The effects of food components on the digestion of DNA by pepsin

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ABSTRACT
Recently, our study found that naked nucleic acids (NAs) can be digested by pepsin. To better understand the fate of dietary DNA in the digestive tract, in this study we investigated the effects of several food compositions on its digestion. The results showed that protein inhibited the digestion of DNA when the protein:DNA ratio was higher than 80:1 (m/m). DNA found in nucleoprotein (NA), which more closely resembles the state of DNA in food, was as efficiently digested as naked DNA. When the carbohydrate:DNA ratio was 50:1–140:1 (m/m), mono-, di- and polysaccharides did not inhibit DNA digestion. NaCl exhibited an inhibitory effect at 300 mM, whereas divalent cations (Ca2+ and Mg2+) exerted a much stronger inhibitory effect even at 50 mM. The polycation compounds (e.g. chitosan and spermine) showed a significant inhibitory effect at N/P (NH3+/PO43−/C0 = 10:1. The close relationship between food composition and DNA digestion suggests that dietary habits and food complexes are important for understanding the in vivo fate of the ingested DNA in the digestive tract.

Introduction
The ingestion of nucleic acids (NAs) as a nutritional supplement and whether the genetically modified (GM) food can affect human’s health have attracted wide attention recently. In 2011, Zhang et al. reported that exogenous plant miRNAs are able to transfer into mice blood and regulated gene expression in vivo (Zhang et al. 2011). Zhang’s study caused some controversies, e.g. whether the exogenous genes could pass through the biological barriers of mammal, including the strong acid environment of stomach and complex condition of gastrointestinal tract where nuclease and associated microbiota are present (O’Neill et al. 2011; Zhang et al. 2012). Discussion over the fate of exogenous NAs in digestive tract encouraged us to reconsider its digestion metabolism. Interestingly, our recently published study found that naked NAs could be digested by porcine pepsin (Liu et al. 2015). This finding revealed the new function of pepsin and doubted the previous understanding that the digestion of NAs started in the intestine (Carver & Allan Walker 1995). Understanding the digestion of NAs is of great importance, on one hand, the digestion products of oligonucleotides, mononucleotides, and even bases are more readily to be absorbed by intestinal epithelial cell, thus function as nutrition supplement or participate in DNA salvage synthesis pathway in vivo. On the other hand, the possibility that mammalian genome is at risk of invasion by foreign DNA also caused some worries (Scrable & Stambrook 1999; Abdelkrim et al. 2005), thus complete digestion of NAs in digestive tract may be helpful to mitigate the possible invading risks of foreign gene.

Our previous study showed that naked DNA could be digested well by pepsin in vitro (Liu et al. 2015). However, compared with naked DNA, digestion of dietary NAs is more complicated. Firstly, DNA in food is tightly packed on histones as nucleoprotein (NP) complex (Bonner et al. 1968), therefore DNA may be less likely to contact pepsin, and protein as the optimum substrate will compete with DNA during digestion (Shobha et al. 2014). Moreover, various dietary components might interact with DNA and affect the activity of pepsin, for example, pepsin activity was reported to be affected by metal cations and pH values (Yoshimasu et al. 2002; Klomklao et al. 2007), thus NA digestion might be inhibited or accelerated. All of these possibilities could affect the fate of dietary DNA in the stomach.

To clearly understand the digestibility of dietary NAs in stomach, in this study, we employed several
common food components, including proteins, carbohydrates, metal cations and polycation compounds (i.e. spermin and chitosan) to evaluate their effects on the digestion of DNA by pepsin (EC 3.4.23.1). In addition, the digestion of NP extracted from Corvina was also investigated. Various concentrations of food components were employed to simulate different dietary intakes.

