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This study investigated some functional and biochemical characteristics of a bacteriolytic protein in the salivary glands of adult Aedes aegypti. A method for the isolation of this protein from other mosquito salivary gland components is also described. Based on some of its biochemical properties, this bacteriolytic protein can be classified as a lysozyme.

This protein is strongly-linked to mosquito sugarfeeding activity because there is a statistically significant ($P < 0.05$) increase in the levels of lytic activity six hours before mosquitoes start to sugar feed. By its bacteriolytic action, it may function as a protective mechanism against bacteria-contaminated sugar meals. Preliminary work suggests that mosquitoes exposed to lyophilized Micrococcus lysodeikticus in their sugar meal respond by increasing the lytic activity in their

salivary glands.

The levels of bacteriolytic activity are apparently not affected by bloodfeeding. In the absence of feeding, as in teneral and bloodfed mosquitoes, salivary bacteriolytic activity increases to a maximum, then levels off. This suggests a regulation of the synthesis of this salivary protein that is independent of the feeding state of the adult mosquito.

A combination of centrifugation, polyacrylamide gel electrophoresis (non-denaturing and denaturing), cation exchange chromatography and gel filtration, was used to isolate the protein from other mosquito salivary gland components. This salivary protein is lysozyme-like in several aspects: 1) it lyses bacterial cell walls of M. lysodeikticus, 2) it is a basic protein with a pI between 7.47 and 8.89, 3) it is thermostable at low pH, and loses its activity at high pH, and 4) it is composed of one polypeptide chain. Its molecular weight is twice that of hen egg white lysozyme. This salivary bacteriolytic protein is the first insect exocrine lysozyme to be characterized.

A Lysozyme-like Protein in the Salivary Glands of Adult *Aedes aegypti*: Functional and Biochemical Characteristics

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INTRODUCTION

Insects have a remarkable system of defense against invasive and pathogenic organisms. These defense mechanisms have largely been investigated in the past three decades. Most have been "vaccination" studies done on larval and pupal stages wherein challenges ranging from ultra-filtered saline to microbes and microbial products have been introduced into the hemocoel in an effort to understand the mechanisms at work in insect immunity. Much of the work therefore has been on the endocrine basis of insect immunity.

The most common route of infection for an insect would be through ingestion of contaminated food not through inoculation. Insects, being the most highly successful group of organisms, are expected to have evolved a defense mechanism that will protect them from potentially pathogenic organisms that may be present in their food. Indeed adult mosquitoes produce a bacteriolytic protein that has been shown to be secretory, thus of exocrine nature. This bacteriolytic protein was studied in this work and a method is described for its isolation.

The concentration of this protein is apparently not affected by blood feeding but is strongly tied to the sugar

feeding behavior of adult mosquitoes. One organ involved in its elaboration is the salivary gland.

REVIEW OF LITERATURE

Mosquito salivary glands

Salivary glands of adult mosquitoes function in two activities namely, sugar and blood feeding,the latter being responsible for the delivery of pathogens to vector mosquitoes. Only female mosquitoes blood feed, and this is also reflected in the morphological differences of the salivary glands of adult mosquitoes, those of the female undergoing dramatic development following emergence while those of the male remain small and show little change (Orr et al., 1961). Female salivary glands consist of three distinct lobes, two lateral and one median (Janzen and Wright, 1971; Orr et al., 1961). The lateral lobes may be divided into distal and proximal portions with a short intermediate region between the two. The median lobe consists only of a short intermediate and a lateral lobe. In each lobe, a single layer of epithelial cells surrounds a central duct which extends throughout the length of the lobe. There are three glandular regions in the salivary glands of female Aedes aegypti (Orr et al., 1961; Janzen and Wright, 1971). The secretory materials from the proximal lateral lobes are involved in sugar feeding and are common to both sexes. These enzymes include a

non-specific esterase (Poehling and Meyer, 1980; Nakayama et al., 1985), a bacteriolytic factor (Rossignol and Lueders, 1986) and alpha-glucosidase (Marinotti and James, 1990). The alpha-glucosidase is involved in sugar metabolism while the bacteriolytic factor is strongly correlated to sugar feeding behavior (Pimentel and Rossignol, 1990) and is presumed to be protective (Rossignol and Lueders, 1986). A salivary bacteriolytic factor may be important because microbial gut infections have been shown to modulate the competence of sandflies as disease vectors (Schlein et al., 1985).

The two other secretory regions, the median and distal lateral lobes, produce proteins released during blood feeding (Poehling, 1979) and are thus female-specific. Both these regions bind a common lectin, RCA 120 (Perrone et al., 1986), and express a female-enriched gene (James et al., 1991), but may be distinguished from each other histochemically and in their binding of other lectins (Perrone et al., 1986). Apyrase is produced here and occurs in both sexes although is twenty-fold "higher" in females (Ribeiro et al., 1984). The adult female appears to be able to control the release of salivary products depending on its feeding activity (Marinotti and James, 1990). A marked decrease in maltase activity in the salivary glands was observed after sugar feeding, while activities of both maltase and apyrase decreased after a

blood meal.

Sugar feeding

The sugar meal sustains the female mosquito until it finds its host, and allows an infected mosquito to live long enough to oviposit, to bite repeatedly, and to become infective (Van Handel, 1984). Various species of mosquitoes have been shown to feed on sugar sources in nature (Bidlingmayer and Hem, 1973; Reisen et al., 1986). Sugar in nature is obtained from nectar and honeydew (Grimstad and DeFoliart, 1974; Magnarelli, 1977). Feeding on flower nectars greatly affects longevity and dispersal potential of mosquitoes and other hematophagous Diptera (Magnarelli, 1978), and therefore, their ability to transmit diseases (Van Handel, 1972). It is assumed that, in nature, plant juices (Mogi and Miyagi, 1989), particularly flower nectars, form the principal food of male mosquitoes and of species not known to suck blood (autogenous)(Van Handel, 1984).

In other dipteran species, sugar is also important, especially in autogenous species. Brody (1939) reported that prior to their first oviposition, screwworms require a carbohydrate but not a protein meal. Carbohydrates were also necessary for these flies to survive to oviposition. Peterson et al. (1987) showed further that 0.3M solutions of sucrose, fructose, glucose, maltose or lactose were better at promoting egg maturation and longevity than 0.1M

solutions.

In Culicoides species, many of which are autogenous in their first ovarian cycle, nectar sugars form the chief source of energy for flight and maintenance activities (Magnarelli and Anderson, 1981). Tabanids, also autogenous in their first ovarian cycle, deposit sugars as yolk during oocyte formation, but compared with the contribution from vertebrate blood, sugar was determined to be supplementary (Bosler and Hansens, 1974; Magnarelli, 1981; Magnarelli, 1987).

In mosquitoes, Van Handel (1984) showed that dietary carbohydrates not only provided immediate flight energy but, with prolonged rest after the sugar meal, also contributed to fat accumulation. Accumulated fat can not be used for flight but can provide energy for survival when food is not available. A blood meal instead of nectar taken by an "exhausted" mosquito requires at least a day before enough glycogen is synthesized from blood proteins to resume flight (Nayar and Van Handel, 1971). Whether a sugar meal is stored as glycogen or fat therefore determines many aspects of mosquito behavior, including flight, survival and mating.

Sugar also affects the gonotrophic condition of mosquitoes. Sugar may promote pre-vitellogenic development by enhancing juvenile hormone secretion (Lea, 1963). Mosquitoes need a critical volume of blood to develop a

batch of eggs. However, partial blood meals may still result in the development of eggs if followed by the ingestion of sugar (Nayar and Sauerman, 1975c; Edman and Lynn, 1975). Sugar was also shown to be extremely important for oogenesis. Adult mosquitoes developed from under-nourished larvae imbibed sugar more efficiently (Nayar and Sauerman, 1975a) and developed ovarian follicles to the pre-vitellogenic stage whereas those that were continued to be sugar-deprived as adults have teneral follicles (Mer, 1936).

The importance of sugar in the gonotrophic cycle has also been investigated in other dipteran species. Mullens (1985) showed that parous females of Culicoides variipennis, vectors of blue-tongue virus, contained sugars more often than did males and nullipars. In contrast, Magnarelli, (1981) showed that in C. melleus and C. hollensis, there is little correlation between the presence of simple sugars and vitellogenesis during the early stages of anautogenous development. The same is true for tabanid species, Tabanus quinquevittatus and Hybomitra lasiophthalma (Leprince and Brigas-Poulin, 1990). In these species, there is no difference between parous and nulliparous females with respect to the presence of total sugar content. In SW Quebec populations of T. quinquevittatus, important losses in fat body reserve following oviposition may stimulate frequent feedings on

sugars. Parous females rely more heavily on carbohydrates to fulfill their energy requirements than do nullipars (Leprince and Lewis, 1986).

The role of sugar in the vectorial capacity of mosquitoes is vital. The ingestion of a single meal of sucrose by Aedes aegypti influenced both egg maturation and the behavior of the gravid mosquitoes (Klowden, 1986). Sugar-deprived females showed a higher frequency of host-seeking even after the blood meal and were less likely to develop eggs. Moreover, Aedes aegypti showed increased blood-feeding frequency at least during the first gonotrophic cycle when sugar was not available (Foster and Eischen, 1987). The expression of autogeny in the crabhole mosquito, Deinocerites cancer, is also affected by sugar availability. The frequency of the occurrence of autogeny was reduced when females did not have access to sugar, and furthermore, sugar-fed females produced more eggs (O'Meara and Petersen, 1985). Indirectly through its effects on longevity, behavior and fecundity of insect vectors, sugar availability is a critical factor in biological transmission of diseases and parasites.

Insect immunity

Insects are the most diverse animals on earth (Daly et al., 1978). More than 10^6 insect species are recognized in the literature and estimates indicate that the number of individual insects is as high as 10^{18} (Wigglesworth, 1964).

They abound in habitats that are also replete with organisms that use insects as a source of nutrition; these include predators, ectoparasites which consume all or part of an insect's body from the outside, endoparasites which enter the host's body before consuming it, and other species capable of colonizing an insect's body cavity (Dunn, 1990).

Partly as protection against these insectivorous organisms, insects have evolved passive physical barriers such as a sclerotized integument (cuticle) and a peritrophic membrane which isolates the midgut epithelium from the ingested food (Dunn, 1986; Dunn, 1990). Many organisms have evolved mechanisms to penetrate these barriers while other organisms gain access to the hemocoel via wounds. Despite the large number of infectious diseases of insects (Burges, 1981) and the broad spectrum of prokaryotic and eukaryotic organisms that are insect parasites, pathogens or potential pathogens, insects have thrived (Dunn, 1990).

Aside from the passive structural barriers, insects have evolved effective, active cellular (Ratcliffe and Rowley, 1979) and humoral defense mechanisms, and some are capable of acquiring a protected (immune) state after surviving bacterial infections (Gotz and Boman, 1985; Ratcliffe et al., 1985; Brehelin, 1986; Dunn, 1986;). There is a large diversity of immune mechanisms in insects

but only humoral immunity will be discussed here.

Humoral immunity has been studied for more than two decades (Gotz and Boman, 1985) and reviews have been published by Chadwick (1975), Chadwick and Aston (1979), Boman (1981), Boman and Hultmark (1981) and Dunn (1986, 1990). Some of the immune humoral factors are normally present in the hemolymph (Boman et al., 1986). Other factors have been proven to be inducible, that is, they require de novo synthesis of RNA and proteins (Gotz and Boman, 1985).

I. Normal Hemolymph Factors

A. Lectins

Early work on insect immunity was limited to vaccination studies, and it was taken for granted that insects produced "antibodies" (Boman et al., 1986). Experimental methods and terminology were borrowed from work on vertebrate immunity (Gotz and Boman, 1985) and this initial search in insects resulted in the discovery of lectins. Lectins are a heterogenous group of glycoproteins that agglutinate vertebrate cells in vitro (Gotz and Boman, 1985; Boman et al., 1986). Lectins are ubiquitous; they are found in plants, microorganisms and on cells and in serum or hemolymph of vertebrates and invertebrates (Gold and Balding, 1975; Marchalonis and Schluter, 1990).

The mechanism for the agglutination of vertebrate cells has in many cases been shown to be a highly specific

multivalent capacity to bind to certain sugar moieties on the cell membranes (Gotz and Boman, 1985). To elucidate the function of lectins in biological systems, Renwrantz and Stahmer (1983) used purified agglutinins (lectins) from Mytilus edulis (bay mussel) and observed enhanced phagocytic uptake of yeast cells by M. edulis hemocytes in vitro.

Insect lectins or agglutinins have been observed to act against microorganisms. The protozoan Tetrahymena pyriformis was shown to be immobilized by lectins in the American cokroach (Seaman and Robert, 1968); lectins increased in the plasma of the lepidopteran, Anticarsia gemmatalis, in response to a fungal infection (Pendland and Boucias, 1985); two specific lectins agglutinated trypanosomes in the assassin bug, vector for Trypanosoma cruzi (Pereira et al., 1981); and Trypanosoma brucei and Leishmania hertigi were agglutinated in the presence of cell-free hemolymph of Schistocerca gregaria and Periplaneta americana (Ingram et al., 1984). From the flesh-fly, Sarcophaga, (Komano et al., 1980; 1981) purified a lectin which is inducible in the larva but constitutively synthesized in the pupa.

The natural function of lectins has remained a puzzle, but it is reasonable to assume that they function as primordial recognition molecules (Marchalonis and Schluter, 1990) by agglutinating invading micro-organisms which carry

the respective sugar residues on their cell surfaces (Gotz and Boman, 1985). The resulting clumps of foreign cells could then be easily susceptible to either phagocytosis or to encapsulation and melanization. Because lectins occur in a variety of organisms, they may function as primordial recognition molecules (Marchalonis and Schluter, 1990).

B. Phenoloxidase

Phenoloxidase (o-diphenol: O_2 oxidoreductase, EC 1.10.3.1) is a highly reactive enzyme that produces quinones which can react with proteins and with the thiol and amino-groups of many compounds. In many organisms as in insects, it is often stored in the plasma in the form of prophenoloxidase and can be activated by materials from the hemocytes (Pye, 1974).

Phenoloxidase is responsible for tanning and sclerotization of the insect cuticle (Richards, 1978). It has also been implicated in insect immunity because this proenzyme when activated initiates a cascade of enzymes and other factors responsible for the initiation of melanin synthesis by the host (Soderhall and Smith, 1986). The synthesis of melanin as a response to foreign material in the hemolymph has been shown to be part of the cellular and humoral defense reactions of insects (Ratcliffe, 1986). Ronald Ross probably observed a melanization reaction manifest in the "black spores" and degenerate malaria oocysts among the mosquitoes he dissected (Harrison, 1978).

Many different classes of substances including carbohydrates, organic solvents, detergents and proteolytic enzymes can activate phenoloxidase but the relationship between phenoloxidase activity and non-self recognition was first demonstrated by Pye (1974). Zymosan, a yeast polysaccharide, and a preparation of damaged Pseudomonas aeruginosa resulted in more prophenoloxidase activation than trypsin injections in immune plasma of Galleria mellonella. The natural control function of phenoloxidase seems to be mediated by the proteolytic cleavage of the enzyme prophenoloxidase (Gotz and Boman, 1985).

Phenoloxidase has also been proposed to participate in other immune mechanisms such as cellular encapsulation (Ratcliffe, 1986) and phagocytosis (Bayne, 1990). Some of the melanin precursors have been found to be fungistatic (Soderhall and Ajaxon, 1982) but the overall role of phenoloxidase as part of the immune response is not very well established (Gotz and Boman, 1985). As understood in crustaceans, it is a complex enzyme system which has been shown to provide opsonins, initiate capsule/nodule formation, participate in coagulation and thus facilitate microbial killing (Soderhall and Smith, 1986). It also mediates communication/cooperation between the different hemocyte populations (Soderhall and Smith, 1986). It appears to bear a certain similarity to the complement pathway of higher animals.

II. Inducible Factors

The detailed studies of Briggs (1958) and Stephens (1959) were the first to demonstrate the presence of antibacterial activity in hemolymph of immunized insects. The first antibacterial factor to be identified in insect hemolymph was lysozyme and it was claimed that it is the main antibacterial factor responsible for the immunity of vaccinated insects (Mohrig and Messner, 1968; Boman et al., 1986;). This is an overly simplistic view of the defense system of organisms as diverse as insects. Boman et al. (1974) showed that lysozyme is only one of several inducible proteins that are part of a multi-component antibacterial system. These workers, using diapausing pupae of Hyalophora cecropia have, over the years, purified 15 different immune protein from insect hemolymph (Boman et al, 1986).

The variety of immune proteins in their hemolymph enable insects to eliminate many kinds of bacteria that gain entry into the hemocoel (Boman et al., 1986). Aside from lysozyme, cecropins, attacins and related compounds have been isolated, purified and characterized (Gotz and Boman, 1985).

A. Cecropins

Cecropins were discovered in 1979 when these peptides were successfully isolated from the Cecropia lysozymes (Boman et al., 1986). Two forms, cecropins A and B were

isolated simultaneously with lysozyme (Hultmark et al., 1980). Cecropin D as well as three main forms believed to be precursors were isolated two years later (Hultmark et al., 1982). These peptides are of low molecular weight (4.2 kD) (Steiner et al., 1981) and are apparently widespread in the Lepidoptera (Hoffman et al., 1981). Cecropins have a wide spectrum of anti-bacterial activity, being active against both Gram-positive and Gram-negative bacteria (Steiner et al., 1981).

All of the isolated cecropins are similar with a strongly basic N-terminal region and a long hydrophobic stretch in the C-terminal half (Boman and Steiner, 1981). The high degree of homology shown by these 5 cecropins so far sequenced suggests that they evolved through gene duplications.

A bactericidal protein termed sarcotoxin I was elicited after wounding of the integument of Sarcophaga peregrina (Okada and Natori, 1983; Okada and Natori, 1985). It was effective against certain Gram-negative and Gram-positive bacteria and had an amino acid composition similar to that of cecropins. Expression of this gene appears to be developmentally regulated in non-wounded embryonic and pupal stages (Nanbu et al., 1988).

B. Attacins

Hultmark and co-workers (1983) first isolated attacins by molecular sieving. The antibacterial fractions showed

a molecular weight considerably larger than the cecropins. Subsequent studies revealed as many as six different components (A to F) which could be fractionated according to their iso-electric point. Boman and co-workers (1986) have not been able to document any real differences in the function of attacins and cecropins.

The N-terminal sequences for five of the attacins indicated that the three basic forms have similar sequences, while the two acidic forms are identical, but slightly different from the basic (Hultmark et al., 1983). These data strongly suggested the existence of only two different genes for attacins, one for the basic and one for the neutral or acidic form. These two main kinds of attacins are very similar with as much as 79% homology on the amino acid level; at the DNA level, the homology is 76% for the coding region, in contrast to only 36% in the region beyond the stop signal (Boman et al. 1986). Thus, as in the case of the cecropins, it seems likely that the attacins have arisen through gene duplications.

In Sarcophaga peregrina, the wound-elicited set of peptides includes an attacin-like protein termed sarcotoxin II (Ando et al., 1983). It has an apparent molecular weight of 26 kD but its activity against bacteria has not been further characterized (Dunn, 1990). Another set of antibacterial proteins produced by S. peregrina are termed sarcotoxins III (Baba et al., 1987).

Other insect bactericidal proteins include sapecins, phormicins and diptericins. Sapecin, was first observed in an S. peregrina embryonic cell line and showed activity against Gram-positive bacteria (Matsuyama and Natori, 1988a). It was subsequently observed to be synthesized by the hemocytes after wounding of the larval integument (Matsuyama and Natori, 1988b). Another flesh fly, Phormia terranovae, produces immune proteins termed phormicins, and wounded larvae also synthesize cecropin-like peptides and broad-spectrum antibacterial proteins termed diptericins (Lambert et al., 1989).

C. Hemolin

Previously called P4 (Rasmuson and Boman, 1979), this 48-kD protein is present in low but significant amounts in the hemolymph of cecropia pupae (Sun et al., 1990). Its concentration in the insect hemolymph increases 18-fold after injection of live bacteria. It does not however exhibit direct bactericidal effects (Andersson and Steiner, 1987). Analysis of the deduced amino acid sequence revealed that hemolin has immunoglobulin-like domains (Sun et al., 1990). It appears that hemolin is a recognition molecule which is strongly induced after bacterial infection. It binds to surface structures common to many bacteria where, with another hemolymph protein, it forms a complex which might constitute an important part of the insect's primary immune response (Sun et al., 1990).

