

Identifying molecular mass of coagulant protein from edible Hibiscus seeds using SDS-PAGE analysis

Alfred Ndahi Jones^a and John Bridgeman^b

^aDepartment of Civil Engineering, Faculty of Engineering, University of Maiduguri, P.M.B 1069, Maiduguri, Borno State, Nigeria.

Corresponding author, E-mail address: alfred_ndahi@yahoo.com

^bFaculty of Engineering and Informatics, University of Bradford, Bradford, West Yorkshire, BD7 1DP, United Kingdom.

E-mail address: j.bridgeman@bradford.ac.uk

Abstract

This study used sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and a jar test apparatus to investigate the molecular weight (MW) and turbidity removal potential of Hibiscus seeds. Three Hibiscus species were assessed, *viz.*; okra crude extract (OCE), sabdariffa crude extract (SCE) and kenaf crude extract (KCE). Furthermore, purified versions of each (i.e. purified okra protein (POP), purified sabdariffa protein (PSP) and purified kenaf protein (PKP)) obtained from anionic exchange were evaluated. The results show that while the crude extracts exhibit multiple presence of proteins with MW sizes varying between 11 and 82 kDa, the purified samples consist of a single coagulant protein band around 39 kDa. In each case, significant turbidity removal was recorded with the purified proteins where POP, PSP and PKP achieved approximately 98, 94 and 90% removal respectively, at a reduced dosage of ≤ 0.6 mg/l. However, OCE and SCE achieved lower turbidity removal of 86 and 85 using 40 mg/l dose respectively while KCE recorded only 73% turbidity removal with 60 mg/l dose. Sludge generation by crude and purified proteins was approximately 25% of

sludge produced by aluminium sulphate and carried the additional benefit of being biodegradable. Therefore, it is evident that the coagulant protein in Hibiscus plant seeds has potential applications for improvements to accessing clean water in developing countries.

Keywords: Protein, Hibiscus seeds, turbidity removal, molecular weight, water treatment

Introduction

Access to clean drinking water is a basic human right which improves quality of life and overall human development (Abia et al., 2015). Conversely, poor water quality and sanitation are major causes of child death in developing countries due to waterborne and food related infections (Bodlund et al., 2014). However, in many developing countries access to improved drinking water is a major challenge due to rapid urbanisation, climate change and lack of effective and efficient water supply schemes. Rapid urbanisation is expected to rise in Africa (UN, 2012, Gretsche et al., 2016), thereby putting pressure on existing stressed water resources and sanitation facilities. In addition, a lack of piped water supply, sanitation and effective waste disposal systems are anticipated to increase child mortality rates by more than 20 times compared with areas having functional facilities (UNICEF, 2002). The sustainable development goal to improve access to water, sanitation and hygiene to the unserved population by the year 2030 (SDG6, UN, 2016), is a recent global commitment to combat this threat. However, combating waterborne diseases is challenging because of the need for water to be free from organic and inorganic particulates and pathogenic organisms to make it potable. Conventional chemicals such as aluminium sulphate (alum) as coagulant and chlorine which is routinely used as disinfectant (Abokifa et al., 2016) are often used to effect treatment. However, such chemicals are costly to import. Therefore, recent work has considered the use of cost-effective natural extracts in drinking water treatment (Beltran-Heredia and Sanchez-Martin, 2009, Bodlund et al., 2014, Jones and Bridgeman, 2016a) in order to increase access

to clean drinking water. *Moringa oleifera* (MO) seed is the natural extract that has received most attention (Jahn Samia, 1998). However, it is noteworthy that other plants have also been studied recently. Notable among them are; purified MO seed protein (Ghebremichael et al., 2006, Sanchez-Martin et al., 2010) and crude MO extract (Jahn, 1986, Ndabigengesere and Narasiah, 1998, Sanchez-Martin et al., 2012, Petersen et al., 2016), *Cactus latifaria spp* (Diaz et al., 1999), and *Cactus Opuntia spp* (Zhang et al., 2006, Miller et al., 2008). Also, the coagulation potential of chestnut and acorn (*Fagaceae*) has been reported (ciban et al., 2009). Common bean (*Phaseolus vulgaris*) seed, a primary source of proteins in Tanzania and many other countries, has also been evaluated as a potential coagulant (Antov et al., 2010, Marobhe et al., 2007).

