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ISOLATION, CHARACTERIZATION AND ANTIOXIDANT ACTIVITY OF FIBRINOGEN-LIKE PROTEIN-1 FROM SERUM AND SYNOVIAL FLUID OF PATIENTS WITH RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation and oxidative stress. Fibrinogen-like protein-1 (FGL1) has been implicated in immune regulation, but its antioxidant role under inflammatory conditions remains underexplored. This study aimed to isolate and purify FGL1 from the serum of healthy controls and from the serum and synovial fluid from inflamed joints of RA patients, and to assess its antioxidant capacity. Purification included ammonium sulfate precipitation (65%), dialysis, and gel filtration chromatography (Sephadex G-75), SDS-PAGE and HPLC. Antioxidant activity was evaluated by DPPH radical scavenging and IC_{50} calculation. SDS-PAGE and HPLC analysis confirmed the successful isolation, identity and high purity of FGL1 from all samples, the protein molecular weight ranged from 68 to 70 kDa. The DPPH assay showed that FGL1 isolated from synovial fluid of RA patients had the highest antioxidant activity (IC_{50} = 2.124 ng/ml), followed by RA serum (2.172 ng/ml) and control serum (2.798 ng/ml). These results indicate the dual role of FGL1 protein in immune response and oxidative balance, making it a promising biomarker and potential therapeutic target in rheumatoid arthritis.

K e y w o r d s: rheumatoid arthritis, fibrinogen-like protein-1, serum, synovial fluid, antioxidant activity, IC_{50}, DPPH.

Rheumatoid arthritis (RA) is a chronic autoimmune disease that attacks the synovial membrane. This immune attack leads to synovial inflammation. It affects approximately 1.5-2% of the world's population [1, 2]. This disease affects approximately 1.5–2% of the global population, with women being more affected than men by a ratio of 3:1 [3]. In recent years, oxidative stress (OS) has been shown to contribute to the development of this disease by damaging lipids, proteins, and DNA, causing synovial inflammation [4]. Metabolism contributes to the production of reactive oxygen species (ROS), but diet, pollution, and an imbalance in the gut microbiome can lead to the overproduction of these species, which play a significant role in causing inflammation and joint tissue destruction [5, 6]. Antioxidants are considered an integrated treatment for patients with rheumatoid arthritis, given the evidence of damage caused by oxidative stress in this disease. Recent interest has increased in studying proteins that may have a regulatory role in inflammatory and oxidative responses. FGL1, also known as hepatocyte-derived fibrinogen-binding protein 1, is one of these proteins and is a member of the fibrinogen family [7, 8]. In contrast to healthy individuals, patients with elevated disease activity of RA exhibited lower levels of TLAG-3 regulatory cells, as demonstrated by a LIMIT study [9]. RA is a chronic inflammatory disorder that is prevalent and can result in a variety of complications, both within and outside of the joints. The synovial inflammatory environment is characterized by immune dysregulation, resulting from a complex network of inflammatory cells, components, degradative enzymes, and angiogenic factors [10]. The primary objective of this study was to evaluate the antioxidant activity of fibrinogen-like protein-1 (FGL1) using a DPPH free radical scavenging assay and determine its IC_{50} value after isolating and purifying the protein from the serum and synovial fluid of rheumatoid arthritis (RA) patients. So, we think FGL1 has more antioxidant activity when under oxidative stress from persistent inflammation in RA. This notion led us to examine how to extract, purify, and measure its

antioxidant capacity in the serum and synovial fluid of people with RA.

Materials and Methods

The study included 120 participants with rheumatoid arthritis of both sexes, comprising 94 women and 26 men aged between 20 and 65 years. 100 samples were selected as a control group of both sexes, with 65 women and 35 men, matched in age. The study was conducted at Al-Jinan Center and outpatient clinics licensed by Anbar Health. Sample collection was conducted from 1 Sep 2022 to 1 Dec 2023. According to Administrative Order 365, dated 7/1/2024, all procedures followed ethical guidelines and were approved by the institutional ethics committee for the University of Mosul and Anbar Health Department.

Excluded from this study were ɜatients with any type of arthritis and other types of disease, such as heart disease, cancer, and kidney disease.

This study collected two types of samples, including serum and synovial fluid (SF). In accordance with the ethical standards of the Institutional Review Board, signed informed consent was obtained from all participants.

A serum sample was collected by withdrawing 5 ml of venous blood, then placed in a gel tube and left for 15 min at 37°C. After that, the sample was centrifuged at 3000 × g, then the serum was separated and stored at -20°C until the required tests were performed [11].

10 ml of synovial fluid was collected from the knee joint of 40 patients (11 males, 29 females) aged 30–65 years, and serum from the same patients was collected; after that, SF samples were then transferred to sterile tubes. Then, they were centrifuged to remove cellular debris, and the supernatant was stored at -20°C until use

Estimation of protein concentration. According to the Lowry method (1973), protein concentration was determined using the Folin-Ciocalteu reagent. The standard curve of bovine serum albumin (BSA) was used at different concentrations to find the concentration of the unknown protein [12-14].

Purification of FGL1. Three steps were followed to isolate and purify the FGL1 protein from the serum of rheumatoid arthritis patients, the serum of healthy controls, and synovial fluid. The method started with protein precipitation by the incremental addition of ammonium sulfate until 65% saturation was achieved. This method was used on 50 cc of serum and synovial fluid. The addition was made gradually at 4°C with magnetic stirrer agitation to ensure homogeneity. The precipitate was allowed to settle for 24 h at the same temperature. A cooled centrifuge at 300 × g was used for 20 min to separate the precipitate from the filtrate. FGL1 protein activity and concentration were measured to assess the efficacy of isolation and purification [15, 16].

Dialysis. Dialysis isolated small molecules from the FGL1 protein at 4°C to protect protein stability. This stage assessed the FGL1 protein concentration to ensure purification success [17].

Lyophilization (freeze dryer). Extracting water from the sample under pressure and low temperature allows the frozen water to sublime directly from solid to gas, creating a concentrated protein solution. The solutions are frozen in plastic containers in a freezer at -20 ˚C and then transferred to lyophilization equipment to remove the water. The protein sediment solution is then refrigerated in sealed containers until needed [18].

Filtration chromatography. Partially purified FGL1 protein was applied to a 65 cm height Sephadex G-75-filled column (0.65×70 cm). Elution was performed at a flow rate of 2 ml per fraction. The optical density of each fraction was measured at 280 nm to assess the protein content using a Shimadzu UV-1800 UV-Vis spectrometer (Kyoto, Japan) equipped with a quartz cuvette (1 cm path length). All measurements were performed in triplicate to ensure accuracy. Fractions exhibiting FGL1 activity were detected, and the active fractions were combined for further study [12, 19, 20].

Determination of the molecular weight of FGL1. Several standard solutions with molecular weights ranging from 204 to 2,000,000 Da were used to characterize the Sephadex G-75 column. Blue Dextran (2,000,000 Da) was used exclusively to determine the void volume (V°), while proteins within the column's effective separation range (e.g., BSA, α-amylase, pepsin) were used to generate a standard curve to estimate the molecular weight of FGL1. 2 ml of each standard was injected, followed by 2 ml of distilled water to wash the column, and then 2 ml of the concentrated protein sample resulting from the membrane separation was passed through. The protein content of each fraction was measured by reading the absorbance at 280 nm. The fractions with the highest absorbance were collected for analysis.[21].

