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ARTICLE

Evaluating the Potential for Enzymatic Acrylamide Mitigation in a Range of Food Products Using an Asparaginase from *Aspergillus oryzae*

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Asparaginase, an enzyme that hydrolyzes asparagine to aspartic acid, presents a potentially very effective means for reducing acrylamide formation in foods via removal of the precursor, asparagine, from the primary ingredients. An extracellular asparaginase amenable to industrial production was cloned and expressed in *Aspergillus oryzae*. This asparaginase was tested in a range of food products, including semisweet biscuits, ginger biscuits, crisp bread, French fries, and sliced potato chips. In dough-based applications, addition of asparaginase resulted in reduction of acrylamide content in the final products of 34−92%. Enzyme dose, dough resting time, and water content were identified as critical parameters. Treating French fries and sliced potato chips was more challenging as the solid nature of these whole-cut products limits enzyme−substrate contact. However, by treating potato pieces with asparaginase after blanching, the acrylamide levels in French fries could be lowered by 60−85% and that in potato chips by up to 60%.

KEYWORDS: Acrylamide; asparaginase; *Aspergillus oryzae*; cereal foods; biscuits; potato; French fries

INTRODUCTION

The discovery in 2002 of high levels of acrylamide in many common food products has caused intense debate and concern. Acrylamide is classified as "probably carcinogenic to humans" (*1*) and might therefore pose a potential health risk through dietary exposure (*2*, *3*). Extensive international research programs have been established to investigate and evaluate this risk. Until the results from these studies are available, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended that appropriate efforts to reduce the concentration of acrylamide in food should continue (*2*).

The predominant formation pathway of acrylamide is via a Maillard reaction between the amino acid asparagine and reducing sugars. Maillard reactions, also known as non-enzymatic browning, typically occur at temperatures above 100 °C and are responsible for important color and flavor development in fried and baked starchy products. Thus, the same reactions that make these foods tasty give rise to a potential carcinogen (*4*, *7*−*11*).

Both cereal- and potato-based food products that have been prepared by heating can have relatively high levels (30−7500 μg/kg) of acrylamide and account for a significant proportion of dietary exposure (*2*, *4*). Therefore, a variety of methods have been developed and tested for reducing acrylamide levels in these foods (*4*−*6*). Because removal of acrylamide from prepared food products is impractical, mitigation strategies have focused on limiting acrylamide formation.

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In cereal food products, the most influential parameters that can be manipulated to reduce acrylamide formation are the time and temperature profiles during baking. Recipe changes such as the addition of citric acid or calcium ions, replacement of reducing sugars with sucrose, omission of ammonium bicarbonate, and use of flour with low asparagine content can also lower acrylamide levels (*4*, *6*, *12*).

For products based on whole potato pieces, reduction strategies have focused on controlling the level of reducing sugars, because this has been found to be the determinant factor for final acrylamide levels in these products (*13*−*18*). Low sugar levels are achieved by cultivar selection and by optimal storage. Product recipe changes are also reported to be useful, including addition of amino acids (*19*) or calcium ions (*4*, *6*, *20*) and lowering of pH (*5*, *21*, *22*). Relevant process changes comprise decreasing cooking temperatures and optimizing heating profiles (*5*). Prolonged blanching and soaking have proven to be quite effective in reducing acrylamide formation by simply washing out the precursors (*21*, *22*).

The majority of these methods will, however, limit not only acrylamide formation but also the formation of desired Maillard products, which can have a negative impact on both product taste and appearance.

Application of the enzyme asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) provides a possible alternative method for acrylamide mitigation that should have a very limited effect on the general formation of Maillard products. Asparaginase can selectively reduce the level of free asparagine by hydrolyzing it to aspartic acid and ammonia, thereby specifically removing one of

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the essential acrylamide precursors. The majority of amino acids will not be affected, and impact on final food product quality should therefore be limited.

The effective use of asparaginase for acrylamide mitigation has been demonstrated in a number of studies done in simple systems of reagents in solution as well as in a number of food products (*10*, *12*, *23*−*25*, *35*), typically using an asparaginase from *Escherichia coli* developed as a pharmaceutical agent (*26*). The studies demonstrate that applying asparaginase to food ingredients results in a reduction in their asparagine levels and subsequently lower levels of acrylamide in the final products when compared to untreated products. However, comparison of the results shows that little direct correlation is observed between the percentage reduction in asparagine levels in the ingredients and the percentage reduction in acrylamide formation in the various products. This is likely due to the impact of both processing conditions and other recipe components, which vary from product to product, on acrylamide formation.

This paper describes the cloning and recombinant expression of a new asparaginase from the fungus *Aspergillus oryzae* and application testing of this enzyme in a range of potato- and cereal-based food products. Five food products were selected as relevant model systems, reflecting the diversity of products reported to contain high levels of acrylamide: ginger biscuits, crisp bread, semisweet biscuits, French fries, and sliced potato chips (referred to as crisps in the United Kingdom). These products represent very different types of foods with respect to raw materials and production processes, parameters that might influence the enzyme activity and acrylamide formation. Acrylamide levels in final products are presented and compared to untreated controls to assess the efficacy of the asparaginase in acrylamide mitigation.

MATERIALS AND METHODS

Sequence Database Searching. Database searching was performed with the TFASTXY program, version 3.2t07 (*27*).

Subcloning and Recombinant Expression. The *A. oryzae* asparaginase coding sequence was amplified from cDNA clone ZY087777 using the Extensor Hi-Fidelity PCR Master Mix (ABgene, Surrey, U.K.) and asparaginase-specific oligonucleotides with appropriate restriction sites added to the primer ends to facilitate subcloning of the PCR product (AoASP7, CAAGGATCCAGCAGTATGGGTGTCAATTTC; AoASP8, ATCAAGCTTCTATTATCCATCCCATCCA). The PCR fragment was restricted with *Bam*HI and *Hin*dIII and cloned into the *Aspergillus* expression vector pMStr57 (*28*). The resulting plasmid was transformed into *A. oryzae* strain BECh2 (*28*). Transformants were selected during regeneration and reisolated twice under selection on Cove minimal plates with sucrose and acetamide (*29*). Transformants were fermented in 500 mL flasks containing 150 mL of DAP2C-1 medium (*28*) shaken at 200 rpm at 30 °C for 4 days. Fungal mycelium was removed from fermentation samples by centrifugation before further analysis. Expression was monitored by protein gel electrophoresis on NuPage Novex 10% Bis-Tris gels (Invitrogen, Carlsbad, CA), with NuPage MES SDS running buffer. For reference, Amersham's LMW-SDS Marker Kit (GE Healthcare, Little Chalfont, U.K.) was used.

