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Original Scientific Paper

Fractionation of Tetanus Toxoid by Sephadex Gel Filtration and Immunochemical Characterization of the Fractions

M. Fleš and B. Pende

Institute of Immunology, Zagreb, Croatia, Yugoslavia

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Three different types of tetanus toxoid were fractionated on Sephadex G-200 gel. The distribution of the fractions was determined spectrophotometrically at 280 m μ . Specific activity of the fractions *in vitro* was determined by flocculation with standard tetanus antitoxin and by means of immunodiffusion and immunoelectrophoresis. In all three types of the toxoid the active component appeared in the first three fractions; about 75% was found in the third fraction. The purity of this fraction, in terms of Lf/mg. of protein nitrogen (PN), was increased about twice in comparison with the starting material. The highest purity, 2400 Lf/mg. PN, was achieved in the third fraction of the toxoid type B-1, which was prepurified, first by ultrafiltration of the toxin, then, after detoxication, by fractional precipitation with ammonium sulfate. This fraction was homogeneous according to immunodiffusion and immunoelectrophoresis tests.

It has been concluded that fractional purification of tetanus toxoid can be effectively achieved by gel filtration on Sephadex G-200, but the effect of purification highly depends on the purity of the starting material.

INTRODUCTION

Toxoids for the preparation of vaccines should contain all antigenic components essential in the development of the active immunity. Although the removal of the nonspecific components should not necessarily give a toxoid of higher immunogenic value¹, it is essential to remove these components, especially the nonspecific proteins, since they often cause allergic reactions.

The most commonly used methods for the large scale purification of tetanus toxoid are fractionation of the crude toxoid by ammonium sulfate², by methanol³ or by trichloroacetic acid⁴. Recently an extensive use of polydextran gels for the fractionation of macromolecules was described⁵. Several authors have published on the separation of tetanus toxoid⁶, toxin⁷ and antitoxin⁸ on Sephadex gels. In our preliminary gel filtration experiments we used Sephadex G-100, but over 80% of the flocculation activity appeared in the first peak, indicating that the more porous gel would be more suitable for the fractionation of our samples of tetanus toxoid. With this purpose we used Sephadex G-200 gel in the experiments described in this paper.

MATERIALS AND METHODS

Starting materials. Three different types of tetanus toxoid preparations (A, B and B-1) were used as starting materials in the fractionation experiments. All three were prepared from the crude tetanus toxin, obtained by cultivation of *Clo-*

stridium tetani strain »Copenhagen 43415« on the modified Legroux-Ramon medium⁹. Type A was prepared from the crude tetanus toxin by detoxication with diluted formaldehyde, ultrafiltration of the toxoid through a collodion membrane and subsequent purification by ammonium sulfate fractionation. The concentration was 2300 Lf/ml. and the purity 1085 Lf/mg. of protein nitrogen (PN). Type B was prepared by ultrafiltration of the crude tetanus toxin, followed by treatment with formaldehyde. This preparation was used without further purification in chromatographic experiments, and had a concentration of 2550 Lf/ml. and a purity of 840 Lf/mg. of PN. Type B-1 was obtained from the crude toxoid type B by fractional precipitation with ammonium sulfate. This toxoid had the highest purity, 1400 Lf/mg. of PN, and its concentration was 2900 Lf/ml. Tetanus toxoid type A and type B-1 represent our routine production lots.

Chromatography on Sephadex G-200. Sephadex gel G-200, 140—400 mesh *Pharmacia Fine Chemicals*, Uppsala, Sweden was suspended in 0.1% sodium chloride and allowed to swell at room temperature for three days with occasional shaking. After repeated decantation, the gel was resuspended in the phosphate buffered saline (0.137 M NaCl, 0.0012 M KH_2PO_4 , 0.02 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, pH 7.5) containing 0.1% formaldehyde as bacterial growth inhibitor. The gel was poured into the glass column (3.1×105 cm.) by the usual technique. The equilibrated gel had a bed height of 80 cm. with a void volume of approximately 200 ml. The toxoid was applied on the top of the gel and eluted with the same buffer solution. The fractions were collected by means of an automatic fraction collector. The distribution of the fractions was determined spectrophotometrically using Beckman DU spectrophotometer.

