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β-Synuclein Protein from *Xenopus laevis*: Overexpression in *Escherichia coli* of the GST-Tagged Protein and Production of Polyclonal Antibodies

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Abstract—This report presents a procedure to obtain and purify recombinant β-synuclein from *Xenopus laevis* expressed in *Escherichia coli* as a glutathione-S-transferase fusion protein. After identification by mass spectrometry, the protein was then used to raise anti-*X. laevis* β-synuclein polyclonal antibodies, which were suitable to detect the presence of β-synuclein in *X. laevis* brain by Western blot. This is the first report of a positive identification of β-synuclein in an amphibian at the protein level.

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Key words: *Xenopus laevis*, β-synuclein, *Escherichia coli*, β-synuclein expression, antibody production

β-Synuclein (also known as phosphoneuroprotein 14 or PNP14) [1, 2] is a member of the synuclein family, which includes α-synuclein (also known as the non-amyloid component of plaques precursor protein or NACP) [3, 4], β-synuclein, and γ-synuclein (also known as breast cancer-specific gene 1 product or BCSG1 and persyn) [5, 6]. The β-synuclein protein is predominately expressed in brain, particularly in the neocortex, hippocampus, striatum, thalamus, and cerebellum; protein immunoreactivity is enriched at presynaptic terminals [7, 8]. Structurally, β-synuclein (amino acids 1-134) shares with α- and γ-synuclein a common design composed of three modular protein domains including a highly conserved N-terminal lipid binding α-helix (residues 7-87), a variable internal hydrophobic NAC domain (residues 61-95), and a C-terminal acidic tail (residues 95-140) composed primarily of glutamate and aspartate residues [9, 10]. Over half of the synuclein molecule (amino acids 7-87) is composed of seven imperfect repeat sequences of 11 amino acids, each with the core consensus sequence motif KTKEGV [9, 10]. Although the three synuclein proteins are highly homologous, β-synuclein lacks the middle region of α-synuclein—the core of the NAC binding domain [11, 12].

Although the physiological functions of β-synuclein are not clearly understood, previous studies have shown that β-synuclein has a neuroprotective effect. β-Synuclein protects the central nervous system from the toxic effects of α-synuclein overexpression [13] through mechanisms involving direct interactions between β-synuclein and Akt [14]. Another report has shown that β-synuclein protects TSM1 neurons from the toxic effects of 6-hydroxydopamine by decreasing the expression of the pro-apoptotic gene *p53* [15]. In addition, β-synuclein has a chaperone-like activity [16] and appears to play a role in cytoskeletal reorganization [17]. It specifically inhibits phospholipase D2 [18] and α-synuclein aggregation [13]. However, compared with the central involvement of α-synuclein in a number of neurodegenerative

Abbreviations: anti-Xen-SYNB polyclonal antibodies) anti-*X. laevis* β-synuclein polyclonal antibodies; ESI mass spectrometry) electrospray ionization mass spectrometry; GST) glutathione-S-transferase; IPTG) isopropyl-β-D-thiogalactopyranoside; ORF) open reading frame; PVDF) polyvinylidene fluoride; SYNB) β-synuclein; xSYNB) *Xenopus laevis* β-synuclein; Xen-GST-SYNB) *X. laevis* GST-β-synuclein fusion protein.

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diseases including Parkinson's disease, dementia with Lewy bodies, Alzheimer's disease, and multiple system atrophy [19], β-synuclein has not been implicated in neurodegenerative disease [20].

Xenopus laevis, as an ideal animal model, has the following features such as ease of growth and breeding, easy-to-manipulate genetic system, and high homology between *X. laevis* and humans [21]. The similarities and shared features between *X. laevis* and mammals have led to the development of many *X. laevis* pathogenesis models and disease-related assays for drug screening [22] and diseases such as tumor [23], organ or tissue development [24], and neurodegenerative diseases. Although β-synuclein has been widely studied in humans (*H. sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), ox (*Bos taurus*) [25], and puffer fish *Fugu rubripes* [19] by means of cDNA cloning, genome database searching, and comparative genomics, no studies have been reported on the expression of β-synuclein at the protein level *in vivo* in *X. laevis*.

The aim of the present work was to investigate whether β-synuclein was expressed at the protein level *in vivo* in *X. laevis*. Although there were already obtained antibodies against β-synuclein from mammals such as pan synuclein antibody from Abcam (England), most of them were predicted to react with *X. laevis* due to sequence homology. To detect β-synuclein in *X. laevis* more effectively, we present here its cloning, overexpression, purification, and the production of polyclonal antibodies, which were suitable to detect the presence of β-synuclein in *X. laevis* brain by Western blot. To our knowledge, this is the first report of a positive identification of β-synuclein in amphibians at the protein level. Additionally, the availability of the polyclonal antibodies would serve as tools to further investigate other unknown functions of β-synuclein. Based on the results, additional information concerning the normal functions of β-synuclein may be obtained using *X. laevis* as an animal model.

