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**The electrophoretic behaviour in relation to the structural integrity of
codfish parvalbumin upon heat-treatment**

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ABSTRACT

This work evaluates the impact of heat processing of parvalbumin, a major fish-allergen, on the consequences for quantitative analysis of this protein embedded in different matrices during heating (either isolated, in an aqueous extract or in whole fillets) to assess potential health risks. It is shown that oligomerization of parvalbumin does occur, but only upon heat treatments above 80°C. This coincides with the ability of the isolated protein to refold up to this temperature in a fully reversible way, as demonstrated by circular dichroism analysis. In autoclaved samples a disintegration of the protein structure is observed. The situation becomes different when parvalbumin is embedded in a matrix with other constituents, like in fish extracts or whole fillets. The electrophoretic analysis of parvalbumin (SDS-PAGE and immunoblotting) is largely determined by complexation with other proteins resulting in insoluble materials caused by the partial unfolding of the parvalbumin at elevated temperatures. This effect is more strongly observed for cod fish extract, compared to whole cod fillets as in the latter situation the integrity of the tissue hampers this inter-protein complexation. Moreover, it is shown by ELISA-analysis of heat-treated samples that using blotting procedures where disintegration of complexes may be promoted, restoring some of the IgG-binding propensity, may provide false outcomes. We conclude that antibody binding to parvalbumin is dominated by the potential to form heat-induced complexes with other proteins. The possible less-soluble or extractable character of these complexes may provide confusing information regarding potential health risks of fish and fish protein-containing food composites when analysing such heat-treated samples by immunochemical assays.

INTRODUCTION

Fish plays an important role in human nutrition as a source for proteins, polyunsaturated fatty acids, vitamins A and D, or iron and calcium¹. But fish is also one of the most frequent causes of food allergy². The rising consumption of fish contributes to the prevalence of fish allergy. Fish-allergic individuals are counselled to follow a strict fish avoidance diet to prevent reactions. The increasing availability of fish-derived ingredients, including as a powdered constituent in complex food products, raises concerns for fish-allergic consumers. Prevalence rates of fish allergy range from 0.2 to 2.3% of the population, reaching up to 8% among fish processing workers¹⁻³. Individuals allergic to ingestion or inhalation, or even skin contact to fish proteins subjected to cooking or other processing, may exhibit IgE-mediated type I responses including symptoms such as urticaria, dermatitis, angioedema, diarrhoea, asthma, or anaphylactic reactions³.

Parvalbumin (PV) is considered to be a pan-allergen for fish-allergic patients. Parvalbumins from many different fish species commonly consumed in Western Europe share similar biochemical and immunochemical characteristics^{4,5} and are reported to cross-react with each other and with parvalbumins in frog meat⁶ or in tropical fish species consumed in Asian-Pacific countries⁷. Recently it was shown that parvalbumin cross-reactivity is related to the molecular origin of this major allergen⁸.

Parvalbumins are proteins conserved in lower vertebrates and are abundant in white muscle. Parvalbumins are found in fast twitch skeletal muscles of higher vertebrates, as well as in a variety of non-muscle tissues, including testis, endocrine glands, skin, and specific neurons⁹, involved in muscle contraction, calcium buffering, and signal transduction between cellular compartments¹⁰⁻¹³. For cod it is estimated that 0.15-0.63% of fish muscle tissue (wet weight) consists of parvalbumin¹⁴. They are typically 10-12 kDa, acidic (pI=4.0-5.2) and structurally characterized by the presence of three typical helix-loop-helix domains, of which two are capable of binding divalent cations, like Ca⁺. The third domain forms a cap that covers the hydrophobic surface of the pair of functional domains^{15,16}. Based on the amino acid sequences, two distinct phylogenetic lineages of parvalbumins, named α and β , have been identified¹⁷. The β lineage is reported to be especially allergenic³ and its IgE binding has been shown to be strongly calcium-dependent¹⁸.

Heat treatment is an important food preparation and preservation procedure. It is required to ensure microbial safety or to obtain desirable organoleptic attributes, improving appearance, texture, flavour and taste of products¹⁶. Parvalbumins are renowned for their strong resistance against heat treatment, associated to the high reversibility in their denaturation in the presence of calcium¹⁹ and other denaturing conditions²⁰. The heat stability of parvalbumin provides a major source of risk for fish-allergic consumers²¹. It was recently reported that heat treatment of cod parvalbumin at high temperatures alters the structure of the protein, losing partially its secondary structure and initiating aggregation²², and thereby affecting antibody reactivity⁸. The thermal stability of parvalbumins is different amongst isotypes, as has been studied in carp²³ and red stingray²⁴, where some isoforms formed aggregates during heat processing.

Allergenicity is described by two key parameters: the potency to sensitize an (atopic) individual and the IgE binding essential to elicit an allergic reaction once an individual is sensitized. Therefore, fish processing may affect structural epitopes responsible for IgE binding to parvalbumin²⁵. Boiling and cooking of fish can also lead to the formation of molecular aggregates and thereby enhancement of IgE reactivity as shown for tuna, salmon, cod, flounder, and whiff^{26, 27}, despite the observation that the allergenicity to certain proteins in tuna and salmon was reduced by heat processing^{28,29}. Also other IgE-binding proteins have been identified in Baltic^{30,19} and Atlantic cod^{31,32}. Some of these proteins were also recognized by monoclonal parvalbumin antibodies^{19,30-32}, associated to dimer or oligomers³¹. It has been suggested that IgE binding for multimers is stronger than to monomeric parvalbumin³³. The occurrence of monomeric and oligomeric parvalbumin has also been demonstrated in different fish extracts⁸. A direct link between their presence and their IgE-binding potential has not been reported.

