Contribution to Assessing Cross-Linking of Protein Hydrolyzate with Diepoxides

by

J. Hrncirik*[1] , J. Pseja[2] , J. Kupec[2] and M. Tupy[2]

[1]*Department of Food Engineering, Faculty of Technology, Tomas Bata University in Zlín*
Mostní 5139, 760 01 Zlín, Czech Republic

[2]*Department of Environmental Engineering, Faculty of Technology, Tomas Bata University*
Zlín, Mostní 5139, 760 01 Zlín, Czech Republic

Abstract

This work summarizes the influence exerted on cross-linking of protein hydrolysate obtained through enzymatic hydrolysis of chrome-tanned leather shavings, by means of diepoxides (1,2:3,4 diepoxybutane and 1,2:7,8 diepoxyoctane). Attention was given to preparation of cross-linked samples, their solubility, intrinsic viscosity and IR spectra. Suitable conditions for cross-linking were found at pH = 9 and 25°C temperature. Solubility of samples decreased proportionately with increasing concentrations of cross-linking agent. Viscosity measurements yielded best results in 1M KCl solution. Determining intrinsic viscosity indices proved this value was influenced more by the approach of the gelation point than by the epoxide content. IR spectra demonstrated that in the region of wave numbers 838, 912 and 1,250 cm^{-1}, peaks of epoxide groups of employed pure cross-linking agents coincide with similar peaks of other groups of cross-linked samples; peaks of epoxide bonds at the mentioned wave numbers are discernible only with highest employed concentrations of cross-linking agents. Future research will address examining various epoxy groups, employing di- or multifunctional amines and determining critical gelation conversions and critical stoichiometric ratios of defined substances.

Resumen

Este trabajo resume la influencia ejercida sobre la reticulación de la proteína hidrolizada obtenida por medio de la hidrólisis enzimática de virutas de cuero curtidas al cromo, por medio de di-epoxidos (1,2:3,4 di-epoxi-butano y 1,2:7,8 di-epoxi-octano). Se prestó atención a la preparación de las muestras reticuladas, su solubilidad, su viscosidad intrínseca y su espectro IR. Las condiciones convenientes para el reticulado fueron encontradas a pH= 9 y 25°C de temperatura. La solubilidad de las muestras disminuyó proporcionalmente con el aumento de la concentración del agente reticulante. Las viscosidades medidas dieron mejores resultados en la solución del 1M KCl. La determinación de los índices de viscosidad intrínseca probó que este valor fue influído más por el acercamiento del punto de gelación que por el contenido del epóxido. Los espectros IR demostraron que en la región de los números de onda 838, 912 y 1.250 cm^{-1}, picos de los grupos de epóxido de reticulantes puros empleados coinciden con los picos similares de otros grupos de muestras reticuladas; los picos de los enlaces de epóxido en los números mencionados de onda son perceptibles solamente con el empleo de concentraciones más altas de agentes de reticulado. La investigación futura se dirigirá al examen de varios grupos epoxi, empleando di-aminas o aminas multifuncionales y determinando conversiones de gelación críticas y cocientes estoiquiométricos críticos de sustancias definidas

Introduction

Possible ways of utilizing chrome shavings in recent years resulted in a number of proposals for their processing; some of these also concern enzymatic hydrolysis for production of protein hydrolysate (ProtH) which we described in greater detail in our last work published in this journal[1]. This ProtH is produced by two-stage (alkaline and enzymatic) hydrolysis of chrome-tanned shavings under atmospheric pressure with following separation of chromium and solid particles. The amount of chromium in this collagen product then attains around 20 - 30 ppm; hydrolysate is in the form of solution or powder. Its physical and chemical characteristics have already been described[2].

For example, ProtH is used as filler in a blend with polyvinyl alcohol for extrusion because it is twice cheaper as that and also contributes to improving biodegradability properties as it increases degradation of resultant modified polymer and even improves mechanical properties and thermal stability[3].

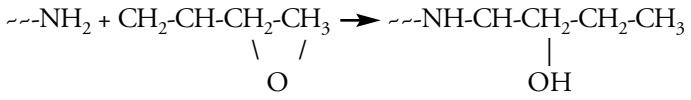
Using thermogravimetric analysis, thermal stability of polyvinylalcohol films was studied in dependence on various ratios of that and ProtH in the blend; it was found that a

* Corresponding Author - E-mail: hrncirik@ft.utb.cz
Manuscript received October 4, 2006, accepted for publication April 18, 2007

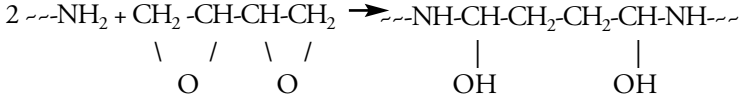
concentration of 5 wt.% ProtH in the mix had no influence on thermal stability. The extent of thermal degradation was much reduced particularly at higher ProtH concentrations; a greater quantity of this product in the blend has a disadvantage in increasing its viscosity and thus impairing processing properties[3].

Regarding practical applicability, cross-linking proteins with substances containing epoxy groups has major significance, particularly in medicine. It offers a number of procedures for adapting final properties of protein to requirements of processor and user. Materials studied most extensively that were derived from collagen (including that itself) include its epoxide-cross-linked modifications. Cross-linking is to be understood as a process producing a three-dimensional structure through chemical linkage of reactive groups in polymer with reactive agent. Formation of such a spatial network results in altered mechanical properties, for example, in elasticity, strength and swelling or solubility.

Basic reactive groups in collagen are -NH_2, -COOH, -OH, -SH; the reaction group in epoxides is epoxy group. In proteins, cross-linking mainly proceeds between an epoxy and amino group, to a lesser extent between epoxy and carboxyl or hydroxyl group; the peptide bond does not participate in epoxide cross-linking[4,5]. In case a monofunctional epoxide participates in cross-linking reaction or a multifunctional epoxide reacts with merely one reactive amino group, the reaction runs, for example, in accordance with following scheme:

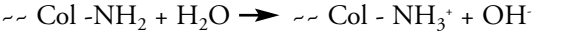


As no cross-linking of protein chains occurs, the case is termed "masking". Real cross-linking already takes place between protein chains, for example:



Tissues modified with monofunctional epoxides (masked) are more elastic and exhibit lower denaturing temperatures than tissues cross-linked with polyfunctional epoxides[4, 5].

Rate of protein cross-linking with epoxides grows with increasing temperature and also with increasing concentration of cross-linking agent, cross-linking also proceeds faster at higher pH of the environment. This may be explained by increased effective reactant concentration. The amino group in collagen (~~Col) produces a balance as follows:



The non-protonized amino group on left side of the equation is the relevant reactive group entering the reaction with epoxy group. At a higher pH, equilibrium is shifted to the left. A strong nucleophile agent[6] is then formed from the ε-amino group in the basic region. In a strongly acid region, even formation of protonized epoxide may be expected.

Because cross-linking proceeds outside the peptide bond, which is easily split by water (subject to hydrolysis), cross-linked collagen is also subject to biodegradation. The produced network admittedly prevents some enzymes in reaching to reactive sites but degradation rate thereby decreases only slightly and protein may be further biodegradable.

