



DIPARTIMENTO DI SCIENZE AGRARIE ALIMENTARI E AMBIENTALI

CORSO DI LAUREA IN: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

PROTEASE INHIBITORS FROM EDIBLE *T. MOLITOR* LARVAE

TIPO TESI: sperimentale

Studente:
AMEL TWFEK

Relatore:
PROF. NADIA RAFFAELLI

Amel

Nadia Raffaelli

ANNO ACCADEMICO 2018-2019

CONTENTS

List of contents	I
Dedication	III
Acknowledgement	VI
Abbreviation	V
1. Introduction	1
1.1. Edible insects	1
1.1.1. Nutritional value of insects	3
1.1.2. <i>Tenebrio Molitor</i> L.	5
1.2. Anti-nutritional factors	7
1.2.1. Protease inhibitors	9
1.2.2. Anti-nutritional factors in edible insects	12
1.3. Aim of the thesis	13
2. Material and Methods	14
2.1. Mealworms	14
2.2. Materials	14

2.3. Preparation of solutions	15
2.3.1. Preparation of buffer solutions	15
2.3.2. Preparation of enzymes solutions	15
2.3.3. Preparation of substrate solutions	15
2.4. <i>T. molitor</i> larvae crude extract preparation	16
2.5. Determination of protein concentrations	16
2.6. Determination of trypsin and Chymotrypsin activities	16
2.7. Determination of Trypsin and Chymotrypsin inhibitor activities	17
3. Results and Discussion	19
4. Conclusion	24
References	25

DEDICATION

In the name of God, peace and blessings be upon the Prophet of God. I dedicate this humble effort to my little family. To my spiritual friend, lovely spouse, Karameldeen Omer, without his support I could not achieve this success. All my respect and love for you. To my friends and colleagues from all over the world. Finally, I would like to dedicate this research to Polytechnic University of Marche and to the greatest institution of science and knowledge, University of Khartoum.

ACKNOWLEDGEMENTS

All praise is due to Allah, The Almighty and The Merciful, who gave me power to carry out this research work. I would like to express a great appreciation to my supervisor Prof. Nadia Raffaelli for her concerns, encouragement and kind guidance with great patience. Without her continual inspiration, it would have not been possible to complete this study. I will not forget to acknowledge Dr. Federica for her assistance and powerful comments and advises on this work. Also, it's a good opportunity to express gratitude to all members of the Biochemistry department. Moreover, I should be grateful to my husband for providing me continuous encouragement and supporting me spiritually throughout the process of researching and writing this thesis. This accomplishment would not have been possible without him. I am also indebted to all my family members, colleagues and friends for their moral support. Finally, many thanks to the Polytechnic University of Marche, Italy and University of Khartoum, Sudan and thanks to anybody who contributed to enriching me with knowledge.

ABBREVIATIONS

S	Substrate
ANF	Anti-nutritional factors
BSA	Bovine serum albumin
BAPNA	N- α -Benzoyl-DL-Arginine-p-nitroanilide hydrochloride
SAAPFPNA	N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-p-nitroanilide
TCEP	Tris-(2-carboxyethyl) phosphine hydrochloride
DMSO	Dimethyl sulfoxide
DMF	Dimethyl formamide
Tris-HCl	Tris-hydrochloric acid
HCl	Hydrochloric acid
NaCl	Sodium chloride
dw	Dry weight
V_{mix}	Volume of mixture
V_{enzyme}	Volume of enzyme

1. Introduction

1.1. Edible insects

Entomophagy, i.e., the use of insects as food, is traditionally practiced in many parts of the world ^[1]. Over 2000 insect species are known to be edible. (List of edible insects of the world (<http://www.ent.wur.nl/UK/Edible+insects/Worldwide+species+list/>) ^[2].

Most of the insects consumed in significant quantities belong to one of the following six orders: Lepidoptera, including butterflies, moths and caterpillars, Coleoptera, or the beetles, Orthoptera, including locusts, crickets, and grasshoppers, Isoptera, including termites, Hymenoptera, including ants, bees, and wasps, and Hemiptera (bugs) ^[3]. Those edible insects may potentially be used at different stages of their development, i.e. egg, larva, pupa and adult stages.

The insects groups those may be used as foods in the European Union (EU) are: yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*) and tropical banded cricket (*Gryllodes sigillatus*) ^[4]. Among them, mealworm larvae (*Tenebrio molitor L.*) are the most produced insects thanks to their cosmopolite status, flexibility in livestock management and environmental sustainability opportunity ^[5].

Due to the increasing cost of animal proteins, food and feed insecurity, population growth, and increasing need for protein-rich food in the developed and less developed countries, alternative sources of protein-rich food are highly needed ^[6], which may be edible insects ^[7]. Various

researchers reported that insects could be an attractive alternative as they are natural food of many vertebrates including human ^[8].

Most edible insects used for food and feed are gathered in the wild, although farming of insects for direct human consumption has recently begun ^[9]. Insects could be farmed in laboratories or in commercial facilities ^[4].

Compared with livestock, breeding insects seems to be more environmentally friendly because of lower greenhouse gas emissions and ammonia, water pollution and land use ^[7]. Taking into consideration that insects have a high fecundity, can be multivoltine, have a high feed conversion efficiency, low space requirement, are omnivorous and are of nutritional value, edible insects can represent an interesting food and feed alternative ^[1, 10].

Edible insects can be fed different types of organic waste, some insect species can biodegrade organic waste and transform it into high-quality insect biomass ^[11, 12]. However, some Western countries are experiencing difficulties in farming edible insects within the scope of their legislation. A growing interest in the consumption of insects has become apparent in traditional media and scientific literature. Despite legal uncertainties, farms devoted to rearing edible insects in Europe, as well as those found elsewhere, are growing rapidly in number. In Europe, there are a number of companies and research projects studying the best way to introduce insects into the diet. Compared to only a few years ago, the availability of edible insects has grown dramatically, and this boom appears to be just at the beginning ^[13].

