IMMUNOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDIES ON TWO GASTRIC ENZYMES IN NEONATE, YOUNG AND ADULT GOATS

H. Amasaki[1] S. Gozawa, R. Akuzawa[2],
K. Suzuki[3], M. Daigo and A. Andren[4]

Department of Veterinary Anatomy,
Nippon Veterinary and Zootechnical College,
Musashino-shi, Tokyo 180, Japan.

Summary

The present paper demonstrates the expressions and amounts of pepsinogen and prochymosin in neonate, young and adult goat's proper gastric glandular regions by the immunochemical and the immunohistochemical analyses with the anti-bovine pepsinogen serum and anti-bovine chymosin serum.

Each bovine serum was demonstrated to have reactivities against corresponding goat's antigen by immunochemical analyses and enzymatic activities.

The anti-pepsinogen was higher in the new born animals than the maternal milk feeding one, suggesting that the maternal milk might control the pepsinogen production in the proper gastric glands. The patterns of prochymosin expression in the goats was similar to that in cattle.

(Key Words: Gastric Proteinase, Milk Feeding, Goat Stomach)

Introduction

The dietary ingredients drastically changes and induces the structural and functional adaptations of the digestive organs during weaning periods in the cattle (Huber, 1968; Tamate et al., 1962). Representative gastric proteinases, chymosin and pepsin, show drastic changes in the activities and amounts in calves after birth (Andrén, 1982; Garnot et al., 1974). Chymosin has high milk clotting activities, but a low protein digestive activity, and is produced mainly before weaning stages (Andrén et al., 1982). The milk clotting and proteolytic activities of pepsin are opposite to chymosin (Andrén et al., 1981; Baudys et al.,

1988; Hsu and Raine, 1984). These adaptive changes in both enzymes have been studied in cattle, but not in goats. If the same phenomena occurs, the experimental control would be much easier in goats than in cattle, since the former is much smaller in size.

The present paper describes the immunochemical and immunohistochemical demonstrations of these two enzymes in the proper gastric glandular mucosa of abomasum in non-feeding neonates, maternal milk feeding young animals and conventional diets feeding adult goats.

Materials and Methods

Animals

In total, 13 male Shiba goats were used in the present examinations; 3 newborn animals before suckling milk 12 hours after birth, 7 young animals fed by maternal milk alone on days 8 and 14 after birth, 3 adult animals maintained by conventional diets more than a year.

Anti-bodies and antigens

The purified bovine antigen and anti-sera used for the analysis of the goat prochymosin and pepsinogen were originally raised against the bovine chymosin, and pepsinogen. Cross-reactivities of the anti-bovine chymosin serum to the prochymo-

sin, and the anti-bovine pepsinogen serum to the bovine pepsin have been described earlier by Andrén et al. (1982, 1981). We confirmed the specific cross reactivities between the bovine and goat's extractions obtained by gel filtration, Sephadex G 100 (Phalmacia, Co., Ltd., U.S.A.). The levels of the proteolic activities of each gel fraction was examined by the hemoglobin digestion test, and the levels of milk clotting activities according to the Arima's report (1967). The levels of immuno-reactivity against the present sera were analysed by the ELISA methods (Johnson et al., 1978).

Preparations of samples for immunochemical examinations

The crude samples were extracted from the proper gastric glandular regions in 0.02 M phosphate buffered saline, pH 6.1 (PBS) with proteinase inhibitor (1 mM ethylendiaminetetraacetic acid disodium salt dihydrate, 1 mM p-chloromercuribenzenesulfonic acid. Sigma chemical company, U.S.A.) (Foltmann et al., 1977). The 2 g of the fresh epithelial layer was separated from the underneath connective tissues manually, miced in 1 ml PBS and was broken by ultrasonic generator (model US-150; Nissei Co., Ltd., Japan). The suspension was precipitated by saturated ammonium sulfate. The precipitate was dialyzed against 0.01 M PBS pH 6.1 at $0\sim4^{\circ}$C and then lyophilized. The samples were stored at -20°C until assayed.

Immuno-blotting (Legender and Matsudaira, 1988)

After electrophoresis with 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), the gel fractions were blotted on the immobilon transfer membranes (Millipore, Co., Ltd., U.S.A.) by Milliblot-SDA (Millipore, Co., Ltd.). The membranes were incubated with the antipepsinogen or anti-chymosin serum. Each membrane was then incubated again with the goat anti-rabbit IgG-HRP (E Y Laboratories Inc., U.S.A.). The color was developed with 3, 3'diamino-benzidine, tetrahydrochloride (DAB) (Woko pure chemical industries, Ltd., Japan) in the presence of hydrogen peroxide. The purified bovine chymosin (Sigma chemical company, U.S.A.) and the purified bovine pepsin were used for the molecular markers.

