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COMBINED USE OF TRYPSIN–AGAROSE AFFINITY CHROMATOGRAPHY AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE PURIFICATION OF SINGLE-CHAIN PROTEASE INHIBITOR FROM CORN SEEDS

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SUMMARY

We have developed a large-scale method for recovering the corn inhibitor of trypsin and activated Hageman factor from a trypsin–agarose column predominantly in the single-chain form. To do so, inhibitor retained by the column was eluted with 1.0 *M* glycine buffer, pH 2.1. We have used reversed-phase high-performance liquid chromatography to further purify the inhibitor eluted from the trypsin–agarose column by separating the single-chain inhibitor from two-chain inhibitor (a small amount of which is present in the preparation after trypsin–agarose chromatography) and from still smaller amounts of another protein (apparently trypsin) that appears as a contaminant during trypsin–agarose chromatography.

INTRODUCTION

We have previously isolated a trypsin inhibitor from opaque-2 corn seeds by affinity chromatography on trypsin–agarose[1]. Two forms of the inhibitor were obtained in approximately equal amounts. One form is the single-chain (virgin) inhibitor, with a molecular weight (known now from the amino acid sequence[2]) of 12 028. The second (modified) form has two polypeptide chains containing 36 and 76 residues[1,2]. The modified form is produced from the virgin inhibitor by trypsin in the affinity chromatography step[1,2]. To avoid exposure to trypsin, we developed an alternate approach to isolate the inhibitor by chromatography on immobilized antibodies against the inhibitor[3].

It has been reported[4,5] that corn trypsin inhibitor is also a specific inhibitor of activated Hageman Factor (Factor XII_a) of the intrinsic blood clotting process. To use the corn inhibitor in physiological studies, large quantities of highly purified inhibitor are needed. Affinity chromatography on trypsin–agarose is well suited for large-scale preparations, but in our previous form of that chromatography, that procedure suffers not only from the fact that it generates heterogeneity by the enzymatic action of the immobilized trypsin, but it also results in a small but worrisome con-

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tamination of the purified inhibitor by trypsin, due to a slow leakage of trypsin from the affinity column.

Here we report a modification in the affinity chromatography procedure that greatly enhances its usefulness by providing predominantly the single-chain form of the inhibitor. We also report a reversed-phase high-performance liquid chromatography (HPLC) purification of the material eluted from the affinity column. This HPLC step removes contaminating trypsin, separates modified inhibitor from single-chain inhibitor, and appears to resolve the single-chain protein into several very closely related species.

MATERIALS AND METHODS

Extraction of the inhibitor from corn seeds

Seeds of opaque-2 corn were generously provided by Dr. Clyde A. Wassom of the Department of Agronomy, Kansas State University. Seeds were ground, defatted with acetone, and extracted with 0.2 *M* sodium chloride as described previously[1].

Immobilization of trypsin

One gram of trypsin (Sigma, bovine Type III) was covalently linked to 100 ml of CNBr activated[6] Sepharose 2B. The coupling reaction was conducted at 4°C for 18 h in 0.2 *M* sodium borate (pH 9.0). From the absorbance of the material that was washed from the agarose after the coupling reaction, we estimate that *ca.* 750 mg of protein was coupled to the agarose.

Trypsin–agarose column chromatography

Chromatography on a column (14 × 2.5 cm I.D.) that contained *ca.* 70 ml of trypsin–agarose was performed as described previously[1], except that material retained by the column after extensive washing with a buffer at pH 8.2 was subsequently eluted from the column by applying 1.0 *M* glycine buffer (pH 2.1), using a BRL adjustable plunger.

Sephadex G-25 chromatography

Glycine buffer was removed from the eluted inhibitor preparation by chromatography on a Sephadex G-25 column (36 × 4.2 cm I.D.) in 0.1 *M* acetic acid. Inhibitor fractions from the Sephadex G-25 column were lyophilized, dissolved in water and again lyophilized to remove residual acetic acid.

Reversed-phase HPLC

Reversed-phase HPLC was carried out with a Beckman Model 332 liquid chromatography system and a 250 × 10 mm I.D. SynChropak RP-P (C_{18}) column (SynChrom). Chromatography was performed at room temperature and at a flow-rate of 3 ml/min. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.07% trifluoroacetic acid in acetonitrile[7]. All solvent components were HPLC grade.

Lyophilized corn inhibitor, isolated as described above, was dissolved at 2 mg/ml in solvent A. Phenylmethanesulfonyl fluoride was added to a final concentration of 1 m*M*. The sample was passed through a Millipore membrane (0.22 μm) before injection of a 5-ml portion onto the HPLC system. Solvent A was applied for

14 min. The mobile phase was then changed to 34.5% solvent B in 2 min and mobile phase of this composition was then applied for *ca.* 40 min. Then the mobile phase was changed to 100% solvent B (over 7 min) to elute the remaining polypeptides. Fractions were collected and lyophilized.