Materials and methods

Materials

Pepsin (P8667, 3200–4500 units/mg) extracted from porcine gastric mucosa was purchased from Sigma-Aldrich Co., Ltd (Shanghai, China), and was dissolved in water to a concentration of 40 mg/mL. Digestion buffers for pepsin were prepared as stock solutions of 60 mM NaCl and 100 mM NaH2PO4 in H2O, and the pH was adjusted to the required values by the addition of 1 M HCl or 1 M NaOH.

kDNA (48.5 kb) was purchased from Thermo Fisher Scientific (Fermentas, Waltham, MA; SD0011) with concentration of 300 ng/mL. The storage buffer for kDNA was 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA. Hemoglobin (HB) (H2625) was purchased from Sigma Co., Ltd. Salmon sperm DNA (D1626) purchased from Sigma was dissolved in ddH2O at a concentration of 300 ng/mL. Bovine serum albumin (BSA), chitosanase of 0.1 U/mL, analytical pure carbohydrates including starch, maltose, glucose and glucosamine were also purchased from Beijing Solarbio Science & Technology Co., Ltd, Beijing, China. Chitosan (CS, deacetylation was 90.25%, 50–100 KDa) was purchased from Solarbio (Beijing, China). All the carbohydrates were dissolved in ddH2O with concentration of 40 mg/mL for storage. All enzymes and solutions were stored at −20°C.

Simulated gastric juice

The simulated in vitro digestions were mainly carried out using 4 mg/mL pepsin which was approximately equal to the amount of 3.2 mg/mL recommended by USP (United States Pharmacopeia). Considering that pH value will be increased to 3.0–6.0 within 2 h after food ingestion (Dressman et al. 1990), the pH value was set at 3.8. All the reactions were carried out at 37°C.

Extraction of NP from Corvina spermary

Corvina was obtained from a local market, and the spermary was excised as soon as the fish was killed. The extraction protocol was based on that explained by Bonner et al., with slight modification (Bonner et al. 1968). The spermary (20 mL) was washed three times in sodium citrate (0.1 mol/L, 60 mL) for 10 min until the washing solution was clear. Then, 20 mL of NaCl (1.0 mol/L) was added, the solution was homogenized using a Selecta Sonopuls for 1–2 min, and 180 mL of NaCl (1.0 mol/L) was added and incubated at room temperature for 1 h. The homogenate was filtered using a piece of gauze and the filtrate was collected, then a double volume of alcohol (95%) was added. The precipitate was collected then drained, and the crude extract was cut into small pieces. Next, a double volume of NaCl (1.0 mol/L) was added and incubated for 10 min, then the mixture was homogenized for 1–2 min and dissolved by an 8× volume of NaCl solution. Finally, the extract was soaked in isopropyl alcohol for 12 h and lyophilized to obtain purified NP. The NP protein content was measured using the bicinchoninic acid (BCA) protein determination method using BSA as a standard. The protein content was measured using a kit (PC0020) purchased from Solarbio (Beijing, China) and was approximately 37%.

Digestion of NP by pepsin

The extracted NP was dissolved in ddH2O (3%, w/v) before use. Aliquot of 4 μL NP (30 mg/mL) was added to 36 μL of mixtures (pH 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0) to a final concentration of 3 mg/mL, the final reaction solution including 25 mM NaH2PO4, 15 mM NaCl and 0.4% (w/v) of pepsin. The mixtures were incubated at 37°C for 5 h. After reaction, samples were extracted by phenol–chloroform–isopentanol (25:24:1/v:v:v) method to remove pepsin and protein in NP (Wilson 1987). The supernatant of 10 μL was used for electrophoresis on 1% agarose gel.

Digestion of salmon sperm DNA

Salmon sperm DNA (300 ng/μL) of 3 μL was digested in 20 μL of buffer (pH 3.8, including 4 mg/mL of pepsin, 25 mM NaH2PO4 and 15 mM NaCl), reactions were incubated at 37°C for 5 h.

Effect of protein on the digestion of DNA by pepsin

HB (M, 67 000 Da) and BSA (M, 66 400 Da) were used to evaluate the influence of protein on DNA digestion. HB (24 mg/mL) was dissolved in 1 mM HCl, and BSA (24 mg/mL) was dissolved in ddH2O immediately before use. The mass ratios of HB:λDNA and BSA:λDNA were both designated as 5:1, 10:1, 20:1,
30:1, 40:1, 60:1, 80:1, 200:1 and 400:1, respectively. All the reactions were carried out in pH 3.8 buffer (including 25 mM NaH2PO4 and 15 mM NaCl) at 37°C for 2 h. The digestion results were analyzed on a 1% agarose gel.

