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Expression and Purification of The Third P Region of α1B Subunit of N-type Calcium Channel of *Rattus norvegicus**

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Abbreviations *E. coli, Escherichia coli*; Cys, cystine; Cav22P, the third P region of the α1B subunit of the N-type calcium channel of *Rattus norvegicus*; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; aa, amino acid(s); ORF, open reading frame; GST, glutathione S-transferase; HIS, poly histine tag; GEB, glutathione elution buffer; TBS, Tris buffer solution; LB, Luria-Bertani; YT, Yeast Extract Tryptone.

Abstract The DNA fragment coding the third P region of α1B subunit of N-type voltage-gated calcium channel of *Rattus norvegicus* (Cav22P for short) was amplified by high fidelity PCR, inserted into vectors pET28b and pGEX-4T-1 respectively, and expressed in *Escherichia coli* Rosetta. The expressed product HIS-Cav22P mostly deposited in an inclusive body. The inclusive body of HIS-Cav22P dissolved in urea buffer. After dilution and dialysis, the refolded protein HIS-Cav22P was finally purified *via* Histrap chelating HP column. Ultraviolet spectroscopy results demonstrated that HIS-Cav22P protein can bind to calcium ions reversely. The expressed product GST-Cav22P was purified from the supernatant of cell lysate using Glutathione Sepharose 4B column. However, GST-Cav22P degraded severely, which caused it difficult to purify the protein Cav22P and the GST pull-down assay. All the results suggest that the active recombinant proteins of Cav22P may act as a molecular target for high through screen of non-narcotic analgesic drugs.

Key words expression, purification, N-type calcium channel
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N-type calcium channel (referred as Cav2.2) is a kind of conserved voltage-gated calcium channel. It can mediate a variety of cell functions of neurons, especially neurotransmitters release. The N-type calcium channel comprises a pore-forming subunit α1 (encoded by the α1B gene) and ancillary subunits β, α2δ and, possibly, γ. The α1 subunit contains four functional districts (Ⅰ～Ⅳ), each of which has six transmembrane segments (S1～S6). S4 is the voltage-sensing region. The cytoplasmic fragments between functional districts Ⅲ and Ⅳ constitute the inactivation gate[1]. The connection loop between S5 and S6 has a special structure known as the P region. The third P region of the α1B subunit of N-type calcium channel has a high similarity with some proteins such as troponin, calmodulin and small albumin, in which a kind of typical Ca^{2+}-binding domains——EF-hand motif——is widely present. Thus, the third P region in the calcium channel is a crucial target to clarify the mechanism of ion selection and voltage sensitivity.

Electrophysiological experiments have demonstrated that ω-conotoxins can selectively inhibit N-type calcium flow[2~4], thereby blocking the release of the neurotransmitters which is triggered by the initial hurt feeling, thus keeping pain signals from transmitting into the brain. The ω-conotoxins consist of three pairs of disulfide bonds whose cross-linking models are characterized as Cys1-Cys4, Cys2-Cys5 and

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Cys3-Cys6[2]. There is a new recombinant conopeptide SO3. This conotoxin was acquired by gene cloning from the South China Sea lineolatus Conus (*Conus striatus*) and peptide synthesis. The conotoxin SO3 also belongs to ω-conotoxins[2].

Site-directed mutagenesis in the EF-hand domain of the third P region of the α1B subunit of the N-type calcium channel (usually with calcium ions) leads to the reduction of the blocking effects of the conotoxins such as MVIIA[5~7]. Basing on computer simulation, Yue *et al*.[8] proposed that the binding site in the N-type calcium channel of conotoxins should be the third P region of the α1B subunit of the N-type calcium channel. Therefore, it is of great scientific interest to demonstrate that the ω-conotoxins interact with the third P region of the α1B subunit of the N-type calcium channel. The study of the structure and biological activity of the third P region of the α1B subunit of the N-type calcium channel will provide useful and reliable theoretical basis for designing safe and effective small compounds for development of non-narcotic analgesic drugs, which are cheaper and have less side effects than chemically synthetic peptides.

In this report, two types of expression vectors for the third P region of the α1B subunit of the N-type calcium channel of *Rattus norvegicus* (Cav22P for short), pET28b-Cav22P and pGEX-4T-Cav22P were constructed respectively. Through optimization of the conditions for expression, purification and refolding, highly pure soluble recombinant proteins of HIS-Cav22P were obtained. It has been demonstrated by ultraviolet spectroscopy analysis that HIS-Cav22P can bind calcium ions reversely. The active recombinant proteins Cav22P provide a basis for detection of the interactions between conotoxins and N-type calcium channel by protein-protein interaction assays such as pull-down assay, SPR and crosslinking. It would give a direct evidence of the molecular mechanism of antalgic effects of the conotoxins and bring a promising prospect for establishment of a technology platform of high through screen of natural non-narcotic analgesic drugs.

