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University of New Hampshire

PH.D.

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THE STRUCTURE OF THE ORB WEB AND ITS
DEGRADATION BY SPIDER DIGESTIVE FLUID PROTEASES

BY

EDWARD J. KAVANAGH
B.A., Marist College, 1974

A DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Zoology

September, 1979

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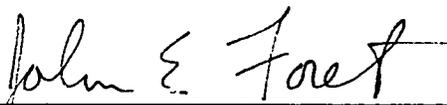
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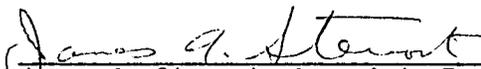
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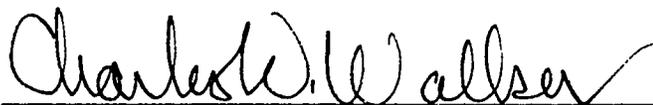
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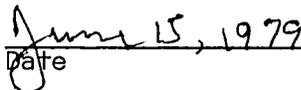
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ABSTRACT

The proteolytic activity of the digestive fluid of the spider Argiope aurantia was studied. General protease activity, defined by digestion of the protein casein, was unaffected by seryl or sulfhydryl protease inhibitors but was inhibited by the metal chelator EDTA. Peak protease activity was observed at pH 7.8, coincident with the pH of the fluid itself. Specific substrates demonstrated elastolytic and chymotryptic activity but no tryptic, carboxypeptidase or leucine amino peptidase activity. The digestive fluid was shown by autoradiography to be uniquely able to digest the radial thread of the orb web of Araneus diadematus.

Selective enzyme digestion studies with A. aurantia digestive fluid and other commercially available proteases together with histological staining and differential isotope localization were used to study the structure of the orb webs of Araneus trifolium and Argiope trifasciata. Three fiber types were present in these webs: (a) a major fiber found throughout the web, (b) a minor fiber found only in radial threads, and (c) the core fibers of the sticky spiral thread. Three nonfibrous secretions were found on these fibers. These included a water soluble viscid coating of the sticky spiral and two adhesives which fasten the threads of the web together; one found only at junctions of sticky spiral and radial threads and the other at all other thread connections. The possible glandular sources of these materials are discussed.

Fractionation of the digestive fluid by ion exchange chromato-

graphy yielded four major protease fractions: A, B, C and D. The protease activities of all fractions were inhibited by Cu, Cd and Ni and EDTA. Zn, Co and Pb ions reversed the chelator inhibition of these proteases. Each protease fraction showed different substrate specificities, variously hydrolyzing casein, elastin and several synthetic ester substrates to different degrees. Two of the fractions, A and B, were able to dissolve the generally protease-resistant large ampullate fiber of spiders' orb webs. Hydroxylapatite chromatography of fractions A, B and fraction D, the fraction most active against casein and elastin, revealed that fractions B and D each contained a single protease while fraction A contained at least two proteases.

The single protease of fraction B, Argiope protease B, was shown by polyacrylamide gel electrophoresis to have been purified to homogeneity and was further characterized. The molecular weight of protease B was estimated to be 16,000 - 18,000 by denaturing techniques and 12,000 by non-denaturing means. Its stability parameters were established and the kinetics of metal ion and metal chelator inhibition was established. The cleavage specificity of the insulin B chain by protease B was also determined.

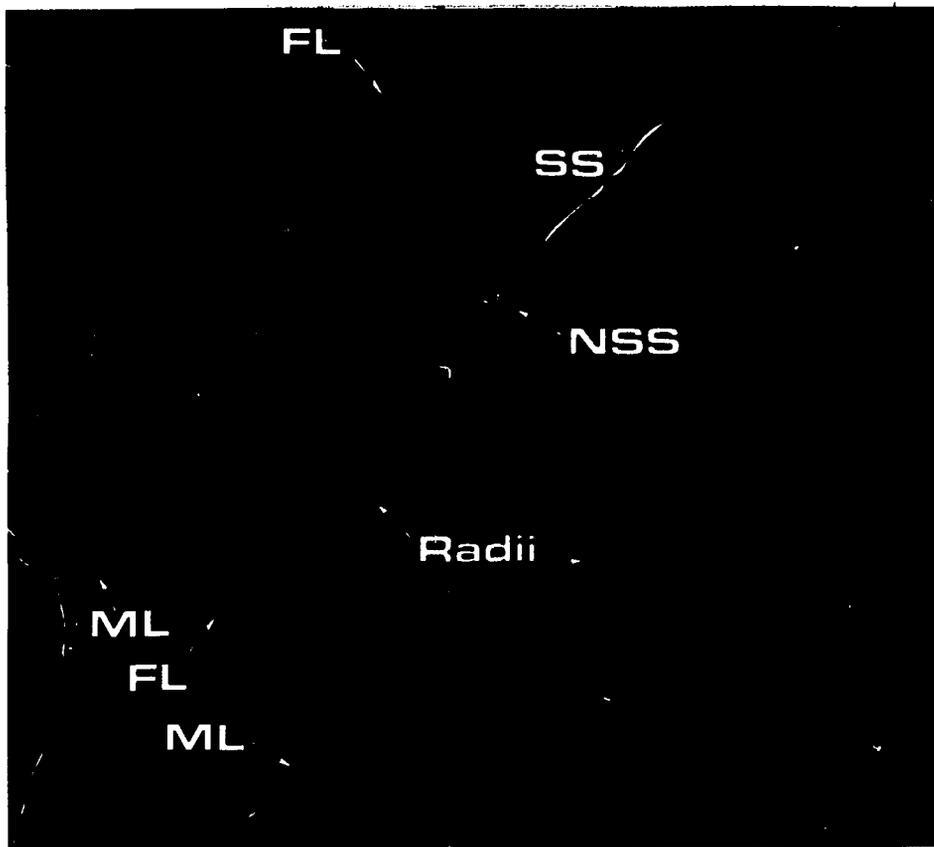
The digestion of reeled silk fibroin by purified protease B was studied. Fibroin digestion products included numerous small peptides of less than 1,200 molecular weight. The amino-terminal amino acids of the digestion products as well as the relative amino acid composition of digested and undigested fibroin were determined by dansylation techniques.

INTRODUCTION

The orb webs produced by members of the genera Araneus and Argiope are complex structures constructed in two basic phases. First a scaffold of linear threads is laid down (Fig. 1). These threads are composed of two to ten parallel fibers (Denny, 1976) derived from the large ampullate glands. During the second phase of web construction, three spiral threads are laid on the linear framework. The first, a tight spiral of dry thread, known as the non-sticky or hub spiral, is placed just outside the center of the web; this serves to reinforce the web (Savory, 1952). Next, the spider makes a sidestep and proceeds toward the periphery of the web laying a second dry thread known as the provisional spiral. Once the latter is completed, the spider returns to the center of the web laying the viscid, prey-catching sticky spiral. During the construction of the sticky spiral the provisional spiral is removed and ingested (Witt, et al., 1968). The sticky spiral is composed of two materials; a pair of core fibers and a viscid covering substance. While the aggregate glands are generally accepted as the source of the viscid substance, both the flagelliform glands (Sekiguchi, 1952) and the large ampullate glands (Peakall, 1964) have been suggested as the source of the core fibers. In addition, glandular secretions may also serve to bind the various threads at their intersections (Jackson, 1971).

Orb weaving spiders have five to seven paired silk glands (Andersen, 1970). Although the functions of the secretions of several glands are well established, there is still uncertainty as to how many glands are employed in web construction (Peakall, 1968). The involvement

Figure 1. A typical orb web and the lines (threads) of which it is composed. Radii run from the center of the web to frame lines (FL) which are connected to the substratum by mooring lines (ML). Attached to the radii are the centrally located hub spiral (HS) and peripheral sticky spiral (SS).

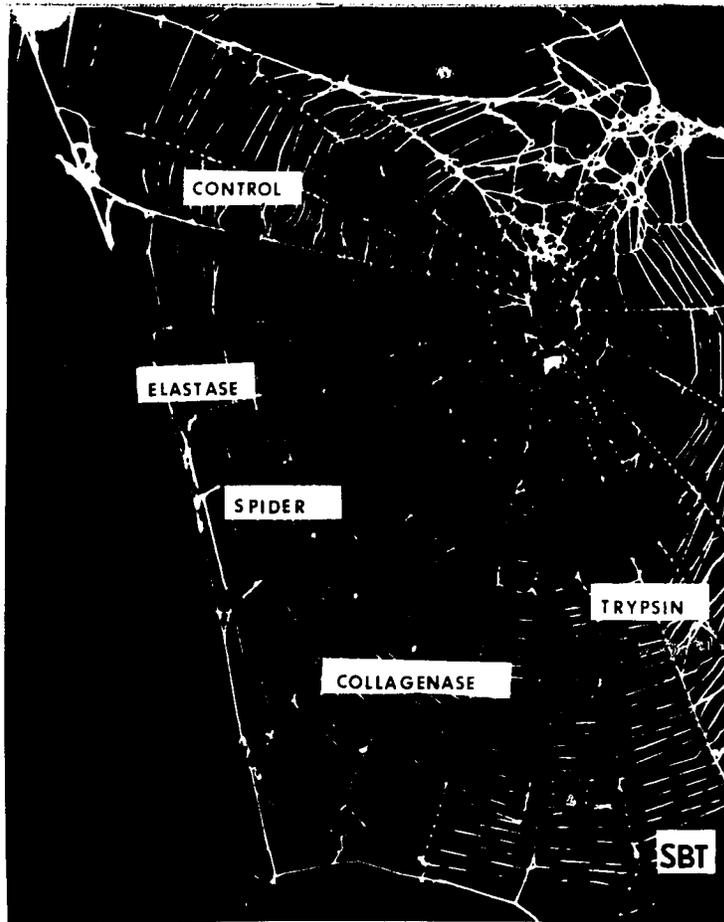


of particular glands in web construction has previously been deduced by: (a) observation of which spinnerets and spinning tubes are the source of a particular silk (Comstock, 1948), (b) observation of luminal contents of glands before and after a particular structure is constructed (Peakall, 1964) and (c) comparison of the amino acid content of silks with that of the glandular luminal contents (Andersen, 1970).

An interesting metabolic aspect of orb webs is their degradation and possible recycling of their constituent amino acids. Behavioral evidence (Breed, et al., 1964; Peakall, 1972) has established that orb weaving spiders regularly ingest their webs. Radioisotope tracer data (Peakall, 1972) has indicated that the constituents of ingested webs are rapidly and efficiently incorporated into new web proteins. Proteases in the digestive fluid that spiders use to digest their prey extracorporally have been shown to be capable of web digestion (Fig. 2) (Tillinghast and Kavanagh, 1977).

The digestive proteases of spiders have not been studied extensively. Pickford (1942) observed alkaline and acidic protease activity in tissue extracts and in the gut fluids of several spiders. Hemolymph and gut fluid esterases and phosphatases of other species have been studied by polyacrylamide electrophoretic techniques (Couch and Benton, 1971). The initial description of A. aurantia digestive fluid noting alkaline general protease, elastase and chymotryptic activities reported in this dissertation was published in 1977 (Tillinghast and Kavanagh, 1977). More recently, Mommsen (1978) observed a single alkaline, casein-digesting protease in the digestive fluid of the European funnel weaver Tegenaria atrica.

Figure 2. The effect of commercial enzymes and Argiope digestive fluid on the radial and spiral fibers of the orb-web of Araneus diadematus. Filter discs were wetted with 0.2 ml 0.1 M TES buffer, pH 7.5, with 0.36 mM CaCl₂ containing 1 mg of commercial enzyme or 50 µl spider digestive fluid. Following incubation with the web at room temperature for 24 hours the discs were removed and the web exposed to Kodak Sb-54 X-ray film for one month (Tillinghast and Kavanagh, 1977).



The present study of spider digestive fluid and web structure is divided into five sections: (a) characteristics of the total protease activity of unfractionated A. aurantia digestive fluid (Tillinghast and Kavanagh, 1977), (b) elucidation of the structure of the orb web using digestive fluid as a tool (Kavanagh and Tillinghast, 1979), (c) separation, purification and description of the major protease fractions of the digestive fluid, (d) physical characterization of the silk fibroin-digesting protease of the fluid Argiope protease B, and (e) studies of silk fibroin digestion by Argiope protease B.

MATERIALS AND METHODS

Collection of Animals and Digestive Fluid

Adult female Argiope and Araneus were captured in fields and wetlands of New Hampshire, Maine and Massachusetts during the months of July and August and identified according to Kaston (1952). Argiope aurantia spiders were milked of their digestive fluid within four hours of capture. Usually, within thirty seconds after being gently restrained on their backs with forceps, A. aurantia spiders would extrude a small drop-let of digestive fluid from their mouthparts which was drawn via suction into a 20 μ l capillary tube. Individual samples, averaging about 25 per spider, were pooled and stored at -20°C for up to eight months without loss of protease activity. Due to the small size of A. aurantia fangs it was impossible to prevent venom contamination of the collected digestive fluid. The other species of spiders collected yielded little or no digestive fluid when similarly handled. Couch and Benton (1971) and Mommsen, (1978) have also reported the collection of miniscule (0.5 - 1.0 μ l) amounts of fluid per spider from other species. The large yields of fluid obtained from A. aurantia may be related to its size (over 3 cm in body length when mature) and its short life span. Argiope aurantia lays its cocoon and then dies in late August or early September. In the weeks preceding this it undergoes rapid growth and feeds voraciously on locusts and other large insects which are prevalent in its habitat. This may account for the large volumes of fluid found in freshly caught mature specimens. Attempts were made to collect quantities

of venom-free digestive fluid from commercially obtained (Connecticut Valley Biological Supply Company) tarantula species by covering the fangs of the animals with intramedic tubing and applying low voltage electric shock (Schanbacher, et al., 1973). Digestive fluid could be obtained in this manner only within several days of when the animal had last eaten which can be as infrequently as three months.

Web Collection and Autoradiography

Webs collected from the field were heavily contaminated with pollen and seeds and were therefore not used in this study. Instead, A. trifolium and A. trifasciata were maintained in cages 20 x 20 x 10 inches with removable glass windows front and back (Witt, et al., 1968) and exposed to a natural light cycle. These species, unlike A. aurantia, regularly build intact orb webs in captivity. Webs were collected on 8 x 10 inch glass plates, wrapped in polyethelene film, (these are called plated webs) and stored in a cool, dry place for up to four months before use.

For autoradiographic studies, spiders were fed one microcurie of either D(U¹⁴C) glucose, inorganic ³⁵S orthophosphate ³²P (Amersham Corp.) in water from a syringe. Existing webs were then removed and subsequent webs produced by the experimental spiders were collected. The plated and wrapped webs were exposed to Kodak SD-54 medical x-ray film for predetermined periods of time prior to development, depending on the isotope used.

Web Staining and Photography

Unfixed plated webs were treated directly with one of a variety of histological stains, including Mallory-Heidenhain's trichrome (Cason

1950), spirit blue (Elder and Owen, 1967), methyl green, coomassie brilliant blue R, hematoxylin and eosin, safranin O and fast green as well as Mallory's phosphotungstic acid hematoxylin (Lillie, 1954) and aldehyde fuchsin (Cameron and Steele, 1959). Usually the natural adhesiveness of the web was sufficient to maintain the web on the plate during treatment with these stains. Plated webs were also stained by the periodic acid Schiff (PAS) method. Plated webs were fixed to their plates by many small droplets of epoxy cement and then dipped in a 0.7% solution of periodic acid (in 7% acetic acid) for two hours. The plates were then soaked in two changes of 0.2% potassium metabisulfite for two hours before being stained overnight with Schiff's reagent.

Photomicrographs were produced with a Nikon LKE research microscope coupled with a Nikon AFM photomicrographic system; Kodak PCF415 photomicrographic color film was employed under bright field conditions.

Protease Digestion of Plated Webs

Discs of filter paper (Whatman 3 MM) wetted with proteases dissolved in 0.05 M Tris-HCl pH 7.8 containing 5 mM calcium chloride and 5 mM sodium azide were placed on plated webs and covered with a weighted plastic disc. After incubation in a moist covered chamber at 37°C for 12 to 24 hours the discs were removed and the plated web was exposed to x-ray film or stained for microscopic observation. The enzymes used included bovine pancreatic trypsin (E.C. 3.5.21.4), porcine pancreatic elastase (E.C. 3.4.31.11), clostridial collagenase (E.C. 3.4.24.3) and those present in the digestive fluid of A. aurantia. Commercial enzymes were obtained from the Sigma Chemical Corporation* (St. Louis, MO). The

*Unless otherwise stated all reagents used in this study were products of the Sigma Chemical Corporation, St. Louis, MO.

digestion of large ampullate fibers (LAF) by the different protease of A. aurantia digestive fluid were performed in this fashion using A. trifolium webs exclusively.

Enzyme Assays

General protease activity (GPA) was assayed by two different modifications of Kunitz's (1947) casein digestion method.

In the studies of unfractionated digestive fluid GPA was assayed as follows. A reaction mixture consisted of 2.5 ml of 1% Hammerstein casein in 0.05 M borate-KCl-NaOH, pH 7.8 and 0.3 ml of distilled water. After equilibration for 10 minutes at 37°C the reaction was initiated by the addition of 0.2 ml diluted enzyme and allowed to incubate for 30 minutes. The reaction was terminated by the addition of 4.0 ml of 5% trichloroacetic acid (TCA) and set aside for 1 hour. The precipitate was removed by filtration and the absorbance of the filtrate at 280 nm was measured. Blank values were obtained by the addition of enzyme immediately after casein precipitation. It was observed that increases in A_{280} values of 0.25 to 1.0. Activity units were expressed as the amount of enzyme (mg) which caused a change of A_{280} of 1.00 per minute.

A second modification of the casein digestion method was used to determine GPA during the separation and study of the individual proteases of A. aurantia digestive fluid. From 20 to 100 μ l enzyme solution was incubated for 20 minutes with 3 ml of 0.5% Hammerstein casein (Schwarz-Mann) in 0.1 M borate-KCl-NaOH buffer, pH 7.8 pre-equilibrated at 37°C. Undigested casein was precipitated by the addition of 3 ml of 5% TCA and removed by filtration through Whatman #1 paper after 30 minutes

incubation at 5°C. Soluble digestion products were estimated spectrophotometrically at 280 nm or by the ninhydrin method of Moore and Stein (1948). Protease units were determined from a standard curve of A_{280} versus enzyme concentration (Fig. 3). One protease unit is defined as that amount of enzyme which in the initial phase of casein digestion, releases one microgram of trichloroacetic acid soluble leucine equivalents per ml per minute and is equivalent to 1.2 μ g of purified Argiope protease B.

