Purification of NAD\textsuperscript{+} glycohydrolase from human serum

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Abstract. In the present study, NAD\textsuperscript{+} glycohydrolase was purified from serum samples collected from healthy individuals using ammonium sulfate fractionation, Affi-Gel blue (Cibacron Blue F3GA) affinity chromatography, Sephadex G-100 column chromatography and isoelectric focusing. The final step was followed by a second Sephadex G-100 column chromatography assay in order to remove the ampholytes from the isoelectric focusing step. In terms of enhancement of specific activity, the NAD\textsuperscript{+} glycohydrolase protein was purified ~480-fold, with a yield of 1% compared with the initial serum fraction. The purified fraction appeared to be homogeneous, with a molecular weight of 39 kDa, as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and also corresponded to the soluble (monomeric) form of surface antigen CD38.

Introduction

NAD\textsuperscript{+} glycohydrolase (NADase; EC 3.2.2.5) is an enzyme that catalyzes the hydrolysis of NAD\textsuperscript{+} to nicotinamide and ADP-ribose. In eukaryotes, particularly mammals, NAD\textsuperscript{+} glycohydrolase activity is largely attributed to surface antigen CD38, a multifaceted protein detected primarily in the activation stages of lymphocytes and several other cell types, and is involved in heterotypic interactions with CD31 on endothelial cells, as well as acting as a receptor that modulates signal transmission. Finally, CD38 is an ectoenzyme with its catalytic site residing on the outer surface of the cell membrane (3). As such, it exhibits three catalytic activities, those of NAD\textsuperscript{+} glycohydrolase, ADP-ribozyme, and cyclic ADP-ribose hydrolase. Cyclic ADP-ribose, the product of CD38 ADP-ribose cyclase activity, has received considerable interest as an inositol 1,4,5-triphosphate (IP-3)-independent Ca\textsuperscript{2+}-mobilizer. Moreover, CD38 expression has gained importance as a prognostic marker in chronic lymphocytic leukemia (CLL) (4,5), HIV infection (6) and cancer (7,8).

A soluble form of CD38 with a molecular weight of 38 kDa also exists. The form was first identified in the supernatants of a mixed lymphocyte culture and CD38\textsuperscript{+} leukemic cell lines (9). The soluble form appears to correspond to the extracellular portion of CD38 and exists partially in dimeric form (10). The presence of elevated levels of a soluble form of CD38 has also been shown in serum samples from cancer patients (11). Soluble CD38 was reported to be decreased in rheumatoid arthritis and systemic lupus erythematosus patients (12), but increased in carriers of viral hepatitis G markers (13). A decrease in the serum levels of the dimeric form of soluble CD38 was observed in burned patients (14). By contrast, CD38 has been implicated to be involved in the prolongation of the lifespan of memory B-cell responses (15). Such data suggests a possible association between CD38 and various physiological and pathological events. The levels of soluble CD38 appear to change depending on the underlying disorder. In view of such variable data, the present study attempted to purify soluble CD38, as the availability of purified protein would enable the identification of its molecular and cellular interactions and eventually the role(s) that CD38 may have in various pathogenic networks.

Materials and methods

Materials. [Carbonyl-\textsuperscript{14}C]NAD\textsuperscript{+}, with a specific activity of 53 mCi/mmol, was purchased from Amersham Life Sciences (Piscataway, NJ, USA). Sephadex G-100, Affi-Gel blue (Cibacron Blue F3GA), monoclonal anti-human CD38 (from a Pig's brain), Amphotelin carrier ampholytes and all chemicals of analytical grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). The AG1-X4 anion exchange resin was a product of Bio-Rad Laboratories (Hercules, CA, USA).

Serum samples were obtained from young and apparently healthy individuals.

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Steps. All procedures were performed at 4°C. The serum samples were fractionated using successive steps of ammonium sulfate precipitation, affinity chromatography (Affi-Gel
Ammonium sulfate fractionation. Well-powdered solid ammonium sulfate was added slowly to the serum samples and was continuously stirred until saturation levels of 35 and 65% were attained. Once each level of saturation had been reached, the stirring was continued for a further 30 min. Thereafter, the samples were centrifuged for 20 min at 10,000 x g and the collected precipitated proteins were dissolved in and dialyzed exhaustively against 10 mM potassium phosphate (pH 7.4). The supernatant obtained following the centrifugation of the precipitated proteins at 65% saturation was also dialyzed against the same buffer.

