Bulgarian Chemical Comunications, Volume 48, Special Issue A (pp. 29-37) 2016

Optimization of the classical method for nucleation and growth of rhombohedral insulin crystals by pH titration and screening

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Submitted on September 8, 2015; Revised on November 23, 2015

Crystallization of hexameric zinc-containing insulin in its rhombohedral space group is accomplished by step titration of an acidic insulin solution containing an excess amount of Zn^{2+} ions either in the presence of acetone or not. During the preparation of the mixture, precipitation was observed when crossing a critical pH value. This precipitate dissolves and produces a clear solution after the addition of acetone. We investigated the influence of the pH on the crystallization of both porcine and recombinant human insulin by means of a systematic study of the pH evolution. Taking into account the volume fractions and concentrations of all components, by varying the ratio between the initial acid solution (0.02M HCl pH 1.55) and the citrate buffer (0.1M pH 6.98) we modulated the rate at which the critical pH was reached. This characterization allows us to precisely tune the conditions for obtaining small crystals in batch crystallization droplets in the presence or absence of acetone.

Keywords: Insulin hexamers, rhombohedral crystals, crystallization screening

INTRODUCTION

Insulin is a hormone present in the blood stream and involved in glucose homeostasis. In a healthy body, insulin is stored as rhombohedral single crystals in the granules of the pancreas beta-cells. This specific crystal form is well known and the building blocks of the crystals are insulin-zinc hexamers [1, 2]. The nanocrystals formed in the granules are released into the blood stream where they dissolve very fast [3] delivering a sufficient amount of insulin monomers, the active form that binds to cell insulin-receptors to control glucose assimilation in the intracellular space [1, 2]. In the case of disorders like *diabetes mellitus* type I, this vital hormone is administered externally, most often subcutaneously, by using medical solutions or suspensions based on recombinant human insulin or different insulin analogues of controlled formulations [4-6]. The type of insulin formulation and the dosage strongly depends on the prescribed medical treatment. Comprehensible, the numerical studies on insulin crystallization have focused in two main directions: 1) investigations of the formation and dissolution of natural (rhombohedral) zinc-containing insulin crystals [3, 7]; 2) the formulation of fast, intermediate and long-period acting insulin drugs [4-6]. The first trend is related to the better understanding of the factors governing the *in-vivo* crystallization and dissolution of insulin, whereas the second one covers the needs of patients with insulin disorders.

The studies on the crystallization of insulin and insulin-complexes have greatly contributed to the optimization of the growth of single protein crystals for crystallographic structure determinations by X-ray and neutron diffraction methods but open questions remains on the nucleation and growth to control both processes that are of fundamental relevance for the pharmaceutical industry. X-ray crystallography has also been used to characterize the insulin-protamine complex, typically used in pharmaceutical formulation [8] although a clear picture of this interaction is still to come. Several of these studies [7-18] have extensively screened different crystallization conditions in order to get crystals of sufficient size for X-ray characterization. Nevertheless, the rhombohedral crystal form has been investigated at the nucleation and growth stage in an effort to understand which parameters affect the formation of insulin crystals *in vivo* [9-13]. Atomic Force Microscopy (AFM) of well faceted crystal faces has enabled the observation of insulin hexamers as building blocks of rhombohedral crystals, the natural storage form in the pancreas gland [9]. Moreover, the role of acetone on the growth of insulin crystals has also been investigated as well as the increased step kinetic coefficient linked to an entropic effect of acetone [14] that is due to the hydrophobic driven assembly of insulin hexamer proposed by Yip et al. in 1998 [10].

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In most studies, insulin from different sources has been crystallized following a well-established strategy used in the pioneering works of Schlichtkrull [15-17] in which an insulin stock solution is prepared by the dissolution of the lyophilized insulin in low concentration hydrochloric acid. This procedure allows for the preparation of stock solutions with high insulin concentration that remains stable for several days at temperatures below 20ºC [18]. The formation of the insulin-zinc hexamer building block is guaranteed by the strong coordination of insulin dimers with Zn^{2+} ions provided in the solution as $ZnCl_2$, while supersaturation is reached by decreasing the solubility of insulin-zinc hexamers driven by increasing the pH, typically above pH 6.00, with citrate buffer. Other additives such as sodium chloride or sodium hydroxide are typically used to stabilize and keep clear the stock solutions while acetone or phenol are essential additives to either control the nucleation and growth or to obtain the desired insulin polymorph, respectively.