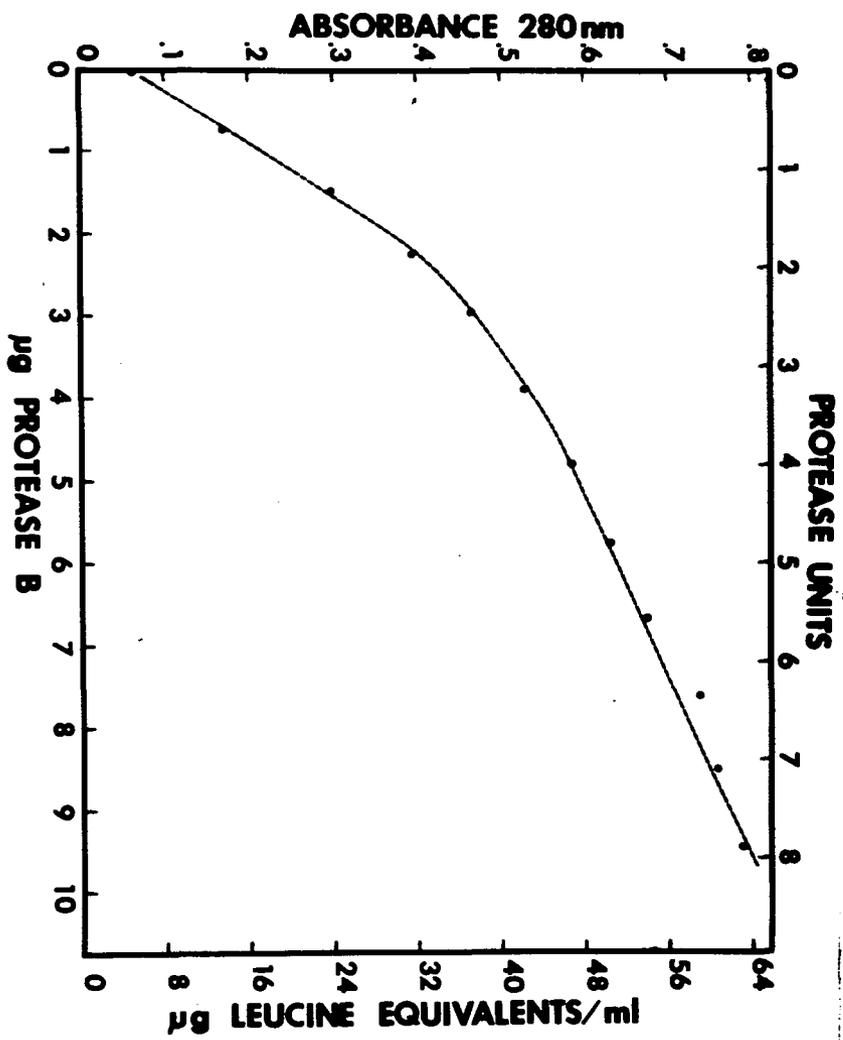
Tryptic activity was assayed according to the procedure of Hummel (1959) using N-toluenesulfonyl-L-arginine methyl ester (TAME) as a substrate and N-benzoyl-DL-arginine-p-nitroanilide HCl as a substrate by the method of Erlanger, et al., (1961).

Chymotryptic activity was assayed with kits purchased from the Worthington Biochemical Corporation (Freehold, NJ) using N-benzoyl-L-tryosine ethyl ester (BTEE) and N acetyl-L-tryosine ethyl ester (ATEE). Absorbance changes were monitored at 30°C and reliability of the assays confirmed by the use of commercial enzymes.

Carboxypeptidase A was assayed by the method of Folk and Schirmer (1963) using hippuryl-L-phenylalanine as substrate; carboxypeptidase B by the procedure of Folk, et al., (1960). Leucine aminopeptidase was assayed according to Mitz and Schlueter (1958).

Elastase activity was assayed by the elasto-orcein method of Sachar, et al., (1955), with N-acetyl(Ala)₃ methyl ester as described by Beith and Meyer (1973) and with Elastin-Congo Red (ECR). The ECR assay was initiated by addition of enzyme aliquots to 5 ml of 0.1 M borate-KCl-NaOH, pH 7.8, pre-equilibrated at 37°C and containing 20 mg of substrate. Digestion was halted by rapid filtration and the absorbance

Figure 3. General protease activity (GPA) standard curve of casein digestion. Incubation of Argiope protease with casein catalyzed the release of trichloroacetic acid soluble digestion products which were estimated as leucine equivalents by ninhydrin assay (Moore and Stein, 1948) or spectrophotometrically at 280 nm. Protease units were defined as the amount of enzyme which released 1.0 μg leucine equivalents/ml/minute.



of the filtrate at 495 nm was determined. The extent of elastin digestion was determined from a standard curve of absorbance versus substrate concentration after complete substrate digestion with pancreatic elastase.

Hydrolysis of N-carbobenzoxy-p-nitrophenyl ester (NCBZ-p-NPE) derivatives of the amino acids alanine, glycine, proline and valine were determined by the method of Visser and Blout (1972) as modified by Mallory and Travis (1975) using a substrate concentration of 1.3×10^{-4} M in 0.05 M Pipes (piperazine-N,N'-bis(2-ethane sulfonic acid) buffer at pH 6.6.

Protein, Phosphate and pH Determinations

Protein concentrations were determined either by the methods of Lowry, et al., (1951) using bovine serum albumin (BSA) as a standard or that of Bradford (1975) using an equal mixture of BSA and ovalbumin as a standard. The pH of pooled samples of digestive fluid was measured with a Radiometer model 26 pH meter. The Fiske-Subbarow method (Qser, 1965) was used for the determination of inorganic phosphate.

Thermal Stability of Spider Digestive Fluid

Spider digestive fluid was diluted to a protein concentration of 0.05 mg/ml in 0.05 M borate-KCl-NaOH, pH 7.8 pre-equilibrated at selected temperatures between 15°C to 70°C at 5°C intervals. The dilute enzyme mixture was maintained at the selected temperature for 20 minutes before being rapidly brought to 25°C for GPA assay.

Inhibitor Studies of Unfractionated Digestive Fluid

Aliquots of digestive fluid, diluted to approximately 250 µg pro-

tein per ml, were added to equal volumes of various inhibitors in 0.05 M Tris-HCl, pH 7.2 to give a final inhibitor concentration of 5 mM or 0.5 mg/ml in the case of the lima bean trypsin inhibitor (LBTI). These mixtures were incubated for one hour at 4°C and then assayed for GPA by the method of Kunitz (1947) as described above (Enzyme Assays).

To evaluate the temperature effects upon EDTA inhibition, digestive fluid was diluted to 8 µg protein/ml in .05 M borate-KCl-NaOH, pH 7.8 with or without the addition of 5 mM CaCl₂ and/or 25 mM EDTA. The samples were incubated at 5°C or 30°C. At various time intervals 0.1 ml aliquots were removed for GPA assay.

Ion Exchange Cellulose and Hydroxylapatite Chromatography

Digestive fluid (6 ml) was centrifuged at 10,000 g for 15 minutes and then dialyzed overnight against 2.0 L of 0.5 M Tris-HCl buffer, pH 8.2, containing 5 mM CaCl₂. The dialysate was applied to a 1.5 x 18 cm column packed with DEAE cellulose (DE-32, Whatman) equilibrated with the same buffer. The column was washed with buffer until thirty 3.5 ml fractions were collected. This was followed by an eluant containing 0.2 M NaCl in the same buffer and an additional thirty fractions were then collected. The first protease peak obtained from DEAE chromatography was dialyzed overnight against 1.0 L of 0.25 M Tris-maleate, pH 6.4 containing 5 mM CaCl₂. The dialysate was then applied to a 1 x 18 cm column packed with CM-cellulose (CM-32, Whatman) equilibrated with the same buffer. After twenty 3.5 ml fractions were collected, a linear gradient of 0 to 0.15 M NaCl was established. Upon completion of the gradient, the column was washed further with 0.15 M NaCl in buffer and additional fractions were collected.

The protease peaks eluted from the CM cellulose columns were dialyzed overnight against 2.0 L of 0.01 M sodium phosphate buffer, pH 6.8. The dialysates were individually applied to a 30 x 6.8 cm column of hydroxyapatite (DNA grade, Bio Rad) which was then washed with three volumes of buffer. A linear gradient from 0.01 M to 0.20 M sodium phosphate buffer at pH 6.8 was then run through the column as 4.0 ml fractions were collected.

Metal Ion Inhibition and Inhibition Reversal of CM-Cellulose Separated Protease Fractions

Samples of the protease fractions (A, B, C and D) obtained from CM-cellulose column chromatography were diluted five-fold in 0.025 M Tris-maleate buffer, pH 6.4, containing individual metal salts (reagent grade products of Merck and Co., and J.T. Baker Chemical Company) at final concentrations of 0.1 or 1.0 mM. These mixtures were incubated for 15 minutes at room temperature before being assayed for GPA. Other samples of the protease fractions, previously dialyzed against metal-free Tris-maleate buffer, were diluted with equal volumes of 20 mM EDTA in pH 6.4 buffer and incubated 30 minutes at room temperature. After removal of unbound EDTA by overnight dialysis against cold Tris-maleate, aliquote of the proteases were diluted four-fold in buffer containing individual metal salts at final concentrations of 0.2 to 1.0 mM. Following incubation at 25°C for 30 minutes these metal-enzyme mixtures were assayed for GPA.

Electrophoretic Methods

Whole digestive fluid was initially fractionated by thin layer agarose electrophoresis using 0.5 M sodium barbital, pH 8.6, containing 2 mM calcium lactate as the electrolyte. Up to 20 μ l of fluid

was applied to slits cut in a 1.6% agarose (Sigma Type: low EEO) slabs, 0.3 x 2 x 20 cm, on glass slides and subjected to a current of 3 mA per slide for 12 hours. Amido black was used as a general protein stain whereas protease activity was detected by the method of Uriel (1960). Purified spider proteases were subjected to cationic polyacrylamide electrophoresis according to Reisfeld, et al., (1962) using a 7.5% gel. Separated proteins were made visible by staining with a 0.2% Brilliant Blue R or Fast Green F.S.C. in 7% acetic acid, 50% methanol for three hours at 37°C (Maizel, 1971). Fast Green was less sensitive than Brilliant Blue R but was completely destained much more rapidly (24 hours as compared to several weeks for Brilliant Blue R in a Bio-Rad Model 170A Diffusion Destainer). Insulin and web digest samples were subjected to high voltage paper electrophoresis (Blackburn, 1968). Samples of 10 to 35 µl were separated on Whatman 3 MM paper for 1 hour at 100 volts/cm using pH 2.2, formic-acetic acid buffer as the electrolyte. After drying, the paper was stained by dipping in calcium acetate-ninhydrin reagent (Blackburn, 1968) followed by development overnight in a dark ammonia-free chamber.

Sodium dodecyl sulfate (SDS) electrophoresis was performed as described by Maizel (1971). While acrylamide concentrations varied with experiments, 12% gels were used to determine the molecular weight of Argiope protease. Samples were prepared by dissolution in or dialysis against stacking gel buffer diluted eight-fold, 1% SDS, and 0.1% 2-mercaptaethanol followed by heating in a boiling water bath for 3 minutes. After cooling the samples were applied to the gels with the addition of a tenth volume of glycerol and 5 µl 0.01% from phenol blue tracking dye.

The gels were run at a 4 mA/tube until the tracking dye was about 5 mm from the ends of the tubes. After removal of gels from the tubes the position of the tracking dye was marked with a stainless steel pin and the gels were stained with Brilliant Blue R as previously described. For molecular weight estimates, the migration of the sample relative to the tracking dye (R_f) was measured and compared to a standard curve established with the proteins bovine serum albumin, ovalbumin, porcine trypsin and myoglobin.

Carbohydrate Analysis

Polyacrylamide gels were analyzed for carbohydrate by staining with the PAS method (Segrest and Jackson, 1972) or with Alcian Blue (Wardi and Michos, 1972). After being fixed overnight in ethanol:acetic acid: water, 45:5:55, gels were incubated in 0.7% periodic acid in 5% acetic acid for two hours. The gels were then soaked in two changes of 0.2% potassium metabisulfite for three hours before being stained overnight in either Schiff's reagent or 0.5% Alcian blue in 3% acetic acid.

The reducible carbohydrate content of Argiope protease B was determined by the method of Dubois, et al., (1956). Two ml aqueous samples in 25 mm wide test tubes were mixed with 50 μ l of 80% analytical grade phenol. Five ml of sulfuric acid was rapidly added with a glass syringe to the tubes which were allowed to stand for one hour before the absorbance of their contents at 490 nm was determined. Glucose samples of 5 to 70 μ g were used to establish a standard curve.

Elemental Analysis

The zinc content of Argiope protease B was determined on an Instrumentation Laboratory (Wilmington, MA) aa/ae spectrophotometer.

Protease samples were dialyzed against 0.05 M Tris-HCl, pH 8.0, in twice distilled water and were assayed for protein concentration and GPA shortly before elemental analysis.

Isoelectric Focusing

Isoelectric focusing in acrylamide gels was carried out using wide range ampholyte (Bio-Lyte 3/10:Bio Rad Laboratories) according to the manufacturer's specifications (Bio Rad, 1974). Gels contained 7.5% acrylamide, 5% glycerol, 2% ampholyte and were photopolymerized using riboflavin-5' phosphate. Protein samples (20-35 μ g) were overlaid on the gels in 40% sucrose which were then run for 9 or 12 hours at 220 volts using 0.06N H_2SO_4 as the anolyte and 0.04N NaOH containing 0.2N $Ca(OH)_2$ as the catholyte. Immediately afterwards, the gels were either stained for protein using Brilliant Blue R/ $CuSO_4$ (Righette and Drysdale, 1974) or sliced into 0.5 mm sections which were placed into 2 ml of 10 mM NaCl. The slices were eluted overnight under nitrogen before the pH of the eluant was determined.

Isoelectric focusing was also performed in agarose tube gels containing linear polyacrylamide polymer to reduce endosomatic flow (Johansson and Hjerten, 1974). Agarose (Sigma Type I) and polyacrylamide polymer were solubilized in boiling distilled water which was allowed to cool to 65°C before ampholyte and protein samples were added. The mixture was then poured into 5 x 125 mm glass tubes which were sealed at both ends with the dialysis tubing after the gel hardened. Final gel concentrations were .8% agarose, 2.5% acrylamide polymer, 3% ampholyte (2% Bio-Lyte 3/10, 1% Bio-Lyte 9/10, Bio Rad Laboratories), and 25 μ g protein per gel tube. The tubes were focused at 220 V for 1 to 2 hours using 1% acetic acid as the anolyte and 2% ethanolamine

as the catholyte. Afterwards the gels were sliced and eluted as previously described or soaked in 10% TCA for two days before staining with Brilliant Blue R.

Thermal and pH Stability of Argiope Protease B

Purified protease was dialyzed overnight against 0.05 M Tris-HCl buffer, pH 8.2 at 4°C. The dialyzate was divided into two tubes one of which was incubated at 25°C and the other at 37°C. At specific time intervals samples were removed for GPA assay. Other samples of freshly purified protease B were diluted ten fold in either 0.05 M Tris-maleate or 0.05 M glycine-NaOH at various pH levels. These dilutions were incubated at 25°C for 30 minutes before being assayed for GPA.

Gel Filtration of Argiope Protease B in Denaturing and Non-denaturing Solvents

Purified protease B (180 µg in 10% glycerol) was applied to a 1.5 x 90 cm column of Sephadex G-100 fine (Pharmacia) equilibrated with 0.125 M borate-KCl-NaOH, pH 7.8. The column was eluted at a rate of 8 ml/hr as 1.0 ml fraction were collected. The elution volume of protease B was determined by GPA assay of these fractions. Elution volumes under the same conditions were obtained for the proteins, bovine serum albumin (BSA), ovalbumin, porcine trypsin and myoglobin. These were detected by porcein assay (Bradford, 1976). The molecular weight of protease B was estimated from a standard curve of molecular weight versus elution volume (Whitaker, 1963).

Gel filtration in a denaturing solvent was performed in a 1.5 x 90 cm column packed with Sepharose 6B (Pharmacia) equilibrated on 0.025 M Tris-HCl, pH 7.5, containing 2% SDS, and 0.5% 2-mercaptoethanol. Mol-

ecular weight standards BSA, ovalbumin and myoglobin (8 mg each), were dissolved in a denaturing buffer of 4% SDS, 1% 2-mercaptoethanol in 0.1 M Tris-HCl, pH 7.5 (Goldsmith and Konigsberg, 1977) and incubated in a boiling water bath for 3 minutes before being applied to the column. The column was eluted at 6 ml/hr while the elution volume of the standards was determined by measuring the A_{230} of the effluent fractions. Purified protease B was lyophilized, dissolved in 1.0 ml of denaturing buffer and was run through the column under the same condition as were the standard proteins.

Kinetics of Copper Ion Inhibition of Argiope Protease B.

Kinetic analysis of Cu inhibition of the protease was carried out using the ester substrate NCBZ-Ala-NPE (Enzyme Assays). Purified protease (40 $\mu\text{g/ml}$) was incubated with various concentrations of CuCl_2 (0-20 μM) for 30 minutes at 25°C before duplicate 30 μl aliquots (1.2 μg enzyme) were assayed. The assay mixture contained various amounts of substrate (3.0×10^{-5} to 5.0×10^{-4} M) and the same concentration of CuCl_2 that the protease had been preincubated with. The reaction was allowed to proceed for 5 minutes and the change in A_{347} followed with a recording spectrophotometer (Beckman DB-GT with a 10 inch recorder). The rate of the reaction was established by drawing a straight line through the linear first 3 minutes of the recording trace.

Iodoacetate Inhibition of Argiope Protease B

Purified protease B (80 $\mu\text{g/ml}$) was incubated with and without 5.0 mM iodoacetic acid in .05M glycine-NaOH buffer, pH 8.4, for 20 minutes at room temperature. Commercial papain (Sigma Chemical Co.)

was treated identically as a positive control. Alternatively freshly purified protease B was incubated with 20 mM EDTA in 0.05 M Tris-HCl, pH 7.2 for 20 minutes at 30°C then divided into two samples. One sample was dialyzed overnight at 5°C against 0.05 M Tris-HCl, pH 7.2 containing 10 mM iodoacetate. The other sample was dialyzed against buffer alone. Aliquots of both samples were then incubated with and without 1.0 mM ZnCl₂ before being assayed for GPA.

Metal Chelator Inhibition of Argiope Protease B

Aliquots (5 µg) of protease B were incubated with various metal chelators at concentrations of 1.1×10^{-4} M to 2.7×10^{-2} M in 0.02 M Pipes, pH 7.0 for one hour at 37°C before being assayed for GPA. Chelators used included o-phenanthroline, EDTA, cysteine, 2-mercaptoethanol sodium azide and potassium cyanide. O-phenanthroline required the addition of 10% acetone for solubilization and due to its high absorbance at 280 nm, GPA was assayed by the ninhydrin reaction.

Argiope Protease B Cleavage of the Oxidized B Chain of Insulin

Oxidized B chain of bovine insulin was purchased from Sigma Chemical Company and used without further purification. Duplicate tubes of insulin B chain (7.5 mg in 1.5 ml, 0.1 M NH₄HCO₃, pH 8.0) were digested with 75 µg of purified Argiope protease B for 4 hours at 30°C before being quickly frozen. Controls of enzyme and insulin alone were treated identically. One tube of digested insulin was lyophilized, solubilized in 0.5 ml buffer and applied to a 1.5 x 90 cm column Sephadex G-25 (Pharmacia) equilibrated with 0.1 M NH₄HCO₃, pH 8.0. The column was eluted at 12 ml/hr at room temperature. Peptides were de-

tected in the effluent fractions by ninhydrin reaction after alkaline hydrolysis (Hirs, 1967). Alternatively, the digestion mixture was fractionated by high voltage paper electrophoresis in parallel after which a guide strip was cut from the paper and stained with cadmium acetate ninhydrin (Blackburn, 1968). Separated peptides located in this manner were cut out of the electrophoretogram and eluted with distilled water.

