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The effect of the extrusion temperature and the residence time on the D-amino acid content of corn extrudates

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ABSTRACT

Racemization of peptide-bonded amino acids results in a decrease of protein digestibility, and long-term negative health consequences due to the intake of dietary D-amino acids cannot be excluded. Temperature and time dependence of racemization has been investigated in strong alkaline solutions mostly in cases of clear proteins. The aim of the research was to determine the amount of D-enantiomers and the level of racemization of amino acids with the highest rate of racemization and occurring in the largest quantities in corn grain extrudates treated with different heat effects (temperature and residence time combinations). Extrusion trials below 144°C with residence times of 28-72 s did not induce significant ($P<0.05$) racemization. Treatment at 171°C and at 200°C induced significant racemization of aspartic acid (2.4% and 6.1%, respectively). In case of serine and glutamic acid the ratio of D-enantiomers increased significantly due to the treatments at 200°C (0.75% and 0.69%, respectively). The L-aspartic acid and L-lyisine content of the products extruded at 200°C were significantly lower than in control and in products produced at lower temperatures. The primary cause of the loss of L-aspartic acid was D-aspartic acid formation. In contrast, racemization of lysine played a minor role in the decrease of L-lysine content.
(Keywords: racemization, D-amino acid, ground corn, extrusion temperature, residence time)

ÖSSZEFOGLALÁS

Extrudált kukoricadara D-aminosav tartalmának alakulása a kezelési hőmérséklet és a tartózkodási idő függvényében

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A fehérjében kötött aminosavak racemizációja csökkentheti a fehérjék emészthetőségét, és nem zárható ki annak a lehetősége sem, hogy a D-aminosavak hosszú távú fogyasztásának kedvezőtlen egészségügyi hatásai vannak. A racemizációs folyamat hőmérséklet- és időfüggését eddig csak lúgos oldatokban vizsgálták, többnyire tiszta fehérje preparátumokból. Célul tűztük ki annak vizsgálatát, hogy hogyan változik a leggyorsabban racemizálódó és a kukorica darában a legnagyobb mennyiségben előforduló aminosavak D-enantiomerjeinek abszolút mennyisége, valamint a

racemizáció mértéke az egyik leggyakrabban alkalmazott termikus művelet, az extrudálás során, a kezelési hőmérséklet és a tartózkodási idő függvényében. A 144ºC alatti hőmérsékleten, 28-75 másodperc tartózkodási idejű extrúziós próbák nem jártak szignifikáns (P<0,05) racemizációval. Az aszparaginsavnál szignifikáns racemizáció következett be a 170ºC-os kezelés (2,4%), és a 200ºC-os kezelés (6,1%) hatására. A szerinnél (0,75%) és a glutaminsavnál (0,69%) csak a 200ºC-os kezelésnél volt jelentősen magasabb a D-enantiomerek aránya, mint a kontrollban. A 200ºC-on kezelt minták L-aszparaginsav és az L-lizin tartalma szignifikánsan kevesebb volt, mint a kontrollban és az alacsonyabb hőmérsékleten kezelt mintákban. Míg az L-aszparaginsav veszteség elsődleges oka a racemizáció volt, az L-lizin tartalom csökkenésben az L-D átalakulás nem játszott jelentős szerepet.
(Kulcsszavak: racemizáció, D-aminosav, kukoricadara, extrudálási hőmérséklet, tartózkodási idő)

INTRODUCTION

Extrusion has gained an increasing role in the production of both food and feed products due to its advantegous properties, that is structure formation, inactivation of heat sensitive antinutritive factors, improvement of microbiological quality and increase of digestibility of nutrients (*Lásztity and Örsi,* 1984*; Ormainé et al.,* 1987; 1988). Simultaneously with the increasing use of this technique, the changes in chemical composition of nutrients due to extrusion is being evaluated. Due to high temperature during treatment the stereospecificity of the amino acids may change. The extent of racemization has been thoroughly studied in clear protein preparations in alkaline medium at high temperatures (*Masters and Friedman,* 1980*, Friedman et al.,* 1981*; Liardon and Ledermann,* 1986*; Liardon and Friedmann,* 1987). The degree of racemization associated with some food processing procedures has been also investigated (*Csapó et al.,* 2000; *Csapó et al.,* 2001), but the effect of extrusion on racemization has not been evaluated yet. The decrease of the amino acid content in corn grain due to extrusion has been investigated, but in these studies the ratio of the enantiomers was not determined. The greatest loss was detected in lysin content, but the loss of some other amino acids such as aspartic acid were also reported (*Ormainé et al.,* 1988 *Ormainé and Czukor,* 1991). Aspartic acid, glutamic acid and serine have been reported to be susceptible to racemization (*Friedman,* 1999), and also occurs in higher quantities in corn. Maise is also rich in leucin and isoleucin that are not supposed to be inclined to racemization. The possible changes in L-lysin content are also the matter of interest, because this amino acid occurs in the lowest quantities related to requirements in seeds. The purpose of the research was to investigate the influence of the heat effect (dependent on the applied temperature and screw speed) on the D-amino acid content of corn grain during extrusion.

