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FACULTY OF ANIMAL SCIENCE

Institute of Chemistry

Department of Biochemistry and Food Chemistry

Head of doctorate school:
DR. PÉTER HORN
Member of the Hungarian Academy of Sciences

Supervisor:
DR. JÁNOS CSAPÓ
Doctor of the Hungarian Academy of Sciences

THE INFLUENCE OF SOME FEED PROCESSING TECHNIQUES AND FOOD MANUFACTURE ON THE D-AMINO ACID CONTENT OF PRODUCTS

ÉVA VARGA-VISI

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1. BACKGROUND OF THE RESEARCH, OBJECTIVES

Protein is one of the most valuable ingredients of foods and feeds, but its digestibility and the availability of amino acids can be deteriorated by numerous chemical changes. One of these processes is racemization of the amino acids, which involves the modification of the spatial arrangement of moieties around the asymmetric carbon atom. The formed D-amino acids can be released from the peptide chain to a lesser degree during digestion, and enzymatic conversion back to the L-form is also limited. Despite the restricted digestion of the D-amino acid containing proteins and the negative discrimination during absorption, some D-amino acids can get into the blood stream of the organism and have been claimed to be harmful for health. Due to the undesirable consequences of D-amino acids in foods and feeds it would be required to assess the magnitude they formed during different processing techniques and studying the factors that may affect their formation.

The D-amino acid content of raw materials is usually low. They can be formed during the manufacture of food and feed if the conditions during operation involve application of heating, alkaline conditions, or fermentation. Higher temperature results in faster racemization of both protein-bound and free amino acids. During fermentation bacteria release free D-amino acids. The free form can be available in a larger ratio than protein bound, because limited proteolysis is not an impediment in the former case. Cheeses were claimed to be the richest sources of the free D-enantiomers among fermented products.

The purpose of the experiments can be summarized as follows:

1. The determination of the degree of amino acid racemization due to different heat treatments.

1.1. The influence of air drying on the D- and L-amino acid content of corn.

1.2. Studying the degree of racemization of amino acids in corn coarse extruded at different temperature and residence time combinations.

1.3. The effect of pressurized steam toasting on the D- and L-amino acid content of fullfat soybeans.

1.4. The determination of the degree of racemization of amino acids in ground fullfat soy due to extruder processing with the application of different temperature and residence time combinations.

2. Studying cheesemaking in the point of view of the formation of free D-amino acids.

2.1. Determination of the free D-amino acid content of different cheeses.

2.2. Studying the D-enantiomer formation process during Cheddar cheese making.

2. MATERIALS AND METHODS

2.1. The conditions of the heat treatments

Dried corn was sampled at the drying plants of "Agria Agricultural and Supplying Co-operative" at Szentgáloskér, and "Kapostáj Agricultural Co-operative" at Zimány. The corn was dried with an industrial Bábolna B1-15 type gravitational drying tower.

The sampling of toasted soy products (hydrothermic soy coarse and 'natúr' hydrothermic soy) was rendered by Bóly Rt. (Bóly-Állomáspuszta). The soy products were produced at the feed processing plant situated in Törökdomb and the soybeans were heat processed with an industrial KAHL HR-1600 type hydrothermic reactor after cracking.

Prior to the extrusion the fullfat soybeans and the corn were ground and the particle size distribution was determined. In the case of maize, the moisture content was determined and adjusted to the required level (18%) during conditioning. The extrusion trials were accomplished at the Department of Biocemistry and Food Technology of the University of Technology and Economics. The extrusion was carried out with an experimental Do-Corder DC 2001 type Brabender machine equipped with a single screw. The temperature of the barrel and the die and the screw speed were kept under control. The applied nominal temperature and screw speed levels were selected to cover the technical applicable wide range of these parameters. The measured zone temperatures were recorded at each level of the treatments. The minimum residence time belonging to the given screw speed and the throughput of the extrusion were also measured and determined. The control samples were taken from the conditioned but not processed bunch immediately prior to the extrusion.

