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Cytochemical and Biochemical Characterization of Alkaline Phosphatase in Swine Adenohypophysis

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Summary

In the glandular cells of swine anterior pituitary gland (S-APG cells), alkaline phosphatase (ALPase) activity was apparent on the cell membrane and in the cytoplasm. The azo dye and lead citrate method gave similar results on the intracellular localization of the enzyme. The best results were obtained when β-glycerophosphate was used as a substrate in the lead citrate method. The ALPase activity on the cell membrane was completely inhibited by the preincubation of 2 mM L-cysteine. The cells with ALPase activity (ALPase cells) were considered as gonadotrophs because they were more in females and castrated male than males, mainly distributed in the zona tuberalis and stained simultaneously with periodic acid-shiff reaction or aldehyde thionin reaction. The diversity of localization and intensity of intracellular ALPase activity reflected the different state of secretory activity of the cells. Anterior pituitary ALPase was electrophoretically yielded as a single band. By the prior incubation with neuraminidase, the mobility of anterior pituitary ALPase was appreciably reduced, indicating that the isoenzyme which is different from liver and intestine ALPase isoenzymes exists in swine anterior pituitary.

Functional modulation in the glandular cells of anterior pituitary gland (APG cells) has been cytochemically estimated as the relative intensity and localization of several enzyme activity. The activities of nucleoside phosphatase (1, 2), thiamine pyrophosphatase (3), acid phosphatase (4, 5) and oxidative enzymes (6-8) are likely to be associated with the synthesis and condensation of secretory granules, and the level of hormone secretion. However, since the reaction products of the enzymes are detected in all types of APG cells, the information is inadequate to evaluate the functional changes of distinct types of APG cells.

In the previous study, we reported that S-APG cells are positive for alkaline phosphatase (ALPase) reaction and indicated the possibility that the ALPase

activity restrict in gonadotrophs (9). In the present study, APG cells showing ALPase activity is detailed and the enzyme localization is discussed with relation to the cell function. In addition, ALPase in the gland is histochemically and biochemically characterized.

Materials and Methods

Animals and Tissues

Crossbred swine (Landrace × Large White) consisted of two each of fetuses with 20 cm body length (fetus I) and with 30 cm body length (fetus II), 1, 2, 3, 4 and 5 months old of females, and four each of 6 months old of males, castrated males and females. The anterior pituitary glands (S-APG), liver and intestine were removed freshly from the animals which were killed in a local slaughterhouse and fixed for 8 hrs in neutral formal-calcium at 4°C. After washing for 24 hrs in cold saline, the tissues were frozen in a mixture of acetone and dry ice and stored at −80°C until use.

Histochemistry

The cryostat sections of 6 μm were prepared and alkaline phosphatase (ALPase) activity was detected by the lead citrate method (10) and the azo dye method (11). More detailed description of the procedures has been described elsewhere (9). In the lead citrate method, β-glycerophosphate was used as a substrate and the final pH was adjusted to 9.2-9.4. Histochemical specificity of ALPase was examined by using various substrates (phosphothreonine, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, phosphoenol pruvate, phosphoserine, ribose phosphate, glucosamine-6-phosphate) and a specific inhibitor of ALPase, L-cysteine (12). The reaction was carried out by the same procedure as the lead citrate method except that each substrate instead of β-glycerophosphate was added to the reaction mixtures. All chemicals were purchased from Sigma Chemical (St. Louis. MO. USA). In the control experiments, incubation without the substrate was performed. The cells positive for ALPase reaction (ALPase cells) was counted on 20 areas by scare micrometer at random. The percentages of ALPase cells per glandular cells of S-APG (S-APG cells) and ALPase cell type per ALPase cells were calculated in the portion of pars distalis except the zona tuberalis (zona distalis : portion I) and the zona tuberalis (portion II). In some experiments, ALPase reaction and periodic acid-shiff (PAS) or aldehyde thionin (AT) reaction were combined for the simultaneous demonstration of ALPase activity and PAS- or AT-positive substances in S-APG cells (13).

