Nitrate Reductase Nanoparticles: Synthesis and Characterization

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Article History:
Received on: 10 Jan 2020
Revised on: 07 Mar 2020
Accepted on: 13 Mar 2020

Keywords:
Homodimer,
FAD,
Mo-MPT,
Nanoaggregate,
Spectroscopy,
Impedance,
Shelf life

ABSTRACT
Nanoparticles of enzyme Nitrate reductase (NaR) a soluble homodimer enzyme of ~100 kDa polypeptide with cofactors – FAD, heme-molybdopterin (Mo-MPT) and electron donor NAD(P)H, catalyses the reduction of nitrate to nitrite has been synthesised. Nanoparticles of Nitrate reductase enzyme have been prepared by chemical desolvation method including glutaraldehyde cross-linking to form the nanoaggregate. Characterisation of NaR nanoparticles has been made by Transmission Electron Microscopy (TEM), UV-Visible Spectroscopy and by electrochemical Impedance Spectroscopic Study (EIS). TEM study revealed the size of globular aggregated was in the range of 20–30 nm. UV Visible spectroscopic studies depicted that the absorption of NaR NPS is much higher at 560 nm than that of the free enzyme, which showed maximum absorption at 540 nm. NaR NPs aggregates formed were more active, highly stable, have a higher shelf life and can be reused repeatedly. Enzyme nanoparticles with 10-100 nm dimensions and exhibit unique physical, chemical and catalytic properties due to increased surface area. Nitrate reductase nanoparticles can be used as a biochemical tool to increase protein production and grain yield by promoting amino acids production in plants. The synthesised NaR NPs are used in the fabrication of enzyme-based nanosensor in the detection of nitrates in drinking water and serum samples.

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ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v11i3.2740

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et al., 1994). Sulfur atom in the coordination sphere protects it from direct attack (Stolz and Basu, 2002). Nitrate binding induces conformational rearrangement in-ring thus reduction of nitrate into nitrite occurs (Su et al., 1996). Nitrate reductase found in cytosol and reduction of nitrate takes place chiefly in green leaves and roots. NaR is labile, and the reduction reaction is affected by oxygen. First nitrate assimilation is initiated by uptake of nitrate from the root system then reduced to nitrite by nitrate reductase and further nitrite is reduced to ammonia by nitrite reductase (Crawford and Arst, 1993). It is a substrate inducible (Bevers and Hageman, 1969) type of enzyme. Its synthesis is induced by NO$_3^-$ in tissues of higher plants. It is synthesised in the cells when its substrate, i.e., NO$_3^-$ is present and disintegrates when NO$_3^-$ is absent. Its activity is affected by nitrate conc., ammonium, carbohydrate level, other environmental factors and its reducing equivalents temperature and light (Kaiser and Brendle-Behnisch, 1991). Light stimulates protein phosphatase which dephosphorylates some serine residues and leads to activation of nitrate reductase enzyme.

Under darkness and in the presence of Mg$^{2+}$, a protein kinase (Huber et al., 1992) is stimulated which phosphorylate the same serine residues of the nitrate reductase and causes inactivation of nitrate reductase enzyme. Mainly regulation of nitrate reductase activity occurs through reversible phosphorylation-dephosphorylation process (Mackintosh, 1992) than synthesis and breakdown of this enzyme which takes hours. It is regulated both at transcriptional and translational level (Nussaume et al., 1995) induced by light, nitrate and a negative feedback mechanism. Nitrate reductase is subject to post-translational modulation (Scheible et al., 1997) involving reversible phosphorylation on 543-serine residue (Bachmann et al., 1996) and binding of Mg$^{2+}$ or another divalent cation and an inhibitor protein which have a regulatory effect on nitrate reductase (Kaiser and Huber, 1994). Nitrate reductase improves the plants’ tolerance in anoxic conditions (Botrel et al., 1997). Increased activity of nitrate reductase is related to dramatically increased nitrite release in roots showing dephosphorylation of the nitrate reductase in anoxic conditions. In anoxia-tolerant, lower vertebrates have an intrinsic ability to increase intracellular nitrite concentration in tissues such as the heart, red and white skeletal muscles which develop different nitrite levels when exposed to profound hypoxia (Dolomato et al., 2011). Liver, muscle and heart tissue possess nitrate reductase activity that supplies nitrite to the masses during severe hypoxia. NaR activity decreases during water stress. Nitrate Reductase activation declines in drought leave compared with well-watered controls. The values of NaR activity are considered low in the presence of Mg$^{2+}$ inhibitor (Venkatesan, 2005).

