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The use of asparaginase to reduce acrylamide levels in cooked food

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RESEARCH HIGHLIGHTS

Strategies to reduce acrylamide in cooked food often rely on a reduction in the Maillard reaction.

Mitigation procedures that modify the Maillard reaction may negatively affect flavour and colour.

Asparaginase may reduce acrylamide formation, while maintaining sensory quality.

This review collates research on the use of enzymes to mitigate acrylamide formation.

25

26   ABSTRACT

27   Strategies proposed for reducing the formation of the suspected carcinogen acrylamide in

28   cooked foods often rely on a reduction in the extent of the Maillard reaction, in which

29   acrylamide is formed from the reaction between asparagine and reducing sugars. However,

30   the Maillard reaction also provides desirable sensory attributes of cooked foods. Mitigation

31   procedures that modify the Maillard reaction may negatively affect flavour and colour. The

32   use of asparaginase to convert asparagine to aspartic acid may provide a means to reduce

33   acrylamide formation, while maintaining sensory quality. This review collates research on the

34   use of enzymes, asparaginase in particular, to mitigate acrylamide formation. Asparaginase is

35   a powerful tool for the food industry and it is likely that its use will increase. However, the

36   potential adverse effects of asparaginase treatment on sensory properties of cooked foods and

37   the need to achieve sufficient enzyme–substrate contact remain areas for future research.

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1. Introduction

It is now over ten years since the Swedish Food Authority and the University of Stockholm confirmed the existence of the suspected carcinogen acrylamide in a variety of heated foods (Tareke, Rydberg, Karlsson, Eriksson, & Tornqvist, 2002). Several months after the announcement, researchers showed that acrylamide is formed from asparagine and reducing sugars during the Maillard reaction (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). As shown in Figure 1, asparagine and reducing sugars take part in a conjugation reaction resulting in the formation of N-glycosylasparagine, which as a result of high temperature treatment will form a decarboxylated Schiff base. The decarboxylated Schiff base may decompose directly to form acrylamide or may hydrolyse to form 3-aminopropionamide (Hedegaard, Frandsen, & Skibsted, 2008). 3-Aminopropionamide is also believed to be an important precursor of acrylamide (Granvogl & Schieberle, 2006).

Since 2002, the food industry worldwide has collaborated with scientists, in order to reduce the levels of acrylamide in cooked foods. Mitigation techniques can be separated into three different types. Firstly, starting materials low in acrylamide precursors can be used to reduce the acrylamide in the final product. Secondly, process conditions may be modified, in order to decrease the amount of acrylamide formation. Thirdly, post-process intervention could be used to reduce acrylamide (Pedreschi, Mariotti, & Granby, 2014). While the third approach is not widely considered, an example is the use of supercritical CO_2 extraction to reduce acrylamide levels in coffee. Almost 80% of the acrylamide was removed using this technique (Banchero, Pellegrino, & Manna, 2013), although further sensory tests are needed to validate the effect on food quality.

This review will describe the main mitigation strategies used for acrylamide but will focus on the use of enzymes, in particular asparaginase, to reduce levels of acrylamide precursors. A more general review on acrylamide mitigation has been recently published (Friedman,

68   2015), while another recent review has covered the latest studies on the sources, purification,

69   and characterisation of L-asparaginase and its application in both the pharmaceutical and

70   food industries (Zuo, Zhang, Jiang, & Mu, 2015a).

71

72   2. **Acrylamide mitigation strategies**

73   *2.1 Raw materials*

74   Decreasing the amounts of acrylamide precursors will have a huge impact on final

75   acrylamide production (Zyzak et al., 2003). However, the effect will be dependent on the

76   relative levels of precursors. If total reducing sugars are present at higher levels than

77   asparagine in a food, reduction in asparagine will have the greater effect on acrylamide

78   formation, and *vice versa*. Numerous papers have demonstrated that acrylamide formation is

79   proportional to reducing sugar concentrations in potato (Elmore, Briddon, Dodson,

80   Muttucumaru, Halford, & Mottram, 2015; Ohara-Takada et al., 2005; Vinci, Mestdagh, & De

81   Meulenaer, 2012), while in cereals, such as rye and wheat, acrylamide formation is

82   proportional to asparagine content (Curtis et al., 2010; Halford, Curtis, Muttucumaru, Postles,

83   Elmore, & Mottram, 2012). Hence potato varieties low in reducing sugars and cereal varieties

84   low in asparagine are sought. Storage may increase levels of reducing sugars in stored

85   potatoes, particularly under low-temperature conditions (Rak, Navarro, & Palta, 2013), while

86   levels of fertilisation, for example nitrogen and sulfur, may have effects on reducing sugars

87   and asparagine levels in both potatoes and cereals (Elmore, Mottram, Muttucumaru, Dodson,

88   Parry, & Halford, 2007; Muttucumaru et al., 2006; Muttucumaru, Powers, Elmore, Mottram,

89   & Halford, 2013). It is clear, however, that little or no acrylamide will form in the absence of

90   asparagine, while other components of the food matrix, such as lipid-derived aldehydes

91   (Zamora & Hidalgo, 2008), amino acids such as serine and threonine (Shu, 1999), and other

4

92   carbonyl-containing molecules (Hamzalioğlu & Gökmen, 2012; Zamora, Delgado, &

93   Hidalgo, 2011), can react with asparagine to form acrylamide.

94   *2.2 Process-based mitigation*

95   Initial mitigation methods involved the control of processing conditions; for instance,

96   lowering pH, reducing cooking temperature and shortening the processing time (Palazoğlu &

97   Gökmen, 2008). Although these methods achieved an effective reduction of acrylamide,

98   sensory properties of the food were compromised. As the Maillard reaction begins when food

99   is heated, the first option in this type of mitigation method is to lower the temperature and

100 time of heating. However, as the Maillard reaction is also responsible for generating desirable

101 taste and smell in cooked food, sensory properties become unacceptable when cooking

102 temperature is substantially reduced (Masi, Dinnella, Barnaba, Navarini, & Monteleone,

103 2013).

