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Biocomposites with a Content of Keratin from Chicken Feathers

Abstract
The market's demand for fibrous materials with increased moisture retention is principally connected with the essential development of sanitary and cosmetic products. Keratin, which is included in feathers, appears to be an original raw material which enables fibrous composite materials of this kind to be manufactured. The aim of our investigation was to obtain different keratin forms from feathers, to identify and determine these forms, and to indicate new application directions connected with fibres and fibrous products. An optimum extraction method for obtaining keratin from chicken feathers was developed, and an attempt was made to obtain stable keratin solutions with the addition of other biopolymers, such as cellulose and alginate.

Key words: *keratin, feathers, fibrous biocomposites, sorption, keratin solutions, cellulose, alginate.*

Introduction

Recently, researchers and manufacturers have been searching for new directions for applying polymers which up to the present have not been well known. Keratin from chicken feathers is a by-product which is available in great amounts, and which is only used in a small degree. The amount of this waste is continuously increasing, in connection with the increase in fowl meat production.

The keratin included in chicken feathers is a very inconvenient and troublesome waste product of the poultry-farming industry, and therefore it is presently the object of intensive investigations in many research centres. Many publications and patents proposing applications for this biopolymer have been issued as a result of these research works. Applications of keratin preparations in the cosmetic industry are the best known and described in literature. However, opportunities to use this interesting protein in other fields have arisen, for example as component of various kinds of composites [1 - 5], as a component of biodegradable nonwovens [6 - 8], and in biotechnology [9].

Considering the hydrophilic properties of keratin, it would be appropriate to use it for manufacturing fibres with increased sorption features, which would in turn be useful for producing textile products dedicated to sanitary & medical applications, and as a technical sorption material. Such applications of keratin from chicken feathers are undoubtedly novel directions of use.

Keratin is insoluble in water, weak acids and bases, as well as in organic solvents. The amino-acid content of keratin is characterised by a high cystine content (and at the same time sulphur), which may change within 2% wt and 18% wt, a significant amount of hydroxyamino-acids, especially serine (about 15% wt), and a lack of hydroxyproline and hydroxylisine, among other substances [10, 11]. The chemical activity of keratin is connected in a significant degree to the cystine content. The disulphide bonds which is formed between two cysteine molecules is responsible for the high strength of keratin and its resistance against the action of proteolitic enzymes. On the other hand, keratin is very reactive, as cystine can easily be reduced, oxidised, and hydrolysed [12 - 15]. In order to precisely determine the possible future applications of keratin, it is necessary to learn in detail the structure and the potential possibilities of this valuable protein.

The aim and scope of the investigation

The above-mentioned reasons formed the outlines defining the aims of the research work which we undertook. We provided and carried out tests estimating sorption properties and evaluating the particles' dimensions, as well as preliminary investigations into preparing spinning solutions of keratin with alginates and biomodified cellulose. The aim of our research work was to obtain various forms of keratin from feathers, to identify and determine these forms, and to define new directions for applications. In particular, the elaboration of an optimum method for extracting keratin from chicken feathers and attempts to obtain keratin solutions with a content of other biopolymers, such as cellulose or alginates, were included in the scope of our research work.

Materials and methods

Materials

Raw material

The raw material for obtaining keratin came from chicken feathers which were characterised by the following contents:
- a sulphur content of 2.9%,
- a nitrogen content of 15.5%, and
- an ash content of about 1%.

The chemical agents used for obtaining the keratin were of analytical purity.

Methods

Preliminary processing of the chicken feathers

Before dissolution, the feathers were washed many times with hot water with detergent addition, dried, filtered, and again washed with ethylene alcohol (Trademark: 'ETOH Antibacterial 96%'), and dried. After drying, the feathers were cut into short segments or milled.

Solubilisation of keratin in alkali medium

The cleaned, dried, and cut feathers were dissolved in an aqueous solution of 5% wt NaOH and 0.1 M solution of Na_2S. The process was conducted under dynamic conditions, at a temperature of 40 °C over 2 hours. After the process was finalised, the keratin solution was filtered in order to separate the insoluble parts of feathers, and next submitted to dialysis. The dialysis was conducted with the use of a 76 × 49 mm cellulose dialysis

sleeve, made by Sigma-Aldrich. The dialysis was performed at environmental temperature over 48 hours.

