

1 **Optimisation of the extraction and purification of chondroitin**
2 **sulphate from head by-products of *Prionace glauca* by**
3 **environmental friendly processes.**

4

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29 **Abstract**

30 The goal of the present work was to optimise the different environmental friendly
31 processes involved in the extraction and purification of chondroitin sulphate (CS)
32 from *Prionace glauca* head wastes. The experimental development was based on
33 second order rotatable designs and evaluated by response surface methodology
34 combined with a previous kinetic approach. The sequential stages optimised were:
35 1) the enzymatic hydrolysis of head cartilage catalysed by alcalase (55.7°C/pH
36 8.2); 2) the chemical treatment of enzyme hydrolysates by means of alkaline-
37 hydroalcoholic saline solutions (NaOH: 0.54 M, EtOH: 1.17 v, NaCl: 2.5%) to end
38 the protein hydrolysis and to precipitate and selectively redissolve CS versus the
39 peptidic material and 3) the selective purification and concentration of CS and the
40 concomitant protein permeation of extracts which were obtained from previous
41 treatment using ultrafiltration and diafiltration (UF-DF) technologies at two different
42 cut-offs.

43

44 **Keywords:** Chondroitin sulphate production; cartilage *P. glauca* wastes; by-
45 products upgrade; process optimisation; response surface methodology.

46 **Introduction**

47 The generation of discards and by-products from fishing activities is one of the
48 most important problems to maintain the sustainability of the marine resources and
49 the extractive marine industry. European Union (EU) has shown a big concern
50 about this problematic; thus, new and more restrictive policies (zero discards) have
51 been legislated in order to minimize the adverse ecological, environmental and
52 pollutant impact of the biomass wasted, to attain their reduction and the
53 development of valorisation alternatives. These measures are within the objectives
54 of the Horizon 2020 European initiative called as *Blue technology* (EU, 2012). It is
55 focused on how technologies can put marine resources to productive use and
56 create sustainable growth for the exploration of bioactive compounds with potential
57 interest in the food, feed or pharmaceutical industries.

58

59 The blue shark (*Prionace glauca*) is a pelagic elasmobranch found throughout the
60 world's oceans, in all tropical and temperate waters. It is principally caught as by-
61 catch on longlines and in gill nets fisheries targeting tuna and swordfish (Carvalho,
62 Murie, Hazin, Hazin, Leite-Mourato, & Burgess, 2011). Catches of this species
63 appears to be on the rise, due to consumer habits which are changing and the
64 meat of this species is now appreciated. In this sense, blue shark was the fourth
65 most important frozen fish species landed in 2013 in the largest fishing port in
66 Europe, located in Vigo (North-West Spain). Fresh and frozen volumes of blue
67 shark auctioned in Vigo fish auction, accounted up to 8600 Tn in 2013.
68 Transformation of this species throughout the manufacture chain, include different

69 presentations as loins, fillets or fish bites, where the cartilage is removed and
70 primarily used for low added-value processes.

71

72 One of the most valuable materials from chondrichthyes fishes, including sharks, is
73 the cartilage. Cartilage is a tissue composed by a matrix of collagen associated
74 with proteoglycans. They are macromolecules with a core protein linked covalently
75 to glycosaminoglycans (GAGs) among which chondroitin sulphate (CS) is the most
76 important (Vázquez, Rodríguez-Amado, Montemayor, Fraguas, González, &
77 Murado, 2013). CS is a polymer constituted by alternating molecules of glucuronic
78 acid and N-acetylgalactosamine sulphated. This material is an essential
79 component of extracellular matrix of connective tissues being a fundamental key
80 for the control of cell development and adhesion or the elasticity of the articular
81 cartilage (Schiraldi, Cimini, & de Rosa, 2010). In the last decade, a numerous and
82 varied type of CS applications has been reported mainly related to the
83 bioengineering of tissues (Chang, Liu, Lin, Chou, & Lin, 2003; Silva et al., 2012;
84 Leite, Sher, & Mano, 2014). But it has also demonstrated to have antiviral
85 properties (Yamada & Sugahara, 2008), to be used as food preservative with
86 emulsifying features (Hamano et al., 1989) and to be administered in nutraceutical
87 formulations to prevent and reduce osteoarthritis (Michel et al., 2005; Volpi, 2009).

88

89 Bovine, porcine and chicken cartilage have been traditional sources for obtaining
90 CS (Nakano, Dodd, & Scott, 1997), however since the bovine spongiform
91 encephalopathy outbreak, the uses of alternative materials have received
92 increasing attention, and so this polymer has been isolated from various tissues

93 from different vertebrate (Kinoshita-Toyoda et al. 2004; Zhao et al., 2013) and
94 invertebrate (Gu et al., 1999) marine species. Recently, much attention has been
95 paid to the isolation of this polysaccharide from elasmobranch sources (Lignot,
96 Lahogue, & Bourseau, 2003; Murado, Fraguas, Montemayor, Vázquez, &
97 González, 2010; Kim et al., 2012; Xie, Ye, & Luo, 2014), due to the benefits in
98 terms of safety, potential bioactivities and structural issues (González, Leyva, &
99 Moraes, 2001) associated with elasmobranch cartilage. According to our
100 knowledge, only one previous work studied the extraction of CS from *P. glauca*
101 cartilage (Xie, Ye, & Luo, 2014) but in this article no optimisation of conditions for
102 CS production is reported. The present work is the first time dealing with a
103 complete optimisation of the extraction conditions for the production of CS from
104 head cartilage by-product of *P. glauca*.

105

106 The main problem of CS recovery from cartilage is the digestion of this tissue and
107 the subsequent elimination of the proteins that are present in the proteoglycan
108 matrix (collagen and aggrecans). The final purity needed is dependent on the type
109 of application, higher for clinical uses and lower in nutraceutical and dietary
110 formulations. The fundamentals of many of the isolation procedures developed for
111 mammalian and marine sources (bovine and porcine trachea, shark fins, etc.) are
112 based on (Vázquez, Rodríguez-Amado, Montemayor, Fraguas, González, &
113 Murado, 2013; Shi, Meng, Li, Chen, Liu, & Bai, 2014): 1) chemical hydrolysis of
114 cartilage; 2) breakdown of proteoglycan core; 3) selective elimination of proteins
115 and CS isolation; 4) final purification of CS. Commonly, the steps of digestion,
116 hydrolysis, differential recovery of GAG and purification, are performed using

117 alkalis, cysteine or guanidine HCl and chemical precipitation by cetylpyridinium
118 chloride, trichloroacetic acid or detergents combined with chromatography of gel
119 filtration for purification (Michelacci, & Horton, 1989; Souza, Kozlowski, Cerqueira,
120 Castelo-Branco, Costa, & Pavão, 2007). However, those processes are not fully
121 efficient for obtaining high CS purity and also they are time-consuming, expensive
122 to be scaled and environmentally unfriendly. Thus, alternatives based on enzyme
123 hydrolysis and membranes technology performances have been explored in recent
124 years (Lignot, Lahogue, & Bourseau, 2003; Murado, Fraguas, Montemayor,
125 Vázquez, & González, 2010).

