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Antitumor Toxic Protein Abrin and Abrus Agglutinin

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ABSTRACT *: Abrus agglutinin was purified from the kernels of Abrus precatorius by Sepharose 4B affinity column chromatography followed by Sephadex G-100 gel filtration column chromatography. About 1.25 g of abrus agglutinin was obtained from 1 kg of the kernels. The LD_{50} of abrus agglutinin is 5 mg/kg of body weight, which is less toxic than that of abrin, 20 μg/kg body weight. The amino acid sequence of abrus agglutinin was determined by protein sequencing techniques and deduced from the nucleotide sequence of a cDNA clone encoding full length of abrus agglutinin. There are 258 residues, 2 residues and 267 residues in the A-chain, the linker peptide and the B-chain of abrus agglutinin, respectively. Abrus agglutinin had high homology to abrin-a (77.8%). The 13 amino acid residues involved in catalytic function, which are highly conserved among abrin and ricin, were also conserved within abrus agglutinin. The protein synthesis inhibitory activity of abrus agglutinin (IC_{50} = 3.5 nM) was weaker than that of abrin-a (0.05 nM). By molecular modeling followed by site-directed mutagenesis showed that Pro199 of abrus agglutinin A-chain located in amphipathic helix H and corresponding to Asn200 of abrin A-chain, can induce bending of helix H. This bending would presumably affect the binding of abrus agglutinin A-chain to its target sequence GpApGpAp, in the tetraloop structure of 28S r-RNA subunit and this could be one of major factors contributing to the relatively weak protein synthesis inhibitory activity and toxicity of abrus agglutinin.*

I. INTRODUCTION

Plant *Abrus precatorius (Leguminous)* is widely growing in tropical and subtropical regions. The seeds are red with a black spot and they are often used as eyes in dolls and necklaces and various jewelry. The seeds contain abrus toxin which was first reported by Warden and Waddell (Warden and Waddel, 1884) and characterized as an "albumose" by Martin (Martin, 1887). Abrin was first isolated from the seeds of *Abrus precatorius* in this laboratory. The yield is about 1.2 g per kg of the seeds and the LD_{50} is about 20 μg/kg body weight (Lin *et al.*, 1981). The distribution of I^{131}-abrin administrated by i.p. injection was shown that the majority of I^{131}-abrin was located at rat liver and kidney (Lin and Tung, 1972). The purified abrin was shown to have a remarkable inhibitory effect on protein and DNA biosynthesis of rat liver but no effects on RNA biosynthesis and mitochondria respiration. It was shown that abrin as well as ricin inhibited the growth of experimental animal tumor when they i.p. administrated (Lin *et al.*, 1970). The possible inhibitory mechanisms of abrin or ricin were demonstrated that both toxins caused the irreversible protein biosynthesis inhibition of the experimental animal tumor cells (Lin *et al.*, 1970). Abrin, one of type II ribosomal inactivation proteins, consists of a toxophoric A chain linked to a cell-binding B chain by a disulfide bond. The B chain, a lectin, binds to cell surface glycoproteins with D(+)-galactopyranose moieties (Olsnes *et al.*, 1974), while A-chain has a N-glycosidase activity that cleaves the C-N glycosidic bond of adenine residue at 4324 of rat 28S rRNA (Endo *et al.*, 1987). Depurination of this site inhibits the binding of the elongation factor 2 to abrin treated ribosomes (Jimenez and Vazguez, 1985). The toxicity of abrin is so high that a single molecule of abrin penetrates into the cells and produces the cell death (Eilclid *et al.*, 1980).

