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1 **Partial depolymerization of tamarind seed xyloglucan and its functionality toward enhancing**  
2 **the solubility of curcumin**

3  
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18  
19 **Abstract**

20 Polysaccharides of tamarind seed, a byproduct of the tamarind pulp industry, displayed a potential  
21 solubility improvement of lipophilic bioactive molecules but their textural characteristics hinder the  
22 dietary formulation. In contrast, the commonly available xyloglucan oligosaccharides (XOSs) with  
23 degrees of polymerization (DPs) of 7, 8, and 9 were too short to maintain their ability. The binding  
24 capacity of the between sizes is unknown due to a lack of appropriate preparation. We prepared  
25 xyloglucan megalosaccharides (XMSs) by partial depolymerization, where term megalosaccharide  
26 (MS) defines the middle chain-length saccharide between DPs 10–100. Digestion with fungal  
27 cellulase enabled reproducible active XMSs. Further identification of pure XMS segments  
28 indicated that XMS-B has an average DP of 17.2 (Gal<sub>3</sub>Glc<sub>8</sub>Xyl<sub>6</sub>) with a branched dimer of XOS 8  
29 and 9 and was free of side-chain arabinose, the residue influencing high viscosity. Curcumin, a  
30 bioactive pigment, has poor bioavailability because of its water insolubility. XMSs with average  
31 DPs of 15.4–24.3 have similarly sufficient capacities to solubilize curcumin. The solubility of  
32 curcumin was improved 180-fold by the addition of 50%, w/v, XMSs, which yielded a clear yellow  
33 liquid. Our findings indicated that XMSs were a promising added-value agent in foods and  
34 pharmaceuticals for the oral intake of curcumin.

36 **Keywords:** acid hydrolysis, cellulase, hydrophobic interaction, tamarind gum, *Trichoderma viride*,  
37 xyloglucan megalosaccharide

38

### 39 **Abbreviations**

40 AN, *Aspergillus niger* cellulase; DP, degree of polymerization; HCl, hydrochloric acid;  
41 HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric  
42 detection;  $K_s$ , equilibrium constant;  $K_c$ , association constant; MW, molecular weight; TV,  
43 *Trichoderma viride* cellulase; XMS, xyloglucan megalosaccharide; XOS, xyloglucan  
44 oligosaccharide; YC, *Trichoderma viride* cellulase.

45

### 46 **1. Introduction**

47 Some polysaccharides display a strong affinity for hydrophobic binding to nonpolar  
48 molecules based on their fine structure of monosaccharide compositions, linkages, and degree of  
49 polymerization (DP). Their intrinsic characteristics, including viscosity and swelling degree, may  
50 slightly promote this binding interaction but highly influence the limitation of the dietary  
51 formulation in health sciences to be mainly a heterogeneous form, e.g., tablet, capsule, powder, etc.  
52 (Sapkal, Narkhede, Babhulkar, Mehetre, & Rathi, 2013; Viral, Dhiren, Mane, & Umesh, 2010).  
53 Xyloglucan is a major structure of plant hemicellulose found abundant in tamarind (*Tamarindus*  
54 *indica* L.) seed gum, which is a byproduct of the tamarind pulp industry. According to the  
55 structural configuration, Janado & Yano (1985) suggested that two aldopentoses, D-xylose and L-  
56 arabinose, predominantly possess superior hydrophobicity among other monosaccharides, where  
57 D-galactose and D-glucose are highly water-soluble aldohexoses facilitating chain solubility. Since  
58 tamarind seed xyloglucan underlies these patterns, we hypothesized that it is a potential source of  
59 botanical host molecules to enhance the water solubility of lipophilic ligands if the appropriate size  
60 preparation strategy can be established. Tamarind seed xyloglucan is a structurally highly branched  
61 heteropolysaccharide with a molecular weight (MW) varying from 650,000 to 2,500,000, and the  
62 monomers of glucose, xylose, galactose, and arabinose are contained in a molar ratio of 45:34:18:3  
63 (Majee, Avlani, & Biswas, 2016; Walker et al., 2017). The structure of tamarind seed xyloglucan  
64 consists fundamentally of a cellulose-like  $\beta$ -(1 $\rightarrow$ 4)-linked glucan backbone where on average, 3  
65 out of 4 glucosyl backbone residues have 1 $\rightarrow$ 6 linked xylose substituents. The branches can have  
66 galactose and arabinose substituents where the galactose substituent dominates the water solubility,  
67 as its abstraction (45% by  $\beta$ -galactosidase) can compose a thermally reversible gel at physiological  
68 temperature and thus utilize as conveyances for nasal drug delivery (Mahajan, Tyagi, Lohiya, &  
69 Nerkar, 2012). A minor amount of arabinose units is present at the side chain with a random  
70 arrangement (Niemann, Carpita, & Whistler, 1997), and the function is not reported even though a  
71 high degree of arabinose substitution in arabinoxylan from rye grain leads to highly viscous

72 solutions (Bengtsson, Andersson, Westerlund, & Åman 1992). Tamarind seed xyloglucan is a non-  
73 nutrient and is widely applied for thickening, stabilizing, and gelling agents in the food industry.  
74 Similarly, xyloglucan oligosaccharides (XOSs) are also obtained from tamarind seed xyloglucan by  
75 exhaustive digestion with fungal endo-(1→4)-β-D-glucanases (Satoh, Tateishi, & Sugiyama, 2013).  
76 They are classified into three different structures for the repeating units of tamarind seed  
77 xyloglucan: heptasaccharide (Glc<sub>4</sub>Xyl<sub>3</sub>, XOS 7, octasaccharide (Glc<sub>4</sub>Xyl<sub>3</sub>Gal, XOS 8), and  
78 nonasaccharide (Glc<sub>4</sub>Xyl<sub>3</sub>Gal<sub>2</sub>, XOS 9). They are likely the active ingredients associated with  
79 flower opening in carnation and inhibitory effects on the absorption of D-glucose in the intestine  
80 (Satoh et al., 2013; Sone & Sato, 1994). From the aforementioned, tamarind seed xyloglucan and  
81 XOSs have various industrial and pharmaceutical applications. However, the potency of their  
82 middle size denoting saccharides with DPs of 10–100, a term “megalosaccharide” (MS) originally  
83 proposed by French and his colleagues (Thoma, Wright, & French, 1959), has not been intensively  
84 used to date since there were no appropriate preparations of the sample sizes. Although tamarind  
85 seed xyloglucan is edible, the consumption is not practically in households due to its poor textural  
86 characteristics of the highly viscous. XMS is probably more soluble and less viscous in water but  
87 its preparation and functionality remain unclear.

