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Original Article

THE FORMATION AND CHARACTERIZATION OF COLLAGEN LIQUID CRYSTALS FROM SNAKEHEAD FISH SKIN *(CHANNA STRIATA)*

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ABSTRACT

Objective: Liquid crystals are special state of matters which have regularity of solid arrangement but had a liquid-like flow characteristics. Collagen is a biopolymer that qualified for requirements as the system of a liquid crystal because it is mesogenic and rigid in a triple-helix section. There are various sources of collagen that have been used; one of them is snakehead fish skin *(Channa striata)*.

Methods: The stages of research were collagen isolation, collagen identification, liquid crystals formation, and characterization. Collagen liquid crystals were formed by lyotropic method using 0.5 M acetic acid and treated with and without sonication at 30, 60, and 80 mg/ml concentrations. The formation of Liquid crystal phase characterized by using Polarization Ligh microscopy.

Results: Mesophase analysis using polarized light microscope showed the presence of cholesteric phase (fingerprint pattern) which seen from the lowest concentration used in this study (30 mg/ml). The increasing of collagen concentration and sonication treatment can trigger the formation of clearly liquid crystal cholesteric phase under polarized light microscope. Infrared spectra of collagen liquid crystals both sonicated or not, showed no change in triple-helix.

Conclusion: The formation of lyotropic liquid crystal of collagen from snakehead fish skin showed the cholesteric pattern without changing the triple-helix collagen structure.

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INTRODUCTION

Liquid crystals in pharmaceutical preparations have played an important role in accelerating drug delivery by increasing their permeability [1]. The liotropic liquid crystals have a similar structural organization with stratum corneum to the stratum corneum, there for it can improve permeability of drug because their affinity to the skin [2]. In recent years, liotropic liquid crystal systems have attracted attention as skin penetration enhancers for high molecular weight agents [3].

In the development of pharmaceutical preparations, there are many systems that have a high penetration-enhancing activity such as liposomes and niosomes. However, the material has poor stability. Therefore, a physically stable liquid crystal system has been developed for the topical formulation [4].

Collagen is a biopolymer that qualified to a liquid crystals system because it is mesogenic. Mesogen is a compound that displays the liquid crystals properties. The basic mesogen property of collagen is amphiphilic and anisotropic polymer, which allows it to form liquid crystals system [5]. The mesogen group consists of rigid rod-like portion of the molecule, which is the triple-helix of collagen [6].

The presence of liquid crystals phase in collagen from rat tail tendon [7] and calfskin [8, 9] has previously been found with characteristic of cholesteric phase or fingerprint pattern under polarizing microscope [10]. Concentration and sonication treatment affect the formation of collagen liquid crystals [11]. Collagen liquid crystals from calfskin were reported to form liquid crystals system spontaneously at 60 mg/ml and assisted by sonication treatment at 30 mg/ml. Sonication treatment has been found to help the formation of clearly fingerprint pattern under polarizing microscope [9].

In this study, the formation of liquid crystals sourced from snakehead fish skin will be carried out to obtain information on the liquid crystal phase of collagen from snakehead fish skin dan its characterization.

MATERIALS AND METHODS

Collagen isolated from snakehead fish skin *(Channa striata)*, glacial acetic acid pro analysis (PT. Smart Lab), selenium (Merck), sulfuric acid (PT. Brataco), sodium hydroxide (PT Brataco), boric acid (PT. Brataco), methyl red, bromcresol green, hexane (PT. Brataco), acrylamide (Vivantis), ammonium persulfate (APS), tetramethyl ethylenediamine (TEMED), isopropanol (PT. Brataco), sodium dodecyl sulfate (Vivantis), tris (Merck), glycerol (Vivantis), beta-mercaptoethanol (Sigma-Aldrich), prestained protein ladder 11–245 kDa (Gangnam Stain), comassie blue staining (MP Biomedicals), buffers pH 4.01 and 6.86 (Mettler Toledo), nitric acid (PT. Brataco), perchloric acid (PT. Brataco), and hydrochloric acid (PT. Brataco).

