



## Histological Changes of Tissues and Cell Wall of Rice Straw Influenced by Chemical Pretreatments

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**ABSTRACT** : Sodium hydroxide (SH) or ammonium bicarbonate (AB) were applied to rice straw to investigate the effects on histological change of stem tissue or cell wall before and after *in sacco* degradation using a scanning electron microscope (SEM) and a transmission electron microscope (TEM). The SEM revealed that, the parenchyma and vascular bundles were distorted by treatment with SH at 30 or 45 g/kg straw dry matter. Faultage between phloem of large vascular bundles and parenchyma occurred with further increasing SH to 60 or 75 g/kg. The cell wall in these stem tissues was crimped when observed by TEM. However, only parenchyma and large vascular tissues were slightly distorted in AB-treated stem. For untreated and AB-treated stems, the initiation of observable ruminal degradation of cell wall was prolonged from 12 h for inner parenchyma to 24 h for sclerenchyma and to 48 h for phloem of small vascular bundles, while the outer epidermis was intact even at 72 h. For SH-treated stem, however, the cell wall from all of the investigated tissues, epidermis, small vascular bundles, sclerenchyma, and parenchyma started to be degraded at 12 h incubation. These results indicate that SH treatment contracts rice straw stem leading to an improvement in rumen degradation, and that the degradation of SH-treated stem is bilateral from inner and outer surface simultaneously. (**Key Words** : Chemical Treatment, Electron Microscope, Histological Changes, Rice Straw Stem, Rumen Degradability)

### INTRODUCTION

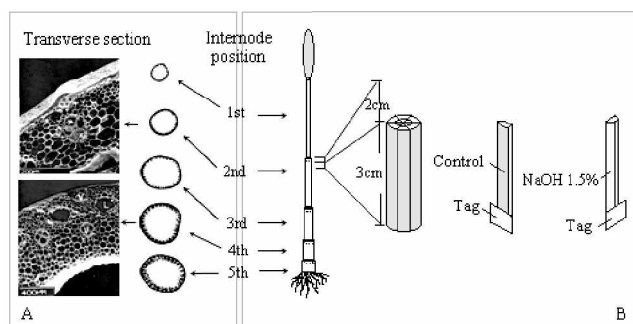
Rice straw (RS) is an important forage for ruminants in many rice-producing countries. Making full use of RS for animal production can save grain, provide additional income to farmers and decrease environment pollution due to burning of straw after harvest (Promkot et al., 2007; Wora-anu et al., 2007). Development and application of chemical treatments for upgrading straw has stimulated intense interest in the developing countries since the 1970's, but there are still some blind spots on the mechanism with which the treatments improve the nutritive value of straw.

Different types of cells of straw are distinct in their digestibility (Akin and Burdick, 1981; Engels and Schuurmans, 1992; Migné et al., 1996), and respond to chemical treatments in a different way (Shen et al., 1999). Structural methods offer effective ways to evaluate digestion of specific cell types and have increased understanding of the contribution of plant and rumen microbial factors in digestion of forages (Akin, 1989; Liu et

al., 2005). However, the subjectivity of the sampling and observing has made some acquired results unbelievable or warped. Straw has distinct structure and chemical composition from the upper internode to base internode during growth, and hence differs in degradability between internodes (Migné et al., 1996). Therefore, sampling site is an important factor for acquiring comparable results. Initial microbial colonization occurs at sites of tissue damage (Bauchop, 1980). Use of a small particle of sample is prone to result in selective degradation by the micro-organisms and impaired observability of treatment effects. On the other hand, the holistic domino effect of treatments could be observed if samples were observed at a long interval.

With electron microscopes based on strict sampling and observing rules, Wang et al. (2007) found that sodium hydroxide and ammonium bicarbonate exerted their influence on epidermal histology of RS stem with different modes, resulting in different degradability of RS stem epidermis. However, these changes of epidermis were not related to straw degradability. Microbial digestion that occurred and progressed from more digestible inner tissue had been observed under microscope (Migné et al., 1996). Histological investigation of both inner and outer tissues of

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Received August 27, 2007; Accepted January 4, 2008



**Figure 1.** Theory (A, Zee, 1981) and practice (B, Wang et al., 2007) of preparing rice straw samples for chemical pretreatment and microscopic studies. Transverse section through lower internode was different to that of upper internode, especially the existence of lysigenous space (signified as L in picture A).

straw stem may reveal selective degradation by rumen microorganisms.