MATERIALS AND METHODS

In this work we used the following materials and reagents: *Escherichia coli* strain DH5α was purchased from TaKaRa (China) and BL21(DE3) from Qiagen (USA). pGEM-T Easy vector system was purchased from Promega (USA). pGEX-4T-1 plasmid was purchased from Pharmacia (USA). Restriction endonucleases, T4 DNA ligase, GeneRuler™ DNA Ladder Mix, and Prestained low range protein molecular weight marker were products of MBI Fermentas (Lithuania).

RT-PCR and cDNA cloning of *Xenopus laevis* β-synuclein (xSYNB). Total RNA from *X. laevis* brain was isolated using the Trizol reagent (Invitrogen, USA). Based on the *xSYNB* cDNA sequence (GenBank accession No. BC084970), a pair of primers were designed as follows: 5′-CAG**GGATCC***ATG*GATGTGCTTATGAAAGG-3′ (forward), 5′-GAA**CTCGAG**TTATGCTTCAGGCTCATATTCC-3′ (reverse). The incorporated 5′*Bam*HI and 3′*Xho*I restriction sites are shown in bold and start codon in italics. RT-PCR for the amplification of the ORF (open reading frame) was performed according to the instruction of One Step RNA PCR Kit (AMV) (TaKaRa). The reaction was carried out using the following reaction cycles: reverse transcription at 50°C for 30 min, initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing for 40 sec at 57°C, extension at 72°C for 1 min, then final extension at 72°C for 10 min followed. The amplification product was analyzed by electrophoresis on 1% agarose gel. The specific RT-PCR product obtained was purified and then used for ligation in pGEM-T Easy vector.

Construction of expression plasmid pGEX-4T/xSYNB. The resulting specific RT-PCR product and pGEM-T Easy vector were ligated at 16°C overnight using T4 DNA ligase to yield the pGEM-T/ORF construct. This construct was transformed into *E. coli* DH5α cells and the resulting colonies were screened by blue white colony selection and PCR. Then the ORF of xSYNB cloned in pGEM-T Easy vector was digested with *Bam*HI and *Xho*I restriction enzymes and run on 1% agarose gel. A 410-bp insert was cut and purified from the agarose gel and subcloned into the expression vector pGEX-4T-1. Successful cloning of coding region of xSYNB was confirmed by both restriction enzyme digestion and DNA sequencing (Invitrogen). The resulting expression plasmid, named as pGEX-4T/xSYNB, has the glutathione-S-transferase (GST) tag and the thrombin recognition site in frame with the 5′ end of the ORF of xSYNB.

Expression and solubility identification of Xen-GST-SYNB (*X. laevis* GST-β-synuclein fusion protein). For expression of the protein, the recombinant plasmid, pGEX-4T/xSYNB was transformed into *E. coli* BL21(DE3) cells, which provide phage T7 RNA polymerase for expression of heterologous genes [26]. A single colony containing plasmid pGEX-4T-1/xSYNB was used to inoculate 5 ml LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.4) containing 100 μg/ml ampicillin and shaken overnight at 37°C. Then the culture was transferred into 25 ml fresh LB broth flask and grown at 37°C with vigorous shaking. When absorbance at 600 nm reached 0.6, a 1-ml aliquots of cells was taken, centrifuged, and frozen at −20°C. To the remaining culture, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM and incubated at 37°C with shaking. A time course of expression to determine the optimal induction time for maximum expression of protein was measured by taking aliquots of cells at 1, 2, 3, and 4 h after induction with IPTG. These samples were analyzed by 12% SDS-PAGE carried out according to Laemmli [27]. Protein gels were stained with

Coomassie brilliant blue R250. The expression was evaluated by comparing these samples with the control (not induced by IPTG) to observe whether an additional band corresponding to the target gene was present.

The solubility of the expressed protein was identified as follows. Collected cells were suspended in PBS buffer (phosphate-buffered saline, pH 7.4) and sonicated (on for 8 sec, off for 10 sec; 100 cycles) on ice, and then a 1-ml aliquot of lysate was taken and centrifuged at 14,000 rpm for 30 min. The pellet was resuspended in PBS buffer. The supernatant and pellet of the recombinant protein were analyzed by SDS-PAGE to test the solubility.

Purification of Xen-GST-SYNB. For large-scale expression and purification, an Erlenmeyer flask containing 1 liter of LB broth supplemented with 100 μg/ml ampicillin was inoculated with 25 ml of the overnight culture (pGEX-4T/xSYNB) and grown at 37°C to A_{600} of 0.6, at which time IPTG was added (final concentration 1 mM) to induce expression of the Xen-GST-SYNB fusion protein, and the cells were grown at 30°C for an additional 4 h before they were harvested by centrifugation. The pellet was resuspended in 25 ml PBS buffer, 200 μg/ml lysozyme was added to the resuspended bacteria, and the bacteria were incubated on ice for 20 min. The bacteria were further disrupted using sonication as described above. The soluble protein from the cell lysate was collected in the supernatant after centrifugation at 14,000 rpm for 30 min at 4°C and stored at 4°C until further use.

