Affinity Purification of Insulin-degrading Enzyme and Its Endogenous Inhibitor from Rat Liver*

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A metallothiol protease called insulin-degrading enzyme (IDE) seems to be implicated in insulin metabolism to terminate the response of cells to hormone, as well as in other biological functions, including muscle differentiation, regulation of growth factor levels, and antigen processing. In order to obtain highly pure and biologically active IDE, we have developed an immunoaffinity method using a monoclonal antibody to this enzyme (9B12). When the cytosolic fraction of rat liver was first applied to a 9B12-coupled Affi-Gel 10 column, more than 97% of the insulin-degrading activity was absorbed. Among various kinds of buffers successfully eluting the enzyme, only the buffer with a high pH (pH 11) could retain the full biological activity of this enzyme. IDE was further purified via two steps of chromatography using Mono Q anion exchange and Superose 12 molecular sieves columns. The final preparation showed a single band at 110 kDa on reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In the eluate from the immunoaffinity column, the inhibitory activity associated with the enzyme was also observed. To better recover this endogenous inhibitor, heat-treated cytosolic fraction was fractionated by ammonium sulfate precipitation and applied to the immunoaffinity column on which IDE had been adsorbed. Then, IDE and its inhibitor could be co-eluted with pH 11 as a complex form. After heat treatment of this fraction, the inhibitor was further purified using the same series of chromatography as IDE to more than 20,000-fold; it showed a 14 kDa band on SDS-PAGE. It inhibited both the insulin degradation by IDE in a competitive manner and the cross-linking of 125I-insulin to IDE. Highly purified IDE and the endogenous inhibitor will be useful tools for better understanding the various biological functions of this enzyme.

Over 40 years have passed since the first description of enzymes that degrade insulin (1). Among the many enzymes subsequently reported, a neutral thiol metalloproteinase with a high affinity for insulin, which we have termed insulin-degrading enzyme (IDE) (2-5), has been generally accepted as playing a physiologically important role in insulin metabolism (6-10). However, the characteristics of this enzyme, including molecular weight, optimum pH, metal requirement, continue to be controversial because of the difficulty of obtaining a homogeneous and biologically active enzyme preparation (11). Many of these problems have been solved through the purification of IDE from human erythrocytes to homogeneity (5) and the production of monoclonal antibodies to the enzyme (6). Another well characterized proteolytic enzyme with specificity for insulin, termed insulin protease (12-15), has been demonstrated to be identical with IDE from its reactivity to monoclonal antibodies to IDE, molecular weight, and cleavage site in insulin (16). A complementary DNA (cDNA) coding for human erythrocyte IDE has been isolated and sequenced. Consequently, although the deduced sequence of IDE shows significant identity in at least two discrete regions with Escherichia coli protease III (17) and does appear to have a zinc-binding site similar to those of the thermolysin superfamily of zinc metalloproteases (18, 19), this enzyme does not show extensive sequence homology with other known proteases. Furthermore, enzymes similar to IDE have been reported to be implicated in regulating growth factor levels (20), muscle differentiation (21), and processing of insulin by antigen-presenting cells (22), suggesting that IDE has a multifaceted biological significance. In order to analyze the potential functions of this novel protease more precisely, the purification of a homogeneous enzyme preserving its biological activity is essential.

IDE is widely distributed in various tissues, and its activity is reported to be regulated by an endogenous inhibitor (23-25). Although many investigators have attempted to purify this protease from various sources and examine its enzymatic characteristics, the purification of its inhibitor and its nature have received less attention. Protease inhibitors seem to be very important since they regulate the biological activity of specific proteases. In studies of the isolation and specificity of protease inhibitors, a purified enzyme must be employed because of the significant contribution of other contaminated proteins.

