Impact of calcium on salivary α-amylase activity, starch paste apparent viscosity and thickness perception

MORRIS, Cecile

Published version

MORRIS, Cecile (2011). Impact of calcium on salivary α-amylase activity, starch paste apparent viscosity and thickness perception. Chemosensory Perception, 3, 112-116.

Impact of calcium on salivary α-amylase activity, starch paste apparent viscosity and thickness perception

Keywords: starch, α-amylase, viscosity, calcium, thickness perception

Abstract

Thickness perception of starch-thickened products during eating has been linked to starch viscosity and salivary amylase activity. Calcium is an essential cofactor for α-amylase and there is anecdotal evidence that adding extra calcium affects amylase activity in processes like mashing of beer. The aims of this paper were to 1) investigate the role of salivary calcium on α-amylase activity and 2) to measure the effect of calcium concentration on apparent viscosity and thickness perception when interacting with salivary α-amylase in starch-based samples. α-Amylase activity in saliva samples from 28 people was assessed using a typical starch pasting cycle (up to 95°C). The activity of the enzyme (as measured by the change in starch apparent viscosity) was maintained by the presence of calcium, probably by protecting the enzyme from heat denaturation. Enhancement of α-amylase activity by calcium at 37°C was also observed although to a smaller extent. Sensory analysis showed a general trend of decreased thickness perception in the presence of calcium but the result was only significant for one pair of samples, suggesting a limited impact of calcium enhanced enzyme activity on perceived thickness.

22

1. Introduction

24 A wide range of thickeners is currently used in processed food to provide body and improved
25 organoleptic properties to food products. Starch is the most commonly used thickener and several
26 studies have focused on thickness perception in starch-thickened products. Salivary α-amylase has
27 some effect on the apparent viscosity and thickness perception of those products due to hydrolysis
28 during eating (Ferry et al., 2006). Natural variation in salivary α-amylase activity has been proposed
29 as one explanation for the observation that individual human assessors rate the perceived thickness
30 of the same starch-thickened products very differently. Recently, genetic factors were shown to
31 play a role in this phenomenon (Mandel et al., 2010) but other factors, such as the variation in
32 human salivary calcium concentration, may also contribute to the variation in perceived thickness, as
33 described below.

34 Human saliva plays several roles during mastication and is also a factor in oral health (Edgar, 1992).
35 Its main functions have been identified as pre-digestion of starch (through α-amylase activity), food
36 bolus lubrication, dilution and clearance and neutralization and buffering (Edgar, 1992). During
37 chewing, some starch is hydrolyzed into glucose and dextrins by salivary α-amylase but the degree
38 of hydrolysis ranges considerably (1 to 27%) depending on food type (Woolnough et al., 2010).

39 The role of calcium in the activation and stabilization of α-amylase has been extensively studied
40 (Bush et al., 1989; Vallee et al., 1959). The proposed stabilization mechanism involves interaction of
41 the cations with some negatively charged amino acid residues, which maintain the 3D structure of
42 the protein (Muralikrishna & Nirmala, 2005). α-Amylases from different sources (including human α-
43 amylase) were found to contain at least 1 mole of calcium per mole of protein but it was also noted
44 that calcium could bind “extrinsically” (non-specifically through polar side chains) with up to 9 to 10
45 moles of calcium per mole of protein (Vallee et al., 1959). Since then, three different binding sites

46 (CaI, CaII and CaIII) have been identified in certain α-amylases (Machius et al., 1998; Suvd et al.,
47 2001). In particular, CaIII, at the interface between domains A and C, has been found in the most
48 thermostable α-amylases and thermostability has been related to the extent of calcium binding and
49 number of binding sites (Kumari et al., 2010). The effect of decreasing calcium contents and the
50 resulting decrease in the activity of α-amylase has been reported (Hsiu et al., 1964; Nielsen et al.,
51 2003) and the loss of activity by calcium depletion is only partially reversible (Nazmi et al., 2008).
52 Human saliva requires at least 1 mole of calcium per mole of protein for full activity (Hsiu et al., 1964)
53 but the effect of excess calcium on α-amylase (as would be the case in natural eating conditions) has
54 rarely been investigated in food systems. Nielsen (2003) found evidence that increasing
55 concentrations of excess calcium were involved in specific inter α-amylase molecular interactions
56 but no indication of the effect on α-amylase activity was given.

