Journal of Cereal Research
Volume 15 (1): 1-13
Homepage: http://epubs.icar.org.in/ejournal/index.php/JWR
Reveiw Article

Exploration of defensive protein Amylase Trypsin Inhibitors from wheat: A novel approach for crop protection

Sapna Chaudhary and Rajarshi Kumar Gaur*

Department of Biotechnology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur-273009, Uttar Pradesh, India

Article history:
Received: 14 Apr., 2023
Revised: 07 May, 2023
Accepted: 07 May, 2023

Citation:
Chaudhary S, RK Gaur. 2023. Exploration of defensive protein Amylase Trypsin Inhibitors from wheat: A novel approach fro crop protection. *Journal of Cereal Research* ***15 (1):*** *1-13. http://doi.org/10.25174/2582-2675/2023/130726*

****Corresponding author:***
E-mail: gaurrajarshi@hotmail.com

Abstract

Insect pests are highly dependent on starch-rich cereals and cause severe damage to the cereal grain and nutrient yield. Amylase trypsin inhibitor protein degrades the digestive enzyme alpha-amylase which plays a key role in carbohydrate metabolism as well as the growth and development of insects. These inhibitor proteins are mainly found in cereal crops like wheat, maize, and barley which are rich sources of starch. Due to the defensive mechanism against pests, Amylase trypsin inhibitor protein could be a prominent candidate for pest management in cereal crops. It could be used in marker-assisted plant breeding and genome mapping. Amylase trypsin inhibitor proteins prevent various diseases such as diabetes but also cause wheat allergy, baker's asthma, and food allergies. In this review, we summarize the identification, characterization, purification, inhibitory mechanism, and various analyses of amylase trypsin inhibitor protein to control pests from cereal crops as a natural defense and reduce human allergies.

Keywords: Wheat, insect pests, defense mechanism, protease inhibitor, amylase trypsin inhibitor protein.

1. Introduction

Grains are very important in the human diet. For the majority of the world's population, grains are the most important source of calories. The three predominating sources of social crops in the world are rice, wheat, and maize (Awika, 2011). Rice, wheat and corn are the three most important crop sources in the world According to FAO 2021 statistics, at 770.4 million tonnes, India ranked second as the world's biggest wheat-producing country, accounting for about 41% of global wheat production (Jakhar *et al*., 2020). About 30.4% of the total diet comes from grains and grain products (Laskowski *et al*., 2019). Wheat (Triticum aestivum L) is the individual ultimate economically important refined crop. It is an important beginning of strength, nutrients, protein, vitamins, and phytochemicals (Bao and Malunga, 2022; Mc Kevith, 2004). Regular consumption of grains, especially whole grains, may prevent chronic sicknesses, including diabetes, coronary heart ailment, and colorectal cancers (Mc Kevith, 2004).

Wheat is one of the most popular grains in the world. In India, wheat is the dominant crop mainly cultivated in the states of Bihar, Haryana, Madhya Pradesh, Punjab, Rajasthan, and Uttar Pradesh. These states are located in the northern plains, a rich wheat-growing region called the "Wheat Bowl of India" (Kulshrestha, 1885). The main cultivated wheat varieties are durum wheat (T. durum) and common wheat (T. aestivum) out of the thousands of wheat varieties. Wheat is a majorly cultivated crop because it has properly yielded capability, grows properly in temperate climatic circumstances, matures in a short period, and conveys all-purpose flour. The supply and demand for grains have increased as the world's population grows rapidly. In both developed and developing countries, insect pests reduce cereal production rates (Dhaliwal *et*

al., 2015; Jakhar *et al*., 2020). In such a situation, reduced crop productivity due to insect pest infestation may lead to food insecurity in the future (Husain *et al*., 2021). Many insect pests such as Tenebrio molitor, Armyworm, Wheat aphid, pink weevil, White borer, Grasshopper, Ghujia weevil, Cereal leaf beetle, and termites are associated with the wheat crop those attacks and cause damage to grain quality, leading to significant yield losses (Farook *et al*., 2018; Kauppi *et al*., 2021). Different wheat cultivars exhibit a differential response to insect pests (Kumar *et al*., 2022). Insects have a digestive enzyme known as alpha-amylase which catalyzes the hydrolysis of starch, and glycogen into the simpler sugar maltose and digests food easily (Da Lage, 2007, 2018). It plays a major role in the survival of insects and animals that are present in their midgut (Franco *et al*., 2002). The main economic loss caused by insects attacking grain is not always the actual material they consume, but also their excrement such as uric acid which is a carrier of pathogenic bacteria, which contaminates the residue with undesirable odors and flavors, rendering the food unfit for human consumption (Deshwal *et al*., 2020; Mandali, 2020). It can also affect the nutritional value of grains by reducing vitamins and carbohydrates and increasing free fatty acids (Mc Kevith *et al*., 2004). Integrated pest management includes various cultural practices, biological control, and chemical control methods to protect cereal crops from harmful insect pests (Singh *et al*., 2020). However, the use of pesticides has its drawbacks, as their excessive use is highly harmful to both human health and the environment (Hossard *et al*., 2014). Genetic resistance to plant insects is another desired goal of most plant breeders. Transgenic crops can be developed that are herbicide-tolerant, reducing the need for herbicides (Mc Kevith *et al*., 2004). However, acquiring this trait is a major challenge due to the interaction between genetic and environmental factors. Therefore, another natural strategy for detecting the tolerant genotype should be explored along with this. In plants, various protease inhibitors are produced but among these, Alpha-amylase inhibitors majorly inhibit the activity of the digestive proteases alpha-amylase and proteinase within the insect gut, thus affecting the insect's digestive system. These inhibitors are mainly found in edible parts of plants such as potato tubers, legume seeds, and most cereal grains such as wheat, millet, and maize (Franco *et al*., 2002). Overexpression of these inhibitory proteins in plant genetic engineering makes significant use of the plant defense against insect pests. In transgenic crops, these proteinase inhibitor genes are used to enhance insect resistance mechanisms (Gatehouse *et al*., 2011). It could be useful in the treatment of diabetes mellitus to control hyperglycemia, reducing the risk of cardiovascular disease and colon cancer (Agarwal, 2016). It additionally has been reported that has a major role in wheat allergic reactions, baker's asthma, and meal hypersensitivity (Oda *et al*.,1997; Geisslitz *et al*.,2022). This review article describes currently emerging sustainable pest management methods that should be tailored to minimize pest infestation and wheat yield loss. Consequently, the main aim of this review is to apprehend the identification, characterization, purification, and study of various biochemical and molecular analyzes of the amylase trypsin inhibitor proteins. We also discussed the potential of amylase trypsin inhibitor proteins, i.e., how induced responses relate to plant resistance or susceptibility to pathogen and insect attack, and the way to use these facts to resist insect growth and enhance crop production.

