Immunotoxins and Neurotrophins: Novel Strategies for the Efficient Expression of Recombinant Proteins

Dem Fachbereich Chemie der Universität Kaiserslautern
zur Erlangung des akademischen Grades
"Doktor der Naturwissenschaften" vorgelegte Dissertation

DISSERTATION
(D 386)

Zhuoyu Li

Betreuer: Prof. Dr. Wolfgang E. Trommer
Prof. Dr. Jingming Yuan

Kaiserslautern 2002

Die vorliegende Arbeit entstand zwischen Dezember 1997 und Juni 2001 im Fachbereich Chemie, Abteilung Biochemie der Unversität Kaiserslautern, Deutschland und Biotechnologie Zentrum der Universität Shanxi, Taiyuan, China.

Promotionskommission:

Vorsitzender:	Prof. Dr. Dr. D Schrenk
1. Berichterstatter:	Prof. Dr. W. E. Trommer
2. Berichterstatter:	Prof. Dr. J. Yuan

Zusammenfassung

Mit der Entwicklung “rekombinanter” DNA-Techniken begann ein neues Zeitalter für die Herstellung von Proteinen, sowohl im wissenschaftlichen Bereich als auch bei der industriellen Nutzung. Allerdings ist die Reinigung der rekombinant produzierten Proteine nicht immer einfach. In dieser Arbeit wurden drei pharmakologisch interessante Proteine exprimiert und es wurde dabei versucht, durch neuartige Genkonstrukte Proteine zu erhalten, deren Aufarbeitung gegenüber der bisherigen Vorgehensweise vereinfacht ist. Bei den rekombinant exprimierten Proteinen handelt es sich um das humane Neurotrophin 3 (hNT3), das Pflanzentoxin Gelonin sowie um ein Fusionsprotein aus Gelonin und der extrazellulären Domaine der α-Untereinheit des humanen Acetylcholin-Rezeptors (Gelonin-AchR).

In dem ersten Projekt wurden die Gene für das Pflanzentoxin Gelonin, die extrazelluläre Domaine der α-Untereinheit des humanen Achetylcholin-Rezeptors sowie für ein Fusionsprotein bestehend aus beiden Komponenten cloniert und die Genprodukte in *E. coli* exprimiert. Das Gen für Gelonin hatte Shi Ya-Wei chemisch synthetisiert und freundlicherweise zur Verfügung gestellt. Es wurde zunächst in den Vektor pUC118 eincloniert und dann in pET28a transferiert und exprimiert. Das rekombinante Gelonin konnte in zwei Schritten gereinigt werden und man erhielt eine homogene Bande im SDS-Gel im Bereich von 28 kD. Die Expression der extrazellulären N-terminalen Domaine der α-Untereinheit des Acetylcholin-Rezeptors (Aminosäuren 1-181) wurde ebenfalls cloniert und in *E. coli* exprimiert. Allerdings entstanden dabei unlösliche Aggregate, die nicht oder in nur sehr geringem Maße renaturiert werden konnten. Selbst bei Coexpression mit dem Chaperonin-System GroELS konnten nur geringe und schlecht reproduzierbare Mengen an löslichem Produkt erhalten werden. Eine Lösung des Problems ergab sich mit der Konstruktion eines Fusionsproteines bestehend aus Gelonin und der AchR-Domaine, da durch die höhere Löslichkeit des Gelonins eine Solubilisierung des Fusionsproteins mit anschließender Reinigung möglich wurde. Die biologische Aktivität des rekombinant produzierten Gelonins sowie der Gelonin-Acetylcholin-Rezeptor-Fusion wurde im *in-vitro* Translationstests gezeigt. Die korrekte Faltung der AchR-Domaine wurde im ELISA mit Hilfe spezifischer monoklonaler Antikörper nachgewiesen.

Die Expression und Reinigung des rekombinanten hNT3 wurde in einem Protein-Selbst-Spleiß-System durchgeführt. Dazu wurde ein 380 bp-Fragment (entspricht einem Protein mit 14 kD) der bekannten hNT3-DNA-Sequenz mit Hilfe von PCR aus menschlichem Vollblut amplifiziert und in den Vektor pTXB1 eincloniert. pTXB1 enthält zusätzlich ein Intein-Fragment,

an dem eine Chitin-Bindungsproteindomaine (CBD) anfusioniert ist. Ein ähnliches Konstrukt, pJLA-hNT3, wurde zusätzlich hergestellt, bei dem die Genexpression unter einen Hitzeschockpromotor gestellt ist. Von beiden Konstrukten konnte das Zielprotein hNT3-intein-CBD entweder nach Induktion mit IPTG oder nach Hitzeschock erhalten werden. Das exprimierte Protein akkumulierte in Aggregatform und wurde nach Denaturierung und Renaturierung als lösliches Fusionsprotein auf einer Chitin-Affinitätssäule gereinigt. Nach Spaltung in Gegenwart von DTT wurde ein 14 kD Protein erhalten, das dem hNT3 entspricht. Sowohl das hNT3 als auch das Fusionsprotein hNT3-intein-CBD zeigten dieselbe biologische Aktivität basierend auf Wachstumsassays an dorsalen Ganglien in Hühnerembryonen.

Abstract

The development of recombinant DNA techniques opened a new era for protein production both in scientific research and industrial application. However, the purification of recombinant proteins is very often quite difficult and inefficient. Therefore, we tried to employ novel techniques for the expression and purification of three pharmacologically interesting proteins: the plant toxin gelonin; a fusion protein of gelonin and the extracellular domain of the α subunit of the acetylcholine receptor (gelonin-AchR) and human neurotrophin 3 (hNT3).

Recombinant gelonin, acetylcholine receptor α subunit and their fusion product, gelonin-AchR were constructed and expressed. The gelonin gene, a 753 bp polynucleotide was chemically synthesized by Ya-Wei Shi *et al.* and was kindly provided to us. The gene was first inserted into the vector pUC118 yielding pUC-gel. It was subsequently transferred into pET28a and pET-gel was expressed in *E. coli*. The product, gelonin was soluble and was purified in two steps showing a homogeneous band corresponding to 28 kD on SDS-PAGE. The expression of the extracellular domain of the α-subunit of AchR always led to insoluble aggregates and even upon coexpression with the chaperonin GroESL, very small and hardly reproducible amounts of soluble material were formed, only. Therefore, recombinant AchR- gelonin was cloned and expressed in the same host. The corresponding fusion protein, gelonin–AchR, again formed aggregates and it had to be solubilized in 6 M Gu-HCl for further purification and refolding. The final product, however, was recognized by several monoclonal antibodies directed against the extracellular domain of the α-subunit of AchR as well as a polyclonal serum against gelonin.

Expression and purification of recombinant hNT3 was achieved by the use of a protein self-splicing system. Based on the reported hNT3 DNA sequence, a 380 bp fragment corresponding to a 14 kD protein was amplified from genomal DNA of human whole blood by PCR. The DNA fragment was cloned into the pTXB1 vector, which contains a DNA fragment of intein and chintin binding domain (CBD). A further construct, pJLA-hNT3, is temperature-inducible. Both constructs expressed the target protein, hNT3-intein-CBD in *E. coli* by the induction with IPTG or temperature, however, as aggregates. After denaturation and renaturation, the soluble fusion protein was slowly loaded on an affinity column of chitin beads. A 14 kD hNT3 could be isolated after cleavage with DTT either at 4˚C or 25˚C for 48 h. Based on nerve fiber out-growth of the dorsal root ganglia of chicken embryos, both, hNT-3-intein-CBD and hNT3 itself exhibit almost the same biological activity.

Contents

Part I Immunotoxins.....1

1A Gene clone, expression and characteristics.....1

1A.1 Ribosome inactivating proteins.....1

1A.1.1 Toxicity.....2

1A.1.2 Membrane translocation.....4

1A.1.3 Antiviral activity.....5

1A.1.4 Ribosome specificities.....6

1A.1.5 Gelonin.....7

1A.1.5.1 The primary structure.....8

1A.1.5.2 The conformation.....8

1A.1.5.3 Catalytic mechanism.....9

1A.1.6 Research goals.....9

1A.2 Results and discussion.....10

1A.2.1 Gene clone of gelonin.....10

1A.2.1.1 Construction of pUC-gel.....12

1A.2.1.2 Extraction and identification of pUC-gel.....12

1A.2.2 Construction of an expression plasmid.....13

1A.2.2.1 Identification of pET-gel gene.....14

1A.2.3 Expression of pET-gel in *E.coli* BL21.....16

1A.2.4 Purification of recombinant gelonin.....17

1A.2.5 ELISA and Western blots.....20

1A.2.6 Reticulocyte lysate activity.....21

1A.3 Summary.....21

1A.4 Literature.....22

1B Co-expression of the extracellular human acetylcholine receptor α subunit fragment and the chaperonin GroESL in *E. coli*..... 24

1B.1 Introduction.....24

1B.1.1 Muscle AchRs.....25

1B.1.2 Research goals.....28

1B.2 Results and discussion……..……..……..……..28
1B.2.1 Identification of the recombinant plasmids……..28
1B.2.2 Co-expression of the recombinant plasmids in *E. coli*……..……...30
1B.2.3 Analysis of Western blots……..……...31
1B.2.4 Purification of Products……..……31
1B.3 Summary……..…..……..….31
1B.4 Literature……..……..……..……32

1C Gene clone and expression of gelonin-acetylcholine receptor α subunit domain……..34
1C.1 Introduction……...34
1C.1.1 Immune system……..34
1C.1.1.1 Antigens and antibodies……..……35
1C.1.1.2 The antibody response……36
1C.1.1.3 Cell mediated immunity……37
1C.1.1.4 Cell recognition of self and non self……37
1C.1.1.5 Autoimmunity and autoimmune disease……38
1C.1.2 Myasthenia gravis……...38
1C.1.2.1 Cellular immune mechanisms in MG and EAMG……..39
1C.1.2.2 Development of specific immunosuppressive therapies for EAMG and MG……..……...40
1C.1.3 Research goals……..……41
1C.2 Results and discussion……..……..……..41
1C.2.1 Cloning gelonin-AchR fragment gene……..……...41
1C.2.1.1 Identification of pET-GA and pJLA-GA……44
1C.2.2 Expression……..45
1C.2.3 Purification and identification of supernatant……47
1C.2.3.1 Isolation of supernatant……...47
1C.2.3.2 Western blots of gelonin-AchR……..……49
1C.2.3.3 Discussion……..……...49
1C.2.4 Denaturation and refolding of inclusion bodies……..…50
1C.2.4.1 Preliminary purification of denatured proteins……50
1C.2.4.2 Refolding of fusion protein……51

1C.2.4.3 ELISA assay……53
1C.2.4.4 Effect of gelonin-AchR fragment on protein synthesis……54
1C.3 Summary……55
1C.4 Literature……56

Part II Neurotrophins……59

2.1 Introduction……59
2.1.1 Neurotrophins……59
2.1.2 Neruotrophin receptors……60
2.1.3 Human neurotrophin 3……61
2.1.3.1 Molecular characteristics of hNT3……61
2.1.4 Purification of the recombinant proteins……62
2.1.5 Research goals……67
2.2 Results and discussion……68
2.2.1 Amplification and identification of human neurotrophin 3 gene……68
2.2.1.1 Preparation of genomic DNA from human whole blood……68
2.2.1.2 Amplification of human neurotrophin 3 gene by PCR……68
2.2.2 Construction of recombinant pGEM-hNT3……69
2.2.2.1 Background of pGEM-T vector……69
2.2.2.2 Construction of pGEM-hNT3……70
2.2.2.3 Confirmation of pGEM-hNT3……70
2.2.2.4 DNA sequence analysis……71
2.2.3 Construction and expression of pTXB-hNT3……72
2.2.3.1 Background of pTXB1……72
2.2.3.2 Construction of pTXB-hNT3……72
2.2.3.3 Identification of pTXB-hNT3……74
2.2.3.4 Expression and purification……74
2.2.4 Construction and expression of pJLA-hNT3……77
2.2.4.1 Construction of pJLA-hNT3……77
2.2.4.2 Identification of pJLA-hNT3……77

2.2.4.3 Expression and purification……78
2.2.5 Bioactivity assay……80
2.3 Summary……81
2.4 Literature……82

Part III Materials and methods……84

3.1 Materials……84
3.1.1 *E. coli* strains……84
3.1.2 Plasmids……84
3.1.3 Enzymes……85
3.1.4 Oligonucleotides……86
3.1.5 Media and antibiotics……88
3.1.6 Chemicals……88
3.1.7 Other materials……89
3.1.8 Equipment……89
3.2 Cultivation and storage……89
3.2.1 Media, agar plates and antibiotics for the cultivation of *E. coli* strains……90
3.2.2 Storage of *E. coli* cultures……91
3.3 General methods……91
3.3.1 Preparation of competent *E. coli* cells……91
3.3.2 Transformation of competent *E. coli* cells with plasmid DNA……92
3.3.3 Plasmid minipreparation……93
3.3.3.1 Alkaline lysis method……93
3.3.3.2 Plasmid minipreparation with QIAprep™ Spin Miniprep kit……94
3.3.4 Purification of PCR products……94
3.3.5 Ligation……94
3.3.6 Agarose gel electrophoresis……95
3.3.7 Estimation of DNA concentration……96
3.3.8 Determination of protein concentration……96
3.3.9 SDS-PAGE gel electrophoresis……96

3.4 Clone of pGEM-hNT3……98
3.4.1 Preparation of human genomic DNA……98
3.4.2 Amplification of hNT3 gene……98
3.4.3 Construction of pGEM-hNT3……99
3.5 Determination of biological activity……100
3.5.1 hNT3 bioactivity assay……100
3.5.1.1 Collection of ganglia……100
3.5.1.2 The preparation of sample……101
3.5.1.3 The assay……101
3.5.2 ELISA……102
3.5.3 Western blots……103
3.5.4 The inhibition of protein synthesis *in vitro*……104
3.6 Literature……105

Abbreviations……106
Acknowledgements……109
Curriculum Vitae……110

Part I Immunotoxins

Conjugates composed of tissue-specific monoclonal antibodies and toxins have found wide interest as potential anti-cancer drugs. Far less common is the similar approach to use conjugates of antigens with toxins for the treatment of autoimmune diseases. In this case, the auto-antigen would direct the toxin to its target, antigen-specific lymphocytes, which would be selectively eliminated resulting in an antigen-specific suppression of the immune system. This approach, as initially suggested by Géza Filipp, was successfully taken in the laboratory of Trommer in Kaiserslautern [1] in the case of experimental autoimmune Myasthenia gravis (EAMG). Myasthenic rats were treated with a conjugate composed of the acetylcholine receptor (AchR) and the plant toxin gelonin leading to a substantial recovery of functional receptors in the neuromuscular endplates of these animals. Neither the receptor nor the toxin alone had comparable effects. However, the antibody titer against the receptor raised during treatment. A possible reason could have been immune reactions against pathophysiologically irrelevant parts of the receptor from *Torpedo* which had been used in the conjugates [1]. Hence, in the current work, recombinant fragments of the α-subunit, which contains the main immunogenic region (MIR) will be employed.

1A Gene clone, expression and characteristics of gelonin

1A.1 Ribosome inactivating proteins

Ribosome inactivating proteins (RIPs) are bio-macromolecules that specifically interfere with eukaryotic protein translation. Most plant and bacterial RIPs exert their effects through catalytically and irreversibly inactivating the 60S subunit of eukaryotic ribosomes [2]. Plant RIPs can be categorized into two groups: type-I RIPs, most of them being glycoproteins such as gelonin, bryodin or trichosanthin, are single-chain proteins with molecular weight of about 30 kD. However, type-II RIPs, such as ricin or abrin, consist of two polypeptide chains (A and B) which are linked by a disulfide bridge. The enzymatic property of type-II RIPs is associated with the A chain, while the B chain is like a lectin, which facilitates entry of the toxin into intact cells by binding non-specifically to galactose moieties on the cell surface [3, 4, 5].

1A.1.1 Toxicity

The potent cytotoxicity of heterodimeric ricin was first described over a century ago. The mechanism by which RIPs inactivate 60S ribosomal subunits was elucidated by Endo and his colleagues in 1987 [1, 6]. The mechanism was first deduced from ricin A chain, but has not been shown to operate for all plant RIPs and for bacterial toxins such as *Shigella* toxin and the Shiga-like toxins from certain enteropathogenic strains of *E. coli*. Ricin A chain is an RNA specific N-glycosidase that hydrolytically cleaves a single N-glycosidic bond from among over four thousand nucleoside bases present in 28S rRNA (4800 bases). A specific adenine residue is removed (A-4324 in the case of rat liver 28S rRNA) resulting in the inability of the ribosome to bind elogation factor 2 and thus terminating the elongation cycle of protein synthesis. Several rRNA sequences surrounding the adenine residue removed by plant RIPs are shown in Table 1A.1. The ricin A chain can specifically depurinate 1500-2000 rat liver ribosomes per minute. The preferred substrate for ricin A chain is 26/28S rRNA in native 60S ribosomal subunits. Whilst the naked 26/28S rRNA can also act as a substrate but the K_{cat} for the latter reaction is approximately 10^5- fold lower than that for the former. This suggests that the native secondary structure of ribosome-associated 26/28S RNA is important for the action of RIPs and that ribosomal proteins may play an important role in maintaining this RNA conformation, and/or in providing a high affinity binding site for the toxin [7].

Table 1A.1 Nucleotide sequence in rRNA surrounding the adenine* removed by RNA N-glycosidase [4]

Ribosome sources	Sequences
Xenopus	5'….AGUACGA*GAGGAAC….3'
Rat	5'….AGUACGA*GAGGAAC….3'
Yeast	5'….AGUACGA*GAGGAAC….3'
Wheat	5'….AGUACGA*GAGGAAC….3'
E.coli	5'….AGUACGA*GAGGACC….3'

Table 1A.2 Some examples of single-chain RIPs [4]

Plant sources	Inhibitor	M_r (dalton)	Glycosylated
Phytolacca Americana (pokeweed) seeds or leaves	Pokeweed antiviral Proteins (PAP)	30,000	No
Triticum aestivum (wheat) seeds	Tritin	30,000	No
Gelonium multiflorum seeds	Gelonin	30,000	Yes
Momordica charantia (bitter ground) seeds	Momordin	31,000	Yes
Saponaria officinalis (soapwort) seeds	Saporin	29,500	No
Dianthus carophyllus (carnation) leaves	Dianthin	30,000	Yes

Single chain RIPs (Table 1A.2) are potent inhibitors in cell-free translation systems, they exhibit the low toxicity to intact cells; IC_{50} value for protein synthesis inhibition by whole cells being 10^5 to 10^7 fold higher than those for ribosomes. In contrast to double chain RIPs (dsRIPs), single chain RIPs (scRIPs) [8] cannot normally enter into cells and cannot therefore readily reach their ribosomal sites. However, the cytotoxity of single chain RIPs can be dramatically enhanced if entry into the cell cytoplasm is facilitated. This can be achieved by conjugating RIPs to antibodies [9], or by incorporating RIPs into some structures such as liposomes or erythrocyte ghosts which can be fused with intact cells.

The most thoroughly studied toxic proteins from plants are the cytotoxic lectins such as ricin and abrin (Table 1A.3). These all are N-glycosylated heterodimers consisting of two polypeptides linked together by a single disulfide bond. The A chain is a RNA-specific N-glycosidase, structurally and functionally corresponding to the single chain RIPs. Cytotoxity is conferred by the B chain, which allows the holotoxin to bind to the cell surface and which ultimately leads to the penetration of the toxic A chain into the cell cytosol.

Table 1A.3 Cytotoxic heterodimeric lectins [4]

Plant sources	Inhibitors	M_r (dalton)	Glycosylated
Ricinus communis	Ricin	65,000	Yes
(castor bean) seeds	A chain	32,000	Yes
	B chain	34,000	Yes
Abrus precatorius	Abrin	65,000	Yes
(jequirity bean) seeds	A chain	30,000	No
	B chain	36,000	Yes
Adenia digitata	Modeccin	63,000	Yes
roots	A chain	28,000	
	B chain	31,000	
Adenia volkensii	Volkensin	62,000	Yes
roots	A chain	29,000	
	B chain	36,000	
Viscum album	Viscumin	60,000	Yes
(mistletoe) leaves	A chain	29,000	
	B chain	32,000	

The binding of cytotoxic lectins to the surface of target cells is opportunistic and is achieved by reversible interactions between the B chain and any carbohydrate moiety containing terminal galactose residues. Such residues occur on both glycoproteins and glycolipids and the abundance of such potential binding sites on most mammalian cells ensures a high concentration of bound toxin at the cell surface. Hela cells for example, possess 3×10^7 binding sites per cell for ricin [10].

1A.1.2 Membrane translocation

At some stages during the entry process, ricin A chain crosses an intracellular membrane into the cytosol, although the actual site at which this translocation step occurs has not been convincingly established. Translocation of a toxic fragment into the cytosol is best understood for *diphtheria* toxin. This bacterial toxin enters the cytosol from acidified endosomes where the low pH induces a conformational change in the B fragment. This change results in the exposure of B chain hydrophobic domains which insert into the endosomal membrane and somehow facilitate transfer of the A fragment into the cytosol. Because of this requirement for low pH for translocation, cells in which endosomal and lysosomal pH has been increased (by treatment with NH_4Cl, for example),

or mutant cell lines defective in the acidification of endosomes, are resistant to *diphtheria* toxin. Although the endosome has also been implicated as the intracellular site of ricin translocation, it appears that the endocytosis of ricin continues beyond the endosomal stage before translocation takes place. Treatments which increase endosomal pH do not reduce ricin toxicity, and the lag time between cellular exposure to ricin and measurable decrease in protein synthetic activity (60-90 min) is considerably longer than that for the bacterial toxin. Several studies using immunoelectron microscopy have shown that endocytosed ricin is first delivered to the endosomes and a fraction subsequently appears within the Golgi complex, in particular the trans-Golgi network [11]. There is now considerable experimental evidence that ricin must reach the Golgi complex in order to exert its cytotoxic effects. A number of treatments that induce morphological changes as the disruption of the Golgi by brefeldin A, inhibit the cytotoxicity of ricin and *pseudomonas* exotoxin A, while having no effect on the cytotoxicity of diphtheria toxin [12].

Ricin B chain is required for the efficient translocation of ricin A chain into the cytosol, beyond its role in the initial cell surface binding of the holotoxin [13]. This B chain requirement is not absolute however, since many immunotoxins containing ricin A chain alone are potently cytotoxic. Clearly the A chain can cross a membrane in the absence of B chain, which argues against the B chain having a direct function in the translocation step. It is possible that the B chain facilitates translocation indirectly by delivering the A chain to a translocationally-competent compartment. If this is the case, the association with the B chain might effectively prevent the A chain from inserting into membranes until such a compartment has been reached. The intracellular role of ricin B chain envisaged above could result from the B chain binding to intracellular galactose-containing components (receptor), particularly in the trans-Golgi. Such galactose binding could reduce the amount of ricin recycled back to the cell surface, it might allow ricin to move from the Golgi to the ER as discussed above, or it could have a more direct role in membrane translocation within the Golgi complex. In these situations, the galactose binding sites are clearly important for the B chain's intracellular role in cellular intoxication [14, 15].

1A.1.3 Antiviral activity

The first single chain RIP to be isolated and studied was pokeweed antiviral protein (PAP). It was known that leaf extracts from several plants, including *Phytolacca americana* (pokeweed), when mixed with a suspension of tobacco mosaic virus, prevent the mechanical transmission of viral infection to other plants. The pokeweed antiviral factor was purified and identified as a single chain RIP. PAP also prevented animal virus replication in mammalian cells, where it was found to

inhibit protein synthesis by virally infected cells at PAP concentrations which did not affect normal cells. All RIPs are known to be more toxic to virus-infected cells than to non-infected cells, apparently because infection permeabilizes the host and allows the toxins to penetrate into the cytosol [16].

From early studies on the antiviral activity of plant extracts it was concluded that while these extracts prevented viral infection in other plant species, they did not prevent infection of the very plants from which they derived. This suggested that the antiviral principle did not act directly on the virus but that its effectiveness was due to some action on the infected plant. Now, it has been clearly shown that pokeweed ribosomes are sensitive to PAP [17]. A study of the intracellular location of PAP has shown that this RIP is heavily sequestered in the cell wall of pokeweed cells [18], a location consistent with its proposed anti-viral role. Thus viral infection of pokeweed cells might provoke a damage limitation exercise in which the infected cells become permeable to their own extra-cellular RIP that enters the cytosol and depurinates the pokeweed ribosomes and thereby prevents viral replication.

1A.1.4 Ribosome specificities

The active site was identified by x-ray analysis of the ricin A chain and confirmed by site-directed mutagenesis. This region is highly conserved in RIPs (Table 1A.4). Their ribosomal substrate specificities however, can be very different, for example, mammalian and yeast ribosomes are very sensitive to ricin A chain, plant ribosomes much less so, and prokaryotic ribosomes are completely insensitive. In spite of the fact that the consensus sequence around the target adenine is also highly conserved in all 23, 26, 28S rRNAs, the sensitivity of a single type of ribosome to different RIPs also varies markedly, for example, wheat germ ribosomes are relatively insensitive to ricin A chain but are 1000 to 10,000 times more sensitive to the single chain RIP dianthin.

The most dramatic variation in sensitivity which has emerged recently is that *E. coli* ribosomes were believed to be completely insensitive to all RIPs. Indeed, *E. coli* has been successfully used as host to produce recombinant ricin A chain and abrin A chain [19]. These proteins were produced cytoplasmically where they accounted for up to 10% of the total bacterial protein without affecting bacterial growth. *Mirabilis* antiviral protein (MAP) cytoplasmically in *E. coli* resulted in severely inhibited growth of the host, caused by the recombinant product, and the yield of product was very low. Subsequently MAP was shown to inhibit protein synthesis by *E. coli* ribosomes *in vitro*, in contrast to the effect of the ricin or abrin A chain [20]. More recently it was shown that both PAP

and dianthin likewise inhibit protein synthesis by *E. coli* ribosomes. These single chain RIPs are able to specifically depurinate *E. coli* 23S rRNA at the expected site (Table 1A.1). The adenine residue -glycosidically removed from 23S rRNA by the RIPs is known to be a key binding residue for EF-G and EF-Tu [21].

Table 1A.4 Comparison of active site region of plant toxins [4]

RIP	Residue position	Amino acid sequence
Ricin A chain	172-185	CIQMISEA*ARFQYI
Abrin A chain	158-171	IIQMVSEA*ARFRYI
PAP	170-183	AIQMVSEA*ARFKYI
MAP	163-176	AIQMVSEA*ARFKYI
Saporin	171-184	AIQMTAEA*ARFRYI
Diarithin	152-165	AIQMTAEA*ARFRYI

1A.1.5 Gelonin

Gelonin extracted from seeds of the plant G*elonium multiflorum* belongs to type-I ribosome inactivating proteins. Gelonin is glycosylated with terminal mannose residues. Due to lack of the B-chain domain binding the cell surface, it normally does not enter intact cells. Even if it enters cells via a different mechanism, e.g. as a conjugate, it exhibits a different intracellular distribution, compared with type-II RIPs. Gelonin is assumed to be trapped in the endosomal/lysosomal compartment, a location explaining its relatively low toxicity. In cell-free systems, however, gelonin has the powerful N-glycosidase activity on eukaryotic ribosomes by releasing adenine-4324 from a vital region of the 28S rRNA unit [20].

Table 1A.5 The amino acid sequence of gelonin precursor

10	20	30	40	50
MKGNMKVYWI	KIAVATWFCC	TTIVLGSTAR	IFSLPTNDEE	ETSKTL**GLDT**
60	70	80	90	100
VSFSTKGATY	ITYVNFLNEL	RVKLKPEGNS	HGIPLLRKKC	DDPGKCFVLV
110	120	130	140	150
ALSNDNGQLA	EIAIDVTSVY	VVGYQVRNRS	YFFKDAPDAA	YEGLFKNTIK

160	170	180	190	200
TRLHFGGSYP	SLEGEKAYRE	TTDLGIEPLR	IGIKKLDENA	IDNYKPTEIA
210	220	230	240	250
SSLLVVIQMV	SEAARFTFIE	NQIRNNFQQR	IRPANNTISL	ENKWGKLSFQ
260	270	280	290	300
IRTSGANGMF	SEAVELERAN	GKKYYVTAVD	QVKPKIALLK	FVD**KDPK**TSL
310	316			
AAELIIQNYE	S L V G F D			

*This sequence is from Swiss-prot P33186. The residue **G** from 47 is the N-terminus of recombinant gelonin and **K** at the stop of 297 is the C-terminus.

1A.1.5.1 The primary structure

Gelonin consists of 251 amino acid residues, similar to other scRIPs such as monorcharin (247 aa) and the A-chain of dsRIPs for instance ricin A-chain (267 aa). Though the conserved amino acid residues among them are less than 40%, their structure is the same from the results of different RIPs cross-reaction with specific antiserum. *In vivo*, an inactive precursor of gelonin is first biosynthesized and then transported to safe compartments within the cell. Finally, the functional gelonin is produced by glycosylation with the post-translational modification. The primary structure of the precursor is as shown above Table 1A.5 [21].

1A.1.5.2 The conformation

Hosur *et al*. published a three-dimensional structure of gelonin at 1.8 Å resolution [22], which is basically identical to the conformation of ricin A and α-momorcharin (Fig 1A.1).

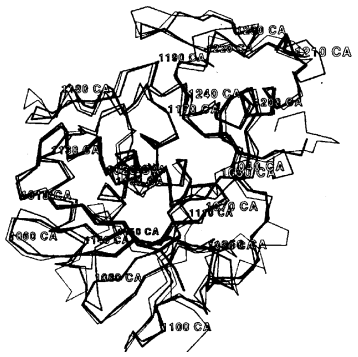


Fig 1A.1 Superposition of gelonin, ricin A and α-momorcharin [22]

The stereoview shows C^{α} tracing: **bold**, gelonin; **Mediun**, ricin A; **light**, α-momorcharin. Every 10th residue of gelonin is marked. Differences are mostly in the loop regions.

As shown in Fig 1A.1, structurally, gelonin belongs to the (α+β) class of proteins. With respect to the β strands, β_1, β_4, β_5, β_6, β_7 and β_8 form a mixed β sheet, and β_2, β_3 as well as β_9, β_{10} form two antiparallel beta ribbons, while the helix regions, except for short segments of type 3_{10} (123 to 125 and 237 to 239) are all of the α-helix type. Thus, there are two distinct structural domains in the gelonin molecule, one large and a small one. The former consists of residues 3-32 and 40-187 while the latter is composed of residues 33-39 and 188-247.

1A.1.5.3 Catalytic mechanism

Active site From the varies studies as X-ray diffraction, site-directed mutagenesis and chemical modification etc, the active site residues of gelonin are Tyr74, Arg169, Gly111, Glu166, Tyr113 and Trp198. There is a number of hydrogen bonding interactions between these residues, such as Arg169/Glu166. In addition, the water molecules also participate in the catalytic reaction, there are at least two hydrogen bonds from water to protein atoms.

Glycosylation Gelonin is a glycoprotein with terminal mannose residues. It is known that Asn82 and Asn189 are two potential glycosylation sites in the N-terminal glycosylation of gelonin.

Catalytic mechanism As a glycosidase, it is the cleavage of the N-glycosidic bond of a specific adenine in 28S rRNA from the 60S subunit of eukaryotic ribosomes. It has been shown that the true substrate is a ribo-oligonuclotide hairpin in which the double helical stem is at least three base pairs long and the connecting hairpin loop contains the sequence GAGA. The first adenine will be removed by RIPs. The removal of adenine is a hydrolysis reaction and involves addition of a water molecule to the substrate. Based on the structure, two kinds of molecular catalytic mechanisms have been suggested. Both include an oxycarbonium ion intermediate which is generated by different chemical steps. However, in both methods a water molecular is required for nucleophilic attack on the C1' atom of the intermediate [23].

1A.1.6 Research goals

Toxins as generally utilized in immunoconjugates belong to a group of enzymes that inhibit the protein biosynthesis [24]. Gelonin, a plant toxin from *Gelonium multiflorum* has successfully been employed in such approaches including its application in antigen-specific immune suppression [1]. However, it is not easily available from natural sources. Recombinant gelonin has been reported [21] but the clone is not available from the authors. In their work the cDNA was obtained from

highly fragmented mRNA isolated from the seeds of *Gelonium multiflorum*. Based on the published sequence of gelonin, Ya-Wei Shi *et al*. synthesized the gene chemically in four fragments which were kindly provided to us [25]. In these constructs some of the bases had been substituted for the corresponding nucleotides in the published DNA sequence in order to yield optimized codons for the same amino acids in *E. coli.* These DNA fragments will now be fused and used for the expression of gelonin.

1A.2 Results and discussion

1A.2.1 Gene clone of gelonin

The gene coding for gelonin was kindly provided by Ya-Wei Shi *et al*. in 4 consecutive fragments as shown in Fig 1A.2. Ya-Wei Shi *et al*. also provided the two recombinant plasmids pUC-gel I and pUC-gel II containing gelonin fragments 1+2 and 3+4 respectively [25].

