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Supplementing Proteolytic Enzymes Increased the In Vitro Nutrient Effective Degradability and Fermentation Characteristics of Pineapple Waste Silage

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Abstract: Pineapple waste silage (PAS) is an abundant agro-industrial by-product characterized by its high fiber content posing a high potential feed value as roughage for ruminants. Studies on its supplementation with proteolytic enzyme (PE) will help extend its utilization as an alternative nutritive feed source for cattle nutrition. Thus, this study aimed to determine the in vitro nutrient degradability and fermentation characteristics of fiber-rich but low-protein PAS supplemented with different levels of PE. Seven treatments were evaluated in this study: PAS without PE and PAS1 to PAS6, which corresponds to incremental levels of PAS supplementation as follows: 0.1%, 0.2%, 0.3%, 1%, 2%, and 4%. The nutrient disappearance, nutrient effective degradability, and fermentation characteristics such as total gas production, ammonia-nitrogen, and pH values were evaluated in vitro. PAS without added PE showed a comparably good nutritive value (dry matter: 94.30%, neutral detergent fiber: 63.66%, acid detergent fiber: 34.78%) to that of commonly used corn silage in South Korea. With the supplementation of PE in PAS, the PE increased the effective degradability of different nutrients such as dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), organic matter (OM), and crude protein (CP). The effect of PE supplementation on the degradation of nutrients was consistent with improvements in in vitro rumen fermentation characteristics. Supplementing PAS with PE increased the total gas production and decreased the pH values, which are characteristics of heightened fiber degradation and fermentation. The ammonia-N concentration of the in vitro-incubated PAS was moderated by the addition of PE, which is likely due to the decrease in pH or in vitro acidosis and has shown a synergistic protease activity effect on nutrient degradation. Overall, supplementing PAS with PE increased the effective degradability of DM, NDF, ADF, OM, and CP, with the most dramatic effects observed in PAS3 and PAS6 (0.3% and 4%, respectively).

Keywords: pineapple waste; silage; proteolytic enzyme; rumen fermentation; nutrient degradability; in vitro

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1. Introduction

The utilization of agro-industrial by-products in South Korea is one of the many programs of the country to achieve net-zero carbon emissions. One of these agro-industrial by-products is pineapple waste silage (PAS), a non-conventional feed resource mainly composed of spent pulp, peels, pomace, crown, and leaves fermented to silage form to preserve its nutritive quality and prolong its storage life to serve as a roughage substitute

during feed scarcity, which can occur during dry periods annually or when the agricultural industry is competing against climate change. Despite its low protein and mineral contents [1], PAS is rich in nutrients such as fiber, cellulose, hemicellulose, pectin, sugars, and bioactive and functional compounds, making it a good roughage for ruminants [2–5]. Some studies of PAS digestibility reported various results, from a high effective degradability of dry matter of 83% [6] to a low in vitro dry-matter digestibility of 26% [7]. To maximize the energy and nutrient availability of PAS, one of the several strategies employed is the addition of exogenous enzymes, although this is not a common practice in ruminant diets. Most studies that have explored the use of exogenous enzymes have mainly focused on the efficacy of fibrolytic enzymes that contain endoglucanase, xylanase, and exoglucanase. Some studies that explored proteolytic enzymes (PEs) reported increased fiber digestion for in vitro alfalfa diets [8] and in vivo total mixed ration of alfalfa hay, barley silage, and concentrate [9], while others have reported that proteases initiate protein degradation in silages and herbages in sacco [10,11], suggesting that protease activity can enhance the utilization of nutrients in roughage. Hence, it was hypothesized in this study that PEs would enhance fiber, protein, and other nutrient degradation if added to fiber-rich low-protein PAS. This study aimed to determine the in vitro nutrient degradability and fermentation characteristics of PAS supplemented with different levels of PEs. Specifically, the in vitro effective degradability of dry matter (EDDM), effective degradability of neutral detergent fiber (EDNDF), effective degradability of acid detergent fiber (EDADF), effective degradability of organic matter (EDOM), and effective degradability of crude protein (EDCP) were examined. In addition, fermentation characteristics, such as the pH, total gas production, and ammonia-nitrogen content, of the in vitro incubated PAS supplemented with PEs were evaluated.

2. Materials and Methods

2.1. Sample Preparation and Chemical Composition Analysis

The PAS was obtained from General Santos Feed Manufacturing Corporation (Philippines) and contained mainly the spent pulp, peels, pomace, cores, crown, and leaves. The samples were weighed and dried at 65 °C for 72 h in a forced-air drying oven to a constant weight to analyze the DM content and then ground in a Wiley mill using a 1 mm screen before analysis. The dried and ground PAS was divided into seven groups and mixed with different levels of bromelain PE (Sein Bio, Seoul, South Korea). The enzyme product is in powder form and is characterized by protease activity derived from a strain of *Bacillus licheniformis* compliant with the specifications for food-grade enzymes, as specified by the manufacturers. The enzyme was added at incremental levels: 0% or without PEs for PAS and 0.1%, 0.2%, 0.3%, 1%, 2%, and 4% PEs for PAS1, PAS2, PAS3, PAS4, PAS5, and PAS6, respectively. The nutrient composition of the samples was determined. The crude protein (CP) calculated as N × 6.25 and the ash were both determined based on the Official Methods of Analysis of the Association of Official Analytical Chemists [12], while the neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the method of Goering and Van Soest [13].