104 Besides temperature, another important parameter, pH, has also been studied. In a model

105 system, the acrylamide content will reach a maximum amount when the pH is around 8,

106 which is near to the pK_a value of asparagine and leads to an enhancement in the initial steps

107 of acrylamide formation (Rydberg, Eriksson, Tareke, Karlsson, Ehrenberg, & Tornqvist,

108 2003). Several authors have reduced acrylamide formation by reducing the pH, using

109 compounds such as citric acid (Gama-Baumgartner, Grob, & Biedermann, 2004), although

110 product quality has suffered generally using this approach (Vinci, Mestdagh, & De

111 Meulenaer, 2012).

112 *2.3 Use of additives*

113 Adding other chemicals prior to or after heating could also decrease the final acrylamide

114 amount in a cooked product. For example, adding glycine before heating will compete with

115 asparagine to lower the final acrylamide amount (Bråthen & Knutsen, 2005). It could also be

added after the Maillard reaction, to react with acrylamide directly, thus lowering its amount in the final product (Liu, Man, Zhu, Hu, & Chen, 2013). However, this method also has negative effects on the sensory properties of the product, as added glycine will react with reducing sugars to increase levels of odour-active alkylpyrazines (Low, Parker, & Mottram, 2007)

The addition of divalent cations has also been shown to be an effective means of reducing acrylamide. Nixtamalisation, the traditional cooking of corn grains in calcium hydroxide solution prior to milling, is traditionally used in the preparation of tortillas (Salazar, Arambula-Villa, Luna-Barcenas, Figueroa-Cardenas, Azuara, & Vazquez-Landaverde, 2014). A calcium chloride dip for potatoes reduced acrylamide in French fries by 95%, with no adverse effects on product quality reported. The effect is due to the divalent cations inhibiting formation of the Schiff base (Gökmen & Şenyuva, 2007). Monovalent cations at low concentrations may also exhibit a mitigation effect. Addition of 1–2% sodium chloride to a bread mix led to a substantial reduction in acrylamide in baked rolls. Higher salt concentrations inhibited yeast growth, resulting in increased acrylamide formation (Claus, Mongili, Weisz, Schieber, & Carle, 2008). A 2% sodium chloride dip pre-treatment (60 min, room temperature) resulted in a 78% reduction in acrylamide in fried potato discs (Sansano, Juan-Borras, Escriche, Andres, & Heredia 2015).

Antioxidants could also be added to inhibit the formation of acrylamide. However, effects were variable in the several studies carried out during the past decade, due to the various types of antioxidants used (Jin, Wu, & Zhang, 2013). For instance, rosemary added to corn or olive oil could effectively lower the amount of acrylamide in fried potato slices (Becalski, Lau, Lewis, & Seaman, 2002), while some commonly used antioxidants, such as BHT, sesamol and Vitamin E, had an enhanced effect on acrylamide formation in cooked meat (Tareke, 2003). The reducing or promoting effects could be attributed to differences in

141   reaction conditions, antioxidant dosage and different reaction pathways.

142   *2.4 Enzymatic approaches for acrylamide reduction*

143   Fermentation methods use specific microorganisms to consume the asparagine or reducing

144   sugar before the food processing step (Sadd, Hamlet, & Liang, 2008). For instance, a starter

145   medium containing lactic acid bacteria was used in the preparation of wholemeal rye bread

146   and substantially reduced acrylamide levels in the final product (Bartkiene, Jakobsone,

147   Juodeikiene, Vidmantiene, Pugajeva, & Bartkevics, 2013b). As well as lowering the pH, the

148   lactic acid bacteria reduced the levels of reducing sugars in the dough.

149   However, there are several points that need to be considered in a fermentation approach.

150   To begin with, the temperature and pH need to be controlled in order to maximise the activity

151   of the microorganism. Even if the reducing sugar consumed was added back after processing,

152   the sensory quality of the final product may still be affected by the fermentation step

153   (Bartkiene, Jakobsone, Juodeikiene, Vidmantiene, Pugajeva, & Bartkevics, 2013a). Secondly,

154   fermentation predominantly works in bakery products, with limited application application in

155   potato-based products and coffee (Kamkar, Qajarbeygi, Jannat, Babaei, Misaghi, & Aghaee,

156   2015).

157   The use of an enzymatic approach to modify reaction pathways was first proposed by

158   Amrein et al. (2004), who used asparaginase to hydrolyse asparagine to aspartic acid and

159   ammonia (Ciesarová, Kiss, & Boegl, 2006). This approach is considered to be effective

160   because asparagine is not considered a major contributor to the overall flavour and colour of

161   cooked foods (Parker, Balagiannis, Higley, Smith, Wedzicha, & Mottram, 2012), so desirable

162   sensory properties are maintained.

3. **Asparaginase**

Asparaginase (L-asparagine amidohydrolases EC 3.5.1.1) is an enzyme widely distributed in animals, plants and living organisms (Wriston, 1985). It has been shown that asparaginase catalyses the hydrolysis of asparagine into aspartic acid and ammonia by hydrolysing the amide group in the side chain of asparagine (Hendriksen, Kornbrust, Ostergaard, & Stringer, 2009). Aspartic acid will then enter the citric acid cycle, playing a vital role in amino acid metabolism. Asparagine is responsible for nitrogen storage in most plants; therefore, asparaginase plays an important role in energy utilisation (Sieciechowicz, Joy, & Ireland, 1988).

