Report of the Appropriate Technology for Processing and Utilization of Ipil-Ipil for Leaf Meal Forage

—Development of the Method of Reduction of Mimosine Level for Bohol Agricultural Promotion Center Project in Philippines—

March, 1986

Japan International Cooperation Agency

PREFACE

This report is compiled the results of the research work of the appropriate technology for processing and utilization of Ipil-Ipil for leaf forage by Japan Scientific Feeds Association trusted by JICA.

This research work was aimed to develop the method of reduction of mimosine level of Ipil-Ipil for the Bohol Agricultural Promotion Centre Project in the Republic of the Philippines (hereinafter referred to as the Project).

The Project has been implemented by the Record of Discussion, signed on Feb 2, 1983. The main activities of the Project are to develop and improve the technology of Planting and cultivating the lowland and upland crops through the analyzes of the different soil characters in between east and west of the Bohol Province. Another is to train the technicians and farmers and to extend them the most appropriate technology developped by the Project.

As 4th years of the cooperation passes, the Project is going to be on the extensible stage of the technology. Through the research work, the results examined are hoped to be useful for the effective utilization of Ipil-Ipil for leaf forage and then highly expected to be helpful to the increase of the agricultural production mainly by the promotion of livestock and the farmers' income in Bohol province.

Finally, I wish to express my sincere thanks to all the cooperators (Japan Scientific Feeds Association, Ryukyu University, Nakazima agriculture and Livestock Co ; ltd and advisory members) and hope cooperation from them to the Project will unchangeably continue onward.

March, 1986

Takashi Tauchi
Director
Agricultural Development Cooperation Department
Japan International Cooperation Agency

CONTENTS

INTRODUCTION

It is indicated by a number of scholars that the stalks and leaves of the Ipil-Ipil (Leucaena Leucocepha de wit) which is an ever green perennial plant in the pulse family widely distributed in the torrid and subtropic zones are very valuable as a rather scarce. Ipil-Ipil is grown in the considerable areas in the Island of Bohol in Philippines as well, and produced in a good yield (5 tons green grass/10a, 6 times harvesting/year). In this connection, for futher increasing the yield and accelerating the effective use of Ipil-Ipil, sternuous exertion is being made for the improvement of the cultivation process of Ipil-Ipil by the Bohol Agricultural Development Center Project in cooperation with Japan. On the other hand, it is reported that, when domestic animsls are fed with Ipil-Ipil for a long time, those morbid symptoms including growth impediment, reproduction difficulty and hair removal, can be developed by the mimosine {B-(N-(3-hydroxy-4 oxypyridyl))-2-aminopropionic acid} which is an amino acid contained in Ipil-Ipil, and that thyroidal tumor can be caused by the 3-hydroxy-(IH)-pyridone which is a decompostion product from mimosine in Rumen. Whereas various measures are reported for weakening the toxic action of mimosine to the domestic animals, actual application of these means are hardly effected due to a variety of probmems, such as the loss of the effective components in Ipil-Ipil. Accordingly, Ipil-Ipil is only use as forage within a limit not developing the toxic symptom due to the minosine for individualdomestic animal, and the value of Ipil-Ipil as forage is not fully utilized still at present.

Under such circumstances, this reserch work has been initiated according to the commission of Japan International Cooperation Agency for settling the problems described above to investigate a measure for fully utilization the value of Ipil-Ipil as a forage source and thus assisting the technical guidance of the agriculture in the actual locale.

Incidentally, kind guidance and cooperation are given by the following committee members on the planning of the present project and examination of the experimental results in the project, and earnest study is made by the members of the reserch team in University of Ryukyus on effective reduction of the mimosine in Ipil-Ipil. Furthermore, earnest cooperation is carried out by Nakajima Agricultural Institure, Ltd., on experimental manufacture and improvement of the pellet machine. In these respects, deep gratitude is expressed to these gentlemen.

Dr. Hiroshi Morimoto
President
Japan Scientific Feeds Association

Committee Members

Muneo Ogata	Japan International Cooperation Agency
Yoshitsugu Kawasaki	University of Ryukyus
Kiyomi Kosaka	National Institute of Animal Industry
Akihiro Takigawa	National Institute of Animal Industry
Fujiya Hongo	University of Ryukyus
Syotaro mizusawa	Nakajima Agricultur-al Institute, Ltd.,
Hiroshi Morimoto	Japan Scientific Feeds Association

I Inverstigation of the Effective Reduction Methods of Mimosine in Ipil-Ipil

I a A Simple and Economical Analysis Methods of Mimosine and A Method for it's Isolation

I b Analysis of Mimosine in the Various Parts in Ipil-Ipil

I c Degradation of Mimosine by Enzymes, Including Commercial Agents

I d A Basic Study on the Effect of the Addition of Metal Salts to Ipil-Ipil Feed

I e Effect of the Mimosine Autolytic Enzyme for the Toxicity Redution of Ipil-Ipil

I f Investigation on the Toxicity of Mimosine Degradation Products and Methods for the Elimination of Both Mimosine and the Degradation Products

Shinkichi Tawata University of Ryukyus
Shiryo Yaga University of Ryukyus

I a A SIMPLE AND ECONOMICAL ANALYSIS METHOD OF MIMOSINE AND A METHOD FOR ITS ISOLATTON

INTRODUCTION

Hegarty et al[1]. reported the purification method of mimosine from Ipil-Ipil seeds by using dialysis, and Hongo et al[2]. also reported an isolation method of mimosine by recrystalization. We tried to isolate mimosine from the leaves of Ipil-Ipil because of the possibility of supplying meterial throughout the year, whereas with seeds supply is limited to August in Okinawa. Recently, Lowry et al[3]. also reposted an isolation method; however, we slightly modified their method.

METHODS AND RESULTS

One Kg of freshly harvested Ipil-Ipil leaves was dipped in 10 liters of boiling water. The boiling was continued for 10 min. After the water extract was cooled to room temperature, 10 liters of 95 % ethanol was added and left to stand for 30 min or until the polysaccharide precipitated. The mixture was centrifuged for 15 min at 8.000 rpm to remove the polysaccharide. The supernatant was passed through a column packed with 1.5 liters of Amberlite IRA 120 (technical grade) in acid form. The resin was washed with deionized water (1 liter) and 80 % ethanol (1.5 liters). Mimosine was then eluted with 2N NH_4OH (1 liter) and the solution was evaporated at 40°C using a rotary evaporator until a sticky residue was obtained. Mimosine was precipitated after adjusting the pH 4.5-5.0 using 6N HCl and allowing this solution to cool in a refrigerator. Mimosine was recrystallized 5 times from ammonia solution and HCl. About 5g of relatively pure mimosine was obtained by this technique.

REFERENCES

1. Hegarty, M.P., and R.D. Court, Aust. J. Agric. Res., 15 : 165-166, 1964
2. Hongo, F., Y. Kawashima, S. Shiroma, and T. Fukazawa, Jpn. J. Zootech. Sci., 54 : 217-223, 1983
3. Lowry, J.B., T.S. Gouw, and B. Tangendjaja, Leucaena Research Reports, 5 : 50, 1984

QUANTITATIVE ANALYSIS OF MIMOSINE

INTRODUCTION

Since mimosine suppresses the growth of ruminants as well as nonruminants, Ipil-Ipil is limited in use as a feed. It is reported that 3-hydroxy-4(1H)-pyridone (DHP), which is identified in plants and animals as one of the metabolites of mimosine, causes also a disease in livestock. It therefore seems to be the indispensable to analyze simultaneously both mimosine and DHP in any given sample for study of the utilization of Ipil-Ipil as fodder. At present, anlysis methods of mimosine and DHP involving paper chromatography[1], gas chromatography[2], amino acid analyzer[3], and colorimetric analysis[4,5] have been reported. However, these methods need either complicated handling or mimosine and DHP have to be analyzed individually. An excellent simultaneous analysis of mimosine and DHP in the extracts of plants and animals is high performance liquid chromatography (HPLC) which is reported by Tangendjaja et al[6]. and it seems to be relatively easy for operation and convenience. They used Water's HPLC (model ALC/GPC 244) equipped with μBondapak C_{18} column for analysis of mimosine and DHP and they detected at 280 nm with good results. Their employed solvent system was 0.2% (w/v) orthophosphoric acid, and levels of 1 ng mimosine and 2 ng DHP were analyzed with a flow rate of 1 ml/min. Since they observed at 280 nm for the detection, nothing was detected except mimosine and DHP. Practically, many kinds of compounds are contained in the extracts of plants and animals, therefore, it is desirable to analyze simultaneously whole compounds which have UV absorption together with mimosine and DHP. Our new method is also a HPLC technique, but the method is different from their metiod, so we describe below an example of analysis and results obtained.

METHODS AND RESULTS

50 ml of 0.1 NHCl was added to 2g of freeze-dried Ipil-Ipil leaves which were made into impalpable powder by Wiley's mill, and the mixture was then homogenized for 10 min by polytron with ice cooling. The obtained mixture of Ipil-Ipil was centrifuged at 12.000 rpm by Hitachi 20 PR, and the supernatant was filtered by sucking through Toyo filterpaper No. 52. The filtrate was brought to a volume of 100 ml by the addition of 0.1 NHCl, and then the solution was passed through the diskpaper for HPLC. 2 μl of the solution was injected to HPLC (Shimadzu LC-6A) for analysis. The column used was Shim-pack CLC-ODS (15 cm x 6 mm i.d.), and the column temperature was 50℃. Mobile phase employed was the mixture solution of 10 mM potassium-di-hydrogen phosphate 10 mM phosphoric acid : acetonitrile (45 : 45 : 10), and finally 0.1% sodium 1-octanesulfonate was added to the mixture as the surface active agent. They were detected at 250 nm with flow rate 1.5 ml/min.

Fig Ia-1 shows an example of analysis using mimosine 7.2 μg and DHP 8.2 μg. Retention times were 2.847 and 5.917 min for mimosine and DHP, respectively. Fig Ia-2 shows the example of analysis of fresh leaves of Ipil-Ipil. Mimosine appears at 3.252 min in this case, and it showed that at least 19 kinds of unknown compounds were also existent in the extract of Ipil-Ipil. In this HPLC analysis, in the case of injection of Ipil-Ipil leaves, not only the changes of mimosine and DHP but also those of other unknown compounds could be observed. Analysis time was very quick, within 10 min, and the analysis values were also accurate.

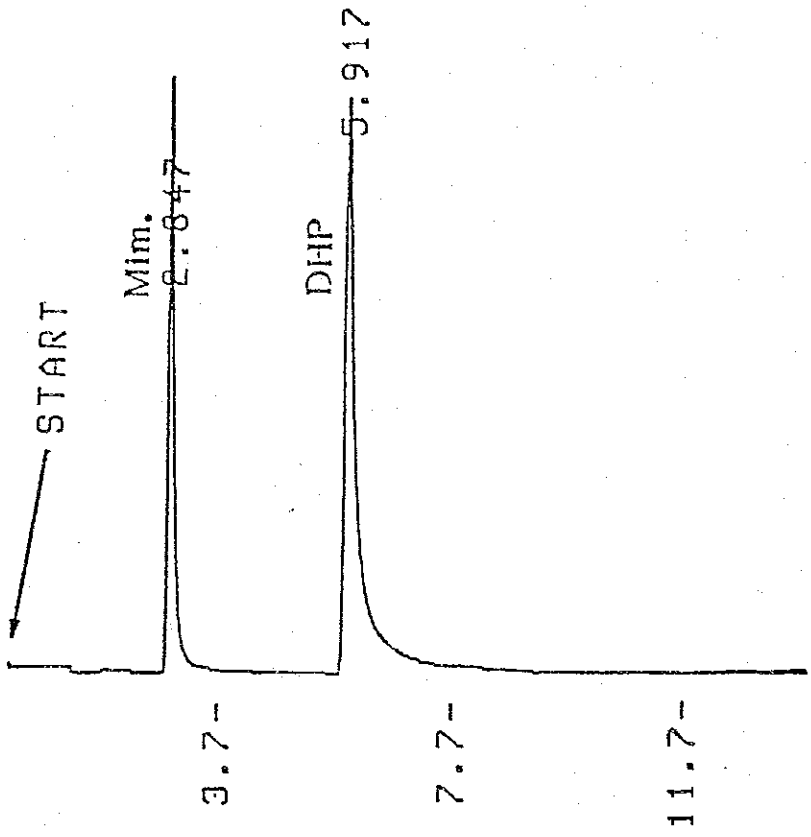
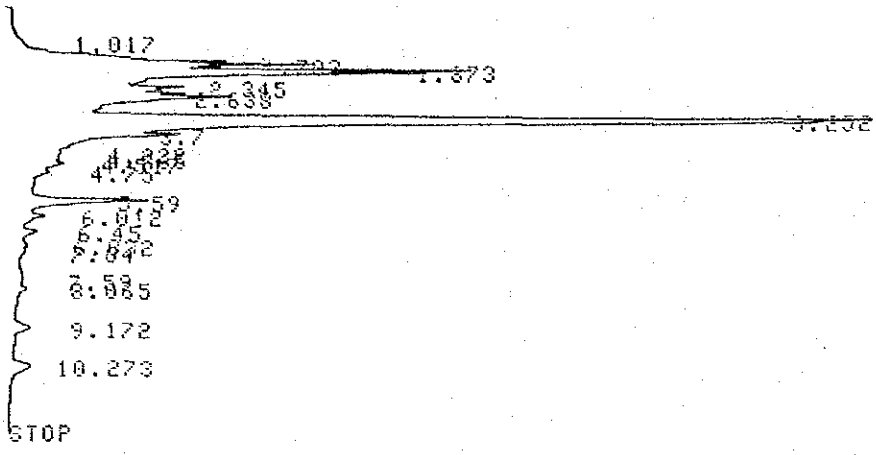


Fig. Ia-1 Typical chromatogram of mimosine and DHP. HPLC conditions: Column; shim-pack CLC-ODS(15 cm x 6 mm i.d.), Mobile phase; 10 mM Potassium di-hydrogen phosphate : 10 mM Phosphoric acid : Acetonitrile (45 : 45 : 10) with 0.1% Sodium 1-octanesulfonate pH=2.9, Detector; UV 250 nm, Flow rate; 1.5ml/min, Column temperature; 50°C; Injection size; 2 µl.

START
DATE 00/00/00 TIME 08:13:57



CHROMATOPAC C-R3A FILE 0
SAMPLE NO 0 METHOD 444
REPORT NO 82 SAMPLE WT 100

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	1.017	80				
2	1.703	8245	V			
3	1.873	13973	V			
4	2.345	3697	V			
5	2.638	8220	V			
6	3.252	28338	V	2	0.0356	MIN
7	3.7	4623	V	2		
8	4.232	479	V			
9	4.365	522	V			
10	4.517	689	V			
11	4.75	316	V			
12	5.59	2200				
13	6.012	281				
14	6.45	285		1		
15	6.832	116		1		
16	7.04	60	V	1		
17	7.59	197				
18	8.065	202	V			
19	9.172	431				
20	10.273	770				
	TOTAL	73723				

Fig. Ia-2 Typical chromatogram of Ipil-ipil extracts.

REFERENCES

1. Hegarty, M.P., R.D. Court, and P.M. Thorne, Aust. J. Agr. Res., 15 : 168-179, 1964
2. Mee, J.M.L., and C.C. Brooks, J. Chromatogr., 62 : 141-143, 1971
3. Mzik, J., J. Chromatogr., 144 : 146, 1977
4. Megarrity, R.G., J. Sci. Food Agr., 29 : 182-186, 1978
5. Matsumoto, H., and G.D. Sherman, Arch. Biochem. Biophys., 33 : 195-200, 1951
6. Tangendjaja, B., and R.B.H. Wills, J. Chromatogr., 202 : 313-318, 1980

I b. ANALYSIS OF MIMOSINE IN THE VARIOUS PARTS OF IPIL-IPIL

INTRODUCTION

Ipil-Ipil is distributed widely in the tropics and sub-tropics, and is highly valuable as a source of protein in the poor feed-producing regions. However, it is reported that Ipil-Ipil causes growth retardation, and hair loss in animals because of the presence of a toxic amino acid, mimosine. In order to find a strain or a part which contains smaller amounts of mimosine, the Okinawa native strain and the introduced strains K72a, K8, and K28 were utilized for the determination of mimosine content. General compositions and lignin contents of each strain were also determined.

MATERIALS AND METHODS

Material

Stems, leaves, seeds and other parts of a native strain of Ipil-Ipil about 3m high, which grows wild on the campus of University of the Ryukyus, were used for the analysis of mimosine. The strains K72a, K8, and K28 which were introduced from Hawaii and Taiwan in 1983, were also used.

Determination of mimosine content

The mimosine contents of all parts of Ipil-Ipil were determined by the method of HPLC which was described previously in Chapter Ia.

Analysis of lignin and general composition of Ipil-Ipil

Lignin was analyzed by the JIS method, and general composition was analyzed by the general feed analysis method as described in the textbook published by the Japan Feed Association[1].

RESULTS AND DISCUSSION

Mimosine contents in the various parts of Ipil-Ipil

Fig. Ib-1 shows the drawing of various parts of Ipil-Ipil. The examined parts are the

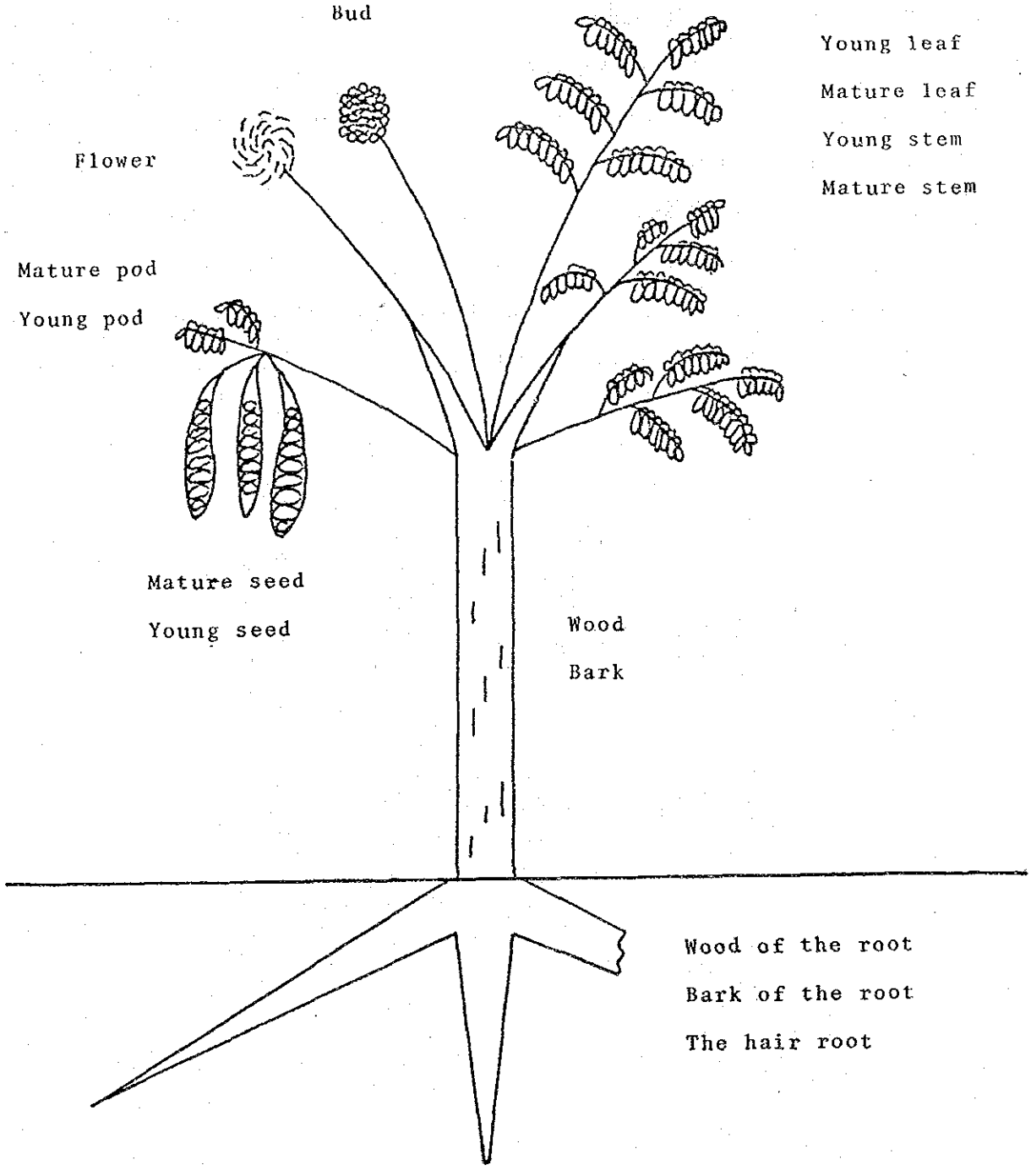


Fig. Ib-1 Analytical sites of the Ipil-ipil.

young leaf, young stem, mature leaf, mature stem, bud, flower, mature pod, young pod, mature seed, young seed, wood, bark, wood of the root, bark of the root, and the root hair. Thus the mimosine contents of all parts of the plant were analyzed. Fig. Ib-2 shows the analytical result of mimosine contents in four different leaves. The mimosine contents of native, K72a, K8, and K28 were 1.25, 1.3, 1.19, and 1.45%, respectively. K28 showed the highest and K8 the lowest among these four. Fig. Ib-3, understandable at a glance, shows the mimosine contents in the various parts of Ipil-Ipil as expressed in graph form. The results of mimosine content analysis in the various parts of each strain are shown in Table Ib-1. The average mimosine content in young leaves of the four strains was 2.66%, with K28 the highest and K8 the lowest. The average value in the mature leaves was 0.48%, with K28 the highest and K8 the lowest, as in the young leaves.

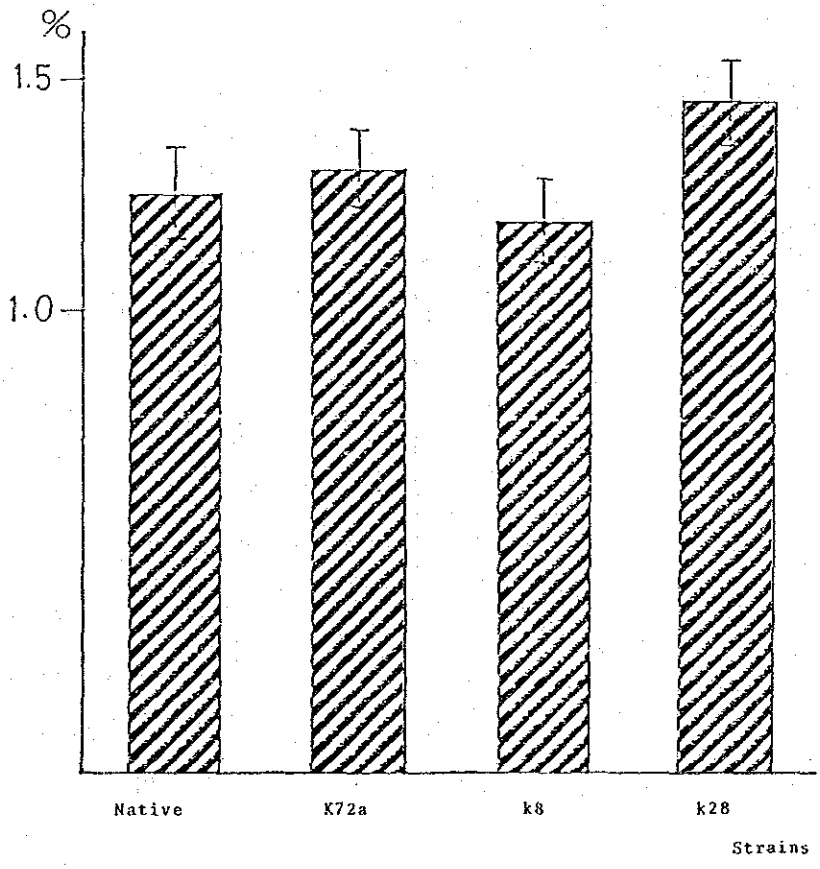
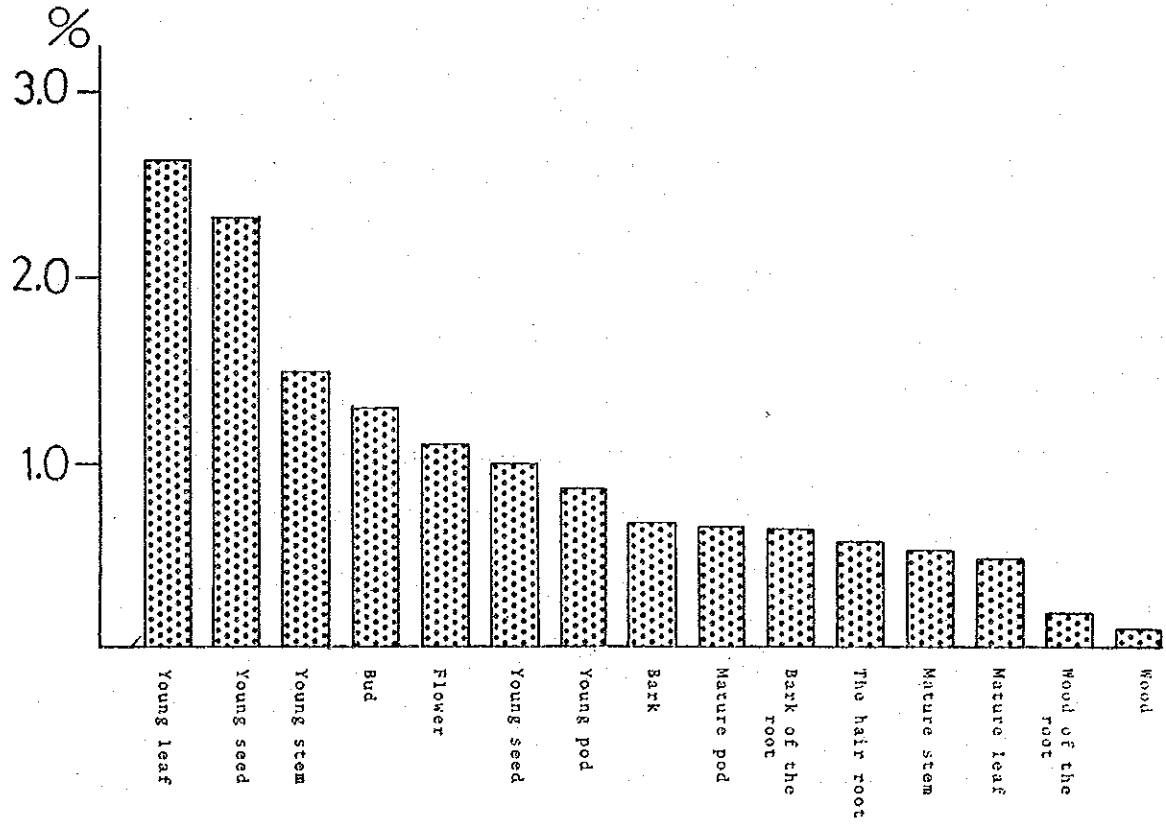


Fig. Ib-2 Mimosine content in the different strains.

