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Purification of Nucleoprotein of H9N2 Avian Influenza Virus Strain by Electroelution

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Abstract

Background: Avian influenza virus (AIV) belongs to the family of Orthomyxoviruses type A and causes avian influenza (AI) infectious disease. Currently, serological diagnostic techniques such as agar gel propagation (AGP), hemagglutination inhibition, and enzyme-linked immunosorbent assay (ELISA) are considered as important tools for the antibodies detection against viral antigens. Due to antigenic variation in the surface of AIV glycoproteins (hemagglutinin and neuraminidase), these proteins cannot be used in serological tests. Development of assays to detect AI surface glycoproteins is problematic because a great variety of combinations of these subtypes are found in nature. The internal antigen determinants on the nucleoprotein (NP) are highly conserved within influenza viruses, making this protein more appropriate for a serological test.
Objectives: In the experimental present study, an effectual method was expanded to purify NP of H9N2 AIV based on Electroelution method.
Methods: AIV strain A/flash chicken/Iran/772/1998 (H9N2) was acquired from the Department of Avian Influenza Reference Laboratory, Razi Vaccine and Serum Research Institute, Iran, about 2 cc, in 2017. Nucleoprotein of AIV (H9N2) was purified by an efficient and simple modified method directly from native polyacrylamide gel electrophoresis (PAGE) according to the Electroelution method. The purified protein concentration was defined by the Lowry method, and the purified NP protein (60 KDa) was examined by Tricine-SDS-PAGE.
Results: The protein concentration of the virus solution was 4.62 mg/mL by the Lowry method. The purified Nucleoprotein concentration was 0.296 mg/mL by Lowry method and the Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis results showed only a 60-KDa protein band in the gel.
Conclusions: The current technique was simple and rapid and made it possible to isolate NP from the H9N2 virus. The Nucleoprotein antigen is an appropriate candidate potential to detect antibodies against all subtypes of AIV and used as the main target antigen for the diagnosis of influenza virus due to its very high scale of sequence preserved among exist strains.

Keywords: Avian Influenza Virus, Enzyme Linked Immunosorbent Assay, Electroelution, H9N2 Subtype, Hemagglutination, Influenza A Virus, Membrane Glycoproteins, Native Polyacrylamide Gel Electrophoresis, Neuraminidase, Nucleoproteins, Sodium Dodecyl Sulfate Antigens

1. Background

Avian influenza (AI), a zoonotic infection initiated by influenza viruses (a member of the Orthomyxoviridae family), separated into five different genera consisting types A, B, and C, as well as isavirus and thogotovirus (1). Types B and C circulate constantly in human subjects, while the type-A infects humans, different warm-blooded animals, and a wide assortment of trained and wild flying creatures (2). Respiratory and infectious diseases are the most common diseases, which are caused by microbial factors, such as viruses, bacteria, and fungi. Influenza A viruses (IAVs) harbor eight gene segments encoding 10 viral proteins: the structural proteins containing neuraminidase (NA), hemagglutinin (HA), nucleoprotein (NP), that encapsulates the ssRNA (-) genome, two matrix proteins (M1, M2), two polymerase basic proteins (PB1, PB2), and polymerase acidic protein (PA1). There are also two non-structural proteins (NS1, NS2) (3). Among the genera of influenza viruses, NP and M are highly conserved (4). Avian influenza virus (AIV) is subtyped according to the antigenic difference of the viral surface glycoproteins (HA, NA) to 17 HA and 10 NA (5). AIVs in birds are classified as either high pathogenic-

