Anaerobic Biological Degradation of Protein Hydrolysate Cross-Linked with Higher-Molecular Diepoxides

by

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Abstract

This work focuses on the influence of cross-linking with higher-molecular glycol diepoxides (and glycerol triglycidyleter) on biologic anaerobic degradation of protein hydrolysate (trade name Hykol) produced from chrome-tanned wastes (shavings). Samples of Hykol cross-linked with selected higher-molecular diepoxides were degraded with anaerobic sludge from a municipal plant at concentrations, in which organic carbon in samples in test bottles was approx. 200 mg/L, dry matter (inoculum) was 4.5g/L, temperature 35° C. Biodegradation of tested modified hydrolysate samples produced recorded quantities of biogas (CH_4, CO_2). Anaerobic degradation rate of the samples was least reduced when polyethylene glycol (600) diglycidyl ether was employed as the cross-linking agent, and most reduced when glycerol triglycidyl ether was applied. Attention was also given to preparation, the determination of solubility, intrinsic viscosity and IR spectra of prepared samples. In comparison with Hykol the degree of degradation decreased approx. from 70-50%. These modified hydrolysates were studied as materials which have the potential to be used as good biodegradable films, e.g. for agricultural applications.

Resumen

Este trabajo se centra en la influencia del reticulamiento por medio de glicoles diepóxidos de alto peso molecular (y glicerol-triglicidil-eter) sobre la degradación biológica anaeróbica del hidrolizado de proteínas (nombre comercial Hykol) producidos a partir de los desechos (virutas) de curtidos al cromo. Las muestras de Hykol reticuladas con los diepóxidos seleccionados de alto peso molecular fueron degradadas con barros anaeróbicos en una planta municipal, a concentraciones en el que el carbono orgánico fue aprox. 200 mg/L en las muestras de ensayos en botellas, materia seca (inóculo) de 4.5g/L, la temperatura 35° C. La biodegradación de las muestras producidas de hidrolizados modificados produjeron cantidades de biogás (CH_4, CO_2) que fueron registradas. La tasa de degradación anaeróbica de las muestras fue menos reducida cuando el polietilenglicol (600) diglicidil-éter fue utilizado como agente reticulante, y más reducida cuando el glicerol-triglicidil eter fue aplicado. También se prestó atención a la preparación, la determinación de la solubilidad y la viscosidad intrínseca y los espectros IR de las muestras preparadas. En comparación con Hykol el grado de degradación se redujo aprox. de 70-50%. Estos hidrolizados modificados fueron estudiados como materiales que tienen el potencial de ser usado como buenas películas biodegradables, por ejemplo, para aplicaciones agrícolas.

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INTRODUCTION

In a previously published article[1] we examined the similar question of biodegradability in connection with cross-linking of commercially produced protein hydrolysate by means of low-molecular diepoxides. In this present contribution, we concentrated to the same extent on higher-molecular diepoxides. The cross-linking mechanism in this case is virtually the same as with low-molecular diepoxides as already described in the mentioned work. Apart from linking of protein chains (real cross-linking), a reaction of merely one amino group and cross-linking agent (so-called masking) may also take place.

In connection with cross-linking with higher-molecular diepoxides (or even polyepoxides containing more epoxy groups), the research quoted in literature is mainly focused on utilizing bio-prostheses in medicine. Greatest attention was given to ethylene glycol diglycidyl ether (E) as to basic member in this series, or to compounds containing more ethylene glycol members and glycerol polyglycidyl ethers (G) with two or more epoxy groups. Tomihata et al.[2] compared various cross-linking agents of this kind in a reaction with gelatin and collagen; they shoved, beside others, improved mechanical properties. They also mention that biodegradation as well as solubility of cross-linked preparations is reduced with increasing network density. Sung et al.[3] tried to replace sterilization of bioprostheses (through heat or radiation) by employing E. They studied masking and also cross-linking, which renders bioprostheses more resistant to collagenases. After cross-linking with E arteries isolated from pigs, a heightened fixation index and denaturing temperature was observed as well as resistance to bacterial collagenases *in vitro*[4]. Tissues treated with monofunctional fixatives are more flexible than when multifunctional cross-linking agents[5] are applied. Other authors[6] demonstrated that splitting of peptide bonds is accompanied with lowered denaturation temperature and strength of samples but fixing with E improves these properties, a disadvantage here being a certain cytotoxicity[7] of epoxides.

Several researchers have reported on higher-molecular diepoxides and their effect on collagen materials [8-12]. Subjects chiefly discussed were collagen fixation [9,12] and mechanism of the reaction in question with particular view to optimizing processes in prosthetics. Tu et al.[10] describe masking at higher pH levels. Imamura[8] observed an anti-calcination action of diepoxides, whose positive influence on fixation of the aorta (with pigs) was confirmed by Shen et al.[11].

As is obvious from the presented survey, this reported research was almost exclusively focused on collagen tissues with prospective utilization of cross-linking to improve their mechanical properties.