Generally, insects were found to be highly nutritious and to represent good sources of proteins, fat, minerals, vitamins, and energy ^[1]. Insects are already used as natural food ingredients, e.g. the red colourant carmine (E120) used in yogurt is an extract of the female cochineal insect ^[10].

1.1.1. Nutritional value of insects

The summary report of the first conference on insects as food and feed stated that insects have good nutritional quality ^[14]. Insects are a nutritionally interesting material and may be included among the common diet of consumers in EU countries in the future ^[7].

The nutritional value of edible insects is very diverse mainly because of the large number and variability of species. Nutritional values can vary considerably even within a species depending on the stage of metamorphosis, origin of the insect ^[7, 15] and it is highly dependent on the feed. This possibly also opens opportunities for regulation, enrichment, and addition of certain food ingredients such as the omega -3-fatty acids DHA and EPA via feed ^[1].

Edible insects offer promise as environmentally sustainable sources of protein. They have been shown to contain a large quantity of protein of good quality and high digestibility ^[16].

Protein content has been reported to vary between 7 and 91% (dw), depending on the insect species, most insects contain around 60% protein. Protein contents could vary by more than 50% within the same insect species, in certain instances. The large variability may arise from extrinsic

factors such as feed and ecology which are likely to affect final composition. Other parameters such as the development stage of the edible insects and the manner in which they are processed (thermal and mechanical treatments) may also affect their protein content.

Edible insect proteins have a good amino acid profile. It has been shown that a significant content of essential amino acids (i.e. Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan and Valine) may be found across different insect species ^[17].

Although edible insects are good in amino acid content, reports indicated that some of them are deficient in some amino acids. As an example, the edible insects like Mormon cricket (*Anabrus simplex* Haldeman) and house cricket (*Acheta domesticus* Linnaeus) are deficient in methionine ^[8]. The essential amino acid indices in *T. molitor*, superworm (*Zophobas morio*) and *A. diaperinus* were found to be within the range of those from soybean and bovine caseins ^[4]. The composition of amino acid in Mealworms (*T. molitor*) is sufficient to meet dietary requirements of humans, except for methionine which is limiting in this species ^[17].

After protein, fat represents the second largest portion of the nutrient composition of edible insects, ranging from 13% for Orthoptera (grasshoppers, crickets, locusts) to 33% for Coleoptera (beetles, grubs). The fatty acids of edible insects are generally comparable with those of poultry and fish in their degree of unsaturation ^[15]. Edible insects contain good quality fatty acid especially long chain omega-3 fatty acids such as alpha-linolenic acid, eicosapentaenoic acid. Different species of insect have different fatty acid profiles ^[16].

In addition, edible insects have also been found to be rich sources of carbohydrates, various vitamins (A, D, thiamine, riboflavin, pantothenic acid, biotin, and folic acid) and minerals (iron, calcium, copper, magnesium, manganese, phosphorous, selenium, and zinc) ^[18].

A wide range of biofunctional components (e.g. chitin, polyphenols, antioxidant enzymes, antimicrobial peptides/proteins, etc.) exist in insects ^[4]. Therefore, insects offer an important nutritional resource for humans and are worthy of development in various bio-prospecting aspects ^[19].

1.1.2. *Tenebrio molitor* L.

Mealworms are the larvae of two species of darkling beetles of the Tenebrionidae family: the yellow mealworm beetle (*Tenebrio molitor* Linnaeus), and the smaller and less common dark or mini mealworm beetle (*Tenebrio obscurus* Fabricius).

Among edible insect species stands out the mealworms *Tenebrio molitor* Linnaeus (1758 – Coleoptera, Tenebrionidae), since it is currently consumed by humans ^[20].

This is an insect species that has one of the highest amounts of protein (from 47.76 to 53.13%) and lipids (27.25 to 38.26%), with energy contributions varying from 379 to 573 kcal/100g. Considering a daily energy value for an adult of 2000 kcal/ day, 100 g of *T. molitor* meet approximately a quarter of the daily energy needed. *T. molitor* is among the largest beetles that infest food products in warehouses, mainly grain warehouses. This species begins to lay eggs from 4 to 17 days after copulation. A single female can generate

an average of 500 eggs. The embryonic development lasts from 4 to 6 days, which can be accelerated with a slight increase in temperature (25 to 27°C). Larval period is about 3 months; at this stage, the insect is consumed. An average mature larva weighs 0.2 g and is 25-35mm long. After this phase, the larva turns into a pupa, a stage that lasts 5 to 6 days and culminates in an adult individual ^[20, 21].

T. molitor is a pest of grain, flour, and food stores, but often not of much importance since populations are quite small. Mealworms are easy to breed and feed and have a valuable protein profile. For these reasons, they are produced industrially as feed for pets and zoo animals, including birds, reptiles, small mammals, batrachians, and fish. They are usually fed live, but they are also sold canned, dried, or in powder form.

The mealworm species can be grown successfully on diets composed of organic by-products. Diet affects mealworm growth, development, and feed conversion efficiency, where diets high in yeast-derived protein appear favorable with respect to reduced larval development time, reduced mortality, and increased weight gain. Dietary protein content had a minor effect on mealworm protein content, whereas larval fat content and fatty acid composition varied over a wider range. They may be easily reared on fresh oats, wheat bran or grain, with sliced potato, carrots, or apple as a moisture source ^[22].

1.2. Anti-nutritional factors

The anti-nutritional factors (ANF) are compounds synthesized in natural food and/or feed stuffs by the normal metabolism of species. ANF reduce the nutrient utilization and/or food intake and exerts effect contrary to optimum nutrition by different mechanisms (e.g. inactivation of some nutrients, diminution of the digestive process, or metabolic utilization of feed) [23, 24].

It is well known that plants generally contain ANF acquired from fertilizer and pesticides and several naturally-occurring chemicals. Some of these chemicals are known as “secondary metabolites” and they have been shown to be highly biologically active. These secondary metabolites are produced as side products of processes leading to the synthesis of primary metabolites. They include saponins, tannins, flavonoids, alkaloids, proteases inhibitors, oxalates, phytates, haemagglutinins (lectins), cyanogenic glycosides, cardiac glycosides, coumarins and gossypol. The list is inexhaustible [24-26].

