Novel Procedure for Large-Scale Purification of Atelocollagen, by Selective Precipitation

by

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Abstract

This paper describes a novel procedure for the purification of raw atelocollagen solutions, based on a multi-stage selective precipitation of the protein fractions, using polyethylene glycols (PEGs) as crowding agents. The precipitation selectivity was provided by the simultaneous effect of pH and crowding agents. Two adjuvants were added to improve the precipitation selectivity: ethanol and trimethylamine-N-oxide (TMAO). An optimal composition of the crowding mixture, consisting of 23% PEG 400, 61% PEG 6000 and 16% PEG 20000, induced a "screening" effect within the isoelectric range of atelocollagen. The optimal recipe for the screening of an atelocollagen sol obtained at plant scale was: 80g/L crowding mixture, 0.2 molar TMAO, 0.3 molar ethanol, pH = 3.8, processing time: 4-6 hr at 5 ÷ 10°C. The proposed procedure is useful in the manufacture of colloidal collagen solutions, in order to replace or to facilitate expensive and denaturing operations, like ultrafiltration.

Keywords: atelocollagen, selective precipitation, crowding agents, collagen-based biomaterials

Resumen

Este trabajo describe un procedimiento novedoso para la purificación de soluciones crudas de atelocolágeno [extracto acuoso de colágeno por uso de proteasas], basado en precipitaciones selectivas en varias etapas de fracciones proteínicas por medio de glicoles polietilénicos (PEGs) como agentes de hacinamiento [a la solubilidad] . . La selectividad de precipitación fue efectuada por el uso simultáneo de agentes de hacinamiento y pH. Dos adyuvantes fueron añadidos para incrementar la selectividad de la precipitación: etanol y óxido-N-trimetíl amina (TMAO). La composición óptima de la mezcla de hacinamiento constituida por 23% PEG 400, 61% PEG 6000 y 16% de PEG 2000, indujeron un effecto de apantallamiento en el rango isoeléctrico de la solución de atelocolágeno. La óptima composición para el apantallamiento de una solución de atelocolágeno a escala de planta fue: 80g/L de las mezcla de hacinamiento, 0,2 molar TMAO, 0,3 molar etanol, pH=3,8, y tiempo de procesamiento: 4-6 horas.entre 5-10°C. El procedimiento propuesto es útil en la preparación de soluciones colagénicas coloidales, en vista de reemplazar o facilitar costosas operaciones con riesgo de denaturación, como lo es el de la ultrafiltración.

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INTRODUCTION

At present, about 6 to 9 percent of the hides resources are qualitatively inappropriate to obtain leathers. Despite their poor quality as raw material for tanneries, these hides contain high amounts of mature collagen. Lately, much attention has been paid to the valorization of such "low quality" hides, for obtaining minimally denatured collagen forms, of high interest for cosmetic, pharmaceutical and biomedical applications.

As a cutting-edge area of the biomedical field, tissue engineering focuses on the restoration, maintenance or improvement of tissue function through the development of biological substitutes. Two main constituents of these biological substitutes are scaffolds and cells; one of the most versatile scaffolds is built from atelocollagen.

The most recent paradigm of tissue engineering stipulates the *in situ* regeneration of injured organs[1], starting from implanted surrogates of extracellular matrices (s-ECM). At present, s-ECMs can be obtained by: **(1)** reassembling of the collagen tri-dimensional structures to form quasi-natural substrates, and **(2)** generating synthetic hydrogels able to mimic the invasive characteristics of collagen[2]. The advantage of fibrillar collagen (types I, II, III) over synthesis challengers consists in its higher cito-sustainability, by virtue of the triple-helical supramolecular structure. Hence, the quasi-native fibrillar collagens, like atelocollagen, represent the most "friendly" partners of cells in tissue engineering approaches.

To act as an appropriate substrate in tissue engineering, fibrillar atelocollagen must preserve its biological activity, i.e. the ability to generate supramolecular aggregates. Aggregation-competent atelocollagen can be obtained by: **(i)** "disassembling" of collagen from natural sources, by solubilizing techniques, **(ii)** genetically-driven biosynthesis of collagen by transgenic techniques[3] and **(iii)** chemical synthesis of engineered self-assembling collagen-like polypeptides [4,5]. Only the first way is feasible for obtaining medium (50 – 500 grams as dry matter (DM)) or large (0.5 – 10 kg as DM) amounts of aggregation-competent molecules, useful in producing scaffolds.