57 Calcium concentration in human saliva varies greatly and published values are: 68 ± 16 ppm (Sewon
58 et al., 2004), 45 – 172 ppm (Salvolini et al., 1999) and 45 ± 22 ppm (Larsen et al., 1999). A similar
59 variation in human salivary α-amylase activity has been reported, with values ranging between 50
60 and 400 $U.mL^{-1}$ (Kivela et al., 1997; Mandel et al., 2010).

61 An indirect measure of α-amylase activity, which is particularly relevant to food application
62 (Gonzalez et al., 2002), can be obtained by measuring the decrease in viscosity of starch pastes with
63 the addition of α-amylase (Collado & Corke, 1999). This assay has been used to study the
64 relationship between α-amylase activity, starch paste mechanical properties and sensory analysis of
65 starch thickness perception (Evans et al., 1986; de Wijk et al., 2004; Mandel et al., 2010).
66 Furthermore, the effect of decreased starch viscosity (due to α-amylase activity) affects aroma
67 release (Ferry et al., 2004; Tietz et al., 2008) and saltiness perception (Ferry et al., 2006).
68 Amylomaltase-treated starches were found to be particularly good fat substitutes in yoghurts and a
69 loss of instrumentally-measured firmness due to α-amylase was reported in those systems (Alting et

al., 2009). It is therefore generally accepted that α-amylase has a significant impact on a number of critical starch attributes during eating (Engelen & Van Der Bilt, 2008), thickness perception being the main one. In reviewing the literature, there appeared to be a great variation in sensory analysis of thickness perception for the same starch-thickened food system which could be due to the natural variation of α-amylase activity between donors. Recently, α-amylase concentration in saliva has been linked to genetic differences (Mandel et al., 2010) and this was proposed as an explanation for the natural variation observed in thickness perception of starch-thickened systems.

The aim of this project was to investigate whether salivary calcium levels affected the sensory perception of thickness in starch-thickened products. The hypothesis was that the natural variation in salivary calcium (and the known interaction between calcium and α-amylase activity) could affect the degree of starch degradation, which could be measured by monitoring viscosity. The effect was studied under two conditions, namely during starch gelatinization (temperatures up to 95°C) and on pre-gelatinized starch pastes at 37°C (eating conditions) with apparent viscosity measured instrumentally. Initially, the relationship between salivary calcium concentration and salivary α-amylase activity was measured in gelatinized starch. Next, the effect of added calcium and salivary α-amylase activity on apparent viscosity in starch-thickened systems was investigated. Sensory data were acquired to support the instrumental data and ultimately answer the question of whether thickness perception can be manipulated by adding calcium to starch-thickened food systems.

2. Materials and Methods

2.1. Materials:

Calcium chloride was purchased from Sigma-Aldrich (223506, purity ≥99%). Corn starch was purchased from Sigma-Aldrich (S4126). For sensory testing, food grade materials were used: corn

92 starch (Leeds KW, Leeds, UK) and calcium chloride (Premier Chemicals, Huntingdon, UK). Sugar
93 (sucrose, Silverspoon) was purchased from the local supermarket.

94 For the determination of calcium in saliva by flame photometry, a certified 1000 ppm Ca solution
95 (Sherwood Scientific Ltd., Cambridge, UK) was diluted to make up standard solutions of 1 to 10 ppm.

96 Saliva: 28 students and staff from the University volunteered to provide un-stimulated saliva. The
97 donors were instructed to collect as much saliva as was comfortable over a period of 10 min. The
98 average saliva collection volume was 5 mL.

99 Viscosity measurements were performed no more than 3 hours after the saliva was collected and
100 the remaining aliquot was frozen (-20°C) for subsequent calcium concentration determination (2 to 6
101 weeks later). Human salivary α-amylase has been reported to be stable for several days at 4°C
102 (Schipper et al., 2007) and randomization of the experiments ensured that the time-dependent
103 proteolysis of others proteins would not impact on the results.