1 Materials and methods

1.1 Prediction of the hydrophobicity and the structure of the protein

Since many factors have influences on recombinant protein expression, simulation and prediction by bioinformatical softwares can help us in choosing feasible methods to acquire soluble and active proteins. Therefore protein hydrophobicity was predicted by Kyte-Doolittle hydropathy plot analysis in the Protscale program on the Expasy server (http://au.expasy.org/tools/protscale.html). Then, the secondary structure of Cav22P was predicted with the software SSpro version 4.5 on the Stratch Protein Predictor server (http://scratch.proteomics.ics.uci.edu/).

1.2 PCR amplification

The fragment coding for the third P region (aa1302 ~ 1388) of the α1B subunit of the N-type calcium channel (GenBank No: AF055477) was amplified with a pair of specific primers: a forward primer with 5′ cg*ggatcc*aaaagggaagttc 3′ and a reverse primer with 5′ cg*ctcgag*cttcatccaaaac 3′. The 5′ *Bam*H Ⅰ and 3′ *Xho* Ⅰ restriction sites are shown in italics and a rare code CTG is changed into CTT. The vector α1B clone, which is a gift from Professor Diane Lipscombe of Brown University, contains the full length of the cDNA of α1B subunit of the N-type calcium channel of the *Rattus norvegicus* to be used as PCR template. PCR was performed using a TGradient Thermocycler (Biometra, Gottingen, Germany) programmed for an initial denaturation (95 ℃ for 5 min), followed by 30 cycles for 95℃ for 30 s, 58℃ for 30 s and 72℃ for 40 s. A final extension step was conducted at 72℃ for 7 min. The PCR product with an expected size was cloned into pGM-T Easy Vector (Tiangen, Beijing, China) and sequenced.

1.3 Vectors construction

The pGM-T Easy Vector containing the aimed fragment was digested by double enzymes *Bam*H Ⅰ and *Xho* Ⅰ. Then, the fragment coding for the third P region of the α1B subunit of the N-type calcium channel was ligated overnight at 16℃ into the pET28b vector (Novagen, Germany) and then transformed into *Escherichia coli*(*E. coli*) JM109. The correct ORF was confirmed by restriction analysis and sequencing. The recombinant vector was named as vector pET28b-Cav22P. The GST fusion protein expression vector was constructed by the same strategy with a different forward primer (5′ cg*ggatcc*aaagggaagttc 3′) and the expression vector of pGEX-4T-1.

1.4 Optimization of fusion protein expression

Escherichia coli Rosetta cells were transformed with the vector pET28b-Cav22P. The recombinant colonies were selected on Luria-Bertani medium (LB) agar plate containing Kanamycin (50 mg/L). To

determine the best condition of the fusion protein expression, the bacteria were induced with 1 mmol/L IPTG when the optical density of the bacteria reached 0.6. The bacteria were then incubated at 37℃, 30℃, 28℃, 25℃ and 20℃ respectively with shaking at 200 r/min till the A_{600} measurements reached 20. Other becteria were induced with 0.4 mmol/L or 0.6 mmol/L IPTG when the A_{600} measurement reached 0.6 and shaken at 37℃ or were induced with 1 mmol/L IPTG when the A_{600} measurement reached 0.8 or 1.0 and shaken at 37℃. The cells of each condition were harvested separately by centrifugation at 4℃ and disrupted by sonication. The proteins in the supernatant and the pellet were separated by centrifugation and analyzed by 15% SDS-PAGE. The same experiment was done with pGEX-4T-Cav22P using Ampicilin at working concentration of 60 mg/L.

1.5 Expression and purification of HIS-Cav22P

Ten of *E. coli* single colonies containing the recombinant vector pET28b-Cav22P were pre-cultured in 100 ml LB medium at 37℃ and 200 r/min for overnight. The bacteria were collected by centrifugation and then diluted in 600 ml of Yeast Extract Tryptone medium (2×YT) and supplemented with Kanamycin of 100 mg/L and glucose to 1%. The culture was grown in a 3 L shaking incubator at 37℃ and 200 r/min till the cells reached midlog growth (A_{600} measurements of 0.6～1.0). Then, the expression of the target protein was induced by adding IPTG to final concentration of 0.4 mmol/L. After incubation at 37℃ for 4 h, the cells were harvested by centrifugation and resuspended in 30 ml Tris-HCl buffer (containing 10 mmol/L PMSF) and were sonicated (5 s work and 5 s rest for 80 cycles, 5 min cool on ice and then another 80 cycles). The lysate was clarified by centrifugation for 30 min at 10 000 r/min and 4℃. Then, 5 ml of the supernatant (added with 50 μmol/L β-mercaptoethanol, 100 μmol/L imidazole, 50 μl Triton X-100) was loaded onto a 1 ml Histrap chelating HP column (Amersham Biosciences, USA). Next, the column was washed with 5 ml 100 mmol/L imidazole elution buffer. Finally, the fusion protein HIS-Cav22P was washed out with 3 ml 110 mmol/L imidazole elution buffer. The inclusive body of HIS-Cav22P was washed by 1 mol/L Urea buffer and centrifuged at 10 000 r/min and 4℃, for 20 min. 40 ml 8 mol/L Urea buffer was added to the pellet to dissolve the inclusive body by shaking the tube overnight. The supernatant was acquired by centrifugation at 4℃ and 10 000 r/min for 30 min. It was then injected extremely slowly at the rate about 1 ml/min into 120 ml Tris-HCl. The solution was then diluted in Tris-HCl buffer, changing with fresh buffer each 4 h for three times. The purification of the refolded protein was done in the same purification procedure as the soluble HIS-Cav22P. The purity of the protein was detected by 15% SDS-PAGE. The protein concentration was determined by Bradford method.

