CHARACTERIZATION OF ALPHA-AMYLASE AND POLYGALACTURONASE FROM *LYGUS* SPP. (HETEROPTERA:MIRIDAE)

A Thesis

Submitted to the Faculty

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by

Amewushika Agblor

In Partial Fulfillment of the

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AMEWUSHIKA AGBLOR

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ABSTRACT

Polygalacturonase and α-amylase were extracted from salivary glands of the legume-destroying insects *Lygus lineolaris* (Palisot de Beauvois) and *L. hesperus* Knight. These enzymes have slightly acidic pH optima and temperature optima of 40 °C, and were strongly activated by chloride ions. Thermal inactivation of both enzymes follows first-order reaction kinetics, from which thermodynamic variables (Ea, ΔH, ΔG, ΔS) have been calculated. The *L. lineolaris* enzymes retained some activity even after exposure to heat at 100 °C for 30 minutes. Michaelis constants obtained respectively for *L. lineolaris* and for *L. hesperus* were 62.5 and 16.4 mg/mL for soluble starch, and 5.9 and 3.2 mg/mL for polygalacturonic acid, possibly reflecting the relative abilities of these insects to attack structural and nutritional components of pulse crops. Specific activities of the α-amylases of *L. lineolaris* and of *L. hesperus* indicate a stronger affinity for starch, as extracted from legumes, compared to that for soluble starch.

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Last, but not the least, I wish to thank my Lord and Saviour Jesus Christ for his manifold blessings.

DEDICATION

To the blessed memory of my beloved parents, Vincent and Georgial Kwamie, who aspired for the greatest things in life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Insects are the most common and widespread of all animals living on earth. It is estimated that there are between 900,000 (Sinha and Watters, 1985) and 1.5 million forms (Hoffman and Henderson, 1966) of insect life on earth. They consume enormous amounts of food and, as a result, remain a constant threat to the world's food supply despite drastic control measures.

Food losses may occur at the pre-harvest (on the field) or post-harvest (during storage) stage of plant development, causing degradation in the quality, and reduction in the yield and processing efficiency of the crop, with subsequent social, economic and nutritional implications. Many insect species feed on different plant parts as they mature in the field. For example, species of *Lygus* inhabit the apical portions of host plants, which provide warm, airy, sunlit niches (Bugg *et al.*, 1990), and feed preferentially on meristematic and young reproductive tissues (Strong, 1970; Summerfield *et al.*, 1982).

The genus *Lygus* belongs to the insect order Hemiptera, suborder Heteroptera and family Miridae, and is characterized by piercing-sucking mouthparts which determine their feeding behaviour (Miles, 1972). Species of *Lygus* normally prefer a particular host plant, but in its absence they attack the seedlings of other plants. As plant maturity approaches, the insects disperse to younger plants because the older tissues become more resistant to stylet penetration (Summerfield *et al.*, 1982), due to the accumulation of lignin and other polysaccharides within the cell walls (Bateman and Millar, 1966).

Approximately forty-three species of *Lygus* have been identified worldwide (Kelton, 1975). Of these, thirty-four occur in North America, seven in Europe and two in China. According to Tingey and Pillemer (1977), at least nine species are of economic importance to agriculture, especially *L. hesperus* Knight, which destroys legume seeds, fruits (eg. strawberries), and vegetables (eg. carrots), and *L. lineolaris* (Palisot de Beauvois), which is believed to destroy over 300 species of host plants in Europe (Fleischer and Gaylor, 1988), such as legume seeds, vegetables and seed alfalfa (Young, 1986).

The damage to crops by species of *Lygus* is enormous and has been estimated to cost millions of dollars (Kelton, 1975). Up to 50% crop damage has been reported (Beirne, 1972). Insects of the Miridae family are notorious for the extent of destruction that single insects can cause (Martin *et al.*, 1988). Hagel (1978) estimated that, in the absence of *Lygus* species, an average yield of 398 pods of field peas could be obtained per 18 plants, but one single insect is capable of reducing the average yield of field peas to 242 pods per 18 plants. Getzin (1983) also reported that one *Lygus* adult could destroy 1.7 fruiting buds per day in cabbage, representing 0.79 kg of seed per hectare per day. Also, single punctures of *Phaseolus* seeds by species of *Lygus* reduced the average seed dry weight by 18%, and three or more punctures reduced it even further (Summerfield *et al.*, 1982).

As plant feeders, species of *Lygus* possess very potent salivary enzymes. Their feeding behaviour, which is a combination of the piercing and sucking action of their mouthparts, as well as the secretion of salivary enzymes, physically damages the plants and causes adverse secondary effects such as necrosis, pod distortion and growth retardation (Strong, 1970). Several enzymes have been implicated in host plant injury, of which the polygalacturonases, α-amylases and proteases are considered to be the most important (Laurema *et al.*, 1985). These enzymes facilitate the penetration of plant tissues by the insect's stylet, and subsequently become involved in the

utilization of the nutritional components of the plant. Secondary microbial infections, a result of the feeding injury (Bateman and Millar, 1966; Summerfield *et al.*, 1982; Arnett, 1985), may also cause further degradation of the food or enhance mycotoxin production during storage.

The objectives of the work described in this thesis are:-

(1) To characterize α-amylase and polygalacturonase from the salivary glands of *L. hesperus* Knight and *L. lineolaris* (Palisot de Beauvois) with respect to the effects of pH, temperature, enzyme concentration, substrate concentration, and the ionic environment.

(2) To compare the activities of the insect enzymes towards the appropriate substrates, in laboratory chemical form, and as extracted from legumes.

(3) To assess the relative abilities of these two insect species to attack structural and nutritional components of pulse crops, thereby causing a reduction in crop yield and quality.

2. LITERATURE REVIEW

2.0 The Genus *Lygus*

Although Strong (1968) reported that *L. lineolaris* (Palisot de Beauvois) and *L. hesperus* Knight are the two most important species of *Lygus*, there are several other species that cause substantial damage to crops, and could be of equal importance. In Finland, *L. rugulipennis* Poppius destroys small grain such as wheat kernels (Nuorteva and Veijola, 1954) and barley (Varis, 1991), strawberries and sugarbeet (Varis *et al.*, 1983), whilst in Japan, Hori (1970a) reported damage to sugarbeet, wheat and legume seeds by *L. disponsi* Linnavuori. *L. lucorum* Meyer-Dür and *L. vosseleris* Poppius are found on cotton; the former species in China (Tingey and Pillemer, 1977), and the latter in Uganda (Strong, 1970). *L. elisus* Van Duzee, *L. desertus* Knight and *L. nigrosignotus* Knight have also been implicated in causing damage to bean plants (Hagel, 1978). Damage to cocoa and sweet potato in Papua-New Guinea (Miles, 1987), and to tea in Sri Lanka (Tingey and Pillemer, 1977), has been reported. Recently, in Western Canada, three species of *Lygus*, namely *L. borealis* (Kelton), *L. elisus* Van Duzee and *L. lineolaris* (Palisot de Beauvois) have been reported to cause severe damage to rapeseed canola (Butts and Lamb, 1990; 1991). Species of *Lygus* are attracted to yellow (Summerfield *et al.* 1982) and as a result, canola plants are highly susceptible to damage (Getzin, 1983).

2.0.0 Life History

Species of *Lygus* overwinter as adults under debris or litter, and emerge in the early spring to feed, mate and oviposit. Oviposition usually lasts three weeks, but in some cases may continue for seven weeks. Nymphs emerge 10-14 days after oviposition, pass through five instars, and 30-45 days later, new adults emerge. Species of *Lygus* have one to four generations per year (Hagel, 1978; Philip and Mengersen, 1989), which increases the incidence of further destruction. During each generation, the adults reach their highest population in the mid-to-late summer (Baker *et al.*, 1946), depending on the temperature (Butler and Wardecker, 1971). *Lygus* damage is reported to be relatively high above 20°C (Fye, 1982); cool weather reduces their feeding activities (Beirne, 1972).

2.1 The Role of the Salivary Glands of *Lygus*

The internal morphology of insects includes the alimentary canal, a straight tube of epithelium cells connecting the mouth to the anus. It has three distinct regions, the foregut, midgut and hindgut, which are connected by valves to regulate the flow of food from one region to the next (Wigglesworth, 1972; Terra, 1990). The mouth contains the salivary glands which extend inwards into the foregut. The main functions of the foregut are storage, and some digestion of food. Digestion occurs mainly in the midgut, and involves the biochemical breakdown of polymeric food substances to monomers by hydrolases. The hindgut has excretory functions.

The salivary gland is of the utmost importance to *Lygus* because it is the principal site of digestion of food. There is evidence to suggest that the activity of the salivary α-amylases from *Lygus* is

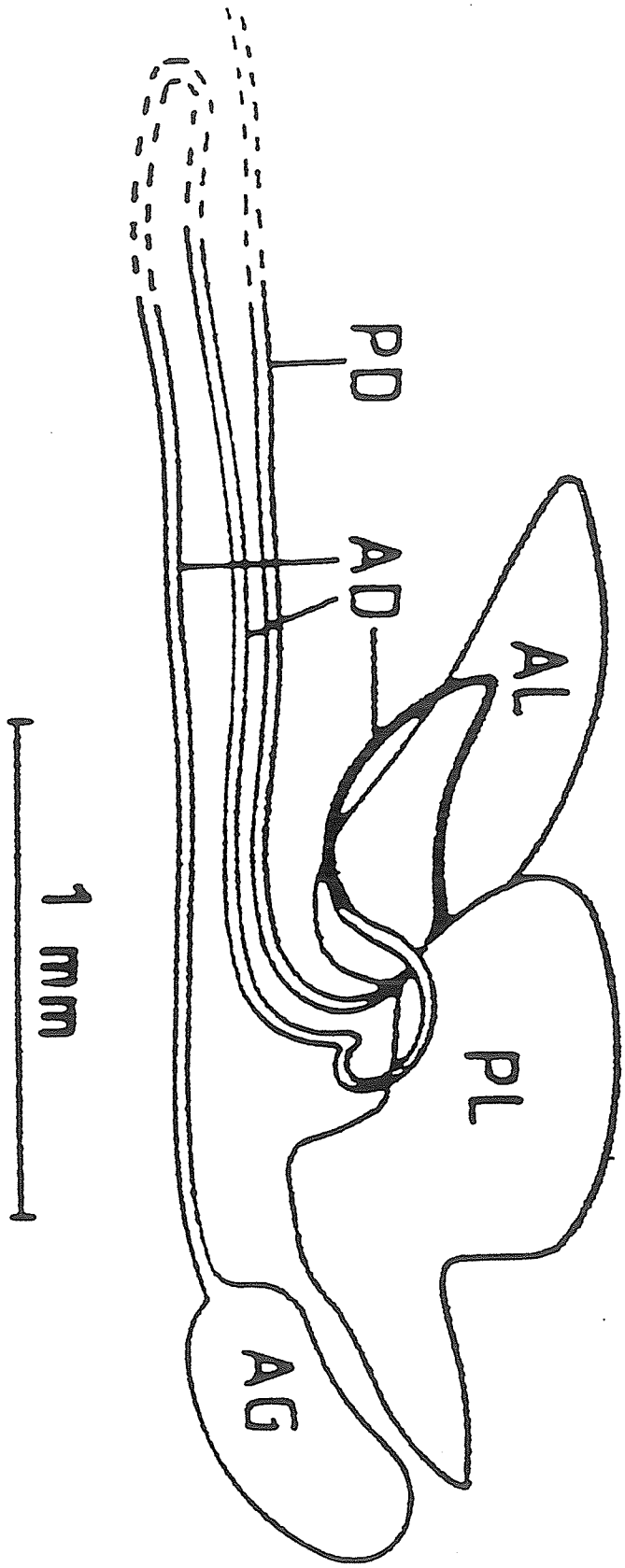
twice as high as the activity of the midgut α-amylases (Hori, 1970a; Takanoma and Hori, 1974). The glands are typically bilobed in nature, and produce saliva, a clear watery fluid, which moistens the food, adjusts the pH and ionic content, and secretes hydrolytic enzymes. The saliva also aids in the mechanical penetration of the stylets into plant tissues (Miles, 1972), and moistens and cleans the mouthparts between feeds (Wigglesworth, 1972). Lying adjacent to the salivary gland is the accessory gland (Baptist, 1941) (see also Figure 1), which produces a watery secretion that dilutes the saliva, and acts as a vehicle for the transfer of several substances such as hydrolyzing enzymes (Miles, 1972) and salivary amino acids (Hori, 1975a). The "watery saliva" itself contains no hydrolyzing enzymes and its secretion ceases when the stylets penetrate the plant (Miles, 1968). The pH of the salivary and accessory gland fluids is thought to be 6.8 to 7.0 (Strong, 1970; Wigglesworth, 1972), and 8.0 to 9.0 respectively (Miles, 1968).

The three most important salivary enzymes are polygalacturonase, α-amylase and protease. Of lesser importance are invertase, phosphatase, trehalase and phenolase, which are thought to be derived from the gland tissue, and are only minor constituents of the saliva. These enzymes are all located in the posterior lobe of the salivary gland except for the α-amylase and protease which are located in the anterior lobe, and phenolase, which is located in the accessory gland (Laurema *et al.*, 1985). Due to low lipid contents in sunflower seeds that are injured by *Lygus*, Laurema *et al.* (1985) suggested that lipases may be present in the salivary glands. However, Baptist (1941) and Nuorteva (1954), reported the absence of lipases from the salivary glands of *Lygus*.

2.1.0 The Feeding Mechanism

Lygus feeds by the lacerate-and-flush method (Miles, 1972). During feeding they lacerate adjacent

Figure 1. The salivary glands of *Lygus*. AD = accessory duct; AG = accessory gland; AL = anterior lobe of the principal gland; PD = Principal duct; PL = posterior lobe of the principal gland (Laurema *et al.*, 1985).

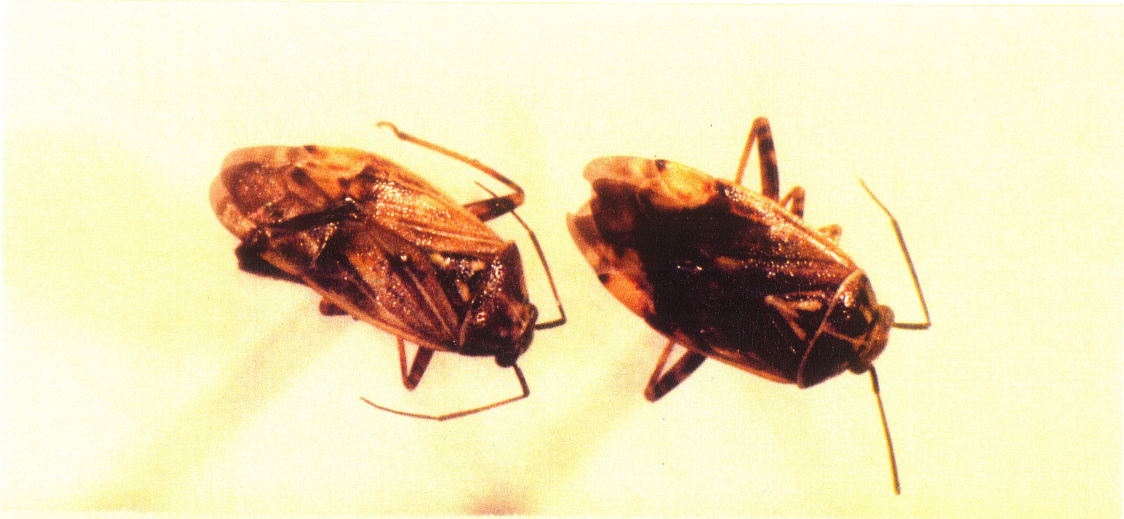

PD
AD
AL
PL
AG
1 mm

pockets of cells, inject saliva to digest the cells, and then ingest the liquefied tissues. The mouthparts of these insects include a proboscis, which is used to pierce and probe plant tissues for food. The proboscis contains four bristles, two of which are modified mandibles, whilst the other two are maxillary stylets (Elmore, 1955). The stylets lie closely together between the two mandibles forming two canals, one of which carries saliva into the plant whilst the other carries liquefied food from the plant (see also Figures 2 and 3).

Immediately prior to feeding, *Lygus* touches the feeding site several times with the labium (lower lip), generating a gustatory stimulus which results in the immediate release of the "watery saliva" (Strong, 1970; Miles, 1972). Once the taste is satisfactory, the stylets penetrate into the plant to a depth of 2 to 3.5 mm (Miles, 1987). Stylet insertion occurs randomly, either inter- or intracellularly (Hori, 1971a) and the frequency of their penetration into plant tissue averages about two probes per hour (Hori, 1971b).

Following the maceration of plant tissues, *Lygus* withdraws the liquefied contents of the plant material. *L. disponsi* Linnavuori adults, for example, withdrew an average of 7-13 mg of plant juice per day (Hori, 1971b), whilst one adult *L. hesperus* Knight digested about 6 mg of plant tissue per hour (Strong and Kruitwagen, 1968). According to Strong (1970), an adult *L. hesperus* Knight could ingest the entire contents of an alfalfa floral bud, approximately 0.25 mm in diameter, in 23 seconds. Feeding time often exceeds one hour (Hori, 1971b; Laurema *et al.*, 1985) during which the tissues within reach of the stylets are depleted of fluids.

Figure 2. Photograph of *L. lineolaris*.



L. lineolaris

Figure 3. Photograph of *L. hesperus*.


L. hesperus

2.2 Nature of Host Plant Injury

2.2.0 Tissue Necrosis and Wilting

Lygus injury is a combination of mechanical and enzymatic processes. The first apparent sign of injury is the puncturing of plant tissues, which appears as a small hole in the seed coat beneath which are loose starch granules (Shull and Wakeland, 1931), and is sometimes referred to as the chalky spot or yeast spot (Summerfield *et al.* 1982; Butts and Lamb, 1990). Following the injury, the area surrounding the punctured cells turns brown (Baker *et al.*, 1946; Middlekauf and Stevenson, 1952; Hori, 1971a) due to the oxidation of phenolic compounds, released from the damaged tissues, to quinones, by the action of polyphenol oxidase. The quinones are further oxidized and produce the characteristic brown discolouration. Tissue necrosis and wilting due to *Lygus* injury have been reported to occur frequently in cotton and tea (Tingey and Pillemer, 1977), beans (Shull and Wakeland, 1931), cabbage seed (Getzin, 1983), tobacco, lettuce and celery (Beirne, 1972; Boivin *et al.*, 1991). Tissue injury, resulting from *Lygus* feeding, is also manifested as leaf crinkling (stunted and rolled leaves), and is commonly found in sugarbeet, onion seedlings, radish and turnips (Fye, 1982), or as swollen nodes in cotton (Tingey and Pillemer, 1977).

2.2.1 Abscission of Fruiting Forms

Lygus feeds preferentially on meristematic plant tissue and developing reproductive organs. The fertilization of a flower is often associated with auxin production to ensure fruit set, and also to regulate plant growth (Strong, 1970). The stylets of the insect, which can reach the auxin-producing sites of the plant, cause the hormonal level in the abscission layer to increase, resulting in the premature drop of buds, flowers, fruits and seeds, and subsequent reduced pod set (Stitt,

1944; Middlekauf and Stevenson, 1952). This commonly occurs in cotton, snapbeans, tomato, alfalfa, tea (Tingey and Pillemer, 1977), and rapeseed canola (Butts and Lamb, 1990). Blasting of floral parts (the dropping of buds and blossoms) has been reported to have severe consequences on the crop yield (Shorey *et al.* 1965; Khattat and Stewart, 1975).

2.2.2 Morphological Deformation

Lygus injury is also manifested by apical seediness, catfacing (shallow or deep depressions) of fruits, misshapen fruit, shrivelled seeds, distorted pods, pitted and aborted seeds (Baker *et al.* 1946; Fye, 1982; Bosque-Perez *et al.*, 1987; Butts and Lamb, 1990). Young pods of lima beans were particularly susceptible to pod distortion and pitted seeds (Elmore, 1955). Other crops which are susceptible to morphological deformation are strawberries, peaches, carrots, apples, cotton, alfalfa, canola and cowpeas.

2.2.3 Retarded Growth

Altered vegetative growth, either in the form of an increase, or of a decrease in vegetative biomass, occurs as a result of *Lygus* injury (Scott, 1969; Fye, 1982, 1984; Getzin, 1983; Philip and Mengersen, 1989). Crops such as field peas, lentils, soyabeans, kidney beans, snap beans, navy beans, cabbage seeds, flax, carrots and cucumber are commonly affected. Retarded growth is also manifested as an abnormal increase in plant height or in root weight (Tingey and Pillemer, 1977). Growth retardation, as a result of *Lygus* injury, can last for at least 10 days (Walstrom, 1983).

2.3 *Lygus* Damage and Legume Quality

Food legumes are indigenous to warmer regions of the world, where they contribute significantly to the diets of close to one billion people (Newman *et al.*, 1986). They provide diversity in the diet, and enhance nutritional quality. In addition, their ability to fix nitrogen in symbiotic associations, with strains of *Rhizobium*, makes them significant in maintaining the nitrogen balance in deficient soils (Brady, 1986).

Lygus damage is an important criterion in the grading of legume seeds. Seeds are downgraded by as much as 20% of their weight (Summerfield *et al.*, 1982), and seed lots containing as little as 3.5% of damaged seed are routinely rejected by food processors. The effect of damage is initially evident in a discoloured appearance.

Damaged seeds of *Phaseolus vulgaris* (common bean), *Phaseolus lunatus* (lima bean), *Vigna unguiculata* (cowpea) and *Lens culinaris* Medik (lentils), became mushy on cooking, tasted less sweet, and were more bland than the undamaged seeds. The damaged seeds also had thicker and tougher testas, and were more susceptible to fungal attack and rapid deterioration during storage (Summerfield *et al.*, 1982). The cell walls of damaged seeds were distorted, cellular contents were disrupted, and tissue integrity was lost. The middle lamella which was extremely prominent in undamaged tissues was unnoticeable in damaged seeds. The absence of starch granules in these tissues, as well as their inability to synthesize storage proteins, was also reported. Large intercellular air spaces, totally absent in undamaged tissues, were observed. These air spaces were thought to be coagulated membranes (Summerfield *et al.*, 1982).

Besides legume seeds, losses in the quality of cereal grains, such as wheat, have also been reported. According to Varis (1991), *Lygus* damage impairs germination and the subsequent

development of the plants that are grown from the grain. An increase in the proteolytic activity, and in diastatic power (reducing sugar content) (Nuorteva, 1954), and a low falling number in damaged wheats have been reported (Rautapää, 1969). The falling number is the time, in seconds, required for a viscometer stirrer to drop a fixed distance through a hot aqueous flour gel being liquefied by the enzyme (Greenaway, 1969). These parameters are undesirable for the bread-making properties of wheat flour.

2.4 Characteristics of Salivary Amylase and Polygalacturonase from *Lygus*

2.4.0 α-Amylase

Starch is the major storage carbohydrate in higher plants. It is a homopolysaccharide of α-D-glucose with two structurally distinct components, linear amylose and branched amylopectin (Meyer and Gibbons, 1951). The anhydroglucose units in amylose are linked by α-(1→4) bonds, whilst in amylopectin, the anhydroglucose units are linked by α-(1→4) and α-(1→6) bonds. Starch also contains some non-carbohydrate constituents such as lipids, proteins, and phosphorus (Biliaderis, 1991).

