Thesis book

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APPLICATION OF MICROBIAL TRANSGLUTAMINASE BY DAIRY AND MEAT PRODUCTS

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A jelölt a Szent István Egyetem Doktori Szabályzatában előírt valamennyi feltételnek eleget tett, az értekezés műhelyvitájában elhangzott észrevételeket és javaslatokat az értekezés átdolgozásakor figyelembe vette, azért az értekezés nyilvános vitára bocsátható.

Az iskolavezető jóváhagyása

A témavezetők jóváhagyása
1. THE BACKGROUND OF RESEARCH WORK, AIMS

Nowadays the food industry is interested in researches, which could satisfy the customer needs and therefore can lead to high profitability. Food products tend to be low-calorie, containing less preservatives and food additives in order to follow the modern nutritional habit of health-conscious customers. However besides these requirements, customers wish to experience the same taste and texture that they have in mind while thinking on traditional meals.

The quality properties of fermented, acid- and rennet coagulated dairy products and Bologna-type sausages are in close relation with their physical attributes. Characteristical properties are for example whey drainage and gelfirmness for yogurt and hardness for cheese. Hardness is also crucial for meat products beside water holding capacity and springiness. Manufacturers can select from a wide range of food additives and exipients to develop the suitable reological characteristics and also in order to achieve favourable sensory quality. However, the use of such additives tends to cause more and more distrust and even rejection by some customers.

The enzyme called microbial transglutaminase (mTG, EC 2.3.2.13), belongs to the family of transferase enzymes, and can be a potential option to improve the texture. This enzyme forms intra and intermolecular covalent binds between 2 aminoacids: glutamine and lysine. Due to this enzyme activity the structure of dairy- and meat proteins can be stabilized. Since Ca-independent mTG can be produced with microbial fermentation on industrial level, this enzyme
represents a real alternative to partially or fully substitute food additives used as texture stabilizers. The role of mTG activity and even more the effects of enzyme treatment are important topics of the food research since the 90’s. As a result of this research interest, several dairy and meat production process have changed and many patents appeared. However it is evident from the scientific literature, that published papers concentrated mainly on existing products, meeting possible customer needs (eg.: non-fat stirred yogurt, non-phosphate Bologna-type sausage). Although after decades of research experience, the functionality of mTG is still not yet clarified. This is shown by the fact, that this enzyme is still used in wide concentration range.

After recognising this lack of knowledge, my main research interest was to investigate the structure-modifying properties of mTG on high-protein foods. I aimed to follow and understand this effect in scientific manner.

In order to clarify the direct effect of mTG activity I examined which classic and instrumental analytical method is suitable for the determination of enzyme activity in modell solutions and in dairy and meat food matrixes.

I have determined the direct effect of mTG through the investigation of circumstances of enzyme treatment (enzyme preparation, mTG concentration, timing of mTG addition) by the production of set-type yogurt, Hungarian cottage cheese, Trappist cheese, and by Bologna-type sausage. I have taken into account the possibilities of generally used production processes. I have also investigated the sol-gel transformation during the fermentation of yogurt model
solution and set-type yogurt to scientifically prove the gelling properties of mTG. I have also followed the effect of applied bulking agent and lactic acid bacteria.

I have investigated the role of mTG to substitute food additives. Therefore I have studied how the concentration of pickling salt and phosphate can be reduced by Bologna-type sausages using mTG.

My scientific study was done on laboratory and small-scale level to reach the mentioned research goals. Based on my results, I would like to give advice on how to produce reduced fat and additive containing dairy and meat product on large scale.
2. MATERIALS AND METHODS

2.1. Manufacturing methods of research products

The enzyme concentration is given in U/g protein as it is defined also by other scientists.

