HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY OF LIPASE IN MOUSE PANCREAS*

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To examine the intracellular localization of the pancreatic lipase in the mouse pancreas, both histochemical and immunohistochemical stainings were applied on the ultra-thin frozen sections. Furthermore, in order to study the mutual relationship of the distribution between lipase and α -amylase as well as α -amylase and chymotrypsinogen A within a zymogen granule, immunohistochemical double staining was performed.

The positive reaction for lipase by the histochemical staining were observed in the peripheral zone of the zymogen granules, as belt-like electron opaque deposits of 20 Å to 40 Å in width. Especially, in the isolated zymogen granules, the positive reaction sites were clearly recognized inside the limiting membrane of the zymogen granule as electron dense deposits of 30 Å to 40 Å in diameter. By the immunohistochemical study, lipase was localized along the limiting membrane of the zymogen granule, while, α -amylase and chymotrypsinogen A were distributed diffusely in a zymogen granule. Moreover, when the isolated zymogen granules were treated with alkaline solution, large amounts of lipase remained in the membrane fraction, but most α -amylase activity (more than 95%) was extracted into the soluble fraction. This fact suggests that lipase is more closely associated with the limiting membrane of the zymogen granule, than α -amylase.

From these results, the localizing pattern of the pancreatic lipase was found to be different from those of α -amylase, chymotrypsinogen A and trypsinogen; the former bound loosely to the inside of the limiting membrane of the zymogen granule, while the latter enzymes were localized diffusely within a zymogen granule.

The intracellular localization of pancreatic lipase (glycerol ester hydrolase, EC 3.1.1.3.) has been studied by many investigators with the histochemical method (1, 9, 10, 20, 23, 24, 36), and with the biochemical method (3, 4, 6, 8, 12, 39).

The histochemical study of the electron microscopic level, showed that pancreatic lipase localized on the limiting membrane of the zymogen granules (23, 24) or on the outer surface of the limiting membrane (36). Biochemical studies reported that pancreatic lipase bound more strongly to the zymogen granule than the other digestive enzymes did (2, 12, 39). However, there have been no reports on intracellular localization of pancreatic lipase by the immunohistochemical methods,

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nor on the elucidation of the mutual relationship of the distribution pattern between the lipase and other enzymes.

The present study was undertaken to examine whether lipase is localized on the surface or in the inside of the limiting membrane of the zymogen granule by aid of both histochemical and immunohistochemical methods, and, also, to demonstrate the mutual relationship in the distribution between lipase and other pancreatic enzymes, such as α -amylase and chymotrypsinogen A, in a zymogen granule, by aid of immunohistochemical double staining method (27).

MATERIALS AND METHODS

Reagents were from the following sources: Ferritin (horse spleen, 2x crystallized) was the product of ICN Pharmaceuticals Inc. (Cleveland, O., U.S.A.). It was further purified by the method as described by Hsu (1967). Pancreatic α -amylase (Type I-A, 2x crystallized from hog pancreas), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl (WSC) and peroxidase (Type VI, from horseradish) were obtained from Sigma Chemical Companies (St. Louis, U.S.A.). Beef pancreatic chymotrypsinogen A was purchased from Miles Laboratories (Elkhart, Ind., U.S.A.). Tween 80 was obtained from Wako Pure Chemical Industries (Japan). The powder of 4-chloro-1-naphthol was the product of K & K Laboratories (Calif., U.S.A.). Sephadex G-25, Sephadex G-100, DEAE-cellulose and CM-cellulose were the products of Pharmacia Fine Chemicals, Inc. (Uppsala, Sweden).