EXPERIMENTAL

Chemicals and material

Glycerol triglycidyl ether (G), M_{eq} epoxy (equivalent weight per epoxy group) = (WPE) = 145 g/mol; Polysciences, USA (alternatively Denacol EX 313, Nagase Chemicals Ltd, Osaka, Japan). Ethylene glycol diglycidyl ether (E), M_{eq} epoxy (WPE) = 112 g/mol, Polysciences, USA (alternatively Denacol EX 810, Nagase Chemicals Ltd, Osaka, Japan). Polyethylene glycol (200) diglycidyl ether (PEG 200), M_{eq} epoxy (WPE) = 195 g/mol, Polysciences, USA (alternatively Denacol EX 821, Nagase Chemicals Ltd, Osaka, Japan). Polyethylene glycol (600) diglycidyl ether (PEG 600), M_{eq} epoxy (WPE) = 400 g/mol, Polysciences, USA (alternatively Denacol 841, Nagase Chemicals Ltd, Osaka, Japan). Protein hydrolyzate (further ProtH) – trade name Hykol E, produced by Kortan, Hradek n/Nisou, Czech Republic; nitrogen content 14.1%, molecular weight M_w approx. $1x10^4$ Da. All other chemicals were of analytical or pure quality, produced or distributed by Lachema (Brno, CZ).

Inoculum

Inoculation was performed with partly digested sludge from anaerobic stabilization operated at the municipal wastewater treatment plant at Zlin-Malenovice. Before use, sludge was left without access of air for 7 days at 35°C, the mechanical impurities were removed, and it was bubbled with nitrogen 10 min and centrifuged 10 min at 3,000 G. It was diluted with a mineral medium to dry matter content of about 4 g/L (approx. 2 g/L organic fraction).

Mineral medium

Medium was prepared according to internal instruction based on EN ISO 11734 and ASTM-D 5210-91 standards for testing biodegradability of organic substances under anaerobic conditions[1].

Samples and their preparation

Cross-linked samples were prepared from a solution of ProtH (dry matter 41.7%) in distilled water. Agents employed for cross-linking were glycerol triglycidyl ether (G), ethylene glycol diglycidyl ether (E), polyethylene glycol (200) diglycidyl ether (PEG 200), polyethylene glycol (600) diglycidyl ether (PEG 600). Cross-linking was performed at pH = 10 (after adding 12.1 M NaOH) in glass ampules for 3 days at a laboratory temperature of 22°C. Data on sample preparation are shown in Table 1.

Composition of samples corresponded to molar ratio of epoxy and amino groups below and also above gelation point. Mass ratio epoxide/ProtH dry matter is given by sample designation, for example, GTGE 08, meaning ProtH is cross-linked with a dose of 8.2% weight GTGE per ProtH dry matter. Neutralizing samples with acid before measuring in viscometer proved unnecessary because solution pH dropped to 7 after strong dilution with solvent (1M KCl).

TABLE I
Composition of samples (quantities of epoxide/ProtH and expected stoichiometric relations oxirane/-NH_2

Cross-linking agent	Sample	Cross-linking agent/ProtH [w/w]	Expected stoichiometric relation oxirane/ -NH2 [mol/mol]
GTGE	GTGE 08	0.082	0.527
	GTGE 16	0.164	1.054
	GTGE 25	0.247	1.581
	GTGE 32	0.329	2.107
EGDGE	EGDGE 07	0.073	0.606
	EGDGE 15	0.146	1.212
	EGDGE 22	0.219	1.818
	EGDGE 29	0.292	2.424
PEG 200	PEG 200/15	0.146	0.697
	PEG 200/29	0.292	1.394
	PEG 200/44	0.438	2.090
	PEG 200/58	0.584	2.786
PEG 600	PEG 600/15	0.146	0.695
	PEG 600/32	0.292	1.394
	PEG 600/48	0.438	2.090
	PEG 600/64	0.584	2.786

Calculation of expected stoichiometric ratio oxirane/-NH_2 is based on WPE values given by manufacturer (M_{eq} epoxy) and equivalent weights of ProtH per one amino group (estimate of expected quantity of amino acids in ProtH chain M_{eq} of -NH_2 = 930 g ProtH dry matter per 1 mol -NH_2 groups [13]).

Apparatus and equipment

The laboratory glassware and equipment were of domestic and foreign origin. The analyzer used was Micro-Oxymax by Columbus Co., Columbus, Ohio, USA. Spectral analyses were performed on UV-VIS spectrometer UNICAM UV 500, Thermo Electron Corp., Cambridge, U.K. and on FT-IR spectrometer Avatar 320, Nicolet Instrument Corp., Madison, WI, USA (with single-reflex ZnSe ATR "Miracle"). Viscosity data were obtained with an Ubbelohde, type U2, viscometer, by Kavalier Glassworks, Držkov, Czech Republic. Carbon analyses were run on total organic carbon analyzer TOC-5000A, Shimadzu Corp., Australia. The refrigerated centrifuge MR23i used was Jouan, S.A., Saint-Herblain, France.

Methods

Determining dissolution rate

Suspensions were prepared (0.1g dry matter weighed per 20 mL distilled water) from all cross-linked samples including pure ProtH. Dissolution was surveyed under stirring at temperature 22° C. Samples in 4-mL quantities were taken from prepared suspensions at time intervals of 10, 30, 60, 90 and 120 min. The withdrawn quantity was filtered through medium density filter paper which had been thoroughly washed with distilled water and dried. Filtrate was diluted fifty-fold and analyzed on carbon analyzer.

