Purification of Snake Antivenom

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Abstract: Thai Red Cross Society (T.R.C.S.) started supplying antivenom purified with pepsin digestion and salting - out of ammonium sulfate 5 years ago. Purification has increased safty, but a great loss in purification has lowed recovery to less than 20% and threatening a stable supply of antivenoms.

In order to increase the recovery rate in antivenom purification, problems of T.R.C.S. antivenom purification method were identified and improvement measures were discussed.

The results were as follows.

1. Purification methd of T.R.C.S. need improvements in pepsin digestion time and the amount of ammonium sulfate in the 2nd time salting - out.
2. Purification with a special attention to pepsin digestion, heating and salting out of ammonium sulfate resulted in 2—3 times higer recovery rate than that of T.R.C.S.. Specific activity showed 2.3 times increased.

Key words: purification, snake antivenom.

I Introduction

Among the variety of snakes, poisonous as well as harmless, inhabiting in tropical Thailand, 8 species listed in Table 1 are the harmful ones to human and animals. There are approximately 6,000 cases of snake bites reported annually.

In treating the victims of snake bite, antivenoms, which are produced 20,000 vials annually by T.R.C.S., are administered with a great effectiveness. Incidentally, only 6 fatal cases were reported in 1989 with 0.1% fatality rate.

T.R.C.S. currently produces 6 kinds of antivenoms for Cobra, King Cobra, Banded Krait, Malayan Pit Viper, Green Pit Viper and Russell's Viper. Major antivenoms are these three types: Cobra, Green Pit Viper and Malayan Pit Viper, which are accountable for a majority of snake bites. Formally the antivenoms without purification were supplied. With technical training at Pastour Institute (France) and Common wealth Institute (Australia), T.R.C.S. started supplying antivenoms purified with pepsin digestion and salting - out of ammonium sulfate 5 years ago. Purification has increased safety by eliminating albumin - a major cause of side effects; however, a great loss in purification has lowered recovery to less than 20%, threatening a stable supply of antivenoms. My training in The 11th JICA Public Health Technologist Course was directed to identify problems of antivenom purification method by T.R.C.S and to consider some improvement measures.

Table I. Poisonous Snakes of Thailand

Neurotoxic snakes	
Cobra	*Naja kaouthia*
Spitting Cobra	*Naja sputatrix*
King Cobra	*Ophiophagus hannah*
Banded Krait	*Bungarus fasciatus*
Malayan Krait	*Bungarus candidus*
Hematotoxic snakes	
Green Pit Viper	*Trimeresurus albolabis*
	Trimeresurus macrops
Malayan Pit Viper	*Agkistrodon rhodostoma*
Russell's Viper	*Vipera russelli*

II Materials and Methods

1. Antivenom

Horse serum immunized with *Trimeresurus elegans* and goat serum immunized with *Trimeresurus flavoviridis* were used for purification experiment. Protein concentration measured 74 mg / ml for horse serum immunized with *T. elegans;* 93 mg / ml for goat surum immunized with *T. flavoviridis*.

Anti - hemorrhagic potency measured 186 u./ ml for horse serum immunized with *T. elegans*; 327 u./ ml for goat serum immunized with *T. flavoviridis*.

2. Estimation of protein

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Protein concentrations were estimated by measuring absorbance at 280 nm by spectrophotometer (Hitachi U－4000). A factor of 1.25 O D_{280} was used to convert absorbance to mg of serum protein per ml.

3. Determination of anti - hemorrhagic potency

Anti - hemorrhagic potency was determined by rabbits skin test based on national standard method. Since no national standard has yet been set for antivenom for *T. elegans*, test toxin and standard antivenom made in Okinawa Prefectural Institute of Health and Environment were used for titration.

4. Electrophoresis

Electrophoresis on a piece of cellulose acetate membrane was conducted. Then protein band was stained with Ponceau 3R. Protein concentration of bands was estimated by measuring absorbance at 500 nm by densitometer (Tokyo Photoelectric Company LTD.)

