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Purification of Insulin-Specific Protease by Affinity Chromatography

(rat-skeletal muscle/sulfhydryl dependence/cytoplasm)

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ABSTRACT **A single enzyme that proteolytically degrades insulin was isolated from rat skeletal muscle. This enzyme was purified 1000-fold by a series of steps, including affinity chromatography on insulin bound to agarose at the NH_2-terminal phenylalanine of the B chain. Insulin linked to agarose at the B-29 lysine residue did not bind the enzyme and, therefore, was not suitable for purification procedures. Insulin linked at the phenylalanine residue was a substrate for the enzyme and was degraded by it; insulin attached to agarose at the lysine residue was not degraded by the enzyme. The purified enzyme preparation yielded one major band on polyacrylamide gel electrophoresis, and elution of this area of the gel yielded insulin-degrading activity. The purified enzyme degraded insulin but not proinsulin, with a K_m for insulin of 22 nM and a K_i for proinsulin of 40 nM. The enzyme is sulfhydryl-dependent, with a physiological pH optimum.**

The mechanisms of degradation and inactivation of insulin are unclear. A preparation, insulinase, that inactivated insulin with some specificity was described and studied by Mirsky (1). Liver-insulinase activity was partially purified by Vaughan (2). Other workers also studied insulin degradation and demonstrated its proteolytic nature (3), but the incomplete purification and characterization of insulinase did not permit a complete evaluation of its physiological significance.

In addition to the proteolytic destruction of insulin, which has also been described in adipose tissue (4), insulin can be inactivated by reductive cleavage of the disulfide bonds into A and B chains by glutathione insulin transhydrogenase (5, 6). The high K_m of this enzyme and the difficulty demonstrating activity in liver fractions untreated with detergent or acetone cast doubt on its physiological importance (7, 8).

An enzyme was partially purified from rat-skeletal muscle that degraded insulin at physiological concentrations (1–0.1 nM). This enzyme was clearly different from glutathione insulin transhydrogenase by virtue of its low K_m and its lack of dependence on glutathione (9). Insulin-degrading activity was demonstrated in the soluble fraction of cells from rat diaphragms (10). Brush partially purified the rat skeletal-muscle enzyme and demonstrated that this sulfhydryl enzyme proteolytically degraded insulin, but not proinsulin (11). A similar enzyme has been demonstrated in other rat tissues (12, 13), and a similar enzyme in the liver has been partially purified (7). Other studies on degradation of insulin by liver have been reported (14).

Using a new purification procedure including affinity chromatography, we have now purified a single enzyme from the supernatant fraction of rat skeletal muscle that proteolytically degrades insulin, with little effect on proinsulin. The properties of this enzyme appear to be similar to those of the partially purified enzyme described earlier by Brush (11), and, therefore, the enzyme described here is called insulin-specific protease (ISP).

Abbreviation: ISP, insulin-specific protease.

MATERIALS AND METHODS

Male Holtzman rats (200–300 g) were used for all enzyme preparations. Porcine insulin, proinsulin, and diacetyl insulin were gifts from Dr. R. Chance of the Eli Lilly Co., Indianapolis, Indiana. [^{125}I]Insulin was purchased from Cambridge Nuclear Corp., Cambridge, Mass., and had 0.3 atom of iodine per mol; it was 96% Cl_3CCOOH-precipitable and 94% immunoprecipitable, and was biologically active in isolated fat cells. Rabbit antiserum globulin to guinea pig serum was purchased from Cappell Laboratories, Downington, Pa. Ammonium sulfate, ultra-pure grade, was obtained from Schwarz/Mann, Orangeburg, N.Y. All other chemicals were of reagent grade and purchased commercially. Porcine proinsulin was lightly iodinated (less than 1 g-atom of iodine per mol of proinsulin) with ^{125}I (15). Insulin–agarose preparations were synthesized (16). Cyanogen bromide-activated agarose was reacted with insulin under two different conditions, with 0.2 M sodium bicarbonate buffer at pH 9 or with 0.2 M sodium citrate at pH 5. Under acidic conditions, the N-terminal phenylalanine of the B chain of insulin binds to agarose; under basic conditions, the reaction is with the lysine residue at position 29 on the B chain (16). Diacetylated insulin was reacted with activated agarose at pH 9.

