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Insulinase-Inhibitory Activity of Protein Hydrolysates.*† (23021)

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During studies of the identity of the factor (or factors) in liver extracts which inhibits activity of insulinase(1), it became apparent that the greatest concentration of this factor occurred in fractions reported by others to be rich in strepogenin activity(2). Since hydrolysates of insulin and a variety of other proteins are also rich in strepogenin activity (2,3), the insulinase-inhibitory activity of such hydrolysates was studied.

Methods. The various proteins used were hydrolyzed for 3 hours with concentrated hydrochloric acid (4 g protein/100 ml acid) at 37°C, neutralized and evaporated to dryness in flash evaporator. The hydrolysates were kept in the dry state until immediately before use.¶ The effect of the presence of various concentrations of the protein hydrolysates on rate of degradation of I^{131} labeled insulin by liver extract was determined by the procedure described previously(4). Inhibitory activity of the 3-hour acid hydrolysates of proteins was computed on the basis of quantity of nitrogen necessary to produce a 50% inhibition of insulinase activity of the extract. Since an hydrolysate of insulin had the greatest inhibitory activity, the activities of other hydrolysates were expressed as percentage of activity of the insulin hydrolysate. The effect of prolonged acid hydrolysis on insulinase-inhibitory activity of crystalline insulin, bovine plasma albumin and casein was determined by treating these proteins as above, with concentrated hydrochloric acid for 72 hours at 37°C. Aliquots were removed at the beginning and at various designated intervals during hydrolysis, neutralized and lyophilized. The quantity of each hydrolysate, in terms of nitrogen, necessary to produce a 50% inhibition of insulinase activity of a liver extract was determined. Maximum insulinase-inhibitory activity of an hydrolysate was attributed to that aliquot which produced a 50% inhibition with the smallest quantity. All other assays were expressed in terms of percentage of aliquot with maximum activity. In addition, tryptic and chymotryptic digests of insulin, bovine plasma albumin, crystalline lysozyme and casein were prepared. The proteins were dissolved or dispersed in distilled water, heated at 100°C for 3 minutes, cooled and adjusted to pH 7.8. A 0.5% solution of each protein was incubated under toluene for 12 hours at 37°C with an amount of crystalline trypsin or crystalline chymotrypsin equal to 1% of weight of the protein. pH was maintained during incubation period by addition of alkali. At the end of incubation period, the reaction mixtures were adjusted to pH 3, heated to 100°C, cooled, neutralized and lyophilized. The action of hydrolysates on degradation of I^{131} labeled insulin was determined as above using the equivalent of 5 mg of protein. The data were expressed in terms of activity of an equivalent amount of a 3-hour acid hydrolysate of crystalline insulin. The type of inhibition produced by some of the hydrolysate of proteins was determined from studies of the effect of single concentration of each hydrolysate on action of

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TABLE I. Insulinase-Inhibitory Activity of 3-Hour Acid Hydrolysates of Various Proteins. Inhibitory activity computed on basis of quantity of hydrolysate (in terms of N) required to produce 50% inhibition of insulinase activity of liver extract and expressed in terms of activity of an hydrolysate of insulin.

