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Effect of transglutaminase and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide on the solubility of fish gelatin–chitosan films

Ilona Kołodziejska *, Barbara Piotrowska, Monika Bulge, Robert Tylingo

Department of Food Chemistry, Technology and Biotechnology, Chemical Faculty, Gdansk University of Technology, G. Narutowicza 11/12, 80-952 Gdańsk, Poland

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Abstract

The possibility of decreasing the water solubility of the films made from fish gelatin and chitosan by modification with TGase was investigated. The effectiveness of enzymatic treatment was also compared with chemical crosslinking using EDC. The treatment of the components with TGase in concentration of 0.2 mg/ml of the film-forming solution limited the solubility of the films at 25 °C from 65% to 28% at pH 6 and from 96% to 37% at pH 3. After 15 min of heating at 100 °C, the modified films were soluble in 23% at pH 6 and in 41% at pH 3. Further decrease of the solubility of the fish gelatin–chitosan films was achieved when enzymatic modification was conducted in the presence of 5–10 mM DTT; the solubility was about twice lower than that without DTT at both studied temperatures and pH values. Generally, the composite films modified with EDC in concentration of 30 mM were distinctly less soluble than films made from the components modified with TGase in the presence of DTT.

Keywords: Fish gelatin–chitosan films; Crosslinking; Transglutaminase; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

1. Introduction

Proteins, polysaccharides and lipids or mixtures of these compounds may be used for production of biodegradable, edible films, and coatings. The properties and possibilities of applications of such materials have been reviewed by Gennadios, Hanna, and Kurth (1997), Kester and Fennema (1986), Krochta and de Mulder-Johnston (1997), Miller and Krochta (1997), Park (1999). The edible films can be used not only as packaging materials but also as carriers of food additives, e.g., antioxidants, antimicrobials, flavoring agents, and pigments (Appendini & Hotchkiss, 2002; Kester & Fennema, 1986; Krochta & de Mulder-Johnston, 1997).

Mixtures of pigskin gelatin and chitosan were already used by Arvanitoyannis, Nakayama, and Aiba (1998) to prepare edible films. However, until now fish gelatin was not applied for this purpose. The gelatin produced from skins of fish living in cold waters does not gel at room temperature – its gelling temperature is below 8–10 °C (Norland, 1990). Our previous experiments showed that fish gelatin films were completely soluble even at room temperature (Piotrowska, Kołodziejska, Januszewska-Jóźwiak, & Wojtasz-Pająk, 2005). This problem arises when packaging material should be resistant to solubilization, especially in contact with acidic food or during heating. Therefore, such films could not be suitable for coating or packaging of many food products.

It was assumed that the solubility of gelatin–chitosan films could be limited by crosslinking with TGase. This enzyme catalyses formation of the covalent linkages between γ-carboxyamide groups of peptide-bound glutamine residues and ε-amino groups of lysine or primary amino groups of a number of components (Folk, 1980). TGase was used for modification of the properties of protein films (Mahmoud & Savello, 1993; Motoki, Aso, Seguro, & Nio, 1987; Yildirim & Hettiarachchy, 1998),

* Corresponding author. Tel.: +48 58 3472656; fax: +48 58 3472694.
E-mail address: i.kolodziejska@chem.pg.gda.pl (I. Kołodziejska).

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including gelatin (de Carvalho & Grosso, 2004). Furthermore, Chen, Embree, Brown, Taylor, and Payne (2003) showed that TGase-catalyzed gelation of type A gelatin was faster and the gels were stronger when the reaction was conducted in the presence of chitosan.

The objective of this study was to determine the possibility of decreasing the water solubility of the films composed of fish gelatin and chitosan by modification with TGase. The effectiveness of enzymatic treatment was compared with chemical crosslinking of components with EDC. The EDC was used for crosslinking of polysaccharides and/or proteins (Kuijpers et al., 2000; Pieper, Hafmans, Veerkamp, & van Kuppevelt, 2000; Wang et al., 2003; Wissink et al., 2001). Although, this reagent participates in reaction between molecules containing free carboxylic and amine groups, similarly to enzymatic reaction – the same amide bonds are formed.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree 73%) was obtained from krill chitin in the Sea Fisheries Institute in Gdynia according to Kołodziejska, Wojtasz-Pająk, Ogonowska, and Sikorski (2000). The source of protein was pigskin gelatin 300 Bloom (BL) from Sigma Chemical Co. and gelatin obtained from the Baltic cod skins as described by Kołodziejska, Kaczorowski, Piotrowska, and Sadowska (2004). EDC was purchased from Sigma Chemical Co. For the enzymatic modification the preparation of commercial TGase (Ajinomoto Co's Transglutaminase Activa®-WM, Japan) containing 99% of maltodextrin and 1% of enzyme was used. Six grams of the dry preparation was mixed with 20 ml of cold water in an ice bath for 15 min and centrifuged at 6000*g* for 15 min at 4 °C. The protein content in the enzyme solution was determined according to Lowry, Rosebrough, Farr, and Randall (1951).

