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Resistance of Soybean Vegetative Storage Proteins (S-VSPs) to Proteolysis by Rumen Microorganisms

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Soybean vegetative storage proteins (S-VSPs) are lysine-rich and, hence, are potentially of high nutritive value for high productive ruminants. Using S-VSPs from wild-type soybean and from transgenic tobacco plants expressing either one of the two S-VSPs subunits (S-VSPα or S-VSPβ) or both, we tested their stability in cow rumen fluid under in situ conditions, using SDS-polyacrylamide gel electrophoresis. Proteolysis and degradation pattern of S-VSPs from transgenic tobacco leaves occurred relatively fast compared with that of wild-type (WT) soybean plants. Comparing the two S-VSPs subunits expressed in transgenic plants, we found that S-VSPα was degraded much faster than S-VSPβ. The degradation pattern of S-VSPs in transgenic tobacco plants expressing both subunits resembled that of WT soybean. In contrast, the degradation pattern of transgenic tobacco plants expressing a single subunit was different. These finding suggest that the quaternary structure of S-VSPs may be an important factor determining their resistance to rumen degradation. Our results also suggest that the stability to rumen proteolysis of a given protein, when expressed in a transgenic plant, may not always be predictable and has to be verified.

KEYWORDS: Soybean vegetative storage proteins; transgenic plants; tobacco; rumen proteolysis

INTRODUCTION

The major function of ruminant protein nutrition is to optimize the efficiency of dietary nitrogen utilization so as to maximize growth and milk production per unit of N consumed. As much as 40% protein is lost from the rumen of animals grazing on temperate legumes (*1*). Essential amino acids that are released by proteolytic digestion in the rumen are deaminated and might be lost through the digestive system, via urinary urea excretion, or reabsorbed and converted to microbial biomass (*2*). Among the essential amino acids, lysine and methionine are generally the two most limiting for protein synthesis both in growing ruminants (*3*) and in lactating dairy cows (*4*). A potential avenue to overcome this loss of amino acids, particularly lysine and methionine, is to identify and overexpress in transgenic plants lysine- and methionine- rich proteins which are resistant to rumen degradation

In vitro and in situ rumen digestion experiments followed by SDS-PAGE analysis revealed that some animal and plant proteins, such as bovine serum albumin and pea (*Pisum sativum*) albumins, are highly stable to rumen proteolysis (*5*); in contrast, other proteins such as casein and vicilin are rapidly degraded (*5*−*7*). Western blot analysis has been used in several studies (*6*−*8*) to follow more accurately the in vitro degradation rates of specific proteins. McNabb et al. (*7*) found that the degradation of vicilin and the Rubisco small subunit occurred in a single phase, whereas degradation of the Rubisco large subunit, ovalbumin, and sunflower albumin 8 was biphasic. The half-life time inside the rumen fluid varied between 10 min for vicilin to 69.3 h for the second component of the sunflower albumin 8. Using similar in situ techniques with proteins having different proportions of sulfur amino acids, Hancock et al. (*8*) showed that the stability of a given protein in the rumen positively correlates with the degree of cross-linking by disulfide bonds.

Soybean vegetative storage proteins (S-VSPα and S-VSPβ) are the major leaf storage proteins of soybean and contain as much as 7% lysine but very low sulfur amino acids and disulfide bonds (*9*−*11*). Previous work from our laboratory has shown that S-VSPα accumulates to high levels in heterologous plants (*12*). If S-VSPs had been shown to be resistant to rumen degradation, transgenic forage crops expressing S-VSPs could become a potential tool to increase the quantity of lysine bypassing the rumen and being available to the small intestine. Such nutritionally improved forage crops may benefit ruminant animal production.

Our study is designed to test the in situ resistance to ruminal proteolysis of S-VSPα and S-VSPβ in both wild type (WT)

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soybean and in transgenic tobacco plants overexpressing these proteins using Western blot analysis.

