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Quantitative comparison on the refinement of horse antivenom by salt fractionation and ion-exchange chromatography

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Abstract

A quantitative comparison was made on the fractionation of pepsin-digested horse antivenoms by ammonium sulfate (AS) fractional precipitation and ion-exchange chromatography on Q-Sepharose. In the precipitation process, pepsin digested horse anti-*Naja kaouthia* serum was precipitated by 30% saturated AS followed by 50% saturated AS. The recovery of antibody activity [as measured by an enzyme-linked immunosorbent assay (ELISA) against the cobra postsynaptic neurotoxin 3] from the 30–50% saturated AS precipitate was 53% with a 1.93-fold purification. For the chromatographic process, the behavior of the horse antitoxin antibody and its $F(ab')_2$ fragments was first studied. The pepsin digested horse serum was then desalted on a Bio-gel P-2 column followed by chromatography on Q-Sepharose using a linear gradient (20 m*M* Tris–HCl, pH 8.0 containing 0.0 to 0.5 *M* NaCl). A peak containing primarily the $F(ab')_2$ antibody could be obtained. This peak constituted 73% of the total antivenom activity with 2.08-fold purification. The total recovery of antibody activity by the chromatographic process was 90%. The yield of antibody activity was about 2-fold higher than that reported previously with other fractionation procedures. The implications of these results for the refining of horse therapeutic antivenoms are discussed. © 1997 Elsevier Science B.V.

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1. Introduction

Therapeutic snake antivenoms used in many countries including Thailand are pepsin-digested, ammonium sulfate (AS) precipitated $F(ab')_2$ fragments. The fractionation procedure, originally developed by Harms in 1948 [1], is simple but the recovery of antivenom activity is very low: probably less than 50%, using rather inaccurate in vivo neutralization assay. Since the antivenoms are difficult to produce [2], are expensive and are in short supply, this inefficient procedure should be improved upon.

Various groups of investigators have reported the use of affinity chromatography in the purification of antivenom antibodies, for example, ovine F(ab) [3], horse IgG [4], avian egg yolk IgY [5]. However, no quantitative data on the yield and recovery of antibody activity were reported in these studies. In our previous study [6] using various affinity adsorbents to purify horse antivenom $F(ab')_2$, a 10–12 fold increase in potency was observed but the operational half-life of all adsorbents (19–108 days) and the recovery of antibody activity (30–35%) were rather low and the process was not considered economically feasible, especially with antivenoms which are in short supply. Fractionation of an-

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tivenom by caprylic acid precipitation of non-immunoglobulin protein of horse plasma has also been extensively studied [7–9]. It was found that 25.8–37.5% of $F(ab')_2$ antivenom activity could be recovered by this procedure [9].

Ion-exchange chromatography has been successfully used in the large scale fractionation of serum proteins and antibodies for therapeutic applications [10–12]. The present study involved the use of ion-exchange chromatography to fractionate horse antivenom antibodies. In this communication, the chromatographic behavior of horse IgG, IgG(T) [13,14] and $F(ab')_2$ fragments on Q-Sepharose were studied using an enzyme-linked immunosorbent assay (ELISA) to monitor the antibody activity. The purification, the yield and the recovery of antibody activity obtained from the chromatographic procedure were compared with those obtained by the conventional AS fractional precipitation procedure. The chromatographic procedure described here gave higher yields than other fractionation schemes.

2. Experimental

2.1. Chemicals and biochemicals

Horse therapeutic antivenom against *Naja kaouthia* (also known as *Naja naja siamensis*) was obtained from Queen Saovabha Memorial Institute, The Thai Red Cross Society (Bangkok, Thailand). *N. kaouthia* postsynaptic neurotoxin 3 was purified as previously described [15]. All other chemicals were of reagent grade and were obtained from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2.2. Pepsin digestion

Pepsin digestion of horse serum was carried out using a pepsin (EC 3.4.23.1, Lot. 78F-8056, specific activity 3470 units/mg protein) to protein ratio of 1:100 (w/w). The digestion was carried out in 100 m*M* sodium citrate buffer, pH 3.8 for 18 h at 37°C. The digestion was stopped by addition of 1 *M* Tris base to bring the pH to 8.0.