All insulin–agarose preparations were immunoassayable by the double antibody method (17), with guinea pig antisera to porcine insulin as the first antibody and rabbit antisera to guinea-pig gamma globulin as the second antibody. The procedure was as described (18), except that the first antibody was incubated on a shaker at 4° and the agarose beads were removed by centrifugation immediately before addition of the second antibody. Removal of agarose produced better pellets and more reproducible results. Insulin–agarose derivatives were also biologically active in isolated fat cells prepared by the method of Rodbell (19).

Enzyme Preparation and Purification. 6–8 Male rats were killed by decapitation. The hind-leg muscles were removed, cleaned of connective tissue, and homogenized in 0.35 M sucrose, 5 ml/g of tissue, in a Waring blendor for 90 sec. The total muscle weight of a typical preparation was about 150 g. The homogenate was centrifuged for 15 min at 13,000 × *g*. The supernatant fraction was then centrifuged at 55,000 ×

g for 90 min. This supernatant solution was fractionated with ammonium sulfate: 0.21 g of solid ammonium sulfate was added per ml of supernatant (30% saturation); the pH was maintained at 6.2 by addition of 0.1 M NH_4OH or 0.1 M HCl as required. The suspension was centrifuged at 17,000 $\times$ g for 20 min. The precipitate was discarded, and 0.21 g of ammonium sulfate was added per ml of supernatant (60% saturation). The protein suspension was centrifuged as above. The precipitate was dissolved in 20 mM sodium acetate (pH 6.2) ($^1/_{15}$ the original volume). This solution was immediately dialyzed against 20 volumes of the same buffer for 3 hr with two changes. All procedures were done at 4°. This preparation, called the 30–60% fraction, could then be frozen and stored without loss of activity. Additional purification, however, resulted in a loss of stability, and all subsequent steps were done during the same day in order to minimize the loss of enzymatic activity.

The enzyme, 2–3 ml of the ammonium sulfate fraction, was passed through a Sephadex G-100 column (1.5 $\times$ 83 cm) in 20 mM acetate buffer (pH 6.2). The peak activity, which eluted with the void volume, was pooled and immediately adsorbed to a QAE–Sephadex column (1.5 $\times$ 7 cm). This column was washed with 0.1 M NaCl in 20 mM acetate buffer; the enzyme was eluted with 0.2 M NaCl in 20 mM acetate buffer. The peak activity was pooled and immediately dialyzed against 20 mM acetate buffer (pH 6.2)—20 volumes for 2 hr with three changes. The enzyme was then adsorbed to Sepharose-bound insulin in a 0.5 $\times$ 6-cm column, washed with 20 mM acetate buffer (pH 6.2), and eluted with 0.2 M NaCl in 20 mM acetate buffer. The purified enzyme was dialyzed, and its activity was studied. The purified enzyme preparation contained about 50 μg of protein.

Assay of Enzymatic Activity. One assay involved the conversion of [^{125}I]insulin to Cl_3CCOOH-soluble products, and the other tested the conversion of immunoassayable insulin to nonimmunoassayable components.

In the trichloroacetic acid method, the enzyme was incubated with 28 pM [^{125}I]insulin in 0.1 M borate buffer (pH 7.5) with 0.4% bovine albumin. After incubation at 37° for 10 min in a metabolic shaker, the reaction was stopped by addition of 1 ml of 10% Cl_3CCOOH. After centrifugation, the supernatant and precipitate were counted in a Packard autogamma spectrometer. One unit of enzyme activity is that amount of enzyme that converts 1 fmol of insulin to acid-soluble material per min (11).

Degradation of immunoreactive insulin was measured by incubation of 25 μl of enzyme with porcine insulin (0.01–10 nM) in 0.13 M borate buffer (pH 7.5) with 0.5% bovine albumin in a total volume of 225 μl. After incubation, the reaction was stopped by addition of 25 μl of 10 mM N-ethyl maleimide, and insulin was assayed by the double antibody immunoassay (18).

Assay of Proteolytic Activity. 2 ml of ISP purified on insulin–agarose, about 50 μg of protein, was dialyzed against 50 mM phosphate buffer (pH 7.5) for 3 hr with three changes. ISP in pH 7.5 buffer was incubated for 20 hr at 37° with 1.5 mg of porcine insulin (11). Similarly prepared enzyme was also incubated with 1.5 mg of bovine-serum albumin under the same conditions. Insulin and albumin that had not been reacted with the enzyme were also assayed. The mixtures were then lyophilized, dissolved in 0.2 ml of 0.64 M acetic acid, and assayed for ninhydrin-reactive material by the method of Moore and Stein (20), as modified by Hirs (21).

