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Development of antivenom through antibody purification for *Aegaeobuthus gibbosus anatolicus* venom: a promising approach for scorpionism treatment

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Abstract. This study focuses on determining the chromatographic and electrophoretic profiles of the product obtained after developing antivenom derived from the venom of the *Aegaeobuthus gibbosus anatolicus* using antibody purification methods. The research extensively explains the antibody purification methods used in the antivenom development process, covering antibody precipitation, dialysis, HPLC, and SDS-PAGE analyses starting from blood-serum samples of immunized animals. In the serum samples of immunized mice, 9 bands ranging from 10 to 300 kDa were observed. After purification, only a single band at 75 kDa was detected. The electrophoretic profile of the spleen from healthy mice showed 7 bands between 11-150 kDa, while spleen samples from immunized mice showed 8 bands in the same range. The HPLC analysis reveals a significant single peak, particularly about the size and shape of the other peaks. This study highlights the potential of a therapeutic approach in the treatment of scorpionism and provides important insights into the development of antivenoms.

Keywords: *Aegaeobuthus gibbosus anatolicus,* antibody, antivenom, HPLC, SDS-PAGE.

Introduction

Scorpion stings, known as scorpionism, present a considerable public health issue globally, particularly for individuals residing in rural regions. *Aegaeobuthus gibbosus anatolicus* is a scorpion species widely found in the Anatolian region, and its stings can lead to severe health complications (Chippaux & Goyffon 2008, Diego-García et al. 2014, Filazi & Özkan 2021). Throughout their evolutionary processes, scorpions have utilized diverse bioactive components gathered in their venoms to capture prey or defend themselves. Scorpion toxins are a mixture of biologically active compounds in venom glands (Koç & Arıkan 2018, Klotz et al. 2023). Despite being water-soluble and antigenic with a heterogeneous structure, they contain various proportions of neurotoxins, cardiotoxins, hemolytic toxins, nephrotoxins, phosphodiesterase, glycos-aminoglycans, phospholipase, hyaluronidase, histamine, serotonin, tryptophan, and cytokine-releasing substances (Abdel-Rahman et al. 2009, Joseph & George 2012, Bermúdez-Méndez et al. 2018). Knowing the toxicity and immunogenicity of specific venom components is important for antivenom development. Therefore, identifying the most important toxins in highly medically significant venoms and designing the most suitable toxin mixtures for immunization are of great importance (Rangel-Santos & Mota 2000, Gutiérrez et al. 2010, Laustsen et al. 2015, 2017).

Antivenoms are biological products derived from the plasma of hyperimmune animals and protect against venom. They may contain F(ab')2 fragments, the entire IgG molecule, or only Fab fragments (İsmail 2014, Chippaux 2012, Boyer et al. 2018). Antibodies play essential roles in immune responses, diagnostics, and therapeutics. The isolation and

purification of antibodies from biological samples is a critical process in biotechnology and medicine. Antibody purification uses chromatography, precipitation, filtration, and affinity-based methods. This process enhances the antibodies' specificity, purity, and efficacy (Bermúdez-Méndez et al. 2018, Gutiérrez et al. 2010, Laustsen et al. 2017). The purification begins by separating antibodies from animal plasma and continues with precipitation, chromatographic, and filtration steps. These steps are aimed at separating antibodies from unwanted components. Fab and F(ab')2-based antivenoms, formed by eliminating the Fc region, provide higher neutralization capacity and safety. These advanced antivenoms offer more effective protection against various venom agents and are continually optimised (Antúnez et al. 2010, Bermúdez-Méndez et al. 2018, Gutiérrez et al. 2010, Laustsen et al. 2017).

The development of species-specific antivenom studies is important as an effective treatment method. The development of a potent antivenom against scorpion venom represents a promising strategy for the treatment of scorpionism. This study provides a comprehensive analysis of the purification process for antibodies derived from laboratory animals immunized with the venom of *Aegaeobuthus gibbosus anatolicus*.