The α-amylases (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) are endo-splitting enzymes, which catalyze the internal random hydrolysis of the α-1,4 glucosidic linkages in polysaccharides, such as starch and glycogen, and their degradation products with a chain length of at least 3 D-glucose residues (Rick and Stegbauer, 1974). α-Amylases occur in animals, higher plants, microorganisms, and insects (Bernfeld, 1951; Whelan, 1964). Their function is to solubilize and digest starch and other related carbohydrates.

The hydrolysis of starch by α-amylases occurs on the surface of the enzyme, and involves an interaction between the functional groups at the active site of the enzyme, and the functional groups of the substrate, to form an enzyme-substrate complex. The active site of the enzyme is divided into two parts: a binding site and a catalytic site. The binding site is made up of a number of subsites, whilst the catalytic site contains two or three electrophilic (proton donor), and nucleophilic (proton acceptor) groups. The number of subsites and their arrangement in conjunction with the catalytic groups determine the type of products formed (Robyt, 1984). A proposed mechanism, for α-amylase catalysis, involves the protonation of the oxygen of the D-glucose bond by hydrogen ions from the amino or imidazole groups of the enzyme. The electron deficient centre at C-1 then attract electrons from donor groups, such as carboxyl groups. This structure is then cleaved on the C-1 side of the bond to form a carbonium ion intermediate, and a neutral D-glucosyl fragment. Finally, a hydroxyl ion is added to the carbonium ion intermediate (Pazur, 1965).

2.4.0.0 Effect of pH on α-amylase activity

The pH optimum of most insect α-amylases lies within the neutral to slightly acid pH range (Baker, 1983). The pH optimum of *L. rugulipennis* α-amylase was 5.0 in the absence, and 6.0 in the presence, of activators such as nitrate and chloride ions (Laurema *et al.*, 1985). Hori (1970a), reported a pH optimum of 5.0 for *L. disponsi* α-amylase.

The respective pH optima for *Sitophilus granarius* and *S. zeamais* α-amylases (stored-product insects) were 5.0 and 4.75 (Baker, 1983). Campos *et al.* (1989), reported an optimum in the range of 5.2 and 6.0 for the α-amylase of the bruchid beetle *Callosobruchus maculatus*. *C. chinensis* α-

amylase was strongly activated between 5.2 and 5.8 (Podoler and Applebaum, 1971), whilst *Tenebrio molitor* α-amylase was most active at 5.8 (Buonocore *et al.*, 1976). The pH optimum of *Rhynchosciara americana* α-amylase was 6.8 (Terra *et al.*, 1977).

The pH optimum of human salivary amylase and of hog pancreatic amylase is 6.9. Sorghum and barley malt α-amylases were most active at pH 4.6, and between 4.5 and 5.4 respectively. Microorganisms such as *Bacillus subtilis*, *B. stearothermophilus*, and *Aspergillus oryzae* have pH optima at 6.0, and in the range of 4.6 to 5.1, and 5.5 to 5.9 respectively (Pazur, 1965). Chiang *et al.* (1979) reported a pH optimum between 6.0 and 7.0 for *B. licheniformis*.

Lygus rugulipennis α-amylase was stable to changes in the pH from 4.0 to 11.0 at 2°C, for 23 hours. Rapid inactivation of the enzyme occurred below pH 4.0 and above pH 11.0 (Laurema *et al.*, 1985). The effect of pH on the stability of *T. molitor* α-amylase was studied at 4°C and at 37°C, for 30 minutes, and for 24 hours. The enzyme was most stable between pH 5.8 and 8.5 (Buonocore *et al.*, 1976). According to Chiang *et al.* (1979), *B. licheniformis* was most stable between pH 6.5 and 10.0 at 25°C for 24 hours.

2.4.0.1 Effect of temperature on α-amylase activity

The optimum temperature for *L. disponsi* α-amylase occurred at 37°C (Hori, 1970a; 1970b). *L. rugulipennis* α-amylase was strongly activated between 35 and 40°C (Laurema *et al.*, 1985). *T. molitor* α-amylase showed an optimum at 37°C (Buonocore *et al.*, 1976), whilst *C. chinensis* α-amylase was most active between 36 and 40°C. Complete inactivation of this enzyme occurred at 60°C (Podoler and Applebaum, 1971). The temperature optima of *R. americana* and of *B.*

licheniformis α-amylases were 50°C (Terra *et al.*, 1977), and 85°C respectively (Chiang *et al.*, 1979). However, the effect of temperature on enzyme activity is time-dependent (Reed, 1975). Short reaction times result in high temperature optima, whilst low temperature optima are associated with long reaction times. According to Sizer (1943), the optimum temperature is not the same for one particular enzyme, but varies from one enzyme source to another.

Most α-amylases, including those of *Lygus*, are relatively stable to thermal denaturation. A heat treatment incubation time of 15 minutes, on crude extracts of *L. rugulipennis*, did not cause any appreciable losses in α-amylase activity between 30 and 40°C. At 50°C, 25% of the activity was lost, whilst at 60°C, only 10% of the activity remained (Laurema *et al.*, 1985). *T. molitor* α-amylase was also relatively stable to heat between 30 and 40°C. Incubating the crude enzyme extract for 20 minutes and for 60 minutes at 50°C, resulted in a 10% loss in activity. Total denaturation of the enzyme occurred at 60°C. At this same temperature, a 5-minute heat treatment resulted in a loss of 25% in enzyme activity (Buonocore *et al.*, 1976).

The α-amylases are metalloproteins, containing one atom of calcium per molecule of enzyme (Whitaker, 1972). It provides stability to the enzyme against thermal denaturation, pH, and proteolysis (Robyt, 1984). Baker (1983) reported that a heat treatment of 20 minutes at 60°C in a NaCl-enzyme mixture resulted in a loss of 95% in the activity of *S. granarius* α-amylase. In a $CaCl_2$-enzyme mixture, only 30% of the enzyme activity was lost.

The effect of calcium on α-amylase activity varies from one enzyme source to another and is thought to be concentration-dependent (Irshad and Sharma, 1981). The activities of maize, barley, wheat, and peanut α-amylases were inhibited by calcium concentrations of 50 mM. Bacterial and hog pancreatic α-amylases were, however, unaffected by calcium concentrations of 250 mM.

Evidence suggests that calcium ions are chelated by various groups on the enzyme, which contributes to the stability of the tertiary, and in some cases the secondary, structure of the enzyme (Whitaker, 1972; Robyt, 1984).

2.4.0.2 Effect of activators and inhibitors

The extent to which starch digestion occurs is contingent upon the presence of certain activators such as chloride and nitrate ions (Hori, 1970b), as well as calcium ions (Podoler and Applebaum, 1971). Chloride is present in the stems, veins and petioles of many plants, whilst nitrates are available from the atmosphere or soil through absorption by the roots. These activators bind to insect α-amylases, causing an increase in the reaction velocity (Terra *et al.*, 1977). α-Amylases from other sources, such as human saliva and pig pancreas, also show strong activation by chloride ions (Bernfeld, 1955). Podoler and Applebaum (1971), however, reported the inhibition of the α-amylase of *C. chinensis* by chloride ions.

The presence of endogenous α-amylase inhibitors, in some members of the graminae and leguminosae families, as plant resistance factors, has been recognized for at least 50 years (Yetter *et al.*, 1979; Campos *et al.*, 1989). These α-amylase inhibitors are thought to affect the catalytic functions of insect enzymes, both *in vitro* and *in vivo* (Gatehouse *et al.*, 1986). They are glycoproteins, containing about 10% carbohydrate, and have been found to be active against mammalian α-amylases, but do not inhibit plant or microbial α-amylases (Powers and Culbertson, 1982). Insect α-amylases have also been reported to be relatively resistant to inhibitor attack, due to the presence of isoenzymes. Four isozymic forms of α-amylase were reported in *C. maculatus*, with molecular weights of 33, 36, 45 and 56 kilodaltons by sodium dodecyl sulphate-polyacrylamide

gel electrophoresis (Campos et al., 1989). This diversity in molecular weights, within one single insect species, could explain the relative resistance of insect α-amylases to endogenous inhibitors.

2.4.0.3 α-Amylase isoenzymes

Many α-amylases exist in isozymic forms. Their enzymic properties are generally similar, and in most cases possess identical molecular weights. Differences occur in amino acid composition, the presence or absence of amide groups, or carbohydrate contents (Terra *et al.*, 1977; Robyt, 1984). Many isozymic forms are separable by polyacrylamide gel electrophoresis, or by ion exchange chromatography.

The absence of α-amylase isoenzymes in *Lygus* has been reported (Laurema *et al.*, 1985), but other insect species such as *S. granarius*, *S. oryzae*, *S. zeamais* (Baker, 1983), *R. americana* (Terra *et al.*, 1977), and *Drosophila melanogaster* (Doane, 1967) possess α-amylase isozymic forms. As many as eight isozymes were reported in *D. melanogaster*. α-Amylase isoenzymes occur in human saliva and pancreas, and in hog pancreas (Robyt, 1984).

2.4.0.4 Molecular weights

At the present time, none of the *Lygus* α-amylases have been characterized on the basis of their molecular weights. Some α-amylases from stored-product insects, such as *T. molitor*, have a molecular weight of 68 000 as determined by SDS-PAGE (Buonocore *et al.*, 1976). The molecular

weights of hog pancreatic and barley malt α-amylase are 51 000 and 59 000 daltons respectively. Some microbial α-amylases, such as *B. subtilis*, *B. stearothermophilus*, and *A. oryzae*, have molecular weights of 49 000, 15 600, and 52 000 daltons respectively (Pazur, 1965).

2.4.0.5 Effect of enzyme concentration

The initial reaction velocity was highly dependent on the concentration of *L. disponsi* α-amylase (Hori, 1970a). A linear relationship was found between the two variables, for enzyme concentrations between 0.1 mL and 1.0 mL, per 2.5 mL of the reaction mixture. *R. americana* midgut α-amylase also showed a linear relationship between the enzyme concentration and initial reaction velocity, up to 0.5 midguts/mL of the reaction mixture (Terra *et al.*, 1977).

2.4.0.6 Effect of substrate concentration

The effect of substrate concentration on *L. disponsi* α-amylase, showed that the initial velocity increased proportionally with substrate concentration up to a maximum of 0.6% (w/v) starch solution (Hori, 1970a). Beyond this, a further increase in the substrate concentration, up to 1.2% starch solution, did not have any effect on the reaction velocity.

The effect of substrate concentration on other insect α-amylase activities has been reported. The K_m of *T. molitor* α-amylase on soluble starch was 1.8 mg/mL and its specific activity was 1600 μmoles maltose/min/mg protein. The K_m was reported to be unaffected by the presence or

absence of activators. Using glycogen as the substrate, the values of the K_m and specific activity were 13.3 mg/mL and 1520 μmoles maltose/min/mg protein respectively (Buonocore *et al.*, 1976). The K_m values for starch, for *S. granarius* and *S. zeamais* α-amylases, were 0.77 and 1.3 mg/mL respectively. Their respective specific activities were 72.8 and 40.2 μmoles maltose/min/mg protein (Baker, 1983). *C. chinensis* α-amylase had a K_m of 2.3 mg/mL for soluble starch (Podoler and Applebaum, 1971), whilst the affinity constant for *R. americana* α-amylase for soluble starch was 1.4 mg/mL (Terra *et al.*, 1977).

The K_m values for other insect α-amylases were reported as 0.9 and 2.5 mg/mL, for two isozymes of *D. melanogaster*, and 0.89 mg/mL for *Locusta migratoria* (Baker, 1983). Garden pea and *B. stearothermophilus* α-amylases were reported to have K_m values of 0.2 and 1.0 mg/mL respectively (Podoler and Applebaum, 1971). The respective K_m values for *B. licheniformis* and Thermophile V-2 were 0.8 and 3.9 mg/mL (Chiang *et al.*, 1979).

2.4.1 Polygalacturonase

The plant cell wall is composed of polymers, including pectic substances which provide rigidity to the plant, and which present a physical barrier against pathogens (Karr and Albersheim, 1970; Collmer and Keen, 1986). Pectin, a water-soluble viscous heteropolysaccharide, occurs mainly in young and in fruit tissues, and together with other polysaccharides and proteins, constitute the adhesive material in the middle lamella of cells (Bateman and Millar, 1966; Pressey, 1986). It is characterized by partially methoxylated D-galacturonic acid residues, bonded by α-D-(1→4) linkages, which are interrupted periodically by 1,2-linked L-rhamnose (Schwimmer, 1981; Morris *et al.*, 1982). The polymer also contains neutral sugars, usually D-galactose and L-arabinose, and

occasionally D-xylose and L-fructose (Rexová-Benková and Markovič, 1976). The absence of methyl groups results in pectic acid or pectate, whilst esterification to varying degrees with methanol produces pectinic acid.

The enzymes responsible for the hydrolytic cleavage of the α-(1→4) bonds between galacturonic acid residues of pectic acid are known as polygalacturonases, or alternatively D-galacturonases (poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15). The polygalacturonases can be exo-(terminal) or endo-(random) splitting in their mode of action (Schwimmer, 1981). Most insect polygalacturonases, including those of *Lygus*, are endo-splitting. The absence of pectin lyases, pectin methylesterases or exopolygalacturonases in *Lygus* has been reported (Laurema *et al.*, 1985).

Polygalacturonases occur in many plant pathogens such as saprophytic fungi, bacteria and yeasts, and in several plant organs, such as fruits, stems, and leaves, and also in some insect species (Rexová-Benková and Markovič, 1976). The only animals that have been found to possess polygalacturonases are snails (Lineweaver and Jansen, 1951). The presence of polygalacturonases in aphids was first detected by McAllan and Cameron (1956). Later, Adams and McAllan (1958) detected polygalacturonases in several insect species with piercing-sucking mouthparts. The presence of polygalacturonase in the salivary glands of *Lygus* has been confirmed by several workers (Laurema and Nuorteva, 1961; Strong and Kruitwagen, 1968; Varis *et al.*, 1983). The occurrence of salivary polygalacturonase is reported to be a special feature of the Miridae family (Takanoma and Hori, 1974).

The principal function of this enzyme is to dissolve the pectic substances in the middle lamella and other parts of the cell wall in order to facilitate the penetration and utilization of plant tissue (Adams and McAllan, 1958; Strong, 1970; Miles, 1972; Laurema *et al.*, 1985). Another possible

function is the release of cell-bound proteins (Strand *et al.*, 1976), since there is considerable evidence to suggest that the plant cell wall contains significant amounts of structural proteins in the intercellular spaces (Bateman and Millar, 1966).

2.4.1.0 Effect of pH on polygalacturonase activity

Few workers have so far attempted to characterize the polygalacturonase of *Lygus*. As a result a broad range of pH optima has been reported. Laurema and Nuorteva (1961) reported a pH optimum of 4.4 to 4.8, whilst Strong and Kruitwagen (1968) recorded an optimum of 7.0 for *L. hesperus* polygalacturonase. An optimum of 8.0 was reported for *L. disponsi* polygalacturonase (Hori, 1975b), and 5.0 for *L. rugulipennis* polygalacturonase (Laurema *et al.*, 1985). Generally, the pH optima of polygalacturonases, like other glycan hydrolases, lies in the weakly acidic region, between 4.0 and 6.5 (Rexová-Benková and Markovič, 1976). These authors indicated that the pH optimum of most endopolygalacturonases depends on the degree of polymerization (d.p.) of the substrate. A decrease in the d.p. causes the pH optimum to shift towards the acidic side. For instance, the pH optimum of a yeast endopolygalacturonase, with high molecular weight substrates, was 4.4, but when tri- and tetrasaccharides were used, the pH optimum was at 3.5. According to Pressey and Advants (1971), the shift in polygalacturonase activity to the acidic side with decreasing substrate molecular weight could be attributed to the presence of isoenzymes which are substrate size specific, or to the inhibition of polygalacturonase by large molecular weight substrates at low pH values.

The pH optima of the polygalacturonases from tomatoes, d'Anjou pears, oat seedlings, peaches, kidney beans, asparagus shoots, and turnip roots range from 4.5 to 5.8 (Pressey, 1986).

Polygalacturonases from some microbial sources such as the yeast *Saccharomyces fragilis*, and the fungus *Corticium rolfsii*, have pH optima of 4.4-4.5 (Phaff, 1966) and 2.5 (Tagawa and Kaji, 1988) respectively. The endopolygalacturonase of *Penicillium italicum* has a pH optimum of 4.5, whilst its exopolygalacturonase has an optimum of 4.0 (Hershenhorn *et al.*, 1990). Karr and Albersheim (1970), and Kester and Visser (1990), reported an optimum in the range of 4.0 and 4.9, for *A. niger* polygalacturonase, whilst Ueda *et al.* (1982) reported an optimum of 4.0 for *A. oryzae* polygalacturonase. *Verticillium albo-atrum* polygalacturonase has a pH optimum between 5.25 and 6.0 (Mussell and Strouse, 1972), whilst Kertesz (1951) reported an optimum of 3.5 for a fungal polygalacturonase.

The effect of pH on the stability of *L. rugulipennis* polygalacturonase was studied at 2°C for 2 hours. The enzyme was most stable between pH 3.0 and 8.0 (Laurema *et al.*, 1985). *C. rolfsii* polygalacturonase was stable between pH 2.5 and 8.0 at 5°C for one month (Tagawa and Kaji, 1988), whilst *A. oryzae* polygalacturonase was stable between pH 2.0 and 6.0 at 30°C for 60 minutes (Ueda *et al.*, 1982). *V. albo-atrum* polygalacturonase was most stable at pH 5.0 for 6 months at 4°C (Mussell and Strouse, 1972).

2.4.1.1 Effect of temperature on polygalacturonase activity

The optimum temperature of *L. rugulipennis* polygalacturonase occurred in the range of 35-40°C (Laurema *et al.*, 1985). Whilst the optimum temperature of *P. italicum* exopolygalacturonase occurred at 40°C, the endopolygalacturonase was most active at 50°C (Hershenhorn *et al.*, 1990). An optimum of 60°C was reported for *A. oryzae* polygalacturonase (Ueda *et al.*, 1982).

According to Strong and Kruitwagen (1968), the polygalacturonase of *L. hesperus* was stable to heat at 100°C for 30 minutes, but *L. rugulipennis* polygalacturonase was inactivated at 60°C after a 15-minute heat treatment (Laurema *et al.*, 1985). Pressey (1986) reported that while one of the two isoenzymes of tomato polygalacturonase was inactivated after 5 minutes at 65°C, the other was inactivated at 90°C after a 5-minute heat treatment. *A. oryzae* polygalacturonase was stable to heat up to 60°C, but complete inactivation occurred at 70°C (Ueda *et al.*, 1982).

2.4.1.2 Effect of activators and inhibitors

Sodium and potassium chlorides increased the catalytic activity of *L. rugulipennis* polygalacturonase (Laurema *et al.*, 1985). These authors indicated that the addition of activators did not affect the pH optimum of the enzyme. Pressey and Advants (1971) reported that NaCl has a dual effect on tomato polygalacturonase; it activates the enzyme, and prevents the aggregation of high molecular weight substrates by hydrogen bonds. The net effect is a shift in the pH towards the acidic side. Other activators of tomato polygalacturonase are cesium chloride and sodium nitrate (Pressey and Advants, 1971).

Polyvalent chlorides such as calcium, barium, magnesium, ferric, and ferrous, were inhibitory towards *L. rugulipennis* polygalacturonase. These salts are thought to cause gelation of the substrate by forming salt bridges between the carboxyl groups of adjacent pectic acid chains (Bateman and Millar, 1966; Pressey and Advants, 1971; Morris *et al.*, 1982). Corden *et al.* (1964) reported that in addition to the above, other polyvalent ions such as silver, aluminium, lead, copper, chromium, cadmium, zinc, cobalt, and nickel, have inhibitory effects on polygalacturonases, particularly from microbial sources.

Some polygalacturonases of mold origin are activated by sodium, potassium, and cesium salts, in addition to certain di- and trivalent ions, such as calcium, and aluminium (Kertesz, 1951). Pressey (1986) also reported that polygalacturonases from peach, d'Anjou pear, pea and oat seedlings were activated by calcium ions. These polygalacturonases are exopolygalacturonases, and require calcium to remove monomer units from the nonreducing ends of the substrate molecule.

2.4.1.3 Polygalacturonase isoenzymes and their molecular weights

The presence of two or three polygalacturonase isoenzymes in *L. rugulipennis* and in *L. hesperus* has been reported. These isozymic forms are thought to be endopolygalacturonases (Strong and Kruitwagen, 1968; Laurema *et al.*, 1985). Pressey (1986) reported the presence of two endopolygalacturonases in tomatoes, which differ in their molecular weight and in their heat stability. Their molecular weights were 100 000 and 44 000 daltons, confirmed by gel filtration chromatography. Pressey and Advants (1971) however, reported that the molecular weight of tomato polygalacturonase was 43 600 as shown by gel filtration chromatography. Other isozymes of polygalacturonase have been reported in peach, papaya, and d'Anjou pears (Pressey, 1986).

Six isoenzymes, five endo- and one exopolygalacturonase, were reported in *A. niger* (Kester and Visser, 1990). The molecular weights of the endopolygalacturonases ranged from 32 000 to 53 000 as shown by gel filtration chromatography, and from 38 000 to 59 000, confirmed by SDS-PAGE. *A. japonica* polygalacturonase has a molecular weight of 35 500 (Rexová-Benková and Markovič, 1976). The respective molecular weights of an exo- and two endopolygalacturonases from *P. italicum* are 60 000, and 36 000 and 38 000 respectively (Hershenhorn *et al.*, 1990). *V. albo-atrum* and *Sclerotium rolfsii* also contain endo- and exopolygalacturonases. The molecular weights of

the endopolygalacturonases were 34 500 and 31 000 daltons respectively, whilst molecular weights of 44 000 and 46 000 were reported for the exopolygalacturonases (Mussell and Strouse, 1972).

2.4.1.4 Effect of substrate concentration

Polygalacturonases are specific towards pectic acid or pectates (Schwimmer, 1981). *L. rugulipennis* polygalacturonase was found to be dependent on substrate concentration, and maximum velocity was reached at 0.4% (w/v) pectic acid (Laurema *et al.*, 1985). The activity of the enzyme at pH 5.0, 0.2% pectic acid, and 35°C, was 21.33 μmoles reducing groups/h, in the presence of 0.2 M KCl, and 1.84 μmoles reducing groups/h in the absence of KCl.

The specific activity of crude enzyme extracts of *V. albo-atrum* at pH 5.0, 39°C, and 1.5% sodium pectate was 1896 μmoles reducing groups/min/mg protein (Mussell and Strouse, 1972), whilst for *P. italicum*, 8 μmoles reducing groups/min/mg protein at pH 4.2, 30°C, and 1.5% sodium pectate was recorded (Hershenhorn *et al.*, 1990). Ueda *et al.* (1982) reported that the specific activity of *A. oryzae* was 0.43 μmoles reducing groups/min/mg protein. The experimental conditions were 0.5% pectic acid, pH 4.0, and a temperature of 40°C. The specific activity of *C. rolfsii* polygalacturonase was 0.53 μmoles reducing groups/min/mg protein at pH 2.5, 1.0% pectic acid and 30°C (Tagawa and Kaji, 1988).

2.4.2 Other Salivary Enzymes from *Lygus*

Salivary proteases are important to *Lygus* because they facilitate the utilization of food proteins, which are required for nutritional purposes. Several insect species belonging to the suborder Heteroptera have proteases which are active in the neutral to alkaline pH range (Laurema *et al.*, 1985). Acid proteases are commonly found in the midgut of several Hemiptera (Baker, 1982). Two types of proteases (acid and alkaline) were found in *L. rugulipennis*. The former had a pH optimum at 3.0 and the latter was at pH 7.6 (Laurema *et al.*, 1985). Hori (1970c) reported that the pH optima of *L. disponsi* proteases occurred at 5.0 and 10.0. The alkaline proteases are thought to be the more important of the two (Chayka, 1982), because it appears that they are more resistant to endogenous inhibitors (Campos et al., 1982).

