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Investigation on chemical cross-linked collagen phosphoric acid hydrolysates with cyanuric chloride by differential scanning calorimetry

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Abstract The process of cross-linking of collagen phosphoric acid hydrolysates (CH) with cyanuric chloride (CY) was studied by the increase in the denaturation temperature using differential scanning calorimetry (DSC). This measurement gave indications concerning the efficiency of the treatment, i.e., the extent of cross-linking of the collagen hydrolysates. The optimal conditions for cross-linking were determined: CH/CY in a ratio 1:1, reaction time 1 h at temperature 50 °C. At these conditions cross-linked structural units with higher thermal stability were formed.

Keywords Differential scanning calorimetry · Thermal denaturation · Collagen hydrolysates · Cyanuric chloride

Abbreviations

DSC	Differential scanning calorimetry
CH	Collagen hydrolysate
CY	Cyanuric chloride

Introduction

Collagenous proteins, a meat production by-products, are an important raw material for the production of leather, gelatine, and glues, but are also widely used in the production of foodstuffs, cosmetics, pharmaceuticals (vaccine, vitamin, and enzyme stabilizers, capsules), and are even used in human medicine (vehiculum of some medicaments, haemostatic agents, vascular replacements, etc.) [1, 2].

Hydrolysates can be defined as proteins that are chemically or enzymatically broken down to peptides of varying size. In aqueous solutions, collagen hydrolysates behave like "substances with positive sorption," but do not display strong surface activity if not further processed. Most strategies introduce stable and covalent intermolecular cross-links between collagen fibrils. Different ways of cross-linking, either chemical or physical, have been carried out and often the method is prescribed by the target application [3, 4]. Better understanding of the physical properties of collagen-based materials is of great importance for subsequent application of these materials. Usually, chemical cross-linking procedures involve the use of reagents such as glutaraldehyde [5], dialdehyde starch [6–8], isocyanates, polyepoxy compounds [9, 10], polyglycidyl ethers [11], succinimide, carbodiimide, etc. [12]. Cross-linking influences the strength, resorbtion rate, and biocompatibility of biomaterials [13, 14]. A higher degree of cross-linking is generally associated with a lower antigenicity. Furthermore, the stability and durability are related to increase in temperature of denaturation [15, 16].

In a previous study, we have reported technologies for processing wastes from pig skins, pig bones, and pig cartilages of breed Bulgarian blue to produce collagen hydrolysates by utilization of different concentrations of phosphoric acid [17]. We have studied the molecular interactions in collagen hydrolysates/cyanuric chloride mixture by free amino group analysis, collagenase digestion and FTIR spectroscopy [18].

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The aim of this study is an investigation by means of differential scanning calorimetry (DSC) on the degree of cross-linking of collagen phosphoric acid hydrolysates (CH) with cyanuric chloride (CY).

Materials and methods

Starting materials

Leather wastes have been obtained from the leather slaughterhouse near Sofia. Lyophilized CH in sponge form was prepared by phosphoric acid hydrolysis of pig leather wastes by a procedure according to reference [17]. The cross-linking of lyophilized CH with CY was prepared as described in [18].

CY was obtained from Merck. All other chemicals used in assays were reagent grade.

Differential scanning calorimetry

The degree of cross-linking of the samples was related to the increase of the denaturation temperature (T_d). The denaturation temperature (T_d) was defined as the temperature at the maximum of excess heat capacity curve. T_d values were determined by means of a high-sensitivity differential scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russia), with sensitivity greater than 4×10^{-6} cal K^{-1} and a noise level less than 5×10^{-7} W. A constant pressure of 2 atm was maintained during all DSC experiments to prevent possible degassing of the solution on heating. Three different heating scan rates (0.25, 0.5, and 1 °C min^{-1}) were used. Prior to the calorimetric experiment, the protein samples (20–30 mg) were dialyzed extensively against the buffer. Each sample run was preceded by a baseline run with buffer-filled cells. The transitions were corrected for the difference in heat capacity between the initial and final states by using a linear chemical base line. The reversibility of the thermal denaturation of the CH, before and after modification with CY, was examined by immediately heating the sample after cooling (second cycle). The calorimetric data were evaluated using the ORIGIN (Micro Cal Software) program package. Each curve was deconvoluted mathematically, using theoretical Gaussian fitting. The calorimetric enthalpy (ΔH_{cal}/J g^{-1}) of the transition was determined as the area under the excess heat capacity curve. Mean values and standard errors were obtained from three independent experiments. The standard error for T_d is less than 0.5 °C in all cases.

