10.1016/J.NUMECD.2015.05.005

319 Bhattarai, R. R., Dhital, S., Mense, A., Gidley, M. J., & Shi, Y. C. (2018). Intact cellular structure
320 in cereal endosperm limits starch digestion in vitro. *Food Hydrocolloids*, *81*, 139–148.
321 https://doi.org/10.1016/J.FOODHYD.2018.02.027

322 Chen, Y. C., Smith, H. A., Hengist, A., Chrzanowski-Smith, O. J., Mikkelsen, U. R., Carroll, H.
323 A., Betts, J. A., Thompson, D., Saunders, J., & Gonzalez, J. T. (2020). Co-ingestion of whey
324 protein hydrolysate with milk minerals rich in calcium potently stimulates glucagon-like
325 peptide-1 secretion: an RCT in healthy adults. *European Journal of Nutrition*, *59*(6), 2449–

2462. https://doi.org/10.1007/S00394-019-02092-4

El-Agamy, E. I. (2009). *Bioactive components in camel milk. Bioactive components in milk and dairy products* . (pp. 159–192). Wiley-Blackwell.

Englyst, H. N., Kingman, S. M., & Cummings, J. H. (1992). Classification and measurement of nutritionally important starch fractions. *European Journal of Clinical Nutrition*, *46 Suppl 2*(SUPPL. 2), S33-50. https://europepmc.org/article/med/1330528

Gangoiti, J., Corwin, S. F., Lamothe, L. M., Vafiadi, C., Hamaker, B. R., & Dijkhuizen, L. (2018). Synthesis of novel α-glucans with potential health benefits through controlled glucose release in the human gastrointestinal tract. *Critical Reviews in Food Science and Nutrition*, *in press*. https://doi.org/10.1080/10408398.2018.1516621

Geerts, B. F., Van Dongen, M. G. J., Flameling, B., Moerland, M. M., De Kam, M. L., Cohen, A. F., Romijn, J. A., Gerhardt, C. C., Kloek, J., & Burggraaf, J. (2011). Hydrolyzed casein decreases postprandial glucose concentrations in T2DM patients irrespective of leucine content. *Taylor & Francis*, *8*(3), 280–292. https://doi.org/10.3109/19390211.2011.593617

Gunnerud, U. J., Östman, E. M., & Björck, I. M. E. (2013). Effects of whey proteins on glycaemia and insulinaemia to an oral glucose load in healthy adults; a dose–response study. *European Journal of Clinical Nutrition 2013 67:7*, *67*(7), 749–753. https://doi.org/10.1038/ejcn.2013.88

Hanefeld, M., & Schaper, F. (2007). The Role of Alpha-Glucosidase Inhibitors (Acarbose). *Pharmacotherapy of Diabetes: New Developments: Improving Life and Prognosis for Diabetic Patients*, 143–152. https://doi.org/10.1007/978-0-387-69737-6_13

Hasek, L. Y., Phillips, R. J., Zhang, G., Kinzig, K. P., Young Kim, C., Powley, T. L., & Hamaker,

B. R. (2018). Dietary slowly digestible starch triggers the gut-brain axis in obese rats with accompanied reduced food intake. *Mol Nutr Food Res*, *62*(5). https://doi.org/10.1002/mnfr.201700117

Hooton, D., Lentle, R., Monro, J., Wickham, M., & Simpson, R. (2015). The secretion and action of brush border enzymes in the mammalian small intestine. In *Reviews of physiology, biochemistry and pharmacology* (Vol. 168, pp. 59–118). https://doi.org/10.1007/112_2015_24

Jafar, S., Kamal, H., Mudgil, P., Hassan, H. M., & Maqsood, S. (2018). Camel whey protein hydrolysates displayed enhanced cholesteryl esterase and lipase inhibitory, anti-hypertensive and anti-haemolytic properties. *LWT*, *98*, 212–218. https://doi.org/10.1016/J.LWT.2018.08.024

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2013). Inhibition of dipeptidyl peptidase (DPP)-IV and α-glucosidase activities by pepsin-treated whey proteins. *Journal of Agricultural and Food Chemistry*, *61*(31), 7500–7506. https://doi.org/10.1021/JF401000S

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2014). Isolation and characterization of peptides with dipeptidyl peptidase-IV inhibitory activity from pepsin-treated bovine whey proteins. *Peptides*, *54*, 39–48. https://doi.org/10.1016/J.PEPTIDES.2014.01.002

Laleye, L. C., Jobe, B., & Wasesa, A. A. H. (2008). Comparative study on heat stability and functionality of camel and bovine milk whey proteins. *Journal of Dairy Science*, *91*(12), 4527–4534. https://doi.org/10.3168/JDS.2008-1446

