



Kinetic properties of glutamate metabolism in the nematode parasite *Haemonchus contortus* (L₃)

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ABSTRACT

The key steps in cell metabolism of all organisms are the synthesis of both glutamate and glutamine because they denote the only means of incorporating inorganic nitrogen into carbon backbones. In this study, an assay for the activity of two key enzymes in nitrogen metabolisms such as glutamate dehydrogenase (GDH) and glutamine synthase (GOGAT) was conducted using homogenates of L3 larvae of *Haemonchus contortus*. GDH was assayed both in the direction of glutamate utilisation and glutamate formation. GOGAT activity was monitored in the direction of glutamine utilisation. The present result showed that *H. contortus* had a high K_m for ammonia (27.22mM) and glutamine (15.04 mM). The high K_m for ammonia suggests a very low affinity for ammonia, meaning that in the reversible amination of 2-oxoglutarate to glutamate, the predominant direction is likely to be glutamate deamination and not the incorporation of ammonia. The activity of GOGAT was also demonstrated but with a high K_m , which indicates a low binding affinity of glutamine to the enzyme. Nevertheless, the presence of the two key enzymes of nitrogen metabolism, i.e. GDH and GOGAT, may provide a potential target for anthelmintic action.

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INTRODUCTION

Gastrointestinal nematodes that live within the farmed livestock can cause health problems in livestock with more economic loss. *Haemonchus contortus* is one of the nematodes. The infectious parasitic cycle begins as adult female worms lay eggs

inside the intestine and will pass out in the faeces. The eggs in faeces may hatch into first-stage larvae (L1) under favourable conditions. Development of L1 into L2 stage occurs by feeding on the faecal bacteria, then L2 stage into L3 which is infective but has the L2 cuticle as a protective sheath. After an appropriate host eats L3 along with pasture, they shed the L2 cuticle and enter the lumen of gastric glands, where they develop and emerge into L4 or immature adult worms after 2 to 4 days (Sinnathamby *et al.*, 2018). Since parasitism can cause host mortality, long-lasting and novel effective methods should be needed in addition to anti-parasitic drugs. With the knowledge of metabolic pathways and the enzymes involved in the pathways which are essential for larval growth, it is possible to predict the underlying mechanism to create a new target of anthelmintic (Magdaleno *et al.*, 2011).

Absorption of nitrogen through nitrogen metabolism is an essential process in a living organism. Even the parasitic larva and parasitic eggs require more nitrogen and its related energy metabolism as nitrogen is the necessary component of major macromolecules such as proteins and nucleic acids, enzymes and hormones (Harper *et al.*, 2010). L-glutamic acid plays a crucial role in the metabolism of amino acids. The key steps in cell metabolism in all organisms are the synthesis of both glutamate and glutamine. This is because these compounds are the only means of incorporating inorganic nitrogen into carbon backbones. Inorganic nitrogen is assimilated in the form of ammonium. Ammonia that is excreted is synthesised from glutamate by glutamate dehydrogenase.

On the other hand, ammonia can also be incorporated into glutamate by reverse reaction where there is an expression of both glutamines:2-oxoglutarate aminotransferase or glutamate synthase (GS-GOGAT) (Scaraffia *et al.*, 2005). The two major pathways for ammonium assimilation are glutamine synthetase (GS) and glutamate synthase (GOGAT) cyclic mechanism. This cycle becomes active when there is limited exogenous nitrogen source, due to the high affinity of GS for ammonium. This pathway consumes more energy, and therefore its regulation is under transcriptional and post-translational levels of control (Reitzer, 2003). Its analogue amino acid can inhibit all those glutamate pathways as it is the common substrate of most enzymes involved in the two mentioned pathways. Inhibition of some glutamate metabolic pathways could lead to diminished parasitic survival (Silber *et al.*, 2005). In the present study, glutamate dehydrogenase (GDH) and glutamine synthase (GOGAT) activity were analysed using homogenates of the L₃ larvae of *H. contortus*. Kinetic activities of such two critical enzymes in L₃ larvae of *H. contortus* was determined. GDH activity was studied both in the direction of glutamate utilisation and glutamate formation, whereas GOGAT activity was evaluated in the direction of glutamine utilisation.

MATERIALS AND METHODS

Procurement of parasites

For the procurement of third-stage larvae (L₃), a faecal sample was collected directly from the rectum of naturally exposed goats to make a pool of positive samples for copro-culture following the procedure of Zajac and Conboy (Zajac and Conboy, 2012). Third stage larvae (L₃) were isolated and collected through Baermann's technique. The larvae were identified according to their morphological charac-

teristics and stored at 4°C until further use (Baermann, 1917).

