Advances Aesthetic Technologies, Inc. –Final Report
February 2017

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Objective: To assess if Hylenex® recombinant (hyaluronidase human injection) degrades/reacts with Algeness® DF, an agarose-based dermal filler

Methods: First, pure agarose (0.2% and 1.5%) was exposed to Hylenex® recombinant (hyaluronidase human injection) and β-agarase. Liquid chromatography/mass spectrometry was used to detect the presence of oligosaccharides, which are known breakdown products of agarose. Finally, the effects of hyaluronidase and β-agarase on Algeness® DF were compared.

Results: Hylenex® recombinant (hyaluronidase human injection) is the so-called gold standard for treating over-injection of hyaluronic acid dermal fillers. Hyaluronidase cleaves the β-1,4 linkage of hyaluronic acid. While the structure of hyaluronic acid and agarose are very different, it appears hyaluronidase slowly hydrolyzes agarose at the β-1,4 bond. As expected, β-agarase cleaved agarose at the β-1,4 glyosidic linkages producing the dimer, tetramer and hexamer neoagarooligosaccharides for 0.2% agarose, and the dimer and tetramer for 1.5% agarose. The presence of the hexamer was also observed in both the 0.2% and 1.5% agarose samples exposed to Hylenex®. Similar results were observed in the enzymatic degradation of Algeness® DF when exposed to β-agarase and Hylenex®. The dimer and tetramer products were observed in the β-agarase/ Algeness® DF reaction, while the hexamer was observed in the Hylenex®/ Algeness® DF reaction.

Conclusions: The enzymatic hydrolysis of pure agarose by β-agarase produced three compounds, neoagarobiose, neoagarotetraose and neoagarohexaose, which result from the cleavage of β-1,4 glycosidic bonds. The enzymatic hydrolysis of agarose by Hylenex® produced one product, neoagarohexaose in low amounts. Confirmation of these findings require additional experimentation however results are promising.

Introduction

Agarose, a linear polymer composed of repeating units of D-galactose and 3,6-anhydro-L-galactopyranose, linked by α-1,3 and β-1,4 glycosidic bonds (Figure 1A). β-agarase hydrolyzes agarose at the β-1,4 bond. The resulting reaction produces a series of neoagarooligosaccharides with repeating disaccharide units (Figure 1B).[1] Depending on the cleavage site, neoagarooligosaccharides of varying molecular mass are produced, including neoagarobiose (324.28 g/mol), neoagarotetraose (630.55 g/mol) and neoagarohexaose (936.82 g/mol).

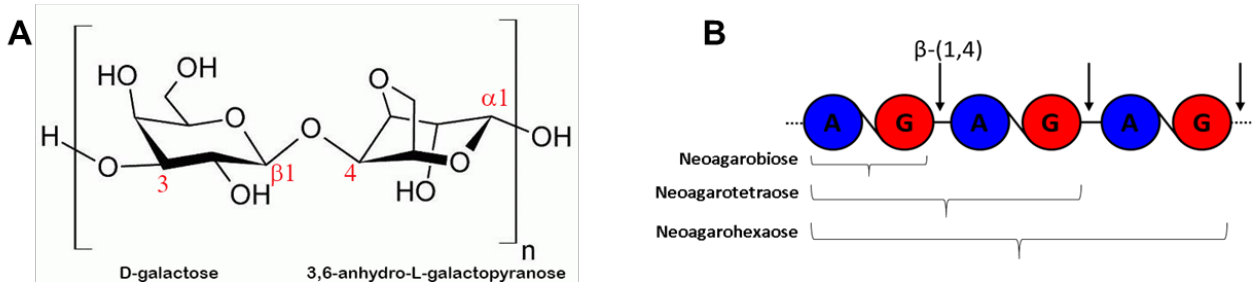

Figure 1. A) Agarose is a polysaccharide that consists of repeating units of D-galactose and 3,6-anhydro-L-galactopyranose. B) β-agarase cleaves agarose at the β-1,4 bonds between D-galactose (G) and 3,6-anhydro-L-galactopyranose (A) producing a series of neoagarooligosaccharides consisting of repeating disaccharide units.

Hylenex® recombinant (hyaluronidase human injection) is an FDA approved enzyme, and current standard for the off-label treatment to overcorrect hyaluronic acid (HA) based dermal fillers.[2-4] Hyaluronidase hydrolyzes HA by cleaving the β-1,4 bond between the glucosamine and glucuronic acid (Figure 2).