Lan-Pidhainy, X., & Wolever, T. M. S. (2010). The hypoglycemic effect of fat and protein is not attenuated by insulin resistance. *The American Journal of Clinical Nutrition*, *91*(1), 98–105.

https://doi.org/10.3945/AJCN.2009.28125

Layman, D. K., Shiue, H., Sather, C., Erickson, D. J., & Baum, J. (2003). Increased Dietary Protein Modifies Glucose and Insulin Homeostasis in Adult Women during Weight Loss. *The Journal of Nutrition*, *133*(2), 405–410. https://doi.org/10.1093/JN/133.2.405

Li, M., Griffin, L. E., Corbin, S., Neilson, A. P., & Ferruzzi, M. G. (2020). Modulating phenolic bioaccessibility and glycemic response of starch-based foods in Wistar rats by physical complexation between starch and phenolic acid. *Journal of Agricultural and Food Chemistry*, *68*(46), 13257–13266. https://doi.org/10.1021/acs.jafc.0c01387

Lopez-Rubio, A., Flanagan, B. M., Shrestha, A. K., Gidley, M. J., & Gilbert, E. P. (2008). Molecular rearrangement of starch during in vitro digestion: Toward a better understanding of enzyme resistant starch formation in processed starches. *Biomacromolecules*, *9*(7), 1951–1958. https://doi.org/10.1021/BM800213H

Luo, S., Zeng, Z., Mei, Y., Huang, K., Wu, J., Liu, C., & Hu, X. (2020). Improving ordered arrangement of the short-chain amylose-lipid complex by narrowing molecular weight distribution of short-chain amylose. *Carbohydrate Polymers*, *240*, 116364. https://doi.org/10.1016/J.CARBPOL.2020.116359

Manders, R. J. F., Praet, S. F. E., Meex, R. C. R., Koopman, R., De Roos, A. L., Wagenmakers, A. J. M., Saris, W. H. M., & Van Loon, L. J. C. (2006). Protein hydrolysate/leucine co-ingestion reduces the prevalence of hyperglycemia in type 2 diabetic patients. *Diab. Care*, *29*, 2721–2722. https://doi.org/10.2337/dc06-1424

Mentlein, R. (2004). Cell-surface peptidases. *International Review of Cytology*, *235*, 165–213. https://doi.org/10.1016/S0074-7696(04)35004-7

Metzler-Zebeli, B. U., Hooda, S., Pieper, R., Zijlstra, R. T., Van Kessel, A. G., Mosenthin, R., & Gänzle, M. G. (2010). Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic escherichia coli in the pig gastrointestinal tract. *Applied and Environmental Microbiology*, *76*(11), 3692–3701. https://doi.org/10.1128/AEM.00257-10

Miao, M., & Hamaker, B. R. (2021). Food matrix effects for modulating starch bioavailability. *Annual Review of Food Science and Technology*, *12*(1). https://doi.org/10.1146/annurev-food-070620-013937

Ngoh, Y. Y., & Gan, C. Y. (2016). Enzyme-assisted extraction and identification of antioxidative and α-amylase inhibitory peptides from Pinto beans (*Phaseolus vulgaris* cv. Pinto). *Food Chemistry*, *190*, 331–337. https://doi.org/10.1016/J.FOODCHEM.2015.05.120

Nongonierma, A. B., & FitzGerald, R. J. (2016). Strategies for the discovery, identification and validation of milk protein-derived bioactive peptides. *Trends in Food Science and Technology*, *50*, 26–43. https://doi.org/10.1016/J.TIFS.2016.01.022

Oku, T., Tanabe, K., Ogawa, S., Sadamori, N., & Nakamura, S. (2011). Similarity of hydrolyzing activity of human and rat small intestinal disaccharidases. *Clinical and Experimental Gastroenterology*, *4*, 155–161. https://doi.org/10.2147/CEG.S19961

Park, Y. W. (2009). *Bioactive components in milk and dairy products*. Wiley-Blackwell.

Perin, D., & Murano, E. (2017). Starch polysaccharides in the human diet: effect of the different source and processing on its absorption. *Natural Product Communications*, *12*(4), 837–853.

Rafiq, S., Huma, N., Pasha, I., Sameen, A., Mukhtar, O., & Khan, M. I. (2015). Chemical composition, nitrogen fractions and amino acids profile of milk from different animal

species. *Asian-Australasian Journal of Animal Sciences*, *29*(7), 1022–1028. https://doi.org/10.5713/AJAS.15.0452

Sánchez-Rivera, L., Ares, I., Miralles, B., Gómez-Ruiz, J. Á., Recio, I., Martínez-Larrañaga, M. R., Anadón, A., & Martínez, M. A. (2014). Bioavailability and kinetics of the antihypertensive casein-derived peptide HLPLP in rats. *Journal of Agricultural and Food Chemistry*, *62*(49), 11869–11875. https://doi.org/10.1021/jf5035256

