Characterization of Low-molecular Weight Collagen Hydrolysates prepared by Combination of Enzymatic and Acid Hydrolysis

by

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Abstract

Formic, phosphoric and nitric acids were used for hydrolysis of the commercially available hydrolysis product of chrome shavings Hykol-E. The aim of the work was to prepare low-molecular weight product (M_w < 5000 g.mol^{-1}) to be used as the plant bio-stimulator. Course of hydrolysis was monitored by following the changes of molecular weight (MW) and molecular weight distribution (MWD) determined by gel permeation chromatography (GPC). The study demonstrated that MW and MWD of hydrolysates are influenced by the choice of hydrolysing acid, its concentration as well as by the time of acid treatment. Using the combination of enzymatic and acid hydrolysis, it is possible to prepare products with the tailor-made, pre-defined molecular weight fitting the intended use.

Resumen

Los ácidos fórmico, fosfórico y nítrico se utilizaron para la hidrólisis del producto disponible comercialmente de virutas de cromo, Hykol-E. El objetivo del trabajo fue preparar un producto de bajo peso molecular (M_w < 5000 g.mol^{-1}) para ser utilizado como un bio-estimulador para plantas. El transcurso de la hidrólisis fue supervisado siguiendo los cambios en el peso molecular (MW) y la distribución del peso molecular (MWD), determinado por cromatografía de permeación de gel (GPC). El estudio demostró que el MW y la MWD de los hidrolizados se ven influidos por la elección del ácido utilizado en la hidrólisis, su concentración, así como por el tiempo del tratamiento. Utilizando una combinación de la hidrólisis enzimática y la ácida, es posible preparar productos hechos a la medida, definiendo previamente su peso molecular más adecuado al uso previsto.

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INTRODUCTION

During processing of raw hides in the tanning process, only of about 20% of the collagen material is utilized. The residual 80% is a "waste",[1] which is, if not tanned like a hide splits, used mainly for the manufacture of edible collagen casings, gelatine and various pharmaceutical preparations, for example for drug encapsulates. More complications are, however, met during processing of chromed leather shavings. Though a sufficient number of suitable laboratory-scale technologies have been developed capable of producing collagen hydrolysates with different quality, only a few of them are implemented in industry. As a hopeful upturn are hence considered the research activities of USDA, proving that the dose of proteolytic, relatively expensive enzyme used for hydrolysis can be significantly reduced and thus the compromise between product quality and price can be achieved.[2-6] The American experience with enzymatic hydrolysis were successfully exploited for the industrial application in Czech Republic, where a manufacturing plant with a daily processing capacity of 3 ton of chrome shaving has been built. Experience from the daily operation of the plant was published.[7] This cooperation also resulted in a common patent resting on the use of organic bases capable of producing high quality and low cost hydrolysates with low ash content. [8].

Successful development of the technology for processing of chrome shavings is only part of the problem. The other and often more complicated problem is to find applications for the isolated products – collagen hydrolysates. In order to find appropriate application for hydrolysates, it is necessary to manufacture them with a sufficiently high utility value and subsequently to find the application in the sector of the market where, due to their specific properties, competitive strength of hydrolysates is superior to existing products. In this respect, the use the collagen hydrolysates prepared from chrome shavings for manufacture of surfactants of N-acyled aminoacid type "Lamepone" seems to be promising.[9-12] Powder hydrolysates of chromed leather waste obtained by the process[7,8] are interesting because of their low chrome content (around 20 ppm) and inherently low ash value (in general approx. 3%), which are difficult to achieve with other methods used for hydrolysis of this raw material. Application of commercially available hydrolysates in surfactants is, however, complicated by their relatively high average molecular weight (in general from 15 000 to 20 000 $g.mol^{-1}$), leading to lower solubility (and hence lower surface activity) of the final products.