A. DNA- GGCCTGGAC ACCGTGAGCT TTAGCACTAA AGGTGCCACT

B DNA- ATG GGCCTGGAT ACCGTGAGCT TCAGCACCAA AGGCGCCACC

Fragment 1 →

TATATTACCT ACGTGAATTT CTTGAATGAG CTACGAGTTA AATTGAAACC

TATATTACCT ATGTGAACTT CCTGAACGAA CTGCGTGTGA AACTGAAACC

CGAAGGTAAC AGCCATGGAA TCCCATTGCT GCGCAAAAAA TGTGATGATC

GGAAGGCAAC AGCCATGGCA TTCCGCTGCT GCGTAAAAAA TGCGATGATC

CTGGAAAGTG TTTCGTTTTG GTAGCGCTTT CAAATGACAA TGGACAGTTG

CGGGCAAATG CTTCGTGCTG GTGGCGCTGA GCAACGATAA CGGCCAGCTA

Fragment 2 →

GCGGAAATAG CTATAGATGT TACAAGTGTT TATGTGGTGG GCTATCAAGT

GCGGAAATTG CGATTGATGT GACCAGCGTG TATGTGGTGG GCTATCAGGT

AAGAAACAGA TCTTACTTCT TTAAAGATGC TCCAGATGCT GCTTACGAAG

GCGTAACCGT AGCTATTTCT TCAAAGATGC GCCGGATGCG GCGTATGAAG

GCCTCTTCAA AAACACAATT AAAACAAGAC TTCATTTTGG CGGCAGCTAT

GCCTGTTCAA AAACACCATT AAAACCCGTC TGCATTTTCGG CGGCAGCTAT

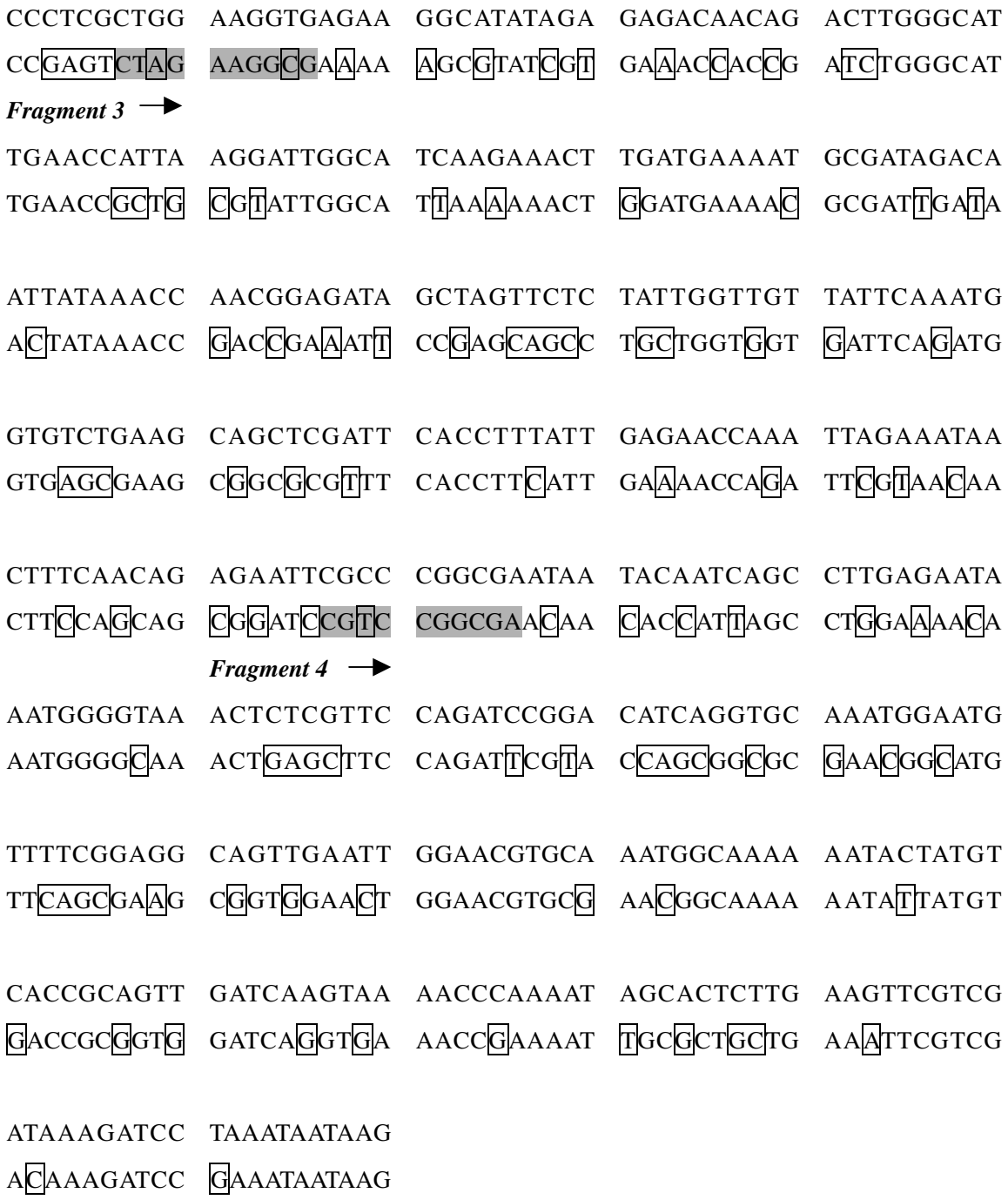


Fig 1A.2 A comparison of DNA sequence between native gelonin gene and synthetic gelonin gene A. Original DNA sequence; B. Synthetic DNA sequence showing the fragments 1-4 provided by Ya-Wei Shi *et al* [25].

1A.2.1.1 Construction of pUC-gel

Two recombinant plasmids harboring parts of the gelonin gene, pUC-gel I with the fragments of gelonin 1 and 2 and pUC-gel II with the fragments of gelonin 3 and 4 were provided by Ya-Wei Shi [25]. Based on the restriction enzyme sites (Fig 1A.3), pUC-gel I and pUC-gel II were separately digested with XbaI and EcoRI at 37°C for 2 h, and then separated by an 1% LM agarose gel electrophoresis, at 80 V, 1.5 h. The large fragment cut from pUC-gel I and the small fragment from pUC-gel II were collected and spun at 13,000 r/m for 30 sec and incubated at 70°C for 10 min and transferred into 42°C incubator for 5 min. Finally, 1 µl 10x β- agarase buffer and 1µl β-agarase were added and the mixture was incubated for 1 h at 42°C before ligation.

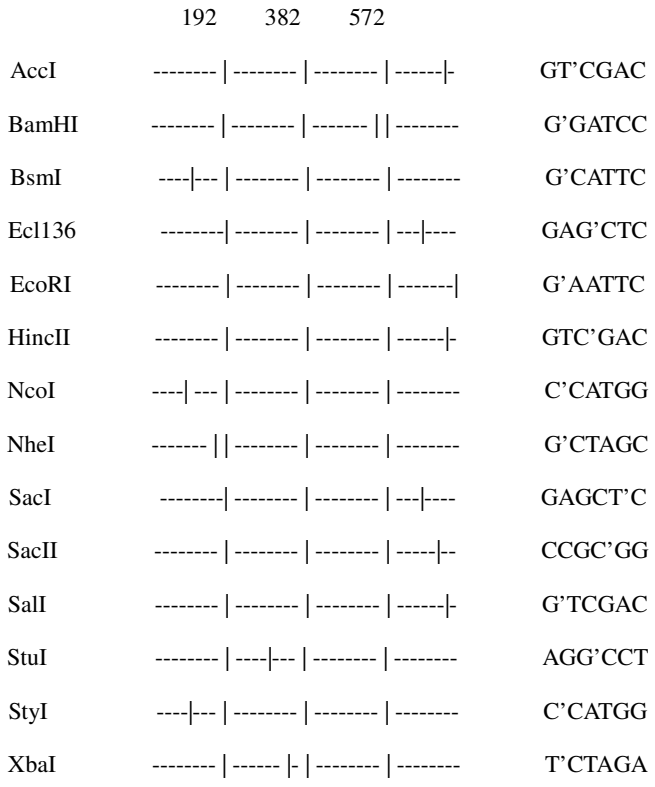


Fig 1A.3 The relative location of the restriction enzyme sites of synthetic gene

1A.2.1.2 Extraction and identification of pUC-gel

After ligation and transformation, a single colony was picked and transferred into 5 ml LB+ampicillin at 37°C and shaking overnight. The plasmids were extracted using the plasmid-mini kit, then digested with XbaI and EcoRI. It was shown from the digestion of the pUC-gel with XbaI/EcoRI on a 1.2% agarose gel that a band corresponding to about 415 bp had formed, which is akin to Gel 3+4 (415 bp) (See lane1 in Fig 1A.4). This indicates that the Gel 3+4 fragment had

been inserted into the vector and the whole gene of gelonin was present (Fig 1A.4). The new recombinant plasmid pUC-gel was constructed, which contains the whole gene of gelonin (Fig 1A.5)

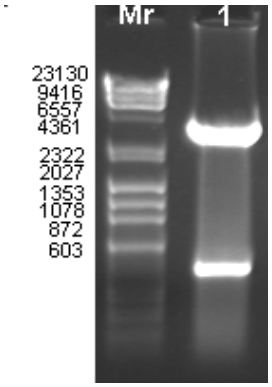


Fig 1A.4 Identification of pUC-gel
Mr. DNA marker
Lane1: pUC-gel cleavaged with XbaI/EcoRI
(Gelonin gene should be about 760 bp)

1A.2.2 Construction of an expression plasmid

For the expression of the recombinant gelonin, plasmid pET28a containing the T7 promoter, kanamycin (Kan) resistance and IPTG induction was chosen as vector. According to MCS of the vector, both pUC-gel and pET28a were digested with NdeI and EcoRI respectively, the gene fragment of gelonin from pUC-gel was then inserted into pET28a to construct a recombinant pET-gel (Fig 1A.5). By the analysis of transformation, plasmid extraction, double enzymatic digestion and DNA sequence assay, it was further confirmed that the vector pET-gel contained a 753 bp DNA fragment, which was the same as that designed in Fig 1A.2.

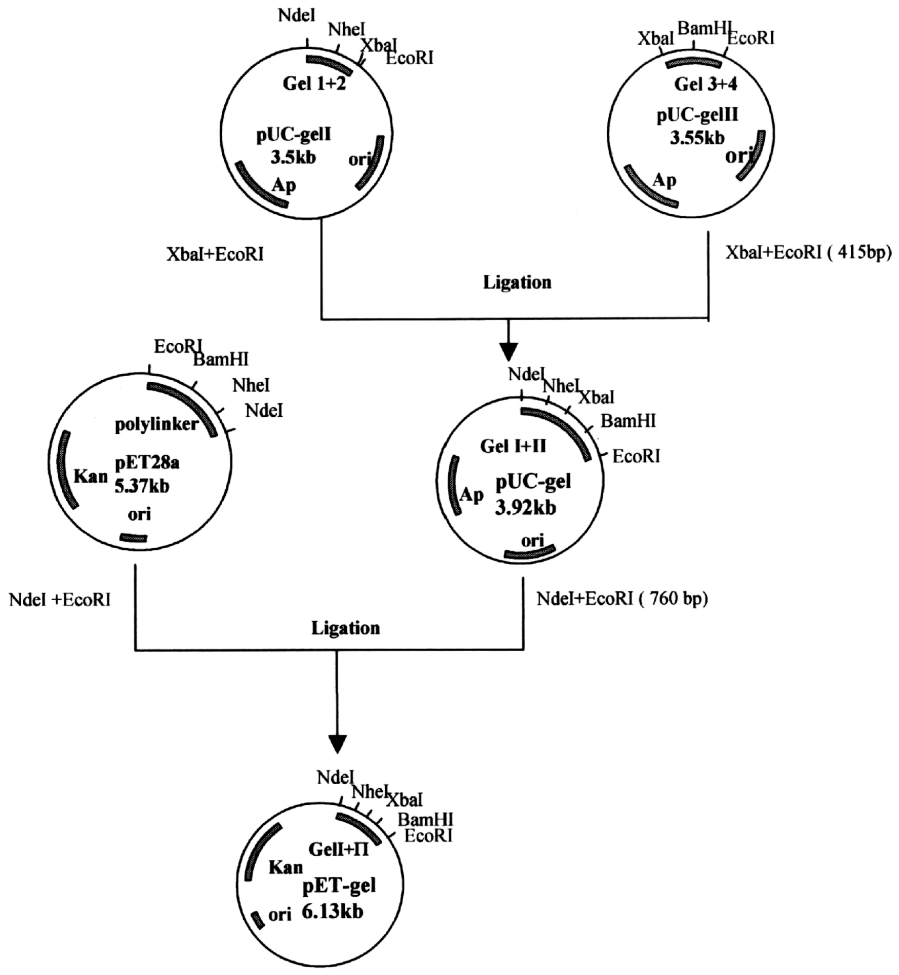


Fig 1A.5 Construction of the expression vector containing the gelonin gene

(pUC-gel I, pUC-gel II were provided by Ya-Wei, Shi [25])

1A.2.2.1 Identification of pET-gel gene

Some single colonies were transferred into 5 ml LB + ampicillin at 37°C in a shaker to reach an optical density OD_{600} of 0.7-0.8. Isopropyl-1-thio-β-D–galactopyranoside (IPTG) was added to a final concentration of 1 mM for induction and the culture was allowed to grow for another 3 h. Then 40 µl broth mixed with 3x sample buffer 10 µl was boiled for 5 min before loading on 12% SDS-PAGE. The expression product should show a band around 28 kD if the recombinant plasmid was correct. It is shown in Fig 1A.6 that strains of 3# (Lane 3 in Fig 1A.6) and 9# (Lane 9 in Fig 1A.6) yielded a new band. It was reasoned that gelonin could be expressed in *E. coli* BL21/pET-gel.

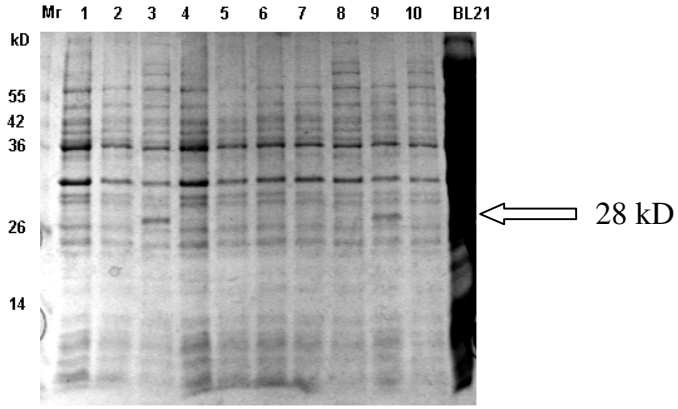


Fig 1A.6 Electrophoretic patterns of single colony culture on SDS-PAGE

Lane1-Lane10 are the different colonies of transformed strains after ligation on LB media plates. (Directly after ligation, the cells of BL21 were transformed with the plasmid pET-gel and plated on LB media plates. Different colonies were picked out and expressed in 5 ml LB culture. According to SDS-PAGE analysis, Lane3 and Lane9 show positive clones.)

Plasmids in 3# (Lane and 9# strains were extracted and digested with NdeI and EcoRI, a band of about 760 bp was detected on 1% agarose gel. After DNA sequence analysis, the gelonin gene was further confirmed (Fig 1A.7).

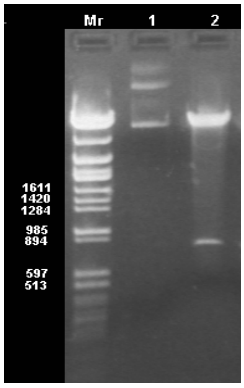


Fig 1A.7 Identification of pET-gel by endonuclease digestion

Mr: λDNA / EcoRI 4771; Lane1: pET-gel (6000 bp);

Lane2: pET-gel digested with NdeI /EcoRI (Gelonin gene should be about 760 bp)

1A.2.3 Expression of pET-gel in *E. coli* BL21

40 µl culture of strain *E. coli* BL21/pET-gel stored at –80˚C was taken into 20 ml LB+16 µl kanamycin (100 mg/ml) medium in shaker, 220 r/m, 37˚C overnight, then used to inoculate 1000 ml LB+800 µl kanamycin (100 mg/ml) medium. The broth was cultured to reach an optical density (OD_{600nm}) of approximately 0.6~0.7 and immediately induced by IPTG (final conc. 1 mM) for another 4 h growing at the same temperature. Finally, the pellets were harvested by centrifugation (6,000 r/m, 10 min), washed twice with 0.02 M Tris-HCl, pH7.6. The pellets were re-suspended in 50 ml, 50 mM phosphate buffer (pH6.5) including 2 mM DTT, 1.5 mM PMSF, 2 mM EDTA. It was sonicated 15 times on ice for 8 sec each. The quantity of crude protein was measured by the coomassie brilliant blue assay. Usually, about 130 mg total protein from 1 L culture can be obtained.

In order to confirm whether inclusion-bodies were formed, the precipitate was dissolved in 8 M urea, the supernatant and precipitate were subjected to 12% SDS-PAGE respectively. As shown in Fig 1A.8, there was only a very weak band in the precipitate at 28 kD (see lane 2 in Fig 1A.8) as compared to the corresponding band from the supernatant (Lane 3). Consequently, recombinant gelonin is rather soluble (Fig 1A.8).

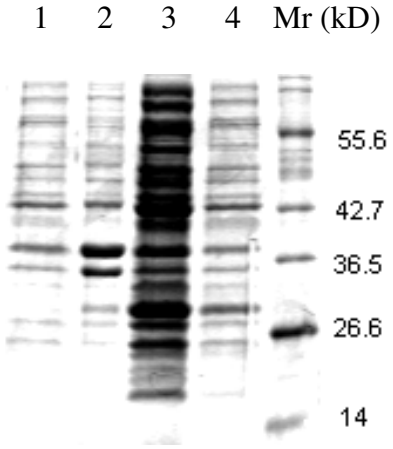


Fig 1A.8 Electrophoretic patterns of *E. coli* BL21/pET-gel culture on 12% SDS-PAGE

Lane1: Culture of *E.coli* BL21/pET-gel uninduction

Lane2: Precipitation after sonication and centrifugation with induction

Lane3: Supernatant after sonication and centrifugation with induction

Lane4: Culture of *E.coli* BL21/pET-gel with induction

To investigate the optimal conditions for induction, we attempted to express recombinant gelonin at different temperatures and times, i.e. 37°C for 4 h, 30°C for 4 h, or 12 °C, overnight. The comparative results were listed in Table 1A.6. In this work, the strain BL21/pET-gel was induced at 37°C for 4 h.

Table 1A.6 The different conditions of induction (100 ml LB medium)

Induced Temperature/time	37°C/4 h	30°C/4 h	30°C/1 h, 12°C/ overnight
Mixture (after sonication)	15 mg	13 mg	13.6 mg
Supernatant	12 mg	11 mg	11.3 mg
pellets	3 mg	2 mg	2.3 mg

1A.2.4 Purification of recombinant gelonin

To compare the different separation conditions, some matrices including Heparin 6ff, SP-Sepharose ff, Q-Sepharose ff, Superdex 75 were tried. Finally, after SDS-PAGE analysis the following process was chosen.

Crude proteins → SP-Sepharose ff → Dialysis → Ultra-filtration → SP-Sepharose (1ml pre-packed column on AKTA purifier, Pharmacia)

The supernatant from sonication and centrifugation was loaded on SP-Sepharose ff, and after washing, a stepwise elution with different concentrations of NaCl (0.2 M, 0.5 M, 1 M, in 50 mM PBS, pH6.5) was performed (Fig 1A.9). The fractions pooled in the different peaks were examined on 12% SDS-PAGE. Gelonin could be eluted in peak 3 with 0.5 M NaCl (see lane 3 in Fig 1A.10).

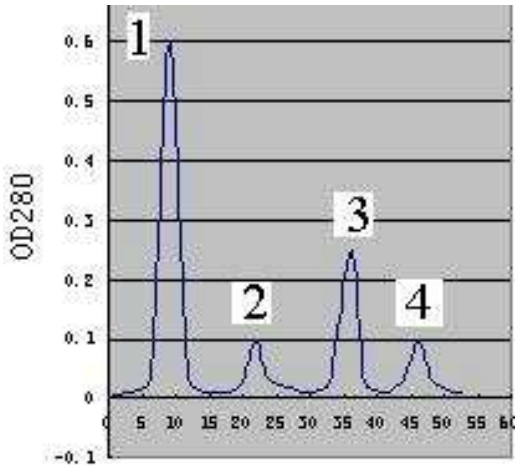


Fig 1A.9 A preliminary elution pattern on SP-Sepharose

Sample: 20 mg proteins were applied to a SP-Sepharose the column (2.2 x 6 cm) equilibrated with 50 mM PBS, pH6.5; Flow rate: 1ml/min; Elution with stepwise of 0.2 M NaCl, 0.5 M NaCl, 1 M NaCl. Peak 1: Flow through fraction; Peak 2: Fraction with 0.2 M NaCl; Peak 3: Fraction with 0.5 M NaCl;

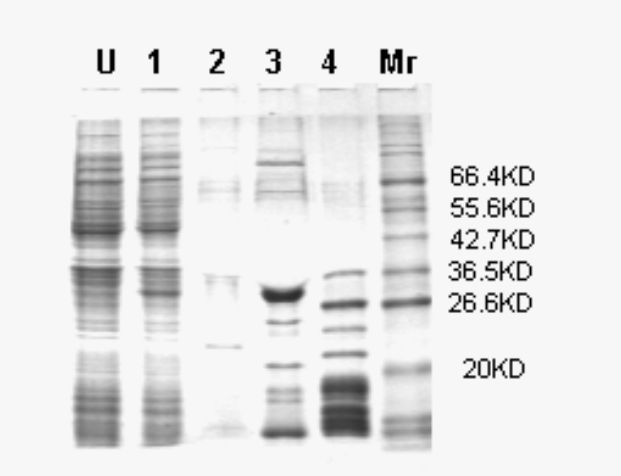


Fig 1A.10 Electrophoretic patterns of each peak pooled from SP-Sepharose ff

LaneU: pET-gel/BL21 uninduced; Lane1: pET-gel/BL21 induced

Lane2: Peak 2; Lane3: Peak 3; Lane4: Peak 4.

In order to purify the protein further, the fraction of peak 3 was first dialyzed against 50 mM Tris-HCl, pH10.5 and then concentrated by Amicon PM10 to 7.5 mg/ml. The concentrated sample was loaded on pre-packed SP-Sepharose (1 ml on AKTA purifier) and eluted with a continuous gradient of 0~1 M NaCl in a buffer of 50 mM Tris-Cl, pH10.5. The fractions pooled from different peaks were examined on 12% SDS-PAGE, which showed that pure gelonin had been obtained (See lane 1 in Fig 1A.12).

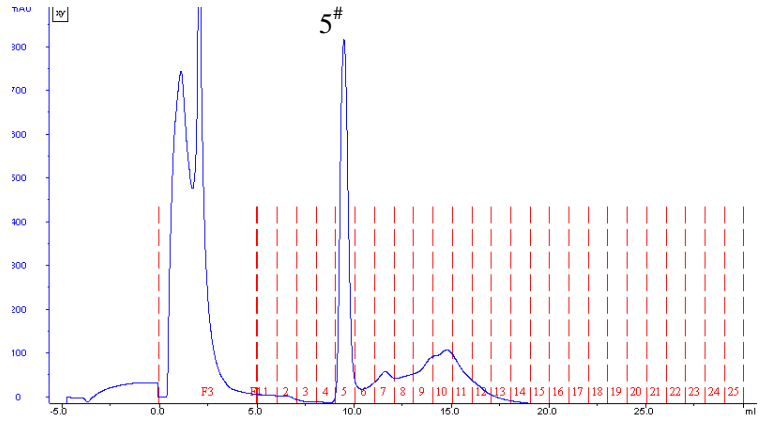


Fig 1A.11 Purification of gelonin on AKTA Purifier

Loading sample: 1.5 mg; Support: Pre-packed SP-Sepharose ff (1 ml).

Equilibration buffer: 50 mM Tris-HCl, pH10.5.

Gradient: 0-1 M NaCl in 50 mM Tris-HCl, pH10.5.

Flow rate: 1 ml/min; Fraction size: 1 ml.

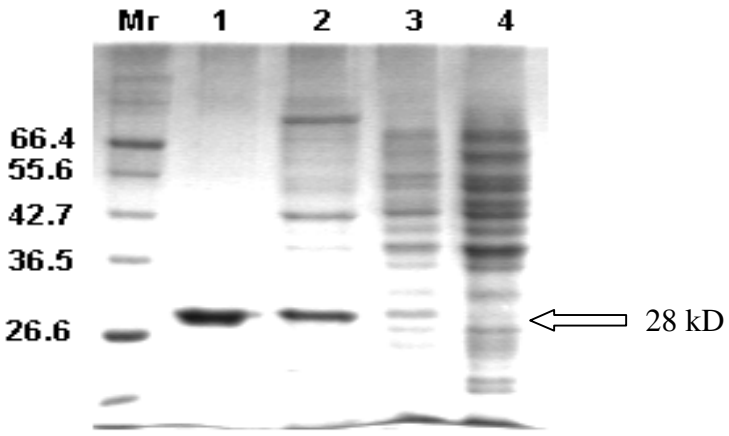


Fig 1A.12 Electrophoretic patterns of gelonin on 12% SDS-PAGE

Lane1: 5# tube from pre-packed SP-Sepharose ff (Fig 1A.11)

Lane2: 0.5 M NaCl fraction from SP-Sepharose ff

Lane3: pET-gel/BL21 induced

Lane4: pET-gel/BL21 uninduced

1A.2.5 ELISA and Western blots

The purified sample was first investigated by ELISA and Western blots (Fig 1A.13). The mouse anti-gelonin polyclonal antiserum was used as first antibody and peroxidase-conjugated sheep anti-mouse Ig was the second antibody with p-nitrophenyl phosphate as substrate. For the ELISA, wells of microtiter plates were coated with 2 μg sample. The sample of the purified gelonin was diluted to 2 μg with 100 μl coating buffer. The coated plates were incubated at 4℃ overnight. After washing, the bound protein was incubated with the first antibody (diluted to 1:1000) and the second antibody (diluted to 1:4000) as described in 3.5.2. The color developed in 30 min or so was measured at 405 nm (Table 1A.7).

Table 1A.7 ELISA assay

Sample	Gelonin (recombinant)	Gelonin (native)[a]	BSA[b]
OD405nm	0.19±0.01	0.17±0.01	0.01±0.01

a. Positive control (Gelonin from seeds); b. Negative control.

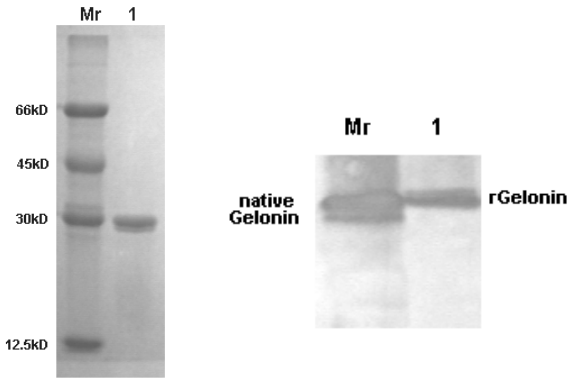


Fig 1A.13 Western blots analysis of gelonin

Left: SDS-PAGE; Right: Western Blots.

Mr.: Standard protein (30 kD gelonin isolated from seeds)

Lane1: Recombinant gelonin

(The first antibody is mouse anti-gelonin polyclonal antiserum)

1A.2.6 Reticulocyte lysate activity

The biological activity of both native gelonin and recombinant gelonin was assayed in a cell free rabbit reticulocyte lysate protein translation system, and was quantified by the incorporation of ^{14}C-valine *in vitro*. Native gelonin inhibited translation by 50% at a concentration of 15 ng/ml and recombinant gelonin at a concentration of 35 ng/ml (Fig 1A.14).

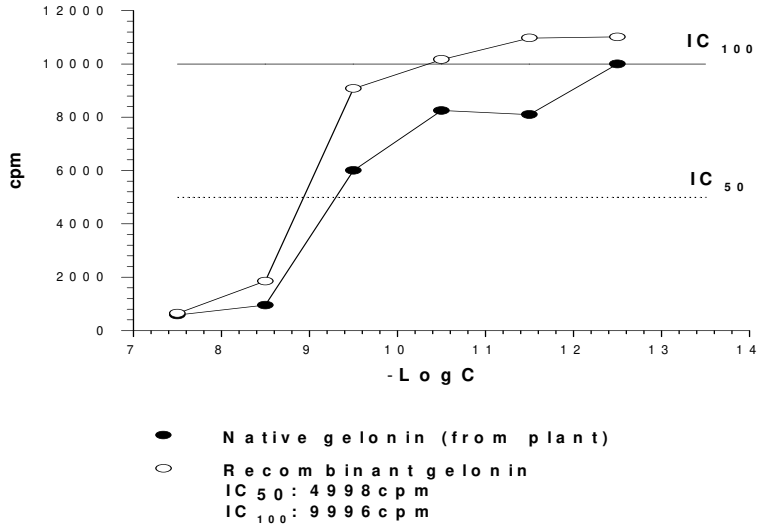


Fig 1A.14 Comparison of inhibition activity of native and recombinant gelonin.

The amount of ^{14}C valine incorporation was measured in relation to the concentration of recombinant gelonin and compared to native gelonin *in vitro* translation assay

(C: concentration of the sample; cpm: count/min of radioactivity)

(IC_{50}: 50% incorporation; IC_{100}: 100% incorporation)

1A.3 Summary

A recombinant plasmid containing the gelonin gene was constructed by molecular cloning. It was been confirmed that the DNA sequence of gelonin in the recombinant was identical to the known sequence by both double endonuclease cleavage and DNA sequence determination.

Engineering strain *E. coli* BL21/pET-gel can express gelonin, a 28 kD protein in LB+kanamycin medium upon induction by IPTG. The expression product of recombinant gelonin is quite soluble according to SDS-PAGE analysis so that it could be conveniently purified in the downstream process.

Comparing the various separation matrices and the different conditions, we decided to choose SP-Sepharose ff as its separating matrix. It was shown from SDS-PAGE analysis that the purification process of gelonin we employed is of high efficiency.

Finally, a preliminary toxicity test suggested from the results of Western blot analysis, ELISA and inhibition of protein synthesis *in vitro*, that the bioactivity of recombinant gelonin is about the same as that of gelonin from native sources.

1A.4 Literature

1. Urbatsch I L, Sterz R K, Trommer W E, *et al*. Antigen-specific therapy of experimental myasthenia gravis with acetylcholine receptor-gelonin conjugates *in vivo*. Eur. J. Immunol., 1993, **23**: 776-779.
2. Stirpe F, Barbieri L. Ribosome inactivating proteins up to date. FEBS Lett. , 1986, **195**: 1-8.
3. Elklid K, Olsnes S. Animal toxicity of *Shigella dysenterial* cytotoxin. J. Immuno, 1983, **130**: 380-384.
4. Barbieri L, Stirp F. Ribosome-inactivating proteins from plants: properties and possible uses. Cancer surveys, 1982, **1**: 489-520.
5. Sperti S, Brigotti M, Zamboni M, *et al*. Requirements for the inactivation of ribosomes by gelonin. Biochem. J. 1991, **277**: 281-284.
6. Endo Y, Tsurugi K. RNA N-glycosidase activity of ricin A chain. Mechaniem of action of the toxic lectin ricin on eukaryotic ribosomes. J. Biol. Chem., 1987, **262**: 8128-8130.
7. Osborn RW, Hartley MR. Dual effect of ricin A chain on protein synthesis in rabit reticulocyte lysate. Inhibition of initiation and translocation. Eur. J. Biochem, 1990, **193**: 401-407.
8. Simmons BM, Stahl PD, Russel JH. Mannose receptor-mediated uptake of ricin toxin and ricin A chain by macrophages. Multiple intracellular pathways for A chain translocation. J. Biol. Chem., 1986, **261**: 7912-7920.
9. Spooner RA, Lord JM. Immunotoxins: status and prospects. Trends biotech., 1990, **8**: 189-193.
10. Sandvig K, Olsnes S, Pihl.A. Kinetics of binding of the toxic lectins abrin and ricin to surface receptors on human cells. J. Biol. Chem., 1976, **251**: 3977-3984.
11. Van Deur B, Sandvig K, Peterson OW, *et al*. Estimation of the amount of internalized ricin that reaches the trans-Golgi network. J. Cell Biol., 1988, **106**: 253-267.
12. Yoshida T, Chen C, Zhang M, *et al*. Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin and *Pseudomonas* toxin. Exp. Cell Res.1991, **192**: 389-395.

