Viscosity Enzyme Stability Assay

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Tradename: ACB Modified Pomegranate Enzyme PF

Code: 20440PF

CAS #: 84961-57-9 & 1686112-10-6 (or) 84775-94-0 (or) 9015-54-7

Test Request Form #: 13565

Lot #: N251001A & N251104E

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:
Viscosity Enzyme Stability Assay

Introduction
Enzymes are biological catalysts for accelerating specific chemical reactions. While often used in solutions, solubilized enzymes are prone to denaturation leading to reduced function over time. Understanding enzymatic activity is important as it can reveal if a product is working and how well it is working. Viscosity analysis of gelatin solutions provides a fast and simple way to observe enzyme activity as changes in protein structure or molecular weight impact viscosity. Therefore, changes in the viscosity of a gelatin solution after the addition of an enzyme product is indicative of enzymatic activity.

Accordingly, a Viscosity Enzyme Stability Assay was conducted to assess the enzymatic activity of **AC Modified Pomegranate Enzyme PF** over time by measuring changes in viscosity at time of manufacture and after stability periods. To demonstrate the superior nature of the product, Bromelain and Papain were also tested. Reduced viscosity is indicative of enzymatic activity.

Assay Principle
The viscosity of an animal protein gelatin solution was evaluated using a rotation viscometer at various speeds. When exposed to an enzymatic solution, viscosity was monitored for changes indicative of enzymatic activity. As reactions occur, viscosity of the gelatin solution will decrease due to changes in protein structure of molecular weight, allowing quantification of enzymatic activity. To understand changes in enzymatic activity over time, samples of different ages were tested. Samples from time of manufacture (Lot #: N251104E) and one month stability (Lot #: N251001A) were tested as well as one month accelerated stability samples using heat treatment (Lot #: N251001A).

Materials
A. Equipment:	Brookfield DVII+ Pro Serial Viscometer RV (RT80605461 RVDV-11T); RV-2 Spindle; Beakers; Stir Plate; Stir Bar; Temperature Probe
B. Reagents:	Bovine Gelatin [Low Bloom (50-125 g) = 20,000-25,000 Da]; DI Water
C. Software:	Excel Analysis ToolPak (Microsoft)

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Methods

A 45% solution of Bovine Gelatin was produced by diluting gelatin powder in DI Water. Next, 175 mL of the Bovine Gelatin Solution was aliquoted into beakers and allowed to sit overnight at room temperature to remove any air bubbles. The enzyme solutions tested are described in Table 1.

Table 1. Enzyme Solutions

1.0% Bromelain in Bovine Gelatin Solution	Time of Manufacture
	One Month Stability
	One Month Accelerated Stability
1.0% Papain in Bovine Gelatin Solution	Time of Manufacture
	One Month Stability
	One Month Accelerated Stability
10.0% **AC Modified Pomegranate Enzyme PF** in Bovine Gelatin Solution	Time of Manufacture
	One Month Stability
	One Month Accelerated Stability

The viscometer was calibrated according to manufacturer instructions and equipped with the RV-2 spindle. Each test sample was tested by lowering the spindle into the beaker until the sample reached the level line on the spindle. Viscosity was measured for 60 seconds at 10, 20, 30, 50, 60, 100 rpm and the final baseline value was recorded in cPs.

Immediately following initial measurements, enzyme solutions (1.0% Bromelain, 1.0% Papain, or 10.0% **AC Modified Pomegranate Enzyme PF** (all (w/w))) were added to each beaker and mixed until uniform, taking care to avoid addition of air bubbles to the solution (Table 1). Viscosity was measured again at each respective speed for 60 seconds. Final viscosity values were recorded.