Collagen isolation

Isolation of collagen from snakehead fish skin *(Channa striata)* was carried out with the combination of acetic acid and papain enzymes from fresh papaya latex *(Carica papaya)* without centrifugation [12]. This method was patented by Nofita in 2017 with patent number IDP000073332.

Identification of collagen

Identification of isolated collagen from snakehead fish skin *(Channa striata)* was to ensure the collagen used in accordance with specified requirements. Identification of collagen includes yield calculation, protein band profile analysis [13], water content, ash content, protein content, fat content pH, heavy metals [14], functional group analysis [15], thermal analysis, and x-ray difratogram analysis [13].

Formation of collagen liquid crystals

The dried collagen were 150, 300, and 400 mg of were dispersed in 5 ml of 0.5 M [8] acetic acid as much as 2 batches, then left overnight at low temperature. One batch of these was sonicated for 10 min [8]. Unsolicited collagen colloidal dispersions at 30, 60, and 80 mg/ml concentrations were written as U-col(30), U-col(60), and U-col(80), while sonicated ones were written as S-col (30), S-col(60), and S-col(80).

Mesophase analysis

S-col(30), U-col(30), S-col(60), U-col(60), S-col(80), and U-col(80) dripped between object and cover glass. Samples were viewed under polarized light microscope at 500x magnification (bar represents 50 µm) [8]. Dried collagen samples and collagen dispersion at 0.25 mg/ml were also observed for comparison.

Functional group analysis

FT-IR specta were obtained with ATR-FTIR spectrometer over the wavenumbers range of 4500–500 cm^{-1}. Samples of sonicated and unsonicated collagen liquid crystals were dripped onto the surface of ZnSe crystals. Then, FTIR spectra was measured at a resolution of 1 cm^{-1} and scanning of 20 scans/sec [16].

RESULTS AND DISCUSSION

Identification of collagen

The yield of isolated collagen was calculated based on the percentage of collagen sample weight produced from the initial weight of snakehead fish skin *(Channa striata)*. If the yield is high, the effectiveness of isolation treatment is also high. The yield of collagen obtained was 18.05%.

Water content test was carried out to determine the amount of water bound to collagen [15]. The lower water content of collagen, the better the quality [10]. Water content result of dried collagen from snakehead fish skin *(Channa striata)* was 6.011±0.813%. This value is still within the standard set by Indonesian National Standard (SNI), namely collagen containing maximum of 12% water [14].

Table 1: Collagen identification results from snakehead fish skin *(Channa striata)*

Analysis	Requirements	Results
Water content	≤ 12%	6.011±0.813 %
Ash content	≤ 1%	0.66±0.802%
Protein content	≥ 75%	92.41%
Fat content	Low	0.326±0.05%
pH	6,5-8	6.68±0.01
Heavy metal	Pb (≤ 0.4 mg/kg)	Pb (0.002 mg/kg)
	Cd (≤ 0.1 mg/kg)	Cd (<0.095 mg/kg)

The ash content test aims to determine the mineral content and purity of collagen by dry ashing method [17]. Ash content result of dried collagen from snakehead fish skin *(Channa striata)* was 0.66±0.802%. This value is included in the requirements set by SNI, namely the maximum collagen ash content of 1% [14].

Collagen is a derivative product of protein, therefore the protein content in collagen is very important [16]. Protein content testing was carried out to determine the amount of crude protein by determining total amount of nitrogen (N) contained in collagen. The higher protein content of collagen, the better the quality [16]. Protein content result of dried collagen from snakehead fish skin *(Channa striata)* as much as 92.41%. This value is in accordance with the standards set by SNI, namely, the protein content of collagen at least about 75%. [14].

Fat content test aims to determine the amount of crude fat contained in collagen. Fat content can affect the quality of collagen during storage, where the lower fat content of collagen, the better the quality [16]. Fat content result of dried collagen from snakehead fish skin *(Channa striata)* was 0.326±0.05%. This value is lower than the previous study which also examined fat content of collagen from snakehead fish skin *(Channa striata)* with the result of 2.05% [14].

pH result of collagen from snakehead fish skin *(Channa striata)* obtained was 6.68±0.01. This value is still within the standard set by SNI, which is the good pH range of collagen is 6.5–8 [14].