Material and methods

The electrostimulation method was utilized to produce crude venom, using 120 scorpions that were collected through nocturnal fieldwork in (27°02' and 27°15'E, 37°36' and 37°42'N) Dilek Peninsula, Aydın, Turkey. The peptide content in the crude venom was determined using a nanodrop spectrophotometer. For this purpose, 2 µl of fractions were dissolved and loaded into the device with 100 µl of distilled water. Measurements were conducted at a wavelength of 280 nanometres (A280). Fifteen male BALB/c mice weighing 20-25 grams and aged 6-8 weeks were utilized for antibody production against the venom antigen. The experimental procedures followed the guidelines and ethical approval (Approval No: 2020-091) of the Ege University Laboratory Animals Practice and Research Center (EGEHAYMER). Weekly blood samples were collected from the mice for 72 days to examine the formation of antibodies against the venom. The blood samples were collected in Eppendorf tubes containing EDTA and centrifuged at 5000 g for 5 minutes. The separated serum samples were stored at -20ºC until further analysis using the ELISA method.

Following the determination of the presence and quantity of antibodies in the serum samples based on ELISA results, antibody purification processes were applied to the serum samples. At the end of the process, the spleens of euthanized mice from both the control and experimental groups were collected and subjected to electrophoretic comparison after homogenization.

Antibody Purification

The process involves several steps to isolate and fractionate antibodies effectively (Horenstein et al. 2005, Grodzki & Berenstein 2010, Guidlolin et al. 2010, Estrada-Gomez et al. 2022).

Antibody purification is the method for isolating and fractionating antibodies from the sera obtained from mouse blood samples. The process involves the following steps:

1. Serum samples were mixed for 2 hours with an ammonium sulfate solution, which was then separated by filtration using cellulose membranes.
2. The immunoglobulins mixture was separated using cellulose membranes with a pore size of 10 µm.
3. Dialysis was performed to remove salts from the obtained IgG antibodies.
4. The obtained IgG solution was sterilized using a 0.22 µm filter membrane and then lyophilized.
5. High-Performance Liquid Chromatography (HPLC) was conducted to check the purity of

the product.

This method was carried out with some modifications to the procedure described by Guidlolin and colleagues (Guidlolin et al. 2010, Estrada-Gomez et al. 2022).

Ammonium sulfate precipitation

A container filled with ice was utilized to hold 20 ml of a serum sample, which was subsequently placed on a magnetic stirrer. While stirring, ammonium sulfate was added to the solution slowly and continuously. After the addition of the salt was completed, the stirring process continued for 2 hours. The resulting upper liquid in the product was discarded after centrifugation at 3000 g for 30 minutes. The next step involved the utilization of dialysis to effectively eradicate the residual ammonium sulfate from the surrounding area (Grodzki & Berenstein 2010, Temizkan & Arda 2021).

Dialysis method

Before using the dialysis membrane, it was washed with ultra-pure water (UPW). Subsequently, one end of the membrane was carefully tied, and the protein solution was transferred into the membrane using a micropipette. The other end was tied after removing the air from the membrane, leaving a small gap. The dialysis membrane was immersed in at least 10 times its volume of dialysis buffer (pure water or the buffer containing the protein), and the dialysis fluid was stirred on a magnetic stirrer at 4°C overnight, renewing it several times. Once the process was complete, one end of the tube was cut, and the sample inside was transferred into a clean glass tube (Figure 1). The obtained product was stored at the temperature of -20°C after the process of lyophilization (Andrew et al. 2002).