The amino terminal amino acid of the digested peptides were determined by Gray's (1972) method. Samples were dried, dissolved in 20 μl of 0.2 M sodium bicarbonate, mixed with an equal volume of 5 mM dansyl chloride in acetone and incubated at 37°C for 30 minutes. The samples were then hydrolyzed in 200 μl of 6 M HCl for 12 hours, dried under vacuum at 60°C, and extracted with water-saturated ethyl acetate. After the ethyl acetate was dried under a nitrogen stream the residue was solubilized with 20 μl of 50% pyridine and chromatographed on micropolyamide thin layer plates (Schleicher and Schuell) according to Woods and Wang (1968). The N-1 and N-2 amino acids of the digestive peptides were determined by Edman degradation (Gray, 1972). Samples were dried, solubilized with 100 μl of 50% pyridine, then incubated with 50 μl of 10% phenylisothiocyanate (Aldrich Chemical Co.) in pyridine under nitrogen for 45 minutes at 45°C. The samples were then dried under vacuum, incubated with 100 μl trifluoroacetic acid under nitrogen for 15 minutes at 45°C, before being thoroughly dried again under vacuum. The residue was dissolved with 200 μl of water and extracted three times with ethyl acetate before the aqueous phase was dansylated as described above. The amino acid composition of

the peptides from insulin was determined by hydrolyzing samples in 6 M HCl for 12 hours followed by dansylation as described by Roberts, et al., (1973). After the hydrolysates had dried they were solubilized in 0.05 mM sodium bicarbonate, adjusted to pH 9.0 with NaOH and combined with an equal volume of 1 mM dansyl chloride in acetone. After the mixtures had incubated in the dark for 30 minutes, 2 μ l samples were spotted directly on polyamide plates and chromatographed as previously described.

Fibroin Digestion by Argiope Protease B

Reeled dragline was used as a source of large ampullate fibroin that was relatively free of other secretions. Argiope aurantia individuals were placed on a clean glass plate over a black surface, which facilitated visualization of silk threads, and allowed to put down an attachment disc and tracking dragline. As the spider was gently restrained with a forceps the line was wrapped on a glass rod (5 mm dia. and then cut away from the attachment disc. The rod was then inserted into the bit of a variable speed electric motor, eight inches above the spider and the dragline was gently reeled from the spinnerets from the spider. Care was taken to immediately part the line if any other silk secretions appeared to attach to it. An average of 50 to 100 μ g of silk fibroin could be collected from each spider. Microscopic examination of reeled silk revealed it to contain mostly large ampullate fibers with minor amounts of minor radial fibers and attachment disc material.

Reeled silk collected from many spiders (30 mg) was soaked for one hour at 30°C in 5.0 ml of 0.1 M NH_4HCO_3 , pH 8.0, containing 0.5% Triton X-100. This first wash was decanted and saved for spectro-

photometric and electrophoretic analysis. The silk fibroin was then washed three more times in buffer without detergent. The washed silk was then digested with .8 mg of Argiope protease B in 6.0 ml of ammonium bicarbonate, pH 8.0, at 30°C for 18 hours. Soluble digestion products were separated from undigested silk by centrifugation, decanted and frozen while the undigested silk was washed three times with 5 ml of distilled water before being stored frozen.

Ninhydrin reactivity of the soluble digestion products was determined before and after alkaline hydrolysis (Hirs, 1967). Peptide mapping (Smith, 1969) and gel chromatography were used to separate the digestion products. In the first technique parallel samples (35 μ l) of the digest were separated by high voltage electrophoresis (electrophoretic methods). After the location of the separated peptides was established by staining of a guide strip, an unstained strip of paper containing the peptide was sewn to a second sheet of Whatman 3 mm which was then subjected to descending paper chromatography in butanol, acetic acid and water; 12:3:5. Alternatively, 3 ml of the digest was concentrated by lyophilization and applied to a 1.5 x 90 cm column of Sephadex G-15 (Pharmacia) equilibrated on .1 M NH_4HCO_3 , pH 8.0. The column was eluted at 5 ml/hr and peptides detected in the elutant fractions by ninhydrin reagent after alkaline hydrolysis (Hirs, 1967). The amino terminal acid, and amino terminal sequence of fractionated and unfractionated digestion products was determined by Edman degradation and dansylation techniques (see Insulin Digestion). The relative amino acid composition of the digested and undigested fibroin was established by acid hydrolysis followed by dansylation identification of the amino acids.

RESULTS

I. The Protease Activity of Unfractionated Digestive Fluid

Digestive Fluid Physical Characteristics

Digestive fluid pooled from freshly captured spiders was usually very cloudy and contained variable amounts of dark brown pigment as well as many particulates. Three different pooled samples of fluid had identical pH values of 7.82. The protein content of 12 different fluid samples was 34.9 ± 8.4 mg/ml while the average inorganic phosphate concentration was 45 mM.

Thermal Stability and pH Optima of General Protease Activity

Digestive fluid diluted to 0.5 mg/ml retained full protease activity (GPA) when incubated for 20 minutes at 5° to 40°C, with 90% of the activity evident after incubation at 50°C. Complete inactivation occurred in 30 minutes at 70°C. The pH optima of fluid GPA corresponded closely to the pH of the fluid itself at approximately pH 7.8 (Fig. 4).

Digestive Fluid Specific Protease Content

Table I presents a partial list of the specific protease content of the digestive fluid of A. aurantia. Elastolytic and "chymotryptic" activities were present while "tryptic", "carboxypeptidase" and "leucine aminopeptidase" activities were not detected.

Inhibition of General Protease Activity

Of the inhibitors studied, iodoacetate, lima bean trypsin inhibitor, phenylmethyl sulfonyl fluoride and EDTA, only the latter had any

Figure 4. pH optimum for general protease activity. One ml of 1.5 ml 0.2 M Borate-KCl-NaOH (■) or phosphate (●) buffer of the desired pH value. To this was added 0.5 ml (30 µg protein) diluted digestive fluid and the mixture incubated for 75 minutes at 37°C. The reaction was stopped with 3.5 ml 5% TCA, filtered and the supernatant $A_{280 \text{ nm}}$ was recorded. (Tillinghast and Kavanagh, 1977).

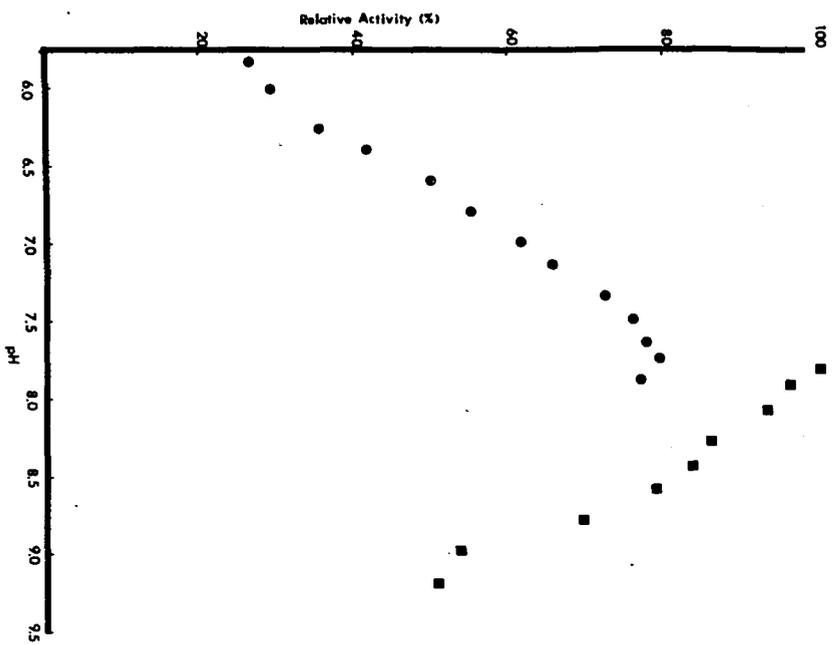


Table I

Specific Substrate Activity of A. aurantia Digestive Fluid

Protease Type	Substrate	Activity	Units	N. Assay
"Trypsin"	BAPA	None		3
"Trypsin"	TAME	None		3
"Chymotrypsin"	BTEE	15.6	Worthington Units/ mg protein	3
"Chymotrypsin"	ATEE	10.5	Worthington Units/mg protein	3
"Carboxypeptidase"	HPA	None		3
"Carboxypeptidase"	HA	None		3
"Leucine Aminopeptidase"	Leucine amide	None		
Elastase	Elastin-Orcein	30.2	mg Elastin-Orcein hydrolyzed/ mg protein 120 minutes	4
"Elastase"	NAA3ME	None		3

BAPA, Benzoyl-Arginine-Nitroanilide; TAME, Toluene Sulfolyl Arginine Methyl Ester; BTEE, Benzoyl-Tyrosine Ethylester; ATEE, Acetyl-Tyrosine Ethyl Ester; HPA, Hippuryl-L-Phenylalanine; HA, Hippuryl-L-Arginine; E-O, Elastin-Orcein; NAA3ME, N-Acetyl-L-Alanyl-L-Alanyl-L-Alanyl-Methyl Ester.

significant inhibitory effect on the digestion of casein by digestive fluid proteases. Inhibition by EDTA was proportional to the concentration of the inhibitor and could be maximized to 80% by exhaustive dialysis of the digestive fluid against 10 mM EDTA buffered at pH 7.8. No decrease in GPA activity was observed after a parallel dialysis of fluid in buffers alone. The inhibitory effect of EDTA showed a temperature dependence with little inhibition at 5°C (Fig. 5).

II. Web Structure

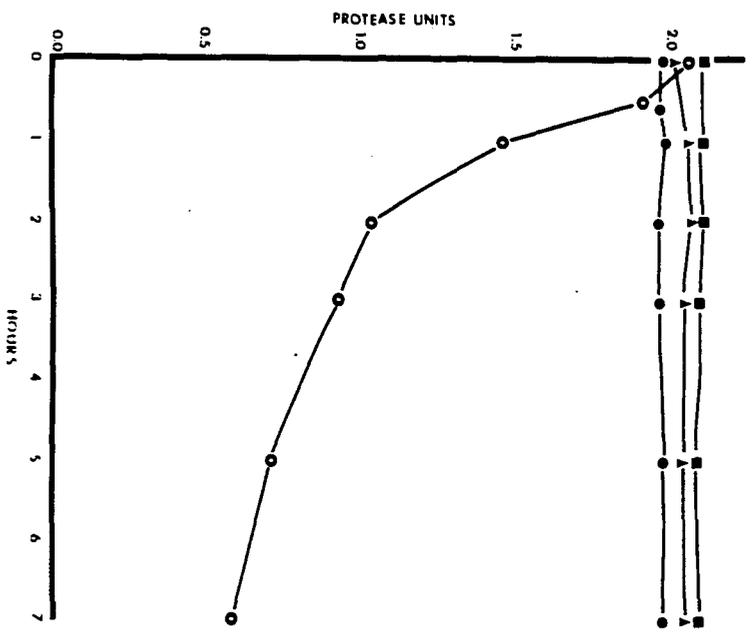
The webs of both Araneus trifolium and Argiope trifasciata reacted identically in all tests and the results for these two species are presented together.

Radii

In nearly all cases examined, orb web radial threads consisted of two pairs of fibers, a major pair and a minor pair, whose size varied with that of the spider. In A. trifolium the average width across both fibers of the major pair was 11.1 μm (8.0-14.0 μm) while the average width of the minor pair of fibers was 6.3 μm (4.8-8.2 μm), although in some individuals the size difference between the two pairs of fibers was not as large. While the two pairs of fibers often separated from each other, rarely did the two fibers of an individual pair separate.

Radial thread fibers could be differentiated by their dye affinities as well as size. The Mallory-Heidenhain trichrome stain, which proved most useful in differentiating web components (Table 2), stained the major fibers of the radius red and the minor fibers yellow to orange (Fig. 6). Although the latter reaction is indicative of elastic

Figure 5. The effect of temperature on protease inhibition by EDTA. Digestive fluid was diluted to 8 μg protein/ ml in 0.1 M Borate buffer, pH 7.8 with the following variations: buffer alone, 30°C (\blacktriangle); with 5 mM CaCl_2 , 30°C (\blacksquare), 25 mM EDTA, 30°C (\odot) or 25 mM EDTA, 5°C (\bullet). At various time intervals 0.1 ml aliquots were removed for protease assay. (Tillinghast and Kavanagh, 1977).



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Table 2

Staining Reactions and Protease Resistance of Orb Web Structures

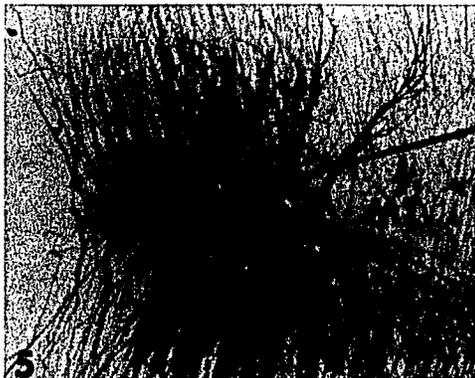
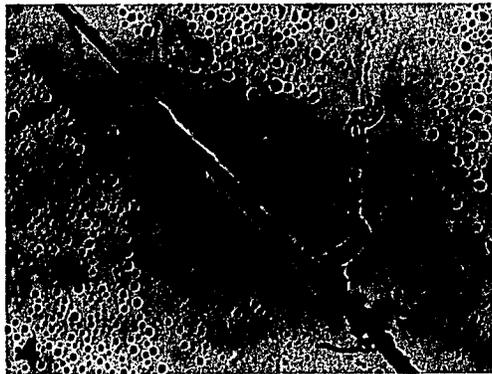
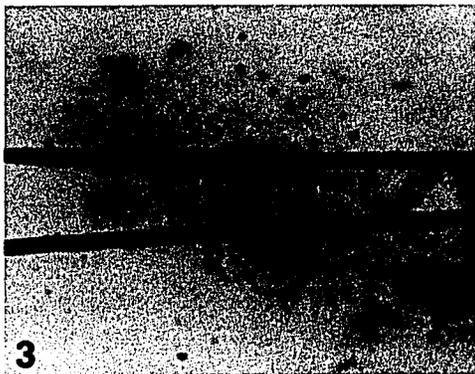
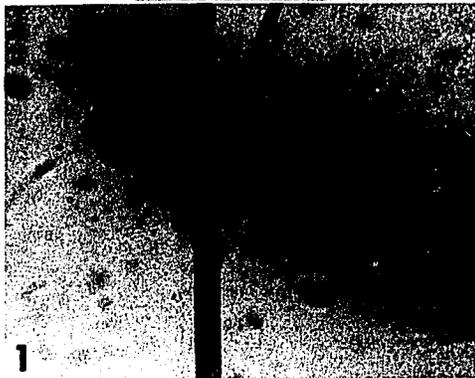
	Major radial fibers	Minor radial fibers	Sticky spiral core fibers	Sticky spiral covering	SS-R cement	HS-R cement
Stain						
Coomassie Blue pH 4.8	+/-	+/-	<u>dark blue</u> violet	<u>dark blue</u> violet	<u>dark blue</u> violet	+/-
Aldehyde Fuchsin	light purple	light purple	dark purple	lost	-	+/-
Mallory-Heidenhain's Trichrome	pink-red	yellow-orange	blue-purple	blue	<u>faint pink</u> purple	bright red
Methyl Green pH 4.8	light blue	dark blue	+/-	+/-	+/-	+/-
Mallory's Phosphotungstic Acid Hematoxylin	pink	blue	pink	pink	pink	pink
Periodic Acid Schiff	+/-	-	-	+/-	pink	-
<hr/>						
Protease Treatment						
Buffer Control	resistant	resistant	resistant	solubilized	resistant	resistant
Pancreatic Proteases (trypsin and elastase)	resistant	resistant	solubilized	solubilized	resistant	resistant
<u>A. aurantia</u> digestive fluid	solubilized	resistant	solubilized	solubilized	slowly solubilized	solubilized

tissue in classical histology (Cason, 1950) several stains known to have affinity for invertebrate elastic tissue such as ocrein, spirit blue and aldehyde fuchsin (Elder and Owen, 1967) failed to stain the minor fibers. Differential staining of the major and minor fibers was also obtained with Mallory's phosphotungstic acid hematoxylin (Fig. 6) and methyl green in 0.1 M sodium acetate pH 4.8 (Table 2). All of these stains demonstrated that major fibers of the radius were found throughout the web and the minor fiber pair was found only in radial threads. Mullen (1969) was able to differentiate the contents of the major and minor ampullate glands of Araneus sericatus by staining formalin-fixed sections with fast green and safranin O. In the present study neither of these stains proved effective in differentiating the radial thread fibers of unfixed orb webs.

The radial threads of the orb are resistant to digestion by pancreatic trypsin, elastase and clostridial collagenase as demonstrated by ^{14}C autoradiographs of enzymes-treated webs (Tillinghast and Kavanagh, 1977). The same technique showed complete dissolution of sections of radial thread treated with the digestive fluid of Argiope aurantia. However, microscopic examination of sections of plated web treated with digestive fluid revealed that whereas the major fibers were digested, the minor fibers remained intact (Fig. 6). Inasmuch as autoradiography was ineffective in demonstrating the digestion of the unlabeled minor fibers, we confirmed that the minor fibers were indeed resistant to the commercial enzymes by microscopic examination (Table 2).

Figure 6:

1. Radial thread fibers of A. trifolium stained with Mallory-Heidenhain trichrome. The pair of major fibers stains red while the pair of minor fibers stains yellow-orange. x 200.
2. Radial thread fibers of A. trifolium stained with Mallory's phosphotungstic acid hematoxylin. The pair of minor fibers stains blue while the pair of major fibers stains pink. x 200.
3. Radial (R) and sticky spiral (SS) threads of A. trifasciata stained with aldehyde fuchsin. x 200.
4. A A. trifolium radial thread entering a section of the web which has been treated with spider digestive fluid. At the edge of the treated area a HS-R junction is partially broken down (A). Farther in the digested fluid treated area the major fibers of the radius are dissolved (B) while the minor fibers are unaffected (C). x 100.
5. Attachment disc of A. trifolium with associated mooring thread (MT) stained with Mallory-Heidenhain trichrome. x 40.
6. HS-R junction stained with Mallory-Heidenhain trichrome. The non-sticky spiral (HS) and radial (R) threads are obscured by the dense cement (C) of the junction. x 200.



The Sticky Spiral

Unlike the fiber pairs of the radii, the two core fibers of the sticky spiral did not generally adhere to each other when stained. They individually range in width from 4.4 to 5.4 μm . These fibers also differ from the radial fibers in that they vary in width along their length and they also show numerous indentations and other flaws rare in the radial fibers.

The core fibers of the sticky spiral could be distinguished from the radial fibers since the former stained specifically with aldehyde fuchsin (Fig. 6). No other stain used showed as clear an affinity for these fibers, including hematoxylin which as Sekiguchi (1952) reported, stains the contents of the flagelliform glands. Microscopic examination of regions of web treated with pancreatic trypsin and elastase showed complete dissolution of the spiral core fibers with no effect upon adjacent radial fibers.

The viscid covering of the sticky spiral is quite soluble and was easily removed during staining procedures. However, Mallory-Heidenhain trichrome preserved the globular arrangement of this material on the core fibers (Fig. 8) and also stained it blue. Fischer and Brander (1960) reported inorganic phosphate to be one of the soluble constituents of the sticky spiral. We have confirmed this assumption by means of autoradiography (Fig. 7). After administration in their diet, radioactively labeled phosphate was seen almost exclusively in association with the viscid spiral and was easily removed by a filter disc wetted with buffer.

Sticky Spiral-Radial Junctions

The sticky spiral is connected to the radii at many points which

Figure 7.