L-Asparaginase has been used as a therapeutic treatment for certain kinds of cancer, such as leukaemia (Bushman, Palmieri, Whinna, & Church, 2000). In leukaemia sufferers malignant cells depend more on exogenous asparagine and glutamine to survive than normal cells. Asparaginase injected into the bloodstream hydrolyses free asparagine into aspartic acid and ammonia, and glutamine to glutamic acid and ammonia (Friedman, 2003). In this way, the growth of malignant cells is inhibited.

L-Asparaginase is an intracellular enzyme which is obtained from a variety of microorganisms: *Escherichia coli, Erwinia carotovora, Bacillus* sp., *Enterobacter aerogenes, Corynebacterium glutamicum, Pseudomonas stutzeri* and *Candida utilis* (Qin & Zhao, 2003). For pharmaceutical uses, L-asparaginase is typically obtained from *Escherichia coli* (Pritsa & Kyriakidis, 2001). However, the production of the enzyme is complex with low yield. Until now, there is no medium that has been specifically established for the optimum production of L-asparaginase from different microorganisms. To maximise enzyme production, each organism has its own optimum conditions. Research on *E. coli* using response surface methodology achieved a 10-fold enhancement in asparaginase production (Kenari, Alemzadeh, & Maghsodi, 2011).

188 Most asparaginases are quite specific for asparagine. Optimal activity is usually achieved

189 at pH 5–7 and 37 °C. However, as glutamine has similar structure to asparagine, some

190 enzymes also have a low activity towards glutamine (Krasotkina, Borisova, Gervaziev, &

191 Sokolov, 2004). A small group of enzymes, called glutaminase-asparaginases, have activities

192 for both asparagine and glutamine but prefer glutamine as a substrate (Roberts, Holcenberg,

193 & Dolowy, 1972). Crystallographic study has shown that both types of asparaginase,

194 common asparaginase and glutaminase-asparaginase, have the same basic structure and

195 catalytic mechanism but differ in working conditions (pH and temperature) (Yao, Yasutake,

196 Morita, & Tanaka, 2005). Researchers believe that glutaminase activity caused by

197 glutaminase-asparaginase will exert serious adverse effects on human health, such as liver

198 dysfunction, pancreatitis and leucopoenia (Mahajan, Saran, Kameswaran, Kumar, & Saxena,

199 2012). Therefore, this specific type of asparaginase should be strictly avoided in the food

200 industry.

201 Commercially, there are two asparaginase products currently available for acrylamide

202 mitigation in the food industry. These are PreventASe[TM] from DSM (Heerlen, The

203 Netherlands) and Acrylaway[®] from Novozymes A/S (Bagsvaerd, Denmark). PreventASe[TM]

204 was the first, launched in 2007. It was obtained after analysing the gene sequence of

205 *Aspergillus niger* and produced recombinant in the *Aspergillus niger* host. It has an acidic

206 profile (optimum pH 4–5, temperature 50 °C). Acrylaway[®] on the other hand, is obtained

207 from *Aspergillus oryzae* and has an almost neutral profile (optimum pH 7, temperature 37

208 °C).

209 Regarding safety, these enzymes are produced by specific fungal strains of *A. oryzae* and

210 *A. niger*, fungi that have been widely used in commercial products for several decades and

211 have been proved to be safe by JECFA (JECFA, 2007). Acrylaway[®] and PreventASe[TM] have

212 shown high specificity and therefore minimum activity towards glutamine and other amino

213 acids. Ultimately, these enzymes will be deactivated during the heating process, ensuring

214 their safe application in foodstuffs (Hendriksen et al., 2009).

215   Asparaginase has received "generally recognized as safe" status from the US government.

216 It has also been given a favourable evaluation as a food additive by the Joint FAO/WHO

217 Expert committee (JEFCA, 2007) and it is currently used in several countries, including

218 United States, Australia, New Zealand, China, Russia, Mexico and several European

219 countries. As different dosages of asparaginase will be used in different types of food, there is

220 no unified standard for the maximum dosage.

221   *3.1.   Use of asparaginase in acrylamide mitigation*

222   As shown in Table 1, over the last decade there have been numerous studies monitoring

223 the reduction of acrylamide formation by means of asparaginase treatment. The first study

224 was carried out by Zyzak et al. in 2003, immediately after the formation mechanism was

225 revealed. However, Zyzak's research was focused on the formation mechanism rather than

226 the mitigation efficiency. He used commercial asparaginase from Aldrich (A2925 from

227 *Erwinia chrysanthemi*), 50 U added to 60 g of mashed potato slurry (15 g potato, 45 g water),

228 to hydrolyse the asparagine, in order to verify that asparagine is indeed the precursor of

229 acrylamide. The asparaginase achieved an 88% asparagine reduction that led to 99%

230 acrylamide reduction in a microwaved mashed potato snack, heated at full power until brown

231 (Zyzak et al., 2003).

232   The following year, the first paper on the use of asparaginase as an acrylamide mitigation

233 method was published. Asparaginase (from *E. coli,* 4 U/kg) added to gingerbread hydrolysed

234 approximately 75% of the free asparagine, leading to a 55% acrylamide reduction in the final

235 product. The acrylamide-reduced product was identical to a control product in both colour

236 and taste (Amrein, Schönbächler, Escher, & Amadò, 2004). Though this enzyme application

formed only a small part of the research, it stressed the advantage of the enzymatic method

on mitigating acrylamide while maintaining the organoleptic properties of the product.