The purification procedures for Xen-GST-SYNB were carried out according to the instruction of GST Purification Kit (Clontech, Japan) with some modifications. The supernatant containing the fusion protein was loaded at room temperature onto a prepacked TALON® 2 ml Disposable Gravity Column to be mixed with Glutathione–Uniflow Resin equilibrated with 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.5). The column was washed by 10 column volumes of equilibration buffer. The Xen-GST-SYNB fusion protein was eluted with 50 mM Tris-HCl containing 10 mM glutathione (pH 8.0), fractions were collected, both the presence of recombinant protein in the eluted fractions and the purity of the protein were assessed by 12% SDS-PAGE, and the protein concentration was determined according to Bradford [28].

Mass spectrometric analysis of the purified Xen-GST-SYNB. A sample of the purified Xen-GST-SYNB was separated in 12% SDS-PAGE and stained with Coomassie brilliant blue R-250. The Coomassie-stained band was excised from SDS-PAGE gels, minced, reduced, alkylated with iodoacetamide, and digested overnight with 50 ng trypsin (Promega, USA). The resulting peptides were extracted with 50% acetonitrile in 5% trifluoroacetic acid and then purified and concentrated using ZipTip® pipette tips (Millipore, USA) following the manufacture's directions. The peptides eluted from the ZipTip® tips were then ready for mass-spectrometric analysis. ESI mass spectra were recorded on a Waters micromass Q-TOF premier (Waters Corporation, USA) using the reflectron detector in positive mode. The device was calibrated using peptides of known masses. Protein database search was performed with MASCOT (http://www.matrixscience.com).

Production of antibodies against Xen-GST-SYNB and Western blot analysis. The recombinant protein identified by mass spectrometry was further used for raising antibodies in a New Zealand white rabbit. The rabbit was first immunized using 100 μg of recombinant protein in Freund's complete adjuvant. After two weeks, the rabbit was boosted three times with 50 μg recombinant protein each in incomplete Freund's adjuvant at a two-week interval, and serum was obtained after the last bleeding. Antibody titer was determined by ELISA and was found to be 1 : 12,800. For Western blot, the purified recombinant protein was separated on 12% SDS-PAGE and electrophoretically transferred onto PVDF (polyvinylidene fluoride) membrane. After overnight blocking with 5% non-fat milk in TBS-T (Tris-buffered saline containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.6, and 0.1% Tween 20), the membrane was incubated with antiserum to the recombinant protein at a dilution of 1 : 2000 for 1 h at room temperature. Washed three times with TBS-T, the membrane was incubated with 1 : 5000 diluted goat anti-rabbit IgG conjugated with horseradish peroxidase (ZSGB-BIO, China) for 1 h. Finally, the membrane was washed two times with TBS-T and once with TBS and then developed in buffer containing 3,3′-diaminobenzidine (DAB) until brownish bands were observed. Color development was then terminated by thorough washing in TBS.

Expression of xSYNB in *Xenopus laevis* brain and other tissues. *Xenopus laevis* was bred in our laboratory. Tissues of *X. laevis* including brain, liver, and heart were excised, washed with cold physiological solution, and homogenized in 500 μl of 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 10 mM EDTA in TBS, pH 7.4, containing a protease inhibitor cocktail. The material was centrifuged at 15,000 rpm for 1 h at 4°C, and the protein concentration was determined according to Bradford [28]. Proteins were separated in 12% SDS-PAGE followed by Western blot as described above.

RESULTS AND DISCUSSION

Cloning of xSYNB and construction of expression plasmid pGEX-4T/xSYNB. The strategies of xSYNB gene cloning and expression vector constructing were described in experimental procedures. On the basis of the cDNA sequence of xSYNB, we designed primers to amplify the xSYNB coding region from total RNA isolated from *X. laevis* brain. The amplified product shown in

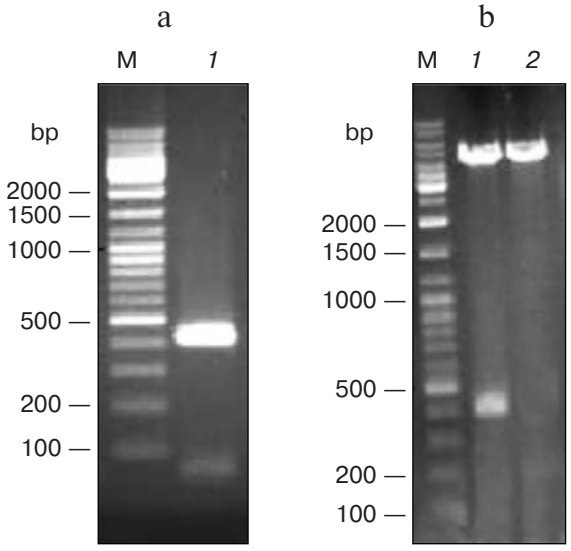

Fig. 1. Cloning of xSYNB and construction of the expression vector pGEX-4T/xSYNB. a) Agarose gel electrophoresis of RT-PCR product of xSYNB. Lanes: M) DNA marker; *1*) RT-PCR product (ORF 405 bp). b) Agarose gel electrophoresis analysis of the expression vector construction. Lanes: M) DNA marker; *1*) double restriction enzyme digestion of pGEX-4T/xSYNB (*Bam*HI, *Xho*I); *2*) pGEX-4T/xSYNB.

