1 # Comparative digestion of thermally treated vertebrates and

2 # invertebrates allergen pairs in real food matrix

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13 ## Abbreviations

14 **CA**, cooked abalone; **CB**, cooked beef; **CC**, cooked chicken; **CO**, cooked oyster; **CP**, cooked

15 pork; **CS**, cooked shrimp; **Ctrl**, control; **GI**, Gastrointestinal; **GP**, gastric phase; **IP**, intestinal

16 phase; **LSB**, Laemmli sample buffer; **MLC**, myosin light chain; **NR**, non-reducing; **OP**, oral

17 phase; **RA**, raw abalone; **RB**, raw beef; **RC**, raw chicken; **RO**, raw oyster; **RS**, raw shrimp;

18 **SAM**, signal accumulation mode; **SCP**, sarcoplasmic calcium-binding protein; **SGF**, simulated

19 gastric fluid; **SIF**, simulated intestinal fluid; **SSF**, simulated salivary fluid; **TM**, tropomyosin;

20 **TPM2**, Human tropomyosin 2

ABSTRACT

The digestion stability of allergen pairs, tropomyosin, TM (fish and seafood allergen), and myosin light chain, MLC (chicken meat allergen) is compared among vertebrates and invertebrates in raw and cooked food matrix under standardized simulated *in vitro* gastrointestinal (GI) digestion. SDS-PAGE followed by multiple TM and MLC-specific antibodies in semidry WB revealed pepsin resistance of invertebrate TMs (abalone, oyster, shrimp) under diet-relevant conditions (raw, cooked). Vertebrate TMs (chicken, pork, beef) were less stable to digestion except that the raw chicken TM was observed pepsin resistant (not diet-relevant). Vertebrate (chicken) MLC was thermally stable. A new 28 kDa protein bound to anti-MLC antibody in cooked chicken and pork; could be the aggregates of MLC. Raw shrimp MLC showed pepsin resistance among invertebrates. A good correlation was observed between combined resistance of TM and MLC to GI digestion following the diet-relevant thermal treatment and reported protein allergenicity among vertebrates and invertebrates.

Keywords: food matrix; *in vitro* gastrointestinal digestion; meat allergen pair; myosin light chain; thermal treatment; tropomyosin

1. Introduction

Shellfish is listed as one of the 14 most common allergenic foods and food groups in Europe (milk, egg, fish, crustaceans, molluscs, tree nuts, peanuts, soy, wheat, lupine, sesame, mustard, celery, and sulfite (for hypersensitivity reasons)); labelling of these foods is mandatory when used as ingredients (Mazzucchelli et al., 2018). Allergies to crustacean shellfish are very common, but molluscan shellfish allergies do not occur that frequently. However, abalone, a marine gastropod commonly consumed in Asia, is one of the 25 allergenic foods in Japan. Tropomyosin (TM) is a common and major allergen in the disc abalone *Haliotis discus* (Emoto et al., 2009). The Korean Ministry of Food and Drug Safety (MFDS) has established food labels for 21 foods, including shrimp, pork, chicken, beef, and mollusc shellfish (clams, mussels, abalone, and oyster) (Suh et al., 2019). Although many shellfish are consumed raw, they are also frequently consumed in cooked form. Therefore, studying the effects of heating on food protein allergenicity is important for identifying reliable diagnostic markers and appropriate measures for specific immunotherapy. Heating has been shown to induce marked effects on the protein profiles and IgE reactivity of shellfish extracts (Abramovitch et al., 2017; Guang- Ming et al., 2010; Mills et al., 2009; Nowak-Wegrzyn & Fiocchi, 2009).

In addition to TM, the most prevalent and thoroughly studied pan-allergen in shellfish allergy, arginine kinase (AK, Lit v 2), myosin light chain kinase (MLC, Lit v 3), and sarcoplasmic calcium-binding protein (SCP, Lit v 4) have been identified as clinically relevant major shellfish allergens. MLC 1, a skeletal muscle isoform, has been identified and listed as an allergen, Gal d 7, in chicken (allergome.org) (Ayuso et al., 2008; Shiomi et al., 2008). The subunit structure of this vertebrate allergen is identical to that of invertebrate allergens, that is, a hexamer of two heavy chains and

58 four light chains (UniProtKB P02604, allergome.org). This calcium ion-binding muscle protein

59 has been documented as a sensitising agent for shrimp and/or chicken allergies in children and

60 adults. Recombinant Gal d 7 has been produced and used for identifying patients with primary

61 sensitisation to poultry meat (not limited to chicken meat) (Klug et al., 2020). No complete

62 confirmatory assessment has been reported for high-risk allergens or pan-allergens. However, the

63 Food and Agriculture Organization (FAO) and Codex Alimentarius 2013 has proposed resistance

64 to pepsin digestion as a predictor of allergenicity, claiming that this characteristic is shared by

65 most allergens (Commission Codex Alimentarius, 2003; FAO/WHO, 2001). Therefore, simulated

66 digestion has gained significant attention in recent years. Numerous comparative studies have

67 revealed the practicality and pitfalls of simulated *in vitro* digestion in assessing allergens

68 (Akkerdaas et al., 2018; Bøgh & Madsen, 2016; Fu et al., 2002; Gámez et al., 2015; Naegeli et al.,

69 2021). The major pitfall is the exclusion of the real food matrix effect on allergenic protein

70 digestion and the lack of research on digestion-resistant allergen peptide fragments. Ofori-Anti et

71 al. (2008) has suggested that the results could be misleading if the possible effect of the food

72 matrices are not considered; digestibility tests are performed using only the purified protein (Ofori-

73 Anti et al., 2008).

74 Previously, the allergenicity of invertebrate TM in shrimp and clams was compared with that of

75 vertebrate TM in fish. The invertebrate TM triggered stronger elicitation of anaphylaxis in a mouse

76 cell model compared with vertebrate TM, regardless of TM thermal processing (Xu et al., 2020).

77 In a case report on fish-sensitised patients, the authors demonstrated that studying TM on a wider

78 scale is clinically relevant because not only invertebrate TM but also vertebrate TM could elicit

79 clinically relevant reactions in patients with allergy (González-Fernández et al., 2018). These

80 studies further emphasise the need for comprehensive research on structurally and functionally

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81 similar proteins of vertebrates and invertebrates in terms of potential allergenicity and cross-

82 reactivity. The effect of thermal treatment on invertebrate TM has been studied. For example, in

83 the case of boiled, fried, and roasted oysters, TM IgE binding with patient sera was the lowest in

84 roasted and fried samples, higher in boiled samples, and the highest in raw extracts, according to

85 immunoblotting studies (Yadzir et al., 2015). Previous research has also focused on the

86 comparison of structure and biophysical features between allergenic (invertebrate, i.e., shrimp)

87 and non-allergenic (vertebrate, i.e., pig) TM. The allergenicity depended not only on sequence but

88 also on contributions of protein structure and dynamics (James et al., 2018). Additionally,

89 considering the high structural similarity between vertebrate and invertebrate TM, it is enigmatic

90 why invertebrate TMs are allergenic, whereas vertebrate TMs are not (www.allergen.org) (Jenkins

91 et al., 2007; Klueber et al., 2020).

92 Numerous studies on *in vitro* digestion of allergenic proteins followed by appropriate IgE

93 measurement assays do not clarify whether a correlation exists between digestion stability and

94 protein allergenicity. Comprehensive reviews on this topic indicate that more than half *in vitro*

95 digested allergenic proteins are not digestion-resistant; the predictability of distinguishing

96 allergenic from non-allergenic protein pairs is not enhanced by sub-optimal pH, low pepsin-to-

97 protein ratio, and resistance to peptic and pancreatic digestion (Akkerdaas et al., 2018; Bøgh &

98 Madsen, 2016). The *in vitro* digestion stability test alone is not a definitive assessment. For

99 improving digestibility testing strategies, physiologically relevant conditions need to be used,

100 taking the food matrix into consideration (Brodkorb et al., 2019). Therefore, the objective of our

101 study was to compare the digestion stability and patterns of homologous allergen pairs (TM, a

102 major allergen of invertebrates, and MLC, a newly established chicken meat allergen) among

103 vertebrates and invertebrates in raw and cooked food matrices under standardised simulated *in*

vitro gastrointestinal (GI) digestion. As a novel approach in our work, we considered the food matrix and used diet-relevant treatments (either raw or cooked) to observe the digestibility of these TM and MLC pairs via specific antibody-based western blotting (WB).

2. Experimental section

All chemicals and enzymes were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared in ultra-pure water filtered through 0.20 μm capsule filter (Arioso water purification system, Human Corporation, Korea).

2.1 Thermal treatment of samples

Three raw invertebrates, abalone (RA), oyster (RO), and shrimp (RS), were collected from the fish market and transferred to the laboratory in an ice cooler. Raw vertebrate samples, chicken (RC), pork, and beef (RB), were collected from a local fresh meat shop and transported to the laboratory in a Styrofoam box with an icepack. The raw samples were subsequently stored at −20 °C until further use. The cooked samples were subjected to diet-relevant thermal treatment, as described in Table S1. Briefly, the invertebrates were thawed and cooked (CA, CO, and CS) by immersion in boiling water for 5 min along with the shell. The vertebrates were cooked (CC, CP, and CB) in water at 180 °C for 30–90 min until the internal temperature reached 73 °C–75 °C, which ensured a safe minimum internal temperature (*Safe Minimum Internal Temperature Chart | Food Safety and Inspection Service*, n.d.).

2.2 Standardized static *in vitro* simulation of GI digestion

Raw and cooked vertebrate and invertebrate samples were digested under simulated conditions developed by the COST INFOGEST network and revised by Brodkorb et al. (2019) with slight

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125 modifications, as described by Khulal et al. (2021). The salivary enzyme amylase was not added

126 during the oral phase (OP) of digestion; the volume was replaced with MilliQ water. The details

127 of the method, including the concentrations of stock solutions of simulated salivary fluid (SSF),

128 simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), are provided in the Supporting

129 Information. In addition to the raw and cooked samples, control/stability sample tubes (Ctrl) were

130 prepared for evaluating food stability during exposure to simulated digestive fluids without

131 enzymes, simultaneously with the gastric phase (GP) and the intestinal phase (IP). In the enzyme-

132 blank tubes, the digestion tube contained quartz sand instead of the food sample with ①pepsin at

133 120 min time (P120') and ②trypsin and chymotrypsin at 120 min time with bile (T120').