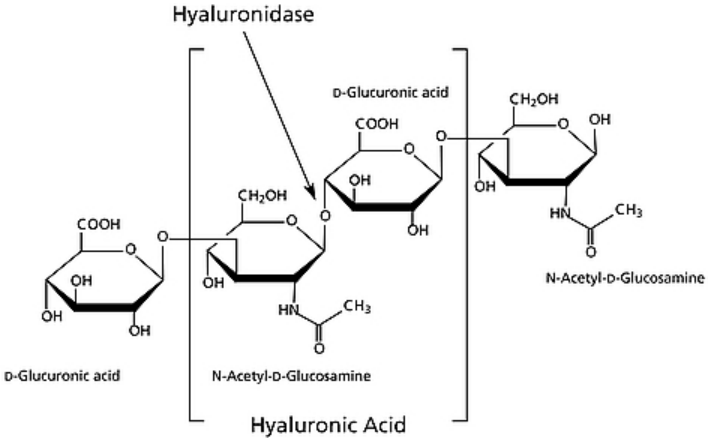

Figure 2. Hyaluronic acid (HA) is composed of repeating units of D-Glucuronic acid and N-Acetyl-D-Glucosamine, linked by β -1,3 and β-1,4 bonds. Hyaluronidase cleaves HA at the β-1,4 linkage, producing a series of repeating disaccharide units. Image from: www.sigmaaldrich.com

The objective of this study was to determine if Hylenex® recombinant hydrolyzes the agarose-based dermal filler, Algeness® DF, at the β-1,4 linkage between D-galactose and 3,6-anhydro-L-

galactopyranose. Neoagarotetraose was used as a reference standard to confirm enzymatic activity in this study.

Experimental

Control Samples – 0.2% Agarose Gel

Agarose powder (0.2g) was added to 100ml of water (99°C) and cooled in a water bath to 42°C. Three control samples were prepared using 1000µl of molten agarose (2mg). Samples were incubated at 42°C for 2 hr, and then either 1) centrifuged at 3000g for 5 min at 4°C, 2) centrifuged at 12000g for 5 min at 4°C or 3) heated at 90°C for 10 min (so-called "kill enzyme" step) and centrifuged at 12000g for 5 min at 4°C. The supernatant was filtered using 0.2 µm Nylon membrane filters. All samples were analyzed by LC/MS to determine if sample preparation methods contributed to the degradation of agarose gel. Additionally, the Molisch test for sugars was conducted on all samples.

Enzyme Degradation – 0.2% Agarose Gel

Agarose gel (0.2%) was exposed to Hylenex® recombinant (hyaluronidase human injection) and β-agarase to compare the effects of enzymatic degradation on the two samples. Briefly, Hylenex® (200µl – 30 units) was added to agarose (200µl – 0.4mg agarose) and incubated at 37°C for 2 hr. Additionally, β-agarase (5µl – 5 units) was added to agarose (1000µl – 2mg agarose) and incubated at 37°C for 2 hr. Samples were centrifuged at 12000g for 5 min at 4°C. The supernatant was filtered using 0.2 µm Nylon membrane filters and analyzed by LC/MS. The Molisch test for sugars was conducted on all samples.

Enzyme Degradation – 1.5% Agarose Gel

Agarose gel (1.5%) was exposed to Hylenex® recombinant (hyaluronidase human injection) and β-agarase to compare the effects of enzymatic degradation. Briefly, Hylenex® (200µl – 30 units) was added to agarose (400µl – 14mg agarose) and incubated at 37°C for either 2 or 48 hr. Additionally, β-agarase (30µl – 30 units) was added to agarose (400µl – 14mg agarose) and incubated at 37°C for either 2 or 48 hr. Samples were centrifuged at 10000g for 5 min at 4°C. Supernatant was filtered using 0.2 µm Nylon membrane filters and analyzed by LC/MS.

Enzyme Degradation - Algeness® DF

Algeness® DF (3.5% agarose) was exposed to Hylenex® recombinant (hyaluronidase human injection) and β-agarase to compare the effects of enzymatic degradation. Briefly, enzyme was added to the dermal filler and incubated at 37°C for 48 hr. Table 1 shows the amount of dermal filler and enzyme used for each reaction. After incubation period, samples were heated at 90°C for 10 min ("kill enzyme") and then centrifuged at 10000g for 5 min at 4°C. Supernatant was filtered using 0.2 µm Nylon membrane filters and analyzed by LC/MS.