Protein hydrolysate	% activity
Insulin, crystalline, Lilly No. 535664	100.0
Pitressin, Parke-Davis, No. 174363	87.1
Pitocin, Parke-Davis, Nos. 182530 and 182531	83.0
Growth hormone (Somar), Armour, No. M-208	81.3
Glucagon, crude, Lilly, E-1399	77.3
Intrinsic factor, Lilly, No. 260-201B-51-3	72.7
Adrenocorticotrophin, Armour, No. 41-L-2	57.8
Prolactin, crude, 6 units/mg, Lilly, No. 953561	42.0
Lysozyme, crystalline, Armour, No. 00325	42.0
Bovine plasma albumin, Fraction V, Pentex, No. B6004	38.1
Trypsin, crystalline, Pentex, No. A3401	38.1
Alpha Amylase, Nutritional Biochemical, No. 2796	38.1
Egg albumin, crystalline, Armour No. E90115, Pentex No. B4902	37.8
Casein, vitamin-free, Nutritional Biochemical	36.4
Serum albumin, human, Lilly, No. 352319B	33.4
Renin substrate, Lilly, No. 235-195B-104	33.4
Chymotrypsin, crystalline, salt-free, Pentex, No. A2701	33.4
Serum albumin, bovine, crystalline, Pentex, No. A1201	32.2
Globin, purified, Nutritional Biochemical	30.8
Catalase, port liver, Pentex, No. A3901	30.0
Chymotrypsin, crystalline, Bios	29.8
Pepsin, 3× crystallized, Pentex, No. C3705	29.0
Ribonuclease, crystalline, salt-free, Pentex	28.3
Beta-Lactoglobulin, crystalline, Pentex, No. A4801	27.6
Gamma globulin, bovine, Pentex, No. C0602	25.8
Tetanus antitoxin, Lilly, No. B-8652B	25.8
Renin, porcine, purified, Lilly, No. 221-194B-213	25.5
Malt diastase, Nutritional Biochemical, analytical grade	24.3
Plasma albumin, bovine, crystalline, Armour, No. M66605	24.0
Fibrinogen, bovine, Armour, No. N1804	24.0
Protamine, (Salmine), Lilly, No. 961271	23.4
Gamma globulin, porcine, Pentex, No. A2001	23.2
Egg albumin, 5× crystallized, Pentex, No. B4903	21.7
Emulsin, Nutritional Biochemical, No. 6076	21.6
Trypsinogen, crystalline, General Biochemicals, No. 28910	20.5
Papain, Lilly, No. 221-267B-31	20.2
Ficin, General Biochemicals, No. 20142	17.3
Immune serum globulin, human, Lilly, No. 8471X342644Z	16.6

extract incubated with different concentrations of labeled insulin. The effect of an acid hydrolysate of bovine plasma albumin on destruction of I^{131} insulin by intact mice was determined by procedure described previously(5).

Results. It is apparent from the data summarized in Table I that a fairly large number of proteins yield insulinase-inhibitory fragments after relatively mild acid hydrolysis. The hydrolysates of 12 other proteins that were studied had no inhibitory activity.‡ A Lineweaver-Burk(6) plot of the effect of hydrolysates of crystalline insulin and bovine plasma albumin on destruction of various concentrations of labeled insulin by fresh liver extract suggests that inhibition produced by various hydrolysates is of the competitive type (Fig. 1).

The peptide nature of fragment (or fragments) responsible for insulinase-inhibitory activity of the protein hydrolysates is indicated by complete loss of inhibitory activity which occurs after prolonged hydrolysis of the proteins. The data illustrated in Fig. 2 reveal that hydrolysis of insulin, bovine plasma albumin and casein with concentrated hydrochloric acid results in maximum inhibitory activity in 2 to 6 hours; thereafter insulinase-inhibitory activity diminishes so that

‡ The 12 proteins were crystalline phosphorylase, crystalline trypsin, edestin, ovamucoid, urease, glucuronidase, hemoglobin, trypsin-inhibitor, protamine sulfate, alpha amylase, beta amylase, and elastin.

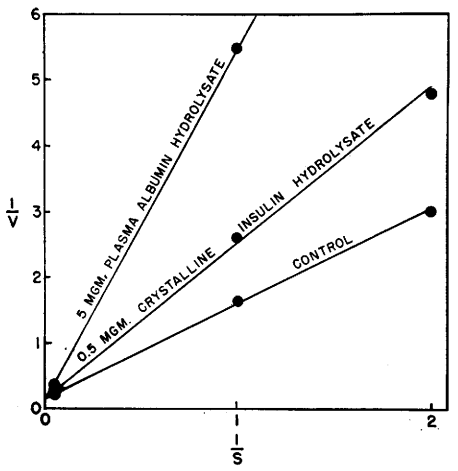

FIG. 1. Inhibition of insulinase activity of rat liver extract by acid hydrolysates of crystalline insulin and bovine plasma albumin. Incubation of 1 ml of liver extract plus 1 ml of phosphate buffer containing various quantities of I^{131} insulin or plus 1 ml phosphate buffer containing designated amounts of protein hydrolysate and various quantities of insulin, at pH 7.8 and 37°C for 30 min. V = units of insulin destroyed in 30 min. S = units of insulin/ml of incubation mixture.

after 48 hours of hydrolysis there is essentially no activity.