2.2. In Vitro Incubation to Measure Fermentation Characteristics

The samples were first incubated in vitro with rumen fluid to determine the fermentation characteristics such as the pH, ammonia-N (NH3-N) concentration, and total gas production of the seven experimental groups. The rumen contents were obtained 2 h after morning feeding (08:00) from two ruminal-cannulated non-lactating Korean native cows (Hanwoo, bodyweight = 520 ± 25 kg) fed a total daily diet of 10 kg/d (6 kg concentrate and 4 kg ryegrass, as fed basis) twice per day in equal volumes. The rumen fluid was hand-squeezed, filtered through eight layers of cheesecloth, and kept in a water bath at 39 °C. The incubation solution was prepared by mixing 50 mL of filtered rumen fluid with 100 mL of McDougall's artificial saliva [14] in a 250 mL Erlenmeyer flask. Duplicates of nylon bags containing one gram of dried and ground PAS samples with or without PE

(weight per surface area: 40 mg/cm^2) were incubated in flasks for 3, 6, 12, 24, 48, and 72 h. The flasks were then sealed with silicone rubber stoppers with a 3-way stopcock and incubated anaerobically in an orbital shaking incubator at a speed of 135 rpm for 24 h at 39 °C. Carbon dioxide (CO_2) was continuously flushed throughout the operation.

The incubation was stopped by removing the bottles from the orbital shaking incubator at 3, 6, 12, 24, 48, and 72 h. The amount of gas produced was read by inserting a calibrated glass syringe into the 3-way stopcock and the pH of the incubated solution was immediately measured. Simultaneously, two aliquots of the incubated solution (1 mL each) were collected from each flask for both volatile fatty acid (VFA) and ammonia analysis. One of the aliquots was immediately frozen for VFA analysis while the other 1 mL aliquot for ammonia analysis was mixed with 0.2 mL phosphoric acid to stop the fermentation. The ammonia concentration was determined using a spectrophotometer, as described by [15]. The in vitro incubation was conducted three times with each treatment in duplicate under similar conditions.

2.3. Estimation of Effective Degradability of Nutrients In Vitro

After incubation, the nylon bags were removed from the flasks, immediately washed in warm tap water until the rinse solution was clear, and then dried at 80 °C for 48 h in a drying oven to a constant weight to determine the EDDM, EDNDF, EDADF, EDOM, and EDCP in the seven experimental groups. The degradability at 0 h of incubation was obtained by rinsing the un-incubated nylon bag samples. Additional triplicate bags were also incubated in autoclaved rumen fluid for 0.5 h to correct for washing losses.

The percentage of DM, NDF, ADF, OM, and CP disappearance at each incubation time was calculated from the portion remaining after incubation, and the rate was fitted to the following equation [16]:

$$Y(t) = a + b\ (1 - e\hat{}ct) \quad (1)$$

where Y(t) is the proportion of the incubated material degraded at time t, t is the incubation time (h), a is the highly soluble and instantly degradable fraction, b is the insoluble and slowly degradable fraction, c is the rate constant of degradation (%h^{-1}), and e = 2.7182 (base for natural logarithm). Parameters a, b, and c were estimated using the Marquardt iterative procedure with the PROC NLIN of SAS on Academics [17]. The effective degradability of all nutrients of the pineapple waste silage samples was calculated using the following equation:

$$ED = a + ((b \times c))/(c + r) \quad (2)$$

where r is the rate constant of passage (%h^{-1}), and a hypothetical value of 0.023/h was used for the estimation of PAS.

2.4. Statistical Analysis

All data were statistically analyzed using procedures in free online SAS on Academics. In total, there were seven treatments for each experimental analysis. Each sample treatment was replicated twice, so there were duplicates in one run of analysis and the experimental analyses were run three times, so there were six data points per sample. In the data analysis, for each variable measured at each time point, the replicates were averaged so the total number of observations was 7 (treatments) × 3 (times) = 21 observations. These 21 observations per dependent variable were subjected to least-squares analysis of variance (ANOVA) using the general linear model procedure in statistical analysis software (SAS) on Academic. Significance in the data was declared at $p < 0.05$, and the data were further subjected to Duncan's multiple range test (DMRT) if differences were detected. To indicate the dispersion of the data from the mean and present the results of this study more precisely, data are presented as mean ± standard deviation.

