ISSI LABORATORIES, INC.
515 BLUE RIDGE AVENUE
PISCATAWAY, NEW JERSEY 08854-5013
Telephone: 732-246-3930 (Voice), 732-247-4977 (Facsimile)
Email: <issi@pipeline.com>

CONFIDENTIAL

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

Dear Mr. Skop:

Phase2®/StarchLite™

This is to report to you on the analytical results obtained at our laboratory on Phase2®/StarchLite™.

Introduction

Ingested starch (amylose and amylopectin) is hydrolyzed into simple sugars (such as glucose, maltose and maltotriose) by an enzyme (α-amylase), resulting in increased availability of the monosaccharides. If this process can be minimized, it would limit the availability of sugars in the blood stream. A glycoprotein from white kidney bean was known to have some inhibitory effect on α-amylase (up to 50% inhibition). In human subjects, the glycemic index was considerably reduced upon ingestion of white kidney bean powder. It would, therefore, be a beneficial situation if the white kidney bean powder is incorporated into certain regular food items, especially to the benefit of obese and diabetic individuals.

Analytical Problem

When the white kidney bean powder (Phase2®/StarchLite™) was incorporated into a prepared food, ingestion of it yielded the desired glycemic effect *in vivo*. However, the available laboratory assay techniques were unable to detect the expected inhibitory effect in vitro. This apparent anomaly had to be resolved, in order to unequivocally establish the inhibitory activity of Phase2®/StarchLite™ in prepared foods.

Objective of Investigation

The objective of the proposed investigation is to determine the fate of Phase2®/StarchLite™ in the processed foods and to develop a method for monitoring its inhibitory activity. At the outset, the investigation rested on the hypothesis that (1) Phase2®/StarchLite™ was active in the prepared food, but was inaccessible for measurement *in vitro*; (2) the source of a-Amylase was not that of human; and (3) the assay reaction mixture was inappropriate for the enzyme-inhibitor complex.

Materials and Methods

Equipment and Materials

1. Stove, domestic style (natural gas)
2. Cooking pan, stainless steel (4 quart size)
3. Mixing bowl, stainless steel (4 quart size)
4. Fork, stainless steel, domestic
5. Balance (Scale), capable of 100 g at 0.01 g increments
6. Water (city tap)
7. Butter, unsweetened, unsalted
8. Reagent grade water
9. Mashed Potatoes (Potato Buds®, Betty Crocker® Brand by General Mills)
10. Phase2®/StarchLite™
11. Plastic storage bags
12. Refrigerator (~5 °C)
13. Microcentrifuge (Centronics Model C0240; Spintron Inc., Metuchen, NJ or equivalent unit)
14. Microcentrifuge plastic tubes for above, 1.5 mL capacity
15. Refrigerated Centrifuge (2000 rpm at 10 °C)
16. 50-mL Capacity plastic centrifuge tubes for above
17. Multiwell plate (96-well)
18. Pipettes (assorted)
19. α-Amylase, human salivary (from volunteers at our laboratory)
20. Substrate (2-Chloro-4-nitrophenyl-α-D-maltotrioside; $C_{24}H_{34}ClNO_{18}$, FW 660)
21. Phosphate Buffered Saline, *p*H 7.4, 10 mM.
22. Microplate Reader (Bio-Rad Model 680 or equivalent unit)
23. Hot surface (~30 °C)
24. Multi-channel pipette, adjustable volume

Preparation of Instant Mashed Potatoes with *Phase2®/StarchLite*™ ("MP")

Pharmachem's Stove-Top Method:

Water-- 300 mL (73%)
Butter-- 27.12 g (6.6%)
Potato Buds-- 71.51 g (17.4%)
StarchLite® -- 12.33 g (3.0%)

"Premix Potato Buds and Phase2®/StarchLite™ until Phase2®/StarchLite™ is completely blended into the mixture. Combine water and butter in a pot. Heat until boiling. Remove pot from heat. Stir in Potato Buds/ StarchLite® mixture until moistened. Let stand for approximately 2 minutes or until liquid is absorbed and whip up with a fork."

A *control* preparation without Phase2®/StarchLite™ was prepared in a similar manner.

The preparations was allowed to cool at room temperature for about an hour, then transferred into plastic bags and kept overnight at ca. 5 °C in a refrigerator.