2.2. The conditions of the cheese manufacture

The Cheddar cheeses were produced in compliance with a standard cheesemaking protocol (*Scott*, 1998) at the experimental plant of the Department of Food Science and Technology, University College (Cork, Ireland). The raw milk was pasteurized and the casein-fat ratio was adjusted, then the milk was inoculated with the bulk cultures of *Lactococcus lactis* subsp. *cremoris* 303 or *Lactococcus lactis* subsp. *cremoris* AM2 single-strain cultures. After renneting when the required consistency of curd was obtained the curd was cut, then the curd/whey mixture was stirred and cooked. After cheddaring the curd was milled and salted then moulded and pressed. The cheeses were vacuum wrapped and stored.

During each cheesemaking process samples were taken at the following stages: after salting, after pressing, and during ripening at the age of one week, 28. days, and nine weeks. Samples were ground, lyophilised and stored frozen until analysis.

2.3. Chemical analysis

The chemical analyses of the samples were carried out at the Department of Biochemistry and Food Chemistry, Institute of Chemistry, University of Kaposvár. The moisture content was determined in accordance with the standard procedure of MSZ ISO 1442. The crude protein content determination based on the Kjeldahl-method and it was accomplished in compliance with the Hungarian Feed Codex (1991) chapter No. 6.1. The amino acid analysis was carried out with an Aminochrom OE-914 type amino acid analyzer. The trypsin inhibitor activity (TIA) of the soy products was determined according to the instructions of the EN ISO 14902 standard.

Prior to the determination of the total amount of the amino acid enantiomers proteins were hydrolyzed under acidic conditions. The amount of free D- and L-amino acids was measured after the

precipitation of proteins with trichloroacetic acid. Before the analyses diastereoisomers were produced from the D- and L-enantiomers with OPA (O-phthaldialdehyde) and TATG (1-thio-β-D-glucose-tetraacetate), in order to accomplish the separation with the use of an achiral stationary phase column. The compounds were separated on a 125 mm x 4 mm i.d. column packed with Superspher 60 RP-8e. The derivatization and the analysis were carried out with a LaChrom type MERCK-Hitachi high performance liquid chromatograph (HPLC) comprising L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and D-7000 AIA data conversion utility for the HPLC system manager. Data analysis was accomplished with the D-7000 HPLC System Manager" (HSM) software.

2.4. Statistical analysis

The evaluation of the different heat treatments based on the D- and L-amino acid content and the degree of racemization in experimental units. In the case of corn-drying the means of the above-mentioned variables belonging to the heat-treated and the control groups were compared with t-test for independent samples. The comparison of the control and the two processed soy products were obtained with one-way analysis of variance.

The influence of temperature and screw speed levels on the D-amino acid content and racemization of the extruded products was evaluated with multiple analysis of variance. In order to estimate the random error the extrusion trials with the full cross-classification of the applied nominal temperature and screw speed levels were repeated three times. The classification of the experimental units based on the applied levels of treatments. If the treatment means differed significantly ($P<0.05$), the comparison was accomplished with the Student-Newman-Keuls range test. If the variance of groups were inhomogeneous (Levene test, $P<0.05$), the comparison of treatment means was obtained with the Tamhane test. In some cases the distribution of variables within the

groups did not follow the normal distribution (Shapiro-Wilk test, $P<0.01$). In that case the influence of the two factors was evaluated independently with the corresponding nonparametric test: the Kruskal-Wallis test, instead of the analysis of variance.

The connection between the measured variables (D-amino acid content and $\frac{D}{D+L}\cdot 100$ ratio) and the applied treatment conditions (temperature and residence time) was evaluated with multiple linear regression. The temperature and the residence time data measured on each experimental unit was used for this analysis. The D-amino acid content and the $\frac{D}{D+L}\cdot 100$ ratio were regarded as dependent variables while the temperature and the residence time of the treatment were independent variables. The inclusion or exclusion of the independent variables in order to identify an appropriate regression model based on the F-test of the partial determination coefficients and it was accomplished with the use of the „forward" program method.