1. Autoradiograph of an A. trifasciata web labeled with radioactive phosphate. Only the sticky spiral shows incorporation of the label, and this is easily removed by buffer (S).
2. Autoradiograph of an A. trifasciata was labeled with radioactive ^{35}S sulfate. Incorporation of the label is seen in radii, HS-R junctions, the sticky spiral and provisional spiral fragments (PSF). In those sections exposed to buffers, the labeling is removed. (Kavanagh and Tillinghast, 1979).

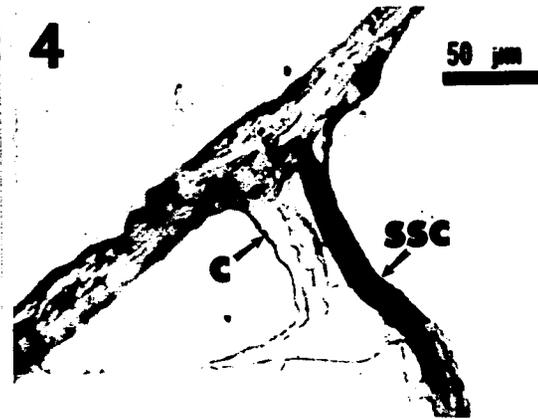
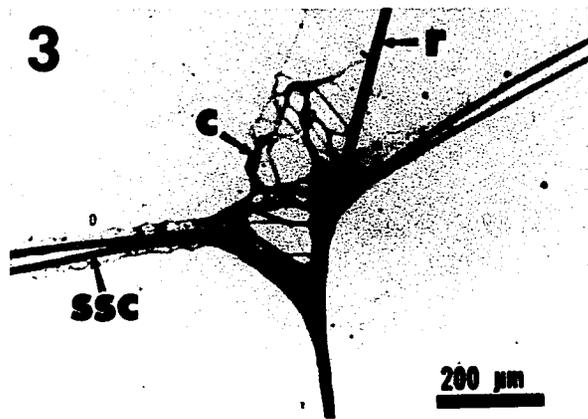
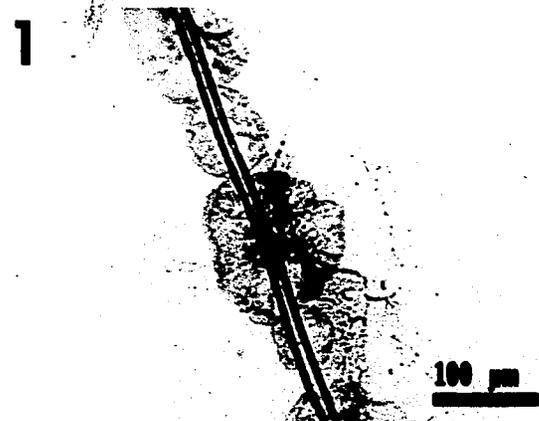


Figure 8.

1. Sticky spiral thread of A. trifasciata stained with Mallory-Heidenhain trichrome. x 100.

- 2 and 3. SS-R junctions of A. trifolium stained with Mallory-Heidenhain trichrome. The sticky spiral core fibers (SSC) run intact through the junctions and do not fuse with the radii (R). The cement which holds the sticky spiral to the radius may be coalesced or drawn into strands. B x 200, C x 100.

4. SS-R junction of A. trifasciata stained with aldehyde fuchsin. The sticky spiral core fibers (SSC) stain darkly while the junction cement (C) which covers the sticky spiral and radial threads is refractory to the stain. x 400. (Kavanagh and Tillinghast, 1979).



have been termed sticky spiral-radial junctions or SS-R's by Jackson (1971). Through the use of histological stains we have been able to observe the sticky spiral core fibers running intact through a number of SS-R's (Fig. 8), a fact not noted by Jackson (1971). In many cases, the spiral core fibers folded in a hairpin fashion on the radius or ran parallel along it for up to two or three millimeters. We have never observed a "melting" of the spiral core fibers around the radii. The latter arrangement would suggest these junctions are formed in part by the solidification of still fluid sticky spiral fiber around the radii (Jackson, 1971).

Treatment of SS-R's with trypsin, elastase or collagenase dissolved the spiral fibers but left the cementing material intact on the radius. Traces of it were even observed on the minor radial fibers after the major fibers had been dissolved by spider digestive fluid. Thus, there is a substance at the SS-R's distinct from the viscid covering of the sticky spiral which serves to bind sticky spiral core fibers to the radial fibers and which is not stained by aldehyde fuchsin (Fig. 8) nor is it susceptible to digestion by pancreatic proteases. In untreated webs this junctional cement was pliable and could be manipulated along the radius with a fine needle. After treatment with acidic staining solutions the cement generally adhered to glass and appeared as either a solid mass or as fine strands (Fig. 8). Eberhard (1976) has observed that in the webs of a number of orb weaving species, the junction of the sticky spiral and the radius is not a completely fixed structure. After a sticky spiral thread was stretched past a certain point the junction partially broke so that the sticky spiral core fibers were able to slide through their attachment to the radius.

Eberhard also observed that if the spiral was pulled further, it would break before the junction cement would slide along the radius, indicating the cement sticks more tightly to the radius than to the sticky spiral.

The cement did not stain strongly with any dye except Brilliant Blue (Table 2). Often the staining was obscured by the viscid sticky spiral covering which tended to accumulate on the junctions. The cement was characterized by protease resistance; it was not dissolved by pancreatic proteases and often only marginally susceptible to digestion by spider digestive fluid. (Fig. 6.4). Traces of cement could be observed on the minor radial fibers after the major fibers were digested away. In untreated webs the junction cement often appears to be composed of both fibrous and liquid elements (Eberhard, 1976). Neither staining nor protease treatment highlighted distinct fibrous elements in the SS-R junction cement.

Hub Spiral

Observations of the hub spiral showed it to consist of a single pair of fibers which were morphologically, and tinctorially identical to the major fibers of the radii.

Hub Spiral Radial Junctions

The hub spiral is attached to the radii at junctions (HS-R's) which differ in shape from SS-R's (Jackson, 1971). Although tension bearing radii run straight through the SS-R's, both the spiral and radial threads in HS-R's are displaced indicating that these junctions are either under greater stress or possibly less balanced than the SS-R junction. The cement holding the hub spiral to the radii is neither pliable

or fixed to glass by acidic stains. With staining, the hub spiral cement resembles the attachment discs which are adhesive zones that anchor mooring threads to the substratum (Fig. 6, 5-6). Both stained bright red when treated with Mallory-Heidenhain trichrome and neither stained by Brilliant Blue R at pH 4.8, a procedure which stained the SS-R cement darkly (Table 2). The hub spiral cement, like most components of the web, was resistant to pancreatic proteases but dissolved by the digestive fluid of A. aurantia.

Provisional Spiral

The remnants of the provisional spiral were readily found on the radii of intact webs; they were particularly evident in ^{35}S web autoradiographs (Fig. 7). It is not clear to us if the provisional spiral is simply broken during sticky spiral construction or partially ingested as suggested by Savory (1952). In any case, the provisional spiral appeared identical in all respects to the hub spiral; it consisted of a single pair of major radial fibers connected to the radii by the non-pliable cement found at HS-R junction.

Hub and Frame

Frame and mooring threads consisted of two to five pairs of fibers in parallel array which were identical in all their staining reactions to the major radial fibers. Occasionally, in Araneus, the bulk of the frame was increased by a number of sticky spiral core fibers which adhered to the frame threads. The cable-like frame and mooring threads were attached to each other by the dense inflexible cement of the HS-R junction. Lastly, the cement is found at less organized connections of threads at the hub or center of the web where three or

more fibers may be held together by a single accumulation of cement.

III. Separation and Purification of Digestive Fluid Protease

Agarose Electrophoresis of Digestive Fluid

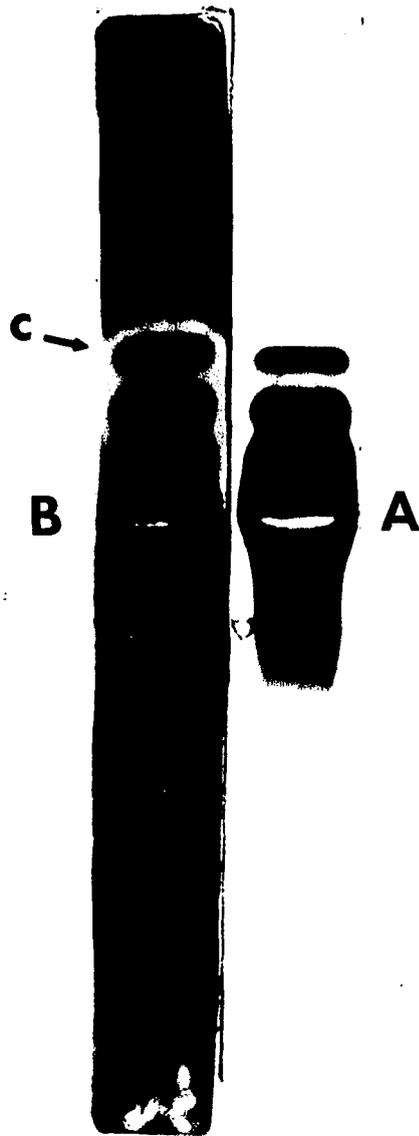
An agarose electrophoresis slide stained for protein (Fig. 9,A), after being run at pH 8.6, showed that spider digestive fluid contained several prominent cationic proteins. On the other hand, the dark brown pigment of the fluid coated the anionic side of the sample slits and distorted the separation of anionic proteins. Negatively staining "halos" around the cationic proteins on a slide impregnated with casein before staining indicated that several cationic proteins were protease active. (Fig. 9,B) whereas the anionic proteins were not.

Ion Exchange Chromatographic Separation of Protease Fractions

Based on the results of agarose electrophoresis a two stage purification scheme was used to separate the basic proteases of the digestive fluid: DEAE cellulose chromatography to remove the proteases from the heavy pigment and CM cellulose chromatography to separate the proteases from each other.

DEAE chromatography of digestive fluid reproducibly yielded protease peaks; one in the void volume of the column and a second upon washing of the column with .2 M NaCl containing buffer (Table 3). The first protease peak was free of pigment, dissolved web LAF (large ampullate fibroin/major radial fiber) and contained 60-80% of the protease units applied to the column. The second protease peak was eluted with some pigment, did not digest LAF and contained 3-4% of the protease units applied to the column.

Figure 9. Agarose electrophoresis of A. aurantia digestive fluid. Digestive fluid samples (20 μ l) were applied to slits (s) cut in agarose slides and were electrophoresed for 3 hours at 3 mA/slide using pH 8.6, veronal buffer as the electrolyte. Slide A was stained with Amido Black directly after electrophoresis. Slide B was incubated in a casein solution before staining. Clearing (C) of the impregnated casein around cathodal proteins indicated them to be proteases.



+

Table 3

Purification Table of Proteases from the Digestive Fluid of Argiope aurantia.

	Protease Units	Protease Units/mg Protein	Fold Purification	% recovery of pro- tease units from crude digestive fluid
crude digestive fluid	31500	180	-	-
DEAE Protease Peak I	21065	533	3.0	66.0
DEAE Protease Peak II	1390	135	-	4.3
Protease Fraction A	1782	265	1.5	5.6
Protease Fraction B	3010	733	4.0	9.6
Protease Fraction C	1350	891	5.0	4.2
Protease Fraction D	5297	1521	8.4	16.8

Hydroxyapatite Purified Protease B		715	4.0	
Hydroxyapatite Purified D		1782	9.9	

The first protease peak obtained from DEAE-cellulose chromatography was fractionated on a CM-cellulose column whose elution profile showed four major protease peaks (Fig. 10) designated as protease fractions A through D. The distribution of protease activity between the four fractions varied from preparation to preparation, however total yield of protease activity and the specific activity of the fractions remained approximately that shown in Table 3.

Protease Fraction Substrate Specificity

The four protease fractions isolated by CM-cellulose chromatography differed in their substrate specificities (Table 4). Only fractions A and B dissolved Araneus LAF. These also hydrolyzed several NCBZ-p-NPE ester derivatives which were not hydrolyzed by the proteases of fractions C and D. These NCBZ-p-NPE ester derivatives, especially that of alanine, have been used as elastase substrates (Geneste and Bender, 1969). Hydrolysis of these substrates does not reflect elastase activity with Argiope proteases, however, since fraction A and B were active against NCBZ-p-NPE esters but had little or no elastase activity, whereas fractions C and D, which do digest elastin, were inactive against these synthetic esters. Hydrolysis of NCBZ-p-NPE esters by Argiope proteases is affected by the assay buffer: their activity was reduced by 50% if 0.5 M sodium phosphate buffer were substituted for the Pipes buffer normally used in the assay of these substrates.

Metal Ions

Although they had different substrate specificities, the four Argiope protease fractions were similarly affected by a number of metal

Figure 10. Elution profile of protease activity of DEAE-filtered digestive fluid from a CM cellulose column. Bars indicate fractions which were pooled as partially purified protease fractions.

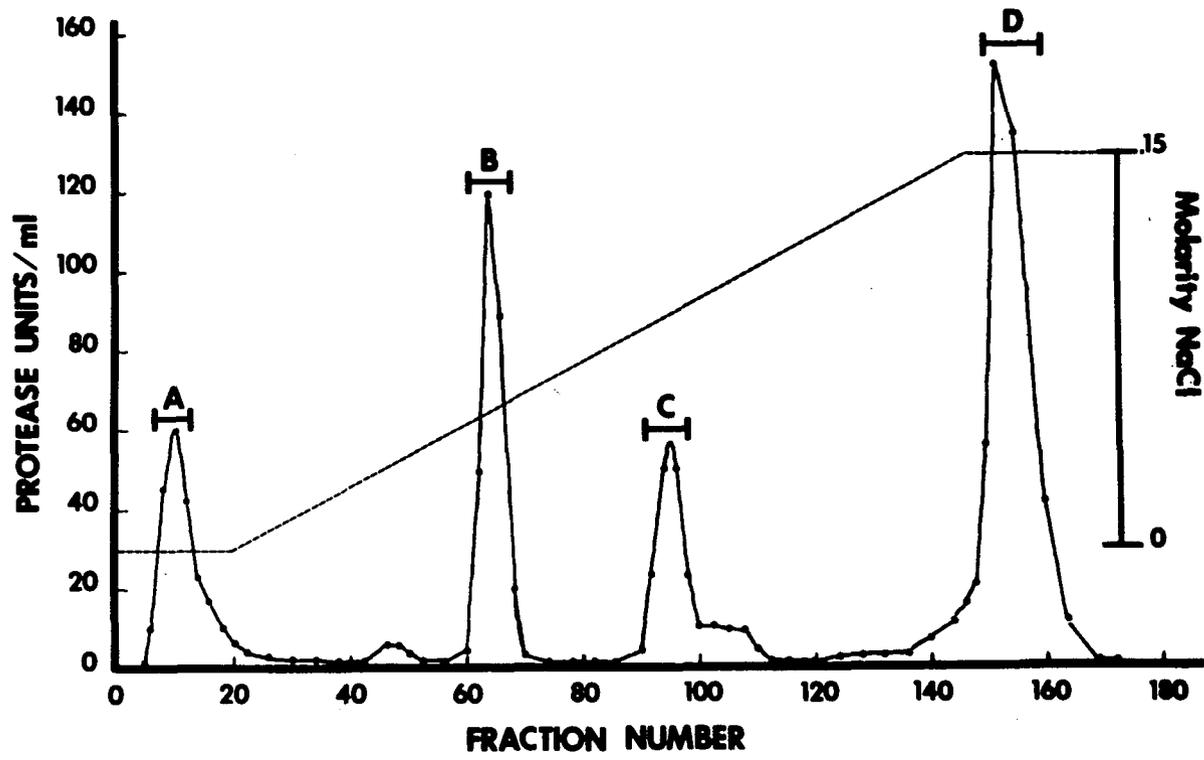


Table 4.

Substrate specificities of Argiope protease fractions. Esterase and elastolytic activity is expressed relative to general protease activity as μg substrate hydrolyzed/min/protease unit. (Fig. 3)

Substrate	A	B	C	D
LAF Digestion	+	+	-	-
NCBZ-Ala-p-NPE	.306	.545	.032	.027
NCBZ-Gly-p-NPE	.194	.086	0	0
NCBZ-Pro-p-NPE	.041	0	0	0
NCBZ-Val-p-NPE	0	0	0	0
Elastin Congo Red	0	44.0	58.1	122.0

ions (Table 5). Inhibitory metal ions in order of increasing effectiveness were Zn, Ni, Hg, Cd and Cu, with Zn showing only mild inhibition at a concentration of 0.1 mM. No metal ions appeared to potentiate protease activity significantly.

All four of the protease fractions were inhibited by the metal chelator EDTA to similar degrees (Table 6). Inhibition by EDTA was reversed in all fractions by several metal ions (Table 6) with only slight variation between the fractions in which ions are most effective in inhibition reversal. Zinc, lead and cobalt were most effective in restoring inhibited protease activity while manganese iron, copper and calcium gave lesser and more variable degrees of inhibition reversal.

Protease Purification by Hydroxyapatite Chromatography

Hydroxyapatite chromatography of protease fraction B and D from CM-cellulose fractionation yielded single protease peaks (Figs. 11, 12) in high yield (usually > 70% recovery). This procedure yields little or no increase in specific activity (Table 3) as both proteases contain only trace contaminants after CM-cellulose chromatography. Both native and SDS polyacrylamide electrophoresis show the hydroxyapatite chromatographed protease, now designated as Argiope protease B and Argiope protease D, to be purified to electrophoretic homogeneity (Fig. 13).

Protease fraction A eluted as two protease peaks, designated as A1 and A2, from a hydroxyapatite column (Fig. 14). These were recovered in low yield (< 30% of the units applied to the column). The first peak, A1, hydrolyzed LAF and NCBZ-p-NPE esters while the second peak, A2, did not. The protease of peak A2 differed from all other proteases studied by not conforming to the calibration curve of casein digestion (Fig. 3). The concentration of A2 could be increased in-

Table 5. The effect of metal ions on the GPA of Argiopo protease fractions. Activity is expressed as a percentage of controls incubated without metal ions.

Protease Fraction ion concentration	A		B		C		D	
	0.1 mM	1.0 mM	0.1 mM	1.0 M	0.1 mM	1.0 mM	0.1 mM	1.0 mM
AgNO ₃	94	*	98	*	104	*	99	*
CaCl ₂	100	98	101	108	102	100	104	102
CdCl ₂	91	55	102	67	78	23	94	46
CoCl ₂	89	88	102	98	92	86	100	99
CuCl ₂	40	34	19	14	19	16	35	27
FeCl ₂	96	90	99	94	99	104	100	95
HgCl ₂	91	54	100	64	78	51	97	72
MgCl ₂	100	100	100	104	101	99	99	103
MnCl ₂	100	100	101	105	100	97	100	92
NiCl ₂	91	69	92	78	100	83	94	76
PbNO ₃	96	90	101	98	97	97	100	92
SrNO ₃	98	90	97	102	97	96	100	100
ZnCl ₂	100	80	97	92	100	94	100	96

*Assay not performed since silver nitrate is not completely soluble at this concentration and pH.

Table 6. Reversal of EDTA inhibition of Argiophe protease fractions by metal ions. The protease fractions were incubated for 2 hours at 30°C in 0.025 M Tris-maleate, pH 6.4, and 10 mM EDTA. After removal of samples to estimate the extent of inhibition, the protease fractions were dialyzed overnight to remove unbound EDTA. Aliquots of the fractions were then incubated for 15 minutes at room temperature with various metal salts at concentration of 0.2 or 1.0 mM before being assayed in duplicate for general protease activity.

