IDENTIFICATION OF SOME DIGESTIVE ENZYMES IN THE COCKROACH Periplaneta americana Linnaeus (Blattidae : Blattodea) BY USING AGAR GEL DIFFUSION BIOASSAY

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Practical physiology courses are integral parts of the subjects leading to the award of B.Sc. hon.;s in Zoology from the Faculty of Science of the University of Khartoum. The number of students taking physiology is relatively high and the costs of some practical procedure are likewise high and the materials are not available every time. Besides, there is a move throughout the word not to use vertebrate animals in biomedical research and teaching.

The Association for Biology Laboratory Education (ABLE) in the USA and Canada has called for the improving the undergraduate biology laboratory experience by promoting the development and dissemination of reliable laboratory exercises - i.e., adoption of “the three R’s” approach (Replacement, Refinement, and Reduction) in biomedical research and teaching.

The present worker has followed the agar-gel diffusion bioassay (AGB) to identify and determine the amylase and chymotrypsin potencies of various sections of the digestive system of the cockroach, Periplaneta Americana Linnaeus. The procedure adopted was more or less akin to that recommend by ABEL. In the AGB used here the standard enzyme (amylase or chymotrypsin) solutions of varying concentrations is allowed to diffuse through agar gel previously seeded with a test compound (starch or casein). After an incubation period of six hours, a positive result can be detected visually with staining procedure (iodine test or treatment with acetic acid). The test compound or organism serves as an indicator to make visible the
effect of the substance of biological activity. The affected zones appear clear from the surrounding agar. The dose/response relationship was found by relating the diameter of the affected zone to the concentration of the enzyme. Through regression analysis the relationship between the response ($y$) and the dose ($x$) quantify the relationship ($y = a + bx$). The equation for amylase was found to be ($y = 8.7275 + 6.8813x$). The equation for chymotrypsin was found to be ($y = -6.6923 + 7.395x$). The unknown concentration of the enzyme in the biological sample could thus be determined. Amylase was found only in tissues of the salivary glands (215 μg/ml) and mesenteric caeca (45 μg/ml). Chymotrypsin was found only in mesenteric caeca (5590.3 μg/ml). The esophagus, the crop, the stomach, the colon and rectum were devoid of digestive enzymes.

It was recommended that the effect of pH, temperature, concentrations of enzymes and substrates can be tested. If Standard enzymes are not available or expensive, then comparative approach is give all the necessary training - i.e., comparing the relative distribution and potency of enzymes of widely or closely species.

It is also recommended that such a work be accompanied by histochemical studies of the various sections of the alimentary canal and if possible the study of the cellular components of the digestive cells with electron microscopy. This will allow correlation of structure with function – i.e., makes the functional identification and nomenclature of digestive cells possible.
المستخلص العربي

تشكل مترات الفسيولوجيا العملية جزء أصيل من المناهج المرسوم الذي يقود إلى نيل درجة البكالوريوس (شرف) في علم الحيوان من كلية علوم جامعة الخرطوم. يتفق عدد الطلاب الذين يدرسون الفسيولوجيا عاماً بعد عام وكذلك تزايد النسبة كثيرة من الأجزاء المقررة من علم الفسيولوجيا العملية. أضيف إلى ذلك أن هناك توجه دولي عام بعدم استخدام الحيوانات السفيرة في الأبحاث الطبية والبيولوجية أو بعض التدريس.

The Association for Biology Laboratory Education (ABLE) في الولايات المتحدة وكندا يحسن تدريب طلاب البيولوجيا عن طريق تطوير ونشر تدريبات في البيولوجيا العملية ويعتمد عليها - بمعنى تبني المدخل الثلاثي "(1) استخدام طرق تجريبية مقبولة أخلاقياً، و(2) توجيه الأداء، و(3) الحصول على أفضل النتائج بأقل عدد من الحيوانات" في البحث والتدريب في العلوم الطبية والبيولوجية.

قامت الباحثة الحالية بتابع اختبار الإنتشار في وسط هلام الأجار لتتعرف على انزيمي الأنيلامين والكيموتيربيسين في الأقسام المختلفة من القناة الهضمية للصرصور الأمريكي Periplaneta Americana وكذلك تقرر تركيز هما بهذه الأقسام.

الببتست الباطنية الطريقة التي توصي بها الرابطة المذكورة وتمكنت لتركيزات متفرقة من الإنزيم العضلي (إم إس أو كيموتيربيسين) من الإنتشار عبر هلام أجار سيرك ومعول بمركب (أي مهاي) مناسب (نها أو كازين) ورصد النتائج بعد ست ساعات. تكمن الباحة من مشاهدات تأثير الأنزيم باستخدام صبغة البود أو المعاملة بمحمض خرابك. إتخاذ التأثير شكل دائرة محددة ظاهرة عن ما حولها. استخدم النها أو الكازين كدليل يسجل تأثير الأنزيم. وقد قدمت العلاقة بين مقدار الجرعة (ص) والإستجابة (الإنتشار) مثالية بقطر المنطقة المترافئة عن طريق تحليل الإنساـر أي حساب معادلة الخط المستقيم (ص = أ + ب ص). كانت المعادلة بالنسبة للإنزيم العضلي (أ = 5.7275 + 0.8813) و (ص) = 6.6923 + 0.7375. ومن معادلة الخط المستقيم تمكنت الباحة من حساب الجرعة في الأقسام المختلفة من القناة الهضمية للصرصور الأمريكي. وجد الأنيلامين فقط في نسبة الغدد اللعابية يتركز.
قدره (215 ميكروجرام/ملليتر) وفي نسخة الأنيبوب الأعورية المساريفية بتركيز قدره (45 ميكروجرام/ملليتر). ووجد الكايمون بسبين فقط في نسخة الأنيبوب الأعورية المساريفية بتركيز قدره (0.3 ميكروجرام/ملليتر). لم يحوى المربي والمحلية والمعدة والقولون والمستقيم على أي نشاط إنزيمي هضمي.