D. Lysozyme (Muramidase; EC 3.2.1.17)

The hemolymph of normal non-immunized insect larvae contains low constitutive levels of the bacteriolytic factor enzyme lysozyme (Anderson and Cook, 1979; Dunn and Drake, 1983; Anderson, 1984; Dunn, 1986; Dunn et al., 1987; Kanost et al., 1988; Dunn, 1990). The level of lysozyme is increased manyfold following injection with bacteria (Chadwick, 1970; Powning and Davidson, 1973; Faye et al., 1975; Anderson and Cook, 1979).

Lysozyme has been purified from "immunized" serum of several lepidopteran larvae (Powning and Davidson, 1973; Hultmark, 1980) and from hemocytes of Locusta (Zachary and Hoffman, 1984). The enzymes from these insects are small (15.3 to 16.2 kDa) basic proteins with properties such as heat stability, pH optima, and ionic strength similar to those of chicken egg white lysozyme (Jolles, 1969). The amino acid sequences of the amino-termini (residues 1 - 34) of three insect lysozymes and the complete sequence of a fourth (Engstrom et al., 1985) have been reported; they exhibit considerable sequence homology both to each other and to the chicken enzyme (Jolles et al., 1979).

Lysozymes are widely distributed enzymes and they are found in a number of organs, tissues, and secretions (spleen, kidney, leucocytes, tears, saliva, milk, serum) of vertebrates (Phillips, 1966; Jolles, 1969). The existence of lysozyme was first demonstrated by Fleming (1922), also

the discoverer of penicillin. Lysozymes also occur in invertebrates, bacteria, phages and plants. Previous studies with chicken egg white lysozyme and other lysozymes have resulted to the elucidation of the following properties of the enzyme (Jolles, 1969):

a. basic protein
b. low molecular weight; the highest being phage lysozyme with 18 kD
c. stability at high temperatures and at low pH
d. lability at high pH
e. lysis of suspensions of Micrococcus lysodeikticus cells
f. its action on appropriate compounds liberates reducing and amino sugars.

A property common to all the lysozymes studied is their ability to rapidly lyse Gram-positive bacteria such as M. lysodeikticus. Enzymes that degrade the peptidoglycans of bacterial cell walls are mainly bacteriolytic endoenzymes (Jolles, 1969). They can be grouped into three classes: carbohydrases, acetylmuramyl-L-alanine amidases, and peptidases (Strominger and Ghuysen, 1967). Carbohydrases include endoacetylglucosaminidases, endoacetylmuramidases, and exoacetylglucosaminidases. Lysozymes belong to the group of endoacetylmuramidases.

Lysozyme from chicken egg white has been extensively

characterized, and insect lysozymes are compared to it in terms of activity, biochemical properties and amino acid sequence. Compared to chicken egg white and human lysozymes, Cecropia lysozyme has been shown to share 40 of 120 amino acids (40% homology) in its primary structure (Marchalonis and Schluter, 1990). This shows that insect lysozymes are homologous to both chicken and human lysozymes. Molecules greater than 25% identical are considered unquestionably homologous to one another (Doolittle, 1989). Furthermore, the active site of the insect lysozyme has also been conserved with respect to the chicken enzyme (Boman, et al., 1986). The following discussion will therefore be mostly on studies on chicken egg white lysozyme.

Chicken egg white lysozyme is a low molecular weight (14.5 kD) cationic protein with bacteriolytic properties, hydrolyzing N-acetylmuramic B-1,4 N-acetylglucosamine linkages of the peptidoglycan constituting the bacterial cell wall (Sharon, 1969). Extensive studies have been done on chicken egg white lysozyme (Flowers and Sharon, 1979); its primary structure, the disposition of its disulfide bonds, its spatial behavior, its active center, specificity and mode of action have all been investigated in detail (Jolles, 1969). In fact, it is the first enzyme molecule whose three dimensional structure was elucidated (Phillips, 1966). The amino acid sequence of the chicken lysozyme was

determined by Canfield (1963).

Chicken lysozyme is a protein composed of 129 amino acids, and is folded into right and left folds leaving a cleft in the middle. It is a single polypeptide chain crosslinked at four places by disulfide bonds (Brown, 1964; Canfield and Liu, 1965). It is described as an "oil drop with a polar coat" because the hydrophobic residues are in the interior and the hydrophilic residues are on the outside (Phillips, 1966). Lysozyme illustrates the mechanism of substrate distortion as a way for activation of its substrate (metzler, 1977; Flowers and Sharon, 1979). X-ray crystallographic studies of the enzyme and its complexes with various inhibitors showed the location of its active site in a hydrophobic cleft with strict steric requirements and consequently activating the substrate by forcing it into the more reactive half-chair conformation (Phillips, 1966; Flowers and Sharon, 1979).

Circulating levels of lysozyme has been used as a diagnostic tool (Grossowicz and Ariel, 1983) in a number of human diseases such as monocytic and myelocytic leukemia (Osserman and Lawlor, 1966) and sarcoidosis (Pascual et al., 1973).

When measuring lysozyme, it is the enzymatic activity that is assessed, hence the term lysozyme level generally means lysozyme activity (Grossowicz and Ariel, 1983). The assay is based on the lysis of a turbid suspension of

Micrococcus lysodeikticus cells, the substrate being the protective cell wall of this Gram-positive bacterium.

Lysozyme assay methods

The current methods for the assay of lysozyme were reviewed by Grossowicz and Ariel (1983), and this review is the main source of the material presented here.

The determination of lysozyme activity include turbidimetric methods, lysoplate assay, immunoassays and other techniques. Factors that affect the enzymatic properties of lysozyme influence its determination (Gorin et al., 1971) and these include pH, temperature, ionic strength, as well as the method of preparation of the substrate, M. lysodeikticus (Smolelis and Hartsell, 1949; Grossowicz and Ariel, 1983). Divalent cations in solution decrease while monovalent cations increase lysozyme activity (Smolelis and Hartsell, 1949). The presence of electropositively charged molecules like protamine and histones enhance the activity (Kaiser, 1953; Skarnes and Watson, 1955) while electronegatively charged molecules like heparin (Kaiser, 1953), hyaluronic acid, DNA, and RNA (Skarnes and Watson, 1955) decrease lysozyme activity. For a lysozyme assay to be reproducible, it is therefore necessary to describe the methodology in detail. There are several methods used for the determination of lysozyme activity.

I. Turbidimetric Method

This assay is based on spectrophotometric measurements of the clearing of a turbid suspension of M. lysodeikticus by lysozyme (Smolelis and Hartsell, 1949; Gorin et al., 1971). The clearing phenomenon is a complex process and only indirectly connected with the enzyme's catalytic reaction (Grossowicz and Ariel, 1983), as such, different approaches have been used to relate the empirical measurements to lysozyme enzymatic activity (Gorin et al., 1971). Smolelis and Hartsell (1949) determined the change in absorbance in a short time interval immediately after the addition of lysozyme; Gorin et al. (1971) related the enzyme activity to the time required to produce a specific absorbance change. Other workers calculated enzymatic activity from the specific rate constant (Smith et al., 1955) or from the plots of initial slopes of transmittance versus time (Selsted and Martinez, 1980).

II. Lysoplate Assay Method

This method was developed by Osserman and Lawlor (1966). A suspension of heat-killed M. lysodeikticus is taken up in a small volume of 0.06M phosphate buffer, pH 6.3. This suspension is added to molten 1% agar or agarose at 60 to 70 C in the same buffer and poured into Petri dishes. After solidification of the agar, 2-mm diameter wells are cut in the agar and samples of lysozyme solutions are placed in the wells. The plates are incubated at room

temperature for 12 to 18 hours during which time clearing zones develop around the wells as a result of bacterial lysis. The diameter of the cleared zones is proportional to the logarithm of lysozyme concentration. The activity of test samples is easily quantified by using a semilogarithmic plot of the diameters of the cleared zones versus standard solutions of lysozyme.

This method has several disadvantages, the primary one being the long period of incubation required. Moreover, the edge of the zone of lysis is often blurred and thus difficult to measure accurately; this often requires repetition of the assay which entails further delay in obtaining the results (Grossowicz and Ariel, 1983).

Greenwald and Moy (1976) observed that the clarity of the plates and the values obtained depend to a large extent on the batch of agar used. Different agar batches contain various amounts of sulfate and carboxyl anions as well as inorganic salts, estimated as ash content. Lysozyme, being a highly cationic protein, is therefore considerably influenced by the ionic composition of the agar gel.

Zucker and co-workers (1970) found a good correlation between the turbidimetric and lysoplate methods for assaying plasma lysozyme activity. However, the visual determination of the end-point readings in the lysoplate assay makes it a less objective and less precise method, especially when using very low lysozyme concentrations.

Moreover, the turbidimetric method provides results more quickly.

III. Immunological Methods

A. Immunochemical Method

The sensitivity of this assay is considerably lower that the lysoplate method especially at high dilutions (Virella, 1977). Goudswaard and Virella (1977) increased the sensitivity of this method by using the laser nephelometer especially for lysozyme concentrations between 1 and 10 mg/liter. Laser nephelometry made this method more accurate and slightly more sensitive than the lysoplate method especially at low lysozyme concentrations. The method is suitable for large series of determinations as in hospitals, and the results are available within 2 to 3 hrs.

B. Radioimmunoassay

Using lysozyme from chicken egg white (Yuzuriha et al., 1978) and humans, Yuzuriha et al. (1979) developed a radioimmunoassay based on the competitive technique. The method involves the distribution of radiolabelled lysozyme between the supernatant and dextran-coated charcoal. ^{125}I-labelled lysozyme is measured in the supernatant after centrifugation of the antigen-antibody complexes. Increased lysozyme concentration in the sample is reflected by increases in the supernatant radioactivity while the bound radioactivity decreases. Another more convenient

radioimmunoassay was developed by the same workers (Yuzuriha et al., 1979) in which the antibody against lysozyme, rather than lysozyme itself, is labeled with ^{125}I. The radio-activity of the antigen-antibody complex increases with increasing lysozyme concentrations. For both methods, the range in sensitivity is 5 to 250 ug of lysozyme per liter. The latter method is more convenient but the former is more precise (Grossowicz and Ariel, 1983). These methods are valuable in their ability to distinguish between lysozymes from different "immunological species", and also measure the enzyme from one species even in the presence of lysozyme from another species.

C. Enzymoimmunoassay

Yuzuriha et al. (1979), working with human lysozyme, developed an enzymoimmunoassay to avoid the use of radioisotopes. This is based on the sandwich technique in which an alkaline phosphatase (AP)-antibody conjugate is used to form a sandwich of human lysozyme between the antibody to lysozyme and the AP-antibody conjugates. Increasing concentrations of lysozyme are reflected as increases in AP activity. The range of sensitivity is the same as for the radioimmunoassay (5 to 250 ug/liter) but the precision is somewhat lower. Nevertheless this enzymo-immunoassay is more convenient and the high reproducibility gives it a satisfactory precision. The results of an immunoassay are always affected by the source of lysozyme

used as a standard, therefore the source of lysozyme must be stated in any immunoassay (Yuzuriha et al., 1979).

D. Inhibition of the inactivation of human lysozyme-bacteriophage conjugate

This immunoassay for human lysozyme was developed by Maron in 1971 (Grossowicz and Ariel, 1983). It is based on the inhibition of the inactivation of human lysozyme-bacteriophage conjugate which is specifically inactivated by antibodies directed against human lysozyme. Free lysozyme in the medium inhibits this inactivation, thus the percentage of inhibition of the inactivation of lysozyme-bacteriophage conjugate is calculated from the number of phage survivors.

IV. Fluorimetric Assay

This method was developed by Mintz et al. (1975) using Bacillus subtilis cell walls labelled with fluorescamine. The fluorescent method is as sensitive as the radioactive methods but considerably less expensive, as sensitive as the turbidimetric method but more time-consuming and the degree of variation between samples is fairly high (Grossowicz and Ariel, 1983).

V. Colorimetric Method

This is insensitive relative to the other methods. Lysozyme is incubated with the substrate, 3,4-dinitrophenyl-tetra-N-acetyl-B-chitotetraoside dissolved in citrate buffer, pH 6.0 for 30 min. at 37 C.

The release of 3,4-dinitrophenol is measured spectrophotometrically at 400 nm (Grossowicz and Ariel, 1983).

VI. Histochemical and Cytochemical Localization of Lysozyme Activity

Speece (1964) devised the first histochemical method by observing morphological changes as determined by staining with alcian blue and basic fuchsin in films of M. lysodeikticus embedded in agar after incubation with lysozyme-containing frozen tissue. Antibody to lysozyme was coupled to a fluorescent dye and the fluorescence of lysozyme-containing cells was measured (Asamer et al., 1969; Glynn and Parkman, 1964).

The first direct cytochemical method was devised by Scholnik and Kass (1973). These workers used the dis-azo dye biebrich scarlet which stains basic proteins like lysozyme. The addition of N-acetylglucosamine oligosaccharides from chitin hydrolysates greatly diminishes the color reaction presumably because of competition between the dye and the oligosaccharides for the active site of lysozyme. This makes the test specific for lysozyme.

Preparation of the substrate for lysozyme assays

Boasson (1938) described one of the earliest procedures to assay lysozyme activity. It was based on the rate of clarification of a suspension of Micrococcus lysodeikticus cells by lysozyme. As such it was necessary to have a stable suspension of M. lysodeikticus cells that shows unaltered susceptibility to lysozyme over time. The phenolized cells used by Boasson (1938) proved to be highly variable. Methods for treating M. lysodeikticus cells to give highly reproducible results have been investigated (Meyer and Hahnel, 1946; Smolelis and Hartsell, 1952). These workers treated M. lysodeikticus cells with any or a combination of the following treatments: acetone, phenol, UV light, heat, distilled water, and ether. Gorin et al. (1971) showed that the same lysozyme solution gave different results when tested against different isolates of M. lysodeikticus, the difference varying between 30 and 100%. On the same bacterial suspension, however, the deviation between individual determinations did not exceed ±5%.

Grossowicz and co-workers (1979) used a stable suspension of M. lysodeikticus cells in glycerol-tris buffer (40:60 by volume) 0.06M, pH 7.5, stored at -20 C. This suspension is prepared from 24-hr-culture cells washed with cold 0.06M tris buffer, pH 7.5. Highly reproducible results were obtained throughout the 8-month period of

storage, the deviations never exceeding 3%. Lysis of the cell suspension was measured spectrophotometrically after an incubation time of 15 minutes. High lysozyme sensitivity and repro-ducibility of results are probably due to elimination of damage to the lipids and proteins adjacent to the cell wall, and to lack of activation of autolytic enzymes; such activation presumably occurs in the various preparations of killed cells (Grossowicz and Ariel, 1983).

Lysozyme has been observed in the hemolymph and other tissues of various species of insects (Table 1). Aside from the hemolymph, recent studies have shown that the enzyme is contained in hemocytes and fat body (Dunn et al., 1985) and synthesized, contained and released from the pericardial complex of Manduca sexta. Zachary and Hoffman (1984) showed that in Locusta, serum lysozyme is synthesized and stored in two hemocyte types, the granulocytes and the coagulocytes. Rossignol and Lueders (1986) demonstrated the presence of a bacteriolytic factor which is lysozyme-like in the salivary glands of Aedes aegypti.

Table 1. Occurrence of lytic activity in insects. (Partially from Kramer et al., 1985).

Species	Tissue Source	Reference
Bombyx mori (silkmoth)	hemolymph	Powning and Davidson, 1973
	hemolymph	Croizier and Croizier, 1978
Ceratitis capitata (Mediterranean fruit fly)	eggs	Fernandez-Souza et al., 1977
Galleria mellonella (greater wax moth)	hemolymph	Powning and Davidson, 1973
	hemolymph	Croizier and Croizier, 1978
Periplaneta americana (American cockroach)	gut and hemolymph	Powning and Irzykiewicz, 1963
	embryonic cell lines E Pa and hemocyte line H Pa 33	Landureau and Jolles, 1970 Bernier et al., 1974
Spodoptera eridania (armyworm)	hemolymph and hemocytes	Anderson and Cook, 1979
Choristoneura fumiferana (spruce budworm)	embryonic cell line	Koga et al. unpublished
Culex quinquefasciatus (house mosquito)	ovary	Turner et al., 1981
Drosophila melanogaster (fruit fly)	embryo	Turner et al., 1981
Hyalophora cecropia (giant silkmoth)	hemolymph	Boman et al., 1985
Manduca sexta (tobacco hornworm)	hemolymph	Dunn et al., 1985
Rhodnius prolixus (assassin bug)	gut	Ribeiro and Pereira, 1984
Aedes aegypti (yellow fever mosquito)	salivary glands	Rossignol and Lueders, 1986

OBJECTIVES OF THE STUDY

Many studies have been done on insect hemolymph antibacterial factors. The synthesis of these hemolymph proteins is part of the insect's immune response to organisms or foreign material that gain access to its hemocoel. However, in nature, insects pick up various organisms which are potentially parasitic or pathogenic, along with contaminated food. The presence of a bacteriolytic factor in the salivary glands of adult Aedes aegypti mosquitoes has been demonstrated (Rossignol and Lueders, 1986). This factor, secreted in the insect saliva, may be a defense mechanism against the possibility of a pathogenic invasion via the oral route.

There are two objectives for this study: 1) to investigate the function of this salivary bacteriolytic factor in adult Aedes aegypti, and 2) to isolate this factor from other components in the mosquito salivary glands.

To investigate the function of this bacteriolytic factor, several experiments were done. First, lytic activity levels were assayed in the salivary glands of different age groups from emergence to 10-day old non-sugar feeding mosquitoes. Salivary lytic activity levels of sugarfeeding mosquitoes were also determined, and these levels were correlated with the age at which adult mosquitoes started to sugar feed. The salivary glands of

bloodfed mosquitoes were also assayed for bacteriolytic activity. This is interesting because A. aegypti mosquitoes do not generally sugar feed after a blood meal (Foster, 1986). To determine whether mosquitoes increase their salivary lytic activity in response to an oral challenge of bacteria, female Aedes aegypti mosquitoes were exposed to lyophilized Micrococcus lysodeikticus cells in their sugar meal and the levels of lytic activity their salivary glands measured.

To isolate this bacteriolytic factor from other salivary gland components, different methods were tried. By a combination of centrifugation, polyacrylamide gel electrophoresis (non-denaturing and denaturing), cation exchange chromatography and gel filtration, the bacteriolytic factor was isolated. In the process, some biochemical characteristics of the protein were also elucidated.

CHAPTER I

AGE DEPENDENCE OF SALIVARY BACTERIOLYTIC ACTIVITY IN ADULT MOSQUITOES

INTRODUCTION

Sugar, mostly in nectar and honey dew, provides a female mosquito with its nutritional requirements. Such feeding plays an important role in allowing a vector to live long enough to oviposit, and possibly to bite repeatedly and to become infective (Van Handel, 1984). Sterility of sugar sources in nature is not assured, so a protection against potentially pathogenic bacteria in this food source would be advantageous. Bacteriolytic activity in salivary glands of both male and female Aedes aegypti (Rossignol and Lueders, 1986) may provide protection against certain bacteria, just as lysozyme in other systems is a strong antibacterial agent (Fleming, 1922).

In the first days after emergence, mosquitoes do not blood feed, and have immature ovaries (Lea, 1963) and gut cells (Rossignol et al., 1982) which develop over the first three days or so in response to hormonal signals. It is still unclear whether or not young mosquitoes require nutrients at all, and whether their vector-related functions await an endocrine signal. The levels of bacteriolytic activity in the salivary glands of newly

emerged and aging mosquitoes were therefore measured. Then the age at which adult mosquitoes start to sugar feed was determined. Whether or not the production of bacteriolytic factor is under control of the corpora allata was also investigated.

MATERIALS AND METHODS

Mosquitoes

Mosquitoes used in this study were Aedes aegypti (Georgia strain). Larvae were fed pelleted Hartz^{R} gerbil and hamster food. Adults were fed dry sucrose; water was given ad libitum. Mosquitoes were held at room temperature with 12-12 hour light-dark cycle. Upon emergence, female mosquitoes were isolated from males and kept in separate rearing cages for different periods as required for the various experiments. Only female mosquitoes were used in all experiments.

Sugar feeding and mosquito age

Mosquitoes of the same age were kept in cylindrical cardboard rearing cages (diameter=8.4 cm, height=6.6 cm) covered with nylon netting. They were given only water until the required age after which they were also given two sugar cubes dyed with Congo red. All mosquitoes had access to sugar cubes for 6 hours. At the end of the experiment, the sugar cubes were removed and the whole cage was kept at 0°C overnight. Whether the mosquitoes sugar fed or not was

then determined by examining the mosquitoes under a dissecting microscope for the presence of the Congo red dye in their digestive tract.