5. High Performance Liquid Chromatography (HPLC)

HPLC was conducted by connecting 2 pieces of gel filteration column (Protein pak 300: waters). The column was eluted with 0.01M ,pH＝7.0, tris - HCl buffer (0.2M NaCl).

III Results and Discussion

1. Study of pepsin digestion

In order to identify a cause for low recovery, pepsin digestion in the process of purification was studied. At T.R.C.S., 0.1% pepsin is added to partially purified antivenom solution (1.0 N HCl, pH ＝ 3.5 － 4.0) and incubated for one hour at 37℃. In the experiment, samples were collected at 1hr, 3 hrs, 5 hrs and 24 hrs after incubation in order to observe progress of pepsin digestion. Degree of digestion was estimated by the increase of 0.44 M TCA soluable protein. That is to say that 0.44 M TCA was added to the the samples and the resulting precipitates were removed by centrifuge (5,000 rpm, 20 min.) in order to measure absorbance of supernatant at 280 nm.

Results are shown in Fig.1. The experiments were repeated twice respectively. The plots of Fig. 1 indicate average of duplulicates. 0.44 M TCA soluable protein made by pepsin digestion showed a rapid increase up to 5 hours and then became stable. It was assumed that pepsin digestion is almost complete within 5 hours. The slopes of time - absorbance curve at 0.1% and 0.3% pepsin concentration were nearly the same.

A very gradual increase of absorbance curve up to 24 hours suggests that the longer the incubation time, the better pepsin digestion is. However, the risk of contamination may also increase with time. Therefore it is adequate to limit pepsin digestion within 5 hours for producing antivenom for treatment. Since pepsin additions in 0.1% and 0.3% concentrations make little difference, use of 0.1% concentration would be adequate from a viewpoint of economic advantage and possible residue after purification.

2. Influence of heating on antivenom

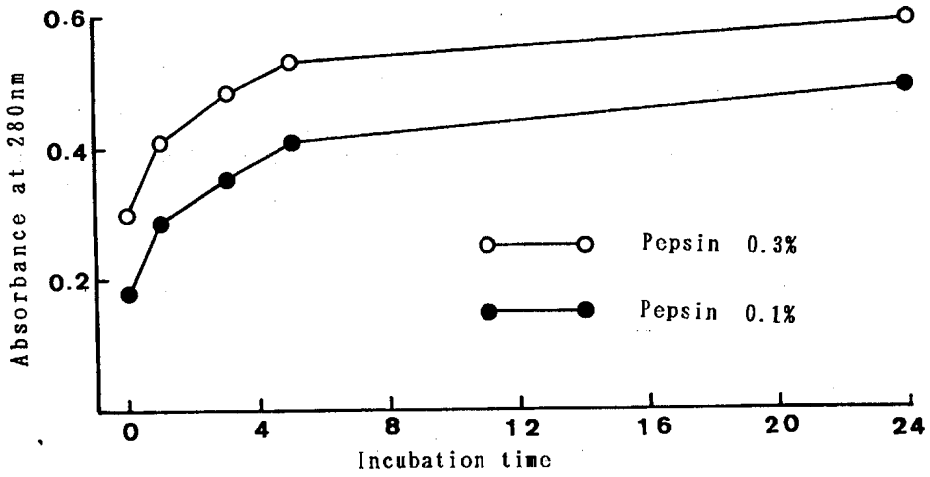

Fig 1. study of Pepsin Digestion

Cr. AV. (50ml)+DW. (50ml)→pH=3.8→ Pepsin. (0.1g or 0.3g)→incubate (37℃)

Samp. (1.5ml)+0.44M TCA(1.5ml)→ cent. (5.000rpm, 20min)→Super→OD_{280}

Influence of heating (52 - 60 ℃, 15 min) on serum protein was examined.

Crude serum and purified serum containing 30 saturate % of ammonium sulfate were divided into 3 groups and were adjusted to pH=4.0, pH=4.5, pH=5.0 with IN HCl respectively and further heated for 15 min. at 52℃, 55℃ and 60℃. The samples were then centrifuged (5.000rpm, 20min.) to collect supernatants.
The absorbance of the supernatant were measured at 280nm.