TABLE 1. *Degradation by ISP of porcine insulin and various insulin–agarose derivatives*

Min of incubation	fmol Substrate remaining			
	Insulin	Insulin–agarose (pH 5)	Insulin–agarose (pH 9)	Diacetyl insulin–agarose
0	100	102	100	190
2	79	60	100	190
5	30	34	101	190
10	11	21	103	190
20	7	12	91	190

Each substrate was incubated with ISP at 37° in 0.13 M borate buffer (pH 7.5) for the indicated time. 0.10 mM N-ethylmaleimide was added to stop the reaction, and the mixture was immunoassayed by the double-antibody immunoassay. The ISP used was partially purified by ammonium sulfate fractionation.

RESULTS

Reaction of ISP with Insulin–Agarose. For determination of the proper procedure for purification of the enzyme by affinity chromatography, preliminary studies on the reaction of ISP with insulin bound to agarose were performed. After they were extensively washed, the various agarose–insulin complexes were incubated with partially purified (by ammonium sulfate fractionation) ISP, and degradation of the bound insulin to nonimmunoassayable components was determined (Table 1). Nonbound insulin and insulin–agarose (pH 5) were readily degraded, while diacetyl insulin–agarose and insulin–agarose (pH 9) were not degraded.

We showed that the degree of degradation was a reflection of the ability to bind ISP by adding ISP to 0.5 ml of the various agarose preparations, mixing well, and centrifuging to remove the supernatant. The supernatant was then assayed for enzymatic activity. Less than 5% of the enzyme added was recovered from the supernatant of insulin–agarose (pH 5), while 70% remained in the supernatant of insulin–agarose (pH 9) and over 80% was recovered from the supernatant of diacetyl insulin–agarose.

The same type of result was obtained when small (0.5 $\times$ 3 cm) columns were prepared with the various agarose preparations. When equal amounts of enzyme were added to each of four columns, none of the enzyme bound to plain agarose or diacetyl insulin–agarose, less than 5% bound to insulin–agarose (pH 9), and 100% of the enzymatic activity bound to the pH 5 preparation. All subsequent studies, therefore, were done with insulin–agarose prepared at pH 5. Three different preparations were made for these studies, and no difference among them was seen.

Recovery of ISP from small columns (0.5 $\times$ 3 cm) of insulin–agarose (pH 5) was examined under several conditions, including different buffers, pH values, and temperatures. The optimum conditions for binding were a pH of 6.2 at 4°. Higher pH values and higher temperatures were associated with decreased binding to the column, possibly due to degradation of insulin–agarose. Lower pH values gave a decreased recovery of the enzyme.

TABLE 2. *Summary of purification of insulin-specific protease from muscle*

	Specific* activity	Fold purification	Yield (%)
Crude homogenate	0.75	1	100
100,000 × *g* supernatant	3.97	5.3	95
$(NH_4)_2SO_4$ 30–60% fraction	8.0	10.7	79
Sephadex G-100	10.6	14.1	39
QAE–Sephadex	98.6	131.4	39
Affinity chromatography	760	1013	20

* fmol of insulin degraded per min per mg of protein.

Purification of ISP. The final enzyme preparation was about 1000-fold purified as compared with the crude homogenate (Table 2). Only a slight purification was seen with passage over Sephadex G-100 (Fig. 1), but this step was necessary to remove an inactive component that was not removed by any subsequent step. Fig. 2 shows the elution pattern from QAE–Sephadex obtained with 0.2 M NaCl. Better resolution was obtained by washing first with 0.1 M NaCl followed by elution with 0.2 M NaCl than was obtained by a linear salt gradient. Fig. 3 depicts elution from the insulin–agarose column. 0.1 M NaCl eluted insulin-degrading activity from the column, but a sharper peak resulted when 0.2 M NaCl was used. Fig. 4 shows the polyacrylamide gel pattern of the enzyme at various stages of purification. On gel *AC* (containing the enzyme purified 1000-fold, obtained after affinity chromatography), only one major band can be seen. This band represents highly concentrated, very pure enzyme. For comparison, about 7–8 times as much enzymatic activity was applied to gel *AC* as was applied to gel *I*. Since a very faint band of protein can be seen above and below the major band on gel *AC*, the enzyme is not homogeneous; but when an unstained gel was sliced and homogenized in acetate buffer, only the section corresponding to the major band contained insulin-degrading activity. Addition of material eluted from other sections of the gel to eluted ISP resulted in neither stimulation nor inhibition of insulin-degrading activity. For experiments in which enzymatic activity was eluted from polyacrylamide gel, electrophoresis was run at 4° and 1.6 mM dithiothreitol was added to the buffer.

