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1 **A NATURAL COAGULANT PROTEIN FROM COPRA (COCOS NUCIFERA):**
2 **ISOLATION, CHARACTERIZATION, AND POTENTIAL FOR WATER**
3 **PURIFICATION**

4 **J. K. FATOMBI^{1,3*}, B. LARTIGES^{2,3}, T. AMINOU¹, O. BARRES³, C. CAILLET³.**

5 *1- Laboratoire d'Expertise et de Recherche en Chimie de l'Environnement et de l'Eau*
6 *(LERCEE) Faculté des Sciences et Techniques/Département de Chimie/Université d'Abomey-*
7 *Calavi, 01 BP 526, COTONOU (Bénin)*

8 *2- University of Toulouse – Geosciences Environment Toulouse – UMR CNRS-UPS-IRD*
9 *5563/234 – 14 Av. Edouard Belin – 31400 Toulouse, France*

10 *3- Université de Lorraine - Laboratoire Environnement et Minéralurgie (LEM) UMR 7569*
11 *CNRS-INPL (ENSG) 15 Avenue du Charmois 54501 Vandœuvre-lès Nancy (France)*

12 Corresponding author: E-mail: jacquesfatombi@yahoo.fr

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* Corresponding author : FATOMBI K. Jacques ; E-mail: jacquesfatombi@yahoo.fr

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22 **Abstract:**

23 A natural coagulant protein was extracted from the endosperm of *Cocos nucifera*, and
24 characterized by Fourier Transform Infrared Spectroscopy, Size Exclusion Chromatography,
25 potentiometric titration, electrophoretic mobility, and then tested for its coagulant properties
26 using silica nanocolloids. The infrared spectra revealed predominant absorption bands at 1649
27 cm^{-1} and 1536 cm^{-1} characteristics of $-\text{C}=\text{O}$ in primary and secondary amides of proteins
28 arranged into an alpha-helical secondary structure, and the presence of triolein when fat was
29 not removed from the extract. The molecular weight determined by size exclusion
30 chromatography was about 5.6 kDa, the charge density of the fat extracted protein being
31 1.05meq.g^{-1} , and the isoelectric point 7.5. The proteinous extracts behaved as effective
32 coagulants of silica particles, the destabilisation mechanism being consistent with an
33 heteroaggregation of oppositely charged colloids. Overall, the characteristics of that natural
34 protein were closely similar to those of *Moringa oleifera* seed extract, thus suggesting that
35 copra can be considered as a potential source of an efficient and sustainable coagulant for
36 water treatment in emerging countries.

37 **Keywords:** *Cocos nucifera* - natural coagulant - water treatment .

38 **Highlights:** •A protein coagulant similar to Moringa Oleifera seed extract, was isolated
39 from copra.

40 •Its potential for water purification was successfully tested with silica
41 nanocolloids.

42

43

44 1- Introduction

45 In his book "The quest for pure water", Baker (1949) [1] relating the history of water
46 purification, reveals that some nuts such as almonds in Egypt, *Strychnos potatorum* in India,
47 or beans in Soudan, have been used in the ancient times to promote the coagulation of turbid
48 waters. Although alum and other hydrolyzable metal salts have long supplanted those natural
49 clarification aids, other nut-derived substances and plant extracts have recently been
50 considered as potential coagulants and water softeners in emerging countries, where the
51 access to classical inorganic coagulants is sometimes difficult [2-3]. Thus, *Opuntia cactaceae*
52 [3-5], leaves of *Cassia alata* [6] and of *Calotropis procera* [7], and the now classical *Moringa*
53 *oleifera* seeds [8-13], have been reported to yield macromolecules of promising coagulating
54 properties that may reconcile a cost-effective water treatment with sustainable development
55 [3, 14]. Until now, only the nature of active agent in *Moringa oleifera* seeds, i.e. a dimeric
56 cationic polypeptide of 6-17 kDa molecular weight with a 10-11 isoelectric point, and the
57 corresponding aggregation mechanism, have been investigated into details [8-11, 15-17].

