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1 **PROTECTIVE ROLE OF BOWMAN-BIRK PROTEASE INHIBITOR ON**
2 **SOYBEAN LUNASIN DIGESTION: EFFECT OF RELEASED PEPTIDES ON**
3 **COLON CANCER GROWTH**
4

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20 **Abstract**

21 Lunasin is a naturally-occurring peptide with demonstrated chemopreventive,
22 antioxidant and anti-inflammatory properties. To exert these activities, orally ingested
23 lunasin is requested to survive proteolytic attack of digestive enzymes to reach target
24 tissues in active form/s. Preliminary studies suggested the protective role of protease
25 inhibitors, such as Bowman-Birk inhibitor and Kunitz-trypsin inhibitor against lunasin's
26 digestion by both pepsin and pancreatin. This work describes in depth the behaviour of
27 lunasin under conditions simulating transit through the gastrointestinal tract in the
28 absence or presence of soybean Bowman-Birk isoinhibitor 1 (IBB1) in both active and
29 inactive state. By liquid chromatography coupled to tandem mass spectrometry (HPLC-
30 MS/MS), the remaining lunasin at the end of gastric and gastro-duodenal phases was
31 quantified. Protection against the action of pepsin was independent of the amount of
32 IBB1 present in the analyzed samples, whereas a IBB1 dose-dependent effect against
33 trypsin and chymotrypsin was observed. Peptides released from lunasin and inactive
34 IBB1 were identified by MS/MS. Remaining lunasin and IBB1 as well as their derived
35 peptides could be responsible for the anti-proliferative activity against colon cancer
36 cells observed for the digests obtained at the end of simulated gastrointestinal digestion.

37

38 **Keywords**

39 Lunasin, Bowman-Birk protease inhibitor, simulated gastrointestinal digestion, colon
40 cancer cells, anti-proliferative activity

41

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42 **1. Introduction**

43 Lunasin is a naturally-occurring peptide corresponding to the small subunit
44 peptide of 2S albumin.[1] Its amino acid sequence
45 (SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD, National
46 Center for Biotechnology Information, NCBI, accession number AAP62458) is
47 characterized by the presence of a predicted α-helix structure, a tri-peptide RGD cell
48 adhesion motif and a continuous sequence of nine aspartic acid (D) residues at the C-
49 terminus. A recent molecular dynamics study of this peptide has suggested the
50 important role played by its α-helicity and the highly negative-charged C-terminal tail
51 in the recognition and binding to the chromatin residue, and thus, in the anti-mitotic
52 activity of lunasin in several mammalian cell lines.[2]

53 Recent studies have revealed the potential role of lunasin against established
54 breast, colon, prostate cancer, and leukemia cell lines, through its ability to inhibit cell
55 proliferation by arresting cell cycle and inducing apoptosis.[3] The chemoprotective
56 effects of lunasin against skin,[4] breast,[5,6] and colon cancer[7,8] have been also evaluated in
57 animal models. Moreover, lunasin has been shown to restrain oxidative status caused by
58 chemical agents in both intestinal Caco-2 and hepatic HepG2 cells, and to inhibit
59 inflammation in cultured RAW 264.7 macrophages. These antioxidant and anti-
60 inflammatory properties have been suggested to contribute on the anti-cancer activity of
61 lunasin.[3]

62 Food-derived peptides are expected to exert their health beneficial properties
63 after being orally taken. Even though these peptides can elicit hormone-like functions
64 locally in the gastrointestinal tract, they are generally required to flow into the blood to
65 exert specific activities at a systemic level. In last years, the number of studies
66 evaluating bioavailability aspects of dietary peptides, such as their resistance to

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67 digestive enzymes and their absorption/distribution rates has notably raised.[9] In
68 addition, the application of peptidomics tools on these analysis has markedly emerged.[10]
69 It is increasingly obvious that gastrointestinal digestion has an important influence on
70 the biological activity of food-derived peptides, by the release of new active fragments
71 from their precursors or, on the contrary, giving rise to fragments with less or null
72 activity.[11-15]

73 Park et al.[16] reported that isolated lunasin, either synthetic or soybean-purified, is
74 easily digested after 2 minutes incubation with simulated gastric or intestinal fluids.
75 However, lunasin survives, at least partially, the attack of digestive enzymes when is
76 present in crude protein extracts purified from soybean or other plants sources. By
77 Western-Blot analysis, Jeong et al.[17] demonstrated that approximately 85% of the
78 original lunasin contained in *Solanum nigrum* L. crude protein extract remained intact
79 after 120 min digestion with simulated gastrointestinal fluids. In the case of lunasin
80 present in soybean crude protein extract, 60% and 80% of initial lunasin was detected
81 by immunoblotting after proteolysis by pepsin and pancreatin for 120 min,
82 respectively.[16] Results from these studies suggested that the presence in sufficient
83 amount of naturally-occurring protease inhibitors, such as Bowman-Birk inhibitor (BBI)
84 or Kunitz-trypsin inhibitor, might exert a protective role against lunasin's proteolysis by
85 digestive enzymes. Hernández-Ledesma et al.[18] also observed the protective role exerted
86 by BBI when lunasin present in soybean-derived foods was subjected to a sequential
87 digestion with pepsin and pancreatin, suggesting the importance of the lunasin:BBI ratio
88 on this protection. However, to our knowledge, the behavior of lunasin during its transit
89 through gastrointestinal tract has not been previously evaluated. Thus, the present study
90 aims to simulate physiological conditions using an *in vitro* digestion model for lunasin
91 in the absence and/or presence of a major soybean Bowman-Birk isoinhibitor (IBB1).

