Effect of Hydroquinone on Ruminal Urease in the Sheep and its Inhibition Kinetics *in vitro***

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ABSTRACT : Effect of hydroquinone (HQ) on rumen urease activity was studied. Hydroquinone at concentrations of 0.01 ppm, 0.1 ppm, 1 ppm, and 10 ppm inhibited urease activity of intact rumen microbes *in vitro* by 25%, 34%, 55% and 64% respectively. In the presence of low concentrations of β-mercaptoethanol, rumen urease could be solubilized and partially purified. The Km for the enzyme was 2×10^{-3} M with Vmax of 319.4 μmoles/mg min. The kinetics of inhibition with partially purified rumen urease was investigated. The result showed that the inhibitory effect was not eliminated by increasing urea concentrations indicating a noncompetitive effect in nature with an inhibition constant 1.2×10^{-5} M. Hydroquinone at the concentration of 10 ppm produced 64% urease inhibition, did not affect ruminal total dehydrogenase and proteolytic enzyme ($p>0.05$), but increased cellulase activity by 28% ($p<0.05$) *in vitro*. These results indicated that hydroquinone was a effective inhibitor of rumen urease and could effectively delay urea hydrolysis without a negative effect. The inhibitor appeared to offer a potential to improve nitrogen utilization by ruminants fed diets containing urea. ***(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 9 : 1216-1220)***

Key Words : Hydroquinone, Ruminal Urease, Inhibition Kinetics, Sheep

INTRODUCTION

Urea fed to ruminants is broken down rapidly to ammonia in the rumen. The rate of ammonia release often exceeds the capacity of the microbes to utilize ammonia and decreases efficiency of its utilization. The efficiency of urea utilization was increased by specific inhibitors of rumen urease (Mahadevan et al., 1976; Chalupa, 1977). Hitherto several urease inhibitors, including acetohydroxamic acid (AHA), phosphoric phenly ester diamide (PPDA) and N-(n-butyl)-thiophosphoric triamide (NBPT) have been investigated. Although these inhibitors are capable of short-term inhibition of microbial urease activity in the rumen, the ruminal microflora are capable of adapting to chronic urease inhibitor administration. AHA reduced production of volatile fatty acids, decreased the ratio of acetate to propionate and reduced viable counts of anaerobic bacteria in rumen fluid at a rate which produced about 50% urease inhibition *in vitro* (Chan and Jones, 1973). Supplementation of NBPT at a level sufficient to cause a 77% reduction in rumen urease activity had a negative effect on nitrogen metabolism in the animal (Ludden et al., 2000a,b). Administration of ruminants with PPDA suppressed digestion of cellulose in unadapted dairy cows fed urea treated with PPDA (Voigt et al., 1980b). As a result, practical use of these potent inhibitors in improving the utilization of dietary urea is limited. Thus, it is imperative to look for other urease inhibitors that could be used for regulating rumen urease activity without negative effects. Hydroquinone (HQ) was chosen as the best soil urease inhibitor that specifically inhibited urease from soil bacteria (Bremner, 1995). The objective of this study was to determine the effect of this inhibitor on the activity of some rumen enzymes, to summarize the kinetics experiments describing the properties of the urease inhibitor and to evaluate its potential value for application to ruminant production.

** Supported by The National Ministry of Education and Heilongjiang Provincial Commission of Science & Technology, P. R. China.

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Received February 22, 2001; Accepted May 10, 2001

MATERIALS AND METHODS

Collection of rumen liquor

Rumen fluid was obtained from four fistulated adult male sheep (Texel×Local breed) weighing 38±1.2 kg and fed 0.6 kg concentrate mixture daily containing soybean meal (15%), corn (79%), urea (2%), mineral mixture and salt (4%). *Leymus chinensis* was fed *ad libitum*. Rumen liquor was transported to the laboratory in thermos flasks and strained through four layers of cheese-cloth.

Washed cell suspension

The rumen liquor (0.5l) was centrifuged at 15,000×g for 15 min at 4°C. The supernatant was discarded. The precipitate (bacteria+protozoa) was washed twice and suspended in phosphate buffer (pH 7.1, 0.05 M) containing 0.02% β- mercaptoethanol (pM buffer).

