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EFFECTS OF BACTERIAL EXOPOLYSACCHARIDE ON RHEOLOGICAL PROPERTIES OF ACID MILK CURD AND ITS CORRELATES

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ABSTRACT

Exopolysaccharide (EPS) is a general term for the forms of bacterial polysaccharides found outside the cell wall of bacteria. The industrial microbiologist has become interested in exploiting microbial exopolymer production due to an increasing interest and need for novel polysaccharides. The use of bacterial EPS in acid milk curd (AMC) production accounts for a quality improvement of the milk curd in terms of stability during the normal operations of storage and transportation. On the other hand, EPS have been used as prebiotic because of its health-promoting effects in human beings. The objective of this study was to evaluate the rheological properties (RP) and their statistical correlations in acid milk curd (AMC) samples added with bacterial EPS. Rheometric properties are suitable parameters to evaluate food quality such as AMC. Some of the RP normally used in this evaluation include elastic modulus (EM), hardness (HR), breaking energy (BE), viscosity (VIS) and stress. Correlation between pairs of these parameters are important to understand in order to predict statistically other parameters in case they cannot be easily determined. Acid milk curd samples were formulated using 10, 15, and 20 % of reconstituted skim milk (RSM), to which 0, 0.2, 0.4, 0.6, 0.8, and 1 % level of EPS were added, respectively. Samples were pasteurized at 60 ° C for 30 min, and at 85 °C for 30s. Afterwards, AMC samples were tempered to 40 °C inoculated with *Lactobacillus delbrueckii* subsp. *bulgaricus* B-5b, and incubated at 37 °C for 16 h. Results indicated that viscosity and hardness showed the strongest and highest correlation among all the parameters evaluated (r=0.9272) and it was comparable to that between viscosity and breaking energy (r=0.8349) and breaking energy and hardness (r=0.7694). However, elastic modulus and viscosity showed a rather low correlation (r=0.5394) and very low correlation (r=0.1830), respectively, suggesting that estimation of elastic modulus from viscosity values would be inaccurate. In general, rheometric properties of acid milk curd increased with higher EPS concentrations until 1 % level of EPS addition. Therefore, it is recommended to use BE and HR values, and BE and VIS values to predict accurate measurements of other RP values.

Key words: Exopolysaccharide (EPS), acid milk curd (AMC), rheometric properties (RP), statistical correlation (SC)

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INTRODUCTION

The term “exopolysaccharides” (EPS) was proposed by Sutherland in 1972 and provides a general term for all forms of bacterial polysaccharides found outside of the cell wall [1,2,3,4,5,6,7]. The production of EPS is found in many species of both Gram positive and Gram negative bacteria and has been the subject of numerous investigations in diverse fields of interest [8]. Microorganisms produce EPS to form a barrier resistant to drying and attack by amoebae, bacteriophages and leucocytes [9]. Researches have classified EPS into two major groups based on their composition and mechanism of synthesis: homopolysaccharides and heteropolysaccharides [10,11].

Exopolysaccharides-producing bacteria are added to food to improve their properties and to develop new kinds of food, as food additive and also to make new foods [6, 12,13,14]. Research on polysaccharides added to milk is widely performed to understand the special interactions between milk proteins and polysaccharides [15]. Electrostatic attractions between charged polysaccharides and proteins at a molecular level are generally considered to be the major driving force for these interactions [16].

Different factors affect the rheometric properties of food products derived from AMC [17,18]. Rheology is formally defined as the study of the flow and the deformation of matter. Rheology applied to a body (solid, liquid or gas) leads to cause movement from its original position or to produce a change in its original shape [19,20]. The study of rheology is important in debris-flow as well as in many other kind of manufacture processes such as formulation and production of paints, polymers, printing inks, paper coatings, ceramics, cosmetics, food systems, pharmaceuticals, agrochemicals and liquid detergents [21]. Rheological properties describe the movement of the body of a food product, and include viscosity (VIS), hardness (HR), elastic modulus (EM), and breaking energy (BE). Elastic modulus reflects the movement of atoms from their equilibrium position by bond stretching, bond compression or by change of spacing between adjacent non bonded atoms. In other words, EM is a measure of the rigidity of the material [22]. *Stress* is the weight of sample in mass units (kg) multiplied by its acceleration due to gravity (about 9.81 m/s^2). Shear is defined as the relative distance through which two parallel planes in the sample travel, divided by the distance separating them. Therefore, EM can be defined as the result of stress divided by the shear [22,23].