Heavy metal analysis aims to ensure the collagen material is safe to use and guaranteed from heavy metal contamination [13]. Heavy metal results of lead (Pb) and cadmium (Cd) obtained were 0.002 and<0.095 mg/kg. These values are still within the range set by SNI, where collagen from fishery ingredients contains less than 0.4 mg/kg lead and 0.1 mg/kg cadmium [14]. This shows that collagen from snakehead fish skin *(Channa striata)* not contained heavy metal cadmium and only small amount of lead heavy metal. It can be said that, collagen from snakehead fish skin *(Channa striata)* is safe to use.

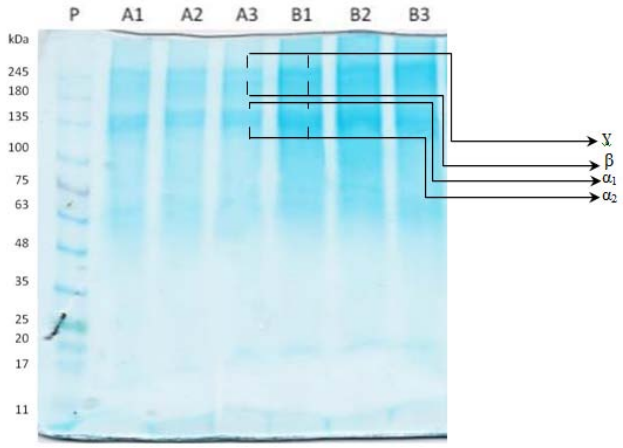

Fig. 1: SDS-PAGE band results of collagen from snakehead fish skin

Description

P: Prestained Protein Ladder

A1–A3: Collagen sample 5 mg/ml

B1–B3: Collagen sample 10 mg/ml

The results obtained were the presence of two protein bands in collagen samples of 5 and 10 mg/ml (A1–B3) around 135 kDa. This indicates there are α_1 and α_2 chains with molecular weights above

and below 135 kDa, respectively. Based on previous studies, it was reported that α chain of collagen from snakehead fish skin is around 130 kDa [19].

There are also β (dimers) and γ (trimers) subunits indicating cross - linking of collagen [19]. There were two protein bands in collagen samples of 5 and 10 mg/ml (A1–B3) around 245 kDa. This indicates that there are β (dimers) and γ (trimers) subunits in samples with molecular weights below and above 245 kDa, respectively. Based on previous studies, it was reported that β subunit of collagen sourced from fish was found at 200–250 kDa [19].

Based on infrared spectra obtained, absorption peaks of collagen from snakehead fish skin (*Channa striata*) include amides A, B, I, II, and III and each gives the characteristics of functional groups as shown in table 2.

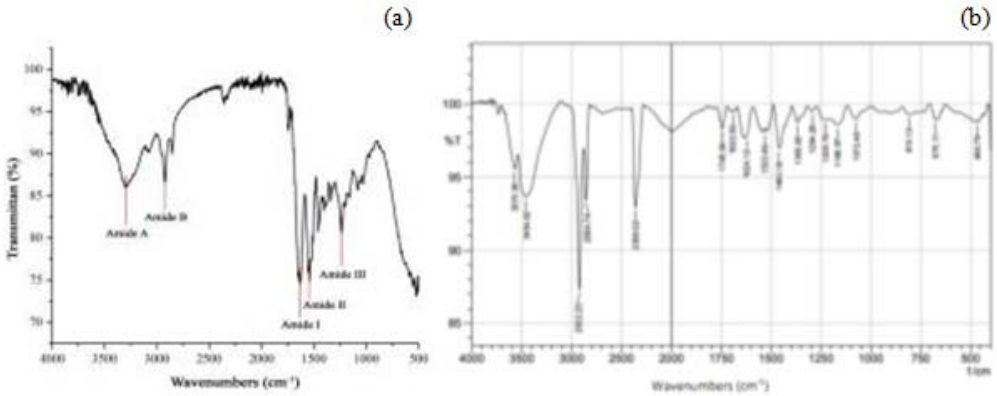

Fig. 2: (a) Infrared spectra results of collagen from snakehead fish skin *(Channa striata)* (b) Infrared spectra of the collagen from snakehead fish skin *(Channa striata)*; from literature [19]

