Revista da Sociedade Brasileira de Medicina Tropical 46(5):605-610, Sep-Oct, 2013
http://dx.doi.org/10.1590/0037-8682-0154-2013

Major Article

Analysis of the chemical components of hydatid fluid from *Echinococcus granulosus*

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ABSTRACT

Introduction: The aim of this study was to explore the environment of *Echinococcus granulosus* (*E. granulosus*) protoscolices and their relationship with their host. **Methods:** Proteins from the hydatid-cyst fluid (HCF) from *E. granulosus* were identified by proteomics. An inductively coupled plasma atomic emission spectrometer (ICP-AES) was used to determine the elements, an automatic biochemical analyzer was used to detect the types and levels of biochemical indices, and an automatic amino acid analyzer was used to detect the types and levels of amino acids in the *E. granulosus* HCF. **Results:** I) Approximately 30 protein spots and 21 peptide mass fingerprints (PMF) were acquired in the two-dimensional gel electrophoresis (2-DE) pattern of hydatid fluid; II) We detected 10 chemical elements in the cyst fluid, including sodium, potassium, calcium, magnesium, copper, and zinc; III) We measured 19 biochemical metabolites in the cyst fluid, and the amount of most of these metabolites was lower than that in normal human serum; IV) We detected 17 free amino acids and measured some of these, including alanine, glycine, and valine. **Conclusions:** We identified and measured many chemical components of the cyst fluid, providing a theoretical basis for developing new drugs to prevent and treat hydatid disease by inhibiting or blocking nutrition, metabolism, and other functions of the pathogen.

Keywords: *Echinococcus granulosus*. Hydatid fluid. Two-dimensional gel electrophoresis. Peptide mass fingerprints. MASCOT software.

INTRODUCTION

Echinococcosis, also called hydatid disease, is a zoonos is caused by the larval stage of *Echinococcus*. *Echinococcosis* affects humans and other mammals, such as sheep, dogs, rodents, and horses[1]. Once *Echinococcus* infects a host, the oncosphere of *Echinococcus* will develop into a cyst. The cyst forms a relatively stable internal environment to avoiddamage to the larvae from the host immune system. Hydatid cyst fluid (HCF) is an important component of the internal environment and fills the entire cyst. HCF is a clear or clear yellow liquid with antigenic properties. HCF provides needed nutrition forlarval growth, playing an important role in their lifecycle of *Echinococcus*.

Only a few comprehensive studies of the chemical composition of HCF in human liver have been reported. Previous studies focused on livestock such as sheep and cattle. Capron and Yarzabal discovered antigen 5 and antigen B in HCF through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis[2,3]. Zhu used improved two-dimensional polyacrylamide electrophoresis to identify 111 proteins in the liver HCF of infected sheep[4]. Chemale attempted to analyze proteins in liver HCF of cattle but failed to establish a 2-DE database because of the effect of highly abundant albumin and immunoglobulin[5]. Forty-eight *E. granulosus* proteins were identified by Aziz[6]; however, many previously identified components of HCF were not included. HCF proteins are composed of 44% albumin, 39% α-globulin and β-globulin, and 17% γ-globulin[7]. Li determined that liver HCF and lung HCF from sheep and yak contained 17 amino acids, but the total protein level was very low, equivalent to a level of approximately 1-2% in serum[8]. Similarly, the cholesterol level was also found to be low (approximately 12% in serum). Polysaccharides, together with proteins and lipids, were present in sheep liver HCF[9]. Other researchers also detected urea, uric acid, proteins and amino acids, lipids, electrolytes, glucose, glycogen, many trace elements, and proteases, for example, in the HCF[10-13]. In summary, these studies established a baseline assessment of the chemical constituents of HCF.

In the past few years, little progress has been made in establishing the chemical constitution of HCF. In this chapter, we report the results of a comprehensive analysis of the environment of the larvae. Because HCF exchanges substances with the host for the survival and reproduction of protoscolexes, an understanding of the larval environment will aid in identifying

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Received 6 August 2013
Accepted 3 October 2013

the essential components of parasite growth and, potentially, in developing novel methods for preventing *E. granulosus* infection.