**Effect of carbohydrates on the digestion of DNA by pepsin**

The effect of carbohydrates on DNA’s digestion was evaluated by adding glucose (monosaccharide), maltose (disaccharide), glucosamine and starch (polysaccharide). The mass ratios of carbohydrate:DNA were designated as 50:1–140:1.

**Effect of salts on the digestion of DNA by pepsin**

NaCl (0–520 mM), KCl (0–450 mM), MgCl2 (0–100 mM) and CaCl2 (0–100 mM) were mixed with pepsin solution to obtain the designated concentrations.

**Effect of polycations on the digestion of DNA by pepsin**

Spermine and CS are two types of polycations. Various concentrations of CS were mixed with λDNA at charge molar ratios [positive charge (CS):negative charge (λDNA)] of 5:1, 10:1, 20:1 and 30:1, respectively. All mixtures were kept at room temperature for 30 min, then digestion buffer (pH 3.8) and pepsin were added and the mixtures were incubated at 37°C for 2 h. After digestion, the tubes were placed on ice to terminate the reactions immediately, and 1 M NaOH was added to adjust the reaction pH value from 3.8 to 6.5 ± 0.5. Next, chitosanase was added to hydrolyze CS at 37°C for 2 h to release the wrapped DNA. Spermine was mixed with λDNA at molar ratios [positive charge (Spermine) to negative charge (λDNA)] of 10:1, 20:1, 40:1, 60:1 and 80:1, respectively.

**Assay of pepsin activity**

Pepsin activity was evaluated by the method described by Anson et al. with a slight modification using HB as the substrate (Anson 1938). Pepsin (0.04 mg/mL) of 50 μL was prepared in HCl of pH 3.0, then mixed with 250 μL (2.5 mg) of 37°C equilibrated HB substrate (pH 3.0), and incubated at 37°C for 10 min. Enzyme reaction was terminated by adding 500 μL of 5% (w/v) TCA, mixed by swirling and incubated at 37°C for 5 min. The reaction mixture was centrifuged at 12 000 rpm for 10 min and the absorbance of oligopeptides content in the supernatant was measured at 280 nm. One unit of activity was defined as the increase of 0.001 in absorbance at 280 nm per minute. The blank was carried out in the same manner, except that the enzyme was added after addition of 5% (w/v) TCA.

**Effect of salts on the activity of pepsin**

Various chemicals were mixed with pepsin solution to obtain the designed concentrations of metal ions (0–600 mM for NaCl and KCl, 0–100 mM for MgCl2 and CaCl2). The mixtures were kept at room temperature for 30 min and the remaining activity was determined using HB as a substrate at pH 3.0 and at 37°C for 10 min. Other conditions were same as the method of assay of pepsin activity.

**Results and discussions**

**Effect of protein on the digestion of DNA by pepsin**

DNA was digested together with HB (Figure 1(A)) or BSA (Figure 1(B)) to evaluate the effect of protein on the digestion of DNA. According to Chinese Dietary Reference Intakes, the recommended protein intake is 55–65 g/d for adults, 18 years and older, and the suggested intake of diet NAs should be less than 1.5 g/d, as recommended by World Health Organization (WHO). Thus, the appropriate mass ratio of protein:λDNA was calculated to be 40:1 (60 g/d:1.5 g/d). Different mass ratios of protein:λDNA were designed ranging from 5:1–400:1, considering the individual differences in food intake. As shown in Figure 1, at 40:1 (Figure 1(A), lane 5), HB did not exert a significant effect on the digestion of λDNA compared with the reaction without HB (lane C). When the mass ratios of protein:λDNA were 80:1 and 200:1, the digestion of λDNA slowed to some extent (Figure 1(A), lane 7, 8). When the ratio of protein:λDNA was increased to 400:1 (Figure 1(A), lane 9), the digestion of λDNA was significantly inhibited. Similar results were observed using BSA as the substrate (Figure 1(B)). The above results indicate that the inhibitory effect of protein on the digestion of DNA by pepsin exhibited concentration dependence. When the ratio of protein:λDNA fell within the range of a recommended diet (less than 80:1), the protein had almost no effect on the digestion of naked DNA. When the ratio of protein:λDNA increased to 80:1 or higher, the digestion of DNA was restricted. These results may be explained by the fact that protein is the optimal substrate of pepsin. As reported in our
previous study, the rate of protein digestion was almost 10,000× faster than that of DNA (Liu et al. 2015). Therefore, when the concentration of protein was much higher than that of DNA, such as 400:1 (protein:DNA), pepsin might be predominantly occupied by protein. Thus, docking between pepsin and DNA could be blocked, and the digestion of DNA would be inhibited. Moreover, the $pK_a$ value of the DNA phosphate group was 1.5 (Nelson et al. 2008), indicating that DNA was negatively charged during the reaction (pH 3.8). However, the proteins employed (i.e. HB and BSA) were positively charged at pH 3.8, thus, negatively charged DNA was preferred for combining with positively charged proteins or peptides (i.e. the digested products), which might further inhibit the binding of DNA and pepsin.