58 Just like *Moringa oleifera* sometimes referred to as "the miracle tree", Coconut palm
59 (*Cocos nucifera*) is a multipurpose tree which is a source of food, beverage, oilseed, fibre,
60 charcoal, and health products, and is revered by people as "the tree of life". It widely grows
61 across the world, (Asia, Africa, Caribbean, Latin America), and the overall coconut
62 production has been estimated to be at least 61.7 million ton/year according to current FAO
63 statistics. In a preliminary study, Fatombi et al. [18] showed that the casein cream extracted
64 from the coconut endosperm possesses a coagulant activity similar to that of *M. oleifera*
65 seeds, the two natural coagulants being tested on Okpara river water and synthetic
66 suspensions prepared with kaolin particles. To better understand the origin of such coagulant
67 activity, an improved characterization of coconut casein cream is needed. In the present paper,
68 Fourier Transform Infrared Spectroscopy, potentiometric titration, electrophoretic mobility,

69 and size exclusion chromatography, are used to characterize the properties of two caseins, i.e.
70 with or without fat, extracted from the cream of *Cocos nucifera*. In addition, the aggregation
71 mechanisms associated with those two natural coagulants are explored using silica
72 nanocolloids, a typical component of surface waters which is frequently used to assess the
73 efficiency of metal hydrolysable coagulants [20, 33]. Jar-tests, electrophoretic mobility
74 measurements, and Transmission Electron Microscopy on ultrathin sections cut from resin-
75 embedded aggregates, were then used to describe the interaction between the silica particles
76 and the two coconut caseins.

77 **2- Experimental section**

78 **2.1- *Extraction of casein from coconut cream***

79 The coconuts were purchased from the local markets of Cotonou (Benin Republic).
80 Mature coconuts were first dehusked and cracked to remove coconut water. About a hundred
81 grams of finely grated coconut endosperm obtained with a vegetable grater, were then added
82 to 500 mL of distilled water. After shaking for 10 min, the mixture thus obtained was filtered
83 through a 80 μ m-mesh sieve, and the liquid collected was separated between an aqueous phase
84 (coconut milk) and an organic phase (coconut cream). Hydrochloric acid 1M solution was
85 then added to 100 mL of coconut cream until the precipitation of casein occurred. The casein
86 precipitate was separated from oil and hydrochloric acid residues by centrifugation at 3500
87 rpm for 20min. The pellet of raw casein was oven-dried at 40°C during 24 hours and is further
88 referred to as CaMG (casein with fat). In addition, a fat-free casein named CaSMG hereafter,
89 was also obtained by extraction with hexane in a soxhlet extractor. The coagulant solution
90 was prepared by adding two grams of either CaMG or CaSMG to 100mL of distilled water,
91 and the mixture was then agitated at room temperature for two hours.

92 **2.2- *Characterization of caseins***

93 The infrared spectra of CaSMG and CaMG caseins were recorded with a Fourier-
94 transform infrared spectrometer (Bruker IFS55) equipped with a DTGS detector in the
95 transmission mode. 1 mg of casein was mixed with about 150 mg KBr (FTIR grade), and a
96 pellet was prepared using a 10 T press. The spectra were recorded in the 4000-400 cm^{-1} range
97 with 64 scans collected at 2 cm^{-1} resolution.

98 The electrophoretic mobility of CaSMG and CaMG suspensions (40mg/L of each
99 casein in NaCl 0.1M) was determined with a zetaphoremeter IV (CAD Instrumentation,
100 France) equipped with a CCD camera. The electrophoretic mobility was obtained by
101 measuring the direction and velocity of particles in the applied electric field (800 V/m).