Sample	Vol. Algeness®	Vol. Hylenex®	Vol. β-agarase
AA	400µl – 14 mg Agarose	-	30µl – 30 units
AH	400µl – 14 mg Agarose	200µl – 30 units	-

Liquid Chromatography/Mass Spectrometry

Separations were performed on an Agilent 1260 LC/MS equipped with a cooled autosampler tray (4°C) and temperature-controlled column compartment (20°C), which held a 2.1 x 100mm i.d., 3.5µm particle size C_{18} Zorbax Eclipse Plus column (Agilent, Santa Clara, CA). Sample injection volumes were 5 uL, except for the Algeness/Hylenex reaction which was 25uL. A gradient elution was employed with a mobile phase composition of 0.5mM ammonium acetate in 18Ω Millipore water (A) and acetonitrile (B), and flow rate of 0.4 mL/min. The gradient profile is shown in Table 2.

Time (min)	% A	% B
0.5	100.0	0.0
5	2.0	98.0
6	2.0	98.0
7	100.0	0.0
8	100.0	0.0

The MS was operated in positive ionization mode for agarose and negative ionization mode for hyaluronic acid, with a fragmentation voltage of 100V. Spectra were recorded in full scan mode from 300 to 2000 *m/z*. Neoagarotetraose, a known degradation product of agarose when exposed to β-agarase, was used to confirm enzyme activity.

Results

Agarose Gel

The analysis of pure agarose and β-agarase and Hylenex® were carried out to determine whether the enzymes hydrolyzed the starting material. After incubation, samples were analyzed by LC/MS to determine the presence of neoagarooligosaccharides, thus confirming if the enzyme produced the expected products. Neoagarotetraose was used as a reference standard. As expected, the analysis of the 0.2% agarose/β-agarase sample produced three breakdown products, neoagarobiose, neoagarotetraose and neoagarohexaose (Figure 3). In contrast, LC/MS analysis of the 0.2% agarose/Hylenex® sample revealed the hexasaccharide only (Figure 4). For the 1.5% agarose, neoagarobiose and neoagarotetraose were observed in both the 2 hr and 48 hr reactions with β-agarase. On the other hand, no products were observed in the 2 hr reaction with Hylenex®, while neoagarohexaose was the only product observed in the 48 hr reaction.

Although results are not quantitative, neoagarotetraose produced the highest signal in the agarose/β-agarase sample. The neoagarohexaose signal from the reaction of agarose/Hylenex® was buried in the sample matrix, but observable using spectral deconvolution data analysis software, at near instrument detection limits. Based on these results, Hylenex® hydrolyzes agarose, however, further studies should be conducted to determine reaction rate and amount. Note: all observations are based on one analysis. Additional experiments should be performed to confirm findings.