Miscellaneous procedures

Trypsin inhibitor activity was assayed as described previously[1]. Sodium dodecyl sulfate (SDS) gel electrophoresis was done according to either Weber and Osborne[8] or Laemmli[9]. Proteins were hydrolyzed in 6 *M* hydrochloric acid at 110°C for 24 h, and amino acid compositions determined in triplicate with a Dionex Microbore amino acid analyzer.

RESULTS AND DISCUSSION

Isolation of corn inhibitor by trypsin–agarose

Elution of the corn inhibitor from a trypsin–agarose column with 0.1 *M* glycine buffer at pH 2.1 resulted in recovery of the inhibitor largely in the single-chain, virgin form. Application of the pH 2.1 buffer resulted in elution of an initial shoulder followed by a large peak (Fig. 1). As is shown by the electrophoretic analysis of Fig. 2, two-chain, modified inhibitor predominates in the first portion of the eluted material (the shoulder). The major peak, whose appearance corresponds with the attainment of pH 2.1 in the effluent stream, consists largely of the virgin inhibitor.

According to the standard mechanism of action of a protein inhibitor of a

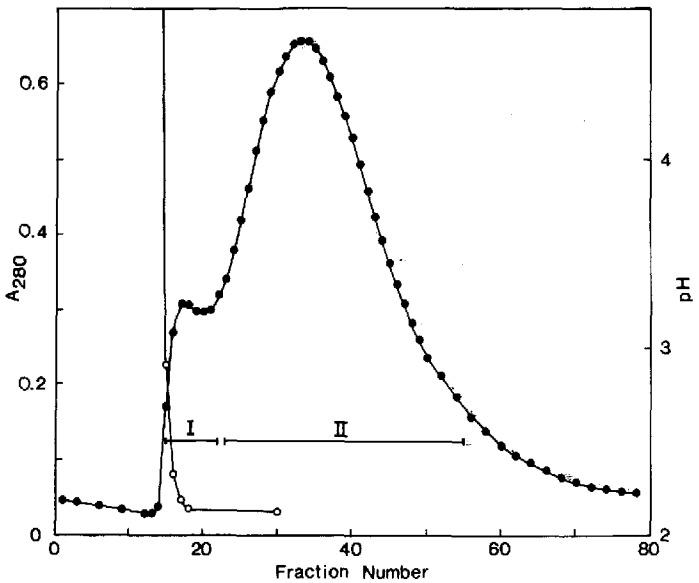

Fig. 1. Elution profile from chromatography of an extract of corn seeds on trypsin–agarose. A salt extract from *ca.* 150 g of dry seeds was loaded onto the column. The inhibitor (apparently the only protein which binds to the column[1]) was eluted with 1 *M* glycine (pH 2.1) at a flow-rate of *ca.* 100 ml/h. Fraction volume, 3.5 ml. About 30 mg of inhibitor were obtained from the column. Line with solid circles shows the profile of A_{280}. Line with open circles shows the pH of eluted fractions.

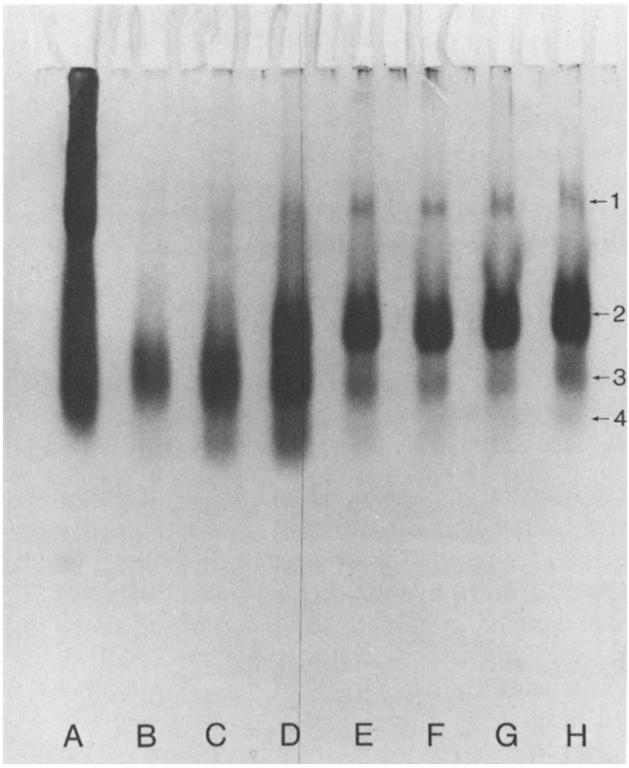

Fig. 2. SDS gel electrophoretic[8] analysis of corn inhibitor fractions from trypsin–agarose chromatography (Fig. 1). The separating gel was polymerized from 15% acrylamide–0.4% bisacrylamide. Lane A, flow-through material from the trypsin–agarose column. Lanes B and C, fractions 15 and 17 (shoulder of main peak from the elution profile shown in Fig. 1). Lane D, fraction 22 of Fig. 1. Lanes E, F, G, and H, fractions 30, 34, 38, and 50 (main peak) of Fig. 1. The band labeled "1" is the polypeptide that we assume to be trypsin. Band 2 is the single-chain (virgin) inhibitor. Bands 3 and 4 are the two chains of the modified inhibitor.

