

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

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

3

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

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7 **Abstract**

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

21

22

23 **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 dextrans 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 (Cal, Call and Calll) have been identified in certain α -amylases (Machius et al., 1998; Suvd et al.,
47 2001). In particular, Calll, 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

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

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

88 **2. Materials and Methods**

89 **2.1. Materials:**

90 Calcium chloride was purchased from Sigma-Aldrich (223506, purity $\geq 99\%$). Corn starch was
91 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 μ L of diluent was added to 40 μ L of 5% lanthanum chloride solution (Sigma-Aldrich
108 298182, purity 99.9%) and 200 μ L 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 μ L 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 μ L) 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

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

145 The control sample was prepared by mixing 6.8% starch, 83.7 % water and 9.5% sugar and heating
146 up to 95°C for 10 min. Calcium chloride was added to half of the starch paste while still hot. The
147 samples were served at room temperature (18-21°C). The sensory testing took place a maximum of
148 6 h after sample preparation.

149 **2.2.4. Statistical analysis**

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

156

157 **3. Results and discussion**

158 **3.1. Natural variation in salivary α -amylase activity and calcium effect:**

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

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

164 **Figure 1** thereabout

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

168 The salivary calcium concentration found in the saliva of 28 donors ranged from 30 to 87 ppm with
169 an average of 55 ppm and a standard deviation of 12 ppm. This was in good agreement with the
170 values reported elsewhere (Larsen et al., 1999; Salvolini et al., 1999; Sewon et al., 2004). Saliva from
171 the 28 donors was analyzed for α -amylase activity using Protocol 1 and the assumption made that
172 end viscosity was a reflection of amylase activity. When salivary calcium concentration of the donors
173 was plotted against the end viscosity from Protocol 1 (Figure 2), no clear trends were observed. The
174 Pearson product moment correlation was -0.2689 (critical value for $\alpha=0.05$ is -0.4683; (O'Mahony,
175 1986)).which indicated that there was no significant correlation between the salivary α -amylase
176 activity and salivary calcium concentration under the conditions of Protocol 1.

177 **Figure 2** thereabout

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

182 **Figure 3** thereabout

183 The significant correlation could be interpreted in two ways: 1) an indication that a greater salivary
184 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).

207 **Figure 5** thereabout

208 However, Protocol 2 offered a better discrimination between the saliva samples and all the
209 treatments were significantly different from one another except the two control samples (with
210 water instead of saliva) which were not significantly different. The effect of added calcium was to
211 enhance salivary α -amylase activity which resulted in an average 77% reduction in peak starch
212 apparent viscosity as measured in the RVA at the highest concentration of calcium chloride. This
213 protection of α -amylase by addition of calcium was reported in barley and malt where the activity of
214 α -amylase in the presence of calcium was increased at high temperatures (70°C) (Bertoft et al.,
215 1984). The mechanism proposed was that calcium protected the enzyme against thermal
216 degradation and allowed it to maintain a higher activity. Indeed brewers have used calcium for a
217 number of reasons (lowering mash pH to optimise enzymatic action, precipitating unwanted
218 nitrogen, facilitating fining and yeast flocculation and preventing the precipitation of oxalate in the
219 beers) for years (Comrie, 1967). The ability of calcium to protect α -amylase from destruction by heat
220 was reported by brewers as far back as 1963 (Harrison, 1963). This protection against thermal
221 degradation explains the discrepancy observed in Figures 2 and 3 where Protocol 2 yielded a
222 significant correlation between enzyme activity and salivary calcium concentration while Protocol 1
223 did not, even though it exhibited a better discriminatory ability between the α -amylase activity of
224 saliva samples (Figure 1). It is likely that, upon heating, the salivary calcium acted as a stabilizer and
225 improved the salivary α -amylase activity thus making the activity dependent on the calcium
226 concentration in saliva. Human salivary α -amylase activity is temperature dependent (Lin et al.,
227 2009), with its activity falling sharply with incubation temperatures greater than 40°C. This also
228 explains the poorer discrimination ability of Protocol 2 (Figure 1): in the absence of added calcium,
229 the only calcium available to protect the enzyme was the salivary free calcium which was not enough
230 to fully protect the human α -amylase against thermal degradation. In contrast, in Protocol 1, the
231 saliva was added after pasting and was not subjected to high temperatures and thermal degradation,

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

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

241 **3.3. Perception of starch thickened systems**

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

248 **Figure 6** hereabout

249 Out of the four pairs tested, only one pair (model system vs. model system with added CaCl_2 at 100
250 ppm) resulted in a significant difference in perceived thickness at $\alpha=0.05$. The sample with no added
251 calcium chloride was perceived as significantly thicker (21 panelists out of 30) than the sample with
252 added calcium chloride. The other two pairs (outside of the pair of dummy samples introduced first)
253 displayed the same trend whereby the samples with no added calcium chloride were selected as
254 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

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350

351

352 **List of Tables and Figures**

353 Table 1 Sample composition for sensory paired comparison tests

354 Figure 1 Discriminative ability of Protocols 1 and 2 to investigate the effect of salivary α -amylase
355 activity on apparent viscosity as measured by A) Protocol 1 and B) Protocol 2. The letters refer to
356 statistically different populations of donors ($\alpha=0.05$).

357 Figure 2 Correlation between salivary calcium concentration and end apparent viscosity as measured
358 by Protocol 1. The points represent the average of 2 determinations (end viscosity) or three
359 determinations (calcium concentration) and the error bars represent 1 SD.

360 Figure 3 Correlation between salivary calcium concentration and peak viscosity as measured by
361 Protocol 2. The points represent the average of two determinations (peak viscosity) or three
362 determinations (calcium concentration) and the error bars represent 1 SD.

363 Figure 4 Typical post-pasting RVA profiles (Protocol 1) for starch samples with and without saliva and
364 calcium chloride.

365 Figure 5 Typical pasting profiles (Protocol 2) for starch samples containing saliva and added calcium
366 chloride.

367 Figure 6 Paired comparison results and associated levels of significance for four pairs: one pair of
368 dummy samples, two pairs of model systems and model systems with added calcium chloride and
369 one pair of commercial soup and commercial soup with added calcium chloride.