At laboratory level, atelocollagen is obtained as colloidal solutions by protected chemical and / or enzyme solubilization of mature collagen. At large scale, it is obtained by the "alkali pre-treatment" pathway, which assures the compromise between the required atelocollagen characteristics and the technical possibilities. Dissolving collagen at plant scale gives rise to a polycompositional, polydisperse colloidal system, containing ten protein fractions, along with tissue debris: **(i)** denaturated non-collagenic proteins, **(ii)** polypeptide fragments, **(iii)** long-catenary hydrolyzed collagen, **(iv)** denaturated collagen α-chains and its di- and tri-aggregates, **(v)** eucollagen forms, **(vi)** fragments of chemically associated collagen and glico-proteins, **(vii)** intact monomeric α-chains detached from the collagen triple-helix, **(viii)** non-altered, pepsin- and papain-resistant triple-helical atelocollagen molecules, **(ix)** supramolecular collagen aggregates, **(x)** incipient renaturated and aggregated fibrillar structures. Amongst these, only the eighth fraction may be of practical interest. An efficient separation of such complex mixture can be done in several steps only, following three processes at least: enzyme treatments, ultrafiltration and selective precipitation.

The classical purification technique at plant scale involves two main stages: (1) precipitation of the quasi-native atelocollagen fraction, with NaCl or salt mixtures; large amounts of concentrated salt solutions result from the process, which must be treated as wastewaters; (2) the protein precipitate is resuspended in acidic solutions and subjected to extensive diafiltration, to reduce the polydispersity of the colloidal system and to eliminate the excessive salts content.

The present paper presents a novel purification procedure, which assures the elimination of all tissue debris and parasitic protein fractions, in a simple, cheap and ecofriendly way. The principle consists in a salt-free, multi-stage selective precipitation of the protein fractions, using inert organic polymers, in the presence of organic low molecular adjuvants. The inert polymers, acting as "crowding agents", reduce the water solubility of all the intact proteins and proteins aggregates. By their kosmotropic action, the adjuvants protect the triple helical topology of atelocollagen and provide the separation of intact atelocollagen molecules. The proposed procedure increases the fractionation selectivity and significantly reduces the inorganic salts consumption. The residual solution may be reused with a minimal adjusting of its composition.

EXPERIMENTAL

Materials and reagents

Low quality, green and preserved young bovine hides (6 to 15kg in green state) were the primary collagen source. *The enzymes* used in the experiments were *pepsin* (EC 3.4.23.1, technical grade, purchased from Serva Electrophoresis GmbH) and *papain* (EC 3.4.22.2, 0.5 units/mg, purchased from Sigma - Aldrich Chemie GmbH).

The crowding agents. A mixture of polyethylene glycol polymers, consisting of PEG 400, PEG 600 and PEG 20.000, provided by Merck Schuchardt OHG, was used as crowding agent. The mixture was formulated according to the DOE principles[6]. A restricted three components mixture design, able to generate a full cubic model was used (depicted in Figure 1, and summarized in Table I).

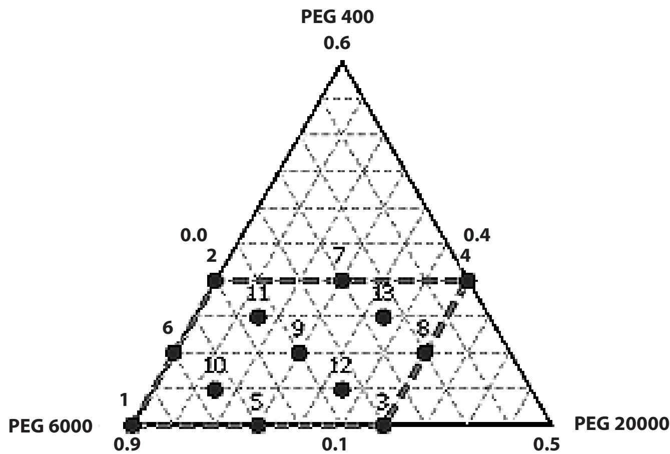

Figure 1. – The experimental design of the tertiary PEGs mixture composition.

