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Short communication

Thermal stability of starch degrading enzymes of teff (*Eragrostis tef*) malt during isothermal mashing

Mekonnen M. Gebremariam, Martin Zarnkow*, Thomas Becker

Institute of Brewing and Beverage Technology, Center of Life and Food Sciences, Technische Universität Muenchen, Germany

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ABSTRACT

Thermal stability of starch degrading enzymes varies from one source to another. This research was aimed to study thermal stability of starch degrading enzymes of teff malt. Isothermal mashing at temperatures ranging between 40 and 75 °C with sampling in 15 min interval for a total of 90 min was conducted. The study showed that deactivation rate constants of alpha- and beta-amylases ranged from 0.0003 to 0.0409 min^{-1}, and 0.002 to 0.032 min^{-1}, respectively. Rate of deactivation of limit dextrinase was not significant at temperatures lower than 60 °C but showed high deactivation at higher temperatures with rate constants ranging from 0.02 to 0.1 min^{-1}. The thermal deactivation energies of alpha-amylase, beta-amylase, and limit dextrinase were found to be 148, 82, and 144 kJ/mol, respectively. The present findings have significant applications in commercial processes where determination of the upper temperature limits for these enzymes is required.

1. Introduction

The demand for gluten-free foods is certainly increasing. Interest in teff has increased noticeably due to its very attractive nutritional profile and gluten-free nature [1,2], making it a suitable substitute for wheat and other cereals in their food applications including in beverage production. The capacity of a cereal to produce malt with amylolytic enzymes is a primary quality criterion for the cereal to be used in brewing industries. Our previous findings showed that teff has the potential to produce malt with high amylolytic enzyme activities [2–4]. Starch degrading enzymes are among the most important enzymes used in food and other industries involving starch hydrolysis. It is well known that germination of cereals causes a significant increase in the enzyme activities. On the other hand, kilning and mashing processes may decrease the activities of the enzymes [5–8]. Thermal stability of the enzymes is a very crucial property in designing processes involving starch hydrolysis as it determines the limits for use of the enzymes [9]. The temperature program in mashing, for example, is normally designed to favor starch degrading enzymes in order to maximize extract yield and formation of fermentable sugars. The relationship between thermostability of starch degrading enzymes and the gelatinization temperature of the starch essentially determines the formation of fermentable sugars during mashing [10,11].

The thermal stability profiles of starch degrading enzymes vary from one cereal to another [12,13]. Studies on barley malt showed that limit dextrinase, which hydrolyses α-1,6-linkages of amylopectin, has somewhat more thermal stability when compared to beta-amylase. But it is readily inactivated at temperatures higher than 65 °C. On the other hand, α-amylase has high stability when compared to the other two, and retains significant amount of its activity at relatively higher temperatures (>65 °C) [14–16]. However, the thermal stabilities of starch degrading enzymes of teff malt have not been studied yet. Thus, this research was aimed to study the thermal stability of amylolytic enzymes of teff malt.

2. Materials and methods

2.1. Thermal stability test

We studied the thermal deactivation of alpha-amylase, beta-amylase, and limit dextrinase during isothermal mashing of teff malt in a 1:4 malt to water ratio at temperatures ranging from 40 to 75 °C with sampling in 15 min interval for a total of 90 min. The samples were cooled down to room temperature, and assayed for alpha-amylase, beta-amylase, and limit dextrinase activities using standard methods [17–20]. Since limit dextrinase did not show any deactivation at temperatures lower than 60 °C, additional experiments were conducted at temperatures of 62, 63, 64, 65, and 70 °C. The malt samples used in these experiments were prepared using a previously developed condition of malting [3,4]. It is necessary to know the enzyme activities in the malt samples in order to determine the absolute residual enzyme activity after thermal exposure. Thus, preliminary experiments were carried out to determine the enzyme activities that can be achieved under the mashing conditions before exposure of the enzymes to higher thermal energy. The activities of all the three types of enzymes considered in this study showed a significant increment throughout the 90 min of thermal exposure at 30 °C but started to decline after 90 min. Hence, the highest enzyme activities recorded at this temperature were used as initial activities (X_o).