2.2. Enzymatic and chemical modifications

The gelatin–chitosan films (4:1, w/w) were obtained by mixing 2% solution of chitosan (pH 5 adjusted with 0.5 M HCl) with 25% solution of pigskin or fish gelatin. The mixture was occasionally stirred during 2 h of incubation at 50 °C and centrifuged at 2000*g* and 20 °C for 15 min.

EDC was added to the film-forming solutions of chitosan and fish gelatin at room temperature to the final concentration ranged from 5 to 50 mM; TGase was added in concentration of 0.2 mg/ml. Film-forming solutions containing pigskin gelatin and chitosan were heated to 50 °C before adding TGase.

In some experiments, the enzymatic crosslinking was conducted in the presence of DTT at concentration 1.25–10 mM and chemical modification with EDC in the presence of *N*-hydroxysuccinimide (NHS) in the ratio of EDC to NHS as 1:0.125, 1:0.17, 1:0.2, 1:0.25, 1:0.34, 1:0.5, and 1:1 (w/w).

The films were formed just after adding of TGase or EDC as described in the Section 2.4. The enzymatic and chemical reactions were running during that process.

2.3. Viscosity

The viscosity of the film-forming solutions, modified with TGase at concentration of 0.2 mg/ml or EDC at concentration of 30 mM, was measured in Brookfield DV-III viscosimeter using the small sample adapter and SC4-27 spindle at 25 rpm.

2.4. Film formation

In all experiments, 40 g of solutions were cast on a square area (255 cm^2) of a polyester surface and spread manually to the outside borders. The films were dried at room temperature for 24–48 h at 35–45% relative humidity (RH) or in two stages: first 12 h at 65–70% RH, followed by 24–48 h at 35–45% RH.

2.5. Solubility measurements

To determine the solubility of the films about 50 mg of the dry sample was immersed in 20 ml of 0.2 M Mc'Ilvaine buffer (pH 3.0 or 6.0). The samples were incubated 24 h at 25 °C or 15 min or 60 min at 100 °C. The insoluble material was separated on a funnel with cotton wool. Nitrogen or, in some experiments, hydroxyproline contents, were determined in the insoluble residues of the films.

The solubility of the films was determined on the base of nitrogen that was dissolved in the buffer and was expressed as the percentage of nitrogen contained in the initial films. Solubility of the particular components of the films was estimated as follows. The solubility of gelatin was determined from hydroxyproline that migrated to the buffer and was expressed as the percentage of hydroxyproline contained in the initial films. The solubility of chitosan was calculated on the base of nitrogen that was dissolved in the buffer, diminished by nitrogen hydroxyproline, and was expressed as the percentage of the chitosan nitrogen contained in the initial films.

Nitrogen was determined according to AOAC method (1990). Hydroxyproline content was assayed after hydrolysis of the material in 6 M HCl for 6 h at 105 °C, using the colorimetric method recommended by ISO (International Standard, 1978).

3. Results and discussion

3.1. Effect of TGase and EDC on the solubility of gelatin–chitosan films

The solubility of unmodified composite films prepared from fish gelatin and chitosan (4:1, w/w) at 25 °C in buffers

of pH 6 and 3, was 65% and 96%, respectively (Table 1). To compare, the films obtained from chitosan and pigskin gelatin, 300 BL, were at this temperature almost insoluble at pH 6 and only in 28% soluble at pH 3. After modification with TGase, the solubility of the films decreased to 28% at pH 6 and 37% at pH 3 (Table 1). Moreover, enzymatic treatment limited film solubility at 100 °C and pH 6, from about 98% (for not crosslinked samples) to 36%. However, the obtained films were not resistant to elevated temperature at pH 3.