Histochemical procedures;

1) Fresh mouse pancreas was cut into small pieces of about 1 mm³ size with razor blades on a plastic plate, in a drop of 3% WSC dissolved in 0.1 M cacodylate buffer, pH 7.2, which contained 1% paraformaldehyde (42). The tissue blocks were further fixed in the same fixative at 4°C for 6 hr. The blocks were subsequently fixed in the 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 hr at 4°C. After fixation, the blocks were washed several times with 0.1 M cacodylate buffer (pH 7.2) containing 8% sucrose and embedded in 20% gelatin solution. The ultra-thin frozen sections were prepared according to the method of Bernhard and Viron (5) with a slight modification, by use of a Porter-Blum MT-2B ultramicrotome equipped with a cryo-kit attachment (Ivan-Sorvall Inc., Newtown, Conn., U.S.A.).

The ultra-thin frozen sections were transferred, with the aid of plastic Marinozzi rings (2), onto the reaction medium (23), which consisted of 1 ml of 5% Tween 80, 1 ml of 10% CaCl₂, 2.5 ml of 0.2 M Tris-maleate buffer, pH 7.2, and 20.5 ml of distilled water. The sections were incubated at 37°C for 60 min. The medium was changed every 30 min. After the reaction, the sections were floated on 2% EDTA solution prepared in 0.1 M cacodylate buffer, pH 7.2, for 10 min to remove the excess calcium. Sections were, then, washed with 0.1 M cacodylate buffer, pH 7.2, for 10 min, treated with 0.1% lead nitrate solution for 15 min, washed with several changes of distilled water, and finally, mounted on carbon-strengthened formval-coated grids. The specimens were post-fixed with 2% osmium tetroxide vapor for 15 min, and examined with either Hitachi HU-12A or JEOL 100-C type electron microscopes.

2) The adult ddy mice (body weight, 25 g) were fasted overnight, but could drink

water freely. The fasted mice were sacrificed by cervical luxation. The pancreas were quickly removed, minced with razor blades on ice-cold plastic plate, and homogenized in 0.3 M sucrose in 10 mM Tris-maleate buffer, pH 6.5 (10%, w/v) by use of a Potter-Elvehjem homogenizer. The zymogen granule fraction was obtained after differential centrifugations by the method as described by Vandermeers-Piret *et al.* (39). The zymogen granule fraction was fixed in 4% paraformaldehyde prepared in 0.1 M cacodylate buffer, pH 7.2, which contained 0.5% glutaraldehyde, at 4°C for 30 min. After fixation, the zymogen granules were collected by centrifugation, and washed well in 0.1 M cacodylate buffer, pH 7.2, to remove the excess fixative. The pellets were subsequently incubated in the reaction medium described above at 37°C for 60 min, changing the medium every 30 min. After incubation, the pellets were washed, dehydrated with an ascending series of alcohols, and embedded in epoxy resin according to the ordinary method.

Ultra-thin sections were cut with Porter-Blum ultramicrotome type MT-1 or MT-2B, stained doubly with uranium acetate and lead acetate, and examined with either Hitachi HU-12A or JEOL 100-C type electron microscope.

Immunohistochemical procedures;

