Development of Simple and Precise Method of Arginine Determination in Rumen Fluid by Spectrophotometer

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Summary: The objective of current study was to build up a convenient, economic and accurate procedure to determine arginine (ARG) concentration in rumen fluid. Rumen fluid was collected from 3 rumen fistulated Chinese Holstein dairy cows and added with or without (control) 1mmol/l unprotected ARG and blank (with only medium) in to syringe system in triplicate as a replicate. All syringes were incubated in water bath at 39 °C for 0, 2, 4, 6, 12 and 24 h and were terminated to measure the ARG concentration. Sakaguchi reaction method was used to analyze the ARG concentration in rumen fluid by determining the rumen degradation rate of protected and unprotected ARG. Temperature, time and absorbance were optimized in the procedure based on Sakaguchi reaction. Color consistency remained 4-6 min. The optimum temperature (0-5) °C was observed for maximum optical density 0.663 at wave length 500 nm. Minimum ARG that could be determined in rumen fluid by spectrophotometer was 4-5 µg/ml. No significance (P>0.05) difference were observed between two results derived from spectrophotometer and amino acid analyzer methods. In conclusion, the spectrophotometer method of ARG determination in rumen fluid based on Sakaguchi reaction is easy, accurate, and economical and could be useful in learning ARG metabolism in the rumen.

Key words: Arginine; Rumen fluid; Sakaguchi reaction; Spectrophotometer, Method development.

Introduction

Arginine (ARG) is well recognized amino acid (AA) for its importance in urea cycle and for precursor of polyamines and nitric oxide. Previous reports showed that abomasal administration of ARG enhanced milk production in dairy cows [1], improved the nitrogen metabolism in heifers [2], and enhanced the immunity and growth performance of pre-ruminant calves [3]. Recently, injection of ARG-HCl was reported to decreased embryonic loss in ewes [4], increased lamb birth weight in gestationally nutrient restricted ewes [5], and improved fetal lamb survival to term in prolific ewes [6]. Parental administration of L-Arg increased the brown adipose tissue in lambs and enhanced neonatal thermogenesis at birth [7]. However parental and abomasal administration of ARG to farm animal is not practical, due to complexity of ARG metabolism in ruminant animals. Therefore, determination of ARG concentration in rumen fluid to understand the rumen degradation, fermentation and intestinal digestibility, will be an essential step before feeding.

Regular methods used for ARG determination in rumen fluid include high performance liquid chromatography (HPLC) [8] and AA analyzer [9]. The use of HPLC and AA analyzer were of high sensitivity and accuracy, but were of high cost, time-consuming, and were not commonly available to small-scale animal nutrition laboratories. Whereas, use of spectrophomter for ARG determination in biological fluid with Sakaguchi reaction is relatively simple, cheap and sensitive [10, 11], but was not used in rumen fluid. Thus, a procedure based on Sakaguchi reaction for the determination of ARG in rumen fluid should be developed for small scale animal nutrition laboratories to study the ARG metabolism in rumen and for intestinal digestibility.

Experimental

Material

Commercial unprotected ARG with purity 98.5% the rumen protected ARG 60% with 40% oil palm layer. 5-Sulfosalicylic acid. Bromine purity 99.5%. Hydorxyquinoline (8-Quinolinol) with 99% purity, Urea purity ≥98%. Sodium Hydroxide NaOH 96% purity and Ethanol purity 99.7% were used for the determination of ARG in rumen fluid. Distilled water was purified by a Millipore system MilliQ.

Method of ARG Determination in Rumen Fluid

For rumen unprotected, Calibrated glass syringes (Model Fortuna, Häberle Laborte –chnik,

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Lonsee-Ettlenschieß, Germany) were used as *in vitro* incubation. The oven dried substrate 200 mg dry matter (DM) basis Chinese wild grass and corn meal with ratio of 50:50 were weighed into 100 ml glass syringes in triplicate. Rumen fluids were collected from three ruminally fistulated cows previously fed the ration (Table-1) and were added 1 mmol/l unprotected ARG with or without (control) as was described by Chacher *et al.* [13]. Rumen liquor-buffer mixture 30 ml (ratio1:2) were added into each syringe [14] and were incubated in water bath at 39°C for 0, 2, 4, 6, 12 and 24 h. Differences in the composition and activity of rumen fluid inoculums were controlled by incubation of buffered ruminal fluid without substrate (Blank). In each point time, 3 syringes from each treatment were terminated and rumen fluid were transferred into to 50 ml tube and remained to be determined.