Fixation Index

Fixation index (FI) is the per cent fraction of amino groups reacted with respective cross-linking agent. A higher value of fixation index signifies a lower number of residual free amino groups and thus higher cross-linking degree of employed material [14].

$$FI = \frac{NH_2\,(v) - NH_2\,(z)}{NH_2\,(v)} .10C \quad \textbf{(1)}$$

where

FI is fixation index (fraction of reacted amino groups) [%]

$NH_2(v)$ quantity of free amino groups in uncross-linked sample (before reaction) [mmol/g]

$NH_2(z)$ quantity of free amino groups in cross-linked sample (after reaction) [mmol/g]

The ninhydrin method was used to determine free amino groups; the conditions were described in a previously published article [1].

Determining Intrinsic Viscosity (IV)

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

$$[\eta] = \lim_{c\_0} (_{\eta spec}/c) \quad \textbf{(2)}$$

where c is concentration of polymer solution; specific viscosity (η_{spec}) is given by relationship

$$\eta_{spec} = \eta_{rel} - 1 \quad \textbf{(3)}$$

Relative viscosity of solution is defined as

$$\eta_{rel} = \eta / \eta_o \quad \textbf{(4)}$$

where η is viscosity of solution and η_o viscosity of solvent. IV depends on molecular weight of polymer in solution according to the Mark-Houwink equation

$$[\eta] = K * M^{a} \quad \textbf{(5)}$$

where

M is molecular weight of polymer,

K and a are constants for a certain polymer-solvent system and temperature.

Dependencies η_{spec} /c on c are nonlinear in systems with greater interaction, that is, in polar systems. Determining [η] is difficult in the case of a polyelectrolyte where dependence η_{spec} /c on c is very nonlinear. Linearity is much improved by increasing ionic strength with added indifferent electrolyte that suppresses mutual interactions of groups in a chain, or it is recommended to extrapolate [η] to infinite ionic strength (I) in coordinates of [η] and $1/\sqrt{I}$; $[\eta] = [\eta]_{\infty} + k / \sqrt{I}$. From this results the demand to maintain a constant, if possible high, ionic strength. For determining [η] of polyelectrolyte, it is recommended to maintain a constant level of pH = pI (isoelectric point) and greater ionic strength, at least I = 0.05 M, preferably 0.5 M. Literature does not mention any particular problems when determining IV of gelatin, the value given for 0.5 M KCl at 20°C is $[\eta] = 4.07 * 10^{-4} * M^{0.52}$ [dL/g], [g/mol]; ProtH should behave likewise. With its value of $M = 1*10^4$ g/mol, we assume [η] = 0.05 dL/g.[13]

When seeking an optimum solvent for measuring sample solutions viscosity, tests were run on influence of pH and ionic strength on IV at a temperature of 25°C. Dependence η_{spec} / c vs. c of ProtH aqueous solutions was strongly nonlinear; for this reason, ProtH was measured when dissolved in 0.5M KCl or 1M KCl.

Measuring IR spectra

Samples prepared by cross-linking ProtH with respective epoxide were dried at 100°C, crushed in agate bowl to fine powder and kept in desiccator over silica gel at temperature 22°C. For actual measurement, sample was spread onto a single-reflex ATR ZnSe crystal to which it was compressed with a force of approx. 40 N over an area of about 16 mm^2 to achieve maximum contact of sample with crystal. Spectra of background and sample were scanned by 32 scans at 4-cm^{-1} resolution.

Determining degree of anaerobic degradation

Sludge diluted with mineral medium was filled into test bottles (volume approx. 250 mL); tested sample was then added, in a total organic carbon concentration (TOC) of approx. 200 mg TOC/L, i.e. 20 mg TOC per test bottle. Bottles were bubbled through with nitrogen, rapidly closed and attached to measuring apparatus. The bottles were placed on an electromagnetic stirrer immersed in water bath at a constant temperature of 35°C, and stirred at a rate of 300 rpm. [15]

Gaseous products arising during anaerobic degradation of tested samples were analyzed for CH_4 and CO_2 contents by means of analyzer Micro-Oxymax, with detection based on single-beam infrared detector. Atmospheres in bottles are periodically (at selected time intervals) circulated through cooler and drying tower to gas sensors, where their concentrations are determined and volumes of produced gases calculated.

Total weight of metabolized carbon of a given sample (after subtracting the blank) corresponds to quantity of inorganic carbon (IC) in aqueous phase and quantity of carbon in produced bio-gas. The extent of total biodegradation is described by equation

$$D_t = (m_t / m_v) * 100 \quad \textbf{(6)}$$

where

D_t is total biodegradation expressed as percentage [%]

m_t total metabolized carbon (biogas + increment of IC in solution after experiment) [mg]

m_v organic carbon in tested sample at start of experiment [mg]

Extent of biodegradation may also be expressed by means of the degree of theoretically attained methane yield, as

$$D_{t\,CH4} = (m_{CH4} / m_{CH4\,th}) *100 \quad (7)$$

where

$D_{t\,CH4}$ is degree of theoretically attained methane yield [%]

m_{CH4} quantity of methane produced during biodegradation (by experiment test) [mg]

$m_{CH4\,th}$ theoretically expected production of methane [mg]

RESULTS AND DISCUSSION

Dissolution rate

Procedure for determining dissolution rate during 2-hour contact with aqueous environment, given in the Experimental section, yielded results similar to those in Fig.1.