Another interesting application field of collagen hydrolysates can be found in agriculture. Besides the successful use of hydrolysates as the nitrogen containing organic fertilizers, their exploitation as biostimulators or modifiers for formulations used in the chemical protection of plants are under thorough investigation. Used as modifiers, hydrolysates can prolong life-time of agrochemicals and enable reduction of the active component concentration, while maintaining the sufficiently long life-time of the product In this way, the negative impact of the protective preparations on environment can be significantly reduced.[13] For this kind of applications, molecular weight and molecular weight distribution of hydrolysates play a key role.[14-17] One of the possible functions of biostimulators is to increase immunity of the plants.[15,16] It is suggested that the efficiency of a biostimulator is closely correlated to the diffusion velocity of biostimulator into the plant body and diffusion velocity depends, then, on the molecular weight.[17] Industrially performed enzymatic hydrolysis however provides hydrolysates having only the limited lower limit of molecular weights and for biostimulating purposes it is necessary molecular weight to be further reduced. This can be achieved by acid hydrolysis of collagen hydrolysates (manufactured by enzymatic hydrolysis) with strong acids.

The presented paper is dealing with description of conditions suitable for acid hydrolysis of collagen hydrolysates. The aim of the presented study was to reduce molecular weight of the samples and prepare low molecular weight hydrolysates, preferably with molecular weight lower than 5000 $g.mol^{-1}$, that can be used as biostimulators. Molecular weight change during hydrolysis can be followed by several analytical methods, for example by gel electrophoresis, which was successfully used when modification of hydrolysates with transglutaminase was studied.[18-20] In this paper, the course of hydrolysis was followed by Gel Permeation Chromatography (GPC) and molecular weight and molecular weight distribution of collagen hydrolysates were determined.

EXPERIMENTAL SECTION

Materials and methods

Starting material

Commercially available collagen hydrolysate Hykol-E supplied by Tanex s.r.o., Hradek nad Nisou, Czech Republic, was used in the study. Hykol-E is manufactured by alkaline hydrolysis (pH ~ 9) of chrome-tanned leather shavings in the presence of proteolytic enzyme Alcalase-DX-L. Hydrolysis is performed at 70° C for 3 to 6 hours. The raw hydrolysate is filtered and dried. Resulting product is yellowish, odourless with neutral pH. Typically, average molecular weight of the product varies from 15 000 to 20 000 $g.mol^{-1}$ dependent on the raw material used. The applied sample was in the liquid form (dry matter content 30 – 45%) and the ash content (mainly sodium sulphate) was 3 to 6 %. Due to low Cr^{III} content of 15 ppm and absence of Cr^{VI}, Hykol-E is suitable for use in the food and cosmetics industry.

Preparation of hydrolysates using acid hydrolysis

Low-molecular weight hydrolysates were prepared by acid hydrolysis. After preliminary testing, three acids – 85%

formic, 85% phosphoric and 65% nitric acids were used in the study. The use of phosphoric acid as hydrolysing agent and potassium hydroxide for neutralization afforded NPK stimulators. When nitric acid and potassium hydroxide for pH adjustment were employed, NK stimulators were formed. Finally, using formic acid leads usually to formation of N stimulator, as an excess formic acid having boiling point of about 100 °C can be removed by distillation. However, in our experiment, pH of the resulting hydrolysates was adjusted using potassium hydroxide so that NK stimulators were also obtained. Abbreviation N stands for nitrogen, P for phosphorus and K for potassium, the elements being introduced to hydrolysates from individual components used during hydrolysis.

For hydrolysis, 150 g of starting material Hykol-E was weighed and mixed with 30 g HCOOH and 21.3 g H_3PO_4, respectively. The mixture was kept boiling under reflux for 8 hours. Samples for analysis were withdrawn at regular, one-hour time intervals and analysed for their molecular weights and molecular weight distributions. The third sample series was prepared in slightly different way compared to previously described samples; 1000 g Hykol-E was mixed with 40 g HNO_3 and refluxed for 10 hours. The sampling was again performed at one-hour time intervals. The concentrations of all the three acids in the test were chosen with the aim to obtain hydrolysates with molecular weight lower than 5000 $g.mol^{-1}$.