The objective of the present study was to reveal the mechanism for improved degradability of chemically pretreated straw. Scanning electron microscope (SEM) and transmission electron microscope (TEM) were applied to investigate the histological changes of stem tissue or cell wall related to different chemical treatments before and after *in sacco* degradation.

## MATERIALS AND METHODS

### Plant materials

The RS used was from late season rice (variety V. *Yiu64*), cultivated in Zhejiang province of China. The RS used in this experiment was the same as that in Wang et al. (2007).

### Chemical pretreatments and stem samples

Effects of treatments with sodium hydroxide (SH) and ammonium bicarbonate (AB) were investigated in this study. Dosages of SH were 15, 30, 45, 60 and 75 g/kg straw DM and dosages of AB were 30, 60, 90, 120 and 150 g/kg DM. For practical considerations and evaluation of pretreatment efficacy, 500 g of chopped RS (3-5 cm, air-dried) and 15 sub-stem samples were treated together in three replicates of each treatment level. The amount of water used to dissolve the SH or AB was controlled to adjust the initial moisture content of the treated RS to about 400 or 500 g/kg, respectively. After the RS and sub-samples of stem were thoroughly mixed with the SH or AB solution, the sealed bags with straw were placed in a container at 35°C for 3 or 10 days, respectively. Untreated RS and stem were used as control treatments.

The 15 sub-stem samples in every pretreatment were selected as follows: each 3 cm stem sample was taken from 2 cm below the second rice stem node, and then it was

longitudinally cut into six parts, which were distributed randomly to the five different dosages in SH or AB as well as the control (Figure 1). To ensure that three stem sub-samples from every pretreatment were used for each incubation time in the rumen of a sheep, 15 sub-stem samples were prepared from 15 random samples.

Chemical pretreatment efficacies were evaluated in terms of the changes of crude protein, ash, neutral detergent fiber (NDFom), and *in sacco* dry matter degradability (DMD) of RS. The details of chemical analysis and measurement of DMD were as described elsewhere (Wang et al., 2007).

### Stem sub-samples for microscopic investigation before degradation

Before they were prepared for microscopic investigation, the untreated and treated sub-stems were immersed in cold water for 12 h to keep their structure intact. The procedures for preparation of samples for SEM and TEM were as described by Liu et al. (2005), except for embedding during preparation of samples for TEM where dehydrated samples were embedded in resin according to the following procedure: acetone/resin (1:1, v/v) for 1 h, acetone/resin (1:3, v/v) for 3 h, pure resin overnight and then samples were transferred to tubes filled with resin and polymerized at 70°C for at least 12 h.

### Stem sub-samples for microscopic investigation after degradation

Based on results from SEM and for practical considerations, the sub-samples of stem treated with SH at 45 g/kg DM and with AB at 90 g/kg DM were used for *in sacco* incubation. Separated from DMD determination, only one tagged sub-stem was put into a nylon bag (40 µm mesh, 2 cm×5 cm inner size) to prevent sub-stems from adhering to each other. All untreated and treated sub-stems were suspended in the rumen of three rumen-fistulated Huzhou sheep for 12, 24, 48 and 72 h (Ørskov et al., 1980). For every pretreatment, 3 incubated sub-stems could be acquired at each time from every sheep. At the end of degradation, bags were immediately immersed in running cold water, gently washed by hand for 20 min and then the samples were immediately prepared for electron microscope observation. The cut ends of all incubated sub-stems had 1 mm cut off to eliminate the cutting effect before materials were prepared for microscopic investigation.

### Statistical analysis

Data for chemical composition and DMD of straw were analyzed by the general linear models procedure of SAS (1999). Linear and quadratic effects of increasing dosage of treatment were determined using orthogonal polynomial contrasts (Steel and Torrie, 1980).

