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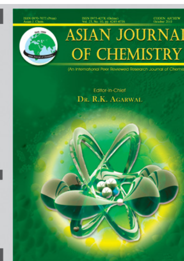


# Processing of Keratin Waste of Meat Industry

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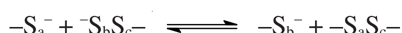
Chicken feathers were processed through hydrolysis in two stages and breakdown efficiency was monitored by factor tests. In the first stage, feathers were incubated in an environment of 0.1 % or 0.3 % aqueous solution of KOH (ratio 1:50) for 24 h at 70 ºC. After adapting pH level to 9, action by proteolytic enzyme polarzyme 12 T (1-5 %, w/w) at temperature 30-50 ºC broke down the feathers in 4-8 h into keratin hydrolyzate. Hydrolysis of feathers in environment of 0.1 % KOH is quite low (max. 20 %). In an environment of 0.3 % KOH the hydrolysis efficiency is more than four-fold. At upper limits of factors under study (8 h hydrolysis at 50 ºC with 5 % addition of enzyme) 88.7 % feathers were degraded. Test results confirmed that applying proteolytic enzyme polarzyme 12 T achieved efficiency of chicken feather degradation similar to formerly used enzyme Savinase Ultra 16 L.

**Key Words: Enzyme hydrolysis, Chicken feathers, Keratin hydrolyzate, Keratin waste, Meat industry.**

## INTRODUCTION

Solid biological waste rich in keratin is a by-product of many industrial branches (particularly poultry, meat and tanning industry). Keratin waste (feathers, antlers, bristles, claws, wool and others) is not adequately utilized, cumulates in the environment or is disposed of by incineration[1-4]. About 8.5 billion tonnes of waste chicken feathers are annually produced worldwide, in India alone it is *ca.* 350 million tonnes. Considering that feathers make up *ca.* 7 % chicken live weight, this source of keratin is significant[5-8].

In order to use waste keratin for producing compostable packages, mulching foils, films and coatings, it has to be transformed into a stable keratin solution. Keratin is a protein exhibiting high chemical resistance because it is strongly crosslinked with disulfide bridges that render its processing difficult. Obtaining soluble products requires cleaving crosslinks and possibly carrying out hydrolysis of peptide bonds. Cleavage of disulfide bridges may proceed through a reaction of thiol anion according to following pattern:



$$-S_a^- + {}^-S_bS_c- \rightleftharpoons -S_b^- + -S_aS_c-$$

With a very high pH level, hydrolysis of disulfide bridges also appears, in accordance with pattern[9]:

$$-SS- + OH^- \longrightarrow -S^- + -SOH$$

Under mild conditions (pH *ca.* 8), even sulphitolysis takes place, following pattern:

$$-SS- + HSO_3^- \longrightarrow -SH + -SSO_3^-$$

Keratin may be extracted from starting material only if disulfide bonds and hydrogen bonds are disrupted. Gousterova *et al.*[10] recommend processing keratin waste in a strongly alkaline environment (KOH, pH ≈ 12) at elevated temperature (120 ºC) and increased pressure (0.2 MPa) leading to preparation of a keratin hydrolyzate that can be employed as fertilizer. Besides alkaline processing of keratin waste in autoclave, they described alkaline hydrolysis of wool (in mixture 0.15 mol $L^{-1}$ KOH and 0.05 mol $L^{-1}$ NaOH) in microwave oven for 1 h. Microwave radiation easily penetrates to the porous bed of keratin. Keratin hydrolyzate (reduced, soluble keratin) may also be prepared through acid hydrolysis. Karthikeyan *et al.*[11] processed waste raw horns first in a steam rendering plant 3 h under high pressure (0.276 MPa). Material thus pre-processed was dried and ground in grinder to horn powder. Twenty kg powder was mixed with 8 L concentrated HCl (11.6 mol $L^{-1}$) and 32 L water. The mix was extracted in mild steel glass-lined reactor, blown through with steam and temperature kept at 95 ± 5 ºC for 3 h. Hydrolyzed horn flour contained 35 % (w/v) solid fraction. Extraction of keratin from wool employing system of urea-reducing agent-surfactant is described in works by Yamauchi *et al.*[12,13]. Dried and degreased (hexane-

