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Expression, purification and preliminary biochemical studies of the N-terminal domain of leucine-rich repeat kinase 2

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ABSTRACT

Leucine-rich repeat kinase 2 gene is a key factor for Parkinson's disease and encodes for a large protein kinase LRRK2 (280 kDa) with multiple domains, including the different repeat sequences at the N-terminus such as ankyrin domain. Here, we successfully expressed and purified two kinds of LRRK2's N-terminal fragments N1 (aa12–320) and N2 (aa12–860). The purified N2 protein was identified by mass spectrometry and N1's molecular weight was determined to be 33.23 kDa. Gel filtration revealed that N1 exhibits as monomer, dimer and tetramer and N2 as oligomer in solution. N1's multiple oligomeric states were further proved by native-page and cross-linking gel experiments. Circular dichroism spectrum indicated that N1 and N2 contain both α helixes and β sheets. The polymerization character of LRRK2 N-terminal region would be speculated to relate with its biological function.

1. Introduction

Parkinson's disease (PD) is a common, age-related neurodegenerative disorder characterized by tremor, rigidity, bradykinesia and postural instability [1]. The incidence of PD increases along with age, usually 0.3% afflicted at age 50 and increases to 4.3% by age of 85 [2]. Recent studies suggested the leucine-rich repeat kinase 2 gene (*lrrk2*, *park8* or *dardarin*) playing an important role in Parkinson's disease [3]. Mutations in this gene are the leading cause of the late-onset, autosomal-dominant Park8 type of Parkinson's disease, such as G2019S [4–6]. Significantly, mutations of *lrrk2* have also been related with sporadic PD with unprecedented 1–2% prevalence [7].

The *lrrk2* gene encodes for a large protein kinase LRRK2 (280 kDa) with multiple domains [1], including a leucine-rich repeat (LRR) domain, a Roc GTPase domain followed by its associated C-terminal of Roc (COR) domain [8], a kinase domain of the tyrosine kinase-like (TKL) subfamily [9], and a C-terminal WD40 domain. Its N-terminal region is predicted to adopt the folds of armadillo repeats (aa180–660) and seven ankyrin repeats (aa690–860) [1]. And the mutation R793M in the predicted ankyrin repeat domain was putatively related with the Parkinson's disease [10,11].

In our study, we cloned different fragments of human LRRK2 (hLRRK2) and tried to express and purify them for further biochemical and structural study. Here we reported the large-scale expression, purification and preliminary biochemical study of two N-terminal fragments N1 (aa12–320) and N2 (aa12–860) of hLRRK2. Gel filtration and chemical cross-linking revealed that N1 exhibits as monomer, dimer and tetramer in solution and N2 as multimer, which took in accord with previous reports on the polymerization form of hLRRK2 [12,13]. To our best knowledge, besides our work, there is only another report on the purification and preliminary biochemical study of hLRRK2's N-terminal fragments with amino acids from 100 to 500 [14]. Our study on the N-terminus of LRRK2 is of great significance for the preparation of PD's detection antibody, detection kit of PD and further structural study, and also gave some clew for LRRK2's biological function study, which is important for the therapeutic development.

2. Materials and methods

2.1. Molecular cloning, protein expression and purification

The cDNA fragments coding for hLRRK2's N1 and N2 were amplified with the primers:

N1-F: 5′-CGGGATCCGACGAGGAAACTCTGAAGAAGTTG A-3′
N1-R: 5′-CCGCTCGAGCCAGTGAGGAGGGCCAAACAGCTGA-3′
N2-F: 5′-CGGGATCCGACGAGGAAACTCTGAAGAAGTTG A-3′
N2-R: 5′-CCGCTCGAGCCGCTGCCTGAGGCTGTTCCTTCTT-3′.

Abbreviations: IPTG, isopropyl-b-D-thiogalactopyranoside; GST, glutathione S-transferase; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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The PCR product of N1 was inserted into the pGEX-6P1 vector and N2 was inserted into the pEXS-DH vector (His-tag, Yujia Zhai and Qiangjun Zhou, Fei Sun's lab, IBP of CAS) by using BamHI and XhoI restriction sites. The recombinant plasmids were transformed into *E. coli* strain rosetta (Novagen) for protein expression. The bacteria were cultured in TB medium (Terrific Broth powder medium) containing 100 μg/ml Ampicillin and induced at 16 °C by 0.5 mM IPTG.

