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ORIGINAL RESEARH

Biochemical properties of γ-glutamyl transpeptidase from *Onchocerca volvulus*

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عزل ودراسة خصائص إنزيم γ-glutamyl transpeptidase من طفيل Onchocerca Voluulus

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الملخص: تمت دراسة أنزيم (γ-glutamyl transpeptidase) من طفيل (Onchocerca Voluulus) المسبب لداء العمى الليلي (Onchocerciasis) المنتشر في عدة مناطق من العالم وخاصة في إفريقيا وأمريكيا اللاتينية. هذا الأنزيم يلعب دورا مهما في المحافظة على الجلوتاثيون (Glutathione) وهو مركب عضوي يحافظ على درجة اختزال الخلية، حيث أنه الأنزيم المسؤول عن تحطيم الجلوتاثيون في الخلية إلى عناصره الأساسية وبالتالي إعطاء الخلية مصدر للأحماض الأمينية مثل الCysteine . لقد أظهرت الدراسة أن هذا الأنزيم مرتبط غشاء الخلية عند الطفيل وقد تم تنقية الأنزيم إلى درجة عالية واستعمل هذا المستخلص في تحديد ميزات البروتين ومن تلك الميزات أنه يستقبل عدة أحماض أمينية وثنائي الببتيدات من أجل ربطها بالمركب العضوي جاما جلوتاميل وقد وجد أن قيمة ميكاليس (Michalis-Menten) لتلك المركبات في نطاق الميليمولار(mM) . وقد وجد أيضا أن المثبط " acivicin" له كفاءة عالية في وقف عمل الأنزيم بقيمة عامل مثبط (ki= 0.59±0.04) وبالتالي يمكن أن يكون هذا الإنزيم مرشح لأن يكون هدفا لتطوير أدوية ضد تلك الطفيليات.

ABSTRACT: γ-glutamyl transpeptidase (GGT) from *Onchocerca volvulus* is involved in the oxidative stress. The enzyme catalyzes the breakdown of glutathione (GSH) and thus provide the parasite with cysteine for the synthesis of GSH and other amnio acids. The enzyme, which was highly purified from *Onchocerca volvulus*, is found to be membrane-bound with a specific activity of 100 U mg^{-1} and a molecular mass of 68 KDa. The apparent K_m-values for the γ-glutamyl donor L-glutamic acid γ-(4-nitroanilide) is 0.023 ± 0.013 mM. The data presented in this study showed that various amino acids and dipeptides served for the γ-glutamyl moieties of the enzyme reaction products and showed K_m values in the mM range. Acivicin was an irreversible inhibitor of the enzyme with a pseudo-first-order kinetics (k_{max}) of 0.34 ± 0.004 min^{-1} and K_I = 0.59 ± 0.04 mM. These finding indicate the physiological role of the GGT of this parasite nematode in the catabolism of GSH. Further studies are required to investigate the use of this enzyme as a potential target for the development of chemotherapy against *O. volvulus.*

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INTRODUCTION

Onchocerciasis is a parasitic disease caused by the filarial worm *Onchocerca volvulus*. It is called river blindness because the blackflies that transmit the disease breed in fast flowing waters and it is considered as world's second leading infectious cause of blindness. The disease is spread over several countries including Africa, the Arabian Peninsula, and Central and South America where about 18 million people are infected with *O. volvulus*. Additionally, about 50 million individuals are at risk of acquiring the parasite. Furthermore, it significantly associated with the socio-economic development in affected communities (see www.who.int/tdr/index.html).

