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Hydrogels of collagen hydrolysate cross-linked with dialdehyde starch

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Abstract Processing hydrogels of collagen hydrolysate (H) cross-linked with dialdehyde starch (DAS) by dipping or casting into biodegradable materials for various applications, is complicated by their marked tendency to aging. One-hour action by temperatures at 60–90 °C reduces sorbed water content in hydrogels by approx. 12%; dependence of the extent of this reduction on temperature (within the mentioned range) was not detected. Effect of thermal action on duration of their disintegration in an aqueous medium and on its pH (within limits 4.8–7.4) was not found either, neither on their gel–sol transition temperature. This supports the view that aging is caused by time-dependent increasing network density of inter-chain hydrogen cross-links. The given temperature interval is satisfactory for processing hydrogels through technologies currently used in processing synthetic plastics (compression molding, injection molding).

Keywords Biodegradable packing materials · Collagen hydrolysate · Dialdehyde starch · Hydrogels

Introduction

Collagen protein, which makes up 20–30% weight of slaughterhouse animals [1], is of relatively minor importance for producing foodstuffs and animal feed, above all for its low nutritive value and deficit in essential amino acids. The essence of its industrial use remains in manufacturing flat materials (leathers) for the clothing, footwear or fancy goods industry. Nevertheless, only about 40–50% weight starting raw hides is thus currently appreciated; hence, production of protein (collagen) waste is considerable. A certain proportion of collagen proteins from meat production is processed into biodegradable and edible casings for meat products or for gelatin and glues. Collagen materials for medical purposes (plasma expanders, haemostatic materials, surgical thread, materials for reconstructive surgery, etc.) are also important products, but volume of collagen waste thus processed (compared to production of protein waste) is substantially smaller. Further sources of collagen proteins (for example, short and long tendons of slaughterhouse cattle) are minimally utilized in industry as a starting raw material and are rather treated as a difficultly applicable meat production waste. Quality of meat products of collagen origin, the same as economics of their manufacture, are to a large extent affected by crosslink density in original collagen, which grows with the age of slaughterhouse animals or is increased on purpose during collagen industrial processing. Refined collagen exhibiting low cross-link density yields through partial hydrolysis with water at temperatures of 60–70 °C superior quality gelatin with high gel rigidity. Higher cross-link density of starting collagen raw material requires conducting hydrolysis at higher temperatures (80–95 °C) and increases extent of hydrolysis of collagen chain peptide bonds. In practice, this shows through a reduced mean molecular weight of obtained gelatin and reduced rigidity of its gel [2], and limits its practical employment. Products of a lower mean molecular weight are then more utilized in technical practice as adhesives for polar, fibrous adherents (glues) or as protective colloids.

An overly high crosslink density causes unsatisfactory mechanical properties of leathers (excessive rigidity), inconvenient rheological properties of edible fibrous

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casings for meat products, and of other collagen products. Processing collagen products displaying unsatisfactory properties and also collagen waste from their manufacture has to be preceded by controlled hydrolysis which, if combined with present manufacturing processes, can lead to environmentally and economically interesting, virtually no-waste production procedures. It is advantageous to perform hydrolysis through biotechnological procedures typical for their minimal energy demand, which use commercially readily available proteases of microbial origin.

Controlled enzymatic hydrolysis was employed to resolve the controversial requirement of high gelatin quality while preserving high yield. It was proposed to combine extracting collagen raw material with water at a temperature of max. 70 °C, with controlled hydrolysis of extracted collagen residue to collagen hydrolysate that is utilizable to greater economic effect than glue in manufacture of acylamide type (Lamepon type) surfactants or as humectants in cosmetic formulations for skin treatment [3].