126

127 In the present study, different processes for the extraction and purification of
128 chondroitin sulphate from *P. glauca* head cartilage by-products have been
129 reported. In this context, a sequential combination of stages of cartilage hydrolysis
130 mediated by alcalase, selective precipitation of CS in alkaline hydroalcoholic
131 medium and, finally, CS purification and protein removing using ultrafiltration and
132 diafiltration (UF-DF) procedures.

133

134 **Materials and Methods**

135 ***Cartilage preparation***

136 Head wastes of blue shark (*Prionace glauca*) were kindly provided by Propegal
137 S.L. (Vigo, Spain) and stored at -20°C. These materials were initially heated in
138 water bath at 80°C/30 min and muscle was manually discarded. The cartilaginous

139 material were then milled and homogenized at ~1 mm size using a grinder
140 (Thermomix TM31, Vorwerk) and stored at -20°C until use.

141

142 ***Analytical determinations***

143 Levels of ash, total carbohydrate, crude protein, moisture and fat were determined
144 in triplicate, from subsamples taken before cartilage storage. Total nitrogen was
145 measured according to the Kjeldahl method (AOAC, 1997) in a DigiPREP HT
146 digester, DigiPREP 500 fully automatic steam distillation (SCP Science, Canada)
147 and a TitroLine easy titration unit (SCHOTT Instruments, Germany) and crude
148 protein content calculated as total nitrogen multiplied by 6.25. Ash was quantified
149 by calcination at 600°C in a muffle furnace and moisture content determined after
150 heating the sample at 105 °C in an oven until constant weight. Fat concentration
151 was obtained by the methodology developed in AOAC (1990). Total carbohydrate
152 content was calculated by the difference between total weight (subtracting protein,
153 fat and ash) and moisture content.

154

155 In CS extracts, total soluble proteins (Pr) were quantified using the method of
156 Lowry et al. (1951). CS concentration, as glucuronic acid, was determined by the
157 method of Van den Hoogen et al. (1998) according with the mathematical
158 modifications reported in Murado et al. (2005). CS purity index (I_p), defined as I_p
159 (%) = $CS \times 100 / (CS + Pr)$, was also calculated in all purification stages.

160

161 ***Alcalase hydrolysis process***

162 Samples of 25 g of cartilage were suspended in 75 mL of distilled water and pre-
163 incubated in the conditions of pH and T defined by the factorial design described in
164 Table 1 (see also experimental design section). After addition of 0.3% (v/w) (7.2
165 AU/kg of cartilage) of alcalase 2.4L (Novozymes S.A.), the kinetics of hydrolysis
166 were carried out in a 100 mL-thermostated reactor connected to a pH-Stat system
167 (Metrohm, UK) with agitation (200 rpm) under the experimental conditions from
168 Table 1. At the end of hydrolysis time (4 h), the enzymatic reactions were stopped
169 by boiling, freezing and centrifuged (6000 g, 20 min) to recover the supernatants
170 (hydrolysates). In these hydrolysates, the concentrations of total proteins and CS
171 were analyzed after performing a chemical treatment with alkaline hydroalcoholic in
172 the suboptimal conditions of 0.5 M (NaOH) and 1.1 v (EtOH).

173

174 The calculation of the degree of hydrolysis (H , in %) was performed according to
175 the pH-Stat protocol reported by Adler-Nissen (1986). It was proportional to the
176 alkali added in order to maintain the pH at the established value and determined by
177 the following equation:

178

$$179 \quad H = \frac{B N_b}{\alpha M_p h_{tot}} \quad [1]$$

180

181 where, B is the volume (mL) of 0.2 M NaOH consumed during hydrolysis; N_b is the
182 normality of NaOH; M_p is the mass (g) of initial protein ($N \times 6.25$); h_{tot} is the total
183 number of peptide bonds available for proteolytic hydrolysis (8.6 meq/g), and α is

184 the average degree of dissociation of the amino groups in the protein substrate and
185 it was calculated as follows:

186

$$187 \quad \alpha = \frac{10^{\text{pH}-\text{pK}}}{1+10^{\text{pH}-\text{pK}}} \quad [2]$$

188

189 The pK value is dependent on the temperature of hydrolysis (in K degrees),
190 therefore it can be also calculated according to the following expression:

191

$$192 \quad \text{pK} = 2400 \left(7.8 + \frac{298 - T}{298 T} \right) \quad [3]$$

193

194 Subsequently, the progress of the hydrolysis data (H) of *P. glauca* cartilage was
195 modelled by the Weibull equation (Vázquez, Lorenzo, Fuciños, & Franco, 2012):

196

$$197 \quad H = H_m \left\{ 1 - \exp \left[-\ln 2 \left(\frac{t}{\tau} \right)^\beta \right] \right\} \quad \text{with} \quad v_m = \frac{H_m \beta \ln 2}{2 \tau} \quad [4]$$

198

199 where, H is the degree of hydrolysis (%); t is the time of hydrolysis (min); H_m is the
200 maximum degree of hydrolysis (%); β is a parameter related with the maximum
201 slope of cartilage hydrolysis (dimensionless); τ is the time required to achieve the
202 semi-maximum degree of hydrolysis (min) and v_m is the maximum hydrolysis rate
203 at the τ -time ($\% \text{ min}^{-1}$).