1) Purification of the pancreatic lipase

Pancreatic lipase was prepared from rat pancreas by the method of Vandermeers *et al.* (38). Adult rats were fasted overnight and sacrificed by cervical fracture. The pancreas was excised as quickly as possible, cut into small pieces with razor blades on ice-cold plastic plate and homogenized in 0.2 M Tris-HCl buffer, pH 8.3, (30%, w/v) with a Potter-Elvehjem homogenizer. The homogenate was left in the deep-freezer (−40°C) for 1 hr. After being kept in the deep-freezer, the homogenate was thawed and centrifuged at 900 g for 20 min. The resultant supernatant was collected. The remaining precipitate was resuspended in the same buffer and then centrifuged at 900 g for 20 min. The supernatant was put together with the above supernatant. The resultant supernatant (about 50 ml) was ultra-centrifuged at 100,000 g for 60 min with a Hitachi 65P ultra-centrifuge (Rotor 65P-444, Hitachi Co. Japan). After ultra-centrifugation, the supernatant was first introduced to a Sephadex G-25 column (60×3.0 cm, flow rate of 50 ml/h) pre-equilibrated with 13 mM Tris-HCl buffer, pH 8.2, in order to remove the excess salt. The fractions which contained crude lipase were collected and applied to a DEAE-cellulose column (20×3.0 cm, flow rate of 15 ml/h) which was previously equilibrated with 13 mM Tris-HCl buffer, pH 8.2. The by-passed proteins were collected and concentrated to about one-fourth of the original volume by lyophilization. The concentrated proteins were submitted to a Sephadex G-100 column (100×3.0 cm, flow rate of 25 ml/h) pre-equilibrated with 5 mM Tris-HCl buffer, pH 7.0, and eluted with the same buffer. The fractions which contained high lipase activity were collected and dialyzed against 5 mM phosphate buffer, pH 6.5, at 4°C overnight. The dialysate was adsorbed to CM-cellulose column (10×1.0 cm), which was equilibrated with 5 mM phosphate buffer, pH 6.5. The column was washed with 200 ml of the same buffer, and then eluted with a linear gradient (flow rate of 12 ml/h), which was generated with 50 ml of 5 mM phosphate buffer, pH 6.5, in the mixing chamber and 50 ml of 5 mM phosphate buffer, pH 6.5, containing 0.1 M NaCl in the reservoir. The resulting eluate was divided into 2 ml fractions. Fractions containing high

specific activity of lipase were collected. The lipase obtained showed a single protein band on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 1) and the molecular weight was calculated to about 38,000 (32).

2) Preparation of antibodies

One milligram of the purified pancreatic lipase was mixed with an equal volume of Freund's complete adjuvant (Difco, Inc., Detroit, U.S.A.) and injected subcutaneously into rabbits at an interval of a week. Four weeks later, 0.5 mg of the antigen was administered intravenously for the booster injection. Seven days after the booster injection, anti-lipase serum was harvested from the marginal ear vein of the sensitized rabbit.

The crude γ -globulin was prepared by precipitation at 50% saturation of am-

FIG. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of lipase, which was purified from rat pancreas. The gel was stained with Coomassie brilliant blue. The purified lipase shows a single protein band and the molecular weight is equivalent to about 38,000.

monium sulfate, and further purified by DEAE-cellulose chromatography according to the method of Peterson and Sober (31). The anti-lipase antibody thus obtained showed only one clear precipitine line against rat pancreatic extract on the Ouchterlony's gel diffusion plate (Fig. 2). Rabbit antisera against beef pancreatic chymotrypsinogen A, and guinea pig antisera against hog pancreatic α -amylase were prepared by the same procedures. These antisera were specific to the homogeneous antigen and did not react with the heterogeneous antigens on the double diffusion test of Ouchterlony.

3) Preparation of labeled antibody

Horseradish peroxidase (HRP) was conjugated to anti-rabbit IgG goat γ -globulin or anti-guinea pig IgG goat γ -globulin by the method of Nakane and Kawaoi (25). The HRP-labeled γ -globulin was purified on a DEAE-cellulose column by the method of Yamashita *et al.* (41). Conjugation and purification of the ferritin-labeled antibodies were carried out by the method of Kraehenbuhl *et al.* (19).

4) Immunohistochemical staining

The zymogen granules were isolated from fresh mouse pancreas by the method described by Vandermeers-Piret *et al.* (39), and fixed, with 3% WSC dissolved in 0.1 M phosphate buffer, pH 7.2, which contained 1% paraformaldehyde (42), or with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, containing 0.5% glutaraldehyde, for 30 min at 4°C. After being rinsed several times with the same buffer as used for the fixative, the pellets were embedded in a 20% gelatin solution. The ultra-thin frozen sections were prepared by the same manner as described before.