Determining the correlations between any two parameters of RP [20] is important to estimate those that cannot be easily measured. Hence, the evaluation of the correlation between pairs of parameters related to physical properties (PP) will help predict other physical properties. This is also helpful in order to save research time, energy and materials if data must be available immediately, without reducing data validity. Quality of dairy products such as AMC obtained by acidification or enzymatic coagulation, acid or fermentation processes, may be described by their RP.

The purpose of this study was to determine the rheological properties and the correlations between pairs of parameters of RP of AMC added with EPS as treatment and then inoculated with *Lactobacillus delbrueckii* subsp. *bulgaricus (LDB)*, a culture starter commonly used in yogurt making.

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MATERIALS AND METHODS

Lactic Acid Bacteria (LAB) Cultures

Lactobacillus delbrueckii subsp. *bulgaricus* B-5b (LDB B-5b) was obtained from Japan Milk Product Technology Association, Tokyo, Japan, which was routinely propagated in 10 % Reconstituted Skim Milk (RSM). Reconstituted skim milk was autoclaved at 121 ºC for 15 min and tempered to 37 ºC prior to inoculation at a 0.1 % level in the RSM, and allowed to incubate at 37 ºC overnight.

Preparation of acid milk curd

Research design was a Factorial Randomized Design with 3 factors. These include acid milk curd concentrations, EPS concentrations and Pasteurization Methods. Acid milk curd samples were prepared in 10 %, 15 % and 20 % of RSM. Curdlan, an EPS obtained from *Alcaligenes faecalis* var. *myxogenes* (Takeda chemical Industries Ltd., Osaka, Japan) was added at 0 %, 0.2 %, 0.4 %, 0.6 %, 0.8 % and 1.0 % levels to the milk, heated at 60 ºC for 30 min and at 85 ºC for 30 s, tempered to 37 ºC, inoculated with 1 % (v/v) *LDB* B-5b, and incubated at 37 ºC for 16 hours.

Evaluation of viscosity and other rheometric properties

Viscosities of curdlan-added AMC samples were measured by using a viscometer (Tokimec Inc., Visconic ED-model) with steady shear rate of 1-100/sec along with a MK 50 rotor assembly and NV sensor system operating at 25 ºC. Viscosity was expressed in millipascals per second [24].

Rheometric properties such as hardness (HR), breaking energy (BE) and elastic modulus (EM)of the AMC samples were determined by using a Sanwa-Riken JK-T 264 type Rheometer, operated with a penetration speed of 1.44 mm/s for the curd knife and a chard speed of 18 cm/min [25]. From the linear range of strain-stress relationship, elastic modulus (stress/strain, dyne/cm) of AMC was calculated on the basis of curd hardness (g dyne), cross section of plunger (0.1963 cm), height of sample (4.9363 cm) and penetration depth of plunger (0.4807 cm) [12].

From the chart, value for X and Y were determined on the methods described by Ohashi *et al*. [26], using the following equations and conditions.

$$HR = x\ g\ dyne$$

$$BE\left(\frac{dyne}{cm^2}\right) = \left(\frac{HR}{A}\right) \times \left(\frac{a}{L}\right)$$

$$EM\left(\frac{dyne}{cm^2}\right) = \left(\frac{HR}{A}\right) \div \left(\frac{a}{L}\right)$$

Where:
HR = Hardness (g dyne)
A = Transversal area of knife
a = knife penetration
L = Height of sample

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Statistical Analysis

Rheological data obtained were analyzed with randomized analysis by ANOVA, and reported by using correlation analysis of RP for all treatments within EPS, RSM and PM [27]. The statistical correlations among the four RP measurements were determined.