The adjuvant chemicals. Reactive grade trimethylamine-N-oxide hydrochloride (TMAO, CAS 1184-78-7, from Sigma - Aldrich) and ethanol were used to assist the crowders mixture. *All the general reagents* are of technical grade, except the p.a. chemicals used in the ultrapurification stage. In the raw solubilization and physical-chemical refining, all the floats and solutions were prepared with deionised water. For enzyme refining and ultrapurification, deionised, pyrogen-free water was used.

Methods

The overall process is depicted in Figure 2. In the first stage, a primary colloidal solution was obtained, which was further physically purified to generate a primary atelocollagen solution. After an enzyme refining and a subsequent dialysis, the final ultrapurification stage was applied, obeying the principles presented in the introductory paragraph.

The primary atelocollagen solution was obtained following a 52-operation plant-scale procedure, based on alkali pre-treatment, pepsin solubilization, acidic precipitation and acid resolubilization processes, as described by Li, Fukunaga, Takenouchi and Nakamura[7]. The characteristics of the resulting solution: 1.6 ÷ 1.8% DM, 1.2 ÷ 1.35% protein content, approx. 75g protein/100g DM, pH 3.1 ÷ 3.3, global isoelectric pH range 4.12 ÷ 4.16, no sediment after 30 minutes centrifugation at 8000g RCF.

The enzyme refining of the primary atelocollagen solution was conducted according to Gunasekaran[8], using papain for the advanced hydrolysis of the telopeptides. The treatment was conducted at low temperature (6 ÷ 10˚C), using 1.8g/L papain powder, under slow mixing, for 12 hours. The enzyme inhibition was induced with 1.2ml/L H_2O_2 32% (v/v).

The ultrapurification procedure comprised five steps, as it follows:

(**1**) *elimination of low molecular protein fractions*, by ultrafiltration over a polyethersulfone membrane (Millipore Biomax discs PBHK15005, filtration area 117 cm^2), having the nominal molecular weight limit (NMWL) of 100 kDa;

(**2**) *selective fractionated precipitation with PEG mixtures*; the generic recipe was: **(a)** a 0.2 molar TMAO solution was obtained by slowly dissolving the TMAO dihydrate in the ultrafiltered atelocollagen solution, under gentle agitation; **(b)** the solution was cooled at 5°C and then ethanol was added, to reach a concentration of 0.3 molar ethanol; **(c)** the pH of the cooled solution was corrected at 3.8, near the atelocollagen isoelectric range, with 0.1 molar NaOH; **(d)** the PEGs were blended separately to get a homogeneous mixture, which was slowly poured into the cold atelocollagen solution, under vigorous stirring; the PEGs mixture mass contribution in the reaction mass was 8% w/v (half of the quantity that agglomerates the whole protein into an amorphous slurry); **(e)** the atelocollagen solution was kept at 20°C for 4 to 6 hr under gentle stirring, when the selective fractionated precipitation took place, resulting in the separation of variable size flocks, stable enough not to be