The present study describes how biologically active IDE has been successfully purified to homogeneity via three brief steps, which include the immunoaffinity method. This enzyme was subsequently employed to purify and characterize an endogenous inhibitor of IDE. It is shown that the inhibitor is naturally associated with IDE in the cytosolic fraction. Furthermore, this inhibitor was purified more than 20,000-fold.
using specific binding to the enzyme, and it showed a 14 kDa band on SDS-PAGE.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals were purchased: porcine monocomponent insulin from Novo (Copenhagen, Denmark); carrier-free NaI\textsuperscript{131} from Amersham Japan (Tokyo, Japan); Affi-Gel 10 from Bio-Rad; Protein G-Sepharose 4 Fast Flow, Superose 12, Mono Q, Sephadex G-25 (PD10), and G-200 from Pharmacia LKB Biotechnology Inc; YM-30 membrane from W. R. Grace & Co. (Danvers, MA); Centrisil 1 from Servorust (Goettingen, Germany). Other chemicals were of reagent grade. \textsuperscript{125}I-insulin (150-200 \textmu Ci/\mu g) was prepared by the chloramine-T method.

Production of Anti-IDE Monoclonal Antibody-coupled Column—Monoclonal antibodies to human erythrocyte IDE were produced as previously described (8). Among four monoclonal antibodies that recognized human IDE, two antibodies (9B12 and 28H1) were found to also recognize rat liver IDE (26). We chose 9B12 to produce the immunoaffinity column because of its high affinity to IDE. This antibody was purified from ascites fluid on Protein G-Sepharose 4 Fast Flow. Eighty mg of 9B12 was coupled with 20 ml of Affi-Gel 10 following the manufacturer’s instructions.

Dissociation of \textsuperscript{125}I-Insulin Cross-linked IDE from Anti-IDE Monoclonal Antibody—Fifty \mu l of anti-IDE monoclonal antibody 9B12 (10 \mu g/ml in Tris-\texttt{HCl}, pH 9.6) was coated on 96-well polyvinyl microtiter plates for 16 h at 4 \textdegree C. The wells were washed with 20 mM phosphate-buffered saline (PBS), pH 7.5, containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 (buffer A). IDE that had been cross-linked to \textsuperscript{125}I-insulin (approximately 1 x 10\textsuperscript{6} cpm) was then added to the wells. After 12 h at 4 \textdegree C, the wells were washed with buffer A, following which 300 \mu l of various kinds of elution buffers were added. After 2 h at 4 \textdegree C, the wells were washed again with buffer A, then the plate from the plate and counted in a \gamma counter. IDE cross-linking to \textsuperscript{125}I-insulin was prepared as described earlier (8). In short, the enzyme in 40 \mu l of phosphate buffer (PB), pH 7.5, was incubated with \textsuperscript{125}I-insulin for 30 min at 4 \textdegree C, and then 3 \mu l of 7.8 M disuccinimidyl suberate (dissolved in dimethyl sulfoxide) was added. After 40 min at 0 \textdegree C, the reaction was stopped by the addition of 1 M Tris, and the free \textsuperscript{125}I-insulin was removed by Sephadex G-200. The effects of various kinds of elution buffers on IDE activity were also tested. Fifty \mu l of partially purified IDE (8) was incubated with 450 \mu l of various elution buffers at 4 \textdegree C for 2 h. The samples were desalted with Sephadex G-25 previously equilibrated with 20 mM PBS, pH 7.5, containing 0.25% BSA. Degradation activities of these samples were tested by the trichloroacetic acid method as described under “Insulin Degradation and Inhibition Assay.”

Purification of IDE—Rat liver was homogenized in a 4-fold volume of 50 mM Tris-HCl, pH 7.5. The homogenate was centrifuged at 2,000 \times g for 1 h, and then the supernatant was centrifuged at 100,000 \times g for 40 min. After the pH was adjusted with NaOH to pH 7.5, the 100,000 \times g supernatant (cytosolic fraction) was applied to the monocloned anti-IDE antibody column. The column was washed with 20 mM PB containing 1 M NaCl, following which it was eluted with 75 mM Na\textsubscript{2}CO\textsubscript{3}, pH 11 (1 M Hepes, pH 7.5) and immediately neutralized with 1 M Hepes. This fraction (preparation A) was dialyzed against 50 mM Tris-HCl (pH 7.0), then applied to a Mono Q anion exchange column pre-equilibrated with 20 mM Tris-HCl (pH 7.0), and eluted with a linear salt gradient (100-400 mM NaCl). The peak of insulin-degrading activity (fraction 15-18) was pooled and used for characterization.