13. Youle RJ, Neville DM. Kinetics of protein synthesis inactivation by ricin-anti-Thy 1 antibody hybrids. J Biol. Chem., 1982, **257**: 1598-1600.
14. Berg K, Selbo PK, Prasmickaite L, *et al*. Photochemical internalization: a novel technology for delivery of macromolecules into cytosol. Cancer Res., 1999, **59**: 1183-1190.
15. Selbo PK, Sandvig K, Kirveliene V, *et al*. Release of gelonin from endosomes and lysosomes to cytosol by photochemical internalization. Biochimical et Biophysical Acta, 2000, **1475**: 307-313.
16. Fernandez-Puentes C, Carrasco L. Viral infection permeabilizes mammalian cells to protein toxins. Cell, 1980, **20**: 769-775.
17. Taylor BE, Irvin JD. Depurination of plant ribosomes by pokeweed antiviral protein. FEBS Lett. 1990, **35**: 882-889.
18. Ready MR, Brown DT, Robertus JD. Extracellular location of pokeweed antiviral protein. Proc. Natl. Acad. Sci. USA, 1986, **83**: 5053-5056.
19. Wood KA, Lord JM, Wawrzynczak EJ. Preproabrin: genome cloning, characterization and the expression of the A-chain in *E.coli*. Eur. J. Biochem., 1991, **198**: 723-732.
20. Michael G, Rosenblum MG, Kohr WA, Beattie KL, *et al*. Amino acid sequence analysis, gene construction, cloning, and expression of gelonin, a toxin derived from *Geloniun multiflorum*. J. Interferon and cytokine Res., 1995, **15**: 547-555.
21. Nolan PA, Garrison DA, Better M. Cloning and expression of a gene encoding gelonin, a ribosome inactivating protein from gelonium multiflorum. Gene, 1993, **134**: 223-227.
22. Hosur MV, Nair B, Satyamurthy P, *et al*. X-ray structure of gelonin at 1.8A resolution. J. Mol. Bio., 1995, **250**: 368-380.
23. Better M, Bernhard SL, Fishwild DM, *et al*. Gelonin analogs with engineered cystine residues form antibody immunoconjugates with unique properties. J. Biol. Chem., 1994, **269**: 9644-9650.
24. Au TK, Collins TL, Lam TB, *et al*. The plant ribosome inactivating proteins luffin and saporin are potent inhibitors of HIV intergrase. FEBS lett., 2000, **471**: 169-172.
25. Shi YW, Wise GJ. The research report for the gene synthesis of gelonin in Kaiserslautern Uni.,1998.

1B Co-expression of the extracellular human acetylcholine receptor α subunit fragment and the chaperonin GroESL in *E. coli*

1B.1 Introduction

Nicotinic acetylcholine receptors (AchRs) are a family of transmembrane glycoproteins including both the muscle-type and neuronal-type AchR [1]. AchRs are known to be a part of the superfamily of neurotransmitter-gated ion channels, each composed of five homologous subunits organized around a central ion channel. cDNAs for 16 types of AchR subunits have been cloned from several species [2, 3]. These include α subunits numbered 1-9, β subunits numbered 1-4, δ, γ, ε subunits. The AchR family contains three branches, one occurs in muscle and the other two are neuronal (Fig 1B.1).

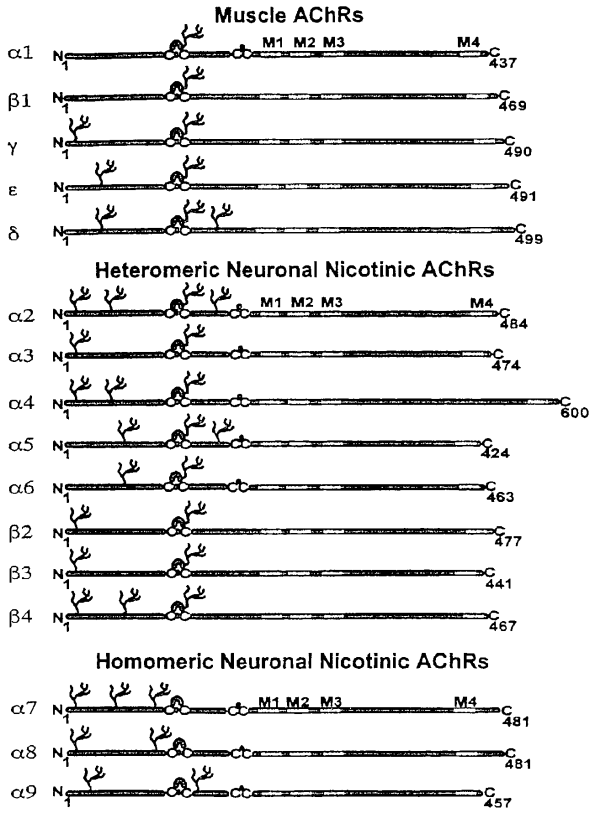


Fig 1B.1 The structure characteristics of AchRs [3]

Numbers: the amino acid residues, Greek symbols: the subunits of AchR

Loop: disulfide site, M1-M4: transmembrane domain, Branches: glycosylation sites

Fig 1B.1 depicts the structures of the subunits comprising these three branches of the AchR family

[4, 5, 6]. The N-terminal extracellular domain consists of about 220 amino acids and contains two homologus disulfide-linked loops. In most AchR subunits there is an N-glycosylation site at 141, some contain additional glycosylation sites in the large extracellular domain, and all contain at least one such site [7, 8, 9].

1B.1.1 Muscle AchRs

In 1973, the first purification of milligram amounts of acetylcholine receptor from electrophorus electric organ resulted in the discovery of EAMG (experimental autoimmune Myasthenia gravis) when it was found that rabbits immunized with that AchR became weak and died [10]. At that time, muscle AchRs were well characterized electrophysiologically as Ach-gated cation channels, and snake venom toxins such as α-bungarotoxin were just coming into use for localizing, quantifying, and affinity purifying AchRs.

The structure of muscle-type AchRs found in the *Torpedo* electric organ has been characterized in some details [4, 11]. The nicotinic acetylcholine receptor (AchR) found at the neuromuscular junction is the autoantigen involved in the autoimmune disease Myasthenia gravis (MG). Owing to its dual importance as a model autoantigen and as a model neurotransmitter receptor, its general and antigenic structures have been extensively studied over the last 26 years. The AchR molecule is a transmembrane glycoprotein (M_r ~290 kD) consisting of five homologous subunits in the stoichimetry $\alpha_2\beta\gamma\delta$ (embryonic) or $\alpha_2\beta\varepsilon\delta$ (adult), which form the action channel. The α subunit carries in its N-terminal extracellular domain the main immunogenic region (MIR), a group of conformationally dependent epitopes that seems to be a major target for the anti-AchR antibodies in MG patients [12, 13].

AchR subunits are thought to be organized like barrel staves around the central ion channel in the order αγαδβ, as shown in Fig 1B.2. The binding sites of acetylcholine (Ach) are formed at the interfaces between α and γ or ε subunits. Because amino acids from both subunits contribute to each of the two Ach binding sites, the properties of each of the Ach binding sites are somewhat different. Both sites must have agonist bound for the ion channel to have a high probability of flickering open for 1 or 2 ms. An antagonist bound to either site prevents activation.

Electron diffraction studies of *Torpedo* electric organ AchRs have reached a resolution of 4.6 Å [11]. The AchR is about 80 Å in diameter and 120 Å long and 65 Å extending on the extracellular surface, 40 Å crossing the lipid bilayer, and 15 Å extending beneath the bilayer into the cytoplasm.

The extracellular vestibule of the channel is about 25 Å in diameter surrounded by walls about 25 Å thick. The actual lumen of the channel through the lipid bilayer is quite narrow. The gate of the channel is thought to be at its cytoplasmic end [13].

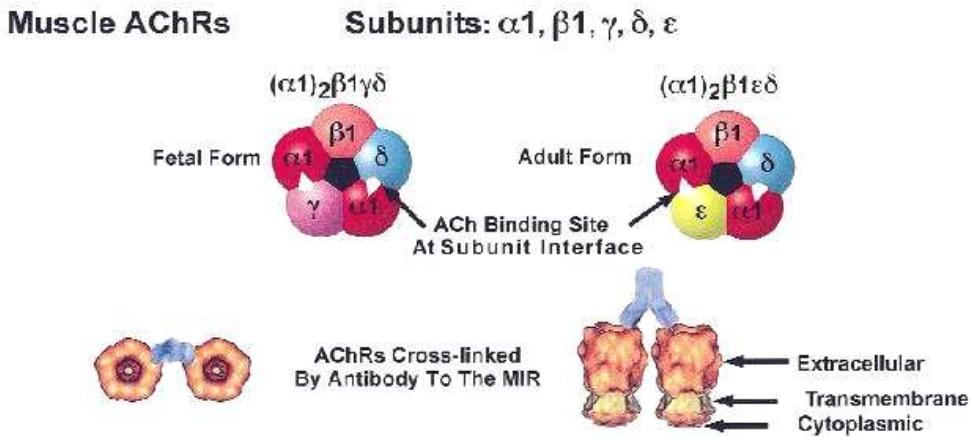


Fig 1B.2 The arrangement of subunits of muscle AchRs [3]

Nicotinic acetylcholine receptors, as well as other members of the ligand-gated ion channel superfamily, present a very simple repertoire of functional properties. The AchR recognizes the neurotransmitter Ach and upon binding, the intrinsically coupled ion channel is opened, augmenting in turn the possibility of cations to cross the lipid membrane. Thus, after channel opening, a new ionic concentration is found at the aqueous solution bathing the opposed faces of the lipid bilayer of the cell. In particular, the extracellular liquid presents now a higher concentration of K^+ (efflux) and the cytoplasmic compartment has a higher content of Na^+ (influx). When the concentration reaches equilibrium a membrane depolarization is produced. Depolarization of the membrane provokes a specific physiological response by each cell that is involved. For instance, if the muscle membrane depolarization is large enough, an action potential propagates from the neuromuscular junction all over the muscle fiber. During the propagating action potential, the release of Ca^{2+} ions from intracellular stores in the muscle cell is stimulated. The final response in the muscle is the contraction of its myofibrils [14].

All these biologically relevant AchR properties are triggered by the binding of the neurotransmitter Ach. Upon binding, the receptor protein undergoes a conformational change. Several lines of experimental evidence suggest that the AchR may exist in a minimum of four interconvertible states. The tetrahedral diagram indicating the existence of at least four receptor states is shown in Fig 1B.3. In the absence of agonists, most *Torpedo* receptors (~80%) are in the resting state (Fig 1B.3, R). An additional about 20% of receptors is in the desensitized state (Fig 1B.3, D). The

resting state is defined by the existence of an activatable closed ion channel. In the presences of agonists, the receptor is activated (Fig 1B.3, A) in the microsecond to millisecond time frame. The state presents an open ion channel and a low affinity for agonists (apparent dissociation constant K_d between 10 μM and 1 mM). Concomitantly, in the prolonged presence of agonists the activated receptor is commuted to an intermediate state (Fig 1B.3, I) in the 1-100 ms time scale and further to a D state in the second to minutes time frame. No energy sources nor an ionic gradient is needed to induce the conformational shift from the R to the D state [14].

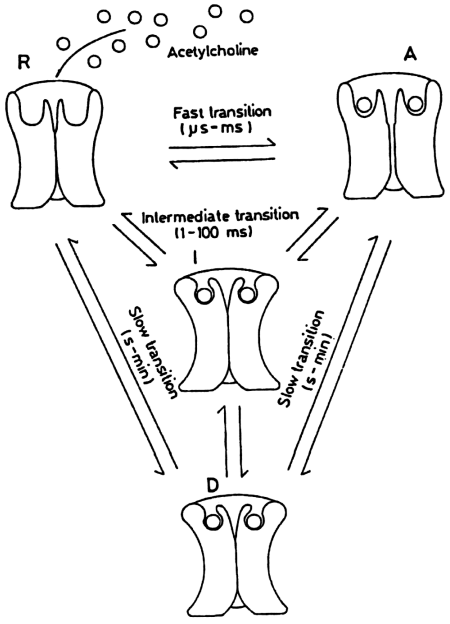


Fig 1B.3 Diagram showing the dynamic of multiple conformational states of the nicotinic acetylcholine receptor [14]

In the absence of the neurotransmitter Ach, shown here as empty circles, the AchR is in the resting state (R), a conformation state where the ion channel is closed. The closed ion channel can be opened upon binding of two Ach molecules to AchR. This active state (A) presents low affinity for agonists. The transition from the R to A state is a fast process that proceeds in the microsecond and millisecond time regime. In the prolonged presence of agonists, the AchR becomes refractive to the agonist's pharmacological action and thus, to the activation of its ion channel. In the *Torpedo* AchR there exists two refractive closed ion channel states, the intermediate (I) and the desensitized (D) state. Both states show high affinity for agonists and some antagonists. The transition from the A to the I state is a slow process that is produced in 1-100 ms time range. The transition from the A to D state has a much slower time course.

1B.1.2 Research goals

The acetylcholine receptor (AchR) is a trans-membrane glycoprotein, consisting of 2α, β, γ, δ subunits. In muscle tissue of adults, the γ-subunit is substituted by the ε-subunit. A mature α-subunit has 437 amino acid residues containing the so-called main immunogenic region (MIR)- a binding site of a polysaccharide. MIR located at residues 66 to 76 of α-subunit and contains an Asn residue as the binding site of polysaccharide. MIR, as the target of AchR antibodies, plays an important role in the autoimmune disease Myasthenia gravis. In order to investigate the mechanism of Myasthenia gravis, researchers have attempted to obtain the relevant proteins by gene technology. Unfortunately, the amount of AchR or its domain when expressed in engineering bacteria usually is very low or the product occurs in the form of inclusion bodies. For improving the efficiency of refolding and solubility of recombinant proteins, two recombinant vectors of co-expressing the AchR α-subunit fragment and the chaperonin GroESL will be performed. In this part, plasmid pPR506 which contains the gene of the α-subunit domain of AchR and plasmid pGE60 containing the gene of the chaperonin GroESL will be co-transferred into *E. coli* DH5α and co-expressed by the induction with IPTG. The receptor fragment could be coupled chemically to gelonin and be employed in the treatment of Myasthenia gravis.

1B.2 Results and discussion

1B.2.1 Identification of the recombinant plasmids

The construction of pPR506 is shown in Fig 1B.4. To verify co-transformation of pPR506 and pGE60, the two plasmids were transferred singly or doubly into *E. coli* DH5α. Afterwards, the plasmids were extracted and analyzed by 1% agarose electrophoresis (Fig 1B.5). The gel confirmed indicated that the molecular weights of pPR506 and pGE60 of about 2.3 kb (Fig 1B.5; A) and 6.0 kb (Fig 1B.5; C) respectively, while the co-transformed plasmid contained both pPR506 and pGE60 (Fig 1B.5; B) as deduced from their electrophoretic patterns.

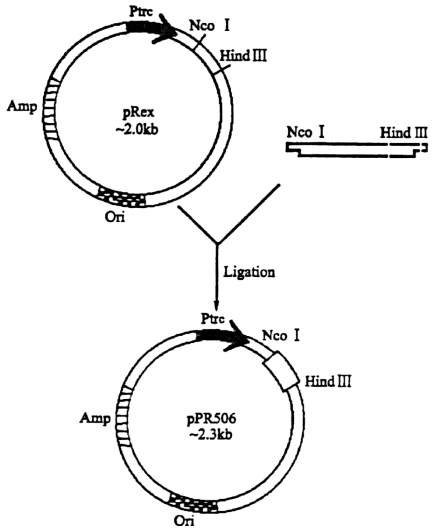


Fig 1B.4 Construction of plasmid pPR506

The target gene is a DNA fragment of AchR α-subunit residues from 1-120

By DNA sequence analysis, the fragment sequence in pPR506 is identical to the DNA sequence deduced from the known amino acid residues of the AchR α-subunit domain. GAA encoding Glu is the first amino acid of the domain and TCC encoding Ser is located at the 3'-terminus [15].

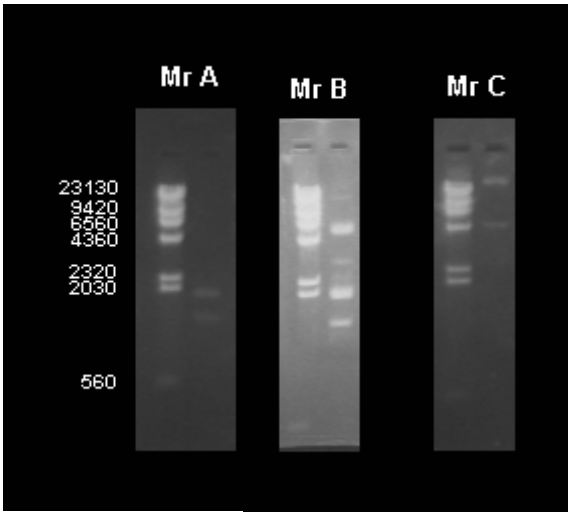


Fig 1B.5 Co-transformation of plasmid pPR506 and pGE60

Mr: λDNA/Hind III; A. pPR506 only (2.0 kb containing the AchR fragment); B. pGE60 (6.0 Kb) and pPR506 (Containing chaperonin GroESL and AchR fragment); C. pGE60 only (Containing charperonin GroESL).

1B.2.2 Co-expression of the recombinant plasmids in *E. coli*

The engineering strain *E. coli* DH5α/pPR506+pGE60 was cultured in LB medium which contained both ampicillin (Amp) and chloromycetin (Chl) at 37˚C, 220 r/m until the optical density (OD_{600nm}) of the culture reached about 0.5~0.6. Subsequently, IPTG was added for induction and fermentation was continued for another 4 h at the same temperature. Pellets were harvested by centrifugation (5000 r/m, 30 min) and sonicated in PBS. The supernatant was analyzed by SDS-PAGE. Electrophoretic analysis of the culture products related that the expression of pPR506 had yield only a 13 kD band equal to the AchR α-subunit domain and the amount of expression was also very low and those of pGE60 had two bands at 60 kD and 10 kD equal to the products of GroEL and GroES respectively, while the co-expression product displayed four bands, of which the 60 kD band could result from GroEL and 10 kD band from GroES as well as a 13 kD band that is likely to be the α-subunit fragment (Fig 1B.6). The new band at 23 kD could be the complex which contains 13 kD α-subunit fragment from the result of immunoblotting assay that both bands of 13 kD and 23 kD were positive (Fig 1B.7).

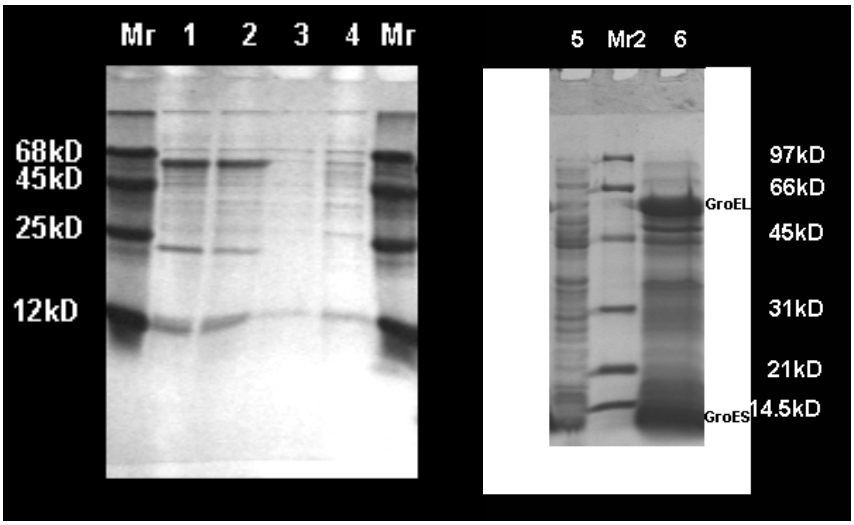


Fig 1B.6 SDS-PAGE analysis for expression product

Lane1 and lane2: Co-expression products (AchR fragment and charperonin GroESL)

Lane3 and Lane4: Expression products by DH5α/pPR506 (Only AchR fragment)

Lane5: Expression products of DH5α (Control, no plasmids presented)

Lane6: Expression products by DH5α/pGE60 (Only charperonin GroEL/GroES)

1B.2.3 Analysis of Western blots

After centrifugation of the sonicated mixture, the supernatant was run on 12% SDS-PAGE, the components on the gel were transferred into a nitrocellulose membrane by sandwich electrophoresis (Fig 1B.6, Lane1, 2, 3, 4 were transferred onto membrane). The membrane was incubated with the primary antibody (mAb35; Part I·1C.2.3.4) and the second antibody (rabbit anti-rat Ig 1:1000) respectively. Finally, the membrane treated as above was developed by BCIP/NBT until the color of the target protein occurred and re-transferred into PBS to be fixed immediately. Upon repetition of the experiments, the western blot was not always positive.

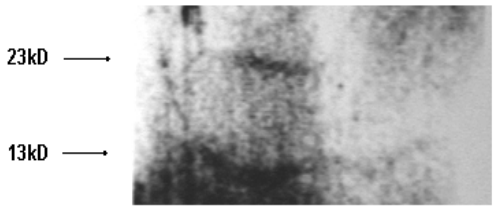


Fig 1B.7 Western blot

The original SDS-PAGE picture was shown in Fig 1B.6.

The first antibody is mAb35 (Part I.C.2.3.4)

1B.2.4 Purification of products

After the supernatant was concentrated by Centriprep, not only some low molecular weight proteins were partly extruded but also the sample was concentrated about 6 folds. The total protein of cells was approximately 62 mg under the condition of the experiments. The concentrated sample (about 5 mg) was loaded on Superose 12 HR10/30 on FPLC. The four eluted peaks were pooled and run on SDS-PAGE respectively (Fig1B.8).

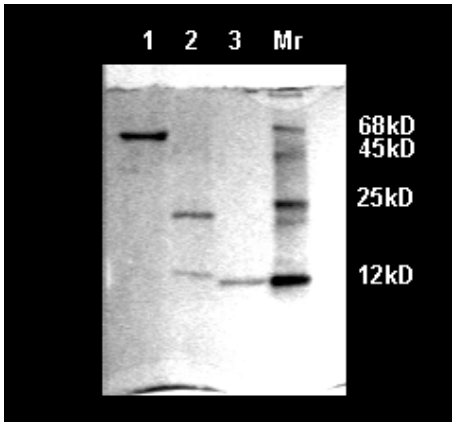


Fig 1B.8 Electrophoretic patterns of each peak from HPLC by SDS-PAGE

(The co-expression products of AchR fragment and GroESL were purified with Superose 12 HR 10/30.)

Lane1: Peak1, 60 kD (GroEL)

Lane2: Peak2, 23 kD and 13 kD (AchR fragment)

Lane3: Peak3, 10 kD (GroES).

1B.3 Summary

As described in previous work, the expression products of the acetylcholine receptor α-subunit or its domain in bacteria usually resulted in inclusion bodies consisting of insoluble aggregates of the proteins. It was then necessary to denature and renature the aggregates to obtain native protein. However, it was reported that molecular chaperonins such as GroESL may facilitate renaturation of denatured proteins to their native conformation. It was shown from our experiments that GroESL slightly improved the correct expression of the acetylcholine receptor α-subunit fragment. Based on the molecular weight of acetylcholine receptor α-subunit domain and GroES, it is very likely that the 13 kD band in the SDS-gel belongs to the α-subunit fragment. The 23 kD would account for a complex between the α subunit fragment and GroES, but there is no further evidence for such a complex besides the Western blot (Fig 1B.7).

Our experiments clearly show that co-expression of the α-subunit fragment of AchR together with GroESL leads to a soluble form of the receptor. However, the yields are very low and not reproducible. The amount of receptor fragment was certainly not sufficient for chemical cross-linking with gelonin. Hence, we tried to express the conjugate as a fusion protein as described in Part I· C

1B.4 Literature

1. Lindstrom J. Neuronal nicotinic acetylcholine receptors. In: Narahashi T, editor. Ion channels. New York: Plenum press, 1996: p377-450.
2. Lindstrom J. Purification and cloning of nicotinic acetylcholine receptors. In Aeneric S, Brioni D, editors. Neuronoal nicotinic receptors: pharmacology and therapeutic opportunities. New York, John Wiley and sons; 1999.
3. Lindstrom J. Acetylcholine receptors and Myasthenia. Muscle and Nerve, 2000, **23**: 453-477.
4. Karlin A, Akabas M. Toward a structure basis for the function of nicotinic acetylcholine receptorsand their cousins. Neuron, 1995, **15**: 1231-1244.
5. LeNovere N, Changeux JP. Molecular evolution of the nicotinc acetylcholine receptor: an example of multi-gene family in excitable cells. J. Mol. Evol., 1995, **40**:155-172.
6. LeNovere N, Corringer JP, Changeux JP. Improved secondary structure predictions for a nicotinic receptor subunit: incoporation of solvent accessibility and experimental data into a two-dimentional representation. Biophys J., 1999, **76**: 2329-2345.

7. Anand R, Bason L, Saedi M, *et al*. Report epitopes: a novel approach to examine transmembrane topology of intergral membrane proteins applied to the α_1 subunit of the nicotinic acetylcholine receptor. Biochemistry, 1993, **32**: 9975-9984.
8. Zhang H, Karlin A. Identification of acetylcholine receptor channel-lining residues in the M1 segment of the β-subunit. Biochemistry, 1997, **36**: 15856-15864.
9. Zhang H, karlin A. Contribution of the β subunit M2 segment to the ion-conducting pathway of the acetylcholine receptor. Biochemistry, 1998, **37**: 7952-7964.
10. Patrick J, Lindstrom J. Autoimmune response to AchR. Science, 1973, **180**: 871-872.
11. Miyazawa A, Fujiyoshi Y, Stowell M, *et al*. Nicotinic acetylcholine receptor at 4.6Å resolution: transverse tunnels in the channel wall. J. Mol. Biol. 1999, **288**: 765-786.
12. Beeson D, Morris A, Vincent A, *et al*. The human muscle nicotinic acetylcholine receptor α-subunit sxists as two isoforms: a novel exon. EMBO J., 1990: **9**: 2101-2106.
13. Wilson G, Karlin A. The location of the gate of the acetylcholine recepor channel. Neuron, 1998, **20**: 1269-1281.
14. Arias HR. Binding sites for exogenous and endogenous non-competitive inhibitors of the nicotinic acetylcholine receptor. BBA, 1998, **1376**: 173-220.
15. Saedi MS, Anand R, Lindstrom J, *et al*. Determination of amino acids critical to the main immunogenic region of intact acetylcholine receptors by *in vitro* mutagenesis. FEBS lett., 1990, **267**: 55-59.

1C Gene clone and expression of gelonin-acetylcholine receptor α subunit domain

1C.1 Introduction

Conjugation between a RIP and an antibody is often used as a therapeutic agent. The antibody could ideally deliver the cytotoxic compounds to the surface of cells, and immunoconjugates consisting of a whole antibody or antibody domains linked to proteins that disrupt cellular protein synthesis have been widely described [1]. Immunoconjugates have typically been constructed *in vitro* with antibodies and cytotoxic proteins by heterobifunctional cross-linking agents [2]. Recent advances in antibody engineering, however, make it possible to express a variety of antibody domains independently in microorganisms, and to express antibody domains as fusion proteins with a variety of proteins such as enzymes, e.g. RIPs. Moreover, in such constructs the solubility of hydrophobic proteins could be improved by the conjugation with highly soluble RIPs.

1C.1.1 Immune system

The immune system protects us from infections by microbes. It is composed of a number of different cell types, tissues and organs. Many of these cells are organized into separate lymphoid organs or glands. Since attack from microbes can come at many different sites of the body, the immune system has a mobile force of cells in the blood stream which are ready to attack the invading microbe wherever it enters the body.

All immunocompetent individuals have many distinct lymphocytes, each of which is specific for a different foreign substance. When an antigen is introduced into an individual, lymphocytes with receptors for this antigen seek out and bind it and are triggered to proliferate and differentiate, giving rise to clones of cells specific for the antigen. These cells or their products specifically react with the antigen to neutralize or eliminate it. The much large number of antigen-specific cells late in the immune response is responsible for the memory of immunity.

There are of two major types of lymphocytes, B cells and T cells. T cells mature under the influence of the thymus and, on stimulation by antigen, give rise to cellular immunity. B cells

mature under the influence of bone marrow and give rise to humoral immunity that involves production of soluble molecules, immunoglobulins. Interactions between T and B cells, as well as antigen presenting cells, are critical to the development of specific immunity.

1C.1.1.1 Antigens and antibodies

Invading organisms have antigens which are recognized by the immune system. Antigens are defined as substances which induce an immune response. They include proteins, carbohydrates, lipids and nulcleic acids. An antigen molecule may contain a number of the same or different antigenic determinants to which individual antibodies or cell responses are made. These antigenic determinants or epitopes are the smallest unit of an antigen to which an antibody or cell can bind (Fig1C.1). For a protein, an antibody binds to a unit which is about three to six amino acids whilst for a carbohydrate it is about five to six sugar residues. Therefore, most large molecules possess many antigenic determinants per molecule. However, these determinants may be identical or different from each other on the same molecule.

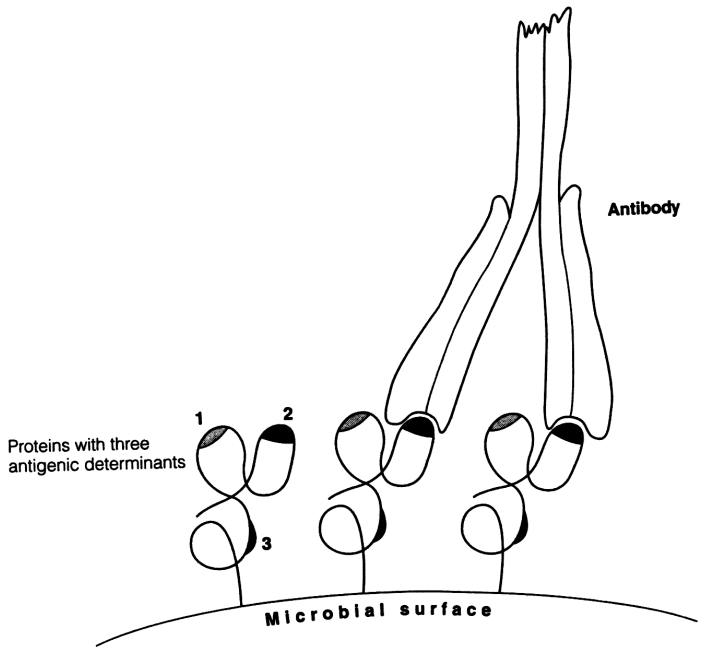


Fig 1C.1 Antigenic determinants (epitopes) recognized by antibodies

Antibodies have a basic unit of four polypeptide chains -two identical pairs of light (L) chains and heavy (H) chains- bound together by covalent disulfide bridges as well as by noncovalent interactions. These molecules can be proteolytically cleaved to yield two Fab fragments (the antigen binding part of the molecules) and an Fc fragment (the part of the molecule responsible for effect functions). Both L- and H-chains are divided into V and C regions determining the fate of

the antigen. The valence of an antibody is the number of antigenic determinants with which it can react (Fig 1C.2).

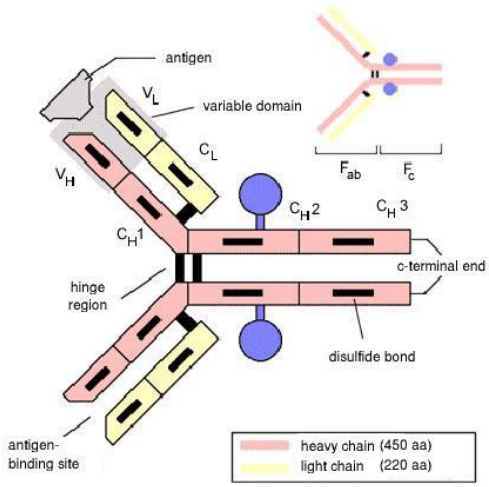


Fig 1C.2 IgG immunoglobulin: basic 4 chain structure representative of all immunoglobulins [3]

Different antibody classes with different biological activities have evolved to deal with antigens with different properties and which enter the body at different sites. There is some overlapping in their function and in where they are produced, but generally there is a division of labor among the different antibody classes, e.g. IgA is most common antibody in mucosal secretions while IgM is mainly found in the plasma, both are most effective at those locations. IgG, immunoglobulins provide the bulk of immunity to most blood borne infectious agents, it is the only antibody class to cross the placenta to provide humoral immunity to the infant. Allergic reactions are predominantly associated with IgE.

1C.1.1.2 The antibody response

Antigen introduced into an individual binds specifically to B cells with receptors for that antigen. In the presence of T cell help, these B cells clonally expand and some differentiate into plasma cells which produce antibodies specific for the antigen that triggers the response. On first exposure to antigen, a primary immune response develops resulting in production of IgM antibodies. This usually is followed by an IgG immune response after a few days. This response is self-limiting and will stop when antigen is no longer available to stimulate B cells. When antigen is reintroduced, there are more antigen specific B cells which have differentiated to more responsive memory B cells, resulting in a more rapid response and usually in IgG antibody production.

