PREPARATION OF *Toxocara canis* ANTIGENS

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Abstract

Studies were conducted on the determination of protein concentration in lyophilized adult and eggs crude antigen of *T. canis* worms. The gel filtration column with sephadex G-200 was used to purify adult crude antigen (ACA). The result of purification was found with molecular weight of 35481.33 dalton. The protein concentration of adult and eggs crude antigen were 449 and 290 µg/ml, respectively. In the present study a new technique for cultivation of 2nd stage larvae of *T. canis* was used with 1640 RPMI media to purify execretory /secretory antigens (E/S) and Tegumental larval antigen (TES). Protein concentration was estimated for E/S and TES antigens and it was 520 and 351 µg/ml, respectively.

Key word

Antigens, *Toxocara canis,* column chromatography

Part of Ph.D thesis

Introduction

Toxocara canis is a remarkable nematode parasite, commonly found in dogs but can invade a wide range of hosts including humans (Lewis and Maizels, 1993). Stirewalt (1963) considered that the products which are secreted by nematodes hold the key to their success as parasite.Welch *et al.* (1983) notified that adult crude antigen of *T. canis* contain at least 12 different proteins and 9 different antigens when purified through affinity column. De Savigny (1975) was the first to demonstrate that *T. canis* larvae could be cultured for long time and the supernatents of the culture contained antigens which is used specifically in diagnosis of human toxocariasis. Infective larvae of *T. canis* survive in their hosts for extended periods in a state of developmental arrest, successfully evading immune destruction. This survival strategy is thought to be mediated by *T. canis* excretory/secretory products which regulate or divert the immune response (Loukas *et al*., 2000).

T. canis larvae secrete at least 50 distinct macromolecules by two dimensional gel analysis technique (Page *et al*., 1992). Furthermore, Robertson *et al.* (1989) reported that *Toxocara* larvae secreted protease with a molecular weight of 12000 dalton. Also, the external surface of *T. canis* larvae is covered by carbohydrate glycocalyx as a surface coat (Maizels and Page, 1990; Maizels, 2004).

The aim of the present study is to detect the protein contents of adult crude, eggs crude, E/S and TES larval antigen and further purified some of these antigens for use in immunodiagnosis at definitive and paratenic hosts (dogs and mice, respectively).

Materials and Methods

Worms Collection

Eighty male and female *T. canis* worms (10-18 cm long) were collected from intestine of dissected stray dogs. Worms were washed several times with normal saline (0.85%). The anterior third of the female worms were dissected, and then uteri and eggs were isolated in a clean Petri dish containing normal saline.

Preparation of Adult Crude Antigen

Adult crude antigen (ACA) of *T. canis* was prepared according to Welch *et al*. (1983) which briefly, the whole worms were fragmented in tissue homogenizer in an ice bath. The worms sludge was reduced to a paste by ultrasonic (Ultrasonic dismembrator). The paste was dispersed in 10 ml distilled water and delipidized with 3 ml n-hexane, the defatted worm homogenate was centrifuged at 10000 rpm for 30 min., the supernatant was collected as a crude antigen. The crude antigen was lyophilized using freeze drier (type Labaconco / England) in Science College/ Basrah University, then stored at -20 C^0 until used for immunological test. The same procedure was used for egg crude antigen (ECA) isolated from the female of *T. canis* worms (1). The protein concentration of lyophilized adult and egg crude antigens was estimated by using the procedure of Bradford (1976). A standardization curve of standard protein (Bovine serum albumin) with 100, 200, 300, 400, 500, 600 and 700 µg/ml fitted (Fig. 1).

