Optimization of microbial transglutaminase activity in ice cream using response surface methodology

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ABSTRACT

In order to determine the maximum activity range of transglutaminase (TG) in ice cream, the central composite design was used and the TG concentration along with the reaction time and temperature were evaluated. The rheological behavior of the ice cream mix that provided the best response and the determination of the protein cross-linking by electrophoresis (SDS-PAGE) were evaluated. It was observed that the TG increased the consistency index and favored the pseudoplastic behavior of the ice cream samples. The maximum response (0.69 Pa s) was obtained using a TG concentration of 4 U g protein at 56.8 °C with a reaction time of 90 min. The protein cross-linking was confirmed by electrophoresis, which showed that κ-casein was more susceptible to attack from TG than whey proteins.

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1. Introduction

The microbial transglutaminase (TG; EC 2.3.2.13) has the capacity to modify protein properties through acyl transfer reactions between the γ-carboxyamide group of peptides bound to glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors), including the ε-amino group of lysine residues in certain proteins (Hinz, Huppertz, Kulozik, & Kelly, 2007). The modification of proteins can occur through cross-linking reactions, deamidation and amine incorporation (Jong & Koppelman, 2002). According to Hiller and Lorenzen (2009), cross-linking seems to be the most dominant reaction and results in the formation of ε-(γ-glutamyl)lysine intra- and inter-molecular isopeptide bonds, and in the formation of polymers with higher-molecular-weights.

The cross-linking between proteins through the action of TG can modify several functional properties of foods, such as solubility, heat stability, and the gelation, emulsifying and rheological proprieties (Hinz et al., 2007). The milk proteins present in ice cream consist of a mixture of αs1-, αs2-, β- and κ-caseins and whey proteins (α-lactoalbumins and β-lactoglobulins). Both casein and whey protein are good acyl donor and/or acceptor substrates for TG (Rodríguez-Nogales, 2006), however, the β- and κ-caseins have greater reactivity with TG than other caseins and whey proteins (Myllärinen, Buchert, & Autio, 2007). The whey proteins in their native globular structure are less prone than caseins to enzymatic cross-linking, mainly due to the stabilization of the globular conformation by disulphide bonds which limit the availability of the cross-linking sites of the TG (Bönisch, Huss, Weitl, & Kulozik, 2007). In general, a progressive increase in temperature disrupts the protein-water system breaking the hydrogen bonds that stabilize the protein structure. This results in the protein unfolding and induces protein–protein interaction, bringing the proteins closer to the TG active sites and leading to the formation of polymers (Nieuwenhuizen et al., 2003; Rodríguez-Nogales, 2006).

Milk proteins are present in ice cream as part of the milk solids-not-fat component and play very important functional roles in the development of the ice cream structure. They contribute to the partial coalescence and fat structure formation, and are adsorbed at the air interface, leading to enhanced aeration and foam stability (Vega & Goff, 2005). Thus, the polymerization of milk proteins by the action of TG can lead to the formation of a more resistant protein film (Pinterits & Arntfield, 2008), improving the functional proprieties of the ice cream.

To determine the best conditions for the TG activity in foods, several authors have used the response surface methodology (RSM) (Gauche, Vieira, Ogliari, & Bordignon-Luiz, 2008; Pinterits & Arntfield, 2008; Rodríguez-Nogales, 2006). This methodology is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables, with the objective of optimizing the response (Abdullah, Salamatina, & Kamaruddin, 2009). The main advantage of RSM is the reduced
number of experimental runs needed to provide statistically acceptable results (Zhang, Ma, Yang, Liu, & Zhang, 2009). The RSM, using central composite design, involves full factorial research to examine simultaneous, systematic and efficient variations in the important components, identifying possible interactions, higher-order effects and determining the optimum operational conditions (Roriz, Osma, Teixeira, & Couto, 2009).

The aim of this study was to evaluate the effects of the transglutaminase enzyme in ice cream, optimizing the conditions for enzymatic activity (reaction concentration, temperature and time) using the response surface methodology to evaluate the rheological profile of the conditions that provided the best response. The polymerization of milk proteins was confirmed through the electrophoresis profile.