3. Results and Discussion

3.1. Chemical Composition of the PAS with or without PEs

PAS without PE showed lower DM, CP, and ADF and higher OM than PAS supplemented with PE, while the NDF content was the same in all treatment groups (Table 1). The DM, CP, and OM contents of PAS in this study are similar to the chemical composition of the pineapple cannery by-product reported in a recent study [6], although the NDF and ADF contents were higher in the present study. The high NDF and ADF contents in this study were similar to that reported for PAS (NDF: 59.48%, ADF: 42.63%) used as roughage feed for beef cattle [18]. Higher DM and CP and lower OM of PAS supplemented with PE were expected. The supplemented PE was in powder form; thus, it increased the surface area of the samples for moisture absorption, thereby reducing the DM and OM of PAS. In addition, PE supplementation significantly increased the protein content of PAS, as the enzymes are proteins. In comparison with the commonly used and locally produced corn silage in South Korea [19], PAS with or without PE showed similar NDF (63.09% PAS vs. 65.90% corn silage), ADF (36.64% PAS vs. 37.05% corn silage), and ash content (7.35% PAS vs. 6.05% corn silage). Overall, PAS with or without PE displayed comparably good nutritive value compared with corn silage.

Table 1. Chemical composition of pineapple waste silage supplemented with different levels of fibrolytic enzymes.

Chemical Composition	PAS	PAS1	PAS2	PAS3	PAS4	PAS5	PAS6
MC, %	5.70 ± 0.36^{a}	3.66 ± 0.16^{b}	3.94 ± 0.29^{b}	3.70 ± 0.19^{b}	3.75 ± 0.28^{b}	4.03 ± 0.18^{c}	3.80 ± 0.34^{b}
NDF, %	63.66 ± 0.37	62.32 ± 2.47	63.94 ± 0.35	62.34 ± 0.53	63.23 ± 0.09	63.41 ± 0.65	62.74 ± 0.93
ADF, %	34.78 ± 1.51^{a}	38.79 ± 0.28^{b}	38.48 ± 0.83^{b}	37.59 ± 0.95^{b}	34.29 ± 0.48^{a}	35.09 ± 0.26^{a}	37.45 ± 0.10^{b}
CP, %	14.18 ± 0.11^{a}	14.92 ± 0.35^{b}	14.90 ± 0.50^{b}	14.10 ± 1.90^{b}	13.12 ± 2.10^{b}	13.97 ± 0.84^{b}	13.79 ± 0.83^{b}
Ash, %	7.71 ± 0.21^{a}	7.85 ± 0.16^{b}	7.76 ± 0.24^{b}	7.95 ± 0.12^{b}	8.10 ± 0.27^{c}	8.12 ± 0.10^{c}	7.95 ± 0.16^{b}

MC, moisture content; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein. Means in the same row with different superscripts ^{a,b,c} are significantly different ($p < 0.05$).

3.2. Nutrient Disappearance and Effective Degradability of In Vitro-Incubated PAS with or without PE

The dry-matter loss of PAS supplemented with PE showed an increasing trend with incubation time while PAS without PE increased up until 12 h, slightly dropped at 24 h, and was steady from 48 h to 72 h (Table 2). This notably indicates that the PE inclusion in pineapple waste silage regardless of the concentration would increase the dry-matter degradation in the rumen. The average dry-matter disappearance values at all incubation periods obtained in this study ranged from 29.11% to 72.7%, with PAS2 showing the highest DM disappearance. The DM disappearance of PAS reached its highest value (40.94%) at 12 h, slightly dropped to 39.05% at 24 h, and remained at 39% from 48 h to 72 h incubation time. The highest DM disappearance observed for PAS supplemented with PE ranged from 65.12% to 72.70% at 48 h to 72 h, respectively. These findings are similar to the results of a previous study in which 80% DM disappearance was achieved by incubating pineapple by-product for 96 h [20]. In terms of effective degradability, PAS1 to PAS5 showed higher values of the highly soluble fraction a than PAS6, which could indicate that the addition of 4% PE or higher in pineapple waste silage will not further increase the effective degradability of highly soluble parts of silages. This also suggests that a 0.1 to 2.0% PE level in pineapple waste silage is effective in degrading the highly soluble parts of the by-product silage. Interestingly, all PAS supplemented with PE showed higher values of the slowly soluble fraction b than PAS alone, indicating that the supplementation of PE in pineapple waste silage increased the effective degradation of the hardly degradable parts of by-product silages. In support of this, supplementing with PE increased the EDDM of pineapple waste silage from 58.57 to 61.56%. The different levels of added PE did not show a significant difference. The EDDM of PAS alone was significantly lowest at 38.21%.

Nonetheless, adding PE to PAS increased the effective DM degradability, which is likely due to protease acting on the degradation of the cell walls of the substrate.

Table 2. Percentage dry-matter (DM) disappearance at different incubation periods and the effective degradability of dry matter of in vitro-incubated pineapple waste silage with or without proteolytic enzyme.