104 ## 2.2. Methods:

105 ### 2.2.1. Flame photometer:

106 The calcium concentration in saliva was determined using the protocol described in Sewon et al.
107 (2004): 1760 µL of diluent was added to 40 µL of 5% lanthanum chloride solution (Sigma-Aldrich
108 298182, purity 99.9%) and 200 µL of saliva. The samples were then analyzed using a Model 410
109 Classic Flame Photometer (Sherwood Scientific Ltd.).

110 ### 2.2.2. Rapid Viscosity Analyser (RVA):

111 Two different protocols were selected to estimate the activity of α-amylase by measuring the
112 change in apparent viscosity of starch pastes in the Rapid Viscosity Analyzer (RVA; Newport Scientific,
113 Warriewood, Australia). Protocol 1 was chosen because it mimicked the conditions during eating
114 and gave an indication of the effect of calcium on starch degradation in vivo (Ferry et al., 2004).

115 Protocol 2 was chosen as it operated at conditions relevant to starch degradation during processing
116 and because of the established correlation between the apparent peak viscosity and α-amylase
117 activity (Collado & Corke, 1999).

118 In Protocol 1, a 10.8% corn starch paste (2.7 g of corn starch in 22.3 g of water) was prepared in the
119 RVA using the following temperature profile: 1 min at 50°C, heating to 95°C over the next 4 min,
120 followed by a 3 min holding period at 95°C then cooling to 37°C. The RVA was then stopped to add
121 50 µL of saliva to the freshly prepared paste. A second run during which the temperature was kept
122 constant at 37°C was started and the decrease in apparent viscosity of the paste was measured for 3
123 min. The end viscosity was used as an indicator of amylase activity (Ferry et al., 2004). A similar
124 protocol was shown to correlate well with α-amylase activity as measured using an enzymatic assay
125 (Mandel et al., 2010). For the first 10 s, the paddle speed was 960 rpm but was then lowered to 160
126 rpm. Control experiments substituted the same volume of water for saliva.

127 Protocol 2 was based on a method developed by Collado & Corke (1999) which demonstrated a
128 significant correlation between the peak pasting viscosity and the endogenous α-amylase activity in
129 sweet potato samples. Therefore, for this protocol, the same paste and the same heating profile as
130 Protocol 1 were used but the peak viscosity was taken as an indicator of α-amylase activity and a
131 larger aliquot (600 µL) of saliva / water (control) was added to the mix prior to gelatinization.

132 For both protocols, the effect of added calcium chloride on salivary α-amylase activity was
133 investigated by adding two different levels of calcium chloride to the mix prior to gelatinization.

134 **2.2.3. Sensory evaluation, paired comparison tests**

135 Thirty panelists (students and staff from the University) were recruited to participate in this study.
136 Four paired comparison tests (Table 1) took place in a single session lasting approximately 20 min.
137 The panelists were instructed to taste the samples in the order presented and indicate which sample
138 was the thickest. The presentation order was balanced between the panelists. Apple slices and water

were available for palate cleansing between each sample. No eating instructions other than to concentrate on texture and thickness were given as this does not appear to have an impact on thickness assessment (de Wijk et al., 2004). No training was provided but a pair of dummy samples (identical to each other and to the control of the model system pairs) was introduced first to familiarize the panelists with the texture of the products and give them the opportunity to decide on their own thickness assessment protocol.

The control sample was prepared by mixing 6.8% starch, 83.7 % water and 9.5% sugar and heating up to 95°C for 10 min. Calcium chloride was added to half of the starch paste while still hot. The samples were served at room temperature (18-21°C). The sensory testing took place a maximum of 6 h after sample preparation.

