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Thermal gelation of ribulose 1,5-bisphosphate carboxylase oxygenase with milk macromolecules

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ABSTRACT

The thermal gelation of rubisco in the presence of ions was previously published. The critical gel concentration varies according to the ionic strength i.e. electrostatic repulsion. Nothing was known about gelation of rubisco with macromolecules. Rubisco was prepared according to laboratory method and casein was obtained by chromatography; the mixture was heated at 80°C to obtain gelation. It was concluded that excluded volume phenomena is the main factor of gelation of rubisco with milk constituents.

KEYWORDS

Casein;
DSC;
Excluded volume;
Gelation;
Ionic strength;
Milk;
Polyanion;
Rubisco.

INTRODUCTION

Gelation is obtained with many proteins: the chondroitin-4-sulfate proteoglycan[1], the α, β poly (N-2-hydroxymethyl)-DL-aspartamide[2] the bovine serum albumin[3] the beta-hairpin oligopeptides[4], the nucleoporin[5]. Gelation occurs in the presence of a protein and an inert substance: hemoglobin S and bovine serum albumin[6], alkaline phosphatase and liver alcohol deshydrogenase[7]. Gelation occurs also with milk protein. The concentration of β-lactoglobulin necessary to form a gel by heating is about 1 % (w/v) independent of the ionic strength[8]. At high pressure a total concentration of at least 4% of sodium caseinate or whey protein concentrate was required for homogenization-induced gelation[9].

Ribulose 1, 5-bisphosphate carboxylase oxygenase (rubisco) is a vegetable enzyme implicated in the fixation of atmospheric CO_2 in green plant leaves. As its weak activity is compensated by its abundance in vegetative cells, it is therefore the most abundant protein on earth. Its thermal gelation varies according to vegetative species. That of lucerne (*Medicago sativa*) is about 1 g/L[10].

Thus, the thermal gelation of rubisco, in the presence of various macromolecules, has not been of interest to researchers. Therefore, the aim of this work was to study gelation of rubisco with milk macromolecules.

MATERIALS AND METHODOLOGY

Preparation of rubisco

The extraction was done by a well-known

method[11]. The stems of lucerne (*Medicago sativa*) were harvested and transported rapidly into a cold room (4-6°C) where leaves (25 g) were finely chopped, then ground for 6 minutes in a motorized grinder containing 100 mL of 0.1 M pH 7.5 Tris/HCl buffer containing 0.2 % β-mercaptoethanol, 5.0 g/L polyethyleneglycol 6000 and 0.37 g/L EDTA, and polyvinylpyrrolidone activated as well as 10 g/L of an insoluble casein.

The activation of polyvinylpyrrolidone was done by heating 25 g in 500 mL of 10 % HCL followed by washing the pasty formed with water until the washing water became neutral. The mixture was then filtered with a 106 μm pore size filter, after centrifuged (7000 g 15 min 4°C). The supernatant was saturated with 40 % and then 55 % of $(NH_4)_2SO_4$. The precipitate was introduced in 15 mM, pH 7.5 N - (2 Hydroxyethyl) piperazine N'-(-2 sulfonic) acid (HEPES). After centrifugation (10 000 g, 4°C, 20 min) and filtration on PD-10 column, the solution was allowed to pass trough CL 6B sepharose gel packed in a 2.6 cm×90 cm column. The chromatograph was attached to a detector set at 280 nm. The linear rate of the elution was 10 $cm \times h^{-1}$; only the first part of the second peak was of rubisco.

Preparation of casein

Total casein had been produced from low fat milk of a homozygote cow for the main caseins αS1, βB and κB. The pH of low fat milk was adjusted to 4.6 by HCl then centrifuged (2,000 g) at ambient temperature. The precipitate obtained was washed several times with distilled water, and then the casein was dissolved at pH 7.0 and lyophilized. The whole casein was fractionated by two successive chromatography on DEAE cellulose containing urea and β–mercaptoethanol, with a NaCl linear gradient[12].

Protein measurements

The concentration of proteins was determined according Beer Lambert's law using the absorption coefficient of 10 and 24 kDa[12], then 17 and 550 kDa[13,14] respectively for αS1 casein and rubisco, and then turbidity was noted.