MATERIALS AND METHODS

Conditioning and extrusion of grain

Commercial corn grain was obtained in a supermarket and the particle size distribution was determined. Prior to extrusion, the grain was hydrated to the desired total moisture of 18% by adding the calculated amount of water. In digestibility studies maize is usually treated at 130–140ºC with the moisture content of 18-20% (*Chae et al. ,* 2000*; Cho et al.,* 2001). After a half an hour mixing, the hydrated grain was allowed to

equilibrate in a tight sealed container overnight. Ten kg of conditioned material was used for each trial. Extrusion was carried out using a Do-Corder DC 2001 type Brabender machine equipped with a 19 mm i. d. barrel (21:1 length to diameter ratio); a screw with the length of 400 mm with increasing screw diameter from 12 to 17 mm, and a cylindrical die which consists of two parts: a 55 mm long by 8 mm i. d. following a 22 mm long by 5 mm. The barrel and the die were heated by electrically controlled split ring resistance heaters, and the screw speed was also kept under control. The barrel and the die temperatures were monitored by thermocouples mounted in shallow wells. Extrusion trials with the full cross-classification of the applied nominal temperature and screw speed levels (*Table 1*) were repeated three times on three different days. From the three reported zone temperatures (T_1, T_2, T_3), one value was calculated (T) to characterize the effect of temperature with the equation of: $T = \frac{T_1 + 2T_2 + T_3}{4}$, which can be deduced from the horizontal temperature graph of the barrel. Minimum residence time was determined by introducing a small amount of dye into the feeding port and measuring the time required for the first colored extrudate to exit the die. Prior to sampling, the machine was allowed to equilibrate to the desired temperature, then appr. 200g sample was collected and allowed to cool down before being homogenized, and sealed in polyethylene bags and stored at -20˚C.

Table 1

Nominal temperature and screw speed levels

Temperature levels (1)	**T_1 (°C) 1. zone (barrel)(2)**	**T_2 (°C) 2. zone (barrel)(3)**	**T_3 (°C) 3. zone (die) (4)**	**Screw speed levels (5)**	**Screw speed (rpm) (6)**
1	110	110	110	**1**	40
2	140	140	140	**2**	80
3	170	170	170	**3**	120
4	200	200	200	**4**	160

1. táblázat: A névleges hőmérséklet és fordulatszám szintek

Hőmérséklet szintek(1), Az 1. zóna hőmérséklete a házon (°C)(2), A 2. zóna hőmérséklete a házon (°C)(3), A matricafej hőmérséklete (°C)(4), Fordulatszám-szintek(5), Fordulatszám (min^{-1})(6)

Chemical analysis

The moisture content was determined based on the standard procedure of ISO MSZ 1442. Prior to amino acid analysis the samples were dissolved in hydrochloric acid (6 M; 5 cm^3) and proteins were hydrolyzed at 105±1°C for 24h. After cooling, the pH was set to pH=7 with sodium hydroxide solution. Diastereomers were produced with OPA (o-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) (Sigma, St. Louis, MO, USA) (*Einarsson et al.*, 1987; *Csapó et al.,* 1995). Derivatization and analysis were carried out with a MERCK-Hitachi HPLC comprising L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and AIA data conversion utility for the D-7000 HPLC system manager. The compounds

were separated on a 125 mm×4 mm i.d. column packed with Superspher 60 RP-8e (MERCK, Darmstadt, Germany). The mobile phase gradient can be seen in *Table 2*, the flow rate was 1 cm^3 min^{-1}, and the oven temperature was 40°C.

Table 2

The mobile phase gradient for the separation of OPA-TATG derivatives of amino acids

Time (min) (1)	Methanol (v/v%) (2)	Phosphate buffer 50mM (v/v%) (3)	Acetonitril (v/v%) (4)
0	28	72	0
10	28	72	0
100	24	36	40
110	24	36	40
115	28	72	0

2. táblázat: Aminosav enantiomerek OPA-TATG származékainak elválasztására alkalmazott gradiensprogram

Idő (min)(1), Metanol (v/v%)(2), Foszfát puffer 50mM (v/v%)(3), Acetonitril (v/v%)(4)

Solvents (acetonitrile and methanol) were HPLC gradient grade (MERCK, Darmstadt, Germany). The derivatives were detected with a fluorescence detector (λ_{ex} 325 nm, λ_{em} 420 nm).