ProtH is closer to gelatin than to collagen, for that reason we looked for literature sources concerning gelatin cross-linking. Authors of some works[5, 7] conducted research into cross-linking of gelatin with 1,2:3,4 diepoxybutane (DEB) and 1,2:7,8 diepoxyoctane (DEO). They investigated solubility of variously cross-linked samples and arrived at the conclusion that if the sample is more densely cross-linked, thus if its s tructure is interconnected to greater degree, the less or at least more slowly does the sample dissolve. The selected solvent was water, also phosphate buffer of pH = 8.2, and a dissolution temperature of 50°C. At first, the sample cross-linked with DEO dissolved faster than that with DEB but after approx. 180 min their solubility was similar.

Tests were run on samples of gelatin film cross-linked with DEB or DEO from the viewpoint of crystallinity[8]. Their degradation took place in soil; all were completely degraded in 6 days[7]. The same authors[9] further investigated breakdown of cross-linked gelatin in river and lake water; it was completely degraded in 4-6 days. It follows that gelatin so cross-linked is degradable in an anaerobic aqueous environment as well as in soil[5].

Gelatin processed with the same cross-linking agents was also studied from the viewpoint of solubilization and biodegradation by alkaline protease[10].

ProtH may be cross-linked with agents already mentioned containing an epoxide group but also with aldehydes[11] or diisocyanates[12].

Authors[13] worked on modifying ProtH among others with epoxycyclohexane. They prepared samples modified with 1, 2, 5 and 10 wt.% cross-linking agent. Sample solubility and biodegradation rate decreased with a growing content of cross-linking agent.

The protein hydrolysate here discussed is a cheap, readily biodegradable substance applicable in technical practice as filler in blends with other biodegradable polymers and some plastics. Modifying it with cross-linking agents is a possibility for altering mechanical and other properties which might lead to its use on a wider scale than hitherto. This study should contribute to clarifying changes that accompany ProtH cross-linking.

EXPERIMENTAL

Chemicals and Material

Current chemicals were of analytical or pure quality, produced or distributed by Lachema Co, Brno, Czech Republic.

1,2:3,4 Diepoxybutane (97%) was produced by Sigma-Aldrich, St. Louis, MO, U.S.A.; toxic with regard of his high volatility and solubility.

1,2:7,8 Diepoxyoctane was produced by MERCK-Schuchard, Hohenbrunn, Germany; less toxic as DEB.

Collagen protein hydrolysate, trade name Hykol E, was produced by Kortan Co, Hrádek n/Nisou, Czech Republic; nitrogen content 14.1%, molecular weight M_w approx. 1.10^4 Da.

Apparatus and Equipment

Current laboratory glass and basic equipment were of domestic and foreign products. Analyzer used was total organic carbon analyzer TOC-5000A by Shimadzu Corp, Australia. The UV-VIS spectrometer was THERMO SPECTRONIC. The cooled centrifuge was type MR23i from Jouan, France. The FT-IR spectrometer was Avatar 320 with single reflex ZnSe ATR "Miracle", U.S.A. Viscosity was measured on an Ubbelohde, TS No 11130 type U1, viscometer by KAVALIER Glassworks, Dr_kov, Czech Republic.

Methods

In cross-linking, mainly free -NH_2 or -COOH groups and partly also -OH groups in the protein chain are able to react with epoxides. It is thus appropriate to determine their quantity and favorable cross-linking conditions. Selected physical and chemical properties of prepared cross-linked samples were examined, in particular solubility, free amino group content, solution viscosity and IR spectra.

Samples and their Preparation

At pH = pK, acid or basic groups of protein hydrolysate are obviously neutralized by half; at these pH levels a virtually maximum concentration of reactive groups is already attained (cross-linking with epoxides should, therefore, proceed at practically maximum rate).

In order to find out if cross-linking may be performed even at pH below 10 (for slowing down potential alkaline hydrolysis of ProtH), samples containing 1.13% weight and 2.27% weight DEB, and 3.30% weight DEO (related to ProtH dry matter) were cross-linked at various pH levels[14,15]. Fixation indices (FI) of these samples were then determined employing the ninhydrin method[16]. Cross-linking was performed in enclosed Petri dishes lined with aluminum film in a fume cupboard for one day at a laboratory temperature of approx. 22°C, and the cross-linked sample was next dried at 80°C for 3 days in a drier. It was subsequently ground in a mortar to fine powder and placed in desiccator over silica gel.

Composition and designation of cross-linked DEB and DEO samples are shown in Table 1.

TABLE I
Composition (quantity of epoxide/ProtH and expected* stoichiometric ratio epoxy/-NH_2) and fixation index of samples

Cross-linking agent	Cross-linking agent/ProtH $w_{E/ProtH}$ [%]	Expected stoichiometric relation epoxy/-NH_2 $x_{E/NH2}$ [%]	FI [%]
	0.38	8.2	22.9
	0.76	16.4	30.1
	1.13	24.4	40.7
DEB	2.27	48.4	55.6
	4.53	100	68.8
	9.06	196	83.8
	18.1	392	85.1
	0.55	7.2	29.7
	1.10	14.4	35.1
	1.65	21.6	42.7
DEO	3.30	41.2	53.2
	6.60	82.4	69.1
	13.2	173	85.5
	26.4	346	100

* Calculating assumed stoichiometric ratio epoxy/-NH2 is based on values of WPE ($M_{eq\text{-}epox}$) and equivalent weight of ProtH ($M_{eq\text{-}ProtH}$) going to one amino group (estimate from assumed presence of amino acids in ProtH chain; $M_{eq\text{-}ProtH}$ = 930g dry matter ProtH per 1 mol -NH_2 groups[1]). Composition of samples corresponded to molar ratio of epoxy and amino groups below and above assumed gelation point.

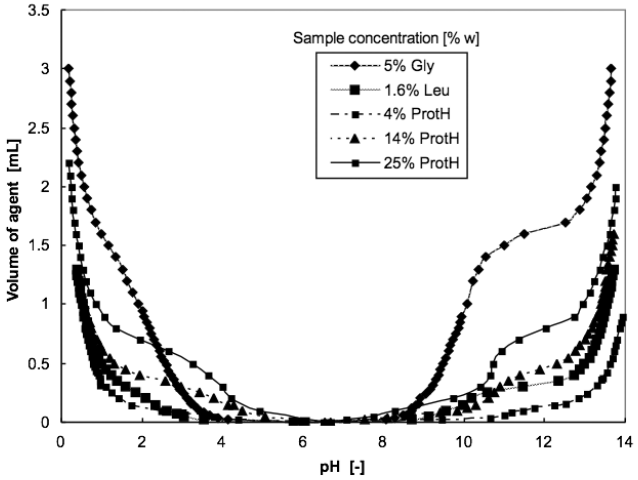


Figure 1: Titration of glycin, L-leucin and ProtH solutions with 5.93M KOH and 6.26M HCl

Determining -NH*2* and -COOH Contents in Protein Hydrolysate through Titration

As ProtH is a protein of strong buffer capacity, $-NH_2$ and -COOH contents cannot be determined under classic analytical conditions, i.e. through titration to methyl orange and phenolphthalein. Outside extreme pH values, the mentioned groups react together to produce ~-COO^- NH_3^+-~ salt. Therefore, analysis had to be performed through potentiometric titration in a concentrated environment of sample and agent.