Gel permeation chromatography

GPC analyses were performed using a PLGPC-50 *(Polymer Laboratories, Church Stretton, United Kingdom)* equipped with a PL differential refractometer (DRI) and on-line viscometer detectors (VIS). Analyses were performed with a column set consisting of two columns connected to series, one TSK GMPWXL column *(Tosoh Bioscience, Stuttgart, Germany)* and one Ultrahydrogel 250 column (*Waters, Milford MA*) at 30 °C with the mobile phase flow rate of 0.8 mL/min. Aqueous 0.1 M $NaNO_3$, 0.2 % NaN_3 and 15 % acetonitrile was used as the mobile phase. The columns were calibrated using narrow molecular weight polysaccharide pullulan standards (*Polymer Laboratories Ltd, Church Stretton, United Kingdom*) with molecular weights ranging from 180 to 788 000 (given by supplier). A 100 μL injection loop was used for all measurements. Universal calibration was applied for the determination of molecular weight from the DRI and the VIS signal. Data processing was performed with Cirrus GPC, Multi Detector Software *(Polymer Laboratories Ltd)*. For GPC analyses, hydrolysates were accurately weighed and dissolved for approximately 10 hours at room temperature in a mobile phase. The concentration ranged from 2.0 to 3.5 $mg.ml^{-1}$. Prior to measurements, the samples were visually controlled for presence of particles and/or foreign matter and filtered through a 0.45 μm Chromafil PP/PET filter.

Molecular weight and molecular weight distribution

Compared to discrete molecules which have well-defined molecular weights, polymers are composed of hundreds to thousand of chains of different molecular weights that result in characteristic molecular weight distribution (MWD). For natural polymers, it will depend on their source and method of isolation, for synthetic polymers on kinetics, conditions and method of preparation.[21] In order to describe MWD, moments or statistical averages of the distribution are calculated. In most cases, number average M_n and weight average M_w molecular weight are determined as the characteristics describing MWD. The magnitude of M_n is sensitive to the presence of low molecular weight species and on the other hand M_w indicates changes in high molecular weight component. The width of MWD can be characterised by polydispersity index, simply determined as ratio of M_w/M_n. Values M_w and M_n can be independently measured by physical methods such as osmometry, end group analysis (M_n) or light scattering or sedimentation (M_w) In addition, these molecular weight averages can be also statistically calculated from gel permeation chromatography measurements.[21]

Weight average molecular weight is defined as

$$\overline{M}_w = \frac{\sum (w_i \cdot M_i)}{\sum w_i} = \frac{\sum N_i \cdot M_i^2}{\sum N_i \cdot M_i} \qquad (1)$$

and number average molecular weight is defined as

$$\overline{M}_n = \frac{\sum w_i}{\sum N_i} = \frac{\sum N_i \cdot M_i}{\sum N_i} \text{ , where} \qquad (2)$$

w_i = is the weigh of i molecules with molecular weight M_i and N_i is the number of i-th molecules with molecular weight M_i.[21]

RESULTS AND DISCUSSION

Gel permeation chromatography is an analytical tool routinely used for characterisation of molecular weight distribution of synthetic and biological polymers. If equipped with a viscosity detector in addition to a concentration detector, GPC can be with advantage used for the absolute molecular weight determination. From the concentration and viscosity signal, limiting viscosity number [η] (LVN) of each eluting fraction can be determined. LVN is a measure of specific volume of the polymer in solution and it is correlated to the molecular weight (M) and the hydrodynamic volume (V_h) of the analysed polymer. In GPC, a certain V_h corresponds to a certain retention volume V_e. Thus, M is found from the measured [η] and a calibration curve of Log M[η] as a

TABLE I
Values of weight average molecular weight M_w and polydispersity index $P = M_w/M_n$ measured for samples prepared by hydrolysis with formic, nitric and phosphoric acid

Time of hydrolysis [h]	formic acid		phosphoric acid		nitric acid	
	M_w [g.mol^{-1}]	$P = \frac{M_w}{M_n}$	M_w [g.mol^{-1}]	$P = \frac{M_w}{M_n}$	M_w [g.mol^{-1}]	$P = \frac{M_w}{M_n}$
1	-	-	1440	2.0	3500	2.5
2	3900	3.9	1400	1.8	3400	2.8
4	3000	2.7	1130	1.6	3100	2.6
8	2200	2.8	1130	1.6	2800	2.2
Hykol-E *(Starting material)*						
Mw = 18400 g. mol $^{-1}$					P = 12.3	

function of V_e. Using GPC, both weight average molecular weight M_w and number average molecular weight M_n are obtained. Results from GPC analyses of prepared hydrolysates are given in Table I. For illustration, examples of numerical values M_w and polydispersity index $P = M_w/M_n$ for one, two, four and eight hours hydrolysis were chosen and tabulated. Table I contains also the corresponding values for starting material Hykol-E. Changes of M_w values as a function of hydrolysis time are, for all three acid in the test, compared in Figure 1.