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ity (HP) or low pathogenicity (LP) according to the clinical signs severity, and rate of mortality in birds (6). Although the H9N2 IAV strain is low pathogenic, it often infects avian species and its mortality rate, among which is reported above 50% (7). The H9N2 strain is broadly dispersed worldwide; it was first identified in Wisconsin in 1966 (8). H9N2 viruses transmit from birds to humans and other mammals (9-12) and cause infections in humans as well as wild and domesticated animals in many Eurasian countries (8, 13). They are also spread to the Middle East and Africa (13, 14). H9N2 human infections have been incessantly reported, but there were no H9N2 vaccines available in humans (15). It has been reported that patients benefit from antiviral treatment, especially in the first 48 hours of illness (16). Currently, serological diagnostic techniques such as agar gel propagation (AGP), hemagglutination inhibition, and enzyme-linked immunosorbent assay (ELISA) are considered as important tools for the antibodies detection against viral antigens (17). These techniques are common and relatively inexpensive, and generate results more rapidly (18, 19). Enhancing the detection efficiency in a small sample size is important for accurate molecular detection (20). The development of assays to detect AI surface glycoproteins is problematic because a great variety of combinations of these subtypes are found in nature (21). Influenza virus also has good candidate proteins such as NP used as the main target antigen for the diagnosis of influenza virus due to its very high scale of sequence preserved among exist strains (22-24).

2. Objectives

In the present experimental study, an effectual method was expanded to purify NP of H9N2 AIV based on electroelution method. To evaluate purified protein specificity, Tricine-SDS-PAGE (Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis) has been used.

3. Methods

3.1. Viruses

AIV strain A/flash chicken/Iran/772/1998 (H9N2) was acquired from the Department of Avian Influenza Reference Laboratory, Razi Vaccine and Serum Research Institute, Iran, about 2 mL, in 2017 and stored at -70°C. In the present experimental study, the amount of present proteins in virus solution was determined by BSA (bovine serum albumin) standard protein (Sigma, St. Louis, MO) using the Lowry et al. method (25).

3.2. Gel Electrophoresis of the H9N2 AIV

SDS-PAGE was used to separate the H9N2 AIV protein bands based on their known molecular weights (26). The virus solution was electrophoresed in a 10% gel. Samples were diluted into sample buffer (5% β-mercaptoethanol), and next heated up to 95°C for 10 minutes. Gels were stained with Coomassie Brilliant Blue R-250.

In order to analyze proteins with biological activity, a non-denaturing system is needed; for this purpose, a non-denaturing system based on the method of Davis was used (27, 28). H9N2 virus was electrophoresed on a 10% native PAGE three times to obtain enough gel for electroelution of NP. The sample was ready in sample buffer without β-mercaptoethanol, SDS, or heating and stained with Coomassie blue. Every time 600 μL viral protein was loaded into 11 lanes.

3.3. Electroelution of Nucleoprotein

Bio-Rad model 422 Electro-Eluter was used to recover NP separated by native PAGE by means of an electric field, based on the manufacturer's manual. Concisely, the 60-KDa protein bands were cut from the four native gels and placed into tubes with a frit at the bottom. The tubes were filled with elution buffer (25 mM Tris /glycine199 mM) and then, silicon adaptors attached to a membrane cap (MWCO of 12000 - 15000 Daltons) were filled with elution buffer and attached to the end of the tubes. Then the elution was performed at 10 mA/tube for five hours. In the end, the eluted proteins were pipetted from the membrane. Dialysis was not performed due to the absence of SDS in the buffer. Then proteins were concentrated with a 6-KDa PEG.

3.4. Estimation of Purified Protein

The purified protein concentration was defined by the Lowry et al. method (25) in the optical density (750 nm) and the protein amount was counted by BSA (Sigma, St. Louis, MO) as the standard.

3.5. Tricine- SDS-PAGE of Purified Nucleoprotein

Tricine-SDS-PAGE gels are usually applied in order to proteins separation below 100 KDa. The purified NP protein (60 KDa) was examined by Tricine-SDS-PAGE (29).

4. Results

4.1. Protein Concentration

The protein concentration of the virus solution was 4.62 mg/mL by the Lowry method.