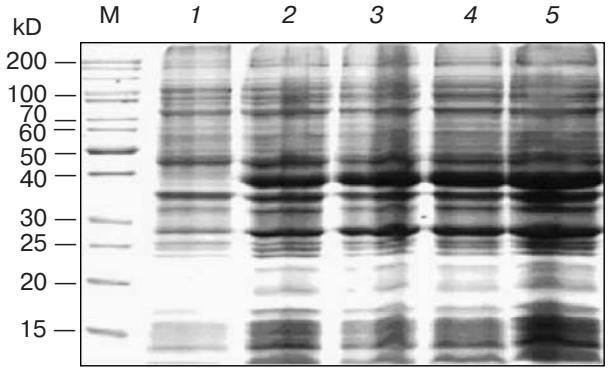

Fig. 2. SDS-PAGE analysis of recombinant xSYNB produced by *E. coli*. Lanes: M) molecular markers; *1*) total bacterial protein as control without IPTG induction; *2-5*) total bacterial protein after IPTG induction (1-4 h). Recombinant xSYNB was expressed 1 h after induction.

Fig. 1a was about 410 bp long, with a *Bam*HI and a *Xho*I site at the 5′ and 3′ end, respectively. The amplified fragment was first cloned into pGEM-T Easy vector then subcloned into the expression vector pGEX-4T-1. The resulting expression plasmid (shown in Fig. 1b), which contains a GST tag and one thrombin recognition site in frame with the 5′ end of the coding region of xSYNB, was named pGEX-4T/xSYNB and predicted to encode a recombinant protein with a molecular weight of 40 kD.

Expression and purification of SYNB from *X. laevis*. The expression of xSYNB was investigated in *E. coli* strain BL21(DE3) as described in the experimental procedures. On analysis of SDS-PAGE, the predicted 40 kD recombinant protein band was visualized as shown in Fig. 2, while overexpressed protein was not detected in the negative control *E. coli* BL21(DE3) cells (not induced by IPTG). The result also confirmed that recombinant protein was in-frame with the GST-tag. A time course of expression to determine the optimal induction time for maximum expression of protein was established by taking aliquot of cells at 1, 2, 3, and 4 h after induction with IPTG and analyzed by SDS-PAGE. The analysis revealed that maximum protein induction could be achieved at the fourth hour (shown in Fig. 2).

To examine the relative distribution of the expressed Xen-GST-SYNB fusion protein in the soluble and insoluble fractions, we examined both the supernatant and the pellet of the cell lysate after sonication to detect the recombinant protein. The result of solubility examination indicates that xSYNB is partially soluble expressed in BL21(DE3) as shown in Fig. 3a. To examine the effect of temperature on xSYNB soluble expression, induction was carried out at 30 and 37°C, respectively. Unfortunately, no observable difference in the solubility of the protein was seen (data not shown). However, the rate of protein synthesis is decreased with slower rate of cell growth, and thus sufficient time for proper protein folding is provided, increasing the possibility of the protein being present in the native form and thus being available for purification under native conditions. So the final induction condition used was 1 mM IPTG, 30°C for 4 h.

GST fusion proteins can be purified from crude bacterial protein extracts by one-step affinity chromatogra-

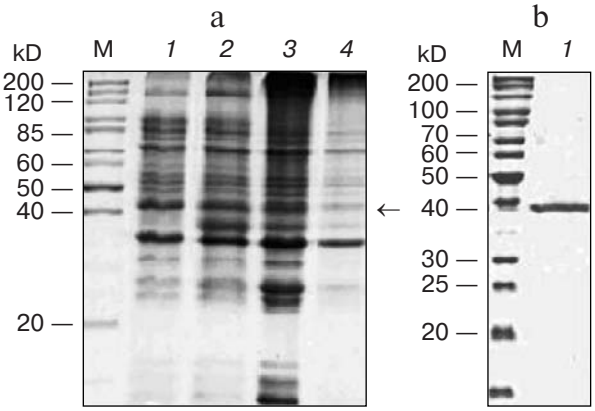

Fig. 3. SDS-PAGE analysis of the expression, solubility identification, and purification of xSYNB in *E. coli* BL21(DE3). a) Lanes: M) molecular markers; *1*) total bacterial protein as control without IPTG induction; *2*) total bacterial protein after IPTG induction; *3*) precipitate of total bacterial protein containing xSYNB after IPTG induction; *4*) supernatant of total bacterial protein containing xSYNB after IPTG induction. Arrow indicates the position of Xen-GST-SYNB on SDS-PAGE (about 40 kD). b) Lanes: M) molecular markers; *1*) affinity-purified Xen-GST-SYNB.

phy using glutathione-Sepharose [29]. For purification of Xen-GST-SYNB, cells collected from 1 liter LB culture were sonicated. As the soluble fraction contained a reasonable amount of Xen-GST-SYNB, the recombinant protein was purified from the supernatant of the lysate by affinity chromatography using a Glutathione–Uniflow Resin column as described in the "Materials and Methods" section. After purification, elution fractions were analyzed for xSYNB production by 12% SDS-PAGE, and protein concentration was determined using Bradford reagent. As expected, the eluted protein showed a single band at the expected molecular mass (40 kD) on SDS-PAGE (Fig. 3b). The purity of the protein was estimated to be higher than 90% by SDS-PAGE.

