ISSN: 2457-0400

Impact Factor: 6.711

WORLD JOURNAL OF ADVANCE HEALTHCARE RESEARCH

www.wjahr.com

Volume: 9, Issue: 12

Page N. 170-174

Year: 2025

Original Article

Coden USA: WJAMA3

PARTIAL PURIFICATION OF SOLUBLE ALPHA-KLOTHO FROM HUMAN PLASMA

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Article Received: 05 November 2025 **Article Revised:** 25 November 2025 **Article Published:** 01 December 2025

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DOI: https://doi.org/10.5281/zenodo.17801079

How to cite this Article: Dezheen Shabaan Kucher, Thikra Ali Allwsh* (2025). Partial Purification Of Soluble Alpha-Klotho From Human Plasma. World Journal of Advance Healthcare Research, 9(12), 170–174.

ABSTRACT

Alpha-Klotho (α-Klotho) is considered of the key regulatory proteins involved in maintaining homeostasis in biological systems, particularly in neurons and neurotransmitters. The present study aimed to isolate and partially purify the α-Klotho protein from the plasma using various biotechnological processes, as well as finding the molecular weight of α-Klotho. The research involved using plasma from a healthy person for partial purification of α-Klotho by using three sequential steps: Saline displacement precipitation, dialysis, and size exclusion chromatography were used with Sephadex gel number G-75. The results showed an increase in the specific concentration and the number of isolations of α-Klotho (22.74) (1.3), respectively ،following first step in precipitation and the recovery was 87% of α-Klotho. Also, the specific concentration increased to (33.13) in dialysis. The recovery amount of the total α-Klotho level reached 82%. In addition, two protein peaks (A and B) were detected, with α-Klotho corresponding to peak B, showing a purification fold of 38.5 compared with the crude plasma after the gel filtration process, with a 71-recovery rate. Electrophoretic (SDS-PAGE) was performed to confirm the purity of the α-Klotho, and its molecular weight was determined by both gel filtration and SDS-PAGE techniques, revealing an approximate molecular weight of 68 and 66 kDa, respectively.

KEYWORDS: Klotho, Gel filtration, Sephadex G-75, Dialysis, SDS-PAGE, plasma, Molecular weight.

INTRODUCTION

The name Klotho originates from Greek mythology, where three goddesses Clotho, Lachesis, and Atropos determined each person's lifespan (Nambidi et al., 2025), scientists named a newly discovered gene Klotho in 1997 after an unexpected scientific breakthrough.

The discovery came about by chance during a traditional gene modification experiment in mice involving microinjection. A new strain of mice was produced that displayed symptoms of accelerated ageing and a significantly shortened lifespan. Initially, this strain was dismissed as unimportant because it did not express the introduced gene as intended. However, further examination revealed that mice inheriting two copies of the modified gene exhibited clear signs of premature ageing (Hajare et al., 2025).

It turned out that the inserted DNA had randomly disrupted the promoter region of a previously unknown gene. This disruption reduced the gene's expression dramatically, revealing its crucial role in aging. The gene was later named Klotho in honour of the mythological figure (zhang et al., 2024).

Zsemlye et al. explained that gene Klotho in humans has been found on chromosome number 13 (Zsemlye et al., 2025) and chromosome 5 in mice (Hong et al., 2025). It encodes a unique protein in three forms: transmembrane, shed, and secreted. Klotho transmembrane is encoded by an mRNA that includes all five exons, producing a protein of approximately 116 kDa, which increases to around 130 kDa after glycosylation. This form spans the cell membrane, with its two extracellular domains (KL1 and KL2) showing weak enzymatic activity, particularly sialidase-like activity, due to the substitution of key catalytic residues (e.g., glutamate replaced by asparagine, alanine, or serine) (Zhang et al., 2024).

Another variant results from cleavage between the KL1 and KL2 domains, producing a ~70 kDa protein that includes only the KL1 domain. Enzymes like BACE1 can also generate this version(Zhang et al., 2024).

Kuro-o and colleagues in 1997 first observed that klotho is linked with controlling ageing and the age-related diseases in mammals (Shen et al., 2025). In a rat model prone to kidney damage, delivering the klotho helped protect the kidneys even though the gene was only active in the liver, suggesting it had effects beyond the tissue where it was expressed (Oishi et al., 2021). What's especially fascinating is that even though klotho is only active in a few specific tissues, (Martín-Vírgala et al., 2023) suggest with it seem to that the Klotho protein may work like a hormone, travelling through the bloodstream and influencing distant organs.