To demonstrate the localization of lipase, immunohistochemical studies were performed by the indirect staining method described in a previous publication (26), by use of both HRP-labeled antibody and ferritin-labeled antibody. In the case

FIG. 2. Ouchterlony's gel diffusion analysis of the purified anti-rat pancreatic lipase rabbit antibody against the rat pancreatic extract. The antiserum was placed in the center well and rat pancreatic extract was filled in the surrounding six wells. The rat pancreatic extract was diluted in a multiple and was placed clockwise from the top. Only one clear precipitine line is observed. This line showed the positive lipase activity.

of HRP-labeled antibody method, 4-chloro-1-naphthol solution (5 mg of 4-chloro-1-naphthol in 100 ml of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.0002% H_2O_2) was employed in place of diaminobenzidine to visualize the reactive sites.

The simultaneous visualization of lipase and α -amylase, as well as α -amylase and chymotrypsinogen A, were also carried out by use of both HRP-labeled antibody and ferritin-labeled antibody (27). To localize lipase in combination with α -amylase, the tissues were first stained with anti-lipase rabbit γ -globulin which reacted with ferritin-labeled anti-rabbit IgG goat γ -globulin, and were secondly stained with anti- α -amylase guinea pig IgG which subsequently reacted with HRP-labeled goat γ -globulin against guinea pig IgG. In a reverse order, ferritin-labeled anti-guinea pig IgG goat γ -globulin was applied after the tissues were treated with anti- α -amylase guinea pig γ -globulin, and HRP-labeled anti-rabbit IgG goat γ -globulin was applied after the tissues were treated with anti-lipase rabbit IgG. In case of simultaneous demonstration of chymotrypsinogen A and α -amylase, the tissues were first treated with anti-chymotrypsinogen A rabbit γ -globulin, washed with PBS, and were then covered with ferritin-labeled anti-rabbit IgG goat γ -globulin. Then, anti- α -amylase guinea pig IgG was placed on the tissues, followed by an application of HRP-labeled anti-guinea pig goat γ -globulin with intervening washing with PBS.

5) Solubilization of enzymes from the zymogen granules

Solubilization of enzymes from the zymogen granules was carried out by the method of Meldolesi *et al.* (22). The zymogen granules were suspended in 10 ml of 0.2 M NaHCO_3 , pH 7.8 or in a mixture of 7 ml of 0.2 M NaHCO_3 and 3 ml of 0.17 M NaCl, pH 7.8, and then left for 1 or 3 hr at 4°C with gently stirring. The suspension was ultra-centrifuged at 100,000 g for 60 min by use of a Hitachi 65P ultra-centrifuge (Hitachi Co. Japan). After ultra-centrifugation, the resultant supernatant was collected (termed "soluble fraction"), and the precipitate was suspended in 10 mM Tris-maleate buffer, pH 6.5, which contained 3% sucrose (termed "membrane fraction"). Enzyme activities of lipase and α -amylase were measured in both soluble and membrane fractions.

The assay for α -amylase was carried out by the procedure of Bernfeld (4), in which soluble starch was used as substrate. The lipase activity was assayed by the method of Verduin *et al.* (40), using olive oil emulsion as substrate.

RESULTS

On the ultra-thin frozen sections of pancreas, positive reaction sites for lipase demonstrated by the histochemical method were recognized inside and outside the limiting membrane of zymogen granules of every acinar cell, as belt-like, highly dense deposits of 20 to 40 Å in width (Figs. 3A, B, C). On the ultra-thin section of the isolated zymogen granules, positive reactions were observed inside the limiting membrane of the zymogen granule, as electron opaque deposits of 30 to 40 Å in diameter (Figs. 4A, B, C, D, E).

