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PURIFICATION OF CYSTOZOITES OF SARCOCYSTIS SP. BY THE CHROMATOGRAPHIC GEL SPHERON

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Abstract. Cystozoites of *Sarcocystis* sp. isolated from beef muscles were purified by elution through Spheron chromatographic gel. Using the number of cystozoites recovered and the purity of suspension as criteria, the effect of length of gel column, rate of elution, and ionic strength of the eluent was evaluated. The optimum purity was achieved at the elution rate of 10 ml/min and column length of 100 mm. The recovery of cystozoites (38—56 %) was in correlation to the ionic strength of the eluent. Of the current isotonic solutions, Tyrode's solution was found to be the best eluent. Preliminary assays with different coccidia indicate that the method could be used for the study of a variety of parasitological topics.

For biochemical and immunological analyses, as well as for in vitro cultivation of sarcocysts, it is essential to recover a sufficient amount of cystozoites which are not contaminated by remnants of muscle tissue. A pure suspension of cystozoites cannot be obtained but from macroscopic cysts of *Sarcocystis gigantea* (Railliet, 1886) Ashford, 1977.

There is a number of methods which were worked out in order to isolate and purify trophozoites of *Toxoplasma gondii*. With all of them it is vital that the material contains a large amount of parasites. That is seldom the case with sarcocysts; as a rule, there is a comparatively low concentration of cystozoites and abundant muscle detritus. Thus the methods convenient for *Toxoplasma* are rarely applicable for collecting purified cystozoites.

Therefore we aimed at working out a simple method for the isolation and purification of *Sarcocystis* cystozoites which would not require pretentious equipment and hardly available substrates. Our assay was based on the method of purifying coccidia sporozoites by elution through a column of glass beads (Wagenbach 1969). In our modification, the glass beads were replaced by Spheron chromatographic gel (Lachema).

MATERIAL AND METHODS

Isolation of *Sarcocystis* cystozoites. Oesophagi as the source of cystozoites were collected from currently slaughtered cattle. After removing the epithelium and connective tissue, the oesophagus musculature was grinded in a meat mill and under permanent stirring exposed to artificial peptic juice (Sharma and Dubey 1981) at 37 °C for 10 minutes. The digest was filtered through several layers of gauze and centrifuged at 3,000 rpm for 10 minutes. The sediment was resuspended in respective eluents and the suspension was administered on the gel column.

Preparation of eluents. The following solutions were employed: Ringer's, Tyrode's, Hanks's, PBS (phosphate buffered saline), and MEM (minimum essential medium).

Primary Ringer's solution was prepared as described by Wagenbach (1969). Solutions of different ionic strengths were obtained by its dilution. Their osmolarity was adjusted by glucose (Lanham and Godfrey 1970). Tyrode's and Hanks's solutions and PBS were prepared as described by Ferenčík et al. (1981), MEM as instructed by the producer (ÚSOL Praha), pH was adjusted to 7.5 by phenol red.

Chromatographic gel and preparation of columns. To the hydrophilic hydroxy-alkyl-metacrylate gel Spheron 1000 (Lachema Brno) with particles of 100—200 μm was allowed to swell in the

respective eluents for 12 hours. After de-aeration, the gel was filled into glass columns (200 mm in length and 25 mm in diameter). The eluents were supplied to columns by means of TS 201 transfusion set. Steady hydrostatic pressure was achieved on the principle of Mariotte's bottle.

Evaluation of properties of columns and course of purification. The length of the gel columns, rate of elution, and ionic strength of eluent were successively altered. The pivot assay was carried out with the primary Ringer's solution. The remaining solutions were tested by comparison to it, and evaluation of the recovery of cystozoites and purity of suspension:

a) Length of column. At a constant flow rate of Ringer's solution, columns of 50, 100 and 150 mm were tested.

b) Rate of elution. With gel columns of 100 mm length, the rate of flow of Ringer's solution was adjusted to 5 ml, 10 ml, and 20 ml/min, respectively.

c) Ionic strength of eluents. At the constant 100 mm length of column and 10 ml/min elution rate, Ringer's solutions of different ionic strengths were tested.

Counting of cystozoites. Coulter Counter model ZF. (Aperture 707, attenuation 32, and treshold 15) was employed for counting.