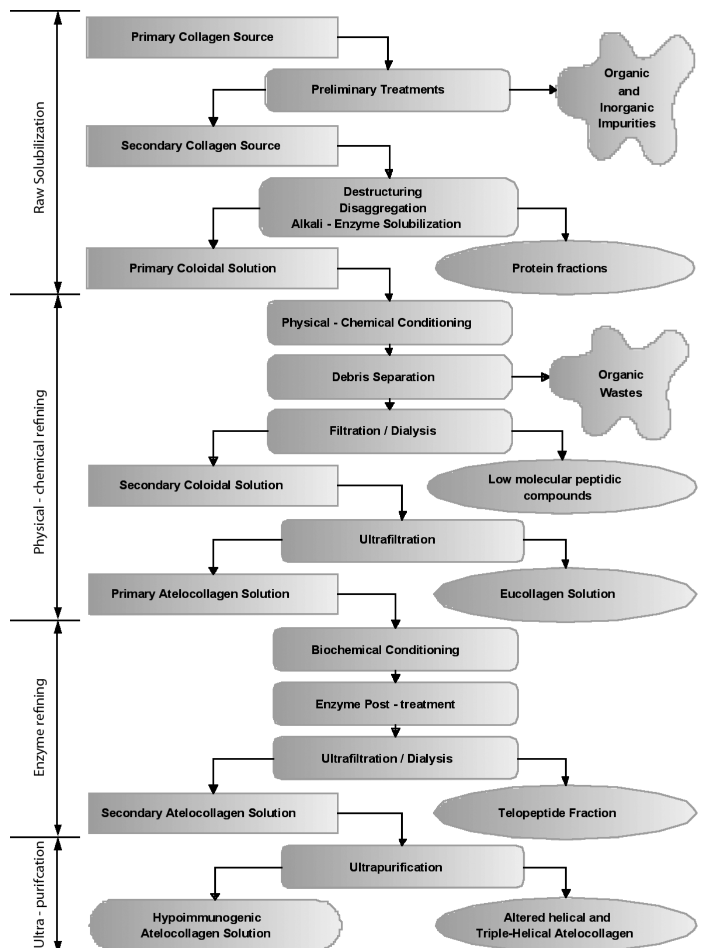

Figure 2. – The generic pathway for the obtaining of atelocollagen solutions at plant-scale.

disrupted during handling of large liquid volumes; the flocculated system was stored for another 2 hr at 20°C;

(**3**) *removing the precipitated undesirable protein fractions and colloidal size debris* by centrifugation at 4000g RCF for 20 min.

(**4**) *total precipitation of the purified triple-helical intact atelocollagen*, by raising the pH of the supernatant to 4.5, with 0.1 molar NaOH; in the presence of PEG mixture and adjuvants, the precipitation was quickly promoted and resulted in a fine, stable suspension, able to separate a white atelocollagen slurry after 20 min centrifugation at 8000g RCF;

(**5**) *controlled solubilization of the atelocollagen* slurry in a volume of 0.1 molar acetic acid, equal to the initial volume of the impure solution; PEGs and adjuvants traces were removed by a final diafiltration over a 100 kDa NMWL membrane (Sartorius Stedim cardriges, PES membrane), with seven times as much 0.1 molar acetic acid as the initial volume of the redissolving solution.

The colloidal system turbidity was determined on a JASCO V-550 UV-VIS double beam scanning spectrophotometer, at 313 nm. The samples were drawn at the end of phase **(e)** of step **(2)** of the ultrapurification procedure, quickly introduced in the 10 mm quartz cuvette, thermostated at 20°C, and the optical density was measured. Turbidity, τ, was expressed as the ratio between the light intensity that passes through the sample cuvette, **I**, and the incident light intensity, I_0, according to relation:

$$\tau = \tau \ln (I / I_0) \quad (1)$$

Total protein nitrogen content was determined as Total Kjeldahl Nitrogen (TKN), on a VELP DK6 programmable heating diges UDK 132 semiautomatic distillation unit, on samples equivalent to 1g DM protein, prelevated from the initial solution and from particular precipitates.

TABLE I
The restricted mixture design matrix and the measured responses values

Mixture design structure							
Number of mixture components: 3					Imposed restrictions to mixture components		
Design degree: 3					Variable	Lower value	Upper value
Design type: extreme vertices					PEG 400	0.100	0.300
Design augmenting: central point, axial points					PEG 6000	0.400	1.000
A priori model: third order, full cubic					PEG 20000	0.000	0.300
Exp. no.	**Order in the experiment**	**Design point (Fig. 2)**	**Mixture components (mass fractions)**			**Measured responses (nondimensional)**	
			PEG 400	**PEG 6000**	**PEG 20000**	**Turbidity** τ	**Precipitate amount % pp**
1.	10	1	0.100	0.900	0.000	3.93	17.06
2.	3	2	0.300	0.700	0.000	3.08	9.38
3.	5	3	0.100	0.600	0.300	2.83	27.30
4.	12	4	0.300	0.400	0.300	1.33	7.25
5.	9	5	0.100	0.750	0.150	3.74	11.33
6.	4	6	0.200	0.800	0.000	3.17	10.57
7.	7	7	0.300	0.550	0.150	2.56	12.09
8.	1	8	0.200	0.500	0.300	1.74	23.12
9.	6	9	0.200	0.650	0.150	4.11	21.31
10.	2	10	0.150	0.775	0.075	4.26	19.25

Undesirable protein fractions and debris, **pp**, separated by precipitation, were calculated as:

$$\%pp = \frac{\left(N_{initial\ sol} - N_{post\text{-}precipitate\ sol}\right)}{N_{initial\ sol}} 100 \qquad (2)$$

where: $N_{initial\ sol}$ - the total protein nitrogen in the initial atelocollagen solution before ultrapurification, and $N_{post\text{-}precipitate\ sol}$ - the same parameter determined for the centrifuged supernatant at the end of step (3). The nitrogen balance was checked by the TKN of the final ultrapurified atelocollagen solution.