The following buffer systems were used: 50 mM acetate buffer pH 4.5, 50 mM phosphate buffer pH 7.5, and 50 mM carbonate buffer pH 9.5.

Results and discussion

Determination of the experimental parameters

Figure 1 presents the DSC curve of non-cross-linked lyophilized CH in 50 mM phosphate buffer, pH 7.5. The thermal denaturation of non-cross-linked CH is partially reversible, i.e. the second scan is not identical with first scan and shows decreased values of a heat capacity and a calorimetric enthalpy. The DSC curve has two main endothermic transitions, centered at 24.0 and 31.7 °C (first scan) and one transition at 26.2 °C (second scan). The enthalpy change at the first scan is $\Delta H_{cal} = 2.1$ J g^{-1} and at the second scan is less $\Delta H_{cal} = 1.7$ J g^{-1}.

The observed incomplete recovery of some fragments in the CH after heating is probable caused by various secondary processes such aggregation of denatured protein or its chemical modifications.

The obtained DSC curve has an asymmetric shape as an indication for the existence of more then one structural unit in the analyzed sample. By means of mathematical deconvolution, the thermodynamic parameters (T_d and ΔH_{cal}) of the individual transitions can be determined.

pH-dependence of the denaturation temperature of non-cross-linked CH

We have investigated the thermal denaturation of non-cross-linked CH in a variety of different buffer systems to role out buffer specific effects.

The deconvolution of the excess heat capacity function of non-cross-linked CH, dissolved in 50 mM phosphate buffer pH 7.5, is shown on Fig. 2. In Table 1, the

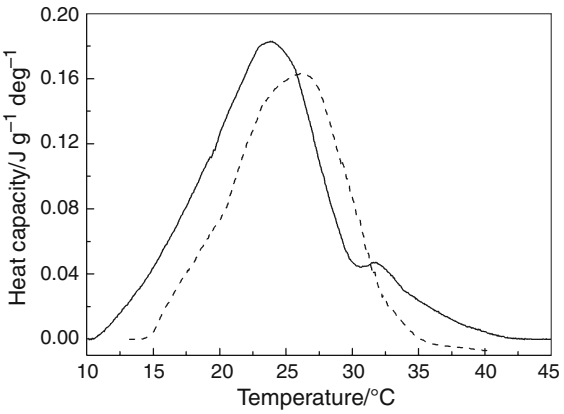

Fig. 1 DSC curves of non-cross-linked lyophilized collagen hydrolysates dissolved in 50 mM phosphate buffer, pH 7.5. The scanning rate was 0.5 °C min^{-1}. Profile of DSC curve at heating to 100 °C (first scan) (*solid line*); profile of DSC curve at heating to 100 °C, subsequent cooling to 10 °C and repeated heating to 100 °C (*dash line*) (second scan)

