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A Novel Glucomannan Extraction Method Using Aqueous Two-Phase System

The process of glucomannan extraction used Aqueous Two-Phase System Method

Enny Sholichah[1,2], Bambang Purwono[1*], Agnes Murdiati[3], Akhmad Syoufian[1], Nok Afifah[2] and Achmat Sarifudin[2]

[1]Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Gadjah Mada, Jl. Sekip Utara, Bulaksumur, Yogyakarta, Indonesia
[2]Research Center for Appropriate Technology, National Research and Innovation Agency Jl. KS. Tubun No. 5 Subang, West Java, Indonesia
[3]Department of Food and Agriculture Product Technology, Faculty of Agriculture Technology Universitas Gadjah Mada, Jl. Flora No.1 Bulak sumur, Yogyakarta, Indonesia

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SUMMARY

Research background. Glucomannan is a polysaccharide compound used widely in food and pharmacies industries. Tuber of *Amorpophallus muelleri* Blume is called as *porang* in Indonesia. Ethanol extraction system is commonly used to extract glucomannan from *porang* flour, however, the method still shows some limitations. Glucomannan obtained from ethanol extraction method contains protein higher than which of the standard glucomannan. The current research explored the salting-out effect of salts of aqueous two-phase system for glucomannan extraction process. Therefore, protein can be removed from glucomannan flour enhancing the purity of the obtained glucomannan.

*Corresponding author: purwono.bambang@ugm.ac.id

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1 *Experimental approach.* A novel glucomannan extraction method using aqueous two-phase
2 system (ATPS) which consisted of salt and ethanol was investigated. The salts of Na_2HPO_4
3 and K_2HPO_4 solution in 3 different concentration levels (1 %, 2 %, and 3 %), mixed with 40 %
4 ethanol with 1:1 ratio, were used to compose ATPS. The performance of ATPS method on
5 glucomannan extraction was observed based on the phase separation and the characteristics
6 of glucomannan including proximate, color, thermal properties and surface morphology of
7 glucomannan. The statistical analysis was performed to test the significant differences
8 between the mean value of each treatment. The statistical significance level (P) was set at
9 0.05.
10 *Results and conclusions.* Results indicated that ATPS showed an ability to separate *porang*
11 flour in solution into 3 parts namely bottom (F1), middle (F2) and top (F3) parts. The bottom
12 (F1) and middle (F2) parts were rich of glucomannan and starch, respectively while the top
13 part consisted of ethanol-soluble compound. Salts impacted the yield of glucomannan and the
14 characteristics of the obtained glucomannan including the color, impurities (protein and ash)
15 content, thermal properties, molecular mass and surface morphology. The increasing salt
16 concentration reduced the yield of glucomannan but increased the yield of the other
17 components. ATPS reduced the protein content and increased the lightness of glucomannan.
18 Glucomannan obtained from ATPS showed higher thermal stability than the control.
19 *Novelty and scientific contribution.* Salting-out effect of salt of ATPS is commonly used in
20 protein precipitation and isolation. However, there was no report found on the implementation
21 of ATPS for glucomannan isolation. This study showed that ATPS method (Na_2HPO_4/ethanol
22 and K_2HPO_4/ethanol) is a potential novel extraction method to be implemented in glucomannan
23 extraction process.
24

25 **Keywords**: aqueous two-phase system (ATPS), glucomannan, Na_2HPO_4, K_2HPO_4, porang
26

27 INTRODUCTION

28 Tuber of *Amorpophallus muelleri* Blume which is locally as known as *porang* is the most
29 common source of glucomannan in Indonesia. Glucomannan is a polysaccharide molecule
30 which has been widely used as ingredient in food and pharmacies industries. Glucomannan
31 can be classified as a functional food since it can form short fatty acid in intestinal system,
32 improving immunity covering therapies for anti-obesity, regulation in lipid metabolism, laxative
33 effect, anti-diabetic, anti-inflammatory, prebiotic to wound dressing applications (*1*). The
34 physicochemical properties of glucomannan are influenced by the purity grade of

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glucomannan. Some global institution such as European Food Safety Authority, Food and Agriculture Organisation (FAO) and the People's Republic of China which concern on glucomannan quality issued the standard requirement for glucomannan. The European Food Safety Authority recommends glucomannan for food additive should contain protein and ash contents less than 1.5 % and 2 %, respectively (*2*). Moreover, FAO (Food and Agriculture Organization) regulation states konjac flour for food additive should contain glucomannan > 75 %, protein content <8 % and ash content <5 % (*3*). Professional Standard of the People's Republic of China for Konjac flour also specified the color of glucomannan was white in order to not affect its purposes (*4*). The major content of Amorphophallus tuber is glucomannan, however, it also contains other components which are known as the impurity components including starch, protein, and ash (*5,6*). Glucomannan content in some Amorphophallus tuber from Vietnam is about 5–9 % (m/m, db) (*7*) while *porang* tuber is about 16 % (m/m, db). Meanwhile, the starch, protein, and ash contents in *porang* tuber are 11.2 %, 4.28-9.5 %, and 0.83-5.69 % respectively (*8*). The protein, starch and other polysaccharides contents reduce the viscosity of glucomannan. Yuan *et al.* (*9*) developing method to reduce of glucomannan viscosity for beverage product by using adding some compounds of differing molecular mass such as dextrin, protein and hydrolysed guar gum. Therefore, the glucomannan content is among the other parameters that need to be analyzed to classify the quality of porang flour (*10*). Research to obtain glucomannan that meets the glucomannan standards is still required.

Purification of glucomannan from the other impurity components has been carried out using mechanical and chemical methods. The mechanical separation method has been used to separate glucomannan from *porang* flour with a yield of 33.39-66.75 % and a glucomannan content of 47.45 – 60.67 % (m/m, db) (*11*). In the chemical separation method, alcohol solvents, including ethanol and isopropyl alcohol, at various concentration levels and extraction temperatures have been successfully used to separate glucomannan from *porang* flour (*12*). The important factors in using ethanol for glucomannan extraction process are concentration of ethanol, extraction time and temperature and also the number of extraction cycle. 50 % of ethanol with 2 extraction cycles produced glucomannan with yield of 11.86-14.59 %. The addition of extraction cycles increases the glucomannan content and decreases the other impurity components in the glucomannan product, however, it significantly increases the amount of wasted ethanol, extraction time and costs. On the other hand, protein contents of isolated glucomannan using ethanol range from 3.8-5.18 % (*6,13*). Ultrasonic assisted extraction method was used for polysaccharide extraction and improved it’s biological activities (*14*). Our previous study indicated that freezing/thawing cycles pre-treatment could

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reduce the ash content of glucomannan but not its protein and color (*15*). Therefore, a method to isolate glucomannan with low ash, protein, and color content need to be developed.

Aqueous Two-Phase System (ATPS) shows an opportunity to separate biomacromolecules including glucomannan, starch, protein and organic color compounds. The Aqueous Two-Phase System (ATPS) extraction method is a liquid-liquid extraction method that involves equilibrium, phase separation and solute concentration in one stage (*16*). The principle of ATPS extraction is the difference in solubility of a substance or material in a two-phase water system. ATPS can be made using a solution system consisting of polymer/polymer, polymer/salt, ionic/salt, and salt/alcohol. The advantages of the ATPS method are more environmentally friendly, faster, easier to process, and produce glucomannan with a high level of yield, purity, and capacity (*17*). An ATPS consisted of Ammonium sulfate and ethanol was used to extract polysaccharide from *Grifola frondosa* (GFPA), *Selaginella doederleinii, Phellinus linteus* (*P. linteus*) (*18*–*20*).

ATPS formed by short-chain alcohols and salts shows many advantages such as low viscosity, high mass transfer efficiency, stable and wide phase formation, and is cheaper than polymers (*21*). The optimum concentration of ethanol for glucomannan extraction is 40-50 %. If it is less than 40 %, the granules of glucomannan can absorb the water molecules more, dissolve and make a sol form (*22*). Salt acts as an agent to form water-rich and ethanol-rich phases which could separate a component based on its solubility in water and alcohol (*23*). The selection of salt type influences the two-phase polarity and the salting-out effect. Furthermore, the salt and alcohol concentrations determine the formation of a two-phase solution (*24*). Phosphate salts are commonly used in the ATPS system to fractionate polysaccharides and proteins (*18*). It was reported that potassium phosphate dibasic (K_2HPO_4) can induce the separation of water-ethanol which bonds by hydrogen bonds (*23*) and it gives more salting-out to salting-in effect based on the anionic and cationic sequences ($SO_4^{2-} < H_2PO_4^- < Cl^- < NO_3^- < ClO_4^- < SCN^-$ and $K^+ < Na^+ < H^+ < Mg^{2+} < Ca^{2+} < Al^{3+}$) (*25*). However, no report on the implementation of ATPS for glucomannan isolation was found. Therefore, this study investigated the isolation of glucomannan from *porang* flour using ATPS which was composed of Na_2HPO_4/ethanol and K_2HPO_4/ethanol.

MATERIALS AND METHODS

Materials

The main material was *porang* flour, which was obtained from a local supplier in Subang, West Java, Indonesia. The chemical reagents were technical grade ethanol,

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analytical grade of potassium hydrogen phosphate (K_2HPO_4) (Merck, Damstadt, Germany), and sodium hydrogen phosphate (Na_2HPO_4) (Merck, Damstadt, Germany), chromatography grade of water (Merck, Damstadt, Germany) and the standards substances of polyethylene oxide/polyethylene glycol for GPC (Agilent, Shropshire, Uk) was provided by PT. Berca Niaga Medika, Indonesia.

Process of glucomannan extraction using ATPS Method

The extraction of glucomannan was conducted based on the schematic diagram of the operation procedure of an alcohol/salt ATPS extraction (*26*). ATPS was made by mixing K_2HPO_4 and Na_2HPO_4 solution at varying concentrations of 1 %; 2 % and 3 % with 40 % of ethanol at a volume ratio of 1:1. The salt concentrations were selected based on the binodal curve of ethanol/K_2HPO_4 and ethanol/Na_2HPO_4 conducted in a preliminary study (Fig. S1and Table S2). *Porang* flour that passes a 40-mesh screen was added to ATPS solution with a solid/liquid ratio of 100 g/250mL, and then it was extracted in a high-speed blender (8010BU Set; Waring Blender Laboratory, Torrington, USA) (18,000 rpm) for 2 min (Table S1). High speed mixing was chosen based on preliminary study about the effect of low speed and high speed mixing on the yield of glucomannan (Table S1). After resting for 30 min, the solution was separated into 3 fractions in which the glucomannan fraction was at the bottom layer. The glucomannan obtained from ATPS extraction namely the treated glucomannan. The control sample of glucomannan was prepared by extracting *porang* flour with 40 % ethanol.