Four isoabrins, abrin-a,-b,-c and -d were isolated from *A. precatorius* and among them, abrin-a is the most toxic (Lin *et al.*, 1981). The cDNA sequences of abrin-a,-b and -d were cloned (Hung *et al.*, 1993), and cDNA of the abrin A-chains was expressed in *Escherichia coli* (Hung *et al.*, 1994). The recombinant abrin A-chains were shown to be active against

the protein biosynthesis of rabbit reticulocyte cell-free system (Hung *et al.*, 1994). Tertiary structure of abrin-a was elucidated by x-ray crystallographic analysis at 2.14 Å resolution (Tahirov *et al.*, 1995). The alignment of amino acid sequences of abrin a A-chain with other ribosomal inactivating proteins (RIPs) revealed that a high degree of sequence homology among the RIPs (Funatsu *et al.*, 1991) and that the conserved amino acid residues clustered together around the well-defined cleft of active site were Tyr74, Tyr113, Glu164, Arg167 and Trp198. By site-directed mutagenesis approaches were employed to study the functions of three conserved amino acids and the results indicated that Arg 167 and Glu 164 are essential for abrin a A-chain catalysis (Hung *et al.*, 1994). Furthermore, Trp198 was found to be important for the reassociation of abrin-a A-chain with B-chain to form intact abrin-a (Chen *et al.*, 1997).

II. PURIFICATION AND PRIMARY STRUCTURE OF ABRUS AGGLUTININ

1. Purification of Abrus Agglutinin

200 grams of kernels of *A. precatorius* was solaced in 1 of acetic acid at 4°C, overnight and then homogenized with a Waring blender. The homogenate was centrifuged at 10,000 g at 4°C for 20 min. The proteins in the supernatant were fractionated with ammonium sulfate and the proteins precipitated between 30 and 90% saturation of ammonium sulfate were collected by centrifugation and dialyzed against 10 mM phosphate buffer, pH 8.0 at 4°C for 2-day with six/changes of the buffer. The supernatant of dialyzate was applied to a Sepharose 4B column (3.0×50 cm) which was pre-equilibrated with 10 mM phosphate buffer, pH 8.0. The column was then eluted with the same buffer until absorbance at 280 nm of the eluent returned to base line, 0.1 M D-galactose in the buffer was then applied for the elution of bound lectins (Fig. 1). The fractions containing proteins were pooled and then applied to a Sephadex G-100 column which was equilibrated with 10 mM phosphate buffer. The column was eluted with the same buffer and two protein peaks were obtained. The first peak contains abrus agglutinin while the second peak, abrin (Fig. 2). From 200 grams of the kernels of *A. precatorius*, 450 mg of

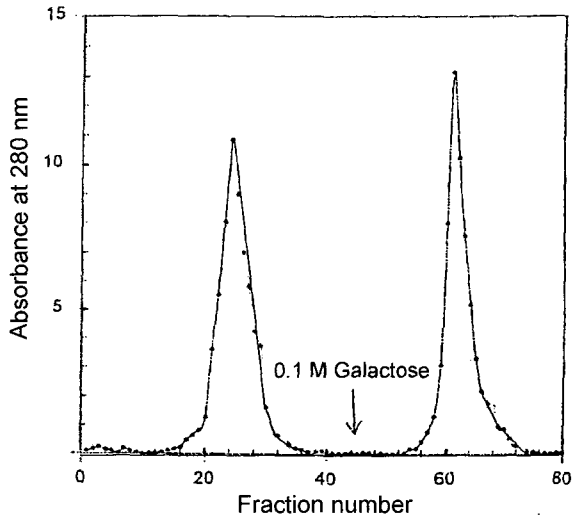

Fig. 1. Purification of abrus agglutinin by Sepharose 6B affinity chromatography. Crude extracts were applied to a Sepharose 6B column (3.0×50 cm), prequilibrated with 10 mM phosphate buffer, pH 8.0. The column was first eluted with 10 mM phosphate buffer, pH 8.0, and then with 0.1 M galactose in 10 mM phosphate buffer, pH 8.0. Each fraction of 5 ml was collected and the flow rate was 20 ml/h.

Abrus agglutinin was obtained. The LD_{50} of abrin is 20 μg/kg body weight, while that of Abrus agglutinin, 5 mg/kg body weight.