Association of IDE and the Endogenous Inhibitor—Rat liver 100,000 \times g supernatant was applied to the monocloned anti-IDE antibody column and washed with 20 mM PB (pH 7.5) containing 1 M NaCl in the same way as IDE was purified. After that, the rat liver 100,000 \times g supernatant that had passed through the monocloned anti-IDE antibody column was heat-treated (80 \textdegree C, 15 min) and then centrifuged at 15,000 \times g for 1 h. The supernatant was fractionated by ammonium sulfate precipitation. The fraction that was precipitated with 50% ammonium sulfate was dissolved in 20 mM PB (pH 7.5) and desalted on the Sephadex G-25 column previously equilibrated with 20 mM PB (pH 7.5). This sample was applied to the monocloned anti-IDE antibody column to which IDE had been coupled and then washed with 20 mM PB containing 1 M NaCl. The column was then eluted with 75 mM Na\textsubscript{2}CO\textsubscript{3} (pH 11) and immediately neutralized with 1 M Hepes. This fraction (preparation B) and preparation A were heat-treated and centrifuged, and then the inhibitory activity of the supernatant was tested.

Inhibitory Effect of the Inhibitor on Binding of \textsuperscript{125}I-Insulin to IDE—Labeled insulin was cross-linked to the enzyme as described above in the presence or absence of the inhibitor. The samples were electrophoresed on a 7.5% reduced SDS-PAGE and autoradiographed. Alternatively, 40 \mu l of pure IDE was added to the 96-well polyvinyl microtiter plates that had been coated with 9B12 as described above. After 16 h at 4 \textdegree C, the wells were washed with buffer A, and then \textsuperscript{125}I-insulin (1 x 10\textsuperscript{6} cpm) and the endogenous inhibitor or various agents that inhibit IDE activity were added. After 2 h at 4 \textdegree C, wells were washed and counted in a \gamma counter.

RESULTS

Dissociation of IDE Cross-linked to \textsuperscript{125}I-Insulin from Anti-IDE Monoclonal Antibody—Among the 17 kinds of buffers we tested, three (pH 2.5, pH 11, and 6 M urea) could dissociate more than 60% of the IDE-insulin complex from the anti-IDE monoclonal antibody, 9B12 (Fig. 1A). Of the three buffers, pH 11 exhibited no inhibitory effect on the insulin-degrading activity of IDE (Fig. 1B). Thus, we selected pH 11 as the elution buffer to dissociate IDE from the anti-IDE monoclonal antibody-coupled column.

Purification of IDE—Table I summarizes the results of each step of purification. When 100-800 ml of rat liver 100,000 \times g supernatant was applied to the anti-IDE column, more than 97% of the insulin-degrading activity was adsorbed to the column. By washing with pH 7.5 and eluting with pH 11 buffer, a large amount of protein could be separated from the enzyme. IDE was further eluted from Mono Q anion exchange chromatography at 200-300 mM NaCl. The elution profile of the insulin-degrading activity on Superose 12 molecular sieve chromatography (the last step of purification) corresponded to the elution of the protein, indicating the purity of the enzyme (Fig. 2). The molecular weight of the enzyme was estimated to be 300,000 by its behavior on this column. With crude homogenate as the starting material, about 15,000-fold purification was obtained. The purity of the preparation (fraction 15-18 from the Superose 12 column) was also indicated by the presence of only a single protein band of 110 kDa on reduced SDS-PAGE (Fig. 2, inset). The shoulder on both the
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Fig. 1. Effects of various kinds of buffers on dissociation of \(^{125}\)I-insulin cross-linked IDE from anti-IDE monoclonal antibody (A) and insulin-degrading activity by IDE (B). A, \(^{125}\)I-insulin cross-linked IDE was adsorbed into 9B12-coated microplate and then dissociated with various kinds of buffers. The control value (0%) dissociated with 10 mM PBS (pH 7.5) was 1450 cpm, and 100% represented 50 cpm as background. 1-3 M NaCl, 0.5-2.5 M MgCl\(_2\), 0.5-1 M KCl, and 1-6 M urea were dissolved in 10 mM PB (pH 7.5). pH 11, 10, 4, and 2.3 were prepared by 75 mM Na\(_2\)CO\(_3\), 50 mM Tris-HCl, 50 mM citrate buffer, and 50 mM glycine-HCl, respectively. B, purified IDE was incubated with various kinds of buffers for 2 h at 4 °C and desalted. \(^{125}\)I-insulin-degrading activity of IDE was then determined by trichloroacetic acid precipitation. The maximal degradation of insulin with PBS was considered to be 100%, and the other values were normalized to this. Each bar represents the mean of triplicate determinations.