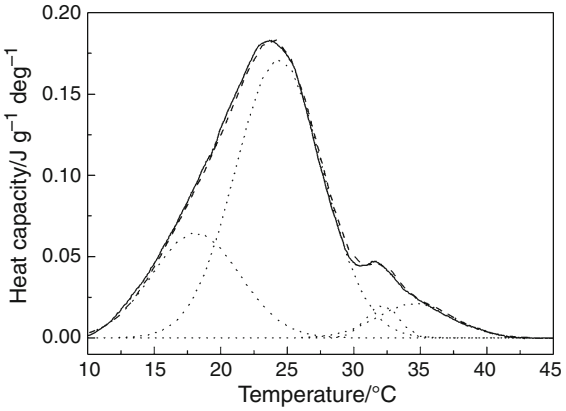

Fig. 2 Deconvolution of the heat capacity function of non-cross-linked lyophilized collagen hydrolysates dissolved in phosphate buffer, pH 7.5: experimental curve (*solid line*); calculated, assuming component transitions (*dash line*); component transitions (*dot line*)

Table 1 Thermodynamic parameters, transition temperature (T_d) and calorimetric enthalpy (ΔH_{cal}), of the heat sorption curves of non-cross-linked lyophilized CH, dissolved in different buffers

Peak	ΔH_{cal}/J g^{-1}	T_d/°C
pH 4.5		
1	2.64 ± 0.4	19.5
2	0.78 ± 0.3	27.4
3	1.93 ± 0.4	30.6
pH 7.5		
1	0.53 ± 0.2	18.1
2	1.41 ± 0.3	24.3
3	0.06 ± 0.2	32.1
4	0.15 ± 0.2	34.5
pH 9.5		
1	0.64 ± 0.2	21.6
2	1.39 ± 0.2	26.8

thermodynamic parameters obtained for the individual structural units are presented.

Three structural units were detected after deconvolution of the DSC curve of non-cross-linked CH, dissolved in acetate buffer pH 4.5 (Table 1). The DSC curve of non-cross-linked CH, dissolved in carbonate buffer with pH 9.5, was deconvoluted into transitions of two individual structural units (Table 1).

The comparison of the data obtained shows that at pH 7.5, in part of the CH more stable structural units appeared for which T_d is higher then other buffers have been used (T_d 32.1 and 34.5 °C). We selected the 50 mM phosphate buffer pH 7.5 as appropriate for calorimetric analysis of CH, because in this buffer more stable structure units were formed. This buffer also is less temperature sensitive.

Influence of heating rate on the denaturation temperature of non-cross-linked CH

The DSC curves of non-cross-linked CH at different heating rates: 0.25, 0.5, and 1 °C min^{-1} are shown in Fig. 3. The profiles of the observed curves are shifted toward higher temperatures approximately with 1 °C with a decrease in the heating rate, while ΔH_{cal} decreases. The temperature of denaturation of CH at heating rate 0.25 °C min^{-1} is 25.9 °C, at heating rate 0.5 °C min^{-1} is 24.9 °C and at heating rate 1 °C min^{-1} is 23.9 °C.

By thermodynamic considerations it is follows the choice of slower rate of scanning, because such a rate the least disturbs the equilibrium state of the investigate system during the phase transition. From the other hand the slowest rate is uncomfortable because of the large duration of the measuring what can collects different mistakes,

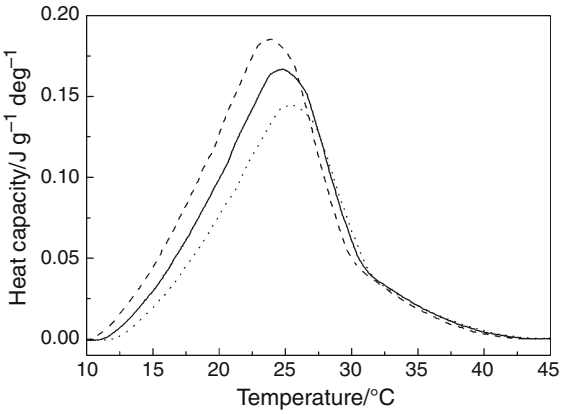

Fig. 3 DSC curves of non-cross-linked collagen hydrolysates dissolved in phosphate buffer, pH 7.5, at different heating rates: 0.25 °C min^{-1} (*dot line*), 0.5 °C min^{-1} (*solid line*), 1.0 °C min^{-1} (*dash line*)