Enzyme-linked immunosorbent assay (ELISA) (Johnson et al., 1978)

The amounts of the prochymosin and pepsinogen in the proper gastric mucosa were analysed by the ELISA method using the alkaline phosphatase labeled 2nd antibody.

Immunohistochemistry (Hsu and Raine, 1984)

The proper gastric glandular region was fixed in Zamboni solution. The samples were dehydrated in a series of ethanol, embedded with a paraffin and serially sectioned at 5 μm.

The anti-sera were prepared as described above. The sections were incubated with the first antibody (anti-chymosin serum; x 4,000 or anti-pepsinogen serum; x 3,000) at 4°C over night, and the avidin-biotin peroxidase complex method (ABC methods; Zymed Laboratories Inc., U.S.A.) was applied for detection of prochymosin and pepsinogen. 3-amino-9-ethylcarbazole (AEC) was used for the coloration. They were counter stained by hematoxilin and observed under a light microscope.

Results

Immuno-blotting test

The molecular size of goat chymosin from the crude extracts were detected at about 37 kDa, and the pepsinogen at about 40 kDa. Each molecular size of goat chymosin and pepsinogen was close to that of the purified bovine antigen, respectively.

The crossreactivity to the anti-chymosin was detected strongly in the animals maintained by maternal milk alone, while that to the anti-pepsinogen serum was in adult animals (figure 1). The protein digestive activities (PA) was equal to that of the ELISA peak reactivities against the anti-pepsinogen serum (figure 2-a). The peak of the milk clotting activities (MCA) was equal to that of the ELISA peak reactivities against the anti-chymosin serum (figure 2-b).

ELISA for the levels of pepsinogen and prochymosin.

The amount of prochymosin was high at maternal milk stage, and was low in the adults. The amount of pepsinogen was almost stabilized in all stages, and was the highest in young goats maintained by maternal milk alone (table 1).

TABLE 1. AMOUNT OF PROCHYMOSIN AND PEP-
SINOGEN IN THE GOAT'S ABOMASUL
MUCOSA OF THE PROPER GASTRIC STO-
MACH BY ELISA (O.D./mg TISSUE) M±SE

Sample	(number)	Prochymosin	Pepsinogen
New born	(3)	0.79±0.003	1.55±0.06
Maternal milk stage	(7)	2.30±0.11*	1.80±0.11*
Adult	(3)	0.03±0.03*	1.60±0.05

*Statistically different (p < 0.05) from the respective
values obtained with new born animals.

2-a

Enzyme assay(Protease activity) and ELISA(Pepsinogen)

2-b

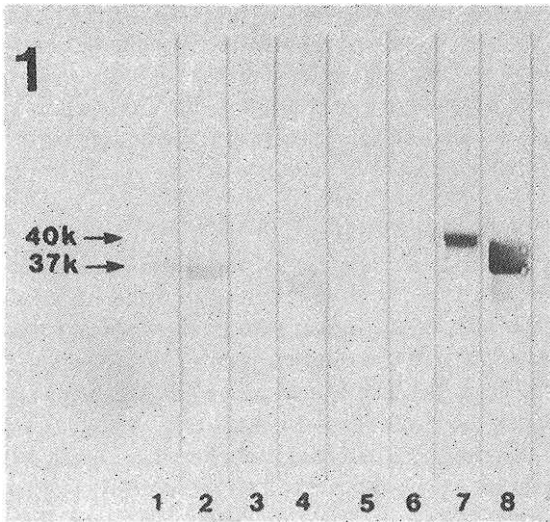Enzyme assay(Milk clotting activity) and ELISA(Prochymosin)

Figure 1. Immunoblots with anti-chymosin and
anti-pepsinogen serum after 10% SDS-
PAGE. Lanes 1-4 with anti-chymosin
serum and lanes 5·8 with anti-pepsino-
gen serum; lanes 1 and 5 represent crude
gastric extract of new born animals,
lanes 2 and 6 that of young animals on
days 8 after birth maintained by mater-
nal milk alone, lanes 3 and 7 that of
adult, lane 4 purified bovine chymosin
and ane 8 purified bovine pepsinogen.
Anti-chymosin reacted bands are
located at about 37 kDa, anc anti-pep-
sinogen at about 40 kDa.