METHODS

Purification of hydatid-cyst fluid

Human HCF was collected after the surgical removal of fertile cysts from patients with cystic hydatid disease in The General Hospital of Ningxia Medical University. In total, 21 cysts of different sizes were isolated in a germ-free environment, and 55ml of cyst fluid was aspirated from these cysts using sterile needles under aseptic conditions and centrifuged at 10,000g for 15min at 4°C to remove particles. The supernatant fluid was stored at -80°C until use.

Electrophoresis and in-gel digestion

First, 20ml of cyst liquid was pre-frozen at -85°C for 5h and then placed in the freeze-dryer (-60°C, vacuum) overnight. After 24h, the cyst liquid was freeze-dried into a powder. The powder was lysed using lysis buffer [9mol/l urea, 4% 3-(3-cholamidopropyl) dimethyl-ammonio-1-propane sulfonate (CHAPS), 1% dithiothreitol (DTT), and 0.5% protease-inhibitor cocktail], fully oscillated and blended at 4°C for 1h, and centrifuged at 12,000g at 4°C for 30min. The final supernatant fluid was stored at -85°C until use. To remove salt, lipids, and undesired detergents, cleanup was performed with the ReadyPrepTM 2-D Cleanup Kit (BIO-RAD) California (USA). Next, the AurumTM Serum Protein Mini Kit (BIO-RAD) was used to remove the most abundant proteins. The protein pellets were re-solubilized in immobilized pH gradient (IPG) rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 50mM DTT, 0.2% Bio-Lyte ampholyte, 0.001% bromophenol blue) and then centrifuged. The supernatant was held at 4°C. The total protein concentration for each sample was determined using the Bradford assay[14]. Areas of the 17cm IPG strips (BIO-RAD) were wetted with the above sample in a rehydration tray, and mineral oil was added to prevent evaporation. The isoelectric focusing (IEF) program was set as follows: step 1: 250V (linear) for 30min; step 2: 500V (rapid) for 1h; step 3: 4,000V (linear) for 4h; step 4: 4,000V (rapid) for 30,000Vh; and step 5: 500V (rapid) for 20h (holding step). A total of 2ml of equilibration buffer I [6mol/l urea, 0.375mol/l Tris-HCL (pH 8.8), 20% glycerol, 2% SDS, 2% DTT] was added to the top of the strip in an 11-cm equilibration tray, followed by gentle rocking for 15min. Equilibration buffer I was discarded and replaced with equilibration buffer II [6mol/l urea, 0.375mol/l Tris-HCL (pH 8.8), 20% glycerol, 2% SDS, 2.5% iodoacetamide], followed by gentle rocking for 15min. IPG was loaded onto an SDS-PAGE gel composed of a 12% separation gel, and the proteins were stained using Coomassie Brilliant Blue. Each protein spot was sliced and destained 3 to 4 times by incubation in 50% acetonitrile and 25mM NH_4HCO_3 for 10min. After destaining, the gel pieces were placed in 10mM DTT/100 mM ammonium bicarbonate solution and deoxidated for 1h at 65°C. The DTT solution was removed after cooling to room temperature. Then, the samples were alkylated with 55mM iodoacetamide/100mM ammonium bicarbonate solution for 45 min at room temperature in the dark and then dried in a vacuum centrifuge. The dried gel pieces were subsequently rehydrated with 20µl of 100mM NH_4HCO_3 containing 12.5ng/µl trypsin (Sigma) for 16h at 37°C and then ultrasonically treated in 15µl of 0.1% trifluoroacetic acid (TFA). The extracts were analyzed by matrix-assisted laser desorption/ionizationtime-of-flight mass spectrometry (MALDI-TOF-MS).

Analysis of inorganic elements and biochemical parameters in hydatid-cyst fluid

Cyst fluid was added to an equal volume of acid digestion mixture (nitric acid and perchloric acid at a ratio of 4:1). Samples were heated in an automatic electric digestive device. After cooling, a clear solution formed and was diluted to 10ml with 1% nitric acid. An equal volume of acid digestion mixture treated the same way served as the control. The blank control, the standard for each element, the elements in the controls, and the elements in the samples were sequentially determined.

The automatic biochemical analyzer (AU-400, Olympus) was calibrated and controlled with quality-control liquid and standard liquid (Olympus) and then used to analyze cyst fluid. The results are shown as the mean ± standard deviation.