Although antibodies produced by a single cell and its daughter cells are identical (homogeneous or monoclonal; mAb), the response to a given antigen involves many different clones of cells and thus, overall, is very heterogeneous (multiclonal). Considering the size of an antigenic determinant, the number of determinants on a molecule, the number of different molecules on the microorganism, the response to a microorganism results in a large number of different antibodies. Even antibodies against a single antigenic determinant are heterogeneous, indicating that the immune system is capable of producing many different antibodies, even to a single well-defined antigenic determinant. This heterogeneity is essential for many of the protective functions of antibodies.

1C.1.1.3 Cell mediated immunity

Cell mediated immunity (or T cell mediated immunity) is due to the direct action of T cells, and can be transferred by cells. T cells have two major roles which are carried out by two distinct subpopulations. T helper cells (Th) help other cells carry out their function, whilst cytotoxic T cells (Tc) directly kill cells infected with intracellular microbes. Both Th and Tc need to interact directly with the cells they are going to kill/help and they do this through specific recognition mechanisms. This is mediated through interaction with MHC (major histocompatibility complex) on the surface of the cell being for help and cytotoxicity.

1C.1.1.4 Cell recognition of self and non self

Cells of the immune system must be able to recognize and eliminate microbes and thus to distinguish what is “foreign” from what is self. A fundamental requirement of the immune system is that it destroys, eliminates or inactivates all foreign viruses, bacteria and parasites without destroying self cells or molecules. Self tolerance is the state of immunological unresponsiveness to self antigens. It is maintained through a number of different mechanisms in central and peripheral lymphoid organs. The fundamental basis for self tolerance is that interaction of antigen with immature clones of lymphocytes already expressing antigen receptors, would result in an unresponsive state. In the thymus, self-reactive T cells are clonally eliminated by negative selection as part of maturation of the T cell repertoire. In the bone marrow, self reactive B cells are also eliminated. Lymphocytes escaping tolerance in the primary lymphoid organs are eliminated or anergized in the peripheral lymphoid organs.

1C.1.1.5 Autoimmunity and autoimmune diseases

Autoimmunity is an acquired immune reactivity to self antigens. The immune system has the capacity to respond to virtually all molecules and/or cells. Although the capacity to respond to self antigen is present in all of us, in most instances such responses result in tolerance or anergy, indicating that the mechanisms must exist to prevent or subdue autoimmune responses. Moreover, autoreactive T and B cells and autoantibodies are found in people who do not have autoimmune diseases, demonstrating that immunological autoreactivity alone is not enough for the development of disease. The mechanisms currently thought to prevent/dampen autoimmune responses include inactivation or deletion of autoreactive T and B cells, active suppression by cells or cytokines, idiotype/anti-idiotype interactions, and the immunosuppressive adrenal hormones, the glucocorticoids.

Autoimmune diseases arise as the result of a breakdown in self-tolerance. Factors predisposing and/or contributing to the development of autoimmune diseases include age, genetics, gender, infections and the nature of the autoantigen. Combinations of these factors are probably important in the development of an autoimmune disease. Autoimmune diseases are quite prevalent in the general population, where it is estimated that approximately 3.5% of individuals are afflicted.

1C.1.2 Myasthenia gravis

Myasthenia gravis is a disorder in which autoantibodies to acetylcholine receptors at the neuromuscular junction of skeletal muscle lead to AchR loss and muscle weakness. It is a classic acquired autoimmune disease. What induces the autoimmune response to muscle AchRs in MG is not known. The mechanisms may differ in various forms of MG. In most MG patients, the immunogen is likely to be native muscle AchR or a closely related molecule because the spectrum of autoantibody specificities in MG and EAMG is very similar [4]. Fetal-type AchRs may often be the immunogen in MG, as indicated by the selective reaction with fetal AchRs found in many MG patient autoantibodies [5, 6, 7]. Denatured AchR subunits or synthetic fragments of AchR subunits are extremely inefficient at provoking EAMG because they lack the MIR [8, 9, 10].

The thymus is suspected to be the site of the initiation of the autoimmune response to AchRs in MG because: (1) thymic myoid cells express fetal AchRs and other muscle proteins and low levels of AchR may also be produced by some other thymic cells [11, 12, 13], (2) 70% of MG patients exhibit thymus lymphoid follicular hyperplasia and exhibit germinal centers producing antibodies

to AchRs [14, 15, 16], (3) thymectomy is often beneficial [17], (4) 10-15% of MG patients exhibit thymoma [18], (5) the thymus is not hyperplastic or thymomatous in EAMG where the autoimmune response is initiated with exogenous AchR [19], (6) transplant of fragments of MG patient thymus to severe combined immunodeficiency (SCID) mice can transfer MG to the mice [20]. However, the thymus is probably not the sole site for induction or maintenance of the autoimmune response to AchR, thymectomy is not uniform or complete in its beneficial effect on MG.

EAMG has been induced in many species by immunization with purified Torpedo electric organ AchR [21]. AchR is quite immunogenic and EAMG has been induced by AchR [22]. Adjuvants are usually used, but it is possible to induce EAMG with AchR even in the absence of adjuvants [23]. Among MG patients, the absolute concentration of antibodies to AchR does not correlate closely with severity [24], but generally patients with only ocular manifestation have lower autoantibody concentrations than do patients with generalized MG, and changes in an individual's autoantibody concentrations usually parallels changes in their clinical state [25]. The basic mechanisms by which autoantibodies to AchR impair neuromuscular transmission appear to be very similar in MG and chronic EAMG [26, 27].

1C.1.2.1 Cellular immune mechanisms in MG and EAMG

EAMG is caused by an antibody mediated autoimmune response, it is not surprising that development of EAMG depends on the presence of B-cells. B-cell-deficient mutant mice are resistant to induction of EAMG, but develop a normal T-lymphocyte-mediated immune response and are susceptible to passively transferred EAMG mediated by a mAb to the MIR [28]. The T-cell response in these mice lacking B-cells developed more slowly than it would have with antigen presentation by B-cells as well as professional antigen-presenting cells. Antigen-specific B-cells can be very efficient at presenting peptides from the native antigens that they bind with high affinity.

Development of EAMG and MG depends on T-lymphocytes to cooperate with B-lymphocytes in developing autoantibody-producing plasma cells, but which T-cell types are involved has been more difficult to determine. CD4+ helper lymphocytes are necessary for the production of antibodies to AchR in both EAMG and MG [29]. Using various methods with human MG patient lymphocytes, different AchR epitopes have been identified. In various strains of rats and mice, different prominent epitopes have also been identified [30]. Some of these have been used to

induce tolerance and reduce induction of EAMG [31]. The dependence of these epitopes on the genetics of the antigen-presenting proteins, T-cell receptors, and other components of the cellular immune processing, recognition, and regulatory systems makes inbred rodent strains exquisitely sensitive to immune regulation by AchR peptides and small mutations of these peptides. However, it is unclear whether such subtleties will prove useful in predicting or manipulating the immune response in the outbred human populations that comprise MG patients.

1C.1.2.2 Development of specific immunosuppressive therapies for EAMG and MG

There is currently no specific immunosuppressive therapy or cure for MG. Nonspecific immunosuppressive therapy of MG with prednisone and other drugs, as well as by thymectomy combined with symptomatic therapy using inhibitors of acetylcholinesterase, permits substantial control of MG and has greatly reduced its mortality. There are significant side effects associated with prolonged nonspecific immunosuppressive therapy. Because so much is known about the antigenic structure of muscle AchRs and the pathological mechanisms of MG, it would seem reasonable to hope for a specific immunosuppressive therapy, because it has been thought that MG is the best characterized autoimmune disease [32]. Successful specific immunosuppression of MG might be valuable not only for its benefit to MG patients, but also as a model for what might be applied to others autoimmune diseases with well characterized autoantigens.

Some exotic approaches to inhibit autoantibodies to the MIR have been reported as potential therapies for MG. Tzartos and coworkers proposed to use monovalent Fab fragments of mAbs to the MIR to compete for binding of autoantibodies [33]. Due to lacking the Fc region, they cannot fix complement and cause disruption of the postsynaptic membrane. Because they are monovalent, they can not crosslink AchRs and cause antigenic modulations, as can bivalent $F(ab)_2$ fragments [34]. Fab fragments can prevent antigenic modulation of AchRs in cultured cells caused by mAbs to the MIR or by MG patient sera [15]. Single chain Fv fragments of MIR mAbs and MG patient autoantibodies have been expressed in bacteria, humanized, and selected for high affinity [35]. Unfortunately, Fab is very quickly cleared from circulation.

In addition there are other approaches taken for specific immunosuppression, such as *Torpedo* AchR coupled with toxic drugs in order to try to kill B-lymphocytes that interact with it. It was reported by Trommer that an AchR-conjugate i.e. AchR-gelonin obtained by chemical coupling could be employed for selective elimination of specific lymphocytes involved in triggering and maintenance of EAMG *in vitro*. The plant toxin gelonin was used which catalytically inhibits the

elongation step of protein synthesis. In their study, it was indicated that a marked improvement of clinical symptoms as well as a significant increase in functional AchR had occurred as compared to treatment with AchR or gelonin alone or untreated rats with EAMG as determined 6-10 weeks later [3].

1C.1.3 Research goals

Myasthenia gravis is an autoimmune disorder characterized by weakness and fatigability of skeletal muscles. The pathogenesis of MG in human and experimental MG in animals (EAMG) results from a reduction of the available acetylcholine receptors at neuromuscular junctions due to an antibody-mediated autoimmune response. Although treatment of MG with general immunosuppressive agents is reasonably effective, it may have numerous adverse side effects. Ideally, treatment of MG should eliminate the specific pathogenic autoimmune response to AchRs, without otherwise suppressing the immune system. The present study aims at a novel strategy for specific immunotherapy of MG

AchR-toxin conjugates have been employed for selective elimination of specific lymphocytes involved in triggering and maintenance of EAMG *in vivo* using the plant toxin ricin. Ricin as well as gelonin catalytically inhibits the elongation step of protein synthesis. In the previous chapter (Part I· 1B), we have described attempts for coexpression of the extracellular domain of the α-subunit of AchR with the chaperonin GroESL. It was then planned to couple the receptor fragment chemically to gelonin. However, since the yields of soluble protein were very low, the expression of a fusion protein composed of gelonin and AchR receptor fragment will be tried and its biological activity be tested.

1C.2 Results and discussion

1C.2.1 Cloning gelonin-AchR fragment gene

Based on our earlier work and the relevant data obtained, a strategy of recombinants pET-GA and pJLA-GA was employed as shown in Fig 1C.3. The beginning two plasmids pUC-gel I and pUC-gel II-AchR were provided by Ya-Wei Shi [25, 1A.4]. In order to obtain a gelonin-AchR fusion gene, the gel II-AchR fragment from pUC-gel II-AchR was inserted into the opened pUC-gel I by double enzymic digestion with XbaI/EcoRI and ligation, to be a fusion construct, named pUC-GA. After identification of the construct, a band of 1196 bp equal to the fragment

expected has been found on the gel which indicated that the recombinant was the one we anticipated (Fig 1C.4).

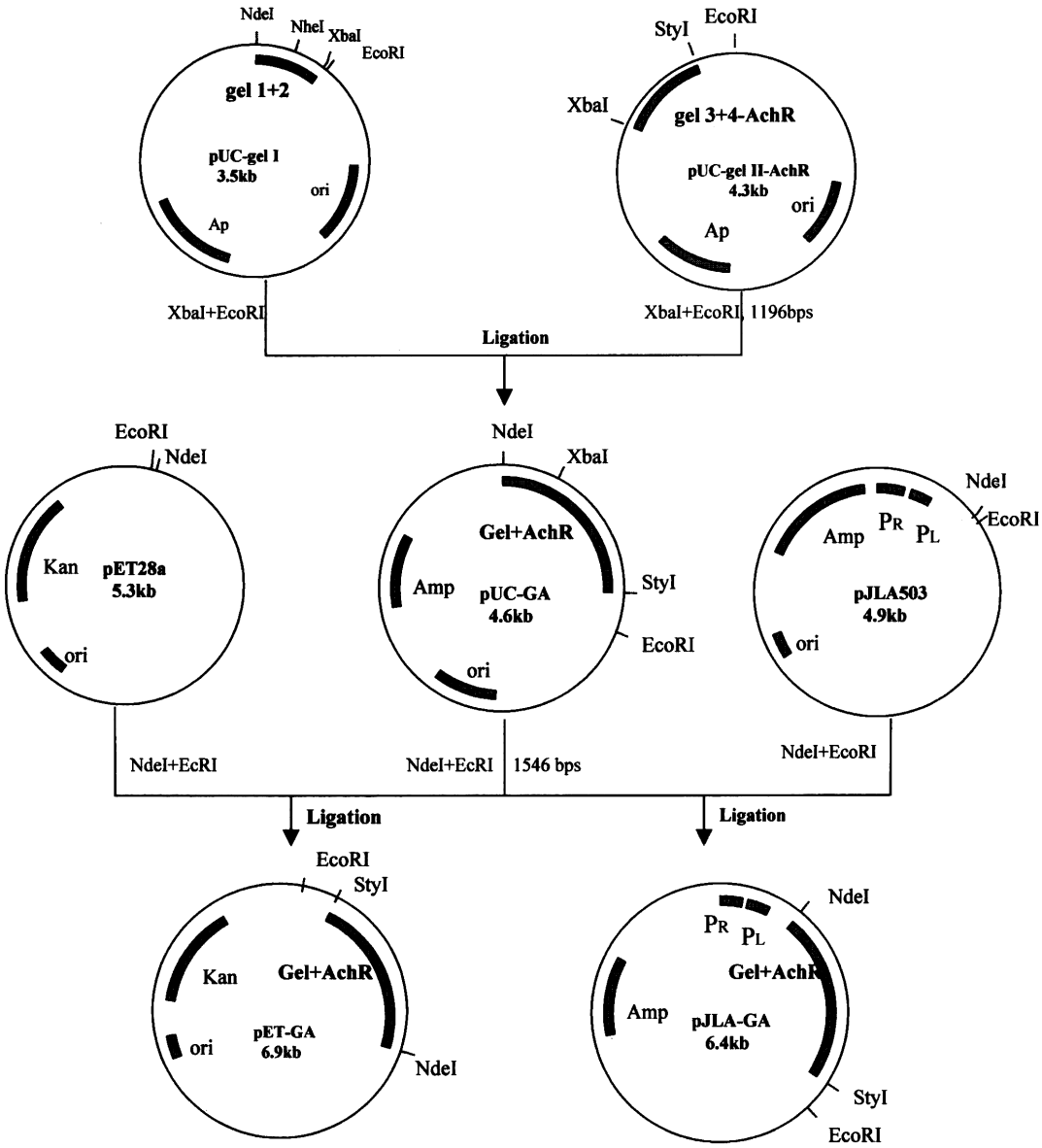


Fig 1C.3 Construction of expression vector for gelonin-AchR

(pUC-gel I, pUC-gel II-AchR were provided by Ya-Wei Shi *et al* [25, in 1A.4])

Table 1C.1 Reaction system of enzymatic digestion of pUC-gel I/pUC-gel II-AchR

Compounds	Volume
PUC-gel I or pUC-gel II-AchR	30 µl
XbaI	2 µl
EcoRI	2 µl
10x buffer2#	4 µl
H_2O_{bidest}	2 µl

* The reaction mixture was incubated at 37℃ for 2 h

Table 1C.2 Ligation system for pUC-GA

Reaction mixture	Volume
PUC-gel I (opened)	0.5 µl
Gel3+4+AchR (1196 bps)	6 µl
10xT_4 DNA ligase buffer	1 µl
T_4 DNA ligase	1 µl
H_2O_{bidest}	1.5 µl

*The reaction mixture was incubated at 26˚C for 2 h and continuously at 16˚C overnight

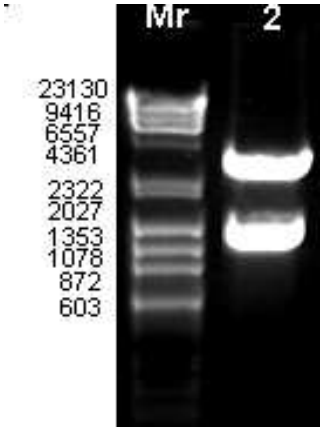


Fig 1C.4 Identification of pUC-GA

Mr. DNA marker (λ DNA/HindIII cut)

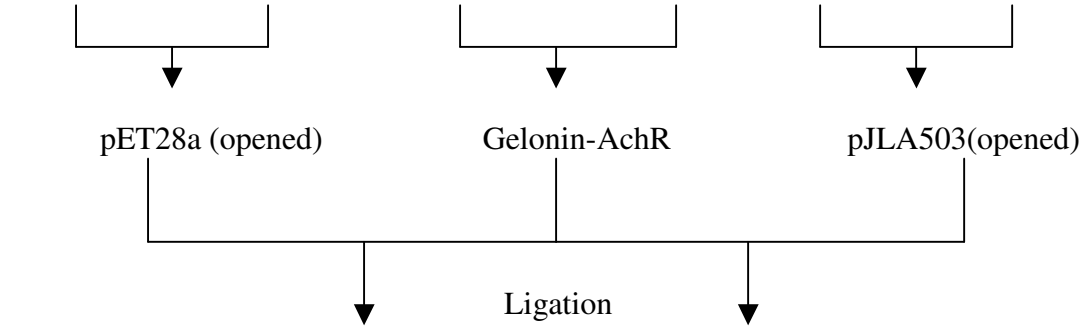
Lane 2: pUC-GA digested with XbaI/EcoRI

(The fragment gel II+AchR should be 1196 bps)

In addition, two expression plamids, pET-GA and pJLA-GA were produced by inserting gelonin-AchR DNA fragment cut by NdeI/EcoRI into the vectors pET28a or pJLA503 following the routine DNA cloning technology. By double cleavage with NdeI and EcoRI as well as DNA sequence determination, the successful production of two expression recombinants, pET-GA and pJLA-GA was demonstrated (Table 1C.3).

Table 1C.3 The ligation system for pET-GA and pJLA-GA

1		2		3	
pET28a	30 µl	pUC-GA	30 µl	pJLA503	30 µl
NdeI	2 µl	NdeI	2 µl	NdeI	2 µl
EcoRI	2 µl	EcoRI	2 µl	EcoRI	2 µl
10x buffer2#	4 µl	10x buffer2#	4 µl	10x buffer2#	4 µl
H_2O_{bidest}	2 µl	H_2O_{bidest}	2 µl	H_2O_{bidest}	2 µl



pET28a (opened)	1 µl	pJLA503 (opened)	0.7 µl
Gel+AchR (1546 bps)	5 µl	Gel+AchR (1546 bps)	7 µl
10x T_4DNA ligase buffer	1 µl	10x T_4DNA ligase buffer	1 µl
T_4DNA ligase	1 µl	T_4DNA ligase	1 µl
ddH_2O	2 µl	ddH_2O	0.3 µl

*Conditions for ligation: 26℃ for 3 h, 18℃ overnight in water bath

1C.2.1.1 Identification of pET-GA and pJLA-GA

Recombinant plasmids of pET-GA or pJLA-GA were transferred into host strain E. coli BL21, DH5α or DHI, respectively. After incubating the strains on LB plates (ampicillin or kanamycin), 20 colonies were separately taken out in 5 ml LB medium with the proper antibiotics at 37°C in a shaker. After 12 h, the plasmids were extracted. The correct recombinants were obtained as shown by cleavage of the recombinant plasmid with NdeI/EcoRI and subsequently agarose gel electrophoresis, a fragment of 1546 bp corresponding to gelonin-AchR was detected (Fig 1C.5 and Fig 1C.6).

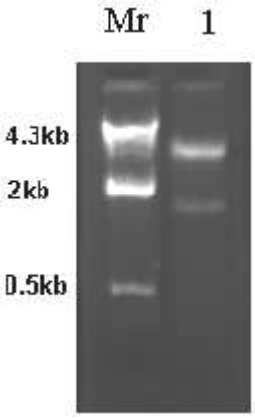


Fig 1C.5 The products of pET-GA by double endonuclease cleavage

Lane1: pET-GA with NdeI/EcoRI

(The fragment of gelonin-AchR should be 1546 bps)

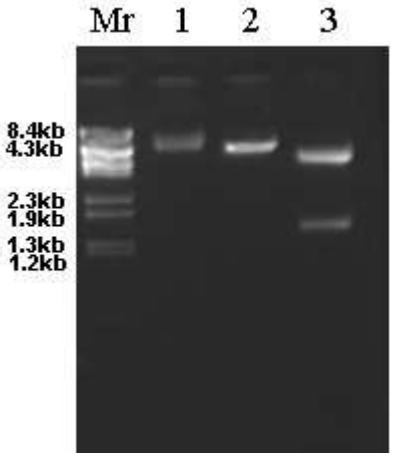


Fig 1C.6 The products of pJLA-GA by double endonuclease cleavage

Lane1: pJLA503

Lane2: pJLA503 with EcoRI

Lane3: pJLA503 with NdeI/EcoRI

(The fragment of gelonin-AchR should be 1546 bps)

1C.2.2 Expression

20 ml LB medium containing kanamycin or ampicillin was inoculated with one colony and shaken at 37°C overnight. After 12 h, the pre-culture was transferred into 1000 ml LB (Kan or Amp). The broth was cultured to reach an optical density (OD600nm) of about 0.7-0.8 and immediately induced with IPTG for strain E. coli BL21/pET-GA at the same temperature for 4 h or induced at 42°C for 3 h for strain E. coli DH5α/pJLA-GA. The cells were harvested by centrifugation and re-suspended in 50 mM phosphate buffer pH7.0. After sonication and centrifugation, the mixture was run on 12% SDS-PAGE to identify the products. The results showed that the fusion product of gelonin-AchR fragment obviously forms inclusion bodies and the concentration of soluble protein is very low (Fig 1C.7). However, the product of strain E. coli DH5α/pJLA-GA by inducing at 42°C could not be confirmed whether it formed the target protein because the corresponding band was not detected on the SDS-PAGE (Fig 1C.8). Therefore, only BL21/pET-GA was used for further studies.

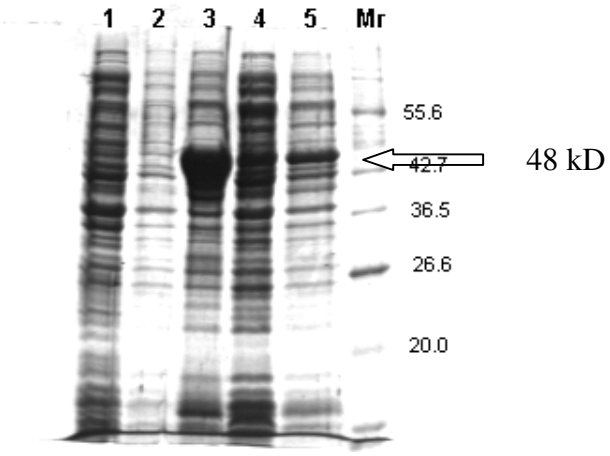


Fig 1C.7 Expression of pET-GA in *E. coli* BL21

(Transformed cells of BL21 with plasmid pET-GA were plated on LB media plates. Single colony was picked and screened for expression on SDS-PAGE)

Lane1: Strain *E.coli* BL21; Lane2: BL21/pET-GA uninduction

Lane3: Precipitate after induction and sonication

Lane4: Supernatant after induction and sonication

Lane5: BL21/pET-GA with induction

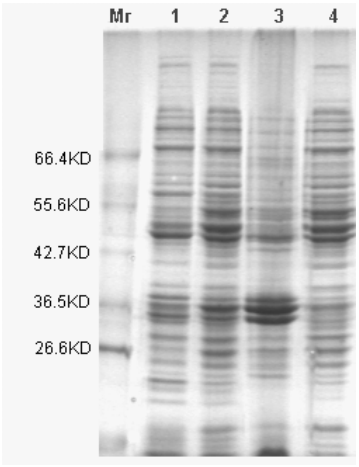


Fig 1C.8 Expression of pJLA-GA in DH5α

(Transformed cells of DH5α with plasmid pJLA-GA were plated on LB media plates. Single colony was picked and screened for expression on SDS-PAGE)

Lane1: Culture of DH5α

Lane2: Culture of DH5α/pJLA-GA with induction

Lane3: Precipitate after induction and sonication

Lane4: Supernatant after induction and sonication

1C.2.3 Purification and identification of supernatant

The pellets of cells from 1 L LB medium were re-suspended in 30 ml, 50 mM, phosphate buffer pH7.2, containing 1 mM PMSF, 20 mg lysozyme (25,000 U/mg), at RT 1-1.5 h. Then 2 mM DTT was added before sonication. It was sonicated under the condition of 30 sec, 15x on ice. Then the mixture was separated by centrifugation (14,000 r/m, 30 min). Two parts were collected, one was the supernatant and the other was the inclusion body. Usually, there are about 130 mg of total protein in 1 L LB to be obtained in supernatant, while 80 mg or so proteins existed in the precipitate. Then the two parts were processed separately.

1C.2.3.1 Isolation of supernatant

Supernatant in 50 mM PBS, pH7.2, 1 mM PMSF and 2 mM DTT, adjusted with 1 M NaCl and 0.25 M $(NH_4)_2SO_4$, was loaded on Phenyl Sepharose 6 ff column and stepwise eluted with 0.5 M NaCl, 50 mM PBS pH7.2 and H_2O (Fig 1C.9). A 48 kD product was eluted in the water fraction as shown by SDS-PAGE analysis (Fig 1C.11).

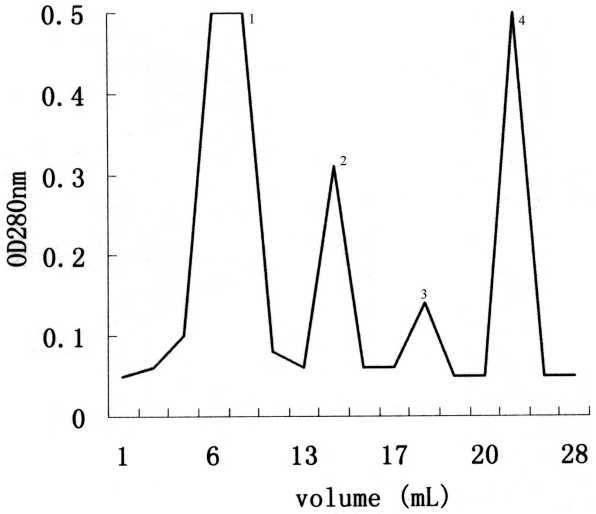


Fig 1C.9 Purification of gelonin-AchR on Phenyl-Sepharose

Sample: Supernatant after sonication; Column: 0.9 x 10 cm

Equilibration buffer: 50 mM PBS, pH7.2, 1M NaCl and 0.25 M $(NH_4)_2SO_4$

Elution buffer: 0.5 M NaCl; 50 mM PBS pH7.2; H_2O

Detection: A_{280nm}; Flow rate: 1min/ml

Peak 1: Flow through; Peak 2: 0.5 M NaCl in 50 mM PBS pH7.2

Peak 3: 50 mM PBS pH7.2; Peak 4: Water

Then the fraction in H_2O was concentrated and loaded on cation and anion-exchange resin. Unfortunately, the product cannot be purified either on cation or on anion resins, even if the pH value was changed from 4 to 10. According to the resolution on SDS-PAGE, the sample from hydrophobic column was applied on Superose12 (AKTA purifier, Pharmacia) (Fig 1C.10). Though the purification efficiency was not satisfying, some pure products were detected by SDS-PAGE (Fig 1C.11).

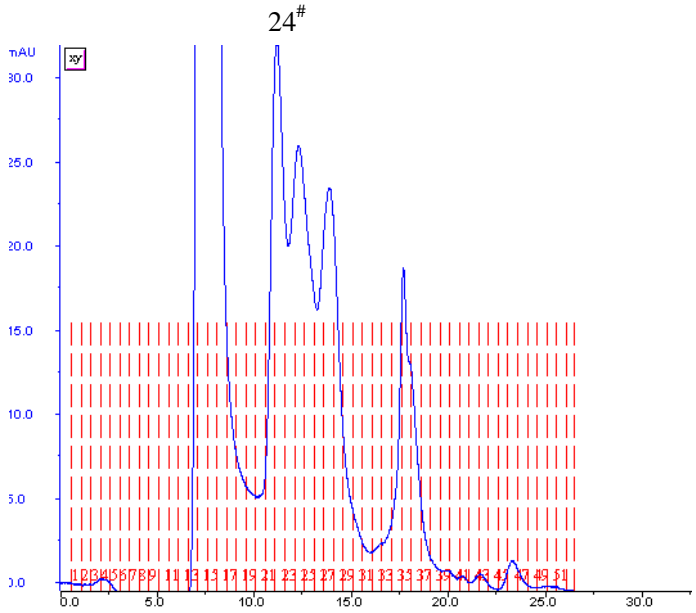


Fig 1C.10 Purification of gelonin-AchR by Superose 12 on AKTA Purifier

Column: Pre-packed Superose 12 HR 10/30 from Phamacia; Sample: 100 µl (4 mg/ml) of peak 4 of previous column (See Fig 1C.9); Elution buffer: 50 mM PBS pH7.2; Flow rate: 0.5 ml/min; Fraction size: 0.5 ml. No $24^{\#}$, $25^{\#}$ containing a 48 kD protein.

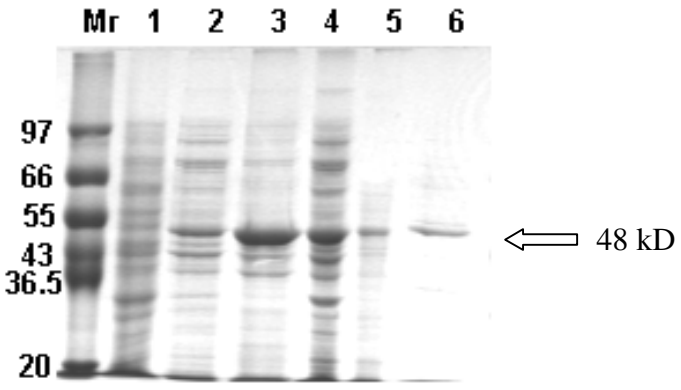


Fig 1C.11 Electrophoretic patterns of fusion protein on 12% SDS-PAGE

Lane1: Uninduced pET-GA/BL21; Lane2: Induced pET-GA/BL21; Lane3: Inclusion bodies; Lane4: Mixture after sonication; Lane5: Peak 4 from phenyl-Sepharose (See Fig 1C.9); Lane6: Fraction $24^{\#}$ from Superose 12 HR 10/30 (See Fig 1C.10).

1C.2.3.2 Western blots of gelonin-AchR

For further confirmation, Western Blots were performed. Gelonin-AchR was run on 12% SDS-PAGE and the native gelonin (30 kD) isolated from seeds of Gelonium multiflorum to which a standard protein marker had been added was used as a positive control for the Western blots. The antigelonin polyclonal antiserum was used as first antibody. Peroxidase-conjugated sheep anti-mouse Ig diluted 1:4000 was added and reacted with the substrate, BCIP/NBT in the western blot assay. It is indicated from Fig 1C.12 that there is a clear band (30 kD) of native gelonin in Lane Mr. and recombinant gelonin-AchR was blotted in Lane 1.

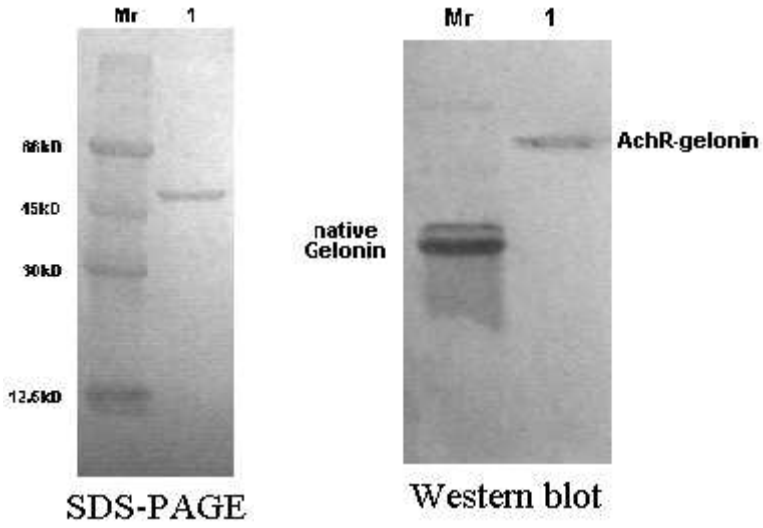


Fig 1C.12 Western Blots of gelonin-AchR

Left: SDS-PAGE; Right: Western blot

Mr. containing native gelonin (30 kD) as control.

Lane1: Pure recombinant gelonin-AchR

The first antibody is mouse anti-gelonin polyclonal antiserum

1C.2.3.3 Discussion

Based on the electrophoretic patterns of the supernatant centrifuged from sonication mixture, a 48 kD protein band was found in spite of limited quantity, compared with the same band from the precipitate. In order to obtain the fusion protein quickly, a process was chosen to purify the soluble part. It was indicated from the experimental results that the step of hydrophobic chromatography was more effective, not only many impurities were washed out, but also the 48 kD band was concentrated. Subsequently, strong and weak cation exchangers

(SP-Sepharose, CM-Sephadex) and strong and weak anion exchangers (Q-Sepharose, DEAE-Sephadex) were tried for the purification, but with little success. Finally, a procedure of gel filtration on Superose 12 was applied to purify the fraction eluted from Phenyl-Sepharose. Though the resolution of gel column was not so ideal, a pure 48 kD product was finally obtained as shown by SDS-PAGE analysis.