Thin Layer Chromatography (TLC) Assay. Fermentation samples were incubated with 0.5% w/v asparagine in 100 mM sodium phosphate buffer, pH 7.0, for 1 h at 37 °C in an Eppendorf thermomixer (Eppendorf, Hamburg, Germany) shaking at 300 rpm. Samples were applied to 20 × 20 cm silica gel 60 aluminum TLC sheets (Merck & Co., Whitehouse Station, NJ) and separated with a 70% isopropanol solution and a 5 h run time. A 0.5% w/v asparagine solution and a 0.25% w/v aspartic acid solution were applied for comparison. TLC sheets were stained with ninhydrin spray reagent (BDH Chemicals, Poole, U.K.) and dried at 100 °C for 10−15 min.

Assay for Characterization of Asparaginase. Fifty microliters of diluted asparaginase was mixed with 450 μL of asparagine substrate [10 mg/mL L-asparagine in 100 mM succinic acid, 100 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)), 100 mM CHES (2-(*N*-cyclohexylamino)ethanesulfonic acid), 100 mM CABS (4-(cyclohexylamino)-1-butanesulfonic acid), 1 mM $CaCl_2$, 150 mM KCl adjusted to reaction pH with HCl or NaOH] in an Eppendorf tube on ice. The assay was initiated by transferring the tube to an Eppendorf thermomixer (Eppendorf, Hamburg, Germany) set to the reaction temperature and incubated for 15 min at 1400 rpm. The reaction was stopped by transferring the tube back to the ice bath and adding 500 μL of 0.5 M trichloroacetic acid. The tube was shaken and centrifuged shortly in an ice-cold centrifuge to precipitate protein. The ammonia concentration in the supernatant was monitored as a measure of the asparaginase activity. A buffer blind was included in the assay (instead of enzyme). Ammonia concentration was measured using an ammonia test kit from Roche Diagnostics, catalog no. 11 112 732 035 (Basel, Switzerland).

Asparaginase Production for Application Testing. Recombinant *A. oryzae* strains over-expressing the *A. oryzae* asparaginase were fermented by Novozymes A/S in pilot scale to produce enzyme batch KSF0083, with an activity of 14280 asparaginase activity units (ASNU)/g, or in production scale to produce Acrylaway L, with an activity of 3500 ASNU/g. One ASNU is the amount of enzyme that produces 1 μmol of ammonia per minute at 37 °C and pH 7.0. The activity is determined relative to an asparaginase standard that was prepared and calibrated for the purpose of standardizing Novozymes' Acrylaway products.

Dry Solids (DS) Determination. Samples were ground and dried at 105 °C to constant weight. DS were calculated as a percentage of the initial weight.

Acrylamide Content. A minimum of 50 g of the samples was sent to Eurofins Danmark A/S (Galten, Denmark) for acrylamide analysis. At Eurofins samples were homogenized and defatted using hexane. Internal standard d_3-acrylamide was added, and samples were extracted using water in an ultrasonic bath for 30 min at 60 °C. Extracted samples were purified, and acrylamide was quantified using LC-MS/MS (*30*).

Asparagine/Aspartic Acid Content. Analysis was done in treated potato strips after drying and before frying. Approximately 0.5 g of sample was taken and 5 mL of boiling water added for asparaginase inactivation. The samples were held for a further 20 min at 100 °C for extraction of amino acids. The samples were then centrifuged, and the liquid extract was removed and filtered (0.2 μm). Filtered samples were analyzed on a Dionex high-pressure liquid chromatograph (HPLC) equipped with an automated sample injector and a fluorescence detector using precolumn derivatization of the samples in borate buffer (0.4 M, Agilent Technologies, Santa Clara, CA) with OPA reagent (*o*-phthalaldehyde, 10 mg/mL, and 3-mercaptopropionic acid, Agilent Technologies). Both the borate buffer and the OPA reagent were diluted 1:3 with water before use. Derivatization was done using the autosampler by adding and mixing the following volumes in a separate vial: 10 μL of borate buffer, 2 μL of sample, 2 μL of OPA reagent, and 130 μL of water. The column was an Agilent Zorbax Eclipse AAA (4.6 × 150 mm, 3.5 μm) with mobile phases A, 20 mM Na_2HPO_4, pH 7.5, and B, 45:45:10 acetonitrile/methanol/Milli-Q water; gradient 0−2 min, 0% B; 2−10 min, 28% B; 10−11 min, 100% B; 11−15 min, 100% B; 15−16 min, 0% B; 16−20 min, 0% B; flow, 2 mL/min; detection, extinction/emission 340/450 nm. Quantification was done by comparison to known standards made in the range of 0.03125−1.25 mM (asparagine and aspartic acid).

Glucose Content. Five potatoes were cut and pressed in a fruit juicer, and the glucose content was measured directly in the juice using a blood sugar automated measuring device (Accu-Chek Aviva, blood glucose meter; Roche, Basel, Switzerland).

Dough pH. pH was measured in a 10% w/v suspension of dough samples in Milli-Q water.

Recipes. All baking recipes are listed in baker's percent according to the following formula:

$$\text{baker's \%} = (\text{ingredient wt}/\text{flour wt}) \times 100\%$$

Semisweet Biscuits. Semisweet biscuits were prepared using 100% wheat flour, 25% tap water, 20.83% sucrose, 16.07% shortening, 1.2% salt, 1.31% glucose, 0.5% sodium

bicarbonate, and 1.07% ammonium bicarbonate. Glucose, sodium bicarbonate, and ammonium bicarbonate were dissolved in water, and asparaginase was added. The rest of the ingredients were mixed in and the dough kneaded in a Bjoern RN Varimixer (A/S Wodschow & Co., Broendby, Denmark) for 2 min at 93 rpm, followed by 13 min at 130 rpm at 35 °C (dough end temperature, 40 °C; dough pH, 8.75). The dough rested for 15 or 30 min at 86% relative humidity (RH) and 40 °C. It was then sheeted 4-fold to a final thickness of 2.8 cm. Biscuits were cut with a hand cutter to a size of 7 × 7 cm. Samples were baked at 260 °C for 5.5 min in a deck oven.