Free amino acid analysis of hydatid-cyst fluid

Cyst fluid was mixed with an equal volume of 10% sulfosalicylic acid and centrifuged at 10,000g for 15min. The supernatant was passed through a 0.45-µm filter, and amino acids were detected by the auto amino-acid analyzer (HITACHI L-8900). The fluid was separated using 4.6mm×60mm sulfonic acid cation resin in the lithium citrate buffer (PH 2.8-4.1) at 0.3ml/min for 30min. The reaction temperature was 135°C. The flow rate of the color development reagent ninhydrin was 0.25ml/min. The results are shown as the average amino acid concentrations ± standard deviation.

RESULTS

Composition of parasite proteins in hydatid-cyst fluid

We identified 30 protein spots using PD Quest 8.0 2D analysis software (**Figure 1**). The molecular weight of most of these proteins ranged from 43 to 97kDa. The isoelectric points of the 21 proteins identified in the cyst fluid ranged from 5 to 9. The data were uploaded to the swiss pro program and searched by Mascot (**Table 1**). Protein scores greater than 75 were considered significant ($p<0.05$). We found three proteins, namely β-hemoglobin (Nº 3), albumin (Nº 12), and serum transferrin (Nº 18), in the cyst fluid.

Inorganic element content and biochemical properties of hydatid-cyst fluid

The mineral elements in the hydatid fluid are shown in **Table 2**, and the biochemical indices of the HCF of *E. granulosus* are shown in **Table 3**. Several biochemical metabolites and proteins were measured in the cyst fluid. The amounts of many biochemical components, such as glucose, α-hydroxybutyric

TABLE 1 - Proteins identified by peptide mass fingerprints. Peptide mass fingerprints from the protein spots cut from the gel (**Figure 1**) used in the MASCOT search.

Spot number	Mass	Isoelectric point	Score	Expected	Matches	Protein name and species
1	15,763	7.52	27	1.1e+03	5	GRPE_BACFN
2	15,680	7.02	36	1.4e+02	3	RS5_CALS8
3	16,102	6.75	80	0.0047	9	HBB_ HUMAN
4	25,238	5.57	28	7.9e+02	6	AHPD_ACIBL
5	42,350	6.12	34	2e+02	9	MINN_BACCN
6	58,634	4.78	30	5.4e+02	9	ACPS_DESDG
7	58,837	4.66	49	7.1	10	PYREL_METBU
8	57,985	6.87	63	0.25	21	SYP_HELPH
9	58,953	9.41	53	2.4	20	PURA_METMA
10	73,357	5.72	59	0.68	36	ALBU_HUMAN
11	73,286	5.80	30	5.8e+02	10	HBBY_MESAU
12	71,317	5.92	121	4.2e-07	33	ALBU_HUMAN
13	75,309	5.93	42	30	10	CMOA_AERS4
14	75,388	6.25	41	45	9	LDHD_STAAC
15	75,462	6.26	32	3.2e+02	3	Y195_BPT7
16	75,394	6.41	51	3.8	14	Y262_STAES
17	75,410	6.41	51	3.8	14	Y262_STAES
18	71,317	5.92	106	1.3e-05	36	ALBU_HUMAN
19	98,230	6.05	25	1.5e+03	4	MT2_YARLI
20	98,782	5.95	35	1.5e+02	9	CB061_MOUSE
21	16,570	9.47	46	13	14	DOT1_EMENI