Extraction of α-Amylase Inhibitor of Phase2®/StarchLite™ from Instant Mashed Potatoes (MP)

Water-soluble constituents in MP were extracted with Phosphate Buffered Saline (PBS) by sonication, shaking and centrifugation, as follows:

1. Weigh out 10 g of Instant Mashed Potatoes *with* Phase2®/StarchLite™ ("SMP") into a 50-mL plastic centrifuge tube.
2. Weigh out 10 g of Instant Mashed Potatoes *without* Phase2®/StarchLite™ ("CMP") into a 50-mL plastic centrifuge tube.

3. Weigh out 10 g of Instant Mashed Potatoes *without* Phase2®/StarchLite™ ("CMP") into a 50-mL plastic centrifuge tube, and add 306 mg of StarchLite.
4. Weigh out 306 mg of StarchLite into a 50-mL plastic centrifuge tube.
5. To all 4 tubes above, add 10 mL of PBS.
6. Mix with a Votex® mixer for 30 sec.
7. Sonicate in an ultrasonic water bath for 5 min.
8. Centrifuge for 1 hr at 2,000 rpm and 10 °C.
9. Pipette out aliquots of the supernatant from each tube into 4 microcentrifuge tubes.
10. Centrifuge with the microcentrifuge for 10 min at room temperature.
11. 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 415 nm. Its formation is directly proportional to the α-Amylase activity. Hitherto, the measurements were mostly made with spectrophotometers that usually require milliliter volumes of reaction mixture. However, with the advent of multi-well plate reader technology, it is now more convenient, efficient, and economical to conduct these tests, up to 96 reactions at a time.

In the present study, assays were carried out with a total volume of 150 µL of reaction mixture per well, as follows:

1. α-Amylase (1,4-α-D-Glucan-glucanohydrolase; E.C. 3.2.1.1); equivalent of 10 µL human saliva
2. 100 µL of sample extract (100 µL PBS for control) (α-Amylase inhibitor was equivalent of 3 mg of Phase2®/StarchLite™)
3. Incubate for the assigned time over a warm plate (~30 °C), covering the multiwell plate loosely with a plastic lid
4. Add 40 µL of the substrate (2-Chloro-4-nitrophenyl-α-D-maltotrioside) solution
5. Measure the Optical Density at 415 nm. If time course values are planned, continue measurements at the selected time intervals.

Results and Discussion

The following 5 reaction conditions were assayed in 2 separate assays, and in duplicate within each assay:

1. Control-- No Phase2®/StarchLite™ and Mashed Potatoes. This is a reaction that would provide the extent of enzymatic hydrolysis of the substrate, free of any influence by the ingredients in the food preparation.
2. SL-- Phase2®/StarchLite™ Only (No Mashed Potatoes). This is a reaction that would provide the extent of enzymatic hydrolysis of the substrate after the enzyme was exposed to Phase2®/StarchLite™, but free of any influence by the ingredients in the food preparation.
3. SL+UT-- Phase2®/StarchLite™ added to Mashed Potatoes prepared without Phase2®/StarchLite™. This is a reaction that would provide the extent of enzymatic hydrolysis of the substrate after the enzyme was exposed to ("*uncooked"*) Phase2®/StarchLite™, but not free of any influence by the other ingredients in the food preparation. (This is a post-preparation addition of Phase2®/StarchLite™.)
4. UT-- Mashed Potatoes Prepared Without Phase2®/StarchLite™. This is a reaction that would provide the extent of enzymatic hydrolysis of the substrate in the total absence of Phase2®/StarchLite™, but not free of any influence by the ingredients in the food preparation.

5. T-- Mashed Potatoes Prepared With Phase2®/StarchLite™. This is a reaction that would provide the extent of enzymatic hydrolysis of the substrate after the enzyme was exposed to Phase2®/StarchLite™, but not free of any influence by the other ingredients in the food preparation.