Ciesarová et al. (2006) set up a model system to examine the importance of all the related

factors, such as temperature, dosage and application time. However, there were insufficient

time and temperature points studied to determine optimum activity. Applying asparaginase to

dried potato powder led to a 90% acrylamide reduction in cooked product. However, instead

of considering the effect of the cut and shape of the potato products, this research focused

more on potato varieties. Although this research showed great success in acrylamide

reduction, the agronomic factors were discussed more than the enzyme itself.

Pedreschi, Kaack, and Granby (2008) were the first to publish results using a commercial

asparaginase (Acrylaway®). They established that the optimum temperature and pH for this

enzyme were 60 °C and 7.0, respectively. A reduction of 67% in acrylamide was achieved in

French fries under these conditions. In this study, the importance of blanching and

temperature control of asparaginase treatment was highlighted. It is known that blanching

will change the microstructure of the potato strips and increase the contact probability of

asparaginase and asparagine (Lisińska, Tajner-Czopek, & Kalum, 2007), so blanching is

highly recommended for increasing the performance of the enzyme.

Another study by the same group focused on the combination of asparaginase

(Acrylaway®) and conventional blanching, alongside their individual usage. Blanching using

hot water at 85 °C to treat the potato tuber samples for 3.5 min was compared with enzymatic

mitigation using an asparaginase solution (10000 ASNU/L) at 50 °C for 20 min. One ASNU

is defined as the amount of asparaginase that produces one micromole of ammonia per

minute under standard conditions (pH 7; 37 °C). Experimental results showed that blanching

and enzyme treatments have a similar effect on acrylamide reduction (17%). By combining

the two methods, almost 90% of acrylamide was mitigated. The authors assumed that the

microstructure of the potato tissues was changed in the blanching process, causing the asparagine in the cell to have a more effective interaction with the enzyme outside the cell (Pedreschi, Mariotti, Granby, & Risum, 2011). Although acrylamide in this research was significantly reduced, no sensory analysis of the product was performed.

In 2009, another study involving Acrylaway® was carried out on a much wider range of foods, including gingerbread, crispbread, semi-sweet biscuits, French fries and crisps (Hendriksen et al., 2009). Again the optimum conditions of temperature and pH were 60 °C and 7.0. In this study, other factors were also taken into consideration, depending on the food matrix. In semi-sweet biscuits, the dosage was the variable and the temperature was set at 40 °C. Asparaginase treatment took place at the dough resting time before the biscuits were baked at 260 °C for 5.5 min (Fig. 2a). For the crispbread trial, the temperature was held at 10, 15,or 20 °C for 30 or 60 min and the dosage was set at 2100 ASNU/kg of flour. Then the crispbread was baked at 250 °C for 11 min (Fig. 2b). By changing the dosage of enzyme and time, the influences of each factor are revealed. Besides dosage and temperature, water content is another important factor in a cereal-based product like gingerbread, as higher water activity will provide sufficient contact for the enzyme with the substrate. Therefore, in order not to compromise final product sensory quality, a higher water content is recommended, although a further drying step may be needed subsequently. For potato-based products, a reduction was achieved in both French fries and crisps. Potatoes made into French fries were treated with 10500 ASNU/L and then fried for 3 min at 175 °C. Sliced potatoes used to make potato crisps were treated with various concentrations of enzyme for 15 min at 40 °C. Then frying was conducted for 2.5 min at 180 °C. In the French fries experiment, the authors prepared a sample set with a one-minute dip and 20 minutes soaking in the enzyme bath. Although the acrylamide reduction in the one-minute dip (59% maximum) was less than for the samples in the 20-min soak (85% maximum), the results were still meaningful for the

287 practical continuous process. Results indicated a broad range of enzyme applications. The

288 authors suggested that by combining modified processing conditions with an enzymatic

289 approach, acrylamide could be mitigated at a fairly low cost. The key point in this research is

290 that the authors tried to assess all related variables and generate specific solutions for each

291 type of product from an enormous data-set.

292 Also in 2009, Kukurová, Morales, Bednáriková, and Ciesarová (2009) used two levels of

293 Acrylaway® (100 U/kg and 500 U/kg flour) in the preparation of fried bread rolls. The

294 asparaginase treatment (15 min, 37 °C) removed at least 96% of the asparagine from the

295 dough; acrylamide could not be quantified in the fried rolls with Acrylaway® added, while

296 levels of 215 μg per kg were present in control rolls fried at 200 °C for 8 min. The same

297 group also studied acrylamide formation in cookies treated with Acrylaway® (500 U/kg

298 flour) and different raising agents (Kukurová, Ciesarová, Mogol, Açar, & Gökmen, 2013).

299 The raising agents increased the pH of the dough, reducing the effectiveness of the

300 asparaginase in reducing asparagine. When applied for less than 30 minutes, the asparagine

301 had no effect on the sensory properties of the cookies.

302 Hendriksen, Budolfsen, and Baumann (2013) used Acrylaway® to study potato and cereal

303 products and also carried out the first experiments on the effect of asparaginase on

304 acrylamide in coffee. For cereal products, the mitigation efficiency reached 95% in lebkuchen

305 and 90% in tortilla chips, whereas in coffee a 70–80% reduction was achieved. In potato

306 products, experiments on potato tuber snack pellets and French fries were carried out. In

307 potato tuber snack pellets, the enzyme was added directly to a dough based on 29% potato

308 starch, 27.6% potato granules, 15% potato flakes, 1.4% salt and 27% water, while in the

309 French fries test, Bintje or Maris Piper potatoes were manually peeled and cut into 8 mm × 8

310 mm strips. Two innovative points of this study stands out. First, asparaginase is added at the

311 disodium acid pyrophosphate (SAPP) dipping stage rather than as a separate step. SAPP is

commonly used in potato processing to prevent after-cooking darkening. Integrating the two treatments could reduce time and cost. Secondly, an industrial scale trial was carried out to test the mitigation efficiency of asparaginase in continuous processing. Such trials will push the industrial application of asparaginase forward. Reductions in potato product were comparatively low, due to the insufficient contact of asparagine and enzyme. However, the industrial scale experiment (8 tonne/h) still achieved satisfactory results; a 43% reduction in 10 mm × 10 mm and 53% reduction in 7 mm × 7 mm potato pieces was achieved. A dye-based experiment indicated that asparaginase could only penetrate 1 mm into the potato, again highlighting the importance of incorporating a blanching step when treating potatoes (Hendriksen et al., 2013).