Mosquito dissection

Mosquitoes were dissected for two purposes: extracting salivary glands to assay bacteriolytic activity at different ages, and removing the corpora allata to determine whether bacteriolytic factor production is under endocrinological control. Allatectomy was based on the method of Lea (1963). The corpora allata of females were removed within three hours after emergence and mosquitoes that survived (55%) this dissection were given only water until salivary gland extraction at various ages. A separate group of mosquitoes had the dorsal neck membranes torn to provide a sham. Sizes of the ten largest follicles in the ovaries were also noted to determine whether or not the allatectomy was successful. Only salivary glands from mosquitoes whose follicles were less than 70 microns in diameter (indicative of successful allatectomy) were included in the analysis.

Mosquitoes were cold anaesthetized and salivary glands were dissected directly into Hayes' saline (Hayes, 1953). The glands were then isolated into 1.5 ml plastic microfuge tubes with 10.0 ul of distilled water, which also achieved homogenization, and stored in -70°C.

Bacteriolytic factor assay

Frozen salivary glands were thawed and centrifuged in a Beckman Microfuge E for 5 minutes. Salivary gland homogenates of 4.0 ul were inoculated into 1.0 mm diameter wells in agarose plates. Agarose plates consisted of 10.0 ml of 0.7% agarose in phosphate buffer (0.067M KH_2PO_4-Na_2HPO_4, pH 6.2) in a 100 mm Petri dish with 0.15 mg/ml Micrococcus lysodeikticus (Sigma) and sodium azide (0.02%) added. For determining bacteriolytic activity, the areas of lysis around the agarose wells were measured after 48 hours incubation at 31 C. Activity was calibrated against chicken egg white lysozyme (Sigma) and expressed as lysozyme unit as defined in Rossignol and Lueders (1986).

RESULTS

Bacteriolytic activity was evident in the 0-6 hour age-group and, based on a Duncan multiple range test, increased significantly within 6 to 12 hours (Fig. I.1). There was a six-fold increase in activity until three days. Bacteriolytic activity increased until day 10, the last day of the experiment. However, the rate of increase in activity declined starting on day 3.

To relate gland physiology with behaviour, sugar feeding activity after emergence was examined. Mosquitoes began sugar feeding after approximately two days (Fig. I.2).

Because several of the early events following emergence are triggered by juvenile hormone release from the corpora allata (Racioppi et al., 1984), allatectomy was done on newly emerged mosquitoes to determine whether the increase in bacteriolytic activity was under the influence of the corpora allata. Differences in the levels of the bacteriolytic activity in allatectomized and sham-operated mosquitoes were not significant (Table I.1). This observation suggests that the accumulation of this bacteriolytic activity in the salivary glands is not under the control of the corpora allata.

DISCUSSION

Bacteriolytic activity in female Aedes aegypti salivary glands is at a low level at emergence, increases six-fold over the first three days and then remains relatively constant. The rise appears not to be mediated by juvenile hormone. The age-dependent increase in salivary bacteriolytic activity may have biological implications.

Newly emerged mosquitoes do not possess much bacteriolytic activity in the first 6 hours. This observation implies that, if the activity is protective, newly-emerged mosquitoes may not safely take a sugar meal and therefore need to synthesize immediately an effective quantity of the lytic factor. A significant increase in

bacteriolytic activity did occur in the 6-12 hour group to a level presumably sufficient for sugar feeding, because some of the 12-18 hour group attempted to sugar feed. However, a significant proportion of adult mosquitoes started sugar feeding only on the second day after emergence. The continued increase in bacteriolytic activity in older mosquitoes may have been due to the fact that these mosquitoes had not been allowed to sugar feed before salivary gland extraction. Thus, bacteriolytic activity may have accumulated in the salivary gland instead of being secreted into the crop. The levelling off of the lytic activity beginning on the third day shows that salivary glands reach maturity after three days from adult emergence. Maturity of the salivary glands does not appear to be a prerequisite to sugar feeding as long as the levels of the lytic factor are at least half of maximum levels.

Three days after emergence, salivary glands are histologically mature; after this time there are no significant morphological changes (Orr et al., 1961). The total protein content of salivary glands continues to increase up to the seventh day of adult development in Anopheles and Culex (Poehling, 1979) and in Aedes aegypti (Racioppi and Spielman, 1987). However, these additional proteins may not be necessary for sugar feeding because a large proportion of mosquitoes started sugar feeding on the second day after emergence.

The accumulation of bacteriolytic activity may be linked to early sugar feeding activity as shown here. Recently, a putative glucosidase was reported in the salivary glands of both male and female Aedes aegypti (James et al., 1989) but it remains to be shown that this glucosidase is exocrine. The protective role of bacterial and fungal inhibitors in the gut of phlebotomine flies has been shown but their source, identity and characteristics are yet undefined (Schlein et al., 1985). These authors also gave experimental evidence that Leishmania major does not survive in Phlebotomus papatasi with gut mycosis. Gut mycoses in populations of sandfly vectors reduce the incidence of Leishmania infection in endemic areas (Schlein et al., 1985). The presence of an anti-fungal or antibacterial agent in the gut of some blood feeding flies may therefore modulate their competence as vectors. Thus, antimicrobial factors within the salivary glands and other tissues may strongly influence the vector biology of an insect.

Table I.1. Bacteriolytic activity in extracts of salivary glands from control, sham-operated and allatectomized three-day old female Aedes aegypti.

Treatment	N	Activity (lysozyme units)	Duncan Grouping*
Control	8	2.20	A
Sham-operated	8	2.18	A
Allatectomized	14	2.05	A

* Means with the same Duncan grouping are not significantly different from each other at α=0.05.

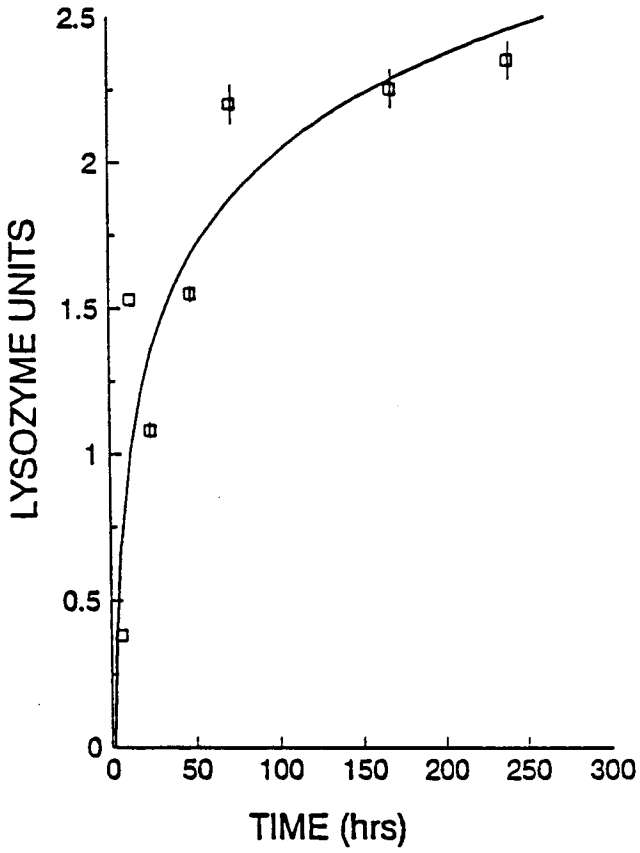

Figure I.1. Bacteriolytic activity in extracts of salivary glands of adult female Aedes aegypti; r^2=0.84 (logarithmic regression); ±S.E.

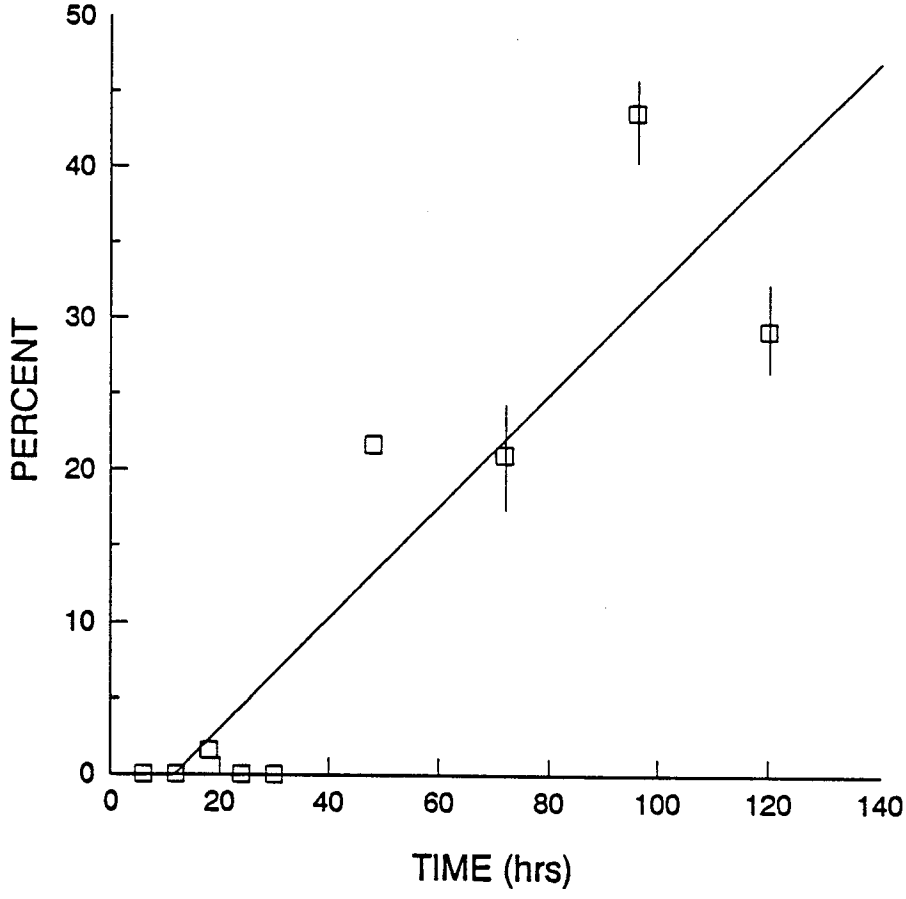

Figure I.2. Percent of female Aedes aegypti mosquitoes of a certain age that fed within 6 hours of exposure to sugar; r^2=0.81; ±S.E.

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CHAPTER II

SALIVARY BACTERIOLYTIC ACTIVITY IN BLOOD-FED AND *MICROCOCCUS LYSODEIKTICUS*-EXPOSED MOSQUITOES

INTRODUCTION

Many changes occur in a mosquito following blood feeding (Racioppi and Spielman, 1986). These include increased synthesis of digestive enzymes and of vitellogenin, release of hormones (Briegel and Lea, 1975; Hagedorn et al., 1973) and disaggregation of whorls of rough endoplasmic reticulum in the midgut (Bertram and Bird, 1961). Synthesis of salivary proteins concurrently decreases (Racioppi and Spielman, 1986). This correlates well with laboratory recordings of sugar feeding activity before, during and after gonotrophic cycles (Foster, 1986). Such work confirmed that a blood meal inhibits sugar feeding. However there is a great variation in the extent of the inhibition in different species. In *Aedes aegypti*, blood feeding nearly always caused a complete cessation of sugar feeding that lasted throughout the period of blood digestion (Foster, 1986).

Rossignol and Lueders (1986) suggested that this bacteriolytic factor may function as a sterilizing agent during sugar feeding. To determine whether or not bacteriolytic activity of salivary glands is reduced or

even eliminated with the cessation of sugar feeding, salivary lytic levels were determined in bloodfed mosquitoes. Because of its possible protective function, bacteriolytic activity of salivary glands was assayed in mosquitoes exposed to different concentrations of Micrococcus lysodeikticus in their sugar meal.

MATERIALS AND METHODS

Bacteriolytic factor and blood feeding

Mosquitoes used in this study were Aedes aegypti (Georgia strain). Rearing procedures have been described (Pimentel and Rossignol, 1990). Upon emergence (day 1) female mosquitoes were isolated from males and kept in separate rearing cages. Sugar cubes were given to appropriate treatments from day 1. Water was given ad libitum.

To determine the effect of blood feeding on the salivary bacteriolytic factor levels, sugar feeding mosquitoes were bloodfed and their salivary glands were assayed daily for lytic activity. Groups of 100 to 110 mosquitoes were fed on an anaesthetized rat 5 days after emergence. This bloodmeal was given in the morning at least 6 hours before the scheduled salivary gland extraction for day 5. Another group was not bloodfed and therefore served as a control treatment. Salivary bacteriolytic activity was assayed everyday from emergence

to day 10 (after oviposition) for both treatments. Sugar feeding in adult mosquitoes has been shown to have a diurnal rhythm (Gillet et al., 1962). To minimize the possible effect of this sugar feeding behavior of mosquitoes on bacteriolytic activity levels, salivary glands were extracted at 1600 to 1800 everyday from 10 mosquitoes in each treatment. The assay for bacteriolytic activity has been described (Pimentel and Rossignol, 1990) but the quantity of Micrococcus lysodeikticus was increased to 0.25 mg/ml to enable more accurate measurement of diameters of lysed areas.

Salivary bacteriolytic activity in the presence of bacteria in the sugar meal

To investigate whether mosquitoes respond to an oral challenge of bacteria by increasing their salivary lytic activity, lyophilized Micrococcus lysodeikticus (Sigma) cells were added to their sugar meal at concentrations of 0.05 and 0.50% w/v. Another group (0.00%) was given only the basal food which consisted of a sterile 10.0% sucrose solution. Mosquitoes used were at least 4 days old. Three groups each of 90 female mosquitoes were not given water and sugar cubes for 6 hours, then exposed to their respective treatments for at least 48 hours before the first batch of mosquitoes were taken for salivary gland extraction.

The solutions were administered to the mosquitoes via inverted 16 ml Wheaton tubes with the open end flush with the nylon net covering the experimental cages. This ensured that mosquitoes had easy access to the sugar solutions at all times. All sugar solutions were dyed with 0.05% w/v Congo red. The presence of the red dye in the crop of mosquitoes indicated that mosquitoes fed on the sugar solutions. Only those that fed were included in the analyses. Levels of salivary lytic activity were assayed in all treatments at intervals of 2 to 3 days for a 15-day period. Ten mosquitoes per treatment were salivariectomized at each day of observation. Mortality in each treatment was also noted.

RESULTS

Bacteriolytic factor and blood feeding

The bloodfed group of mosquitoes showed increases in their salivary bacteriolytic activity starting on day 5, the day of the bloodmeal, and maintained higher levels throughout the remainder of the experiment (Fig. II.1). Salivary bacteriolytic activity increased on days 6 and 7, and fluctuated at that high level until day 10, the day of oviposition and the last day of sampling. The bloodfed mosquitoes showed an average increase of 55% over the non-bloodfed ones.

Before blood feeding, the two treatments showed variable daily fluctuations of salivary bacteriolytic activity but after day 5, the non-bloodfed group showed a generally low level of bacteriolytic activity while the bloodfed group maintained their bacteriolytic activity at a high level.

Salivary bacteriolytic activity in the presence of bacteria in the sugar meal

Table II.1 presents the mean levels of salivary bacteriolytic activity of mosquitoes exposed to different concentrations of Micrococcus lysodeikticus in their sugar meal. The corresponding Duncan groupings for the different treatments show that the presence of higher levels of M. lysodeikticus up to 0.05% w/v does not have a statistically significant effect on the levels of salivary lytic activity. However, the mean salivary bacteriolytic activity of mosquitoes exposed to higher quantities of bacteria were correspondingly higher than that for mosquitoes not exposed to any bacteria in their sugar meal. Only after day 10 was the salivary lytic activity of the group exposed to the highest concentration of bacteria consistently higher than the other treatment groups, and this increasing trend toward the end of the experiment, is also suggested by the regression lines presented in Figure II.2. Mortality data for all the treatments are presented in Table II.2. It is apparent that the group exposed to

the highest concentration of bacteria had the highest mortality and that this mortality occurred early in the experiment. Later, mortality in the high treatment group approximated that of the other treatments.

DISCUSSION

Bacteriolytic factor and blood feeding

The data suggest that bloodfed mosquitoes generally have higher levels of the bacteriolytic factor in their salivary glands than non-bloodfed mosquitoes no matter what the levels of the salivary bacteriolytic activity were before the bloodmeal. This is surprising in view of the virtual absence of feeding activity in bloodfed mosquitoes especially Aedes aegypti (Foster, 1986). This increase in the levels of the lytic factor may be due to either or both the induction of higher salivary protein synthetic rates by blood feeding or simply the accumulation of the lytic protein in the salivary glands because of the absence of feeding. Because of the gradual increase in the level of the salivary bacteriolytic factor, it is more likely that the increase is due to accumulation. It appears that there is a continuous synthesis of the bacteriolytic factor whether the mosquito is feeding or not, and this synthesis may be independent of the feeding state of the adult mosquito. In Rhodnius prolixus, Ribeiro and Pereira (1984) showed that a bloodmeal in the gut induces the

presence of glycosidases (including the bacteriolytic enzyme lysozyme) in the crop and intestine. However, they did not determine the source of the glycosidase activity or whether the enzymes were synthesized de novo. This study demonstrates that a bloodmeal results in higher levels of bacteriolytic activity in the salivary glands of Aedes aegypti during the gonotrophic cycle. It remains to be shown however, whether a bloodmeal also results in a higher bacteriolytic activity in the mosquito gut.

A lower salivary protein content has been reported in mosquitoes after blood feeding and this is due to either or both a lowered protein synthesis (Racioppi and Spielman, 1986) or the emptying of the contents of the salivary glands whether the mosquito mouthparts are placed in oil, sugar or in a vertebrate host (Racioppi and Spielman, 1987). Mosquitoes regulate salivation (Spielman et al., 1986; James et al., 1990) but electrophoretic evidence shows that a third of total (Poehling, 1979) and individual salivary proteins (Racioppi and Spielman, 1987) are lost during blood feeding. Racioppi and Spielman (1987) showed that adult mosquitoes continuously synthesize salivary protein and although these authors did not determine the nature of the proteins synthesized, it is apparent from this study that bacteriolytic factor may be one of the continuously synthesized salivary proteins.

Salivary bacteriolytic activity in the presence of bacteria in the sugar meal

Insects have the cuticle and other passive structural barriers against penetration of the hemocoel by pathogens and endoparasites (Dunn, 1986). In addition, the hemolymph of insects contains immune proteins including low levels of the bacteriolytic enzyme, lysozyme. The levels of these immune proteins increase manyfold following infection by bacteria (Boman et al., 1986; Dunn, 1986). This immune response was investigated by injecting foreign substances including bacteria into the insect hemolymph.

Another route of entry of pathogenic organisms may be through the ingestion of contaminated food. A bacteriolytic factor in the salivary gland may be one mechanism of defense against a pathogenic invasion via the oral route.

In these experiments, mosquitoes fed sugar without bacteria had the lowest mean level of salivary lytic activity while mosquitoes exposed to higher concentrations of Micrococcus lysodeikticus correspondingly increased the levels of bacteriolytic activity in their salivary glands. The differences were, however, not statistically significant. This may be due to several reasons including 1) M. lysodeikticus is not a pathogenic bacterium, and this is evidenced by the low mortality rates observed even after prolonged exposure of the mosquitoes to bacteria; the high

initial mortality observed in the group given the highest quantity of bacteria may be due to other causes, 2) the quantities of bacteria included in the sugar meal were too low to induce higher rates of the bacteriolytic enzyme, and, 3) mosquitoes may not respond to the presence of bacteria in their meal. More studies are therefore needed to determine whether mosquitoes and other insects respond to an oral challenge of microorganisms, especially those that are potentially pathogenic.

Table II.1. Mean levels of salivary bacteriolytic activity of mosquitoes exposed to different concentrations of Micrococcus lysodeikticus in their sugar meal.

% Bacteria (w/v)	N	Mean ±S.E.	Duncan grouping*
0.00	85	0.27 ±0.02	A
0.05	38	0.28 ±0.02	A
0.50	39	0.32 ±0.05	A

* Means with the same letter are not significantly different at $\alpha = 0.05$.

Table II.2. Percentage mortality of mosquitoes exposed to different concentrations of Micrococcus lysodeikticus in their sugar meal.