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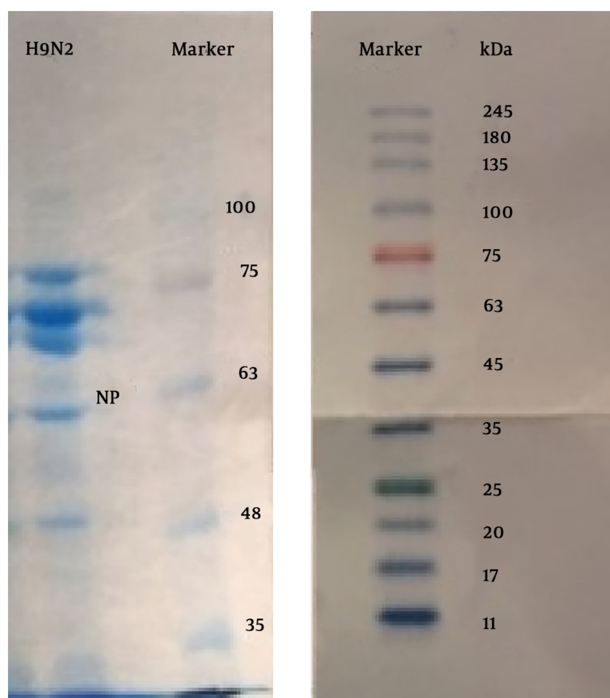

Figure 1. SDS-PAGE of the Avian influenza virus (H9N2)

4.2. Electrophoresis Analysis

Figures 1 and 2, respectively, show the results of SDS-PAGE and native PAGE gel of the H9N2 AIV. After separation of influenza virus proteins in polyacrylamide gel, the protein band of 60-KDa was cut and purified from the gel by electroelution.

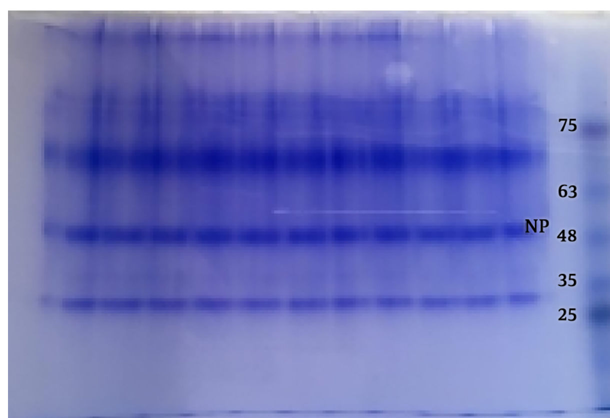

Figure 2. Native PAGE of the Avian influenza virus (H9N2)

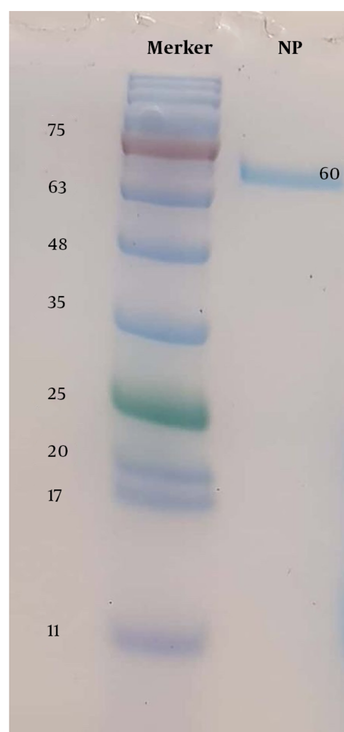

Figure 3. Tricine-SDS-PAGE of purified NP protein

4.3. Characterization of the Purified Nucleoprotein

The protein concentration of the purified protein was 0.296 mg/mL by the Lowry et al. method, and the results of Tricine-SDS-PAGE showed only a 60-KDa protein band in the gel (Figure 3).

5. Discussion

AI is recognized as an etiological infectious factor with great economic losses that outbreaks contributed progressively in the world. The development of a new, rapid, and accurate diagnostic tools is needed to detect antibodies against AIV immunodominant proteins by serological methods (19). The NP of AIV has a highly conserved sequence across the subtypes and strains of IAV; therefore, it is used as a basis for classification and diagnosis of AI. The NP of AIV is an internal antigenic determinant and is applied successfully in an indirect ELISA test to detect AIV-specific antibodies (18, 30, 31).