1C.2.4 Denaturation and refolding of inclusion bodies

The inclusion bodies were washed three times with 40 ml B-PER reagent which was diluted with PBS by 1:10 and centrifuged at the same condition, then were solubilized in 6 M guanidine hydrochloride (GuHCl), 20 mM Tris-HCl (pH8.0), 5 mM DTT, 2 mM EDTA, incubated at room temperature for 2 h and stored at 4°C overnight. After centrifugation, the solution containing denatured proteins was collected and stored at 4°C until purification.

1C.2.4.1 Preliminary purification of denatured proteins

The denatured proteins were separated by size-exclusion chromatography as follows: Sephacryl S-200 column (2.2x100 cm) pre-equilibrated with 5 M GuHCl, 20 mM Tris-HCl pH8.0 (column buffer) was passed with 22 mg (11 mg/ml) of denatured proteins. The column was eluted with column buffer at the flow rate of 0.5 ml/min. Fractions were further examined by SDS-PAGE.

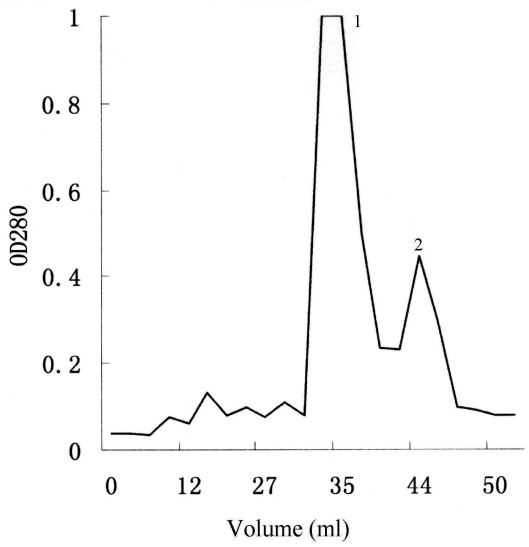


Fig1C.13 A profile of denatured mixture of gelonin-AchR fragment on Sephacryl S-200

Sample: 22 mg (11 mg/ml in 6 M GuHCL); Equilibration buffer: 5 M GuHCL in 20 mM Tris-HCL pH8.2; Column: 2.2 x 100 cm; Flow rate: 1 ml/min; Fraction: 3 ml; Detection: 280 nm (OD: 1.0)

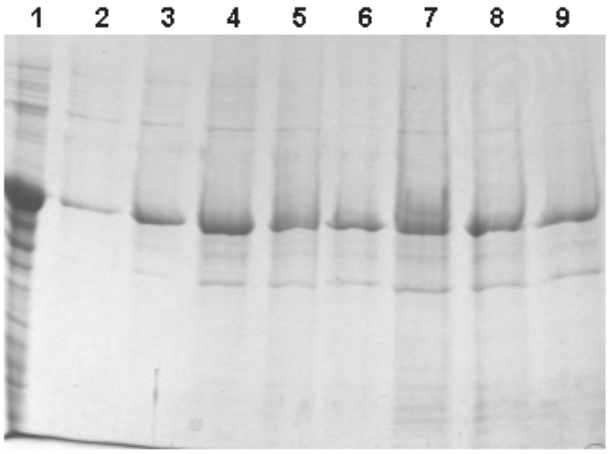


Fig1C.14 Electrophoretic patterns of the fractions on SDS-PAGE

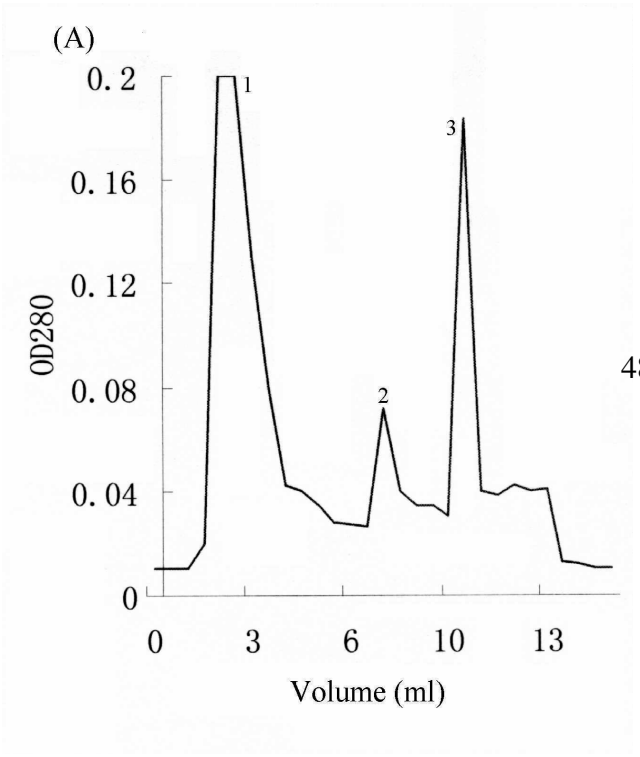
Lane1: Denatured mixture of gelonin-AchR fragment

Lane2-9: Different fractions from peak 1 in Fig 1C.13

1C.2.4.2 Refolding of fusion protein

Method 1

The denatured sample was stepwise diluted from 5 M, 3 M to 1 M guanidine in 20 mM Tris-HCl pH8.2, then dialyzed against 20 mM Tris-HCL (pH8.2), 2 h and the precipitate was spun down at 14,000 r/m for 30 min. 1 mM GSH (glutathione) and 0.1 mM GSSG (oxidized glutathione) were added into the diluted solution for refolding, gently shaking at 4˚C for 12 h. After that, this solution was dialyzed against 20 mM Tris-HCL, pH8.2 thoroughly. This soluble protein was purified on Q-Sepharose column, eluted with a stepwise gradient: 0.1 M NaCl, 0.2 M NaCl, 0.5 M NaCl and the target protein could be eluted from 0.1 M NaCl As Fig 1C.15. Normally 100 μg soluble protein could be obtained form 1 mg denatured protein.



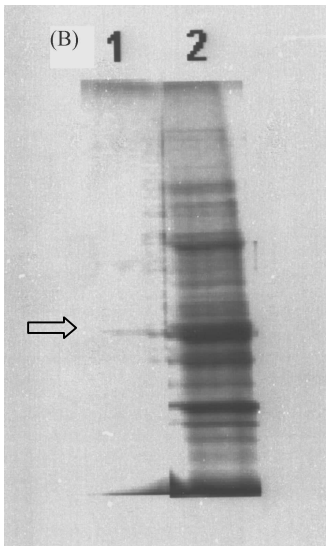


Fig 1C.15A An elution diagram of renatured gelonin-AchR fragment

Sample: Refolded gelonin-AchR fragment fusion protein; Flow rate: 1min/ml

Matrix: Q-Sepharose (3×4 cm); Equilibration buffer: 20 mM Tris-HCl, pH8.2

Elution: Stepwise with 0.1M NaCl, 0.2M NaCl, 0.5M NaCl;

Peak 1: 0.1M NaCl, Peak 2: 0.2M NaCl, Peak 3: 0.5M NaCl;

Detection: 280 nm (OD: 0.2).

Fig 1C.15B Electrophoretic patterns of refolded gelonin-AchR fragment protein eluted from Q-Sepharose column

Lane1: Fraction of refolded gelonin-AchR fragment

Lane2: Mixture after sonication.

Method 2

The denatured inclusion bodies were mixed with the final concentration of 20 mM Tris-HCl pH8.0, 5 mM DTT, 2 mM EDTA, 2 mM GSH and 0.2 mM GSSG by rapid votexing at room temperature to a final concentration of 400 μg /ml protein and 1 M GuHCL. Incubation was performed at 4ºC for about 16 h. Normally more than 200 μg refolded protein could be obtained from 1 mg denatured protein as Fig 1C.16.

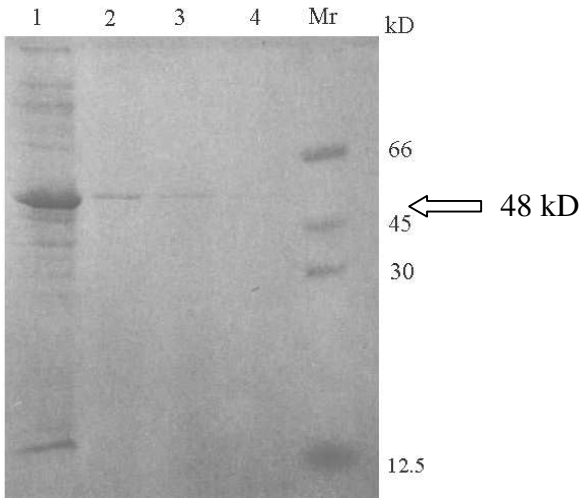


Fig 1C.16 Electrophoretic patterns of refolded protein

Lane1: Denatured gelonin-AchR fragment fusion protein

Lane 2-4: Refolding fusion protein in different amount (5 μg, 3 μg, 1 μg)

1C.2.4.3 ELISA assay

The mAb (# 35, 192, 198) used in this study are directed against the extracellular region of AchR α subunit. The antibodies were derived from rats immunized with intact AchR with either human muscle (mAb 192, 198) or *Electrophorus* electricus electric organ (mAb 35). The mAb 35 exhibits nice cross reactivity with human AchR.

The different fractions eluted from Sephacryl S-200 were analyzed by SDS-PAGE. The fraction corresponding to the immunotoxin (48 kD) was pooled and refolded as described in 1C.3.4.2. In order to examine whether the product is a toxin, the refolded protein was assessed by ELISA. Wells of microtiter plates were coated with 2 μg samples either refolded or the unfolded fusion protein in refolding buffer or PBS buffer plus coating buffer till 100 μl incubated at 4°C overnight. The coated wells were washed 3 times with PBST for 1.5 h at room temperature. Monoclonal antibodies diluted with PBS were added to each well and incubated for 2 h at room temperature. Afterwards each well was washed with PBST for 3 times before adding 100 μl of peroxidase-conjugated rabbit anti-rat Ig (dilution 1:1000) in PBS for 1 h at room temperature. The wells were continuously washed with PBST for four times respectively. The bound mAb was measured by peroxidase activity with P-nitrophenyl phosphate as the substrate. The color developed in 30 min or so was measured at 405 nm.

The three mAbs (# 192, 198, 35) [36], partly or completely recognize the native conformation of human AchR (Table 1C.4), were used for a binding assay. The binding ability of mAb 198 is significantly higher than the background level which refers using BSA as the negative control and positive control of AchR isolated from Electrophorus electric organs. However, the binding affinity of mAb 198 to the denatured protein was rather low. Binding of mAb 198, 192 was increased with decreasing concentrations of GuHCl (Table 1C.5).

Table 1C.4 ELISA assay

Antibodies	Fusion protein (Recombinant)	AchR[a] (*Torpedo*)	BSA[b]
mAb192	0.11±0.01	-	0.01±0.01
mAb198	0.71±0.01	0.53±0.01	0.01±0.01
mAb35	0.2 0±0.02	0.13±0.01	0.01±0.01
Polyclonal antigelonin	0.12±0.02	-	0.01±0.01

a. Positive control; b. Negative control

Table 1C.5 mAb binding by the various refolding conditions

Refolding protein in	MAb192	MAb198
5M GuHCl	0.03±0.01	0.22±0.02
2.5M GuHCl	0.03±0.01	0.29±0.01
1.25M GuHCl	0.11±0.01	0.71±0.02
1M GuHCl	0.10±0.01	0.64±0.01
BSA (control)	0.01±0.01	0.05±0.01

mAb 35 reacts with native AchR of human and *Torpedo*

mAb 192 reacts with native human AchR

mAb 198 reacts with AchR of human and *Torpedo* either native or denatured

1C.2.4.4 Effect of gelonin-AchR fragment on protein synthesis

To examine the toxicity of the fusion protein, an *in vitro* translation assay in a reticulocyte lysate containing globin mRNA was performed with a series of samples diluted from 3.16 x 10^{-8} M to 3.16 x 10^{-13} M. The protein synthesis was measured by the incorporation of ^{14}C-valine into this

protein. It was shown from Fig 1C.17 that gelonin alone and gelonin-AchR possess similar toxicity with IC_{50} of gelonin at 35 ng/ml and of gelonin-AchR at 30 ng/ml respectively.

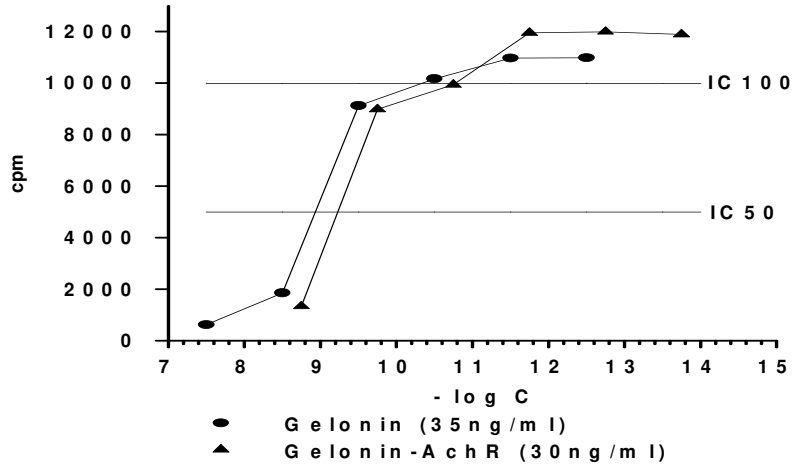


Fig 1C.17 Comparison of the cell-free protein synthesis (rabbit reticulocyte) inhibitory activity of recombinant gelonin and its fusion protein (Refer to previous Fig 1A.14 in 1A.2.6).

1C.3 Summary

Immunotoxins as first proposed by Paul Ehrlich as 'magic bullets' in cancer treatment are conjugates composed of tissue-specific antibodies and a toxin. Similarly, conjugates of auto-antigens with toxins could be used for treatment of autoimmune diseases. The auto-antigen moiety would direct the toxin to lymphocytes with an affinity and specificity for this macromolecule.

In part I·1C a conjugate of the extracellular portion of the α-subunit of the AchR (amino acids 1-181) with the single chain RIP gelonin was constructed and expressed as fusion protein for a potential treatment of Myasthenia gravis. This autoimmune disease is characterized by autoantibodies mainly against the AchR from the neuromuscular endplate.

In this study, we constructed a recombinant plasmid with the vector pET28a and the gene of gelonin and α-subunit fragment of AchR. The fused protein was expressed in *E. coli* and obtained mainly in the precipitate in the form of inclusion bodies as shown by SDS-PAGE. After solubilizing the inclusion bodies in denaturing buffer containing 6 M GuHCl or 8 M urea, several refolding conditions were tried to obtain the functional protein. However, most of these procedures

were unsatisfactory. The most efficient method by which the fusion protein was obtained in a native-like conformation, was to dilute the denatured protein into 1 M GuHCl with refolding buffer containing GSH, GSSG and DTT at room temperature. The purified and refolded fusion protein binds mAb 35, 192, 198 as well as polyclonal antigelonin. It also inhibits protein biosynthesis with an IC_{50} similar to gelonin itself. Given the specificity of the AchR for target lymphocytes, the conjugate could be an effective therapeutic agent for the treatment of the autoimmune disease Myasthenia gravis.

1C.4 Literature

1. Better M, Bernhard S L, Williams R E, *et al*. T cell targeted immunofusion proteins from *Escherichia coli*. J. of Biological Chemistry, 1995, **270**: 14951-14957.
2. Urbatsch I L, Sterz R K, Trommer W E, *et al*. Antigen-specific therapy of experimental myasthenia gravis with acetylcholine receptor-gelonin conjugates *in vivo*. Eur. J. Immunol., 1993, **23**: 776-779.
3. Hossann M. Experiments for the synthesis of acetylcholine receptor-gelonin conjugate. Diploma Thesis; 2001 in Kaiserslautern University.
4. Tzartos S, Seybold M, Lindstrom J. Specificities of antibodies to acetylcholine receptors in sera from myasthenia gravis patients measured by monoclonal antibodies. Proc. Natl. Acad. Sci. USA, 1982, **79**: 188-192.
5. Vincent A, Willcox N, Hill M, *et al*. Determinant spreading and immune responses to acetylcholine receptors in myasthenia gravis. Immunol. Rev., 1998, **164**: 157-168.
6. Weinberg C, Hall Z. Antibodies from patients with myasthenia gravis recognize determinants unique to extrajunctional acetylcholine receptors. Proc. Natl. Acad. Sci. USA, 1979, **76**: 504-508.
7. Kaminski H. Acetylcholine receptor epitopes in ocular myasthenia. Ann. NY. Acad. Sci., 1998: **841**: 309-319.
8. Bartfeld D, Fuchs S. Specific immunosuppression of experimental autoimmune myasthenia gravis by denatured acetylcholine receptor. Proc. Natl. Acad. Sci. USA, 1978, **75**: 4006-4010.
9. Lindstrom J, Einarson B, Merlie J. Immunization of rats with polypeptide chains from Torpedo acetylcholine receptor causes an autoimmune response to receptors in rat muscle. Proc. Natl. Acad. Sci. USA, 1978: **75**: 769-769-773.
10. Lindstrom J, Peng X, Kuryatov A, *et al*. Molecular ad antigenic structure of nicotinic acetylcholine receptor. Ann. NY. Acad. Sci., 1998: **841**: 71-86.

11. Kao I, Drachman D. Thymic muscle cells bear acetylcholine receptors: possible relation to myasthenia gravis. Science, 1977, **195**: 74-75.
12. Nelson S, Conti-Troconi B. Adult thymuses express an embryonic nicotinic acetylcholine receptor-like protein. J. Neuroimmunol, 1990, **29**: 81-92.
13. Schluep M, Willcox N, Vincent A, *et al*. Acetylcholine receptors in human thymic myoid cells in situ: an immunohistological study. Ann. Neurol, 1987, **22**: 212-222.
14. Farrar J, Portolano S, Willcox N, *et al*. Diverse Fabs specific for receptor epitopes from a myasthenia gravis combinatorial library. Int. Immunol., 1997, **9**: 1311-1318.
15. Craus Y, debates M, Parren P, *et al*. Huamn anti-nicotinic acetylcholine receptor recombinant Fab fragments isolated from gravis patients reflect predominant specifictties in serum and block the action of pathogenic serum antibodies. J. Immunol., 1997, **158**: 1919-1929.
16. Thomas J, Willcox N, Newsom-Davis J. Immunohistological studies of the thymus in myasthenia gravis. Correlation with clinic state and thymocyte culture response. J Neuroimmunol., 1982, **98**: 2358-2363.
17. Drachman D. Myasthenia gravis. N. Engl. J. Med., 1994, **330**: 1797-1810.
18. Willcox H. Thymic tumors with myasthenia gravis or bone marrow dyscrasias. In: Peckham M, editor. Oxford Textbook of oncology. Oxford University press, 1995, 1562-1568.
19. Meinl E, Klinkurt W, Wekerle H. The thymus in myasthenia gravis: changes typical for the human disease are absent in experimental autoimmune myasthenia gravis of the Lewis rat. Am. J. Pathol., 1991, **139**:995-1008.
20. Schoenbeck S, Padberg F, Hohlfeld R, *et al*. Transplantation of thymic autoimmune microenvironment to SCID mice: a new model of myasthenia gravis. J. Clin. Invest, 1992, **90**: 245-250.
21. Lindstrom J, Shelton GD, Fujii Y. Myasthenia gravis. Adv. Immunol. 1988, **42**: 283-284.
22. Lindstrom J, Einarson B. Lennon V, *et al*. Pathological mechanisms in EAMG. Immunogenicity of muscle acetylcholine receptor and quantitative extraction of muscle acetylcholine receptor and quantitative extraction of receptor and antibody–receptor complexes from muscles of rats with experimental autoimmune myasthenia gravis. J. Exp. Med., 1976: **144**: 726-738.
23. Jermy A, Beeson D, Vincent A. Pathogenic autoimmunity to affinity-purified mouse acetylcholine receptor induced without adjuvant in BALB/c mice. Eur. J. Immunol., 1993, **23**: 973-976.
24. Lindstrom J, Seybold M, Lennon V. Antibody to acetylcholine receptor in myasthenia gravis: prevalence, clinic, correlates, and diagnostic value. Neurology, 1976, **26**: 1054-1059.

25. Seybold M, Lindstrom J. Patterns of acetylcholine receptor antibody fluctuation in myasthenia gravis. Ann. NY. Acad. Sci., 1981, **377**: 292-306.
26. Engel A. Acquired autoimmune myasthenia gravis. In: Engel A, Franzini-Armstrong C, editors. Myology, 2nd ed. New York, 1994, 1769-1797.
27. Lindstrom J. Experimental induction and treatment of myasthenia gravis. In: Engel A, editor. Myasthenia gravis and myasthenic disorders. Oxford University Press, 1999, 111-130.
28. Dedhia V, Goluszko E, Wu B, *et al*. The effect of B cell deficiency on the immune response to acetylcholine rceptor and development of experimental autoimmune myasthenia gravis. Clin. Immunol. Immunopathol, 1998, **87**: 266-276.
29. Conti-Fine B, Protti M, bellone M, *et al*. Myasthenia gravis: the immunobiology of an autoimmune disease. Austin, TX: RG Landes, 1997.
30. Oshima M, Ashizawa T, Pollack M, *et al*. Autoimmune T cell recognition of human acetylcholine receptor. The sites of T cell recognition in myasthenia gravis on the extracellular part of the α subunit. Eur, J. Immunol., 1990, **20**: 2563-2569.
31. Barchan D, Asher O, Tzartos S, *et al*. Modulation of the anti-acetylcholine receptor response and experimental autoimmune myasthenia gravis by recombinant fragments of the AchR. Eur. J. Immunol., 1998, **28**: 616-624.
32. Younger D, Worrall B, Penn A. Myasthenia gravis: historical perspective and overview. Neurology , 1997, **48**: 1-7.
33. Tzartos S, barkas T, Cung M, *et al*. Anatomy of the antigenic structure of a large membrane autoantigen, the muscle-type nicotinic acetylcholine receptor. Immunol Rev. 1998, **163**: 89-120.
34. Loutrari H, kokla A, Tzartos S. Passive transfer of experimental myasthenia acetylcholine via antigenic modulation of acetylcholine receptor. Eur. J. Immunol., 1994, **22**: 2449-2452.
35. Mamalaki A, Trakas N, Tzartos S. Bacterial expression of single chain Fv fragment which efficiently protects the acetylcholine receptor against antigenic modulation caused by myasthenic antibodies. Eur. J. Immunol., 1993, **23**: 1839-1845.
36. Tsouloufis T, Mamalaki A, Tzartos S. Recognition of conformationally dependent epitopes on the N-terminal extracellular domain of the human muscle acetylcholine receptor α subunit expressed in *E.coli*: implications for myasthenia gravis therapeutic approaches. International Immunol., 2000, **12**: 1255-1265.

Part II Neurotrophins

2.1 Introduction

2.1.1 Neurotrophins

The development and maintenance of the nervous system depend on proteins by the name of neurotrophic factors (Table 2.1). The neurotrophic factors, a subclass of cell growth factors, act on neurons as well as other non-neuronal cells. As depicted in Figure 2.1, the subclass contains at least three distinct families: neurotrophins, i.e. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophins 3, 4/5 (NT3, NT4/5); the neuropoietic cytokine, i.e. ciliary neurotrophic factor (CNTF) and the fibroblast growth factors (FGF), i.e. acidic and basic FGF [1]. The role of neurotrophic factors in cell development has been investigated extensively in cultured cells from different brain regions. These studies indicate that survival of cultured embryonic neurons from different brain regions may require one or more neurotrophins.

Table 2.1 Examples of proteins reported to have neurotrophin properties

Proteins with well-documented neurotrophin activity	Proteins with putative neurotrophin activity
Acidic fibroblast growth factor (aFGF)	Cholinergic neuronal differentiation factor (CDF)
Basic fibroblast growth factor (bFGF)	Epidermal growth factor (EGF)
Brain-derived neurotrophic factor (BDNF)	Heparin binding neurotrophic factor (HBNF)
Ciliary neruotrophic factor (CTNF)	Insulin
Interleukin 1, 3 and 6 (IL-1, 3, 6)	Insulin like growth factor (IGFs)
Neurotrophin 3 (NT3)	Protease nexin I and II
Nerve growth factor (NGF)	Transforming growth factor alpha (TGFα)
Glia-derived neurotrophic factor (GDNF)	

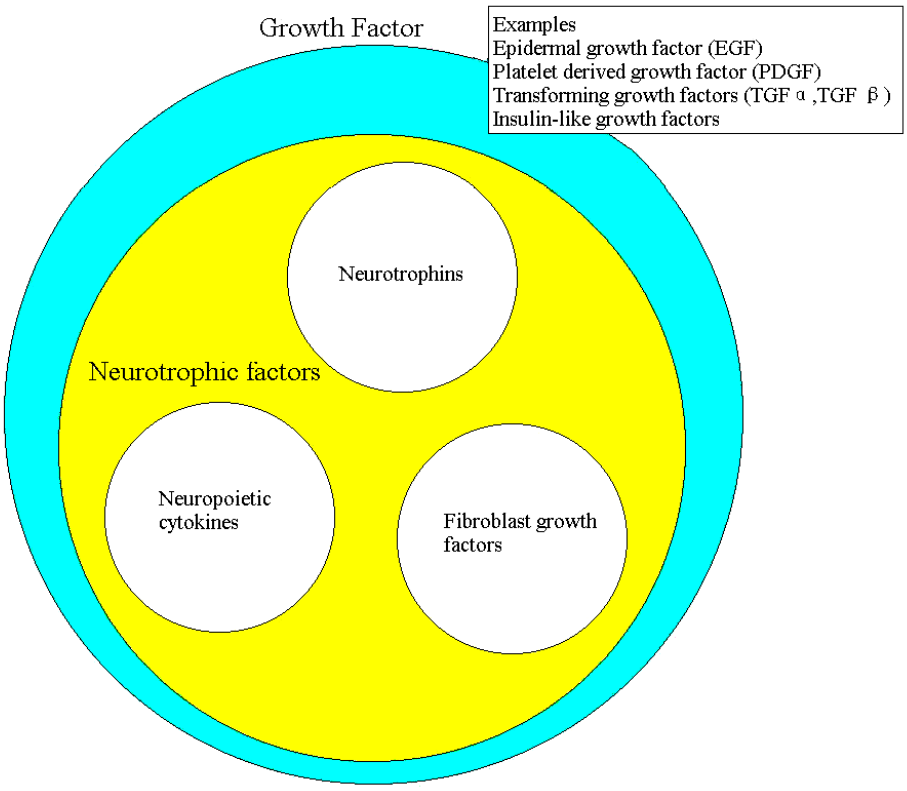


Fig 2.1 Classification of neurotrophic factors

2.1.2 Neurotrophin receptors

The members of the neurotrophin family are all specific for their various receptors. Based on the binding ability of neurotrophins and their receptors, they can be divided into two classes: high affinity receptors which are tyrosine receptor kinases (Trk) and the low affinity receptor p75 [2]. High affinity receptors encoded by the Trk primitive cancer gene are trans-membrane proteins with molecular weights of 120-160 kD. They exist in three subclasses, TrkA, TrkB and TrkC. As indicated in Fig 2.2, TrkA could bind to NGF, TrkB has been demonstrated to bind brain-derived neurotrophic factor (BDNF) and neurotrophin 4/5 as well as TrkC bind to neurotrophin 3. The low affinity receptor, p75 with a molecular weight of 75 kD, is composed of an extra-cellular region, a transmembrane structure and an intra-cellular region. All the members of neurotrophins can interact with the receptor p75 as described in Fig 2.2.

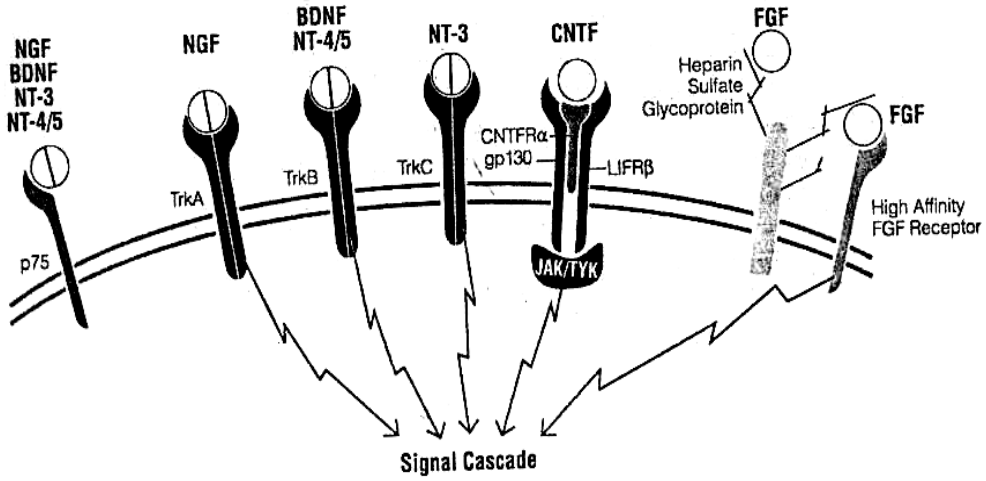


Fig 2.2 Neurotrophins and their receptors [2]

2.1.3 Human neurotrophin 3

The human neurotrophin 3 (hNT3) is one of the most important members of the neurotrophin family. It can provide nutrition for neurons and repair damaged neuronal cells. Recently, neurotrophin research has made some progress with respect to the structure and function of the receptors including hNT3 and its mechanism in the central and peripheral nervous system.

2.1.3.1 Molecular characteristics of hNT3

The human brain-derived neurotrophic factor, BDNF and NT3 are relatively small proteins with 119 to 130 amino acids. The conserved regions (60% overall homology) and the disulfide bond sites of the four proteins are the same (see Fig 2.3), leading to very similar 3D structures [3, 4].

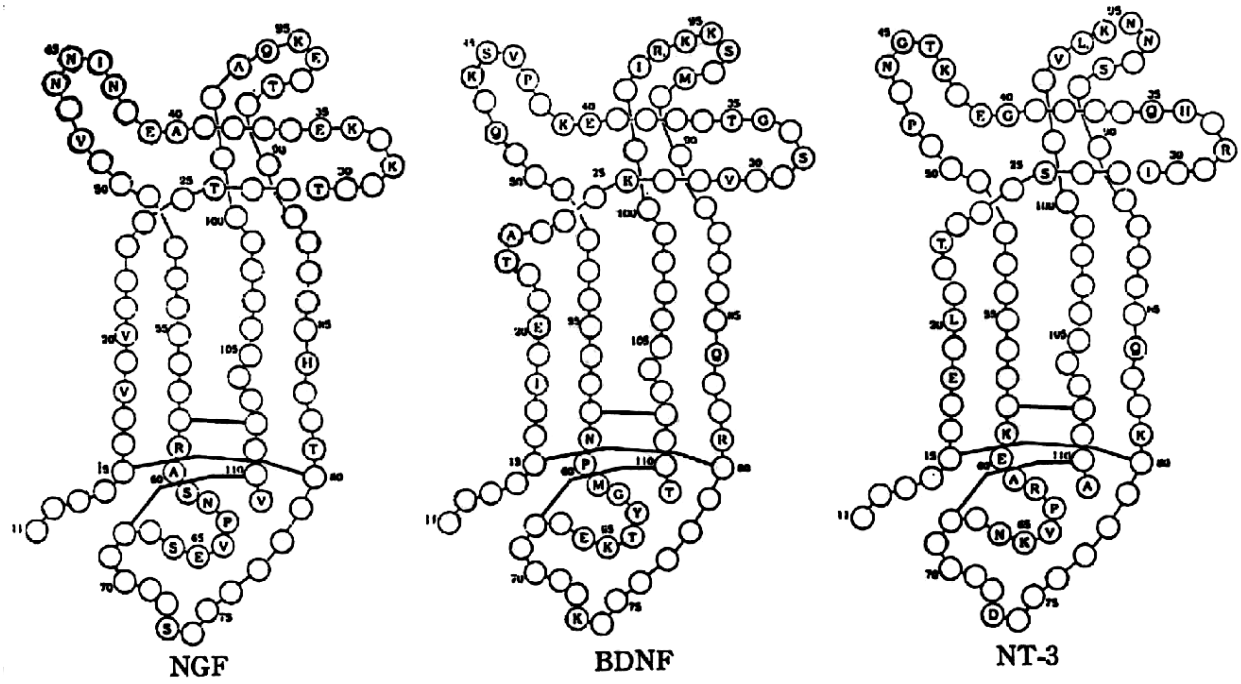


Fig 2.3 Comparison of three dimensional structure of NGF, BDNF and NT3 [4]

The variable amino acids are predominantly located in four regions: three hairpin regions (residues 29-35, 43-49, 92-97) in the upper ring and the 58-68 amino acid region in the lower ring of these molecules. Each member of the neurotrophins has independent bioactivity and affinity to different receptors [5].