In this work we present a systematic study for the preparation of supersaturated insulin solutions based on the most common method that contains $ZnCl_2$ and sodium citrate buffer as the main components, and either with or without acetone. Based on these results, a comprehensive protocol for the preparation of crystallizing solutions of hexameric insulin to produce a controllable nucleation density is presented.

EXPERIMENTAL

For the preparation of the protein stock solutions, lyophilized porcine (Sigma Aldrich, Ref. I5523) and human recombinant insulin (Roche Diagnostics GmbH, Ref. 11376497001) were used without further purification. Insulin stock solutions (20 mg/ml) were prepared by dissolving the insulin in 0.02 M HCl (J. T. Baker, analytical grade). The citrate buffers at 0.065, 0.1, 0.15 and 0.2 M were prepared at pH 6.98 by titration of the buffer salt with a citric acid solution. Zinc chloride stock solution was prepared at 0.05 M concentration. The buffer components and zinc chloride were of analytical grade from Sigma. Acetone (Chromasolv plus for HPLC > 99.9%) was used as provided by the supplier (Sigma Aldrich, Ref. 650501). All solutions were prepared with MilliQ water.

The titration experiments were monitored with a GRISSON pH-meter equipped with a standard pH microelectrode under continuous stirring in 1.5 mL eppendorf tubes with insulin and 10 mL glass vessel without insulin.

The insulin stock solutions were prepared in small eppendorf tubes by precise gravimetric measurements of small amounts of lyophilized protein and the addition of diluted hydrochloric acid solution (0.02 M) to obtain a final concentration of 20 mg/ml. The final concentration of insulin stock solution was determined using a CARY 1E UV-Visible Spectrophotometer and an extinction coefficient of 1.04 ml mg^{-1} cm^{-1} [19] with 0.02M HCl as reference sample.

The crystallization screening was performed in micro-batch crystallization plates with 12x8 cylindrical reservoirs (Hampton Research). The crystallization droplets (20µL) were prepared in a single PCR eppendorf tube for appropriate mixing of the solution components before transferring each sample to the plate under a layer of several millimetres of paraffin oil (Hampton Research). Two stock solutions of human and porcine insulin were prepared and the concentration was determined spectrophotometrically at 19.0 and 17.0 mg/ml respectively. In the crystallization experiments, the final protein concentrations were varied from 0.95 to 7.6 mg/ml and from 0.85 to 6.8 mg/ml for human and porcine insulin respectively (Table 1). The volume portion of HCl (40% v/v) and the concentration of $ZnCl_2$ (5.0 mM) were kept constant while the amount of citrate buffer was varied to study the pH effect in the presence of acetone (15% v/v, samples 1 to 6) or without acetone (samples 7 to 12). Table 1 summarizes the final composition of the mixtures represented both in concentrations and in percentage of volume. Figure 1 also shows schematically the recipe volume ratio used in this study.

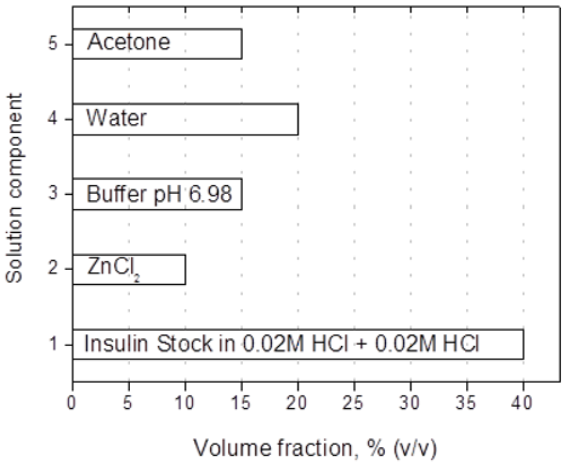

Fig. 1. Volume fractions of each component of the standard mixture for the crystallization of insulin

All experiments were performed at constant room temperature (24ºC) and the evolution of the crystallization experiments over time were followed by optical microscopy (Nikon AZ100).

Table 1. Final concentrations of the components-mix in each batch experiment.