Table I

| Steps                  | Protein | Volume | Total protein | -Fold | Recovery |
|------------------------|---------|--------|---------------|-------|----------|
| Crude homogenate       | 32      | 350    | 11,200        | 1     | 100      |
| 100,000 \(\times\) g supernatant | 12      | 300    | 3,600         | 2.8   | 90       |
| Mono anti-IDE          | \(4.0 \times 10^{-2}\) | 80     | 3.2           | 672   | 19       |
| Mono Q                 | \(1.0 \times 10^{-2}\) | 10     | 1.0           | 4,700 | 4.2      |
| Superose 12            | \(3.0 \times 10^{-3}\) | 6.4    | 0.02          | 15,000 | 2.5      |

* Calculated on the assumption that \(A_{380}\) = 10.

Fig. 2. Elution profile of IDE on Sepharose 12 column chromatography (the last step of purification). Ten \(\mu\)l of each fraction was tested for insulin-degrading activity. IDE activity (●) and the protein elution profile (——) are exactly parallel. Markers used for the determination of the mass of the enzyme were thyroglobulin (670 kDa), apoferritin (443 kDa), \(\gamma\)-globulin (158 kDa), albumin (66 kDa), and ovalbumin (45 kDa). Eighty \(\mu\)l of peak fraction (fraction 15-18) was applied to reduced 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue (inset).

Fig. 3. Effect of heat treatment on insulin-degrading activity and inhibitory activity of rat liver 100,000 \(\times\) g supernatant. Rat liver 100,000 \(\times\) g supernatant was heat-treated (80 °C) for various durations and centrifuged at 15,000 \(\times\) g for 15 min, and then both the insulin-degrading activity (●) and the inhibitory activity (○) in the supernatant were tested. To determine the insulin-degrading activity, each supernatant after heat treatment was diluted 30-fold with 20 mM PBS and 50 \(\mu\)l of each fraction was assayed for insulin-degrading activity. The degradation activity at 0 min was considered to be 100%, and the other values were normalized to this. For the determination of inhibitory activity, the protein concentration of the supernatant was adjusted to 100 \(\mu\)g/ml, and the inhibitory effect of each fraction on the insulin-degrading activity of purified IDE was assayed by trichloroacetic acid precipitation.

The Effect of Heat Treatment on Insulin-degrading Activity and Its Inhibitory Activity in 100,000 \(\times\) g Supernatant—At first, we could not detect an inhibitory activity for IDE in 100,000 \(\times\) g supernatant, because this fraction contains a large amount of insulin-degrading activity. However, the inhibitory activity became detectable after heat treatment, because IDE immediately lost its activity by heat treatment at 80 °C, whereas the inhibitory activity showed a heat stability (Fig. 3). The specific activity of the inhibitor was increased by heat treatment, since other proteins were precipitated after this treatment. Fig. 4 shows the dose-response curve of inhibition of IDE activity by heat-treated 100,000 \(\times\) g supernatant. Between 30–70% inhibition, the inhibitory activity was linearly dependent upon protein concentration. Both the level of purification and the recovery of the inhibitor were calculated with this standard curve.

