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Identification and Extraction of Superoxide-Generating Protein Assemblies from *Helianthus tuberosus*, *Daucus sativus*, and *Solanum tuberosum*

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ABSTRACT

In this study, isoforms of NPC-Fe(III) complexes were extracted and purified from *Helianthus tuberosus* (Armenian Jerusalem artichoke), *Solanum tuberosum* (potato), and *Daucus sativus* (carrot). For the 1st time, the physicochemical properties of these complexes were determined, including their specific content, O_2^--producing activity, steady-state concentration of O_2^-, and fluorescence intensity. These complexes exhibited peak optical absorbance in both the visible and UV spectra. Hybrid complexes between the isolated NPC-Fe(III) complexes and NADPH oxidase (Nox) were generated, with subsequent observation of O_2^- production by these hybrid complexes. Under aerobic conditions, the complexes continually produced O_2^- by transferring electrons from NPC to Fe(III), which then reduced O_2 to O_2^-. Finally, O_2 stabilized the O_2^-. Overall, the findings suggest that these isoforms of NPC-Fe(III) complexes represent novel prooxidant components from the studied plants, with NPC acting as a bioelectric source for the generation of O_2^-.

Keywords: O2–-producing NPC-Fe(III) thermostable complex, *Solanum tuberosum*, *Helianthus tuberosus*, Isolation, *Daucus sativus*

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Introduction

Research has highlighted the antioxidant properties of *Daucus sativus*, which, in dry conditions, accumulates a variety of phenolic compounds and vitamins [1, 2]. Both *Solanum tuberosum* and *Helianthus tuberosus* are also known to exhibit significant antioxidant activity [3, 4]. However, the prooxidant potential of these root crops has not been studied until now [5]. Aerobic organisms, including these root vegetables, maintain a delicate balance between antioxidant and prooxidant mechanisms [6, 7]. Additionally, isoforms of O_2^--producing complexes, such as the NPC-Fe(III) complexes, have recently been isolated and purified from several plant systems for the first time [8-12].

This study aims to isolate, purify, and characterize the O_2^--producing NPC-Fe(III) complexes from *Helianthus tuberosus*, *Daucus sativus*, and *Solanum tuberosum* sourced from Armenia, focusing on their specific properties.

Materials and Methods

Extraction and purification of O_2^--generating NPC-Fe(III) complexes from Helianthus tuberosus, Daucus sativus, and Solanum tuberosum

To isolate and purify the isoforms of O_2^--producing NPC-Fe(III) complexes, 50-100 g of *Helianthus tuberosus*, *Daucus sativus*, and *Solanum tuberosum* were processed using a standard procedure [8]. Initially, the NPC-Fe(III) complexes were extracted at pH = 9.5 with ferri Hb (50 μM). The complexes were then precipitated at pH = 4.8, followed by solubilization in water at pH = 9.5. Purification involved ion-exchange chromatography on DE-52 cellulose and gel filtration on Sephadex G-100 or G-200 at pH = 9.5. To remove other proteins, the aqueous solutions were subjected to thermal treatment in boiling water for 10-12 minutes, and centrifugation was employed to clear any residues. After elution according to a standard protocol, the NPC-Fe(III) complexes were deionized, lyophilized, and stored at -10 °C under anaerobic conditions.

Electrophoretic analysis of these isoforms was performed on polyacrylamide gels (PAAG) with concentrations of 7% or 10%, depending on the isoelectric properties of the proteins.

Quantification of NPC in the isoforms of NPC-Fe(III) complexes from Helianthus tuberosus, Daucus sativus, and Solanum tuberosum

The amount of NPC, a NADPH-associated component, in the NPC-Fe(III) isoforms from *Helianthus tuberosus*, *Daucus sativus*, and *Solanum tuberosum* was determined by measuring the fluorescence intensity (F) at wavelengths of 450-460 nm upon excitation at 370 nanometers.

Separation of NPC from aqueous solutions of NPC-Fe(III) complexes from Helianthus tuberosus, Daucus sativus, and Solanum tuberosum

To isolate NPC from the aqueous solutions of NPC-Fe(III) complexes, the solutions were treated with 10^{-4} M EDTA for 10 minutes at room temperature. Following this, ion exchange chromatography on DE-52 cellulose at pH = 9.5 was performed. Under these conditions, NPC was eluted, while Fe(III) remained bound to the cellulose due to the EDTA interaction. Fe(III) content was assessed using the orthophenanthroline method. The isolated NPC exhibited solely reductive properties.