**Table 1.** Effects of treatment with sodium hydroxide (SH) and ammonium bicarbonate (AB) on chemical composition (g/kg DM) and *in sacco* degradability (g/kg) of rice straw

Indices	Rice straw treated by													Effect <sup>b</sup>			
	None		SH (g/kg DM)						AB (g/kg DM)					SH		AB	
	0	15	30	45	60	75	RMSE <sup>a</sup>	30	60	90	120	150	RMSE	L	Q	L	Q
NDFom <sup>cd</sup>	727	695	680	644	609	576	17.2	737	725	709	709	701	12.2	**	NS	**	NS
CP <sup>d</sup>	55	66	57	61	57	56	2.8	60	71	90	93	101	4.1	NS	*	**	NS
Ash <sup>d</sup>	123	147	151	160	181	180	11.2	121	126	120	124	125	6.4	**	NS	NS	NS
DMD <sup>e</sup>	327	448	546	620	649	739	28.0	338	383	395	421	441	23.1	**	*	**	NS

<sup>a</sup>RMSE = Root mean squares error. <sup>b</sup>L = Linear; Q = Quadratic; \* p<0.05; \*\* p<0.01; NS = p>0.05.

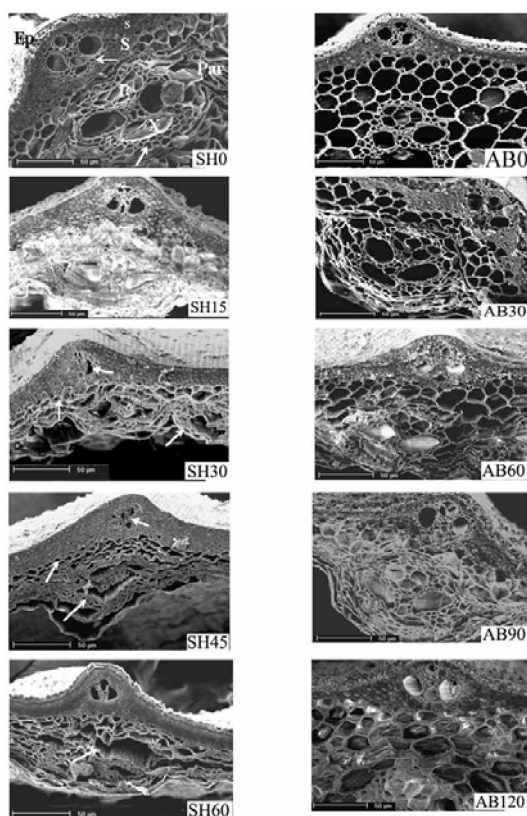
<sup>c</sup>NDFom = Neutral detergent fiber not assayed with a heat stable amylase and expressed exclusive of residual ash.

<sup>d</sup>From Wang et al. (2007). <sup>e</sup>DMD = *In sacco* DM degradability at 48 h of incubation.

## RESULTS AND DISCUSSION

### Chemical composition and *in sacco* degradability of rice straw

The results for chemical composition and the increased



**Figure 2.** Histological changes in transverse section of rice straw stem after treatment with sodium hydroxide at levels of 0 (SH0), 15 (SH15), 30 (SH30), 45 (SH45) and 60 g/kg DM (SH60), or ammonia bicarbonate at 0 (AB0), 30 (AB30), 60 (AB60), 90 (AB90) and 120 g kg/DM (AB120). Ep = epidermis; S = sclerenchyma; Par = parenchyma; P = phloem of vascular bundle; X = xylem of vascular bundle (Bars, 50  $\mu$ m). The parenchyma and vascular bundles were distorted by treatment with SH at 30 or 45 g/kg straw dry matter; faults between phloem of large vascular bundles and parenchyma occurred with further increasing SH to 60 or 75 g/kg. However, only parenchyma and large vascular tissues were slightly distorted in AB-treated stem.