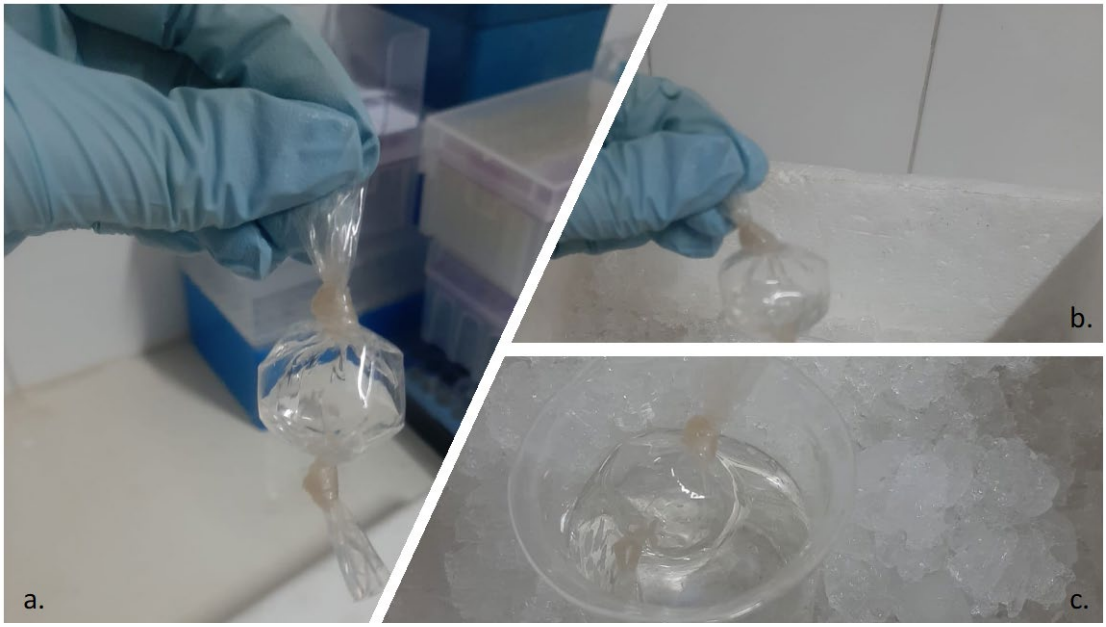

Figure 1. Dialysis Stages: a. Transfer of the protein solution into the membrane and removal of the air inside, followed by tying the other end. b. Dialysis membrane with the protein solution inside, removing the air, and tying the other end. c. Immersion of the dialysis membrane into at least 10 times its volume of dialysis buffer (pure water).

High-Performance Liquid Chromatography (HPLC)

In the study, the purity analysis of the product obtained after dialysis was performed using HPLC. The Agilent 1260 Infinity HPLC system and a Diode Array Detector (DAD) were

employed as the devices in the study. A 5 μm mobile phase, a 150*4.6 mm C18 reverse-phase analytical column, and a flow rate of 0.8 ml per minute for 60 minutes were used for separation, monitoring absorbance at 280 nm. Linear gradient application of the mobile phase was carried out during separations. The mobile phase was determined as Acetonitrile: Water (80:20, v:v), and the injection volume was set at 20 μl/2 mg (Ali 2022, Darcy et al. 2011).

Electrophoretic Profiling through SDS-PAGE Studies

In the study, comparative purity and molecular weight analysis were conducted through Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) on serum samples subjected to purification analyses and spleen samples obtained from mice. Tris-Trisine Gel (10%-16%) For this we used electrophoresis. The protein concentration was determined using the Bicinchoninic Acid (BCA) protein concentration determination method (Walker 2009). An undenatured solution was used for protein concentration determination at each purification stage following the BCA kit instructions. For gel preparation, 10% separating gel and 5% stacking gel were applied to the gel maker. The purified antibody protein was mixed with SDS-PAGE Sample Loading Buffer (5×) in a 4:1 ratio and subjected to a boiling water bath for 10 minutes. Following the SDS-PAGE method (Haider et al. 2012), the gel was placed into the electrophoresis tank, and the sample was loaded for electrophoresis. Electrophoresis conditions included 80 V for the stacking gel and 120 V for the separating gel. Bromophenol blue was monitored during electrophoresis, and the process was stopped when it was approximately 1 cm from the bottom. The gel was removed, washed, fixed, stained with Coomassie brilliant blue, destained, and bands were observed and photographed. An image was captured using the ChemiDoc™ MP Imaging System from Biorad. The experiment was repeated at least three times to confirm the presence of protein bands at different kDa levels to ensure statistical analysis and data reliability (Özkan & Ciftci 2010, Kırdök & Arikan 2024).

Results

The following results were obtained by purifying and fractionating antibodies from mouse serum samples. Initially, High-Performance Liquid Chromatography (HPLC) was conducted to confirm the purity of the product.