Table 2: Analysis of absorption peaks and functional groups of collagen from snakehead fish skin *(Channa striata)*

Absorption peak	Wavenumber	Peak region	Functional group
Amide A	3304.06 cm^{-1}	3350–3550 cm^{-1}	N-H streching
Amide B	2922.16 cm^{-1}	2915–2935 cm^{-1}	CH_2 streching asymmetric
Amide I	1635.64 cm^{-1}	1600–1700 cm^{-1}	C=O streching
Amide II	1541.12 cm^{-1}	1480–1575 cm^{-1}	CN streching, NH bending
Amide III	1236.37 cm^{-1}	1229–1301 cm^{-1}	CN streching, NH bending

Absorption peak of amide I collagen from snakehead fish skin *(Channa striata)* indicates the presence of β-sheet structure and has not yet been denatured into gelatin which has α-helix structure [17]. Triple-helix as characteristic of collagen can also be seen from intensity ratio between the peak of amide III and 1450 cm^{-1} [23], which is 1.17. This intensity ratio indicates collagen from snakehead fish skin *(Channa striata)* still has a triple-helix structure and has not been degraded to gelatin because its value is close to 1.0 [19].

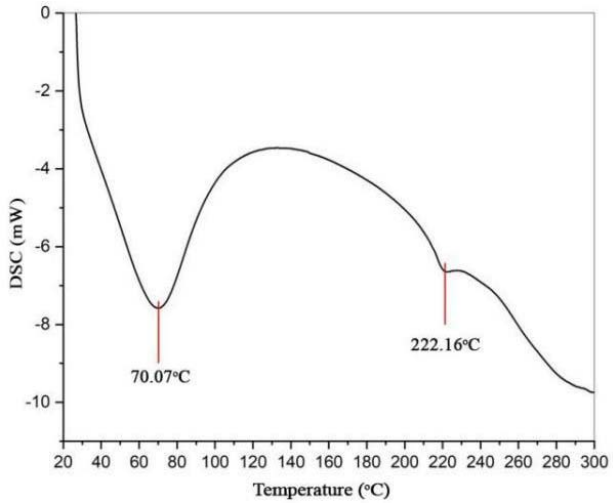

Fig. 3: DSC thermogram results of collagen from snakehead fish skin

Thermal properties of collagen from snakehead fish skin *(Channa striata)* is have two endothermic peaks, namely at 70.07 °C which indicates the glass transition temperature (Tg) and 222.16 °C which indicates the peak melting temperature (T-max). Glass transition (Tg) represents temperature when hydrogen bonds in collagen are broken so that it turns into gelatin [17]. The wide glass transition peak (Tg) can be attributed to the evaporation of water during the DSC process because the collagen still contains a small amount of water [19].

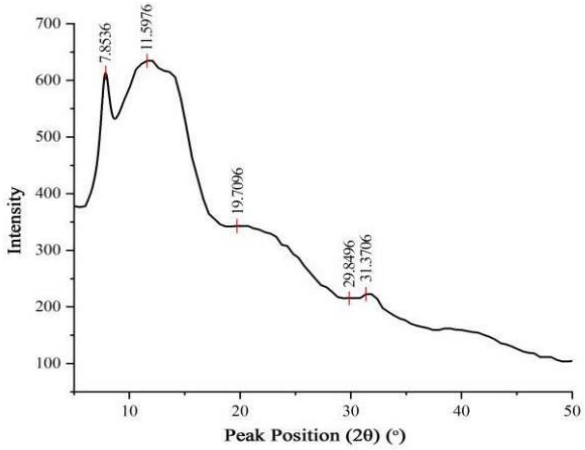

Fig. 4: X-ray diffractogram results of collagen from snakehead fish skin

The first characteristic peak of collagen appears in the range of 5–10° [13]. Sharp peak of 7.8536 °indicates the longest distance between the triple-helix is 1.132 nm. The second characteristic peak of collagen appear at 16–25° in the form of broad peaks. The peak of 19.7096° indicates the presence of amorphous scattering resulting from the disorganized collagen component [26]. The third characteristic peak appears at 30–35° [13]. This small peak also appears in the results obtained, namely at a peak of 31.3706°.