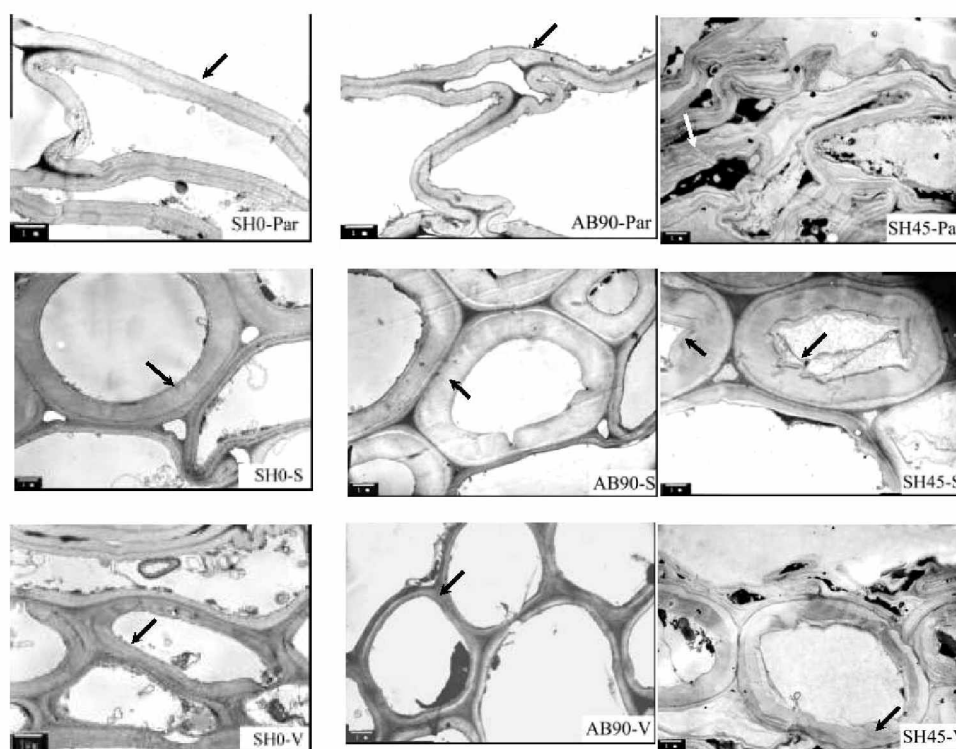
*in sacco* DMD (Table 1) in pretreated RS are comparable to other results (Liu et al. 2002; Suksombat, 2004) which indicated that both treatments in this experiment were effective, i.e. the pretreated-stems could be used for histological investigation.

### Histological changes of tissues and cell wall of rice straw influenced by pretreatment

*The changes of tissues* : Histological changes of tissues of untreated, SH and AB-treated RSs are shown in Figure 2. The cells were clearly observable in or around the vascular bundles in untreated-stem (Figure 2, SH0, arrow). When SH increased to 30 or 45 g/kg DM, the parenchyma and the vascular bundles were so distorted that the figure of cells was irregular in the connection between the rind and pith regions, and the cells could not be clearly observed in or around the vascular bundles (Figure 2, SH30 and SH45, arrow). Faults could be observed between phloem of large vascular bundles and the parenchyma under SEM with increasing dosage of SH to 60 (Figure 2, SH60) or 75 g/kg DM (picture not shown). Contrary to SH treatment, only slight distortion of parenchyma and large vascular bundles could be observed in AB-treated stems (Figure 2, AB30 to AB120).

*The changes of cell wall* : The TEM of cell wall from sclerenchyma, parenchyma and vascular bundles of different straws are presented in Figure 3. All the cell walls were crimped with SH treatment, while these changes could not be observed in AB-treated cell walls (Figure 3, arrow).

The histological changes after treatment indicate the modification of stem structure. The distorted figure of SH-treated parenchyma was reflective of the contraction of stem, while the crimp in SH-treated vascular bundles might be the swelling character of the contracted stem after immersion in water during preparation of stem samples. The contracted stem of SH-treated straw may be caused by high permeation of sodium ions (Bergen, 1972; Meryman, 1973). It has been speculated that cellulose within the cell wall matrix may be physically restrained from swelling and alkali treatments may reduce the strength of inter-molecular hydrogen bonds that bind cellulose molecules together and



**Figure 3.** Transmission electron microscope of the cells of the parenchyma (SH0-Par) and sclerenchyma connected to parenchyma (between rind and pith, SH0-S), and periphery cells of larger vascular bundles pore (SH0-V) in untreated stem and stem treated with 90 g ammonium bicarbonate (AB90-Par, AB90-S and AB90-V) or with 45 g sodium hydroxide per kilogram DM (SH45-Par, SH45-S and SH45-V) (Bars, 1  $\mu$ m). All the cell walls were crimped with SH treatment, while these changes could not be observed in AB-treated cell walls.

remove these restraints to a certain extent (Han and Garrett, 1986). The contraction and swelling was consistent with the results of volumetric weight and swelling capacity changes (Wang et al., 2006). Liquid ammonia has the ability to form an ammonia-cellulose complex and to decrease the crystallinity of cellulose (Isogai and Usuda, 1992). Goto and Yokoe (1996) observed that gaseous ammonia-treated barley straw had 14% lower ratio of crystalline to amorphous regions than did untreated straw. In this study, lack of effect of AB on histological changes suggests the weakness of AB on cell wall matrix.