MATERIALS AND METHODS

Plant Material. The following plant material was used in this study: *Nicotiana tabacum* L. cv. Samsun NN, soybean (*Glycine max* Merr.), homozygous transgenic tobacco plants expressing S-VSPα (line W_2) or S-VSPβ (line K_2) and F_1 made by crossing between lines W_2 and K_2 (line K_2/W_2). All plants were grown in a controlled chamber for 14 h daylength (28/24 °C day/night temperature). For each group of plants, total leaf material of several mature plants was pooled and dried at 65 °C for 72 h.

Construction of Chimeric Genes. An *Eco*RI DNA fragment of pKSH1 (the coding DNA sequence of S-VSPα) or of pKSH3 (the coding DNA sequence of S-VSPβ) (*11*) were subcloned between the cauliflower mosaic virus (CaMV 35S) promoter and the Ω translation enhancer (*13*), and the octopine synthase teminator (*14*). The chimeric gene was inserted into the *Sma*I-*Sac*I sites of the binary vector pBINPLUS (*15*) to form the pBIN234 or pBIN13, respectively. In the same way, the *Eco*RI fragment of pKSH3, the coding sequence of S-VSPβ (*11*), was cloned into the *Sma*I-*Sac*I sites of the binary vector pBINPLUS (*15*) to form the pBIN23. The chimeric S-VSPα and S-VSPβ gene constructs were introduced into *N. tabacum* cv. Samsun NN.

Plant Transformation. Tobacco plants were grown under sterile conditions in Magenta boxes containing solidified (0.8% agar) Nitsch medium (*16*). Leaf disks were incubated for 1 h in an overnight culture of *Agrobacterium tumefaciens* containing the S-VSPα or S-VSPβ gene constructs. After the leaf disks were blotted on sterile paper, they were cultured on a solidified (1% agar) MS (*17*) medium supplemented with 2 mg/L kinetin (6-furfurylaminopurine) and 0.8 mg/L indole-3-acetic acid (Duchefa). After 48 h of co-cultivation, the disks were transferred to MS medium containing 500 mg/L carbenecillin and 100 mg/L kanamycin. Regenerated shoots were transferred to solidified (0.8%) Nitsch medium supplemented with 100 mg/L kanamycin for rooting. Regenerated shoots that rooted in the presence of 100 mg/L kanamycin were transferred to the greenhouse inside peat pellets (Jiffy 7) for establishment. After about one month the plants were transferred to 3L pots.

Production of Antibodies. Polyclonal antibodies were raised against a recombinant S-VSPβ, produced as a His-tagged protein in bacteria. The recombinant S-VSPβ was purified using His-bind TM Buffer kit (Novagen) according to the manufacturer's protocol, and the purified protein was injected into rabbits. The anti-VSPβ antiserum detected both S-VSPα and S-VSPβ in Western blots of soybean leaves.

In situ Incubation. The in situ technique was based on measuring the protein loss from a nylon-fiber (Dacron) bag suspended inside the rumen (*18*). In situ incubation of the different plant materials was carried out in an Israeli Holstein female cow between the second and third lactation fitted with a rumen fistula, and maintained on a diet of 20 kg DM/day containing 16.8% crude protein, 32.95% NDF, 19.6% ADF, and 1.765 Nel/kg DM. Each bag contained a known amount (4 g) of dried samples and was inserted inside the rumen through the fistula. At the end of each incubation interval, bags were removed from the rumen, washed thoroughly under running tap water until the rinsing water was colorless, and dried to constant weight at 100 °C. Samples from each group of plants were collected at 3, 6, 9, 12, 24, and 36 h and stored at −70 °C. The samples were then analyzed by SDS-polyacrylamide gel electrophoresis. The data presented in this report were derived from three independent replicates.