For coffee, increased acrylamide mitigation could be achieved by incubating the wetted green beans. Typically, green coffee beans are steamed to decrease the caffeine content. The decaffeination process is usually carried out by a water or solvent partition system. Firstly, green coffee beans are steamed to make the caffeine available. Then, a solvent is used to extract the caffeine. Finally, the green beans are steamed again to remove any residual solvent (Spiller, 1997). Hence, asparaginase could be infused during these steps with minor changes to the processing conditions. A laboratory-scale experiment indicated that a low dosage (2000–6000 ASNU) of asparaginase could achieve 55–74% acrylamide reduction in coffee beans (Hendriksen et al., 2013), while work in our laboratory showed that both the steaming step and the asparaginase treatment caused a reduction in free asparagine when the coffee was roasted, which was reflected in acrylamide losses from 69% to 86% using dosages of 2600 to 20000 ASNU, respectively (Xu, Khalid, Oruna-Concha, & Elmore, 2015).

The effect of asparaginase on acrylamide mitigation in biscuits has also been examined by Anese, Quarta, and Frias (2011). The authors used asparaginase levels from 100 to 900 ASNU (Acrylaway®) with 20–54 °C incubation temperature and 10–30 min incubation time,

337 15 treatments in all. By analysing the results from each treatment, the influence of each factor

338 was considered and optimum conditions could be obtained. This study not only demonstrated

339 a method that could assess the effect of the enzyme but compared the effect obtained with the

340 cost for each treatment. This paper also contained valuable advice on the practical application

341 of asparaginase. For instance, acrylamide development was at a minimum at intermediate

342 asparaginase concentrations and increased asparaginase addition did not significantly affect

343 the colour of the final product.

344   We are only aware of one publication where PreventASe™ was used as the asparaginase

345 source. A solution of 500 ASNU in 10 mL of water was spread onto the surface of a

346 wheat/oat bread loaf prior to baking. The enzyme was effective during a proofing step of 15

347 min at 32 °C. This treatment led to a 46% reduction in the acrylamide content of the baked

348 bread crust (Ciesarová et al., 2014).

349   *3.2.   New sources of asparaginase*

350   Recent papers have identified new sources of asparaginase for acrylamide reduction.

351 Tuncel, Yılmaz, and Şener (2010) used asparaginase from a vegetable source. They

352 germinated pea flour to enhance asparaginase activity and remove beany flavour. The pea

353 flour was finely ground and then added to wheat flour in white wheat bread, wheat bran bread

354 and wholegrain white bread at three different levels (1%, 3% and 5%). The bread was baked

355 at 220 °C for 22–25 min. In white wheat bread acrylamide reduction was less than 10% in all

356 cases, while addition of 5% pea flour to bran bread and grain bread reduced acrylamide levels

357 in crust by 57% and 68%, respectively. Although the sensory panel showed detectable

358 differences in the final products, there was no significant negative impact on sensory

359 properties. The extraction of asparaginase from fungus is relatively costly; therefore this

360 approach provides an effective alternative means to produce asparaginase.

361   Asparaginase with low glutaminase activity was successfully extracted from *Bacillus*

362   *licheniformis* and was used to reduce acrylamide in fried potato strips by up to 80%

363   (Mahajan, Saran, Kameswaran, Kumar, & Saxena, 2012).

364   Asparaginase produced from *Cladosporium* sp. was used in the crumb and crust of sweet

365   bread (Kumar, Shimray, Indrani, & Manonmani, 2014). The dosage used varied from 50–300

366   U. However, the units were not defined. Reductions of 97% and 73% were achieved at 300 U

367   in bread crust and crumb, respectively. The authors also measured the formation of 5-

368   (hydroxymethyl)furfural, which is another potential toxicant formed in the Maillard reaction,

369   and it was also decreased. This research also showed a new possible source for asparaginase,

370   although yield data were not disclosed.

371   Asparaginase has also been extracted from *Rhizomucor miehei* (Huang, Liu, Sun, Yan, &

372   Jiang, 2014). The extracted asparaginase, designated as RmAsnase, was optimally active at

373   pH 7.0 and 45 °C and was stable at this temperature for 30 min. RmAsnase was cloned and

374   expressed in *Escherichia coli* and proved highly specific towards asparagine. The researchers

375   demonstrated that a low concentration of asparaginase (0.5 U/g flour) had much better

376   mitigation efficiency in bread (40%) than biscuits (15%). However, an 80% reduction was

377   achieved in both products at 10 U/g flour. Overall, this new enzyme showed remarkable

378   potential both as an acrylamide mitigator and also in leukaemia therapy.