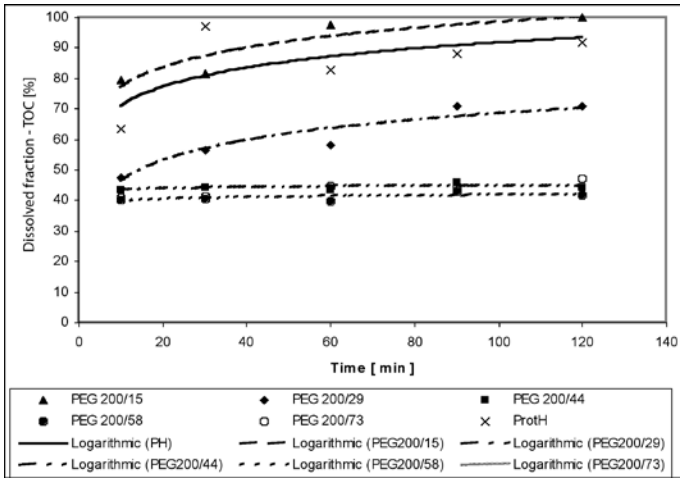

Figure 1: Dissolution kinetics of ProtH samples cross-linked with polyethylene glycol (200) diglycidyl ether

Dissolution of samples cross-linked with the other employed cross-linking agents exhibited same trends and similar courses during the first 120 min. Least soluble were samples cross-linked with GTGE, most soluble were those cross-linked with PEG (600). The influence of increasing quantity of cross-linking agent on decreasing dissolution rate and dissolution is quite obvious. Individual points were interpolated by logarithmic regression y = a + b* ln (x). Samples with PEG 600 and E were somewhat more soluble. However, viewed otherwise, for instance from the perspective of mechanical resistance, glycerin triglycidyl ether could be regarded as the most suitable cross-linking agent for its higher number of functional groups and thus potential formation of a stronger network in sample; this was confirmed by solubility measurements.

Formation of a network requires using a substance with a greater number of cross-linking functional groups. If functionality of cross-linking substances is only slightly greater than 2, they have to be mixed in an accurate stoichiometric ratio and the reaction must run to high conversion; otherwise a network cannot form. As ProtH does not exhibit overly high functionality, a network might not form as long as bis-epoxide is applied in an inconvenient stoichiometric ratio or the reaction does not proceed to high conversion. Employed diepoxides may be polluted with monofunctional substances or even their WPE may rise through homopolymeration and or a non-stoichiometric mixture get prepared. Even hydrolysis of ProtH may occur and an increase in content of monofunctional substances in ProtH able to react with epoxide. The substantial degree of collagen hydrolysis when producing ProtH (comparatively small molecules and thus low functionality when compared, for example with gelatin) is accordingly the principal cause of trouble when realizing a network.

Fixation index

Values of FI are shown in Fig.2. It is apparent that FI grows with increasing addition of employed cross-linking agent.

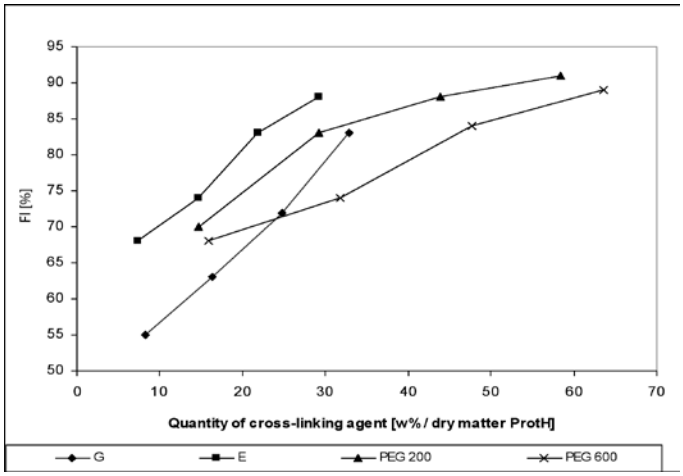

Figure 2: Dependence of FI of samples on quantities of cross-linking agents

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 i.e. the amino group is either mono- or bifunctional). b) colored products formed by reaction of analytical agent with non-reacted amino groups have the same extinction coefficient at selected wavelength, independently of amino group location (type of amino acid).

Values of FI are not as expected. When both conditions a and b are met, FI should increase linearly with epoxide dosage. With epoxide in excess, FI should attain 100%. 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%.

When FI yields correct values, it should enable to determine 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, possible kinetic and steric limitations are not substantial. When comparing changes in fixation indices (Fig.2), cross-linking agent E appears to be the best. As many as 67% free amino groups already get occupied at low concentrations (7% wt.), as opposed to the relatively "worst" cross-linking agent G which at a same concentration displays a 12-% lower FI value. But that is not quite in accord with WPE values quoted by manufacturer. The curve for G should lie between values for E and PEG 200.

Determining intrinsic viscosity

Buffer 1M $NaHCO_3$ + 1M K_2CO_3 precipitated ProtH in the system and could not be applied. When 1M KCNS was used as solvent, linearity of graphical extrapolation to IV values was not better than when 1M KCl was applied. Accordingly, 1M KCl at pH = 7 appeared as the most satisfactory solvent. Examples showing extrapolation [η] = [IV] from dependency η_{spec} /c (on axis) vs. c with ProtH samples cross-linked with EGDGE are shown in Fig.3.