Table 3: Position of peaks and distance between collagen molecule chains of snakehead fish skin *(Channa striata)*

Peak position (2θ) (°)	Distance between molecular chains (nm)	Collagen characteristic Peak Region (°)
7.8536	1.132	5–10
19.7096	0.450	16–25
31.3706	0.285	30–35

Mesophase analysis

Mesophase analysis using polarizing microscope is the main characterization to determine the presence of collagen liquid crystals from snakehead fish skin *(Channa striata).* Characteristic of type I collagen liquid crystals under polarizing microscope is cholesteric phase that resembles a fingerprint pattern [11].

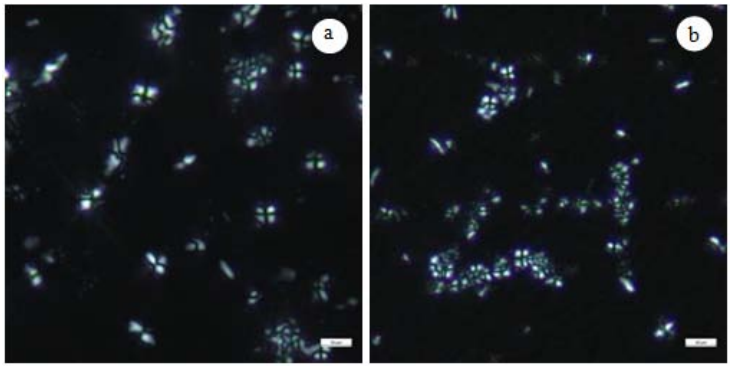

Fig. 5: Observations on polarizing microscope with 500x magnification (a) Dried collagen from snakehead fish skin *(Channa striata)* (b) Colloidal dispersion of collagen from snakehead fish skin *(Channa striata)* 0.25 mg/ml

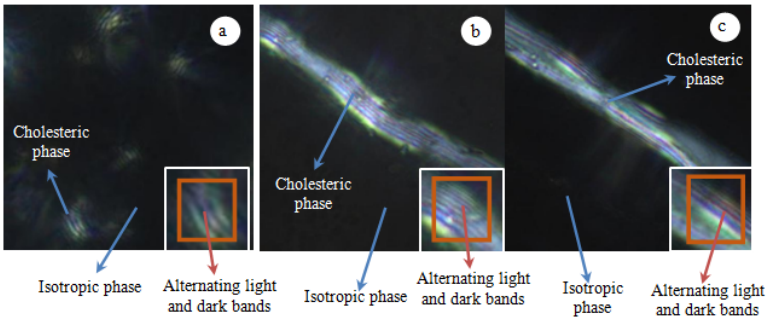

Fig. 6: Observation of the cholesteric phase of collagen liquid crystals from snakehead fish skin without sonication on polarizing microscope with 500x magnification (a) 30 mg/ml (b) 60 mg/ml (c) 80 mg/ml

Observation of collagen colloidal dispersion at 0.25 mg/ml was not much different from the observation of dried collagen in fig. 7(a). Collagen colloidal dispersion with very low concentration (0.25 mg/ml) still resembles dried collagen and has not been able to form liquid crystals. This indicates that collagen liquid crystals cannot be formed with very low collagen concentrations.

In the observation of collagen liquid crystals at 30 mg/ml, collagen has started to form a liquid crystals system. It is characterized by the presence of short and thin cholesteric phase under polarizing microscope. Collagen exhibits the behavior of liquid crystals system that undergoes a spontaneous phase transition from an isotropic phase to liquid crystals by increasing concentration [23].