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

	Optical Density (415 nm)		
Sample	*Assay 1*	*Assay 2*	*Mean*
Control	2.033	2.244	2.139
SL	0.088	0.096	0.092
SL+UT	0.161	0.149	0.155
UT	2.094	2.694	2.394
T	0.152	0.160	0.156

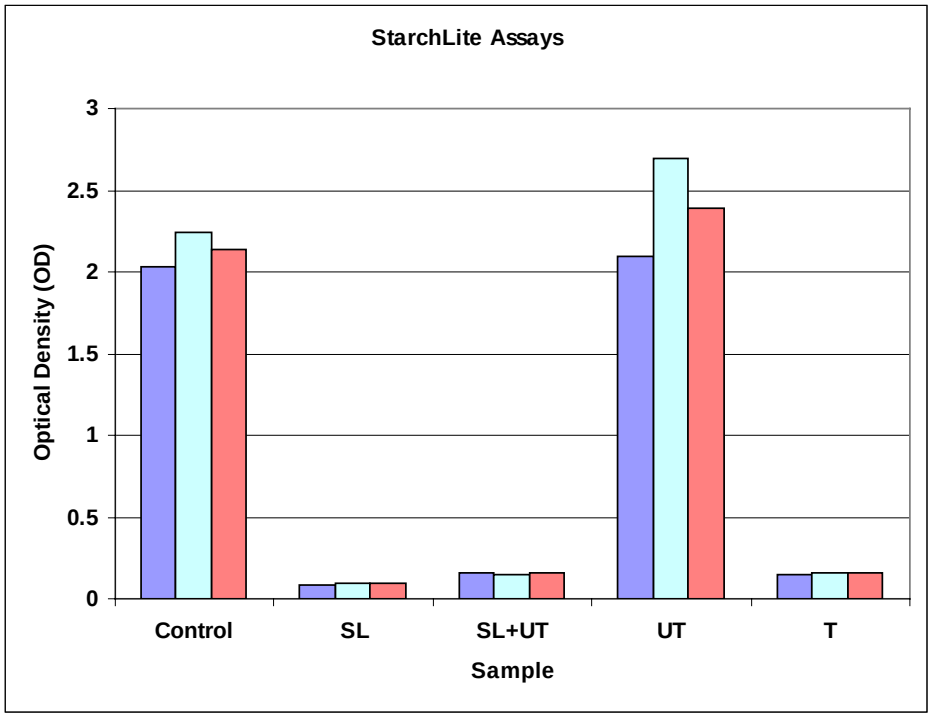

Figure 1. Inhibition of α-Amylase Activity by Phase2®/StarchLite™ in Instant Mashed Potatoes.

Control = No Phase2®/StarchLite™ - No Mashed Potatoes
SL = Phase2®/StarchLite™ Only-No Mashed Potatoes
SL+UT = Phase2®/StarchLite™ Added to Mashed Potatoes Prepared Without Phase2®/StarchLite™
UT = Mashed Potatoes Prepared Without Phase2®/StarchLite™
T = Mashed Potatoes Prepared With Phase2®/StarchLite™

Vertical bars represent Assay 1, Assay 2, and their Mean values, respectively.

Inhibition of α-Amylase by Phase2®/StarchLite™ in Instant Mashed Potatoes (MP)

By a direct comparison of the α-Amylase activity between the Instant Mashed Potatoes prepared with and without Phase2®/StarchLite™ (samples T and UT, respectively), the extent of inhibition was calculated as:

100–([0.156/2.394] x 100)
= 100–(0.06516 x 100)
= 100–6.516
= 93.5%

Interference of Other Ingredients in Instant Mashed Potatoes (MP)

There was a slight increase in the α-Amylase activity when the reaction mixture contained MP (see SL 0.92 *versus* SL+UT 0.155 or T 0.156). It appears that the potato starch was responsible for this situation. Since potato starch is a substrate by itself, it is not surprising that it could add to the overall reaction velocity, as could be expected under the *first order kinetics*. Nonetheless, the observed effect was negligible and, with the employment of appropriate control, had no negative impact on the assay outcome.

Effect of Incubation Time on Inhibition

An independent assay was carried out to determine the optimum time of exposure (incubation) to achieve a desired level of inhibition. Incubation times of 0, 30, 60 and 120 min were studied. Following the incubation, the substrate was added and activity read (OD_{415}). Following the initial reading, the readings were continued through 2 min, 5 min and 10 min.