In many of these studies, the coagulant compound was shown to be a cationic protein. For instance, Ndabigengesere et al. (1995) identified the coagulant compound in MO seed extract as a cationic protein with a MW of 13kDa and having isoelectric point (IEP) of 10 and 11 while Gassenschmidt et al. (1995) reported the MW of purified MO protein as consisting of dimeric bands of 6.5 and 14 kDa, each showing high turbidity removal efficiency. Conversely, Ghebremichael et al. (2005) showed that the MW of the coagulant protein in purified MO seed to be less than 6.5 kDa, achieving significant turbidity reduction in water. Similarly, Antov et al. (2012) observed that the only fraction of common bean (*Phaseolus vulgaris*) extract that has turbidity removal potential is a MW of less than 10 kDa. However, Montoya et al. (2008) observed that the coagulant compound in common bean has a dimeric protein with MW of approximately 20 and 50 kDa, while Morales-de León et al. (2007) reported the MW of common bean between 26 and 49 kDa. Additionally, Bodlund et al. (2014) conducted a peptide sequence of two types of *Mustard* seeds which recorded 70 and 85% turbidity removal performance consist of MW of 6.5 and 9 kDa respectively. Furthermore, Marobhe and Renman, (2013) showed that purified *Parkinsonia aculeata* seeds are cationic proteins with MW of 6.5

kDa, similar to that of MO which reduced the turbidity of Charco dam water from 880 NTU to less than 13 NTU. One major advantage of using natural extract in water treatment is the low sludge production compared to traditional chemicals such as alum. Ndabigengesere and Narasiah (1998) compared the quality of water treated with shelled MO seeds and alum and observed sludge volume production was 5 times lower in MO-treated water than alum counterpart. Similarly, Yin (2010) reported similar findings in most natural extracts studied, thus, reducing the cost associated with sludge handling and disposal. Such sludge is also biodegradable and could serve as nutrients on agricultural farmlands. Therefore, the work reported here aimed to investigate the MW of coagulant protein in both crude and purified Hibiscus seeds (namely: *okra*, *sabdariffa* and *kenaf*) using the SDS-PAGE analysis technique and to consider their turbidity removal efficiency in water treatment.

Materials and methods

Seed collection and crude extract preparation

Hibiscus seeds were obtained from a market in Nigeria. The seed kernels were manually removed from the seedpod, followed by washing according to Jones and Bridgeman (2016a) and then ground into a fine powder using a Tema laboratory disc mill. The ground seed powders were then sieved following Jones and Bridgeman (2016b). The powder retained in the 212 μm , and 300 μm sieve sizes were combined and used in the preparation of the crude seed extracts (CSEs) to make 2% (w/v) suspension. The suspension was further processed using a magnetic stirrer for 15 min then centrifuged at 4500 rpm for 10 min (Heraeus Megafuge16, Thermo Scientific, Germany). Further filtration was conducted using a Whatman No. 42 filter paper prior to use as coagulants.

Lipid extraction from the seeds

Lipid extraction and purification of the bioactive compounds in the seeds was performed using the ground seed powders, sized between 212 and 300 μm and then defatted using high-grade hexane in an electro-thermal Soxhlet extractor. 20 g of the seed powder was used during the extraction. The extraction was performed using 1% w/v of seed sample in 2 L of solvent volume (hexane) and heated to 60 $^{\circ}\text{C}$. The process was run continually for 8 hrs with each complete cycle taking approximately 2-3 min. The residues obtained from the extraction thimble were removed and dried overnight at room temperature of 19 ± 2 $^{\circ}\text{C}$. The dried residue was ground again into a fine powder using a pestle and mortar and was used in the subsequent purification processes. Each of these samples was kept in a plastic container at 4 $^{\circ}\text{C}$ in a refrigerator until use.