Study the purity and weight determination of FGL1. The SDS-Page electrophoresis technique was used; protein standards were used to determine the molecular weight of FGL1 after electrophoresis, and migratory protein distances were calculated [22]. The purity of FGL1 was determined using the SDS-Page electrophoresis technique; in reference, proteins with known molecular weights in the range of 11 to 180 kDa and migrating protein distances were estimated. SDS polyacrylamide gel (8×8 cm, 0.75 mm) in the presence of reference proteins with known molecular weights and migrating protein distances was estimated. The distances of protein migration were determined after electrophoresis to determine the protein molecular weight using the modified approach [23, 24]. The presence of subunits was evaluated in 12% polyacrylamide gels (8×8 cm, 0.75 mm) containing 0.1% SDS. In brief, 5% stacking and 12% running gels were run at room temperature for 30-40 min at 80 V and 1-1.5 h at 100 V in 25 mmol/l Tris-HCl, 0.2 mol/l glycine buffer (pH 8.3) containing 0.1% SDS. Loading of 20–25 μg of protein in each lane was chosen to ensure accurate molecular weight estimation by the appearance of clear bands without gel saturation, after 3 min in a boiling water bath with 2% SDS, 5% 2-mercaptoethanol, 25% glycerol, and 0.1% bromophenol blue in 60 mmol/l Tris-HCl (pH 6.8). Preserved molecular weight standards were used and stained under the same conditions. The gels were stained in glacial acetic acid for 3 h: methanol: distilled water (10:45:45, v/v/v) with 0.25 percent Coomassie brilliant blue R-250, then glacial acetic acid: methanol: distilled water (10:45:45, v/v/v) without Coomassie brilliant blue R-250 for 20 min per time [22].

Reversed phase-high performance liquid chromatography (RP-HPLC). Reversed-phase high-performance liquid chromatography (RP-HPLC, Shimadzu) was used to show the extent of purity of peak A, which was separated from gel filtration. This analysis was performed in the State Company for Drug Industries and Medical Appliances in Nineveh. Chromatographic Condition [25]. Column (stainless steel) C18 25 cm x 4.6 mm Mobile phase and solvent Acetonitrile in distilled water (90:10) Flow rate 1.0 ml/min. Temperature Ambient191

Estimation of FGL1 as an antioxidant by scavenging free radicals. The 1,1-diphenyl-2-picrylhydrazyl DPPH assay is a reliable method for determining the potential antioxidant capacity of several proteins [26]. DPPH-mediated free radical scavenging activity is generally measured in terms of the percentage of inhibition of previously formed free radicals by the antioxidant, and this assay is based on measuring the antioxidant's reducing capacity toward the DPPH radical [27]. DPPH is stable, commercially available organic nitrogen radical that reacts with hydrogen/electron-donating compounds and has a UV-visible absorption peak in the 515–520 nm range. Upon reduction, the color of the radical solution changes according to the number of paired electrons, and the reaction progress is conveniently monitored by spectrophotometry [28, 29].

This assay is performed according to a modified method of Munteanu and Apetrei 2021 [30].

A series of different concentrations of FGL1 protein were prepared (e.g., 1, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.5, 4.5, 5.0, 5.5, 6.0 ng/ml) using DMSO (dimethyl sulfoxide) as a solvent, and DPPH solution was prepared at a concentration of 0.1 mg/ 100 ml of methanol. This procedure was performed in the dark to avoid the effect of light at room temperature for 30 min. Optical density was assayed at 517 nm.

Statistical analysis. Data were analyzed using GraphPad Prism (version 10). The percentage of free radical scavenging activity (FRSA) of the DPPH reagent by the isolated and purified FGL1 protein from the three sources (patient serum, synovial fluid, and healthy serum) was calculated using the following equation:

Free radical scavenging % = $[(A_0–A_1)/A_0]\times100$, were A_0 – blank absorbance (DPPH without sample), and A_1 – sample absorbance.

The relationship between concentration and percentage of scavenging was then plotted using a nonlinear regression model of the type: Dose-response – Inhibition: log(inhibitor) vs. response – Variable slope (four parameters) to determine the IC_{50} value, which represents the concentration of protein required to remove 50% of the free radicals [31, 32].

Results and Discussion

Purification of fibrinogen-like protein-1. Three steps were employed to purify the FGL1 protein from healthy serum, serum, and synovial fluid from RA.

Table 1 illustrates the progressive purification of FGL1 from healthy serum, RA serum, and RA synovial fluid. Specific protein concentration, purification folds, and recovery percentages improved at each stage, with the highest purity consistently achieved in synovial fluid.

Table 1. Purification of FGL1 from healthy serum, RA serum, and RA synovial fluid

Sample source	Volume, ml	Protein concentration, mg/ml	Total protein amount, mg	FGL1 concentration, ng/ml	Total FGL1 amount, ng	Specific protein	Purification, folds	Recovery, %
Crude enzyme (serum)								
Healthy serum	50	60	3000	2.6	130	0.04	1	100
RA serum	50	63	3150	12.5	625	0.20	1	100
RA synovial fluid	50	61	3050	62	3100	1.02	1	100
Ammonium sulfate precipitation (65%)								
Healthy serum	32	42	1344	3.3	105.6	0.08	1.81	81.2
RA serum	34	44	1496	14	476	0.32	1.60	76.2
RA snovial fluid	33	45	1485	73.5	2425.5	1.63	1.61	78.2
After dialysis								
Healthy serum	34	35	1190	3	102	0.09	1.98	78.5
RA serum	37	38	1406	13	481	0.34	1.72	77.0
RA synovial fluid	35	36	1260	69	2415	1.92	1.89	77.9
Gel filtration chromatography (Sephadex G-75)								
Healthy serum	12	11	132	8.4	100.8	0.76	17.62	77.5
RA serum	12	10	120	41	492	4.10	20.66	78.7
RA synovial fluid	12	9	108	197	2364	21.89	21.54	76.3

Notably, the final gel filtration step using Sephadex G-75 resulted in significant enrichment of FGL1 and effective removal of contaminants across all samples. RA synovial fluid yielded the highest specific activity (21.89) and purification fold (21.54), confirming its suitability as an optimal source for FGL1 isolation.

In the crude sample, the specific efficiency was essentially high, reaching 1.02, significantly higher than that recorded in the serum of healthy subjects (0.04) and the serum of patients (0.20). This indicates the high local abundance of FGL1 in the inflamed joint, supporting the data FGL1 is abundantly secreted within the chronic inflammatory environment of affected joints. Following the 65% ammonium sulfate precipitation step, the specific activity improved to 1.63, with a 1.61-fold increase in purification times, and the recovery rate remained at 78.2%. This reflects the success of this step in reducing nonspecific proteins without significantly affecting the amount of FGL1.

Dialysis increased the specific concentration to 1.92, purification times by 1.89-fold and recovery rate by 77.9%.

This demonstrates the effective removal of small-sized contaminants and improved purity while retaining the target protein.