Different methods such as cloning and expression of AIV NP, size exclusion chromatography, and high-performance liquid chromatography are applied to purify nucleoprotein. Bhat et al., expressed the NP gene of AIV in *Escherichia coli* to produce a 6x-His-tagged recombinant NP

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antigen of ~ 61 KDa molecular weight as the soluble fraction (32). Express nucleoprotein in the *E. coli* expression system usually is difficult and has not been more often applied in order to produce AIV recombinant NP, since it should be usually recuperated from the insoluble fraction and needs multiple purification steps (33).

Accordingly, the current study developed an efficient and reproducible method to purify NP from the native PAGE gel, based on electroelution method in which less time is spent to elute several protein bands. In other protein purification techniques such as column chromatography, contamination by other nonspecific proteins could often seem through purification of the protein, while in the current study method, it is avoided. Sa-Pereira et al., purified xylanase from the polyacrylamide gel by electroelution method; some properties of this enzyme such as specific activity are presented (34). Eamsobhana et al., purified a 31-KDa glycoprotein antigen by electroelution of *Parastrongylus cantonensis* worms. The produced 31-KDa *P. cantonensis* antigen had a high degree of purity and specify (35).

Moreover, it avoids the longtime purification of the NP protein. The high resolution of this technique caused protein purity with a high level of purification. This technique does not need an expensive device and reagents and is appropriate for soluble proteins and membrane-bound proteins (36). In conclusion, the current technique was simple and rapid and made it possible to isolate NP from the H9N2 virus.

In the present study, NP of H9N2 AIV strain was purified from native PAGE gel by electroelution method. Purification of the NP protein was successful and it was detectable on Tricine-SDS-PAGE.

The purified NP could be used in serological techniques to diagnose the infection caused by this virus and was a suitable candidate to produce monoclonal antibodies to detect AIV NP.

Footnotes

Authors' Contribution: Maryam Hashemi, Rasool Madani, and Mahmoudreza Aghamaali developed the original idea and the protocol, abstracted and analyzed data. Maryam Hashemi and Tara Emami contributed to the development of the protocol, abstracted data, and prepared the manuscript.

Conflict of Interests: The authors of the present review declare no conflict of interest.

Ethical Approval: The ethical code is 95002-9251-18-18-12. AIV strain A/flash chicken/Iran/772/1998 (H9N2) was acquired from the Department of Avian Influenza Reference Laboratory, Razi Vaccine and Serum Research Institute, Iran. There are not Human or animal subject this work.

