Molecular Cloning of Phospholipase A2 from a Thai Russell's Viper Venom Gland cDNA Library

ISSARANG NUCHPRAYOON, M.D., Ph.D.*,
SUNUTCHA SUNTRARACHUN, M.Sc.**,
NARUMOL PAKMANEE, M.Sc.**,
SURANG NUCHPRAYOON, M.D., Ph.D.*,
ARKHOM SAI-NGAM, M.Sc.*,
JUREEPORN NOIPHROM, B.Sc.**,
LAWAN CHANHOME, D.V.M.**,
VISITH SITPRIJA, M.D., Ph.D.**

Abstract

Snake venom contains several toxins. Russell's viper (*D. russellii*, RV) is a venomous snake prevalent in northern and central Thailand. RV bites can cause disseminated coagulation, hemolysis, and edema of the bitten limbs. To identify protein components of RV venom, we made a cDNA library from RV venom glands, and randomly sequenced cloned cDNA. We were able to clone a cDNA encoding RV phospholipase A2 (PLA2). PLA2 is an active enzyme found in several species of snake venom worldwide. PLA2 is thought to be toxic to cell membrane, thereby, can cause local cell and tissue damage, as well as systemic effects in snake bite victims. This PLA2 cDNA clone would facilitate *in vivo* studies of the pathophysiology of RV bite.

Key word : Russell's Viper Venom, Phospholipase A2, Molecular Genetics

NUCHPRAYOON I, SAI-NGAM A, SUNTRARACHUN S, et al
J Med Assoc Thai 2001; 84 (Suppl 1): S99-S105

Snake bite is a common medical emergency in Thailand and Southeast Asia. Russell's viper (*Daboia russellii*, RV, formerly called *Vipera russelli*) is a venomous snake prevalent in northern and central Thailand as well as in Myanmar, India, Sri Lanka, China, Taiwan, and Indonesia. Russell's viper bites can cause disseminated intravascular coagulation (DIC), hemolysis, and severe necrosis and edema of the bitten limb. People bitten by a RV who show signs of coagulopathy must be promptly treated with anti-venom(1).

RV is widely distributed in East and Southeast Asia. At least 5 subspecies of RV have been recognized based on minor differences in color and

* Department of Pediatrics, Faculty of Medicine, Chulalongorn University, Bangkok 10330,
** Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok 10330, Thailand.

markings; *D. russellii russellii* in India, *D. russellii pulchella* in Sri Lanka, *D. russellii siamensis* in Myanmar, Thailand, and China, *D. russellii formosensis* in Taiwan, and *D. russellii limitis* in Indonesia(2). Coagulation factor X activator protein has been identified and believed to be the key component that causes DIC(3).

Snake venom is a mixture of several proteins, many of which are toxins. Although a few proteins in RV venom have been identified, most components have not been characterized. Purified components may be useful in studying and developing monoclonal antibodies for diagnosis and/or treatment of RV bites.

To identify protein components of RV venom, we made a cDNA library from RV venom glands, and randomly determined DNA sequences of the cloned cDNAs. We identified a cDNA clone encoding a full-length phospholipase A2, and compared it with the other RV PLA2.

MATERIAL AND METHOD

Animal specimens

Two adult Russell's vipers, *D. russellii siamensis*, weighing 0.70 and 1.16 kg, were obtained from Angthong province, Thailand.

Poly (A)+ RNA isolation

Four venom glands (0.21, 0.24, 0.38 and 0.40 g) were dissected from the two RVs under ether anesthesia and kept immediately in liquid nitrogen until use. Total RNA was isolated by TRIzol LS reagent (Gibco BRL, Grand Island, NY) according to manufacturer's recommendation. A yield of 800 µg of total RNA was then purified for poly (A)+ RNA by PolyAT Tract system (Promega, Madison, WI) according to the manufacturer's recommendation. A yield of poly (A)+ RNA was approximately 8 µg.

cDNA library construction

A cDNA library was constructed using ZAP express cDNA Synthesis Kit and ZAP express cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendation. Briefly, 5 µg of poly (A)+ RNA was used as substrate to construct the double-stranded cDNA. The cDNA was then modified for ligating to the vector arms. The ligated DNA was packed with packaging extract to generate the infectious phage particle.