2. Wheat: Source of protein

The wheat kernel contains 8-10% of the protein, differentiated into Gluten and Non-Gluten proteins (Biesiekierski, 2017). Osborne (1907) first developed a comprehensive wheat protein fractionation scheme based on protein functionality and different solubility in different solvents. Wheat is categorized on the basis of cytogenetic research and growth habits. On the basis of their growth habits, it is divided into two categories: spring wheat, and winter wheat/facultative which account for 65% and 35%, respectively, of the total global wheat production area (Oyewole, 2016). The basic chromosomal number of Triticum and related species is x=7. In terms of chromosomal number, wheat is classified (Tadesse *et al*.,2016) as given in (Table 1). They mainly fall into different classes such as albumin, globulin, gliadin, and glutenin. These are water-soluble, saline-soluble, ethanol-soluble, and other dilute and alkali-soluble, respectively (Figure 1). Gluten-containing gliadins and glutenin are Prolamins-type proteins recognized as the major wheat storage proteins, together makeup about 80% of the remaining protein in grain (Biesiekierski, 2017; Uthayakumaran and Wrigley, 2017). Whereas, Non-Gluten proteins are metabolic and structural proteins

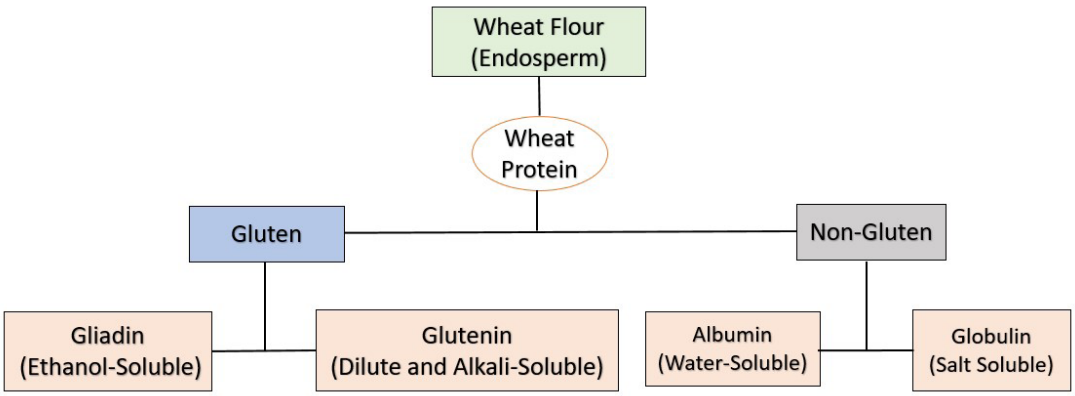

Figure 1: Classification of Wheat Protein

Table 1: Different polyploid wheat varieties are classified based on the number of chromosomes in each cell.

Types of Polyploidies	No. of sets of chromosomes	Wheat varieties
Diploid (AA)	2n=2x=14 (2 sets of 7 chromosomes in each cell)	*Einkorn* (T. *monococcum*)
Tetraploid (AABB)	2n=4x=28 (4 sets of 7 chromosomes in each cell)	Emmer (*T. dicoccum*), *Durum* wheat (*T. durum).*
Hexaploid (AABBDD)	2n=6x=42 (6 sets of 7 chromosomes in each cell)	Spelt Wheat (*T. Spelta*), Common or Bread Wheat (*T. aestivum*).

named albumins and globulin in wheat endosperm make up 20% of all cereal proteins, of which the amylase trypsin inhibitor protein is the maximum considerable protein within the water-soluble (albumin) fraction of wheat grain that functions as a plant defense protein (Cousineau, 2012; Zilic *et al*., 2011) (Figure 2). They are also classified based on the polypeptide chain as monomeric or polymeric proteins. The monomeric proteins are Albumin, Globulin, and Gliadin whereas polymeric proteins are Glutenin.

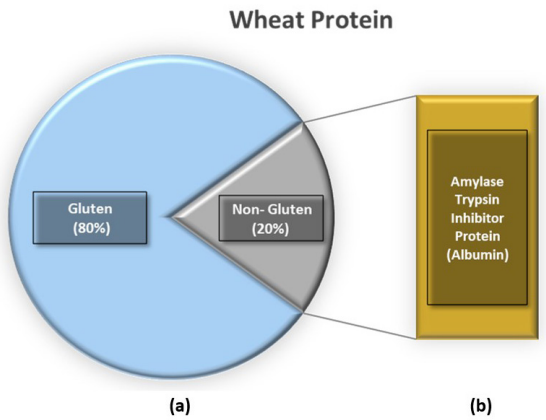

Figure 2: Distribution of Wheat Protein (a) Gluten (80%) and Non-Gluten (20%) (b) Albumin fraction containing defensive protein Amylase Trypsin Inhibitors.

3. Protease Inhibitor: Plant Defense

Plants have defense mechanisms such as morphological, biochemical, and molecular mechanisms against herbivores and insect pests (War *et al*., 2012). Several compounds used in plant defense include alkaloids, antibiotics, terpenes, and some proteins such as chitinase, lectins, vicilins, systemins, and enzyme inhibitors (Franco *et al*., 2002). Plant protease inhibitors are proteins produced by plants that inhibit the activity of the digestive proteases of herbivores, phytopathogens, larval proteolytic enzymes, and microbial pathogen proteases (Rodríguez-Sifuentes *et al*., 2020). These inhibitory proteins are induced by insect attacks or plant damage, and target to attach at a specific active position of the substrate. Plant proteinase inhibitor protein is mainly found in food storage parts of the plants such as potato tubers, seeds of legumes, and most cereal grains including rice, wheat, maize, millet, and barley (Birk, 1976, Buonocore *et al*., 1977; Call *et al*., 2021; Geisslitz *et al*., 2022; Granum, 1979; Limas *et al*., 2004; Shivaraj, 1981). Besides this, it is also present in leguminous seeds such as kidney beans, peanuts, black gram, and chickpeas (Le Berre-Anton *et al*., 1997; Birk, 1996).

Protease inhibitors are generally classified based on the sequence specificity of the reactive site, structural function, and biochemical properties (Clemente *et al*., 2019; Priya *et al*., 2013). Families of Plant proteases include Serpin (Serine PI), Cysteine PIs, and Aspartyl and carboxypeptidase inhibitor family. Serin proteases inhibitors are further classified into the Bowman-Birk inhibitors (BBIs) family, Cereal trypsin/alpha-amylase inhibitors. Kunitz family, Mustard (Sinapis) trypsin inhibitors (MSI), Potato type I PIs (PI 1), Potato type II PIs (PI 2), and Squash inhibitors. Cysteine PIs family includes (CYS) cystatin superfamily. Aspartyl and Metallo-carboxypeptidase inhibitor family are also strong protease inhibitors of aspartyl and carboxypeptidase (Christeller and Laing, 2005; Gitlin-Domagalska *et al*., 2020; Habib and Fazili, 2007; Jamal *et al*., 2012;). Most of the plant protease inhibitor families contain inhibitors of serine protease and some also contain cysteine protease that targets trypsin and chymotrypsin/ subtilisin.

Amylase Trypsin Inhibitor Protein: Structure and Function

Cereal alpha-amylase trypsin inhibitor proteins having 16 families are present in cereals like ragi, barley, wheat, and maize which mainly target insect protease, Alpha-amylase, and Trypsin. Alpha-amylase (α-1,4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) is a crucial digestive enzyme that performs a key role in the maturation and development of insects and animals (Franco *et al*., 2002). It catalyzes the hydrolysis of α-d-(1→4)-glucan linkages in starch, glycogen, and different carbohydrates. The growth and development of insects are rather dependent on starch. The maximum activity is obtained at 40-50°C and the range of pH- optimum is 4-10 for different alpha-amylases of insects (Kaur *et al*., 2014; Talley *et al*., 2010). Different insect alpha-amylases have efficient composition, physicochemical properties, and specificity toward various substrates.