In the final step, gel filtration using Sephadex G-75, the greatest improvement in purity was achieved, with the specific concentration jumping to 21.89, the number of purifications increasing to 21.54, and a high recovery rate of 76.3%. This achievement is one of the most remarkable purification results, and demonstrates that synovial fluid is the best source for isolating FGL1, due to its relatively high concentration and low complexity of nonspecific proteins.

Gel filtration chromatography results using a Sephadex G-75 column (0.65×70 cm) showed significant variation in the elution volumes (Ve) and peak range of FGL1 depending on the biological source (serum from healthy subjects, serum from patients with RA, synovial fluid). Each fraction was collected at a volume of 2 ml, allowing accurate tracking of the protein peak appearance.

In Fig. 1 (FGL1 isolation from healthy subjects' serum), the protein band appeared in fractions 10 to 12, with a clear peak at fraction 11, i.e., at an elution

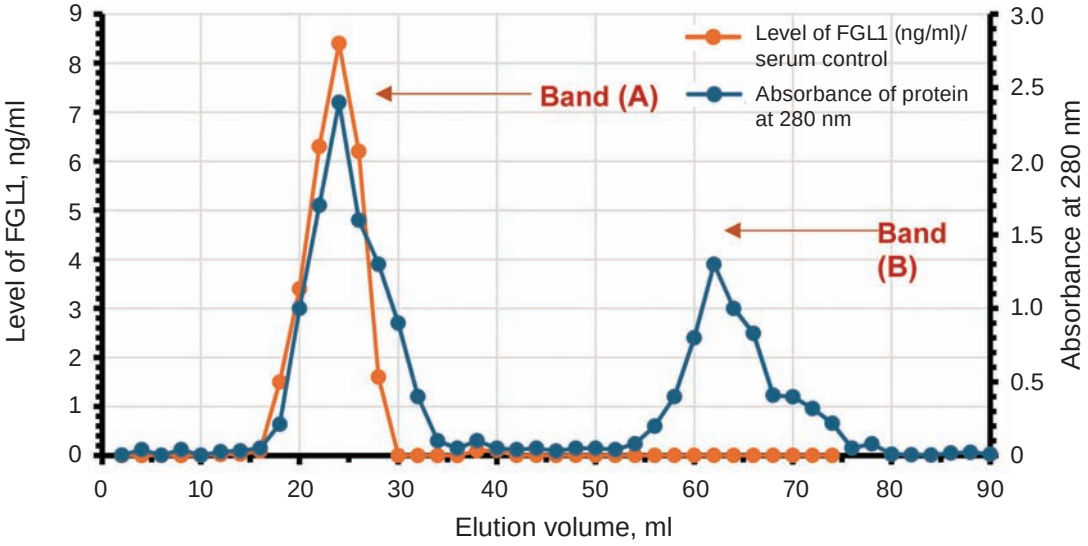

Fig. 1. Gel filtration chromatography of fibrinogen-like protein-1 (FGL1); protein extracted from the serum of a control using a Sephadex G-75 column (0.65×70 cm); 2 ml for each elution fraction

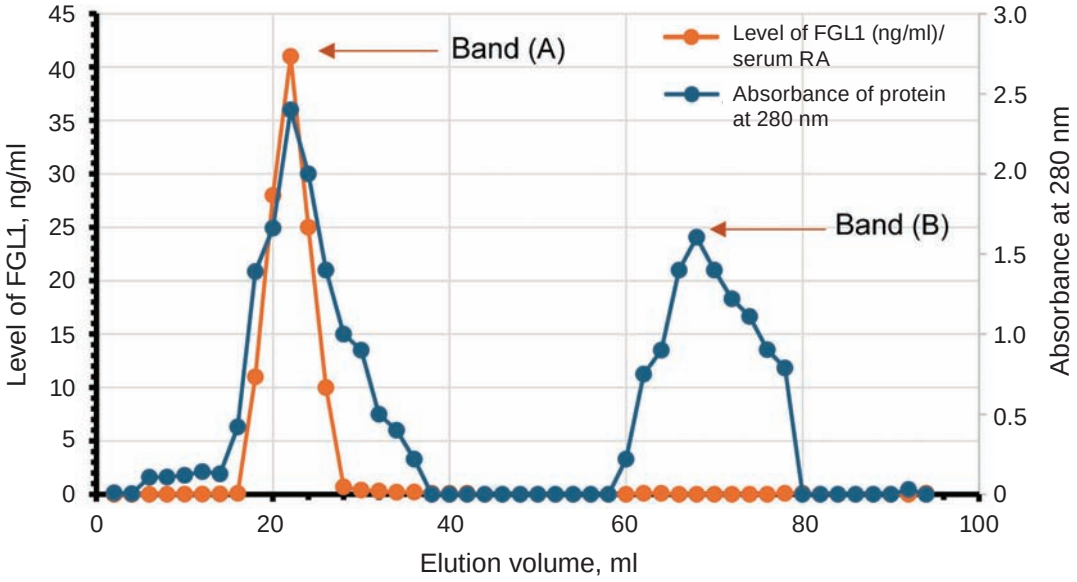

Fig. 2. Gel filtration chromatography of fibrinogen-like protein-1 (FGL1); protein extracted from serum of rheumatoid arthritis (RA) patients using a Sephadex G-75 column (0.65×70 cm), 2 ml for each elution fraction

volume of approximately 22 ml. This pattern reflects low protein concentration and poor initial purity, resulting in slight band scattering. These results are consistent with those reported by [33], which demonstrated that FGL1 is present at low concentrations in the blood under physiological conditions.

Fig. 2 (FGL1) from RA patients' serum shows a protein band in fractions 9-11 with a high peak at fraction 10 (Ve ≈ 20 ml). A dense, clear band indicated severe inflammatory reactions and increased FGL1 in RA patients' blood. [5, 34] found that chronic inflammatory illnesses overexpress this protein, which regulates immunological reactivity. Fig. 3 (FGL1 isolation from synovial fluid) shows a distinct band in fractions 8-10, peaking at fraction 9 (Ve ≈ 18 ml). The fact that protein is pure and abundant in the joint environment may explain its quick column evacuation.

Estimated of molecular weight of FGL1 by gel filtration. The study relied on gel filtration chro-