The aim of this work is to evaluate the impact of heat processing of parvalbumin (PV) on structural motifs in relation to the matrix in which PV is embedded (isolated, in an aqueous extract or in whole fillets). In particular, the heat-induced effects on the PV aggregation state were evaluated by electrophoresis and immunoblotting. Circular dichroism analysis was applied to assess the reversibility of structural motifs in the proteins.

106 MATERIALS and METHODS

107 *Materials*

108 Atlantic Cod (*Gadus morhua*) was purchased as frozen fillet produced by Albert Heijn (Zaandam ,
109 The Netherlands). The fillets were stored at -20°C until further use. Three types of parvalbumin
110 batches were prepared: sliced pieces of cod fillet, cod fillet protein extract, and purified parvalbumin.
111 Cod fillet samples were prepared as ~5 g pieces. Cod fillet protein extracts were from defrosted 20 g
112 portions of cod muscle as described elsewhere ³⁴. The thawed fillet was homogenized in 60 ml of
113 deionized water and 6.4 ml 1M Tris-HCl (pH 9) using an Ultra Turrax blender (IKA, Staufen,
114 Germany). Thereafter the pH was maintained around pH 8 using 1M NaOH. The slurry was stirred for
115 2 hours on ice and then centrifuged for 30 min at 1800g at 4°C. The supernatant was collected and
116 stored in the freezer. Purified parvalbumin was obtained as described earlier by Koppelman and
117 coworkers ³⁵.

118
119 *Heating of protein samples*

120 Aliquots of purified parvalbumin, cod fillet protein extracts and pieces of cod fillet were heated at 20,
121 40, 50, 60, 70, 80 or 100°C for 2 hours in a water bath. This incubation time was selected sufficiently
122 long to avoid uncertainties in warming up of the samples, and to represent processing-relevant times.
123 For cod fillet pieces, the heating time was extended by five minutes, as a control experiment showed
124 that this time was needed to obtain the set temperature at the interior of the pieces. Alternatively
125 samples were heated in an autoclave for 2 hours at 120°C. After the heating step, the samples were
126 cooled to room temperature. After centrifugation (3612g in a table-top centrifuge at 4°C for 30 min) of
127 the extracts and purified parvalbumin, a sample was collected from the supernatant (20 µl). The
128 remaining mass (30 µl) was kept separately, and referred to as total sample. The volume ratio for the
129 supernatant was kept at 2/5th of the sample volume to be sure that the supernatant did not contain any
130 pelleted material. As a consequence, the total sample contains the pelleted material and part of the
131 supernatant. Both samples, supernatant and total sample, were immediately mixed with Laemmli
132 buffer (1:1 vol.:vol.).

Protein concentration determination

Protein concentrations were determined using the Bradford assay kit (Sigma-Aldrich, USA). Bovine serum albumin standards (Sigma-Aldrich, USA) were used and the absorbance was determined at 595nm using a UV/Vis Spectrophotometer (PerkinElmer, USA) in a quartz cell with a path length of 1 cm. It is assumed that in the assay also pelleted protein becomes accessible for Coomassie-binding.

SDS-PAGE analysis

Protein profiles for pure PV, raw extracts and cod fillet extracts were obtained using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Fish proteins already diluted in Laemmli sample buffer containing 2-mercaptoethanol were heated for 10min at 70°C, loaded on a 12%Bis-Tris gel and subjected to SDS-PAGE using the XCell Surelock Min-Cell (Invitrogen, USA) at 200v. Novex Sharp Pre-stained Protein standards (Novex, USA) were used to estimate the molecular weights of individual proteins. Proteins were visualized by SimplyBlue SafeStain (Invitrogen, USA).

Immunoblotting

PV and fish protein extracts were separated using SDS-PAGE as described above, and then transferred to an activated PVDF membrane (Invitrogen, USA) using the XCell II Blot Module (Invitrogen, USA) for 1h at 30V. After blocking with 5% (w/v) skim milk in TBST for 1h at 4°C, membranes were incubated overnight with the primary polyclonal Rabbit anti-parvalbumin antibody ¹⁴, diluted 1:2500 in the blocking buffer. Membranes were subsequently washed in TBST (diluted Tris-buffered saline) and incubated with the secondary Goat anti-Rabbit IgG antibody (Thermo Fisher Scientific) for 1h. The protein-antigen interaction was visualised using DAB substrate (Roche, Germany). The staining reaction was stopped after 10 min with TBST.