Examples of these titrations in a higher-concentrated medium are presented in titrations of 5-% glycin solution and 1.6-% L-leucin solution (L-leucin of higher concentration in water could not be prepared). Titration analyses were also run on ProtH solutions of 4-, 14- and 25-wt.% concentrations. Titration agents were 5.93M KOH and 6.26M HCl solutions (Fig. 1).

Determining Non-Reacted Amino Groups

When determining fixation index, free ammonium ions interfere, or those coming in the sample, for example, from ProtH production. The interfering effect caused by ammonium ions is obvious from equations describing mechanism of reactions in which ninhydrin reacts with free amino acid[16].

Composition of samples is mostly presented as weight ratio $w_{E/ProtH}$ of employed quantity of cross-linking agent (epoxide) mepox related to weight of dry matter $m_{ProtH\text{-}dry\ matter}$ of cross-linked ProtH. Calculation is performed from relationship:

$$w_{E/ProtH} = \frac{m_{epox}}{m_{ProtH\text{-}dry\ matter}} \cdot 100\% \qquad /1/$$

or as the molar stoichiometric ratio of quantity of epoxy groups of cross-linking agent to $-NH_2$ groups calculated as percentage from relationship:

$$x\ E/NH2 = \frac{m_{epox}\ /M_{ekv\text{-}epox}}{m_{ProtH\text{-}dry\ matter.}\ /\ M_{ekv\text{-}ProtH}} \cdot 100\% \qquad /2/$$

Equivalent molecular weight of employed epoxide cross-linking agents is $M_{Eq\text{-}epox} = 1/2\ M_{epox}$ because the epoxides in question are bifunctional epoxides. Equivalent weight of ProtH related to one amino group $M_{Eq\text{-}ProtH}$ (estimate from assumed quantity of amino acids in a chain of ProtH; M eq -NH_2 = 930g dry matter ProtH /1mol -NH_2 groups[1]; or 1,075 g dry matter ProtH /1 mol -NH2 groups from titration with KOH solution).

Greatest network density may be theoretically achieved with 100-% conversion of -NH_2 groups in an equimolar system (i.e. at $x_{E/NH2}$ = 100%), that is with weight rations of epoxide:

$$w_{theor\ DEB\ /\ ProtH} = \frac{M_{ekv\text{-}DEB}}{M_{ekv\text{-}ProtH}} .100\% = \frac{43}{1075} . 100\% = 4.0\ wt.\%\ per\ DEB \qquad /3/$$

$$w_{theor\ DEO\ /\ ProtH} = \frac{M_{ekv\text{-}DEO}}{M_{ekv\text{-}ProtH}} .100\% = \frac{71}{1075} . 100\% = 6.6\ wt.\%\ per\ DEO \qquad /4/$$

Determining Dissolution Rate of Cross-linked Samples by Means of TOC[15]

Suspensions were prepared (weighed-in quantity 0.375g dry matter / 75mL distilled water) from all cross-linked samples including pure ProtH. Dissolution was surveyed at laboratory temperature under stirring at 200 rpm.

A non-dissolved sample fraction settled on bottom of beaker before sampling. Samples containing 6.6, 13.2 and 26.4 wt.% DEO dissolved and settled with difficulty; for determining max. solubility (sol content), all suspensions that had dissolved 90 min were subjected to sonic treatment for another 1.5 hrs. Quantities of 10 mL were withdrawn from prepared suspensions at intervals of 10, 30, 60, 90 min and filtered through medium-density filter paper that had been thoroughly washed with boiling distilled water and dried. Filtrate was diluted 25x and analyzed on carbon analyzer.

Determining Intrinsic Viscosity

Intrinsic viscosity [η] (or IV) is defined by relationship

$$[\eta] = \lim_{c\_0}\ \eta_{spec} / c \qquad /5/$$

here c is concentration of polymer solution; the relationship valid for specific viscosity (ηspec) is

$$\eta_{spec} = \eta_{rel}\text{-}1 \qquad /6/$$

ηrel is relative viscosity of solution defined by expression

$$\eta_{rel}=\eta/\eta o \qquad /7/$$

where η is solution viscosity and ηo is solvent viscosity.

Intrinsic viscosity depends on molecular weight of polymer in solution, in accordance with the Mark - Houwink equation

$$[\eta] = K\ .\ M^a \qquad /8/$$

where M is molar weight of polymer, K, a are constants for a certain system of polymer-solvent and temperature.

Dependencies η_{spec}/c vs. c are nonlinear in systems of greater interaction, that is, in polar systems. Difficult determining appears in the case of a polyelectrolyte, where dependency η_{spec}/c vs. c is very nonlinear. Linearity is always improved by increasing ionic strength (I) on addition of indifferent electrolyte, which suppresses interactions between groups in the chain. Another recommended possibility is extrapolation of [η] to infinite ionic strength in coordinates [η],1/√I ; [η] = [η]∞ + k / √I.

From here stems the requirement to keep up a constant, as high as possible ionic strength. Determining [η] of polyelectrolyte is convenient at a constant pH = I (isoelectric point) and greater ionic strength, at least I = 0.05M, or rather 0.5M. No particular problems concerning determination of gelatin limit viscosity index are mentioned in literature17; the level given at 20°C in 0.5M KCl is $4.07.10^{-4}$. $M^{0.52}$ [dL/g], [g/mol]. ProtH should behave similarly; with its M = 1.10^4 g/mol, anticipated [η] = 0.05 dL/g.

Measuring IR Spectra

Samples prepared by cross-linking ProtH with respective epoxide were dried at 100°C, crushed to powder in agate mortar and kept in a desiccator over silica gel at laboratory temperature. In actual measurement, sample was spread onto a single-reflex ATR ZnSe crystal, to which it was compressed with approx. 40 N force over an approx. 16 mm2 area so as to achieve maximal contact of sample with crystal. Spectra of background and also sample were scanned by 32 scans at 4 cm^{-1} resolution.

Spectra were expressed and further processed in the usual format of absorbance as a function of wave number. To simplify comparisons of sample spectra, spectra were normalized (sum of absorbances over whole spectrum was unit).

DISCUSSION

Determining -NH_2 and -COOH Contents in Protein Hydrolysate through Titration

As the amino group and carboxyl group of amino acids are subject to interaction with ready formation of ~-COO^- NH_3^+-~ salt, decomposition of this salt occurs at end point. With reference substance glycin, theoretical content of -COOH groups is 60 wt.%; content determined through titration was 62.7 wt.% , which verified the method. With glycin, values pK ; pK_A = 2.34; pK_B = 9.60 were read from values in Fig.1 and these correspond to tabulated logarithms pK of dissociation constants. Values determined for ProtH were pK_A = 2.20 and pK_B = 10.60. Titration of polyelectrolyte is more distinct at higher salinity produced by added indifferent electrolyte (for example KCl) and yields higher pK_A and pK_B values.

Isoelectric point of ProtH and employed amino acids was in the around of pH = 6.5. With ProtH, acidimetric and alcalimetric analyses determined 5.12 wt.% -COOH and 1.35 wt.% -NH_2. Equivalent molecular weights calculated from these for -COOH and -NH_2 groups in ProtH are 716 g/mol -COOH and 1,075 g/mol -NH_2.