Purification using Ion Exchange Column

Isolation of the bio-active protein compounds responsible for coagulation activity was performed using an ion exchange column (IEC). A Hi-Trap Q HP (1 ml) anion column, (GE Healthcare, Sweden) was used for the purification of the protein. The column was connected to a pump (Watson-Marlow Breeder pump 323, UK) and the pump head adjusted to a flow rate of 1 ml per min. The preservatives were washed with 10 ml of DI water, followed by 10 column volume of 1 M of NaCl dissolved in the phosphate buffer. The column was then equilibrated with the phosphate buffer before loading the protein in order to bring the column to the required pH. 5g of oil-free powder was dissolved in 0.1 M phosphate buffer and mixed thoroughly for 1 h using a magnetic stirrer (Stuart Scientific, UK). The suspension was centrifuged at 20,000 rpm at 4 $^{\circ}\text{C}$ for 40 min and the supernatant subsequently decanted. The supernatant was then filtered through a 0.45 μm membrane filter before injection onto the IEX column. The filtered supernatant was injected using the peristaltic pump onto the IEC to separate the protein of interest from the contaminants.

The sample was loaded onto the column at a flow rate of 1 ml per min, where the protein of interest was bound to the column matrix throughout the loading process. The weakly-bound contaminants were washed away with the starting buffer using 10 CV. The bound proteins were eluted beginning with 0.3, 0.5 and 1.0 M of NaCl-phosphate buffers and the various fractions collected. The collected protein fractions were analysed for coagulation activity using a standard jar tester (Phipps and Bird, 7790-900B USA).

SDS-PAGE analysis

SDS-PAGE analysis is the most widely used technique to separate and characterize proteins by electrophoresis to denature the protein using polyacrylamide gel as a support medium where it is reduced to a linear molecule. Protein is caused to migrate through a gel matrix, mainly influenced by an electrical field. Here, charged species are moved according to their net charges in the applied electric field. The MW of each protein is obtained, proportional to their net charges. This is also the same as the amino acid composition of the protein (sum of positive and negative charges). The analysis gives a better understanding of the molecular size of the coagulant protein (s) in the seeds.

Table 1 shows how the gel used for the SDS-PAGE analysis was prepared. For proper protein MW analysis, the stacking gel was prepared to concentrate the protein band while the running gel allows all the proteins to migrate simultaneously.

Table 1 SDS-PAGE for gel preparation used in the molecular weight analysis.

The protein sample was resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Protran BA-85, Pierce Protein Biology). The membrane was blocked in 5% milk/1X Tris-buffered Saline-TWEEN 20 (TBST) and incubated on a rocker at room temperature for 30 min. After blocking, the membranes were incubated with primary antibody polyclonal goat anti-

GFP (AbD Serotec) 1:2000 of antibody in TBST (dilution buffer) overnight at 4°C. At the end of the incubation, the membranes were washed three times with TBST at 5 min interval each and then incubated with secondary antibody (polyclonal anti-Goat HRP) 1:1000 of antibody in TBST for 1 h at room temperature on a rocker. The membranes were washed again as completed at the end of the primary antibody incubation. The blots were then incubated with West Pico Chemiluminescent Substrate (Pierce) and then visualized with Gene Snap Software (SynGene) and the proteins stained with either Ponceau S solution or Coomassie dye Brilliant Blue R-250.

Collection of water sample and jar test experiment

River water samples were collected from the Bourn Brook river adjacent to the University of Birmingham railway station in a set of 1 L sterilised plastic containers. Jar tests were performed using a jar test apparatus (Phipps and Bird, 7790-900B, USA) comprising six 1L beakers following Jones and Bridgeman (2016a) to determine the performance of each of the coagulants (crude and purified proteins). Briefly, the coagulant was dosed during rapid mixing at 200 rpm for 1 min. Thereafter, the water was flocculated at a reduced speed of 30 rpm for 30 min. The suspension was allowed to stand undisturbed for 1 h to allow sedimentation. A 10 ml final water sample was then drawn via syringe, 2cm from the top water surface in the beakers. Both initial and final water turbidity were measured using a turbidity meter (HI 93703, Hanna). All experiments were conducted in triplicate.

Results

SDS-PAGE analysis was conducted on the extracts in order to obtain information on their MW and the turbidity removal performance of the different protein fractions. The various bands and sizes of proteins are shown in Figs 1 and 2. Fig 1 shows some similar distinct protein bands across all the extracts. Each extract, however, consists of multiple heterogeneous bands, having

varied protein composition and physical characteristics. The various protein bands in OCE (lane 1), SCE (lane 2) and KCE (lane 3) in Fig 1 were compared in order to provide information regarding their MW. The results show that the band between 40 and 46 kDa is similar for SCE and KCE. However, the concentration of protein band with MW of 46 kDa was found in all the extracts, including OCE.