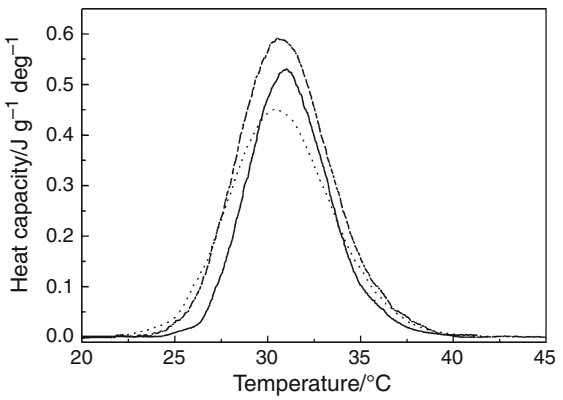

Fig. 4 DSC curves of cross-linked collagen hydrolysates obtained at a CH/CY 3:1, temperature 20 °C and different reaction time—1 h (*solid line*); 3 h (*dash line*); 6 h (*dot line*). Scanning rate: 0.5 °C min^{-1}

caused of apparatuses imperfection, for example instability of the base line and accumulation of external disturbance. An important consideration at a choice of the rate is a circumstance that in the scientific literature well-known rates at the DSC measuring is often 0.5 and 1 °C min^{-1} what allows comparison of the results obtained from different authors. From these considerations we selected the most suitable scanning rate of 0.5 °C min^{-1} for measuring of T_d of non-cross-linked and cross-linked collagen hydrolysates. Privalov also has shown that the denaturation of collagen is very slow process in comparison with the denaturation of other proteins [19]. He has studied a collagen by DSC at low heating rate, generally from 0.25 to 0.5 °C min^{-1}, and in strongly diluted solutions.

Table 2 Thermodynamic parameters, transition temperature (T_d) and calorimetric enthalpy (ΔH_{cal}), of the heat sorption curves of cross-linked collagen hydrolysates obtained at different ratio CH/CY and different reaction time, at 20 °C

Peak	ΔH_{cal}/J g^{-1}	T_d/°C
CH/CY 1:1		
1 h		
1	1.47 ± 0.1	23.3
2	1.15 ± 0.3	32.5
3	0.98 ± 0.2	37.5
3 h		
1	2.59 ± 0.3	25.7
2	1.27 ± 0.2	30.2
6 h		
1	2.96 ± 0.3	25.6
2	0.49 ± 0.3	36.3
3	1.28 ± 0.3	35.4
CH/CY 2:1		
1 h		
1	0.38 ± 0.2	24.6
2	1.97 ± 0.4	31.0
3	0.10 ± 0.1	36.0
3 h		
1	4.86 ± 0.1	29.8
2	0.56 ± 0.1	34.8
6 h		
1	0.13 ± 0.2	21.5
2	1.38 ± 0.2	30.6
CH/CY 3:1		
1 h		
1	3.08 ± 0.1	30.9
2	0.42 ± 0.1	34.0
3 h		
1	3.16 ± 0.1	30.7
2	0.54 ± 0.1	34.4
6 h		
1	2.83 ± 0.1	30.4
2	0.32 ± 0.1	34.8

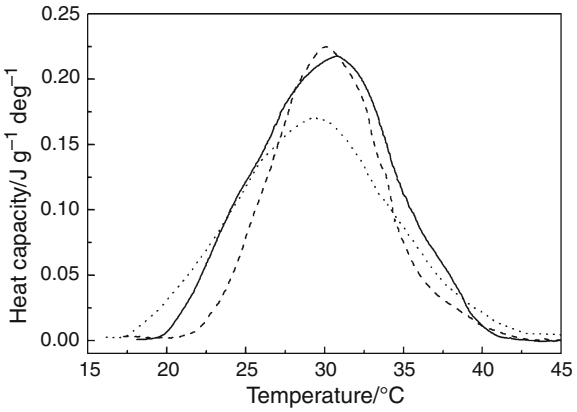

Fig. 5 DSC curves of cross-linked collagen hydrolysates obtained at a ratio CH/CY 2:1, temperature 20 °C and different reaction time—1 h (*solid line*); 3 h (*dash line*); 6 h (*dot line*). Scanning rate: 0.5 °C min^{-1}