134 After digestion, the digesta at the GP and IP endpoints were centrifuged at 11400 × g for 25 min

135 (Labogene mini microcentrifuge, Bio-Medical Science Co., Ltd., Seoul, Korea) to separate the

136 supernatant from the pellets. The total soluble protein content in the digesta supernatant was

137 determined by conducting a bicinchoninic acid (BCA) assay in triplicate with bovine serum

138 albumin (BSA) as the standard and characterised using sodium dodecyl sulfate-polyacrylamide gel

139 electrophoresis (SDS-PAGE). The digestibility experiments were repeated twice with the

140 biological replicate samples, each consisting of two technical replicates.

141 **2.3 SDS-PAGE**

142 All digesta supernatant samples were mixed well with Laemmli sample buffer (LSB) (1610747,

143 Bio-Rad Laboratories, Hercules, CA, USA) for non-reducing SDS-PAGE and β-mercaptoethanol

144 with LSB for reducing SDS-PAGE to achieve a final protein concentration of 1 μg/μL. The

145 samples were denatured by heating at 95 °C for 5 min at 400 rpm on a thermoshaker (Thermo

146 Fischer Scientific, Waltham, MA, USA) and cooled to room temperature (25 °C–27 °C);

147 subsequently, 20 μg of total protein was loaded per well and resolved on 4–20 % precast gels

148   (Mini-PROTEAN TGX, Any kD, 30 µL, Bio-Rad Laboratories, Hercules, CA, USA). Pre-stained

149   standards (1610376, Bio-Rad Precision Plus Protein Dual Xtra Standard, 2-250 kDa, Bio-Rad

150   Laboratories, Hercules, CA, USA) were used as molecular weight markers. Proteins were resolved

151   at 200 V for 30 min using a Bio-Rad Mini-PROTEAN tetra system and stained with Coomassie

152   brilliant blue-R250 (CBBR), followed by destaining. Gel visualisation, image export, and protein

153   band semi-quantification were performed using a gel documentation unit: Chemidoc XRS+ and

154   Image lab software (version 6.0; Bio-Rad Laboratories, Hercules, CA, USA).

2.4 Allergen-specific antibody based WB

156   SDS-PAGE stain-free gels (Mini-PROTEAN TGX, stain-free gel, any kD, 30 µL Bio-Rad

157   Laboratories, Hercules, CA, USA) were used for WB, with the pre-stained Western C standard

158   (10–250 kDa) as a protein marker (1610376, 250 µL, Bio-Rad Laboratories, Hercules, CA, USA).

159   The samples were resolved under reducing conditions and transferred to 0.20 µm polyvinylidene

160   fluoride (PVDF) membranes (Trans-Blot turbo mini 0.2 µm PVDF transfer packs, Bio-Rad

161   Laboratories, Hercules, CA, USA). PVDF membranes were blocked for 2 h with 3 % BSA in 1×

162   TBST (20 mM Tris, 150 mM NaCl, 0.1 % Tween 20) and washed with 1× TBST three times for

163   10 min each (membrane washing kept the same between incubations) prior to probing with the

164   respective primary antibodies: rabbit anti-shrimp TM IgG pAb at 1:1000 (PA-SHM, Indoor

165   Biotechnologies, Charlottesville, VA, USA), rabbit anti-human IgG TPM2 pAb at 1:1000 (LS-

166   C81187, LifeSpan BioSciences, Seattle, WA, USA), mouse anti-chicken MLC IgM isotype mAb

167   (M4401, Sigma Aldrich, St. Louis, MO, USA) at 1:500 dilution or rabbit anti-human MLC2

168   isoform pAb (3672S, Cell Signalling Technology, Danvers, MA, USA) at 1:800 dilution prepared

169   in 1 % BSA in 1× TBST for 1 h 30 min at room temperature (25 °C–27 °C). The blots were

170   incubated for 1 h 30 min at RT with the secondary antibodies, goat anti-rabbit IgG-AP pAb

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171 (A3687, Merck, Darmstadt, Germany) at 1:15000 or goat anti-mouse IgG-AP pAb (A3562, Merck,

172 Darmstadt, Germany) at 1:1000 dilution in 1 % BSA in TBST, followed by TBST/TBS washing.

173 They were developed using a chemiluminescent substrate (1705018, Immune-Star AP substrate,

174 Bio-Rad Laboratories, Hercules, CA, USA) in the dark by immersing the membrane in the

175 substrate for 5 min. Precision protein StrepTactin-AP Conjugate (1610382, 300 µL, Bio-Rad

176 Laboratories, Hercules, CA, USA) was used at a 1:5000 dilution during secondary antibody

177 incubation for visualising the protein marker. Table S2 presents information on the antibodies,

178 along with a list of cross-reactive specificities. The final best image captured with Chemidoc XRS+

179 was selected among the set number of images obtained under signal accumulation mode (SAM)

180 within the selected exposure time range from 5 s to 300 s.

181 **2.5 Statistical analysis**

182 Grouped graph charts of duplicate data and their means and standard deviations (error bars shown)

183 were created using GraphPad Prism 9.0.1 (GraphPad, San Diego, CA, USA). Two-way ANOVA

184 and Dunnett's multiple comparison test were performed at a 95 % confidence level.

185 **3. Results**

186 **3.1 SDS-PAGE and TM-specific antibody-based WB of raw and thermally treated digesta**

187 [Here for Figure 1]

188 Figure 1 presents the theoretical Mw of the allergen pair TM and MLC and their allergenicity

189 based on data available until date from allergen databases. Additionally, the shrimp allergen TM

190 shares 54.58 % sequence identity with non-allergenic chicken TM and 55.99 % sequence identity

191 with pork and beef TM, according to multiple sequence alignment on the UniProt database. The

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192   allergen chicken MLC shares less than 50 % sequence identity with the minor allergen MLC in

193   invertebrates (i.e. 20.67 % with shrimp MLC, 30.67 % with oyster MLC, and 29.53 % with abalone

194   MLC) (uniprot.org; detailed matrix is provided in the Supporting Information).

195   We compared the effect of thermal treatment and simulated GI digestion on our proteins of interest

196   at ≈37 kDa and ≈20 kDa in invertebrates and vertebrates in a real food matrix by conducting

197   reducing and non-reducing (NR) SDS-PAGE followed by WB.

198   [Here for Figure 2A and 2B]

199   SDS-PAGE revealed a slightly different protein profile based on the SDS sample treatment

200   (reducing vs. NR), exclusively in terms of band intensity. In the case of both invertebrates (Figure

201   2A) and vertebrates (Figure 2B), the reducing SDS-PAGE gels demonstrated sharp and intense

202   ≈37 kDa (red boxes) and ≈20 kDa bands (yellow boxes) corresponding to the monomeric TM and

203   MLC. The NR SDS-PAGE gels showed distinctive higher Mw bands corresponding to TM

204   disulphide-linked dimers (≈75 kDa) in control lanes of RA and CA (Figure 2A), RC, CC, CP, RB,

205   and CB (Figure 2B). TM disulphide-linked dimers (≈75 kDa) was not observed in shrimp. Thermal

206   treatment did not impair the TM extraction as it is thermostable, but MLC extraction was reduced

207   in cooked shrimp (CS). No effect of thermal treatment on the TM and MLC was observed in terms

208   of enhanced aggregation.

209   To determine whether the highlighted protein of interest based on their Mw as determined using

210   SDS-PAGE is indeed TM and MLC, we subsequently performed specific antibody-based WB.

211   Considering that the objective of our study was to observe how the tested allergenic proteins

212   degrade as their digestion peptides appear, reducing SDS-PAGE is more appropriate than NR

213   SDS-PAGE.

3.1.1 TM-specific antibody-based WB of raw and thermally treated digesta

3.1.1.1 Effect of thermal treatment

Reducing SDS-PAGE revealed a prominent ≈37 kDa band separated in both RA ctrl and CA ctrl (Figure 2A). Abalone, when cooked and separated in SDS gel (CA), revealed additional smaller (<15 kDa) and heavier (≈150 kDa) protein bands in contrast to its raw counterpart, RA. This 37 kDa band was confirmed to be TM upon recognition by the anti-shrimp TM pAb (Figure 3).

[Here for Figure 3]

Abalone TM, including the 75 kDa band, showed thermal resistance, since these can be observed in both the RA and CA control lanes of the blot (Figure 3A). The 25 kDa and 20 kDa bands were of lower intensity in CA than in RA. In the case of oysters, a smeared ≈37 kDa band was observed on the gels, which was comparatively more intense in the CO control lane than in the RO control lane. The blot in Figure 3(A-D) confirms the oyster TM reactivity with respect to shrimp TM pAb, where, instead of a specific sharp TM band at 37 kDa, the smeared lane above 37 kDa represents the bound TM; smearing is visible in both the RO and CO control lanes, indicating its thermal resistance. Additionally, smaller proteins (<15 kDa) were observed in SDS-PAGE gels of the RO ctrl but not in the CO ctrl, suggesting that the smaller protein might be affected by thermal treatment. In the case of shrimp, TM appeared intact upon thermal treatment as it persisted in both RS and CS control lanes (Figure 2A), as confirmed by the blot (Figure 3B) showing the strong staining of the TM band by the anti-shrimp TM pAb. However, the ≈20 kDa band appeared to have slightly lost its intensity in the CS lane than in the RS lane, suggesting a mild degrading effect of thermal treatment on the ≈20 kDa shrimp protein. Collectively, our results indicated that under

235 reducing conditions, invertebrate TM staining was the most intense in shrimp and least visible in

236 oysters at 37 kDa.

237 Although vertebrate TM was theoretically determined to be ≈32 kDa (Figure 1), the 37 kDa band

238 was recognised as TM in vertebrates by both anti-shrimp TM pAb and anti-human TPM2 pAb

239 (validated for WB and claimed reactivity to chicken and bovine) (Figure 4). SDS-PAGE clearly

240 illustrates a single intense ≈37 kDa band in RC ctrl that fades in the CC ctrl (Figure 2B). Two

241 closely separated bands at ≈37 kDa in the case of pork, CP ctrl, beef, RB, and CB ctrl (Figure 2B)

242 could easily be inferred as TM isomers, mildly resistant to diet-related thermal treatment, as

243 confirmed by the blot against anti-shrimp TM pAb (Figure 3C, D). However, the blots also

244 revealed the reactivity of shrimp TM pAb to a single vertebrate TM band rather than the

245 aforementioned double bands. These blots provided significant insights into the shared allergen

246 epitopes among these sets of invertebrates and vertebrates in terms of TM. For clarity and

247 confirmation, Figure 4 shows the antibody binding pattern to vertebrate TM using an anti-human

248 TPM2 antibody. This additionally suggests the mild resistance of vertebrate TM to thermal

249 treatment. Furthermore, the chicken protein of ≈100 kDa appeared to be thermally unstable, as

250 observed in the RC control lane but not in the CC control lane. Notably, heavier proteins (> 250

251 kDa) were exclusively observed in CP ctrl. Overall, vertebrate TM was significantly degraded

252 upon thermal treatment.