Separation of ATPS phases

Porang flour in K_2HPO_4/ethanol and Na_2HPO_4/ethanol of ATPS separated in 3 parts. The bottom part (F1) and the middle part (F2) were salt-rich phases and the top part (F3) was ethanol-rich phase. Separation of F1 was filtered using a filter (Vitamax; Madato, Taiwan), dried the residue and obtained glucomannan fraction as the F1. The glucomannan fraction was added with 40 % of ethanol, mixed in a high speed mixer and filtered. This step was repeated by using 70 % of ethanol in order to wash the glucomannan fraction. After the washing step, the glucomannan was dried at a temperature of 50 °C for 12 h. Iodine test was used to confirm that starch component was separated from the glucomannan fraction. The following step was centrifuging the suspension of F2 and F1 parts at 4,000 rpm for 30 min and drying the residue to get the F2 (starch fraction). The yield of F1 and F2 was measured by weighing the dried F1 and F2 divided by the initial weight (porang flour). The F3 was calculated by subtracting F1 and F2 from 100 %. The control treatment was conducted the same as the ATPS separation

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Proximate contents of glucomannan

The proximate content of the glucomannan was including moisture, ash, protein, and carbohydrate. The moisture and ash contents were assayed by using a gravimetric method based on determination of moisture and ash content in animal feed: AOAC official method 942.05 revisited (*27*,*28*). Nitrogen combustion method was used to measure protein content using a protein analyzer (Buchi Dumaster, D480; Elementar Analysenseysteme, Hanau, Germany). The calculation of protein content used a nitrogen conversion factor of 5.7 according to the standards of the U.S. Food Chemical Codex (FCC) and European Commission (*2*). Glucomannan content was calculated as the percentage of carbohydrates which was determined by a by-different method (*3*).

Color

A spectrophotometer (CM700D; Minolta Konica, Osaka, Japan) was used to measure the color of the glucomannan sample. The sample was set in a cuvette, then the color parameter reading was carried out. The data were reported including value of L* (lightness index), a* (red to green index), and b* (yellow to blue index). The change color of treated sample compared to control and calculated by using following equation:

$$\Delta E = \sqrt{(\Delta L^2} + \Delta a^2 + \Delta b^2 \qquad /1/$$

Fourier Transform Infra Red (FTIR) spectroscopy

FTIR spectrophotometer (Alpha II; Bruker, Ettlingen, Germany) was used to identify the functional groups in the glucomannan obtained. The analysis was carried out in the infrared region, namely wave numbers 400-4,000 cm^{-1} with a resolution of 4 cm^{-1}.

Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H NMR spectra was read on NMR (JNM ECZR500; Jeol, Tokyo, Japan). The preparation sample based on (*29*) with modification. A total of 40 mg of sample was dissolved in D_2O (40mg/mL) and mixed for 1 h, and the measurement was run at a temperature of 25 °C at 500 MHz. Chemical shifts are expressed in ppm and use trimethylsilane (TMS) as the reference standard. ^{13}C_CPMAS (solid NMR) of spectra were recorded on a NMR (JNM-ECZ500R/S1 DPX200; Jeol, Tokyo, Japan) that was operated at a frequency of 125.76 MHz and employed a solid-state probe equipped with 4 mm (o.d) spinner. The spectra were recorded at 5000 Scan, relaxation delay 15 s, spin rate10 kHz. The integration value of

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anomeric proton from ^{1}H-NMR spectra was used to calculate the ratio of mannose and glucose. The results of the integration of anomeric carbon area at a chemical shift of 105 ppm and methyl carbon at 21 ppm were used to calculate the degree of acetylation (DA) with the following equation (*30*):

$$DA = \frac{100 \cdot I_A}{I_{Ac}} \qquad /2/$$

Where :

DA : degree of acetylation

I_A : Integrated area of chemical shift at 105 ppm

I_{Ac} : Integrated area of chemical shift at 21 ppm

Molecular Mass

The molecular mass of the glucomannan sample was determined by using a Gel Permeation Chromatography/Size Exclusion Chromatography (GPC/SEC) system (1260 Infinity II; Agilent Technologies, Wadbronn,Germany) with column PL 2080-0700 and two detectors of a refractive index (RI) detector and a viscometer detector. The mobile phase consisted of water and 0.02 % NaN_3. The glucomannan sample was dissolved in water (1 mg/mL), stirred, and filtered using Millipore 0.45µL. The flow rate of eluent was 0.5 mL/min and the columns and detectors were maintained at 35 °C (*15*). Prior to utilization, the GPC/SEC was calibrated by using standard substances of polyethylene oxide/polyethylene glycol (Agilent, Shropshire, UK).

Thermal analysis

The thermal properties of the sample were assessed by a DTA-TG apparatus (DTG-60; Shimadzu, Kyoto, Japan). The sample of 5 mg was placed on the aluminium sample pan, sealed, and heated from a temperature of 25 °C to 450 °C with an average heating rate of 10 °C/min (*7*).

Morphological and residual mineral analysis using SEM-EDX

The morphological properties of the glucomannan sample were observed by a Scanning Electron Microscope (JSM-IT300LV; Jeol, Tokyo, Japan). Prior to analysis, the sample was sieved through an 80-mesh screen. The sample was located on a metal stub which was previously covered with double-sided adhesive tape. An air blower pump dust cleaner rubber was used to remove the excess sample from the metal stub. Prior to

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observation, the sample was coated with gold and it was examined with an accelerating voltage of 2 kV at magnification levels of 100, 500, and 1,000 times.

Statistical analysis

A completely randomized design (CRD) with 2 factors including the salt (Na_2HPO_4 and K_2HPO_4) and salt concentration level (1; 2 and 3 %) was used as the experimental design. The effects of ATPS extraction including the separation, proximate, color, and thermal properties of glucomannan, were observed. Multivariate Variance analysis (MANOVA) and then followed by a post-hoc Duncan test were performed to test the significant differences between the mean values of each treatment (*15*). The statistical significance level (p) was set at 0.05. The statistical analysis was conducted by using SPSS version 26 (*31*).

RESULTS AND DISCUSSION

ATPS method for glucomannan extraction

The ATPS composed of ethanol, salt and water, where the binodal curves (Fig. S1) were constructed to determine the compositions of Na_2HPO_4/ethanol and K_2HPO_4/ethanol used in this experiment. It shows that the binodal curve limited the two ATPS zones namely monophasic region (lower side) and biphasic region (upper side). Ethanol with concentration of 40 % (*22*) was chosen as the starting concentration of ethanol used in the further calculation of the salt concentrations. Then, the concentrations of salts of 1, 2, and 3 % (*32*) were selected based on their presence in the monophasic region and near with critical point of the binodal curve. Based on these, the compositions of ATPS used in this study were presented in the Table S2.

Fig. S2 illustrates the proposed mechanism of glucomannan extraction using the ATPS system based on the visual observation during the extraction process. Extraction of glucomannan from *porang* flour using ATPS made from Na_2HPO_4/ethanol and K_2HPO_4/ethanol produced 3 parts, namely the bottom (F1), middle (F2) and top part (F3). The bottom and middle parts (F1 and F2) were rich in salt-water phases. Based on the iodine testing (*5*), the color of the bottom part (F1) and top part (F3) did not alter when they were tested with iodine, while the middle part (F2) showed a dark blue color. This result indicated that the bottom part was rich in glucomannan and the middle part was high in starch content. The top part (F3) was the ethanol-rich phase which contained many simple sugar components, dyes and other components. The formation of three layers of the extraction was greatly influenced by the salts in the ATPS composition. The K_2HPO_4 and Na_2HPO_4 are dissociated into K^+, Na^+, and HPO_4^{2-}

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1 ions when they are dissolved in water. The ions have the capability to break the hydrogen bond
2 between water and ethanol because of the hydration ion mechanism (*33*). Hydration ion
3 capacity depends on the Gibb energy of hydration ion (*18*). Furthermore, both salts have
4 salting-out effect more than other salts based on the ionic strength sequences of SO_4^{2-}<$H_2PO_4^-$
5 <Cl^-<NO_3^-<ClO_4^-<SCN^- and K^+<Na^+<H^+<Mg^{2+}<Ca^{2+}<Al^{3+} (*23*,*25*). When the salts are added
6 into the glucomannan solution, there are competitions between the ions and the glucomannan
7 molecules to bind the water. This phenomenon reduces the glucomannan solubility in the
8 solution, and eventually the glucomannan molecules precipitate in the bottom phase. The
9 similar result was reported during polysaccharide extraction of *Lycium barbarum L* with ATPS
10 (*34*). The F1 and F2 (Fig. S2) which consisted of polysaccharides were separated into 2
11 fractions due to the difference of their water absorption capacity and molecular mass. The
12 water absorption capacity of glucomannan, starch and cellulose are 50-100 g/g, 0.884-0.951
13 g/g, and 40 g/g respectively (*5*,*35*,*36*).

14 The percentages of the results of ATPS separation are shown in Table 1. Results
15 indicated that the yield of glucomannan (F1) from ATPS extraction did not significantly change
16 but tend become lower than that of the control. However, the percentages of the middle part
17 (F2) and the top part ethanol (F3) from ATPS extraction were higher than that of the control.
18 These might be occurred due to some glucomannan molecules were not separated from the
19 starch in the middle part (F2) since they almost have similar molecular mass of 10^6 Dalton.
20 Other possibility is that there is an interaction between glucomannan and the other
21 polysaccharide molecules through an ion bridging mechanism. Glucomannan interacts with
22 xanthan gum via Na^+ and Ca^{2+} ions (*36*). In our experiment, ATPS with K_2HPO_4 yielded higher
23 F2 part than that with Na_2HPO_4. The cation of K^+ has bigger atomic size than Na^+, therefore,
24 they might have different ionic strength when they interact with starch and protein molecules
25 according to the lyotropic sequence (*25*). Results also showed that increasing salt
26 concentration up to 3 % tended to increase the yield of F1 and F2. These might be due to the
27 increase of the salting-out effect of salt. The top part (F3) of the ATPS extraction was higher
28 than that of the control (ethanol extraction). F3 contained non-polar components such as
29 flavonoids, polyphenols, ethanol soluble alkaloids as also reported by Wan *et al.* and Xi *et al.*
30 (*23*,*26*). Moreover, the F3 also contained other organic compounds such as carotenoids,
31 oligosaccharides, and monosaccharides which were soluble in the ethanol phase.
32

33 *Physicochemical properties*

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1 Results of proximate analysis (Table 2) indicated that the protein content of
2 glucomannan extracted using ATPS was significantly lower than that of the control
3 glucomannan ($p<0.05$). Cheng *et al.* (*37*) reported that protein could be denatured in the
4 aqueous-organic solvents and precipitated in the aqueous phase. Furthermore, the salting-out
5 effect of K_2HPO_4 and Na_2HPO_4 caused the solubility of protein in water decrease and
6 precipitated in the middle part (F2) (Table 3). Therefore, the protein content of glucomannan
7 obtained from the ATPS method was lower than that obtained by the conventional extraction
8 method (control). These results were similar to the report of Antune *et al.* (*18*) in that the
9 separation of polysaccharides with ATPS resulted in the lower protein content. The ATPS
10 extraction effectively reduced the protein content until <0.5 %. Therefore, the protein content
11 obtained from this study was lower than that obtained by ethanol extraction (3.8-4.4 %) (*13*),
12 FTC-pre-treatment methods (1.4-2.3 %) (*15*) and microwave assisted extraction (0.82 %) (*38*).
13 Results (Table 2) also showed that the ash content of glucomannan extracted by
14 Na_2HPO_4/ethanol ATPS was lower than that obtained from K_2HPO_4/ethanol. This result
15 indicated that glucomannan contained residue of potassium and phosphate from ATPS. The
16 result of minerals measurement and SEM-EDX observation showed that the K dan P elements
17 in the glucomannan obtained by K_2HPO_4/ethanol ATPS (Table 3) were higher than that by
18 ethanol method. K^+ ions might have higher ionic interaction with glucomannan molecules than
19 the Na+ ions because the atomic size of K^+ ions is higher than the Na^+ ions. The degree of
20 ionic binding is directly related to the nuclear charge effect that depends on the size and charge
21 of the dissolved ions (*39*).
22 Table 4 indicates that glucomannan from the ATPS extraction was brighter in color than
23 that of the control ($p<0.05$). Results indicated that the lightness values of glucomannan from
24 ATPS method increased significantly compared to those of the control. Moreover, the change
25 value (ΔE) exhibited the treated samples were different with the control. Results also indicated
26 that higher salt percentages of ATPS tended to produce glucomannan with lower lightness
27 values. Among the treated samples, glucomannan produced from ATPS of Na_2HPO_4 2 %
28 showed the highest level of lightness and the lowest values of a and b and the highest value
29 of change value (ΔE). ATPS can inhibit browning reactions by inhibiting mechanisms of the
30 activity of oxidizing enzymes (*22*). Moreover, the salt addition impacted the increasing polarity
31 of the bottom phase which resulted in an increase in the solubility of organic compounds in the
32 ethanol-rich phase (top phase) including the carotenoid compounds. The color of glucomannan
33 is influenced by the natural yellow-orange color characteristics of *porang* tuber (*5*). *Porang*
34 tuber contains organic compounds such as carotenoids, polyphenols, and other color