ANF in plants may be classified on the basis of their chemical structure, the specific actions they bring about or their biosynthetic origin. Although the following classification does not encompass all the known groups of anti-nutritional factors, it does present the list of those frequently found in human foods and animal feedstuffs. ANF may be divided into two major categories. They are:

- I. Proteins (such as lectins and protease inhibitors) which are sensitive to normal processing temperatures.

II. Other substances which are stable or resistant to these temperatures and which include, among many others, polyphenolic compounds (mainly condensed tannins), non-protein amino acids and galactomannan gums [27].

ANF may occur endogenously or may be formed during heat/alkaline processing of proteins. Examples of major naturally occurring anti-nutritional factors include trypsin inhibitors and haemagglutinins in legumes, tannins in legumes and cereals, phytates in cereals and oilseeds, glucosinolates in mustard and canola protein products and gossypol in cottonseed protein products. Examples of important ANF formed during the heat/alkaline treatments of protein products include Maillard reaction products, oxidized forms of sulphur amino acids and D-amino acids [28].

The biochemical and toxicological/adverse effects of ANF involve interfering with processes such as digestion, absorption and utilization of nutrients (e.g. vitamins and minerals). ANF can also affect the body's metabolic rate or exert direct toxic effects. The bioavailability of the essential nutrients could be reduced by the presence of ANF [26, 27]. For example, phytate renders minerals, especially divalent cations, unavailable and inhibits proteolytic and amylolytic enzymes. Polyphenol affects the mineral bioavailability and protein and carbohydrate digestibility of food grains [29].

Mineral deficiencies, manifesting as different disease conditions such as goiter, rickets or one form of metabolic dysfunction or the other, may be a result of the interactions between the nutrient and anti-nutrient components of the diet in both animals and humans [27, 30].

However, some ANF may exert beneficial health effects at low concentrations. For example, when used at low levels, phytate, lectins, tannins, amylase inhibitors and saponins have also been shown to reduce the blood glucose and insulin responses to starchy foods and/or the plasma cholesterol and triglycerides. In addition, phytates, tannins, saponins, protease inhibitors and oxalates have been related to reduce cancer risks. This implies that ANF might not be always harmful even though they lack nutritive value. Despite of this, the balance between beneficial and hazardous effects of anti-nutrients rely on their concentration, chemical structure, time of exposure and interaction with other dietary components [23].

Due to this, they can be considered as ANF with negative effects or non-nutritive compounds with positive effects on health [23]. It is therefore essential to evaluate the composition of different materials used in the diet to ascertain their nutritive values [27].

1.2.1. Protease inhibitors

Protease inhibitors are substances of proteinic or polypeptidic nature forming relatively stable complexes with proteases and regulating their proteolytic activity, for blocking these in emergency cases, or for signaling receptor interactions or clearance [31-34].

In addition to their roles in regulating endogenous proteolytic activities, they are important for protecting fluids or tissues from degradation by unwanted or foreign proteolytic activities [32].

A common feature for all protease inhibitors is the presence of a disulphide linkage in their conformation make it readily exposed to tryptic attack. Protease inhibitors function by combining stoichiometrically with the active enzyme to form tightly bound enzyme substrate-like complexes. These inhibitors differ from normal substrates in that the complex formed is stable because of a very complementary fit, and the accumulation of weak, non-covalent bonds at the contact zone. The affinity of enzyme for the inhibitor is much greater than that for natural substrates ^[35].

Protease inhibitors are widespread in nature and occur in various tissues of microorganisms, plants, and animals. Protease inhibitors occur in most legumes and cereals, in certain vegetables- such as cabbage, cucumbers, potatoes, tomatoes, and spinach- and in certain fruits- such as apples, bananas, pineapples and raisins. The quantity of inhibitor depends on variety and physiological status of the plant and on levels of insect infestations or damage. The majority of the protease inhibitors differ in specificity and inhibiting capacity. Many are able to inhibit one or two enzymes. Inhibitors of plant origin are known to be effective against enzymes of several protease classes. Of interest with regard to mammalian protein digestion and nutrition are inhibitors of:

- The aspartic proteases (pepsin),
- The serine proteases (chymotrypsin, trypsin and pancreatic elastase),
- The metallo-carboxy- peptidases ^[31, 36].

Among common food and feed protein products, soya beans are the most concentrated source of trypsin and chymotrypsin inhibitors. Protease inhibitors isolated from soya bean fall into two main categories including the

Kunitz inhibitor and the Bowman-Birk inhibitor. The Kunitz inhibitor has a molecular weight of about 21.5 kDa with two disulphide bridges and possesses a specificity directed mainly against trypsin. The Bowman-Birk inhibitor has a molecular weight of about 8 kDa with a high proportion of disulphide bonds and the capability of inhibiting chymotrypsin and trypsin at independent binding sites. Inhibition of trypsin and chymotrypsin in humans by soya bean extracts has been reported [28, 36].

From the nutritional perspective the inhibition of the mammalian serine proteases trypsin and chymotrypsin is most important [31]. Protease inhibitors inhibit digestive enzymes cause substantial reductions in protein and amino acid digestibility values (up to 50 %) and protein quality (up to 100 %) in animal models. Exposure to trypsin inhibitors results in increased synthesis and secretion of proteases (such as trypsin, chymotrypsin and elastase) and pancreatic hypertrophy and hyperplasia in animal models. The increased secretion of proteases supported the suggestion that the growth depression caused by trypsin inhibitors was the consequence of an endogenous loss of amino acids. In fact, trypsin and chymotrypsin are particularly rich in sulphur-containing amino acids. Therefore, the effect of a hyperactive pancreas would be to divert these amino acids from the synthesis of body tissue proteins to the synthesis of enzymes, which are subsequently lost in the faeces [28, 33, 36, 37].

1.2.2. Anti-nutritional factors in edible insects

Edible insects are highly nutritious. However, for an extensive and safe utilization of insects as food and food ingredients, ANF and harmful ingredients of insects should also be considered.