2.3. Ammonium sulfate precipitation of horse antivenom proteins

Precipitation of immunoglobulins from serum was carried out at 50% saturated AS at 4°C overnight. The precipitate was redissolved and dialyzed overnight in 20 m*M* Tris–HCl, pH 8.0.

AS precipitation of horse antivenom $F(ab')_2$ from pepsin-digested serum was carried out using a procedure from the Queen Saovabha Memorial Institute. The first precipitation was carried out at 30% saturated AS at 25°C and pH 5.0; the mixture was then heated at 55°C for 1 h. The precipitate, separated by centrifugation at 10 000 *g* for 10 min, was dialyzed in 150 m*M* phosphate buffered saline (PBS), pH 7.4. The protein content and the antibody activity in the dialyzed and dissolved precipitate were estimated. The 30% saturated AS supernatant was adjusted to pH 5.0 and sodium pyrophosphate was added to give a final concentration of 4.5 m*M*. The solution was then made 50% saturated AS and allowed to stand at 25°C for 60 min. The precipitate which formed was separated by centrifugation, dissolved and dialyzed as described above. The protein concentration and antibody activity were estimated. These experiments were carried out in triplicate.

2.4. Assays of antibodies against N. kaouthia neurotoxin 3 and venom

Specific antibody against *N. kaouthia* postsynaptic neurotoxin 3 was assayed by ELISA. The procedure was modified from that described by Rungsiwongse and Ratanabanangkoon [16]. A polyvinyl microtiter plate (Cat. No. 1550, Costar) was coated overnight with 50 μl/well of 5 μg/ml of *N. kaouthia* neurotoxin 3 in 0.05 *M* carbonate–bicarbonate buffer, pH 9.6. After incubation overnight at 4°C, the unbound antigen was removed and the plate was washed four times for 3 min intervals with 0.05% Tween 20 in normal saline (NSST). Appropriate starting dilutions of samples, including positive and negative references, were made in a diluting buffer (0.15 *M* PBS, 0.05% Tween 20, 0.5% BSA). A 50 μl of sample dilution was added to each well and incubated at room temperature for 1 h. After four washes with NSST, 50 μl/well of 1:200 diluted rabbit anti

$F(ab')_2$ in diluting buffer was added and incubated at room temperature for 1 h. After four washes with NSST, 50 μl/well of 1:1000 diluted swine anti-rabbit IgG-HRP conjugate (Lot. 125, Dako-immunoglobulins, Glostrup, Denmark) in diluting buffer was added and incubated at room temperature for 1 h. After five washes with NSST, 100 μl/well of freshly prepared chromogenic substrate solution (40 mg% *o*-phenylenediamine and 0.003% hydrogen peroxide in 0.075 *M* citrate phosphate buffer, pH 5.0) was added, The enzyme–substrate reaction was allowed to take place at room temperature in the dark for 30 min and was terminated by the addition of 25 μl of 2 *M* sulfuric acid. The absorbance at 490 nm was read against substrate blank using a Minireader II photometer (Dynatech Laboratories, Alexandria, VA, USA).

When the antibody against *N. kaouthia* venom was assayed, the crude venom at a concentration of 10 μg/ml was used to coat the plate. The remaining assay procedure was as described above.

2.5. Quantitative estimation of antibody activity against N. kaouthia postsynaptic neurotoxin 3

The assay was carried out by an ELISA as described in Section 2.4. The yield of immunoreactive antibodies from each pooled fraction was determined by testing 2-fold dilutions by ELISA [17]. The dilution giving as ELISA absorbance of 1.0 was set to contain 100 antibody units/ml.

2.6. Chromatography on Q-Sepharose

Q-Sepharose FF was equilibrated in 20 m*M* Tris–HCl, pH 8.0 and packed in a 3.7×0.9 cm I.D. column. The flow-rate was 25 ml/h and fractions of 1 ml were collected.

2.7. Miscellaneous procedures

Protein concentration was determined by the procedure of Lowry et al. [18] using bovine serum albumin (BSA) as the standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli [19].

3. Results

3.1. Chromatography of pepsin digested serum on a Sephadex G-150 column

When the pepsin digested serum was chromatographed on a Sephadex G-150 (121×0.9 cm I.D.) column, a major protein peak at M_w 100 kDa and a broad peak of smaller peptides were observed (data not shown). When the chromatographic fractions were assayed for antibody activity, a symmetrical peak of antibody activity was found to coincide exactly with the 100 kDa peak and no activity was found in either the higher or lower M_w fractions. These results indicated that the IgG(T) was completely digested by pepsin under the conditions employed and that only the $F(ab')_2$ antibody remained in the sample studied.