Extraction of keratin

After the dialysis, 2 N hydrochloric acid was added to the keratin solution in order to precipitate the keratin at pH 4.2. The precipitated keratin was centrifuged, washed several times with distilled water in order to obtain neutral pH, and dried by lyophilisation. In order to obtain a micro-spherical keratin form, after dialysis the solution was directly transmitted to spatter-drying. Drying was carried out over some seconds at different temperatures selected within the ranges of 140 °C to 160 °C at the inlet, and within 85 °C to 90 °C at the outlet.

Lyophilisation of keratin

The lyophilisation was carried out over 25 hours with the use of an Alpha 1-4 type lyophilisator from Christ Co., at an initial plate temperature of -20 °C and a final temperature of 10 °C, whereas the preparation temperature was within the range of 2 °C to 6 °C.

Chemical modification of keratin

Monochloroacetic acid was used for keratin modification [4]. Various amounts of monochloroacetic acid (1, 2, or 4g depending on the variant performed) were added to alkali solutions of keratin at environmental temperature over 1 hour. The keratin modified was precipitated with 2 N HCl from these solutions. After centrifugation and being carefully washed, the keratin sediment was subjected to lyophilisation.

Assessing the water retention value (WRV)

The water retention value was assessed in accordance with the standard method [16].

Assessing the sorption coefficient

The sorption coefficient was in accordance with the standard method [17].

Testing the moisture absorption

The moisture absorption was determined on keratin samples dried to a constant mass preliminary with the use of an exsiccator at a relative humidity of 65% (NH_4NO_3) and at an environmental temperature of 20 - 21 °C. The moisture sorption was monitored by assessing the sample mass as a function of time. After stabilising the sample mass at a constant level, which means at full saturation by moisture under the given conditions, the keratin samples were placed into an exsiccator with a relative humidity of 93 °C (KNO_3) and for the second time the sample mass changes were estimated as a function of time. After stabilisation of the sample mass, the samples were repeatedly placed into the exsiccator of 65% RH in order to estimate the moisture desorption of the samples tested.

Chromatographic investigations

Chromatographic system

For the gel permeation chromatography (GPC) analysis, we used a module HP1050 liquid chromatograph from Hewlett Packard equipped with the following devices:

- a Viscotek DG 700 four-channel vacuum degasifier,
- a HP 1050 isocratic pump from Hewlett Packard,
- a HP 1047 refractometric detector from Hewlett Packard, and
- a system of appropriate columns.

The PL Caliber™ GPC/SEC software program from Polymer Laboratories Ltd was used.

Preparing keratin solutions

A keratin sample of 5 mg was placed in a graduated flask of 10 cm^3 volume, 7 cm^3 of the solvent (0.05 mol/dcm^3 Tris + 0.02% NaN_3 + HCl) was added, and then set for 16 hours for dissolution. Next, the keratin solution was mixed by shaking for about 30 minutes, and added the solvent to the volume of 10 cm^{-3}. After mixing and filtration (0.45 μm, Milex), clear keratin solution were obtained, suitable for the GPC analysis.

Chromatographic parameters of keratin analysis

- Columns: 1 × TSKgel PW_{XL} guard (7.8 mm × 4 cm from TosoHaas, 2 × TSKgel GM PW_{XL} guard (7.8 mm × 4 cm from TosoHaas,
- Eluent: 0.05 mol/dcm^3 Tris-HCL (pH 8.5) + 0.02% NaN_3,
- Column temperature: 30 °C,
- Flow speed of eluent: 0.5 cm^3/min,
- Volume of sample: 100 μl,
- Calibration: set of standard proteins within the range of molecular weights from 17 kDa to 158 kDa and polydispersion of Mw/Mn ≤ 1.2 (BioRad).

Determining the content of sulfhydril groups in keratin

The method applied uses the spectrophotometric technique for visible radiation [18]. The basis of this measurement is the colour reaction with the Ellman reagent (DTNB), i.e. 5,5'-dithiobis (2-nitro-benzoic acid). Cysteine was used as the standard substance. The calibration curve was elaborated for four standards with concentrations from 3.3 to 13.2 mmol/l. The measurements were carried out at a wavelength of 485 nm in a cell of 1 cm thickness, using the Ellman reagent as a carrier.

Determining method

The cell was filled with 0.1 cm^3 of the tested keratin solution and 2.4 cm^3 of the Ellman reagent. Absorption measurements were carried out after 10 minutes, using the Ellman reagent as the blank test; the content of the sulfhydril groups was measured in mmol/l.