serine protease[10], in its simplest form, trypsin (T) converts a virgin inhibitor (I) into its modified form (I*). Both I and I* form a complex (C) with trypsin:

$$T + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} T + I^*$$

When the complex, C, is dissociated by a rapid change to low pH[10], the I:I* ratio is determined by the ratio of two dissociation rate constants (k_{-1}:k_{-2}), and not by equilibrium constants. Such kinetically controlled dissociation resulted in recovery of a preponderance of the virgin form in the case of the soybean trypsin inhibitor (Kunitz)[10], since, for that protein's interaction with trypsin, k_{-1} is greater than k_{-2} at pH 2.

It may be that k_{-1} is greater than k_{-2} for many protein inhibitors of serine proteases[11]. Sealock and Laskowski[11] used 0.1 *N* hydrochloric acid to dissociate the trypsin–bovine trypsin inhibitor (Kazal) complex on a trypsin–agarose column. The

eluted material contained predominantly (90%) virgin inhibitor and a trace amount of trypsin. Our results with the corn inhibitor are readily explained if we assume that k_{-1} is also greater than k_{-2} at pH 2 in the trypsin–corn inhibitor system. Analysis of small fractions in our effluent stream demonstrates that when that stream is at intermediate pH values, the modified inhibitor predominates. After the effluent stream achieves pH 2.1, it contains predominantly the virgin form of the inhibitor. Use of a concentrated buffer at low pH and of a flow adaptor on the chromatography column aided in making a rapid transition from pH 8.2 (used for loading the inhibitor and removal of unbound material) to pH 2.1. A still higher proportion of virgin inhibitor might have been obtained if an eluent of still lower pH had been used, but that would have risked damage to the column. Use of an eluent of pH 2.1 allows the trypsin–agarose column to be used repeatedly.

Modified inhibitor collected from a large-scale trypsin–agarose chromatography, as just described, could be converted in high yield into virgin inhibitor by chromatography on a small (10 × 1.5 cm I.D.) trypsin–agarose column (results not shown). The small diameter of that column results in a more sudden pH drop than that we can achieve on the larger column. The larger column, although it does produce as high a proportion of the inhibitor in the virgin form, does provide a larger quantity of inhibitor in that form in each use and is therefore preferred for large-scale work. To our knowledge, this is the first application of trypsin–agarose chromatography on a large scale in which elution of predominantly single-chain form has been obtained by lowering the pH in a protease–inhibitor system where equilibria favor the modified form.

Purification of the inhibitor by reversed-phase HPLC

Corn inhibitor from the main peak of a large-scale trypsin–agarose affinity chromatography was purified further by reversed-phase HPLC. The preparation (before HPLC) contains a small amount of a polypeptide with a molecular weight of *ca.* 23000 (as judged by SDS gel electrophoresis) (Fig. 2). The polypeptide's mobility is indistinguishable from that of single-chain trypsin (results not shown). It appears that trypsin slowly leaks from the trypsin–agarose. (Note that Sealock and Laskowski observed trypsin contamination in material they eluted with 0.1 *M* hydrochloric acid from a trypsin–agarose column[11].) We designate this contaminating polypeptide as "polypeptide 1".

Reversed-phase HPLC was carried out to remove polypeptide 1 from the corn inhibitor and to separate the virgin form of the inhibitor from the modified form. We obtained an additional result, however: fractionation of what are apparently multiple forms of the single-chain inhibitor.

Fig. 3 shows the HPLC elution profile. Chromatography was carried out as described in Materials and Methods. SDS gel electrophoretic analysis (Fig. 4) shows that virgin inhibitor was effectively separated from modified-form inhibitor and from polypeptide 1. The modified inhibitor eluted from the column first (fraction A), followed by virgin inhibitor (designated as fractions B, C, D. E, and F). Fraction G, obtained after the mobile phase was changed to 100% solvent B, was composed of polypeptide 1 as well as some virgin inhibitor and modified inhibitor. In later work we have found that twice as much sample can be applied to this HPLC column without significantly decreasing resolution.

