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Review: Amylopectin synthesis and hydrolysis - Understanding isoamylase and limit dextrinase and their impact on starch structure on barley (*Hordeum vulgare*) quality

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1 **Review: Amylopectin synthesis and hydrolysis - understanding isoamylase and limit**

2 **dextrinase and their impact on starch structure on barley (*Hordeum vulgare*) quality**

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15

16 **Abstract**

17 **Background**

18 Starch contributes to barley grain and malt quality which in turn contributes to beer quality

19 and flavour; through fermentable sugar profiles, rates of fermentation and Mallard reactions.

20 Both amylopectin and amylose are enzymatically degraded to release maltose, maltotriose and

21 higher order sugars.

22 **Scope and Approach**

23 Amylopectin is highly branched [α-(1→6) glycoside bond branch points] with numerous

24 short branches while amylose is a long chained polymer with a few side branches. During

25 grain development, the final level of branching is controlled by two enzymes namely;

26 isoamylase and limit dextrinase (LD). Mutations in either of these genes can also result in

27 changes to structure, content, and granule formation and size. During the malting free LD will

28 to cleave the α-(1→6) bonds but during mashing processes, bound LD is release, resulting in

29 chains of various length available for other starch degrading enzymes to hydrolyse.

30 **Findings and conclusions**

31 While there is a good understanding of most of the individual aspects in amylopectin

32 formation, structure and degradation; the story remains incomplete, as most of this

33 understanding has been gained from experiments with only a limited number of barley

34 varieties, limitations in the technology for structural measurement, and since no data is

35 available to link structure to fermentable sugar profiles.

36

37 **Keywords:**

38 barley; amylopectin; starch structure; isoamylase; limit dextrinase; malting quality; brewing

39

40 **Introduction**

2

41   Barley (*Hordeum vulgare* L.) like many other significant cereal crops belongs to the grass

42   family *Poaceae*. The species believed to have originated in the Fertile Crescent of the Middle

43   East, with archaeological evidence suggesting cultivation dated as far back as 7000 to 6000

44   B.C. (Wendorf, Schild et al. 1979, Zohary and Hopf 2000). Barley has a very wide and

45   diverse geographical distribution, with cultivation in areas from within the Arctic Circle, to

46   the tropics and from sea level to the high plains of the Ganges (Körnicke 1985). The

47   estimated global production area is around 57.2 million ha, yielding approximately 133

48   million metric ton of grain annually. This makes barley the fourth largest grain crop produced

49   globally.

50   Barley is used as a food source for animals and human but the more significant use is in

51   the production for alcoholic beverages such as beer and whiskey. Pre-history records suggest

52   barley was used to make a wine approximately 10,000 years ago. Regardless of the use, the

53   primary purpose is to utilize starch as an energy source. While starch is a very simple

54   molecule in terms of its chemical composition, ie glucose, its structure is more complex,

55   being comprised of two polymers, amylose and amylopectin with the latter being three times

56   more abundant. This review will focus on amylopectin, its structural development in barley

57   and its influence on malting and brewing.

58

59   **Grain quality and end use**

60   Barley is grown for feed, food or used in industrial applications such as malting (Ullrich

61   2011, Gous, Gilbert et al. 2015). Complex genetic, physiochemical properties, and their

62   associated interactions have resulted in continued attempts to improve barley grain quality

63   (Gous, Gilbert et al. 2015). A number of these quality traits directly determine potential end

64   use. Foremost of these quality traits is grain size, with plump grain desired by both maltsters

65   and the animal industries alike (Fox, Panozzo et al. 2003, Gous, Gilbert et al. 2015). Grain

66 plumpness is determined by the endosperm, which contains starch, non-starch

67 polysaccharides, protein and lipids (Fox, Panozzo et al. 2003), and is very much influenced by

68 the genotype and growing environment (Fox, Kelly et al. 2006). As such, plumpness is often

69 used as a proxy for potential starch content by many users.

70 Starch and protein are the most important compositional components of the endosperm,

71 and are often targeted by industry as traits of interest, due to their significant contribution to

72 nutritional and commercial value (Fox, Panozzo et al. 2003, Gous, Gilbert et al. 2015). Starch

73 is the most abundant component of the endosperm at around 65% (by weight); with additional

74 carbohydrates, such as sugars and non-starch polysaccharides (β-glucan and arabinoxylans)

75 contributing up to approximately 80% of total dry grain weight (Fox, Panozzo et al. 2003,

76 Gous, Gilbert et al. 2015).

77

78 **Starch**

79 Barley starch is a complex polymer comprised of a mixture of amylose and amylopectin,

80 both of which are built from glucose molecules linked via α-(1→4) glycosidic bonds forming

81 linear chains (for amylose); while some chains have α-(1→6) glycosidic branches forming

82 amylopectin (Vilaplana and Gilbert 2010, Gous, Hasjim et al. 2013) (Figure 1). These

83 polymers exist in a ratio of approximately 25% amylose and 75% amylopectin of total starch

84 in the grain (Newman and Newman 1992), but genetic mutations do allow for 'waxy' type

85 varieties which contain 100% amylopectin. While conversely, high amylose barley varieties

86 exist with amylopectin levels of up to 40% (Swanston, Ellis et al. 1995, MacGregor, Bazin et

87 al. 2002, Morell, Kosar-Hashemi et al. 2003). Waxy and high-amylose starches have

88 numerous applications in both industrial and food industries. Waxy starches are used in the

89 food industry as emulsifiers, thickeners and freeze-thaw stabilizers (Beckles and

90 Thitisaksakul 2014). However due to the high rate of conversion waxy-starches are also as

4

91 livestock feed (Beckles and Thitisaksakul 2014). Its industrial application is as an additive in

92 the paper and textile manufacturing process (Beckles and Thitisaksakul 2014). High-amylose

93 starches on the other hand are used as edible films, confectionary and bioplastics. It also has

94 health benefits due to its low digestibility, making it ideal as a colon drug delivery system

95 (Beckles and Thitisaksakul 2014).

96   Amylopectin is a highly branched polymer with numerous short-chained branches with a

97 high molecular weight of 10^{7-9} Da (Vilaplana and Gilbert 2010), making up approximately

98 35% by weight of total grain composition. This makes amylopectin the most abundant single

99 component in barley, ahead of protein (approximately 10% by weight) and amylose (10%

100 weight). Amylose is an almost linear polymer with few long-chain branches and a moderate

101 molecular weight of 10^{5-6} Da (Vilaplana and Gilbert 2010).