Mass spectrometric identification of xSYNB. To further characterize the overexpressed protein, the purified Xen-GST-SYNB was subjected to mass spectrometric analysis. Identity between the xSYNB of the purified fusion protein and the expected product was confirmed as shown in Fig. 4, the result suggesting that the part of the purified Xen-GST-SYNB fusion protein excluding the GST tag was unambiguously xSYNB.

Production of antibodies against Xen-GST-SYNB and Western blot analysis. Although antibodies against β-synuclein from mammals (e.g. pan synuclein antibodies from Abcam, which were obtained against the synthetic peptide AKEGVVAAAEKTKQGV corresponding to amino acids 11-26 of the human synuclein protein) were available, they were predicted to react with *X. laevis* due to sequence homology. We decided to produce polyclonal antibodies against the full-length β-synuclein from *X. laevis*. This decision was based on the following considerations: (i) potentially more effective detection of β-synuclein in *X. laevis* compared with the commercial antibodies described above; (ii) enhancement of immunogenicity when the full-length β-synuclein was used as the optimal immunogen; (iii) other applications of the antibodies in investigating the unknown functions of β-synuclein. The polyclonal antibodies can be used to study the interacting protein of β-synuclein. Because of the involvements of the synuclein family in some neurodegenerative diseases, research on members of the synuclein family and their interacting proteins have been carried out.

Previous studies have shown that synphilin and myocilin are interacting proteins of α-synuclein and γ-synuclein, respectively [30-33]. However, to date no studies have been reported on an interacting protein of β-synuclein. The potential binding partners of β-synuclein can be obtained using co-immunoprecipitation with the polyclonal antibodies against β-synuclein or GST-pull

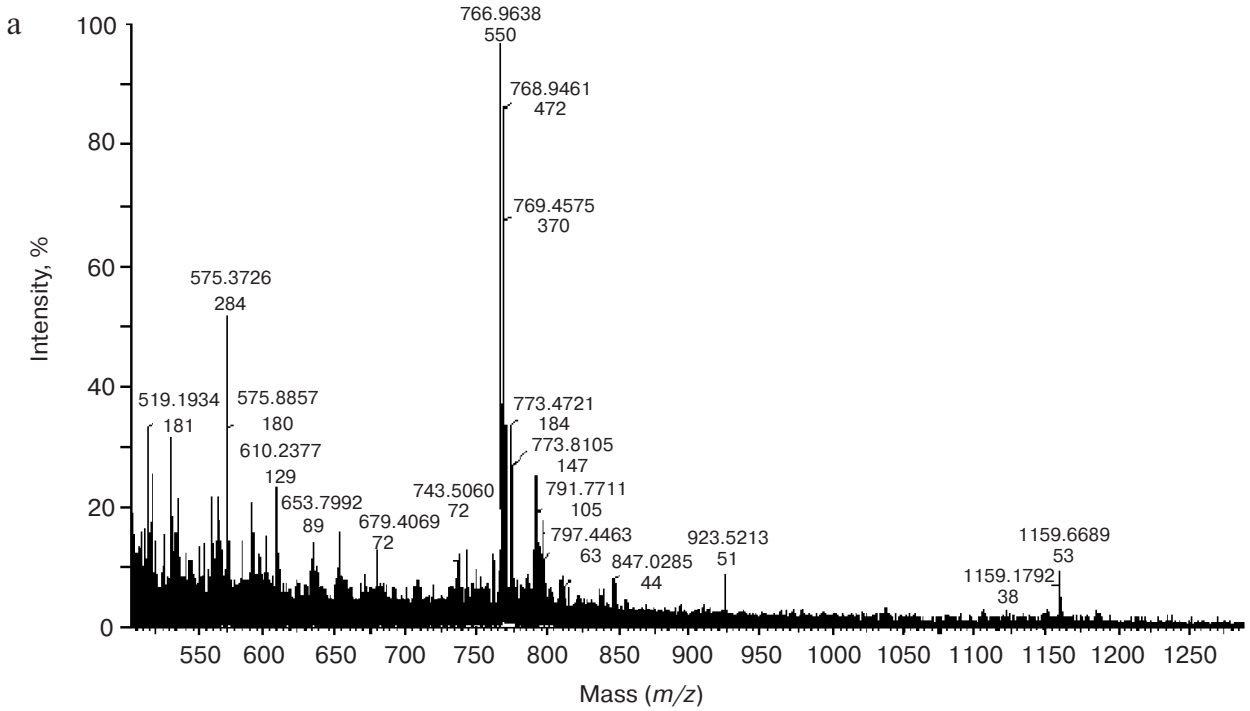

Fig. 4. ESI mass spectrum of the tryptic digest of Xen-GST-SYNB. a) ESI spectrum. Masses are indicated above the peaks. b) Matched peptide fragments of xSYNB. The matched portions are shown in bold and underlined.