2. Materials and methods

2.1. Materials

The following ingredients were used to manufacture the ice cream: skimmed cow’s milk (67 g/100 g), sucrose (17 g/100 g), cream (8 g/100 g), skimmed milk powder (7 g/100 g), Emustab® emulsifier (Duas Rodas, Jaraguá do Sul, SC, Brazil) (0.5 g/100 g), and Super Liga Neutra® stabilizer (Duas Rodas, Jaraguá do Sul, SC, Brazil) (0.5 g/100 g). The microbial transglutaminase, composed of lactose, maltodextrin and transglutaminase, was provided by Ajinomoto® (Ajinomoto, São Paulo, SP, Brazil). The TG had an enzymatic activity of 100 U g⁻¹, as per manufacturer’s data, and it was used in the original form without further purification.

All reagents used were of analytical grade. Ultrapure water (Direct-Q water purification system, Millipore Corp, Molsheim, France) was used in the electrophoresis solutions.

2.2. Methods

2.2.1. Enzymatic cross-linking and ice cream preparation

The milk was submitted to heat treatment at 78 °C for 15 min for denaturation of the whey proteins (Rodriguez-Nogales, 2006). After cooling (25 °C), TG was added in the milk, before the mix of the ingredients, and its concentrations were calculated considering the milk protein content (3.90 g/100 g), quantified by the Kjeldahl method (AOAC, 2005). For TG deactivation, the samples were submitted to heat treatment at 80 °C for 2 min, according to Rodriguez-Nogales (2006). The best conditions for the enzymatic activity were determined by experimental design using a central composite design, described in the Response Surface Methodology section.

The ingredients, with the exception of the emulsifier, were mixed and pasteurized at 85 °C for 15 min with constant stirring. After the pasteurization, the ice cream mix was rapidly cooled to 50 °C and homogenized for 3 min. For ageing, the ice cream mix was then stored at 4 ± 1 °C for 24 h. The emulsifier was then added and the mixture beaten (Bellagi, Britania, Curitiba, Brazil) at 815 rpm, at 4 °C for 5 min. It was then cooled in a freezer (CVU30, Consul, Whirlpool S.A., São Paulo, Brazil) at −20 ± 1 °C and stored under this condition until the analysis was carried out.

The control sample was prepared following the procedure described above, but without addition of the TG enzyme.

2.2.2. Response surface methodology

The parameters tested in the experimental design to determine the best conditions for the experiment were: concentration, temperature and enzymatic activity time. To evaluate the effects and the interactions of these three variables, the response surface methodology was used by with a central composite design. The independent variables were TG concentration (x₁), temperature (x₂) and reaction time (x₃) and their levels were coded as −1, 0 and 1. The design was constructed based on a 3³ factorial design with 4 replications of the center point to estimate the experimental error, leading to 18 experiments, carried out in random order and in triplicate. Table 1 shows the factors and the levels of the variables used in the design.

The consistency index values (Pa s⁰) of the ice cream samples subjected to enzymatic treatment with TG were the selected responses (Y) for this research. In order to estimate the response, an empirical model composed of a second-order polynomial was constructed (1):

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \epsilon$$  

(1)

where  \(y\) is the predicted response, \(\beta_0\) the model constant, \(\beta_i\) the coefficients of the linear effects, \(\beta_{ii}\) the coefficients of the quadratic effects, \(\beta_{ij}\) the coefficients of interaction between the factors, \(x_t\) and \(x_j\) the independent coded variables, \(\epsilon\) the error, \(k\) the number of the variables considered, and \(i\) and \(j\) the coded factors of system.

The coefficients were calculated by regression analysis and their significance was verified using analysis of variance (ANOVA) with the Statistic (version 7.0) software program.

2.2.3. Rheological measurements

The rheological measurements of the samples of melted ice cream were carried out with a Brookfield rotational rheometer with a concentric cylinder (Brookfield Engineering Laboratories model DV-III Ultra, Stoughton, MA, USA) and a ULA spindle. The rheometer was thermostatically controlled by a water circulator (TECNAL model TE-184, SP, Brazil) at 4.0 °C ± 0.1, and the samples were left to stand for 15 min to ensure stability. The Power Law model (2) was applied to describe the flow behavior of the samples, and the consistency index (K) was used as the parameter to verify an alteration in the viscosity of the ice cream samples treated with TG:

$$\sigma = K \gamma^n$$  

(2)

where  \(\sigma\) is the shear stress (Pa),  \(K\) the consistency index (Pa s¹),  \(\gamma\) the shear rate (s⁻¹), and  \(n\) the flow behavior index (adimensional). The measurements were performed in triplicate for each sample and the data evaluated using Origin® software version 6.0 (Microcal Software Inc., Northampton, MA, USA). The Tukey test was applied when a difference between the values was verified at a significance level of 5%.