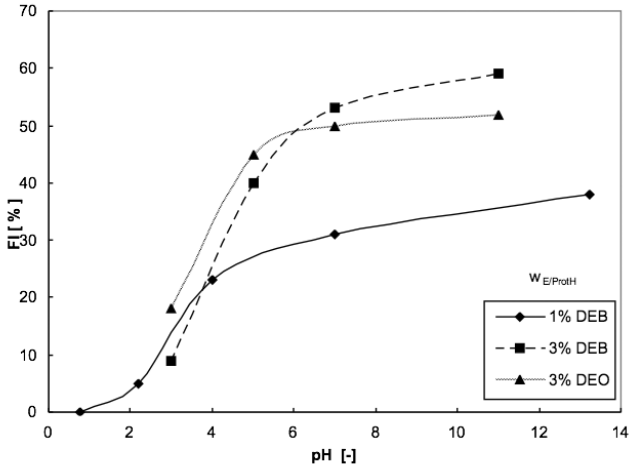


Figure 2: Influence of pH on conversion of -NH_2 groups (fixation index - FI) of ProtH with DEB and DEO

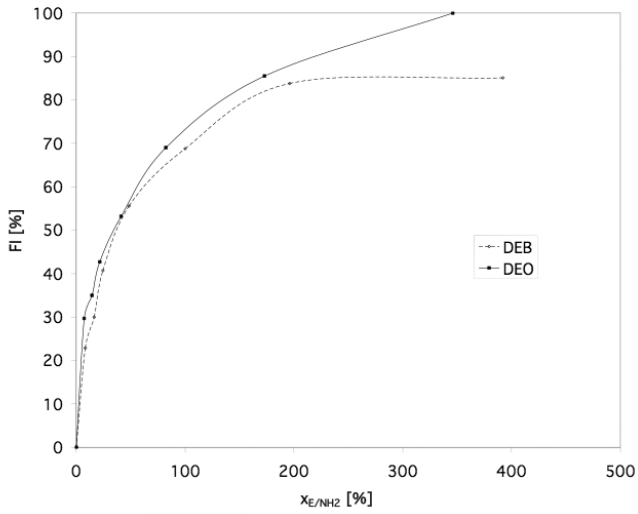


Figure 3: Dependence of fixation index (conversion of -NH_2 groups) on starting composition according to ratio of epoxy / -NH_2 groups after adding cross-linking agent (DEB, DEO)

Determining Non-Reacted Amino Groups

Content of free (so far non-reacted) amino groups of cross-linked samples was determined and these were used to express fixation index[15] (conversion of amino groups in cross-linking reaction).

It follows from Fig.2 that cross-linking of -NH_2 groups in an acid environment are inhibited. At low pH levels even breakdown of epoxide as well as of ProtH through hydrolysis may occur. At pH = 7 and higher, fixation index is favorable, and pH = 9.0 was thus chosen for sample cross-linking, when high conversion of amino groups takes place and samples do not bear the load of an extremely basic environment. In addition, the high pH suppresses possible bonding of epoxide to -COOH groups, signifying that practically only -NH_2 groups react with epoxide.

In cross-linking performed at pH = 9 a high conversion of amino

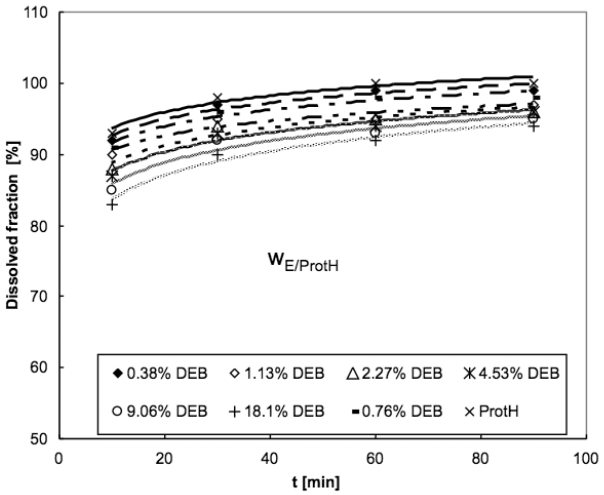


Figure 4: Dissolution of DEB-cross-linked ProtH samples in water at 25°C

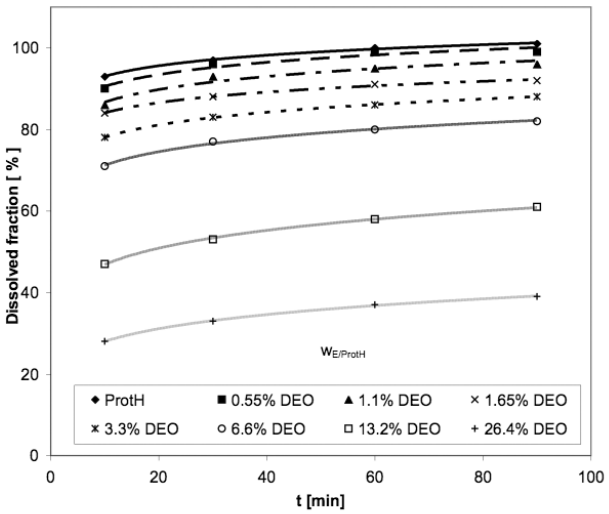


Figure 5: Dissolution of DEO-cross-linked ProtH samples in water at 25°C

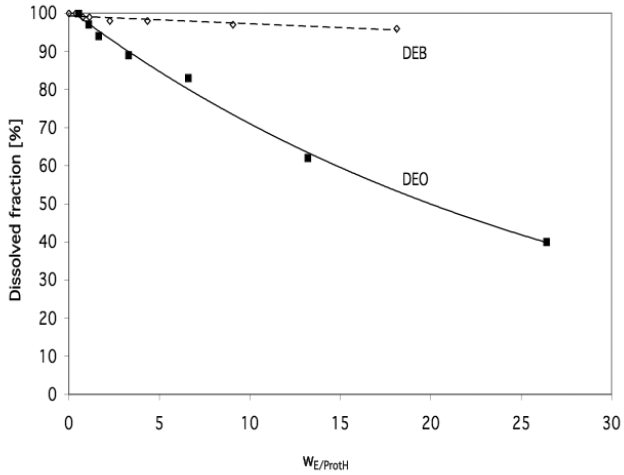


Figure 6: Solubility of cross-linked samples (in water at 25°C after 90 min and sonic treatment 1.5 h)

groups takes place and at the same time bonding of epoxides with -COOH groups is suppressed. Determination of amino groups serving to calculate fixation index is interfered (owing to the chemistry of the ninhydrin reaction) by free ammonium ions present from production of ProtH. Fig.3 shows obvious growth of FI with growing content of cross-linking agent.

From Fig.3 it follows that fixation index displays a strongly nonlinear character of growth with increasing quantity of epoxide in the mixture, and approaches the anticipated theoretical 100 % conversion level of $-NH_2$ groups always more slowly. It attains its maximum slower with samples cross-linked with DEB. However, with the equimolar composition assumed, conversion of $-NH_2$ groups reaches merely about 70% in the case of DEO and only 65% with DEB.

Simple and obvious conditions to be met are as follows:

a) Epoxides react at sufficient rate, i.e. in virtually quantitative manner with $-NH_2$ (with one or both hydrogen atoms of N, meaning the amino group is either mono- or bifunctional).
b) Colored products formed by reaction of agent with amino groups have the same extinction coefficient at selected wavelength, independently of amino group location (type of amino acid).