يمكن عن طريق هذا التدريب دراسة تأثير تركيز أيون الهيدروجين ودرجة الحرارة وتركيز الإنزيم والمياه على سرعة التفاعل. إذا كانت الإنزيمات المغذية غير متوفرة بسبب علّة شنها يمكن اللجوء إلى الدراسة المقارنة حيث يسند على التركيز القياسي للهرمون بدلالة تركيزه في حيوان آخر قريب أو بعيد النسب التطوري. كما أن مثل هذا الدراسة يُفضل أن تكون مصحوبة بدراسة الأنسجة المفرزة للإنزيمات بتقنيات علم الأنسجة الكيميائي أو دراستها بالمجهر الإلكتروني فهذا يُمكن من الربط بين التركيب والوظيفة مما يسمح بنسبية الخلايا تسمية وظيفية.
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 THE INSECT:
1.1.1 SCIENTIFIC CLASSIFICATION
Phylum: Arthropoda
Subphylum: Hexapoda
Class: Insecta
Subclass: Pterygota
Infraorder: Neoptera
Superorder: Dictyoptera
Order: Blattodea
Family: Blattidae
Genus: Periplaneta
Species: Periplaneta americana Linnaeus

1.1.2 GENERAL BIOLOGY:
The American cockroach (Fig. 1.1) is an ancient cosmopolitan unmistakable large (30 to 50 mm) reddish brown synanthropic insect pest found in dark damp places such as kitchens, stores, manholes, sewers, ships, hospitals, hostels, barracks, markets, groceries, basements, furnace rooms etc... (Rust et al., 1991; Kettle, 1994). Apart from their presence being aesthetically unacceptable they produce a characteristic offensive odour. The American cockroach, like other domestic cockroaches, is a scavenger attracted to any organic material which may serve as food. It feeds on human food, excreta, sputa and when food is scarce, the bindings of books and even paper (Kettle, 1994). This wide-ranging feeding habit make cockroaches potential mechanical vectors of pathogens. The relationship of cockroaches with pathogenic organisms and other life forms has been comprehensively reviewed by Roth and Willis (1957, 1960). At least 22 species of pathogenic human bacteria, virus, fungi, and protozoans, as well as five species of
helminthic worms, have been isolated from field collected American
cockroaches (Rust et. al., 1991).

The American cockroach, being a hemi-
metabolous insect, has
three life stages: the
egg, a variable number
of nymphal instars, and
adult (Fig. 1.2). The life
cycle from egg to adult
averages about 600 days
while the adult life span
may be another 400
days. The immatures
emerge from the egg case in about six to eight weeks and mature in about six
to twelve months. Adults can live up to one year and an adult female will
produce an average of 150 young in her lifetime. Environmental factors such
as temperature and humidity can increase or decrease the developmental
time of the American cockroach. Outdoors the female shows a preference
for moist, concealed oviposition sites (Bell and Adiyodi 1981).

1.1.3 DIGESTIVE SYSTEM AND DIGESTION IN INSECTS (Fig. 1.2):
An insect uses its digestive system to extract nutrients and other substances
from the food it consumes. Most of this food is ingested in the form of
macromolecules and other complex substances which must be broken down
by catabolic reactions into smaller molecules before being used by cells of
the body for energy, growth, or reproduction.
The insect's digestive system is a closed system, with one long enclosed coiled tube called the alimentary canal which runs lengthwise through the body. The alimentary canal only allows food to enter the mouth, and then gets processed as it travels toward the anus. The insects alimentary canal has specific sections for grinding and food storage, enzyme production and nutrient absorption (McGavin, 2001; Triplehorn & Johnson, 2005). Sphincters control the food and fluid movement between three regions. The three regions include the foregut (stomatodeum) the midgut (mesenteron), and the hindgut (proctodeum).

In addition to the alimentary canal, insects also have paired salivary glands and salivary reservoirs. These structures usually reside in the thorax (adjacent to the fore-gut). The salivary glands produce saliva, the salivary ducts lead from the glands to the reservoirs and then forward through the head to an opening, the salivarium, behind the hypopharynx; movements of the mouthparts help mix saliva with food in the buccal cavity. Saliva mixes with food which travels through salivary tubes into the mouth, beginning the process of breaking it down (Elzinga, 2004).

The stomatodeum and proctodeum are invaginations of the epidermis and are lined with cuticle (intima). The mesenteron is not lined with cuticle but with rapidly dividing and therefore constantly replaced, epithelial cells (McGavin, 2001; Triplehorn & Johnson, 2005). The cuticle sheds with every moult along with the exoskeleton (Triplehorn & Johnson, 2005). Food is moved down the gut by muscular contractions called peristalsis (Elzinga, 2004).

1. **Stomatodeum** (foregut): This region stores, grinds and transports food to the next region (Gullan & Cranston, 2005). Included in this are the buccal cavity, the pharynx, the oesophagus, the crop (stores food), and
proventriculus or gizzard (grinds food) (Tripehorn & Johnson, 2005). Salivary secretions from the labial glands dilute the ingested food. In mosquitoes (Diptera), which are blood-feeding insects, anticoagulants and blood thinners are also released here.

2. **Mesenteron:** Digestive enzymes in this region are produced and secreted into the lumen and here nutrients are absorbed into the insect’s body. Food is enveloped by this part of the gut as it arrives from the foregut by the peritrophic membrane which is a mucopolysaccharide layer secreted from the midgut’s epithelial cells (McGavin, 2001). It is thought that this membrane prevents food pathogens from contacting the epithelium and attacking the insects’ body (McGavin, 2001). It also acts as a filter allowing small molecules through, but preventing large molecules and particles of food from reaching the midgut cells (Gullan & Cranston, 2005). After the large substances are broken down into smaller ones, digestion and consequent nutrient absorption takes place at the surface the epithelium (McGavin, 2001). Microscopic projections from the mid-gut wall (microvilli), increase surface area and allow for maximum absorption of nutrients.