ELISA for parvalbumin

A sandwich ELISA (enzyme-linked immunosorbent assay) based on an affinity-purified polyclonal antibodies (IgG) raised against purified cod parvalbumin was used with all samples ¹⁴. Purified cod parvalbumin was used as standard. First, a wide dilution range using 5-fold serial dilutions was tested

162 to determine the dilution that resulted in a half-maximal signal. Subsequently, a narrow dilution range
163 (serial dilution of 2-fold) around this dilution was tested. Then, 5 dilutions in this area were tested in
164 triplicate. Calibration curves were fitted using a four parameter logistic function and had a correlation
165 coefficient (R^2) of at least 0.998. Results are expressed in μg parvalbumin per ml in un-diluted sample.

167 *Circular dichroism*

168 The different samples, heated for 2 hrs at indicated temperatures at a concentration of 2 mg/ml were
169 diluted to 0.2 mg/ml with deionized water (pH 6.8) and transferred to a quartz cuvet with a 1 mm
170 optical path. Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-715
171 spectropolarimeter in the spectral range from 190 to 260 nm with a resolution of 0.2 nm, a band width
172 of 2 nm, scan speed of 100 nm/min and an instrumental response time of 0.125 sec. The instrument
173 was equipped with a computer-controlled Peltier-element to set the temperature or apply temperature
174 ramps between 20 and 95 (± 0.1) $^{\circ}\text{C}$. 16 spectra were accumulated and averaged. The spectrum of a
175 protein-free samples was subtracted. Alternatively, the ellipticity at 222 nm was monitored during
176 applied heating and cooling ramps of 1 $^{\circ}\text{C}/\text{min}$.

RESULTS and DISCUSSION

Electrophoretic analysis of heat processed purified parvalbumin

Figure 1 shows that purified parvalbumin in aqueous buffer without heat treatment runs on an SDS-PAGE gel (upper panels) as a single band at approximately 12 kDa, as reported previously³⁶. Heating the sample and subsequent centrifugation and analysis using gel electrophoresis under reducing conditions shows that parvalbumin can be found in both the supernatant and total sample. With heat treatments of up to 80°C even for prolonged 2-hour incubation for two hours, no traces of multimeric protein were observed on the SDS-PAGE gel. Incubation at 100°C leads to a small (estimated less than 1%) contribution of dimeric protein in the supernatant. The total sample under this condition contains a more significant contribution (~5%) of aggregated proteins, as judged from visual inspection of the gel. Monomeric proteins are the predominant fraction but dimers, trimers, and other aggregates up to hexamers can be distinguished at both non-reducing and reducing conditions with a predominance of the apparent dimeric and trimeric forms. Cod parvalbumin oligomers have been previously reported³¹. Analysis of the autoclaved sample showed that degradation of the protein had occurred into smaller (<12kDa) peptide fragments in both the supernatant and the total sample. It is likely that the observed degradation products arise from the supernatant contribution to the total sample rather than its insoluble components.

The polyclonal rabbit anti-parvalbumin serum recognizes parvalbumin including all of the oligomeric structures observed in the 100°C-treated samples as shown by the immunoblot analysis (lower panels in Figure 1). Interestingly, the intensity of staining on the blot decreases progressively with heat treatment (compare with upper panels) especially at temperatures above 80°C. This effect appears to be stronger for the supernatant compared to the total sample. Moreover, the autoclaved sample does not show any significant IgG binding, except for a very faint band in the total sample.

Heat-induced conformational changes of parvalbumin

Parvalbumin is rather temperature-resistant, especially due to its refolding propensity¹⁹. To evaluate this in more detail, the secondary structure of the protein was studied as a function of temperature

using circular dichroism. Figure 2 shows the CD-spectra of samples that were heat-treated at different temperatures for 2 hrs and subsequently cooled to 20°C. Typically the spectra show three extremes, at 198, 208 and 222 nm, indicative for highly structured proteins, as described before³³. The shape of the spectra only deviated from that of the native material when the sample was heated above 80°C. Despite the similar protein-concentration the autoclaved sample (dashed line in Figure 2, which is a redrawn spectrum where the intensity was amplified 5 times to allow comparison with the other spectra) showed a low intensity, likely to be caused by disintegration of the protein in smaller fragments as illustrated in Figure 1. This degradation will lower the number of chiral peptide bonds as detected with CD. When analysis of these spectra was conducted using a shape-analysis curve fitting procedure, it was observed that the native protein bears a high secondary structure content with approximately 30% α -helix and 42% β -strands (Figure 3). The proportion of these two secondary structure contributions remains constant up to 70°C and decreases gradually at higher incubation temperatures to about half their proportions for the autoclaved sample, illustrating the occurrence of some irreversible conformational change. When the temperature profile is shown at an ellipticity of 222 nm during heating from 20 to 95°C (Figure 4), the secondary structure was in fact gradually reduced already at low temperatures, with a small cooperative unfolding starting between 70 and 80°C, in line with previous reports³⁷. Moreover, no cooperative refolding step was observed upon subsequent cooling and the secondary structure gradually increased again, as evidenced by the increase in absolute ellipticity (Figure 4). The difference at 20°C prior to heating and after cooling reflects the irreversible unfolding as illustrated in Figure 2. By taking spectra at intervals of 10°C during heating and cooling and subsequent analysis for the secondary structure content, this gradual unfolding-refolding was evident (results not shown). The spectrum of parvalbumin at 95°C (not shown) shows a much more profound degree of unfolding (up to about 65% random coil) that partly refolds during cooling. At this point, it cannot be concluded whether this partial refolding reflects an ensemble-averaged behaviour of unfolded and fully refolded contributions or whether all denatured proteins refold to a non-native state containing a less defined secondary structure.