Measurement of O_2^--producing activity units in NPC-Fe(III) complexes

To determine the activity units of the O_2^--producing NPC-Fe(III) complexes from *Helianthus tuberosus*, *Daucus sativus*, and *Solanum tuberosum*, the increase in adrenochrome absorption at 500 nm was monitored until it reached 50%. These values were reported as specific activity units (U/mg) for each complex.

Determination of stationary O_2^- concentrations in NPC-Fe(III) complexes from helianthus tuberosus, Daucus sativus, and Solanum tuberosum

The stationary concentration of O_2^- produced by NPC-Fe(III) complexes was assessed through the optical absorbance of adrenochrome at 500 nm. The molar extinction coefficient (750 $M^{-1} \cdot cm^{-1}$) of the generated O_2^- was used to calculate its concentration. Absorbance values (A500/E) allowed for the determination of O_2^- levels, with a control sample prepared using only oxygen-induced oxidation of adrenaline [13-15].

Gas-Phase O_2^- production by NPC-Fe(III) complex isoforms from helianthus tuberosus, Daucus sativus, and Solanum tuberosum

The generation of gas-phase O_2^- was tested by exposing aqueous solutions of NPC-Fe(III) complexes to oxygen at 0.1 atmospheres for varying times. The produced O_2^- was transferred via glass or silicone tubing (at least 1 meter in length) [10]. The concentration of O_2^- was determined using the adrenochrome assay described above.

The following laboratory equipment was used in these experiments: cellulose DE-52 (Whatman, UK), Sephadex G-100 and G-200 (Pharmacia, Sweden), adrenaline (Sigma, USA), Cary 60 spectrophotometer, Cary Eclipse spectrofluorimeter (USA), and Janetzki centrifuges K-70D and K-24 (Germany).

To ensure consistency and reliability, the isolation of NPC-Fe(III) complex isoforms was repeated six times to calculate the mean values and confirm experimental reproducibility.

Results and Discussion

During electrophoresis of the O_2^--producing NPC-Fe(III) isoforms on PAAG, aggregation was observed at the point where the complexes exited the PAAG tubes. This aggregation was caused by the interaction of the NPC-Fe(III) isoforms with the heterogeneous PAAG phase under the applied electrical field. Notably, no water-soluble proteins with acidic or basic properties, which could have been stained with amido black, were detected in the PAAG tubes. This provides an initial indication of the purity of the NPC-Fe(III) isoforms. The second indicator of their purity was the symmetrical elution pattern from G-100 or G-200 Sephadex. A third confirmation of purity was the stability of the A280/A420 ratio during subsequent purification stages of the NPC-Fe(III) complexes.
The high thermal stability of these isoforms from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus* is likely due to the transient temperature spikes reaching 280-300 °C, occurring in nanoseconds, which facilitate redox metabolic processes [15]. The optical absorption spectra of these isoforms, observed in opalescent aqueous solutions at pH = 9.5, are shown in **Figure 1**.

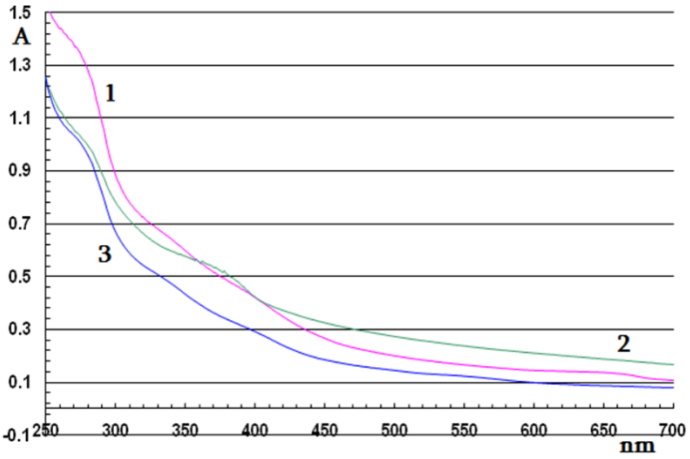

Figure 1. Optical absorption spectra of aqueous solutions containing isoforms of O_2^--producing NPC-Fe(III) complexes derived from *Helianthus tuberosus* (1), *Solanum tuberosum* (2), and *Daucus sativus* (3) at pH = 9.5.