Evaluation of the course of purification. The eluate containing cystozoites was successively caught into calibrated test tubes. To prevent the sedimentation, 2 ml of a methanol-glycerol mixture (1 : 1) was added to each sample. The number of cystozoites in each sample was expressed as the percentage of total number of cystozoites in 100 ml.

Rate of recovered cystozoites. A suspension consisting of a known number of cystozoites and digested musculature from bovine fetuses (void of sarcocyst was prepared). It was supplied on the column, and the cystozoites were counted after elution.

Purity of suspension. The purity was tested microscopically at 500× magnification and recorded as the relation of cystozoites number to the number of foreign particles.

For the statistical evaluation of all results, limits of confidence were calculated.

Vitality of purified cystozoites. In order to find out whether and how the cystozoites survived the process of purification, we made use of tissue cultivation and assays in definitive hosts:

a) Tissue cultures. A monolayer of dog kidney cells (MDKC) on a object carrier was kept submerged in MEM with 10 % of bovine serum in plastic dishes. Purified cystozoites were resuspended in the same mixture and administered on the MDKC. After two hours of incubation, the supernatant was decanted and fresh cultivation substrate was supplied on the layer. 2, 5, 12, and 24 hours after inoculation, the object carrier with the monolayer was removed, stabilized by Bouin's fixative and dyed by Ehrlich's hematoxylin.

b) Assays in definitive hosts. Two kittens and two pups were orally administered a suspension of purified cystozoites. The infection was repeated three times at intervals of 24 hours, each dose containing 8 to 10 million cystozoites. The faeces were microscopically examined daily till day 20 after the first administration of cystozoites.

RESULTS

Effect of the length of column. The effect of the length of gel columns on the recovery of cystozoites and purity of suspension is evident from Table 1.

The column of 50 mm yielded the highest amount of cystozoites, however, to the detriment of purity. With columns of 100 and 150 mm, there were no significant differences in the recovery and purity. We therefore preferred columns of 100 mm which were more economic.

Table 1. Effect of length of gel column on the recovery of cystozoites and purity of suspension

Number of columns	Length of gel column (mm)	Recovery of cystozoites (%)	Purity of suspension
		$\bar{x} \pm s_{\bar{x}} \cdot t$	$\bar{x} \pm s_{\bar{x}} \cdot t$
5	50	41.4 ± 1.22	143 ± 81
5	100	38.5 ± 1.08	222 ± 32
5	150	38.2 ± 0.92	243 ± 52

Eluent — Ringer's solution, elution rate — 10 ml/min, purity of suspension — number of cystozoites/1 foreign particle.

Effect of elution rate. The effect of the rate of flow of the eluent on the recovery of cystozoites and purity of suspension is illustrated in Table 2. The yield of cystozoites is in direct proportion to the elution rate. The difference between the elution rates of 5 ml/min and 10 ml/min was insignificant, and therefore the rate of 10 ml/min was used.

Table 2. Effect of elution rate on the recovery of cystozoites and purity of suspension

Number of columns	Elution rate (ml/min.)	Recovery of cystozoites (%)	Purity of suspension
		$\bar{x} \pm s_{\bar{x}} \cdot t$	$\bar{x} \pm s_{\bar{x}} \cdot t$
5	5	33.6 ± 0.91	283 ± 68
5	10	38.5 ± 1.08	222 ± 32
5	15	58.5 ± 3.66	72 ± 52

Eluent — Ringer's solution, length of gel columns — 100 mm, purity of suspension — number of cystozoites/1 foreign particle.

Effect of ionic strength. The effect of the ionic strength of eluent on the recovery of cystozoites and purity of suspension is demonstrated in Table 3. There was a direct relation between the purity of the suspension and ionic strength of the eluent, and an indirect relation between the recovery of cystozoites and ionic strength. No significant differences were registered when eluents of 0.220 and 0.330 ionic strength were employed. With regard to the purity we consider the solution of the highest ionic strength the most convenient.

Table 3. Effect of ionic strength of the eluent on the recovery of cystozoites and purity of suspension

Number of columns	Ionic strength of eluent	Recovery of cystozoites (%)	Purity of suspension
		$\bar{x} \pm s_{\bar{x}} \cdot t$	$\bar{x} \pm s_{\bar{x}} \cdot t$
5	0.330	38.5 ± 1.08	222 ± 32
5	0.220	39.6 ± 2.82	172 ± 63
5	0.110	44.6 ± 2.98	92 ± 89

Eluent — Ringer's solution, length of gel columns — 100 mm, elution rate — 10 ml/min, purity of suspension — number of cystozoites/1 foreign particle.

