International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Physicochemical Characteristics of Carboxymethyl Chitosan from Silkworm *(Bombyx mori* L.) Pupa

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Abstract

Carboxymethyl chitosan (CMC) is one of chitosan derivatives which is able to give health benefits as well as antioxidant source. Instead crustacean, silkworm pupa is also an alternative source of chitosan. This experimental study was carried out to find the optimum molarity of NaOH (5 M, 10 M, and 15 M) on alkalization process in CMC silkworm pupa production based on its physicochemical characteristics. The best CMC characteristics were yield about 113.79 % - 115.94 %, alkalinity levels about 4.01 - 4.22, moisture contents about 10.80 % - 11.99 %, ash contents about 0.04 % - 0.37 %, nitrogen totals about 4.06 % - 4.76 %, solubility about 93.39 % - 99.28 % and viscosity about 14.22 - 106.92 mPa.s. These CMC characteristic proved that CMC made from silkworm pupa was high quality which was produced by applying NaOH 10 M in alkalization process.

Keywords: Bombyx mori L.; carboxymethyl chitosan; characteristics; NaOH; molarity.

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1. Introduction

Stress oxidative has a major role in non-communicable diseases (NCD) development, such as cancer and cardiovascular diseases. Prevention of NCD could be done by daily consumption pattern modification, such as rising healthy and rich antioxidant content of food intake.

CMC is a chitosan derivate which is able to give health benefits as well as antioxidant source. Investigation and development of chitosan and its derivate as a source of food material rich in antioxidants still focused on crustaceans shell basis, whereas a research carried out in 2012 proved silkworm pupa was a potential source of chitosan [1, 2, 3].

Silkworm pupa is a reeling industry waste which is potentially to be a source of an edible material rich in antioxidant, called chitosan. To improve solubility level of chitosan made from crustaceans shell could be done by chemical modification. By this modification could be produce CMC which is proved has higher antioxidant activity than chitosan due to lower molecule weight which will increase intermolecular and intra-molecular hydrogen bonds dissolution [4, 5].

Silkworm pupa was well understood had potency as source of chitosan. However, not yet known about its potency and characteristic as a CMC source. This research was attempt to produce CMC from silkworm pupa chitosan using three different molarities of NaOH on alkalization stage and to observe, physicochemical properties and appearances of CMC. Henceforth, give more choices of food material rich in antioxidant as a way of non communicable diseases prevention.

2. Materials and Methods

2.1 Materials

The main material used in this research was silkworm pupa which was kindly given by the central silk industry in Pati – Central Java, Indonesia.

2.2 Sample Preparation

Research was done into two stages, i.e. chitosan production from silkworm pupa and CMC production from previous stage product (chitosan) using three NaOH molarities on alkalization process (5 M, 10 M, 15 M)

2.2.1 Chitosan preparation

Chitosan production was started by washing pupa shells with water, continued by drying until surely dry. Afterwards, dried shells were subjected to demineralization, deproteination, and deasetilation processes (in a series). Demineralization process was conducted by dry silkworm pupa shells submersion into 1 N HCl for 3 days, continued by heating at 100 ^{o}C for 2 hours and washing until pH 7 using deionized water. Deproteination process was conducted by submersion into NaOH 3 N (1:10) for 3 days, continued by heating at 100 ^{o}C for 2 hours and washing until pH 7 using deionized water. Deasetilation process was conducted by submersion into

NaOH 50 % (1:20) for 3 days, continued by heating at 140 ^oC for 2 hours and washing until pH 7 using deionized water. The end process by drying using oven at 50 ^oC for 24 hours [6].

2.2.2 Carboxymethyl chitosan (CMC) production

CMC was prepared from previous chitosan production by subjecting chitosan to alkalization, and carboxymethylation processes (in a series). Alkalization process was conducted by dissolving 10 g chitosan into 100 mL isopropyl alcohol 40 ^oC, stirred up for 30 minutes; continued by adding 12 mL NaOH (5 M, 10 M, 15 M) into the solutions, and stirred up for 90 minutes. Afterwards, subjected into carboxymethylation process by adding 30 g monochloroacetic acid (divided into 4 parts, each 7.5 g) within 5 minutes, continued by boiling the solution at 60 ^oC for 4 hours. Final process was washing using ethanol 96 % and drying using oven at 50 ^oC (48 hour) [7].