Nanotechnology is a fascinating branch of applied science which is widely used in almost all fields with improved properties of particles at nano scale (Ferrario-Méry et al., 1998). Synthesis of enzyme nanoparticles at larger scale enhances the use of enzymes by removing the only limitation of reusability associated with their use in any reaction. Enzyme nanoparticles are prepared by converting enzyme into nano-aggregates by cross-linking strategy (Unkles, 2001). For this enzyme, the molecule is surrounded by the organic/inorganic network that results in a stable catalyst with no mass transfer limitation (Kim and Grate, 2003). Immobilisation of enzyme nanoparticles resulted in a loss of enzyme activity and denaturation due to change in various physicochemical conditions. The cross-linking method of the enzyme via glutaraldehyde performed reaction with amine residue was initially developed in the 1960s. This method stabilises enzyme by converting it in the form of nanoaggregate and their successful use in biocatalyst industry. There are some drawbacks associated with this cross-linking technology such as low enzyme activity retention, little stability and inferior reproducitively (Ruan et al., 1998).

Further, the results could be better obtained by precipitating and cross-linking the enzyme on physical support that helps in the improvement of the technique. Addition of different types of salts, organic solvents, and acid helps in enzyme aggregation and precipitation. These Enzyme nanoparticles are very tiny particles and have different properties than their larger particle counterparts thus possess unique structural and catalytic properties which enhance the rate of reaction. This nanoaggregate are supramolecular structure held by non-covalent interactions with the much higher and increased catalytic activity of then the native enzyme (Harin and Schreier, 1999). Recently, several enzyme nanoparticles have been developed, but till now no nitrate reductase nanoparticles has been reported. Hence in the present study, NaR NPs were synthesised by the glutaraldehyde method and characterised by using Transmission electron microscopy (TEM), UV-visible spectroscopy and Electrochemical Impedance Spectroscopic (EIS).
Graph 1: Graphical Abstract

Figure 1: TEM image of prepared NaR Nanoparticles
Figure 2: UV visible spectra of free NaR enzyme and NaR Nanoparticles

Figure 3: Nyquist plot of Electrochemical Impedence Spectroscopic of free NaR and NaR Nanoparticles coated Au electrode.
MATERIALS AND METHODS

Chemicals
Enzyme Nitrate Reductase from Aspergillus from Sigma–Aldrich, N-ethyl-N-(3-dimethyl amine
propyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), Potassium nitrate, potassium dihydrogen
phosphate and dipotassium hydrogen phosphate, N-(1-naphthyl)-ethylenediamine hydrochloride
(NED), NADH were purchased from Himedia. All other chemicals were of analytical reagent (AG)
grade. Double Distilled water (DW) is used in all experiments. Instruments –Cold centrifuge, stirrer,
UV-visible spectroscopy was carried out at Department of M.D. University, Rohtak, Transmission
electron microscopy was carried out at AIIMS New Delhi.

Enzyme assay
The enzyme activity was measured as the rate of nitrite production. One unit of NaR is deϐined as the
amount of enzyme required to generate one μmol of nitrite/min. Assay of NaR was done as described
by (Campbell and Smarrelli, 1978) with modiϐication.

Synthesis of NaRNPs Nanoparticles
The reaction mixture contained 1.3 mL of 0.1 PB (pH 7.5), 0.2 mL of 0.2 M KNO3 and 0.2 mL of 0.4 mM
NADH. After incubation at 30º C for 2 minutes, 0.3 ml of the enzyme was added, the tubes were shaken
and incubated for 30 minutes. 1 mL of 0.02% N-(1-naphthyl)-ethylenediamine hydrochloride (NED)
was added to the test tubes to develop the coloured complex, and As450 nm was read against the blank. In
blank, the crude enzyme was replaced by the buffer solution. Synthesis of NaR nanoparticles
Nitrate reductase NPs were prepared by desolva-
tion method as described by (Kundu et al., 2013).
3 mL of absolute ethanol was added to 1.5 mL of
enzyme solution (1mg/mL) at a dropping rate of
0.1 to 0.2 mL/minute under constant stirring at 500
rpm resulting into small particles of nano size. After
that, 1.8 mL of 2.5% glutaraldehyde was added to the
nanoparticles suspension under continuous stirring
at 4ºC for 24h. The amino group were introduced by
the addition of 0.12 gm cysteamine dihydrochloride
with constant stirring for 5-6 hour, forming enzyme
nanoparticles by cross-linking. NaR nanoparticles
(NPs) were dispersed in 0.1M phosphate buffer and
sonicated stored at 4ºC. The amino group of cyste-
amine reacts with –CHO group of glutaraldehyde
cross-linked to form nanoparticles.