2.2.4. Gel electrophoresis

To confirm the polymerization of the milk proteins present in mix by TG, polyacrylamide gel electrophoresis was performed following the methodology described by Laemmli (1970). A Mini Protein Electrophoresis Cell (Bio-Rad Laboratories, California, USA) and a discontinuous system composed by a stacking gel (3.5 g/100 g) and a separating gel (12 g/100 g) were used for the identification of the cross-linking proteins. The samples were diluted (1:6 ratio) with buffer solution (pH 6.5) containing:

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Independent variables and their levels used for the experimental design.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors</td>
<td>Levels</td>
</tr>
<tr>
<td>Transglutaminase (U g⁻¹ protein)</td>
<td>0.64 2 4 6 7.36</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>23.2 30 40 50 56.8</td>
</tr>
<tr>
<td>Time (min)</td>
<td>39.6 60 90 120 140.4</td>
</tr>
</tbody>
</table>
0.015 mol L⁻¹ Tris–HCl, 25 g/100 g glycerol, 1 g/100 g SDS, 2.5 g/100 g β-mercaptoethanol and 0.1 g/100 g bromophenol blue. The electrophoresis was carried out with run buffer (pH 8.3) composed of Tris base (15 g L⁻¹), glycine (72 g L⁻¹) and SDS (5 g L⁻¹). After the run, the gel was stained with the dye Coomassie Brilliant Blue at 1 g/100 g in a mixture of methanol (45 g/100 g), glacial acetic acid (10 g/100 g) and distilled water (45 g/100 g). For the decolorization of the gel a solution containing methanol (50 g/100 g), glacial acetic acid (10 g/100 g) and distilled water (40 g/100 g) and a solution of 7.5 g/100 g acetic acid were prepared. The protein fractions were identified using the molecular weight standard (Natural Unstained Standards-Broad Range, Bio-Rad) of 6400 to 200,000 g mol⁻¹.

The samples used were: a) control sample; b) sample that showed the highest consistency according to the experimental design with preheated milk before incubation with TG; c) and the sample that showed the highest consistency according to the experimental design but without pre-heating treatment of milk before incubation with TG (this sample to analyze the efficiency of denaturation of whey proteins in the TG activity).

3. Results and discussion

3.1. Response surface methodology

A central composite design, analyzed using the response surface methodology, was used to elucidate the main effects and the interactions of the factors studied: influence of TG concentration, temperature and enzymatic reaction time in ice cream samples. Table 2 shows the factors and experimental and predicted response values of the consistency index (K) obtained from the experimental design. The factor values were stipulated by preliminary studies (data not shown). The consistency index was used as a response in this study, since this index increased with the polymerization of the milk proteins by the TG, due to the proteins of high-molecular-weight formed during the reaction which led to changes in the functional properties of the milk products (Hinz et al., 2007). Moreover, this index is correlated with the viscosity (Wilcox & Swaisgood, 2002), texture, melting resistance and smoothness of the ice cream (Kuş, Altan, & Kaya, 2005).

The predicted values for the consistency index are close to the experimental values demonstrating that the model is applicable.

The maximum response \( K = 0.69 \text{ Pa s}^n \) was obtained with a TG concentration of 4 U g⁻¹ protein, at 56.8 °C with a 90 min reaction time, which indicates that the highest degree of milk protein cross-linking in ice cream was obtained under these conditions (Table 2).

Analysis of variance (ANOVA) was used to determine the relation between the response and the significant variables, to obtain the optimum conditions for the TG activity. These results showed that the model was significant \( (F = 3.49) \) and the lack-of-fit \( (p < 0.05) \) and the lack-of-fit \( (p > 0.05) \) indicated that the quadratic model was valid for this study. The quadratic effects of the factors TG concentration and reaction time were significant \( (p < 0.05) \), however, the temperature and the interaction effects between the variables were not significant in this study. These same factors were evaluated by Gauche et al. (2008) in milk whey protein cross-linking, using the central composite design. The authors observed that the consistency index was significant for the 3 factors studied, obtaining a maximum response with a TG concentration of 30 U g⁻¹ protein at 36 °C with a 128 min reaction time.

