PURIFICATION AND CHARACTERIZATION OF INHIBITORS OF INSULIN SPECIFIC PROTEASE IN HUMAN SERUM

James S. Brush

with the technical assistance of

Ramila J. Shah

Veterans Administration Hospital and the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38104

Received June 5,1973

Summary

The presence in human serum of inhibitory activity to rat liver insulin specific protease has been detected in an alpha$_1$ globulin preparation (Cohn Fraction IV$_1$). Separation into four components and partial purification (40 to 107 fold) has been achieved by heat denaturation of non-active protein, Sephadex G-100 gel filtration and ion-exchange chromatography upon QAE Sephadex. Each of the inhibitors was found to be competitive in nature. The molecular weight of the inhibitors is between 4,000-7,000 and the activity is destroyed for the most part by chymotrypsin.

Introduction

In studies upon the metabolism of insulin in the rat diaphragm it was shown that insulin is rapidly degraded to products of low molecular weight by a soluble enzyme present in the cytoplasmic compartment of the tissue (1). The same or a very similar enzyme was partially purified and characterized in both rat muscle (2) and liver (3). It was shown to be a sulfhydryl-dependent enzyme which degrades insulin proteolytically with great specificity (2,3). Examination of modifiers and inhibitors of the enzyme have given some indication of its mode of inhibition (4).

In the present investigation the presence of four distinct polypeptide inhibitors in human serum of the liver enzyme, insulin specific protease, have been detected. These inhibitors have now been considerably purified from a serum alpha$_1$ globulin preparation and characterized. The mode of inhibition by these inhibitors has been examined using enzyme kinetic methods.

Materials and Methods

Human alpha$_1$ globulin (Cohn Fraction IV$_1$) was obtained from Miles Laboratories, Inc., Research Division, Kankakee, Illinois).

Three times crystallized chymotrypsin was obtained from Sigma Chemical Co. (St. Louis, Missouri).

The insulin specific protease preparation used for the routine assay of inhibitor was the dialyzed 100,000 x g supernatant solution of rat liver homogenate prepared as previously described (3) and stored at -60°. The enzyme used in the kinetic experiments was purified 5-7 fold by adsorption and elution from calcium phosphate gel (3).

Radioiodinated ^{125}I insulin was obtained from Cambridge Nuclear Corp. (Cambridge, Massachusetts). The enzymatic activity was determined by measuring the net per cent of ^{125}I insulin converted to trichloroacetic acid soluble form as previously described (2, 3).

A column of Sephadex G-100 1.5 x 22 cm was used for the purification of the inhibitors and for the estimation of their molecular weights. It was eluted with a solution containing 0.10 M NaCl and 0.05 M tris HCl buffer, pH 7.8 (NT buffer).

Calibration of the column was carried out using myoglobin

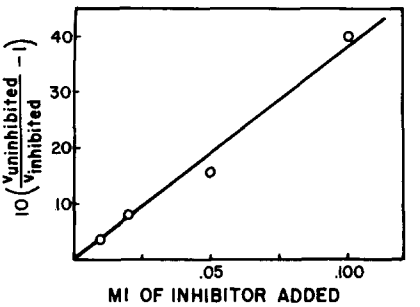

Figure 1. Linearity of assay for insulin specific protease crude inhibitor. Each point is the average of duplicate determinations. See Materials and Methods for details.

(M.W., 16,000) and ^{125}I insulin whose molecular weight is 6,000 at the dilute concentrations used (1). ^{125}I labeled insulin was measured in each fraction by counting in a Packard Autogamma spectrometer. Protein concentrations were determined from the absorbance at 280 mμ and 260 mμ (5).

A rate equation for enzyme inhibition has been derived which expresses the rate of an inhibited reaction as a linear function of inhibitor concentration, and which is independent of the type of inhibition. Adjusted to a standard reaction rate arbitrarily fixed as the rate for an amount of uninhibited enzyme converting one-tenth of the ^{125}I insulin present to trichloroacetic acid soluble form in 10 min the expression for the units of inhibition becomes the following:

$$10\left(\frac{v_{uninhibited}}{v_{inhibited}} - 1\right) = \text{Units of Inhibition}$$

The assay of trypsin inhibitory activity was carried out according to the method of Eriksson (6).