Table-1: List of Ingredients and nutrients chemical composition of basal diet fed to cows during rumen fluid collection period

Ingredients	% DM
Corn silage	**17.00**
Chinese wild rye	**10.00**
Alfalfa hay	**28.00**
Corn grain ground	**27.00**
Wheat bran	**05.00**
Soybean meal	**05.00**
Cotton seed meal	**04.00**
Ca Carbonate	**00.25**
Ca Diphosphate	**01.25**
Sodium Carbonate	**01.00**
Na Cl Salt	**00.50**
Premix	**01.00**
Total	**100.00**
Nutrients Composition	**Contents%**
NE_L Mcal/kg	**01.56**
CP% DM	**15.10**
NDF% DM	**34.40**
ADF% DM	**34.40**
Ca% DM	**00.92**
P% DM	**00.62**

Forge to concentrate ratio 55:45

Note: The premix added into ration contain

Vit: A 60000 IU	Vit: D 15000 IU	Vit: E 3000 IU
Fe: 1500mg	CU: 1500mg	Zn: 7500mg
Mn: 1500mg	Se: 50mg I: 50mg	Co: 20 mg

Rumen-protected ARG (1.5g) was weight into nylon bags (46 μm porosity) and were incubated in 3 rumen fistulated cows in triplicate for 0, 2, 4, 6, 8, 12, and 24 h as described by Wang *et al.,* [15]. At each time point 3 bags were taken out and washed with tape water then dried at 45°C for 30 minutes, individual ARG samples were then transferred to 15 ml tube, containing 10 ml water and were heated at 90 °C for 10 minutes in water bath. The solid (upper) was discarded and liquid (lower) was collected into separate tubes and stored at 4 °C as was described by Wang *et al*., [15] for later ARG analysis.

The determination of ARG in rumen fluid was carried out by means of Sakaguchi's reaction [16] with the minor modification of Wang *et al* [10]. Five ml rumen sample in duplicate was collected from all the treatments in to eppendorf tube and was mixed immediately with an equal volume (1 ml) of 4% (w/v) 5-Sulfosalicylic acid for deproteinisation. Samples were centrifuged at 3000 rpm for 10 minutes at 20 °C, and were stored at 4 °C until analysis. The samples were diluted before analysis. Another incubated sample was analyzed by Beckman 121 MB AA analyzer. The resin was eluted with 2.0 mol/l of ammonium hydroxide solution, followed by analysis of ARG by ninhydrin solution [9].

Stock solution was made by dissolving 100mg ARG into 100ml deionized water, standard solution was prepared by diluting the 0, 2, 4, 6, 8, 10 and 12 μg/ml of stock solution into deionized water. Hydroxyquinoline was prepared by addition of 0.2 g of 8-Quinolinol in 100 ml of 95% ethanol and stored in cooled place, the solution 0.2% was diluted with deionized water before use. For the preparation of sodium hypobromite, 0.68 ml was dissolved into 100 ml cooled 5% sodium hydroxide and was stored in cool and dark place. Solution of 2 mol/l NaOH was prepared by dissolving 8 g of NaOH in 100ml deionized water and stored in cooled placed. Urea solution (40% w/v) was prepared according to quantity of sample required.

The standard line developed for ARG determination was based with Sakaguchi reaction and absorbance was measured through spectrophotometer All reagents and solutions were cooled on ice before determination. Then 1.0 ml 0.2% diluted 8-hydroxyquinoline and 1.0 ml of 2 M NaOH were added sequentially to a tube containing 5 ml of ARG sample with concentration of (0-12 μg/ml ARG) respectively. The solution was mixed and placed in ice for 10 minutes, and 0.5 ml of cold sodium hypobromite was added to develop the color by vigorously shaking for 30 sec. After that 1.0 ml of urea solution (40% w/v) added instantly and then solution was mixed uniformly. Finally 200 μl were taken in 96 well plate and OD value was measured at WL 500 nm by spectrophotometer Standard curve with the concentration of 0, 2, 4, 6, 8, 10 and 12 μg/ml were build and equation $Y = 0.0509x + 0.0524$ with regression ($r^2 = 0.9983$) was obtained as shown in Fig. 1.

The concentration of ARG in rumen fluid was determined by estimating the rumen degradation rates of protected and unprotected ARG, by incubating the samples in rumen fluid for 24 h. The parameters were optimized by Sakaguchi reaction with the

concentrations of 8-ydroquinoline and sodium hydrobromite. Temperature, time and absorbance were considered as important factors.

