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Wheat Alpha Amylase

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Abstract

Alpha amylase (α -amylase) (EC 3.2.1.1) is an endo-amylolytic enzyme that plays an important role in seed germination in wheat. However, excessive α -amylase in post-harvest wheat grains has a negative effect on wheat yield and end-products quality. A wide range of studies have been carried out on wheat alpha amylase due to its important biological roles in post-harvest sprouting. In this article, past researches in the aspects of its biochemical activity assay, suppressor, genetic mechanism, and expression regulation in wheat plants were reviewed. Its impacts on breadmaking quality as well as a range of human health related issues were also covered.

α -AMYLASE IN WHEAT

α -Amylase plays a key role in hydrolysing starch during seed germination process in plants. The activity of α -amylase was found to have a positive correlation of Pre-harvest sprouting (PHS) rates [1]. From the wheat breeding point of view, it is of great importance to maintain a lower α -amylase activity in order to improve PHS tolerance. Seed germination starts with water absorption, which increases the hormonal level in seed endosperm which in turn invokes the activation of α -amylase and other proteolytic enzymes in the aleurone and embryo. Starch in endosperm will thus be hydrolyzed into simple sugars to provide fuel needed for germination [2]. Amylases are the enzymes responsible for this process. There are three major types of amylase based on their mode of action, including endo-amylases (α -amylases), exo-amylases (β -amylase, glucoamylases, α -glucosidases), and debranching enzymes (iso-amylases and limit dextrinase). α -Amylase is the key in converting starch into soluble maltodextrins that are subsequently hydrolyzed to maltose and glucose [3,4].

Starch consists of linear amylose with mainly α -1, 4 bonds and branched amylopectin linked with α -1, 4 and α -1, 6 bonds. α -amylase breaks down starch by cleaving α -1, 4 bonds in the starch [5]. The α -amylase protein consists of a large glycosyl hydrolase superfamily (GHS) domain, a C terminal β -sheet domain, along with several well-characterized active sites, catalytic sites, and calcium-binding sites [6]. In wheat, a signal peptide with a length of 24 amino acid (aa) was identified at the N-terminal end of protein. Wheat α -amylase proteins belong to the glycosyl hydrolase 13 (GH13) family according to their amino acid sequences, which is characterized by the presence of three domains with domain A being the main catalytic site known as the TIM-barrel [7].

HPLC identified 8 different kinds of α -amylase in wheat. Among them, 2 kinds of α -amylase were better clarified functionally and genetically, including α -amylase-1 and α -amylase-2 [8]. Analysis on wheat α -amylase isoenzyme indicated that there are at least 22 isoenzymes [8]. Slight differences in the enzymatic properties may make one isozyme better suited to a particular substrate or intracellular environment than another, making them adsorb and degrade starch granules at different rates [9].

During wheat grain development, there exist 3 phases of α -amylase formation: (1) pre-mature state in the absence

of germination; (2) excessive deposition of α -amylase in the endosperm after maturity; and (3) germination after breaking down the dormancy. The activity of α -amylase changes through this seed developing process, being hundreds folds higher in germination seeds and before maturity than that in mature seeds [10]. In most varieties, activity of α -amylase reaches the peak at around 14 days after anthesis. As for germination seeds, activity can be tested one day after imbibition of water, it peaks at around 7-8 days and disappears after 12 days [11]. Based on their different modes of enzyme accumulation, α -amylase synthesis routes in wheat can be differentiated as retained pericarp α -amylase activity (RPAA), pre-maturity α -amylase activity (PMAA), prematurity sprouting (PrMS) and post-maturity sprouting (PoMS), and the frequency of occurrence is in the order PoMS>PMAA>PrMS>RPAA. Among those, PMAA is also often called late maturity α -amylase (LMA), which is the most frequent route of alpha amylase accumulation, closely followed by PoMS. Grains with excessive PoMS usually present visible sprouting with severely impaired end use quality [12].