2.2.4. Statistical analysis

The Analysis of Variance was performed using SPSS (Chicago, U.S.A.). The protocols ability to discriminate between donors' salivary α-amylase activity was evaluated using a 1 way ANOVA while a 2 way ANOVA (fixed factors: sample and donor) was used to evaluate the effect of added calcium chloride on viscosity. Where appropriate, a Tukey's HSD test was used to determine which samples were significantly different from one another. Pearson's coefficients were calculated using Excel (Microsoft, Seattle, U.S.A.). The significance level for all the tests was selected as 5%.

3. Results and discussion

3.1. Natural variation in salivary α-amylase activity and calcium effect:

Figure 1 (A and B) shows the data obtained for amylase activity in saliva from volunteers using Protocol 1 and 2 respectively. Protocol 1 measured the end viscosity of the starch paste 3 min after the introduction of saliva/water once the paste had cooled down to 37°C after gelatinization. In

contrast, Protocol 2 measured the effect of salivary amylase during a high temperature gelatinization (pasting) cycle.

Figure 1 thereabout

Visual inspection of Figure 1 plus one way Analysis of Variance, followed by Tukey's HSD test on the end viscosity (Protocol 1) and the peak viscosity (Protocol 2), revealed that Protocol 1 provided better discrimination between the donors and there were fewer subgroups compared to Protocol 2.

The salivary calcium concentration found in the saliva of 28 donors ranged from 30 to 87 ppm with an average of 55 ppm and a standard deviation of 12 ppm. This was in good agreement with the values reported elsewhere (Larsen et al., 1999; Salvolini et al., 1999; Sewon et al., 2004). Saliva from the 28 donors was analyzed for α-amylase activity using Protocol 1 and the assumption made that end viscosity was a reflection of amylase activity. When salivary calcium concentration of the donors was plotted against the end viscosity from Protocol 1 (Figure 2), no clear trends were observed. The Pearson product moment correlation was -0.2689 (critical value for α=0.05 is -0.4683; (O'Mahony, 1986)).which indicated that there was no significant correlation between the salivary α-amylase activity and salivary calcium concentration under the conditions of Protocol 1.

Figure 2 thereabout

In contrast, for Protocol 2 (Figure 3), a roughly linear trend was observed between peak viscosity and salivary calcium concentration. The Pearson product moment correlation was -0.5521 (critical value for α=0.05 is -0.4555, (O'Mahony, 1986)) which indicated a significant correlation between the starch viscosity and salivary calcium concentration under the conditions of Protocol 2.

Figure 3 thereabout

The significant correlation could be interpreted in two ways: 1) an indication that a greater salivary calcium concentration resulted in enhanced α-amylase activity or 2) that the donors with highest

185 calcium concentration also had a greater salivary α-amylase concentration. However, this latter
186 explanation is not supported by the results from Protocol 1, where increased salivary calcium did not
187 correlate with increased α-amylase activity. This suggests that the first explanation is valid and that
188 calcium only affects α-amylase activity at temperatures around 95°C. A potential mechanism is that
189 the excess calcium in saliva helps stabilize the α-amylase and protects it against the heat
190 denaturation which could be experienced during Protocol 2.

191 To further investigate the role of free calcium, calcium chloride was added to the starch paste at two
192 levels, prior to gelatinization and using the same protocols.

193 ### 3.2. Effect of added $CaCl_2$ to the starch system

194 Figure 4 and 5 show typical RVA profiles for Protocols 1 and 2 respectively for five samples, starch
195 paste, paste + $CaCl_2$ (level 2), starch + saliva, starch + saliva + $CaCl_2$ (level 1) and starch + saliva +
196 $CaCl_2$ (level 2).

197 Figure 4 shows that the two samples without saliva show little change when incubated at 37°C while
198 the addition of saliva caused a significant decrease in end viscosity. A two way ANOVA revealed
199 significant differences among the sample set ($p<0.001$) and the donors ($p<0.001$).

200 **Figure 4** thereabout

201 A Tukey's HSD test showed that while the two control samples (without saliva) were not significantly
202 different from one another, they were significantly different to the three other samples. Among the
203 samples tested with saliva, the end viscosity of the sample without calcium chloride was significantly
204 higher than the sample with the highest level of calcium chloride added ($p=0.006$), suggesting that
205 the salivary α-amylase activity was increased on average by 24% by the addition of 20 ppm of $CaCl_2$.