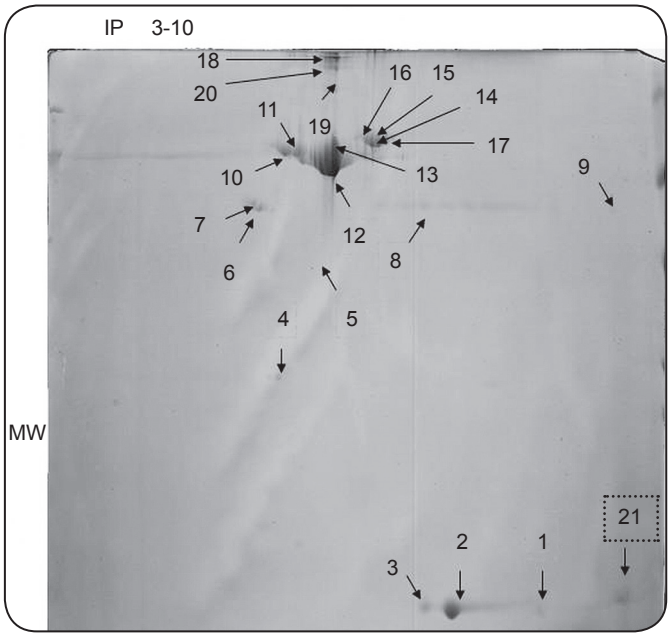

FIGURE 1 - 2-DE gel of HCF protein extracts that were first run on a 17cm pH 3-10 IPG strip in the first dimension and then run on a 20×20-cm 12% SDS-PAGE gel in the second dimension; 400µg of total proteins was loaded. 2-DE: two-dimensional gel electrophoresis; HCF: hydatid-cyst fluid; IPG: immobilized pH gradient; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IP: isoelectric point; MW: molecular weight.

TABLE 2 - The mineral element composition of hydatid fluid. The elements were determined using the inductively coupled plasma atomic emission spectrometer.

Element	$\overline{\chi}_{\pm s}$ (mg/l)	Analytical line (nm)
Calcium	113.93±34.05	315.88
Potassium	216.76±2.85	766.49
Magnesium	44.21±4.05	285.21
Ferrum	—	238.20
Copper	0.01±0.01	324.75
Chromium	—	267.71
Cadmium	—	214.44
Zinc	0.12±0.05	213.86
Selenium	—	196.03
Sodium	1554.46±61.09	589.59

acid, triglycerides, uric acid, and creatine kinase, are lower than those in normal human serum, with each component typically constituting a serum equivalent of approximately 1% of the HCF. The cholesterol level is also lower in cyst fluid; however,

TABLE 3 - Biochemical indices of the hydatid-cyst fluid of *Echinococcus granulosus.* Biochemical indexes of hydatid-cyst fluid detected by the automatic biochemical analyzer (AU-400, Olympus).

Content	Result	Human serum references
Total protein	0.55±0.13	64-82g/l
Albumin	0.10±0.08	34-50g/l
Globulin	0.50±0.18	20-45g/l
Glutamic-pyruvictransaminase	0.90±0.32	0-40u/l
Glutamic-oxalacetic transaminase	0.80±0.22	0-40u/l
Glutamic-oxalacetic transaminase/glutamic-pyruvictransaminase	0.92±0.18	0.36-0.68
Alkaline phosphatase	—	50-136u/l
Transglutaminase	—	0-40u/l
Lactic dehydrogenase	0.58±0.43	109-245u/l
Creatine kinase	0.48±0.10	25-200u/l
Isoenzyme of creatine kinase	0.18±0.10	0-24u/l
Urea	5.08±0.28	2.5-6.5mmol/l
Creatinine	25.10±0.39	44-150μmol/l
Uric acid	55.93±8.58	135-425μmol/l
Total cholesterol	0.02±0.01	3.1-5.7mmol/l
High-density lipoprotein	0.01±0.01	0.78-2mmol/l
Low-densitylipoprotein	—	0-3.7mmol/l
Triglyceride	0.02±0.01	0.56-1.71mmol/l
Glucose	1.65±0.26	3.8-6.1mmol/l
A-hydroxybutyric acid	0.20±0.08	95-250mmol/l
Ph	7.72±0.12	7.35-7.45

the ratio of glutamic-oxalacetic transaminase to glutamic-pyruvictransaminase is higher than that in normal human serum, and the levels of both enzymes are relatively high. The levels of urea, low-density lipoprotein, glutamic-pyruvic transaminase, the isoenzyme of creatine kinase, transglutaminase, and glutamic-oxalacetic transaminase are similar in cyst fluid and in normal human serum.

Analysis of amino acids in hydatid-cyst fluid

The results of the amino acid analysis in the HCF of *E. granulosus* are shown in **Table 4**. We measured the levels of 17 amino acids in the cyst fluid. The level of alanine was highest, followed by glycine. We failed to detect aspartic acid, most likely due to its low concentration.