The variables that were evaluated during the examination of cheesemaking steps were the D- and L-amino acid content of samples, the D-amino acid composition and the $\frac{D}{D+L}\cdot 100$ ratio. The influence of the processing steps and the starter strains on the above variables was evaluated with multiple analysis of variance. If there was significant difference among the groups, the treatment means were compared with the Student-Newman-Keuls test.

Data analysis was carried out with the use of SPSS for Windows 10.0 (1999) statistical program.

3. RESULTS AND DISCUSSION

3.1. The determination of the degree of amino acid racemization due to different heat treatments

3.1.1.The influence of corn drying on the formation of D-amino acids

The D-amino acid content of the samples before and after drying did not differ significantly, not even at the applied highest temperatures (100ºC drying air temperature and 40-45ºC kernel temperature).

3.1.2. The influence of the extrusion on the racemization of amino acids in corn

The racemization of aspartic acid, serine, glutamic acid and leucine remained below 1% in samples processed at low nominal temperature levels (≤140ºC) and the quantities of their D-enantiomers did not differ from the control. At 170ºC the concentration of D-aspartic acid (15.6 mg/100g) was significantly higher than in control samples, and due to the 200ºC treatment there was an even more significant increase (38.5 mg/100g). The levels of D-serine and D-glutamic acid emerged significantly only at the treatment at 200ºC related to the other applied temperatures (3.2 and 9.7 mg/100g, respectively). The same pattern of change was observed in the case of the degree of racemization ($\frac{D}{D+L} \cdot 100$). In samples extruded at 170ºC, 2.4% of aspartic acid, at 200ºC, 6.1% of that were present as D-enantiomer.

The samples extruded at different speed rates and therefore contacted with heat at distinct residence times showed no difference in D-amino acid content and state of racemization ($P>0.05$). In the time and temperature range, which was under investigation in this study, effects due to differences in temperature were more emphasized than those of residence time. Owing to the temperature dependence of the reaction rate constans and the first order reaction kinetic equitation of racemization, it

can be concluded that the highest possible prolongation of residence time, which was three fold longer than the shortest one, exerted less effect on the D-amino acid formation than increasing extrusion temperature with 10ºC.

Among the L-enantiomers under the scope of the investigation, there was a 24% loss of L-lysine and that of 7.7% of L-aspartic acid in samples treated at 200ºC related to control. Within the concentration decrease of L-aspartic acid 78% of that can be assigned to the D-amino acid formation. In contrast with this not more than 2% of the decrease of L-lysine level can be associated with racemization.

3.1.3. The influence of toasting on the D- and L-amino acid content of fullfat soybeans

Pressurized steam cooking of fullfat soy did not result in significant increase of the amount of D-enantiomers or the decrease of the concentration of the L-amino acids ($P>0.05$), while the trypsin inhibitor activity (TIA) was reduced to the required level ($TIA<1.5$ mg/g) and the results of the urease test ($\Delta pH<0.2$) also verified the adequate intensity of the heat treatment.

3.1.4. The effect of the extruder processing on the racemization of amino acids in fullfat soy

In the case of the treatment of dry fullfat soy, the samples heated at lower temperatures (≤140ºC) contained already more D-serine and D-glutamic acid than the control, and their amount continuously rose with the increase of temperature, together with the amount of D-phenylalanine. The $\frac{D}{D+L}\cdot 100$ value which is the measure of racemization, followed a similar tendency in the case of the above-mentioned amino acids, but in this case the difference between samples treated at the lowest and the highest levels of temperature was higher (e. g. glutamic acid: 0.57%, 100ºC; 1.43%; 220ºC) than in the case of the absolute

amount of the D-amino acids (e. g. glutamic acid: 40 mg/100g, 100ºC; 89 mg/100g, 220ºC). This can be explained by the fact that parallel with the increase of the D-enantiomers, the amount of the L-amino acids decreased due to racemization and other chemical changes.