L. rugulipennis alkaline protease is most stable between pH 4.0 and 10.0 for 48 hours at 2°C, whilst the acid protease is most stable between pH 2.0 to 6.0 for 6 hours at the same temperature. A 15-minute heat treatment at 40°C resulted in a loss of 25% in the activity for the acid protease, whilst all of the activity remained for the alkaline protease. Complete inactivation of both enzymes occurred at 70°C (Laurema *et al.*, 1985).

Other carbohydrases present in *Lygus* are invertase, trehalase, and maltase (Baptist, 1941; Elmore, 1955; Strong, 1970; Hori, 1975c; Laurema *et al.*, 1985). The pH optimum of trehalase and invertase is between 5.5 and 6.0. The function of trehalase is in the conversion of haemolymph trehalose to glucose; invertase is required for the hydrolysis of sucrose (Laurema *et al.*, 1985). Trehalase was most stable between pH 4.0 and 7.0 at 2°C for 21 hours, and for 15 minutes between 30 and 40°C. Complete inactivation occurred at 70°C.

The presence of other minor enzymes such as acid phosphatase has been demonstrated. They are

nonsecretable constituents of the glandular cells, with a pH optimum of 4.0 (Varis *et al.*, 1983; Laurema *et al.*, 1985). Acid phosphatase was most stable at pH values between 2.0 and 8.0, for 20 hours at 2°C, and was inactivated after a 15-minute heat treatment at 60°C. The acid phosphatase of *L. disponsi* has an optimum at pH 5.5 (Hori, 1974).

The exact function of phenolases has not been confirmed (Laurema *et al.*, 1985), but Strong (1970) suggested that they assist in the conversion of phenolic compounds released from damaged tissues to quinones initiating tissue darkening. These enzymes are located in the accessory glands (Miles, 1968).

3. MATERIALS AND METHODS

3.0 Source Material

Live adult insects (*Lygus lineolaris*) were obtained from Dr. C.G. Jackson of the Honey Bee Biology and Biological Control Laboratory, Tucson, Arizona, and adults of *Lygus hesperus*, also in live form, were obtained from Dr. G.L. Snodgrass of the Southern Insect Management Laboratory, Stoneville, Mississippi. All insects were kept in frozen storage (approximately -10°C) where they remained alive for five to seven days. Immediately after the death of the insects, the following stage of this study was undertaken.

3.1 Preparation of Crude Enzyme Extract

Insect heads (each containing a pair of salivary glands) were detached from the insect bodies, and placed directly into cold sodium monobasic-dibasic phosphate buffer, 0.01M, pH 7.0 (Hori, 1970a), in an ultra micro homogenizer (conical 08 418C; total capacity, 15 mL) in the proportion of two heads per mL of buffer. The mixture (containing 20 heads in 10 mL of buffer) was homogenized for three minutes, and the resulting homogenate was centrifuged at 26 000 x g for five minutes at 2-4°C. The supernatant was filtered through Whatman no. 4 filter paper to remove floating debris, and the filtrate was stored at 4°C and used as the crude enzyme extract. Due to

unpleasant odours arising after one week of storage at this temperature, the extract was prepared on an "as is required" basis.

3.2 Protein Determination

Protein was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as the standard. Protein concentrations of all crude enzyme extracts were calculated relative to the standard. The relationship between the protein concentration (mg/mL) and the absorbance (640 nm) can be represented by the linear regression equation: $Y = 0.079 + 1.204(X)$, where Y is the protein concentration, and X is the absorbance. The coefficient of determination (r^2) obtained from the regression equation was 0.980.

3.3 Enzyme Assays

These were carried out using the procedure of Bernfeld (1955), employing the dinitrosalicylic acid reagent (DNS). This reducing sugar reagent was prepared by suspending 20 g of 3,5-dinitrosalicylic acid in approximately 400 mL of water. Sodium hydroxide (320 mL; 2.5N) was added with warming and stirring, to dissolve the acid. Sodium potassium tartrate (600 g) was then gradually added to the solution. When fully dissolved, the orange solution was made up to two litres with distilled water, filtered through Whatman no. 4 filter paper to remove impurities, and stored in a closed brown bottle at room temperature to protect the solution from light and from atmospheric carbon dioxide. The DNS reagent was stable for at least six months (Whelan,

1964).

3.3.0 Determination of α-Amylase Activity

Alpha-amylase activity was determined using 1% (w/v) soluble starch (Baker Chemical Company; reagent grade) as the substrate (Rick and Stegbauer, 1974), in sodium acetate-acetic acid buffer, 0.01M, pH 5.0 (Varis *et al.*, 1983), containing 20 mM sodium chloride (Baker, 1983) to activate the enzyme.

The reaction mixture, consisting of 5.0 mL of substrate and 1.0 mL of the crude enzyme preparation, was incubated at 35°C for two hours. At timed intervals (10, 30, 60, 90, and 120 minutes), 0.5 mL aliquots were withdrawn from the reaction vessel and placed into tubes containing 1.0 mL of DNS reagent, thereby terminating the reaction. The tubes were held at 100°C for 10 minutes for colour development, and cooled to room temperature for 20-60 minutes (Rick and Stegbauer, 1974). The absorbance was read at 550 nm in a LKB Ultrospec II, using maltose as the standard.

A maltose standard solution (1.0 mg/mL) was used to establish a calibration curve (Rick and Stegbauer, 1974). Tubes contained 0.2-1.0 mL of maltose standard solution, which in each case was diluted to a final volume of 1.0 mL with distilled water. A blank, containing 1.0 mL of distilled water only, was also prepared. To all the tubes, 1.0 mL of starch solution (1% w/v) and 1.0 mL of DNS reagent was added, mixed thoroughly and held at 100°C for 10 minutes. The tubes were cooled and the absorbance was read at 550 nm. The regression equation of the relationship between the absorbance and maltose concentration can be represented as: Y = 0 +

1.09(X), where Y is the absorbance at 550 nm and X is the maltose concentration (mg/mL). The coefficient of determination (r^2) obtained from the regression equation was 0.995.

The specific activity of α-amylase was calculated as μmoles maltose/h/mg protein at 35°C, under the described experimental conditions.

3.3.1 Determination of Polygalacturonase Activity

Polygalacturonase activity was determined using 0.2% (w/v) polygalacturonic (pectic) acid (Sigma Chemical Company; reagent grade) as the substrate, adjusted to a pH of 5.0 with 1.0N sodium hydroxide (Laurema *et al.*, 1985).

The reaction mixture, consisting of 5.0 mL of substrate in a sodium acetate-acetic acid buffer, 0.01M, pH 5.0, containing 20 mM NaCl, and 1.0 mL of enzyme was incubated at 35°C for two hours. Aliquots (0.5 mL) of the reaction mixture were withdrawn from the reaction vessel at timed intervals (10, 30, 60, 90, and 120 minutes) and placed into tubes containing 1.0 mL of DNS reagent, to terminate the reaction. The tubes were held at 100°C for 10 minutes, cooled for 20-60 minutes, and the absorbance read at 550 nm in a LKB Ultrospec II. The production of reducing groups was expressed as maltose.

The specific activity of polygalacturonase was calculated as μmoles reducing groups/h/mg protein at 35°C, under the described experimental conditions.

3.4 Effect of pH on Enzyme Activity

The effect of pH on the activities of the two enzymes was determined in the range of 3.0 to 8.0 using the following buffer systems:- citric acid-sodium citrate buffer, 0.01M, pH 3.0; acetic acid-sodium acetate buffer, 0.01M, pH 4.0 and 5.0; sodium monobasic-dibasic phosphate buffer, 0.01M, pH 6.0, 7.0, and 8.0. At timed intervals, 0.5 mL aliquots of the reaction mixture were withdrawn from the reaction vessel, and the reaction was terminated with 1.0 mL of DNS reagent and assayed for enzyme activity as previously described.

3.5 Effect of Temperature on Enzyme Activity

3.5.0 Optimum Temperature

The activity of each enzyme was determined at temperatures of 30, 35, 40, 50 and 60°C. At timed intervals, 0.5 mL aliquots of the reaction mixture were withdrawn from the reaction vessel and the reaction terminated with the DNS reagent. Enzyme activity was assayed as described previously.

3.5.1 Thermal Stability

Aliquots of crude enzyme extracts were variously held at 60-100°C for 5-30 minutes, cooled rapidly under running cold tap water, and assayed for residual enzyme activity at 30°C. A control experiment, undertaken concurrently, contained untreated enzyme. Aliquots of 0.5 mL were

withdrawn from the reaction mixtures and the reaction terminated with DNS reagent. Enzyme activity was assayed as described previously.

3.6 Effect of Enzyme Concentration

The effect of enzyme concentration on activity was studied by varying the amount of enzyme present in a series of reaction mixtures, whilst all other experimental conditions remained constant. Seven amounts of enzyme extract, from 0.25 mL to 3.0 mL, were used in reaction mixtures each containing 5.0 mL of substrate solution. The reaction was terminated by the addition of the DNS reagent and assayed for enzyme activity in the usual way.

3.7 Effect of Substrate Concentration

The effect of substrate concentration was determined by holding the enzyme concentration and all other experimental conditions constant, while varying the concentration of the substrate. Starch solutions at five different concentrations (1.0 to 5.0% (w/v)), and pectic acid solutions at five concentrations (0.2 to 1.0% (w/v)), in sodium acetate-acetic acid buffer, 0.01M, pH 5.0, containing 20 mM NaCl, were used. The reaction was stopped by adding 1.0 mL of DNS reagent and thereafter assayed for enzyme activity. Lineweaver-Burk (double-reciprocal) plots were derived from the data relating the reaction velocity to the substrate concentration.

3.8 Effect of Chloride Ions on Enzyme Activity

The effect of chloride ions on the enzymic activity was determined by varying the concentration of sodium chloride, incorporated in the substrate solutions, from 0.02M to 1.0M, whilst all other experimental conditions remained constant. Aliquots (0.5 mL) of the reaction mixture were removed at timed intervals, the reaction terminated and assayed for enzyme activity in the usual way.

3.9 Effect of α-Amylase Activity on Legume Starches

Two grams each of dry, mature field peas (*Pisum sativum*), cowpeas (*Vigna unguiculata*), lima beans (*Phaseolus lunatus*), and white kidney beans (*Phaseolus vulgaris*), containing approximately 50% starch (Watt and Merrill, 1975), were soaked overnight in water at room temperature. Frozen immature field peas (9.37 g), were allowed to thaw to room temperature for two hours. The fully-hydrated seeds were drained, blotted dry and ground in a mortar and pestle into a smooth paste. The paste was boiled in 30 mL of water for one minute, cooled, and centrifuged at 26 000 x g for five minutes at 2-4°C. The supernatant was made up to a final volume of 100 mL with sodium acetate-acetic acid buffer, 0.01M, pH 5.0, containing 20 mM NaCl. The starch solutions, of which the approximate final concentrations were 1% (w/v) (based on calculations), were used as substrates to determine the α-amylase activities from *L. lineolaris* and *L. hesperus*.

4. RESULTS

4.0 Effect of pH on Enzyme Activity

4.0.0 α-Amylase

The hydrolysis of soluble starch by the α-amylases of *L. lineolaris* and of *L. hesperus* was studied in the range of pH 3.0 to 8.0. Their pH-activity curves are shown in Figures 4 and 5 respectively. *L. lineolaris* α-amylase had an optimum of 5.0, whilst *L. hesperus* was most active at pH 6.0. From these results it would appear that whilst *L. lineolaris* α-amylase was very sensitive at middle range pH values particularly between pH 4.0 and 6.0, *L. hesperus* α-amylase was very sensitive at extreme pH values.

4.0.1 Polygalacturonase

Figures 6 and 7 are pH-activity curves of the polygalacturonases of *L. lineolaris* and of *L. hesperus* respectively. Maximum activity occurred at pH 5.0 in both cases. *L. lineolaris* polygalacturonase, in addition, showed strong activation at pH 6.0, which may indicate the presence of an isoenzyme, of which the optimum is at pH 6.0. *L. hesperus* polygalacturonase, on the other hand, showed one single distinct peak, although the enzyme was also fairly active at pH 6.0. Over the more alkaline pH range, *L. lineolaris* polygalacturonase was more sensitive to change than *L. hesperus*

Figure 4. pH-Activity curve of *L. lineolaris* α-amylase. Each point represents the mean of triplicate determinations. Vertical bars indicate the standard deviations of the mean.

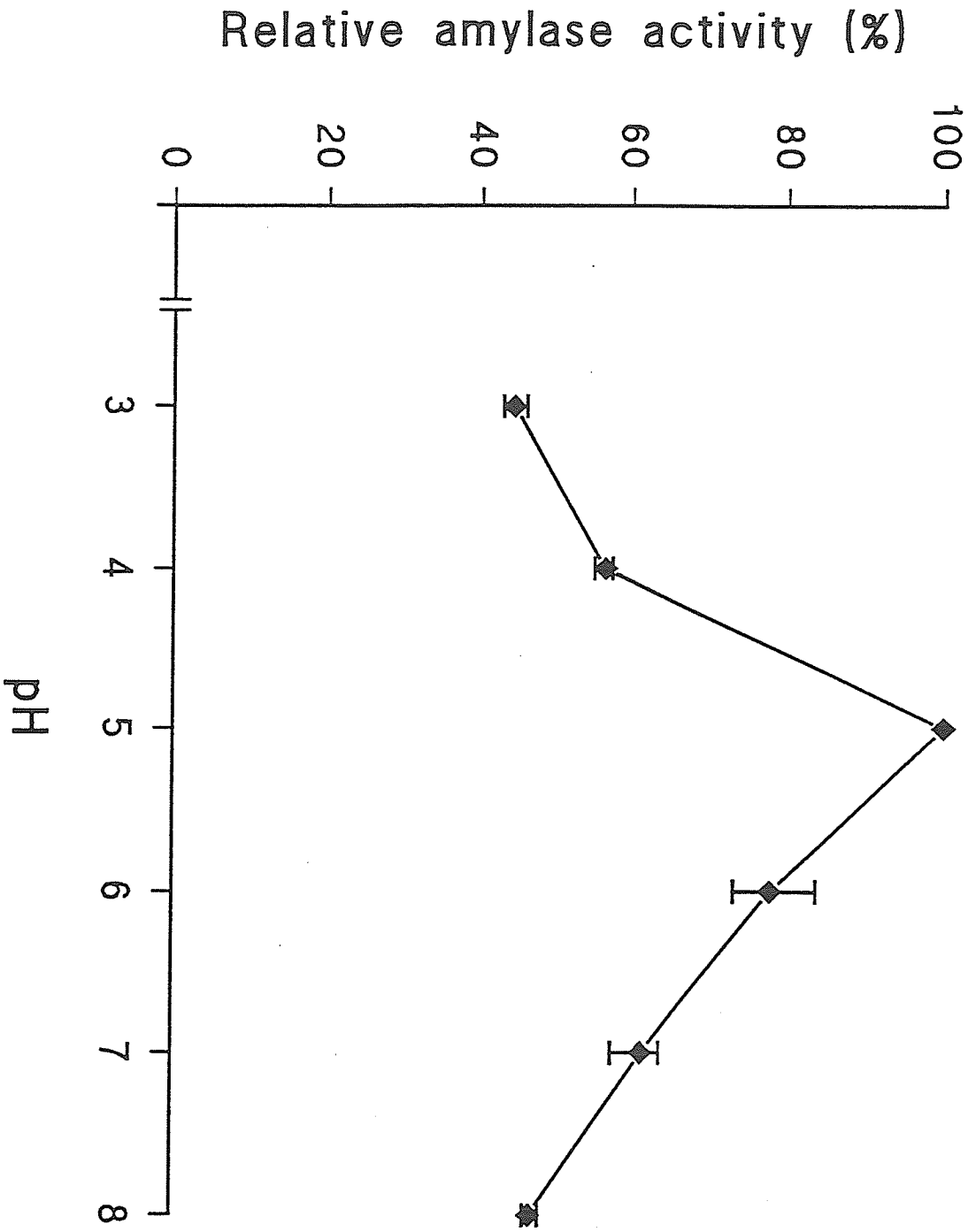

Relative amylase activity (%)
0
20
40
60
80
100
pH
3
4
5
6
7
8

Figure 5. pH-Activity curve of *L. hesperus* α-amylase. Each point represents the mean of triplicate determinations. Vertical bars indicate the standard deviations of the mean.

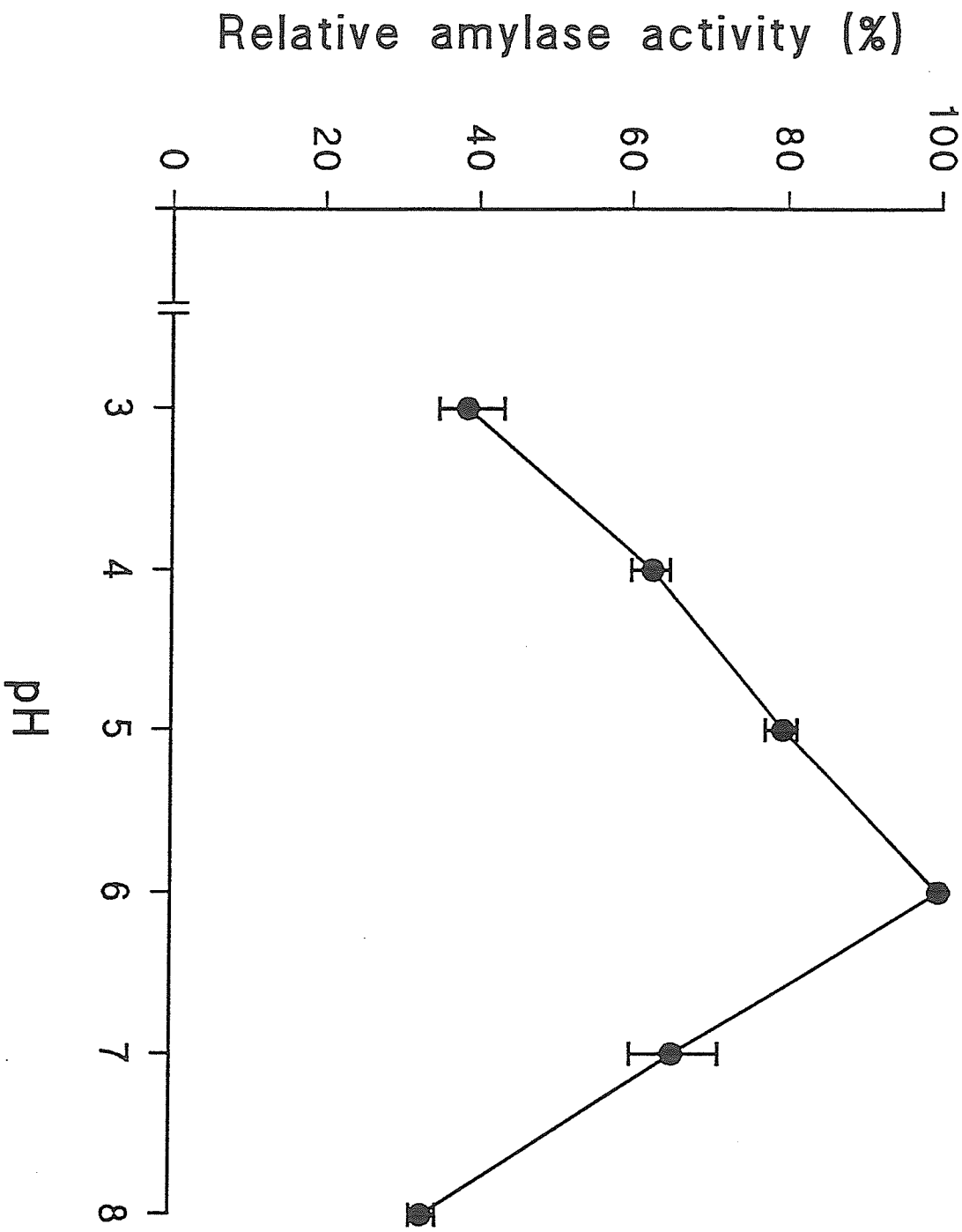

Relative amylase activity (%)
0
20
40
60
80
100
pH
3
4
5
6
7
8

Figure 6. pH-Activity curve of *L. lineolaris* polygalacturonase. Each point represents the mean of triplicate determinations. Vertical bars indicate the standard deviations of the mean.