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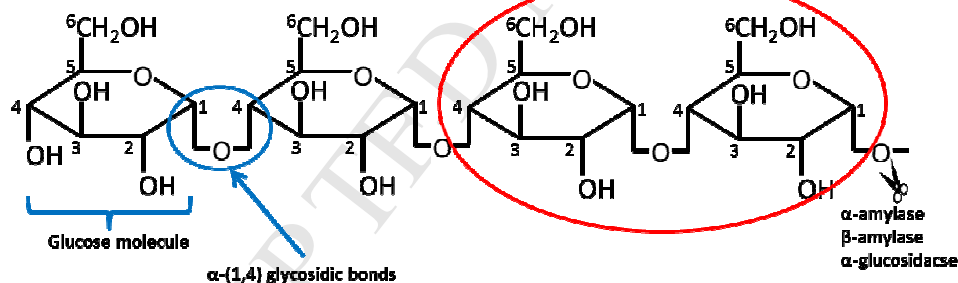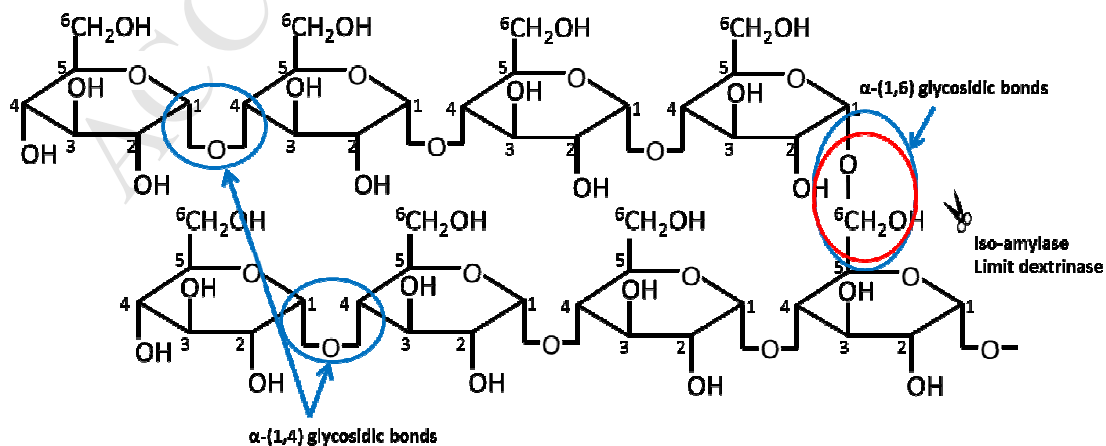
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104   **Figure 1:** a) Shows glucose molecules linked together with α-(1→4) linkages to form

105   amylose which is cut by α-amylase, β-amylase and α-glucosidase to form maltose (glucose +

106   glucose) from the non-reducing end. b) Shows an amylopectin chain made up of chains

107   formed by α-(1→4) linkages and branched by α-(1→6) linkages. These branches are cut by

108   debranching enzymes Isoamylase and limit dextrinase, resulting in more straight chains for

109   hydrolysis by α-amylase and β-amylase.

110

111   Figure 2 shows the development of the amylose and amylopectin from initial chains

112   (structure level 1) to the final structure of the endosperm (structure level 6). Structure levels 3

113   and 4; show the lamella layers with amorphous lamella and crystalline lamella that form the

114   granule. Most of the branch points are located in the amorphous lamellae, while the outer

115   chains are present in the crystalline lamellae which form double helices (Tester, Karkalas et

116   al. 2004, Gous, Gilbert et al. 2015).

117   The branches associated within amylopectin can be categorized into A, B and C chains,

118   depending on their lengths and relative position (Nakamura 2002) (Figure 2). A-chains are

119   comprised of short branches found on the outer fringes of the amylopectin molecule, while B-

120   chains are longer with one or more branches occupying the inner molecule. The C-chain

121   consists of a reducing terminal glucose residue and serves as an important factor in the

122   production of the B-chains (Wang, Henry et al. 2014, Gous, Gilbert et al. 2015). Although

123   amylopectin's shorter branches are confined to a single lamella, some of the longer B-chains

124   are trans-lamellar and span more than one crystalline lamella (Tester, Karkalas et al. 2004,

125   Wang, Henry et al. 2014).

126   Starch granules exist as either large A type granules or smaller B type granules. A type

127   granule are approximately four times larger than B type, while B type granules are

128   approximately four times as abundant as A type granules (Figure 2 – Level 5).

129

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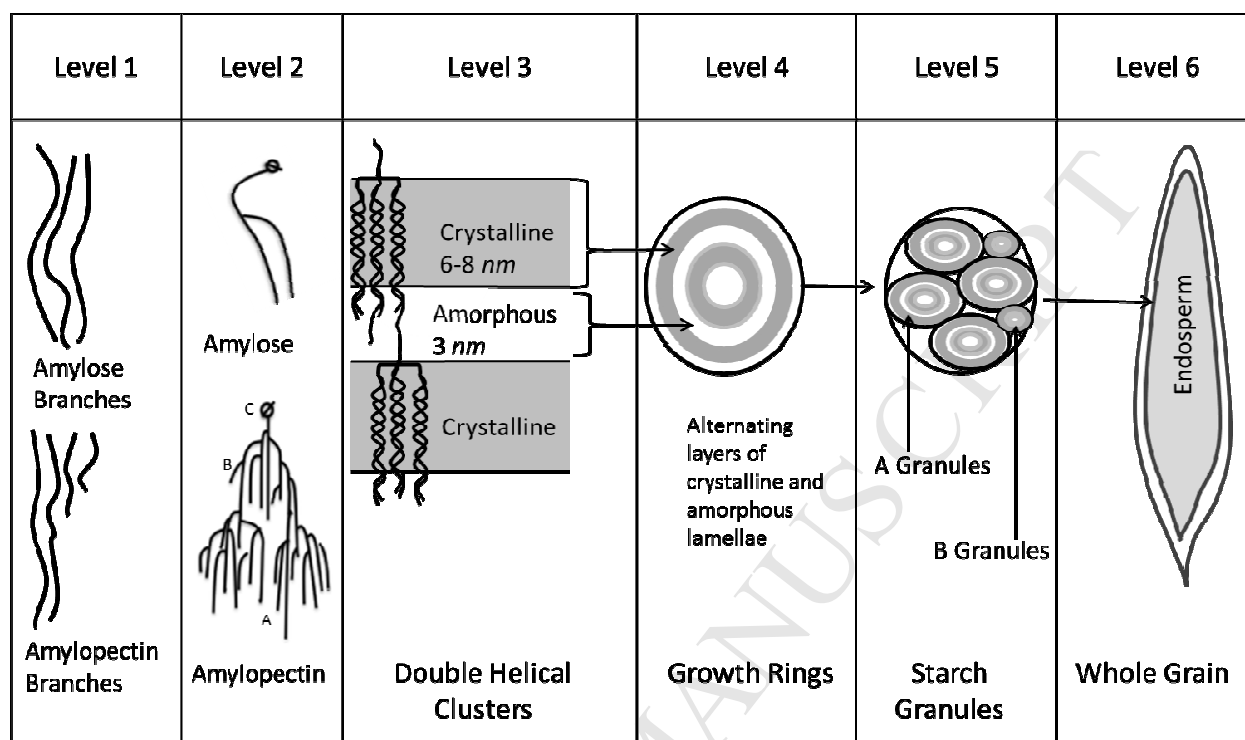
Figure 2: The six levels of starch structure in cereal grain (modified from Gilbert 2014). Modified with A, B and C chains labelled on the amylopectin polymer and the width of the crystalline and amorphous layers in nanometers (nm).