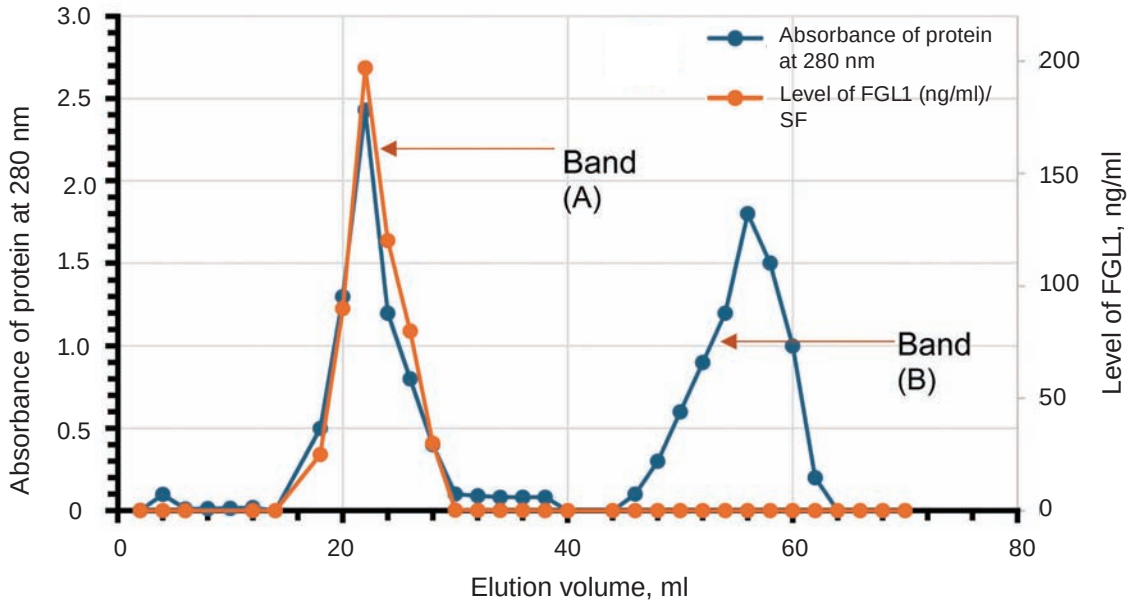

Fig. 3. Gel filtration chromatography of fibrinogen-like protein-1 (FGL1); protein extracted from serum of synovial fluid using a Sephadex G-75 column (0.65×70 cm); 2 ml for each elution fraction

Table 2. Decimal logarithm of molecular weight of FGL1 protein extracted from serum of rheumatoid arthritis (RA) patients using a Sephadex G-75 column with dimensions (0.65×70 cm)

No	Standard name and unknown	The molecular weight of markers and FGL1 (Dalton)	Log of M. Wt of the standards and the unknown	Elution volume, ml
1	Blue dextran	2000000	6.3	14
2	Hexokinase	100000	5.00	18
3	FGL1 Serum control	68410	4.8888	24
4	FGL1 (RA) synovial fluid	70144	4.8582	22
5	FGL1 (RA)	701000	4.8276	22
6	α –amylase	58000	4.76	28
7	Egg albumin	45000	4.65	34
8	Pepsin	36000	4.56	46
9	Insulin	5750	3.76	96

matography (GFC) using a Sephadex G-75 column (0.65×70 cm) to estimate the molecular weight of FGL1 extracted from the three sources. To achieve this purpose, a set of standard proteins with known molecular weights was passed through the column. The elution volume of each protein was recorded, the logarithm of the molecular weight (log MW) was calculated, and the relationship between log MW and Ve was plotted, as shown in Table 2 and Fig. 4. The results showed that FGL1 extracted from the serum of healthy individuals appeared at Ve ≈ 24 ml, while FGL1 from the serum and synovial fluid of rheumatoid arthritis patients appeared at Ve ≈ 22 ml. Based on the position of these values on the graph generated by the standard proteins, the molecular weight of FGL1 was estimated, ranging from 68 to 70 kDa. This result is consistent with a previous study [35]. In a paper published in Frontiers, a recombinant

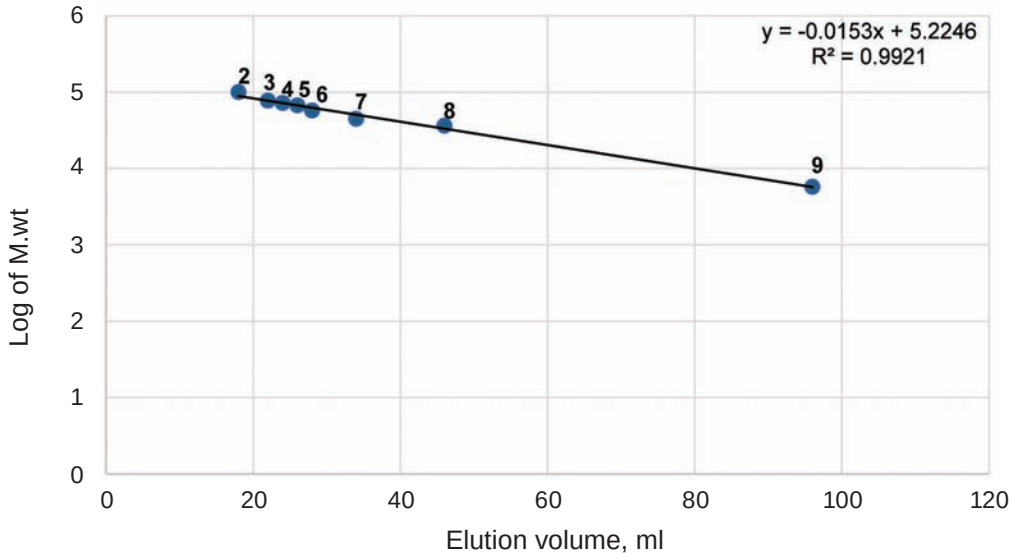

Fig. 4. Log molecular weight of purified FGL1 protein produced from blood serum of patients with rheumatoid arthritis (RA) using a Sephadex G-75 column (dimensions: 0.65×70 cm) at a flow rate of 2 ml per fraction. 2 – Hexokinase, 3 – FGL1 serum control, 4 – FGL1 (RA) synovial fluid, 5 – FGL1 (RA), 6 – α-amylase, 7 – egg albumin , 8 – pepsin, 9 – insulin

FGL1 protein was isolated and purified, showing a similar molecular weight of 67-68 kDa. SDS-PAGE analyzed the purity of the protein, and the results showed the efficacy of FGL1 as an anti-inflammatory agent in animal models of arthritis, opening up prospects for its use as a novel treatment for inflammatory diseases [34]. However, a study showed that the molecular weight of the FGL1 protein isolated from bacteria (such as *E. coli*) is about 34 kDa. This weight represents the basic conformation of the protein without post-translational modifications, such as glycosylation, which does not occur in bacterial systems [36].

In contrast, when FGL1 is produced in eukaryotic cells (such as mammalian cells), it undergoes glycosylation, which increases its molecular weight to about 55-60 kDa [37]. The reviewed studies estimate the molecular weight of FGL1 to be about 68 kDa as a homodimer complex connected by disulfide links. The molecular weight of specific domains or unaltered transcripts may decrease due to varying degrees of post-translational modification, such as glycosylation [7].

Molecular weight estimation of FGL1 protein using gel filtration and SDS-PAGE. SDS-PAGE electrophoresis was performed to measure the purity and molecular weight of FGL1 protein from RA patients' serum and synovial fluid after gel filtration [38, 39] chromatography (Hu. Both RA blood and synovial fluid samples showed separate protein bands with an estimated molecular weight of 68 kDa, which matches the FGL1 molecular weight previously reported [40]. However, healthy serum showed a single 70 kDa band.

Although similar in size, this minor shift could be related to post-translational changes or isoform variants that take place under normal physiologic settings. These changes are known to marginally modify protein migration on SDS-PAGE gels. The existence of similar 68 kDa band in both patient-derived specimens, as well as the molecular weight's congruence with gel filtration results, indicates that the protein separated is FGL1. Additionally, the strong, single band in RA samples, with no significant contamination or numerous bands, shows a high level of purity after the gel filtration procedure. Meanwhile, the greater molecular weight band observed in the control serum could be the physiological structure of FGL1 in non-inflammatory settings, possibly glycosylated or less active. This finding lends support to the theory that FGL1 becomes overproduced or structurally altered during prolonged inflammation, particularly in autoimmune disorders such as RA, and may contribute to its high diagnostic value in severe cases [41].