253 *3.1.1.2 Effect of simulated in vitro gastrointestinal (GI) digestion*

254 Upon comparing the effect of GI digestion on invertebrate TM in real food matrix using SDS-

255 PAGE (Figure 2A), the ≈37 kDa band was shown to persist in the GP digesta lanes of all

256 invertebrates; the bands for RA, CA, RO, and CO showed lower intensity than the bands in their

12

257   respective control lanes, whereas the bands for RS and CS lanes exhibited relatively higher

258   intensity. Furthermore, according to the results of the blotting (Figure 3 (A, B)), the bound TM in

259   their GP lanes confirmed that the invertebrate TM was not completely digested by pepsin. The

260   relative quantity histograms representing semi-quantitative densitometric data for duplicate

261   analysis (Figure 3E) reinforced the observation that all invertebrate TM resist peptic digestion.

262   The raw shrimp TM at the GP showed the highest relative quantity of 0.81 ($\approx$81 %) with respect

263   to its control, which declined to 0.46 ($\approx$46 %) after thermal treatment, that is, CS GP. The abalone

264   TM displayed a similar pattern; RA GP exhibited a relative quantity of 0.44 ($\approx$44 %), whereas after

265   thermal treatment, CA GP showed a decline to 0.25 ($\approx$25 %). The oyster TM, however, appeared

266   to be undigested even after thermal treatment according to the densitometric data, and both RO

267   and CO GP showed a relative quantity of 0.47 and 0.46, respectively. However, TM was

268   completely digested by the intestinal enzymes trypsin and chymotrypsin in abalone and raw

269   oysters. The persistent faint band recognition in the RS IP lane (Figure 3B) at a relative quantity

270   of 0.20 ($\approx$20 %) (Figure 3E) might indicate that raw TM could be trypsin resistant, in contrast to

271   thermally treated shrimp TM, which was completely digested. In addition, the thermally stable

272    $\approx$75 kDa protein of RA, CA, RO, and CO ctrl recognised by the anti-shrimp TM pAb was no longer

273   observed in the GP and IP lanes. This $\approx$75 kDa protein was readily digested by pepsin. Similarly,

274   the higher Mw proteins (>37 kDa) in the RS and CS control lanes recognised by the pAb showed

275   thermal stability; however, they were digested by pepsin, trypsin, and chymotrypsin.

276   Upon comparing the effect of GI digestion on vertebrate TM in a real food matrix, when probed

277   with anti-shrimp TM pAb, raw chicken TM (RC Ctrl, Figure 3C) exhibited the strongest reactivity

278   to shrimp TM pAb, followed by weak staining of the band when digested by pepsin (CC GP lane).

279   The densitometric semi-quantitative relative quantity histogram (Figure 3E) confirmed the TM at

280   RC GP at 0.11 (≈11 %). Although cooked chicken, pork, and beef TM showed mild resistance to

281   thermal treatment, their TM does not persist in the GP and IP of GI digestion. Pork and beef digesta

282   TM did not show any reactivity to shrimp TM. Therefore, to further study the TM of vertebrates,

283   another set of WB experiments were conducted with vertebrate specific anti-TPM2 pAb for

284   invertebrate and vertebrate samples.

285   [Here for Figure 4]

286   This anti-human TPM2 pAb did not show any specific binding to invertebrate TM (Figure S2) on

287   raw nor thermally treated digesta. Hence, the relative quantity histogram in Figure 3F does not

288   indicate the quantity for the invertebrate group. The vertebrate TM reacted with the anti-human

289   TPM2 pAb (Figure 4), on the basis of which the raw chicken TM band was observed in the GP

290   lane, and trace amounts remained in the IP lane. The relative quantities of RC TM at GP and IP

291   based on densitometric data were 0.74 and 0.23, respectively (Figure 3F). Intense band reactivity

292   was observed in thermally treated chicken, pork, and beef (CC ctrl, CP ctrl, and CB ctrl),

293   reinforcing their thermal resistance, as was observed for the anti-shrimp TM pAb. Pepsin

294   significantly digested TM in cooked chicken (CC GP, Figure 4) with a relative quantity of 0.05

295   (≈5 %) (Figure 3F). Additionally, thermally treated pork TM, recognised by vertebrate TPM2,

296   resisted pepsin digestion (CP GP lane with a relative quantity of 0.27); however, it was completely

297   digested by trypsin and chymotrypsin in SIF (CP IP lane). In the case of raw beef, the vertebrate

298   TM reactivity suggested resistance to GI digestion (RB GP and RB IP lanes with relative quantities

299   of 0.51 and 0.19, respectively). Thermally treated beef was almost completely digested by the GP

300   endpoint (faint binding in the CB GP lane of 0.08 relative quantity). Additionally, in pork and beef,

301   a thermally resistant 75 kDa band was observed, which appeared to be easily digested by intestinal

14

302 enzymes (Figure 4). The application of this anti-human TPM2 pAb WB revealed supplemental

303 vertebrate TM behaviour upon thermal treatment and GI digestion, specifically in pork and beef.

304 Our results emphasise that TM, a strong shellfish allergen, is strongly or mildly but surely heat-

305 and pepsin-resistant. In contrast, the vertebrate TM reactive to shrimp TM antibody could be

306 partially heat resistant but readily digested by pepsin in human GP, except in raw chicken (raw

307 chicken is not diet relevant in real life).

3.2 MLC-specific antibody-based WB of raw and thermally treated digesta

309 The MLC bands in all invertebrates with theoretical Mw of ≈18–20 kDa and vertebrates with ≈21–

310 22 kDa (Figure 1) are visible in the ctrl lanes of raw and cooked samples (RA, CA, RO, CO, RS,

311 CS, RC, CC, CP, RB, and CB) in the SDS PAGE electropherogram in Figure 2A and Figure 2B.

312 Figure 5 (A-D) shows the results for MLC antibody-based semi-dry WB of invertebrate samples

313 against i) rabbit anti-human MLC2 IgG isoform pAb (Figure 5A, 5B) and the vertebrate samples

314 against ii) mouse anti-chicken MLC IgM isotype mAb (Figure 5C, 5D). Two primary MLC

315 antibodies were used for determining differences in MLC recognition and intensity. MLC

316 recognition was more prominent in vertebrate samples than in invertebrate samples. Figure 5A

317 does not show specific binding to bands corresponding to 18–20 kDa for RA, CA, RO, and CO. A

318 distinct 20 kDa band was recognised in the RS control lane by anti-human MLC2 pAb (Figure

319 5B), which showed a reduced intensity in the GP (relative quantity of 0.29, ≈29 %) in relation to

320 its ctrl (Figure 5E). This result suggests that raw shrimp MLC2 appears to be mildly pepsin-

321 resistant, possibly along with the smaller fragment of approximately 14 kDa protein (RS GP lane).

322 However, lack of further binding with the cooked shrimp indicated that this myosin protein band

323 might not be thermally resistant. This MLC2 isoform pAb did not react with vertebrate protein

324 bands between 18–25 kDa. However, a higher Mw protein recognition was observed (results not

325 shown).

326 [Here for Figure 5]

327 Upon comparing the effects of thermal treatment and/or GI digestion on vertebrate MLC2 in a real

328 food matrix, when probed with anti-chicken MLC mAb, intense MLC band recognition was

329 observed below the theoretical Mw of 22 kDa (Figure 1) in the case of raw chicken (RC Ctrl,

330 Figure 4C); this protein was not completely degraded by pepsin digestion (faint band remains in

331 RC GP) but was completely digested in the IP. Although this commercially available MLC IgM

332 isotype mAb derived from mouse cells using chicken lens membrane as the immunogen was

333 claimed to be the most reactive with chicken gizzard, intestine, and pig stomach MLC at 20 kDa

334 protein, it did not specifically recognize the 20 kDa band in chicken nor pork samples. The chicken

335 23 kDa MLC is a recently discovered and IUIS-listed major meat allergen for patients primarily

336 sensitised to chicken meat (allergen.org) (Klug et al., 2020). An additional ≈75 kDa and slightly

337 higher Mw proteins were also recognised in the case of RC ctrl, which strongly persisted in GP.

338 The 3 bands between 18–25 kDa reacts with this MLC mAb in the case of cooked chicken (CC

339 ctrl) that faded away completely upon GI digestion; however, a single band appeared to bind with

340 the MLC antibody in CC GP at approximately 28 kDa, indicating pepsin-resistant protein. Similar

341 to chicken, cooked pork exhibited a single 20 kDa protein band that disappears upon pepsin

342 digestion (CP GP). Upon pepsin digestion, CP showed a protein band (≈14 kDa) with an additional

343 band of ≈28 kDa. Interesting binding patterns were observed in the case of beef (red meat). In

344 addition to the ≈18 kDa band, the ≈24 kDa band bound to the MLC mAb in the case of raw beef

345 (RB Ctrl); however, between the two, only the ≈24 kDa protein was mildly pepsin-resistant (RB

16

346   GP). Both protein bands were thermally resistant (CB Ctrl), as indicated by strong mAb binding.

347   Two additional pepsin-resistant bands were observed at ≈65 and 74 kDa in raw beef; however,

348   thermally treated beef MLC was not pepsin-resistant. MLC was also completely digested by

349   trypsin and chymotrypsin. According to our results, thermal treatment appeared to alter the MLC

350   protein profile in vertebrates, since the number and Mw of recognised MLC bands (18–25 kDa) in

351   RC ctrl and RB ctrl differed from those in the thermally treated CC ctrl and CB ctrl. The MLC

352   protein band recognised at ≈28 kDa in the GP of chicken and pork appeared to be resistant to both

353   thermal treatment and peptic digestion. This anti-chicken MLC mAb did not show any specific

354   reactivity with the MLC of invertebrate samples (blots not shown).

4. Discussion

356   In this study, raw and thermally treated invertebrate and vertebrate food sources were subjected to

357   an *in vitro* static INFOGEST protocol that mimicked protein digestion in the human GI tract under

358   simulated physiological conditions (Brodkorb et al., 2019). We aimed to understand the influence

359   of a real food matrix and diet-relevant thermal treatment on the digestion stability of TM and

360   MLC2 pairs via WB with specific antibodies. We detected TM at 37 kDa by SDS-PAGE and

361   confirmed it by WB with anti-shrimp TM pAb; although multiple bands were observed, we

362   focused on only the 37 kDa band for comparing the thermal and GI digestion stability among the

363   samples. For MLC, chicken MLC1 at 23 kDa, as listed in the IUIS nomenclature as a meat allergen

364   (Klug et al., 2020), was used for comparison. In our study, the recognised Mw of both TM and

365   MLC2 from all the samples on SDS-PAGE electropherograms and blots varied from the predicted

366   Mw from the allergen databases. This phenomenon could be due to multiple factors such as protein

367   mobility in the gel, thermal treatment-induced fragmentation, cross-linking, aggregation, protein

368   solubility, and denaturation due to enzymatic digestion.