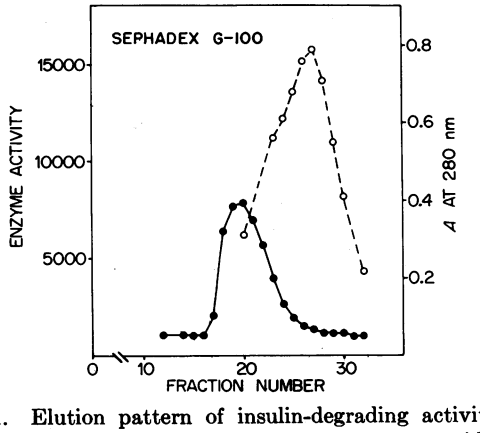

FIG. 1. Elution pattern of insulin-degrading activity from Sephadex G-100. O– – –O, *A* 280 nm; ●——●, cpm acid-soluble degradation products.

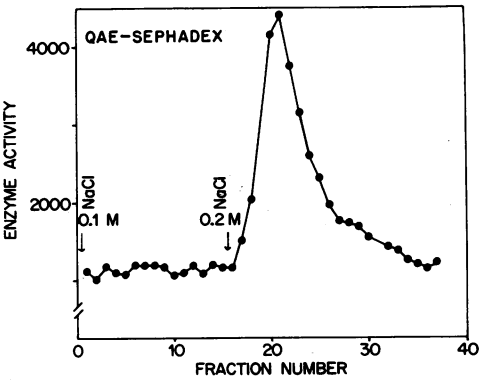

FIG. 2. Elution of insulin-degrading activity from QAE–Sephadex. Enzyme activity was adsorbed to the column in 20 mM acetate buffer (pH 6.2). The column was washed with 0.1 M NaCl in 20 mM acetate, and the activity was eluted with 0.2 M NaCl in 20 mM acetate (pH 6.2). Enzyme activity in cpm of acid-soluble degradation products.

Properties of the Purified Enzyme. Enzyme activity was linear with protein concentration (Fig. 5), and the pH optimum was 7.4 (data not shown). The enzyme degraded insulin both to acid-soluble products and to nonimmunoassayable components (Fig. 6). Proinsulin was not converted to nonimmunoassayable components to any appreciable extent by the purified enzyme (Fig. 6), and less than 1% of [^{125}I]-proinsulin was degraded to acid-soluble products under conditions that converted 30% of [^{125}I]insulin to acid-soluble material (data not shown). The K_i for unlabeled proinsulin was 40 nM. The K_m for insulin was 22 nM (Fig. 7). Similar K_m values were obtained by the immunoassay method and the trichloroacetic-acid assay.

The enzyme was a protease that converted insulin to ninhydrin-positive material after incubation. An 8.7-fold increase in ninhydrin-reactive material, measured as leucine equivalents, was seen after reaction of insulin with the enzyme. Reaction of ISP with bovine-serum albumin produced only a 1.6-fold increase in leucine equivalents. ISP was also shown to be sulfhydryl-dependent, since glutathione and dithiothreitol stimulated activity and *N*-ethyl maleimide and *p*-hydroxymercuribenzoate inhibited it completely (Table 3). The purified enzyme was unstable, losing activity when stored either frozen or at 4°.

DISCUSSION

Binding of an enzyme to its substrate or to a competitive inhibitor offers a degree of specificity that is not present in

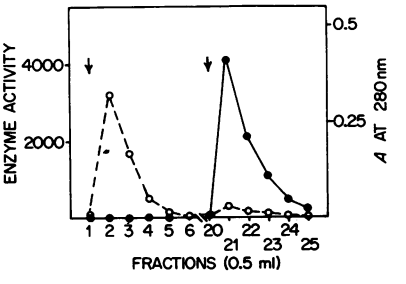

FIG. 3. Elution of insulin-specific protease from insulin–agarose. O– – –O, *A* 280 nm; ●——●, cpm acid-soluble degradation products. *Left arrow*, enzyme added; *right arrow*, elution with 0.2 M NaCl in 20 mM acetate, pH 6.2.