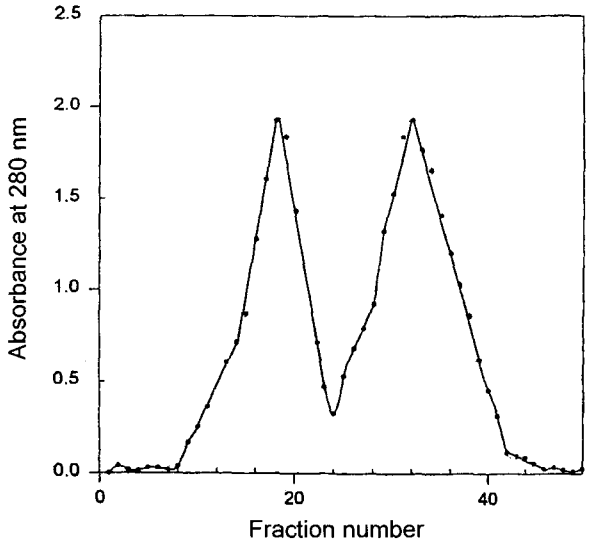

Fig. 2. Purification of abrus agglutinin by gel filtration chromatography. The fractions eluted with 0.1 M galactose by Sepharose 6B affinity column chromatography were applied to a Sephadex G-100 column (2.2×100 cm) which was equilibrated with 10 mM phosphate buffer, pH 8.0. The column was eluted with 10 mM phosphate buffer, pH 8.0. Each fraction of 5 ml was collected and the folw rate was 20 ml/h. The first protein peak is aburs agglutinin and the second peak, abrin.

2. Separation of A and B Subunits of Abrus Agglutinin

10 mg of abrus agglutinin was reduced with 5 % b-mercaptoethanol in 8 M urea, 10 mM Tris-HCl buffer, pH 8.6 at 37°C for 16 h. The reaction products was loaded onto a Sephadex G-150 column (3.0×100 cm) and eluted with 10 mM Tris-HCl buffer, pH 8.6. Two protein peaks were obtained, and the first peak is the A-chain of abrus agglutinin while the second peak, the B-chain of abrus agglutinin. From 10 mg of abrus agglutinin, 1.5 mg of A-chain and 2.5 mg of B-chain were obtained (Fig. 3). The N-terminal amino acid sequences of the A-and B-chain of abrus agglutinin were determined with an ABI 476A amino acid sequencer, and the N-terminal amino acid sequences of abrus agglutinin A- and B-chain were as following.

A-chain: Q D P I K F T T G S A T

B-chain: V V E Q S K I C S S

3. Determination of Amino Acid Sequence of Abrus Agglutinin

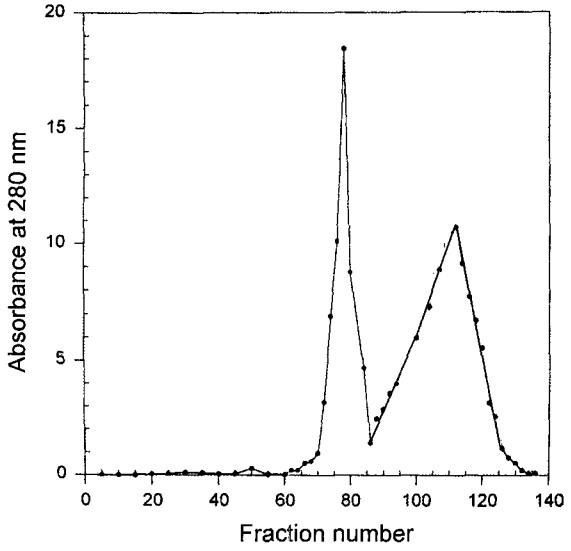

Fig. 3. Separation of subunits of abrus agglutinin. Aburs agglutinin was reduced with 5% 2-mercaptoethanol in 10 mM Tris-HCl buffer, pH 8.6 containing 8 M urea at 37°C for 12 h. The reaction products were fractionated with a Sephadex G-150 column (3.0×100 cm) pre-equilibrated and eluted with 10 mM Tris-HCl pH 8.6 containing 6 M urea and 1% 2-mercaptoethanol.Each fraction of 1 m*l* was collected and flow rate was 4 ml/h. The first protein peak is A-chain and the second peak, B chain.