3.2. Ammonium sulfate fractional precipitation of horse antivenom $F(ab')_2$

Pepsin digested antivenom serum (1.35 ml containing 100.13 mg protein) was fractionated by AS precipitation as described in Section 2.3. The results for protein content and antibody activity recoveries are shown in Table 1.

3.3. Chromatography of ammonium sulfate precipitated horse antivenom immunoglobulins on Q-Sepharose

AS precipitated serum protein (35 mg) was redissolved and dialyzed in 20 m*M* Tris–HCl pH 8.0. It was then chromatographed on Q-Sepharose. The flow-through contained 6.34% of the protein with no antibody activity (Fig. 1). The bound proteins were eluted with a linear sodium chloride (0–0.5 *M*, 60 ml each) gradient. Two peaks (26.7% of the protein) with no antibody activity were eluted at the beginning of the gradient. These peaks were shown to be IgG by SDS–PAGE. The main antibody peak, the IgG(T) was eluted at 0.15 *M* NaCl and contained 37.8% of the total protein. The total recovery of protein from the column was 115% while the recovery of antibody activity was 95%.

A similar chromatographic profile was obtained

Table 1
Quantitative estimations of protein and antibody activity of protein fractions in AS precipitation of pepsin-digested horse antivenom

Sample	Total protein (mg)	Protein recovery (%)	Total activity (units)	Recovery of activity (%)	Specific activity (unit/mg)	Purification (fold)
1. Digested serum	100.13	–	457 193	–	4566	–
2. First AS precipitate (30% saturated AS)	22.42	22.39	50 007	10.94	2230.5	0.49
3. Second AS precipitate (50% saturated AS)	27.66	27.64	243 821	53.33	8821.3	1.93

The results are averages of triplicate experiments.

when 0.4 ml of dialyzed horse antivenom serum was used (data not shown). The main antibody peak was eluted at about 0.15 *M* NaCl. The IgG peak showed no antibody activity. The major difference of this profile, when compared to Fig. 1, was the presence of albumin following the IgG(T) peak. By applying 1, 1.5, 2.0 and 2.5 ml of horse serum to the column, the antibody activities of the flow-through peaks as calculated from the ELISA results, were 1.9%, 10.6%, 12.8% and 27.8%, respectively. At the same time, the IgG(T) peak, accounted for 90.6%, 83.3%, 76.1% and 42.2% of the total antibody activity, respectively. The corresponding percentages of recovered antibody activity were 92.5, 93.9, 88.9 and 70. The capacity of the anion-exchanger was calculated to be 2.0 ml of serum per ml of packed gel.

3.4. Chromatography of pepsin-digested antivenom serum on Q-Sepharose

Pepsin digested serum (1 ml containing 75 mg protein) was chromatographed on a Biogel P-2 column (40×2.6 cm I.D.) to change the buffer to 20 m*M* Tris–HCl, pH 8.0. The protein peak (25 ml)

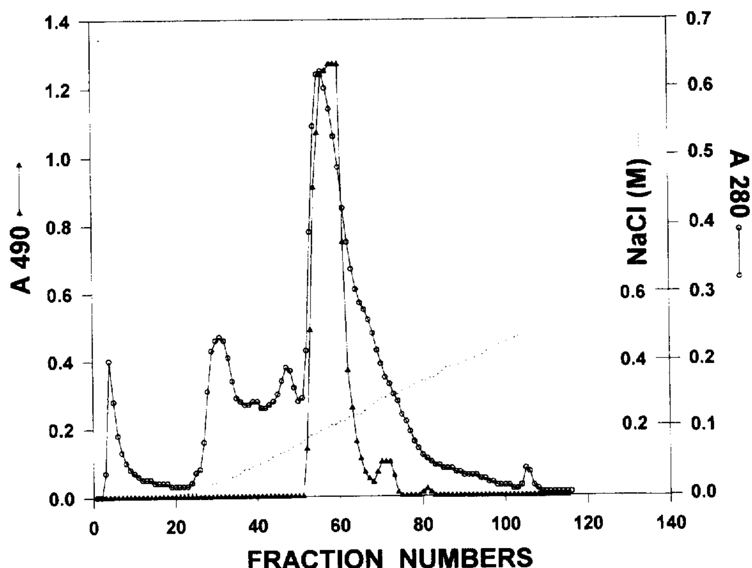

Fig. 1. Chromatographic profile of AS precipitated horse antivenom proteins on Q-Sepharose. The antibody activity of each fraction is shown by the ELISA absorbance at 490 nm.