369   Invertebrate TM is a well-known major shrimp allergen that is cross-reactive between crustaceans

370   and molluscs (González-Fernández et al., 2018, 2017; James et al., 2018; James & Nanda, 2020;

371   Kamemura et al., 2019; Klueber et al., 2020; Liu et al., 2013; L. Xu et al., 2020; L. L. Xu et al.,

372   2020). In the present study, we investigated the stability of combined thermal and GI digestion in

373   a real food matrix and its correlation with their established allergenicity. Allergens sensitising via

374   the GI tract must hypothetically resist GI digestion, and this resistance might rely not only on their

375   intrinsic properties but also on the food matrix (Pekar et al., 2018) Therefore, we chose to study

376   TM and MLC2 in their matrix instead of their purified extracts. Another reason for this objective

377   was that the effect of the food matrix could lead to decreased protein digestibility in the stomach

378   and preservation of allergenic epitopes for interactions with the immune system in the intestine

379   (Nowak-Wegrzyn & Fiocchi, 2009).

380   TM from abalone, oyster, and shrimp showed antibody recognition even after diet-relevant thermal

381   treatment and peptic digestion at the GP, confirming its conserved thermal stability, as well as

382   resistance against simulated gastric digestion. In contrast, vertebrate TM (recognised by shrimp

383   anti-TM pAb) from chicken, pork, and beef in their matrix appeared to be partially heat resistant;

384   however, they were readily digested by pepsin in GP, except for the trivial faint band in raw

385   chicken GP (raw chicken is not diet relevant in real life). All TMs, except that from raw shrimp,

386   were completely digested in the IP of the simulated GI digestion (Figure 2A, 2B, Figure 3). Till

387   date, two theories have been proposed: one, that food allergens are more resistant to digestion than

388   unproven allergens, weak allergens, or non-allergenic proteins and, the other, that food allergens

389 are not essentially digestion-resistant compared to non-allergenic proteins, thus concluding that *in*

390 *vitro* digestibility and protein allergenicity are independent parameters (Akkerdaas et al., 2018;

391 Apostolovic et al., 2016; Bøgh & Madsen, 2016; Fu et al., 2002; Gámez et al., 2015). Our results

392 regarding TM suggest that while invertebrate TM with proven strong allergenicity is essentially

393 resistant to gastric digestion (resistant to even intestinal digestion in the case of raw shrimp), their

394 unproven, weak, or non-allergenic vertebrate TM counterparts are not resistant to peptic digestion.

395 Both invertebrate and vertebrate TMs were thermally stable; vertebrate TMs were mildly resistant

396 to thermal treatment compared to invertebrate TMs (considering that all samples were similarly

397 thermally treated by boiling). Therefore, in our study, resistance to thermal treatment could not

398 serve as a differentiating parameter between allergen and weak/non-allergenic TM. However, RC

399 TM resisted gastric digestion compared to CC TM, suggesting that the combination of thermal

400 treatment and peptic digestion could degrade TM, lowering its allergenicity. This could

401 additionally support the results of Klueber et al. (Klueber et al., 2020), who explained that chicken

402 TM, although bound to IgE from patients allergic to shrimp, did not show positive skin reactivity.

403 In contrast, fish (vertebrate) TM cannot be ignored, since fish species containing salmon, catfish,

404 and tilapia have been listed as food allergens by the WHO/IUIS Allergen Nomenclature Sub-

405 committee. Currently, the clinical relevance of fish TM as an allergen is unknown because of its

406 high homology with human TM, and self-protein autoreactivity might be the reason for allergic

407 reactions as well (Keshavarz et al., 2020). Nevertheless, future research on comparative

408 digestibility, including studies on fish TM, may add valuable data to our current research.

409 MLC, a minor shrimp allergen (MLC2 isoform), was recently identified as a major chicken meat

410 allergen (MLC1) that forms a myosin protein complex with its heavy chains (Klug et al., 2020).

411 Allergenic MLC isoforms differ between MLC1 and MLC2 in different geographical locations

412   worldwide (Yang et al., 2020). Therefore, in our study, we investigated MLC2 between 18–22

413   kDa in invertebrates and vertebrates.

414   In invertebrates, only the raw shrimp recognised MLC2 ab, which resisted peptic digestion;

415   however, it was thermally denatured. Interestingly, since MLC has been recognised as a minor

416   allergen in shrimp (Figure 1, allergen.org), this result additionally suggests that the allergen MLC

417   must survive gastric digestion.

418   The vertebrate MLC mAb bound to multiple vertebrate protein bands in chicken, pork, and beef,

419   despite the monoclonal specificity (no non-specific binding due to the secondary antibody, Figure

420   S2). This could be attributed to the presence of similar linear epitopes on the proteins that could

421   be recognised by the MLC mAb. Chicken, pork, and beef MLC2 ($\approx$20 kDa) are thermally stable;

422   however, the visible differences in the bound protein bands suggest that this could be the effect of

423   the thermal treatment. Notably, the MLC from all samples was easily digested by pepsin, and in

424   all cases, protein breakdown appeared to occur through a common intermediate, labelled here as

425   the $\approx$14 kDa fragment (Figure 5). A similar case regarding the 13 kDa band revealed that a variant

426   of the myosin complex in cooked chicken strongly exhibited IgE reactivity to patient sera with

427   poultry meat allergy (Klug et al., 2020). Studies on the behaviour of chicken breast MLC protein

428   under thermal treatment demonstrated that the light chains detach from the myosin complex and

429   solubilise; heavy chains, in contrast, degrade and aggregate. As a result, light chains are more

430   likely to sensitise vulnerable individuals, considering their solubility (Klug et al., 2020; Smyth et

431   al., 1996). Similarly, a study on beef MLC heat denaturation revealed that pH largely influences

432   its unfolding and aggregability. In addition, the heavy chain and light chain myosin isoforms,

433   regardless of species, unfold and aggregate in diverse patterns (Vega-Warner & Smith, 2001).

434   Accordingly, in our results (Figure 5), we have speculated that the ≈28 kDa band recognized by

435   MLC mAb in cooked vertebrate gastric digesta (CC, CP in GP lanes) could most likely be MLC

436   aggregates formed under the thermal treatment and GI digestion.

437   Apart from the known allergens TM and MLC, abalone (RA and CA) proteins of approximately

438   50 kDa and oyster (RO and CO) proteins of approximately 75 kDa were visible in IP lanes under

439   reducing conditions (Figure 2A, 2B). The 75 kDa band recognized by the anti-shrimp TM pAb

440   could be the TM dimer, whereas the 49 kDa IgE-binding protein in abalone was discovered and

441   designated as Hal m 1 allergen on the basis of WB against an abalone-sensitive patient's sera,

442   which was found to be extremely heat stable (Lopata et al., 1997). Similarly, a 100 kDa allergen

443   was detected in the disc abalone, *H. discus*, and identified as paramyosin on the basis of the

444   determined amino acid sequences of the peptide fragments. The cross-reactivity between

445   paramyosin and TM was demonstrated by inhibition immunoblotting and ELISA (Suzuki et al.,

446   2011).

447   While the allergenicity of these allergenic proteins can only be confirmed after their divalent

448   reactivity to IgE antibodies on the mast cell surface or basophils, their digestibility pattern and

449   stability is a vital preliminary step before tests such as histamine release tests and skin prick tests.

450   WB, despite being useful molecular assay, has the limitation of not allowing the detection of small

451   protein fragments. These low-Mw fragments could be digestion-resistant peptides that still contain

452   IgE-binding allergenic epitopes. To further confirm these possibilities, proteomics-based

453   approaches, such as LC-MS/MS, are vital, and they must be considered in future research, since

454   the LC-MS/MS approach by James et al. revealed that shrimp TM exhibited a greater stability of

455   fragments overlapping with IgE epitopes than pork TM despite similar SDS-PAGE profiles (James

456 et al., 2018). Additionally, human digestion varies greatly among individuals, along with other

457 parameters such as gastric pH, bile, and fluid composition, thus allowing or suppressing food

458 allergenic protein digestion in the gut and leading to subsequent absorption and sensitisation. The

459 best application of the simulated static digestion of proteins is to obtain relative and comparative

460 results. Such information on allergenic protein digestion behaviour in any species can be pivotal

461 for the development of rapid and accurate allergy tests and, therefore, could contribute to effective

462 patient management. The implementation of simulated digestion followed by the specific

463 antibody-based WB in case of known allergens in lieu of processing techniques can thus facilitate

464 the collection of vital information (cross-reactivity, stability, binding-site availability, and

465 allergenic protein post-translational modifications) required for taking reasonable further steps

466 towards ground-breaking strategies for the management and/or assessment of patients with allergy.

Conclusions

468 Standardised simulated *in vitro* digestion of raw and thermally treated invertebrate and vertebrate

469 samples showed a good correlation between the combined resistance of TM and MLC to digestion

470 following thermal treatment and allergenicity among vertebrates and invertebrates. The resolved

471 TM and MLC proteins, among other non/unproven allergenic proteins, were clearly identified by

472 WB using multiple specific pAbs and mAbs. Under thermal treatments that mimic human eating

473 habits, invertebrate TMs were resistant to gastric digestion in their matrix, whereas vertebrate TMs

474 were not stable, degrading during the gastric phase, except in raw chicken (irrelevant diet

475 condition). Vertebrate (Chicken) MLC, recognised as a major allergen, was thermally stable. A

476 single ≈28 kDa protein appeared and bound to the MLC antibody in cooked chicken and pork,

477 probably because of the MLC aggregates resulting from thermal treatment in combination with GI

478 digestion. Among the invertebrate samples, only raw shrimp MLC showed pepsin resistance.

479 Therefore, digestibility studies on food allergens and novel foods should include testing solid food

480 digestion in relation to all diet-related thermal treatments for gaining more profound insights into

481 allergen behaviour in the GI tract.

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Conflict of interest statement

490 Authors declare there is no conflict.

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Figure Legends

Figure 1. WHO/IUIS recognized major (invertebrates) and unrecognized/minor or non-allergen (vertebrates) TM pair ; major (vertebrates) and unrecognized/minor or non-allergen (invertebrates) MLC pair along with their molecular weight (source: allergen.org, allergome.org, uniprot.org).