Currently, the chemotherapeutic approaches to control parasite transmission and to treat onchocerciasis depend on the use of drugs that kills only microfilariae but not the adult parasites. This necessitates the continuous use of these microfilaricides until the adult worms die. Therefore, searching new chemical leads towards development of new drugs that effectively kill the adult worms would greatly support the control and treatment of *O. volvulus* infections.[1] Furthermore, the potential development of drug-resistant strains of the nematode also demands the identification of alternative drug candidates to control the disease.[2]

γ-glutamyl transpeptidase (GGT), which catalyzes the transfer of the γ-glutamyl moiety of γ-glutamyl peptides to appropriate acceptors, is distributed in a wide range of living cells. The enzyme catlayzes the first step in the glutathione (GSH) breakdown. Thus, its physiological function is associated with its action in cleaving extracellular GSH providing the cells with access to additional cysteine, the rate limiting amino acid, for GSH *de novo* synthesis. Thus GGT enzyme is critical for maintaining GSH and cysteine homeostasis.[3,4] GGT plays also roles in antioxidant defense, detoxification and inflammation processes. For example, GGT has been found to be involved in resistance of some types of tumors to drugs such as cisplatin[5] and thus inhibition of this protein would enhance the efficacy of anticancer drugs.

Although the role of the GGT of mammals and microbes has extensively studied, there is little information about the parasite enzyme.[6-10] In filarial parasites, the enzyme has been reported to be associated with the pathology of pulmonary eosinophilia through its potent allergenicity and the induction of antibodies against the host pulmonary epithelium.[11] However, this property seems to be independent of exact parasite infection since soluble extracts from *Ascaris suum,*[12-13] *Nippostrongylus brasiliensis*[14] and *Brugia*[15] induced TH2 CD4+ responses. Thus the molecular nature of the nematode allergen (GGT) is still unclear. Therefore, the aim of this study is to isolate the enzyme from *O. volvulus* and characterize it in the aim of understanding its role and its biochemical and molecular properties especially a strong allergy is associated with infection with this parasite.

MATERIALS AND METHODS

Parasites:

Onchocercomas were isolated from Liberian patients. The nodules had been surgically removed from the patients using local anaesthesia and aseptic conditions. By microdissection, the worms were isolated from the nodules and, to eliminate host tissue, incubated in RPMI 1640 containing 0.5% collagenase (Clostridium histolyticum). The collagenase digestion was carried out for 24 h in a 37°C shaking incubator. Parasites are kept at -196°C until use.

Purification of γ-glutamyl transpeptidase:

All steps were carried out at 4◦C. The total worms were homogenized in 3 volume of 50 mM Tris-HCl buffer (pH 7.5) at 1400 rev./min with a glass/glass homogenizer. The homogenate was centrifuged at 100,000 g for 1 h. The pellet was suspended in 5 vol. of 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M sodium chloride, 0.1 mM phenylmethylsulfonyl Fluoride and 1% Triton X-100 at 1400 rev./min with glass/glass homogenizer. The suspended pellet was stirred gently for 1 h and subsequently centrifuged at 100,000 g for 1 h. The supernatant of the suspended pellet was further partially purified applying DEAE-cellulose column previously equilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.1% Triton X-100 (buffer A). The column was thereafter washed with buffer A, followed by a gradient of NaCl (0.0-0.6 M). The enzyme was eluted between 0.45 and 0.55 M NaCl. The eluent was concentrated to 1 ml using 10 microconcentrator and subjected to gel filtration column (Superdex 200) previously equilibrated with buffer A plus 0.15 M NaCl at a flow rate of 1 ml/min. The enzyme activity was detected in the void volume and this fraction was used for enzymatic charactyerization. Aliquot of the void volume was treated with bromelain (0.008 mg) for 45 min at 37 °C and re-subjected to gel filtration chromatography under the same conditions.

Assay and kinetic characterization of γ-glutamyl transpeptidase (GGT):

The standard assay for GGT was performed relying on the hydrolysis of the substrate analogue, L-glutamic acid γ-(4-nitroanilide) (GNA, MaxDiscovery GGT enzymatic Assay Kit) by the *O. volvulus* GGT and the dipeptide Gly.Gly according to the procedure described in the kit. Under these conditions, various amino acids and dipeptides were tested as acceptors at concentration range 0.1-20 mM. One unit (U) of the enzyme activity is defined as the amount that catalyzes the formation of 1 μmole of GNA per min at 37 °C. Protein was determined according to Bradford.[16]