The average of young stem was 1.50%, and of mature stems 0.54 %. Thus mimosine contents varied even in the same plant, therefore, there was no accurate average value of mimosine content in the plant, though it was found that the value seems to be relative to the variety of the plant. Krishnamurthy[2] and Hongo[3] also reported that the mimosine content in the various parts of Ipil-Ipil differed even in the same strain by the analytical conditions and the circumstances of growing the plant. They did not analyze the root and flower of the plant. Mimosine cootent of the bud was relatively higher than that of the flower, and as for the mimosine content in seeds, the mature seeds showed values twice as high as those of the young seeds. Mimosine content in the bark was higher than in the wood. Among the parts of the root, the bark and the hair root were high, and the woody parts of the root were similar to the wood. The average values of each K28 and K8 strain were 1.14 and 0.08%, respectively, which were in accordance with the results of analysis of leaves alone.

Fig. Ib-3 Mimosine content of the various parts of Ipil-ipil.



General composition of the parts of wood and bark in four strains of Ipil-Ipil

Table Ib-2 shows the general composition of the wood and the bark. The greater part of wood were the crude fibers and the water soluble carbohydrates. Protein and lipid were about 3% and less than 1%, respectively. The composition in the bark showed relatively high values of crude ash and protein in comparison to the wood. The difference between the strains was not significant except the protein content in the bark of K28 showed about 6% higher than that of K8.

Table Ib-1 Mimosine content of the various parts of Ipil-ipil.

	Native	K 72a	K 8	K 28	Average
Young leaf	2.71	2.53	2.54	2.88	2.66
Mature leaf	0.38	0.61	0.27	0.67	0.48
Young stem	1.41	1.48	1.56	1.54	1.50
Mature stem	0.48	0.58	0.39	0.72	0.54
Bud	1.43	1.35	1.05	1.52	1.34
Flower	1.30	1.97	1.03	1.39	1.17
Mature seed	2.53	3.19	1.83	2.25	2.37
Young seed	1.41	0.71	0.92	0.96	1.00
Mature pod	0.70	0.67	0.68	0.68	0.68
Young pod	0.90	0.89	0.74	1.02	0.89
Bark	0.43	0.96	0.27	1.11	0.69
Wood	0.10	0.11	0.09	0.10	0.10
Wood of the Root	0.13	0.09	0.36	0.71	0.18
Bark of the Root	0.88	0.25	0.76	0.73	0.66
The hair root	0.97	0.19	0.71	0.85	0.57
Average	1.05	1.04	0.88	1.14	

Table Ib-2 General composition of the wood and bark of Ipil-ipil.

	Crude Ash	Crude Fiber	Crude Lipid	Crude Protein	Water Soluble Carbohydrate
The Woody Parts					
Native	0.88	56.13	0.45	2.75	39.78
K 72a	1.48	56.16	0.44	2.75	39.16
K 8	1.10	55.83	0.29	2.25	40.62
K 28	1.47	56.00	0.54	4.21	37.78
Average	1.23	56.03	0.43	2.99	39.34
The Parts of Bark					
Native	6.60	21.77	1.85	10.77	59.02
K 72a	7.25	24.80	1.57	10.82	55.56
K 8	6.53	19.31	1.11	8.83	63.76
K 28	6.85	23.44	1.70	14.00	54.00
Average	6.81	22.33	1.56	11.11	58.09

Lignin content in wood

The lignin contents of wood were shown in Fig. Ib-4. The lignin content of wood in the native strain was the highest, and K8 was the lowest. The lignin content of bark in K72a showed the highest, and was lowest in K8. The average of the lignin content of the woody parts of the four strains was about 20%, and the rest consisted of cellulose and hemi-cellulose. If the lignin in the wood can be eliminated or degraded, the wood could be a significant resource for crude feed.

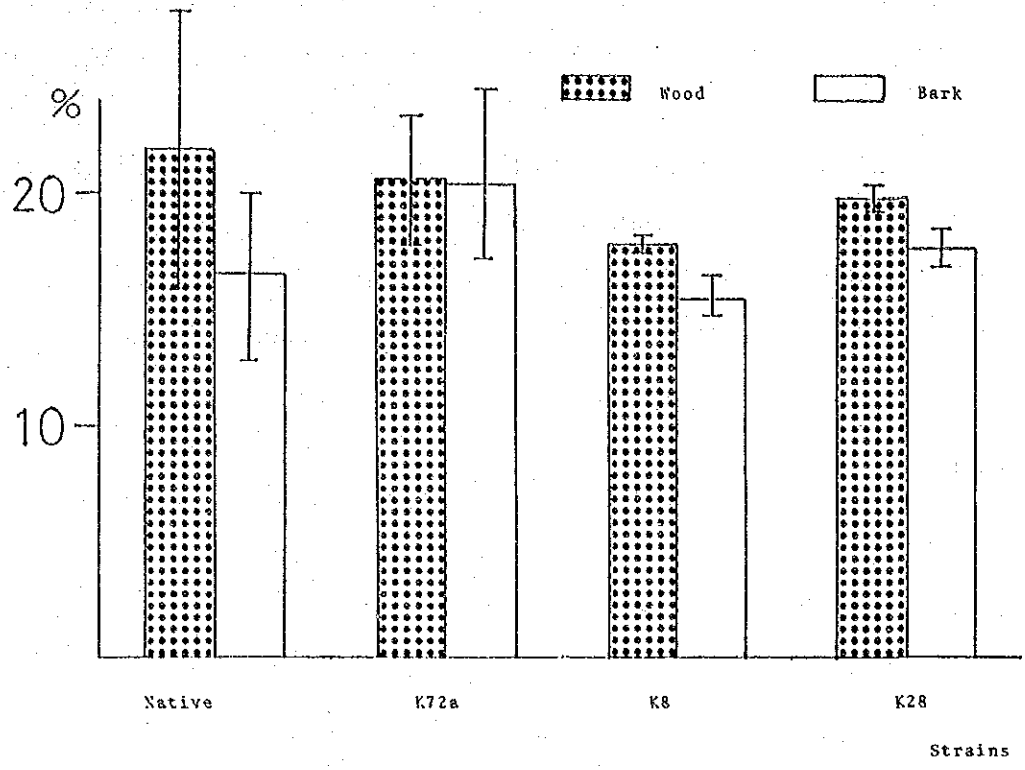


Fig. Ib-4 Lignin content in the various strains of Ipil-ipil.

REFERENCES

1. Shiryobunseki kijunkenkyukai hen, Shiryobunseki kijun cyukai, Nihonshiryokyo-kai-hakkou, p 6-16

2. Krishnamurshy, K., and M.K. Munegowda, Leucaena Research Reports, 4 : 27, 1983

3. Hongo, F., S. Tawata, Y. Watanabe, and S. Shiroma, Jpn. J. Zootech. Sci., 57 : 223-230, 1986

I c. Degradation of Mimosine by Enzymes, including Commercial Agents

INTRODUCTION

The animals fed Ipil-Ipil exhibit goitrogen[1]. Goitrogen occurs because of 3-hyroxy-4(1H)-pyridone (DHP)[2] which is produced by the degradation of mimosine through autolysis[3] in the plant or by the interaction of microorganisms[4] in the rumen. Investigation of Ipil-Ipil toxicity problem in a ruminant in the northern part of Australia proved[5] that there are no microorganisms in the rumen which have the ability to degrade mimosine to form DHP.

Recently, a ruminant was found that exhibited no disease resulting from Ipil-Ipil feeding in certain regions of Hawaii and Indonesia. This was due to the presence of rumen microorganisms which can degrade not only mimosine but also DHP. It is reported[6] that less than 1% of DHP was detected in the urine in one case, even after feeding a lot of Ipil-Ipil. In Okinawa prefecture, the rumen microorganisms in goat[7] and cattle[8] are clarified to degrade mimosine, for the reduction toxicity of Ipil-Ipil.

The effect of mimosine on the protease which is produced by the rumen microorganisms in Japanese black cattle was investigated, and furthermore, the effect on the degradation of mimosine by commercial enzymes was also investigated.

MATERIALS AND METHODS

1. Materials

Mimosine was obtained as described in Chapter Ia. The bovine rumen contents of Japanese black cattle were provided as needed by the Okinawa Livestock Processing Co. Ltd. The enzymes such as trypsin (bovine pancreases), α-chymotrypsin (bovine pancreas) and pepsin (porcine stomach) were purchased fron Sigma.

2. Treatment of bovine rumen content

The supernatant of bovine content was prepared by the metnod described by Kandatsu et al[10,11]. The rumen content was squeezed through two layers of gauze for separation of digestal solids, and the filtered rumen fluid was allowed to stand for 60 min at 38℃, before the upper solid phase was removed by skimming. The lower liquid phase was centrifuged at 1,000 rpm for 10 min to obtain the protozoa fraction which was washed with 0.5M phosphate buffer (pH 7.0). Again the supernatant was centrifuged, this time at 12,000 rpm for 20 min, and the precipitate (bacteria fraction) was removed. The resultant supernatant used was a protozoa-and bacteria-free fraction (PBFF). The proteolytic activities of each three fractions were measured.

3. Preparation of crude enzymes

To make acetone powder, a 20-fold volume of acetone was added to each of the fractions (protozoa, bacteria and PBFF) which were washed and dried under pressure. The obtained acetone powders were dissolved in 0.05N phosphate buffer (pH 7.0), and centrifuged at 15,000 rpm for 10 min and the precipitate was removed. The supernatant was dialyzed with phosphate beffer at 5℃ for 24 hrs, and subsequently used as a crude enzyme without further purification.

4. Preparation of substrate

To 1.0g of Hammerstene casein, 70 ml of water was added. To the solution, 5.0 ml of 0.1N HCl was added for acid protease, and 5.0 ml of 0.1N NaOH was added for alkaline protease. A buffer was prepared with citrate-HCl for pH 1~5, with citrate-NaOH for pH 5~6, and with Na_2PO_4-NaOH for pH 8~11 solutions. The prepared solutions were heated for 5 min at 60℃, and after being cooled down to room temperature, they brought up to a quantity of 50 ml with the addition of distilled water.

5. Assay of protease activity

The protease activity was measured as follows. Enzyme solution 1ml was added at

37℃ to 0.5 ml of 2.0% milk casein solution containing 0.05M lactate buffer at each optimum pH. After incubation for 10 min, the reaction was stopped by the addition of 2.5 ml of 10% perhydrochloric acid, followed by centrifugation at 15,000 rpm for 30 min. The optical density of the supernatant was measured at 280 nm.

6. Determination of protein content

Biuret reagent 4.0 ml was added to each 1 ml solution of bovine serum albumin (1 ~10 mg) ; the solutions were stood for 30 min and then the optical density was measured at 540 nm to provide a calibration curve. Biuret reagent 4.0 ml was also added to 1 ml of crude enzyme for the determination of the protein content from the calibration curve.

7. Effect of mimosine and metal ions on proteolytic enzyme

The proteolytic activity of the protozoa, bacteria and microorganisms-free fractions was measured and the effect of mimosine was determined. Mimosine was dissolved in buffer 0.1N citrate-NaOH (pH 5.0) and Toris (pH 7.2) to make a final concentration of 0~4 mM. The metal ions, $CaCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$ and $HgCl_2$ were also dissolved in the buffers to make 5.0 mM solutions. Each 1.0 ml of inhibitors were added to the crude enzymes for the determination of the inhibition rates.

8. Effect of mimosine on pepsin, trypsin and α-chymotrypsin

Pepsin was dissolved in 0.2N KCL-HCl buffer (pH 2.0), and trypsin and α-chymotrypsin were dissolved in 0.2N phosphate buffer (pH 7.0). Each enzyme 2.0 ml was added to 2 ml of phosphate buffer, to which were added 1.0 ml of casein (2.0% concentration in an acid and in a neutral buffer) and 1 ml of mimosine (concentrations of 0, 2.5, 5.0 and 10 mM). Each of the mixtures was incubated for 30 min at 38℃, the reaction was then stopped with the addition of 16% trichloroacetic acid, followed by centrifugation at 5,000 rpm for 30 min. The optical density of the supernatant was measured at 280 nm. The degradation rate of mimosine by trypsin, α-chymotrypsin,

pepsin, pancreatine and papain were examined under each of the optimum conditions. 2 ml of 0.25 mM mimosine was added to 2 ml of the buffer, and the mixture was incubated for 30 min at 24 hrs. The reduction rates of mimosine were determined by HPLC after the removal of protein by 16% tca addition.

RESULTS AND DISCUSSION

1. Effect of pH and temperature on proteolytic activity

Fig. Ic-1 shows the effect of pH on the proteolytic activity of the protozoa fraction from four different rumen sources. The proteolytic activity of each of the four different rumen sources was slightly different, as shown in Fig. Ic-1, though in all cases, it showed peaks at pH 3.4 and 7.1. Fig. Ic-2 shows the effect of temperature on the proteolytic activity of the protozoa fraction. The crude enzymes with optimum pH 3.4 and 7.1 had optimum temperatures at 45℃ and 70℃, respectively. All values of optimum pH,

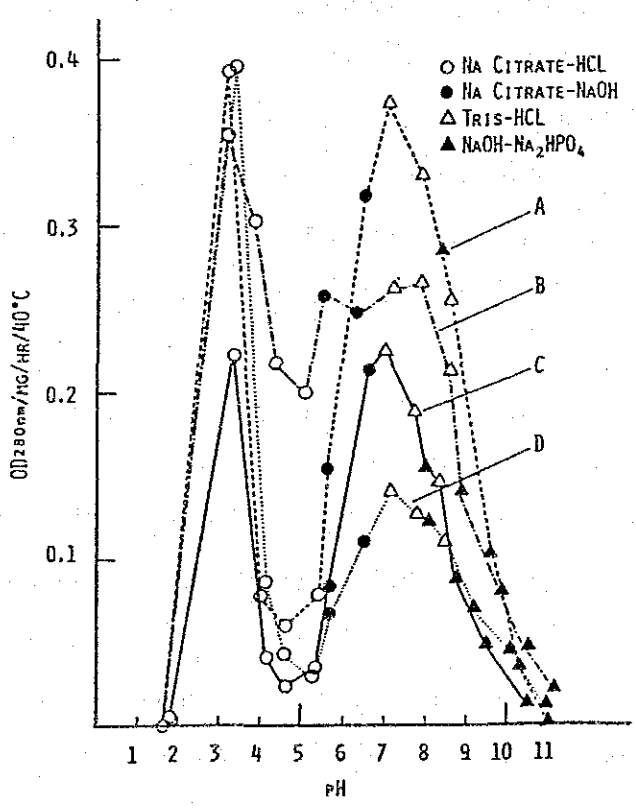


Fig. Ic-1 Effect of pH on proteolytic activity of PF from different rumen souces.

temperature and specific activity obtained from each of the protozoa, bacteria and PBFF fractions are shown in Table Ic-1. The proteolytic activity of each of the three fractions showed two peaks at acid area (pH 3.4~5.2) and neutral area (pH 7.0~7.8) at the corresponding optimum temperatures.

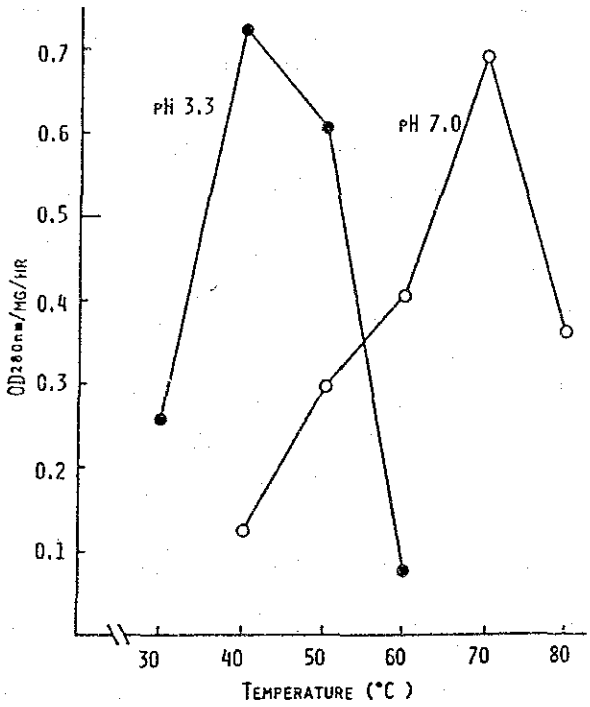


Fig. Ic-2 Effect of temperature on proteolytic activity of PF.

Table Ic-1 Optimum pH, temperature, specific activity.

	pH	temperature(°C)	specific activity(mg/hr)
Protozoa	3.4	45	0.32
	7.1	70	0.60
Bacteria	5.2	40	0.79
	7.0	50	0.90
Rumen liquid	5.2	50	0.78
	7.8	40	0.38

2. Effect of dialysis on proteolytic activity

Table Ic-2 shows the effect of dialysis on proteolytic activity of the protozoa, bacteria and PBFF fractions. The undialyzed crude enzymes had low activity, and the enzymes after being dialyzed had high activity, indicating significant purification by means of dialysis.

Table Ic-2 Effect of dialysis on proteolytic activity of PF, BF and PBFF.

	Reaction pH	Treatment	Exptl.	Blanks	Difference	Specific Activity
Protozoa	3.4	Dialyzed	1.02	0.33	0.69	0.46
		Undialyzed	0.31	0.31	0	-
	7.1	Dialyzed	0.56	0.32	0.24	0.16
		Undialyzed	0.29	0.34	0	-
Bacteria	5.4	Dialyzed	1.14	0.88	0.26	0.86
		Undialyzed	1.82	1.72	0.10	0.27
	7.0	Dialyzed	0.91	0.85	0.06	0.20
		Undialyzed	1.75	1.72	0.03	0.09
Microorganism-free fraction	5.6	Dialyzed	1.15	0.79	0.36	0.36
		Undialyzed	1.48	1.30	0.18	0.13
	8.0	Dialyzed	0.91	0.77	0.14	0.14
		Undialyzed	1.40	1.30	0.10	0.07

3. Effect of mimosine and metal ions on proteolytic enzymes

To elucidate the function of mimosine in digestive organs in ruminats, the effect of mimosine on the digestive proteolytic enzymes of Japanese black cattle rumen content was examined. Table Ic-3 shows the inhibition effect of the addition of a final concentration of 4.0 mM mimosine and 5.0 mM of various metal ions to acid proteases and neutral proteases. Mimosine inhibited acid proteolytic activity by 0~20%, but the neutral proteases were not inhibited by mimosine. The proteolytic activity was also inhibited by 5.0 mM of Hg^{++}, and slightly inhibited by Zn^{++}, but it was not inhibited

Table Ic-3 Effect of various metal ions and mimosine on the activity of proteolytic enzymes.

	Relative activity (percentage)						
	No addition	$CaCl_2$	$ZnCl_2$	$MnCl_2$	$MgCl_2$	$HgCl_2$	Mimosine
Protozoa fraction							
pH 3.3	100	94	92	97	95	0	20
pH 7.1	100	95	98	71	93	0	70
Bacteria fraction							
pH 5.2	100	95	95	96	95	80	0
pH 7.1	100	97	20	75	98	0	100
Microorganism-free fraction							
pH 5.2	100	98	80	67	94	0	0
pH 7.1	100	98	19	77	93	0	100

Concentrations: 5 mM for each metal ion addition; 4 mM for mimosine.

by Ca^{++} and Mg^{++}.

Although the crude inhibitors of trypsin and α-chymotrypsin were found by Fukuda et al[11]. Ipil-Ipil the effect of mimosine was not examined. Since mimosine was found to inhibit neutral protfases in the rumen of Japanese black cattle, the effect of mimosine on proteolytic enzymes such as pepsin, trypsin and α-chymotrypsin was examined. Commercially available enzymes were used with 2% Hammerstene casein substrate. Mimosine (concentrations of 0~5.0 mM) was incubated with the corresponding enzymes at 38℃ for 30 min for determination of inhibition rates. The inhibition effect of 5.0 mM mimosine was 22% on α-chymotrypsin. However, pepsin and trypsin were not affected. Table Ic-4 shows the results of mimosine degradation rate by the commercial proteinases. The commercial enzymes such as trypsin, α-chymotrypsin, pepsin, pancreatine and papain did not decompose mimosine, therefore, it was found to be difficult for the utilization of commercial enzymes to degrade mimosine.

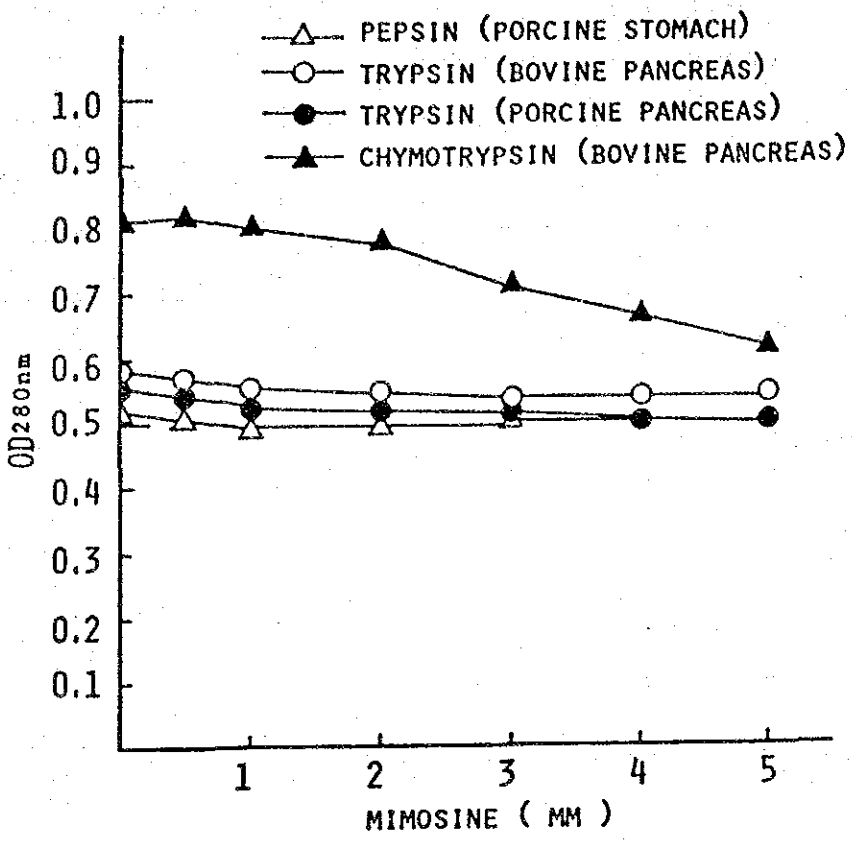


Fig. Ic-3 Effect of mimosine on proteolytic activity of pepsin, trypsin and chymotrypsin.

Table Ic-4 Degradation of mimosine by commercial enzymes.

	Retention time	Area	Degradation rate (%)
Trypsin	2.97	2279	103
α-Chymotrypsin	2.98	2304	105
Pepsin	2.97	2268	103
Pancreatin	2.98	2272	103
Papain	2.98	2294	104
Control	2.98	2209	100

It is desirable to use the endogenous enzyme in Ipil-Ipil for the degradation of mimosine.

REFERENCES

1) Jones, R.J.. Wild Anim. Rev., **31**: 13-23, 1979.

2) Lawry, B.J., Maryanto, and B. Tangendjaja, J. Sci. Food Agric., **34**: 529-533, 1983.

3) Hegarty, M.P., P.G. Schimckel, and R.D. Court, Aust. J. Agric. Res., **15**: 153-167, 1964.

4) Hrgarty, M.P., C.P. Lee, G.S. Christie, R.D. Court, and K.P. Haydock, Aust. J. Biol. Sci., **32**: 27-40, 1979

5) Jones, R.J., Aust. Vet. J., **57**: 55-56, 1981.

6) Lowry, J.B., Leucaena Res. Reports, **2**: 31, 1981.

7) Shiroma, S. and A. Akashi, Jpn. J. Zootech. Sci., **47**: 739-747, 1976.

8) Hongo, F. and S. Shiroma, Sci. Bull. Coll. Agr. Univ. Ryukyus, **26**: 379-387, 1979.

9) Hongo, F., Y. Kawashima, S. Shiroma and T. Fukazawa, Jpn. J. Zootech. Sci., **54**: 217-223, 1983.

10) Kandatsu, M. and N. Takahashi, Nippon Nogei Kagaku Kaishi, **30**: 96-99, 1956.

11) Shinchi, S. and M. Kandatsu, Jpn. J. Zootech. Sci., **54**: 290-296, 1983.

12) Fukuda, N., T. Koja, I. Chinen, F. Hongo, S. Shiroma and H. Yomo, Nippon Nogei Kagaku Kaishi, **54**: 1015-1019, 1980.

I d A BACIC STUDY ON THE EFFECT OF THE ADDITION OF METAL SALTS TO IPIL-IPIL FEED

INTRODUCTION

Yoshida and Matsumoto[1] first reported that the toxicity of mimosine on animals decreased in the case of ferrous sulfate addition to Ipil-Ipil feed. Acamovic et al[2]. also reported the effect of iron and alminum, and they reported that Fe (III) decreased the toxicity of mimosine more than Al (III) and Fe (II)[3]. It is well kown that the addition of iron salts to Ipil-Ipil feed increases the mimosine content in the excrements rather than in the urine in animals. Though not yet confirmed, this seems to be due to the inhibition of mimosine absorption by the formation of chelating bond with iron atom within the small intestine, which is one of the digestive organs in animals. Therefore, in this experiment, the effect of the various kinds of acids and bases on the mimosine permeability in the dialysis membrane and the absorption in the small intestine were investigated. The chemical stucture of the complex formed by mimosine and the iron atom was assumed by determination of the maximum complex formation rate in the mixture ratio between mimosine and iron atoms.

MATERIALS AND METHODS

Materials

The dialysis membrane used was cellulose membrane 20/32 which i6 produced by Sanko Junyaku Co. Ltd. The small intestine of swine was offered by Okinawa Prefecture Meet Center.

Membrane permeability of mimosine

Fig Id-1 shows the method on mimosine permeability of dialysis membrane. Thus, 5 mM mimosine was packed in dialysis membrane with equivalent of acids (HCl, H_2SO_4,

HCOOH, CH_3COOH, CCl_3COOH, $CH_3CH(OH)COOH$) and bases (NaOH, KOH, KCl, $CaCl_2$, NaCl, CH_3COONa, $MgCl_2$, $ZnCl_2$, $MnCl_2$, $FeCl_2$, $FeCl_3$, $FeSO_4$), and each mixture was dialyzed for three hours at room temperature with stirring and using the distilled water as the solution outside of the membrane. After the dialysis, the mimosine content of the outside solution and of the inside solution were determined consecutively by HPLC at 0.5, 1, 2, and 3 hrs. Next, only 5 mM mimosine solution was subsequently packed in the dialysis membrane, and each equivalent molar of acids and bases was used as the solution outside of the membrane. The dialysis was continued for 3 hrs at room temperature, and the changes of mimosine were examined as described above.

Permeability of mimosine in the small intestine of swine

10 mM mimosine solution was packed in the small intestine of swine with each of the equivalent molar of acids and bases. Distilled water was used as outside solution with stirring at room temperature, and the changes of mimosine content were determined regularly by HPLC for 3 hrs.