204

205 ***Chemical treatment using alkaline hydroalcoholic media***

206 Three litres of hydrolysate prepared in the best conditions for the alcalase
207 hydrolysis of cartilage (55.7°C/pH 8.2 using Tris-HCl buffer 0.1M/1% (v/w) of
208 alcalase/200 rpm) were used to assess the joint influence of NaOH concentration
209 and ethanol (EtOH) volume in the extraction of CS. Thus, the precipitations of CS
210 were performed by adding to the hydrolysates, slowly and with middle agitation at
211 room temperature, different hydroalcoholic solutions of NaOH in the proportions
212 defined for the experimental design which are summarized in Table 1. In all the
213 alkaline hydroalcoholic mixtures, 2.5% of NaCl was added for a better redissolution
214 of CS in water. After 2 h in agitation, the suspensions were centrifuged (6000 g, 20
215 min) and the sediments were redissolved with water and neutralized with 6 M HCl.

216

217 ***Experimental designs and statistical analysis***

218 Enzyme hydrolysis of head cartilage and selective precipitation of CS were studied
219 using a rotatable second order designs with quintuple replication in the centre of
220 the experimental domains (Box, Hunter, & Hunter, 2005) (Table 1). In the first
221 case, the combined effect of T and pH on the parameters describing the hydrolysis
222 degree of cartilages and final CS concentration and purity was evaluated. In the
223 second step, NaOH concentration and volumes of EtOH required for the final
224 alkaline proteolysis of proteoglycan and selective precipitation of CS were
225 optimised.

226

227 The experimental results of the factorial designs were modelled by second-order
228 polynomial equations as:

229

$$230 \quad Y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{\substack{j=2 \\ j>i}}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2 \quad [5]$$

231

232 where Y represents the response to be modelled; b_0 is the constant coefficient, b_i is
233 the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the
234 coefficients of squared effect, n is the number of variables and X_i and X_j define the
235 independent variables.

236

237 The goodness-of-fit was established as the adjusted determination coefficient (R_{adj}^2),
238 the statistical significance of the coefficients was verified by means of the Student
239 t-test ($\alpha=0.05$) and the model consistency by the Fisher F test ($\alpha=0.05$) using the
240 following mean squares ratios:

241

$F1 = \text{Model} / \text{Total error}$

$F2 = (\text{Model} + \text{Lack of fitting}) / \text{Model}$

$F3 = \text{Total error} / \text{Experimental error}$

$F4 = \text{Lack of fitting} / \text{Experimental error}$

the model is acceptable when

$$F1 \geq F_{den}^{num}$$

$$F2 \leq F_{den}^{num}$$

$$F3 \leq F_{den}^{num}$$

$$F4 \leq F_{den}^{num}$$

242

243

244 **Ultrafiltration-diafiltration process**

245 Ultrafiltration-diafiltration (UF-DF) of neutralized extracts of CS was performed
246 employing spiral polyethersulfone membranes of 0.56 m² (Prep/Scale-TFF,
247 Millipore Corporation, Bedford, MA, USA) with 100 and 30 kDa molecular weight
248 cut-offs (MWCO). A sequential cascade of 100 to 30 kDa membrane was
249 executed, in both cases operating with total recirculation mode for the retentates at
250 40°C and inlet constant pressure (~1 bar). Firstly, UF obtained after 100 kDa
251 membrane was immediately followed by DF procedure. The final retentate (after
252 DF) was finally dried. Permeate obtained after UF was passed through 30 kDa
253 membrane for concentration (UF step) and subsequent desalination and protein
254 elimination by DF. Permeate of the 30 kDa-UF was discarded. The samples taken
255 from all UF-DF kinetics were analyzed in terms of CS and protein content.

256

257 For modelling the membrane process, a constant volume was fixed for the DF step
258 (filtration flow = water intake flow), where the concentration of a permeable solute
259 in the retentate was predicted by using the first-order equation (Amado, Vázquez,
260 González, & Murado, 2013):

261

$$262 \quad R = R_f + R_0 \exp [-(1-s)D] \quad [6]$$

263

264 where, R is the concentration of permeable protein or CS in the retentate (% from
265 the level at initial DF), R_0 is the permeate concentration (%), R_f is the asymptotic
266 and retentate concentration (%), D is the relative diavolume (volume of added
267 water/constant retentate volume) and s is the specific retention of protein or CS

268 with variation between 0 (the solute is filtered as the solvent) and 1 (the solute is
269 totally retained). Thus, using normalized values (%): $R_o + R_f = 100$, with $R_o = 0$ if all
270 protein or CS are permeable. In addition, the percentage of protein or CS
271 eliminated by three diavolumes (R_{3D}) was calculated by substituting in equation [6]
272 the value of parameter D by 3.

273

274 ***Numerical methods for non-linear curves modelling***

275 Degree of hydrolysis data and DF kinetics were modelled by minimisation the sum
276 of quadratic differences between observed and predicted values, using the non
277 linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the
278 Microsoft Excel spreadsheet. Confidence intervals from the parametric estimates
279 (Student's t test) and consistence of mathematical models (Fisher's F test) were
280 evaluated by "SolverAid" macro, freely available from de Levie's Excellaneous
281 website: <http://www.bowdoin.edu/~rdelevie/excellaneous/>.

282

283 **3. Results and Discussion**

284 The percentage of fresh cartilage was 15.33 ± 2.96 % (w/w of wet head). The
285 chemical composition of head cartilages from *P. glauca* expressed as percentage
286 of dry weight was as follows (average \pm SD, in % of dry weight): 54.44 ± 0.70 ,
287 33.55 ± 0.52 , 11.75 ± 0.23 and 0.26 ± 0.01 for protein, ash, carbohydrates and fat,
288 respectively. The content of moisture (%) was 79.01 ± 0.46 of total weight.

289

290 *3.1. Optimisation of alcalase hydrolysis on P. glauca head cartilages.*

291 A varied set of enzyme hydrolysis trends were generated for the different
292 conditions of pH and T tested (Figure 1). In some pH and T levels the activity of
293 alcalase to solubilise head cartilages was null and therefore no degree of
294 hydrolysis was observed. The kinetics data were accurately modelled by Weibull
295 equation [4]. The numerical value of the parameters and the corresponding
296 statistical analysis of fittings are showed in Table A (supplementary material).