Western Blot Analysis. Extraction of total soluble proteins, Western blot analysis, and quantitative estimations of S-VSPs levels were done according to Guenoune et al. (*5*), with the exception that proteins were extracted from 1 g of dried leaf samples using 2 mL of phosphate buffer saline (PBS). Extracts were centrifuged at 15 000*g* for 30 min at 4 °C. Protein concentration was measured by the Bradford method (Bio Rad) (*19*) and equal amounts (20 μg) of proteins were separated on 10% SDS-polyacrylamide gels (*20*). Proteins in the polyacrylamide gels were transferred to nitrocellulose membranes, stained with Ponceau-S, and reacted with anti S-VSPs serum using the ECL kit (Biological Industries, Bet Haemek Israel) as recommended by the manufacturer. The relative amount of S-VSPs in each sample was determined by scanning autoradiograms in an Umax 1220S scanner and calculated by the TINA (version 2.1) program (Raytest, Germany).

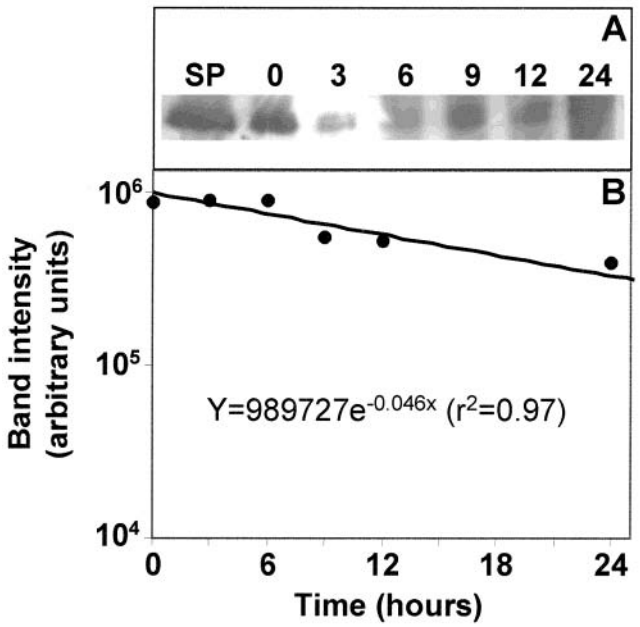

Figure 1. Time course degradation of S-VSPs from WT soybean leaves inside the rumen (in situ). (A) Western blot analysis with anti-S-VSP antibodies. Samples were removed at the time points indicated and electrophoretically separated on SDS-PAGE (12% gel). SP, untreated WT soybean leaves; time 0, incubation for 1 h in water at 39 °C. (B) Proteolysis curve. The curve was obtained by interpolation of the data for a linear curve. The S-VSPs levels at each time point represent the mean values of three replicates. The SE bars are obscured by the symbols.

Calculation of Data. The disappearance of a given protein, as determined by changes in protein band intensity, was plotted (log scale) against the incubation time and curve representing fitted. Proteolysis in the in situ incubations obeyed first-order kinetics, and a single experimental component (eq 1; (*21*)) was fitted

$$Y_{(t)} = Y_0\, e^{-kt} \quad (1)$$

where (t) is time in hours; $Y_{(t)}$ is the absolute quantity at time t; Y_0 is the absolute quantity at time 0; and k = the fractional degradation of the protein (h^{-1}). The half-life ($t_{1/2}$ (hours)) was calculated from eq 2 (*22*):

$$t_{1/2}\,(\mathrm{h}) = \frac{\log_e 0.5}{k} = \frac{0.693}{k} \quad (2)$$

Statistical Analysis. The proteolysis of different plant materials was compared by analysis of variance, using *F*-test analysis and the JMP program, version 3.1.5.

