TRANSLATION OF MESSENGER RNA FOR CORN TRYPSIN INHIBITOR

by

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B.A., Otterbein College, 1976

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

Kansas State University

Manhattan, Kansas

1983

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ACKNOWLEDGMENTS

I wish to express my gratitude to my major professor, Dr. Gerald Reeck, for his help while I was a student, and also to Dr. S. Muthukrishnan for his guidance during the completion of this project.

INTRODUCTION

Protein Inhibitors of Serine Proteases

In a review article that concerns protein inhibitors of proteinases, Laskowski and Kato (1) have described corn trypsin inhibitor as belonging to the serine proteinase inhibitors, a group of proteins that prevents proteolysis by competitive inhibition for enzymes such as trypsin, chymotrypsin, plasmin, elastase and kallikrein. The serine proteinase inhibitors follow a standard mechanism, the main features of which are discussed below.

The reactive site, a peptide bond on the surface of the inhibitor molecule binds to the enzyme as would a substrate. It is enclosed inside one or more disulfide loops so that in most cases the two peptide chains are still held together when the native form of the inhibitor (single chain) is converted to the modified form (split chain).

In most cases, the active site residue P_1 can be matched with that of the enzyme it inhibits. For example, inhibitors with Lys and Arg P_1 residues usually affect trypsin and enzymes related in specificity. Those with Ala and Ser P_1 residues interact with elastase-like enzymes. If substitution of the active site residue by another residue occurs, inhibition often continues to take place but may be directed against a different enzyme. Substituting a Lys or an Arg residue in a trypsin inhibitor can change its specificity to that of a

chymotrypsin inhibitor. Inhibitory activity is retained upon hydrolysis of the active site, as a stable intermediate can still be formed between the enzyme and either the modified or the virgin inhibitor. A common feature of the proteinase inhibitors is the existence of several homologous reactive sites on the same polypeptide chain. It is referred to as multiheadedness and is the result of gene duplication (1).

Richardson in a review article on proteinases inhibitors (2) reported they are small-sized with molecular weights usually ranging from 4,000 to 25,000. He goes on to describe the techniques available for their isolation and purification. Separation from other proteins can be achieved by precipitating those by heat without denaturing the inhibitors which are usually resistant to heat. Another method is to segregate the enzyme-inhibitor complex and to then remove the enzyme. Two other techniques that are available are ion exchange chromatography and affinity chromatography; the latter uses columns packed with insoluble resins or polymerized dextrans to which the proteinase enzymes are bound. Crude extracts of plants at neutral pH can be loaded onto the column. The inhibitor binds to the proteinases and is eventually eluted with higher ionic concentration/low pH buffers.

The function of proteinase inhibitors is not well defined. They may act as storage proteins or may be part of a control mechanism for metabolism and protein degradation. They may also have evolved as a defense mechanism against destructive agents such as insects (1,2).

Two of the best studied inhibitors of proteinase of the serine family are the Bowman-Birk inhibitor and the Kunitz inhibitor both found in soybean (1,2). The Bowman-Birk inhibitor family is characterized by 2 homologous regions comprising 71 amino acid residues in total. One region has an inhibitory site against trypsin and the other against chymotrypsin. Reduction of the disulfide bonds changes the conformation of the protein and destroys its inhibitory function. The Bowman-Birk inhibitor molecule has a tendency to dimerize or trimerize.

The Kunitz inhibitor consists of 181 amino acid residues. It has only one active site which inhibits trypsin (single headedness) and two disulfide bridges. The binding region spans 12 amino acids. The molecule can be subjected to a variety of enzymatic and chemical treatments without loss of inhibitory activity such as the substitution of Arg 63 by Lys 63. If, however, Arg 63 is replaced by Lys 63, the molecule becomes a chymotrypsin inhibitor. The two forms of the molecule appearing on polyacrylamide gel electrophoresis represent the two co-dominant alleles at a single locus (1).

Subtilisin inhibitors have been isolated from the Streptomyces species of bacteria, from barley and recently from the inner bark (latex) of Hevea brasiliensis (3). 1.1 to 12% of the protein content found in the aqueous serum that results from centrifuging the latex of Hevea brasiliensis consists of the inhibitor. In comparison, the percentage of inhibitor obtained from the total proteins of cereal grains

is 5 to 10%. The subtilisin inhibitor from Hevea brasiliensis, named HP-In, was found to have a molecular weight of 11,370 by amino acid analysis and to dimerize readily as do some other proteinase inhibitors (soybean, potato). It does not have any homologous regions, however. The conspicuous absence of cysteine may account for the ease with which the molecule can be denatured since no cysteine bridges are present. Conversely, a large glycine content (21%) was observed.

Corn Inhibitor of Trypsin and Activated Hageman Factor

Corn trypsin inhibitor was first isolated by Hochstrasser et al. (4) in 1967 using an affinity chromatography column packed with a trypsin resin. The inhibitor was released with 6M urea and purified by CM-cellulose and Sephadex G-75 chromatography.

A corn trypsin inhibitor protein was later isolated by Swartz et al. (5) from ground opaque-2 corn seeds by passing a corn extract through a trypsin Sepharose column and eluting the bound inhibitor with a solution of 1M acetic acid and 0.02M $CaCl_2$. Subsequent chromatography of the inhibitor on DEAE cellulose with an NaCl gradient in the presence of 8M urea resulted in elution of two peaks; the protein in the major peak had a molecular weight of approximately 12,000, and the protein in the minor peak was formed of 2 components with molecular weights of 6,000 and 4,000. The moelcular weight of the native inhibitor was found to be 12,500 by

sedimentation equilibrium. It consists of a single polypeptide chain which is partially converted to a split chain (the modified inhibitor) while being purified.

Corfman and Reeck (6) reported in 1982 how purification of the inhibitor can be achieved by chromatography on an immunoadsorbent that contained antibody against corn trypsin inhibitor. The bound material was eluted with 1M propionic acid. Polyacrylamide gel electrophoresis of the purified inhibitor showed the presence of two bands, one of which co-migrated with the single chain species mentioned earlier (5) while the other one did so with the two-chain component. Only the single-chain form was observed if soybean trypsin inhibitor (Kunitz) was added to the initial extract. Those results indicated that degradation of the native form of the protein to the modified form is not contingent upon the presence of exogenous trypsin. Double immunodiffusion experiments did not reveal any differences between inhibitors purified by either one of these methods and in both cases, amino acid analysis gave similar results.

Current interest in CTI also arises from its ability to selectively inhibit the Hageman factor HF_f or prekallikrein activator in the blood clotting pathway without inhibiting plasma kallikrein (7,23). HF_f seems to be the agent responsible for the hypotension caused by the plasma protein fraction used for blood volume expansion during surgery (8,23).

The amino acid sequence of CTI (also called CHFI for corn Hageman factor fragment inhibitor) is:

Ser Ala Gly Thr Ser Cys Val Pro Gly Trp Ala Ile Pro
His Asn Pro Leu Pro Ser Cys (20)
Cys Trp Tyr Val Thr Ser Arg Arg Cys Gly Ile Gly Pro
Arg Pro Arg Leu Pro Trp Pro (40)
Glu Leu Lys Arg Arg Cys Cys Arg Glu Leu Ala Asp Ile
Pro Ala Tyr Cys Arg Cys Thr (60)
Ala Leu Ser Ile Leu Met Asp Gly Ala Ile Pro Pro Gly
Pro Asp Ala Gln Leu Glu Gly (80)
Ala Leu Glu Asp Leu Pro Gly Cys Pro Arg $\frac{\text{Ala}}{\text{Glu}}$ Val Gln
Gln Gly Phe Ala Ala Thr Leu (100)
Val Thr Glu Ala Glu Cys Asn Leu Gly Thr Ile Ser (112)

Its reactive site is Arg 36 - Leu 37 (W. A. Mahoney and G. R. Reeck, personal communication).

Messenger RNA's for Plant Inhibitors

Two studies of interest with respect to plants in RNA translation of proteinase inhibitors are those of Nelson and Ryan (9) with tomato plants and of Foard *et al.* (10) with soybeans.