Association of IDE and the Endogenous Inhibitor—When the pH 11 eluate from the anti-IDE column to which only 100,000 \(\times\) g supernatant had been previously applied (preparation A) was heat-treated, inhibitory activity was detected (Fig. 5). This result indicates that some inhibitor is naturally associated with IDE in cytosolic fraction. A pass-through fraction from the column was heat-treated, fractionated by ammonium sulfate precipitation, and then recycled over a column with bound IDE. A fraction of the inhibitory activity...
was adsorbed to the column, and this activity, which eluted from the column (preparation B), was increased when compared with the activity obtained from preparation A (Fig. 5). Fig. 5 also shows that it is not likely that the inhibitor is denatured or degraded products of IDE caused by heat treatment, since heat-treated pure IDE did not have any inhibitory activity. Fig. 6A shows the elution profile of the insulin-degrading activity and inhibitory activity in preparation A on Superose 12. Insulin-degrading activity and inhibitory activity were detectable at $M_\text{r} 300,000$ and 11,000, respectively. Fig. 6B shows the inhibitory activity of preparation B on Superose 12 column. Each fraction was heat-treated and centrifuged, and inhibitory activity in the supernatant was tested. A major peak of inhibitory activity at $M_\text{r} 300,000$ and a minor peak at $M_\text{r} 11,000$ were obtained. In addition, when the protein eluted at $M_\text{r} 300,000$ (fraction 12-22 in Fig. 6B) was applied to Superose 12 after heat treatment and removal of precipitated protein with centrifugation, a single peak of inhibitory activity at $M_\text{r} 11,000$ was obtained (Fig. 6C). On the other hand, when preparation B that had been previously heat-treated was applied to Superose 12, a single peak of inhibitory activity was also eluted at $M_\text{r} 11,000$ (data not shown).

Purification of the Endogenous Inhibitor of IDE—For further purification, preparation B was heat-treated and applied to a Mono Q anion exchange column. The inhibitor was eluted as a single peak at 100–150 mM NaCl from the column (data not shown). The peak of inhibitory activity was collected, concentrated, and then applied to the Superose 12 column (Fig. 7A). A single peak of inhibitory activity was eluted at $M_\text{r} 11,000$. The molecular weight of the inhibitor was calibrated to be 11,500 by its behavior on Superose 12 (Fig. 7B) and showed a 14 kDa band on 18% reduced SDS-PAGE with silver staining (Fig. 7C). Table II summarizes the results of each step of the purification. The inhibitor was purified more than 20,000-fold.

Characterization of the Inhibitor—Fig. 8 presents a kinetic analysis of the degradation of insulin by IDE in the presence and absence of the endogenous inhibitor. The $K_m$ for insulin...
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was 80 nM. Addition of the inhibitor altered the $K_a$ but had no effect on $V_{max}$, indicating that the inhibition manner is competitive with a $K_i$ of 180 nM. Fig. 9A shows that the inhibitor decreased the cross-linking of $^{125}$I-insulin to IDE. The labeled insulin was cross-linked to IDE and showed a single band at 110 kDa on autoradiography (lane 1). Addition of the inhibitor reduced the 110 kDa band in a dose-dependent manner, and 20 μl of an inhibitor sample reduced the 110 kDa band to an undetectable level (lane 2), in the same way as the addition of a large amount of unlabeled insulin (lane 1) did. Fig. 9B shows the inhibitory effect of the inhibitor and various agents that inhibit IDE activity (5, 21) on binding of $^{125}$I-insulin to IDE in the plate assay. The endogenous inhibitor inhibited the binding of insulin to IDE in a dose-dependent manner similar to that found in the cross-linking study. Among the agents tested, p-hydroxymercuribenzoate and bacitracin could also inhibit the binding of insulin to IDE.

DISCUSSION

In the present study, we developed an immunoaffinity purification method for IDE using a column coupled with monoclonal antibody to IDE, 9B12 (8). We selected the rat liver as the source of IDE for purification, since the cytosolic fraction of this tissue contains abundant IDE when compared with other tissues, such as the kidney, brain, and skeletal muscle (26). We also employed the immunoaffinity procedure as a first step of purification, since the liver contains many proteins, including several proteases besides IDE. As shown in Table I, a large amount of protein could be separated from IDE by immunoaffinity purification, after which IDE was easily purified to homogeneity with two additional conventional steps. The enzyme characteristics of the purified sample, such as molecular weight, optimal pH for insulin degradation, and metal requirement, are identical with those of human erythrocyte IDE (data not shown).

Although many studies have indicated the importance of IDE in insulin degradation in the intact cell, some properties

FIG. 8. Lineweaver-Burk analysis of IDE with insulin as a substrate in the presence and absence of purified inhibitor. Purified IDE was incubated for 10 min at 37 °C in 20 mM PB (pH 7.5) containing 0.25% BSA, 0.1 ng/ml $^{125}$I-insulin, and various concentrations of unlabeled insulin (0.025–0.84 μM). The concentration of inhibitor (final preparation, 0.15 μg/ml protein concentration) was estimated to be approximately 14 nM. (The molecular weight of the inhibitor used was 11,000).