Starch Biosynthesis

The enzymatic stepwise process in which amylose and amylopectin are synthesised is shown in Figure 3. Since this review is focused on debranching enzymes (iso-amylase and limit dextrinase), all other starch synthesizing enzymes will only be briefly discussed. Starch is synthesised and stored in a granular form in plastids during photosynthesis; whereas for long-term storage, starch is stored in amyloplasts such as those found in the grain endosperm (Wang, Henry et al. 2014). Starch synthase enzymes elongate chains by catalysing the transfer of glucose units from ADP-glucose to the non-reducing ends via α-$(1\rightarrow4)$ linkages (Fujita, Yoshida et al. 2006, Wang, Henry et al. 2014). Starch structure varies between botanical

7

145 organs, plant species and varieties, and environmental conditions, with these structural

146 differences brought about by differences in starch biosynthesis, involving multiple enzymes

147 (Gous, Gilbert et al. 2015). These multifaceted biosynthetic pathways involve ADP-glucose

148 pyrophosphorylase (AGPase), starch synthases (SSs), starch branching enzymes (SBE), and

149 debranching enzymes (DBE), of which several isoforms play distinct roles (Wang, Henry et

150 al. 2014) (Figure 3). The presence and pleiotropic effects of these enzymes and isoforms,

151 complicates starch biosynthetic pathways (Wang, Henry et al. 2014). The biosynthetic

152 interactions of all starch synthesis genes in a single cultivar, under the influence of external

153 conditions, are not fully understood due to their complexity, although various relationships

154 have been proposed (Jane, Chen et al. 1999, Kharabian-Masouleh, Waters et al. 2012, Witt,

155 Doutch et al. 2012, Syahariza, Sar et al. 2013, Witt and Gilbert 2014).

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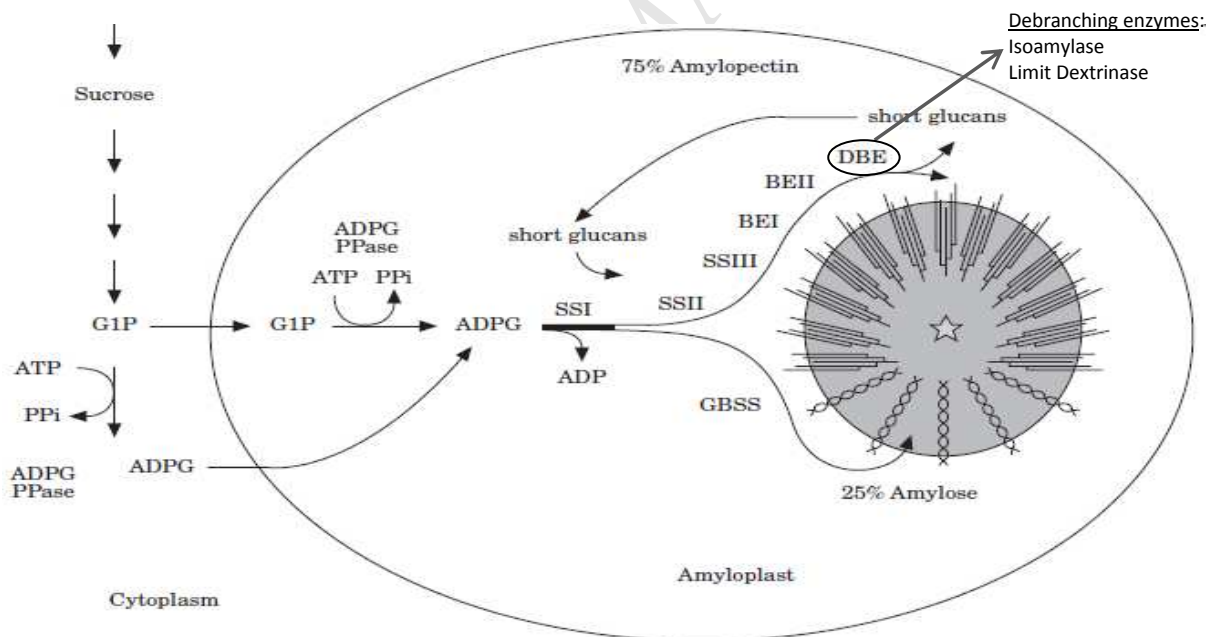
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159 **Figure 3**: Starch synthesis pathway. (key genes: SS starch synthase, BE starch branching

160 enzyme, DBE starch debranching enzyme, GBSS granule bound starch synthase)

161

8

162   Debranching is the last step in the synthesis of amylopectin. A physiological relationship

163   between the branching and debranching enzymes has been observed in barley (Sun, Sathish et

164   al. 1998, Sun, Sathish et al. 1999) which has been proposed to balance the structure when

165   forming the layers in starch granules (Wu and Gilbert 2010). In barley, the dominant DBEs

166   are isoamylases. These enzymes are present in all tissues of the developing caryopsis but are

167   most abundant in the developing endosperm (Radchuk, Borisjuk et al. 2009). Another DBE

168   also present in the developing endosperm (Sissons, Lance et al. 1992, Sissons, Lance et al.

169   1992, Radchuk, Borisjuk et al. 2009) or more specifically in barley as limit dextrinase (LD).

170   Limit dextrinase is predominantly involved in debranching amylopectin during germination

171   and is an important malting quality trait (discussed below).

172

173   The enzymatic steps in the synthesis of typical starch remain fixed. However, changes to

174   specific enzymes in the starch synthesis pathway, such as in one or more of the starch

175   synthase genes, may result in a higher amylopectin (waxy) or amylose content. Additionally,

176   the growing environment can have a major influence on enzyme activity, final granule size

177   and starch structure. Excessively high field temperatures during grain fill may reduce the size

178   of the large A granules and/or increasing the ratio of small B granules by either reducing the

179   activity of the starch synthase enzymesor impacting on starch granule initiation as suggest

180   previously (MacLeod and Duffus 1988).