206 The same conclusions could be drawn when Protocol 2 was used (Figure 5).

Figure 5 thereabout

However, Protocol 2 offered a better discrimination between the saliva samples and all the treatments were significantly different from one another except the two control samples (with water instead of saliva) which were not significantly different. The effect of added calcium was to enhance salivary α-amylase activity which resulted in an average 77% reduction in peak starch apparent viscosity as measured in the RVA at the highest concentration of calcium chloride. This protection of α-amylase by addition of calcium was reported in barley and malt where the activity of α-amylase in the presence of calcium was increased at high temperatures (70°C) (Bertoft et al., 1984). The mechanism proposed was that calcium protected the enzyme against thermal degradation and allowed it to maintain a higher activity. Indeed brewers have used calcium for a number of reasons (lowering mash pH to optimise enzymatic action, precipitating unwanted nitrogen, facilitating fining and yeast flocculation and preventing the precipitation of oxalate in the beers) for years (Comrie, 1967). The ability of calcium to protect α-amylase from destruction by heat was reported by brewers as far back as 1963 (Harrison, 1963). This protection against thermal degradation explains the discrepancy observed in Figures 2 and 3 where Protocol 2 yielded a significant correlation between enzyme activity and salivary calcium concentration while Protocol 1 did not, even though it exhibited a better discriminatory ability between the α-amylase activity of saliva samples (Figure 1). It is likely that, upon heating, the salivary calcium acted as a stabilizer and improved the salivary α-amylase activity thus making the activity dependent on the calcium concentration in saliva. Human salivary α-amylase activity is temperature dependent (Lin et al., 2009), with its activity falling sharply with incubation temperatures greater than 40°C. This also explains the poorer discrimination ability of Protocol 2 (Figure 1): in the absence of added calcium, the only calcium available to protect the enzyme was the salivary free calcium which was not enough to fully protect the human α-amylase against thermal degradation. In contrast, in Protocol 1, the saliva was added after pasting and was not subjected to high temperatures and thermal degradation,

hence the smaller (but significant) difference observed between samples containing saliva on the one hand and saliva + calcium on the other. The mechanism through which this is achieved in Protocol 1 is likely to be enzyme stabilization.

While the impact of α-amylase inhibition (using acarbose an anti-diabetic drug or by lowering the pH) on perceived thickness has already been reported (de Wijk et al., 2004; Heinzerling et al., 2008), the effect of α-amylase calcium-stabilization on thickness perception is less well known. Considering that an increase in α-amylase activity was observed with both protocols upon addition of calcium chloride to the food matrix, sensory data was acquired to test whether this difference in viscosity could be perceived.

3.3. Perception of starch thickened systems

Four samples were prepared for sensory analysis using paired comparison tests. The model system contained corn starch, water and sugar, the latter to make the pastes more palatable for the panelists. A commercial starch-thickened soup was also studied as it was hypothesized that salivary α-amylase and calcium might have an effect on starch hydrolysis and perception during eating. Panelists were presented with the two model system samples as a "dummy pair" to measure the panel's performance on sensory thickness discrimination. The results are presented in Figure 6.

Figure 6 hereabout

Out of the four pairs tested, only one pair (model system vs. model system with added $CaCl_2$ at 100 ppm) resulted in a significant difference in perceived thickness at α=0.05. The sample with no added calcium chloride was perceived as significantly thicker (21 panelists out of 30) than the sample with added calcium chloride. The other two pairs (outside of the pair of dummy samples introduced first) displayed the same trend whereby the samples with no added calcium chloride were selected as being the thickest more times (respectively 17 and 19 times out of 30) than the sample with added

255 calcium chloride. This supports the instrumental findings showing that adding calcium chloride to
256 the food has an effect on apparent starch viscosity which is borderline perceivable by panelists and
257 may not be noticeable in real eating conditions.