The significant regression coefficients were negative, indicating that a response surface with a maximum point was obtained in the experimental design. A quadratic model, built through the regression analysis, described the mathematic relation between the independent and response variables (3).

\[
K = 0.573896 - 0.110819 \cdot \text{TG}^2 - 0.091332 \cdot t^2 \tag{3}
\]

where K is the consistency index (Pa sⁿ), TG the concentration of transglutaminase (U g⁻¹ protein) and t the time (min).

For the graphical representation of functions of this design, graphs are used which describe the individual and cumulative effects of the variables tested and their effect on the response (Prakash, Tat, & Hasan, 2009). Fig. 1 shows the contours (A) in a two-dimensional plane and the response surface graph (B) in a three–dimensional plane for the regression model fitted to the data.

It was observed that the optimum region of enzymatic activity of TG was obtained for a TG concentration of 4 U g⁻¹ protein with 90 min of reaction (Fig. 1). The consistency index increased progressively until a TG concentration of 4 U g⁻¹ protein, with the response decreasing at concentrations above one. According to Lorenzen (2007), this decrease may be related with the cross-linking degree, because the enzymatic reaction of TG is dependent on the availability of reactive glutamine residues located at the protein surface. The κ-casein molecule is the most reactive of the casein molecules because it is located at the surface of the casein micelle, and has in its composition 14 glutamine residues, but only 4 of these residues are reactive (Bönisch, Heidebach, & Kulozik, 2008). The β-casein, located within the micelle, has a disordered, flexible and open structure, becoming accessible to attack by TG (Rodriguez-Nogales, 2006). However, it has only 5 reactive glutamine residues out of a total the 21 residues (Christensen, Sørensen, Højrup, Petersen, & Rasmussen, 1996). The whey proteins also have limited glutamine residues available to TG due to their compact globular structure. For example, α-lactalbumin has 6 glutamines but only 5 of these react with TG. The availability of these residues is dependent on the temperature, pH and the presence or absence of Ca²⁺ (Nieuwenhuizen et al., 2003). The β-lactoglobulin has 7 glutamines residues but only 4 are reactive (Nieuwenhuizen, Dekker, Groneveld, Koster, & Jong, 2004).

Thus, a limited number of reactive glutamine residues of the caseins are available to TG, and TG concentrations above 4 U g⁻¹ protein may be higher than that required for this reaction in ice cream. According to Jong and Koppelman (2002), the saturation of the protein substrate may occur and the over-formed polymers can

<table>
<thead>
<tr>
<th>Test</th>
<th>Factor</th>
<th>Transglutaminase (U g⁻¹ protein)</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Consistency index (Pa sⁿ)</th>
<th>Predicted</th>
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<td>0.35 ± 0.04</td>
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<td>6</td>
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* Mean values ± standard deviation.
inhibit the uniform development of protein network, which explains the decrease in the consistency index of the ice cream. Bönisch et al. (2008) observed that at TG concentrations above 5 U g\(^{-1}\) protein, there was interference between the protein cross-linking and the rennet coagulation process, where an increase in the degree of cross-linking reduced the aggregation of para-k-casein micelles, reducing the elasticity of the samples.

### 3.2. Adequacy of the model

It is important to assess the fitted model to ensure that it provides sufficient approximation to the results obtained in the experimental conditions. The normality of the data, checked using a normal probability plot of the residuals and the difference between the observed and predicted values from the regression, showed that the experimental points were normally distributed around the line, indicating that the normality assumption was satisfied. A determination coefficient value (\(R^2\)) of 0.85 was obtained for this model, which indicates a good fit between the observed and the predicted response values. The plots of the residual versus the predicted values (plots not shown) showed that the residuals were scattered randomly around zero and didn’t show outliers, because all the values lie within the accepted range (\(-3\) and \(+3\)) to validate the model (Roriz et al., 2009). Thus, the variance analysis results were valid, since the model assumptions were satisfied.

### 3.3. Rheological measurements

The Power Law model was used to calculate the consistency index (K) and the flow behavior index (n) of the control ice cream samples (without enzymatic treatment with TG) and of the samples that presented the maximum response for the consistency index in the experimental design. The model adequately fitted the data curve of the shear stress versus the shear rate for all samples (\(R^2 > 0.98\)).