Insulin	**Porcine**			**Human**			**Porcine**			**Human**			**Recipe components**
Sample	1	2	3	4	5	6	7	8	9	10	11	12	
	8	8	8	8	8	8	8	8	8	8	8	8	mM HCl
	5	5	5	5	5	5	5	5	5	5	5	5	mM $ZnCl_2$
A to H	15	25	35	15	25	35	15	35	50	15	35	50	mM Buffer
	20	10	0	20	10	0	35	15	0	35	15	0	Water (%)
	15	15	15	15	15	15	0	0	0	0	0	0	Acetone (%)
A	0.85	0.85	0.85	0.95	0.95	0.95	0.85	0.85	0.85	0.95	0.95	0.95	
B	1.70	1.70	1.70	1.90	1.90	1.90	1.70	1.70	1.70	1.90	1.90	1.90	
C	2.55	2.55	2.55	2.85	2.85	2.85	2.55	2.55	2.55	2.85	2.85	2.85	
D	3.40	3.40	3.40	3.80	3.80	3.80	3.40	3.40	3.40	3.80	3.80	3.80	Insulin
E	4.25	4.25	4.25	4.75	4.75	4.75	4.25	4.25	4.25	4.75	4.75	4.75	(mg/ml)
F	5.10	5.10	5.10	5.70	5.70	5.70	5.10	5.10	5.10	5.70	5.70	5.70	
G	5.95	5.95	5.95	6.65	6.65	6.65	5.95	5.95	5.95	6.65	6.65	6.65	
H	6.8	6.8	6.8	7.60	7.60	7.60	6.8	6.8	6.8	7.60	7.60	7.60	
Volumetric composition (%v/v)													
Sample	1	2	3	4	5	6	7	8	9	10	11	12	
	40	40	40	40	40	40	40	40	40	40	40	40	0.02M HCl
	10	10	10	10	10	10	10	10	10	10	10	10	0.05M$ZnCl_2$
A to H	15	25	35	15	25	35	15	35	50	15	35	50	0.1M Buffer
	20	10	0	20	10	0	35	15	0	35	15	0	Water
	15	15	15	15	15	15	0	0	0	0	0	0	Acetone

RESULTS AND DISCUSSION

1. Dissecting the insulin-hexamer crystallization procedure

There are a number of publications in which the titration of an acidic (HCl) insulin solution containing an excess of Zn^{2+} is titrated with citrate buffer in the presence or absence of acetone to crystallize insulin in its rhombohedral form. Table 2 summarizes the final composition and relevant notes of a representative group of publications using this procedure and modifications thereof [7, 9, 12-16, 20-23]. It is very impressive than even though all the consulted works seem to follow a similar protocol, none of them achieve an identical final composition or follows an exact preparationprotocol. Without taking into account the sample heating prior to crystallization, as used in several published protocols, we can find different approaches to clear the crystallizing solution. For example, increasing the pH by adding NaOH, lowering the pH with HCl or using the combination of NaOH and NaCl to stabilize the solution. This variability may hinder the proper interpretation of the observed output.

Therefore, following the previously published composition of the crystallization cocktail, in this study we decide to fix the final volume and to study the influence of the most relevant variables, protein concentration, pH and acetone, while keeping the rest of the components at a fixed concentration to provide a reproducible protocol for future investigations.

In order to characterize the described system we started our study by titrating the HCl solution containing the zinc chloride with citrate buffer in the absence of insulin. The buffer solution was added stepwise in aliquots of 250 μL while following the pH evolution of the system. At the final step acetone was added (Figure 2). Four trisodium citrate buffer concentrations, 65, 100, 150 and 200 mM, at a fixed pH of 6.98, were tested in order to evaluate the influence of the initial buffer concentration on the pH evolution of the titration system. The components' volume ratios were kept constant as depicted in the experimental section and corresponded to 8:2:7:3 for HCl, $ZnCl_2$, trisodium citrate and acetone, respectively, and therefore the final buffer concentrations were 22.75, 35, 52.5 and 70 mM.

In all cases the minimum reported pH for crystallization (pH 5.65) [7] was achieved. As expected, the lower citrate buffer concentration only overcomes this value in the final steps and the maximum attainable pH was 6.5 with the highest buffer concentration. Note that the titration behaviour with the 150 mM citrate buffer was similar to that of the 200 mM buffer and the titration slopes are steeper even with the minimal volume of the drop. However, our preliminary screenings have showed that the final sodium

Table 2. Representative compositions of crystallization solutions published to date.