88 Curcumin is a yellow lipid-soluble phenolic pigment present in the spice turmeric  
89 (*Curcuma longa*). It has various therapeutic potential and biological activities, such as anti-  
90 inflammatory and antioxidant activities, as denoted by over 6,000 citations (Prasad, Tyagi, &  
91 Aggarwal, 2014). However, practical water insolubility (0.54 μM or 0.2 μg/mL), which is  
92 eventually responsible for poor stability and poor bioavailability, has been highlighted as a major  
93 issue. Various types of curcumin forms have been designed to improve bioavailability, including  
94 chemical modification, nanoparticles, micelles, liposomes, and lipidic nanoparticles possessing an  
95 internal cubic phase structure (Yadav et al., 2020; Wu et al., 2020; Victorelli et al., 2022). In  
96 addition, some additives, such as resveratrol/lactoglobulin, cyclodextrin, steviol glycoside, and  
97 poly-(lactic-co-glycolic acid), are considerably attributed to the increased solubility of curcumin  
98 (Zhang et al., 2022; Mangolim et al., 2014; Nguyen et al., 2017; Sharma et al., 2021). Here, we  
99 proposed a different approach to solubilize curcumin for its potent use. The objective of this study  
100 was to establish preparation methods for functional XMSs from tamarind seed polysaccharides and  
101 provide a systematic study of the real binding capacity between curcumin and the fine structures of  
102 XMSs.

103 In this study, we first evaluated the solubilizing ability of curcumin by tamarind seed  
104 xyloglucan compared with other water-soluble plant and algae polysaccharides, including λ-  
105 carrageenan, gum arabic, guar gum, pectin, and amylopectin, to screen the functional  
106 polysaccharide source. XMS was then prepared by acid and enzyme digestions of tamarind seed  
107 xyloglucan and further fractionated by methanol since they are uncomplicated implements. The

108 complex formation of XMS and curcumin was verified by a fluorescent approach to provide  
109 fundamental knowledge of the real binding.

110

## 111 **2. Materials and methods**

112 **2.1. Preparation of XMSs and their isolation.** Acid and enzyme hydrolyses were carried out as  
113 follows: a 1% w/v slurry of tamarind seed xyloglucan (high purity grade, Tokyo Chemical Industry,  
114 Tokyo, Japan, 2 g) was prepared in 200 ml of water (for acid) and 50 mM sodium phosphate buffer,  
115 pH 6.0 (for the enzyme) and subsequently heated by microwave and vigorously stirred until boiling  
116 2–3 times to have a homogeneous form; and then partially hydrolyzed by hydrochloric acid (HCl)  
117 solution or cellulases. The former was performed by the addition of hot HCl (4 M; preheated at  
118 80 °C) to obtain a final concentration of 0.5 M and incubated for 2, 4, and 5 h at 80 °C, followed by  
119 neutralization with 4.0 M sodium hydroxide in an ice bath. The latter was performed  
120 with *Trichoderma viride* cellulase (TV, Sigma–Aldrich, Tokyo, Japan), *Aspergillus niger* cellulase  
121 (AN, Nacalai Tesque, Kyoto, Japan), and *Trichoderma viride* cellulase from Seshin Pharmaceutical,  
122 Tokyo, Japan (YC). The reaction mixtures were carried out at 37 °C for 2 h utilizing individual  
123 fungal cellulases of 9.5 units, and the samples were autoclaved at 121 °C for 15 min to inactivate  
124 the enzyme. Thereafter, 90%, v/v, methanol was integrated while mixing, and the digested samples  
125 were placed in ice water for an additional 2 h. The precipitant was isolated by centrifugation at  
126 13,000 ×g for 30 min, evaporated, and dried under a vacuum. The enzyme activity determined for  
127 the above three commercial cellulases (TV, YC, and AN) was 0.033, 0.027, and 0.012 unit/mg  
128 solid, respectively, whereas one unit is the amount of enzyme that liberates 1 μmol of glucose from  
129 sodium carboxymethyl cellulose (Sigma, 0.5%, w/v) per minute in 50 mM sodium phosphate  
130 buffer pH 6.0 and 37 °C. For the time course study, the samples were withdrawn at different time  
131 intervals until 24 h. Next, the reaction mixture containing 15 g of tamarind seed xyloglucan was  
132 prepared again with YC, but the incubation time was fixed at 2 h to collect various sizes of MSs.  
133 After autoclaving, the sample was centrifuged at 11,300 ×g for 30 min and then filtered through a  
134 membrane filter (0.2 μm mixed cellulose ester, Advantec, Tokyo, Japan) to remove the insoluble  
135 pellet, and the supernatant was desalted by a manually packed ion-exchange column (amberlite  
136 MB4, Organo, Tokyo, Japan). The product sizes in the supernatant were isolated into four fractions  
137 by sequential methanol precipitation (≤ 60, 60–70, 80–95%, v/v). The shortest fragment containing  
138 XOSs was not precipitated. Thus, it was collected after evaporation and dialyzed with a  
139 Spectra/Por®6 membrane (Rancho Dominguez, CA, USA) with a MW cutoff of 1 kD against water  
140 to discard glucose and oligosaccharides having an average DP less than 6 (referred to as > 95%,  
141 v/v).

142 The yield was expressed as the ratio (%) of the amount of the obtained xyloglucan  
143 hydrolyses divided by the amount of the substrate used (15 g). Based on the obtained functional

144 size results, the methanol concentration was considerably adjusted to 60–75%, v/v to obtain MSs  
145 with an average DP of 19. The pellet was dried by lyophilizer (Eyela FDU-1200, Tokyo, Japan)  
146 and profiled by Dionex high-performance anion exchange chromatography with pulsed  
147 amperometric detection (HPAEC-PAD, CarboPac™ PA1 Column 4 × 250 mm, Dionex Co.,  
148 Sunnyvale, CA, USA) with an eluent of 16 mM sodium hydroxide for 20 min and further  
149 incremented to 200 mM with a sodium acetate gradient of 0–100 mM for 40 min and from 100–  
150 250 mM for 20 min. Purification of XOSs and XMS-A and XMS-B from YC digests was  
151 performed by preparative HPLC on an Imtakt Unison US-Amino column (20 × 250 mm) with an  
152 eluent of 70%, v/v, acetonitrile in water at a flow rate of 4 mL/min for XOS 7–9 and 65%, v/v, at 2  
153 mL/min for XMS-A and XMS-B. Upon evaporation of the solvent, the purities of XOSs, XMS-A,  
154 and XMS-B were determined by HPAEC-PAD analysis. These samples were also used as external  
155 standards to quantify the concentration of peaks presented in Fig. 3.