102 The charge density of coconut caseins was determined by potentiometric titration (Titrand
103 809 Metrohm) at 25°C under an inert nitrogen atmosphere. The titration was carried out with
104 100 mg of a given casein suspended into 50 mL NaNO_3 0.1M solution as a function of titrant
105 volume (HNO_3 0.1M or NaOH 0.1M). Before analysis, the suspension was allowed to
106 stabilize for 1h under magnetic stirring and nitrogen stream. A quick step titration in acid-to-
107 basis direction from natural pH to pH 10 was first recorded in order to reach the pH
108 considered as the pH origin of the following titration curve used for the charge density
109 determination. The second titration was then performed in the basic-to-acid direction from pH
110 10 to pH 3. The reference curve was recorded by titration of the pure electrolyte solution
111 NaNO_3 0.1 M under the same conditions. In the case of a solid/electrolyte interface, protons
112 or hydroxyls can be used to probe the surface charge density. Hence, the proton consumption
113 (PC in $\mu\text{mol/g}$) was calculated according to the method reported by Prelot et al. [19], i.e. $\text{PC} =$
114 $\Delta V C/m$, where ΔV is the difference between the volume of titrant added to the suspension
115 (V_s) and that added to the reference electrolyte (V_r) ($\Delta V = V_s - V_r$), C being the titrant
116 concentration and m the mass of solid (g).

117

118 The molecular weight of macromolecules contained in CaSMG and in CaMG was
119 inferred using size exclusion chromatography equipped with a GF-250 9.4x250mm column
120 that can separate soluble macromolecules in water in the 4kDa-400 kDa molecular weight
121 range. The column was calibrated with standard proteins: thyroglobulin 670 kDa, IgG 160
122 kDa, apo-transferrin 78 kDa, bovine albumin 66 kDa, chicken albumin 44.3 kDa,
123 myoglobin 17.6 kDa, ribonuclease A 13.7 kDa, and insulin 5.73 kDa. A few micrograms of
124 each standard protein were dissolved in NaCl 0.2M solution, and the calibration curve was
125 obtained from a plot of the logarithm of the molecular weight versus elution time. CaSMG
126 and CaMG samples were prepared by dissolving 50 mg of each casein in 2.5mL NaCl 0.2M
127 solution. After shaking for two hours, the suspension was filtered on a membrane (0.45µm
128 pore size). About 100µL of filtrate were injected in the column, the elution being performed
129 with NaCl 0.2M solution at a 1 mL.min⁻¹ rate. The proteins were detected by UV
130 spectrophotometry with the detection wavelength set at a 215.8nm.

131 **2.3- *Preparation of colloidal suspensions and aggregation procedure***

132 The silica suspensions were prepared by diluting Ludox HS 40 (Aldrich) at 100, 250,
133 500 and 750mg/L with deionised water (Millipore, MilliQ). 336mg/L of NaHCO₃ were added
134 to give a buffer capacity similar to that of a surface water [20]. The pH of silica suspensions
135 was adjusted at 6 with dropwise addition of HCl 0.1N solution. The Ludox silica used in this
136 study is an alkaline aqueous dispersion of non porous spherical particles 14 nm in diameter.
137 Unlike kaolin particles used in our previous study [18] that present a shape factor and two
138 types of surfaces (edge and basal), the spherical precipitated Ludox silica with an
139 energetically homogeneous surface [35] makes a better model for investigating the
140 aggregation mechanisms with the coconut caseins. In addition, it was previously used to

141 assess the coagulating properties of WAC HB (Water Aluminum Chloride High Basicity)
142 [20].

143 Mixing of the silica suspensions with the natural coagulant was carried out in a 150mL
144 glass beaker reactor equipped with four Plexiglas baffles to avoid the formation of a vortex at
145 high mixing speed. The reactor is fitted with a rectangular paddle located at 1/3 of the beaker
146 height from the bottom. The synthetic suspension was agitated at 250 rpm for 2min; the
147 selected volume of CaMG or CaSMG coagulant solution was then added with an Eppendorf
148 micropipette (100-1000 μ L). The stirring rate was maintained at 250 rpm for 3min, and then
149 was decreased at 60 rpm for 20 min. At the end of stirring, the aggregated suspension was
150 allowed to settle for 30 min in a graduated Imhoff cone.

151 50 mL of supernatant were siphoned out from 25mm under the free water surface. The
152 supernatant was analysed for pH (WTW LF 340), residual turbidity (HACH 2100P), and
153 electrophoretic mobility (CAD Instrumentation Zetaphoremeter). The sludge volume was
154 measured by reading the graduation marks on the side of the Imhoff cone.