Ginger Biscuits. Ginger biscuits were prepared using 100% wheat flour, 10% tap water, 33.33% sucrose, 20.5% shortening, 25% cane syrup, 0.83% salt, 0.83% sodium bicarbonate, and 0.28% ammonium bicarbonate. Sodium bicarbonate and ammonium bicarbonate were dissolved in water, and asparaginase was added. All ingredients were mixed together in a Bjoern RN Varimixer (A/S Wodschow & Co.) at slow speed for 3 min. Holding time of the dough was 30 min at 25 °C and 70% RH, and dough pH was 7.8. The dough was sheeted, cut, and baked at 190 °C for 13 min in a deck oven. To test the effect of water activity on enzyme performance, the calculated water content in the dough (added water + water in flour and cane syrup) was varied between 11 and 19% by regulating the amount of added water. Baking time was adjusted accordingly, to achieve a similar dry matter in all final products, which ranged from 94.8 to 95.3%. Dough samples with calculated water content of <15% could not be sheeted. Instead, 10 g of these samples was pressed into a round metal form (D_i = 53 mm) using a metal stamp.

Crisp Bread. One hundred percent rye flour, 65% water, and 1.15% salt were mixed in a Bjoern RN Varimixer (A/S Wodschow & Co.) at slow speed for 4 min. Enzyme was added with the water at 2100 ASNU/kg of flour. The dough was held for 30 or 60 min at 65% RH and 10, 15, or 20 °C. This was achieved by adjusting the water temperature before addition, tempering mixing vessels, and subsequent resting in thermostated cabinets. Dough pH was 6.4. The dough was sheeted to a final thickness of 4 mm, cut to a size of 10 × 6.5 cm, and baked in a rack oven at 250 °C for 11 min.

French Fries. Large-sized Bintje baking potatoes were used for all experiments. Potatoes were stored at optimal temperature, between 8 and 10 °C, at the supplier until delivery. However, in the laboratory, storing at 8−10 °C resulted in sprouting, so potatoes had to be stored colder, at 4 °C, to avoid this. For investigating the effect of raw material variation, potatoes were purchased over a period of 7 months from October to April corresponding to different ages (i.e., time from harvest to experiment) of the potatoes. Batch 1 was obtained in October and was stored cold (~4 °C) for 3 weeks, batch 2 was obtained in December and stored cold for 4 weeks, batch 3 was obtained in April and stored cold for 1.5 weeks, and batch 4, also from April, was processed immediately without cold storage.

In each experiment, a blank, a control sample, and one or more enzyme-treated samples were included. Samples consisted of 150 or 300 g of potato strips that were prepared for processing by manually peeling potatoes and cutting them into 8 × 8 mm strips. To minimize effects from variations among individual potatoes, the strips from each potato were distributed evenly across all test samples. The enzyme-treated samples were made by incubating blanched potato strips in enzyme solution, the blank sample was incubated in water without enzyme, whereas the control sample was made without an extra incubation step. The control sample was included to mimic the existing industrial product.

Two different processes were used for treating potato strips with asparaginase, a 20 min soaking process, and a 1 min dipping process. In experiments using the soaking process, the strips were blanched by pouring water at 90−95 °C over them and subsequently holding them in water at 70 °C for 20 min with a water to potato ratio of 2:1 (w/w). All potato strips used in a single experiment were blanched simultaneously. After blanching, the potatoes were drained and put in plastic bags and placed in the refrigerator at 5 °C for cooling and temporary storage (<2 h) to facilitate sequential processing of the different samples. The enzyme-treated and blank samples were then immersed in deionized water at 40 °C and allowed to equilibrate for 5 min. Incubation continued for an additional 20 min at 40 °C either with or without asparaginase at a concentration of 10500 ASNU/L. The water/potato ratio for the soaking process was 2:1 w/w. The pH was not adjusted, but fell within the range of 5.5−6.5. In experiments using the shorter treatment time of 1 min, the blanching technique used was the same as described above, but the raw potato strips were held temporarily (<2 h) in deionized water before blanching to facilitate processing of the different samples. Samples were blanched individually and treated immediately afterward. For enzyme-treated and blank samples, potatoes were drained and dipped in water for 1 min at 55 °C either with or without asparaginase added to a final concentration of 10500 ASNU/L (water/potato ratio 2:1 w/w, pH 5.5−6.5). All samples (blank, enzyme-treated, control) were further processed by drying in a ventilated heating chamber at 85 °C for 10 min, corresponding to a weight loss of 12−15%, par fried for 1 min at 175 °C, and blast frozen. The second frying was for 3 min at 175 °C to make the final product.

Sliced Potato Chips. Slices (1.4 mm) were made from Bintje baking potatoes and given a short blanching at 80 °C for 1 min, cooled, and incubated with enzyme at various concentrations for 15 min at 40 °C (water/potato ratio 5:1). Frying was done at 180 °C for 2.5 min.

Statistical Methods. One-way analysis of variance was performed in Microsoft Office Excel.

RESULTS AND DISCUSSION

Cloning, Recombinant Expression, and Characterization of a Fungal Asparaginase. To identify a microbial asparaginase for recombinant industrial production, the published peptide sequence of an asparaginase from *Saccharomyces cerevisiae*, ASP II (sequence accession no. P11163) (*31*), was used to search Novozymes' sequence database, which includes whole and partial gene sequences from a variety of fungi. DNA clones corresponding to these sequences are organized in gridded libraries (*32*). A cDNA clone from *A. oryzae* was thus identified that encodes a peptide with 50% identity to ASP II throughout a 330 amino acid overlap when aligned with the sequence comparison program FASTAP (*33*) and includes the 4 active site residues identified in the sequence annotation for P11163. The sequence of the *A. oryzae* cDNA is available via sequence accession no. ADO07178. To obtain the putative enzyme for further investigations, the cDNA was expressed recombinantly in a high-yielding generally recognized as safe (GRAS) expression system, which relies on *A. oryzae* as an expression host (*28*).