Preparation of homogenates

H. contortus sample (1g) was transferred to a frozen, chilled mortar for at least 15min and homogenised with a chilled pestle. When homogenates were to be used for more than one assay, aliquots were kept in Eppendorf tubes on ice until required. The protein concentrations of homogenate were determined by the Bradford method (Bradford, 1976).

Glutamate dehydrogenase (GDH) assay

GDH activity at 30°C was estimated in the direction of glutamate utilisation in homogenates of sheathed L₃ using the protocol for continuous assays. To the reaction mixture (total volume of 3ml) containing 50µg homogenate protein in 100mM phosphate buffer, 0.2mM NAD⁺ was added at pH 7.5 where the reaction was initiated by adding 5mM glutamate. The K_m and V_{max} were determined for GDH activity for the substrate glutamate and the cofactor NAD⁺/NADP⁺ in the direction of glutamate utilisation. Same kinetic parameters were determined for the substrate α-ketoglutarate, ammonia and the cofactor NADH/NADPH in the direction of glutamate formation.

The direction of glutamate utilisation

For the substrate glutamate, varying concentrations of glutamate from 0 to 20mM was added to the respective reaction mixture to initiate the reaction. For the cofactor, NAD⁺/NADP⁺, varying concentrations of NAD⁺ (or NADP⁺) from 0 to 10mM was added to the respective reaction mixture to initiate the reaction.

The direction of glutamate formation

For the substrate α-KG, varying concentrations of α-KG from 0-20mM was added to the respective reaction mixture to initiate the reaction. For the substrate ammonia, varying concentrations of ammonia from 0 to 150mM was added to the respective reaction mixture to initiate the reaction. For the cofactor NADH, varying concentrations of NADH (or NADPH) from 0 to 2mM was added to the respective reaction mixture to initiate the reaction.

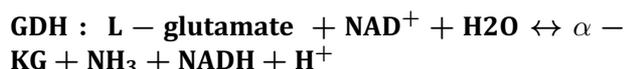
Glutamate synthase (GOGAT)

GOGAT activity was monitored in the direction of glutamine utilisation. K_m and V_{max} for substrate glutamine were determined by adding 0.2mM NADH to the reaction mixture (3ml) containing 20mM of α-KG and 50µg homogenate protein prepared in 100mM phosphate buffer at pH 7.5. The reaction was initiated by the addition of glutamine in concentrations from 0-3mM. GOGAT activity was calculated

from the rate of utilisation of NADH, and readings were observed at 340nm.

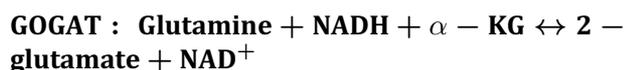
RESULTS

GDH catalyses the reversible oxidative deamination of L-glutamate to α -KG and ammonia with either NAD^+ or NADP^+ as a cofactor. There are three enzyme dehydrogenase groups based on cofactor requirements such as NAD^+ specific (EC 1.4.1.2), NADP^+ specific (EC 1.4.1.4) or dual cofactor specific (EC 1.4.1.3) which can use either cofactor when an enzyme is present in higher organisms. Glutamate dehydrogenase (GDH) was assayed both in the direction of glutamate utilisation and formation,



GDH activity was calculated from the rate of utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm. GDH homogenate activity monitored in the direction of glutamate formation with increasing concentration of 2-oxoglutarate, ammonia, NADH, NAD and glutamate were shown in Figures 1, 2, 3, 4 and 5 respectively.

GOGAT activity was monitored in the direction of glutamine utilisation,



Enzyme GOGAT was assayed with an increasing concentration of glutamine at 30°C. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH and was monitored spectrophotometrically at 340nm, as shown in Figure 6.

DISCUSSION

The present study on *L3H.contortus* confirms the presence of significant activity of GDH and GOGAT. In the deaminating direction, the K_m obtained for NAD was 1.705, which is comparatively similar to K_m obtained in *T.cruzi* as described by (Cazzulo *et al.*, 1979). In the aminating direction, the K_m obtained for NADH was 0.55 that was much higher than any other species. The results are in similar to the previous reports where other helminths such as *H.diminuta*, *F.hepatica* and *O.cuniculi* GDHs seemed to be more active with NAD^+ or NADH than with NADP (Mustafa *et al.*, 1978; Prichard and Schofield, 1968; Hutchinson and Fernando, 1975). The GDH of *C.callunae*, on the other hand, explicitly needed NADPH and NADP^+ as coenzymes in the amination and deamination reactions, respectively (Ertan, 1992).

