International Journal of Bio-Science and Bio-Technology
Vol.8, No.5 (2016), pp. 165-178
http://dx.doi.org/10.14257/ijbsbt.2016.8.5.15

Fabrication of Nanoparticles of Sulfated Low Methoxyl Pectin by Cross-linking Reaction on Ionotropic Gelation Technique

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Abstract

The sulfation process on pectin produces a number of sulfate groups replacing hydroxyl groups within low methoxyl pectin (LMP) chains. A structure alteration due to the sulfation process may results in changes in LMP characteristics. The LMP sulfation process was aimed to reach an increase in pectin functionality in terms of bioactivities. To gain sulfated LMP (sLMP) performance, the ionic gelation process was undertaken so as sLMP nanoparticles were obtained through cross-linking reactions with divalent cations. The sulfation process on sLMP was conducted by employing different volume of SO_3-DMF solvent. According to the Fourier Transform Infrared (FTIR) spectroscopy analysis, pectin was successfully sulfated indicated by the presence of –S groups within pectin chains. The sulfation process with solution of SO_3-DMF 9.0 mL indicated the best results, i.e. sulfated percentage of 43.63% with the sulfate content of 0.302% and degree of sulfation of 0.0155. Sulfated LMP formed nanoparticles in a formula combination of LMP:sLMP (a ratio of 1:1) with a composition of pectin of 2% and $CaCl_2$ of 0.8%. The nanoparticles of sulfated pectin was relatively stable at cool temperature storage.

Keywords*: pectin sulfation, SO_3-DMF, sulfate content, nanoparticles, stability*

1. Introduction

Biochemistry researchers and nutritionists nowadays pay great attention to polysaccharide since it has a broad spectrum of biological activities [1]. It was reported that some polysaccharide activities already discovered comprising an immunity increase, antitumor, antiviral, antioxidant and hypoglycemic effects. Numerous evidences show that biological activities of polysaccharide are relied on its structure, among others, on the sulfate degree [2]. Sulfate and hexauronic acid within the sulfated-polysaccharide fraction of an *Ophiopogon japonicus* tuber root are effective indicators of antioxidant and immunomodulatory activities [3].

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ISSN: 2233-7849 IJBSBT

Sulfated polysaccharide is a polysaccharide highly containing sulfate groups. Some polysaccharides reported containing naturally sulfates are glucan, chitosan, dextran, pullulan and others [4]. Natural sulfated polysaccharides such as fucan and galactan originating from sea invertebrates were reported having excellent biological activities [5, 6]. However, these polysaccharides commonly are found in a limited number. Based on the many benefits offered by the biological activity of the sulfated polysaccharide, it is necessary to increase its availability.

Sulfated polysaccharide is more often obtained through chemical modifications [7]. Chemical modifications on polysaccharide are performed to attain new functions, engender more active derivatives, or enhance its functions. Modifications through sulfation is replacement of hydroxyl groups within polymers with sulfate groups for the purpose of enhancing effects of polysaccharide physiological functions, for example, anticoagulant, antitumor and anti-HIV functions [8,-9]. Sulfation reactions involve entire primary and secondary hydroxyls within polysaccharide. Jung *et al*. [4] working sulfation on polysaccharide extracted from *Pleurotus eryngii* fungus reported that it was effective in inhibiting cancer cell growth. According to [10], sulfation on derived polysaccharide of an *Auricularia auricular* fungus showed antioxidant activity, and it indicated a novel type of immunopotentiator [11].

There are many chemical agent able to be used for either the process of sulfonation or sulfation the polysaccharides. These compounds include liquid or gas-phase sulfur trioxide (SO_3), concentrated sulfate-acid solution (93-98%), oleum, chlorosulfonic acid and sulfur dioxide. In addition, pyridine, piperidine N-sulfonate acid or complexes of sulfur trioxide and pyridine, tri-ethylamine or DMF may be used [12], formamide and DMSO are also regularly used as solvents in sulfated polysaccharide reactions. As stated by [13], chlorosulfonic acid is the most optimal reagent for chemical sulfatation. Chlorosulfonic acid is made of chloride acid and sulfur trioxide by the reaction of SO_3 + HCl = $ClSO_2OH$.