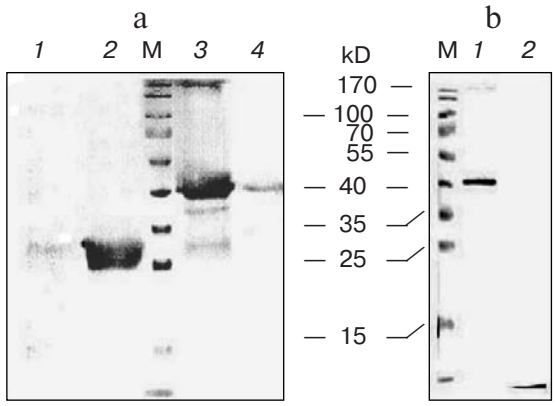

Fig. 5. Western blot analysis of reactivities of polyclonal antibodies raised against Xen-GST-SYNB. a) Western blot analysis using polyclonal antiserum to recombinant protein expressed in *E. coli*. Lanes: *1*) cells carrying pGEX-4T-1 vector before IPTG induction; *2*) cells carrying pGEX-4T-1 vector after IPTG induction; M) prestained protein molecular weight markers; *3*) cells carrying pGEX-4T/xSYNB vector after IPTG induction; *4*) cells carrying pGEX-4T/xSYNB vector before IPTG induction. b) Western blot analysis using purified Xen-GST-SYNB. Lanes: M) prestained protein molecular weight markers; *1*) polyclonal antiserum; *2*) pre-immune serum.

down with the purified Xen-GST-SYNB in cell-free *X. laevis* egg extracts. In addition, the polyclonal antibodies would serve as a tool to further investigate unknown functions of β-synuclein in the early development of *X. laevis*. Additional information about physiological functions of β-synuclein can be obtained by injecting embryos with the polyclonal antibodies, which works by interfering with the endogenous β-synuclein. The blocking effect of the polyclonal antibodies against the full-length β-synuclein on endogenous β-synuclein may be better than that of those antibodies against a special peptide of synucleins, thus providing possible additional evidence in investigating the unknown function of β-synuclein.

Base on these considerations, we subsequently used the entire fusion proteins for animal injections. After repeating the immunization of rabbits with the purified Xen-GST-SYNB protein, we generated the polyclonal antibodies against Xen-GST-SYNB.

When GST and Xen-GST-SYNB expressed in *E. coli* strain BL21(DE3) were subjected to Western blot analysis with the polyclonal antibodies, we observed two main bands whose molecular weights were 26 and 40 kD, respectively. As shown in Fig. 5a, the 26 kD band corresponds to the GST tag, whereas the 40 kD band corresponds to the calculated molecular weight of GST-SYNB. The result showed that the anti-Xen-SYNB polyclonal antibodies could not only recognize GST protein but also recognize GST-SYNB. As the polyclonal antiserum was generated with GST-SYNB fusion protein, anti-GST was also present in the antisera. The specificity of the antibodies was further checked by immunoblot analysis using the purified Xen-GST-SYNB. As expected, a 40 kD band, which corresponded to GST-xSYNB, was observed. The same sample was also incubated with the pre-immune serum, but no reaction was observed (shown in Fig. 5b). The results indicate that the polyclonal antibodies are specific for the antigen (Xen-GST-SYNB). These results provide direct evidence that the polyclonal antibodies can react with the xSYNB *in vitro*, probably leading to better specificity of reacting with *X. laevis* compared with the prediction that the commercial

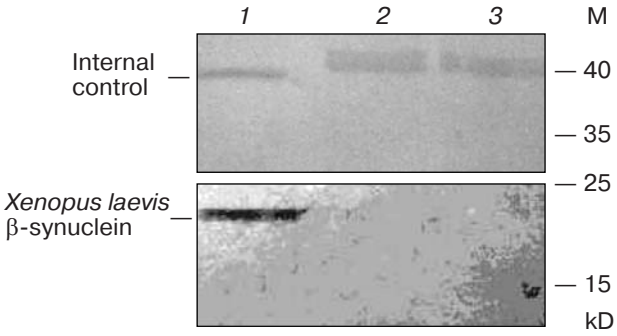

Fig. 6. Western blot analysis of β-synuclein expression in *X. laevis* tissues. Proteins extracted from brain, heart, and liver of *X. laevis* were separated in 12% SDS-PAGE, followed by Western blot onto a PVDF membrane using anti xSYNB polyclonal antibodies (rabbit antiserum). M, protein molecular mass markers. Lanes: *1*) brain; *2*) heart; *3*) liver. The apparent nonspecific band of the three tissues was used as an internal control. Experiments were repeated three times with similar results.