The amino acid residues important for binding to p75 and the Trk receptor are located in the variable regions of neurotrophin 3. Those regions can be divided into seven different parts: the amino terminal region (residues 1-9), a carboxy terminal region (residues 111-118) and the loop regions I, II, III, IV, V (residues 23-25, 40-49, 59-66, 79-88, 94-98). By knockout and targeted gene-mutation, amino acid mediated interactions of NT3 and TrkC were determined, which include Arg8 and Tyr11 of the amino-terminal region, Thr22 of region I, amino acids 39-48 of region II and Lys80 and Gln83 of region IV [6].

2.1.4 Purification of the recombinant proteins

Since the 90's of the last century, the discovery of protein self-splicing has changed our view of the flow of genetic information from DNA to protein [7, 8]. Protein splicing occurs at the post-translation level of protein biosynthesis. After synthesis of a precursor protein, an internal

protein domain (termed the intein) is precisely excised from the precursor and two external domains (termed exteins) are ligated together forming a peptide bond between the exteins [9, 10, 11]. Thus, two proteins are derived from a single gene, one of which is not collinear with the original open reading frame. After a 454-residue intein in the 69 kD vacuolar membrane ATPase subunit of Saccharomyces cerevisa (Sce VMA intein) was initially discovered, almost 100 inteins have been identified in eubacterial, archae and eukaryotic unicellular organisms. The alignment of inteins has revealed that most inteins are bi-functional proteins, containing an endonuclease domain and a splicing element, while a few inteins (termed mini-intein) ranging in size from 134 to 198 amino acids lack the endonuclease region. Intein as a splicing element has some conserved amino acids at the junctions, which play an important role in protein splicing as shown in Table 2.2.

Table 2.2 Comparison of conserved amino acids at the intein splice junctions [12]

N-domain (N-Extein)		Protein splicing element (Intein)			C-domain (C-Extein)	
Sce	AILYVG	C	FAKGT (454) NQVVV	H N	C	GERGN
Ctr	VIIYVG	C	FTKGT (471) NMALV	H N	C	GERGN
Mtu	KVVKNK	C	LAEGT (440) EGVVV	H N	C	SPPFK
TliI2	KVLYAD	S	VSGES (390) NNILV	H N	T	DGFYA
TliI1	IKLLAN	S	ILPNE (538) GLLYA	H N	S	YYGYM
PspI1	IKILAN	S	ILPEE (537) GFLYA	H N	S	YYGYY
MIP	RGTRRA	S	ILPEE (537) GSLYA	H N	S	GNSAF

Sce: *Saccharomyces cerevisiae* vacuolar ATPase subunit intein

Ctr: *Candida tropicalis* vacuolar ATPase subunit intein

Mtu: *Mycobacterium tuberculosis* RecA intein

Tli I1 / I 2: *Thermococcus litoralis* DNA polymerase intein-1 and intein-2

PsP: *Pyrococcus sp*. GB-D DNA polymerase intein

MIP: Psp pol. intein inserted between MBP and paramyosin

Residues with hydroxyl or thiol groups (Ser, Cys, Thr) are present at both splicing junctions. The hydrophobic amino acids His-Asn at the C-terminus of the intein are essential residues for the function of inteins. Though the correct conformation of intein is necessary for protein splicing, the residues at the junctions are crucial [12, 13, 14, 15]. Based on the protein splicing mechanism proposed by Xu and coworkers [16, 17], a branched intermediate is formed as shown in Fig 2.4.

The splice process comprises four tightly coupled nucleophilic displacements via a branched intermediate. The amino acid residues (Cys, Ser, Thr) at two splice junctions and an invariant Asn at the intein C-terminus are essential for the intein-catalyzed splicing reaction. Splicing is initiated by an N-S (or N-O) acyl rearrangement involving Cys (or Ser) at the N-terminus of the intein, generating a reactive thioester (ester) bond. Transesterification occurs at the thioester (ester) formed in step1 and the side chain of residue (Ser, Cys or Thr) at the beginning of the C-extein. Finally, the ester (or thioester) linkage between the exteins undergoes a spontaneous acyl rearrangement to form a native peptide bond via a branched intermediated [18, 19]. It was reported from Xu's and Chong's work at New England Biolabs that the formation of a thioester linking the N-extein and the intein is still possible if the conserved Asn at C-terminus of the intein is mutated to Ala to block C-terminal cleavage. Conversely, if the CyS1 at N-terminus of the intein is mutated to Ala1 to block N-terminal cleavage, splicing at C-terminus of the intein is also performed (Fig 2.5). Based on this principle, a target protein could be obtained from a fusion product, protein-intein or intein-protein, by a thioester bond cleavage. Therefore, this system may facilitate the purification of recombinant proteins, which may not easily be accessible otherwise.

Purification of recombinant proteins by the use of affinity tags is a convenient and widely used technology. A variety of affinity tags have been employed, including Schistosoma glutathione S-transferase (GST), E. coli maltose-binding protein (MBP), Staphylococcus protein A, polyhistidine and calmodulin-binding peptide etc. After affinity absorption, the tag is usually cleaved from the fusion protein by treatment with a site-specific protease [20, 21]. However, the use of proteases has limited the application of many purification systems. First, cleavage by proteases is not always specific, resulting in cleavage at secondary sites; second, the cleavage is sometimes inefficient due to the inaccessibility of the cleavage site in the fusion protein; third, additional purification steps are required to obtain the target protein. To avoid and improve the limitations, a protein splicing system in vitro was introduced to purify recombinant proteins by intein self excision as described above. In order to make affinity absorption possible, an affinity tag in the form of a chitin binding domain (CBD) coding region was inserted into the open reading frame of the intein at the N-terminus or C-terminus [22, 23]. In such case, the expression product has an extremely high affinity to chitin beads. During protein purification, the fusion protein consisting of a target protein-intein-CBD complex or a CBD-intein-target protein complex, can be cleaved by thiol reagents such as DTT or β-ME directly in chitin bead columns. Using the self-cleavable intein system, a target protein could then be obtained from crude extract in a one step purification procedure as shown in Fig 2.5 and 2.6.

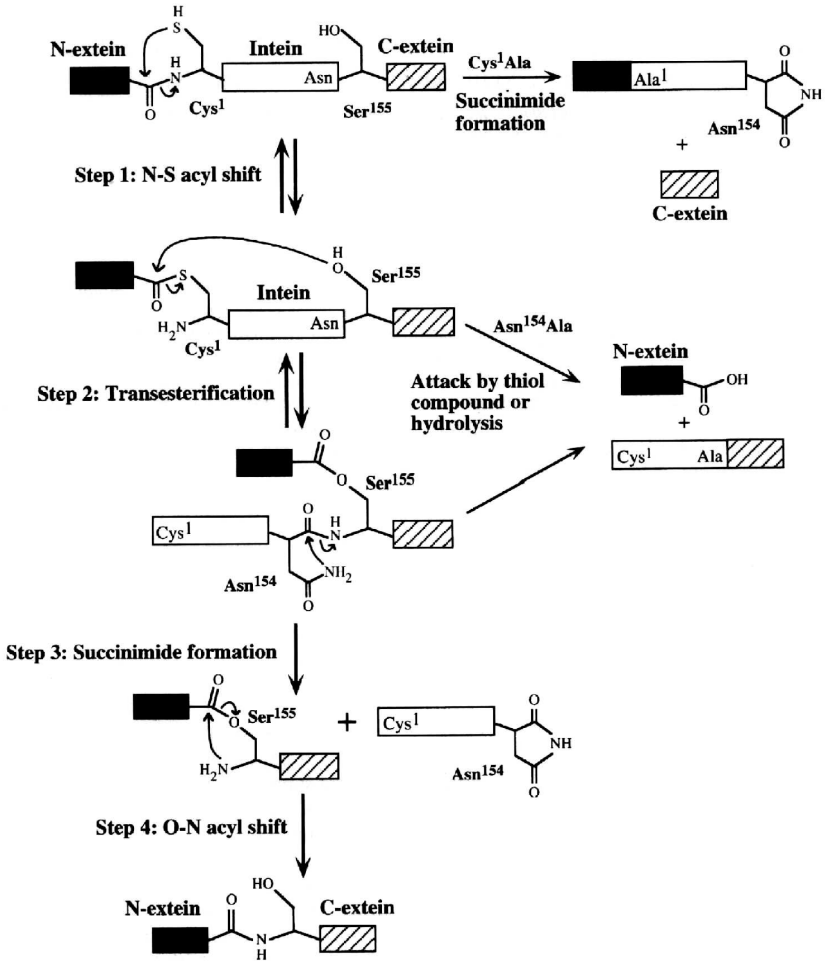


Fig 2.4 Proposed protein splicing and cleavage pathway for the mini-intein [23]

Protein splicing occurs via four concerted nucleophilic replacements:

Step1: Formation of a linear thioester intermediate at the N-terminus of the intein by an N-S acyl rearrangement at Cys^{1}.

Step2: Formation of branched intermediate by transesterification involving attack by the side chain of Ser^{155} on the thioester formed in step1.

Step3: Excision of the intein by peptide bond cleavage coupled to Succinimide formation by Asn^{154} at the C-treminus of intein.

Step4: Spontaneous O-N acyl rearrangement of the transitory ligated exteins to form a stable amide bond. Protein splicing for other inteins presumably proceeds by four analogues chemical steps, except that the Cys residues may be replaced by Ser or Thr or vice versa. N-terminal splice junction cleavage can occur by hydrolysis or nucleophilic attack of the thioester or ester linkage. Cyclization of Asn^{154} can proceed independently when the normal route of the splicing reaction is inhibited

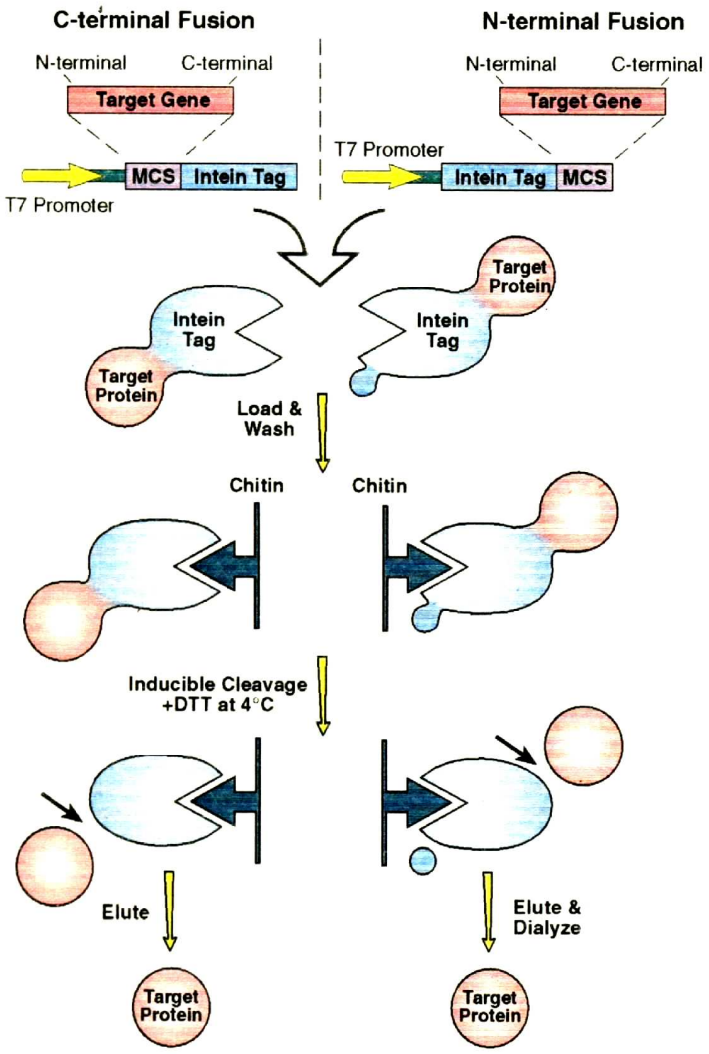


Fig 2.5 Intein-mediated protein purification system [23]

The target proteins fused to intein can be cleaved at N-terminus or C-terminus of the intein by DTT. In order to form an affinity tag, a chitin binding domain (CBD) was co-expressed with the fusion protein together.

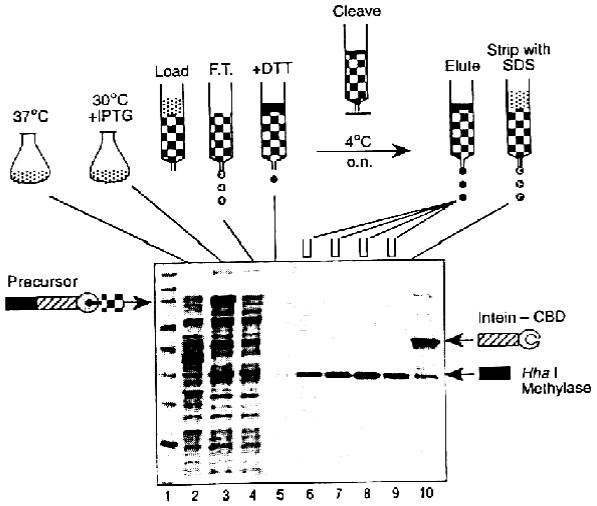


Fig 2.6 A schematic representation for single column purification of recombinant proteins using a self-cleavable affinity tag [24]

Upper: A diagram for the process of culture, induction, affinity absorption and DTT cleavage

Lower: The electrophoresis patterns of sample from the corresponding parts on SDS-PAGE.

1. Protein marker;
2. Uninduced cells;
3. Clarified crude extract from induced cells;
4. Flow through;
5. Quick DTT wash;

6-9. Fractions of eluted protein after stopping column flow and inducing a self-cleavage reaction at 4°C overnight;

10. SDS stripping of remaining proteins bond to chintin beads column.

2.1.5 Research goals

The human neurotrophic factor 3 (hNT3) is a member of the expanding family of neurotrophins which can stimulate the growth, differentiation and survival of peripheral and central neurons during the development or the damage of nerve cells. hNT3 is a 13.6 kD protein corresponding to 119 amino acid residues and usually exists as a dimer *in vivo*. The dimer can bind to cell surface receptors and perform its biological function. Therefore, hNT3 could serve as a potential therapeutic agent for nerve diseases, such as peripheral neuritis. Because the amount of hNT3 in cells or organelles is very limited, it is imperative to obtain hNT3 by gene engineering technology in higher amounts.

Protein splicing is a post translational processing event involving the precise excision of an internal protein segment, the intein, from a primary translation product with concomitant ligation of the flanking sequence, the exteins. Since the mechanism of protein splicing *in vitro* was reported, the research and application of protein splicing has been largely expanded to intein-mediated protein purification. The goal of this work was to amplify the hNT3 gene from genomic DNA of human whole blood by PCR and inserted it into an expression vector pTXB1 which contains an intein-chitin binding domain (CBD) construct. After expression we could purify the fusion protein of hNT3-intein-CBD with a chintin bead column and try to get pure hNT3 under intein splicing conditions by a one step procedure. Finally, the biological activity of hNT3 and hNT3-intein-CBD can be determined by the outgrowth of nerve fiber round dorsal root ganglia of chicken embryos.

2.2 Results and discussion

2.2.1 Amplification and identification of the human neurotrophin 3 gene

2.2.1.1 Preparation of genomic DNA from human whole blood

Fresh human whole blood was rapidly mixed with buffer in an Eppendorf tube, and the supernatant was removed by centrifugation. The precipitate was resuspended in potassium containing buffer. The mixture was incubated to digest the cells. After inactivation of protease K, the sample was used directly as the template for PCR (see 3.4.1).

2.2.1.2 Amplification of human neurotrophin 3 gene by PCR

The primers used in amplification of human neurotrophin 3 gene by PCR were designed according to the reported DNA sequence for the human neurotrophin 3 gene [3], and synthesized by Shanghai Biotechnol. Co. Ltd (see 3.4.2)

Primer A FW. 33 mers (10 μmol / L):
5'-GAA TCC **CAT ATG** TAC GCG GAG CAT AAG AGT CAC-3'

Primer B RV. 34 mers (10 μmol / L):
5'-GCA **GTC GAC** TCA TGT TCT TCC GAT TTT TCT CGA C-3'

The amplified product was analyzed on a 1.5% agarose gel. As shown in Fig 2.7, the PCR reaction yielded a 380 bp fragment which corresponds to the reported size for the human neurotrophin 3 gene.

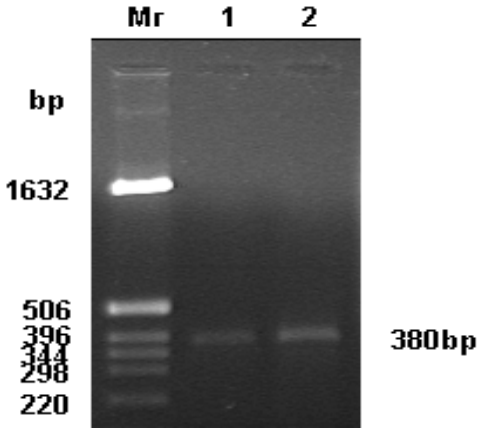


Fig 2.7 hNT3 gene amplified by PCR

Mr: pBR322 DNA/HinfI cut;

Lane1-2: hNT3 products by PCR (380 bp)

2.2.2 Construction of recombinant pGEM-hNT3

2.2.2.1 Background of pGEM-T vector

The pGEM-T Easy Vector is a high-copy plasmid widely used for direct cloning of PCR products. It contains a multiple cloning site and permits blue/white screening of recombinants. It can express a foreign gene from the promoters, T7 and SP6. Most importantly, this linear vector contains an extra thymidine (T) base at each of the 3' ends. This allows for direct ligation with DNA fragment products from PCR reactions using Taq polymerase, which normally adds a single extra adenine (A) to the 3'-end of an amplified DNA strand.

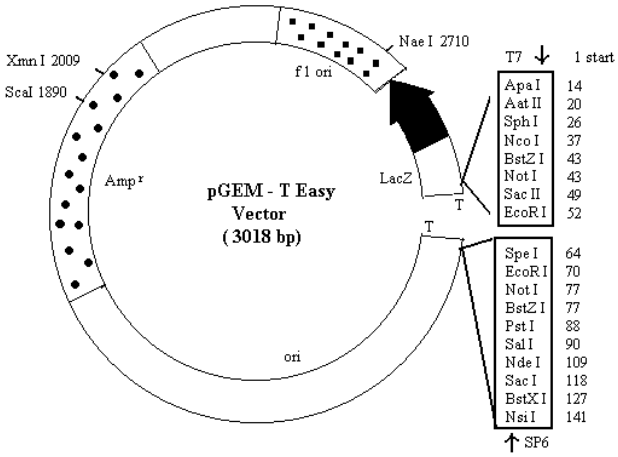


Fig 2.8 pGEM-T easy vector map (See 3.1.2)

2.2.2.2 Construction of pGEM-hNT3

The conditions for the ligation reaction are described in part III (3.4.3). The reaction mixture was then transformed into *E. coli* DH 5α by a heat shock procedure (see 3.3.2) and plated on LB agar medium plates supplemented with ampicillin and Xgal for white/blue screening. After incubation, a number of blue single colonies were picked and grown in LB (ampicillin) medium. The recombinant plasmids were extracted from the cells as described in Part III (3.3.3) and the insertion of hNT3 gene was confirmed as described below.

2.2.2.3 Confirmation of pGEM-hNT3

The plasmid pGEM-hNT3 was digested with NdeI and/or SalI and resolved on a 1.5% agarose gel (see 3.4.3, Table 3.6). The results showed that the single digestion (with NdeI) yielded a single band, while the double digestion (with NdeI and SalI) produced an extra band around 380 bp. The results suggested that the human neurotrophin 3 fragment amplified by PCR was correctly inserted into the pGEM-T vector (Fig 2.9).

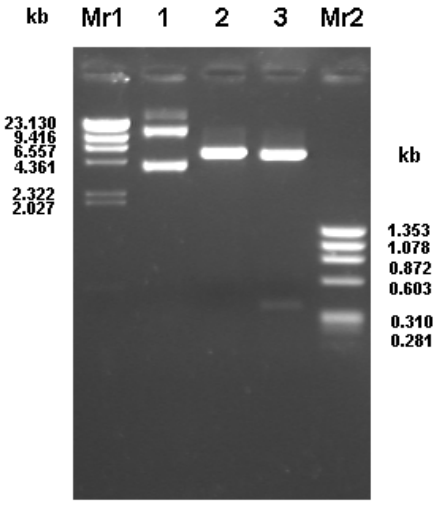


Fig 2.9 Electrophoresis patterns of pGEM-hNT3 cut by restriction endonucleases

Mr_1: λ DNA/HindIII cut

Lane1: pGEM-hNT3

Lane2: pGEM-hNT3 cut by SalI (only one band occurring)

Lane3: pGEM-hNT3 cut by NdeI/SalI (a 380 bp band corresponding to hNT3)

2.2.2.4 DNA sequence analysis

DNA sequence analysis for pGEM-hNT3 was performed with an ABI Prism 377 DNA Sequencer. A primer corresponding to the T7 promoter sequence was used as the starter. The result (Fig 2.10) showed that the sequence of the cloned hNT3 and the sites of restriction digestion were identical to those that were reported. Meanwhile, the upstream and downstream sequences of the gene were also identical to the precursor of hNT3 in genomic DNA sequence.

DNA sequence of hNT3

CATTGGGCCC GACGTCGCAT GCTCCCGGCC GCCATGGCCG CGGGATTGAA
TCC**CATATG**T ACGCGGAGCA TAAGAGTCAC CGAGGGGAGT ACTCGGTATG
TGACAGTGAG AGTCTGTGGG TGACCGACAA GTCATCGGCC ATCGACATTC
GGGGACACCA GGTCACGGTG CTGGGGGAGA TCAAAACGGG CAACTCTCCC
GTCAAACAAT ATTTTTATGA AACGCGATGT AAGGAAGCCA GGCCGGTCAA
AAACGGTTGC AGGGGTATTG ATGATAAACA CTGGAACTCT CAGTGCAAAA
CATCCCAAAC CTACGTCCGA GCACTGACTT CAGAGAACAA TAAACTCGTG
GGCTGGCGGT GGATACGGAT AGACACGTCC TGTGTGTGTG CCTTGTCGAG
AAAAATCGGA **AGAACA**TGAG TCGACTGCAA TCACTAGTGC GGCCGCCTGC
AGGTCGACCA TATGGGAGAG CTCCCAACGC GTTGGATGCA TAGCTTGAGT
ATTCTATAGT GTCACCTAAA TAGCTTGGCG TAATCATGGT

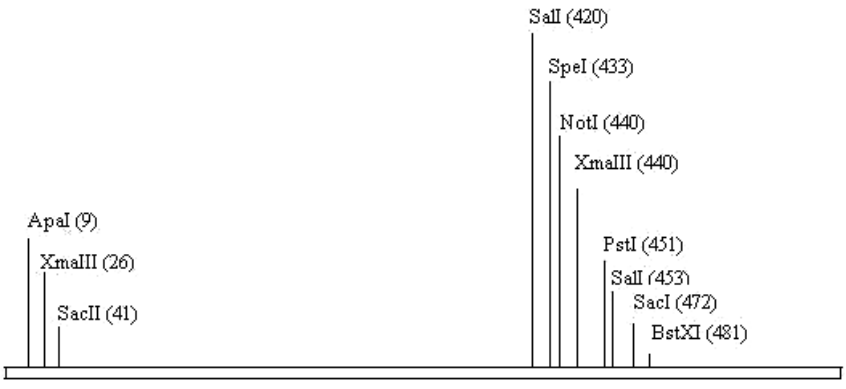
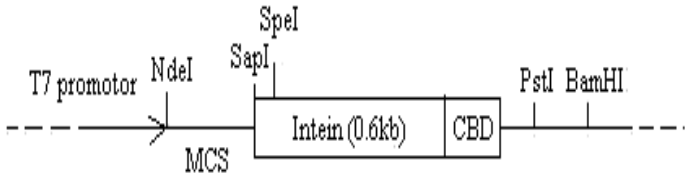


Fig 2.10 The DNA sequence of hNT3 (upper panel) and the corresponding restriction enzyme sites in hNT3 (lower panel)

2.2.3 Construction and expression of pTXB-hNT3 [25]

2.2.3.1 Background of pTXB1

The pTXB1 vector used in this study is a substitute of pTYB (New England Biolabs) catalogue # 6701). In this vector, the *Sce* VMA1 intein is replaced with the mini-intein (see Fig 2.4). In order to confine the self-splicing to the N-terminus of the intein only, the conserved Asn residue at the C-terminus of the intein has been mutated into Ala to block the C-terminal splicing. This vector also contains the ampicillin resistance gene and a chitin binding domain coding region. Protein expression of the engineered strain is IPTG-inducible.



MCS: -Nde1-Nhe1-Nru1- Sal1-Not1-EcoR1-Xho1-Sap1-

Fig 2.11 A diagram of inserted sites in MCS of pTXB1

The target gene was inserted into the MCS between NdeI and SapI

2.2.3.2 Construction of pTXB-hNT3

2.2.3.2.1 hNT3 gene amplified from pGEM-hNT3 by PCR

pGEM-hNT3 was used as the template to amplify the hNT-3 gene for subcloning into the pTXB1 vector. The primers used were as follows:

FW primer (33 mers)
5'-GGA GCT **CATATG** TAC GCG GAG CAT AAG AGT CAC-3'
RV primer (34 mers)
5'-GGT C**GC TCT TC**G GCA TGT TCT TCC CAT TTT TCT C-3'

The primers were designed to introduce a codon (ATG) at the 5'-end of the open reading frame of the target gene, and an extra Cys codon (TGC) at the 3'-end of the open reading frame of the target gene. The latter design is to produce a protein splice site at the N-terminus of the intein. The amplified product was resolved on a 1% agarose gel (Fig 2.12).

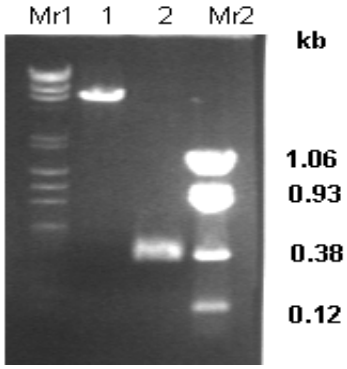


Fig 2.12 Electrophoretic patterns of the PCR product on 1% agarose gel.

Mr1: λDNA/HindIII cut and ΦX174 DNA/HaeIII cut;

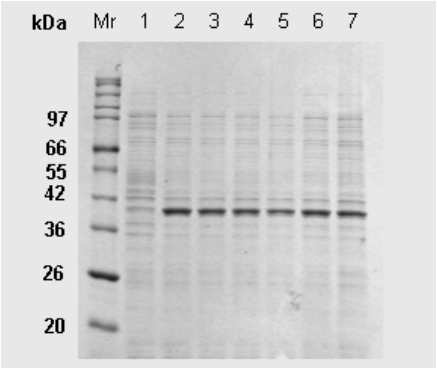
Lane1: Recombinant plasmid pTXB-hNT3

Lane2: hNT3 PCR products (380 bp)

Mr2: pBR322 DNA/BstN I cut

2.2.3.2.2 Construction and screening of pTXB-hNT3

The PCR product was digested with NdeI/SapI, and then force-cloned into the identically restricted endonuclease sites of pTXB1. The ligation reaction was performed at 12 ^{o}C overnight using T_4 DNA ligase. The mixture was then transformed into *E. coli* 2566 and plated on LB agar medium plates supplemented with ampicillin. Several single colonies were picked and grown in LB medium supplemented with ampicillin, and the protein expression was initiated by the addition of IPTG. The positive colonies were screened by SDS-PAGE. As shown in Fig 2.13, a prominent 41 kD band corresponding to the molecular weight of hNT3-intein-CBD fusion product was clearly visible for all of the colonies screened.



Mr: Broad range 7702# from NEB;

Lane1: *E. coli* 2566/pTXB-hNT3 Uninduced;

Lane2-7: *E. coli* 2566/pTXB-hNT3 induced with IPTG

Fig 2.13 Electrophoresis patterns of expression products on 12% SDS-PAGE

(Transformed cells of *E. coli* 2566 with plasmid pTXB-hNT3 were plated on LB media plates. Single colony was picked and screened for expression on SDS-PAGE)

2.2.3.3 Identification of pTXB-hNT3

The recombinant plasmid was extracted from several positive colonies and subjected to enzyme digestion analysis. As the SapI site in the hNT3 gene was deleted after subcloning into pTXB1, NdeI, SpeI and PstI in the open reading frame of the fusion construct were used to determine whether the hNT3 gene was inserted into the pTXB1 vector. The results (Fig 2.14) showed that three bands corresponding to 0.4 kb, 1.2 kb, and 0.8 kb were generated following the digestion by NdeI/SpeI, NdeI/PstI, and SpeI/PstI respectively, confirming the correct insertion of the human neurotrophin 3 gene. Meanwhile, DNA sequence analysis of the recombinant plasmid also indicated that the sequence of human neurotrophin 3 was the same as reported previously (Fig 2.10).

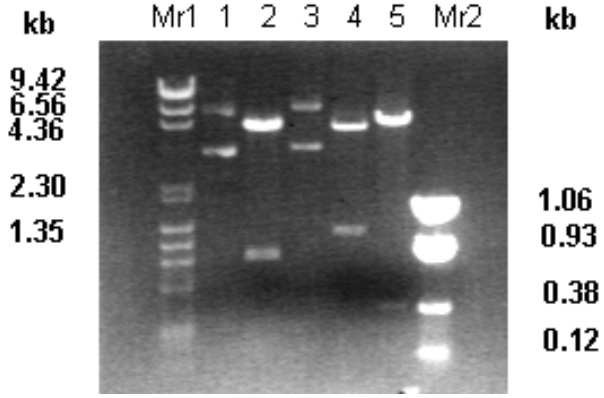


Fig 2.14 Enzymatic cleavage diagram of pTXB-hNT3 on 1% agarose gel

Mr1: λ DNA/HindIII cut and ΦX174 DNA/HaeIII cut;

Lane1 and Lane3: Recombinant plasmid pTXB-hNT3

Lane2: pTXB-hNT3 cut by SpeI/PstI (ca.0.8 kb fragment)

Lane4: pTXB-hNT3 cut by NdeI/PstI (ca.1.2 kb fragment)

Lane5: pTXB-hNT3 cut by NdeI/SpeI (ca.0.4 kb fragment)

Mr2: pBR322 DNA/BstNI cut

2.2.3.4 Expression and purification

20 ml LB medium supplemented with ampicillin was inoculated by a single colony of the engineered strain, *E. coli* 2566/pTXB-hNT3 and incubated at 37 ^{o}C overnight. This small culture was used to inoculate 1000 ml LB broth supplemented with ampicillin. When the culture reached an OD_{600nm} of 0.6-0.7, the inducer IPTG was added to a final concentration of 1 mM and the culture was continuously incubated at 30 ^{o}C for another 3 h. Cells were then harvested by centrifugation and the pellets were stored at –70 ^{o}C or used immediately.

The pellets from 1 L culture were re-suspended in 30-35 ml of ice cold 0.05 M Hepes buffer containing 0.5 M NaCl and 0.1 mM EDTA (pH 8.0), which were then subjected to sonication and centrifugation. The target protein was found mainly in the form of aggregates. To remove the non-target proteins, the inclusion bodies were washed once time with 0.05 M Hepes buffer and three times with B-PER (Bacterial Protein Extract Reagent) diluted by 1:10 with the Hepes buffer after centrifugation.

2.2.3.4.1 Denaturation and renaturation of inclusion bodies

The function of a protein critically depends on its three dimensional structure. Inclusion bodies, on the other hand, are merely aggregates of unfolded polypeptide chains, which have no defined conformation or function. To obtain functional proteins, these inclusion bodies can be solubilized with 8 M urea or 6 M guanidine hydrochloride (GuHCl) and the soluble protein can often be refolded under stringent conditions such as low temperature and low protein concentration etc. In this experiment, the inclusion bodies from 1 L culture were solubilized in 6 ml of 0.05 M Hepes buffer containing 1 mM EDTA, 100 mM DTT, and 8 M urea (pH 8.0) at room temperature for 2 h, followed by centrifugation at 20,000 r/m for 20 min to remove precipitates. After determination of the total protein concentration, the solution was gradually diluted by stepwise addition of 0.05 M Hepes buffer containing 2 mM glutathione (GSH), 0.2 mM oxidized glutathione (GSSG) and 0.5 M L-arginine (pH 8.0) to reduce the urea concentration to 1 M. The protein concentration was then further adjusted to 100 μg/ml by dilution, and the polypeptides were allowed to refold at 16 ^{o}C for 24 h with slow stirring. Subsequently, the refolded protein was applied to a chintin beads column for affinity purification as described in the next step.