Results

In Figure 1 are shown the results of an experiment demonstrating that the expression relating inhibitor concentration

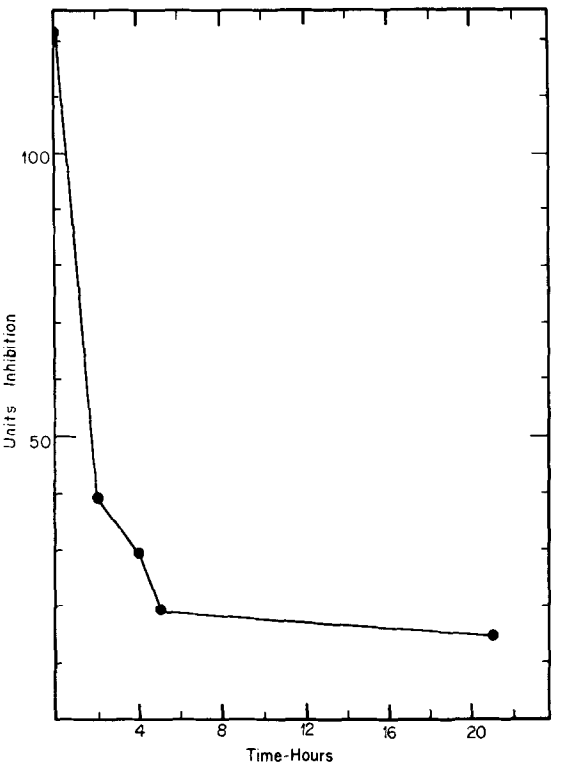

Figure 2. Chymotryptic cleavage of plasma inhibitors. Normal human plasma (20 ml) was diluted with NT buffer to 120 ml and heated at 100° for 15 min. The denatured protein present was removed by centrifugation. The supernatant was dialyzed twice against 6 l of deionized distilled water and lyophilized. It was redissolved in 4 ml of 0.9% NaCl and an insoluble residue removed by centrifugation. An aliquot of this solution containing approximately 1200 units of inhibitor was incubated with 30.6 mg of chymotrypsin in 0.1 M tris buffer, pH 7.8 at 37°. At the indicated times 1.0 ml aliquots of the mixture were transferred to test tubes containing 2 μmoles of phenylmethyl sulfonyl fluoride (PMSF) and the inhibitory activity determined in the presence of 2mM of PMSF and then frozen.

to enzyme activity is experimentally linear as predicted.

In Figure 2 are shown the results of an experiment demonstrating the degradation by chymotrypsin of inhibitory activity partially purified by the heating procedure described below. The inhibitor of chymotryptic activity, phenylmethylsulfonyl fluoride (7), has no effect upon the activity of the enzyme (2) and was used to stop chymotryptic hydrolysis at the indicated times. Of the total starting activity 84% was destroyed

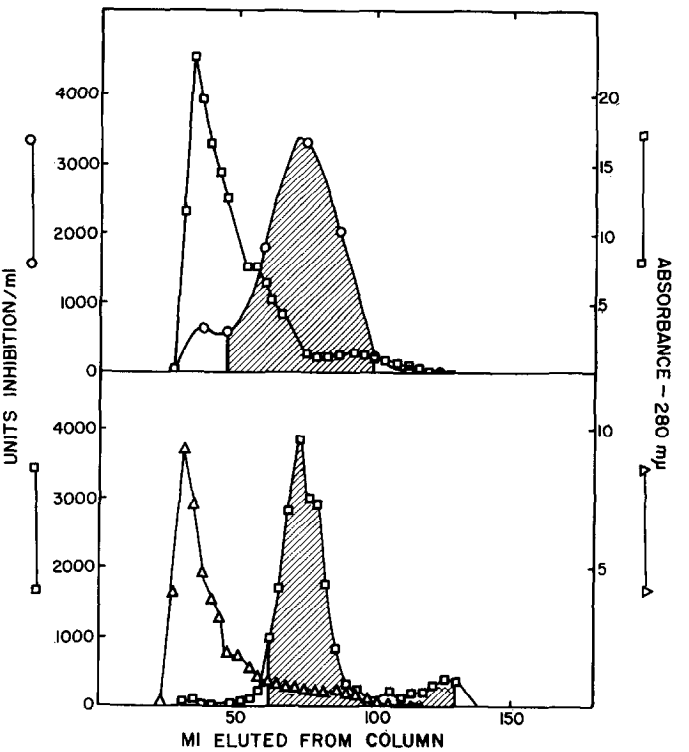

Figure 3. Gel filtration of insulin specific protease inhibitors upon Sephadex G-100. A. First passage through column. B. Second passage through column.

during the first 5 hrs of incubation, but only an additional 3.7% in the succeeding 16 hrs. This probably indicates that at least one of the component inhibitors is resistant to chymotryptic hydrolysis.

The purification procedure for the inhibitors was the following:

Step 1; 4.9 gm of lyophilized alpha$_1$ globulin (Cohn Fraction IV$_1$) was dissolved in 219 ml of NT buffer. Undissolved material was removed by centrifugation.