Maximum complex formation rates

Each different volume of 1 mM solution of $FeCl_2$, $FeCl_3$, and $FeSO_4$ was mixed with 1mM solution of mimosine to make the molar ratio 0-1.0 mixture solutions. After being fully mixed, they were left standing for 15 min. Optical denxities of the mixture solutions were determined by spectrophotometer (Hitachi 2010 type) at 535 nm, and the maximum complex formation rate was decided by Fig Id-9.

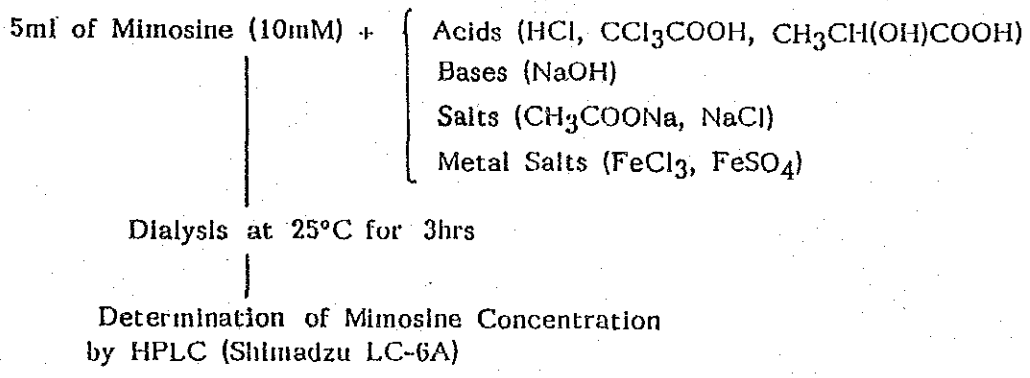


Fig. Id-1 Permeability of mimosine in the dialysis membrane.

RESULTS

Effect of acids and bases on permeability of mimosine in the dialysis

When mimosine was packed with equivalent molar of bases, the permeability was inhibited remarkably. Effect of NaOH and KOH are shown in Fig Id-2. Control

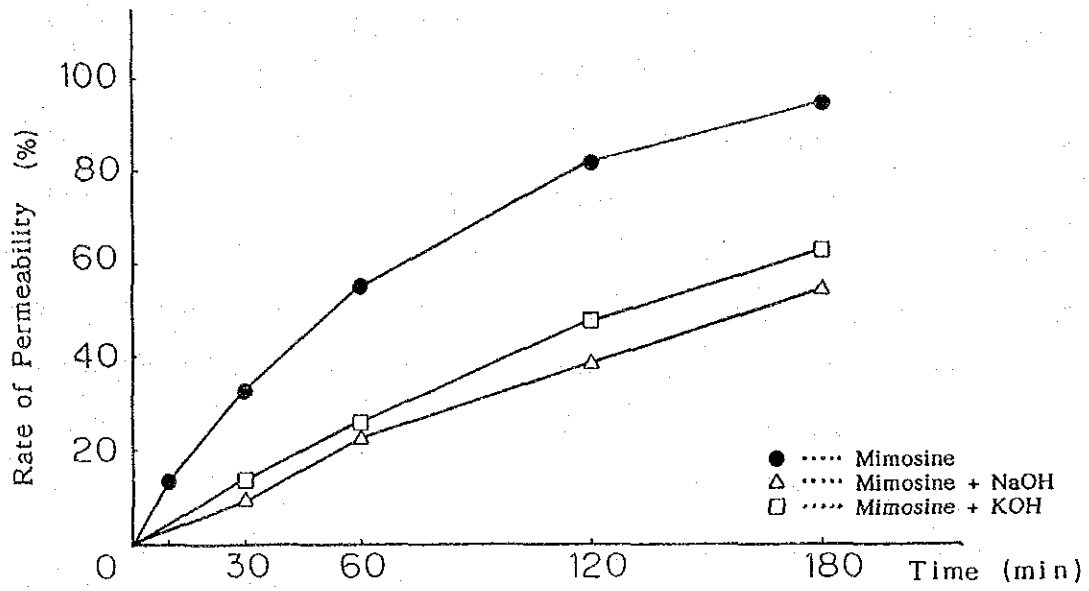


Fig. Id-2 Effect of bases on permeability of mimosine in the dialysis membrane.

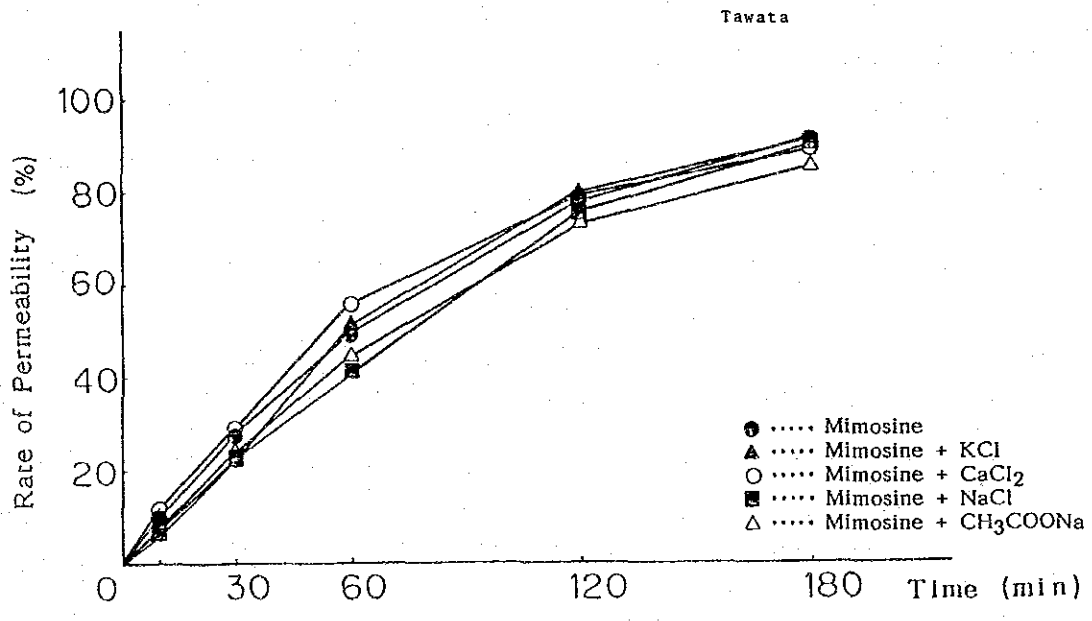


Fig. Id-3 Effect of salts on permeability of mimosine in the dialysis membrane.

represents the permeable rate of mimosine alone in the dialysis membrane. The neutral bases such as KCl, $CaCl_2$, NaCl, and CH_3COONa did not affect the permeability of mimosine, as shown in Fig Id-3. The effect of metal salts on the mimosine permeability were shown in Fig Id-4. Although $MgCl_2$, $ZnCl_2$, and $MnCl_2$ did not affect the permeability, iron salts such as $FeCl_2$, $FeCl_3$, and $FeSO_4$ showed remarkable inhibition.

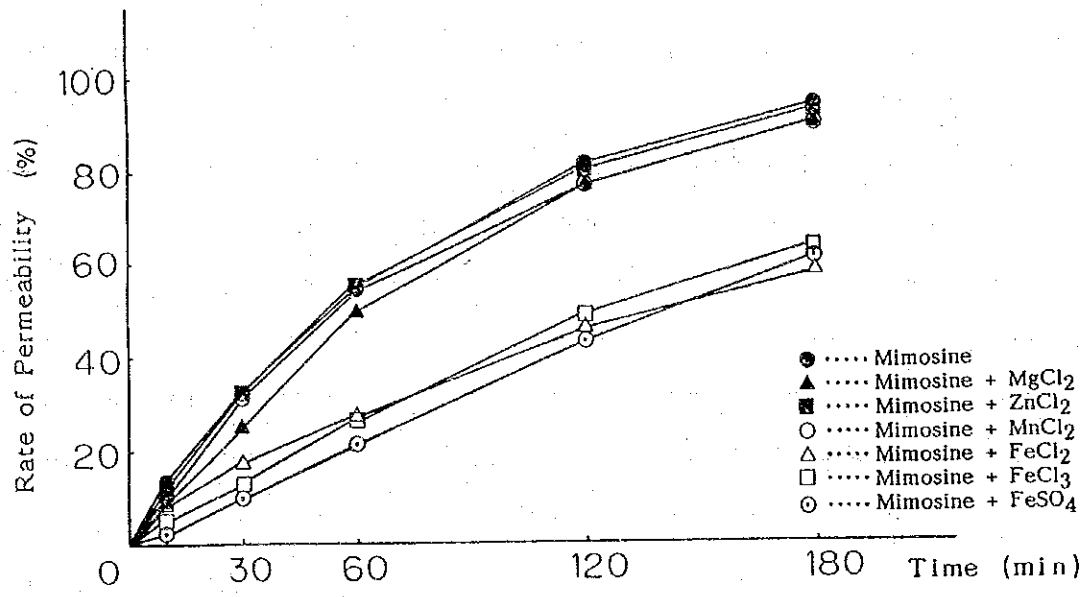


Fig. Id-4 Effect of metal salts on permeability of mimosine in the dialysis membrane.

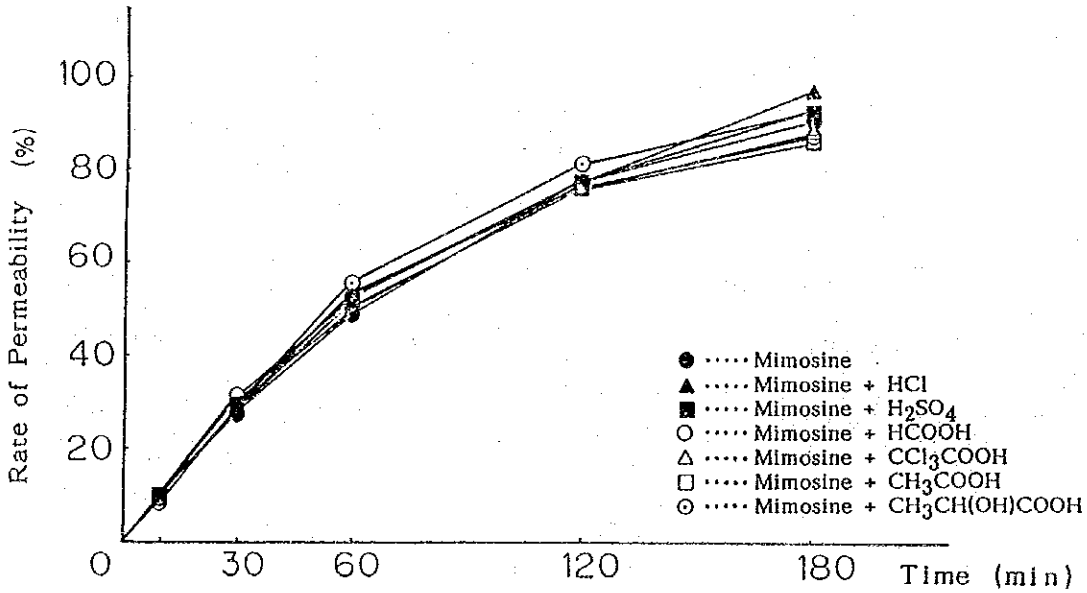


Fig. Id-5 Effect of acids on permeability of mimosine in the dialysis membrane.

The effect of acids is shown in Fig Id-5. HCl and $CH_3CH(OH)COOH$ increased a little the permeability of mimosine. In this experiment, it was found that alkali and iron salts inhibited 30-40% mimosine permeability, but neutral salts and acids did not affect. Mext, 5 mM of mimosine alone was packed in the dialysis membrane, and each of equivalent molar of the various salts as the outside solution was used for the investigation of mimosine permeability. The result is shown in Fig Id-6. When NaCl and CH_3COONa were used as the outside solution, they did not affect the mimosine permeability. $FeCl_3$ and $FeSO_4$ in the outside of the membrane inhibited the mimosine permeability as strong as they were in the inside solution. It was found that NaOH in the outside solution inhibited 20% more than did NaOH in the inside solution. HCl and CCl_3COOH in the outside solution promoted somewhat the mimosine permeability, as shown in Fig Id-7.

Formation of the complex

Ultra violet absorption spectrum of 0.025 mM mimosine is shown in the left side in Fig Id-8. The visible absorption spectrum of the complex, which was formed by mixing for each 1 mM of $FeCl_3$ and mimosine in the ratio of 2 : 8, is shown in the right side

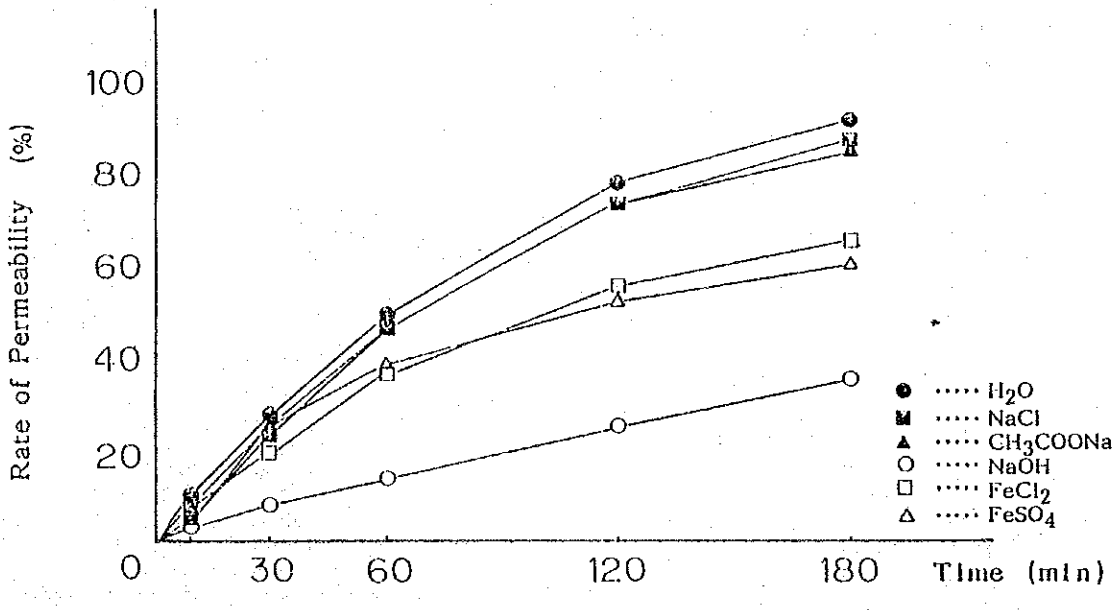


Fig. Id-6 Effect of salts in the outside solutions of the membrane on changes of mimosine permeability rate.

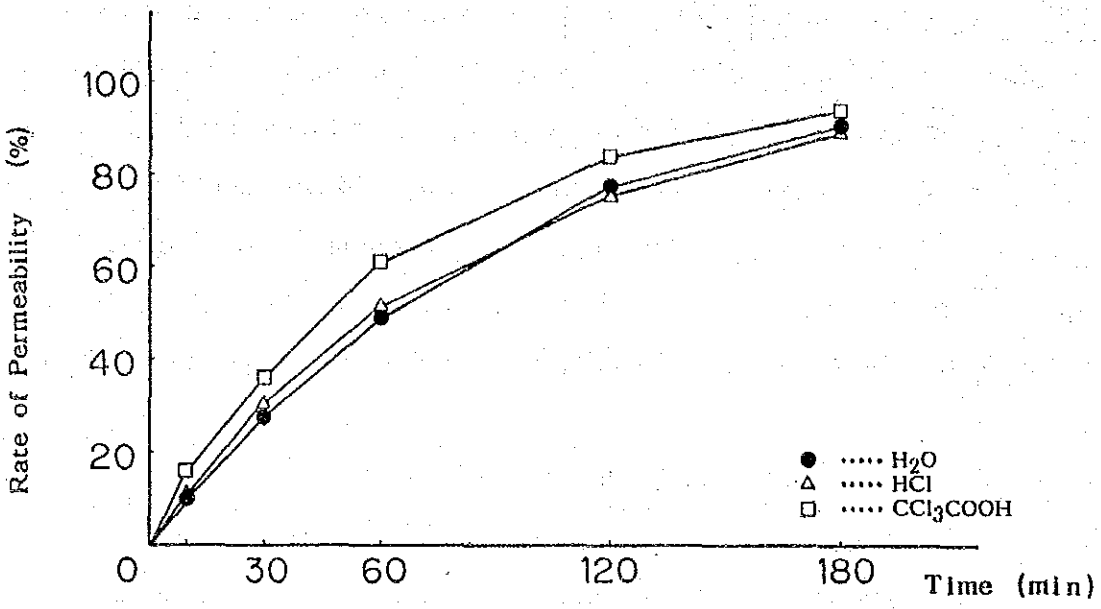


Fig. Id-7 Effect of acids in the outside solutions of the membrane on changes of mimosine permeability rate.

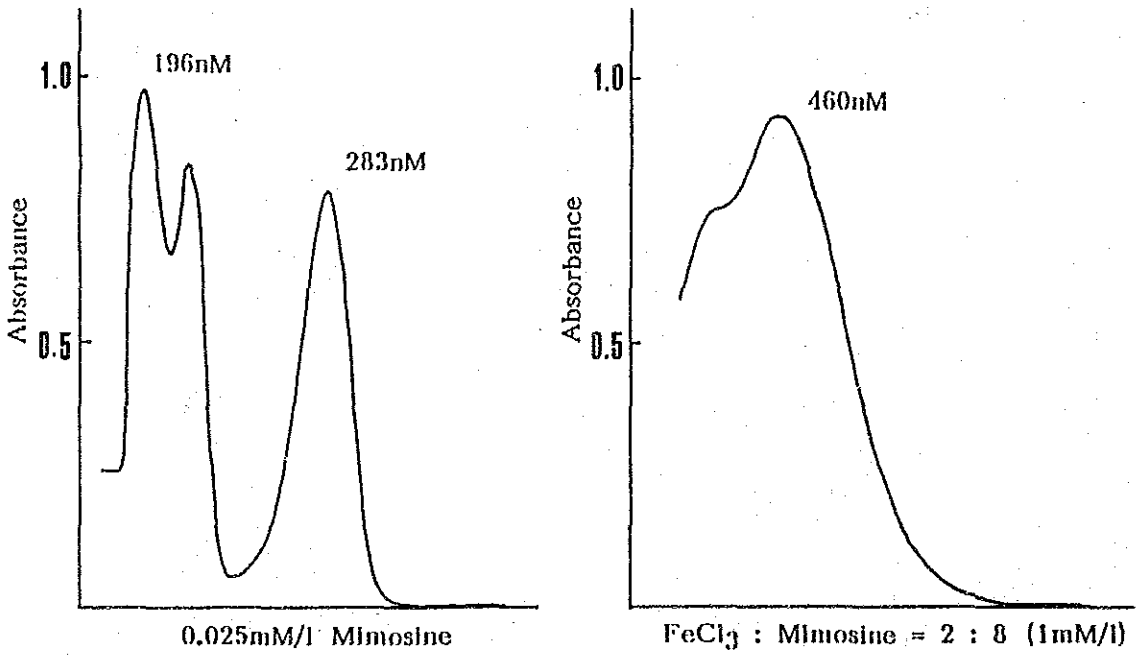


Fig. Id-8 Ultraviolet and visible rays absorption of mimosine.

in Fig Id-8. As mimosine forms the complex with the iron atom, it changes to have the maximum absorption at 460 nm, which means the mimosine molecule has the ability of forming chelating bond. By using Job's continuation variation method, the maximum formation rate of the complex in the ratio of mimosine and iron salts was determined as shown in Fig Id-9. It was found that the maximum complex formation rate between mimosine and $FeSO_4$ was the ratio of 6 : 4, and was the ratio of 3 : 7 in the case of $FeCl_3$. Fig Id-10 shows the presumed chemical structure of the complex which was formed by the mixing of mimosine and $FeSO_4$.

Mimosine permeability in the small intestine of swine

With mimosine and equivalent molar of acids and bases were packed in thd small intestine of swine, and the changes of mimosine content were determined on regularly. The results are shown in Figs Id-11 and Id-12. NaOH had no effect at all on the permeability in the small intestine, and that was absolutely different in the case of the dialysis membrand. Although the neutral salts as NaCl and CH_3COONa had no effect

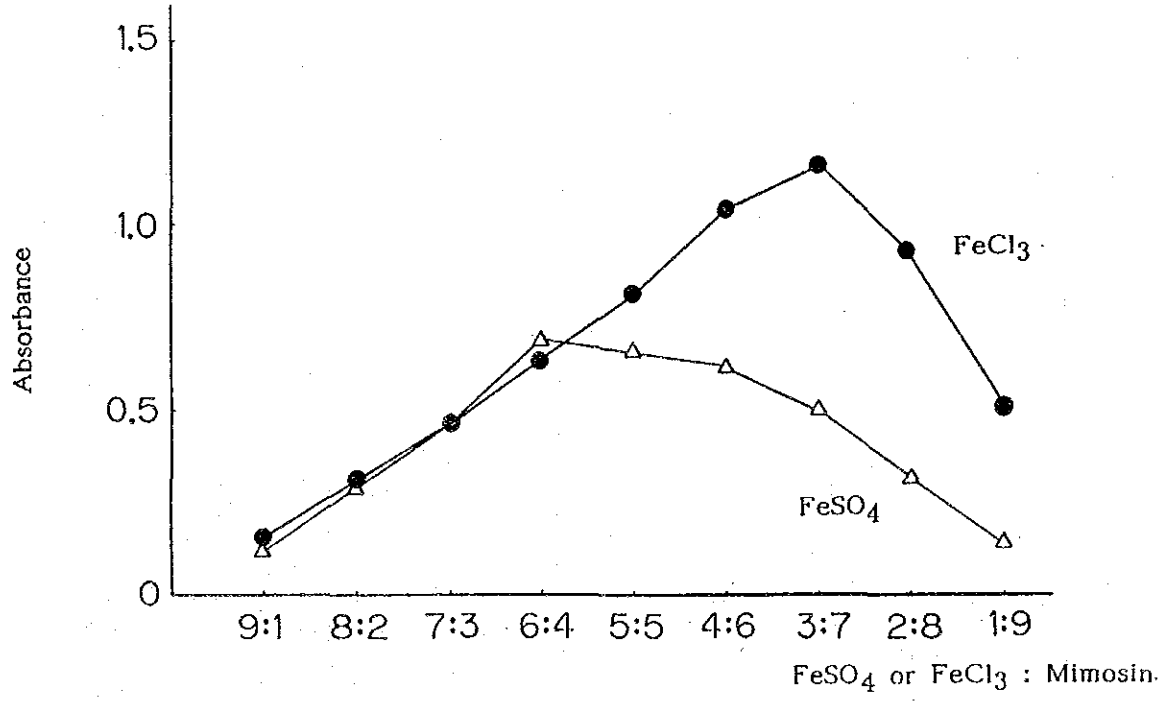
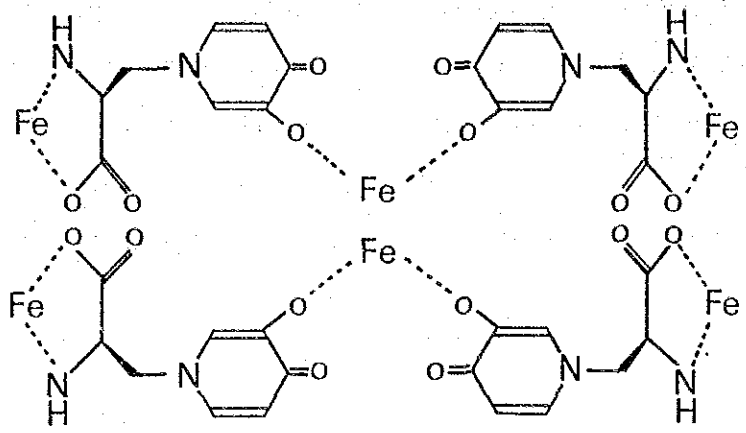


Fig. Id-9 Maximum formation rates of the complex between mimosine and metal ions.



Mimosine : $FeSO_4$ (4:6)

Fig. Id-10 Presumed chemical structure of the complex between mimosine and ferrous sulfate.

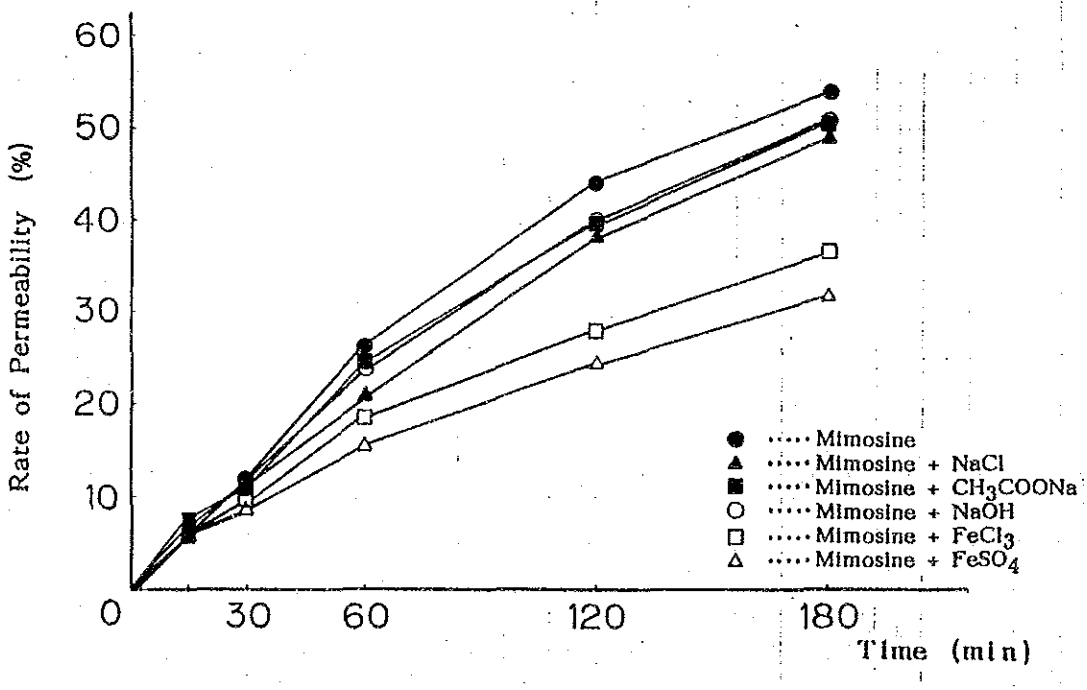


Fig. Id-11 Effect of salts on permeability of mimosine in the small intestine of swine.

on the permeability, $FeCl_3$ and $FeSO_4$ inhibited the mimosine permeability as well as the dialysis membrane. Furthermore, acids such as HCl and CCl_3COOH had the tendency of promoting the mimosine permeability for the first half hour of permeation. Although $CH_3CH(OH)COOH$ did not show any effect on the dialysis membrane, it showed remarkable inhibition on the small intestine, as can be seen in Fig Id-12.