The effects of α -amylase on wheat end use quality

Studies have shown that high α -amylase in wheat lead to grain yield and economic loss and reduced quality, including low falling number, low viscosity, sticky crumb and collapsed loaves. In breadmaking process, excessive levels of natural α -amylase activity in wheat leads to more rapidly degraded starch in flour during mixing and fermentation, which causes the reducing of water holding capacity and this eventually results in sticky dough, decreased loaf volume, compact interior, and dark crust in breadmaking process [13,14]. The extreme stickiness of dough also causes requirement of special handling which can disrupt the bakery operations [15]. Pan bread is more sensitive to high α -amylase activity compared to flat bread and bun [16]. During breadmaking, adding barley α -amylase inhibitor improves the baking quality of sprout damaged wheat flour [17]. For noodle quality, hydrolysis of the protein and starch in the damaged flour resulted in products with less elasticity, darker colour and weaker strength [16]. For Speciality batters, high α -amylase in sprouted wheat generally reduces the quality of batters for many uses, and often causes the loss of shape, light and viscous character [18].

By comparing the α -amylase activity in different parts of already sprouted wheat grains, a study showed the germ and aleurone of sprouted wheat grain contained a significantly

higher α -amylase activity than the starchy endosperm. Through removing the germ prior to milling and adjusting pearling procedure, it is possible to produce flour with lower α -amylase activities [19]. In addition, wheat varieties with stronger gluten have more tolerance of germination damage. An extra-strong gluten wheat variety Victoria INTA was found to have less sensitivity to higher α -amylase activity and possesses a lower level of deterioration of breadmaking quality from sprouting [20]. Despite improving milling process and selecting extra-strong wheat; during breadmaking, adding barley α -amylase inhibitor was also proved to improve the baking quality of sprout damaged wheat flour [17].

However, a small portion of α -amylase activity in flour has been shown to enhance bread quality [21]. The bakery industry uses α -amylase to improve the textural properties of bread and to reduce elasticity. α -amylase was reported to attenuate the negative effects of high damaged starch on dough properties [22].

Assay of α -amylase activity

Accurate assay of α -amylase activity is of great importance in monitoring wheat grain breadmaking quality as well as in breeding new varieties. A wide array of analytical methods are available for the measurement of α -amylase activity [23-25]. The measurement of α -amylase usually requires extraction of the enzyme from the sample matrix followed by measurement of hydrolysis rate of dye-labelled starch or other model substrates under controlled conditions. Separating specific α -amylase isozymes can be achieved by isoelectric focusing in a liquid column [26].

The most common method for measuring α -amylase activity is the falling number (FN) method [27], which is a viscometric assay that is assessed as the time required for a plunger to fall through a heated slurry of whole meal and water resulted from the rapid gelatinization and liquefaction of the starch by α -amylase. This method is widely accepted as a standardized method for assessing wheat grain breadmaking quality in relation to the preharvest sprouting trait. FN values of 300 seconds or in some cases 350 seconds are required for inclusion of the delivered wheat grain in high-quality grades by the wheat industries in most countries [28].

Whilst falling number is used universally to assess the wheat grain breadmaking quality and α -amylase activity, other methods such as the Rapid Visco Analyser [29] and near-infrared (NIR) analysis [30] are preferred sometimes as they provide additional important information about protein and starch properties that are relevant to processing and which can be very useful for breadmaking [31]. Enzyme-linked immunosorbent assays (ELISA) provides an alternative method for detection of preharvest sprouting through the use of antibodies that are specific for α -amylase isozymes [32].

LATE MATURITY AMYLASE (LMA)

Late maturity amylase (LMA) is also called prematurity α -amylase in UK. Wheat germplasm from the UK, Japan, China, Australia, North America, South Africa were all reported to have LMA damage [28]. Since wheat grains affected by LMA are usually without visual sprouting, the presence of LMA is unlikely to affect

nutritional value. However, significantly lower FN value suggests inferior end product quality [33,34]. LMA is usually activated by a cool temperature shock (average minimum 8°C, maximum 26°C) during the middle to later stages of grain development and ripening (25-30 days after anthesis) [35].