A reduction of disulphide bonds in the native proteins in the presence of reducing agents, e.g., DTT, increases reactivity of TGase (Nielsen, 1995). Fish collagen and gelatin do not contain disulphide bonds (Gudmundsson & Hafsteinsson, 1997; Norland, 1990; Piez & Gross, 1960). However, TGase belongs to the thiol enzymes and there is the possibility of decreasing its activity as the result of – SH groups oxidation for example during film formation. In such case, the addition of reducing substances to the reaction mixture should increase the activity of the enzyme by breaking of disulphide bonds or by preventing against their formation. It was checked therefore, whether further limitation of the solubility of the films can be achieved by enzymatic modification of the components in the presence of DTT. This hypothesis was confirmed by about two times lower solubility of films at 25 °C modified with TGase and DTT at concentration of 5–10 mM, as compared with that for samples prepared without DTT (Table 2). Moreover, the DTT addition significantly influenced on the solubility of films at elevated temperatures. After 1 h incubation of the samples at 100 °C the solubility of films prepared from components enzymatically modified in the presence of DTT in a concentration of 5 mM was decreased to 40% at pH 3 and to 17% at pH 6 (Table 2). The films solubility depended also on the incubation time, after 15 min of heating at 100 °C only amounted to 23% at pH 3 and 11% at pH 6 (Table 3).

In order to compare the effect of chemical and enzymatic crosslinking, the mixture of gelatin and chitosan was treated with EDC at different concentrations. The solubility of the composite fish gelatin–chitosan films modified with EDC at concentration of 30 mM and preliminary dried for 12 h at RH about 65% and then at RH 35–45% decreased to about 30% at both investigated pH values. Higher concentrations of EDC did not cause any further reduction of the films' solubility (Fig. 1a). More significant decrease in the solubility was achieved when the films were dried all time at RH values of 35–45% (Fig. 1b). The ezymatically modified films were more soluble in buffers of pH 6 or 3 at 25 °C than films modified with EDC. However, in the presence of DTT, the differences in the solubility were much lower (Table 4). During heating at 100 °C for 15 min at pH 3 the composite films modified with EDC were less soluble than films prepared from components modified with TGase and DTT. However, there were no differences in their solubility at pH 6 (Table 4).

The crosslinking of molecules with EDC is often conducted in the presence of NHS to increase the efficiency of the formation of amide bonds (Kuijpers et al., 2000). However, addition of NHS in different concentrations and EDC in concentration of 30 mM to film-forming

Table 2
Effect of concentration of DTT on the solubility of fish gelatin–chitosan films modified with TGase in concentration of 0.2 mg/ml[a]

DTT concentration (mM)	Solubility (%)[b]			
	pH 3		pH 6	
	25 °C	100 °C	25 °C	100 °C
0	37 ± 1.1	99 ± 0.1	28 ± 0.9	36 ± 2.8
1.25	26 ± 1.2	86 ± 1.1	19 ± 0.6	22 ± 1.6
2.50	19 ± 2.7	45 ± 5.7	16 ± 0.5	20 ± 0.8
5.00	17 ± 0.3	40 ± 0.2	17 ± 0.4	17 ± 1.6
7.50	19 ± 0.4	44 ± 0.4	15 ± 1.0	16 ± 1.9
10.00	17 ± 0.4	39 ± 4.0	14 ± 0.3	16 ± 0.9

[a] 24 h at 25 °C, 1 h at 100 °C.
[b] Mean values ± standard deviation of five determinations.

Table 3
Effect of concentration of DTT on the solubility of fish gelatin–chitosan films modified with TGase in concentration of 0.2 mg/ml[a]

DTT concentration (mM)	Solubility (%)[b]	
	pH 3	pH 6
0	41 ± 1.2	23 ± 2.0
1.25	30 ± 1.5	16 ± 1.6
2.50	25 ± 1.2	13 ± 2.4
5.00	22 ± 1.7	12 ± 0.9
7.50	23 ± 1.6	11 ± 2.5
10.00	23 ± 1.0	11 ± 1.4

[a] 15 min at 100 °C.
[b] Mean values ± standard deviation of five determinations.

Table 1
Effect of pH and temperature on the solubility of gelatin–chitosan films[a]

Films	TG concentration (mg/ml)	Solubility (%)[b]			
		pH 3		pH 6	
		25 °C	100 °C	25 °C	100 °C
Pigskin gelatin–chitosan	0	28 ± 3.1	97 ± 1.1	3 ± 0.4	86 ± 1.2
Fish gelatin–chitosan	0	96 ± 1.1	97 ± 0.6	65 ± 1.7	98 ± 0.0
	0.2	37 ± 1.1	99 ± 0.1	28 ± 0.9	36 ± 2.8

[a] 24 h at 25 °C, 1 h at 100 °C.
[b] Mean values ± standard deviation of five determinations.