% Bacteria (w/v)	Duration of exposure (days)			
	5	11	13	15
0.00	0	1.1	0	0
0.05	0	3.3	0	1.1
0.50	26.7	2.2	1.1	-

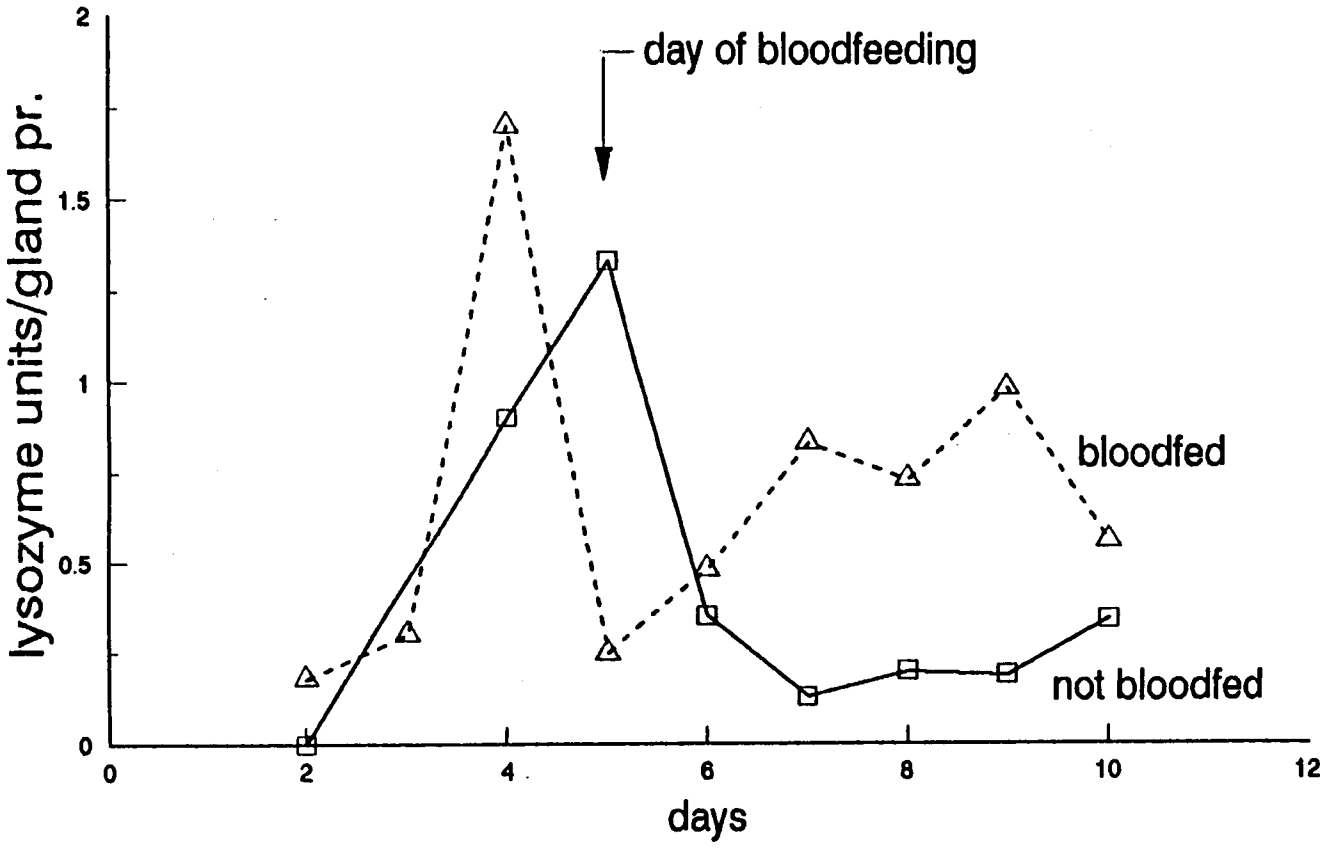

Figure II.1 Bacteriolytic activity in bloodfed and non-bloodfed mosquitoes.

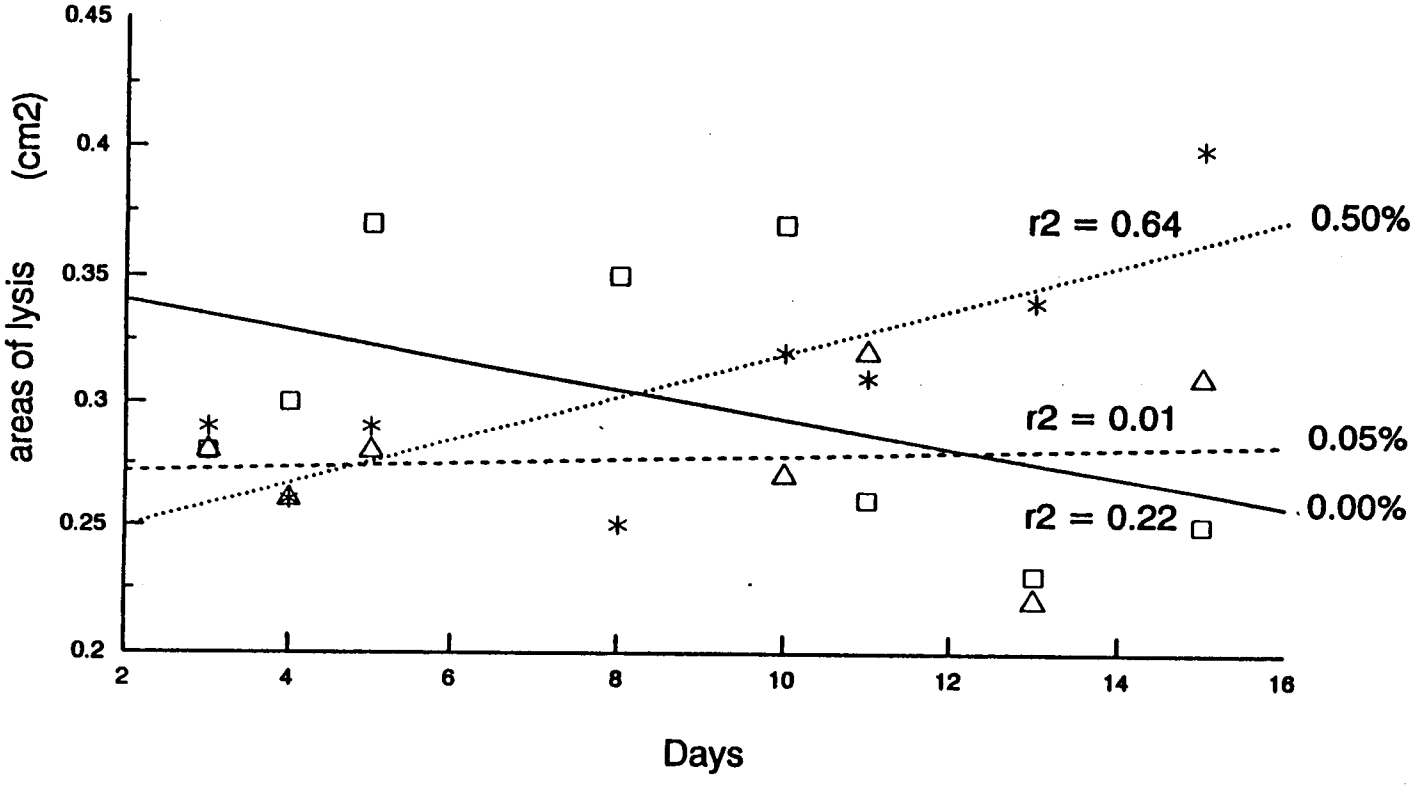

Figure II.2 Regression lines to show the trends in bacteriolytic activity levels in mosquitoes fed different concentrations of lyophilized Micrococcus lysodeikticus in their sugar meal.

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CHAPTER III

ISOLATION AND CHARACTERIZATION OF A LYSOZYME-LIKE PROTEIN IN THE SALIVARY GLANDS OF ADULT AEDES AEGYPTI

INTRODUCTION

Rossignol and Lueders (1986) demonstrated the presence of a bacteriolytic enzyme in the salivary glands of adult Aedes aegypti. It was lysozyme-like in its pH of optimum activity and in its ability to lyse Micrococcus lysodeikticus cells although the products of the digestion of bacterial cell walls were not similar to those of digestion by hen egg white lysozyme in terms of their relative mobilities when subjected to thin layer chromatography. The possibility exists that this difference in the products of digestion may be due to other lytic enzymes present in whole mosquito salivary gland homogenates which may have interfered with the digestion or may have further digested the products from the salivary bacteriolytic enzyme. It is therefore necessary to isolate this protein from other salivary gland components before a full characterization of its activity is possible.

MATERIALS AND METHODS

Mosquitoes

Mosquitoes used were Aedes aegypti (Georgia strain). Rearing procedures have been described (Pimentel and Rossignol, 1990). Only female mosquitoes were used.

Preliminary experiments

I. Centrifugation

To have an idea of the molecular weight of the salivary bacteriolytic factor, mosquito salivary glands were centrifuged at 13000g for various lengths of time through a cellulose acetate filter (PGC cat. #342-140) with molecular weight cut-off of 5 kD. The fraction retained and the filtrate were assayed for lytic activity using the lysoplate method as in Pimentel and Rossignol (1990).

II. Non-denaturing Electrophoresis

This was done to determine the behavior of the native protein when subjected to an electric field under different conditions of pH and gel concentrations. This behavior gives an idea of the mass and net charge of the native protein and thus some possible method(s) of isolation. Based on the method of Hultmark et al. (1980), non-denaturing electrophoresis could also be used as an isolation method. The polyacrylamide gel after non-denaturing PAGE is overlaid with an agarose gel (0.7% in 0.067M KH_2PO_4-Na_2HPO_4, pH 6.2) containing lyophilized Micrococcus lysodeikticus (1.0 mg/ml) and incubated for at

least 24 hours at 31 C. The bacteriolytic protein is then located where lysis occurs on the overlaid agarose gel. A piece of the polyacrylamide gel at that location is collected and the protein(s) eluted and subjected to SDS-PAGE.

Two different non-denaturing electrophoresis methods were used. One method is described in the Hoefer Manual (1989) for the separation of serum proteins in their native state. A discontinuous buffer system was used (lower chamber: 63 mM tris, 50 mM HCl, pH 7.47; upper chamber: 37.6 mM tris, 40 mM glycine, pH 8.89). The upper buffer is the cathode and the lower buffer is the anode. The other method is that of Reisig et al. (1962) for the separation of basic proteins. This is a discontinuous polyacrylamide gel electrophoresis wherein both the gel and the buffers are pH 4, the upper buffer is the anode and the lower buffer is the cathode. The running conditions will be indicated for each run, all gels are 8 x 10 cm, 0.75 mm thick.

Purification of salivary lysozyme

A combination of cation exchange chromatography on carboxymethylcellulose, gel filtration on Sephadex G-50 and SDS-PAGE was used to isolate this salivary protein. The lytic activity of samples was determined by the incubation with Micrococcus lysodeikticus (lysoplate method) as described in Pimentel and Rossignol (1990).

I. Cation Exchange Chromatography

A. Without Acidification

This method was adapted from Jolles et al. (1962). Samples ranging from 50 to 88 pairs of salivary glands in 10.0 ul distilled water per pair were centrifuged at 13000g for 30 minutes at 4 C. The supernatant was collected, its initial lysozyme level determined and the rest of the sample applied to a column of carboxymethylcellulose (CMC, 13 x 0.75 cm. diam., 3.0 ml bed volume) previously equilibrated at least overnight with 0.01M Na_2HPO_4, pH 5.5. Fractions were immediately collected and the sample was eluted with a gradient of concentration (0.01 to 0.5 M Na_2HPO_4) and pH (5.5 to 7.5). A total of 80 fractions were collected for each run; the fraction volumes and the total buffer volume used were also noted.

The presence of protein in the fractions was determined by optical density readings at 280 nm using a solution of 50.0 ul sample and 150.0 ul distilled water. Ten ul from the peak fraction and two or three fractions before and after the peak were checked for lytic activity by the lysoplate method. If positive for lytic activity, the fractions were pooled and subjected to SDS-PAGE to determine purity and, if impure, further purified by passing the sample through a column of Sephadex G-50 using the same buffer throughout the run. The A280 and the lytic activity of the fractions collected from the SG-50 column

were again determined by the lysoplate method and, if positive for lysis, the fractions were concentrated by centrifugation through a cellulose acetate filter with molecular weight cut-off (MWCO) of 5 kD. The samples were again subjected to SDS-PAGE to determine purity. Molecular weights were also determined by running molecular weight standards along with the sample containing the bacteriolytic protein.

B. With Acidification

The general procedure was adapted from Zachary and Hoffman (1984) except that the samples were only acidified to 0.20 M instead of 0.25 M. Samples of salivary glands ranging from 100 to 225 pairs in 10.0 ul distilled water per pair were pooled, acidified with 1.0 M acetic acid to a final concentration of 0.20 M and boiled for 5.0 min. After cooling and centrifugation (13000g, 20 min.), the supernatant was collected and diluted with an equal volume of 0.4 M ammonium acetate, pH 6.5. This solution was applied to a CMC column (13 x 0.75 cm diam., 3.0 ml bed volume) equilibrated for at least two days with 0.4 M ammonium acetate, pH 6.5. The sample was eluted with a gradient (0.4 to 3.0 M) of ammonium acetate at a constant pH of 6.5. Eighty fractions were collected for each run; the fraction volume for each run ranged from 5 to 10 drops. Bacteriolytic activity was assayed by the lysoplate method as in Pimentel and Rossignol (1990) except that Micrococcus

lysodeikticus in the agarose was increased to 0.25 mg/ml. Active fractions were pooled, lyophilized and kept at -85 C until needed.

II. Gel Filtration

Gel filtration on Sephadex G-50 was done on pooled CMC fractions to further purify the sample or to confirm the molecular weight obtained from SDS-PAGE. A Sephadex G-50 column (9 x 0.75 cm diam., 5 ml bed volume) was equilibrated with 0.4 M ammonium acetate, pH 6.5 for at least three days. For confirmation of the molecular weight (MW) obtained from SDS-PAGE, samples containing the pure protein were passed through a SG-50 column (9 x 0.75 cm diam., 3 ml bed volume) standardized by determining the elution volumes of blue dextran and other proteins namely chicken serum albumin (MW=45 kD), carbonic anhydrase (MW=29 kD), cytochrome C (MW=12.4 kD) and aprotinin (MW=6.5 kD); volume for all standards and unknown samples was 50.0 ul. The protein was eluted with the equilibration buffer. Fraction volumes and total number of fractions were noted for each run. The A280 was determined and the lytic activity of the peak fractions assayed.

III. SDS-PAGE

Polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) was used to determine purity of the samples from the different chromatography runs, and also to determine the molecular weight of the proteins

separated. The procedure was adapted from Laemmli (1970). The stacking gel was 2.7%T (T, total acrylamide), pH 6.8, followed by a separating gel, either 10 or 15%T, pH 8.8. The running buffer consisted of 0.025 M tris pH 8.3, 0.192 M glycine, with 0.1% SDS. A constant current (10 mAmp per 8 x 10 cm. gel) was applied until the tracking dye, bromophenol blue, was 0.5 cm from the gel bottom.

To locate the proteins in the gel silver staining was done using Sigma^R Silver Stain AG-25 kit. The relative mobilities of the proteins were then compared with those of molecular weight standards run on the same gel.

RESULTS

Preliminary experiments

I. Centrifugation

The results of centrifugation through a cellulose acetate filter with a MWCO of 5 kD are shown in Table III.1. Lytic activity was restricted to the fraction that did not pass through the filter (retentate). Thus the bacteriolytic protein must have a molecular weight above 5 kD. SDS-PAGE (15%T) of the retentate showed almost identical bands as in unfiltered (MWCO=5kD) mosquito salivary glands (Fig. III.1). Most of the proteins in the adult mosquito salivary glands therefore have molecular weights greater than 5 kD.

II. Non-denaturing electrophoresis

Early attempts to isolate the bacteriolytic protein was based on the method of Hultmark et al., (1980) which involves separating a protein mixture through a polyacrylamide gel under non-denaturing conditions, then incubating the gel with an agar gel containing lyophilized cells and cell walls of Micrococcus lysodeikticus. The lytic protein is then located where lysis on the overlaid agar gel occurs.

This procedure was done not only in an attempt to isolate the protein(s) but also to observe the behavior of the native protein under different conditions of pH to obtain an indication of its native charge. This information was of importance in the determination of the method(s) to be used for isolation and purification of the bacteriolytic protein.

A. With Normal Polarity

Hen egg white lysozyme (HEWL) and mosquito salivary gland homogenates (MSGH) (Fig. III.2) were subjected to non-denaturing electrophoresis according to the method described in the Hoefer Manual (1989) for separation of serum proteins. A discontinuous buffer system was used (lower chamber, LC, pH 7.47; upper chamber, UC, pH 8.89) and the gels were run at 7 ma per 2 gels for 3 hours at room temperature.

Overnight incubation with an agar gel overlay showed

lysis only at the origin of the gel. The proteins, including the pure sample of HEWL did not migrate into the gel, thus at the buffer pH used, the bacteriolytic protein(s) must have a net positive charge (like HEWL) and therefore would not migrate toward the anode. Consequently, non-denaturing electrophoresis with reversed polarity was tried.

B. With Reversed Polarity

HEWL and MSGH were subjected to non-denaturing PAGE as in Figure III.2, except that the electrodes from the power supply were reversed and the run was for only 2.5 hrs. Only HEWL migrated into the gel (Fig. III.3) while the salivary lytic protein remained at the origin as evidenced by the lysis on the overlaid agar gel containing M. lysodeikticus.

From these two electrophoretic runs, it can be concluded that, at the pH of the buffers used (8.89 LC, 7.47 UC), the salivary lytic protein(s) must be around the isoelectric pH, thus possess a net zero charge and therefore would not move to either anode or cathode when subjected to an electric field. The salivary bacteriolytic protein therefore appears to be a basic protein.

C. Normal Polarity in Acidic or Basic Conditions

This was done to confirm the basic nature of the bacteriolytic protein and at the same time determine the best pH conditions for the electrophoretic migration of the

lytic proteins. The same buffers were used except for the pH. Gel A was subjected to a pH 4.03 UC buffer (pH adjusted with HCl) while gel B was subjected to a pH 9.03 UC buffer (Fig. III.4). Lower chamber buffers were the same for both gels. Current was maintained at 11 ma for 2 gels; this was increased to 21 ma after 1 hr because the dye front, a combination of bromophenol blue and methyl green was not migrating for both gels. The increase in the applied current forced the dye front to move into the gel.

Overnight incubation with agar gel overlay showed lytic activity for both gels with gel A showing lysis in the whole lane while gel B showed only one spot of lysed area. Gel A shows a possible contamination of the whole lane rather than a migration of the lytic proteins. Gel B showed a migration of the protein components because of the presence of areas of concentrated lysis. Thus, basic conditions favor migration of the salivary lytic protein to the anode. At pH 9.03, the lytic protein must therefore have a negative charge.

The migration of the lytic protein under basic conditions agrees with the previous finding that the pI of the lytic protein(s) must be in the pH range 7.47 to 8.89 and therefore at pH 9.03 the protein(s) would have a net negative charge, thus would tend to migrate toward the anode. The center of the area of lysis on the gel B was collected (sample M, for middle) for further analysis by

SDS-PAGE.

D. Non-denaturing Electrophoresis in Acidic Conditions

To investigate further the results obtained in gel A, the salivary gland homogenates were run at pH 4.03 according to the method of Reisig et al. (1962) with normal polarity for 3 hours. Overnight incubation with substrate showed no lysis anywhere on the overlaid agar gel. Another run with reversed polarity and pH 4.03 showed lysis only at the origin of the gel. There was some degree of separation because some proteins did migrate into the gel toward the anode but the lytic protein(s) stayed at the origin (Fig. III.5). This is further evidence that the lytic protein is indeed a basic protein; at pH 4.03, it has a net positive charge and therefore would not move toward the anode. A piece of the gel origin, approximately 2 x 5 mm, was collected (sample O, for origin) for further analysis by SDS-PAGE.

The results from the non-denaturing PAGE of the MSGH demonstrate that at pH 4.03, the bacteriolytic protein has a net positive charge and therefore diffused into the upper buffer chamber during normal electrode electrophoresis, thus lysis was not observed at all in the gel. However, the proteins did have the tendency to move toward the cathode during reverse electrophoresis, thus did not diffuse into the upper chamber but remained on the gel

origin. Three hours was apparently not long enough for the bacteriolytic protein to migrate into the gel, and it therefore barely migrated from the origin.