After protein expression, the bacteria were harvested by centrifugation, N1 was resuspended in PBS and N2 was resuspended in lysis buffer (50 mM Tris, pH 8.0, 0.3 M NaCl, 10% glycerol, 20 mM imidazole, 1 mg/ml lysozyme, 10 μg/ml RNase A, 1 mM PMSF), incubated on ice for 30 min and then broken by sonication at 4 °C (The maker of the sonicator is Ningbo Scientz Biotechnology Co., Ltd. and the model of sonicator is JY92-2D). After centrifugation (15,455 *g*, 35 min), the supernatants were loaded into GST column for N1 and Ni-NTA column for N2, then washed by PBS and washing buffer (50 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 10% glycerol, 100 mM imidazole) respectively. GST fused N1 protein was incised by precision protease and N2 protein was eluted by 500 mM imidazole. After affinity chromatography, we used ultra-filtration (Millipore) for protein desalting and concentration. Further purification was carried out by using gel filtration chromatography (Superdex-200). The fraction eluted from the Superdex-200 column was pooled and analyzed by SDS-PAGE. Protein concentration was determined using the BCA Protein Assay Kit (Novagen). The protein was buffered in 50 mM Tris, 10% glycerin, 150 mM NaCl, pH8.0 and fast frozen using liquid nitrogen and stored at −80 °C.

2.2. Mass spectrometry

Protein identification was carried out using peptide mapping fingerprint analysis and this work was completed by Proteomics Center, Protein Sciences Research Platform, Chinese Academy of Sciences.

2.3. Gel filtration

Superdex-200 size exclusion chromatography column (GE Healthcare, China) was mounted on Biologic Duoflow (Bio-Rad, USA) and used to analyze the homogeneity and apparent types of polymerization of N1 and N2 (the protein concentration used was 10 mg/ml and the loading volume was 0.3 ml). The buffer B as stated above was used and the flow rate was 0.5 ml/min. Standard molecular weight markers used for calibration were Ribonuclease A (13.7 kDa, elution volume is 16.56 ml), BSA (monomer is 67.0 kDa, elution volume is 13.35 ml and dimer is 134.0 kDa, elution volume is 11.68 ml), GST (dimer is 52 kDa, elution volume is 14.13 ml) and precision protease (dimer is 92 kDa, elution volume is 12.64 ml). Apparent molecular weights (MW) were deduced by plotting Kav versus log (MW) with $Kav = (Ve - V_0) / (Vt - V_0)$ where Ve is the elution volume of the target, Vt is the total column bed volume and V_0 is the void volume.

2.4. Native-page

The purified N1 and N2 protein were diluted by 50 mM Tris, 150 mM NaCl and 10% glycerol, pH 8.0 to different concentrations and loaded into the native-page. The assay was performed at the condition of 60 voltage and 4 °C for 10 h.

2.5. Chemical cross-linking

Protein was diluted to 5 mg/ml by 25 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM EDTA and 1 mM DTT. Ethylene glycolbis (succinimidylsuccinate) was dissolved in DMSO to a concentration of 50 mM and then added to 10 μl of protein sample with a final concentration of 5 mM. After the mixture was incubated on ice for 2 h, the reaction was quenched for 15 min by adding 1 M Tris–HCl (pH 7.5) to a final concentration of 50 mM. An equal volume of 2×SDS-PAGE sample buffer was added and a small amount was analyzed on a 12% SDS-PAGE.

2.6. Circular dichroism spectroscopy

Circular dichroism (CD) spectrum was measured at 16 °C with an Aviv 62DS CD spectropolarimeter and the protein sample was buffered in PBS (pH7.4) with the concentration of 0.5 mg/ml. The intensities of the CD absorption were expressed in units of millidegree. The spectra were recorded over the wavelength range of 190–250 nm with a bandwidth of 1 nm, a scan speed of 50 nm/min and a 5 s response time. The spectra were corrected by subtracting the buffer baseline and were averaged over three scans.

3. Results

3.1. N1 and N2 were expressed in soluble form and purified in high purity

The expression vector pEXS-DH (for N2) that encodes for 8-His-tag at either the N- or C-terminus followed by a TEV protease cleavage site at the N-terminus was constructed in our laboratory. And the pGEX-6P1 vector (Novagen, for N1) was also used for protein expression. Different kinds of medium such as LB, 2YT and TB were used for screening the best expression condition of LRRK2. Finally, we found that N1 and N2 expressed in TB medium at 16 °C could yield the most amounts of soluble protein.