Step 2; the solubilized protein from Step 1 was heated at 100^{o} for 15 min and denatured protein removed by centrifugation at 4^{o}. The volume of the supernatant was 165 ml and the units of activity/ml were unchanged by the heating procedure.

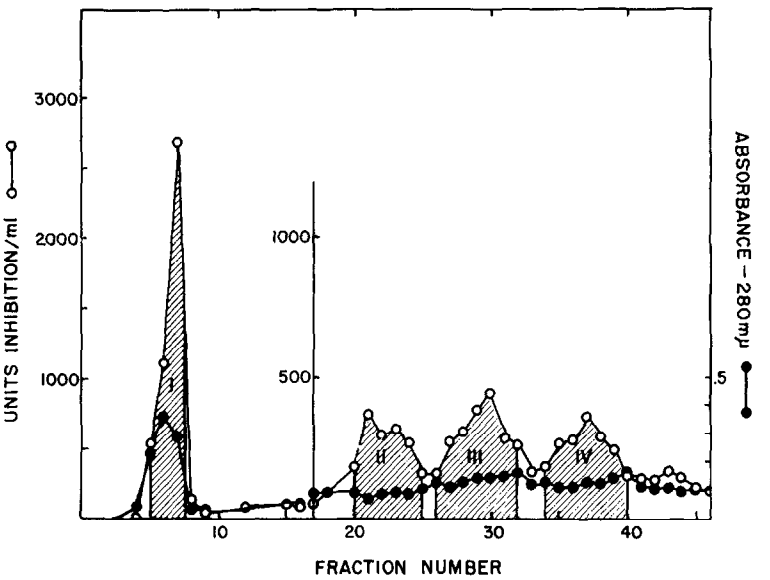

Figure 4. Fractionation of insulin specific protease inhibitors upon QAE Sephadex A25. A linear gradient of NaCl from zero to 0.35 M was the eluting solution.

Step 3; the supernatant from Step 2 was dialyzed twice against 4 l of deionized water and lyophilized. The lyophilized material was redissolved in 8 ml of NT buffer and placed upon the Sephadex G-100 column and eluted with NT buffer. The pattern of elution is shown in Figure 3A. A small peak of inhibitory activity appears in the first peak of translucent protein, but the bulk of the activity is of lower molecular weight. The activity in the shaded area was pooled, dialyzed as before, lyophilized and redissolved in 3 ml of NT buffer and again passed through the Sephadex G-100 column. The results obtained upon this second passage shown in Figure 3B demonstrate a much improved purification. In this case the inhibitor activity curve seems to indicate three different areas of activity -- an initial sharp peak followed by an unresolved shoulder of activity, and finally material in the large initial peak and shoulder are estimated to be in the range of 4000 to 7000.

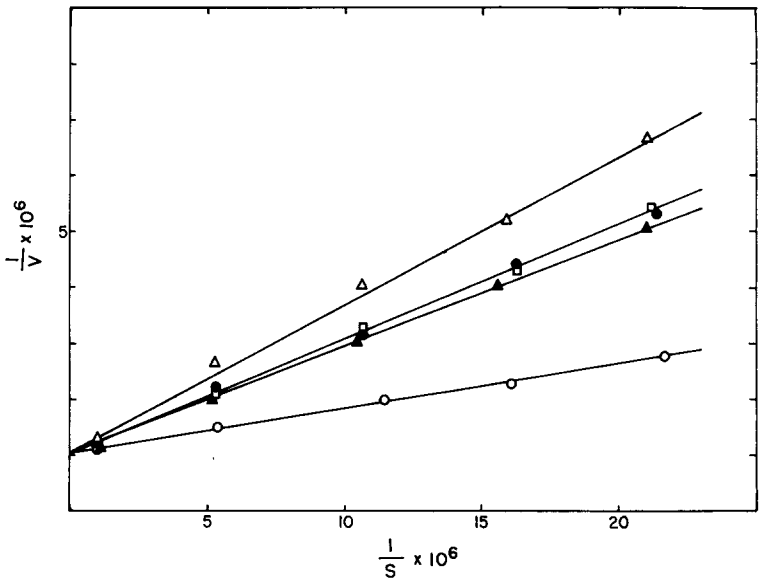

Figure 5. Patterns of inhibition of insulin specific protease by purified and separated serum inhibitors. 0 no inhibitor. ▲ + 3 µg peak I. ◘ + 5 µg peak II. ● + 6 µg peak III. △ + 12 µg peak IV. Units of S are moles/l. Units of v are moles/l/10 min/ml enzyme.