DISCUSSION

Three proteins, β-hemoglobin, albumin, and serum transferrin, were found in the cyst fluid. Transferrins are iron-binding blood-plasma glycoproteins that control the level of free iron in biological fluids and participate in the body's resistance to infection. Thus, the transferrin in cyst fluid is likely able to transport the iron required for the growth of *E. granulosus.* Albumin is the most abundant protein in plasma. Its main function in mammals is to maintain oncotic pressure. It is also a transport protein for, e.g., fatty acids, unconjugated bilirubin, and thyroid hormones. Albumin is also a nutrient for cells. Human albumin in the cyst fluid provides energy to the larvae. Hemoglobin is a composite protein containing iron, which is composed of ferroheme and globin and plays an important role in transporting oxygen and carbon dioxide. β-hemoglobin is a subtype of hemoglobin that is detected in cyst fluid. β-hemoglobin provides necessary energy to larvae by transporting oxygen and carbon dioxide.

The concentrations of inorganic elements in cyst fluid vary across hosts. The concentration of Na^{2+} in the cyst fluid was approximately half that in healthy human serum, while the levels of K^{+}, Mg^{2+}, and Ca^{2+} were higher in the cyst fluid (**Table 2**). One important role of Ca^{2+} is to control the pH and thus prevent acidity in the HCF, and Mg^{2+} and Ca^{2+} are found in calcareous bodies in the cyst[15]. These results established the concentrations of inorganic elements in cyst fluid and indicated that the entrance

TABLE 4 - Analysis of amino acids in the hydatid-cyst fluid of *Echinococcus granulosus*. Analysis of amino acids in hydatid-cyst fluid detected by the auto-amino-acid analyzer (Hitachi L-8900).

Amino acids	Content (nmol/μl)
Glycine (GLY)	4.49±0.59
Leucine (LEU)	0.99±0.29
Methionine (MET)	0.23±0.06
Tyrosine (TYR)	0.19±0.04
Histidine (HIS)	0.51±0.06
Threonine (THR)	0.39±0.04
Alanine (ALA)	13.33±2.83
Isoleucine (ILE)	0.63±0.15
Cysteine (CYS)	0.17±0.04
Lysine (LYS)	0.77±0.02
Aspartate (ASP)	—
Valine (VAL)	3.21±0.98
Phenylalanine (PHE)	0.11±0.03
Proline (PRO)	0.86±0.28
Serine (SER)	0.34±0.06
Glutamic (GLU)	0.24±0.03
Arginine (ARG)	0.14±0.02
Ammonia (residue) NH_3	0.42±0.06

of these elements into the cyst is strictly controlled to meet the requirements of parasite growth.

We found that the levels of both glutamic-oxalacetic transaminase and glutamic-pyruvictransaminase are high in cyst fluid (**Table 4**), suggesting a high level of transamination in larvae. High activity of these enzymes may also be related to a liver disorder caused by liver echinococcosis. The level of uric acid in cyst fluid is also very high. Uric acid and ascorbic acid are strong reducing agents (electron donors) and potent antioxidants. In humans, over half the antioxidant capacity of blood plasma is derived from uric acid[16], and plasma uric acid levels correlate with longevity in primates and other mammals[17]. Thus, uric acid may play a protective role during the growth of protoscolexes.

Alanine is enriched in cyst fluid compared with other amino acids. High levels of alanine were also reported in cyst fluid in other studies[18]. In our assays, aspartic acid was shown to be absent from cyst fluid. The intake of amino acids in hydatid disease could occur in two steps: amino acids first cross the cyst wall by free diffusion and then enter the cyst fluid through free diffusion and active transport mediated by specific receptors in the germinal layer[19]. The absence of aspartic acid suggests that the cyst membrane does not contain aspartic acid receptors and that the larvae cannot synthesize this amino acid. Therefore, the larvae must obtain aspartic acid through transamination, as is consistent with the high levels of transaminases found in the HCF. The levels of amino acids vary remarkably in cyst fluid from different hosts (pigs, sheep, cattle, and humans)[20,21].

These results indicate that the entrance of all organic and inorganic chemicals depends on parasite requirements. The chemical composition of cyst fluid plays an important role in protecting against hydatid disease and providing nutritional material. Knowledge of parasite nutrition can aid in identifying new ways to prevent hydatid disease by changing the nutrient composition of cyst fluid or blocking nutrition and metabolic pathways.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work was supported by grants from the National Natural Science Foundation of China (Nº 30960360).

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