Amylase trypsin inhibitor proteins are usually directed against animal-derived alpha-amylase, which includes a wide range of insects and microorganisms, but rarely against plant-derived amylase. (Buonocore *et al*., 1977; Gatehouse *et al*., 1986; Mundy *et al*., 1983; Wilson *et al*., 1997). Most of these suppressor proteins in cereals are individuals of a large superfamily known as the storage proteins prolamins, whose members are believed to be derived from the same 20 amino acid ancestor (Kreis, 1985).

Kneen and Sandstedt (1946) first studied the water-soluble amylase inhibitor protein in a wheat kernel that was not soluble in ammonium sulfate and 90% ethanol. Cantagalli *et al*. (1971) isolated three albumin proteins according to their electrophoretic mobility of 0.28,0.34, and 0.39 from wheat seeds. Their amino acid composition is very similar. The purified albumins have a specific and common function that shows similar chemical and physical characteristics. Carrano *et al*. (1989) isolated a new water-soluble monomeric amylase inhibitor protein coded as 0.14 which is inactive against animal amylases from wheat flour by a chromatographic procedure. Low molecular weight, excessive resistance to cleavage and denaturation, and conserved properties of intrachain disulfide bonds are features of these inhibitor proteins (Geisslitz *et al*., 2021). ATI inhibits the action of a protease enzyme in insects, alpha-amylase is concerned with starch breakdown, and trypsin is implicated in proteolysis (Geisslitz, 2022). Due to trypsin inhibitors, dietary protein may be less easily digested and may be expelled in the stool (Poerio *et al*., 1989). Alpha-amylase trypsin inhibitors are considered to be a part of the protective chemical compounds of plants against pathogens and pests (Silano *et al*., 1975). Alpha amylases of insects have conserved domain structure, with the variable in length, disulfide bond numbers, and their secondary structure. Therefore, Alpha-amylase inhibitor proteins have a highly specific reactive site that binds specifically to the substrate's active site. The loop variations adjacent to the enzyme active site determine the inhibitor's specificity for alpha-amylases. Their conformation may have an impact on specificity and binding affinities (Feng *et al*., 1996; Rane *et al*., 2020).

4. Mechanism of action of Amylase Trypsin inhibitors over proteases of plants pathogen

Amylase Trypsin inhibitors (ATIs) are a class of plant proteins that play an essential function in a plant's natural defense mechanism against pests and phytopathogens. Buonocore *et al*. (1985) characterized a tetrameric inhibitor that is active on mammalian and avian alpha-amylase from wheat kernel. Alpha-amylase inhibitor proteins are the most prominent candidate for the management of insect pests as they may be particularly dependent on starchy food for their survival and growth (Franco *et al*.,

2002). Plants defend themselves without delay with the aid of constitutively expressing amylase and protease inhibitors and by inducing these inhibitors in response to mechanical injury or pest attack (Stevens *et al*., 2012). Priya *et al.* (2012) evaluated the specificity of the ATIs and identified six wheat genotypes that have an extensively high potential of a-amylase trypsin inhibitors towards insects but low inhibition capability toward pancreatic and salivary amylases.

Plant pathogens feed on plants and can easily degrade plant protein to obtain nutrients for their growth and survival. Plant Protease inhibitors such as ATIs tend to inhibit the mechanism of pathogen digestive enzymes and serve as a plant defense mechanism leading to malnutrition, delayed larval development, and even lethality causing death and opening up new methods of pest control (Kaur *et al*., 2014; Rodríguez-Sifuentes *et al*., 2020; War *et al*., 2012, Zhu-Salzman *et al*., 2014) described in (Figure 3). Wisessing *et al.* (2010) found that a monomer having a molecular weight of 27 k Da, an alpha-amylase inhibitor isolated from Mung bean seeds inhibits an insect Callosobruchus maculate. Gonzalez-Ruiz *et al.* (2019) observed inverse relations between R. dominica and alpha-amylase inhibitors of wheat. El-Latif *et al.* (2020) purified alpha-amylase inhibitors from durum wheat that were highly effective against the development of Tribolium castaneum and Callosobruchus maculatus both in vivo and in vitro. Recently, Capocchi *et al.* (2021) reported for the first time a new monomeric, an inhibitory alpha-amylase protein isolated from tetraploid emmer wheat acts more strongly against coleopteran insect pests.

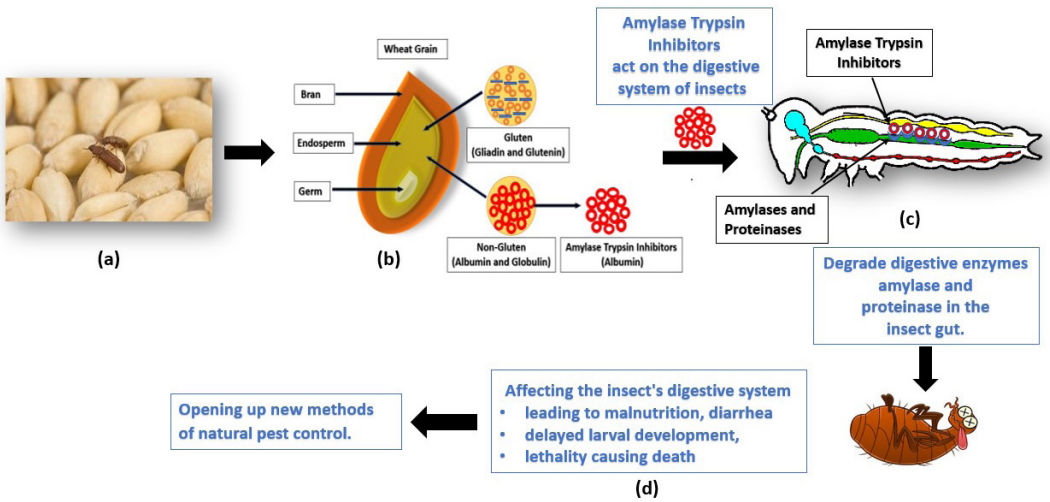

Figure 3: Mechanism of action of Amylase Trypsin inhibitors over proteases of plants pathogen: (a) Insects pest feed on cereal grains and cause damage. (b) Wheat grains have different types of protein as described in the text. (c) Amylase Trypsin Inhibitor Protein acts on the digestive system of insects and inhibits the function of their digestive enzyme. (d) By affecting the digestive system of insects, it serves as a plant defensive mechanism and opens a novel pest control method.