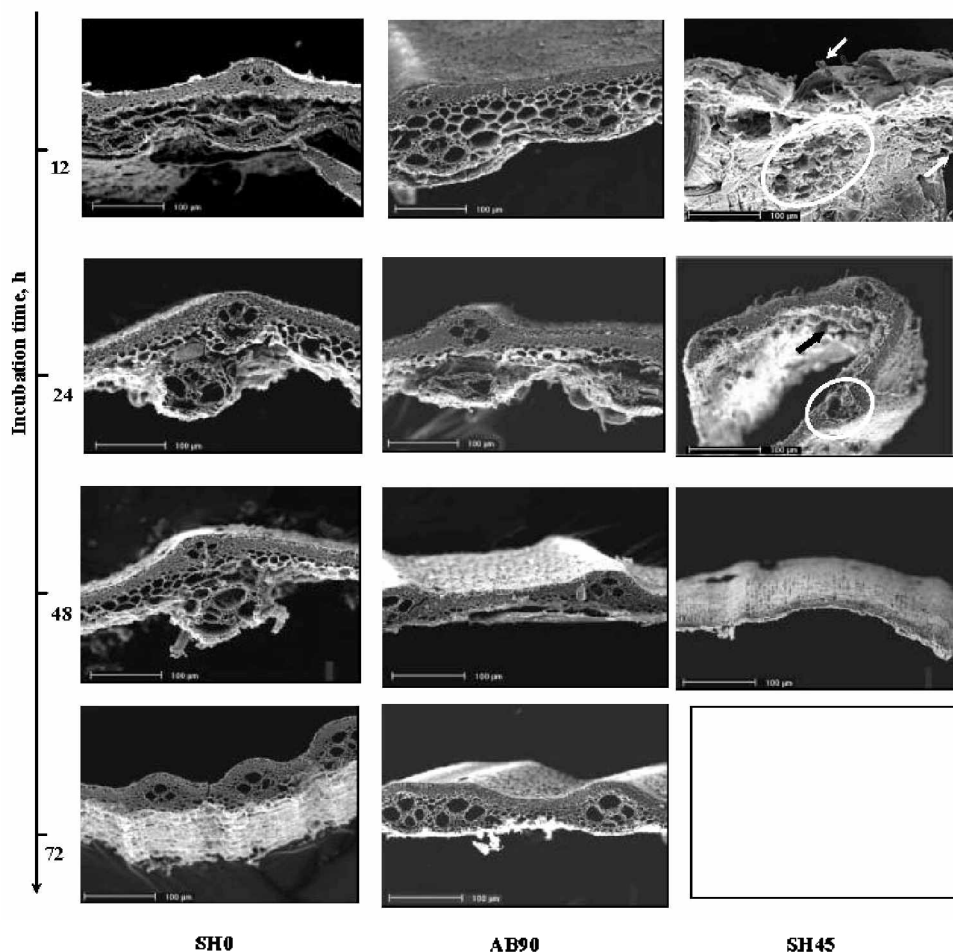
#### **Histological changes of tissues and cell wall of pretreated rice straw after rumen incubation**

The degradability of all the sub-stem samples observed in three animals was similar for every pretreatment. Therefore, only the results from one sub-stem sample obtained in the same animal are shown for every pretreatment. The SEM and TEM pictures for every pretreatment at each incubation time were from a single sub-stem sample.