1.6 Expression and purification of GST-Cav22P and Cav22P

The same method as the expression operations of HIS-Cav22P was used for expression and purification of GST-Cav22P. The 2×YT medium for *E.coli* Rosetta containing pGEX-4T-Cav22P was supplied with Ampicilin of 120 mg/L. When the A_{600} measurements reached 0.6, IPTG was added to final concentration at 0.1 mmol/L and the bacteria were incubated at 25℃ for 8 h. Then the bacteria were harvested by centrifugation. The cells was resuspended in 30 ml PMSF-containing PBS buffer and lysed *via* sonication. After centrifugation, 10 ml of the supernatant was filtered and injected through the pre-balanced 1 ml Glutathione Sepharose 4B column. The column was then washed with 30 ml PBS and then added 1 ml GEB for 10 min incubation at 4℃. The fractions were collected by repeat 10 times. Some GST can be separated by molecule sieve sepharose G-75 at 4℃. The recombinant protein GST-Cav22P was digested by thrombin at 4℃ for 24 h, 16℃ for 12 h, 22℃ for 10 h, and 37℃ for 4 h respectively. The digested protein solution was filtered *via* Glutathione Sepharose 4B column to collect the protein Cav22P. The purity of the protein was detected by 15% SDS-PAGE.

1.7 Ultraviolet spectroscopy

To observe the calcium ion binding activities of the recombinant proteins of Cav22P, two kinds of UV spectrums of the proteins were obtained by standard program of Ultrospec 3000 UV/Visible Spectrophotometer (Amersham, Piscataway, NJ, USA) at room temperature: (i) Wavescan from 200 nm to 400 nm of the recombinant protein HIS-Cav22P (50 mg/L) with EGTA(0.1 mmol/L) in PBS buffer as the reference solution was EGTA(0.1 mmol/L) in PBS buffer; (ii) Wavescan from 200 nm to 400 nm of the recombinant protein HIS-Cav22P(50 mg/L)with $CaCl_2$ (0.1 mmol/L) in PBS buffer as the reference solution was $CaCl_2$ (0.1 mmol/L) in PBS buffer. EGTA is added to chelate the calcium ion in the solution. The

spectrums were collected and compared by overlapping the curves.

1.8 GST pull-down assay

First, 3 μg GST and 3 μg GST-Cav22P were separately put into two 1.5 ml EP tubes. Then, conotoxin SO3 was added into each tube at the molar ratio (protein∶peptide=1∶10). Next, 500 μl binding buffer [9] was added and mixed well. The tubes were then shaken at 4℃ for 2 h. After that, 50 μl well-regenerated 50% Glutathione Sepharose 4B beads was added in each tube, mixed well and shaken at 4℃ for 2 h. After centrifugation at 5 000 r/min and 4℃ for 5 min, the precipitant of each tube was resuspended with binding buffer, washed 3 min and centrifuged twice at 500 *g* for 5 min. The precipitant was added 50 μl GEB buffer and then loading buffer to be loaded in 15% SDS-PAGE gel at 10 mA for 50 min.

2 Results

2.1 Prediction of the hydrophobicity and the structure of Cav22P

Before constructing expression vector, Kyte-Doolittle hydrophobicity plot of the amino acid sequence of third P region of the α1B subunit of N-type calcium channel of *Rattus norvegicus* (Cav22P for short) was carried out. A small peak above 1.8 was presented in the position of 50～55 aa when a window size was set to 9 (Figure 1). It indicates that there may be a short transmembrane fragment in the Cav22P. The amino acid residues in the fragment (46～58 aa) were predicted to be hydrophobic. That suggests that Cav22P may have low solubility and would be expressed mostly in inclusive body. It is also shown that the first half of the protein Cav22P is very hydrophilic so that the recombinant protein Cav22P would be easier to be expressed than the proteins that have long hydrophobic regions near their N-terminals.