379   Asparaginase from food-grade *Bacillus subtilis* was applied to potato chips (Onishi,

380   Prihanto, Yano, Takagi, Umekawa, & Wakayama, 2015). Compared to a control sample, 40

381   U of asparaginase led to 80% reduction in acrylamide. One unit of the enzyme was defined as

382   the amount that catalysed the formation of 1 μmol ammonia per min; additional details were

383   not provided by the authors. They suggested that BAsnase, as the enzyme was christened,

384   could be used to spray potatoes prior to cooking at home. Similar reductions in acrylamide in

385   French fries were also obtained using an asparaginase from *Thermococcus zilligii*. The

purified enzyme displayed a maximum activity at pH 8.5 and 90 °C (Zuo, Zhang, Jiang, & Mu, 2015b) and retained 70% of its original activity after 2 hours incubation at 85 °C. Another asparaginase with high temperature stability was recently isolated from *Pyrococcus furiosus* (Kundu, Bansal, & Mishra, 2013). In addition, at the end of 2013 Novozymes launched Acrylaway® HighT, which is specifically designed for higher temperature processing (http://www.novozymes.com/en/news/news-archive/Pages/New-Novozymes'-solution-enables-acrylamide-mitigation-in-even-more-product-categories--.aspx.) Enzymes stable at such temperatures can be incorporated into the blanching step of a commercial process, which would increase their applicability.

Immobilised asparaginase may not convert as much asparagine as free asparaginase but its stability is improved, meaning that blanching water containing immobilised enzyme can be re-used several times without loss of asparaginase activity. The asparaginase is immobilised by crosslinking with glutaraldehyde on an inert silica-based carrier (Hendriksen, Puder, & Olsen, 2014).

3.3. *Asparaginase activity under different conditions*

To quantify the effect of asparaginase in food applications, its activity needs to be determined. For the two commercially available enzymes (Acrylaway® by Novozymes and PreventASe™ by DSM), two different methods to determine the activity of the enzyme have been used. Both methods are based on measuring the ammonia that is generated from the asparagine hydrolysis. However, in the method used to measure the activity of Acrylaway, ammonia subsequently reacts with α-ketoglutarate to form L-glutamic acid. The reaction is catalysed by glutamate dehydrogenase in the presence of NADH, which is oxidised to NAD+ with the concomitant loss of absorbance measured at 340 nm. The asparaginase activity is measured as the rate of NADH consumption under standard conditions (pH = 7; 37 °C). The

activity of asparaginase is expressed in ASNU activity units. One ASNU is defined as the amount of asparaginase that produces one micromole of ammonia per minute under standard conditions (Hendriksen et al., 2009). The activity of PreventASe™ is measured by a different method. The liberated ammonia subsequently reacts with phenol nitroprusside and alkaline hypochlorite resulting in a blue colour. This is known as the Berthelot reaction (Rhine, Sims, Mulvaney, & Pratt, 1998). The activity of asparaginase is determined by measuring the absorbance of the reaction mixture at 600 nm. Asparaginase activity is expressed in ASPU activity units. One ASPU is defined as the amount of asparaginase that liberates one micromole of ammonia from L-asparagine per minute under standard conditions (pH = 5.0; 37 °C).

By setting up a standard activity determination method, the activity of the enzyme under different conditions of pH and temperature can be measured. From the work done by Hendriksen's group in Novozymes, it was shown that Acrylaway® has almost two times the activity at 60 °C compared to its activity at 37 °C. Also, the activity of the enzyme at pH 7 is almost two times its activity at pH 5. These differences in activity should be considered prior to the application of the enzyme to a substrate.

3.4. Practicalities of asparaginase application

The practicalities of asparaginase application can be assessed from three different aspects: raw material composition, processing and commerce.

The amount of asparagine in a foodstuff should be considered when deciding the dosage of asparaginase. For example, concentrations of free asparagine in potato may vary by a factor of 10 or more, and can be affected by variety and growing conditions, (Halford et al., 2012b).

When using asparaginase in food manufacture, factors like temperature, time and substrate

434 ratio are of importance. For example, the optimal temperature for Acrylaway® is around 60

435 °C and its activity will decrease significantly above this temperature (Hendriksen et al.,

436 2009). Hence asparaginase will be denatured and inactivated during food processing. The

437 dwell time for enzyme and foodstuff before heat treatment should be optimised before

438 application. Enzyme–substrate ratio is also an important factor and dosage of the enzyme

439 should be determined, so that maximum mitigation is achieved with minimum enzyme

440 concentration. Extra water may be used to ensure the delivery of the enzyme (Whitehurst &

441 Van Oort, 2009).

442 Even though asparaginase has advantages over other mitigation methods, its use by

443 manufacturers may not be commercially viable at present. There are also issues with the

444 industrial application of asparaginase in a continuous process, which can achieve good results

445 in a relatively short time. Pilot-scale experiments have been carried out using a continuous

446 process for French fries production (Hendriksen et al., 2009). High levels of acrylamide

447 reduction were achieved (60–85% in French Fries and 60% in potato chips). However, more

448 research is needed to better incorporate asparaginase usage into industrial-scale food

449 production. To maximise the overall effect of the enzymatic method, pre- and post-treatment

450 procedures may need to be adapted; for instance, reduction of starting material dimensions

451 and blanching before enzyme treatment and modification of the process conditions after the

452 enzyme treatment by, for example, changing cooking temperature and pH.

453

454 4. Conclusions

455 Asparaginase has become a powerful tool for acrylamide mitigation in the food industry.

456 With the success of commercial products, it is likely that asparaginase will be used more and

457 more. The first commercially available "acrylamide-free" product, biscuits treated with

458   Preventase™, was announced to launch shortly in Germany for Christmas, 2008. This

459   information was released by DSM Food Specialities, although the manufacturer's name was

460   not disclosed (Foodingredients1st, 2008). However, there was no more news subsequently.