The approximate molecular weight of FGL1 purified from RA patient serum, synovial fluid, and serum from healthy controls was estimated using

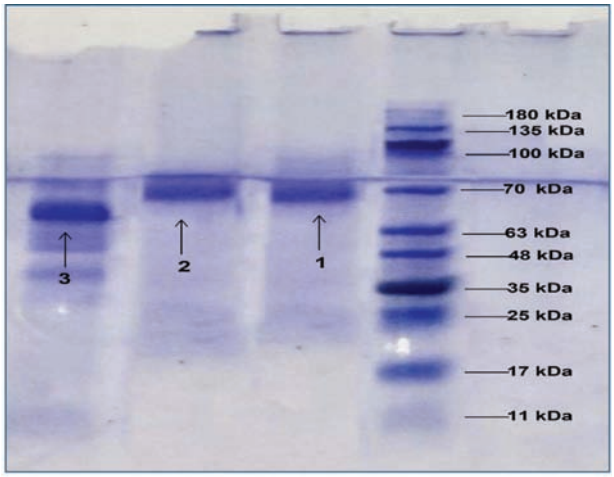

Fig. 5. SDS-PAGE analysis of purified FGL1 protein. Lane 1 – FGL1 from serum of healthy control. Lane 2 – FGL1 from serum of RA patient. Lane 3 – FGL1 from synovial fluid of RA patient

two complementary techniques: gel filtration chromatography on a Sephadex G-75 column, and electrophoresis using SDS-PAGE.

SDS-PAGE confirmed the pure material estimate. Gel filtration is shown by a distinct 68-70 kDa major band in Fig. 5. Both methods purify and identify proteins.

In [42], FGL1 was found to have a molecular mass of ~70 kDa on SDS-PAGE, and in [43,] its mature state was confirmed by Western blot. In [44], the molecular mass of FGL1 is reported to be 68-72 kDa but varies by tissue. The protein isoform bands differ.

According to this research, protein purification, chromatography, and electrophoresis can identify compounds, molecular weight banding and SDS-PAGE separated proteins as estimated by FGL1 protein gel filtration.

Moreover, Fig. 6 shows an examination of the calculated molecular weight of the FGL1 protein in a variety of samples: serum from healthy people, serum from RA patients, and synovial fluid from the same patients, employing two analyzing techniques: gel filtration chromatography using Sephadex G-75 and SDS-PAGE electrophoresis.

The results revealed that the molecular weight of FGL1 in the blood of healthy persons was approximately 68,400 Da by gel filtration and 68,000 Da by SDS-PAGE, demonstrating a good correlation between the two methods under normal physiological conditions.

Conversely, RA patients' serum protein molecular weight increased to 70,200 Da by gel filtration with 69,500 Da by SDS-PAGE. The same individuals' synovial fluid samples showed a rise in molecular weights to 70,400 Da (gel filtration) and 70,000 Da (SDS-PAGE).

These findings show that the FGL1 proteins may undergo structural abnormalities or modifications after translation (like glycosylation) in patients, resulting in an apparent increase in molecular

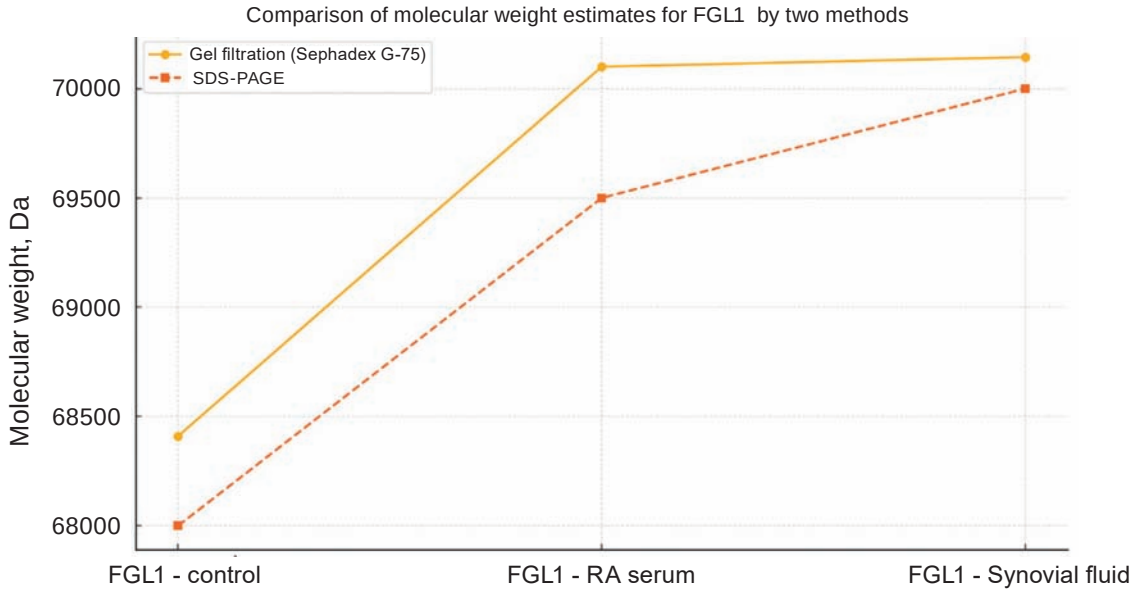

Fig. 6. Comparison of molecular weight estimates of FGL1 protein by gel filtration chromatography and SDS-PAGE

weight, particularly in inflammatory settings such as serum and synovial fluid in RA patients. Furthermore, the close agreement between the two methodologies verifies the correctness of the measures and the efficacy of the purification operations that preceded the analysis.

Analytical comparison of purification steps for FGL1 from three sources. The purification results of FGL1 from the three sources show significant differences in purification efficiency and target protein abundance, reflecting the biological environment and FGL1 concentration differences in each source. Initially, the specificity in healthy subjects' serum was very low (0.04), compared to RA patients' serum (0.20) and synovial fluid (1.02), indicating that FGL1 is expressed at a higher intensity in the inflammatory joint environment, a context of chronic inflammation, and interacts with the LAG-3 receptor [43].

In terms of improvement in specificity during the purification steps, the highest improvement was in synovial fluid, rising from 1.02 to 21.89, followed by patients' serum (from 0.20 to 4.10), and then healthy subjects' serum (from 0.04 to 0.76). These results reflect the ability of gel separation to achieve effective protein purification, especially when the initial concentration is higher, such as in synovial fluid.

In terms of purification folds, the highest value was recorded in synovial fluid (21.54-fold), followed by patient serum (20.66-fold), and finally, healthy serum (17.62-fold). This order indicates that purification efficiency is directly related to the protein abundance in the sample and the low complexity of other protein components in the biological medium.

Recovery percentages were similar across all samples, ranging from 76.3% in synovial fluid to 78.7% in patient serum and 77.5% in healthy serum.