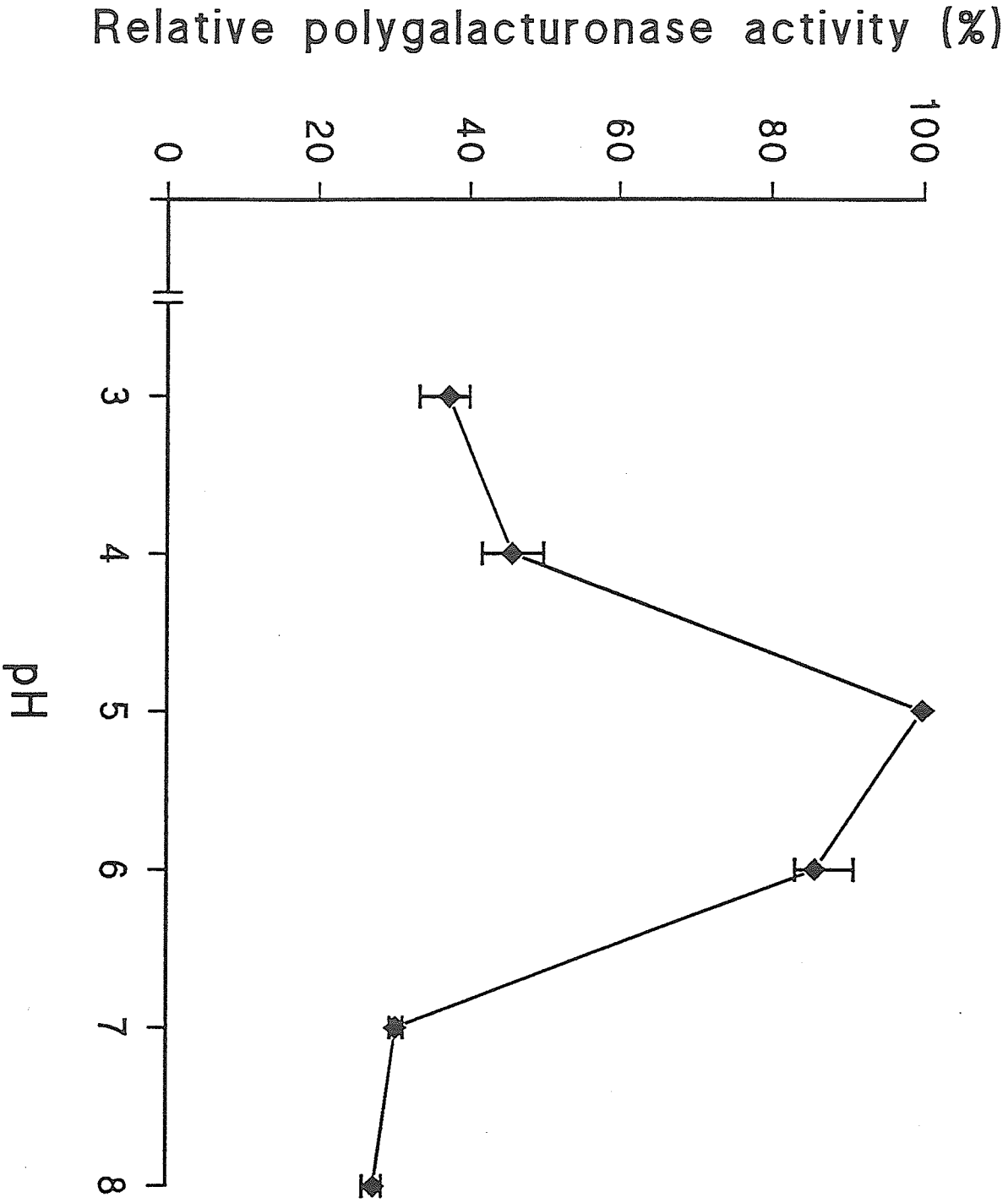
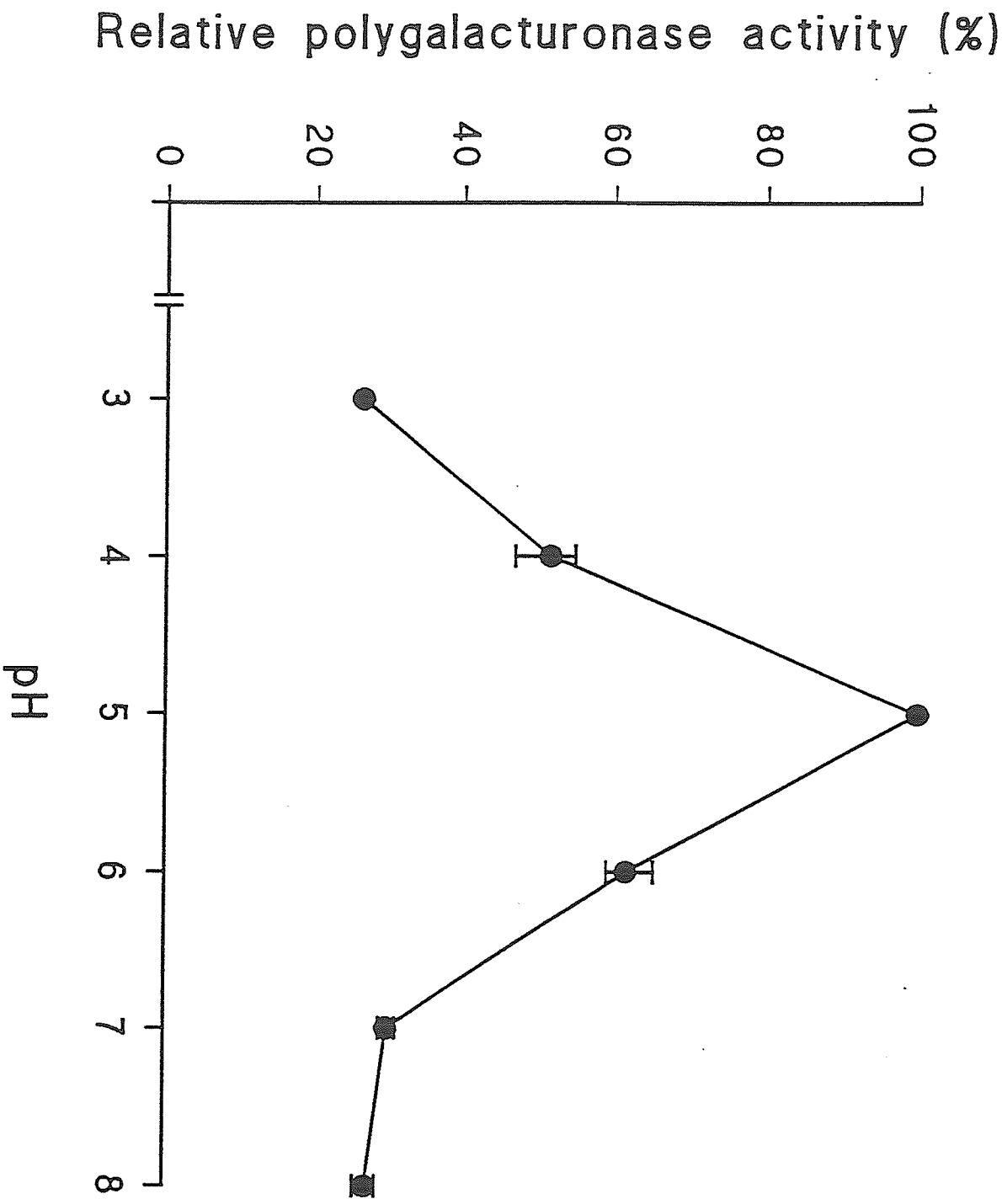

Relative polygalacturonase activity (%)
0
20
40
60
80
100
pH
3
4
5
6
7
8

Figure 7. pH-Activity curve of *L. hesperus* polygalacturonase. Each point is based on triplicate determinations. Vertical bars indicate standard deviations of the mean.


Relative polygalacturonase activity (%)
0
20
40
60
80
100
pH
3
4
5
6
7
8

polygalacturonase, while the reverse was true over the acidic range particularly between pH 3.0 to 4.0.

4.1 Effect of Temperature on Enzyme Activity

4.1.0 α-Amylase

The effect of temperature on enzyme activity was studied at 30, 35, 40, 50 and 60°C. Temperature-activity curves for *L. lineolaris* and for *L. hesperus* α-amylases are shown in Figures 8 and 9 respectively. In both cases, there was an increase in enzyme activity from 30 to 40°C. Their temperature optima occurred at 40°C. Although this was the optimum, the enzymes were also strongly activated at 35°C. In accordance with the Arrhenius rule, at temperatures below the optimum, the rate of enzyme reactions increases with temperature so that a rise in 10°C doubles, or in some cases triples, the rate of the enzyme reaction (Sizer, 1943; Berk, 1976). This is commonly referred to as the Q_{10} rule, and was observed for both *L. lineolaris* and *L. hesperus* α-amylase. Above 40°C, a decrease in the enzyme activity occurred, due to thermal denaturation. However, complete inactivation did not occur at 60°C; about 50% of the enzyme activity remained, which is indicative of the thermal stability of both *L. lineolaris* and *L. hesperus* α-amylase.

4.1.1 Polygalacturonase

The effect of temperature on *L. lineolaris* and on *L. hesperus* polygalacturonases is shown in Figures 10 and 11 respectively. Although the optimum temperature occurred at 40°C in both

Figure 8. Temperature-Activity curve of *L. lineolaris* α-amylase. Each point is based on the mean of triplicate determinations. Vertical bars indicate the standard deviations of the mean.

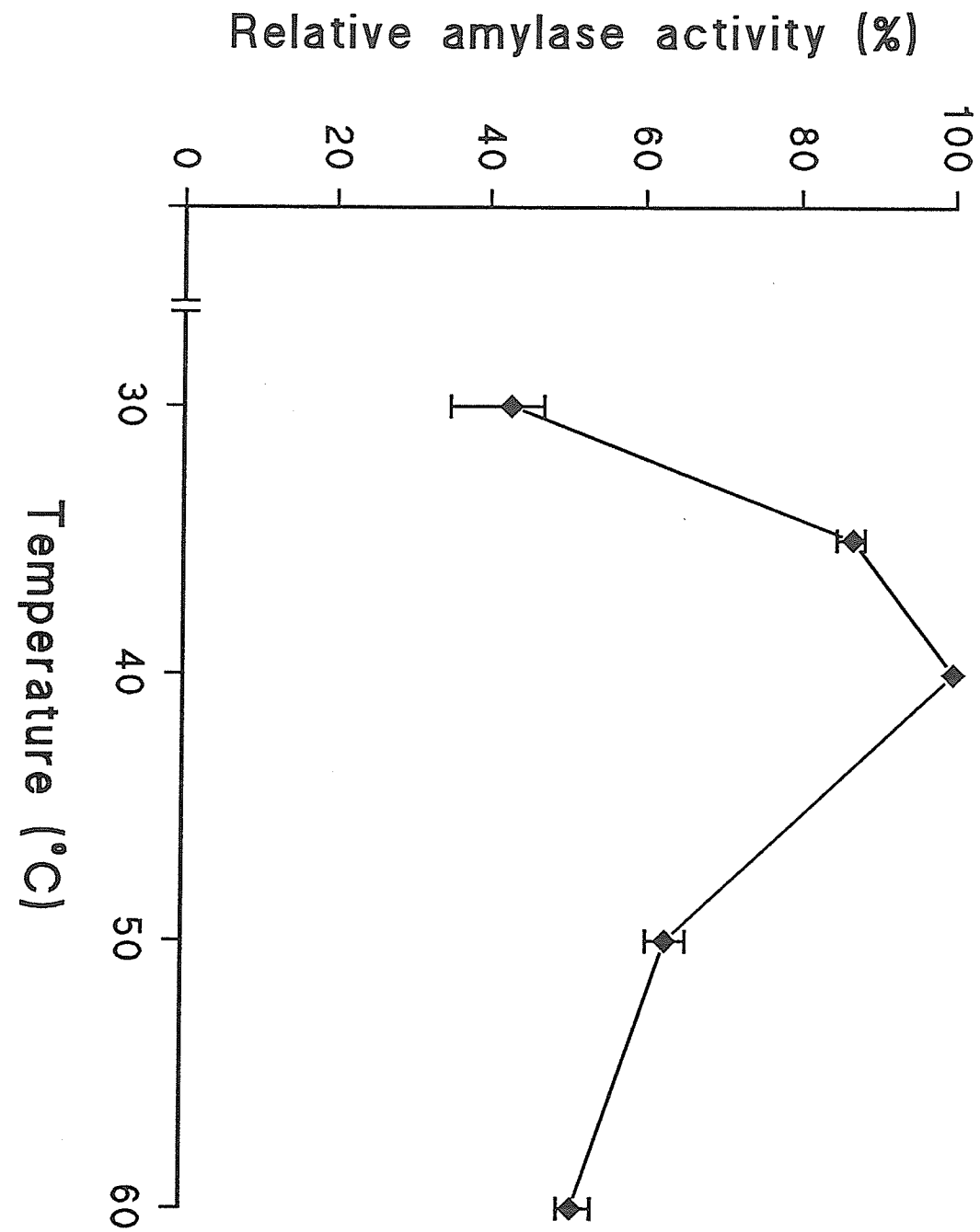

Relative amylase activity (%)
0
20
40
60
80
100
Temperature (°C)
30
40
50
60

Figure 9. Temperature-Activity curve of *L. hesperus* α-amylase. Each point represents the mean of triplicate determinations. Vertical bars indicate the standard deviations of the mean.

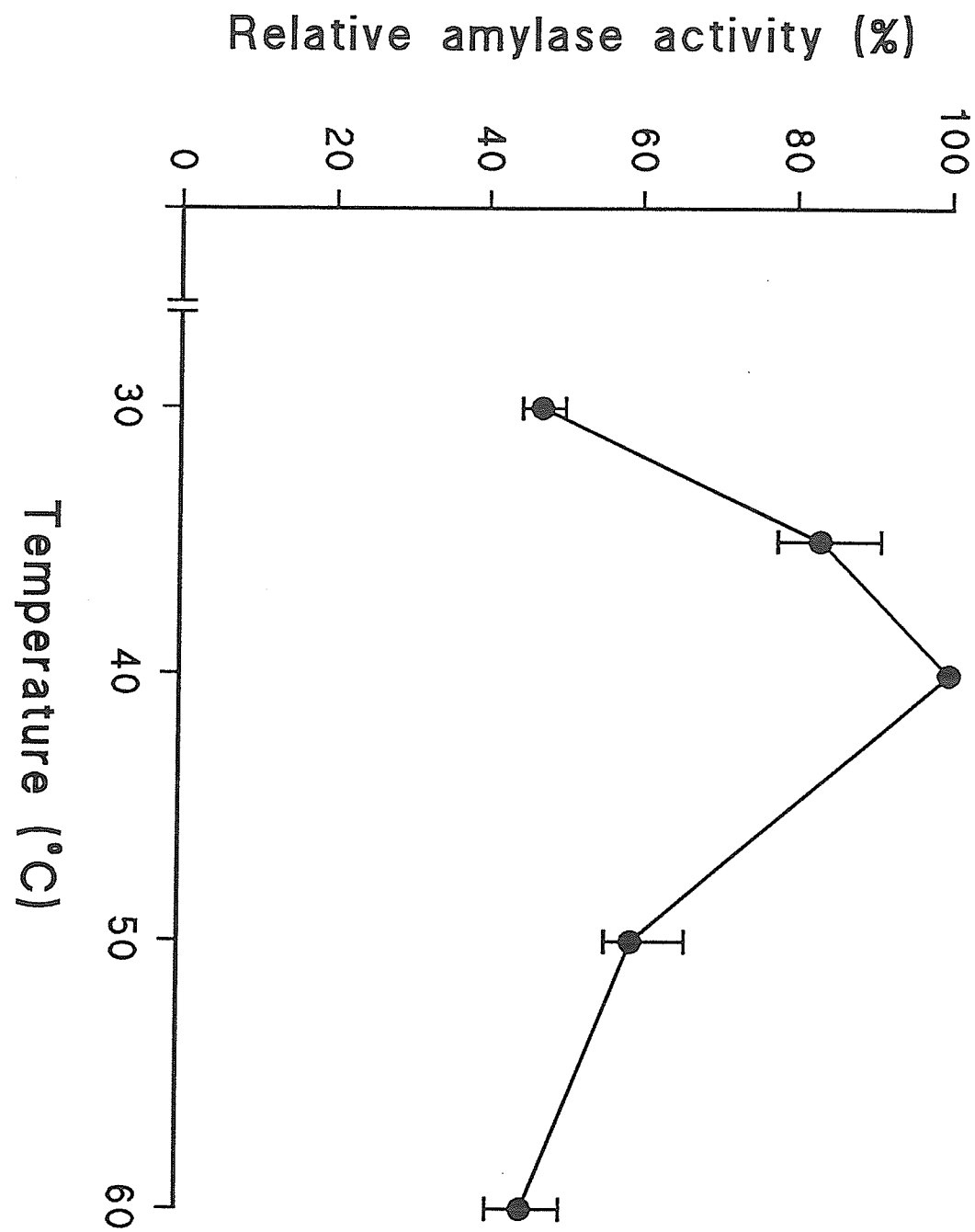

Relative amylase activity (%)
0
20
40
60
80
100
Temperature (°C)
30
40
50
60

Figure 10. Temperature-Activity curve of *L. lineolaris* polygalacturonase. Each point represents the mean of triplicate determinations. Vertical bars indicate the standard deviations of the mean.

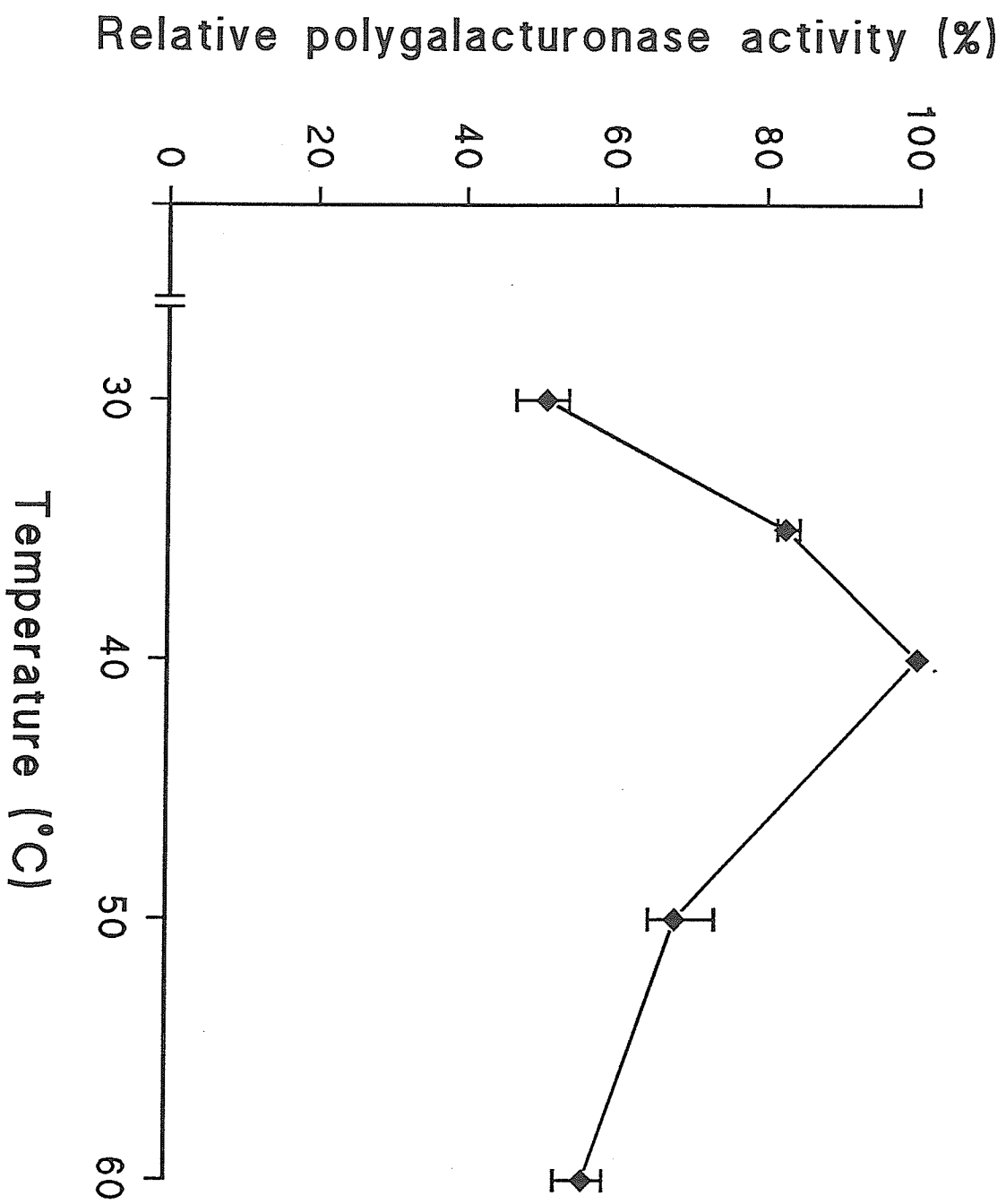
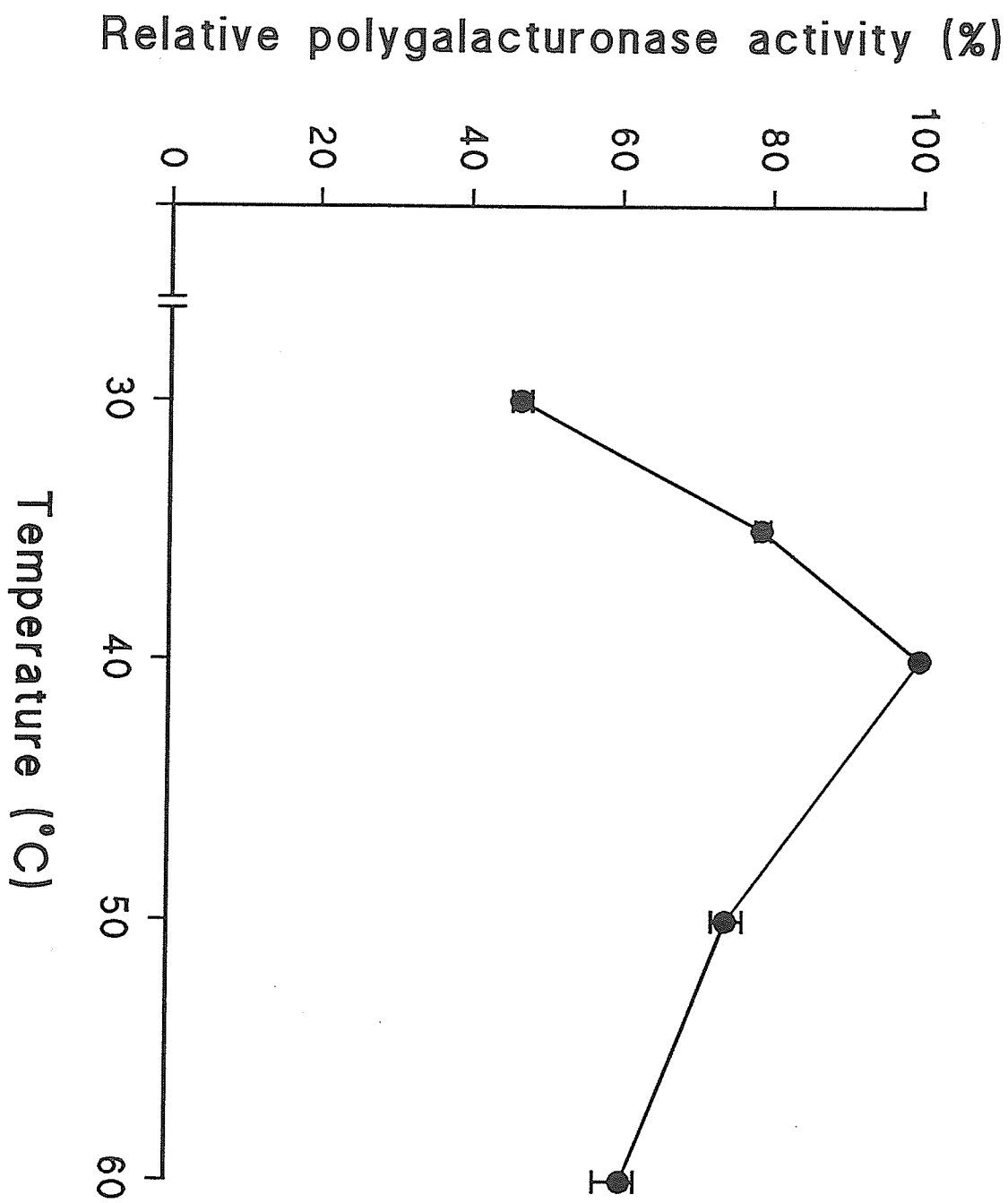

Relative polygalacturonase activity (%)
0
20
40
60
80
100
Temperature (°C)
30
40
50
60

Figure 11. Temperature-Activity curve of *L. hesperus* polygalacturonase. Each point represents the mean of triplicate determinations. Vertical bars indicate the standard deviations of the mean.


Relative polygalacturonase activity (%)
0
20
40
60
80
100
Temperature (°C)
30
40
50
60

cases, the enzymes were also strongly activated between 35 and 40°C. In accordance with the Q_{10} rule, a doubling in the rate of the enzyme reaction was observed between 30 and 40°C. The enzyme activity began to decline after 40°C, due to thermal denaturation but by 60°C, about 60% of the activity still remained, indicating that *L. lineolaris* and *L. hesperus* polygalacturonases are also relatively heat stable. However, they appeared to be more heat stable than *L. lineolaris* and *L. hesperus* α-amylases.

4.2 Effect of Temperature on Enzyme Stability

4.2.0 α-Amylase

Table 1 presents the residual activities of *L. lineolaris* α-amylase when exposed to various time-temperature regimes. These results also confirm the relative thermal stability of the enzyme. Losses in enzyme activity were minimal between 60 and 90°C, but began to decline rapidly between 90 and 100°C. A heat treatment at 100°C for 30 minutes did not fully inactivate the enzyme.