*The changes of tissues* : Histological changes of tissues after rumen incubation are shown in Figure 4. Parenchyma of RS was more degradable than sclerenchyma, and the

large vascular bundles were separated from the parenchyma before their degradation could be observed in untreated stem (Figure 4, SH0). After *in sacco* degradation for 12 h, the SH-treated stem was so fragile that it cracked during preparation for microscopic investigation. Sporangia were also observed on the exo- or endo-stem surface (Figure 4 SH45, arrows and ring). At 24 h of incubation, both parenchyma and large vascular bundles were colonized by sporangia of rumen fungi (arrow in Figure 4, SH45) and slight degradation of small vascular bundles could be observed under SEM (ring in Figure 4, SH45). At 48 h of incubation, only a thin layer of sclerenchyma was observed (Figure 4, SH45), and the sclerenchyma layer became so thin that it was impossible to keep its integrity for microscopic observation at 72 h incubation. The degradation of SH-treated stem was consistent with the increase of DMD detected with whole straw in this study. However, with the limitation of microscopy, the AB-treated stem (Figure 4, AB90) could not be observed to be more degradable than the untreated one under SEM (Figure 4 SH0), in spite of a slight increase in DMD of AB-treated RS (395 vs. 327 g/kg, Table 1).

*The changes of cell wall* : Cell wall from epidermis, small vascular bundles, sclerenchyma, and parenchyma

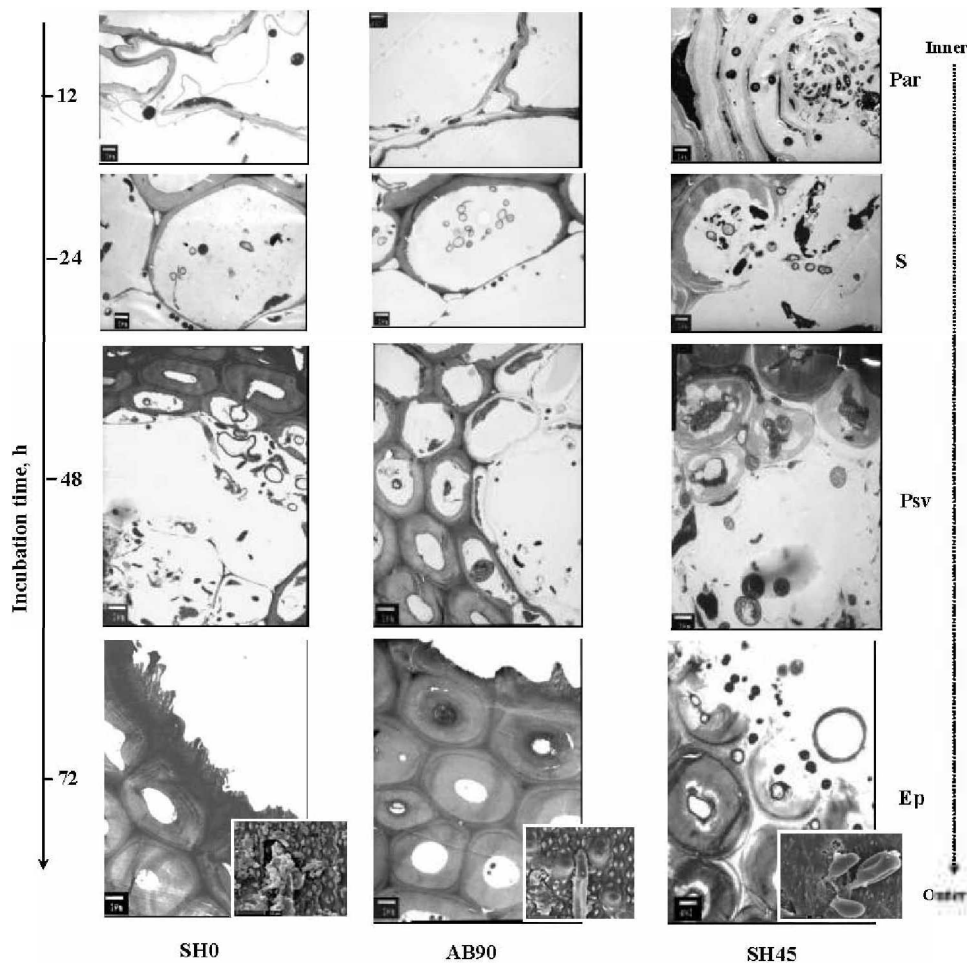


**Figure 4.** Scanning electron microscope of the untreated (SH0) stem and rice straw stem treated with 90 g ammonium bicarbonate (AB90) or 45 g sodium hydroxide per kilogram DM (SH45) after rumen incubation for 12, 24, 48 and 72 h (Bars, 100 µm). The sclerenchyma after 72 h of rumen incubation was too thin to prepare the sub-sample for microscopic investigation. Arrow and ring at 12 h and arrow at 24 h: sporangia of rumen fungi; ring at 24 h: the degraded small vascular bundle.