Similarly to the extrusion of maize, changes in residence time did not exert a significant effect on the D-amino acid content and on the degree of racemization. Within the studied temperature-time combinations this phenomenon can also be explained with the domination of the temperature effect.

Due to treatments above 140ºC the concentration of most of the L-amino acids decreased. Likewise in the case of the corn extrusion, the loss of L-lysine (21%; 220ºC) was the highest degree among L-amino acids under study, and in this process racemization showed to play a minor role. In soy samples treated at 220ºC the concentration decrease of L-lysine, L-serine, L-glutamic acid and L-aspartic acid was 21, 17, 10 and 8.6%, respectively. Some part of the loss cannot be assigned to racemization (92, 76, 87 and 75%, respectively), but other reactions e. g. crosslink formation and side-chain alteration. In contrast with this, when samples were treated at 220ºC, the concentration decrease of L-phenylalanine was practically identical with the increase of the amount of D-phenylalanine.

The heat treatment of ground soy resulted in larger amount of D-amino acids than that of maize, presumably this can be explained by the four-fold higher protein content of soybeans related to that of corn. When comparing the $\frac{D}{D+L} \cdot 100$ ratio which is independent on the absolute amount of protein it can be seen that heat treatment with approximately equal intensity produced a higher degree of racemization in soy proteins than that of corn proteins.

3.2. The determination of the amount of the D-amino acids in cheeses and studying the process of their formation

3.2.1. The determination of the free D-amino acid content of different cheeses

The free D-amino acid content of fresh Mozzarella cheese (6.7 mg/100g) was far less than that of ripened cheeses (25-85 mg/100g), but a longer ripening period is not absolutely accompanied with a higher level of D-amino acids. Data on the D-amino acid content of cheeses processed with different technologies suggested that with the analysis of different products an unambiguous relation cannot be found between the length of the ripening period and the D-amino acid content of cheeses. In order to evaluate how D-enantiomers formed during cheesemaking, e. g. ripening, sampling during processing is required.

3.2.2. Changes in the free D-amino acid content during Cheddar cheese manufacture

During the ripening period the concentration of free D-alanine continuously increased ($P=0.002$) in the dry matter content of the cheese. This can be attributed to processes connected with lysis of bacteria and responsible for the release of D-alanine from the cell wall and the cytoplazm. During ripening the increase of D-alanine followed the liberation of L-alanine which originated from milk proteins. One might speculate that part of the free D-alanine content derived from the free L-alanine pool of cheese due to the bacterial alanine racemase if this enzyme can operate outside the bacteria. If it is possible, the L→D transformation may become more intensive during ripening. On one hand with the increasing intensity of lysis more and more alanine racemase gets out of the cells, on the other hand due to proteolytic activity the amount of free L-alanine increases during ripening providing more substrate for alanine racemase.

The dry matter of curd contained significantly larger amount of D-aspartic acid after pressing than before pressing in the case of both strains, and more D-glutamic acid in the case of strain ‘303’ ($P<0.05$). The pressure which was exerted during pressing (75 kPa) was four orders of magnitude lower than required in order to reduce the cell count in raw milk, therefore the applied pressure by itself cannot be responsible for releasing the D-enantiomers. Although, it cannot be excluded that power impulses together with other factors, e.g. the increase of osmotic pressure promoted the destruction of cells.

The D-amino acid ‘pattern’ of the product continuously changed during ripening ($P<0.01$), due to the increase of D-alanine, and the decrease of D-glutamic acid ratio within the D-amino acid pool. But the selection of strain for inoculation did not exert an effect on the free D-amino acid composition of Cheddar cheese.