A. oryzae transformants were tested for expression of the putative asparaginase by monitoring small fermentation cultures with SDS-PAGE. Many transformants were observed to express an extracellular protein that was not seen in the untransformed control strain (**Figure 1A**). This protein resolves as a double band at around 35−40 kDa and a smear from about 40 to 65 kDa. Four different N-terminal sequences were determined for the

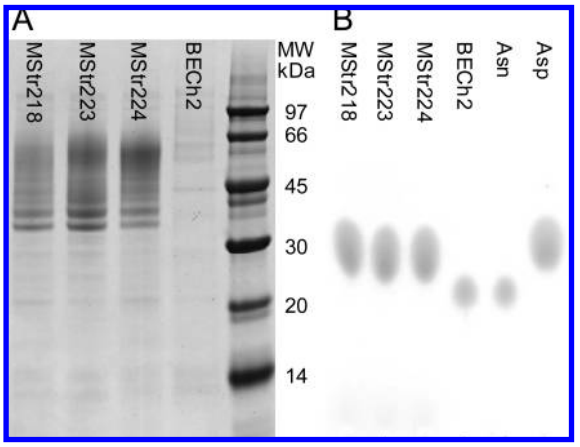

Figure 1. Analyses of fermentation supernatants of the untransformed *Aspergillus oryzae* strain, BECh2, and transformants MStr218, MStr223, and MStr224, which express the putative *A. oryzae* asparaginase: (**A**) SDS-PAGE (MW, molecular weight standard); (**B**) TLC of the supernatants incubated with asparagine [standards are asparagine (Asn) and aspartic acid (Asp)].

recombinantly expressed protein (*28*), which predict mature proteins in the size range of 32−37 kDa. The greater protein sizes and the very broad size range observed in SDS-PAGE indicate that the putative asparaginase from *A. oryzae* is glycosylated and that there is a great variety in degree of glycosylation. ASP II is reported to be heavily glycosylated (*31*).

To test for asparaginase activity, culture broths containing the recombinant protein seen in SDS-PAGE analysis were incubated with asparagine, and the resulting reaction products were resolved with TLC. As can be seen in **Figure 1B**, culture broths from the transformed strains could readily hydrolyze asparagine to aspartate, whereas the broth from untransformed *Aspergillus* could not. Combined results from gel electrophoresis and activity assay indicate that the expressed protein is an asparaginase.

The activity of the *A. oryzae* asparaginase was measured at a range of pH values at 37 °C (**Figure 2A**) and at a range of temperatures at pH 7 (**Figure 2B**). The asparaginase was seen to be most active in the neutral pH range and at temperatures up to 60 °C, conditions amenable for treating food ingredients or products. The *A. oryzae* asparaginase was shown to be stable between pH 4 and 8 for 2 h at 37 °C in buffer (*28*).

Asparaginase Application Testing. To evaluate the potential of the *A. oryzae* asparaginase as a tool for general acrylamide mitigation in foods, a range of application trials were performed for both cereal- and potato-based food products. For the cereal-based foods, semisweet biscuits, crisp bread, and ginger biscuits were chosen as model systems: semisweet biscuits as a well-known and typical wheat flour based biscuit; crisp bread as a rye flour based product characterized by both high water content and low dough temperature; and finally ginger biscuits as a short dough product characterized by a surprisingly high level of acrylamide compared to other biscuits types. The European Commission database of acrylamide levels in commercial products reports that gingerbread samples have contained up to 7834 μg/kg, with a median measured value of 303 μg/kg; biscuit samples have contained up to 3324 μg/kg, with a median of 145 μg/kg; and crisp bread samples have contained up to 2838 μg/kg, with a median of 244 μg/kg (*34*).

A number of variables can influence the effectiveness of enzyme-catalyzed reactions, including the dose of the enzyme, the length of reaction time, water content of the reaction environment, and the temperature and pH at which the reaction

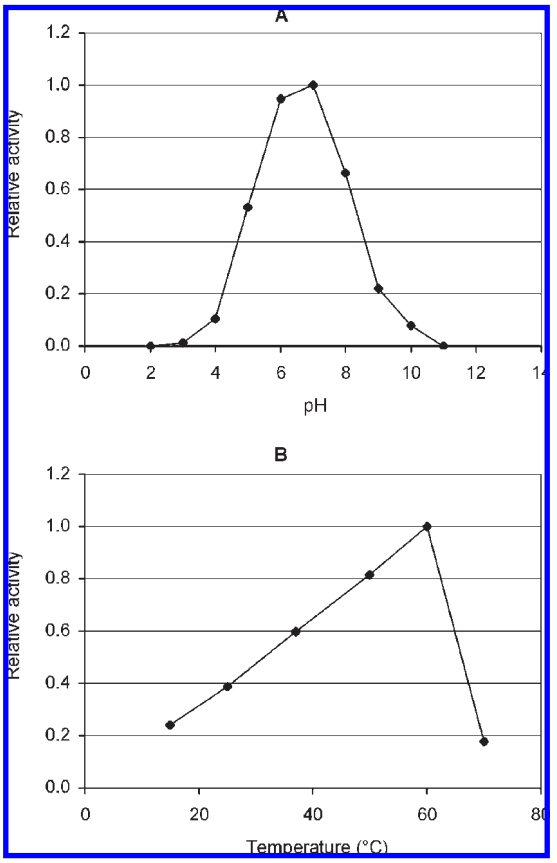

Figure 2. (**A**) pH activity profile at 37 °C. Activities are represented relative to pH optimum. (**B**) Temperature activity profile at pH 7. Activities are represented relative to temperature optimum.

occurs. For most bakery products, asparaginase can be added directly to the dough, and incubation will occur at room temperature or slightly higher and at dough pH. The two most important variables for these systems are therefore enzyme dose and reaction time, which is roughly equal to the resting time for the dough.

Semisweet Biscuits. Results from testing asparaginase in semisweet biscuits while varying both enzyme dose and reaction time are shown in **Figure 3**. In the control samples, acrylamide levels of 370 and 460 μg/kg were measured for 15 and 30 min resting times, respectively. This compares well with reported levels for similar commercial products (*34*). The difference between the measured control values probably reflects experimental variation rather than the difference in resting time, because this would not be expected to affect acrylamide levels.