Among of various types of polysaccharides, pectin is a polysaccharide that is widely used in human's lives. Pectin comprises one of polysaccharide types widely applied as functional components in foods and pharmaceuticals. Pectin has effects able to decrease cholesterol levels [14], decelerate gastric emptying [15], induce apoptosis of colonic cancer cells [16], be a combined material to extend hormonal and antibiotic works, as well as be an injection material to prevent bleeding [17]. It is upon the fact that pectin is resistant to protease and amylase, enzymes active in the upper gastrointestinal tract. Moreover, pectin is found as a wonderful biopolymer as it is highly degradable and biocompatible. This polysaccharide is recorded non-toxic and abundantly available.

Pectin, especially LMP, is capable to form unique gels with divalent cations and cross-links in the ionic gelation process, therefore make it ideal as a bioactive delivery agent. This polysaccharide is able to develop aggregation at low pH. The level of acidity of solution contributes to the ionization level of pectin molecules and the electrostatic interaction with cations. At pH 4 the ionization level of pectin molecules is considered high [18]. This consequently enables strong bonds between negative charges of hydroxyl ions within pectin and positive charges of calcium ions, thereby resulting in small particle sizes. Pectin mostly is applied as a colon-specific delivery device, since at neutral pH or nearly neutral in the colon, pectin aggregates will be dissociated and form long chains. Based on those physicochemical properties, the LMP can be used as a candidate of sulfated polysaccharide by giving the sulfur ions in the side chain of free carboxyl groups through sulfation process. In the form of sulfated pectin, hopefully it has a good biology activities for human's health.

Biodegradable and biocompatible polymers that are suitable for human use can be prepared as particle complexes in various sizes. Nowadays, nanoparticles have attracted the attention of researcher to be applied as a delivery agent of macromolecular bioactives such as peptides, proteins and gens due to its capability protecting labile components from

enzymatic reactions. Moreover, the endocytic uptake of nanoparticles leads to broken barrier permeability as a consequence of epithelial absorption. Particles whose charges and sizes are compatible are able to enter into mammalian cells through different routes, and in other words this may enhance opportunities to enter into cells. By arranging some important process parameters, LMP can be fabricated into nanoparticles by means of crosslinking in the ionic gelation process. Low methoxyl pectin in the form of nanoparticles are expected as a delivery agents that are more resistant to harsh condition for sensitive bioactives into targeted organs in the human body.

Pursuant to literatures, it appears that specific studies on sulfation of LMP prepared for producing nanoparticles have never been reported. However, to do this research there is a fundamental problem. The addition of sulfate groups into the pectin chain is likely to affect the physicochemical properties of sulfated LMP. According to [4], solubility of sulfated polysaccharide in water undergoes an increase. The solubility changes caused difficulties in the formation of particles of ionic gelation in the cross-linking reaction between sLMP and cation. It is caused by a reduction the anionic groups of LMP after the sulfation process. Therefore, it is necessary to study extensively for searching the techniques, methods or ways to overcome the problems. This work was aimed to chemically modify low methoxyl pectin derived from lime (*Citrus aurantifolia* Swingle) peel through the sulfation process as a material for production of a relatively stable nanoparticle functioning as a colon-specific delivery agent.

2. Materials and Methods

2.1. Preparation of Low Methoxyl Pectin for the Sulfatation Process

This research employed LMP (extracted from Citrus aurantifolia Swingle lime peel) with the DE of 45.77% for the sulfation process. Pectin sulfation was carried out in accordance with method of [19] with a slight modification. The reagent for SO_3-DMF sulfation was prepared by dripping 50 mL of $HClSO_3$ into an Erlenmeyer containing 300 mL of DMF (N,N-dimethyl formamide) and cooled in a container filled by iced water. Dried pectin (1.0 g) was added into 40 mL of formamide, followed by the mixture stirred at 80^{o}C within 3 hours for the purpose of distribution in the solvent. Next, the SO_3-DMF reagent (sulfation reagent volumes of 6.0; 7.5; 9.0; 10.5; and 12 mL) was added into the mixture. After 3 hours, the mixture was cooled at room temperature and precipitated with 75% alcohol for 24 hours. Precipitates then were filtered and rinsed three times in 60% alcohol and put into 100 mL of aquadest. The solution then were neutralized with 1 mol/mL of NaOH solution and dialyzed in flowing water for 72 hours using a 2000 NMWCO (nominal molecular weight cut-off) pore-sized dialyzing membrane or 2000 kda. The mixture then was dried in an oven at 50^{o}C. Dried sulfated-pectin was characterized upon structure parameters employing the FTIR, the percentage of sulfated pectin products as well as the sulfate content and degree of substitution.