The indirect dependence of the recovery on the ionic strength is also obvious from Fig. 1, which illustrates the course of elution of cystozoites at different ionic strenghts of the eluent.

Our conclusion concerning the effects of the ionic strength was supported by an assay performed with Tyrode's solution as eluent: the mean recovery of cystozoites

was 56 %, and one foreign particle was associated to 381 cystozoites (Fig. 2). The rest of the eluent was tested orientationally, without exactly determining the recovery and purity. Nevertheless, the tests showed that also other isotonic solutions were convenient for the purification of cystozoites.

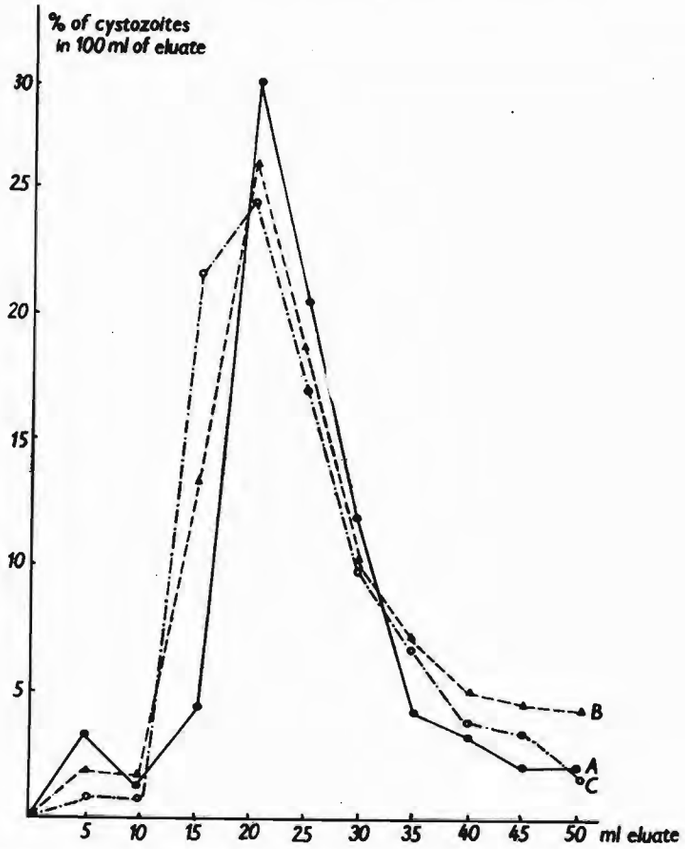

Fig. 1. Course of elution of cystozoites of *Sarcocystis* sp. at different ionic strength of eluent. Eluent-Ringer's solution. Ionic strength: A — 0.110, B — 0.220, C — 0.330. Length of gel columns — 100 mm, elution rate — 10 ml/min.

Effect of proportion of cystozoites in the initial material. It had to be clarified whether or not the intensity of infestation of muscle tissue by the cystozoites affected the recovery and purity (Table 4). Neither the recovery of cystozoites nor the purity of suspension were influenced by the initial intensity of infestation. The reverse test with different amounts of musculature and constant number of cystozoites was not performed; we only made experience that more than 50 g of muscle tissue fouled the gel column.

Vitality of purified cystozoites. Both in vitro and in vivo tests made it evident that cystozoites suffered no harm by the process of isolation and purification. In tissue