Preparation of cell free extract

The suspension was divided into ten aliquots of 25 ml,

and subject to ultrasonic disruption at 600 Watts for 4 min with intermittent cooling (0-4°C) by Ultrasonic Dismembrator (Model JY 92-II, Ningbo Medical Instrument Co., China). The cell debris was removed by centrifugation at 20,000 × g for 20 min at 4°C. The supernatant kept was frozen for 3 to 5 h and thawed. Precipitate was removed by centrifugation at 12,000×g for 15 min at 4°C to get cell-free extract (CFE).

Salting-out treatment

The resulting cell-free extract was brought to 65% ammonium sulfate saturation by adding 4.6 g of salt to 108 ml of solution. The resulting material was allowed to stand for 30 min after being centrifuged at 17,000×g for 15 minutes at 0°C. The supernatant was treated by adding 7.42 g of ammonium sulfate to 106 ml of solution in order to increase ammonium sulfate saturation to 75%. The precipitate was harvested by centrifugation as in the previous step and resuspended in 10 ml of PM buffer.

Calcium phosphate gel treatment

The enzyme solution from the salting-out treatment was added with constant stirring to calcium phosphate gel (wet weight 4.54 g) pre-equilibrated with PM buffer. After 15 min the gel was removed by centrifugation at 15,000×g for 10 min at 4°C and discarded. The urease was not absorbed on the gel under the conditions of PM buffer, but some other nonurease proteins were absorbed. The supernatant having urease activity was taken to the next step for further purification.

Acetone fractionation

Pre-chilled acetone was added to the same volume of the supernatant obtained from calcium phosphate gel treatment. The precipitate was discarded after centrifugation, and the supernatant was treated with the same volume of pre-chilled acetone. The precipitate obtained after centrifugation was again suspended in 10 ml of PM-buffer. Efforts to purify the rumen urease further by Sephadex G-200 and DEAE- Sephadex A-25 columns were not successful. The urease solution obtained after acetone precipitation had to be used for studying the kinetics of inhibition.

Protein determination

The protein content of urease obtained after acetone fractionation was estimated by the method of Lowry et al. (1951) using casein as a standard. The total urease activity of every purification fraction was estimated according to the following urease assay procedure. The total urease activities obtained at each step were expressed as micromoles of ammonia produced per minute per milliliter of the partially purified urease solution. The protein content of urease solution determined was 0.98 mg/ml.

Ruminal enzymes assay

Strained rumen fluid (10 ml) from each fistulated sheep was pre-incubated with 1 ml of the inhibitor solution in phosphate buffer for 15 min at 39°C, and the final concentration of the inhibitor in test was 10 ppm. The enzyme activities of the blanks without the inhibitor and tests were assayed at the same time.

Urease activity was assayed by the electrochemical method. The test tubes capped with a stopper fitted with a Bunsen valve were used, and 8 ml of phosphate buffer (pH 7.1, 0.05 M), 1 ml of urea solution (20 mg/ml) and 1 ml of strained rumen liquor were added to them in order. Tubes were incubated for 15 min at 39°C. The reaction was terminated by addition of 1 ml of 10% trichloroacetic acid, and ammonia readings were taken by ammonia gas-sensing electrode (Expandable ionAnalyzer EA 940, Orion Research Inc., USA). The urease activity (units) was expressed as micromoles of ammonia released per minute per milliliter of ruminal fluid.

Cellulase relative activity of rumen fluid with cotton (5 mg) as substrate was determined by the method of cotton dissolution described by Stewart (1977). Cellulase relative activity is defined as disappearance rate of cotton which was incubated with rumen fluid (2 ml) for 48 h.

Proteolytic activity was estimated by incubating rumen fluid (4 ml) under anaerobic condition with 1 ml of 1% casein solution (pH 6.5). Four hours later, 1 ml of the reaction mixture was taken out, and put in 5 ml of trichloroacetic acid (10%). Contents were shaken, centrifuged, and 1 ml of the supernatant was collected. Free tyrosine in the supernatant was determined by the Folin phenol method described by Lowry (1951) (1 unit enzyme activity=1 μg of tyrosine produced per min at 39°C).