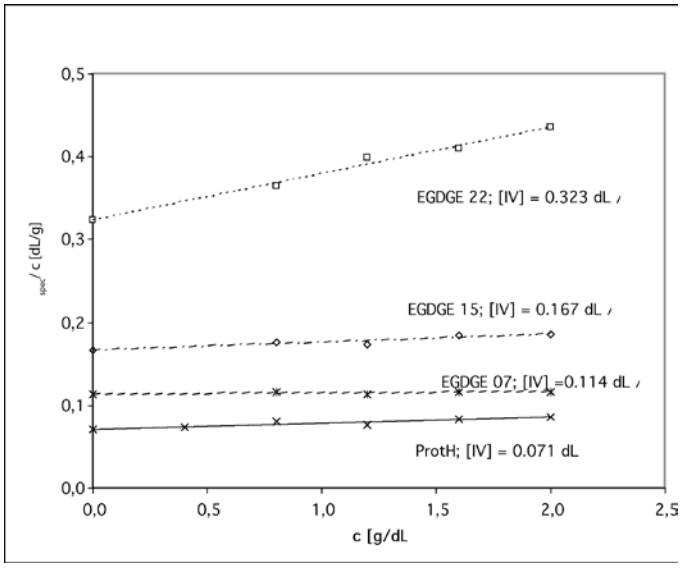

Figure 3: Extrapolation [η] = [IV] from dependence η_{spec} / c vs. c (on axis η_{spec} / c) of ProtH and samples EGDGE series dissolved in v 1M KCl (25°C)

Trends of the dependence were similar when different cross-linking agents were employed. Values of obtained intrinsic viscosities are summarized in Table 2 for all measured systems.

Table 2 indicates an obvious trend of intrinsic viscosity to grow with increasing additions of epoxide cross-linking agents. Samples containing greatest epoxide additions (except for PEG 600) could not be measured because they passed from solution to gel (cross-linking took place to greater extent than expected). From samples that immediately cross-linked to gel it was not possible to easily prepare a solution by extracting soluble fractions and determining its concentration for evaluating outflow times. IV of ProtH is naturally the lowest because no linking of ProtH molecules through epoxide cross-linking agent occurred. The objective was also to determine epoxide dosage needed to attain gelation point. It is interesting to determine how markedly IV in the vicinity of gelation point changes, or how viscosity of a system with composition approaching gelation point alters with time, i.e. whether cross-linking under given conditions does not proceed too slowly or even distinct hydrolysis of proteins occurs.

From IV measurements it follows that sample IV, when close to gelation point, strongly depends on epoxide dosage. Distance to gelation point may be assessed from magnitude of ratio IV_{sample}/ IV_{ProtH}; if greater than 3, the system is quite near gelation point. Before gelation point, mean molecular weight of ProtH increased approx. tenfold due to cross-linking. Assessing the distance to gelation point by merely stating a loss in fluidity is less helpful than knowledge of system viscosity or system IV. Gelation point may also be approached in systems containing excess epoxides. When functionality of ProtH is low, the interval between these gelation points is narrow.

An interesting value is the dosage of epoxide for achieving maximal cross-linking degree (minimal sol content). That is equivalent to knowing real weight of ProtH pertinent to one amino group, or also to knowing WPE of cross-linking agent (equivalent weight per epoxy group). If epoxide dosage is higher, the system contains free epoxy groups, which may prove undesirable. Employing IR spectra, determining free epoxides present in slightly surplus quantities in systems cross-linked with glycidyl ethers is not easy. Because the constants of the Mark-Houwink equation for this ProtH are not known - particularly not so for cross-linked samples, determining molecular weight of samples was not possible.

IR spectra

Cross-linking of proteins with epoxides utilizes ability of epoxides to react with groups containing an active hydrogen, for example, -NH_2, -SH, -COOH, -OH. The epoxide ring, rich in energy, opens at the same time to form hydroxy

TABLE II
Intrinsic viscosity [IV] = [n]; [dL/g] of samples (25°C, 1M-KCl, pH = 7)

A-Sample	GTGE 08	GTGE 16	GTGE 25	GTGE33	
$B = [IV] = \lim_{c \to 0} (n_{spec}/c)$	0.109	0.144	0.247	-	
A	PEG 200/15	PEG 200/29	PEG 200/44	PEG 200/58	
B	0.103	0.16	0.275	-	
A	PEG 600/16	PEG 600/32	PEG 600/48	PEG 600/64	PEG 600/80
B	0.078	0.091	0.111	0.116	0.119
A	EGDGE 07	EGDGE 15	EGDGE 22	EGDGE 29	
B	0.114	0.167	0.323	-	
A	ProtH				
B	0.071				

ethers. Instead of a tight (formally ether) bond in epoxide a less tensioned ether bond is formed, which may be distinguished in the spectrum from epoxide. In an acid environment, homopolymeration proceeds transforming epoxide into polyether. Presence of cross-linking agents and chemical reaction leads to changes in spectra, as long as new bands do not strongly overlap with spectral bands of other C-O bonds (e.g. in alcohols). Bands corresponding to hydrogen in $-NH_2$ and -OH groups are usually very wide, their position is dependent on concentration, environment, temperature, etc. They may also overlap with bands of water (bound or moisture). Very similar spectra are displayed by samples cross-linked with PEG 200 (Fig.4) and PEG 600. Similar enough to them are spectra of samples cross-linked with EGDGE and GTGE.