III. SDS-PAGE

Pieces of the non-denaturing polyacrylamide gels (samples O and M) that showed lysis on the agar gel overlay were macerated, redissolved and subjected to SDS-PAGE (15%T) resulting in several bands with molecular weights ranging from 14.3 to greater than 66 kD (Fig. III.6 and Table III.2). The bands in the two samples were relatively consistent with each other.

The results of these preliminary experiments indicate that the mosquito salivary bacteriolytic protein(s) has a molecular weight greater than 14.3 kD. It is a basic protein, with a pI between 7.47 and 8.89, thus it is negatively charged at pH 9.03 and positively charged at 4.03 and will move accordingly when subjected to an electric field during non-denaturing electrophoresis.

Isolation and purification

Because the bacteriolytic protein(s) has a positive charge below pH 7.5 (the usual pH used for separation of proteins), the method of isolation chosen is cation exchange chromatography. This is also the method usually used for the isolation of lysozymes from other sources (e.g. Jolles et al, 1962; Li-Chan et al., 1986).

The general procedure involves fractionating the

mosquito salivary gland homogenates by passing the sample through a carboxymethylcellulose (CMC) column, a cationic exchanger. Each resulting fraction is assayed for the presence of protein by its absorbance at 280 nm. The peak fractions and those around them are assayed for lytic activity, and the active fractions either subjected immediately to SDS-PAGE to determine purity or further purified by passage through a column of Sephadex G-50 (SG-50). This is a molecular sieve and will sort the components of the fractions according to their molecular weight and shape. The A280 of the SG-50 fractions are again determined, their lytic activities assayed. Positive fractions are then subjected to SDS-PAGE to determine purity. If the sample shows only one band after SDS-PAGE, the molecular weight is determined by comparing its relative mobility to the relative mobilities of proteins of known molecular weights run on the same gel.

The methods used were either modified from Jolles et al., (1962) or Zachary and Hoffman (1984). These different cation exchange methods differed in two major aspects: 1) the pre-treatment of the salivary gland homogenates before application to the CMC column; the Zachary and Hoffman procedure involved an acidification of the sample; and 2) the composition of the buffer solutions and the gradients used to elute the lytic proteins from the CMC column.

I. Modified Jolles Method

This method was modified from Jolles et al. (1962). Figure III.7 contains a flow diagram of the method modified as a result of several trials. Eighty fractions were collected, 4 drops per fraction using 11.0 ml total elution buffer with a concentration gradient from 0.01 to 0.1M Na_2HPO_4, pH gradient 5.5 to 7.5, elution rate 55 drops/min. The MSGH did fractionate according to their affinity with the carboxymethylcellulose (Fig. III.8), but lysoplate assay of the fractions produced negative results. The original sample (50 prs salivary glands in 50 ul phosphate buffer: 0.067 M KH_2PO_4-Na_2HPO_4, pH 6.2) had a lytic activity comparable to 0.1 unit of HEWL. None of this lysozyme activity was recovered in any of the fractions. Possible reasons may be, 1) the protein in the fractions was too dilute; or 2) the protein was denatured during the separation due to buffer, pH, length of chromatography time, temperature, etc.

SDS-PAGE (10%) of the A280 peak fractions (1, 3, 5, 33, 45) did not show any bands after silver staining. The proteins were either too dilute for detection or too small to be resolved by 10% polyacrylamide gels. A trial run with the standards alone using 20% gel resulted in a very brittle gel and the proteins stacked around the origin. A 15% polyacrylamide gel was therefore formulated and determined to give the best resolution especially for the

lower molecular weight proteins.

Jolles' procedure was further modified in the following ways: 1) increased number of salivary glands in the sample (88 prs in 100 ul Hayes saline), 2) sample centrifuged for one hour to remove all impurities and cell wall debris, 3) lesser gradients of concentration and pH (Table III.3), 4) slower elution rate: 30 drops/min.

Peak A280 were in fractions 15, 20, 27, and 53 (Fig. III.9). Fractions before and after these were saved for further study. All saved fractions were negative for lytic activity but SDS-PAGE (15%) of fractions 15, 20, 27, and 53 showed several bands ranging from 27.5 to 65 kD (Table III.4). The separation was therefore not very efficient and because of the apparent loss of activity after elution, the method was not used in further trials.

II. Modified Zachary and Hoffman Method

A. Preliminary Trials

From the preceding chromatography runs, it became apparent that the buffer system somehow caused a loss in the activity of the lytic protein(s). A search for another method which allows stabilization of the protein(s) at low pH (because lysozymes are known to be thermostable at low pH) resulted in the use of the method of Zachary and Hoffman (1984). Figure III.10 shows a flow diagram of the method.

One chromatographic run was made following the method

of Zachary and Hoffman (1984). 235 pairs of salivary glands in 1.50 ml distilled water was acidified to 0.25 M by the addition of 1.0 M acetic acid. The results are presented in Figure III.11. Lytic activity was eluted at buffer concentrations of 0.4 to 1.2 M ammonium acetate, but none of the fractions showed any bands in SDS-PAGE after silver staining. To concentrate the proteins, the fractions were lyophilized and evaporated to dryness and subsequently reconstituted. The fractions showed a whitish precipitate that would not dissolve and under SDS-PAGE (15%T), none of the proteins migrated into the gel. The precipitate probably interfered with the proteins in the fractions.

Another run with the same method was done on 200 pairs of salivary glands in 1.8 ml distilled water but this time, acidified to 0.5 M. The results are presented in Figure III.12. Lytic activity was eluted using a buffer gradient from 0.4 to 2.0 M ammonium acetate. However, the proteins would not resolve in SDS-PAGE even with further concentration by lyophilization and reconstitution of the samples. The white precipitate was again observed, especially after lyophilization.

The third run with the same method was done using 400 pairs of salivary glands in 5.8 ml distilled water acidified to 0.4 M. The results are presented in Figure III.13. Lytic activity was recovered in the fractions,

during a buffer gradient of 0.4 to 1.6M ammonium acetate. The proteins still would not resolve or even migrate into the gel. The white precipitate was again present. It is possible that this white precipitate is composed of proteins clumped together because of high acidification of the mosquito salivary gland homogenate (MSGH), thereby preventing migration of individual proteins into the separating gel.

It is apparent from the preceding experiments that the acidification of the sample to 0.25M acetic acid and higher may be causing the proteins to precipitate from solution. A lower acid concentration was then necessary to keep the proteins in solution for them to be resolved in SDS-PAGE. As will be shown in subsequent results, acidification of the MSGH to 0.2M was determined to be optimal for keeping the proteins in solution.

B. Trial One

The results of this chromatography are shown in Figure III.14. Only the peak fractions (1, 15, 20, 43, 45, 50) were tested for lytic activity (Table III.5). Fractions 15 and 20 showed lytic activity corresponding to <0.1 unit of HEWL. To confirm the presence of lytic activity, the peak fractions were concentrated by centrifugation through cellulose acetate (MWCO = 5 kD) for 2.5 min at 13000g. The results are presented in Table III.6. Fractions 15 and 20 were indeed bacteriolytic and following concentration, both

fractions had increased lytic activity to approximately 0.1 unit of HEWL. With 15% concentration, fraction 1 has also become lytic. This result may have been due to contamination from the cellulose acetate filter not thoroughly washed after centrifugation of the lytic samples. Fractions 15, 20 and 43, 45 and 50 as control were subjected to non-denaturing polyacrylamide electrophoresis (7%) in acid conditions for 3 hrs, but after overnight incubation with agar gel overlay did not produce any lysis. The fractions were probably too dilute in the lytic proteins. With SDS-PAGE (15%T), only fraction 15 yielded bands corresponding to molecular weights of 29,000 and 34,000 D (Fig. III.15).

Inspite of the presence of lytic activity as determined by the lysoplate assay, non-denaturing PAGE of fractions 15 and 20 using the Hoefer method did not produce any lysis after incubation with an agar gel overlay. To investigate this further, another method of non-denaturing PAGE was tried. Fractions 15 and 20 were subjected to non-denaturing PAGE according to the method of Gabriel (1971). This electrophoresis was done twice, and on both times, no lysis was observed after incubation with an agar gel overlay.

To determine the efficiency of separation of the lytic proteins by means of the Zachary and Hoffman (1984) method, some fractions were subjected to SDS-PAGE (15%T) (Fig.

III.16). The O-sample was again electrophoresed together with these fractions. Indeed, lytic fraction 15 and fractions around it (14 and 16) had two bands corresponding to those of the lytic O-sample (Table III.7).

Bands with Rf 0.28 and 0.36 corresponding to molecular weights of 38,000 and 31,000 respectively are common to all the samples; band 28 is the most prominent in fractions 14, 15 and 16. Although fractions 14 and 16 were not checked for lytic activity, fraction 15 and the O-sample were positive for lytic activity, and therefore, the lytic protein(s) may be both or either of the 31 kD and/or the 38 kD protein.

To further purify the protein, samples around fraction 15 that had an A280 greater than 0.01 were pooled. Total volume of the pooled sample was 1.41 ml, A280 of 0.057. This was passed through a SG-50 column, 3.0 ml bed volume, 7 drops per min using 4.0 ml of 0.4 M ammonium acetate buffer. Each SG-50 fraction was 5 drops and the A280 of 125.0 ul subsamples of some of the fractions were determined (Table III.8). Lytic activity was present in all the assayed fractions (20 to 26). SDS-PAGE (15%T) of 15.0 ul subsamples to determine the purity of the SG-50 fractions resulted in faint bands around 66 kD (Fig. III.17). There are two possible explanations for this result: 1) the lytic proteins dimerized upon passage through SG-50 - lysozymes have been observed to dimerize in

the presence of glucose (Cho et al., 1984); 2) the two bands are impurities because the pooled CMC fractions that were positive for lytic activity contained only proteins with a maximum of 38 kD. Moreover, in SDS-PAGE, all the samples were treated with ß-mercaptoethanol, a reducing agent, thus eliminating the possibility of a dimer connected by disulfide bonds. Even the lanes for the molecular weight markers also showed the faint bands, thus these are definitely impurities. No other bands were observed probably because the samples were too dilute in the proteins after passage through SG-50 chromatography. The fractions were therefore concentrated by centrifugation through cellulose acetate (MWCO = 5 kD) (Table III.9) in preparation for SDS-PAGE. Figure III.18 shows several bands in SG-50 fractions 11 and 21, but all of the fractions again showed the 66 kD impurity band.

C. Trial Two

One hundred pairs of salivary glands (775 ul) were acidified to 0.20 M and subjected to CMC chromatography by the Zachary and Hoffman (1984) method. The results are presented in Figure III.19. Lytic fractions were from 29 to 51, eluted with a buffer gradient of 0.8 to 1.2 M ammonium acetate, 0.4 M, pH 6.5. However, SDS-PAGE (15%) showed that all of the fractions contained several proteins (Fig. III.20). Because of the apparent loss of the protein as it passed through SG-50, no further purification of the

samples was attempted.

D. Trial Three

The purpose of this run was to use more salivary glands in the CMC chromatography so that enough of the active lytic protein could still be recovered after passing through SG-50.

One hundred and twenty six pairs of salivary glands in 1,200 ul distilled water were subjected to CMC chromatography as in the first runs. The sample was eluted with the buffer conditions as shown in Table III.9. The results are presented in Figure III.21. Fractions 26 to 65 were lytic; the lytic protein eluted at a buffer gradient of 0.8 to 1.6 M ammonium acetate. Groups of fractions were pooled for further purification: 26 to 30, 31 to 40, and 41 to 55. The A280 of these pooled samples ranged from 0.014 to 0.002. Each pooled sample was passed through the same SG-50 column used in the first trial, and the conditions of the elution are shown in Table III.9. A 100.0 ul subsample of the resulting SG-50 fractions was centrifuged through cellulose acetate filter with MWCO of 5,000 D until the indicated concentrations, then assayed for lytic activity (Table III.10).

SDS-PAGE (15%) of centrifuged and uncentrifuged SG-50 fractions from each pooled CMC sample are shown in Figure III.22. Gel A showed high molecular weight bands of 58 kD and 68 kD. Gels A and B showed the same intensity of

staining inspite of the fact that gel B samples were concentrated by centrifugation; bands that developed in B had molecular weights higher than 66 kD.

Some of the SG-50 samples were evaporated to almost dryness at -20 C and reconstituted as indicated in Table III.11. Lytic activity is also presented. SDS-PAGE (15%) reveals that the samples were not pure and contained only high molecular weight proteins (Fig. III.23).

E. Trial Four

The sample consisted of 255 pairs of salivary glands in 2.35 ml distilled water. This chromatography was done with twice the volume of 0.4 M ammonium acetate used in the other runs. From previous runs, it was observed that the lytic protein eluted starting at a buffer concentration of 0.8M ammonium acetate, and therefore, an additional volume of 0.4M buffer will serve as a wash medium to remove all proteins not bonded by the CMC cation exchanger. This will therefore eliminate other proteins that may appear as impurities when the CMC fractions are subjected to SDS-PAGE. The results of the CMC run are presented in Figure III.24. Lytic activity was observed in fractions 28 to 64, (except 50 to 59) corresponding to a buffer gradient of 0.4 to 1.6 M ammonium acetate. The early elution of the lytic protein may have been due to the overloading of the CMC column with the lytic protein thus elution started toward the end of the 0.4 M wash. Active fractions from 28

to 41 direct from CMC subjected to SDS-PAGE (15%), after silver staining, resulted in one faint band with a relative mobility corresponding to a molecular weight of 32.5 kD (Fig. III.25). All the other lytic fractions did not result in any bands; the protein may have been too dilute and this may be one reason for the apparent lack of lytic activity in fractions 50 to 59. Another reason may be the insensitivity of the lysoplate assay as a method of determining lysozyme activity.

CMC fractions 28, 29, 30, 31, 33 were pooled, lyophilized and reconstituted to 100 ul for SG-50 chromatography to confirm the molecular weight as obtained by SDS-PAGE (Table III.12). Run number 1 resulted in one actively lytic fraction with an apparent molecular weight of 39 kD.

CMC fractions 34, 35, 36, and 37 were pooled, lyophilized and reconstituted to 120.0 ul, of which 8.0 ul was incubated for assay of lytic activity, 12.0 ul was subjected to SDS-PAGE for purity and molecular weight determination, and two 50.0 ul subsamples were used for SG-50 chromatography as a double check on the molecular weight obtained in run number 1. This pooled sample was highly lytic and gave a very distinct band after SDS-PAGE corresponding to a molecular weight of 36 kD (Fig. III.26). The two SG-50 runs did not show any protein bands on SDS-PAGE (Fig. III.26) although fraction 24 from run number 2 and fraction 27 from run number 3 were lytic and eluted

from the SG-50 column with apparent molecular weights of 33 kD and 21 kD respectively.

The mean molecular weight resulting from these three SG-50 fractionations of a pure sample of the lytic protein is 31 kD. From SDS-PAGE results, the mean molecular weight is 34.25 kD. These estimates of the molecular weight of the salivary bacteriolytic protein are relatively consistent with each other inspite of the different method of determination used. The unknown protein therefore has a molecular weight of approximately 31 to 35 kD.

DISCUSSION

Preliminary experiments

Using a centrifuge with a cellulose acetate filter, it was established that the bacteriolytic protein in the salivary glands of adult Aedes aegypti has a molecular weight greater than 5 kD. The banding pattern of SDS-PAGE-subjected samples of the centrifuge-filtered mosquito salivary glands was almost identical to unfiltered gland samples. Most of the proteins in the mosquito salivary glands therefore have molecular weights greater than 5 kD.

Non-denaturing polyacrylamide gel electrophoresis was used as a preliminary method to observe the migratory behavior of the native protein under different conditions of pH and direction of electrical polarity. This gave information on the native charge of the protein under

different pH conditions, and these data were vital for determining the method(s) of isolation used.

Non-denaturing electrophoresis was used with a discontinuous buffer system of different pH in the lower (7.47) and upper chamber (8.89). The proteins did not move from the origin under normal or reversed electrophoresis as evidenced by lysis of bacteria in the overlaid agar gel. This result indicated that the bacteriolytic protein must be around its isoelectric pH (pI) at the pH of the buffers used. Like hen egg white lysozyme, the mosquito salivary bacteriolytic protein is therefore a basic protein with a pI between 7.47 to 8.89.

The high pI of the protein was further evidenced by its having a positive charge when in an acidic environment (pH 4.03), and a negative charge when exposed to a basic environment (9.03). When exposed to an electric field, it therefore moved toward the cathode or anode depending on the existing pH conditions of a non-denaturing electrophoresis.

Isolation and purification

Cation exchange chromatography was the method of isolation used because the mosquito salivary bacteriolytic protein was determined to be of positive charge at pH below 7.5. Several methods and various modifications were tried with buffers of different compositions and pH. From these trials, it was determined that the protein was most stable

when it was first acidified before applying the sample into a cation exchange column. Like many lysozymes from other sources therefore (Jolles, 1969), the mosquito salivary bacteriolytic protein is thermostable at low pH as evidenced by the retention of lytic activity even after boiling of the mosquito salivary gland homogenates for 5 min. This thermostability at low pH enabled maintenance of lytic activity even after long periods of chromatography through either carboxymethylcellulose or Sephadex G-50. In this regard, it is lysozyme-like.

Therefore, the best method for isolating the bacteriolytic factor in the salivary glands of adult Aedes aegypti mosquitoes is a modification of the method of Zachary and Hoffman (1984). The mosquito salivary gland homogenate is first acidified to 0.2 M with 1.0 M acetic acid. Then, all other lytic proteins are denatured by boiling the acidified sample for 5 minutes. Centrifuging the sample for 20 minutes removes all denatured proteins and other debris. The supernatant is diluted with 0.4 M ammonium acetate before it is applied to a cation exchange column. The lysozyme-like protein generally eluted at a buffer gradient of 0.8 to 1.6 M ammonium acetate, pH 6.5.

This protein is lysozyme-like in its ability to lyse M. lysodeikticus, stability at high temperatures and low pH. Like lysozyme, it is a basic protein, its pI is between 7.47 and 8.89. As determined from SDS-PAGE of pure

fractions with lytic activity, the protein is composed of one polypeptide chain with an apparent molecular weight between 31 and 35 kD. Pieces of gels with lytic activity and were collected from non-denaturing electrophoretic runs contained a 33 kD protein (sample M) and a 31 kD protein (sample O).

Three methods of isolation, non-denaturing polyacrylamide electrophoresis, cation exchange chromatography on carboxymethylcellulose and SG-50 chromatography all resulted in lytic samples that contained a protein in the 31 to 35 kD molecular weight range. These independent methods of molecular weight determination therefore clearly demonstrated the presence of a lysozyme-like protein in the salivary glands of adult Aedes aegypti mosquitoes. This protein has a molecular weight approximately twice that reported for hen egg white lysozyme and other known lysozymes (Jolles, 1969). That the protein is a dimer is not a possibility because samples reduced by ß-mercaptoethanol showed the same relative mobility as those without the reducing agent when subjected to SDS-PAGE.

By the use of a combination of different methods - cation exchange chromatography on carboxymethylcellulose, gel filtration on Sephadex G-50, and denaturing (SDS) as well as non-denaturing polyacrylamide gel electrophoresis - the bacteriolytic protein in the salivary glands of adult Aedes aegypti was isolated and characterized.

This mosquito salivary bacteriolytic protein is lysozyme-like in several aspects: 1) it lyses bacterial cell walls of Micrococcus lysodeikticus, 2) it is a basic protein with a pI between 7.47 and 8.89, 3) it is thermostable at low pH and therefore maintains its lytic activity even after boiling as long as it is in an acid solution, 4) it is composed of one polypeptide chain. It has a molecular weight almost twice that of hen egg white lysozyme. This salivary bacteriolytic protein is the first exocrine lysozyme reported in an adult dipteran species.

Table III.1. Lytic activity of fractions of salivary glands centrifuged through a cellulose acetate filter (MWCO = 5 kD).

No. of Gland Pairs	Sample Volume (ul)	Volume Inoculated (ul)	Mean Diam. of Lysis (cm.)
5	50	5.0	0.50
effluent	15	4.0	0.00
retentate	25	4.0	0.85
10	50	5.0	1.35
effluent	20	4.0	0.00
retentate	20	4.0	1.40

Table III.2. Rf values and molecular weights of protein bands from samples O and M, pieces of polyacrylamide gels that contained the lytic protein(s) after non-denaturing electrophoresis.

Sample	Rf	Comments	Mol. Weight X 1000
O	0.03		>66
	0.19		56
	0.27	quite prominent	42
	0.33	quite prominent	36
	0.64	most prominent	14.3
M	0.03		>66
	0.13		66
	0.19		56
	0.27	quite prominent	42
	0.33	quite prominent	36
	0.60	quite prominent	15.5
	0.64	most prominent	14.3

Table III.3. Gradient of elution buffer, Na_2HPO_4, used in the modified Jolles method of cation exchange chromatography.

