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CHEMICAL COMPOSITION OF PHASE I *COXIELLA BURNETII* SOLUBLE ANTIGEN PREPARED BY TRICHLOROACETIC ACID EXTRACTION

M. LUKÁČOVÁ, R. BREZINA, Š. SCHRAMEK, J. PASTOREK

Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava, Czechoslovakia

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Summary. — Optimal conditions of extraction (time and temperature) by trichloroacetic acid of soluble antigen from phase I *Coxiella burnetii* (TCAE), possessing protective properties and used as a chemovaccine against Q fever in men, were studied. Extracts prepared under various conditions were analysed for their polysaccharide, protein and phosphorus contents. Forty-five min of extraction at 0 °C were sufficient to obtain a soluble antigen reacting in immunodiffusion with hyperimmune rabbit antiserum. The polysaccharide contents decreased with prolonged extraction at 0 °C. At higher extraction temperatures (37 and 100 °C), the polysaccharide contents increased while that of proteins decreased. TCAE prepared at 100 °C gave no positive immunodiffusion reaction.

Key words: *Coxiella burnetii phase I*; *trichloroacetic acid extraction*; *soluble antigen*

Introduction

Extraction by trichloroacetic acid of purified phase I *Coxiella burnetii* suspensions yields soluble antigen (TCAE) possessing phase I antigen reactivity (Brezina and Urvölgyi, 1961, 1962). Various extraction conditions have been employed: 6 hr at 0 °C with 8 % trichloroacetic acid (Brezina *et al.*, 1962), later 4 hr at 0—4 °C with 6 % trichloroacetic acid (Brezina, 1977) or 6 hr at 4 °C with 10 % trichloroacetic acid (Kazár *et al.*, 1978). At present, the chemovaccine is prepared by extraction at 0 °C for 4 hr with 10 % trichloroacetic acid. TCAE represents a protein — lipopolysaccharide complex; its chromatography on DEAE-cellulose yielded five fractions, three of which were active in the complement-fixation reaction (Brezina and Schramek, 1968). By its properties it resembled extracts obtained by the same method from Gram-negative bacteria (Schramek and Brezina, 1974). Basic chemical analyses of TCAE were reported by Anacker *et al.* (1963) and Schramek and Brezina (1974). In laboratory animals (mice and rabbits), TCAE induces the formation of antibodies against antigens 1 and 2, which

was demonstrated by complement fixation and microagglutination reactions and the opsonization phagocytosis and serum protective tests (Kazár *et al.*, 1978). It also induces the cell-mediated component of immunity (Kazár *et al.*, 1983). It offers efficient protection against Q fever and has been employed as a chemovaccine for human use (Cracea *et al.*, 1973; Brezina *et al.*, 1974). While vaccination with killed *C. burnetii* suspensions may result in unwanted local and total reactions in the vaccines, such side effects have not been recorded after vaccination with TCAE (Brezina *et al.*, 1974). The use of TCAE as a chemovaccine in men has been discussed in detail by Brezina (1977). It offers protection against Q fever (Brezina *et al.*, 1981; Kazár *et al.*, 1982, 1983). So far, the effects of extraction conditions on the chemical composition of TCAE have not been tested. They were the subject of the present investigations.

Materials and Methods

C. burnetii strain Nine Mile in phase I (3 EP) was propagated in chick embryo yolk sacs and purified by differential centrifugation and ether treatment (Ormsbee, 1962). The kinetics of extraction with trichloroacetic acid was investigated as follows: suspensions of 20 mg of *C. burnetii* cells in 20 ml of 10 % trichloroacetic acid were stirred at 0, 37 and 100 °C for 45 min and 2 and 4 hr. One-ml samples were taken at regular intervals, neutralized, centrifuged and the supernatants were assayed for polysaccharide contents. After the end of extraction, the suspensions were immediately adjusted to pH 7 with 30 % NaOH and centrifuged. The supernatants were dialysed against tap water and then against distilled water. Dialysis was checked by measuring the conductivity of the latter. After dialysis, the extract was filtered through a membrane filter with pores of 0.2 μm diameter, concentrated *in vacuo* and lyophilized. For chemical analyses and immunodiffusion reactions, 1 mg/ml TCAE suspensions in distilled water were used.

Chemical analyses. Polysaccharide contents (total sugars) were determined spectrophotometrically by the phenol — sulphuric acid reaction, using glucose as standard (Dubois *et al.*, 1956). Proteins were determined spectrophotometrically with Coomassie Brilliant Blue G-250, using bovine serum albumin as standard (Bradford, 1976). Phosphorus was assayed spectrophotometrically based on the reaction with ammonium molybdate (Lowry *et al.*, 1954).