Localization of lipase was also examined immunohistochemically on the ultra-thin frozen sections of the pellets of isolated zymogen granules, by use of both ferritin-labeled antibody and HRP-labeled antibody methods. The ferritin particles were accumulated inside the limiting membrane of the zymogen granule (Fig. 5A), and

FIG. 3A. Localization of lipase is shown histochemically by Gomori's "Tween" technique on the ultra-thin frozen sections of mouse pancreas, fixed in 3% WSC, containing 1% paraformaldehyde, and further fixed in 0.5% glutaraldehyde. Positive reaction of lipase are noticeable along the limiting membrane of every zymogen granules as homogeneous belt-like deposits of 20 Å to 40 Å in width. Lipase activity is not observable in the core region of the zymogen granule. $\times 12,000$

FIG. 3B. Same as Fig. 3A. Positive reaction deposits of lipase are present only along the limiting membrane of the zymogen granules. $\times 44,000$

FIG. 3C. Enlarged figure of a part of Fig. 3A. Reaction products are recognized as homogeneous deposits only along the limiting membrane of the zymogen granules. $\times 48,000$

FIG. 5A. Localization of lipase is demonstrated by ferritin-labeled antibody method on the ultra-thin frozen sections of the isolated zymogen granules, which are fixed in 3% WSC, containing 1% paraformaldehyde. Ferritin particles are recognizable inside the limiting membrane of the zymogen granule, but are not observable at the central region of the zymogen granule. $\times 48,000$

FIG. 5B. Localization of lipase is shown by HRP-labeled antibody method on the ultra-thin frozen sections of the isolated zymogen granules. Reaction products of 4-chloro-1-naphthol for HRP-reaction are recognizable along the limiting membrane of the zymogen granule, but not observable in the core region of the zymogen granule. $\times 48,000$

FIG. 4A. This picture shows the lipase activity of the isolated zymogen granules. The isolated zymogen granules are fixed in 4% paraformaldehyde containing 0.5% glutaraldehyde. The pellets are washed well, stained with Gomori's "Tween" technique for lipase and embedded in epoxy resin. Positive reaction of lipase is distinguishable inside the limiting membrane of the zymogen granule, as electron opaque deposits of 30 Å to 40 Å in diameter. $\times 44,000$

FIG. 4B. Same as Fig. 4A. $\times 48,000$

FIG. 4C. Same as Fig. 4A. $\times 38,000$

FIG. 4D. Same as Fig. 4A. $\times 44,000$

FIG. 4E. Same as Fig. 4A. $\times 48,000$

the reaction products of 4-chloro-1-naphthol were recognized around the limiting membrane of the zymogen granule (Fig. 5B).

To examine the mutual relationship of the distribution between lipase and other pancreatic enzymes, the simultaneous demonstration of lipase and α -amylase was performed on an identical ultra-thin frozen section of the isolated zymogen granules, by combined staining with HRP-labeled antibody method for the demonstration of lipase and ferritin-labeled antibody method for α -amylase. The reaction products of 4-chloro-1-naphthol for HRP-reaction which showed the location of lipase were recognized on the limiting membrane of the zymogen granule, while ferritin particles which represented the site of α -amylase were noticed scatteringly in the zymogen granules (Fig. 6A). In a reverse order, in which lipase was shown by ferritin-labeled antibody method while α -amylase was demonstrated by HRP-labeled antibody method, the ferritin particles were accumulated inside the limiting membrane of the zymogen granule, while the reaction products of 4-chloro-1-naphthol were recognized diffusely in the zymogen granules (Figs. 6B, C). In the case of the simultaneous staining of α -amylase and chymotrypsinogen A performed on an identical ultra-thin frozen sections of the isolated zymogen granules by combination with ferritin-labeled antibody and HRP-labeled antibody methods, both ferritin particles, which showed the sites of chymotrypsinogen A, and the reaction products of 4-chloro-1-naphthol for HRP-reaction, which represented the sites of α -amylase, were recognized diffusely and in the mixed pattern within an identical zymogen granule (Fig. 7)