181

182   **Starch debranching enzymes are involved in both synthesise and degradation**

183   In general, starch debranching enzyme (EC 3.2.1.61) hydrolyses α-(1$\rightarrow$6) glycosidic

184   linkages during amylopectin synthesis (Myers, Morell et al. 2000, Wang, Henry et al. 2014).

185   As mentioned above, two genes for DBE have been identified in barley, the first being

186   isoamylase and the second being limit dextrinase (Doehlert and C.A. 1991, Wang, Henry et

187 al. 2014). At least three different isoforms of the isoamylase (Isa) that can debranch

188 amylopectin have been identified, and have been classified as *Isa1*, *Isa2*, and *Isa3* (Nakamura

189 2002). These differing isoforms have been reported to be present and active in differing barley

190 tissue including the pericarp, aleurone but specifically the endosperm, during grain filling

191 (Radchuk, Borisjuk et al. 2009). The *Isa1* isoform is the most active in the endosperm and for

192 the longest period of time during grain filling. The down regulation and absence of *Isa1*

193 modifies the well-ordered structure of amylopectin (Nakamura 2002), resulting in the

194 formation of a 'sugary' amylopectin, with lots of short chains (Burton, Jenner et al. 2002).

195

196   The expression of a mutant anti-*Isa1* gene has shown to lower starch gelatinization

197 temperature and viscosity by producing a less crystalline starch structure (Fujita et al. 2006).

198 However, in contrast to *Isa1*, the absence of *Isa2* does not result in severely abnormal starch

199 morphology, despite *Isa2* being required together with *Isa1* for activity of the Isa heteromeric

200 enzyme (H.S., Iqbal et al. 2009, Kubo, Colleoni et al. 2010). Thus, changes in Isa expression

201 and subsequently any variation in the regulation of the protein expression, will change the

202 physiochemical properties of starch. Consequently, these changes may impact on final grain

203 quality and end use.

204

205   The locus for *Isa1* is on 7HS around the centromere (Burton, Jenner et al. 2002) while the

206 locus for the LD gene is at around 50 centiMorgans also on 7HS (Burton, Jenner et al. 2002)

207 (Figure 4).

208

209 **Starch molecular characterisation**

210   Size exclusion chromatography (SEC), also known as gel permeation chromatography, is

211 commonly used to characterize starch polymer structure. In SEC, molecules in a mobile

212 solvent (eluent) are separated by molecular size, due to their hydrodynamic volume (V_h), or

213 their corresponding hydrodynamic radius (R_h) (Cave, Seabrook et al. 2009, Gous, Gilbert et

214 al. 2015). For linear polymers like debranched starch, there is a unique relationship between

215 R_h and molecular weight, however this does not hold true for complex branched polymers

216 (Gous, Gilbert et al. 2015).

217

218   To determine the R_h or weight-average molecular weight ($\bar{M}_W$), three different types of

219 detectors are commonly used in SEC (Vilaplana and Gilbert 2010). The differential refractive

220 index (DRI), multi-angle laser light scattering (MALLS), and viscometry detector, are either

221 used individually or in combination to provide comprehensive starch structural information

222 (Gous, Gilbert et al. 2015). The DRI provides the weight distribution of molecules as

223 functions of R_h, while MALLS detector provides the $\bar{M}_W$ and the z-average size ($R_{g,z}$) as the

224 radius of gyration while the viscometry detector provides the distribution of molecules (Gous,

225 Gilbert et al. 2015). It is however essential that starch samples used for molecular structural

226 characterization be prepared without aggregation, loss, degradation or retrogradation. To

227 prevent these negative effects, samples are dissolved in an eluent comprised of dimethyl

228 sulfoxide and lithium bromide (Hasjim, Lavau et al. 2010, Vilaplana and Gilbert 2010).

229

230   To accurately determine V_h from elution time, it is essential that calibrations be

231 performed using narrowly-dispersed linear glucan pullulan or dextran standards with known

232 molecular weights. Although SEC is commonly used in starch characterization it is restricted

233 by band broadening, shear scission, and low recovery of larger molecules like that of

234 amylopectin (Cave, Seabrook et al. 2009). Part of the problem is that the appropriate

235 standards are not available for molecules greater than ~ 50 nm in size (Gous, Gilbert et al.

236 2015). It is also problematic that shear scission is unavoidable in SEC, making the data

11

237 generated for large molecules only semi-quantitative (Gous, Gilbert et al. 2015). Regardless

238 of these limitations, qualitative and semi-quantitative comparisons of size distributions can

239 still be performed with SEC data. Furthermore, when the SEC samples are analysed under the

240 same conditions, the effects of shear scission would be similar across all samples (Gilbert, Wu

241 et al. 2013, Gous, Gilbert et al. 2015), so that relative size distributions are at least

242 determined.

243

244 **Starch and its role in Malting and Brewing quality**

245   The fermentation of sweet liquids into alcoholic beverages such as beer has been

246 conducted by humans for many millennia (Bamforth 2008). Thousands of years ago, we

247 started to understand the process of using germinated grain (malted) to enable this process.

248 Where today, commercial malting is an industrial process carried out in nearly every country

249 of the world where there are breweries. Malt is derived from the germination and then drying

250 of cereal grain in a process that takes between 6 to 8 days, depending upon the type of cereal

251 and malt quality required (Figure 5a). During this process, some internal components such as

252 proteins are reduced to amino acids and cell wall components (β-glucan and arabinoxylan) are

253 reduced to their base sugar units. More importantly, starch degrading enzymes (SDE) are

254 released and/or synthesised. These enzymes include

255   i.   limit dextrinase to cut α-(1→6) linkages on amylopectin or amylose,

256   ii.   α-amylase to hydrolyse chains into smaller fragments,

257   iii. β-amylase cut maltose and, to a minor extent,

258   iv.   α-glucosidase cut glucose from the non-reducing ends of the chain fragments

259   (respectively).

260

261 However, during malting the master's objective is to have as little starch as possible degraded

262 to minimize malting losses. The majority of starch hydrolysis occurs during the first stage of

263 brewing, called mashing where the starch has been gelatinized to enable the efficient access of

264 the starch hydrolysing enzymes (Figure 5b). The enzymic breakdown of starch into simpler

265 sugars including maltose and glucose provides a major part of the food-energy source for

266 yeast and the fermentation process (Figure 5b).

a).