Initial			Modified		
Conc. (M)	pH	Vol. (ml)	Conc. (M)	pH	Vol. (ml)
0.01	5.5	1.0	0.01	5.5	1.0
0.05	6.5	1.0	0.025	6.0	1.0
0.1	7.5	1.0	0.05	6.5	1.0
0.01	5.5	8.0	0.075	7.0	1.0
			0.1	7.5	5.0
			0.5	8.4	1.6

Table III.4. Rf values and molecular weights of protein bands in fractions with peak A280 after elution of the mosquito salivary gland homogenate by the modified Jolles (1962) procedure.

Fraction	Rf	Mol. Weight X 1000
20	0.04	65
	0.10	50
	0.16	44
	0.25	35
	0.33	29
	0.35	27.5
53	0.10	50
	0.16	44
	0.25	35
	0.34	28

Table III.5. Lysoplate assay of peak A280 fractions eluted in the first trial using the modified Zachary and Hoffman (1984) method. Volumes for standard solutions of HEWL are 4.0 ul while volumes used for fractions are 10.0 ul each.

Sample/Fraction	Mean lysis diam. (cm)
HEWL, 0.1 unit	1.37
0.5	2.30
1.0	2.40
1.5	2.53
Fraction 1	0.0
15	0.83
20	0.87
43	0.0
45	0.0
50	0.0

Table III.6. Lysoplate assay of peak A280 fractions eluted in the first trial using the modified Zachary and Hoffman (1984) method. Samples were concentrated by centrifugation through a cellulose acetate filter (MWCO = 5 kD).

Fraction	Original Vol. (ul)	Final Vol. (ul)	% Conc.	Mean lysis diam. (cm)
1	65.0	55.0	15	1.35
15	65.0	33.0	46	1.83
20	55.0	43.0	22	1.10
43	33.8	22.0	27	0
45	65.0	50.0	23	0
50	75.0	40.0	47	0

Table III.7. Rf values and molecular weights of the different proteins eluted in the first trial using Zachary and Hoffman (1984) method and subjected to SDS-PAGE (15%).

Fraction/Sample	Rf	MW X 1000
14	0.20	48
	0.28	38
	0.36	31
15	0.28	38
	0.36	31
16	0.28	38
	0.36	31
Sample O	0.10	62
	0.17	51
	0.28	38
	0.36	31

Table III.8. Lytic fractions from SG-50 run of pooled samples from first trial using the Zachary and Hoffman method. Samples are concentrated in preparation for SDS-PAGE (15%).

Fraction	Original Vol. (ul)	Final Volume (ul)	% Conc.
11	100	40	60
19	150	100	33
21	103	45	56
22	103	70	32
23	103	70	32
24	100	54	46
25	90	50	44
26	100	75	25

Table III.9. Buffer conditions for elution of proteins in the third trial run using the modified Zachary and Hoffman method. constant pH (6.5) and a constant volume (3.0 ml) were used at each concentration. Sample fractionated until fraction 11.

Ammonium acetate (M)	Until Fraction No.
0.4	25
0.8	43
1.2	54
1.6	67
2.0	80

Table III.10. SG-50 fractions of pooled samples from the third CMC run using the modified Zachary and Hoffman method. Each fraction was originally 100.0 ul.

CMC samples pooled	SG-50 fractions	Final Vol. (ul)	% Conc	Mean lysis diam. (cm)
26 - 30	10	80	20	0.78
	11	40	60	0.68
	12	40	60	fungi infested
	13	62	38	0.68
	14	60	40	0.79
	15	80	20	0.69
	16	80	20	0.68
	17	80	20	0.30
31 - 40	30	70	30	0.75
	31	50	50	0.91
	32	50	50	1.08
	33	53	47	0.38
	34	72	28	1.33
	35	70	30	0.99
	36	63	37	0.72
	37	72	28	0.81
41 - 55	55	83	17	0.56
	56	70	30	0.68
	57	61	39	0.70
	58	90	10	0.53
	59	60	40	0.30
	60	90	10	0.58
	61	90	10	0.60
	62	90	10	0.55

Table III.11. SG-50 fractions lyophilized (-20 C) to almost dryness then reconstituted with 0.4 M ammonium acetate in preparation for SDS-PAGE (10% and 15%).

Fraction	Volume Added (ul)	Mean lysis diam. (cm)
16	35.0	0.79
17	50.0	0.74
35	40.0	1.08
36	35.0	1.08
58	45.0	0.91
59	35.0	1.10

Table III.12. Estimated molecular weights from peak fractions obtained from SG-50 chromatography of the pure sample of bacteriolytic protein obtained in the fourth trial CMC run.

Run Number	Fraction	Ve/Vo	Lytic Activity	MW X 1000
1	22.5	1.05	+	39
	26	1.21	-	24
	29	1.35	-	16.2
	31	1.44	-	12.2
2	24	1.12	+	24
	30	1.40	-	14.2
3	27	1.26	+	21

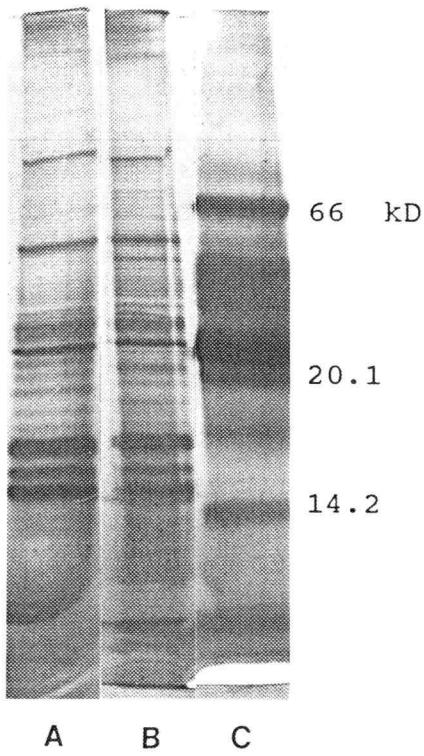

Figure III.1 SDS-PAGE (10%) of homogenates of mosquito salivary glands with (A) and without (B) centrifugation through cellulose acetate (MWCO = 5kD); C, molecular weight markers.

	Lane				
	1	2	3	4	5
Sample	HEWL	II	II	I	HEWL
Vol. (ul)	30.0	20.0	20.0	20.0	10.0
Initial Lysis Diam (cm) per 3.5 ul	2.75	0.75	0.75	0.35	2.75
	incubated with substrate				stained

Figure III.2. Lay-out for non-denaturing electrophoresis (7%) of hen egg white lysozyme (HEWL) and mosquito salivary gland homogenates, using normal electrode positions.

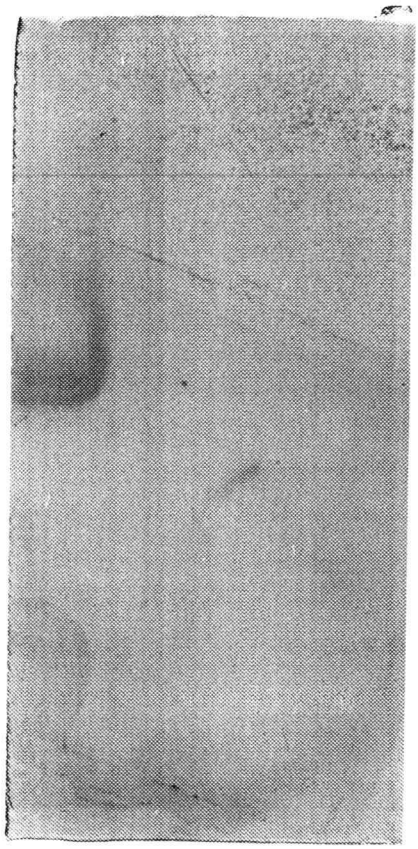

Figure III.3. Protein band of hen egg white lysozyme after non-denaturing electrophoresis using reversed electrodes. This indicates the location of the lysed areas in Micrococcus lysodeikticus-containing agarose gel overlaid on the polyacrylamide gel.

	lane 1	2	3	4	5
Gel A Vol. (ul)	15.0	15.0	none	5.0	none
Gel B Vol. (ul)	10.0	8.0	none	2.0	none
	incubated with substrate			stained	

Figure III.4. Lay-out for non-denaturing electrophoresis of salivary gland homogenates using acidic (pH 4.03, Gel A) and basic (pH 9.03, Gel B) buffers in the upper buffer chamber.

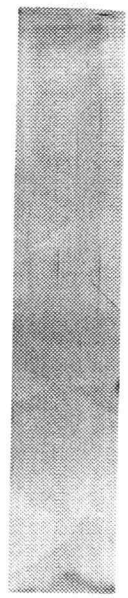

Figure III.5. Protein bands obtained after non-denaturing electrophoresis (reversed electrodes, pH 4.03) of a homogenate of mosquito salivary glands. Only the gel origin showed lysis in the overlaid agarose gel containing lyophilized cells of Micrococcus lysodeikticus.

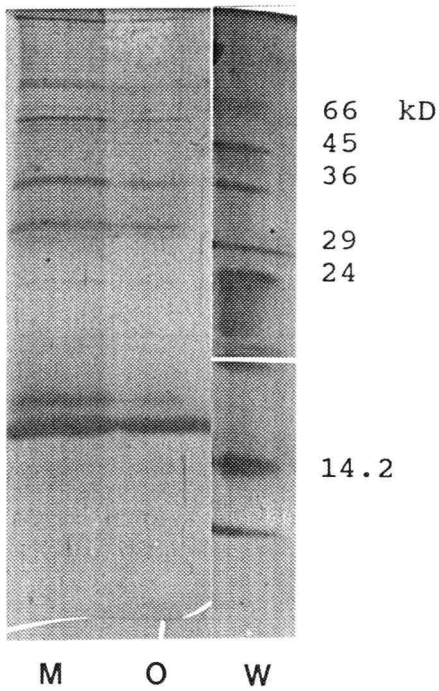

Figure III.6. SDS-PAGE (15%) of samples M and O that showed lysis in the overlaid Micrococcus lysodeikticus-containing agarose gel; W, molecular weight markers.

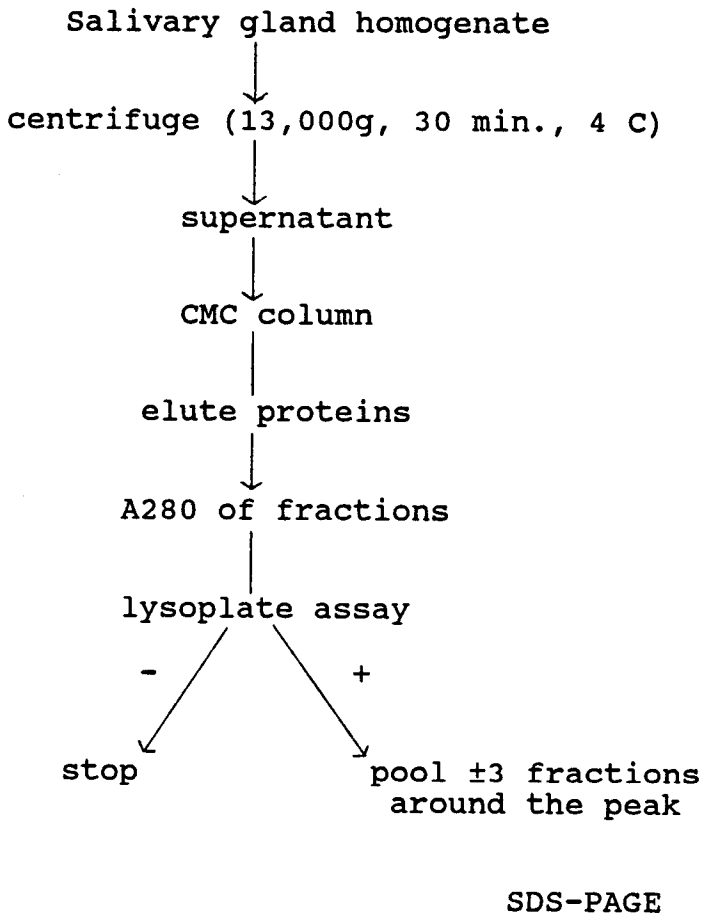

Figure III.7. Flow diagram of the isolation procedure using cation exchange chromatography on carboxymethylcellulose as modified from Jolles (1962).

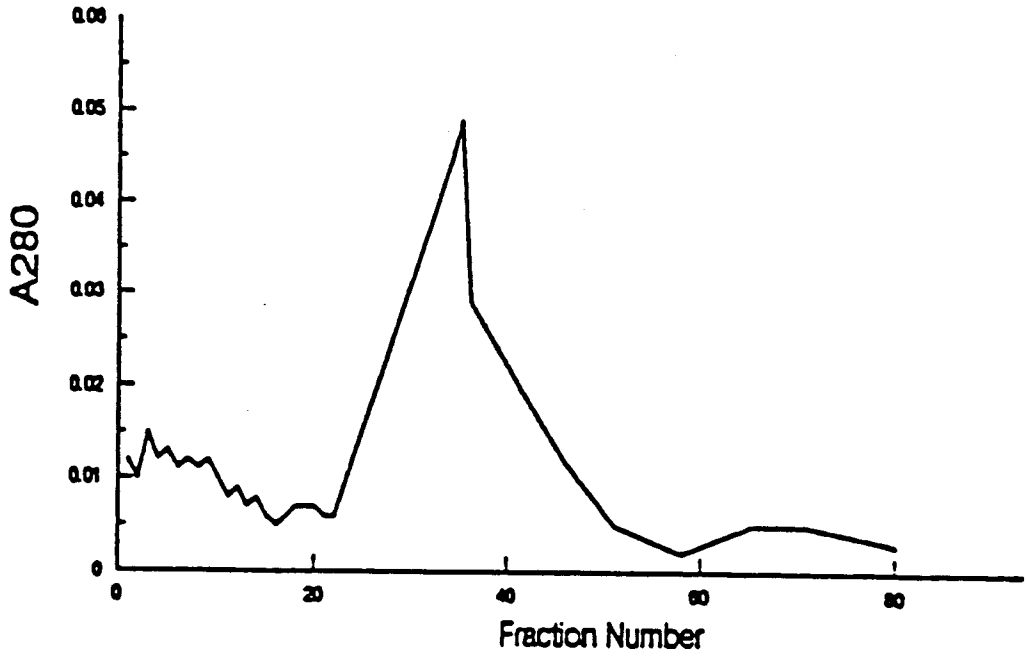

Figure III.8. Absorbance (280 nm) of the fractions eluted from homogenate of mosquito salivary glands (50 prs. in phosphate buffer, 0.067M KH_2PO_4-Na_2HPO_4, pH 6.2) applied to a carboxymethylcellulose column in the first trial using the procedure of Jolles (1962).

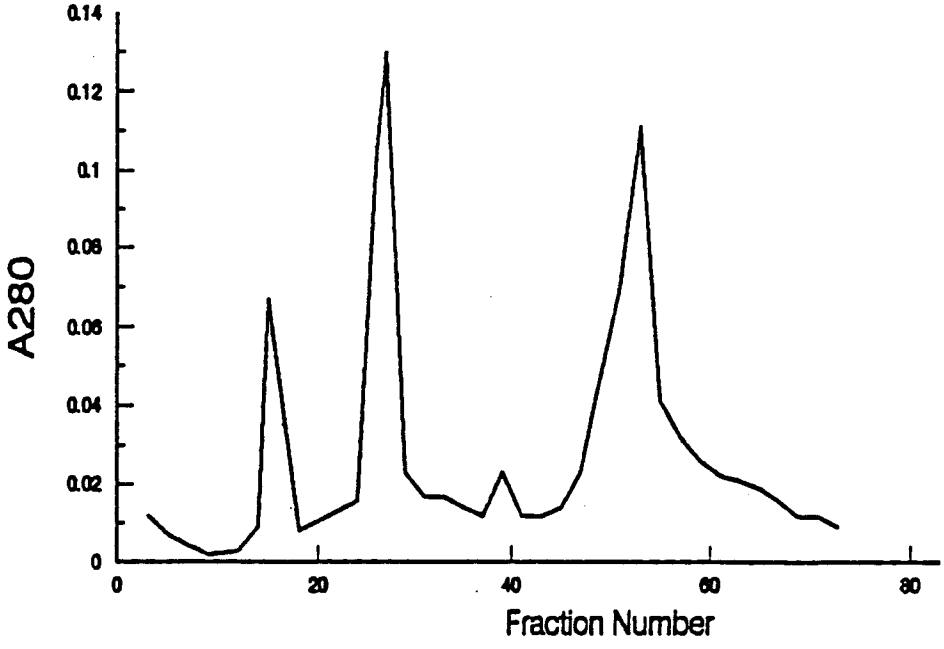

Figure III.9. Absorbance (280 nm) of the fractions from a homogenate of mosquito salivary glands (88 prs. in 100.0 ul Hayes saline) applied to a carboxymethylcellulose column in the second trial using the procedure of Jolles (1962).

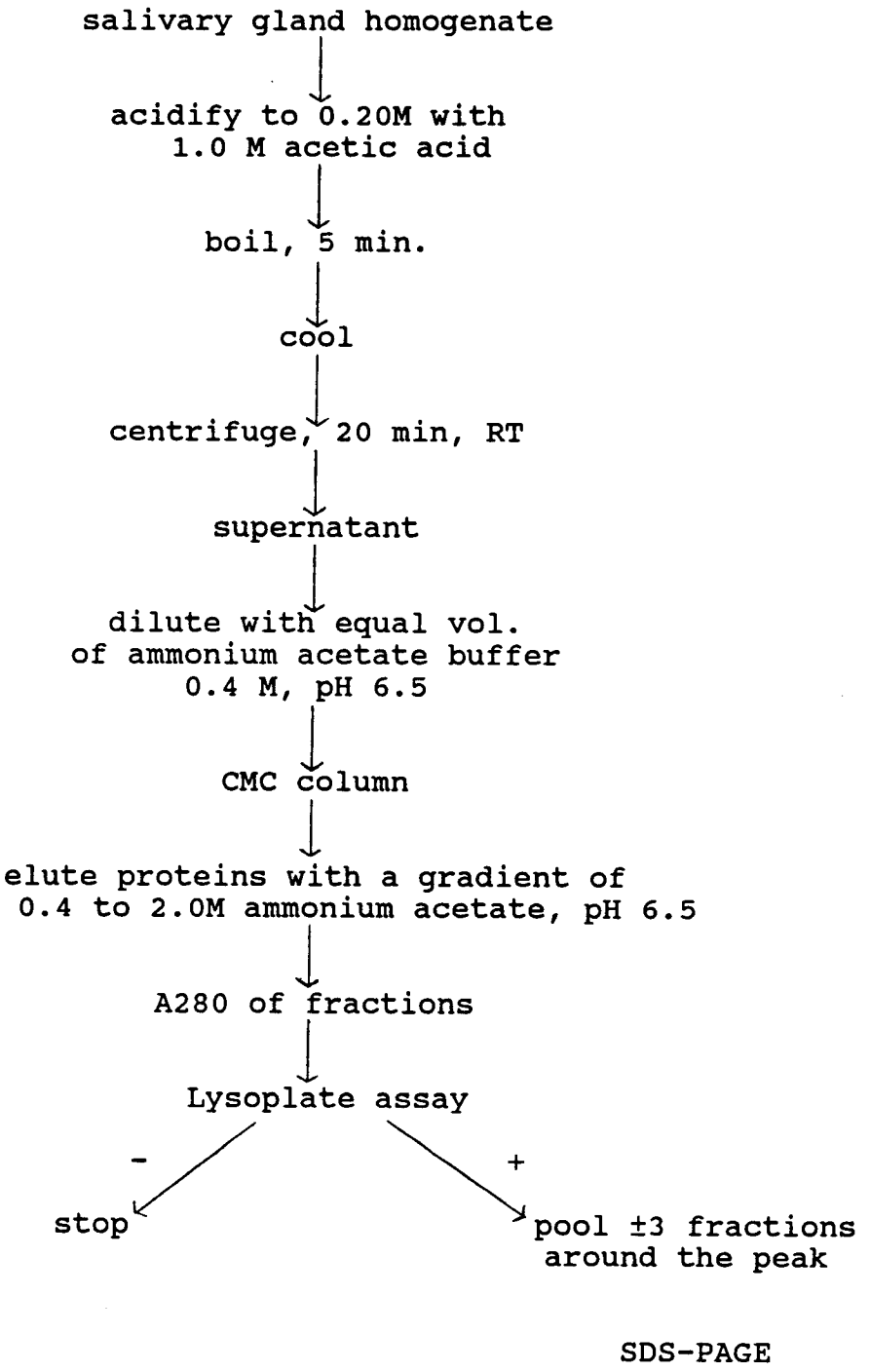

Figure III.10. Flow diagram of the method of sample preparation and handling for the chromatography procedure as modified from Zachary and Hoffman (1984).