Figure 1 Shows SDS-PAGE analysis of the crude extracts. lanes 1, 2, 3 are OCE, SCE and 3 KCE while lane 4 is protein marker (7-175 kDa).

There is also clear evidence of similarity in protein band between 17 and 21 kDa in SCE and KCE, but the densest protein band above 17 kDa was observed in SCE. Interestingly, however, a faint protein band can be discerned even above the 50 kDa distinct band in SCE and KCE, which was not visible in OCE. Most notably, the protein concentration of the band with MW of 17 kDa is more discernible in OCE extract than in the SCE and KCE samples. Similarly, the protein bands above 7 kDa are faint but visible in OCE compared to the SCE and KCE samples. Overall, there are clear similarities in protein bands between SCE and KCE compared to the OCE sample.

Figure 2 SDS-PAGE analysis of Hibiscus eluted with 0.3M NaCl. Lane 1: Marker; Lane 2: POP; Lane 3: unabsorbed OK; Lane 4: PSP; Lane 5: unabsorbed SB; Lane 6: PKP; Lane 7: unabsorbed KE.

Fig 2 presents the SDS-PAGE analysis of purified protein fractions eluted with 0.3 M NaCl (the other fractions (0.1 and 0.5 M NaCl) did not show any coagulation activity when tested). The SDS-PAGE results show a single protein band across all the samples. The fraction eluted with the 0.3 M was considered in this work because of its coagulation activity. It is logical to conclude that most, if not all, of the bands that were visible in the crude extracts were eliminated after the purification, except the protein bands with MW of 39 kDa (width, 28-39

kDa). The single densest protein band with MW of between 28 and 39 kDa was considered to be responsible for the coagulation activity.

The concentration of the proteins across this band was similar except that the band was moderately dense in PSP but slightly wider in POP. It is also likely that there exists a small unit of coagulant protein with MW of 11 kDa as seen in the SDS-PAGE analysis. However, its presence may not have any significant influence on the coagulation activity because it is observed in a similar band even in the contaminant ladder in lanes 3 (unabsorbed OK), 5 (unabsorbed SB) and 7 (unabsorbed KE).

The results of the test show that protein fraction eluted with 0.3 M NaCl were found to have coagulation activity and so this was used in the subsequent coagulation investigations. Fig. 3 presents the performance of POP, PSP and PKP in turbidity removal. Fig 4 shows results for investigating the performance of OCE, SCE and KCE in water with initial turbidity of 50 NTU. In the work reported here, a 40 mg/l dose of both OCE and SCE was used to achieve a final residual turbidity of less than 7 NTU, while a 60 mg/l dose of KCE caused a reduction in turbidity from 50 to 13.75 NTU. Turbidity removal performance was observed to follow the order *OCE* (86%) > *SCE* (85%) > *KCE*(73%).

At the optimum coagulant dose, the purified protein dosage was reduced significantly ($p < 0.05$) compared with the crude extracts in Fig 4. A maximum turbidity removal of approximately 98% was achieved with 0.5 mg/l dose of POP while a dosage 0.6 mg/l was used to achieve 94 and 90% turbidity removal using PSP and PKP respectively. Interestingly, however, purified protein was found to perform optimally at a reduced dosage compared with the crude extracts in Fig 4 to achieve minimal residual turbidity.

Figure 3 Performance of POP, PSP and PSP as coagulants in water of NTU =50.

Figure 4 The performance of OCE, SCE and KCE as coagulants in water of NTU = 50.

The sludge production of all the natural coagulants was considered in relation to the performance of aluminium sulphate as a coagulant. Using an initial turbidity of 100 NTU, 0.5 mg/l dose of POP produced 0.5 ml of sludge after 1 hr sedimentation. A 0.6 mg/l dose of PSP and PKP and 5 mg/l of AS produced 0.54, 0.4 ml and 2.1 ml of sludge respectively. The ratio of sludge volume generated by AS to POP, PSP and PKP was 4:1, 4:1. and 5:1. This is similar to that found by Ndabigengesere et al. (1995) who reported that the volume of sludge produced by AS was five times higher than that of MO seed extracts.