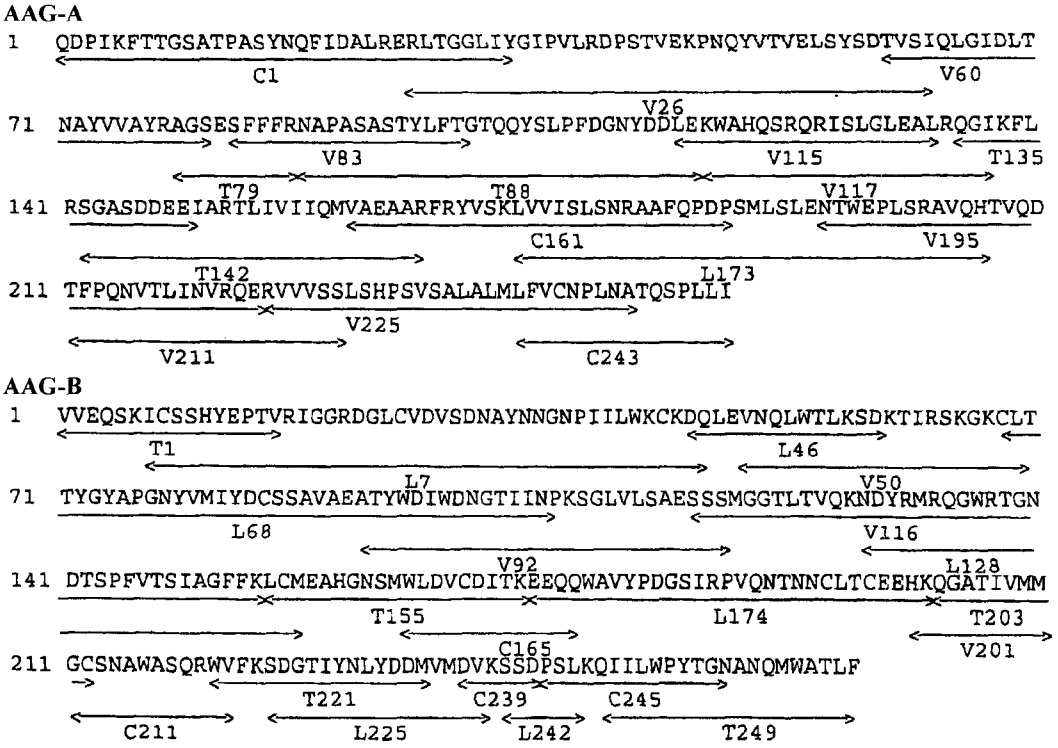

Fig. 4. Complete amino acid sequence of abrus agglutinin. The peptides generated by trypsin, S *aureus* V8 endoproteinase, or Lys-C endoproteinase digestion and cyanogen bromide cleavage of abrus agglutinin were purified by HPLC. The amino acid sequences of purified peptides were determined with an ABI 476A amino acid autoseqencer. The designation of peptides are as following: T, trypsin; L, Lys-C endoproteinase; V. S. *aureus* V8 endoproteinase; and C, cyanogen bromide.

The complete primary structure of abrus agglutinin was determined by sequencing the peptides generated by Lys-C endo-peptidase, *S.aureus* V-8 endoproteinase and L-l-tosylamido-2-phenylethyl chloromethyl ketonetreated trypsin digestion and cyanogen bromide treatment (Fig. 4). Abrus agglutinin is composed of 525 amino and residues : 258 residues in A-chain, 267 residues in B-chain. Alignment of amino acid sequences of abrus agglutinin and abrin-a -b and -d showed high homology. There are 27 similar amino acid residues (10.8%) and 168 identical residues (66.9%) between the A-chains of abrus agglutinin and abrin-a while B chain had 17 similar amino acid residues (6.4%) and 214 invariant residues (80.2%) (Fig. 5). There are 13 amino acid residues at the proposed active site, including two catalytic residues, Gln163 and Arg166, which are highly conserved among type I and type II Rips (Funatsu *et al.*, 1991), and these 13