Total dehydrogenase activity was determined by the colorimetric method described by Liuxijie (1993). Rumen fluid (4 ml) was incubated with 0.2 ml of 1.5% 2, 3, 5-triphenylte trazolium chloride (TTC) solution for 10 min at 39°C under anaerobic conditions. Ten minutes later, 5 ml of isopropyl alcohol (AR) was added to the reaction mixture. Contents were mixed, centrifuged at 3,600×g for 15 min, and absorbency of the supernatant was monitored at 485 nm using spectrophotometer (UV-2401PC, Shimadazu, Japan). One unit is the amount of the enzyme that caused 0.1 of absorbency increase per min at 39°C.

Effect of different HQ concentrations on rate of ammonia formation

Rate of ammonia formation was determined by the same procedure as the urease assay mentioned above.The final concentration of the inhibitor in the four tests was 0.01 ppm,

0.1 ppm, 1 ppm and 10 ppm of the reaction mixture. The blank contained 1.0 ml of phosphate buffer in place of the inhibitor. An aliquot (2 ml) was taken from the reaction mixture at 15, 30, 45, 60 and 90 min of incubation to determine ammonia concentration.

Kinetics of urease inhibition by HQ

The urease solution obtained after acetone precipitation was incubated with increasing concentrations of urea (1×10^{-2} M to 8×10^{-2} M) and inhibitor (0, 5 and 20 ppm) in a final volume of 10 ml of buffer (pH 7.1, 0.05 M) for 15 min at 39°C. Ammonia formed was measured as above.

Statistical analysis

All data of rumen enzyme activity were analyzed using the ANOVA procedure of SAS (1989) according to a randomized complete block design (blocked by individual). Student's t-test was done based on a paired design.

RESULTS AND DISCUSSION

Urease activity of every purification fraction was calculated and shown in table 1.The preliminary results showed that urease activity increased as purification proceeded. Highest urease activity was found in the acetone precipitation. Urease solution of 30-fold purification was obtained compared with the washed cell suspension. The specific activity of the urease was calculated as 96.8 units/mg based on the protein content of urease solution. This supported the early conclusion that rumen fluid urease is intracellular (Jones et al., 1964; Brent et al., 1967).

The inhibitory effects of HQ on rate of urea hydrolysis by rumen microbes *in vitro* are shown in figure 1, and the effect of inhibitor on ruminal urease activity was presented in table 2. Figure 1 illustrates the changes in ammonia

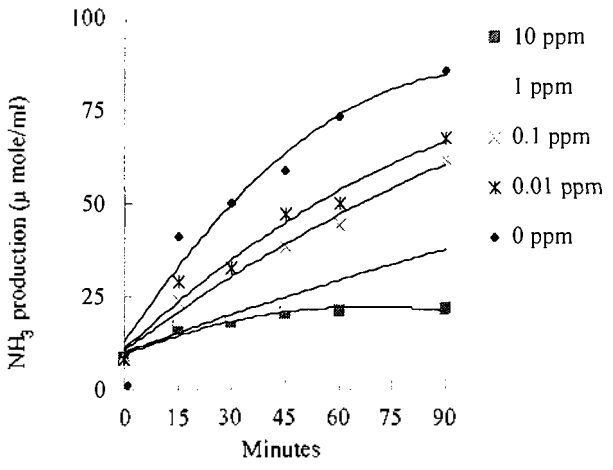

Figure 1. Effect of HQ on the rate of urea hydrolysis in rumen liquid

Table 1. Purification of urease from various rumen fluid fractions

	Volume (ml)	Urease activity [a] (units/ml)
Washed suspension cell	250	3.2
Cell free extract	100	12.1
Salting-out treatment	100	73.3
Calcium phosphate gel	25	82.1
Acetone fractionation	10	95.0

[a] One activity unit was defined as producing 1.0 μmole ammonia from urea per min per ml of urease solution from various rumen fluid fractions.