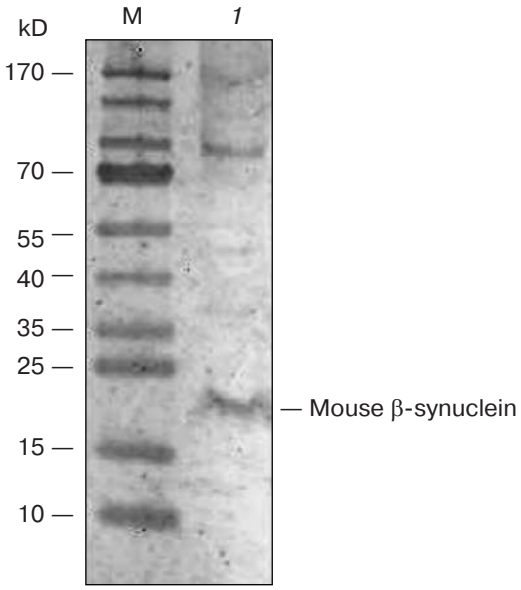

Fig. 7. Western blot analysis of β-synuclein expression in *M. musculus* brain. Protein extracted from *M. musculus* brain was separated in 12% SDS-PAGE followed by Western blot onto a PVDF membrane using anti-xSYNB polyclonal antibodies. Lanes: M) protein molecular mass markers; *1*) *M. musculus* brain.

antibodies react with synucleins of *X. laevis* due to sequence homology.

Expression of SYNB in *X. laevis* brain and other tissues. To further examine whether β-synuclein was expressed at the protein level *in vivo* in *X. laevis*, we decided to detect it by western blot analysis using the polyclonal antibodies produced in this work. Western blot analysis of the total proteins extracted from *X. laevis* brain, heart, and liver were immunodecorated using the polyclonal antibodies against xSYNB. As shown in Fig. 6, an immunoreactive band with a molecular weight of about 19 kD was detected in brain, while no immunoreactive bands (the corresponding position) were detected in liver and heart. Using amino acid alignments, we found that in *X. laevis* β-synuclein was 58.6 and 51.0% identical to α- and γ-synuclein, respectively, which was similar to that of *H. sapiens* and *M. musculus*, i.e. in *H. sapiens* the identities of β-synuclein to α- and γ-synuclein were 59.3 and 47%, while they were 57.9 and 48.5% in *M. musculus*. Based on the alignment results and the use of full-length recombinant β-synuclein as immunogen, it seems that additional cross-reactivity may exist, i.e. the polyclonal antibodies against β-synuclein possibly recognize both α- and γ-synuclein. However, our data has shown that there is only one brain-specific immunoreactive band with a molecular weight of about 19 kD. Due to high degree of homology of β-synuclein between *M. musculus* and *X. laevis*, we decided to examine whether there was a similar result in *M. musculus*. Western blot analysis of the total proteins extracted from *M. musculus* brain with the poly-