Affinity (Cibacron Blue F3GA) chromatography. Affinity chromatography was performed with an Affi-Gel blue column as follows (16). The column (1x50 cm) was equilibrated with buffer A [10 mM potassium phosphate, pH 7.4, containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulphonate (CHAPS)]. Subsequent to adding the sample, the column was washed with buffer A and enzymatic activity was eluted with the same buffer containing 1.5 M NaCl.

Gel filtration chromatography. Fractionation was performed at 4°C on a Sephadex G-100 Superfine gel. The column (1x50 cm) was equilibrated with 50 mM Tris-HCl, pH 7.4 and 100 mM KCl and calibrated using bovine serum albumin (M, 66 kDa), ovalbumin (M, 45 kDa) and carbonic anhydrase (M, 29 kDa) as molecular weight markers. The column was eluted with the same buffer at a flow rate of 0.05 ml/min. The amount of protein in the 1-ml column fractions was monitored by absorption measurements at 280 nm.

Isoelectric focusing. Following the gel filtration chromatography, the fractions with molecular weights of 35-40 kDa and the NAD⁺ glycohydrolase activity were collected, dialyzed against 10 mM Tris-HCl (pH 7.4) and subjected to isoelectric focusing with an LKB 8100-1 column, according to the manufacturer’s instructions. Briefly, half of the dialyzed protein sample was mixed with 1.8 ml carrier ampholytes (pH range, 3.5-10.0) in a total volume of 60 ml. This solution also contained 28 g sucrose (dense solution). The other half of the protein sample was mixed with 0.6 ml carrier ampholytes (pH range, 3.5-10.0), again in a total volume of 60 ml (light solution). The two solutions were mixed in a series of reaction tubes so that a density gradient was formed and, thereafter, layered in a stepwise manner in the column over the anode solution (0.2 ml phosphoric acid, 12 g sucrose and 14 ml dH₂O). Finally, the cathode solution (0.2 ml ethylenediamine and 10 ml dH₂O) was added to the top of the gradient. The isoelectric focusing was performed for 24 h at 4°C and the operational power level was maintained at <W. Upon completion, the column content was harvested in 1-ml fractions and their A₃₈₀, pH-values and enzymatic activities were determined.

Gel filtration chromatography. Following isoelectric focusing, the fractions with NAD⁺ glycohydrolase activity were once again subjected to Sephadex G-100 chromatography, as described previously, in order to remove the ampholytes.

NAD⁺ glycohydrolase activity assay. NAD⁺ glycohydrolase activity was determined by separating [carbonyl-¹⁴C]nicotinamide from [carbonyl-¹⁴C]NAD⁺ with a BioRad AG-1X4 anion exchange resin (17). The reaction mixture (20 µl), containing 12 µl serum fractions, 7 µl mix (10 mM NaCl, 500 µM ZnCl₂, 50 µM CaCl₂ and 20 mM Tris-HCl, pH 9.0) and 10 µM [carbonyl-¹⁴C]NAD⁺, was incubated for 30 min at 37°C. The samples were then added to the BioRad AG-1X4 column and the [carbonyl-¹⁴C]nicotinamide that was released due to NAD⁺ glycohydrolase action was eluted with dH₂O. The [carbonyl-¹⁴C]NAD⁺ that was retained on the column was subsequently eluted with 0.5 M NaCl. The radioactivity that was eluted with dH₂O and 0.5 M NaCl was determined in Bray’s solution using a liquid scintillation counter (Packard Tri-Carb 1000TR, Meriden, CT, USA). The specific activity of the NAD⁺ glycohydrolase activity was defined as the nmol of [carbonyl-¹⁴C]nicotinamide released under the aforementioned reaction conditions per mg of protein.