At this point it is unclear whether the observed irreversible conformational changes above 80 °C can be held entirely responsible for the diminished staining intensity on the immunoblot. Although the

correlation between the structural integrity and the immunoblot band intensity is striking, a more extensive and detailed analysis would be required to determine if these two observations were linked or coincidental. The absence of IgG binding with the autoclaved sample is remarkable, and can be partially linked to the degradation of the protein into smaller fragments (Figure 1) with more limited IgG-binding potential. Alternatively, as Figure 1 still shows a significant population of protein in the autoclaved sample that runs with an apparent molecular mass as the native protein, and in view of the presence of a far-UV CD spectrum, it can also be concluded that irreversible unfolding of the protein beyond a certain level of the native protein results in a strongly diminished IgG binding.

Electrophoretic behaviour of heated parvalbumin embedded in matrices

Figures 5 and 6 show the electrophoretic analysis of parvalbumin heated either in an extract or embedded in a whole fillet and incubated for two hours at temperatures ranging from 20°C up to 100°C. Samples not subjected to heat-treatment (lanes next to the marker-lane) and those treated in the autoclave (AC) (2 hrs at 120°C) (right lanes) are also included. With the cod fish protein extract (Figure 5), a large number of distinctive bands are observed from the non-heated sample with dominant bands, next to that of parvalbumin, at around 16, 36 and 40 kDa. The vast majority of these proteins, with the exception of parvalbumin, lose their solubility when incubated at temperatures above 50°C. Parvalbumin is very heat resistant and remains soluble at high temperatures, as similarly observed for the pure protein (see Figure 2). Also proteins can be observed at higher molecular weights in the total sample at elevated temperatures, but there are no indications for extensive aggregation phenomena, since no material can be observed at the top of the (stacking) gel shown in Figure 5. As observed for purified parvalbumin, the autoclave treatment resulted in degradation of the proteins, and no apparent aggregation of the material. Immunoblot analysis of these samples shows that the IgG is specific for parvalbumin and does not show any significant cross-sensitivity for other extracted fish proteins. In the supernatant, a pattern similar to the pure parvalbumin is observed where at higher temperatures the relative staining intensity to protein bands on the blot is reduced. Analysis of the total sample suggests that the heat treatment resulted in a complexation of parvalbumin with other fish muscle proteins. For example, bands at approximate molecular weights of 20, 40, and 60

kDa were observed (Figure 5) at heat treatments of 60°C and above. Identification of these other proteins, however, is currently unresolved.

When parvalbumin is heated in the matrix of whole cod fish fillet (Figure 6), the extensive complexation of parvalbumin with other fish muscle proteins is not as evident as observed for the fish extract (compare with Figure 5). Such complexation could be hampered by the spatial organization of proteins within the tissue, providing a stable network that becomes disrupted in the extraction process. Less extensive complexation was observed after heating of the whole cod fish fillet where only two main contributions were observed at 40 and 200 kDa in addition to monomeric parvalbumin.

ELISA-analysis

Table 1 presents the analysis of the parvalbumin content using the IgG ELISA in the whole cod fish fillet when heated for prolonged times (2 hours) at various temperatures. At 50°C, IgG binding was reduced by 29%, whereas IgG binding was reduced by 93% when heated at 100°C. The reduced reactivity in ELISA at moderate incubation temperatures (50 and 80°C) is not in line with the staining intensities observed in the immunoblotting of these samples (Figure 6). Most likely this is related to the blotting procedure where the proteins are exposed to SDS, an unfolding agent, and mercaptoethanol, a reducing agent. The protein-dissociative conditions in the immunoblot analysis might promote the recognition by IgG. Clearly, autoclave treatment caused the complete loss of IgG binding in the ELISA, as similarly observed for the immunoblot analysis. Recent work showed that heating purified parvalbumin (i.e. in absence of a matrix) for 20 min at 100°C had no significant influence on IgG binding as assessed by ELISA, whereas the same heat treatment of whole cod fillet reduced the reactivity to only 14% (results not shown, ³⁴). This suggests that the matrix plays an important role for the stability of immunogenicity of parvalbumin. Our observation that autoclave treatment of cod fish fillet strongly reduced the IgG-reactivity of parvalbumin is in line with the observation that canned salmon was less reactive than non-heated salmon in an oral food challenge in fish-allergic patients ³⁸, although at this stage we cannot be sure that the same mechanism plays a role.

Concluding remarks

290 This work shows that the matrix affects the aggregation behaviour of parvalbumin upon heating. Less
291 aggregation was observed with parvalbumin embedded in whole cod fillets where the tissue
292 orientation of the protein likely hampers protein complexation and aggregation. Dimerization or
293 oligomerization of parvalbumin does occur, but only to a limited extent and does not interfere
294 substantially with a quantitative analysis of the allergenic potential of a heat-treated material.
295 Sampling conditions, like storage time and incubation buffers may affect the aggregation process of
296 parvalbumin, and thereby the degree of oligomerization.