Three separate experiments were performed for each condition and test speed. Data is displayed as averages of all three experiments and was analyzed using a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. Percent change is expressed relative to the Bovine Gelatin Solution and calculated by the following equation:

$$Percent\ Change\ (\%) = \frac{Viscosity_{Test\ Article} - Viscosity_{Bovine\ Gelatin}}{Viscosity_{Bovine\ Gelatin}} \times 100$$

Results

The data obtained met criteria for a valid assay. After addition of fresh Bromelain, Papain, and **AC Modified Pomegranate Enzyme PF**, viscosity of the Bovine Gelatin solution decreased at all viscometer speeds tested. After one month stability and one month accelerated stability, both Bromelain and Papain demonstrated higher viscosities, while **AC Modified Pomegranate Enzyme PF** continued to exhibit decreased viscosity across all viscometer speeds compared to the Bovine Gelatin solution.

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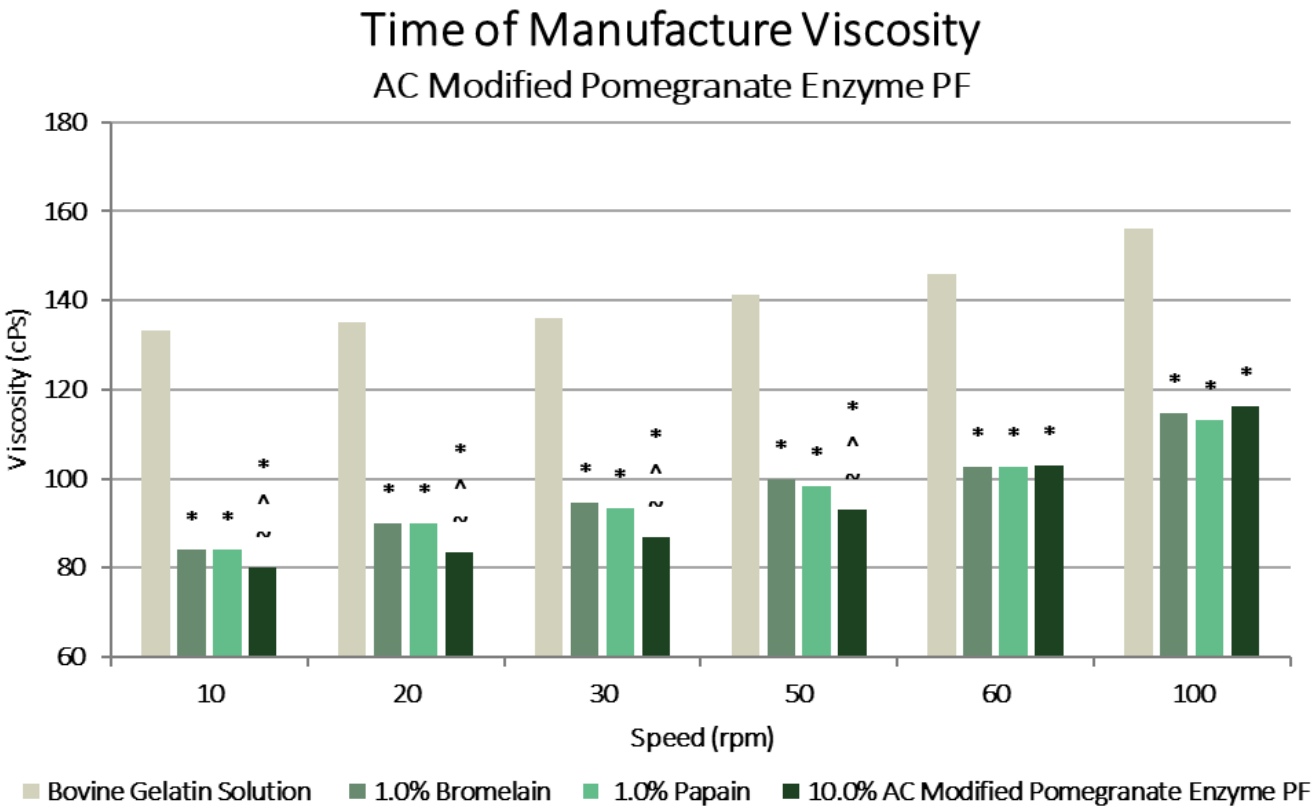

Figure 1. Time of Manufacture Viscosity of Bovine Gelatin Solutions at various speeds. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed. ^ indicates significance ($p \leq 0.05$) compared to 1.0% Bromelain for each test speed. ~ indicates significance ($p \leq 0.05$) compared to 1.0% Papain for each test speed.