Results and Discussion

Purification of NAD⁺ glycohydrolase

Ammonium sulfate precipitation. Serum (60 ml) was subjected to fractionation with ammonium sulfate at concentrations that were increased in a stepwise manner, first to 35 and then to 65% saturation. Following each saturation step, the precipitated proteins were collected by centrifugation, as well as the supernatant fraction obtained following centrifugation of the proteins precipitated at 65% ammonium sulfate saturation, were dialyzed against 10 mM potassium phosphate, pH 7.4, and then assayed for NAD⁺ glycohydrolase activity. Bars represent the total activity present in each fraction. In this pilot fractionation performed with 20 ml serum, the yield was considerably higher than that attained when larger serum volumes were processed (see Table I for comparison).
and assayed for NAD$^+$ glycohydrolase activity (Fig. 1). The supernatant fraction obtained following the centrifugation of the proteins precipitated at 65% ammonium sulfate saturation was subjected to dialysis and a subsequent activity test. NAD$^+$ glycohydrolase activity was observed almost exclusively in the 65% supernatant. This step resulted in the removal of a significant portion of the serum proteins and in an ~20-fold enrichment of the enzymatic activity, with a yield of 32% (Table I).

**Affi-Gel blue affinity chromatography.** The 65% supernatant fraction was then injected into the Affi-Gel blue column from which proteins were successively eluted with buffer A and buffer A plus 1.5 M NaCl. A stepwise elution approach at lower NaCl concentrations failed to isolate the activity in a well-defined concentration range. Instead, the activity was observed to be spread over all the attempted concentrations. Moreover, the 1.5 M NaCl-eluate appeared to contain the bulk of the protein that was applied to the column, aside from the NAD$^+$ glycohydrolase activity. Thus, this step did not result in a meaningful enrichment of the enzymatic activity (Fig. 2).

**Sephadex G-100 column chromatography.** Following Affi-Gel blue affinity chromatography, the fractions with NAD$^+$ glycohydrolase activity were pooled and a 5 ml aliquot was applied to the Sephadex G-100 column. Fractions of 1 ml were collected and their NAD$^+$ glycohydrolase activities were determined. The major peak (35-40 kDa) revealed a relatively high specific activity and appeared to be the most suitable for the further purification steps (Fig. 3).

**Isoelectric focusing.** The fractions from the Sephadex G-100 column chromatography that corresponded to the minor activity peak within the molecular weight range of 35-40 kDa were pooled and a 5 ml aliquot was applied to the Sephadex G-100 column. Fractions of 1 ml were collected and applied once more to the Sephadex G-100 column in order to remove the ampholytes.

**Assessment of purification.** A SDS-PAGE analysis of the active fraction obtained at the end of the purification proce-

| Fraction                | Total protein (mg) | Total activity (nmol) | Specific activity (nmol/mg) | Purification (fold) | Yield (%) |
|-------------------------|--------------------|-----------------------|-----------------------------|---------------------|-----------|
| Serum                   | 4920               | 285                   | 0.058                       | 1                   | 100       |
| Ammonium sulfate        | 80                 | 92                    | 1.15                        | 19.8                | 32.3      |
| Affi-Gel Blue           | 17                 | 21                    | 1.24                        | 21.4                | 7.4       |
| Sephadex G-100 (1)      | 0.94               | 3.3                   | 3.5                         | 60.3                | 1.15      |
| Isoelectric focusing    | 0.49               | 3.5                   | 7.1                         | 122.4               | 1.23      |
| Sephadex G-100 (2)      | 0.1                | 2.8                   | 28                          | 483                 | 0.97      |

Table I. Purification of NAD$^+$ glycohydrolase from human serum.
Albumin for Cibacron Blue F3GA has resulted in an expansion of its affinity for Cibacron Blue F3GA. However, the present study demonstrated that Affi-Gel blue glycohydrolase from calf spleens and thyroid glands has a marked affinity for Cibacron Blue F3GA. The purification of proteins with dinucleotide fold has previously been used successfully in the development of chromatographic methods for albumin depletion in the preparation of low abundance proteins for proteomic analysis. In the present study, a stepwise development of Affi-Gel blue affinity chromatography, with the aim of differentially separating NAD^+ glycohydrolase from serum albumin, was not successful. Moreover, the co-elution of the major, high molecular weight (possibly dimeric) form of this protein together with serum albumin in gel filtration chromatography dissuaded us from concentrating on its purification. The purification of this dimeric form was not attempted due to the low yield of the present procedure.