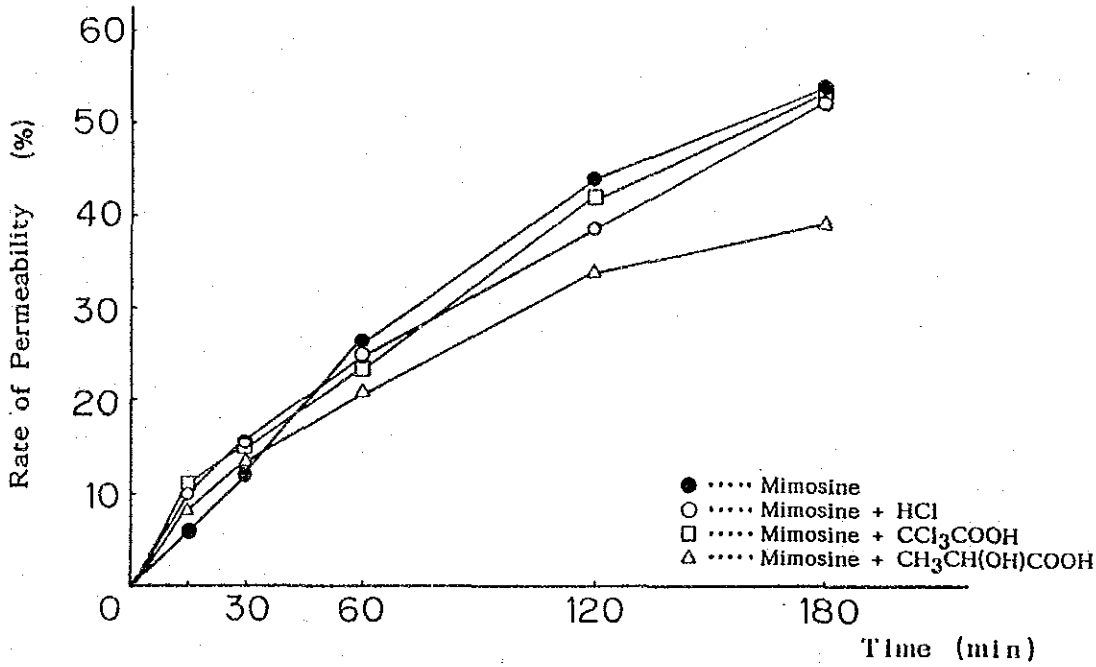


Fig. Id-12 Effect of acids on permeability of mimosine in the small intestine of swine.

DISCUSSION

As a result of this experiment, it was found that mimosine permeability is affected conspicuously by the presence of a reagent. Previously, only compounds such as those containing the iron atom, which has the ability of forming chelating bond with the mimosine molecule have been utilized as a reagent to reduce the toxicity of Ipil-Ipil, but it is suggested here that there is some future possibility for the discovery even of a suitable organic compound for this purpose. On the other hand, there is a compound which promotes mimosine permeability. These are, for example, HCl and

$CH_3CH(OH)COOH$ in the dialysis membrane, and CCl_3COOH in the small intestine of swine. It seems, therefore, to be of interest to conduct animal experimentation using these reagents. Furthermore, since this simple method can provide results very easily in a short time, the method seems to be applicable to finding a compound more satisfactory than $FeSO_4$ and other such compounds.

REFERENCES

1. Matsumoto, H., E.G. Smith, G.D. Sherman, Arch. Biochem. Biophys., 33 : 201-211, 1951
2. Acamovic, T., and J.P.F. D'Mello, Leucaena Newsletter, 1 : 38, 1980
3. Acamovic, T., and J.P.F. D'Mello, Leucaena Research Reports, 2 : 60-61, 1981

I e EFFECT OF THE MIMOSINE AUTOLYTIC ENZYME FOR THE TOXICITY REDUCTION OF IPIL-IPIL

INTRODUCTION

It is known that mimosine is decomposed by rumen microorganisms[1] and heating[2]. Hongo et al.[3] also reported that mimosine was decomposed by the ensiling of Ipil-Ipil or by treatment with various organic acids. Recently, Lowry et al.[4] reported that mimosine was converted to DHP by the action of an enzyme present in the finely cut leaves of Ipil-Ipil. Tangendjaja et al.[5] reported that when the intact fresh leaves of Ipil-Ipil were heated at 70℃ for 10 min, about 80 % of the mimosine was decomposed. This also seems to be the effect of the enzymes. Although DHP is reported to cause goitrogen in livestock[6], it is lower in toxicity than mimosine; therefore, it seems to be more practical to convert mimosine to DHP for the use of Ipil-Ipil as feed for livestock. In this experiment, the possibility of the preparation of a safer feed from Ipil-Ipil was investigated by the utilization of enzymes in Ipil-Ipil for the degradation of mimosine to DHP.

MATERIALS AND METHODS

Reduction of mimosine in Ipil-Ipil by the method of pressure

The changes of mimosine in the finely cut Ipil-Ipil were examined by the condition of buried in refrigerator at 10℃ or at room temperature. The leaves of Ipil-Ipil were finely cut at 3-5 mm length, they were hermetically sealed in a polyethylene bag which was then encased in a cylindrical container under the pressure of a weight. After containments of 0, 1, 2, 3, 5, 7, 14, and 21 days, 5g of each of the contents was removed to which was added 50 ml of 0.1N HCl, and then homogenized after cooling for 5 min. The mixture was centrifuged at 16.000 rpm for 15 min, the supernatant fluid was brought

to a constant volume at 100 ml with 0.1N HCl, then 2 μl of the solution was injected to HPLC for analysis of mimosine and DHP. The method is shown in Fig Ie-1.

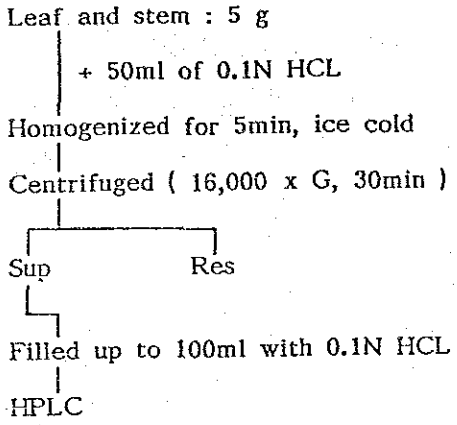


Fig. Ie-1 Analysis method of mimosine and DHP in Ipil-ipil.

Subsequently, the weight of the stone was controlled at 0, 10, 20, 30, 40, and 50 g/cm to determine the effect of pressure on the changes of mimosine and DHP. Furthermore, the cut leaves of Ipil-Ipil were separated by length into three classes, long (more than 5 mm), medium (3-5 mm) and short (less than 3 mm), to determine the effect of cut on the results of the pressure method.

Determination of the mimosine degradation enzyme in Ipil-Ipil

80 ml of phosphate buffer (pH 7.0) was added to 40g og Ipil-Ipil leaves, cooled, and it homogenized for 10 min by polytron, and then centrifuged at 16.000 rpm. The obtained supernatant was used as thd crude enzyme. Each 4 ml of this solution was taken in test tubes which were then heated at 60℃. After removal of protein by the addition of 10 % TCA, the mixture was centrifuged, and then the mimosine and DHP contents were measured. The method is shown in Fig Ie-2.

Effect of the crude enzyme on Ipil-Ipil leaves

400 ml of water was added to 200g of Ipil-Ipil leaves for the preparation of water -extracted crude enzyme. Finely cut Ipil-Ipil leaves 1g was leached in 50 ml of this solution and heated at 40℃. The contents of mimosine and DHP in the supernatant of this solution were measured regularly. The method is shown in Fig Ie-3.

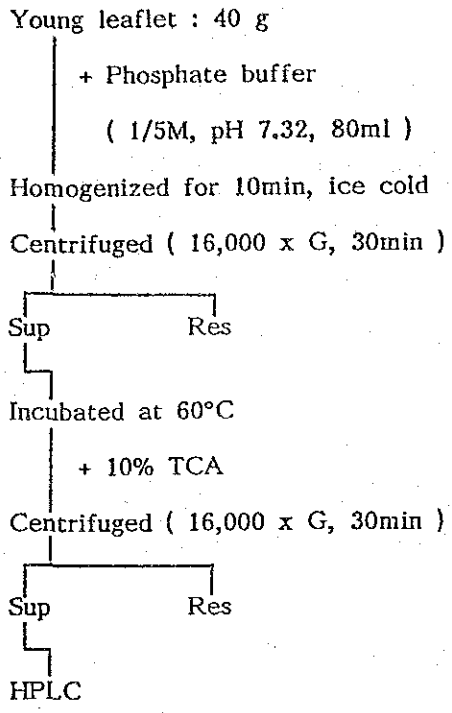


Fig. Ie-2 Measurement of activity of the mimosine degradation enzyme in Ipil-ipil

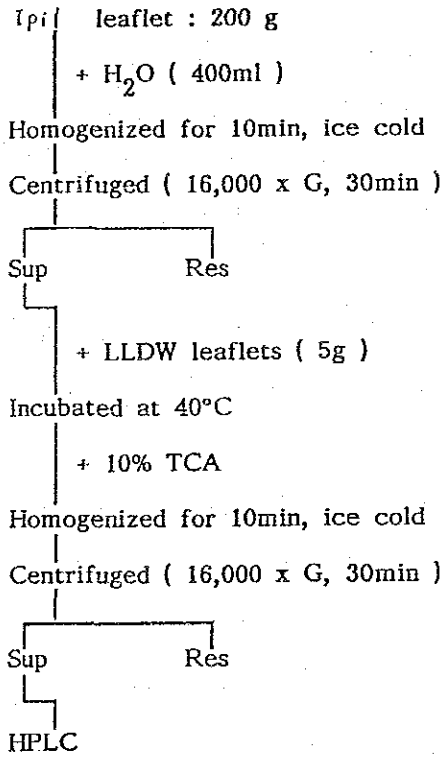


Fig. Ie-3 Extraction of the mimosine degradation enzyme from Ipil-ipil.

RESULTS

Changes of mimosine, DHP, and pH in Ipil-Ipil by the pressure method

Fig Ie-4 shows the effect of the period of containment under pressure on the percentage content of mimosine and DHP, and on pH when the initial contents of mimosine and DHP in the finely-cut Ipil-Ipil leaves were 100 % and 0 %, respectively. The left vertical axis shows the percentage content of mimosine and DHP, and the right vertical axis shows the changes of pH, and the transversal axis shows the days of containment. Mimosine content decreases over time, dropping to 24 % at the seventh day, and 10 % at the 28th day, after which time there was no further decrease. DHP content showed the reverse trend of increasing proportionately with the decrease of mimosine. The initial value of pH was 6.2, but it decreased slowly during the containment, showing 6.0 at the 7th day, and 5.7 at the 21st day. After the 28th day, the value remained constant at about 5.3.

Decrease of mimosine under the pressurized containment at a cool temperature

Fig Ie-5 shows the changes of mimosine and DHP under conditions of containment

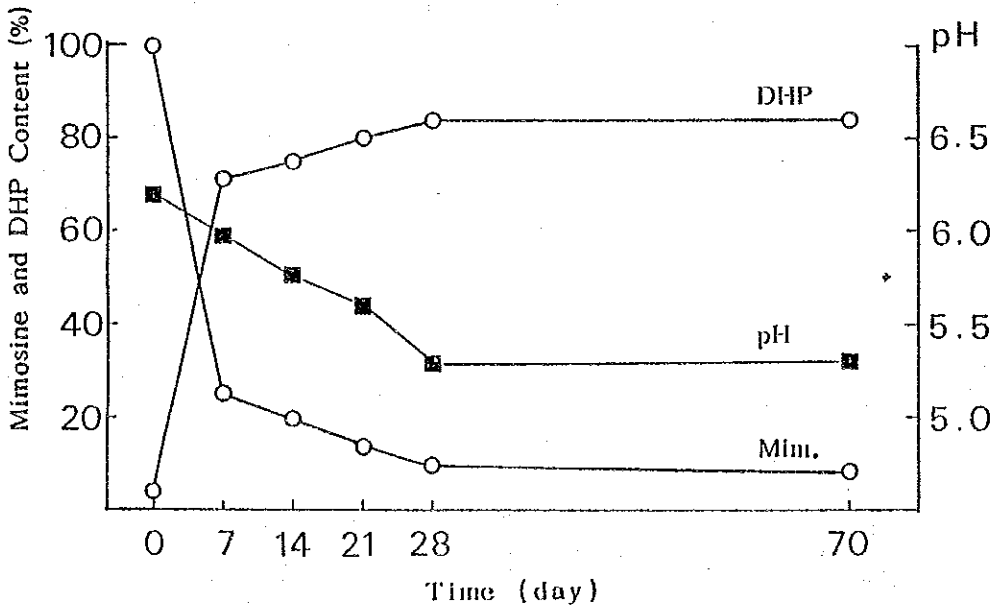


Fig. Ie-4 Changes in pH, mimosine and DHP under the pressure conditions.

under pressure at 10℃ in a refrigerator. Mimosine and DHP did not show any notable change until the 5th day of containment. From that time until the 21st day, there was a reduction of about 35 % of the mimosine and concurrent formation of 30 % DHP. The results of similar experimentation at room temperature (25℃) are shown in Fig Ie-6. The degradation of mimosine reached a maximum at 7th day, after which time there was no significant change.

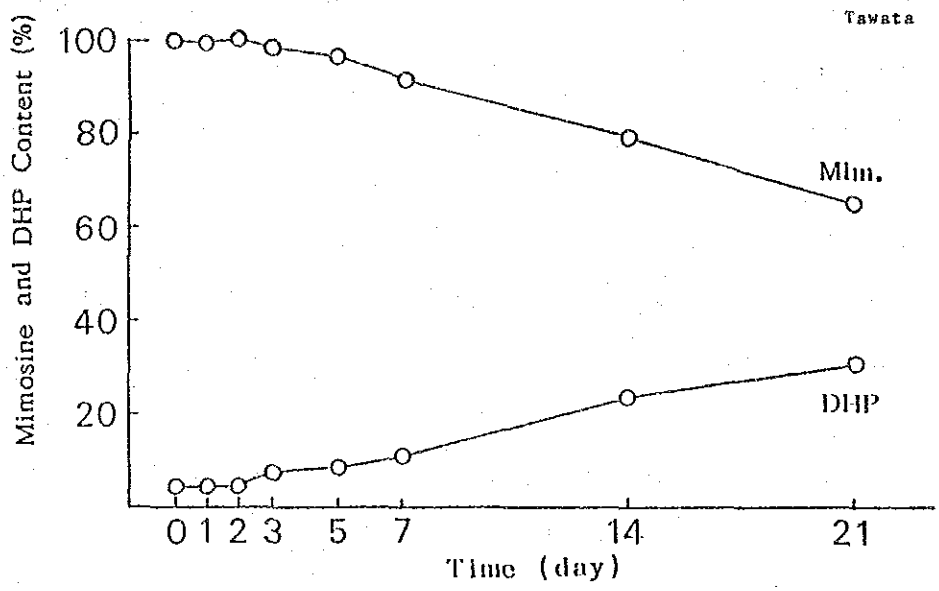


Fig. Ie-5 Changes in mimosine and DHP in the case of the standing at 10°C.

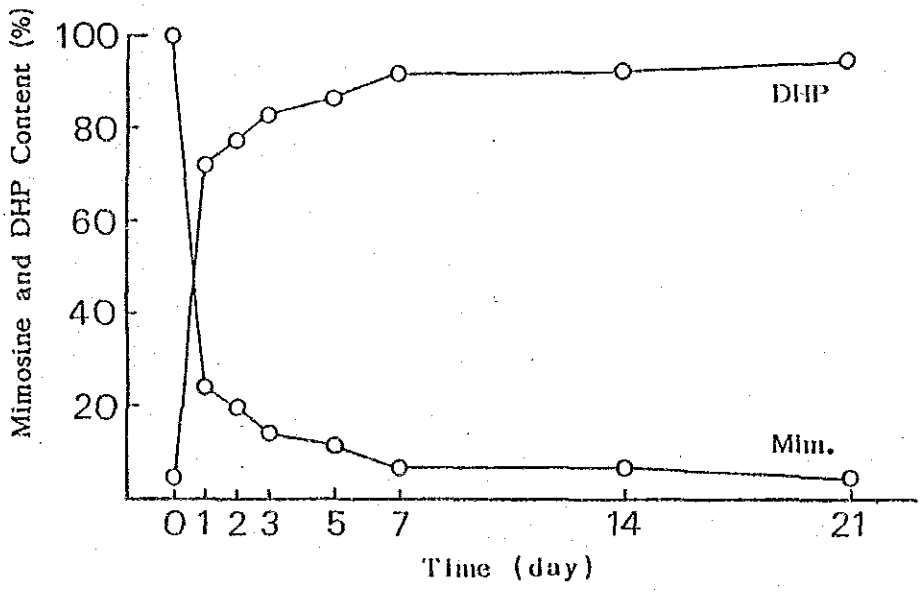


Fig. Ie-6 Changes in mimosine and DHP in the case of the standing at room temperature(25°C).

Reduction of mimosine by the weight of pressing

Fig Ie-7 shows the changes of mimosine according to differing pressure weights of 0, 10, 20, 30, 40, and 50 g/cm². The weight most effective on the reduction of mimosine was 40 g/cm², at which pressure about 86 % of the mimosine was decreased on the 21st day. In the experiment without pressure weight, mimosine decreased significantly more slowly than in the experiments using pressure. It was also observed that in the experiment without a pressure weight, the substance became moldy and there were signs of decomposition after the 14th day. Fig Ie-8 shows graphically the condition of DHP formation. Again, the amount of DHP formation was the highest with 40 g/cm² of pressure weight, and as with the mimosine reruction, about 85 % DHP was reached on the 21st day. Fig Ie-9 shows the changes of pH in each experiment using the pressure method. In the experiment without added weight, pH immediately started to decreasd and after the 7th day, the pH showed a constant value. In the experiment with weights, pH did not decrease the first two days, and after the third day, decreased rapidly, then decreased very slowly. Fig Ie-10 shows the change of mimosine and DHP according to the different cut sizes of Ipil-Ipil leaves. When the size of the length was separated in the three classes such as more than 5 mm, 3-5 mm, and less than 3 mm, the higher degradation of mimosine and increase of DHP corresponded to the shorter length of

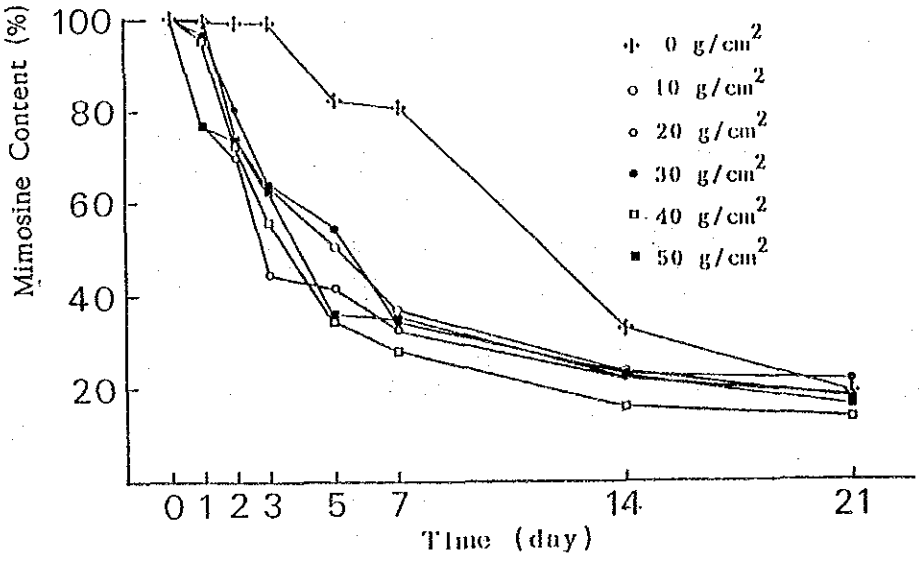


Fig. Ie-7 Changes in mimosine under the different pressures.

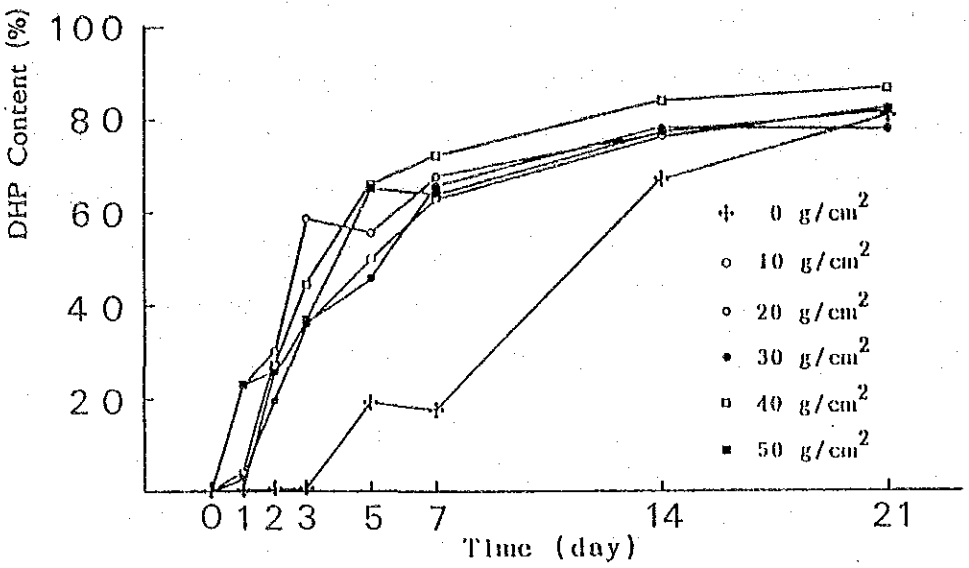


Fig. Ie-8 Changes in DHP under the different pressures.

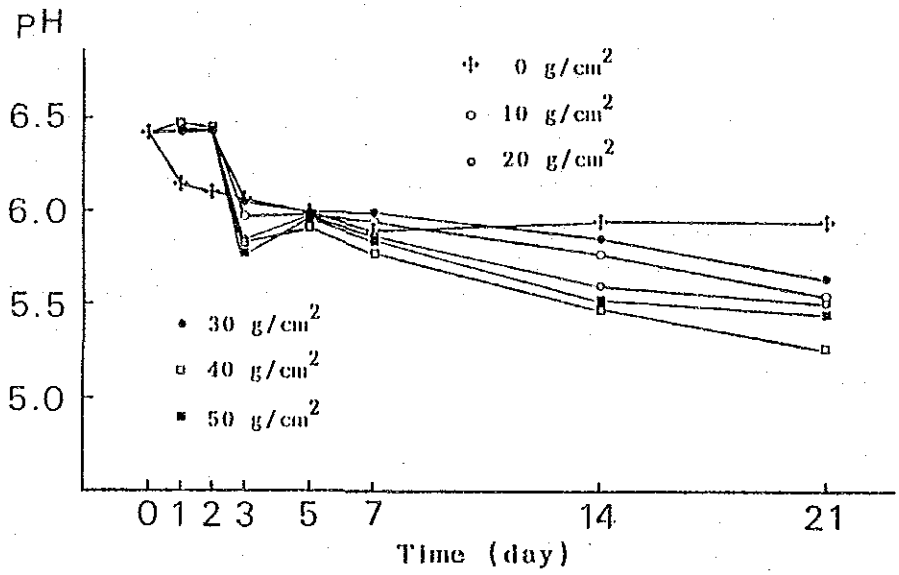


Fig. Ie-9 . Changes in pH under the different pressures.

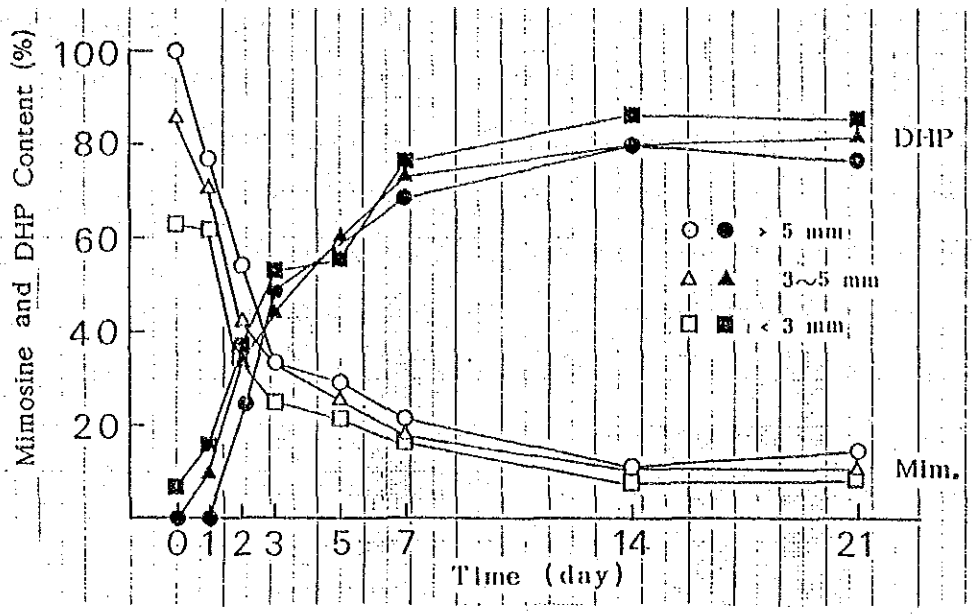


Fig. Ie-10 Changes in mimosine and DHP under the different cutting sizes.

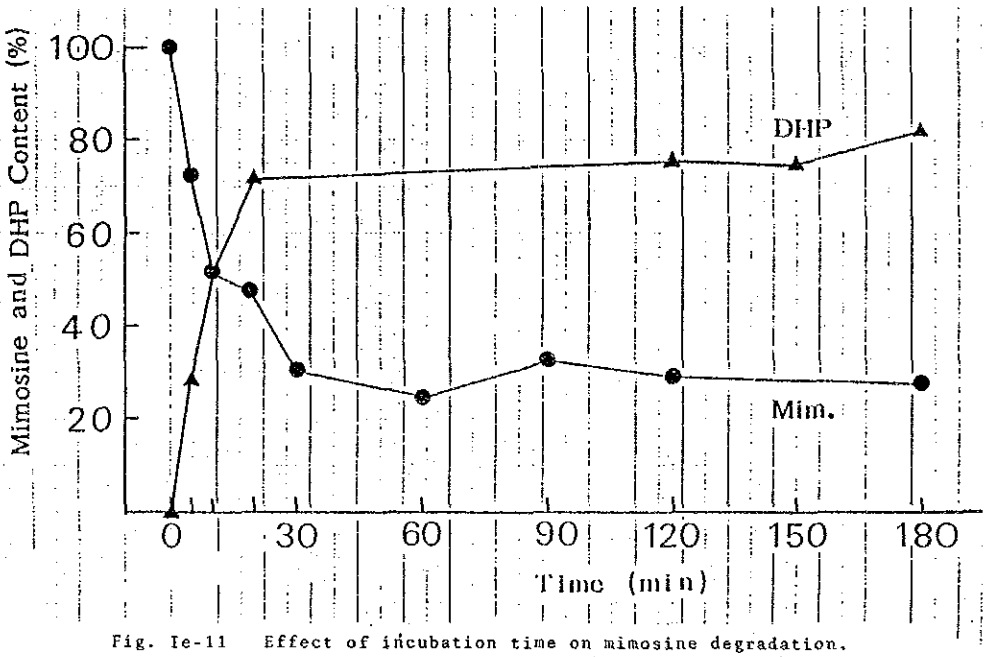


Fig. Ie-11 Effect of incubation time on mimosine degradation.

leaves. However, in the three experiments, only about 5 % difference between them was observed until 7th day of containment after which there seemed to be no significant diffrence. Fig Ie-11 shows the results of investigation of the degradation of mimosine by the crude enzyme extracted from the leaves of Ipil-Ipil. The reduction of mimosine was initially very rapid, and reached about 70 % reduction at 30 min; after that the level of mimosine was almost constant. DHP also formed in a short period, after which it remained constant. Fig Ie-12 shows the effect of the crude enzyme extracted by water on the mimosine content in the finely cut Ipil-Ipil leaves. The mimosine content decreased gradually to about 96 % at 30 min and to about 35 % at 120 min; that is, decreases of about 4 % and 35 % at 30 and 120 mins, respectively.