Nelson and Ryan (9) observed an increase in the level of proteinase inhibitors I and II following wounding of the leaves of the plant. They surmised that a putative wound hormone, the proteinase inhibitor-inducing factor (PIIF)

was released throughout the plant and signaled the need for proteinase inhibitor accumulation by turning on inactive forms of mRNA or the synthesis of new mRNA. Using a non wounded plant as a control and a cell free reticulocyte lysate system *in vitro*, they discovered that the mRNA originating from wounded plants alone directed the incorporation of ^{35}S Met into immunoprecipitable inhibitors I and II. Furthermore, as the inhibitors are rarely observed in unwounded plants, Nelson and Ryan inferred that their occurrence is primarily a response to cell damage.

The molecular weights of the inhibitors synthesized *in vitro* was calculated by means of the electrophoretic mobility of the proteins. They were found to be 2,000 to 3,000 daltons higher than those obtained for proteins synthesized *in vivo* in wounded plants. This suggests that some processing takes place in the plant, resulting in the clipping of part of the preinhibitor protein before or while the newly synthesized proteins are transported from the leaf cytoplasm, the site of synthesis, to the vacuole where they are eventually stored.

In a preliminary step towards the study of the genes coding for the Bowman-Birk proteinase inhibitor, Foard *et al.* (10) isolated mRNA from soybean and fractionated it on a sucrose gradient in order to identify those fractions that were most active in protein translation. The highest A_{280} reading was obtained in Fraction 18 while maximal translational activity was observed in fraction 14. Fractions 11-14

were the most enriched in the mRNA coding for synthesis of the Bowman-Birk inhibitor. Translation was carried out using a wheat germ S23 system.

Newly synthesized inhibitor proteins were isolated by immunoprecipitation and the radioactive eluates were subsequently resolved by 15% polyacrylamide gel electrophoresis. The labeled protein bands that appeared on the gel comigrated with the trimer of the Bowman-Birk inhibitor, not with the monomer. The molecular weight of the monomer is 8,000. Resolution of the products by HPLC and amino acid analysis by Edman degradation and CNBr clearage enabled the researchers to demonstrate that the protein bands they had observed constituted indeed the Bowman-Birk inhibitor.

The large size of the immunoprecipitable proteins observed in SDS gel patterns suggests that the inhibitor is either synthesized as a monomer that self-associates later or else directly as a trimer. It has been shown by Millar et al. (11) that the Bowman-Birk inhibitor shifts reversibly from monomer to dimer and trimer. Therefore, it seems that the conditions used for in vitro synthesis of the inhibitor are favorable to the existence of multimeric forms of that protein. A complete understanding of this phenomenon however, will require additional experiments to be carried out.

Messenger RNA's for Corn Seed Proteins

Zein proteins have been the most extensively studied proteins in corn (for review, see reference 12) and for that reason will be briefly mentioned here. Zein genes can be considered to be part of a multigene family as they exhibit the properties of multiplicity, sequence homology and overlapping phenotypic functions which are 3 of the 4 conditions required for such classification (12). A family is understood to represent the entire zein mRNA population while a subfamily corresponds to subdivisions of that population and includes many genes. Knowledge of the nucleotide sequence of zein cDNA's and genes allows the amino acid sequences of zein proteins to be determined. Those proteins are synthesized in the endosperm of the corn kernel and are stored in protein bodies. One dimensional gel electrophoresis has revealed two major classes of zein proteins of respectively 19 and 22 kilodaltons. However, isoelectric focusing and two-dimensional gel electrophoresis have resolved those protein samples into patterns of greater complexity and such that as many as 100 products of a multigene family may be present (12).

The use of cDNA clones in hybridization-release translation experiments has provided a tool for studying zein mRNA. Three cDNA clones, A20, A30, and B49 are together capable of hybridization with most of the zein mRNA. A20 binds mRNA

that corresponds to 19 kd, A30 binds that of 19 and 22 kd while B49 binds mostly the 22 kd class and some of the 19 kd (12).

A20 and A30 inserts of 1,000 bases that contained the nucleotide sequence coding for the zein proteins were used in experiments carried out to obtain the amino acid sequence of the corresponding protein. The amino acid sequence anticipated from the nucleotide sequence of the A30 cDNA consisted of 213 amino acids, had a molecular weight of 23,329 and included a 20 amino acid unit that was repeated 7 or 8 times, which is in agreement with the known sequence (12).

A striking homology is observed between A20 and A30 cDNA's but not with B49. Also the nucleotide sequence of A30 is 97% homologous to a genomic clone Z4 that codes for a zein of the 22 kd class. It does however, differ from the A30 sequence in that it carries an internal repeat of 96 nucleotides which is found only once in A30 (12).

Research Undertaken for this Thesis

In this study, I am reporting how I isolated mRNA from corn kernels, carried out the translation of poly(A)$^+$ mRNA into proteins and removed the corn trypsin inhibitor (CTI) proteins or pre-proteins by immunoprecipitation. I attempted then to characterize those proteins or pre-proteins by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials

A. Corn

The corn used in this study was obtained from Dr. Clyde Wassom of the Department of Agronomy at Kansas State University. It was of the Funk hybrid variety (normal genotype) with F2 seeds. The ears of corn were harvested three weeks after pollination.

B. RNA Isolation

Cesium chloride, ultra pure: BRL

Guanidine monothiocyanate, Baker Grade: J. T. Baker Chemical Co.

N-lauroyl sarcosine: Sigma Chemical Co.

Oligo (dT)-cellulose (type 3): Collaborative Research, Inc.

Phenol, liquefied, 88%: Fisher Scientific Company.

Proteinase K: Boehringer Mannheim.

Sodium dodecyl sulfate (SDS), electrophoresis grade: Polysciences, Inc. or Bio-Rad Laboratory.

Trishydroxymethylamino methane (Tris), ultra pure grade: Sigma Chemical Company.

Tubing, for oligo (dT) column chromatography, sterile K50L 84 cm, 3.3 ml capacity: Pharmaseal, Inc.

C. RNA Translation

Amino acids, L form: Sigma Chemical Co.

ATP: Calbiochem.

Casamino acids: Difco Laboratories

Creatine phosphate (CP): Sigma Chemical Co.

Creatine phosphokinase (CP kinase): Sigma Chemical Co.

2,5-Diphenyloxazole (PPO): New England Nuclear.

1,4-Di-2(5-phenyloxazolyl)-benzene (POPOP): Sigma Chemical Co.

GTP: Sigma Chemical Co.

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES): Sigma Chemical Co.

^{35}S Methionine, Catalog number NEG-009H translation grade, specific activity 1050 or 1200 Ci/mmole: New England Nuclear.

Micrococcal nuclease: Boehringer Mannheim.

Phenylmethane sulfonylfluoride (PMSF): Sigma Chemical Co.

Ribonuclease-A from bovine pancreas, type 1-A: Sigma Chemical Co.

Ribonucleic acid, transfer, type V, from wheat germ: Sigma Chemical Co.

Spermidine: Sigma Chemical Co.

D. Immunoprecipitation

Antibody to corn trypsin inhibitor: obtained from Mei-Guey Lei of this laboratory.

Bovine serum albumin (BSA), (Nucleic Acid Enzyme Grade): BRL

Coomassie brilliant blue, R: Sigma Chemical Co.

Methionine, L form: Sigma Chemical Co.

Nonidet P-40 (NP-40): BRL

Protein A-Sepharose Cl-4B: Sigma Chemical Co.

E. Gel Electrophoresis

Acrylamide, ultra pure electrophoresis grade: Sigma Chemical Co. or BRL

Ammonium Persulfate: Sigma Chemical Co.

Ampholine pH 5-7 (40%): LKB

Bis-acrylamide: Eastman Kodak Company

Urea, ultra pure grade: Schwarz/Mann.

F. Autoradiography

Cronex intensifying screen: E. I. due Pont de Nemours and Co. (Inc.).

Enhance: New England Nuclear

X-Ray film XAR5: Eastman Kodak Company

Methods

A. Corn

Kernels were removed from some of the corn four hours after the ears were harvested. The kernels were first frozen in liquid nitrogen and then kept at -20°C. Some ears of corn

were frozen whole. They were placed into a -20°C freezer as soon as they were brought back to the lab, approximately one hour after being harvested.