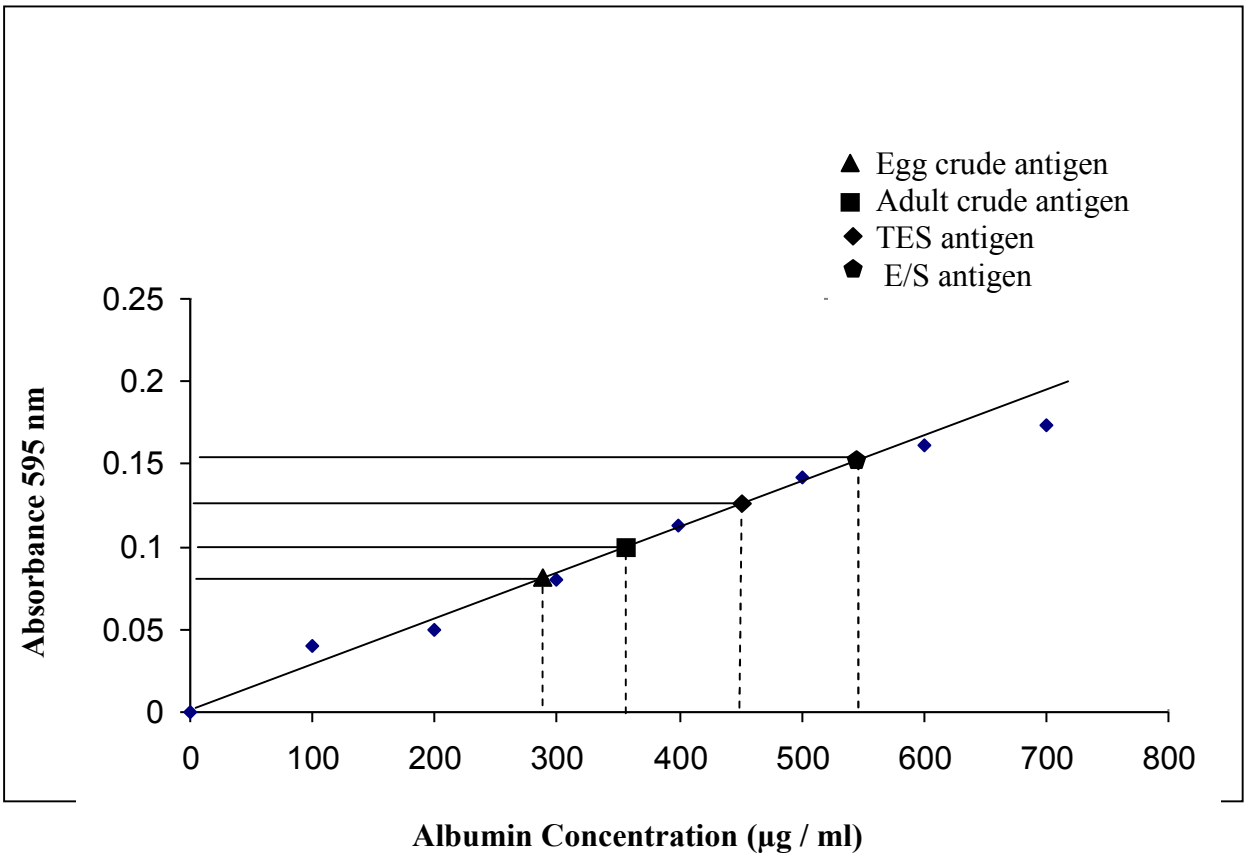

Figure (1) : Standard curve of bovine serum albumin.

Purification of Adult Crude Antigen of *T. canis*

The procedure of Belew *et al*. (1968) was followed using 16 x 1.6 cm of sephadex G-200 column.

According to Leslie and Frank (1976), two grams of sephadex G-200 were melted in 100 ml distilled water using water bath (Guwina-Hofmann GmbH/ Berlin) for 5 hours to give enough sowllen of the gel. The gel was poured in the column (vertical level) using a clean glass rod to avoid air bubbles and left to settle with opened outlet. Sodium azid solution 0.05% was put on the surface of column. The column packing was equilibrated with (0.05) M acetate buffer pH 5.5 (pH Hanna instruments), the gel was eluted with the same buffer at flow rate of 300 ml/ hour which was adjusted by using perstatic pump (LKB Bromma 2120 Vario-perpex II pump). Fractions (5 ml each) were collected in sterile glass tubes by using Fraction Collector (LKB Bromma 2070 ultrasonic /

England). A blue dextran with molecular weight of 2000 kilodalton was used for evaluating the void volume (Vo). Protein elution profiles were recorded by spectrophotometer at 280 nm with (Ultrospac Biochem 4050/ LKB). Fractions were monitored for protein by calibration with standard molecular weight proteins: Bovin serum albumine (67 kilodalton), Ovalbumin (45 kilodalton) and Trypsin (23 kilodalton). Fractions were stored at -20 C^0 in glass container for immunological test. The elution volume (Ve) for calibration proteins was determined spectrophotometry at 280 nm. The calibration curve was fitted by measuring the elution volume (Ve) of several standard proteins, their corresponding (Kav) values (distribution coefficient) was calculated by using the equation below:

