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HIGH AFFINITY OF RECOMBINANT DJ-1 (PARK7) PROTEIN TO NI-NTA

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ABSTRACT

The DJ-1 protein also known as Parkinson disease protein 7 (PARK7) is a glyoxalase associated with hereditary form of Parkinson's disease. Glyoxalase DJ-1 consists of 189 amino acids and has a molecular weight of 20 kDa. The physiological role of DJ-1 is unclear but recent evidence suggest that it can detoxify reactive electrophiles formed in glycolysis. Expression and purification of the recombinant DJ-1 protein in *Escherichia coli* revealed an abnormally high affinity of rDJ-1 for Ni-NTA, which was accompanied by a color change of Ni-NTA from blue to blue-violet. rDJ-1 formed the strong complex with Ni-NTA, which was destroyed only by 2400 mM imidazole. Experiments with the mutant DJ-1 protein containing the Cys106Ser substitution confirmed the key role of cysteine at position 106 for the formation of this complex. The discovered phenomenon shows the potential of using the DJ-1-tag as a protein tag for obtaining high-purity protein preparations and for bioanalytical applications.

Key words: Parkinson disease, recombinant protein, affinity chromatography, DJ-1 protein.

INTRODUCTION

The DJ-1protein, also known as Parkinson disease protein 7 (PARK7) is a glyoxalase that is associated with Parkinson's disease [1]. Glyoxalase DJ-1 consists of 189 amino acids and has a molecular weight of 20 kDa. The physiological role of DJ-1 is assumed to be the protection of brain cells from oxidative stress however the underlying mechanisms are unclear. One of the suggested mechanisms indicates that under oxidative stress, DJ-1 inhibits the aggregation of α-synuclein due to its chaperone activity [1, 2] signaling the presence of oxidative stress. Additionally, DJ-1 acts as a positive regulator of transcription. It is known that mutations in DJ-1 can cause an early onset of Parkinson's disease [3], which is associated with oxidative stress of cells [4, 5].

Previously, when obtaining polyclonal antibodies against DJ-1, we cloned the *park7* gene in *E.coli* cells and carried out expression and purification of the recombinant DJ-1 protein bearing the hexahistidine tag by immobilized metal-affinity chromatography using Ni-NTA [6]. An advantage of using Ni-NTA is its high binding capacity, which is typically in the range of 50 mg of protein per mL of resin. This is the highest capacity among all other affinity matrices that typically bind 3–10 mg of protein per mL of resin. Another advantage of Ni NTA is its cost: Ni-NTA is the cheapest affinity matrix among all other affinity resins. The third advantage of Ni-NTA is the ease of regeneration of the matrix with Ni^{2+} ions, which again affects the cost of recombinant protein purification. These characteristics make Ni-NTA popular choice for the purification of recombinant proteins [7]. During the purification of recombinant DJ-1, an abnormally high affinity of rDJ-1 for Ni-NTA was observed, which manifested itself in the need for a higher concentration of imidazole when eluting the recombinant protein from the column. We attribute this phenomenon to the modification of cysteine at position 106, and to verify this hypothesis, experiments were carried out to introduce a mutation in the sequence of the *park7* gene followed by purification of two variants of the recombinant protein DJ-1.

The hypothesis of this study is that the recombinant DJ-1 protein is modified at the cysteine in position 106 when expressed in *E.coli* cells and this modification increases the affinity of rDJ-1 for Ni-NTA. Therefore, a higher concentration of imidazole is required to elute rDJ-1 from the Ni-NTA column. To verify this hypothesis, mutations were entered into the gene at position 106 replacing catalytic cysteine with isosteric serine (C106S). The aim of this work was to study the affinity of the recombinant DJ-1 protein to Ni-NTA. To achieve the aim, there were the following tasks: obtaining the mutant sequence of the *park7* gene; expression of *park7* and *park7mut* genes in *E.coli* cells; obtaining two recombinant proteins DJ-1 and DJ-1mut and comparing the degree of affinity for Ni-NTA.