297 Heat treatment as such, and especially the structural refolding propensity of the protein is thus only
298 partially responsible for loss of IgG reactivity. Antibody binding to parvalbumin is dominated by the
299 potential of the protein to form heat-induced insoluble complexes with itself and other proteins and
300 immunoblot analysis of such heat-treated samples may provide confusing information regarding the
301 potential health risks of heat-processed fish and fish-containing composite foods. Especially when
302 parvalbumin enters the food chain as fish-extract like in fish meal great a thorough immunochemical
303 analysis is essential.

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FIGURE CAPTIONS

Figure 1. Heating of purified cod parvalbumin. Top panels: SDS-PAGE analysis with protein stain; bottom panels: immunoblotting with anti-parvalbumin antiserum; left panels: supernatant; right panels: total sample. In all lanes 10 μ g of total protein was applied. The incubation temperatures (2 hrs) are indicated.

Figure 2. Far-UV circular dichroism spectra of 0.2 mg/mL purified parvalbumin samples recorded at 20 °C, pre-treated for 2 hrs at a concentration of 2 mg/mL at indicated temperatures. The dashed line represents the redrawn spectrum of the autoclaved sample (2 hrs at 120°C) where the intensity was multiplied by a factor of 5.

Figure 3. Secondary structure analysis of the spectra shown in Figure 2 using a non-linear regression fitting procedure with reference spectra as described in the method section. The alpha-helix contributions are represented by the black bars, beta-strands by the dark grey, and non-structured contributions by the light grey bars.

Figure 4. Temperature scans of 0.2 mg/mL purified parvalbumin with a heating and cooling rate of 1°C/min. The ellipticity is monitored at 222 nm.

Figure 5. Heating of cod protein extract. Top panels: SDS-PAGE analysis with protein stain; bottom panels: Immunoblotting with anti-parvalbumin antiserum; left panels: supernatant; right panels: total sample. In all lanes 10 μ g of total protein was applied. The incubation temperatures (2 hrs) are indicated.

Figure 6. Heating of whole cod fillet. Top panels: SDS-PAGE analysis with protein stain; bottom panels: immunoblotting with anti-parvalbumin antiserum; left panels: supernatant; right panels: total

445 sample. In all lanes 10 μg of total protein was applied. The incubation temperatures (2 hrs) are
446 indicated.
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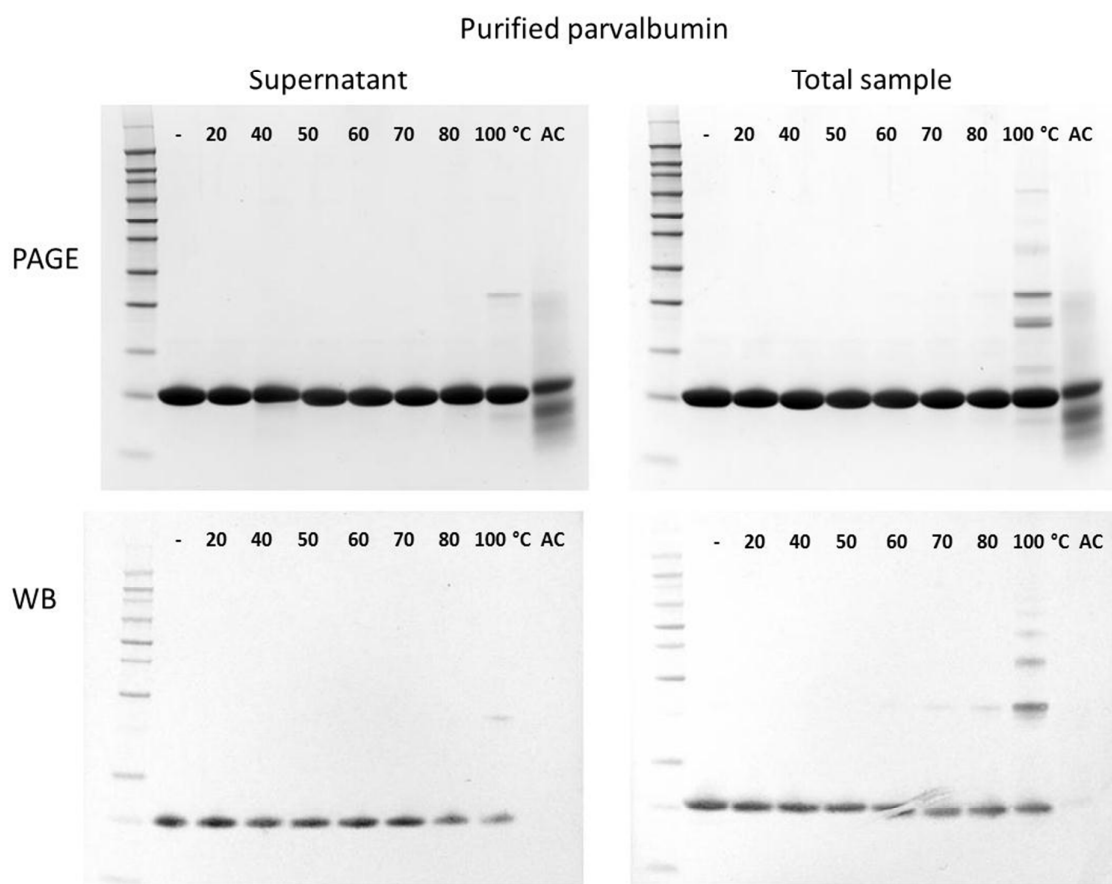