2.2. The Analysis of Sulfated LMP Profile

a. FTIR (Fourier Transform Infrared) Spectroscopy

A two mg of sample and 200 mg of KBr were ground and mixed until homogenous and put in a disc molding apparatus, molded into KBr pellets. The pellet sample was put into a sample pan in the FTIR apparatus, and measured at 4000 cm-1 to 450 cm-1 wave length.

b. Degree of Substitution

This step was started by making of $BaCl_2$-gelatin solution. One gram of gelatin and 200 mL of aquadest were mixed then heated at 60^o-70^{o}C until the gelatin was dissolved. The gelatin solution was stored in the refrigerator within 6 hours or overnight. One gram of $BaCl_2$ was mixed into the solution and homogenized, then stored again in the refrigerator

for one week. Meanwhile, the mother liquor of Na_2SO_4 (concentration of 100 ppm) was made in separately flask. A 0.0148 gram of Na_2SO_4 was put into a 100 mL- flask then dissolved in aquadest up to the mark terra. From the mother liquor the standard solution was prepared. Each of the 5, 10, 15, 20, and 25 mL volumes from the mother liquor was put into a 100 mL-flask at each concentration of 5, 10, 15, 20, and 25 ppm then dissolved in aquadest up to the terra mark. Absorbance was measured by a UV-VIS spectrophotometer at 420-nm wave length. The sample solution was prepared with following steps. Two miligrams of sample was dissolved in 2 mL of aquadest then 5 ml of Trichloro Acetic acid of 4% was added in a closed tube. The mixture was heat in a water bath at 100^{o}C within 4 hours then cooled at room temperature. Before the tube was opened, the solution was shaken and 1 ml was taken before put into a centrifuge. Two mililiter of aquadest and 1 ml of the $BaCl_2$-gelatin solution were added and centrifuged within 1 minute. Absorbance was measured employing a UV-VIS spectrophotometer at 420 nm wave length. The sulfur content contained in the sample was obtain after the sulfate concentration was calculated, followed by the sulfate degree of substitution calculated using this following formula:

$$DS = \frac{162 \text{ x } S\% / 32}{100 - (80/32 \text{ X } S\%)} \quad (1)$$

Descriptions:

DS	=	degree of substitution
162	=	the molecular weight of glucose
80	=	molecular weight of sulfate groups
32	=	the molecular weight of sulfur atom
S%	=	concentration of sulfuric

2.3. The Ionic Gelation Cross-linking Process in Sulfated LMP Nanoparticle Formation

In order to reach the nanoparticle sLMP this study was designed with steps as follows:

a. Determination of a ratio between sulfated LMP and cation concentrations producing the nanoparticle size

The study used a pectin and cation concentration range of 0.01-1% and the pectin:cation volume ratio of 1:10, the observation was done macroscopically followed by size measurement. The best result was considered according to achievement of the nanometric size and the relatively low value of PDI (PolyDiversity Index).

b. Preparation of sulfated LMP nanoparticles

This study was conducted by proving occurrence of the ionic gelation process using sLMP, which was marked by the bead formation. The analysis and size measurement were carried out macroscopically. The best result was envisaged based on achievement of the nanometric scale, negative charges and relatively low PDI value.

c. Observation on sulfated LMP nanoparticle stability

Nanoparticles selected in the (a) step were subjected to measurement of sizes and zeta potentials in 8 points (7 weeks) in order to reach relatively stable sLMP nanoparticles. sLMP nanoparticle storage was done at cool temperature (5^{o}C), room temperature (27-33^{o}C) and 40^{o}C. A sample observation was also performed macroscopically.

d. Production of sLMP nanoparticles

Procedures of sLMP nanoparticle formation [18] were as follows. Both of sulfated low methoxyl pectin and cations employing were dissolved in distilled water to reach the treatment concentrations. Further, 2.0 mL of the pectin solution (LMP:sLMP=1:1) was dripped into 2 mL of the cation solution while mixing by a stirrer, at 25ºC. Mixing was continued for 20 minutes. Nanoparticles produced then were collected for the purpose of size and zeta potential analyses.

e. Characterization of sulfated LMP nanoparticle physical-properties

Particle sizes and zeta potentials were measured from new samples on a Zetasizer Nano ZS apparatus. For the purpose of analysis, each sample was dissolved in pure water at a suitable concentration and placed in the electrophoretic cell and the analysis was performed at 25ºC.