Determining the sulphur and nitrogen content

The nitrogen content was determined by the Kjejdahl method, whereas the sulphur content was assessed by the Sheniger standard method [19, 20].

Estimating the keratin particle sizes

The keratin particle sizes were estimated with scanning electron microscopy (SEM). Sample photos were taken by a Quanta 200 scanning electron microscope made by FEI, at magnifications of 2000× and 5000×. The sample tested was spread on the table and glued with carbon glue. The keratin preparation structure was tested under high vacuum in natural state, without sputtering a gold layer on the sample. We estimated the size of the particles which the keratin preparations obtained were composed of.

Microscope analysis of the solutions

The solutions containing keratin, as well as the cellulose and alginate solutions, were evaluated with the use of a Biolar-type polarisation microscope made by ZPO, Warsaw. The images were recorded with a computer analyser made by IMAL Co.

Estimating keratin properties: results and discussion

It is generally known that the keratin included in feathers is resistant to the action of polar solvents thanks to the high content of disulphide bonds and the great amount of hydrophobic amino-acids. Therefore, keratin is a very difficult subject for analytical research.

As the result of the process of extracting keratin from chicken feathers with the

use of NaOH and Na_2S, solutions are obtained from which keratin is isolated by dialysis. Keratin may be precipitated by hydrochloric acid at pH ≈ 4.5 and subjected to lyophilisation, or the keratin solution after the dialysis can be directed to spatter-drying. In addition, keratin may be modified by monochloracetic acid. We applied all these methods, and the keratin forms obtained are marked as shown in Tables 1 and 2.

Basic properties of the keratin preparations

The properties of non-modified and modified keratin are presented in Table 3. The keratins obtained have the form of white or beige powder depending on the extracting agent (NaOH or Na_2S). They are characterised by nitrogen content from 9.5 to 15.2%, and sulphur content from 1.70 to 2.37%. The dissolution process efficiency (the percentage value of extracted keratin per 100 g of feathers) was within the range of 30 – 40%.

Cystine, cysteine and small amounts of metionine are sulphuric amino-acids which are composed of proteins. Therefore the amount of sulphur in keratin is mainly decided by the sulphur of the disulphide cystine bonds (-SS-) as well as that originating in the free sulfhydryl groups (-SH-) of cysteine. The keratin can be extracted from the feathers by breaking the disulphide bonds in cystine, which results in the creation of sulfhydryl groups (-SH-) of cysteine. One investigation [18] has indicated that if all disulphide bonds break, the amount of cysteine equals 720 μmol/g of feathers. After extracting the feathers, we obtained a keratin solution with a sulfhydril group content of 360 μmol/g, which indicates that about 50% of the disulphide bonds were broken, and at the same time the native keratin was significantly structurally modified. To protect the cysteine remains before the secondary creation of intra- and intermolecular disulphide bonds between the molecules of the dissolved keratin, monochloroacetic acid was added to the solution of this protein. The keratin solutions were diluted 10 times before the measurements were made. The amounts of sulfhydril groups in the keratin solutions are measured in mmol/l. Dividing this result by the amount of feathers dissolved in 1 l of the solution, we obtain the result in μmol of cysteine per g of feathers. These results are presented in Table 4.

Table 1. *The keratin forms differentiated by processing; Remarks: * - modification by 1, 2, or 4 g of monochloroacetic acid per 1000 ml keratin solution; ** - additional processing: ∅ - solution after dialysis subjected to ultrasounds; ⊕ - processing in greatlaboratory scale; A – D: all dried by lyophilisation; R – all spatter-dried, differentiated by keratin concentration in the solution and by initial & final drying temperature, all presented in Table 2.*

Keratin form designation	Disintegrating	Dissolution by:	Drying by:	Modified *	Additional processing **
A	Cut	Na_2S	Lyoph.	-	-
B	Milled	Na_2S	Lyoph.	-	-
B/m1	Milled	Na_2S	Lyoph.	1 g/ 1000 ml	-
B/m2	Milled	Na_2S	Lyoph.	2 g/ 1000 ml	-
B/m4	Milled	Na_2S	Lyoph.	4 g/ 1000 ml	-
C	Milled	NaOH	Lyoph.	-	-
D	Cut	NaOH	Lyoph.	-	-
K I	Cut	Na_2S	Spatter	-	-
K II	Cut	Na_2S	Spatter	-	-
K III	Cut	Na_2S	Spatter	-	-
K IV	Cut	Na_2S	Spatter	-	-
K V	Cut	Na_2S	Spatter	-	∅
K VI	Cut	Na_2S	Spatter	-	∅
K VIII	Cut	Na_2S	Spatter	-	⊕