Sartorius, T., Weidner, A., Dharsono, T., Wilhelm, M., Boulier, A., & Brown, C. S. (2019). Postprandial effects of a proprietary milk protein hydrolysate containing bioactive peptides in prediabetic subjects. *Nutrients*, *11*(7), 1700–1717. https://doi.org/10.3390/NU11071700

Simsek, M., Quezada-Calvillo, R., Nichols, B. L., & Hamaker, B. R. (2017). Phenolic compounds increase the transcription of mouse intestinal maltase-glucoamylase and sucrase-isomaltase. *Food & Function*, *8*(5), 1915–1924. https://doi.org/10.1039/C7FO00015D

Siow, H. L., & Gan, C. Y. (2016). Extraction, identification, and structure–activity relationship of antioxidative and α-amylase inhibitory peptides from cumin seeds (*Cuminum cyminum*). *Journal of Functional Foods*, *22*, 1–12. https://doi.org/10.1016/J.JFF.2016.01.011

Sun, L., Warren, F. J., & Gidley, M. J. (2018). Soluble polysaccharides reduce binding and inhibitory activity of tea polyphenols against porcine pancreatic α-amylase. *Food Hydrocolloids*, *79*, 63–70. https://doi.org/10.1016/J.FOODHYD.2017.12.011

Ten Have, G. A. M., Van Der Pijl, P. C., Kies, A. K., & Deutz, N. E. P. (2015). Enhanced lacto-tri-Peptide bio-availability by co-ingestion of macronutrients. *PLOS ONE*, *10*(6), e0130638. https://doi.org/10.1371/JOURNAL.PONE.0130638

Tsunehiro, J., Okamoto, K., Furuyama, Y., Yatake, T., & Kaneko, T. (1999). Digestibility of the

hydrogenated derivative of an isomaltooligosaccharide mixture by rats. *Bioscience, Biotechnology, and Biochemistry*, *63*, 1515–1521. https://doi.org/10.1271/bbb.63.1515

Uchida, M., Ohshiba, Y., & Mogami, O. (2011). Novel dipeptidyl peptidase-4-inhibiting peptide derived from β-lactoglobulin. *Journal of Pharmacological Sciences*, *117*(1), 63–66. https://doi.org/10.1254/JPHS.11089SC

Wolever, T. M. S. (2017). Effect of macronutrients on the glycemic index. *The American Journal of Clinical Nutrition*, *106*(2), 704–705. https://doi.org/10.3945/AJCN.117.158055

Xu, Q., Hong, H., Wu, J., & Yan, X. (2019). Bioavailability of bioactive peptides derived from food proteins across the intestinal epithelial membrane: A review. *Trends in Food Science & Technology*, *86*, 399–411. https://doi.org/10.1016/J.TIFS.2019.02.050

Yoshioka, M., Erickson, R. H., & Kim, Y. S. (1988). Digestion and assimilation of proline-containing peptides by rat intestinal brush border membrane carboxypeptidases. Role of the combined action of angiotensin-converting enzyme and carboxypeptidase P. *The Journal of Clinical Investigation*, *81*(4), 1090–1095. https://doi.org/10.1172/JCI113421

Yu, Z., Yin, Y., Zhao, W., Liu, J., & Chen, F. (2012). Anti-diabetic activity peptides from albumin against α-glucosidase and α-amylase. *Food Chemistry*, *135*(3), 2078–2085. https://doi.org/10.1016/J.FOODCHEM.2012.06.088

Zambrowicz, A., Pokora, M., Setner, B., Dąbrowska, A., Szołtysik, M., Babij, K., Szewczuk, Z., Trziszka, T., Lubec, G., & Chrzanowska, J. (2015). Multifunctional peptides derived from an egg yolk protein hydrolysate: isolation and characterization. *Amino Acids*, *47*(2), 369. https://doi.org/10.1007/S00726-014-1869-X

Zhang, G., Hasek, L. Y., Lee, B. H., & Hamaker, B. R. (2015). Gut feedback mechanisms and

food intake: a physiological approach to slow carbohydrate bioavailability. *Food & Function*, *6*(4), 1072–1089. https://doi.org/10.1039/C4FO00803K

Zhou, X., Ying, Y., Hu, B., Pang, Y., & Bao, J. (2018). Physicochemical properties and digestibility of endosperm starches in four indica rice mutants. *Carbohydrate Polymers*, *195*, 1–8. https://doi.org/10.1016/J.CARBPOL.2018.04.070

Figure legends

Figure 1. Flow diagram for the isolation of bioactive peptide from camel milk whey protein.

Figure 2. Fractionation of peptides obtained by hydrolysis of camel whey with flavourzyme on Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC). The column was eluted with 5% isopropanol. The volume of each fraction was 5mL. Fractions are labeled with S, W, F1, F2 and F3 to designate elution during sample loading, washing, and eluate that was pooled in fractions F1, F2 and F3. The fraction that was selected for peptide sequencing is underlined.