Electrophoresis

Biochemically, ALPase from anterior pituitary gland (APG-ALPase), liver

(liver ALPase) and intestine (intestine ALPase) of swine was prepared in a partially purified form by the procedure described for the human intestinal enzyme (14). The activity was assayed 37°C by using p-nitrophenyl phosphate in which the release of p-nitrophenol was measured at 410 nm (15). The specific activities were expressed as μ moles of p-nitrophenol released per min per mg protein. Protein was measured by the method of Lowry *et al.* (16) with bovine serum albumin as a standard. Polyacrylamide gel disc electrophoresis was accomplished following the method developed by Ornstein and Davis (17) but employing 10% acrylamide in the lower gel and 3.3% acrylamide in the spacer and sample gels. Spacer gel solution (200 μl) and sample gel solution (100 μl) containing an appropriate dilution of the enzyme were layered above a lower gel. After the run, the enzyme portion was detected by incubating the gel cylinder in the medium of azo dye method for 20 min at room temperature. To investigate the binding of sialic acid to ALPase, the enzyme was pretreated with neuraminidase (Wako Pure Chemical Ind., Osaka Japan) by the method of Saraswathi and Bachhawat (18).

Statistical Analysis

A statistical difference in the percentage of ALPase cells was determined by student's t test.

Results

Histochemical Characters of ALPase in S-APG Cells

The reaction product of ALPase in S-APG cells was detected along the cell membrane as well as in the cytoplasm by the lead citrate method (Fig. 1) and the azo dye method (Fig. 2). The activity was also observed in the Golgi area by the both methods (inset in Fig. 1-2). The lead citrate method gave finer reaction product although the intensity was slightly weaker than that with the azo dye method. The preincubation with 2 mM L-cysteine completely inhibited ALPase activity in S-APG cells. Replacement of the substrate from β-glycerophosphate to monophosphate compounds led to the less reaction intensity (Table 1). For the ALPase activity on the cell membrane, β-glycerophosphate yielded the highest intensity. Phosphothreonine and glucose-1-phosphate gave the moderute intensity but the background was also stained. Using five monophosphate compounds, the reaction product was not observed. For the cytoplasmic activity, the similar results were obtained in S-APG cell, liver and intestine when β-glycerophosphate, phosphothreonine, glucose-1-phosphate and phosphoenol pruvate were used as a sabstrate (Table 1). However, the activities except for β-glycerophosphate remained slightly after the preincubation of 2 mM L-cysteine.

TABLE 1. *Spectrum of Phosphate-splitting with ALPase Localized in S-APG Cells*

Substrate	APG-cells		Liver	Intestine
	Mb	Cy	Cy	Cy
B-Glycerophosphate	⋕	⋕	⋕	⋕
Phosphothreonine	⋕	+	+	⋕
Glucose-1-phosphate	⋕	+	+	⋕
Glucose-6-phosphate	−	−	+	+
Fructose-6-phosphate	−	−	⊥	+
Phosphoenol pruvate	⊥	⋕	⋕	⋕
Phosphoserine	−	−	⋕	⋕
Ribose phosphate	−	−	−	−
Glucosamine-6-phosphate	−	⋕	⊥	⊥

The intensity of ALPase activity: −, negative; ⊥, very weak; +, weak; ⋕, moderate; ⋕ ~ ⋕, strong. Enzyme distribution: Mb, cell membrane; Cy, cytoplasm.

Diversity of Localization and Intensity of ALPase Activity

ALPase cells were mainly distributed in portion II although they occurred throughout the gland. The number was significantly more in portion I of females and in portion II of females and castrated males (Table 2). In APG of adult swine, ALPase cells were divided into four types by the localization and intensity of intracellular activity as follows; i) First cell type (Type 1 ALPase cell) which revealed the strong activity in the cytoplasm (Fig. 3), ii) Second cell type (Type 2 ALPase cell) which retained the activity on the membrane and in the cytoplasm but the intensity changed from cell to cell (Fig. 4), iii) Third cell type (Type 3 ALPase cell) in which the strong activity was constantly localized on the cell membrane (Fig. 5) and iv) Forth cell type (Type 4 ALPase cell) which showed the

TABLE 2. *Percentages of ALPase Cells in S-APG*

Sexes	% of ALPase cells	
	portion I	portion II
ML	12.1±2.46	21.9±2.94
FL	16.3±1.83*	34.1±2.86**
CM	13.9±1.64	30.9±3.55**

Values are the mean percentages for four animals.
Sexes: ML, males; FL, females; CM, castrated males.
**, <0.01; *, <0.05 compared to males.