2.1.1. Acid casein modell solution

The oscillatory shear measurement was applied on acid casein model solutions. The 2.7% w/v% acid casein (Sigma-Aldrich GmbH, Germany) model solution was diluted in phosphate buffer. The phosphate buffer (pH 6.8) constituted of 0.2 M NaH$_2$PO$_4$ and 0.2 M Na$_2$HPO$_4$. The acid casein model solution was preserved with sodium azide (0.3 g kg$^{-1}$). The 3 U/g protein mTG enzyme solution was prepared with Activa MP (Ajinomoto Foods Europe SAS, Hamburg, Germany) powder (enzyme activity = 100 U/g) diluted in 10 ml distilled water. This enzyme solution was added to 2.7 w/v% acid casein model solution. The enzyme-treatment was at 40 °C until definite time (1; 2; 3; 4; 5 hours). Enzyme inactivation was achieved by heat treatment (85 °C, 15 min) and subsequent cooling in ice water. The gelation process was induced by (3.5%; 4%; 4.5%) GDL (glukono-delta-lakton) directly before the oscillatory shear measurement.

2.1.2. Yogurt model solution

The SANS measurement was made on yogurt model solution to define the gelation process and the influence of mTG enzyme based on neutron-scattering A 10% skim milk powder (SMP) solution (yogurt model solution) was
prepared using skim milk powder from Tutti Hungary Ltd., Budapest, Hungary (0.14% fat, 33% protein). The solvent heavy water (99.5% D₂O) was used to increase contrast in neutron scattering experiments. Solutions were stirred on MLW RH3 magnetic stirrer (VEB MLW Prüfgeräte-Werk, Freital, Germany) 500 rpm, 5 min at 40 °C. A control solution was prepared based on addition of a 0.3% lyophilized DVS (DVS = Direct Vat Set) yogurt culture (YF L811; Christian Hansen, Hørsholm, Denmark) to a heavy water SMP solution at 20 °C. An mTG treated solution prepared using the same amount of YF-L811 DVS yogurt culture and 0.3% Activa YG (Ajinomoto Foods Europe SAS, Hamburg, Germany) powder (enzyme activity = 104 U/g of protein), which were added simultaneously to the heavy water SMP solution. Yogurt model solutions were kept at 43 °C, which is the optimal temperature of the applied DVS yogurt culture. Structural changes in sol-gel transformation were analyzed based on sampling of yogurt model solutions at 0, 15, 30, 60, 100, and 140 min during fermentation. Yogurt model samples were heat-treated at 70 °C for 10 min to stop fermentation and enzyme activity. Fat globules and casein micelles were eliminated using centrifugation (Mikro 120; Hettich Zentrifugen, Tuttlingen, Germany) at 1400 rpm for 10 min at 20 °C. The supernatant was placed into quartz cuvettes for SANS measurements.

2.1.3. Cheese model solution

Low-fat, semi-hard cheese made of 2.2% fat pasteurized milk was used as a sample for fluorescent measurement. Sampling was done during the manufacturing and every
week during 4-weeks ripening. The steps of sample preparation were the following: 1.2 g sample was taken from the middle of the cheese. These samples were homogenized in 5 mL Milli-Q water on ice using ultra-turrax T25 digital homogenizer (IKA, Staufen, Germany) at 1000 rpm for 2 min. The homogenized enzyme-treated sample was centrifuged (10 min, 5 ºC, 5000 rpm). Supernatants were frozen and stored until measurement.

2.1.4. Manufacturing process of set-type yogurt
The yogurt milk was 1.5% fat UHT milk (fat: 1.40%±0.08, non-fat dry matter: 8.48%±0.05, protein: 3.10%±0.02) was obtained from Alföldi Tej Ltd. (Székesfehérvár, Hungary). The manufacturing process was based on general practice of the Department of Refrigeration and Livestock Products Technology.

2.1.5. Manufacturing process of Hungarian cottage cheese
The Hungarian cottage cheese milk was 1.2% fat pasteurized milk (fat: 1.28%±0.08, non-fat dry matter: 9.07%±0.05, protein: 3.42%±0.04). The manufacturing steps were according to a company’s common practise, which asked me to conduct the experiments.

