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Analysis of Electropherogram Profile of Crude Cartilage Oligomeric Matrix Protein for Rheumatoid Arthritis Diagnosis

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ABSTRACT

Cartilage oligomeric matrix protein (COMP) is a potential biomarker to monitor the development of cartilage damage and injury in rheumatoid arthritis (RA). The most accurate method of determining COMP levels is the enzyme-linked immunosorbent assay, but this method is expensive and requires trained analysts. An alternative method to solve this problem is analyzing the electropherogram profile of crude COMP. This method separates proteins based on molecular weight, so it can differentiate pentamer COMP from its fragments. This study was conducted to analyze the electroferogram profile of crude COMP of RA patients and normal individuals, so it can be applied to RA diagnosis. COMP was precipitated on its isoelectric pH, then crude COMP was dissolved and electrophoresed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).The electropherogram profiles of RA patients were dominated by pentamer and dimerbands, whereas the electropherogram profiles of normal individual were dominated by tetramer, trimer, and dimer bands. Increased pentamer COMP in serum was indicated by the cartilage damage. The electropherogram profile of crude COMP of RA patient can be differentiated from normal individuals, so it can be used for the RA diagnosis.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by erosive arthritis in symmetric synovial joints. This disease causes pain due to joint damage and dysfunction, decreased quality of life, and even disability (Schuna, 2007). The RA prevalence varies in different populations, generally 0.5-1.0% in the world (Sack *et al*., 2010) and 0.1-0.3% in Indonesia (Nainggolan, 2009). Epidemiological data suggest that genetic predisposition and exposure to unknown environmental factors play a role in RA triggering (Schuna, 2007). Early diagnosis and therapy in aggressive RA patients is essential to prevent disability due to late treatment (Vittecoq *et al.,* 2003). Cartilage oligomeric matrix protein (COMP) is a secreted glycoprotein compound that found in extracelullar matrix of skeletal tissue (Holden *et al.,* 2005). COMP determines the fiber composition of collagen type II in cartilage and in collaboration with other matrix proteins, to stabilize the collagen tissue (Halasz *et al.,* 2007). The importance of this function was observed from the presence of COMP and its proteolytic fragments which released into synovial fluid and serum during skeletal dysplasia (Holden *et al*., 2001). So that COMP can be used as a potential biomarker to monitor the development of cartilage damage and During skeletal dysplasia, COMP and its proteolytic fragments were observed in synovial fluid and serum (Holden *et al*., 2001).

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This fact was supported the development of COMP as a biomarker to monitor the development of cartilage damage and injury (Vilim *et al.*, 2002) and support the RA diagnosis (Tseng *et al*., 2009). The most accurate method of determining COMP levels is the enzyme-linked immunosorbent assay (ELISA), but this method is expensive and requires trained analysts. An alternative method to solve this problem is analysis electropherogram profile of crude COMP. The objective of this study was to analyze the electropherogram profile of crude COMP of RA patients and normal individuals, so it can be applied to RA diagnosis. This study was the first study that uses the crude COMP which is isolated from serum of RA patient and normal individuals then analyzed the electropherogram profile of crude COMP.

MATERIALS AND METHODS

Subjects

The study was conducted after being approved by Health Research Ethics Committee of Government Hospital of Dr. Hasan Sadikin Bandung, West Java, Indonesia, No. LB.04.01/A05/EC/075III/2016. The RA patients in Rheumatology Clinic and normal individuals were recruited after getting an explanation and signed the informed consent. This study was a follow-up study, all serum of RA patients and individuals normal were collected from previous studies (Saptarini *et al*., 2017).

Materials

All chemical reagents for buffer are of analytical grade (Merck). Acrylamide, N'N'-bis-methylene-acrylamide, glycerol, blue bromophenol, Tris-base, sodium dodecyl sulphate, N,N,N',N'-tetramethylethenicaminamine, and ammonium persulfate are electrophoresis grade (Sigma Aldrich). Brilliant blue Coomassie R-250 and protein marker # 26614 (Thermo Scientific).

Precipitation of Serum COMP

Precipitation of serum COMP was conducted by converting the serum pH to an isoelectric pH of COMP (pI 4.36 ± 0.01) using 0.1 M citric acid solution pH 1.88 ± 0.01. The solution was centrifuged at 12,000 rpm and 4 °C, the precipitated COMP was separated from the supernatant. The precipitated COMP was reconstituted with citrate buffer pH 7.4 ± 0.01 and stored at -80 °C.