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References

1. Ducatez MF, Pelletier C, Meyer G. Influenza D virus in cattle, France, 2011-2014. *Emerg Infect Dis*. 2015;**21**(2):368–71. doi: 10.3201/eid2102.141449. [PubMed: 25628038]. [PubMed Central: PMC4313661].
2. Li H, Liu X, Chen F, Zuo K, Wu C, Yan Y, et al. Avian Influenza Virus Subtype H9N2 affects intestinal microbiota, barrier structure injury, and inflammatory intestinal disease in the Chicken ileum. *Viruses*. 2018;**10**(5). doi: 10.3390/v10050270. [PubMed: 29783653]. [PubMed Central: PMC5977263].
3. Wong SS, Yuen KY. Avian influenza virus infections in humans. *Chest*. 2006;**129**(1):156–68. doi: 10.1378/chest.129.1.156. [PubMed: 16424427].
4. Van Reeth K, De Vleeschauwer A, Kyriakis K, Pensaert M, editors. Influenza in birds, pigs and humans: Old theories versus current viewpoints. *Proceedings of the 19th IPVS Congress*. Copenhagen, Denmark. 2006.
5. Mair CM, Ludwig K, Herrmann A, Sieben C. Receptor binding and pH stability - how influenza A virus hemagglutinin affects host-specific virus infection. *Biochim Biophys Acta*. 2014;**1838**(4):1153–68. doi: 10.1016/j.bbamem.2013.10.004. [PubMed: 24161712].
6. Nguyen LT, Nakaishi K, Motojima K, Ohkawara A, Minato E, Maruyama J, et al. Rapid and broad detection of H5 hemagglutinin by an immunochromatographic kit using novel monoclonal antibody against highly pathogenic avian influenza virus belonging to the genetic clade 2.3.4.4. *PLoS One*. 2017;**12**(8). e0182228. doi: 10.1371/journal.pone.0182228. [PubMed: 28787440]. [PubMed Central: PMC5546692].
7. Nili H, Asasi K. Avian influenza (H9N2) outbreak in Iran. *Avian Dis*. 2003;**47**(3 Suppl):828–31. doi: 10.1637/0005-2086-47.s3.828. [PubMed: 14575072].
8. Homme PJ, Easterday BC. Avian influenza virus infections. I. Characteristics of influenza A-turkey-Wisconsin-1966 virus. *Avian Dis*. 1970;**14**(1):66–74. [PubMed: 4314007].
9. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, Xu KM, et al. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol*. 2005;**43**(11):5760–7. doi: 10.1128/JCM.43.11.5760-5767.2005. [PubMed: 16272514]. [PubMed Central: PMC1287799].
10. Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, et al. Avian-to-human transmission of H9N2 subtype influenza A viruses: Relationship between H9N2 and H5N1 human isolates. *Proc Natl Acad Sci U S A*. 2000;**97**(17):9654–8. doi: 10.1073/pnas.160270697. [PubMed: 10920197]. [PubMed Central: PMC16920].
11. Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, et al. Human infection with influenza H9N2. *Lancet*. 1999;**354**(9182):916–7. doi: 10.1016/s0140-6736(99)03311-5. [PubMed: 10489954].
12. Xu C, Fan W, Wei R, Zhao H. Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. *Microbes Infect*. 2004;**6**(10):919–25. doi: 10.1016/j.micinf.2004.04.015. [PubMed: 15310468].