Parameters	PAS	PAS1	PAS2	PAS3	PAS4	PAS5	PAS6
0 h	30.40	30.69	32.85	32.28	31.17	29.13	29.11
3 h	36.68	45.52	39.34	45.44	45.06	47.54	45.75
6 h	37.64	47.07	40.81	39.55	46.14	42.60	39.11
12 h	40.94	44.29	46.12	47.26	52.86	52.96	54.81
24 h	39.05	59.38	58.69	61.80	64.91	60.98	63.17
48 h	39.95	67.29	72.70	67.44	66.55	68.86	65.12
72 h	39.23	71.85	71.87	71.97	70.83	66.29	70.37
a, %	$31.78 \pm 1.39^{c,d}$	$33.55 \pm 0.54^{a,b}$	$31.53 \pm 1.77^{c,d}$	33.97 ± 0.58^{a}	$33.44 \pm 0.61^{a,b}$	$32.58 \pm 0.57^{b,c}$	31.35 ± 0.66^{d}
b, %	26.85 ± 2.69^{d}	37.68 ± 2.41^{b}	41.48 ± 3.30^{a}	37.37 ± 3.52^{b}	34.88 ± 2.28^{c}	32.57 ± 2.47^{c}	$35.28 \pm 2.33^{b,c}$
c, %/h	0.45 ± 0.22^{a}	0.07 ± 0.05^{b}	0.10 ± 0.12^{b}	0.06 ± 0.06^{b}	$0.29 \pm 0.40^{a,b}$	$0.32 \pm 0.43^{a,b}$	$0.29 \pm 0.41^{a,b}$
EDDM, %	38.21 ± 1.32^{b}	60.04 ± 3.83^{a}	60.43 ± 3.55^{a}	58.57 ± 6.94^{a}	61.56 ± 2.82^{a}	59.75 ± 2.24^{a}	60.44 ± 2.94^{a}

EDDM, effective degradability of dry matter; a, b, c in the first column corresponds to: a, highly soluble fraction of sample; b, insoluble and slowly soluble fraction of sample at time infinity; c, rate constants of degradation of fraction b. Means in the same row with different superscripts ^{a,b,c} are significantly different ($p < 0.05$).

As shown in Table 3, the addition of PE to PAS resulted in an increase in organic-matter (OM) disappearance. The PAS without PE supplementation showed a 24.30% average OM disappearance, which is half of the average OM disappearance of PAS with PE (49.70%) at all incubation times. Among the concentration levels, PAS with 4% PE resulted in the highest EDOM (61.95%). The EDOM of PAS1 to PAS5 ranged from 55.80% to 57.64%, indicating that 0.1% to 2% PE treatments do not vary in their degradation of organic matter in pineapple waste silage. The high OM degradability of PAS could be due to the effective attack and degradation of fibers by PE, making the organic nutrients of the silage available for digestion and utilization. The high effective dry-matter (EDDM) and organic-matter degradability (EDOM) with the addition of PE was similar to the increased DM and OM digestibility of the total mixed rations when supplemented with exogenous PE [21].

Table 3. Percentage organic-matter (OM) disappearance at different incubation periods and the effective degradability of organic matter of in vitro-incubated pineapple waste silage with or without proteolytic enzyme.

Parameters	PAS	PAS1	PAS2	PAS3	PAS4	PAS5	PAS6
0 h	28.81	27.05	27.34	30.56	23.10	21.69	24.58
3 h	21.53	39.61	37.08	35.49	49.13	41.92	40.09
6 h	25.32	41.38	38.44	51.48	36.27	37.21	36.28
12 h	21.87	44.62	43.89	44.78	48.41	51.55	53.2
24 h	23.01	55.42	53.37	56.46	62.06	60.61	70.59
48 h	23.31	64.52	65.36	63.82	62.67	64.99	74.28
72 h	26.25	70.56	69.37	70.97	67.24	62.13	67.81
a, %	28.67 ± 0.82^{a}	36.75 ± 0.30^{d}	$29.74 \pm 2.10^{b,c}$	33.41 ± 0.4^{d}	$28.58 \pm 1.07^{b,c}$	24.30 ± 0.52^{b}	24.68 ± 0.72^{b}
b, %	21.44 ± 0.37^{a}	38.54 ± 91.12^{b}	43.14 ± 3.35^{c}	37.44 ± 1.30^{b}	37.14 ± 2.48^{b}	39.31 ± 4.12^{b}	47.88 ± 4.04^{c}
c, %/h	0.38 ± 0.14^{d}	0.001 ± 0.00^{a}	0.04 ± 0.15^{b}	0.04 ± 0.02^{b}	$0.08 \pm 0.45^{b,c}$	0.10 ± 0.77^{c}	$0.08 \pm 0.81^{b,c}$
EDOM, %	23.60 ± 0.16^{a}	$57.49 \pm 0.95^{c,d}$	$55.80 \pm 4.66^{b,c}$	$57.64 \pm 2.64^{c,d}$	$57.25 \pm 3.01^{c,d}$	56.34 ± 1.59^{b}	61.95 ± 2.29^{d}

EDOM, effective degradability of organic matter; a, b, c in the first column corresponds to: a, highly soluble fraction of sample; b, insoluble and slowly soluble fraction of sample at time infinity; c, rate constants of degradation of fraction b. Means in the same row with different superscripts ^{a,b,c,d} are significantly different ($p < 0.05$).