5. Molecular characterization of Amylase Trypsin Inhibitor Protein

ATI amylase trypsin inhibitors contain similar proteins called isomers that are distinguished according to their electrophoretic motility. Based on the molecular level, they are classified as 12 k Da, 24 k Da and 24 k Da. The 12 k Da subfamily contains 0.28 monomeric proteins, 24 k Da are homo dimeric proteins referred to as 0.19 and 0.53 which are more common in hexaploid wheat, whereas, 60 k Da are hetero tetrameric proteins known as chloroform and methanol mixture (CM) proteins such as CM1, CM2, CM3, CM16, and CM17 (Buonocore *et al*., 1985; Gomez 1989). Isoforms 0.19, CM1, and CM17 are mainly present in hexaploid wheat, while other isoforms CM2, CM3, and CM16 are abundantly present in tetraploid wheat (Geisslitz *et al*., 2022; Møller and Svensson, 2022). Monomeric and dimeric ATIs were placed on chromosomes 6 and 3 respectively. CM1, CM3, and CM16; CM2, and a trypsin inhibitor are placed on chromosomes 4 and 7 respectively. Genes are located on chromosomes 3, 4, and 7 for the monomeric, dimeric, and tetrameric subfamilies of ATI, respectively (Bose *et al*., 2020) (Table 2).

Table 2: Genes of different ATIs located in the respective site of a chromosome

ATI	Chromosomes
0.28	6 B
0.19	1B and 3B
CM1	1A, 1B, and 4B
CM 2	1B, 7B, and 1D
CM 3	1A, 5A, 4B and 6D
CM 16	5A, 6B, and 7B,
CM 17	1B, 4B and 7D

Petrucci *et al*., (1976) characterized a 0.19 wheat grain amylase inhibitor through circular dichroism spectra with approximately 50% ordered structure. Maeda *et al.* (1983) isolated a 0.53 alpha-amylase inhibitor from the wheat kernel that consists of two equal subunits of 124 amino acids, each with 9 cysteine residues. Human salivary alpha-amylase is inhibited by inhibitor 0.53, which is 500 times more potent than human pancreatic alpha-amylase. According to Gomez *et al.* (1991), a homologous variant of 0.28, WMAI-1 and WMAI-2 are encoded by a gene on the short arm of chromosomes 6D and 6B, respectively. Multiple gene families on chromosomes 3BS and 3DS encode a dimer inhibitor of wheat alpha-amylase identified at the 24 k Da molecular level (Sanchez-monge *et al*., 1989; Wang *et al*., 2006). Sharma *et al.* (2013) investigated the molecular diversity of alpha-amylase dimeric inhibitors (WDAI) isolated from three genotypes of Indian bread wheat. Pandey *et al.* (2016) used the CTAB procedure defined by Murray and Thompson (1980), for the isolation of genomic DNA from younger leaves of approximately two-week-old seedlings and amplified PCR by using particular primers PSF and PSR of the inhibitor gene of alpha-amylase (Sharma *et al*., 2013). Pandey *et al.* (2016) investigated the molecular evolution and sequencing of amylase inhibitor genes in wheat and relatively wild species that showed high sequence conservation, similar structures, and homology to other inhibitors using BLAST and multiple sequence alignments. Hassouni *et al.* (2021) studied 30 QTL and identified more than 10% of the ATI genotypic variation. Recently, Simonetti *et al.* (2022), analyzed the sequences of four ATIs genes in ten genotypes of modern and ancient wheat with distinct ploidy stages.

6. Purification and Proteome Analysis of Amylase Trypsin Inhibitor Protein

Proteomic strategies have been applied for the identification of wheat inhibitor proteins or allergen proteins. According to the method of Laemmli (1970), protein separation was performed by conventional SDS-PAGE. Gomez *et al*., (1991) purified and characterized 12.5kD wheat monomeric amylase inhibitors (WMAI) from T. aestivum using salt extraction, gel filtration, and RP-HPLC. Heidari and Heidarizadeh (2005) purified amylase inhibitor from wheat by an anion exchange fast protein liquid chromatography (FPLC). Two-dimensional electrophoresis and IgE immunoblot analysis were used to identify wheat allergens (Akagawa *et al*., 2007). Hailegiorgis *et al.* (2020) analyzed protein fraction by using discontinuous SDS-PAGE and estimated the total protein content of the durum wheat varieties through the micro-Kjeldahl method. Various types of chromatographic techniques and detection methods are used for the purification and detection of proteins and peptides including MALDI-TOF Mass Spectrometry, HPLC and DNS (Call *et al*., 2021; Dupont *et al*., 2011, He Li, 2021; Sagu *et al*., 2020). Many studies have been performed for the qualitative and quantitative characterization of alpha-amylase trypsin inhibitors using HPLC fractionation and Mass Spectrometry tools. Oda and Fukuyama (1997) used X-ray crystallography to determine the tertiary and quaternary shape of 0.19 alpha AIs from wheat kernels and reported that 0.19 AI has an excessive alpha helix content material. Each subunit has 5 alpha-helices organized in an up-and-down pattern, maintaining the helix packing modes. Alpha-amylase inhibitor, which is recognized by Ig E on 2-Dimensional Electrophoresis, was one of 19 possible wheat allergens identified using three methods, including MALDI-TOF and QTOF LCQ(DECA) nLC-MS/MS IT methodology (Sotkovsky *et al*.,2008). By using 2- Dimensional Electrophoresis and tandem MALDI- TOF/TOF-MS, a low level of accumulation of 11 alpha-amylase inhibitor proteins (spots 94-102, 119, and 120) was identified during the early growth of grains (Guo *et al*.,2012). By another LTQ-Orbi Trap analysis on peptides, the identification of the CM3 protein was verified (Prandi,2013). Sagu, (2019) used a new approach to alpha-amylase trypsin extraction from wheat by using the Plackett-Burman design, and optimization is realized

using the Doehlert design. Liquid chromatography-multiple reaction monitoring-mass spectrometry (LC-MRM-MS) was used to identify and quantify 18 ATIs in different wheat cultivars (Bose *et al*.,2020). Sagu *et al.* (2020) investigated the identification and quantification of ATI in 46 wheat cultivar samples using two extraction systems based primarily on an ammonium bicarbonate buffer and chloroform/methanol (CM) mixture, with three factors optimized: amylase tryptic digestion, gel chromatographic separation, and targeted tandem mass spectrometric analysis (HPLC-MS/MS). Thirteen individual/common biomarkers have been discovered. The first comprehensive and precise proteomic profiling and qualitative assessment using a shotgun method show a close similarity between the molecular level of metabolic and chloroform-methanol (CM) protein fractions isolated from developed grains of ancient and contemporary Italian durum wheat genotypes (Francesco *et al*.,2020).

A targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique was used for the comparative analysis of the ATIs in ancient and modern wheat species (Geisslitz *et al*.,2020). Sielaff *et al*., (2021) provided a unique data-independent acquisition LC-MS method for the quantitative proteome evaluation of ATI isolated from grain flour with the aid of combining Qcon CAT technology with short microflow LC gradients and data-independent acquisition (DIA). The method offers strong as well as particular quantification of recognized ATI proteins throughout wheat flour in the context of massive breeding projects whereas concurrently quantifying the proteome in the same LC-MS experiment by using Top3 intensity-based label-free Quantification (LFQ). For the purpose of creating higher-quality, healthier wheat, Afzal *et al*., (2021) used LC-MS-based label-free quantitative (LFQ) proteomics to identify 756 proteins across 150 wheat varieties. Gonzalez-Ruiz *et al.* (2021) used size-exclusion chromatography to isolate wheat albumin inhibitory protein against the alpha-amylase isoforms of R. dominica to distinguish their susceptibility to inhibition. Call *et al.* (2021) analyzed changes in ATIs protein, and carbohydrates during seed development from harvested caryopses and characterized ATIs and carbohydrates by MALDI-TOF-MS and HPAEC-PAD. For the purpose of identifying the proteins in each of the fractions and establishing their purity, Sagu *et al.* (2022) carried out a wide range of analyses. This includes protein content determination, SDS PAGE, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and targeted LC-MS / MS.