cDNA identification

For excision of the phagemid, the XL1-BLUE MRF' cells were coinfected with an aliquot of the cDNA library and the ExAssist interference-resistant helper phage. The pBK-CMV excised phagemid was plated in freshly grown XLOLR cells in LB-kanamycin agar plates (50 µg/ml) and allowed to grow overnight at 37°C. Plasmid DNA was isolated by alkaline miniprep(4) and double-digested with *EcoR* I and *Xho* I (New England Biolabs, Beverly, MA) for 2 hours at 37°C. The product was analyzed by 1.2 per cent agarose gel electrophoresis. Ten randomly chosen plasmids carrying cDNA inserts were subjected to DNA sequencing using ABI Prism 310 Genetic Analyser (Perkin-Elmer, Norwalk, CT). The sequencing was performed in a 5' direction using T3 sequencing primer. For RVV012, the sequencing was performed in both directions using T3 and T7 primers.

Sequence analysis

The cDNA sequences and predicted amino acid sequences were compared to the sequences in the GENBANK database using BLAST program (5). The sequence alignments were performed using CLUSTAL X program.

RESULTS

We successfully made a cDNA library from *D. russellii siamensis* venom gland. Despite our efforts to use as few snakes as possible, it needed 4 venom glands to obtain adequate RNA for library construction. These two snakes were verified by a taxonomist to be *D. russellii siamensis* from the same region. The constructed cDNA library contained approximately 1.0×10^6 plaque-forming units per µg of vector arms. Ten cDNA clones which were randomly picked for a preliminary screening of the DNA sequence of RV cDNA library contained cDNA inserts varying in length from 0.6-2.0 kb (average length = 1.1 kb). The identification of each clone is shown in Table 1. One clone, RVV012, with 610 bp in length showed a strongly significant homology to the PLA2 gene of those from several snake venoms. RVV012 possesses 60 nucleotides of 5' untranslated region

Table 1. Identification of ten clones picked randomly from RV cDNA library.

Clone	Approx. length (kb)	Homologous sequences (expected value)[a]
RVV003	0.9	(X78971)[b] mettalloprotease, *Echis pyramidum* (2.4)
RVV005	1.3	(AF155739)[b] axotrophin, *Mus musculus* (6e-44)
RVV009	1.1	No significant homology found
RVV010	1.4	No significant homology found
RVV011	0.8	No significant homology found
RVV012	0.6	(S29299)[b] phospholipase A2, *D. russellii* (0.0)
RVV013	1.3	No significant homology found
RVV015	1.1	(A42972)[b] coagulation factor X activating enzyme heavy chain, *D. russellii* (0.0)
RVV016	1.0	(AL353819)[b] related to carboxylic and transport protein JEN1, *Neurospora crassa* (4.0)
RVV020	2.0	(AK023676)[b] unnamed protein product, *Homo sapiens* (9e-13)

Note : [a] The homologous sequences represent the highest score when performed BLASTX search.
[b] GENBANK accession number.

(UTR), an open reading frame encod 138 amino acid residues of PLA2 including the first 48 nucleotides of signal peptide, and 111 nucleotides of 3'-UTR. The nucleotide and predicted amino acid sequences of RVV012 are shown in Fig. 1.

The predicted 138 amino acid residues of RVV012 were 100 per cent identical to a previously reported PLA2 of *D. russellii formosensis* (GENBANK accession # S29299) which was isolated from a Taiwan RV (Fig. 2A, f1). Comparison of amino acid sequences of PLA2s among *D. russellii* subspecies (ssp. *formosensis*, ssp. *russellii* and ssp. *siamensis*) showed a highly significant homology (Fig. 2). In addition to f1, RVV012 is significantly homologous to other reported *D. russellii formosensis* PLA2s (96/138 = 69% identity, Fig. 2A, f2) and *D. russellii russellii* (81/121 = 67% identity, Fig. 2A, r1). Partial amino acid sequences of RVV012 was identical to one of six reported PLA2s of *D. russellii siamensis* obtained from N-terminal sequencing (Fig. 2B, S4).

In addition to RVV012, we identified a clone (RVV015) encoding part of the coagulation factor X activating enzyme heavy chain gene. Another cDNA clone (RVV003) was remotely homologous to a metalloprotease from an African viper, *Echis pyramidum*. The remaining seven clones have no significant homology to known snake venom components (Table 1).