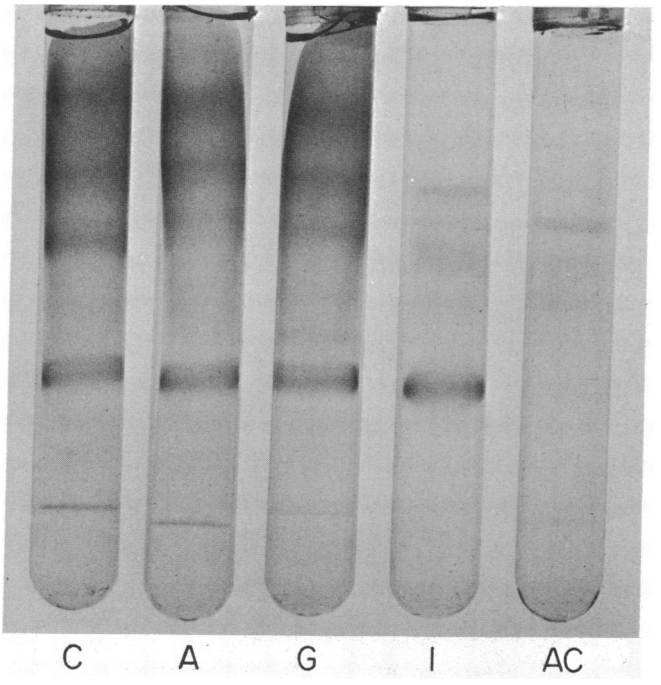

FIG. 4. Polyacrylamide gel pattern of various preparations of insulin-degrading activity. Gel C represents the crude 100,000 × *g* fraction; *A* is the 30–60% $(NH_4)_2SO_4$ fraction; *G* is the peak from the G-100 Sephadex column; *I* represents the QAE–Sephadex peak; and *AC* is the enzyme from affinity chromatography.

most purification procedures. Insulin reacts readily with cyanogen bromide-activated agarose, which has been used for purification of insulin antibodies (22) and for studies on the action of insulin (16). Several problems had to be overcome before agarose–insulin could be used to purify an insulin-degrading enzyme. We had to be able to bind the enzyme to the substrate without degrading the insulin and, thereby, breaking the bond. The combination of a decrease in the temperature and the pH was a practical and convenient way to bind ISP to insulin–agarose with little degradation, since the pH optimum of ISP is 7.4–7.6.

Disruption of the enzyme–substrate bond without destruction of the enzyme was another problem. Our attempts to elute active enzyme by changing the pH, increasing the temperature, or adding *N*-ethyl maleimide or insulin were unsuccessful. High salt concentration eluted protein from the column but with rapid loss of enzymatic activity. Elution

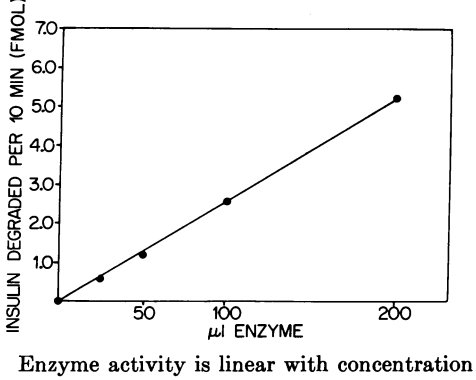

FIG. 5. Enzyme activity is linear with concentration of protein.

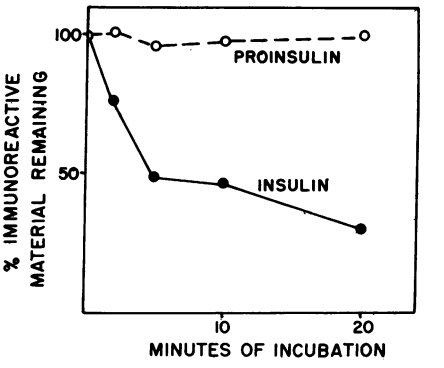

FIG. 6. Degradation of immunoreactive insulin and proinsulin by ISP. Insulin or proinsulin was incubated at 37° with purified enzyme in 0.13 M borate buffer (pH 7.5) with 0.3% bovine-serum albumin. Immunoreactive material remaining was measured.

with 0.2 M NaCl followed by immediate dialysis gave the best yields of enzyme.