Step 4; the activity in the shaded area of Figure 3B was combined, dialyzed, lyophilized and further purified by ion-exchange chromatography upon QAE Sephadex A-25 resin: The results obtained are shown in Figure 4. Four distinct peaks of activity were obtained in this case. An initial very large peak of activity is apparently material which does not bind to the column and is designated peak I. Three much smaller peaks followed in succession and are designated peaks II, III and IV.

In Table I is summarized the purification achieved at each step. The individual peaks varied considerably in their purity from 42 fold for peak IV to 107 fold for peak I.

Antitrypsin inhibitory activity comprises a large part of the alpha$_1$ globulin fraction (8). This activity was measured at the first and second steps of the purification

TABLE I

Purification of Insulin Specific Protease Inhibitors From Human Serum

Fraction	Total Activity (units)	Specific Activity (Units/mg protein)	Yield Per cent	Fold Purification
1. Cohn Fraction IV$_1$ Alpha$_1$ Globulin	145,900	46	100	1.0
2. Heat denaturation	108,500	100	74	3.9
3. Sephadex G-100				
a. First passage	106,100	468	73	10.2
b. Second passage	72,500	2850	50	62.1
4. QAE Sephadex				
Peak I	13,400	4900	9	107
Peak II	5,280	2850	4	62
Peak III	6,670	2650	5	58
Peak IV	5,650	1950	4	42

procedure. It was low in the crude preparation (7 μg of trypsin inhibitory capacity/mg protein) but this activity was undetectable after the heat denaturation step.

Peaks I through IV were tested for their mode of inhibition by the enzyme kinetic methods previously described (2, 3). Figure 5 shows that each of the inhibitors seems to be of competitive type. Peaks I through IV were also tested for their ability to react with guinea pig insulin antisera and were found to be unreactive.

Discussion

In contrast to the previously described trypsin and chymotrypsin inhibitor present in the alpha$_1$ globulin

fraction (9), the inhibitors of the insulin protease are heat stable under the conditions used. They are, therefore, distinctly different in this property. Their ability to be destroyed by chymotrypsin indicates that they are polypeptides. Gel filtration upon Sephadex G-100 indicated some resolution of at least two classes of inhibitors whose molecular weights are in a range (4000-7000) which includes that of insulin (6000). Ion-exchange chromatography upon QAE Sephadex resolved the activity into four distinct peaks with 43% of the total activity appearing in the first peak.

When each of the four peaks was tested to determine the nature of the inhibition which is produced, each of the inhibitors were of the competitive type. In previous studies (3, 4) none of the inhibitors studied has been of this type except beef proinsulin (2) whose amino acid sequence is identical to insulin with the addition only of the peptide connecting the A and B chains (11). This was true even of the nonapeptide bradykinin (4) whose pattern of inhibition was consistent with a mechanism in which its complexing with the enzyme does not prevent subsequent binding of the substrate in a catalytically inactive ternary complex.

At its present state of purity the active material of peak I is at least as potent an inhibitor per mg of protein as proinsulin which heretofore was the most active inhibitor described (2).

Insulin specific protease is a highly specific enzyme degrading essentially a single protein substrate under physiological conditions (1, 2, 3). The recognition by the enzyme of insulin as a distinct protein requires that it recognize and associate with multiple points within the molecule. The present four serum

inhibitors may be somewhat similar to the structure of insulin, therefore.

Acknowledgement

Appreciation is expressed to Dr. John Griffin and Mrs. Paula White for the performance of the trypsin inhibitor assays, and to Mrs. Mary Alice Bobal for the performance of the assay of inhibitory fractions for insulin content using guinea pig insulin antisera. This work was supported by a Veterans Administration Research Grant.

References

1. Brush, J. S. and Kitabchi, A. E. (1970) Biochim. Biophys. Acta 215, 134-144.
2. Brush, J. S. (1971) Diabetes 20, 140-145.
3. Burghen, G. A., Kitabchi, A. E. and Brush, J. S. (1972) Endocrinology 91, 633-642.
4. Brush, J. S., (1973) Fed. Proc. 32, 503 Abs.
5. Colowick, S. P. and Kaplan, N. O. (1957) Methods in Enzymology, Vol. III, p. 454.
6. Eriksson, S. (1965) Acta Med. Scand. 177: Suppl. 1-85.
7. Fahrney, E. E. and Gold, A. M. (1963), J. Am. Chem. Soc. 85, 997-1000.
8. Vogel, R., Trautschold, I. and Werle, E. (1968) Natural Proteinase Inhibitors, p. 59, Academic Press, New York.
9. Bundy, H. F. and Mehl, J. W. (1959), J. Biol. Chem. 234, 1124-1128.