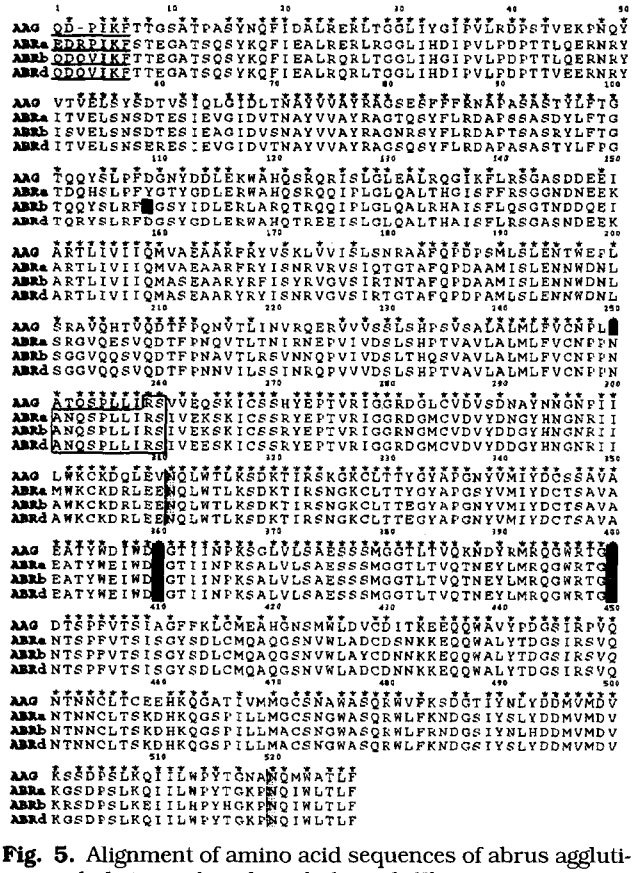

Fig. 5. Alignment of amino acid sequences of abrus agglutinin and abrin-a, abrin-b and abrin-d. The invariant amino acid residues are marked by asterisks. Sites of potential asparagine-linked N-glycosylation are shown in black. The linkers between A and B chains are boxed. The six or seven N-terminal amino acid residues are underlined.

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AAG   LFVCNPLNATQ -S PLL I RS VV - - EQSKC IS S HY
ABRa  LFVCNPPNANQ -S PLLI RS I V - - E ESKC IS S RY
ACTI  FKI KPYRDDYKLVYCEGNSDDES CKDLG IS I DD
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Fig. 6. Alignment of amino acid sequences of linker regions of three plant proteins. The linkers are underlined, AAG; abrus agglutinin; ABRa, abrin a and ACTI, *Acacia confusa* trypsin inhibitor.

residues were completely conserved within AAG-A. Two putative galactose binding residue, Asn51 and Asn260 were present in abrus agglutinin B-chain. B-chain of abrus agglutinin has two putative N-linked oligosaccharide glycosylation sites at Asn100 and Asn140. There is a new glycosylation site, Asn250 in abrus agglutinin, not present in the B-chain of abrin-a. It is interesting to note that the internal linker peptide of abrus agglutinin is a dipeptide which is removed during the post-translation modification of primary translation product by proteolysis whereas abrin-a, -b and -d have a decapeptide as linker peptide (Hung *et al.*, 1992). This difference could be due the new putative N-glycosylation site at the Asn 250 in the A-chain of abrus agglutinin, which could cause the steric hindrance by oligosaccharide at this putative glycosylation site, Ans250-Ala251-Thr252 and could render abrus agglutinin A-chain resistance to proteolytic digestion at Asn250-Ala251 during post-transnational processing. Several plant lectins and tyrpsin inhibitors have been reported to share similar post-translational processing. The C-terminal cleavage site of abrus agglutinin is cleaved at Ser260-Val261 which is the same as those of abrin-a and *Acacia confusa* trypsin inhibitor where as the N-terminal ceavage site differs from those abrin-a and *A. confusa* trypsin inhibitor (Fig. 6).