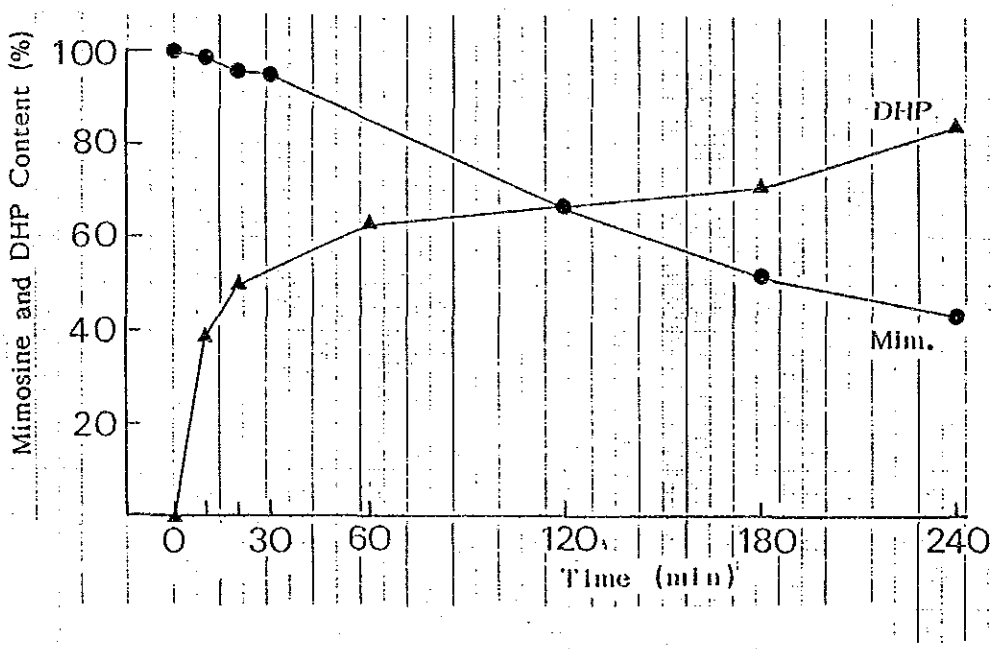


Fig. Ie-12 Effect on mimosine degradation by extracts from Ipil-ipil leaves.

DISCUSSION

This experiment confirmed that the mimosine degradation enzyme is present in the leaves of Ipil-Ipil. It is also confirmed that the rates of mimosine degradation were dependent upon the temperature, pressure weight, and size of cut leaves. Although the crude enzyme extracted from Ipil-Ipil leaves showed week activity, it was clearly established that mimosine in the leaves of Ipil-Ipil was degraded by the enzyme.

REFERENCES

1. Tangendjaja, B., J.P. Hogan, and R.B.H. Wills, Aust. J. Agric. Res., 34 : 289-293, 1983
2. Smith, L.K., and L. Fowden, J. Exp. Bot., 17 : 750-760, 1966
3. Hongo, F., S. Tawata, Y. Watanabe, and S. Shiroma, Jpn. J. Zootech. Sci., 57 : 223-230, 1986
4. Lowry, J.B., Naryanto, and B. Tangendjaja, J. Sci. Food Agric., 34 : 529-533, 1983
5. Tangendjaja, B., J.B. Lowry, and R.B.H. Wills, J. Sci. Food Agric., 35 : 613-616, 1984
6. Hegarty, M.F., C.P. Lee, G.S. Christie, R.D. Court, and K.P. Haydock, Aust. J. Biol. Sci., 32 : 27-40, 1979

I f INVESTIGATION ON THE TOXICITY OF MIMOSINE DEGRADATION PRODUCTS AND METHODS FOR THE ELIMINATION OF BOTH MIMOSINE AND THE DEGRADATION PRODUCTS

INTRODUCTION

It is known that one of the mimosine degradation products, 3-hydroxy-4(1H)pyridone (DHP), causes goitrogen in mouse and livestock[1]. In present methods of use of untreated Ipil-Ipil, it is reported that feed with less than 30 % Ipil-Ipil for cattle and less than 10 % for swine and less than 5 % for poultry do not affect the growth of the livestock at all. In addition to adjustments of the amount of Ipil-Ipil fed to individual animals, various treatments of Ipil-Ipil have been reported, including the reduction of mimosine in Ipil-Ipil leaves by heating[2], by the utilization of endogenous mimosine degradation enzyme[3], and by the addition of iron salts to make a mimosine complex for the purpose of inhibition of absorption in the body[4]. However, each of these methods is considered to entail problems such as the loss of nutrients from Ipil-Ipil, the formation of DHP, and the surplus absorption of the iron atom in the body.

The present study was carried out to determine an effective method of reruction of mimosine and DHP without any loss of protein in Ipil-Ipil for the ultimate purpose of utilizing Ipil-Ipil as a safe feed.

METHOD

100g of Ipil-Ipil leaves in a nylon bag were leached for 24 hrs at room temperature in 1.000 ml each of 0.1N solution of salts and acids. The eluted mimosine and DHP in the medium, and the remaining mimosine and DHP in the Ipil-Ipil leaves were simultaneously determined by HPLC. The changes of mimosine content after and before the leaching were compared. Additionally, the general compositions were compared

before and after the leaching and also the level of protein loss was examined.

RESULTS

Reduction of mimosine by the method of leaching

Degradation or removal of mimosine is an indispensable condition for feeding Ipil -Ipil to livestock. At present, it is known that there are some methods for detoxifying of Ipil-Ipil, as by the addition od iron salts of the heating of Ipil-Ipil leaves, but both

Table If-1 Mimosine reduction by the leaching method with each 0.1N solution.

Untreated Ipil	100 (%)
H_2O	7.2
HCl	31.2
NaOH	3.6
NaCl	12.0
KCl	13.2
$CaCl_2$	13.2
CH_3COONa	8.7
$CuCl_2$	31.5
$ZnCl_2$	39.4
$MgCl_2$	10.7
$FeCl_3$	36.1
$FeSO_4$	27.4
Formic acid	11.6
Acetic acid	8.8
Lactic acid	7.8
Fumaric acid	10.2
Succinic Acid	8.2
Malic acid	10.2
Tartaric acid	11.2

these methods introduce a further problem by the formation of DHP which is a mimosine degradation product. Table If-1 shows the results of HPLC analysis of the residual mimosine content in Ipil-Ipil which was leached for 24 hrs at 25℃ in water and in various 0.1N solutions of acids and salts. It was confirmed that water was the most effective for the reduction of mimosine : as much 92.8 % of the mimosine was removed, as shown in the Table. Metal salts such as $CaCl_2$, $ZnCl_2$ and $FeSO_4$ instead inhibited the elution of mimosine. Organic acids and CH_3COONa were more effective on elution than were NaCl, KCl and $CaCl_2$. Since NaOH discolors Ipil-Ipil, and has to be washed out with a large amount of water after treatment, we decided to investigate primarily with CH_3COONa or with sea water because of it is readily availabe in great quantities. Taking the mimosine content in untreated Ipil-Ipil to be 100 %, Fig If-1 shows the mimosine contents which were leached for 24 hrs at 25℃ in 0-100 % sea water or in 0.01-1.0N CH_3COONa. In this case, the most effective reagent was 0.05N CH_3COONa in that it removed 94.4 % of the mimosine, and was the most effective reagent among the reagents used. In the case of 60 % sea water, 90.1 % of the mimosine was removed ; therefore, the utilization of sea water seemed to be an effective method for saving water.

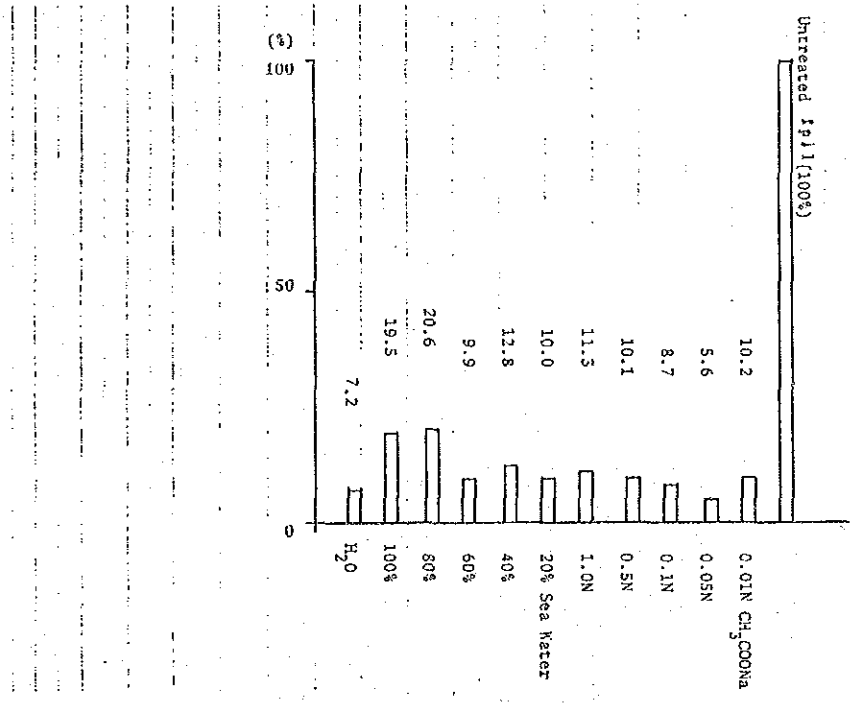


Fig. If-1 Mimosine reduction by the leaching method with sea water and CH_3COONa.

A typical example of HPLC analysis of mimosine and DHP is shown in Fig If-2. The left spectrum in the Fig shows the HPLC analysis of the medium of the Ipil-Ipil which was leached by 0.05N CH_3COONa for 24 hrs at 25℃. The middle spectrum shows the untreated Ipil-Ipil and exibits an absence of DHP. A large amount of mimosine seemed to be degraded to form DHP during the leaching. The degradation seemed to be due to the presence of the endogenous enzyme in Ipil-Ipil. The right spectrum in the Fig shows that small amounts of both mimosine and DHP are present in Ipil-Ipil after the leaching. The residue rate of mimosine is 4.5 %, and it appears that most of the DHP was also transfered in the solution. Fig If-3 shows the results of the residual mimosine and DHP in the treated Ipil-Ipil, and the eluted mimosine and DHP in the medium which was leached in 0.01-1.0N CH_3COONa solutions and water. In the treatment by 0.05N CH_3COONa, the formation rate of DHP in Ipil-Ipil was about half that in the water treatment. If the toxicity of DHP is considered, the most effective

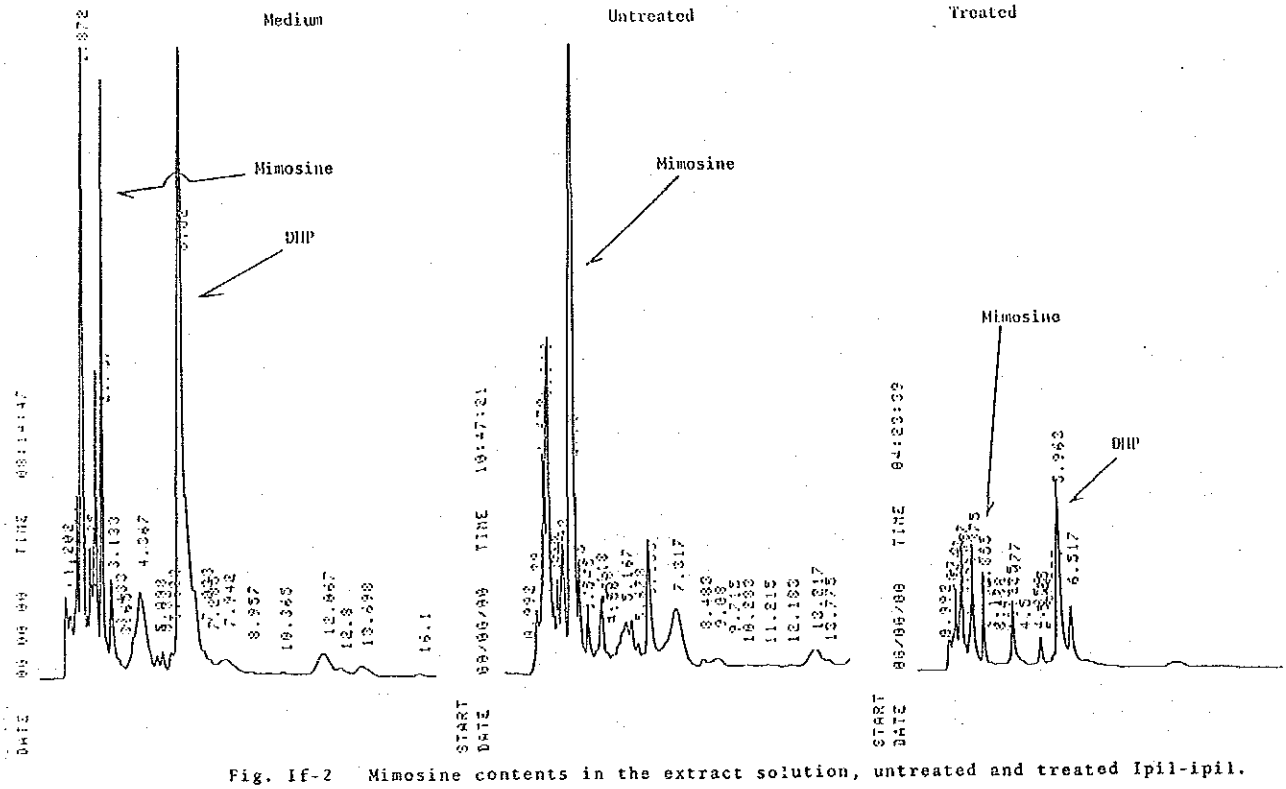


Fig. If-2 Mimosine contents in the extract solution, untreated and treated Ipil-ipil.

leaching reagent might be 0.05N CH_3COONa. Accordingly, it was confirmed that a safer feed of Ipil-Ipil could be prepared by this method. A comparison of the general composition of 0.05N CH_3COONa treated Ipil-Ipil and untreated Ipil-Ipil are shown in Table If-2. The total weight of Ipil-Ipil after the leaching decreased about 3 %. In individual composition, crude ash 4.1 %, water soluble carbohydrate 1.8 %, and crude protein 0.9 % were decreased, respectively. By contrast, crude fiber and crude fat, which are the sources of nutrients in feed for livestock, were increased by 4.7 % and 2.1 %, respectively. A small amount of various free amino acids and about 1 % mimosine are

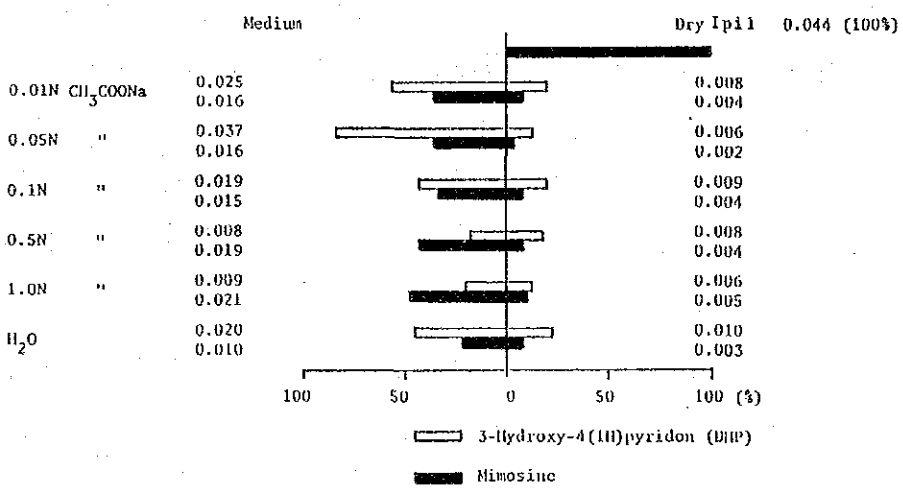


Fig. If-3 Analyses of mimosine and DHP in the extract solutions and treated Ipil-ipil leaves.

Table If-2 General composition of untreated and treated Ipil-ipil.

	Crude ash	Crude fiber	Crude fat	Crude protein	Water soluble Carbohydrate	Mimosine (%)
Treated Ipil	5.4	17.8	8.3	20.8	47.7	5.6
Untreated Ipil	9.5	13.1	6.2	21.7	49.5	100

Ipil : Leucaena leucocephala de Wit

Untreated : Dry basis Ipil leaves

Treated : Ipil leaves was leached in 0.05N CH_3COONa for 24 hrs at 25°C

present in untreated Ipil-Ipil, and these are considered to be eluted in the medium, together with other inorganic compounds such as K, Ca, and P, and others. Therefore it is considered that polymer compounds such as proteins, which are present in the cells, did not elute at all.

DISCUSSION

From the studies mentioned above, it can be seen that from Ipil-Ipil, which is presently an unused resource that grows wild everywhere in Okinawa, it is possible to remove about 95 % of mimosine by leaching without any loss of the nutrients, important for use as a livestock feed, such as crude fiber, crude fat and crude protein; therefore, it is considered that the absolute utilization of Ipil-Ipil could be possible in the near future. The leaching method seemed to have been investigated by Szyzka et al.[5], though they did not report it in detail. As the method is relatively simple and removes simultaneously both mimosine and DHP, it is considered to be the most superior method. It is believed to be a method practically applicable fop both the small scale treatment of a local farmer and the large scale treatment of a commercial enterprise.

REFERENCES

1. Hegarty, M.P., C.P. Lee, G.S. Christie, R.D. Court, and K.P. Haydock, Aust. J. Biol. Sci., 32 : 27-40, 1979
2. Tangendjaja, B., J.B. Lowry, and B.H.W. Wills, J. Sci. Food Agric., 35 : 613-616, 1984
3. Lowry, J.B., Maryanto, and B. Tangendjaja, J. Sci. Food Agric, 34 : 529-533, 1983
4. Matsumoto, H., E.G. Smith, G.D. Sherman, Arch. Biochem. Biophys., 33 : 201-211, 1951
5. Szyzka, M., U.T. Meulen, and E.H.A. El-Harith, Leucaena Research Reports, 4 : 13, 1083

II Comfirmation of A Presevation Method of Ipil-Ipil and Preparation of Feeding Level for Pratical Utilization

II a Studies on Silage of Ipil-Ipil

·····Fujiya Hongo University of Ryukyus

II b Investigation on the Effects of Toxicity Reduction and Nutritive Values of the Ensiling Ipil-Ipil Leaves on Sheep

·····Katsunori Sunagawa University of Ryukyus

II c Histopathological Effect of Grazing Ipil-Ipil in Sheep

·····Yoshitsugu Kawashima University of Ryukyus

II d Improving and Testing of Pellet Mathine

·····Syotaro mizusawa Nakajima Agricultural Institute, Ltd.,

II a STUDIES ON SILAGE OF IPIL-IPIL (*Leucaena leucocephala de Wit*)

INTRODUCTION

Leucaena leucocephala de Wit is one of species of the family Leguminosoe, popularly known as "Ipil-Ipil" in the philippines. The plant has been widely distributed in the tropics and subtropics. It can thrive satisfactorily even in nitrogen poor soils and hillscopes because of possessing a deep-rooted, with an aggressive the taproot that exploits water and minerals below the root zone of many agricultural crop plants. The plant is high palatability for domestic animals and, to be advantageous, the vegetative part of the plant is high in protein.

Therefore, many studies[1-4] have been pointed out that plant is highly valuable feed materials for domestic animals in tropical districts. It has been reported[5] that Ipil-Ipil grows naturally over about 1,200 ha on Okinawa in Japan. It is able to reap 6 times in a year, and the total in one year harvest is about 5t/10a in Okinawa. As the plant's yield of naturalized type here is lower than of the type reported from the tropics (K8, K28 and K72a types), introductions of another type of Ipil-Ipil with higher yielding ability than the naturalized type here are considered as a pasture legume.

In Okinawa, napier grass is called as the highest yielder, and its annual yield is 25 ~30 t/10a. However, the grass has serious weak points from the point of view of a constant feed for domestic animals. One of them is low in the nitrogen content, and another is a partial productivity at the limited time of hot and rainy season from May to September. Therefore, it seems that the usage of Ipil-Ipil for foliage is significantly important from the point of compensating the seasonal qualitative and quantitative shortages of the napier grass as mentioned above. Unfortunately, Ipil-Ipil contains mimosine, which produces toxicity in many animals fed leaf meal. Fear of the mimosine toxicity amoung animals feed Ipil-Ipilis still an obstacle to the use of the crop for cattle

forage. Matsumoto et al[6]. have shown that 65 % of the mimosine content in the vegetative part of the plant decreased by heating at 85℃ for 48 hr. However, the method for decreasing mimosine level is appeared to be impractical because of causing the lost of the remaining effective components by heat treatment for a long hours.

It is well known that mimosine is discomposed to 3-hydroxy-4(1H)-pyridone (DHP) by intra-rumen microbial group. It seems that lactic bacteria, one of intrarumen microbial group, play the breakdown of mimosine to DHP. From this idea the study was conducted to prove whether the treatment of ensilage of Ipil-Ipil with the heighest activity of lactic bacteria has an effect on reducing mimosine content or not.

MATERIALS AND METHODS

1. Materials

Vegitative parts of Ipil-Ipil were collected from the brush-field of Ipil-Ipil at a cutting height of about 2.3m in the vicinity of the agricultural department of Ryukyu University at the beginning of July, 1985. The main chemical composition of these materials were shown in the Table 1.

Table 1. Chemical composition of ensiled LLdW materials (%)

	Moisture	Crude protein	Crude fat	Nitrogen free extract	Crude fiber	Crude ash.	Water soluble carbohydrate	Mimosine
Wet basis	65.8	6.6	1.6	17.0	6.3	2.7	1.4	0.9
Dry basis	——	21.8	5.6	50.7	12.8	9.1	4.1	2.6

LLdW: Leucaena leucocephala de Wit

2. Silage making

Vegitative parts were cut into pieces with 5~10 mm long immediately after sampling and divided into an untreated group (control group) and test group. Im test groups, the addition of 2.5, 5, 10 and 15 % of molasses to vegitative parts were done, and then each silage was made according to Kemble's method[7] by using 1,000 ml glass bottle as a container. For chemical analysis, samples were taken out of the glass bottles just before package (control) and at 7, 14, 21 and 70 days after the package.

3. Analysis of silage

The analysis of silage was conducted by the following method. The pH of the extract of silage was measured by pH meter. The extract was obtained from the suspension of about 10g of the silage in 20 ml of water after standing for 24 hr at cool room. Moisture[8] was calculated from the difference between fresh weight and the air-drying of the silage at 100℃ for 18 hr. Lactic acid was determined by colorimetry according to Barker-Summerson method[9] modified by Barnett[10]. Volatile fatty acid was determined by gas liquid chromatograph (Hitachi, 073 type) on the extract prepared by a process comprising: titrating the distillate, which was obtained by applying steam distillation to the aqueous extract of silage, with 0.1N sodium hydroxyde solution, dripping a small quantity of alkali to the titrated solution before evaporation to dryness, and adding phosphoric acid to the dry matter to perform ether extraction. The content of mimosine was measured by the following method. 5g of silage, to which 50 ml of 0.1N hydrochloric acid was added, was homogenized under cooling and subjected to centrifugal separation (16,000 x g, 30 min) to be separated into supernatant solution and residue while the same operation was further applied to the residue three times to obtain supernatant solution which was, in turn, mixed with the previously obtained supernatant solution to prepare mimosine extract. This extract is neutralized by 0.1N sodium hydroxide solution and evaporated to dryness at about 50℃ under reduced pressure. Further, the resulting dry matter was brought to constant volume (50 ml) by citric buffer (pH 2.2) for the analysis of amino acid and each 0.5 ml was injected in amino acid automatic analyzer (Hitachi liquid chromatograph, 034 type) and the analysis of mimosine was performed by the one column method (column : 9 x 400 mm, ninhydrin flow rate : 30 ml/hr, column temperature ; 55℃, wavelength : 440 nm, 570 nm and 640 nm, simultaneous measurement) for performing three-stage elution using three kinds of citric buffers (pH 3.25, pH 4.25 and pH 5.28). The chromatogram obtained was compared with that of standard mimosine preliminarly analyzed under the same condition and mimosine was identified from elution position and the content thereof was calculated by so called HW method.

RESULTS AND DISCUSSION

1. Changes of fermentative component in Ipil-Ipil silage

The moisture content at the packing time was about 66 %. In Fig. 1, the pH value in each group just before packing showed the maximum level of 6.3 and lowed with the lapse of the ensiling period. However, the pH values both in control and in the test groups receiving 2.5 % of molasses were gradually decreased. The changes of pH value at the period form 0 day to 70 days in case of receiving 2.5 % of molasses were within 5.3~5.5. On the other hand, the pH value of 10 and 15 % molasses addition groups lowered immediately to 4.4 and 4.0, respectively. In 10 % molasses addition group, the value decreased to 4.0 at 21 day and it showed almost no change up to 70 days.

The content of lactic acids in 0 % (control), 2.5- and 5 % molasses addition groups after packing for 70 days were extremely low in these three groups, and the value were 0.25, 0.30, 0.70 %, respectively. Acetic acid was also low values of 0.20, 0.23 and 0.22 % in all groups while butyric acids were slight in quantity in control. In 10- and 15 %- addition groups, the contents of organic acids markedly increased up to 7 days. For example, the contents of lactic acid in both groups were 2.24 and 2.48 %, respectively. On 21 days the content of lactic acid in 10 % molasses addition group was 3.42 % while the values in the 15 % molasses-addition group reached 3.20 % on 14 days. No conspicuous changes were shown up to 70 days in both groups. Acetic acid in the 15 %-addition group showed 0.22 % at 14 day storage in the 10 %-addition group. In the 15 %-addition group, the values were 0.24 % at 7 days storage, then stable values were seen up to 70 days.

From these results, it seemed that it is necessary for Ipil-Ipil to add at least 10 % of molasses when silage is prepared by using the vegitative parts of Ipil-Ipil. In both 10 and 15 % molasses addition groups the pH values and the content of organic acids was shown stable values after 14 days storage in the former group but 21 days storage in the latter group. The quality of silage on 21 day storage 10 %-molasses addition and on 14 day storage 15 %-molasses addition was evaluated according to quality

discrimination standards by Freak's method. As a result, both silage were evaluated as "excellent" and light green and good quality silages having aromatic sour flavor were obtained.