FI values reached above were not as expected. Both conditions are met, FI should increases linearly with epoxide dosage. Essentially, with FI = 0% - 100% and with epoxide in excess, FI should attain 100%. Otherwise said, if the lowest dose of epoxide already achieves FI > 50%, there is no reason why a double dose of epoxide should not achieve FI = 100%.

If FI yields correct values, it should enable to determine correct equivalent weight pertaining to one $-NH_2$ group in ProtH. However, there is always the question of $-NH_2$ acting on epoxide either as a monofunctional or bifunctional group. Bifunctional is the more probable answer, i.e. if possible kinetic and steric limitations are not substantial.

Solubility of Cross-Linked Samples

From Figs.4 and 5 it follows that samples prepared with a greater DEO addition were only partly soluble even under great dilution and after sonic treatment (1.5 hrs) as opposed to readily soluble samples cross-linked with DEB. DEB-cross-linked samples exhibit virtually complete water solubility after 90 min at 25°C even with an 18.1 wt.% addition of DEB. Practically complete water solubility of DEO-cross-linked samples already comes to an end with a dose of 6.6 wt.% (Fig.6). A major fraction of insoluble gel forms with higher DEO dosage.

Intrinsic Viscosity

When seeking an optimal solvent for measuring viscosity of sample solutions, tests were carried out on influence of ionic strength on intrinsic viscosity, at a temperature of 25°C. Dependence η_{spec}/c vs. c of ProtH aqueous solutions was nonlinear, the sample was thus measured when dissolved in 0.5M or 1M KCl. Buffer 1M $NaHCO_3$ + 1M K_2CO_3 precipitated

ProtH from the system and could not be used. 1M KCNS applied as solvent did not produce better linearity of yields than 1M KCl; therefore, 1M KCl at a neutral pH = 7 proved to be the most suitable (Fig.7).

Samples cross-linked with 6.6 wt.% , 13.2 wt.% and 26.4 wt.% DEO were difficultly dissolved and filtered in order to achieve required concentration. Before measurements in viscometer, it was not necessary to neutralize samples with acid because solution pH dropped to a level of 7 (Fig.8) after strong dilution with 1M KCl.

Values of obtained IV are summarized for all measured systems in Fig.9.

The trend of IV increasing with a growing addition of epoxide cross-linking agents is apparent only when DEO is employed. Samples with greatest epoxide additions (except for DEB) were difficult to measure because they passed from solution into gel (cross-linking took place to greater extent than expected). From samples that had cross-linked into gel it was not possible to prepare a concentrated solution with ease by extracting soluble fractions. Intrinsic viscosity of ProtH is, of course, the lowest because linking of ProtH molecules with epoxide cross-linking agent did not take place.

It is interesting to determine epoxide dosage necessary to attain gelation point. It is also interesting to find how markedly IV alters in the vicinity of gelation point or how viscosity of the system having a composition near to gelation point alters with time, i.e. whether cross-linking under given conditions does not proceed too slowly or whether even marked hydrolysis of proteins does not occur.

From IV measurements it follows that intrinsic viscosity of a sample near gelation point strongly depends on epoxide dosage. Distance to gelation point (before gelation) may be assessed from magnitude of ratio (IV sample) / (IV ProtH) ; if the ratio > 3, the system is quite near gelation point (average M_w of ProtH increased approximately tenfold through cross-linking before gelation point). Assessing the distance to gelation point by merely stating a drop in fluidity is less productive than knowing system viscosity or IV.

Gelation point may be also approached in systems containing an excess of epoxides. If functionality of ProtH is low, the interval of composition between gelation points when amino groups or epoxy groups are in excess is narrow.

An interesting value is epoxide dosage for reaching maximal cross-linking degree (minimal sol content). That is equivalent to knowing real weight of ProtH per one amino group, and at the same time also to knowing WPE (equivalent weight per epoxy group) of cross-linking agent. With higher epoxide dosage, free epoxy groups are present in the system, which may prove undesirable. Determining presence of free epoxides by means of IR spectra under a small surplus of epoxides is not easy.

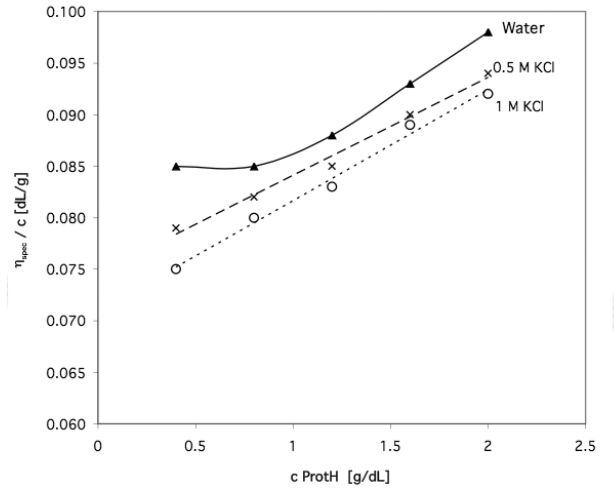


Figure 7: Dependence η_{spec}/c vs. c of ProtH in water, 0.5M and 1M KCl for determining IV= [η] (25°C, pH = 7)

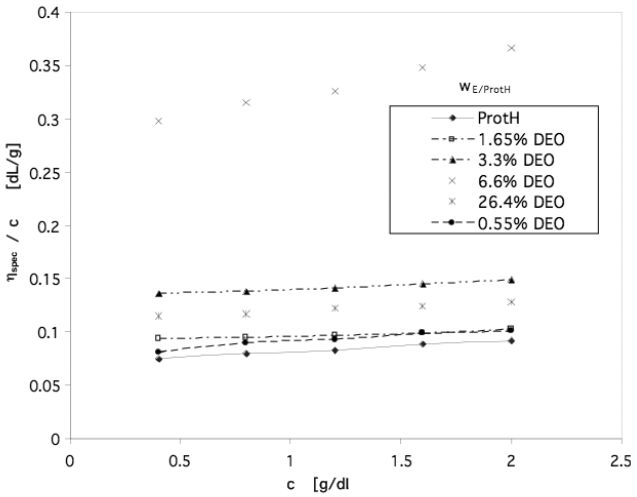


Figure 8: Dependence η_{spec}/c vs. c (in 1M KCl, 25°C, pH=7) for ProtH cross-linked with DEO at pH=9

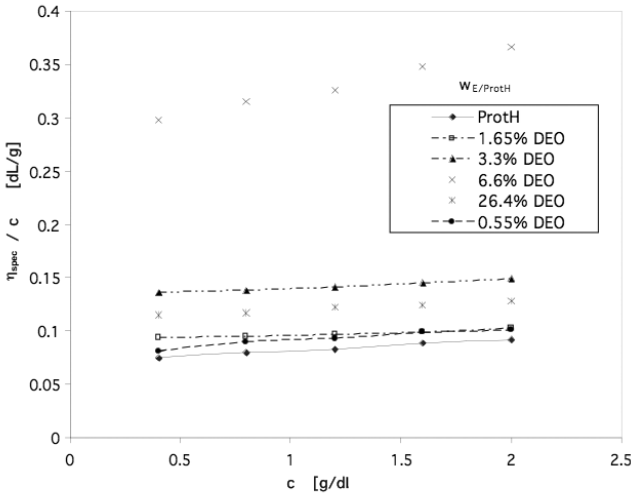


Figure 9: Dependence of intrinsic viscosities (in 1M KCl at 25°C, pH=7) on cross-linked agent dose (% weight epoxide/ProtH)