Results and Discussion

In confined aqueous medium, all proteins tend to associate, or even to crystallize. Being a dissymetric molecule and having a strict spatial distribution of hydrophilic and hydrophobic domains, intact atelocollagen will generate labile unregulated fibrillar aggregates at relatively low concentrations of crowding agents, before all the lower molecular fractions present in the secondary atelocollagen solution, but after the spatially extended supramolecular aggregates.

To stabilize the native triple-helical conformation of atelocollagen and to facilitate the selective separation of randomly-folded denaturated species, two kinds of low molecular compounds were added to the primary atelocollagen solution, prior to PEGs addition: a *kosmotrope* (the trimethylamine-N-oxide) which was an efficient stabilizer for the highly-ordered collagen domains, rich in intramolecular hydrogen bonds, and *an alcohol* (the ethanol) which acted weakly antagonically, by decreasing the strength of hydrogen bonding and electrostatic interactions[9], but showed poor efficiency in destabilizing the low molecular and denaturated protein forms. The competition between these two adjuvants, based on their weak asymmetric antagonism, increased the selectivity of precipitation mediated by the crowding agents.

In a crowded medium and in the presence of kosmotropes, the atelocollagen association equilibria were highly influenced by several nonspecific interactions, able to dictate the precipitation rate and magnitude, within ordinary variation ranges of the chemical parameters. For small pH variation of the crowded atelocollagen solution, the precipitation of supramolecular aggregated collagen species is substantially increased, in contrast with the unaffected collagen macromolecules, or with hydrolytically degraded ones. The effect of pH variation on the collagen solution turbidity, in different process stages, is given in Figure 3. Comparing to the initial atelocollagen solution, the general content of the collagen species and the polydispersity of the colloidal system are decreased, both by precipitation in the presence of PEG mixture (which drastically reduce the supramolecular agregates content), and by final diafiltration (which eliminates all the polypeptides resulted by atelocollagen unfolding or by accidental hydrolysis).

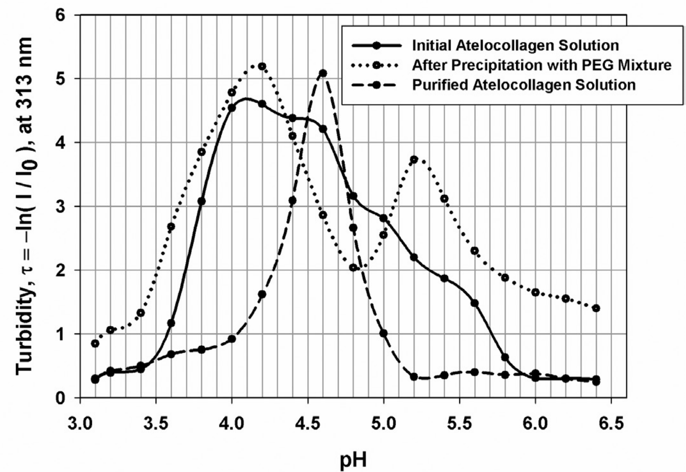

Figure 3. – Turbidity of atelocollagen solutions vs. pH, within the global isoelectric domain and in near vicinity. (Values in ordinate are nondimensional and normalized.)