Determination of the denaturation temperature

A Setaram (DSC 111) programmed differential calorimeter incorporated to an automated system and calibrated with indium and gallium was used. Its temperature varied from 298.0 K (≈ 25 °C) to 383.0 K (≈ 110 °C). The speed of temperature rise was 3.00 $K \times min^{-1}$ and the frequency of acquisition was a measure each 0.80 sec.

Determination of the critical gel concentration (C_{gel})

One mL of a well-known protein concentration was introduced in a test tube then covered by 1 mL of paraffin oil. This test tube was put in an ethylene glycol bath (80°C, 1 h)[8]. Then, the test tube was put upside down; the reaction was positive if the gel did not break.

Statistical analysis and graphs drawing

All measurements were repeated at least three times and the averages were compared by analysis of variance (*XLSTAT* software). Graphs were scanned then redrawn using Ungraph software (Biosoft, United Kingdom).

RESULTS AND DISCUSSION

After the treatment of Lucerne leaves with $(NH_4)_2SO_4$, the fractionation of the extract gave a chromatogram in figure 1. The first half of the 2nd peak was that of rubisco[15].

The fractionation of the whole casein revealed four peaks, the last of which (around 40th fraction) contains αS1 casein (Figure 2). It was generally agreed that gelation was a two steps process, where the denaturizing protein was induced by heating and the gel formation was the consequence of aggregation of the denaturizing molecules when the concentration was larger than the percolation threshold[16,17]. The thermal denaturizing of rubisco occured around 67.7°C[18]. After thermal denaturizing, rubisco molecules aggregated to form a three dimensional network. Gelation occured when this network reached an undefined size.

The mixture of rubisco and αS1 casein gave a thermogram (Figure 3) indicating a denaturizing temperature around 68.8°C. When the ratio αS1 casein / rubisco increased, the denaturizing temperature of rubisco increased from 67.3 to 68.5°C then decreases (TABLE 1).

The rubisco C_{gel} with 2.5 mg/mL αS1 casein was about 1.0±0.5 mg/mL. The gelation also occurred be-

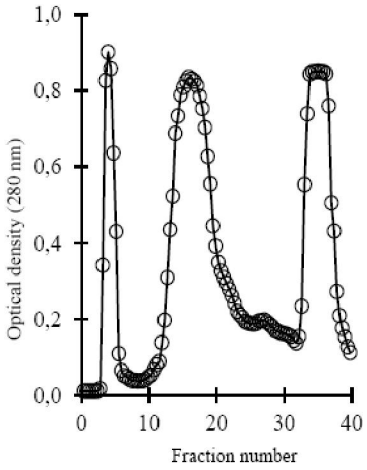

Figure 1 : Gel filtration of a $(NH_4)_2SO_4$ precipitate. The sample was dissolved in 12 mL, 15 mM pH 7.5 HEPES. A 2.6×90 cm column packed with CL-6B Sepharose. The linear rate was 14 cm×h^{-1} and the fraction volume was 10 mL

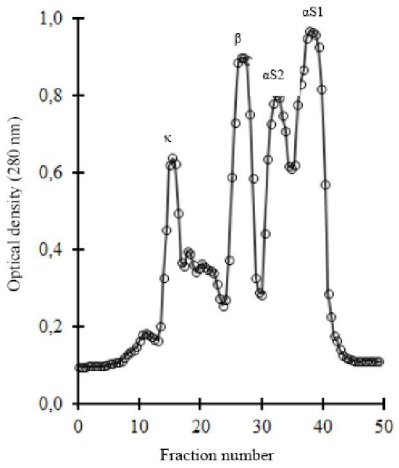

Figure 2 : Chromatogramm by ions exchanged of casein. 1.65×5 cm column was packed with DEAE cellulose. The elution was done with a linear gradient of 0 to 0.5 M NaCl. The linear rate was 12 cm×h^{-1} and the fraction volume was 10 mL. αS1: αS1 casein, αS2: αS2 casein, β: β-casein, κ: κ-casein