Figure 2A. SDS-PAGE electropherogram of the thermally treated and digested invertebrate samples under NR condition and reducing condition. Red arrow (→) and red boxes (▢) represents monomeric TM band at around 37 kDa, whereas oval red box (○) represents TM disulphide-linked dimers. Yellow arrow (→) and yellow box (▭) shows the 18-20 kDa band (protein of interest). Mw: molecular weight of standard protein, Std: precision plus dual xtra standard (2-250 kDa), TM: Natural shrimp tropomyosin standard, P120': pepsin enzyme control without food matrix after 120', T120': trypsin and chymotrypsin control without food matrix after 120', Ctrl: supernatant without enzymes but just the digestion fluids , GP: supernatant with the pepsin inhibitor at the end of gastric phase, IP: supernatant with protease inhibitor collected at the end of intestinal digestion. RA: Raw Abalone, CA: Cooked Abalone, RO: Raw oyster, CO: Cooked oyster, RS: Raw shrimp, CS: Cooked shrimp.

Figure 2B. SDS-PAGE electropherogram of the thermally treated and digested vertebrate samples under non-reducing condition and reducing condition. Red arrow (→) and red boxes (▭) represents monomeric TM band at around 37 kDa, whereas oval red box (○) represents TM disulphide-linked dimers. Yellow arrow (→) and yellow box (▭) shows the 18-20 kDa band (protein of interest). Mw: molecular weight of standard protein, Std: precision plus dual xtra standard (2-250 kDa), TM: Natural shrimp tropomyosin standard, P120': pepsin enzyme control without food matrix after 120', T120': trypsin and chymotrypsin control without food matrix after

665   120', Ctrl: supernatant without enzymes but just the digestion fluids , GP: supernatant with the

666   pepsin inhibitor at the end of gastric phase, IP: supernatant with protease inhibitor collected at the

667   end of intestinal digestion. RC: Raw Chicken, CC: Cooked chicken, CP: Cooked pork, RB: Raw

668   Beef, CB: Cooked beef.

669   **Figure 3.** TM specific antibody-based WB of raw and thermally treated digesta. PVDF blots of

670   raw and thermally treated invertebrates digesta (**A-B),** vertebrates digesta (**C-D)** probed with anti-

671   shrimp TM pAb. Comparative densitometric relative quantity histograms of TM recognition by

672   specific antibodies; rabbit anti shrimp TM **(E)** and anti-human TPM2 antibody **(F)** in GP and IP

673   digestion endpoints relative to individual sample's Ctrl sample. (*The replicate data are shown with*

674   *the mean and error bars).* Mw: molecular weight of standard protein (2-250 kDa), TM: Natural

675   shrimp tropomyosin standard, P120': pepsin enzyme control without food matrix after 120',

676   T120': trypsin and chymotrypsin control without food matrix after 120', Ctrl: supernatant without

677   enzymes but just the digestion fluids , GP: supernatant with the pepsin inhibitor at the end of gastric

678   phase, IP: supernatant with protease inhibitor collected at the end of intestinal digestion. RA: Raw

679   Abalone, CA: Cooked Abalone, RO: Raw oyster, CO: Cooked oyster, RS: Raw shrimp, CS:

680   Cooked shrimp, RC: Raw Chicken, CC: Cooked chicken, CP: Cooked pork, RB: Raw Beef, CB:

681   Cooked beef.

682   **Figure 4.** Blots of vertebrates digesta probed with anti-human TPM2 pAb. Mw: molecular weight

683   of standard protein (10-250 kDa), TM: Natural shrimp tropomyosin standard, P120': pepsin

684   enzyme control without food matrix after 120', T120': trypsin and chymotrypsin control without
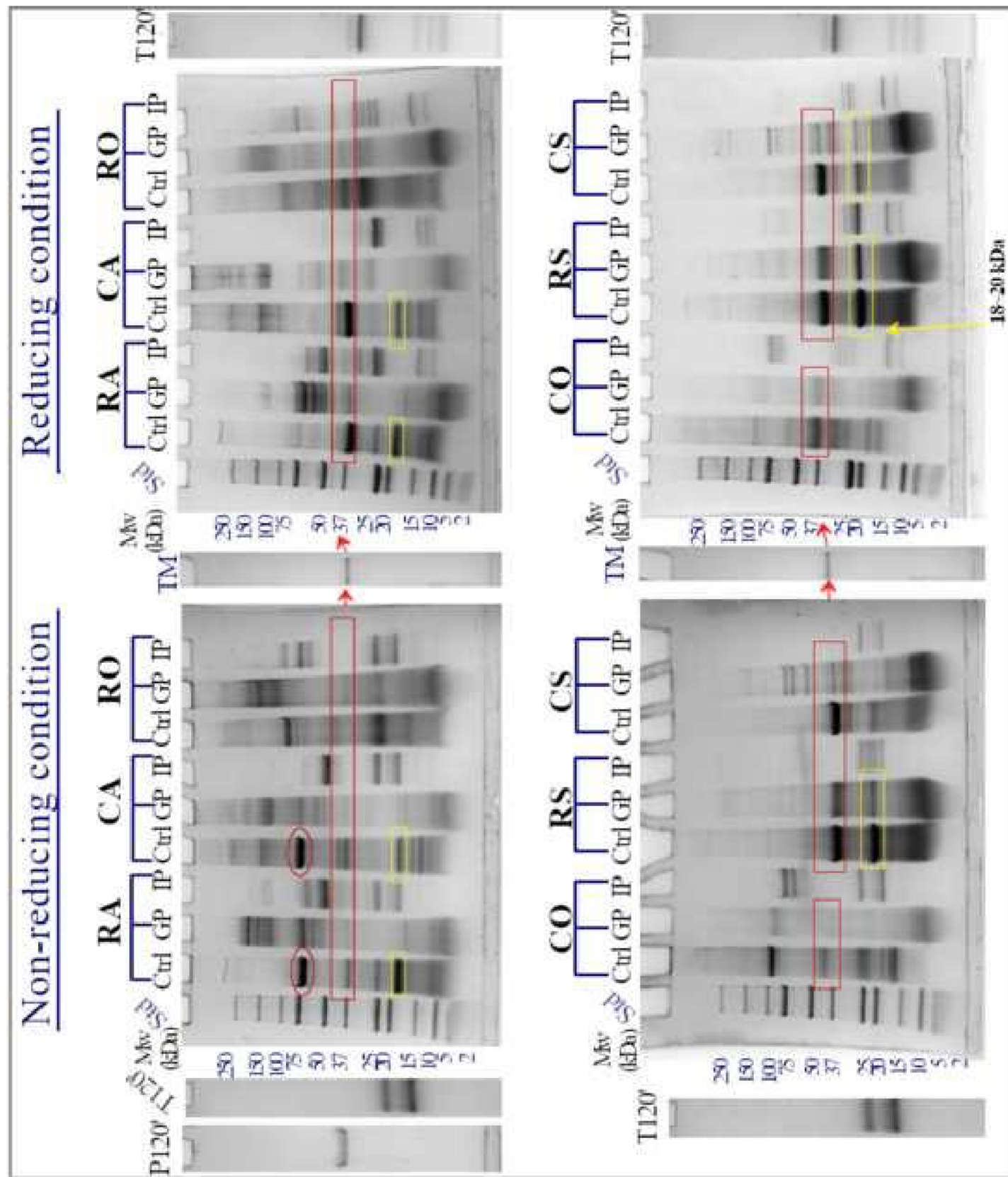
685   food matrix after 120', Ctrl: supernatant without enzymes but just the digestion fluids , GP:

686   supernatant with the pepsin inhibitor at the end of gastric phase, IP: supernatant with protease

687   inhibitor collected at the end of intestinal digestion. RC: Raw Chicken, CC: Cooked chicken, CP:

688   Cooked pork, RB: Raw Beef, CB: Cooked beef.

689   **Figure 5**. MLC specific antibody-based WB of raw and thermally treated digesta. Blots of

690   invertebrates digesta probed with anti-human MLC2 isoform pAb (**A-B**); blots of vertebrates

691   digesta probed with anti-chicken MLC mAb (**C-D**). Comparative densitometric relative quantity

692   histograms of MLC recognition by specific antibodies; rabbit anti human MLC2 isoform (**E**) and

693   mouse anti-chicken MLC mAb (**F**) in GP and IP digestion endpoints relative to individual sample's

694   Ctrl sample. Ctrl: supernatant without enzymes but just the digestion fluids , GP: supernatant with

695   the pepsin inhibitor at the end of gastric phase, IP: supernatant with protease inhibitor collected at

696   the end of intestinal digestion. RA: Raw Abalone, CA: Cooked Abalone, RO: Raw oyster, CO:

697   Cooked oyster, RS: Raw shrimp, CS: Cooked shrimp, RC: Raw Chicken, CC: Cooked chicken,

698   CP: Cooked pork, RB: Raw Beef, CB: Cooked beef. P120': pepsin enzyme control without food

699   matrix after 120', T120': trypsin and chymotrypsin control without food matrix after 120'.