RESULTS

Rumen Degradation of WT S−VSPs. The time course for the in situ rumen degradation of S-VSPs (both S-VSPα and S-VSPβ) from WT soybean leaves is presented in **Figure 1**. The Western blot analysis (**Figure 1A**) demonstrates that WT S-VSPs was highly stable to rumen degradation. The S-VSPs level was little, if any, affected by rumen proteolysis, until 6 h incubation. It declined significantly thereafter with increasing incubation time (**Figure 1B** and **Table 1**), reaching the level of 40% from initial amount of protein after 24 h incubation (**Table 1**). Proteolysis of WT S-VSPs followed first-order kinetics, and

Table 1. Relative Stability of S-VSPs[a] to Rumen Degradation in Wild-Type Soybean and in Transgenic Tobacco Plants

	wild-type soybean	transgenic tobacco[b]		
		line W_2	line K_2	line K_2/W_2
0	100 ± 12.6 Aa[c]	51 ± 4.0 Ca	74 ± 11.8 Ba	96 ± 3.9 Aa
3	97 ± 6.6 Aa	24 ± 3.1 Cb	46 ± 2.9 Bb	94 ± 2.5 Aa
6	96 ± 4.4 Aa	0 Cc	46 ± 2.9 Bb	72 ± 3.3 Ab
9	64 ± 7.4 Ab	0 Cc	47 ± 2.2 Bb	71 ± 3.6 Bb
12	54 ± 5.4 Ab	0 Cc	45 ± 4.2 Ab	21 ± 3.7 Bc
24	31 ± 1.0 Bc	0 Dc	42 ± 1.4 Ab	12 ± 1.7 Cc

[a] % of started amount of protein. [b] Line W_2 expressing S-VSPα; line K_2 expressing S-VSPβ; line K_2/W_2 expressing both S-VSPs. [c] Capital letters indicate significant differences between plants (row-wise) and small letters indicate significant differences between incubation times within the same plant (column-wise).

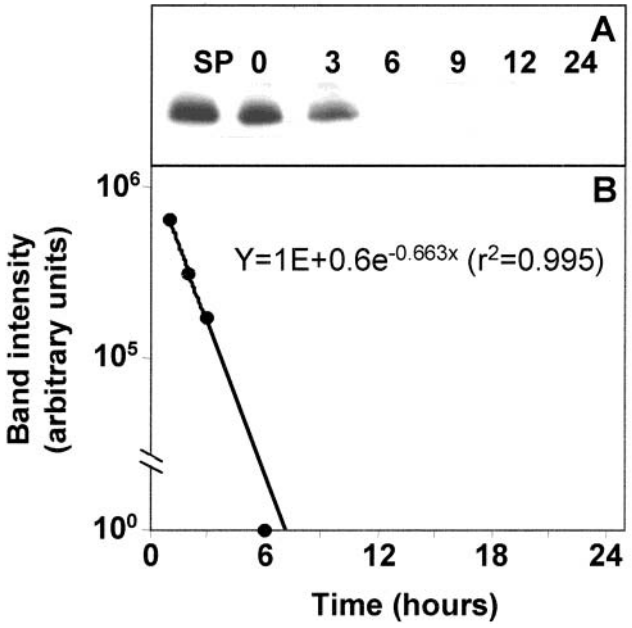

Figure 2. Time course degradation of S-VSPα from transgenic tobacco leaves inside the rumen (in situ). (A) Western blot analysis with anti-S-VSP antibodies. Samples were removed at the time points indicated and electrophoretically separated on SDS-PAGE (12% gel). SP, untreated transgenic tobacco line W_2 expressing S-VSPα; time 0, incubation for 1 h in water at 39 °C. (B) Proteolysis curve. The curve was obtained by interpolation of the data for a linear curve. The S-VSPα levels at each time point represent the mean values of three replicates. The SE bars are obscured by the symbols.

the degradation rate was 0.046 h^{-1}, with a half-life of 15.1 h (**Figure 1B**).

