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# STRUCTURAL CHARACTERIZATION OF *FASCIOLA GIGANTICA* PARTIALLY PURIFIED WORM ANTIGEN AND ITS POTENCY IN DIAGNOSIS OF FASCIOLIASIS

(With One Table and 5 Figures)

By

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## الوصف التركيبى لمولد الضد المنقى جزئياً من ديدان الفاشيولا جيجنتيكا وقدرته على تشخيص مرض الفاشيولا

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اشتمل هذا البحث على عمل تنقية جزئية عن طريق جهاز التحليل الكروماتوجرافى للمستخلص الخام للطور البالغ لديدان الفاشيولا جيجينتيكا باستخدام سيفدكس G200 وقد نتج عن هذا التحليل فصل ٤ أجزاء (مركبات) من هذا المستخلص الخام. وقد أظهر جهاز الفصل الكهربائى (electrophoresis) صورة بسيطة لكل من هذه الأجزاء الأربعة مقارنة بصورة المستخلص الخام. وقد تم اختبار الكفاءة المناعية للأجزاء الأربعة المفصولة من المستخلص الخام مع الأجسام المناعية المحضرة فى الأرانب باستخدام اختبار الاليزا وقد أظهر هذا الاختبار تفوق المركب الثانى (F2). وقد نجح هذا المركب (F2) فى تشخيص مرض الفاشيولا بنسبة ١٠٠%. وقد تم عمل وصف تركيبى لهذا الجزء (F2) عن طريق استخدام جهاز الفصل الكهربى (electrophoresis) ونقطة التعادل الكهربى ( isoelectric point) وأيضا التحليل الأمينى لهذا المركب وقد أظهر التحليل الكهربائى أن هذا الجزء (F2) يحتوى على ثلاثة مركبات ذات أوزان جزيئية ١٠٠، ١٠٦، ١٣٣ كيلو دالتون وكانت نقط التعادل الكهربى لهذه المركبات هى ٥,٢ ، ٥,٩ ، ٦,٥ ، ٧,٢ وقد أظهر التحليل الأمينى لهذا المركب (F2) أنه يحتوى على ١٧ حمض أمينى وكانت نسبة Lysine و Alanine و Proline كبيرة مقارنة بباقى الأحماض الأمينية.

## SUMMARY

Chromatographic analysis of *Fasciola gigantica* adult crude extract was undertaken using Sephadex G-200. Four fractions were isolated by this approach. The isolated fractions showed simple electrophoretic profile, as judged by SDS-PAGE, compared to the complex profile of crude

extract. Fraction 2(F2), exhibited the most potent activities against rabbits hyperimmune serum in ELISA. This fraction was utilized in diagnosis of fascioliasis among buffaloes recording 100% sensitivity. Structural characterization of the isolated fraction, F2, by SDS polyacrylamide gel electrophoresis, isoelectric focusing and amino acid analysis showed that F2 consists of only three polypeptides of 133 KD, 106 KD and 100 KD with isoelectric points of 7.2, 6.5, 5.9 and 5.2. Moreover, 17 amino acids were identified in F2 with high proportions of only three of them (Lysine, Alanine and Proline).

***Key words:*** *Fasciola gigantica, gel filteration, ELISA, diagnosis.*

## INTRODUCTION

*Fasciola species* are parasitic trematodes with a world wide distribution, which infect a wide variety of mammals, including humans (Mezo *et al.*, 2003). Infection with *Fasciola gigantica* is considered one of the most causes of economic loss of animals in Egypt.

The parasitological diagnosis of fascioliasis based on the demonstration of the eggs in feces is usually unsatisfactory due to false passage of eggs and failure of immature worm to maturation. Disease occur as early as 3 weeks post infection while parasitological diagnosis is only possible at about 10-14 weeks after infection when eggs begin to appear in feces (Hillyer, 1981; Ibarra *et al.*, 1998 and Hasseeb *et al.*, 2003). So, serological diagnosis is reliable and potent alternative approach to overcome the deficiencies of fascioliasis diagnosis by parasitological means (Hillyer, 1981 AND Hillyer *et al.*, 1992). Also the advantage of the serological tests is its capability to detect IgG anti *Fasciola* antibodies from the second week post infection , which is remarkably early in the diagnosis of this diseases (Ibarra *et al.*, 1998). Trials have been proposed serodiagnosis of *F.gigantica* infection in ruminants (Swarup *et al*, 1987 and Fagbemi & Obarisiagbon, 1990). Serodiagnosis of fascioliasis is carried out by ELISA (Clery *et al.*, 1996; Sanchez-Andrade *et al.*, 2000; Paz-Silva *et al.*, 2003 and Velusamy *et al.*, 2004).