7. Amylase Trypsin Inhibitor: Human Health

ATI in wheat performs a vital position in lowering the danger of diabetes, cardiovascular ailment, and colon cancer (Brouns *et al*., 2019; Geisslitz *et al*., 2022). However, eating too much whole wheat can have serious health consequences. Ingestion of wheat may cause hypersensitivity. The most important low-molecular-weight wheat allergens in food allergy are α-amylase inhibitors (James *et al.* 1997; Pastorello *et al.* 2007). Junker *et al.* (2012) concluded that modern wheat (hexaploid), CM3, and 0.19 species of ATI are major innate immune response activators in dendritic cells, macrophages, monocytes, and TLR4, and which is highly resistant to the intestinal proteolysis. According to a study published at United European Gastroenterology Week 2016, regular consumption of wheat containing amylase trypsin inhibitor (ATI) may lead to systemic inflammation and non-celiac gluten sensitivity. Schuppan *et al.* (2015) found that unlike the inflammation caused by celiac disease, the intestinal inflammation that occurs in non-celiac gluten sensitivity is likely caused by ATI found in wheat rather than gluten proteins. Cuccioloni *et al.* (2017) used an approach based on surface plasmon resonance biosensors and molecular docking methods to study the interaction between CM3 and TLR4. Zevallos *et al.* (2017) characterized the biological activities of ATI and found that their uptake promoted mild intestinal inflammation through infiltration and myeloid cell activation. Yolanda *et al.* (2018) analyzed the function of ATI within the activation of innate immunity and the improvement of symptomatic traits of non-celiac gluten sensitivity. Guilherme et al. (2020) evaluated the effects of his ATI on rodent models and found effects on the mouse gut microbiota and metabolism that exacerbate the pathological features of Alzheimer's disease.

Therefore, this particular wheat ingredient (ATIs) can also have adverse effects on the human body,

including wheat allergies, bread asthma, celiac disease (CD), and food allergies (Tatham *et al*., 2008).

Conclusion

Cereal Amylase trypsin inhibitor, a widely known protease inhibitor against serin as well as digestive enzyme alpha-amylase and trypsin of insect pests and animals, have been identified as promising pest management candidates in cereal crops. Its main role is to provide protection against the pest by inhibiting their digestive enzyme. The losses caused by pests in cereal crops are around 21.3 percent. In India, the cereal crops like wheat are widely attacked by *Tenebrio Molitor*, Armyworm, and many more pests that cause severe damage to the crop and decline their yield and nutrients. The inhibitory mechanism acting on mammalian and insect alpha-amylases is an essential step within the development of high-affinity/selective α-amylase inhibitors with potential in fields ranging from diabetes treatment to plant protection. Many researchers have demonstrated the isolation, characterization, and other analyses of the inhibitor protein. The mechanism of action of these inhibitor proteins will understand through structural, physiochemical, and molecular analyses. In plant genetic engineering, overexpression of the inhibitor protein could be exploiting the plant's defense mechanism against pests. In transgenic crops, these proteinase inhibitor genes are used to enhance insect resistance. PCR markers based on SNPs for a huge number of genes associated with agronomic and yield-related characters can extensively improve the effectiveness of marker-assisted breeding and mapping of genes in wheat. Screening inhibitors in grains and identification of their evolutionary relationship can assist to distinguish new insecticidal determinants. Amylase trypsin inhibitors isolated from wheat could improve their yield quality by inhibiting the digestive enzyme of pests and also be profitable to decrease the use of pesticides. It could be the development of environmentally secure, durable, and effective biological methods for pest control. Various identification and quantification techniques for wheat inhibitor proteins are used which will offer a novel insight into a rational program of concrete bioinsecticides. Furthermore, detection technologies and management strategies are needed for better results to control pests and lower their adverse effect on humans.

Author Contributions

SC prepared the manuscript and R.K. Gaur helped in preparing the final version of the manuscript and correspond to the journal.

Ethical Approval

This article does not contain any studies involving human or animal participants performed by any of the authors.

Conflicts of Interest:

The authors declare no conflict of interest.

8. References

1. Afzal M, M Sielaff, V Curella, M Neerukonda, K El Hassouni, D Schuppan, S Tenzer and C Longin. 2021. Characterization of 150 Wheat Cultivars by LC-MS-Based Label-Free Quantitative Proteomics Unravels Possibilities to Design Wheat Better for Baking Quality and Human Health. *Plants* **10(3)**:424.
2. Agarwal P. 2016. Alpha-amylase inhibition can treat diabetes mellitus. Research and Reviews, *Journal of Medical and Health Sciences.* 5.
3. Akagawa M, T Handoyo, T Ishii, S Kumazawa, N Morita and K Suyama. 2007. Proteomic analysis of wheat flour allergens. *Journal of Agricultural and Food Chemistry* **55(17):**6863-6870.
4. Awika JM. 2011. Major Cereal Grains Production and Use around the World. In: Advances in Cereal Science: Implications to Food Processing and Health Promotion. *ACS Symposium* Series 1-13.
5. Bao J and LN Malunga. 2022. Editorial: Compositional Diversity in Cereals in Relation to Their Nutritional Quality and Health Benefits. *Frontiers in Nutrition* **8:**819923.
6. Biesiekierski JR. 2017. What is gluten? *Journal of Gastroenterology and Hepatology* **32:** 78-81.
7. Birk Y. 1976. Proteinase inhibitors from cereal grains. Methods in Enzymology **45:** 723-728.
8. Birk Y. 1996. Protein proteinase inhibitors in legume seeds-overview. *Archivos latinoamericanos de nutricion* **44**(4 Suppl 1) 26S–30S.
9. Brouns F, GV Rooy, P Shewry, S Rustgi, and D Jonkers. 2019. Adverse Reactions to Wheat or Wheat Components. *Comprehensive reviews in food science and food safety*, **18(5):**1437-1452.

10. Bose U, A Juhász and JA Broadbent. 2020. Identification and Quantitation of Amylase Trypsin Inhibitors Across Cultivars Representing the Diversity of Bread Wheat, *Journal of Proteome Research* **19(5):**2136-2148.

11. Buonocore V, TC Petrucci and V Silano. 1977. Wheat protein inhibitors of α-amylase. *Phytochemistry* **16:** 811-820.

12. Buonocore V, MGD Biasi, P Giardina. 1985. Purification and properties of an α-amylase tetrameric inhibitor from wheat kernel, *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **831(1):**40-4.

13. Call L, E Haider and S D'Amico. 2021. Synthesis and accumulation of amylase-trypsin inhibitors and changes in carbohydrate profile during grain development of bread wheat (Triticum aestivum L.). *BMC Plant Biology* **21:** 113.