Figure 3. Inhibition of starch hydrolysis by peptides obtained by hydrolysis of camel whey with flavourzyme and fractionation on an Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC). The inhibitory activity is expressed as % reduction relate to starch hydrolysis without peptide addition. Fractions are labeled with S, W, F1, F2 and F3 to designate elution during sample loading, washing, and eluate that was pooled in fractions F1, F2 and F3.

Figure 4. Panel A: Fractionation of peptides obtained by hydrolysis of camel whey with flavourzyme on SP- Sepharose fast flow cation exchange chromatography (CEX). **Panel B:** Fractionation of F1 eluting from CEX on an Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC). The volume of each fraction was 5mL. Fractions are labeled with S, W, F1, F2 and F3 to designate elution during sample loading, washing, and eluate that was pooled in fractions F1, F2 and F3. Fractions eluting from the HIC column are designated with an asterisk; fractions that were selected for further fractionation or peptide sequencing are underlined.

Figure 5. Inhibition of starch hydrolysis by peptides obtained by hydrolysis of camel whey with flavourzyme and fractionation. **Panel A.** Fractions obtained with a SP- Sepharose fast flow cation exchange (CEX) column. **Panel B.** Fractions after separation on CEX and subsequent separation

of fraction F1 on an Octyl Sepharose CL-4B column (hydrophobic interaction chromatography; HIC). The inhibitory activity is expressed as % reduction relate to starch hydrolysis without peptide addition. Fractions are labeled with S, W, F1, F2 and F3 to designate elution during sample loading, washing, and eluate that was pooled in fractions F1, F2 and F3.

Table 1. Peptide recovery after chromatography on SP-Sepharose fast flow column (cation exchange chromatography; CEX) and on Octyl Sepharose CL-4B column (hydrophobic interaction chromatography; HIC), and inhibition of starch digestion of collected fractions (peptides) at the ratio 1:3 (w/w peptide: starch) respectively. The intact and hydrolyzed casein and whey protein sample analyses were performed in triplicate and fractions were collected in duplicate.

Sample	Amount of protein or % of protein recovered after chromatography	Inhibition of starch hydrolysis
Camel milk	--	not determined
Casein	--	7.6% ±1.1
Whey	--	10.1% ±0.9
Hydrolyzed casein	--	11.3% ±0.9
Hydrolyzed whey	250mg, corresponding to 34mg whey protein	16.5% ±0.2
Fraction 1 after HIC	32%	26.9% ±0.1
Fraction 1 after CEX	86%	24.1% ±0.1
Fraction F1* after CEX and HIC	45%	32.8% ±0.4
Fraction F2* after CEX and HIC	09%	35.7% ±0.3

Table 2. Sequences of peptides in camel whey protein hydrolysates in fraction F1 obtained after separation on an Octyl Sepharose CL-4B hydrophobic interaction column. Peptides were sequenced by LC-MS/MS after trypsin hydrolysis.

Sequence	RT (min)	m/z	pI
VTMQNLNDR	13.30	1090.53	6.61
IRDWYQR[a)]	13.35	1036.53	9.84
LVPVICHR	13.47	993.57	8.86
GFSSGSAVVSGGSR	13.54	1254.61	10.84
LASYLDKVR	14.64	1064.61	9.74
YFCDNQETISSK	14.65	1491.64	3.93
ALEEANADLEVK	16.31	1301.66	3.54
IRLENEIQTYR	16.60	1434.77	6.96
FLEQQNQVLQTK	16.82	1475.79	6.61
FASFIDKVR	17.27	1082.60	9.87
RHPEYAVSLLLR	18.90	1453.83	9.54
DAEAWFNEK	19.64	1109.49	3.69
VLDELTLAR	19.79	1029.59	3.93
EYGLFQINNK	20.68	1225.62	6.79
WELLQQVNTSTR	22.06	1475.75	6.74
VVSVLTIQHQDWLTGK	22.25	1824.01	7.77
NMFETPFLAR	23.81	1225.60	6.44
LALDIEIATYR	24.15	1277.71	3.39
FLEQQNQVLQTKWELLQQVNTSTR	24.72	2932.52	6.61
SLDLDSIIAEVK	27.38	1302.72	6.61
VNLFDIPLEVQYVR	29.23	1704.94	6.61
LALDVEIATYR	57.04	1263.70	6.61

[a)]Sequences that were not identified after additional fractionation on a cation exchange column (Table 3) are underlined.

Table 3. Sequences of peptides identified in camel whey protein hydrolysates in the pooled fractions F1* and F2* after separation on SP-Sepharose fast flow column (CEX), followed by separation on an Octyl Sepharose CL-4B column (HIC). Peptides were sequenced by LC-MS/MS after trypsin hydrolysis unless otherwise specified.