Similarly, when OCE, SCE and KCE samples were tested, the results showed that sludge production was 1.1ml in water treated with OCE and 1.3 ml in SCE and KCE treated waters. Furthermore, the volumes of sludge produced with the CSEs were observed to be lower than the sludge volume generated by AS during the treatment. Not only is the sludge volume produced with either purified or crude proteins smaller than that produced when using aluminium sulphate, their sludge is biodegradable and non-toxic, which offers further advantages of using natural coagulants as an alternative to synthetic coagulants.

Interestingly, the cost of purifying Hibiscus seed proteins for water treatment was compared with that of the synthetic water treatment sachets being supplied by Oxfam GB. Oxfam Supply

Operation is one of the NGOs currently producing FCF/3 water sachet to make water accessible in developing countries. Each pack contains 6000 sachets powder (5g) for household water treatment. One sachet can treat between 15 - 20 litres of water. The cost of one pack is £312.00, excluding logistics. Therefore,

- 1 sachet of FCF/3 cost $(31200/6000) = 5.2$ pence, (cost of treating approximately 20 L of water).

Similarly, the approximate cost of purifying 2.5 kg is 60 pence for *Okra* and *sabdariffa* proteins respectively while the cost of purifying 2.5 kg of *kenaf* protein is 40 pence.

- Thus, the estimated cost of purifying *okra* and *kenaf* proteins to treat 20 litres of water is 0.05 pence while that of *sabdariffa* protein is 0.06 pence compared with 5.2 pence using FCF/3 water treatment sachet

Discussion

The SDS-PAGE analysis of the crude extracts shows multiple protein bands in each of the extracts indicating that there are various heterogeneous protein compounds in the seeds. The MW of these proteins varies according to size and bands, each having different physical characteristics. Although observations of the protein profile show the physical appearance of the various bands to be somewhat similar, they have varied coagulation behaviour. The concentration of the bands indicates much overlapping across the protein compounds that may or may not have coagulation activity. Determination of the particular protein band, size or MW responsible for the coagulation activity in the extracts is challenging. This is because Ghebremichael et al. (2005) and (Bodlund et al., 2014) have shown in their separate studies that the unbound proteins were comprised of contaminants and proteins with little turbidity removal potential.

269 Additionally, the presence of such proteins may render the coagulation process ineffective and
270 could contribute to the overall NOM in the treated water, therefore, negating the objective of
271 water treatment using Hibiscus seeds. The presence of NOM in water poses challenges for
272 water treatment, creating odour, taste, colour and encouraging bacterial growth (Bolto, 1995,
273 Broin et al., 2002, Ali et al., 2010). Thus, NOM removal is necessary to minimise health
274 concerns related to disinfection byproduct formation in the treated water, especially if chlorine
275 is used as disinfectant (Singer, 1999, Bridgeman et al., 2011, Liu et al., 2014).

276 To facilitate the removal of the overlapping proteins in the band with limited coagulation
277 activity and other compounds, protein purification was adopted. The purification of the proteins
278 presented an identical single protein band with low MW of 39 kDa in the SDS-PAGE analyses.
279 The work reported here found that only the protein fraction eluted with the low ionic strength
280 of 0.3 M NaCl solution has coagulation activity, thus indicating the MW of POP, PSP and PKP
281 in the SDS-PAGE analysis which can coagulate water. Contaminants that were not bound to
282 the matrix were eluted as unabsorbed. It was evident that all the other protein bands seen in the
283 crude extracts were contaminants or proteins which affect the overall turbidity reduction
284 potential. It is noteworthy that the proteins that were unabsorbed and weakly bound to the
285 matrix, and those proteins that were strongly bound to the matrix that were either eluted with
286 the starting buffer or with the high ionic strength of 0.5 and 1.0 M NaCl, were non-coagulant
287 proteins as they did not coagulate particle in water (results not shown). This is because the
288 results obtained from jar test experiments showed no evidence of coagulation when these
289 fractions were employed.