High-performance liquid chromatography (HPLC) as a method for purity evaluation of isolated FGL1 protein. The purity of the FGL1 protein was investigated using the HPLC method, as shown in Fig. 7, *A-D*. To ensure the purity and identity of FGL1 purified from various sources, a direct comparison was made with commercially synthesized FGL1 protein from an international business utilizing high-performance liquid chromatography (HPLC) at 280 nm. The original sample (commercial protein) showed a single, strong protein peak with a retention time (Rt) of 1.839 min, indicating 100% purity. This represents a pure protein and acts like the "chromatographic signature" of FGL1. When this sample was compared to protein obtained from RA patients' plasma, a remarkable match was found, with a prominent peak occurring at Rt = 1.837 min, accounting for 77.25% of the overall absorbance.

This peak, in terms of time, location, and spectral characteristics, matches the commercial protein, supporting the identity of the isolated protein and demonstrating the success of the purification process. The two additional peaks appearing in the sample (1.370 and 1.563 min) are attributed to conjugated proteins or residual serum impurities.

A similar chromatographic pattern emerged in synovial fluid, with a broad protein peak at Rt = 1.822 min, with an absorption of 67.03%. Despite five additional peaks, the central peak remained closest in time to the reference protein, indicating that FGL1 is the predominant component in this sample, albeit within an environment rich in other joint proteins.

In contrast, the serum sample from healthy subjects showed two distinct peaks, one at Rt = 1.840 min, with an absorption of 74.23%, almost identical to the commercial protein, indicating the presence of FGL1 at a low concentration in this sample. The second peak (at 1.525 min) may reflect the presence of a common plasma protein or another protein component usually present in blood. Similar retention times between the isolated protein from all three samples and the commercial protein show a similar structure between the isolated and synthesized FGL1. Variations in peak height and size reveal biological source concentrations and purity. These findings support earlier findings that FGL1 concentrations are higher in inflammatory disorders such as RA than in blood [43]. Thus, HPLC is a robust and reliable method for verifying protein purity and identifying it by comparing the retention time to a reference protein, making it valuable in diagnostics and analytical research.

Estimation of FGL1 as an antioxidant by scavenging free radicals. FGL1 is a protein previously shown to be associated with inflammatory and immune responses, particularly in diseases such as rheumatoid arthritis (RA) [45, 46]. This experiment aimed to evaluate the ability of FGL1 to scavenge free radicals using a DPPH reagent in samples from patients with RA, healthy controls, and synovial fluid, to understand the potential role of FGL1 as an antioxidant in these different environments [47].

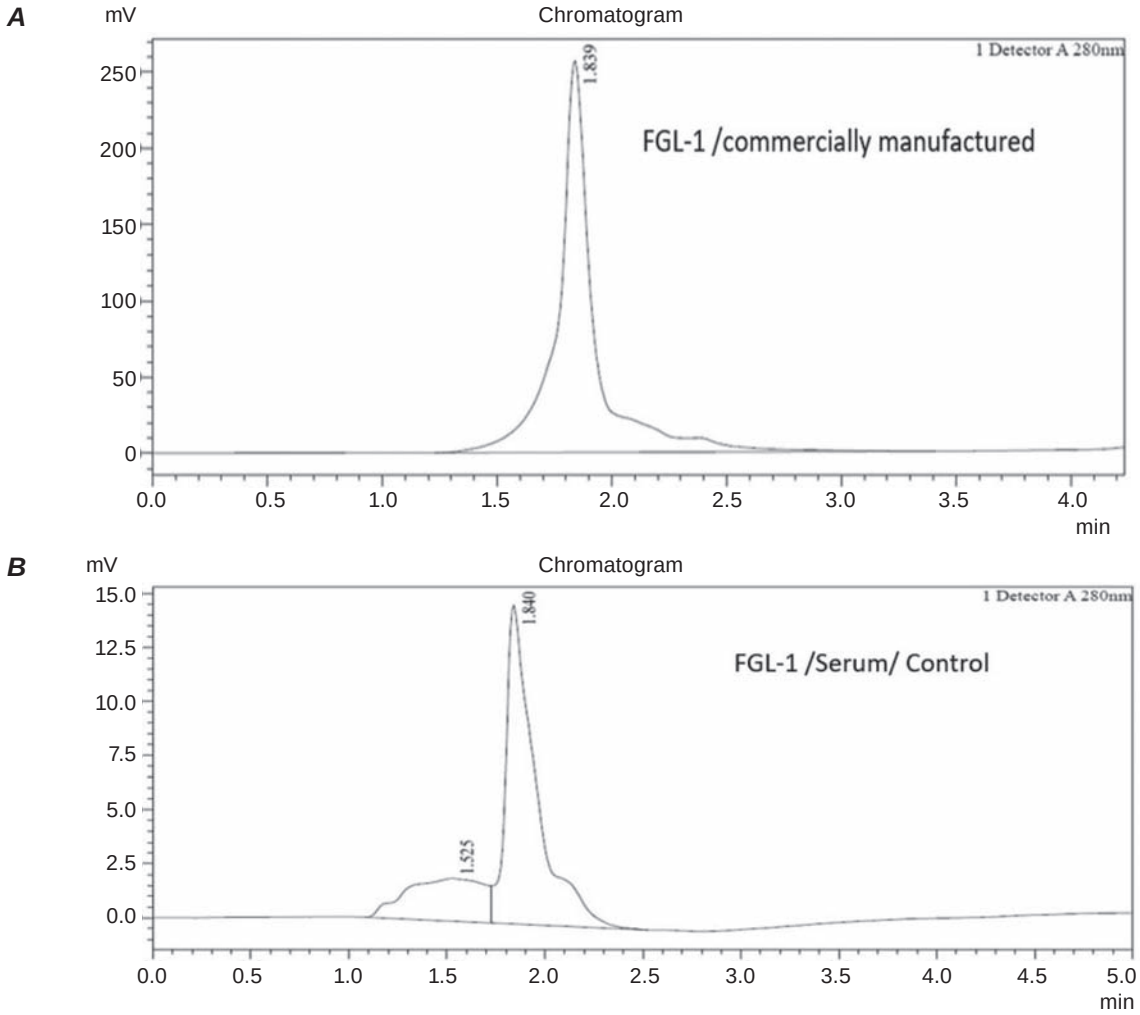

Fig. 7. ***A*** *– HPLC chromatogram of commercially synthesized FGL1 protein at 280 nm wavelength.* ***B*** *– HPLC chromatogram of FGL1 protein isolated from serum of healthy subjects at a wavelength of 280 nm*

Furthermore, there is no linear relationship between antioxidant concentration and DPPH radical scavenging activity. This means that the effect of increasing the concentration is not proportional or constant with the activity, as shown in several studies [48, 49]. Therefore, I suggest using the concentration that inhibits 50% (IC_{50}), which is the concentration required to achieve a 50% antioxidant effect. This means that FGL1 achieves 50% inhibition of DPPH radical activity at this concentration. It is a parameter commonly used to express antioxidant capacity [50]. GraphPad Prism analysis software was used to calculate the IC50 value in this study, which is consistent with a study that used this software [51].