To examine the activity of the inhibitor acivicin ((αS, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; Sigma) on the parasite GGT, the partial purified enzyme preparation (0.01 mg/ml) was incubated in 20 mM Tris buffer, pH 7.4, at 4 °C with various concentrations of acivicin (0.025-0.25 mM). At the indicated time, an aliquot was removed and enzymatic activity at standard conditions was measured by monitoring the hydrolysis of GNA using spectrophotometer. Data were plotted as a function of time and fit to a single-order decay with Prism (Graph Pad Software version 5). The observed rate constant (k_{obs}) at a particular reagent concentration (R) can be described by

$$1/K_{obs}=1/K_{max}+(K_I/K_{max})(1/[$$

where k_{max} is equal to the maximal inactivation rate at saturating concentrations of the reagent and $K_I= (k_{-1} + k_{max})/k_1$ and represents the reagent concentration that results in half of the maximal inactivation rate.[17] A double-reciprocal plot was used to calculate k_{max} and K_I of the inhibitor.

RESULTS

Enzyme purification and Molecular mass

The purification scheme of the enzyme involving anion exchanger and gel filtration chromatography led to a highly purified enzyme preparation with 400-fold purification with a specific activity of 100 U/mg protein. Table 1 summarizes the purification procedure. The enzyme was eluted in the void volume of the gel filtration chromatography. Bromelain treatment of the enzyme and subsequent application to the same column resulted in a single peak of enzyme activity of molecular mass 68 KDa.

Kinetic properties of the enzyme

The optimal pH and temperature of the

Table 1. *Purification procedure of γ-glutamyl transpeptidase from O. volvulus.*

Purification step	Total protein (mg)	Total activity (U)	Specific Activity (U mg-1)	Yield (%)	Purification fold
Homoginate	120	90	0.75	100	1
Triton-solubilized membrane extract	30	70	2.33	78	4
DEAE-cellulose	1	60	60.33	66	120
Gel filtration	0.3	30	100.00	33	400

enzyme were 8.5 and 37°C, respectively. The apparent K_m-values for L-glutamic acid γ-(4-nitroanilide) is 0.023 ± 0.013 mM (n=3) Table 2 gives data on the ability of various amino acids and dipeptides to serve as acceptors for γ-glutamyl moiety of the enzyme reaction products. The best acceptor is L-Gly.Gly followed by L-Ser.Gly and L-Ala.Gly. The K_m-vlaues for acceptors are in mM range. Enzyme inhibition showed that the enzyme is inhibited by acivicin in time-dependent manner. The double-reciprocal plot (Figure 1) was used to calculate k_{max} = 0.34 ± 0.004 min^{-1} and K_I = 0.59 ± 0.04 mM.

DISCUSSION

Oxidative stress is under focus as a potential drug target against parasites and other diseases as cancer and cardiovascular diseases. GGT is an important enzyme in this condition where it maintains the steady-state concentration of both intracellular and extracellular glutathione. Thus, targeting the parasite GGT is important in the approach of chemotherapy.

Purification of the enzyme from the *O. volvulus* led to 400-fold purity and resulted in highly purified preparation. The limitation to achieve 100% purified enzyme relied on the difficulty to obtain enough worms from the source.