Analysis. Specific flocculation activity was determined by the method of Ramon¹⁰ with standard tetanus antitoxin (Yugoslav substandard, produced by Institute of Immunology, Zagreb, Yugoslavia). Protein nitrogen was determined by the micro-Kjeldahl method¹¹. Diffusion in agar gel was performed by the method of Ouchterlony¹², and immunoelectrophoresis by the method of Scheidegger¹³.

RESULTS AND DISCUSSION

A 10 ml. sample of tetanus toxoid type A was applied to the Sephadex G-200 column and eluted at a rate of about 20 ml. per hour. The 5 ml. fractions of the effluent were collected and the optical density of each fraction determined at a wave length of 280 m μ . Fig. 1, which represents the fractionation pattern of the effluent, indicates that the tetanus toxoid type A can be separated into five fractions. The gel filtration was repeated five times, identical

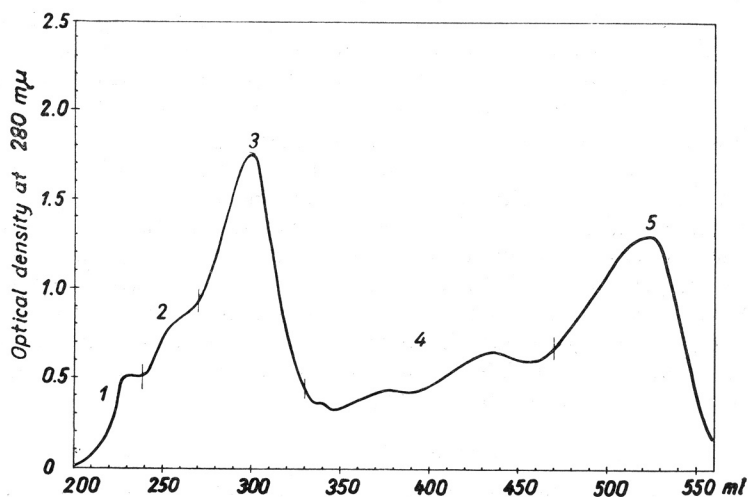


Fig. 1. Gel filtration of tetanus toxoid type A on Sephadex G-200.

fractions were collected and their antigenic activity was determined *in vitro* by flocculation with standard tetanus antitoxin. It is evident from the column 4 of Table I that the flocculation activity was recovered with 100%, most of which (71.5%) was accumulated in the third fraction. The fifth fraction was practically inactive. The protein nitrogen was determined for each fraction and, as evidenced by the column 7 of Table I it was recovered almost quantitatively. Column 8, which shows the purity of all five fractions, expressed in terms of flocculation units per mg. of PN, indicates that fraction 3 is twice as pure as the starting material. The large amount of nonspecific proteins is removed with the last two fractions, which contain together 39.2% of PN, but only 5.2% of the flocculation activity.

TABLE I
Fractionation of tetanus toxoid type A on Sephadex G-200.

Fraction	1 ml.	2 Lf/ml.	3 Lf	4 % Lf	5 mg. PN/ml.	6 mg. PN	7 % PN	8 Lf/mg. PN
Starting material	50	2300	115 000		2.120	106.0		1085
1	185	38	7 050	6.1	0.045	8.3	7.8	850
2	160	136	21 800	18.9	0.088	14.1	13.3	1550
3	283	290	82 000	71.5	0.144	40.8	38.5	2000
4	745	8	5 950	5.2	0.035	26.1	24.6	228
5	550	—	—	—	0.028	15.4	14.6	—
Total recovered			116 780	101.7		104.7	98.8	

The chromatographic procedure for tetanus toxoid type B was performed in the same manner as described for type A. From the fractionation pattern (Fig. 2) it is evident that the first part of the effluent was separated in three