Controlled enzymatic breakdown technique was applied to processing both collagen waste from chrome-tanned leather manufacture, and to waste from casings manufacture with overly high crosslink density [4]. Obtained collagen hydrolysates exhibit, as compared to gelatin, lower mean molecular mass (20–35 kDa) and a wider distribution [5]. Their aqueous solutions form hydrogels only at dry matter concentrations of 28–52% (w/w), which puts them in some industrial applications at a disadvantage against gelatin. Lower mean molecular weight of such hydrolysates and the higher hydrophilicity associated with that may be eliminated by cross-linking protein chains of hydrolysates with specific enzymes (for example, aminotransferases of bacterial origin *Streptoverticilium* sp., *Streptomyces* sp. and others) or low-molecular aldehydes (for example, formaldehyde, glutaraldehyde) [6–9]. However, aldehydes chiefly interesting are polymeric; as opposed to low-molecular aldehydes they are biocompatible, displaying virtually zero toxicity to live organisms. Various authors proposed employing dialdehyde starch [10], alginate dialdehydes [8], dialdehydes derived from various dextrans, often prepared immediately in reaction mixtures through partial oxidation [9, 11].

Draye and coworkers [11] were probably the first to observe aging of such hydrogels, showing through slower dissolution in an aqueous environment and associated slower release of active substances from biodegradable packings of such kind. Aging of these hydrogels, according to the cited authors, shows most distinctly during the first week of storage. On the other hand, inclination to aging complicates dipping and casting technologies currently used for manufacturing biodegradable protein-based packing materials.

Aging of gelatin gel was detected already earlier through dynamic measurements by Nijenhuis [12]. Kozlov and Burdygina [13] demonstrated that gelatin containing less than 2% (w/w) water behaves like a fragile, highly cross-linked polymer and dissolves poorly in water, and ascribed this altered behavior to higher density of inter-chain hydrogen bonds due to excessively reduced water content. This aging process is obviously less marked than with collagen hydrolysate cross linked with DAS, and that is why mentions concerning aging of soft (SGC) or hard (HGC) gelatin capsules associated with their slowed disintegration in an aqueous environment were not found.

Hydrogels of collagen hydrolysate cross-linked with dialdehyde starch reveal that their aging, in addition, is accompanied with a loss in thermo-reversibility: hydrogels prepared from a 25–27% (w/w) collagen hydrolysate (w/w) and 15–20% dialdehyde starch (related to weight of hydrolysate in mixture) preserve thermo-reversibility for approx. 300 h, with gel rigidity gradually increasing. Exceeding mentioned limits leads to thermo-irreversible gels in a considerably shorter time (approx. 5–10 h). Their solubility in an aqueous environment is also considerably retarded, but keeps retained. The loss in thermo-reversibility as well as retarded dissolution, consequent of hydrogel aging, were thus attributed, the same as with gelatin, to (time-dependent) increasing density of hydrogen bonds in hydrogels [14, 15].

Distinct inclination of these hydrogels to aging becomes the limiting factor when processing them into biodegradable or biocompatible products through the most customary technology of dipping; hence, procedures winning interest are technologies usual in synthetic plastics processing (compression molding, injection molding, and extrusion).

Exposure of hydrogels to higher temperatures during their processing into biocompatible or biodegradable products may affect their aging process as well as disintegration (dissolution) of produced materials in an aqueous environment, which is regarded as their significant parameter. The mentioned effects were studied to greater detail in the presented work.

Experimental and results

Starting materials

Collagen hydrolysate (H) was prepared by controlled enzymatic hydrolysis of collagen waste from meat casings production according to [4]. Basic characteristics of starting hydrolysate may be seen in data of Table 1.

Dialdehyde starch (DAS)—CAS 9047-50-1, obtained by oxidizing starch. Sigma-Aldrich Biochemicals and Reagents, 2004–2005, p. 1569, No. P 9265.

Hydrogels were prepared by cross-linking collagen hydrolysate (H) with dialdehyde starch (DAS) to provide

Table 1 Basic characteristics of enzymatic hydrolysate of collagen waste from casings manufacture

Dry substance, %	92.99
Amide nitrogen in dry substance, %	14.85
Ash in dry substance, %	4.94
Ca content in dry substance, ppm	27456.62
Mg content in dry substance, ppm	4798.00
Primary amino-groups in dry substance, mmol NH_2 per g	0.216
Av. molecular mass (numerical mean, M_N), kDa	17.75

for both thermo-reversible and thermo-irreversible gels [14]. Composition of reaction mixtures may be seen in data of Table 2.