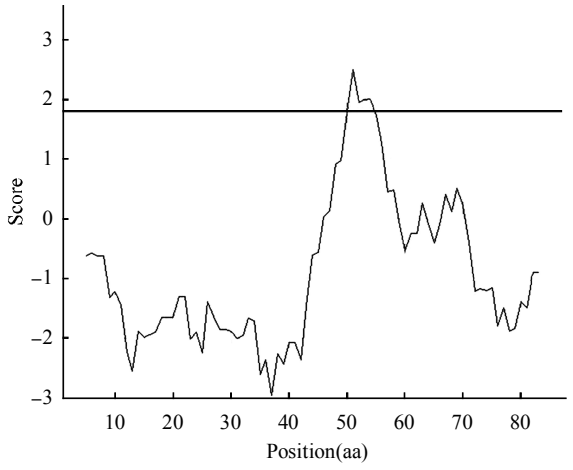

Fig. 1 Kyte-Doolittle hydrophobicity plot analysis of Cav22P

Window size is set at 9. Straight line indicates the threshold (value=1.8) for hydrophobic region.

As the protein Cav22P may be expressed mostly in the inclusive body, methods of refolding proteins need to be chosen. Hence the secondary structure of Cav22P was predicted as shown in Figure 2. Note that Cav22P consists of mainly α-helixes (32%) and extended strands (15%). The α-helixes are in the putative EF-hand motif and the second half part of the protein Cav22P. Usually, α-helixes are easier to be refolded than other kinds of secondary structures. Therefore, it should be practical to refold the recombinant Cav22P protein in the inclusive body into the state with natural conformation.

Predicted secondary structure:

1～35aa CCCEEEECCCCCCCHHHHCCCEEEECCCCCCCCCE

36～70aa EEECCCCCHHHHHHHHHHHHHHHCCCCCHHHHHHH

71～87aa HHCCCCCCCCCCCECC

Fig. 2 Secondary structure prediction of Cav22P

Letters H, E and C indicate α-helix (in the shadow), extended strand and the rest, respectively.

2.2 Expression of fusion protein HIS-Cav22P

A great deal of HIS-Cav22P protein was expressed in the presence of 0.4 mmol/L IPTG at 37℃ for 3 h. The molecular mass of HIS-Cav22P in the gel is about 15 ku that is very close to 14.925 ku, the molecular mass of HIS-Cav22P predicted with the software ExPASy (http://au.expasy.org/cgi-bin/pi_tool). The recombinant protein HIS-Cav22P is mostly in the inclusive body, the weight of which reached 20% of the total proteins in the pallet of the bacterial lysate (Figure 3). The results in lanes *3*, *4*, *8* and *9* in Figure 3b indicate that the amount of the soluble HIS-Cav22P decreases at low induction temperature and high initial A_{600} measurements. The proportion of the amount of HIS-Cav22P in the supernatant is not high enough for efficient purification. Therefore, GST fusion tag was used to increase the solubility of the recombinant protein.

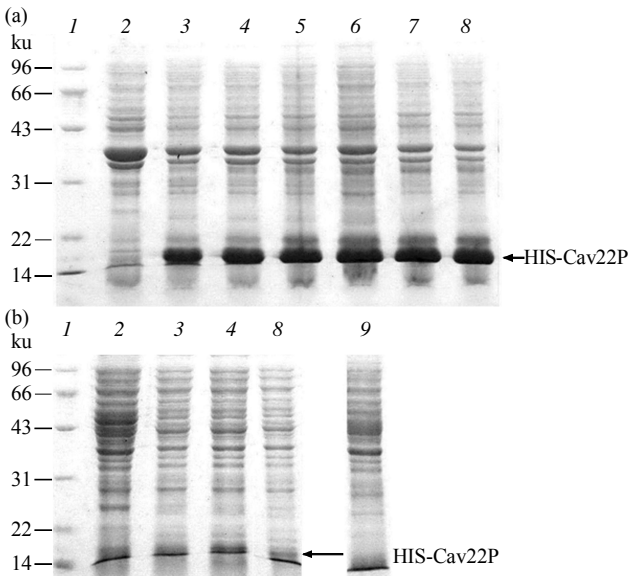

Fig. 3 SDS-PAGE analysis of the optimal conditions for HIS-Cav22P expression

The proteins in the pellet (Figure 3a) and the supernatant (Figure 3b) of the lysate of the bacteria with pET-28b-Cav22P vector are shown. The bacteria were incubated at 37℃ and induced at A_{600} of 0.6 with 1 mmol/L IPTG for 3 h (lane *4*). Others were prepared using the same method except that un-induced (lane *2*), incubated at 30℃ for 6 h (lane *3*), 0.6 and 0.4 mmol/IPTG (lanes *5* and *6*, respectively), initial A_{600} measurement of 0.8 and 1.0 (lanes *7* and *8*, respectively), incubated at 25℃ for 8 h (lane *9*). Protein molecular mass markers are shown in lane *1*. The induced HIS-Cav22P protein is indicated by the arrow on the right of the gel.