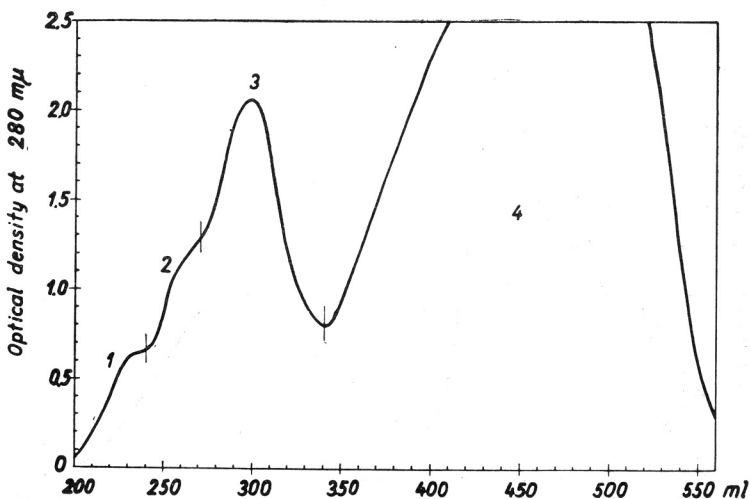


Fig. 2. Gel filtration of tetanus toxoid type B on Sephadex G-200.

fractions similar to the first part of the pattern obtained with the toxoid type A, while the second part of the effluent consists of only one fraction. The specific flocculation activity and protein nitrogen was determined for each fraction and the numerical values are given in Table II. The major portion of the flocculation

TABLE II
Fractionation of tetanus toxoid type B on Sephadex G-200.

Fraction	1 ml.	2 Lf/ml.	3 Lf	4 % Lf	5 mg. PN/ml.	6 mg. PN	7 % PN	8 Lf/mg. PN
Starting material	30	2550	76 500		3.04	91.2		840
1	145	16	2 320	3.0	0.027	3.9	4.3	590
2	112	126	14 110	18.4	0.100	11.2	12.3	1260
3	225	260	58 500	76.5	0.172	38.7	42.4	1510
4	696	5	3 480	4.5	0.048	33.4	36.6	104
Total recovered			78 310	102.4		87.2	95.6	

activity was again contained in the third and second fraction, 76.5% and 18.4% respectively, while most of the protein nitrogen was found in fractions 3 and 4. Column 8 of Table II indicates that the purity of the fraction 3 was increased almost twice and the purity of the fraction 2 was increased by a factor of approximately 1.5 in comparison with the purity of the starting material.

Fractionation of tetanus toxoid type B-1 was performed in the manner previously described. The effluent showed four peaks in the optical density diagram (Fig. 3), and was collected in four pools. The first three were similar to the fractions obtained in gel filtration of the toxoid type A and B, while the fourth fraction had a much lower optical density than in the case of the toxoid type B.

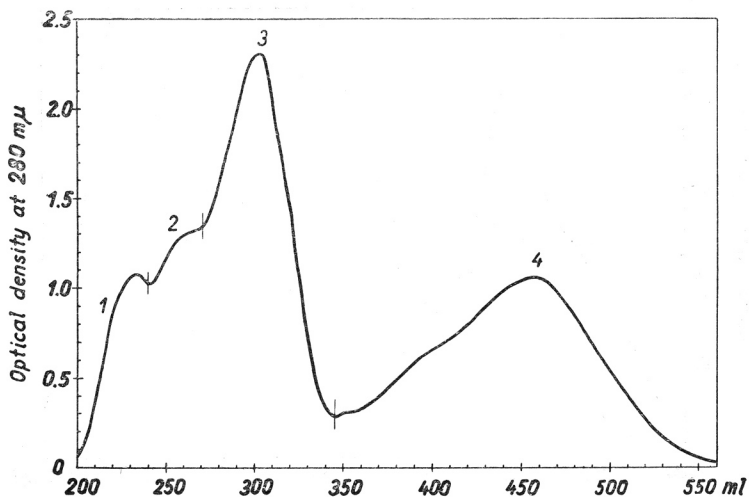


Fig. 3. Gel filtration of tetanus toxoid type B-1 on Sephadex G-200.

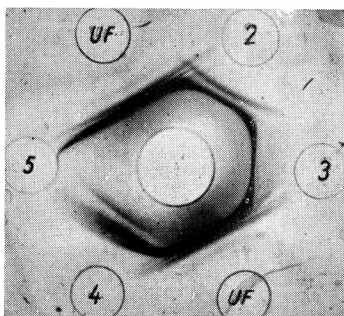


Fig. 4. Immunoprecipitation of the fractions of tetanus toxoid type A.

