5. Evidence is presented showing that the carbohydrate–peptide bond in egg albumin consists of a linkage of the β-carboxyl group of an aspartic acid residue with the 1-amino group of 2-acetamido-1-amino-1,2-dideoxyglucose. This has been compared and contrasted with carbohydrate–peptide bonds in other glycoproteins.

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A Pure Trypsin Inhibitor from Soya Beans

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An acetone-insoluble trypsin inhibitor (Bowman, 1946) has been separated from soya beans and purified by dialysis, freeze-drying and fractionation on a diethylaminoethylcellulose column (Birk, 1961*a*, *b*). Rackis, Sasame, Mann, Anderson & Smith (1961) reported the isolation of a similar inhibitor from soya-bean-whey proteins. Applebaum, Janković & Birk (1961) have shown that the freeze-dried preparation exhibits amylase activity. The latter could not be separated completely from the trypsin inhibitor on diethylaminoethylcellulose columns. Preliminary experiments indicate that the complex formed between the inhibitor purified on diethylaminoethylcellulose and trypsin shows neither trypsin nor trypsin-inhibiting activity, but exhibits all the amylase activity of the inhibitor. These findings suggest that the inhibitor and amylase are two different materials.

This paper is devoted to further fractionation of the purified inhibitor on a carboxymethylcellulose column and to the characterization of the pure inhibitor. A rapid method for preparing the pure inhibitor on this cation-exchanger directly from the dialysed preparation, omitting purification on diethylaminoethylcellulose, is also given.

EXPERIMENTAL

Abbreviations and designations. CM-cellulose, carboxymethylcellulose; DEAE-cellulose, diethylaminoethylcellulose. 'Crude inhibitor', Bowman's (1946) crude, acetone-insoluble inhibitor. 'Non-diffusible inhibitor', dialysed and

freeze-dried crude inhibitor. 'Purified inhibitor A', 'non-diffusible inhibitor' purified on DEAE-cellulose (Birk, 1961*b*). 'Purified inhibitor AA', 'non-diffusible inhibitor' fractionated and purified on CM-cellulose.

Assay of trypsin- and α-chymotrypsin-inhibiting activity. This was by the casein-digestion method at pH 7·6 (Kunitz, 1947). The enzymes were commercial crystalline preparations of Worthington Biochemical Corp. Inhibitor (in 0·5 ml. of solution) was preincubated with enzyme for 30 min. at 37° and the remaining proteolytic activity was then determined. One unit of inhibition (T.I.U.) (Kunitz, 1947) is equivalent to a decrease of 1·0/min. in E_{280} of a trichloroacetic acid filtrate in excess of the appropriate blank readings.

Assay of amylase activity. This was by the method of Bernfeld (1955) for assay of sweet-potato β-amylase with dinitrosalicylic acid reagent, with these modifications: the substrate was dissolved in 0·2M- instead of 0·016M-buffer and the reaction was at 30° instead of 20°. The extinction of the solution containing the brown reduction product was determined at 550 mμ. Amylase activity was expressed in terms of mg. of maltose liberated in the reaction mixture in 3 min. at 30°.

Chromatography on CM-cellulose columns. CM-cellulose (Whatman no. 70) was equilibrated with 5 mM-sodium acetate buffer, pH 4·0, the starting buffer, and the suspension was poured into a column (2·2 cm. × 45 cm.) and allowed to settle under gravity. Purified inhibitor A or non-diffusible inhibitor (1 g. in 100 ml. of starting buffer) was applied to the column. Elution was performed with these agents successively: (*a*) starting buffer; (*b*) gradient to 0·14M-NaCl in starting buffer, mixing chamber volume 1000 ml.; (*c*) a combined gradient to 0·45M-NaCl and to 0·2M-sodium acetate, mixing chamber volume 500 ml., thus introducing also a change in pH, as indicated in Fig. 1. The rate of flow was adjusted to 200 ml./hr. and 8 ml. fractions were collected. The protein content of the initial solution and of each effluent tube was estimated by determining E_{280}. Every second tube was assayed for trypsin-inhibiting activity and for amylase. For preparative purposes the step of freeze-drying in the preparation of non-diffusible inhibitor was omitted. Acetate buffer (0·2M, pH 4·0) was added to the non-diffusible preparation to bring the solution to 5 mM-buffer.