The average percentage NDF disappearance (Table 4) and ADF disappearance (Table 5) of PAS without PE were 42.69% and 33.40%, respectively. The effect of PE added to PAS on NDF disappearance generally showed an increasing trend with increasing incubation time. The highest percentage NDF disappearance (79.61%) was observed in PAS supplemented with 4% PE (PAS6). Subsequently, PAS6 showed the highest EDNDF (61.58%). The EDNDF

of PAS supplemented with other levels of PE (0.1% to 2.0%) showed lower values than PAS6, ranging from 58.87 to 60.76%, but higher than PAS alone (42.80%). The EDNDF of PAS supplemented with PE was better than the neutral detergent fiber digestibility (NDFD) of commonly used corn silage (51.3%) in South Korea [19]. Adding PE increased the NDF degradability of PAS, similar to findings in in vitro studies of alfalfa hay and total mixed ration, which also showed increased in vitro NDF degradability [22,23]. However, some studies have reported that PE was more effective in alfalfa hay than in corn silage [23] or showed minimal fiber-digestion efficacy in barley silage [24], which was attributed to the higher quality of silages or fermentation acids produced during the ensiling process. Contrary to the cited literature, supplementation of PAS with PE significantly increased the effective NDF degradability, likely due to the synergistic effect of the protease activity of the added PE and the existing digestive enzymes produced by the ruminal microorganisms in vitro. In addition, adding PE to PAS also increased the effective ADF degradability, strengthening the effective fiber-degradation effect of the PE in PAS. Supplementing PAS with PE increased the percentage ADF disappearance. The ADF loss of PAS with PE ranged from 30.01 to 79.02%. The addition of PE to PAS increased ADF disappearance with increasing incubation time. However, the incremental addition of PE to PAS did not show a linear increase with the highly insoluble and highly soluble fractions, thus resulting in a nonlinear increase in EDADF. Nonetheless, adding 2% PE increased the highly soluble and insoluble fractions of PAS, consequently resulting in an increased EDADF of PAS5. Other concentration levels of PE (PAS1–PAS4 and PAS6) added to pineapple waste silage showed a higher EDADF in comparison to PAS alone, indicating that the addition of PE could increase the degradation of ADF in by-product silages. Lignin, which acts as a restricting barrier to enzyme activities, could have been acted upon and degraded by the added PEs in addition to the digestive enzymes produced by the ruminal microorganisms.

Table 4. Percentage neutral detergent fiber (NDF) disappearance at different incubation periods and the effective degradability of neutral detergent fiber of in vitro-incubated pineapple waste silage with or without proteolytic enzyme.

Parameters	PAS	PAS1	PAS2	PAS3	PAS4	PAS5	PAS6
0 h	24.89	31.58	24.96	25.83	27.81	26.71	29.17
3 h	43.07	49.15	47.33	44.76	43.92	42.85	55.19
6 h	46.46	56.43	51.94	49.64	41.14	43.80	60.88
12 h	46.92	55.93	56.3	56.05	57.5	56.57	62.89
24 h	46.83	63.14	60.38	61.66	57.19	66.30	79.61
48 h	45.83	59.44	62.97	62.12	64.29	67.91	70.65
72 h	44.80	69.59	69.32	70.85	68.68	69.68	68.94
a, %	29.81 ± 4.40 ^{a}	34.13 ± 2.86 ^{b}	28.67 ± 3.23 ^{b}	29.13 ± 2.16 ^{b}	30.56 ± 0.75 ^{b}	28.92 ± 0.74 ^{b}	33.33 ± 4.63 ^{a}
b, %	13.11 ± 7.44 ^{c}	28.13 ± 4.09 ^{b}	33.78 ± 4.69 ^{a}	35.01 ± 3.48 ^{a}	34.09 ± 2.19 ^{a}	36.84 ± 4.23 ^{a}	28.44 ± 9.61 ^{b}
c, %/h	2.41 ± 0.24 ^{b}	0.35 ± 0.34 ^{c}	0.42 ± 0.48 ^{c}	0.27 ± 0.40 ^{c}	0.24 ± 0.35 ^{c}	0.49 ± 0.68 ^{c}	3.35 ± 0.16 ^{a}
EDNDF, %	42.80 ± 3.83 ^{c}	59.93 ± 0.55 ^{a,b}	59.53 ± 0.60 ^{a,b}	59.82 ± 0.81 ^{a,b}	58.87 ± 2.26 ^{b}	60.76 ± 1.50 ^{a,b}	61.58 ± 5.86 ^{a}

EDNDF, effective degradability of neutral detergent fiber; a, b, c in the first column corresponds to: a, highly soluble fraction of sample; b, insoluble and slowly soluble fraction of sample at time infinity; c, rate constants of degradation of fraction b. Means in the same row with different superscripts ^{a,b,c} are significantly different ($p < 0.05$).