Insulin-degrading activity could be considerably purified by application of the 30–60% ammonium sulfate fraction directly on an insulin–agarose column. The material eluted had a specific activity of 276, a 368-fold purification over the muscle homogenate. Polyacrylamide gel electrophoresis of this preparation showed several bands of protein in addition to the major one representing ISP. Elution from unstained polyacrylamide gels of the areas corresponding to the other major protein bands did not reveal any insulin-degrading activity; combination of these eluted materials with eluted ISP neither stimulated nor inhibited ISP activity. Thus, several cytoplasmic components bind to insulin–agarose and are eluted with NaCl. Therefore, several steps before affinity chromatography were necessary to achieve maximal purification.

ISP failed to bind and degrade some preparations of insulin–agarose although these preparations had both immunological and biological activity; three separate batches of insulin–agarose, prepared under conditions that bound the lysine at B-29 to agarose, had immunoassayable insulin attached to the agarose and also stimulated antilipolysis and glucose uptake in isolated fat cells. These findings suggest that the membrane receptor may recognize a different site on the insulin molecule than the site that binds to the degrading enzyme. Membrane binding and degradation are independent (8). This observation also appears to be true for proinsulin.

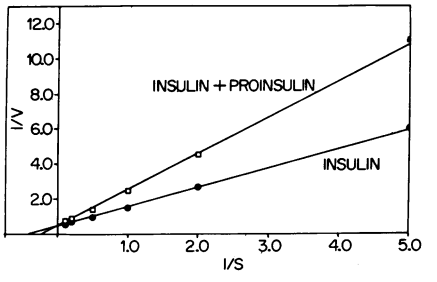

FIG. 7. Kinetic study of insulin degradation and the effect of proinsulin. Velocity is expressed as mol × 10^{-11} of insulin degraded per ml per min. Incubation time was 5 min. Substrate concentration is expressed as mol × 10^{-8} per liter. Proinsulin concentration was 0.27 μM. Assay was by the trichloroacetic-acid method.

TABLE 3. *Insulin-degrading activity of the purified enzyme under various conditions*

	Relative activity (%)
Freshly prepared enzyme	100
After 12 hr at 4°	55
Frozen and thawed	75
Glutathione (1 mM)	133
Dithiothreitol (1 mM)	155
N-Ethylmaleimide (1 mM)	0
p-Hydroxymercuribenzoate (1 mM)	0
$ZnCl_2$ (10 mM)	0

Activity was measured by conversion of [^{125}I]insulin to Cl_3CCOOH-soluble material in the presence of each of the above after incubation with purified ISP for 10 min at 37°. Percent stimulation by glutathione and dithiothreitol was calculated from control tubes containing these materials but without enzyme.

Proinsulin is degraded by ISP at 1/30–1/50 the rate of insulin (11), while the binding of proinsulin to isolated cell membranes and the biological activity of proinsulin (23, 24) are about 1/10 that of insulin (25).

Examination of the three-dimensional structure of insulin suggests another possibility for failure of ISP to degrade these preparations. The B-1 phenylalanine is located at one end of the molecule, and the B-29 lysine at the other end (26). Phenylalanine-bound insulin is degraded, while lysine-bound insulin is not. This result may indicate that ISP recognizes a site in the region of, and perhaps including, the B-29 lysine and that when the large agarose molecule interferes with this site, neither binding nor degradation occurs. Attachment of agarose to B-1 phenylalanine at the opposite end of the insulin molecule, however, leaves the B-29 region free to be recognized and degraded. The relatively poor degradation of proinsulin may also be partly explained by interference with this site, since the connecting peptide may obscure the B-29 region.

Since the enzyme is in cytoplasm and not in blood (18), insulin degradation would presumably occur from the cytoplasm. This process might require entrance of insulin into cells (10), or it might be related in some way to the membrane, although membrane binding and degradation are separate processes (8). ISP may be loosely associated with the membrane and the process of homogenization might break the association. The explanation for failure of the purified enzyme to degrade 100% of the insulin (Fig. 6) is not clear, since the less purified preparation degrades essentially all of the insulin added (18). This could be due to inhibition by degradation products, but additional information will be required to answer this question. The low K_m of the enzyme insures maximal destruction of insulin with little degradation of other proteins (7).

The present demonstration that insulin is proteolytically degraded by a single specific enzyme in peripheral tissue suggests that degradation of insulin may be important in control of amounts of insulin and may possibly influence its action.

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