DISCUSSION

Although DIC and renal failure are common findings among all RV bites, certain clinical findings vary among geographical regions[2]. RV bites in India and Sri Lanka also have prominent neurological symptoms, with ptosis and external ophthalmoplegia[1]. RV bites in Myanmar often present with conjunctival edema and other signs of vascular leakage[8]. Some of these findings may be explained by variations in phospholipase A2 (PLA2) in RV venom from different subspecies.

PLA2 is an enzyme that can catalyze phospholipid hydrolysis to produce free fatty acid and lysophospholipid. PLA2 has been found in reptiles and mammals of several species, and has been classified in several groups based on molecular weight, amino acid sequence and homology, calcium dependence, and cellular localization[9]. Low molecular weight (~14 kilodalton) snake venom PLA2s has been classified into two groups. Calcium-dependent group I PLA2s is found in cobras and kraits, while calcium-independent group II PLA2s is found in vipers.

PLA2 is a major constituent of RV venom, up to 70 per cent of dry weight in some specimens [2]. RV PLA2 has been isolated biochemically from Indian *D. russellii russellii* and 7 isozymes have been identified. A purified major component of *D. russellii russellii* venom PLA2, VRV-PL-VIIIa, is a basic protein that causes neurotoxic symptoms, as well as myonecrosis when intramuscularly injected into mice[10]. VRV-PL-VI induces only edema when injected in mouse foot pad[11]. VRV-PL-V also causes neurotoxic symptoms[12]. VRV-PL-IIIb has an anticoagulant effect through inhibition of platelet aggregation[13]. Of these *D. russellii russellii* PLA2s, partial protein sequence has been available for VRV-PL-VIIIa[14]. Monoclonal antibodies against VRV-PL-VIIIa have been shown to

```
  1 ggtgcttctgaaccccttcaactctgagaaaaggctgccaactgtctggattcaggagg
 61 ATGAGGACTCTCTGGATAGTGGCCGTGTGCCTGATAGGCGTTGAAGGGAACCTTTTCCAG
    M  R  T  L  W  I  V  A  V  C  L  I  G  V  E  G  N  L  F  Q
121 TTTGGGGAGATGATCTTGGAAAAGACGGGGAAAGAAGTTGTTCATTCCTACGCCATTTAC
    F  G  E  M  I  L  E  K  T  G  K  E  V  V  H  S  Y  A  I  Y
181 GGATGCTACTGCGGCTGGGGAGGCCAAGGCAGGGCACAGGACGCCACCGACCGCTGCTGC
    G  C  Y  C  G  W  G  G  Q  G  R  A  Q  D  A  T  D  R  C  C
241 TTTGTGCACGACTGCTGTTACGGGACAGTGAATGACTGCAACCCCAAAACGGCCACCTAT
    F  V  H  D  C  C  Y  G  T  V  N  D  C  N  P  K  T  A  T  Y
301 TCCTACAGCTTTGAGAACGGGGATATCGTCTGCGGAGACAACGACCTGTGCCTGAGGACT
    S  Y  S  F  E  N  G  D  I  V  C  G  D  N  D  L  C  L  R  T
361 GTTTGTGAGTGTGACAGGGCCGCGGCAATCTGCCTTGGACAGAATGTGAATACATACGAC
    V  C  E  C  D  R  A  A  A  I  C  L  G  Q  N  V  N  T  Y  D
421 AAAAACTATGAGTACTACTCAATCTCTCATTGCACGGAGGAGTCAGAGCAATGCTAAgtc
    K  N  Y  E  Y  Y  S  I  S  H  C  T  E  E  S  E  Q  C  *
481 tctgcaggacgggaaaaagccctccaattacacaattgtggttgtgctactctattattc
541 tgaatgcaatactgagcaataaacaggtgccagctctgcactaaatcgaaaaaaaaaaaa
601 aaaaaaaaaa
```

Fig. 1. The nucleotide and predicted amino acid sequences of clone RVV012. 5' untranslated region (UTR) and 3'-UTR are shown in the lowercase letters, the coding sequences are shown in the uppercase letters. The uppercase letters (bold) indicate amino acid sequences. Underlined sequence is the signal peptide, * represents stop codon.

inhibit toxicities of the whole *D. russellii russellii* venom to some degree[14] suggesting that PLA2s are important components of venom from this subspecie.