290 Comparative analysis of the crude and the purified protein has shown the presence of many
291 macromolecules in the extracts while the purified proteins consist of a single small size protein.
292 This observation was seen in MO protein as having two distinct protein bands when it was
293 isolated and characterised by Gassenschmidt et al. (1995), Ghebremichael et al. (2005) and

Ghebremichael et al. (2006) with increased adsorption capacity. It is thought that the elimination of other non-coagulant proteins, are the reasons for the increased coagulation activity, initially retarded due to protein-protein complexes (Bodlund et al., 2014). Assessment of the coagulation activity of POP, PSP and PKP showed increased water treatability of the proteins, producing turbid-free water compared to the crude extracts at a reduced dosage. The low dose requirement of the purified coagulant protein samples was caused by increased adsorption action of the proteins (Gassenschmidt et al., 1995). The low sludge volume production is another advantage of using natural extracts as it provides savings due to cost associated with the requirement for disposal facilities and its beneficial use in agriculture.

Furthermore, because of the variation in performance of the different Hibiscus species, it is important in this work to compare the cost benefit of using Hibiscus protein for people in developing countries with that of FCF/3: Sachet water treatment (Butyl Group, 2016) (combined flocculant and disinfectant) being supplied by Oxfam GB One FCF/3 sachet can treat between 15-20 litres at a cost of 5.2 pence (Butyl Group, 2016), excluding logistics, while the cost of treating approximately 20 litres of water were found to be 0.05 pence for *Okra* and *Kenaf*, and 0.06 pence for *Sabdariffa* proteins respectively. It is assumed that the per capita water consumption in developing countries is equivalent to 20/L/c/d in this study. Thus, the potential for cost saving is significant. It is noteworthy that the column used in the purification process is inexpensive and could be reuse several times provided it is washed properly and preserved with the same elution buffer. Additionally, for cost implication of using Hibiscus proteins, British pound was adopted here because the work was conducted in the United Kingdom.

Conclusion

SDS-PAGE analysis is a suitable tool to identify protein fractions with coagulation potential for drinking water treatment. Hibiscus seeds are biodegradable low-cost plants which consist of a single protein band (MW of 39 kDa) with significant turbidity removal potential that could help people in developing countries have access to turbid-free water for domestic use. The purification protocol adopted here is economical and can be scaled-up in a relatively straightforward manner using locally available materials with significant cost savings compared to imported materials. Currently, a large-scale purification technique using locally available facilities is on-going in Nigeria (not part of this study) to produce purified Hibiscus and other naturally-occurring plant proteins for large water treatment that could be adopted in developing countries.

Overall, the cost of treating water using Hibiscus protein is significantly cheaper than the use of synthetic water treatment sachet. The seeds can be used to effect robust and efficient water treatment, producing low sludge volume, thereby eliminating the difficulty arising from sludge disposal challenge as with the conventional chemicals.

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439 Table 1 SDS-PAGE for gel preparation used in the molecular weight analysis.

S/ No	Running Gel		Stacking Gel	
1	54µl	20% SDS	20µl	20% SDS
2	3.0ML	29: 1 40% Acrylamide	0.3ml	29:1 40% Acrylamide
3	2.9ml	dH ₂ O	2.85ml	dH ₂ O
4	54ul	10% APS	38ul	10% APS
5	4.0ml	1M Tris, pH 8.8	0.57ml	1M Tris, pH 6.8
6	10µl	TEMED	5µl	TEMED