Table 6

	Protease Fraction			
	A	B	C	D
	% Inhibition			
10 mM EDTA	67	89	77	89
	% Reversal of Inhibition			
CaCl ₂				
0.2 mM	18	0	0	0
1.0 mM	87	0	0	0
CdCl ₂				
0.2 mM	8	0	0	14
1.0 mM	0	7	0	10
CoCl ₂				
0.2 mM	72	85	66	89
1.0 mM	63	76	61	88
CuCl ₂				
0.2 mM	0	0	8	0
1.0 mM	0	3	3	24
FeCl ₂				
0.2 mM	0	0	0	0
1.0 mM	21	7	15	24
HgCl ₂				
0.2 mM	0	0	0	0
1.0 mM	0	0	0	0
MgCl ₂				
0.2 mM	0	0	0	0
1.0 mM	0	0	0	0
MnCl ₂				
0.2 mM	56	24	13	41
1.0 mM	60	24	26	19
NiCl ₂				
0.2 mM	-	15	10	30
1.0 mM	11	9	14	32
PbNO ₃				
0.2 mM	75	93	63	81
1.0 mM	94	76	60	82
SrNO ₃				
0.2 mM	0	0	0	0
1.0 mM	0	0	0	0
ZnCl ₂				
0.2 mM	83	100	90	97
1.0 mM	100	93	80	87

Figure 11. Elution profile of Argiope protease B from a hydroxy-apatite column.

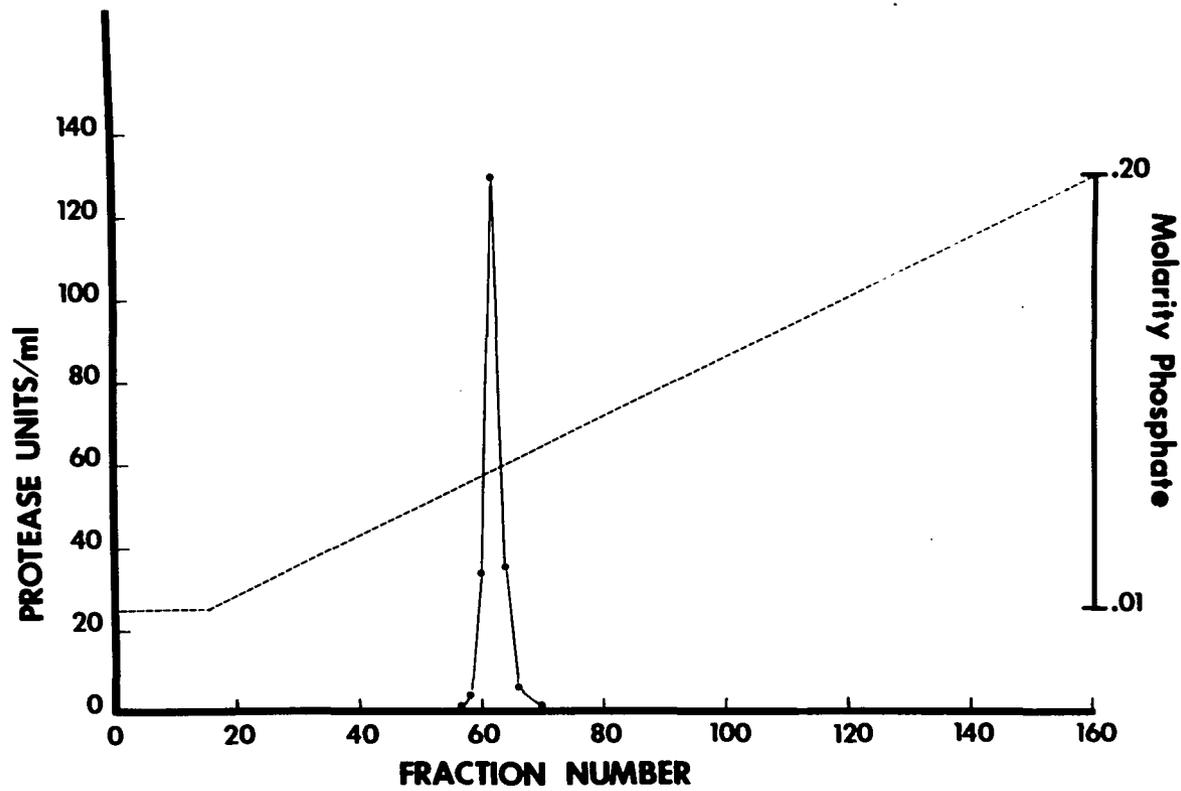


Figure 12. Elution profile of Argiope protease D from a hydroxy-apatite column.

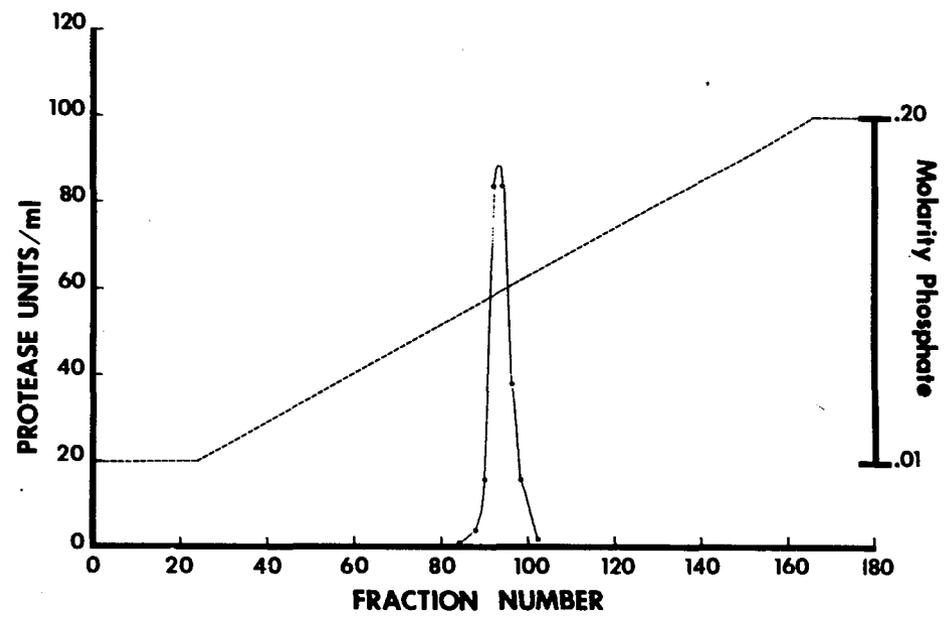


Figure 13. Photograph of polyacrylamide gels containing Argiope protease B and Argiope protease D. Protease B is shown in a pH 4.3 cationic gel (A) stained with Fast Green FSC and in an SDS 15% acrylamide gel (B) stained with Brilliant Blue R. Protease D is also shown in an SDS 15% acrylamide gel (C) stained with Brilliant Blue R.

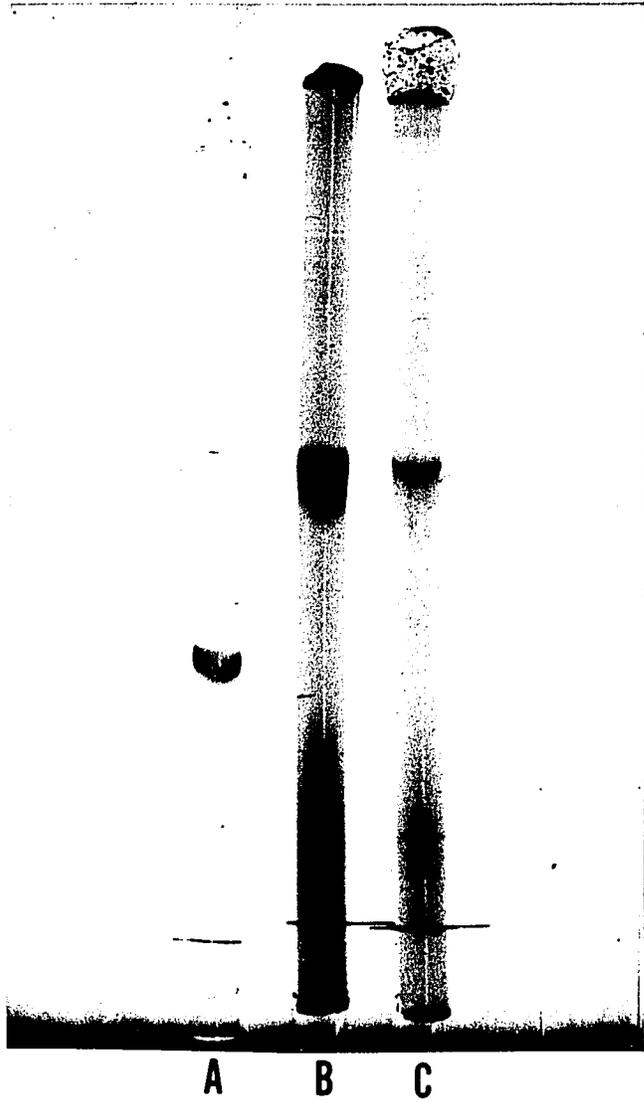
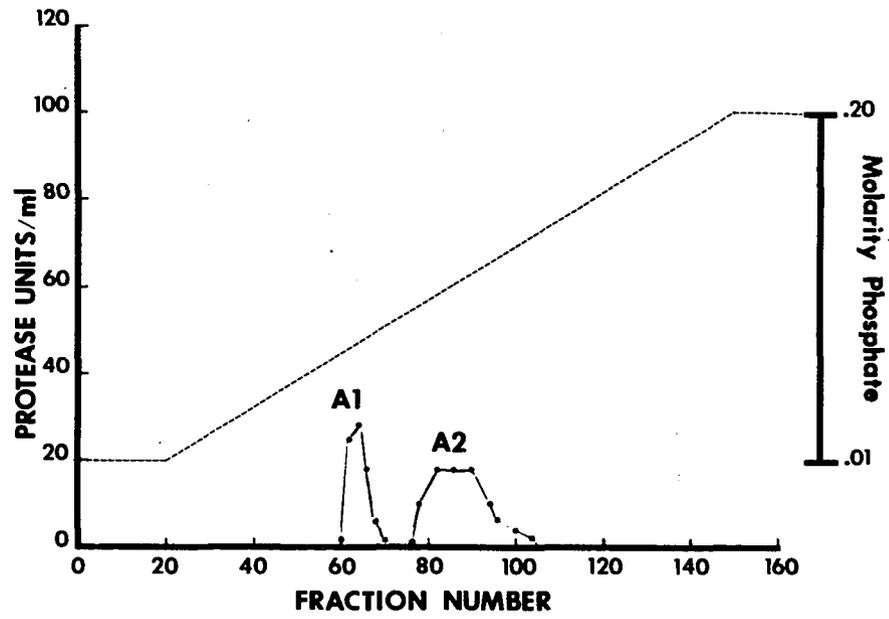


Figure 14. Elution profile of proteases from hydroxyapatite filtration of protease fraction A. The first protease peak, A1, was esterolytic and dissolved LAF. The second peak, A2, was not esterolytic or active against LAF and showed limited activity against casein.



definitely without increasing the release of TCA soluble leucine equivalents above 20 $\mu\text{g/ml}$ suggesting this protease has a limited action on the casein substrate.

IV. Characterization of Silk Fibroin Digesting Argiope Protease B Molecular Weight

Both Argiope protease B and Argiope protease D were subjected to SDS electrophoresis in 12% gels (Fig. 13) along with several separate standard proteins used as references. The molecular weights of proteases B and D were estimated to be 17,600 and 20,200 respectively from the standard curve (Fig. 15).

When protease B was chromatographed on a Sephadex G-100 column in a non-denaturing solvent it eluted significantly below myoglobin (Fig. 16) indicating a molecular weight of approximately 12,000. On the other hand, when protease B was chromatographed on a Sepharose 6B column in a denaturing solvent it eluted only slightly below myoglobin indicating a molecular weight of 16,400 (Fig. 17).

Thermal and pH Stability

Protease B, buffered at pH 8.2, was found to be stable at room temperature for up to 24 hours and showed only moderate loss in GPA at 37°C (Fig. 18). The protease could be stored for months at 5°C without loss in GPA. Thymol (.01%) or 5 mM sodium azide and 5 mM CaCl_2 were added as antimicrobials.

The protease was stable in alkaline and neutral buffer but was inactivated by incubation in acidic buffer (Fig. 19).

Figure 15. Standard curve of the log molecular weight of standard proteins (bovine serum albumin, BSA; ovalbumin, Ob; trypsin, Tr and myoglobin, MB) versus their migration relative to bromphenol blue, R_f , in SDS polyacrylamide gels. B+ and D+ indicate the R_f values obtained for protease B and protease D.

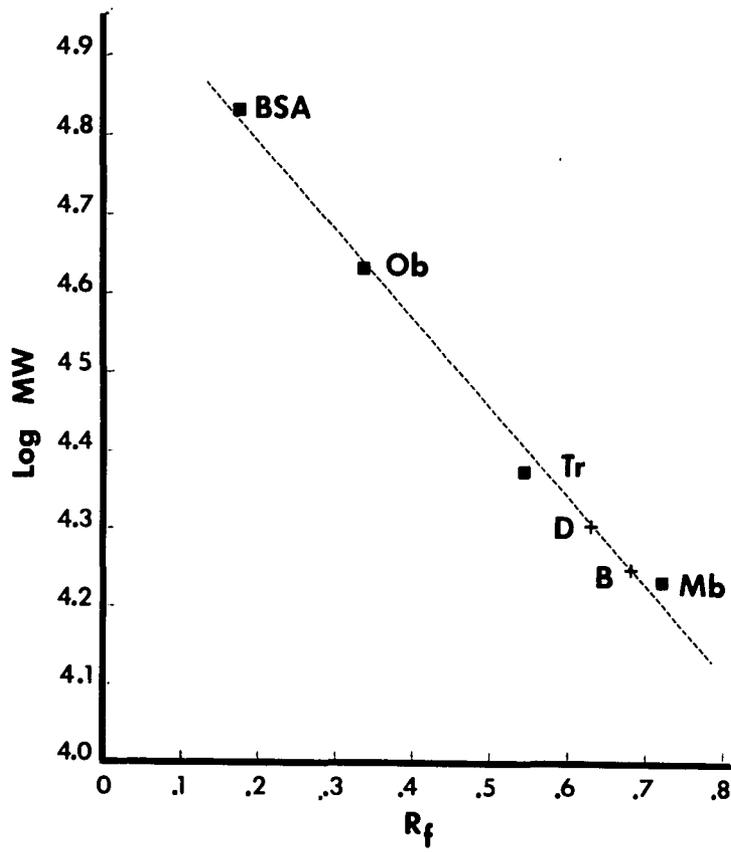


Figure 16. Standard curve of the log molecular weight of standard proteins (bovine serum albumin, BSA; ovalbumin, Ob; trypsin, Tr and myoglobin, Mb) versus their elution volume (fraction number) from a column of Sephadex G-100. B+ indicates the elution volume of protease B detected by its GPA.

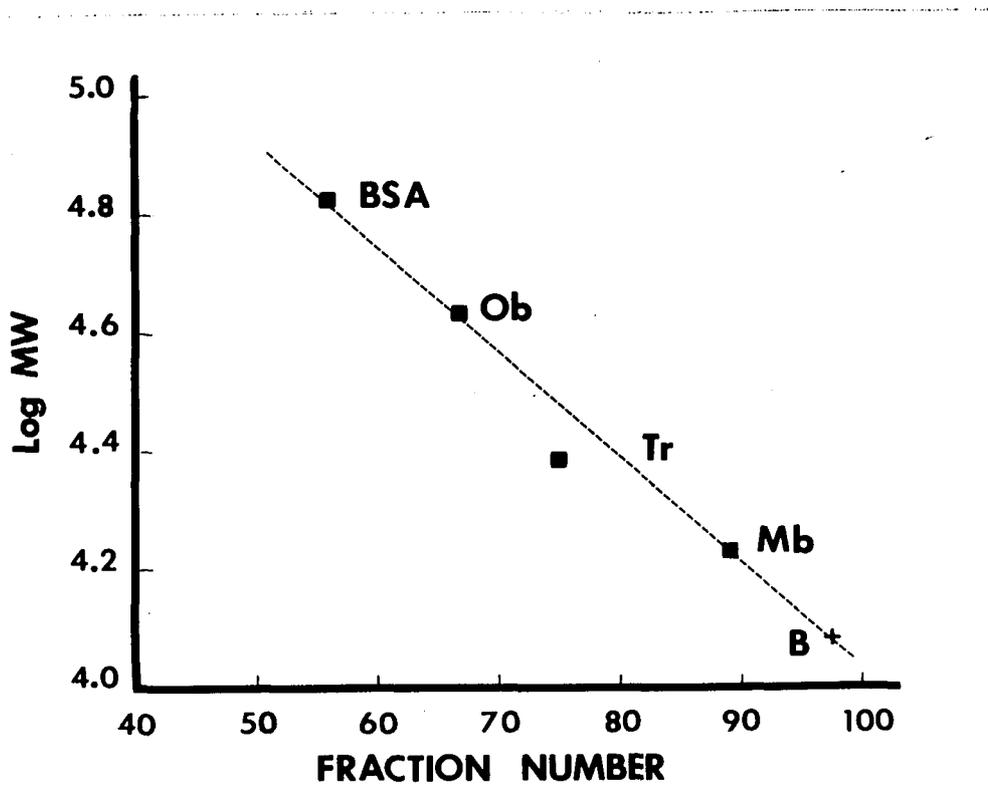


Figure 17. Standard curve of the log molecular weight of standard proteins (bovine serum albumin, BSA; ovalbumin, Ob and myoglobin, Mb) versus their elution volume (fraction number) in a denaturing solvent from a Sepharose 6B column. B+ indicated the elution volume determined for protease B.

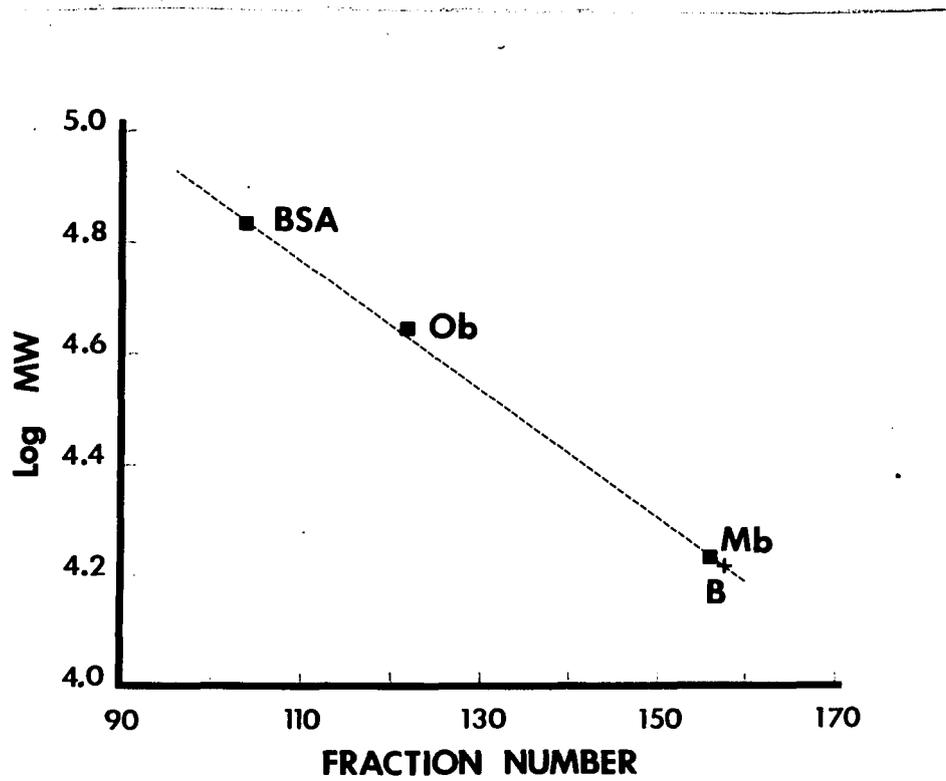


Figure 18. The thermal stability of Argiope protease B. Activity is expressed as a percentage of control maintained at 4°C.