![Graph](image)

FIG. 7. Elution profile of endogenous inhibitor on Superose 12 column chromatography (the last step of purification) and determination of the molecular weight of the inhibitor. A, preparation B was heat-treated, and the denatured protein was removed with centrifugation. The supernatant was desalted with Sephadex G-25 previously equilibrated with 50 mM Tris-HCl (pH 8.0), and then applied to a Mono Q anion exchange column and eluted with linear salt gradient. The peak of inhibitory activity from Mono Q was collected, concentrated, and applied to Superose 12 column. The fractions 27–29 and 24–26 were pooled and concentrated. The fractions were then applied to a Superose 12 column. The fractions 27–29 were pooled and concentrated and applied to Superose 12 column. The fractions 27–29 and 24–26 were pooled and concentrated and applied to Superose 12 column. The fractions 27–29 and 24–26 were pooled and concentrated and applied to Superose 12 column. The fractions 27–29 and 24–26 were pooled and concentrated and applied to Superose 12 column.

![Graph](image)

TABLE II

| Steps        | Protein conc. (μg/ml) | Volume (ml) | Total protein (mg) | -Fold Recovery | % |
|--------------|----------------------|-------------|--------------------|----------------|---|
| Heat treatment| 2.8                  | 435         | 1,218              | 5.9            | 100 |
| 50% (NH4)2SO4| 3.2                  | 105         | 336                | 14.2           | 66.4 |
| Mono anti-IDE| 5.7 × 10^{-3}        | 60          | 0.34               | 309            | 1.5 |
| Mono Q       | 0.3 × 10^{-3}        | 20          | 6.0 × 10^{-3}      | 1,720          | 0.14 |
| Superose 12  | 0.15 × 10^{-3}       | 0.75        | 1.13 × 10^{-3}     | 21,800         | 0.03 |

a Calculated on the assumption that $A_{390.15} = 10$. 

![Graph](image)
of this enzyme still remain ambiguous. When and how do IDE contact insulin that is endocytosed via a receptor-mediated pathway? How is IDE regulated in its biological activities in the cell? Since IDE is primarily cytosolic (27), high substrate specificity and/or elaborate regulatory (especially inhibitory) mechanisms are necessary in order not to degrade intracellular proteins. Shortly after the first report of IDE, Mirsky and Periutti (23) described an “insulinase inhibitor” extracted from beef liver homogenate. However, very little is known about the endogenous inhibitor of this enzyme even now. Ryan and Duckworth (24) characterized the endogenous inhibitor of this enzyme by anion exchange chromatography from rat skeletal muscle. The molecular weight of the inhibitor was 67,000 or 80,000–120,000, and the inhibition manner was noncompetitive. McKenzie and Burghen (25) documented the endogenous inhibitor of the same enzyme of rat liver. The inhibition manner of their inhibitor was competitive and a 15,000-Da dialysis membrane permitted free passage of the inhibitor, whereas a 3,500-Da membrane retained it. One explanation for this controversy would be that different tissues may contain different types of inhibitors, since this is the case in a certain protease inhibitor (28). However, we recently purified an IDE inhibitor from human erythrocytes and its properties, such as molecular weight, its inhibitory manner, and its behavior on anion exchange column, were the same as those of the inhibitor from rat liver. Another explanation could be the purity of the inhibitor and the enzyme employed for the inhibitor assay. The enzyme that McKenzie and Burghen (25) used was about 700-fold pure, Ryan and Duckworth (24) did not mention the purity of the enzyme they used, and neither study mentioned the purity of the inhibitor.