3. **Proctodeum** (hindgut): This is divided into three sections; the anterior is the ileum, the middle portion, the colon, and the wider, posterior section is the rectum (Gullan & Cranston, 2005). This extends from the pyloric valve which is located between the mid and the hindgut to the anus (Tripehorn & Johnson, 2005). Here absorption of water, salts and other beneficial substances take place before excretion (Gullan & Cranston, 2005). The main organs of osmoregulation and excretion are the blind-ended Malpighian tubules at the anterior end of the hindgut (McGavin, 2001; Tripehorn & Johnson, 2005; Gullan and Cranston, 2005)
1.2 AGAR GEL DIFFUSION BIOASSAY (AGB)

The generalization written below was abridged from Crans (1925), Weitz (1960), Swingl (1925), Wilson and Walker (2001), Brantl (2002) and Siriysatien (2010). Before the advent of Enzyme-linked immunosorbent assay (ELISA), electrophoresis, chromatography, spectrophotometry and related molecular biology techniques in separating and identification of biological compounds, the agar gel diffusion bioassays (the most used descendants of the original precipitation reaction) were extensively used in serology, identification of animal and human blood, arthropod blood meal identification, forensic medicine, immunology, epidemiology, taxonomy, and in general, identification of compounds with some biological activity.

One form or the other of AGB is still in use as a trustworthy technique.

In the agar gel diffusion bioassay (AGB) a substance with biological activity (e.g., enzyme, antibody, serum, vitamin, antibiotic, drug, toxin, pesticide, ...) is allowed to diffuse through agar gel previously seeded with a test compound (e.g., starch, protein or antiserum, antigen...) or with organism (e.g., a species of a bacterium or a fungus). After an incubation period, a
positive result can be detected visually with or without special staining procedure. The test compound or organism serves as an indicator to make visible the effect of the substance of biological activity. The method of conducting the assay may be:
1. linear diffusion in a tube or channel,
2. radial diffusion (use of Petri plates) or
3. Bioautography (in early technique before agar gel is replaced by silica gel).

1.3 AIMS AND OBJECTIVES
1.3.1 Aims:
The use of vertebrate animals has been a tradition in zoology labs. Yet, there are good and current reasons to find alternatives to the use of vertebrates when possible. One reason is the cost of the specimens; another is the concerns dealing with procedures on vertebrates (Watson and Omoto, 2005). The far fetching goal of the present work is to contribute, support, encourage and adopt the objectives of some international associations that address their efforts to minimize the costs of lab training of undergraduates and at the same time concerned with the ethical issues of animal experimentation. In other words, many concerned biological institutions seek alternative strategies to the use of vertebrates in undergraduate physiology and biochemistry laboratories. In America and Canada these associations include: Association for Biology Laboratory Education or ABLE (http://www.ableweb.org); Bioscience Educational Net or BEN (www.biosciednet.org/portal) and American Institute of Biological Sciences or AIBS (http://www.aibs.org).

1.3.2 Objectives:
The near-fetching goal of the present work is to investigate the distribution of the digestive enzymes in the American cockroach, *Periplaneta americana* following the procedure recommended by Watson (2003) and Watson and Omoto. (2005) in the Proceedings of the 24th and 26th Workshop / Conference of the Association for Biology Laboratory Education (ABLE) to confirm its applicability and suitability in training undergraduates in the Department of Zoology, Faculty of Science, University of Khartoum.
CHAPTER TWO
MATERIAL AND METHODS
(All figures are placed at the end of the chapter)

2.1 COLLECTION AND MAINTENANCE OF ADULT COCKROACHES:
Adults of Periplaneta americana were collected from highly infested houses in El Hag Yousif town (Sharq-Al-Nil Locality, Khartoum state) either from manholes by hanging bated cardboard boxes (Fig. 2.1A) or from kitchens by using baited jam jars (Fig. 2.1B). The traps were set before sunset and the catch was collect next morning.
In the laboratory, only sound and active cockroaches were kept in a maintenance cage made of rectangular plastic container (Fig. 2.2). The cockroaches were watered and fed, ad libitum, on bread pieces, smashed potato and small amount of yeast as a source of vitamins.

2.2 PROCEDURE:
The procedure pertinent to removal of the digestive system and all steps involved in the agar gel diffusion bioassay was more or less similar to that followed by Watson (2003). The procedure followed by the mentioned author is copied in the appendix. The differences between the procedure followed by the present author and Watson (2003) were minor:

1. Anesthesia of cockroaches was not done with carbon dioxide (CO₂), instead by keeping the insects in a chilling chamber (Fig. 2.3) for at least 20 minutes. The chamber consisted of a plastic bucket 20 cm in diameter and 25 cm deep containing salted crushed ice and wrapped with a jacket of cotton wool as a heat insulator. An empty cylindrical glass jam jar (8 cm in diameter and 15 cm deep) was placed in the crushed ice to receive a plastic bag containing the cockroaches to be anesthetized (5 - 10 at a time). The cockroaches become fully
anesthetized after 10 – 15 minutes and remain so for about 15 minutes — i.e., enough time to dissect out the digestive system.

2. Dissection of the cockroaches was done in small dissecting dish made of glass Petri dish (15 cm in diameter and 2 cm deep) with a layer of bee wax, 1 cm deep.

3. In the present work five sections of the digestive system (Fig.2.5) were processed instead of the three (foregut, mid gut and hindgut) tested by Watson (2003). The parts processed were: (1) the salivary glands, (2) esophagus and crop, (3) mesenteric caeca, (4) stomach and (5) the hind gut. The gizzard was excluded from the bioassays because it is highly chitinized and bears tough teeth which may break the mortar of the glass homogenizer.

4. A glass tissue Dounce homogenizer (Fig. 2.6) was used to grind and homogenize the tissues of the digestive system instead of using mortar and pestle.