2.4. Statistical Analysis

The analysis of sulfated low methoxyl pectin samples was conducted in three replicates in a completely randomized design (CRD). The statistical analysis was done by employing the statistical analysis of SPSS for windows (ver. 17). A Duncan Test was applied to evaluate any significant difference among mean values at the 95% confidence level ($P<0.05$).

3. Results and Discussion

3.1. Sulfated LMP Characterization

a. The FTIR spectroscopy analysis

An analysis of the FTIR spectroscopy was applied to observe molecular vibrations and polar bonds between different atoms. The FTIR spectroscopy analysis allows to be done against polysaccharide structures such as monosaccharide, glycosidic bonds and functional groups [21]. Figure 1 was FTIR profile of unsulfated LMP (P0), while the presence of sulfur groups within sLMP in this study was presented on Figure 2.

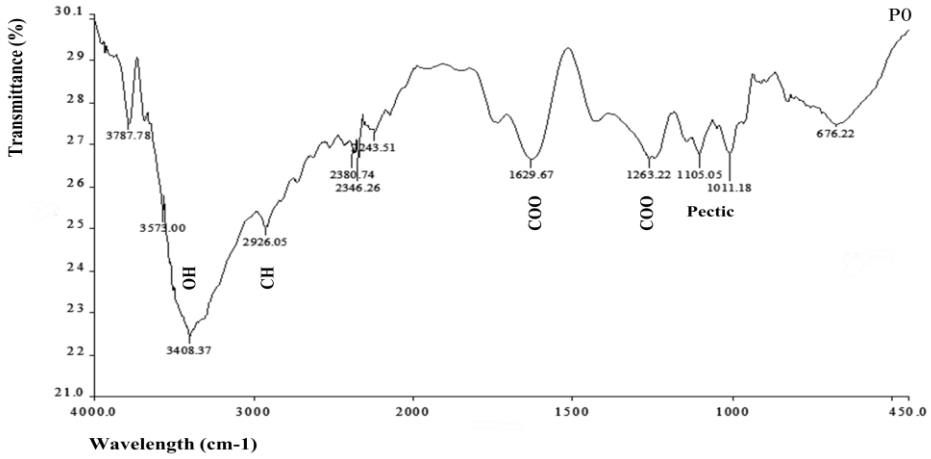


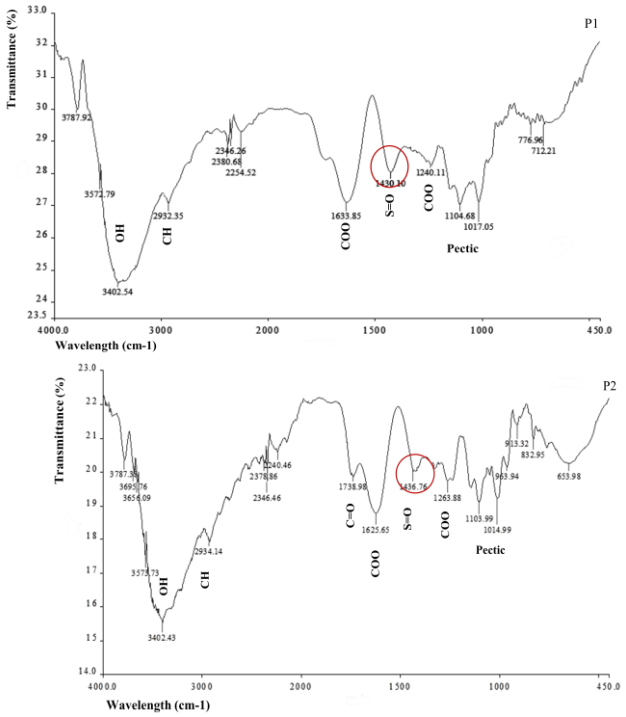
Figure 1. Fourier Transform Infrared of unsulfated low methoxyl pectin

The FTIR spectra in the region between 4000-400 cm-1 identified the major chemical groups in the pectin and provided structural information of pectin. The spectral data obtained were analyzed by comparing the FTIR spectra in the following characteristic