Acid hydrolysates of proteins, which are effective as insulinase-inhibitors *in vitro* may also decrease destruction of insulin by the intact animal. Thus, subcutaneous administration of 4 mg of an hydrolysate of bovine plasma albumin/g body weight to 24 mice resulted in reduction of rate of insulin destruction from 15.8 ± 0.5 to 10.5 ± 1.1 units/

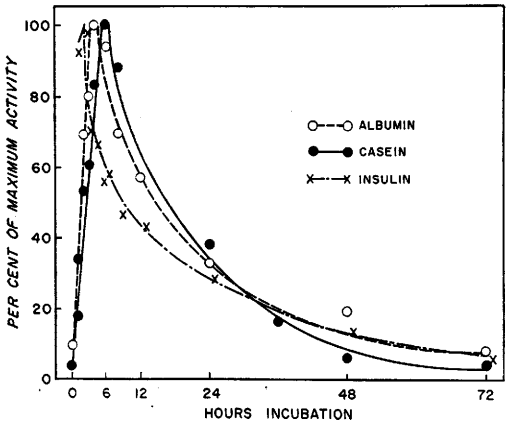

FIG. 2. Effect of prolonged acid hydrolysis of crystalline insulin, bovine plasma albumin and casein on insulinase-inhibitory activity. (See text.)

100 g/hour ($P<0.001$). Administration of such hydrolysates to mice or rats, however, does not produce a decrease in blood sugar concentration.

Enzymatic digests of various proteins are effective as inhibitors of the destruction of insulin by potent extracts of liver (Table II). Thus, incubation of crystalline insulin, casein, albumin and lysozyme with crystalline trypsin and crystalline chymotrypsin at 37°C and pH 7.8 for 12 hours yields products which inhibit insulinase (Table II). It is interesting that whereas a tryptic digest of bovine plasma albumin is more active than a chymotryptic digest in inhibition of insulinase, a chymotryptic digest of crystalline lysozyme is more active than a tryptic digest.

TABLE II. Insulinase-Inhibitory Activity of Tryptic and Chymotryptic Digests of Proteins Expressed in Terms of Inhibition Produced by Equivalent Amount of 3-Hour Acid Hydrolysate of Crystalline Insulin.

	Inhibition (%)		
Protein	Untreated	Tryptic digest	Chymotryptic digest
Crystalline insulin	100.3*	98.1	102.1
Bovine plasma albumin	19.0	52.0	19.8
Crystalline lysozyme	18.1	20.8	106.0
Casein	7.5	48.6	57.3

* Reflects dilution of labeled insulin.

Discussion. Our studies reveal that an acid or an enzymatic hydrolysis of various proteins yields peptides which can competitively inhibit the action of insulinase *in vitro* and *in vivo*. The inability of these hydrolysates to lower blood sugar concentration of mice and rats may be due to the presence of glucogenic as well as insulinase-inhibitory peptides. Although the hydrolysates which exhibit greatest insulinase-inhibitory activity are also those which have the greatest strepogenin activity(2,3), the 2 activities are not related. Thus, purified polypeptides§ which have a fair degree of strepogenin activity, exhibit no insulinase-inhibitory activity.

§ We are indebted to Dr. W. Woolley for providing samples of serylhistidylleucylvalylglutamic acid and serylhistidylleucylvalylglutamylalanylleucine.

The nature of peptides responsible for inhibition of insulinase remains unknown. In view of the observation that a variety of natural and synthetic plant growth regulators exert an insulinase-inhibitory and hypoglycemic action(7,8), it is interesting to note that enzymatic digests of plant and animal proteins are rich in auxins which apparently are newly formed during protein digestion(9, 10). Whether or not auxin activity and insulinase-inhibitory activity of protein hydrolysates are related awaits further study.

Tomizawa and Williams have reported that addition of casein, α-corticotrophin trichloracetate, glucagon and growth hormone to the incubation mixture of liver extract and labeled insulin results in decrease in rate of insulin destruction. They interpret this observation to mean that added proteins compete with insulin for a non-specific enzyme system(11). The data reported herein do not support the conclusion of Tomizawa and Williams. Thus, acid hydrolysates of glucagon, corticotrophin, growth hormone and casein are among the most potent inhibitors of insulinase. These proteins are also degraded by extracts of liver which hydrolyze insulin(12). Consequently, it is probable that inhibition of insulin destruction which occurs *in vitro* in the presence of the above proteins is due to release of insulinase-inhibitory peptides rather than to competition between insulin and any of the aforementioned proteins.

Conclusion. Destruction of insulin by extracts of rat liver is inhibited competitively by 3-hour acid hydrolysates and by tryptic and chymotryptic digests of a variety of proteins. Insulinase-inhibitory activity is attributed to a peptide (or peptides) which loses activity on complete hydrolysis.

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