The curves in Figure 3 confirm the presence of different macromolecular species, which arise during the ultrapurification operations. Before the selective precipitation, the broad multimodal distribution of protein fractions indicates the presence of a heterogenous mixture of intact, altered and associated atelocollagen species, each of them having a well-defined isoelectric point. After the treatment with crowding agents, two dominant species, with distinct isoelectric points, remain in the solution, but the distribution is still broad, at least at lower isoelectric pH values. The curve corresponding to final ultrapurified atelocollagen solution shows a narrow dispersion of the macromolecular characteristics, suggesting that only very similar atelocollagen macromolecules are present in the colloidal system. These macromolecules were hidden in the lower broad fraction of the previously discussed curve. The effective abundance of the macromolecular species cannot be directly derived from Figure 3, because the depicted turbidity variations are nondimensional (being logarithmed ratios between light intensities) and are normalized by reporting to the biggest amplitude (in order to ease the interpretation). Despite this inconvenience, curves in Figure 3 reveal the ability of atelocollagen and collagen derived polypeptides to precipitate by simply adjusting the pH of the crowded colloidal system.

The global isoelectric domain of the purified atelocollagen increased by 0.4 units on the pH scale (from 4.2 to 4.6), as a result of the removal of the chemically affected collagenic forms (especially the deamidated polypeptides, the deamidated α- and β-subunits chains and the deamidated γ-units, all of them hydrolytically and/or thermally denaturated). The new value of the isoelectric point remains in the acidic range because the intact triple helical atelocollagen was obtained through an alkali treatment, which is deamidating *per se*.

The effect of the PEGs mixture recipe on the precipitated collagen fraction was assessed through the total protein nitrogen content of the sediment separated at the end of step (3). A "screening" effect was distinguished in the isoelectric domain and in the near vicinity, induced by an optimal PEG mixture, consisting of 23% PEG 400, 61% PEG 6000 and 16% PEG 20000. This optimal composition was obtained by a ternary mixture formulation study, conducted accordingly to the experimental design given in Table I. Figures 4 and 5 present the dependence of the solution turbidity and the total amount of separated protein fractions on the composition of the crowding agents mixture. The optimal range of the PEGs mixture composition is given in Figure 6.

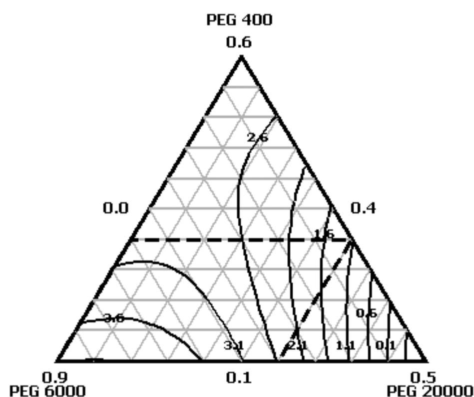

Figure 4. – The dependence of colloidal solution turbidity on PEG mixture composition, near the global isoelectric point (pH 3.8).

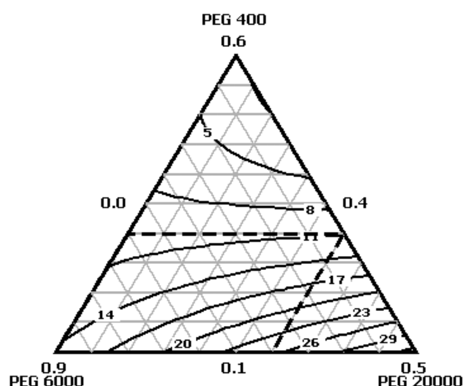

Figure 5. – The dependence of precipitated protein fraction on PEG mixture composition, near the global isoelectric point (pH 3.8).

There are two reasons for mixing the three different molecular weight PEGs. First of all, they selectively reduce the proteins solubility according to the relation between their own hydrodynamic radii ($r(\text{Å}) = 0.29 \cdot M_w^{0.454}$) and the protein specific volume in water solutions[10,11,12]. Secondly, from an engineer's point of view, the impact of mixtures on immediate, non-selective, protein precipitation is strongly diminished by the "enveloping" of high molecular weight solid PEG particles by the low molecular, liquid PEG 400. Direct solution of PEG 20000 in a concentrated atelocollagen sol results in early precipitation of intact triple-helical molecules, together with undesired protein fractions. Hence, important distinction can be made between the effects of the three PEGs on the selective precipitation of the protein fractions.