MATERIALS AND METHODS

Strains, DNA, vectors and reagents

The vector pPARK7 was used for expression the *park7* gene and for site directed mutagenesis. DpnI endonuclease and Pfu DNA Polymerase were purchased from Thermo Scientific. *E.coli* strain DH5α was used for plasmid preparation and site directed mutagenesis. *E.coli* strain BL21(DE3)-RIPL was used to express *park7* gene and its mutant.

Media

Lennox (LB) Broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with 100 μg/mL was used for cultivation of DH5α cells transformed with plasmids. The Super Optimal Broth with catabolite repression (1% of tryptone, 0.5% of yeast extract, 0.05% of NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose, pH 7.5) was used for *E.coli* transformation. The LB with 1.5% agar and 150 μg/mL ampicillin was used for recombinant strains selection. Terrific Broth (TB) (1.2% tryptone, 2.4% yeast extract, 0.5% glycerol, 170 mM KH_2PO_4, 720 mM K_2HPO_4) with 100 μg/mL ampicillin was used for cultivation *E.coli* strain-producers. The media was prepared in accordance with Maniatis protocol [8].

Site-directed mutagenesis

Site-directed mutagenesis was performed for pPARK7 vector to obtain mutant form of DJ-1 protein with using DJ-1mutC106Sfw (5'-TCTGCAGGTCCTACTGCTCT-GTTGGCTCAT-3') and DJ-1mutC106Srv (5'-AGTAG-GACCTGCAGAGATGGCGGCTATCAG-3') olygonucleotides. PCR mixture (50 µL final volume) contained 5 µL of 10X Pfu–pol Buffer, 4 µL of dNTPs (2 mM stock solution), 1 µL of each primer (10 µM stock solution), 1 µL of plasmide (100 ng), 1 µL of Pfu DNA polymerase (2 U/ul), and 37 µL of nuclease-free water. The following amplification parameters were used: initial denaturation at 95 °C for 5 min; then 30 cycles of 95 °C for 1 min, 70 °C for 1 min, and 72 °C for 2 min/kb; and final extension at 72 °C for 10 min. The amplification product was purified by phenol-chloroform method and then treated by 1 ul of the DpnI (10U/ul) in 1X Tango Buffer for degradation initial pPARK7 vector. The DpnI was heat inactivation at 80 °C for 20 min. The mutant plasmid was purified by phenol-chloroform method and the DH5α competent cells were transformed with the mutant plasmid. The recombinant colonies were selected on LB agar with ampicillin. The plasmid DNA was extracted with MiniPrep (Thermo Scientific) and sequenced.

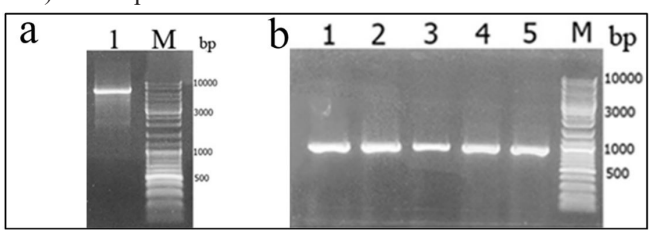

Figure 1 — Results of cite-directed mutagenesis (a) and PCR-screening (b) for park7mut gene

Sequencing

The nucleotide sequence of open reading frame (ORF) was determined. The *park7mut* gene was amplified and sequenced. The amplification was performed with a universal primer pair: T7Fw (5`-TAATACGACTCACTATAGGG-3`) and T7Rv (5'-GCTAGTTATTGCTCAGCGG-3'). Sequencing was carried out by Sanger method [9] on an ABI 3730xl Genetic Analyzer (Applied Biosystems) using BigDye Terminator v3.1 (Applied Biosystems). The sequences were analyzed with Vector NTI software.