Insulin (mg/ml)	HCl (mM)	$ZnCl_2$ (mM)	Citrate (mM)	Acetone (% v/v)	Notes	Ref.
4.8	NG	7 ‡	NG	-	pH_{Final} 5.65; pH_{Buffer} 6.98, 0.624% (w/v) Cl^-	[7]
NG	20 $	150 $	200 $	-	pH_{Final} 8.5; 750 mM NaOH for complete dissolution plus 5% phenol and 1.0 M NaCl	[9] $
0.75-5.0	10 or 13	6	50	0 or 15	pH_{Final} NG; Acetone was replaced with HCl (20 mM)	[12] [14]&
NG	NG	5	50	-	pH_{Final} 7 (citric buffer)	[13]
0.5%#	NG	0.04%	50	15	pH_{Final} 6.0-6.3 (sodium citrate)	[15]
0.5%#	NG	2-5%	50	15	pH_{Final} 6.0	[16]
2.0	1	5	50	15	pH_{Final} NG; pH_{buffer} 6.98	[20]
4-8	1	5	50	15	pH_{Final} NG; pH_{buffer} 6.98	[21]
5	10	7.5$	50	16.6	pH_{Final} 6.39; adjusted from pH 10.6 to pH_{Final} with 1.0 M NaOH and HCl	[22]
1.25	10	1.8	50	-	pH_{Final} 6.1	[23]*

NG: Not given. *The volume of insulin is stated as 100 ml but it should be a mistake. We have used 10 mL in our calculations. #As appears in the original article. $Zinc acetate was used instead of zinc chloride. Given concentrations correspond to stock solution. ‡From zinc sulfate. &Acetone 0 or 20% (v/v).

citrate concentrations in the crystallizations samples should be approximately 50 mM to avoid the growth of imperfect insulin crystals. This observation validates the final concentration of 50 mM of citrate buffer used in most of the reported experiments. Finally, acetone was added following the classical protocol provoking a rise of the pH of approximately 0.2 units. Since zinc ions are needed to stabilize the formation of the insulin-hexamer, we also investigated the possible role of zinc chloride, used in most procedures, on the pH.

In Figure 2b the influence of adding the zinc chloride after titration of the samples is shown to emulate the protocol used by Nanev and co-workers [21]. In this case the pH briefly dropped, which could cause aggregation of the protein if the citrate buffer concentration is not high enough e.g. 65 mM. We have also studied the titration behaviour of HCl solutions, containing zinc chloride or just water, as a function of the citrate buffer concentration. The results clearly show that the pH evolution is retarded in the presence of zinc chloride. This effect was stronger at the lowest buffer concentration and therefore the inclusion of zinc chloride impedes the ability of the buffer to increase the pH. As we will explain in the next section, this may play a relevant role on the formation of insulin-hexamer aggregates during the titration of the crystallizing solution.

2. Stepwise titration of the insulin crystallizing solution

In order to correlate the behaviour of the crystallizing solutions with the evolution of the pH, we monitored the pH during the titration of insulin dissolved in 0.02 M hydrochloric acid, with trisodium citrate buffer (100 mM). Figure 3 shows the evolution of the pH of human and porcine insulin solutions (20 mg/ml stock solution) in the presence of zinc chloride, titrated with 100 mM trisodium citrate following the volume ratios described above (Figure 1). The initial pH of the 20 mM hydrochloric stock solution was 1.55-1.58, and it increases to approximately pH 2.0 after adding 20 μL of the insulin stock solution (in 20 mM HCl) and 10 μL zinc chloride stock solution. At this point a clear difference was observed between human and porcine insulin. While for human insulin the pH rises to 1.90, in the case of porcine insulin it increases to pH 2.25, 0.35 units of difference that was maintained during the whole experiment. This increment cannot be attributed to a different pI since porcine insulin differs from human insulin only in having alanine instead of threonine at the carboxyl terminal of the B-chain (B30). As deduced from Figure 2 the pH increments cannot be insulin source. The titration was started by stepwise addition of 1 μL of citrate buffer (100 mM, pH 6.98). The pH of both types of insulin solutions changed at the same rate until the pH reached 3.8, which coincided with the observation of a cloudy