GDH also has a very low affinity for ammonia, and its

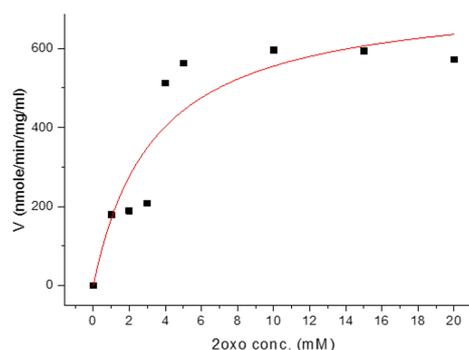


Figure 1: Homogenate activity monitored in the direction of glutamate formation with increasing concentration of 2-oxoglutarate at 30°C

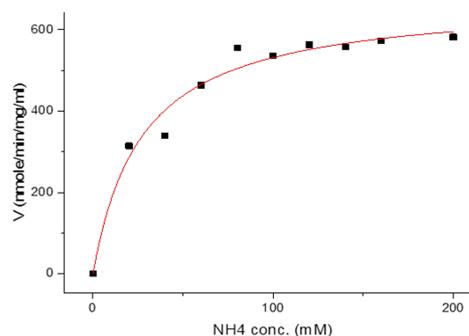


Figure 2: Homogenate activity monitored in the direction of glutamate formation with increasing concentration of ammonia (NH_4) at 30°C

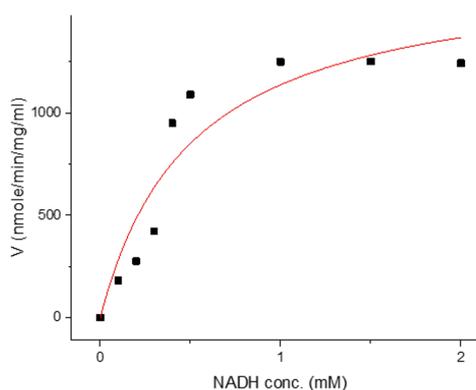


Figure 3: Homogenate activity monitored in the direction of glutamate formation with increasing concentration of NADH at 30°C

affinity for ammonia usually determines the direc-

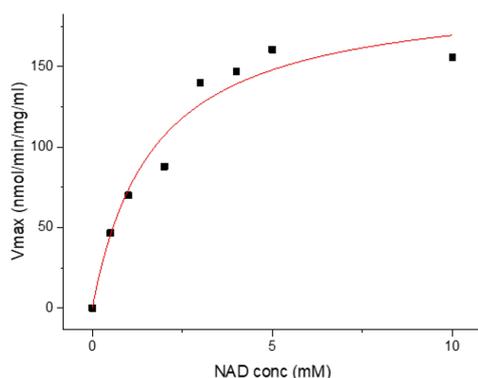


Figure 4: Homogenate activity monitored in the direction of glutamate utilisation with increasing concentration of NAD at 30°C

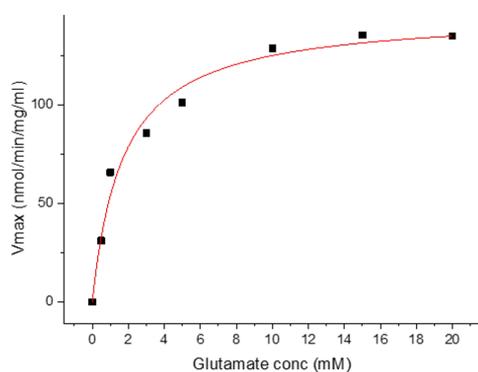


Figure 5: Homogenate activity monitored in the direction of glutamate utilisation with increasing concentration of glutamate at 30°C