2.3 Expression of fusion protein GST-Cav22P

GST-Cav22P was highly expressed by induction of IPTG at very low final concentration of 0.2 mmol/L. The molecular mass of the recombinant protein GST-Cav22P in the gel is about 36 ku, in accordance with the predicted molecular mass of Cav22P that is 10.379 ku while GST is about 26 ku. When cultured at 37℃, GST-Cav22P was mostly in the inclusive body (lanes *2* and *3* in Figure 4a). The proportion of soluble GST-Cav22P in the supernatant of the bacteria lysate became higher at lower temperature and reached 15% at the temperature of 25℃. However, the incubation time of the cells increased exponentially. Since almost equal amount of GST protein and GST-Cav22P were in the whole proteins of the bacterial lysate, it is very arduous to purify GST-Cav22P. Sometimes, *E.coli* bacteria with GST fusion expression recombinant vectors do produce GST protein. It may be due to the ribosome leaves down from the plasmid when the GST is synthesized. In our study, the GST was degraded from GST-Cav22P because the amount of GST increased and GST-Cav22P decreased during the storage at –20℃ after GST-Cav22P was separated from GST *via* molecule sieve sepharose. It resulted in difficulties in the thrombin digestion and purification of Cav22P.

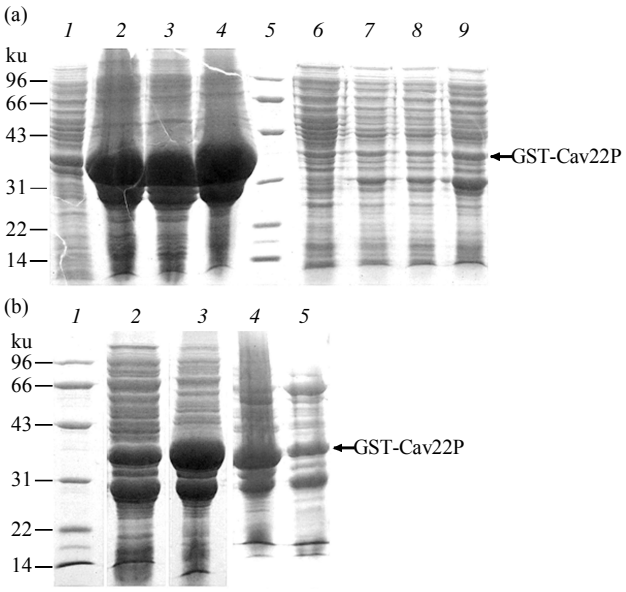

Fig. 4 SDS-PAGE analysis of the optimal conditions for GST-Cav22P expression

The proteins in the pellet of the lysate of the bacteria with pGEX-4T-Cav22P vector are shown in lanes *1*～*4* in Figure 4a and lanes *3* and *4* in Figure 4b. The proteins in the supernatant are shown in lanes *6*～*9* in Figure 4a and lanes *2* and *5* in Figure 4b. (a) The bacteria in Figure 4a were prepared in the following procedure: incubated at 37℃ and induced at A_{600} of 0.6 by 0.2 mmol/L IPTG for 3 h (lanes *2* and *7*), except that un-induced (lanes *1* and *6*), 0.4 mmol/L IPTG (lanes *3* and *8*), incubated at 30℃ for 6 h (lanes *4* and *9*). (b) The bacteria in Figure 4b were prepared at nearly the same method above except that incubated at 20℃ for 18 h (lanes *4* and *5*), 25℃ for 9 h (lanes *2* and *3*). The protein molecular mass marker is shown in lane *1*. The induced GST-Cav22P protein is indicated by the arrow on the right of the gel.

2.4 Purification of HIS-Cav22P

The recombinant protein HIS-Cav22P in the inclusive body was efficiently purified by Ni column. After dissolved by 8 mol/L urea buffer (lane *2* in Figure 5a) and diluted in 2 mol/L urea to let the protein correctly refolded and then diluted with Tris-HCl buffer (lane *3* in Figure 5a), the recombinant protein HIS-Cav22P took up about half amount of the whole proteins in the refolded solution. During the purification *via* Ni column, the impurity was washed away with the Tris-HCl buffer supplemented with imidazole at final concentration of less than

100 nmol/L. The soluble HIS-Cav22P was collected with Tris-HCl buffer supplemented with imidazole at final concentration of 110 nmol/L. The purity of the recombinant protein HIS-Cav22P is about 80%.