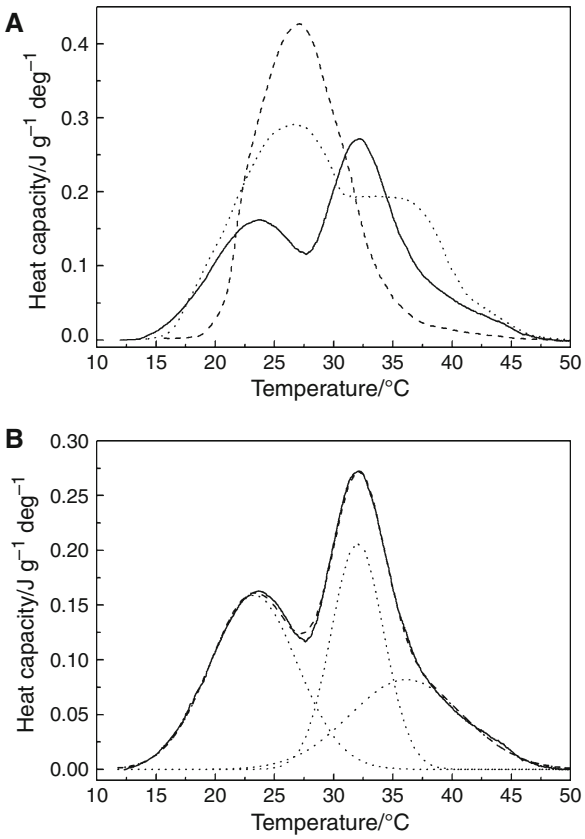

Fig. 6 **a** DSC curves of cross-linked collagen hydrolysates obtained at a ratio CH/CY 1:1, temperature 20 °C and different reaction time: 1 h (*solid line*); 3 h (*dash line*); 6 h (*dot line*). Scanning rate: 0.5 °C min^{-1}. **b** Deconvolution of the heat capacity functions of cross-linked collagen hydrolysates—CH/CY 1:1, reaction time 1 h, at 20 °C: experimental curve (*solid line*); calculated, assuming component transitions (*dash line*); component transitions (*dot line*)