Rechromatography of fraction II (fractions 104–119; Fig. 1) from the CM-cellulose column was performed on a CM-cellulose column (1·1 cm. × 28 cm.) prepared as described above. The effluent in tubes 104–119 from the first column was combined and dialysed to remove the NaCl. Acetate buffer (0·2M, pH 4·0) was added to the solution to adjust its buffer molarity to 5 mM, and the solution was then applied to the column. Elution was performed with starting buffer and gradients as described above, the mixing chamber of (*b*) being this time 300 ml. and that of (*c*) 150 ml. The protein content and activities of the effluent tubes were determined as in the first CM-cellulose fractionation (see Fig. 2). The contents of tubes 20–28 were combined and dialysed. The non-diffusible material was freeze-dried and designated purified inhibitor AA. Its protein content was determined by the micro-Kjeldahl method.

Chromatography on DEAE-cellulose columns. DEAE-cellulose (Carl Schleicher and Schüll) was equilibrated with 0·01M-sodium phosphate, pH 7·6, the starting buffer. The suspension of the adsorbent was poured into a column (1·1 cm. × 20 cm.) and allowed to settle under gravity. A sample containing 14 mg. of purified inhibitor AA in 5 ml. of starting buffer was applied to the column. Elution was primarily performed with 80 ml. of starting buffer and then with a gradient of NaCl (0–0·26M) in starting buffer, the mixing chamber volume being 200 ml. Protein content and activities were determined as described above.

All column-chromatography was done at room temperature.

Ultracentrifugal analysis. Measurements of sedimentation velocity were made in a Spinco model E ultracentrifuge, equipped with a phase-plate schlieren optical system, 12 mm. cell, operating at 59 780 rev./min. and at 23·8°. The solvent employed was 0·9% NaCl. Measurements of diffusion were performed in the same Spinco model E ultracentrifuge, again in 0·9% NaCl. The boundary between the solvent and the solution was obtained with a synthetic-boundary cell, capillary type, and operating at 9341 rev./min.

Determination of isoelectric point. Samples (1 ml.) of 0·5% purified inhibitor AA in water were added to 0·1M-acetate buffers (6 ml.) of various pH values containing 30% of $(NH_4)_2SO_4$; the latter salt was added because preliminary experiments showed that no precipitation of protein occurred in its absence even after 48 hr. The

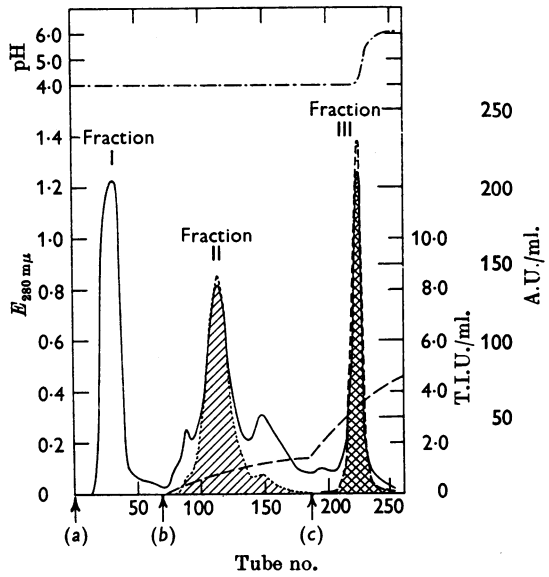

Fig. 1. Chromatography of the dialysis residue of 'crude inhibitor' (1 g. in 130 ml.) on a CM-cellulose column (2·2 cm. × 45 cm.): (*a*) 5 mM-sodium acetate, pH 4·0, starting buffer, total volume of eluting agent 560 ml.; (*b*) gradient to 0·14M-NaCl in starting buffer, mixing chamber volume 1000 ml., total volume of eluting agent 944 ml.; (*c*) combined gradient to 0·45M-NaCl and to 0·2M-sodium acetate, mixing chamber volume 500 ml., total volume of eluting agent 496 ml. Flow rate 200 ml./hr., with effluent collected in 8 ml. fractions. ▨, Trypsin-inhibiting activity (T.I.U., see text); ▩, amylase activity (A.U.: mg. of maltose/3 min. at 30°); ----, molarity of NaCl; -·-·-, pH.

mixtures were lightly agitated. The pH and the turbidity (as E_{600}) of the various mixtures were measured immediately.