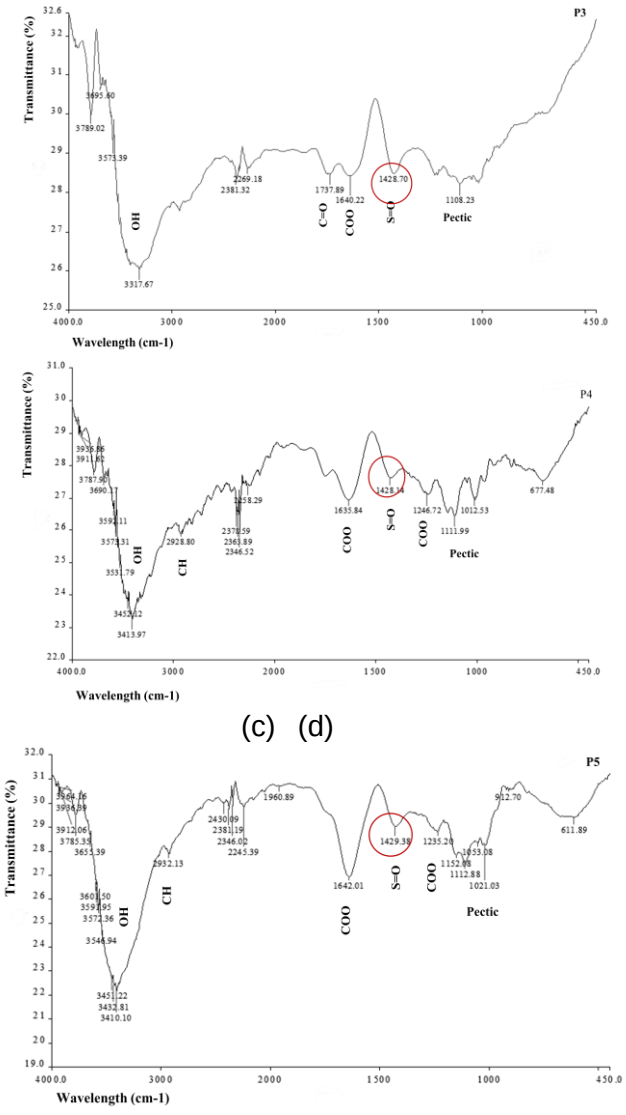
regions, OH stretching band envelope 3600-3100 cm-1, CH stretch at 3000-2800 cm-1, the fingerprint region of spectra under ca. 2000 cm-1, including the band contributing to resonant absorption energy of pyranose cycle vibrations 1200-950 cm-1, as well as the region 1200-1800 cm-1 featuring the state of carboxylic groups (COO). According to [22], the region 1200-950 cm-1 polysaccharide has a strong absorption as fingerprint, is characteristic of each polymer. The region featuring the state of carboxylic groups of pectin at 1750-1350 cm-1. The band at ca. 1750 cm-1 is assigned to stretching C=O mode non-ionized methylated or protonated carboxyl.

The absorption band characteristics appearing in the FTIR spectra of pectin showed in Figures 1 and 2. For the pyranose cycle vibrations region as characteristic for peptic substances of pectin, there were almost identical spectral parts with: bands at 1105 and 1011 cm-1 (P0); 1104 and 1017 cm-1 (P1); 1103 and 1014 cm-1 (P2); 1108 cm-1 (P3); 1111 and 1012 cm-1 (P4); 1152, 1112, 1053 and 1021 cm-1 (P5). All peptic polysaccharides characterized mainly by these peaks, those absorbances were the galacturonic acid. The peak in 3600-3200 cm-1 region showed that there were too many OH groups in the pectin molecule.

The band centered at 1738 cm-1 in P2 spectral and 1737 cm-1 in P3 spectral parts have been utilized to probe the DE in pectin. These bands have been assigned to the C=O stretching vibration of methyl ester. There are two bands in the lime LMP spectrum within region (a major one and a less intense one). Those two bands correspond, respectively, to asymmetrical and symmetrical stretching vibrations due to the COO- group of polygalacturonic acid. The bands were: 1629 and 1263 (P0); 1633 and 1240 cm-1 (P1); 1625 and 1263 cm-1 (P2); 1640 cm-1 (P3); 1635 and 1246 cm-1 (P4); and 1642 and 1235 cm-1 (P5). The spectral parts at 1430 cm-1 (P1), 1436 cm-1 (P2); 1428 cm-1 (P3); 1428 cm-1 (P4); and 1429 cm-1 (P5) were indicating the existence of strong vibration of S=O (sulfate) bonds in sLMP.