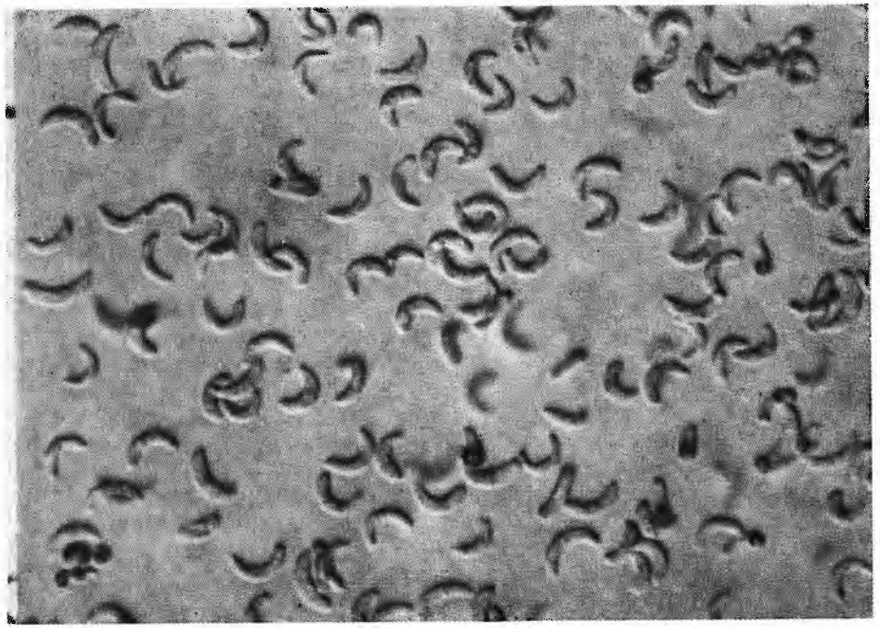

Fig. 2. Cystozoites of *Sarcocystis* sp. after purification by Spheron chromatographic gel. Native (×950).

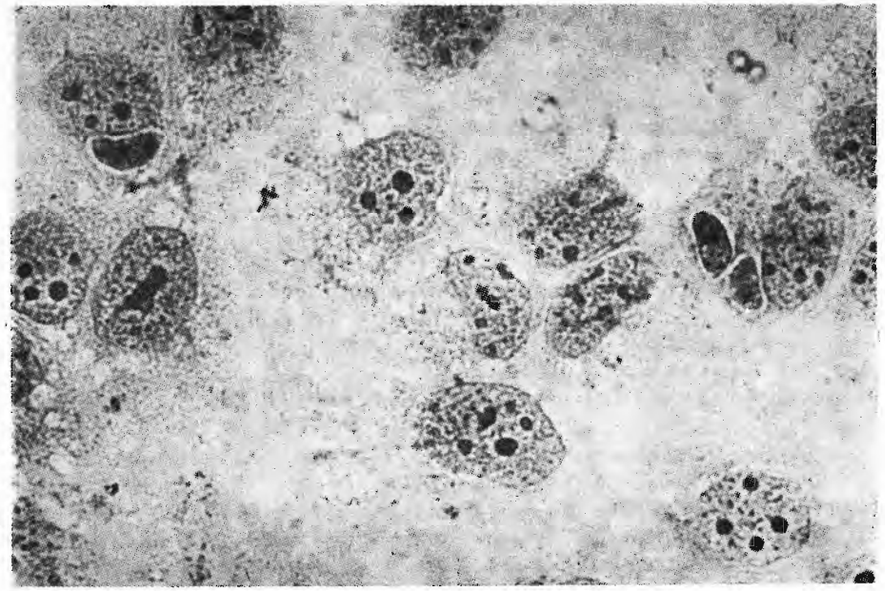

Fig. 3. Cystozoites of *Sarcocystis* sp. within MDCK cells 2 h p.i. Ehrlich's hematoxylin (×1 250).

Table 4. Effect of proportion of cystozoites in initial material on the recovery of cystozoites and purity of suspension

Number of columns	Amount of musculature from bovine fetuses	Number of cystozoites per column	Recovery of cystozoites (%)	Purity of suspension
	(g)	(10^3)	$\bar{x} \pm s_{\bar{x}} \cdot t$	$\bar{x} \pm s_{\bar{x}} \cdot t$
5	50	1.260	38.5 ± 1.08	222 ± 32
5	50	582	39.8 ± 1.84	218 ± 56
5	50	306	39.2 ± 2.52	240 ± 42

Eluent — Ringer's solution, length of gel columns — 100 mm, elution rate — 10 ml/min, purity of suspension — number of cystozoites/1 foreign particle.

cultures, the purified cystozoites entered the cells after supplying them on a monolayer of dog kidney cells (Fig. 3).

Faeces from experimental kittens and pups contained sporocysts from day 11 after the first oral dose of purified cystozoites. The elimination of sporocysts remained more or less constant over the whole period of the assay.

DISCUSSION

Considering the morphological and biological similarities, the purification of cystozoites of *Sarcocystis* sp. and trophozoites of *Toxoplasma gondii* is supposed to be equal. With *Toxoplasma*, rough material to be purified is mostly peritoneal exudation from experimentally infected mice or *Toxoplasma* grown on tissue cultures rich in trophozoites (Gracheva et al. 1973, Valkoun and Jíra 1975).