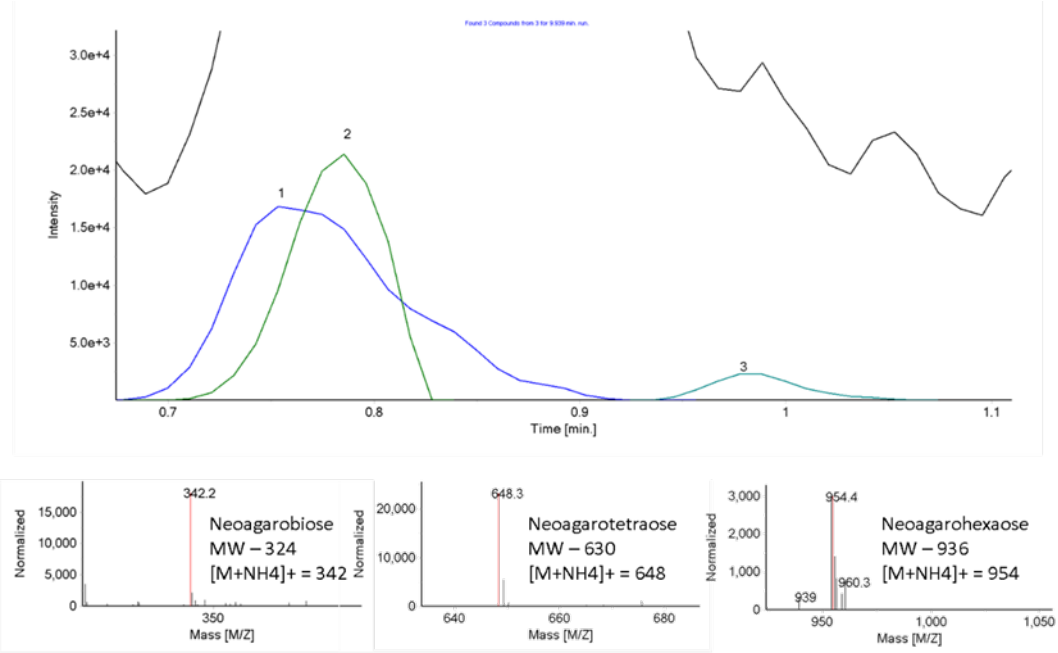

Figure 3. Ion Analytics Deconvolution Software (Robbat) confirmed the presence of neoagarooligosaccharides in the 0.2% agarose/β-agarase sample.

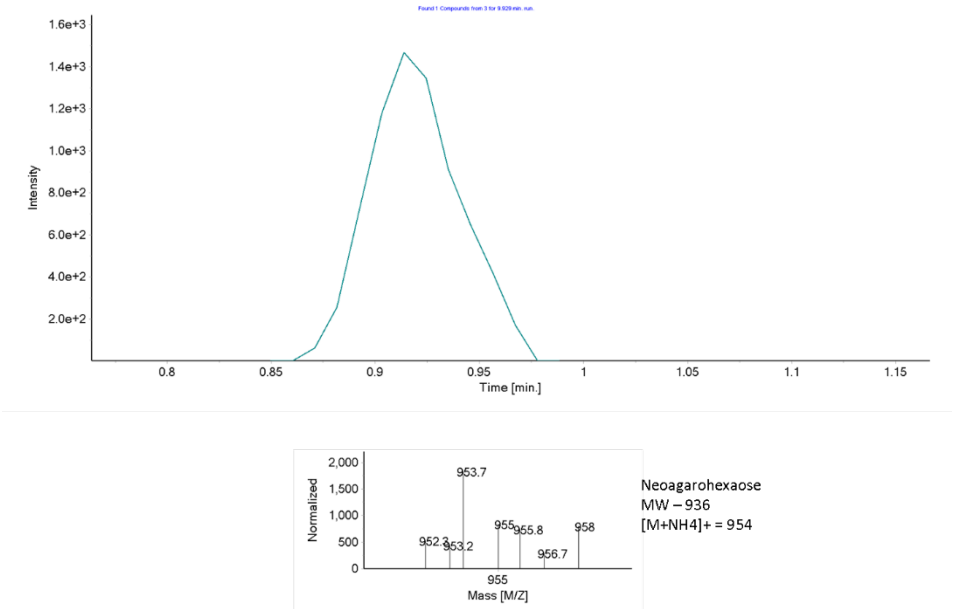

Figure 4 Neoagarohexaose was the only compound identified in the 0.2% agarose/ Hylenex® sample by LC/MS.

Algeness® DF

The Molisch test is a qualitative analysis indicating the presence of carbohydrates in a sample. A positive result is indicated by a purple ring between the sample and the acid. The absence of sugars results in a clear solution. The presence of monosaccharides yields a faster reaction, while disaccharides and polysaccharides are slower to react. Immediately upon adding the Molisch reagent to the test tube, the Algeness®/β-agarase sample turned dark purple (Figure 5). The