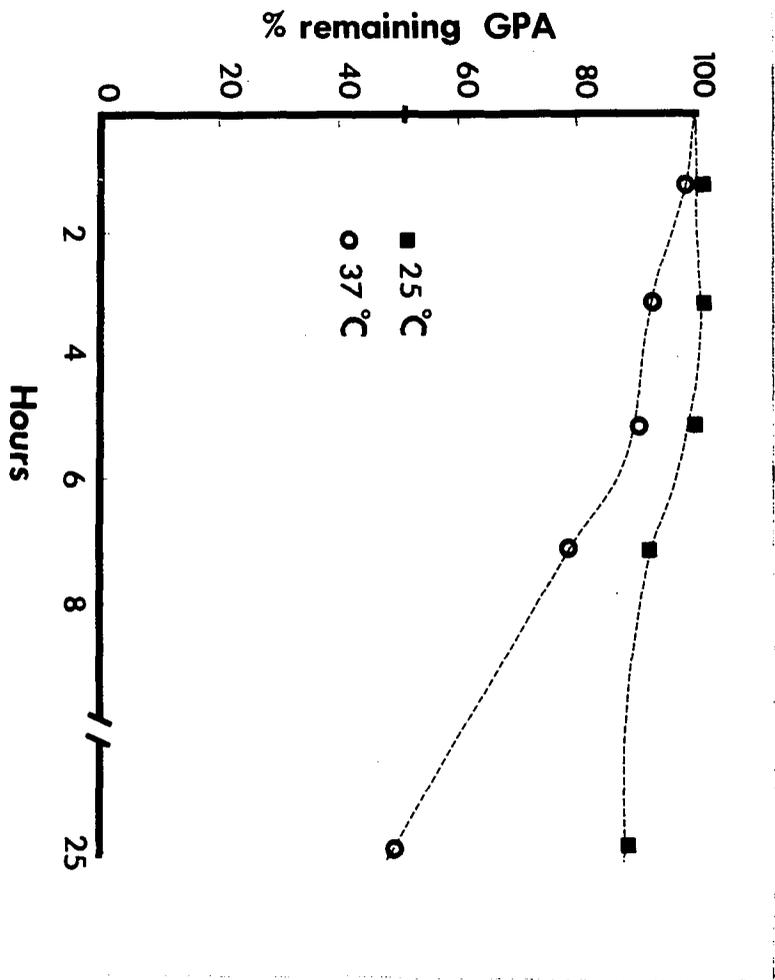
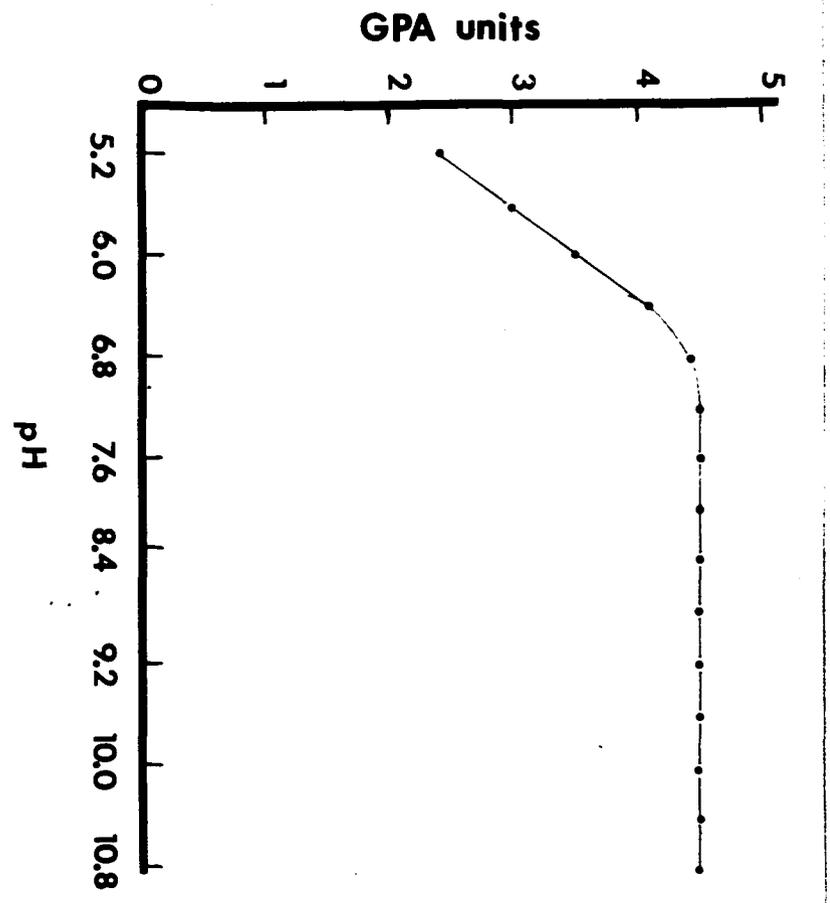


Figure 19. The stability of Argiope protease B versus pH.



Carbohydrate Content

Both staining and chemical assay procedures indicated a trace of carbohydrate content in protease B. Faint reactivity was seen with PAS and Alcian blue staining of polyacrylamide gels when more than 15 μg of enzyme was present. The phenol-sulfuric acid assay indicated the protease had a reducing sugar content of 8 $\mu\text{g}/\text{mg}$ protein or 0.8%.

Elemental Analysis

A 130 $\mu\text{g}/\text{ml}$ sample of protease B (1.2 $\mu\text{g}/\text{GPA}$ unit) was found to contain 0.25 $\mu\text{g}/\text{ml}$ of zinc ion when either zinc chloride or zinc acetate were used as standards. If a molecular weight of 17,000 is assumed for the protease then the molar ratio of zinc to protease was 0.5.

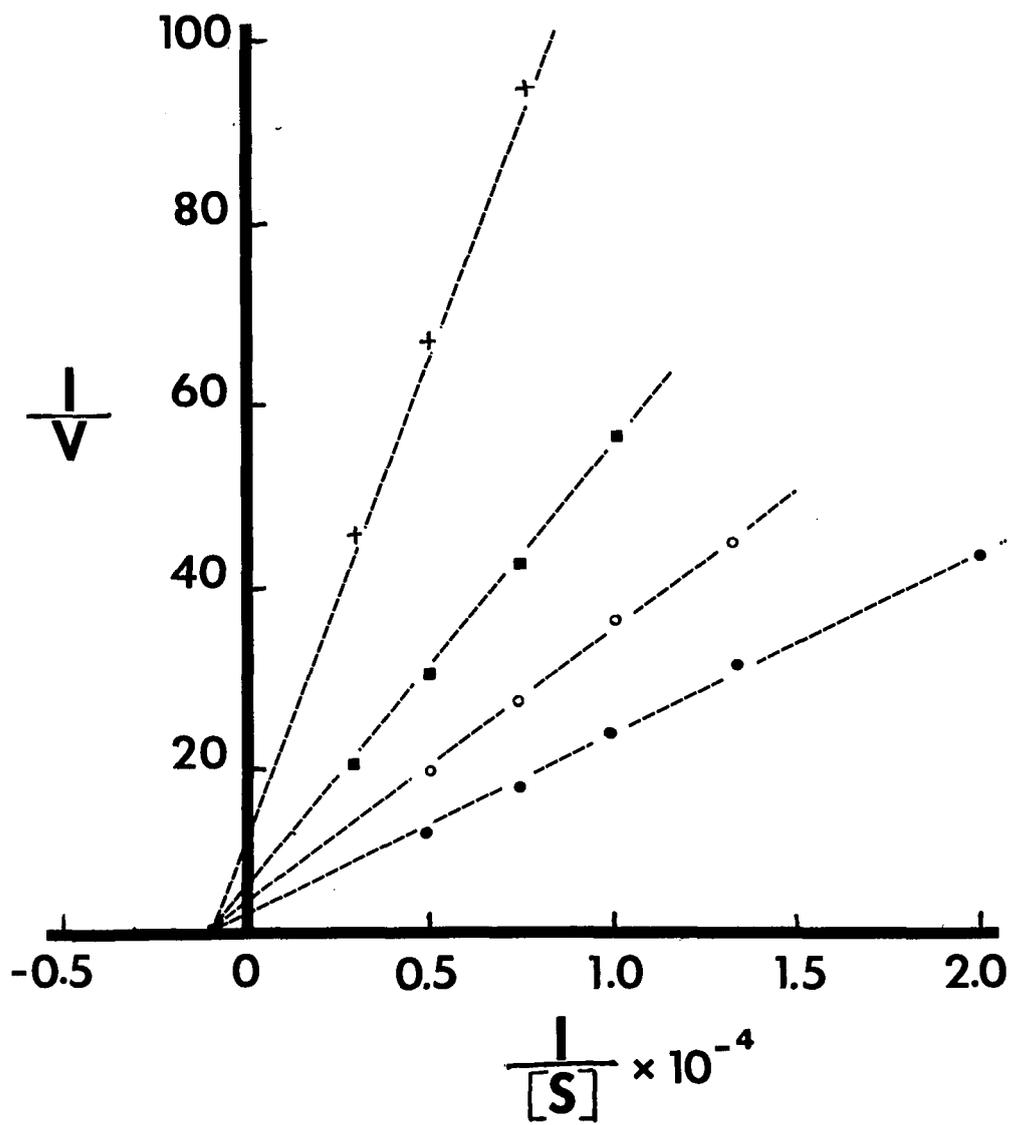
Copper Ion Inhibition

Purified protease B was used to make kinetic determinations with the assay of the substrate NCBZ-alanine-NBE as described in "Materials and Methods". A Lineweaver-Burke plot of substrate concentration dependence (Fig. 20) gave an apparent K_m of 1.0×10^{-3} M for this substrate at pH 6.5. Similar plots of the same concentration of enzyme incubated and assayed with various concentrations of CuCl_2 showed that copper did not effect the apparent K_m (Fig. 20).

Isoelectric Point Estimation

Purified proteases B and D were focused on wide range ampholyte polyacrylamide gels for periods of 9 or 12 hours. After, eluants of gel slices indicated that stable pH gradients had formed (Fig. 21). The gels for both proteases had a single protein band upon staining (Fig. 22). However, these bands were curved toward the cathodal end

Figure 20. The effect of Cu^{++} ion on the esterolytic of Argiope protease B. The protease was assayed against NCBZ-ala-NPE at substrate concentration from 3.0×10^{-5} M to 2.0×10^{-4} and at the rate of the reaction ($\Delta A_{347}/\text{min}$) determined. Assays were performed without CuCl_2 (), with 1.0 μM CuCl_2 (), 1.5 μM CuCl_2 () and 2.0 μM CuCl_2 ().



of the gels indicating they had not reached their isoelectric position and were still migrating cathodally. The protease were not detected in gels which had been focused for 12 hours under the same conditions and had most likely migrated off the cathodal ends of the gels.

Attempts were made to extend the basic end of the polyacrylamide gel pH gradient by the addition of basic narrow range ampholytes. These gels either failed to polymerize or liquified during focusing. Both ampholyte manufacturers, Brinkman and Bio-Rad Laboratories, were contacted with regard to this problem. The technical staff of Brinkman saw no reason for this problem while the staff of Bio-Rad Laboratories communicated their experience that the utility of polyacrylamide gels for basic pH electrofocusing is limited due to liquification of acrylamide at high pH. Electrofocusing in thin layer granular supports, such as "Pevicon" were recommended. However, the apparatus for thin layer electrofocusing was not available.

Electrofocusing in agarose fortified with linear polyacrylamide polymer proved capable of resolving protein incorporated in gels into discrete bands. However, the pH gradients which formed were not stable and pI measurements were not possible.

Iodacetate and Metal Chelator Inhibitor

Purified protease B was not inhibited by 5 mM iodacetic acid at either pH 6.4 or 8.4, nor did iodoacetic acid inhibit the reversal of EDTA inhibition by zinc ion at pH 7.2. The stability of the inhibitor was verified by its ability to inhibit the sulfhydryl enzyme papain.

Various metal chelators inhibited the GPA of protease B (Fig. 23). O-phenanthroline was the most potent inhibitor and showed a hyperbolic

Figure 21. Polyacrylamide isoelectric focusing pH gradient. Gels which had been focused for 9 hours were sliced into 0.5 cm sections and eluted overnight in 2 ml of 10 mM NaCl. Arrows indicate the location of protease B (B) and protease D (D) in the gels after 9 hours of focusing.

Figure 22. Polyacrylamide isoelectric focusing gels. Argiope protease B (B) and Argiope protease D (D) focused as single bands at the cathodal ends of the gels (marked by steel wire). The faint band behind protease B (a) was an artifact found in gels run without sample protein.

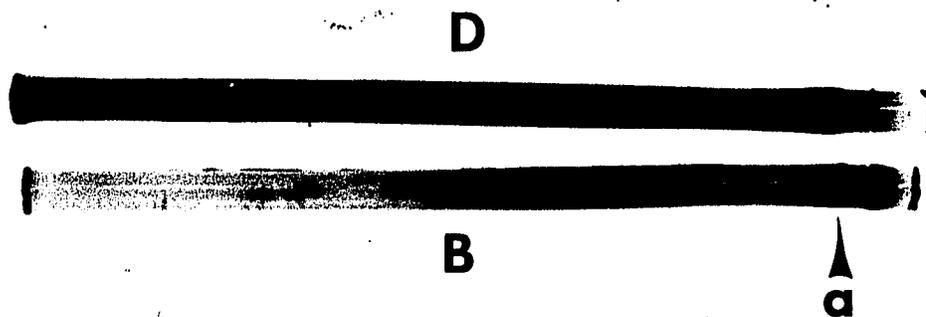
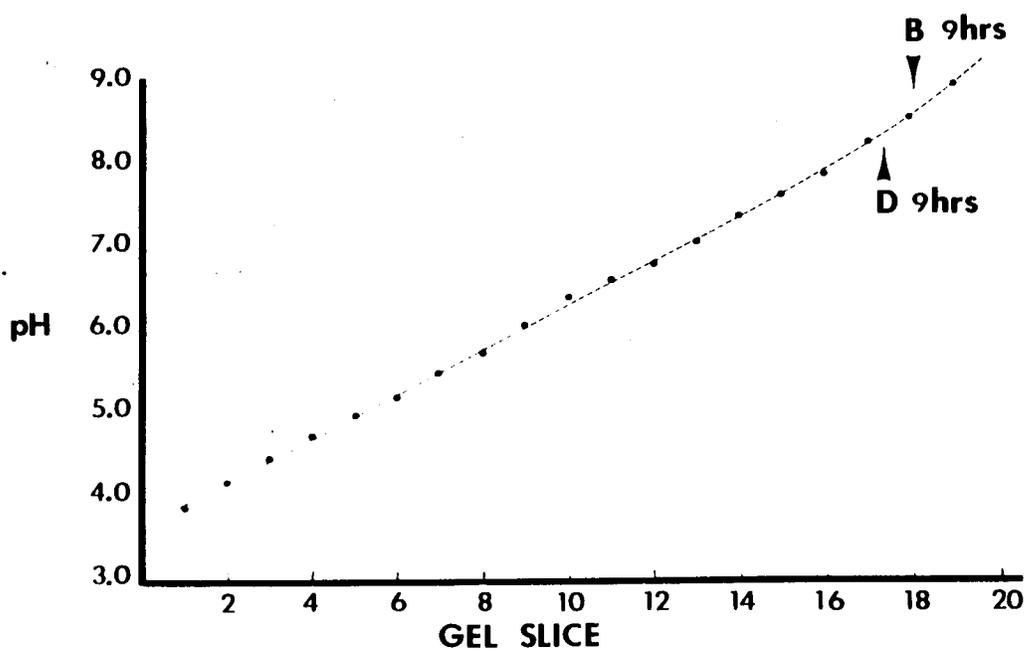
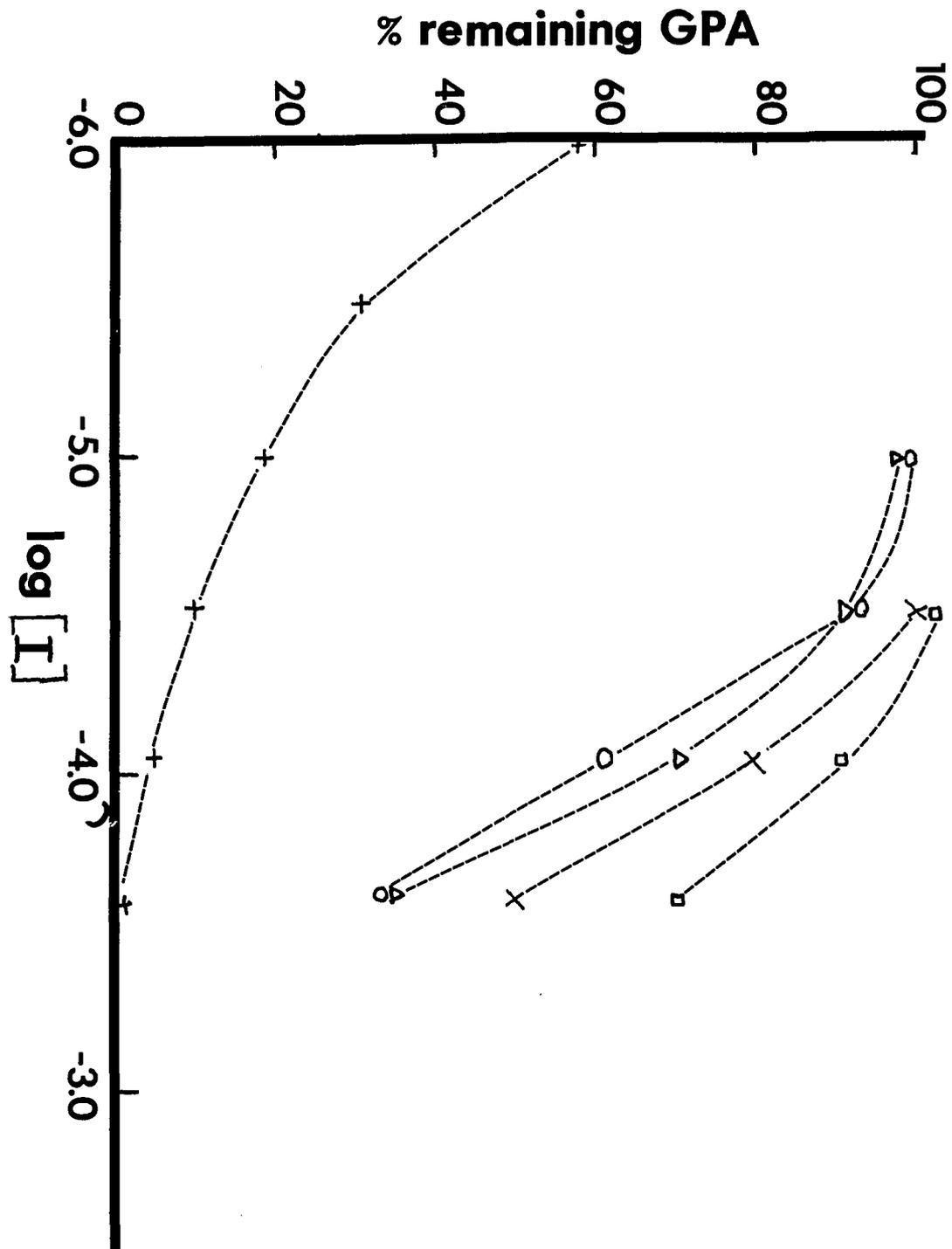


Figure 23. The effect of various metal chelators, o-phenanthroline (+), EDTA (Δ), KCN (x), cysteine (o) and 2-mercaptoethanol (), on the activity of protease B. Protease activity is expressed as a percent of a control incubated without chelator.



concentration versus inhibition curve. Other less potent inhibitors which showed parabolic inhibition curves were cysteine, EDTA, cyanide and mercaptoethanol.