Working procedure

Weighed amount of H (corresponding to 25–30% solution of 20-mL volume) was dissolved in 10 mL water. Solution pH level was adjusted with 1 N NaOH to 11.0 and the solution was left to stand overnight to eliminate possible buffer effect of hydrolysate. Subsequently, solution pH level was rechecked and adjusted if necessary. Volume was filled to 20 mL, and under good stirring in a water bath heated to 60 °C the amount of DAS corresponding to 10–20% per H content in solution was gradually added, always in doses of 0.1 g. A following dose was always added on complete dissolution of previous dose. After adding all DAS, reaction mixture was heated another 60 min, then left to cool freely to room temperature (25 °C). Gel was obtained by cooling the reaction mixture to 10 °C for 12–16 h in a refrigerator.

Hydrogels were evaluated through methods of thermal analysis: thermogravimetry (TG) and differential scanning calorimetry (DSC). Derivative TG curves of gels (TGA Q500—TA Instruments, New Castle, Del, U.S.A.—at $dT/dt = 5\ °C\ min^{-1}$, $\Delta T = 25–200\ °C$, N_2 flow rate = 150 mL min^{-1}) show 2 maximums in temperature ranges 35–60 °C and 102–155 °C. Actual TG curves reveal a noticeable decrease in weight of measured gel samples up to temperatures of 150–160 °C, which is not accompanied with pronounced peaks on the derivational record. Thermal breakdown of H and DAS was detected only at temperatures above 200 °C, for that reason the loss in weight of measured samples at 102–150 °C can be attributed to loss of more strongly (structurally) bound water. On corresponding DSC curves (DSC 2000—TA Instruments, New Castle, Del., U.S.A.), obtained under same conditions ($dT/dt = 5\ °C\ min^{-1}$, $\Delta T = 25–200\ °C$, N_2 flow rate 150 mL min^{-1}) two endothermal minimums may also be detected in temperature regions $35.6 \pm 5.0\ °C$ and $108.4 \pm 4.1\ °C$. In the case of protein materials, the first of them is usually attributed to combined gel melting and water from it evaporating [16]. The second, less prominent, is undoubtedly related only to evaporation of water from gels. In temperature interval 120–200 °C no further peaks can be detected on DSC curves. Curves of gel thermal analysis have a typical course which is presented for illustration in Fig. 1a–d.

Disintegration of gels in an aqueous environment was examined employing technique by Einerson et al. [17] originally applied to protein films. Studying degree of gel swelling through gravimetry is associated with certain problems; therefore, original method was modified in that a gel sample of 0.1 g weight immersed in 10 mL water of pH

Table 2 Organization of factor experiment 3^3 for evaluating influence of temperature on gels of collagen hydrolysate crosslinked with DAS

Run	Factor A	Factor B	Factor C	Mp_{gel}	Water (%)				Gel disintegration time (h)	
	% H	% DAS	°C/1 h	°C	Sorbed	Structural	Total	Start. gel	pH = 4.8	pH = 7.4
1	27.5	15	75	38.0	40.0	20.5	61.1	68.4	2.8	3.0
2	30.0	15	90	36.5	34.7	6.6	58.2	71.25	3.0	2.0
3	27.5	20	90	33.0	42.0	10.4	52.0	67.0	56.0	56.0
4	30.0	10	75	35.0	52.6	7.4	57.2	65.5	0.5	0.5
5	30.0	15	60	39.8	45.4	11.8	60.0	67.0	0.5	0.5
6	25.0	20	75	31.8	39.2	12.8	46.4	64.0	48.0	48.0
7	25.0	15	60	40.4	64.2	1.9	66.8	71.25	0.5	0.5
8	27.5	15	75	30.4	38.7	8.6	47.3	68.37	1.0	1.0
9	27.5	10	90	38.5	46.8	6.2	65.1	69.75	0.5	0.5
10	27.5	10	60	36.1	62.9	2.2	56.6	72.5	0.5	0.5
11	27.5	20	60	36.1	49.7	8.7	58.4	67.0	4.0	4.0
12	25.0	15	90	32.8	52.8	5.4	52.0	70.0	1.5	1.5
13	25.0	10	75	30.8	52.8	3.8	41.3	65.5	1.0	1.0
14	30.0	20	75	33.8	43.6	6.4	53.0	69.75	6.0	6.0
15	27.5	15	75	37.5	56.2	4.9	61.1	68.38	2.5	2.5