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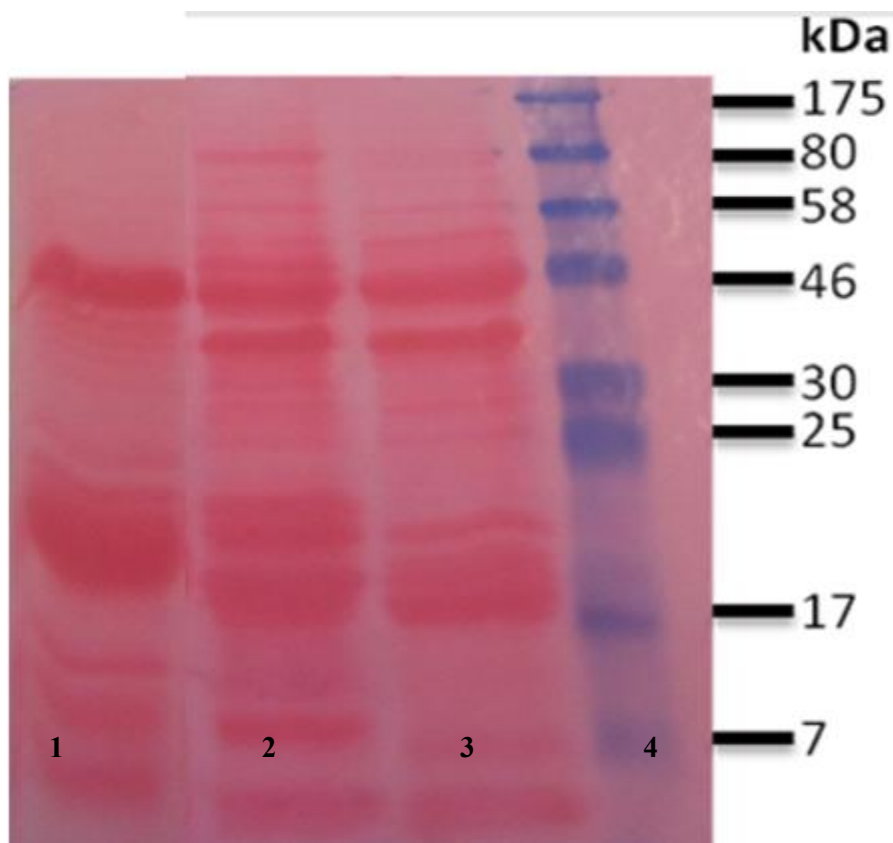


Figure 1 Shows SDS-PAGE analysis of the crude extracts. lanes 1, 2, 3 are OCE, SCE and 3 KCE while lane 4 is protein marker (7-175 kDa).

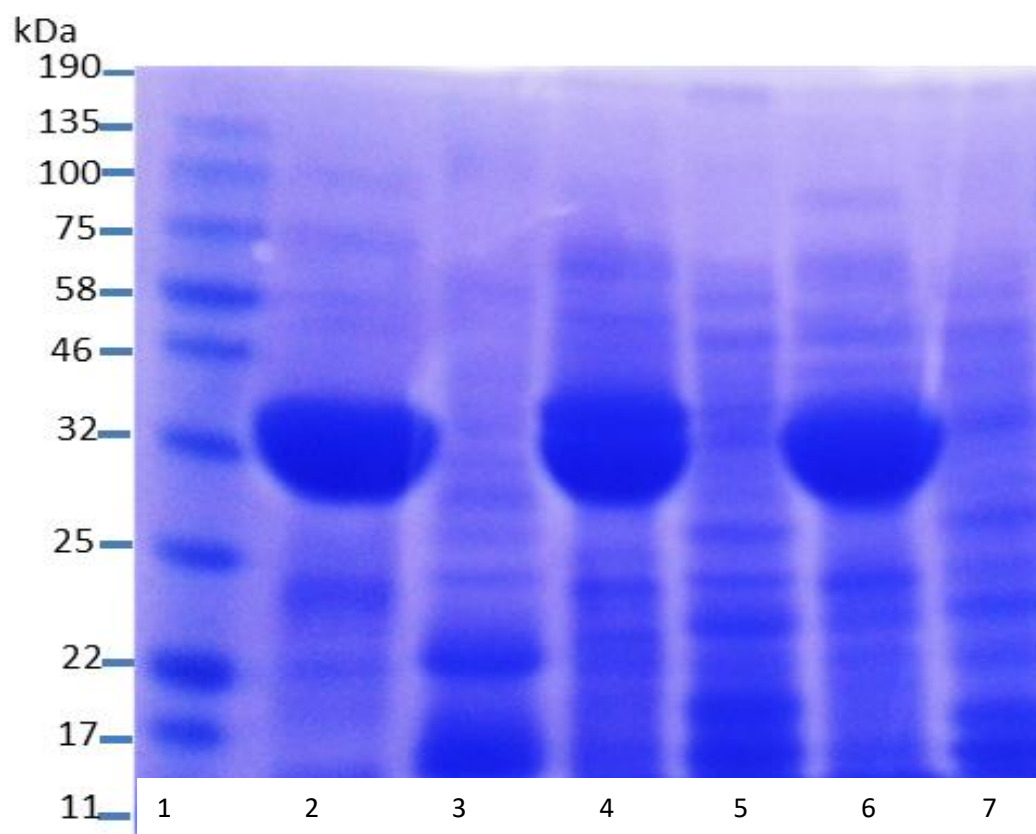


Figure 2 SDS-PAGE analysis of Hibiscus eluted with 0.3M NaCl. Lane 1: Marker; Lane 2: POP; Lane 3: unabsorbed OK; Lane 4: PSP; Lane 5: unabsorbed SB; Lane 6: PKP; Lane 7: unabsorbed KE.