155 Transmission electron Microscopy observation of aggregates was performed with a
156 Philips CM 20 operating at 200 kV, on ultrathin slices obtained from resin-embedded
157 samples. The sludge was first fixed with 2% osmium tetroxide. After one hour of reaction,
158 excess of treated water was removed and replaced with solutions of acetone in water (10%,
159 20%, 40%, 60%, 80%, 90% and then 100%). The samples were then impregnated in an epoxy
160 resin (Epon 812, Euromedex). Impregnated samples were placed in molds and polymerized in
161 an oven at 60°C for 24 hours. The blocks obtained were cut using a Reichert OM U2
162 ultramicrotome. The sections were mounted on a carbon-coated copper grid and stained with
163 lead citrate and uranyl acetate.

164 **3- Results and discussion**

165 **3.1- *Physicochemical characterization of CaMG and CaSMG caseins.***

166 Both caseins were analysed by FTIR to assess the nature of active functional groups in
167 CaSMG and CaMG coagulants. As illustrated in figure 1, similar absorption bands at 1649
168 cm^{-1} , 1536 cm^{-1} , and 1237 cm^{-1} , can be recognized in both CaSMG and CaMG spectra. These
169 peaks are characteristic of carbonyl C=O stretching vibrations in primary, secondary, and
170 tertiary amides, respectively [21], the positions of those infrared bands being consistent with
171 the presence of α -helices in the secondary structure of proteins [17, 22]. The rather broad
172 peaks around 3411 cm^{-1} and 3290 cm^{-1} can be assigned to the stretching vibrations of O-H
173 and N-H and to intermolecular hydrogen bonds within the caseins macromolecules,
174 respectively [23].

175 **Figure 1: FTIR spectra of CaMG and CaSMG**

176 The CaMG spectrum also reveals intense infrared bands at 1746, 2924, 2854, and
177 2956 cm^{-1} . The strong peak at 1746 cm^{-1} , attributed to the C=O stretching vibration of the
178 carbonyl groups in methyl esters and triglycerides, has been identified in the infrared
179 spectrum of cashew nut kernel [24]. The classical bands at 2924, 2854, and 2956 cm^{-1} are
180 assigned to C-H asymmetric and symmetric stretching in CH_2 and C-H asymmetric stretching
181 in CH_3 , respectively. Those infrared signals are typical of compounds containing long alkyl
182 chains, and their absence in the CaSMG spectrum validates the fat extraction from the casein.
183 The remaining bands at 1467 cm^{-1} and 1162 cm^{-1} likely identify the CH_2 scissors deformation
184 vibration and the C=O bending vibration, respectively. Interestingly, the infrared bands
185 typical of the fats contained in CaMG and removed during the hexane treatment, are
186 comparable with those of triolein, a triglyceride of oleic acid found in a number of oily nuts
187 [24-25]. On the other hand, the abundant presence of amide functional groups in the caseins is
188 consistent with a proteinous nature of those compounds. Moreover, the infrared spectrum of

189 CaSMG is similar to that of purified active protein in *Moringa oleifera* seeds used for
190 aggregation of colloidal particles [17].

191 **Figure 2: Size exclusion chromatograms of CaMG and CaSMG**

192 The size exclusion chromatograms of CaMG and CaSMG are shown in figure 2.
193 CaMG is eluted as two main peaks detected at retention times of 14.15 and 16.8 min, whereas
194 CaSMG appears as a single peak at about 14 min with a minor shoulder around 16.2 min.
195 Obviously, the purified compound in CaSMG chromatogram corresponds to the proteinous
196 matter identified by FTIR. From the calibration curve relating the molecular weight of
197 standard proteins to elution time, a molecular weight of about 5.6 kDa can be calculated.
198 Interestingly, the flocculating activity of *M. oleifera* seeds has been attributed to proteins of
199 molecular weight ranging from 6 to 17 kDa [8, 9, 11, 13, 16, 26, 27]. The second peak at 16.8
200 min retention time on CaMG chromatogram is tentatively attributed to the triolein-like fat.