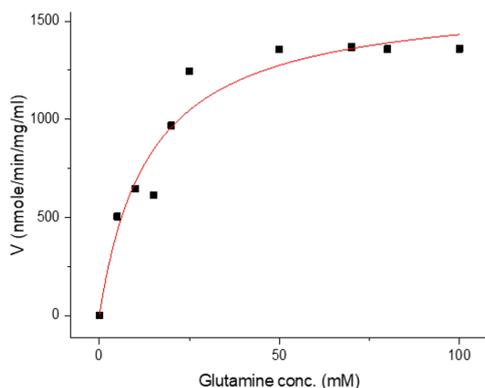


Figure 6: Enzyme assayed with increasing concentration of glutamine at 30°C

tion it dominates (Muhamad, 2006). The present result showed that *H.contortus* had a high K_m as 27.22mM for ammonia, as shown in Figure 2. Even with comparatively high enzyme activity, this very low affinity for ammonia would propose that in the reversible amination of 2-oxoglutarate to glutamate, the predominant direction is likely to be glutamate deamination and not the incorporation of ammonia. Nevertheless, the K_m value on *H.contortus* has also been reported (Rhodes and Ferguson, 1973), where the K_m value was higher than the K_m value obtained in the present study. The difference in the K_m value might be due to the insensitivity of the larvae towards the enzyme. This is because even purified enzyme preparations are often heterogeneous. Also, it can be assumed that the high K_m values for all the substrates and cofactors could be due to the different sensitivities of the larval stage toward the enzyme GDH.

Figure 1, Glutamate dehydrogenase (GDH) activity of the L_3 larvae of *H.contortus*. Homogenate activity monitored in the direction of glutamate formation with increasing concentration of 2-oxoglutarate at 30°C. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Figure 2, Glutamate dehydrogenase (GDH) activity of the L_3 larvae of *H.contortus*. Homogenate activity monitored in the direction of glutamate formation with increasing concentration of ammonia (NH_4) at 30°C. GDH activity was calculated from the rate of utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Figure 3, Glutamate dehydrogenase (GDH) activity of the L_3 larvae of *H.contortus*. Homogenate activity monitored in the direction of glutamate formation with increasing concentration of NADH at 30°C. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Figure 4, Glutamate dehydrogenase (GDH) activity of the L_3 larvae of *H.contortus*. Homogenate activity monitored in the direction of glutamate utilisation with increasing concentration of NAD at 30°C. GDH activity was calculated from the rate of NADH utilisation through the reduction of NAD, which was monitored spectrophotometrically at 340 nm.

Figure 5, Glutamate dehydrogenase (GDH) activity of the L_3 larvae of *H.contortus*. Homogenate activity monitored in the direction of glutamate utilisation with increasing concentration of glutamate at 30°C. GDH activity was calculated from the rate of NADH utilisation through the reduction of NAD, which was

monitored spectrophotometrically at 340 nm.

Figure 6, Glutamate synthase (GOGAT) activity of the L₃ larvae of *H. contortus*. Enzyme assayed with increasing concentration of glutamine at 30°C. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340nm.

GOGAT, on the other hand, is an enzyme that manufactures glutamate from glutamine and α -ketoglutarate, and thus along with glutamine synthetase (GS) plays a central role in the regulation of nitrogen assimilation in photosynthetic eukaryotes and prokaryotes (Blanco and Blanco, 2017). Glutamate and glutamine are the crucial intermediates in the biosynthesis of cell materials of organisms. When ammonia is available in excess, glutamate dehydrogenase catalyses the assimilation of ammonia, whereas at limited concentrations of ammonia a combined system of GS and GOGAT is active. This was generally supported by the findings that GS was repressed and GDH was induced when ammonia was present in excess and that the affinity of GS for ammonia was higher than that of GDH (Tempest *et al.*, 1970). The enzyme is often described as not present in animals. However, the demonstration of GOGAT in L₃ *O. circumcincta* was reported by (Muhamad, 2006), which makes it the most interesting findings as GOGAT enzymes were believed not to be expressed in the sheep host. In the present study, the expression of GOGAT was demonstrated but with a high K_m, which indicates its low-affinity binding to the enzyme. However, concerning the chemotaxis study, glutamine attracted the highest migration from the larvae. This suggests that the larvae may have a high need for glutamine that can be used for many other systems.

GOGAT is of more interest because it was assumed that GOGAT utilises cytosolic α -ketoglutarate. GOGAT also can withdraw α -ketoglutarate from the mitochondrial pool, suggesting its relation to the transport of α -ketoglutarate between cytosol and mitochondria (Larsson *et al.*, 1998). In yeast, data from earlier mathematical models have also indicated that the GOGAT pathway plays a more critical physiological role than is generally assumed (van Riel *et al.*, 2000). Indeed, GOGAT has been proposed to be an essential gene for the *Mycobacterium tuberculosis* (*M. Tuberculosis*) life cycle and has been suggested to be a useful platform for further research targeting therapeutic intervention in *M. tuberculosis* (Cheung and Tanner, 2011)

CONCLUSIONS

The understanding of this enzymatic activity is central to gaining insights into the catabolic and anabolic pathways of these worms and may serve as a target for anthelmintic inhibition. Therefore, the activity of the two key enzymes of nitrogen metabolism, i.e. GDH and GOGAT may serve as a target for such aim.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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