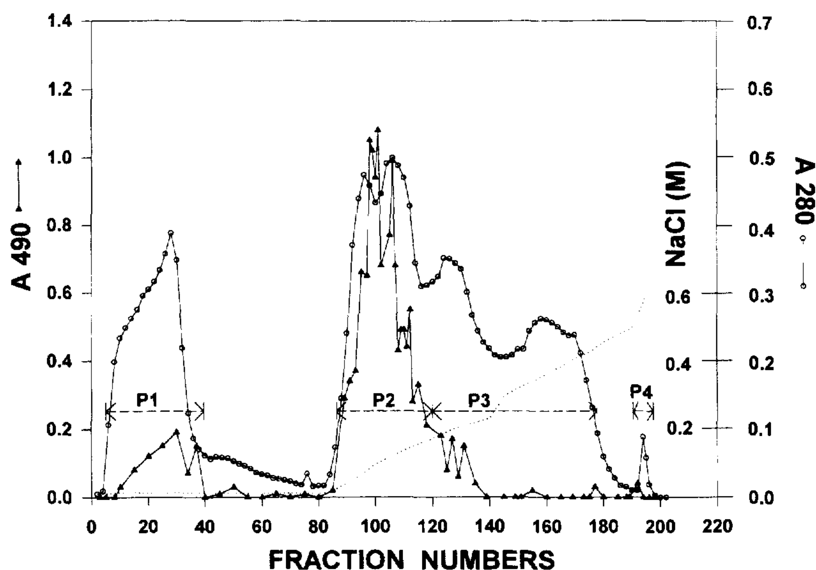

Fig. 2. Chromatographic profile of pepsin digested horse antivenom serum on Q-Sepharose. The protein peaks were pooled as shown.

from this column was then applied to a Q-Sepharose column (3.7×0.9 cm I.D). The chromatographic profile is shown in Fig. 2. About one-third of the protein was in the flow-through. Eluted at the beginning of the gradient (0–0.5 *M* NaCl, 40 ml each) was the $F(ab')_2$ peak with almost all the antibody activity. When the fractions of the chromatographic profile were pooled as shown in Fig. 2, the protein and antibody activity recovered in each pooled peak were as shown in Table 2.

3.5. SDS–PAGE of antivenom preparations obtained from salt precipitation and Q-Sepharose chromatography

When the final products obtained from 50% AS precipitation and from Q-Sepharose chromatography (P2 fraction) were subjected to SDS–PAGE, the two preparations showed very similar protein patterns (Fig. 3). Two prominent bands at about 25 kDa were most likely from the heavy and light chains of IgG

Table 2
Quantitative estimations of protein and antibody activity of protein fractions obtained from Q-Sepharose chromatography of pepsin digested horse antivenom

Sample	Total protein (mg)	Protein recovery (%)	Total activity (units)	Recovery of activity (%)	Specific activity (unit/mg)	Purification (fold)
1. Digested serum after Biogel P-2	54.60	–	236 265	–	4327.20	–
2. P1 of Q-Seph.[a]	18.39	33.68	21 157	8.95	1150	0.26
3. P2 of Q-Seph.	19.12	35.02	172 237	72.90	9008	2.08
4. P3 of Q-Seph.	16.59	30.38	17 287	7.32	1042	0.24
5. P4 of Q-Seph.	0.31	0.56	–	–	–	–
P1+P2+P3+P4	54.41	99.64	210 681	89.17	–	–

The results are averages of quadruplicate experiments.
[a] Protein peaks were pooled as shown in Fig. 2.