dichloromethane 1:1 mix) wool (10 g) is mixed with 8-M urea (180 mL), 2-mercaptoethanol (15 mL) and sodium dodecyl sulphate (6 g) and is shaken at 60 ºC for 6 h. During the process, aqueous phase is kept at an approximately neutral level. The mixture is quickly filtered and filtrate is dialyzed (cellulose thimble) 2-3 times against de-aerated water. A colourless, clear keratin hydrolyzate solution is thus obtained; its yield borders on 50 %. Extraction of keratin employing the method of urea-reducing agent is also described by Schrooyen[14,15].

Reduced forms of keratin (keratin hydrolyzates) are employed, for example, in the food industry for preparing packaging materials (films). Keratin films display lower permeability for water vapour and gases than collagen films cross-linked with glutaraldehyde. Keratin hydrolyzates are also suitable as edible coatings, for example, for meat, poultry and fish. Aqueous solutions of reduced keratin are used for preparing packaging materials for microcapsules. Materials encapsulated in the food industry are, for example, colouring agents, flavours, aromas, medicines, oils, fats. Encapsulated substance preserves its activity longer and its release in a specific place may be controlled[16-19]. Microcapsules are also utilized in non-food applications, for example, in the textile industry, graphic and chemical industries and in agriculture[20-23]. Keratin hydrolyzates are also found as ingredients in cosmetics (shampoos, hair conditioners, nutrient serums for hair tips, nail varnishes, mascaras) or find application in medicine as substrates for cell culture and tissue engineering[24-29].

The objective of this contribution is processing of waste chicken feathers through enzymatic hydrolysis into keratin hydrolyzate and studying effect of technological conditions (amount of enzyme, temperature and time of hydrolysis) on breakdown efficiency. Due to the fact that we had already earlier dealt with breakdown of chicken feathers through enzymatic hydrolysis (using enzyme Savinase Ultra 16 L supplied by Novozymes A/S Bagsvaerd, Denmark), the object of our contribution was also to verify breakdown efficiency when using a different alcalase (Polarzyme 12 T) from same supplier.

## EXPERIMENTAL

White-coloured poultry feathers were supplied from a poultry farm in the Czech Republic. Raw feathers contained no impurities. Feathers (air-dry) in 100 g amounts were cut into small pieces of less than 5 mm length. A homogenized sample of feathers was then conditioned for 72 h in dessicator filled with dry silica gel (22 ± 1 ºC) and first analyses were performed[30]: dry matter = 93.2 %; in dry matter: fat = 1.15 %, inorganic solids = 1.01 %; in dry matter of degreased sample: total Kjeldahl nitrogen = 14.6 %, sulphur = 2.00 %.

Polarzyme 12 T, Greasex 50 L were supplied by Novozymes A/S Bagsvaerd, Denmark. Facilitated dosage of enzyme Polarzyme 12 T is enabled by preparing stock solutions of enzyme from a concentrated solution, pipetting 2 mL concentrated enzyme solution into a 50 mL volumetric flask, filling with distilled water and mixing contents. KOH, NaOH, $H_2SO_4$, $H_3PO_4$, 30 % $H_2O_2$, $BaCl_2$ and *n*-hexane (all in analytical grade) were supplied by Petr Lukes, the Czech Republic.

Heater plate Schott Ceran 93020 (Germany), magnetic stirrer with temperature control Ika ETS-D4 fuzzy (Germany), drier WTB Binder E/B 28 (Germany), incubator WTC Binder B53 (Germany), electronic balances KERN 770/GS/GJ and 440-47 (Germany), rotary vacuum evaporator Laborota 4000 (Heidolph Instruments, Germany), pH-meter Picollo HI 1295 (Germany), mineralization apparatus Hach Digesdahl (USA), muffle furnace Nabertherm L 9/S 27 (Germany), Parnas-Wagner distillation apparatus, Soxhlet extraction apparatus, filter paper Filpap KA-1 of 9 cm diameter (the Czech Republic).