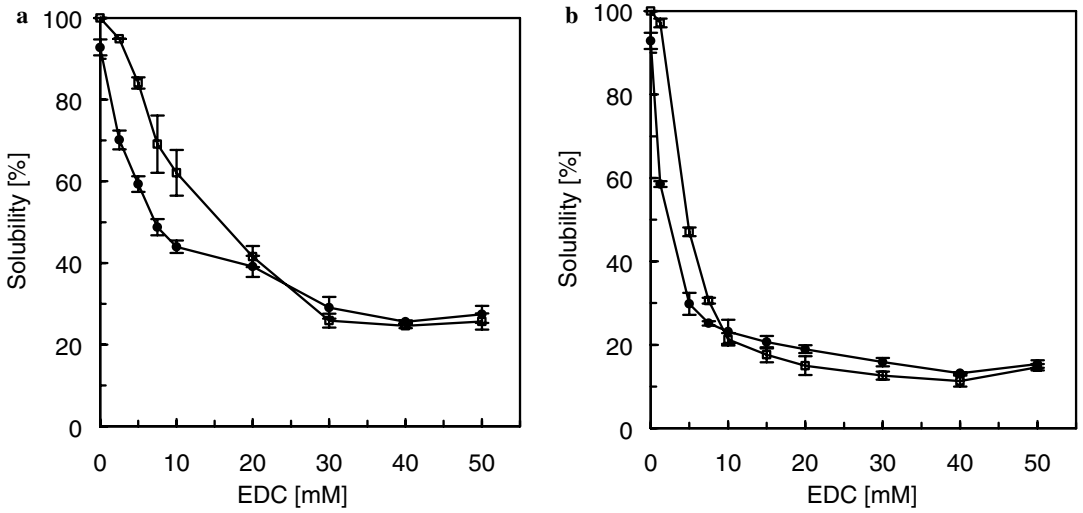

Fig. 1. Effect of EDC on the solubility of fish gelatin–chitosan films in buffer of pH 3 (□) and pH 6 (●), (25 °C, 24 h): (a) – films dried first for 12 h at RH about 65% and next at RH 35–45%; (b) – films dried for 24–48 h at RH 35–45%.

Table 4
Solubility of fish gelatine–chitosan films: unmodified; modified with TGase, 0.2 mg/ml (TGase); modified with TGase, 0.2 mg/ml, in the presence of DTT, 5 mM (TGase/DTT); modified with EDC, 30 mM (EDC)

Films	Conditions	Solubility (%)[a]	
		pH 3	pH 6
Unmodified	25 °C, 24 h	96 ± 1.1	65 ± 1.7
TGase		37 ± 1.1	28 ± 0.9
TGase/DTT		17 ± 0.3	17 ± 0.4
EDC		10 ± 0.9	12 ± 1.4
Unmodified	100 °C, 15 min	96 ± 1.2	92 ± 1.3
TGase		41 ± 1.2	23 ± 1.0
TGase/DTT		22 ± 1.7	11 ± 1.4
EDC		11 ± 0.5	12 ± 0.8

[a] Mean values ± standard deviation of five determinations.

solution did not affect the solubility of films in buffers at pH 3 and 6 at 25 °C (Table 5).

Determination of the films solubility on the base of nitrogen that was dissolved in the buffer solution did not answer the question what was the source of nitrogen, chitosan or gelatin. Therefore, in some experiments solubility of the particular components of the films was estimated.

The amounts of gelatin migrated at 25 °C into the buffers at pH 3 and 6 from the composite films modified with TGase were similar (Table 6). Furthermore, gelatin from fish gelatin–chitosan films modified with EDC or TGase in the presence of DTT was almost completely insoluble in both buffers. Chitosan was responsible in greater extent than gelatin for the higher solubility at pH 3 of the enzymatically or chemically modified films (Table 6). However, at pH 3 the solubility of chitosan from films crosslinked with EDC was two times lower than that from films modified with TGase in the presence of DTT (Table 6). According to Chiou and Wu (2004) EDC has ability to activate the hydroxyl groups of chitosan which then might react with