2.3 Carboxymethyl Chitosan (CMC) Physicochemical Characterization

Characteristics were determined by characterization of copolymer i.e. physicochemical analysis and appearance observation. Characterization of copolymer CMC was determined by Fourier Transform Infrared (FT-IR), the dried samples were pressed with spectroscopy grade KBr (Potassium Bromide pellets) and FT-IR spectra were recorded on FT-IR spectrometer within number range of 400 – 4000 cm^{-1}. Physicochemical properties were determined by analyzing yields, alkalinity, moisture, ash, nitrogen total, solubility, and viscosity values. Yield value was determined by percentage of total CMC obtained to total chitosan weight; alkalinity was determined by measuring the pH value of 1 g CMC in 100 mL deionized water; proximate analysis by AOAC was performed to measure moisture, ash, and nitrogen total contents [8]; solubility was determined by solution filtration of 1 g CMC in 100 mL deionized water using whatman 42, paper filter then was dried in oven at 105 ^oC for 3 hours and desiccators for 15 minutes; and viscosity was analyzed using Viscometer Brookfield type LV. Meanwhile, appearance determination was carried out by observing colour, odour, and form.

2.4 Statistical Analysis

All data analysis except appearances of CMC, were expressed as means ± SD. Data were analyzed by an analysis of variance (p = 0.05). If the result of ANOVA was significantly, then followed by Duncan's multiple range test. The results were processed by computer programs: Microsoft Excel for Windows and SPSS System for Window V 20.0

3. Results and Discussion

In this study, chitin was silkworm pupa shells, a natural polysaccharides composed at β (1→4) 2-acetamido-2-deoxy β-D-glucose (N-acetylglucosamine) bonds whereas chitosan (β – (1, 4)-*N*-acetyl-D-glucosamine) is a derivate product of chitin, after deacetylation process with acidic solutions [9, 10]. Both are potential substances as antitumor, anticancer, antioxidant, antibacterial, etc [4]. Chitosan was obtained from chitin of silkworm pupa was subjected to be processed as CMC. CMC is a chitosan derivate that is modified with hydrophilic group addition to improve the solubility level to be higher than chitosan.

Principally, CMC production is base-acid reactions. Chitosan bound to Na^+ from NaOH is reacted to monochlorocacetic acid; exchange between water soluble Na^+ with Cl^- that released by monochloroacetic acid; form NaCl solutions; meanwhile chitosan that release Na^+ will be reactive toward carboxyl of monochloroacetic acid, then forming CMC [11, 12]. In addition, according to FT-IR, substitution of chitosan by monochloroacetic acid had been occurred successfully.

Physicochemical characteristics were including level of yields, alkalinities, moistures, ashes, total nitrogen, solubility, and viscosities of three groups CMC based on NaOH molarities on alkalization process, i.e. 5 M, 10 M, and 15 M (Table 1). Meanwhile, Direct observation had done over CMC of silkworm pupa appearances, i.e. colour, odour, and form (Table 2).

3.1 Characterization of copolymer

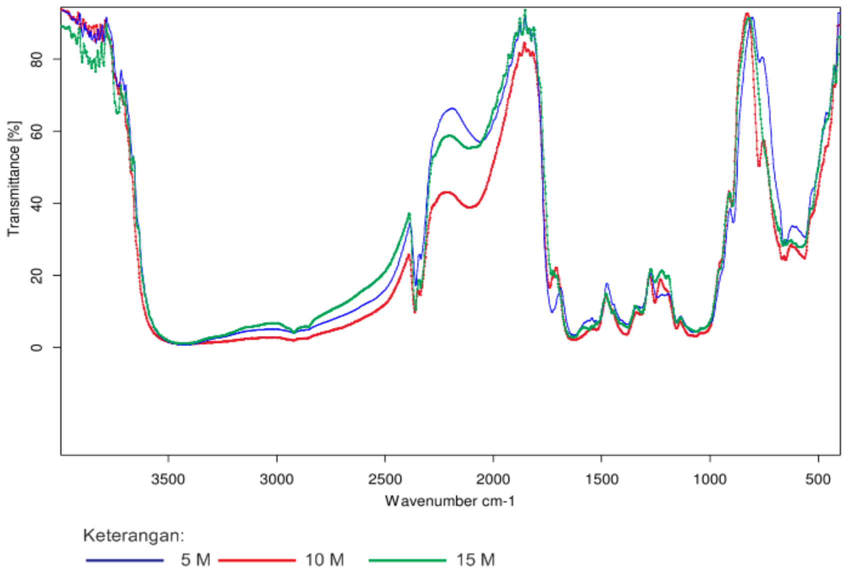

Figure 1: FT-IR Spectra of Carboxymethyl Chitosan Copolymer of Silkworm Pupa

Copolymer characterization was determined by FT-IR spectra analysis. The carboxymethylation in CMC production provoked structural changes which were clearly identified by C=O, C-O, and C-O-C groups existences. Those are such main features of CMC molecules. The vibration of O-H and C-O explain that chitosan has been substituted by carboxyl group of monochloroacetic acid [13].

