July 19, 2007

Mr. Gregory Drew  
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Dear Mr. Drew:

**Phase2®/StarchLite™ in Chewing Gum**

This has reference to your samples of chewing gum that were processed with- and without Phase2®/StarchLite™.

**Objective of Investigation**

The objective of the analytical efforts is to assess the efficacy of Phase2®/StarchLite™ in the gum towards inhibiting the human salivary α-Amylase.

When Phase2®/StarchLite™ was incorporated into the chewing gum, there appears to be an intimate physical association of it with the gum components. This situation prevented the measurement of inhibitory activity, since Phase2®/StarchLite™ was not present as an aqueous solution. It was, therefore, necessary to first release Phase2®/StarchLite™ from the gum before an assay could be conducted. The following were the practical issues to be resolved:

1. The assay methodology requires that all reagents must be in aqueous solutions.
2. The chewing gum per se is insoluble in water, although there may be some minor constituents that may be water-soluble.
3. Phase2®/StarchLite™ is soluble in water, but only after it is released from the gum.
4. Blending or squeezing in aqueous medium did not release Phase2®/StarchLite™ from the gum.

**Experimental Strategy**

It became evident that a valid assay for Phase2®/StarchLite™ could not be conducted without releasing it from the gum.

The following aspects were, therefore, included in the present investigation.
(1) Apply a suitable method to “break up” the gum.
(2) Extract Phase2®/StarchLite™ into an aqueous buffer.
(3) Assay the aqueous buffer extract for the α-Amylase inhibition.

Materials and Methods

Sample Preparation

The Chewing Gum had an average weight of 1 g/piece. Using a kitchen cheese grater, the pieces were individually grated into particles of ca. 1-3 mm.

Extraction Method

1. Place 1 g of chewing gum particles into a 50-mL capacity plastic vial (centrifuge tube).
2. Add ca. 10 g of clean sand (ca. 1-mm size).
3. Add 10 stainless steel balls (4-mm diameter).
4. Add 5 mL of Potassium Phosphat e Buffer Saline (PBS) (pH 7.4, 10 mM).
5. Shake in an Orbit Shaker for 140 min at 300 rpm.
6. Pipette out 1 mL-aliquots of the supernatant into 4 microcentrifuge tubes.
7. Centrifuge with a microcentrifuge for 20 min at room temperature (at ca. 14,000 g).
8. Use the clear supernatant for assays.

Assay Method

The assay method is based on the principle that the hydrolysis of 2-Chloro-4-nitrophenyl-α-D-maltotrioside, catalyzed by α-Amylase, yields 2-Chloro-4-nitrophenol that is quantitatively measured by its absorbance at 405 nm. Its formation is directly proportional to the α-Amylase activity.

In the present study, assays were carried out with a total volume of 150 μL of reaction mixture per a microplate well, and measuring the Optical Density with a Microplate Reader (Bio-Rad Model 680), as follows:

1. α-Amylase (1,4-α-D-Glucan-glucanohydrolase; E.C. 3.2.1.1); equivalent of 1 μL human saliva.
2. 100 μL of sample extract (α-Amylase inhibitor was equivalent of 2.5 mg of Phase2®/StarchLite™).
3. Incubate for 30 min over a warm plate (~30 °C), covering the microplate loosely with a plastic lid.
4. Add 160 μL of the substrate (2-Chloro-4-nitrophenyl-α-D-maltotrioside) solution.
5. Measure the Optical Density at 405 nm at 5 min.

Measurements were made with 3 replications of each of the following:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme (μL)</th>
<th>Buffer (PBS) (μL)</th>
<th>Gum Extract (μL)</th>
<th>Substrate (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Blank (Background)</td>
<td>None</td>
<td>110</td>
<td>None</td>
<td>160</td>
</tr>
<tr>
<td>2. Untreated Gum</td>
<td>None</td>
<td>10</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td>3. Uninhibited Enzyme</td>
<td>10</td>
<td>100</td>
<td>None</td>
<td>160</td>
</tr>
<tr>
<td>4. Treated Gum</td>
<td>10</td>
<td>None</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td>5. Phase2/StarchLite</td>
<td>10</td>
<td>None</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td>6. Untreated Gum plus Phase2/StarchLite</td>
<td>10</td>
<td>None</td>
<td>100</td>
<td>160</td>
</tr>
</tbody>
</table>
**Results**

The assays were conducted in triplicate for the Untreated (Chewing Gum without Phase2<sup>®</sup>/StarchLite™) and Treated (Chewing Gum with Phase2<sup>®</sup>/StarchLite™), along with the experimental controls.

The Optical density values, which are directly related to the α-Amylase activity, are summarized in the following Table 2 and Figure 1.

<table>
<thead>
<tr>
<th>Replication</th>
<th>Enzyme (Uninhibited)</th>
<th>Untreated Chewing Gum</th>
<th>Treated Chewing Gum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.915</td>
<td>1.809</td>
<td>0.246</td>
</tr>
<tr>
<td>2</td>
<td>1.862</td>
<td>1.915</td>
<td>0.218</td>
</tr>
<tr>
<td>3</td>
<td>1.925</td>
<td>1.951</td>
<td>0.289</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>1.901</strong></td>
<td><strong>1.892</strong></td>
<td><strong>0.251</strong></td>
</tr>
<tr>
<td><strong>Activity Remaining</strong></td>
<td></td>
<td></td>
<td>99.53%  13.20%</td>
</tr>
</tbody>
</table>

**Figure 1.** Inhibition of α-Amylase Activity by Phase2<sup>®</sup>/StarchLite™ in Chewing Gum.
The data obtained with the recovery of Phase2®/StarchLite that was spiked (mixed) with Untreated Gum (see Reactions 5 and 6 in Table 1) are summarized below in Table 3.

Table 3

<table>
<thead>
<tr>
<th>Replication</th>
<th>Phase2®/StarchLite</th>
<th>Chewing Gum Spiked with Phase2®/StarchLite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.156</td>
<td>0.146</td>
</tr>
<tr>
<td>2</td>
<td>0.166</td>
<td>0.129</td>
</tr>
<tr>
<td>3</td>
<td>0.156</td>
<td>0.170</td>
</tr>
<tr>
<td>Mean</td>
<td>0.159</td>
<td>0.148</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td>93.08%</td>
</tr>
</tbody>
</table>

As could be seen from the above data, about 7% of Phase2®/StarchLite was left behind after the extraction.

Conclusions

(1) The \( \alpha \)-Amylase of human saliva was significantly inhibited by the inhibitor in Phase2®/StarchLite™. The mean inhibition of \( \alpha \)-Amylase activity was 86.80%, calculated as follows:

\[
\frac{100-13.20}{13.20} = 86.80\%
\]

(2) The remainder of 12.88% is most likely the amount of Phase2®/StarchLite™ that was still unextractable from the Chewing Gum. At least, about 7% of it could be attributed to the unextractability, as was seen in the spiked experiment. Since the spiking of the Untreated Chewing Gum does not mimic the actual formulation of the product, it is reasonable to expect that an additional 5% of the activity could be still resident in the Treated Gum.

(3) The inhibitor in Phase2®/StarchLite™ was unaffected during the processing/manufacturing of the Chewing Gum.

Respectfully submitted:

Yesu T. Das, Ph.D.