The key step for N1 and N2 purification was affinity chromatography and it was difficult to remove the protein contamination in the followed purification steps. And it was also found that heavy sonication would yield a redundant contaminated protein with molecular weight close to N2, which could not be easily removed during purification. The optimal program for breaking bacteria by sonication is to suspend the cells in corresponding buffer with the concentration from 1 mg/ml to 2 mg/ml and run sonication for only 3 cycles with the power 400 W, 8 min/cycle and 5 s stop between two cycles. After affinity chromatography (Fig. 1) and size exclusion chromatography, 95% purity of N1 and N2 protein was obtained (Fig. 2). The yield rate of N1 and N2 protein was nearly 25 mg and 5 mg per liter TB culture media respectively. The purified N2 protein was identified by peptide fingerprinting mass spectrometry (Table 1). N1's molecular weight was determined as 33.2 kDa by MALDI-TOF mass spectrometry (data not shown).

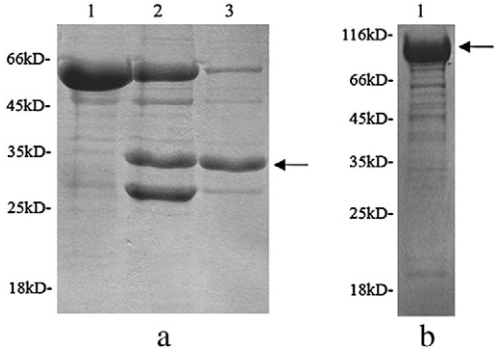

Fig. 1. SDS-PAGE of purified N1 and N2 protein after affinity chromatography. Samples were separated by SDS-PAGE and detected by coomassie blue staining. a) Lane 1, GST-fusion protein of N1; lane 2, GST-fusion protein of N1 was incised by precision protease; lane 3, flow through after being incised by precision protease; b) lane 1, purified N2 after Ni-NTA affinity chromatography. Targeted proteins were labeled by arrow.

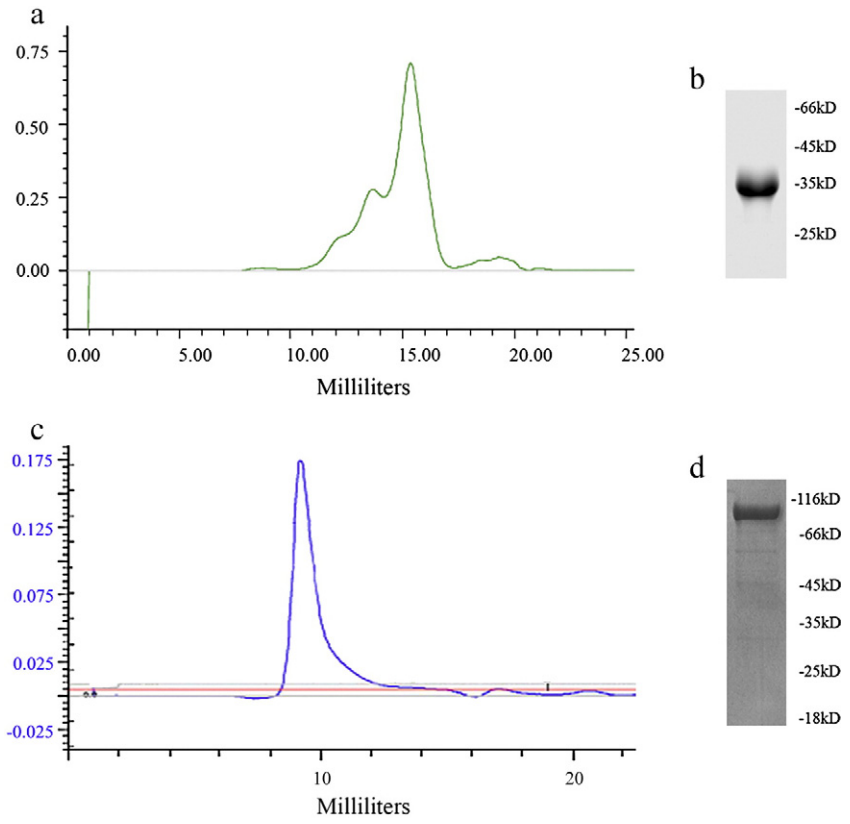

Fig. 2. Gel filtration chromatography. a) Superdex-200 map of N1; b) purified N1; c) Superdex-200 map of N2; d) purified N2.