**Degreasing poultry feathers:** Dry feathers were mixed with water (preheated to 40 ± 2 ºC) in ratio 1:75. Lipolytic enzyme Greasex 50 L was added in a dose of 1.7 % (related to weighed-in dry feathers) and the contents were stirred 5 min. Level of mixture pH was then adjusted to 9.0 ± 0.2 by adding 1 % NaOH solution, the mix was then incubated 24 h at 40 ± 0.5 ºC; during the first 6 h of incubation contents were shortly stirred in 1 h intervals. The mixture was then filtered through a sieve and degreased feathers were washed with a stream of fresh water (*ca.* 30 ºC warm) and dried at 103 ± 2 ºC to constant weight.

**Planning and evaluating feather breakdown test:** It was decided to process feathers into keratin hydrolyzate through 2-stage alkaline-enzymatic hydrolysis. In the first stage, feathers were incubated 24 h in alkaline environment (0.1 or 0.3 % KOH water solution) at 70 ºC. Feathers were mixed with KOH solutions in ratio 1:50. In the 2nd stage, after lowering pH to 9.0 ± 0.1, the swelled and partly degraded feathers were hydrolyzed with added proteolytic enzyme (Polarzyme 12 T). In order to examine influence of studied factors during hydrolysis (temperature, time and enzyme dose) on hydrolysis efficiency (expressed in our case by amount of degraded feathers in %), experiments were planned according to method of factor tests. Factor tests $2^3$ were employed (3 studied factors on two levels - minimal and maximal) with two repetitions in the middle of test. Examined factors were: factor A - time of hydrolysis 2nd stage: bottom limit 4 h, upper limit 8 h; factor B - dose of enzyme (related to weighed dry feathers, w/w): bottom limit 1 %, upper limit 5 %; factor C - temperature of hydrolysis 2nd stage: bottom limit 30 ± 0.2 ºC, upper limit 50 ± 0.2 ºC. Data evaluation ran on statistical program STATGRAPHICS (Statistical Graphics System-Version: 6.0. Manugistic, Inc. and Statistical Graphics Co., USA, 1992). Survey of factor tests organization including results is summarized in Table-1.

### General procedure

**Alkaline-enzymatic hydrolysis of poultry feathers:** Two-stage alkaline-enzymatic hydrolysis of degreased and dried (at 103 ºC) poultry feathers were carried out in accord with the working procedure as follows. Hydrolysis 1st stage: Feathers were weighed (2 g) into an Erlenmeyer flask and 100 mL preheated (70 ± 1 ºC) water KOH solution (0.1 or 0.3 %) was added. The flask was stoppered and its contents were vigorously shaken for 30 sec. It was then put in an incubator with temperature set at 70 ± 0.2 ºC and contents were incubated 24 h. During first 8 h of incubation, contents were shortly (*ca.* 30 sec) shaken in 1 h intervals; care was always taken that all feathers after shaking were wetted with KOH solution and did not remain on flask walls. On finishing first stage of hydrolysis,

TABLE-1
RESULTS OF DEGRADING POULTRY FEATHERS THROUGH 2-STAGE ALKALINE-ENZYMATIC HYDROLYSIS IN ENVIRONMENT OF 0.1 or 0.3 % KOH

| Run | Factors under study | | | Degraded feathers (%)[a] | |
|---|---|---|---|---|---|
| | Factor A: Time of hydrolysis 2nd stage (h) | Factor B: Dose of enzyme (%) | Factor C: Temperature of hydrolysis 2nd stage (ºC) | Hydrolysis in environment of 0.1 % KOH | Hydrolysis in environment of 0.3 % KOH |
| 1 | 4 | 1 | 30 | 12.67 | 71.40 |
| 2 | 4 | 1 | 50 | 14.61 | 76.86 |
| 3 | 4 | 5 | 30 | 14.14 | 77.72 |
| 4 | 4 | 5 | 50 | 15.30 | 84.57 |
| 5 | 6 | 3 | 40 | 16.15 | 76.36 |
| 6 | 6 | 3 | 40 | 16.03 | 76.48 |
| 7 | 8 | 1 | 30 | 16.68 | 75.67 |
| 8 | 8 | 1 | 50 | 18.63 | 83.77 |
| 9 | 8 | 5 | 30 | 17.91 | 82.29 |
| 10 | 8 | 5 | 50 | 20.11 | 88.71 |