Klotho protein has been found to affect the insulin pathway and also insulin-like growth factor-1acts as an antioxidant through its interaction with regulatory proteins (Typiak and Piwkowska 2021).

MATERIALS AND METHODS

Separation of α-Klotho from plasma

The Blood Bank of Duhok helped us get plasma (30 mL) from a healthy 18-year-old donor and will be employed in the next steps of purification (Thikra and Jehan, 2014).

1. Using ammonium sulfate to separate and precipitate protein

The first stage in purifying plasma is to use (NH4)2SO4 to make proteins precipitate. This happens depending on how saturated, at a saturation level of 65%, (NH4)2SO4 was slowly added to plasma at 4°C while an electric stirrer was constantly used to mix for 60 minutes (Robyt and White, 1987).

2. Separation by Cooling Centrifuge

The precipitate was put in a cold centrifuge for 35 minutes at 10000 xg. After separating, the precipitate was dissolved in distilled water (Rana and Thikra, 2021).

3. Dialysis

Cellophane tubes are used as a semipermeable membrane for this process (Robitt and White, 1987). The dialysis process was performed by placing the precipitate solution in a cellophane tube, which was then placed in a 0.1 M ammonium bicarbonate (NH_4HCO_3) solution (Plummer, 1978). The temperature was maintained at 4°C for 24 hours.

4. Size exclusion chromatography (Gel Filtration Chromatography)

It is separate compounds based on their molecular weights and sizes. It is also called molecular sieve chromatography. Sephadex G-75 was used, and protein peaks were identified by measuring the absorption at 280 nm (Thikra and Jehan, 2014). This technique can also be used to determine the molecular weight of protein peaks (Andrews, 1965).

The steps involved are as follows.

Preparation of Sephadex G-75 Gel and Column

Distilled water was added to the powder of Sephadex gel (G-75), then allowed to swell for 72 hours until complete swelling occurred (Robyt and White, 1987).

Column packing

The Sephadex G-75 was placed in a glass column 90 cm high and 1.5 cm wide, resulting in a gel height of approximately 82 cm.

Addition of standard proteins

Standard proteins with molecular weights ranging from 2000,000 to 204 Daltons were used to evaluate the approximate molecular weight of the separated protein peaks. Tryptophan (Trp) and blue dextrin were used to determine the column size, in addition to the use of standard proteins, egg albumin, trypsin, insulin, bovine serum albumin (BSA), casein, and pepsin.

Adding samples

The proteinous sample (2mL) was put on top of Sephadex G-75, followed by (2mL) of distilled water. Collect the displaced liquid in 3 minutes with 60 mL/h. Proteins were detected by measuring absorbance at 280 nm, also α-Klotho was detected by determining its concentration. The proteinous peak in which α-Klotho was found (peak B) was collected, lyophilized, and kept in the freezer in a tight sample tube for further experiments.

Freeze-Drying Technique (Lyophilization) Lyophilizer

The protein fractions obtained from gel filtration chromatography were freeze-dried using a lyophilizer to achieve concentration (Al-Barhawi & Al-Wash, 2022).

Estimation of Total Protein Using the Modified Lowry Method

The assay by Schacterle and Pollack (1973) is used to determine protein concentration. This is a modification of the Folin-Lowry method.

SDS-PAGE electrophoresis for evaluating the molecular weight

Sodium dodecyl sulfate (SDS) gives proteins a negative charge, thus making proteins separation dependent on size and molecular weight (Laemmli, 1970).

Estimation of Soluble Alpha-Klotho Concentration

Human Alpha-Klotho concentration (α-Klotho) was evaluated by using a kit from Sunlong Company (China), dependent on an Enzyme-Linked Immunosorbent Assay (ELISA).

RESULTS AND DISCUSSION

Saline Displacement

Table (1) shows that the use of ammonium sulfate (0-65%) resulted in an increase in the specific concentration (22.74) and the number of isolations (1.3) of α-Klotho compared to the specific concentration (17.57) and number of isolations (1) of plasma. The recovery rate also reached 87%, indicating that ammonium sulfate salt (0-65%) is a good precipitator for proteins in general (Thikra and Noori, 2023) and for Klotho in particular.

Dialysis

Table (1) also shows that dialysis increases the specific concentration (33.13) while decreasing the recovery rate (82%) of α-Klotho, highlighting the importance of dialysis for removing interfering substances (Thikra and Liqaa, 2019).

Table 1: Steps of Purification for plasma α-Klotho.