3.2. Oligomerization of N1 and N2 characterized by gel filtration, native-page and cross-linking experiments

In order to study the oligomerization states, the apparent molecular weight of N1 and N2 was measured by gel filtration using Superdex-200 column that was calibrated with the standard molecular weight markers Ribonuclease A, BSA, GST and precision protease (PPase) (Fig. 3a). N1 appeared as three peaks in the gel filtration profile and their molecular weights were calculated to be 143.0 kDa, 76.8 kDa and 33.2 kDa, which approximately correspond to tetramer (140.8 kDa) dimer (70.4 kDa) and monomer (35.2 kDa) of N1 respectively. In addition, when we repeated the gel filtration assay using the second peak corresponding to N1 dimer, both monomer and dimer appeared again (Fig. 3b), which revealed that N1's oligomerization state had a dynamic balance. Interestingly, N2's peak was calculated to be 735.8 kDa that was approximately 8 folds of N2's theoretical molecular weight (95.3 kDa) (Fig. 3a). There were two bands in the result of N1's native-page, we speculated that the two bands were N1's monomer and dimer, while the tetramer could not be seen due to its low abundance (Fig. 3c). Cross-linking gel of N1 gave the similar result with gel filtration that N1 exists dominantly as monomer with dimer and tetramer existing (Fig. 3d). We also tried to analyze N2's multimerization by cross-linking assay, but N2 formed large polymers and could not be well resolved in SDS-PAGE. So we didn't show N2's result here.

Table 1
Peptides identified by peptide fingerprint mass spectrometry of N2. The identified peptides were shown in bold.

1	MASGSCQGCE	EDEETLKKLI	VRLNNVQEGK	QIETLVQILE	DLLVFTYSEH
51	ASKLFQGK**NI**	**HVPLLIVLDS**	**YMRVASVQQV**	**GWSLLCK**LIE	VCPGTMQSLM
101	GPQDVGNDWE	VLGVHQLILK	**MLTVHNASVN**	**LSVIGLK**TLD	LLLTSGKITL
151	LILDEESDIF	MLIFDAMHSF	PANDEVQKLG	CK**ALHVLFER**	VSEEQLTEFV
201	ENKDYMILLS	ALTNFKDEEE	IVLHVLHCLH	SLAIPCNNVE	VLMSGNVRCY
251	NIVVEAMKAF	PMSER**IQEVS**	**CCLLHR**LTLG	NFFNILVLNE	VHEFVVKAVQ
301	QYPENAALQI	SALSCLALLT	ETIFLNQDLE	EK**NENQENDD**	**EGEEDKLFWL**
351	**EACYK**ALTWH	RKNKHVQEAA	CWALNNLLMY	QNSLHEK**IGD**	**EDGHFPAHR**E
401	VMLSMLMHSS	SK**EVFQASAN**	**ALSTLLEQNV**	**NFR**KILLSK**G**	**IHLNVLELMQ**
451	**K**HIHSPEVAE	SGCKMLNHLF	EGSNTSLDIM	AAVVPKILTV	MK**RHETSLPV**
501	**QLEALRAILH**	**FIVPGMPEES**	**REDTEFHHK**L	NMVKKQCFKN	DIHKLVLAAL
551	NR**FIGNPGIQ**	**K**CGLKVISSI	VHFPDALEML	SLEGAMDSVL	HTLQMYPDDQ
601	EIQCLGLSLI	GYLITKKNVF	IGTGHLLAKI	LVSSLYRFKD	VAEIQTKGFQ
651	TILAILKLSA	SFSKLLVHHS	FDLVIFHQMS	SNIMEQKDQQ	FLNLCCKCFA
701	KVAMDDYLKN	VMLERACDQN	NSIMVECLLL	LGADANQAKE	GSSLICQVCE
751	KESSPKLVEL	LLNSGSREQD	VRKALTISIG	KGDSQIISLL	LRRLALDVAN
801	NSICLGGFCI	GKVEPSWLGP	LFPDKTSNLR	KQTNIASTLA	RMVIRYQMKS
851	AVEEGTASGS				