201 Figure 3a shows the proton binding isotherms inferred from the potentiometric
202 titration curves of CaMG and CaSMG. Curves with similar shape are obtained for the two
203 caseins. The negative values obtained above pH 3 for CaMG and pH 3.4 for CaSMG, indicate
204 a release of protons from caseins, whereas the positive values correspond to an adsorption of
205 protons. At first approximation, it can be considered that the fat associated with CaMG casein
206 do not present any major titratable functional groups, and hence, that the difference between
207 the two curves correspond to the weight of fat in casein. Therefore, CaMG should contain
208 almost equivalent amounts of protein and fat. The net charge densities obtained from the
209 titration curves of CaMG and CaSMG, are 0.47 and 1.09 meq/g, respectively. Such values are
210 lower than that of chitosan (4.5 meq/g) [32], but remain close to the 1.2-7.3 meq/g charge
211 density range of usual commercial cationic polyelectrolytes [28].

212 **Figure 3: a) Variation with pH of proton consumption for CaMG and CaSMG; b)**
213 **Evolution of electrophoretic mobility as a function of pH for CaMG and CaSMG**
214 **caseins.**

215 The electrophoretic mobility of both CaMG and CaSMG caseins as a function of pH is
216 shown in figure 3b. An identical Point of Zero Mobility (PZM) of 7.5 is found for both
217 coagulants, which, in accordance with the potentiometric titration results presented above,
218 indicates that the fat in CaMG does not significantly contribute to the charge of that extract.
219 The difference between the pH at no proton release (~ 3.5) and the point of zero mobility (7.5)
220 likely originates from the organization of the protein coagulant as a soft particle permeable
221 towards ions and hydric fluxes [35]. The PZM of active protein in coconut casein is lower
222 than the value ~10 reported in the literature for the purified protein contained in *Moringa*
223 *oleifera* seeds [8, 13, 29], thus suggesting a higher content in carboxylic groups in coconut
224 caseins.

225 **3.2- Jar-test results**

226 Figure 4a compares the performance of CaSMG and CaMG in terms of turbidity
227 removal and sludge volume in the case of 250 mg/L silica nanocolloids suspensions
228 aggregated at pH 6. Similar patterns are obtained for both coagulants: the residual turbidities
229 increase steadily at low casein concentration as small non-settleable aggregates are formed,
230 and after reaching a maximum, decrease steeply with further addition of coagulants as settling
231 aggregates are then produced. At higher casein concentrations, the supernatant turbidities
232 remain low and practically constant in the range of dosages investigated. In parallel, the
233 sludge volumes rapidly increase after the respective maxima in residual turbidity, to reach an
234 identical and steady plateau value. In other words, the amount of sludge obtained during the
235 jar-test does not depend on the type of casein used. This implies that the quantity of silica

236 particles is a main determining parameter for sludge volume, as confirmed by the proportional
237 relationship between these two variables established for a series of jar-tests performed with
238 varying concentrations in silica colloids (figure 4b). The supernatant pH of the treated water
239 remained essentially constant with only a small decrease of 0.3 pH units at high coagulant
240 concentration.

241 **Figure 4: a) Variation of residual turbidity (solid curves) and sludge volume**
242 **(hyphenated curves) as a function of casein concentration for both CaMG and CaSMG ;**
243 **OCC indicates the Optimum Coagulant Concentration. b) Variation of maximum sludge**
244 **as a function of silica concentration for casein CaSMG.**

245 The optimum coagulant concentration (OCCs) is defined as the minimum dosage
246 needed to attain the low residual turbidity value. It may seem paradoxical to obtain a residual
247 turbidity at OCC higher than that of the untreated suspension. However, the light scattered by
248 a few non-settleable fractal aggregates in the micron size range is certainly much higher than
249 that of all dispersed elementary nanoparticles. According to figure 4a, CaSMG is more
250 effective than CaMG, especially that a slightly lower residual turbidity and a smaller sludge
251 volume are obtained at the OCC. However, the difference between the two OCCs, 80 mg/L
252 and 100 mg/L for CaSMG and CaMG respectively, is much less than expected if charge
253 neutralization was the direct aggregation mechanism. Indeed, as the charge density of CaMG
254 is about half that of CaSMG, the corresponding OCC should be double of that associated with
255 CaSMG. In comparison, the OCC recorded with WAC HB – a commercial aluminum
256 chlorosulfate coagulant - to treat a 250 mg silica suspension at pH 6 was about 3 mg/L of Al.