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume

Vo = void volume = elution volume of blue dextran

Vt = bed volume of column

Purification of Larval Antigens

Eggs Culture

The procedure of Bowman *et al*. (1987) was used. Breifly, the females of *T. canis* were dissected and the anterior one third of the uterus was placed in 1% sodium hypochlorite, stirred on a magnetic stirrer (Ikamag Reo Drehzahil electronic) for 10 min. and poured through two layers of gauze to remove any large pieces of tissue, the resulting material was centrifuged for 3 min. at 2000 rpm. The supernatent was discarded and pellets were washed twice with distilled water and once with 0.5% formalin. The pellets were resuspended in (0.5%) formalin and placed in a conical flask. Formalin (0.5%) was added to make a final volume approximately one cm deep, the flask was covered with parafilm

and stored at room temperature with gentle agitation once a week. The eggs were used after at least 40 days, before their examination under (40x) magnification to ensure that they had reached to infective stage. The eggs were washed with distilled water to remove the formalin.

Collection of *T. canis* Larvae

The procedure of Sugane and Oshima (1984) was used with some modification due to local limitation. Embryonated eggs of *T. canis* suspended in 0.85% normal saline were stirred with ultrasonic dismemberator for 10 min. in an ice bath. The larvae and eggs shell debris were placed in two layers of nylon mesh, then larvae were placed in clean glass Petri dish.

Cultivation of Second Stage Larvae of *T. canis*

For cultivation of 2^{nd} stage larvae to release execratory/ secrotery antigen (E/S) a modified procedure of Bowman *et al*. (1987) was used in briefly: The concentration of larvae were adjusted to 1 x 10^3 ml in RPMI 1640 media (Sigma Chemical Co.) with 500 µg/ml streptomycin and 1% glutamine. The larvae with culture media were placed in clean conical flask and incubated at 37 C^0 in incubator (Termaks/ England) and gently agitated once a day. The culture media was replaced by fresh media once a week (De Savigny, 1975). Cultivation of this study took about two weeks only and then the activity of the larvae was reduced.

Isolation of Partially Purified E/S Antigen of *T. canis* Larvae

Partially purified E/S antigen was isolated as described by Oshima (1983), Briefly: culture media of *T. canis* was centrifuged in cold centrifuge (Chilsprin 2 Fisons/ MSE) at 4000 rpm for 30 minute, then dialysed against distilled water by dialyses bag (Cut-off 12-14 KD Spectrum Medical Industries) for 24 hours at 4 C^0. The purified E/S antigens were kept in a clean container at -20 C^0 until used for immunological test. The protein concentration was measured according to Bradford (1976).

Isolation of the Tegumental Larvae Antigen

A modified procedure of Oshima (1983) was used for isolation of the tegumental larvae of *T. canis*. Larvae with culture media were stirred for 30 minutes at 4000 rpm in cold centrifuge, the sediment was ultrasonic for 10 minutes in an ice bath, then dialysed against distilled water for 24 hours at 4 C^0. The tegumental larvae antigen (TES antigen) was placed in clean container at -20 C^0 until used. The protein concentration was measured according to the method of Bradford (1976).

Results

Protein Concentration

The protein concentration of lyophilized adult and egg crude antigens, (E/S and TES larval antigens) were found to be 449 and 290, 520 and 351 µg/ml, respectively using standard curve of bovine serum albumin (Figure 1).

Gel Filtration

The purified (ACA) of *T. canis* using gel filtration on sephadex-G200 column showed that the elution peak of blue dextrane was obtained at fraction number 25 (Vo=125 ml). The peaks of standard proteins (Bovine Serum Albumin, Ovalbumin and Trypsin) were detected at fraction number (37, 50 and 70) respectively, while the peak of antigen was obtained at fraction number 56 (Figure 2). The (Kav) of antigen (43.05) was plotted on standard curve (Figure 3) and the molecular weight of the antigen was found to be 35481.33 dalton.

Egg and Adult Worm

Plate (1) shows *T. canis* unembryonated egg, embryonated egg with 2^{nd} stage larva inside egg shell after 40 days culture in 0.5% formalin and anterior and posterior ends of adult male.