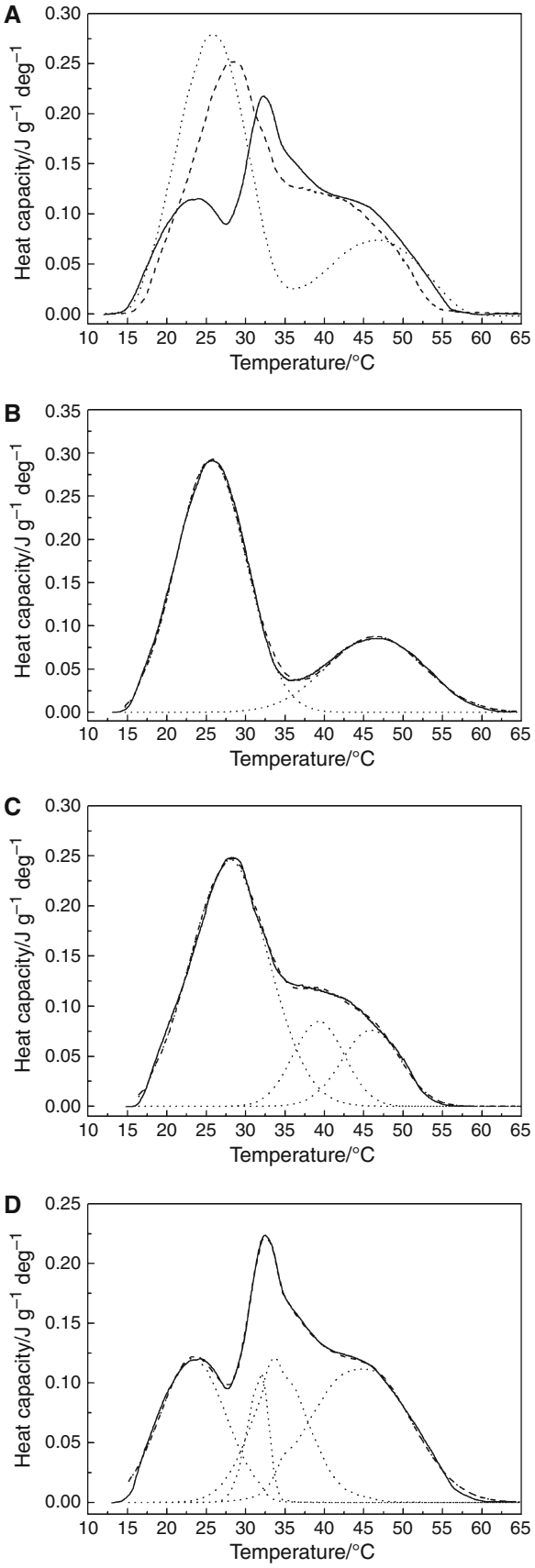

Fig. 7 **a** DSC curves of cross-linked collagen hydrolysates obtained at different ratio CH/CY, reaction time 1 h, at 50 °C—CH/CY 1:1 (*solid line*); CH/CY 2:1(*dash line*); CH/CY 3:1 (*dot line*). Scanning rate: 0.5 °C min^{-1}. **b** Deconvolution of the heat capacity functions of cross-linked collagen hydrolysates—CH/CY 3:1, reaction time 1 h, at 50 °C: experimental curve (*solid line*); calculated, assuming component transitions (*dash line*); component transitions (*dot line*). **c** Deconvolution of the heat capacity functions of cross-linked collagen hydrolysates—CH/CY 2:1, reaction time 1 h, at 50 °C: experimental curve (*solid line*); calculated, assuming component transitions (*dash line*); component transitions (*dot line*). **d** Deconvolution of the heat capacity functions of cross-linked collagen hydrolysates—CH/CY 1:1, reaction time 1 h, at 50 °C: experimental curve (*solid line*); calculated, assuming component transitions (*dash line*); component transitions (*dot line*)

Influence of the reaction time on the cross-linking of CH with CY

The process of modification of CH with CY at different ratios (CH/CY 3:1, 2:1 and 1:1), at temperature 20 °C and reaction time (1, 3, and 6 h) was studied by means of DSC.

The DSC measurements of cross-linked collagen hydrolysates, obtained at a ratio CH/CY 3:1 and reaction time (1, 3, and 6 h) show relatively cooperative transitions between 25 and 35 °C (Fig. 4). The thermodynamic parameters for two individual structural units, determined by deconvolution of the experimental DSC curves are shown in Table 2. The main transitions have T_d around 31 °C. As can be seen, the cross-linking at 3:1 ratio CH/CY finishes after 1 h reaction time.

The DSC curves of cross-linked collagen hydrolysates, obtained at a ratio CH/CY 2:1, at temperature 20 °C and different reaction time (1, 3, and 6 h) are presented on Fig. 5. The thermodynamic parameters for the individual structural units, determined by deconvolution of the experimental DSC curves are shown in Table 2. It seems that at ratio CH/CY 2:1 the heterogeneity of the obtained cross-linked collagen hydrolysates increases. It can be observed again, that the increase of the reaction time up to 6 h has not brought to formation of structures with higher thermostability.