From spectra of epoxides only (pure cross-linking agents) it follows that in the range of wave numbers 838, 912 and 1,250 cm^{-1} there should appear peaks of epoxide bond (cyclic

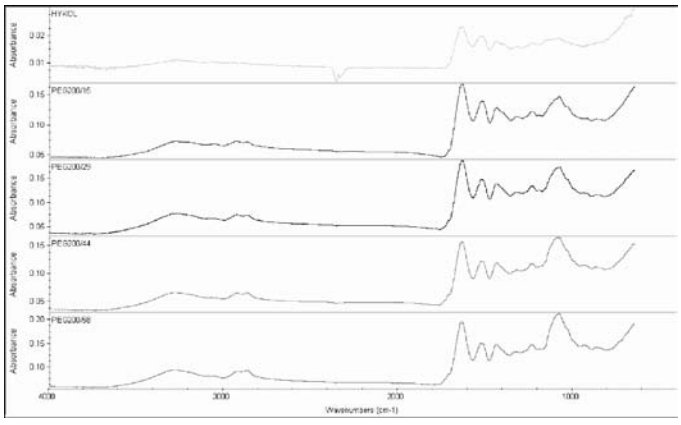

Figure 4: IR absorbance spectra of ProtH and samples of PEG 200 series

ethers). Only in the case of high epoxide concentrations in samples there should be found peaks of epoxides that are in excess (non-reacted). However, epoxide bond peaks strongly overlap with peaks of ether bonds belonging to cross-linking agents themselves.[16] In the vicinity of wave numbers 1,080 cm^{-1} a vibration of the C-O alcohol bond is found. Bonds of this kind are produced by cross-linking and their peaks in cross-linked samples should increase. Spectra indicate that peaks of these bonds are apparently on the increase.

The most significant peaks for ProtH are found in the 1,400 – 1,650 cm^{-1} range of wave numbers. The height of these peaks should display a decreasing tendency with increasing quantity of epoxides. Representative spectra were not standardized and, therefore, the decrease of these peaks was not quite distinct. Peaks in the 2,300 cm^{-1} range of the ProtH spectrum can be attributed to CO_2 , a mere impurity in the background. Further to be seen in the spectra are peaks belonging to the C-H bond in the 2,900 cm^{-1} range of wave numbers, and wide peaks of -NH- bonds in amines and of -OH bonds of water or hydroxy ethers.

Anaerobic degradation of soluble fractions of cross-linked samples (experiment 1)

Anaerobic degradation of soluble fractions of ProtH samples cross-linked with various epoxides was an experiment where all prepared samples were measured, except for those most cross-linked exhibiting very low solubility. Two further experiments set up were blanks (bottles containing sludge without substrate). Sodium acetate in two bottles served as model substance. All 30 measuring positions in the Micro-Oxymax analyzer were occupied. Employed inoculum was anaerobic sludge from the municipal wastewater treatment

TABLE III
Dissolved fractions of ProtH samples cross-linked with various epoxides

Sample	G 08	G 16	G 24	G 32	E 07	E 15	E 22	E29
Dissolved fraction [%]	100	86	76	53	100	100	100	75
Sample	PEG 200/15	PEG 200/29	PEG 200/44	PEG 200/58	PEG 600/16	PEG 600/32	PEG 600/48	PEG 600/64
Dissolved fraction [%]	100	92	71	75	100	100	100	100

plant; dry matter of raw sludge was 20.3 g/L and was dosed so that its concentration in test bottles was 4.5 g/L. Suspensions of samples were prepared (weighed-in quantity 0.2 g/ 50 mL), and were next subjected to sonic treatment lasting 1.5 hours that helped – at least partly – to dissolve samples. Solutions of samples were then filtered and the fraction of dissolved quantity was determined (Table 3).

Before determining dissolved carbon values on the mentioned carbon analyzer, the solid fraction of sludge suspension was separated by centrifugation at 20,000 G for 10 min at 15°C. For determining degradations accurately, filtrate was dosed into measuring bottles so that final TOC concentration was approx. 200 mg/L. The experiment was terminated after 234 hours. TOC and IC values were determined both at the start (time t = 0 h) and end of experiment (t = 234 h). Calculation of D_t utilized the classic balance of carbon in gaseous and liquid phase. Inorganic carbon virtually corresponds only to dissolved CO_2 and HCO_3^- because solubility of CH_4 under given conditions is negligible. Values of D_t are shown in Fig.5.

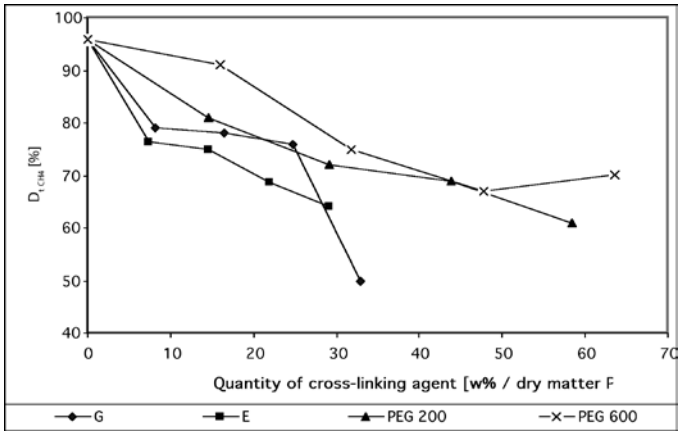

Figure 5: Experiment 1. Biodegradation degree (234 h, 35°C) dissolved fraction (sol) of ProtH cross-linked with various cross-linking agents

When comparing total degradations (Fig.5) it is obvious that samples best biodegradable were cross-linked by means of PEG 600. Due to their complete solubility (see Table 3), their actual cross-linking may be well regarded with doubt. Values of fixation indices in Fig.2 for samples cross-linked with this epoxide prove that reactions of amine groups really occurred, but it does not signify a network was formed. During the build-up of a network, IV of measured samples would increase, their complete solubility would disappear with formation of network, a gel fraction would appear and dissolution rate of these samples would go down. A higher degree of reactions in the sample should produce visible changes in IR spectra of cross-linked samples.