Table 2. *Spatter-drying conditions of keratin preparations.*

Keratin form designation	Keratin concentration in solution, % wt.	Drying temperature	
		Initial, °C	Final, °C
K I	1.0	85	147
K II	0.75	83	153
K III	0.5	83	153
K IV	0.5	85	128
K V	1.0	78	153
K VI	0.5	78	153
K VIII	1.0	85	147

Table 3. *Basic properties of keratin preparations.* ***Remark:*** *efficiency - percentage value of extracted keratin per 100 g feathers.*

Keratin form designation	Colour	Nitrogen content, %	Sulphur content, %	Humidity, %	Efficiency*, %
A	white	15.20	2.30	5.9	40
B	white	14.80	2.10	6.0	38
C	beige	9.53	2.10	6.1	40
D	beige	11.40	1.71	5.8	41
K I	white	15.23	2.30	4.2	30
K VIII	white	15.09	2.07	5.9	37
K IV	white	14.46	2.10	6.0	35
B/m1	white	14.79	2.37	5.9	35
B/m2	white	14.90	2.17	6.9	30
B/m4	white	14.07	2.07	6.2	32

Table 4*. Content of sulfhydril groups (SH) in keratin solutions; *) Amount of monochloroacetic acid in g/1,000 cm³ of keratin solution.*

Type of solution	Amount of monochloroacetic acid*, g	Amount of SH groups, μmol/g
keratin solution after filtration	0.0	360
solution of Bm/1	1.0	358
Solution of Bm/2	2.0	335
Solution of Bm/4	4.0	305

On the basis of the results obtained, we could state that adding monochloroacetic acid in an amount of 4 g/1000 cm³ causes a modification of the sulfhydril groups (SH) of the cystein remains at the level of 15% in relation to unmodified keratin.

Sorption properties

Considering the possibilities of applying keratin as an addition to products for increased moisture absorption, we carried out tests in order to estimation the usability of keratin for such applications.

Table 5 presents the sorption properties (water retention value – WRV, and sorption coefficient) of unmodified and modified keratins.

On the basis of the results obtained, it is clearly apparent that modification of the keratin structures and the type of drying employed influence the sorption features of this protein. The highest sorption parameters were obtained for spatter-dried keratins, and slightly lower for modified keratins. In the case of modified and lyophilised keratins, the water retention value and the sorption coefficient reached higher values in comparison to those keratin samples which were lyophilised but not modified.

Another method of estimating the sorption properties is to test the moisture absorption of the samples prepared. The sorption and de-sorption process was tested for selected keratin samples, and the dependencies of the moisture content as a function of time are presented in Figure 1.

The sorption process under the condition of 65% RH is not intensive, and a greater jump does not appear until the samples are placed in the exsiccator of 93% RH. The highest absorbing capacity of about 45% was observed for spatter-dried keratin, whereas the keratin preparations dried by lyophilisation are characterised by a significantly smaller absorbing capacity at the level of about 20%, not essentially more than the keratin which was sputter-dried under conditions of 65% RH. Lyophilised keratin after the de-sorption process bonds about 15% of moisture, whereas the spatter-dried only bonds about 20%. These keratin properties, especially those of spatter-dried keratin, indicate the possibilities of its application as an addition to increase the hygroscopic properties of different kinds of products, such as hygienic products.

Molecular weight tests of keratin by the GPC method

Keratin is a very difficult object to test, considering chromatographic analysis, as it is insoluble in typical solvents. Electrophoresis in a Polyacryloamide Gel (PAGE) is a standard method for determining the molecular weight of proteins. The method of High Performance Size Exclusion Chromatography / High Performance Gel Filtration Chromatography (HPSEC/HPGFC) is used significantly less often. The main reason for this is the difficulty in choosing an appropriate solvent which would fulfil the demands for eluents in the HPSEC/HPGFC method.

Investigation was carried out in order to select a composition of the keratin solvent which would be useful for HPSEC/HPGFC analysis. Finally the following composition content was elaborated: 0.5 mol/dcm^3 Tris-HCl (pH 8.5) + 0.02% NaN_3.