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13. Alexander DJ. A review of avian influenza in different bird species. *Vet Microbiol*. 2000;**74**(1-2):3–13. doi: 10.1016/s0378-1135(00)00160-7. [PubMed: 10799774].
14. Noda T, Sagara H, Yen A, Takada A, Kida H, Cheng RH, et al. Architecture of ribonucleoprotein complexes in influenza A virus particles. *Nature*. 2006;**439**(7075):490–2. doi: 10.1038/nature04378. [PubMed: 16437116].
15. Lim YH, Mo IP, Koo BS, Choi YK, Lee SC, Kang SY. Production and characterization of monoclonal antibodies against an avian influenza virus (H9N2). *Korean J Vet Serv*. 2017;**40**(3):187–92.
16. Gouya MM, Nabavi M, Soroush M, Haghdoust AA, Ghalehee S, Hemmati P, et al. Mortality from pandemic influenza A (H1N1) in Iran. *Iran Red Crescent Med J*. 2011;**13**(10):698–701. [PubMed: 22737407]. [PubMed Central: PMC3371880].
17. Borzi MM, Silva KR, Montassier MdFS, Fernando FS, Tamanine MdLF, dos Santos RM, et al. Development and application of an enzyme-linked immunosorbent assay (ELISA) using a soluble recombinant nucleoprotein for the detection of antibodies to avian influenza virus. *Afr J Microbiol Res*. 2017;**11**(18):697–704. doi: 10.5897/ajmr2016.8407.
18. Le Van P. *Expression of avian influenza virus (H9N2) genes and its diagnostic application*. South Korea: Chungbuk National University; 2006.
19. Watson DS, Reddy SM, Brahmakshatriya V, Lupiani B. A multiplexed immunoassay for detection of antibodies against avian influenza virus. *J Immunol Methods*. 2009;**340**(2):123–31. doi: 10.1016/j.jim.2008.10.007. [PubMed: 19000692].
20. Kim JW, Lee CY, Nguyen TT, Kim IH, Kwon HJ, Kim JH. An optimized molecular method for detection of influenza A virus using improved generic primers and concentration of the viral genomic RNA and nucleoprotein complex. *J Vet Diagn Invest*. 2019;**31**(2):175–83. doi: 10.1177/1040638719830760. [PubMed: 30795722].
21. Moghadami M, Kazeroni PA, Honarvar B, Ebrahimi M, Bakhtiari H, Akbarpour MA, et al. Influenza A (H1N1) virus pandemic in Fars province: A report from southern Iran, July-December 2009. *Iran Red Crescent Med J*. 2010;**12**(3):231.
22. van Wyke KL, Hinshaw VS, Bean WJ Jr, Webster RG. Antigenic variation of influenza A virus nucleoprotein detected with monoclonal antibodies. *J Virol*. 1980;**35**(1):24–30. [PubMed: 6157838]. [PubMed Central: PMC288779].
23. van Wyke KL, Yewdell JW, Reck LJ, Murphy BR. Antigenic characterization of influenza A virus matrix protein with monoclonal antibodies. *J Virol*. 1984;**49**(1):248–52. [PubMed: 6197538]. [PubMed Central: PMC255448].
24. Schild GC, Oxford JS, Newman RW. Evidence for antigenic variation in influenza A nucleoprotein. *Virology*. 1979;**93**(2):569–73. doi: 10.1016/0042-6822(79)90259-9. [PubMed: 88124].
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;**193**(1):265–75. [PubMed: 14907713].
26. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;**227**(5259):680–5. doi: 10.1038/227680a0. [PubMed: 5432063].
27. Davis BJ. Disc electrophoresis. Ii. Method and application to human serum proteins. *Ann N Y Acad Sci*. 1964;**121**:404–27. doi: 10.1111/j.1749-6632.1964.tb14213.x. [PubMed: 14240539].
28. Walker JM. Nondenaturing polyacrylamide gel electrophoresis of proteins. *The protein protocols handbook*. Germany: Springer; 2009. p. 171–6. doi: 10.1007/978-1-59745-198-7_20.
29. Schagger H. Tricine-SDS-PAGE. *Nat Protoc*. 2006;**1**(1):16–22. doi: 10.1038/nprot.2006.4. [PubMed: 17406207].
30. Wu R, Hu S, Xiao Y, Li Z, Shi D, Bi D. Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. *Vet Res Commun*. 2007;**31**(5):631–41. doi: 10.1007/s11259-007-3510-x. [PubMed: 17225082].
31. Yang M, Berhane Y, Salo T, Li M, Hole K, Clavijo A. Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. *J Virol Methods*. 2008;**147**(2):265–74. doi: 10.1016/j.jviromet.2007.09.016. [PubMed: 18006085].
32. Bhat S, Bhatia S, Sood R, Bhatnagar H, Pateriya A, Venkatesh G. Production and characterization of monoclonal antibodies against nucleoprotein of avian influenza virus. *Monoclon Antib Immunodiagn Immunother*. 2013;**32**(6):413–8. doi: 10.1089/mab.2013.0053. [PubMed: 24328746].
33. Jin M, Wang G, Zhang R, Zhao S, Li H, Tan Y, et al. Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. *Avian Dis*. 2004;**48**(4):870–8. doi: 10.1637/7226-062204r. [PubMed: 15666868].
34. Sa-Pereira P, Duarte J, Costa-Ferreira M. Electroelution as a simple and fast protein purification method: Isolation of an extracellular xylanase from Bacillus sp. CCMI 966. *Enzyme Microb Technol*. 2000;**27**(1-2):95–9. [PubMed: 10862907].
35. Eamsobhana P, Yoolek A, Suvouttho S, Suvouttho S. Purification of a specific immunodiagnostic Parastrongylus cantonensis antigen by electroelution from SDS-polyacrylamide gels. *Southeast Asian J Trop Med Public Health*. 2001;**32**(2):308–13. [PubMed: 11556581].
36. Ohhashi T, Moritani C, Andoh H, Satoh S, Ohmori S, Lottspeich F, et al. Preparative high-yield electroelution of proteins after separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and its application to analysis of amino acid sequences and to raise antibodies. *J Chromatogr*. 1991;**585**(1):153–9. doi: 10.1016/0021-9673(91)85069-r. [PubMed: 1666109].

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