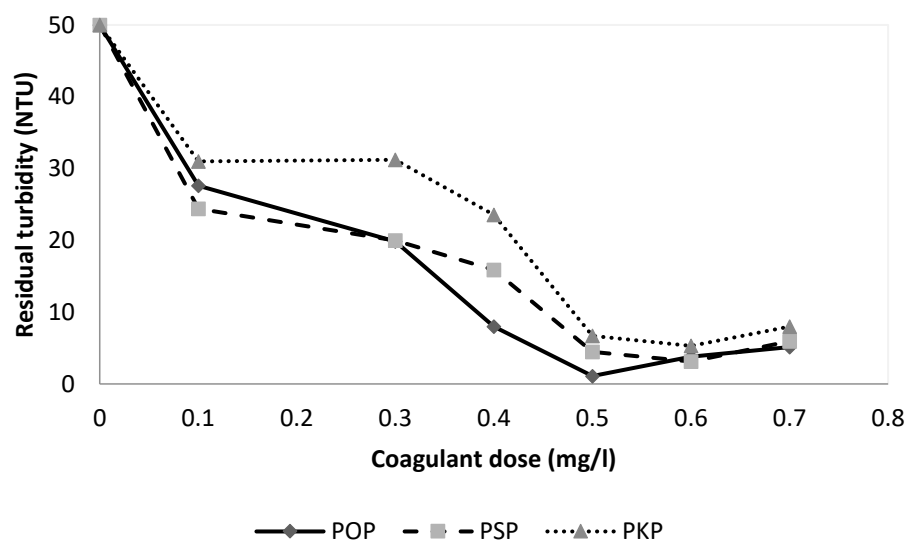


Figure 3 Performance of POP, PSP and PSP as coagulants in water of NTU =50.

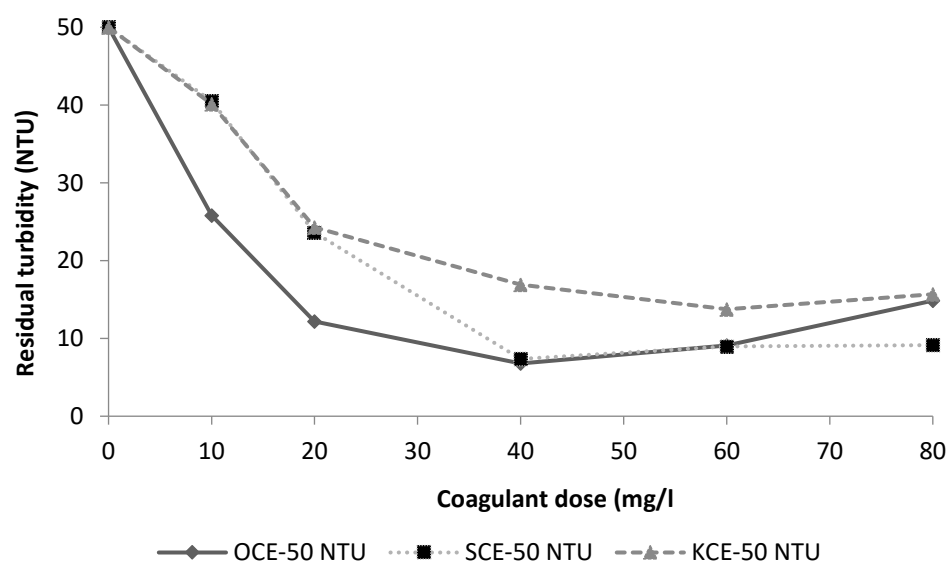


Figure 4 The performance of OCE, SCE and KCE as coagulants in water of NTU = 50.

List of Figure captions

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