The current study aims to partially purified antigen from *Fasciola gigantica* by gel filteration chromatography. The resulting fractions analyzed by ELISA in order to evaluate the most sensitive fraction and assess its potency in diagnosis of fascioliasis among naturally infected buffaloes. Also, identifying the structural characterization for the most potent fraction.

## MATERIALS and METHODS

**Parasites:** *Fasciola gigantica* adult worms were collected from condemned livers of buffaloes slaughtered in Cairo abattoir.

**Antigen preparation:** *F. gigantica* adult worms were washed thoroughly with distilled water to eliminate traces of bile and blood. The antigen was prepared by minor modifications of the method described by Nordon and Strand (1984). One volume adult worms was suspended in three volumes in lysis buffer. Suspension of the parasite was homogenized in an ice bath. Homogenate was sonicated at 28μ at 60 pulses in an MSE sonicator. The sonicate was centrifuged at 10.000 g for 1 hr and supernatants collected, assayed for protein content by method of Lowry *et al.* (1951) and aliquoted and stored at –20°C until use.

**Rabbit hyperimmune serum:** The rabbit immune serum was prepared according to the technique adopted by Fagbemi *et al.* (1995). Briefly, rabbits were immunized subcutaneously with ≅ 40 μg of whole worm extract of *F. gigantica* in Freund's complete adjuvant. A booster dose of ≅ 40 μg in Freund's incomplete adjuvant was given on day 14. Second and third booster doses were given on day 21 and 28 and serum samples were collected 4 days after last immunization.

**Buffalo sera collection:** Sera from 54 naturally infected buffaloes were collected and analyzed for antibodies detection. The infection was ascertained by coprological examinations according to Thienpont *et al.* (1979). 37 sample sera from coprologically negative buffaloes were also collected and analyzed for antibody detection. Buffaloes sera were heat inactivated at 56°C for 30 minutes for decomplementation before analysis.

**Gel filteration chromatography:** Sephadex G-200 column was used for the fractionation of *F. gigantica* extract. The column was equilibrated and eluted with 0.05 M PBS containing 0.02% $NaN_3$. The flow rate was 12 ml/h and fractions were collected in 1 ml aliquots. Column eluates were monitored at 280 nm. The column was calibrated with molecular weight standards from Bio-Rad Laboratories. Blue dextran (2000 KD) was used to determine the void volume.

**Enzyme linked immunosorbent assay (ELISA):**

ELISA was adopted to evaluate *F. gigantica* fractions activities utilizing rabbit hyperimmune sera according to Santigo *et al.* (1986). It was also used to evaluate the potency of the selected fraction in fascioliasis diagnosis. The assay was performed as described by Wijffels *et al.* (1994). The optimum antigen concentration and sera dilution were

determined by checkerboard titration. ELISA plates were coated with 50 µg/ml of each antigen. The selected dilution of sera was 1:100. Antibovine IgG horse-radish peroxidase conjugate was used. ELISA OD cutoff values were calculated by the method of Allan *et al.* (1992).

**SDS-polyacrylamid gel electrophoresis (SDS-PAGE):**

20 µg of crude extract and also of pure fractions collected after gel filteration were electrophoresed, under reducing conditions, on 7.5% SDS-PAGE as described by Laemmli (1970). Gel was stained with silver stain according Wray *et al.* (1981). High and low molecular weight standards were electrophoresed in the same gel.

**Isoelectric focusing (IEF):** IEF of the isolated fraction was performed as described by O'Farrell (1975) in slab gel supplemented with urea and ampholine. Gels were stained with commasie blue and photographed wet. Isoelectric focusing of a particular protein can be determined by mixing a mixture of proteins of known isoelectric points on the same gels ranged from 4.9-8.4.

**Amino acid analysis:** Free amino acid composition of the isolated fraction was determined after hydrolysis in HCl prior to analysis with amino acid analyzer (GBC Australia).

## RESULTS

**Fractionation of *F. gigantica* crude extract on sephadex G-200 column chromatography:**

Gel filteration was adopted for the fractionation of *F. gigantica* extract. The elution profile is shown in Fig. (1) where four fractions were eluted from the column at different absorbance values and elution volumes. Each fraction was collected lyophilized and analyzed separately.