Figure 1

Figure 1.bmp

Figure 2A

Background

Figure 2B

Figure 2_final.tif

Figure 3

Figure 3.bmp
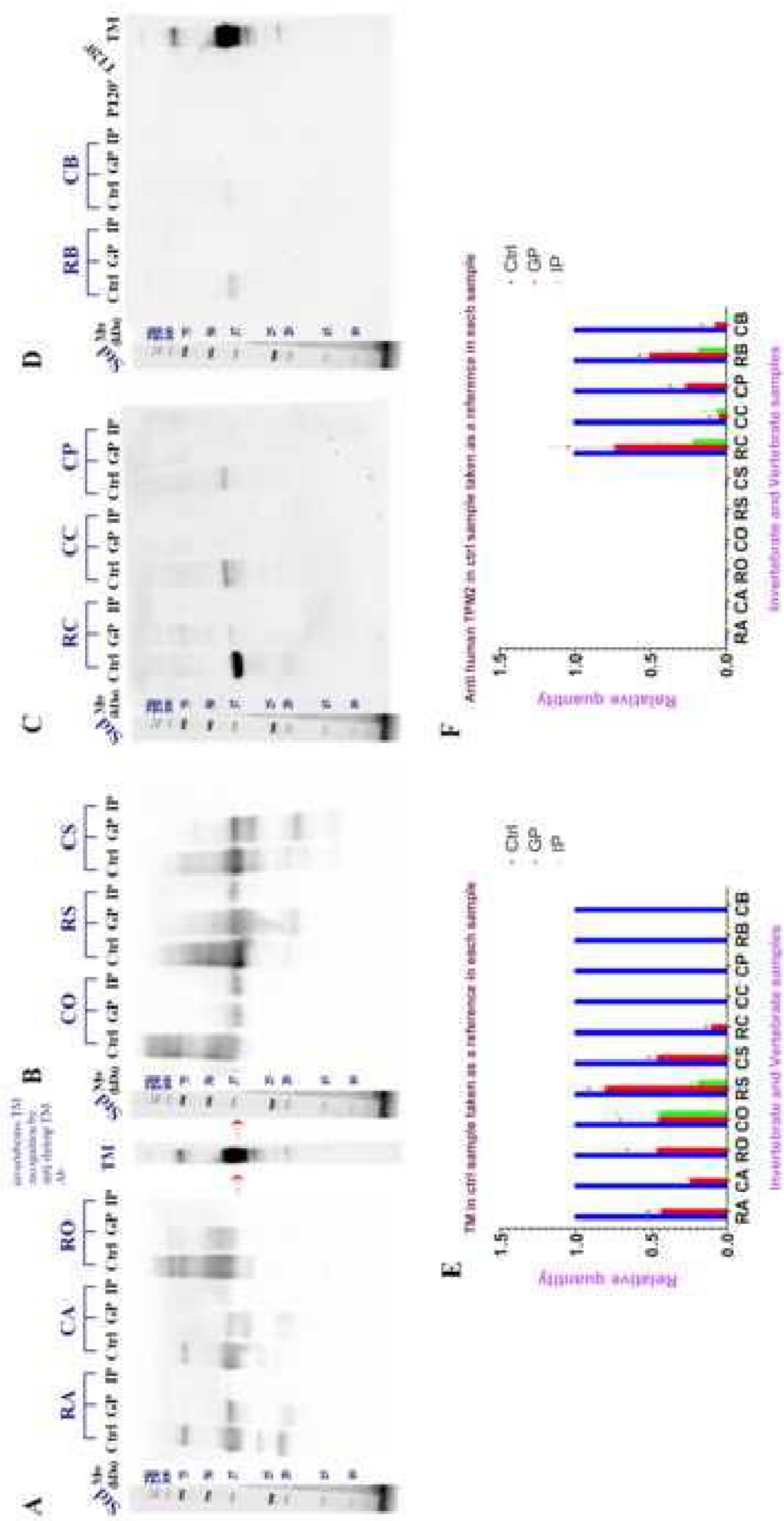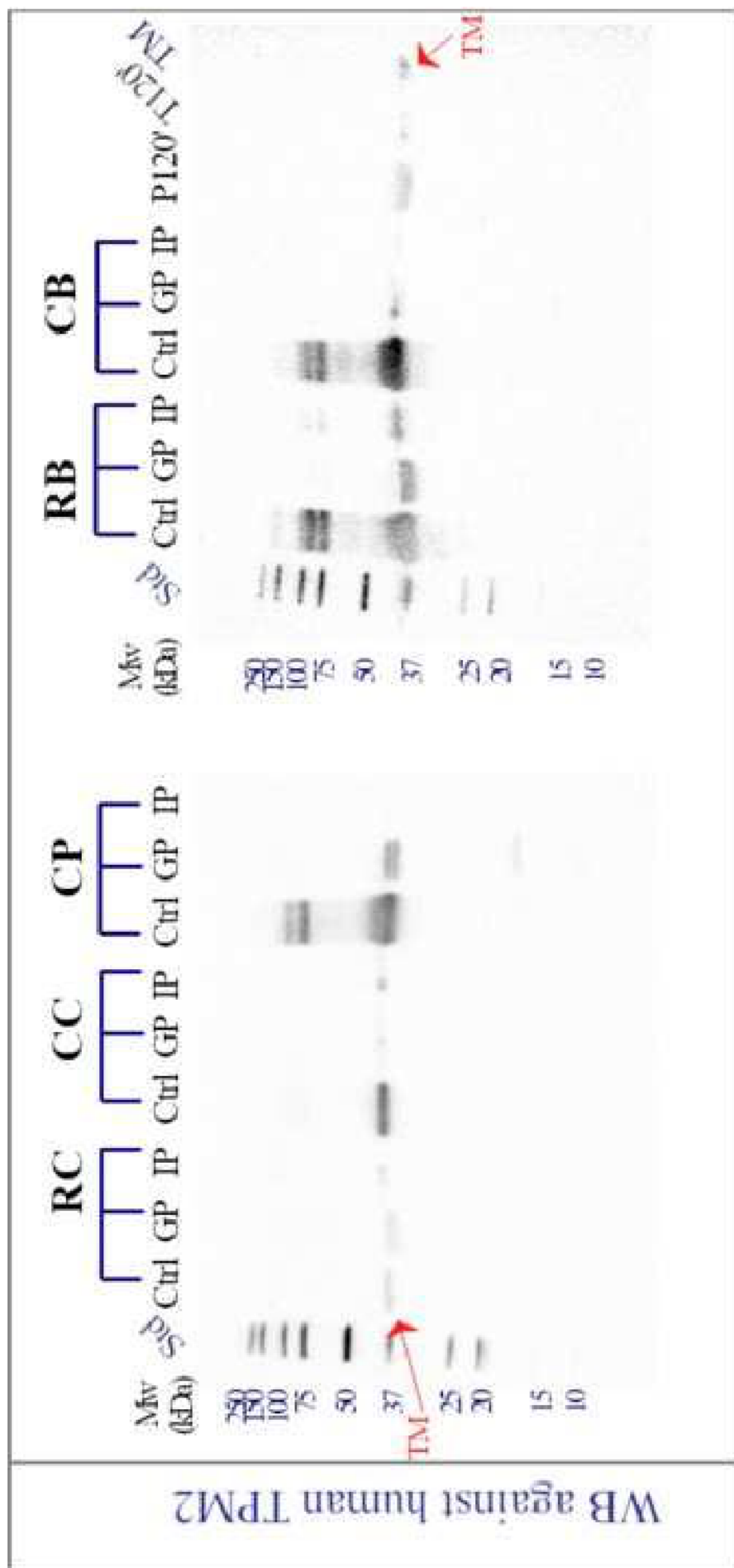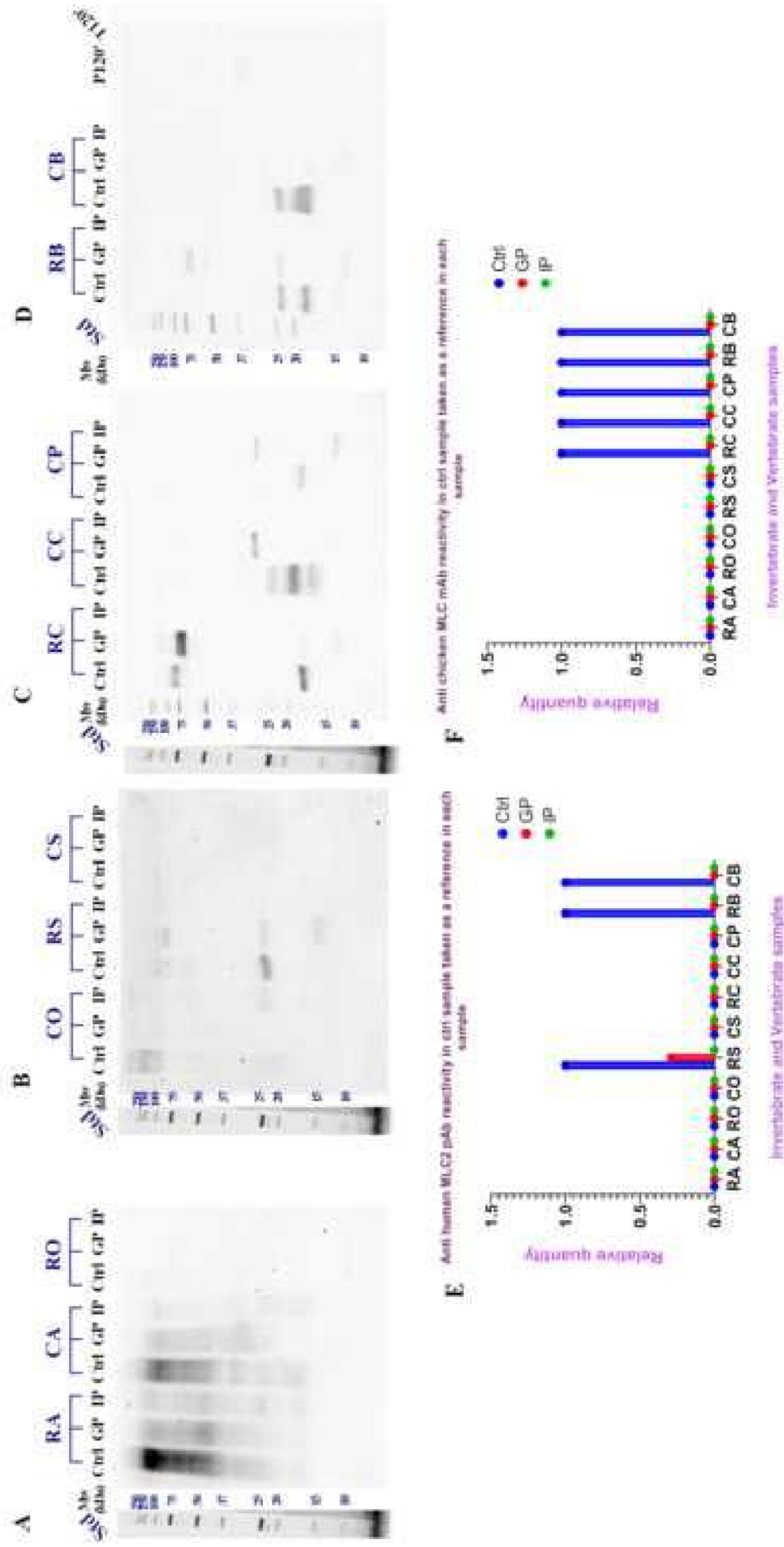
Figure 4

Figure 5

Figure 4.bmp

1 **Supporting information**

2 **Comparative digestion of thermally treated vertebrates and**

3 **invertebrates allergen pairs in real food matrix**

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13 **Abbreviations**

14 **CA**, cooked abalone; **CB**, cooked beef; **CC**, cooked chicken; **CO**, cooked oyster; **CP**, cooked

15 pork; **CS**, cooked shrimp; **Ctrl**, control; **GI**, Gastrointestinal; **GP**, gastric phase; **IP**, intestinal

16 phase; **LSB**, Laemmli sample buffer; **MLC**, myosin light chain; **NR**, non-reducing; **OP**, oral