Purification of DJ-1 and DJ-1mut

Competent cells of *E.coli* BL21(DE3)-RIPL strain were transformed by pPARK and pPARKmutC106S vectors and were seeded on the LB agar plates with ampicillin. The plates were cultured at 37 °C for overnight. A single colony of the each strain was inoculated into TB with ampicillin. At OD_{600}=0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added at 0.3 mM. The cultures were incubated at 18 °C and 130 rpm shaking for 16 hours. The cells of recombinant strains were harvested at 4 °C, 6000×g for 7 minutes. The pellet was suspended in 500 mM NaCl, 20 mM Tris-HCl (pH 8.0) with 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by ultrasonic sonication. The lysate was centrifuged at 4 °C, 40000×g for 60 minutes. The clarified lysate was loaded onto the column with Ni-NTA agarose (Invitrogen). The column was washed with buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM Imidazole). The protein was eluted by 150–3000 mM Imidazole in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl. The fraction were photographed and analyzed by SDS-PAGE [10]. The protein concentration was determined by Bradford method [11].

RESULTS AND DISCUSSION

The mutation was introduced by site-directed mutagenesis using DJ-1mutC106Sfw and DJ-1mutC106Srv oligonucleotides. Figure 1a shows a linearized pPARK7 vector carrying the Cys106Ser substitution. Positive clones were obtained by transformation DH5α competent cell with the purified vector. PCR screening of five of them showed a positive result (Figure 1b). Sequencing of the open reading frame for mutant vector confirmed the introduced mutation.

The plasmid pPARK7mutC106S was obtained, the plasmid has in ORF the rDJ-1mutC106S protein, in which cysteine at position 106 was replaced by serine. The recombinant DJ-1 and DJ-1mut proteins were obtained by plasmid expression of the two vectors: pPARK7 and pPARK7mutC106S in BL21(DE3)-RIPL cells. Figure 2 shows the results of chromatographic purification of rDJ-1 and rDJ-1mut proteins using Ni-NTA column.

As follows from Figure 2, the affinity of rDJ-1 and rDJ-1mut containing 6His-tag to Ni-NTA is different. In the case of rDJ-1 (wild type), protein elution starts at 150 mM imidazole and continues up to 2400 mM. Elution of rDJ-1 ended at 3000 mM. Mutant rDJ-1mut elutes differently. Recombinant DJ-1mut also starts to elute at 150 mM, but the elution ends already at 300 mM imidazole (Figure 2b).

Purification of recombinant rDJ-1 and rDJ-1mut from Ni-NTA column revealed another effect. As known, when NTA is activated by Ni^{2+} ions, it acquires a blue color, which does not

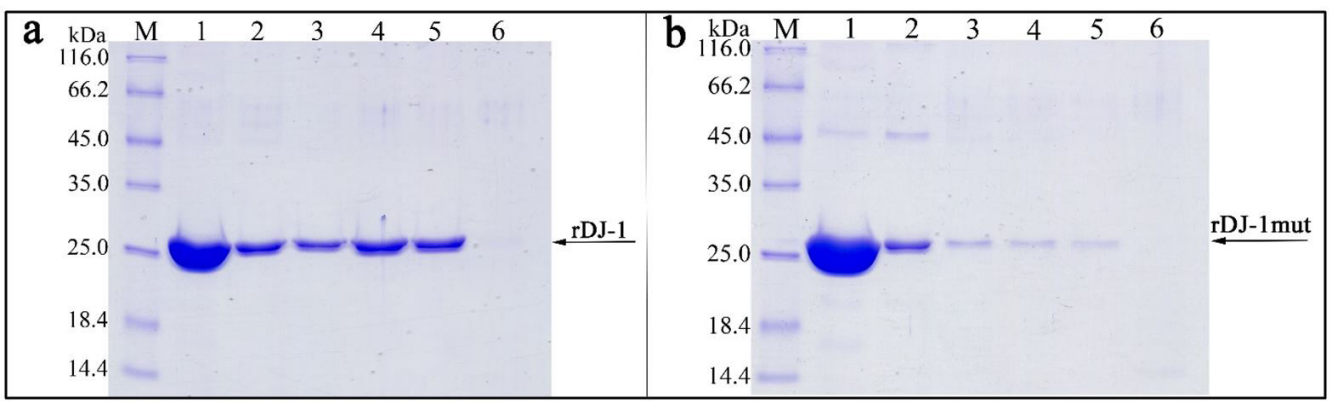

Figure 2 — Result of purification of rDJ-1 (a) and rDJ-1mut (b): 1–150 mM; 2–300 mM; 3–600 mM; 4–1200 mM; 5–2400 mM; 5–3000 mM; M — protein marker