Table 2. Percent Change of Time of Manufacture Viscosity of Bovine Gelatin Solution at various speeds. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed.

Viscometer Speed (rpm)	1.0% Bromelain	1.0% Papain	10.0% AC Modified Pomegranate Enzyme PF
10	37%	37%	40%
20	33%	33%	38%
30	30%	31%	36%
50	29%	30%	34%
60	30%	30%	29%
100	26%	27%	26%

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Table 3. Results from one-way ANOVA Statistical Analysis between conditions compared at Time of Manufacture at each speed. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed. ^ indicates significance ($p \leq 0.05$) compared to 1.0% Bromelain for each test speed. ~ indicates significance ($p \leq 0.05$) compared to 1.0% Papain for each test speed.

Viscometer Speed (rpm)	Bovine Gelatin vs 1.0% Bromelain	Bovine Gelatin vs 1.0% Papain	Bovine Gelatin vs 10.0% AC Modified Pomegranate Enzyme PF	1.0% Bromelain vs 10.0% AC Modified Pomegranate Enzyme PF	1.0% Papain vs 10.0% AC Modified Pomegranate Enzyme PF
10	< 0.001*	< 0.001*	< 0.001*	0.036^	0.023~
20	< 0.001*	< 0.001*	< 0.001*	0.031^	0.021~
30	< 0.001*	< 0.001*	< 0.001*	0.030^	0.027~
50	< 0.001*	< 0.001*	< 0.001*	0.037^	0.024~
60	< 0.001*	< 0.001*	< 0.001*	> 0.05	> 0.05
100	< 0.001*	< 0.001*	< 0.001*	> 0.05	> 0.05