Cleavage of the B Chain of Insulin

Separately incubated controls of protease B and insulin B chain showed neither was susceptible to hydrolysis during the incubation period. Gel chromatography of insulin B chain digested by protease B on G-25 Sephadex showed the protease was intact in the void volume. There was no remaining intact insulin and a large unresolved peak of digestion products was obtained. High voltage electrophoresis of the digestion products reveal four major digestion fractions; two major and two minor (Fig. 24). Dansylation of the eluted fractions showed them to consist of two or three peptides. The major cleavage sites in the insulin B chain was determined by the amino terminal sequence and amino acid composition of these separated peptides (Fig. 25).

V. Silk Fibroin Digestion by Argiope Protease B

Removal and Analysis of Soluble Fibroin Components

We have observed in autoradiographic studies of orb webs that even non-sticky fibers are covered with soluble components. The reeled dragline used to study fibroin digestion was therefore washed with non-ionic detergent (Triton X-100) before being exposed to protease B. Detergent soaks of reeled silk fibroin characteristically turned light green within one hour of incubation. The absorption spectrum of the soluble components released showed a broad absorption between 200 and 450 nm with peaks at 245, 290 and 325 nm (Fig. 26). The peak at 245 nm was lost when the soak was frozen and thawed probably due to the loss

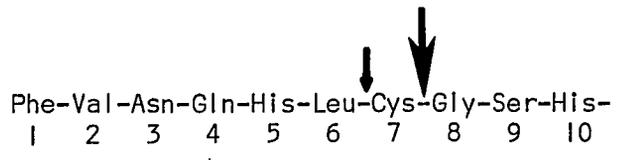
Figure 24. High voltage paper electrophoresis of protease B digested insulin B chain. Separated peptides were made visible by staining with cadmium acetate ninhydrin (Blackburn, 1968).

8).



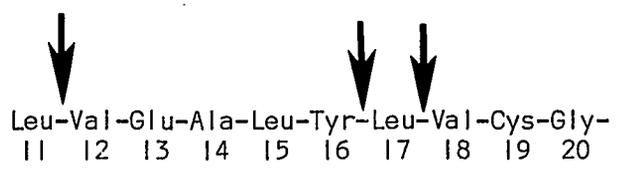
Figure 25. Cleavage specificity of protease B on the B chain of bovine insulin. Large arrows mark major sites and small arrows mark minor sites.

Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-
1 2 3 4 5 6 7 8 9 10



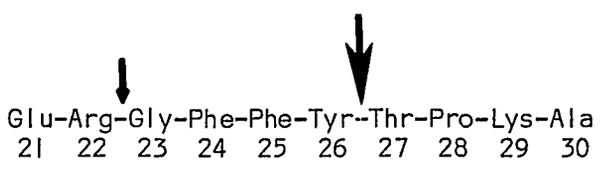
A horizontal sequence of ten amino acids: Phe, Val, Asn, Gln, His, Leu, Cys, Gly, Ser, His. Below each amino acid is a number from 1 to 10. Three arrows point downwards to the amino acids at positions 6, 7, and 8.

Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-
11 12 13 14 15 16 17 18 19 20



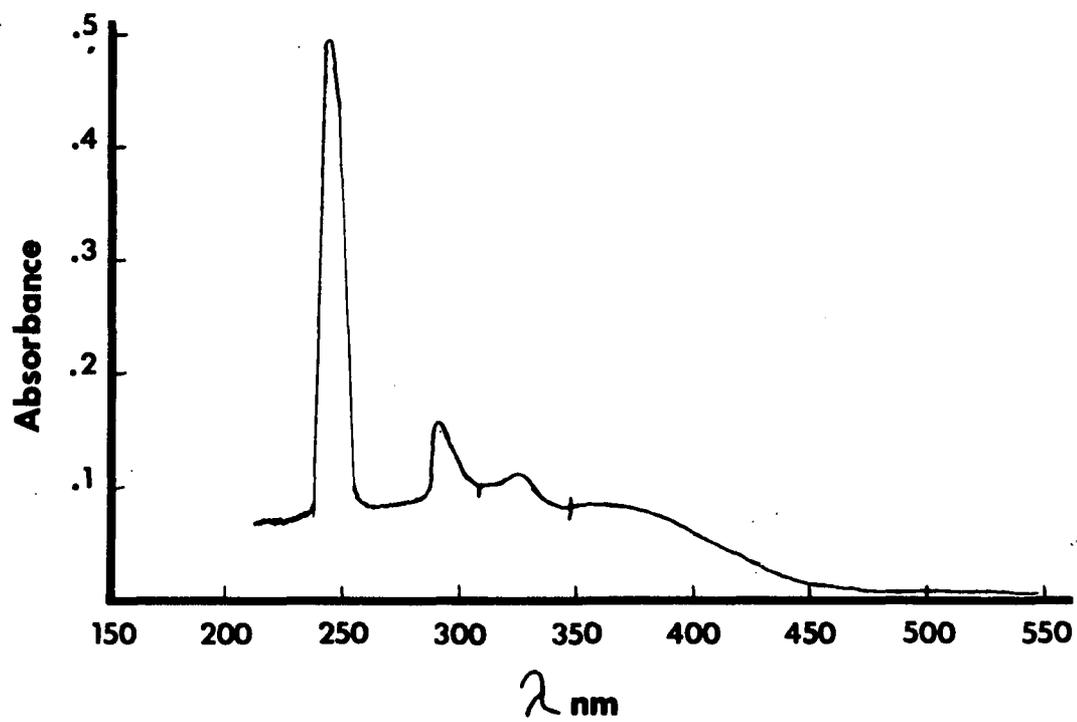
A horizontal sequence of ten amino acids: Leu, Val, Glu, Ala, Leu, Tyr, Leu, Val, Cys, Gly. Below each amino acid is a number from 11 to 20. Three arrows point downwards to the amino acids at positions 12, 16, and 18.

Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala
21 22 23 24 25 26 27 28 29 30



A horizontal sequence of ten amino acids: Glu, Arg, Gly, Phe, Phe, Tyr, Thr, Pro, Lys, Ala. Below each amino acid is a number from 21 to 30. Two arrows point downwards to the amino acids at positions 22 and 26.

Figure 26. Absorption spectrum of the Triton X-100 solubilized covering of reeled A. aurantia fibroin.



of a precipitable component. Analysis of the material removed from the fibroin by Triton X-100 wash by SDS electrophoresis in 7.5% gels showed two bands stainable by Fast Green (Fig. 27). These bands were also lightly stained by Alcian Blue carbohydrate staining.

Analysis of Solubilized Fibroin Digestion Products

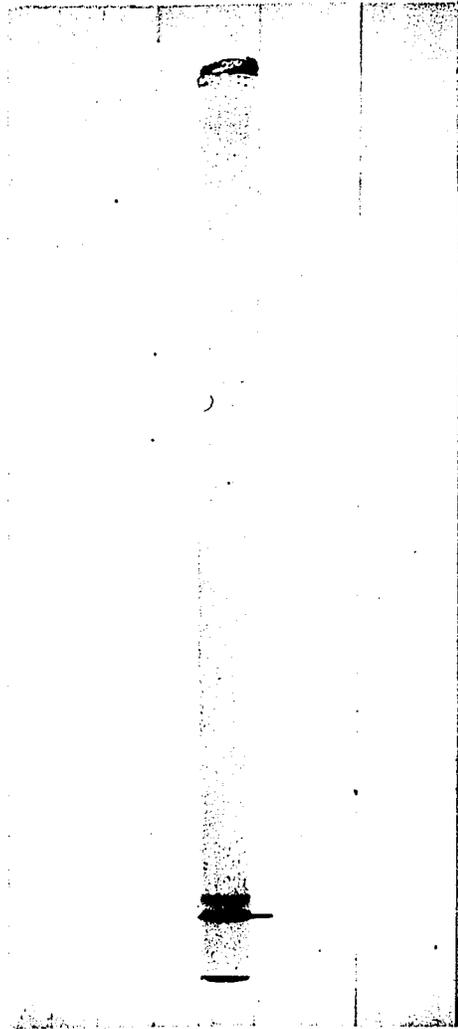
Analysis of separately incubated controls of washed fibroin and protease B showed no release of extraneous protein or ninhydrin-reactive substances. A 30 mg mass of washed fibroin incubated with protease B became unraveled and "fluffy" in appearance although a considerable amount of fiber remained undigested at the end of the incubation period with no loss in the GPA activity of the protease. The undigested fibroin was removed from the protease and the solubilized digestion products by centrifugation and was exposed to a fresh enzyme preparation for 12 hours before being washed in preparation for amino acid analysis.

Separation of the first fibroin digestion 5,8 and 15% SDS gels revealed no protein other than the protease. Both the first and second digests contained ninhydrin reactive substances; 4.4 total mg of leucine equivalents in the first digest and 0.45 total mg in the second digest. The first digest was reassayed for leucine equivalents after alkaline hydrolysis (Hirs, 1967) and was found to contain 27 mg.

Separation of Digestion Products

Cadmium acetate ninhydrin staining of high voltage paper electrophoretograms of the digest mixture revealed a smear of unresolved spots. This smear was not satisfactorily resolved by descending paper chromatography in a second dimension using butanol, acetic acid and water although a minimum of twelve spots were observed. The bulk of the

Figure 27. / Photograph of an SDS polyacrylamide gel (7.5%) containing surfactant-solubilized fibroin coverings stained with Fast Green FSC. Two bands are visible; one which migrated with the tracking dye (marked with a stainless steel wire) and second just above the first.



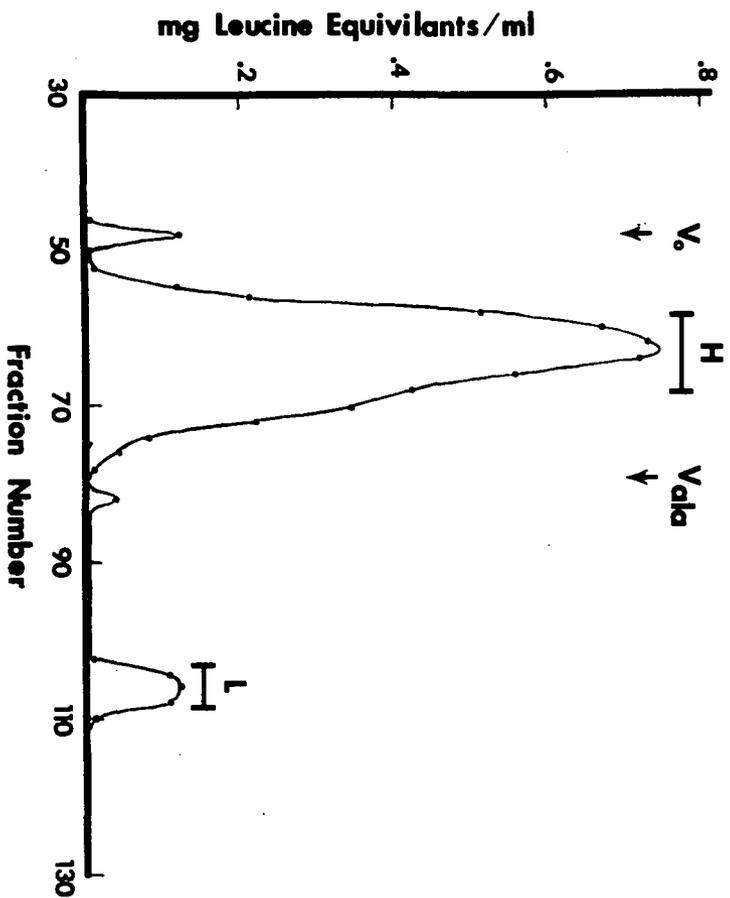
initial fibroin digest was concentrated by lyophilization and chromatographed on a column of G-15 Sephadex. The elution profile of the digest showed three peaks (Fig. 28); one in the void volume corresponding to active protease B, a major peak of digestion products and a minor peak of digestion products which eluted beyond the elution volume of alanine. The two digestion product peaks were collected and designated as fraction H and fraction L (Fig. 28).

Amino Acid Composition of the Digestion Products

Unfractionated fibroin digestion products and the washed undigested fibroin were hydrolyzed for 12 hours and their constituent amino acids were identified by dansylation. The separation pattern of dansyl-amino acids on polyamide plates were identical for both samples with glycine as the major spot with lesser but major amounts of alanine proline and cysteic acid, as well as detectable amounts of phenylalanine, valine, leucine, tryosine, aspartic acid, glutamic acid, histidine, lysine and arginine. The amino acid composition of the two digest fractions separated by gel filtration was similarly determined by hydrolysis and dansylation. The composition of the H fraction closely resembled that of the unfractionated digest while the L fraction contained only proline, glycine and alanine.

Edman degradation and dansylation was used to determine the amino terminal amino acid sequence of the digest fractions. The H fraction contained peptides with alanine and glycine as the N terminal amino acids with proline, alanine and glycine as the N-1 amino acids. Digest fraction L contained the single tripeptide gly-pro-tyr.

Figure 28. Elution profile of fibroin digestion products from a column of Sephadex G-15. Arrows mark the previously determined void volume (V_0) and elution volume of alanine (V_{ala}). Solid bars indicate those elutant fractions which were combined as digest fractions H and L for further study.



DISCUSSION

1. The Protease Activity of Unfractionated Digestive Fluid

In the field A. aurantia paralyzes its prey with venom, swathes it in silk and injects digestive fluid to dissolve it. This process may take several hours in the heat of summer. It is therefore not surprising that the protease activity of the digestive fluid is stable for hours at 37°C. The protease activity appears to be fully potentiated in that it is buffered at its optimum pH, 7.8 and zymogens are not evident. Thus, fluid incubation with or without Ca⁺⁺ at 37°C, repeated freezing and thawing, detergents or the addition of exogenous trypsin, failed to increase the protease activity of the fluid.

Proteolytic enzymes of invertebrate origin have not been examined as extensively as those from vertebrate and microbial sources. Invertebrate tryptic proteases (Neurath, et.al., 1976) and collagenases (Eisen and Jeffrey, 1969; Phillips and Dresden, 1973; Boulard and Garrone, 1978) have received attention but there is little information on invertebrate metalloproteases or those which digest fibrous proteins other than collagen. Keratin digesting Tineola bisseliela larvae contain a number of proteases including several of which are metal chelator sensitive as well as several seryl proteases (Ward, 1975). Silkworm imagoes escape from their cocoons with the aid of a trypsin-like serine protease which digests the nonfibrous components of the cocoon (Kafatos and Williams, 1964). They also possess a midgut protease capable of digesting chemically solubilized fibroin (Eguchi and Iwamoto, 1975). No "tryptic" and slight "chymotryptic" activity were observed in the digestive fluid of A. aurantia (Table I). These observations were made on the activity of

the digestive fluid against several synthetic ester substrates. The latter were designed to differentiate the activity of vertebrate pancreatic proteases. Several plant and invertebrate proteases which do not cleave peptide chains with the same specificity as trypsin and chymotrypsin also cleave these substrates (Zwilling, et.al., 1972). The lack of inhibition of digestive fluid protease activity by the serine enzyme inhibitor phenylmethylsulfonyl fluoride argues against the presence of a true chymotrypsin in the fluid. Similarly, the elastin digesting proteases of the digestive fluid also differ from pancreatic elastases by not being inhibited by seryl enzyme inhibitors and by not hydrolyzing the substrate NAAME (Berth and Meyer, 1973).

Mommsen (1978) has recently observed a very active "carboxypeptidase A" enzyme in the digestive fluid of the spider Tegenaria atrica. We did not detect any hydrolysis of the carboxypeptidase A substrate hippuryl-L-phenylalanine by A. aurantia digestive fluid which had been frozen and thawed. Mommsen also observed that the digestive fluid of T. atrica, contains only alkaline protease activity against peptide substrates. This agrees with the work presented here. The acidic protease activity earlier observed by Pickford (1942) in tarantula body homogenates was likely due to lysosomal rather than digestive fluid protease.

Marked inhibition of digestive fluid protease activity by EDTA suggested that most of the protease present were metalloproteases. That tightly bound metal(s) were necessary for protease activity was indicated by the stability of the protease activity after dialysis and that elevated temperatures were needed for EDTA inhibition (Fig. 5).

It is probable that the digestive fluid analyzed in this study

was contaminated with venom when it was collected. Proteases have been reported in spider venoms (Kaiser and Raab, 1967; Mebs, 1970). However, no proteases have been detected in venom samples which had been carefully collected so as to avoid digestive fluid contamination (McCrone, 1969; Perret, 1977). It is therefore unlikely that the proteases examined in this study were secreted from venom glands and that previous observations of venom proteases are actually descriptions of digestive fluid proteases.

II. Web Structure

On the basis of the present study, we believe that there are three fiber types in the orb web of Araneus trifolium and Argiope trifasciata. For convenience, we shall refer to them as the major, minor and sticky spiral core (s.s.c.) fibers.

The major fibers, which stain red with Mallory-Heidenhain trichrome were resistant to the commercial proteases employed here but are susceptible to digestion by spider proteases. They are found in the radii (major radial fibers) and in the webs of the two species examined they are usually the only components of the provisional spiral, hub spiral, frame threads and mooring threads. Since the large ampullate glands are the largest of the spider silk glands, they have long been regarded as the principle source of orb web fibroin. The widespread use of the major fiber is consistent with so large a reservoir. The provisional spiral, previously thought to be derived from the minor ampullate glands (Andersen, 1970; Koor, 1977) appears instead to be derived from the major ampullate gland.

The minor fibers (minor radial fibers) of the radii of A. trifasciata and A. trifolium webs are similar to or smaller in size

than the major fibers, stain blue with Mallory's phosphotungstic acid hematoxylin and were resistant to all proteases tested. Several workers have made reference to small or thin elements in the webs of spiders. DeWilde (1943) was able to separate "thin elements" from the trailing line, radii and frame threads of Araneus diadematus. More recently, Work (1976, 1977) has separated "minor ampullate fibers" from the dragline and mooring threads of A. diadematus. On the other hand, Comstock (1948) reported that a spider's dragline usually consists of two pairs of fibers; no difference in the size of the latter was noted by Comstock. Denny (1976) has reported that frame, radial and mooring threads of Araneus sericatus consist of strands of identical diameter.

The minor fibers observed in A. trifolium and A. trifasciata were restricted to the radial thread. They were never observed in the frame and mooring threads. The numerous attachment discs left by spiders on the glass walls of the laboratory cages were observed to secure only major fiber pairs as identified by staining.