We suggest that the soluble monomer of CD38 was purified from the human serum in the present study since the molecular weight of the purified protein matched that reported for soluble monomeric CD38. It is notable that the second gel filtration step performed on the Sephadex G-100 column with the aim of depleting the ampholytes, actually led to a final, clear enrichment of this monomeric form of soluble NAD^+ glycohydrolase. Following successive purification steps, the fractions corresponding to the minor peak of NAD^+ glycohydrolase activity within the molecular weight range of 35–40 kDa were combined, dialyzed against 10 mM Tris-HCl, pH 7.4 and mixed with ampholytes, then subjected to isoelectric focusing. The pH, A280 and NAD^+ glycohydrolase activity of the fractions were determined at the end of the process. The purification of this dimeric form was not attempted due to the low yield of the present procedure.

However, a change in the purification protocol, with the isoelectric focusing step directly following ammonium sulfate fractionation, may enable the efficient separation of the two forms of NAD^+ glycohydrolase from serum albumin. The fairly low isoelectric point (pl) of serum albumin (4.4) provides an opportunity which may be exploited.

In the present attempt to purify NAD^+ glycohydrolase (soluble CD38) from human serum, the isolation of its monomeric form in an apparently homogeneous state was achieved. However, the abundance of serum albumin and its co-elution in Affi-Gel blue affinity chromatography, as well as gel filtration with Sephadex G-100, prevented the similar purification of the putative dimer of NAD^+ glycohydrolase. This result demonstrates the difficulties that are likely to be encountered in the purification of low abundance proteins from serum when a differential depletion of serum albumin is not possible.

The information gained in this first attempt provides valuable information for designing future approaches that should take into account the similar chromatographic behavior of the NAD^+ glycohydrolase dimer and serum albumin. Therefore, we propose that purification protocols that initially deplete serum albumin, particularly by making use of its low pl-value, are likely to have an improved prospect for the purification of the monomeric and dimeric forms of NAD^+ glycohydrolase.
At present, research is in progress to aid in the implementation of this strategy.