5. In the present work 25 cockroaches, regardless of sex, were dissected. Ground tissues of every one section of the alimentary canal were pooled together. The enzyme extracts were bioassayed for protease in the same day they were prepared while the enzyme extracts were bioassayed for amylase within 2 - 3 days after preparation. In the latter case, the extracts were kept in labeled stoppered tubes and placed in the freezer.

6. The Petri dishes were strictly labeled to prevent confusion. The top half of every Petri dish was marked with a permanent marker with circles and numbers denoting the actual wells at the bottom part of dish. A short line was drawn such that it extended from the top to the bottom half of the dish so that the top and bottom lines can be
matched in order to identify the wells and their contents properly. In addition, the top and bottom halves of Petri dishes were marked as ST for gels with starch or CA for gels with casein (Figs 2.6, 2.7).

1. The standard chymotrypsin used was a Sigma-Aldrich α-Chymotrypsin from bovine pancreas, Product Number C 3142, 40 units/mg protein, Storage Temperature - 0 °C, Expiry date November 2012. Five wide logarithmically spaced doses were used instead of the three logarithmically spaced doses (20, 200, 2000 µg/ml) used by Watson (2003). The doses used in the present work are shown in Table (2.1) below:

**Table (2.1). The logarithmically spaced doses* of chymotrypsin used in the present work**

<table>
<thead>
<tr>
<th>Dose of chymotrypsin</th>
<th>Log</th>
<th>Unit**/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.625</td>
<td>1.1938</td>
<td>0.625</td>
</tr>
<tr>
<td>62.5</td>
<td>1.7599</td>
<td>2.5</td>
</tr>
<tr>
<td>250</td>
<td>2.3980</td>
<td>10</td>
</tr>
<tr>
<td>1000</td>
<td>3.0000</td>
<td>40</td>
</tr>
<tr>
<td>4000</td>
<td>3.6020</td>
<td>160</td>
</tr>
</tbody>
</table>

*, The log interval chosen was Log 4 = 0.6020; **, international unit defined as the minimum dose that produce measurable or observable response on an agreed upon concentration of a substrate given an agreed upon time.

The number of doses was increased in the present work to yield data enough to apply correlation and regression analyses instead of just plotting graphs.

2. The protease enzyme pepsin was not tried.

3. The amylase enzyme used in the present work was β-Amylase from barley Type II-B, 80 units/mg protein, Sigma-Aldrich, product Number A7130, Storage Temperature 2-8 °C, Expiry date November
2012. The five logarithmically spaced doses used by Watson (2003) was also used in the present study and are shown in table (2.2) below:

Table (2.2). The logarithmically spaced doses* of amylase used in the present work

<table>
<thead>
<tr>
<th>µg/ml</th>
<th>Log</th>
<th>Unit**/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>-0.0969</td>
<td>0.064</td>
</tr>
<tr>
<td>4.0</td>
<td>0.6020</td>
<td>0.320</td>
</tr>
<tr>
<td>20.0</td>
<td>1.3010</td>
<td>1.600</td>
</tr>
<tr>
<td>100.0</td>
<td>2.0000</td>
<td>8.000</td>
</tr>
<tr>
<td>500.0</td>
<td>2.6990</td>
<td>40.000</td>
</tr>
</tbody>
</table>

*, The log interval chosen was Log 5 = 0.6990; **, see table (2.1) above

4. The protocol followed in the present investigation, although largely similar to that recommended by Watson (2003) is different in the number and arrangement of wells in the agar gel substrate. The controls (including the boiled tissues) and the standard enzymes were assayed in a single Petri dish whereas the not boiled extracts of the various sections of the cockroach alimentary canal was tested in a separate Petri dish. Figures (2.6, 2.7) illustrate the protocol followed in the present work.

5. Since preparation of agar gels is time consuming, Agar Petri plates with starch or casein were prepared two days ahead of time.

6. Bioassays were run as replicates of five and the numerical data obtained were subjected to correlation and regression analyses.

7. Glass tubing (3 mm in diameter) was used to dig the wells in the agar gel instead of using Pasteur pipettes.

8. A more concentrated iodine solution (4% iodine in 2.5% potassium iodide) was used to stain starch instead of Lugol's solution. The later
gave faint bluish colouration to the unaffected starch-containing regions. Besides, with the solution used, a deep iodine brownish colour persisted in the enzyme-affected (starch-free) regions giving very clear rings.

9. Among the difficulties encountered:

a. Preparation and spreading of agar are sensitive processes that need good past experience, otherwise lumps of agar may form and the quantity of agar may run short and do not cover the whole Petri dish.

b. Digging wells in an agar gel of about 2.5 mm in thickness was, again troublesome. Making wells of the same size in specified place and distributing them in specified places such that the resulting rings do not overlap need skill. Besides, the well-digging device may slip this way or another.

c. Keeping Petri dishes in the fridge freezer to stop further reactions (awaiting photography), cause the agar layer to crack.

2.3 statistics:

The regression equation \( y = a + bx \) was calculated using probit double transformation regression analysis according to Finney (1947 and 1952) to determine the quantitative relationship between response (diameter of the affected region – rings) and the dose of the standard enzyme. In the equation (Fig. 2.8):

\[
y = \text{response as determined (mm) by the diameter of the affected zone around the well containing enzymatic activity.}
\]

\[
a = \text{The intersect of the regression line with X axis – i.e., the dose which produce 0% response.}
\]
b = the slope of the regression line – i.e., the tan of the angle the regression line makes with the horizontal. (see Figure 2.8).

10. From the equation, the concentration of an enzyme-containing solution or extract can be determined.

**Fig. (2.1).** Trapping cockroaches. **A,** from a manhole; **B,** from a kitchen.

**Fig. (2.2).** Cockroaches' maintenance cage

**Fig. (2.3).** Chilling chamber for anesthetizing cockroaches
\textbf{Fig. (2.4). Dissecting dish}

\textbf{Fig. (2.5). Diagram of the digestive tract of the cockroach.} Parts 1 to 4 were excised for enzymatic activity testing; part *, the gizzard, was discarded since it is highly chitinized, bears hard teeth and my break the glass tube of the homogenizer.