Table 5
Effect of NHS on the solubility of fish gelatin–chitosan films modified with EDC in concentration of 30 mM[a]

EDC:NHS	Solubility (%)[b]	
	pH 3	pH 6
1:0	10 ± 0.9	12 ± 1.4
1:0.125	13 ± 1.4	14 ± 1.7
1:0.17	11 ± 1.3	13 ± 1.0
1:0.2	11 ± 1.1	12 ± 0.7
1:0.25	9 ± 2.1	11 ± 0.6
1:0.34	11 ± 1.1	12 ± 1.5
1:0.5	11 ± 0.5	12 ± 0.7
1:1	14 ± 1.0	14 ± 0.3

[a] 24 h at 25 °C.
[b] Mean values ± standard deviation of five determinations.

Table 6
Solubility of particular components of fish gelatine–chitosan films: unmodified; modified with TGase, 0.2 mg/ml (TGase); modified with TGase, 0.2 mg/ml, in the presence of DTT, 5 mM (TGase/DTT); modified with EDC, 30 mM (EDC)

Films	Conditions	Solubility (%)[a]	
		Chitosan	Gelatin
Unmodified	pH 6, 25 °C, 24 h	1 ± 0.3	98 ± 0.8
TGase		19 ± 1.1	26 ± 1.1
TGase/DTT		29 ± 0.9	7 ± 1.7
EDC		20 ± 3.0	8 ± 1.3
Unmodified	pH 3, 25 °C, 24 h	80 ± 2.5	100 ± 0.7
TGase		46 ± 0.6	25 ± 0.5
TGase/DTT		43 ± 0.7	1 ± 1.5
EDC		22 ± 0.3	4 ± 1.1
Unmodified	pH 3, 100 °C, 15 min	88 ± 0.8	100 ± 2.2
TGase		53 ± 0.5	27 ± 0.7
TGase/DTT		45 ± 1.0	5 ± 0.3
EDC		14 ± 1.2	9 ± 0.9

[a] Mean values ± standard deviation of five determinations.

the amino groups of the protein. Moreover, Wang et al. (2003) reported that in the presence of EDC ester bonds, between carboxylic groups of collagen and hydroxyl groups of chitosan, could also be formed. These results indicate that crosslinking of the polymers with EDC may be caused not only by amide bonds formation but also other linkages may occur. That is probably why the solubility of chitosan from chemically modified films was lower than that of enzymatically modified.

The solubility of chitosan from films heated for 15 min at 100 °C in buffer solution of pH 3 was about 88% of its initial content, while under these conditions gelatin was completely dissolved (Table 6). The solubility of the components of the heated films was limited after their enzymatic or chemical modification to a similar level as that obtained at 25 °C (Table 6). These results suggest that some quantity of chitosan in composite films participate in the crosslinking reactions because films prepared only from chitosan were completely dissolved at 25 °C and at 100 °C at pH 3 (Piotrowska et al., 2005). However, after enzymatic modification, the solubility of chitosan from the films increased at pH 6 and 25 °C. Under these conditions chitosan in the one-component films (Piotrowska et al., 2005) or in the unmodified fish gelatin–chitosan films (Table 6) was almost not soluble.

3.2. Effect of enzymatic modifications on the viscosity of the film-forming solutions

The viscosity of fish gelatin–chitosan mixtures (4:1, w/w) was measured under the same conditions as were used during film preparation – at room temperature using TGase in concentration of 0.2 mg/ml. A sudden increase of the viscosity of fish gelatin–chitosan solution modified with TGase was observed after 6 h of incubation at 25 °C, while in the samples modified additionally in the presence of DTT this effect was observed after reaction time shortened to about 2.5 h (Fig. 2). Moreover, the viscosity of 4% of fish gelatin solution modified with TGase achieved maximal value of 700 MPa*s, when in the samples containing gelatin and chitosan the enhancement of viscosity was much higher (Fig. 2). Chen et al. (2003) reported that addition of chitosan in a concentration of 0.32–5% solution of enzymatically modified gelatin reduced time of the gel formation at 35 °C from 4 to 1.5 h. This phenomenon indicates that the amine groups of chitosan participate in crosslinking reactions with gelatin. However, it is also possible that this polymer affects the crosslinking of proteins indirectly. Mariniello et al. (2003) reported that addition of pectin facilitated formation of TGase-catalyzed covalent bonds between molecules of proteins of defatted soy flour. According to these authors the electrostatic interactions between protein and pectin molecules change the conformation of soy proteins, thus assuring a better affinity of TGase to the substrate. On the other hand, between chitosan and gelatin in the film-forming solutions at pH 6 there are rather electrostatic repulsive forces, because