Table 2. Effect of various concentrations of hydroquinone on rumen urease[b]

Final HQ concentration (ppm)	Rumen activity [c] (μmoles/mg protein)	Inhibition (%)
0	1.6±0.32	-
0.01	1.1±0.31	24.7
0.1	1.0±0.24	34.2
1	0.7±0.26**	55.1
10	0.6±0.13**	62.9

[b] Mean±SE (n=4)

[c] In micromoles of ammonia produced per minute per milligram protein in the presence of an excess of urea during 15-minute incubation.

**Values bearing double asterisk differ from zero control significantly ($p<0.01$).

concentrations over the incubation time. As seen from fgure 1, increasing the concentrations of HQ linearly depressed the rate of urea hydrolysis and subsequent NH_3 production ($p<0.05$). At the inhibitor concentrations of 1 ppm and 10 ppm, the urease activity was significantly different from the control ($p<0.01$). There was no significant difference between HQ treatments in urease activity within 15 min of incubation ($p>0.05$).

In the present study, regression analysis of ammonia production (Y) on reaction time (t) is as follows:

$Y=-0.0074t^2+1.4601t+12.658$ (0 ppm, $R^2=0.96$, n=6)
$Y=-0.003t^2+0.89t+10.9$ (0.01 ppm, $R^2=0.96$, n=6)
$Y=-0.0018t^2+0.7164t+10.452$ (0.1ppm, $R^2=0.96$, n=6)
$Y=-0.0005t^2+0.3477t+10.159$ (1.0 ppm, $R^2=0.96$, n=6)
$Y=-0.0027t^2+0.3724t+9.5018$ (10 ppm, $R^2=0.96$, n=6).

With incubation time increasing, ammonia production increased quadratically ($p<0.05$) but reaction rate decreased in the presence of the same HQ concentrations. This result followed a general pattern of urea hydrolysis by urease.

At an inhibitor concentrations of 1.0 ppm (1×10^{-5} M), about 55% inhibition of urease activity was observed. At an inhibitor concentration of 10 ppm (1×10^{-4} M), the urease

activity was decreased by 63% (table 2). A parallel observation was made by Cook (1976) where AHA resulted in 50% inhibition of sheep rumen urease at a concentration of 5×10^{-5} M. Markkar et al. (1981) reported that AHA concentrations of between 5×10^{-5} M and 1×10^{-4} M inhibited urease activity of intact rumen by 50%. This experiment gave a similar effect of urease inhibition at a lower dose. From the comparisons made in the other study (unpublished data) between HQ and AHA, it was obvious that HQ was the most effective inhibitor of rumen urease when the two inhibitor were present in equimolar concentrations. The present study suggest that HQ is, an effective urease inhibitor, which is in accordance with the results of Nimenya et al. (2000), who reported that increasing HQ concentrations resulted in a strong dose-related decrease in NH_3 production from cattle urine.

Apart from HQ effect on urease activity in the rumen, the effect of HQ on activity of cellulase, total dehydrogenase and proteolytic enzymes which is closely related to rumen microbial metabolism was investigated in the present study (table 3). The results revealed that HQ was a specific inhibitor of both plant urease and rumen bacteria urease and did not have an inhibitory effect on cellulase, total dehydrogenase and proteinase. There were not significant differences between the control and the treatment in total dehydrogenase and proteinase activities in the rumen ($p>0.05$), but rumen cellulase activity increased significantly ($p<0.05$). Makkar et al. (1981) demonstrated that addition of AHA at a level sufficient to produce a 74% inhibition of urease had no effect on the activity of celluloytic or protelytic enzymes *in vitro*. This is in contrast to that observed *in vitro* (Ludden et al., 2000), in that NBPT depressed fiber digestibility.