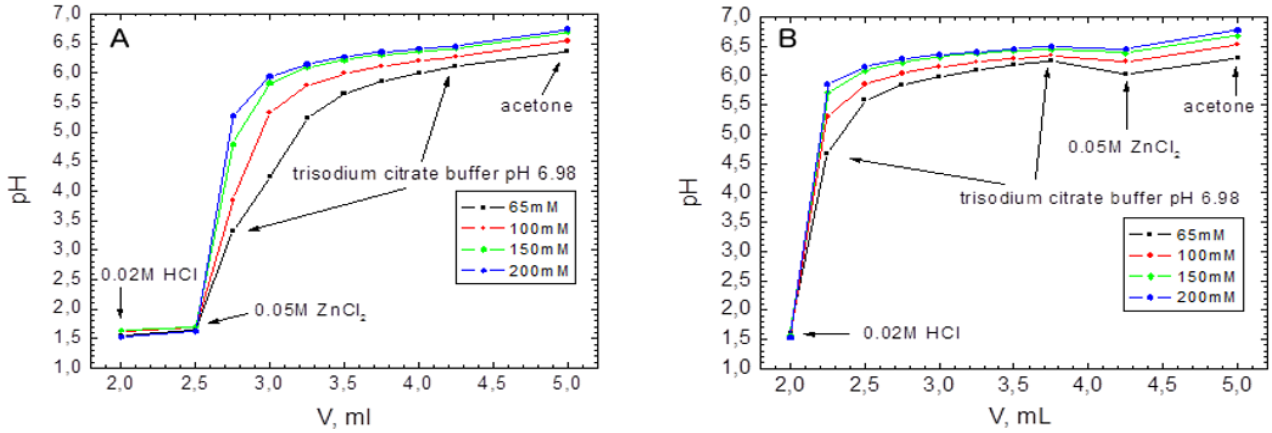

Fig. 2. Titration curves of hydrochloric acid solution with citrate buffer in presence of zinc chloride (A) or adding the zinc chloride before the acetone (B). The incorporation points of zinc chloride and acetone are indicated with arrows. The titration interval with citrate buffer is flanked with two arrows.

precipitation in both types of insulin. At this point the rate of variation of the pH was slower in the case of human insulin. In both cases an inflexion point was detected at a pH near 4.5 that is softer in the case of porcine insulin. Beyond pH 5.0 the change of pH was identical for both preparations. At pH 5.72 the insulin solution becomes visibly clear and the precipitate completely dissolves at pH 5.84. When the total volume reached 80 µL the change in pH was small and the titration was finished by adding 5 µL of citrate buffer. As the last step of the procedure, acetone was added to the insulin crystallization mixture which increased the pH 0.25-0.27 units while in the absence of insulin it was 0.2 pH units.

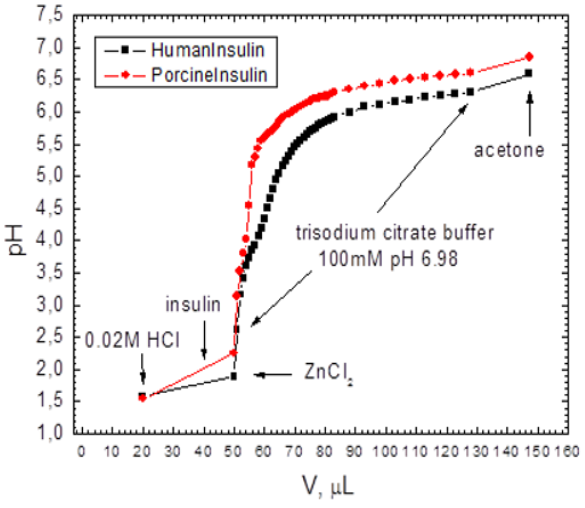

Fig. 3. Titration curves of insulin (porcine and human) dissolved in HCl solution with citric buffer.

The behaviour of the insulin crystallizing solution in the pH range 3.4 to 5.8 has already been described in the literature pointing out that approximately 99% of the total insulin forms aggregates in this range of pH [8] and also fits with the early studies of Wintersteiner and Abramson who demonstrated that the solubility of insulin in acetate buffer was very low in the pH range 4.8 to 6.2 (approximately $4 \cdot 10^{-3}$ mg/ml) [24]. This aggregation driven by the change in pH suggests the possibility of exerting a precise control over the nucleation rate of insulin crystals by a precise control of the pH.

3. Micro-batch under oil crystallization of porcine and human insulin

We have carried out a study on the influence of acetone, buffer and protein concentrations on the crystallization of insulin (human and porcine). This investigation was carried out using the batch under-oil method. The set up was designed to cover, as much as possible, all the variations found for that particular procedure for the preparation of supersaturated insulin solutions. Besides the comparison between porcine and human insulin and the influence of acetone (0% or 15% v/v), the ratio between the final concentrations of HCl and citrate buffer, i.e. final pH, were also addressed in this multi-parameter aggregation screening.