[a]Related to degreased and dried (at 103 ºC) feathers.

mixture pH was adjusted to level 9.0 ± 0.1 with added 0.15 mL 10 % $H_3PO_4$ (mixture with 0.1 % KOH) or 0.25 mL 10 % $H_3PO_4$ (mixture with 0.3 % KOH). Hydrolysis 2nd stage: After adjusting pH, temperature in flask was adapted to the level according to plan of experiment for factor C. Enzyme (factor B) was added with pipette-amounts were 1 mL enzyme pipetted from stock solution (corresponding to 1 % dose of enzyme per weighed dried feathers-bottom limit) or 5 mL (corresponds to 5 % dose of enzyme per weighed dried feathers-upper limit). A small cylindrical stirring element (8 mm × 40 mm) was placed in the flask and the contents were stirred at 600 rpm on a magnetic stirrer at planned temperature (factor C) for the time as planned (factor A). After finishing 2nd stage of hydrolysis, undegraded fraction of feathers was separated from keratin hydrolyzate by filtering through low-density filter paper (Filpap KA-1) on a Buchner funnel with slight under-pressure; it was then additionally washed with 30 mL distilled water (washing water was added to keratin hydrolyzate). After drying the non-degraded feather fraction in a drier at 103 ± 2 ºC to constant weight and cooling in a dessicator (filled with dried silica gel), the amount of decomposed feathers was determined through gravimetry. After filtration, keratin hydrolyzate was heated to boiling point and boiled for 10 min to inactivate enzyme.

## RESULTS AND DISCUSSION

**Breakdown of Feathers:** Results of poultry feathers broken down by 2-stage alkaline-enzymatic hydrolysis in an environment of 0.1 or 0.3 % KOH are given in Table-1.

Amount of degraded poultry feathers (y) monitored by a 3-factor test is described by following nonlinear equation:

$$y = k + x_1A + x_2B + x_3C + x_{12}AB + x_{13}AC + x_{23}BC$$

A, B, C-studied factors in hydrolysis (A = time of hydrolysis 2nd stage, B = dose of enzyme, C = temperature of hydrolysis 2nd stage); AB, AC, BC - interaction of factors; k, $x_1$, $x_2$, $x_3$, $x_{12}$, $x_{13}$, $x_{23}$ - regression coefficients.

In breakdown of feathers in an environment of 0.1 % KOH the regression equation has form as follows: y = 6.943000 + 0.724062A + 0.33375B + 0.0611875C + 0.0171875AB + 0.0065625AC - 0.0033125BC; correlation factor: $R^2$ = 0.995857.

In breakdown of feathers in an environment of 0.3 % KOH the regression equation has form as follows: y = 55.41930 + 0.922187A + 2.135B + 0.257938C - 0.0771875AB + 0.0138125AC - 0.001812BC; correlation factor: $R^2$ = 0.905886.

Statistical significance of particular factors for monitored quantities was evaluated by standard procedure applying the Fisher test of significance[31]. Results of evaluation are listed in Table-2.

In case chicken feathers are hydrolyzed in an environment of 0.1 % KOH, all 3 factors under study exert a statistically significant influence on course of hydrolysis. The most important factor is time of hydrolysis second stage (factor A) followed by temperature of hydrolysis second stage (factor C) and dose of enzyme (factor B) (Table-2). Graphical presentation indicating influence of temperature and time of hydrolysis second stage at two selected additions of enzyme on amount of degraded feathers are shown in Figs. 1 and 2. Graphs unambiguously indicate positive influence of prolonged hydrolysis duration and elevated temperatures on break down efficiency. In the case of a 5 % addition of enzyme, *ca.* 20 % feathers were degraded in 8 h of hydrolysis at 50 ºC (Fig. 2). Nevertheless, quantity of decomposed feathers at upper limits of studied factors is quite low; further prolonging time of hydrolysis, increasing temperature and increasing addition of enzyme is economically and technologically unacceptable.