**Electrophoretic profile of *F. gigantica* crude and pure antigens:**

The four isolated fractions were separately electrophoresed on SDS-PAGE. At the same time, *F. gigantica* crude extract was electrophoresed under the same conditions for comparative purposes. The crude extract was resolved into 12 component (Fig. 2 Lane. A). While the four separated fractions showed simple electrophoretic profile, Fig. 2 Lanes B, C, D. and E.

**Antigenic activities of isolated fractions:**

The antigenic activities of each fraction was evaluated by ELISA in which rabbit hyperimmune serum was utilized. As shown in Fig. 3, fraction number 2 showed the most potent activities compared with the

three other fractions. Based on these results, fraction number 2 was selected to diagnose fascioliasis among naturally infected buffaloes.

**Parasitological examination:**

A total of 91 buffaloes slaughtered in Cairo abattoir were examined parasitologically for detection fascioliasis. The examination revealed 54 buffaloes infected with *Fasciola gigantica* and 37 buffaloes gave negative results.

**Diagnosis of buffaloes fascioliasis by *F. gigantica* fractions 2:**

Sample sera collected from naturally infected and noninfected buffaloes, as proved by coprological examination, were assayed against fraction 2 in ELISA. The assay confirmed the infection and recorded 100% sensitivity (Fig. 4). The cutoff value was 0.23.

**Structural characterization of fraction 2:**

a) **SDS- polyacrylamide gel electrophoresis (SDS-PAGE):** The electrophoretic profile of the isolated fraction 2 is shown in (Fig. 2. Lane C). This fraction was resolved, under reducing conditions, into 3 bands of molecular weight of 133KD, 106KD, 100KD.

b) **Isoelectric focusing (IEF):** For further characterization of the partially purified fraction, isoelectric point of the fraction was identified by isoelectric focusing technique. As shown in Fig. 5, the components have PIs of 7.2, 6.5, 5.9 and 5.2.

c) **Amino acid analysis:** For additional structural characterization of the isolated fraction 2, analysis of its free amino acids was undertaken. The fraction exhibited 17 amino acids as shown in table 1. The fraction is rich in Lysine (8), Alanine (7.5) and Proline (5.2) While Isoleucine (0.6), Methionine (0.8) were present in minute amounts.

## DISCUSSION

Immunodiagnosis of parasitic infection faces the problem of cross-reaction. Production of purified antigen (s) is only way to minimize the cross reactivity.

In the current research, a partial purification of *F. gigantica* crude extract by gel filteration using sephadex G200 was undertaken. The purification process resulted in four fractions of different protein content and antigenic activities and the fraction 2 was the most potent fractions as judged by ELISA. Previously, comparable results were obtained, where two fractions were obtained by Carlos *et al.* (1988). They were purified *F. hepatica* antigen by gel filteration and HPLC and evaluated the putative potency of both fractions. Rhee *et al.* (1986) used

sephadex G100 column chromatography to obtain the most specific and sensitive fractions from crude antigen of *F. hepatica* for immunodiagnosis of bovine fascioliasis. Seven fractionated antigens were obtained and the fifth antigen was suggested to be the specific antigen for the immunodiagnosis of bovine fascioliasis. Cervi *et al.* (1992) revealed that *F. hepatica* total antigen gave four fractions in a sephadex G-100 column and these fractions showed the presence of different antigenic components. Osman *et al.* (1992) indicated that four peaks were obtained when *Fasciola* somatic antigen was fractionated by column chromatography and these fractions evaluated by ELISA.

The electrophoretic profile of *F. gigantica* crude extracts as studied by SDS-PAGE, in the current research, exhibited a complex profile of 12 components in both high and low molecular weight ranges (24-205 KD), while the four isolated fractions were showed simple profiles. Electrophoretic make up of *Fasciola* crude extract by SDS-PAGE was previously probed. Yadav and Gupta (1995) indicated that mature *F. hepatica* antigen separated out in 9 bands in the range 12-95 KD. SDS analysis of *F. hepatica* total antigen showed glycoproteins bands ranging from 14 to 94 KD (Cervi *et al.*, 1992). Electrophoretic profile of *F. gigantica* surface and tegument antigens was showed by Krailas *et al.* (2002) and they demonstrated that the proteins had molecular weights of 20-97 KD. The contradiction in the results may be account for the difference in the species of parasite or the type of antigen used.