2.1.6. Manufacturing process of Trappist cheese
Semi-hard cheese samples were made from 1.5% fat pasteurized cows’ milk (fat: 1.42%±0.11, non-fat dry matter: 9.13%±0.01, protein: 3.34%±0.01), which was manufactured by Fuchs Tej Ltd. (Valkó, Hungary). The
milk fat level was set to 2.2%, 2.8%, 3.5% and 5% fat with 30% fat whipping cream (fat: 30.89%±0.34, non-fat dry matter: 4.65%±0.25, protein: 1.76%±0.11) manufactured by Fuchs Tej Ltd. (Valkó, Hungary)

The manufacturing steps were based on general practice of the Department of Refrigeration and Livestock Products Technology.

2.1.7. Manufacturing process of Bologna-type sausage

The pork meat batter (protein: 18.11%±0.23) was manufactured in 5.5 L Robot-Coupe R502 cutter. The manufacturing process was based on general practice of the Department of Refrigeration and Livestock Products Technology. The Activa TG-H-NF (nominal activity: 32-52 U/g, actual activity: 42 U/g) preparation contained only maltodextrin beside the enzyme. The meat batters were stuffed into 21 mm diameter cellulose casings. The smoking, heat treatment, shower and drainage was done in CS350 EL type smoking machine.

2.2. Determination of enzyme activity of the trial products

2.2.1. Hydroxamate method

I applied the colorimetric hydroxamate assay to directly determine the enzyme activity. This method is generally accepted and applied according to the scientific literature GROSSOVITZ et al. 1950; FOLK és COLE 1966). The measurement is based on the enzymatic incorporation of
hydroxylamine into the glutamine peptide Cbz-Gln-Gly. This is followed by ferric chloride staining. The enzyme activity leads to hydroxamate production, which binds the Fe$^{3+}$ ions and results a red colored complex. The enzyme activity can be defined due to this color development by absorbance at 525 nm. The enzyme activity: 1 U (unit) will catalyse the formation of 1 µmole of hydroxamate per min from Z-Gln-Gly-OH and hydroxylamine at pH 6.0 at 37 °C.

2.2.2. Fluorescent method

The principle of the measurement is the dansylated dipeptide ZQG-DNS, which is used for fluorescent labelling. The ZQG-DNS reacts specifically with the active mTG, which leads to the increase of fluorescent intensity (PASTERNACK et al. 1997). Relative fluorescence intensity (rfu, relative fluorescence unit) was measured continuously for 5 min using an Enspire Multimode Reader fluorimeter (PerkinElmer, Massachusetts, USA). The excitation wavelength was 340 nm and the absorption wavelength was 532 nm during the measurement. Sampling was done during the manufacture and weekly during 4 weeks of ripening in order to define the residual enzyme activity of the cheese samples.
2.3. The physical and chemical analysis of the products

2.3.1. Determination of dry matter content

2.3.2. Determination of fat content

2.3.3. Determination of protein content

2.3.4. Determination of TBA-number by Bologna-type sausage

2.3.5. Determination of syneresis

2.3.6. Determination of Water Holding Capacity (WHC)

2.3.7. Determination of Soxhlet-Henkel acidity (SH°)

2.4. Analytical measurement methods of products

2.4.1. Determination of pH

2.4.2. Color measurement

2.4.3. Following sol – gel transformation with neutron scattering methods

The Yellow Submarine instrument is a pin-hole type SANS machine located at the cold neutron source channel of the 10 MW Budapest Research Reactor at the Budapest Neutron Centre in Hungary. The instrument setting parameters used were a sample to detector distance = 5.6 m, wavelength = 1.13 nm, and a beam aperture size = 12 mm. Scattered neutrons were detected using a 64 x 64 pixel (1 cm × 1 cm pixel size) 2 dimensional position sensitive Commissariat a l'Energie Atomique-Laboratoire d'Electronique et de L'Informatique (CEA-LETI) detector
(Grenoble, France) filled with BF3 gas. Yogurt samples were placed in 5 mm thick quartz cuvettes designed for neutron experiments with low neutron absorption and neutron scattering properties. A defined Q range from 0.06 1/nm to 0.3 1/nm was established.