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References

1. Deaglio S, Aydin S, Vaisitti T, Bergui L and Malavasi F: CD38 at the junction between prognostic marker and therapeutic target. Trends Mol Med 14: 210-218, 2008.
2. Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, Vaisitti T and Aydin A: Evolution and function of ADP-ribosyl cyclase/CD38 gene family in physiology and pathology. Physiol Rev 88: 841-886, 2008.
3. Coşkun O and Nurten R: Purification of NAD⁺ glycohydrolase enzyme from erythrocyte membrane. Türkiye Klinikleri J Cardiovasc Sci 22: 318-323, 2010 (In Turkish).
4. Damle RN, Wasil T, Faiss F, Ghiozzo F, Valetto A, Allen SL, Buchbinder A, Budman D, Dittmar K, Kolitz J, Lichtman SM, Schulman P, Vinciguerra VP, Rei KR, Ferrarini M and Chiorazzi N: Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 94: 1840-1847, 1999.
5. Matrai Z: CD38 as a prognostic marker in CLL. Hematology 10: 39-46, 2005.
6. Liu Z, Cumberland WG, Hultin LE, Prince HE, Detels R and Giorgi JV: Elevated CD38 expression on CD8⁺ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4⁺ cell count, soluble immune activation markers, or combination of HLA-DR and CD38 expression. J Acquir Immune Defic Syndr Hum Retrovirol 16: 83-92, 1997.
7. Albeniz I, Demir O, Nurten R and Bermek E: NAD glycohydrolase activities and ADP-ribose uptake in erythrocytes from normal subjects and cancer patients. Biosci Rep 24: 41-53, 2004.
8. Albeniz I, Demir O, Türkör-Sener L, Yaşçintepe L, Nurten R and Bermek E: Erythrocyte CD38 as a prognostic marker in cancer. Hematology 12: 409-414, 2007.
9. Funaro A, Horenstein AL, Calosso L, Morra M, Tarocco RP, Franco L, De Flora A and Malavasi F: Identification and characterization of an active soluble form of human CD38 in normal and pathological fluids. Int Immunol 8: 1643-1650, 1996.
10. Mallone R, Ferrua S, Morra M, Zocchi E, Mehta K, Notarangelo LD and Malavasi F: Characterization of a CD38-like 78-kilodalton soluble protein released from B cell lines derived from patients with X-linked agammaglobulinemia. J Clin Invest 101: 2821-2830, 1998.
11. Korkut C, Yaşçintepe L, Kiremit-Korkut N, Uzun-Altnöz S, İşsever S, Gümüşel F, Tiryaki D and Bermek E: Serum proteins with NAD⁺ glycohydrolase activity and anti-CD38 reactivity - elevated levels in serum of tumour patients. Cancer Lett 126: 105-109, 1998.
12. Kong KO, Leung BP, Chng HH, Thong BY, Koh ET, Leong KP, Badsha H, Lian TY, Khoo KM and Howe HS: Usefulness of serum soluble CD38 and CD157 levels in differentiating SLE, RA and healthy adults and their relationship with disease activity. Ann Acad Med Singapore 32 (5 Suppl): SI6-SI7, 2003.
13. Kravchenko GA, Novikov DV, Ptitsyna IuS and Novikov VV: Serum levels of soluble forms of membranous antigens of immune system cells in carriers of virus hepatitis G markers. Vopr Virusol 50: 19-22, 2005 (In Russian).
14. Lebedev MI, Egorova NV, Shokima MM, Vilkov SA, Baryshnikov AJ and Novikov VV: Serum levels of different forms of soluble CD38 antigen in burn patients. Burns 30: 552-556, 2004.
15. Liu QX, Hart DN, MacPherson GG, Good MF and Wykes MN: Soluble CD38 significantly prolongs the lifespan of memory B-cell responses. Immunology 125: 14-20, 2008.
16. Muller-Stefferl H, Schennett-Gusse I, Tarnus C and Schuber F: Calf spleen NAD⁺ glycohydrolase: solubilization, purification, and properties of the intact form of the enzyme. Arch Biochem Biophys 304: 154-162, 1993.
17. Kim H, Jacobson EL and Jacobson MK: Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases. Science 261, 1330-1333, 1993.
18. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
19. Morrissey JH: Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117: 307-310, 1981.
20. Thompson ST and Stellwagen E: Binding of Cibacron blue F3GA to proteins containing the dimedone fold. Proc Natl Acad Sci USA 73: 361-365, 1976.
21. De Wolf MJ, Van Dessel GA, Lagrou AR, Hilderson HJ and Dierick WS: Topology, purification and characterization of thyroidal NAD⁺ glycohydrolase. Biochem J 226: 415-427, 1985.
22. Ahmed N, Barker G, Oliva K, Garlin D, Talmadge K, Georgiou H, Quinn M and Rice G: An approach to remove albumin for the proteomic analysis of low abundance biomarkers in human serum. Proteomics 3: 1980-1987, 2003.
23. Colantonio DA, Dunkinson C, Bovenkamp DE and Van Eyke JE: Effective removal of albumin from serum. Proteomics 5: 3831-3835, 2005.
24. Ma ZY, Guan YP and Liu HZ: Affinity adsorption of albumin on Cibacron blue F3GA-coupled non-porous micrometer-sized magnetic polymer microspheres. React Funct Polym 66: 618-624, 2006.
25. Altıntaş EB and Denizli A: Efficient removal of albumin from human serum by monosize dye-affinity beads. J Chromatogr B Analyt Technol Biomed Life Sci 832: 216-223, 2006.
26. Sahab ZJ, Iczkowski KA and Sang QX: Anion exchange fractionation of serum proteins versus albumin elimination. Anal Biochem 368: 24-32, 2007.
27. Liu R, Guan YP and Liu HZ: Usefulness of serum soluble CD38 and CD157 levels in differentiating SLE, RA and healthy adults and their relationship with disease activity. Ann Acad Med Singapore 32 (5 Suppl): SI6-SI7, 2003.