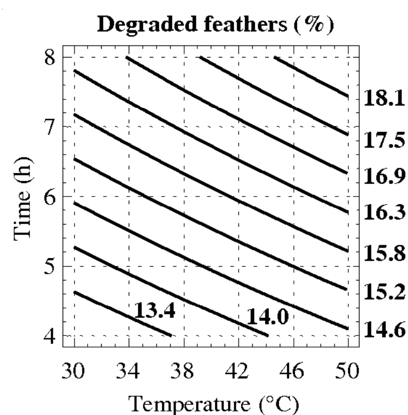



Fig. 1. Influence of temperature and time of hydrolysis second stage on amount of degraded feathers, with 1 % addition of enzyme in environment of 0.1 % KOH

TABLE-2
RESULTS OF ANALYSIS OF SCATTER AND OF FISCHER TEST OF STATISTICAL SIGNIFICANCE OF EXAMINED FACTORS ON AMOUNT OF DEGRADED FEATHERS

| Factors under study | Hydrolysis of feathers in environment of | | | |
|---|---|---|---|---|
| | 0.1 % KOH | | 0.3 % KOH | |
| | Sum of squares | F-ratio | Sum of squares | F-ratio |
| A: time of hydrolysis 2nd stage | 34.4865 | 562.17[a] | 49.4515 | 6.41 |
| B: dose of enzyme | 2.9646 | 48.33[a] | 81.8560 | 10.62[a] |
| C: temperature of hydrolysis 2nd stage | 6.5703 | 107.10[a] | 89.9811 | 11.67[a] |
| Interaction AB | 0.0378 | 0.62 | 0.7626 | 0.10 |
| AC | 0.1378 | 2.25 | 0.6105 | 0.08 |
| BC | 0.0351 | 0.57 | 0.0105 | 0.00 |
| Total error | 0.1840 | – | 23.1337 | – |
| Total error (corr.) | 44.4161 | – | 245.8059 | – |

[a]Statistically significant factor; $F_{crit}^{95\%}{}_{(1;3)} = 10.13$

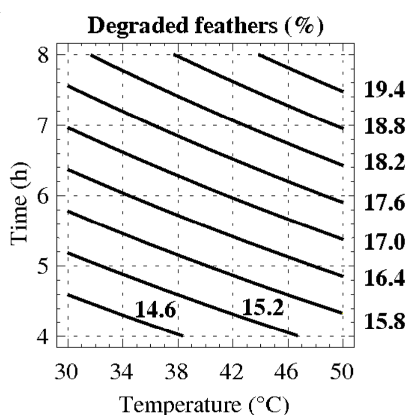



Fig. 2. Influence of temperature and time of hydrolysis second stage on amount of degraded feathers, with 5 % addition of enzyme in environment of 0.1 % KOH

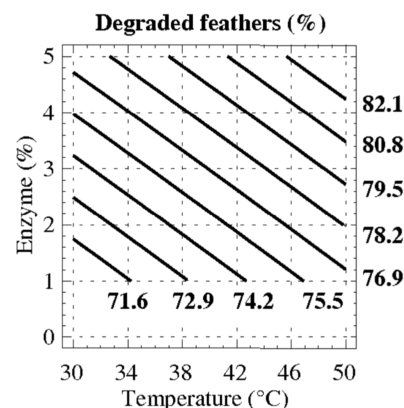



Fig. 3. Influence of temperature of hydrolysis second stage and of enzyme addition on amount of degraded feathers after 4 h of hydrolysis second stage in environment of 0.3 % KOH