Evaluation of the Chromatogram Obtained with HPLC

Components are eluted from the column at different times (different retention times) due to their varying mobilities. Therefore, there are different retention times. When interpreting the HPLC results, considering the size and shape of the peaks on the obtained graph, a major peak point was observed prominently. The chromatogram is shown in Figure 2. Since a C18 reverse-phase column was used, the peak observed at shorter retention times corresponds to a component with higher polarity. Therefore, components with retention times below 20 minutes are more polar than those above 20 minutes. The component observed between 2.5-5 minutes in the chromatogram has been separated in terms of polarity. If the purity of the compound is high, the peak is more distinct and narrower. If the purity of the compound is low, the peak is wider and less distinct. Therefore, the single major peak observed between 2.5-5 minutes indicates the purity of our sample. Minor peaks are present in the chromatogram; however, as stated, the major peak has been considered as the basis.

Evaluation of SDS-PAGE Studies

The GelAnalyzer 19.1 program was utilized to determine the molecular weights of bands in the electrophoretic profile. In the serum samples of

immunized mice, 9 bands ranging from 10 to 300 kDa were observed. These protein bands were determined to be approx. 15, 22, 32, 48, 75, 104, 178, 265, and 297 kDa (Figure 3c). After the purification analysis, a single band at 75 kDa was observed in the serum samples (Figure 3b).

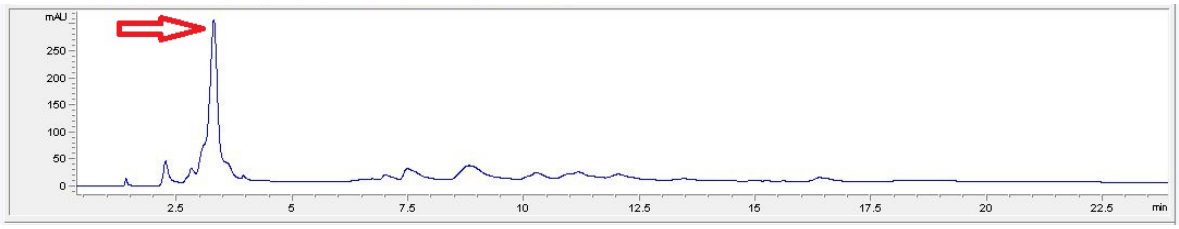

Figure 2. Chromatogram of the lyophilised sample at 280 nm wavelength obtained by HPLC separation after antibody purification processes.

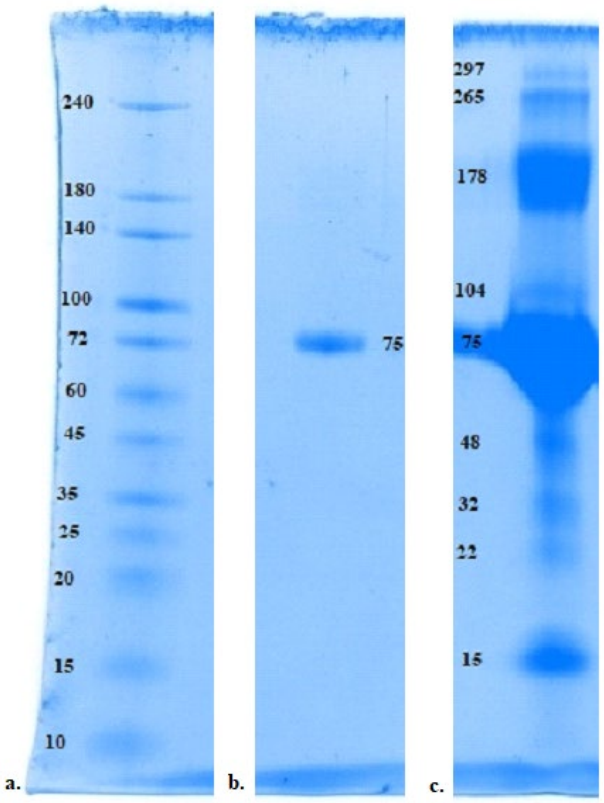

Figure 3.a. Electrophoretic profile of the Protein Marker (Molecular Weight Standard 250-10kDa). b. Electrophoretic profile of the purified serum sample c. Electrophoretic profile of the serum sample

The electrophoretic profile of spleens obtained from healthy mice was examined after homogenization. Seven different bands, ranging from approximately 11-150 kDa, were observed on a 10% Tris-tricine gel electrophoresis (Figure 4b). These protein bands were determined to be approximately 11, 17, 23, 29, 52, 70, 110, and 150 kDa in weight.