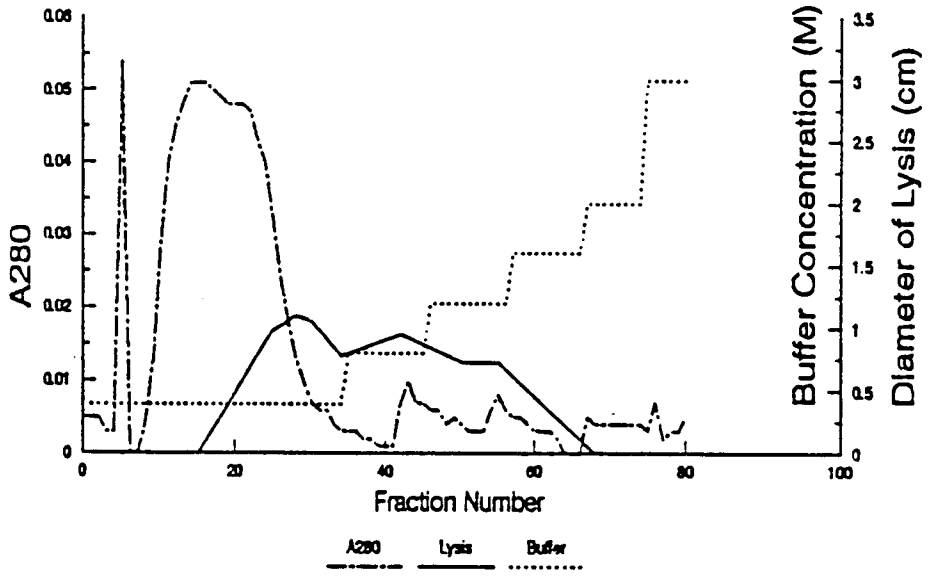

Figure III.11. Elution profile of the proteins from a homogenate of mosquito salivary glands (235 prs. in 1.5 ml distilled water) acidified to 0.25M with acetic acid before being applied to a carboxymethylcellulose column using the procedure of Zachary and Hoffman (1984).

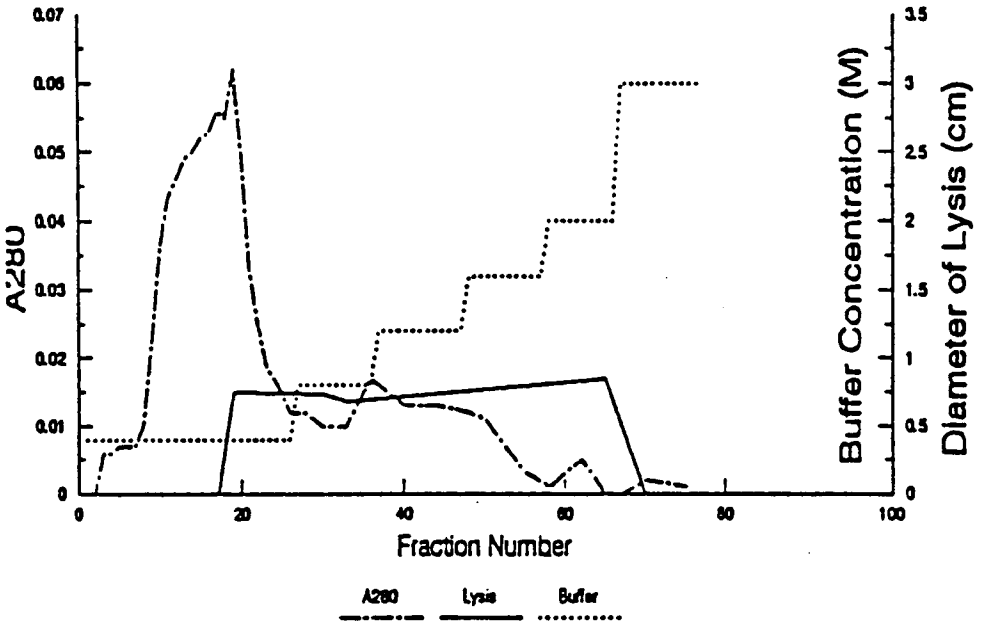

Figure III.12. Elution profile of the proteins from a homogenate of mosquito salivary glands (200 prs. in 1.8 ml distilled water) acidified to 0.5M with acetic acid before being applied to a carboxymethylcellulose column using the procedure of Zachary and Hoffman (1984).

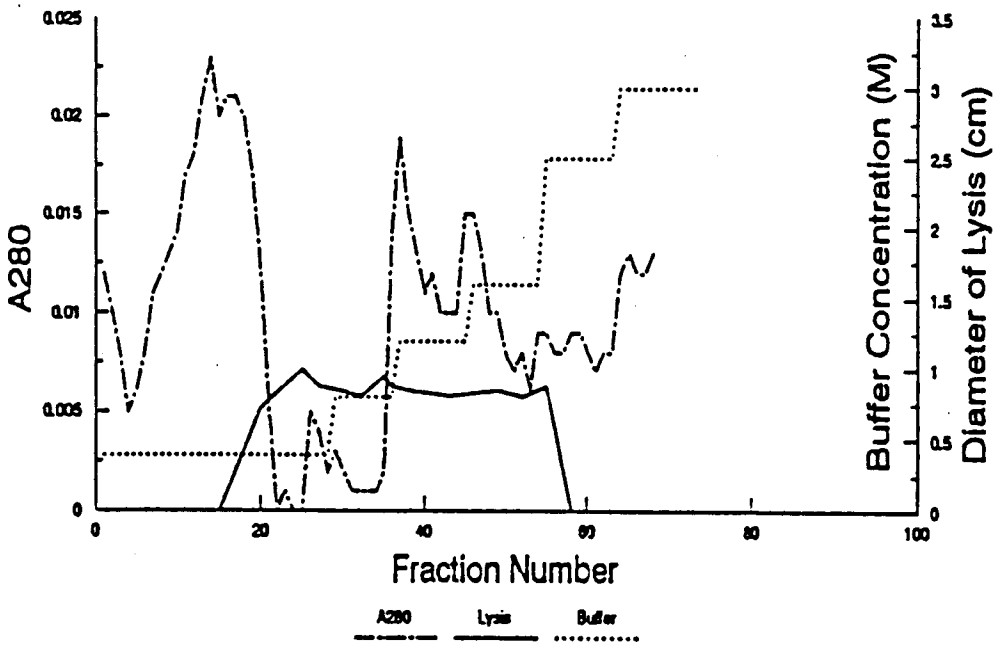

Figure III.13. Elution profile of the proteins from a homogenate of mosquito salivary glands (400 prs. in 5.8 ml distilled water) acidified to 0.4M with acetic acid before being applied to a carboxymethylcellulose column using the procedure of Zachary and Hoffman (1984).

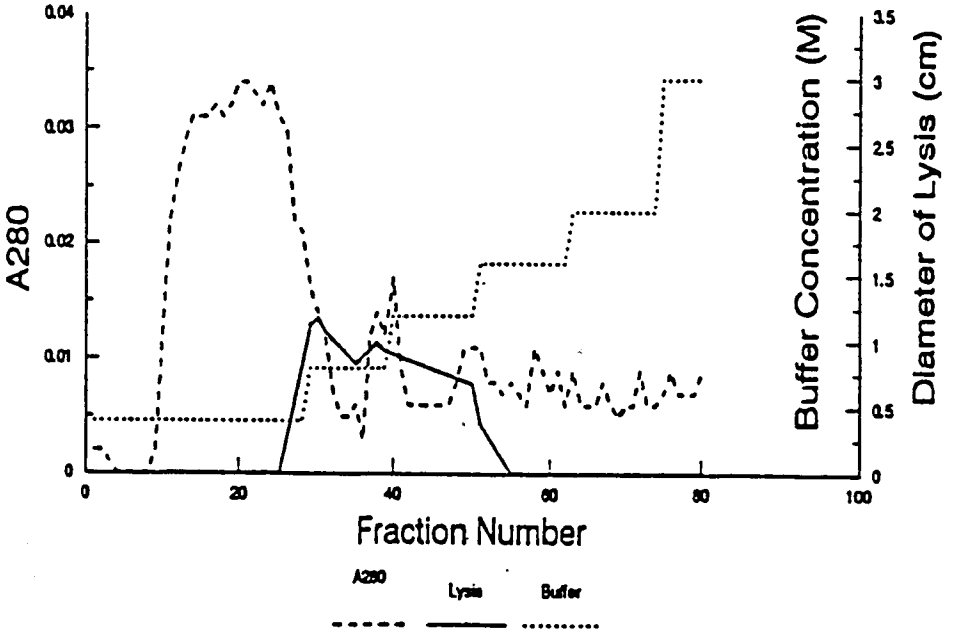

Figure III.14. Elution profile of the proteins from a homogenate of mosquito salivary glands (100 prs. in 1 ml. distilled water) acidified to 0.20M with acetic acid before being applied to a carboxymethylcellulose column.

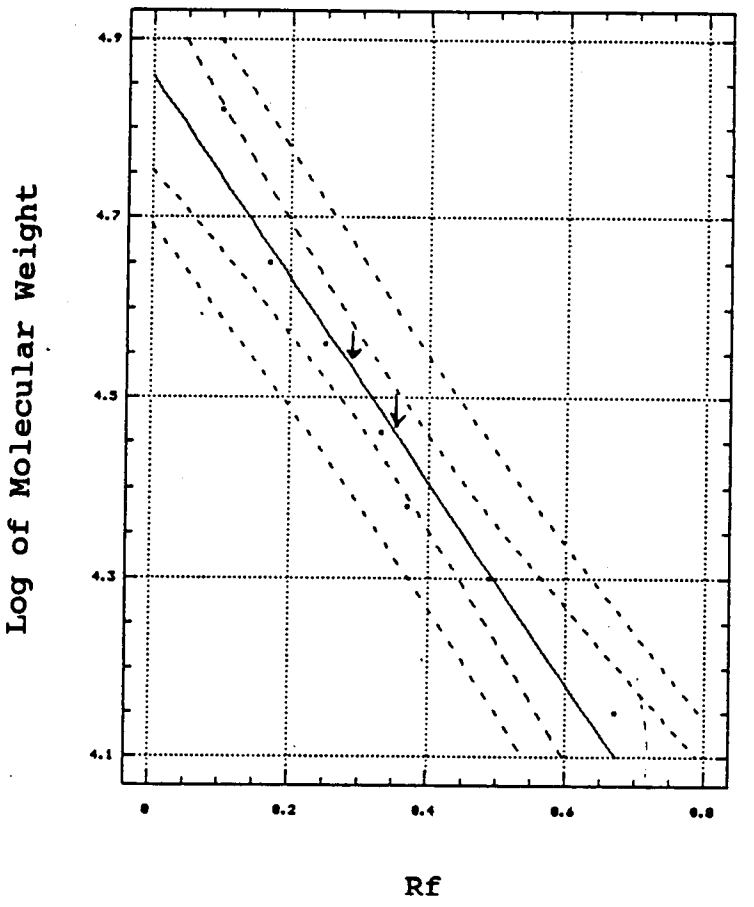

Figure III.15. Arrows indicate the range of molecular weights after SDS-PAGE (15%) of proteins contained in the lytic fraction 15 from the chromatography of salivary gland homogenates acidified to 0.20M.

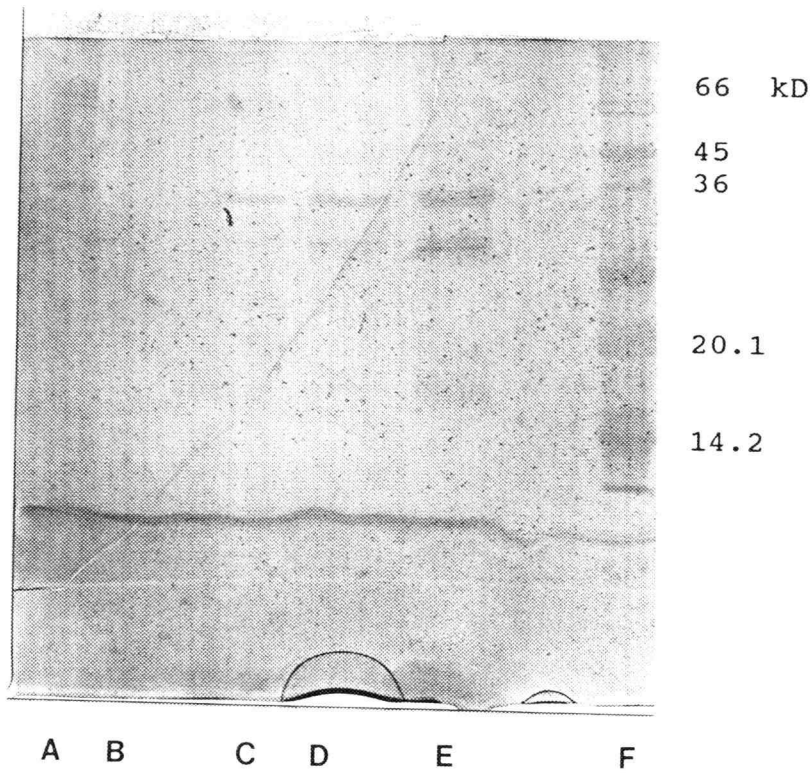

Figure III.16. SDS-PAGE (15%) of fractions obtained from the first trial using the modified Zachary and Hoffman (1984) method: A, sample O; B, fraction 20; C, fraction 16; D, fraction 15; E, fraction 14; F, molecular weight markers.

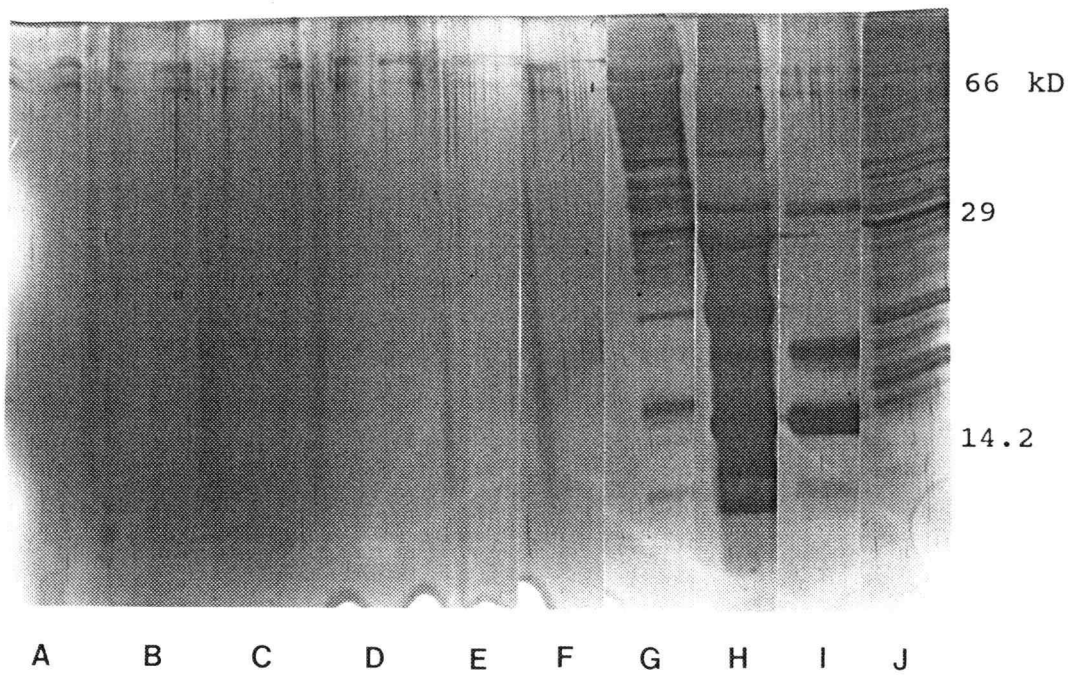

Figure III.17. Protein bands (SDS-PAGE, 15%) in lytic SG-50 fractions collected from pooled active fractions resulting from carboxymethylcellulose chromatography, trial one: A to F, samples; G, bovine serum albumin; H, molecular weight markers; I, lactalbumin marker; J, homogenate of 2 prs of salivary glands.

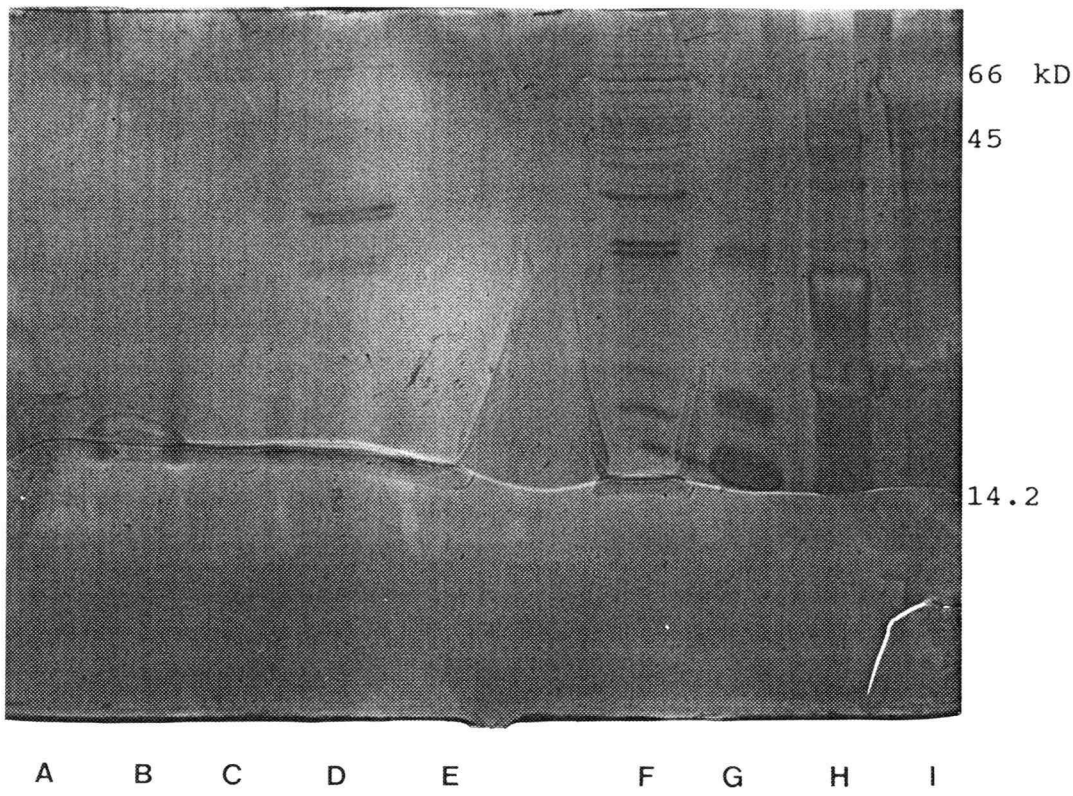

Figure III.18. Protein bands (SDS-PAGE, 15%) in concentrated lytic fractions collected from pooled active fractions resulting from first trial using carboxymethylcellulose chromatography: A to E, fractions 21, 23, 24, 11, 19 respectively; F, salivary gland homogenate; G, lactalbumin; H, molecular weight markers; I, bovine serum albumin.