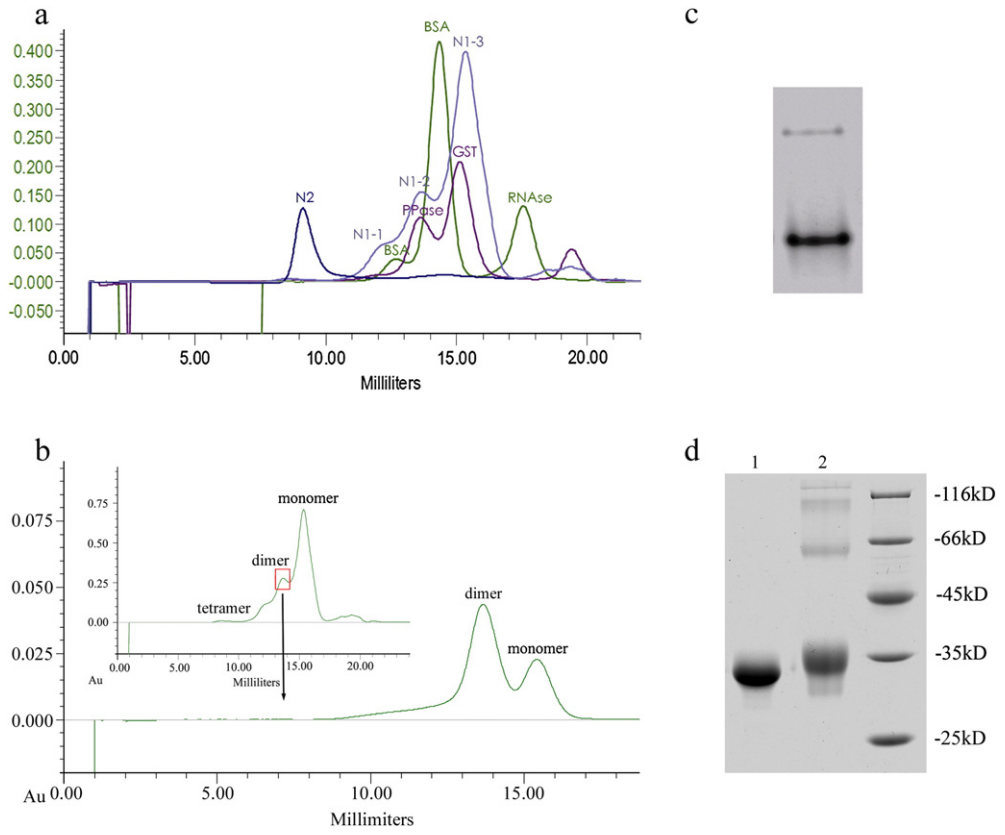

Fig. 3. Oligomerization state of N1 and N2. (a) Gel filtration profile of different molecules. Because the sample loading and elution started at volume 1.0 ml, the elution volume of each peak equals the corresponding readout volume minus 1.0 ml. Ribonuclease A (13.7 kDa, elution volume (EV) is 16.56 ml), BSA (monomer is 67.0 kDa, EV is 13.35 ml and dimer is 134.0 kDa, EV is 11.68 ml), GST (dimer is 52 kDa, EV is 14.13 ml) and precision protease (PPase, dimer is 92 kDa, EV is 12.64 ml) were used as standard molecular weight markers; the peak of N1–3 representing N1 monomer was eluted at 14.77 ml, N1–2 representing dimer at 12.97 ml and N1–1 representing tetramer at 11.64 ml. (b) The dynamic oligomerization balance of N1. Up-left, the gel filtration profile of N1; down-right, the peak for N1 dimer in up-left profile was collected and applied for second gel filtration chromatography; (c) native-page of N1; (d) cross-linking gel of N1. Lane 1, purified N1; lane 2, cross-linked N1. The four bands represent the tetramer, trimer, dimer and monomer of N1 from up to bottom. The appearance of N1 trimer in this cross-linking gel was believed due to the unsaturated cross-linking within N1 tetramer.

3.3. Secondary structure of N1 and N2

Circular dichroism (CD) spectra revealed that N1 and N2 were well folded with the typical curves from 205 nm to 225 nm (Fig. 4). The K2d software was used to calculate the secondary structure of N1 and N2, with the result that almost one third of N1 and N2 structures were random coils and the ratios of alpha helices/beta sheets were 25/14.6% for N1 and 22.2/18.3% for N2 respectively (Table 2). The observed secondary structure content of the N-terminal region of LRRK2 by CD spectrum was similar to the previous prediction [1].