III. MOLECULAR CLONING AND SITE-DIRECTED MUTAGENESIS OF ABRUS AGGLUTININ

1. Molecular Cloning of Abrus Agglutinin

Total cellular RNA was extracted from 15 g of fresh maturing seeds of *A. precatorius* by guanidium thiocyanate procedure and 275 mg of total RNA was obtained (Sambrook *et al.*, 1989). The poly (A^+) RNA rich fraction was abstained by oligo(dT)-cellulose column chromatography (Kaku *et al.*, 1996). The poly (A^+)-rich m-RNA was reverse-transcribed with the

Marathon cDNA amplification kit. The resulting cDNAs ligated to Marathon adaptors for 5' and 3'-RACE. For the first PCR, AAG cDNA, was amplified with sense degenerate primer A and antisense degenerate primer B, corresponding to amino acids 119-127 and 222-228 of the B-chain of abrus agglutinin, respectively. The amplified product was sequenced and used to design the specific antisense primer GSP-1, corresponding to amino acids 129-135 of AAG-B. GSP-1 was then used with the sense degenerate primer C, derived from amino acid 1-8 of the A chain of abrus agglutinin, for the second PCR. The products of second PCR step was sequenced and used to design the specific antisense primer, GSP-2, corresponding to 11-17 of the A-chain of abrus agglutinin. In the third PCR step, GSP-2 was used along with the Marathon primer AP-1. The product of this step was used as template for the fourth PCR step, in which GSP-2 was used along the sense primer AP-2 to yield the 5' end of abrus agglutinin cDNA. For 3' end of abrus agglutinin cDNA was obtained by amplification of a specific sense primer, GSP-3 corresponding to amino acid 174-182 of the chain of abrus agglutinin and Marathon primers of AP-1 and AP-2 in consecutive PCR steps. The full length cDNA of abrus agglutinin was obtained by amplifying *A. precatorius* cDNA with the sense primer GSP-4, which encodes the first sense N-terminal amino acid residues of the A-chain of abrus agglutinin and antisense primer GSP-5 which encodes the last eight C-terminal amino acid residues and the stop coden of the B-chain abrus agglutinin. The PCR product was then ligated into the T vector pTAAG cDNA (Fig. 7 and Table 1).

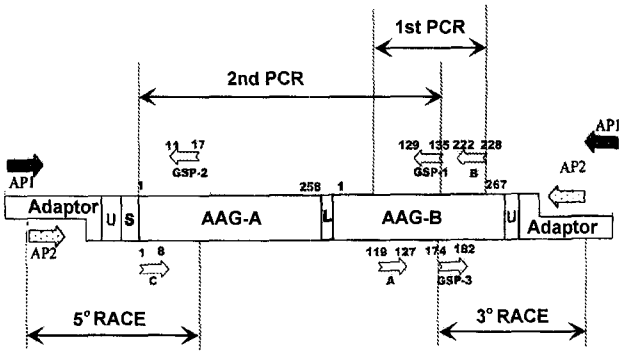

Fig. 7. cDNA cloning of abrus agglutinin. The schematic diagram shows the cloning strategy for abrus agglutinin cDNA. U, untranslated region; S, signal peptide and L, linker.

Sequence analysis abrus agglutinin cDNA showed that it contained 2047 base pairs, with an open rending frame encoding a preproprotein with 547 amino acid residues : a 20-residues signal peptide, a 258-residues the A-chain, a 2-residues linker peptide, a 267-residue the B-chain. The complete amino acid sequence of abrus agglutinin deduced from the nucleotide sequence of abrus agglutinin cDNA was identical to that determined by the protein sequencing techniques except that there were two extra residues, Arg259 and Ser260, which is the internal linker bet-

Table 1. The sequences of the primers used for cloning AAG cDNA and site-directed mutagenesis