257 TEM examination of silica nanocolloids aggregated with CaSMG casein at the OCC
258 (figure 5) reveals relatively compact clusters of particles and small organic fibrils originating
259 from the unfiltered coagulant solution before addition. The cationic protein, which according

260 to its 5.6 kDa molecular weight should be in the nanometer size range [30], likely corresponds
261 to the dark heavy metal-stained grains present at the surface of some silica nanocolloids. It
262 should be noted that the silica particles embedded in the bulk of the resin ultrathin section can
263 be visualized because of mass contrast, but that the coagulant organic matter associated with
264 them can not be stained with heavy metal salts.

265 **Figure 5: TEM micrograph of resin-embedded sludge (CaSMG + silica suspension at**
266 **120mg/L) pH=6 (a: compact clusters of silica colloids; b: small organic fibrils; c:**
267 **coagulant protein)**

268 Figure 6a shows the variation of electrophoretic mobility of particles remaining in the
269 supernatant after settling, as a function of casein concentration. For both CaSMG and CaMG,
270 the electrophoretic mobility of residual aggregates increases with coagulant concentration first
271 rapidly up to the OCC, and then more slowly at higher dosages. However, even above OCCs,
272 the complete neutralization of silica colloids can not be achieved. Actually, the mobility
273 curves do not provide any precise indication regarding the optimal coagulation range. Similar
274 electrokinetic behaviors were reported by Jerri et al. [31] for *Moringa oleifera* cationic protein
275 adsorbed on sand granules, and for *Opuntia* spp. coagulant with kaolin particles [3].

276 **Figure 6: a) Variation of electrophoretic mobility of treated water at pH=6; b) Evolution**
277 **of OCC as a function of silica nanocolloid concentration at pH=6.**

278 Nevertheless, as illustrated in figure 6b, the optimum coagulant concentration is
279 proportional to the amount of silica particles in suspension, such stoichiometric relation being
280 usually interpreted as resulting from a charge neutralization mechanism [28]. Moreover, the
281 ratio of slopes of OCC-silica amount curves for CaSMG and CaMG is about 2, i.e.
282 approximately the ratio of charge densities measured for the two caseins. The aggregation
283 mechanism of silica particles by caseins is therefore related to charge neutralization, even

284 though the charge of colloids can not be reversed at high dosages. A key observation made by
285 Kwaambwa et al. [10] is that the coagulant protein from *Moringa oleifera* seeds has been
286 shown by neutron reflectivity to adsorb cooperatively at the surface of silicon oxide.
287 Therefore, silica nanocolloids could be aggregated by clusters of coconut protein of increasing
288 size with coagulant concentration, the aggregation phenomenon being equivalent to an
289 heteroaggregation of oppositely charged colloids. It should be noted that the residual fat
290 associated with the protein coagulant could play a significant role in such cluster formation.

291 **4- Conclusion**

292 This study demonstrates that an effective coagulant protein can be extracted from the
293 coconut endosperm. This water soluble protein has a 5.6 kDa molecular weight and a 7.5
294 isoelectric point. The destabilisation mechanism of silica particles seems to be related to an
295 heteroaggregation between oppositely charged colloids, the protein component likely forming
296 clusters of increasing size with coagulant concentration. Preliminary jar-tests conducted at pH
297 8 with the fat free casein (CaSMG) showed only a 25% increase of the Optimum Coagulant
298 Concentration for a 250 mg/L silica suspension. This suggests that the natural coagulant
299 protein extracted from copra can certainly be effective over the range of natural water pH.

300 Further work is obviously needed to check whether the coconut protein also possesses
301 efficient antimicrobial and water softening properties, just like the protein extracted from
302 *Moringa oleifera* seeds. Interestingly, *Cocos nucifera* is essentially grown in the intertropical
303 zone where precipitations exceed 2000 mm, whereas *Moringa oleifera* is also present in semi-
304 arid areas. This suggests that both trees can be considered as a potential source of natural
305 coagulant in complementary geographical areas.

306

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