The results presented in Figure 12 suggest that the thermal inactivation of *L. lineolaris* α-amylase follows first-order reaction kinetics. The rate constants (k (min^{-1})) were obtained, by linear regression, from the slope of the line relating the Ln of the residual activity (%) to the heat treatment incubation time (min). The increase in the rate constants suggests an increase in thermal inactivation as the exposure of the enzyme to various temperatures and heating times increased.

Thermodynamic variables calculated from the enzyme inactivation data are presented in Table 2.

TABLE 1. Residual Activities of *L. lineolaris* α-Amylase.

Time (min)	Residual Activity (%) 60°C	70°C	80°C	90°C	94°C	100°C
0	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]
5	99.6[a]	98.3[a]	92.4[a]	89.4[a]	86.5[a]	75.7[ab]
10	98.1[a]	96.5[a]	89.4[a]	86.3[a]	74.0[ab]	65.1[bc]
15	96.1[a]	92.4[a]	86.8[a]	81.6[ab]	69.0[b]	54.0[c]
20	94.8[a]	88.9[a]	84.2[ab]	80.0[ab]	62.7[bc]	39.1[d]
25	90.9[a]	86.8[a]	82.4[ab]	74.0[ab]	54.0[bc]	32.5[d]
30	88.9[a]	84.2[ab]	75.9[ab]	69.0[b]	39.0[d]	24.0[e]

[1] Results are the means of triplicate determinations. Mean values followed by the same letter are not significantly different from each other ($P < 0.05$; Tukey test).

Figure 12. Thermal inactivation of *L. lineolaris* α-amylase. Each point represents the mean of triplicate determinations. First order rate constants (k) are expressed as min^{-1}; r^2 is the coefficient of determination.

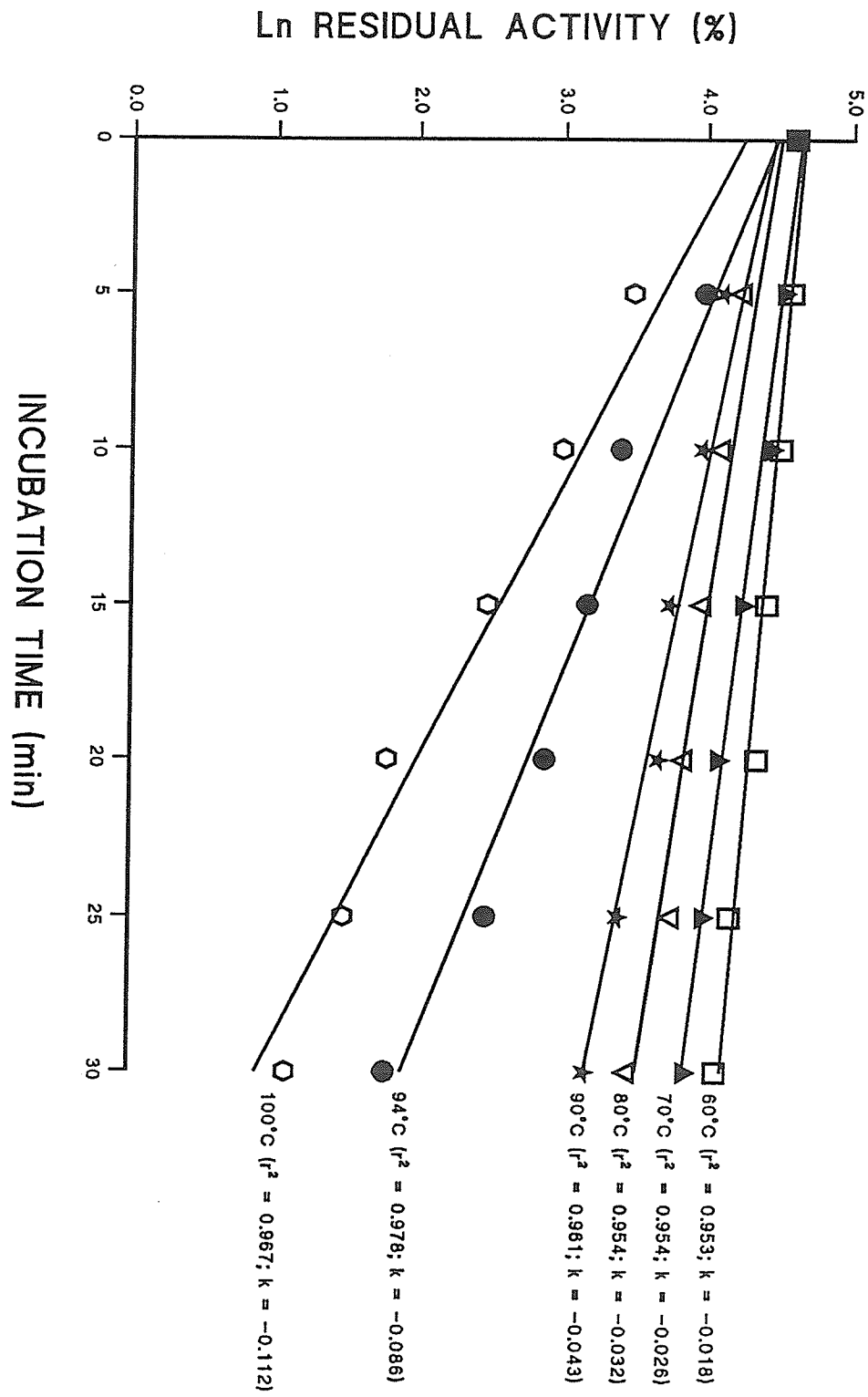

Ln RESIDUAL ACTIVITY (%)
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
25
30
INCUBATION TIME (min)
60°C (r² = 0.953; k = −0.018)
70°C (r² = 0.954; k = −0.026)
80°C (r² = 0.954; k = −0.032)
90°C (r² = 0.981; k = −0.043)
94°C (r² = 0.978; k = −0.086)
100°C (r² = 0.967; k = −0.112)

TABLE 2. Thermodynamic Variables for the Inactivation of *L. lineolaris* α-Amylase and Polygalacturonase.

	E_a^* (kJ mole^{-1})	ΔH (kJ mole^{-1})	ΔG^* (kJ mole^{-1})	ΔS (J mole^{-1} degree^{-1})
[1]	44.861	41.927	108.472	-188.513
[2]	38.770	35.835	108.871	-206.890

* Calculated at 80°C.
[1] *L. lineolaris* α-amylase.
[2] *L. lineolaris* polygalacturonase.

The enthalpy of activation (ΔH) was calculated according to the Eyring transition state theory (Gray, 1971), by a plot relating Ln (k/T) to 1/T (Figure 13), where T is the absolute temperature (°K), and k, is the rate constant in s^{-1}. The slope of the line, determined by linear regression, is equal to the enthalpy of activation divided by R, the gas constant (= 8.314 J $mole^{-1}$ $degree^{-1}$). The energy of activation (E_a), was calculated from the relationship $E_a = \Delta H + RT$. The intercept of the plot relating Ln (k/T) to 1/T is equal to Ln (K_B/h) + $\Delta S/R$, where K_B is the Boltzmann constant (= 1.380 x 10^{-23} J K^{-1} $mole^{-1}$), and h is the Planck constant (= 6.625 x 10^{-3} J s $mole^{-1}$). The entropy of activation (ΔS) could therefore be calculated. Finally, the Gibbs free energy of activation (ΔG), was calculated from the relationship $\Delta G = \Delta H - T\Delta S$ (Henderson *et al.*, 1991).

4.2.1 Polygalacturonase

Table 3 presents the residual activities of *L. lineolaris* polygalacturonase when exposed to various time-temperature regimes. Like the corresponding α-amylase, the polygalacturonase was relatively stable between 60 and 90°C, but as the temperature was increased to 100°C, rapid inactivation occurred. At this temperature, however, much of the enzyme activity remained. It also appears from these results, that *L. lineolaris* polygalacturonase is more heat stable than *L. lineolaris* α-amylase.

The first-order rate constants (k), expressed in min^{-1}, are shown in Figure 14. The increase in rate constants as temperatures increased also suggests a corresponding loss in enzyme activity, and therefore the thermal denaturation of the enzyme. Thermodynamic variables calculated for *L. lineolaris* polygalacturonase are also presented in Table 2. The energy of activation (E_a), and the enthalpy of activation (ΔH), were both lower than the respective values for *L. lineolaris* α-

Figure 13. Determination of the enthalpy of activation (ΔH) for the thermal denaturation of *L. lineolaris* α-amylase. Each point represents the mean of triplicate determinations. The slope of the line is the enthalpy divided by the gas constant (R); r^2 is the coefficient of determination.

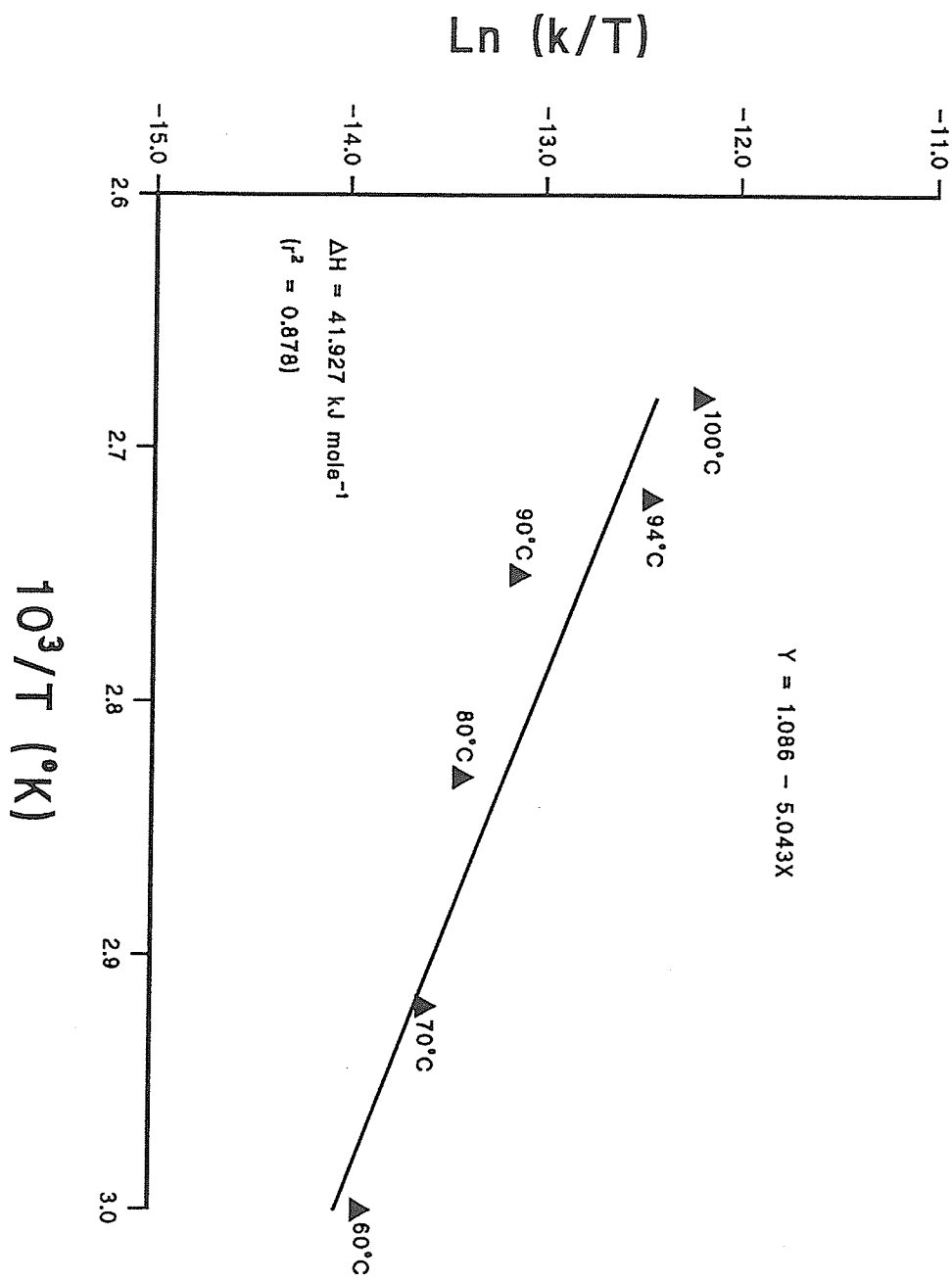
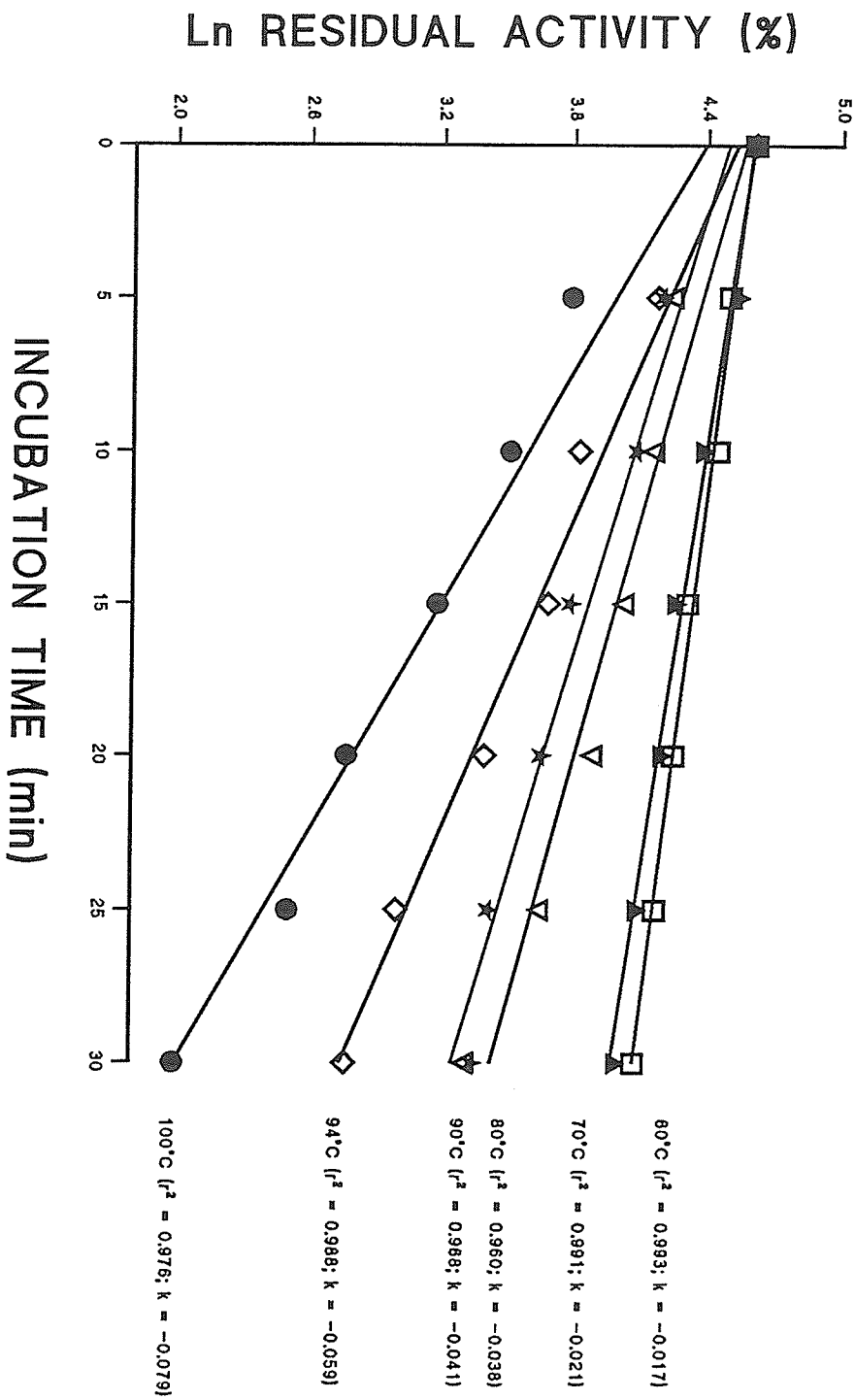

Ln (k/T)
-11.0
-12.0
-13.0
-14.0
-15.0
2.6
2.7
2.8
2.9
3.0
10³/T (°K)
ΔH = 41.927 kJ mole⁻¹
(r² = 0.878)
Y = 1.086 - 5.043X
100°C
94°C
90°C
80°C
70°C
60°C

TABLE 3. Residual Activities of *L. lineolaris* Polygalacturonase.

Time (min)	Residual Activity (%) 60°C	70°C	80°C	90°C	94°C	100°C
0	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]
5	97.6[a]	98.3[a]	92.4[ab]	91.1[ab]	90.7[ab]	82.2[bc]
10	96.5[a]	95.0[a]	90.7[ab]	88.7[ab]	83.1[bc]	76.1[cd]
15	93.7[a]	92.4[ab]	87.9[b]	82.2[bc]	80.0[c]	69.0[d]
20	92.4[ab]	90.9[ab]	84.8[bc]	79.4[c]	73.8[cd]	60.1[e]
25	90.7[ab]	88.7[ab]	79.7[c]	74.2[cd]	65.1[e]	54.4[e]
30	88.7[ab]	86.8[b]	72.7[d]	72.2[d]	60.1[e]	43.4[f]

[1] Results are the means of triplicate determinations. Mean values followed by the same letter are not signicantly different from each other ($P < 0.05$; Tukey test).

Figure 14. Thermal inactivation of *L. lineolaris* polygalacturonase. Each point represents the mean of triplicate determinations. First order rate constants (k) are expressed as min-1; r^2 is the coefficient of determination.


Ln RESIDUAL ACTIVITY (%)
5.0
4.4
3.8
3.2
2.6
2.0
0
5
10
15
20
25
30
INCUBATION TIME (min)
80°C (r² = 0.993; k = −0.017)
70°C (r² = 0.991; k = −0.021)
80°C (r² = 0.960; k = −0.038)
90°C (r² = 0.988; k = −0.041)
94°C (r² = 0.988; k = −0.059)
100°C (r² = 0.976; k = −0.079)

amylase. Although the Gibbs free energy of activation (ΔG) for both enzymes were very similar, the entropy of activation (ΔS), was higher for the polygalacturonase than for the α-amylase. The entropy of activation had a negative value, in both cases, suggesting a decrease in entropy from the reacting materials to the activated complex or transition state (Gray, 1971; Dannenberg and Kessler, 1988). The plot of the relationship between Ln (k/T) and 1/T is illustrated in Figure 15.

4.3 Effect of Enzyme Concentration

4.3.0 α-Amylase

The effect of enzyme concentration on the initial velocities of *L. lineolaris* and of *L. hesperus* α-amylases are presented in Figures 16 and 17. As shown, a direct relationship exists between the enzyme concentration and the initial velocity. One ml of crude enzyme extract contained 0.834 $\pm$ 0.001 mg/ml of protein, for *L. lineolaris*, and 0.680 $\pm$ 0.001 mg/ml of protein, for *L. hesperus*. The slope of the lines, obtained by linear regression, for both *L. lineolaris* and *L. hesperus* α-amylase, differed only slightly from one another, suggesting that the enzyme reaction proceeds at similar rates.

4.3.1 Polygalacturonase

Figures 18 and 19 respectively, illustrate the relationship between *L. lineolaris* and *L. hesperus* polygalacturonase concentration, and the initial reaction velocity. As observed for the α-amylases, a straight line relationship was obtained, indicative of a direct dependency of one variable upon

Figure 15. Determination of the enthalpy of activation (ΔH) for the thermal denaturation of *L. lineolaris* polygalacturonase. Each point represents the mean of triplicate determinations. The slope of the line is the enthalpy divided by the gas constant (R); r^2 is the coefficient of determination.

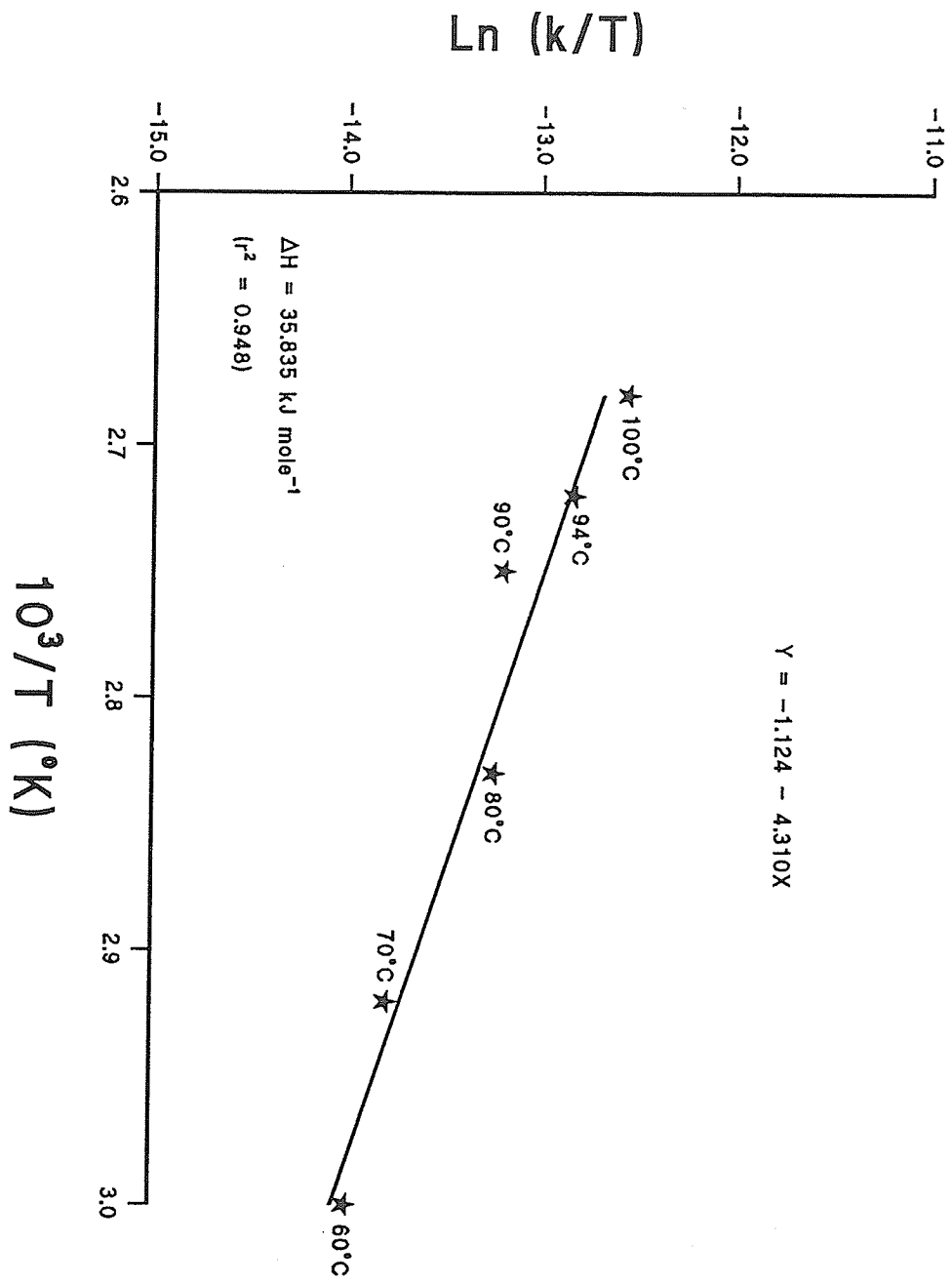

Ln (k/T)
-11.0
-12.0
-13.0
-14.0
-15.0
2.6
2.7
2.8
2.9
3.0
$10^3/T$ (°K)
Y = -1.124 - 4.310X
ΔH = 35.835 kJ mole^{-1}
(r^2 = 0.948)
100°C
94°C
90°C
80°C
70°C
60°C

Figure 16. Effect of enzyme concentration on the initial velocity of *L. lineolaris* α-amylase. Each point represents the mean of triplicate determinations; r^2 is the coefficient of determination.