An anti-IDE monoclonal antibody made it easy to obtain a large amount of pure IDE. Using this enzyme preparation, we could test the inhibitory activity of the inhibitor without contaminating nonspecific proteases that may degrade insulin. The affinity technique employed in this study not only provided a purification of the inhibitor but also directly demonstrated the binding of the enzyme and the inhibitor. It is of interest that the NaHCO₃-eluted fraction from the anti-IDE column to which only the 100,000 × g supernatant had been applied (preparation A) contained inhibitory activity (Fig. 5). It may suggest that the inhibitor was partially associated with IDE in the cytosolic fraction. When the 100,000 × g supernatant was applied to the anti-IDE column, IDE and the inhibitor were eluted with 75 mM NaHCO₃ concomitantly, retaining its biological activity. The inhibitory activity eluted at M, 11,000 in Fig. 6A was due to the inhibitor that was partially dissociated from IDE during the step of chromatography. Conversely, it is likely that the insulin-degrading activity eluted at M, 300,000, was due to free IDE. These results suggest the possibility that the inhibitor could be purified from the cytosolic fraction without heat treatment. As shown in Fig. 5, however, the inhibitory activity from preparation B was much enhanced when compared with the activity from preparation A. Furthermore, heat treatment was a useful step to remove other irrelevant proteins. We used, therefore, the preparation B for further purification of the endogenous inhibitor. The inhibitory activity which eluted at M, 300,000 in Fig. 6B is likely to be identical with the inhibitory activity that eluted at M, 11,000, because the molecular weight of the former was shifted to 11,000 after heat treatment (Fig. 6C). Hence, it is likely that large amounts of inhibitor associate with IDE and are eluted as a 300,000-kDa mass from the anti-IDE column. Table II demonstrates that the percent recovery of inhibitor obtained from the immunoadsorption column comprises only a few percent of the total inhibitory activity in the cytosol, and thus, the physiological significance of this inhibitor may not be immediately obvious. However, the presence of naturally associated inhibitor with IDE in the cytosol may indicate the significant interaction of the enzyme and the inhibitor. In order to obtain a higher yield of the inhibitor, it would be necessary to clarify the associating and/or disso-
cating mechanism(s) of both proteins.

In regard to the nature of endogenous inhibitor, it is unlikely that the inhibitor is denatured or partially degraded IDE by heat treatment, because heat-treated pure IDE has no inhibitory activity. Fig. 7C also shows the difference in molecular weight between the inhibitor and proinsulin, IGF-I, which are barely or more slowly degraded by IDE than insulin (29, 30) and may lower an apparent IDE activity by competing with 125I-insulin in the inhibitory assay. The molecular mass of the inhibitor in both its native and reduced conditions were approximately 11–14 kDa, and the manner of inhibition was competitive. These data, as well as its heat stability, are consistent with the data of McKenzie and Burghen (25). As shown in Fig. 9, the inhibitor could inhibit the binding of insulin to IDE, also supporting the competitive inhibition of the inhibitor. Moreover, among the agents that inhibit the insulin-degrading activity by IDE in the cell (5, 21), only p-hydroxymercuroibenzoate and bacitracin could inhibit the insulin binding to IDE. Since these agents do not compete with the binding of insulin to insulin receptors (data not shown), these two agents may interact to a certain motif of amino acid side of the insulin-binding site on IDE. The reason why the insulin binding to IDE in the presence of N-ethylmaleimide (NEM) or 1,10-phenanthroline increased is not clearly understood. One explanation is that the addition of the two agents may inhibit the degradation of insulin bound to IDE and thus cause the increase of apparent insulin binding. One unique property of the inhibitor is its heat stability. This is similar to calpastatin (28), the endogenous inhibitor of calpain, or calcium-dependent neutral protease, which is also a proteolytic enzyme and exists in the cytosolic fraction of various mammalian tissues (31, 32). Heat treatment is often employed to purify these inhibitors. Recently, many thiol protease inhibitors called cystatin have been purified, most of which are proteins of molecular weight between the inhibitor and proinsulin, IGF-II (35–37). Since IDE has some similarities to these protease inhibitors.

Subcutaneous tissue contains IDE, it may be natural that insulin injected into this tissue can be degraded. Moreover, there are some clinical reports available concerning an excessive amount of insulin degradation in patients that have been shown, in certain cases, to result in insulin resistance (35–37). The endogenous inhibitor of IDE, therefore, could be used for treatment of such patients and utilized as an additional agent of insulin that may increase the absorption rate of insulin in patients that have used for treatment of such patients and utilized as an additional agent of insulin that may increase the absorption rate of insulin. One unique property of the inhibitor is its heat stability. Since IDE may have some similarities to these protease inhibitors.

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