14. Cantagalli P, G Di Giorgio, G Morisi, F Pocchiari and V Silano. 1971. Purification and properties of three albumins from Triticum aestivum seeds. *Journal of the Science of Food and Agriculture* **22:** 256-25.

15. Capocchi A, CG Athanassiou, and G Benelli. 2021. A new monomeric α-amylase inhibitor from the tetraploid emmer wheat is mostly active against stored product pests. *Journal of Pest Science.*

16. Carrano L, N Gianpaolo, V Buonocore, C Caporale and E Poerio. 1989. An effective purification procedure of amylase and trypsin inhibitors from wheat flour; isolation of a new water-soluble protein, *Plant Science* **65(1):**25-31.

17. Christeller J, and W Laing. 2005. Plant serine proteinase inhibitors. *Protein and peptide letters* **12(5):** 439–447.

18. Clemente M, MG Corigliano, SA Pariani, EF Sánchez-López, VA Sander and VA Ramos-Duarte. 2019. Plant Serine Protease Inhibitors: Biotechnology Application in Agriculture and Molecular Farming. *International Journal of Molecular Sciences* **20(6):**1345.

19. Cousineau J. 2012. Production and Characterization of Wheat Gluten Films.

20. Cuccioloni M, M Mozzicafreddo and L Bonfili. 2017. Interfering with the high-affinity interaction between wheat amylase trypsin inhibitor CM3 and toll-like receptor 4: in silico and biosensor-based studies. *Scientific Reports* **7:**13169.

21. Da Lage JL, EGJ Danchin and D Casane. 2007. Where do animal α-amylases come from? An interkingdom trip, *FEBS Letters* 581.

22. JL Da Lage. 2018. The Amylases of Insects. International Journal of Insect Science.

23. Deshwal R, V Vaibhav and N Kumar. 2020. Stored grain insect pests and their management: An overview, Journal of Entomology and Zoology Studies **8(5):** 969-974

24. Dhaliwal GS, V Jindal and B Mohindru. 2015. Crop Losses due to insect pests: Global and Indian Scenario. *Indian journal of Entomology* **77:** 165-168.

25. Dupont FM, WH Vensel, CK Tanaka, WJ Hurkman and SB Altenbach. 2011. Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases, and tandem mass spectrometry. *Proteome Sciences.* **9**, 10.

26. El-latif AOA, N Mohieldeen, AMA Salman, and EN Elpidina. 2020. Isolation and purification of α-amylase inhibitors and their in vitro and in vivo effects on Tribolium castaneum (Herbst) and Callosobruchus maculatus (F.). *Journal of Plant Protection Researc*h **60(4):** 377-387.

27. Poerio E, L Carrano, AM Garzillo, V Buonocore. 1989. A trypsin inhibitor from the water-soluble protein fraction of wheat kernel. *Phytochemistry* **28(5):**1307-1311.

28. Farook B, Umer and Khan, Zakir and Ahad. 2019. A review on insect pest complex of wheat (Triticum aestivum L.). *Journal of Entomology and zoology studies* **7:** 1292-1298.

29. Feng GH, M Richardson, MS Chen, KJ Kramer, TD Morgan and GR Reeck. 1996. alpha-Amylase inhibitors from wheat: amino acid sequences and patterns of inhibition of insect and human alpha-amylases. *Insect biochemistry and molecular biology* **26:** 419-426.

30. Franco OL, DJ Rigden, FR Melo and MF Grossi-De-Sá. 2002. Plant alpha-amylase inhibitors and their interaction with insect alpha-amylases. *European Journal of Biochemistry* **269(2):** 397-412.

31. Francesco AD, R Saletti, V Cunsolo, B Svensson, V Muccilli, PD Vita and F Salvatore. 2020. Qualitative proteomic comparison of metabolic and CM-like protein fractions in old and modern wheat Italian genotypes by a shotgun approach, *Journal of Proteomics* **211:** 103530.

32. Gatehouse AM, KA Fenton, I Jepson and DJ Pavey. 1986. The effects of α-amylase inhibitors on insect storage pests: Inhibition of α-amylase in vitro and effects on development in vivo. *Journal of the Science of Food and Agriculture* **37:** 727-734.

33. Gatehouse JA. 2011. Prospects for Using Proteinase Inhibitors to Protect Transgenic Plants Against Attack by Herbivorous Insects, *Current Protein & Peptide Science* **12(5).**

34. Geisslitz S, CFH Longin, and P Koehler. 2020. Comparative quantitative LC-MS/ MS analysis of 13 amylase/trypsin inhibitors in ancient and modern Triticum species, *Scientific Reports* **10:**14570.

35. Geisslitz S. 2021. Wheat ATIs: Characteristics and Role in Human Disease. *Frontiers in Nutrition* **8:**667370.

36. Geisslitz S, P Weegels and P Shewry. 2022. Wheat amylase/trypsin inhibitors (ATIs): occurrence, function and health aspects. *European Journal of Nutrition.*

37. Gitlin-Domagalska A, A Maciejewska, and D Dębowski. 2020. Bowman-Birk Inhibitors: Insights into Family of Multifunctional Proteins and Peptides with Potential Therapeutical Applications. *Pharmaceuticals*, **13(12),** 421.

38. Granum PE. 1979. Studies on α-amylase inhibitors in foods, *Food Chemistry* **4(3):**173-178.

39. Gomez L, R Sanchez-Monge, F Garcia-Olmedo and G Salcedo. 1989. Wheat tetrameric inhibitors of insect alpha-amylases: Alloploid heterosis at the molecular level. *Proceedings of the National Academy of Sciences of the United States of America*, **86(9):** 3242–3246.

40. Gomez L, R Sanchez-Monge, C Lopez-Otín and G Salcedo. 1991. Wheat Inhibitors of Heterologous alpha -Amylases: Characterization of Major Components from the Monomeric Class. *Plant Physiology* **96(3)**:768–774.

41. González-Ruiz CR, CL Del Toro-Sánchez, YI Cornejo-Ramírez, F Rodríguez-Félix, FJ Wong-Corral, E Márquez-Ríos, JL Cárdenas-López and FJ Cinco-Moroyoqui. 2019. Differential biochemical and kinetic properties of α-amylases from Rhyzopertha dominica (F.) progenies reared on wheat varieties differing in α-amylase inhibitory activity, *Journal of Stored Products Research* **90:** 101748.

42. Guilherme DSM, VF Zevallos, A Pesi, NM Stoye, VTT Nguyen, K Radyushkin, A Schwiertz, U Schmitt, D Schuppan and K Endres. 2020. Dietary Wheat Amylase Trypsin Inhibitors Impact Alzheimer's Disease Pathology in 5xFAD Model Mice. *International Journal of Molecular Sciences* **21(17):**6288.

43. Guo G, D Lv, X Yan, S Subburaj, P Ge, X Li, Y Hu and Y Yan. 2012. Proteome characterization of developing grains in bread wheat cultivars (Triticum aestivum L.). *BMC Plant Biology* **12:** 147.

44. Habib H and KM Fazili. 2007. Plant Protease Inhibitors: A Defense Strategy in Plants. *Biotechnology and Molecular Biology Reviews*, **2:** 68-85.