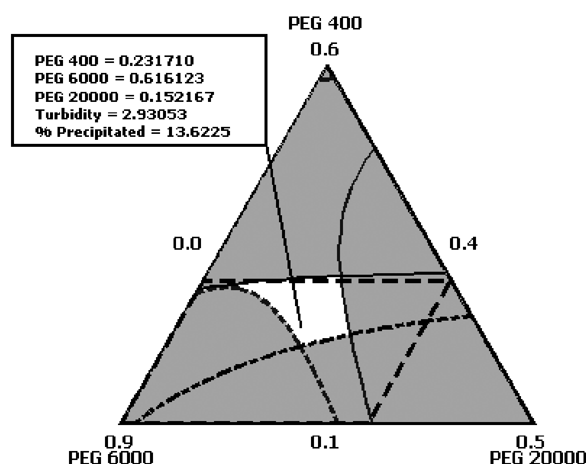

Figure 6. – Optimal domain of the PEG mixture composition.

Table II presents the statistically valid coefficients of the polynomial models fitted to the experimental data. The main effects coefficients describe the individual influence of each mixture component on the measured parameters (the solution turbidity, and the precipitate amount). The negative values reflect process inhibition. The interaction effects coefficients describe the mutual conditioning among the PEG species in the mixture. Negative values reflect an antagonic action of one PEG over the other. Raising the PEG 20000 weight fraction in the mixture increases the precipitation yield (see Table II and Figure 5), but reduces the solution turbidity (see Table II and Figure 4), due to the flocks volume growth. At the same time, the fractionated precipitation becomes less selective. The interaction between PEG 400 and PEG 20000 is antagonic both as it concerns the turbidity and the precipitation yield. This conclusion confirms the initial assumption on the protective role of PEG 400 in fractionated precipitation. The major antagonic inhibiting effect is related to the precipitate amount (compare PEG 400·PEG 20000 coefficients value in Table II). Two unexpected interaction

TABLE II

Coefficients of the mathematical models fitted to mixture formulation experimental data

The mathematical model of solution turbidity			The mathematical model of precipitate yield		
Main effects	PEG 400	6.56	Main effects	PEG 400	-6.7
	PEG 6000	4.97		PEG 6000	16.8
	PEG 20000	-17.30		PEG 20000	70.5
Interaction effects	PEG 400 · PEG 6000	-11.91	Interaction effects	PEG 400 · PEG 6000	0.0
	PEG 400 · PEG 20000	8.07		PEG 400 · PEG 20000	-167.1
	PEG 6000 · PEG 20000	25.59		PEG 6000 · PEG 20000	0.0
Overall adequacy of the model, R^2 (the coefficient of multiple determination)		97.38	Overall adequacy of the model, R^2 (the coefficient of multiple determination)		95.41

effects on turbidity induction were detected: an antagonism between PEG 400 and PEG 6000 and a synergetic potentiation between PEG 400 and PEG 20000. Assuming that PEG 400 decreases the crowding efficiency of higher weight PEGs, by aggregation limiting, it follows that PEG 20000 is less sensitive comparing to PEG 6000. The opposite signs of the mentioned interaction effects are to be revalidated by further studies.

Conclusions

Low quality, but undegraded young bovine hides, can be used for the extraction of highly pure quasi-native atelocollagen.

The removal of the parasitic protein fraction can be done by a salt-free procedure, consisting in the isoelectric precipitation of atelocollagen supramolecular aggregates in a crowded medium, under the assistance of kosmotropes. As crowding agents, a ternary mixture of polyethylene glycols is used.

The proposed procedure is based on the "screening" effect induced by the macromolecular crowding mixture, during the pH-driven precipitation of protein forms present in the impure atelocollagen solutions. A mildly crowded medium (80g/L optimized PEG mixture) and the presence of kosmotrope agents provided a fractionated precipitation at a pH closely below the global isoelectric point of atelocollagen solution. The optimal composition of the crowding agents mixture was: 23% w/w PEG 400, 61% w/w PEG 6000 and 16% w/w PEG 20000.

The experimental procedure comprised five steps and assured a sharp separation of undesired protein forms and a good-yield isolation of the intact triple-helical atelocollagen.

The procedure can be easily scaled-up and can be inserted both in early and final stages of the colloidal collagen manufacture by corium disaggregation and solubilization, in order to replace or to facilitate expensive and potentially denaturing operations, like ultrafiltration.

Acknowledgments

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