**Digestion of NP by pepsin**

Although the digestion of naked DNA in the presence of naked protein revealed the concentration-dependent trend in inhibition, it was not sufficient to reveal protein’s *in vivo* effect on the digestion of DNA in food. As we know, most DNA in food exists in the form of NP, but is not present in the naked form (Kinsella 1987). We therefore employed NP extracted from *Corvina* spermary as the substrate. We found that the DNA in NP was hydrolyzed by pepsin within the pH range of 2.0–5.0 (Figure 2, lanes 1, 3, 5, 7, 9, 11). Moreover, we noticed that the digestion rate of DNA in NP was slightly slower compared to that of naked salmon sperm DNA under the same conditions (Supporting Information Figure S1). This may be due to the abundance of protein, which is the primary substrate of pepsin. Taken together, these results indicate that both naked DNA and the DNA packed in NP are digested by pepsin. The inhibitory effect did not occur when the concentration ratio of protein:DNA fell within the reasonable range.

**Effect of carbohydrates on the digestion of DNA**

Carbohydrates are the main energy source for the human body and are thus one of the most important components of food. Moreover, pepsin was reported to exhibit hydrolysis activity on guar galactomannan (Shobha et al. 2014). This finding implies that carbohydrates could also be a substrate of pepsin, which might effect on the digestion of DNA by pepsin. Thus, several types of monosaccharide, oligosaccharide and
polysaccharide at various concentrations were employed to assess their effects on the digestion of DNA by pepsin. The DRI of carbohydrates is 120 g/d. As mentioned earlier, the WHO recommended NA intake is less than 1.5 g/d, so the appropriate mass ratio of carbohydrate to DNA was calculated to be 80:1 (120 g/d:1.5 g/d), and the mass ratios of carbohydrate:DNA were determined to be 50:1–140:1 in our study. Several types of carbohydrate, such as monosaccharide (glucose and glucosamine), disaccharide (maltose) and polysaccharide (starch) were employed in the study, and the results are shown in Figure 3. At a carbohydrate:DNA ratio of 140:1, the digestion of λDNA was almost the same as in the absence of carbohydrates (Figure 3, lane C). This suggests that carbohydrates had no effect on the digestion of naked DNA. Furthermore, the results of treating a 140:1 DNA to carbohydrate mixture with acidic buffer (pH 3.8) only (pepsin was not added) demonstrated that no impurities in the carbohydrates affected the digestion of DNA (Figure 3, lanes C₁, C₂, C₃, and C₄).

Because protein, carbohydrate and DNA are all the substrates of pepsin, we examined the digestion of DNA together with both protein and carbohydrate. The mass ratio of the three substrates was 1:40:80 (λDNA:HB:glucose). At this ratio, neither HB (Figure 1(A), lane 5) nor glucose (Figure 3) exerted a significant effect on DNA digestion. When the three substrates were digested together, DNA was still digested as efficiently as when in the naked form (Supporting Information Figure S2, lane 2). Thus, the two major components of food – protein and carbohydrates – did not affect the digestion of DNA at the recommended concentrations.