(a) (b)



(c) (d)

(e)

Figure 2. Fourier Transform Infrared Profile of Pectin using: (a) 6 mL (P1); (b) 7.5 mL (P2); (c) 9.0 mL (P3); (d) 10.5 mL (P4); and (e) 12.0 mL (P5)

Based on Figure 2, the FTIR spectra of the sulfation reaction in LMP chain was characterized by those peaks as identified as group of S=O (sulphate). Vibrational str*etc*h of the bands was very strong and intense. All of those bands exhibited the presence of bonds with sulfuric compounds. These bonds indicated that polysaccharide have been successfully sulfated [23]. On the basis of FTIR analysis, LMP derived from lime peel had been completely sulfated in all treatments compared with unsulfation LMP. There was no any vibration band indicating the presence of sulfate groups on unsulfated LMP.

b. Yield of sulfated pectin, sulfate contents and degree of substitutions

In accordance with ANOVA analysis results, treatments were known affecting ($P<0.05$) sulfated pectin yield percentages, sulfate contents and degree of substitutions. Figure 3 presents sLMP characteristics.

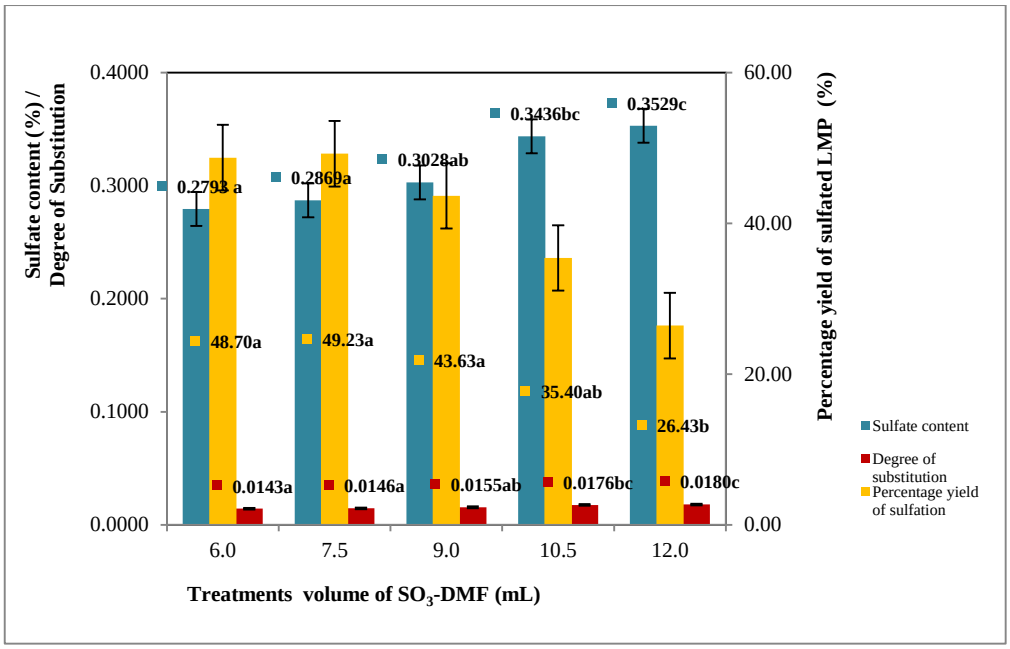


Figure 3. Characteristics of Sulfated Low Methoxyl Pectin from Lime Peel

According to Figure 3, the use of 12.0 mL of SO_3-DMF volume for the sulfatation process indicated the lowest yield percentage (26.43%) compared to other treatments. There was a tendency that as the use of SO_3-DMF volume was higher the percentage of sulfated pectin tended to be lower. The use of 12 –mL SO_3-DMF volume yielding the lowest sulfated pectin (0.264437 g compared to 1 gram of the initial pectin weight) was likely due to the fact that reactions of the polysaccharide changing to other low molecular weight compounds (<2000 kda) passing through pores of dialysis at the time of the dialysis process in flowing water occurred.