Observation of collagen liquid crystals from snakehead fish skin *(Channa striata)* at 60 and 80 mg/ml in fig. 8(b) and (c) looks quite different from the concentration of 30 mg/ml in fig. 8(a). At higher concentrations, the orientation of the cholesteric pattern formed is more clearly visible. This is because the triple-helix is rigid molecule. The higher concentration of collagen, the higher the rigidity, so that it will help the formation of regularity liquid crystals phase [24].

Cholesteric liquid crystals is reported to have regular texture which appears bright and coexists with isotropic phase which appears dark under polarizing microscope [10]. Cholesteric phase is characterized by alternating light-dark bands and appearing as fine regular laminae, which are often described as fingerprint patterns [8].

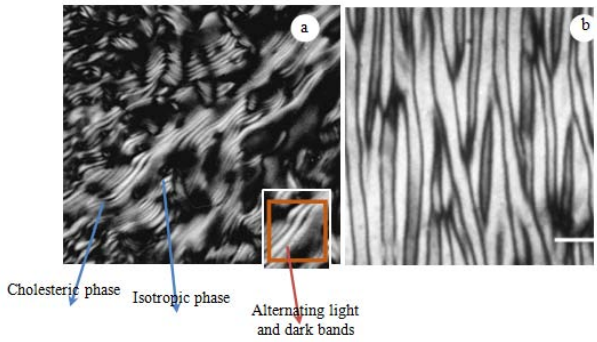

Fig. 7: (a) Cholesteric phase of collagen liquid crystals from calf skin at 50 mg/ml on polarizing microscope with 875x magnification (8) (b) Band patterns of collagen liquid crystals from rat tail tendons at 40 mg/ml

Observations of collagen liquid crystals from snakehead fish skin *(Channa striata)* at 60 and 80 mg/ml without sonication were not much different from the results of observations collagen liquid crystals from calf skin in fig. 9(a) and rat tail tendon in fig. 9(b) [8, 7]. This shows that collagen liquid crystals from snakehead fish skin *(Channa striata)* are able to form more regular liquid crystals phase at 60 and 80 mg/ml compared to 30 mg/ml concentrations. When collagen is dispersed in acid solution to form a liquid crystals system at high concentration, the cholesteric texture looks like band patterns [7].

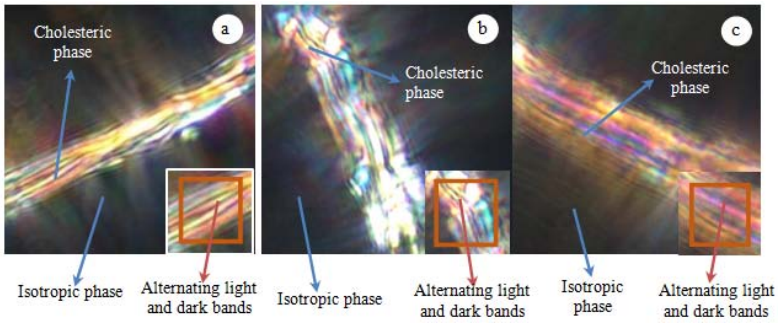

Fig. 8: Observation of the cholesteric phase of collagen liquid crystals from snakehead fish skin with sonication on polarizing microscope with 500x magnification (a) 30 mg/ml (b) 60 mg/ml (c) 80 mg/ml

Observations of collagen liquid crystals from snakehead fish skin *(Channa striata)* at 30, 60, and 80 mg/ml with sonication treatment are shown in fig. 10(a), (b), and (c). In sonicated liquid crystals, alternating light and dark bands are more clearly visible compared to unsolicited liquid crystals under polarizing microscope. This proves that sonication treatment can help the formation of liquid crystals cholesteric phase.

Collagen liquid crystals without prior sonication treatment have very high viscosity, so it will prevent the formation of alternating dark-light bands under polarizing microscope [11]. Ultrasonic waves provided by sonication treatment will help to homogenize the highly concentrated collagen molecules. Then, collected collagen molecules are partially dispersed by ultrasonic waves and liquid crystals phase will be more easily obtained [22].