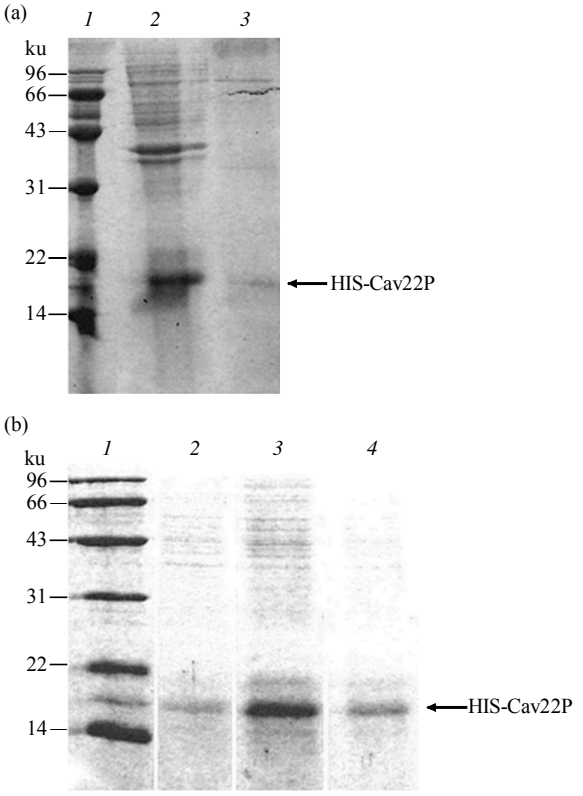

Fig. 5 SDS-PAGE analysis of the purified HIS-Cav22P

Protein molecular mass markers are shown in lane *1*. Lane *2* in Figure 5a represents the proteins in the pellet of the lysate of the bacteria with pET28b-Cav22P plasmid solved in 8 mol/L Urea. Lane *3* in Figure 5a is the result of the refolded solution of HIS-Cav22P. Lanes *2*～*4* in Figure 5b are the results of the solution acquired by washing the Ni column with Tris-HCl supplemented with imidazole at final concentration of 100 nmol/L, 150 nmol/L, and 110 nmol/L, respectively. The HIS-Cav22P protein is indicated by the arrow on the right of the gel.

2.5 Purification of GST-Cav22P and Cav22P

The purification of the recombinant protein GST-Cav22P was troublesome. The band for the protein of 36 ku that matches alone with the position of the expressed GST-Cav22P is just about one sixth thick of the band for 26 ku GST (shown in lane *2* in Figure 6). While in the whole proteins of induced bacteria with pGEX-4T-Cav22P plasmid, the amount of GST-Cav22P is nearly the same as GST alone (shown in lane *2* in Figure 4b). There may be two reasons. One is that the affinity of GST-Cav22P to the Glutathione Sepharose 4B column is not so strong as GST. The other is that the GST-Cav22P degrades severely. Molecule sieve sepharose was used to separate the GST-Cav22P from the GST, however the GST-Cav22P degraded severely that it was difficult to acquire high concentration of GST-Cav22P even though strong protein enzymes inhibitors were added and the purification was conducted at 4℃. After digested by thrombin at 4℃ for 24 h, the GST-Cav22P was loaded onto the Glutathione Sepharose 4B column and the Cav22P was finally purified. There are some GST proteins that didn't bind to the column and were washed out with Cav22P (lane *4* in Figure6). This may due to most GST-Cav22P degraded into GST or truncated proteins and the GST is so much that its amount exceeded the binding capacity of the column.

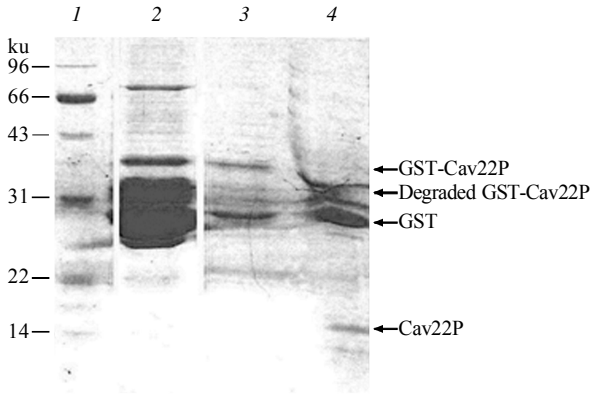

Fig. 6 SDS-PAGE analysis of the purified GST-Cav22P and Cav22P

Lane *1* presents protein molecular mass marker. Lane *2* is the result of GST-Cav22P purified *via* Glutathione Sepharose 4B column. Lane *3* is the result of GST-Cav22P purified *via* molecule sieve sepharose. Lane *4* shows the result of Cav22P purified *via* Glutathione Sepharose 4B column.