Whilst, Figure 3 suggests that the higher SO_3-DMF volume use yielded the increasing sulfate content and degree of substitution, consecutively from the treatment of the 6.0-mL SO_3-DMF volume with the sulfate content of 0.2793% and DS of 0.0143 to 12 mL with the sulfate content of 0.3529% and DS of 0.0180. The degree of substitution is the average number of groups per anhydroglucose unit substituted by other groups. In this term, it indicated the number of sulfate groups (S) substituting hydroxyl (OH) groups on galacturonic acid structures within the polygalacturonic chain of pectin compounds. Accordingly, the success of OH group replacement by –S groups on sulfated pectin structures after the sulfation synthesis was performed was expressed as the degree of substitution. In the process of pectin sulfation, OH groups within polysaccharide chains were replaced by –S available in sulfation agent (SO_3-DMF). Based on [24], the presence of free hydroxyl groups in an abundant number provide a certain substrate so as sulfation reactions occur. Jung *et al*. [4] reported their work that the sulfate content of a *Pleurotus eryngii* fungus polysaccharide derivation increased in line with the sulfation agent ratio. The DS value is highly affected by the sulfate content since the substitution process in the synthesis occurs between hydroxyl groups and sulfate groups. The DS value of polysaccharide is a notable parameter to evaluate its bioactivity [24]. This is because sulfate groups play an essential role in determining polysaccharide bioactivities.

Based on Figure 3 above, the sulfate content and the degree of substitution resulted from the treatment of 6.0-9.0-mL SO_3-DMF volume were lower than the treatment of 10.5-mL and 12.0-mL SO_3-DMF volume. The use of 10.5-mL SO_3-DMF volume produced values of the sulfate content and the degree of substitution relatively similar to the 12.0-mL treatment. Based on decision of balance result on characteristics *i.e.* sulfated percentage of 43.63% with the sulfate content of 0.302% and degree of sulfation of

0.0155, it was selected the treatment of SO_3-DMF volume of 9.0 mL. Sulfated pectin using 9.0-mL SO_3-DMF volume showed better results than other treatments. Pectin which was resulted from 9.0-mL SO_3-DMF volume possibility of providing sulfate content sufficient and safe for use in the production of sLMP nanoparticles with good characteristics and bioactivity.

3.2. Sulfated LMP Nanoparticle Characterization

To prove occurrence of the ionic gelation process from sLMP, the preliminary research step was carried out with the bead formation experiment using sLMP and the $CaCl_2$ solution at low concentrations (<1.0%). Macroscopically, the sLMP solution when dripping into the cation solution would be dispersed (bead not formed) and immediately settled. Pursuant to [4], solubility of sulfated polysaccharide in water undergoes an increase. This fact is due to the presence of groups containing sulfur within polymer structures enhancing the number of ionic groups. Then, the $CaCl_2$ concentration was adjusted to 2% and 3 %. Results obtained were similar to the previous. Thus, preparation of a solution combination between non-sulfated LMP and sLMP at different ratios was performed. Observation results are presented in Table 1.

Table 1. Macroscopic Observation of Beads from Combination Sulfated and non Sulfated Low Methoxyl Pectin

Cation Concent.	**Ratio of LMP:sLMP 0.3%**				
	1 : 1	**1 : 2**	**2 : 1**	**1 : 4**	**4 : 1**
	Macroscopically observation				
$CaCl_2$ 2%	**Spherical plate**	**Concave plate**	**Spherical plate**	**Liquid, broken spherical plate**	**Spherical plate**
$CaCl_2$ 3%	**Spherical plate ϕ = 0.75 cm**	**Dispersed**	**Spherical plate ϕ = 0.5 cm**	**Dispersed**	**Spherical plate ϕ = 0.5 cm**

Table 1 demonstrates that the ionic gelation occurring at ratios of LMP:sLMP 1:1, 2:1 and 4:1. However, to investigate sLMP effects in order to have good performance in biological activities the ratio of LMP:sLMP at 1:1 was selected (to obtain more sulfate groups within the particles). Measurement results in a composition of the pectin 2% and the cation 0.8% at 1:1 ratio were: (1) 643.5 (PDI 0.501); (2) 590.8 (PDI 0.728); (3) 577.5 (PDI 0.679) with a 603.9 (PDI 0.636) mean value. These results demonstrated that pectin particle sizes had reached the nanometric sizes (ranging 500 - <1000 nm).

To observe LMP:sLMP nanoparticle stability an observation on particle sizes during storing at 5^{o}C cool temperature, 27^o-30^{o}C room temperature and 40^{o}C room temperature in 8 points (T0-T7) were carried out. The profile on a rate of particle size increase during storage time is presented in Figure 4.