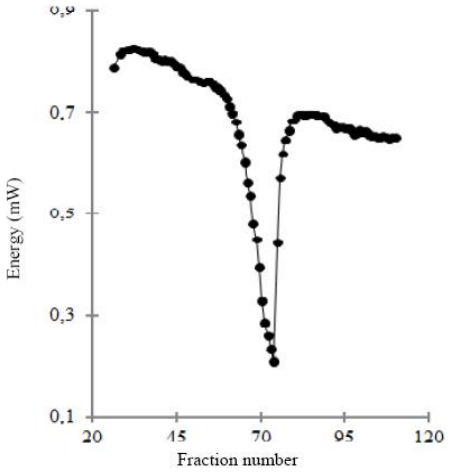

Figure 3 : Thermogramm of a mixture of rubisco and αS_1 casein. Analysis was done between 298.0 K (≈ 25 °C) and 383.0 K (≈ 110 °C). The frequency of acquisition was 0.80 second

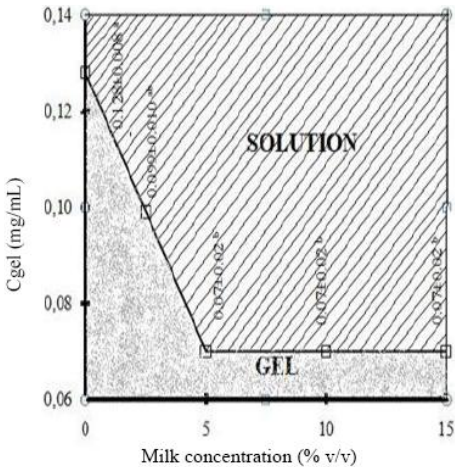

Figure 4 : Phase diagram: effect of milk on the critical gel concentration of rubsico. The same superscripted letters indicate that there is no significant difference ($p<0.05$)

tween α-lactalbumine and bovine serum albumin[19].

The αS1casein diminished very significantly the C_{gel} of rubisco while the Tm was increased by αS1 casein. This behavior was analogous to that observed when the salt concentration increased in the case of NaCl, NaH_2PO_4 or Na_2SO_4: the Tm increased and the C_{gel} decreased[10]. This behavior was different from that observed once the $MgCl_2$ or $CaCl_2$ concentration increased because the Tm and the C_{gel} reduced simultaneously[10].

The αS1casein is a polyanion like those of sulfate ions or phosphate; but this analogy does not suffice to explain all the observed effects in the presence of the αS1 casein which is a macromolecule and possess distinct properties from those of polyvalent anions. The phase diagram of the mixture rubisco - whole casein is shown in table 2. Meanwhile by increasing the concentration of the whole casein (0 to 1 mg/mL), the rubisco C_{gel} decreased from 5.5 to 1.5 mg/mL.

It seems that in diluted milk, casein still finds its self in native micelle forms meanwhile in the preparation of αS1 or whole casein, the micelle structure was greatly modified. The rubisco C_{gel} with whole casein in the presence of either 20 or 100 mM NaCl was respectively 7.0±0.5 and 1.0±0.5 mg/mL[10].

Moreover, it is known that the rubisco C_{gel} is influ-

enced by the salt concentration; between 0 and 100 mM NaCl, the ionic strength (electrostatic repulsion) is high, the less is the C_{gel}; it is the main factor conducting the gelation. Moreover, the ionic strength explains why the phase diagram corresponding to 100 M NaCl was below that of 20 M NaCl (TABLE 2). The fact that the two phase diagram (20 and 100 mM NaCl) are not the same permits to think that the ionic strength is not the only phenomena that explains the mixture rubisco / total casein phase diagram.

In the cases of 20 and 100 mM NaCl phase diagram, and despite the ionic strength, the rubisco C_{gel} increased above 5 and 2.5 mg/mL respectively with the increase of whole casein concentration up to 5 mg/mL. When casein concentration increased, the bridges between denaturized rubisco had difficulties in establishing, so it needed more denaturized rubisco to reach C_{gel} (excluded volume phenomena). Casein has a flexible conformation in solution which portrays some of its organic polymer properties. The volume that it occupies in solution is great.