Statistical analysis

Data analysis was carried out with the use of SPSS for Windows 10.0 (1999) statistical program. There were four levels of temperature factor and four levels of screw speed factor. The number of replication was three, sampling was repeated on three different days with the full cross-classification of the applied levels of factors. The influence of temperature and residence time on the D-amino acid content of the extruded products was evaluated with multiple analysis of variance. The equation of the used linear model was the following:

$Y_{ijk}=\mu+T_i+F_j+TF_{ij}+e_{ijk}$ with

Y_{ijk} = the k^{th} observation in the ij^{th} treatment combination,
μ = the least squares mean,
T_i = the effect of the i^{th} class of factor T (temperature) expressed as a deviation from μ,
F_j = the effect of the j^{th} class of factor F (screw speed) expressed as a deviation from μ,
TF_{ij} = the interaction effect of the i^{th} class of factor T and the j^{th} class of factor F expressed as a deviation from $\mu+T_i+F_j$ and
e_{ijk} = the random error associated with the k^{th} observation in the ij^{th} treatment combination.

If treatment means differed significantly ($P<0.05$), the comparison of that was accomplished with the Student-Newman-Keuls test.

Trials treated at the same temperature with different screw speed were compared to each other with the use of one-way anova, where the equation of the modell was the following:

$Y_{jk}=\mu+F_j+e_{jk}$ with

Y_{jk} = the k^{th} observation in the j^{th} treatment,
μ = the least squares mean,
F_j = the effect of the j^{th} treatment (screw speed) and
e_{jk} = the random error associated with the k^{th} observation in the j^{th} treatment.

RESULTS AND DISCUSSION

Based on the measured true-temperature-data obtained during extrusion, the higher the nominal temperature was, the better accuracy and precision was obtained (*Table 3*). The residence time can be influenced not only the screw speed but also the temperature due to the viscosity changes of the dough (*Phillips et al.,* 1984). In this particular case it was practically independent on temperature, and thus each screw speed levels can be regarded as represent residence time levels (*Table 3*).

Table 3

Nominal and measured properties of extrusion

Levels (1)	**Nominal tempera-ture (ºC) (2)**	**Measured temperature (T) average±s.d. (ºC) (n=12) (3)**	**Levels (4)**	**Screw speed (s^{-1}) (5)**	**Residence time (s) average±s.d. (n=12) (6)**	**Throughput (kg/h) average±s.d. (n=12) (7)**
1	110	129±6.0	**1**	40	75±3	2.5±0.2
2	140	144±1.8	**2**	80	47±2	4.1±0.1
3	170	171±1.0	**3**	120	34±3	5.8±0.4
4	200	200±1.2	**4**	160	28±3	7.0±0.8

3. táblázat: Az extrúzió névleges és tényleges paraméterei

Szintek(1), Névleges hőmérséklet(2), A mért tényleges hőmérséklet, átlag±szórás(3), Szintek(4), Fordulatszám(5), Tartózkodási idő, átlag±szórás(6), Tömegáram, átlag±szórás(7)

Due to the differences in moisture content of controls and extrudates, the amino acid data were calculated to 100% dry matter content. The amount of D-isoleucine and D-lysine were below the detection level, and thus solely the quantity of the L-enantiomer of these amino acids was determined. The control samples were also analysed for D-amino acid content. They probably formed during the acidic hydrolysis of proteins which has to be done before the amino acid analysis (*Masters and Friedman,* 1980; *Csapó et al.,* 1997). Therefore, based on the method of *de Vrese et al.* (2000), the amount of D-amino

acids found in controls was subtracted from the D-amino acid content of extrudates, and thus the amount the D-amino acid formed during extrusion was determined.

Groups of extrudates treated at different temperatures differed significantly both in D-amino acid content ($P<0.05$) (*Table4*) and in the degree of racemization ($P<0.05$) (*Table 5*) at least at two levels. Samples extrudated at 129˚C and at 144˚C did not differ in D-aspartic acid, D-serine, D-glutamic acid and D-leucine content. In case of aspartic acid, which has been reported to be very prone to racemization, a significant D-enantiomer concentration increase was detected at 171˚C, and an even more significant one at 200˚C. The levels of D-serine and D-glutamic acid emerged significantly only at the treatment at 200˚C related to the other applied temperatures. In the case of leucine, at the first two levels there was a smaller amount of D-enantiomer, than at the next two levels.