The summary of results is shown in Table 3 in which “O” represent clear drops; “FC” well-faceted crystals; “RC” rough crystals, ranging from 1 (low) to 3 (high) the number of observed crystals; “CP” crystalline precipitate, ranging from 1 (light) to 3 (heavy) depending on the size and the number of micro-particles; “AP” amorphous precipitates, ranging from 1 (light) to 3 (heavy) depending on the visible density of aggregates and “CG” for crystalline granule-like tiny aggregates. An example of each type of precipitate is shown in Figure 4. The approximate pH values of each drop (last row of Table 3) were determined from similar experiments without insulin.

When comparing the results from porcine and human insulin it can be seen that, regardless of the final crystallization conditions, the level of

Table 3. Crystallization results of human and porcine insulin as a function of the final pH (see last row), protein concentration (increasing from A to H) and used or not of acetone at 15% v/v, samples 1-6 and 7-12, respectively.

Insulin	**Porcine**			**Human**			**Porcine**			**Human**		
Sample	1	2	3	4	5	6	7	8	9	10	11	12
A	O	O	O	CG1	FC2	O	O	O	O	AP1	O	O
B	FC2	FC1	O	CG1	FC2	FC2	O	O	O	AP2	O	O
C	FC3	FC1	O	CP3	CP1	CP1	RC1	O	FC1	AP3	O	O
D	CP1	FC1	FC2	CP3	CP1	FC3	RC1	O	O	AP3	O	FC1
E	CP2	FC3	FC1	CP3	CP2	FC2	RC1	O	RC1	AP3	FC1	RC1
F	FC3	FC2	FC2	CP3	CP3	CG	RC3	FC1	FC1	AP3	RC1	RC1
G	CP2	FC2	FC2	CP3	CP3	CP3	RC1	FC2	FC2	AP3	RC1	FC2
H	CP3	FC3	FC2	CP3	CP3	CP3	RC1	FC2	RC1	AP3	RC1	FC2
acetone		+			+			-			-	
pH*	6,07	6,45	6,59	6,07	6,45	6,59	5,87	6,34	6,48	5,87	6,34	6,48

O: clear droplet or light precipitate; well faceted (FC) or rough (RC) insulin crystals XC1: 1-10; XC2: 10-100; XC3: 100-1000 insulin crystals; crystalline insulin precipitates CP1: transition of the insulin crystals to small crystalline particles; CP2: fine crystalline insulin particles; CP3: heavy crystalline insulin precipitation; CG: precipitate in the form of crystalline granules; amorphous insulin precipitation AP1: initial stage of aggregation; AP2: slightly aggregation; AP3: full aggregation.

* Measured from separate experiments without insulin.

supersaturation reached with human insulin was always higher than that for porcine insulin.

The direct consequence, as observed in comparative single drops, is that the nucleation density was always higher in experiments with human insulin. The lower solubility of human insulin is also reflected by the prevalence of aggregation in samples 4 (with acetone, pH 6.07) and 10 (without acetone, pH 5.87). In these two rows the final concentration of citrate buffer is the lowest among all the experiments indicating the relevant role of the pH on the crystallization of insulin. In the case of porcine insulin under identical conditions (samples 1 and 7, pH 6.07 and 5.87, respectively) most of the drops produced crystals. This can be understood considering that the final pH of porcine insulin is slightly higher, as already shown during the titration experiments (Figure 3). The solubility differences of porcine and human insulin are clearly illustrated in Figure 5 in which the output of low (A), medium (D) and high (H) protein concentration experiments are compared.

The role of acetone was also considered in this study due to its prevalence in many crystallization studies (see Table 2), at a concentration of 15% (v/v). At this concentration two different effects have been described by Vekilov and co-workers: the increase of insulin solubility as acetone concentration rises [14] and the increase of the step kinetic coefficient [12], i.e. higher growth rate as a direct consequence of higher acetone concentration. In our study, the use of acetone reduced the solubility of insulin regardless of the hormone source. At all tested protein concentrations, the number of crystals was higher in the presence of acetone.

This effect is more pronounced in the case of human insulin. In Figure 6 we compared both proteins crystallized in the presence (3 and 6) and in the absence of acetone (8 and 11) while keeping constant the citrate buffer concentration at 35 mM. The observed increase of nucleation density cannot be directly related to the change of pH, 6.59 and 6.34 for samples 3-6 and 8-11, respectively, high enough to be out of the aggregation point detected during the titration experiments. It is also worth mentioning that crystals grown in the absence of acetone have higher propensity to show a higher amount of visible defects including rough faces (See picture H11 of Figure 6 as an example) which will need further investigation.