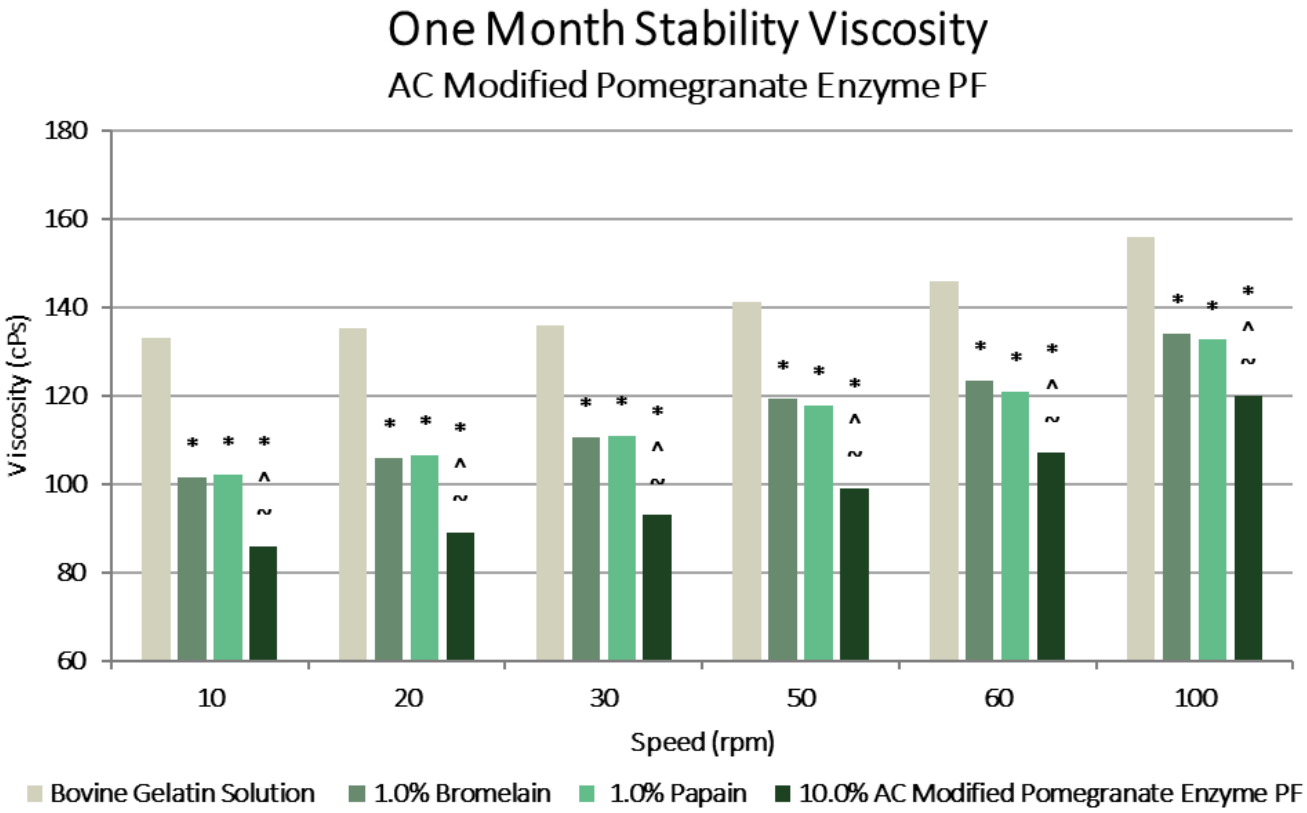

Figure 2. One Month Stability Viscosity of Bovine Gelatin Solutions at various speeds. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed. ^ indicates significance ($p \leq 0.05$) compared to 1.0% Bromelain for each test speed. ~ indicates significance ($p \leq 0.05$) compared to 1.0% Papain for each test speed.

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Table 4. Percent Change of One Month Stability Viscosity of Bovine Gelatin Solution at various speeds. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed.

Viscometer Speed (rpm)	1.0% Bromelain	1.0% Papain	10.0% **AC Modified Pomegranate Enzyme PF**
10	24%	23%	35%
20	22%	21%	34%
30	19%	18%	32%
50	16%	17%	30%
60	16%	17%	27%
100	14%	15%	23%

Table 5. Results from one-way ANOVA Statistical Analysis between conditions compared after One Month Stability at each speed. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed. ^ indicates significance ($p \leq 0.05$) compared to 1.0% Bromelain for each test speed. ~ indicates significance ($p \leq 0.05$) compared to 1.0% Papain for each test speed.

Viscometer Speed (rpm)	Bovine Gelatin vs 1.0% Bromelain	Bovine Gelatin vs 1.0% Papain	Bovine Gelatin vs 10.0% **AC Modified Pomegranate Enzyme PF**	1.0% Bromelain vs 10.0% **AC Modified Pomegranate Enzyme PF**	1.0% Papain vs 10.0% **AC Modified Pomegranate Enzyme PF**
10	< 0.001*	< 0.001*	< 0.001*	0.009^	0.002~
20	< 0.001*	< 0.001*	< 0.001*	0.007^	0.007~
30	< 0.001*	< 0.001*	< 0.001*	0.011^	0.005~
50	< 0.001*	< 0.001*	< 0.001*	< 0.001^	0.003~
60	< 0.001*	< 0.001*	< 0.001*	0.002^	0.001~
100	< 0.001*	< 0.001*	< 0.001*	0.005^	0.001~