Table 4

Influence of the extrusion temperature on the D-amino acid content (mg /100g dry matter)[1, 2] (n=12)

Examined amino acids(1)	Temperature (T) (2)			
	129ºC	**144ºC**	**171ºC**	**200ºC**
D-Asp	$5.1^{a} \pm 2.6$	$6.0^{a} \pm 4.4$	$15.6^{b} \pm 9.0$	$38.5^{c} \pm 22.8$
D-Ser	$0.58^{a} \pm 0.65$	$0.93^{a} \pm 1.1$	$1.2^{a} \pm 1.0$	$3.2^{b} \pm 1.4$
D-Glu	$2.2^{a} \pm 4.5$	$2.4^{a} \pm 4.4$	$2.9^{a} \pm 4.6$	$9.7^{b} \pm 7.1$
D-Leu	$1.3^{a} \pm 2.3$	$3.4^{a} \pm 1.7$	$6.3^{b} \pm 2.7$	$6.6^{b} \pm 3.2$

[abc]Averages in one row with common supercript do not differ. a,b,c=P≤0.05 hz([abc]*Az azonos betűvel jelölt és egy sorban lévő átlagok közt nincs különbség.*) [1]Corrected with control values. ([1]*A kezeletlen kontrollal korrigált értékek.*) [2]Averages and standard deviations of samples extruded at the same temperature, with different screw speeds. ([2]*Azonos hőmérsékleten, de különböző fordulatszámmal extrudált minták mérési eredményeinek átlaga és szórása.*)

4. táblázat: Az extrudálási hőmérséklet hatása a D-aminosav tartalomra (mg/100g szárazanyag)

Vizsgált aminosavak(1), Hőmérséklet(2)

The same pattern of change was observed in the case of the degree of racemization, when not only the amount of the D-enantiomer, but also the quantity of that related to the total amount of the particular amino acid was taken into consideration (*Table 5*). The degree of racemization did not changed with the increase of extrusion temperature from 129˚C to 144˚C. The extent of racemization of aspartic acid increased both at 171˚C and at 200˚C, that of serine and glutamic acid emerged only at 200˚C.

Samples extruded at different speed rates and therefore contacted with heat at distinct residence times, showed no difference in D-amino acid content and racemization state when they were analyzed together with the influence of the temperature. The impact of the temperature on racemization was greater in the used intervals than the influence of the residence time (screw speed), therefore the D-amino acid content of samples extruded at the same residence time but at different temperatures ranged from zero to ten milligrams. When trials treated at the same temperature with different

residence times were compared to each other, the D-aspartic acid content of the samples extruded at 200˚C for 75 s was higher (P=0.033), than that of the other samples extruded at the same temperature with less residence time. But in case of the other temperatures there were no significant differences in D-aspartic acid content among groups with different residence times, and the amount of the D-enantiomers of the other amino acids did not differed either.

Table 5

Influence of the extrusion temperature on the degree of racemization

$$\left(\frac{D}{D+L}\cdot 100\right)^{1,2}$$ **(n=12)**

Examined amino acids(1)	Temperature (T) (2)			
	129ºC	**144ºC**	**171ºC**	**200ºC**
Asp	$0.82^{a} \pm 0.43$	$0.94^{a} \pm 0.66$	$2.42^{b} \pm 1.3$	$6.10^{c} \pm 3.3$
Ser	$0.13^{a} \pm 0.13$	$0.21^{a} \pm 0.24$	$0.28^{a} \pm 0.22$	$0.75^{b} \pm 0.33$
Glu	$0.16^{a} \pm 0.32$	$0.17^{a} \pm 0.32$	$0.20^{a} \pm 0.31$	$0.69^{b} \pm 0.52$
Leu	$0.13^{a} \pm 0.24$	$0.33^{a} \pm 0.17$	$0.65^{b} \pm 0.26$	$0.66^{b} \pm 0.29$

[abc]Averages in one row with common supercript do not differ. a,b,c=P≤0.05 (*[abc]Az azonos betűvel jelölt és egy sorban lévő átlagok közt nincs különbség.*) [1]Corrected with control values. (*[1]A kezeletlen kontrollal korrigált értékek.*) [2]Averages and standard deviations of samples extruded at the same temperature, with different screw speeds. (*[2]Azonos hőmérsékleten, de különböző fordulatszámmal extrudált minták mérési eredményeinek átlaga és szórása.*)

5. táblázat: Az extrudálási hőmérséklet hatása a racemizáció mértékére

Lásd a 4. táblázatban(1, 2)

Disparity among extrudates was evaluated based on data adjusted with control values. Differences among the control and the extrudates were also evaluated - with the use of the original data. Control samples did not differ significantly (P<0.05) from extrudates treated at 129˚C and at 144˚C with respect to the racemization state and D-amino acid content. But samples treated at 171˚C and at 200˚C diverged from control in the same manner as these high temperature extrudates differed from low temperature extrudates in the previous analysis.