\textbf{Fig. (2.6) Glass tissue Dounce homogenizer}
Fig. (2.7). Distribution of wells containing the test materials. A, standard enzyme (chymotrypsin) solutions; B, the test extracts.
Fig. (2.8). Distribution of wells containing the test materials. A, standard enzyme (amylase) solutions; B, the test extracts.
Fig. (2.9). Dose/response regression line

Regression equation: $y = ax + b$

$b = \tan \Theta = \text{slope}$

$a = \text{intersect}$
CHAPTER THREE
RESULTS
(All tables and figures are shown at the end of the chapter)

3.1 General picture of the effects of standard amylase, controls and the tested sections of the alimentary canal of the cockroach, Preplaneta Americana on 1% starch following agar gel diffusion technique.

Table (3.1) shows that only standard amylase solutions and the not boiled extracts of the salivary glands and mesenteric caeca had an appreciable effects on starch. The photographs in (Fig. 3.a and b) show examples of typical results of the agar gel diffusion assay.

3.2 Dose/response regression analysis of the effect of standard amylase solutions on 1% starch

The response, as measured by the diameter of the clear zone around the well containing the enzyme (in mm), was found to form a linear relationship with the log dose of the enzyme (table 3.2 and (Fig. 3.2). The regression equation obtained \( y = 8.7275 + 6.8813 \times \) could thus be used to calculate the enzymatic potency of any amylase-containing solution or extract of unknown concentration.

3.3 Measuring amylase activity in crude water extract of salivary glands and mesenteric caeca of the of the cockroach, *P. Americana*

Table (3.3) shows the mean diameters of the clear zones around the wells containing extract of salivary glands and mesenteric caeca of the of the cockroach. These were 24.78 mm and 20.00 mm respectively. Applying the equation obtained from the bioassay with standard amylase (see section 3.2), the log concentrations of amylase in the salivary glands and mesenteric caeca in the extract prepared were 2.3328 (\( \approx 215 \mu g/ml \)) and 1.6546 (\( \approx 45 \mu g/ml \)). Applying t test to compare the two means showed that the salivary
glands of the cockroach contain significantly higher amount of amylase activity than the mesenteric caeca.

3.4 General picture of the effects of standard chymotrypsin, controls and the tested sections of the alimentary canal of the cockroach, Preplaneta Americana on 12% casein following agar gel diffusion technique.
Table (3.4) shows that only four of the standard chymotrypsin solutions and the not boiled extract of mesenteric caeca had an appreciable effects on casein. Chymotrypsin of concentration of 15.625 µg/ml didn’t produce an appreciable clear zone around the well. The photographs in (Fig. 3.1b) show typical results of the agar gel diffusion assay.

3.5 Dose/response regression analysis of the effect of standard chymotrypsin solutions on 12% casein
The response, as measured by the diameter of the clear zone around the well containing the enzyme (in mm), was found to form a linear relationship with the log dose of the enzyme (table 3.5 and (Fig. 3.3). The regression equation obtained \( y = -6.6923 + 7.395 x \) could thus be used to calculate the enzymatic potency of any chymotrypsin-containing solution or extract of unknown concentration.

3.6 Measuring chymotrypsin activity in crude water extract of mesenteric caeca of the cockroach, P. Americana
Table (3.6) shows the mean diameters of the clear zones around the wells containing extract of mesenteric caeca of the cockroach. The mean diameter of the affected area was found to be 21.02 mm. Applying the equation obtained from the bioassay with standard chymotrypsin (see section 3.5), the log concentrations of chymotrypsin activity in the mesenteric caeca in the extract prepared were 3.7474 (= 5590.3 µg/ml).
Fig. (3.1a). Examples of Petri dishes with agar gel containing 1% starch as substrate and wells containing test extracts – iodine treated.
Yellowish rings in agar gel demonstrate the presence of amylase - Wells without rings had gel buffer and boiled gut sections in them.

A. Standard amylase solutions and controls
1 Amylase (0.8 µg/ml)
2 Amylase (4.0 µg/ml)
3 Amylase (20 µg/ml)
4 Amylase (100 µg/ml)
5 Amylase (500 µg/ml)
6 agar gel buffer
7 Salivary glands extract (boiled)
8 esophagus + crop extract (boiled)
9 Mesenteric caeca extract (boiled)
10 Stomach extract (boiled)

B. Not boiled sections of the cockroach gut
1x Salivary glands extract (not boiled)
2x esophagus + crop extract (not boiled)
3x Mesenteric caeca extract (not boiled)
4x Stomach extract (not boiled)
5x colon + rectum (not boiled)
Fig. (3.1b). Examples of Petri dishes with agar gel containing 12% casein as substrate and wells containing test extracts — acetic acid treated.

Grey rings in agar gel demonstrate the presence of protease - Wells without rings had gel buffer and boiled gut sections in them.

A. Standard chymotrypsin solutions and controls
   1 Chymotrypsin (16 ug/ml)
   2 Chymotrypsin (62 ug/ml)
   3 Chymotrypsin (250 ug/ml)
   4 Chymotrypsin (1000 µg/ml)
   5 Chymotrypsin (4000 µg/ml)
   6 agar gel buffer
   7 Salivary glands extract (boiled)
   8 esophagus + crop extract (boiled)
   9 Mesenteric caeca extract (boiled)
   10 Stomach extract (boiled)
   11 colon + rectum (boiled)

B. Not boiled sections of the cockroach gut
   1x Salivary glands extract (boiled)
   2x esophagus + crop extract (boiled)
   3x Mesenteric caeca extract (boiled)
   4x Stomach extract (boiled)
   5x colon + rectum (boiled)
Table (3.1). Amylase activity of standard amylase and tested sections of the alimentary canal of the cockroach, *P. Americana*

<table>
<thead>
<tr>
<th>Standard amylase µg/ml</th>
<th>Tested section of alimentary canal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0*</td>
<td>B</td>
</tr>
<tr>
<td>0.8</td>
<td>+</td>
</tr>
<tr>
<td>4.0</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>

*, control (agar gel buffer); B. Boiled; NB, Not Boiled; -, No reaction; +, Positive reaction; Es. + cr., esophagus + crop extract; Hingut, colon + rectum extract; Mes. cc., Mesenteric caeca extract; Sal. gl., Salivary glands extract; Stom., Stomach extract.