B. RNA Isolation

Isolation of RNA from corn was done by following the method of Rutter (13) with some modifications. Corn kernels that had been frozen in liquid nitrogen were used. Upon removal from the freezer, 80 grams of corn were immediately placed into a 100 ml filtered solution of 4 M guanidine monothiocyanate in 50 mM Tris-Cl pH 7.5 and 10 mM EDTA. They were coarsely ground with a mortar and pestle and subsequently homogenized in an Omnimixer while kept on ice. The corn solution was filtered through a cheese cloth and centrifuged for 10 minutes at 10,000 g in order to remove all insoluble material. The supernatant was heated at 60°C for 2 minutes and cesium chloride was added to a final concentration of 0.1 g 1 ml of solution. The solution was loaded in four polyallomer centrifuge tubes (25.4 x 89 mm) above a 6 ml cushion of half saturated sterile cesium chloride in 10 mM EDTA. The tubes were centrifuged at 25,000 rpm in an SW 27 rotor for 16 hours. This step insures separation of DNA from RNA. Guanidine monothiocyanate dissociates the ribonucleoprotein complex and denatures the proteins. The cesium chloride gradient is used to isolate the RNA from the DNA. The RNA forms a pellet; the DNA remains on top. After

centrifugation, the tubes were emptied with a Pasteur pipet down to the level of the cesium chloride sterile cushion. At that point the tubes were sectioned with a sterile razor blade so that the solution remaining in the tube could be removed easily without disturbing the small clear translucent RNA pellet visible at the bottom. Sterile techniques and materials were used from that point on. The pellets were removed with a nickel spatula and taken up in 10 ml of 0.5% sarcosyl, 5 mM EDTA and 5% β-mercaptoethanol. The solution was kept at 60° to 65°C for 4 hours and was vortexed every 5 to 10 minutes to dissolve the RNA present. The dissolved RNA was extracted with a 1:1 mixture of phenol-chloroform which separates the RNA from the proteins by denaturing those and pulling them away from the ribonucleoprotein complex. The top aqueous layer, which contained the RNA, was carefully removed and adjusted to a 0.2 M NaCl concentration, then precipitated with ethanol and left at -20°C overnight. It was centrifuged the next day at 10,000 g for 45 minutes. The supernatant was poured off and the total RNA pellet was air dried.

The subsequent isolation of mRNA from total RNA was carried out by modifying the procedure of Aviv and Leder (14). Poly(A)$^+$ RNA fractionation was accomplished by putting the total RNA obtained earlier over an oligo (dT)-cellulose chromatography column. The column itself consisted of a sterile 3 ml syringe, without needle, connected to a piece of sterile tubing that could be opened or closed by means of a

small valve. The total RNA pellet was taken up in a 5 ml solution of 10 mM Tris-Cl pH 7.5 and was vortexed (for approximately one hour) until as much as possible of the pellet was dissolved. At that point, a 0.5 ml aliquot was removed and an A_{260} reading was taken in order to determine the RNA content. An absorbance of 1 at 260 nm (in a 1 cm cell) corresponds to 40 µg of RNA per ml. The aliquot was then frozen at -20°C until the RNA fractionation was completed.

The oligo (dT) column was washed with approximately 30 ml of a 10 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 0.2% SDS solution (high salt buffer or HSB). The sample adjusted to 0.5 M NaCl was then loaded onto the column and 50 drops fractions (1.5 ml) were collected until the A_{260} baseline was reached, at which point all the poly(A)$^-$RNA has been eluted; less than 100 ml of HSB were needed to effect that.

A medium salt buffer (MSB) was applied next. It was a 10 mM Tris-Cl (pH 7.5), 0.1 M NaCl solution. As with HSB, 50 drops fractions were collected until the A_{260} baseline was reached, and this required less than 100 ml of buffer.

Finally, the poly(A)$^+$RNA was eluted by means of a 10 mM Tris-Cl pH 7.5 buffer (low salt buffer or LSB); it was normally released within the first 3 or 4 fractions collected. The poly(A)$^+$ RNA fractions obtained were pooled and readjusted to the same NaCl, Tris-Cl and SDS concentration as that of the high salt buffer (HSB). The sample was loaded onto the oligo (dT) column a second time and was eluted by the method previously described.

After fractionation of the total RNA, a small amount (0.1 mg or so) of proteinase K in low salt buffer was passed through the column in order to destroy any contaminating proteins that might be present. The column was stored at $-20^{o}C$.

An alternative to collecting small volume fractions is to let a given (large) volume of buffer pass through the column and to collect the first 10 ml directly into a centrifuge tube. This approach was used a number of times in the course of this study.

The poly(A)$^{-}$ and the poly(A)$^{+}$ fractions were pooled separately and along with the total RNA aliquot saved earlier, were adjusted to a 0.2 M NaCl concentration, precipitated with 100% ethanol and kept either at $-20^{o}C$ overnight or at $-70^{o}C$ or in dry ice for one hour. The fractions were then centrifuged in a Beckman ultracentrifuge at 25,000 rpm for 1 hour using an SW 27 rotor. The supernatant of each tube was carefully removed with a sterile Pasteur pipet and the pellet was let to air dry overnight. The following day the pellet was dissolved in 0.5 ml of sterile deionized water and an A_{260} reading was taken to determine the RNA concentration. The samples were kept at $-20^{o}C$.

C. Translation

The method used for the translation of mRNA into proteins was a modified version of the procedure given by Pelham and Jackson (15). The wheat germ cell free extracts

were prepared according to the technique of Roberts and Paterson (16). The buffer used for the wheat germ cell free extracts was a solution of 0.02 M HEPES (pH 7.6), 0.1 M KAc, 0.001 M $Mg(Ac)_2$, 0.0002 M $Cacl_2$. The wheat germ pellets were stored in liquid nitrogen.

The procedure used for the translation was as follows: immediately after being removed from the liquid nitrogen, 200 λ of thawed wheat germ pellets were mixed with 2 λ of 0.1 M $CaCl_2$ and 1 λ of a diluted (1 to 3) solution of micrococcal nuclease whose stock solution concentration was 0.5 mg/ml in 10 mM HEPES buffer, pH 7.6. After vortexing, the wheat germ mix was incubated at room temperature (25°C) for 10 minutes and 8 λ of a 0.1 M EGTA solution were added. The mixture was vortexed again and kept on ice until used. That period of time was kept to a minimum; that is, the wheat germ mixture was added to the other reagents required for protein synthesis almost immediately.

The presence of micrococcal nuclease is necessary to ensure that all endogenous mRNA has been destroyed. It requires the presence of calcium ions for activity and is subsequently inactivated by EGTA which chelates those ions.

A 10 x mix stock solution containing all the reagents necessary for protein synthesis was prepared. It consisted of 50 λ of 1 M Hepes buffer (pH 7.6), 8 λ of 1 M DTT, 50 λ of 100 mM ATP (pH 7.6), 25 λ of 40 mM GTP (pH 7.6), 50 λ

of 1 M CP, 25 λ of 2 mg/ml CP kinase, 50 λ of 6 M KAc, 50 λ of 10 mM spermidine, 100 λ of an unlabeled mixture of all amino acids with the exception of methionine, and 92 λ of sterile water.

The standard reaction mixture used for the translation of each RNA sample was made up using 15 λ of the wheat germ mixture, 1 λ of tRNA (the stock was 10 mg tRNA from wheat germ per ml of 10 mM HEPES pH 7.6), 1 λ of a PMSF solution of 7 mg/ml in isopropanol which was diluted 1 to 70, 3 λ of ^{35}S methionine (∿1,200 Ci per mmole), 5 λ from the 10 x mix, a standard amount of RNA and enough water to obtain a total volume of 50 λ. Five samples were run for translation: a sample without RNA (-RNA) in order to obtain the background radioactivity, a 2 μg standard sample of RNA from wheat germ (control sample), a poly$(A)^+$ RNA sample containing 1 μg of RNA per 50 λ reaction mixture and the size of which was scaled up 8 times, that is 8 μg of poly$(A)^+$ RNA were used for a 400 λ reaction mixture, a 2 μg total RNA sample and a 2 μg poly$(A)^-$RNA sample.

Upon addition of all the reagents, the vials were vortexed and incubated at 25°C for one hour. After that time, 10 λ of a stock solution of ribonuclease (1 mg/ml in 50 mM EDTA pH 8.0) were added per 50 λ reaction mixture. The vials were vortexed and incubated at 37°C for 15 minutes. At that point, the process has been completed. A 5 λ aliquot was removed from each sample and spotted onto a small square of

filter paper, washed 4 times in 5% TCA, 1% Casamino acids over ice and washed in 80% EtOH. The filter papers were dried under a lamp and each one was placed into scintillation counting vial; 10 ml of the scintillation cocktail 0 "Scintran" (6 g PPO and 0.2g POPOP in toluene with a total volume of 2.5 liters) were added to each sample and they were placed into a Beckman LS 8000 counter for radioactive counting. The original translation samples were kept at $-20^{o}C$ until further usage.