ANF are usually present in plant materials but many phytophagous insects have been identified to retain these ANF in quite a good amount. Hence, it is recommended to analyze these anti-nutrients if a phytophagous insect is being considered as food, though this kind of study is not much frequent in the literature.

Anti-nutrients such as hydrocyanide, oxalates, phytates and tannins have been reported in edible insects eaten in Nigeria ^[38, 39]. In particular, the presence of hydrocyanide (HCN) (2.187 to 3.203 mg/kg), oxalate (13.20 to 28.40 mg/kg), phytate (0.28 to 0.289 mg/kg) and tannin (0.329 to 0.430 mg/kg) were found in four edible insects: *G. lucens* (cricket), *H. meles* (yam beetle), *R. phoenicis* (palm weevil) and *Z. variegatus* (grasshopper), respectively ^[38]. Tannin (14.3 mg/100 g), phytic acid (178 mg/100 g) and oxalate (2.1 mg/100 g) have also been reported in *O.monoceros* ^[39].

However, in all these species the content of the anti-nutrients is negligible and is considered to be under the tolerance limit in humans when consumed. Much higher amounts have been observed in various plant food materials ^[8, 40, 41].

1.3. Aim of the thesis

T. molitor is a highly nutritional edible insect particularly efficient in transforming diet substrate with low nutritional value in a rich protein product.

Previous work has demonstrated that *T. molitor* larvae reared on wheatmeal represent a better protein source than those reared on flour. They contain a higher protein content and are richer of essential amino acids. Substitution of 25% wheatmeal with olive pomace does not negatively affect the protein quality, and a further increase of olive pomace results in a slow decrease of the protein content, with larvae reared on 75% olive pomace showing a content only slightly lower than that observed in larvae collected on flour. These results suggest that *T. molitor* larvae might constitute a valuable solution in the managing of solid waste produced by olive oil industry, which represents a severe environmental challenge in the Mediterranean area. The aim of this thesis is to determine the presence of protease inhibitors in larvae reared on the different substrates. Protease inhibitors represent one of the most abundant anti-nutritional factors, and nothing is known about their possible presence in edible insects. The experimental research has been focused on the determination of the activity of trypsin and chymotrypsin inhibitors in larvae of *T. molitor* reared on wheat flour, wheatmeal and wheatmeal enriched with different amounts of olive pomace.

2. Material and Methods

2.1. Mealworms

T. molitor larvae were reared by the Entomology group of the Department D3A on six diet substrates consisting of:

S₁: 100% wheat flour.

S₂: 100% wheatmeal flour.

S₃: 75% wheatmeal flour - 25% olive pomace.

S₄: 50% wheatmeal flour -50% olive pomace.

S₅: 25% wheatmeal flour -75% olive pomace.

S₆: 100% olive pomace.

The larvae were frozen and stored at -20°C until analysis time. Three replicates of each substrate were analyzed.

2.2. Materials

Bradford reagent, bovine serum albumin (BSA), bovine trypsin, bovine chymotrypsin, N- α -Benzoyl-DL-Arginine-p-nitroanilide (BAPNA) hydrochloride, N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-p-nitroanilide (SAAPFPNA), dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), Tris-hydrochloric acid (Tris-HCl), Hydrochloric acid (HCl) and sodium chloride (NaCl) were obtained from Sigma-Aldrich Co. All reagents and chemicals were of analytical grade.

2.3. Preparation of solutions

2.3.1. Preparation of buffer solutions

- Extraction buffer: 50.0 mM Tris-HCl, 50.0 mM NaCl, pH 8.0 was prepared and stored at 4°C until analysis time. At moment of experiment 1.0 M of Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) was mixed with Tris-HCl / NaCl buffer to be final concentration of TCEP 1.0 mM.
- Activity assay buffer: 300.0 mM Tris- HCl, pH 8.0 was prepared and stored at 4°C until analysis time.
- 1.0 mM HCl was prepared, stored at room temperature until analysis time and was used for dilution of trypsin and chymotrypsin enzymes.

2.3.2. Preparation of enzymes solutions

- 80 U/ml of bovine chymotrypsin was prepared in 1.0 mM HCl and stored at -20°C. At moment of experiment the solution was diluted to 0.4 U/ml with 1.0 mM HCl and kept on ice while in use.
- 500 U/ml of bovine trypsin was prepared in 1.0 mM HCl and stored at -20°C.

2.3.3. Preparation of substrate solutions

- 1.1 mg BAPNA was dissolved in 2.5 ml of 1.0 % (v/v) of DMSO and 100.0 mM Tris-HCl, pH 8.0 and stored at -20°C until analysis time.
- 1.9 mg SAAPFPNA was dissolved in 1.0 ml of 100 % (v/v) of DMF and stored at -20°C until analysis time.

2.4. *T. molitor* larvae crude extract preparation

Approximately 0.2 g of *T. molitor* larvae were weighed, for each 0.1 g of larvae 300 µl of extraction buffer were added. After mechanical grinding and potter homogenization the suspension was centrifuged for 15 min at 16000xg, at 4°C to remove lipids and insoluble compounds. The supernatant represents the crude extract and contains water-soluble proteins. The extract was boiled at 100°C for 3 min to precipitate the endogenous enzymes. After centrifugation for 3 min the boiled crude extract was stored at -20°C until analysis time.

2.5. Determination of protein concentrations

Protein concentration was measured according to the Bradford method, using BSA as standard.

2.6. Determination of trypsin and Chymotrypsin activities

The activity analyses were carried out in the cuvette of the spectrophotometer. The activity assay is based on the hydrolysis of chromogenic substrates BAPNA and SAAPFPNA by trypsin and chymotrypsin, respectively. Trypsin and chymotrypsin split their substrates to yield yellow dye of paranitroaniline. The activity was measured by monitoring the change in the absorption of paranitroaniline at 410nm.

The trypsin activity assay mixture consisted of 10 U of bovine trypsin, 50 mM Tris-HCl, pH 8.0 and 0.3 mM BAPNA substrate in a final volume of 500 µl. After addition of the substrate, the reaction was monitored at the spectrophotometer at 410 nm, at 37°C for 10 minutes. The activity value was

expressed as Units/ml (U/ml). One Unit is defined as the amount of enzyme that hydrolyzes 1 μ mol of BAPNA per minute at 37°C.