2.4.4. Methods of texture measurement

2.4.4.1. Oscillation viscosimeter

Acid-induced gelation was monitored using a strain-controlled ARES RFS3 rheometer (TA Instruments, Germany) with a concentric cylinder geometry (di = 32 mm; do = 34 mm; h = 33.5 mm). The temperature equilibrated samples were mixed with 35, 40 or 45 g kg\(^{-1}\) glucono-δ-lactone as acidulant and immediately transferred into the rheometer. The storage modulus G' was recorded at a frequency of \(\omega = 1.0\) rad s\(^{-1}\) and a deformation of \(\gamma = 0.003\); gelation temperature was kept constant at 20, 30 or 40 °C by a computer controlled circulator. From the curves, G'\(_{max}\) was extracted as an indicator of maximum gel stiffness. All results shown are mean values of duplicate measurements.

2.4.4.2. Determination of apparent viscosity

As the shear rate (D, 1/s) measured between 10-100 s\(^{-1}\) models the chewing and swallowing properties of yogurt (STEFFE 1996; MEZGER 2006) the shear stress measured at 57.2 1/s shear rate was determined during fermentation with Rheomat 115 (Contraves, Switzerland) rotational
viscometer using the conical measure head type 145. The samples were measured at the fermentation temperature of 43 °C. Shear stress (τ, Pa) was calculated from the read values of the instrument (α) multiplied with the factor of the measure system type 145 (z = 195.5). Apparent viscosity (η, Pa*s) is the shear stress (τ) divided by the instrument factor (z = 195.5).

2.4.4.3. Texture measurement with SMS texture analyzer

The texture measurements were done with SMS TA. XT Plus (Stable Micro Systems, Godalming, Great Britain) texture analyser using 500N force measurement cell. The official software of the instrument called Texture Exponent 32 was applied for data evaluation.

2.4.4.3.1. Determination of gelfirmness

The structure of yogurt was measured with TA. XT Plus (Stable Micro Systems, Great Britain) texture analyser. The samples were tempered to 10°C, the measurement was made with a 20 mm diameter cylinder probe. The gel strength was the force recorded at 10 mm penetration depth. Data evaluation was performed with the software of the Texture Exponent 32 instrument.

2.4.4.3.2. Determination of hardness and adhesiveness

Hardness and adhesiveness was measured with the conical measuring head of TA. XTPlus (Stable Micro Systems, Great Britain). The cross-head pushed the 90°
cone probe of spreadability rig with 2 mm/sec speed into the sampling holder. The meat batter samples were tempered to 12 °C and the measuring time was 90 sec. Three replicates of each samples were evaluated using the official software of the instrument called Texture Exponent 32.

2.4.4.3.3. TPA method

Hardness was also measured with TA. XTPlus (Stable Micro Systems, Great Britain). Cheese and Bologna-type sausage cores (diam. = 12 mm, height = 12 mm) were axially compressed to 70% of their original height with 35 mm cylinder probe at a crosshead speed of 2 mm s⁻¹. The samples were tempered to 12 °C and the measuring time was 2 min. Force–time deformation curves were evaluated with Texture Exponent 32 as the given software of TA.XTPlus and the hardness parameter was selected for analysis. Measurements were done in 10 parallels.

2.4.4.3.4. Measurement with Kramer-type shear cell

The texture properties of Hungarian cottage cheese was measured with Kramer shear cell. The Kramer shear cell simulates the effect of different stresses (shear, push, chewing), which all can be measured on the sample at once. The Hungarian cottage cheese samples were stored at 10 °C until measurement. I put 80 kg sample into the shear cell to initiate the texture analysis. The test began, when the texture analyser measured 0.049N resistance of the sample.
2.5. Sensory measurement methods

2.5.1. Hungarian Standard (MSZ) Scoring Test -100 points

Sensory measurement of Hungarian cottage cheese and Trappist cheese was done scoring test (100 points). The test evaluation was according to the Hungarian Standard (MSZ) 12280-87 and to the directive No. 2-104. of Codex Alimetarius Hungaricus, prepared by Kálmánné Koncz dr. This scoring test is used on national competitions of Hungarian handmade cheese products since several years. Good examples are the “Sajtmustra” - Cheesemuster and the “Újbor és Sajtfesztivál” – Young wine and Cheese Festival. I have asked 10 trained panellist to participate in my sensory test. The sensory characteristics of cheeses were scored accordingly: look (15 point), inner color (15 point), odor (15 point), taste (25 point), texture (15 point), cheeseholes (15 point). The result is presented as a percentage of the maximum point for each attribute.

