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CONFIDENTIAL

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Mr. Gregory Drew
Pharmachem Laboratories, Inc.
265 Harrison Avenue
Kearny, NJ 07032
201-246-1000 (Fax 8105)

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 issued 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 Phosphate 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-nirophenol 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 ^{o}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 1

Reaction	Enzyme (µL)	Buffer (PBS) (µL)	Gum Extract (µL)	Substrate (µL)
1. Blank (Background)	None	110	None	160
2. Untreated Gum	None	10	100	160
3. Uninhibited Enzyme	10	100	None	160
4. Treated Gum	10	None	100	160
5. Phase2/StarchLite	10	None	100	160
6. Untreated Gum *plus* Phase2/StarchLite	10	None	100	160

Results

The assays were conducted in triplicate for the Untreated (Chewing Gum without Phase2®/StarchLite™) and Treated (Chewing Gum with Phase2®/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 2

Optical Density (405 nm)

Replication	Enzyme (Uninhibited)	Untreated Chewing Gum	Treated Chewing Gum
1	1.915	1.809	0.246
2	1.862	1.915	0.218
3	1.925	1.951	0.289
Mean	*1.901*	*1.892*	*0.251*
Activity Remaining		*99.53%*	*13.20%*

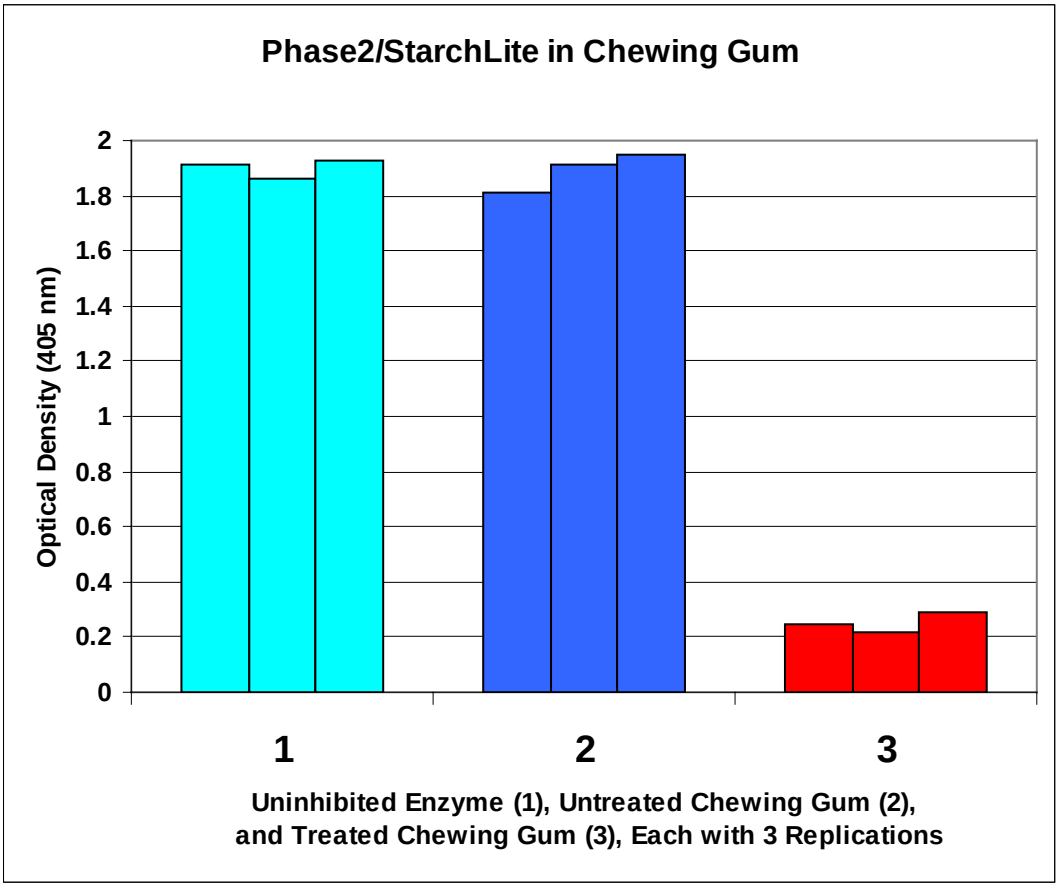

Figure 1. Inhibition of α-Amylase Activity by Phase2®/StarchLite™ in Chewing Gum.

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

Optical Density (405 nm)

Replication	Phase2®/ StarchLite	Chewing Gum Spiked with Phase2®/StarchLite
1	0.156	0.146
2	0.166	0.129
3	0.156	0.170
Mean	*0.159*	*0.148*
Recovery		*93.08%*

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

Conclusions

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

[100-13.20] = 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

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