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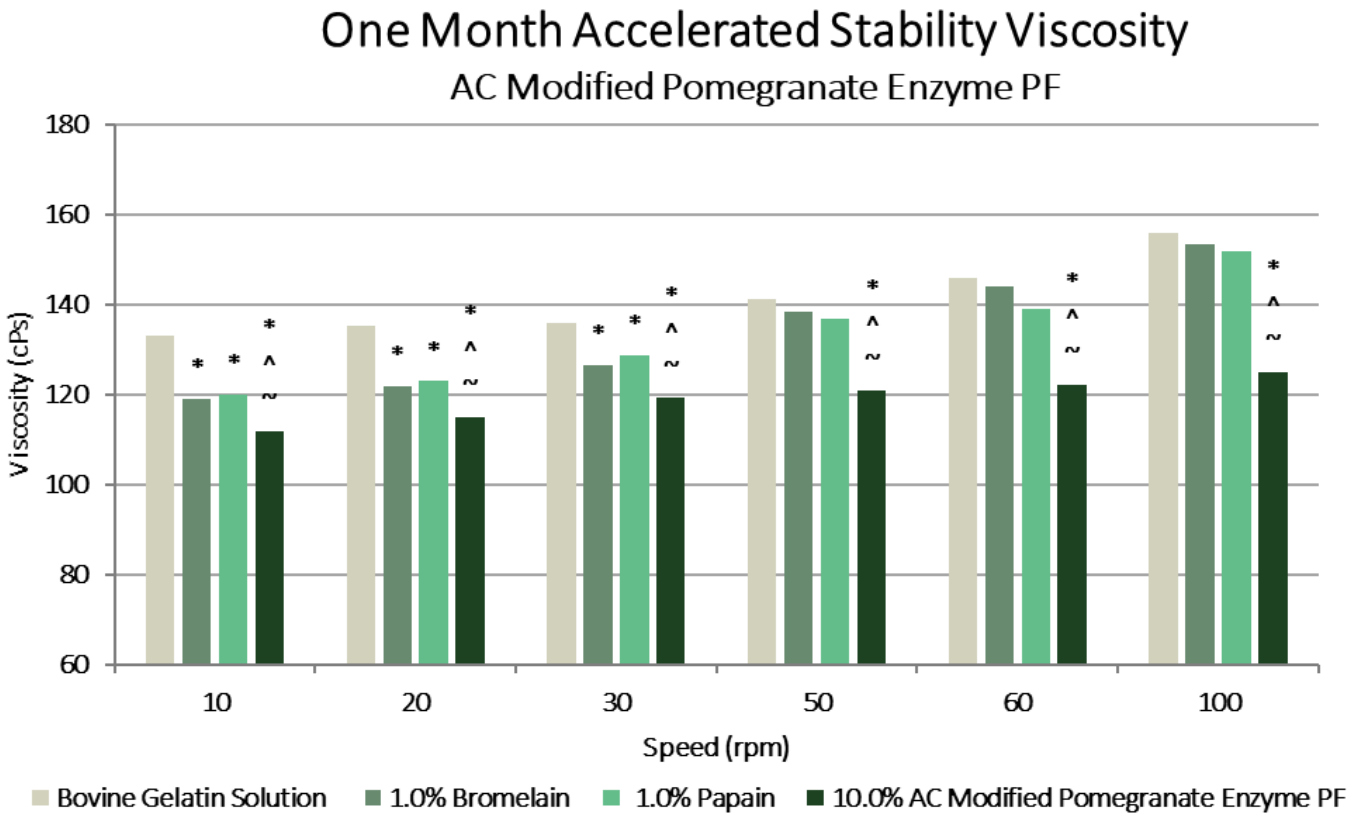

Figure 3. One Month Accelerated Stability Viscosity of Bovine Gelatin Solutions at various speeds. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed. ^ indicates significance ($p \leq 0.05$) compared to 1.0% Bromelain for each test speed. ~ indicates significance ($p \leq 0.05$) compared to 1.0% Papain for each test speed.