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1 compounds which are susceptible to oxidation reactions. The oxidizing reaction occurs more
2 intensively during the processing stages of *porang* chip and flour particularly when the sliced
3 *porang* tubers are exposed to air (*40*). Therefore, the color of glucomannan from ATPS
4 exhibited higher lightness and lower a and b values.
5
6 *Structural properties*
7 Results showed that ATPS extraction produced glucomannan with molecular mass (*M*)
8 ranging from $1.55·10^6$ to $2.9·10^6$ g/mol and molecular number (*Mn*) was $5.42·10^5$-$9.95·10^5$
9 (Table 5). This result indicated that the molecular weight of glucomannan was not affected by
10 the ATPS extraction method ($p>0.05$). Jiang *et al.* (*41*) reported that salt did not influence the
11 degradation of molecular weight of polysaccharide. The molecular mass (*M*) of glucomannan
12 depends on the species of Amorphophallus. For instance, the molecular mass of glucomannan
13 isolated from *Amorphophallus paeoniifolius, Amorphophallus panomensis and*
14 *Amorphophallus tonkinensis,* and *Amorphophallus konjac* are $1.115·10^6$, $1.023·10^6$, $1.043·10^6$,
15 $9.1·10^5$ g/mol, respectively (*7,13*).
16 Table 5 indicates that the isolated glucomannan has PDI ranging from 2.27-3.35 which
17 was similar to the previous studies (*5,42*). This result showed that isolated glucomannan has
18 a broad molecular mass distribution. This result also indicated that the synthesis of
19 glucomannan occurred by an uncontrolled reaction mechanism namely chain reaction. The
20 chain reaction mechanism leads to polymer chains with widely varying molecular mass
21 indicated by PDI of between 1.5 and 20 (*43*). Qi *et al*. (*44*) reported that the biosynthesis
22 pathway of glucomannan in plant occurred by enzymatic mechanisms producing glucomannan
23 molecules with varying chain lengths.
24 The FTIR spectra of glucomannan obtained from ATPS extraction (Fig. 1) show the
25 groups of glucomannan structure. The wide peak at 2900-3600 cm^{-1} is a typical peak from the
26 OH group originating from glucomannan monomers, both glucose and mannose. In addition,
27 the broad peak indicates the large number of hydrogen bonds or bound water molecules. The
28 -CH- aliphatic, C=O of the acetyl, C-H bending, and C-O-C group appear at 2800-2900 cm^{-1},
29 1724 cm^{-1}, 1200-1400 cm^{-1}, and 1000-1100 cm^{-1} respectively. The protein content as another
30 component of glucomannan was detected by the presence of the amide group peak -CONH–
31 at a wave number of 1640 cm^{-1}.The FTIR spectra of glucomannan obtained showed the same
32 pattern as glucomannan obtained from extraction using ethanol (*45*). The bound water
33 indicated by peak at 1611 cm^{-1} and 1411 cm^{-1} (*29*). The peaks of 878 cm^{-1} and 800 cm^{-1} were

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attributed to β-glucosidic and β-mannosidic linkages, respectively. This result was in line with the glucomannan extracted from *Amorphophallus konjac* (*13*).

NMR spectra of glucomannan revealed the chemical shift of proton and carbon of glucomannan. The proton and carbon spectra patterns of glucomannan were identical with those of control glucomannan (Fig. 2a-2d). The difference is the appearance of a proton from the salt residue (Na_2HPO_4 or K_2HPO_4) at a chemical shift of 5.703 ppm. Chemical shift of anomeric proton (H1) of glucomannan control are seen at chemical shifts 5.128 ppm for H1-mannose and 5.213 ppm for H1 glucose (Fig. 2a). Meanwhile, protons (H2-H6) are at a chemical shift of 3.822-4.580 ppm. Proton anomeric of the treated glucomannan (Fig. 2b) shows the chemical shift at 5.053 ppm and 5.268-5.280 for H_1-mannose and glucose, respectively. Meanwhile, the proton shift from H2-H6 (mannose/glucose) is 3.933-4.637 ppm and the proton of methyl group (-CH3) of acetyl appeare at δ 2.7 ppm. Enomoto-Rogers *et al.* (*46*) reported that 1.9 ppm, 2.0 ppm and 2.1 ppm are the chemical shift values for proton of the acetyl group and the chemical shift is 3.3-4.1 ppm are H2-H6 protons from polysaccharides (*47*). The ^1H NMR spectra of glucomannan control and treated glucomannan were similar with the previous study (*48*).

The ratio of glucose and mannose in the glucomannan was calculated by the ratio of the integration of H1 of glucose and mannose. The control glucomannan and treated glucomannan obtained had mannose/glucose ratios of 1.09/1.00 (1.09) and 0.71/1.0 (0.71), respectively. Meanwhile, the ratio of H1 mannose/glucose of glucomannan extracted from *Amorphopallus panomensis* -Vietnam and *Amorphophallus konjac* was 1.00/0.13 and 1.6/1, respectively (*49*,*50*). This result exbited that the species of the Amorphophallus influenced the chemical structure of glucomannan (*42*).

The ^{13}C NMR spectra of anomeric carbon of control glucomannan and treated glucomannan were 102.941-105.147 ppm (Fig. 2c) and 103.554-105.392 ppm (Fig. 2d). Meanwhile, the chemical shift of C2-C5 is 50.489-82.843 ppm with overlapping peaks indicating that the carbon atoms of the pyranose ring, namely glucose and mannose, have almost the same character (*50*). C6 has chemical shift at 62.070 ppm for control glucomannan and 61.396-62.254 ppm for treated glucomannan. The acetyl group appeared as C=O at chemical shift at 170 ppm and CH_3 at 21 ppm. The α and β glucose and mannose configuration could be *determined* by using the chemical shift of anomeric proton (H1)/carbon (C1) in 90–110 ppm and 4.5–5.5 ppm (*29*). Chemical shift of anomeric carbon 98-108 ppm and 101-105 ppm indicated the α-glycoside or β-glycoside bonds, respectively (*48*). According to the chemical shift of anomeric carbon, the β-glycosides bonds contructed both glucomannan

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structures. The involvement of C4 in the formation of glycosidic bonds is shown at a chemical shift of 79.24 ppm (*49*,*51*). Therefore, based on the proton and carbon shift values, the α-glycoside bonds formed are β(1→4)-glycoside and β(1→6)-glycoside. These bonds indicate that the structure of glucomannan has a straight chain as a backbone and branched structure (*48*).

The peaks for acetyl carbon $-CH_3$ and C=O at chemical shifts of 21 ppm and 170 ppm respectively were low intensity. The ratio of peak areas at 21 ppm and 105 ppm indicated the degree of acetylation (DA) of glucomannan (*30*). The result showed that the DA of control glucomannan and treated glucomannan were 4.46 and 1.88. The reducing value of DA of treated glucomannan due to deacetylation process is due to interaction with Na_2HPO_4 salt (*52*).

Thermal Properties

Results indicated that the glucomannan extraction by ATPS influenced the thermal properties of the obtained glucomannan (Fig. 3a-3d). The TGA and DSC thermogram of all samples exhibited consistent thermal degradation patterns of glucomannan in which the first and second were dehydration and degradation patterns, respectively. The dehydration process of the control glucomannan required higher energy and occurred in higher temperature of than that of the treated samples (Table S3). Fig. 3a-3b and Table S3 shows that the onset temperature of degradation process of the control glucomannan was higher than that of glucomannan from the ATPS extraction. This indicated that the glucomannan from the ATPS extraction was easier to degrade than the control glucomannan in starting point. The protein content of the control glucomannan was higher than that of the treated samples. Protein and glucomannan could interact through hydrogen bond between hydroxyl group (-OH) and amine group (-NH-) (*53*), this interaction could enhance the thermal stability of protein.

Thermal gravimetric analysis (Fig. 3c-3d and Table S4) showed that the weight loss of the control glucomannan was higher than the treated samples. This result indicated that phosphate residue might be act as the stabilizer of glucomannan molecules. This result was similar to the previous results in that there was an impact of salt in potato starch and iota-carrageenan solutions (*54*). Deng *et al.* (*55*) reported that the leftover phosphate in the glucomannan sample prevents weight loss during the degradation step.

Morphological characteristic

The morphological surface of glucomannan particles is shown in Fig. 4. The morphological surface of the particles of the control glucomannan was different from those of

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the treated samples. The particles of the treated samples were relatively uniform in size compared to the particles of the control glucomannan. The morphological surface of control glucomannan was similar to that of the purified konjac glucomannan as reported by Yanuriati *et al.* (*5*). However, there were no significant differences among the particles of the treated samples. This result emphasized that the ATPS extraction method could produce uniform glucomannan particles and the extraction method did not destroy the glucomannan particles. The surface of glucomannan particles exhibited wrinkled surfaces. Some impurities can be trapped in the surface of glucomannan, including starch, cellulose, protein, and soluble sugar (*56*). The presence of the phosphate group might result in glucomannan particles with rougher surfaces and larger size (*55*).

CONCLUSIONS

ATPS (Aqueous Two Phase System) has shown as a novel green method to isolate glucomannan from porang (*Amorphophallus muelleri* Blume) flour over the conventional glucomannan isolation using ethanol extraction method. The ATPS extraction method separated glucomannan from the other components to become 3 parts including the bottom part (glucomannan), the middle part (starch and other water-soluble compounds), and the top part (ethanol-soluble compound). The ATPS method produced glucomannan with a brighter color, lower protein content and stable thermal properties than control sample. ATPS with higher salt percentages inclined to produce glucomannan with lower lightness values. The glucomannan particles with uniform shapes were observed by SEM-EDX.

Glucomannan obtained from ATPS using Na_2HPO_4 showed better properties in terms of ash and protein content, color, molecular mass, PDI, and thermal properties than those obtained from K_2HPO_4. Therefore, ATPS from Na_2HPO_4, ethanol, and water mixture showed as a promising new method for the glucomannan extraction process. Optimization scalling up capacity of ATPS extraction method is recommended as future research before the industrial application of ATPS for the glucomannan extraction method.