On Fig. 6 the DSC curves of cross-linked collagen hydrolysates, obtained at a ratio CH/CY 1:1 at 20 °C and different reaction time are presented. Figure 6a shows the observed excess heat absorption results from three independent cooperative transitions. Table 2 summarizes the thermodynamic parameters for the individual structural units. Reaction time of 1 h is enough for the process of cross-linking of CH with CY. The increase of the time of cross-linking of CH with CY at a ratio 1:1 up to 6 h has not led to formation of structural units with increased thermostability.

Table 3 Thermodynamic parameters, transition temperature (T_d) and calorimetric enthalpy (ΔH_{cal}), of the heat sorption curves of cross-linked collagen hydrolysates at different ratio CH/CY, reaction time 1 h and temperature 50 °C

Peak	ΔH_{cal}/J g^{-1}	T_d/°C
CH/CY 1:1		
1	1.27 ± 0.2	23.3
2	0.48 ± 0.3	32.1
3	1.23 ± 0.4	35.2
4	1.60 ± 0.2	45.3
CH/CY 2:1		
1	3.03 ± 0.3	27.9
2	0.69 ± 0.2	39.3
3	0.71 ± 0.2	45.9
CH/CY 3:1		
1	3.25 ± 0.2	25.6
2	1.34 ± 0.2	46.5

Influence of the temperature on the cross-linking of CH with CY

In order to study the influence of the reaction temperature on the process of cross-linking of CH with CY, we performed the modification at 50 °C. Figure 7 presents the DSC curves of cross-linked collagen hydrolysates, obtained at different ratio CH/CY, reaction time 1 h and a temperature 50 °C.

Changes occurred when CH was cross-linked with CY at a ratio CH/CY 3:1, 1 h reaction time and at a temperature 50 °C (Fig. 7a; Table 3). Structural units with increased thermal stability (T_d 46.5 °C) were formed in comparison with the products obtained at the same reaction conditions, but at a temperature 20 °C (Fig. 4; Table 2).

The DSC curve of cross-linked CH with CY at a ratio 2:1, reaction time 1 h, was characterized by three endothermic transitions with T_d, respectively, 24.6, 31.0, and 36.0 °C (Table 2), when the reaction was carried out at 20 °C, but with transitions at 27.9, 39.3, and 45.9 °C, when the cross-linking passed at temperature 50 °C (Fig. 7b; Table 3).

We had observed that the cross-linked CH, obtained at a ratio CH/CY 1:1, reaction time 1 h, at a temperature 20 °C, was characterized by three main endothermic transitions with T_d 23.3, 32.5, and 37.5 °C, respectively (Fig. 6a; Table 2). When the process of cross-linking was carried out at 50 °C and the same ratio CH/CY and reaction time, the DSC curve of the cross-linked CH could be deconvoluted into four endothermic transitions with T_d 23.3, 32.1, 35.2, and 45.3 °C (Fig. 7c). In comparison, the DSC curve of non cross-linked CH was characterized by two transitions with T_d of 24.0 and 31.7 °C (Fig. 1). Therefore, the cross-linking of CH with CY at a ratio 1:1, reaction time 1 h, at temperature 50 °C brings to formation of more thermostable structural units. The thermodynamic parameters of individual transitions are presented in Table 3.

Experiments with further increased quantity of cross-linking reagent cyanuric chloride toward the collagen hydrolysate (ratio CH/CY 1:3, 1:5, 1:9) did not lead to formation of units with higher thermostability (data not shown).

Conclusions

This study by means of DSC enables us to determine the optimal conditions for cross-linking of collagen hydrolysate with cyanuric chloride. The ratio CH/CY 1:1 is sufficient to produce a population of relatively thermostable structures of cross-linked collagen hydrolysates. The process of cross-linking has been completed after the first hour of reaction time. Structural units with higher thermostability have been obtained at a reaction temperature of 50 °C.

These results can be utilized in a number of practical applications for such biodegradable materials.

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