Steps of Purification	Volume (ml)	Conc. Of Protein (mg)	α-Klotho Conc. (pg)	Specific concentration	Number of isolations	Recovery %
Blood Plasma	30	230	4041	17.57	1	100
Proteinous precipitate solution	25	155	3525	22.74	1.3	87
Dialysis	23	103.5	3325	33.13	1.8	82
peak B Size exclusion / G-75	5	4.24	2869	676.65	38.5	71

Gel filtration chromatography

Figure (1) shows the appearance of peaks A and B, as well as their respective elution volumes (66 and 99 mL). The highest specific concentration of alpha-klotho was found in the second peak (B), and therefore, this peak was considered the isolated alpha-klotho, as indicated in Table (1), where the number of isolations reached 38.5.

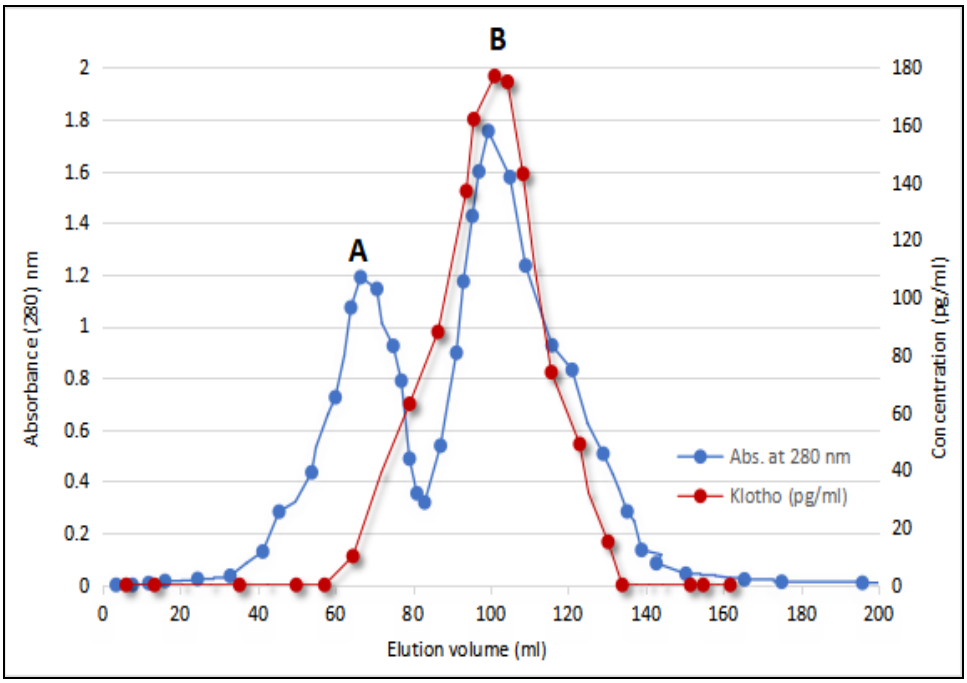

Figure 1: A separation column by Sephadex G-75 in gel filtration chromatography.

Determination of isolated α-Klotho 's (peak B) molecular weight by

1- Size exclusion chromatography technique

It was found that the isolated (peak B) α-Klotho has an approximate molecular weight of 68,000 Daltons, according to the use of the calibration curve shown in Figure 2 for the gel filtration technique.