Efficiency of feather breakdown in an environment of 0.3 % KOH is quite different, ranging from 71.4 % (bottom limits of factors under study) up to 88.7 % (upper limits of factors under study) (Table-1). From analyzed results of the Fisher test it follows that statistically significant factors are temperature of hydrolysis second stage (factor C) and dose of enzyme (factor B) (Table-2). Contour graphs representing influence of hydrolysis second stage and of dose of enzyme after hydrolysis second stage lasting 4 and 8 h, on amount of degraded feathers are shown in Figs. 3 and 4. It is obvious in Fig. 3 that already in 4 h of enzymatic hydrolysis, 71.6-82.1 % feathers may be broken down depending on temperature (x-axis) and dose of enzyme (y-axis). Prolonging duration of enzymatic hydrolysis to 8 h (Fig. 4) brings about an increase up to 76.6-87.1 % in quantity of decomposed feathers. Feather hydrolysis efficiency in an environment of 0.3 % KOH under same conditions of hydrolysis (time, temperature and dose of enzyme) is *ca.* 4.0-5.5 times greater than in an environment of 0.1 % KOH.

**Characteristics of keratin hydrolyzate:** For characterizing keratin hydrolyzate (KH) in the solid state, film was prepared in such manner that keratin hydrolyzate solution was thickened on a rotating vacuum drier to a 4 % solution at a temperature of 80 ºC. Twenty five mL of this solution was cast onto a silicon plate measuring 270 mm × 210 mm × 1.5 mm, this was placed for 48 h in an air-circulation drier with temperature set at 35 ± 1 ºC. After evaporation of solvent (aqueous phase) the film

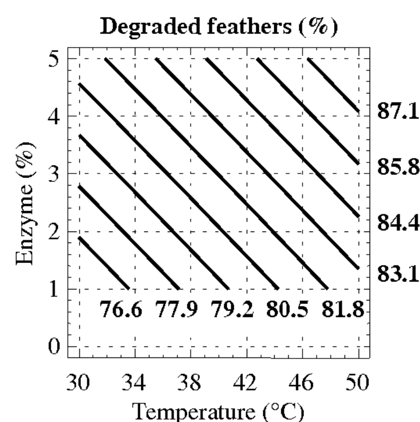



Fig. 4. Influence of temperature of hydrolysis second stage and of enzyme addition on amount of degraded feathers after 8 h of hydrolysis second stage in environment of 0.3 % KOH

was separated from silicon plate. Obtained keratin hydrolyzate film was ground in grinding mortar to powder and that was kept in a desiccator (filled with dried silica gel). Composition of keratin hydrolyzates produced through alkaline-enzymatic hydrolysis in an environment of 0.1 and 0.3 % KOH at maximal levels of factors under study (8 h time of hydrolysis second stage at 50 ºC with 5 % dose of enzyme) is shown in Table-3. For comparing thermal properties of these two keratin hydrolyzates with raw feathers, a determination also performed was thermogravimetric analysis on instrument TGA Q500 (TA

TABLE-3
COMPOSITION OF KERATIN HYDROLYZATES

| Parameter | Hydrolysis in environment of | |
|---|---|---|
| | 0.1 % KOH | 0.3 % KOH |
| Total Kjeldahl nitrogen (%)[a] | 10.4 | 12.3 |
| Primary $-NH_2$ groups (mmol $g^{-1}$)[a] | 0.36 | 0.37 |
| Inorganic solids (%)[a] | 21.3 | 14.0 |
| Sulfur (%)[a] | 2.86 | 1.75 |

[a]Dry matter.

Instruments, USA). Weighed-in quantity of raw feathers was 2 mg, with keratin hydrolyzates it was 5 mg. Measurements were performed in open Al dishes in temperature interval 20-400 ºC, dT/dt = 10 ºC $min^{-1}$ in protective nitrogen atmosphere (150 mL $min^{-1}$). TG curves are shown summarized in Fig. 5. Raw feathers exhibit first more significant drop in weight at 100 ºC, which is related to evaporation of free moisture (6 % loss). Powdery keratin hydrolyzates have 3.2 % moisture content (hydrolyzate prepared in an environment of 0.1 % KOH) or 5.2 % (hydrolyzate prepared in an environment of 0.3 % KOH). Samples of KH show a visible drop in weight with continued heating, the recorded drop in weight was 9.2 or 9.9 % at 165 ºC; raw feathers displayed essentially no decrease in weight (6.4 % loss) at this temperature. While a 9.2 % drop in weight was recorded with raw feathers at 225 ºC, the decrease in weight with keratin hydrolyzates was 16.7 or 20.8 %. At higher temperatures (275 ºC) the beginning of breakdown is already visible-the loss in weight recorded with keratin hydrolyzates was 26.8 or 31.5 %; loss in weight with raw feathers 27.1 %. At high temperatures (350 ºC) marked degradation of organic material is already quite apparent 42 to 48 % drop in weight with keratin hydrolyzates and 57.2 % drop in weight with raw feathers.