In samples treated with asparaginase, a clear reduction of acrylamide levels in the final biscuits was observed, showing an increasing effect with increasing amount of enzyme. Treatment with 525 ASNU/kg of flour and a dough resting time of 15 min resulted in an acrylamide level of 130 μg/kg, equivalent to a reduction of 65% when compared to the corresponding control. Dough treated with twice the amount of enzyme and the same resting time had 58 μg/kg acrylamide in the final biscuit, equal to an 84% reduction in acrylamide content. In all cases the effect of the asparaginase is greater in samples made with a longer resting time, illustrating that the system operated within the dynamic response range of both enzyme dosage and resting time. Vass et al. (*24*) have reported similar results in a wheat flour based cracker showing reduction in acrylamide content of 85% when using an asparaginase from *E. coli*. Amrein et al. (*35*) found

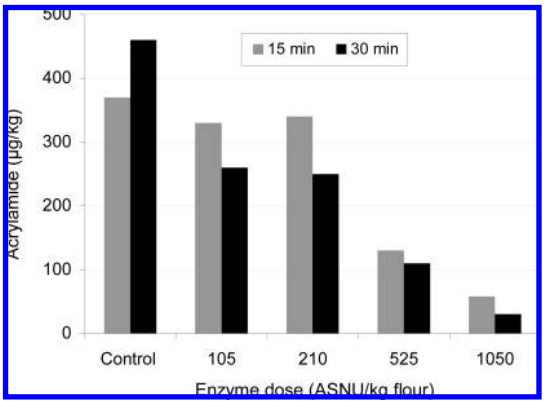

Figure 3. Acrylamide levels in semisweet biscuits as a function of dough resting time (at 40 °C) and asparaginase dose. The values shown represent single determinations.

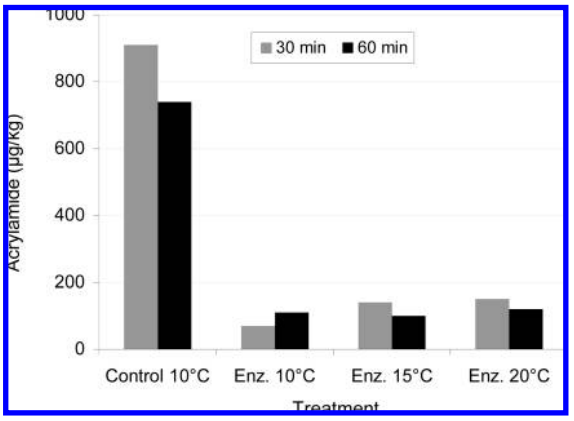

Figure 4. Acrylamide levels in crisp bread as a function of dough resting time and temperature. Asparaginase dose = 2100 ASNU/kg of flour. The values shown represent single determinations.

60−90% reduction in acrylamide levels in hazelnut biscuits when testing the *A.oryzae* asparaginase in pilot scale.

The results presented for this experiment are based on single determinations. To address experimental variation, results from several separate trials in semisweet biscuits were compared. In eight trials in which semisweet biscuits were made using the same recipe and process, including the trial described above, 10 control samples were made. The acrylamide levels in these ranged from 120 to 400 μg/kg, with an average of 238 μg/kg and a standard deviation of 41% (data not shown). The acrylamide levels in corresponding enzyme-treated samples made with 1050 ASNU/kg of flour and a 15 min holding time were also quite variable and ranged from 19 to 70 μg/kg, with an average of 43 μg/kg and a standard deviation of 18% (data not shown). Thus, experimental and analytical variations limit the usefulness of directly comparing the absolute acrylamide measurements from different experiments. However, the relative reduction in acrylamide levels in these treated samples calculated within each experiment ranged from 68 to 91%, with an average of 80% and a standard deviation of 8%. Despite high levels of experimental variation, the effect of enzyme treatment was thus relatively consistent and the result for the single experiment shown in **Figure 3** representative of the results obtained in repeated trials. Other studies have found variations in acrylamide content of around 17% deriving alone from variations in the baking process (*12*, *24*), as well as variations in the acrylamide quantification of 3−12% depending on the food matrix (*30*).

Crisp Bread. Crisp bread production presents the challenge of low dough temperatures during preparation, indicating that a high dosing of asparaginase would likely be needed to achieve a similar degree of reduction of acylamide levels as those observed for semisweet biscuits. From the temperature activity curve for asparaginase (**Figure 2B**) it was estimated that at ∼10 °C the enzyme would have only 30% of the activity it has at 37 °C, suggesting that roughly 3 times more enzyme might be needed in crisp bread dough. Additionally, the crisp bread recipe chosen for this study utilizes rye flour, which typically contains more free asparagine than wheat flour (*5*, *35*), further indicating that a higher enzyme dosing would be required. To investigate the impact of lower dough temperature, experiments were carried out at various dough temperatures and two resting times while enzyme dose was kept constant at 2100 ASNU/kg of flour. This dose corresponds to twice the effective dose in semisweet biscuits and was expected to compensate at least partially for the lower temperature.

Results are illustrated in **Figure 4**. Acrylamide levels measured in the untreated biscuits were 910 and 740 μg/kg. Again the difference in these values probably reflect experimental variation rather than a difference caused by resting time variation. Compared to the reported average level of 244 μg/kg for similar commercial products the values are high, but within the range reported (maximum = 2838 μg/kg) (*34*). Reductions in acrylamide content up to 84−92% were obtained for all asparaginase-treated samples, irrespective of dough temperature and resting time. The high level of acrylamide mitigation achieved with the 10 °C treatment and the lack of dynamic response to higher dough temperatures indicate that the applied enzyme dose was in excess. In comparison to the results in semisweet biscuits, roughly the same level of reduction was achieved in crisp bread using only twice the amount of asparaginase, despite the expected reduction in enzyme activity to one-third due to the lower temperature. This suggests that the asparaginase is more active in crisp bread dough due to factors other than temperature. One of these factors could be water activity, as the water content of crisp bread dough is higher than that of semisweet biscuit dough. Alternatively, the lower pH in the crisp bread dough might be advantageous for the enzyme.

A repetition trial in crisp bread resulted in acrylamide levels of 1100 μg/kg for the control, 260 μg/kg for the enzyme-treated sample at 10 °C, and 140 μg/kg at 20 °C, corresponding to a 76−87% reduction in acrylamide, which is comparable to the results shown in **Figure 4**.