17 phase; **RA**, raw abalone; **RB**, raw beef; **RC**, raw chicken; **RO**, raw oyster; **RS**, raw shrimp;

18 **SAM**, signal accumulation mode; **SCP**, sarcoplasmic calcium-binding protein; **SGF**, simulated

19 gastric fluid; **SIF**, simulated intestinal fluid; **SSF**, simulated salivary fluid; **TM**, tropomyosin;

20 **TPM2**, Human tropomyosin 2

Material and methods

Table S1 The cooking time and temperature for invertebrate and vertebrate samples

S.n.	Samples (Label) (geographical origin)		Mode of Consumption	Average Cooking Temperature and Time
1	Abalone (A) [Incheon fish market]	Raw abalone RA Cooked abalone CA	Frequently Raw, sometimes slightly cooked	Cover and heat half the shell of abalone in the beaker containing boiling water for 5 minutes.
2	Pacific Oyster (O) [Koje island]	Raw oyster RO Cooked oyster CO	Frequently Raw, sometimes lightly cooked	Drop the thawed oysters with shell into the beaker containing boiling water and boil for 5 minutes.
3	Shrimp (S) [Koje island]	Raw shrimp RS Cooked shrimp CS	Cooked	Drop the thawed shrimps with the heads, tails and shells into the boiling water and boil for 5 minutes.
4	Chicken (C) {Boneless, skinless breast} [Songdo]	Raw chicken RC Cooked chicken CC	Cooked	5 g in a beaker full of boiled water at 180°C in oven for 30 minutes (internal temperature checked to 75°C).
5	Pork (P) {Jowl (boneless)} [Songdo]	Cooked pork CP	Cooked	5 g in a beaker full of boiled water at 180°C for 1 h 30 minutes (internal temperature checked to 73°C).
6	Beef (B) {Shoulder steak cut (boneless)} [Songdo]	Raw beef RB Cooked beef CB	Raw (Steak tartare, carpaccio, kitfo) and cooked	

Standardized static in-vitro simulation of gastrointestinal digestion

24 The raw and cooked vertebrate and invertebrate samples were first cut into smaller pieces then

25 homogenized at 4000 rpm for 50 sec-3 sec rest-50 sec (T 25 digital Ultra turrax homogenizer, IKA

26 Korea Ltd.). 240 mg of the homogenized solid food was then weighed in a 2 ml Eppendorf

27 centrifuge tubes for the simulated gastrointestinal digestion. The COST INFOGEST 2.0 static

28 digestion protocol revised by Brodkorb et al (2019) was adopted and slightly modified in this

29 research. We mimicked the successive oral phase (OP), gastric (GP) and intestinal (IP) human

30 digestive phases of our samples in real food matrix keeping in mind the parameters as the

31 electrolytes in Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated

32 Intestinal Fluid (SIF), enzymes and their activity, bile and its concentration, pH and time of

33 digestion are in accordance with the available physiological data based on an international

34 consensus developed by the COST INFOGEST network.[1]

35 Stock solutions of simulated digestive fluids for 125 ml (4x concentration) of SSF, SGF and 250

36 ml (2x concentration) of SIF were prepared. [**Note**: $CaCl_2(H_2O)_2$ was added immediately before use

37 to avoid the precipitation in stock solutions during storage.]

38 In addition to the raw and cooked samples, control/stability sample tubes (Ctrl) were also prepared

39 to evaluate food stability during exposure to simulated digestive fluids without enzymes,

40 simultaneously with the oral, gastric and intestinal phases. The enzyme-blank tubes, i.e., the

41 digestion tube containing quartz sand instead of food sample with ①pepsin at 120' time (P120')

42 and ②Trypsin and Chymotrypsin at 120' time with bile (T120') were also run. This proved to be

43 the essential step in identifying enzyme and degradation products, bile salts during gel analysis of

44 the GP and IP digesta. After the digestion, the ctrl and the digesta at the GP and IP endpoints were

45 centrifuged at 11400 xg for 25 min (Labogene mini micro centrifuge, BMS Korea Ltd) to separate

46 supernatant from the pellets. The enzymes: pepsin for gastric digestion and individual intestinal

47 enzymes trypsin and chymotrypsin activity, bile concentration was determined following the

48 protocol by Brodkorb et al, 2019.

49 **Oral phase (OP)**: 240 mg of each sample in eppendorf microtube was mixed with 240 ul SSF

50 solution (50:50 ratio of sample to SSF). Human salivary α-amylase was not added to the sample.

51 CaCl$_2$ (2.4 µL, 150 mM) was added separately to avoid precipitation in stock solution to achieve

52 final concentration of 0.75 mM. The reaction mixture was incubated for 2 minutes at 37 °C with

53 agitation. All reagents were previously pre-warmed at 37 °C for 5 minutes.

54 **Gastric phase (GP)**: The OP digesta was then mixed with 480 µL of SGF stock solution including

55 3µL of CaCl2 (25 mM) to achieve a final concentration of 75 µM in the digestion mixture. 150 µL

56 porcine pepsin in 10 mM HCl (Enzyme activity of 2500 U/mg, #P7012, Sigma-Aldrich) was added

57 to achieve a final concentration of 2000 U/mL in the digestion mixture. Gastric lipase enzyme was

58 not added in gastric phase. The mixture was adjusted to pH 3 with 1 M HCl, then milliQ water

59 was added such that the final volume of reaction mixture was 960 µL (sample to SGF ratio should

60 be 50:50). The digestion mixture was incubated for 120 minutes at 37 °C with continuous agitation

61 (400 rpm) in thermoshaker. Control samples were run in parallel: Pepsin enzyme controls at 120'

62 without food sample (sample replaced by quartz sand) were also included. Digestion in one

63 replicate tube of each sample was stopped by addition of 20 µL 48 uM protease inhibitor pepstatin

64 A (#P5318, Sigma Aldrich) to achieve the final concentration of 1 µM in the final reaction mixture

65 (the other replicate tube was further continued with the intestinal phase as described below). The

66 GP endpoint samples were centrifuged at 13,500 rpm for 20 minutes; the supernatant was separated

67 from pellet (insoluble solids) and immediately frozen at −20 °C. Protein concentration of

68 supernatant was determined using BCA assay (Thermo Fisher Scientific Inc., Bremen, Germany).

69   **Intestinal phase (IP)**: The resulting 960 μL of complete gastric digesta was mixed with 960μL of

70   SIF stock solution including 23μL of CaCl2 (25 mM) to achieve a final concentration of 300 μM

71   in the digestion mixture. Individual enzymes porcine trypsin (Measured activity was 194.44

72   TAME U/mg, cat #T0303, Sigma-Aldrich) and α-chymotrypsin from bovine pancreas (Measured

73   activity was 27 BTEE U/mg, Cat #C7762, Sigma-Aldrich) (to obtain final activity ratio of 4:1 in

74   the digesta) prepared in SIF was added. The bile extracts porcine (measured concentration was

75   256 mM, cat # B8631, Sigma-Aldrich) was prepared in SIF and added to achieve final

76   concentration of 10mM in the final digesta. The amylase, pancreatic lipase and the colipase

77   enzymes were not added. The mixture was adjusted to pH 7 with 1 M NaOH, then milliQ water

78   was added, such that the final volume of reaction mixture was 1920 μL. The reaction mixture was

79   incubated for 120 minutes at 37 °C with continuous agitation (400 rpm) in thermoshaker. Control

80   samples were run in parallel: trypsin and chymotrypsin enzyme controls at 0' and 120' without

81   CM sample (sample replaced by sand) were also included. The enzyme activity in the final

82   digestion mixture was stopped by addition of 20 μL of 480 mM AEBSF/Pefabloc SC (Cat #76307,

83   Sigma Aldrich) to obtain 5 mM final concentration in the reaction mixture. The samples were

84   centrifuged at 10,000 xg for 20 minutes; the supernatant was separated from pellets (insoluble

85   solids) and immediately frozen at −20 °C until further assessment. Protein concentration of

86   supernatant was determined using BCA assay (Thermo Fisher Scientific Inc., Bremen, Germany)

87   after necessary dilutions.

88   **Allergen specific antibody based Western blot (WB)**

89   Table S2: Details on the TM standard, primary antibodies, and secondary antibodies as per the

90   manufacturer

S.n.	Antibodies	Catalogue number/Manufacturer Details on specificity	Species cross-reactivity by manufacturer
1.i)	Polyclonal rabbit anti-shrimp TM	PA-SHM/Indoor Bio	-
1.ii)	Polyclonal rabbit anti-human TPM2	LS-C81187/31266/ LSBio: LifeSpan BioSciences	**H**uman, **M**ouse, **R**at, Chicken, **B**ovine, Fish sp
1.iii)	Mouse anti-myosin light chain (MLC) IgM isotype monoclonal antibody	M4401/ Sigma Aldrich	**H**uman, **C**hicken, Bovine, **P**ig, Rabbit
1. iv)	Polyclonal rabbit anti-human MLC2	3672S/ Cell signaling Technology	**H**uman, **M**ouse, **R**at, (**C**hicken, **B**ovine, **P**ig)
2. i)	2∘ Ab for WB---- Goat Anti-rabbit IgG, AP conjugated	A3687/sigma Aldrich	-
2.ii)	Goat anti-mouse IgG, AP-conjugated	A3562/Merck, Germany	Reacts with mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM. (By ODD)
3.	Tropomyosin standard----- Natural shrimp TM	NA-STM-1/Indoor Bio	-

91

Results and discussions

93   [Here for Figure S1]