It is possible that in the concentration zone from 0.25 to 2 g/L, where the smallest value of the C_{gel} was observed, these effects of excluded volume that predominates in the gelation of rubisco will be facilitated by its high local concentration. The effects of excluded volume could be stopped above a casein concentration of 2 g/L, as this causes the increase in the viscosity of casein solutions. Solutions present in the medium, and which did not gelate on their own could interfere with the thermal denaturizing or upon aggregation. The influence of solutes on aggregation can manifest in two ways: by their effects on the conformation attained after heating, or could be by a direct effect on the aggregative mechanism: pontage between proteins or steric exclusion[20].

The gelation due to excluded volume has been observed with polymer ionic molecules[21], nucleoporins[22], polymer surfactant and protein surfactant[23]. A gelation of rubisco occurs in presence of milk (Figure 4).

Two parts can be distinguished on this phase diagram: the decrease of C_{gel} when the milk concentration increases from 0 to 5 % (v/v), and the stagnation of C_{gel} while the milk concentration raised from 5 to 15 % (v/v). Milk is a mixture of many compounds that

TABLE 1. Variation of denaturizing temperature of the mixture of αS1 casein/rubisco

α casein/rubisco (ratio)	Denaturazing temperature (°C)
0	67.3 ± 0.5^{a}
3	67.7 ± 0.5^{ab}
6	68.1 ± 0.1^{abc}
9	68.5 ± 0.4^{c}
15	68.2 ± 0.2^{bc}

Mean are followed by standard deviation; the same superscripted letters indicate that there is no difference between two means (p<0.05)

TABLE 2 : Effect of whole casein on the denaturizing temperature of rubisco

*CC (mg/mL)	C_{gel} (mg/mL)[&]
0.00	5.43 ± 0.24^{f}
	1.32 ± 0.20^{abcde}
0.05	5.43 ± 0.24^{f}
	1.27 ± 0.23^{abcde}
0.30	1.81 ± 0.11^{bcdef}
	0.91 ± 0.13^{a}
0.50	1.86 ± 0.30^{cdef}
	0.94 ± 0.14^{ab}
0.70	1.81 ± 0.33^{bcdef}
	1.15 ± 0.08^{abc}
1.00	1.37 ± 0.06^{abcde}
	0.91 ± 0.10^{a}
2.00	1.79 ± 0.22^{abcdef}
	1.29 ± 0.21^{abcde}
2.40	2.58 ± 0.48^{def}
	1.26 ± 0.24^{abcd}
3.40	3.21 ± 0.21^{f}
	2.27 ± 0.13^{cdef}
5.00	5.46 ± 0.43^{f}
	2.66 ± 0.21^{ef}

***Casein concentration (mg/mL), & Mean are followed by standard deviation; the same superscripted letters indicate that there is no difference between two means (p<0.05)**

interact in rubisco gelation: milk fat globules, lactose, protein (whole casein and whey proteins) and ions (Ca^{2+}, Mg^{2+}, Na^{+}, Cl^{-}, SO_4^{2-}, $H_2PO_4^{-}$)[24].

It is well known that some of these ions bind with denaturized rubisco and modify their gelling behavior according to Hofmeister series so as other macromolecules[25-27]. With all theses salts, electrostatic repulsion between rubisco molecules are progressively screened so that C_{gel} is lowered until electrostatic repulsions are

no longer the limiting factor[10]. Between 5 and 15 % (v/v) milk concentration, the volume excluded due to different compounds (milk fat globules, lactose and protein) must be the main factor of gelation procedure.

CONCLUSION

The analysis of the conditions of denaturation and gelation supposes that the electrostatic repulsion could be an essential factor limiting gelation. The reduction in repulsion permits the reduction of the rubisco C_{gel} to 1.5 mg/mL. The presence of casein or milk cause more reduction in the limit to about 0.8 mg/mL. It seems ions or macromolecules intervene by different gelation mechanisms. These solutes can intervene principally on the thermal denaturation by volume excluded mechanisms.

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