TABLE 3. *Distiribution of ALPase Cell Type*

	Sex	Type of ALPase cell			
		I	II	III	IV
Portion I	ML	39-44	22-28	20-26	3-7
	FL	12-17	20-27	46-52	6-12
	CM	7-11	12-17	31-38	31-39
Portion II	ML	20-26	39-47	25-31	5-8
	FL	10-17	18-26	44-50	9-16
	CM	6-9	12-18	33-39	34-42

Values are the range of percentages for four animals. Sexes: ML, males; FL, females; CM, castrated males.

very weak activity in the cytoplasm (Fig. 3). The Golgi area was frequently positive for ALPase reaction in type 2 and 3 ALPase cells. ALPase cells were generally round or oval in shape. The size of type 1 ALPase cell was relatively smaller than that of other 3 types. The high percentage of type 1 ALPase cell was observed in portion I of males. In females, type 3 ALPase cell was dominant to other types. In castrated males, the percentage of type 4 ALPase cell was extremely higher than that in males and females (Table 3). By the combination method for ALPase and PAS or AT reaction, ALPase cells were stained with PAS or AT reaction (Fig. 6). The staining intensity was relatively weak in type 2 and 3 ALPase cells, and very weak in type 1 and 4 ALPase cells.

Cytodifferentiation of ALPase Cells

In fetus I, S-APG cells did not show any ALPase activity although the sinusoids were positively reacted (Fig. 7). ALPase cells showing diffusely the weak activity in the cytoplasm was observed first in fetus II (Fig. 8). The substantial activity on the cell membrane and in the Golgi area was detected in the APG cells of 2 months old in which the cells as well as type 3 ALPase cell occasionally occurred in the zona tuberalis (Fig. 9)