RESULTS AND DISCUSSION

Rheological Properties

Rheology is formally defined as the study of the flow and deformation of matter. Elastic modulus reflects the forces required to move atoms from their equilibrium position by bond stretching, bond compression, or by change of spacing between adjacent non bonded atoms. In other words, elastic modulus is a measure of the rigidity of material. Stress is the weight of sample in kg multiplied by the acceleration due to gravity (about 9.81 m/s^2). Shear is defined as the relative distance through which two parallel planes in the sample travel, divided by the distance separating them. Elastic modulus (modulus of elasticity) therefore, be defined as the stress divided the shear.

There was a significant effect of pasteurization method treatment ($P<0.05$) on viscosity, hardness, breaking energy and elastic modulus. Likewise, the treatment of milk concentration and EPS concentration significantly affected the rheological properties of AMC. There was no significant difference between 0% and 0.2% EPS concentrations, but there were significant differences in other concentrations in viscosity, hardness and breaking energy.

Rheometric parameters (VIS, HR, BE and EM) of acid milk curd generally increased with increasing EPS concentration (Table 1). The HR values varied between 4.7 – 23.3 g dyne, this phenomena can be explained by the fact that during the initial phase of milk clotting by lactic acid bacteria fermentation [28], the rate of increase of the average molecular weight of casein micelles aggregates rises rapidly, supported by EPS as a thickening material in the acid milk curd [29]. This table shows the effect of EPS concentration on HR of AMC samples. By heating for 30 min at 60ºC using 10 % milk and 15 % RSM, viscosity values continued to increase with increasing EPS concentration. Same results were observed with 20 % RSM. The increase in viscosity is due to an increase in casein concentration as the milk concentration increases. This also occurs due to an increase in the EPS concentration, resulting in the bonding of casein with polysaccharide molecules. The individual caseins may associate with themselves or form associations with other milk fractions through hydrophobic or electrostatic interactions [30]. Permanent contact of micelles would occur only when both interaction areas contain enzyme-modified κ-casein. Decreasing κ-casein hydrolysis would reduce interactions accordingly, and thus, a weaker network forms [31]. These data indicate that HR generally increases along as viscosity increases, due to an increase in milk solids and EPS concentration. When there is a fermentation process by *Lactobacillus. delbrueckii* subsp. *bulgaricus*, which converts lactose into lactic acid, there will be a decrease in pH, eventually reaching the isoelectric point of casein. The isoelectric point of *κ*-casein is 5.45-5.77; β-casein 4.83-5.07, and para-κ-casein, evidently exposing a cluster of positively charged group [32].

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At the initial pH of milk (6.64), electrostatic interactions can occur between positively charged para-κ-casein and negatively charged β-casein [29]. This interaction increases the association of micelles and a stronger curd formation. In addition, this molecular bond is further strengthened by the presence of EPS as a binder. Under these conditions, more para-κ-casein molecules become available, which allows for an effective collision to result in an irreversible fusion of the micelles. Sulfhydryl groups can facilitate intermolecular bonding by oxidation of -SH groups or shedding between -SH groups and S-S bonds [33].

As shown in Table 1, rheometric parameters in AMC samples increase as the concentration of EPS increase, and there is a direct relationship between VIS, HR, BE and EM. During the initial phase of the milk clotting, the rate of increase of the average molecular weight of casein micelles aggregates rises rapidly. The effect of EPS curdlan concentration on viscosity, hardness and breaking energy is that the AMC in 10 % skim milk heated at 60 ^{o}C for 30 min has a higher rheometric property value compared to that heated at 85 ^{o}C for 30 s. The increase in curd hardness at higher milk concentrations was due to a higher casein content and the larger change in casein particle size on the LTLT heating of milk (low-temperature-long-time) compared to HTST heating (high-temperature-short-time process). Viscosity values of acid milk curd heated at 60 ^{o}C were greater than acid milk curd heated at 85 ^{o}C, and statistically significantly different both acid milk curds with addition of curdlan and with no added curdlan.

These results probably are related to the complex constituted by denatured β-lactoglobulin and κ-casein, which is formed when heated, and which inhibits micelle fusion [34,35]. Curdlan gel strength also increases when heating temperature increased to 80-100 ^{o}C and forms a firm, resilient and thermo irreversible gel when heated in aqueous media at a temperature higher than 80 ^{o}C [36].