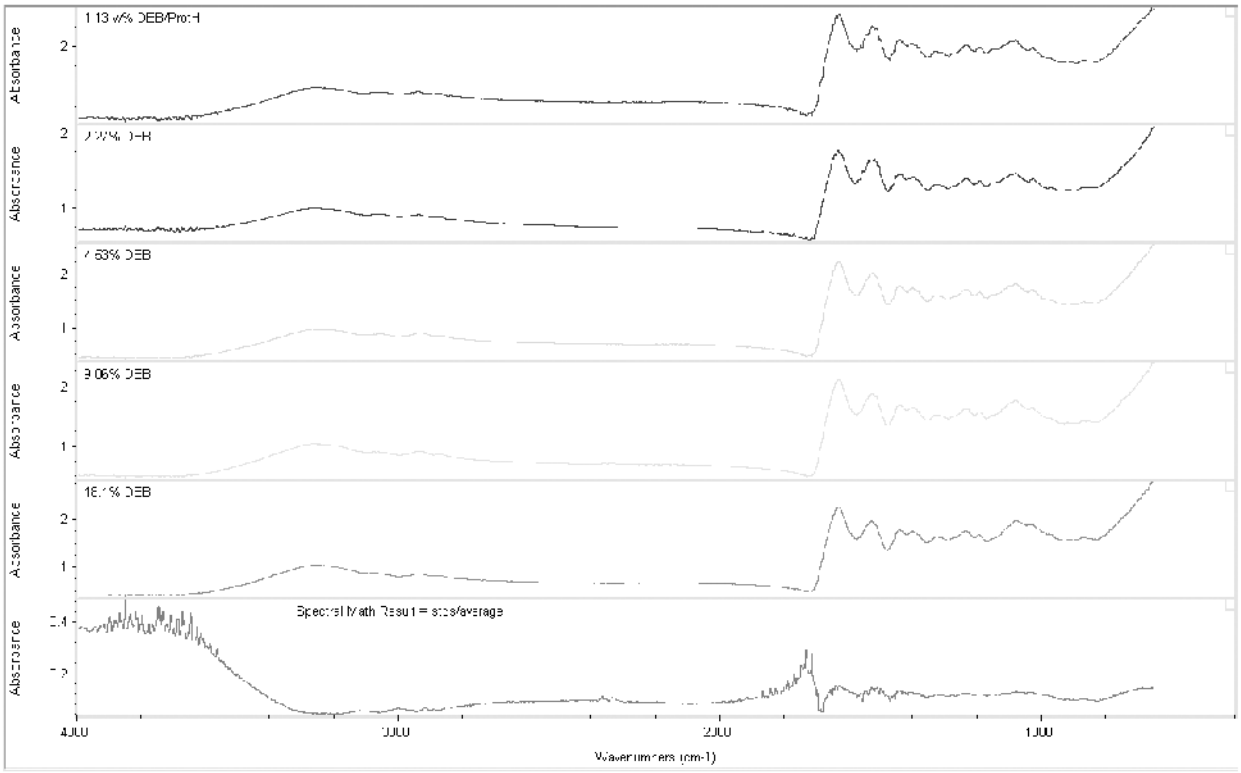


Figure 10: FTIR absorption spectra of ProtH samples cross-linked with DEB (powder on ZnSe crystal, no ATR correction)

As constants of the Mark-Houwink equation for ProtH are not known, all the less so for cross-linked samples, molecular weights of samples could not be determined from IV.

Formation of a spatial network (loss of fluidity, i.e. infinite viscosity) occurs at gelation point. This takes place in practice merely if reacting substances possess high functionality, are blended in a favorable ratio and only at sufficiently high conversion of functional groups, that is, after sufficient time (depending on reaction rate). Diepoxides reacting with amines are only bifunctional. ProtH, according to preliminary calculations (assuming M_w = approx. 1.10^4), is approx. decemfunctional per amino groups.

Most favorable conditions for network buildup come about with real stoichiometry of $x_{E/NH2}$, i.e. epoxy/-NH_2 = 100% and high conversion, with cross-linking time and pH being favorable.

In the case of ProtH, quite short chains bearing epoxide groups have to connect two spatially distant amino groups. In a non-swelled state, amino groups in pure ProtH are at a mutual average distance corresponding to more than approx. 3.27 bonds of fully straightened C-C chain (or 4 bonds of real but hardly probable, maximally straightened conformation of zigzag C-C chain having usual valence angles of 109°). When DEB is employed, maximal distance between reactive sites of epoxy groups (DEB bridges between N atoms) is 5 zigzag bonds; when DEO is used it is 9 bonds. Overcoming 90 % conversion of -NH_2 would require spanning a distance greater than 8.5 zigzag bonds, i.e. with at least DEO. These considerations, nevertheless, merely apply to dry ProtH. Formation of a network with short connecting bridges thus requires forming quite improbable chain conformations, and shows by slowing down the reaction. Conversely, even under conditions for quite rapid reaction of epoxy groups with ProtH and with optimal stoichiometry, free epoxy groups non-reacted for steric reasons should remain in the system. Average distances between amino groups in a solution increase. Cross-linking of a 40 % ProtH solution would initially require spanning an average of 5.9 zigzag bonds, at 50 % conversion as many as 7.4 and at 90% even 13 zigzag elongated bonds. In the case of DEB, therefore, a limited cross-linking course may be expected. But chains in solution are freely mobile, reacting groups approach each other with ease and possible steric limitations show only after gelation point. Hence, cause of the low cross-linking effect by DEB in solution will rather be its lower reactivity (negative substitution effect of neighboring polar groups whether in DEB or in cross-linking intermediates). Another possibility opening up is a potential intramolecular reaction closing the pentamerous ring to nitrogen of secondary amine through which the first epoxide group was attached. Cyclization is preferred for steric as well as kinetic reasons in the case of DEB, not of DEO. This explains that with a steric or ensuing kinetic limitation to the cross-linking reaction, it will be chiefly DEB to behave more as monofunctional agent, without ability to form a network.