156

157 **2.2. Determination of MW, monosaccharide composition, and DP.** The MW of polysaccharides  
158 or MSs was individually determined by a gel filtration column of Shodex OHpak SB-806 HQ or  
159 SB-803 HQ (8.0 × 300 mm), respectively using the same HPLC apparatus described previously  
160 (Lang et al., 2022a). The saccharide samples were dissolved with 5 mg/mL in sodium nitrate  
161 solution (0.1 M), centrifuged, and eluted with the same solution at 0.5 mL/min and 40 °C. The  
162 signals were detected by a refractive index detector (RI 2031 Plus, JASCO, Tokyo, Japan). The  
163 molecular mass markers used were Shodex standard P-82 pullulans (P-800–P-5, Showa Denko  
164 K.K., Kanagawa, Japan) and maltoheptaose (G7, Nihon Shokuhin Kako, Tokyo, Japan). The  
165 calibration curves were constructed using the retention times and MWs (obtained from the  
166 manufacturer instruction) of P-800, P-400, P-200, and P-100 for polysaccharides and P-100, P-50,  
167 P-20, P-10, P-5, and G7 for the MS-size samples.

168 Molecular mass of XMS-A and XMS-B was obtained from ESI mass spectra using Thermo  
169 Scientific Exactive spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples  
170 were subjected to a nanospray in a positive ion mode, and the recorded mass range was at  $m/z$   
171 1,000 to  $m/z$  1,500. The major molecular ion on saccharide analysis is usually derived from  $[M +$   
172  $H]^+$  or  $[M + Na]^+$ : one proton or one sodium is added, and then the ion detected is one ( $z = 1$ ).  
173 However, in the case of  $[M + 2H]^{2+}$  or  $[M + 2Na]^{2+}$  for XMSs ( $z = 2$ ), molecular mass by ESI mass  
174 spectrometry (positive mode) was calculated as follows: molecular mass = ( $m/z$  value ×  $z$ ) – 23,  
175 where 23 is the mass of the adduct ion of  $Na^+$ .

176 For monosaccharide composition analysis, dried XMS (0.5 mg) was hydrolyzed at 120 °C  
177 in 0.1 mL of 2 M trifluoroacetic acid for 2 h in a glass vial. The acid was then removed by nitrogen  
178 flushing. The samples were subsequently redissolved in water at the appropriate dilution and  
179 analyzed by HPAEC-PAD under isocratic conditions of 16 mM sodium hydroxide, and *myo*-

180 inositol was utilized as an internal standard [see Fig. S1 containing a typical example of XMS  
181 (average DP 63.7 in Table 2)]. The molar concentration of each monosaccharide was determined  
182 from peak area using known sugar standard (arabinose, galactose, glucose, or xylose). Molar  
183 percentage of monosaccharide (MOS%) was calculated from the equation:  $MOS\% = \{[MOS] /$   
184  $([Ara] + [Gal] + [Glc] + [Xyl])\} \times 100$ , where MOS indicates the monosaccharide of Ara, Gal, Glc,  
185 or Xyl.

186 DP was calculated by the summation of hexose (glucose and galactose) and pentose  
187 (arabinose and xylose) molar percentages, and the equation was expressed as follows:  $DP = (MW -$   
188  $18) / \{(\%hexose \times 162/100) + (\%pentose \times 132/100)\}$ , whereas MW was obtained from the above  
189 gel filtration HPLC analyses.

190 Accordingly, the XMS average DP 63.7 has the molar concentrations of 2.17, 2.05, 4.15,  
191 and 3.29 mM for Ara, Gal, Glc, and Xyl, respectively. Based on the above equation, MOS% of Ara  
192 =  $\{2.17 / (2.17 + 2.05 + 4.15 + 3.29)\} \times 100 = 18.6\%$ . With the same calculation, MOS% of Gal,  
193 Glc, and Xyl was obtained as 17.5, 35.8, and 28.1, respectively. The MW of XMS average DP 63.7  
194 determined by the gel filtration HPLC was 9,445 Da and  $\%hexose = 17.5 + 35.8 = 53.3$   
195 where  $\%pentose = 18.6 + 28.1 = 46.7$ . Following the above calculation,  $DP = (9,445 - 18) / \{(53.3 \times$   
196  $162/100) + (46.6 \times 132/100)\} = 63.7$  (Table 2).

197

198 **2.3. Curcumin solubility enhancement test.** The curcumin (Nacalai) working standard was  
199 prepared by dissolving 1 mg/mL curcumin in absolute ethanol and subsequently diluting it with  
200 10%, v/v, ethanol in water. The absorbance of the solution was determined at 440 nm by a UV-Vis  
201 spectrophotometer (U-2900, Hitachi, Japan), and the calibration curve was constructed with 0.5–10  
202  $\mu\text{g/mL}$ . Phase solubility studies were prepared as follows: an excess quantity of curcumin (1 mg)  
203 was taken into microcentrifuge tubes, and a fixed volume (100  $\mu\text{L}$ ) of distilled water, or water  
204 containing two concentrations of 0.5 and 1.0%, w/v, for polysaccharides including tamarind seed  
205 xyloglucan,  $\lambda$ -carrageenan (disulfate galactose: monosulfate galactose: galactose, 1: 2.3: 3.3, Wako  
206 Pure Chemical Industries, Osaka, Japan), amylopectin (Wako), gum arabic (rhamnose: arabinose:  
207 galactose: glucuronic acid, 1.5: 3.1: 7.9: 1, Nacalai), pectin (rhamnose: arabinose: glucose:  
208 galacturonic acid, 1: 1.4: 19.6: 18.0, Nacalai), and guar gum (mannose: galactose, 1.5: 1, Tokyo  
209 Chemical Industry Co., Ltd.). The obtained monosaccharide compositions were determined in our  
210 laboratory. The concentrations were 5 and 10%, w/v, for XOSs and XMSs. The tubes were  
211 frequently mixed by vortexing at 25 °C for 6 h. Subsequently, the supernatant was taken by  
212 centrifugation (12,000  $\times g$ , 25 °C, 10 min) and carefully transferred to the incipient tubes, where the  
213 method was repeated twice. A portion of the supernatant was diluted with 10% v/v ethanol, and the  
214 curcumin concentration was determined accordingly. The solubility experiments were conducted in  
215 triplicate. The equilibrium constant ( $K_s$ ) of curcumin complexed with XMS (average DP 19, 0–50%,

216 w/v) was determined from the phase solubility diagram according to our previous works (Lang et  
217 al., 2014).

218

219 **2.4. Hydrophobic binding capacity.** Curcumin is a non-fluorescent compound in aqueous media  
220 but yields a large fluorescence in a nonpolar state or some regions of low polarity. The binding  
221 affinity of curcumin and XMS was determined by the following method. A fixed volume (0.9 mL)  
222 of pure curcumin in water (0.54  $\mu\text{M}$ ) was mixed with an XMS average DP of 19 (0.1 mL, 0–20  
223 mg/mL). Complex formation was monitored at an excitation wavelength of 420 nm and an  
224 emission intensity of 522.8 nm. The association constant ( $K_c$ ) was determined according to  
225 previous methods (Lang et al., 2022b).