The Malting Process

Steeping	Germination	Kilning

barley seeds → (H₂O, air, H₂O) → steeped barley → (water spray, air) → green malt → (warm air) → malt

≈12% H₂O   ≈45% H₂O   ≈45% H₂O   ≈4% H₂O

raise moisture content   enzyme induction, cell wall digestion   drying, flavor development

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b).

The Brewing Process

Malt

MILLING   MASHING   LAUTERING   BOILING   WHIRLPOOLING

FILTERING   MATURING   FERMENTING   COOLING

PACKAGING   DISTRIBUTION

LAGER LORRY

www.ibdasiapac.com.au

267

268   **Figure 5**: Flow diagrams for the (a) malting and (b) brewing processes. Figure 5a sourced

269   from www.scitechconnect.elsevier.com and Figure 5 b sourced from www.ibdasiapac.com.au.

270

271   A number of parameters indicate malt quality including hot water extract (the amount of

272   available solubilised material); diastatic power (combined measure of starch degrading

273   enzyme activity) and fermentability of the wort (extract sugars utilized by yeast for

274   fermentation). Extract and diastase can be predicted somewhat from the analysis of the barley

275   grain (MacGregor 1996), which would suggest a probable association with starch and protein

276   content, respectively. For the following discussing, only the above three parameters

277   mentioned above will be discussed as they relate most to the hydrolysis of starch and the final

278   profile of the important fermentable sugars.

279

280   **Hot Water Extract**

281   The hot water extract (HWE), or wort, produced from the mashing and lautering stages, is

282   the one of most important brewing traits as it contains numerous sugars, amino acids,

283   peptides, lipids, vitamins and minerals that could be used by the yeast and or contribute to

284   beer quality (Figure 5b). The quality of the HWE is influenced by a number of factors. Firstly,

285   barley grain composition is a contributing factor but composition is affected by numerous

286   environmental factors including; growing conditions, temperature, fertiliser use, nitrogen

287   availability and moisture. In general, it is well known that higher protein is negatively

288   correlated with extract (Bishop 1930, Briggs 1978). These factors do not directly impact on

289   HWE, however their effect is observed on the content and compositions of components that

290   do contribute to HWE, such as starch quality and presumably access of starch hydrolysing

291   enzymes to starch during mashing.

292

14

293   Secondly, both physiological and biochemical components of the grain can influence

294   HWE. The type of barley i.e. two or six rowed, with the plumper two rowed having higher

295   level of extract. The husk thickness, grain size, protein, starch, non-starch polysaccharide

296   contents all impact on extract levels. Also high protein content is related to higher enzyme

297   production (Arends, Fox et al. 1995). Barley cultivars with the optimum combination of these

298   traits consistently produce higher extract.

299

300   Thirdly, the malting process (grain modification) is singularly the greatest aspect

301   affecting hot water extract. During malting, enzymes that degrade proteins, non-starch

302   polysaccharides and starch, are either synthesised or released from their bound forms. The

303   objective during the malting process for most maltsters is to maintain high extract levels and

304   yet achieve this at low levels of protein modification to ensure the desirable foam stability in

305   the resultant beer.

306

307   Finally, the mashing process influences HWE, where there are a number of variables

308   that affects the level of extract, such as pH, mash time, mash temperature, grist (particle) size

309   and grist to liquor ratio. While these aspects determine the quality of the final HWE (and

310   fermentable sugar profiles), most these aspects are determined by the genetic attributes of the

311   starting barley. For example, high diastatic power (DP) barley varieties produce high levels of

312   malt DP under optimal conditions.

313

314   **Diastatic Power**

315   Diastatic power is the term used to describe the collective activity of SDE in malt. Four

316   enzymes, α-amylase, β-amylase, limit dextrinase and α-glucosidase, have been identified

317   during malting and mashing (Osman 2002, Briggs, Boulton et al. 2004), although little

318   attention has been paid to α-glucosidase. There are genetic and environmental effects on α-

319   amylase, β-amylase and limit dextrinase (Arends, Fox et al. 1995) with each having an

320   optimal pH and temperature range. Industry methods used to measure DP vary considerably

321   in a number of aspects including; substrate, pH and assay temperature which may in turn

322   differing impacts one or more enzyme. Most methods only provide data on the enzymatic

323   potential under these conditions, which are far removed from industrial mashing conditions

324   (Henry and McLean 1984). The relationships between DP level, individual DP enzymes and

325   either HWE and/or fermentability have been shown (Evans, van Wegen et al. 2003, Evans, Li

326   et al. 2008, Evans, Dambergs et al. 2010). These studies did demonstrate that multi linear

327   equations of parameters including Kohlbach Indext (KI), α-amylase, LD, β-amylase and its

328   thermostability could predict 70-90% of variation in fermentability compared to <50% or less

329   for DP. These results explain why DP has been found to be potentially a misleading measure

330   of fermentability in commercial brews (Evans, Li et al. 2007). It is clear that the prediction of

331   fermentability will be further improved by inclusion of measures of starch structure and

332   complexity, as well as inclusion of sugars produced from non-starch components.

333

334   Low DP barley varieties only produce low to moderate levels of SDE which also affects

335   the fermentable sugar profile and may leave a higher level of unfermentable dextrins. Such

336   varieties are being increasingly sought after by craft brewers and brewers that brew with

337   100% malt and do not include starch adjuncts such as rice or corn grits. The basis for this low

338   DP malt selection is that dextrins and limit dextrins may have a positive effect on the

339   mouthfeel of the beer (Langstaff and Lewis 1993).

340

341   While there is a synergistic relationship between the individual DP enzymes in mashing

342   (Evans 2012), LD is critical for maximising fermentability as it is responsible for the

343 hydrolysis the branches of amylase and amylopectin. This results in shorter chains which can

344 then be hydrolysed by both the amylases. As previously indicated, the role of this debranching

345 enzyme is to hydrolyse α-(1→6) linkages. This follows from LD's physiological role which

346 is to produce suitable substrates for amylases, to ensure the complete hydrolysis of starch into

347 sugars to supply the growing embryo during germination. Humans have learned to co-opt this

348 perfectly designed hydrolysis of starch into sugars for fermentation. Specific information on

349 LD is discussed below.

350

351 **Fermentability**

352   The fermentation of wort is probably the most critical phase of the brewing process as

353 uncontrolled or slow fermentations cause delays in the final processing of beer. Several

354 factors impact wort fermentability, but the main purpose of fermentation is the utilisation of

355 the fermentable sugars to produce alcohol. Maltose is usually the most abundant sugar

356 produced during mashing, followed by maltotriose. The particular mashing style can

357 influence the sugar profile with the lower temperature congress mash (45°C ramp to 70°C)

358 resulting in a ~~higher~~ lower level of maltose compared to the high temperature infusion style