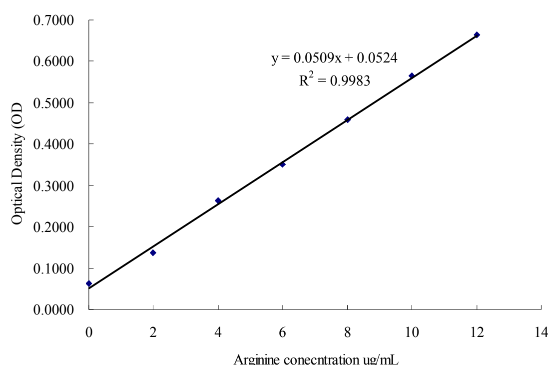

Fig. 1: Standard line at different Arginine concentration

Data Calculation and Statistical Analysis

The 1mmol/l ARG in rumen fluid determined by Spectrophotometer was calculated by subtracting the concentrations of ARG µg/ml in the control and blank samples as method applied by Chacher *et al.,* [13]. The concentration of ARG in rumen fluid by AA analyzer was estimated according to method described by Broderick and Kang ([9]). The data of ARG concentration in rumen fluid obtained by two methods were analyzed with one way analysis of variance using PROC GLM (General Linear Model) procedure of SAS [17] package. Results were subjected to multiple range tests, at $P<0.05$ and standard error of means (SEM).

Results and Discussion

In the current study Sakaguchi method was used to analyze the ARG concentration in rumen fluid by determining the rumen degradation of ARG.

Effect of Temperature and Time on Colour Development and Determination Stability

The effect of temperature on colour development and determination stability was recorded as average OD values 0.6635 ± 0.0046 (SD), 0.6630 ± 0.0033, 0.6308 ± 0.0053 and 0.5901 ± 0.0040, with loss of 0.00, 0.03, 4.92 and 11.06% for 0, 5, 10 and 15 °C, respectively, at 12 µg/ml ARG concentrations.

The observed OD valued for color development and consistencies by time were recorded as 0.6633 ± 0.0035 (SD), 0.6610 ± 0.0044, 0.6450 ± 0.0055, 0.5957 ± 0.0105, with loss of 0.00, 0.35, 2.71 and 10.20 % within 0, 5, 10 and 15 min, and respectively.

The temperature was one of the most critical factor affecting the colour development and stability. The results indicated negative co-relationship (r^2 = -0.093) between temperature and OD values (Fig 2). In current study maximum OD (0.663) was observed in the range of 0-5°C, when the ARG concentration was 12 µg/ml, while Ceriotti and Spandrio [12] reported an optimized with maximum OD (0.80), at 8 µg/ml ARG concentration and tempreture of 10-15°C. The reasons of optimum temperature 10-15°C and OD (0.80) value obtained by Ceriotti and Spandrio, [12] at lower ARG concentration (8 µg/ml) might be due to addition of butanol also differences in wave length and instrument used. Moreover, excess urea used instead of butanol and samples were stabilized on ice. It was tested that addition of butanol ended a complexity and has drawback to get samples from mixture that ultimately affect the linearity. In the present study ARG determination without butanol was proved to be easy and accurate for Sakaguchi reaction.

The color developed by the reaction of ARG with 8-hydroxyquinoline in rumen fluid was stabilized by addition of excessive urea on ice instant. No decreases in color were observed within 4 min. Colour began to decrease after 5, 10 and 15 min with loss of 0.35, 2.71 and 10.20 %, respectively. It was indicated that the values should be obtained with in 4-6 min after development of color (Fig. 2). The reported research showed maximum time for colour stability was about 3-4 min [10]. The difference in results between the current study and the studies conducted by Ceriotti and Spandrio, [12] and Wang *et al*., [10] might be due to difference in experimental procedure.

Absorptive Wavelength

The results of selection of absorbance tested at different WL were recorded as OD value of 0.0010, 0.0500, 0.1000, 0.1570, 0.2500, 0.3500, 0.4500, 0.5350, 0.5985, 0.6451, 0.6633, 0.6100, 0.4980, 0.2500, 0.0004 and 0.0000, for WL 350, 365, 380, 395, 410, 425, 440, 455, 470, 485, 500, 515, 530, 545, 560 and 575 nm, respectively.

The optimized concentrations of sodium hypobromite and 8-hydroquinoline for ARG quantitative determination were 0.4 and 0.02 % respectively. In the present study 8-hydroxyquinoline was selected because chromomeric product formed had much higher apparent molar absorption and the OD values than those with other Sakaguchi reagents. The WL 500 nm which had maximum OD value of (0.663) (Fig. 2) was selected for ARG quantitative analysis in the rumen fluid based on Sakaguchi reagents, which was consisted with study conducted by Wang *et al.,* [10].