The difference of the distribution pattern between lipase and α -amylase within a zymogen granule was also confirmed by the following experiments; the isolated zymogen granules were suspended in a mixture of 7 ml of 0.2 M NaHCO_3 and 3 ml of 0.17 M NaCl, pH 7.8, for 1 or 3 hr at 4°C. Then, the suspension was ultra-centrifuged at 100,000 g for 60 min, in order to recover the disrupted membrane

FIG. 6A. Simultaneous demonstration of lipase and α -amylase on an identical ultra-thin frozen section of the isolated zymogen granules from the mouse pancreas. The pellet was fixed in 3% WSC which contained 1% paraformaldehyde. Ferritin-labeled anti-guinea pig IgG goat γ -globulin was applied to the section which was preliminarily treated with anti- α -amylase guinea pig IgG, and subsequently, HRP-labeled anti-rabbit IgG γ -globulin was applied to the identical section which was previously reacted with anti-lipase rabbit IgG. Reaction products of 4-chloro-1-naphthol for HRP-reaction, which show the location of lipase, are accumulated along the limiting membrane of the zymogen granule. On the other hand, ferritin particles which represent the site of α -amylase are localized scatteringly in the zymogen granule. The reaction products of both 4-chloro-1-naphthol and ferritin particles are clearly demonstrated in an identical zymogen granule, showing that both lipase and α -amylase are concurrently localized in an identical zymogen granule. $\times 60,000$

FIG. 6B. Simultaneous demonstration of lipase and α -amylase. The staining procedure is in a reverse order of the specimen shown in Fig. 6A; lipase was detected by ferritin-labeled antibody and α -amylase was represented by HRP-labeled antibody. Ferritin particles which demonstrate the location of lipase are recognizable along the limiting membrane of the zymogen granule. Reaction products of 4-chloro-1-naphthol for HRP-reaction which show the site of α -amylase are present diffusely in the zymogen granule. Both ferritin particles and reaction products of 4-chloro-1-naphthol are seen in a zymogen granule, indicating that both lipase and α -amylase are synchronously present in an identical zymogen granule. $\times 44,000$

FIG. 6C. Simultaneous demonstration of lipase and α -amylase. The staining procedure is the same as the specimen shown in Fig. 6B. The same feature is observable as in Fig. 6B. $\times 60,000$

FIG. 7. Simultaneous demonstration of chymotrypsinogen A and α -amylase on an identical ultra-thin frozen section of the isolated zymogen granules from the mouse pancreas, fixed in 3% WSC containing 1% paraformaldehyde. Ferritin-labeled anti-rabbit IgG goat γ -globulin was applied to the section which was previously reacted with anti-chymotrypsinogen A rabbit γ -globulin, and subsequently, HRP-labeled anti-guinea pig IgG goat γ -globulin was applied to the same section which was treated in advance with anti- α -amylase guinea pig IgG. Both ferritin particles which show the site of chymotrypsinogen A and the reaction products of 4-chloro-1-naphthol for HRP-reaction which represent the place of α -amylase are localized doubly and diffusely within the same zymogen granule, indicating that both chymotrypsinogen A and α -amylase simultaneously coexist in the same pattern in an identical zymogen granule. $\times 44,000$

of the zymogen granules and a few undisrupted zymogen granules as a pellet. In the case of one-hr treatment, 54.8% of lipase activity was observed in the membrane fraction, while 46.2% of the activity was recognized in the soluble fraction.

FIG. 8. Per cent activity of lipase and α -amylase in both membrane fraction and soluble fraction, which are obtained after alkaline treatment of the isolated zymogen granules, is shown. ■; membrane fraction □; soluble fraction.

FIG. 8A. The case of one hr-treatment with a mixture of 0.2 M NaHCO_3 and 0.17 M NaCl, pH 7.8. The activity of lipase in the membrane fraction is 54.8% and that of the soluble fraction is 46.2%, while 0.9% of α -amylase activity is recognized in the membrane fraction and 99.1% of the activity is in the soluble fraction.