D. Immunoprecipitation

Immunoprecipitation was done using a modification of the procedure described by Kessler (17); 100 λ of protein A-Sepharose beads were left to stir gently in the cold overnight with 100 λ of affinity purified monospecific antibody for corn trypsin inhibitor. The antibody was provided by Mei-Guey Lei, a graduate student in this laboratory. It was obtained by following the method indicated by Corfman (6,18) using a corn trypsin inhibitor affinity column to purify the antibody. The column was packed with corn trypsin inhibitor that was covalently linked to cyanogen bromide-activated Sepharose 4B. It was equilibrated with borate saline buffer and loaded with the partially purified antibody preparation. Elution of the antibody was done with 1 M propionic acid. It was then dialyzed against 0.1 M borate

saline buffer. Its concentration was found to be 0.5 mg/ml (A_{280} = 0.8; the extinction coefficient for IgG is 1.5).

The poly$(A)^+$ RNA translation sample was diluted with 9 times its volume of NP-40 buffer pH 7.5, vortexed, and centrifuged in an Eppendorf centrifuge. The supernatant was removed with a pipet; 2.5 mg of cold methionine were added to it along with 30 λ of PMSF (7 mg/ml in isopropanol). The PMSF was added in order to prevent protease action. 30 λ of the protein A-Sepharose that were linked to the antibody were then added to the translation supernatant. The vial was left to stir slowly at 4°C overnight. The next day, the sample was centrifuged in an Eppendorf centrifuge and the supernatant was discarded. The pellet, which consisted of the protein A-Sepharose beads, was washed 9 times in a solution of NP-40 buffer, (pH 7.5) containing 1 mg/ml of cold methionine. The pellet was then taken up in 40 λ of gel electrophoresis buffer, vortexed, heated at 100°C for 5 minutes and centrifuged for 5 minutes. A 2 λ aliquot to which 100 μg of BSA carrier had been added, was removed for radioactivity counting. The aliquot with the BSA was treated as described earlier in the translation section.

E. SDS Polyacrylamide Gel Electrophoresis

One dimensional SDS gel electrophoresis was carried out by the method of Laemmli (19) using one half the concentration

of bis-acrylamide. The concentration of acrylamide was 15%, that of bis-acrylamide 0.2%. Electrophoresis was done at 25 mA for 3 hours at 4°C.

The protein samples were dissolved in a sample buffer composed of 20% glycerol, 10% β-mercaptoehtanol, 3% SDS, 0.0625 M Tris-Cl pH 7.5 and 0.01% bromphenol blue.

The gel stain was made up of 0.25% Coomassie brilliant blue R, 10% acetic acid and 50% ethanol. The gels were stained overnight. The destain solution was 20% acetic acid and 20% methanol.

F. Two-Dimensional Gel Electrophoresis

The procedure used was that of O'Farrell (20). The first dimension is isoelectric focusing (IEF), a process which gives good resolution of neutral and acidic proteins. It was accomplished by using glass tubes (20 x 2.5 mm inside diameter) into which a gel-forming solution was poured to a height of 16.5 cm.

The gel was made up of 9.2 M urea, 2% Nonidet P-40 (NP-40), 4% acrylamide/bis-acrylamide obtained from a 30% stock solution that was composed of 28.4% acrylamide and 1.6% bis-acrylamide and 1 or 2% Ampholines (consisting of 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10) and 5% β-mercaptoethanol. The anode (upper) solution was 0.02 M NaOH and the cathode solution was 0.01 M H_3PO_4.

Electrophoresis was carried out at an initial voltage of 300 volts which was increased gradually to 1,000 volts within the first hour. After that, the voltage was kept at 1,000 volts for 20 hours. The gels were removed from the glass tubes by means of a syringe to which a short piece of Tygon tubing had been attached; this was connected to the glass tubes and pressure was slowly applied onto the syringe to force the gels out. Those were equilibrated for 1 hour in equilibration buffer (10% glycerol, 5% ß-mercaptoethanol, 2.3% SDS, 0.0625 M Tris-Cl pH 6.8 and a small amount of bromphenol blue). The second dimension was either run immediately or else the gels were frozen over dry ice in methanol and stored at -70°C until further usage.

The second dimension is discontinuous SDS slab gel electrophoresis (210 x 150 x 0.8 mm). The slabs were composed of 15% acrylamide and 0.2% bis-acrylamide. Electrophoresis was run at room temperature at 7 mA per gel for 14-16 hours. Gels were stained in 0.25% Coomassie brlliant blue R/10% acetic acid/50% methanol and destained with 20% acetic acid/ 20% methanol.

F. Autoradiography

After destaining, the gels were shaken at low speed into 100 ml of Enhance at room temperature for one hour. They were then placed into a 200 ml water/5 ml glycerol

solution and kept shaking at room temperature for one hour. They were dried in a gel dryer for an hour. Autoradiography was carried out by placing an X-ray Kodak film on top of a Saran wrap sheet covering the gel and placing the film against an intensifying Cronex screen for one week or longer at -70° C.

G. pH Gradient

Measurement of the pH gradient for corn trypsin inhibitor was accomplished by isoelectric focusing 30 µg of CTI using the first dimension of the two-dimensional gel electrophoresis technique. After removal of the gel from its glass tube, it was cut into 1 cm sections with a razor blade. These were placed into numbered test-tubes which contained 0.5 ml of 0.02 M KCl (degased). The tubes were stoppered and well shaken and kept at 4°C overnight. The pH in each tube was measured the next day and a plot of pH vs tube number was drawn.

Another gel identical to the first one was run along with it; it was stained and destained in the solutions listed under the two-dimensional gel section. The distance between the CTI spot and the top of the gel was measured and the pI for CTI was determined from the plot.

H. Corn Trypsin Inhibitor

Corn trypsin inhibitor (CTI) was provided by Mei-Guey Lei, a graduate student. She followed the isolation procedure

described by Swartz et al. (5) with the following modifications: following the trypsin-Sepharose step, the trypsin inhibitor was eluted with 1 M glycine at pH 2.0. This results in recovering exclusively single-chain inhibitor. It was put over a Sephadex G-25 column and it was eluted with 0.1 M acetic acid and was then lyophilized.

RESULTS

In a typical preparation (100 g of corn), the isolation of RNA and the subsequent fractionation of total RNA into its components resulted in the following amounts of poly(A)$^-$ RNA and poly(A)$^+$ RNA being recovered:

Table 1: Corn RNA recovery (numbers marked with an asteriks add up to more than 100% because of variation in absorbance and contamination of the fractions.

	Total RNA	poly(A)$^-$	poly(A)$^+$
µg/prep	2,090 µg	2,076 µg	60 µg
Percentage	100% (presumed)	99%*	2.87%*

A plot of A_{260} vs fraction number (Figure 1) illustrates this pattern of recovery from chromatography on oligo (dT)-cellulose.

The data obtained from the translation of mRNA into protein using a wheat germ cell free extract is summarized in Table 2.