359 (constant 65°C) (Evans et al. 2005). In addition, grist:liquor ratio and pH also impact on sugar

360 production. A recent studied showed little difference between fermentability and individual

361 fermentable sugars when derived from low and high temperature mash under varying grist to

362 liquor ratios (1:2, 1:3 and 1:4) (Fox 2016). All these factors, ie.grist:lqiour, pH, mash

363 temperature, also influence the activity of the individual DP enzymes. However, current malt

364 parameters (such as HWE and DP) are unreliable indicators of fermentability during actual

365 brewing conditions. To account for other possible variables, recent efforts have identified a

366 number of other malt factors to predict fermentability, where α-amylase ~~and total LD (activity~~

367 ~~and thermostability)~~, Kolbach Index, and the total β-amylase (activity and thermostability)

17

368   were combined (Evans et al. 2005, 2008, 2010). However, from these studies, LD and it

369   thermostable isoform, failed to show any contribution to fermentation. While these studies

370   showed several malt parameters that could give an indication of proved to be important

371   variables that influences fermentable sugar production, there was no measure of fermentable

372   sugars. Further these studies did not quantify the barley starch structure, which would help

373   explain the level of efficiency of the enzymes in producing fermentable sugars.

374

375   **Limit dextrinase**

376   Compared to Isa, there is less information available on the role of LD in starch

377   biosynthesis. Its bi-functional role in starch synthesis and then degradation has been reported

378   in barley (Dinges et al. 2003). The primary function of LD is the hydrolysis of α-$(1\rightarrow6)$

379   linkages in α-limit dextrins of amylopectin (Bojstrup et al. 2014; Huang et al. 2014). In

380   barley, three different isoforms of LD are found which are (i) insoluble when bound, (ii)

381   inactive when soluble (latent) and (iii) active when free, where only active free LD

382   contributes to starch mobilization and digestion (MacGregor 2004).

383

384   Limit dextrinase expression is regulated by a single gene (Burton, Zhang et al. 1999,

385   Kristensen, F. et al. 1999), with peak expression five days post germination. An extended

386   germination period may however be required for optimal LD expression and mobilisation

387   during malting (Kristensen, F. et al. 1999), resulting in superior wort sugars and

388   fermentability (Bamforth 2003). Without free LD available in the HWE, excessive levels of

389   branched dextrins could slow fermentation (MacGregor 2004). During germination LD level

390   increases, with maximum activity reached after eight days. Limit dextrinase will survive

391   kilning with up to around 80% activity. The observed increase in total limit dextrinase activity

392   during germination is due to a bound form being released by the action of proteinase

393   (Longstaff and Bryce 1993). Purified limit dextrinase has an optimal pH of 5.5 and

394   temperature at 50°C (Sissons, Lance et al. 1992), while under Congress mashing conditions,

395   LD has a similar pH optimum but higher temperature optimum of between 60°C to 63°C

396   (Stenholm and Home 1999).

397

398   ~~to 75°C, which is comparable to a kilning regime for lager malt, with up to 75% of viable~~

399   ~~enzyme remaining in solution~~ (Bamforth 2003). ~~However, only 13% of LD survived kilning;~~

400   ~~comparable to ale malt production with a maximum temperature of 95°C is reached~~

401   ~~(Bamforth 2003).~~ Purity of the unbound enzyme form may influence activity; with a near total

402   loss in LD activity in under 10 min at 65°C ~~when pure~~. Low level of free LD activity is

403   attributed to a combination of endogenous inhibitors and a limit dextrinase inhibitor (LDI)

404   bound in key endosperm components (Huang et al. 2014). The release of LD from its inhibiter

405   promotes starch digestion and increases fermentable sugars formation. It is suggest that LD

406   bound to the LDI is ~~the~~ a limiting factor for complete starch digestion during brewing

407   (Bamforth 2003, Huang, Cai et al. 2014).

408

409   The LDI is synthesised during grain fill and later than the synthesis of LD with a decrease

410   in the free LD form and an increase in the bound LD form (MacGregor 2004). ~~. and is~~

411   ~~gradually degraded during malting. However, during malting, LD activity is not only~~

412   ~~determined by enzyme concentration but also by the presence of the LDI (MacGregor 2004).~~

413   When LD was inhibited, there was a reduced number of B granules formed and changes to the

414   chain length of the amylopectin molecule (Stahl, Coates et al. 2004). LD inhibition also

415   effects of the expression of starch synthases and starch degrading amylases (Stahl, Coates et

416   al. 2004), supporting the concept of a physiological balance between the genetic control of

417   genes involved in starch synthesis and degradation.

418   *In-vitro* experiments showed the release of LD can be promoted by the addition of a

419   reducing agent such as dithiothreitol, which is not feasible in brewing because of its toxicity

420   and its strong foul odour. The addition of exogenous protease may however remove LDI

421   (Longstaff and Bryce 1993). It was also demonstrated an increase in LD release was

422   attainable by decreasing the mash pH; with significantly increased LD activity obtained with

423   pH less than 5.0 (Longstaff and Bryce 1993). Although LD may increase starch hydrolysis,

424   resulting in improved fermentable sugar profiles, high dextrin levels may alter starch

425   gelatinisation properties which may have a negative impact on wort filtration and final

426   product quality (Bamforth 2003).

427

Genetic Variation in Isa and LD

429   Both the Isa and LD genes are located on chromosome 7HS (Li et al. 1999). In addition,

430   this region has been associated with increased HWE and DP in molecular mapping

431   populations (Elia et al. 2010) **(refs)**, ~~regardless of which marker technology was applied~~. This

432   locus has been identified in a number of diverse populations including those where a wild

433   parent was used. Nevertheless, there can be some inconsistency in the identification of the

434   QTL between populations. Interestingly, where a QTL for HWE was reported, it was for an

435   infusion style of HWE method (high temperature mashing style) (Islamovic, Obert et al.

436   2014).