FIG. 8B. The case of three hr-treatment with a mixture of 0.2 M NaHCO_3 and 0.17 M NaCl, pH 7.8. Twenty-four per cent of lipase activity is present in the membrane fraction and 76% of the activity is in the soluble fraction. On the other hand, only 0.2% of α -amylase activity is noticeable in the membrane fraction and 99.8% of it is in the soluble fraction.

FIG. 8C. The case of one hr-treatment with 0.2 M NaHCO_3 , pH 7.8. The activity of lipase in the membrane fraction is 50.9% and that of the soluble fraction is 49.1%, while 0.5% of α -amylase activity is present in the membrane fraction and 99.5% is in the soluble fraction.

FIG. 8D. The case of three hr-treatment with a 0.2 M NaHCO_3 , pH 7.8. Thirty per cent of lipase activity is recognized in the membrane fraction and 70% is in the soluble fraction. On the contrary, α -amylase activity is the same as in Fig. 8C.

On the contrary, only 0.9% of α -amylase activity was recognized in the membrane fraction, while 99.1% of its activity was present in the soluble fraction (Fig. 8A). In the case of three-hr treatment, 24% of lipase activity was present in the membrane fraction, while only 0.2% of α -amylase activity was noticed in the membrane fraction (Fig. 8B). Furthermore, the isolated zymogen granules were treated with 0.2 M NaHCO_3 alone, pH 7.8, for 1 or 3 hr at 4°C. and the membrane fraction was gathered by ultra-centrifugation. After one-hr treatment, 50.9% of lipase activity was observed in the membrane fraction, but only 0.5% of α -amylase activity was recognized in the membrane fraction (Fig. 8C). In the case of three-hr treatment, 30% of lipase activity was recognized in the membrane fraction and 70% of its activity was present in the soluble fraction, while α -amylase was almost completely extracted from the zymogen granules (Fig. 8D).

These results suggest that lipase is more closely associated with the limiting membrane of the zymogen granules than α -amylase.

DISCUSSION

The enzymatic and biochemical properties of secretory proteins from pancreatic exocrine cells have been studied extensively for over three decades (7, 11, 15, 17, 16, 30, 33, 35, 37). Furthermore, by the recently advanced immunohistochemical studies, it is well established that the precursors of proteolytic enzymes, α -amylase and hydrolyzing nucleotides are enclosed in the zymogen granules (18, 27, 28). Also, it has become clear that the plural antigens, which have different functions, simultaneously coexist in an identical zymogen granules by aid of immunohistochemical method (27).

It has been reported that pancreatic lipase binds to the zymogen granule relatively more strongly than the other enzymes in dog (12) and in rat pancreas (2, 3). Vandermeers-Piret *et al.* (39) have also described that lipase has a strong tendency to remain in the membrane fraction after treatment of the zymogen granules at acidic pH (pH 6.5).

At the light microscopic level, pancreatic lipase has been localized around the zymogen granules in the apical portion of the acinar cells by use of the histochemical technique (1, 20). Seligman *et al.* (36), who used an aromatic thioester of nonanoic acid as a substrate, have shown with light and electron microscopy that lipase did not localize inside the zymogen granule but was present on the outer surface of the zymogen granule. Murata *et al.* (23) and Nagata *et al.* (24), who used modified Gomori's (9) "Tween" technique for lipase, have reported that lipase was localized on the limiting membrane of the zymogen granules.

In this study, positive reaction products on the ultra-thin frozen section by use of "Tween 80" as a substrate for lipase histochemistry, were recognized on the limiting membrane of the zymogen granules (Figs. 3A, B, C). This result partly coincides with those reported by Seligman *et al.* (36), Murata *et al.* (23) and by Nagata *et al.* (24). But, positive reaction products were observed inside the limiting membrane in the histochemical study on the isolated zymogen granules (Figs. 4A, B, C, D, E). In the immunohistochemical studies, lipase was recognized outside as well as inside the limiting membrane of the zymogen granules (Figs. 5A; 6A, B, C), while α -amylase and chymotrypsinogen A were localized diffusely within the

zymogen granules (Figs. 6A, B, C; 7).