Some of the solutions obtained were characterised by a minimal opalisation, which means that a part of the sample (below 5%) is in a state of suspension and not a solution. Tests were carried out on five keratin samples. Table 6 listed the numerical results of the molecular characteristics of selected keratin samples, whereas in Figure 2 the differential molecular weight distribution dependencies of these samples are presented.

All the tested samples were differentiated by the kind of preparing keratin. The data presented in Table 6 and Figure 2 indicate essential differences in their molecular characteristics, which means that the method of obtaining the keratin preparations significantly influences their molecular weight distribution. Keratin obtained with the use of sodium sulphide and spatter-dried (K IV and K VIII) is degraded to a higher degree, but is more uniform in its molecular structure than all the other keratin samples. The polydispersion degree is at the level of 2.2 to 2.6. The keratin modified by monochloroacetic acid (B/m4) has a relatively high molecular weight, but is molecularly non-uniform (Mw/Mn = 5.6). From the data presented, it is clear that the drying temperature has the greatest influence on keratin degradation. Keratin preparations dried at temperatures from 85 °C to 147 °C (K VIII) are characterised by the lowest molecular weight.

Table 5. *WRV and sorption coefficient of unmodified and modified keratins.*

Type of solution	WRV, %	Sorption coefficient, %
A	105.4	130.5
D	65.1	87.5
K I	153.4	186.2
K VIII	155.5	188.5
B/m1	131.4	155.7
B/m2	138.5	160.0
B/m4	140.2	160.5