Table 4 and Fig. 8,*A–C* illustrate the percentage of free radicals that the FGL1 protein from three samples – patients (RA), controls, and synovial fluid (SF) could scavenge. We examined the protein at 1.2 and 6.0 ng/ml levels and found the IC_{50} for each group. Fig. 8, *A* shows the graph of the sample taken from synovial fluid. It has an IC_{50} value of (3.03×10^{-11} mol/l), which suggests a moderate ascending response that makes FGL1 better at scavenging free radicals. Fig. 8,*B* also shows the graph of the protein taken out of the serum of arthritis patients. Its IC_{50} value is (3.10×10^{-11} mol/l), about the same as synovial fluid. Fig. 8C shows the response curve for serum from healthy people. The IC_{50} value (3.99×10^{-11} mol/l) was greater, which means that FGL1 has a lesser antioxidant capacity than synovial fluid and serum from people with rheumatoid arthritis.

The results show that the ability of FGL1 to combat free radicals varies significantly depending on where the sample came from. The protein from synovial fluid had the lowest IC_{50} value, which means

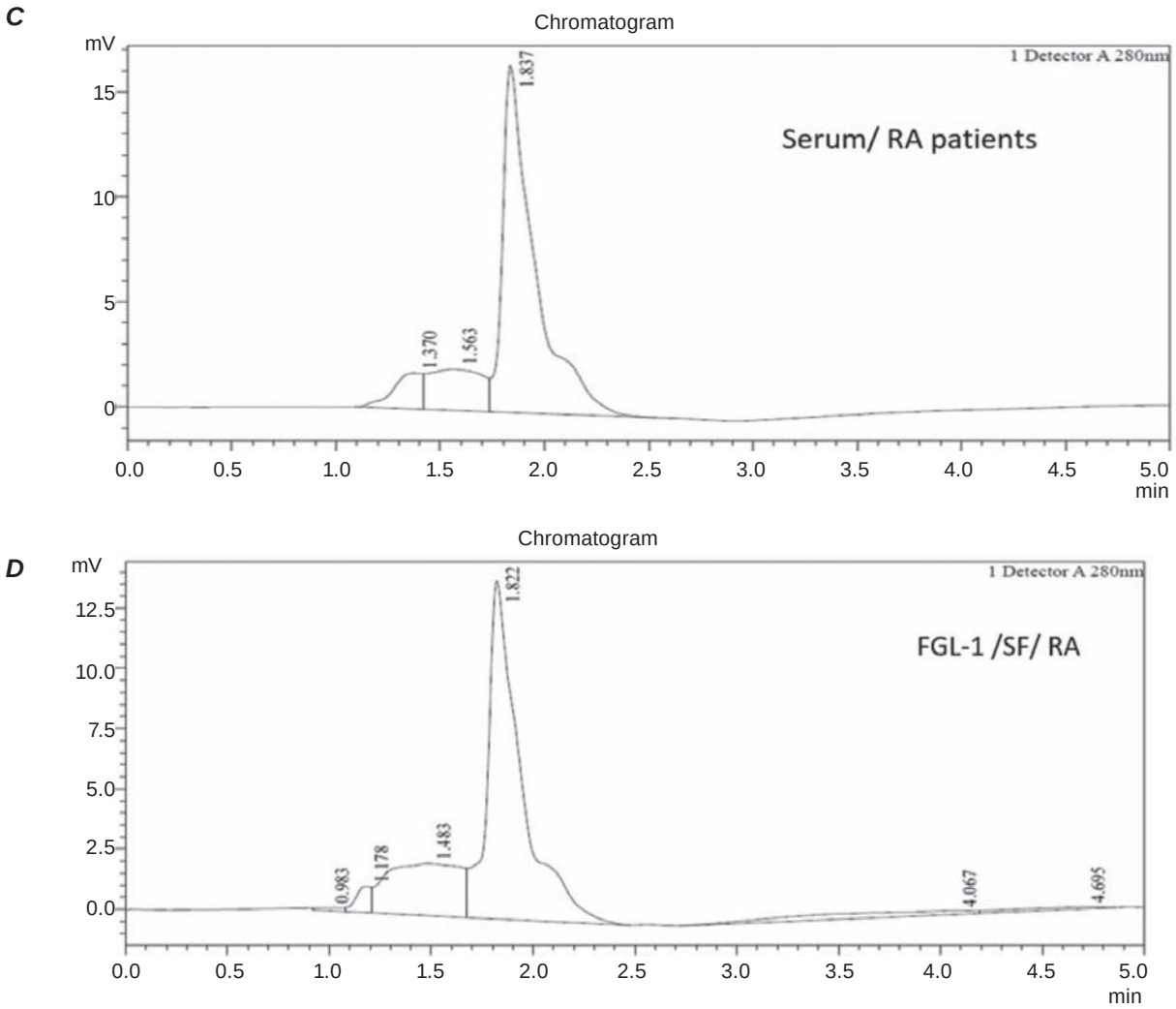

Fig. 8. ***C*** *– HPLC chromatogram of FGL1 protein isolated from serum of rheumatoid arthritis patients at a wavelength of 280 nm.* ***D*** *– HPLC chromatogram of FGL1 protein isolated from synovial fluid of rheumatoid arthritis patients at 280 nm wavelength*

it was better at eliminating free radicals. The protein from rheumatoid arthritis patients' serum came next, while the protein from healthy persons' serum came last. These discrepancies could be caused by changes in the structure of the protein or by alterations that occur after translation when there is inflammation, especially in the joints. Research by [45], backs up this idea by saying that proteins made during the acute phase may become antioxidants since they are constantly exposed to oxidative stress. The fact that the protein taken from the synovial fluid and serum of patients had a lower IC_{50} value than that of healthy controls may also explain why rheumatoid arthritis, which is a long-term inflammatory condition, causes significant changes in the functional and structural expression of some proteins, including FGL1. In this scenario, the inflammatory environment is an important element. For example, inflammatory cytokines like interleukin-6 (IL-6) are much higher in the tissues and fluids of people with rheumatoid arthritis [52]. These cytokines directly affect FGL1 because they take part in post-translational alterations, such as glycosylation, which modify the protein's characteristics and function. The protein that comes from the blood of healthy people is often in its natural state because it is made in the body without any alterations caused by disease or inflammation. This could explain why it has a high IC_{50} value and a poor ability to scavenge free radicals. This aligns with the work by Planavila et al. (2015), which found

Table 4. Percentage of free radical scavenging by FGL1 isolated from different sources

FGL1 concentration, ng/ml	Log concentration	Percentage of free radical scavenging by FGL1 isolated from different sources		
		RA synovial fluid, %	RA serum, %	Control, %
6.0	0.778	45.3	26.2	56.2
5.0	0.699	48.4	51.6	48.4
4.0	0.602	32.7	38.6	40.2
3.5	0.544	49.9	64.1	31.6
3.0	0.477	41.1	33.5	18.7
2.8	0.447	46.0	57.7	7.6
2.6	0.415	32.8	35.3	62.1
2.4	0.380	39.9	46.8	36.0
2.2	0.342	34.1	32.0	29.8
1.4	0.146	35.6	36.0	63.0
1.3	0.114	29.8	29.8	31.2
1.2	0.097	35.8	37.0	58.5
$IC_{50} \approx$		3.03×10^{-11} mol/l	3.10×10^{-11} mol/l	3.99×10^{-11} mol/l