FT-IR spectra reflection of CMC with some NaOH molarities (5 M, 10 M, and 15 M) shown that the carboxyl group substitution had been executed well since the carboxyl group and amine in CMC just identified. According to FT-IR spectra showed in Figure 1, the band stretched at 3132.83 cm^{-1} (5 M NaOH); 3417.42 cm^{-1} (10 M NaOH); and 3417.42 cm^{-1} (15 M NaOH) were attributed for O-H and N-H group. The asymmetrical stretched attributed at 1733.66 cm^{-1} (5 M NaOH); 1740.44 cm^{-1} (10 M NaOH); and 1827.63 cm^{-1} (15 M NaOH) were representing the carboxylate group (C=O). Amine groups ($-NH_2$) was indicated at stretching vibration at 1631.07 cm^{-1} (5 M NaOH); 1631.72 cm^{-1} (10 M NaOH); and 1631.36 cm^{-1} (15 M NaOH). The C-O band was stretched at 1381.94 cm^{-1} (5 M NaOH); 1254.11 cm^{-1} (10 M NaOH); and 1256.99 cm^{-1} (15 M NaOH). The C-O-

C absorption signal of the secondary hydroxyl group had been shifted to 1065.33 cm^{-1} (5 M NaOH); 1065.33 cm^{-1} (10 M NaOH); and 1065.84 cm^{-1} (15 M NaOH) [14, 15, 16].

3.2 Physicochemical characteristics

Yields. CMC obtained from silkworm pupa had yield level were >100 %. yields of CMC treated with 5 M, 10 M, and 15 M statistically were not significant differed ($p>0.05$). Alkalization process, notably sodium hydroxide molarity was not influenced significantly toward yield levels. It is convinced, the influential factor on CMC's yields is in carboxymethylation process which is the usage of monochloroacetic acid certainly affects the yield level due to the competition between substitution Cl^- from monochloroacetic acid with NaOH that creates sodium klorida (NaCl) and sodium glycolate ($HOCH_2$-COONa) meanwhile occurred substitution H^+ in atom C_6 with CH_2COO^- from monochloroacetic acid [11, 12].

Alkalinity (pH). Alkalinity is a parameter of H^+ concentration in a substance. In this study, CMC had alkalinity level about 4. Alkalinity level was increased along with NaOH molarity improvement. There was singificant differences between CMC treated using 5 M NaOH and CMC treated using 10 M ($p <0.05$); between CMC treated using 5 M NaOH and CMC treated using 15 M NaOH ($p<0.05$); no significant differences between CMC treated using 10 M NaOH and 15 M NaOH. It was needed an effort to neutralize alkalinity level of CMC. The reason was NaOH addition was caused precipitation so that could not be separated and drew by ethanol 96 %.

Moisture. Moisture contents of CMC tended to increase along with NaOH molarity improvement. However, no significant difference was observed in CMC moisture contents ($p>0.05$). The result showed moisture content of CMC about 11 %.

Ash. It is revealed that ash content in three groups of CMC about less than 1 % and statistically significant differed ($p<0.05$) among them. It was confirmed that molarity of NaOH in alkalization process intentionally influenced the ash content of CMC. Ash content would be increased when excess Na^+ was occurred.

Total nitrogen. Total nitrogen explains the amount of protein in a substance. Total nitrogen of three groups CMC about 4 % and no significant differences ($p>0.05$) was found among them. It showed that NaOH molarities did not obviously affect the nitrogen total neither protein level.

Solubility. The result of CMC solubility analysis showed that solubility tended to decrease along with the increase of NaOH molarities (5 M, 10 M, 15 M). However, there was no significant difference found between CMC treated using 5 M NaOH and CMC treated using 10 M NaOH ($p>0.05$) but significant difference found between CMC treated using 5 M NaOH and CMC treated using 15 M NaOH ($p<0.05$); between CMC treated using 10 M NaOH and CMC treated using 15 M NaOH ($p<0.05$) In this study, CMC solubility about 97 %. It is believed that the increased of NaOH molarities mildly improved the solubility of CMC until particular molarities level and decreased when the NaOH molarities applied is continuously improved. It was approved that solubility of CMC was increase in NaOH 2.5 – 7.5 M. However, in case NaOH molarity higher than 7.5 M, CMC solubility seems to be decline [17].