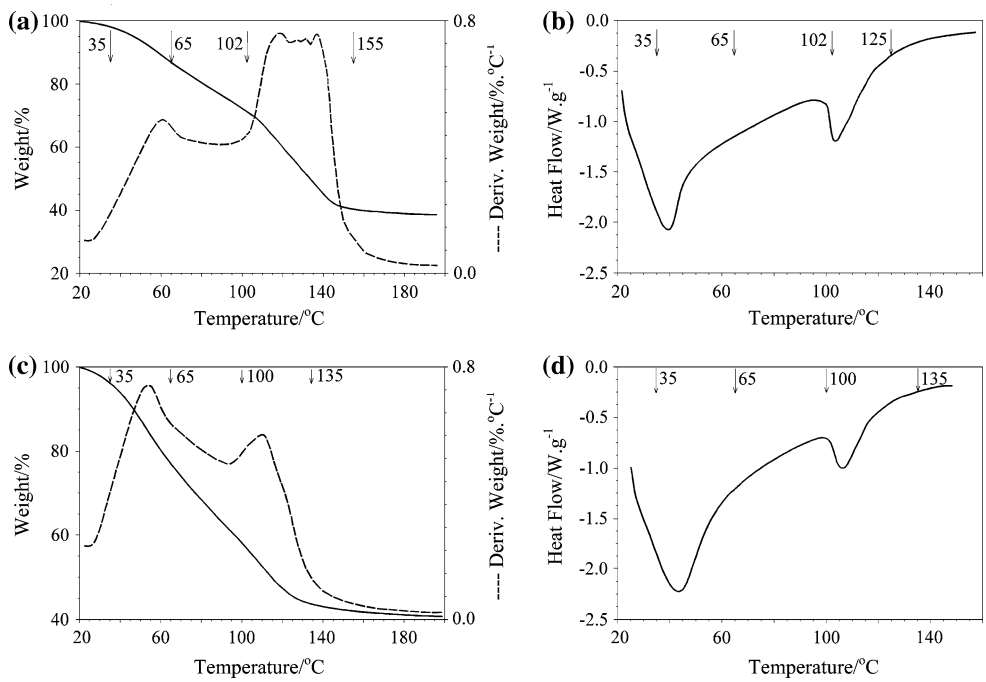

Fig. 1 Typical TG and DSC curves of hydrogels of collagen hydrolysate crosslinked with dialdehyde starch, exposed to treatment of 75–90 °C for 1 h. **a** TG and DTG curves of gel 27.5% H, 15% DAS, treated for 1 h at 75 °C. **b** DSC curve of gel 27.5% H, 15% DAS, treated for 1 h at 75 °C. **c** TG and DTG curve of gel 25 H, 15 DAS, treated for 1 h at 90 °C. **d** DSC curve of gel 25% H, 15% DAS, treated for 1 h at 90 °C

4.5 or 7.4 (simulating pH of environment in stomach and intestines) was visually examined at half-hour intervals. The time after which gel presence could not be visually detected in the solution was considered to be its disintegration time in given environment.

In order to assess effect of short-term (1-h) action by temperatures of 60–90 °C (in a forced-ventilation thermostat) on hydrogel properties, the organization was employed of a factor experiment of 3^3 type having two repetitions in the center, in which factors under study were:

- A: % H in reaction mixture (lower limit 25%, upper limit 30%),
- B: % DAS, related to H content in reaction mixture (lower limit 10%, upper limit 20%),
- C: temperature of 1-h action on prepared gel (lower limit 60 °C, upper limit 90 °C).

Quantities under study were:

- temperature of gel melting (Mp_{gel}),
- % water sorbed by gel (released into temperature 120 °C, W_S),
- % water bound structurally in gel (released in temperature interval 120–150 °C, $W_{Structural}$),
- time of gel disintegration in aqueous environment (pH 4.8 and 7.4) in hours (t_D).

Organization of factor experiment and obtained results are presented in Table 2.

Analysis of variance executed in standard manner [18, 19] by the Fisher test of mean squares of particular factors and mean residual square is obvious in data of Table 3.