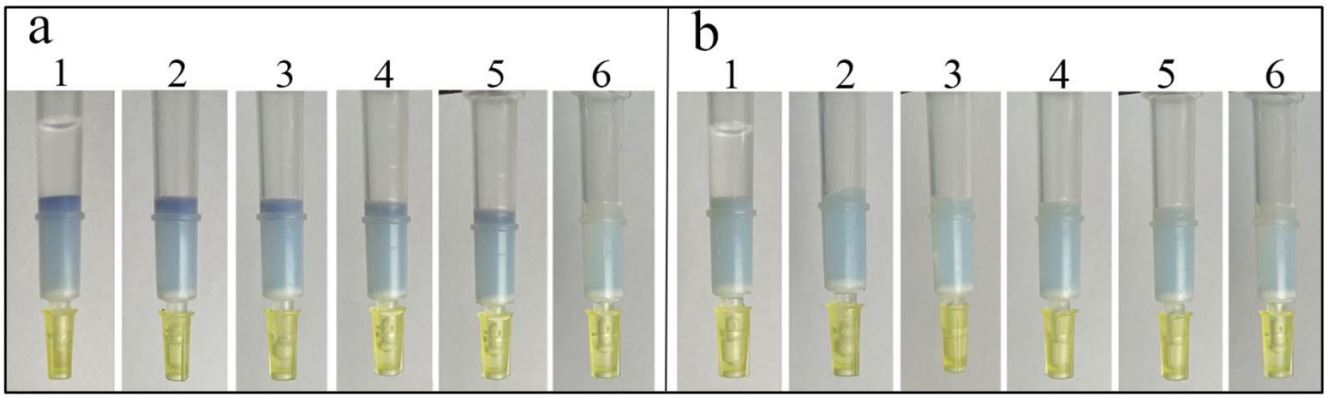

Figure 3 — Color change of Ni-NTA with increasing concentration of imidazole in the elution buffer for proteins rDJ-1 (a) and rDJ-1mut (b): 1–150 mM; 2–300 mM; 3–600 mM; 4–1200 mM; 5–2400 mM; 6–3000 mM

change when any proteins with 6His-tag are purified. However, when loading the lysate with rDJ-1, the color of Ni-NTA column turned to blue-violet, which was maintained when using 150–2400 mM imidazole elution buffer (Figure 3a). For the rDJ-1mut mutant, the Ni-NTA column did not change color and retained its blue color (Figure 3b). The color change with wild-type rDJ-1 well correlates with purification results and indicates that rDJ-1 forms a strong complex with Ni-NTA that is resistant to high concentrations of imidazole. Since the point mutation at position 106 changes the property of the DJ-1 protein, it can be concluded that cysteine at position 106 plays a decisive role in enhancing the affinity for the formation of the complex, which manifests itself in a color change.

The observed effect associated with a change in the color of the recombinant DJ-1 protein containing 6His-tag, accompanied by an increase in affinity for Ni-NTA agarose, may have potential applications. For example, DJ-1 fused with 6His may be used as a protein tag (DJ-1-tag). If the task is to obtain highly pure protein in case of low expression, then the target protein can be fused with DJ-1-tag. This will make it possible to achieve a high level of accumulation of water-soluble protein in the cytosolic fraction, and the high affinity for Ni^{2+} makes it possible to obtain high-purity recombinant proteins. Changing the color of the protein allows controlling the cleaning process in real time.

The use of DJ-1-tag as the protein tag has another advantage. Expression of the *park7* gene in *E.coli* BL21(DE3)-RIPL cells makes it possible to obtain rDJ-1 up to 10 mg per 1 liter of induced culture, which is a very high indicator. And almost all recombinant protein is in a water-soluble form. Therefore, the use of DJ-1 as a protein label will increase the expression level of the target gene. And the fusion protein will be converted into a water-soluble fraction, just like when using maltose-binding protein tag (MBP-tag) [12–14] and thioredoxin tag (Trx-tag) [15, 16].