Our clone of PLA2, 610 bp in length is the most complete report of PLA2 cDNAs to date. The 5'-UTR (60 bp) and 3' UTR (111 bp) of RVV012 is longer than the report by Tsai[7] (15 bp 5'-UTR and 12 bp 3'-UTR, GENBANK accession # X68386). PLA2's cDNA-predicted amino acid sequences of RVV012 is homologous to all reported PLA2 protein sequences from ano-

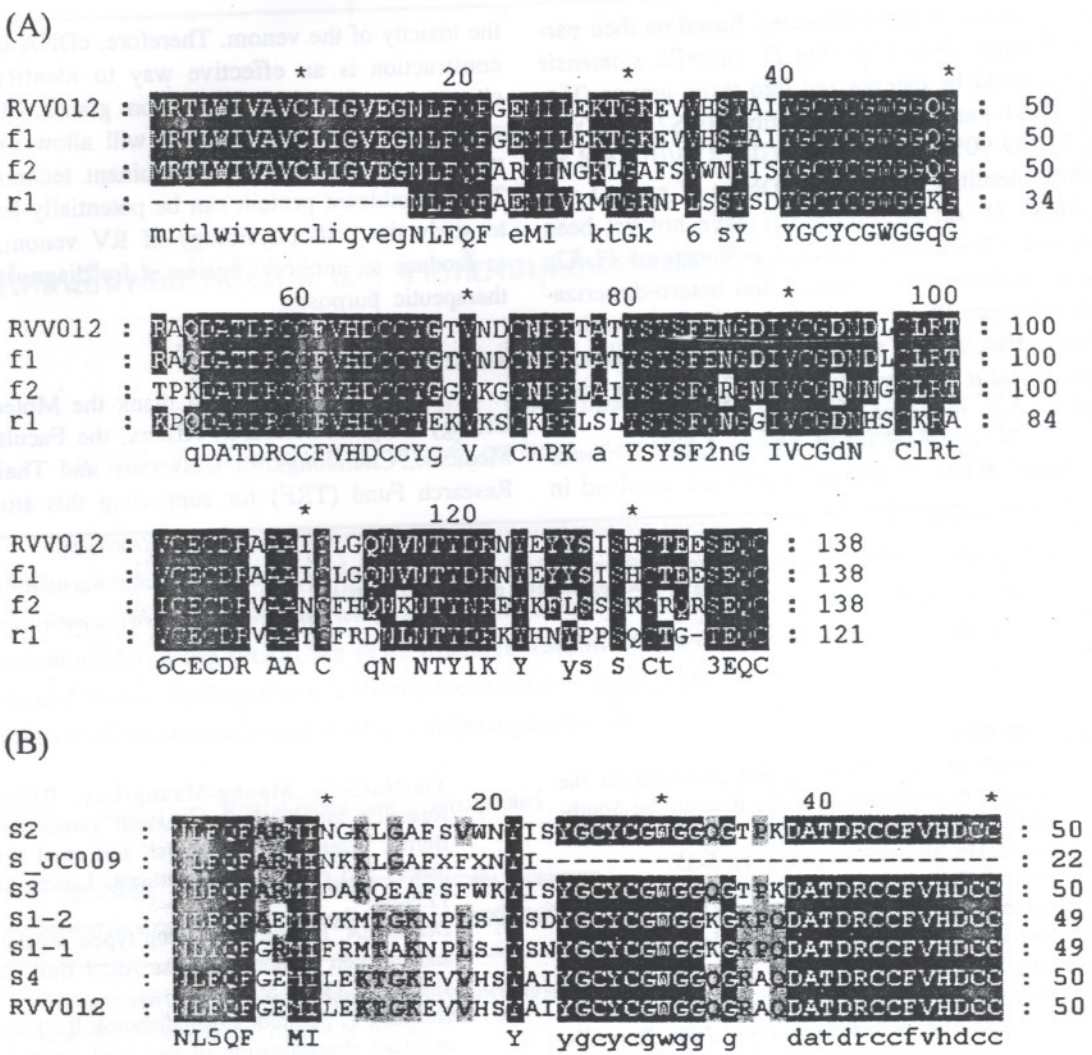

Fig. 2. Comparison of *D. russellii*'s PLA2s. (A) Aligment of mature PLA2's cDNA-predicted amino acid sequences of RVV012, f1; *D. russellii formosensis* GENBANK accession # S29299, f2; *D. russellii formosensis* GENBANK accession # S29299, r1; *D. russellii russellii* GENBANK accession # 2392602. (B) Alignment PLA2's cDNA-predicted amino acid sequences of RVV012 and N-terminal sequencing sequences of various *D. russellii siamensis* mature PLA2s superscript (>) and S_JC0009; GENBANK accession # JC0009.

ther RV subspecies (Fig. 2) suggesting similar functions. Geographical variation of clinical effects could be explained by some of these differences between PLA2 of Thai *D. russellii siamensis* and Indian *D. russellii russellii*. Complementary DNA sequence of *D. russellii siamensis* PLA2 in this study has been found to be identical to a neurotoxic cDNA of Taiwan viper (*D. russellii formosensis*) PLA2[6]. However, since *D. russellii formosensis* PLA2 has been reported to be neurotoxic[6], it does not explain the lack of neurotoxicity in Thai *D. russellii siamensis* RV bites. It is possible that neurotoxicity of PLA2 in mice does not predict neurotoxicity in man or *D. russellii formosensis* venom neurotoxicity could be due to other protein components than PLA2.