The chymotrypsin activity assay mixture consisted of 8 mU of bovine chymotrypsin, 50 mM Tris-HCl, pH 8.0 and 0.1mM SAAPFPNA substrate in a final volume of 500 μ l. After addition of the substrate, the reaction was monitored at the spectrophometer at 410 nm, at 37°C for 10 minutes. The activity value was expressed as U/ml. One Unit is defined as the amount of enzyme that hydrolyzes 1 μ mol of SAAPFPNA per minute at 37°C.

The enzyme activity (U/ml) was calculated by equation (1).

$$U/ml = \frac{\Delta Abs / \text{min} * V_{mix}}{\epsilon * V_{enzyme}} \quad eq. (1)$$

2.7. Determination of Trypsin and Chymotrypsin inhibitor activities

Determination of the enzymatic inhibition against trypsin and chymotrypsin is based on measuring the decrease in enzymatic activity of the proteases caused by samples of *T. molitor* larvae..

The enzymatic assays to assess the trypsin inhibitor activity were carried out on crude extracts of larvae before and after boiling. The assay mixtures contained 10U of bovine trypsin, 50 mM Tris-HCl, pH 8.0, 0.3 mM BAPNA and different amount of larvae crude extracts in a final volume of 500 μ l. A control mixture without the extract was prepared in parallel. After the substrate was added the reaction was monitored at the spectrophometer at 410 nm, at 37°C for 10 minutes. The residual enzymatic activity in all mixtures was measured.

The enzymatic assays to assess the chymotrypsin inhibitor activity were carried out on crude extracts of larvae before and after boiling. The assay mixtures contained 8 mU of bovine chymotrypsin, 50 mM Tris-HCl, pH 8.0, 0.1 mM SAAPFPNA and different amounts of larvae crude extract in a final volume of 500 μ l. A control mixture without the crude extract was prepared in parallel. After the substrate was added the reaction was monitored at the spectrophotometer at 410 nm, at 37°C for 10 minutes. The residual enzymatic activity in all mixtures was measured.

The protease activity values measured in each assay mixture and expressed as nmoles of substrate consumed during the reaction time, were plotted against the amount (mg) of larvae in each mixture. The amount of larvae in each mixture was calculated from the amount of added crude extract. From the slope of the obtained curves, the amount of protease inhibited per mg of larvae was calculated by equation (3)

$$\begin{aligned} &ng \text{ chymotrypsin inhibited/mg larvae} \\ &= \frac{\text{slope} \times 1000}{40} \qquad \qquad \qquad eq. (3) \end{aligned}$$

The equation was based on the specific activity of chymotrypsin, that is 40 mU/ μ g.

3. Results and Discussion

In this study, 15 samples of edible *T. molitor* larvae reared on diet consisting of wheat flour (S₁), wheatmeal flour (S₂), and wheatmeal partially replaced with three different percentages of olive pomace (S₃, S₄, S₅), have been analyzed for the presence of inhibitors of trypsin and chymotrypsin.

The first step of analysis involves the homogenization of mealworms for the preparation of the extract. It consists of reducing the insects to a small particle size by mechanical grinding. Homogenization is carried out in Tris-HCl buffer, at pH 8.0, with NaCl and TCEP as anti-browning agent. This step allows for an increase in surface area between the insect particles and the extraction solvent which generally results in a more efficient extraction of the protein. Protein solubility in water is dependent on pH, the addition of NaCl increases water solubility of yellow mealworm proteins [4]. The supernatant containing water-soluble proteins is retained for subsequent steps.

A centrifugation step at 4°C of insect homogenates allows for lipid and insoluble compounds removal. Lipids form a layer at the top of the supernatant. The supernatants represent the mealworm extracts.

The protein concentration of the extracts was determined using the Bradford method, as described in section 2.5.

The trypsin inhibitor activity was analyzed in all extracts, as reported in section 2.7. No inhibitor activity was determined in any extract.

The chymotrypsin inhibitor activity was also analyzed in all samples as reported in section 2.7. Figure 1 shows the effect of increasing the amount of the S₁ extract on the activity of chymotrypsin. It is evident a progressive decrease of the protease activity, indicating the presence of the inhibitor.

From the slope of the curve an inhibitor activity value of 1.52 ng chymotrypsin inhibited/mg larva was calculated. The presence of the inhibitor was observed in all tested extracts (not shown).

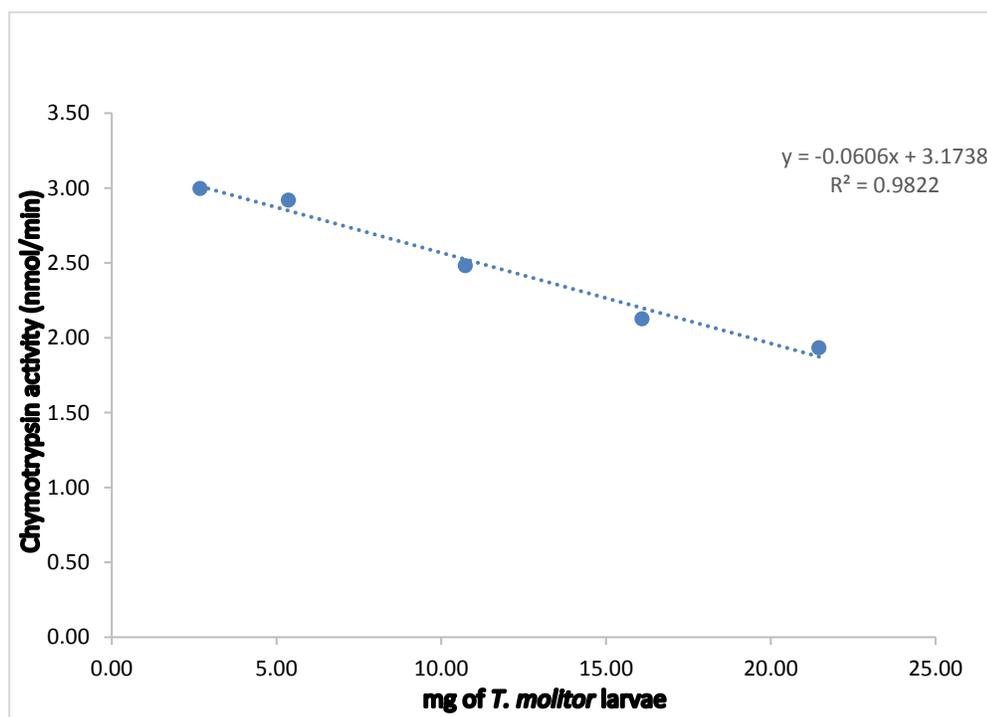


Fig. 1 – Effect of S₁ extract on chymotrypsin activity

Table 1. shows the inhibitor activity values determined in all samples.