The results show that one-hour treatment with formic acid caused reduction in molecular weight from 18400 to 3900 g.mol^{-1}. Prolonging the hydrolysis time to 8 hours, molecular weight further decreased to 2000 g.mol^{-1}. Together with molecular weight lowering, polydispersity index P decreased indicating thus narrowing of molecular weight distribution during hydrolysis. For non-treated sample a broad molecular weight distribution covering almost the entire calibration range is characteristic resulting in polydispersity index P = 12.3, however successive degradation caused by acid hydrolysis lowered P to value of 3.9. Comparing molecular weight of the samples hydrolysed 2 and 8 hours, respectively only a moderate decrease of M_w is observed. The most dramatic drop of molecular weight, hence, occurs within the first hour of hydrolysis. Shape of the distribution curves indicates that reduction of molecular weight takes place through a gradual splitting of high molecular weight fractions and formation of shorter chain units with a lower molecular weight. This is illustrated in Figure 2, where differential distribution curves of Hykol-E hydrolysed with formic acid for 2 and 8 hours, respectively are plotted. Reduction of the high-molecular weight tail of the distribution is notable.

Resulting molecular weight of hydrolysates was also found to be influenced by the acid concentration. The above reported results were obtained on the samples prepared using 30 g formic acid, related to 150 g of starting material. However, as an additional test, increased amount of 159 g formic acid was used for hydrolysis of the same amount of starting material. It was observed that the increased amount of formic acid caused more prominent molecular weight reduction and corresponding shift of the entire molecular weight distribution to a low-molecular weight region, when compared to the sample hydrolysed the same time with 30 g formic acid (Figure 3). Expressed in numerical values, weight average molecular weight of hydrolysates after six hours treatment with 30 g formic acid was 2600 g.mol^{-1} whereas when 159 g formic acid was used, M_w value dropped to 800 g.mol^{-1}.

Correspondingly to formic acid, phosphoric acid caused also hydrolysis of starting material resulting in decrease of molecular weight and narrowing molecular weight distribution. After two-hour hydrolysis, M_w decreased from starting 18400 to 1440 g.mol^{-1} and polydispersity index from 12.3 to 2.0. Additional decrease in molecular weight and polydispersity can be observed after 8 hours hydrolysis. Here M_w dropped to 1130 g.mol^{-1} and P to 1.6. The shape of distribution curves of phosphoric acid hydrolysates is different compared to distribution curves of the formic acid treated samples. In accord with numerical values, distribution curves reflect substantial shift of the entire distribution into a low-molecular weight region (Figure 4)

Also the third sample series where nitric acid was applied follows the trend already observed. After one hour, molecular weight decreased to 3500 g.mol^{-1} and after 8 hours M_w value further decreased to 2700 g.mol^{-1}. Also here, the most

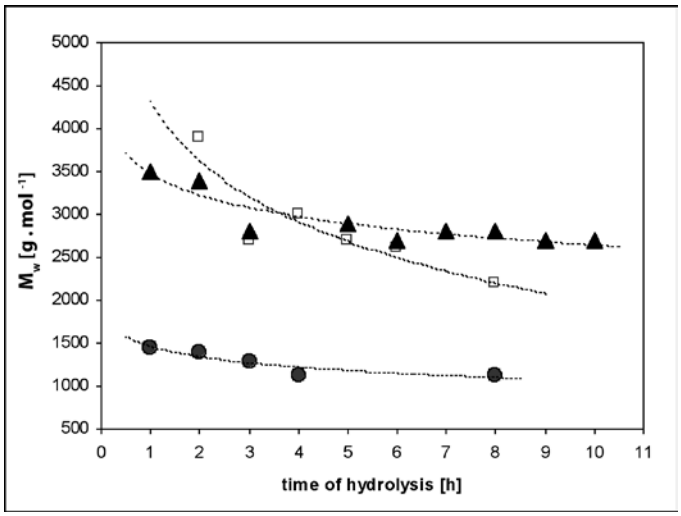

Figure 1: Development of weight average molecular weight Mw with time measured for samples prepared by acid hydrolysis: ▲nitric acid ▫formic acid •phosphoric acid