There are several PLA2 sequences obtained from each RV subspecies N-terminal amino acid sequencing[7]. These findings suggest that there are several PLA2 genes that arise from gene duplication

among snakes in this subspecies. Based on their partial primary sequences, the *D. russellii siamensis* PLA2 could be categorized into three groups (Fig. 2B); (I) S1-1 and S1-2 (83% identity), (II) S2, S_JC0009 an S3 (89-90% identity), and (III) RVV012 and S4 (100% identity). Full-length cDNAs of group I and II PLA2 *D. russellii siamensis* have not yet been reported. Conceivably, several isoforms of PLA2s could be derived from homo- and hetero-dimerization of these PLA2 proteins, resulting in various clinical effects. It is not yet known whether *D. russellii russellii* from various regions will have identical PLA2s sequence.

In addition to PLA2, at least 3 clones (RVV003, RVV012 and RVV015) are involved in the toxicity of the venom. Therefore, cDNA library construction is an effective way to identify and clone genes of interest from venom glands. Cloning of *D. russellii siamensis* PLA2 will allow expression of this enzyme by recombinant technology. The recombinant protein can be potentially helpful to study the pathophysiology of RV venom, and to produce an antibody against it for diagnostic or therapeutic purposes.

ACKNOWLEDGEMENT

The authors wish to thank the Molecular Biology Program Research Affairs, the Faculty of Medicine, Chulalongkorn University and Thailand Research Fund (TRF) for supporting this study.

(Received for publication on March 21, 2001)