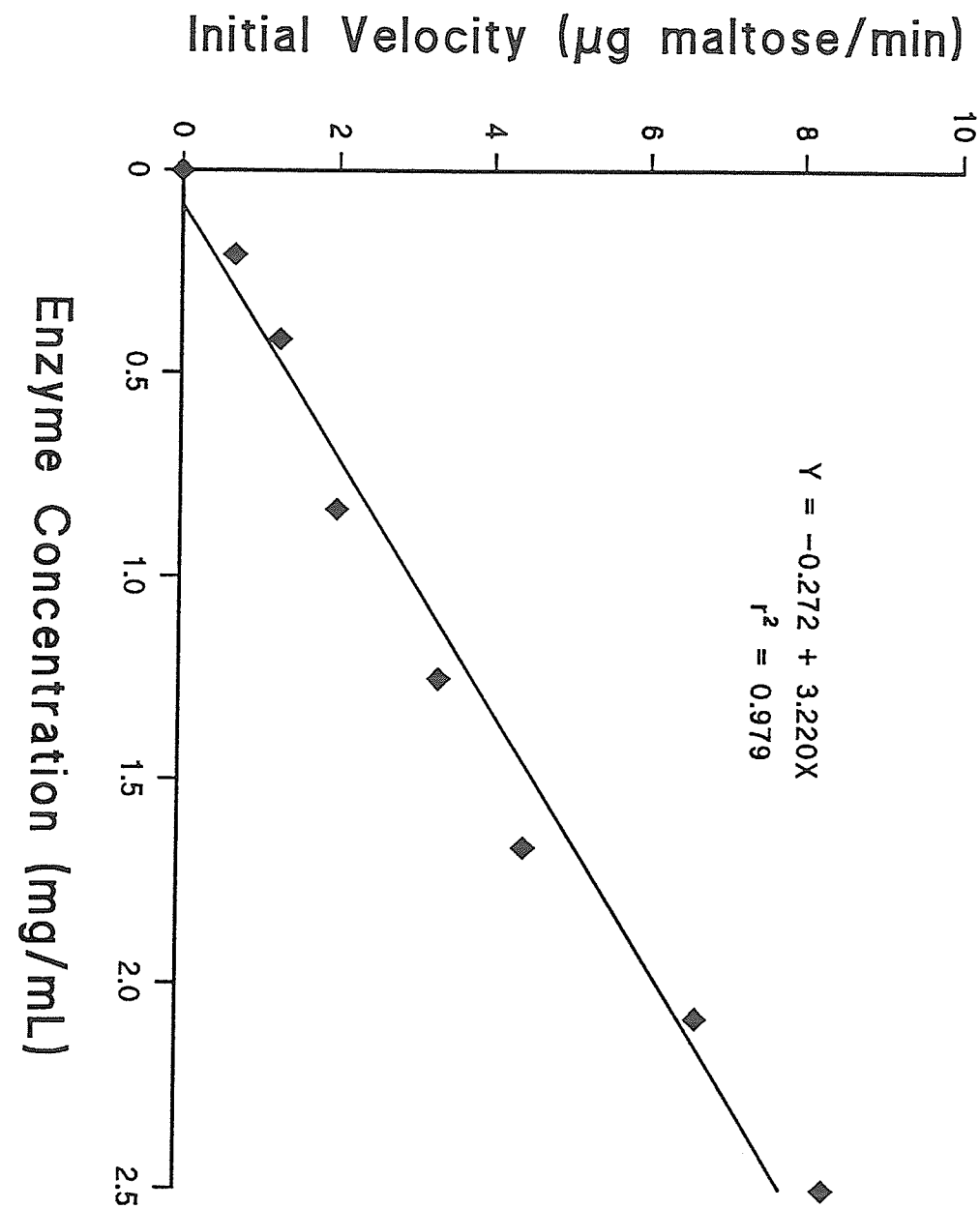

Initial Velocity (μg maltose/min)
0
2
4
6
8
10
Y = −0.272 + 3.220X
r² = 0.979
Enzyme Concentration (mg/mL)
0
0.5
1.0
1.5
2.0
2.5

Figure 17. Effect of enzyme concentration on the initial velocity of *L. hesperus* α-amylase. Each point is based on the mean of triplicate determinations; r^2 is the coefficient of determination.

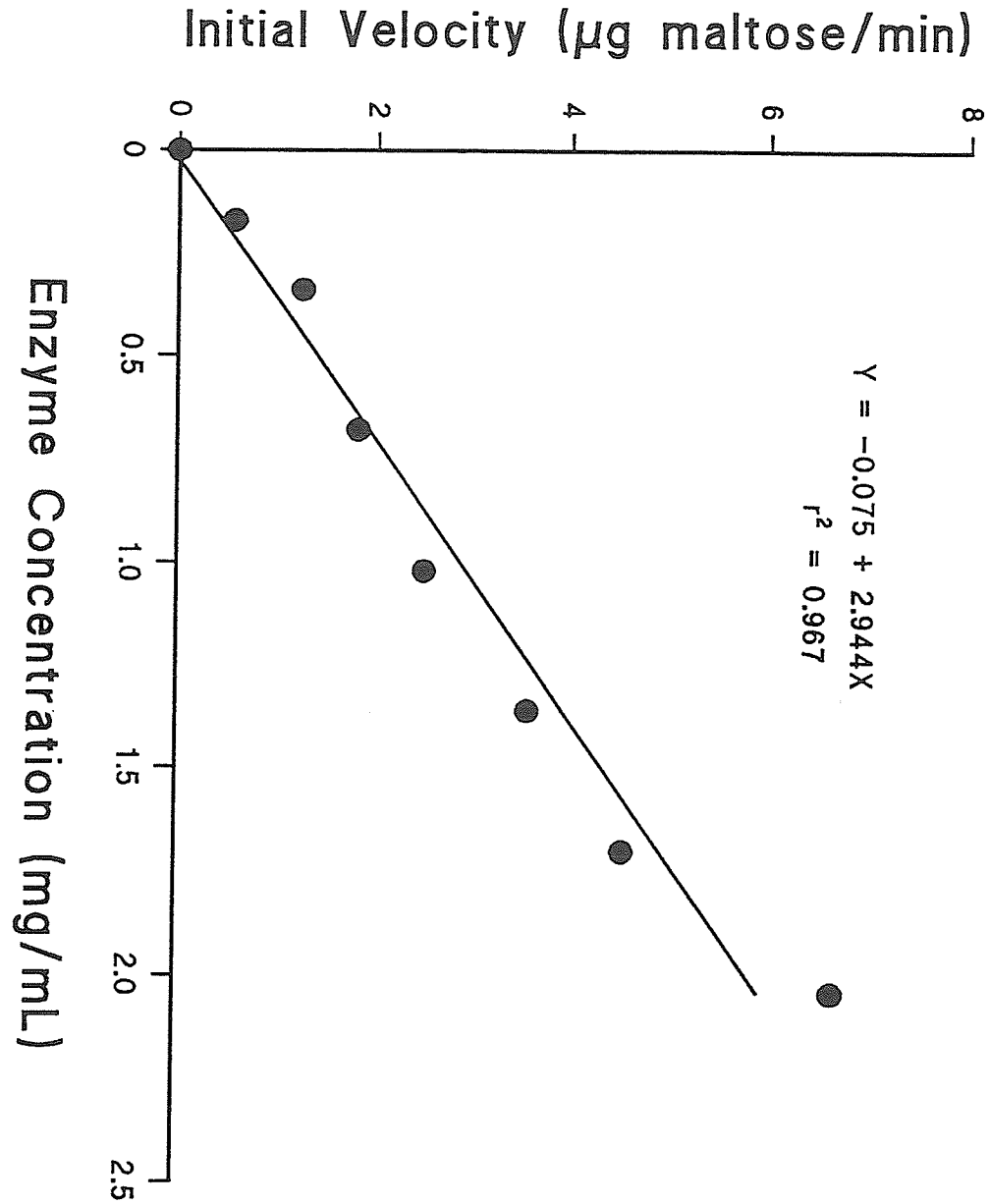

Initial Velocity (µg maltose/min)
0
2
4
6
8
Y = −0.075 + 2.944X
r² = 0.967
Enzyme Concentration (mg/mL)
0
0.5
1.0
1.5
2.0
2.5

Figure 18. Effect of enzyme concentration on the initial velocity of *L. lineolaris* polygalacturonase. Each point is based on triplicate determinations; r^2 is the coefficient of determination.

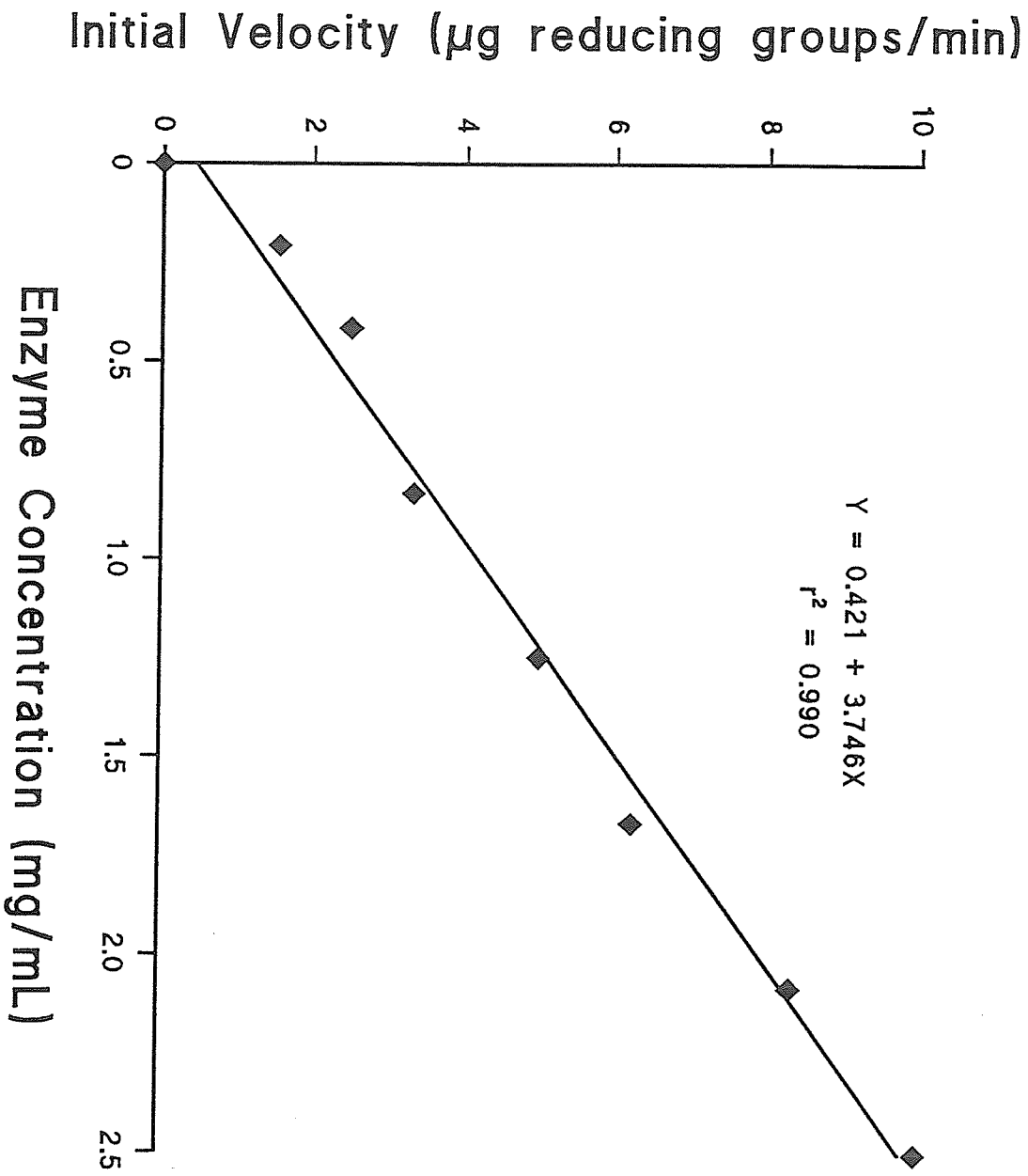
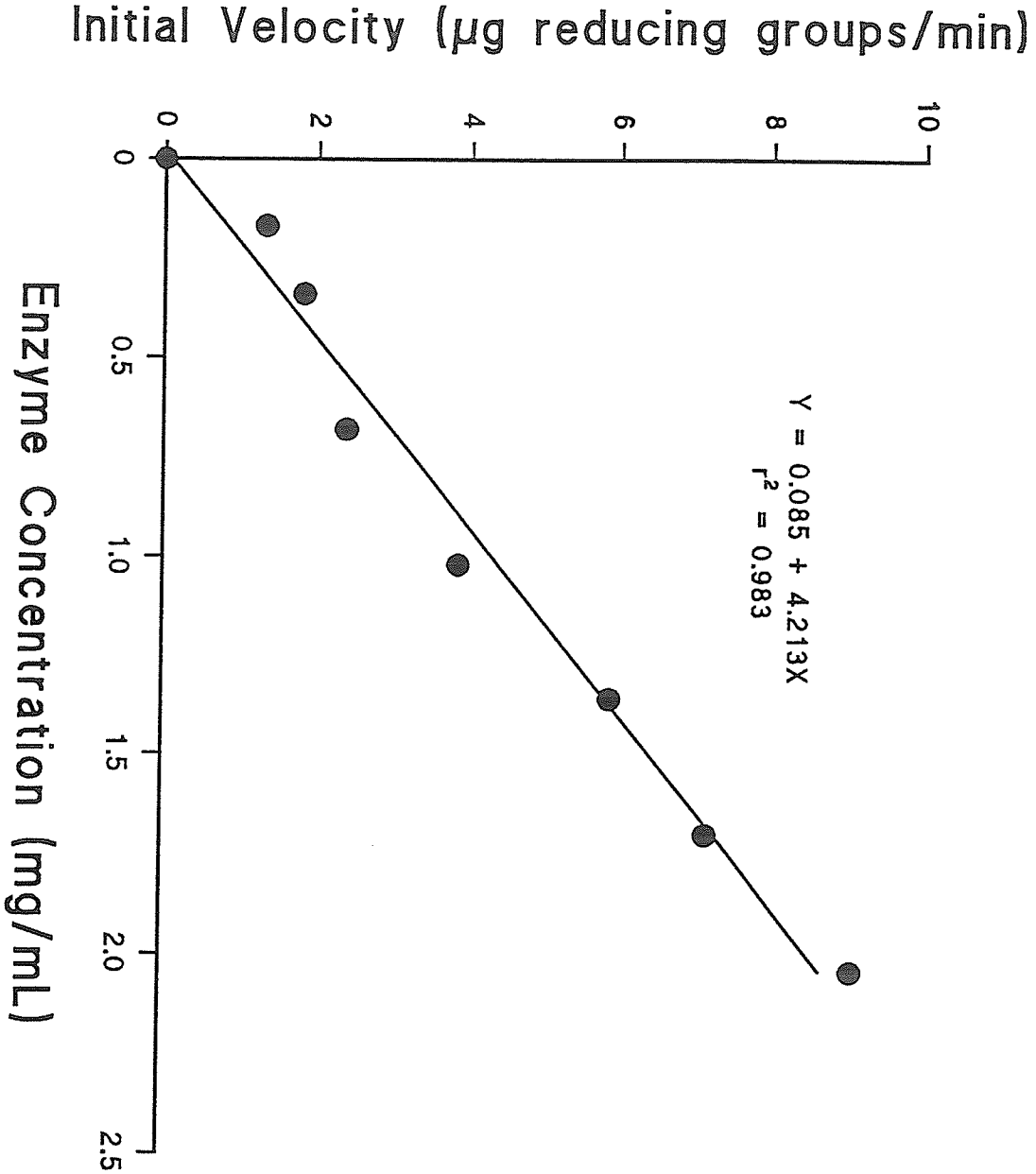

Initial Velocity (μg reducing groups/min)
0
2
4
6
8
10
Y = 0.421 + 3.746X
r² = 0.990
Enzyme Concentration (mg/mL)
0
0.5
1.0
1.5
2.0
2.5

Figure 19. Effect of enzyme concentration on the initial velocity of *L. hesperus* polygalacturonase. Each point is based on triplicate determinations; r^2 is the coefficient of determination.


Initial Velocity (µg reducing groups/min)
0
2
4
6
8
10
0
0.5
1.0
1.5
2.0
2.5
Enzyme Concentration (mg/mL)
Y = 0.085 + 4.213X
r² = 0.983

the other. The slope of the lines, obtained by linear regression, was higher for *L. hesperus* than for *L. lineolaris* polygalacturonase. This gives some indication that the reaction catalyzed by *L. hesperus* polygalacturonase, was accomplished at a faster rate than the same reaction catalyzed by *L. lineolaris* polygalacturonase.

4.4 Effect of Substrate Concentration

4.4.0 α-Amylase

Increasing the concentration of soluble starch, from 1.0 to 5.0% (w/v), resulted in corresponding increases in the initial velocities of *L. lineolaris* and of *L. hesperus* α-amylases up to a maximum point. Beyond this, a further increase in substrate concentration resulted in no further increase in initial velocity. The shape of the curve was a rectangular hyperbola (not shown). Figures 20 and 21 are Lineweaver-Burk (double-reciprocal) plots of the effect of substrate concentration on the initial velocities of *L. lineolaris* and *L. hesperus* α-amylases.

Two intercepts were obtained from the plot; one on the ordinate ($1/V_{max}$), and the other on the abscissa ($-1/K_m$), where V_{max} is the maximum reaction velocity and K_m is the Michaelis-Menten constant. The slope of the line, obtained by linear regression, is V_{max}/K_m. The K_m, a measure of the affinity between and enzyme and substrate (Berk, 1976), was greater for *L. hesperus* α-amylase than it was for *L. lineolaris* α-amylase, indicating a stronger affinity of *L. hesperus* α-amylase for soluble starch, compared to *L. lineolaris* α-amylase. This affinity was also displayed in their specific activities whose respective values are 11.157 and 7.067 μmoles maltose /h/mg protein.

Figure 20. Effect of substrate concentration on the initial velocity of *L. lineolaris* α-amylase. Each point represents the mean of triplicate determinations; r^2 is the coefficient of determination; V_{max} is the maximum reaction velocity; K_m is the Michaelis-Menten constant.

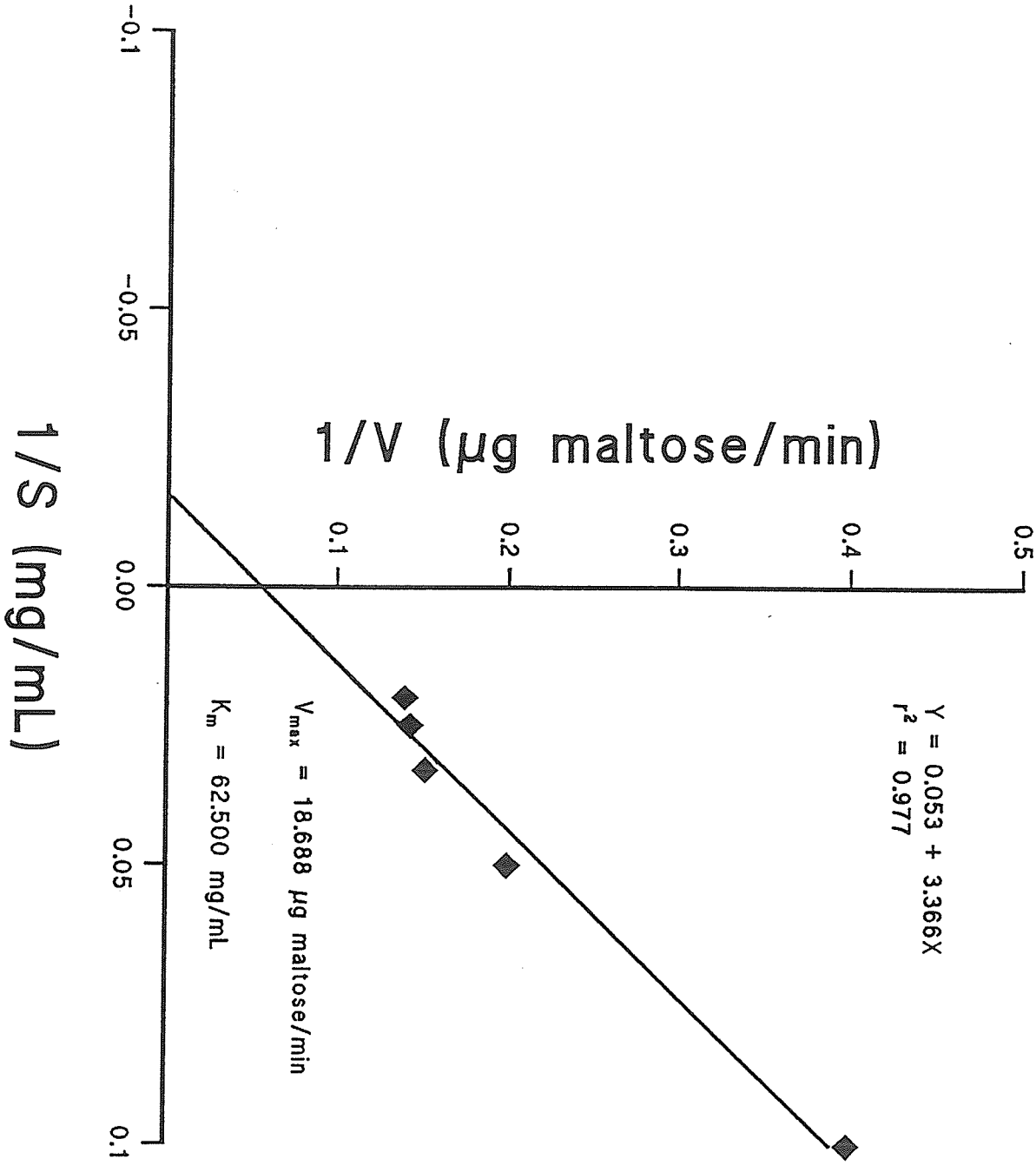
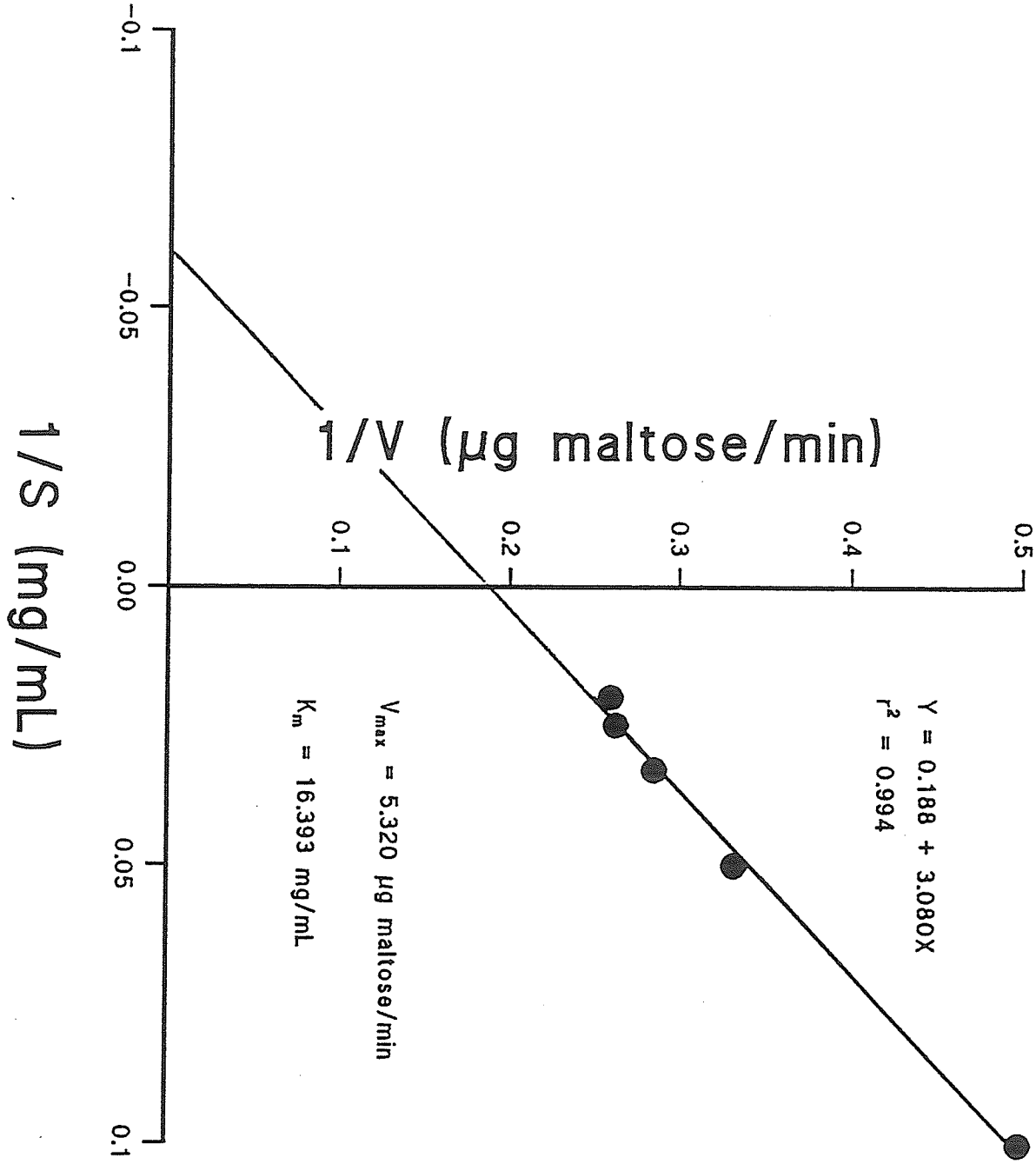

1/V (µg maltose/min)
0.5
0.4
0.3
0.2
0.1
0.00
0.05
0.1
-0.05
-0.1
1/S (mg/mL)
Y = 0.053 + 3.366X
r² = 0.977
Vmax = 18.688 µg maltose/min
Km = 62.500 mg/mL

Figure 21. Effect of substrate concentration on the initial velocity of *L. hesperus* α-amylase. Each point represents the mean of triplicate determinations; r^2 is the coefficient of determination; V_{max} is the maximum reaction velocity; K_m is the Michaelis-Menten constant.