226

### 227 **3. Results and discussion**

228 **3.1. Solubilizing ability of polysaccharides to curcumin.** Some polysaccharides (e.g.,  $\alpha$ -(1 $\rightarrow$ 4)-  
229 glucan) exhibit remarkable hydrophobic character arising from stereochemical constraints on the  
230 chain (Sundari, Raman, & Balasubramanian, 1991). Nevertheless, Viral et al. (2010) reported that  
231 guar gum enhanced the solubility of the licofelone drug approximately 1.2-fold compared to the  
232 pure drug (9  $\mu\text{g/mL}$ ) by claiming the swelling nature of the carrier. The MWs of six water-soluble  
233 polysaccharides were determined by gel filtration HPLC (Fig. S2A): amylopectin (1,086 kDa),  
234 tamarind seed xyloglucan (1,075 kDa),  $\lambda$ -carrageenan (1,046 kDa), guar gum (980 kDa), pectin  
235 (242 and 21 kDa), and gum arabic (164 kDa). We quantified curcumin solubility with the liquid  
236 portion of six polysaccharides with the formulation of 0.5 and 1%, w/v, and the results in Fig. S2B  
237 indicate the best solubility obtained with tamarind seed xyloglucan (48.4-fold of curcumin  
238 solubilized in water, 1-fold = 0.2  $\mu\text{g/mL}$  or 0.54  $\mu\text{M}$  curcumin at 25  $^\circ\text{C}$ ). This is many-fold greater  
239 than the ability of  $\lambda$ -carrageenan > guar gum > gum arabic > pectin > amylopectin (7.9-, 2.6-, 3.1-,  
240 1.5- and 1-fold). D-xylose is a five-carbon sugar and has a relatively high partition coefficient to  
241 polystyrene gel by ordering: (D-xylose, D-arabinose) > D-glucose > D-galactose (Janado & Yano,  
242 1985). In addition, the structural features of xyloglucan are a linear flexible coil conformation and  
243 chain fairly stiff (Park & Cosgrove, 2015). These reasons might be a crucial contribution to  
244 hydrophobic bonding with hydrophobic ligands because the three-dimensional shape of the  
245 cellulose backbone with a ribbon-like conformation was suggested for incapability (Sundari et al.,  
246 1991). Nevertheless, xyloglucan molecules facilely aggregate even in very dilute solutions (Kozioł,  
247 Cybulska, Pieczywek, & Zdunek, 2015) and form a viscous gel after interaction with curcumin.  
248 This polysaccharide property might limit the application of xyloglucan as a carrier. We further  
249 performed the partial depolymerization of tamarind seed xyloglucan by two hydrolysis methods, as  
250 the smaller chains are usually more soluble and less viscous than the larger ones (Guo et al., 2017).

251 Acid-depolymerized products from other five polysaccharides displayed the same curcumin-  
 252 solubilizing ability as their parental chains.  
 253  
 254 **3.2. Solubilizing activity of XMSs obtained from partial hydrolyses to curcumin.** Tamarind  
 255 seed xyloglucan has an average MW of 1,075 kDa with mol% values of 48.1, 32.5, 14.4, and 5.0  
 256 for glucose, xylose, galactose, and arabinose, respectively (Table 1). Varying the incubation time  
 257 from 2–5 h in HCl hydrolysis resulted in three samples with a plausible yield of 30.7–53.7% (Table  
 258 1), and their average DPs of 97.1, 34.3, and 29.6 decreased consistently with the incubation time.  
 259 The elution patterns obtained by gel filtration HPLC are shown in Fig. 1A, revealing a single peak.  
 260 Compared with the parent xyloglucan, the sugar composition of these fragments decreased in  
 261 galactose content (8.3–3.4 mol%) and was free of arabinose residues as they were acid-labile. The  
 262 glucose residues became predominant (57.6–67.3 mol%) corresponding to the main chain of heat-  
 263 insusceptible  $\beta$ -(1→4)-cellulose that is always aggregated. TV catalyzed the hydrolysis of tamarind  
 264 seed xyloglucan to a mixture of shorter chains predicated on an incubation time (40 min, 1 h, and 2  
 265 h). Three samples with average DPs of 121.7, 73.1, and 28.0 have a monomer composition similar  
 266 to that of the parent chain (with only a lower arabinose content) and provided better yields of 40.3–  
 267 78.2% (Table 1). The results obtained from the phase solubility test for curcumin in Fig. 1B  
 268 denoted that the moderate chain length (average DP 28.0) obtained from cellulase hydrolysis has a  
 269 better capacity to improve the curcumin solubility to  $31.9 \pm 0.29$ -fold at 10%, w/v, than the longer  
 270 average DP of 73.1 and 121.7. The longer XMS resulted in subsequent precipitation with curcumin  
 271 binding.

272

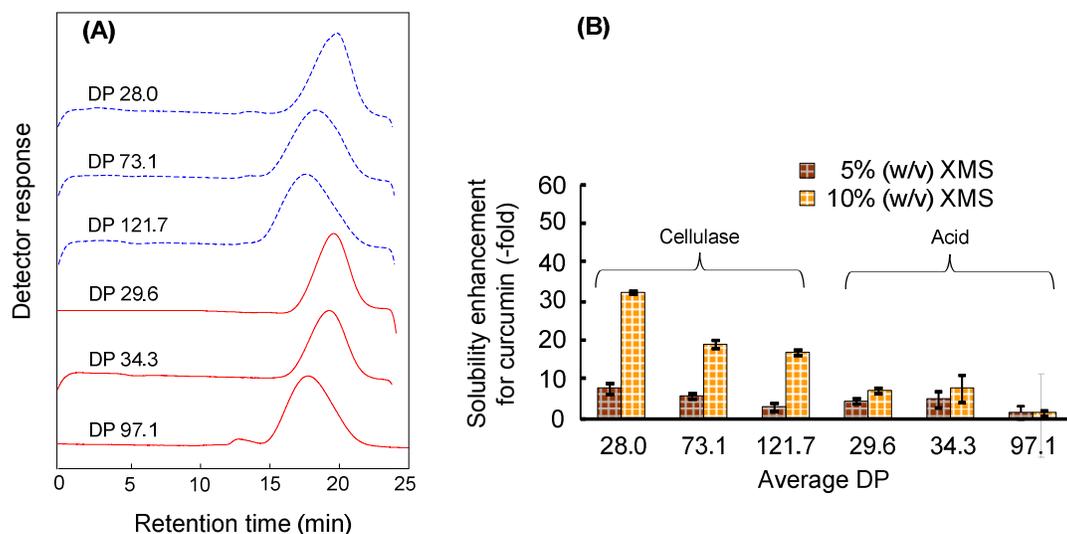
273 **Table 1.** Properties of XMSs prepared from the partial hydrolyses of tamarind seed xyloglucan.