Table 6. Percent Change of One Month Accelerated Stability Viscosity of Bovine Gelatin Solution at various speeds. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed.

Viscometer Speed (rpm)	1.0% Bromelain	1.0% Papain	10.0% AC Modified Pomegranate Enzyme PF
10	11%	10%	16%
20	10%	9%	15%
30	7%	5%	12%
50	2%	3%	15%
60	1%	5%	16%
100	2%	3%	20%

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Table 7. Results from one-way ANOVA Statistical Analysis between conditions compared after One Month Accelerated Stability at each speed. * indicates significance ($p \leq 0.05$) compared to Bovine Gelatin Solution for each test speed. ^ indicates significance ($p \leq 0.05$) compared to 1.0% Bromelain for each test speed. ~ indicates significance ($p \leq 0.05$) compared to 1.0% Papain for each test speed.

Viscometer Speed (rpm)	Bovine Gelatin vs 1.0% Bromelain	Bovine Gelatin vs 1.0% Papain	Bovine Gelatin vs 10.0% AC Modified Pomegranate Enzyme PF	1.0% Bromelain vs 10.0% AC Modified Pomegranate Enzyme PF	1.0% Papain vs 10.0% AC Modified Pomegranate Enzyme PF
10	0.011*	0.021*	< 0.001*	0.001^	0.021~
20	0.020*	0.033*	< 0.001*	0.010^	0.010~
30	0.019*	0.031*	< 0.001*	0.002^	0.003~
50	> 0.05	> 0.05	< 0.001*	< 0.001^	< 0.001~
60	> 0.05	> 0.05	< 0.001*	< 0.001^	< 0.001~
100	> 0.05	> 0.05	< 0.001*	< 0.001^	< 0.001~

Discussion

As demonstrated in Figure 1 and Tables 2 and 3, viscosity significantly decreased at all test speeds when fresh solutions of 1.0% Bromelain, 1.0% Papain, and 10.0% **AC Modified Pomegranate Enzyme PF** were added to the Bovine Gelatin Solution. These data indicate a change in molecular weight due to the enzymatic activity of Bromelain, Papain, and **AC Modified Pomegranate Enzyme PF** at time of manufacture. Moreover, the viscosity of **AC Modified Pomegranate Enzyme PF** was lower or comparable to Bromelain and Papain indicating similar enzymatic activity at time of manufacture.

After one month of stability, the viscosity was significantly reduced at all test speeds when 1.0% Bromelain, 1.0% Papain, and 10.0% **AC Modified Pomegranate Enzyme PF** were added to the Bovine Gelatin Solution (Figure 2; Tables 4 and 5). The reductions in viscosity were less drastic than at time of manufacture, indicating a decline in enzymatic activity of all three batches. Viscosity of 10.0% **AC Modified Pomegranate Enzyme PF** was significantly lower than both 1.0% Bromelain and 1.0% Papain indicating superior stability compared to the raw enzymes.

Finally, the accelerated stability batches of 1.0% Bromelain and 1.0% Papain experienced reduced enzymatic activity and did not significantly alter viscosity of the Bovine Gelatin Solution at higher speeds (Figure 3; Tables 6 and 7). Comparatively, the accelerated stability of 10.0% **AC Modified Pomegranate Enzyme PF** significantly reduced viscosity compared to the Bovine Gelatin Solution as well as the 1.0% Bromelain and 1.0% Papain solutions. This data indicates enzymatic stability of **AC Modified Pomegranate Enzyme PF**.

Enzymes are a specialized class of proteins responsible for catalyzing chemical reactions. The proteins present within **AC Modified Pomegranate Enzyme PF** demonstrate a clear proteolytic ability to convert Bovine Gelatin to lower molecular weight fractions, which is then expressed as a decrease in viscosity. Furthermore, **AC Modified Pomegranate Enzyme PF** maintained proteolytic capabilities overtime better than individual enzymes alone indicating improved enzymatic stability.