The majority of indirect diagnostic methods based on antibodies detection are well described in the literatures (Welch *et al.*, 1987). ELISA is considered the most diagnostic method because it is highly sensitive and specific when compared to diagnosing *Fasciola* species by coprological means, (Ibarra *et al.*, 1998; Fagbemi *et al.*, 1997 and Hasseeb *et al.*, 2003). ELISA have been found to be suitable for diagnosis of fascioliasis due to high sensitivity, possibility of processing many sera samples and its capability to detect IgG anti *Fasciola* antibodies early post infection (Arriaga de Morilla *et al.*, 1989 and Ibarra *et al.*, 1998). In the present study, as judged by ELISA, fraction 2 (100-133 KD) showed high sensitivity in diagnosing fascioliasis. Where detection of antibody sera collected from 54 buffaloes, proved parasitologically infected with *F.gigantica*, reactive to the fraction 2 and revealed 100% sensitivity. Rhee *et al.* (1986) revealed that fraction number fifth, resulting from *F. hepatica* fractionation by gel filteration chromatography, had putative potency in the immunodiagnosis of

fascioliasis. Carlos *et al.* (1988) found that a fraction with high molecular weight, 150-160 KD to be very reactive with sera from early fascioliasis.

The identification of antigens is of fundamental importance, not only to obtain a better understanding to mechanisms of immunity but aslo to facilitate the preparation of purified specific antigen suitable for immunodiagnosis (Carlos *et al.*, 1988).

The important facet of the present study is the structural characterization of the fraction 2, which was found to be consisted of only three polypeptides of molecular weights 100, 106, 133 KD. Carlos *et al.* (1988) characterized the separated fractions electrophoretically and found to be of molecular weight ranged from 150-160KD. Espino *et al.* (1993) reported that the immunogenic purified fraction of *F. hepatica* had molecular weight ranged from 13KD-37 KD.

The purified fraction isolated in the present study was also characterized according to its isoelectric points by isoelectric focusing technique. The assay recorded four components of approximately PIs 7.2, 6.5, 5.9 and 5.2. These four components had three molecular weight as showed by electrophoresis analysis introduced the possibility that one of the three polypeptides splitted to two isoelectric points during isoelectric focusing assay. This assumption must be investigated in further studies.

Identification of the amino acid composition of the pure antigen and its structural details is considered an essential step during purification process (Abdel Rahman, 2000). Previously, studies related to purification of *F. gigantica* adult extract have been concerned with evaluation of pure fractions in serodiagnosis. Nevertheless, no definitive information regarding the molecular nature or isoelectric points were introduced.

So, the current research dealt with further information on the structure of the pure antigen, its free amino acid composition was analyzed. It was concluded that, this fraction is consisted of 17 amino acids with high proportions of only three of them (Lysine, Alanine and Proline). Moreover, there is apparently little amount of Isoleucine and Methionine. Amino acid analysis of *Schistosoma mansoni* cercarial preparations was previously studied by Caulfield *et al.* (1987). They observed that threonine, serine and glutamic acids comprised 44% of the amino acid residues of the protein. After purification of cercarial preparations on sepharose column chromatography, free amino acids, predominantly glycine and serine were found to comprise 17% of the

total protein. These amino acid presented in low proportion in the current research although both are Trematoda, this may account for the difference in the stage of parasites used in both studies. Cox *et al.*, 1990 observed high amount of glutamic and asparagin in *Haemonchus contortus* third stage larvae. From these previous studies together with the present one we can detect that, each helminth has its own amino acid structure and unique proportion of each amino acid.

Further purification of adult worm antigens of *F. gigantica* would be of significance in the diagnosis process. Also, ELISA technique used in this study offered a diagnostic alternative method for detecting early infection of *F. gigantica* in animals and it is recommended for seroepidemiological survey for *F. gigantica* infection.