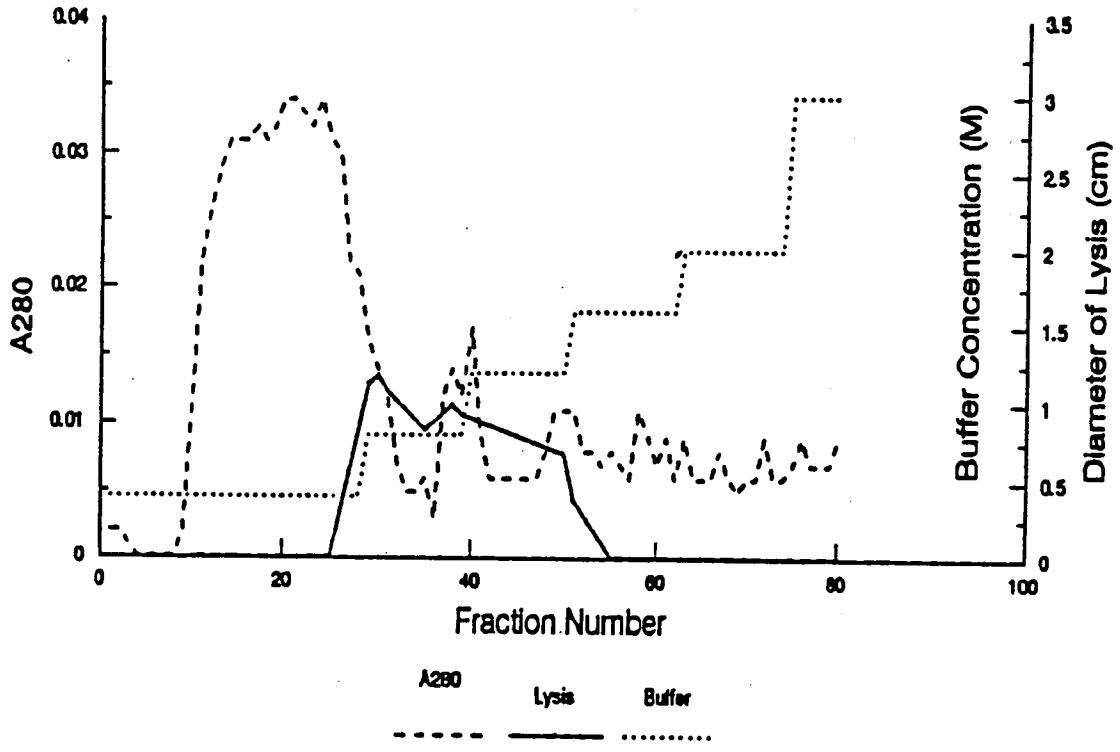

Figure III.19. Elution profile of the proteins from a homogenate of mosquito salivary glands (100 prs. in 775 ul distilled water) in the second trial with acidification to 0.20M before chromatography on carboxymethylcellulose.

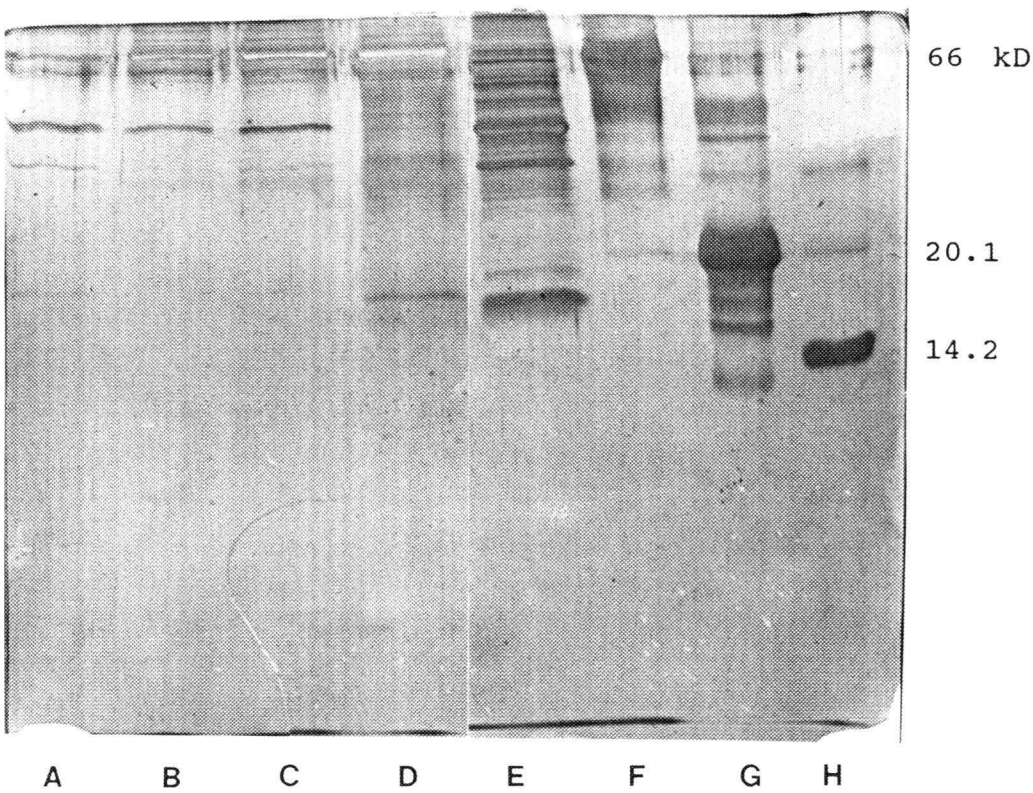

Figure III.20. Protein bands (SDS-PAGE, 15%) in concentrated lytic fractions that were collected from pooled active fractions resulting from the third trial with acidification to 0.20M before chromatography on carboxymethylcellulose: A to D, fractions 29, 35, 38, 45 respectively; E, salivary gland homogenate; F, bovine serum albumin; G, trypsin inhibitor marker; H, lactalbumin marker.

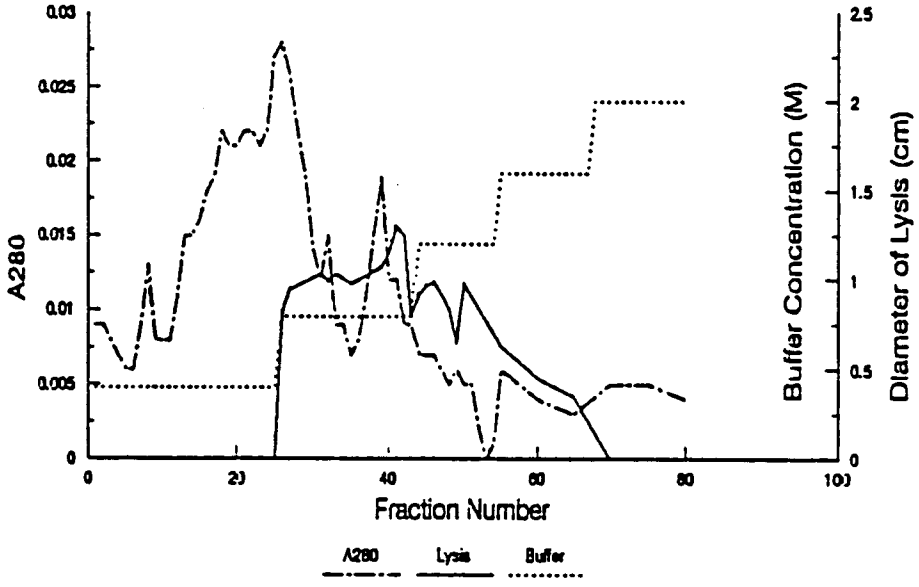

Figure III.21. Elution profile of the proteins from a homogenate of mosquito salivary glands (126 prs. in 1.2 ml distilled water) in the third trial with acidification to 0.20M before chromatography on carboxymethylcellulose.

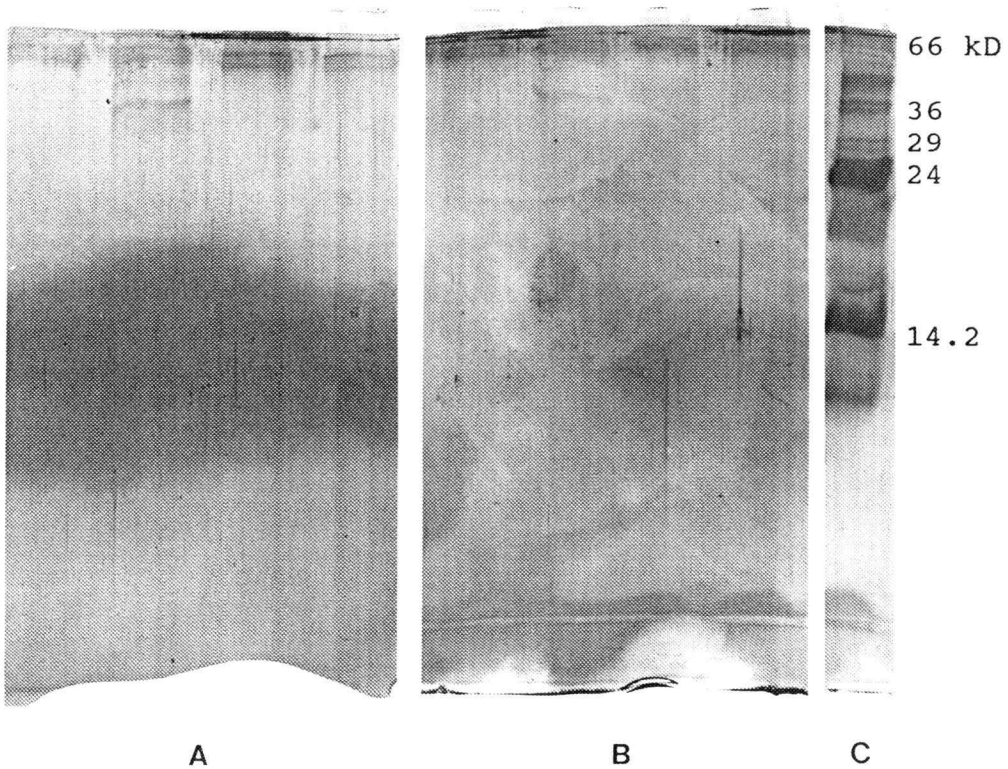

Figure III.22. A comparison of protein bands (SDS-PAGE, 15%T) in some SG-50 fractions from the third chromatography trial: A, unconcentrated; B, concentrated by centrifugation through a cellulose acetate filter (MWCO = 5 kD): from left to right, fractions 17, 35, 36, 58; C, molecular weight markers.

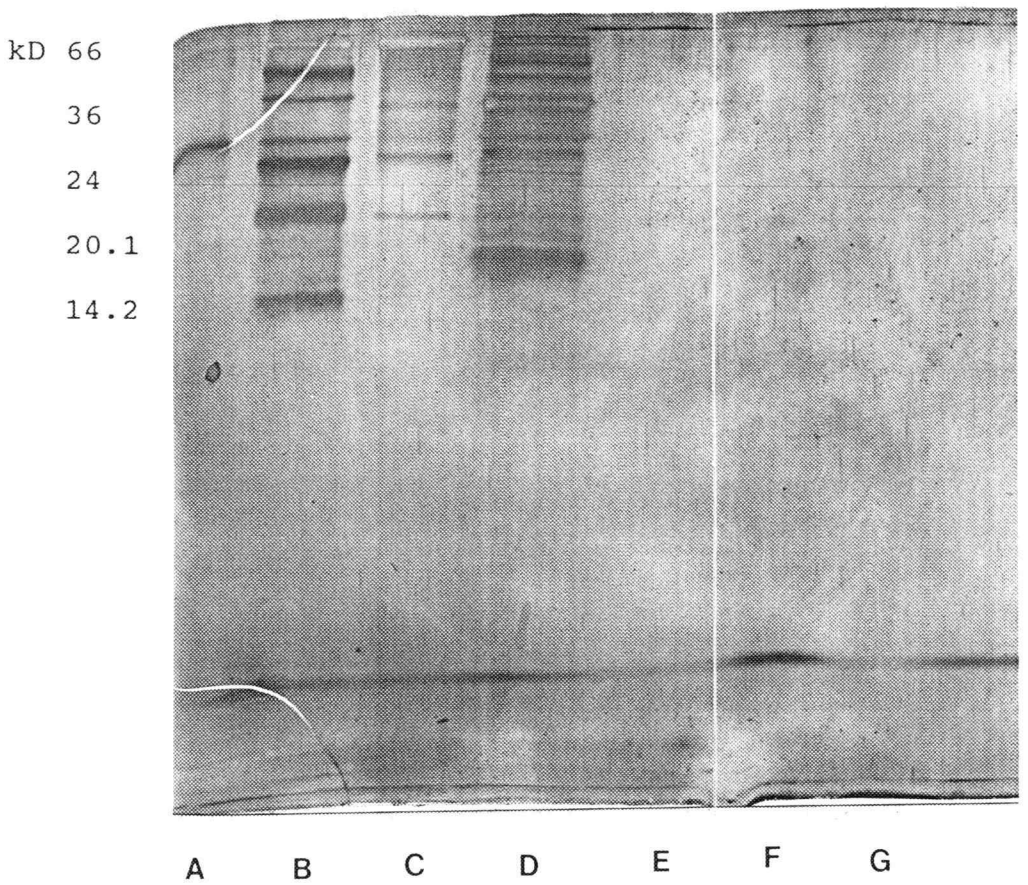

Figure III.23. Protein bands (SDS-PAGE, 15%) in concentrated SG-50 fractions from the third trial using carboxymethylcellulose chromatography. Samples were concentrated by lyophilization and subsequently reconstituted: A, carbonic anhydrase marker; B, molecular weight markers; C, bovine serum albumin; D, salivary gland homogenate; E, F, G, SG-50 fractions 58, 35, 16, respectively.

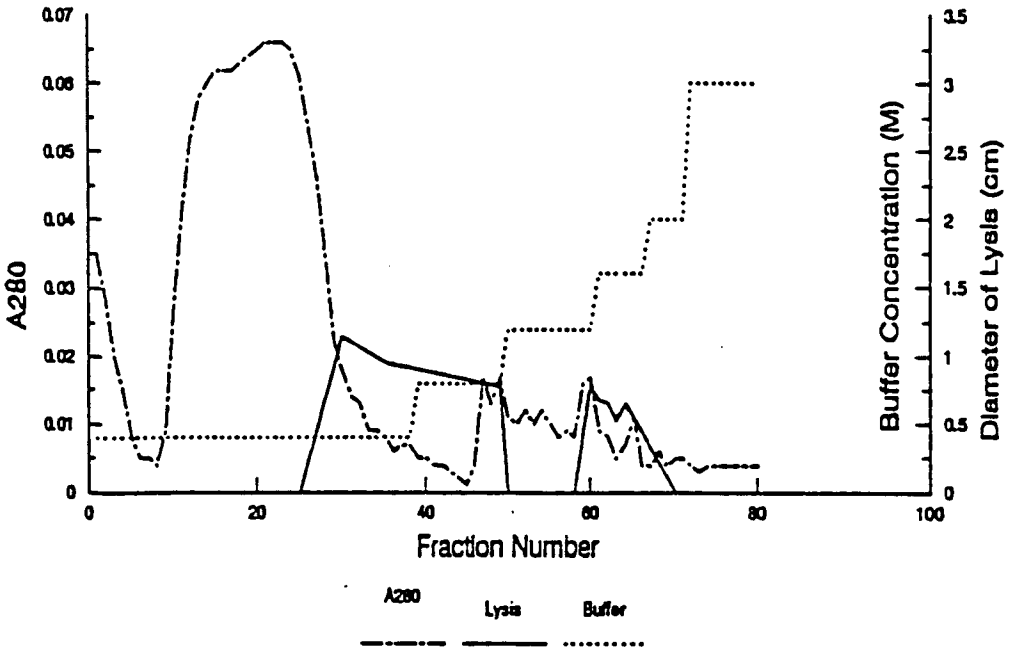

Figure III.24. Elution profile of the proteins in a homogenate of mosquito salivary glands (255 prs. in 2.35 ml distilled water) acidified to 0.20M before chromatography on carboxymethylcellulose in the fourth trial. Additional 4.0 ml of 0.4 M ammonium acetate buffer was used at the start of the run to wash away irrelevant proteins.

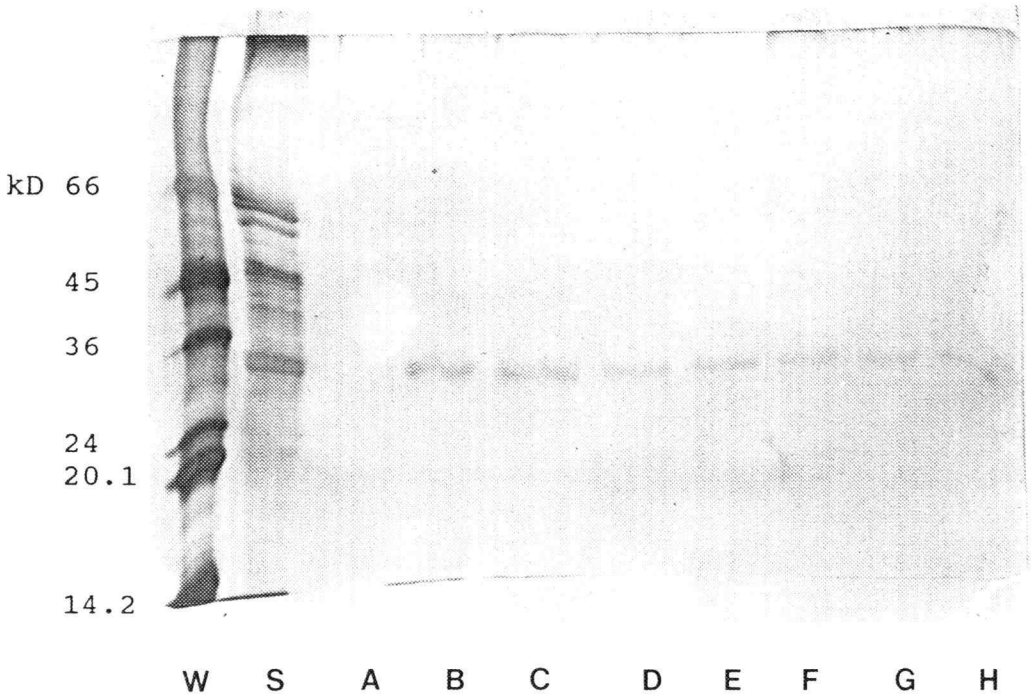

Figure III.25. Protein band (SDS-PAGE, 10%) in lytic fractions collected from the fourth chromatography trial on carboxymethylcellulose with additional initial buffer to wash off the irrelevant proteins: W, molecular weight markers; S, salivary gland homogenate; A to H, fractions 25, 28, 30, 32, 35, 37, 39, 41, respectively.

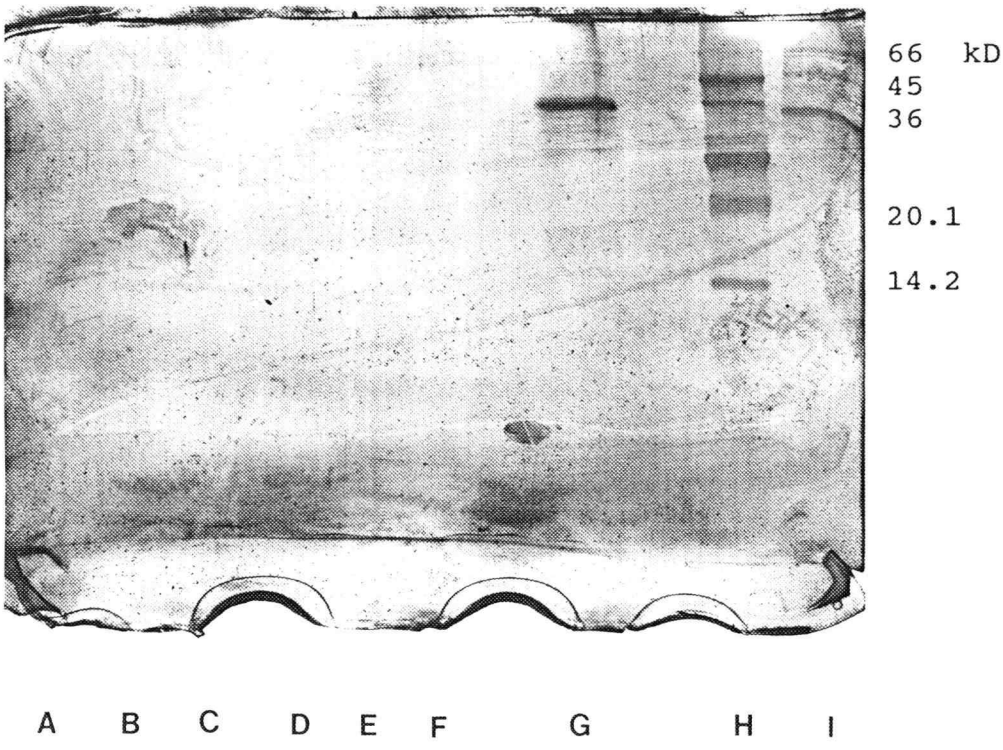

Figure III.26. SDS-PAGE (15%) of pooled carboxymethyl-cellulose lytic fractions that showed one protein band, and the resulting protein bands before (G) and after (A to F) gel filtration of the pooled sample through SG-50: H, molecular weight markers; I, salivary gland homogenate.

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