2.2.3.4.2 One step purification of refolded hNT3-intein-CBD

In the vector pTXB1, a 0.2 kb chitin binding domain (CBD) coding region is located at the C-terminus of the intein for producing an affinity tag. Therefore, the fusion product, hNT3-intein-CBD can bind to the chitin beads column by affinity adsorption. The refolded proteins dialyzed against 0.05 M Hepes and 1M urea (pH8.0) were slowly applied to the column with 10-15 ml bed volume of chitin beads at a flow rate of 0.5 ml/min. Then the column was washed to remove the non-specifically absorbed proteins. 50 mM DTT was then added to start the self-splicing procedure. To allow for the efficient cleavage, the column was closed after 30-40 ml DTT buffer had entered, and the reaction was allowed to proceed for 48 h at 25ºC. On the basis of the mechanism of intein-inducible excision, the fusion protein, hNT3-intein-CBD was spliced by

DTT directly in the affinity column and the cleaved hNT3 was eluted from the column. The results of SDS-PAGE showed that a 14 kD band corresponding to human neurotrophin 3 had occurred (see lane 9 of Fig 2.15). The recovery of refolded human neurotrophin 3 is about 10% of the total proteins loaded on the affinity column. Because of incomplete cleavage under the above conditions, most of hNT3-intein-CBD fusion product was retained in the column. This part could be extracted with in 1% SDS and subsequently investigated by SDS-PAGE (see lane 12 in Fig 2.15).

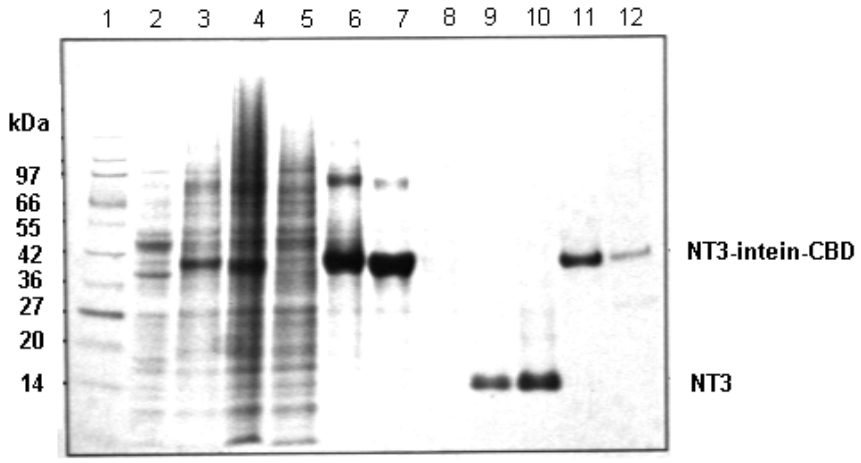


Fig 2.15 The electrophoresis patterns of the expression product and its renaturation on SDS-PAGE

1. Protein marker: The broad protein marker from NEB
2. *E. coli* 2566/pTXB-hNT3 Culture of un-induced cells
3. *E. coli* 2566/pTXB-hNT3 Culture of induced cells (Fusion protein: 41 kD)
4. Mixture of induced cells after sonication
5. Supernatant after step 4 centrifugation
6. Precipitates from step 4 dissolved in 8 M urea
7. Loading sample on affinity column after refolding
8. Flow through and wash the column with column buffer

9-10 Spliced sample eluted from the column cleaved by DTT (hNT3: 14 kD)

11. Fusion protein control
12. Strip out from affinity beads in 1% SDS

2.2.4 Construction and expression of pJLA-hNT3

2.2.4.1 Construction of pJLA-hNT3

The previous experiments showed that it was possible to obtain hNT3 from plamid pTXB-hNT3, however, the yield of 10% of refolded product was low. Therefore we constructed a new plasmid where the expression of the target protein was under the control of a heat inducible promoter. The vector pJLA503 is a plasmid containing a heat inducible promoter. The expression of engineered strains can be achieved by swiftly shifting the incubation temperature to 42° C. According to both polylinkers of pTXB1 and pJLA503, the hNT3-intein-CBD DNA fragment from pTXB-hNT3 cut by NdeI/BamHI was inserted into the opened pJLA503 using T_4 DNA ligase as shown in Fig 2.16.

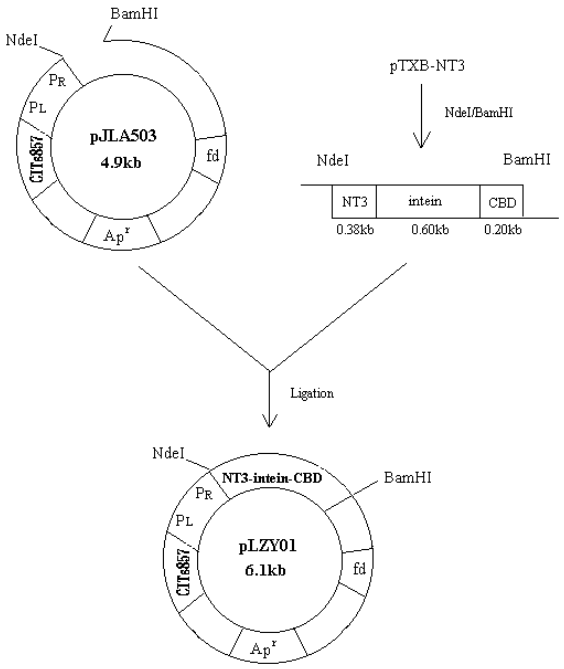


Fig 2.16 Construction of pJLA-hNT3

2.2.4.2 Identification of pJLA-hNT3

After the recombinant was transformed into host *E. coli* BL21, the engineered strain was cultured in LB medium supplemented with ampicillin. The recombinant plasmid was extracted and digested by NdeI and BamHI as described in Part III (3.3.3). The results in Fig 2.17, lane 2 showed two bands on a 1% agarose gel, one corresponds to the original opened pJLA503 of about 5.0 kb and the other is the fusion gene, hNT3-intein-CBD, about 1.2 kb, lane 3 showed only one band that was found after single cleavage with Nde1 (Fig 2.17).

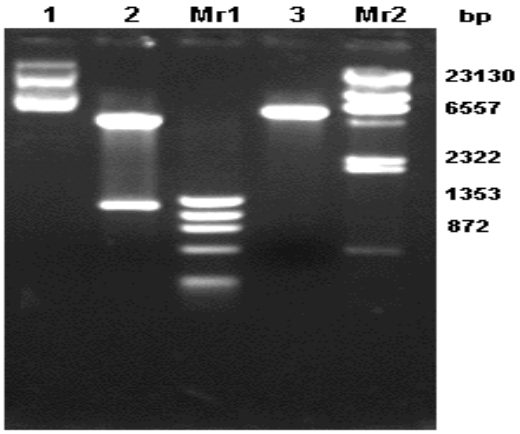


Fig 2.17 Identification of pJLA-hNT3

Lane1: pJLA-hNT3 only

Lane2: pJLA-hNT3 digested with NdeI/BamHI (hNT3-intein-CBD, about 1.2 kb)

Mr1: ΦX174DNA with HaeIII cut

Lane3: pJLA-hNT3 digested with NdeI (5.0 kb)

Mr2: λDNA with HindIII cut

2.2.4.3 Expression and purification

The engineered strain *E. coli* BL21/pJLA-hNT3 was cultured in LB medium supplemented with ampicillin at 37° C. When the culture reached an OD_{600nm} of 0.8-0.9, the incubation temperature was immediately raised to 42° C for another 3 h. The pellets were treated as described in 2.2.3.4. The target protein, hNT3-intein-CBD (41 kD) was also found in the form of aggregates or "inclusion bodies" (see lane1 in Fig 2.18). The amount of inclusion bodies could reach nearly 50% of the total protein in host cells as judged by optical density scanning of SDS-PAGE in Fig 2. 18. According to the principle of protein splicing, the sonicated cells were directly cleaved by DTT and run on SDS-PAGE. After staining with coomassie brilliant blue (CBB), three bands can be seen on the gel (see lane2 in Fig 2.19). Based on their molecular weights, 41 kD corresponds to hNT3-intein-CBD, the 14 kD and 27 kD bands obviously correspond to hNT3 and intein-CBD respectively. It is indicated that the fusion protein can also be excised by DTT, though the cleavage reaction is quite incomplete (Fig 2.19A). After denaturation and renaturation as well as affinity absorption as described in section 2.1.4, a 14 kD band product, hNT3 was also obtained (Fig 2.19B).

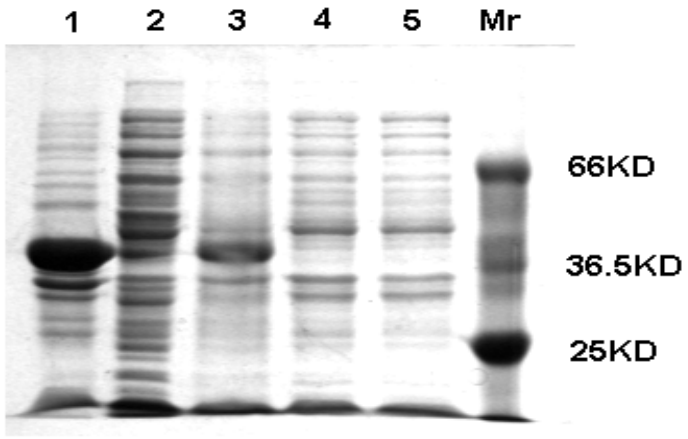


Fig 2.18 The expression products of engineered strain *E. coli* BL21/pJLA-hNT3

Lane1: Precipitates after sonication and centrifugation (fusion protein: 41 kD)

Lane2: Supernatant after sonication and centrifugation

Lane3: Mixture culture after induction (fusion protein: 41 kD)

Lane4: Mixture culture uninduced

Lane5: Strain *E. coli* BL21 without recombinant plasmids

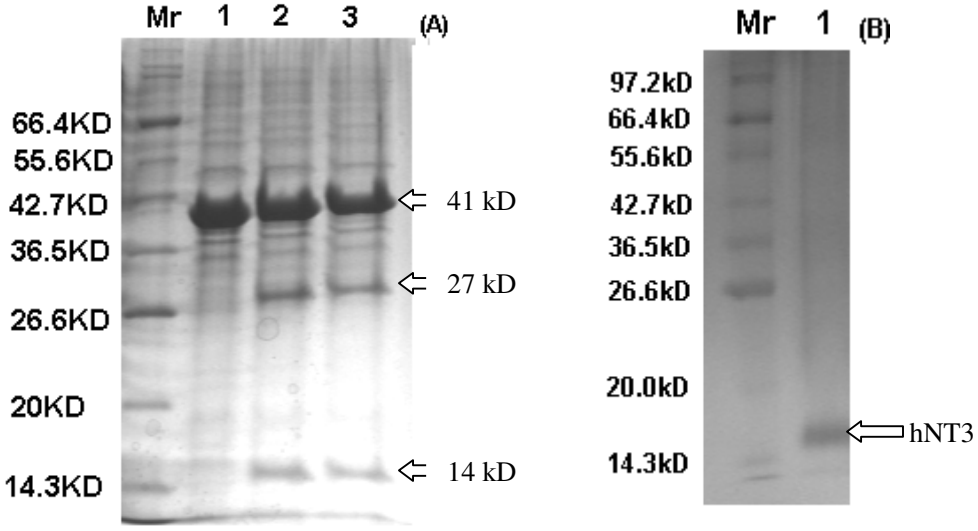


Fig 2.19 Electrophoresis patterns of the fusion product cleaved by DTT

A. Mixture culture after sonication (Lane1, hNT3-intein-CBD) directly added 100 mM DTT (Lane2, containing hNT3-intein-CBD, Intein-CBD, hNT3) or 50 mM DTT (Lane3 same as lane 2) for splicing. B. The refolded protein (hNT3-intein-CBD) was subjected to the column and eluted from a chitin bead column after DTT cleavage (Lane1, only 14 kD hNT3)

2.2.5 Bioactivity assay

The biological activity of neurotrophin 3 in the conditioned media was measured by its ability to stimulate the outgrowth of nerve fibers of the dorsal root ganglia obtained from 8-day-old chicken embryos. The detailed protocol is described in 3.5.4

In general, several drops of collagen from guinea pig tail were spread out on the bottom of each sterile culture bottles. After drying up in an incubator at 37° C, 3 ml DMEM medium was added to each bottle and then incubated in 5% CO_2-incubator overnight. The following day, two separate ganglia were taken into each culture bottle and mixed with 500 µl diluted sample. The diluted procedure results in a series of dilution factors of 1 (original sample), 3×10^{-1}, 10^{-1}, 3×10^{-2}, 10^{-2} etc. Finally, the cultured bottles were incubated in a 5% CO_2-incubator up to 24 h.

The patterns of fiber outgrowth from the explanted ganglia in different amounts of neurotrophin 3 were examined with a phase contrast microscope. One biological unit is defined as the concentration of neurotrophin 3 which corresponds to the outgrowth of nerve fibers caused by 15 ng human nerve growth factor under the same conditions. We found that 80 ng of hNT3-intein-CBD complex and 25 ng of the recombinant hNT3 equaled one biological unit. Based on the molecular weights, hNT3-intein-CBD and hNT3 are almost equally active in stimulating nerve cell growth.

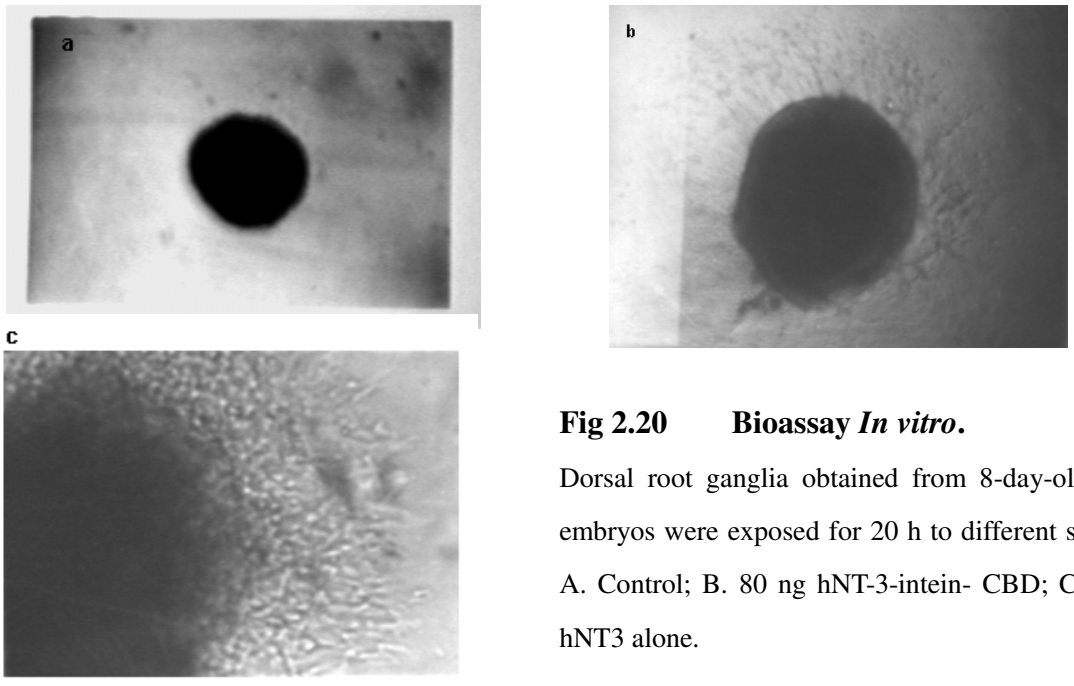


Fig 2.20 Bioassay *In vitro*.

Dorsal root ganglia obtained from 8-day-old chick embryos were exposed for 20 h to different samples. A. Control; B. 80 ng hNT-3-intein- CBD; C. 25 ng hNT3 alone.

2.3 Summary

This part deals with the cloning and expression of the hNT3 gene, especially employing a new method to swiftly purify the expression product, hNT3 by intein-inducible excision. hNT3 is one of the important members of the neurotrophin family. Due to its various potential applications, it is important to be able to obtain rather high amounts of the protein. Most likely, molecular cloning technology is an ideal process to reach the goal. However, many protocols for purifying recombinant proteins have some limitations, for instance, high cost proteases, complicated purification processes, etc. In this case, we tried to introduce a new purification strategy, using a protein self-splicing system which has been developed since 1990s of the last century to purify target proteins from crude expression products by a one step operation. The recombinant hNT3 has been expressed and purified by other researchers, however, hNT3 fusion with the intein gene and purification by the protein splicing system is a rather new approach.

As far as the up-stream process is concerned, first a pair of primers was designed according to the reported DNA sequence of the hNT3 gene. The total DNA of fresh human whole blood was used as the template to amplify the gene by PCR. The amplified gene was inserted into the vector pGEM-T which could directly be ligated with the PCR product. Then the correct structure of pGEM-hNT3 was confirmed by double cleavage with NdeI/SalI and DNA sequence analysis. After that, a second pair of primers was designed on the basis of MCS of pTXB1, and pGEM-hNT3 served as the template to amplify the hNT3 gene by PCR. The amplified hNT3 DNA fragment was inserted into vector pTXB1which contains the intein DNA fragment attached to a chitin-binding domain (CBD) coding region. The engineered strain E. coli 2566/pTXB-hNT3 was cultured in LB medium supplemented with ampicillin. After induction with IPTG, the target protein, hNT3-intein-CBD was mainly found in the form of inclusion bodies. In addition, the other construct, pJLA-hNT3 with temperature induction was tried. However, the expression product of the engineered strain E. coliBL21/pJLA-hNT3 also aggregated just as in case of the above engineered strain.

Protein purification and bioactivity assay are the two main steps in downstream processes. Because the expression products of both engineered strains mentioned above are all “inclusion bodies,” it was necessary to make the aggregates soluble under the conditions of denaturation and renaturation. The inclusion bodies were dissolved in 8 M urea and then the solution of unfolded polypeptides was diluted stepwise into 1 M urea with Hepes buffer and GSH, GSSG, L-arginine for refolding. Finally the refolded protein was applied to an affinity column of chitin beads and

washed to remove non-specifically absorbed proteins. In order to obtain pure hNT3 by intein-inducible excision, DTT was loaded onto the affinity column and kept in the column for 48 h at 25℃. Then the column was eluted with Hepes buffer, and hNT3 was collected as monitored proteins with coomassie brilliant blue. By the preliminary assay of the biological activity, it was shown from the results of the nerve fiber outgrowth of the dorsal root ganglia of chicken embryo that hNT3 and hNT3-intein-CBD exhibit the same bioactivity. One biological unit for the fusion protein, hNT3-intein-CBD and its cleavage product, hNT3, required about 80 ng or 25 ng, respectively, compared to 15 ng of the standard sample, human nerve growth factor.

2.4 Literature

1. Lipton P, Kalil R. Neurotrophic factors. Promega Notes, 1996, 50: 18-23.
2. Ragsdale C, Woodgett J. Trk neurotrophic receptors. Nature, 1991, 350: 660-661.
3. Rosenthal A. Goeddel DV, Nguyen T, *et al*. Primary structure and biological activity of a novel human neurotrophic factor. Neuron, 1995, **141**: 767-773.
4. Maisonpierre P, Bellusic I, Squinto S, *et al*. Neurotrophin-3: a novel factor related to NGF and BDNF. Science, 1990, **247**: 1446-1451.
5. McDonald NQ, Lapatto R, Murray-Rust J, *et al*. New protein fold revealed by a 2.3A resolution crystal structure of nerve growth factor. Nature, 1991, **354**: 411-414.
6. Negro A, Tavella A, Grandi C, *et al*. Production and characterization of recombinant rat brain-derived neurotrophic factor and neurotrophin-3 from insect cells. J. Neurochem, 1994, **62**(2): 471-477.
7. Perler FB, Davis E O, Dean G E, *et al*. Protein splicing elemens: inteins and exteins - a definition of terms and recommended nomenclature. Nucleic Acids Res, 1994, **22**: 1125-1127.
8. Gu H H, Xu J, Gallagher M, and Dean G E. Peptide splicing in the vacuolar ATPase subunit A from *Candida tropicalis.* J. Biol. Chem, 1993, **268**: 7372-7381.
9. Chong S, Montello GE, Zhang A, *et al*. Utilizing the C-terminal cleavage activity of a protein splicing element to purify recombinant proteins in a single chromatographic step. Nucleic Acids Res, 1998, **26**: 5109-5115.
10. Perler FB: The Intein database. Nucleic Acids Res, 2000, **28**: 344-345.
11. Perler FB, Adam E. Protein splicing and its applications. Current opinion in Biotechnol, 2000, **11**: 377-383.
12. Chong S, Xu MQ. Protein splicing of the *Saccharomyces cerevisiae* VMA intein without the endonuclease motifs. J. Biol. Chem., 1997, **272**:15587-15590.

13. Xu MQ, Comb D G, Paulus H, *et al*. Protein splicing: an analysis of the branched intermediated and its resolution by succinimide formation. EMBO J., 1994, **13**(22): 5517-5522.
14. Xu MQ, Perler FB The mechanism of protein splicing and its modulation by mutation. EMBO J., 1996, **15** (19): 5146-5153.
15. Pietrokovski S, Modular organization of inteins and C-terminal autocatalytic domains. Protein Sci., 1998, **7**: 64-71.
16. Chong S, Yang S, Paulus H, *et al*. Protein splicing involving the *Saccharomyces cerevisiae* VMA intein: The steps in the splicing pathway, side reactions leading to protein cleavage and establishment of an in vitro splicing system. J. Biol. Chem., 1996, **271**: 22159-22168.
17. Shao Y, Xu MQ, Paulus H. Protein splicing: evidence for an N-O acyl rearrangement as the initial step in the splicing process. Biochemistry, 1996, **15**: 3810-3815.
18. Dawson P E, Muir T W, Clark-lewis I., *et al*. Synthesis of protein by native chemical ligation. Science, 1994, **266**: 776-779.
19. Tam J P, Lu YA, Liu CF, *et al*. Peptide synthesis using unprotected peptides through orthogonal coupling methods. Proc. Natl. Acad. Sci. USA, 1995, **922**: 12485-12489.
20. Muir TW, Sondhi D, Cole PA. Expressed protein ligation: a general method for protein engineering. Proc. Natl. Acad. Sci. USA, 1998, **95**: 6705-6710.
21. Southworth MW, Amaya K, Evans Tc., *et al*. Purification of proteins fused to either the amino or carboxyl terminus of the *Mycobacterium* xenopi gyrase a intein. Bio. Techniques, 1999, **27**:110-120.
22. Xu MQ, Perler F B. The mechanism of protein splicing and its modulation by mutation. EMBO J., 1996, **15**: 5146-5153.
23. Chong S, Mersha F B, COMB D G, *et al*. Single- column purification of free recombinant proteins using a self-cleavage affinity tag derived from a protein splicing element. Gene, 1997, **192**: 271-281.
24. The handbook for one step protein purification system of New England Biolabs.. Impact I.
25. Yuan JM, Li ZY, Wang YM, *et al*. One step purification for recombinant human neurotrophic factor-3 with the splicing function of intein. Chin. J. Biochem. Mol. Biol, 2000, **16**(3): 335-339.

Part III Materials and methods

3.1 Materials

3.1.1 E. coli strains

***E. coli* strains**	**Genotype**	**Refs**
BL21 (DE3)	F$^-$*ompTgal (dcm) (lon) hsdS$_B$* ($r_B^-m_B^-$; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	[1]
DH5α	F$^-$ */endA1 hsdR17 ($r_k^-m_k^-$) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ (laclZYA-argF) U169 deoR* (Φ 80*dlac Δ(lacZ)M15*)	[2]
DHI	F$^-$ *supE44recA1 endA1gyrA96* (Nal^r) *Thi1 hsdR17* ($r_k^-m_k^+$)	[3]
ER2566	F$^-$ λ$^-$ *fhuA2 (lon) ompT LacZ::T7 genel gal sulA11 Δ (mcrC–mrr) 114 :: lS10R (mcr73::miniTn10)2 R (zgb-210 ::*Tn10*)1 (Tet^s) end A1 (d cm)*	[4]

3.1.2 Plasmids

Plasmids	**Genotype**	**Refs**
pGEM-T	Amp^r, f1 ori, T7 promoter, SP6 transcription, $MCS_{10\text{-}113,}$ LacZ, Lac operate	[5]
pGEM-hNT3	Amp^r, f1 ori, T7 promoter, SP6 transcription, LacZ, Lac operator, hNT3 (380 bp)	This work
pTXB1	Amp^{r,} Ptac, T7 promoter, M13ori, $MCS_{1525\text{-}1575}$, LacZ, Intein-CBD	[6]

pTXB-hNT3	Amp^r, Ptac, T7 promoter, M13ori, LacZ, Intein – CBD – hNT3	This work
pJLA503	Amp^r, $P_RP_{L,}$ cIts857, induction by shifting temperature, fd-transcription terminal, MCS_{12-358}	[7]
pJLA-hNT3	Amp^r, $P_RP_{L,}$ cIts857, induction by shifting temperature, fd-transcription terminal, hNT3	This work
pUC118	Amp^r, LacZ, LacP, MCS_{8-376}	[8]
pUC-gel I	Amp^r, LacZ, LacP, gelonin1+2 (345 bp)	Ya-Wei Shi *et al*. Provided
pUC-gel II	Amp^r, LacZ, LacP, gelonin3+4 (415 bp)	Ya-Wei Shi *et al*. Provided
pUC-gelII-AchR	Amp^r, LacZ, LacP, gelonin3+4+AchR (1196 bp)	Ya-Wei Shi *et al* provided
pUC-gel	Amp^r, LacZ, LacP, gelonin (760 bp)	This work
pET28a	Kan^r, f1 origin, T7 promoter, Lac operator, $MCS_{158-203}$, LacI, pBR322 origin	[9]
pET-gel	Kan^r, f1 origin, T7 promoter, Lac operator, LacI, pBR322 origin, gelonin (760 bp)	This work
pET19b-AchR	Amp^r, LacZ, LacP, AchR (1-181 aa)	In Trommer's lab
pPR506	Amp^r, Ptrc, pBR322 origin, AchR (1-120 aa)	In Trommer's lab
pGE60	Chl^r, Plac, Phs, pBR322 origin, GroES, GroEL	In Trommer's lab
pUC-GA	Amp^r, LacZ, LacP, gelonin+AchR (1546 bp)	This work
pET-GA	Kan^r, f1 origin, T7 promoter, Lac operator, LacI, pBR322 origin, gelonin+AchR (1546 bp)	This work
pJLA-GA	Amp^r, $P_RP_{L,}$ cIts857, induction by shifting temperature, fd-transcription terminal, gelonin+AchR (1546 bp)	This work

Further details about these plasmids are given in the relevant chapters of this thesis

3.1.3 Enzymes

β-agarase	10 U/µl	New England Biolabs
EcoRI	20 U/µl	New England Biolabs
HindIII	20 U/µl	New England Biolabs
KpnI	10 U/µl	New England Biolabs

NcoI	20 U/µl	New England Biolabs
NdeI	20 U/µl	New England Biolabs
PstI	10 U/µl	MBI Fermentas
SalI	20 U/µl	New England Biolabs
SpeI	10 U/µl	New England Biolabs
XhoI	10 U/µl	MBI Fermentas
XbaI	20 U/µl	New England Biolabs
T4 DNA Ligase	20 U/µl	New England Biolabs
T4 DNA Polymerase	5 U/µl	New England Biolabs
Taq Polymerase	5 U/µl	New England Biolabs
Pyrophosphatase	4 U/µl	MBI Fermentas

The digestions were carried out in the New England Biolabs buffer system.

10 x NEB buffer 1:

100 mM Bis Tris-Propane-HCl
100 mM $MgCl_2$
10 mM Dithiothreitol
pH 7.0

10 x NEB buffer 2 :

500 mM NaCl
100 mM Tri-HCl
10 mM Dithiothreitol
pH 7.9

10 x NEB buffer 3 :

1 M NaCl
500 mM Tris-HCl
100 mM $MgCl_2$
10 mM Dithiothreitol
pH 7.9

10 x NEB buffer 4 :

500 mM Potassium acetate
200 mM Tris-acetate
100 mM Magnesium acetate
10 mM Dithiothreitol
pH 7.9

3.1.4 Oligonucleotides

The oligonucleotide fragments for gelonin were synthesized by MWG Biotech AG, Ebersberg. The delivered lyophilisate was solubilized in H_2O_{bidest} prior to use. The concentration was checked by UV absorption measurement (See 3.3.7)

Gelonin 1 fragment: 1×120 mer and 1×119 mer

5'-CCCTGCAGTA	ATAACATATG	GGCCTGGATA	CCGTGAGCTT
CAGCACCAAA	GGCGCCACCT	ATATTACCTA	TGTGAACTTC
CTGAACGAAC	TGCGTGTGAA	ACTGAAACCG	GAAGGCAACA-3'

3'-TGACTTTGGC	CTTCCGTTGT	CGGTACCGTA	AGGCGACGAC
GCATTTTTTA	CGCTACTAGG	CCCGTTTACG	AAGCACGACC
ACCGCGACTC	GTTGCTATTG	CCGGTCGATC	GCCATGGGG-5'

Gelonin 2 fragment: 1×97 mer and 1×98 mer

5'-AAGAATTCGC	TAGCGGAAAT	TGCGATTGAT	GTGACCAGCG
TGTATGTGGT	GGGCTATCAG	GTGCGTAACC	GTAGCTATTT
CTTCAAAGAT	GCGCCGG-3'		

3'-AAAGAAGTT	CTACGCGGCC	TACGCCGCAT	ACTTCCGGAC
AAGTTTTTGT	GGTAATTTTG	GGCAGACGTA	AAGCCGCCGT
CGATAGGCTC	AGATCTCG-5'		

Gelonin 3 fragment: 1×120 mer and 1×119 mer

5'-AATCTAGAAG	GCGAAAAAGC	GTATCGTGAA	ACCACCGATC
TGGGCATTGA	ACCGCTGCGT	ATTGGCATTA	AAAAACTGGA
TGAAAACGCG	ATTGATAACT	ATAAACCGAC	CGAAATTG-3'

3'-GATATTTGGC	TGGCTTTAAC	GCTCGTCGGA	CGACCACCAC
TAAGTCTACC	ACTCGCTTCG	CCGCGCAAAG	TGGAAGTAAC
TTTTGGTCTA	AGCATTGTTG	AAGGTCGTCG	CTTAAGGC-5'

Gelonin 4 fragment: 1×120 mer and 1×119 mer

5'-CGGAATTCGT	CCGGCGAACA	ACACCATTAG	CCTGGAAAAC
AAATGGGGCA	AACTGAGCTT	CCAGATTCGT	ACCAGCGGCG
CGAACGGCAT	GTTCAGCGAA	GCGGTGGAAC	TGGAACGTGC-3'

3'-CGCCACCTTG	ACCTTGCACG	CTTGCCGTTT	TTTATAATAC
ACTGGCGCCA	CCTAGTCCAC	TTTGGCTTTT	AACGCGACGA
CTTTAAGCAG	CTGTTTCTAG	GCTTTATTAT	TCCATGGGG-5'

Shadow indicates the complement bps

3.1.5 Media and antibiotics

Agar	Oxoid LTD, England
Tryptone peptone	Oxoid LTD, England
Yeast extract	Oxoid LTD, England
Ampicillin	Amersco
Kanamycin-sulfat	Amersco
Chloramphenicol	Amersco

3.1.6 Chemicals

30% Acrylamide-bis	Sangon Company, Canada
B-PER Bacterial Reagent	Pierce
DTT	Sigma
EDTA	Sigma
GSH	Sigma
GSSG	Sigma
IPTG	American Bioanal
L-arginine·HCl	Sigma
L-valine	Sigma
Phenol/Chloroform/Isoamylalcohol (P/C/I)	Sangon Company, Canada
PMSF	Amersco
Tris (tris(hydroxymethyl)aminomethane)	Sangon Company, Canada

X-gal	American Bioanal
NBT	Promega
BCIP	Promega
1640	Sangon company, Canada
DMEM	Sangon company, Canada
Rabbit reticulocyte lysate translation systems	Promega

The chemicals not specified here were available in the groups and were of AR grade.

3.1.7 Other materials

Agarose	American Bioanal
BSA (10 mg/ml)	New England Biolabs
Cell free lysate	Promega
λ-DNA	New England Biolabs
λ-DNA/*Eco* 47I-ladder	New England Biolabs
λ-DNA/*Hind* III-ladder	New England Biolabs
Nerve growth factor	Boehringer
QIAprep Spin Miniprep Kit	Qiagen
QIAGEN Nucleotide Removal Kit	Qiagen
QIAGEN Gel Extraction Kit	Qiagen
SP-Sepharose ff	Pharmacia
Q- Sepharose ff	Pharmacia
Phenyl-Sepharose	Pharmacia
Superose 12	Pharmacia

3.1.8 Equipment

Centrifuges	Table centrifuge	SanYo
	SP-21 with a fixed angle rotors	MSE
	Biofuge28RS	Heraeus
Incubator	Air shaker THZ-82	JiangSu, China
Thermocycler	Gene ATAQ PCR System	Phamacia
Other equipment:	GDS	Gene company
	pH meter 720	Orion
	CO_2-Incubator	Heto

Autoclave	SanYo
Chromatography system	LKB
AKTA purifier	Pharmacia
CT60e Freezing dryer	Heto
Sonicator	Zhejian, Xin-zhi

3.2 Cultivation and storage

3.2.1 Media, agar plates and antibiotics for the cultivation of *E. coli* strains

Media were autoclaved in a 10 L autoclave at 121°C and 1.5 bar for 20 min. For preparation of the agar plates, the agar was added to the medium before autoclaving. The plates were stored at 4°C for up to two months.