258 4. Conclusion

259 While it is documented that calcium protects α-amylase against heat denaturation by stabilizing its
260 structure, this has only been exploited by brewers who have added calcium at the mashing stage to
261 improve starch conversion. In this paper, we report that adding calcium chloride and human salivary
262 α-amylase to a starch-thickened food system results in decreased apparent viscosity and thickness
263 perception, which we propose is due to stabilization of the amylase enzyme. The effect is
264 pronounced during starch pasting at high temperatures but even though it has a lesser effect at
265 mouth temperature, a 24% increase in α-amylase activity is observed. Different salivary calcium
266 concentrations may therefore result in different α-amylase activities and may be partly responsible
267 for the natural variation in thickness perception of starch thickened products.

268

5. References

Alting AC, Fred van de V, Kanning MW, Burgering M, Mulleners L, Sein A & Buwalda P (2009) Improved creaminess of low-fat yoghurt: The impact of amylomaltase-treated starch domains. Food Hydrocolloids, 23(3), 980-987.

Bertoft E, Andtfolk C & Kulp SE (1984) Effect of Ph, Temperature, and Calcium-Ions on Barley Malt Alpha-Amylase Isoenzymes. Journal of the Institute of Brewing, 90(5), 298-302.

Bush DS, Sticher L, Vanhuystee R, Wagner D & Jones RL (1989) The Calcium Requirement for Stability and Enzymatic-Activity of 2 Isoforms of Barley Aleurone Alpha-Amylase. Journal of Biological Chemistry, 264(32), 19392-19398.

Collado LS & Corke H (1999) Accurate estimation of sweetpotato amylase activity by flour viscosity analysis. Journal of Agricultural and Food Chemistry, 47(3), 832-835.

Comrie AAD (1967) Brewing liquor - A review*. Journal of the Institute of Brewing, 73, 335-341.

de Wijk RA, Prinz JF, Engelen L & Weenen H (2004) The role of [alpha]-amylase in the perception of oral texture and flavour in custards. Physiology & Behavior, 83(1), 81-91.

Edgar WM (1992) Saliva - Its Secretion, Composition and Functions. British Dental Journal, 172(8), 305-312.

Engelen L & Van Der Bilt A (2008) Oral physiology and texture perception of semisolids. Journal of Texture Studies, 39(1), 83-113.

Evans ID, Haisman DR, Elson EL, Pasternak C & McConnaughey WB (1986) The Effect of Salivary Amylase on the Viscosity Behavior of Gelatinized Starch Suspensions and the Mechanical-Properties of Gelatinized Starch Granules. Journal of the Science of Food and Agriculture, 37(6), 573-590.

290 Ferry AL, Hort J, Mitchell JR, Lagarrigue S & Pamies B (2004) Effect of amylase activity on starch paste
291 viscosity and its implications for flavor perception. Journal of Texture Studies, 35(5), 511-524.

292 Ferry ALS, Mitchell JR, Hort J, Hill SE, Taylor AJ, Lagarrigue S & Valles-Pamies B (2006) In-mouth
293 amylase activity can reduce perception of saltiness in starch-thickened foods. Journal of Agricultural
294 and Food Chemistry, 54(23), 8869-8873.

295 Gonzalez CF, Farina JI & Figueroa LIC (2002) A critical assessment of a viscometric assay for
296 measuring Saccharomycopsis fibuligera alpha-amylase activity on gelatinised cassava starch. Enzyme
297 and Microbial Technology, 30(2), 169-175.

298 Harrison JGL, S.; Stewart, E.D.; Siebenberg, J.; Brenner, M.W.; (1963) Brewery liquor composition -
299 present day views. Journal of the Institute of Brewing, 69, 323-331.

300 Heinzerling CI, Smit G & Dransfield E (2008) Modelling oral conditions and thickness perception of a
301 starch product. International Dairy Journal, 18(8), 867-873.

302 Hsiu J, Fischer H & Stein EA (1964) Alpha-Amylases as Calcium-Metalloenzymes .2. Calcium +
303 Catalytic Activity. Biochemistry, 3(1), 61-66.

304 Kivela J, Parkkila S, Metteri J, Parkkila AK, Toivanen A & Rajaniemi H (1997) Salivary carbonic
305 anhydrase VI concentration and its relation to basic characteristics of saliva in young men. Acta
306 Physiologica Scandinavica, 161(2), 221-225.