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CONFLICT OF INTEREST

The authors declare that they have no competing financial interests or personal relationships that could have appeared to affect the research reported in this paper.

AUTHORS' CONTRIBUTION

Enny Sholichah contributed to investigation, and writing of the original draft. Bambang Purwono contributed to conceptualization, supervision, and review. Agnes Murdiati and Akhmad Syoufian supervised and reviewed the manuscript. Achmat Sarifudin and Nok Afifah participated in Investigation, and writing and reviewing the manuscript.

ORCID ID

B. Purwono https://orcid.org/0000-0001-6284-7782
E. Sholichah https://orcid.org/0000-0002-9902-7873
A. Murdiati https://orcid.org/0000-0001-5664-7792
A. Syoufian https://orcid.org/0000-0002-2615-9437
N. Afifah https://orcid.org/0000-0001-5774-7226
A. Sarifudin https://orcid.org/0000-0002-9964-517X

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23
24 Table 1. Result of separation in ATPS extraction

Sample	*m*(The bottom part-F1)/%	*m*(The middle part-F2)/%	*m*(The top part-F3)/%
Control	(54.40±2.97)[a]	(24.70±4.10)[a]	(20.90±7.07)[a]
Na_2HPO_4-1	(51.23±2.91)[a]	(24.35±2.58)[a]	(24.42±5.49)[a]
Na_2HPO_4-2	(44.27±5.18)[a]	(26.94±4.51)[a]	(28.79±0.67)[a]
Na_2HPO_4-3	(51.46±0.73)[a]	(23.73±4.39)[a]	(24.81±3.66)[a]
K_2HPO_4-1	(46.62±9.07)[a]	(27.76±3.29)[a]	(25.62±5.78)[a]
K_2HPO_4-2	(46.53±1.15)[a]	(27.84±0.25)[a]	(26.45±2.56)[a]
K_2HPO_4-3	(49.05±0.64)[a]	(27.57±1.10)[a]	(22.93±2.38)[a]

25 The average value marked by different letter notations in column showed a noticeable difference according
26 to post-hoc tests of Duncan at a significant level of 5%. F1 : glucomannan; F2 : Starch and water soluble
27 compound and F3 : ethanol soluble compound
28

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1 Table 2. Proximate content on dry basis of glucomannan obtained from ethanol extraction (control) and ATPS
2 extraction

Sample	*m*(Moisture content)/%	*m*(Ash)/%	*m*(Protein)/%	*m*(Glucomannan)/%
Porang flour	(7.39±0.58)	(4.09±0.57)	(9.70±2.81)	(78.43±1.01)
Control	$(7.22±0.88)^{b}$	$(0.79±0.01)^{b}$	$(0.95±0.21)^{b}$	$(91.03±0.69)^{cd}$
Na_2HPO_4-1	$(13.04±0.35)^{d}$	$(0.44±0.18)^{a}$	$(0.51±0.12)^{a}$	$(86.01±0.65)^{b}$
Na_2HPO_4-2	$(8.52±0.04)^{c}$	$(0.41±0.07)^{a}$	$(0.43±0.01)^{a}$	$(90.64±0.12)^{c}$
Na_2HPO_4-3	$(13.91±0.13)^{d}$	$(1.26±0.20)^{bc}$	$(0.51±0.01)^{a}$	$(84.32±0.34)^{a}$
K_2HPO_4-1	$(8.52±0.00)^{a}$	$(0.75±0.02)^{ab}$	$(0.57±0.12)^{a}$	$(90.16±0.10)^{c}$
K_2HPO_4-2	$(6.01±0.22)^{a}$	$(1.49±0.62)^{cd}$	$(0.57±0.00)^{a}$	$(91.94±0.40)^{d}$
K_2HPO_4-3	$(7.05±0.10)^{b}$	$(2.05±0.26)^{d}$	$(0.58±0.07)^{a}$	$(90.31±0.29)^{c}$

3 The average value marked by different letter notations in column showed a noticeable difference according
4 to post-hoc tests of Duncan at a significant level of 5%.
5
6
7
8 Table 3. Sodium, Potassium and Phosphor content observed by SEM-EDX and Protein of middle part (F2)

Samples	The bottom part (F1)			*m*(Protein of the middle part (F2)/%
	Na /%	K/%	P/%	
Na_2HPO_4-1	0.35	0.13	0.19	$(7.89±0.05)^{c}$
Na_2HPO_4-2	0.42	0.21	0.21	$(7.92±0.01)^{c}$
Na_2HPO_4-3	0.26	0.11	0.10	$(6.38±0.14)^{a}$
K_2HPO_4-1	0	0.66	0.18	$(9.66±0.14)^{e}$
K_2HPO_4-2	0	0.97	0.27	$(8.60±0.01)^{d}$
K_2HPO_4-3	0	1.07	0.31	$(7.43±0.04)^{b}$

9 F1 : glucomannan and F2 : Starch and water soluble compound
10
11
12
13 Table 4. Color of glucomannan obtained from ethanol extraction (control) and ATPS extraction

Sample	*L*	*a*	*b*	*ΔE*
Control	$(73.57±0.00)^{a}$	$(4.53±0.02)^{g}$	$(10.48±0.00)^{c}$	0
Na_2HPO_4-1	$(79.16±0.00)^{f}$	$(3.25±0.01)^{c}$	$(10.30±0.01)^{b}$	$(15.88±0.05)^{d}$
Na_2HPO_4-2	$(80.80±0.01)^{g}$	$(2.71±0.00)^{a}$	$(9.56±0.01)^{a}$	$(26.57±0.07)^{e}$
Na_2HPO_4-3	$(78.14±0.01)^{d}$	$(3.85±0.01)^{d}$	$(10.89±0.01)^{d}$	$(11.43±0.03)^{c}$
K_2HPO_4-1	$(79.11±0.01)^{e}$	$(3.11±0.00)^{b}$	$(10.29±0.00)^{b}$	$(15.92±0.01)^{d}$
K_2HPO_4-2	$(75.49±0.00)^{b}$	$(4.18±0.00)^{f}$	$(11.89±0.01)^{f}$	$(2.77±0.01)^{a}$
K_2HPO_4-3	$(76.18±0.01)^{c}$	$(4.01±0.01)^{e}$	$(11.46±0.01)^{e}$	$(3.57±0.04)^{b}$

14 The average value marked by different letter in column notations showed a noticeable difference according to
15 post-hoc tests of Duncan at a significant level of 5%.
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1 Table 5. Molecular mass of glucomannan obtained from ethanol extraction (control) and ATPS extraction

Sample	*Mw*/(g/mol)	*Mn*/(g/mol)	PDI
Control	$(2.25\cdot10^6\pm1.34\cdot10^5)^{b}$	$(7.48\cdot10^5\pm2.97\cdot10)^{4ab}$	$(3.01\pm0.29)^{ab}$
Na_2HPO_4-1	$(1.55\cdot10^6\pm5.94\cdot10^5)^{ab}$	$(6.70\cdot10^5\pm8.49\cdot10)^{4ab}$	$(2.27\pm0.59)^{a}$
Na_2HPO_4-2	$(2.30\cdot10^6\pm2.12\cdot10)^{5b}$	$(9.95\cdot10^5\pm4.95\cdot10)^{4b}$	$(2.41\pm0.26)^{ab}$
Na_2HPO_4-3	$(2.07\cdot10^6\pm4.03\cdot10^5)^{ab}$	$(7.28\cdot10^5\pm1.68\cdot10)^{5ab}$	$(2.86\pm0.11)^{ab}$
K_2HPO_4-1	$(2.9\cdot10^6\pm3.11\cdot10^5)^{a}$	$(6.38\cdot10^5\pm3.29\cdot10)^{5ab}$	$(2.49\pm0.41)^{ab}$
K_2HPO_4-2	$(1.8\cdot10^6\pm3.61\cdot10^5)^{ab}$	$(7.47\cdot10^5\pm2.07\cdot10)^{5ab}$	$(2.83\pm0.35)^{ab}$
K_2HPO_4-3	$(1.7\cdot10^6\pm9.19\cdot10^4)^{ab}$	$(5.42\cdot10^5\pm6.72\cdot10)^{4a}$	$(3.35\pm0.59)^{b}$

2 The average value marked by different letter in column notations in showed a noticeable difference according to
3 post-hoc tests of Duncan at a significant level of 5%. M-: the mass of average molecular mass, Mn:the number of
4 average molecular mass, and PDI: polydiversity index.
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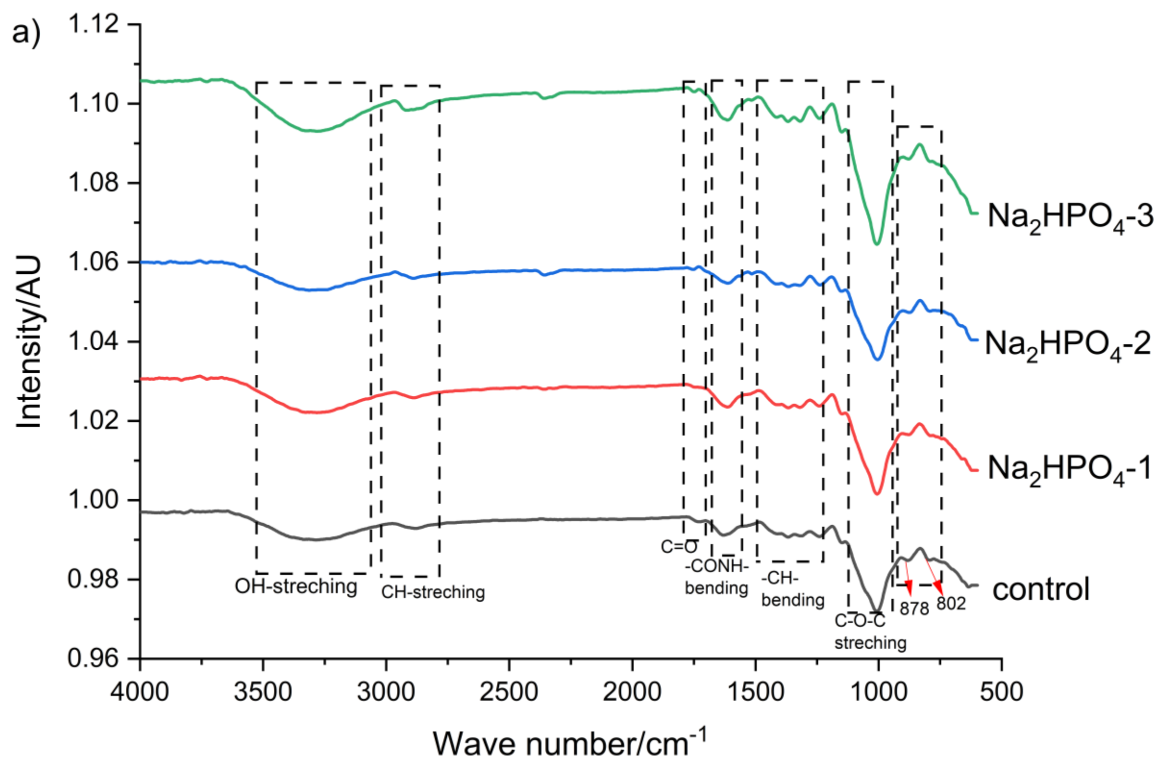