In terms of protein degradation (Table 6), PAS supplemented with PE showed a higher CP disappearance than PAS without PE at all incubation times. It can be noted that there is only a slight increase in the percentage CP disappearance from 0 h to 3 h incubation time. This could indicate that the added PE did not immediately act on proteolysis in the in vitro-incubated substrate PAS. From 6 h to 72 h, PAS with PE showed high CP disappearance ranging from 41.16–84.71%, indicating protein breakdown or degradation by the added PE. The CP disappearance of PAS without PE was the highest (52.26%) at 72 h incubation time. In terms of effective degradability, adding 0.3% PE increased the EDCP of PAS the most, while adding 0.1%, 0.2%, and 4% showed comparably high EDCP results, and adding 1% and 2% PE only slightly increased the EDCP of PAS compared to PAS without added PE. The incremental addition of PE to PAS did not result in a linear increase in the effective

degradability of CP (EDCP), which is likely caused by the effect of the incubation period as observed. In a similar study, it was observed that protein degradation was only numerically increased despite increasing protease activity [9], suggesting that supplementation with PE would not ensure effective CP degradability. Nonetheless, the addition of PE, regardless of the level, increased the effective CP degradability of PAS compared to PAS alone.

Table 5. Percentage acid detergent fiber (ADF) disappearance at different incubation periods and the effective degradability of acid detergent fiber of in vitro-incubated pineapple waste silage with or without proteolytic enzyme.

Parameters	PAS	PAS1	PAS2	PAS3	PAS4	PAS5	PAS6
0 h	31.39	30.46	33.06	30.01	33.9	34.65	33.54
3 h	32.13	35.25	33.73	35.11	43.23	43.55	45.65
6 h	30.36	42.92	45.88	49.83	45.33	47.91	47.89
12 h	33.06	48.87	51.23	56.32	55.72	51.46	59.88
24 h	36.1	52.06	61.38	73.82	67.09	69.35	66.22
48 h	35.06	58.36	62.54	73.89	71.78	77.43	70.85
72 h	35.71	68.4	66.64	71.87	79.02	77.87	78.56
a, %	30.77 ± 0.05^{d}	32.34 ± 0.17^{c}	31.15 ± 0.49^{d}	28.21 ± 0.39^{e}	34.30 ± 1.11^{b}	34.70 ± 1.24^{b}	35.29 ± 0.71^{a}
b, %	31.54 ± 10.02^{c}	$34.54 \pm 0.38^{b,c}$	32.43 ± 2.32^{c}	41.03 ± 5.24^{a}	41.97 ± 1.88^{a}	43.61 ± 2.16^{a}	$39.07 \pm 1.76^{a,b}$
c, %/h	0.06 ± 0.03^{b}	0.04 ± 0.01^{b}	$0.29 \pm 0.41^{a,b}$	0.51 ± 0.76^{a}	0.13 ± 0.19^{b}	0.13 ± 0.19^{b}	$0.20 \pm 0.29^{a,b}$
EDADF, %	53.29 ± 7.26^{c}	$54.85 \pm 0.86^{b,c}$	57.56 ± 3.42^{b}	63.33 ± 2.47^{a}	65.94 ± 4.19^{a}	66.98 ± 4.76^{a}	66.29 ± 3.56^{a}

EDADF, effective degradability of acid detergent fiber; a, b, c in the first column corresponds to: a, highly soluble fraction of sample; b, insoluble and slowly soluble fraction of sample at time infinity; c, rate constants of degradation of fraction b. Means in the same row with different superscripts ^{a,b,c,d,e} are significantly different ($p < 0.05$).

Table 6. Percentage crude protein disappearance at different incubation periods and the effective degradability of crude protein of in vitro-incubated pineapple waste silage with or without proteolytic enzyme.

Parameters	PAS	PAS1	PAS2	PAS3	PAS4	PAS5	PAS6
0 h	30.58	32.66	33.82	35.24	30.32	31.14	31.75
3 h	33.66	32.43	35.08	35.61	35.88	35.17	39.29
6 h	47.86	52.37	49.75	41.16	42.66	45.07	44.55
12 h	49.12	56.04	51.42	53.06	55.27	44.18	53.74
24 h	55.33	63.32	63.18	66.96	62.56	59.55	64.93
48 h	49.41	64.75	78.02	83.37	65.71	70.22	68.79
72 h	52.26	84.71	82.47	82.96	82.25	75.01	77.58
a, %	$30.55 \pm 1.69^{c,d}$	33.36 ± 0.53^{a}	32.08 ± 2.31^{b}	$31.87 \pm 0.71^{b,c}$	$31.27 \pm 0.54^{b,c}$	29.86 ± 2.25^{d}	$31.84 \pm 1.07^{b,c}$
b, %	22.02 ± 1.47^{f}	$45.58 \pm 1.66^{c,d}$	50.44 ± 3.18^{b}	55.62 ± 3.10^{a}	46.83 ± 2.27^{c}	$44.04 \pm 3.73^{d,e}$	42.31 ± 1.74^{e}
c, %/h	0.38 ± 0.46^{a}	0.05 ± 0.04^{b}	0.12 ± 0.21^{b}	0.04 ± 0.01^{b}	0.06 ± 0.05^{b}	0.12 ± 0.17^{b}	0.13 ± 0.19^{b}
EDCP, %	50.46 ± 1.04^{d}	$64.10 \pm 3.98^{a,b,c}$	$66.26 \pm 6.29^{a,b}$	67.21 ± 3.81^{a}	$62.44 \pm 5.19^{b,c}$	60.73 ± 5.82^{c}	$63.86 \pm 4.08^{a,b,c}$