Observation of collagen liquid crystals from snakehead fish skin with sonication treatment gave dominant orange color. This could be due to sonication treatment requiring low heating at 30 °C. As the temperature increases, liquid crystals will appear from orange, yellow, green, blue, and purple spectrums sequentially as the temperature increases [25, 27].

Functional group analysis

Functional group analysis aims to determine functional groups contained in collagen liquid crystals and ensure the presence of collagen in liquid crystals. In addition, functional group analysis was also used to ensure the stability of triple-helix collagen when formed into liquid crystals system.

Collagen liquid crystals from snakehead fish skin *(Channa striata)* both sonicated or not, have four absorption peaks located in collagen absorption region, namely amide A, amide I, amide II, and amide III.

Absorption peaks of amide I and III are the main characteristic peaks

of collagen, where amide I uptake is the determinant of collagen protein's secondary structure. In addition, amide III is a peak that can characterize the presence of a triple-helix by looking at the intensity ratio [26].

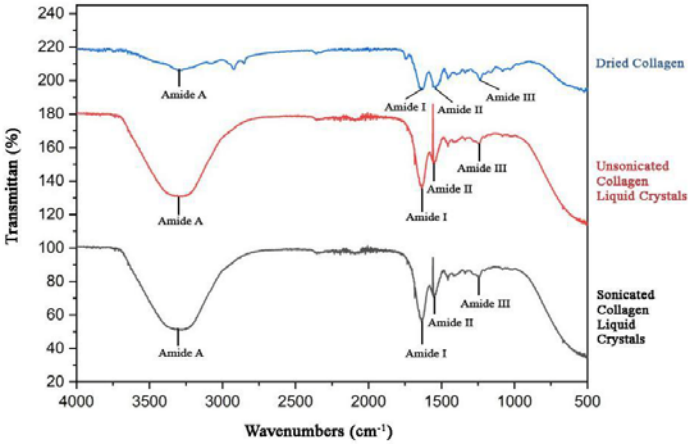

Fig. 9: Infrared spectra of dried collagen and collagen liquid crystals from snakehead fish skin *(Channa striata)*

Table 4: Anaysis of absorption peaks and functional groups of collagen liquid crystals from snakehead fish skin *(Channa striata)*

Absorption peaks	Collagen from snakehead fish skin wavenumbers (cm^{-1})			Absorption peak region (cm^{-1})	Functional group
	Collagen liquid crystals		Dried collagen		
	Without sonication	With sonication			
Amide A	3305.99	3302.13	3304.06	3350–3550	N-H streching
Amide I	1635.64	1635.64	1635.64	1600–1700	C=O streching
Amide II	1560.41	1560.41	1541.12	1480–1575	CN streching, NH bending
Amide III	1242.16	1246.02	1236.37	1229–1301	CN streching, NH bending

Absorption peak of amide I collagen liquid crystals from snakehead fish skin *(Channa striata)* indicates the presence of β-sheet structure and has not been denatured into gelatin which has α-helix structure [17]. Triple-helix as the characteristic of collagen can also be seen from the intensity ratio between the peak of amide III and 1450 cm^{-1} [26]. The intensity ratio are 1.167 and 1.163, which indicates collagen liquid crystals from snakehead fish skin *(Channa striata),* both sonicated or not, still have triple-helix structure and has not been degraded to gelatin because the value is close to 1.0 [18]. This indicates collagen liquid crystals from snakehead fish skin *(Channa striata)* still have characteristics of collagen functional groups in them.

CONCLUSION

Collagen from snakehead fish skin *(Channa striata)* can be formed into liquid crystals system with characteristic of cholesteric phase (fingerprint pattern) under polarizing microscope. Sonication treatment and increased the concentration can assist the formation of cholesteric phase (fingerprint pattern) collagen liquid crystals from snakehead fish skin. The formation of collagen liquid crystals with and without sonication treatment does not affect the stability of triple-helix.

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It can be declared by authors.

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AUTHORS CONTRIBUTIONS

Each authors contributed equally in the research and article preparation. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTERESTS

The authors declare there is no conflict of interest

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