Immunodiffusion reactions were carried out in Petri dishes in 1 % agar dissolved in physiological saline (Ouchterlony, 1948). Hyperimmune rabbit serum containing antibodies against antigens 1 and 2 was obtained by immunizing rabbits with two intraperitoneal doses of 2 mg each of *C. burnetii* in phase I with complete Freund's adjuvant given at an interval of 20 days. Immunization of rabbits with phase II *C. burnetii* was carried out similarly.

Sodium dodecyl sulphate — polyacrylamide gel electrophoresis (SDS-Page) was carried out according to Laemmli (1970), using 8—18 % acrylamide gradients. Silver staining of the gels was employed (Tsai and Frasch, 1982) as for lipopolysaccharides (LPS).

Results

Kinetics of polysaccharide extraction with trichloroacetic acid

Fig. 1 illustrates the course of polysaccharide extraction from *C. burnetii* under various conditions. The curves at 0 and 37 °C reached their maxima after 45 min and 2 hr, respectively. The polysaccharide contents of TCAE decreased with longer extraction time. Both curves showed a similar course. The course of the 100 °C curve was different: the polysaccharide contents first increased and then remained at the same level. Similarly, the phosphorus contents of the 0 °C extract did not change with time (results not shown).

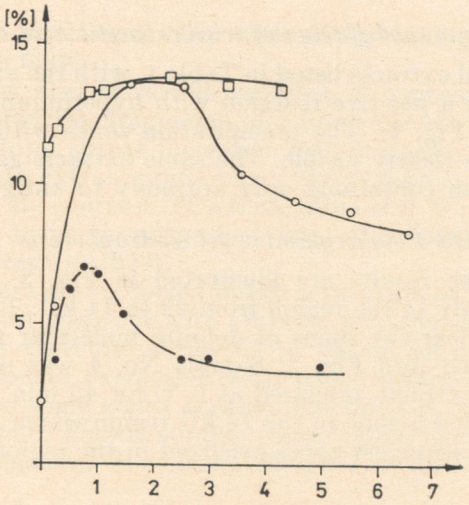

Fig. 1.
Relationship between polysaccharide contents and temperature and time extraction of *C. burnetii* with trichloroacetic acid
□——□ 100 °C
○——○ 37 °C
●——● 0 °C
Abscissa: time in hr; ordinate: polysaccharide content in *C. burnetii* (per cents).

Chemical analyses of trichloroacetic acid extracts prepared under various conditions

The extraction conditions, the yields of extracts and their composition are summarized in Table 1. The highest yield was obtained at 37 °C; the yields obtained after various extraction times at 0 °C were mutually similar, showing a decreasing tendency with time. The yield at 100 °C was the lowest. The polysaccharide content was the highest at 37 °C; at 0 °C, it decreased with the time of extraction.

The protein contents decreased with increasing extraction temperature and time. Phosphorus contents was less affected by the conditions of extraction; it was the highest at 100 °C.

Table 1. Effect of temperature and time of extraction of C. burnetii with trichloroacetic acid on the yield and composition of lyophilized extracts

Extract No.	Temperature °C	Time	Yield %*	Total sugars %**	Protein %**	Phosphorus %**
1	0	45 min	16.6	40.3	16	1.1
2	0	2 hr	14.2	28.2	8.2	1.05
3	0	4 hr	13.9	23.7	8.1	1.08
4	37	2 hr	23.0	57.8	8.0	1.3
5	100	2 hr	10.9	51.0	5.1	2.0

* In per cent related to *C. burnetii* mass.
** In per cent related to the mass of lyophilized extract.
An analysis of purified *C. burnetii* cells yielded the following results: sugars 15.7 %, proteins 36.9 % and phosphorus 2.6 %.

Immunodiffusion of trichloroacetic acid extracts

All extracts listed in Table 1, with the exception of that obtained at 100 °C, gave a positive reaction with hyperimmune serum against antigens 1 and 2 (see Fig. 2). The precipitation zones with extracts prepared at 0 and 37 °C were clearly visible. The same extracts gave no reaction with hyperimmune serum containing only antibody to antigen 2.

SDS-Polyacrylamide gel electrophoresis

The results are illustrated in Fig. 3. The TCAEs shown yielded zones mainly in the region from 22 to 14 kD. The extract prepared at 100 °C gave no clear-cut zones of definite molecular masses and was, therefore, not included into Fig. 3. Sample No. 4 was not filtered. Samples 3 + 7 and 5, i.e. extracts prepared at 0 °C for 45 min and at 37 °C for 2 hr respectively, yielded a zone in the 15 kD region which did not occur in the other samples. The strongest zones occurred in the regions of 16 and 14.2 kD.