The effect of HQ on the kinetics of partially purified rumen urease is shown in figure 2. The double reciprocal plot of Lineweaver-Burk shown that mean values of Michaelis constant (Km)for 0 ppm, 5 ppm and 20 ppm HQ in final reaction volume were similar (2.3×10^{-3} M, 2.0×10^{-3} M and 1.8×10^{-3} M respectively), while values for maximum velocity (Vmax) differed, with coefficient of variation being 24%, indicating that HQ was a noncompetitive inhibitor of ruminal urease. With respect to nature of HQ inhibition of rumen urease there has been no report in the literature published so far. Calculations based on figure 2 yield a Km Value of 2.0×10^{-3} M with Vmax of 319.44 μmoles/mg/min. As HQ inhibition of rumen urease reached equilibrium state, the Michaelis-Menten equation could be applied for calculation of Ki:

$$\frac{1}{v}=\frac{Km}{V\,max}\left(1+\frac{[I]}{Ki}\right)\cdot\frac{1}{[S]}+\frac{1}{V\,max}\left(1+\frac{[I]}{Ki}\right)$$

Where I is HQ concentrations in moles per liter, Ki the inhibition constant in moles per liter, S urea concentrations in moles per liter, v velocity in μ moles NH_3 per min, Km Michaelis constant, Vmax maximum velocity.

The Ki calculated from Dixon plots was 1.2×10^{-5} M. A noncompetitive inhibitor of ruminal urease can form a complex with the urease at or near the metal ion. The sulfhydryl groups in the vicinity of the metal ion played an important role in the complex formation (Makkar et al., 1981). The metal ion nickel is required for space construction of the enzyme action (Dixon et al., 1975; Spears et al., 1978). We speculated the complex formation appeared to bring about metal ion displacement at the active site and subsequent decrease of activity of rumen urease. However, the mechanism of urease inhibition by HQ is not yet established.

Table 3. The effect of hydroquinone on rumen enzyme activity [d]

Item	Control	Treatment (10 ppm HQ)	T-test
Urease (units/ml)[e]	1.6±0.32	0.6±0.03	**
Total dehydrogenase (units/ml)[f]	9.5±0.12	10.0±0.07	NS
Cellulase (%)	54.5±10.93	70.0±5.02	*
Proteolytic enzyme (units/ml)[g]	12.9± 1.06	12.2±0.86	NS

[d] Mean±SE (n=4).

[e] Expressed as micromoles of ammonia released per minute per milliliter of ruminal fluid.

[f] Assayed as amount of 0.1 of absorbency increase monitored at 485 nm using spectrophotometer.

[g] Defined as micrograms of tyrosine produced per min per milliliter of ruminal fluid.

** $p<0.01$;* $p<0.05$; NS: Non-significant.

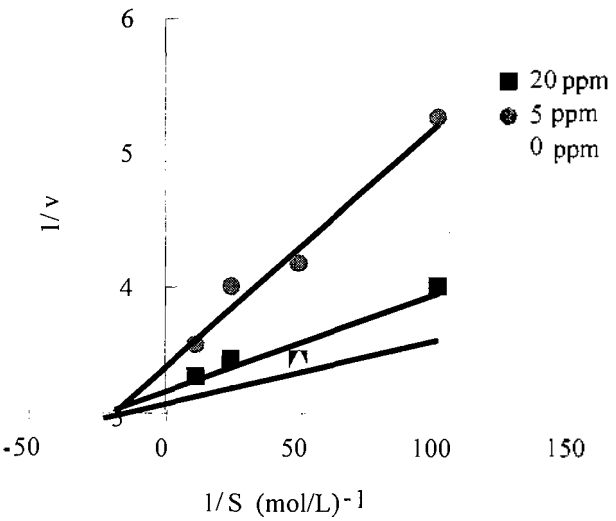

Figure 2. Double reciprocal Lineweaver-Burke plots without (0 ppm) and with (5, 20 ppm) HQ

IMPLICATIONS

HQ inhibited the urease of ruminal fluid *in vitro,* but had no inhibitory effect on cellulase, total dehydrogenase and proteinase. This demonstrated that HQ is a specific urease inhibitor. A 63% decrease in ruminal urease activity *in vitro* could be attained with HQ at a concentrations of 10 ppm. Kinetics studies on the partially purified urease showed that HQ inhibition of ruminal urease was noncompetitive. We obtained a Km value (0.002 M), Vmax value (319.44 μmol/min) and Ki value (1.2×10^{-5} M) for urea or the inhibitor with partially purified enzyme. The above results suggested that HQ is one of the most effective inhibitors of ruminal urease and its application to ruminant practical ration would be promising.

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