Effect of total solids content (10, 15 and 20 % of RSM) on the physical properties of curdlan-added acid milk curd can be explained by the considerable decrease of pore dimensions as total solids contents increased from 10 to 20 %. The concentration of lactose was converted into lactic acid, consequently, acidity increased. With an increased lactose concentration, the hydration of casein (and other proteins) decreases, what might result in smaller size of casein particles.

Rheometric Correlation

Figure 1 shows the plot of VIS and HR of AMC made from RSM 10 %, 15 % and 20 % added EPS curdlan from 0-1 % with pasteurization under Long-temperature Long-Time process (LTLT), at 65^{o}C for 30 min and under High-Temperature-Short-Time (HTST), at 85 ^{o}C for 30 s. Media were fermented by *LDB* B-5b at 37 ^{o}C for 16 h. The results of the study illustrate the increase in VIS along with the increased concentration of RSM used as a basis for making AMC. It can be seen in Figure 1 that HR also increased as increasing EPS curdlan concentration. It is believed that a rise of rigidity of milk coagulated at a higher temperature., and apparently, the rate of acid production also affects gel firmness [24].

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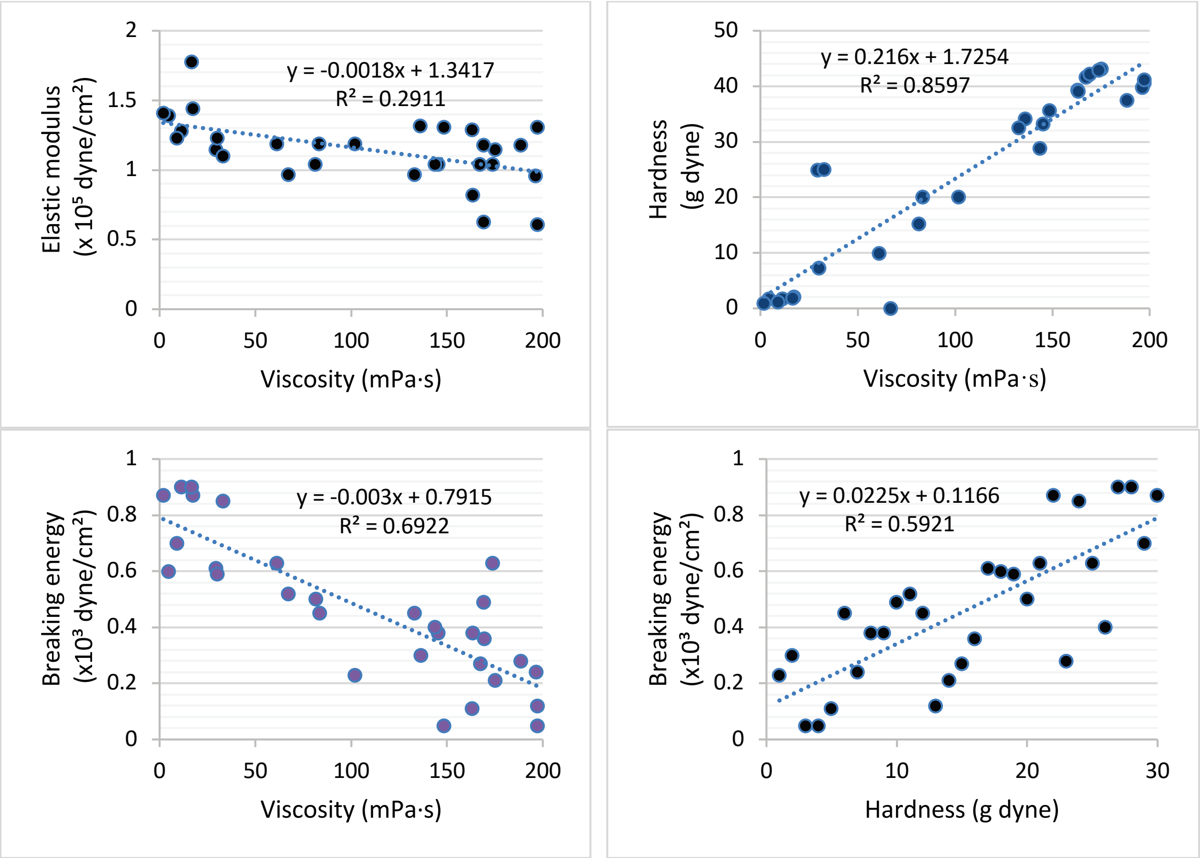
An increase of VIS at the beginning of the milk coagulation process indicates the formation of casein micelles (CM) aggregates [37]. The rate of increase of the VIS or rigidity of the milk gel reaches a maximum (inflection point) depending of the RSM concentration [38]. The individual casein may self-associate or form associations with other fractions through hydrophobic or electronic interactions. Sulfhydryl groups and disulfide bonds are important during gel formation [39]. Results indicate that during the initial phase of milk clotting, the rate of increase of the average molecular weight of CM aggregates rises rapidly. Increased VIS and HR due to this are also influenced by the presence of EPS which acts as a binding agent between casein molecules in the three-dimensional structure of the acid milk molecular network [40].