2.5.2. Texture Profile Analysis

Texture Profile Analysis was applied by set-type yogurt samples in order to find correlation between the enzyme concentration and the attributes of yogurt (porcelain, spoonability, smooth after stirring, whey drainage – color and amount of whey).

2.5.3. Difference test

Difference test was applied by set-type yogurt and Bologna-type sausages 1 day after manufacturing. I have asked 10 trained panellist to participate in my sensory test.
3. RESULTS AND DISCUSSION

Methodological theses

1. I have proved, that the applied small-angle neutron scattering method is able to follow the effect of mTG on sol-gel transformation already after 40 min of fermentation. This could be achieved due to the decreasing quantity of whey protein aggregates from the yogurt.

2. The mTG activity can be determined directly from cheese made of 2.2% fat milk at the following manufacturing stages: after cutting the cheese curd, and at the end of up-heating. This could be achieved due to the enzyme specific incorporation of dansylated ZQG-DNS, which causes a measurable increase in the fluorescent intensity.

Technological theses

1. I have proved that the gelfirmness of set-type yogurt made of 1.5% pasteurised milk, increases linearly with increasing enzyme concentration. Moreover I showed, that this tendency can be divided into 2 stages at 1 U/g protein enzyme concentration.
2. I have identified, that in case of Hungarian cottage cheese is made of 1.2% pasteurised milk, to which mTG enzyme is added in 0.04 U/g protein concentration at 30 °C, simultaneously with inoculation, then this product can have 11% higher yield by calculating with 25% dry matter content.

3. I have identified, that in case Trappist cheese is made of 2.8% pasteurised milk, to which mTG enzyme is added in 0.12 U/g protein concentration at 30 °C, simultaneously with inoculation, then this product can have 11% higher yield in laboratory scale by calculating with 58% dry matter content.

4. I have identified, that in case of Trappist cheese, the higher the fat content of cheesemilk the less the hardness decreases by enzyme-treated cheese. This statement applies if 0.12 U/g protein enzyme was added simultaneously with inoculation.

5. I have identified that the addition of pickling salt and phosphate can be decreased by enzyme treatment of Bologna-type sausages, based on their technofunctional characteristics and product features.
4. CONCLUSIONS AND RECOMMENDATIONS

I have investigated the structure-modifying properties of microbial transglutaminase (mTG) in my doctoral thesis. This research was done on milkprotein model solutions, on set-type yogurt, on Hungarian cottage cheese and on Bologna-type sausage.

The structure-modifying property of mTG was studied in model solutions in one-substrate system (acid casein or skin milk powder). The oscillatory measurement revealed that the maximum gelfirmness can be reached after 4 hours of enzyme treatment in acid casein model solution. After this point, there is no significant change in gelfirmness. The small-angle neutron scattering revealed that more whey proteins are retained in the enzyme-treated yogurt as in control sample. This is due to the polymerised protein network, which is the result of mTG activity. This confirms the possibility of yield enhancement with mTG.

I have also studied how the suitable texture can be achieved by low-fat set-type yogurt using different starter cultures and enzyme treatment. I confirm that the enzyme concentration has no influence on the process of fermentation. The gelfirmness of the final product was in exponential correlation with the enzyme concentration. However 1.0 U/g protein enzyme addition was needed to recognise significant change according to objective and subjective texture analysis. Further studies revealed that the applied starter culture also influences the efficiency of enzyme treatment. According to my results the traditional
yogurt made of 1:1 ratio of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* is more effective in terms of gelfirmness, spoonability and whey drainage as if it is used in combination with *Bifidobacterium animalis subsp. lactis* probiotic lactic acid bacteria. Furthermore these traditionally applied 2 starter cultures are more effective if *Lactobacillus delbrueckii subsp. lactis* is also involved in their fermentation.