**Effect of metal elements on the digestion of DNA**

NaCl is the most prevalent flavoring used around the world, and metal elements such as calcium, magnesium and potassium are considered the necessary elements for human health. In food, they are inevitably ingested together with DNA and are digested with DNA in the gastric track. Moreover, it has been reported that pepsin activity for digesting protein can be blocked by high concentrations of NaCl. We therefore investigated the effect of NaCl and other metal elements on the digestion of DNA by pepsin. Monovalent Na⁺ and K⁺ cations and divalent Ca²⁺ and Mg²⁺ cations were studied, and the results are shown in Figure 4. When the concentration of NaCl or KCl was lower than 300 mM, no obvious effect was observed. At increased NaCl and KCl concentrations greater than 300 mM, the digestion of λDNA was significantly inhibited, and no digestion was observed when the salt concentration was over 400 mM (Figure 4(A), lane 8 and Figure 4(B), lane 6). For divalent cations, the digestion of λDNA was strongly inhibited when the concentration of Ca²⁺ (or Mg²⁺) was as low as 50 mM (Figure 4(C), lane 6 and Figure 4(D), lane 6), and DNA digestion was completely inhibited when Ca²⁺ (or Mg²⁺) was present at 100 mM (Figure 4(C), lane 7 and Figure 4(D), lane 7). These results show that divalent cation have a much stronger influence on the digestion of DNA than monovalent cations.

To investigate whether the inhibitory effect of metal elements on DNA digestion is due to a reduction in pepsin activity, we measured pepsin activity at different cation concentrations (Table 1). A mild continuous decrease in pepsin activity was observed with increase...
in concentrations of monovalent cations (NaCl and KCl); the relative activity of pepsin was decreased to 84% when the NaCl concentration was 600 mM, and a similar result was obtained for KCl. Interestingly, the activity of pepsin was increased slightly in the presence of divalent cations, and the relative activity was 108% when CaCl₂ (or MgCl₂) was present at 50 mM, although DNA digestion was strongly inhibited at this concentration (Figure 4(C,D), lane 6). The effect on pepsin activity was in agreement with the results of a study conducted by Klomklao et al., who found that NaCl could block pepsin activity and CaCl₂ could promote pepsin activity (Klomklao et al. 2007).

As shown in Table 1, even the lowest relative activity of pepsin was >84% (600 mM NaCl). We therefore suggested that the inactivation of pepsin activity was not the main reason for the cation-driven inhibition of DNA digestion. The inhibitory effect might be due to a change in the charge density of the solution. High concentration of metal ions could result in the presence of a high concentration of positive charges around both

| Table 1. Effect of metal elements on the activity of pepsin from porcine stomacha. |
|----------------------------------|----------------------------------|
| Relative activity (%) NaCl KCl | Relative activity (%) b CaCl₂ MgCl₂ |
| Concentration (mM) 0 10 50 100 | Concentration (mM) 0 10 50 100 |
| NaCl | 100 | 99.8 ± 1.2 | 99.1 ± 0.8 | 98.4 ± 0.6 | 97.3 ± 1.0 | 88.0 ± 1.3 | 84.6 ± 1.2 |
| KCl | 100 | 102.3 ± 1.8 | 100.9 ± 1.5 | 96.4 ± 0.9 | 93.9 ± 1.1 | 90.2 ± 0.7 | 87.6 ± 0.3 |
| CaCl₂ | 100 | 110.1 ± 1.0 | 111.4 ± 1.1 | 110.8 ± 0.6 | 118.5 ± 0.5 | 108.2 ± 0.9 | -- |
| MgCl₂ | 100 | 118.0 ± 1.3 | 115.1 ± 0.7 | 110.5 ± 0.8 | 110.9 ± 1.0 | 99.7 ± 1.2 | -- |

aEach enzyme solution was incubated with metal elements solutions of fixed concentration at 37°C for 30 min and the residual activity was assessed using HB as a substrate for 10 min at pH 3.0 and 37°C.
bMean ± SD from triplicate determines.
DNA and pepsin, which might prevent the interaction between DNA and pepsin.