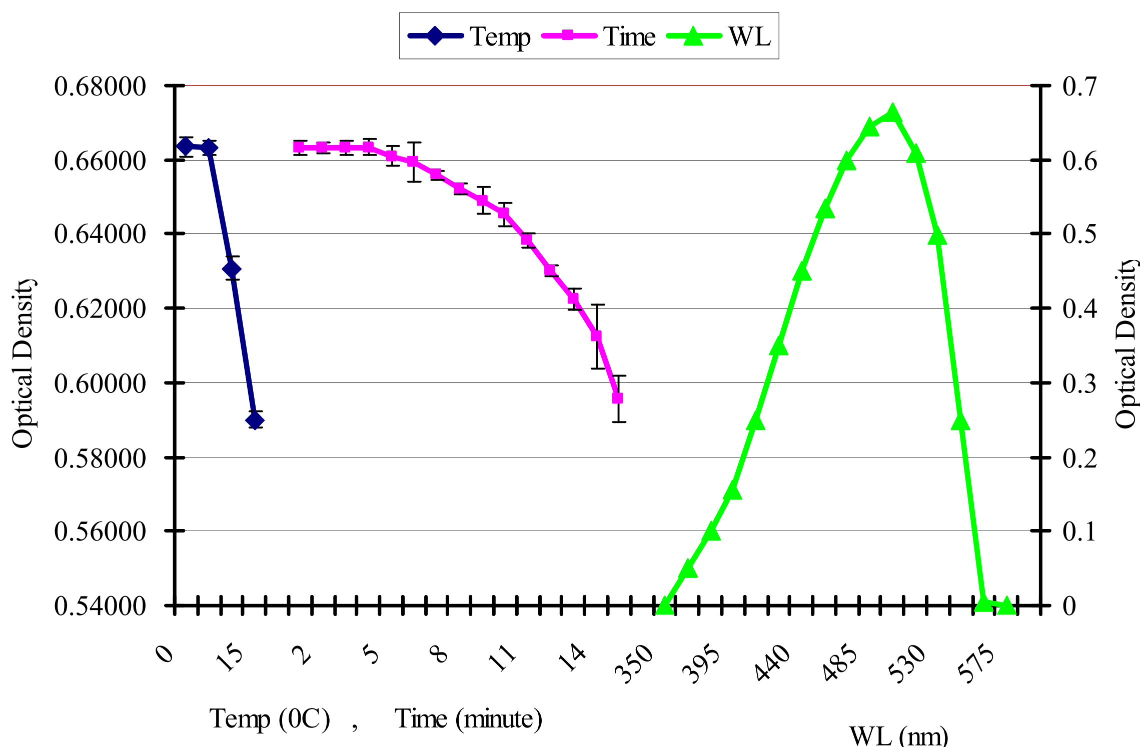

Fig. 1: The effect of temperature, time and absorbance on optical density.

Measurement of ARG Concentration in Rumen Fluid

Concentration of 1mmol/l ARG in rumen fluid determined by spectrophotometer were 174.2, 118.4, 92.0, 82.2, 1.3, and 0.0 µg/ml at 0, 2, 4, 6, 12 and 24 h of incubation period, respectively. The results obtained from AA analyzer were 114.0, 95.2, 87.6 and 0.0 µg/ml at 0, 2, 4, 6, 12 and 24 h, respectively (Table-2). No significances (P >0.05) difference were observed between results of spectrophotometer and AA analyzer during 0, 2, 4 and 6 h. However, very minute amount of ARG concentration was tested by spectrophotometer during 12 hours.

Table-2: Measurement of arginine concentration in rumen fluid by Spectrophotometer and Amino acid analyzer at different incubation period

Incubation (hours)	Arginine concentration µg/mL		SEM	P value
	Spectrophotometer	Amino acid analyzer		
0	174.2	174.2	0.00	1.000
2	118.4	114.0	4.59	0.714
4	92.1	95.2	4.88	0.803
6	82.2	87.6	2.66	0.457
12	1.25	0.00	0.043	0.001

P value (P<0.05) showed significant difference, SEM standard error of mean.

The current method is simple, efficient and suitable for small level laboratories to measure the ARG content in rumen fluid. Moreover, the composition of rumen fluid is complicated, but 1mmol/l ARG in rumen fluid can be directly measured by Sakaguchi reagents without separation compared with the study conducted by Wang *et al.,* [10], who used the resin to separate the ARG from grape juice. Additionally, rumen protected ARG in rumen fluid was determined in the current study. The current procedure provided better improvement and reproducibility compared with other methods, and could be applicable in routine determination of ARG in rumen fluid to understand the rumen degradation and intestinal digestibility.

Conclusion

In rumen fluid, the minimum ARG content that can be determined was 5-6 µg/ml by spectrophotometer. The improved technique was proved to be useful in the process of ARG determination in rumen fluid. This method provided better improvement and reproducibility compared to other methods, therefore, the method could be applicable in routine determination of ARG in rumen fluid. The current technique is of higher efficiency and application and may be helpful to learn ARG metabolism in rumen or rumen protected ARG products stability and intestinal digestibility. This is first study for the determination of ARG in rumen

fluid with Sakaguchi reaction through spectrophotometer.

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