Conversely, 8 distinct bands were observed in the 11-150 kDa range in spleen samples obtained from immunized mice. These protein bands were determined to be approximately 11, 17, 23, 29, 52, 75, 110, and 150 kDa in weight (Figure 4c).

After examining the spleen's electrophoretic profile obtained from healthy mice after homogenization, two distinct bands were observed at approx. 14.3 and 23 kDa following 16% Tris-Tricine gel electrophoresis (Figure 5b). In the spleen samples from mice immunized by venom injection, two distinct bands were observed at 14.3 and 23 kDa (Figure 5c).

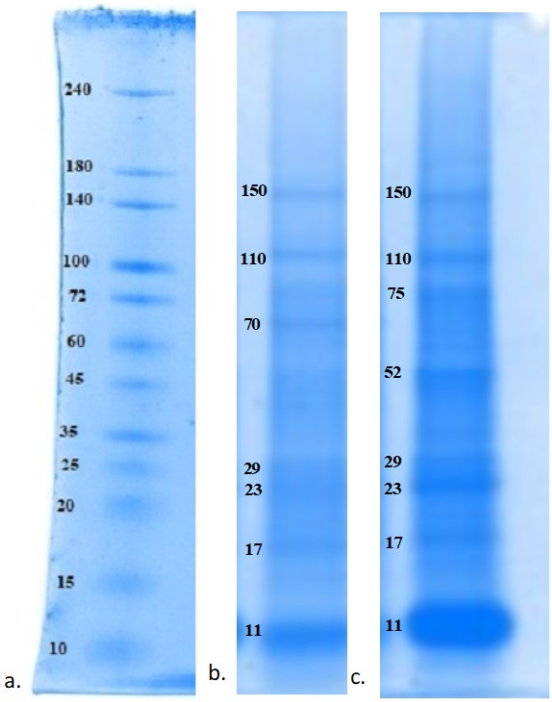

Figure 4.a. Electrophoretic profile of the Protein Marker (Molecular Weight Standard 250 kDa - 10 kDa) b. Electrophoretic profile of the spleen obtained from healthy mice after homogenization c. Electrophoretic profile of the spleen obtained from mice immunized by venom injection

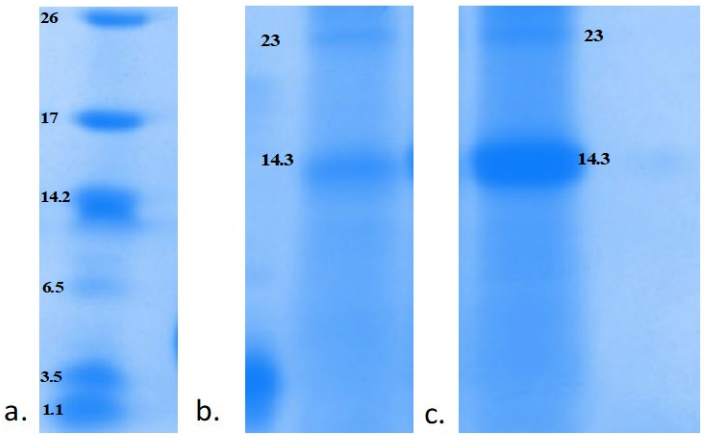

Figure 5. a. Electrophoretic profile of the Protein Marker (Molecular Weight Standard 26 kDa - 1 kDa) b. Electrophoretic profile of the spleen obtained from healthy mice after homogenization c. Electrophoretic profile of the spleen obtained from mice immunized by venom injection.

Discussion

Antibodies have been at the forefront of technology for over a century. Their commercial success and unanticipated growth are demanding innovation across all sectors (Gagnon 2012). Antibody purification aims to separate antibody molecules from other proteins and components present in a mixture to obtain pure antibody samples. This should enable more effective use of antibodies, increase their reliability in biological research and medical applications, and facilitate antibody-based product development.

The essential step of antibody purification, ammonium sulfate precipitation, is a common technique employed in antivenom production to isolate antibodies from animal plasma. Traditionally, substances like ammonium sulfate or caprylic acid precipitate antibodies in excessively immunized animal plasma. The preferred ammonium sulfate precipitation in our study proves to be an effective method, allowing the concentration of the starting material and the precipitation of the desired antibodies (Grodzki & Berenstein 2010, Guidlolin et al. 2010, Gagnon 2012, El-Naggar et al. 2016, Estrada-Gomez et al. 2022).