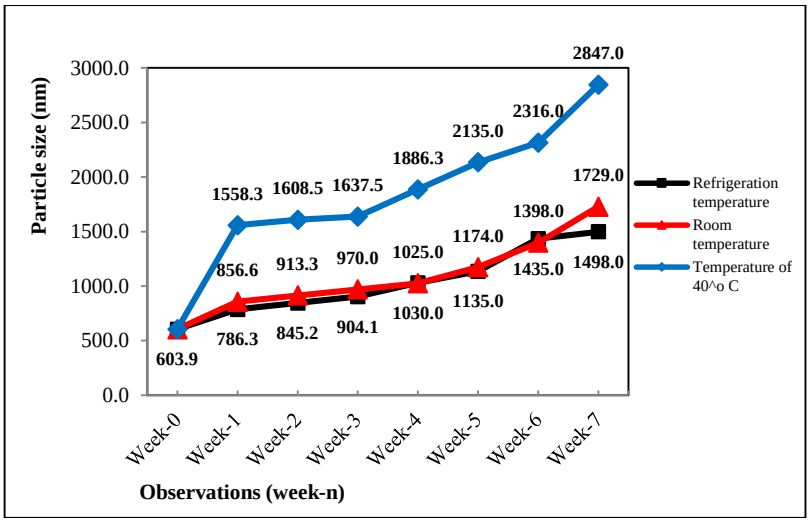


Figure 4. Profile of Increasing Rate of Pectin Nanoparticle Size during Storage in Refrigeration Temperature, Room Temperature and Temperature of 40ºC for 7 Weeks

Based on Figure 4, it is evident that particle sizes underwent an increase during storage and rose sharply in hot-temperature storage. Dramatically increase in the size occurred in the particles storage at 40oC *i.e.* from 603.9 nm (Week-0) to 2847 nm (Week-7) with an increase of 2243.1 nm. The storaging particles in cold and room temperature have a trend of increasing size relatively similar until Week-6 with sloping pattern, i.e being 1398 nm (an increase of 794.1 nm) and 1435 nm (an increase of 831.1 nm) respectivelly. However, at Week-7 the particle size on room temperature storage rising more sharply (be 1729 nm) compared to cold temperature (be 1498 nm). One of factors depicting nanoparticle stability is the zeta potential. This value commonly is incorporated to show charge properties of a nanoparticle surface [25]. The zeta potential reflects electric potential of particles and is affected by the particle composition with medium where particles are dispersed [26]. A nanoparticle with the higher zeta potential than the absolute value of 30 mV appears stable in the suspension, so as the surface charges prevent occurrence of aggregation, results in good colloidal stability [27]. Jonassen *et al*. [20] reported that the chitosan nanoparticle/TPP zeta potential values of <+30mV produced instability during storage in liquid resulting in a particle aggregation thereby allowing greater sizes. The zeta potential values of LMP:sLMP nanoparticles less than -30 mV ranged -15.2 to 16.6 mV. This might be one causing factor of colloidal nanoparticle instability. Particles were aggregated during storage so as the sizes tended to increase.

Nanoparticle sizes of LMP:sLMP experiencing an increase might be also caused by the presence of aggregation induced by Ca at high temperature and aggregation due to hydrogen bonds at low temperature [28]. Perticles of LMP:sLMP stored at cool temperature and room temperature approached the value of one μm (1000 nm) after storing for 4 weeks, whereas at hot temperature it had risen after stirring for one weeks. In addition, the storage at 20ºC and 40ºC experience a decrease in gel strength even though there are calcium ions [29]. Continuous heating at high temperature will lead to a pectin de-polymerization process which is a degradation resulted from hydrolysis on glycoside bonds [30], or β-elimination [31] contributing to a viscosity decrease. Macroscopically, a solution containing sulfated pectin nanoparticles becomes more dilute. Conforming to storability test results in three storage conditions it had better that LMP:sLMP nanoparticles are stored at cool temperature condition (5oC).

4. Conclusion

Pectin was successfully sulfated shown by the presence of –S groups within pectin chains, in accordance with the FTIR analysis. The sulfation process with SO_3-DMF 9.0 mL indicated the best results, *i.e.* 43.63% of the sulfated percentage with the 0.302%-sulfate content and the 0.0155-sulfation degree. Nanoparticles of sLMP could produced using combination of unsulfated LMP and sLMP. Sulfated pectin (sulfation using SO_3-DMF 9.0 mL) formed nanoparticles in a formula combination of LMP:sLMP (a ratio of 1:1) with a composition of pectin of 2% and $CaCl_2$ of 0.8%. Nanoparticles of sulfated pectin (LMP:sLMP) was relatively stable at cool temperature storage.

Acknowledgements

The author expresses gratitude to Indonesian Agency for Research and Development that had provided research funds. Also thanks to Ida Farida who had assisted this research in the ICAPRD laboratory.

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