Discussion

Evaluation of the extraction of *C. burnetii* by trichloroacetic acid based on sugar contents of the extracts showed that shorter extraction times are preferrable. At 0 and 37 °C, the polysaccharide contents decreased with extraction time. Longer action of trichloroacetic acid evidently exerted an adverse effect on the polysaccharides, ending in their destruction. Extraction at 100 °C proceeded differently from that at 0 or 37 °C: *C. burnetii* cells were largely destroyed. Nermut *et al.* (1972) treated *C. burnetii* cells with trichloroacetic acid in the cold and at 60 °C. At the latter temperature, the cell wall was completely separated. In the cold this process was only partial. This means that at high temperatures (100 °C) trichloroactic acid induces profound changes in the cell wall of *C. burnetii*.

Polysaccharide and protein contents of TCAEs prepared under various conditions varied (see Table 1). The results were the most favourable for the extract prepared at 0 °C for 45 min — it contained more polysaccharides and proteins than extracts prepared by extractions lasting longer. At 0 °C, the phosphorus contents remained unchanged. Extracts prepared at 37 and 100 °C contained more polysaccharides and phosphorus and less proteins. Anacker *et al.* (1963) and Schramek and Brezina (1974) analysed TCAEs prepared at 0 °C. They used the tryptophan and anthron methods, respectively, for polysaccharide determination, and found 25.2 % (Anacker *et al.*) and 18—22 % (Schramek and Brezina) polysaccharides in their TCAEs. In *C. burnetii* we found 15.7 % polysaccharides by our method (see Table 1) as compared to 7.5 % reported by Anacker *et al.* Schramek and Brezina used Lowry's method and found 23—27 % of proteins in TCAE. Our results obtained by Bradford's method with Coomassie Brilliant Blue G-250 were lower — many factors interfere in Lowry's method. The lower values of protein contents in TCAEs were supported by the results of PAGE. Staining of gels with Coomassie Brilliant Blue R 250 yielded blue protein zones only when 5-fold

amounts of samples were loaded on the gels than with silver staining, and even then the protein zones were faint.

Trichloroacetic acid as a strong acid causes degradation of proteins and polysaccharides; at higher temperatures, fragments of a low molecular mass result. Although the amount of total sugars increased with increasing temperature of extraction, PAGE of the extract prepared at 100 °C gave no clear-cut separation of the components. By contrast, TCAE prepared at 0 °C proved to be thermostable, retaining its antigenic properties even after heating for 2 hr at 100 °C (Schramek and Brezina, 1974).

The conditions of extraction were also reflected in the separation of TCAE by PAGE. The zones were the most clear-cut with extracts prepared at 0 °C for 45 min. Forty-five min were therefore sufficient to obtain an extract adequate to those obtained by longer extraction with trichloroacetic acid. Following longer extraction times some zones became less distinct (21 and 17 kD), this confirming the results of kinetic studies. Since TCAE contained less proteins and more polysaccharides, the zones observed in PAGE represented mainly the latter.

The results obtained with extraction at 37 °C were interesting. This temperature favourably affected the extraction of polysaccharides, the zones in PAGE were similar to those obtained with extract prepared at 0 °C for 45 min. At 37 °C, the yield of TCAE was the highest.

In pilot tests on the immunogenicity in mice of extracts prepared at 0 °C for 45 min and at 37 °C for 2 hr, the results were similar to those obtained by Kazár *et al.* (1978) with standard vaccine preparations (0 °C, 4 hr).

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Legend to Figures (Plates XI—XII):

Fig. 2. Immunodiffusion reactions of TCAE (1 mg/ml) with hyperimmune rabbit serum against *C. burnetii* antigens 1 and 2 (central well). Peripheral wells: I — TCAE 0 °C, 45 min: II — TCAE 100 °C, 2 hr; III — TCAE 0 °C,4 hr; IV TCAE 37 °C, 2 hr; V — saline; VI — TCAE — 0 °C, 2 hr.

Fig. 3. SDS-PAGE of trichloroacetic acid extracts from *C. burnetii* Ag-LPS stain.
1 — Markers; 2 — TCAE 0 °C, 4 hr; 3 — TCAE 0 °C, 45 min; 4 — standard vaccine, 0 °C, 4 hr; 5 — TCAE 37 °C, 2 hr; 6 — TCAE 0 °C, 2 hr; 7 — TCAE 0 °C, 45 min.