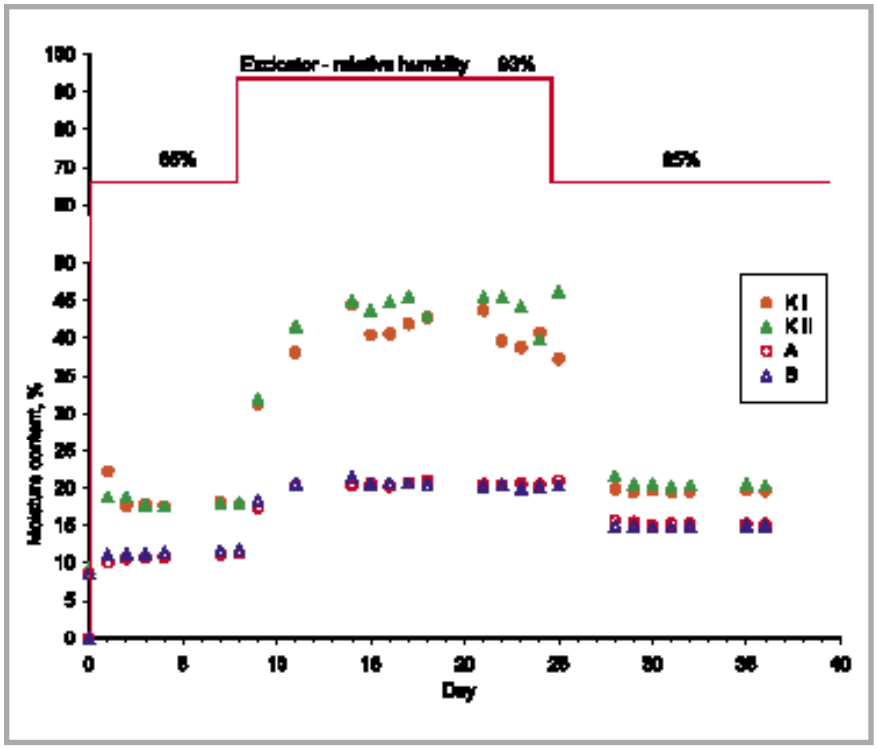

Figure 1. *Sorption and de-sorption of selected keratin preparations.*

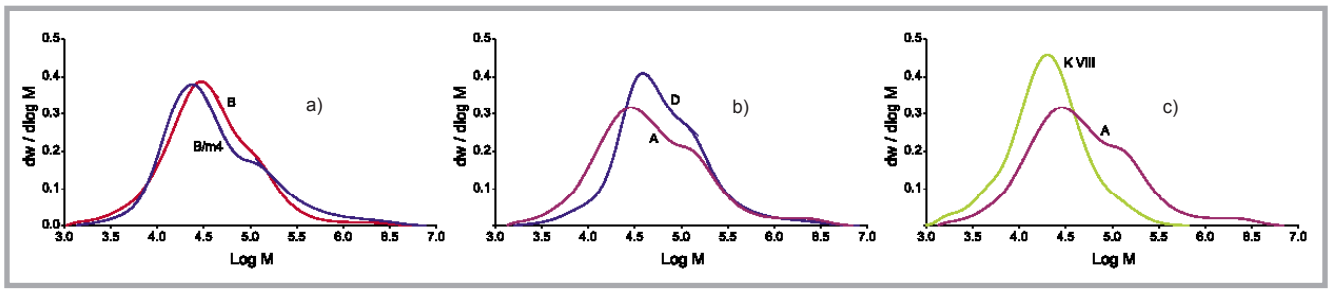

Figure 2. *Differential molecular weight distributions of selected keratin samples; a) comparison of unmodified and modified keratin (B and Bm4); b) comparison of keratin dissolved in Na_2S and NaOH (A and D); c) comparison of lyophilised and spatter-dried keratin (A and K VIII).*

Microscopic observations of keratin

The particle sizes of the keratin preparations obtained, for samples dried under different conditions according to Table 2, were measured with the use of a scanning electron microscope. The results are listed in Table 7.

On the basis of the results obtained, we stated that in all cases, irrespective of the drying conditions, the keratin preparations obtained are characterised by particle sizes below 20 μm. The

Table 6. *Numerical parameters of the molecular characteristic of keratin.*

Keratin form	Mn, kDa	Mw, kDa	Mw/Mn, (-)
K IV	12.5	27.3	2.2
K VIII	12.4	32.2	2.6
D	34.8	137.0	3.9
A	22.2	144.4	6.5
B	19.6	86.2	4.4
B/m4	22.4	130.3	5.8

average diameter values are within the range of 6.2 to 9.2 μm, at a maximum standard deviation of 4.64. The shape of the microspheres would significantly facilitate the introduction of keratin into the solutions of other polymers in order to manufacture biocomposites.

The estimation of the appearance of keratin preparations obtained from chicken feathers was also carried out with the use of SEM. The photos of keratin preparations and of a chicken feather are presented in Figures 3 and 4.

The SEM photos show a wet keratin preparation, the so-called 'never dry' form, spatter-dried and dried by lyophilisation, and modified by monochloroacetic acid. Essential differences in the particle structure are clearly visible. The modified (Figure 3.c) and spatter-dried keratin (Figure 4.d) show the best-developed surface and smaller particles . These keratin forms are also characterised by the best sorption properties. A decidedly more packed structure of the preparation is visible in the case of keratin which is non-modified and dried by lyophilisation (Figure 3.d). For better visualisation of these differences, the keratin preparations in Figure 3.b to 3.d are presented at higher magnitude that those in Figure 4.

Keratin solutions with biomodified cellulose and sodium alginate

Considering the hydrophilic properties of keratin extracted from feathers, we

Table 7*. Particle sizes of spatter-dried keratin preparations.*

Keratin form	Number of measurements	Minimum diameter, μm	Maximum diameter, μm	Average value, μm	Standard deviation, μm
K I	13	3.83	16.08	9.2	4.23
K II	16	2.88	13.,29	6.6	3.19
K III	24	3.26	19.45	6.4	4.64
K IV	33	1.68	15.33	6.2	3.32
K V	22	3.56	12.28	6.9	2.31
K VI	13	4.82	13.12	8.4	2.31
K VIII	17	4.78	13.04	8.3	3.18