Ginger Biscuits. The importance of dough water content was further investigated in ginger biscuits, which are typically prepared from dough with relatively low water content. In **Figure 5** the acrylamide content in ginger biscuits made from dough of various water contents and either with or without asparaginase is illustrated. For dough treated with enzyme, the dose was held constant at 1000 ASNU/kg of flour. Acrylamide levels measured in the control biscuits were rather constant, despite the differences in dough water content, and varied between 530 and 640 μg/kg. Control levels compare well with the reported values from the European acrylamide database (*34*). A clear correlation between the acrylamide level in the final biscuits and the water content in the enzyme-treated dough was seen. For dough with a calculated water content of 15%, corresponding to the standard recipe, a reduction in acrylamide content of 34% was measured as compared to the corresponding control. At the slightly lower water content of 13%, the reduction achieved was only 19%, whereas the enzyme had little if any effect at 11% water content. At the highest water content of 19%, the same dose of asparaginase resulted in a 90% reduction. The reason for this is most likely that enzyme−substrate contact is limited in the low-water dough because of limited diffusion. For comparison,

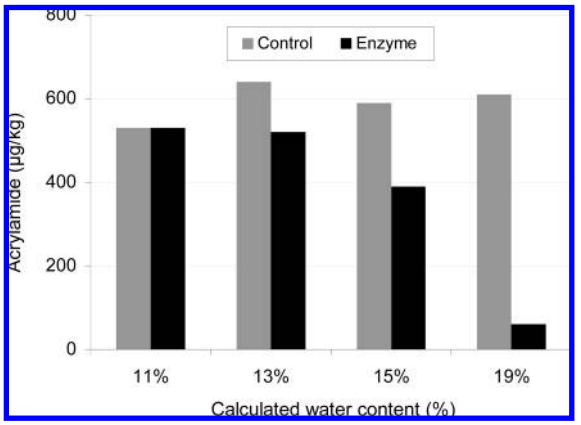

Figure 5. Acrylamide levels in ginger biscuits made from dough of various calculated water contents and with or without enzyme. Asparaginase dose = 1000 ASNU/kg of flour. The values shown represent single determinations.

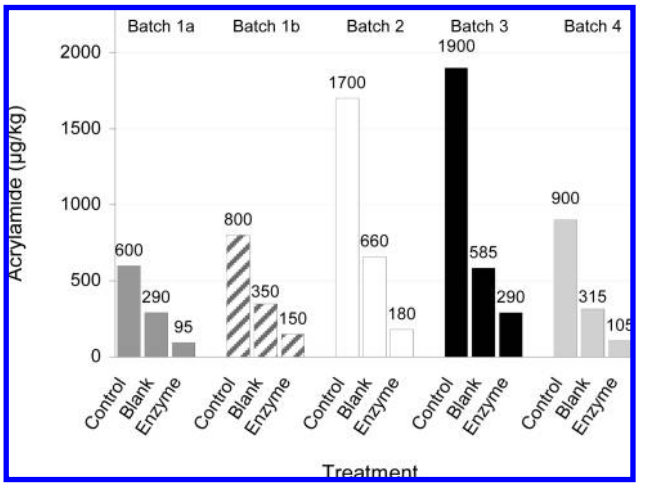

Figure 6. Acrylamide levels in French fries made from different potato batches. The batches varied with respect to age and storage conditions. Fries were made from blanched potato strips soaked in water (blank) or in enzyme solution (enzyme) at 40 °C for 20 min. Asparaginase dose = 10500 ASNU/L of water. Control samples were blanched strips that had no extra soak in water or enzyme solution, but were dried right after blanching. Batches 1a and 1b are replicates made from the same batch but processed within 2 days. For batches 3 and 4 duplicate samples were made, and the values presented are averages, whereas all other values represent single determinations. Moisture content of all final products was around 50%.

the semisweet biscuit dough in this study has a calculated moisture content of ~20% and the crisp bread dough of ~48%.

Similar conclusions were made by Amrein et al. (*12*) when testing asparaginase from *E. coli* in gingerbread. They found that the acrylamide content was reduced by 55%, with approximately 75% of the free asparagine hydrolyzed by the enzyme. Low mobility of the substrate and enzyme in the dough was suggested as an explanation for the incomplete hydrolysis of asparagine and partial reduction in acrylamide formation.

Application Testing in Potato Products. Another category of food products characterized by having relatively high levels of acrylamide are French fries and sliced potato chips. Compared to dough-based products, these foods consist of solid cut pieces of vegetable, making contact between enzyme and substrate far from optimal and introduction of an enzyme treatment potentially more complex. Levels of acrylamide in commercial products vary considerably, reflecting substantial differences in raw materials and processing. Reported levels in French fries range from 5 to 4653 μg/kg, with a median of 186 μg/kg, and in sliced potato chips from 5 to 4215 μg/kg, with a median of 528 μg/kg (*34*).

French Fries. When the industrial process used for French fry production is considered, the most obvious application point for an enzyme is during blanching, when peeled, cut potato strips are held in hot water (70−85 °C) for 10−30 min. In this processing step, the indigenous enzymes of the potato are inactivated and sugars and asparagine are washed out. However, typical blanching temperatures are too high for the *A. oryzae* asparaginase, so an extra process step at a lower temperature must be introduced immediately after blanching.

Reducing sugar content of potatoes varies widely with potato cultivar, age, and storage time and conditions, and acrylamide levels measured in final potato products typically show a similar degree of variation, with higher amounts of acrylamide formed from potatoes with high sugar levels (*13*−*18*). To investigate the effect of this natural raw material variation on asparaginase-mediated acrylamide mitigation, enzyme treatment was tested on several different batches of potatoes. The potatoes were of the same cultivar, but the batches varied with respect to age and storage conditions and covered some of the typical seasonal raw material variation (see Materials and Methods). Dry solids in the batches varied from 18 to 26% (data not shown). **Figure 6** shows the results of treating potato strips from these batches for 20 min after blanching. Acrylamide levels in control samples from the different potato batches varied widely, as expected given the diversity of the potato batches used, and ranged from 600 to 1900 μg/kg. This is within the reported range for commercial products, but above the median value of 186 μg/kg (*34*).