Aging of prepared hydrogels was investigated by measuring rigidity of their gels applying technique according to standard BS 757 1959 [20]. Reaction mixtures were tempered 16–18 h at 10 °C and gel rigidity in degrees Bloom [g] was measured by means of standard apparatus (LFRA Texture Analyser, Stewens, Dunmow, Essex, U.K.). A typical time dependence of gel rigidity before and after 1 h treatment by temperature 90 °C is presented in Fig. 2.

Discussion

Aging of DAS cross-linked hydrogels of H appearing in their gradually increasing strength, even up to loss of thermo-reversibility [14], may be a source of non-homogeneity in processed lots and make production of biodegradable packing and other material by dipping technology difficult. For this reason, techniques usual in synthetic plastics processing (compression molding, injection molding) are gaining in importance. Altered gel properties, whether brought about by aging or heating, may furthermore even limit application potential of such materials.

A wide endothermal peak of DSC curves with minimum at 35.28 ± 2.63 °C associated with gel–sol transition of gels H cross-linked with 10–20% DAS does not exhibit after 1-h action of 60–90 °C temperatures any determinable shift of thermal coordinate of its minimum. The considerable width of this endothermal peak (covering temperature range of approx. 30–100 °C) shows it does not correspond to a pure gel–sol phase transition but rather to its combination with evaporation of water more loosely bound by

Table 3 Fisher test of statistical significance of observed factors from Table 2 for characteristics of gels

Factor	Mp_{gel}	Water content in gel (%)		Gel disintegration time (h)	
		Sorbed	Structural	pH 4.8	pH 7.4
A = % H	0.04	1.65	0.26	2.02	1.93
B = % DAS	1.37	2.55	1.33	14.27*·**	14.30*·**
C = °C for 1 h	0.58	3.26	0.06	3.41	3.54
Interaction					
AB	0.24	0.07	0.76	3.95	3.96
AC	1.11	0.00	0.57	0.00	0.01
BC	1.45	0.22	0.04	6.21**	6.22**
AA	1.00	0.01	0.49	0.05	0.04
BB	0.00	0.11	0.30	5.65**	5.59**
CC	0.93	0.66	0.89	0.00	0.00

* Statistically significant factors at 95% probability level: $F_{krit}^{(1,5)} = 6.61$

** Statistically significant factors at 90% probability level: $F_{krit}^{(1,5)} = 4.061$

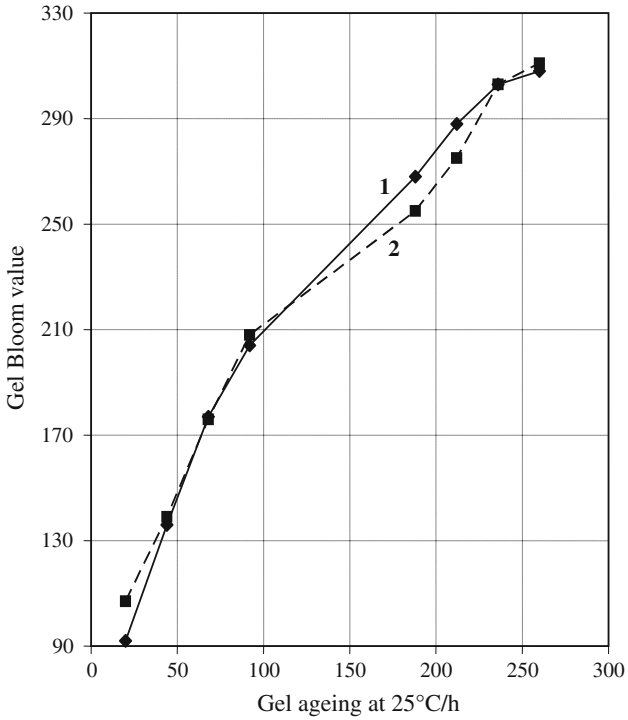

Fig. 2 Rigidities of heat treated and untreated gels time dependence. Gel composition: 27.5% H, 15% DAS. (1) treated for 1 h at 90°C, (2) untreated

hydrogel. This is also in accord with course of TG curves obtained under same conditions as DSC curves—in the given temperature region they display a virtually linear drop of sample weight with temperature (see Fig. 1a). Temperature levels of gel–sol phase transition determined through DSC technique are, therefore, somewhat problematic and do not afford reliable guidance to estimating temperatures suitable for processing these hydrogels through plastics techniques (compression molding, injection molding).