Electrophoresis of Crude COMP

Analysis of electropherogram profile of crude COMP was conducted by electrophoresis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with separating gel of 10% polyacrylamide and retaining gel of 5% polyacrylamide (Laemli, 1970). The electropherograms were scanned and analyzed by Gel Analyzer software. The bands on the electropherograms were marked and compared with protein marker. A plot of logarithmic molecular weight versus Rf was generated from the bands of proteins marker to determine the molecular weight of the unknown protein. If the curve is nearly linear, it can be described by the formula $y = mx + b$, where y is the logarithmic molecular weight, m is the slope, x is the Rf, and b is the y-intercept (http://www.gelanalyzer.com).

Statistical Analysis

Statistical analyzes were conducted by R i386 3.2.0 software for Windows under license from the GNU General Public License (http://cran.r-project.org). The electropherogram profile was grouped based on the bands composition, then analysis of variance was performed to the electropherogram profile groups against COMP levels of serum and crude COMP. The statistically significant difference was expressed if $p<0.05$.

RESULTS AND DISCUSSION

Precipitation of Serum COMP

Eight normal individual serum (code P5 - P12) could not be used for partial purification, because of a little volume of serum. COMP will precipitate at its isoelectric point (pI 4.36 ± 0.01). At isoelectric points, proteins have a zero net charge, so the electrostatic repulsion between protein molecules is gone which lead to aggregates formation (Harrison *et al.,* 2015). Weak and dilute acid (0.1 M citric acid solution) was used to achieve the isoelectric point. It was because weak or dilute acids minimize hydrolysis and denaturation, but still made the aggregates. Protein precipitation is used to concentrate and purify proteins from various contaminants (Harrison *et al.,* 2015). COMP precipitation was accelerated by centrifugation. The colour of crude COMP was whitish bone. After the filtrate was decanted, the crude COMP was reconstituted with phosphate buffer.

Electrophoresis of Crude COMP

Protein and sodium dodecyl sulfate form an anionic complex with a constant negative charge per unit mass. This results in the electrophoresis separation in polyacrylamide gel depending on the protein molecular weight. There is a linear relationship between the logarithm of molecular weight and the relative migration distance of the protein-SDS complex. Tris-glycine buffer systems separate proteins with high pH, thus minimize the protein aggregation and make a good separation (Garfin, 2009). The electropherogram profile of RA patients (Fig. 1) and normal individuals (Fig. 2) showed the presence of bands with molecular weights bigger than 200 kDa with various intensities. These bands were predicted as a pentamer form of COMP. The intensity of these band was proportional to COMP level in crude COMP. The higher the COMP level, the darker the band intensity. COMP levels in the crude COMP have been determined (Saptarini *et al*., 2017). The electropherogram of two RA patients without disease-modifying antirheumatic drugs (DMARDs), i.e. RP23 and RL27, were different from the RA patient electroferogram with prescribed DMARDs. DMARDs, such as methotrexate, was proven to modify the development of joint damage (Lindqvist *et al*., 2005), which resulting decreased COMP levels along with improved joints condition in RA patients.

The RP23 electropherogram showed that pentamer form more than tetramer form of COMP. The RL27 electropherogram showed that tetramer form more than pentamer form of COMP. The difference in electropherogram profile may be due to disease duration of RP23 (12 months) are longer than RL27 (3 months) and COMP level of RP23 (22.78 μg/mL) higher than RL27 (16.53 μg/mL).

Image analysis software allows easy and fast protein analysis. Gel Analyzer software was used to create all graphs from electropherogram, then determine the equation (Table 1). Each equation was used to estimate the unknown protein molecular weight on each band. Estimated protein volume can be determined by comparing the band intensity of the sample to the protein marker. All samples (RA patients and normal individuals) had proteins with molecular weights ranging from 54 to 60 kDa which estimated as fragments of the degraded COMP (Fig. 3). COMP levels were estimated to be comparable to the tetramer and pentamer forms of COMP, due to the limitation of capture antibodies in ELISA kits, i.e. mouse anti-COMP monoclonal antibody, which do not react with monomer, dimer, or trimer forms of COMP. Specific electropherogram profiles can be used to differentiate crude COMP of RA patients from normal individuals. Electropherogram of RA patients were dominated by pentamer and dimer bands, whereas electropherograms of normal individuals were dominated by tetramer, trimer, and dimer bands (Table 2). COMP was found in pentamer and its fragments. This was consistent with the literature that COMP and its proteolytic fragments were released into synovial fluid and serum (Holden *et al*., 2001). The electropherogram profile based on the band composition were categorized into four groups for RA patients and six groups for normal individuals (Table 3). Analysis of variance in each RA patient group showed no significant difference with COMP level of serum (p = 0.56) and crude COMP (p = 0.28). It was the same results for normal individuals, i.e no significant difference with COMP levels of serum (p = 0.59) and crude COMP (p = 0.32). Analysis of variance the electropherogram profile group of RA patients and normal individuals showed significant difference in COMP levels of crude COMP (p = 0.55 x 10^{-3}). It was showed that the electropherogram profile of RA patients can be differentiated from normal individuals, because of the different distribution of COMP oligomers between RA patients and normal individuals.