Table (3.2). Dose/response regression analysis of standard amylase solutions on 1% starch

<table>
<thead>
<tr>
<th>Dose µg/ml</th>
<th>Log</th>
<th>Response mm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0**</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>-0.097</td>
<td>8.6</td>
</tr>
<tr>
<td>4.0</td>
<td>0.6020</td>
<td>12.4</td>
</tr>
<tr>
<td>20.0</td>
<td>1.3010</td>
<td>17.8</td>
</tr>
<tr>
<td>100.0</td>
<td>2.0000</td>
<td>21.5</td>
</tr>
<tr>
<td>500.0</td>
<td>2.6990</td>
<td>28.1</td>
</tr>
</tbody>
</table>

*, Diameter of the affected area of the agar gel; **, Control = blank buffer

Regression equation: \( y = 8.7275 + 6.8813 \times \)

Slope = 81.7°

Log 95% fiducial limits = ±0.2653

Correlation coefficient (r) = +0.9815

![Graph](https://via.placeholder.com/150)

**Fig. (3.2). Dose/response regression line of standard amylase on 1% starch**
Table (3.3). Mean response (mm) of amylase activity of crude water extract of salivary glands and mesenteric caeca of the cockroach, *P. Americana*

<table>
<thead>
<tr>
<th>Response (= Diameter of clear zone in the agar gel (mm))</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>± Standard error</th>
<th>± 95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary glands extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R₁</td>
<td>26.1</td>
<td>24.4</td>
<td>23.8</td>
<td>25.0</td>
</tr>
<tr>
<td>R₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric caeca</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R₁</td>
<td>19.4</td>
<td>20.2</td>
<td>19.8</td>
<td>21.0</td>
</tr>
</tbody>
</table>

*, applying t test to compare between the two means showed that the difference was very highly significant (***). The calculated t value (0.866) was higher than the tabulated t (8.610) at p ≤ 0.001

Table (3.4). Chymotrypsin activity of standard enzyme solutions and extracts of the tested sections of the alimentary canal of the cockroach, *P. Americana*

<table>
<thead>
<tr>
<th>Standard chymotrypsin µg/ml</th>
<th>Tested section of alimentary canal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0*</td>
<td>B</td>
</tr>
<tr>
<td>1.938</td>
<td>1.756</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*, control (agar gel buffer); B. Boiled; NB, Not Boiled; –, No reaction; +, Positive reaction; Es. + cr., esophagus + crop extract; Him.gut, colon + rectum extract; Mes. cc., Mesenteric caeca extract; Sal. gl., Salivary glands extract; Stom., Stomach extract;
Table (3.5). Dose/response regression analysis of standard chymotrypsin solutions on 12\% casein

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>Log</th>
<th>Response mm*</th>
<th>Empirical</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0**</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.625</td>
<td>1.1938</td>
<td>0</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>62.5</td>
<td>1.7599</td>
<td>7.0</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>2.3980</td>
<td>10.1</td>
<td>15.6</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3.0000</td>
<td>15.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4000</td>
<td>3.6020</td>
<td>20.4</td>
<td>19.9</td>
<td></td>
</tr>
</tbody>
</table>

*, Diameter of the affected area of the agar gel;  
**, Control = blank buffer  
Regression equation: \( y = -6.6923 + 7.395x \)  
Slope = 82.3°  
Log 95\% fiducial limits ± 0.2455  
Correlation coefficient (r) = + 0.9824

Fig. (3.3). Dose/response regression line of standard chymotrypsin on 12\% casein
Table (3.6). Mean response (mm) of chymotrypsin activity of crude water extract of mesenteric caeca of the of the cockroach, *P. Americana*

<table>
<thead>
<tr>
<th>Response (= Diameter of clear zone in the agar gel (mm))</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Standard error</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁</td>
<td>R₂</td>
<td>R₃</td>
<td>R₄</td>
<td>R₅</td>
</tr>
</tbody>
</table>
Swingle (1925) in his review "digestive enzymes of an insect" has reviewed published works on cockroach digestive enzymes since 1876 and up to 1925. The pioneering work of Wigglesworth, V.B. (1927a,b,c) has largely established the main points of the subject. These early workers identified the enzymes by simple colorimetric analysis. Advances in biochemical techniques including molecular biology techniques have added a lot of details pertinent to digestive enzymes of the cockroaches (Adiyodi, 1981; Girard and Jouanin, 1999; Wilson and Walker, 2001; Gilbert, 2011). Yet, the general account mentioned below remains well established and agrees in general terms with the results of the present work. The salivary glands produce only an amylase. The hepatic caeca of the midgut produce an amylase, invertase, maltase, lipase and protease. The remainder of the midgut (stomach) secretes the same enzymes though much less abundantly. The hind-gut does not secrete digestive enzymes. The greater part, if not the whole, of digestion takes place in the crop, the enzymes passing backwards from the salivary glands and forwards from the mid-gut. The crop is lined by a membrane of chitin and is generally regarded as playing no part in the secretion of digestive ferments.