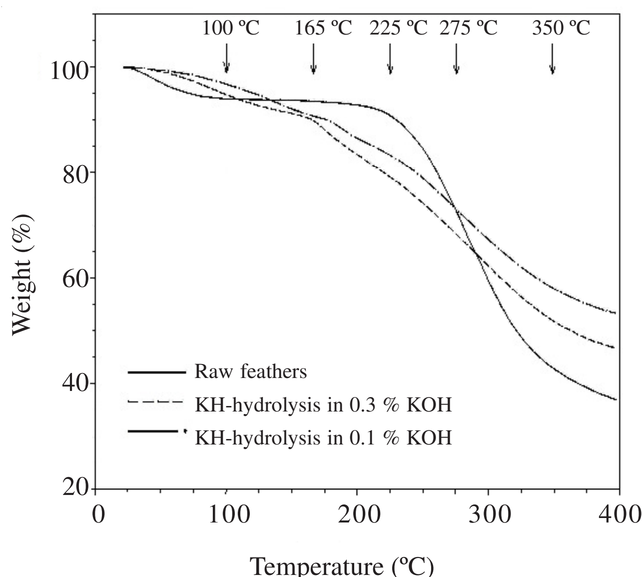



Fig. 5. TG curves of raw feathers and keratin hydrolysates prepared through hydrolysis in environment of 0.1 and 0.3 % KOH

302 **Conclusion**

303 Chicken feathers were processed through hydrolysis in 304 two stages. Feathers were incubated in the first stage in an environment of 0.1 % or 0.3 % aqueous solution of KOH for 24 h at a temperature of 70 ºC; after adapting pH (≈ 9) in the second stage, they were degraded into keratin hydrolyzate through action of proteolytic enzyme Polarzyme 12 T (1-5 %, w/w) at 30-50 ºC in 4-8 h. Efficiency of feather hydrolysis in an environment of 0.1 % KOH is quite low-second stage of hydrolysis at 50 ºC in 5 h with 5 % addition of enzyme (w/w) broke down *ca.* 20 % feathers. In environment of 0.3 % KOH the hydrolysis efficiency is more than fourfold. At bottom limits of studied factors in second stage of hydrolysis (4 h at 30 ºC and 1 % addition of enzyme) feather degradation was 71.4 % feathers; at upper limits (8 h at 50 ºC and 5 % addition of enzyme) quantity of degraded feathers was 88.7 %. Hydrolysis in an environment of 0.3 % KOH has advantage of higher hydrolysis efficiency and also superior quality of prepared keratin hydrolyzate containing *ca.* 20 % more nitrogen and *ca.* 30 % less ash substances than hydrolyzate prepared in an environment of 0.1 % KOH.

Test results also confirmed that applying proteolytic enzyme Polarzyme 12 T (Novozymes A/S Bagsvaerd, Denmark) produced efficiency of chicken feather breakdown similar to that of formerly employed Savinase Ultra 16 L of same manufacturer. Hydrolysis in an environment of 0.3 % KOH at maximal levels of studied factors (8 h in second stage of hydrolysis at 50 ºC and 5 % dose of enzyme) effected 88.7 % feather breakdown when applying enzyme Polarzyme 12 T; with earlier used enzyme Savinase Ultra 16 L the amount attained 90.8 % degraded feathers in the same environment after 8 hours of hydrolysis in same environment with 5 % dose of enzyme, but at higher applied temperature (70 ºC).

Keratin hydrolyzate may be utilized, for example, for preparing (micro) capsules in the chemical industry to preserve longer-duration activity of encapsulated substances (colouring agents, stabilizers, catalysts, aromas) and to ensure their controlled release.

## ACKNOWLEDGEMENTS

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