CONCLUSION

Expression of wild-type *park7* and mutant *park7mut* genes in *E.coli* cells showed an increased affinity of the wild-type DJ-1 protein for Ni-NTA, which appears itself as resistance to high imidazole concentrations up to 2400 mM. The determining role of cysteine at position 106 for increasing the affinity of rDJ-1 to Ni-NTA has been established. Ni-NTA color-changing effect found when interacting with wild-type rDJ-1. Binding of Ni-NTA to rDJ-1 results in a color change from blue to blue-violet. Ni-NTA is blue only after complete elution of rDJ-1 with 2400 mM imidazole. Upon interaction of Ni-NTA with mutant rDJ-1 (with the Cys106Ser mutation), no complex is formed, no color change occurs, and the rDJ-1mut elutes at 300 mM imidazole. This property of recombinant DJ-1 has the prospect of using this protein as the tag for bioanalytical applications.

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ВЫСОКАЯ АФФИННОСТЬ РЕКОМБИНАНТНОГО БЕЛКА DJ (PARK7) К NI-NTA

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АБСТРАКТ

Белок DJ-1 представляет собой дегликазу, ассоциированную с болезнью Паркинсона (Parkinson disease protein 7, PARK7). Дегликаза DJ-1 состоит из 189 аминокислотных остатка и имеет молекулярную массу 20 кДа. Физиологическая роль DJ-1 заключается в том, что в условиях окисления DJ-1 ингибирует агрегацию α-синуклеина за счет своей шаперонной активности. Экспрессия и очистка рекомбинантного белка DJ-1 в *E.coli* выявила аномально высокую аффинность rDJ-1 к Ni-NTA, что сопровождалось изменением цвета Ni-NTA с голубого на сине-фиолетовый. Связываясь с Ni-NTA rDJ-1 образует прочный комплекс, который разрушается только при 2400 мМ имидазола. Эксперименты с мутантным белком DJ-1, содержащим замену Cys106Ser, подтвердили ключевую роль цистеина в положении 106 для образования данного комплекса. Обнаруженный эффект имеет потенциал для применения DJ-1 в качестве белковой метки при получении белковых препаратов высокой чистоты и для биоаналитических приложений.

Ключевые слова: болезнь Паркинсона, рекомбинантный белок, аффинная хроматография, белок Dj-1

NI-NTA-ҒА РЕКОМБИНАНТТЫ DJ-1 (PARK7) АҚУЫЗЫНЫҢ ЖОҒАРЫ АФФИНДІГІ

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ТҮІН

DJ-1 протеині Паркинсон ауруымен байланысты дегиликаза болып табылады (Parkinson disease protein 7, PARK7). DJ-1 дегликазасы 189 амин қышқыл қалдықтарынан тұрып, 20 кДа молекулалық салмаққа ие. DJ-1-ң физиологиялық рөлі тотығу жағдайында, ол шаперондық белсенділігіне байланысты α-синуклеиннің агрегациясын тежейді. *E.coli* жасушаларында рекомбинантты DJ-1 ақуызының экспрессиясы және тазартылуы Ni-NTA түсінің көгілдірден көк-күлгінге дейін өзгеруімен қатар жүретін rDJ-1-нің Ni-NTA-ға әдеттен тыс жоғары аффиндігін анықтады. Ni-NTA-н байланысу арқылы rDJ-1 мықты комплекс түзеді, бұл комплекс тек 2400 Мм имидазол жағдайында ыдырайды. Құрамында Cys106Ser алмастыруы бар мутантты DJ-1 ақуызымен жүргізілген тәжірибелер 106-позициядағы цистеиннің осы кешеннің түзілуінде негізгі рөлін растады. Табылған әсердің жоғары тазалықтағы ақуыз препараттарын алу және биоаналитикалық қолданыс үшін ақуыз белгісі ретінде пайдалану мүмкіндігі бар.

Кілтті сөздер: Паркинсон ауруы, рекомбинантты ақуыз, аффинді хроматография, Dj-1 ақуызы.