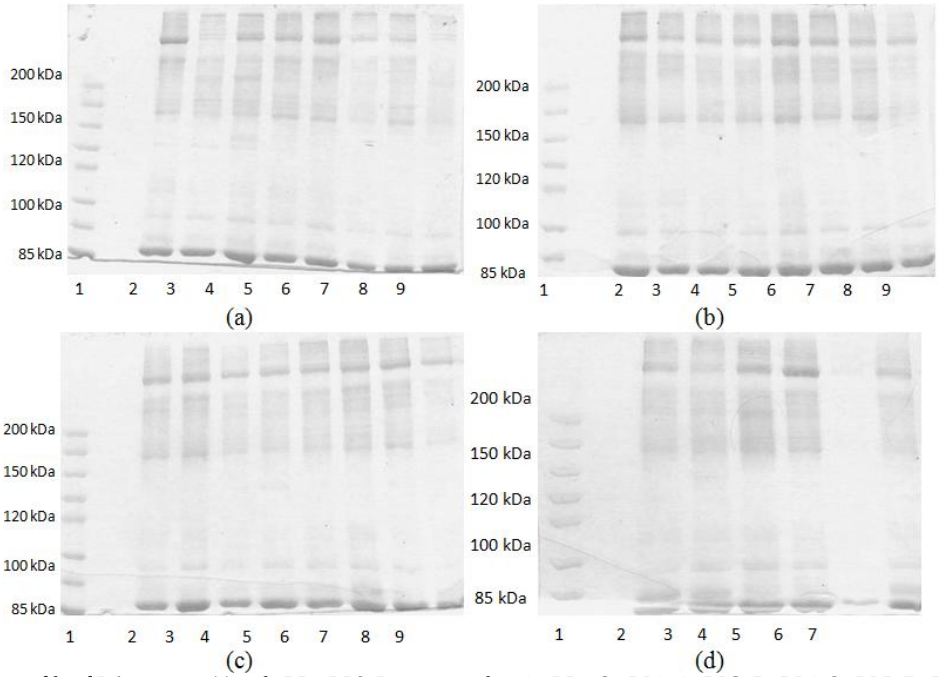

Fig. 1: Electropherogram profile of RA patients. (a) code RL1-RP8: Lane 1= marker, 2= RL1, 3= RP2, 4= RL3, 5= RP4, 6= RP5, 7= RP6, 8= RP7, 9= RP8. (b) codeRL9-RP16. Lane 1= marker, 2= RL9, 3= RP10, 4= RL11, 5= RP12, 6= RP13, 7= RP14, 8= RP15, 9= RP16. (c) code RP17-RP24. Lane 1= marker, 2= RP17, 3= RP18, 4= RP19, 5= RP20, 6= RP21, 7= RP22, 8= RP23, 9= RP24. (d) code RP25-RP30. Lane 1= marker, 2= RP25, 3= RP26, 4= RL27, 5= RP28, 6= RP29, 7= RP30. R = RA patient, P= female, L=male.

Table 1: Equation of electropherogram of RA patients and normal individuals.

Subject	Subject code*	Equation of marker protein	Linearity (R^2)
RA patient	RL1-RP8	$y = 667.01 \times 10^{-4.53x} + 47.12$	0.994
	RL9 – RP16	$y = 451.99 \times 10^{-3.52x} + 41.35$	0.996
	RL9 – RP16	$y = 762.24 \times 10^{-4.75x} + 47.65$	0.994
	RP17 – RP24	$y = 496.23 \times 10^{-3.55x} + 40.75$	0.995
Normal individual	P1 – P16	$y = 948.67 \times 10^{-5.34x} + 50.01$	0.993
	P17 – P24	$y = 480.83 \times 10^{-3.84x} + 42.58$	0.997
	P25 – L30	$y = 558.74 \times 10^{-4.07x} + 40.88$	0.992

* R= RA patient, P= female, L= male.