Table 1 – ng of Chymotrypsin inhibited per mg of *T. molitor* larvae

Sample	R₁	R₂	R₃	Mean ± SD
S₁	1.52	1.27	0.89	1.23 ± 0.32
S₂	1.83	2.34	2.78	2.32 ± 0.48
S₃	1.26	2.17	1.63	1.67 ± 0.43
S₄	2.36	2.79	1.60	2.4 ± 0.60
S₅	2.70	1.61	2.12	2.14 ± 0.55

There is no large variation in the contents of chymotrypsin inhibitors in larvae reared on the different substrates, as also shown in Figure 2. The observed small variations are likely due to the method of determination.

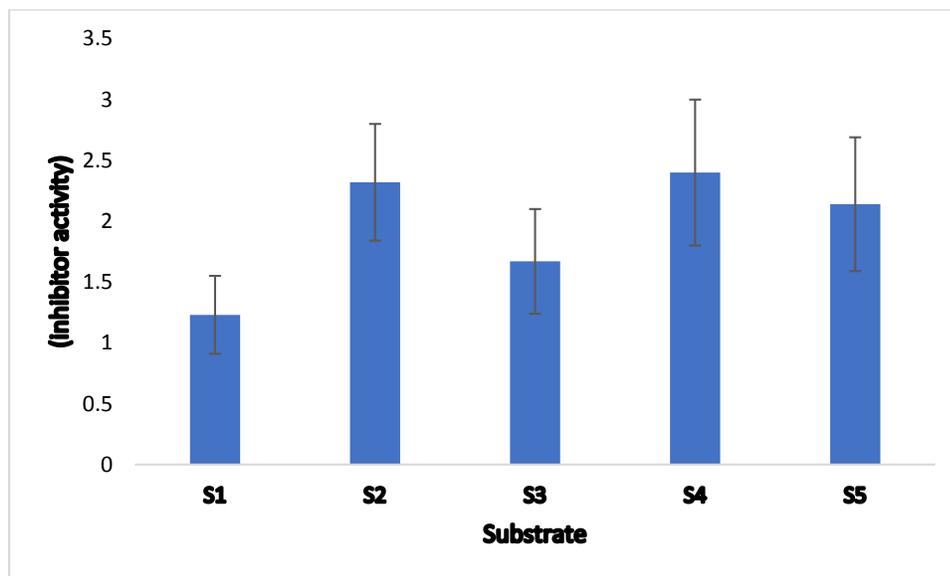


Fig. 2 – Inhibitor activity (ng chymotrypsin inhibited/mg larva) in *T. molitor* grown in different substrates

Even though the results of this work indicate the presence of chymotrypsin inhibitors in *T. molitor*, the contents of such inhibitors per mg of *T. molitor* larvae is significantly lower when compared to chymotrypsin inhibitor in legumes, as shown in Table 2.

Table 2 – Chymotrypsin inhibitor in legumes ^[42] and *T. molitor* larvae

g of chymotrypsin inhibited / Kg	
Soybeans	12 ± 1.8
Kidney beans	9.1 ± 0.9
Cowbeans	9.2 ± 1.1
Lupin seeds	1.4 ± 0.8
<i>T. molitor</i> larvae	$1.23 \times 10^{-3} \pm 0.32 \times 10^{-3}$

When assessing the presence of the inhibitor in the boiled extracts, we found that the inhibitor was still present. Figures 3 and 4 show the changes in chymotrypsin activity in the presence of extracts before and after boiling. Extracts were prepared from larvae reared on S₂ substrate.

There is no significant variation in the inhibition which indicates that the mealworm inhibitor is heat resistant. On the other hand, legumes chymotrypsin inhibitors are denatured with heat.