|                       | Digestion<br>Time | Average<br>DP | Yield<br>(%) | Monosaccharide composition (MOS%) |      |      |      |
|-----------------------|-------------------|---------------|--------------|-----------------------------------|------|------|------|
|                       |                   |               |              | Ara                               | Gal  | Glc  | Xyl  |
| Intact xyloglucan     | -                 | 7,128         | -            | 5.0                               | 14.4 | 48.1 | 32.5 |
| HCl hydrolysis        | 2 h               | 97.1          | 53.7         | 0.0                               | 8.3  | 57.6 | 34.1 |
|                       | 4 h               | 34.3          | 35.7         | 0.0                               | 4.4  | 63.0 | 32.6 |
|                       | 5 h               | 29.6          | 30.7         | 0.0                               | 3.4  | 67.3 | 29.3 |
| Cellulase* hydrolysis | 40 min            | 121.7         | 78.2         | 2.1                               | 15.7 | 46.9 | 35.3 |
|                       | 1 h               | 73.1          | 79.5         | 2.6                               | 15.5 | 46.8 | 35.1 |
|                       | 2 h               | 28.0          | 40.3         | 0.4                               | 16.4 | 48.8 | 34.4 |

274 \* is *Trichoderma viride* cellulase from Sigma (TV).

275

276

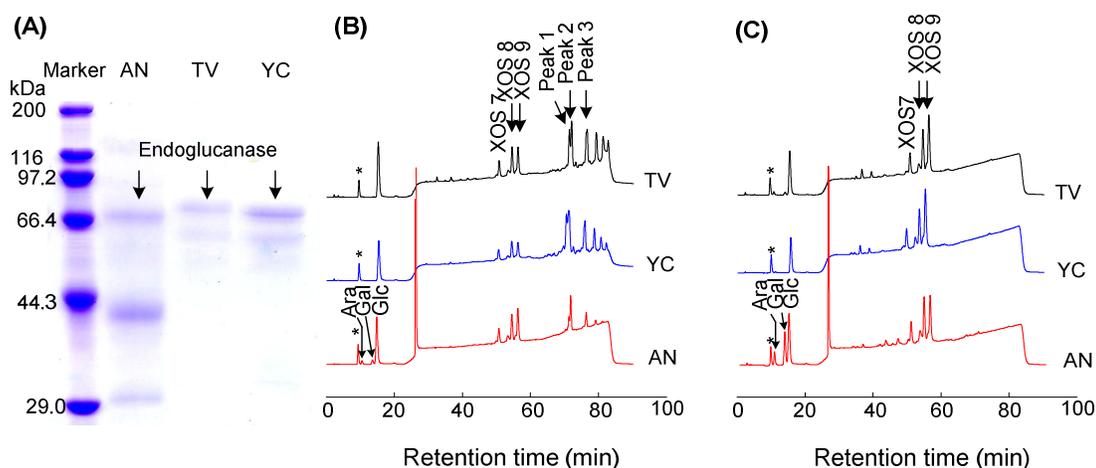


277  
 278 **Fig. 1.** (A) HPLC gel filtration elution profiles of tamarind seed xyloglucan hydrolysates obtained  
 279 from TV digestion (average DPs of 28.0, 73.1, and 121.7, dashed lines) and acid hydrolysis  
 280 (average DPs of 29.6, 34.3, and 97.1, dark lines). (B) Solubility enhancement of curcumin by the  
 281 addition of six XMSs obtained from cellulase and acid hydrolysis with 5%, w/v, and 10%, w/v,  
 282 compared with the solubility of curcumin in water (1-fold).

283

284 **3.3. Cleavage action of three fungal cellulases.** It is important to obtain an adequate amount of  
 285 active XMS samples and reproducible methods. Therefore, the action of cellulase was reconsidered,  
 286 and three commercial sources of fungal cellulase were compared. Their purity was visualized by  
 287 SDS-PAGE analysis (Fig. 2A). It reveals that all three proteins have the largest monomer with a  
 288 similar size of ca. 67 kDa corresponding to monomeric endoglucanase protein (Irshad et al., 2013).  
 289 AN obviously has additional two bands (MWs of 30 and 40 kDa), agreeing with the report of Das,  
 290 Bhattacharya, Roopa, & Yashoda (2011), which did not identify the cellulase activity in both  
 291 proteins.

292



293

294 **Fig. 2.** (A) SDS-PAGE image comparing the commercial cellulase proteins. Lane 1, molecular  
 295 mass marker; Lane 2, AN; Lane 3, TV, and Lane 4, YC. Arrows indicate endoglucanases. HPAEC-  
 296 PAD analysis of the cellulase reaction mixture from TV, YC, and AN with incubation times of 2 h  
 297 (B) and 24 h (C). Peaks labeled: peak 1 or XMS-A, average DP 15.8; peak 2 or XMS-B, average  
 298 DP 16.6; and peak 3 or XMS-C, average DP 22.8. XOS 7, 8, and 9 are xyloglucan heptaose,  
 299 octaose, and nonaose, respectively. \* represents the internal standard myo-inositol.

300

301 The enzymatic products at 2 h were mainly composed of three groups of monosaccharides  
 302 (at retention times of 10–16 min), oligosaccharides, XOSs 7, 8, and 9 (50–57 min), and XMSs (70–  
 303 85 min), in which the last one showed a large variety of peaks (Fig. 2B). After 24 h, the sugars with  
 304 DPs higher than XOS 9 were no longer present (Fig. 2C). The most commonly observed  
 305 monosaccharide, in all cases, was glucose (15.94 min) and with small amounts of galactose (14.02  
 306 min) and arabinose (11.40 min) occurring in AN. This result indicated that AN has a side reaction  
 307 of other hydrolytic activities, likely  $\beta$ -D-galactosidase and  $\alpha$ -L-arabinosidase, removing some  
 308 galactosyl and arabinose residues (Fig. 2B and 2C). Another side reaction of  $\alpha$ -D-xylosidase was  
 309 not detected. YC was more highly restricted in its specificity to endo-(1 $\rightarrow$ 4)- $\beta$ -D-glucanase with  
 310 no side-reaction and exhibited the highest contents of representative XMS-A (peak 1, 70.68 min)  
 311 and XMS-B (peak 2, 71.27 min) compared with the other two cellulases (Fig. 2B and Fig. 3). The  
 312 mechanism of cellulase action on xyloglucan has not been fully established. We proposed a  
 313 mechanism involving the cleavage of random internal  $\beta$ -(1 $\rightarrow$ 4)-glucan at the unsubstituted parts  
 314 and further access to more highly substituted regions. Highly branched regions with arabinose  
 315 substitution of the main chain might be less accessible. This is evidenced by traces of insoluble  
 316 residuals that remained from hydrolysis with a high ratio of arabinose (16.7 mol%) indicated in  
 317 Table 2 according to the next session. We propose an alternative way to utilize fungal cellulase for  
 318 the removal of arabinose-substituted regions in the case of high-viscosity samples.