2.6 The Ca^{2+} binding activity of Cav22P

The activity of the recombinant protein Cav22P was evaluated in order to decide whether the functional study can be started. Since there is a putative EF-hand motif, which may be a typical Ca^{2+} binding domain in the protein Cav22P, the wavescan from 200 to 400 nm by ultra-spectrometry was performed to detect the Ca^{2+} binding activity of the protein Cav22P. The experiment was based on the fact that the conformation and absorption characteristics of proteins change when the proteins bind to metal ions. Figure 7 shows that the largest peak in the overlapped spectrums of the refolded HIS-Cav22P with

0.1 mmol/L $CaCl_2$ shifted to the left of that of the refolded HIS-Cav22P in normal buffer. When the refolded HIS-Cav22P was added with EGTA to 0.1 mmol/L to chelate the free calcium ions, the right part of the curve of HIS-Cav22P became higher. The results indicate the interaction between His-Cav22P and calcium ion. The calcium induced conformational change and the detailed mechanisms need further study. Immuno-spectroscopy and circular dichroism chiroptical spectroscopy are more sensitive for detection of the conformational changes of proteins, however, the immunochemical signal of HIS-Cav22P is very weak to detect.

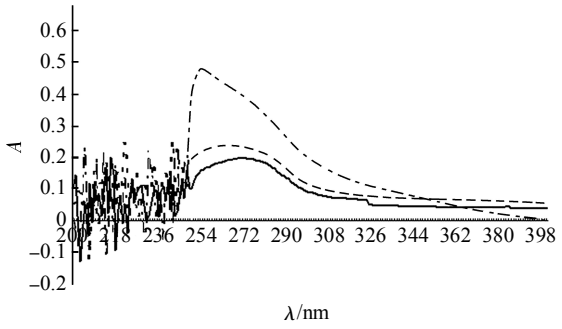

Fig. 7 Wavescan spectrums of refolded HIS-Cav22P with $CaCl_2$ or EGTA

—: His-Cav22P; – – –: His-Cav22P+Ca^{2+}; –·–: His-Cav22P+EGTA.

2.7 The *in vitro* interaction between GST-Cav22P and SO3

GST pull-down assay was conducted to detect the *in vitro* interaction between the protein Cav22P and the 25 amino acid peptide SO3. As shown in Figure 8, there was a slight band with a molecular mass of less than 5 ku pulled down by GST-Cav22P, while no signal was observed in the GST control. The conotoxin SO3 is about 2.6 ku. It can not be detected in the SDS-PAGE gel by Coomassie Brilliant Blue G-250 unless its concentration is higher than 2.5 mg/L and 20 μl for loading. Another problem is related with the long-time incubation of GST-Cav22P with SO3, which led to the degradation of GST-Cav22P into GST and Cav22P. Therefore, other protein-protein interaction assays are needed to confirm the interaction between Cav22P and SO3.

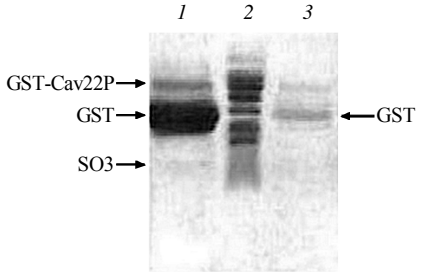

Fig. 8 GST pull-down assay of GST-Cav22P and SO3

Lane *1* shows that GST-Cav22P can track conotoxin SO3. Lane *2* presents standard protein molecular mass marker. Lane *3* shows that GST can not track conotoxin SO3.

3 Discussion

In this paper, recombinant proteins were constructed to study the biological activity of the third P region of the α1B subunit of the N-type calcium channel. PCR with specific primers was adopted to amplify the aimed fragment sequence. The cDNA templated was synthesized by reverse transcriptase from mRNA. However it is hard and expensive to get human hippocampal tissue, where the N-type calcium channel mRNA is highly expressed. Basing upon the fact that the N-type calcium channels are highly conserved in mammals, we started with the hippocampal tissue from rats to extract total RNA. But the cDNA amplified by high fidelity DNA polymerase still has 3 to 5 nucleotides mutations. Therefore vector α1B clone was chosen as the PCR template.

Although the approaches of cloning of N-type calcium channel of the cDNA, construction of expression plasmid, and purification of N-type calcium channel subunits are mature[10], the whole α1B subunit (over 2 000 amino acids) is not suitable for protein-protein interaction assays with the conotoxin SO3 (just 25 amino acids). It is difficult to exclude certain interfere of non-specific binding. A large number of literatures showed that recombinant proteins also bind to the target proteins when a fragment of an ion channel subunit was inserted into fusion protein expression vector[11~14]. The fragment was then cut into smaller fragments to fusion expression and more precise binding region of the ion channel subunit was determined by protein-protein interaction methods such as GST pull-down assay. These results indicate that fragments of the ion channel subunits can have their native conformations as that in the whole subunit, thus it can bind to their targets independently. Of course, the result of *in vitro* GST pull-down assay needs negative control and co-immunoprecipitation experiments, in which two types of proteins interact with each other in the cell physiological conditions.