297

298 The parameters H_m and v_m from equation [4] as well as the CS concentration and
299 I_p index at the end of the hydrolysis time were the dependent variables used to
300 study the joint influence of pH and T on cartilage hydrolysis using alcalase. For the
301 partial purification of CS in the hydrolysis samples, we have applied the suboptimal
302 conditions of chemical treatment reported in Material and Methods (NaOH 0.5 M
303 and EtOH 1.1 v). Table 2 shows the second order equations defining the predicted
304 responses for the enzyme action on cartilage solubilisation affected by the
305 aforementioned independent variables.

306

307 In general, the goodness of fitting and the correlation among data and expected
308 values were good and superior to 70% (94% for I_p). Moreover, they were
309 completely consistent by F-Fisher test evaluation and confirmed their adequate
310 capacity of prediction of the effects studied. In all responses, the quadratic
311 coefficients were always statistically significant and negatives. In Figure 2 (Top) a
312 representation of the theoretical dome surfaces generated by the models from
313 Table 2 are displayed. The optimal values of T and pH that maximize the

314 responses can be calculated as follows (H_m as example) (Wardhani, Vázquez, &
315 Pandiella, 2010):

316

$$317 \quad H_m = 13.0 - 3.72pHT - 3.01T^2 - 6.28pH^2$$

$$318 \quad \frac{\partial H_m}{\partial T} = -3.72pH - 6.02T \quad \text{and} \quad \frac{\partial H_m}{\partial pH} = -3.72T - 12.56pH$$

319

320 Equating both derivatives to zero, we obtain: $T_{opt} = 0$ (55°C in natural value) and
321 $pH_{opt} = 0$ (9.0 in natural value). These are the conditions that lead to the maximum
322 degree of hydrolysis: 13%. The optima values for the rest of the responses are
323 summarized in Table B (supplementary material). In this context, the best options
324 for cartilage hydrolysis were defined as the average of such optimal data: $T =$
325 55.7°C and $pH = 8.2$.

326

327 Based on these optimal values, a set of longer kinetics of hydrolysis were
328 performed with increasing alcalase concentrations and the results established that
329 8 h of hydrolysis and 1% (v/w) of alcalase are the best conditions for both variables
330 (data not shown). Different types of proteases have been tested, in general with
331 good results, for cartilage hydrolysis (Lignot, Lahogue, & Bourseau, 2003; Kim et
332 al., 2012) being papain the most commonly utilised (Garnjanagoonchorn,
333 Wongekalak, & Engkagul, 2007; Gargiulo, Lanzetta, Parrilli, & De Castro, 2009;
334 Murado, Fraguas, Montemayor, Vázquez, & González, 2010). Nevertheless,
335 alcalase has been successfully applied in a great number of fish applications
336 (Safari, Motamedzadegan, Ovissipour, Regenstein, Gildberg, & Rasco 2012;

337 Amado, Vázquez, Murado, & González, 2013) including, recently, the purification
338 of GAGs (Kim et al., 2012; Xie, Ye, & Luo, 2014) showing better results for CS
339 content than those reported by other proteases (Gu et al., 1999) Conventional
340 digestion of cartilage was performed previously using concentrations of alcalase
341 higher than 2% in comparison to our results, that established 1% (24 AU/kg) as the
342 most optimal and cost-effective concentration of enzyme to be applied (Kim et al.,
343 2012).

344

345 *3.2. Optimisation of chemical treatment of alcalase hydrolysates.*

346 In this step, hydrolysates obtained taking into account the previous optimal
347 conditions for cartilage hydrolysis were processed with alkaline hydroalcoholic
348 solutions, in order to optimise the joint effect of EtOH and NaOH concentrations on
349 CS recovery. This chemical treatment helps to liberate CS from the proteoglycan
350 matrix and produce the selective precipitation of CS. Additionally, the constant salt
351 concentration added to the solutions improves the aqueous redissolution of GAG
352 (Murado, Fraguas, Montemayor, Vázquez, & González, 2010; Murado,
353 Montemayor, Cabo, Vázquez, & González, 2012).

354

355 The results for CS and I_p responses (experimental and predicted) from such
356 treatment on *P. glauca* head hydrolysates are listed in Table C (supplementary
357 material). The corresponding empirical models obtained by application of surface
358 response methodology and multivariable analysis to those experimental data are
359 summarized in Table 3. The adjusted coefficients of determination were superior to
360 0.83 suggesting a good correlation among experimental data and theoretical

361 responses. In all cases, the unique non significant coefficient was the linear
362 interaction among E and N ($P < 0.05$). The mathematical and graphical results for
363 both responses were similar showing indistinguishable convex surfaces (Figure 3).
364 This resulted in almost equal optima conditions of alkali and alcohol: 0.53 M and
365 1.17 v for CS, 0.54 M and 1.18 v for I_p -index.

366

367 Different solvents have been checked for the selective precipitation of GAGs and
368 proteins from a digested cartilage (Gargiulo, Lanzetta, Parrilli, & De Castro, 2009;
369 Shi, Meng, Li, Chen, Liu, & Bai, 2014). Cetylpyridinium chloride,
370 cetyltrimethylammonium bromide, guanidine HCl and alcoholic solutions are the
371 most employed (Takai & Kono, 2003; Souza, Kozlowski, Cerqueira, Castelo-
372 Branco, Costa, & Pavão, 2007; Vázquez, Rodríguez-Amado, Montemayor,
373 Fraguas, González, & Murado, 2013). In general, all of them produced acceptable
374 results, but current tendencies towards the application of “green” chemicals make
375 the alcohols the most adequate and economical choice. Although methanol and
376 isopropyl alcohol solutions have been used for precipitation of various shark
377 cartilages, ethanol is the first option reported in the bibliography (Michelacci, &
378 Horton, 1989; Tadashi, 2006; Kim et al., 2012). The results here obtained for *P.*
379 *glauca* were in concordance with those previously found for *Raja clavata*;
380 nevertheless, the optimal concentration of NaOH in blue shark is 2.5-times higher
381 than in ray (Murado, Fraguas, Montemayor, Vázquez, & González, 2010).