319

320 **Table 2.** Properties of XMSs fractionated from the enzyme<sup>a</sup> hydrolysis of tamarind seed  
 321 xyloglucan<sup>b</sup>.

| Methanol<br>Precipitation (%) | Amount<br>(g) | Yield<br>(%) | DP                        | Monosaccharide composition (MOS%) |      |      |      |
|-------------------------------|---------------|--------------|---------------------------|-----------------------------------|------|------|------|
|                               |               |              |                           | Ara                               | Gal  | Glc  | Xyl  |
| Insoluble part                | 0.31          | 2.1          | N.D.                      | 16.7                              | 12.6 | 35.0 | 35.7 |
| ≤ 60                          | 2.51          | 16.7         | 63.7                      | 18.6                              | 17.5 | 35.8 | 28.1 |
| 60–70                         | 2.31          | 15.4         | 24.3                      | 0.3                               | 17.8 | 51.1 | 30.8 |
| 80–95                         | 1.91          | 12.7         | 15.4                      | 0.5                               | 17.5 | 48.7 | 33.3 |
| > 95                          | 1.20          | 8.0          | 9.0                       | -                                 | 16.4 | 47.8 | 35.9 |
| XMS-A                         | N.D.          | N.D.         | 15.8 (16.2 <sup>c</sup> ) | -                                 | 12.6 | 50.1 | 37.0 |
| XMS-B                         | N.D.          | N.D.         | 16.6 (17.2 <sup>c</sup> ) | -                                 | 17.0 | 48.2 | 34.5 |

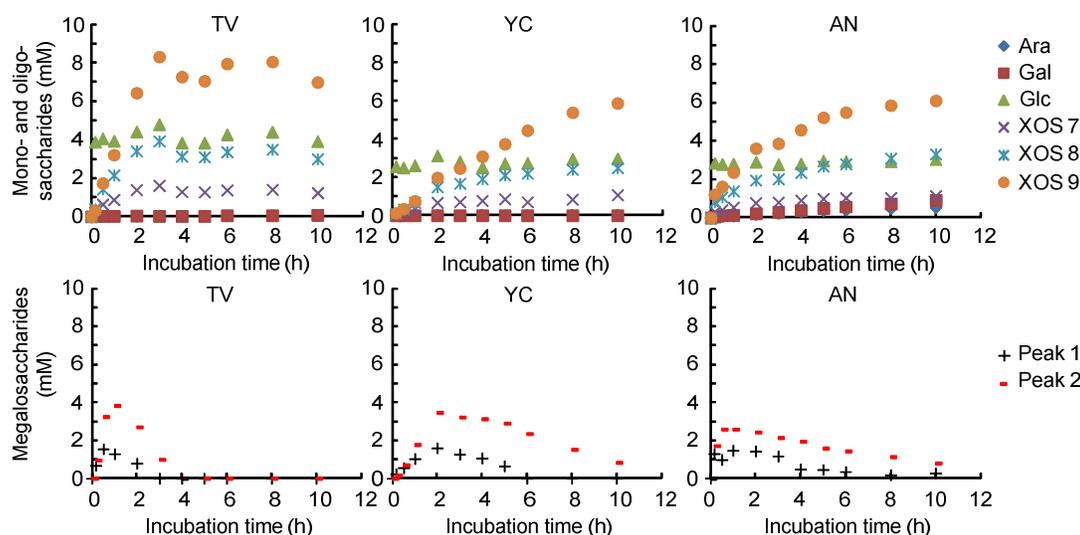
322 <sup>a</sup> is *Trichoderma viride* cellulase from Seshin Pharmaceutical (YC).

323 <sup>b</sup> = 15 g.

324 <sup>c</sup> is DP calculated using mass spectrometry data (MWs of XMS-A and XMS-B: 2,454.78 and  
 325 2,616.82, respectively).

326 N.D. = not determined.

327



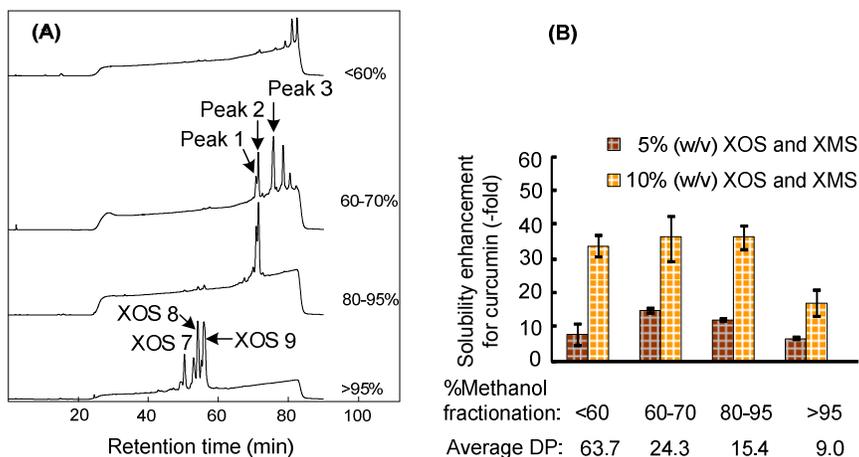
328

329 **Fig. 3.** Time course production of monosaccharides, XOSs, and XMSs from tamarind seed  
 330 xyloglucan by cellulase digestion. The cellulases utilized were the commercially available TV, YC,  
 331 and AN.

332

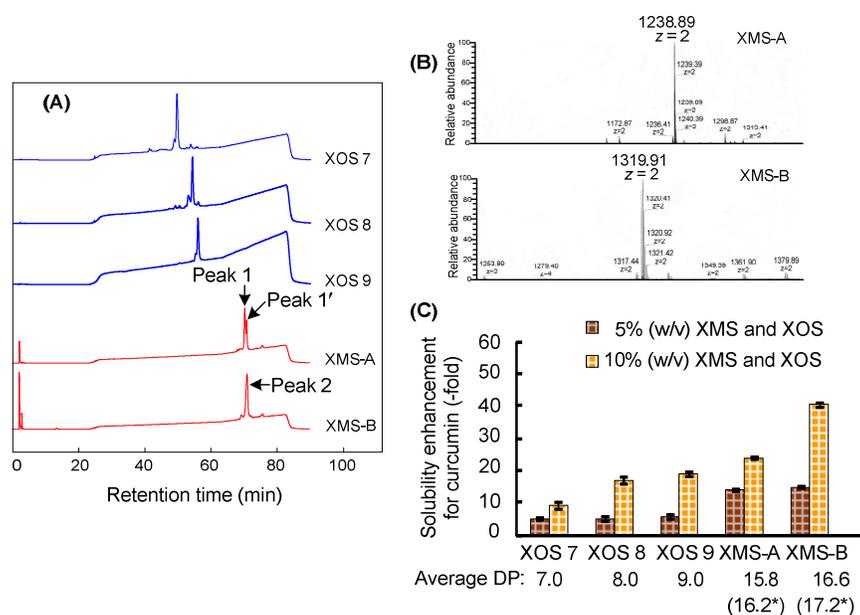
333 **3.4. Isolation of active XMSs.** Four fragments from YC hydrolysates were obtained by sequential  
 334 methanol precipitation, and the chromatograms are presented in Fig. 4A. The largest fragment with  
 335 an average DP of 63.7 was taken by ≤ 60%, v/v, methanol. The arabinose content appeared to be