Finally, we compared the effect of the final pH of the solution, shown in Table 3, on the nucleation of porcine and human insulin. As we mentioned earlier, there was a critical point during the titration of insulin that lies beyond the isoelectric point of the protein (pI 5.30-5.35 as referenced by the suppliers), identified above pH 5.40 as the maximum flocculation point [24]. Accordingly, the closer to this pH value the lower the solubility of insulin in solution and the higher the nucleation density. Our observations are therefore in good agreement with the study of Wintersteiner and Abramson [24] that successfully explained the crystallization behaviour of insulin in the pH range 5.60-5.65 previously reported by Abel and co-workers [25].

However, as deduced from our results, this pH range, 5.60-5.65, is too close to the isoelectric point

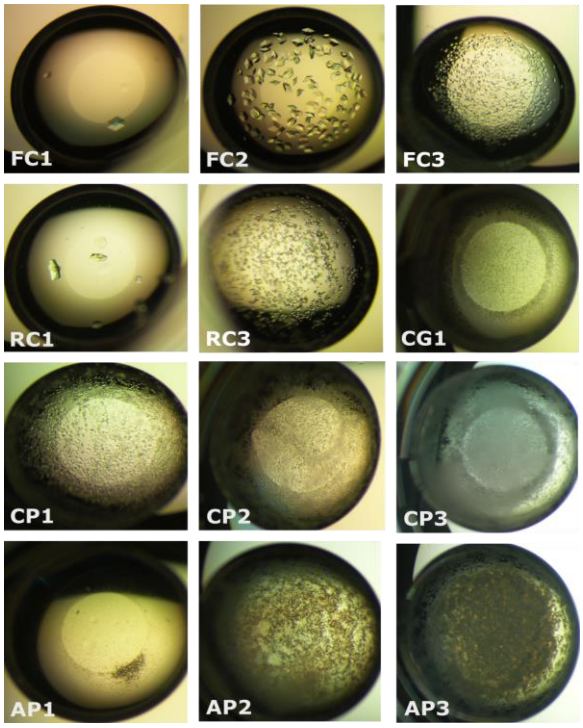

Fig. 4. Selected pictures of the crystallization events corresponding to the notation used in the text and Table 3, i.e. FC1 to FC3, and RC1 to RC3, corresponding to low and high number of crystal and rough crystals, respectively; CP1 to CP3 and AP1 to AP3 corresponding to light and heavy micro-particles and amorphous precipitated, respectively.

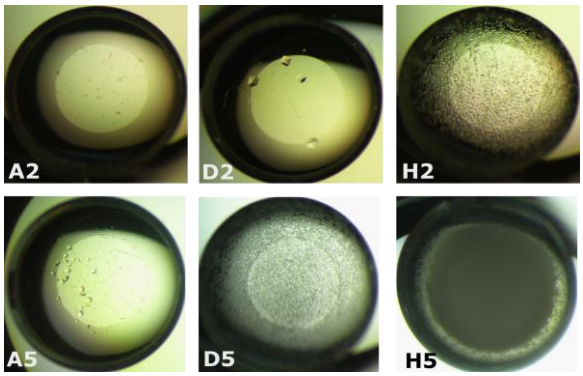

Fig. 5. Comparison between samples from the crystallization plate with porcine (A2 to H2) and human (A5 to H5) insulin concentration 1,0 (A), 4,0 (D) and 8,0 (H) mg/ml respectively.

to exert a reasonable control on the nucleation of insulin. In this line, human insulin seems to be more sensitive to pH changes than porcine insulin that, at this point, can only be attributed to a possible different composition of the lyophilized powder. This is clearly observed at the lowest pH used in this study, 5.87 (Table 3), for which human insulin is fully precipitated (samples 10) while porcine insulin still produces crystals (samples 7). This observation is confirmed in the presence of 15% (v/v) acetone, samples 1 and 4, for porcine and human insulin, respectively, but enhanced due to the reduction of the solubility of insulin.