382

383 3.3. Membrane procedures for CS purification

384 A sequence of UF-DF using membranes of 100 and 30 kDa was utilised for the
385 final CS purification step. In this context, batches of neutralised CS solutions
386 obtained under the conditions optimised in previous sections were firstly processed
387 at 100 kDa performing complete UF and DF stages. The most remarkable kinetics
388 of CS and protein concentration are depicted in Figure 3 (Top, left). Experimental
389 data of concentration factor showed acceptable correlation with the corresponding
390 theoretical values in the case of total proteins however, such correlation was low
391 for CS data. This result indicates a low retention of CS molecules and good
392 concentration of proteins at 100 kDa. These results were confirmed in the process
393 of diafiltration (Figure 3, bottom-left) in which most of CS was permeated after 3
394 diavolumes meanwhile proteins were mainly concentrated. The kinetics of DF were
395 perfectly described by equation [6], in both cases obtaining very high R^2 values
396 (Table D, supplementary material). As expected, the coefficient of retention (s) for
397 proteins was higher than for CS. Furthermore, in the case of GAG R_f parameters
398 were lower and R_{3D} parameters were higher, compared to proteins.

399

400 Permeates from UF at 100 kDa were subsequently concentrated by 30 kDa cut-off.
401 In the first step of UF, total correlation agreement between CS experimental and
402 predicted concentration factor profiles (8-fold) was found (Figure 3, top-right).
403 Proteins were not retained and the filtrate flows were maintained in an average
404 value of 578 ± 57 mL/min. Experimental data of retention obtained in DF stage
405 were accurately modelled using equation [6] (Figure 3, bottom-right and Table D
406 supplementary material). Parameter concerning CS and protein retention at 30 kDa

407 indicated null permeation ($s = 1$, $R_f = 100\%$ and $R_{3D} = 0\%$) for CS, and complete
408 permeation of proteins after 3.5 volumes of water diafiltration. In addition, the
409 desalination of CS retentate at 30 kDa was total (data not shown). The purity of CS
410 retentate (in terms of I_p -values) after drying was of 98.5%. The final yield of CS
411 was $12.08 \pm 0.72 \%$ (w/w of dry cartilage).

412

413 Although the application of membrane technologies (UF-DF) is one of the most
414 common techniques utilized for the recovery of high-value macromolecules
415 obtaining by microbial or enzymatic processes, its use in the purification of CS from
416 biological tissues has not been broadly reported. Other protocols based on
417 chromatographic separation, using different types of columns and elution solvents
418 and gradients, have been usually applied at lab-scale for CS purification (Souza,
419 Kozłowski, Cerqueira, Castelo-Branco, Costa, & Pavão, 2007; Gargiulo, Lanzetta,
420 Parrilli, & De Castro, 2009; Xie, Ye, & Luo, 2014). However, the efficiency of UF-
421 DF, its commercial cost, reproducibility and facility of equipment scaling is higher in
422 comparison with chromatography procedure (Nishigori, Takeda, & Ohori, 2000).
423 Lignot et al. (2003), using skate cartilage as substrate and ceramic membranes of
424 UF, indicated that more than 40% of salts were not dialysed at 0.1 μm pore-size
425 and predicted that 7 diavolumes of water are required for total protein and salt
426 elimination. In the present study, we have demonstrated that at least 3.5
427 diavolumes should be performed to reject both disposable solutes. On the other
428 hand, the high predictive capacity of equation [6] to simulate diafiltration kinetics
429 followed the similar good agreement among experimental and calculate data, that
430 in the assessment of DF dynamics for: total proteins from shrimp wastewaters

431 (Amado, Vázquez, González, Esteban-Fernández, Carrera, & Piñeiro, 2014), CS
432 from rays (Lignot, Lahogue, & Bourseau, 2003; Murado, Fraguas, Montemayor,
433 Vázquez, & González, 2010) and small-spotted catshark (Blanco, Fraguas, Sotelo,
434 Pérez-Martín & Vázquez, 2015).

435

436 **Conclusions**

437 A set of biological, chemical and physical processes, including enzyme hydrolysis,
438 selective precipitation and redissolution of polysaccharides and separation by
439 membranes (UF-DF) have been optimised to extract and purify CS from fishing by-
440 products of *P. glauca*. To obtain this purpose, a combination of kinetic and
441 response surface methodologies based on rotatable factorial designs was applied.
442 The optimal conditions for CS isolation were 55.7°C/ pH 8.2/ 8 h/ 1% alcalase 24
443 AU/kg for enzyme cartilage hydrolysis and NaOH 0.54 M, EtOH 1.17 v, NaCl 2.5%
444 for alkaline hydroalcoholic saline treatment. UF-DF performances at 30 kDa
445 utilising at least 3 diavolumes of water in the diafiltration were needed for yielding
446 CS of high purity (>98%). Chemical characterisation works should be further
447 conducted in order to know the type of CS present in *P. glauca* head cartilage.

448

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FIGURES CAPTIONS

Figure 1. Cartilage hydrolysis over time of *P. glauca* heads by alcalase in each one of the experimental conditions defined in Table 1. The experimental data (symbols) were fitted to the Weibull equation [4] (continuous line).

Figure 2. Top: Predicted response surfaces by empirical equations summarized in Table 2 corresponding to the joint effect of pH and T on alcalase hydrolysis for the different dependent variables quantified. Bottom: Theoretical surfaces calculated by second order equations described in Table 3 to model the combined influence of $NaOH$ and $EtOH$ on the selective treatment of CS from hydrolysate cartilages of *P. glauca*.

Figure 3. UF-DF process for CS purification from *P. glauca* head cartilages at 100 kDa and 100→30 kDa sequences. Top: concentration of retained protein (○) and CS (●) in linear relation with the factor of volumetric concentration (f_c) showing experimental data (points) and theoretical profiles corresponding to a completely retained solute (discontinuous line). Bottom: progress of protein (○) and CS (●) retention with the increase of diavolume from DF process (D). Equation [6] was used to fit the experimental data. Error bars are the confidence intervals ($\alpha = 0.05$; $n = 2$).

TABLES CAPTIONS

Table 1. Experimental domains and codification of independent variables in the factorial rotatable designs executed to study the cartilage hydrolysis by alcalase and the subsequent chemical treatment of the hydrolysates in alkaline hydroalcoholic solutions.

Table 2. Second order equations describing the effect of T and pH on alcalase cartilage hydrolysis, CS production and I_p -index (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R^2_{adj}) and F-values (F_1 , F_2 , F_3 and F_4) is also shown. S: significant; NS: non-significant.

Table 3. Second order equations describing the effect of N and E on selective precipitation of CS (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R^2_{adj}) and F-values (F_1 and F_2) is also shown. S: significant.