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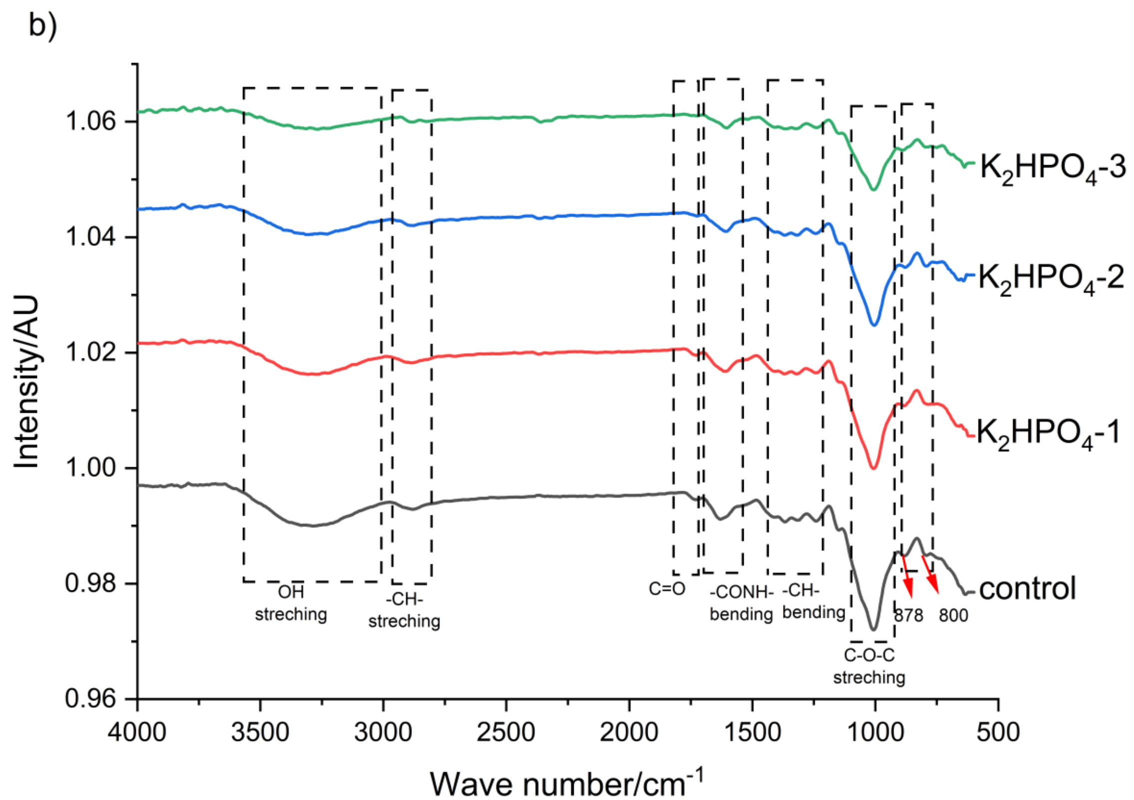

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2 Fig. 1. Spectra FTIR of glucomannan, a) glucomannan obtained from Na_2HPO_4/ethanol-ATPS, b) glucomannan
3 obtained from K_2HPO_4/ethanol-ATPS

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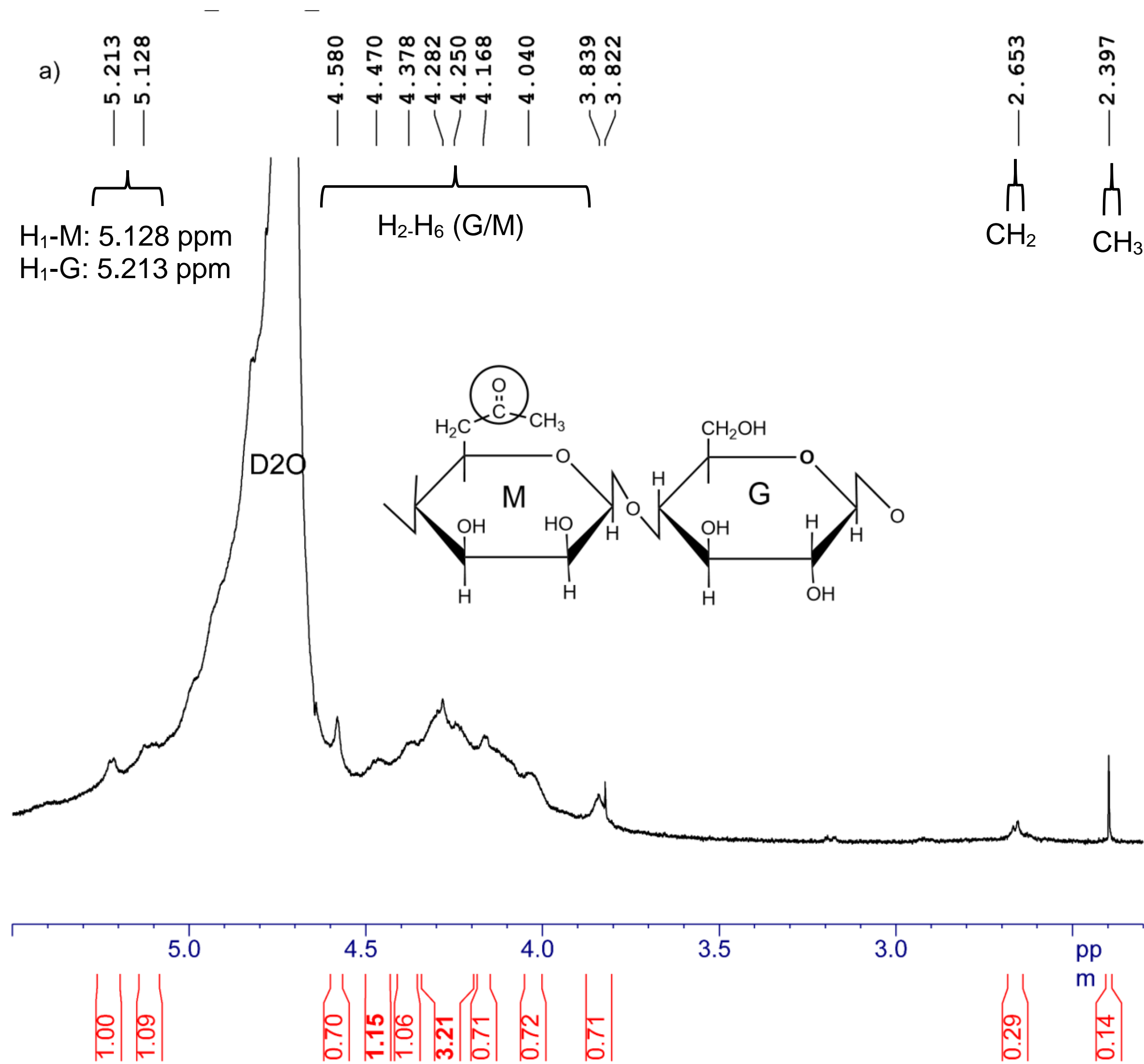

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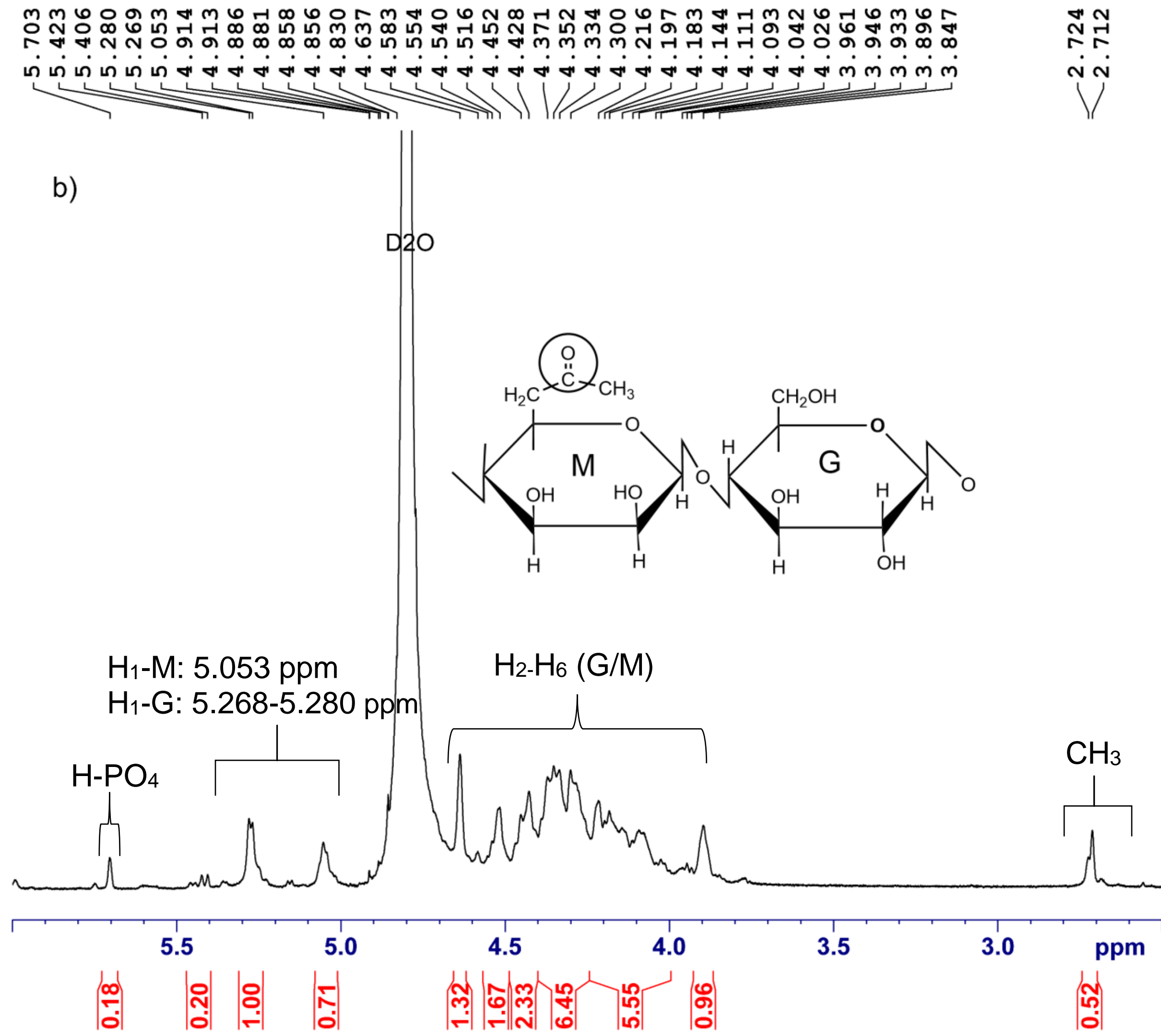