336 the average highest (Table 2). Only these samples showed high viscosity after being dissolved in  
337 water. A fragment of 60–70%, v/v, methanol with an average DP of 24.3 showed the main  
338 composition with XMS-C (peak 3 denoted in Fig. 2B, 75.8 min) combined with a mixture of  
339 slightly shorter and larger sizes. A fragment of 80–95%, v/v, with an average DP of 15.4 shows  
340 mainly pure XMS-A and XMS-B. A short fragment from > 95%, v/v, methanol contained only  
341 XOSs with an average DP of 9.0. Fig. 4B presents the results of phase solubility enhancement tests  
342 for curcumin, which were almost similar between XMSs with average DPs of 24.3 ( $36.1 \pm 6.87$ -  
343 fold) and 15.4 ( $36.6 \pm 3.63$ -fold) but twice as large as XOSs ( $17.0 \pm 3.84$ -fold) at 10%, w/v. Next,  
344 the samples were purified by preparative HPLC. The purity of these fractions of XOS 7–9, XMS-A,  
345 and XMS-B appeared in HPAEC-PAD chromatograms (Fig. 5A). XMS-A fraction displays two  
346 peaks (peak 1 and peak 1'), and the saccharide of peak 1' is different from XMS-B of peak 2 since  
347 molecular masses of XMS-A and XMS-B are distinct (Fig. 5B). ESI mass spectrometry allowed the  
348 MW determination of the purified XMS-A and XMS-B and ascertained their DPs as follows.  
349 Fragment  $[M + 2H]^{2+}$  or  $[M + 2Na]^{2+}$  ions of 1,238.89 and 1,319.91 were observed for XMS-A and  
350 XMS-B, respectively (Fig. 5B), corresponding to MWs of 2,454.78 and 2,616.82. These results  
351 agree with the average MWs of 2,391 and 2,529 obtained by gel chromatography in our analysis.  
352 Calculations based on the sugar content in Table 2 present new average DP values of 16.2 and 17.2  
353 for XMS-A and XMS-B, respectively. The results of the structure analysis of XMS-B by cellulase  
354 hydrolysis showed that the composition led to further hydrolysis to an equimolar mixture of XOS 8  
355 and 9. This structure could be characterized by the presence of a  $\beta$ -(1→4)-glycosidic linkage in the  
356 chain, and in conclusion, XMS-B was a branched  $\text{Gal}_3\text{Glc}_8\text{Xyl}_6$ . The curcumin solubility was  $24.1$   
357  $\pm 0.4$ -fold for XMS-A and  $40.9 \pm 0.7$ -fold for XMS-B. Purified XOS 9 was  $18.9 \pm 0.64$ -fold, and  
358 its capacity was better than those of shorter sized XOS 8 and XOS 7. This observation suggested  
359 that xyloglucan with a longer chain length was more effective in dissolving curcumin, as it  
360 contained a larger amount of xylosyl substitution for a better conformation, promoting hydrophobic  
361 interactions, and galactosyl substituents assisted in water solubility. In addition, the ideal chain  
362 should not appear to be influenced by the high viscosity arising from arabinose.  
363



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**Fig. 4.** (A) HPAEC-PAD elution profiles of YC-digested XMSs obtained from fractionation by methanol. (B) The solubility enhancement of curcumin with the addition of XOS and XMS at 5 and 10%, w/v, compared with the solubility of curcumin in water (1-fold).



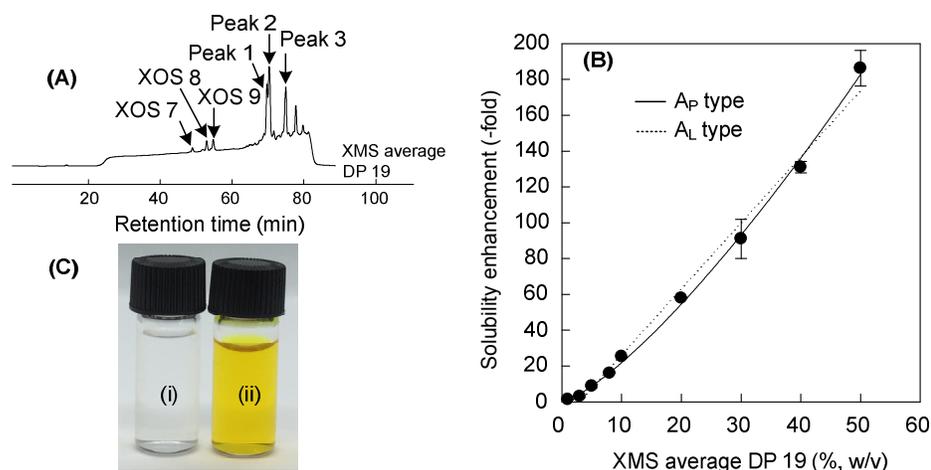
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**Fig. 5.** (A) HPAEC-PAD elution profiles of purified tamarind seed xyloglucan hydrolysates by preparative HPLC. (B) Electrospray ionization mass spectrometry analysis of purified XMS-A and XMS-B separated by HPLC. (C) The solubility enhancement of the corresponding samples. \* is new DPs calculated using mass spectrometry data.