461   The potential adverse effects of asparaginase treatment on sensory properties of cooked

462   foods and the need to achieve sufficient enzyme–substrate contact are areas for future

463   research. However, if the application of asparaginase becomes commercially attractive, its

464   use alongside raw materials low in asparagine may provide the solution to the acrylamide

465   problem.

466

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709   Figure 1 Mechanism of acrylamide formation (adapted from Parker, Balagiannis, Higley,

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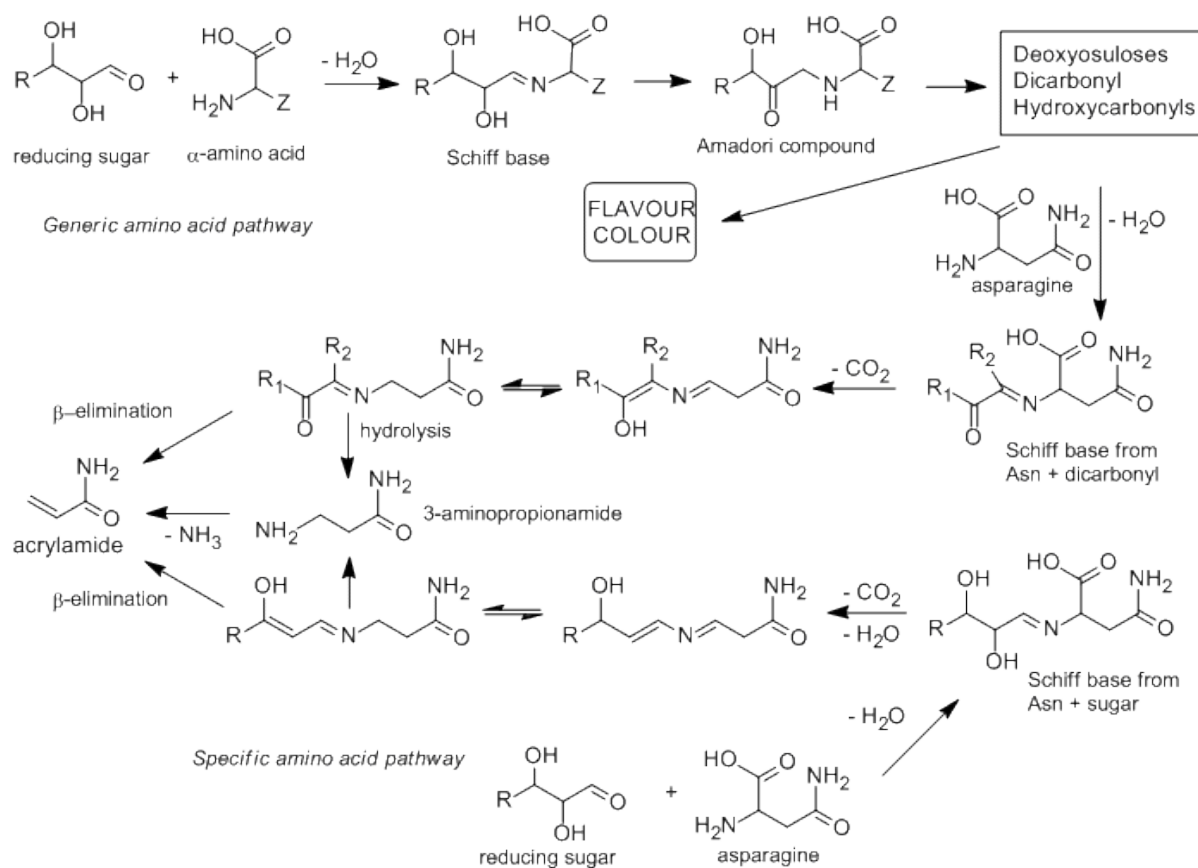
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716 Figure 2 (a) Effect of asparaginase (Acrylaway®) dose at pH 7 and 40 °C on acrylamide formation in semi-sweet biscuits; biscuits were baked at

717 260 °C for 5.5 min. (b) Effect of asparaginase (Acrylaway®; 2100 ASNU/kg of flour) incubation conditions on acrylamide formation in

718 crispbread; crispbread was baked at 250 °C for 11 min (adapted from Hendriksen, Kornbrust, Ostergaard, and Stringer (2009)).