The consistency index value is based on the variation in the shear rate and indicates the viscosity of a fluid (Karaca, Güven, Yasar, Kaya, & Kahyaoglu, 2009). It was observed that the TG interfered in the rheological behavior of the ice cream samples increasing the consistency index (0.62 Pa s\(^{n}\)) in order to control sample (0.29 Pa s\(^{n}\)), and consequently, increasing the viscosity of these products.

All samples showed pseudoplastic behavior, the shear stress increasing with an increase in shear rate (Fig. 2A) (Karaca et al., 2009). The rheological properties of most ice cream are generally described as pseudoplastic (Aime, Arntfield, Malcolmson, & Ryland, 2001; González-Tomás, Bayarri, Taylor, & Costell, 2008; Soukoulis, Chandrinos, & Tzia, 2008) where the viscosity decreases with increasing shear rate (Fig. 2B) (Braun & Rosen, 2000, p. 505). This decrease is partly due to the aggregation of fat globules which decrease in size during shearing, reducing as a consequence the viscosity of ice cream (Nazaruddin, Syaliza, & Rosnani, 2008).

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**Fig. 1.** Contour graph (A) and response surface graph (B) for the consistency index of the transglutaminase concentration (U g\(^{-1}\) protein) versus reaction time (min), at a fixed temperature of 40 °C.

**Fig. 2.** Rheological behavior of (■) control sample and of (○) the sample submitted to treatment with TG (4 U g\(^{-1}\), 56.8 °C and 90 min).
The degree of the pseudoplastic or dilatant characteristic can be evaluated using data on the flow behavior index (n). For the ice cream submitted to enzymatic treatment, there was an increase in the pseudoplastic properties as the flow behavior index approached zero (0.91–0.70). This behavior was also evidenced by Gauche et al. (2008) with the cross-linking of whey proteins through the action of TG. The favoring of a pseudoplastic behavior occurred, probably due to higher-molecular-weight polymers formed during the cross-linking reaction promoted by TG.

Innocente, Comparin, and Corradini (2002) affirm that with an increase in the shear rate, large polymer molecules tend to disentangle and possibly align in the flow field, offering less resistance to flow.

3.4. Gel electrophoresis

The inter-molecular cross-linking of the milk proteins through the action of TG was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This is considered an acceptable method to monitor the cross-linking of proteins by TG, because it shows inter-molecular cross-linking through the formation of new high-molecular-weight bands (Pinterits & Arntfield, 2008).

Through the electrophoresis (Fig. 3) it was possible observed that the enzyme treatment promoted the appearance of new high-molecular-weight protein bands. The formation of these bands was accompanied by a loss of intensity of the κ- and β-caseins bands indicating that larger proteins were formed by the cross-linking of low-molecular-weight polymers, as confirmed by Sharma, Lorenzen, and Qvist (2001) and Moon, Hong, Huppertz, Fox, and Kelly (2008). The main milk proteins involved in the cross-linking were the caseins. The casein fractions are considered good substrates for TG due to their low degree of tertiary structure, flexibility, random-coil arrangement and the absence of disulphide bonds in the β-casein leaving the reactive groups exposed to the enzyme (Özrenk, 2006). With regard to whey proteins, there occurred a greater decrease in the α-lactalbumin band than in the β-lactoglobulin band (Fig. 3, lanes 3 and 4). These proteins tend to form less efficient cross-linking than caseins, mainly due to their compact globular structure being more stable and the high presence of disulphide bonds that inhibit the enzyme action (Bönisch et al., 2007). Moreover, the cross-linking of milk proteins was most notable when the milk pre-heating stage was performed, demonstrating that the denaturation of the whey proteins facilitated the attack of the TG.

4. Conclusions

The application of the response surface methodology was effective in optimizing the parameters for the transglutaminase activity in ice cream. The enzymatic treatment increased considerably the consistency index, until a maximum TG concentration at 4 U g⁻¹ protein with 90 min of reaction, and increased the pseudoplastic properties in relation to the control sample, due to the high-molecular-weight polymers formed during the cross-linking reaction. These polymers were confirmed with the aid of electrophoresis, which showed that the κ-caseins were more susceptible to attack from the TG than the whey proteins in the ice cream. These results demonstrated that TG can improve the functional properties of ice cream, where few cross-links are needed to achieve optimal results and, thus, regulation of the degree of cross-linking is crucial to achieving optimal protein functionality.

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References


* These references are representative of transglutaminase effects on milk proteins and of studies of ice creams characteristics.