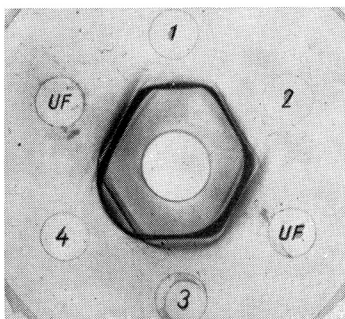


Fig. 5. Immunoprecipitation of the fractions of tetanus toxoid type B.

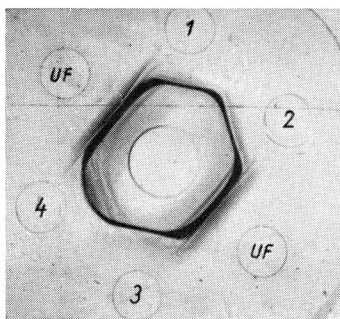


Fig. 6. Immunoprecipitation of the fractions of tetanus toxoid type B-1.

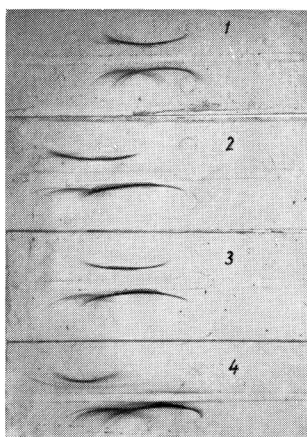


Fig. 7. Immunoelectrophoretic patterns of the fractions of tetanus toxoid type B-1 (upper well of each microslide), compared with the crude ultrafiltrate (bottom well of each microslide). Anode is on the left.

Table III represents the data for specific flocculation activity, protein nitrogen content and purity of the different fractions of tetanus toxoid type B-1. As in previous cases, the third fraction contains most of the antigenic activity *in vitro* and represents the fraction of highest purity.

TABLE III
Fractionation of tetanus toxoid type B-1 on Sephadex G-200.

Fraction	1 ml.	2 Lf/ml.	3 Lf	4 % Lf	5 mg. PN/ml.	6 mg. PN	7 % PN	8 Lf/mg. PN
Starting material	40	2950	118 000		2.100	84.0		1400
1	165	30	4 950	4.2	0.029	4.8	5.7	1030
2	126	188	23 700	20.1	0.110	13.9	16.5	1710
3	293	300	87 900	74.5	0.125	36.6	43.6	2400
4	708	3.5	2 480	2.1	0.038	26.9	32.0	92
Total recovered			119 040	100.9		82.2	97.8	

Immunochemical analyses of each type of tetanus toxoid were performed by gel diffusion technique and results are presented in Figs. 4—6. The fractions and crude ultrafiltrate, all at concentration of 500 Lf/ml., were placed in the outer wells. The center wells contained the standard horse tetanus antitoxin in concentration of 250 I. U./ml. Precipitation lines fully correspond to the flocculation units and degree of purity obtained for individual fractions.

Tetanus toxoid type A (Fig. 4) has a homogeneous fraction 3, while fraction 2 consists of two components. Fraction 4 shows several precipitation lines, but they are all very diffuse, which indicates the presence of comparatively small quantities of antigenic material and is likely to represent the degradation products of the toxoid. Fraction 5 produced no precipitation at all, thus representing a material without antigenic properties. Fraction 1 is not presented in Fig. 4 due to lack of the material.

Tetanus toxoid type B (Fig. 5) shows the lowest degree of purity of all fractions.

Fraction 3 of the toxoid type B-1 (Fig. 6) shows a single specific precipitation line, indicating the homogeneous material. Both fractions 1 and 2 show beside the main precipitation line and additional weak line indicating a two component system. Fraction 4 gives a high number of precipitation lines representing the contaminating material from the crude ultrafiltrate.