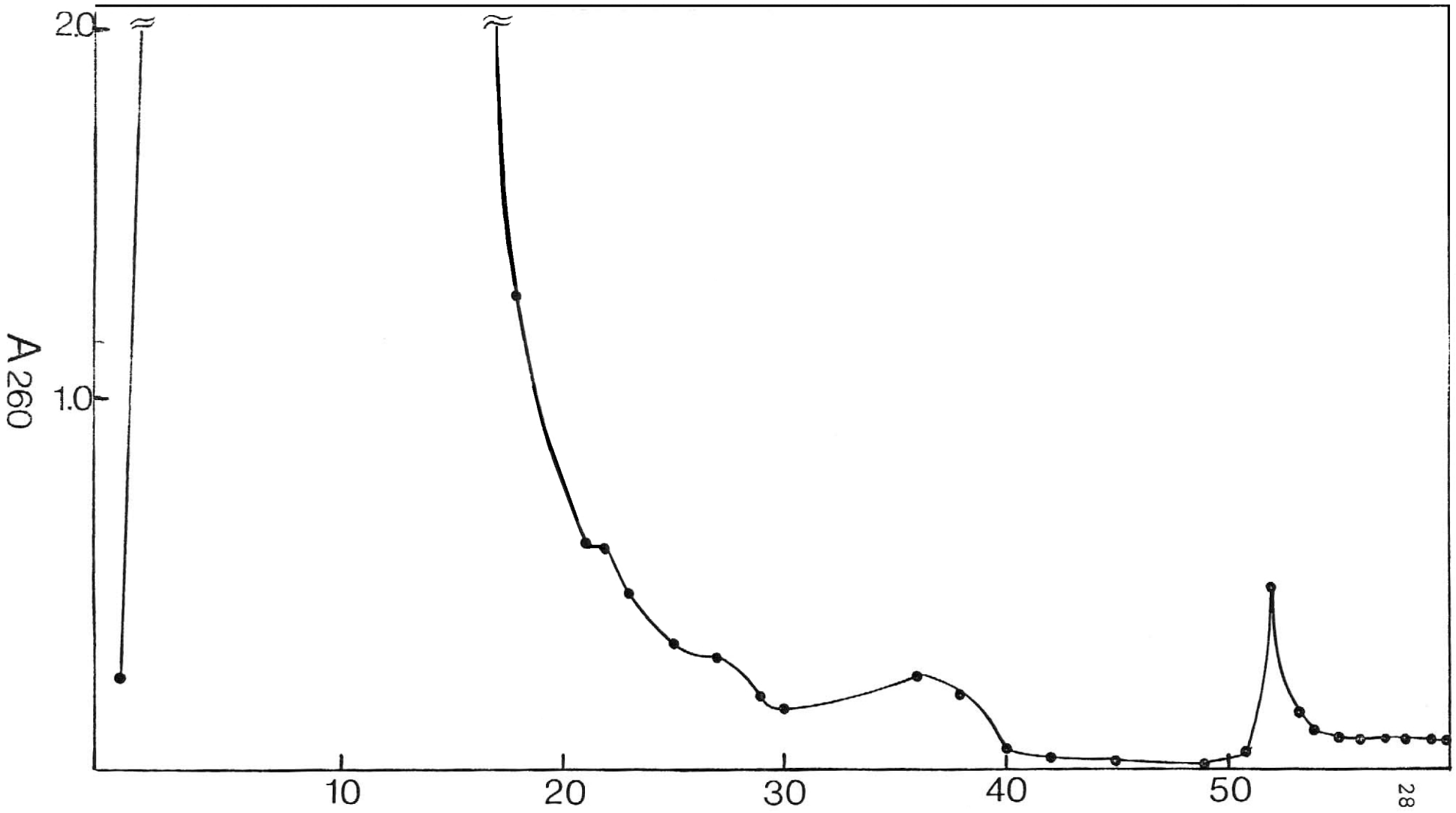
The data of Table 2 illustrates that the poly(A)$^+$ RNA function was the most active in protein translation (51,000 cpm/5 λ spotted). This is in agreement with what would normally be expected.

The first and second eluates of the selective immunoprecipitation of *in vitro* translation of poly(A)$^+$ RNA were found to have 3,000 cpm and 1,600 cpm respectively in a volume of 40 λ each. The electrophoretogram of the eluate

Figure 1. Oligo (dT)-cellulose chromatography of total RNA. Fractions 1 through 30 correspond to the poly(A)$^-$ RNA eluate which consists of transfer RNA and ribosomal RNA (HSB); fractions 31 through 49 correspond to the poly(A)$^-$ RNA eluate which consists of ribosomal RNA that did bind to the column (MSB); fractions 50 through 60 which correspond to the poly(A)$^+$ RNA eluate, that is the messenger RNA (LSB). Fraction volume was 1.5 ml.

The RNA recovery was the same for all corn samples analyzed (corn kernels frozen in liquid nitrogen, whole ears frozen after being cut, whole ears kept on stalks at room temperature for 4 hours, then cut and frozen).

The corn used was 3 weeks old, stage at which it is the most active in protein synthesis.



FRACTION NUMBER

Table 2. Incorporation of radioactive label in corn RNA translation

		Wheat Germ total RNA (control)	total RNA	poly(A)$^-$ RNA	poly(A)$^+$ RNA
Amount of RNA used per 50 λ reaction volume.	0	2 μg	2 μg	2 μg	1 μg
cpm/5 λ (translation)	14,000	36,000	22,000	17,000	51,000
Specific activity of ^{35}S Met.		1,050 or 1,164 Ci/mmole			
cpm/μg RNA	---	18,000	11,000	8,500	51,000

is shown on Figure 2 and the corresponding autoradiogram appears on Figure 3. The corn trypsin inhibitor standards (Figure 2, tracks A and D) consisted of 2 and 5 μg samples respectively. Protein molecular weight standards were also run on the gel (tracks B and E, 7.5 μg each) in order to obtain some information on the molecular weight of the CTI mRNA translation products. The first eluate of the immunoprecipitation was run on track C (3,000 cpm/40 λ). 25 λ samples of poly$(A)^+$, total and poly$(A)^-$ RNA translation samples were each loaded on tracks F, G, H. The mobility of the molecular weight standards was measured (21) and the plot of log of molecular weight vs protein mobility was drawn (Figure 4). The plot was used to calculate the apparent molecular weight of the CTI standard and of each one of the bands appearing on the autoradiogram. The molecular weight obtained for the corn trypsin inhibitor standard was 11,900 while those of the four bands detected on the autoradiogram were respectively, 16,000, 13,600, 12,900, 12,200, from top to bottom, corresponding to a migration distance of 6.8, 7.4, 7.6, 7.8 cm each and 7.9 cm for the CTI standard. They were tentatively identified as being various forms of the corn trypsin inhibitor or precursor proteins of corn trypsin inhibitor.

The two-dimensional gel electrophoresis pattern of the translation of poly$(A)^+$ RNA is shown on Figure 5. 50 λ corresponding to 500,000 counts of the translation mixture were

Fig. 2. SDS polyacrylamide gel electrophoresis (protein staining).

Track A: 2 μg of corn trypsin inhibitor standard;

Track B and E: protein molecular weight standards (BRL); 7.5 μg in each track.

Track D: 5 μg of CTI standard (see arrow).

Track C: corn trypsin inhibitor immunoprecipitation eluate; 2,400 counts loaded onto the gel.

Track F: sample of translation of corn poly(A)$^+$ mRNA; 1,017,500 counts loaded (25 λ).

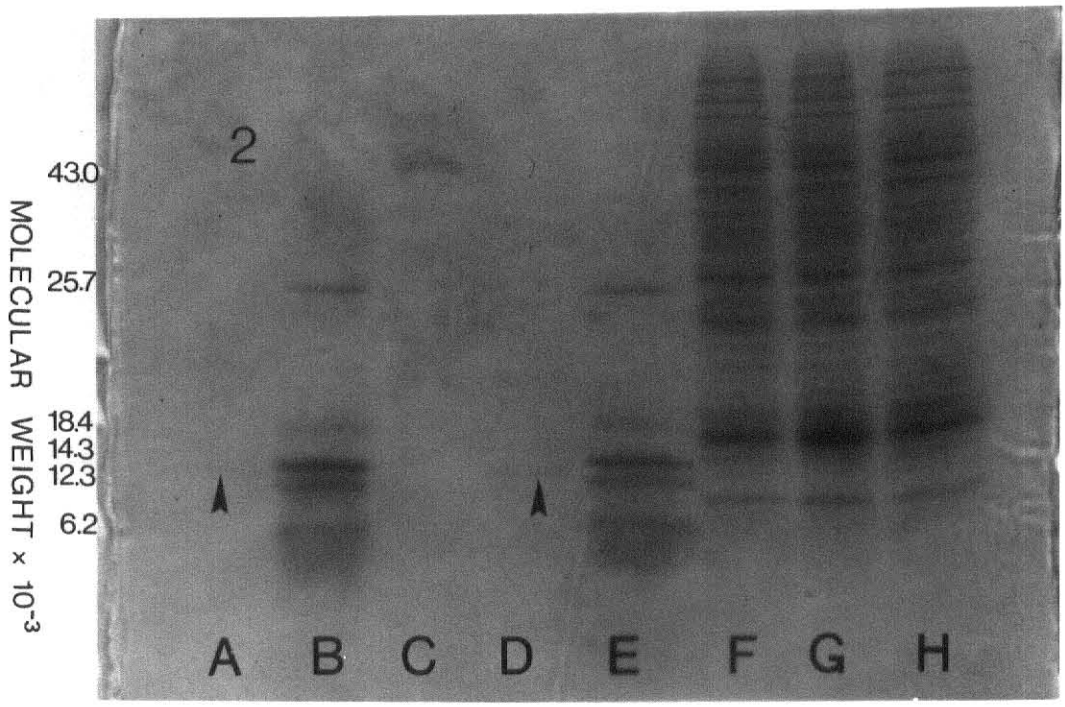
Track G: sample of translation of corn total mRNA; 300,000 counts loaded onto the gel (25 λ).