Primer	Sequence
Primer-A	5' A T G G G A G G A A C A T T A A C A G T A C A A A A 3'
	T T T C T T T G
	C C C C C C
	G G G G G G
Primer-B	5' A G T A C C A T C A C A C T T A A A A A A C 3'
	T T G T G T T G T
	C C C C C
	G G G
AP-1	5' C C A T C C T A A T A C G A C T C A C T A T A G G G C 3'
AP-2	5' A C T C A C T A T A G G G C T C G A G C G G C 3'
GSP-1	5' G C C C T G T C G C A T T C G A T A A T C 3'
GSP-2	5' A T T G T A G C T T G C T G G A G T G G 3'
GSP-3	5' G A A G A G C A G C A A T G G G C A G T T T A C C C 3'
GSP-4	5' C A A G A C C C C A T T A A G T T T A C G 3'
GSP-5	5' T T A A A A C A A A G T A G C C C A C A T T T G G T T 3'
GSP-6	5' G G G A T C C C A A G A C C C C A T T A A G T T T A C G A C T 3'
GSP-7	5' A G A A T T C T T A T A T T A A G A G A G G T G A T T G G G 3'
GSP-8	5' C A C A T G G G A A A A C T T G T C A A G A G C 3'
GSP-9	5' A A T T G G G A C C C T C T G T C A C G A G G 3'

ween the A-and B-chains of abrus agglutinin. These results suggest that the precursor synthesized from the open reading frame of the abrus agglutinin mRNA is post-translationally cleaved into A-and B-chains which are linked by a disulfide bond.

2. Expression of Abrus Agglutinin in *E. Coli*

Abrus agglutinin expression vector was constructed by amplifying pTAAG cDNA with the sense primer GSP-6, which encodes the first seven N-terminal amino and residues of the A-chain of abrus agglutinin behind a *Bam*H1 restriction site, and the antisense primer GSP-7, which encodes the last seven amino acid residues of the A-chain with a stop codon following an *Eco*RI restriction site. The amplified *Bam*H1-*Eco*RI fragment was ligated to pGEX-2T to obtain the expression vector pGEX-AAG-A (Smith and Johnson, 1988). For expression, the expression vector was introduced into *E.coli* TG-1 by $CaCl_2$-mediated transformation. The cultured cells were grown to a cell density 4×10^8 cells/ml at 37°C and then induced with 0.5 mM isopropyl-this-β-D-galactoside at 30°C for 3 h. The cells were treated with lysozyme (0.2 mg/ml) and lysed by freeze/thawing trice. The cell lysate was treated with DNase and the supernatant was applied to a glutathione-Sepharose 4B column (2 ml). The column was washed with 10 mM phosphate buffer and then eluted with 50 mM Tris/HCl, pH 8.0 containing 5 mM glutathione to obtain glutathione S-transferase-abrus agglutinin A-chain fusion protein. The fusion proteins were treated thrombin to liberate the recombinant A-chain of abrus agglutinin which was purified with a Mono Q column (1.6×50 mm). The yield of recombinant abrus agglutinin A-chain was 1.0 mg/liter of induced culture, and the recombinant A-chain was shown to be homogeneous upon analysis by 10% SDS-PAGE, with an estimated molecular mass of 29 KDa (Fig. 8). The recombinant A-chain was demonstrated to be as active as native A-chain of abrus agglutinin and the IC_{50} of the recombinant A-chain was found to 3.5 nM which is the same as that of native A-chain as shown by their inhibitory effects on the protein biosynthesis of rabbit reticulocyte cell free system (Fig. 9). By molecular modeling of the A-chain of abrus agglutinin (Peitsch, 1995; 1996; Guex and Peitsch, 1997), one of the putative