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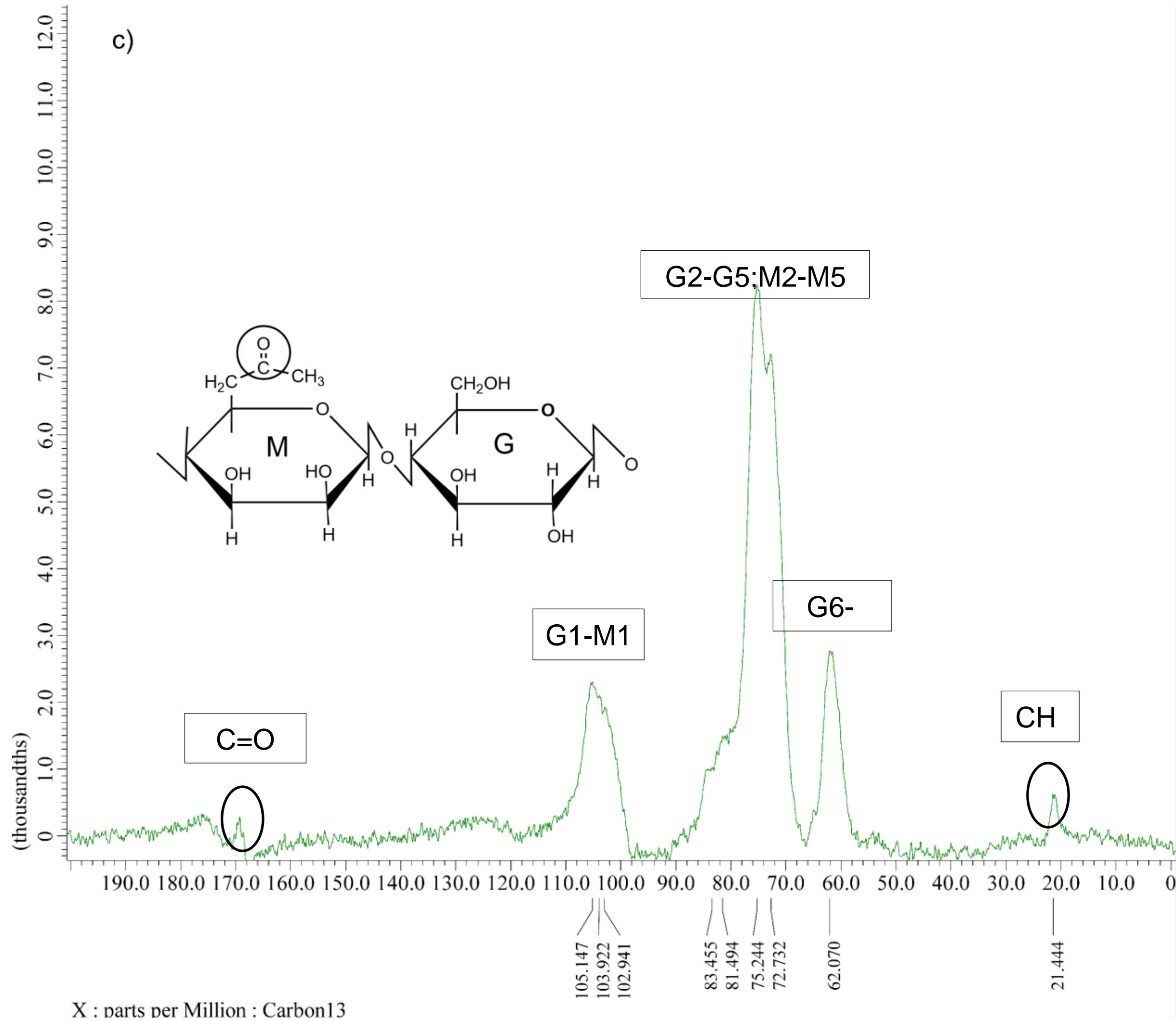

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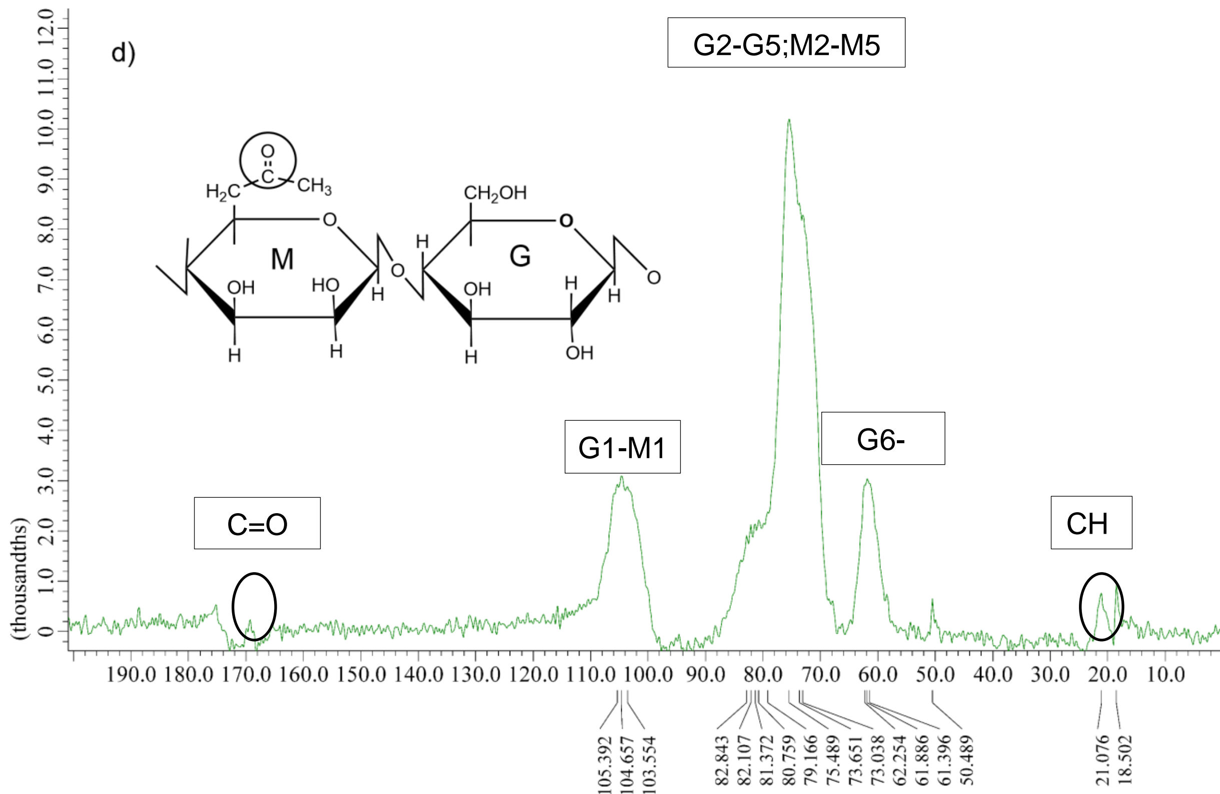

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2 Fig. 2. NMR spectra of glucomannan, a) H-NMR of control glucomannan b) H-NMR of glucomannan obtained
3 from ATPS extraction, c) C-NMR of control glucomannan and d) C-NMR of glucomannan obtained from ATPS
4 extraction

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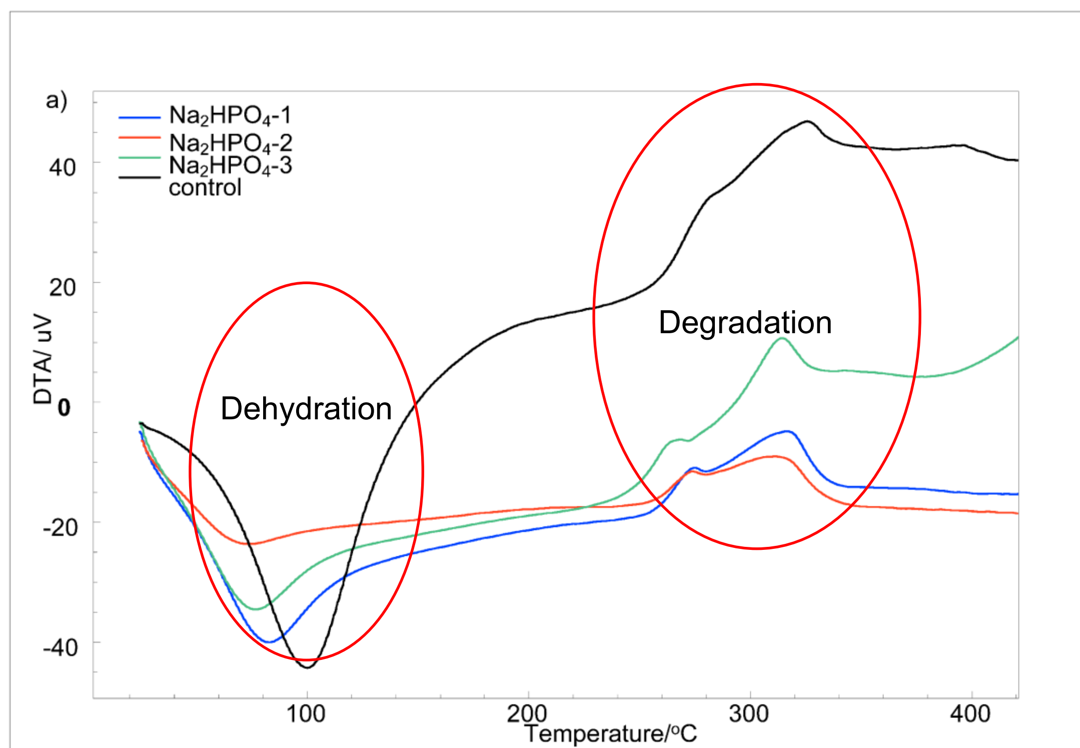

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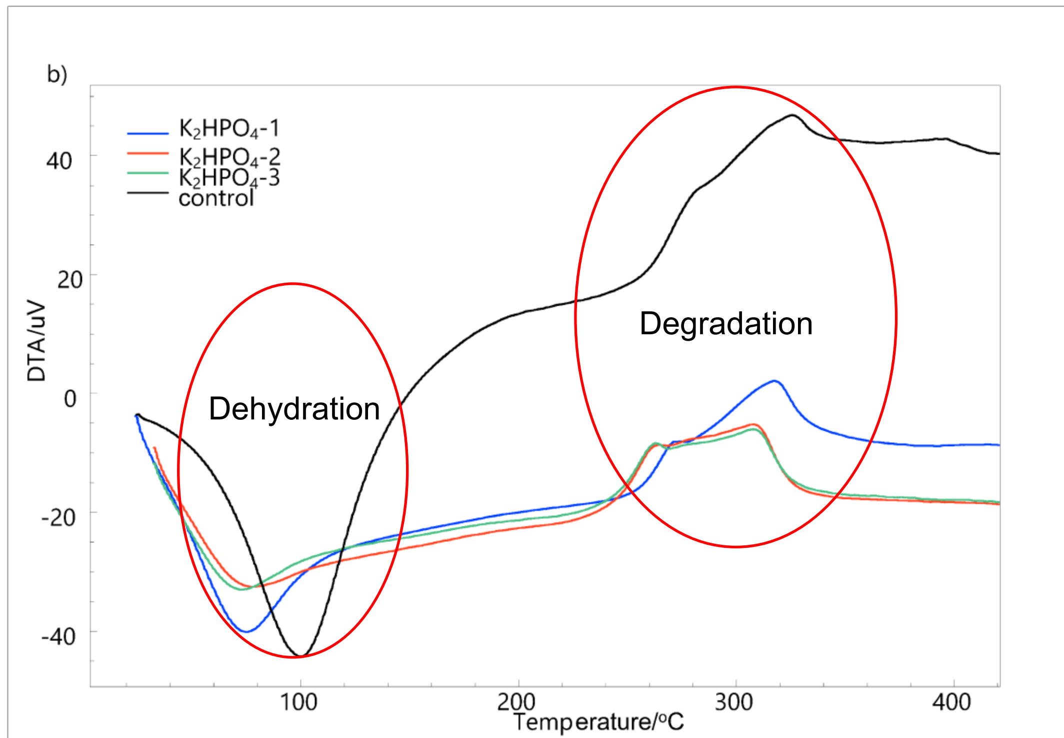