Track H: sample of translation of corn poly(A)$^-$ mRNA; 600,000 counts loaded onto the gel (25 λ).

Samples A, B, C, D each had 40 λ of sample buffer added. Samples E, F, G each had 25 λ of sample buffer added.

CTI standard migrates as a single band.

Fig. 3. Autoradiogram of the SDS polyacrylamide gel from Fig. 2. Four bands can be observed in track C, above the bands of the CTI standard. Their respective molecular weights were calculated to be 16,000, 13,600, 12,900, 12,200 (see arrows).


2
43.0
25.7
18.4
14.3
12.3
6.2
MOLECULAR WEIGHT x 10^{-3}
A B C D E F G H

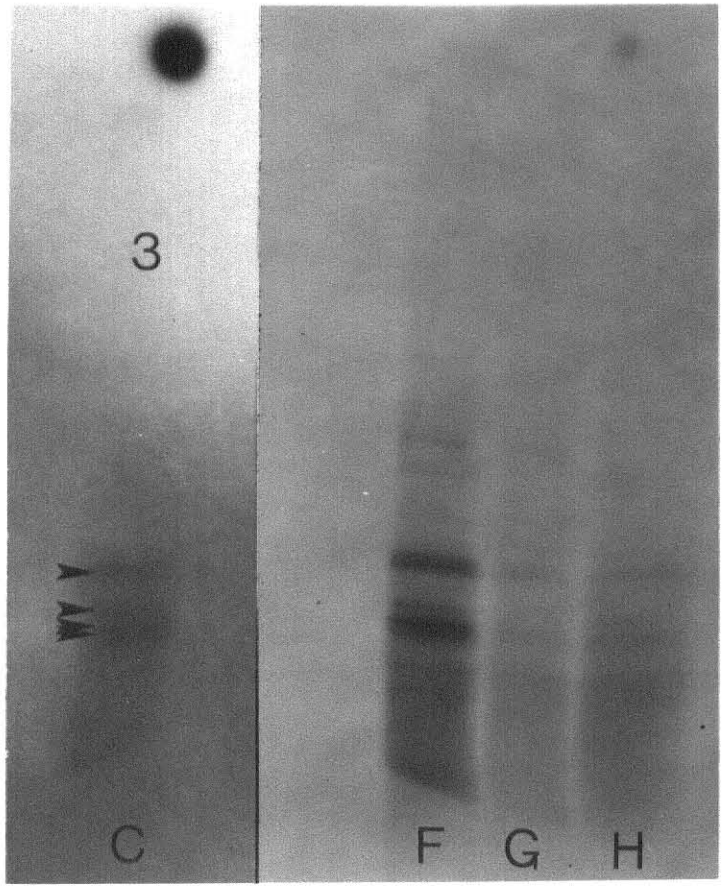

3
C
F G H

Fig. 4. Plot of the log of molecular weight vs mobility. The proteins used as standards were ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), lysozyme (14,300), cytochrome c (12,300) and bovine trypsin inhibitor (6,200). A least squares fit of the data was used for the plot because of the scatter of the original point. Arrows 1, 2, 3, 4 indicate the location of the immunoprecipitation bands observed on the autoradiogram; arrow 5 represents the standard corn trypsin inhibitor. The molecular weights calculated from the plot for bands 1, 2, 3, 4 were 16,000, 13,600, 12,900, 12,200, respectively. That obtained for the standard was 11,900.

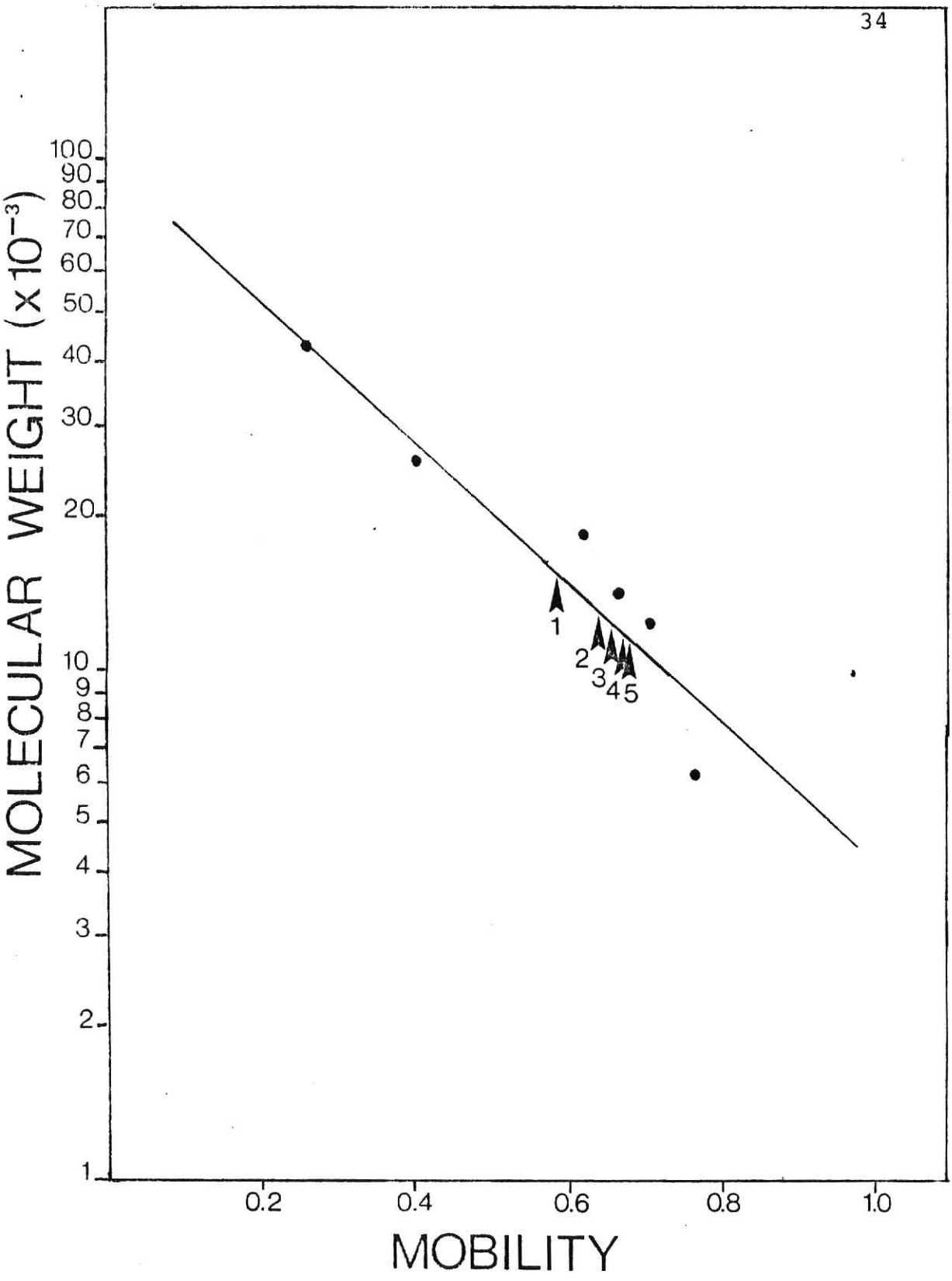
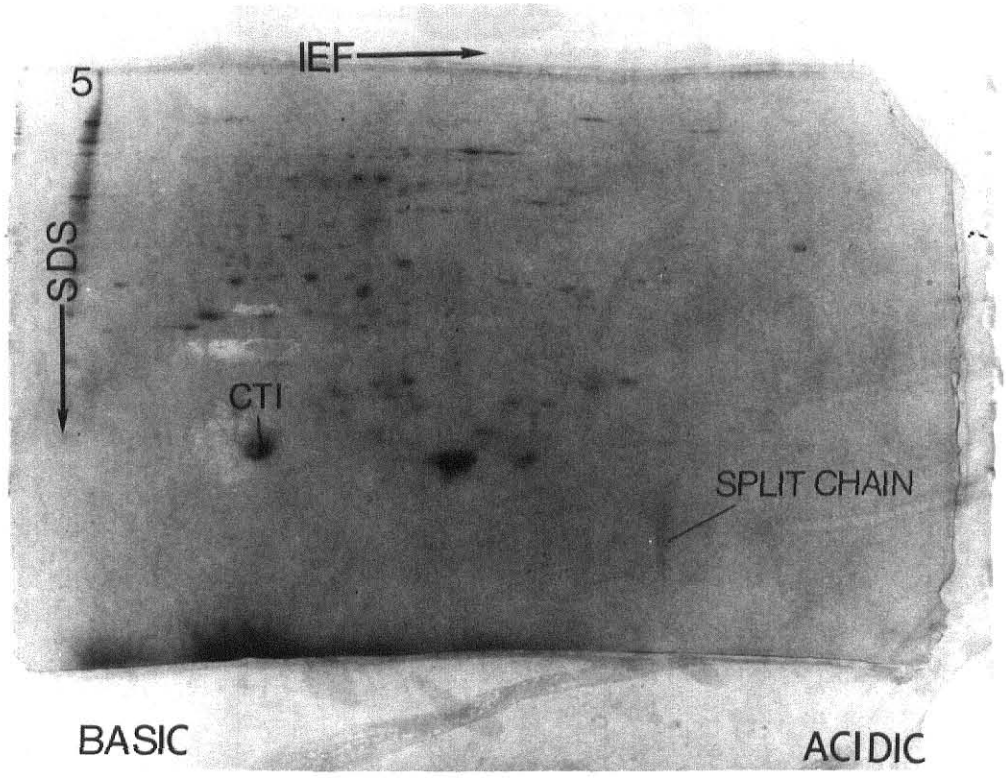
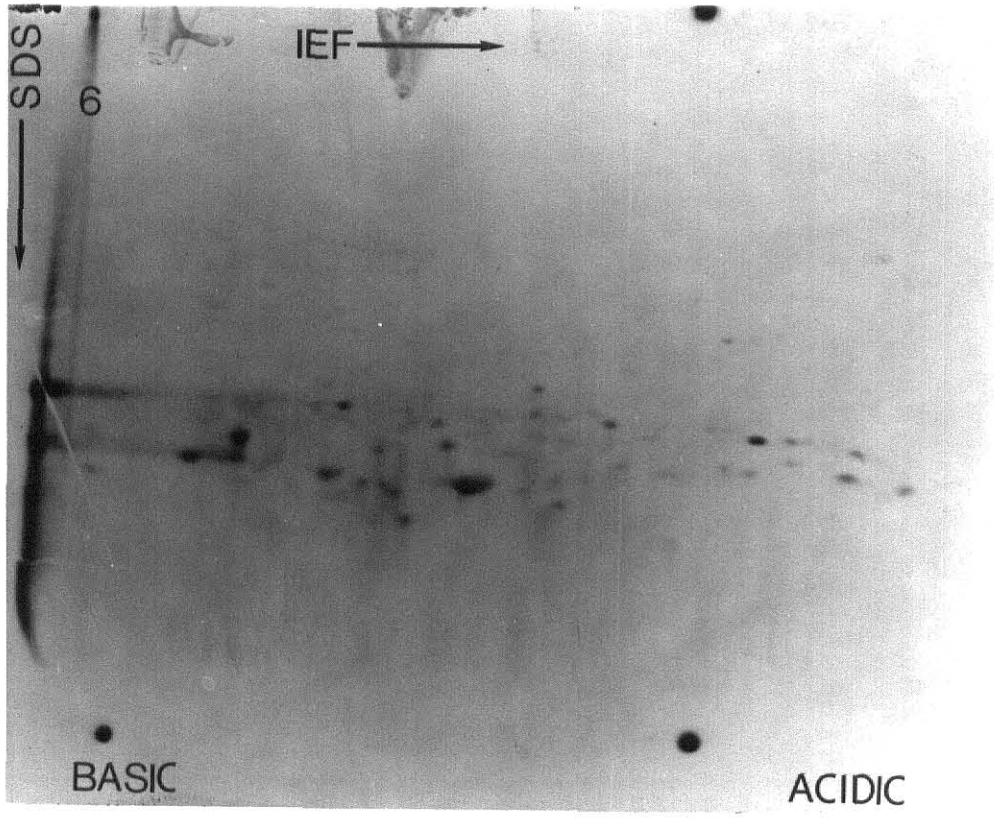