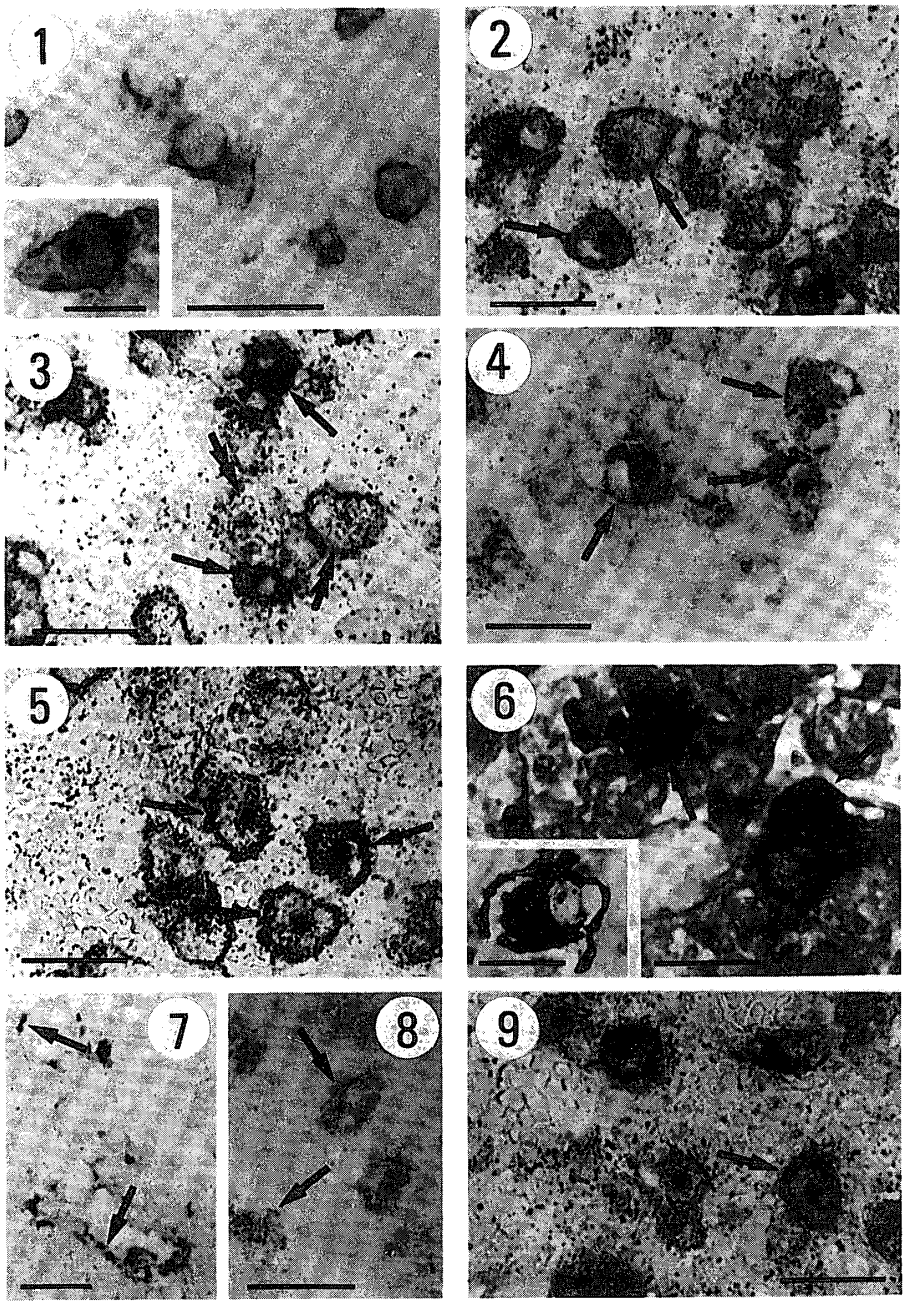
Electrophoretical Characters of APG-ALPase

To investigate the distinctiveness of isoenzyme of ALPase in S-APG, APG-ALPase, liver ALPase and intestine ALPase were partially purified. Finally, the specific activity was 1.32 μmoles/min/mg protein in APG-ALPase, 0.86 μmoles/min/mg protein in liver ALPase and 15.1 μmoles/min/mg protein in intestine ALPase. After the electrophoresis, the site of APG-ALPase was yielded as a single sharp band in the gel cylinder (Fig. 10). The mobility was similar to that of intestine ALPase. In liver ALPase, two bounds were clearly distinguished. A neuraminidase preincubation caused a marked decrease in the mobility of

APG-ALPase as well as liver ALPase. However, intestine ALPase was resistant to the neuraminidase treatment (Fig. 10).

Discussion

The present study confirmed the intracellular localization of ALPase activity in S-APG cells since the methodological data provided the uniform and reproducible product for the visualization. The satisfactory localization of ALPase