Water content in hydrogels is admittedly lower than in the initial reaction mixtures by an average 11–12% after 1-h thermal action by temperatures of 60–90 °C (comp. Table 2), but proving a statistically significant influence by temperatures of the selected interval or by content of H or DAS in the starting reaction mixture, was not successful. Also, content of water sorbed by hydrogels (being released in TG up to a temperature of 120 °C) or structurally (more strongly) bound (released in TG in temperature interval 120–150 °C) was not influenced by these factors in statistically significant manner. The statistical insignificance of temperature for these quantities is probably associated with selected level of temperature and duration of its action on hydrogels.

Nevertheless, experiments confirmed the significant influence of DAS (cross-linking agent) on disintegration rate of hydrogels (within pH limits 4.8–7.4 heedless of pH of aqueous environment). Data in Table 2 demonstrate that a surpassed 15% limit of DAS content (related to H content in reaction mixture)—with statistically insignificant effect of the 1-h action by temperatures in the observed range (60–90 °C)—markedly prolongs time of hydrogel disintegration in an aqueous environment. Even when this limit DAS concentration is surpassed, hydrogels remain soluble in an aqueous environment. The strong time dependence of hydrogel rigidity (aging) also remains preserved after the 1-h action by temperatures in the selected interval, as dependences presented in Fig. 2 show. This is in accord with the concept of a dominant role played by inter-chain hydrogen cross-links during gel aging (see [13, 14].

In orientation experiments, extruding hydrogels into air (RH = 40%, 25 °C) produced fibers (diam. approx. 0.1 mm) losing plasticity quite quickly. While water content in starting hydrogels is in limits 45–60% (see Table 2), TG curves of air-dry fibers merely correspond to an approx. 7.3% (w/w) content of present water. Conditioning fibers (RH = 60%, 25 °C) establishes in them an equilibrium water content around 20%, and their plasticity is renewed. Extruding fibers into 90% ethanol or 90% acetone directly

Table 4 Water content in fibers (Ø ≈ 0.1 mm) produced by extruding hydrogel 27.5% H + 15% DAS at 80 °C

	% water in fibers Ø 0.1 mm		
	Sorbed	Structural	Total
Air-dry (RV = 40%, 25 °C)	1.23	6.4	7.27
Conditioned (RV = 60%, 25 °C, 72 h)	9.75	10.0	19.75
Extruded directly into 90% ethanol	15.2	8.0	23.2
Extruded directly into 80% ethanol	14.6	9.4	24.0
Extruded directly into 90% acetone	12.0	11.0	23.6
Extruded directly into 80% acetone	11.1	13.3	24.4

establishes an equilibrium fiber water content around 23%, which enables to eliminate time demanding conditioning. This is well illustrated by values listed in Table 4.

In the case of other extruded shapes, for example, films, the decrease (owing to less surface extension) in equilibrium water content need not be so great, but the problem in question is quite serious and has to be resolved by adding hydrophile plasticizers [21] that may alter sorption characteristics of extruded materials for water to such an extent that its equilibrium content in a usual environment (characterized by rel. hum. 40%, 25 °C) ranges around 15% (w/w). Choice of suitable plasticizers and their concentrations will then readily lead to fibers, applicable, for example, as haemostatics or surgical suturing materials, or films suitable as biodegradable packing for foodstuffs, pharmaceuticals or cosmetic products.

Conclusion

Hydrogels of collagen hydrolysate cross-linked with dialdehyde starch have strong tendency to aging, which shows through an increase in gel rigidity in time and eventually in lost gel thermo-reversibility. When processing hydrogels into biodegradable (biocompatible) materials, hydrogel aging complicates applying dipping and/or casting (and drying) film technologies.

One hour heating of hydrogels to 60–90 °C does not affect their characteristics except for reducing water content by approx. 12%. Disintegration of such gels in aqueous environment does not depend on pH, disintegration time increases with an increasing content of cross-linking agent (DAS) in hydrogel. Hydrogels may be processed through techniques combining temperature and pressure effects usual in processing synthetic plastics.

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