In **Figure 1**, a distinct peak in absorbance is seen at 280 nm for the proteins. A faint absorbance is also detected at 420 nanometers, 480 nanometers, and 520 nanometers within the visible spectrum. **Figure 2** displays the spectrofluorimetric data for the O_2^--producing complexes from *Helianthus tuberosus* (1), *Solanum tuberosum* (2), and *Daucus sativus* (3) at pH = 9.5.

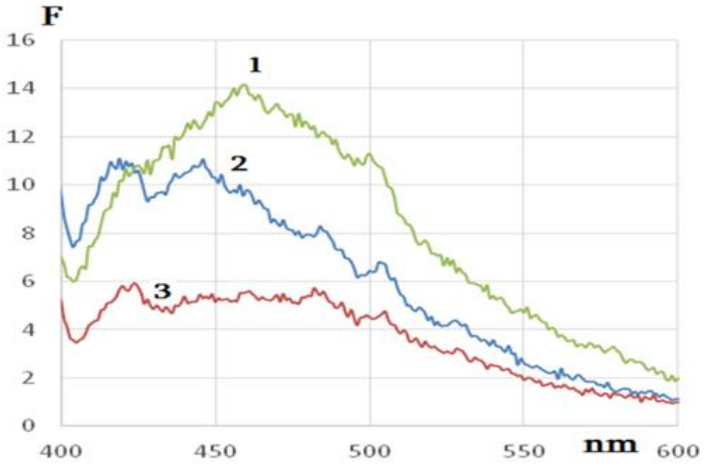

Figure 2. Spectrofluorimetric data of the isoforms of O_2^--producing NPC-Fe(III) complexes (mg/ml) from *Helianthus tuberosus* (1), *Solanum tuberosum* (2), and *Daucus sativus* (3) at 450-460 nm with excitation at 370 nm, pH = 9.5; The "F" represents the opalescence intensity in relative units.

The spectrofluorimetric profiles of NPC isoforms derived from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus* exhibited comparable results. Moreover, NPC acted as an electron donor, facilitating the reduction of potassium permanganate while inhibiting the oxidation process of adrenaline to adrenochrome. Selected physicochemical characteristics of NPC-Fe(III) isoforms from these root crops are shown in **Figures 3a-3d**.