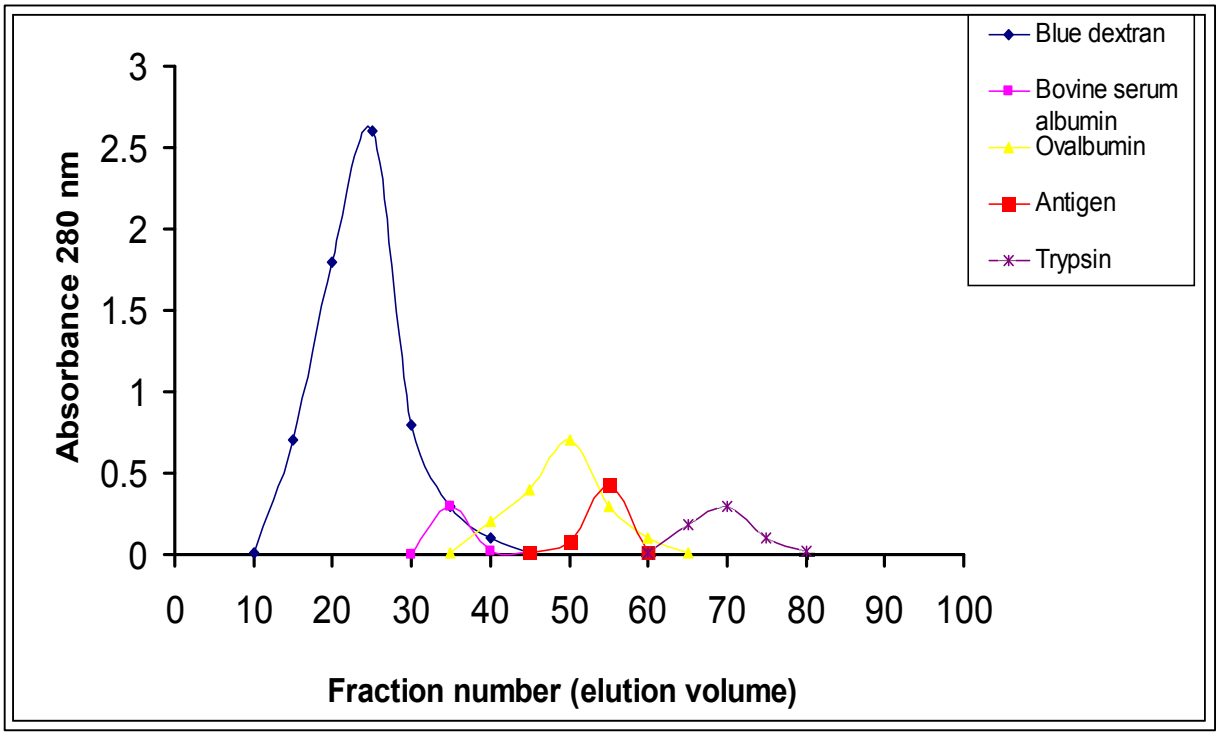

Figure (2): Elution of blue dextran, standard proteins and antigen fractions on sephadex G-200 column chromatography.