α -Amylase inhibitors

Amylase inhibitors are substances that bind to α -amylase and make them inactive. They are important research subjects regarding pre-harvest sprouting, plants pathogen resistance, insect tolerance and human health. There are proteinaceous and non-proteinaceous inhibitors [36]. Two multiple amylase inhibitor gene families with MW 12kDa and 24kDa have been extensively investigated [37]. Amylase inhibitors of the cereal family are composed of 120-130 aa. The bifunctional proteinaceous α -amylase/subtilisin inhibitors (ASI) and α -amylase/trypsin (ATI) have been identified and characterized in wheat [38,39]. ASIs were reported to be reductively inactivated by thioredoxin (*trx*) [40], and transformed wheat lines with anti-*trxs* genes to have reduced α -amylase activity and better pre-harvest sprouting resistance [41,42]. ATIs appear to play important roles in promoting adaptive immunity of celiac disease and other immune-mediated diseases within and outside the GI tract; they may however serve as prime candidates of severe forms of non-celiac gluten (wheat) sensitivity [43].

A major wheat flour allergen with a molecular weight of 15 kDa exists, which is an α -amylase inhibitor that plays an important role in the pathogenesis of baker's asthma disease [44]. The presence of α -amylase inhibitory activity is an important cause of baker's asthma. It has been demonstrated that the most prominent allergenic components are glycoproteins which belong to the subunits of tetrameric α -amylase inhibitors [44]. In addition, α -amylase inhibitors are the potential targets in the developing compounds for the treatment of diabetes and postprandial hyperglycemia [45]. The α -amylase inhibitors also have been reported to have effects similar to insulin in reducing α -amylase activity as well as glucose level [46].

Three α -amylase inhibitor loci (*Isa-1*) were identified in common wheat and located on the long arms of chromosomes 2A, 2B and 2D. The most frequent electrophoretic pattern of common wheat cultivars consisted of two isoforms, encoded by the *Isa-B1b*, *Isa-D1a* alleles and the *Isa-A1* null allele [47]. Dimeric α -amylase inhibitors formed three groups and were clustered with 0.19 inhibitors. It is predicted that dimeric α -amylase inhibitors co-localized into chloroplast and mitochondria [48].

In plants vegetative organs and seeds, several kinds of amylase inhibitors were found to regulate a number of phytophagous insects [49,50]. α -amylase inhibitors improve plant insects tolerance through altering the digestive action of alpha amylases and proteinases in the gut of insects [51]. Wheat kernels contain several types of α -amylase inhibitors that block the digestive α -amylases of various gramnivorious insets [52,53].

As for pathogen resistance, two winter wheat lines with different Fusarium Head Blight (FHB) resistance were analyzed to identify crucial proteins associated with resistance to FHB. Monomeric α -amylase and dimeric α -amylase inhibitors were found both highly accumulated in the more resistant line

after inoculation and in the control conditions. Higher level of α -amylase enzyme activity in more susceptible lines after *F. culmorum* infection confirmed that the inhibition of pathogen amylase activity could be one of the most crucial mechanisms to prevent infection progress [54]. Applying wheat α -amylase inhibitors to *in vitro* culture containing a pathogen mycelium disc resulted in a reduction in both pathogen growth and α -amylase activity, suggesting that α -amylase inhibitor contributed to resistance against pathogen attack, acting in a diversified manner for different fungal species [55].

Heredity of α -amylase

The length of wheat α -amylase gene is 1,472 bp with 3 exons and 2 introns [56]. According to their pI values, α -amylases identified in wheat can be categorized into three major groups: α -amylase-1, α -amylase-2 and α -amylase-3. These different kinds of α -amylases are controlled by different genes (Table 1). α -amylase-1 has high pI and is encoded by α -Amy1 gene that is located on chromosome 6A, 6B and 6D; while α -amylase-2 has low pI and it is controlled by gene α -Am2 on chromosome 7A, 7B and 7D [57,58]. α -amylase-3 has high pI and only exists on the out layer of pericarp and few gene copies of α -Amy3 were observed on chromosome 5 [59]. Unlike the above three gene family, α -Amy4 is a recently discovered new gene family, gene copies were identified on chromosome 2 and 3 [60].