Figures

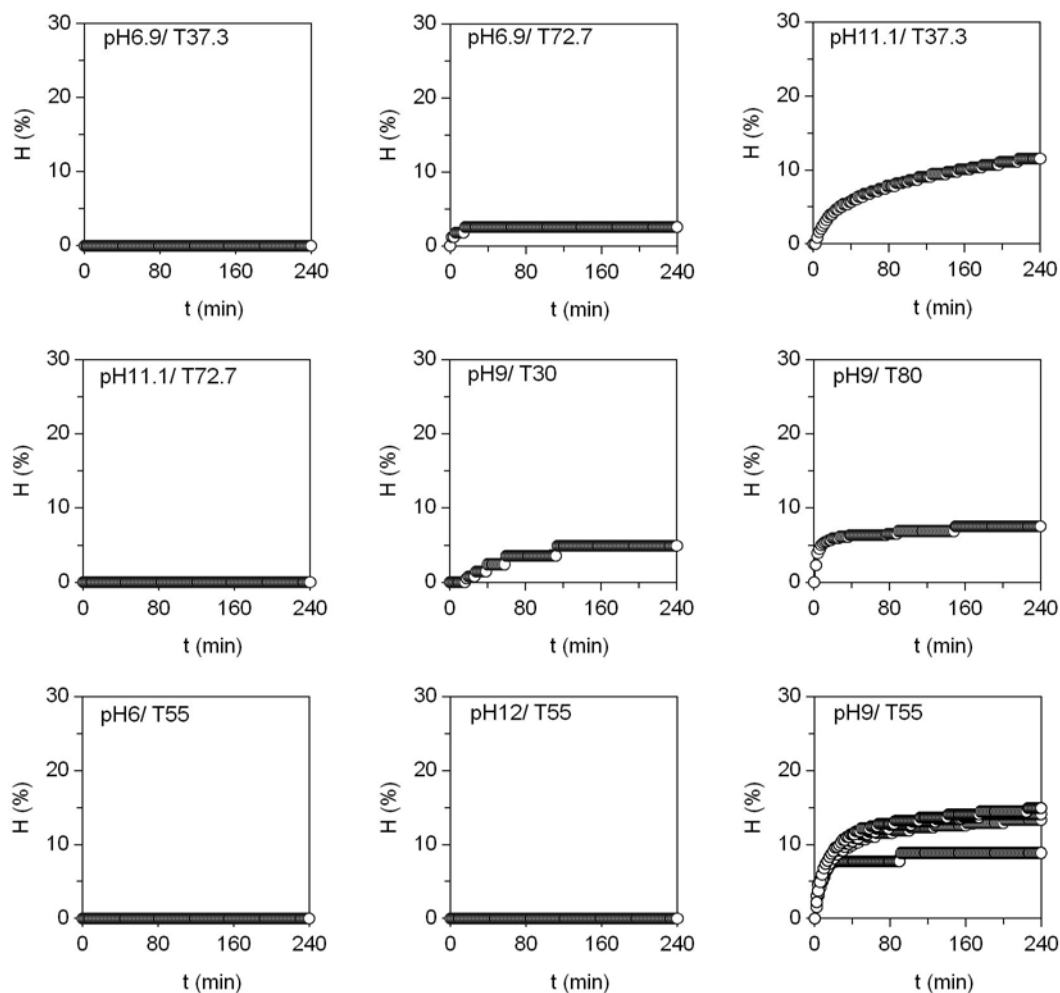
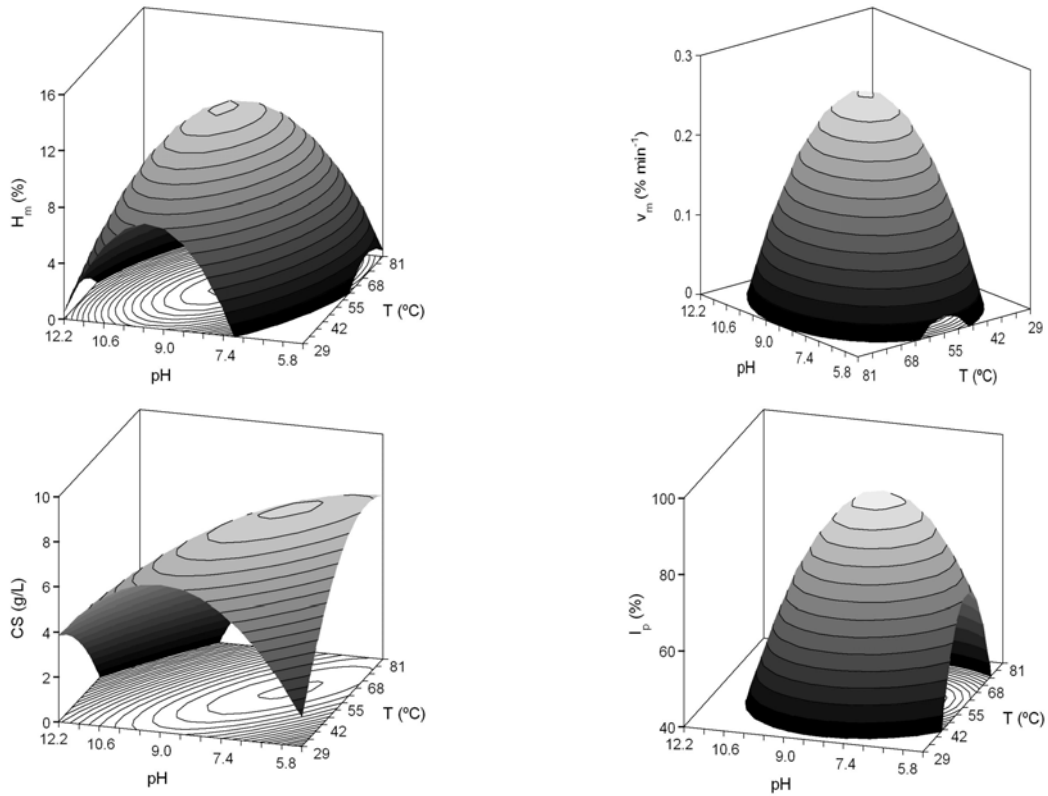


Figure 1. Cartilage hydrolysis over time of *Prionace glauca* head by alcalase in each one of the experimental conditions defined in Table 1. The experimental data (symbols) were fitted to the Weibull equation [4] (continuous line).