Figure 1 represents the correlation between HR and VIS (Y = 0.216X + 1.7254; R^2 = 0.8597). Accordingly, HR value can be predicted from the VIS value, an increase of 1 mPa·s in VIS causes an increase of 0.216 g dyne HR of AMC. The relationship between VIS and HR is a positive relationship (85.97 %).



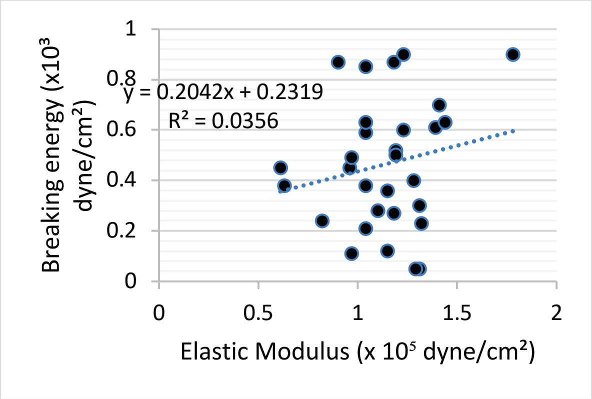


Figure 1: Correlation of pairs of Rheological Properties of acid milk curd by EPS addition

Figure 1 also presents the negative relationship (69.20 %) between VIS and BE, where VIS increases as the BE decreases. An increase of 1 mPa·s in VIS causes a decrease of 0.003 x 10^3 dyne/cm^2 BE. It can be explained that by increasing the concentration of skim milk (SM) and also by increasing the concentration of EPS added in making yogurt the viscosity increases, but the BE decreases, meaning that the energy used in measuring VIS will decrease, only a slight disconnection of energy occurs in the measurement VIS, which shows that the product texture is evenly distributed throughout the product.

This Figure 1 also explains the effect of EPS concentration on BE of 10 %, 15 % and 20 % RSM culture media. Breaking energy values were increased by increased EPS concentration. An increase of 1 mPa·s in VIS causes a decrease of 0.001 x 10^5 dyne/cm^2 EM. There is a negative relationship between VIS and EM (29.10 %). Moreover, EM increased with increasing EPS concentration. Studies on the RP of ultra-filtered skim milk were conducted by Hallstrom and Dejmek [61] and they found that the heat pretreatment at 90 °C for 5 min of the skim milk causes an increase in protein voluminosity compared to what happens at 65 °C for 30 min due to heat denaturation of the whey protein.

A positive relationship (59.20 %) between HR and BE of AMC added with bacterial EPS can be also observed in Figure 1. An increase of 1 g dyne of HR correlates with an increase of 0.022 x 10^3 dyne/cm^2 of BE. The physical properties (PP) of AMC products, such as cheese or yogurt, are influenced by a number of factors including milk composition, milk quality, temperature, bacterial starter, pH, calcium and other mineral concentrations, ripening processes [17] and the presence of additives such as EPS.