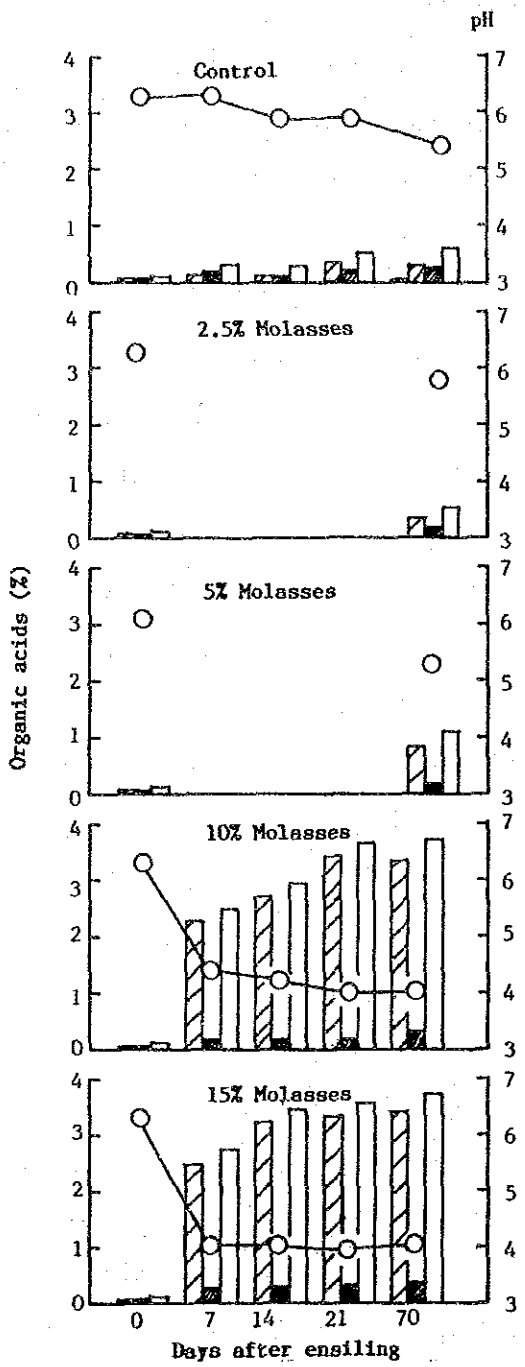


Fig. 1. Effect of the addition of molasses on pH, organic acid composition in ensiled Leucaena silage. —○— : pH, ▨ : Lactic acid, ▩ : Acetic acid, ■ : Butyric acid, □ : Total acid

2. Changes of mimosine content in ensiling process

The mimosine content in the vegetative parts of Ipil-Ipil subjected to the silage was about 0.9 % (about 2.6 % in dry matter). The content of mimosine in 0 % (control) and in the 10 %-molasses addition group decreased with the lapse of the ensiling period (Fig. 2). In these groups, mimosine content was markedly reduced during early period, up to 7 days after storage, when fermentative activity is processed vigorously. In 14 days after storage, the values reached to about 0.1 % and showed a constant low value thereafter. In control, mimosine content showed somewhat high value up to 14 days after storage but maintained low level after 21 days. The reduction ratio of mimosine content at 70 days after ensiling was about 90 % in both groups.

From these results, the effect of silage on marked reducing of mimosine content was elucidated. It suggests that the reduction in mimosine content of silage with the lapse of ensiling period may be due to the decomposition of mimosine by lactic bacteria vigorously propagated in the early stage of fermentation. However, the marked reduction in mimosine content was similarly confirmed even in control group showing extremely slow fermentation. It appeared to be responsible for the decomposition by bacteria coexisting in the packed materials.

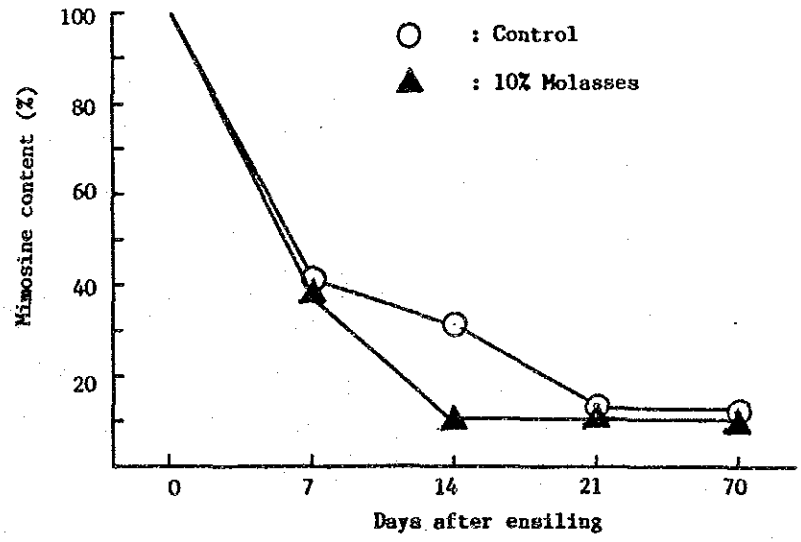


Fig. 2. Changes of mimosine content in Leucaena silage

CONCLUSION

This study was conducted to establish the usefulness of Ipil-Ipil through the decomposition of mimosine by processing silage. The results obtained are as follows.

(1) When silage was made by using only the vegitative parts of Ipil-Ipil, no good quality silage were obtained because the pH value slowly and the composition organic acids gradually decreased with the lapse of ensiling period. However, when molasses were added to it at the ratio of 10 % and 15 %, the pH value in 10 % addition lowered to 4.0 on 21 days after preservation whereas it decreased on 14 days after shortage in the 15 %- addition group. The content of organic acids in both groups increased to 3.4 %, and then the stable values were maintained up to 70 days.

(2) From these results, the mimosine content was markedly reduced during the early period of fermentation, up to about 7 days in groups using only vegitative parts and adding of molasses to vegitative parts. The reduction ratio of mimosine was about 90 % up to 70 days after ensiling. On basis of these results, the pellet which is made from the silage is required for establishing the efficient utilization of Ipil -Ipil.

REFERENCES

1) Skerman, P.J., in Tropical Forage Legumes. (Riveros, F. ed.) 510-519. Food and Agriculture Organization of the United Nations Rome. 1977.

2) Gray, S.G., Grasslands, 2 : 19-20. 1968.

3) Jones, R.J., Wld. Anim. Rev., 31 : 13-23. 1979.

4) Brewbaker, J.L., D.L. Plucknett and V. Gonzalez, Hawaii Agr. Exp. Sta. Res. Bull., 166 : 1-29. 1972.

5) Shinjho, A. and M. Nakasone, Studies on Anim. Husbandry., 30 : 75-76. 1976.

6) Matsumoto, H., E.G. Smith and G.D. Sherman, Arch. Biochem. Biophys., 33 : 201 -211. 1951.
7) Kemble, A.R., J. Sci. Food Agric., 7 : 125-130. 1965.
8) National Glassland Research Institute, Experimental Methods of Silage, 37-60. 1975.
9) Barker, S.B. and W.H. Summerson, J. Biol. Chem., 138 : 535-554. 1941.
10) Barnett, A.J.G., J. Biol. Chem., 49 : 527-529. 1951.

II b Investigation on the Effects of Toxicity Reduction and Nutritive Values of the Ensiling Ipil-Ipil Leaves on Sheep

INTRODUCTION

The utilization of Ipil-Ipil as a feedstuff for livestock has been limited because mimosine causes low weight gains, hair loss and goiter on animal. Jones[1] reported that rations containing up to 40% of Ipil-Ipil resulted in good live weight gain over a period of 112 days in growing steers. Matsuda[2,3] reported that swine fed diets containing up to 10% of Ipil-Ipil leaves and up to 5% for poultry maintained normal growth rate. The air-dried Ipil-Ipil leaf was found by Reis et al[4]. to contain about 4.4% mimosine. Szyszka et al.[5] indicated that the tolerance daily ingestions of mimosine for broiler, rabbit, cattle, goat and sheep were 0.16, 0.23, 0.18 and 0.14 g/kg body weight/day, respectively. It was clarified that the mimosine content varied markedly in each part even in the same strain (Hongo, unpublished). Hongo et al.[6] also reported that more than 90% mimosine in Ipil-Ipil was degraded during the fermentation process of silage. From their experimental results, it can be expected that the combination rate of Ipil-Ipil silage could be increased into rations for a productive livestock.

The present study was carried out in order to investigate the effects of diets containing 66% of Ipil-Ipil silage on live weight gain, thyroid function, hair loss, hair growth, and also the nutritive value on sheep.

MATERIALS AND METHODS

1. Animals

Eight cross-bred wethers (Suffolk × Southdown, 1.5 age, body weight: 37.0~42.5 kg) were used in the experiment. All animals were raised in metabolic cages before 4 months from the start of experiment. A daily ration of 1,200g (alfalfa pellets) was

fed in twice by 600g serving at 9 : 00 o'clock and 17 : 00 o'clock daily. Animals were allowed to take water *ad libitum*. Supatonin (12g/3ml/head) was injected intramuscularly every two months in order to protect sheep from the infectious cerebrospinal filaria.

2. Making of Ipil-Ipil silage pellets and hay pellets

The leaves and green stems of 1~2m high Ipil-Ipil, which grows wild near campus of Ryukyu Univ., were chopped with a hammermill. Molasses were added at a level of 100g/kg Ipil-Ipil and they were ensiled for two weeks. The silage was dried by the sum for 3 days and made pellets using a Nakajima pelleting machine (Nakajima Chikunoki Co.). The Ipil-Ipil hay pellets were made using by the chopped Ipil-Ipil hay similarly.

3. Experimental procedures

The animals were assigned into three groups, that is, two (body weight : 37.0 and 38.0 kg). three (39.5±0.87kg) and three wethers (39.5±2.65kg) to A, H and S groups on the basis of feed, respectively. Each animal in A group fed twice (9 : 00 and 17 : 00 o'clock) a daily 600g alfalfa pellets, in H group, they were fed 200g of the alfalfa pellets and 400g of the Ipil-Ipil hay pellets, and in S group, they were fed 200g of the alfalfa pellets and 400g of the Ipil-Ipil silage pellets. All animals were allowed water *ad libitum*. The feeding period was ten weeks (30th in Sept.~9th in Dec. 1985). Respiratory rate, heart rate, retal temperature, food intake, water intake, feces excretions and body weight were measured at intervals of a week before feeding at 9 : 00 o'clock. Then, blood samples (20ml) were obtained from each animal by venip uncture, and daily feces (100g) were collected. Food and water intake were also observed before feeding at evening. Hair loss was observed ma croscopically. All sheeps were defleeced by the use of a depilatory agent 10cm^2 on the left or right side of the abdomen, and the pelage growth was observed.

4. Analytical methods

Mimosine and DHP (3-hydroxy-4(1H)-pyridone) contents of the pellets and plasma were determined by the same method as described by Hongo et al. Plasma thyroxine (T_4) and triiodothyronine (T_3) levels were assayed with a radioimmunoassay by the method of Chopra.[7] The activities of plasma GOT (glutamate oxaloacetate transaminase) and GPT (glutamate pyruvate transaminase) were assayed with Unikit GOT and GPT (Chugai Pharm. Co.) by a Blood Analyzer Mark II. Feces were dried at 70°C and grinded, and the feces were frozen at −20°C until they were used for analysis. Chemical Composition of feed and feces were analyzed according to the textbook published by Nippon Shiryo Kyokai.

5. Calculation of digestibilities

Digestibilities of the component of alfalfa, Ipil-Ipil silage and Ipil-Ipil hay pellets were calculated by the following equations.

Digestibility of the components of alfalfa pellets: Digestibility (%) = [food intake × component contents (%) − feces extraction × component contents (%) /food intake × component contents (%)] × 100

Digestibility of the components of Ipil-Ipil silage and Ipil-Ipil hay pellets: Digestibility (%) = [a − (b − c)/a] × 100

a: intake of the component of Ipil-Ipil silage or Ipil-Ipil hay = food intake × mixing rate of silage or hay × the components of silage or hay

b: the components of feces = the amounts of excreted feces × the component of feces

c: the components of feces derived from alfalfa feeding (basal feed) = food intake × mixing percentage of alfalfa × the components of alfalfa × (100 − the digestibilities of the component of alfalfa) / 100

6. Statistical analysis

Results are expressed as mean ± SD of 2 or 3 sheep. Statistical significance of the

difference between means was analyzed by the Student's test.

RESULTS AND DISCUSSION

1. Chemical composition of alfalfa, Ipil-Ipil hay and Ipil-Ipil silage pellets.

The results are shown in Table 1. The crude protain contents of Ipil-Ipil hay (Ipil-Ipil) and Ipil-Ipil silage (silage) were similar to alfalfa, but the crude fat and nitrogen free extract (NFE) contents of Ipil-Ipil and silage were lower in comparison to that of alfalfa. In the fiber contents, on the other hand, Ipil-Ipil and silage were higher than alfalfa. By ensiling of Ipil-Ipil, the mimosine content decreased to 1/3 (0.26%) of the average on dry basis, on the other hand, the DHP content of Ipil-Ipil increased two-fold (0.75%) to the average of the untreated Ipil-Ipil.

Table 1. Chemical composition of alfalfa, ipil-ipil and ipil-ipil silage pellets.

	Alfalfa pellet	Ipil-ipil silage pellet	Ipil-ipil pellet
Moisture (%)	13.56±0.21	12.24±0.18	14.47±2.49
Crude ash (%)	7.89±0.37	7.84±1.30	5.87±0.19
Crude protein (%)	17.17±4.90	19.12±2.59	16.61±1.66
Crude fat (%)	2.61±0.09	2.01±0.31	2.27±0.17
Crude fiber (%)	17.98±1.43	20.99±1.30	23.87±1.23
NFE (%)	40.78±5.87	37.80±2.65	36.92±0.89
Mimosine (%)		0.26±0.02	0.75±0.06
DHP (%)		0.46±0.06	0.26±0.01

Ipil-ipil and ipil-ipil silage pellets contain leaf and green stem.

Values are means ± S.D. from three determinations.

2. Environmental temperature, relative humidity and physiological responses of sheep in A, H and S groups

The environmental temperatures and relative humidities of room in which sheep were housed are given in Fig.1. Heart rate remained constant at 65 ± 5 beats / min in three groups during the experimental period. Respiratory rate was 94 ± 12 breaths / min from 1 to 5 weeks, but decreased to 66 ± 9 breaths / min after 6 week. Rectal temperature was 39.2 ± 0.16°C from 1 to 7 weeks, but also decreased to 39.0 ± 0.15°C after weeks. No differences in respiratory rate and heart rate were found between sheep of A, H and S groups. Rectal temperature of sheep in A group was higher than that of sheep in H and S groups.

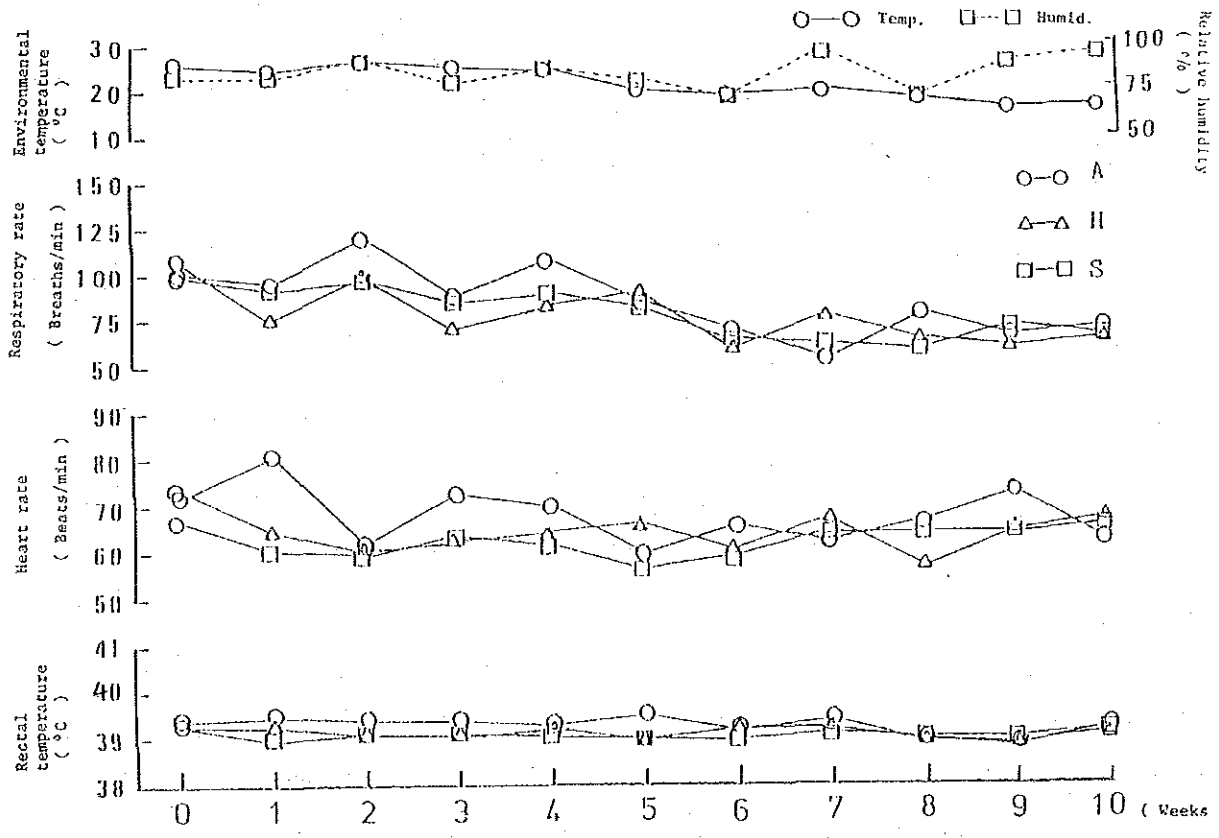


Fig.1. Respiratory rate, heart rate and rectal temperature in sheep fed alfalfa (A), alfalfa 34% + ipil-ipil 66% (H) and alfalfa 34% + ipil-ipil silage 66% (S).

3. Feed intake of sheep in A, H and S groups

Feed intake of sheep in A group remained constant during the experimental period. Feed intake of sheep in H gpoup decreased through 1 to 3 weeks, but feed intake of sheep after 4 weeks were similar to that of 0 weeks. Feed intake of sheep in S group slightly decreased at 1 weeks, but feed intake after 2 weeks were similar to that of 0 weeks. When the daily intake of mimosine for sheep was calculated from values in Table 1 and Fig.2, those values in H and S groups were 155.6, 52.0mg/kg/ body weight/ day, respectively. The daily intake of DHP for sheep in H and S groups were 53.9, 91.9mg/kg/day, respectively.

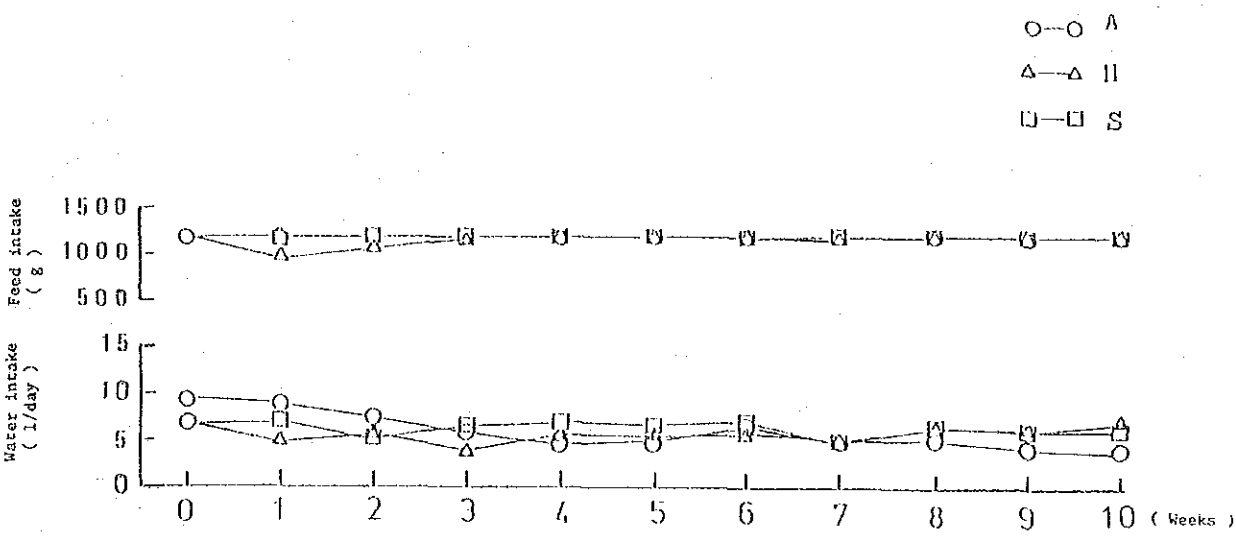


Fig.2. Feed and water intake of sheep fed alfalfa (A), alfalfa 34% + ipil-ipil 66% (H) and alfalfa 34% + ipil-ipil silage 66% (S).

4. Plasma concentrations of substances influenced by mimosine toxicity in A, H and S groups

Fig.3 shows the concentrations of mimosine and DHP in plasma. Plasma mimosine concentrations increased at 1 weeks, but decreased at 2 weeks and remained constant from 2 to 7 weeks in both groups H and S. DHP concentrations of sheep in S group were similar to those of sheep in H group at every weeks except for 1 weeks. The mean plasma mimosine concentrations of sheep in H and S groups were 147.3, 144.

1μg/100ml, respectively. These concentrations were much lower in comparison to values 247, 1980 μg/100 ml) obtained from Merino wethers that 200 or 300 mg of mimosine given orally for 2 days.[4] Published data on plasma DHP concntration in sheep is sparse. Fig.4 shows plasma T_4 and T_3 levels of sheep in A, H and S groups. Plasma T_4 levels of sheep in H and S groups were lower than that of sheep in A group at every weeks. Plasma T_4 levels of sheep in S group were lower than that of sheep in H group after 2 weeks because of the difference of T_4 level at 0 weeks. Plasma T_3 levels of sheep in H and S group were lower than that of sheep in A group at every weeks.

Values of sheep in S group were higher than that of sheep in H group at every weeks. Hegarty[8] reported that the circulating DHP prevents iodination of tyrosine, the first step in the synthesis of thyroxine, resulting in goiter and reduced levels of T_4 in the serum. It seems that a slight decline in T_4 levels of group H and S support the results obtained by Hegarty. The plasma T_3 levels in S group were, however, higher than that in H group although the daily intake of DHP for sheep in S group increased two-fold that in H group. Fig.5 shows plasma GOT and GPT activities of sheep in A, H and S groups. GOT activities in each animal were different from each other at 0 weeks, respectively. But values at each weeks in sheep in H group were higher than that at 0 weeks.

The changes of GPT activities of sheep in A, H and S groups were similar to those of GOT activities. Table 2 compares means of values from 1 to 10 weeks with that of values at 0 weeks in A, H and S groups, respectively. It is known that GOT and GPT are distributed, primarily, in heart muscle, skeltal muscle, liver and kidney in sheep.[9] Increases in the activities of GOT and GPT in H group suggest that mimosihe has caused some active lesions on those organs.

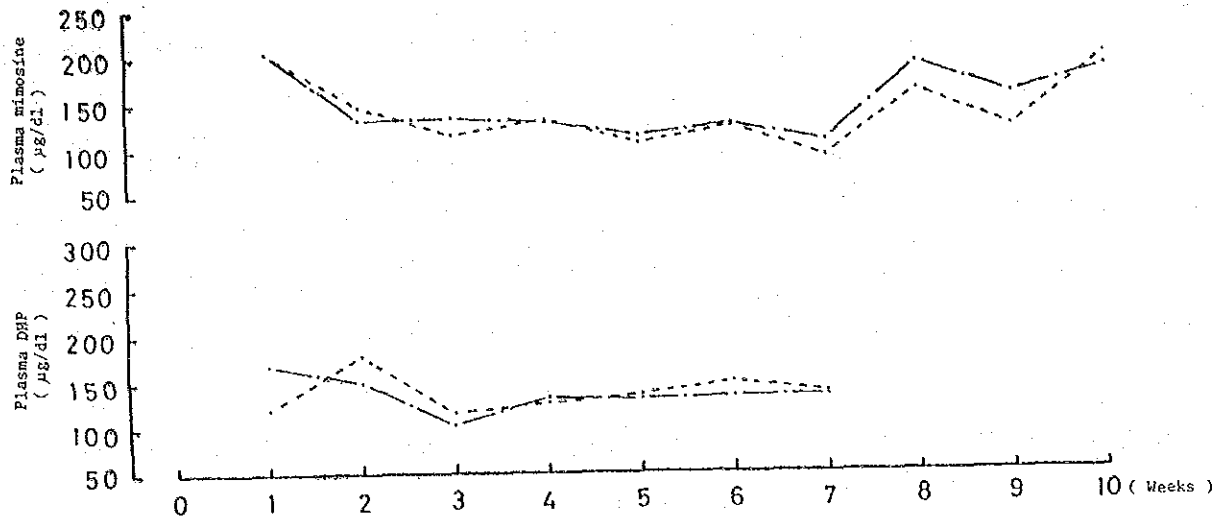


Fig.3. Plasma mimosine and 3-hydroxy-4(1H)-pyridone (DHP) concentrations in sheep fed alfalfa 34 % + ipil-ipil 66 % (H) and alfalfa 34 % + ipil-ipil silage 66 % (S).

H —— —, S - - -

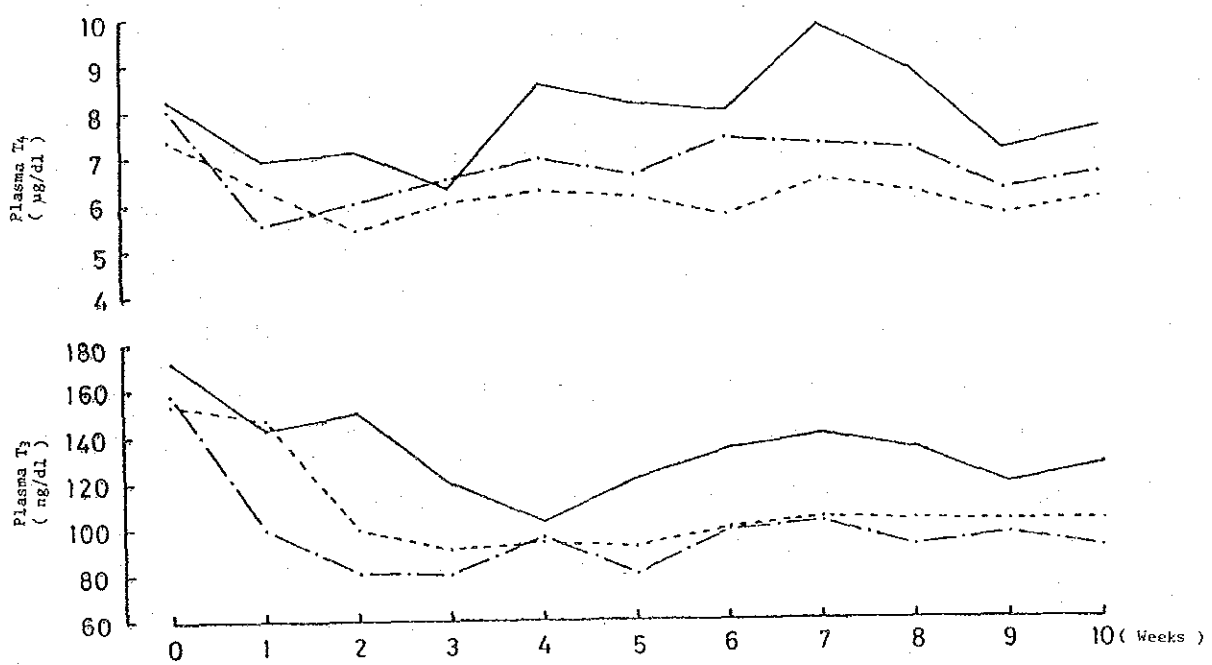


Fig.4. Plasma thyroxine (T_4) and tri-iodothyronine (T_3) levels in sheep fed alfalfa (A), alfalfa 34 % + ipil-ipil 66 % (H) and alfalfa 34 % + ipil-ipil silage 66 % (S).