Rumen Degradation of S−VSPs from Transgenic Tobacco Plants. The time course degradation of S-VSPs from transgenic tobacco leaves expressing S-VSPα, S-VSPβ, or both, under in situ conditions in rumen fluid, is shown in **Figures 2**, **3**, and **4**, respectively. The Western blot analysis shows that S-VSPα was extremely sensitive to rumen proteolysis. It was completely degraded between 3 and 6 h of incubation (**Figure 2A**). Proteolysis of S-VSPα also followed first-order kinetics. The degradation rate was 0.663 h^{-1}, with a half-life of 1.05 h (**Figure 2B**). In contrast, S-VSPβ from transgenic tobacco plants was much more resistant than S-VSPα to in situ rumen proteolysis (**Figure 3A**). S-VSPβ was significantly degraded to 50% of its initial level during the first 3 h of incubation and remained stable up to 24 h of incubation (**Figure 3A** and **Table 1**). The proteolysis of this protein from F_1 plants also followed first-order kinetics, (**Figure 3B**). The degradation rate was 0.0509 h^{-1}, with a half-life of 13.6 h. Western blot analysis of S-VSPs in transgenic tobacco plants expressing both S-VSP genes showed high stability to rumen proteolysis up to 9 h. Continuous degradation was noted thereafter (**Figure 4A**). The degradation pattern of these proteins resembled that of S-VSPs in WT soybean (**Table 1**), although the degradation rate of 0.07 h^{-1} and half-life of 9.9 h were faster (**Figure 4B**).

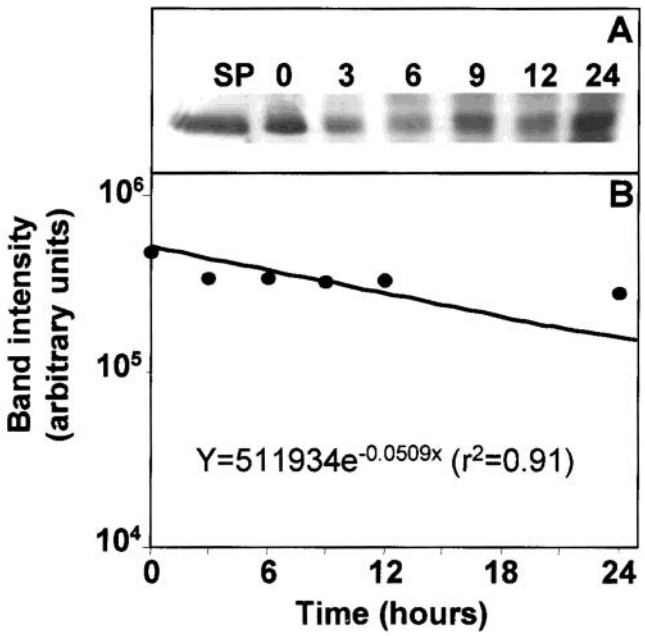

Figure 3. Time course degradation of S-VSPβ from transgenic tobacco leaves inside the rumen (in situ). (A) Western blot analysis with anti-S-VSP antibodies. Samples were removed at the time points indicated and electrophoretically separated on SDS-PAGE (12% gel). SP, untreated transgenic tobacco line K_2 expressing S-VSPβ; time 0, incubation for 1 h in water at 39 °C. (B) Proteolysis curve. The curve was obtained by interpolation of the data for a linear curve. The S-VSPα levels at each time point represent the mean values of three replicates. The SE bars are obscured by the symbols.

DISCUSSION

Liveweight gain and milk production from dairy cattle appear to be limited by amino acids absorbed from the small intestine (*22*). An ideal dietary protein for ruminant feeding should contain high levels of essential amino acids that are not provided by microbial protein and be relatively resistant to rumen proteolysis (*23*). Therefore, establishing the relative degradability of proteins in the rumen is an important prerequisite for genetic engineering studies aimed at producing plants containing proteins of high nutritive value. In the present work, we used SDS-PAGE to follow the degradation rate of lysine-rich S-VSPs in WT soybean and in transgenic tobacco leaves incubated in situ. Our results indicate that S-VSPs of WT soybean were highly stable to rumen proteolysis (**Figure 1A** and **B**). To test whether the rumen stability of S-VSPs in WT soybean exists also in heterologous plants, we overexpressed S-VSPα, S-VSPβ, or both, in transgenic tobacco plants. By using these transgenic tobacco plants, we could study the rumen stability of each of these subunits. The in-rumen time course degradation of each of the two subunits in transgenic tobacco plants differed from that found for S-VSPs of WT soybean. Both subunits were more sensitive to rumen proteolysis than the WT S-VSPs of soybean (**Table 1**). The two subunits also differed from each other in