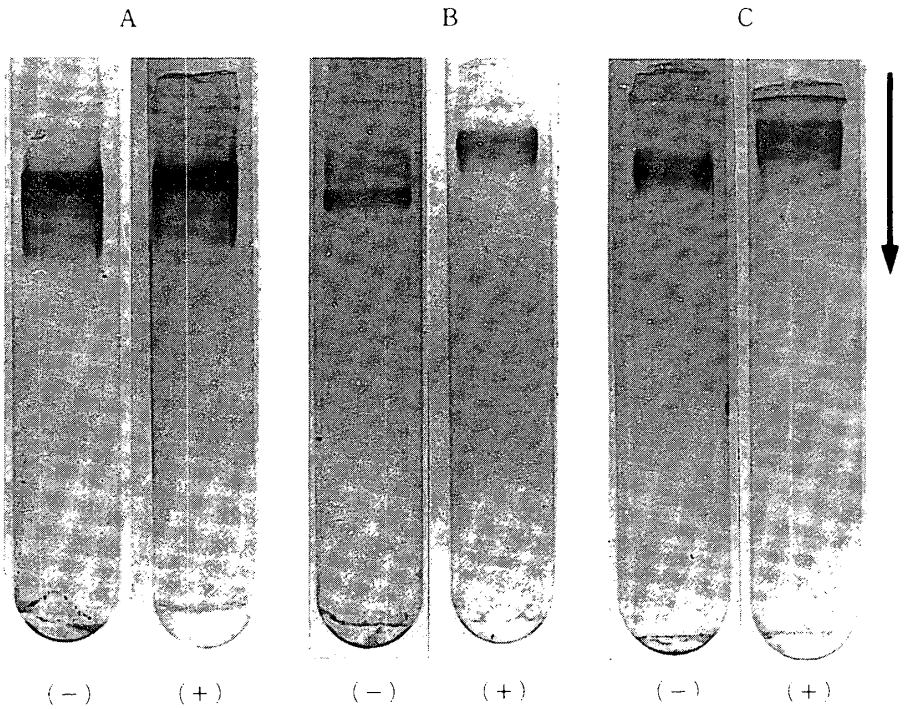


FIG. 10. Polyacrylamide gel enzymograms on swine ALPase with (+) or without (−) the pretreatment of neuraminidase.
A, intestine ALPase; B, liver ALPase; C, APG-ALPase.
The arrow indicates the direction of migration.

activity in S-APG cells was found when β-glycerophosphate was used as a substrate in the lead citrate method. The activity was completely inhibited by L-cysteine as been reported in intestine (12). The intracellular localization was identical to that obtained with the azo dye method. In addition, the lead citrate

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FIG. 1-2. ALPase activity in S-APG cells detected by the lead citrate method (Fig. 1) and the azo dye method (Fig. 2). The activity is apparent on the cell membrane, and in the cytoplasm and Golgi area (inset in Fig. 1 and arrow in Fig. 2).

FIG. 3-5. ALPase cell types classified by the localization and intensity of the enzyme activity. Type 1 ALPase cell, single arrowhead in Fig. 3; type 2 ALPase cell, arrow in Fig. 4; type 3 ALPase cell, arrow in Fig. 5; type 4 ALPase cell, double arrowhead in Fig. 3.

FIG. 6. A combination method of ALPase and PAS reaction. ALPase cells are positive for PAS reaction (arrow) and the cells with the membrane activity contains PAS-positive substances (inset).

FIG. 7-9. ALPase activity during the cytodifferentiation of S-APG cells. The sinusoids are occasionally positive in the gland of fetus I (arrow in Fig. 7). S-APG cells of fetus II and 2 months old swine show the diffuse activity (arrow in Fig. 8) and the activities on the cell membrane and in the Golgi area (arrow in Fig. 9), respectively
Scale bar in Figs. 1 and 7 is 50 μm, in Figs. 2, 3, 4, 5, 6, 8 and 9 is 25 μm, and in inset of Figs. 1 and 6 is 12.5 μm.

method gave finer reaction product than the azo dye method. The findings indicate that β-glycerophosphate is the substrate of choice for the demonstration of ALPase or monoester phosphohydrolase (10) in S-APG cells.

Since ALPase cells were stained with PAS or AT reactions, it is apparent that the cells produce glycoprotein hormone. The morphological findings of ALPase cells provided the possibility that the cells are correspond with the gonadotrophs of S-APG as judged by conventional histology (13). However, immunocytochemical and ultracytochemical studies are further needed to identify the cell type positive for ALPase reaction.

The diffuse activity of ALPase was first detected in S-APG cells of fetus II which were not stained with PAS- and AT-reaction. The activity extremely increased in type 1 ALPase cell with the substances stained with PAS- or AT-reaction. The highest activity on the cell membrane was found in type 3 ALPase cell which were also stained with PAS- or AT-reaction. Accordingly, it is speculated that ALPase are synthesized prior to the onset of hormone synthesis and that gonadotroph-like cells being in state of the increased secretory activity exhibit strongly the membrane activity which may play an important role in the membrane transports of substances for the maintenance of hormone synthesis since ALPase activity is associated with a selective transport of certain metabolites in various cells (19-21). Castration causes the elongation and the degranulation of secretory granules of gonadotrophs which result in the cell degeneration (13, 22). Type 4 ALPase cell in castrated males was relatively large in size, had the weak cytoplasmic activity of ALPase and reduced the reactivity of PAS or AT. The findings show that type 4 ALPase cell is elongated gonadotrophs which have declined the secretory activity. Taken together, the results strongly suggest that the diversity of intracellular ALPase activity may be associated with the function and cytodifferentiation of gonadotroph-like cells in swine.