A ——, H —— —, S - - -

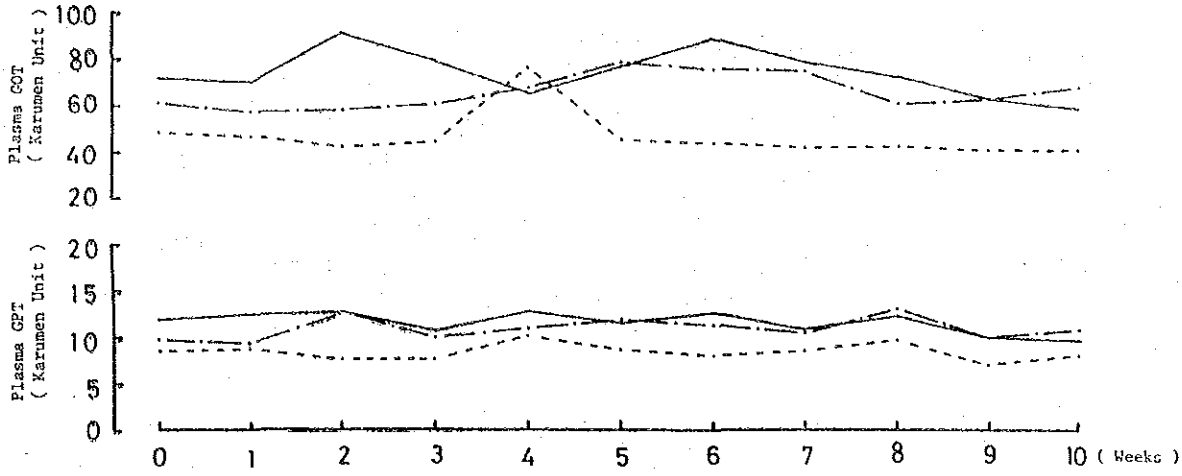


Fig. 5. Plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate trasaminase (GPT) activities in sheep fed alfalfa (A), alfalfa 34 % + ipil-ipil 66 % (H), alfalfa 34 % + ipil-ipil silage 66 % (S). A ——, H — —, S – – –

Table 2. Plasma components in sheep fed alfalfa, alfalfa 34% + ipil-ipil 66% and alfalfa 34% + ipil-ipil silage 66%.

Component	Group	Week 0: Feed: Alfalfa 100%	Week 1~10: Feed: Alfalfa 34% + Ipil-ipil silage 66%	Week 1~10: Feed: Alfalfa 34% + Ipil-ipil 66%	Week 1~10: Feed: Alfalfa 100%
mimosine (μg/dl)	A				
	H			14.73±40.9	
	S		144.1±48.8		
DHP (μg/dl)	A				
	H			142.7 ±53.9	
	S		136.7±20.8		
T_4 (μg/dl)	A	8.3± 1.8			7.7±1.6
	H	8.0± 1.2		** 6.6 ± 0.9	
	S	7.4± 1.6	** 6.1± 1.1		
T_3 (ng/dl)	A	172 ± 4			131±17
	H	158 ±48		** 92 ±15	
	S	153 ±50	** 103 ±23		
GOT (Karmen Unit)	A	73 ±27			74±26
	H	61 ±15		66 ± 9	
	S	49 ± 9	46 ±14		
GPT (Karmen Unit)	A	12 ± 3			12± 2
	H	10 ± 0		** 11 ± 2	
	S	9 ± 2	9 ± 1		

Values that are significantly different from component at O week are indicated by **($P < 0.01$).

5. Obsevations on the pelage growth and hair loss of sheep

None of sheep in H or S groups showed hair loss, and the growth of their pelages were not different from that of sheep in A group. Reis et al.[4] Showed that defleecing ensued when the concentration of mimisine in plasma was maintained above 0.1 mmol/l for at least 30 h. This value was fourteen times the mimosine concentration in H group. The mimosine content of Ipil-Ipil hay pellet was much lower in comparison to values reported by Reis et al. This mimosine degradation of Ipil-Ipil hay pellet was suspected to be due to biochemical changes during sun drying and to heat treatment in making of pellets.[10]

6. Digestibilities of the components of alfalfa, Ipil-Ipil and Ipil-Ipil silage pellets

Fig.6~10 show the digestibilities of the components of alfalfa, Ipil-Ipil and Ipil-Ipil silage at every weeks. Table 3 compares means of values from 1 to 10 weeks with that of values at 0 weeks in sheep fed alfalfa, Ipil-Ipil and Ipil-Ipil silage, respectively. The digestibilities of crude protein and NFE of Ipil-Ipil silage were significantly lower in comparison to values of alfalfa, but the digestibilities of crude fat of Ipil-Ipil silage was significantly higher than value of alfalfa. The digestiblmity of crude fiber of Ipil-Ipil silage, on the other hand, was similar to that of alfalfa. The digestibilities of the components of Ipil-Ipil were similar to values of the components of Ipil-Ipil silage. The low protein digestibilities of Ipil-Ipil hay and silage pellets were suspected to be due to the fact that Ipil-Ipil included more amounts of low soluble protein in the rumen.[11]

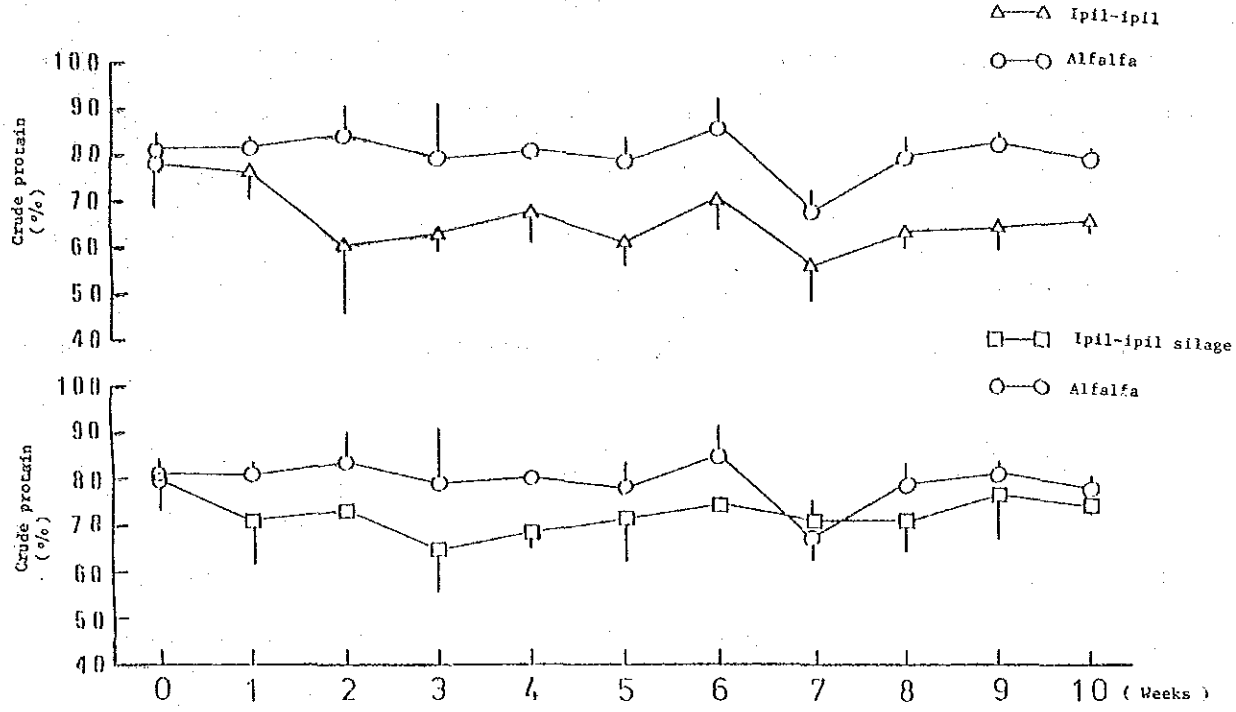


Fig.6. Digestibility of crude protain. The bars indicate the S.D. of means for three animals.

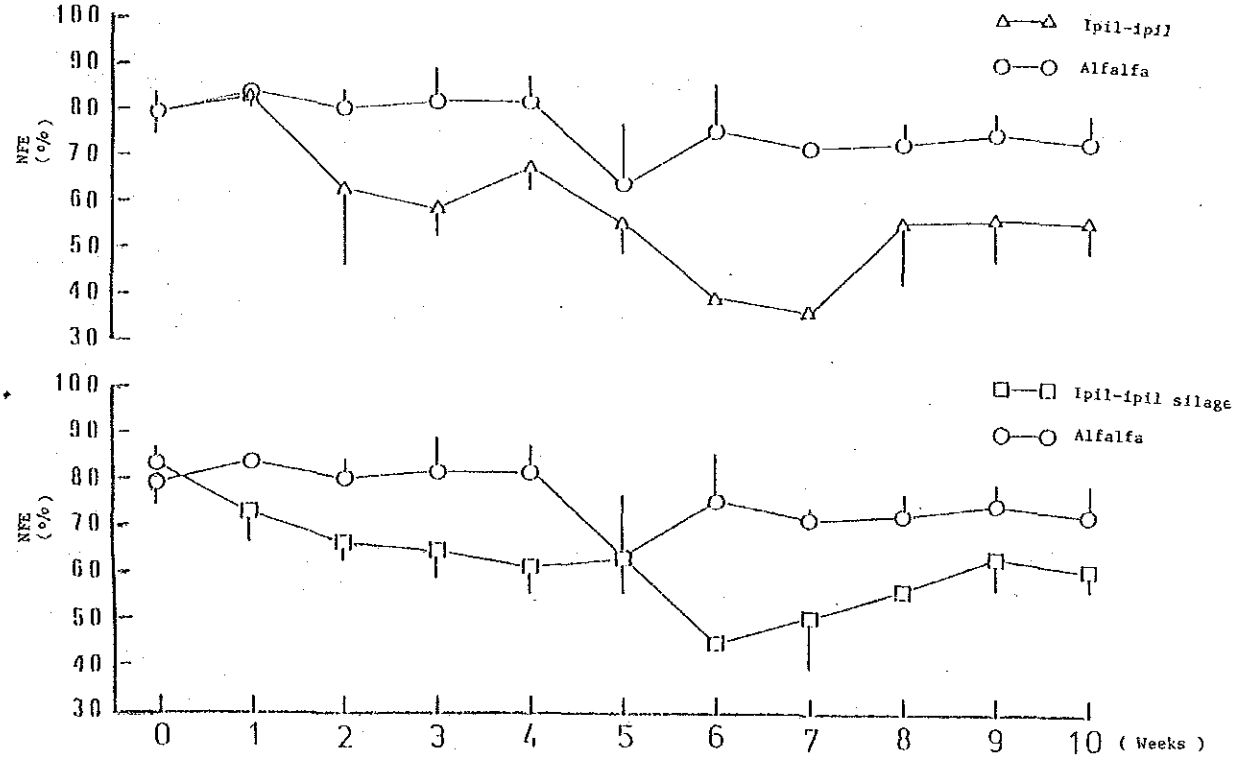


Fig.7. Digestibility of nitrogen free extract (NFE). The bars indicate the S.D. of means for three animals.

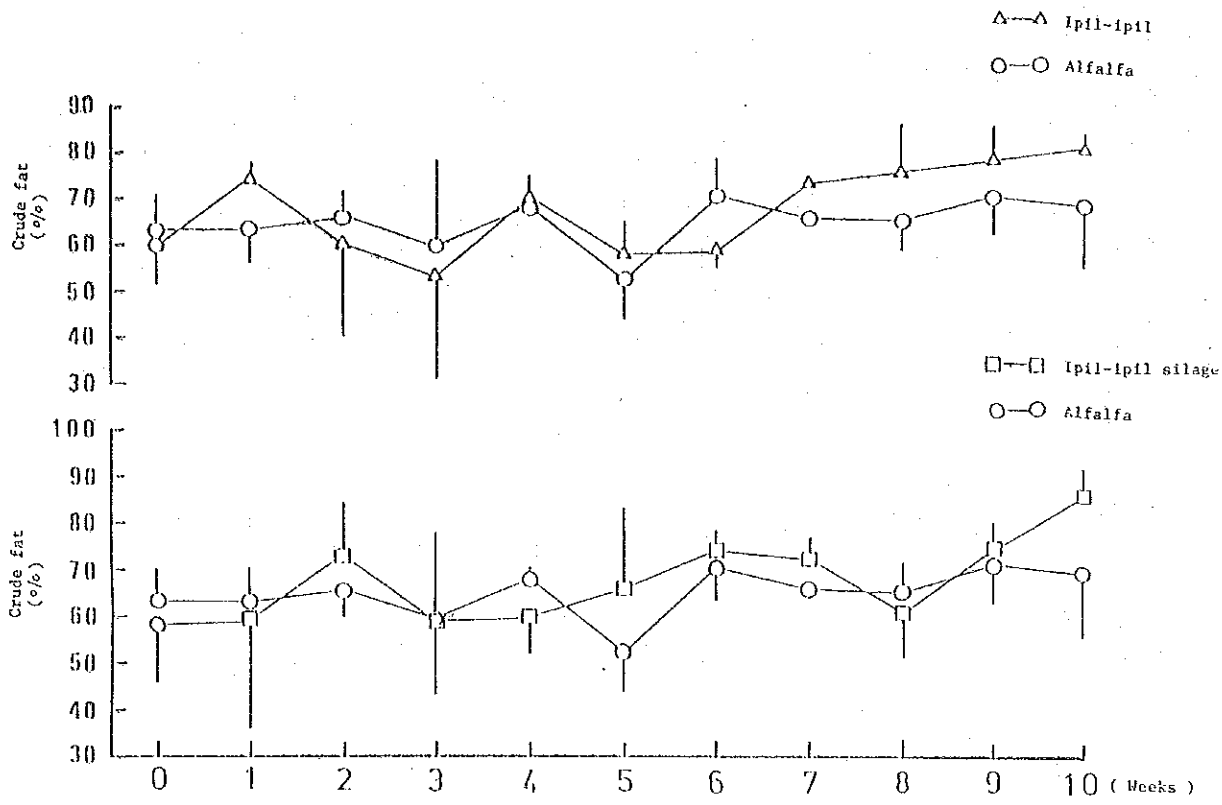


Fig.8. Digestibility of crude fat. The bars indicate the S.D. of means for three animals.

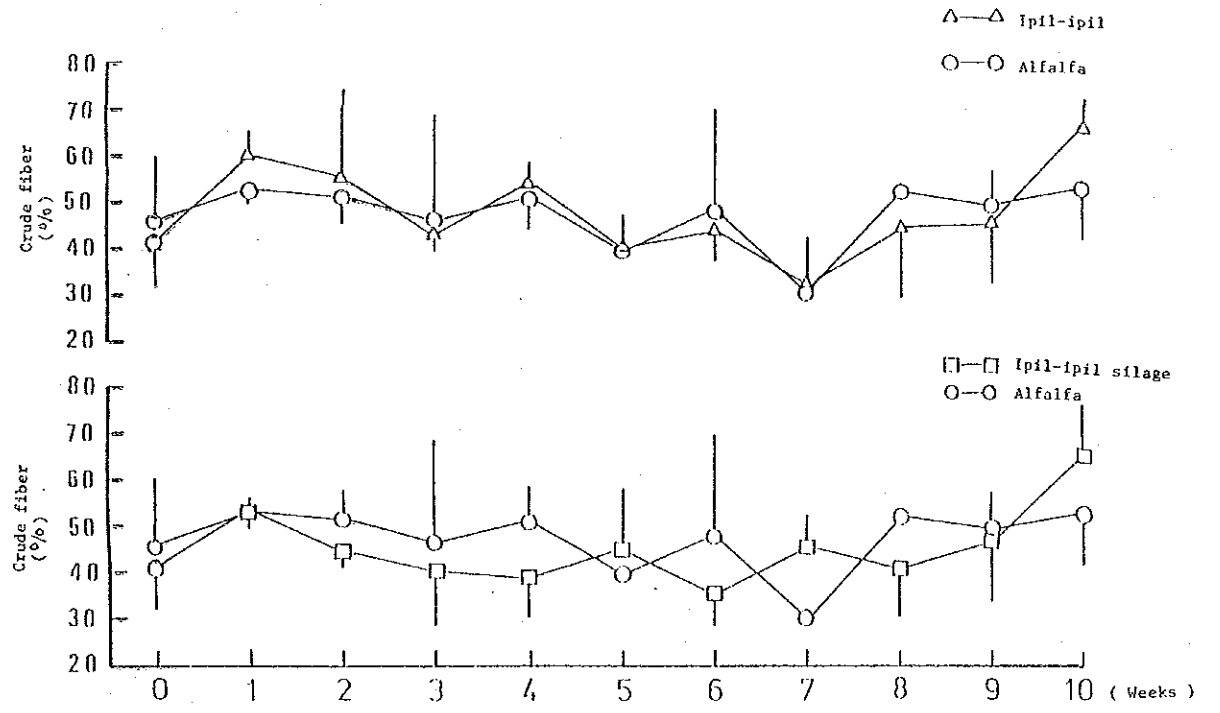


Fig.9. Digestibility of crude fiber. The bars indicate the S.D. of means for three animals.

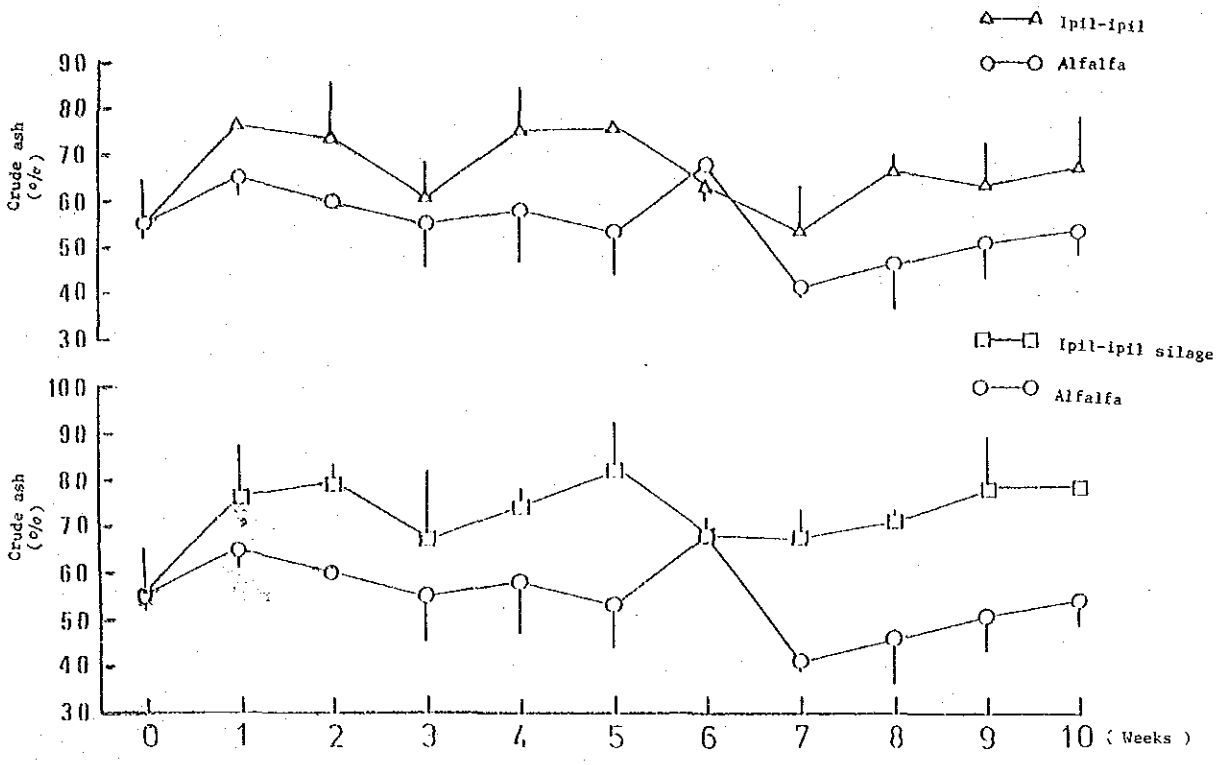


Fig.10. Digestibility of crude ash. The bars indicate the S.D. of means for three animals.

Table 3. Digestibility of the components of alfalfa, ipil-ipil and ipil-ipil silage pellets.

Group \ Week		0	1 ~10		
	Feed	Alfalfa	Ipil-ipil silage	Ipil-ipil	Alfalfa
Crude ash	A	55.19± 3.13			54.60± 9.16
	H	55.01± 9.61		** 67.02± 9.87	
	S	55.86±10.03	** 74.37± 8.44		
Crude protain	A	81.53± 2.76			79.57± 6.15
	H	78.04± 9.17		** 84.38± 7.56	
	S	79.82± 6.07	** 72.08± 6.47		
Crude fat	A	63.61± 6.89			65.45± 8.53
	H	59.85± 8.00		** 68.50±13.26	
	S	58.74±12.50	* 68.55±13.47		
Crude fiber	A	45.92±14.06			47.85±10.23
	H	41.49± 9.74		* 47.97±12.53	
	S	41.57± 9.23	45.57±11.43		
NFE	A	79.36± 4.58			75.94± 7.67
	H	79.59± 4.39		** 58.26±12.75	
	S	83.57± 3.20	** 60.61± 9.29		

Values are means ±S.D. (%) from three animals. Values that are significantly different from digestibility of alfalfa at 0 week are indicated by *($P < 0.05$) and **($P < 0.01$).

A: Alfalfa, H: Ipil-ipil, S: Ipil-ipil silage

7. Nutitive values of alfalfa, Ipil-Ipil and Ipil-Ipil silage

Table 4 shows nutritive values of alfalfa, Ipil-Ipil and Ipil-Ipil silage. The DCP values of Ipil-Ipil silage were similar to those of alfalfa and were higher than the DCP values of Ipil-Ipil. The TDN values of Ipil-Ipil silage and Ipil-Ipil were lower in comparison to values of alfalfa.

Table 4. Nutritive value of alfalfa, ipil-ipil and ipil-ipil silage pellets.

	DM	DCP	TDN
Ipil-ipil silage	87.76±0.18	13.78±1.24	49.86±5.34
Ipil-ipil	85.53±2.49	10.69±1.26	48.01±7.40
Alfalfa	86.44±0.21	13.66±1.06	57.74±5.10

(%, Means± S.D.)

8. Bodyweight changes in sheep fed alfalfa pellet, rations incorporated with Ipil-Ipil or Ipil-Ipil silage pellets

Fig.11 shows the DCP and TDN values in feeding rations that were included with Ipil-Ipil or Ipil-Ipil silage, and bodyweight changes in sheep fed these diets. The DCP intake of sheep in A, H and S groups averaged 4.37, 3.54 and 4.15 g/kg/day during the experimental period, respectively. The TDN intake of sheep in A, H and S groups averaged 18.46, 15.56 and 15.95 g/kg/day during the experimental period, respectively. Mean cummulative liveweight gains of sheep in A group increased gradually during the experimental period. Mean cummulative liveweight gains of sheep in group S and H both were variable from 1 to 3 weeks, but increased gradually after 3 weeks. The gains of sheep in group A, S and H were 4.8 ± 1.06, 4.3 ± 1.90 and 0.7 ± 1.80 kg (Mean ± SD) for ten weeks, respectively.

These results show that ensiling of Ipil-Ipil alleviates the low plasma T_3 levels and the high GOT, GPT levels of Ipil-Ipil toxicities, and that nutritive values of Ipil-Ipil

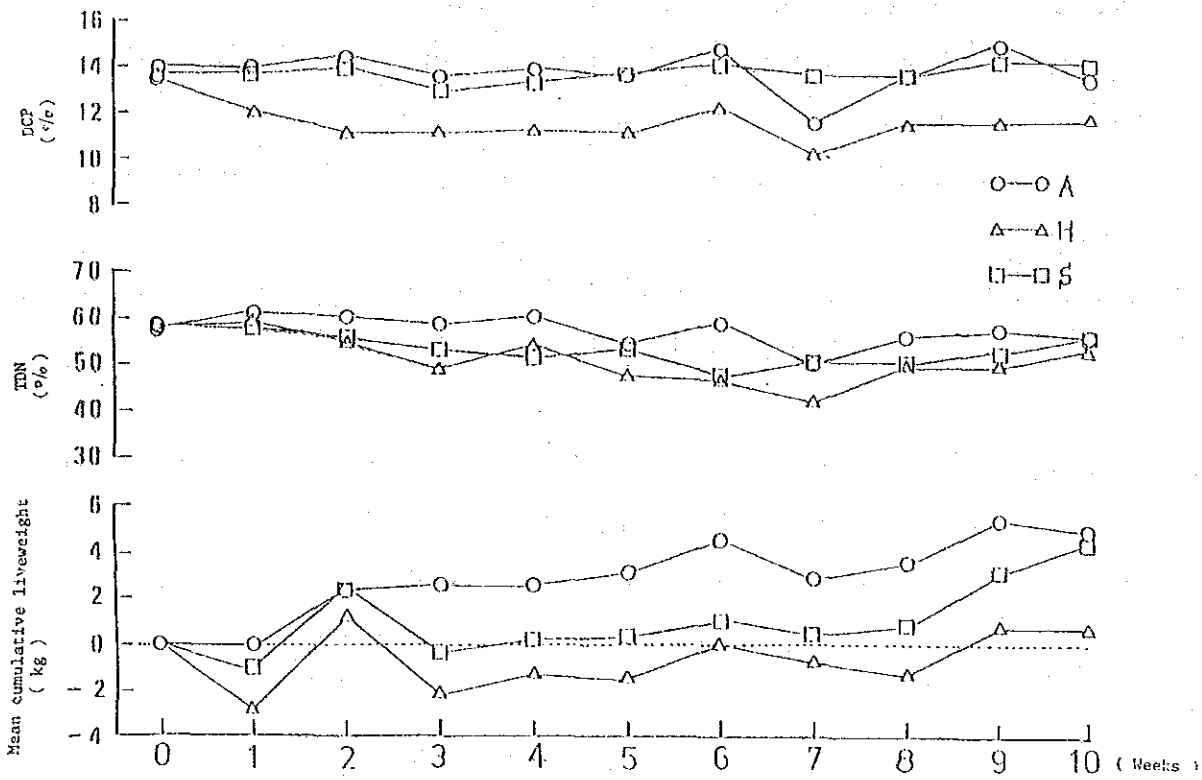


Fig.11. Mean cumulative liveweight change of sheep fed alfalfa (A), alfalfa 34% + Ipil-Ipil 66% (H) and alfalfa 34% + Ipil-Ipil silage 66% (S).

silage are similar to that of alfalfa in sheep. Thus, it seems that sheep are capable to use, with efficiency, levels of 66% of Ipil-Ipil silage in the diet.