Figure 2. The crossreactivities between the bovine
and goat specimens from the crude ex-
traction of the goat's stomach in the
maternal milk stage obtained by gel
filtration, Sephadex G 100 (▲), 2-a:
reactivities against of the anti-bovine
pepsinogen serum. The ELISA peak of
the reactivities against the anti-pepsino-
gen serum (Bar) is equal to the peak of
the proteolic activities (●). 2-b: reacti-
vities against the anti-chymosin serum.
The anti-chymosin serum (Bar) is equal
to the peak of the milk clotting activi-
ties (●).

Immunohistochemistry

The cells immunoreacted by the anti-chymosin
serum and the anti-pepsinogen serum were mainly
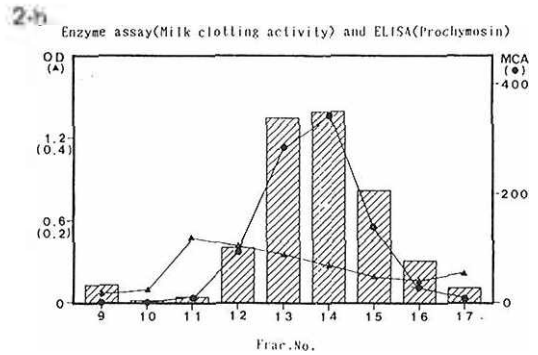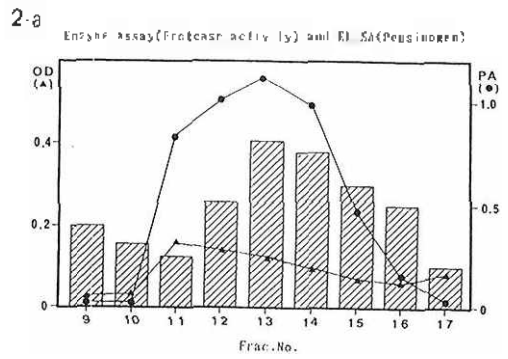located in the gastric chief cells. The cells reacted
against anti-chymosin serum was detected at
luminal surface area of the goat of 8 days after
birth (figure 3-b).

The immunoreactivity against the anti-chymo-
sin serum was strongest in young animals main-
tained by maternal milk alone, while the reactivity
against the anti-pepsinogen serum was stronger in
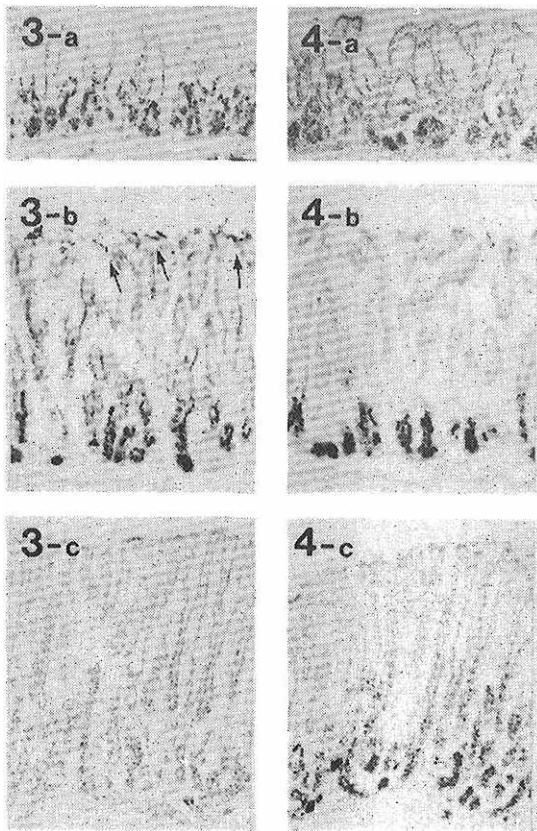young than in adult (figures 3-a, 3-b, 3-c, 4-a,
4-b, 4-c).