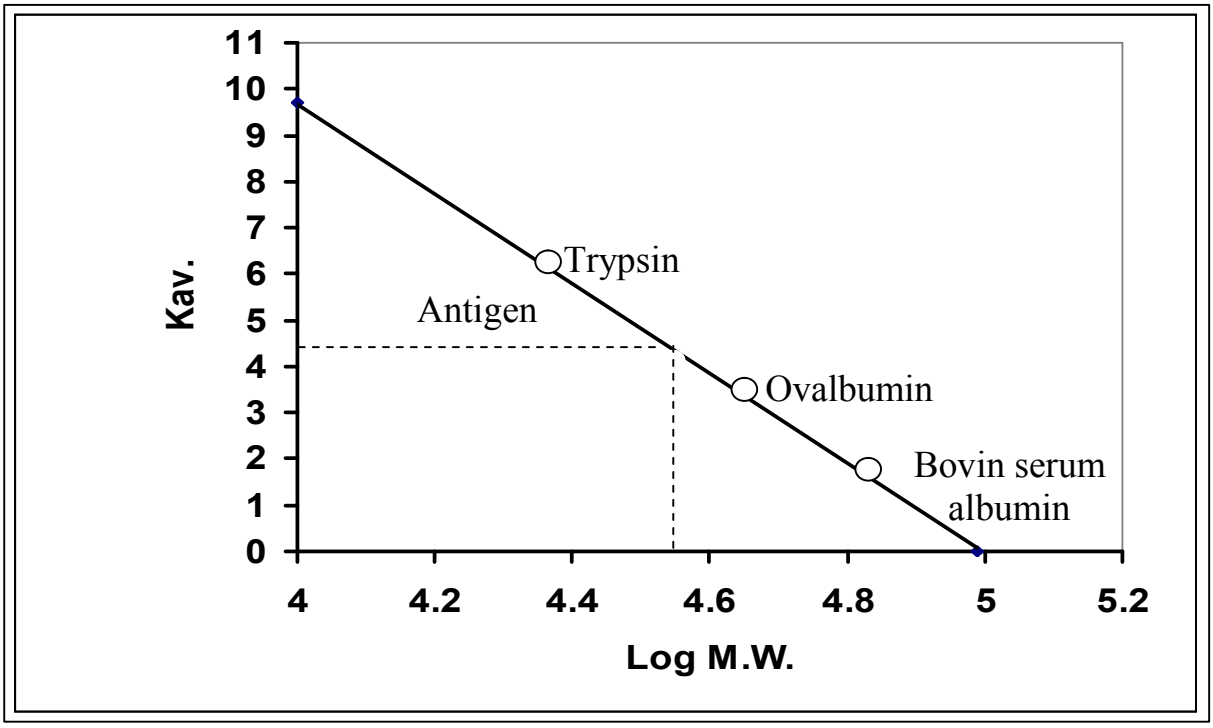

Figure (3): Standard curve of the standard proteins eluted on sephadex G-200 in column chromatography.