The results are shown in Fig.2

A large amount of precipitation was observed in crude serum of pH=4.5 and p=4.0 when heated at 52 ℃ or higher; however, precipitation was scarcely observed in the purified serum.
Pepsin - digested immunoglobulin was stable in these pH range.

At T.R.C.S. antivenom is adjusted to pH=4.5 with 1.0N NaOH after digestion and then heated for 1 hour at 52 - 55℃. When pepsin digestion is sufficiently done, heating does not affect recovery rate. Judging from the results shown above, digestion time given at T.R.C.S. seems too short. Enzyme activation method tried this time will serve as a reference for my improvement effort.

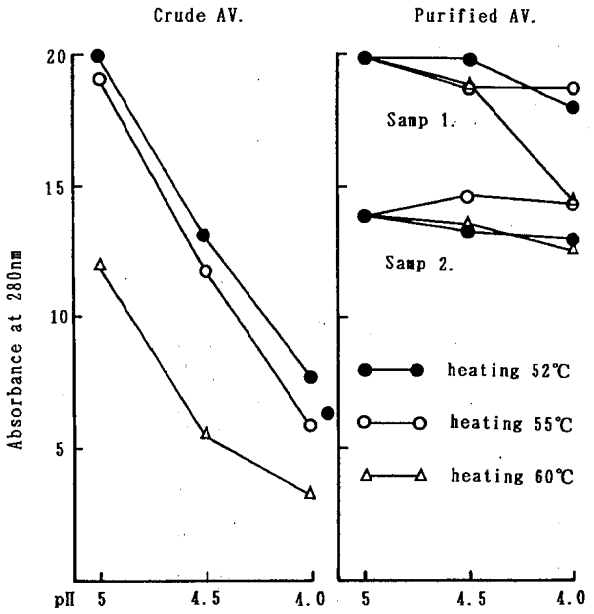

Fig 2. Influence of Heating to Antivenom
Samp 1: Pepsin digestion 5hr.
Samp 2: Pepsin digestion 24hr.

3. The process for purification of antivenom

Based on results of pepsin digestion and heating procedure, purification of antivenom was experimented. The process of purification is shown in Fig.3.

The purification of antivenom was conducted based on the method of T.R.C.S.. Special care was taken this time to pepsin digestion, reaction time and 2nd time salting - out of ammonium sulfate.
When the amount of ammonium sulfate is excessive, digested immunoglobulin together with non - digested globulin will precipitate, resulting in a main cause of low recovery.

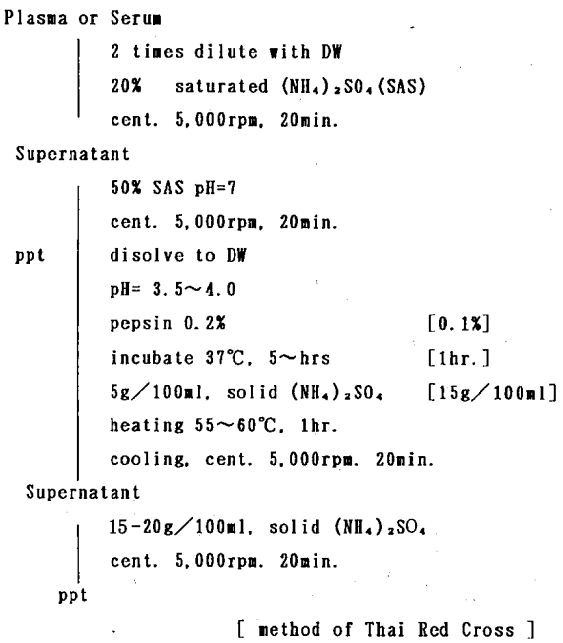

Fig 3. Purification Procedure for Antivenom Production

In the reactive solution sample, 15 saturate % of residual ammonium sulfate of first time salting - out was measured in the pepsin - digested serum. If normal amount of ammonium sulfate (30 saturate %) is added, ammonium sulfate concentraion in the serum will reach 45 saturate % and the digested globulin will also removed together with non - useful matters.