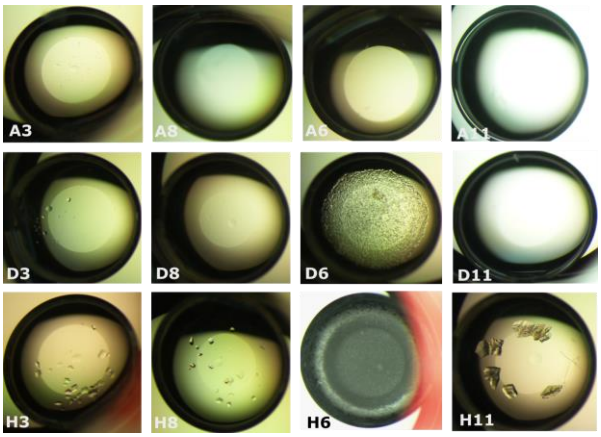

Fig. 6. Comparison of porcine (A3-H3 and A8-H8) and human (A6-H6 and A11-H11) insulin crystallized in the presence (numbers 3 and 6) and in the absence of acetone (numbers 8 and 11).

CONCLUSIONS

Crystallization of proteins is a multiparameter and multicomponent process in which the role of each precipitant-component is unknown. Applying a fixed volume-ratio procedure we have been able to study the main variables of the precipitant cocktail influencing the nucleation behavior of human and porcine insulin. We have identified the pH as the variable that exerts a fine control on the nucleation of insulin crystals. It has been demonstrated that acetone is not an essential additive for the crystallization of insulin although its presence reduced the solubility and facilitated the formation of well-faceted crystals. Porcine and human insulin showed, in general, similar crystallization behavior but with remarkable differences explained from their dissimilar titration behavior.

The formulation of the crystallizing insulin solution that has been used provides a complete picture of the nucleation behavior, it is useful for further fundamental studies and should be taken into consideration for the mass production of insulin crystals.

Acknowledgements. *This research was funded by the projects "Science-Business" BG051PO001-3.3.05-0001 (FVH) and Short Term Scientific Mission (STSM) program of the COST initiative project CM1306-26833 (FVH), by the MICINN (Spain) projects BIO2010-16800 (JAG) and "Factoría Española de Cristalización" Consolider-Ingenio 2010 (JAG & MCM). FVH is thankful to the Laboratorio de Estudios Cristalográficos, IACT (CSIC-UGR) as receiving institution of the COST project.*

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ОПТИМИЗАЦИЯ НА КЛАСИЧЕСКИЯ ПОДХОД ЗА ЗАРАЖДАНЕ И РАСТЕЖ НА РОМБОЕДРИЧНИ КРИСТАЛИ ОТ ИНСУЛИН ЧРЕЗ ПРОВЕЖДАНЕ НА pH ТИТРУВАНЕ И КРИСТАЛИЗАЦИОНЕН СКРИНИНГ

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Постъпила на 8 септември,2015 г. коригирана на 23 ноември, 2015 г.

(Резюме)

Осъществена е кристализация на инсулин в присъствие на цинкови йони под формата на хексамерни единици, които образуват ромбоедрични кристали, чрез постъпково титруване на разтворения инсулин в разредена солна киселина с натриево цитратен буфер и ацетон. При смесване на разтворите се наблюдава характерна преципитация на инсулиновите молекули, поради преодоляване на критични стойности на pH в близост до изоелектричната точка на този белтъчен хормон. При последващо повишаване на pH на разтвора получените белтъчни агрегати се разтварят обратимо, като процеса е подпомогнат и от благоприятното въздействие на добавка от ацетон. В настоящата работа е изследвано влиянието на pH върху кристализацията на две търговски субстанции лиофилизиран инсулин: свински, изолиран от панкреас, и човешки рекомбинантен. Изследвана е агрегационната способност на тези субстанции чрез провеждане на паралелно pH титруване в рамките на използваната рецепта за кристализация. Осъществен е стриктен контрол на всички кристализационни компоненти по отношение на техните концентрации чрез използване на едни и същи обемни части на изходните разтвори. pH на кристализационните смеси е моделирано чрез фиксиране на количеството на солната киселина (0.02M HCl pH 1.55) и единствено вариране на количеството на използвания буфер (0.1M pH 6.98). Този подход допринася за финно оптимизиране и подбор на условията за получаване на инсулинови кристали в малки "batch"- кристализационни системи (20 μL), както в присъствие, така и в отсъствие на ацетон. Скринингът е проведен в комерсиално 12x8 кристализационно плате. На базата на всички (96 бр.) наблюдавани проби са определени оптималните условия за получване на добре остенени инсулинови кристали. Освен тях, под внимание са взети и всички останали комбинации за формиране на аморфни и кристални преципитати, кристални суспензии и финни инсулинови гранули, които в частност представляват интерес за фармацевтичната индустрия. Отчетени са и условията, при които добре остененните кристали се израждат в други форми или нарастват с груби дефекти.