307 Kumari A, Rosenkranz T, Kayastha AM & Fitter J (2010) The effect of calcium binding on the
308 unfolding barrier: A kinetic study on homologous alpha-amylases. Biophysical Chemistry, 151(1-2),
309 54-60.

310 Larsen MJ, Jensen AF, Madsen DM & Pearce EIF (1999) Individual variations of pH, buffer capacity,
311 and concentrations of calcium and phosphate in unstimulated whole saliva. Archives of Oral Biology,
312 44(2), 111-117.

313 Lin J, Lin YS, Kuo ST, Jiang CM & Wu MC (2009) Purification of alpha-amylase from human saliva by
314 superparamagnetic particles. Journal of the Science of Food and Agriculture, 89(4), 574-578.

315 Machius M, Declerck N, Huber R & Wiegand G (1998) Activation of Bacillus licheniformis alpha-
316 amylase through a disorder -> order transition of the substrate-binding site mediated by a calcium-
317 sodium-calcium metal triad. Structure, 6(3), 281-292.

318 Mandel AL, des Gachons CP, Plank KL, Alarcon S & Breslin PAS (2010) Individual Differences in AMY1
319 Gene Copy Number, Salivary alpha-Amylase Levels, and the Perception of Oral Starch. Plos One,
320 5(10),

321 Muralikrishna G & Nirmala M (2005) Cereal alpha-amylases - an overview. Carbohydrate Polymers,
322 60(2), 163-173.

323 Nazmi AR, Reinisch T & Hinz HJ (2008) Calorimetric studies on renaturation by CaCl2 addition of
324 metal-free alpha-amylase from Bacillus licheniformis (BLA). Journal of Thermal Analysis and
325 Calorimetry, 91(1), 141-149.

326 Nielsen AD, Fuglsang CC & Westh P (2003) Effect of calcium ions on the irreversible denaturation of a
327 recombinant Bacillus halmapalus alpha-amylase: a calorimetric investigation. Biochemical Journal,
328 373(2), 337-343.

329 O'Mahony M (1986) Sensory Evaluation of Food. Statistical Methods and Procedures. Marcel Dekker,
330 Inc., New York. Basel.

331 Salvolini E, Mazzanti L, Martarelli D, Di Giorgio R, Fratto G & Curatola G (1999) Changes in the
332 composition of human unstimulated whole saliva with age. Aging-Clinical and Experimental Research,
333 11(2), 119-122.

334 Schipper RG, Silletti E & Vingerhoeds MH (2007) Saliva as research material: biochemical,
335 physicochemical and practical aspects. Archives of Oral Biology, 52(12), 1114-1135.

336 Sewon L, Laine M, Karjalainen S, Doroguinskaia A & Lehtonen-Veromaa M (2004) Salivary calcium
337 reflects skeletal bone density of heavy smokers. Archives of Oral Biology, 49(5), 355-358.

338 Suvd D, Fujimoto Z, Takase K, Matsumura M & Mizuno H (2001) Crystal structure of Bacillus
339 stearothermaphilus alpha-amylase: Possible factors determining the thermostability. Journal of
340 Biochemistry, 129(3), 461-468.

341 Tietz M, Buettner A & Conde-Petit B (2008) Changes in structure and aroma release from starch-
342 aroma systems upon alpha-amylase addition. European Food Research and Technology, 227(5),
343 1439-1446.

344 Vallee BL, Stein EA, Sumerwell WN & Fischer EH (1959) Metal Content of Alpha-Amylases of Various
345 Origins. Journal of Biological Chemistry, 234(11), 2901-2905.

346 Woolnough JW, Bird AR, Monro JA & Brennan CS (2010) The Effect of a Brief Salivary alpha-Amylase
347 Exposure During Chewing on Subsequent in Vitro Starch Digestion Curve Profiles. International
348 Journal of Molecular Sciences, 11(8), 2780-2790.

349
350

351

352 ## List of Tables and Figures