Some methods are based on destroying host cells (Tryon et al. 1978, Mirovský and Valkoun 1981). Hoshimo-Shimizu et al. (1980) isolated trophozoites from host cells by means of lectins. Masihi and Jíra (1979) separated different *Toxoplasma* stages by density gradient centrifugation.

To separate *Toxoplasma* from host cells in tissue cultures, Valkoun (1981) employed paper filtres and density gradient centrifugation followed by trypsin digestion. Cornellissen et al. (1981) and Blewett et al. (1983) isolated tissue cysts and trophozoites of *T. gondii* using Percoll colloidal silica gel solution for the density gradient centrifugation, and so did Bos and Panhuijzen (1982) to separate sporozoites of *Eimeria tenella*. The best results in separating coccidia sporozoites were achieved by Stotish et al. (1977) by centrifugal elutriation. The method, however, requested a special device.

Our assays were modelled on Wagenbach's (1969) method of purifying coccidia sporozoites by a column of glass beads. We modified it by replacing glass beads by chromatographic gel. Notoriously, chromatographic gels have been used to separate different *Trypanosoma* and *Plasmodium* stages (Lanham and Godfrey 1970, Mack et al. 1978, Alvarenga and Breuer 1979, Villalta and Leon 1979). Grimwood et al. (1979) also applied the principle of chromatography when working with a column of glass wool to separate *Toxoplasma* trophozoites and debris of tissue from cultures. Likewise, Kazuyki et al. (1977) purified suspensions of trophozoites by cellulose powder.

We employed the Spheron chromatographic gel (Lachema) whose advantage is a chemical and mechanical stability. Biologically, Spheron is inert and not prone to any microorganisms. Like Wagenbach (1969) we found that the column length of 100 mm and elution rate of 10 ml/min were optimum parameters.

Čech et al. (1977) used Spheron for the separation of the virus of tobacco mosaic and its extraction from infected leaves. Our intention was to separate *Sarcocystis* cystozoites from muscle tissue. In both cases the purification was successful probably due to the interactions between the hydrophobic groups from muscle debris and hydrophilic groups from chromatographic gel. The assumption was corroborated by our results concerning the effect of ionic strength on the recovery of cystozoites and purity of their suspension. The elevation of the ionic strength resulted in an increased intensity of interaction between the gel and hydrophobic protein groups. The same reason can be assumed for the comparatively smaller recovery and increased purity of cystozoite suspension when the eluent displayed higher ionic strength. The relationship between the course of purification and ionic strength is illustrated in Fig. 1. There is a comparatively increased recovery, elevated number of foreign particles, and retarded maximum elution associated with lower ionic strength of the eluent; the reason for it is again the likely intervention of ionic strength affecting links between the chromatographic gel and tissue debris. The registered recovery of cystozoites ranged from 38 to 56 %, depending on the eluent used. The authors who purififfed trophozoites of *T. gondii* reported recoveries of 75 to 99 %. Our results are comparable with the reports concerning isolation of protozoa by chromatographic methods.

The vitality of cystozoites was in no way affected by the process of purification by Spheron. It was verified both by tissue cultivating and biological assay. The results with tissue cultures were in agreement with the results reported by Becker et al. (1979).

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ОЧИСТКА ЦИСТОЗОИТОВ *SARCOCYSTIS* SP. С ПОМОЩЬЮ ХРОМАТОГРАФИЧЕСКОГО ГЕЛЯ СФЕРОНА

Б. Коудела

Резюме. Цистозоиты *Sarcocystis* sp., выделенные из мышцев крупного рогатого скота очищали при помощи хроматографического геля Spheron. Изучали влияние длины колонны геля, скорости элюции и ионной силы элюента на количество полученных цистозоитов и чистоту суспензии. Оптимальная чистота получилась при скорости элюции 10 мл/мин и длине колонны 100 мм. Выделенное количество цистозоитов (38—56 %) находится в соотношении с ионной силой элюента. Среди обычно применяемых изотонических растворов раствор Тайрода оказался самым удобным элюентом. Предварительные опыты с разными кокцидиями показывают, что этот метод можно использовать при изучении разных паразитологических проблем.

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