Exopolysaccharides function as stabilizers which increase the VIS and HR of fermented milks such as yogurt and other AMC. Exopolysaccharides bind water and interact with milk proteins that form the microstructure three-dimensional networks [40]. This affects other RP such as elasticity EM and breaking energy BE. Figure 5 explains the positive relationship between EM and BE (35 %). Every increase of 1 x 10^5 dyne/cm^2 EM correlates with an increase of 0.204 x 10^3 dyne/cm^2 BE.

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Statistical correlation among the three RP parameter and VIS of the AMC containing curdlan EPS with different concentration are shown in Figure1. These relations can be summarized as follows:

$$HR = 0.216\ VIS + 1.7254$$

$$BE = -0.003\ VIS + 0.791$$

$$EM = -0.001\ VIS + 1.341$$

$$BE = 0.022\ HR + 0.116$$

$$BE = 0.204\ EM + 0.231$$

Where VIS, HR, BE and EM represent viscosity (mPa·s), hardness (g dyne), breaking energy (x 10^3 dyne/cm^2), and elastic modulus (x 10^5 dyne/cm^2), respectively.
BE and HR showed the best correlation (r=0.943) and it was comparable to that between VIS and HR (r=0.897). However, EM and VIS had a very low correlation (r=0.649) suggesting that estimation of EM from VIS and vice versa, would be inaccurate.

CONCLUSION

Rheometric parameters are very important physical properties to evaluate the quality of dairy products and the measurement of each RP parameter can be correlated with each other. In this study, BE and HR showed the best correlation (r=0.943) and it was comparable to that between VIS and HR (r=0.897). However, EM and VIS had a very low correlation (r=0.649) suggesting that estimation of EM from VIS and vice versa, would be rather inaccurate. According to these results, it is recommended to use BE and HR, and VIS and HR to predict each other and to get accurate measurements to predict other rheometric properties.

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Table 1: Rheological properties of acid milk curd with fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* B-5b addition EPS

PT (°C)	MC (%)	EPSC (%)	Vis (mPa·s)	HR (g/cm²)	BE ($x10^3$dyne/cm²)	EM ($x10^5$dyne/cm²)
60	10	0	0.346	5.6	0.148	2.316
		0.2	0.464	5.1	0.35	0.796
		0.4	0.830	4.7	0.282	0.824
		0.6	1.582	5.45	0.362	1.224
		0.8	1.946	9.3	0.772	1.160
		1.0	4.044	23.3	2.214	2.554
	15	0	0.446	10.9	3.902	3.288
		0.2	0.446	13.7	11.976	1.632
		0.4	0.844	15.3	14.762	1.564
		0.6	1.920	18.7	18.252	2.110
		0.8	2.766	29.3	30.694	2.820
		1.0	4.702	6.50	8.406	0.52
	20	0	0.516	14.9	1.826	2.48
		0.2	0.858	14.7	2.146	2.394
		0.4	1.116	17.0	2.226	2.534
		0.6	1.492	25.5	2.466	2.678
		0.8	3.122	30.1	2.75	2.748
		1.0	3.512	40.0	5.162	3.73
85	10	0	0.178	3.84	0.368	2.7
		0.2	0.606	4.00	0.190	0.934
		0.4	0.902	7.30	0.512	1.100
		0.6	2.130	13.75	1.340	1.460
		0.8	3.138	13.84	1.802	1.926
		1.0	3.324	26.44	3.410	2.500
	15	0	0.666	12.52	4.888	3.462
		0.2	0.552	20.80	12.046	3.064
		0.4	1.254	18.60	19.090	1.892
		0.6	2.196	23.16	19.288	2.670
		0.8	2.846	34.20	34.820	3.384
		1.0	3.244	44.30	56.374	3.604
	20	0	0.556	20.10	9.448	5.600
		0.2	0.950	31.20	1.018	0.944
		0.4	2.172	23.70	7.080	3.940
		0.6	2.328	25.50	6.180	4.980
		0.8	2.742	42.00	12.560	5.308
		1.0	4.520	40.00	6.940	6.666

Note: PT=Pasteurization Temperature, MC=Milk Concentration, EPSC= EPS Concentration, VIS=Viscosity, HR=Hardness, BE=Breaking Energy, EM= Elastic Modulus

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