Ito (2002) developed a dynamic variant of ammonium sulfate precipitation. In this method, antibody precipitation and resuspension processes are integrated. A semi-permeable membrane separates the water channel from the ammonium sulfate channel, creating an ammonium sulfate gradient in the water channel, leading to the deposition of proteins as precipitates. The gradual reduction in salt concentration in the ammonium sulfate channel allows precipitated species to dissolve in order of their solubility. This method has been successfully applied to fractionate monoclonal antibodies, although its suitability for commercial-scale fractionation has not yet been evaluated (Ito 2002).

Antibody precipitation is often integrated with other techniques to separate crude proteins. This process combines dialysis and gel chromatography (Andrew et al. 2002). In a study published by Mariam et al. (2015), it was concluded that the optimum precipitation rate for IgG in rabbit serum was 40%. The results emphasized the importance of carefully selecting the concentration in the protein precipitation and purification processes. Our study correlates with these findings. The study demonstrated a thick band of albumin at 57 kDa through the gel image of crude serum, indicating low IgG concentration in the crude serum. Compared to the initial serum sample, the intensity of the IgG band was higher in all samples obtained from a low precipitation process at different saturation percentages, while IgG concentrations obtained through ammonium sulfate precipitation at different saturation percentages were almost similar. This indicates that a low concentration, such as 37% and 40%, is sufficient to precipitate most IgG from the serum. As the concentration in the serum increases, there is a tendency for albumin to co-precipitate with IgG, resulting in a decrease in IgG purity. In the overall assessment of the study, purified antibodies, along with studies on F(ab')2 fragments or Fab fragments, are used to enhance the effectiveness and optimize the safety of antivenoms. At this point, Klotz et al. (2023) discussed the use of F(ab')2 antivenom in treating severe scorpion envenomation caused by *Centruroides sculpturatus* in Arizona. Researchers, noting that more than 1000 scorpion stings are reported annually in Arizona, particularly highlighted the vulnerability of small children. By stating that F(ab')2 antivenom is effective in treating severe toxic effects of scorpion stings, they reported the use of F(ab')2 antivenom in 252 patients in emergency services across 15 counties in Arizona from January 2017 to December 2021, with symptoms improving in all patients within 4 hours. Thus, these studies underscore the importance of antivenom-antibody usage. Improved antivenoms have the potential to provide more effective protection,

especially against weakly immunogenic and toxic components. Therefore, the processes of antivenom development progress successfully by combining antibody purification methods with a detailed analysis of venom composition.

HPLC is the most accurate analytical method widely used for the quantitative and qualitative analysis of drug products and is used to determine drug product stability (Ali 2022). In this study, due to the use of a C18 reverse-phase column, the peak observed at shorter retention times corresponds to a component with higher polarity. Therefore, components with retention times shorter than 20 minutes exhibit higher polarity compared to those with retention times longer than 20 minutes. Looking at these results, it is possible to say that they correlate with other studies conducted. A study by Andrew & Titus (2001) emphasized the successful purification of IgG through size exclusion (SE) chromatography following ammonium sulfate precipitation. While this economical method could offer a suitable option for antibody purification, it may not be universally suitable for all rat antibody subclasses. To overcome these challenges, a specific protocol for affinity chromatography using anti-rat antibodies was developed for the purification of rat antibodies.

Precipitation, dialysis, electrophoresis, HPLC, gel chromatography, and other methods should be selected based on the properties of proteins and the purpose of separation. The combination of these methods can be an effective strategy to achieve the targeted purification. The processes of antivenom purification are critical steps that can directly influence the efficacy of antivenom. This process not only involves obtaining the pure form or specific fractions of antibodies that enhance immunity against toxins but also significantly enhances the capacity of antivenoms to bind to poisons and neutralize their effects within the body. This improves the dose-response relationship of antivenoms. It also reduces potential side effects. Purified antibodies are essential tools for advancing biomedical science and improving human health due to their high specificity and affinity. An exciting area for future research and innovation is the development of universal purification platforms capable of purifying antibodies from diverse sources with high purity and yield. In the coming years, antibody purification technology will play a key role in advancing biotechnology, medicine, and healthcare by addressing these challenges.

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