Viscosity. Three groups of CMC had viscosity values statistically significant differed ($p<0.05$) each others. It was approved that NaOH molarity had a prominent role toward viscosity level of CMC. Higher NaOH molarity applied would increase the CMC viscosity value. However, it was suggest that CMC should have viscosity value less than 100 mPa.s, normal value was 60 mPa.s. [18].

Table 1: Physico-chemical properties of carboxymethyl chitosan silkworm pupa

Parameter	**NaOH**		
	5M	**10M**	**15M**
Yields (%)	114.96 ± 4.83^a	115.94 ± 2.86^a	113.79 ± 3.67^a
Alkalinity (pH)	4.01 ± 0.01^a	4.15 ± 0.05^b	4.22 ± 0.02^b
Moisture (%)	10.80 ± 0.13^a	11.37 ± 0.63^a	11.99 ± 0.16^a
Ash (%)	0.19 ± 0.01^a	0.04 ± 0.00^b	0.37 ± 0.01^c
Nitrogen total (%)	4.06 ± 0.79^a	4.76 ± 0.59^a	4.27 ± 0.29^a
Solubility (%)	99.28 ± 0.49^a	98.36 ± 0.37^a	93.39 ± 0.24^b
Viscosity (mPa.s)	14.22 ± 1.61^a	59.67 ± 1.15^b	106.92 ± 5.69^c

[a] Mean ($\bar{x}$) ± standard deviation (SD)

[b] Figures followed by different letters in the same row showed a significant difference between intervention groups (ANOVA test, $p<0.05$)

It is undoubtable that NaOH molarities in alkalization, continued by carboxymethylation using single dose of monochloroacetic acid, and finished by washing using ethanol 96 % influenced the alkalinity (pH), ash content, and viscosity of CMC silkworm pupa. When NaOH was added to chitosan solution, some of NaOH would cause precipitation and could not draw easily by ethanol 96 %, excess H^+ which was trapped in the matrixes was occurred, led the alkalinity of CMC was less than 7. Meanwhile, when NaOH was added to chitosan and continued by monochloroacetic acid to chitosan, it would form CMC with some water (H_2O), NaCl as co-product, and sodium glycolate ($HOCH_2$-COONa) as by-product [11, 19]. Furthermore, when NaOH molarity was increased, it let some Na^+ from NaOH was remained and counted as ash content of CMC [19]. However, optimum NaOH molarity would be produced CMC with less than 0.1 % ash content. Other than that, excess NaOH created more by-product sodium glycolate led to increase the rheology capacity that was responsible to viscosity improvement.

The others precious physicochemical properties to specify characteristics of CMC but statistically were not influenced by NaOH molarities, such as yields, moistures, and totals nitrogen. A single dose of monocholoroacetic acetic acid (30 g) was highly responsible to yields and moistures values. Reaction occurred in CMC preparation may allowed substitution between H^+ on atom C_6 with CH_2COO^- which led increase of molecule weight, therefore caused yield value become >100% of chitosan. Meanwhile H^+ substitution on atom C_6 with CH_2COO^- of monochloroacetic acid was also increased the moisture value due to more occurrences H^+ bound to water. Hence, amount of water out is lesser when it is dried, led CMC become hygroscopic [13]. In addition, NaOH molarities did not give significant effect on total nitrogen of CMC. In this case, NaOH works as OH^- activator and not reacted with amino group of chitosan [20].

3.3 Appearances

Observation over CMC of silkworm pupa appearances showed that CMC has no odour, the colour is beige, and powder form.

Table 2: Appearance observation of carboxymethyl chitosan silkworm pupa

Parameters	Observation result
Colour	Beige
Odour	No odour
Form	Powder

Basic material of CMC affected of its appearances, particularly the colour. CMC obtained from silkworm pupa shells was not white as CMC of crustaceans shell. Silkworm CMC colour was beige due to the basic mater, pupa colour was brown, whereas crustacean shells colour is white. In addition, demineralization was fully responsible to decline the pigment contents in substances, even alkalization take a small part in pigment omission too. The odour was depended on the processes passed in chitosan preparations. Deproteination in chitosan process would omit the protein and fat content that was responsible to create odour.

4. Conclusion

NaOH molarity used in alkalization process of silkworm (*Bombyx mory* L.) pupa CMC preparation was influenced toward some characteristics, i.e. ash content, alkalinity and viscosity. Alkalization using 10M NaOH is proved as a precious in CMC of silkworm pupa preparation with a high value of physicochemical characteristics.

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