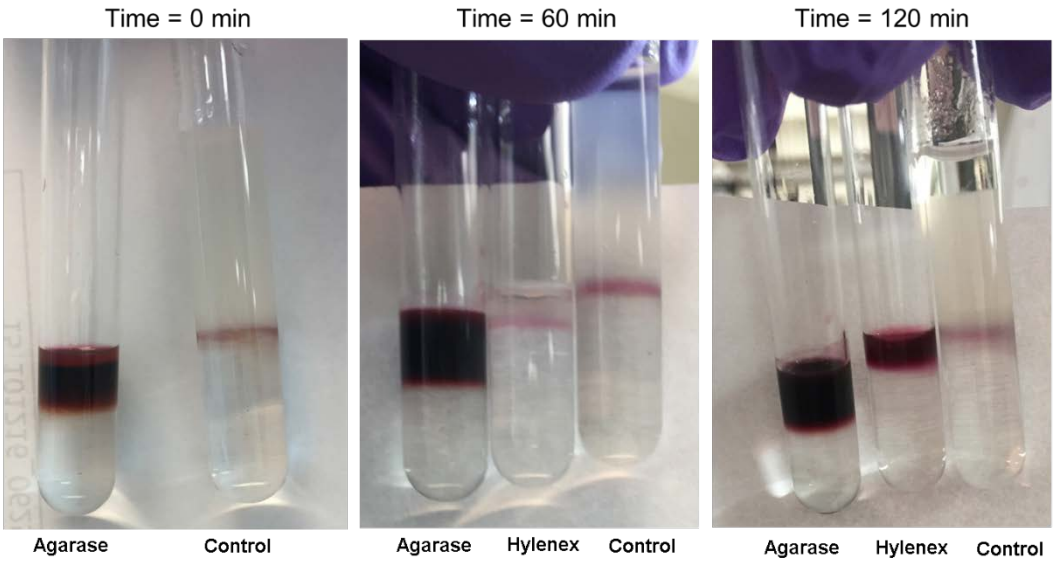

Figure 3. Molisch test results for Algeness®/ β-agarase, Algeness®/ Hylenex® and Algeness®/water (control). At time = 0 min (left), the Algeness®/ β-agarase reacted immediately, while the control formed a small purple ring. After 60 min (middle), the Algeness®/ Hylenex® began to form a purple ring. At 120 min (right), the Algeness®/ Hylenex® had a strong positive signal.

control (Algeness®/water) formed a light purple ring at the interface of the sample and acid, presumably due to concentrated acid hydrolysis. After 1 hr, the Algeness®/β-agarase and the control remained the same, while the Algeness®/Hylenex® formed a light purple ring. The agarose/Hylenex® turned a darker shade of purple after 2 hr. Based on these observations, the Algeness®/β-agarase produces smaller chain sugars compared to Algeness®/Hylenex®.

For the LC/MS analyses, reactions were incubated for 48 hr at 37°C, then heated to kill the enzyme prior to analysis. Neoagarohexaose was the only product observed in the Algeness®/Hylenex® reaction. Similar to the pure agarose experiments, detection of the compound occurred at the detection limit of the instrument and hidden by other components in the sample (Figure 6). Overall, these results in combination with those observed for the pure agarose suggest that Hylenex® hydrolyzes agarose. However, further studies should be conducted to determine reaction rate and amount. Note: all observations are based on one analysis and should be repeated to confirm findings.

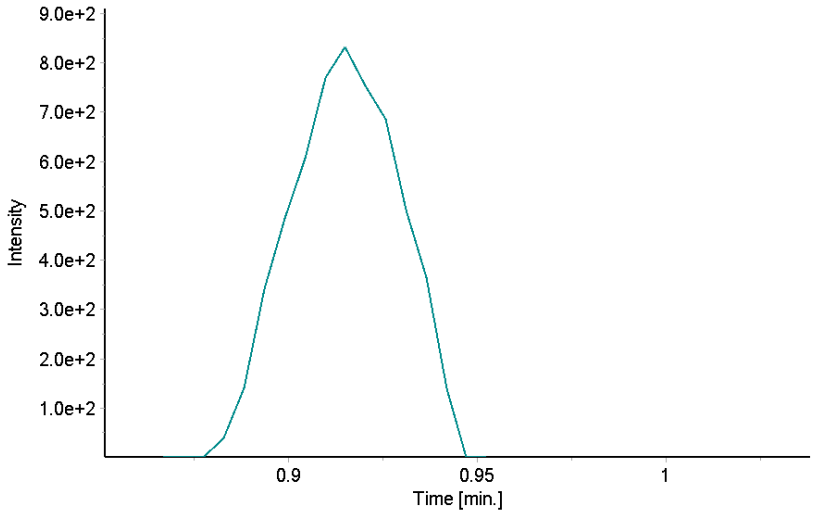

Figure 4. Neoagarohexaose was the only compound identified in the Algeness DF ®/Hylenex® sample by LC/MS. The ion signal was buried in the matrix noise

References

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