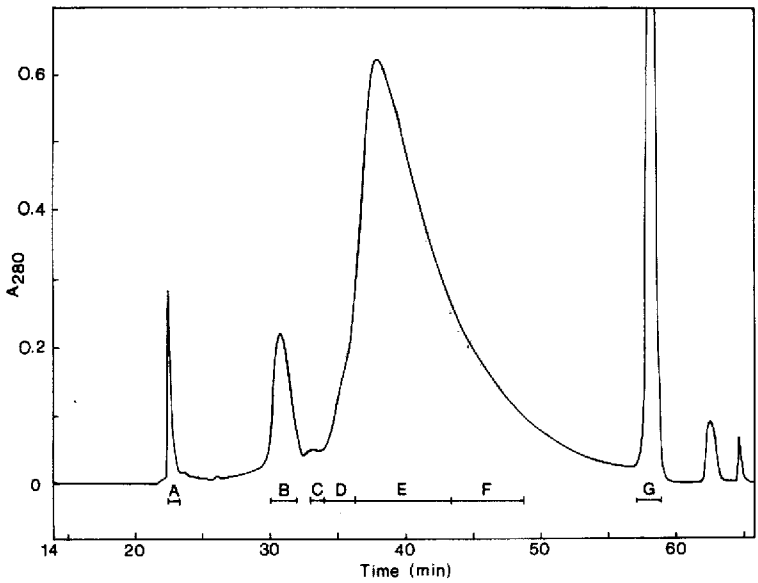

Fig. 3. Reversed-phase HPLC of material in main peak from trypsin–agarose column. See text for experimental procedure.Times given on the absicca are times after injection of sample. The arrow indicates the point at which the mobile phase was changed (over 7 min) to 100% solvent B.

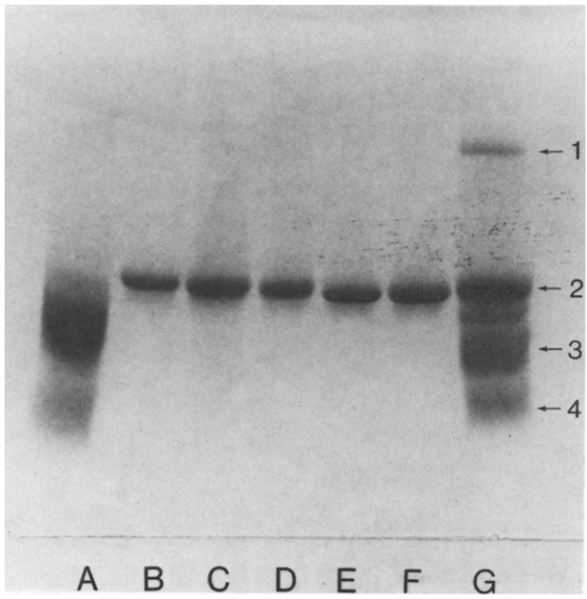

Fig. 4. SDS gel electrophoretic[9] analysis of fractions separated by reversed-phase HPLC. The separating gel was polymerized from 15% acrylamide–0.4% bisacrylamide. Samples in lanes A, B, C, D, E, F, and G were from fractions A, B, C, D, E, F, and G (Fig. 3), respectively. "1" indicates polypeptide 1. "2" is the virgin inhibitor. "3 and 4" indicate the modified inhibitor.

TABLE I

AMINO ACID COMPOSITION OF CORN INHIBITOR FRACTIONS*

Amino acid	*Fraction B*	*Fraction E*	*Amino acid*	*Fraction B*	*Fraction E*
Asp	5.8	5.7	Met	1.3	1.0
Thr	6.4	6.5	Ile	4.2	4.0
Ser	5.3	5.3	Leu	9.2	9.4
Glu	9.2	9.1	Tyr	1.7	1.6
Pro	12.0	12.1	Phe	1.3	0.8
Gly	10.0	11.8	His	2.3	1.8
Ala	11.1	11.2	Lys	1.1	0.8
Cys	7.3	7.0	Arg	7.6	8.1
Val	4.3	3.7			

* Values are in mol%; Trp was not determined.

The HPLC separation reveals heterogeneity in the corn inhibitor. Fractions B, D, E, and F (Fig. 3) contain exclusively virgin inhibitor. We have re-run fractions B and E on reversed-phase HPLC, and they run as single peaks with the same respective elution positions as in the initial HPLC run. Thus, they appear to be distinct species. Fraction B has a slightly lower mobility on SDS gel than does fraction E. These two fractions have essentially the same specific activity. Amino acid analysis (Table I) shows that the two fractions have essentially identical amino acid compositions, although fraction E has a slightly higher content of glycine.

Other fractions have not been examined in as much detail, but it does appear that the material in fraction D is different in electrophoretic mobility than is fraction E, and fraction D seems clearly distinct from fraction B chromatographically. Thus there would appear to be at least three distinct chemical species as revealed by this HPLC system. We hope to further investigate microheterogeneity in the corn inhibitor by recombinant DNA methods.

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