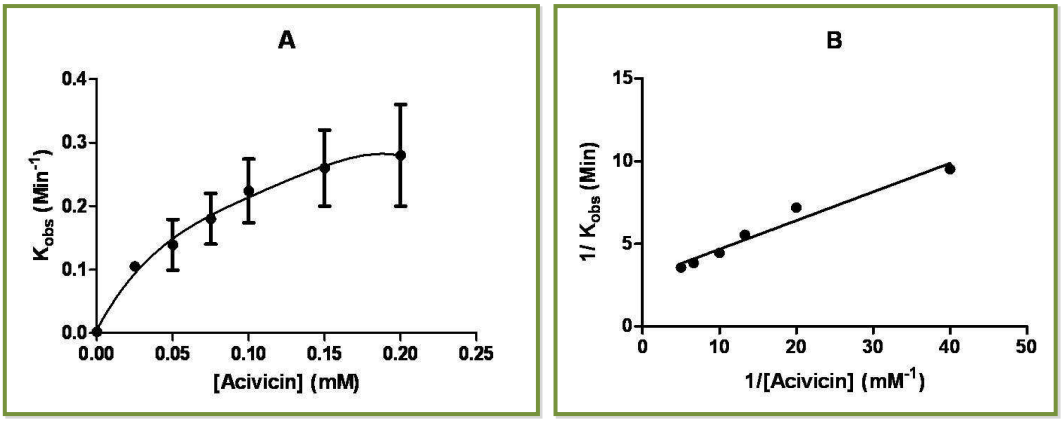

Figure 1. ***Inactivation of Onchocerca volvulus γ-glutamyl transpeptidase (OVGGT) by acivicin.*** OVGGT (0.1 mg/ml) was incubated in 20 mM Tris buffer, pH 7.4, at 4 °C with various concentrations of the inhibitor (0.025-0.25 mM) for 0, 5, 7.5, 10 and 15 min. K_{obs} was calculated from the slope of ln [$E_t/E_{\square}$] versus time where E_t and $E_{\square}$ are enzymatic activities at time 0 and time *t* respectively. **(A)** Dependence of rate of OVGGT inactivation of acivicin and the rate of concentration and the rate of inactivation was determined. **(B)** Double reciprocal plot used to calculate K_{max} and K_I.

Acceptor	Activity (U mg-1)	%	Km (mM)	Vmax (U mg-1)
L-Gly.Gly	7.75 ± 0.09	(100)	0.91 ± 002	100 ± 7
L-Ala.Gly	3.10 ± 0.30	(59)	0.60 ± 0.01	44 ± 1
L-Cys.Gly	2.30 ± 0.20	(28)	0.99 ± 0.02	31 ± 2
L-Gln.Gly	2.10 ± 0.10	(33)	0.60 ± 0.03	36 ± 1
L-Met.Gly	2.80 ± 0.10	(47)	0.44 ± 0.05	40 ± 2
L-Ser.Gly	4.75 ± 0.20	(74)	0.92 ± 0.03	66 ± 3
L-Cys	1.40 ± 0.10	(31)	1.12 ± 0.07	29 ± 1
L-Met	3.95 ± 0.30	(51)	1.00 ± 0.06	33 ± 1
L-Ser	1.85 ± 0.10	(17)	0.85 ± 0.06	27 ± 1

Table 2. *Activity, apparent Km-values of γ-glutamyl transpeptidase from O. volvulus for various amino acids and di-peptides.* The assay of the enzyme was done under standard assay conditions. The final concentration of acceptors was 10 mM. Figures in parentheses correspond to the percentage of activity to L-Gly.Gly. The values, derived from triplicate determinations, represent the mean ± SD of three independent experiments.

The data presented in this study show that GGT from *O. volvulus* exists predominantly in association with the cell membrane. In this regard, the nematode enzyme resembles its counterparts from mammals, *S. cerevisiae*[6,7,18,19] whereas it occurs in the periplasmic space in *E. coli.*[20] However, GGT from Radish Cotyledon was found in two forms: soluble and membrane bound.[21] In the parasite *Trypansoma cruzi*, the enzyme was found to be cytosolic.[22]

A wide range of amino acids and dipeptides were found to be acceptors to the γ-glutamyl moiety of the reactions products of the GGT enzyme. Similar results were reported for the enzymes isolated from various sources.[6,7,18,19,22]

Two distinct forms of the GGT enzyme from *O. volvulus* were determined by gel filtration chromatography corresponding to the void volume and a protein of molecular mass 68 KDa. Re-application of the void volume enzyme onto gel filtration after treatment with bromelain showed identical protein position of 68 KDa indicative that the soluble form is a bound form of the enzyme resulted from endogenous proteolysis. In this regard, the *O. volvulus* is similar to the *A. suum* GGT.[6]

Acivicin, which is commonly used mechanism-based inhibitor of GGT,[23-24] inhibits the GGT from *O. volvulus* irreversibly. This is in agreement with results described for other enzymes of rat kidney, *A. suum, E. coli, H. pylori.*[6,22-24]

The results presented here are of great value since no other study has touched this enzyme from *O. volvulus* and elucidation of the native enzyme properties will hopefully help in the development of chemotherapy against this parasite. However, further studies are required to achieve this goal.

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