The electrophoretic distribution of ALPase showed that a slowly migrating ALPase exists in S-APG. The pattern of mobility was similar to intestinal ALPase but not to liver ALPase. The neuraminidase treatment revealed that the mobility of APG-ALPase was appreciably reduced unlike intestine ALPase. The valuable information demonstrate that APG-ALPase is different from intestine and liver ALPase and has available terminal sialic acid groups as well as placental, liver, kidney and bone ALPase (23). However, immunological investigation using the antibody will be required to further characterize ALPase in APG cells, since the soluble and membranous forms of intestinal ALPase have been identified as distinct isozymes (24).

References

1) Sobel, H.J., *J. Endocrinol.*, **30**, 323 (1964)
2) Smith, R.E. and Farquhar, M.G., *J. Histochem. Cytochem.*, **18**, 237 (1970)
3) Mikami, S., *Proc. Japan Acad.*, **48**, 121 (1972)
4) Sobel, H.J., *Endocrinol.*, **69**, 1108 (1961)
5) Smith, R.E. and Farquhar, M.G., *J. Cell Biol.*, **31**, 319 (1966)
6) Balogh, K.Jr. and Cohen, R.B., *Endocrinol.*, **70**, 874 (1962)
7) Packman, P.M. and Robins, E., *Endocrinol.*, **87**, 13 (1970)
8) Yamaguchi, T., Hoshino, T. and Tamate, H., *Tohoku J. Agr. Res.*, **24**, 128 (1973b)
9) Yamaguchi, T., Hoshino, T. and Tamate, H., *Tohoku J. exp. Med.*, **133**, 167 (1981)
10) Mayahara, H., Hirano, H., Saito,T., and Ogawa, K., *Histochemie*, **11**, 88 (1967)
11) Burstone, M.S., *J. Nat. Cancer Inst.*, **20**, 601 (1958)
12) Halbhuber, K.J., Gossrau, R., Moller, U. and Zimmermann, N., *Histochemistry*, **90**, 67 (1988)
13) Yamaguchi, T., Hoshino, T. and Tamate, H., *Tohoku J. Agr. Res.*, **24**, 41 (1973a)
14) Schlamowitz, M. and Bodansky, O., *J. Biol. Chem.*, **234**, 1433 (1959)
15) Bessey, O.A., Lowry, O.H. and Brock, M.J., *J. Biol. Chem.*, **164**, 321 (1946)
16) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., *J. Biol. Chem.*, **193**, 265 (1951)
17) Ornstein, L. and Davis, B., *Disk Electrophoresis, Part I and II*. Distillation Products Industries, Rochester, N.Y. (1962)
18) Saraswathi, S. and Bachhawat, B. K., *Biochim. Biophys. Acta.*, **212**, 170 (1970)
19) Hugon, J. and Borgers, M., *J. Histochem. Cytochem.*, **14**. 629 (1966)
20) Hugon, J. and Borgers, M., *Histochemie*, **12**, 42 (1968)
21) Yoshioka, T. and Inomata, K., *Acta Histochem. Cytochem.*, **16**, 77 (1983)
22) Yoshimura, F. and Harumiya, K., *Endocrinol. Jap.*, **12**, 119 (1965)
23) Fishman, W.H., Inglis, N.R. and Ghosh, N.K., *Clin. Chim. Acta*, **19**, 71 (1968)
24) Yedlin, S.T., Young, G.P., Seetharam, B., Seetharam, S. and Alpers, D.H., *J. Biol. Chem.*, **256**, 5620 (1981)