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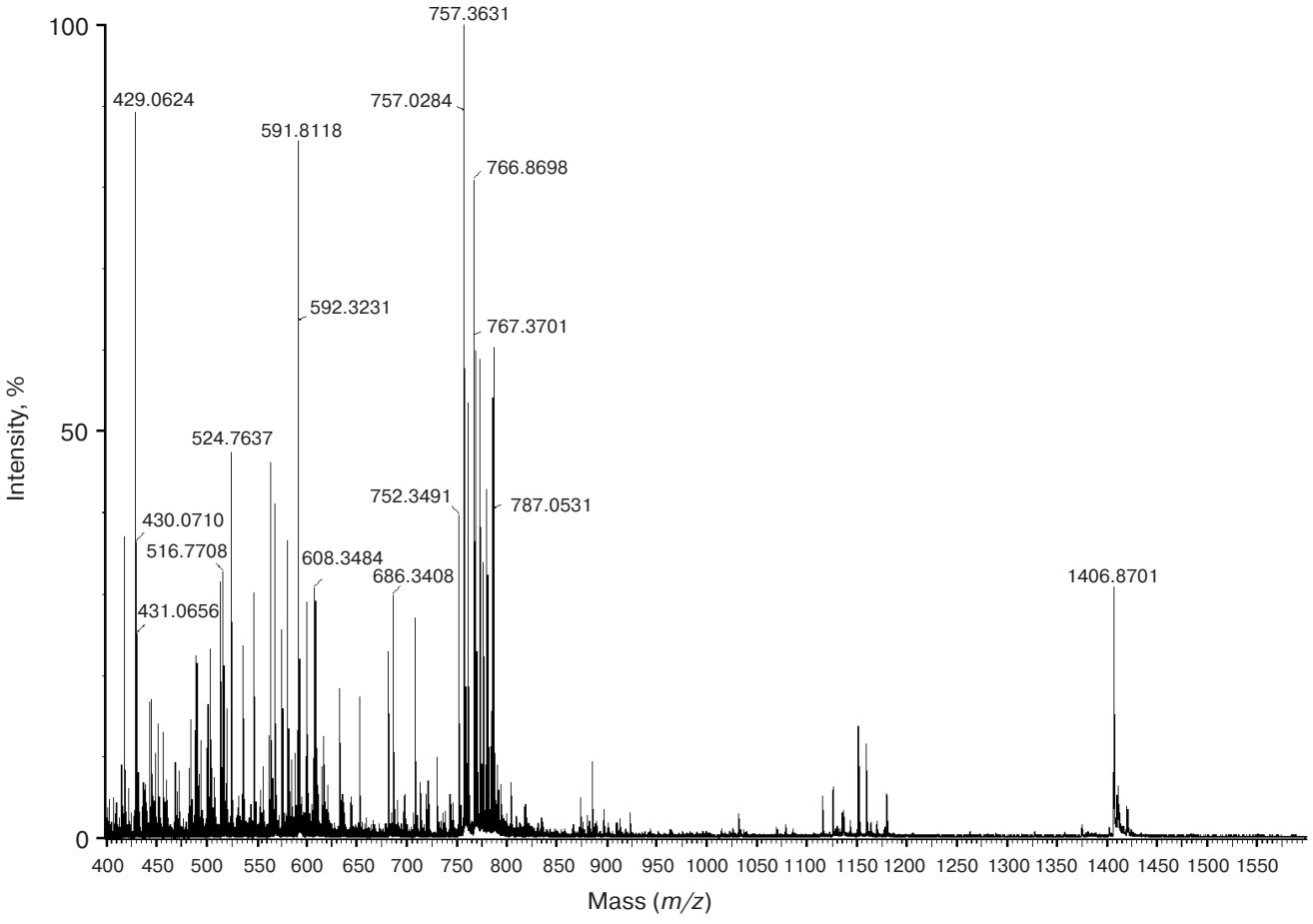

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Fig. 8. ESI mass spectrum of the tryptic digest of *X. laevis* β-synuclein. a) ESI spectrum. Masses are indicated above the peaks. b) Matched peptide fragments of *X. laevis* β-synuclein. The matched portions are shown in bold and underlined. The peptide shown in italics is different from that of *X. laevis* α-synuclein.

clonal antibodies against xSYNB showed that there is also only one immunoreactive band with a molecular weight of about 19 kD in *M. musculus* (shown in Fig. 7). This result is very surprising given the extent of sequence conservation and the high similarity among α-, β-, and γ-synuclein.

However, some rational explanations for this conundrum may be offered as follows: (i) the expression and intracellular localization of α- and β-synuclein are similar, both proteins being expressed within the central nervous system and concentrated within presynaptic terminals [4, 8, 34, 35]. By contrast, γ-synuclein is most abundant within the peripheral nervous system and is mainly cytosolic [34, 36]; (ii) on SDS-PAGE, both native and recombinant α- and β-synuclein run with an apparent molecular mass of about 19 kD, thus leading to difficulty in distinguishing between α- and β-synuclein [4]; similar results were also observed by other authors [37, 38]; (iii) with 78% similarity, β-synuclein has been called an "almost carbon copy" of α-synuclein [39]. So, it was not trivial to generate polyclonal antibodies that clearly distinguish the two forms [4, 40]. To further investigate whether *X. laevis* β-synuclein was indeed included in the protein corresponding to the brain-specific immunoreactive band, the protein sample on SDS-PAGE, whose position corresponded to the position of the brain-specific immunoreactive band of western blot, was subjected to mass spectrometric analysis. As expected, identity between the protein and *X. laevis* β-synuclein was confirmed as shown in Fig. 8. The result indicated that *X. laevis* β-synuclein was indeed expressed at the protein level in *X. laevis* brain.

The observed molecular mass of β-synuclein is higher than the calculated molecular mass of *X. laevis* β-synuclein (14 kD). Jakes et al. have shown that although the synucleins are small proteins having a calculated molecular mass close to 14 kD, their apparent molecular mass is ~19-20 kD, indicating that they are post-translationally modified [4]. Nakajo and colleagues have shown that β-synuclein is phosphorylated in the rat brain, presumably by the Ca^{2+}-calmodulin protein kinase II, and a possible phosphorylation site is the serine residue at position 118, which is conserved throughout species in the β-synuclein sequence but not in the α- or γ-synuclein [2, 41]. Based on these result and the previous observations by other authors, it is suggested that β-synuclein may be post-translationally phosphorylated *in vivo* in *X. laevis* as shown in other species [2, 4, 30]. The result that no immunoreactive bands (the corresponding position) were detected in the case of heart and liver suggested that in adult *X. laevis* β-synuclein was specifically expressed in brain. This confirms previous observations by other authors reporting that the expression of β-synuclein is tissue-specific, i.e. only expressed in brain in mammals [7, 8]. To our knowledge, it is the first time that β-synuclein is detected at the protein level in *X. laevis*.

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