I have identified by Hungarian cottage cheese, that the dry matter, yield, structure and sensory judgement are influenced by the applied enzyme preparation, enzyme concentration and the timing of enzyme addition. According to my results, the best yield improvement (11%) could be achieved with the enzyme preparation containing maltodextrin used in 0.04 U/g protein level. The dry matter content of the product (25%) was also in accordance with Codex Alimentarius Hungaricus.

I have identified by Trappist cheese, that the dry matter, yield, structure and sensory judgement are influenced by the applied enzyme preparation, enzyme concentration and the timing of enzyme addition. According to my results, the best yield improvement (11%) could be achieved by cheese made of 2.8% fat milk, when the mTG (0.12 U/g protein) was added simultaneously with inoculation. I have also realised, that the elasticity decreased better during ripening by the enzyme-treated cheese, than by the control sample, if the cheese was made of 2.2% fat milk.

I came to the conclusion, that the enzyme activity can be determined during cheese manufacture with the applied fluorescent method. According to my results, the enzyme
activity can be determined directly during manufacture due to fluorescent labelling. The method is suitable to detect and quantify enzyme activity in the enzyme concentration applied according to the recommendation of manufacturers of enzyme preparation.

I have chosen the Bologna-type sausages from meat products as it is a widespread and popular product type in Hungary. The structure of Bologna-type sausage was studied also in raw and cooked form. I have examined the effect of 0-4 U/g protein enzyme concentration to find out, what is the optimal level. I have realised, that the enzyme concentration has an influence already on the meat batter. This turned to be harder and more concise in case of overdosage, which led to less compressbility by stuffing the casings. I have also realised, that the meat batter got even harder with time due to the enzyme treatment, therefore I would advise maximum 60 min waiting time until cooking. After the heat treatment, the hardness of Bologna-type sausage was mainly influenced by the enzyme treatment. I have also recognised, that the higher the mTG concentration, the less the TBA-number in the final product. The sensory evaluation proved that the overdosage of mTG leads to less compressability by stuffing and as a consequence: panelists recognised air bubbles on the cutting surface of such products. These air bubbles are disadvantageous also because mechanically bonded water can gather in them. According to the above, maximum 0.6 U/g protein enzyme concentration is optimal to produce suitable Bologna-type sausage.
Further studies were conducted to examine the possibility of reducing the concentration of pickling salt and phosphate, assuming the structure-modifying and water holding capacity of mTG. The enzyme treatment led to significant increase in hardness both in raw and cooked state in the examined concentration range of 1.2-1.8% pickling salt. This effect was independent from the concentration of pickling salt. Furthermore, I have identified, that there is a linear correlation between the concentration of pickling salt and the hardness of raw and cooked Bologna-type sausage. This correlation was tighter by enzyme treatment. The mTG affected the oxidability of the product, which was shown by the decreasing TBA-numbers, which were even lower, than the control values. The TBA-numbers of enzyme-treated products were also lower independent from the applied pickling salt concentration. The sensory evaluation proved, that the mTG led to better judgement in the total examined pickling salt concentration range. Furthermore pickling salt level above 1.4% caused higher scores for color, odor and taste by enzyme-treated products than by control samples.

The structure-modifying property of mTG was significant in the total examined phosphate concentration range (0-0.7%). The enzyme caused 11-20% increase in hardness in the final product. The water holding capacity improved greatly at lower phosphate levels (0-0.3%) due to enzyme treatment. I have determined, that 0.3% phosphate concentration is needed in order to achieve better scores for homogeneity of cutting surface, springiness and crunchiness by enzyme-treated samples than by control ones.
5. PUBLICATION LIST

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* http://taplalkozasmarketing.hu/kiadvanyaink/tanszeki-anyagok_6
International conference (summary)
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**Patent**
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