EDCP, effective degradability of crude protein; a, b, c in the first column corresponds to: a, highly soluble fraction of sample; b, insoluble and slowly soluble fraction of sample at time infinity; c, rate constants of degradation of fraction b. Means in the same row with different superscripts ^{a,b,c,d,e,} is significantly different at $p < 0.05$.

Altogether, it was observed that fraction a, or the highly soluble fractions in all ED nutrients, was high, indicating that highly soluble sugars and nitrogen compounds such as sucrose, fructose, and glucose in PAS were effectively degraded. In addition, fiber and lignin in PAS were also effectively degraded, as indicated by the high b or slowly degradable fractions in all ED nutrients [25]. However, it is worth emphasizing that these highly soluble and slowly degradable fractions in PAS were highly degraded when supplemented with different levels of PEs, as indicated by the high a and b values for all ED nutrients. Consequently, the nutrients DM, NDF, ADF, OM, and CP in PAS with PE were effectively degraded. The most likely mechanism by which PEs enhance fiber degradation is through the attack of cell wall proteins or through nitrogen-containing components acting as physical restricting barriers to degradation, thus allowing for more extensive microbial access to fiber degradation [22,26]. In general, PE supplementation increased EDDM, EDNDF, EDADF, EDOM, and EDCP. Although the effective degradability of each nutrient is not constant for one level or concentration of PE, it can be suggested that PAS

with 4% PE could best increase the effective degradability of all nutrients. However, it should be noted that supplementation of 0.1 to 2.0% PE does not vary greatly compared to the 4% level in effectively degrading NDF, ADF, CP, and OM. Due to the fact that EDDM was comparable at all concentration levels of PE, it is finally suggested that the 0.1% PE level is best because it is both nutrient-fermentation- and cost-effective. In addition, PAS supplemented with PE showed better nutrient degradation than corn silage produced locally and normally used in South Korea.

3.3. Fermentation Characteristics of In Vitro-Incubated PAS with or without PE

The total gas production derived from the in vitro measurements shown in Figure 1a shows an increase with increasing incubation time. The total gas produced was lowest in PAS without PE, whereas the total gas production was highest in PAS supplemented with 4% PE. Other treatments, such as PAS supplemented with 0.1% to 2% PE, also resulted in higher total gas production than PAS without PE. These findings support the highly effective degradability of nutrients, especially the high EDDM in PAS with PE. A high EDDM is highly correlated with total gas production [27], as gas is produced from the fermentation of the highly degraded fraction of the roughage. The total gas production could also indirectly indicate the protease activity of PE in PAS. The PE could have attacked the cell wall proteins of the PAS, making the nutrients of the substrate accessible for microbial fermentation, thus increasing the total gas production. The ammonia-N concentration of PAS without PE was clearly the highest from 6 h to 72 h of incubation time and peaked at 24 h, as presented in Figure 1b. The addition of 0.1 to 0.3% PE to PAS increased the ammonia-N concentration at 6 h, which then gradually decreased until the end of the incubation period. Surprisingly, adding 1, 2, and 4% PE to PAS showed a steady ammonia-N concentration ranging from 3.05 to 5.77 mg $\times$ 100 mL^{-1}. The optimum ruminal ammonia-N concentration for maximum microbial synthesis of proteins is 5–8 mg $\times$ 100 mL^{-1} [28]. All treatment groups, except PAS5, were above the optimum concentration, indicating that dietary protein had reached the level at which protein was converted to digestible energy and that the excess dietary protein was converted to ammonia, supporting the highly effective CP degradation observed in the study. The high ammonia-N concentration of PAS without PE could have suppressed existing enzyme activities in the in vitro culture, thus lowering the effective degradation of nutrients compared to PAS with PE. High concentrations of ammonia-N have been reported to suppress bacterial protease activity via a mechanism analogous to the classical feedback inhibition [29]. This also suggests that the higher ammonia-N concentration in PAS with lower PE (0.1%, 0.2%, and 0.3%) could have contributed to the suppression of the protease activity of the added PE; hence, the addition of higher PE (1%, 2%, and 4%) resulted in a higher effective degradation of nutrients.