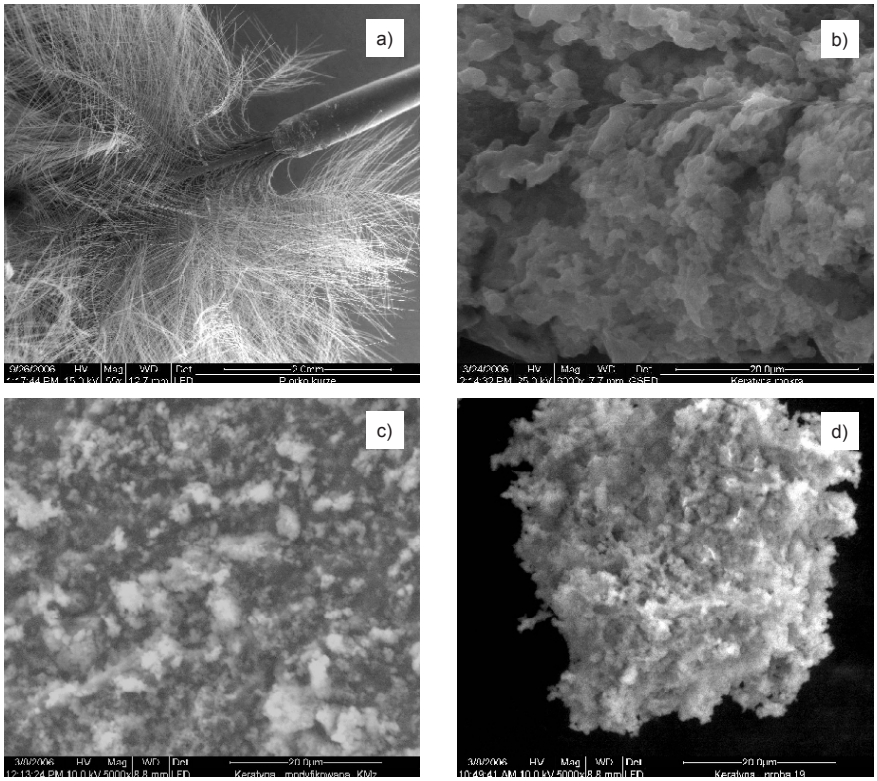

Figure 3. *SEM photos of a) chicken feather, b) wet keratin preparation, called 'never dry', c) keratin preparation modified after lyophilisation, d) keratin preparation non-modified after lyophilisation.*

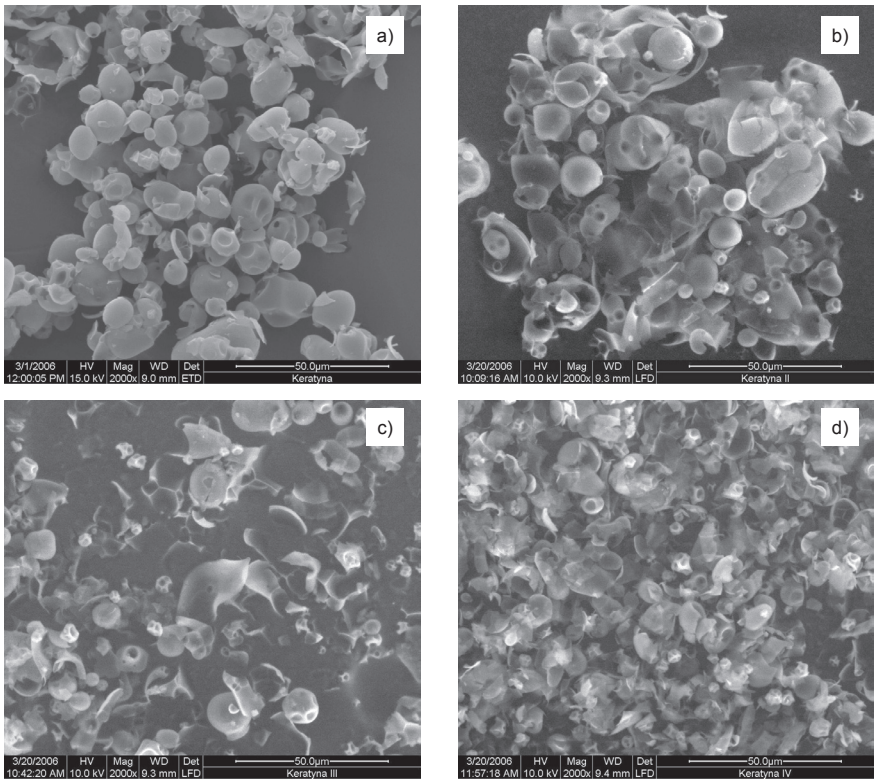

Figure 4. *SEM photos (magnification 2000×) of spatter-dried keratin preparations; a) K I, b) K II, c) K III, d) K IV.*

assume that within the scope of further investigations this biopolymer will be used to obtain composite fibrous materials with increased sorption properties. Our preliminary investigations indicated that alkali solutions of keratin from feathers are compatible with cellulose and alginate solutions. With the aim of preliminarily evaluating the quality of keratin solutions, as well as keratin-cellulose and keratin-alginate solutions, all of them were analysed by an optical microscope.