437   In two feed grain studies, Abdel-Haleem, Bowman et al. (2010) identified a QTL for total

438   starch content; while in a similar region Gous, Lawson et al. (2012) identified a QTL for dry

439   matter disappearance. The same region has been associated with increased grain size in

440   barley, presumably through increased total starch content. This region has also been

441   associated with QTL for a combined measure of starch degrading enzymes namely diastatic

442 power, specifically β-amylase. ~~but QTL for neither of the individual amylases have been~~

443 ~~reported at this loci. It could be then proposed this diastatic power QTL could be LD.~~

444

445   At the gene level, single point mutations (single nucleotide polymorphism [SNP]) as well

446 as sequence deletions have been identified for *Isa1*, resulting in changes in starch tructure and

447 granule size. As the *Isa1* is responsible for hydrolysising the α-1,6 branches from

448 amylopectin, changes in amylopectin structure have been identified in wild types where there

449 was a deletion in the *Isa1* gene (Burton, Jenner et al. 2002). The limit dextrinase gene ~~LD~~

450 ~~form~~ also has single point mutations resulting in an amino acid substitution giving increased

451 thermostability in an *in-vitro* assay although this has yet to be confirmed under mashing

452 conditions ~~in the mash~~ (Yang, Westcott et al. 2009). Figure 4 shows the amino acid sequence

453 from studies sequencing the limit dextrinase gene. Substitutions at 233 Thr/Ala and 885

454 Ala/Ser resulted in an increase thermostability of approximately 10°C. However, the samples

455 tested were all from a single field experiment and the LD activity was assayed at 57°C and not

456 during a mashing experiment, so variation in expression in the same varieties from differing

457 locations would be expected due to environmental influence ~~on protein and diastase~~ (Arends,

458 Fox et al. 1995). While LD thermostability was assayed in many barley varieties, the

459 thermostable form seems to be less common in barleys bred specifically for malting quality.

460 The thermostable LD form coupled with the thermostable β-amylase form, such as Sd2H,

461 could provide malts with increased total enzymatic power (diastase) but also allow the

462 thermostable enzymes to be more active in high temperature mash systems. In addition, they

463 could provide a higher level of fermentable sugars and also be more suited to high gravity

464 mashing where the ratio of malt grist to water can be as low as 1:1.9.

465

466   With regards to gene expression, a single study of LD expression during germination

467   (four days) of four USA malting varieties (of which two were 2-rowed and the other two were

468   6-rowed), showed LD expression levels differed between four varieties. LD was positively

469   correlated with fine extract, based on a ramping style method (Congress), using long term

470   malting quality data of the four varieties. While the two 6-rowed varieties had the highest

471   level of diastase, they didn't have the highest level of α-amylase. This may have been due to

472   either a lower starch to protein ratio or higher protein. The former wasn't reported. Both the

473   two 2-rowed varieties had the highest level of amylase and one of these, Harrington, had the

474   highest level of LD (Lapitan, Hess et al. 2009). Harrington has the low thermostable allele

475   (Yang, Westcott et al. 2009), however in any low temperature mashing, the slow ramping

476   could be conducive for optimal activity of LD. But the major drawback with this mashing

477   style is the low temperature hasn't provided conditions for starch to gelatinise, hence there is

478   no starch degrading enzyme activity. This was suggested in a study where the same samples

479   used in the Yang, Westcott et al. (2009) study had previously been tested for malting quality

480   using a Congress or infusion mash (Evans, van Wegen et al. 2003). However, individual

481   HWE or LD results were not reported.

482

483   **Challenges and implications**

484   Grain quality and composition plays an integral role in brewing and often determines

485   malt quality. Initially, plump grained varieties are selected, in order to obtain the best quality

486   malt, with relatively high levels of SDE activity (e.g. α-amylase, β- amylase and LD) for

487   fermentable sugar production (Fox, Panozzo et al. 2003). Breeders have selected genotypes

488   with plump grain kernels conferring high starch and relatively low protein content, with

489   commercial cultivars receiving a premium for grain size and protein, but not directly starch.

490   Starch is readily hydrolysed into maltose, maltotriose, sucrose, glucose and fructose by ~~Isa1~~

491   and LD during mashing. It is these wort sugars that are fermented by yeast during

492   fermentation. As indicated in earlier sections, numerous studies have investigated the impact

493   of SDE on fermentability (Buttimer and Briggs 2000, Kanauchi and Bamforth 2008).

494   However, little is still known on how allelic variation in SDE expression impacts on starch

495   structure and the variation in structure on fermentability; with starch structural studies

496   predominantly focused on SSs. Alternatively, some studies have attempted to quantify and

497   understand the impact of starch structural changes on malt quality and brewing efficiency

498   (MacGregor 1996, MacGregor, Bazin et al. 1999, Izydorczyk, MacGregor et al. 2001).

499

500   In depth studies on starch structure in brewing have been restricted by several limitations,

501   both technical and environmental. Barley grain quality is in largely determined by genotype,

502   environmental conditions and their subsequent interactions, which also contribute to potential

503   starch structural changes (Gous, Hasjim et al. 2013, Gous, Gilbert et al. 2015).

504   Characterisation of these structural changes is complicated by technical limitations resulting

505   in the incomplete starch dissolution, retrogradation and shear scission etc. It was shown

506   however that an increase in LD release by the endosperm during mashing malting will result

507   in an increase in fermentable sugar production. An extensive search of the literature could not

508   identify any discernible information linking SNP in LD and SDE expression, starch molecular

509   structure and properties. Most of the studies focused on either how starch structural changes

510   affected functional properties; or the identification of SNPs and their impact on fermentation.

511   With the notable absence of comprehensive studies on allelic variation on LD and SDE

512   expression, their impact on starch structure, and how these structural changes impact on grain

513   quality. At most, studies on LD focused on fermentable sugar production without linking its

514   function to Isa and SDE expression and activity.

515

516 **Conclusions**

517 The basis for amylopectin composition is the linkage of thousands of glucose molecules but

518 the final structure can be extremely variable in the number of branches and length of chains.

519 Environment has a major influence on the structure, but of most interest is the action of limit

520 dextrinase in controlling the level of branching during amylopectin synthesis and then needed

521 for complete debranching to assist amylases to hydrolysis the chains into smaller, fermentable

522 glucose based sugars such as maltose and maltotriose. While the relationship between limit

523 dextrinase and amylopectin structure is starting to be understood, ~~understand,~~ there is still a

524 significant gap in the knowledge of any environmental impact of amylopectin structure, the

525 possible rate of hydrolysis and final profile of fermentable sugars for brewing. The efforts by

526 barley breeders to increase SDE has been done with little attention paid to the substrates. It

527 will now possible to understand structure and the full process of amylopectin synthesis and

528 degradation into fermentable sugars.