From both histochemical and immunohistochemical results, the localization pattern of lipase was found to be different from that of other enzymes in the zymogen granules; namely, lipase seems to bind to the inside of the limiting membrane of the zymogen granule, but the other enzymes are diffusely distributed within the zymogen granule (18, 19, 27, 28).

When the isolated zymogen granules are suspended in a mixture of 0.2 M NaHCO_3 and 0.17 M NaCl , pH 7.8, or 0.2 M NaHCO_3 , pH 7.8, they are disrupted and their contents are released (22). Therefore, after the isolated zymogen granules were treated under the above conditions, the suspension could be separated into the membrane fraction and the soluble fraction by ultra-centrifugation. To examine which enzyme is more closely associated with the membrane and which is more easily released from the zymogen granule, the activities of lipase and α -amylase were measured in each fraction. Fiftyfour point eight per cent of lipase activity remained in the membrane fraction, while more than 99% of α -amylase activity was released into the soluble fraction after one-hr treatment with a mixture of 0.2 M NaHCO_3 and 0.17 M NaCl . Under other experimental conditions, larger amounts of lipase remained in the membrane fraction than that of α -amylase (Figs. 8B, C, D). Similar results were obtained by several authors in some animals other than mice. Hokin (12), in dog pancreas, Arnesjo *et al.* (2, 3) and Vandermeers-Piret *et al.* (39), in rat pancreas, have reported that lipase tends to bind more strongly to the zymogen granule-fraction than the other enzymes did.

From the results of the present histochemical and immunohistochemical studies on the isolated zymogen granules, together with those of the extraction experiment, it is concluded that lipase is loosely associated with the inside of the limiting membrane of the zymogen granule and occupies the marginal zone of the zymogen granule. Enzymes other than lipase were localized diffusely in the zymogen granule, as far as the enzymes studied in this experiment. The exact localization of lipase around the limiting membrane of the zymogen granule was not clearly shown in detail, when the histochemical and immunohistochemical methods were applied on the ultra-thin frozen section of pancreas, in place of the pellet of the isolated zymogen granules. Since lipase is found to be localized inside the limiting membrane, a question arises, as to how lipase is discharged into the lumen. In general, the discharge of the enzymes from the pancreas is thought to take the following course; the limiting membrane of the granule fuses firstly to the luminal membrane, followed by fission within the area of fusion, and finally the bulk of the granule contents discharges into the lumen (14, 29). But, according to the histochemical study of Ono (unpublished data), lipase activity was recognized along the inner leaflet of the membrane of the zymogen granules and continuously along the outer surface of the luminal membrane at the place of fission which occurred after the fusion of both kinds of membranes, when the secretion was stimulated by pilocarpine administration. This observation suggests that lipase may be discharged along both kinds of membrane at the place of fission, keeping its loose connection with the limiting membrane of the granule and without being mixed with the other enzymes within the zymogen granule prior to the discharge. Eventually, lipase may fall out of the luminal membrane after fission and be mixed with the other contents of the zymogen granule, near the place of fission.

Finally, as partly reported in the previous report (27), simultaneous demonstration of two different enzymes, α -amylase and lipase or chymotrypsinogen A and α -amylase, on an identical ultra-thin frozen section of the isolated zymogen granules showed that two kinds of enzymes coexist within a single zymogen granule. And, there was no difference in the distribution of each combination of two kinds of enzymes, in each zymogen granule. This fact gives some proof that the content of each zymogen granule is heterogeneous, and that the distribution of enzymes in each granule is quite similar with each other at least at a certain phase of the secretion. This, together with the result of the previous paper, supports the view of Scheele and Palade (34) and that of Tartakoff *et al.* (37), as far as the parallel phase in the secretion of the pancreatic enzymes.

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