Figure 1

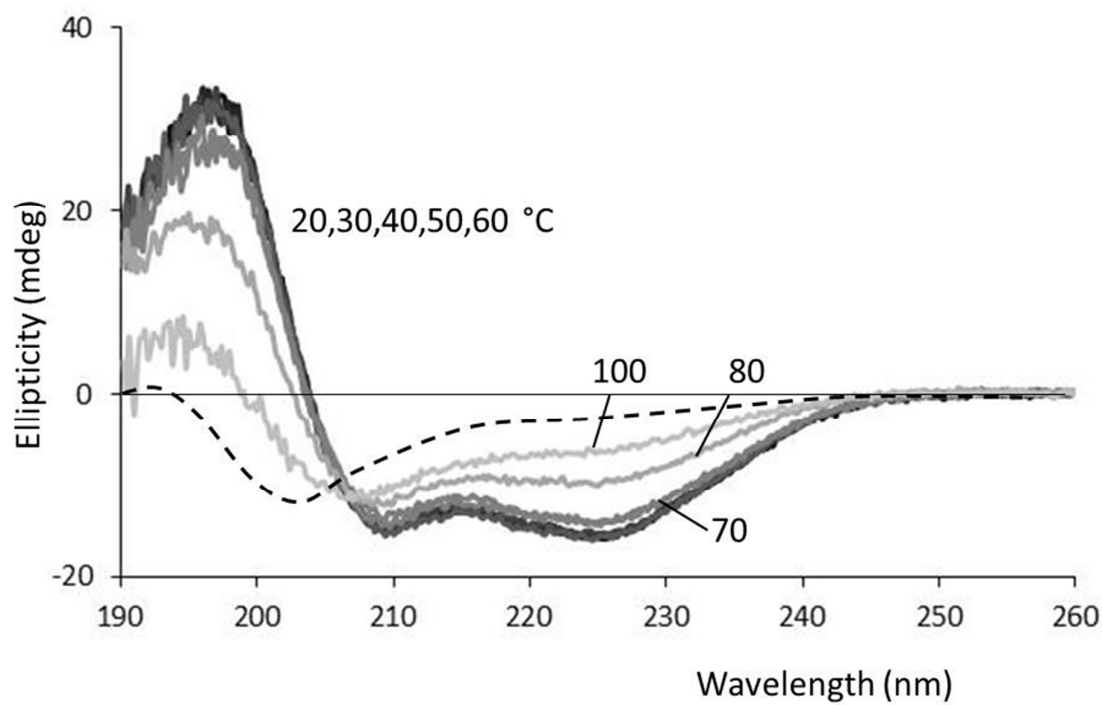


Figure 2

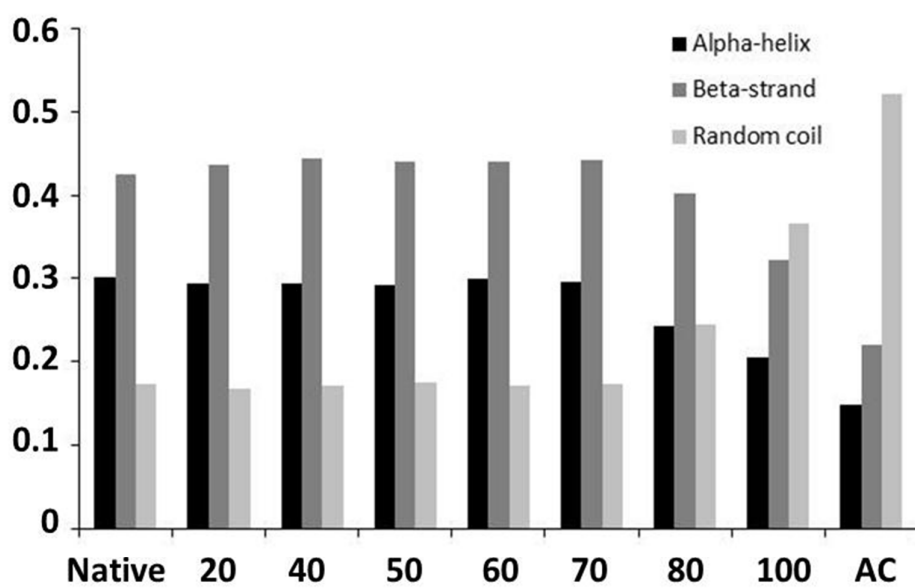


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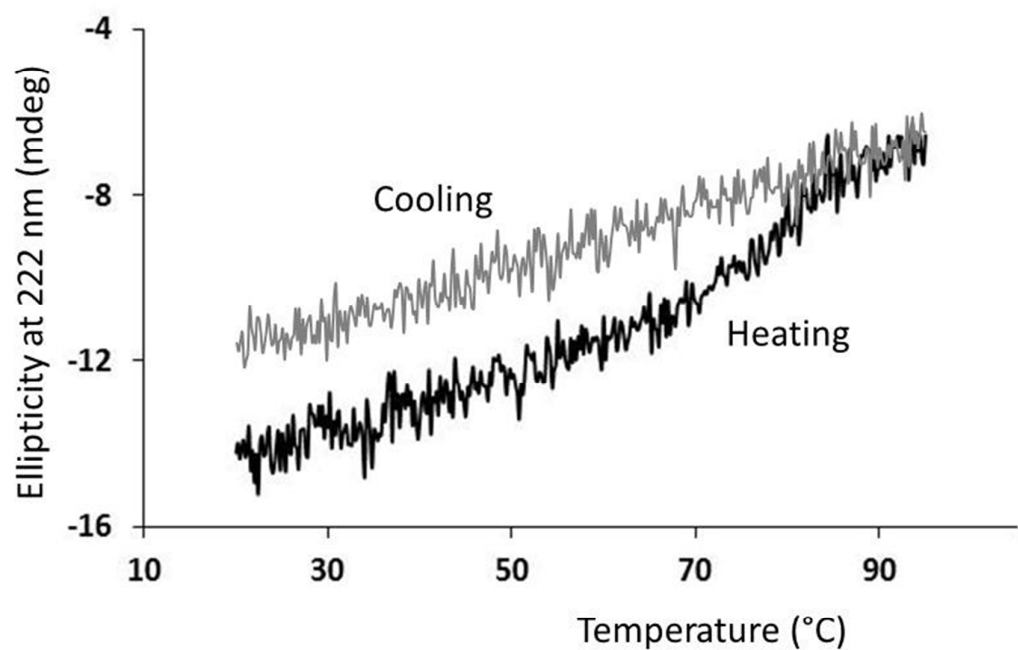