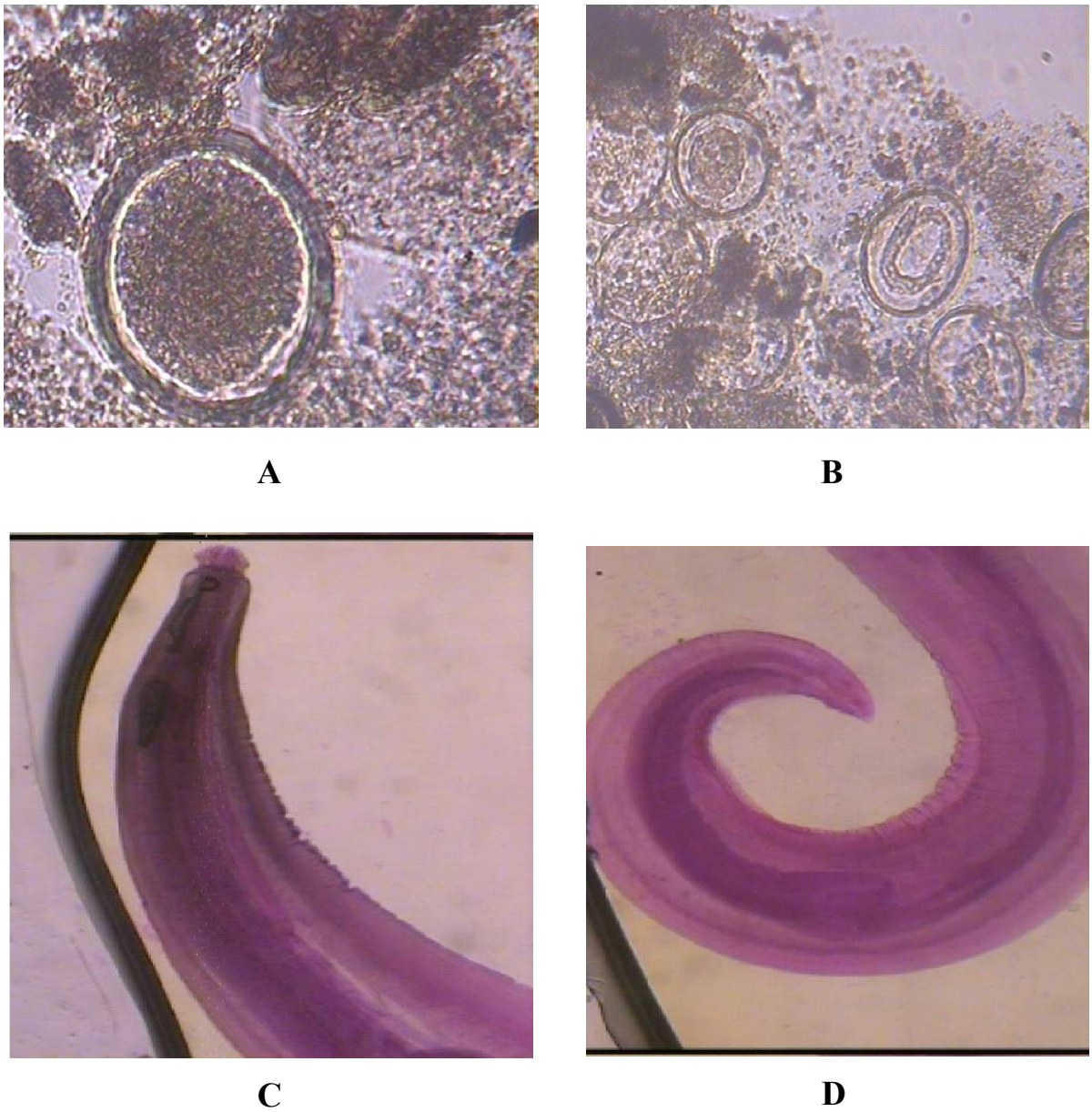

Plate (1): ***Toxocara canis*** A: unembryonated egg, B: embryonated eggs, C and D: anterior and posterior ends of adult male.(4X).

Discussion

Toxocariasis is an important zoonosis caused by the infection of humans with ascarid nematod larvae of *T. canis* from dogs and *T. cati* from cats (Page and Maizels, 1992).

In the present study, the protein concentration of lyophilized adult worms of *T. canis* antigen was higher twice than crude eggs antigen. This may be due to the tegumented surface of the worms which consists of carbohydrates, proteins and glycoprotiens, in addition to the proteins and lipids of internal body. Eggs and their shells consist of lipids, few amounts of protein and carbohydrates.

The protein concentration of E/S antigens of *T. canis* larvae in the current study was higher than TES, adult crude and eggs crude antigens. This may be demonstrated on the basis that E/S antigen is a mixture of many substances like proteins protolytic enzymes and glycoproteins released from larvae to their media which increase protein concentration. The E/S and TES protein concentrations were different as compared with other studies. It might be due to the method of maintaining larvae. For example, De Savigny (1975) used Eagle's minimum essential medium with Hank's salts and the larvae were maintained at 10^7 larvae per ml in tissue culture flask while in the present study, a total of 10^3 larvae per ml of RPMI media were used. Furthermore, the greater complexity of the TES lots may simply reflect technical modifications in production as well as different methods for resolution and detection of constituents used by various authors.

Niedfeld *et al*. (1993) showed that the protein concentration of E/S antigen of *T. canis* larvae was 600 µg/ml by using the method of Lowry *et al*. (1951). Moreover, Oshima (1983) purified E/S antigen from *T. canis* larvae and showed that the antigen was mainly of glycoprotein in nature and had a molecular weight of 35000 dalton and 15% of it containing carbohydrates. Also, he isolated seven fractions of protein bands from 2nd stage larvae of *T. canis* using sephadex G-25 gel filtration. Badley *et al*. (1987) isolated 15 bands of *T. canis* in SDS-PAGE using silver stain.

De Savigny (1975) founded that $1x10^7$ larvae of *T. canis* produced about 0.4-0.9 mg each week of culture or about 4-9 pg protein per larva per day.

Furthermore, the major TES proteins were designted as TES-32, 55 and 70 according to their molecular weight on SDS-PAGE (Maizles and Roberston, 1991).

Robertson *et al.* (1989) pointed out that *Toxocara* larvae secrete a serine protease which migrated around 120000 dalton on substrate gels. Loukas *et al*. (1998) identified Cathepsin L as enzyme secreted from *Toxocara* larvae. Moreover, Tetteh *et al*. (1999) characterised as asparaginyl endopeptidase (legumain) as a cystine proteases with no structural homology to the papain like enzymes in addition to the Cathepsin Z (Falcon *et al*., 2000). Page *et al.* (1992) showed that the external surface of *T. canis* larvae is coated with a carbohydrate rich glycocalyx envelope (10 nm in thickness) and detached by a similar distance from the epicuticle. Niedfeld *et al*. (1993) showed the presence of lipid in the execretory /secretory product from *T. canis* larvae and the fatty acids like myristic, palmitic and stearic, in addition to quantities of oleic and linoleic acids and cholesterol which have a highly important role in the host-parasite relationship. Biochemical analysis coupled with electron microscopy showed that the surface coat of *T. canis* larvae consists of TES -120 set of secreted mucins. TES glycoproteins like TES-32 and TES-70 were not found in the surface coat.