Cheeses from cheesemaking trials with *Lactococcus lactis* subsp. *cremoris* 303 contained more D-amino acid than in the case of trials with *Lactococcus lactis* subsp. *cremoris* AM2 ($P<0.01$). It is possible that strain ’303’ is more susceptible to lysis than strain ‘AM2’ during this sort of Cheddar making protocol and therefore releases more D-amino acid from cells or/and releases more enzymes which form D-amino acid.

4. CONCLUSION

4.1. The determination of the degree of amino acid racemization due to different heat treatments

The studied industrial drying processes did not increase significantly the amount of the D-amino acids in corn, not even when the highest air temperature was applied.

The racemization of the amino acids under the scope of the investigation remained less than 1% and the amount of their D-enantiomers did not differ from the control when extrusion was carried out with the experimental Brabender machine at lower temperatures (140°C) with the residence time of 28-72s. In samples treated at 200°C the degree of racemization of aspartic acid, serine, glutamic acid and leucine was significantly higher than that of the control, but among the above-mentioned amino acids the racemization of aspartic acid was significantly of the highest degree. High temperature extrusion of corn (200°C) resulted in concentration decrease of L-lysine and L-aspartic acid. In the case of L-aspartic acid about three-quarters of the loss can be assigned to the D-amino acid formation. On the contrary racemization probably is not the primary cause of the loss of L-lysine, because the L→D transformation was not significant in this case. Most probably, decomposition in Maillard reaction, crosslink formation and side-chain alteration made the products of lysine undetectable for amino acid analysis.

Pressurized steam cooking of fullfat soybeans did not result in significant formation of D-amino acids or loss of the L-amino acids while the aim of the treatment was attained, the level of trypsin inhibitors was reduced to the required level.

In the case of the extruder processing of dry fullfat soy, notable racemization was detected even at lower temperatures (≤140ºC). The concentration of more L-amino acids decreased, and their loss can be assigned to the L→D conversion to a different degree. While the

transformation of L-phenylalanine nearly completely can be attributed to the racemization, the main cause of the loss of L-lysine is not L→D conversion. Treatments in the same order of magnitude resulted in the formation of more D-amino acids and larger degree of racemization of amino acids in fullfat soy than in maize.

4.2. Determination of the amount of the D-amino acids and the process of their formation during a fermentation technology

When steps of the Cheddar cheese manufacture was studied, among the free D-amino acids that appear in larger quantities (D-alanine, D-aspartic acid and D-glutamic acid) the amount of D-alanine continuously increased during ripening. The rise of the free D-alanine content is supposed to be associated with the increasing number of dead bacterial cells that are exposed to lysis during ripening. The increase of the concentration of D-alanine followed the liberation of L-alanine originated from milk proteins. Due to this tendency the question of the origin of free D-alanine may arise. Some part of it could be originated from bacteria, but besides this it may be formed from free L-alanine if the bacterial alanine racemase could operate outside the bacteria.

During the pressing of the curd, the D-glutamic acid and D-aspartic acid content related to dry matter increased. The intensity of the pressure applied was more magnitudes lower than the one which can cause severe cell damage, therefore the power impulse by itself cannot be accountable for this phenomenon.

The composition of D-amino acids continuously changed during manufacturing, but it was independent of the strain applied for inoculation. On the other hand, the absolute amount of D-amino acids was different among cheeses produced by different single-strain starter cultures, raising the issue that strains might differ in the susceptibility to autolysis.

5. NEW SCIENTIFIC RESULTS

During experimental extrusion of maize at low temperatures (110-140 °C) the quantity of L-amino acid enantiomers under study remained practically unchanged and the amount of D-enantiomers did not increase. At higher temperatures (170-200 °C) the racemization of amino acids became significant.

Pressurized steam cooking (toasting) of fullfat soybeans did not result in significant changes in the concentration of amino acid enantiomers under the scope of the study.

Extruder processing of dry fullfat soy showed to produce significant racemization of amino acids even at lower temperatures (<140 °C).

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