Late maturity amylase (LMA), which also has a high pI, is completely independent of pre harvest sprouting and can be expressed in sprouting tolerant or dormant genotypes [61,62]. Unlike α -amylase-1, which is detected during seed germination, LMA is expressed at the later stage of grain maturity. This trait appeared to be multigenic. The relevant QTL were identified on chromosomes 7B, 3B, 3A, 2D, and 6B [58,62-64]. Further fine mapping of the LMA QTL on 7B and 3B has been conducted [33]. According to Barrero et al. [34], gene α -Amy1 and α -Amy2 were expressed around 23 DPA in +LMA double haploid lines. Abundant allelic variations exist in both α -Amy1 and α -Amy2 gene families [65,66], and different genes are not expressed equally in LMA. The relationship between gene expression of different α -Amy genes and LMA is worthy of a further study.

The presence of dwarfing or gibberellic acid (GA) insensitivity genes can reduce the expression of LMA genes [61]. Individual presence of *Rht1* or *Rht2* gene will partially inhibit the gene expression, while in the case of *Rht3* and the combination *Rht1+Rht2*, gene expression of LMA appears to be almost completely inhibited. The masking effects of individual presence of *Rht1* or *Rht2* gene makes it more difficult to select LMA tolerance wheat lines in breeding program.

REGULATION OF GENE EXPRESSION BY HORMONES AND SUGAR

The plant hormones abscisic acid (ABA) and gibberellic

acid play major and opposing roles in the development and germination of cereal grains [67]. In wheat, the up-regulation of α -amylase by GA was shown to be achieved via interaction of different regulatory proteins with gibberellin response elements (GAREs) and the motif TAACAACTCCGG in promoters of α -amylase genes was vital for its function [68,69]. On the other hand, the ABA-induced down-regulation was regulated via interactions with the ABA response elements (ABRES) [70].

The mechanism of α -amylase synthesis through GA signalling in wheat is still yet to be unravelled. However, studies on rice, arabidopsis and barley suggested that GA signalling process is quite conserved, it is therefore believed that wheat shares similar pathway [71]. Exogenous GA can bind to the GID1 receptor, this promotes binding to DELLA proteins, which refer to a kind of proteins that contain a consensus amino acid sequence D-E-L-L-A in the N-terminal and have been known to rapidly degrade upon the application of GA [72] and *Rht-D1* & *-B1* genes in wheat encode for DELLA homologues [73]. This complex, GA-GID1-DELLA, will attract a specific ubiquitin E3 ligase complex (SCF^{GID2}) to further form a larger SCF complex, which stimulates the poly ubiquitination of DELLA targeting its destruction through the 26S proteasome [74] followed by the induction of α -Amy gene transcription factor GAMYB. Finally, binding of GAMYB protein promotes the expression of α -Amy genes (Figure 1).

Exogenous GA treatment induced the expression of α -Amy1 and α -Amy2 in mature wheat aleurone in one study, along with the upregulation of the GAMYB transcription factor and the ABA catabolic gene ABA89OH-1, showing the evidence of the above-mentioned GA regulation mechanism and the existence of a cross-talk response between GA and ABA [34]. The use of the hormone-biosynthesis inhibitors also confirmed the effects of altered abscisic acid and gibberellin levels on pre-maturity α -amylase formation in wheat grains, with an association between GA levels at mid-grain development and PMA formation was observed [75].

With better understanding of α -amylase gene regulation mechanism by hormones, breeders will be able to improve wheat α -amylase character by manipulating the related genes. Application of altered GA expression in wheat was proved to be effective by introducing a gibberellin 2-oxidase gene (*PcGA2ox1*) originated from legume. Expression of two GA biosynthesis genes (*TaGA20ox1* and *TaGA3ox2*) was up-regulated, and that of two α -amylase gene families (α -Amy1 and α -Amy2) down regulated in transgenic wheat plants [76]. Another example was a transgenic study on gene *trxs*. When a foreign *trxs* gene, which is known to regulate the balances of ABA and GAs, was introduced into wheat, the ABA and ABA/GAs contents in transgenic lines increased significantly ($P < 0.01$) by 18.39% and 23.47%, respectively, at 10–30 day after anthesis while the GAs content decreased [77].