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**Table 1:** Analysis of free amino acids of *F. gigantica* isolated fraction (F2)

| Amino acids | Content (µg/100 mg) |
|---|---|
| Aspartic | 3.81 |
| Glutamic | 3.33 |
| Serine | 3.57 |
| Glycine | 3.83 |
| Histadine | 2.41 |
| Arginine | 1.85 |
| Threonine | 4.87 |
| Alanine | 7.51 |
| Proline | 5.24 |
| Tyrosine | 4.51 |
| Valine | 3.84 |
| Methionine | 0.82 |
| Cystein | 1.08 |
| Isoleucine | 0.61 |
| Leucine | 2.07 |
| Phenylalanine | 3.84 |
| Lysine | 8.04 |

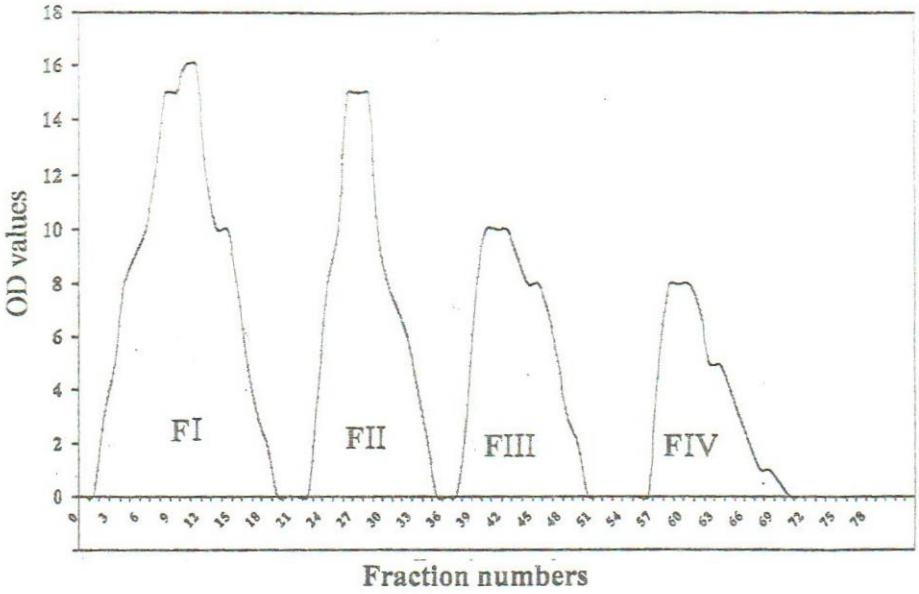


Fig. 1 : Elution profile of *Fasciola gigantica* using gel filteration chromatography

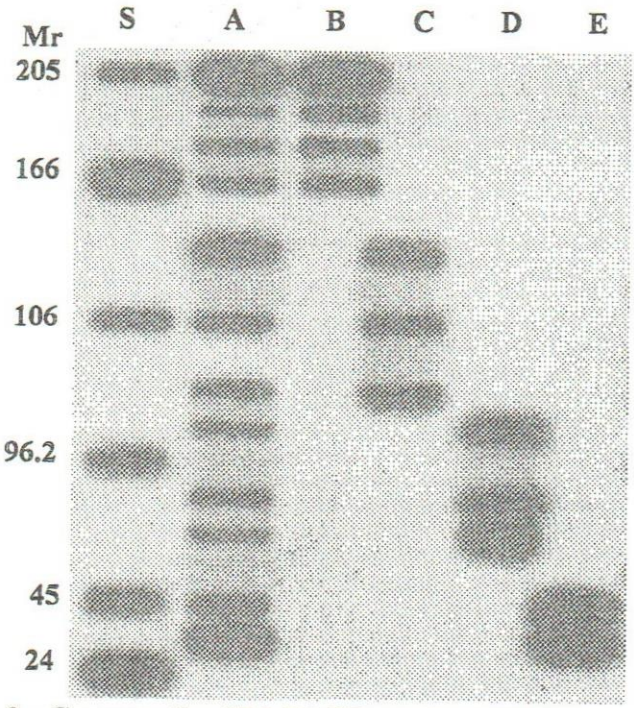


Fig. 2 : Comparative SDS-PAGE profile of *F. gigantica* crude extract (Lane A) and isolated fraction 1 (Lane B) fraction 2 (Lane C) fractions 3 (Lane D) fraction 4 (Lane E). molecualr weight standars (Lane S) indicated in KDa