Analyses of amino acids. Samples of protein (5 mg.) were hydrolysed with 6N-HCl (5 ml.) in a sealed glass tube at 110° for 18 hr. Excess of HCl was removed by evaporating the hydrolysate to dryness repeatedly.

The resulting amino acid hydrolysates were redissolved in water and their contents of methionine and tyrosine were determined according to the quantitative paper-chromatographic method of McFarren (1951).

Tryptophan was determined in the intact protein by 'procedure L' described by Spies & Chambers (1949).

RESULTS AND DISCUSSION

Purification of the inhibitor on a CM-cellulose column. Fig. 1 shows the elution diagram of non-diffusible inhibitor on CM-cellulose. About 95% of the protein applied was eluted. The distinct separation between the trypsin inhibitor and the considerably purified amylase is obvious. The same chromatographic pattern and activity distribution were obtained when purified inhibitor A was chromatographed on CM-cellulose. Since no difference was found between the specific activities of fraction II from both non-diffusible inhibitor and purified inhibitor A fractionated on CM-cellulose, it was decided to abandon the step of chromatography on DEAE-cellulose. The dialysis residue of fraction II (tubes 104–119 from the column) was rechromatographed on a CM-cellulose column (Fig. 2). It appeared as a single peak in its original position in the effluent solution in 96% yield. Its specific activity remained the same. The purity of this preparation of trypsin inhibitor was further substantiated by its chromatography on DEAE-cellulose, from which it emerged again as a single peak. The specific activity of the effluents obtained after rechromatography on each type of column (CM- and DEAE-cellulose) was the same, i.e. $7{\cdot}15 \times 10^{-3}$ T.I.U./μg., in each effluent tube of the peak. Purified inhibitor AA is therefore probably a single pure protein. The purified inhibitor has a nitrogen content of 15·9% and $E^{1\%}_{1\,cm.}$ at 280 mμ was 4·80.

Fig. 1 shows that soya-bean amylase is also highly purified by chromatography on CM-cellulose.

Effect of pure inhibitor on the proteolytic activity of trypsin and α-chymotrypsin. A direct proportionality between the amount of inhibitor used and the amount of trypsin inhibited was observed: a preparation containing 1 μg. of purified inhibitor AA/ml. produced an inhibition of 7×10^{-3} T.I.U./ml. with casein used as substrate. In contrast, the lack of proportionality between the amount of crystalline soya-bean trypsin inhibitor used and chymotrypsin inhibited (Kunitz, 1947) holds true also for the effect of purified inhibitor AA on chymotrypsin (Fig. 3). However, the chymotrypsin-inhibiting activity of purified inhibitor AA is about 13 times as high as that of the crystalline soya-bean trypsin inhibitor prepared by Kunitz (1947) for the following amounts: 3 μg. of purified inhibitor AA or 40 μg. of crystalline soya-bean trypsin inhibitor inhibit 5 μg. of chymotrypsin in a reaction mixture including 12·5 μg. of the latter.

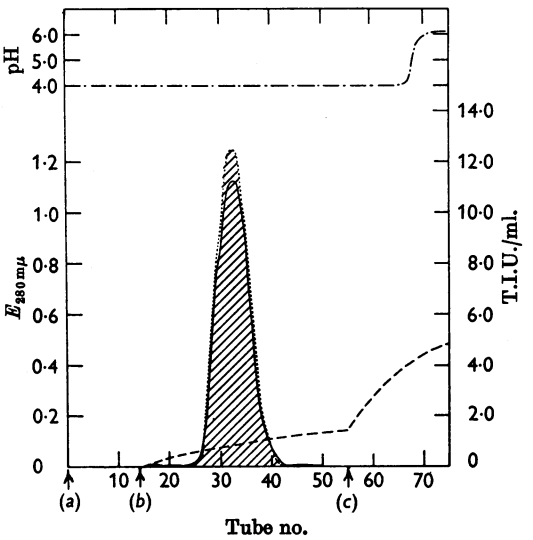

Fig. 2. Rechromatography of the dialysis residue of fraction II (tubes 104–119, Fig. 1) on a CM-cellulose column (1·1 cm. × 28 cm.): (*a*) 0·05M-sodium acetate, pH 4·0, starting buffer; (*b*) gradient to 0·14M-NaCl in starting buffer, mixing chamber volume 300 ml.; (*c*) combined gradient to 0·45M-NaCl and to 0·2M-sodium acetate, mixing chamber volume 150 ml. Flow rate 200 ml./hr., with effluent collected in 8 ml. fractions. ⊾, Trypsin-inhibiting activity (T.I.U., see text); ----, molarity of NaCl; -·-·-, pH.