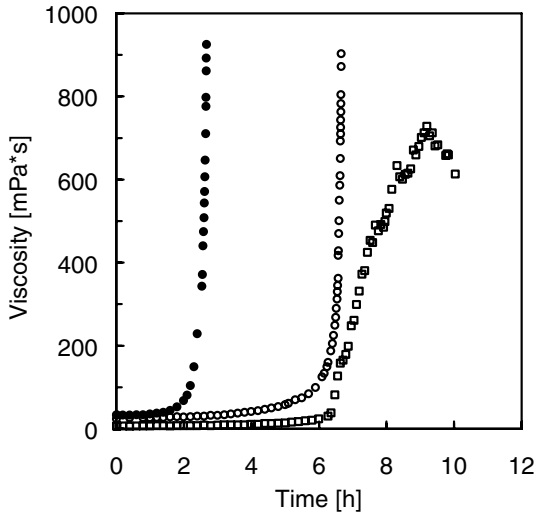

Fig. 2. Effect of TGase on the viscosity of fish gelatin solution (□), fish gelatin–chitosan solutions (○) and TGase in the presence of DTT on the viscosity of fish gelatin–chitosan solutions (●) at pH 6 and 25 °C; in film-forming solutions concentration of gelatin was 4%, chitosan 1%, TGase 0.2 mg/ml, DTT 5 μmol/ml.

chitosan is positively charged at this pH and the isoelectric point of gelatin is about 7.

After addition of EDC in concentration of 30 mM to the solution of fish gelatin–chitosan (4:1, w/w) the viscosity increased after a few minutes (Fig. 3), while in the case of enzymatic modification, conducted even in the presence of DTT, this effect was observed after 2 h (Fig. 2). The rate of crosslinking reaction depended on the pH of the film-forming solutions. It was also found that the time after which the increase in viscosity occurred was shorter in solution of gelatin and chitosan modified with EDC than in solution containing only gelatin modified in the same way.

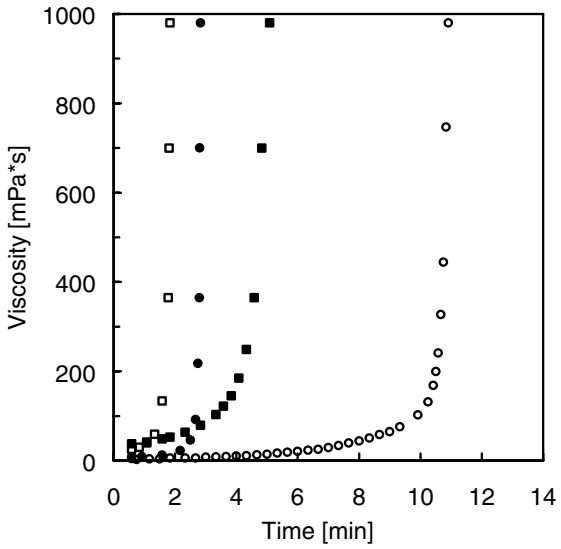

Fig. 3. Effect of EDC at concentration of 30 mM on the viscosity of 5% fish gelatin solution, at pH 6.0 (○), at pH 5.4 (●) and fish gelatin–chitosan solutions (1:4), at pH 6.0 (■), at pH 5.4 (□) at 25 °C.

Reported results suggest that chitosan aids the chemical and enzymatic crosslinking reactions and the detailed studies on this area are in progress.

4. Conclusions

Modifications of fish gelatin and chitosan with TGase allow to obtain films with a low solubility in water medium at pH 6 even during heating at 100 °C for 1 h and at pH 3 for 15 min. Further, decrease of the solubility of fish gelatin–chitosan films is possible when the enzymatic modification is conducted in the presence of DTT. Our unpublished data show that this effect can be also achieved with other reducing substances – cysteine and glutathione. The composite films modified with TGase even in the presence of reducing substance are, as a rule, more soluble than films made from components modified with EDC. However, enzymatic modification of film components can be an alternative to chemical crosslinking with using substances that are not admitted in foods. Furthermore, incorporation of chitosan into fish gelatin films may serve also other functions of the packaging. The polymer partially dissolved after contact with the moist products may act as an antimicrobial agent extending the shelf life of the packed food.

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