Table 1: Four groups of α -amylase isoenzymes in wheat grain.

Category	Gene family	PI	Chromosome group	Expression tissue	Expression stage
Malt	α -Amy1	6.1-6.9	6	aleurone	later stage of seed development
Green	α -Amy2	4.7-6	7	endosperm, embryo, pericarp	prior to anthesis
Unknown	α -Amy3	>10	5	pericarp	seed developing
Unknown	α -Amy4	6-6.3	2 and 3	unknown	seed developing

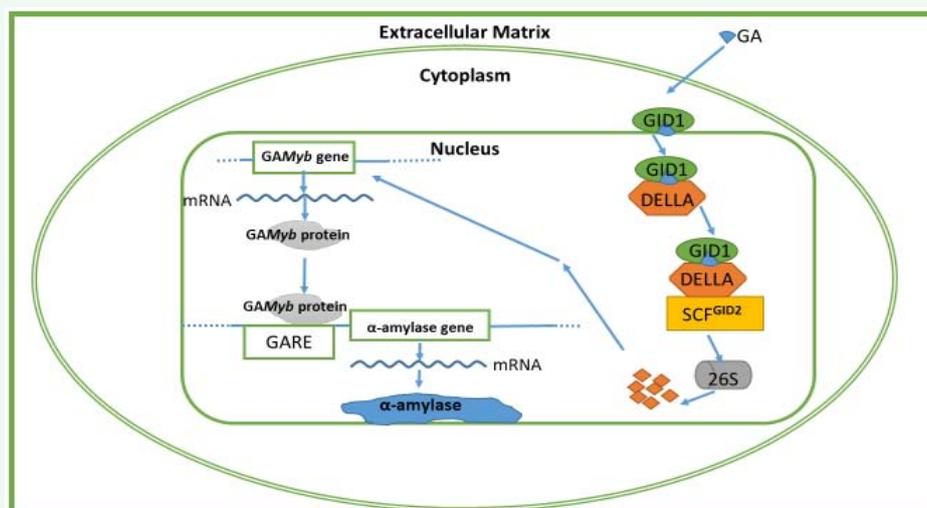


Figure 1 Simplified GA response pathway for α -amylase synthesis in wheat [83-85].

Studies showed that the expression of α -amylase genes were enhanced by sugar starvation and repressed by sugar provision [78,79]. An TATCCA element on the upstream of the transcription starting site was proved to be essential for this sugar regulation mechanism. Since this element is a component of the GA response complex, the regulation of α -amylase gene expression by GA and sugar may share a common regulatory protein that binds to this element. It was confirmed that in rice and barley MYB proteins are the relevant regulatory proteins [80]. The expression of α -amylase gene (α -Amy2) was observed to be inhibited in the cell culture of wheat embryos by sucrose and glucose [81]. Mannose, raffinose, and galactose (60-70% at the concentration of 10 mM) were also observed to show a pronounced suppression of α -amylase genes [82]. In addition, glucose phosphates exerted a somewhat greater effect than unmodified glucose, and this sugar repression of α -amylase synthesis in the embryo is specific while the effect on the cells of the aleurone layer is nonspecific (osmotic) [82].

SUMMARY

The roles of α -amylase in wheat germination, pre-harvest sprouting, and wheat end product quality are of great importance. In modern breeding programmes, selecting wheat materials with both low α -amylase activity and low α -Amy gene expression should be taken into consideration in PHS resistance breeding. Studies on regulation of α -Amy genes and inhibitors require more attention as they can provide potential approaches in the manipulation of α -Amy genes. More studies on α -amylase inhibitors are also essential to reduce human's baker's asthma disease meanwhile maintain the wheat's pathogen resistance.

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