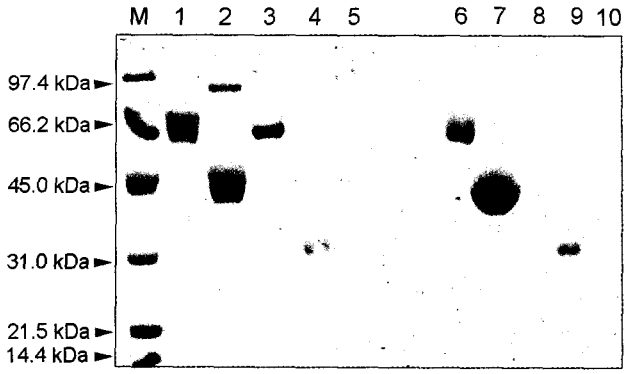

Fig. 8. SDS-PAGE analysis of native and recombinant abrus agglutinin. Samples of purified proteins were analyzed by 10% SDS-PAGE and Coomassie Blue staining. Lane 1. Abrus agglutinin; Lane 2, molecular mass markers (phosphorylase b, 97.4 kDa, serum albumin, 66.2 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21.5 kDa and lysozyme, 14.4 kDa); Lane 3, abrus agglutinin reduced with 2-mercaptoethanol; Lane 4, abrus agglutinin B-chain; Lane 5, abrus agglutinin A-chain; Lane 6, recombinant abrus agglutinin A-chain; Lane 7, recombinant abrus agglutinin P199N; Lane 8, recombinant abrus A-chain; Lane 9, recombinant A-chain N200P.

substrate binding sites of the A-chain of abrin a, Asn200, which corresponding to Arg213 of ricin A-chain (Monzingo and Robertus, 1992), was substituted with Pro199 in the A-chain of abrus agglutinin. By site-directed mutagenesis, expression plasmids carring abrus agglutinin A-chain P199N and abrin-a

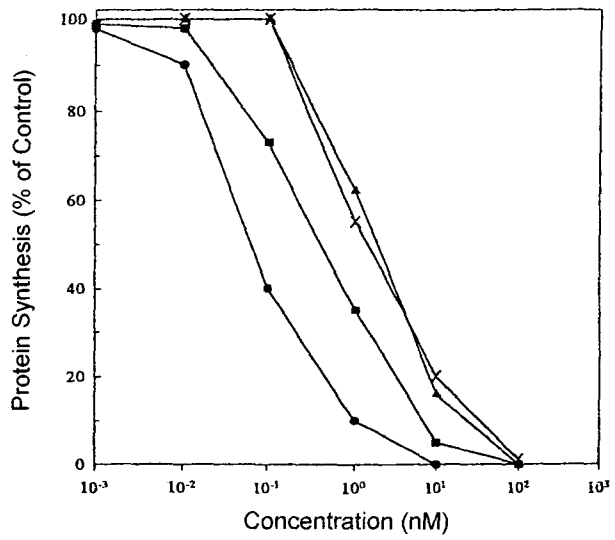

Fig. 9. Effects of recombinant abrus A-chain and recombinant abrus agglutinin A-chain and their mutants in protein synthesis. The inhibitory effects of recombinant proteins on protein biosynthesis were measured with the rabbit reticulocyte system, recombinant abrin a A-chain; recombinant abrus agglutinin A-chain; X, recombinant abrin a A-chain N200P; recombinant abrus agglutinin A-chain P199N.

tory effection the protein biosynthesis. The recombinant P199N of abrus agglutinin A-chain P199N (IC_{50} = 0.53 nM) was 7-fold more potent than wild type abrus agglutinin A-chain where as recombinant abrin a A-chain N200P (IC_{50} = 2.3 nM) was 46-fold less potent than wild type abrin a A-chain (Fig. 9). Since Arg213 of ricin, corresponding to Asn200 of abrin A-chain, is required for binding to the GpApGpAp sequence of the tetraloop located at the 3'-terminal region of 28 s rRNA (Monzingo and Robertus, 1992), the substitution of this residue in abrus agglutinin A-chain Pro199 could cause bending of amphipathic helix H of abrus agglutinin A-chain thus hindering the interaction between abrus agglutinin A-chain and its substrate at the centered GpApGpAp sequence. This suggests that Asn200 of abrin-a A-chain is important for its inhibitory activity (Lin *et al.*, 2000).

IV. TOXICITY AND ANTITUMOR ACTIVITY OF ABRUS AGGLUTININ A-CHAIN AND ABRIN A-CHAIN

Abrus agglutinin and abrin a have similar therapeutic indexes for the treatment of experimental mice with tumor (Lin *et al.*, 1981). Since abrus agglutinin is less toxic than abrin-a, it suggests that abrus-agglutinin could an ideal and better protein for the preparation of immunotoxin for the clinical treatment of cancer.

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