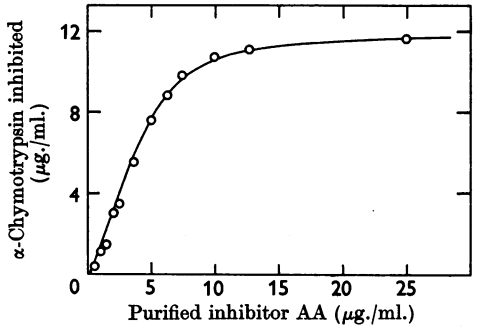

Fig. 3. Effect of purified inhibitor AA on the digestion of casein by α-chymotrypsin: 12·5 μg. of α-chymotrypsin/ml. of 0·5% casein.

Sedimentation and diffusion. Measurements of the sedimentation velocity of purified inhibitor AA, at five 8 min. intervals, showed a single symmetrical boundary. This single component in a solution containing 1 g./100 ml. had $S_{20,w}$ 2·3s and $D_{20,w}$ $9{\cdot}03 \times 10^{-7}$ cm.^{2}/sec. These sedimentation and diffusion coefficients, together with an assumed partial specific volume of 0·75 ml./g., indicate that purified inhibitor AA has mol.wt. 24 000.

Isoelectric point. Determinations of the solubility of the purified inhibitor in 0·1 M-sodium acetate buffers in the range pH 3·90–4·55 indicated an isoelectric point of 4·2.

Amino acid content. The tyrosine and methionine contents of an hydrolysate of purified inhibitor AA were 1·9 and 1·0 g. respectively/100 g. of inhibitor hydrolysed. The complete absence of tryptophan from purified inhibitor AA is of special interest as Kunitz's crystalline soya-bean trypsin inhibitor is apparently the only pure trypsin inhibitor that contains significant quantities of tryptophan (Laskowski & Laskowski, 1954). The non-identity of purified inhibitor A with the crystalline soya-bean trypsin inhibitor of Kunitz, which was established by the distinctly different behaviour on a DEAE-cellulose column (Birk, 1961*b*), is further substantiated by the present work.

SUMMARY

1. Further purification of an acetone-insoluble trypsin inhibitor on a carboxymethylcellulose column and its separation from a highly active amylase has been described.

2. The purity of the inhibitor has been established by rechromatography on carboxymethyl- and diethylaminoethyl-cellulose and by ultracentrifugal analysis.

3. The molecular weight of the pure inhibitor was determined as 24 000 and its isoelectric point was pH 4·2.

4. The non-identity of the pure inhibitor with the crystalline soya-bean trypsin inhibitor has been further substantiated by its higher specific activity (13-fold) against chymotrypsin and by the absence of tryptophan.

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The Photo-assimilation of Acetate by *Rhodospirillum rubrum*

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Rhodospirillum rubrum is a photosynthetic bacterium that grows anaerobically in the light in the presence of one of a number of simple organic compounds together with sodium hydrogen carbonate and growth factors. It was shown to be capable of growing on acetate as major carbon source with yeast autolysate as a source of growth factors. Under appropriate conditions growth was proportional to the concentration of acetate, and approximately 70% of the acetate carbon atoms were converted into cell carbon atoms (van Niel, 1944). Cutinelli, Ehrensvard, Reio, Saluste & Stjernholm (1951) examined the incorporation of isotopically labelled acetate and hydrogen carbonate into cell components of growing cultures of *R. rubrum*. The organism was grown anaerobically in the light on a medium containing yeast extract, acetate and inorganic salts (including hydrogen carbonate). Separate batches of cells were grown in media containing isotopically labelled acetate, as $^{13}CH_3 \cdot {}^{14}CO_2H$ in the presence of hydrogen carbonate, and in media containing $NaH^{14}CO_3$ in