MOLECULAR WEIGHT (x10⁻³)
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0.2
0.4
0.6
0.8
1.0
MOBILITY
1
2
3
4
5

Fig. 5. Two-dimensional gel electrophoresis of the translation of poly(A)$^+$ RNA; 530,000 counts (50 λ) loaded onto the gel along with 30 μg of CTI standard.
Ampholine: pH 5-7. Electrophoresis for the first dimension was carried out to equilibrium.

Fig. 6. Autoradiogram of the two-dimensional gel from Figure 5.

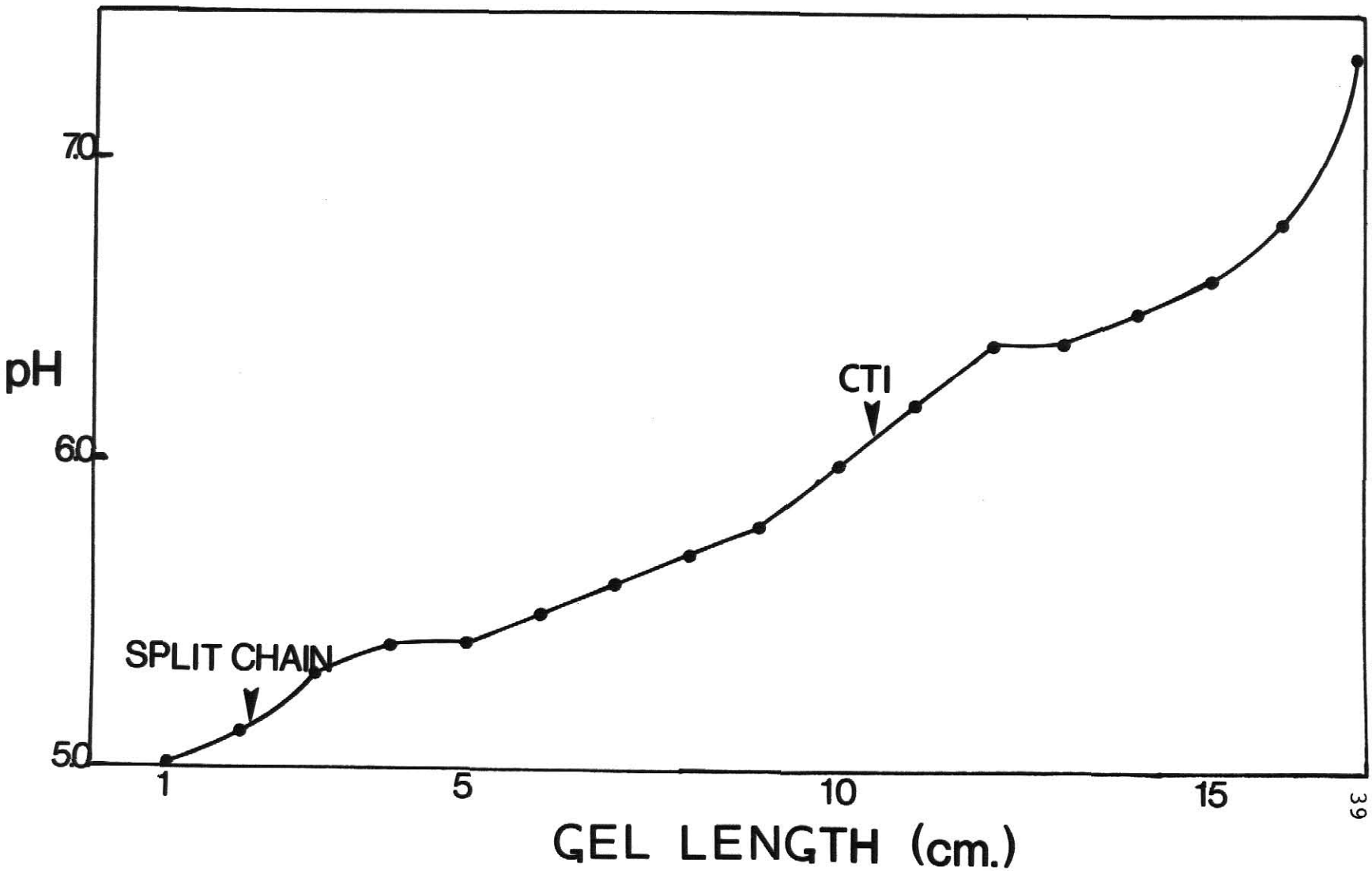

IEF
5
SDS
CTI
SPLIT CHAIN
BASIC
ACIDIC


SDS
IEF
6
BASIC
ACIDIC

loaded onto the gel along with 30 µg of cold CTI standard. The standard which consists of the native form and of the split chain is labeled on the picture. Autoradiography of this gel is shown on Figure 6 and reveals spots in the vicinity of the standard. The film was exposed for four weeks at -70°C.