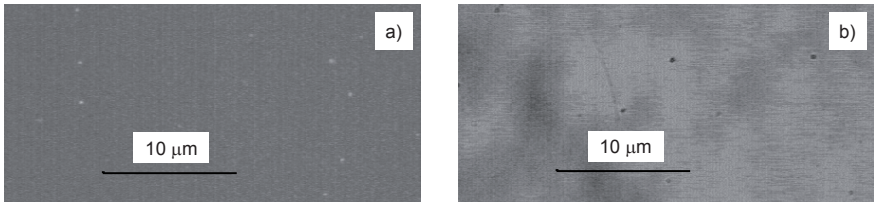

Figure 5. *Microscopic photos; a) solution of keratin after lyophylisation, b) solution of keratin after spatter drying.*

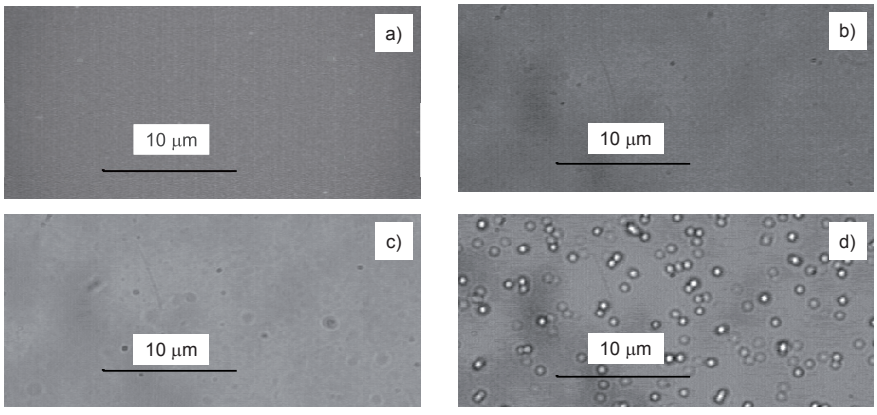

Figure 6. *Microscopic photos; a) cellulose solution, b) cellulose-keratin solution with keratin content below 12%, c) alginate solution, d) alginate-keratin solution with keratin content over 15%.*

Clear, aqueous solutions of sodium alginate and alkali solutions of biomodified cellulose [21] with properties typical of spinning solutions used for fibre spinning were prepared. Alkali keratin solutions with different concentration within the range from 5% to 20% were also prepared. The keratin solutions were mixed in different ratios with the polysaccharide spinning solutions. This stage of research was limited to microscopic observations of the solutions (Figures 5 and 6) and the preliminary estimation of their abilities to obtain fibrous forms from the two-component solutions.

The presence of non-solute particles was not stated by an optical microscope in the alkali solutions of keratin which had been lyophilised and that spatter-dried . Also in keratin-cellulose solutions with a concentration not exceeding 10-12%, such particles were not stated (see Figure 6.b). Above this concentration level, many particles of sizes from 2 µm to 3 µm could be seen in the solution. On the other hand, many particles are visible in keratin-alginate solutions with keratin content above 15% (Figure 6.d).

The preliminary tests of coagulation carried out with keratin-cellulose and keratin-alginate solutions were successful, and allowed us to obtain fibrous products. Further investigations into spinning test are planned.

Summary

- The method of drying the keratin preparations has an essential influence on their properties.
- Spatter-dried keratin preparations are characterised by better sorption properties than lyophilised keratins. The moisture absorption of spatter-dried keratin, of about 45%, is significantly higher than that of lyophilised keratin, which is equal to about 20%.
- Modification with monochloroacetic acid also influences the keratin sorption properties.
- As the result of spatter-drying, keratin preparates were obtained which were characterised by particle sizes below 20 µm. The average diameter values are within the range of 6.2 to 9.2 µm at a maximum standard deviation of 4.62.
- Solutions with the content of sulfhydril groups of 360 µmol/g were obtained after extracting the keratin from feathers, which indicates that

about 50% of the disulphide bonds were broken, and at the same the native keratin was significantly structurally modified.

- Spatter-dried keratin is characterised by the highest molecular uniformity, with a polydispersion coefficient at the level of 2.2 to 2.6, whereas the modified keratin has a relatively high molecular weight, but is mostly non-uniform in its molecular structure (Mw/Mn = 5.8).
- The preliminary tests with the preparation and coagulation of keratin-(sodium alginate) and keratin-(biomodified cellulose) spinning solutions which we carried out proved that our method for obtaining fibres from these solutions is very promising.

Conclusion

Further investigation into obtaining fibres and fibrous products from spinning solutions with keratin content should be carried out.

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