ALCALASE HYDROLYSIS



ALKALINE HYDROALCOHOLIC TREATMENT

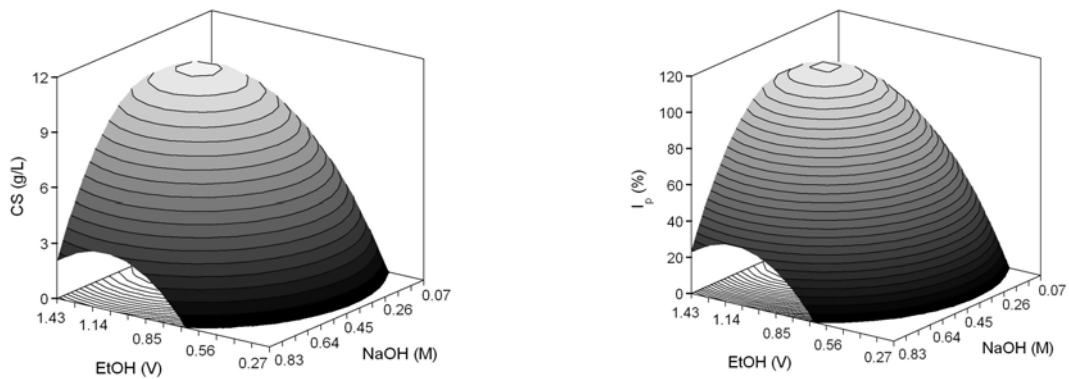


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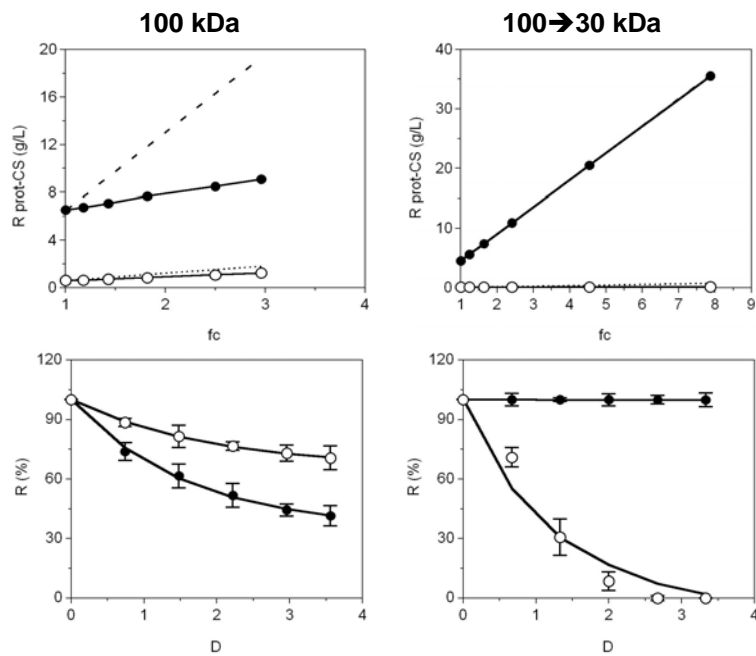


Figure 3. UF-DF process for CS purification from *P. glauca* head cartilages at 100 kDa and 100→30 kDa sequences. Top: concentration of retained protein (○) and CS (●) in linear relation with the factor of volumetric concentration (f_c) showing experimental data (points) and theoretical profiles corresponding to a completely retained solute (discontinuous line). Bottom: progress of protein (○) and CS (●) retention with the increase of diavolume from DF process (D). Equation [6] was used to fit the experimental data. Error bars are the confidence intervals ($\alpha = 0.05$; $n = 2$).

TABLES

Table 1. Experimental domains and codification of independent variables in the factorial rotatable designs executed to study the cartilage hydrolysis by alcalase and the subsequent chemical treatment of the hydrolyzates in alkaline hydroalcoholic solutions.

Coded values	Natural values			
	Alcalase hydrolysis		Chemical treatment	
	T (°C) : <i>T</i>	pH	NaOH (M): <i>N</i>	EtOH (v): <i>E</i>
-1.41	30.0	6.0	0.10	0.30
-1	37.3	6.9	0.20	0.46
0	55.5	9.0	0.45	0.85
+1	72.7	11.1	0.70	1.24
+1.41	80.0	12.0	0.80	1.40

Codification: $V_c = (V_n - V_0) / \Delta V_n$; Decodification: $V_n = V_0 + (\Delta V_n \times V_c)$
 V_n =natural value of the variable to codify
 V_0 =natural value in the centre of the domain
 V_c =codified value of the variable ; ΔV_n = increment of V_n per unit of V_c .

Table 2. Second order equations describing the effect of T and pH on alcalase cartilage hydrolysis, CS production and I_p -index (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R_{adj}^2) and F-values (F_1 , F_2 , F_3 and F_4) is also shown. S: significant; NS: non-significant.

Parameters	H_m	V_m	CS	I_p
b_0 (intercept)	13.00	0.264	7.34	86.87
b_1 (T)	-	-	-1.07	-
b_2 (pH)	-	-	-2.21	-17.38
b_{12} ($T \times pH$)	-3.72	-	-2.04	-
b_{11} (T^2)	-3.01	-0.095	-1.11	-30.35
b_{22} (pH^2)	-6.28	-0.140	-1.72	-11.84
R_{adj}^2	0.720	0.776	0.812	0.939
$F1$	7.72 [$F_9^3 = 3.86$] \Rightarrow S	21.80 [$F_{10}^2 = 4.10$] \Rightarrow S	11.33 [$F_7^5 = 3.97$] \Rightarrow S	62.20 [$F_9^3 = 3.86$] \Rightarrow S
$F2$	0.50 [$F_3^8 = 8.85$] \Rightarrow S	0.26 [$F_2^8 = 19.37$] \Rightarrow S	0.68 [$F_4^8 = 6.04$] \Rightarrow S	0.39 [$F_3^8 = 8.85$] \Rightarrow S
$F3$	2.91 [$F_4^9 = 6.00$] \Rightarrow S	0.51 [$F_4^{10} = 5.96$] \Rightarrow S	2.09 [$F_4^7 = 6.09$] \Rightarrow S	2.08 [$F_4^9 = 6.00$] \Rightarrow S
$F4$	4.44 [$F_4^5 = 6.26$] \Rightarrow S	0.18 [$F_4^6 = 6.16$] \Rightarrow S	3.54 [$F_4^3 = 6.59$] \Rightarrow S	2.94 [$F_4^5 = 6.26$] \Rightarrow S

Table 3. Second order equations describing the effect of N and E on selective precipitation of CS (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R_{adj}^2) and F-values (F_1 , F_2 , F_3 and F_4) is also shown. S: significant.