Membrane proteins, such as ion channels, are strongly hydrophobic, that makes their recombinant expression in *E. coli* very difficult. The reason may be due to the fact that most transmembrane regions are highly hydrophobic so that they are difficult to be dissolved in the water. Some recombinant proteins have negative or even poisoning effects on normal physiological activity of *E. coli*. In our study, at first, the third P region and the two transmembrane segments at its both ends were inserted together into the His-tag fusion protein expression vector. Thus, the expressed product can be seen only in the precipitant part of the bacterial lysate. Besides, the difference between the un-induced cells and induced cells was not distinct until 1% glucose was added into the culture medium to repress low level basal expression (data not shown). It has been reported that *E. coli* C41 and C43 have been applied to membrane protein expression because of their strong resistance to hydrophobic protein[15]. However, neither of the strains worked on the protein Cav22P. The hydrophobic characteristics of the third P region of the α1B subunit of N-type calcium channel of *Rattus norvegicus* were analyzed by using the ProtScale. The result (shown in Figure 1) indicates that the second half of the protein is more highly hydrophobic. That is the reason why the protein is expressed mostly in the inclusive body.

The solubility of the induced expressed protein may be increased by lowering the induction temperature or the IPTG concentration, reducing the induction time, increasing initial A_{600} measurements and so on[16]. In addition, GST-tag usually promotes soluble expression. However, the huge GST-tag degrades easily. Therefore, it is always difficult to cut the GST-tag by enzymes for purified aimed proteins.

It is much easier to get a great amount of HIS-Cav22P from the inclusive body than from the supernatant of cell lysate. As shown in the Figure 2, the main structure of Cav22P must be α-helixes that can be easily refolded in most cases. Thus, renaturation methods referred to protein refolding database (http://refold.med.monash.edu.au/) were used to acquire a large amount of soluble recombinant protein HIS-Cav22P. When the soluble recombinant protein HIS-Cav22P was purified *via* Histrap chelating HP column, some impurity still stayed in the Ni column even after the column was washed by the Tris-HCl buffer with very high concentration of imidazole. That is the reason why the vector for GST fusion protein of Cav22P was constructed and the soluble GST-Cav22P was expressed and purified. However, the protein GST-Cav22P degraded quickly.

Most of the difficulties we met in the expression and purification of the recombinant proteins of Cav22P are common problems in membrane proteins production. As membrane proteins take up about one third of the whole proteomics and they are always very ideal targets for drugs because of their important functions in cell-adhesion, surface recognition, signal transduction and transportation, it is fundemantal to acquire a large amount of active recombinant proteins of membrane proteins for further functional study. In our experiment, the α-helix protein was successfully refolded. It is also worth to try to express the protein together with molecular chaperons to make the goaled protein be folded correctly and easily.

This study provides a basis for further study on the relationship of structure and interaction between conotoxin SO3 and the N-type voltage-sensitive calcium channel α1B subunit, thus gives helpful insight to screen cheap inorganic compounds instead of synthetic drugs. Compared with the physiological effects of the calcium ion and neural signal transduction, the interaction between SO3 and the N-type calcium channel is a more fundamental mechanism of the analgesic effect of SO3. Therefore, it is promising for establishing a high-throughput screening platform of marine natural analgesic drug.

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大鼠 N 型钙通道ⅢP 区原核表达、纯化及活性研究*

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摘要 高保真 PCR 克隆出编码大鼠海马组织 N 型钙通道 α1B 亚基ⅢP 区的 DNA 序列，分别与表达载体 pET28b 和 pGEX-4T-1 连接，转化入能补充稀有密码子 tRNAs 的大肠杆菌 Rosetta 菌株中，通过优化表达条件，实现了高效诱导表达．再通过低温诱导，将包涵体用尿素溶解再稀释后透析法复性、酶切、亲和柱层析等方法得到较纯的可溶性目的蛋白 HIS-Cav22P、GST-Cav22P 和 Cav22P，紫外吸收光谱检测实验证明了重组蛋白 HIS-Cav22P 能和钙离子可逆地结合．最后通过 GST 沉降实验证明了芋螺毒素 SO3 与 Cav22P 存在体外相互结合作用．上述结果为揭示芋螺毒素 SO3 特异性阻断大鼠海马组织 N 型钙流的分子机制提供了直接的依据，也为建立筛选新的非吗啡型天然镇痛药物的技术平台提供了基础．

关键词 纯化，表达，N 型钙通道

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