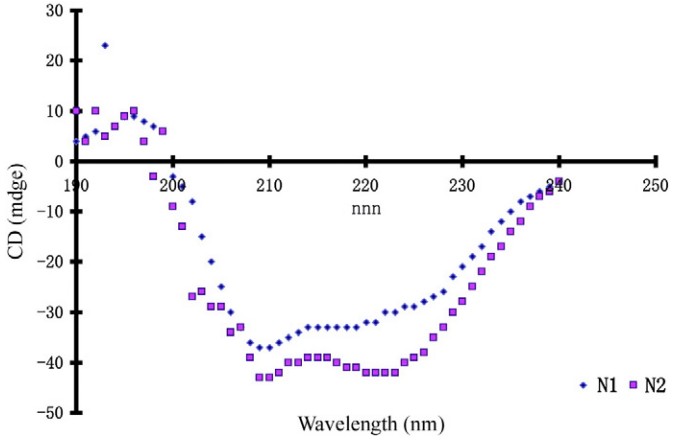

Fig. 4. Circular dichroism spectra of N1 and N2. CD spectrum was measured at far UV 190–250 nm for N1 and N2.

4. Discussion

Previous attempts to isolate and purify the LRRK2 protein were mostly performed in eukaryotic expression system, such as insect cell and mammalian cell expression system, except for the report on the structure study of the ROC domain [12] as well as another report on substrate specificity and inhibitors of LRRK2 [14]. However, the protein yield rate by the eukaryotic expression system was still low and not worthy for further biochemical and structural study.

The biochemical character of the N-terminal region of LRRK2 has not yet been extensively investigated and the previous studies mostly focused on the instability of the N-terminus of LRRK2 *in vivo*. In this report, the N-terminal region of LRRK2 (aa12–320 and 12–860) could be efficiently expressed and purified by *E. coli* expression system, which is a good complementary of the previous work about the expression of the N-terminus of LRRK2 (aa100–500) [14]. Here, N1's molecular weight was determined as 33.23 kDa by MALDI-TOF mass

Table 2
Secondary structure compositions determined by circular dichroism.[a]

Ratio %	N1	N2
Helix	25.0	22.2
Beta	14.6	18.3
Turn	25.6	22.7
Random	34.8	36.8
Total	100	100

[a] Circular dichroism spectra were analyzed using K2d software (http://www.embl-heidelberg.de/_andrade/k2d/) to calculate the secondary structure.

spectrometry. Gel filtration revealed that N1 presents as monomer, dimer and tetramer, and N2 as multimer in solution. N1 oligomerization balance was further proved by cross-linking gel experiments. The polymerization character of LRRK2's N-terminal region would be speculated to relate with its biological function. It has been shown by different groups that active LRRK2 is dimer via major contacts in the ROC and COR domains [12,15]. In addition, the WD40 domain located at C-terminus is required for dimerization and when WD40 domain was deleted, LRRK2 preferred to form polymer than dimer [16]. In our study, we found that the N-terminus of LRRK2 could also form dimer or polymer, which indicated that LRRK2 has an additional interface existing in the N-terminus, and this finding was in accord with and further confirmed the previous suggestion [17].

The CD spectrum indicated well folded N1 and N2 that comprise 25% alpha helices, 14.6% beta sheets and 22.2% alpha helices, 18.3% beta sheets respectively. The alpha helix and beta sheet composition fraction of N1 and N2 is in accord with previous prediction that N-terminal region of LRRK2 contains seven ankyrin repeats sequences [1]. The N2 fragment appears as an oligomer that might reflect non-specific protein aggregation, but its CD spectra showed that it is well folded and promising for further structural study.

The two protein's (N1 and N2) molecular weight were both larger than 20 kDa, so, crystallization method was more suitable for their structural study than NMR. During the study we got some sheet and spicula crystal of N2, while no crystal of N1 could be obtained yet although N1 was more stable than N2. In the future, we will try our best to optimize crystallization of N2 and analyze its 3D structure.

The polymerization character of LRRK2's N-terminal region would be speculated to relate with its biological function. So our biochemical study on the N-terminus of LRRK2 will shed importance on the intrinsic mechanism of LRRK2 during its related pathology progress *in vivo*. The purified N-terminal fragments of LRRK2 could also be used for preparation of antibody and detection kit of PD. The further structural study of the soluble N-terminal domain is surely important for investigating the function of this highly “drug-able” protein.

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