Sequence	Retention time (min)	m/z	pI[a)]
NKYEDEINKR	10.72	1308.66	6.61
VTMQNLNDR	13.25	1090.54	6.61
GFSSGSAVVSGGSR	13.43	1254.61	10.84
YEELQVTAGR	15.32	1165.59	4.15
ALEEANADLEVK	16.20	1301.67	3.54
YEELQITAGR	16.79	1179.61	4.15
WTLLQEQGTK	18.67	1203.64	6.73
DAEAWFNEK	19.41	1109.49	3.69
VLDELTLAR	19.59	1029.59	3.93
GSLGGGFSSGGFSGGSFSR	20.23	1707.77	10.84
WELLQQVNTSTR	21.84	1475.75	6.74
LALDIEIATYR	24.00	1277.71	3.93
SLDLDSIIAEVK	27.17	1302.72	3.54
KKAGVLDYETFTK[b)]	6.35	1499.81	9.44
KHSTKGLGK [b)]	14.76	955.57	10.98

[a)]calculated on https://pepcalc.com/

[b)] these peptides were identified in samples that were not hydrolyzed with trypsin prior to LC-MS/MS analysis.

Table 4. Starch digestibility inhibition (%) of synthesized peptides identified in camel whey protein hydrolysates after separation by cation exchange chromatography and hydrophobic interaction chromatogram or just by hydrophobic interaction chromatography. Peptides synthesized by Canada Peptide company, Pointe-Claire, Quebec, Canada.

Camel Peptide sequences	Uniprot Acc. #	Camel Protein[a)]	Peptide source (column)	pI	Starch digestibility inhibition%
LALDIEIATYR	S9WX05	Keratin like protein	HIC and CEX+HIC	3.93	37.4 ± 1.4[a]
VLDELTLAR	S9XAP9	Keratin like protein	HIC and CEX+HIC	3.93	33.6 ± 2.4[a]
DAEAWFNEK	S9WUY9	Keratin like protein	HIC and CEX+HIC	3.69	5.5 ± 2.1[b]
WTLLQEQGTK	S9Y6J1	Keratin like protein	CEX+HIC	6.73	9.5 ± 1.3[b]
YEELQVTAGR	S9W9S8	F rod domain-containing protein	CEX+HIC	4.15	4.6 ± 1.5[b]
KHSTKGLGK	S9XLY6	Poly [ADP-ribose] polymerase	CEX+HIC	10.98	6.7 ± 1.2[b]

[a)] Identified with NCBI BLASTp against *Camelus bactrianus* (taxid:9837) proteins

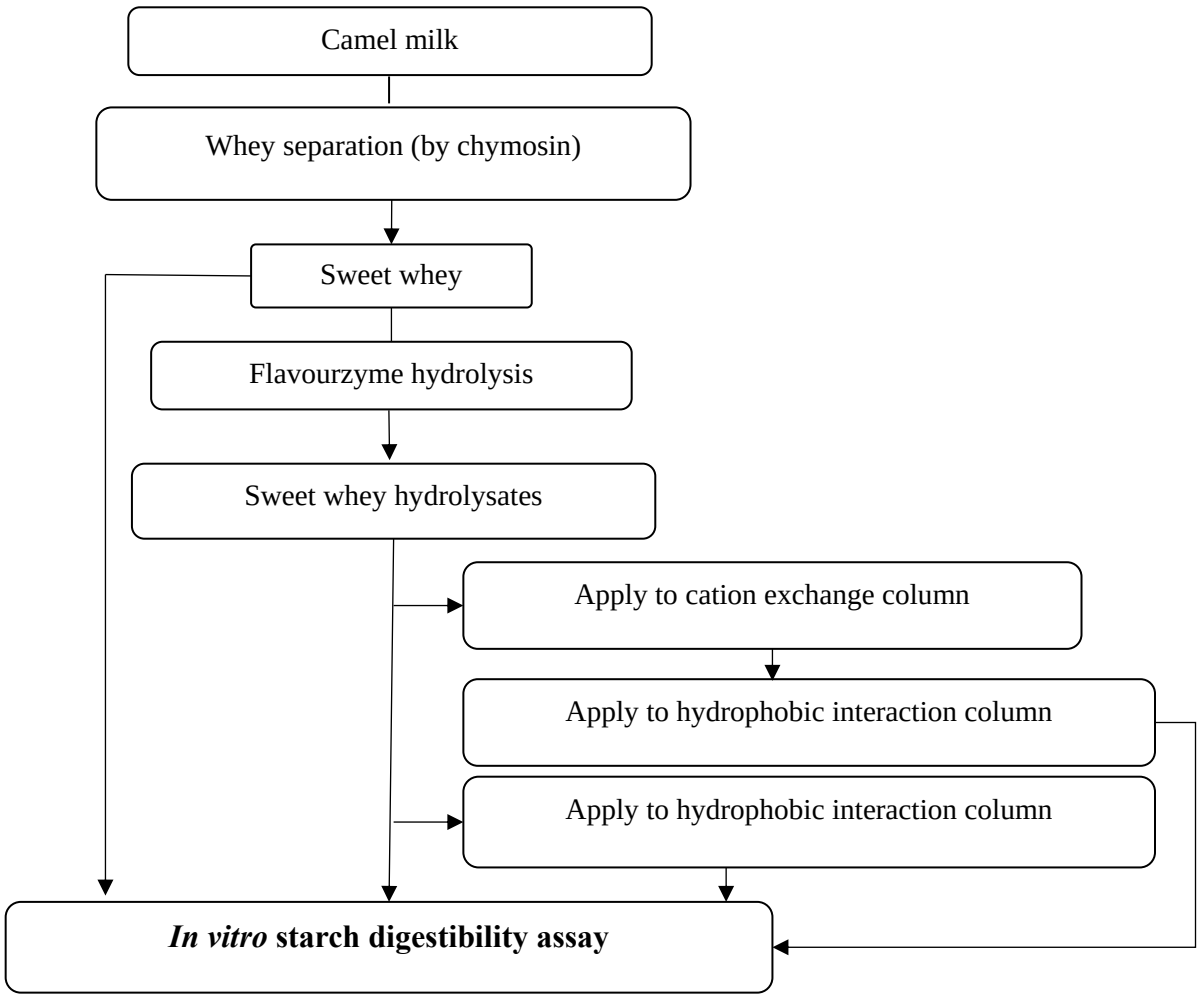
Camel milk
Whey separation (by chymosin)
Sweet whey
Flavourzyme hydrolysis
Sweet whey hydrolysates
Apply to cation exchange column
Apply to hydrophobic interaction column
Apply to hydrophobic interaction column
In vitro starch digestibility assay