375 **3.5. Solubilizing ability of XMS average DP 19 to curcumin.** Based on the results of Fig. 5C,  
376 XMS-B yielded the best curcumin solubilization, implying that XMS-B-rich fraction is suitable for  
377 practical preparation of functional MS. The XMS fraction with 60–75%, v/v, methanol was  
378 considered to improve more XMS-B than 60–70%, v/v, methanol fraction (Fig. 4). As expected,

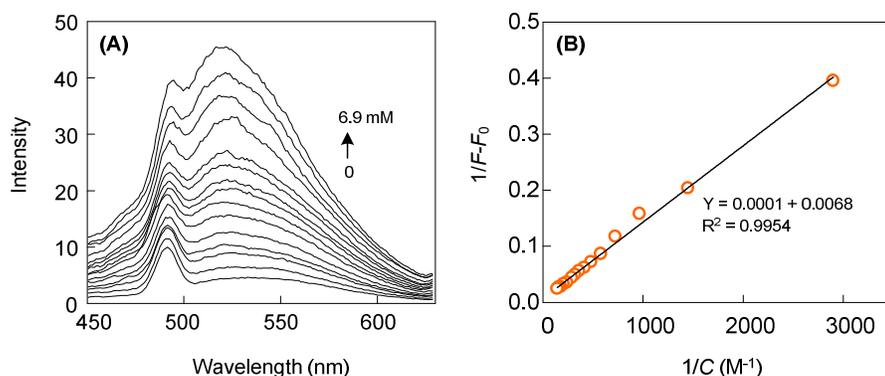
379 the sample (XMS average DP 19; 20% yield) contained more XMS-B (Fig. 6A). The product was  
 380 highly soluble in water at room temperature. We achieved a solubility enhancement of ca.  $25.3 \pm$   
 381  $0.4$ -fold that of curcumin at 10%, w/v, XMS, which was lower than expected probably due to some  
 382 contaminants of XOSs (Fig. 6B). To discard XOSs, the dialysis using a membrane with a MW  
 383 cutoff of 2 kD against water is recommendable for removal of saccharides having an average DP  
 384 less than 12 (Lang et al., 2022b). Nevertheless, the higher solubility enhancement was more  
 385 accessible at higher XMS concentrations, as evidenced by the phase solubility diagram (Fig. 6B).  
 386 The increase in nonlinear type as a function of XMS concentrations corresponded to the  $A_P$ -type  
 387 profile. This can be implied by the fact that the complex is first-order concerning curcumin but  
 388 second- or higher-order concerning XMS concentration. The maximum solubility of curcumin was  
 389 achieved at 97  $\mu\text{M}$  or more than a 180-fold increase over curcumin solubility in water with 50%,  
 390 w/v, XMS average DP 19, and the yellow color could be visually perceived as transparent (Fig. 6C).  
 391 Fluorescence spectra were recorded for curcumin solutions in increasing concentrations of XMS  
 392 average DP 19 (1–20 mg/mL or equivalent to 0.34–6.9 mM). Curcumin in water excited at 420 nm  
 393 was weakly fluorescent and had a broad spectrum centered at approximately 530 nm. During the  
 394 titrations, curcumins exhibited a blueshift of the spectral band to shorter wavelengths (Fig. 7A).  
 395 This phenomenon mainly indicated the movement of curcumin from a polar to a less polar  
 396 environment. This observation is in good accordance with the binding of curcumin and  $\beta$ -  
 397 lactoglobulin protein, which hydrophobic interactions contribute (Sneharani, Karakkat, Singh, &  
 398 Rao, 2010). Fig. 7B shows the double reciprocal plots of the results obtained by integrating the  
 399 XMS dropwise. The binding constant was estimated to be  $68 \text{ M}^{-1}$ .

400



401  
 402 **Fig. 6.** (A) HPAEC-PAD chromatogram of XMS average DP 19 fractionated by 60–75%, v/v,  
 403 methanol. (B) Phase solubility diagram of curcumin with the addition of XMS. Average DP 19  
 404 from 0–50%, w/v. (C) Curcumin solubilized in (i) water and (ii) 50%, w/v, XMS average DP 19.

405



406  
 407 **Fig. 7.** (A) Fluorescence spectra of curcumin with increasing concentrations of XMS average DP  
 408 19 in the direction of the arrow from 0–20 mg/mL (equivalent to 0–6.9 mM). (B) Benesi-  
 409 Hildebrand double reciprocal plot of the emission intensity at 522.8 nm against the series of XMS  
 410 concentrations where the  $I_0$  value was 4.

411

412 Xyloglucan enhanced curcumin solubility by 48.4-fold at its low concentration of 1%, w/v.  
 413 Its hydrophobic coil-structure contributes to curcumin-solubilizing ability. However, the  
 414 concentration is difficult to increase more because of being highly viscous and forming insoluble  
 415 curcumin-xyloglucan complex. XMS average DP 19 increased the curcumin solubility by 25- or  
 416 180-fold at its 10 or 50% (w/v) concentration, respectively. XMS with molecular mass  $\leq 10$  kDa  
 417 does not form coil structure (Hayashi, 1989; Tabuchi, Mori, Kamisaka, & Hoson, 2001). We have  
 418 no reasonable explanation about curcumin-solubilizing mechanism mediated by XMS. The longer  
 419 size or monosaccharides (arabinose, galactose, xylose) of XMS might contribute to hydrophobicity  
 420 and interact with curcumin. Formed curcumin-XMS complex becomes water-soluble due to high  
 421 aqueous solution of XMS.

422

#### 423 4. Conclusion

424 The aqueous solubility of curcumin was improved remarkably by tamarind seed  
 425 xyloglucan solution compared with those of the five polysaccharides. The appropriate size  
 426 preparation strategy could be established in this study, not only in accordance with the  
 427 polysaccharide shortening method (Guo, Hu, Wang, & Ai, 2017), but our methods also selectively  
 428 maintained (e.g., xylose) or removed (e.g., arabinose) the fine composition of tamarind seed  
 429 xyloglucan. The partial depolymerization by YC cellulase at 2 h of incubation enabled the  
 430 reproducible preparation of functional XMS. The active sizes with average DPs of 15.4–24.3  
 431 showed a similar capacity for curcumin solubility to ca. 36–40-fold, and hence, the XMS isolation  
 432 with 60–75% methanol precipitation was approached. The longer DPs caused the precipitation of  
 433 gel complexes and subsequently minimized curcumin solubility in the aqueous phase. The typical  
 434 MS with average DP 19 was readily dissolved in water at room temperature, and the higher the

435 XMS concentration was, the higher the amount of dissolved curcumin. The daily dose of curcumin  
436 was determined accordingly. The XMS functionality and textural property thus fit well in  
437 prevalence common household requisites. The health science formulation development will be  
438 further clarified in the following work.

439

#### 440 **Figure captions**

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469

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476

#### 477 **CRedit authorship contribution statement**

478 Weeranuch Lang: Conceptualization, Investigation, Formal analysis, Writing - original  
479 draft - review & editing. Takayoshi Tagami: Resources, Supervision. Hye-Jin Kang: Formal  
480 analysis, Investigation. Masayuki Okuyama: Data curation, Resources. Nobuo Sakairi:  
481 Conceptualization, Supervision. Atsuo Kimura: Writing - review & editing. Resources, Supervision,  
482 Funding acquisition, Project administration.

483

484 **Declaration of Competing Interest.** The authors report no declarations of interest.

485

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