NaCl is the most popular flavoring used around the world, and the ingested concentration of NaCl might differ with eating habits. To investigate whether this difference could inhibit DNA digestion, we determined the average daily intake of NaCl. References reported that approximately 6-15 g NaCl should be ingested through meals a day (Ge 2011; Sacks et al. 2001). Our gastric residual volume (GRV) is approximately 50-250 mL, and the stomach expends approximately 1000 mL during meal ingestion due to endogenous secretions (Maltby et al. 2004; Marshall & West 2006). Thus, we calculated the concentration of NaCl in each meal to be 34-85 mM. With the addition of normal saline at approximately 150 mM, the NaCl concentration in stomach during meal digestion was approximately 190-240 mM. We noticed that this concentration was close to 300 mM, demonstrating its inhibitory effect on DNA digestion. This implies that these experiments reflect the inhibition of DNA digestion caused by the salt ingested with our meals. Therefore, if we ingest appropriate concentrations of NaCl, the digestion of DNA from food will not be significantly affected. However, if we ingest more than the recommended allowance of NaCl, the digestion of DNA could be markedly inhibited. Thus, different eating habits among individuals might cause an inhibitory effect on DNA digestion.

Effect of polycations on the digestion of DNA

Polycations have been reported to have a protective effect on DNA (Chen et al. 2004; Chen et al. 2013; Gamboa & Leong 2013). To investigate their effect on DNA digestion, we employed two polycation compounds: CS and spermine. As shown in Figure 5(A), when the molar ratio of N:P (NH$_4^+$:PO$_4^{2-}$) was 5:1 (Figure 5(A), lane 1), the digestion of DNA decreased compared to digestion in the absence of CS (Figure 5(A), lane C). With N:P increased to 10:1, the digestion of DNA was restrained completely (Figure 5(A), lane 2). In the presence of spermine and a molar ratio of N:P increasing from 10:1-80:1 (Figure 5(B), lanes 1-5), the digestion of DNA was strongly inhibited and was entirely restrained at a molar ratio of 80:1. These results indicated that both CS and spermine could protect DNA from pepsin degradation.

To improve the stability of drugs during stomach and intestinal digestion, CS-based drugs have been widely employed (Dhadwar et al. 2010; Mao et al. 2010). Our results are consistent with these reports. Our results imply that polycations in food or medicine will slow the digestion of DNA significantly and protect the ingested DNA as it passes through the gastric tract to the small intestine.

Conclusion

In conclusion, our study demonstrates that common food components, including protein, carbohydrate, metal cations, and polycation compounds have a close relationship with the digestion of DNA. Carbohydrates, including monosaccharides, disaccharides and polysaccharides, did not show an observed effect on the digestion of DNA. Protein mixed with naked DNA or combined with DNA in an NA complex did not exert a significant inhibitory effect on DNA digestion when its concentration was less than 80 times that of DNA. Monovalent cations, divalent cations and polycation...
compounds exhibited a significant inhibitory effect on DNA digestion in a concentration-dependent manner. The significant inhibitory effect of metal cations and polycations indicated that eating habits could be important for the digestion of dietary nucleic acids. Although food composition results in different inhibitory effects, they all had a minimal influence on DNA digestion at or below the recommended intake concentrations. Thus, efficient DNA digestion depends on the general diet. As pepsin is the dominant enzyme in the stomach, the digestion of ingested DNA is prospective.

Our study demonstrated the digestion of dietary NAs by pepsin, and further revealed the NA digestion in the gastrointestinal tract. Digestion of NAs in the stomach may accelerate its further digestion in the intestine, which is important for the utilization of DNA and the maintenance of health. For example, mono-ribonucleotide was added into the infant milk powder as a nutritional supplement (Yu 1998; Singhal et al. 2008). However, NAs that are absorbed must escape nucleases in the first barrier of gastrointestinal tract, cellular compartments and in the bloodstream(Sioud 2005; Zhang et al. 2012). For this reason, delivery of oligonucleotide therapeutics does not occur orally. Hope our findings can help to evaluate more precisely the release and digestion of oral delivery DNA/RNA drugs in digestive tract. The digestion of DNA in the stomach requires further investigation because food components are complicated and DNA is occluded in food. In addition, in vivo digestion is less controlled than in vitro digestion. Thus, more sophisticated in vitro and in vivo models are required.

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Disclosure statement

The authors declare no competing financial interests.

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