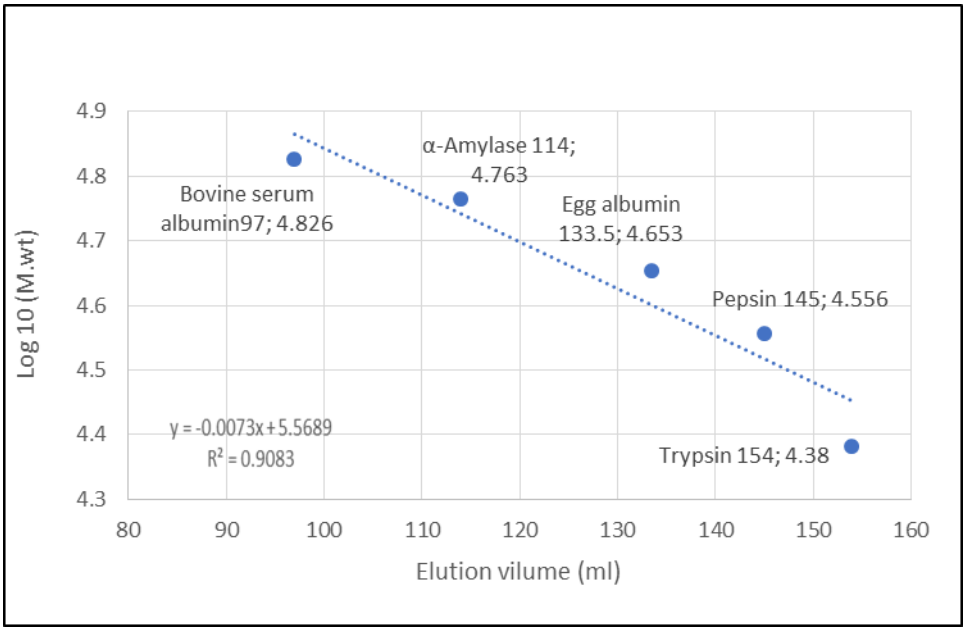

Figure 2: Calibration curve for gel filtration chromatography of molecular weight.

2- Polyacrylamide gel electrophoresis using sodium dodecyl sulfate

Figure 3 shows that the isolated (peak B) α-Klotho has an approximate molecular weight of 66,000 Daltons.

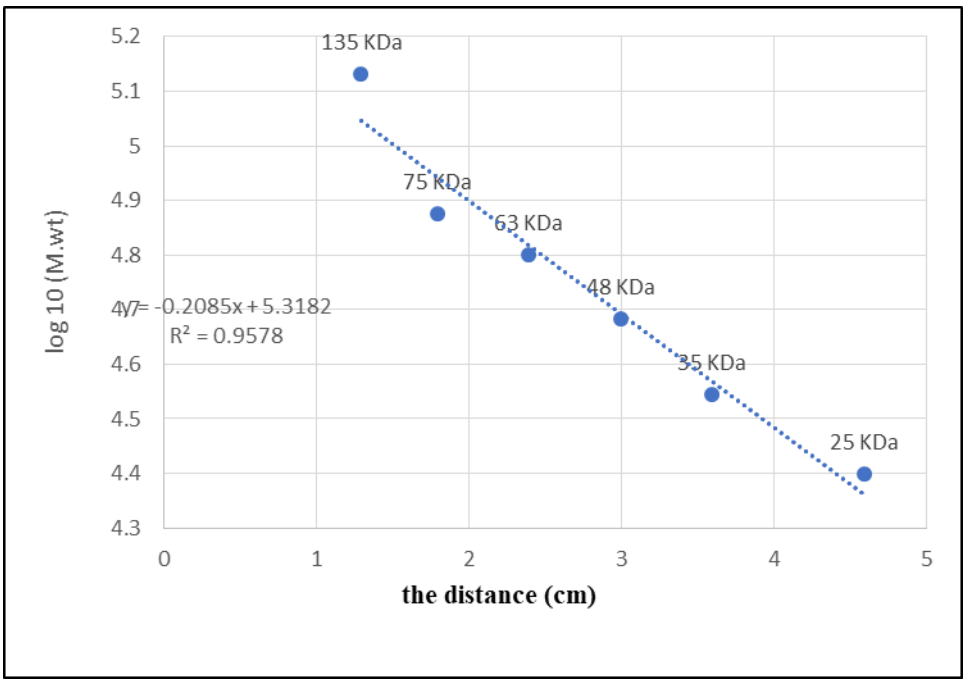

Figure 3: Standard curve of SDS-PAGE for molecular weight evaluation.

In this study, the approximate molecular weight of alpha-klotho, determined by size exclusion chromatography, was 68,000 Da, while its molecular weight, determined by SDS-PAGE, was 66,000 Da.

The close match between these results adds confidence to the data and supports the interpretation that the protein analyzed represents the soluble or cleaved form of α-Klotho, rather than the larger transmembrane form. Previous reports have shown that secreted or shed forms of Klotho usually migrate in the 65–70 kDa range (Wang et al., 2008), depending on experimental conditions and post-translational modifications (Zhong et al., 2020).

CONCLUSION

The study concluded that blood serum is a good source for isolating and purifying alpha-Klotho using ammonium sulfate precipitation, cryocentrifugation,

dialysis, in addition to size exclusion chromatography. Two peaks protein (A and B) showed, with alpha-Klotho present at the second peak (B), exhibiting a purity level 38.5 times greater than that of blood plasma after filtration. Electrophoresis was used to verify the purity of peak isolated for alpha-Klotho. Furthermore, the approximate molecular weight of alpha-Klotho, determined using two methods—gel filtration and SDS-PAGE—was found to be 68 kDa and 66 kDa, respectively.

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