REFERENCES

1. Warrell DA ed. WHO/SEARO guideline for the clinical management of snake bites in the Southeast Asian regions. Southeast Asian J Trop Med Public Health 1999; 30 (Suppl 1): 5-35.
2. Warrell DA. Snake venoms in science and clinical medicine-1. Russell's viper: biology, venom and treatment of bites. Trans R Soc Trop Med Hyg 1989; 83: 732-40.
3. Takeya H, Nishida, S, Miyata T, et al. Coagulation factor X activating enzyme from Russell's viper venom (RVV-X)-a novel metalloproteinase with disintegrin (platelet aggregation inhibitor)-like and c-type lectin-like domains. J Biol Chem 1992; 267: 14109-17.
4. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: A Laboratory Manual. Cold Spring Habor Laboratory, Cold Spring Habor, NY 1982.
5. Altschul SF, Madden TL, Schäffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25: 3389-02.
6. Wang YM, Lu PJ, Ho CL, et al. Characterization and molecular cloning of neurotoxic phospholipase A2 from Taiwan viper (*Vipera russelli formosensis*). Eur J Biochem 1992; 209: 635-41.
7. Tsai IH, Lu PJ, Su JC. Two types of Russell's viper revealed by variation in phospholipase A2 from venom of the subspecies. Toxicon 1996; 34: 99-09.
8. Myint-Lwin, Philllips RE, Tun-Pe, Warrell DA, Tin-Nu-Swe, Maung-Maung-Lay. Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: Haemostatic, vascular, and renal disturbances and response to treatment. Lancet 1985: 1259-64.
9. Dennis EA. Diversity of group types, regulation, and function of phospholipase A2. J Biol Chem 1994; 269: 13057-60.
10. Gowda TV, Schmidt J, Middlebrook JL. Primary sequence determination of the most basic myonecrotic phospholipase A2 from the venom of Vipera russelli. Toxicon 1994; 32: 665-73.
11. Vishwanath BS, Kini RM, Gowda TV. Purification and partial biochemical characterization of an edema inducing phospholipase A2 from *Vipera russelli* (Russell's viper) snake venom. Toxicon 1988; 26: 713-20.
12. Kasturi S, Gowda TV. Analysis of *Vipera russelli* venom using polyclonal antibodies prepared against its purified toxic phospholipase A2 VRV PL-V. Biochem Int 1992; 27: 155-64.
13. Prasad BN, Kemparaju K, Bhatt KG, et al. A platelet aggregation inhibitor phospholipase A2 from Russelli's viper (*Vipera russelli*) venom: isolation and characterization. Toxicon 1996; 34: 1173-85.
14. Gowda TV, Middlebrook JL. Monoclonal antibodies to VRV-PL-VIIIa, a basic multitoxic phospholipase A2 from *Vipera russelli* venom. Toxicon 1994; 32: 955-64.

การโคลนยีนฟอสโฟลัยเปส เอทู จากห้องสมุดยีนของต่อมพิษงูแมวเซา

อิศรางค์ นุชประยูร, พ.บ., ปร.ด., อาคม ใสงาม, วท.ม.*,*
*สุนุชชา สุนทราชุน, วท.ม.**, จุรีพร น้อยพรหม, วท.บ.**, นฤมล พักมณี, วท.ม.**,*
*ลาวัณย์ จันทร์โฮม, ส.พ.บ.**, สุรางค์ นุชประยูร, พ.บ., ปร.ด.*, วิศิษฏ์ สิตปรีชา, พ.บ., ปร.ด.***

งูแมวเซาเป็นงูพิษที่พบได้ชุกชุมทางภาคเหนือและภาคกลางของไทย ผู้ที่ถูกงูแมวเซากัดอาจมีอาการเลือดไม่แข็งตัว เม็ดเลือดแดงแตกและมีอาการบวมบริเวณที่ถูกงูกัด เนื่องจากในพิษงูมีสารพิษมากมายหลายชนิด เราจึงได้สร้างห้องสมุดยีนจากต่อมพิษงูแมวเซา เพื่อจะทำการวิเคราะห์โปรตีนที่เป็นองค์ประกอบในการทำให้เกิดพิษ ในการศึกษาขั้นต้น เราสามารถโคลนยีนฟอสโฟลัยเปส เอทู (PLA2) โดย PLA2 เป็นเอ็นซัยม์ที่พบได้ในพิษงูหลายชนิดทั่วโลก และคาดว่าทำให้เกิดพิษต่อเยื่อหุ้มเซลล์ ส่งผลให้เนื้อเยื่อถูกทำลาย รวมทั้งการเกิดอาการต่าง ๆ ของผู้ที่ถูกงูแมวเซากัด การศึกษาวิจัยยีน PLA2 อาจช่วยให้สามารถศึกษาพยาธิกำเนิดของอาการต่าง ๆ ที่เกิดจากงูแมวเซากัดได้

คำสำคัญ : พิษงูแมวเซา, ฟอสโฟลัยเปส เอทู, อณูพันธุศาสตร์

อิศรางค์ นุชประยูร, อาคม ใสงาม, สุนุชชา สุนทราชุน, และคณะ
จดหมายเหตุทางแพทย์ ฯ 2544; 84 (ฉบับพิเศษ 1): S99–S105

* ภาควิชากุมารเวชศาสตร์, คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย,
** สถานเสาวภา, สภากาชาดไทย, กรุงเทพ ฯ 10330