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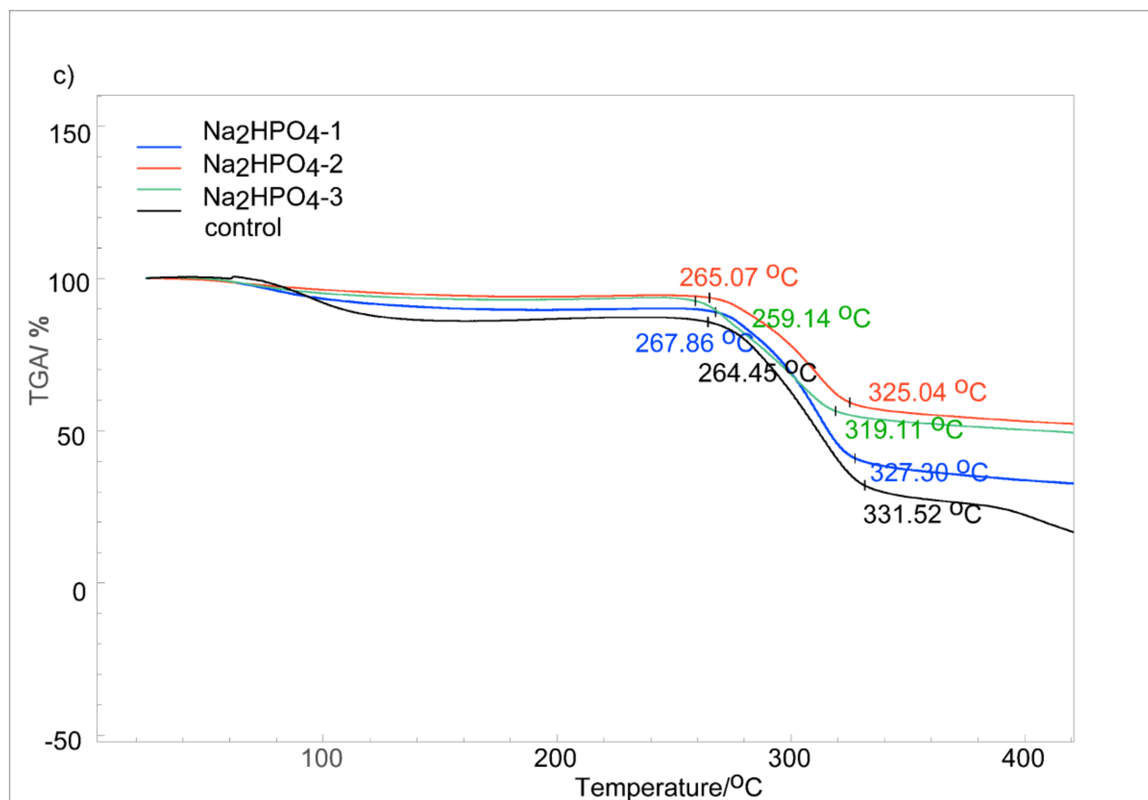

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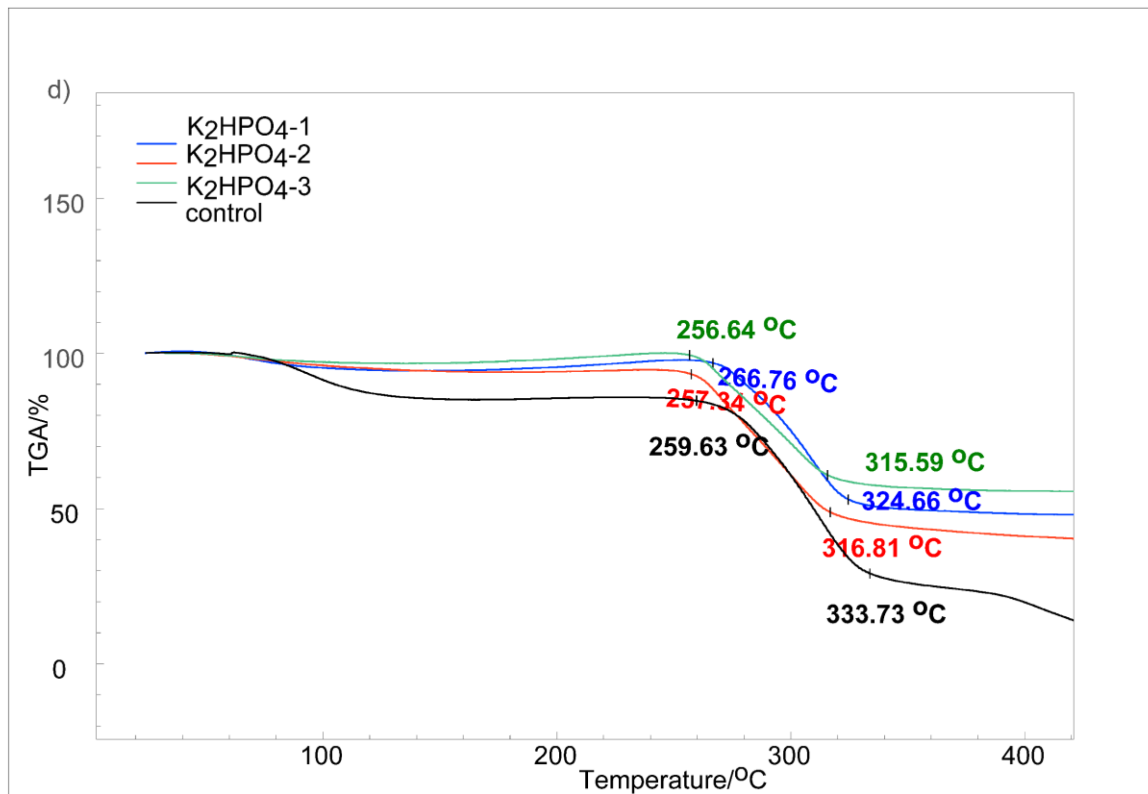

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2 Fig. 3. a) DSC thermogram of control glucomannan and glucomannan obtained from Na_2HPO_4/ethanol-ATPS, b)
3 DSC thermogram of control glucomannan and glucomannan obtained from K_2HPO_4/ethanol-ATPS, c) TGA of
4 control glucomannan and glucomannan obtained from Na_2HPO_4/ethanol-ATPS, and d) TGA of control
5 glucomannan and glucomannan obtained from K_2HPO_4/ethanol-ATPS, and

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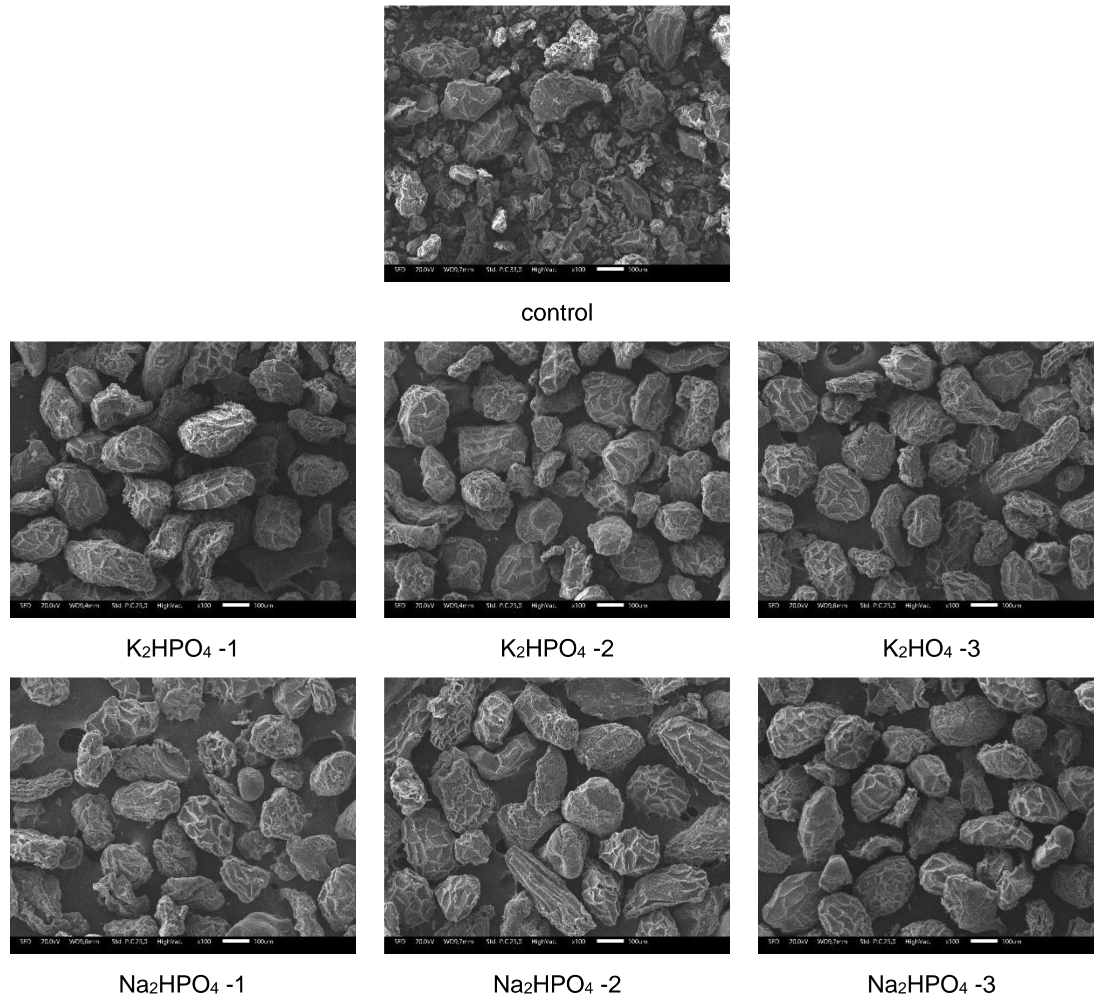

Fig. 4. Morphological surface of the control glucomannan and glucomannan extracted from ATPS observed with 100x magnification

SUPPLEMENTARY MATERIALS

Table S1. The effect of low speed and high speed mixing on glucomannan extraction from Porang flour