Acknowledgments

The author is grateful to Nippon Shiryo Kyokai and JICA, for their assistance on this experiment.

REFERENCES

1) Jones, R. J., World Anim. Rev., 31 : 13-23. 1979.

2) Matsuda, Y., J. Okinawa Anim. Husband., 2 : 4-10. 1967.

3) Matsuda, Y., Sci. Bull. Coll. Agri. Univ. Ryukyus, 7 : 389-392. 1960.

4) Reis, P. J., D. A. Tunks and M. P. Hegarty, Aust. J. Biol. Sci., 28 : 495-501. 1975.
5) Szyszka, M. U. ter Meulen, Boonlom Cheva-Isarakul, S. Posri and N. Potikanond, Leuc. Res. Repts. 5 : 5-11. 1984.
6) Hongo, F., S. Tawata, Y. Watanabe and S. Shiroma, Jpn. J. Zootech. Sci., 57 : 223-230. 1986.
7) Chopra, I. J., J. Clin. Endocrinol. Metab. 34 : 938-947. 1972.
8) Hegarty, M. P., Schinckel, P. G. and Court, R. D., Aust. J. Agric. Res., 15 : 168-1964.
9) Tomoda, I., J. Jpn. Vet. Med. Assoc., 31 : 728-739. 1978.
10) Lowry, J. B., B. Tangendjaja and B. Maryanto, J. Sci. Food Agric., 34 : 529- . 1983.
11) Alvarez, F. J., A. Wilson and T. R. Preston, Trop. Anim. Prod., 2 : 288-291. 1977.

II c HISTOPATHOLOGICAL EFFCT OF GRAZING IPIL-IPIL IN SHEEP

INTRODUCTION

Sheep used in this experiment were divided into three groups ; A group (alfalfa only), S group (66 % of Ipil-Ipil silage combined with 33 % of alfalfa) and H group (66 % of Ipil-Ipil hay combined with 33 % alfalfa). Mimosine and DHP content in the diet of S group were 0.26 ± 0.02 % and 0.46 ± 0.06 %, respectively. In the diet of H group, mimosine and DHP content were 0.75±0.06 and 0.26±0.01 %, respectively. Eighteen tissues and organs obtained from tongue, esophagus, rumen, reticulum, omasum, abomasum, small intestine, rectum, liver, pancreas, lung, kidney, bladder, hypophysis, thyroid gland, pineal gland, adrenal gland, heart, cerebrum, cerebellum and spinal cord were investigated histopathologically. Some materials are fixed in Bouin solution and then dehydrated by ethanol, embedding in paraffin and cut into thin slices by 4-5 micrometer. Slices are stained with H-E solutions and inspected the praparations histopathologically.

RESULTS AND DISCUSSION

1. S GROUP

1) Rumen ; thickening and swelling of the cells in horny layers.

2) Liver ; light activation of Kupper's cells.

3) Lung ; light interstitial pneumonia (swelling of alveoli pulmonis and slight infiltration of round cells and degeneration of epithelium in bronchioli terminales).

4) Cerebrum, cerebellum and spinal cord ; Appearance of unknown crystalloid substances (tentative name ; X-corpuscle).

Size and shape of X-corpuscle are irregular (10 X 10 - 100 X 125 micrometer). These are slightly stained by hematoxylin and have many striped pattern at intervals of 2.5 micrometer. The significance of many striped pattern in this corpscle must

re-examine in future.

2. H GROUP

1) Thyroid gland; hypothyroidism (swelling of follicles and presence of connective tissue are observed).

2) Rumen; findings are as same as S group.

3) Ileum; partial necrosis and hemorrhages on the epithelium of mucous membrane.

4) Liver; findings are as same as S group.

5) Cerebrum, cerebellum andspinal cord; findings are as same as S group.

The influence of feeding various concentrations of Ipil-Ipil on tissues and organs in sheep have not been reported. Ipil-Ipil contains a toxic amino acid, mimosine which causes hair loss in various laboratory and domestic animals (Owen 1958). Ipil-Ipil is also known to cause fleece shedding, excessive salivation and also loss of hooves in sheep (Hegarty, Schinckel and Court 1964). But, in this study, these cases were not observed between S and H groups. It is considerd that the period of feeding and observation of 60 days might be too short or contents of mimosine and DHP might be also low level in the diet used in this experiment.

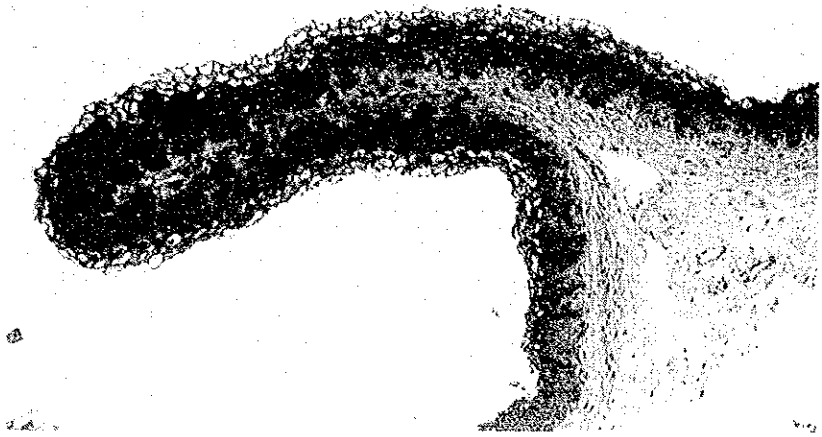
Weight of thyroid gland in A, S and H groups are 2.85g, 3.60g and 2.90g, respectively. But, from histopathological findings of thyroid gland, this organs both in A and S groups were normal, but in H group hypothyroidism is recognized. It is generally assumed that Ipil-Ipil silage is more excellent diet than Ipil-Ipil hay.

REFERENCES

Hegarty, M.P., Schinckel, P.G., and Court, R.D. (1964) ; Reaction of sheep to the consumption of Leucaena glauca Benth. and to its toxic principle mimosine. Australian Journal of Agricultural Reseach 15 : 153-67.

Owen, L.N. (1958) ; Hair loss and other toxic effects of Leucaena glauca (Jumbey). Veterinary Record 70 : 454.

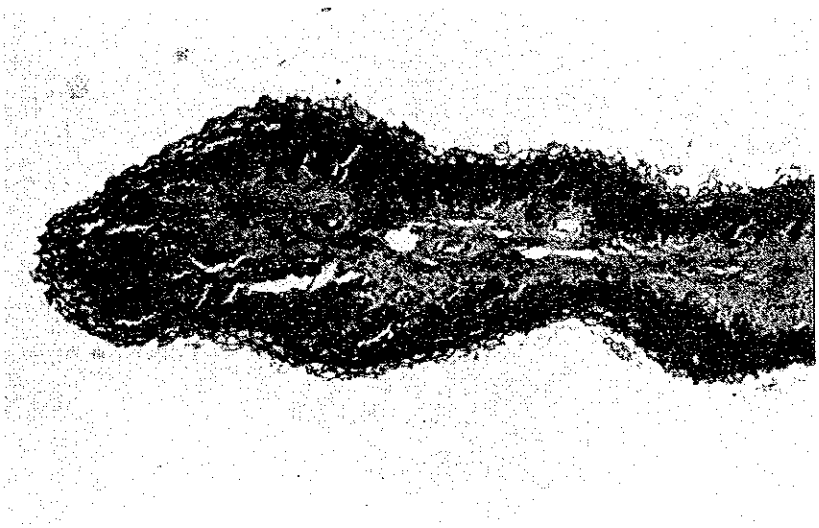
Fig.1



rumen

alfalfa group

Fig.2

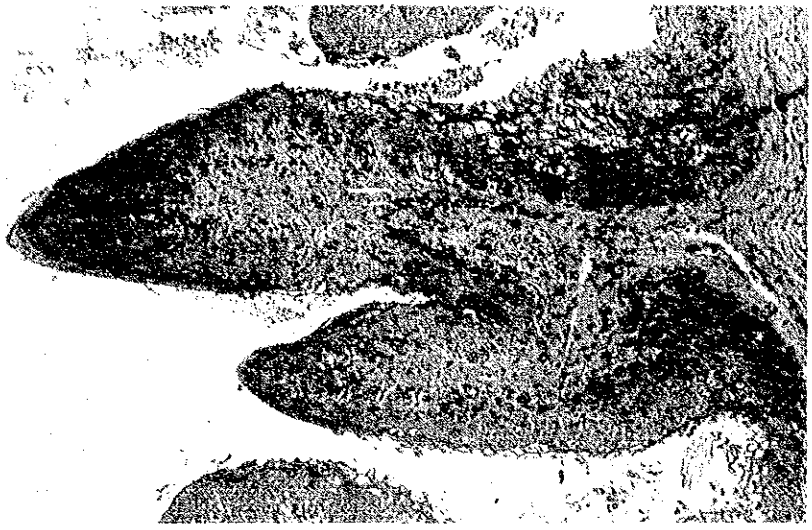


silage group

Fig.3

haylage group

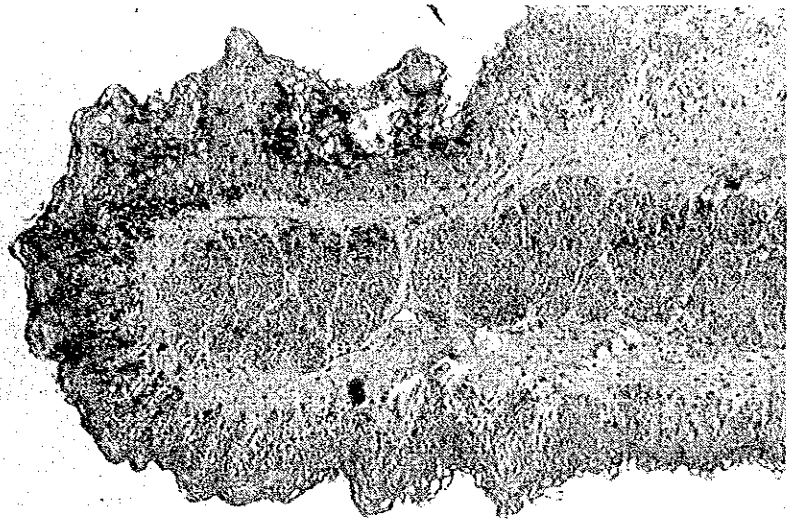
Fig.4



reticulum

alfalfa group

Fig.5

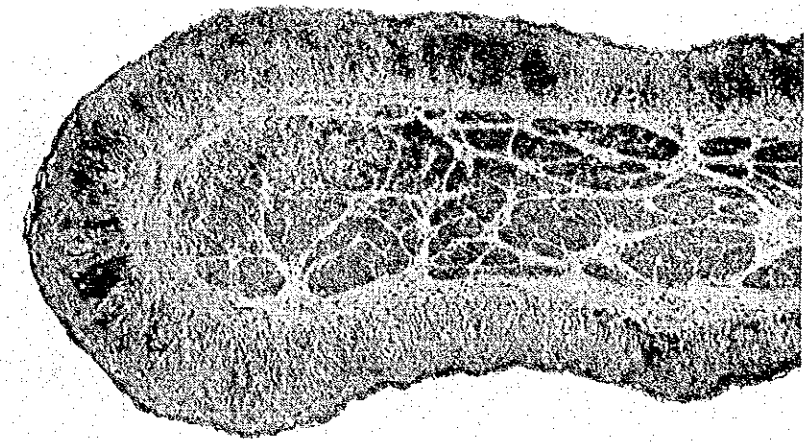


silage group

Fig.6

haylage group

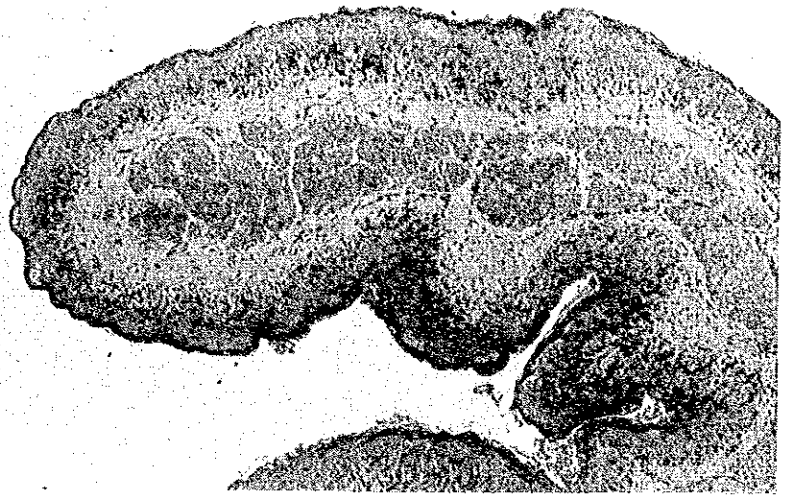
Fig.7



omasum

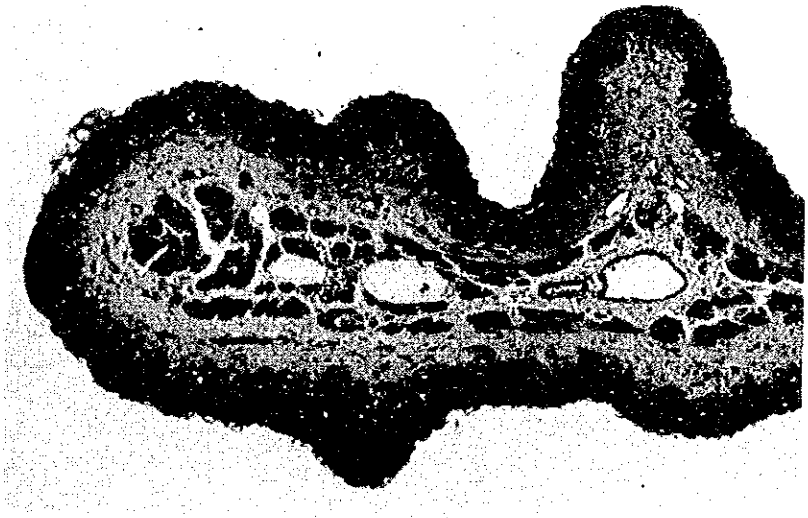
alfalfa group

Fig.8



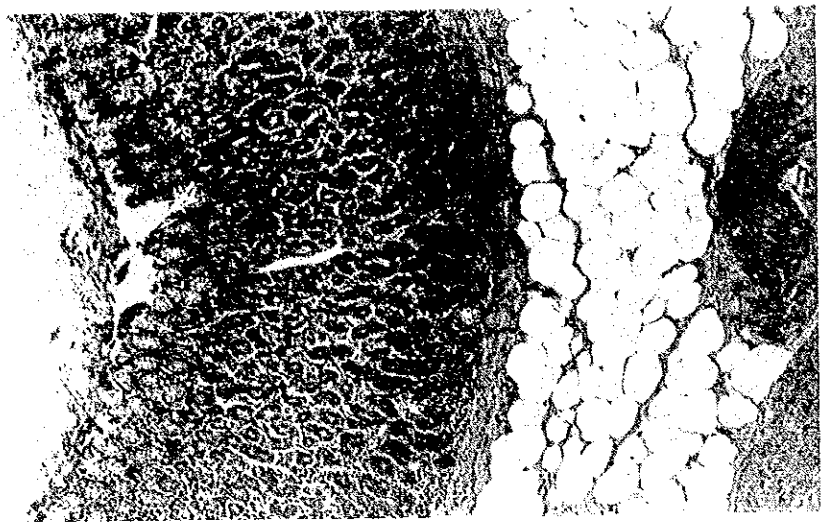
silage group

Fig.9



haylage group

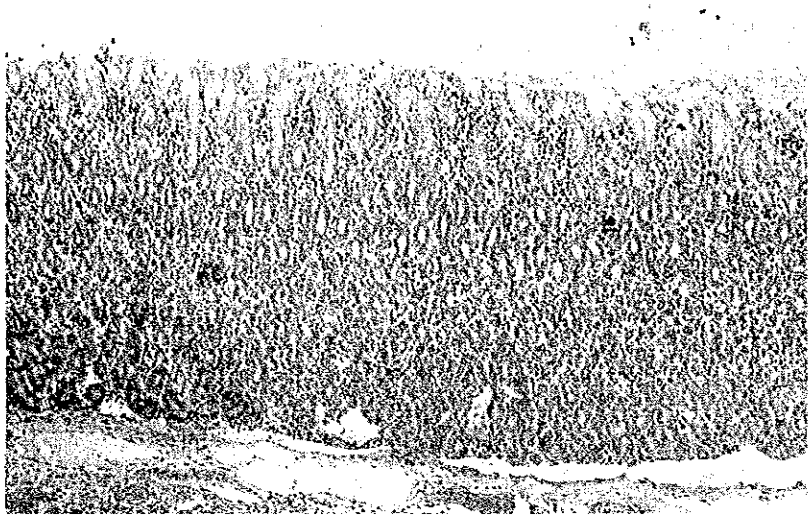
Fig.10



abomasum

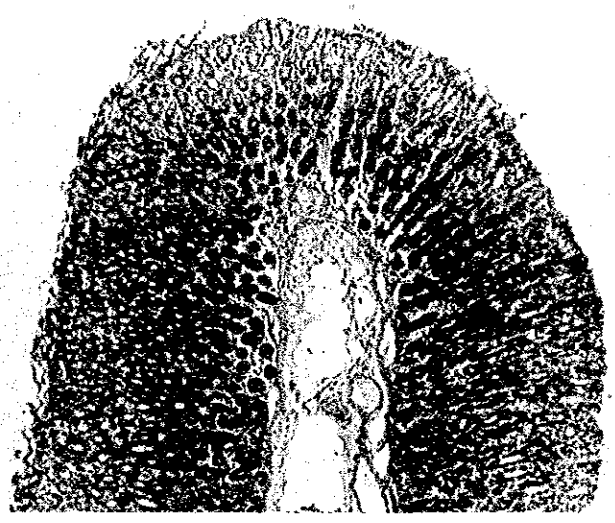
alfalfa group

Fig.11



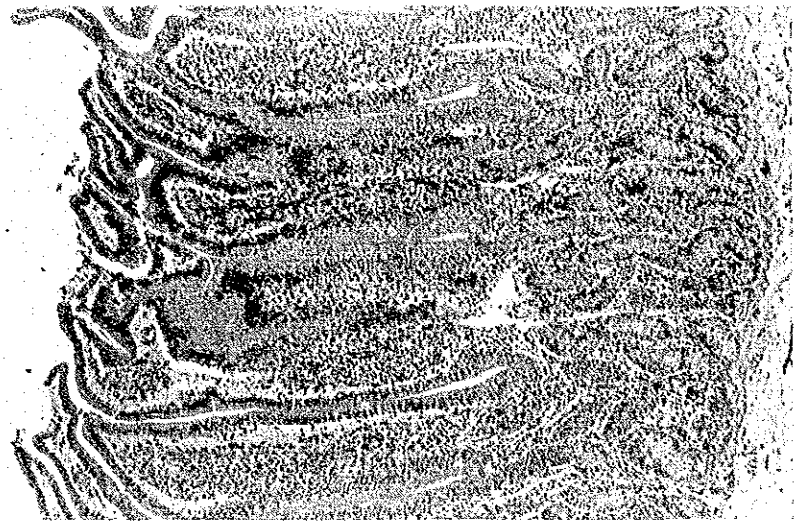
silage group

Fig.12



haylage group

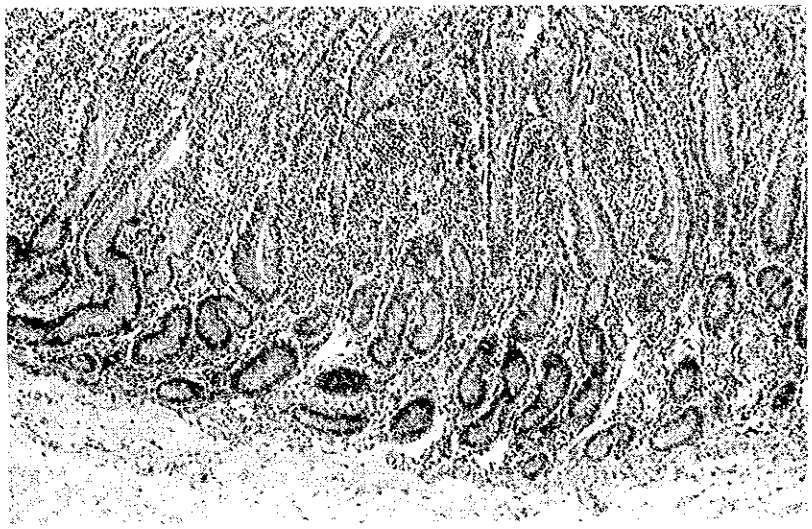
Fig.13



ileum

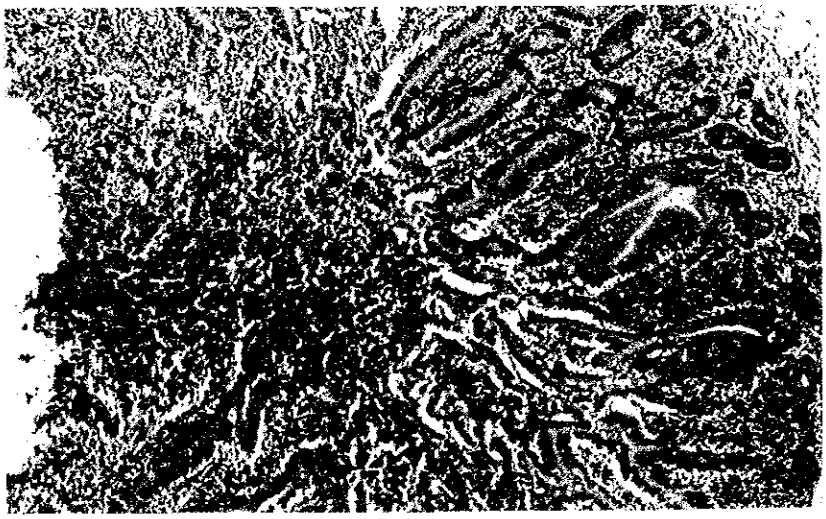
alfalfa group

Fig.14



silage group

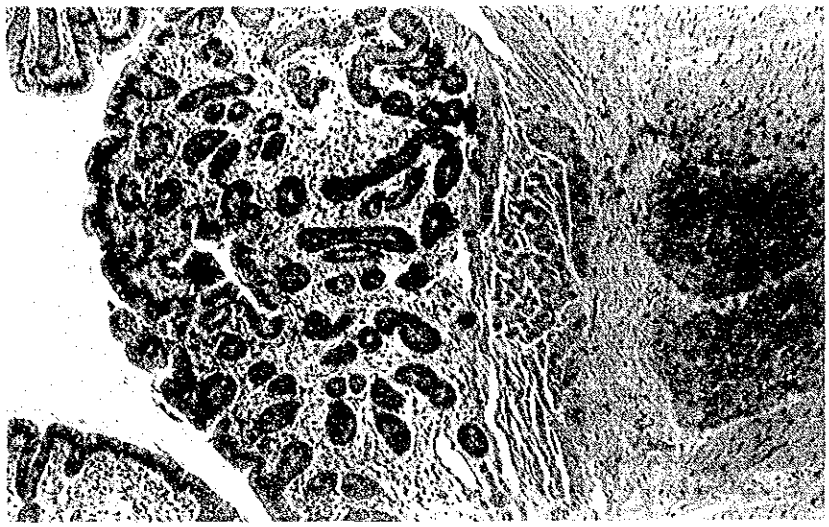
Fig.15



haylage group

hamorrhage and degeneration of epithelium

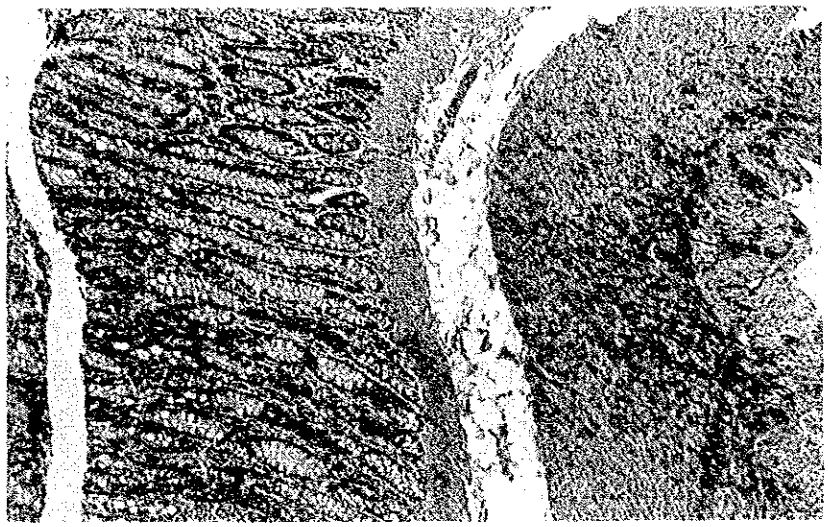
Fig.16



rectum

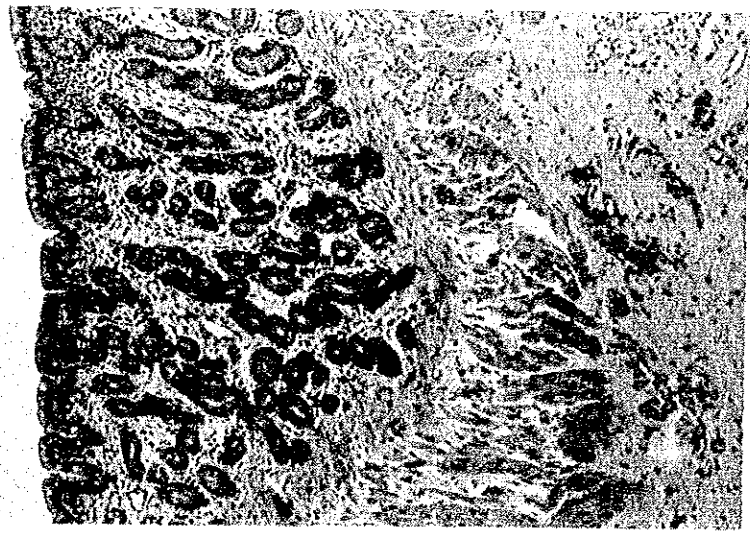
alfalfa group

Fig.17



silage group

Fig.18



haylage group