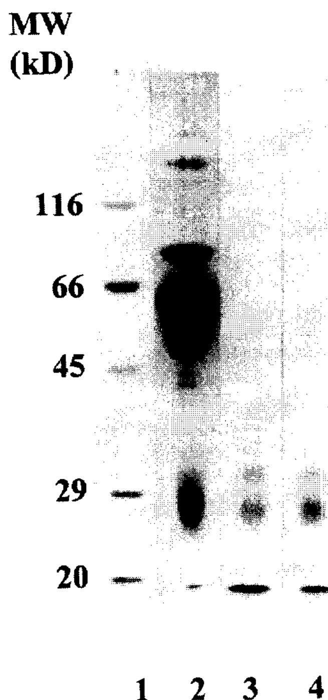

Fig. 3. SDS–PAGE of horse antivenom $F(ab')_2$ preparations: $F(ab')_2$ peak (P2) obtained from Q-Sepharose chromatography (lane 3), $F(ab')_2$ fraction from AS precipitation (lane 4), horse antivenom serum (lane 2) and standard protein markers (lane 1). The electrophoresis was carried out in 12% acrylamide under reducing conditions.

and/or IgG(T). A faint band at 50 kDa was also noted. This was most likely undigested heavy chain of IgG since it was found to be relatively pepsin resistant (KR, unpublished observations). An intense band of low M_w peptides was also observed.

4. Discussion

Although the refining of horse antivenom by AS precipitation has been carried out for almost 50 years, the recovery of antibody activity has not been accurately estimated. This is due to inaccuracy resulting from high variation in the in vivo neutralization assay using mice. Because of the recent development of an accurate ELISA which correlated highly with the neutralization assay [16], it is possible to study and improve each step in the refining process.

It should be mentioned here that in various studies to fractionate antivenom antibody or its fragments [3–5] no quantitative data on the yield and recovery of antibody activity were reported. This information is essential in determining the economic feasibility of the process since most snakebites occur in developing countries with low per capita income. Also, processes giving low yield will have limited use with antivenoms which are difficult to produce and are in short supply [2].

The pepsin digestion conditions employed completely cleaved the IgG(T) to give $F(ab')_2$. Thus, the quantitative comparison carried out here deals purely with $F(ab')_2$ and is not affected by the contamination of intact IgG(T). This is important, because the ELISA color intensities for intact IgG(T) and $F(ab')_2$ antibodies are vastly different. This is due to the presence of the Fc protein.

Fractional AS precipitation resulted in about 53% recovery of the antitoxin $F(ab')_2$ in 30% to 50% AS precipitates. The overall recovery of antibody activity in the 30% and 50% AS precipitates totalled about 64%. The antibody activity in the 30% AS precipitate was likely to be underestimated since it was not possible to completely redissolved all the precipitate for protein and antibody assays.

It should be pointed out that horse antitoxin antibody resides in an immunoglobulin class called IgG(T) with a M_w of about 160 kDa, whereas horse IgG, with a M_w of about 150 kDa, exhibits no antitoxin activity [13,14]. This information is relevant to the study of antibody recovery, which should be determined from the antibody activity, rather than from the total horse antivenom immunoglobulins as the IgG(T) constitutes only a part of the horse immunoglobulin [14].

The Q-Sepharose chromatographic conditions described here can be used to cleanly separate IgG(T) from IgG; AS precipitated immunoglobulins or dialyzed serum could be used as starting material, and this procedure is much simpler than that recently described using hydrophobic chromatography together with protein A affinity chromatography [14].

Ion-exchange chromatography has been extensively used for the fractionation of animal $F(ab')_2$ [20]. Q-Sepharose, when used to fractionate antitoxin $F(ab')_2$ from pepsin digests, resulted in some antibody activity in the flow-through peak (Fig. 2). This

was not due to overloading of the column, since the phenomenon was also observed when a minimal amount of pepsin digest was applied to the column. The nature of this antibody species in the flow-through fractions is not known at present.

The overall recovery of antibody activity from the Q-Sepharose chromatographic process was about 90%. The $F(ab')_2$ pooled peak contained 73% of the total antibody activity. This yield of antibody activity is considerably higher than that achieved by AS fractional precipitation and 2-fold higher than that previously reported [9]. The degree of purification was also slightly higher at 2.08-fold.

The antivenom fractionation under the chromatographic conditions described here could result in a 36% higher antibody yield than that by salt fractionation. Considering the high cost of production of horse antivenom sera, the significantly higher recovery of antibody may offset the additional material and operational costs of chromatography.

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