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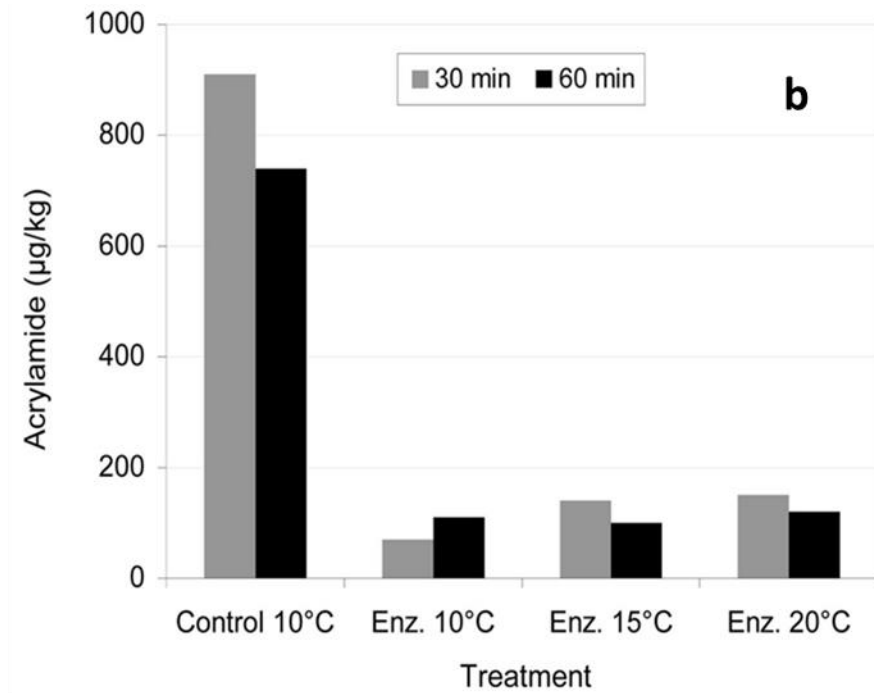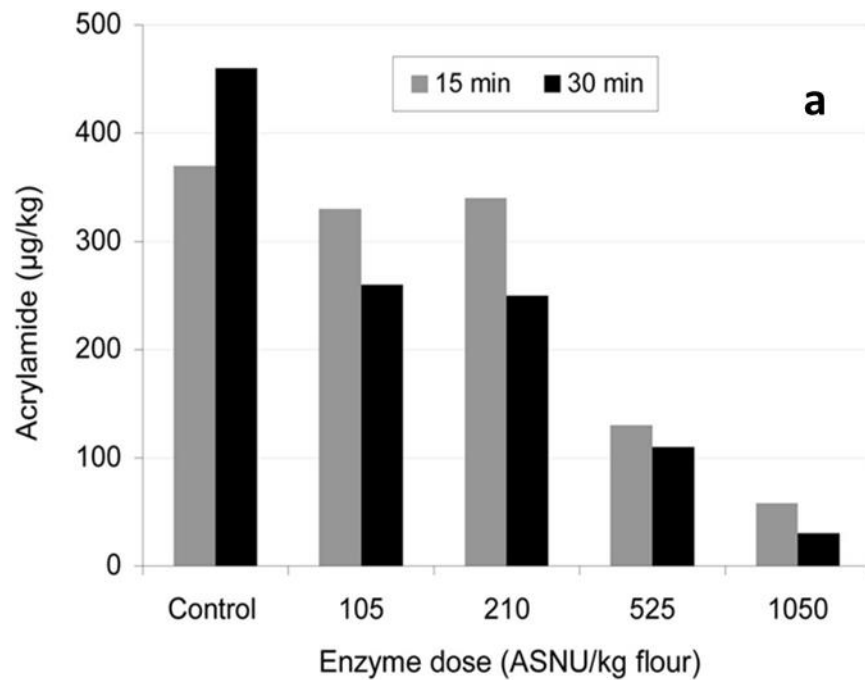
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Table 1 Results published on enzymatic mitigation of acrylamide using asparaginase

foodstuff	enzyme source	enzyme dosage	processing conditions	acrylamide reduction	reference
potato	*Escherichia coli*	not stated	not stated	99%	Zyzak et al. (2003)
gingerbread	*E. coli*	4 U/kg	various time/temperature combinations	55%	Amrein et al.(2004)
potato	*E. coli*	0.2–1 U/g	180 °C, 20 min	50–90%	Ciesarová et al. (2006)
French fries	*A. oryzae*	10000 ASNU/L*	175 °C, 3 min	67%	Pedreschi et al. (2008)
semi-sweet biscuits, ginger biscuits, crispbread, French fries, potato crisps	*A. oryzae*	various dosages	various time/temperature combinations	semi-sweet biscuits: 65–84% ginger biscuits: 34–90% crispbread: 84–92% French fries: 59% potato crisps: 60%	Hendriksen et al. (2009)
fried dough model system	*A. oryzae*	100, 500, 1000 U/kg	180 or 200 °C; 4, 6 or 8 min	90%	Kukurová et al. (2009)
bread	enzymes from *Pisum sativum* L.	not stated	220 °C, 22–25 min	wheat bran bread: 57% whole-grain bread: 68%	Tuncel et al. (2010)
potato chips	*A. oryzae*	10000 ASNU/L	170 °C, 5 min	90%	Pedreschi et al. (2011)
biscuits	*A. oryzae*	100–900 U/kg	200 °C, final moisture content 2%	7–88%	Anese et al. (2011a)
biscuits	*A. oryzae*	900 U/kg	200 °C, final moisture content 2%	69%	Anese et al. (2011b)
potato	*Bacillus licheniformis*	30 IU/mL	175 °C, 15 min	80%	Mahajan et al. (2012)
lebkuchen, tortilla chips, potato snack, French fries, coffee	*A. oryzae*	various dosages	lebkuchen: 200 °C, 14 min tortilla chips: 190 °C, 60 s	lebkuchen: 95% tortilla chips: 90%	Hendriksen et al. (2013).

			French fries: 175 °C, 3 min others not specified	potato snack: 40% French fries: 57% coffee: 55–74%	
cookies	*A. oryzae*	500 U/kg	205 °C, 11 or 15 min	23–75%	Kukurová et al. (2013)
wheat-oat bread	*Aspergillus niger*	500 U	220, 230 and 250 °C; 10, 30 and 40 min	90%	Ciesarová et al. (2014)
sweet bread	*Cladosporium sp.*	50–300 U	220 °C; 25 min	sweet bread crust: 97% sweet bread crumb: 73%	Kumar et al. (2014)
biscuits, bread	*Rhizomucor miehei*	0.5–10 U	200 °C; 15 min	biscuits: 81.6% bread: 94.2%	Huang et al. (2014)
potato crisps	*Bacillus subtilis*	0–40 U	170 °C; 90 s	80%	Onishi et al. (2015)
French fries	*Thermococcus zilligii*	0–20 U	175 °C; 5 min	80%	Zuo et al. (2015b)

* ASNU is defined as the amount of asparaginase that produces 1 µmol of ammonia per min under the conditions of the assay (pH = 7 ± 0.005; 37 ± 0.5 °C) using

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