Treatment with asparaginase resulted in a lower amount of acrylamide in the final products for all batches tested as compared to the control for each batch. Acrylamide levels in the enzyme-treated samples were between 95 and 290 μg/kg, or 80−90% lower than the values from the respective controls. The enzyme was thus effective in reducing acrylamide formation, and performance was relatively consistent, regardless of potato batch, age, and conditions of storage.

In the blank samples, which were treated with water alone, a noticeable reduction in acrylamide content was also observed. Acrylamide levels were between 290 and 660 μg/kg in the different batches. This corresponded to a reduction in acrylamide content in the individual samples of up to 60% that was obtained simply by leaching out more sugar and asparagine than that already removed during blanching. Similar results have been found in other experiments in which prolonged blanching or soaking have shown reductions in acrylamide content from 25 to 70% depending upon treatment temperature and time (*17*, *21*, *22*).

Experimental reproducibility is exemplified by the results shown for batch 1. This batch was processed in two separate experiments (batches 1a and 1b in **Figure 6**) made within 2 days. Acrylamide levels in the controls were 600 and 800 μg/kg. The difference in these values reflects experimental variation, including both minor day-to-day differences in processing and analytical variation in acrylamide analysis. The analytical variation (CV) at Eurofins is reported to be 10% for potato products, whereas acrylamide concentrations in potato chips analyzed in different packages of one batch were found to range between plus and minus 50% of the mean (*30*). In another study evaluating the potential of acrylamide formation in potatoes, process variations up to 36% on reported acrylamide levels were found (*13*).

The calculated reductions in acrylamide levels obtained in the enzyme-treated and the blank samples relative to the controls

Table 1. Calculated Total Percent Reduction in Acrylamide Content of Blank (= Water) and Enzyme Treatments Relative to the Control in French Fries Prepared from Different Potato Batches[a]

batch	blank, %	enzyme, %
1a	69	85
1b	65	88
2	61	89
3	56	81
4	52	84
av	60.6	85.4
SD	6.8	3.2

[a] Reductions are calculated based on the data shown in **Figure 6**.

for the different potato batches are given in **Table 1**. A comparison of these results shows that there is little difference in effect within the treatments, but a significant difference between treatments ($\alpha = 5\%$, $F = 54.3$, $F_{crit} = 5.32$). Inclusion of asparaginase consistently results in significantly lower levels of acrylamide formation. On the basis of this comparison, it can be concluded that for the conditions of treatment used in this experiment, the raw material variations had little effect on relative acrylamide mitigation and that the asparaginase provided a significant and stable reduction in acrylamide formation irrespective of control acrylamide level.

Glucose concentrations measured directly in potato juice made from two of the batches substantiated the correlation between cold storage time, sugar content, and acrylamide formation reported in earlier studies (*13*−*18*). French fries from batch 3 (stored at 4 °C for 1.5 weeks) had an acrylamide content of 1900 μg/kg and a glucose content in the potato juice of 34 mmol/L, whereas fries from batch 4 (no storage at 4 °C) had an acrylamide content of 900 μg/kg and a correspondingly lower glucose level of 12 mmol/L. Additionally, some effect of potato age seems apparent in the results in **Figure 6**, showing that acrylamide levels increased with increasing age of cold-stored batches (batches 1−3).

As an alternative to the 20 min enzyme treatment, a short 1 min dipping treatment was tested in which the asparaginase is allowed to react during the subsequent drying step. Results from such an experiment are shown in **Figure 7**. As seen from the figure, acrylamide levels are reduced as the enzyme dose is increased, reaching a maximum effect of 59% reduction at 10500 ASNU/L versus the control. This is lower than obtained with the 20 min soak (~85%) but still a substantial reduction. Considering the very short treatment time of only 1 min, it is evident that the enzyme is still active during drying, even though drying air temperature is higher than what can be tolerated by the enzyme. However, due to water evaporation from the surface of the potato strips, product temperature will be lower than the drying temperature. A short 1 min dip in water also reduced acrylamide content, but only by 26% as compared to the 60% reduction obtained after 20 min in water (**Table 1**), illustrating the importance of incubation time for having an effect of this extra wash. An evaluation across all treatments showed that the effect of a 1 min dip at the highest enzyme dose was comparable to a 20 min soak in water, clearly illustrating that the time required to achieve a specific acrylamide reduction is decreased significantly by the use of the asparaginase. Apart from the direct savings in process time obtained by implementing a short enzyme treatment step, the alternative long-term blanching or soaking treatment might also lead to wash-out of desired compounds, such as flavor compounds, vitamins, and minerals, and further affect final product properties like crispness.

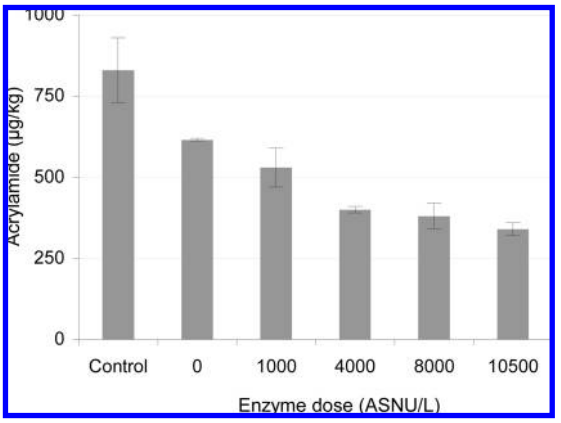

Figure 7. Acrylamide levels in French fries as a function of enzyme dosage. Fries were made from blanched potato strips dipped in enzyme solution at the given concentration (0, 1000, 4000, 8000, or 10500 ASNU/L) at 55 °C for 1 min. Control, no dip. Bars indicate minimum and maximum of duplicate samples.

Table 2. Asparagine and Aspartic Acid Contents in Treated Potato Strips after Drying before Frying[s]

sample	asparagine, g/kg	aspartic acid, g/kg
control	1.50	0.26
blank	1.32	0.21
enzyme	0.59	0.67

[s] Treatment was a 1 min dip in water (= blank) or in enzyme (10500 ASNU/L). Control sample had no extra treatment. Data are from duplicate samples given in grams per kilogram of sample.