45. Hailegiorgis D, F Mekonnen, F Hailu, CA Lee and SJ Yun. 2020. Composition and Molecular Weight Distribution of Albumin and Globulin Protein Isolates from Durum Wheat Genotypes. *American Journal of Plant Sciences* **11:** 137-147.

46. Hassouni K, M Sielaff and V Curella. 2021. Genetic architecture underlying the expression of eight α-amylase trypsin inhibitors. *Theoretical and Applied Genetics* **134:** 3427-3441.

47. He Li. 2021. Proteinaceous α-amylase inhibitors: purification, detection methods, types and mechanisms, *International Journal of Food Properties* **24(1):**277-290

48. Heidari R, S Zareae and M Heidarizadeh. 2005. Extraction, Purification, and Inhibitory Effect of Alpha-Amylase Inhibitor from Wheat (Triticum

aestivum Var. Zarrin). Pakistan *Journal of Nutrition*, **4:** 101-105.

49. Hossard L, A Philibert, M Bertrand, C Colnenne-David, P Debaeke, NM Munier-Jolain, MH Jeuffroy, G Richard and D Makowski. 2014. Effects of halving pesticide use on wheat production. *Scientific Reports* 4.
50. Husain, Dilbar, and Asrar, Muhammad, and Khalid. 2021. Insect pests of economic importance attacking wheat crop (Triticum aestivum L.) in Punjab, Pakistan. *International Journal of Tropical Insect Science.*
51. Jamal F, PK Pandey, D Singh and MY Khan. 2013. Serine protease inhibitors in plants: nature's arsenal crafted for insect predators. *Phytochemistry Reviews* **12:**13.
52. James JM, Sixbey JP, Helm RM, Bannon GA, Burks and AW (1997). Wheat alpha-amylase inhibitor: a second route of allergic sensitization. *The Journal of allergy and clinical immunology* **99(2):**239-244.
53. Jakhar A, T Goswami and SK Sharma. 2020. Status of Insect Pests of Cereals in India and Their Management.
54. Junker Y, S Zeissig, SJ Kim, D Barisani, H Wieser, DA Leffler, V Zevallos, TA Libermann, S Dillon, TL Freitag, CP Kelly and D Schuppan. 2012. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. *Journal of Experimental Medicine* **209(13):**2395-408.
55. Kaur R, N Kaur and AK Gupta. 2014. Structural features, substrate specificity, kinetic properties of insect α-amylase and specificity of plant α-amylase inhibitors, *Pesticide Biochemistry and Physiology*, **116:**83-93.
56. Kauppi K, A Rajala, E Huusela, J Kaseva, P Ruuttunen, H Jalli and L Alakukku. 2021. Impact of Pests on Cereal Grain and Nutrient Yield in Boreal Growing Conditions. *Agronomy* **11(3):** 592.
57. Kneen E and RM Sandstedt. 1946. Distribution and general properties of an amylase inhibitor in cereals. *Archives of Biochemistry and Biophysics* **9:** 235-249.
58. Kreis M, BG Forde, S Rahman, BJ Miflin and PR Shewry. 1985. Molecular evolution of the seed storage proteins of barley, rye, and wheat. *Journal of Molecular Biology* **183 (3)**:499-502.
59. Kulshrestha VP. 1985. History and Ethnobotany of wheat in India. *Journal d'agriculture traditionnelle et de botanique appliquée, 32 année,* 61-71.
60. Kumar R, SS Yadav, S Yadav, P Ranga, H Kumar and V Singh. 2022. Differential response of wheat cultivars to grain damage by Rice Weevil, Sitophilus oryzae (L.) (Coleoptera: Curculionidae). *Journal of Cereal Research* **14 (Spl-2):** 55-62.
61. Laemmli U. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227,** 680–685.
62. Laskowski W, H Górska-Warsewicz, K Rejman, M Czeczotko and J Zwolińska. 2019. How Important are Cereals and Cereal Products in the Average Polish Diet? *Nutrients*, **11**.
63. Le Berre-Anton V, C Bompard-Gilles, F Payan and P Rougé. 1997. Characterization and functional properties of the alpha-amylase inhibitor (alpha-AI) from kidney bean (Phaseolus vulgaris) seeds. *Biochimica et biophysica acta* **1343(1):** 31-40.
64. Limas GG, M Salinas, I Moneo, S Fischer, BD Wittmann-Liebold and E Méndez. 2004. Purification and characterization of ten new rice NaCl-soluble proteins: identification of four protein-synthesis inhibitors and two immunoglobulin-binding proteins. *Planta.* **181:** 1-9.
65. Maeda K, T Hase and H Matsubara. 1983. Complete amino acid sequence of an alpha-amylase inhibitor in the wheat kernel. *Biochimica et biophysica acta* **743(1):**52–57.
66. McKevith B. 2004. Nutritional aspects of cereals. *Nutrition Bulletin* **29:** 111-142.
67. Mandali R. 2020. Stored grain pests and their identification, *National Institute of Plant Health Management.*
68. Møller MS and B Svensson. 2022. Structure, Function and Protein Engineering of Cereal-Type Inhibitors Acting on Amylolytic Enzymes. *Frontiers in Molecular Biosciences* **9:**868568.
69. Mundy J, Svendsen IB and J Hejgaard. 1983. Barley α-amylase/subtilisin inhibitor.I. Isolation and characterization. *Carlsberg Research Communications* **48:**81-90.

70. Murray M, WF Thompson. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8:**4321-4326.

71. Oda Y and K Fukuyama. 1997. 0.19 alpha-amylase inhibitor from wheat. *International Archives of Allergy and Immunology* **144:**10-22.

72. Osborne TB. 1907. The Proteins of the Wheat Kernel. Carnegie Institution of Washington, Publication No. 84, Judd & Detweiler, Inc., Washington DC.

73. Oyewole, C (2016) The wheat crop.

74. Pandey B, M Saini and P Sharma. 2016. Molecular phylogenetic and sequence variation analysis of dimeric α-amylase inhibitor genes in wheat and its wild relative species, *Plant Gene* **6:** 48-58.

75. Pastorello EA, L Farioli and A Conti. 2007. Wheat IgE-Mediated Food Allergy in European Patients: alpha-Amylase Inhibitors, Lipid Transfer Proteins and Low-Molecular-Weight Glutenin Allergenic Molecules Recognized by Double-Blind, Placebo-Controlled Food Challenge, *International Archives of Allergy and Immunology* **144(1):**10-22.

76. Petrucci T, A Rab, M Tomasi and V Silano. 1976. Further characterization studies of the alpha-amylase protein inhibitor of gel electrophoretic mobility 0.19 from the wheat kernel. *Biochimica et biophysica acta* **420(2):**288–297.

77. Prandi B. 2013. LC/MS analysis of proteolytic peptides in wheat extracts for determining the content of the allergen amylase/trypsin inhibitor CM3: Influence of growing area and variety, *Food Chemistry* **140:** 141-146.

78. Priya S, S Kumar, N Kaur and AK Gupta. 2013. Specificity of α-amylase and trypsin inhibitor proteins in wheat against insect pests, *New Zealand Journal of Crop and Horticultural Science* **41(1):**49-5.