Therefore, the amount of 2nd time salting - out of ammonium sulfate was set to 15 saturate % in this experiment, considering the amount of existing ammonium sulfate residue.

Table 2-a. Summary of Purification of Antivenom
(Horse serum immunized with T. elegans)

	vol	Protein			Anti hemorrhagic potency			
	(ml)	mg/ml	total mg	%	u./ml (u./mg)	S.A.	Total u.	%
Cr. AV.	50	74	3,700	100.0	186 (2.51)	1.0	9,300	100.0
Pu. AV.	30	24	720	19.5	137 (5.71)	2.3	4,110	44.2

Table 2-b. Summary of Purification of Antivenom
(Goat serum immunized with T. flavoviridis)

	vol	Protein			Anti hemorrhagic potency			
	(ml)	mg/ml	total mg	%	u./ml (u./mg)	S.A.	Total u.	%
Cr. AV.	50	93	4,650	100.0	327 (3.52)	1.0	16,350	100.0
Pu. AV.	41	31	1,271	27.4	253 (8.16)	2.3	10,373	63.4

The anti-hemorrhagic potency was determined by rabbits skin test.
Potency of Samples were calculated as relative potency to standard AV

Specific activity and recovery rate before and after purification are indicated in Table 2.

Specific activity showed 2.3 times increase in both horse and goat serum. It seem quite difficult to achieve a greater increase by non - specific purification method such as ammonium sulfate fractionation, due to the fact that more than 70 % of total protein turns to globulin in highly immunized animals. Recovery rate was 44. 2% for horse serum immunized with *T. elegans* and 63.4% for goat serum immunized with *T. flavoviridis*. The results were 2 — 3 times higher than those of T.R.C.S.

The results of a series of experiments indicate that purification method of T.R.C.S. has problems in pepsin digestion time and amount of ammonium sulfate for 2nd time salting - out. Since higher recovery rate is a critical factor in stable supply of antivenom, I would like to make efforts for immediate improvement upon returning to Thailand.

4. Electrophoresis

Electrophoresis on cellulose acetate membrane was conducted in order to examine the purity of purified antivenom.

First, a piece of cellulose acetate mambrane (5×6cm) was smeared with sample serum and placed under a constant current of 3mA, 120 V for 40 min. in 0.06M veronal buffer (pH=8.6, I=0.06) at room temperature. Then, the sample was stained with a 0.4% of Ponceau 3R (3% Trichloroacetic acid solution) for 15 min. Unnecessary part other than protein bands was decolored by 3% acetic acid.

After staining, the cellulose acetate membrane was made transparent by decahydro - naphthalene and then densitometry was conducted at 500 nm.

As shown in Fig.4, no peak for albumin was found in the purified sample indicating perfect removal of albumin

5. HPLC on gel filteration column

HPLC was conducted using 2 gel filtration columns, protein pak 300 (7.8 × 300mm). The results and elution system are shown in Fig.5.

The purified serum showed a big peak with shoulder on the right (fra - 1) and small peak (fra - 2). Fra - 1 is presumably pepsin - digested immunoglo - bulin and fra - 2 is a piece of serum fragment generated by digestion.

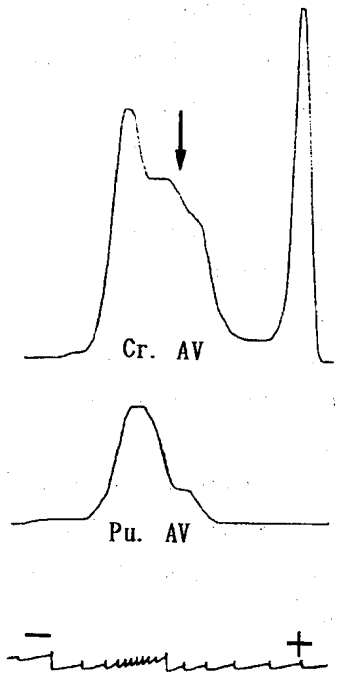

Fig 4. Electrophoresis of Antivenom

Fra - 1, main constituent of purified serum eluted later due to the immunoglobulin crude serum. This result confirmed that the molecular weight of immunoglobulin became smaller by pepsin digestion.