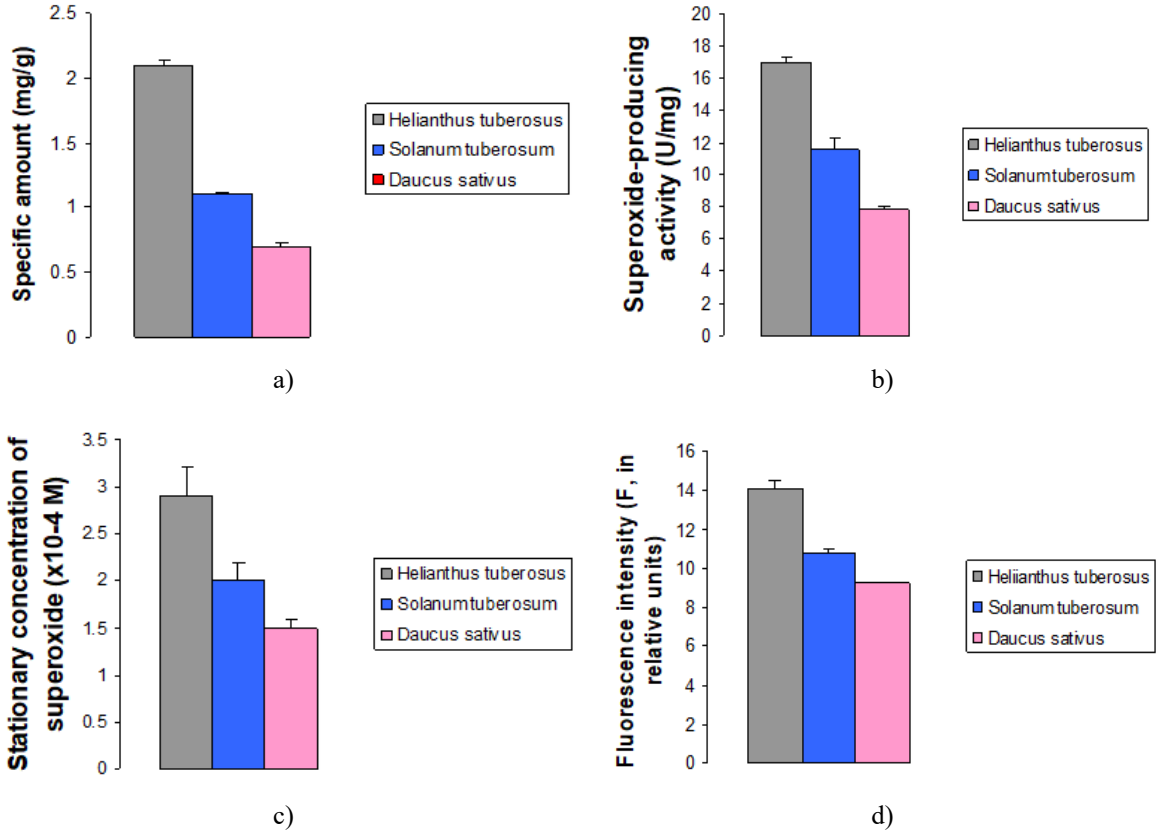

Figure 3. a) the specific contents of isoforms of NPC-Fe(III) from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus*; b) The superoxide-producing activities of the isoforms of NPC- Fe(III) from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus*; c) The stationary concentration of produced O_2^- by isoforms of NPC-Fe(III) from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus*; and d) The fluorescence intensity of the aqueous solutions of the isoforms of NPC-Fe(III) from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus*.

The NPC derived from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus* complexes can act as a substrate for Nox enzymes present in erythrocyte and leukocyte membranes. Therefore, Nox can utilize both free NADPH and NADPH bound to a protein such as NPC. Isolated NPCs exhibit reductive behavior, specifically reducing KMnO4. The hybrid complexes (hNPC-Nox) produce O_2^- continuously for 48-72 hours or longer under aerobic conditions at room temperature, similar to the O_2^--producing NLP-Nox associates, where NLP is an NADPH-containing lipoprotein found on the outer membrane layers of cells. Nox is localized on the biomembrane surface [16].

The O_2^--producing NPC-Fe(III) complexes from *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus*, including the hybrid hNPC-Nox, remain stable even after vacuum lyophilization and can be stored anaerobically at -10°C for two years without significant loss of activity.

The main conclusions of this research are: 1) *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus* maintain a physiological balance between antioxidant systems and the prooxidant isoforms of O_2^--producing NPC-Fe(III) complexes, and 2) the produced O_2^- gas is stabilized by O_2 and transferred into inert materials like glass or silicone tubes.

The practical applications of these findings include: 1) utilizing the liquid-phase O_2^- from these complexes in biochemistry to assess its effects on a variety of biological systems (e.g., enzymes, proteins, lipoproteins, biomembranes, DNA), acting as a biologically beneficial, thermostable, and continuously active agent; 2) using

the quantitative and qualitative alterations in these complexes as a new method for assessing food quality in food chemistry; 3) applying gas-phase O_2^- for therapeutic purposes, such as oxygen masks for treating lung infections; 4) leveraging liquid and gas-phase O_2^- at effective concentrations to regulate cell and microorganism proliferation and apoptosis; 5) preparing and lyophilizing NPC-Fe(III) isoforms, NPC, and hybrid NPC-Nox associates for commercial purposes, using the universal method described; 6) exploring the use of these NPCs in treating immunodeficiencies by stimulating the O_2^--producing activity of immune cells (leukocytes and erythrocytes); and 7) commercial production of these NPC-Fe(III) isoforms.

Conclusion

In this study, we successfully isolated and purified novel prooxidant components, including the O_2^--generating NPC-Fe(III) complexes, NPC, and hybrid NPC-Nox associates, from Armenian varieties of *Helianthus tuberosus*, *Solanum tuberosum*, and *Daucus sativus*. We determined their physicochemical characteristics and the mechanisms by which they exert influence, revealing both fundamental and practical implications for the first time.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: The experimental procedures adhered to the European Communities Council Directive (2010/63/UE) and received approval from the Ethics Committee of Yerevan State Medical University, following the guidelines outlined in the approval document (N10-2/22-IRB APPROVAL, May 19, 2022).

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