Diepoxides in a stoichiometric reaction with decemfunctional ProtH at optimal stoichiometry would produce first fractions of gel at a conversion of about 11%. Higher conversion is

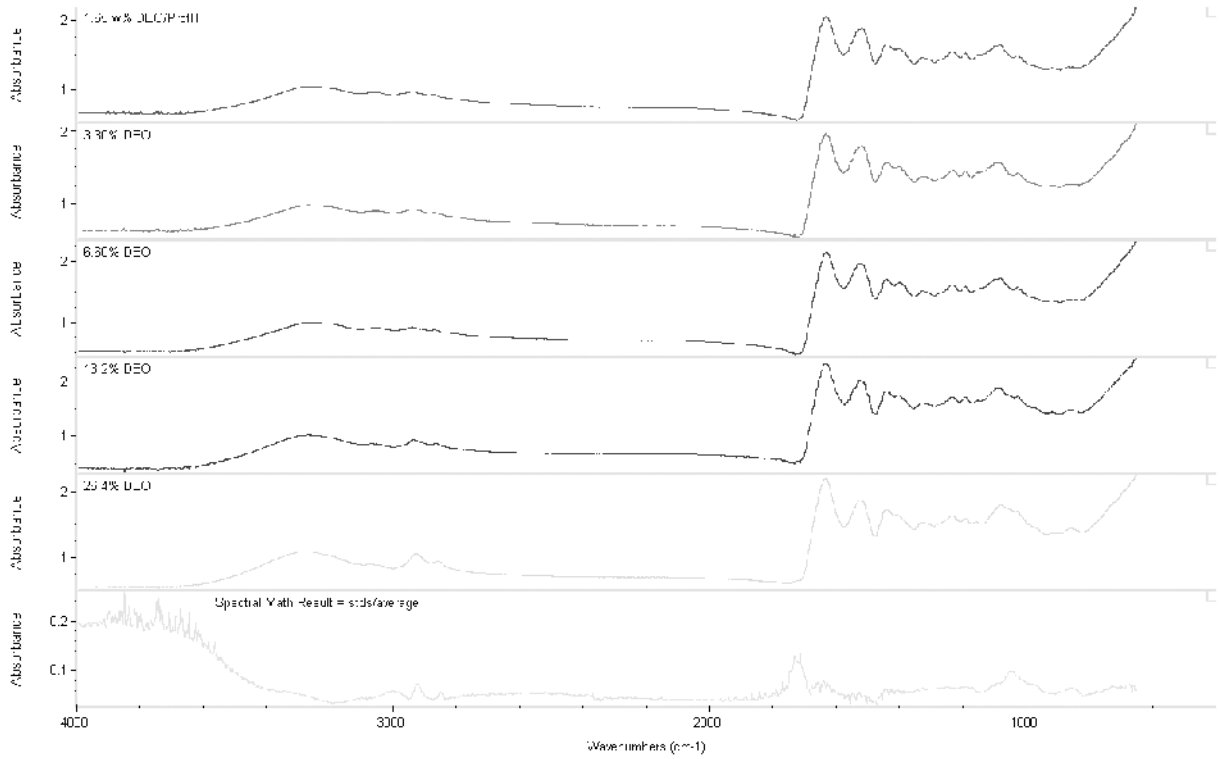


Figure 11: FTIR absorption spectra of ProtH samples cross-linked with DEO (powder on ZnSe crystal, no ATR correction)

necessary in the case of steric limitations. If steric limitations are very great, too short diepoxides will not produce a network even at 100 % amino group conversion.

Gel formation was not found in cross-linking with DEB. More limited solubility of DEO samples and formation of gel occurred only after their drying (their deswelling could have shown). Hence, expected increments in IV when cross-linking ProtH with DEB did not appear, probably due to steric or kinetic reasons.

IR Spectra

Cross-linking of proteins with epoxides utilizes ability of epoxides to react with groups containing an active hydrogen, for example, in -NH_2, -SH, -COOH, -OH groups. There, the energy-rich epoxide ring opens to produce hydroxy derivatives. Instead of a tight (formally ether) bond in epoxide, a less tight C-O bond arises which is discernible in the spectrum from the epoxide bond. In a strongly acid environment, homopolymerizationation of epoxide to polyether takes place.

Present cross-linking agents and the chemical reaction lead to significant changes in spectra unless new bands strongly coincide with spectral bands of other C-O bonds (for example these bonds of alcohols). Bands corresponding to hydrogen of -NH_2, -OH groups are usually very wide, their position depends on concentration, medium, temperature, etc. They may also coincide with bands of water (bound or humidity). Spectra of samples cross-linked with DEB and DEO are very similar.

From spectra of sole epoxides (pure cross-linking agents) it follows that peaks of the epoxide bond (cyclic ethers) should appear in the region of wave numbers 838, 912 and 1,250 cm^{-1}. In the case of high epoxide concentrations in samples, peaks of excess (non-reacted) epoxides[16] should be visible. Vibration of the C-O alcohol bond is found in the range of wave number 1,080 cm^{-1}. These bonds are cross-linking products and their peaks in cross-linked samples should increase with growing epoxide additions. In spectra, a visible growth of peaks of these bonds is obvious merely with samples containing DEO.

The most significant peaks of ProtH are found in wave number region 1,400 - 1,650 cm^{-1}. Height of these peaks should display a decreasing tendency with increasing quantity of epoxides. Illustrative samples were normalized; decrease of these peaks is not quite discernible because doses of cross-linking agent are quite small.

In the range of wave numbers 2,300 cm^{-1} in ProtH spectrum, residues to be sometimes found are peaks of CO_2, which is a mere impurity on the read-off background. In the spectra there are also visible peaks of C-H bonds in the region of wave numbers 2,930 cm^{-1} and 2,860 cm^{-1}, and wide peaks of N-H bonds in amines and O-H bonds of water or hydroxy ethers in the region above 3,000 cm^{-1}.

Conclusions were drawn from comparisons of uncorrected spectra. Those were normalized, i.e. multiplied by a constant to acquire a unit area and offer superior comparison. Naturally, the dependence in question is that of absorbance on wave

number, not transmittance on wave number because the functional groups under consideration absorb light in accordance with the Lambert-Beer law. An overall view of all spectra indicates that the basic line is quite unstable, particularly with large wave numbers.

Absorbance of samples in the region of 1,700 cm^{-1} is very high, however, peaks coincide. Regions of promising wave numbers, where changes in spectrum produced through reaction with epoxides can be best studied, are usually sought from differential spectra of epoxide-modified samples against pure ProtH. These regions are also evident as peaks in the calculated spectrum of standard deviations/average from collected spectra of samples cross-linked with various DEB or DEO doses.

Most important changes may be seen in the system of ProtH with DEO. A wide peak in the region of 3,000- 3,500 cm^{-1} may represent absorbance of newly formed -OH groups after cross-linking. At the same time, however, a visible decrease in peaks of reacted -NH_2 groups should also be seen in these regions.

Hydrophilic character of samples decreases more through cross-linking with DEO than with DEB; content of hydrocarbon groups increases and oxygen content decreases. Peaks of C-H bonds of reacted epoxides were not distinct in the case of DEB. Content of C-H bonds (either in CH, CH_2 or CH_3) in ProtH was similar to that in DEB. In this way, it would be possible to explain practical absence of changes in peaks in the 2,800 - 3,000 cm^{-1} region of samples cross-linked with DEB. Anticipated changes of these peaks can be well seen with samples containing 13.2 and 26.4 wt.% DEO. Samples containing DEB and DEO give rise to a new peak at wave number 1,050 cm^{-1}. This can be a C-O bond from the hydroxyl group produced after reaction with amine. This peak is visible only at dosages above 4.5 wt.% DEB and 6.6 wt.% DEO.

Diminishing peaks, i.e. peaks associated with decreased reaction groups in ProtH in the region of 1,100 cm^{-1} and 1,500 cm^{-1} are not unambiguously tabulated as peaks corresponding to -NH_2 group. They would be expected at wave number around 1,590 cm^{-1} if, however, solutions in question were diluted solutions of amines.