Figure 3 and 4. Slides of goat's proper gastric mucosa stained by the anti-chymosin and anti-pepsinogen serum with ABC methods respectively. Figures 3-a, 3-b, and 3-c treated with anti-chymosin serum, and 4-a, 4-b and 4-c with anti-pepsinogen serum. a; new born goat before starting diets. b; young goat on day 8 after birth maintained by maternal milk alone. c; adult goat maintained by conventional diets. x160. Arrows: the surface cells reacted by the anti-chymosin serum.

crude extraction of the goat's stomach. The molecular sizes by the SDS-PAGE of goat's pepsinogen and chymosin have been reported that there is a considerable homology in the amino-acid sequences of these two gastric enzymes in the cow (Baudys et al., 1988; Foltmann et al., 1977). The molecular weight of goat chymosin and pepsin obtained in the present study supports these previous reports. Each anti-bovine serum (pepsinogen and chymosin) was further demonstrated to react against the goat's antigen (pepsin and chymosin) by its enzymatic activities.

Enzyme-linked immunosorbent assay (ELISA)

Higher pepsinogen levels in the maternal milk feeding stage than in the new born animals suggest that the maternal milk might control the pepsinogen production in proper gastric glands as in cattle (Andrén et al., 1981, 1982).

Immunohistochemistry

Positive reactions against anti-pepsinogen serum and the anti-chymosin serum were detected at the chief cells of the proper gastric mucosa in all stage examined. The levels of reaction against these antibodies in the histological specimens were equivalent to the result of the ELISA.

Therefore, it is concluded that these two proteinases were produced and secreted mainly by the gastric chief cells. Some epithelial surface mucosal cells sometimes had a weak reaction against those anti-bodies in the present study. This result may suggest that some mucosal cells may have capabilities to produce both mucous and proteinases at a time. This might suggest that mucous cells and the chief cells have a close relationship in their origin. Futher study on expression of the m-RNA of pepsinogen and prochymosin would be necessary to rule out the possibility that the mucous cells might incorporate the enzymes.

Acknowledgements

Discussion

Gel filtration and Immuno-blotting (SDS-PAGE)

Test for the cross-reactivities of the anti-sera against the goat antigens.

The present results suggested that the anti-bovine chymosin and anti-pepsinogen sera recognized the goat's chymosin and pepsin from the

Literature Cited

Andrén, A., L. Björck and O. Claesson. 1982. Immuno-histochemical studies on the development of pro-

chymosin- and pepsinogen-containing cells in bovine abomasal mucosa. J. Physiol. 327:247-264.

Andrén, A., L. Björck and O. Claesson. 1981. Effect of supplementary milk-feeding on content of chymosin in the abomasal mucosa of concentrate-fed calves. Swed. J. Res. 11:11-15.

Arima, K., S. Iwasaki and G. Yamura. 1967. Milk clotting enzyme from microorganisms. Part I. Screening test and the identification of the potent fungus. Agr. Biol. Chem. 31(5): 540-545.

Baudys, M., T.G. Erdene, V. Kostaka, M. Pavlik and B. Foltmann. 1988. Comparison between prochymosin and pepsinogen from lamb and calf. Comp. Biochem. Physiol. 89B(2):358-391.

Foltmann, B., V.B. Pedersen, H. Jacobsen, D. Kauffman and G. Wybrandt. 1977. The complete amino acid sequence of prochymosin. Pro. Natl. Acad. Sci. 74(6):2321-2324.

Garnot, P., E. Valles, J.L. Thapon, R. Toullec, R. Tomassone and B. Ribadean-Dumas. 1974. Influence of dietary proteins on rennin and pepsin content of

preruminant calf vell. J. Dairy Sci. 41:19-23.

Hsu, S. and M. Raine. 1984. The use of avidin-biotin-peroxidase complex (ABC) in diagnostic and research pathology. In: Advances in Immunochemistry, R.A. DeLellis, Ed., Mason Publishing USA, Inc., New York, pp.31-42.

Huber, J.T. 1968. Development of the digestive and metabolic apparatus of the calf. J. Dairy Sci 52. 1303-1315.

Johnson, G.D., E.J. Holborow and J. Dorling. 1978 Immunofluorescence and immunoenzyme techniques. In: Handbook of experimental immunology. (Weir, D.M. ed.) 3rd Ed., Blackwell Scientific Publications, Oxford.

Legender, N. and P. Matsudaira. 1988. Direct protein microsequencing from immobion-P transfer membrane. Biotechniques 6(2):154-159.

Tamate, H., A.D McGilliard, N.L. Jacobson and R. Getty. 1962. Effect of various dietaries on the anatomical development of the stomach in the calf. J. Dairy Sci. 45:408-420.