Results from measuring the content of asparagine and aspartic acid in treated potato strips after a 1 min treatment are given in **Table 2**. Levels of asparagine in the blank sample dropped slightly compared to the control, due to the extra wash in water. The asparagine content in the enzyme-treated sample decreased by approximately 60% as compared to the control, whereas the level of aspartic acid increased, illustrating the direct effect of the enzyme on the intermediate treated product. Data from other groups similarly show a direct effect of asparaginase in food ingredients (*11*, *12*, *24*, *36*).

Sliced Potato Chips. Enzyme treatment of sliced potato chips was also tested. Blanching is optional in the industrial production of potato chips, so in this experiment both unblanched and blanched potato slices were tested. Treatment of the blanched slices in water without asparaginase gave an acrylamide level of 1750 μg/kg, which was reduced to around 710 μg/kg with the enzyme (**Figure 8**). If the slices were not blanched prior to the asparaginase treatment, no reduction in acrylamide levels was observed (data not shown). Thus, it seems that asparaginase is ineffective in treating raw potato pieces but that auxiliary processing, such as blanching, can facilitate treatment. Blanching very likely reduces the structural integrity of the potato pieces, weakening the cell wall and membrane, thereby improving substrate diffusion and enzyme−substrate contact.

In this experiment baking potatoes were used instead of industrial chip potatoes, due to difficulties in obtaining these. Baking potatoes have a lower DS and higher sugar content than chip potatoes, boosting the potential for acrylamide formation. Furthermore, frying to a specified moisture level of $<2\%$ was not possible because it resulted in burned product. To compensate, frying time was instead established by targeting an acceptable final product color based on visual examination. The final product therefore did not match typical industrial product

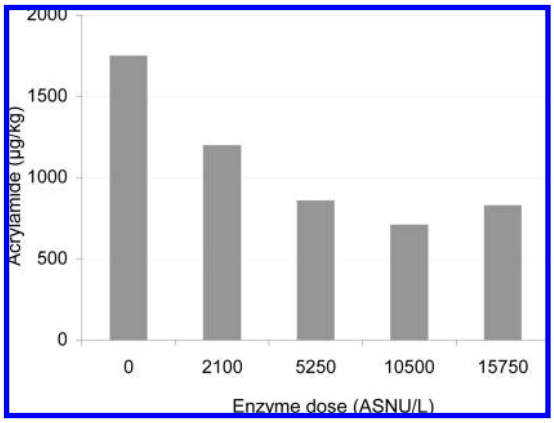

Figure 8. Acrylamide levels in sliced potato chips as a function of enzyme dose. Treatment conditions include 15 min in a water bath at the given enzyme concentration at 40 °C applied after blanching at 80 °C for 1 min. Results represent single determinations.

specifications concerning moisture level, which is indeed a critical factor when looking at acrylamide levels, because low moisture content is strongly correlated with high acrylamide levels (*21*). However, the results presented indicate a possible approach for working with thin potato slices.

The reductions achieved in both French fries and sliced potato chips illustrate that it is indeed possible to affect final acrylamide levels in fried potato products by selectively manipulating the asparagine level, even though reducing sugars have been shown to be the most important and limiting factor for acrylamide formation in this type of product (*13*−*18*). A few other reports on asparaginase treatment of potato-based products have been published and are consistent with this conclusion. Ciesarova et al. (*25*) have tested asparaginase from *E. coli* in roasted pancakes made from either peeled, shredded, fresh potatoes or a dried potato premix. In the fresh potatoes reductions in acrylamide contents from 45 to 97% were achieved, whereas in the dry premix reductions ranged from 70 to 97%. Maximum reductions were thus higher than the results from this study. The reason for this is probably a combination of a longer treatment time and more importantly the much smaller size of the shredded potato pieces, a feature that will clearly facilitate enzyme−substrate contact. Pedreschi et al. (*36*) tested the asparaginase from *A. oryzae* in treatment of French fries. When a 20 min soak at 40 °C and 10000 ASNU/L was used for blanched potato strips, a 62% reduction in acrylamide content was observed, comparable to the results of the present study.

High-level recombinant expression of the extracellular *A. oryzae* asparaginase allows for production of sufficient amounts of enzyme to enable broad industrial implementation. Results presented in this study demonstrate that it is possible to effectively reduce acrylamide formation in a range of food products by applying asparaginase during production. The conditions tested show that the enzyme can be applied to wheat, rye, and potato products, in dough or to solid potato pieces. They also demonstrate that the *A. oryzae* asparaginase can work effectively from 10 to 55 °C, as used for crisp bread and potato experiments, respectively, and at pH values from 5.5 to 8.75, as applied in the treatment of potatoes at one extreme and semisweet biscuits at the other. In dough-based products, key parameters influencing enzyme effectiveness investigated were enzyme dose, dough resting time, and water content. In the treatment of French fries and sliced potato chips, key parameters were enzyme dose and the method of application, because appropriate pretreatment, for example, blanching, was indicated for optimal performance.

The recipes used in this study were chosen and established in the laboratory to resemble industrial processes. The results obtained, therefore, indicate that the *A.oryzae* asparaginase has the potential to be effective under industrial conditions. Industrial implementation in dough-based products will require some initial trials to establish the effective enzyme dosage for a specific product, but only minimal changes to existing production lines will in most cases be needed, because asparaginase can be added in the same manner as other ingredients. Enzymes are already in broad use in the baking industry, and enzyme formulation, storage, and addition techniques are well established (*37*). For French fry production industrial implementation will require modification of current production lines to include an enzyme bath. Of the two treatments considered in this study, a 20 min soak or 1 min dip in enzyme solution, the process with the shorter retention time was nearly as effective, should be easier to implement, and would be less likely to affect product quality. A 1 min enzyme treatment could be operated as a continuous process, if appropriate cooling of the blanched potatoes is implemented and effective temperature control to ensure optimal enzyme performance and stability in the bath established. The short retention time in the enzyme bath exploits the subsequent existing drying step used in industrial processing to provide time for the enzyme to act, and thus allows for enzyme treatment, whereas total production time as such will not be severely affected.

ABBREVIATIONS USED

TLC, thin layer chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CABS, 4-(cyclohexylamino)-1-butanesulfonic acid; ASNU, asparaginase activity unit; DS, dry solids; HPLC, high-pressure liquid chromatography; OPA reagent, *o*-phthalaldehyde and 3-mercaptopropionic acid; RH, relative humidity; GRAS, generally recognized as safe.

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