Anaerobic degradation of mixtures of soluble and insoluble (sol + gel) fractions of cross-linked samples (experiment 2)

Anaerobic degradation of these ProtH samples cross-linked with various epoxides was an experiment all cross-linked samples were subjected to, including samples with greatest contents of cross-linking agent as well as two blank tests. Sodium acetate in two measuring points again served as model substance. Employed inoculum was anaerobic sludge; dry matter in test bottles was 4.5 g/L. Suspensions of samples (weighed-in quantity 0.25 g/l0 mL) were subjected to sonic treatment. This lasted 10 min and helped – again only in part – to dissolve samples. Samples including insoluble fraction were then dosed into test bottles so that final concentration of starting TOC of sample (sol + gel) was 0.2 g/L.

Due to incomplete sample solubility and hence to the impossibility of determining starting TOC values with accuracy, the calculations used theoretical values of TOC per bottle (from weighed-in quantity and elemental analysis). Calculations of total degradations applied only values obtained from the methane analyzer because IC values exhibited great scatter. The test was terminated after 325 hours. CH_4 values were read off at time t = 125 hours to suppress drift of apparatus signal. We assumed all accessible substrate carbon should have been consumed by this time. The potential toxic effects of employed cross-linking agents

were verified with a dose of 10 μL cross-linking agent per 100-mL content of bottle. This quantity was selected to represent an estimated top limit dose of potentially free cross-linking agents.[17] As obvious in Fig.6, the employed cross-linking agents degraded quite well. Inhibition did not occur as CH_4 production was greater than with the blank. Hence, cross-linking agents at the tested concentrations did not produce a toxic effect and could be utilized by anaerobic microorganisms as a source of carbon.

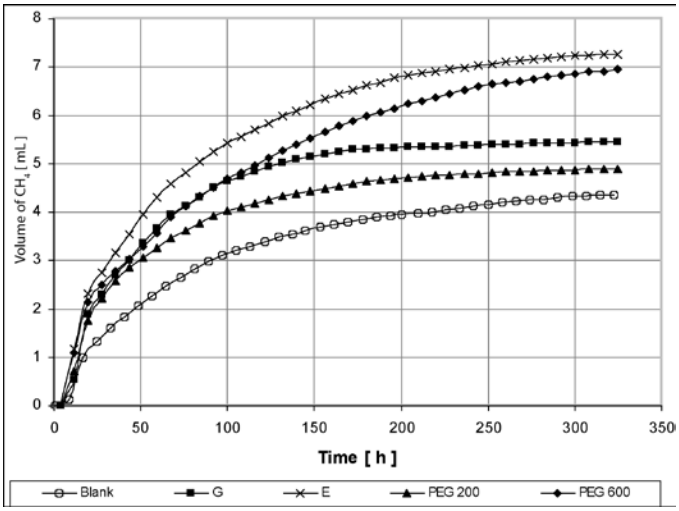

Figure 6: Production of CH_4 - toxicity testing of epoxides used as cross-linking agents (10 μL epoxide per bottle)

It appeared - the same as when examining biodegradation of soluble fractions of samples - that the most readily biodegradable samples are those cross-linked by means of PEG 600 (Fig.7). Total biodegradations were evaluated after 125 hours on all samples, because CH_4 production in most cases did not further increase as opposed to the previous experiment, when total biodegradation was evaluated at the very end of anaerobic degradation after 234 hours.

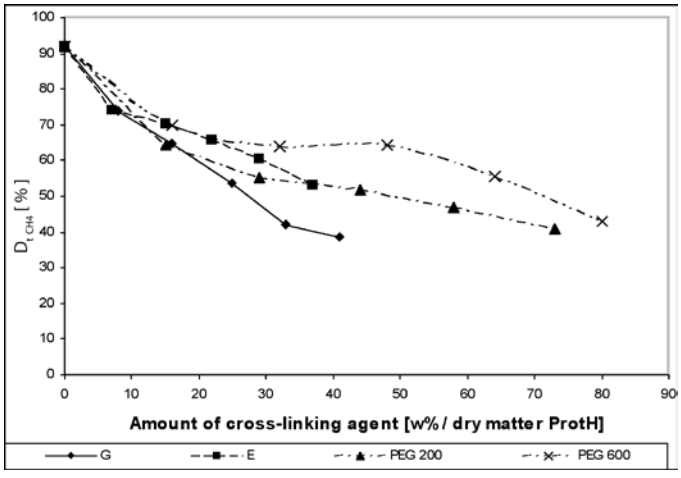

Figure 7: Experiment 2. Degree of theoretical production of methane by samples of ProtH cross-linked with various epoxides (gel+sol) after 125 h, 35°C; for NaAc $D_{t\,CH4}$ = 100%

It again follows from the dependence that with increasing cross-linking of ProtH, its biodegradation capability decreases, which we interpret as reduced ability of microorganisms to penetrate into structure of the modified protein and to degrade it biologically. Uncross-linked ProtH exhibits greatest biodegradation. Cross-linked samples display greatest biodegradation as samples containing lowest fraction of epoxides, and that with all kinds of employed cross-linking agents. Biodegradation was worse when GTGE cross-linking was applied than that seen by applying PEG 600. When comparing total degradations it is obvious that samples most readily biodegradable were those cross-linked by means of PEG 600 because of their faster and complete solubility.