The role of the minor fibers in A. trifasciata and A. trifolium radii is unclear. Two possible functions are: reinforcement of the thread and location of prey ensnared in the web. Orb web radii are laid down in a single round trip by the spider from the hub to a frame thread and back. Since the line laid down by the spider on the way to the frame thread is retrieved on its return to the hub (Witt, et.al., 1968), the inclusion of a second pair of fibers in the permanent radius put down on the return trip, might serve to reinforce the thread. Alternatively, when an insect strikes an orb web the spider will often pluck one or more radii before rushing to the ensnared prey. It is

possible the minor fibers have physical properties different from the major fibers which are especially suited for transmitting information to the spider's very sophisticated vibrational senses.

The glandular source of the minor fibers is also unclear. DeWilde (1943) and Kaston (1964) have suggested that thin components of the web and attachment discs are a product of the pyriform glands. Andersen (1970) has noted the amino acid composition of the pyriform glands is more characteristic of a glue than a fibroin even though it closely resembles that of spider mite webbing which is fibroin (Table 7). Work (1976, 1977) maintains the minor fibers which are found in Araneus diadematus and which exit from spigots on the median spinnerets are a product of the small ampullate glands. In any case, the minor fibers we observed in this study, and the attachment discs, are probably derived from separate glands since the two materials differ in their staining properties (Table 1).

The third fiber type in the orb web, the s.s.c. fiber, differs from the major and minor fibers in its possession of numerous indentations, intense staining with aldehyde fuchsin, and susceptibility to digestion by a wide variety of proteases. The source of this fiber is controversial. Peakall (1968, 1969) has suggested it is a product of the large ampullate glands. Alternatively, Sekiguchi (1952) has provided evidence that the flagelliform gland is the source of the s.s.c. fibers. Studies of the physical properties of frame and sticky spiral threads indicate separate glands are involved in their construction. Although there is disagreement as to whether the s.s.c. fibers are weaker (DeWilde, 1943) or not (Denny, 1976), it is generally agreed they are much more elastic than frame fibers. The s.s.c. fibers also

Table 7

Amino Acid Composition of Various Silks
residues per 100 total residues

	<u>Araneus diadematus</u> (a)					<u>Bombyx mori</u> (b)	<u>Tetranychus cinnabarinus</u> (c)
	Large ampul- late	Small ampul- late	Flagel- liform	Piri- form	Aggre- gate		
Asparatic acid	1.04	1.91	2.68	10.52	9.18	1.3	10.4
Threonine	0.91	1.35	2.48	4.44	7.58	.9	4.5
Serine	7.41	5.08	3.08	14.84	6.80	12.1	12.7
Glutamic acid	11.49	1.59	2.89	10.38	9.75	1.0	13.3
Proline	15.77	trace	20.54	7.78	10.81	.3	5.9
Glycine	37.24	42.77	44.16	7.76	14.48	44.5	13.8
Alanine	17.60	36.75	8.29	9.87	6.19	29.3	9.8
Valine	1.15	1.73	6.68	5.35	5.75	2.2	4.6
Cystine	--	--	--	--	--	.2	.4
Methionine	--	--	--	--	--	.1	.6
Isoleucine	0.63	0.67	1.01	3.71	4.71	.7	3.4
Leucine	1.27	0.96	1.40	5.39	5.50	.5	5.7
Tyrosine	3.92	4.71	2.56	2.20	2.22	5.2	2.7
Phenylalanine	0.45	0.41	1.08	2.29	3.83	.6	3.5
Lysine	0.54	0.39	1.35	9.01	7.43	.3	4.4
Histidine	trace	trace	0.68	2.83	2.38	.2	1.4
Arginine	0.57	1.69	1.13	3.63	3.40	.5	2.9

(a) Andersen, 1970; (b) Lucas, et.al., 1958; (c) Hazan, et.al., 1974.

differ from major fibers in their dye affinities and susceptibility to protease digestion (Tillinghast and Kavanagh, 1977).

The controversy over the origin of the s.s.c. fibers results from the difficulty in locating the flagelliform glands, which are easily mistaken for ampullate glands (Kovoor, 1977). Peakall (1964) was not able to locate them in A. diadematus, while Mullen (1968) seems to have mistaken them for a second pair of minor ampullate glands in A. sericatus. A suspicion that the large ampullate glands are the source of the s.s.c. fibers was based in part on the work of Koenig (1951) who experimentally removed radii from the web just prior to spiral thread construction. He noted that spiders continued to replace excised radii only a finite number of times before proceeding to build a reduced sticky spiral in spite of missing radii. Presumably, some portion of the contents of the large ampullate gland was conserved for sticky spiral construction.

Junctions and the Sticky Spiral

In this report we have provided photographic evidence (Fig. 8) that the core fibers of the sticky spiral pass intact through intersections with radial threads. These core fibers are fastened to the radii by a uniquely pliable cementing substance rather than by a fusion of the former to the latter. This cement is not merely an accumulation of the viscid covering of the sticky spiral thread since it differs markedly from that substance in its resistance to dissolution by buffer and pancreatic enzymes (Table I). As a result of the pliability of this substance, the sticky spiral radial junction is a moveable structure which buffers the web against breakage during the capture of prey (Eberhard, 1976). Staining and protease treatment

have failed to demonstrate fibrous and liquid elements in this cement. This cement is the only web component which reacts detectably with PAS staining indicating that its unique properties may be due to the presence of carbohydrates.

At all other intersections, threads are fastened together by another dense inflexible cement. This cement differs from that of the SS-R junction in its staining reactions (Table I) and morphology. It is likely that it is the same material used to form attachment discs.

The viscid covering of the sticky spiral stains differently from the two cements described above (Table I). Apart from some free amino acids, the amides of taurine and gammaaminobutyric acid have been reported (Fischer and Brander, 1960) as well as pyrrolidone (Schildknecht, et al., 1972) and inorganic phosphate (reported by both groups of researchers). The relationship of these materials to the staining properties and adhesive qualities of the viscid spiral is unclear. Although the aggregate glands are thought to secrete some of this substance (Peakall, 1964), the origin of other materials present is obscure (Andersen, 1970).

It is apparent that there are water soluble substances on the web other than that covering the sticky spiral. Autoradiographs of webs spun by spiders fed radioactive sulfur show a highlighting of the HS-R junction (Fig. 7) which is easily removed by soaking in buffer. Such a highlighting of the HS-R junction is not observed in autoradiographs of webs spun by spiders fed radioactive phosphate although both isotopes are incorporated into the sticky spiral covering. The widespread distribution of sulfur shown by autoradiography suggests that

low molecular weight compounds are not confined to the sticky spiral covering. The origin of these compounds is unknown. The silk glands of several species of spiders possess different regions containing cells filled with secretion granules distinct in their content of sulfhydryls, reducing groups and glycoproteins (Kovoor, 1977).

Whether any one gland is capable of secreting more than one type of material remains an unanswered question.

General Comments

Although histological stains have proved useful in differentiating the components of the orb web, it is difficult to assign specific characteristics to the various fibers and nonfibrous substances. For example, the reaction of the minor fibers with Mallory-Heidenhain trichrome and the s.s.c. fibers with aldehyde fuchsin are both indicative of elastic tissue. Such "elastic stains" of classical histology have been recently shown to be nonspecific and capable of staining forms of collagen as well (Puchtler, et.al., 1976). Also, invertebrate "elastic tissue" differs in affinity for dyes used successfully for vertebrates (Elder and Owen, 1967). It is of interest to note that of all the web components, minor fibers stain with methyl green and phosphotungstic acid hematoxylin which are cationic dyes and with orange G (trichrome component) which is an anionic dye; this indicates both strong anionic and cationic component in this fiber. If, as suggested by Work (1976, 1977) the minor fibers are a product of the small ampullate glands, then such results seem incompatible with largely nonpolar and uncharged amino acid contents of these glands (Table 8).

The resistance of the two types of radial fibers to digestion by proteases is likely a function of their molecular structure. X-ray

diffraction analysis of the frame silk of Araneus diadematus shows it to be a partially crystalline substance (Warwicker, 1960; Lucas, 1964) and it has been suggested that its molecular configuration is similar to that of the silk of Bombyx mori. Amino acid sequence analysis of the latter has revealed long stretches of short side chain amino acids packed into highly ordered β -pleated sheet crystallites alternating with bulky side chain amino acid, non-crystalline regions (Lucas, 1964). The subunit structure of silk fibroin is unclear. Sprague (1975) solubilized the contents of B. mori silk glands and determined that its unextruded fibroin was composed of equal amounts of two similar polypeptide chain whose molecular weight was estimated by SDS electrophoresis at 350,000 daltons. Numerous analyses of the amino acid content of insect and arachnid silks (Lucas and Rudall, 1968); Andersen, 1970; Hazan, et al., 1974) have failed to detect the presence of modified amino acids such as desmosine, or hydroxylysine which may serve as subunit crosslinks. Insect fibroins have been shown to contain trace amounts of carbohydrates (Sinochara, et al., 1971; Sinochara, 1977) which may play a structural role in the molecule. It is likely that the major and minor radial fibers share some common structural element which renders them equally refractory to pancreatic and bacterial proteases. Yet, sufficient structural differences must exist between the two radial fiber types to account for their difference in susceptibility to dissolution by the digestive proteases of Argiope aurantia.

In conclusion, at least six separate secretions, three of which are fibrous, are required for the construction of the orb web. The large ampullate glands secrete large fibers which make up the bulk of the web while the core of the sticky spiral thread is composed of a

pair of fibers derived from the flagelliform glands. A third fiber of unknown origin is found only in the radial threads. Three non-fibrous secretions are found on the threads of the web: a viscid water soluble coating of the sticky spiral, a pliable cement found only at the junctions of the sticky spiral, and a nonpliable cement found at all other thread intersections.

III. Separation and Purification of Digestive Fluid Proteases Metalloproteases

In a preliminary experiment (Fig. 9), agarose electrophoresis demonstrated the presence of several cationic, casein-hydrolyzing proteases in the digestive fluid of A. aurantia and allowed for the design of a purification protocol. Mommsen (1978) has since separated the digestive enzymes of T. atrica by polyacrylamide gel electrophoresis and observed only one casein digesting protease. It is possible that Mommsen did not detect other proteases inasmuch as most proteases in A. aurantia migrate cationically at pH 8.6 and are inactivated at pH 4.3 by cationic polyacrylamide electrophoresis.

DEAE-cellulose ion exchange chromatography of A. aurantia digestive fluid separated protease activity into two peaks (Table 3). The larger of the two peaks was further studied because it contained LAF (large ampullate fibroin/major radial fiber) digesting protease activity. Chromatography of the major DEAE-cellulose peak on CM-cellulose resolved four protease fractions; A, B, C and D. Two of the fractions A and B, digested LAF while fraction D was especially active against elastin fibers.

Although these protease fractions differed in their substrate specificities (Table 4) they were similarly affected by metal ions.

Each was significantly inhibited by EDTA in a reversible manner with Zn, Co and Pb ions being the most effective metals in reversing chelator inhibition. Metal ions can reverse chelator inhibition of enzymes by either replacing an ion which had been extracted from the enzyme by a chelator or by removal of a chelator bound to an enzyme metal complex by mass action (Vallee and Wacker, 1970). The latter mode was probably not the case with A. aurantia proteases since the order of effectiveness of inhibition reversal by metal ions (Table 6) did not resemble the order of their stability constants in EDTA binding (Table 8). Zinc dependent metalloenzymes, such as observed in A. aurantia, are widespread throughout nature (Vallee, 1976). Cobalt has a similar ionic radius to zinc and can replace zinc in many metalloenzymes (Riorden, 1977). Lead does not have a similar ionic radius to zinc but has been clearly shown to be incorporated into and to restore the activity of the zinc enzyme, alkaline phosphatase, after chelator inhibition (Sabbioni, et al., 1976).

Other metal ions, especially Cu^{++} , Cd^{++} , Hg^{++} and Ni^{++} inhibited the protease activity of all fractions. Spiders have high levels of copper in their hemolymph (Stewart and Martin, 1970). Inhibition of their digestive proteases may explain our observation that in crowded groups in the laboratory or in the field, A. aurantia individuals may kill and swath its neighbor but does not consume the carcass.

Protease fractions B and D contained only trace contaminants after CM-cellulose chromatography and these proteases, fibroin-digesting Argiope protease B and the elastase, Argiope protease D, were purified to homogeneity by hydroxyapatite chromatography (Figs. 11, 12). Fraction A eluted in the void volume of the CM-cellulose column and was a

Table 8. Stability constants ($\log K_1$) of 1:1 complexes of metals with EDTA. (Sillen and Martell, 1964).

<u>Metal</u>	<u>Log K_1</u>
Mg ²⁺	9.12
Ca ²⁺	11.0
Mn ²⁺	13.48
Fe ²⁺	14.3
Fe ³⁺	24.33
Co ²⁺	16.1
Ni ²⁺	17.5
Cu ²⁺	18.7
Zn ²⁺	16.4
Cd ²⁺	16.6
Hg ²⁺	22.1

complex mixture. Hydroxyapatite chromatography of this fraction resolved two unpure protease activity peaks. One peak, A1, was esterolytic and active against LAF but was recovered in small amounts. The second peak, A2, was not esterolytic or LAF active and was capable of only limited digestion of casein. SDS electrophoresis of A2 fraction revealed several proteins, with the most predominant having a molecular weight of 40,000, the approximate molecular weight reported for the protease of T. atrica digestive fluid (Mommson, 1978).

IV. Characterization of Argiope Protease B

As the major fibroin digesting protease of the digestive fluid protease B was further investigated. It migrated as a single band in native and SDS electrophoretic gels (Fig. 13) as well as electrofocusing gels (Fig. 22) and was indicated to contain trace amounts of carbohydrate by PAS staining and reducing sugar assay. The molecular weight of the protease was estimated by several techniques. Two similar values were obtained in denaturing systems; 17,600 in SDS polyacrylamide gels and 16,400 by gel chromatography in an SDS-mercaptoethanol buffer. A much lower value of 12,000 was obtained by gel chromatography on a Sephadex gel in a non-denaturing solvent. Møbs (1970) used Sephadex thin layer gel filtration to estimate the molecular weight of a spider "venom" protease at 11,000. Other workers have used Sephadex gel filtration to determine the molecular weight of a crayfish protease at 11,000 (Pfliederer, et al., 1967) and a wasp larval protease at 12,500 (Sonneborn and Pfliederer, 1969) while a "microprotease" (MW 7,800-15,600) have been to be secreted by *Bacillus cereus* (Schenk and Bjorksten, 1973). However, it has become apparent that gel filtration in Sephadex gels often yields

anomalously high estimates of molecular weight for proteases (Voordour, *et al.*, 1974). Recent work (Holmquist, 1977) has shown the microprotease of *B. cereus* to have a true molecular weight of 34,000. Using an average molecular weight of 17,000 from denaturing techniques the zinc content of protease B was estimated at one zinc atom per two polypeptide chains. Attempts were made to determine if the native enzyme exists as a dimer. Molecular weight estimation by sedimentation velocity in an ultracentrifuge proved fruitless due to the small quantities of protease available. No difference in the migration of the protease in SDS gels was observed when it was prepared in the absence of mercaptoethanol.

Protease B is noncompetitively inhibited by copper ions (Fig. 20) indicating that the Cu^{++} does not bind at the protease substrate binding but can combine with either the protease alone or with a protease substrate complex. Heavy metals usually inhibit enzymes by forming metal mercaptides with free sulfhydryl groups (Vallee and Wacker, 1970) and thereby inducing a conformational change in the enzyme. That protease B was not inhibited by iodoacetic acid suggested that copper and other metal ions react with a ligand other than a sulfhydryl group. A variety of metal chelators inhibit protease B (Fig. 23) confirming the necessity of a metal ion for its activity. The most potent inhibitor was O-henanthroline which may be due to its ability to form highly stable 3 to 1 complexes with metal ions (Vallee and Wacker, 1970).

The exact isoelectric point of protease B was not established in this study. Wide range electrofocusing confirmed the homogeneity of hydroxyapatite protease B and protease D (Fig. 22) and established that the isoelectric points of these two proteins were greater than pH 8.2. However, attempts to establish pH gradients in the alkaline range were

Our studies have established that the digestive fluid of A. aurantia is able to dissolve all but one of the components of the orb web (Kavanagh and Tillinghast, 1979). This digestion occurs slowly. A web rolled into a ball placed directly in digestive fluid will take many hours to disassemble and the process is not hastened by reducing or anaerobic conditions. It is possible that other endopeptidases, not used for prey digestion, found deep in the gut are responsible for complete web digestion. This may be the case for digestion of the minor radial fiber of the web. Another possibility is that web fibroins are slowly slowly digested in the gut by proteases such as protease B. Peakall (1972) did not identify the fibers radioactively labeled in the webs in his study. Only soluble nonfibrous components of the web may have been rapidly digested and recycled. A. diadematus will continue to build normal webs for up to a week despite having its web removed daily indicating that digestion of the fibroin of an old web is not necessary for the construction of a new one (Witt, et al., 1968).

In this study we have attempted to elucidate the digestion of large ampullate fibroin, the main component of the orb web. Whole spider digestive fluid is a very heterogeneous mixture whose complexity obscured the results of early fibroin digestion studies (Tillinghast and Kavanagh, 1977). Fibroin digestion was therefore studied with purified, stable, protease B. Even so, whole digestive fluid may contain solubilizing substances which aid in LAF digestion by spiders in nature.

During this study, A. trifolium webs were used in the LAF assay to establish the fibroin dissolving capacity of protease fractions A and B. Though whole digestive fluid dissolves the LAF of A. trifasciata partially purified fractions A and B were only marginally effective against the

untreated webs of this species. If, however, a section of an A. trifasciata web was presoaked in the non-ionic detergent Triton X-100 then fractions A and B, but not C and D, would dissolve the LAF. This result suggests differences in the nonfibroin, surfactant soluble components of the webs of these two species of spider. Natural surfactants have been reported in spider gut fluid (Collatz and Mommsen, 1974). Reeled dragline, instead of orb webs, was used as a source of relatively pure large ampullate fibroin in this study. Even this fibroin was coated with surfactant-soluble substances (Fig. 26) which included two glycoproteins. The exact nature of these substances is presently being studied (Tillinghast, personal communication).

Digestion of the washed dragline fibroin by protease B, as evidenced by the visible disappearance of the silk fibers and the release of ninhydrin reactive substances, occurs gradually over 12 hours with many fibers remaining which are very slowly attacked by a fresh preparation of proteases. Why these remaining fibers are so slowly attacked by protease B was not indicated by their amino acid content which appeared to be identical to that of the solubilized digestion products. Ninhydrin assay (Moore and Stein, 1948) before and after alkaline hydrolysis of the digestive products indicated that latter were small peptides. The small size of the digestion products was further suggested by the lack of any detectable protein bands (exclusive of protease B) on 5, 8 and 15% SDS polyacrylamide gel separations of the digest mixture. Paper electrophoretic and chromatographic techniques failed to resolve the digest mixture, but indicated the presence of a number of similar peptides. Gel filtration of the digest mixture showed the bulk of the digest peptides to have a similar molecular weight of approximately 800 - 1,000.

Cleavage of the LAF molecule by protease B occurs only on the amino side of alanine and glycine residues. Proline, alanine and glycine were the only N-1 amino acids found in the fibroin digestion products. The one digest fragment which was purified, the tripeptide gly-pro-tyr, had tryosine as its carboxy terminal amino acid: protease B cleaved the insulin B chain on the carboxy side of both available tryosine residues. Further clarification of the digestion of large ampullate fibroin will require the use of more sophisitcated separation techniques to isolate all of the digestion peptides.

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