94
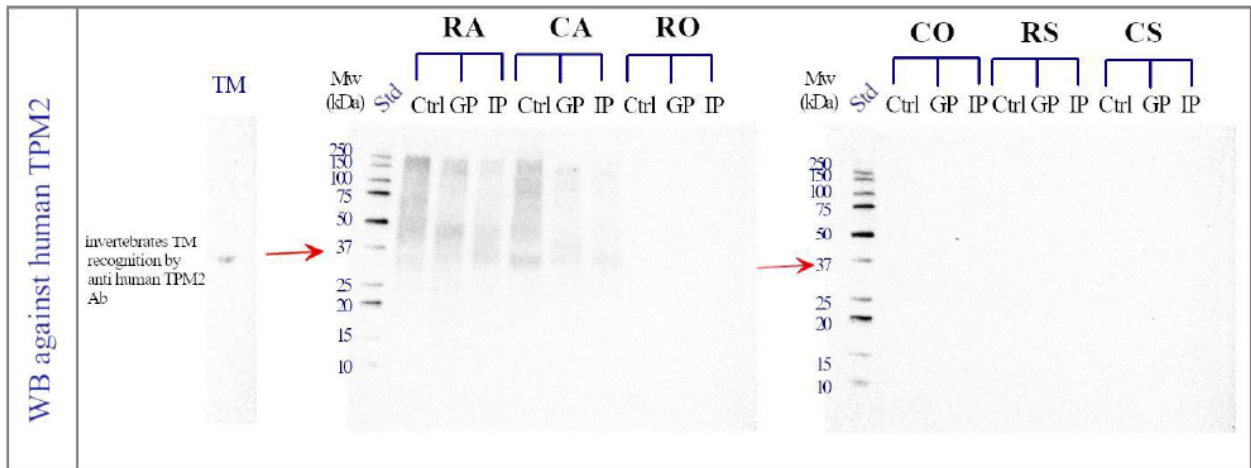
Figure S1. TM specific semi-dry western blot (WB) of invertebrate samples against rabbit anti human tropomyosin 2
(TPM2) pAb. Mw: molecular weight of standard protein, Std: Unstained Western C standard (10-250 kDa), P120':
pepsin enzyme control without food matrix after 120', T120': trypsin and chymotrypsin control without food matrix
after 120', Ctrl: supernatant without enzymes but just the digestion fluids , GP: supernatant with the pepsin inhibitor
at the end of gastric phase, IP: supernatant with protease inhibitor collected at the end of intestinal digestion. RA: Raw
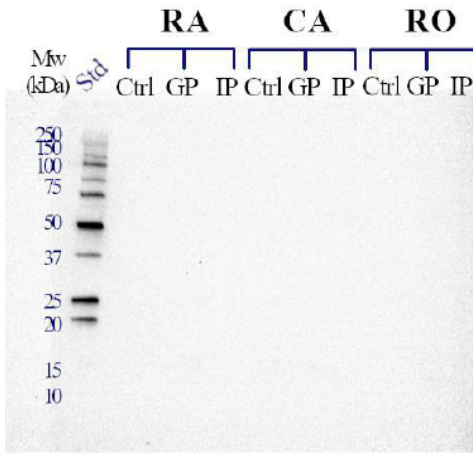Abalone, CA: Cooked Abalone, RO: Raw oyster, CO: Cooked oyster, RS: Raw shrimp, CS: Cooked shrimp.

Secondary Ab control blot without primary antibody.
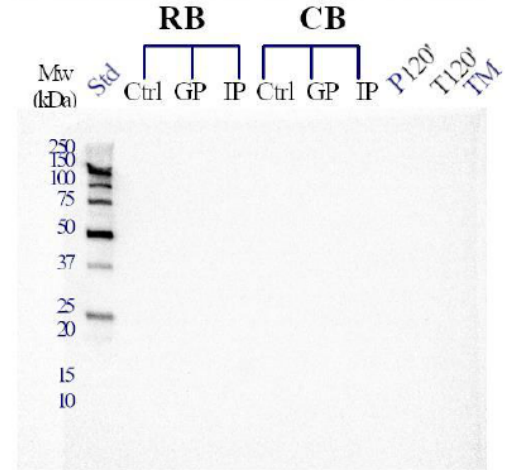
Non-specific binding was <u>not</u> observed.

[Here for Figure S2]

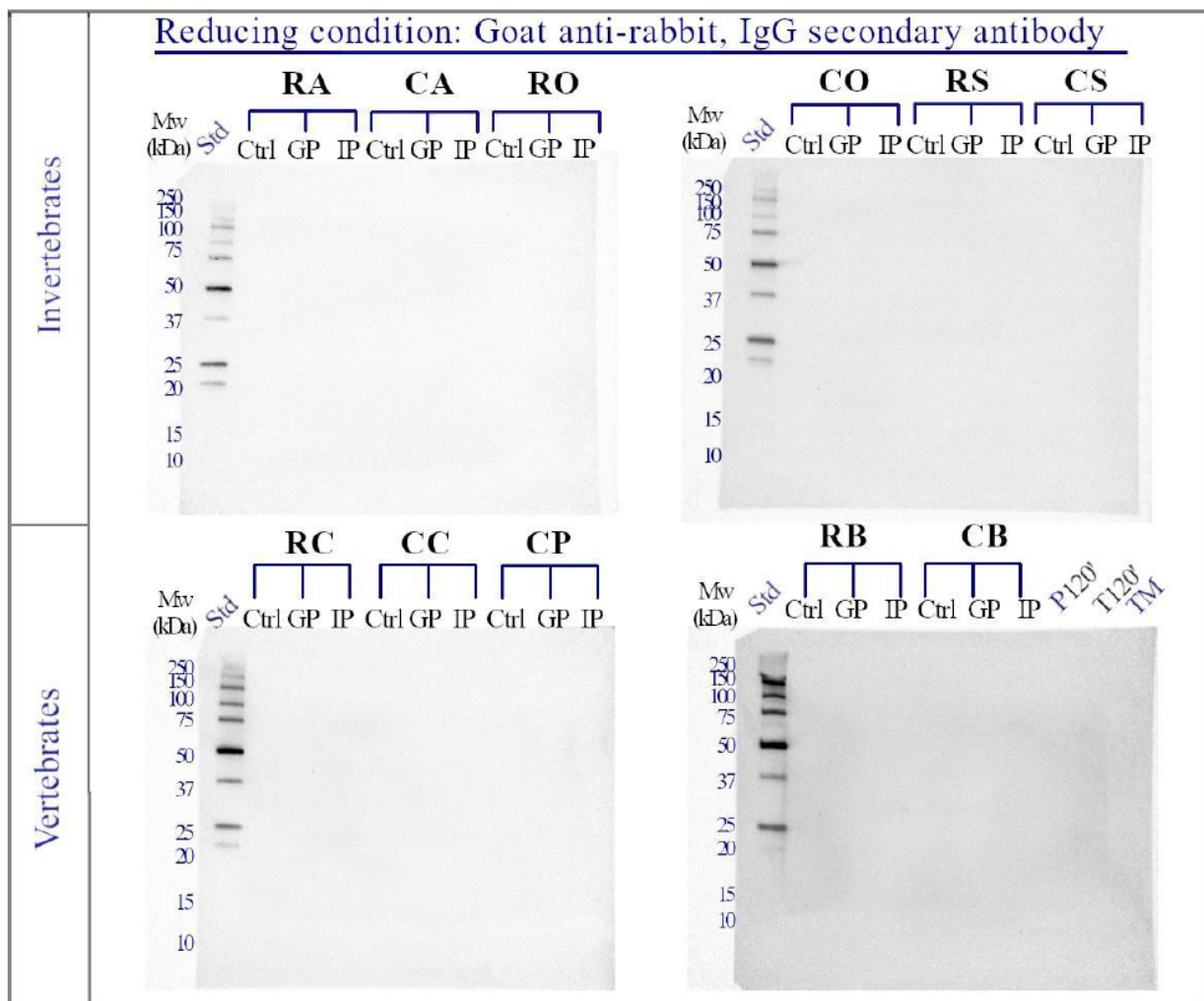Reducing condition: Goat anti-mouse, IgG secondary antibody
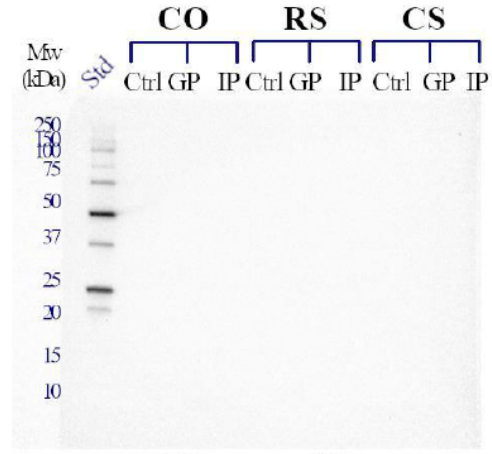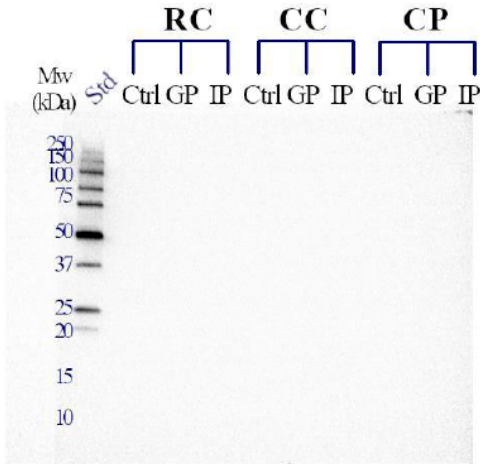
Figure S2. Secondary antibody control blot (without primary antibodies), goat anti-mouse, IgG antibody and goat anti-rabbit, IgG antibody. Ctrl: supernatant without enzymes but just the digestion fluids , GP: supernatant with the pepsin inhibitor at the end of gastric phase, IP: supernatant with protease inhibitor collected at the end of intestinal digestion; B) upon thermal treatment. RA: Raw Abalone, CA: Cooked Abalone, RO: Raw oyster, CO: Cooked oyster, RS: Raw shrimp, CS: Cooked shrimp, RC: Raw Chicken, CC: Cooked chicken, CP: Cooked pork, RB: Raw Beef, CB: Cooked beef. P120': pepsin enzyme control without food matrix after 120', T120': trypsin and chymotrypsin control without food matrix after 120'.

Sequence identity matrix by multiple sequence alignment (uniprot.org**)**

```
Shrimp TM Percent Identity  Matrix against vertebrates TM - created by
Clustal2.1

    1: sp|Q3Y8M6|TPM_PENAT   100.00   55.99   54.58   55.99
```

```
118      2: sp|A1XQV4|TPM3_PIG        55.99   100.00    85.56    90.14
119      3: sp|P19352|TPM2_CHICK      54.58    85.56   100.00    84.51
120      4: sp|Q5KR49|TPM1_BOVIN      55.99    90.14    84.51   100.00
121
122  Chicken MLC Percent Identity  Matrix against invertebrates MLC- created by
123  Clustal2.1
124
125      1: tr|Q70MN9|Q70MN9_CRAGI   100.00    69.43    30.67    20.67
126      2: tr|B6RB34|B6RB34_HALDI    69.43   100.00    29.53    20.13
127      3: sp|P02612|MLRM_CHICK      30.67    29.53   100.00    37.43
128      4: sp|B7SNI3|MLR_PENVA       20.67    20.13    37.43   100.00
```

References

[1]   A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carrière, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C. Martins, S. Marze, D. J. McClements, O. Ménard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies, I. Recio, *Nature Protocols* **2019**, *14*, 991–1014.