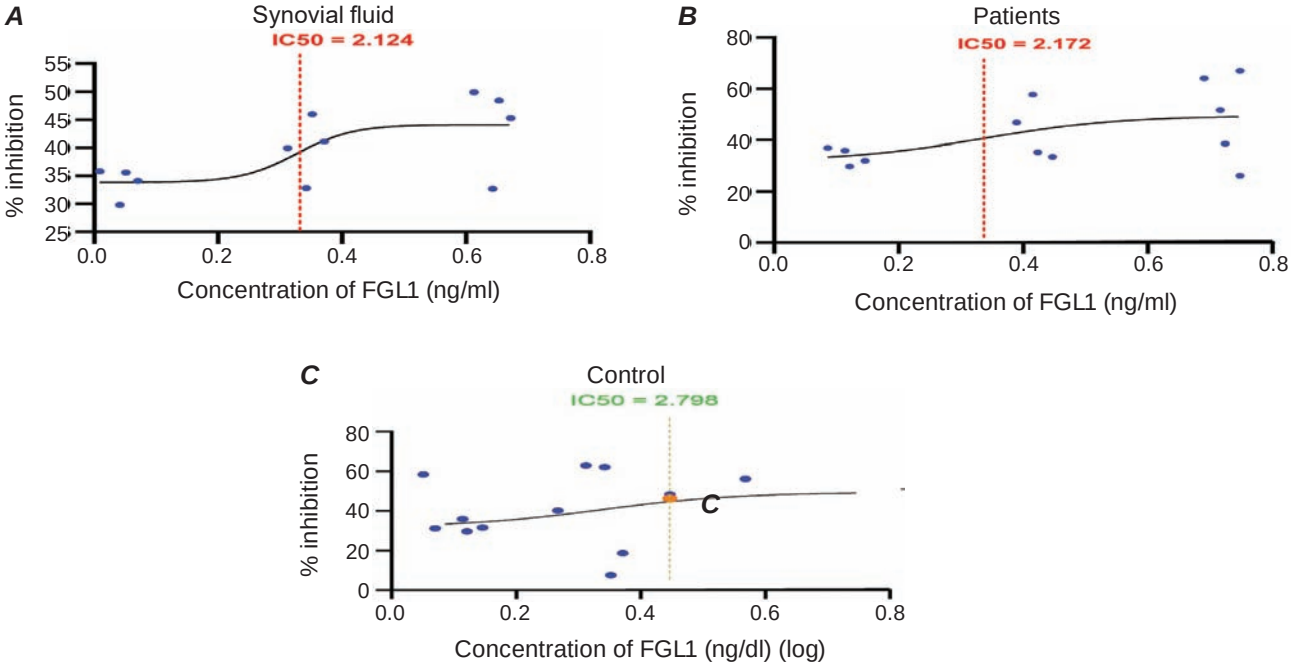

Fig. 8. ***A*** *– Free radical scavenging curve of FGL1 protein extracted from synovial fluid and assessment of IC_{50} value.* ***B*** *– Free radical scavenging curve of FGL1 protein extracted from synovial fluid and assessment of IC_{50} value.* ***C*** *– Free radical scavenging curve of FGL1 protein extracted from synovial fluid and assessment of IC_{50} value*

that FGL1 does not exhibit antioxidant characteristics unless it is present in an inflammatory environment [53].

The results of this study suggest that fibrinogen-like protein-1 may serve as a promising biomarker for assessing disease severity in rheumatoid arthritis and other inflammatory conditions. Elevated levels of the protein in patients' synovial fluid, compared to serum, showed a clear association with chronic inflammatory conditions in the joint. This may also help distinguish between mild and advanced disease. In [52], it was shown that FGL1 had a high predictive value for RA disease activity, with an area under the curve (AUC) of approximately 0.9. Furthermore, the protein's high antioxidant activity may open new avenues for its use as a potential therapeutic target by regulating the oxidative stress associated with the disease.

Conclusions. We conclude that FGL1 likely has a second biological function beyond its role as a component associated with the inflammatory response. The research demonstrated that FGL1 isolated from the three samples could neutralize free radicals, highlighting its antioxidant properties.

In addition, the IC_{50} values indicated that FGL1 isolated from synovial fluid was the most active in scavenging free radicals, followed by serum from rheumatoid arthritis patients, then serum from controls. This is because the inflammatory environment, such as in affected joints, may induce structural changes or post-translational modifications in the FGL1 protein, enhancing its antioxidant capacity. Furthermore, the protein was isolated and purified using several purification procedures, including ammonium sulfate precipitation, dialysis, and gel filtration chromatography using Sephadex G-75, demonstrating high purity and satisfactory extraction rates. SDS-PAGE and gel permeation chromatography revealed that the molecular weight of FGL1 protein ranged between 68 and 70 kDa in all samples, with slight differences observed between healthy controls and patients, reflecting the impact of structural modifications associated with inflammatory conditions. High-performance liquid chromatography (HPLC) confirmed that the retention time of the purified FGL1 protein was consistent with that of the standard protein.

This study provides potential evidence that the FGL1 protein is involved in immune and inflammatory regulation and may also possess antioxidant properties under specific conditions. This provides future opportunities to use this protein as a biomarker of inflammation and oxidative stress, and potentially as a therapeutic target to mitigate damage caused by oxidative stress in chronic inflammatory conditions such as rheumatoid arthritis.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ВИДІЛЕННЯ, ХАРАКТЕРИСТИКА ТА АНТИОКСИДАНТНА АКТИВНІСТЬ ФІБРИНОГЕН-ПОДІБНОГО ПРОТЕЇНУ-1 ІЗ СИРОВАТКИ ТА СИНОВІАЛЬНОЇ РІДИНИ ПАЦІЄНТІВ ІЗ РЕВМАТОЇДНИМ АРТРИТОМ

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Ревматоїдний артрит (РА) – це автоімунне захворювання, що характеризується хронічним запаленням і окислювальним стресом. Фібриноген-подібний протеїн-1 (FGL1) залучений у регуляції імунітету, але його антиоксидантна роль у разі запалення залишається недостатньо вивченою. Метою цього дослідження було виділення, очищення та оцінка антиоксидантної активності FGL1 із сироватки крові здорових людей, а також із сироватки та синовіальної рідини пацієнтів із РА. Очищення включало осадження сульфатом амонію (65%), діаліз, гель-фільтраційну хроматографію (Sephadex G-75), SDS-PAGE і HPLC. Антиоксидантну активність оцінювали за допомогою поглинання радикалів DPPH і розрахунком значення IC_{50}. Аналіз SDS-PAGE і HPLC підтвердив успішне виділення, ідентичність і високу чистоту FGL1 з усіх зразків. Молекулярна маса протеїну варіювала від 68 до 70 кДа. DPPH-аналіз показав, що FGL1, виділений із синовіальної рідини хворих на РА, мав найвищу антиоксидантну активність (IC_{50} = 2,124 нг/мл), у сироватці хворих на РА значення IC_{50} = 2,172 нг/мл і у контрольній групі – IC_{50} = 2,798 нг/мл. Ці результати вказують на подвійну роль протеїну

FGL1 в імунній відповіді та окислювальному балансі, що робить його перспективним біомаркером і потенційною терапевтичною мішенню при ревматоїдному артриті.

Ключові слова: ревматоїдний артрит, фібриноген-подібний протеїн-1, сироватка крові, синовіальна рідина, антиоксидантна активність, IC_{50}, DPPH.

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