Figure1.

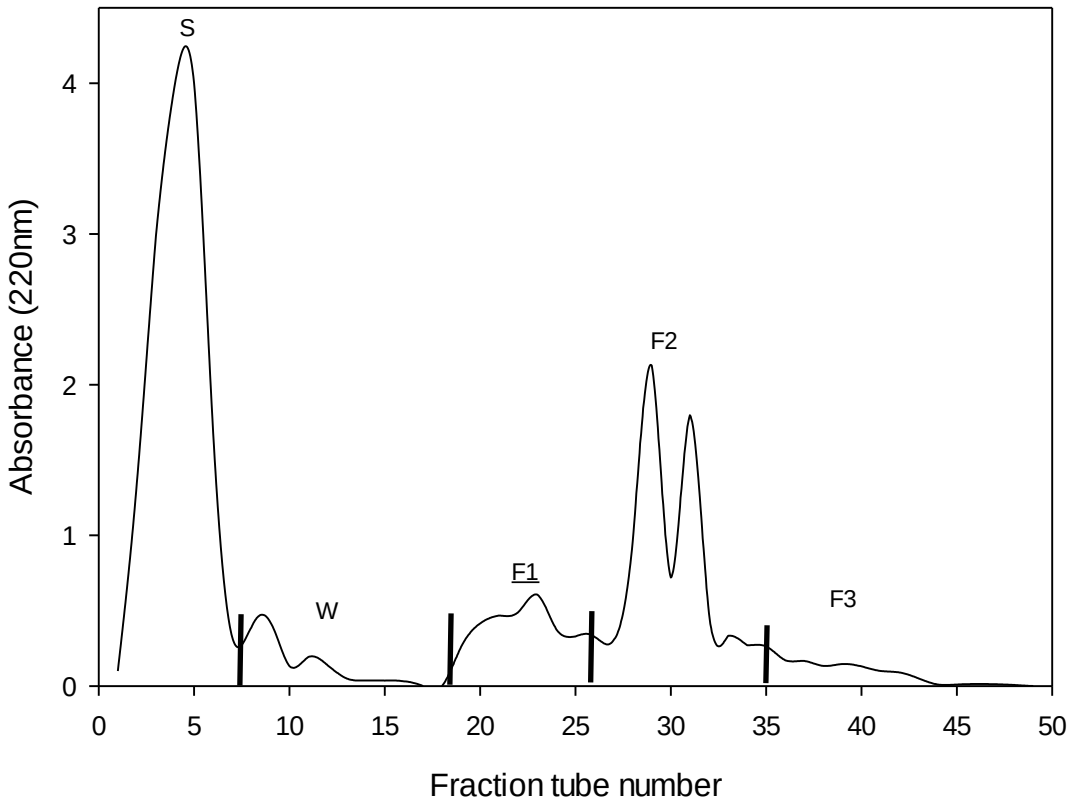
S
W
F1
F2
F3
Absorbance (220nm)
Fraction tube number
0
1
2
3
4
0
5
10
15
20
25
30
35
40
45
50

Figure 2.