The major aim of this work, as mentioned previously, is to introduce the procedure and analysis presented in this work as part of training in an introductory physiology practical course to (a) cut the costs involved and (b) avoid the use of vertebrates in scientific research and experimentation for ethical reasons. Invertebrate animals offer scientists many advantages over vertebrates, including their short life cycle, simple anatomy and the ease
with which large numbers of individuals may be studied. Invertebrates are often cost-effective, as thousands of them can be housed in a single room. Besides, mostly collection, breeding and maintenance of invertebrates is much easier, less troublesome and much cheaper than vertebrates. With the exception of some cephalopods, invertebrate species are not protected under most animal research legislation (Paul, 1995; Lipinski et al., 2004). Hester and Harrison, 2006; Malcolm, 2006; Rollin, 2006; Russell and Burch, 2007).

The Association for Biology Laboratory Education or ABLE in the USA and Canada has called for the improving the undergraduate biology laboratory experience by promoting the development and dissemination of interesting, innovative, and reliable laboratory exercises - i.e., adoption of “the three R’s” (Replacement, Refinement, and Reduction) in biomedical research and teaching. ABEL has convened 34 workshop/conference; the 1st was at the University of Calgary, 1979 and the last (the 34th) was at the University of North Carolina at Chapel Hill June 19-22, 2012. Many scientific bodies help promoting the concepts adopted by ABEL. These include Bioscience Educational Net or BEN, American Institute of Biological Sciences and AAST or American Academy for Science and Technology. Many colleges and departments of biological sciences in USA, Canada, Europe and Australia have followed the procedures published by ABEL with or without modifications. Many educational institutes collect the results obtained by the students and analyze them for confirmation and applicability of the procedures (St. Mary’s College, 1992; McMahon, 2003). According to such analysis the students have come to know practically what is meant by a bioassay, how to determine qualitatively and quantitatively the potency of a sample of biological material by means of bioassays as well as the techniques of weighing and diluting accurately chemicals, extracting
materials from biological samples, statistical analysis using relevant computer statistical packages and discussing and interpreting numerical results – i.e., become trained in the scientific method.

The most expensive item in the described procedure is the price of the standard enzymes which may cost more than $200. As way out, the comparative approach will suffice - i.e., comparing the distribution and comparative potency of the digestive enzymes of widely or closely related species. In such a case, various logarithmically spaced doses of the animal tissues from various segments of the alimentary canal are tested and for each test tissue the dose/response equation is calculated so that the potencies can be compared.

It is also recommended that such a work be accompanied by histochemical studies of the various sections of the alimentary canal and if possible the study of the cellular components of the digestive cells with electron microscopy. This will allow correlation of structure with function – i.e., makes the functional identification and nomenclature of digestive cell types possible. For example, a cell type is better to be described as chymotrysin-secreting cell instead of describing it as parietal or zymogen cell.
REFERENCES

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ISBN-10: 0412161400

AIBS or American Institute of Biological Sciences. http://www.aibs.org


BEN or Bioscience Educational Net. www.biosciednet.org/portal


ISBN-10: 0123847478

ISBN-10: 0123847478


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Triplehorn & Johnson, 2005).


APPENDIX

The procedures shown here were copied from:

Materials:

- Agar powder- 10 gm
  Agar gel buffer- 0.1M NaCl, 10 mM Tris, pH 7.4 - 500 mL
- Enzyme extraction buffer- 20 mM NaCl, 0.02%, 3 drops of ‘Dawn’ detergent, 10 mM Tris, pH 8- 100 mL
- Starch solution- 1% - 50 mL
- Skim milk- 20 mL or 12% casein (pH=8, titrate with 6N NaOH)
- 3 large glass test tubes
- 10 1.5 mL tubes
- 3 petri dishes per group (20)
- 18 glass Pasteur pipettes
- 10 small transfer pipettes: one pipette per student group, one for the starch solution and one for each of the amylase and chymotrypsin solutions.
- Amylase- 4 mg/mL - 10 mL
- Chymotrypsin-10mg/mL- dilutions needed for various standards - 10 mL
- Pepsin- 2mg/mL - 10 mL
- IKI solution (Lugol’s iodine solution)
- Large beaker-2 to boil water
- Cockroaches, CO₂ tank
- 20 scissors and 20 forceps
- Insect saline (500 mL), and 20 petri dishes for cockroach dissection
- 3 glass tissue grinders
- Hot plates
- Acetic acid – 5% 500 mL
- Microcentrifuge tubes
- Centrifuge
- Rulers

Student Outline

Removal and processing of the digestive tract of the cockroach.
Anesthetize 20-25 cockroaches with carbon dioxide (2-3 cockroaches per students for 10 groups of students). Frozen cockroaches may be used. Cut the anus free from the body wall with scissors. Remove the complete digestive tract of the cockroach by holding the cockroach in insect saline and slowly pulling off the head and attached gut with forceps. Should the gut break between the proventriculus and midgut, open the abdomen centrally and dissect out the posterior gut portion. Refer to the diagram and identify (do not isolate the three regions until they are rinsed) the foregut, midgut, and hindgut. Slit open the gut and, holding it with a pair of forceps, vigorously rinse in a dish of insect saline to remove any contained food. Using a scalpel, isolate the three regions of the gut to
be studied (foregut, midgut, and hindgut). Obtain four test tubes and label them 1-4. Collectively, weigh the isolated sections of cockroaches’ gut, be sure not to mix up the different sections of the intestine. Place the isolated sections of gut in separate labeled glass tissue grinders containing enzyme extraction buffer (1 to 1.5 mL of extracting buffer per gram of tissue). After grinding, allow the tissue to settle. Transfer the supernatant by Pasteur pipette into separate test tubes (1-foregut, 2-midgut, and 3-hindgut). Bring the volume of each extract to 2 mL with enzyme extraction buffer. Pipette 1 mL of each of the extracts (1-3) into the 4th tube. Heat this combined extract for 5 minutes. Alternatives to vertebrates 325 in a beaker of boiling water to denature the enzymes present. Cool and bring to about 3 mL with enzyme extraction buffer. This will serve as a suitable control for the enzyme tests. Shake the remaining 3 tissue extracts, and divide the homogenized gut extract into 10 portions (for 10 groups of students) by placing 1 mL of each extract in a labeled 1.5-mL microcentrifuge tube. Cap and shake the tubes vigorously for 3 minutes. Centrifuge the tubes for 5 minutes and remove the supernatant fraction (the liquid) with a pipette. Place these solutions in clean labeled 1.5-mL microcentrifuge tubes. The extracted enzyme is now in these tubes.