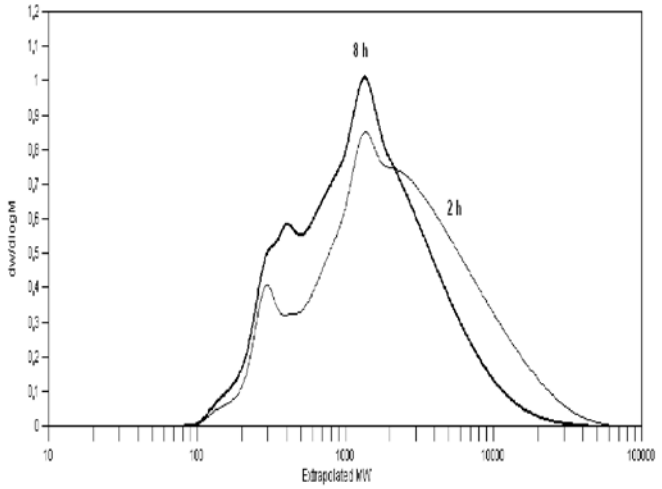

Figure 2: Differential distribution curves recorded for samples hydrolysed with formic acid for 2 hours and 8 hours

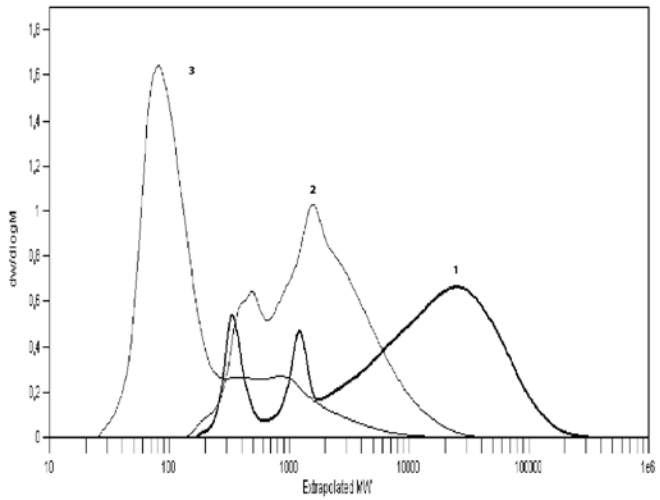

Figure 3: Differential distribution curves recorded for (1) starting material and samples hydrolysed with (2) 30 g formic acid and (3) 159 g formic acid . Hydrolysis time 6 hours.

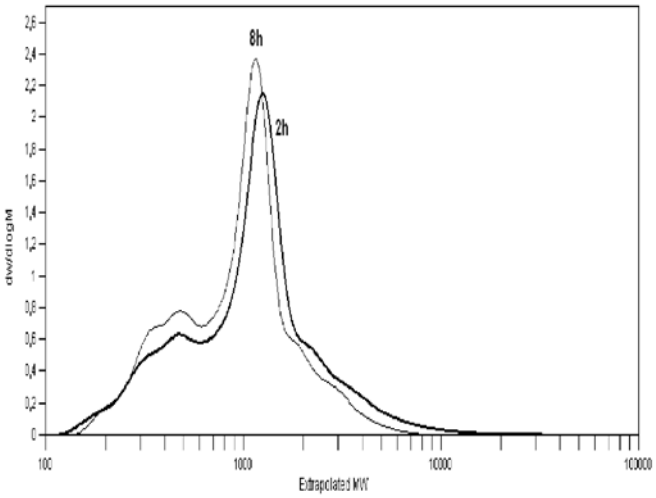

Figure 4: Differential distribution curves recorded for samples hydrolysed with phosphoric acid for 2 hours and 8 hours