Welch *et al.* (1983) showed that by using immunoelectrophoresis and immunodiffusion techniques the pure adult antigens of *T. canis* contained fewer but more specific proteins than crude antigens, and adult *T. canis* crude antigens are a mixture of proteins when purified showed at least 12 different proteins and 9 of them were different antigens. Also, Sarimehmetoglu *et al.* (2001) showed that the protein bands of E/S antigens of 2nd stage larvae of *T. canis* were 24, 28 and 48 kilodalton by using SDS-PAGE. Yokoi *et al.* (2002) reported that many E/S molecules did not have any cross-reactivity with adult *T. canis* antigens. Inuo *et al*. (1995) suggested that adult *T. canis* derived antigens had the ability

to activate human peripheral blood mononuclear cells as conventional antigens, which may be involved in the host defense against helminth infection.

Refrences

Badley, J. E.; Grieve, R. B.; Bowman, D. D.; Glickman, L. T. & Rockey, J. H. (1987). Analysis of *Toxocara canis* larval excretory-secretory Antigens: Physicochemical characterization and antibody recognition. J. Parasitol., 73: 593-600.

Belew, M.; Porath, J. & Fohlman, J. (1968). Adsorption phenomena on sephacryl S-200 superfine. J. Chromatogr., 147: 205-212.

Bowman, D. D.; Mika-Grieve, M. & Grieve, R. B. (1987). Circulating excretory-secretory antigen levels and specific antibody responses in mice infected with *Toxocara canis*. Am. J. Trop. Med. Hyg., 36: 75-82.

Bradford, M. M. (1976). A rapid and sensitive method for the quantiation of microgram quantities of protein utilizating the principle of protein dye binding. Anal. Biochem., 72-248.

De Savigny, D. H. (1975). *In vitro* maintenance of *Toxocara canis* larvae and a simple method for the production of *Toxocara* E/S antigen for use in serodiagnostic tests for visceral larva migrans. J. Parasitol., 61: 781-782.

Falcon, F.; Tetteh, K. K.; Hunt, P.; Blaxter, M. L.; Loukas, A. C. & Maizels, R. M. (2000). The new subfamily of cathepsin Z-like protease genes includes Tc-cpz-1, a cysteine protease gene expressed in *Toxocara canis* adults and infective larvae. Exp. Parasitol., 94: 201-207.

Inuo, G.; Akao, N.; Kohsaka, H.; Saito, I.; Miyasaka, N. & Fujita, K. (1995). *Toxocara canis* adult worm antigen induces proliferative response of healthy human peripheral blood mononuclear cells. Parasite Immunol., 17: 77-84.

Leslie, H. & Frank, C. H. (1976). Isolation and structure of immunoglobuline in : Practical immunology, 3rd ed., Blackwell Sci. Publi., Oxford.

Lewis, J. W. & Maizels, R. M. (1993). *Toxocara* and toxocariasis: Clinical, epidemiological and molecular perspectives. Institute of Biology. London. (Cited from **Maizels, 2004**).

Loukas, A.; Selzer, P. M. & Maizels, R. M. (1998). Characterisation of Tc-cpI-1,a cathepsin L-like cysteine protease from *Toxocara canis* infective larvae .Mol. Biochem. Parasitol., 92: 275-289.

Loukas, A.; Hintz, M.; linder, D.; Mullin, P.; Parkinsan, J.; Tetteh, K. K. & Maizels, R. M. (2000). A family of secreted mucins from the parasitic nematode *Toxocara canis* bears diverse mucin domains but shares similar flam king six-cystine repeat motifs. J. Bio. Chem., 275: 39600-39607.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phinol reagent. J. Biol. Chem., 193: 265-275.

Maizels, R. M. (2004). Biology of *Toxocara canis*. Rick Maizels *Toxocara* page. Univ. Edinburgh. www.*Toxocara*.htm.