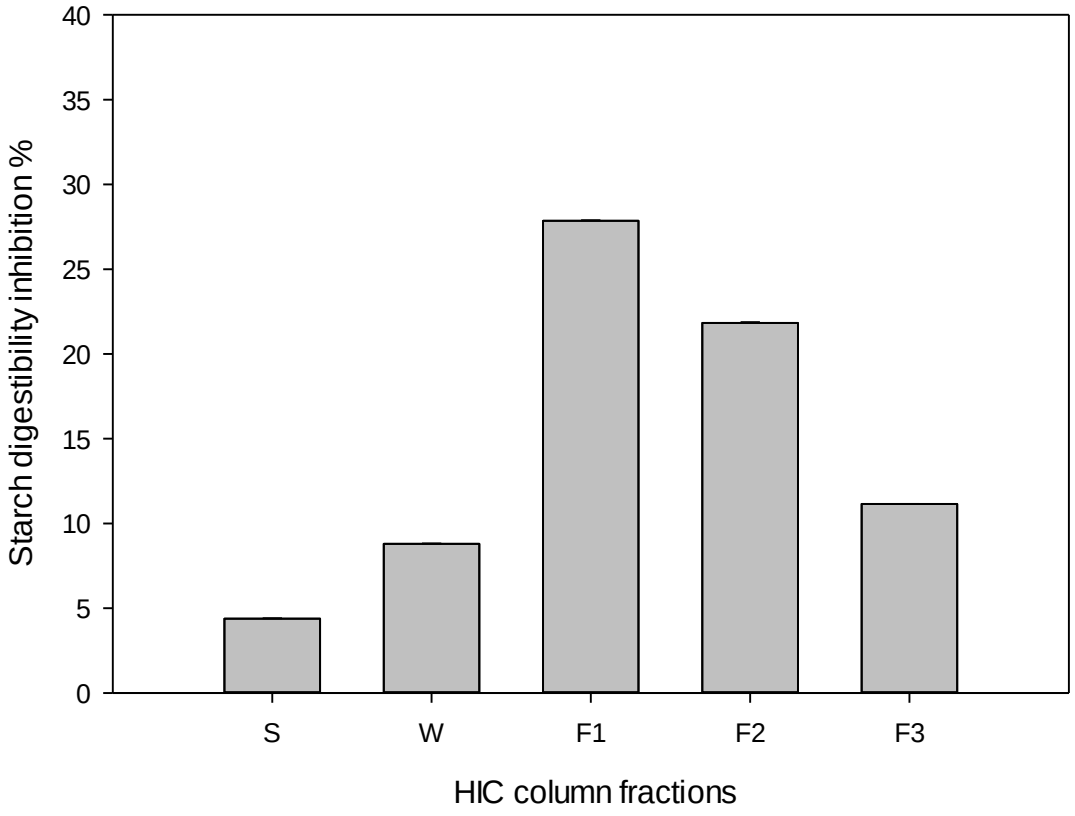
40
35
30
25
20
15
10
5
0
Starch digestibility inhibition %
S
W
F1
F2
F3
HIC column fractions

Figure 3.