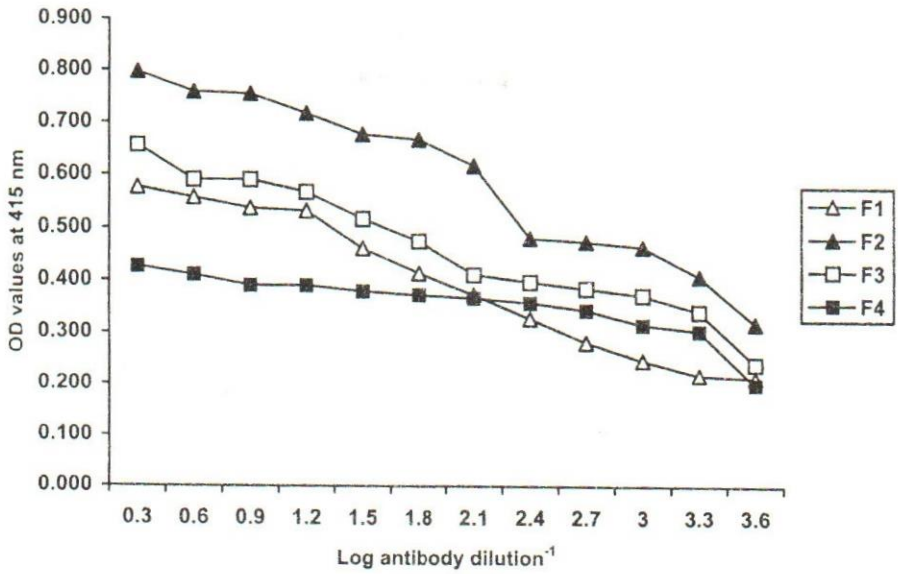


Fig. 3: Evaluation of antigenic activities of *F. gigantica* fractions, resulted from gel filteration chromatography, by ELISA.

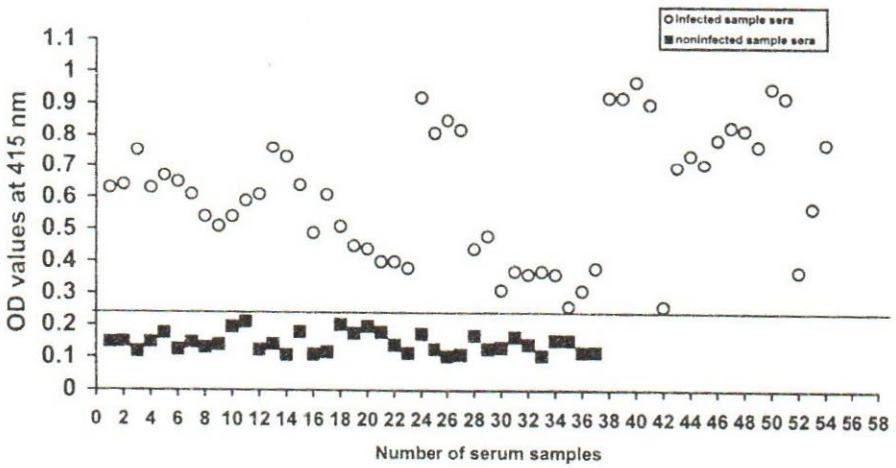


Fig. 4 : ELISA evaluation of the potency of Fasciola gigantica isolated fraction 2 in the diagnosis of fascioliasis in buffaloes
Horizontal line show cutoff value

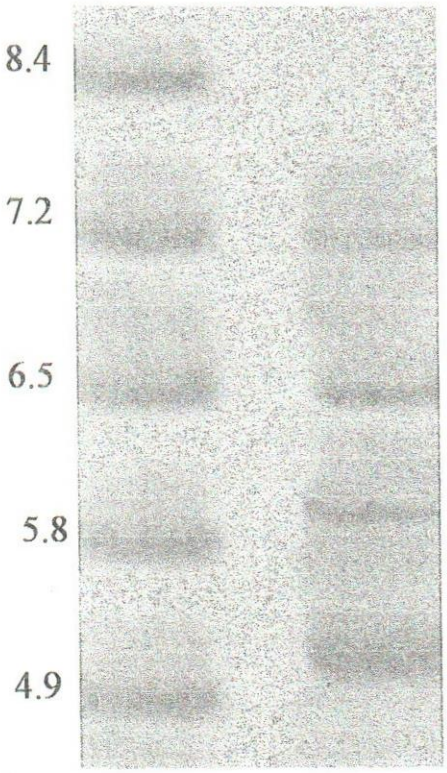


Fig. 5 : Isoelectric focusin of *F.gigantica* fraction 2 (Lane B) isoelectric focusing standards (Lane A).