Isoelectric focusing of a 30 µg sample of CTI standard gave the gel pattern shown on Figure 7. Both the single chain and the split chain forms of CTI are present on the gel. Upon measuring the pH gradient of the gel, respective pH's of 6.05 to 6.15 were found for the native form and of 5.1 to 5.15 for the split chain.

Fig. 7. pH gradient of corn trypsin inhibitor. The pH of the single chain occurs between 6.05 and 6.15; that of the split chain is between 5.1 and 5.15. The actual gel is represented above the plot.


7.0
6.0
5.0
pH
SPLIT CHAIN
CTI
1
5
10
15
GEL LENGTH (cm.)

DISCUSSION

The recovery of poly(A)$^+$ RNA from total RNA was close to 3%. The data on Table 1 shows that of all the samples analyzed, poly(A)$^+$ mRNA is the most efficient for protein synthesis; it has the highest cpm per μg of RNA.

The four bands that appear on the autoradiogram of the immunoprecipitation eluate (Fig. 3) are presumably multiple forms of corn trypsin inhibitor precursors. They are also present on the lanes corresponding to total RNA, poly(A)$^+$ RNA, translation samples of the same autoradiogram. On those samples and especially on the poly(A)$^+$ RNA track, the bands are darker which suggests that other protein bands of the same molecular weight may coexist with the inhibitor or possibly that the antibody-linked protein A-Sepharose beads are not binding all of the corn trypsin inhibitor present in the translation products.

In a previous publication, Swartz et al. (5) determined the molecular weight of CTI to be 11,000 by SDS polyacrylamide gel electrophoresis and 12,500 by sedimentation equilibrium. In this study, the molecular weights of the inhibitor bands were calculated to be 16,000, 13,600, 12,900, 12,200 by SDS gel electrophoresis as compared to 11,900 for the CTI standard. Thus, the protein bands observed here have a higher molecular weight than that of the standard. In this context, it may be noted that other researchers (22) have reported the size of zein proteins to be underestimated

in polyacrylamide gel electrophoresis presumably because of incomplete denaturation of the proteins by SDS. The same phenomenon may be operating in the case of CTI.

The use of HPLC for corn trypsin inhibitor may provide some insight into the significance of the various bands observed. A graduate student in this laboratory, Mei-Guey Lei obtained two peaks when putting a CTI standard sample through the HPLC column. She identified a major peak and a minor peak for which two slightly different apparent molecular weights were observed. Isoelectric focusing revealed the presence of five protein bands for each peak as well as for the original sample; in all three cases, the location of the bands was identical. The nature of the various bands has not been determined but they are likely to correspond to multiple forms of CTI and may be related to the heterogeneity observed on the autoradiogram represented on Figure 3. Alternatively, some multiple forms of CTI may not appear on isoelectric focusing because the amount of CTI present in the standard may be too small to allow detection by conventional means.

Hojima et al. (7) have reported the existence of three variants of CTI with respective pI's of 5.1, 6.3 and 7.7 observed on isoelectric focusing. The single chain inhibitor had a pI of 6.3 and had retained all of its ability to inhibit HF_f as well as trypsin following adsorption on anhydrotrypsin agarose and elution with 0.5 M HOAc. However, if the same sample was applied onto a trypsin agarose column and eluted

with 0.5 M HOAc, part of the inhibitor recovered was transformed into the two-chain form; inhibition against trypsin remained the same but that against HF_f was only 60% of what it originally was. It seems that the single chain form described by those researchers is identical to the one reported here with a pI of 6.1 while the two-chain form presumably matches the one I observed at pI 5.1. The third band mentioned in their publication (pI 7.7) could not have been seen in this study since a pH gradient of 5 to 7 was used.

The multiple bands of the autoradiogram on Figure 3 may also correspond to protein bands with the same antigenic determinant as CTI. Alternatively, they may represent proteins that were translated from 4 different mRNA's arising themselves from four different genes or from multiple forms of mRNA. Other possibilities could be some interference from wheat germ processing or tight binding between the inhibitor and another compound or group that would prevent dissociation in SDS. As mentioned earlier, it has been inferred in other studies that zein proteins do not completely denature in SDS (22).

It is likely, however, that one or more precursor proteins of corn trypsin inhibitor appear on the autoradiogram in Figure 3. A similar assumption was made by Nelson and Ryan (9) when working with proteinase inhibitors I and II in the leaves of tomato plants and observing higher molecular weight inhibitor protein following synthesis. Those authors suggested that the preinhibitors could potentially

be processed during or after synthesis and could be signal peptides that would play a role in transporting the inhibitor molecules throughout the cell.

It is interesting to note that the two-dimensional gel pattern of the translation and its autoradiogram show that the presumed corn trypsin inhibitor spots are located higher than the standard CTI spot which corroborates the evidence for the existence of a higher molecular weight precursor and that of isoforms of corn trypsin inhibitor.

An additional feature worthy of attention is the position of those spots that are close to the standard on the autoradiogram (Fig. 6) of the translation gel pattern and may correspond to the newly synthesized corn trypsin inhibitor proteins. They are located at a slightly higher pH than the standard which suggests that the presumed signal peptide is basic.

Some of the intense protein spots observed on the translation gel pattern (Fig. 5) are likely to be zein proteins since those are the major storage proteins found in corn, accounting for 50 to 60 percent of all its endosperm proteins (22). Zein proteins have been classified as belonging to four molecular weight groups: 22,000, 19,000, 15,000, and 10,000 (for review, see reference 22). The spots on the gel pattern (Fig. 5) have not been positively identified as zein proteins, however, since no standards were run on polyacrylamide gel electrophoresis but their presence is conspicuous

and is worth mentioning. In addition, it is known that the mRNA that codes for those proteins also codes for a signal peptide which ensures their transport throughout the cell and is later removed (22). This phenomenon may resemble closely what takes place in the case of corn trypsin and other protease inhibitors.

In this thesis, I have described the translation of messenger RNA for corn trypsin inhibitor. Following selective immunoprecipitation, I observed 4 bands on SDS polyacrylamide gel electrophoresis, each with an apparent molecular weight higher than that of the corn trypsin inhibitor (CTI) standard. In order to clarify those results, the next step to be undertaken would be to isolate and to purify those bands, to determine the sequence of the proteins obtained and to compare it with that of the known corn trypsin inhibitor standard.

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TRANSLATION OF MESSENGER RNA FOR CORN TRYPSIN INHIBITOR

by

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B.A., Otterbein College, 1976

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

Kansas State University
Manhattan, Kansas

1983

ABSTRACT

The research project undertaken for this thesis consisted of isolating messenger RNA from corn kernels and of fractionating it by oligo (dT)-cellulose chromatography in order to obtain the poly(A)$^+$ RNA fraction. The RNA was translated into proteins by use of a cell free wheat germ system and radioactively labeled ^{35}S Methionine. Selective immunoprecipitation was carried out by means of Protein A-Sepharose beads that were linked to the antibody for corn trypsin inhibitor. The proteins that did bind to the beads were subsequently eluted and were run on SDS polyacrylamide gel electrophoresis. Autoradiography revealed the presence of four bands, each with an apparent molecular weight higher than that of the standard (12,000). They were presumed to be multiple forms of corn trypsin inhibitor or precursor proteins for corn trypsin inhibitor.

The pH gradient of the corn trypsin inhibitor was determined (6.1), a value which is close to that reported in the literature.

The translation system was run on a two-dimensional polyacrylamide gel electrophoresis sytem and an autoradiogram was taken.