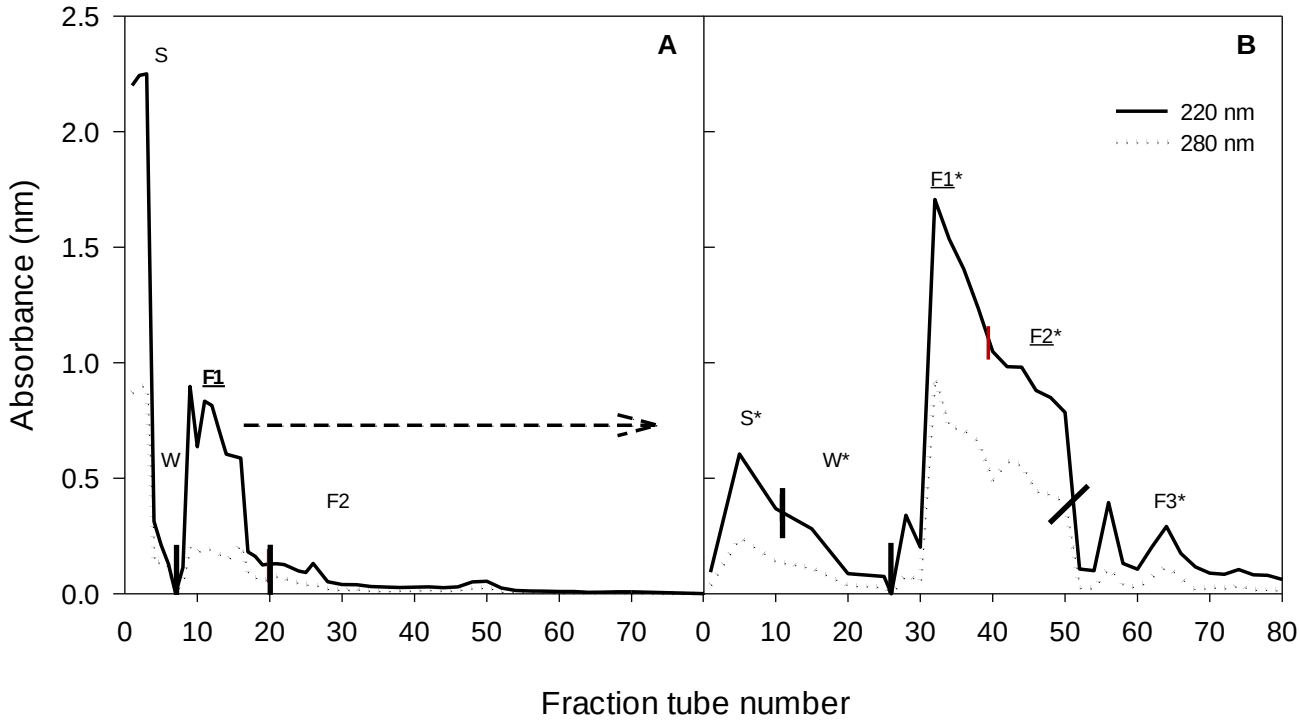
A
B
S
W
F1
F2
S*
W*
F1*
F2*
F3*
220 nm
280 nm
Absorbance (nm)
Fraction tube number
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
50
60
70
80

Figure 4.

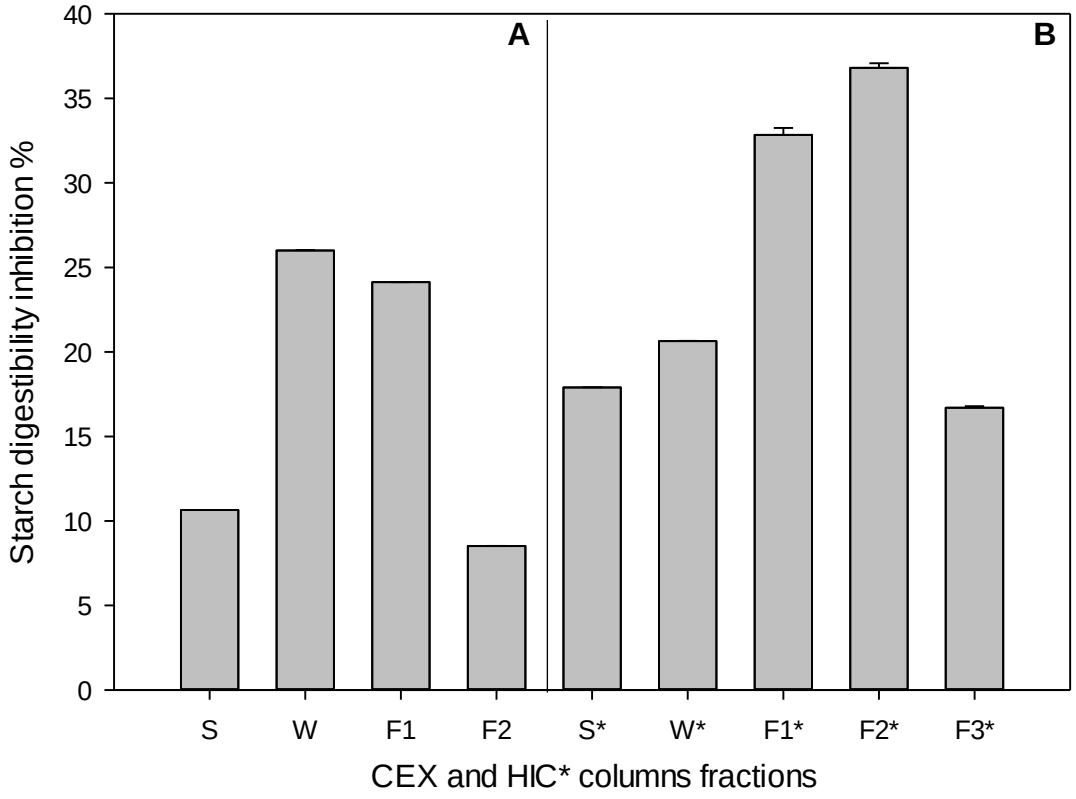
A
B
Starch digestibility inhibition %
40
35
30
25
20
15
10
5
0
S
W
F1
F2
S*
W*
F1*
F2*
F3*
CEX and HIC* columns fractions

Figure 5.