Maizels, R. M. & Page, A. P. (1990). Surface associated glycoproteins from *Toxocara canis* L2 parasites. Acta Trop., 47: 355-364.

Maizels, R. M. & Robertson, B. D. (1991). *Toxocara canis:* Secreted glycoconjugate antigens in immunobiology and immunodiagnosis. In: Parasitic nematodes. Antigens, membranes and genes(ed. Kennedy; M.) 95-115pp. Taylor and Francis Ltd, London: (Cited from **Maizels, 2004**).

Niedfeld, G.; Pezzani, B.; Minvielle, M. & Basualdo Farjat, J. A. (1993). Presence of lipids in the secretory/excretory product from *Toxocara canis*. Vet. Parasitol., 51: 155-158.

Oshima, T. (1983). Role and behaviour of eosinophils in parasitic infections as observed in mice infected with *Toxocara canis*. Asian Parasitol. Symp., 9: 165-173.

Page, A. P. & Maizels, R. M. (1992). Biosynthesis and glycosylation of serine/ threonine-rich secreted proteins from *Toxocara canis* larvae. Parasitology, 105: 297-308.

Page, A. P.; Hamilton, A. J. & Maizels, R. M. (1992). *Toxocara canis*: Monoclonal antibodies to carbohydrate epitopse of secreted (TES) antigens localize to different secretion-related structures in infective larvae. Exp. Parasitol., 75: 56-71.

Robertson, B. D.; Bianco, A. E.; Mckerrow, J. H. & Maizels, R. M. (1989). *Toxocara canis*: Protolytic enzymes screted by the infective larvae *in vitro*. Exp. Parasitol., 69: 30-36.

Sarimehmetoglu, H. O.; Burgu, A.; Aycicek, H.; Gonenc, B.; Tanyuksel, M. & Kara, M. (2001). Application of Western blotting procedure for the immunodiagnosis of visceral larva migrans in mice by using excretory/ secretory antigens. Deut. Tier. Woche., 108: 390-392. (English Summary).

Stirewalt, M. A. (1963). Chemical biology of secretions of larval helminthes. Ann. New York Acad. Sci., 113: 36-53.

Sugane, K. & Oshima, T. (1984). Interelationship of eosinophilia and IgE antibody production to larval ES antigen in *Toxocara canis* infected mice. Parasite Immunol., 6: 409-420.

Tetteh, K. K.; Loukas, A.; Tripp, C. & Maizels, R. M. (1999). Identification of abundantly-expressed novel and conserved genes

from infective stage larvae of *Toxocara canis* by an expressed sequence tag strategy. Infec. Immunol., 67: 4771-4779.

Welch, J. S.; Symons, M. H. & Dobson, C. (1983). Immunodiagnosis of parasitic zoonoses: Purification of *Toxocara canis* antigens by affinity chromatoghraphy. Int. J. Parasitol., 13: 171-178.

Yokoi, K.; Kobayashi, F.; Sakai, J.; Usui, M. & Tsuji, M. (2002). Sandwich ELISA detection of excretory-secretory antigens of *Toxocara canis* larvae using a specific monoclonal antibody. South Asia J. Trop. Med. Publ. Health., 33: 33-37.

تحضير مستضدات من طفيلي الـ

Toxocara canis

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الخـــلاصـــة

ركزت الدراسة الحالية على تحديد تركيز البروتين لمستضدات الديدان والبيوض الخام لطفيلي الـ *Toxocara canis*. أستعمل عمود الفصل الهلامي مع مادة السيفادكس G-200 لتنقية مستضدات الديدان الخام (ACA) . كانت نتيجة التنقية هي الحصول على مستضد بوزن جزيئي مقداره 35481.33 دالتون.

كان تركيز البروتين للديدان الخام والبيوض هو 449، 290 مايكروغرام \ مل على التوالي.أستعمل في الدراسة الحالية تقنية جديدة لاستزراع يرقات الطور الثاني لطفيلي *Toxocara canis* مع الوسط الزرعي RPMI 1640 media للحصول على المستضدات الأفرازية- الأخراجية

(E/S),والمستضدات الجسمية (TES)ليرقات الطور الثاني للطفيلي. قدر تركيز البروتين المستضدين أعلاه وكانت النتيجة 520 ,351مايكروغرام/ مل على التوالي.