The amount of the L-enantiomers did not differ significantly among the control group and groups extruded at different temperature, with two exception. There were less L-aspartic acid and L-lysine in groups treated at 200˚C, than in the others (*Table 6*). Due to extrusion at 200ºC with a Brabender 20 DN type machine, the lysine content of corn grain (with 14% moisture content) decreased with 44%, and that of aspartic acid with 11% (*Ormainé and Czukor,* 1991). In the present study conducted at 200ºC, the total (L+D) lysine content decreased with 24% and aspartic acid with 2%. The smaller loss probably can be attributed to the higher water content used during extrusion (18% v. s. 14%) and there were also some differences in the geometrical construction of the extruders used.

Table 6

Concentration of L-amino acids in extrudates treated at different temperatures (g/100g dry matter)[1] (n=12)

Examined amino acids (1)	Control (2)	Temperature (T) (3)			
		129ºC	144ºC	171ºC	200ºC
L-Asp	0.65[a]±0.06	0.65[a]±0.02	0.65[a]±0.03	0.65[a]±0.03	0.60[b]±0.03
L-Ser	0.45[a]±0.03	0.45[a]±0.05	0.49[a]±0.05	0.46[a]±0.05	0.46[a]±0.04
L-Glu	1.60[a]±0.14	1.61[a]±0.08	1.63[a]±0.11	1.58 [a]±0.12	1.56[a]±0.13
L-Leu	1.04[a]±0.05	1.04[a]±0.07	1.09[a]±0.08	1.02[a]±0.09	1.02[a]±0.11
L-His	0.15[a]±0.01	0.15[a]±0.02	0.16[a]±0.02	0.16[a]±0.01	0.15[a]±0.01
L-Ile	0.28[a]±0.01	0.28[a]±0.02	0.29[a]±0.02	0.27[a]±0.02	0.27[a]±0.02
L-Lys	0.21[a]±0.05	0.21[a]±0.04	0.21[a]±0.02	0.20[a]±0.02	0.16[b]±0.03

[abc]Averages in one row with common supercript do not differ. a,b,c=P≤0.05 ([*abc*]*Az azonos betűvel jelölt és egy sorban lévő átlagok közt nincs különbség.*) [1]Averages and standard deviations of samples extruded at the same temperature, with different residence times. ([1]*Azonos hőmérsékleten, de különböző fordulatszámmal extrudált minták mérési eredményeinek átlaga és szórása.*)

6. táblázat: Az L-aminosavak koncentrációja különböző hőmérsékleten kezelt extrudátumokban (g/100g szárazanyag)

Vizsgált aminosavak(1), Kontroll(2), Hőmérséklet (T)(3)

CONCLUSIONS

In the time and temperature range which was under investigation in this study, effects due to differences in temperature were more emphasized than that of residence time. This can be explained by the fact that while 10˚C temperature increase resulted in 2.2-5.5 fold increase in the first order reaction rate constans (k) of amino acid racemization, for the same increase the reaction time (t) should be increased in the same degree (four-fold, on average), due to their relationship in the first order reaction kinetic equitation ($\ln\frac{A_0}{A} = k \cdot t$). In this case the longest residence time was only 2.7 fold longer than the shortest, while extrusion temperature ranged between 129˚C and 200˚C.

When extruding on this Brabender machine below 144˚C, with the residence time of 28-75s, the amount of D-amino acids under study remained less than 1% and did not differ from control. As the result of the treatments at 200˚C, among amino acids under study, the racemization of aspartic acid was the most emphasized. Although the increase of D-amino acid content of serine, glutamic acid and leucine was also significant, their increments were far less than that of aspartic acid.

The amount of L-aspartic acid changed from 0.65 g/100 g to 0.60 g/100 g due to treatment at 200ºC, while 0.039 g/100g of D-aspartic acid formed. This means that about three-quarters of the loss of the L-enantiomer can be assigned to the D-amino acid formation. Contrast racemization probably is not the primary cause of the loss of L-lysin because the amount of the D-enantiomer was less than 0.001 g/100 g, even at 200ºC. This quantity is less than 2% of the decrease of the L-lysine content (*Table 6*). Most

probably, crosslink formation and side-chain alteration made products of lysine undetectable for amino acid analysis.

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