observed under TEM are shown in Figure 5. Degradation of AB-treated stem cell wall (AB90) was similar to that of the untreated (SH0). None of the cell wall layers of untreated and AB-treated epidermis was digestible, even if they were incubated in the rumen for 72 h. However, cell wall degradation in the small vascular bundles could be observed at 48 h incubation. The sclerenchyma cells connected to parenchyma tissues (between the rind and pith) were degraded earlier than those of small vascular bundles at 24 h rumen incubation, while degradation of parenchyma cells could be clearly observed at 12 h incubation. These changes indicated that the degradation of untreated and AB-treated stems was from inner to outer side. Contrary to untreated and AB-treated stems, in SH-treated stem the degradation of cell wall could be clearly observed in all the investigated tissues, i.e., epidermis, small vascular bundles, sclerenchyma and parenchyma at 12 h incubation (Figure 5, SH45), suggesting that the SH-treated stems are degraded bilaterally, from inner and outer surface simultaneously.

Akin et al. (1974) found that rumen bacteria preferentially degrade the region below the cuticle of epidermal cells. Eventually, bacteria degrade all the epidermal cell wall except the protective cuticle. The bacteria do not attach to the cuticle, which is dissociated from the epidermis during degradation. In order to eliminate the selective degradation effect by rumen microorganisms, the cut end of the stem was removed before the preparation of samples for microscopy in the present study. With this cutting operation, it was observed that none of the cell wall layers of untreated and AB-treated epidermis was digestible, even if they were incubated in the rumen for 72 h (Figure 5, SH0 and AB90), whereas the degradation of epidermis in SH-treated stem could be clearly observed at 12 h incubation (Figure 5, SH45).

Histological pictures revealed the different degradation between SH and AB-treated straw, and the important roles of microorganisms in the rumen degradation. However, it is difficult to quantify microbial population from either SEM



**Figure 5.** Transmission electron microscope of cells in the epidermis (Ep, to show the epidermis clearly, the SEM photos were attached at the right side), phloem of small vascular bundle (Psv), sclerenchyma connected to parenchyma (between rind and pith, S) and parenchyma (Par) of rice straw stem without (SH0) or with 45 g sodium hydroxide (SH45) and 90 g ammonium bicarbonate per kilogram DM treatment (AB90) after rumen incubation (Bars, 1  $\mu$ m). All the pictures for "SH 45" were from 12 h incubation. For SH0 and AB90, the start of observable degradation of cell wall was prolonged from 12 h for inner parenchyma to 24 h for sclerenchyma, and to 48 h for phloem of small vascular bundles, while the outer epidermis was intact even at 72 h. For SH45, the degradation of cell wall from all of the investigated tissues, epidermis, small vascular bundles, sclerenchyma and parenchyma had been started at 12 h incubation.

or TEM pictures directly. Using real-time PCR, Lee et al. (2007) revealed the change of cellulolytic bacterial adhered to the treated straw quantitatively. Fluorescence *in situ* hybridization (FISH), as a technique allowing simultaneous visualization, identification, enumeration and localization of individual microbial cells, is being widely used in many fields of microbiology (Moter and Göbel, 2000). Further work is needed to use these techniques to quantitatively explore the interaction of rumen microorganisms.

## CONCLUSIONS

Treatment with sodium hydroxide resulted in contracted parenchyma, sclerenchyma and vascular bundles of rice straw, had a strong effect on stem structure and hence improved degradation may be expected from the treated

straw. Effect of treatment with ammonium bicarbonate on stem structure was inferior to that of sodium hydroxide. The degradation of the stems treated with sodium hydroxide and ammonium bicarbonate showed different modes: the sodium hydroxide-treated straw was degraded bilaterally from inner and outer surface simultaneously, while the stem treated with ammonium bicarbonate was degraded only from the inner side.

## ACKNOWLEDGMENTS

This research was supported by a grant from the National Natural Science Foundation of China (No.30270943). Authors thank Ms. Junying Li and Mr. Liping He of the Center of Analysis and Measurement of Zhejiang University for assistance in use of electron microscopes.

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