* Corresponding author at: Weihenstephaner Steig 20, 85354 Freising, Germany. Tel.: +49 8161713263; fax: +49 8161713883.
E-mail address: martin.zarnkow@wzw.tum.de (M. Zarnkow).

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2.2. Enzyme activity assay

The wort samples were assayed for alpha-amylase, beta-amylase, and limit dextrinase activities. The enzyme activities were determined spectrophotometrically following standard methods [17–20]. According to these procedures, determination of the enzyme activities of malt involves extraction of the enzymes from the malt using extraction buffers followed by incubating the extract with standard substrate. However, in this study the extracts produced at the different mashing temperatures were incubated with the standard substrate.

2.2.1. Alpha-amylase

For the determination of alpha-amylase activity, teff malt extract (0.2 ml) was incubated for 10 min at 40 °C with 0.2 ml of pre-equilibrated substrate mixture consisting of non-reducing-end blocked p-nitrophenyl maltoheptaoside (BPNPG7) as well as excess levels of gamma-amylase and glucoamylase. The substrate is absolutely resistant to hydrolysis by exo-enzymes such as β-amylase, gamma-amylase and glucoamylase. On hydrolysis of the oligosaccharide by endo-acting α-amylase, the excess quantities of glucoamylase present in the mixture give instantaneous and quantitative hydrolysis of the p-nitrophenyl maltosaccharide fragment to glucose and free p-nitrophenol. The reaction was terminated by the addition of trizma base solution. Then the absorbance of p-nitrophenol in the solution was measured at 410 nm against distilled water [17]. One unit of enzyme activity is defined as the amount of enzyme which releases 1 μmole of p-nitrophenol/min under the defined assay conditions.

2.2.2. Beta-amylase

For beta-amylase measurement, teff malt extract (0.2 ml) was incubated with 0.2 ml of pre-equilibrated substrate mixture of β-glucosidase and p-nitrophenyl-β-D-maltotrioside (PNPβ-G3) at 40 °C for 10 min. The substrate (PNPβ-G3) is rapidly hydrolysed by β-amylase, but not cleaved by cereal α-amylase, which requires a longer stretch of α-1,4-linked D-glucosyl residues to satisfy the substrate sub-site binding requirements [21]. The rate of release of p-nitrophenol by the β-glucosidase present in the substrate mixture relates directly to the rate of release of maltose by β-amylase. The reaction was terminated and color developed on addition of a high pH trizma base solution. The absorbance of the solution was determined at 410 nm against distilled water [18].

2.2.3. Limit dextrinase

The substrate in the determination of limit dextrinase was azurine-crosslinked-pullulan, which is hydrolysed by limit dextrinase and pullulanase, but is resistant to attack by other commonly occurring amylolytic enzymes such as α-amylase, β-amylase and amyloglucosidase. A pre-equilibrated teff malt extract at 40 °C for 5 min was incubated with the substrate at 40 °C for 10 min. After terminating the reaction by trizma base solution, the absorbance was determined at 590 nm [19]. Hydrolysis by limit-dextrinase (pullulanase) produces water soluble dyed fragments, and the rate of release of these substances or the increase in absorbance at 590 nm can be related directly to enzyme activity. Enzyme activity in U/ml was determined by reference to standard curve (Absorbance at 590 nm versus activity in U/ml). One Unit of activity is defined as the amount of enzyme required to release 1 μmole of glucose reducing-sugar equivalents per min from pullulan under the defined assay conditions.

2.3. Enzyme kinetics data analysis

At higher temperatures the enzyme stability decreases by thermal denaturation; as a result the enzymatic activity starts to decline. The thermal energy applied for the denaturation process is termed as the energy of activation of thermal denaturation reaction, or as it is usually called energy of deactivation [22]. Since the active enzymes proceed directly to inactive state without providing significant amount of stable intermediates, thermal deactivation of enzymes is considered as a first order reaction [23–26]. Thus, the deactivation rate constants of the enzymes were determined from residual enzyme activities, which represent the ratio of the enzyme activity at time t to the highest enzyme activity determined from a sample before thermal exposure, using a first order reaction expression.

$$\frac{dX}{dt} = -kX \tag{1}$$

where X represents the maximum enzyme activity following thermal exposure of the teff malt samples for a certain time t.