The lines obtained by immunoelectrophoresis confirm the results obtained by gel diffusion. Fig. 7 shows immunoelectrophoretic lines of all four fractions of the tetanus toxoid type B-1. Fractions 1 and 2 are similar by their types of lines, fraction 3 again consists of a single component, while fraction 4 gives at least 5 precipitation lines. In respect to the ultrafiltrate all individual fractions show a more or less increased electronegativity, possibly due to the increased mobility as a consequence of the removal of the corresponding components from the mixture.

Several other lots of tetanus toxoid of all three types were chromatographed on the same column with similar results. In a single run, without recycling,

the specific flocculation material of the highest purity appeared always in the third fraction, but the absolute value of its purity varied and was dependent on the purity of the starting material. Best results were obtained with toxoid type B-1, from which most of the nonspecific low-molecular polypeptides were removed prior to the action of formaldehyde. This is in accordance with the findings of Fulthorp and Thomson¹, who have shown that purified or ultrafiltered tetanus toxin preparations have a relatively small capacity for fixing formaldehyde, while crude toxic filtrates have a considerable capacity to do so. The difference in the formaldehyde-fixing power of crude toxic filtrates and purified toxins is explained by the authors as being due to the formation of methylene cross linkages between the amino groups of different molecules, which explains the fact that it is not possible to purify the toxoid to the same degree as can be effected with the toxin, and that soluble coloured products present in crude toxic filtrates, which can be ultrafiltered out before treatment with formalin, can no longer be removed in this way after detoxication.

Turpin and Raynaud¹⁴ have found that both tetanus toxin and toxoid may exist in different states of aggregation. They have isolated two molecular species of toxoid having sedimentation coefficients of 4.9 S and 7.2 S respectively. In our preliminary ultracentrifugal studies our third fraction of B-1 type appeared as a single peak and had a sedimentation coefficient of 7.5 S (at the concentration of 0.15%). Since the first and second fractions represent the higher molecular weight species containing substantial amount of the specific flocculation activity, it can be expected that aggregations higher than those described by Turpin and Renaud might exist.

It can be concluded from the results of our work that fractional purification of tetanus toxoid can be effectively achieved by gel filtration on Sephadex G-200, but the effect of purification is highly dependent on the quality of the starting material.

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IZVOD

Frakcioniranje tetanus toksoida filtracijom kroz Sephadex gel i imunokemijska karakterizacija frakcija*M. Fleš i B. Pende*

Provedeno je frakcioniranje tri tipa tetanus toksoida filtracijom kroz Sephadex G-200 gel. Tip A je priređen detoksikacijom sirovog tetanus toksina, ultrafiltracijom dobivenoga sirovog toksoida i frakcioniranim taloženjem pomoću amonijum-sulfata. Tip B je priređen ultrafiltracijom sirovog tetanus toksina i detoksikacijom ultrafiltrata. Tip B-1 je dobiven iz tetanus toksoida tipa B frakcioniranim taloženjem sa amonijum-sulfatom.

Distribucija frakcija načinjena je na temelju mjerenja apsorpcije svjetlosti eluata kod 280 m μ . Tip A je razdijeljen u 5 frakcija, a B i B-1 u 4 frakcije. Specifična aktivnost frakcija *in vitro* određena je flokulacijom sa standardnim tetanus anti-toksinom, imunodifuzijom u agar gelu i imunoelektroforetski. Kod sva tri tipa toksoida aktivna komponenta se pojavljuje u prve tri frakcije, od čega se oko 75% nalazi u trećoj, a oko 20% u drugoj frakciji. Četvrta i peta frakcija su slabo aktivne i predstavljaju nespecifični materijal. Čistoća treće frakcije izražena u Lf/mg proteinskog nitrogena (PN) povećana je oko dva puta, a druge frakcije oko 1,5 puta s obzirom na ishodni materijal. Najveća čistoća (2400 Lf/mg PN) postignuta je u trećoj frakciji toksoida tipa B-1. Ova frakcija se pokazala homogena i u testovima imunodifuzije i imunoelektroforeze.

Ovim radom je pokazano da gel filtracija predstavlja brzu i ekonomičnu metodu purifikacije tetanus toksoida, ali da apsolutna vrijednost čistoće frakcija ovisi o načinu priređivanja i čistoći ishodnog materijala.

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