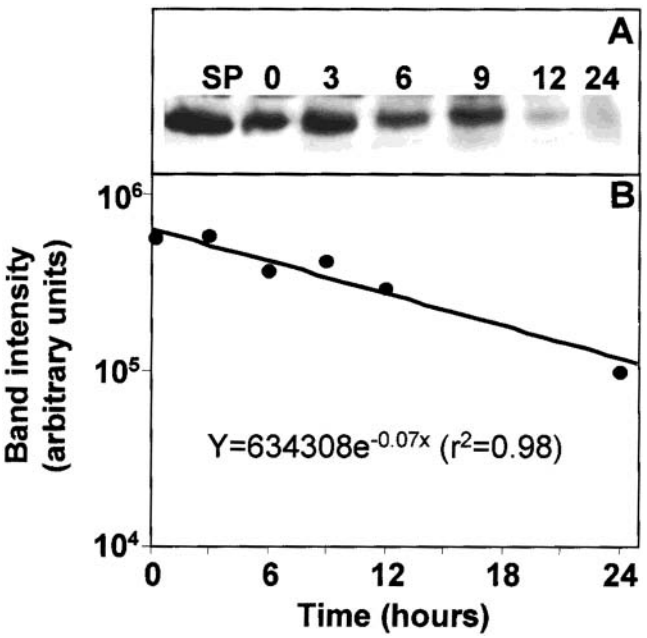

Figure 4. Time course degradation of S-VSPs (S-VSPα and S-VSPβ) from F_1 tobacco leaves inside the rumen (in situ). (A) Western blot analysis with anti-S-VSP antibodies. Samples were removed at the time points indicated and electrophoretically separated on SDS-PAGE (12% gel). SP, untreated F_1 line K_2/W_2 expressing both S-VSPα and S-VSPβ in the same plant; time 0, incubation for 1 h in water at 39 °C. (B) Proteolysis curve. The curve was obtained by interpolation of the data for a linear curve. The S-VSPα levels at each time point represent the mean values of three replicates. The SE bars are obscured by the symbols.

ruminal degradability. S-VSPβ was partially degraded in the first 3 h of incubation in rumen fluid, and remained stable till 24 h incubation (**Figure 3A** and **3B** and **Table 1**), whereas S-VSPα was unstable to rumen proteolysis and was completely degraded between 3 and 6 h (**Figure 2A** and **2B** and **Table 1**). S-VSPs are found in WT soybean both as homo- and heterodimers (*25*). Therefore, these differences in stability and degradation pattern between S-VSP subunits in transgenic tobacco and WT soybean plants may be accounted for by the quaternary structure of the S-VSPs. This is because, in WT soybean, there are three possible interactions between the S-VSP subunits ($\alpha\alpha$, $\beta\beta$, and $\alpha\beta$), but only one form of interaction in the transgenic tobacco plants expressing a single subunit. Indeed, the degradation pattern of S-VSPs in transgenic tobacco plants expressing both subunits, and having three possible interactions, resembled that of WT soybean. Further study is required to understand the interaction between rumen stability and protein structure.

Although the transgene S-VSPβ is less stable to rumen proteolysis than the native S-VSPs, it was still sufficiently stable to rumen proteolysis. Thus, our data also suggests that S-VSPβ-like proteins are potential candidates for improving the nutritional quality of forage crops utilized for ruminants.

In searches for stable proteins as targets for expression in transgenic plants for ruminant feeding, it was assumed to date that the stability of a given protein will be similar when produced in different plant species. Our results, however, suggest that this is not always the case. This is because the transgene protein may be expressed in different cell types and different subcellular locations, and may interact with other endogenous proteins and other factors in a different manner, compared with that of the WT host plants.

ABBREVIATIONS USED

S-VSPs, soybean vegetative proteins; DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber.

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