529

530

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745 **Table 1.** Abbreviations and definitions

AA	α-amylase	Enzyme that cuts randomly along the chains of amylose and amylopectin
AM	Amylose	Straight chain polymer with glucose unit, linked with α-(1→4) links. Can be over 10000 glucose molecules in a single chain. One of two polymers that make starch in plants.
AP	Amylopectin	Large branched polymer mostly α-(1→4) glucose links, with braches through α-(1→6) linkage. The larger polymer to make starch in plants. When 100% amylopectin, the starch is termed 'waxy'.
BA	β-amylase	Enzyme that cuts maltose from the glucose chains (maltose - two glucose joined together)
CLD	Chain length distribution	Distribution of glucose chains of varying lengths
DB	Degree of branching	Number of branches on amylopectin
DBE	Debranching enzymes	Enzymes that cut (cleave) the α-(1→6) linkage from the α-(1→4) chains
DPn	Degree of polymerization	Number of glucose molecules joined together
DP	Diastatic Power	Combined activity of starch degrading enzymes in malt. These enzymes are α-amylase, β-amylase, limit dextrinase and -glucosidase.
GBSS	Granule bound starch synthase	Enzyme that adds glucose molecules to lengthen the chains, specifically amylose.
HWE	Hot-water extract	Concentration of solutes extracted from malt in hot water, measured using specific gravity (^{o}Plato and % sucrose equivalent)
Isa	Isoamylase	One of the α-(1→6) debranching enzymes, active during grain filling.
LD	Limit dextrinase	Another of the α-(1→6) debranching enzymes, active during grain filling but more active during germination.
SBE	Starch branching enzymes	Enzyme that attaches chain in the 6 position to form the branches on amylopectin
SDE	Starch degrading enzymes	
SEC	Size-exclusion chromatography	Method to measure the number of glucose molecules in a chain, specifically amylose
SS	Starch synthases	Enzyme that adds? glucose molecules to lengthen the chains. Makes amylopectin specifically.

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748   10   20   30   40   50   60   70   80   90   100

749 MAVGETGASV SAAEAEAEAT QAFMPDARAY WVTSDLIAWN VGELEAQSVC LYASRAAAMS LSPSNGGIQG YDSKVELQPE SAGLPETVTQ KFPFISSYRA

750 MAVGETGASV SAAEAEAEAT QAFMPDARAY WVTSDLIAWN VGELEAQSVC LYASRAAAMS LSPSNGGIQG YDSKVELQPE SAGLPETVTQ KFPFISSYRA

751   110   120   130   140   150   160   170   180   190   200

752 FKVPSSVDVA SLVKCQLVVA SFGADGKHVD VTGLQLPGVL DDMFAYTGPL GAVFSEDSVS LHLWAPTAQG VSVCFFDGPA GPALETVQLK ESNGVWSVTG

753 F**R**VPSSVDVA SLVKCQLVVA SFGADGKHVD VTGLQLPGVL DDMFAYTGPL GAVFSEDSVS LHLWAPTAQG VSVCFFDGPA GPALETVQLK ESNGVWSVTG

754   210   220   230   240   250   260   270   280   290   300

755 PREWENRYYL YEVDVYHPTK AQVLKCLAGD PYTRSLSANG ARTWLVDINN ETLKPASWDE LADEKPKLDS FSDITIYELH IRDFSAHDGT VDSDSRGGFR

756 PREWENRYYL YEVDVYHPTK AQVLKCLAGD PY**A**R**S**LSANG ARTWLVDINN ETLKPASWDE LADEKPKLDS FSDITIYELH IRDFSAHDGT VDSDSRG**A**FR

757   310   320   330   340   350   360   370   380   390   400

758 AFAYQASAGM EHLRKLSDAG LTHVHLLPSF HFAGVDDIKS NWKFVDECEL ATFPPGSDMQ QAAVVAIQEE DPYNWGYNPV LWGVPKGSYA SDPDGPSRII

759 AFAYQASAGM EHL**C**KLSDAG LTHVHLLPSF HFAGVDDIKS NWKFVDECEL ATFPPGSDMQ QAAVVAIQEE DPYNWGYNPV LWGVPKGSYA SDPDGPSRII

760   410   420   430   440   450   460   470   480   490   500

761 EYRQMVQALN RIGLRVVMDV VYNHLDSSGP CGISSVLDKI VPGYYVRRDT NGQIENSAAM NNTASEHFMV DRLIVDDLLN WAVNYKVDGF RFDLMGHIMK

762 EYRQMVQALN RIGLRVVMDV VYNHLDSSGP CGISSVLDKI VPGYYVRRDT NGQIENSAAM NNTASEHFMV DRLIVDDLLN WAVNYKVDGF RFDLMGHIMK

763   510   520   530   540   550   560   570   580   590   600

764 RTMVTKSALQ SLTTDAHGVD GSKIYLYGEG WDFAEVARNQ RGINGSQLNM SGTGIGSFND RIRDAINGGN PFGNPLQQGF NTGLFLEPNG FYQGNEADTR

765 RTMVTKSALQ SLTTDAHGVD GSKIYLYGEG WDFAEVARNQ RGINGSQLNM SGTGIGSFND RIRDAINGGN PFGNPLQQGF NTGLFLEPNG FYQGNEADTR

766   610   620   630   640   650   660   670   680   690   700

767 RSLATYADQI QIGLAGNLRD YVLISHTGEA KKGSEIHTFD GLPVGYTASP IETINYVSAH DNETLFDVIS VKTPMILSVD ERCRINHLAS SMMALSQGIP

768 RSLATYADQI QIGLAGNLRD YVLISHTGEA KKGSEIHTFD GLPVGYTASP IETINYVSAH DNETLFDVIS VKTPMILSVD ERCRINHLAS SMMALSQGIP

769   710   720   730   740   750   760   770   780   790   800

770 FFHAGDEILR SKSIDRDSYN SGDWFNKLDF TYETNNWGVG LPPSEKNEDN WPLMKPRLEN PSFKPAKGHI LAALDSFVDI LKIRYSSPLF RLSTANDIKQ

771 FFHAGDEILR SKSIDRDSYN SGDWFNKLDF TYETNNWGVG LPPSEKNEDN WPLMKPRLEN PSFKPAKGHI LAALDSFVDI LKIRYSSPLF RLSTANDIKQ

772   810   820   830   840   850   860   870   880   890   900

773 RVRFHNTGPS LVPGVIVMGI EDARGESPEM AQLDTNFSYV VTVFNVCPHE VSMDIPALAS MGFELHPVQV NSSDTLVRKS AYEAATGRFT VPGRTVSVFV

774 RVRFHNTGPS LVPGVIVMGI EDARGESPEM AQLDTNFSYV VTVFNVCPHE VSMDIPALAS MGFELHPVQV NSSDTLVRKS AYEA**S**TCRFT VPGRTVSVFV

775 **Figure 4.** Protein sequence for LD. Highlighted amino acids show thermolabile (top) and thermostable (bottom) sequence.

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- Amylopectin is the most abundant polymer in barley
- Amylopectin is highly branched as a results of branching and debranching enzymes
- Limit dextrinase is one of the debranching enzymes
- Limit dextrinase acts during grain filling and post-harvest germination
- The role of limit dextrinase in both these modes is yet to be clearly defined