**Preparation of the agar gels:**

1. Label three large tubes, A, B and C. Dispense 18 mL of agar gel buffer into tubes A and B, and 19.5 mL to tube C. Add 0.32 g of agar to each tube.
2. Place the tubes into a boiling water bath and allow the agar suspensions to come to a vigorous boil. After boiling for 4 minutes, remove the test tubes from the bath and cool at room temperature for about 3 minutes. The solution should be clear.
3. Add 2 mL of skim milk or 1 mL of 12% casein to tubes A and B. Add four drops of concentrated NaOH to the skim milk so that the pH is around 8. Add 0.5 mL of 1% starch solution to tube C and swirl the tube until the agar forms a suspension. Skim milk (casein) and starch will serve as the substrates for the enzymes used in this experiment. Each tube should have 20 mL of fluid.
4. Pour the melted agar into three Petri dishes, one tube per dish. Label the dishes: A, B and C; these dishes: A, B and C for milk (casein), and C for starch substrate.
5. Let the agar cool at least 15 minutes. The gels can be used or stored in the refrigerator for up to a week.
6. Make 9 wells in each of the three agar plates using the large end of a Pasteur pipette.
7. Add about 5 mL of vinegar to agar plate B. After 15 minutes, discard the acid. The acid will denature and precipitate the casein in the milk, which will cause the protein to turn white.
8. Prepare the appropriate dilutions of chymotrypsin and amylase solutions.
Sample Application:
Using the transfer pipettes, carefully fill the wells (about 0.1 ml) of the plates as indicated below. Separate pipettes should be used to disperse the chymotrypsin and pepsin solutions. (Plate B was treated with 5% acetic acid before extracts were placed in the wells):

<table>
<thead>
<tr>
<th>Well number</th>
<th>Proteolytic enzymes (A and B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agar gel buffer</td>
</tr>
<tr>
<td>2</td>
<td>Chymotrypsin (20 ug/mL)</td>
</tr>
<tr>
<td>3</td>
<td>Chymotrypsin (200 ug/mL)</td>
</tr>
<tr>
<td>4</td>
<td>Chymotrypsin (2000 ug/mL)</td>
</tr>
<tr>
<td>5</td>
<td>Pepsin (2 mg/mL)</td>
</tr>
<tr>
<td>6</td>
<td>Standard foregut extract (not boiled)</td>
</tr>
<tr>
<td>7</td>
<td>Standard midgut extract (not boiled)</td>
</tr>
<tr>
<td>8</td>
<td>Standard hindgut extract (not boiled)</td>
</tr>
<tr>
<td>9</td>
<td>Boiled intestinal extract</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Well number</th>
<th>Amylase C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agar gel buffer</td>
</tr>
<tr>
<td>2</td>
<td>Amylase- 4 ug/mL</td>
</tr>
<tr>
<td>3</td>
<td>Amylase- 20 ug/mL</td>
</tr>
<tr>
<td>4</td>
<td>Amylase- 100 ug/mL</td>
</tr>
<tr>
<td>5</td>
<td>Amylase- 500 ug/mL</td>
</tr>
<tr>
<td>6</td>
<td>Standard foregut extract (not boiled)</td>
</tr>
<tr>
<td>7</td>
<td>Standard midgut extract (not boiled)</td>
</tr>
<tr>
<td>8</td>
<td>Standard hindgut extract (not boiled)</td>
</tr>
<tr>
<td>9</td>
<td>Boiled intestinal extract</td>
</tr>
</tbody>
</table>
After all samples (about 0.1 mL) have been loaded in the wells, place the lids on the dishes. Do not overfill the wells. The dishes should not be moved at this time. The plates should remain at room temperature for 12-24 hours.

Analysis of agar plates:

A. Detection of proteolytic enzyme activity (A and B):
1. Place about 5 mL of vinegar or 4% acetic acid onto agar in plate A. The transparent rings around the sample wells where the casein has been degraded by the enzyme indicate protease activity on both plates A and B.
2. After 20 minutes, discard the acid on plate A, and measure the diameter of the clear rings around each well.
3. The amount of chymotrypsin in the tissue extracts can be compared to the diameters of the rings around the sample wells containing known concentrations of the enzymes.

B. Detection of amylase activity (C):
1. Place about 5 mL of the Lugol's iodine solution onto the agar in the plate.
2. After 10-20 minutes, discard the iodine and fill the petri dish with water.
3. After 10-20 minutes, measure the diameter in mm of the clear rings around each well and record your results below (Table 1). The amount of amylase in the tissue extracts can be compared to the diameters of the clear rings around the sample wells containing known concentrations of the enzymes.

<table>
<thead>
<tr>
<th>Well number</th>
<th>Diameter of clear rings- mm</th>
<th>Protease activity</th>
<th>Amylase activity</th>
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<td>Plate A</td>
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</table>

Table 1. Diameter of rings, measuring protease and amylase activities

Note: Detection of the enzymatic effect can be done after 3, 6, 12 or 24 hours.
Suggestions for Data Analysis and Discussion:
1. Determine the amount (if any) of amylase and chymotrypsin activity in the cockroach gut extracts, using Excel [diameter of ring (cm) on Y axis, and concentration of enzyme on (log) X axis].
2. Explain the basis for the formation of rings in the agar-starch gels.
3. Describe the effects of the heat treatment on the activity of the enzymes in the cockroach gut extract.
4. Relate the findings from the cockroach foregut, midgut, and hindgut to the site of action of proteolytic and carbohydrate enzymes in the digestive tract.