79. Rane AS, RS Joshi and AP Giri (2020) Molecular determinant for specificity: Differential interaction of α-amylases with their proteinaceous inhibitors, *Biochimica et Biophysica Acta (BBA) GeneralSubjects*,**1864(12):**129703.

80. Rodríguez-Sifuentes L, JE Marszalek, C Chuck-Hernández and SO Serna-Saldívar. 2020. Legumes Protease Inhibitors as Biopesticides and Their Defense Mechanisms against Biotic Factors. *International Journal of Molecular Sciences*. **21(9):**3322.

81. Sagu ST. 2019. A New Approach of Extraction of alpha-Amylase/trypsin Inhibitors from Wheat (Triticum aestivum L.), Based on Optimization Using Plackett-Burman and Box-Behnken Designs. *Molecules*, 24.

82. Sagu ST, L Zimmermann, E Landgräber, T Homann, G Huschek, H Özpinar, FJ Schweigert and HM Rawel. 2020. Comprehensive Characterization and Relative Quantification of α-Amylase/Trypsin Inhibitors from Wheat Cultivars by Targeted HPLC-MS/MS. *Foods* **9(10):**144.

83. Sagu ST, E Landgräber, IM Henkel, G Huschek, T Homann, S Butler and OK Schlüter. 2021. Effect of Cereal α-Amylase/Trypsin Inhibitors on Developmental Characteristics and Abundance of Digestive Enzymes of Mealworm Larvae (Tenebrio molitor L.). *Insects*, **12(5):** 454.

84. Sagu ST, G Huschek, TW Braga, M Rackiewicz, T Homann and HM Rawel. 2022. Design of Experiment (DoE) for Optimization of HPLC Conditions for the Simultaneous Fractionation of Seven α-Amylase/Trypsin Inhibitors from Wheat (Triticum aestivum L.). *Processes* **10(2):** 25.

85. Sanchez-monge R, L Gomez, F García-olmedo And G Salcedo. 1989. New dimeric inhibitor of heterologous α-amylases encoded by a duplicated gene in the short arm of chromosome 3B of wheat (Triticum aestivum L.). *European Journal of Biochemistry* **183:** 37-40.

86. Schuppan D, G Pickert, M Ashfaq-Khan and V Zevallos. 2015. Non-celiac wheat sensitivity: differential diagnosis, triggers and implications. *Best Practice and Research in Clinical Gastroenterology* **29:** 469-476.

87. Sielaff M, V Curella, M Neerukonda, M Afzal, K Hassouni, U Distler, D Schuppan, CFH Longin and S Tenzer. 2021. Hybrid QconCAT-Based Targeted Absolute and Data-Independent Acquisition-Based Label-Free Quantification Enables In-Depth Proteomic Characterization of Wheat Amylase/Trypsin Inhibitor Extracts. *Journal of Proteome Research* **20 (3):** 1544-1557.

88. Silano V, M Furia, L Gianfreda and A Macri. 1975. Inhibition of amylases from different origins by albumins from the wheat kernel, *Biochimica et Biophysica Acta (BBA)-Enzymology* **391 (1):** 170-178.

89. Simonetti E, S Bosi and L Negri. 2022. Molecular phylogenetic analysis of amylase trypsin inhibitors (ATIs) from a selection of ancient and modern wheat, *Journal of Cereal Science* **105:**103441.

90. Singh B and P Jasrotia. 2020. Impact of integrated pest management (IPM) module on major insect-pests of wheat and their natural enemies in North-western plains of India. *Journal of Cereal Research* **12(2):**114-119.

91. Sharma P, M Saini, S Sheoran and I Sharma. 2013. Molecular analysis of dimeric α-amylase inhibitor genes in Indian wheat. *Indian Journal of Biotechnology* 12.

92. Shivaraj. 1981. Natural plant enzyme inhibitors: Characterization of an unusual a-amylase/trypsin inhibitor from ragi (Eleusine coracana Geartn.), *Biochemical Journal* **193:** 29-36.

93. Stevens J, K Dunse, J Fox, S Evans, and M Anderson. 2012. Biotechnological Approaches for the Control of Insect Pests in Crop Plants. In (Ed.), *Pesticides - Advances in Chemical and Botanical Pesticides.* Intech Open.

94. Sotkovský P, M Hubálek, L Hernychová, P Novák, M Havranová, I Setinová, A Kitanovicová, M Fuchs, J Stulík, and L Tucková. 2008. Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. *Proteomics,* **8(8):** 1677–1691.

95. Tadesse W, A Amri, F Ogbonnaya, M Sanchez-Garcia, Q Sohail and M Baum. 2016. Wheat. *Genetic and Genomic Resources for Grain Cereals Improvement*, 81-124.

96. Talley K, and E Alexov. 2010. On the pH optimum of activity and stability of proteins. *Proteins,* **78(12):** 2699-2706.

97. Tatham AS and P R Shewry. 2008. Allergens to wheat and related cereals. *Clinical and experimental allergy* **38:** 1712-1726.

98. Uthayakumaran S and C Wrigley. 2017. Wheat: Grain-Quality Characteristics and Management of Quality Requirements, Food Science, Technology and Nutrition, *Cereal Grains* (Second Edition) **5**: 91-134.

99. Wang JR, ZH Yan, YM Wei, E Nevo, BR Baum and YL Zheng. 2006. Molecular characterization of dimeric alpha-amylase inhibitor genes in wheat and development of genome allele-specific primers for the genes located on chromosome 3BS and 3DS, *Journal of Cereal Science* **43(3):**360-368.

100. War AR, MG Paulraj and T Ahmad. 2012. Mechanisms of plant defense against insect herbivores, Plant Signaling & Behavior **7(10):**1306-132.

101. Wilson KA**.** 1997. The Protease Inhibitors of Seeds. In: Larkins, B.A., Vasil, I.K. (eds) Cellular and Molecular Biology of Plant Seed Development. *Advances in Cellular and Molecular Biology of Plants* 4, *Springer.*

102. Wisessing A, A Engkagul, A Wongpiyasatid and K Choowongkomon. 2010. Biochemical characterization of the alpha-amylase inhibitor in mungbeans and its application in inhibiting the growth of Callosobruchus maculatus. *Journal of agricultural and food chemistry* **58(4):** 2131–2137.

103. Yolanda RO, M Jordi and M Lara. 2018. Amylase-Trypsin Inhibitors in Wheat and Other Cereals as Potential Activators of the Effects of Nonceliac Gluten Sensitivity. *Journal of Medicinal Food* **21(3):** 207-214.

104. Zhu-Salzman, Keyan, Zeng and Ren. 2014. Insect Response to Plant Defensive Protease Inhibitors. *Annual review of entomology* **60.**

105. Zevallos VF. 2017. Nutritional Wheat Amylase-Trypsin Inhibitors Promote Intestinal Inflammation via the Activation of Myeloid Cells. *Gastroenterology* **152:**113.

106. Zilić S, M Barać, M Pešić, D Dodig and D Ignjatović-Micić. 2011. Characterization of proteins from grains of different bread and durum wheat genotypes. *International journal of molecular sciences* **12(9):** 5878–5894.