Figure 4

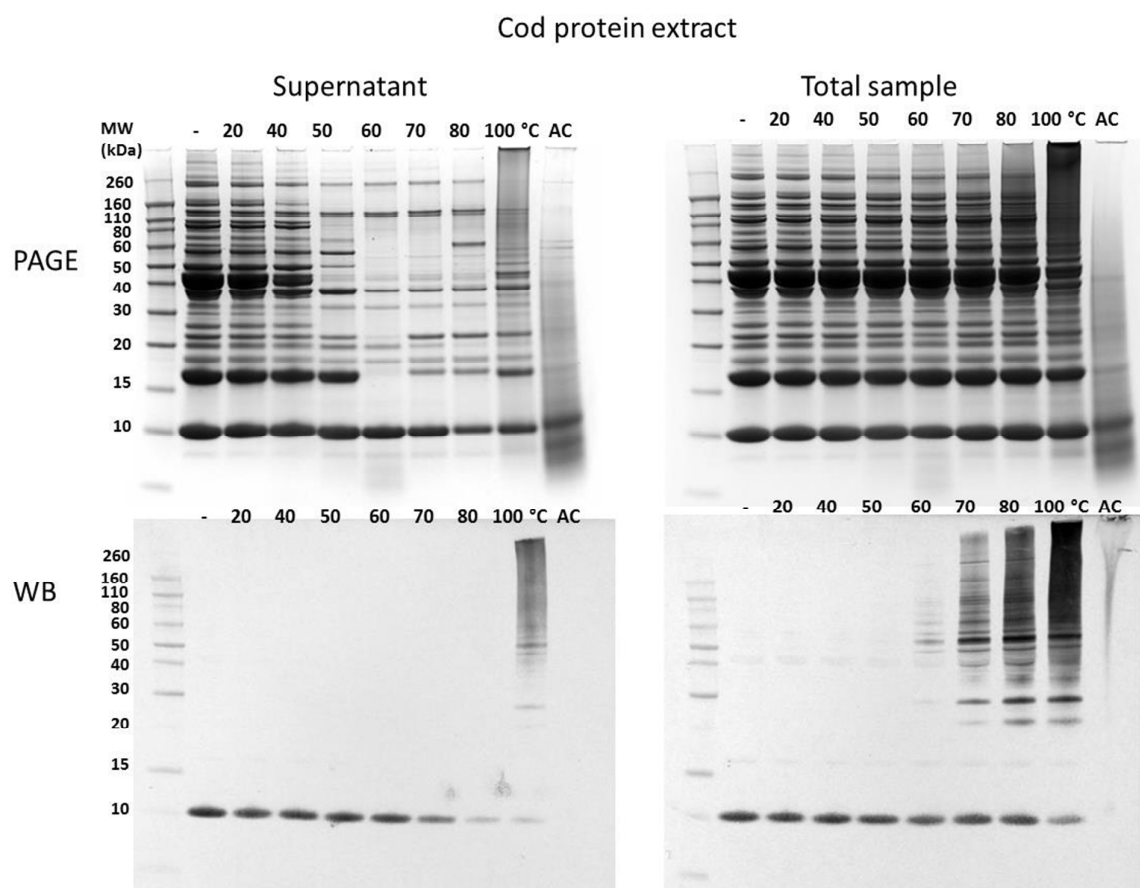


Figure 5

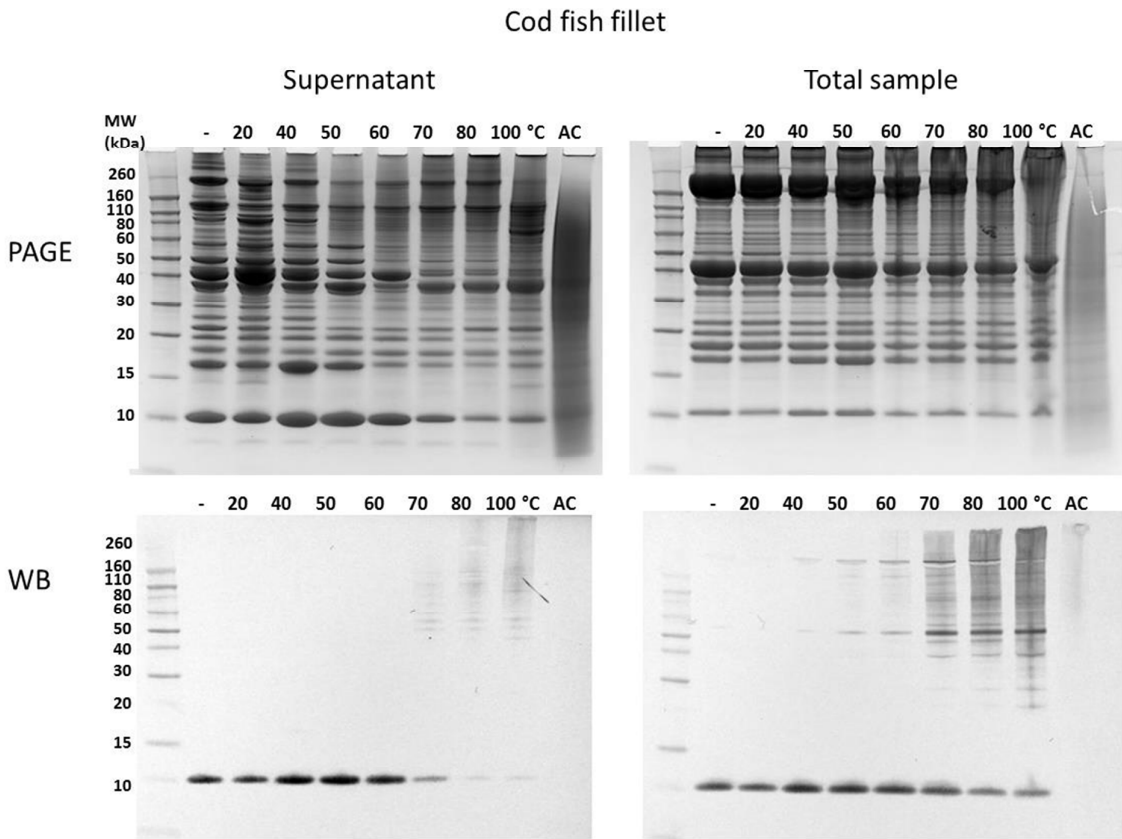
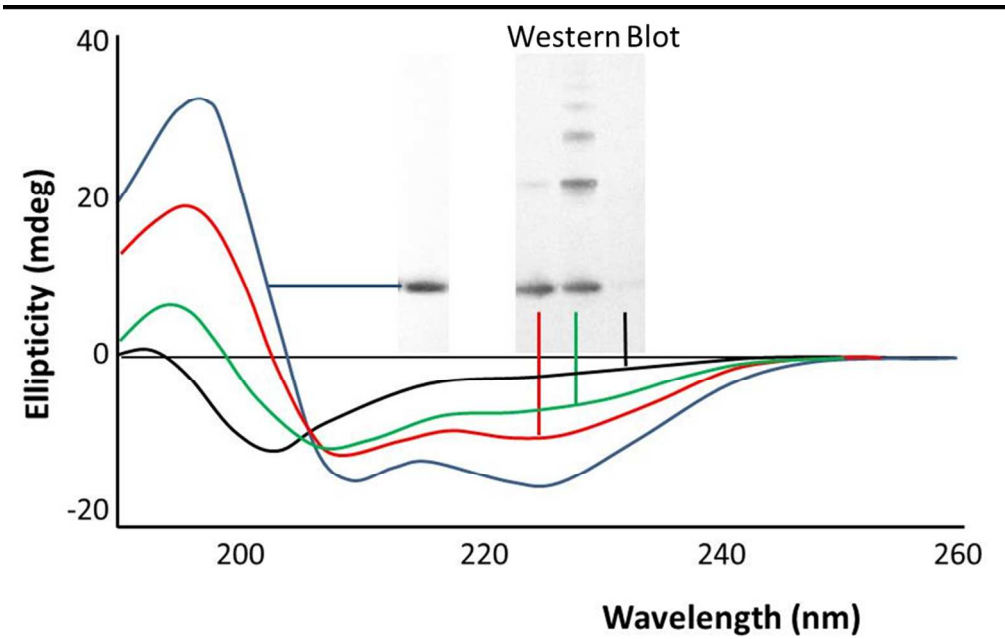


Figure 6

Table 1 Content of parvalbumin extracted from whole cod-fillet incubated for 2 hours at different temperatures as detected by IgE-binding in an ELISA-assay.

	Parvalbumin content in extract ($\mu\text{g/mL}$)
Not heated	388.2
Heated at 50°C	275.4
Heated at 80°C	45.3
Heated at 100°C	29.0
Autoclaved (120 °C)	0.5



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