The results of these observations are summarized below in Figure 2:

Incubation	*Optical Density Reading Time (min)*			
Time (min)	*Initial*	*2 min*	*5 min*	*10 min*
0	0.890	1.435	1.844	2.011
30	0.184	0.195	0.217	0.247
60	0.173	0.175	0.184	0.199
120	0.164	0.164	0.170	0.184

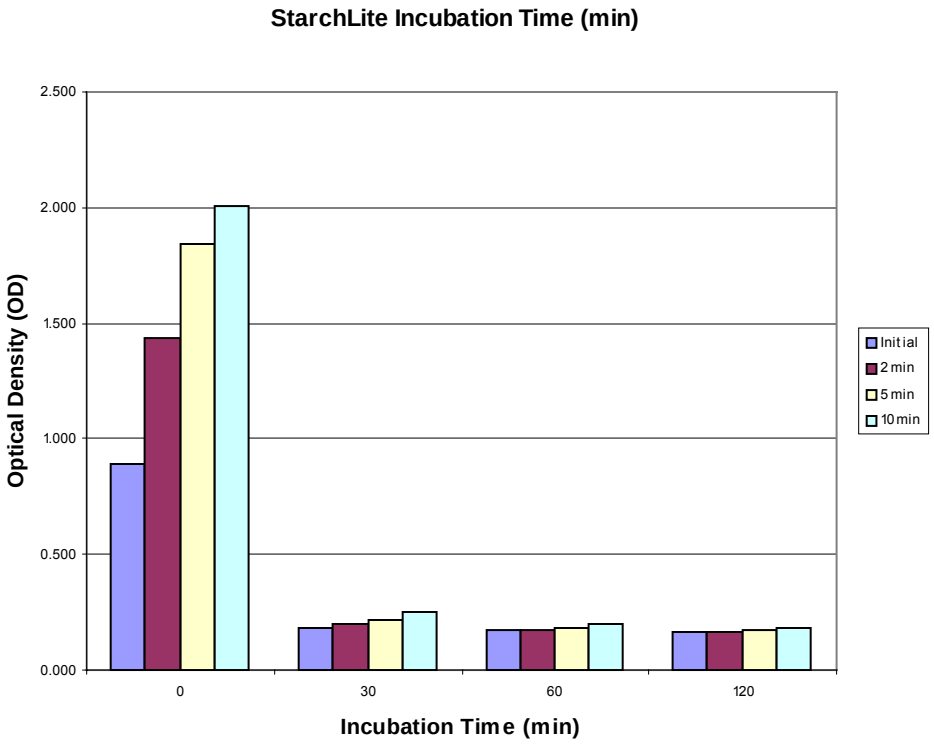

Figure 2. Effect of Time of Incubation on α-Amylase Activity by Phase2®/StarchLite™ in Instant Mashed Potatoes.

Vertical bars at each Incubation Time represent 4 readings taken initially, and after 2 min, 5 min and 10 min, respectively.

As could be seen above, most of the inhibition (nearly 88%) occurred by 30-min incubation. Additional incubations (up to 2 hours) did not contribute significantly. It appears, therefore, that a 30-min incubation would be adequate, although an hour of incubation was employed in present study.

With regards to the time-course reaction of the substrate hydrolysis, the readings taken at the 4 time intervals showed a clear linear rate, from 0.890 to 2.011 over the 10-min period in the *uninhibited* reaction (see 0-min incubation data above). For comparative purposes, it appears adequate to make readings at 5 min following the addition of substrate. In the present study, readings were taken at 4 min for routine evaluation of the activity levels.

It is also interesting to note that the reading time intervals did not matter in case of inhibited reactions (see 30-min, 60-min and 120-min incubation data above). This situation suggests that the substrate *per se* had no influence on the integrity of the enzyme-inhibitor complex.

Conclusions

1. The α-Amylase of human saliva was significantly inhibited by the inhibitor in Phase2®/StarchLite™.
2. The inhibitor in Phase2®/StarchLite™ was unaffected during the preparation of Instant Mashed Potatoes.
3. Exposure of α-Amylase to Phase2®/StarchLite™ for 30 min resulted in significant inhibition.
4. The assay method has been adapted for a multiwell plate reader.

Respectfully Submitted

Yesu T. Das

Yesu T. Das, Ph.D.
ISSI Laboratories, Inc.