The values of the deactivation rate constants (k) were determined from a plot of residual activity data $\ln(X/X_0)$ as a function of time (t) at a particular temperature [25,27], where X_0 represents the maximum enzyme activity before thermal exposure (in our experiments the maximum activity determined at 30 °C).

The energies for thermal deactivation of the enzymes were determined from linearization of the Arrhenius equation (Eq. (2)), and by plotting the natural logarithm of the deactivation rate constant values against the reciprocal of the absolute temperature.

$$k = k_0 \exp\left(-\frac{E_d}{RT}\right) \tag{2}$$

where R is universal gas constant (8.314 J/mol K); k is rate constant; and T is temperature in Kelvin. The values of the deactivation energies (E_d) and the pre-exponential factors (k_0) were estimated from the slope and intercept of the plot of $\ln k$ versus $1/T$, respectively.

2.4. Statistical analysis

All the experiments were conducted in triplicate, unless otherwise stated. All statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Tukey's HSD test at $\alpha = 0.05$ using SPSS for windows.

3. Results and discussion

Thermal deactivation kinetics of alpha-amylase, beta-amylase, and limit dextrinase from teff malt were determined by incubating the enzymes at different temperatures ranging between 40 and 75 °C for a total of 90 min with sampling in 15 min interval. The time–temperature data were used to determine the residual enzyme activities. The proportions of the residual enzyme activities are related exponentially to both the activation energy and the rate constant. From the plot of semi logarithm of enzyme activity residue as a function of time, the slope tells us the rate at which the enzyme activity is changing with the change in time. The statistical analysis showed that there was no significant decrease in the alpha-amylase activity at 40 and 50 °C during the 90 min of mashing (Fig. 1). However, at higher temperatures it was significantly decreased ($p < 0.05$).

The rate constants for the deactivation of alpha-amylase in the wort samples determined from the plot of residual activity data against time (Fig. 1A) ranged from 0.0003 min^{-1} at 40 °C to 0.04 min^{-1} at 75 °C. The deactivation rate constant (k) increased with an increase in the mashing temperature (Fig. 1B), normally following an Arrhenius relationship (Eq. (2)). The rate constants were used for the determination of Arrhenius energy (E_d) and the pre-exponential factor (k_0). The deactivation energies and the pre-exponential factors of the enzymes considered in this study were determined by fitting the data using linear regression to an Arrhenius-type equation (Eq. (2)). The deactivation energy of alpha-amylase in teff malt was found to be 148 kJ/mol. The result showed that its thermal stability is low when compared to the stability of alpha-amylase in jowar (*Sorghum bicolor*), where higher deactivation energy (189.54 kJ/mol) was reported [6]. Based on the Arrhenius type equation developed in this study, it can be estimated that all the alpha-amylase in teff malt will be deactivated at a mashing temperature of 78 °C.

The deactivation rate constants for beta-amylase were in the range of 0.002–0.032 min^{-1}. The highest value was recorded for samples mashed at 70 °C and the lowest was for samples mashed at 40 °C (Fig. 2A). The high values of the deactivation rate constants for beta-amylase at lower temperatures when compared to those of alpha-amylase are good indicators for its less tolerance to thermal exposure at these temperatures. The statistical analysis also confirmed the low stability of beta-amylase at the studied temperatures when compared to alpha-amylase. Beta-amylase activities were significantly decreased ($p < 0.05$) at all studied temperatures during the 90 min of thermal exposure (Fig. 2). Thermal deactivation of alpha-amylase (Fig. 1A), and beta-amylase (Fig. 2A) after 65 °C displayed very dramatic differences. Other researchers [26] also reported considerable reduction, 35 and 57%, in the alpha-amylase activities of millets within a short time (20 min) of incubation at 65 and 70 °C, respectively. This can be explained by partial unfolding and covalent alterations of the protein structures in the aqueous medium [28]. The Arrhenius plot of the deactivation rate constant for beta-amylase is shown in Fig. 2B. The deactivation energy was found to be 82 kJ/mol.

One of the possible factors, among others, for low levels of limit dextrinase activity in cereals [12,29] is the less thermostability of