1/V (µg maltose/min)
0.5
0.4
0.3
0.2
0.1
0.00
0.05
0.1
−0.1
−0.05
1/S (mg/mL)
Y = 0.188 + 3.080X
r² = 0.994
Vmax = 5.320 µg maltose/min
Km = 16.393 mg/mL

4.4.1 Polygalacturonase

The effect of substrate concentration on the initial velocities of *L. lineolaris* and of *L. hesperus* polygalacturonases is presented in Lineweaver-Burk plots in Figures 22 and 23 respectively. The respective specific activities of the enzymes were 11.359 and 17.028 μmoles reducing groups/h/mg protein. *L. hesperus* polygalacturonase appeared to have a stronger affinity for the substrate than *L. lineolaris* polygalacturonase, as shown by their K_m values and specific activities. Overall, the α-amylases had lower specific activities and lower K_m values compared to the polygalacturonases, suggesting that the polygalacturonases are capable of more damage to the structural and nutritional components of host plants, than the α-amylases from the same species of *Lygus*.

4.5 Effect of Chloride Ions on Enzyme Activity

4.5.0 α-Amylase

An increase in the concentration of sodium chloride, from 20 to 100 mM, resulted in an overall increase in the activities of both *L. lineolaris* and *L. hesperus* α-amylase (Table 4). The activity of the control is expressed as 1.000. The increase in *L. lineolaris* α-amylase activity with NaCl concentration was linear and the linear regression equation relating the two variables is: $Y = -0.010 + 0.063X$, where Y is the α-amylase activity, and X is the NaCl concentration. The coefficient of determination (r^2) was 0.977.

The linear regression equation of the line for *L. hesperus* α-amylase is: $Y = -0.207 + 0.065X$. The value of r^2, the coefficient of determination, obtained from the linear regression equation was

Figure 22. Effect of substrate concentration on the initial velocity of *L. lineolaris* polygalacturonase. Each point represents the mean of triplicate determinations; r^2 is the coefficient of determination; V_{max} is the maximum velocity; K_m is the Michaelis-Menten constant.

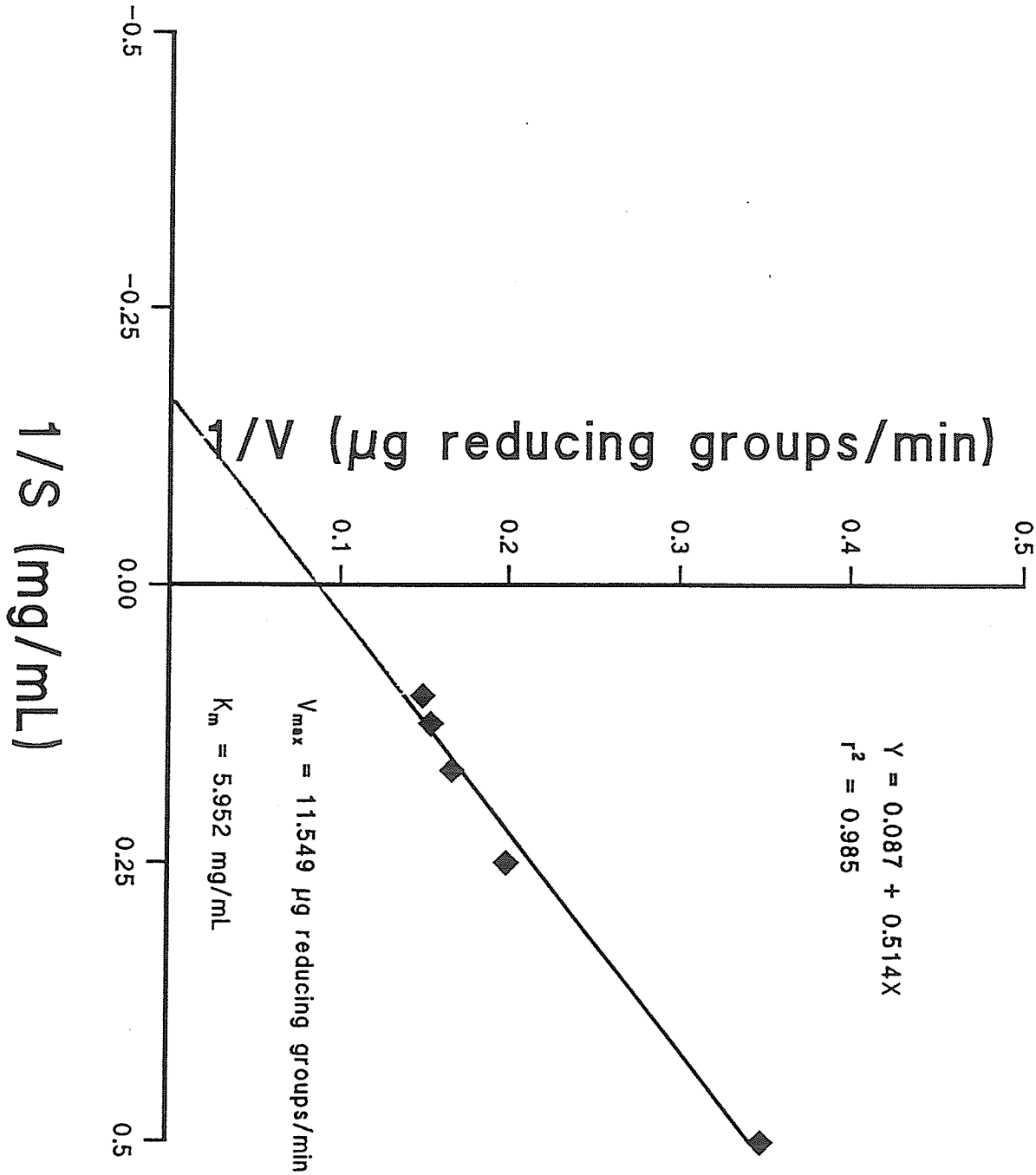

1/V (µg reducing groups/min)
0.5
0.4
0.3
0.2
0.1
0.00
0.25
0.5
−0.5
−0.25
1/S (mg/mL)
Y = 0.087 + 0.514X
r² = 0.985
Vmax = 11.549 µg reducing groups/min
Km = 5.952 mg/mL

Figure 23. Effect of substrate concentration on the initial velocity of *L. hesperus* polygalacturonase. Each point is based on triplicate determinations; r^2 is the coefficient of determination; V_{max} is the maximum velocity; K_m is the Michaelis-Menten constant.

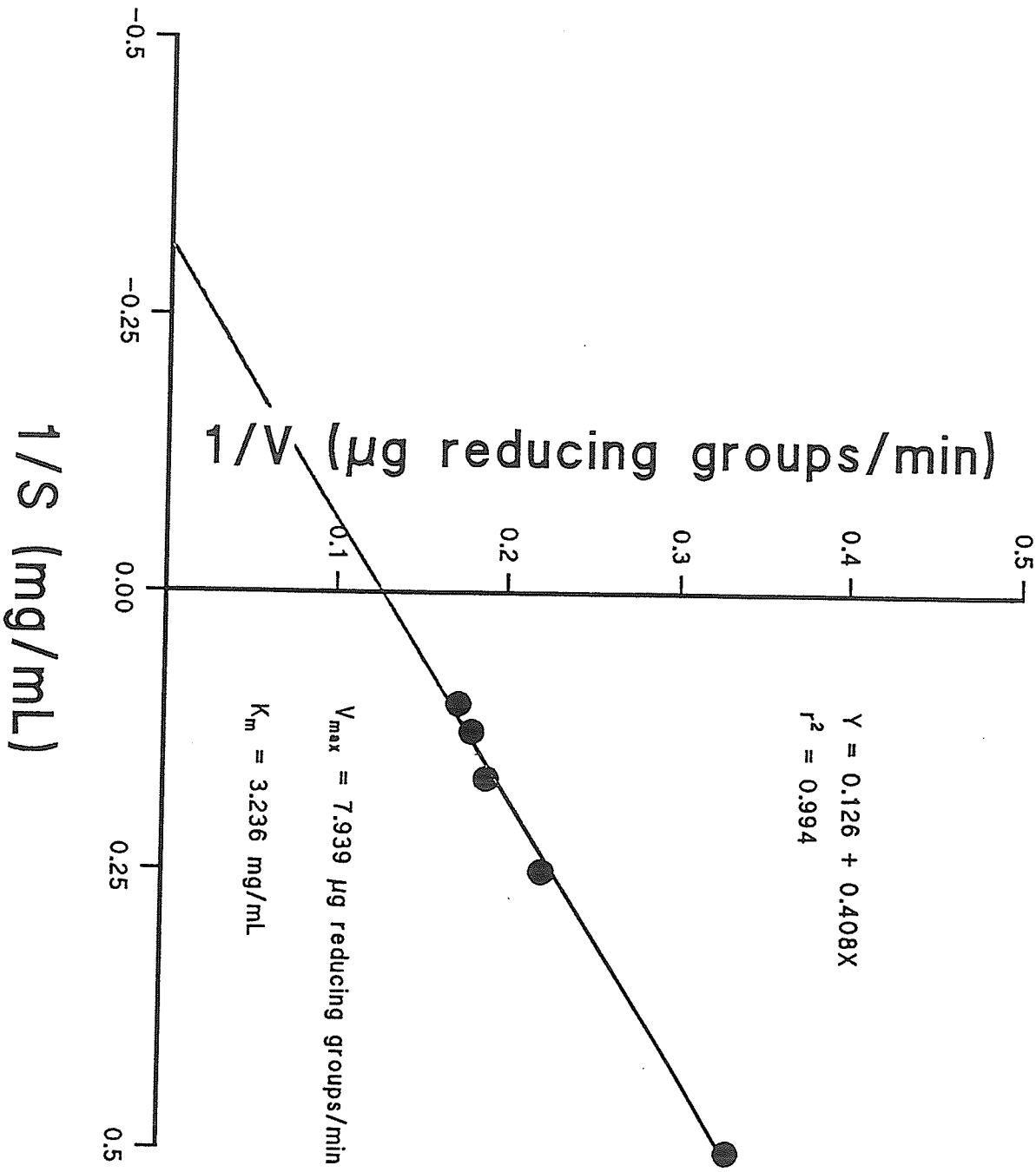

1/S (mg/mL)
-0.5
-0.25
0.00
0.25
0.5
1/V (µg reducing groups/min)
0.1
0.2
0.3
0.4
0.5
Y = 0.126 + 0.408X
r² = 0.994
Vmax = 7.939 µg reducing groups/min
Km = 3.236 mg/mL

TABLE 4. Effect of Sodium Chloride Concentration on the Activities of *L. lineolaris* and *L. hesperus* α-Amylase and Polygalacturonase.

NaCl Conc. (mM)	α-Amylase Activity (μg maltose/min)		Polygalacturonase Activity (μg reducing groups/min)	
	L. lineolaris	*L. hesperus*	*L. lineolaris*	*L. hesperus*
20	1.000	1.000	1.000	1.000
40	2.535	2.667	1.179	1.157
60	4.250	3.735	1.750	1.491
80	5.035	4.652	2.250	2.046
100	6.060	6.556	2.417	2.324

0.984. The slope of the lines were not greatly different from one another, sugggesting that the effect of NaCl concentration on both *L. lineolaris* and *L. hesperus* α-amylase was very similar.

4.5.1 Polygalacturonase

The effect of NaCl concentration on *L. lineolaris* and on *L. hesperus* polygalacturonase activities is also presented in Table 4. The increases in enzymic activities with NaCl concentration were also linear. The linear regression equation of the relationship between the two variables is: Y = 0.548 + 0.019X, where Y is *L. lineolaris* polygalacturonase activity, and X is the NaCl concentration. The coefficient of determination, r^2, was 0.966.

The linear regression equation of the same relationship for *L. hesperus* polygalacturonase is: Y = 0.542 + 0.018X. An r^2 (coefficient of determination) of 0.969 was obtained from the linear regression equation. The slope of the lines suggest that the increase in activity with increasing NaCl concentration was similar for the two enzymes. The enzyme activities from Table 4, however, indicate a stronger activation of the α-amylases by chloride ions than the polygalacturonases. Overall, it would appear that of the two species, *L. lineolaris* was more strongly activated by chloride ions than *L. hesperus*.

4.6 Effect of α-Amylase Activity on Legume Starches

A comparison was made between the specific activities of *L. lineolaris* and of *L. hesperus* α-

amylases on gelatinized starch, of a final concentration of approximately 1% (w/v), from several legumes, and on 1% (w/v) soluble starch (Baker Chemical Company; reagent grade). The results are presented in Table 5. Figures 24, 25, and 26 are progress curves showing the hydrolysis of starches by *L. lineolaris* and *L. hesperus* α-amylases as a function of time. Figures 27 and 28 show physical evidence of *Lygus* damage to the pods and to the mature seeds of field peas respectively.

TABLE 5. Specific Activities of *L. lineolaris* and *L. hesperus* α-Amylase on Legume Starches.

Starch Source	*L. lineolaris* (μmoles maltose/h/mg protein)	*L. hesperus* (μmoles maltose/h/mg protein)
Lima beans (*Phaseolus lunatus*)	20.194 ± 0.153[a]	12.693 ± 0.319[d]
Cowpeas (*Vigna unguiculata*)	20.194 ± 0.220[a]	17.647 ± 0.040[b]
Field peas (*Pisum sativum*), mature	17.670 ± 0.249[b]	20.743 ± 0.358[a]
Field peas (*Pisum sativum*), immature	16.913 ± 0.187[b]	16.099 ± 0.213[b]
Kidney beans (*Phaseolus vulgaris*)	15.146 ± 0.080[bc]	19.814 ± 0.296[ab]
Soluble Starch (reagent grade)	7.067 ± 0.012[e]	11.157 ± 0.005[d]

[1] Results are the means of three independent determinations ± standard deviations. Means followed by the same letters are not significantly different from each other ($P < 0.05$; Tukey test).

Figure 24. Hydrolysis of starch (lima beans (*Phaseolus lunatus*)) by *L. lineolaris* and *L. hesperus* α-amylase as a function of time. Each point is the mean of triplicate determinations.

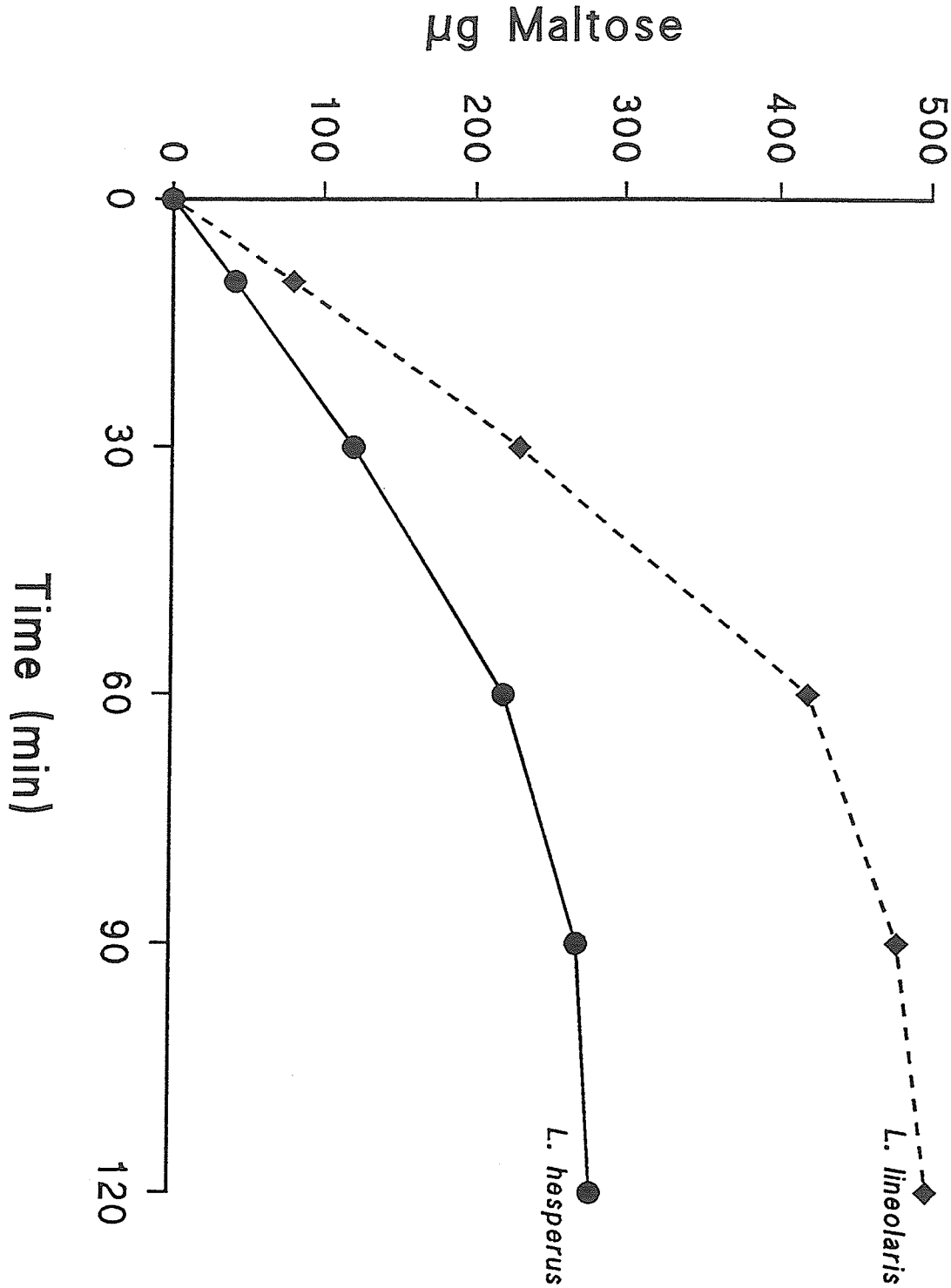

µg Maltose
0
100
200
300
400
500
Time (min)
0
30
60
90
120
L. lineolaris
L. hesperus

Figure 25. Hydrolysis of starch (white kidney beans (*Phaseolus vulgaris*)) by *L. lineolaris* and *L. hesperus* α-amylase as a function of time.

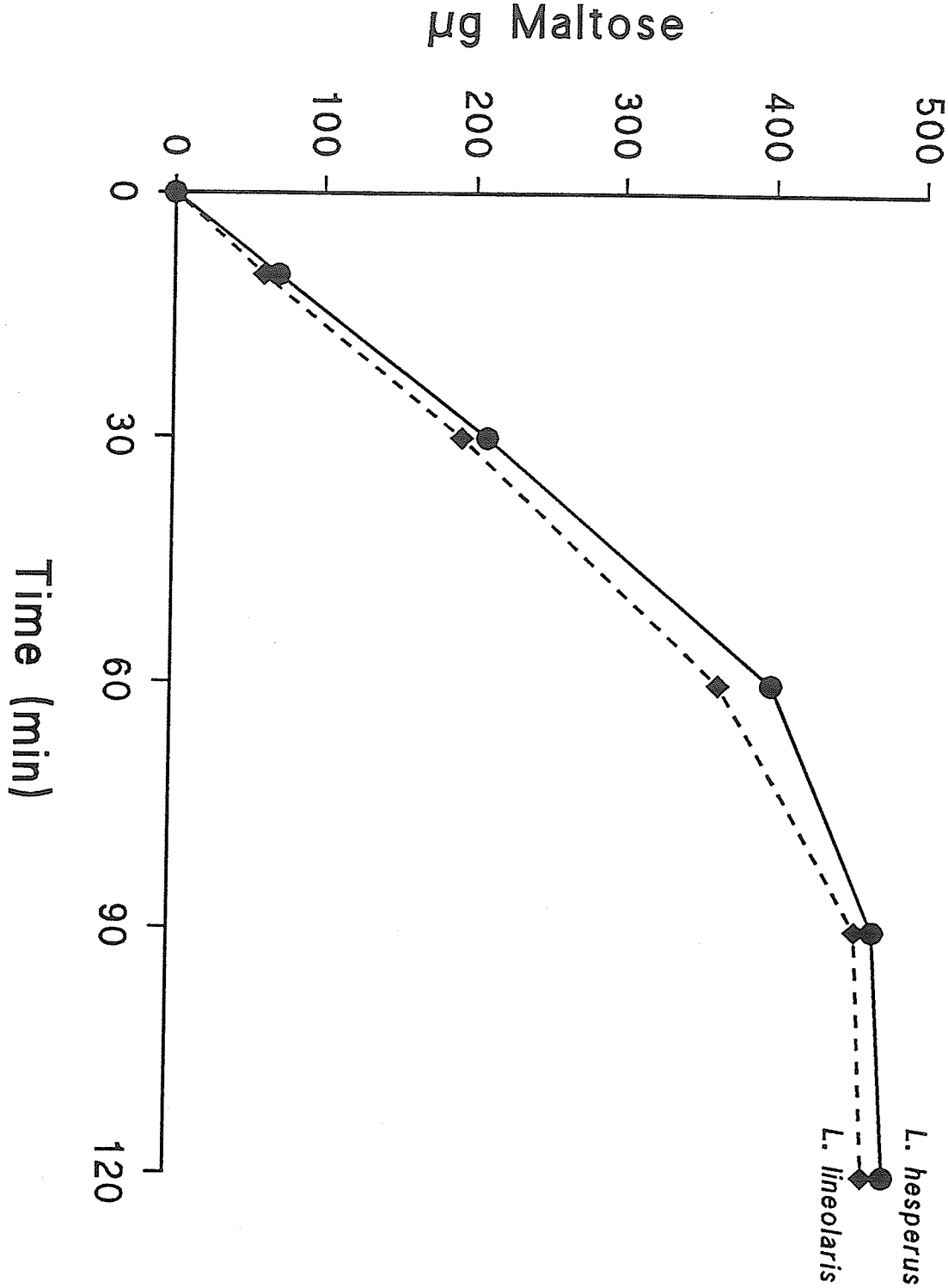

µg Maltose
0
100
200
300
400
500
Time (min)
0
30
60
90
120
L. hesperus
L. lineolaris

Figure 26. Hydrolysis of starch (mature and immature field peas (*Pisum sativum*)) by *L. hesperus* α-amylase as a function of time.