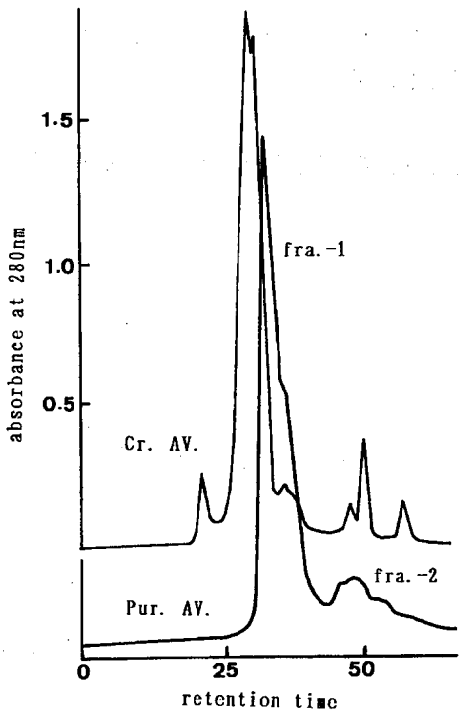

Fig 5. HPLC of Antivenom
Column: Protein pak 300(7.8×300mm)×2
Buffer: 10mM, pH=7.0 tris-HCl(0.2M NaCl)
Flow : 0.5ml/min.

Ⅳ Summary

In order to raise recovery rate in antivenom purification, problems of T.R.C.S. antivenom purification method were identified and improvement measures were discussed.

1. For sufficient pepsin digestion, incubation at 37 ℃ for at least 3 − 5 hours is necessary.
2. Heating (52−60℃) after digestion can cause dena - tured precipitation in non - digested immunoglbulin and cause almost no effect on pepsin - digested im - munoglbulin.
3. A careful attention should be paid to the 2nd time salting - out of ammonium sulfate because of the residual ammonium sulfate of the first time salting - out.
4. Purification with a special attention to pepsin digestion, heating and salting - out of ammonium sulfate resulted in 2 − 3 times higher recovery rate that of T.R.C.S.:44% for horse serum immunized with *T.elegans*; 63.7% for goat serum immunized with *T.flavoviridis*. Specific activity in both antivenoms showed 2.3 times increase.
5. Purification method of T.R.C.S. need improve - ments in pepsin digestion time and the amount of ammonium sulfate in the 2nd time salting - out.

Ⅴ Acknowledgement

I would like to extend my deep appreciation to JICA / OIC staff for their kind assistance that have made my traning a successful one.

毒蛇抗毒素の精製実験

ジュタテップ・ワングサイ、野崎真敏

私が所属するタイ赤十字社では，５年程前から精製された抗毒素を提供しているが，精製過程でのロスが大きく，最近では回収率が２０％を割るまでに低下し，安定供給が危ぶまれている．したがって，今回の研修（公衆衛生技術コース：JICA）では，タイ赤十字社で行っている抗毒素製造法の問題点について検討を行い，下記の結論を得た．

1.一連の精製の手順を点検した結果，タイ赤十字社の方法はペプシン消化時間と２回目の塩析の際の硫安の添加量に問題があることがわかった．

2.ペプシン消化，加熱処理，硫安の添加量に注意しながら抗毒素の精製を行った結果，回収率は，抗サキシマハブ馬抗毒素が４４．２％，抗ハブ山羊抗毒素が６３．４％で，タイ赤十字社より２～３倍高かった．力価は，馬，山羊いずれの抗毒素でも出発時の２．３倍に上昇した．