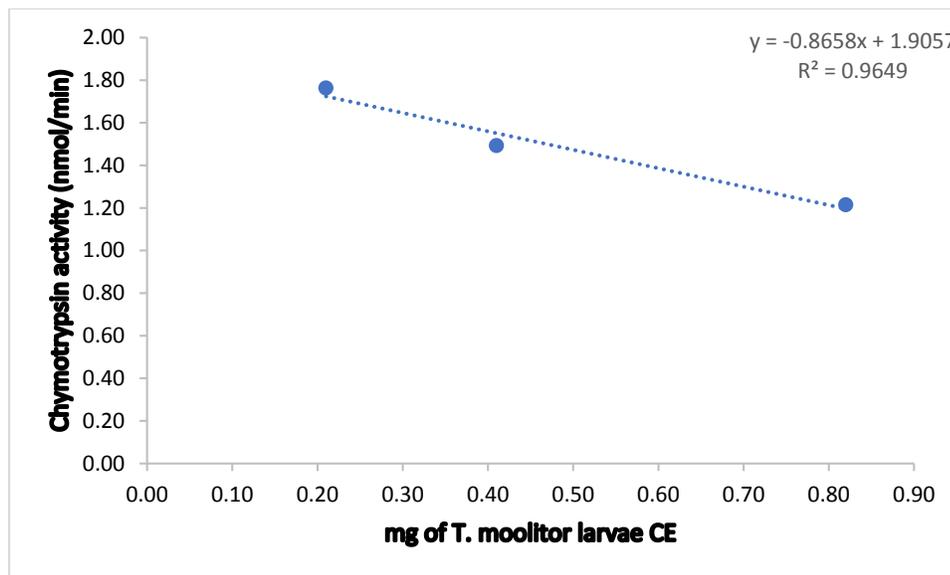


Fig. 3 – Effect of S₂ extract on chymotrypsin activity

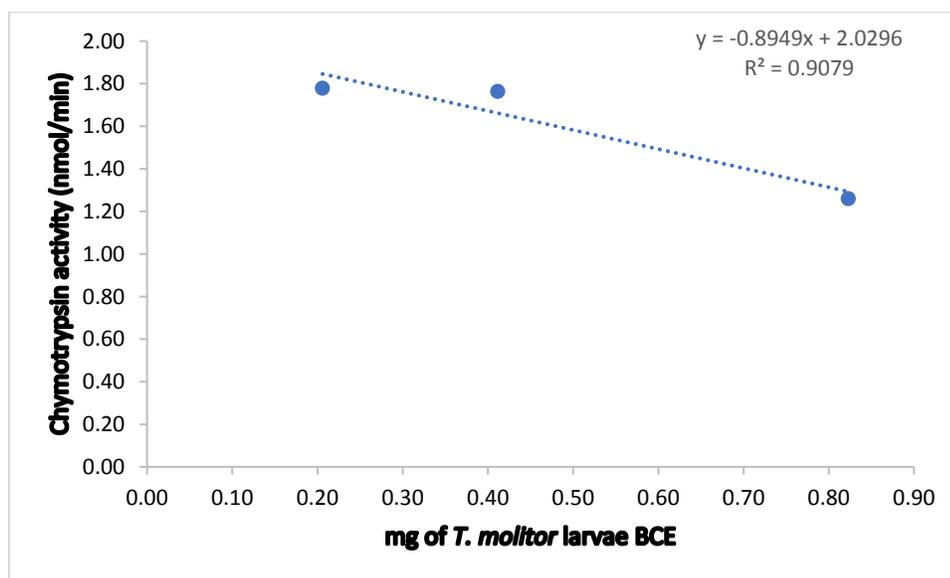


Fig. 4 – Effect of S₂ boiled extract on chymotrypsin activity

4. Conclusion

Edible insects are highly nutritious. However, for an extensive and safe utilization of insects as food and food ingredients, topics such as allergenic and toxic risks as well as presence of anti-nutrients need to be addressed. It is mandatory to know the chemical composition of insects to select the most appropriate species to be reared as food and feed. In addition, research is required on the impacts of rearing and feed substrates on the nutrient composition of insects.

The nutritional value of protein is limited by the presence of anti-nutritional factors; therefore their determination is important for assessing the overall nutritional quality of *T. molitor* larvae. In this study we found the absence of trypsin inhibitor and a very low content of chymotrypsin inhibitor, which does not affect the protein quality of larvae. In fact, the amount of inhibitor is of about one order of magnitude lower than that present in legumes. Thus mealworm larvae can be safely used in the production of nutritional food for human consumption, as well as in animal feed production.

References

1. B. A. Rumpold and O. K. Schluter, Nutritional composition and safety aspects of edible insects, *Molecular Nutrition and Food Research*, 2013 (57) 802-823.
2. <http://www.ent.wur.nl/UK/Edible+insects/Worldwide+species+list/>
3. S. G. F. Bukkens, The nutritional value of edible insects, *Ecology of Food and Nutrition*, 1997 (36) 2-4, 287-319.
4. A. B. Nongonierma, R. J. FitzGerald, Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review, *Innovative Food Science and Emerging Technologies*, 2017.
5. R. C. Megido^a, C. Poelaert^b, M. Ernensa, M. Liotta^a, C. Blecker^c, S. Danthinec, E. Tytecad, É. Haubruge^a, T. Alabia^e, J. Bindelle^b and F. Francisa, Effect of household cooking techniques on the microbiological load and the nutritional quality of mealworms (*Tenebrio molitor* L. 1758), *Food Research International*, 2018 (106) 503-508.
6. T. Bernard and H. M. Womeni, *Entomophagy: Insects as Food*, 2017.
7. L. Kouřimská and A. Adamkova, Nutritional and sensory quality of edible insects, *NFS Journal*, 2016 (4) 22-26.
8. A. Ganguly, R. Chakravorty, M. Das, M. Gupta, D. K. Mandal, P. Haldar, J. Ramos-Elorduy and J. M. P. Moreno, A preliminary study on the estimation of nutrients and anti-nutrients in *Oedaleus abruptus* (Thunberg) (Orthoptera: Acrididae), *International Journal of Nutrition and Metabolism*, 2013 (3) 5, 50-65.

9. V. Varelas and M. Langton, Forest biomass waste as a potential innovative source for rearing edible insects for food and feed: A review, *Innovative Food Science and Emerging Technologies*, 2017 (41) 193-205.
10. Y. Liya, C. M. M. Lakemond, L. M. C. Sagis, E. Verena, A. Huis and B. A. J. S. Martinus, Extraction and characterisation of protein fractions from five insect species, *Food Chemistry*, 2013 (141) 3341-3348.
11. A. Huis, Potential of Insects as Food and Feed in Assuring Food Security, *Annual Review of Entomology*, 2013 (58) 563-583.
12. R. L. Panini, L. E. L. Freitas, A. M. Guimaraes, C. Rios, M. F. O. Silva, F. N. Vieira, D. M. Fracalossi, R. I. Samuels, E. S. Prudencio, C. P. Silva and R. Amboni, Potential use of mealworms as an alternative protein source for Pacific white shrimp: Digestibility and performance, *Aquaculture*, 2017 (473) 115-120.
13. S. Belluco, A. Halloran and A. Ricci, New protein sources and food legislation: the case of edible insects and EU law, *Food Security*, 2017 (9) 803-814.
14. C. L. R. Payne, P. Scarborough, M. Rayner and K. Nonaka, A systematic review of nutrient composition data available for twelve commercially available edible insects, and comparison with reference values, *Trends in Food Science and Technology*, 2016 (47) 69-77.
15. A. Huis, Edible insects are the future, Conference on 'The future of animal products in the human diet: health and environmental concerns', *Proceedings of the Nutrition Society*, 2015, 1-12.
16. P. Raksakantong, N. Meeso, J. Kubola and S. Siriamornpun, Fatty acids and proximate composition of eight Thai edible terricolous insects, *Food Research International*, 2010 (43) 350-355.