Porang flour	*m*(Yield)/%				*m*(Glucomannan content)/%				m(Starch content)/%			
	glucomannan		impurities		glucomannan		impurities		glucomannan		impurities	
	Low speed	High speed	Low speed	High speed	Low speed	High speed	Low speed	High speed	Low speed	High speed	Low speed	High speed
Mesh 60	(63.26±1.09)^{aA}	(59.39±0.96)^{bB}	(13.23±0.36)^{a}	(22.42±1.73)^{a}	(7.84±0.08)^{a}	(8.99±0.28)^{a}	(1.67±0.33)^{a}	(4.08±0.16)^{a}	(20.68±0.55)^{b}	(6.52±0.49)^{b}	(28.43±0.72)^{b}	(31.45±0.49)^{b}
Mesh 80	(63.61±1.26)^{a}	(61.99±1.00)^{a}	(12.89±0.46)^{a}	(21.89±1.64)^{a}	(7.66±0.02)^{bB}	(8.68±0.08)^{ab}	(1.26±0.70)^{aA}	(0.93±0.31)^{cC}	(22.23±1.04)^{aA}	(7.12±0.49)^{abAB}	(28.48±0.28)^{bB}	(32.08±0.49)^{abAB}
Mesh 100	(65.81±1.34)^{a}	(62.68±0.69)^{a}	(12.80±0.09)^{a}	(20.57±1.46)^{a}	(7.46±0.04)^{cC}	(8.44±10.10)^{bB}	(0.70±1.46)^{aA}	(1.76±0.16)^{bB}	(22.68±0.57)^{a}	(8.00±0.49)^{a}	(31.04±0.32)^{a}	(33.24±0.99)^{a}

Low speed: magnetic stirrer 400rpm for30 min
High speed: waring blender laboratorium speed (18,000 rpm) for 2 min

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1 Table S2. Composition of ATPS

ATPS	*m/m*(Salt fraction)	*m/m*(Water fraction)	*m/m*(Ethanol fraction)
Na_2HPO_4-1	0.52	80.32	19.16
Na_2HPO_4-2	1.04	79.90	19.07
Na_2HPO_4-3	1.56	79.48	18.97
K_2HPO_4-1	0.68	80.19	19.13
K_2HPO_4-2	1.36	79.63	19.00
K_2HPO_4-3	2.04	79.08	18.88

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3

4 Table S3. DSC peaks of glucomannan obtained from ethanol extraction (control) and ATPS extraction

Sample	peak 1		peak 2		peak 3	
	Temperature/°C	ΔH/(mcal)	Temperature /°C	ΔH/(mcal)	Temperature/°C	ΔH/(mcal)
Control	99.87	- 3060	293.09	56.94	325.58	223.18
Na_2HPO_4-1	83.19	-1310	274.87	27.83	316.37	280.99
Na_2HPO_4-2	73.74	-402.89	273.63	0.33	310.95	190.10
Na_2HPO_4-3	76.72	-1080	268.23	9.41	314.32	278.49
K_2HPO_4-1	75.28	-1690	274.21	34.39	317.40	313.19
K_2HPO_4-2	77.97	-613.11	264.56	34.74	307.71	124.75
K_2HPO_4-3	72.69	-770.57	262.73	47.22	307.61	162.77

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8 Table S4. TGA thermogram of glucomannan obtained from ethanol extraction (control) and ATPS extraction

Sampel	Temperature/°C	*w*(Weight loss)/%
Control	299.63 - 333.73	55.69
Na_2HPO_4-1	267.86 – 327.30	47.92
Na_2HPO_4-2	265.07 – 325.04	34.28
Na_2HPO_4-3	259.14 – 319.11	36.14
K_2HPO_4-1	266.76 – 324.66	44.18
K_2HPO_4-2	257.34 – 316.81	44.49
K_2HPO_4-3	256.64 – 315.59	38.64

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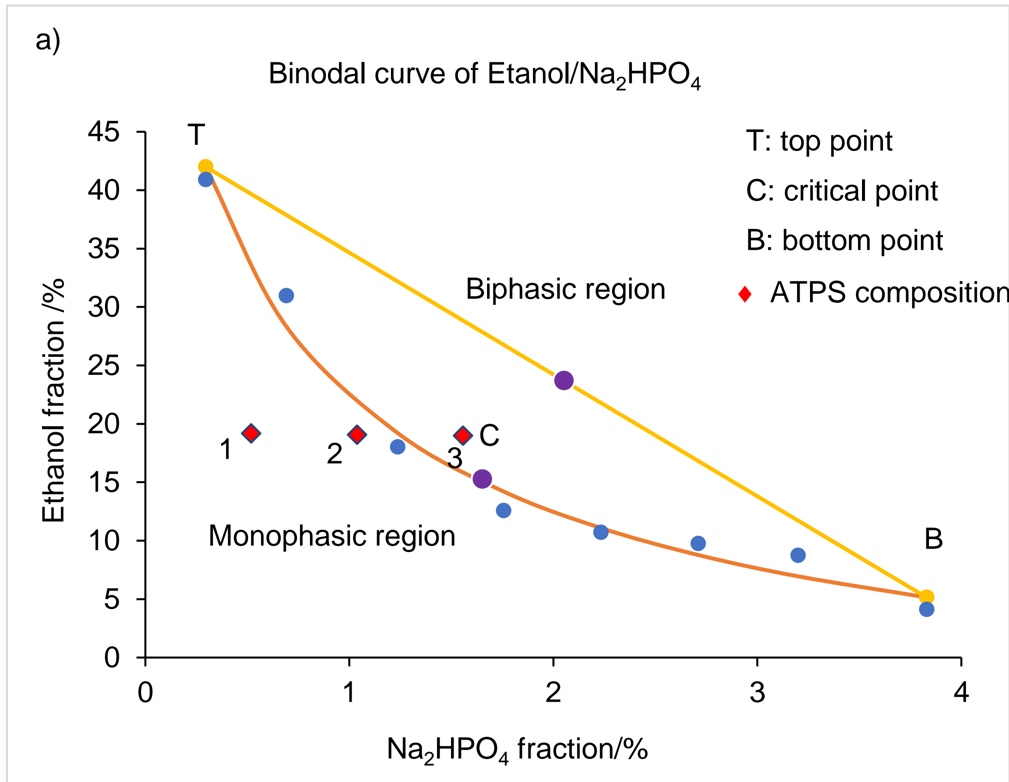

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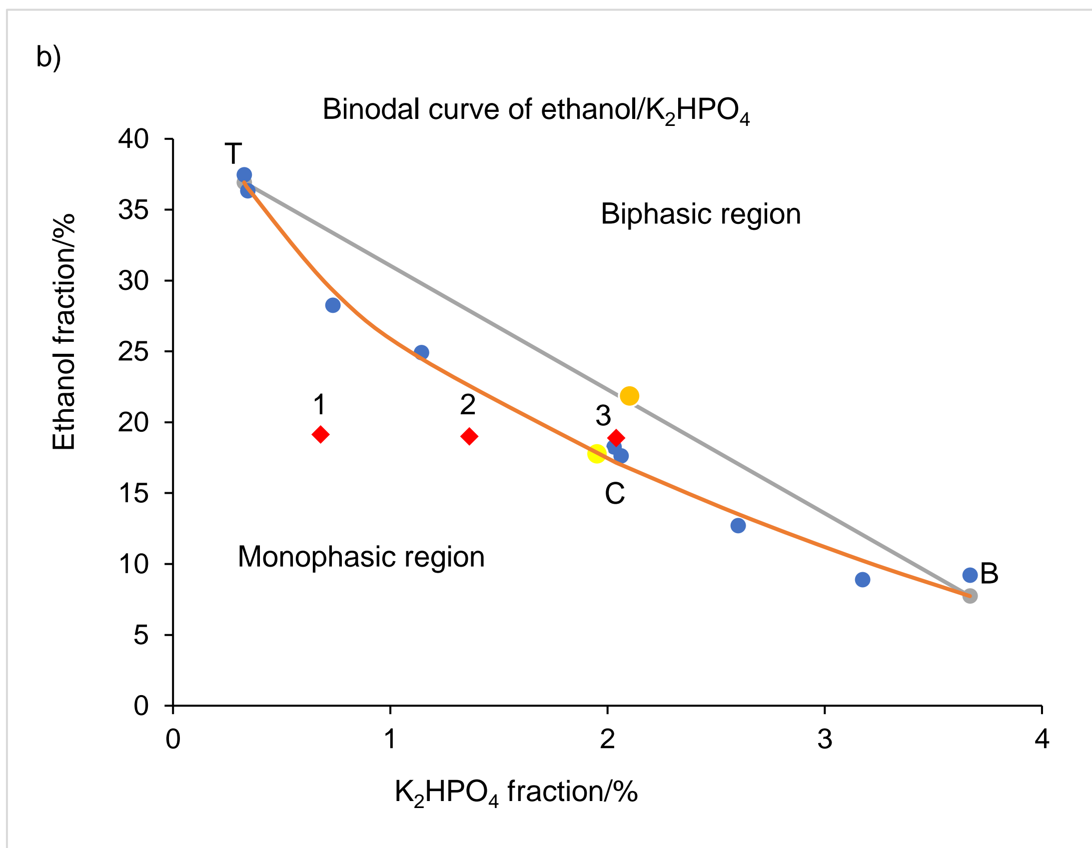

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Salt	TLL	C-point	
		X (salt fraction)	Y (ethanol fraction)
Na_2HPO_4	37.02	1.65	15.27
K_2HPO_4	29.39	1.95	17.79

1 Fig. S1. Binodal curve of ATPS (ethanol/salt) a) Ethanol/Na_2HPO_4-ATPS and b) Ethanol/K_2HPO_4-ATPS
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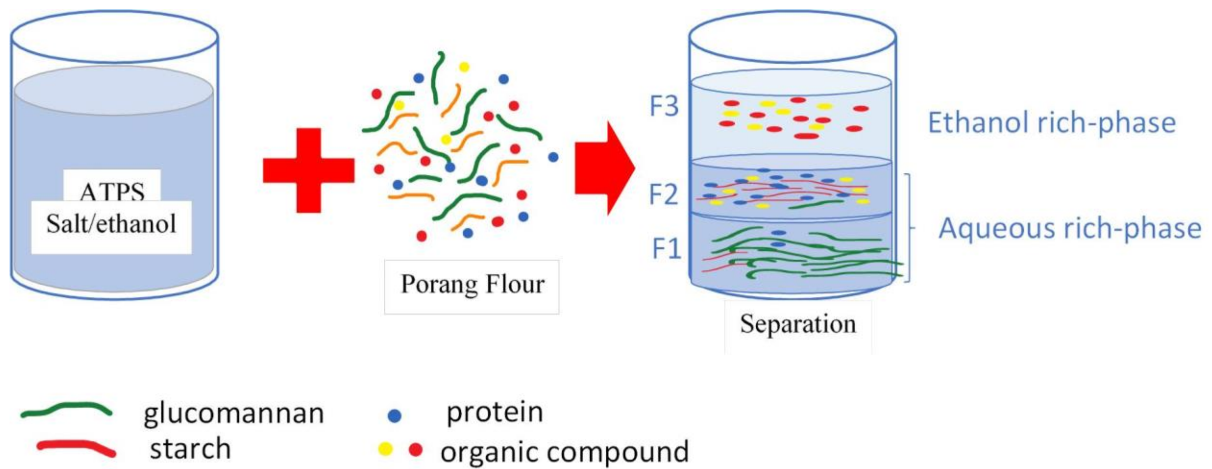

6
7 Fig. S2. Proposed mechanism of glucomannan extraction using ATPS

8 - F1 : glucomannan
9 - F2 : Starch and water soluble compound
10 - F3 : ethanol soluble compound