Characterisation of NaR NPs

The aggregated nitrate reductase nanoparticles
were studied by Transmission electron microscopy
(TEM) at AIIMS New Delhi. Nanoaggregates were
immobilised on a gold electrode by dispersing it. UV
visible spectroscopy of both free Nitrate Reductase
and Nitrate Reductase NPs were measured at dif-
ferent wavelengths in spectronic-20 at Department
of Environment Science, Maharishi Dayanand Uni-
versity, Rohtak. EIS study of enzyme immobilised
electrode was done at National Physical Laboratory
(NPL) New Delhi.

RESULTS AND DISCUSSION

Synthesis and Characterisation of NaR NPs
Transmission Electron Microscopic (TEM) stud-
ies
The TEM images and selected area electron diffrac-
tion patterns of NaR nanoparticles reveal the spher-
ical particles with an average size of 20 – 30 nm,
which is in close agreement with the crystalline
structure complexity. It indicates that the synthe-
sised NaR nanoparticles are not a single crystal,
instead are the aggregates of several single crystals. The TEM images showed NaR NPs aggregates with
an average diameter of about 20-30 nm (Figure 1).

UV Visible spectroscopic studies
UV visible spectra of free nitrate reductase and
nitrate reductase nanoparticles were studied sepa-
rateley. Free nitrate reductase showed a character-
istic absorption peak at 540 nm, and nitrate reduc-
tase nanoparticles showed an absorption peak of
560 nm (Figure 2). The peak is shifted towards
higher wavelength, which is indicative of struc-
tural conformation of the enzyme after nanoparti-
cles synthesis. Similar reports of shifting in absorp-
tion maxima peaks were studied previously by var-
ious researchers for the synthesis of other enzyme
nanoparticles.

Electrochemical Impedance Spectroscopic (EIS)
Studies
EIS study conϐirms the enzyme nanoparticles forma-
tion and their immobilisation on the electrode. At
higher frequencies, the diameter of semicircle sug-
gests the charge transfer resistance (RCT). There
is the poor electrical conductivity of the enzyme
at a lower frequency due to hindrance to elec-
tron transfer. The Rct of enzyme nanoparticles
coated electrode was lower due to decreased resis-
tance and higher charge conductivity at higher fre-
quency(Figure 3).

Practical application of synthesised NaR NPs
Enzyme nanoparticles with 10-100 nm dimensions
and exhibit unique physical, chemical and catalytic
properties due to increased surface area. Enzyme nanoparticles exhibit good shelf life and stability so could be used again and again without leaching. It is easy to immobilise these aggregates on specific support with a high proportion of active enzyme activity. Their practical utility lies in improving yield and water stress tolerance of plants, to study nitrate transporter and in making improved nanosensor.

CONCLUSIONS

The synthesised NaR NPS enzyme nanoparticles exhibit increased surface area and have better chemical and catalytic action. The thiol group on their surface helps in attachment over the specific surface, thus no denaturation and loss of bioactivity in media. Nitrate reductase nanoparticles can be used as a biochemical tool to increase protein production and grain yield by promoting amino acids production in plants. During nitrogen assimilation, NaR nanoparticles can be used to study the involvement of nitrate transporters in nitrate sensing in plants and fungi. In plants, the importance of regulating nitrate reductase activity by nanoparticles is to limit the amount of nitric oxide being produced during the drought, which has many damaging effects. A rapid and specific method based on nitrate reductase biosensor can be used for the determination of nitrate in meat and fishery products. The synthesised NaR NPs are used in the fabrication of enzyme-based nanosensor in the detection of nitrates in drinking water and serum samples. The nanoparticles based improved nanosensor achieves superiority in terms of linear range, sensitivity and response time. Enzyme nanoaggregates provide enhanced shelf life and operational stability to the nanosensor with no leaching action. Thus enzyme nanoaggregate with higher shelf life and stability plays an essential role as an industrial and biomedical catalyst.

Funding Support
None.

Conflict of Interest
None.

REFERENCES

Bachmann, M., Shiraishi, N., Campbell, W. H., Yoo, B. C., Harmon, A. C., Huber, S. C. 1996. Identification of Ser-543 as the Major Regulatory Phosphorylation Site in Spinach Leaf Nitrate Reductase. The Plant Cell, 8(3):505–505.

Beevers, L., Hageman, R. H. 1969. Nitrate Reduction in Higher Plants. Annual Review of Plant Physiology, 20(1):495–522.

Botrel, Magne, C., Kaiser, W. M. 1997. Nitrate reduction, nitrite reduction and ammonium assimilation in barley roots in response to anoxia.

Campbell, W. H., Smarrelli, J. 1978. Purification and Kinetics of Higher Plant NADH:Nitrate Reductase. Plant Physiology, 61(4):611–616.

Crawford, N. M., Arst, H. N. 1993. The Molecular Genetics of Nitrate Assimilation in Fungi and Plants. Annual Review of Genetics, 27(1):115–146.