CONCLUSIONS

This research examined the influence that cross-linking ProtH with selected diglycidyl or triglycidyl compounds exerted on biological anaerobic degradation. Suitable cross-linking conditions selected from those under survey were in the region of pH = 10 and 22°C. Samples exhibited very different solubility which decreased with increasing dose of cross-linking agent; solubility was lowest when GTGE was used, greatest with PEG 600. When determining the number of free amino groups (FI by ninhydrin method) obtained values did not meet the expectation that they would linearly change with dose of cross-linking agent. The most convenient solvent for evaluating viscosity measurements of sample solutions was 1M KCl; from these measurements it followed that IV of samples near gelation point grows much more quickly than dosage of diepoxides. From measurements of FTIR spectra of pure cross-linking agents it followed that absorption peaks of the epoxide bond (cyclic ethers) should be found in the region of wave numbers 838, 912 and 1,250 cm^{-1}. Wave numbers at which typical epoxide group peaks should appear were found by comparing spectra of 6 epoxide cross-linking agents (diepoxybutane, diepoxyoctane, PEG 200, PEG 600, EGDGE and GTGE). If the epoxide concentrations in samples are high, peaks of epoxides in excess (or unreacted) should be visible. Epoxide peaks strongly overlap with peaks of ether bonds of cross-linking agents themselves, a merely slight growth of peaks from spectra in the wave number range mentioned above is perceptible and that only at highest doses of cross-linking agents. Determining the extent of biodegradation under anaerobic conditions confirmed the assumption that biodegradation decreases with an increasing quantity of cross-linking agent. If samples were assessed from the viewpoint of readiness to biodegradation, polyethylene glycol (600) diglycidyl ether would turn out as the most suitable, conversely, glycerin triglycidyl ether as the worst. For further research into these problems, it would be appropriate to investigate cross-linking mechanism in greater detail, to employ strictly bi- or trifunctional cross-linking agents and analyze epoxy groups, i.e. to know also epoxide

concentration; possibly also to use convenient bifunctional or multifunctional amines and measure critical gelation conversions or stoichiometric ratios of defined substances.

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REFERENCES

1. Pšeja, J., Hrnčiřík, J., Kupec, J., Charvátová, J., Hruzík, P. and Tupý, M., J. *Polymer. Environ.* **14** (3), 231-234, 2006
2. Tomihata, K., Burczak, K., Shiraki, K. and Ikada, Z. *Am. Chem. Soc.* Chapter **24**, 275-286, 1994
3. Sung, H.W., Hsu, H.L. and Hsu, Ch.S.*J. Biomed. Mater. Res.* **37**, 376-383, 1997
4. Sung, H.W., Hsu,Ch.S.and Lee,Y.S. Biomaterials 17, 2357-2365, 1996
5. Sung,H.W., Hsu, H.-L., Shih, Ch.Ch. and Lin D.S. *Biomaterials* **17**, 1405-1410, 1996
6. Sung H.W., Chen W.Z., Tsai, Ch.Ch. and Hsu, H.L. J. *Biomater. Sci. Polymer Edn.* **8**, 587-600, 1997
7. Sung, H.W., Huang, R.N., Huang, L.L.H., Tsai, Ch.Ch. and Chiu, Ch.T. *J. Biomed. Material. Res.*.**42**, 560-567, 1998
8. Imamura, E., Sawatani, O., Koyanagi, H., Noishiki, Z. and Miyata, T.*J. Card. Surgery* **4**, 50-57, 1989
9. Tu, R., Lu, C.L., Thyagarajan, K., Wang, E., Nguyen, H., Shen, S., Hata, C. and Quijano R.C. *J. Biomed. Materials Res.* **27**, 3-9, 1993
10. Tu, R., Quijano, R.C., Lu, C.L., Shen, S., Wang, C., Hata, C.and Lin, D.: *Int. J. Artificial Organs* **16**, 537-544, 1993
11. Shen, S.H., Sung, H.W., Tu, R., Hata, C., Lin, D., Noishiki, Y. and Quijano, R.C. *J. Appl. Biomaterials* **5**, 159-162, 1994
12. Tu, R., Shen, S.H., Lin, D., Hata, C., Thyagarajan, K., Noishiki, Z. and Quijano, R. *J.Biomed. Material Res.* **28**, 677-684, 1994
13. Hrnčiřík, J., Dvořáčková, M., Hruška, F., Kupec J. and Kolomazník, K. J. *Am. Leather Chem. Assn.* **100**, 1-7, 2005
14. Kawahara, K., Yakube, Z., Ohide,T. and Kida, K. *Chemosphere* **39**, 2007-2018, 1999
15. Pšeja, J. *Diploma Thesis* (in Czech), 2003, FT UTB in Zlín, ZLÍN,Czech Republic
16. Nosková, R.: *Diploma Thesis* (in Czech), 2006, FT UTB in Zlín, ZLÍN, Czech Republic
17. Olšák, J.: *Diploma Thesis* (in Czech), 2006, FT UTB in Zlín, ZLÍN, Czech Republic