Measuring spectra of proteins cross-linked with DEB and DEO in the solid phase is difficult because peaks coincide or there are peaks related to moisture present in our field of interest. Water content also possibly affects height and width of peaks of groups interacting with water. Verifying of results and their improved interpreting necessitates measuring spectra in H_2O and D_2O solutions, optimally also with model substances (amines, amino acids, epoxides) or at various pH levels.

It is appropriate to measure spectra in the solid phase also employing KBr tablet technique, and that at various concentrations. The basic problem when measuring solutions will be extraordinary great water absorbance in the region of interesting wave numbers. When D2O is used, complications will be caused by immediate hydrogen and deuterium exchange in the -NH_2 and -COOH group and rapid exchange in -OH group and peptide bond -NH-CO-. Spectra together with essential comment are presented in following Figs. 10 and 11.

CONCLUSIONS

This work focused on identifying suitable conditions for cross-linking protein hydrolysate with 1,2:3,4 diepoxybutane and 1,2:7,8 diepoxyoctane, and measuring properties of prepared samples. Cross-linking that proved most suitable was conducted at temperature 25°C and pH 9, where the -COOH groups is suppressed. Properties under observation were solubility, free amino group content, intrinsic viscosity and IR spectra. In spectra of samples, a peak corresponding to dose of epoxide employed as cross-linking agent was visible in wave number range 2,900 cm^{-1}. Solubility of samples decreased with increasing concentrations of cross-linking agent; lower solubility was seen with application of DEO and greater with DEB. When investigating number of free amino groups (fixation index) through the ninhydrin method, the values obtained did not linearly change with dose of cross-linking agent as expected. The most suitable solvent for evaluating measurements of sample solution viscosity levels was 1M KCl. From IV measurements it followed that intrinsic viscosity of a sample near the gelation point grows much more rapidly than dosage of epoxide is increased.

From measurements of IR spectra of pure solvents it followed that absorption peaks of the epoxide bond (cyclic ethers) should be found in wave number range 838, 912 and 1,250 cm^{-1}. Wave numbers at which peaks of epoxide groups were to appear were found by comparing spectra of epoxide cross-linking agents DEB and DEO. Where epoxides were present in samples in high concentrations, peaks of excess epoxides (or unreacted) should have been visible. Epoxide peaks are strongly overlapped by peaks due to other bonds (cross-linking agents as well as of ProtH); only a slight increase of expected peaks at highest epoxide doses is discernible in spectra of the wave number range mentioned above.

Formation of a network requires employing substances displaying a greater number of cross-linking functional groups. If functionality of cross-linking substances is merely slightly greater than 2, they have to be mixed in an accurate stoichiometric ratio and the reaction must run to high conversion, otherwise the network cannot develop. As ProtH does not show overly high functionality, the network will not form if diepoxide is used in unsuitable stoichiometric ratio or the reaction does not proceed to high conversion. Diepoxides used cannot be practically contaminated with monofunctional substances, and possible homopolymerization is insignificant. However hydrolysis of ProtH may take place or increase in content of monofunctional substances contained in ProtH which are able to react with epoxide. The considerable degree of collagen hydrolysis in ProtH production (quite small molecules and hence low functionality as compared to, e.g. gelatin) is thus a hindrance to realizing a network.

For further research into these problems it would prove suitable to investigate cross-linking mechanism in greater detail, employing strictly bi- or trifunctional cross-linking agents, to analyze epoxy groups, hence also to know epoxide concentrations; possibly also to apply suitable bifunctional as well as multifunctional amines and with defined substances measure critical gelation conversions or stoichiometric ratios.

ACKNOWLEDGEMENT

This work was accomplished with financial support for research by the Ministry of Youth, Education and Sports of the Czech Republic, No 708835101.

REFERENCES

1. Hrncirík, J., Dvorácková, M., Hru_ka, F., Kupec, J. and Kolomazník, K.: Kinetics of alkaline hydrolysis of insoluble protein fraction following enzymatic hydrolysis of chrome-tanned shavings. *JALCA* **100**, 1-7, 2005
2. Langmaier, F., Mládek, M., Kolomazník, K. and Maly, A.: Hydrolysates of chrome waste as raw materials for the production of surfactants. *Tenside Surf. Det.* **39**, 47-51, 2000
3. Alexy P., Bakos D., Crkonová G., Kolomazník K., Krsiak M.: Blends of polyvinyl alcohol with collagen hydrolysate: Thermal degradation and processing properties. *Macromol. Symposia*, **170**, 41 - 49, 2001
4. Imamura E., M. D., Sawatani O., Koykanagi H, Noishiki Y., Miyata T.: Epoxy compounds as a new cross-linking agent for porcine aortic leaflets: Subcutaneous implant studies in rats. *J. Card. Surg.* **4**, 50 - 57, 1989
5. Patil R. D., Dalev P. G., Mark J. E., Vassileva E., Fakirov S.: Biodegradation of chemically modified gelatin films in simulated natural environment. *Biotechnik & Biotech.* **15**, 116 - 123, 2001
6. Tu R., Lu C. L., Thyagarajan K., Ngyyen H., Shen S., Hata C., Quijano R. C.: Kinetic study of collagen fixation with polyepoxy fixatives., *J.Biomed Mater. Res.* **27**, 3-9, 1993
7. Patil R. D., Dalev P. G., Mark J. E., Vassileva E., Fakirov S.: Biodegradation of chemically modifited gelatin films in soil. *J. Appl., Polymer. Sci.* **78**, 1341 - 1347, 2000
8. Patil R.D., Mark J.E., Apostolov A., Vassileva E., Fakirov S.: Crystallization of water in some crosslinked gelatins. *Eu. Polym. J.* **36**, 1055-1061, 2000
9. Patil R.D., Dalev P.G., Mark J.E., Vassileva E., Fakirov S.: Biodegradation of chemically modified gelatin films in lake river waters. *J. Appl. Polym. Sci.* **76**, 29-37, 2000
10. Patil R.D., Mark J.E.: Solubilization and biodegradation of cross-linked gelatins by alkaline protease. *Polym.-Plast. Technol. Eng.* **39**, 683-697, 2000
11. Charvátová K., Kresálková M., Kupec J.: Influence of waste protein cross-linking by dialdehydes on anaerobic degradation (in Czech). *Plasty a kaucuk* **39**, 267-269, 2002
12. Apostolov A.A., Boneva D., Vassileva E., Mark J.E., Fakirov S.: Mechanical properties of native and cross-linked gelatins in bending deformation. *J.Appl. Polym.Sci.* **76**, 2041-2048, 2000
13. Jelínek K., Pseja J., Kupec J.: Influence of waste protein cross-linking by monofunctional epoxides on anaerobic biodegradation (in Czech). *Plasty a kaucuk* **40**, 260-262, 2003
14. Pseja J., Hrncirík J., Kupec J., Charvátová H., Hruzík P., Tupy J.: Effect of cross-linking waste protein with diepoxides on its biodegradation under anaerobic conditions. *J. Polym. Environ.* **14** (3), 231-237, 2006
15. Tupy J.: *Thesis* (in Czech), FT UTB Zlín, 2005
16. Charvátová H.: *Thesis* (in Czech), FT UTB, Zlín, 2004
17. Ionesen J.B., Trandafir V., Demetresca I., Iovu H., Zqirian G.: *Eur. Cell. Mater.* **5** (1),10-11, 2003