Dolomtov, S. I., Shekk, P. V., Zukow, W., Kryukova, M. I. 2011. Features of nitrogen metabolism in fishes. Reviews in Fish Biology and Fisheries, 21(4):733–737.

Evans, H. J., Nason, A. 1953. Pyridine Nucleotide: Nitrate Reductase from Extracts of Higher Plants. Plant Physiology, 28(2):233–254.

Ferrario-Méry, S., Valadier, M.-H., Foyer, C. H. 1998. Overexpression of Nitrate Reductase in Tobacco Delays Drought-Induced Decreases in Nitrate Reductase Activity and mRNA. Plant Physiology, 117(1):293–302.

Haring, D., Schreier 1999. Cross-linked enzyme crystal. Curr Opin Chem Biol, 3(1):35–38.

Hewitt, E. J. J., Nicholas 1964. Enzymes of inorganic nitrogen metabolism. Modern Methods of Plant Analysis, 7:67–172.

Huber, J. L., Huber, S. C., Campbell, W. H., Redinbaugh, M. G. 1992. Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. Archives of Biochemistry and Biophysics, 296(1):58–65.

Kaiser, W. M., Brendle-Behnisch, E. 1991. Rapid Modulation of Spinach Leaf Nitrate Reductase Activity by Photosynthesis. Plant Physiology, 96(2):363–367.

Kaiser, W. M., Huber, S. C. 1994. Posttranslational Regulation of Nitrate Reductase in Higher Plants. Plant Physiology, 106(3):817–821.

Kim, J., Grate, J. W. 2003. Single-Enzyme Nanoparticles Armored by a Nanometer-Scale Organic/Inorganic Network. Nano Letters, 3(9):1219–1222.

Kundu, N., Yadav, S., Pundir, C. S. 2013. Preparation and Characterization of Glucose Oxidase Nanoparticles and Their Application in Dissolved Oxygen Metric Determination of Serum Glucose. Journal of Nanoscience and Nanotechnology, 13(3):1710–1716.

Lu, G., Campbell, W. H., Schneider, G., Lindqvist, Y. 1994. Crystal structure of the FAD-containing fragment of corn nitrate reductase at 2.5Å reso-
olution: relationship to other flavoprotein reductases. *Structure*, 2(9):809–821.

Mackintosh, C. 1992. Regulation of spinach-leaf nitrate reductase by reversible phosphorylation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1137(1):121–126.

Nussaume, L., Vincentz, M., Meyer, C., Boutin, J. P., Caboche, M. 1995. Post-transcriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. *The Plant Cell*, 7(5):611–621.

Ratnam, K., Shiraishi, N., Campbell, W. H., Hille, R. 1997. Spectroscopic and Kinetic Characterization of the Recombinant Cytochrome c Reductase Fragment of Nitrate Reductase. *Journal of Biological Chemistry*, 272(4):2122–2128.

Ruan, J., Wu, X., Ye, Y., Härdter, R. 1998. Effect of potassium, magnesium and sulphur applied in different forms of fertilisers on free amino acid content in leaves of tea (Camellia sinensis L.). *Journal of the Science of Food and Agriculture*, 76(3):389–396.

Scheible, W.-R., González-Fontes, A., Morcuende, R., Lauerer, M., Geiger, M., Glaab, J., Gojon, A., Schulze, E.-D., Stitt, M. 1997. Tobacco mutants with a decreased number of functional nia genes compensate by modifying the diurnal regulation of transcription, post-translational modification and turnover of nitrate reductase. *Planta*, 203(3):304–319.

Shiraishi, N., Campbell, W. H. 1996. Expression of nitrate reductase FAD-containing fragments in Pichia. *Flavins and Flavoproteins*, pages 931–934.

Skipper, L., Campbell, W. H., Mertens, J. A., Lowe, D. J. 2001. Pre-steady-state Kinetic Analysis of Recombinant Arabidopsis NADH:Nitrate Reductase. *Journal of Biological Chemistry*, 276(29):26995–27002.

Stolz, J. F., Basu, P. 2002. Evolution of Nitrate Reductase: Molecular and Structural Variations on a Common Function. *ChemBioChem*, 3(2-3):198–206.

Su, W., Huber, S. C., Crawford, N. M. 1996. Identification in vitro of a Post-Translational Regulatory Site in the Hinge 1 Region of Arabidopsis Nitrate Reductase. *The Plant Cell*, 8(3):519–519.

Unkles, S. E. 2001. Apparent genetic redundancy facilitates ecological plasticity for nitrate transport. *The EMBO Journal*, 20(22):6246–6255.

Venkatesan, S. 2005. Impact of genotype and micronutrient applications on nitrate reductase activity of tea leaves. *Journal of the Science of Food and Agriculture*, 85(3):513–516.