The incubation of the agar plates was performed at 37 °C in an incubator. The liquid cultures were incubated at 37°C and 250 r/m in an air shaker. Erlenmeyer flasks or test tubes were used as containers, which were filled maximal to 10% to ensure an adequate aeration of the culture medium.

Media

LB medium:

10 g	Tryptone peptone
5 g	Yeast extract
10 g	NaCl
	Final volume to 1000 ml with H_2O
	pH 7.2 with NaOH
15 g	Agar (for plates only)

Antibiotics and IPTG

The solution of antibiotics and IPTG were filtered sterile through a 0.45 µm filter and added to the pre-autoclaved medium at a temperature under 50 °C.

Table 3.1 Supplements

Supplement	**Stock solution**	**Concentration in the medium**
Ampicillin	100 mg/ml in H_2O_{bidest}	100 µg/ml in liquid cultures 70 µg/ml in solid media
Kanamycin	100 mg/ml in H_2O_{bidest}	80 µg/ml in liquid or solid media
Chloramphenicol	50 mg/ml in 50% EtOH	50 µg/ml in liquid or solid media
IPTG	1 M in H_2O_{bidest}	1 mM in liquid culture

3.2.2 Storage of *E. coli* cultures

The agar plates with *E. coli* cultures or the liquid cultures could be stored for a short period of time (for plates up to two weeks or for liquid cultures up to two days) at 4 °C in a refrigerated room. For storage of longer periods of time, the cultures were stored in 40% glycerol at - 80 °C, where they should survive for 1-2 years.

Preparation of glycerol cultures

Sterile tubes (2 ml, with a screw cap) were filled with 500 µl of 80% glycerol and autoclaved. The same volume of overnight culture grown in LB media was added to the sterile tubes, the tubes were mixed and stored at – 80 °C.

For the cultivation from single colony, a small portion of the top of the deep-frozen glycerol culture was streaked on agar plates with a sterile stick. During inoculation the thawing of the glycerol cultures should be avoided to ensure as long a survival of the cells as possible.

3.3 General methods

3.3.1 Preparation of competent *E. coli* cells

To achieve a high transformation efficiency the competent cells were prepared according to the protocol in the handbook. With the cells more than 10^7 transformants per µg of added DNA can be obtained depending on the plasmid or the *E. coli* strains [10].

10 ml LB medium were inoculated with a single colony of a bacterial strain and incubated in the shaker at 37°C and 250 r/m overnight. 250 ml of LB medium with 2 ml of fresh overnight culture were incubated with rigorous shaking (250 r/m) at 37°C until the cells had reached OD_{600} about 0.5. Finally the cells were cooled down on an ice bath for 30 min. In order to achieve high competence of the cells, it is important that the cells should reach the exponential growth phase.

The next steps were carried out in a refrigerated room and pre-cooled solutions as well as equipment were used. The liquid culture was transferred to two cold 250 ml centrifuge tubes and centrifuged at 5000 r/m at 2°C for 5 min. The supernatant was discarded. The cell pellets were carefully resuspended in 25 ml of 0.1 M $CaCl_2$ cold solution and incubated on an ice bath for 30 min. The suspension was precipitated by recentrifugation as above and the supernatant was discarded. After resuspending the pellets in 10 ml of 0.1 M $CaCl_2$ solution, they were incubated on an ice bath for 2 h. The cell suspension was divided in 100-200 µl aliquots (containing 15% glycerol) in cold microcentrifuge tubes and frozen quickly. The competent cells were stored at -80°C. A transformation with a known amount of plasmid DNA was performed to check the competence of the cells.

3.3.2 Transformation of competent *E. coli* cells with plasmid DNA

An aliquot of competent cells was thawed in an ice bath. If it was necessary to divide the cells into smaller aliquots, cooled pipette tips and cold microcentrifuge tubes were used. The volume of the required cell suspension was dependent on the volume and the concentration of the DNA solution which was used in the transformation.

After the addition of the DNA solution to the competent cells, the solution was carefully mixed with a pipette tip. The mixture was incubated on an ice bath for 1 h and afterwards for heat shock at 42°C for 90 sec. After cooling on ice for 2-5 min, 0.8 ml of LB medium without antibiotics was added and incubated at 37°C and 200 r/m for 45 min. Depending on the expected transformation efficiency, 100-150 µl of the cell culture was spread on the agar plates which contained the appropriate antibiotics. To check the viability of the competent cells, 100 µl of competent cells were spread on LB plates without antibiotics [10].

3.3.3 Plasmid minipreparation

3.3.3.1 Alkaline lysis method

Solution I:	50 mM	Glucose
	10 mM	EDTA
	25 mM	Tris-HCl, pH 8.0
Solution II:	0.2 M	NaOH
	1 %	SDS
	Prepare freshly from stock solution immediately before use.	
Solution III:	3 M	Potassium acetate
	5 M	Glacial acetic acid
	Mix 60 ml of 5 M potassium acetat, 11.5 ml glacial acetic acid and 38.5 ml water.	

1.5 ml of an overnight culture was transferred to a microcentrifuge tube and centrifuged with 13,000 r/m in a table centrifuge for 30 sec. The medium was totally removed with a pipette. The pellet was suspended in 100 µl of solution I and incubated at room temperature for 5 min. Afterwards 200 µl of solution II was added and the mixture was shaken by inverting the tube rapidly but carefully six to eight times and incubated on ice for 5 min. The solution was neutralized and the dodecyl sulfate precipitated as potassium salt by the addition of 150 µl of solution III. The mixture was vortexed for 10 sec. Afterwards the tubes were incubated on ice for 5 min. During centrifugation with 13,000 r/m for 5 min a cell pellet which consists of cell fragments, potassium dodecyl sulfate and attached proteins or chromosomal DNA was formed.

The supernatant which consists of plasmid DNA and RNA was transferred to a new microcentrifuge tube and mixed with an equal volume of phenol/chloroform/isoamyl alcohol. After 2 min of centrifugation at 8,000 r/m, the upper aqueous phase was transferred to a new tube. The plasmid DNA and RNA was precipitated by adding two volumes of 99% ethanol and incubating at 4°C for at least 30 min. After centrifugation at 13,000 r/m for 15 min the supernatant was removed and the pellet was washed by adding 1 ml of 70% ethanol. The pellet was briefly vortexed and centrifuged again at 13,000 r/m for 5 min. The supernatant was removed and the pellet was dried in an desiccator for at least 20 min. The DNA was suspended in 20 µl 10 mM Tris-Cl (pH8.0) that contained 20 µl/ml RNase and stored at -20°C [10].

3.3.3.2 Plasmid minipreparation with QIAprep™ Spin Miniprep Kit

The QIAprep™ Spin Miniprep Kit was used if DNA of high purity was necessary. 2-10 ml of LB medium with a proper antibiotic addition was inoculated with a single colony and incubated at 37°C and 300 r/m for 10-12 h. 2-5 ml of the overnight culture was divided in 2.5 ml microcentrifuge tubes and centrifuged at 13,000 r/m for 1 min. The media were removed and the pellets were resuspended in 250 µl of resuspension buffer P1 until no cell clumps were visible. 250 µl of lysis buffer P2 was added and the tubes was inverted 4-6 times to mix. After addition of 350 µl of neutralization buffer N_3, the mixture was inverted and centrifuged at 13,000 r/m for 10 min. The supernatants were applied from the tube to a QIAprep™ spin column placed in a 2 ml collection tube. The columns were centrifuged at 13,000 r/m for 1 min and the flow-through was discarded. Each column was washed by adding 0.75 ml of washing buffer PE and centrifuging for 1 min at 13,000 r/m. The flow-through was discarded again and the residual washing buffer was removed by centrifuging as before. The spin column was placed in a fresh 1.5 ml microcentrifuge tube and 50 µl of elution buffer EB (10 mM Tris, pH7.5) was added to the center of the column. After 1 min, the tube was centrifuged as before. The plasmid preparations were stored at –20°C.

3.3.4 Purification of PCR products

The PCR product was run on a 1% agarose gel. The gel slice containing the target gene was cut and melted at 70 °C in a water bath. 1 ml of resin was then added and mixed for 20 sec, after that the mixture was transferred into a syringe barrel connected to a Wizard Mini-column. The column was washed one time with 80% iso-propanol, followed by centrifugation at 10,000 r/m for 2 min to completely remove residual iso-propanol. The Mini-column was then transferred to a new 1.5 ml Eppendorf tube, and 50 µl of TE buffer was added. The eluted DNA was collected by centrifugation after 1 min incubation.

3.3.5 Ligation

Ligation were carried out with the T_4 DNA ligase only. This enzyme is able to ligate both “stick” and “blunt ends”. The ligations were carried out in a total volume of 20 µl for sticky ends and 50 µl for blunt ends. For blunt end ligation, the solution contained 0.5 mM ATP, 5mM $MgCl_2$ and at least 50 U ligase/ml. For sticky end ligations the supplied ligation buffer was used. The ligation were carried out at 16˚C overnight.

3.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used for electrophoretic separation, identification and isolation of nucleic acids. The separation is based on the different electrophoretic mobility of the DNA molecules. This mobility is dependent on the size and conformation of the molecules. The DNA fragments in the gel become visible through UV fluorescence of the intercalative agent ethidium bromide [10].

A DNA marker was used as a reference for the determination of the size of linear DNA fragments. The λ DNA /HindIII was cleaved to the bands of 23130, 9416, 6557, 4361, 2322, 2027, 564, 125 bp in agarose gel electrophoresis. For the determination of small DNA fragments, pBR322/BstNI or ΦX174/HaeIII was used as the marker. The cleaved fragments are from 1857 bp-100 bp or so. 0.9–1% agarose gels are mainly used to separate 0.2-9 kb fragments. To determine smaller fragments (from 50 bp up), 3-4% agarose gels were used.

For all gels TAE-buffer was used as the electrophoretic solution. For isolating a DNA fragment, the band was cut out as precisely as possible with a scalpel. It was made sure that the DNA was exposed to UV light as short as possible to prevent any mutations.

Electrophoresis buffer

TAE buffer: 0.04 M Tris-acetic acid
0.001 M EDTA
pH 8.3 with acetic acid

Loading buffer: 0.25 % Bromophenol blue
40% Sucrose

Preparation of the gel and electrophoresis

An adequate amount of agarose in 40 ml TAE buffer was heated in a microwave oven until a clear solution formed. After cooling the solution down to 50-60°C, 2 µl of ethidum bromide stock solution (10 mg/ml) was added. The mixture was poured on a gel tray, which had been sealed with tape. The gel comb was placed in position in the gel. After the gel had solidified (approximately 30 min), the comb was gently removed and the gel was transferred to the electrophoresis chamber

which was filled with TAE buffer. If necessary, the gel was stored in TAE buffer in 2 days. After loading the gel, the electrophoresis was carried out at 80 V (40-50 mA) for 90-120 min.

3.3.7 Estimation of DNA concentration

The DNA solutions were scanned in the range 230-330 nm and the concentration was estimated according to the following relation:

1 OD_{260} = 33 µg/ml ssDNA

1 OD_{260} = 50 µg/ml dsDNA

3.3.8 Determination of protein concentration

Protein concentration was determined by the methods of Lowry [11] or Bradford [12] using bovine serum albumin as a standard.

3.3.9 SDS-PAGE gel electrophoresis

SDS-PAGE is one of the most powerful and convenient methods for examining macromolecules. Acrylamide and bis-acrylamide polymerize to form a sieving matrix, which serves to separate macromolecules by size with polyacrylamide gel electrophoresis (PAGE). The polymerization of acrylamide forms long chains which are cross-linked together by the addition of the bis-acrylamide. The pore size of the matrix is inversely related to the total concentration of acrylamide and bis-acrylamide in solution and the percent of the total monomer which is the cross-linker. SDS is a strong protein denaturing agent, it can cause proteins to assume a rodlike shape. SDS-treated proteins have identical charge to mass ratios and similar shapes. The molecular separation is based on gel filtration and therefore, the electrophoretic mobility of the molecules will be achieved. [10].

Gel preparation and electrophoresis

First, it is necessary to select the appropriate acrylamide percentage on the basis of the size of proteins to be separated. The gel apparatus was assembled following the instruction. The gel and gel buffer were prepared as described in table 3.2 and 3.3. The resolving gel mixture was poured into the assembled gel plate, leaving sufficient space at the top for the stacking gel to be added later. The gel was gently overlaid with 200-400 µl H_2O and polymerised for 30 min or so. After

polymerisation, the overlay was removed. The stacking gel component was filled into the remaining space in the gel apparatus with the gel solution and the comb was inserted immediately. After the upper gel has polymerised, the comb was removed. The wells were rinsed with water to remove unpolymerized acrylaminde. The gel reservoirs were filled with running buffer. Now samples were loaded on the gel and it was run usually with constant 18 mA or so. When the blue dye was at the bottom of the gel, it was stopped. The protein bands can be detected after staining with CBB and destaining with solvent.

Electrophoresis buffer

Table 3.2 Stacking gel buffer

Components	2 ml	4 ml
H_2O_{bidest}	1.4	2.7
30% Acrylamide-bis	0.33	0.67
1.0 M Tris (pH6.8)	0.25	0.5
10% SDS	0.02	0.04
10% Ammonium persulfate	0.02	0.04
TEMED	0.002	0.004

Table 3.3 12% Separating gel

Components	5 ml	10 ml	15 ml
H_2O_{bidest}	1.9	4.0	5.9
30% Acrylaminde-bis	1.7	3.3	5.0
1.0 M Tris (pH6.8)	1.3	2.5	3.8
10% SDS	0.05	0.1	0.15
10% Ammonium persulfate	0.05	0.1	0.15
TEMED	0.002	0.004	0.006

Tank buffer: 25 mM Tris
250 mM Glycine
0.1% SDS
pH8.3

Staining solution:

0.25 g Coomassie blue R-250

100 ml Destaining solution

Destaining solution:

450 ml Methanol

450 ml H_2O_{bidest}

Acetic acid 10 ml

Sample treatment

To prepare the sample for electrophoresis, the protein solution was mixed with 5X sample loading buffer according to the ratio of 1:5 in an Eppendorf tube. The tube was put in boiling water for 1-3 min, then cooled down to room temperature. A standard protein marker was used as a reference for the determination of the size of proteins.

5X sample loading buffer: 250 mM Tris-HCl, pH6.8
25% (β-ME)
10% SDS
0.05% Bromophenol blue
50% Glycerol
Store at –20˚C

3.4 Clone of pGEM-hNT3

3.4.1 Preparation of human genomic DNA

The mixture of 50 µl human whole blood containing anticoagulant and 0.5 ml TE was centrifuged at 13,000 r/m for 10 sec. The supernatant was discarded and the precipitate was washed with 0.5 ml TE. After centrifugation, the precipitate resuspended in K^+ buffer (50 mM KCl; 10 mM Tris-HCl; 7.5 mM MgCl; 0.5% Tween 20; 100 µg/ml proteinase K) was incubated at 56˚C for 45 min to digest the cells, then incubated at 95˚C for 10 min to inactivate proteinase K.

3.4.2 Amplification of hNT3 gene

To amplify the hNT3 gene from the template of human genomic DNA, 10 µl of 10 x Taq buffer, 8 µl of primer FW (10 µM see 2.2.1.2), 8 µl of primer RV (10 µM, see 2.2.1.2), 5 µl dNTPs (4 mM each), 10 µl of $MgSO_4$ (25 mM), 5 µl template of human genomic DNA, 54 µl H_2O_{bidest} and 1 µl Taq were mixed (Table 3.4). The PCR was carried out with the following temperature profile:

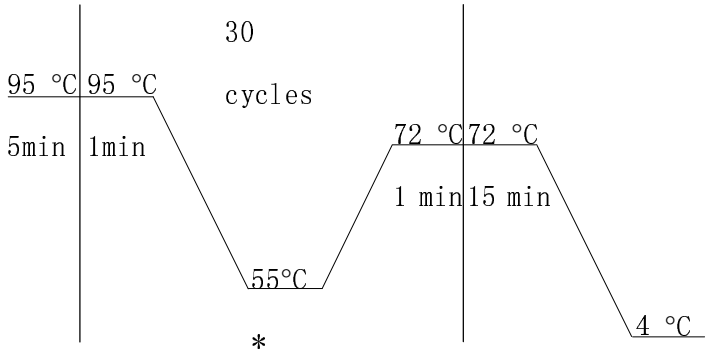


Table 3.4 The PCR reaction system

Materials	Volume (μl)
10 x PCR Buffer	10
Genome DNA	5
dNTP (4mM)	5
Primer (FW)	8
Primer (RV)	8
$MgSO_4$ (25 mM)	10
dd H_2O	53
Taq DNA polymerase	1
Total volume.	100

3.4.3 Construction of pGEM-hNT3

The conditions for the ligation reaction are listed in Table 3.5. The reaction mixture was then transformed into *E. coli* DH 5α by a heat shock procedure and plated on LB agar medium plates supplemented with ampicillin and Xgal for white/blue screening. After incubation at 37 ^{o}C overnight, a number of white single colonies were picked and grown in LB (ampicillin) medium. The recombinant plasmids were extracted from the cells as described in Part III (3.3.3) and the insertion of hNT3 gene was confirmed as described in Table 3.6.

Table 3.5 Ligation reaction system

Materim Materials	St. reaction	Posi.reaction	Control
T4 DNA Ligase 10 x buffer	2 µl	1 µl	1 µl
pGEM-T Vector (50 ng)	1 µl	1 µl	1 µl
PCR product	15 µl	-	-
Control Insert DNA	-	1 µl	-
T4 DNA Ligase (3 Weiss unit/µl)	1 µl	1 µl	1 µl
Add H_2O_{bidest} to final volume	20 µl	20 µl	20 µl

* The reaction mixture was incubated at 16ºC water bath overnight

Table 3.6 Enzyme reaction system for double cleavage by NdeI/SalI

Reactants	Double cleavage	Single cleavage
Plasmid	5 µl	5 µl
10 x SalI buffer	2 µl	2 µl
NdeI	1 µl	-
SalI	1 µl	1 µl
dd H_2O	11 µl	12 µl
Total	20 µl	20 µl

3.5 Determination of biological activity

3.5.1 hNT3 bio-activity assay [13]

3.5.1.1 Collection of ganglia

Fertilized white leghorn chicken eggs are put on storage at 10˚C after delivery from the supplier under refrigerated conditions; the period of the cold storage should not be extended beyond 3-4 days. Eggs are transferred into an egg incubator at 38˚C and rotated every day. After 8 days of incubation, eggs are taken out and the embryos are removed to a frosted glass slide under a dissecting microscope. The embryo is rinsed with sterilized 1640 medium, positioned on its back

with outstretched limbs, and opened up to expose the ventral aspect of the spine with its bilateral arrays of dorsal root ganglia (DRG's). Roots and nerve emerging from the ganglia are cut and the individual ganglia are collected at room temperature into a small plate with 1640 medium.

3.5.1.2 The preparation of sample

Sample

In general, the sample was diluted with PBS buffer in a series of concentrations. The procedure was continued to dilute the content of each tube one after one until the desired dilution range was achieved. This resulted in a series of dilution factors of 1 (original sample), 3×10^{-1}, 10^{-1}, 3×10^{-2}, 10^{-2}, etc. A more fine value of the sample activity may require a more precise dilution technique and narrow dilution intervals (twofold or less) around the previously established activity level. This could make the result more reliable.

Preparation of collagen from rat tail

The tail from a rat about the weight of 250 g was taken out and put into 75% ethanol for 30 min. After that, the skin and muscle were removed. The tendons were cut into small pieces and dissolved by 150 ml 0.1% HAc by shaking gently, at 4°C for 48 h. The extraction solution was centrifuged at 4000 r/m, 30 min. The supernatant was divided into 10 ml portion, put into small bottles and stored at -20°C.

3.5.1.3 The assay

In general, the following procedure is convenient: A series of sterile culture bottles (20×10×5 mm) were streaked out with collagen of rat tail. 3 ml DMEM was added into a bottle and incubated in CO_2-incubator overnight. Next day, the individual ganglia collected at room temperature under a dissecting micro-scope was given into this culture bottle, usually two ganglia per bottle, and mixed with 500 µl of diluted sample. The patterns of fiber outgrowth elicited from the explanted ganglia by the different gradient of sample concentration are examined by phase contrast microscopy at a magnification in 24 h. One biological unit of the sample is defined as having the same outgrowth response as 1 biological unit of sample of nerver growth factor (15 ng) [16]. All of steps described above were performed under sterile condition.

3.5.2 ELISA

ELISA provides a sensitive and quantitative detection of special antigens or antibodies. Wells of microtiter plates were coated with 2 µg protein in 100 µl coating buffer and incubated at 4˚C overnight. The coated plates were washed 3 times with 200 µl PBST at room temperature. After washing, the wells were saturated with 200 µl of 1% BSA in PBS for 1.5 h at room temperature and washed with PBST three times. The first antibody diluted in PBS was added to each well and incubated for 2 h at room temperature (100 µl/per well). Afterwards, each well was washed with PBST three times before adding 100 µl of secondary antibody diluted with PBST for 1 h at room temperature. The wells were continuously washed with PBST four times respectively. The first antibody bound was measured by peroxidase activity with P-nitrophenyl phosphate as the substrate. The color developed about 30 min was measured at 405 nm using a microtiter plate reader [14].

Solution

Coating buffer:
A: 0.2M Na_2CO_3
B: 0.2M $NaHCO_3$
Make up fresh: 17 ml A+8 ml B
pH10.6
Final volume to 100 ml

PBS buffer:
2.5 mM Na_2HPO_4
2 mM NaH_2PO_4
0.14 M NaCl
pH7.2
Final volume to1000 ml

PBST: 100 ml PBS+0.45 ml Tween 20

Block solution: 1% BSA in PBS buffer

Substrate buffer:
0.2 g NaN_3
0.1 g $MgCl_2.6H_2O$
97 ml DEA
pH9.8
Final volume to1000 ml

Substrate:
10 mg P-nitrophenyl phoshate +10 ml Substrate buffer

3.5.3 Western blots

Western blot analysis involves in gel electrophoresis the presence of SDS which results in denaturation and separation of molecules on the basis of size. These molecules are then transferred to another matrix to form a pattern on the matrix identical to that on the gel. In general, antigens immobilized (blotted) on a membrane from SDS-PAGE are detected with primary antibodies. The appropriate enzyme conjugated with the secondary antibody results in the deposition of colour substrate on the membrane at the reaction site. This colour provides a visual indication of potential primary antibody recognition.

Western blots were made by electrophoresis transfer of proteins from SDS-PAGE into nitrocellulose membrane by sandwich electrophoresis. After transfer, the membrane was incubated in 1% milk powder (free lipid) in TBST for 1 h to saturate nonspecific protein binding sites. The membrane was washed with TBS three times. To bind primary antibody, the blocking solution was replaced with TBST containing the appropriate dilution of primary antibody and incubated for 2 h with gentle agitation. In order to remove unbound antibody, the membrane was washed three times with TBS for 10-15 min. Finally, the membrane was incubated in TBST containing the appropriate dilution of anti-Ig AP conjugate for 1 h with gentle agitation. The membrane was washed four times to remove the unbound secondary antibody in TBS and reacted with BCIP/NBT substrate until the color of the target protein occurred and re-transferred into PBS to be fixed immediately. This procedure was done at RT [15].

Solutions

TBS:
10 mM Tris-HCl, pH8.0
150 mM NaCl

TBST:
TBS+0.05% Tween 20

AP buffer:
100 mM Tris-HCl, pH9.5
100 mM NaCl
5 mM MgCl

AP color development solution:
10 ml AP buffer
66 µl NBT
33 µl BCIP

3.5.4 The inhibition of protein synthesis *in vitro*

The rabbit reticulocyte lysate translation system plays an important role in the identification of the investigation of transcriptional and translational regulation. The procedure was performed on 96 wells of microtiter plates. To examine the toxicity of the fusion protein, a series of samples were diluted as follows $3.16x10^{-8}$ M, 3.16×10^{-9} M, 3.16×10^{-10} M, 3.16×10^{-11} M, 3.16×10^{-12} M to $3.16x10^{-13}$ M. 5 µl solution taken out from each diluted sample was added into a well of the test plate and mixed with 40 µl complement lysate, 37ºC incubator for 5 min. Then 10 µl master mixture containing L-(U-^{14}C)-valine, 50 µCi was added into each well, incubator 10 min again. After that, two parallel 5 µl culture from each well were added into 1 ml pre-cool double distilled water, mixed with 500 µl valine (1mg/ml) at 37ºC for 15 min (each sample should be repeated). Finally, the protein precipitated by 4 ml 25% TCA (two times) was dried on glass microfiber filters with vacuum. The microfiber was incubated with 5 ml of radioactivity solution of β-counter (Beckman LS1701) for 2 h at RT. It was then assayed for the radioactivity [16].

Solution A:

250 µl H_2O_{bidest}
250 µl Glycerol
2.5 mg Creatinkinase

Solution B:

242 mg Tris
373 mg KCl
90 ml Ethylene glycol
10 ml H_2O_{bidest}
pH8.2
65.2 mg Hemin

Solution C:

6.7 mg Creatinphosphate
100 µl H_2O_{bidest}

Solution D:

9.5 mg $MgCl.6H_2O$
1.45 g KCl
10 ml H_2O_{bidest}

Solution E: L-(U-^{14}C) valine (50 µCi/ml=1.85 MBq/ml) was purchased from Amersham

Solution F: *Amino acids solution*

7.5 mM: alanine; leucine
5 mM: aspartate; glutamate; glycine; histidine; lysine; serine
3.75 mM: arginine; asparagine; glutamine; isoleucine; phenylalanine; proline; threonine; thryptophan; tyrosine
2.5 mM: cysteine; methionine

Complement Lysate:

970 µl Lysate (Promega)
10 µl Solution A
20 µ l Solution B
20 µl Solution F

Master Mix:

50 µl Solution C
50 µl Solution D
80 µl Solution E

Valine solution:

1 M NaOH
0.5 M H_2O_2
100 mg Valine
Final volume to 100 ml

3.6 Literature

1. Kunkel T A, *et al*. Meth Enzymol, 1987, **154**: 367-382.
2. Woodcock D M, *et al*. Nucl. Acids. Res., 1989, **17**: 3469-3478.
3. Bachmann B J. In *E.coli* and Salmonella typhimurium, Cellular and Molecular Biology, ed. Neidhardt F C, et al. ASM, 1987, 1190-1219.
4. Chong S, *et al*. Gene, 1977, **192**:277-281.
5. Promega catalog, 2000, 158-159.
6. New England Biolabs catalog, 1997, ＃6500.
7. Schauder B, *et al*. Gene, 1987, **52**: 279-283.
8. New England Biolabs catalog, 1999, 224.
9. Novagen catalog, 1993, 154-155.
10. Sambrook J, *et al*. Molecular Cloning*:* A Laboratory Manual, 2nd, Ed., Cold Spring Harbor Laboratory Press, New York, 1989.
11. Lowery O H, Rosenberg N J, Farr A L, *et al*. J. Biol.Chem., 1951, **193**:265-275.
12. Bradford M M. Anal. Biochem., 1976, **72**: 248-254.
13. Levi-Montalcini R. Nerve growth factor. Physiol. Rev., 1968, 48: 534.
14. Harlow E. Antibodies, A laboratory manual. Cold Spring Harbor Laboratory, New York, 1988, Chapter 14, Immunoassay: 553-612.
15. Promega protocols and applications.1996, 298-300.
16. Promega: rabbit reticulocyte lysate systems.1992, #232

Abbreviations

AID	Acquired immune deficiency
AchR	Nicotinic acetylcholine receptor
Ach	acetylcholine
ATP	Adenosine-5'-triphosphate
Amp	Ampicillin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BDNF	Brain-derived neurotrophic factor
bp	Base pairs
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CBD	Chitin binding domain
cccDNA	Cycle close covalent DNA
cDNAs	Complement DNA
Chl	Chloramphenicol
CIAP	Calf intestine alkaline phosphatase
CNTF	Ciliary neurotrophic factor
CySH	Cysteine
DEA	Diethanolamine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside-5'-triphosphate
dsDNA	Double strand DNA
DTT	1,4-dithiothreitol
EAMG	Experimental autoimmune Myasthenia gravis
E. coli	*Escherichia coli*
EDTA	Ethylendiamin-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immuno sorbent assays
EtBr	Ethidium bromide
EtOH	Ethanol
FGF	Fibroblast growth factors
FPLC	Fast performance liquid chromatography
GSH	Glutathione
GSSG	Oxidized glutathione

GST	Glutathione S- transferase
GuHCl	Guanidine hydrochloride
HAc	Acetic acid
hNT3	Human Neurotrophins 3
HIV	Human immunodeficiency virus
IPTG	Isopropyl- β -thiogalactopyranoside
KAc	Potassium acetate
Kan	Kanamycin
Kb	Kilo bases
KD	Kilo dalton
KDEL	lysine-aspartate-glutamate-leucine
LM	Low melt
MAb	Monoclonal antibody
MG	Myasthenia gravis
MIR	Main immunogenic region
Mops	3-(N-morpholino)-1-propane sulfonic acid
MW	Molecular weight
β-ME	β-Mercaptoethanol
OD	Optical density
RIPs	Ribosome inactivating proteins
scRIPs	Single chain ribosome inactivating proteins
dsRIPs	Double chain ribosome inactivating proteins
NBT	Nitroblue tetrazolium
NGF	Nerve growth factor
NT-3 and NT4/5	Neurotrophins 3, 4/5
P/C/I	Phenol/chloroform/isoamylalcohol
PCR	Polymerase chain reaction
pI	Isoelectric point
PMSF	Phenylmethyl sulfonyl fluoride
RNase	Ribonuclease
r/m (rpm)	Rounds per minute
rRNA	Ribosomal ribonucleic acid
SCID	Severe combined immunodeficiency
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis

ssDNA	Single strand DNA
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
Trk molecules	Tyrosine receptor kinases
UV	Ultraviolet
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Acknowledgements

First of all, I would like sincerely to thank my Ph.D supervisors, Prof. Dr. W E. Trommer and Prof. Dr. J M. Yuan for encouraging me to finish my Ph.D project in their labs, and for providing patient guidance, numerous and excellent ideas, and continous efforts to revise my thesis. I am also indebted to Dr. P D. Vogel and Dr. J G. Wise for their great help on designing experiments, establishing methods and solving some problems in this project. In a word, their selflessness and invaluable supervision have made a deep impression upon me in my life.

I wish to thank Dr. Reinhard Philipp and Ms. Carolin Fluck for countless kind help during the past years.

I would thank my colleagues, Ya-Wei Shi for cloning the fragments synthesized chemically of gelonin gene; Elke Litmaianski for lots of technical supports and Martin Hossann for the test of protein synthesis inhibition.

I am very lucky to have all the colleagues in Prof.Trommer's lab. and Prof Yuan's lab. and get along very well with them. I would like to express my gratitude to them. In particular, Jun-Hu Fan, Ya-Mei Wang, Xiu-Qing Yang in Prof. Yuan's lab.

Finally, I am heartfully indebted to my husband, Han-Qing and my daughter, Jing-Jing for their deep understanding, wholehearted support and continuous encouragement in finishing my research project or my leaving for Germany.

The financial and moral supports from International Buro (BMSF) in Germany and Chemistry Department of Kaiserslautern University in Germany, as well as Department of Foreign Affairs and Biotechnol. Center of Shanxi University in China are greatfully acknowledged.

Curriculum Vitae

Personal Data

Name	Zhuoyu Li
Address	Wu-Cheng street 36, Taiyuan 030006, Shanxi, China
Birthday	17/12/1964
Nationality	China
Marital status	Married

Education background

09/72~07/77	Wu-Yi elementary school, Taiyuan, Shanxi province, China
09/77~07/80	Jingsan junior middle school, Taiyuan, Shanxi province, China
09/80~07/82	Jingsan high middle school, Taiyuan, Shanxi province, China
09/82~07/86	Department of Biology, Shanxi University, China. **Degree: B. Sc** Thesis: DNA sequence analysis of Spinach.
09/93~07/97	Biotechnol. Center, Shanxi University, China. **Degree: M. Sc** Thesis: Isolation, Purification and Bioactivity Assay of Human Nerve Growth Factor. Supervisor: Prof. Dr. Jingming Yuan
12/97~03/02	Department of Chemistry/Biochemistry, Kaiserslautern, Germany Biotechnol. Center, Shanxi University, Taiyuan, China. **Degree: Ph. D** Thesis: Immunotoxins and Neurotrophins: Novel Strategies for the Efficient Expression of Recombinant Proteins. Supervisors: Prof. Dr. Wolfgang E. Trommer Prof. Dr. Jingming Yuan