Parameters	CS	I_p
b_0 (intercept)	9.14	89.24
b_1 (N)	1.56	16.09
b_2 (E)	3.85	38.50
b_{12} ($N \times E$)	-	-
b_{11} (N^2)	-2.36	-22.18
b_{22} (E^2)	-2.31	-22.16
R_{adj}^2	0.843	0.842
F_1	17.11 [$F_8^4 = 3.84$] \Rightarrow S	17.03 [$F_8^4 = 3.84$] \Rightarrow S
F_2	0.56 [$F_4^8 = 6.04$] \Rightarrow S	0.56 [$F_4^8 = 6.04$] \Rightarrow S

SUPPLEMENTARY MATERIAL

Table A. Parametric estimations corresponding to the Weibull equation [4] applied to the enzymatic hydrolysis kinetics at the experimental conditions studied. Independent variables are expressed in natural values in brackets. Numerical values of the parameters are shown with their confidence intervals. Determination coefficients (R^2) and p-values from F-Fisher test are also summarized.

Experimental conditions	$H_m(\%)$	$v_m(\% \text{ min}^{-1})$	$\tau(\text{min})$	β	R^2	p-value
T:-1 (37.3°C) / pH:-1 (6.9)	-	-	-	-	-	-
T:1 (72.7°C) / pH:-1 (6.9)	-	-	-	-	-	-
T:-1 (37.3°C) / pH:1 (11.1)	14.86 ± 0.88	0.045 ± 0.006	71.9 ± 10.3	0.63 ± 0.03	0.996	<0.001
T:1 (72.7°C) / pH:1 (11.1)	-	-	-	-	-	-
T:-1.41 (30.0°C) / pH:0 (9.0)	4.99 ± 0.11	0.049 ± 0.004	51.8 ± 2.5	1.46 ± 0.13	0.967	<0.001
T:1.41 (80.0°C) / pH:0 (9.0)	9.04 ± 1.10	0.140 ± 0.086	6.0 ± 3.4	0.27 ± 0.05	0.951	<0.001
T:0 (55.0°C) / pH:-1.41 (6.0)	-	-	-	-	-	-
T:0 (55.0°C) / pH:1.41 (12.0)	1.00 ± 0.12	0.010 ± 0.006	12.2 ± 1.8	0.54 ± 0.21	0.932	<0.001
T:0 (55.0°C) / pH:0 (9.0)	13.39 ± 0.08	0.231 ± 0.006	13.4 ± 0.4	0.67 ± 0.02	0.995	<0.001
T:0 (55.0°C) / pH:0 (9.0)	13.40 ± 0.11	0.209 ± 0.007	14.1 ± 0.5	0.63 ± 0.02	0.994	<0.001
T:0 (55.0°C) / pH:0 (9.0)	9.14 ± 0.28	0.420 ± 0.071	2.9 ± 0.7	0.38 ± 0.06	0.919	<0.001
T:0 (55.0°C) / pH:0 (9.0)	14.45 ± 0.11	0.207 ± 0.006	14.8 ± 0.4	0.61 ± 0.02	0.996	<0.001
T:0 (55.0°C) / pH:0 (9.0)	14.75 ± 0.13	0.252 ± 0.010	12.3 ± 0.5	0.61 ± 0.02	0.992	<0.001

Table B. Optima values of the two independent variables (T_{opt} and pH_{opt}) to obtain the maximum responses from the equations defined in Table 1 and for the different dependent variables evaluated.

	H_m	v_m	CS	I_p
T_{opt} (°C)	55.0	55.0	59.2	53.6
pH_{opt}	9.0	9.0	7.34	7.43
Y_{max}	13.0	0.264	8.08	93.1

Table C. Summary of the independent variables (*NaOH*: *N*, *EtOH*: *E*) in the response surface design with the corresponding experimental (Y_e) and predicted (Y_p) results of selective precipitation of CS from *P. glauca* wastes. Natural values of experimental conditions are in brackets.

Independent variables		CS (g/L)		I_p (%)	
X_1 : <i>N</i>	X_2 : <i>E</i>	Y_e	Y_p	Y_e	Y_p
-1 (0.20)	-1 (0.46)	0.00	-0.94	0.00	-9.7
1 (0.70)	-1 (0.46)	0.00	2.18	0.00	22.5
-1 (0.20)	1 (1.24)	8.97	6.76	89.3	67.3
1 (0.70)	1 (1.24)	8.84	9.88	90.5	99.5
-1.41 (0.10)	0 (0.85)	0.00	2.24	0.00	22.5
1.41 (0.80)	0 (0.85)	8.92	6.64	90.2	67.8
0 (0.45)	-1.41 (0.30)	0.00	-0.88	0.00	-9.1
0 (0.45)	1.41 (1.40)	9.13	9.98	90.3	99.5
0 (0.45)	0 (0.85)	9.75	9.14	89.6	89.2
0 (0.45)	0 (0.85)	9.20	9.14	89.3	89.2
0 (0.45)	0 (0.85)	8.89	9.14	89.0	89.2
0 (0.45)	0 (0.85)	8.75	9.14	89.0	89.2
0 (0.45)	0 (0.85)	9.13	9.14	89.3	89.2

Table D. Parametric estimates from UF-DF purification data of CS and proteins fitted to the equation [6]. Determination coefficients (R^2) are also shown. NS: non-significant.

UF-DF sequence		CS	Proteins
100 kDa	R_0	64.84 ± 8.30	34.34 ± 2.75
	R_f	34.65 ± 8.52	65.66 ± 2.97
	s	0.378 ± 0.203	0.472 ± 0.087
	R^2	0.998	0.999
	R_{3D}	54.81	27.30
100→30 kDa	R_0	0.00 ± 0.00	100.0 ± 31.20
	R_f	100.0 ± 1.80	0.00 ± 0.00
	s	1.00 ± 0.00	0.107 (NS)
	R^2	1.000	0.962
	R_{3D}	0.0	93.14