17. A. Thevenot, J. L. Rivera, A. Wilfart, F. Maillard, M. Hassouna, T. Senga-Kiesse, S. L. Feon and J. Aubin, Mealworm meal for animal feed: Environmental assessment and sensitivity analysis to guide future prospects, *Journal of Cleaner Production*, 2018 (170) 1260- 1267.
18. R. Kohlera, L. Kariukia, C. Lamberta and H. K. Biesalski, Protein, amino acid and mineral composition of some edible insects from Thailand, *Journal of Asia-Pacific Entomology*, 2019 (22) 372-378.
19. T. Shantibala, R. K. Lokeshwari and H. Debaraj, Nutritional and anti-nutritional composition of the five species of aquatic edible insects consumed in Manipur, India, *Journal of Insect Science*, 2014 (14) 14.
20. A. V. Alves, E. J. Sanjinez-Argandona, A. M. Linzmeier, C. A. L. Cardoso and M. L. R. Macedo, Food Value of Mealworm Grown on *Acrocomia aculeata* Pulp Flour, *PLoS ONE*, 2016 (11) 3.
21. X. Zhao, J. V. Luis, D. P. Johansson, R. Landberg and M. Langton, Yellow Mealworm Protein for Food Purposes - Extraction and Functional Properties, *PLoS ONE*, 2016 (11) 2.
22. Mariod, *Unconventional Oilseeds and Oil Sources*, 2017 (50) 331-336.
23. H. F. Gemedé and N. Ratta, Antinutritional factors in plant foods: Potential health benefits and adverse effects, *International Journal of Nutrition and Food Sciences* (2014) 3, 4, 284-289
24. K. O. Soetan and O. E. Oyewole, The need for adequate processing to reduce the anti- nutritional factors in plants used as human foods and animal feeds: A review, *African Journal of Food Science*, 2009 (3) 9, 223-232.
25. S. I. SHIM, W. J. JUN and B. H. KANG, Evaluation of nutritional and anti-nutritional components in Korean wild legumes, *Plant Foods for Human Nutrition*, 2003 (58) 1-11.

26. P. Bora, Anti-Nutritional factors in foods and their effects, *Journal of Academia and Industrial Research*, 2014 (3) 6, 285-290.
27. K. O. Soetan, A. S. Akinrinde, S. B. Adisa, Comparative studies on the proximate composition, mineral and anti-nutritional factors in the seeds and leaves of African locust bean, *Annals Food Science and Technology*, 2014 (15) 1, 70-74.
28. G. S. Gilani, C. W. Xiao and K. A. Cockel, Impact of anti-nutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality, *British Journal of Nutrition*, 2012 (108) 315-332.
29. A. Sharma and A. C. Kapoor, Levels of anti-nutritional factors in pearl millet as affected by processing treatments and various types of fermentation, *Plant Foods for Human Nutrition*, 1996 (49) 241-252.
30. A. R. Ejoh, V. N. Djuikwo, I. Gouado and C. M. Mbofung , Effect of different postharvest treatments on anti-nutritional factors in some commonly consumed leafy vegetables in Cameroon, *Journal of Food Processing and Preservation*, 2009 (33) 161-174.
31. H. D. Belitz and J. K. P. Weder, Protein inhibitors of hydrolases in plant foodstuffs, *Food Reviews International*, 1990 (6) 2, 151-211.
32. C. A. Ryan, Protease inhibitors in plants: Genes for improving defenses against insects and pathogens, *Annual Reviews Phytopathology*, 1990 (28) 425-449.
33. R. Dvorak, A. Pechova, L. Pavlata, J. Filipek, J. Dostalova, Z. Reblova, B. Klejdus, K. Kovarcik and J. Poul, Reduction in the content of anti-nutritional substances in pea seeds (*Pisum sativum* L.) by different treatments, *Czech Journal of Animal Science*, 2005 (50) 11, 519-527.

34. B. Wolfram and H. Robert, Natural protein proteinase inhibitors and their interaction with proteinases, *European Journal of Biochemistry*, 1992 (204) 433-451.
35. C. Hawtin and C. Webb, Faba Bean Improvement, Faba Bean Conference, 1981, 333-334.
36. J. Huisman, Anti-nutritional effects of legume seeds in piglets, rats and chickens, 1990 (1) 10-15.
37. L. L. Murdock and R. E. Shade, Lectins and protease inhibitors as plant defenses against insects, *Journal of Agricultural and Food Chemistry*, 2002 (50) 6605-6611.
38. E. A. Ekop, A. I. Udoh and P. E. Akpan, Proximate and anti-nutrient composition of four edible insects in Akwa Ibom State, Nigeria, *World Journal of Applied Science and Technology*, 2010 (2) 2, 224-231.
39. I. Ifie and C. H. Emeruwa, Nutritional and anti-nutritional characteristics of the larva of *Oryctes monoceros*, *Agriculture and Biology Journal of North America*, 2011 (2) 1, 42-46.
40. O. T. Alamu, A. O. Amao, C. I. Nwokedi, O. A. Oke and I. O. Lawa, Diversity and nutritional status of edible insects in Nigeria: A review, *International Journal of Biodiversity and Conservation*, 2013 (5) 4, 215-222.
41. M. Das and S. K. Mandal, Assessment of nutritional quality and anti-nutritional composition of two edible grasshoppers (Orthoptera: Acrididae) - A search for new food alternative, *International Journal of Medicine and Pharmaceutical Sciences*, 2013 (3) 5, 31-48.
42. F. M. Lajolo and M. I. Genovese, Nutritional significance of lectins and enzyme inhibitors from legumes, *Journal of Agricultural and Food Chemistry*, 2002 (50) 6592-6598.