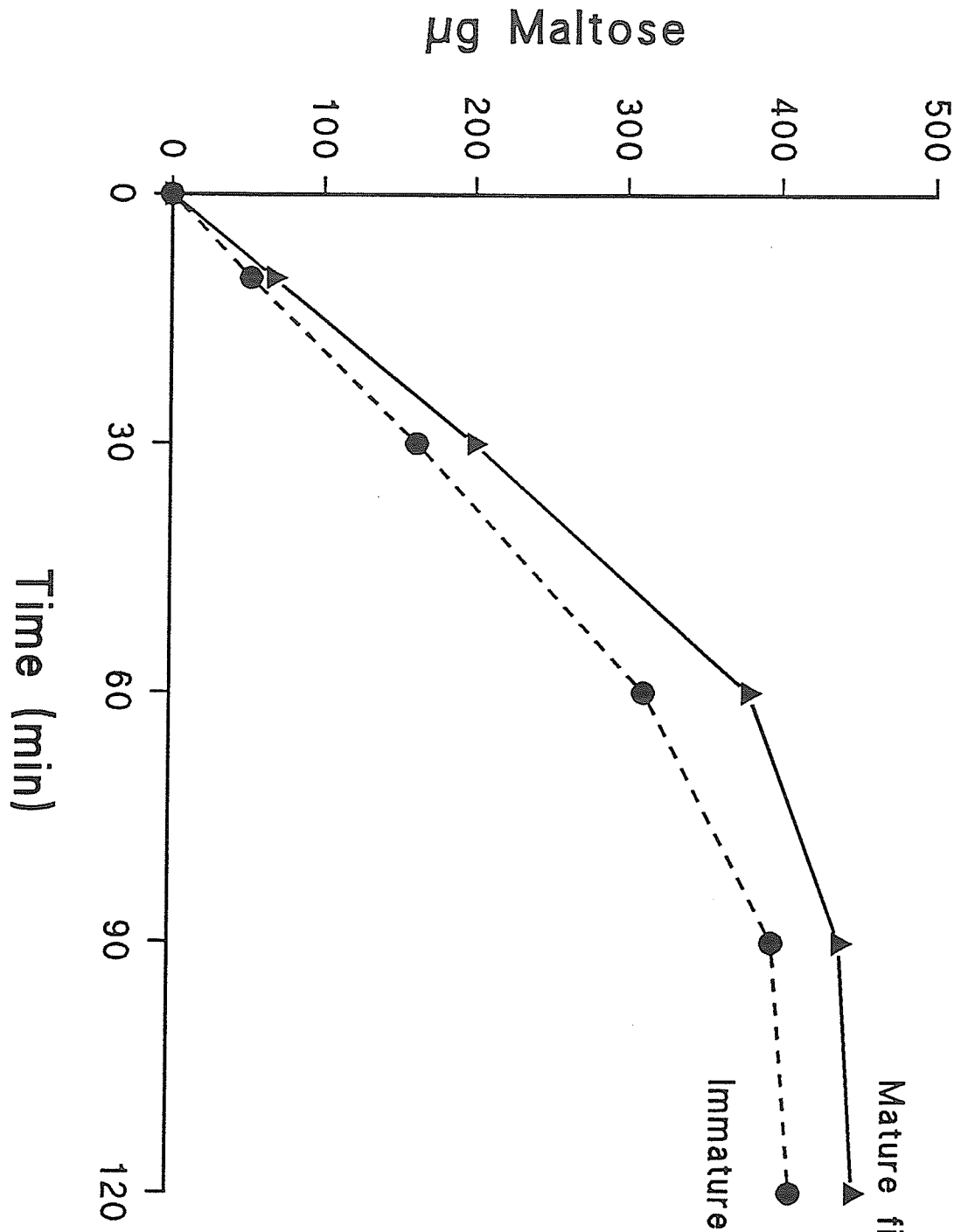
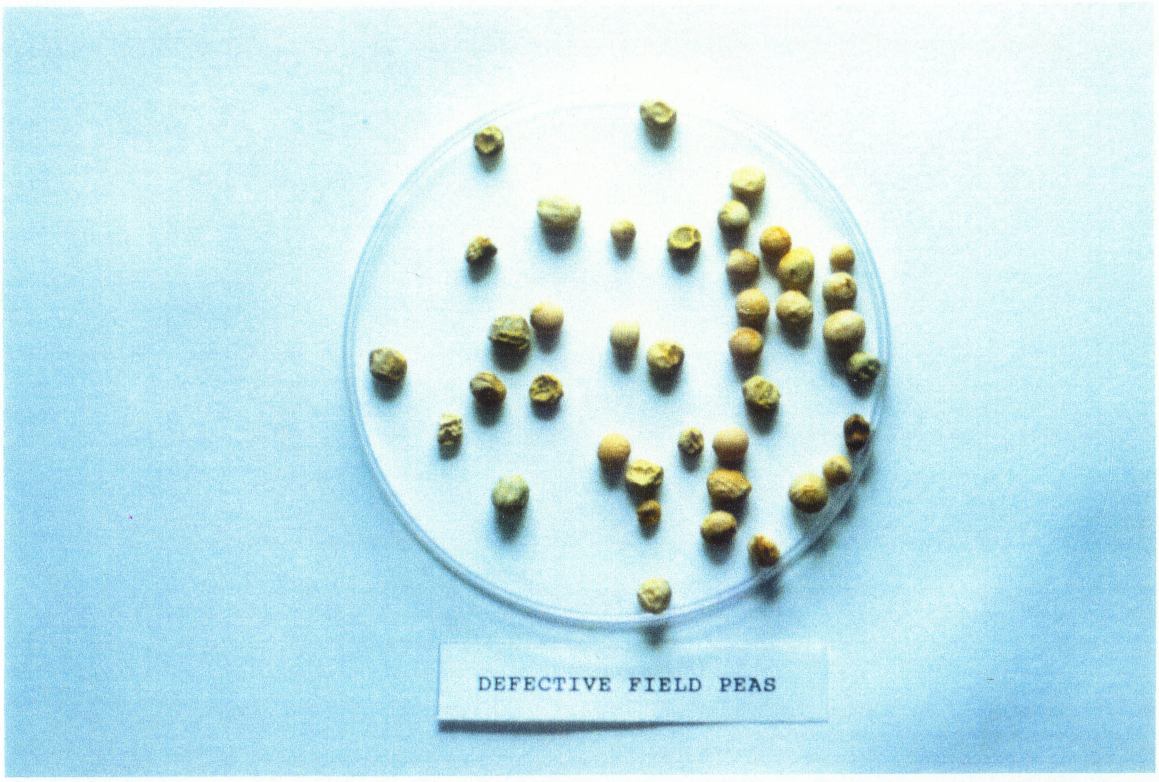

µg Maltose
500
400
300
200
100
0
0
30
60
90
120
Time (min)
Mature field peas
Immature field peas

Figure 27. Photograph of *Lygus* damage to pods of field peas. Bean pods were collected from a farm in Dugald, Manitoba, in the summer of 1989.


DAMAGED PODS

Figure 28. Photograph of *Lygus* damage to seeds of mature field peas. Evidence of necrotic spots, discoloured appearance and pitting.


DEFECTIVE FIELD PEAS

5. DISCUSSION

The ability of an enzyme to catalyze chemical and biochemical reactions depends to a great extent on the conditions in its vicinity, because properties of enzymes are altered under different conditions. Kinetic studies, particularly for crude enzyme preparations, are presently the only approach to obtain detailed information about the optimum conditions for the activity of an enzyme, or the effect of various factors on the enzyme (Dixon and Webb, 1979).

A study of the salivary enzymes of *Lygus* is essential in assessing the extent of host plant injury. Besides, they have only been partially characterized. Several factors determine the initial velocity of an enzyme reaction. Of these, the pH, temperature, enzyme concentration, substrate concentration, and the presence of activators, are among the most important.

Most enzymes are active over a narrow or broad range of pH, and in most cases a sharp optimum is observed. The pH affects the ionization states of amino acid side groups at the active site of the enzyme, which subsequently alter the primary and tertiary structure of the macromolecule. An optimum is observed because one of these ionizable groups is in its catalytically active form at that pH (Godfrey and Reichelt, 1983).

The pH optima of the α-amylases of *L. lineolaris* and of *L. hesperus* occurred in the slightly acidic region, in common with most other insect α-amylases, such as *L. rugulipennis* (pH 6.0) (Laurema *et al.*, 1985), *L. disponsi* (pH 5.0) (Hori, 1970a), *S. granarius* (pH 4.75), *S. zeamais* (pH 5.0)

(Baker, 1983), *C. maculatus* (pH 5.2-6.0) (Campos *et al.*, 1989), and *T. molitor* (pH 5.8) (Buonocore *et al.*, 1976).

The polygalacturonases of *L. lineolaris* and of *L. hesperus* showed maximum activity at pH 5.0. *L. lineolaris* polygalacturonase, however, contained an isoenzyme of which the pH optimum occurred at pH 6.0. The presence of polygalacturonase isoenzymes in *Lygus*, and in some plants and microorganisms, is not uncommon. Strong and Kruitwagen (1968) and Laurema *et al.* (1985) reported the presence of two or three polygalacturonase isoenzymes in *L. hesperus* and in *L. rugulipennis*. Their respective pH optima were 7.0, although strong activation of the enzyme occurred between 5.6 and 7.9, and at 5.0. Hori (1975b) indicated that although *L. disponsi* polygalacturonase was more strongly activated on the alkaline side (pH optimum at 8.0), some activity occurred at pH 6.2. Differences arising among the pH optima of the polygalacturonases from these species of *Lygus* could be attributed to the method of investigation. Whilst Laurema *et al.* (1985) used the reducing sugar (DNS) method, Strong and Kruitwagen (1968) determined the effect of pH on *L. hesperus* polygalacturonase by viscosity, and Hori (1975b) employed a procedure involving paper chromatography.

The polygalacturonase of *Adelphocoris seticornis*, a member of the Miridae family, has an optimum between pH 4.4 and 5.6 (Laurema and Nuorteva, 1961). Most polygalacturonases from other sources, such as plants and microorganisms, also show pH optima in the slightly acidic region (Pressey, 1986; Kester and Visser, 1990).

The sensitivity of *L. hesperus* α-amylase and polygalacturonase at extreme pH values, particularly in the acidic region, could limit host plant selection. *L. lineolaris*, on the other hand, can feed on a more diverse selection of plants because the activities of its α-amylase and polygalacturonase are more sensitive to change over the more alkaline pH range. *L. lineolaris* also has an added

advantage since it contains a polygalacturonase isoenzyme. On the basis of pH effects on enzyme activity, it would appear that *L. lineolaris* is the more dangerous species.

With respect to enzymes, changes in temperature affect two independent processes, namely the catalyzed reaction itself, and the thermal inactivation of the enzyme (Laidler and Peterman, 1983). At lower temperatures, particularly below 30°C, the rate of inactivation is negligible compared to the rate of the catalyzed reaction. The reaction rate therefore increases with a rise in temperature, as with all chemical reactions. At higher temperatures, an unfolding of the protein molecule occurs which becomes irreversible at about 60-70°C for most enzymes (Sizer, 1943; Dixon and Webb, 1979). This causes denaturation of the protein and subsequent inactivation of the enzyme. Some enzymes, through adaptability and selectivity, have developed higher structural stability which enables them to effectively withstand higher temperatures (Laidler and Peterman, 1983). The α-amylase and polygalacturonase of *L. lineolaris* were not completely inactivated by a heat treatment at 100°C for 30 minutes. Strong and Kruitwagen (1968) reported that the polygalacturonase of *L. hesperus* was not completely inactivated when exposed to similar experimental conditions. The α-amylase and polygalacturonase from *L. rugulipennis* were completely inactivated between 60-70°C after a 15-minute heat treatment (Laurema *et al.*, 1985).

Insects are well-adapted to their environment. Differences between species of *Lygus* may be attributed to climatic conditions. *L. rugulipennis* is mainly found in Finland (Varis, 1991) and in Alaska (Kelton, 1975), whilst *L. lineolaris* and *L. hesperus* are found in some parts of Europe and in North America (Tingey and Pillemer, 1977). The highest incidence of *Lygus* damage occurs in the midsummer when temperatures are relatively high (Baker *et al.*, 1946). In order to survive at these temperatures, their salivary enzymes need to be well-adapted to their environment. In comparison, certain bacteria, such as *Bacillus stearothermophilus*, live at high temperatures, and in order to survive, their enzymes, particularly the α-amylases, are heat-stable. According to

Dixon and Webb (1979), a heat treatment for one hour at 90°C caused a loss of only 10% in the *B. stearothermophilus* α-amylase activity. A heat treatment for 30 minutes at 90°C caused losses of 31% and 28% in the activities of *L. lineolaris* α-amylase and polygalacturonase respectively.

The activation energy (E_a), and the enthalpy of activation (ΔH), were both lower for the polygalacturonase of *L. lineolaris* than for the α-amylase. A comparison of the reactions involving *L. lineolaris* α-amylase and polygalacturonase indicates that the latter reaction proceeds more rapidly than the former because a lower energy barrier would have to be overcome to reach the activated complex (Gutfreund, 1951; Gray, 1971; Ainsworth, 1977).

The energies of activation for the α-amylases of *C. chinensis* and of garden-pea were 31.760 kJ $mole^{-1}$, and 29.462 kJ $mole^{-1}$ for barley malt α-amylase (Podoler and Applebaum, 1971). These energies of activation compare with those of *L. lineolaris* α-amylase and polygalacturonase, of which the respective values were 44.861 and 38.770 kJ $mole^{-1}$.

The magnitude of the entropy of activation (ΔS) depends on two factors, namely solvent, and structural effects (Gray, 1971; Laidler and Peterman, 1983). The former factor refers to the interaction between the solvent (water) and the reactants, whilst the latter refers to conformational changes that could occur in the structure of the enzyme. It is proposed that during the course of the reaction, hydrogen bonds are broken, resulting in an unfolding of the enzyme molecule. This unfolding is thought to be important for the formation of the enzyme-substrate complex, and results in an increase in the entropy of activation. Decreases in entropy for the irreversible thermal inactivation of an enzyme may be accounted for by the possible aggregation of enzyme molecules (which have previously partially unfolded). Such aggregation reactions, which are predominant during protein heat denaturation, are always associated with a marked increase in order (Dannenberg and Kessler, 1988; Owusu *et al.*, 1992).

Solvent effects can be explained on the basis of polarity changes that occur during the course of the reaction, and may lead to an increase or a decrease in solvent binding (Gray, 1971; Dixon and Webb, 1979; Laidler and Peterman, 1983). It is thought that molecules of the solvent, such as water, arrange themselves in an "orderly" manner around each charged species. During the formation of the enzyme-substrate complex, an interaction between negatively- and positively-charged species results in the neutralization of charges. The result is that the "orderly" arrangement of the solvent molecules around the charged species is destroyed, and an increase in the entropy occurs. Similarly, if the reaction involves the production of charged species, an increase in water-binding occurs, which gives rise to a decrease in the entropy of activation.

Many reactions involving water result in an increase in polarity when the activated complex is formed, which leads to a negative entropy of activation. According to Gray (1971), negative entropies are associated with slow reaction rates. The negative entropies of activation for *L. lineolaris* α-amylase and polygalacturonase could be attributed to increases in the polarity during the reaction, or to the aggregation of enzyme molecules which are predominant during protein heat denaturation and could lead a tightening of the enzyme structure. The latter reason could also explain their relative heat stabilities.

The effect of enzyme concentration on the initial reaction velocity is based on the assumption that, under a given set of conditions, two enzyme molecules acting independently in solution will transform twice as much substrate as one molecule of enzyme in a given time period (Dixon and Webb, 1979). Although the reaction velocities were directly proportional to the enzyme concentrations in all cases, differences were observed in the rates of reactions of the α-amylases and polygalacturonases of *L. hesperus* and of *L. lineolaris*. The reaction of *L. hesperus* α-amylase on soluble starch proceeded at a slightly slower rate than the reaction of *L. lineolaris* α-amylase. The converse was true for the polygalacturonases.

The substrate concentration is also one of the most important factors affecting the initial reaction velocity. Results obtained from Lineweaver-Burk plots indicated that the polygalacturonases of *L. hesperus* and of *L. lineolaris* had stronger affinities for the substrates than their α-amylases. The specific activities and K_m values also reveal differences between the two species of *Lygus*. With respect to the substrate concentration, which reflects the relative abilities of these insects to destroy legumes, it would appear that *L. hesperus* is more destructive than *L. lineolaris*.

It can therefore be deduced that the polygalacturonases are the more important of the two enzymes, in terms of substrates, than their α-amylases. This supports the expectation that, in terms of the feeding behaviour of *Lygus*, which involves the penetration of plant tissues by the insect's stylets, the action of polygalacturonase precedes that of α-amylase.

Collmer and Keen (1986) suggested that polygalacturonases kill plant cells by rendering them osmotically fragile. Some secondary effects of *Lygus* injury include tissue necrosis, growth retardation, and morphological deformation. Some members of the Miridae family, particularly *L. lineolaris*, have been implicated in the transmission of virus diseases, such as potato mosaic, and spinach blight diseases, while feeding, but Leach (1940) suggested that these diseases were purely mechanical and incidental. Other hemipterous insects, particularly of the Pentatomidae family, have been found to transmit fungal diseases in cotton, lima beans and soya beans (Miles, 1972). However, there is evidence to suggest that secondary microbial infections result from *Lygus* injury (Summerfield *et al.*, 1982; Arnett, 1985).

Chloride ions have been reported in plant tissue, particularly in the stems, leaves and petioles (Hori, 1970b). These ions bind to insect α-amylases and cause an increase in the reaction velocity (Terra *et al.*, 1977). The α-amylases of *L. lineolaris* and of *L. hesperus* were more strongly activated by chloride ions than the polygalacturonases. Whilst the action of polygalacturonase

is restricted to the periphery of the plant cells where the pectic substances are concentrated, the α-amylases degrade starch reserves within the plants. This could explain the overall effect of chloride ions on the α-amylases and polygalacturonases of *L. lineolaris* and of *L. hesperus*. Of these two species, *L. lineolaris* was more strongly activated, probably because it is more sensitive to changes in its environment.

Although *Lygus* prefers a particular host plant, it also attacks other plants (Fye, 1984). *L. lineolaris*, for instance, was found to prefer lima beans and cowpeas to kidney beans, whilst *L. hesperus* was found to prefer mature field peas to immature field peas. Species of *Lygus* prefer young plant tissues because they are less resistant to stylet penetration (Summerfield *et al.*, 1982). These results indicate that in the absence of their stylets, *Lygus* prefers mature plant tissues because the starch content of such plants is higher than in immature plants (Watt and Merrill, 1975).

Banks and Greenwood (1975) reported that several factors determine the gelatinization properties of the starch granule. These include the amylose content, granule size, molecular weight distribution, structural characteristics of the starch components, and the amount and type of impurity present. Some of these factors could account for the differences in the specific activities of the α-amylases of the two insect species towards legume starches.

Differences were also observed between the specific activities of these insect species towards soluble starch, and starch as extracted from legumes. According to Meredith and Pomeranz (1985), the ability of α-amylases to digest native starch is limited. When starch granules are heated in water, gelatinization occurs resulting in a disruption of the granular structure (Biliaderis, 1991). This process renders the starch molecules more susceptible to attack by α-amylases.

In terms of the overall effects of enzyme and of substrate concentrations on activity, it would appear that of the two insect species, *L. hesperus* is more destructive towards legume crops than *L. lineolaris*. However, *L. lineolaris* has some advantages over *L. hesperus*; it contains a polygalacturonase isoenzyme, of which the pH optimum is at 6.0, is also more sensitive in the middle pH range and more strongly activated by the ionic environment. This could probably explain why, economically, *L. lineolaris* is the most important species of *Lygus*, especially on the Canadian Prairies (Philip and Mengersen, 1989).

6. CONTRIBUTIONS TO KNOWLEDGE

1. The α-amylases and polygalacturonases of *L. lineolaris* and of *L. hesperus* have slightly acidic pH optima, and temperature optima of 40°C. Although both enzymes were activated by chloride ions, overall, it appeared that the α-amylases were more strongly activated than the polygalacturonases.

2. *L. lineolaris* α-amylase and polygalacturonase retained 24% and 43%, respectively, of their activity even after exposure to a heat treatment at 100°C for 30 minutes.

3. The energies of activation (E_a) for the reactions involving *L. lineolaris* α-amylase and polygalacturonase suggest that the latter reaction proceeds in preference to the former because a lower energy barrier had to be surmounted in order to reach the activated complex.

4. Decreases in the entropies of activation (ΔS) for the irreversible thermal denaturation of the *L. lineolaris* enzymes may be attributed to increases in polarity during the formation of the enzyme-substrate complex, or to possible aggregation reactions which occur frequently during protein heat denaturation.

5. With respect to the effect of enzyme and of substrate concentrations on activity, it would appear that the polygalacturonases are more destructive towards legume crops than the

α-amylases. This supports the expectation that, in terms of the feeding behaviour of *Lygus*, which involves the penetration of plant tissues by the insect's stylets, the action of polygalacturonase precedes that of α-amylase.

6. In terms of the relative abilities of these insects to destroy legume crops, it would appear that *L. hesperus* is more destructive than *L. lineolaris*.

7. It is suggested that further work be carried out to characterize the proteases of *Lygus* in order to provide a comprehensive study on the salivary enzymes of *Lygus*.

8. It is further suggested that a confirmation be made of the presence or absence of lipases in *Lygus*, and hence their possible role in tissue degradation.

9. It is also recommended that chemical methods of eliminating *Lygus* populations should be carefully examined, to perhaps incorporate the knowledge gained in this study, in order to develop an effective pest management package.

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