The pH of the in vitro-incubated PAS gradually decreased from 6.13 at 0 h incubation to 5.45–5.74 at 72 h. It has been reported that a pH between 6 and 9 is the optimum level to ensure the growth of fiber-digesting cellulolytic bacteria [30]. The pH of all groups fell below the minimum pH level, indicative of fermentation and effective degradation of nutrients converted to organic acids for use in energy production. However, the literature reported that a rumen pH below 6 washes out proteolytic organisms [26], which could decrease nutrient degradation. In this study, the addition of PE could have replaced the apparently washed-out proteolytic organisms when the pH fell below minimum levels, as evidenced by the high nutrient effective degradation of PAS with PE.

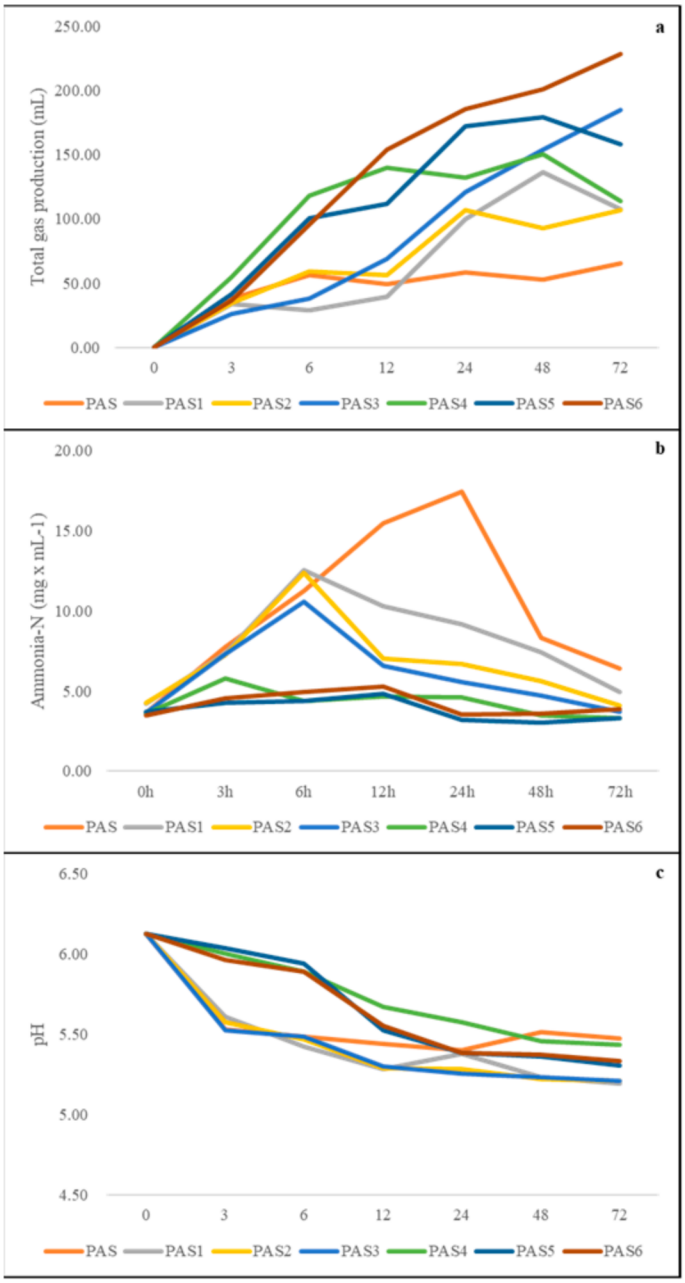

Figure 1. Total gas production (**a**), ammonia-N concentration (**b**), and pH values (**c**) of pineapple waste silage supplemented with or without proteolytic enzymes.

4. Conclusions

Supplementing PAS with PE increased nutrients' effective degradability, such as the EDDM, EDNDF, EDADF, EDOM, and EDCP, in non-conventional feed roughage. The effects of PE addition on the effective degradation of nutrients were also consistent with an improvement in the in vitro rumen fermentation. Supplementing PAS with PE increased the total gas production, decreased the pH values, and increased fiber degradation and fermentation. The moderate ammonia-N concentration of in vitro-incubated PAS with added PE showed a synergistic protease-activity effect on nutrient degradation. Overall, along with favorable fermentation characteristics, supplementation of PAS with PE increased the effective degradability of nutrients compared to that without PE, with the most dramatic effect observed in PAS3 and PAS6 (0.3% and 4%, respectively). However, it should be noted that supplementation of 0.1 to 2.0% PE does not vary greatly from the 4% level in effectively degrading NDF, ADF, CP, and OM. Due to the fact that EDDM was comparable

at all concentrations of PE, it is finally suggested that the 0.1% PE level is best because of its good nutrient degradability and cost-effectivity. Further research is needed to investigate the protease activity and mechanism of PE in the degradation of nutrients.

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