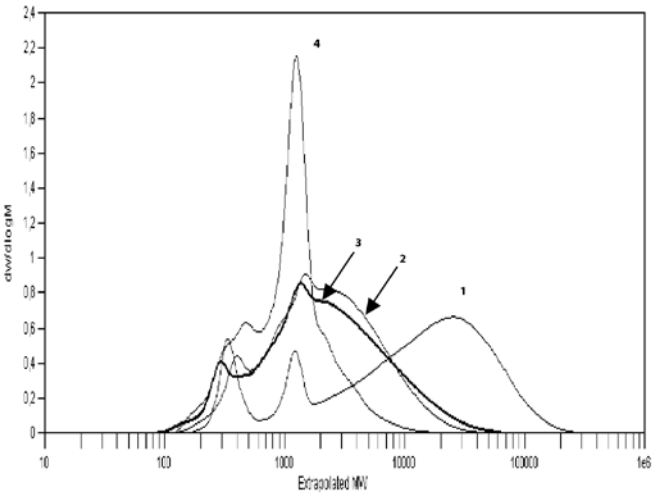

Figure 5: Comparison of differential distribution curves recorded for (1) staring material and samples hydrolysed with (2) nitric acid, (3) formic acid and (4) phosphoric acid for 2 hours.

prominent decrease of molecular weight occurred within the first hour of hydrolysis. Hereinafter molecular weight decreased only moderately. Shape of the distribution curves of nitric acid hydrolysates is similar to that observed for formic acid hydrolysates.

Comparing formic, nitric and phosphoric acids acting as hydrolysing agents, it is obvious that hydrolysates prepared with phosphoric acid show generally the lowest molecular weight and the narrowest molecular weight distribution. Obviously, distribution curves of phosphoric acid hydrolysates show a shift towards a lower molecular weight region and it is also notable presence of increased amount of low molecular weight species. Value of M_w measured after two-hours hydrolysis was reduced to 8 per cent of the M_w determined for starting material.

Products prepared by hydrolysis with nitric and formic acids gave similar trends in terms of molecular weight and

molecular weight distribution. M_w was however higher compared to phosphoric acid hydrolysates. Here M_w decreased to about 21 per cent of the starting value after two-hour hydrolysis. Figure 5, illustrates differences among differential distribution curves of the samples treated for 2 hours with all the used acids.

Three of the low-molecular weight samples prepared by hydrolysis with formic acid were applied to biostimulation testing. Preliminary tests suggest that collagen hydrolysates are able to induce defense related genes in oilseed rape plants. The induced genes include one crucial for plant defense responses to biotrophic pathogens[22] and one thought to be triggered by necrotrophic pathogens and damages caused by insects.[23] Lower molecular weight samples showed better action and hence, low-molecular weight is an advantage. However, mechanism of plant defense responses stimulation by hydrolysates is poorly understood and will be clarified in further work.

CONCLUSIONS

In the study, formic, phosphoric and nitric acids were used for hydrolysis of the commercial starting material Hykol-E with the aim to prepare low-molecular weight products to be used as biostimulators. Course of hydrolysis was monitored by following the changes of molecular weight and molecular weight distribution measured by gel permeation chromatography. The study demonstrated that MW and MWD of hydrolysates is influenced by the choice of hydrolysing acid, its concentration as well as by the time of acid treatment. Irrespective of the acid used, the most significant decrease in molecular weight was observed already after one-hour hydrolysis. During prolonged hydrolysis time, continuous, gradual decrease of molecular weight was further measured. The reduction was, however only shallow and not so prominent as in the beginning of the reaction. Of all the three acids in the test, phosphoric acid is the most effective one. Nitric and formic acids act approximately with the same efficacy when used in the given amounts. From the practical point of view, molecular weight of hydrolysates can simply be controlled with acid concentration, acid type and ratio between amount of starting material and acid. Using the combination of enzymatic and acid hydrolysis, it is possible to prepare low molecular weight products with the tailor-made, pre-defined molecular weight fitting the intended use.

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