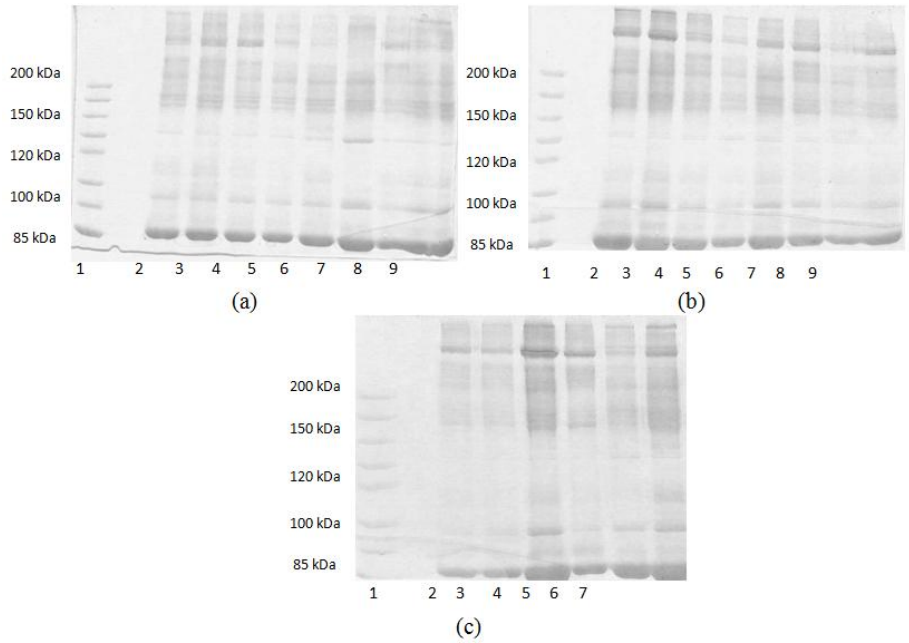

Fig. 2: Electropherogram profile of normal individuals (a) code P1-P16. Lane 1= marker, 2= P1, 3= P2, 4= P3, 5= P4, 6= P13, 7= L14, 8= P15, 9= P16. (b) code P17-P24. Lane 1= marker, 2= P17, 3= L18, 4= P19, 5= L20, 6= P21, 7= L22, 8= P23, 9= P24. (c) code P25-L30.Lane 1= marker, 2= P25, 3= P26, 4= P27, 5= P28, 6= P29, 7= L30. P= female, P= male.

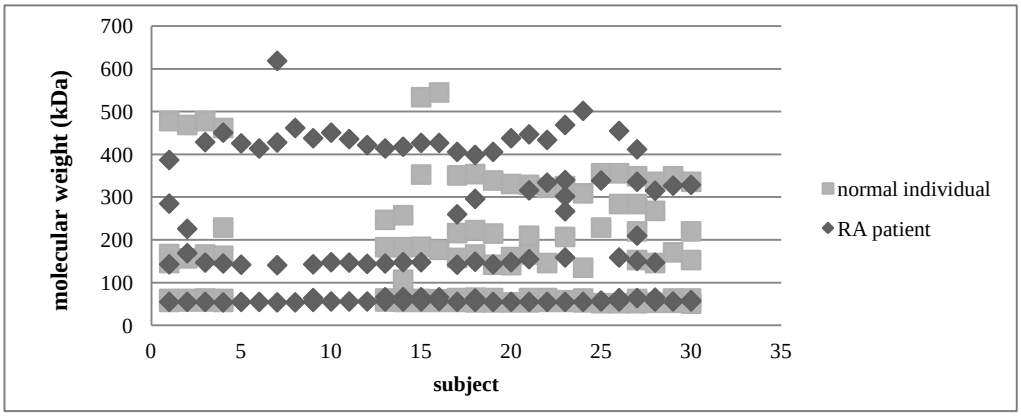

Fig. 3: Distribution of estimated protein molecular weight of crude COMP in RA patients and normal individuals.

Table 2: Distribution of estimated COMP forms in the samples.

Estimated COMP form	Molecular weight (kDa)	Percentage (%)	
		RA patient	Normal individual
Monomer fragment	54-60	100.0	100.0
Dimer	141-184	73.3	68.2
Trimer	207-284	20.0	59.2
Tetramer	302-362	13.3	68.2
Pentamer	387-478	96.7	22.7

Table 3: The subject groups based on the bands composition.

Subject	Groups based on the bands composition	Number of subject (n)	Subject code*
RA patient	Penta-, monomer	7	RP6, RP8, RP16, RP24, RP25, RP29, RP30
	Penta-, di-, monomer	16	RL3, RP4, RP5, RP7, RP7, RL9, RP10, RP11, RP12, RP13, RP14, RP15, RP19, RP20, RP21, RP26, RP28
	Penta-, tri-, di-, monomer	3	RL1, RP17, RP18
	Irregular	4	RP2, RP22, RP23, RP27
Normal individual	Penta-, di-, monomer	4	P1, P2, P3, P16
	Tetra-, tri-, di-, monomer	6	P17, L18, P21, P27, P28, L30
	Tetra-, tri-, monomer	3	P23, P25, P26
	Tetra-, di-, monomer	4	L20, P22, P24, P29
	Tri-, di-, monomer	3	P13, L14, P19
	Irregular	2	P4, P15

*R = RA patient, P = female, L = male.

CONCLUSION

The electropherogram profile of crude COMP of RA patient can be differentiated from normal individuals, so it can be used for the RA diagnosis.

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Conflict of Interests: There are no conflicts of interest.

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