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92 HPLC-MS/MS analysis was carried out to quantify remaining intact lunasin and to
93 identify the peptides released during the digestive process. The anti-proliferative effect
94 against colon cancer cells (HT-29 and Caco-2) of digests obtained at the end of the
95 digestive process was also evaluated.
96
97 **2. Materials and Methods**
98 *2.1. Reagents*
99 Peptide lunasin (>95% of purity) was synthesized by Chengdu KaiJie Biopharm
100 Co., Ltd. (Chengdu, Sichuan, P. R. China). BBI from soybean (T9777), *N*-*α*-benzoyl-
101 DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl-L-tyrosine ethyl ester (BTEE),
102 porcine pepsin (EC 3.4.23.1), pancreatic bovine trypsin (EC 232-650-8), pancreatic
103 bovine α-chymotrypsin (EC 232-671-2, Type I-S), pancreatic porcine lipase (EC 232-
104 619-9, Type VI-S), pancreatic porcine colipase (EC 259-490-1), phosphatidylcholine
105 (PC), sodium taurocholate, sodium glycodeoxycholate, trifluoroacetic acid (TFA), and
106 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased
107 from Sigma Chemical Co. (Madrid, Spain). Other chemicals used were of HPLC grade.
108
109 *2.2. Purification of isoinhibitor soybean IBB1*
110 IBB1, was purified from commercially available soybean BBI following the
111 protocol of Arques et al.[19] Briefly, the sample consisted in a mixture of protease
112 inhibitors that was loaded onto a MonoS 5/50 GL cation exchange column (GE
113 Healthcare, Uppsala, Sweden), connected to an AKTA FPLC system (GE Healthcare),
114 using a linear gradient of 0-0.22 M NaCl in 25 mM sodium acetate buffer, pH 4.4, at a
115 flow rate of 1 mL/min. The elution was monitored at 280 nm and 0.5 mL fractions were
116 collected. Measurements of trypsin inhibitory activity of eluted samples were carried

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117 out in flat-bottom microtitre plates by using BAPNA as specific substrate, and assay
118 products measured at OD_{405nm}, as previously described.[20] The unbound sample,
119 containing both trypsin and chymotrypsin inhibitory activity- the latter measured by
120 using BTEE as specific substrate-,[21] was dialyzed extensively against distilled water and
121 freeze-dried until use. The identification of IBB1 was carried out by peptide mass
122 fingerprinting, as previously reported.[22]

123 To abolish the inhibitory activity of soybean IBB1, chemical inactivation *via*
124 reduction of disulphide bonds and subsequent alkylation of the cysteinyl sulfhydryl
125 groups was carried out.[22] Ten milligrams of soybean IBB1 were dissolved in 50 mM
126 Tris-HCl (pH 8.2) and reduced with 100 µL 0.5 M dithiothreitol (DTT) for 2 min at
127 100ºC, and alkylated with 500 µL of 0.25 M iodoacetamide for 15 min at 50°C under
128 dark conditions. In order to remove residual DTT and iodoacetamide, samples were
129 dialyzed extensively against distilled water and freeze-dried.

130

131 *2.3. Measurement of protease inhibitory activities*

132 IBB1 and its inactive form were assessed for trypsin and chymotrypsin
133 inhibitory activities. Trypsin inhibitory activity was measured using a modified small-
134 scale quantitative assay with BAPNA as specific substrate using 50 mM-Tris pH 7.5 as
135 enzyme assay buffer. One trypsin inhibitor unit was defined as that which gives a
136 reduction in absorbance at 410 nm of 0.01, relative to trypsin control reactions, in 10
137 min in a define assay volume of 10 mL.[23] Chymotrypsin inhibitory activity was
138 measured by using BTEE as specific substrate. One chymotrypsin inhibitor unit was
139 defined as that which gives a reduction in absorbance at 256 nm of 0.01, relative to
140 chymotrypsin control reactions, in 5 min in a defined assay volume of 10 mL, as
141 described previously.[21] Specific trypsin and chymotrypsin inhibitory activity, expressed

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142 as inhibitor units per mg of protein, were calculated and used to assess the chemical
143 inactivation of IBB1.
144

145 *2.4. In vitro simulated gastrointestinal digestion*

146 The *in vitro* digestibility of lunasin in the presence or absence of IBB1, either
147 active or inactive, was evaluated by using an *in vitro* model system mimicking *in vivo*
148 gastric and duodenal digestion according to Moreno et al.[24] with some modifications.[25]
149 The digestions were assessed in the following conditions: lunasin in the absence of
150 IBB1, lunasin in the presence of IBB1 [lunasin:IBB1 ratios of 1:1 and 1:2 (w:w)] and
151 lunasin in the presence of chemically inactivated IBB1 [lunasin:IBB1 ratio of 1:2
152 (w:w)].

153 Lunasin and IBB1 were dissolved in simulated gastric fluid (SGF, 35 mM NaCl,
154 pH 2.0) containing phospholipid vesicles at total protein concentration of 1.5 mg/mL.
155 The solution was adjusted to pH 2.0, preheated for 15 min at 37ºC, and subjected to
156 gastric digestion for 60 min at 37ºC by adding 182 units of porcine pepsin per mg of
157 protein. Gastric reaction was stopped by rising pH up to 7.0-7.5. For intestinal
158 digestion, the pH of the gastric digest was adjusted to 6.5-6.8 by addition of 1 M $CaCl_2$,
159 0.25 M Bis-Tris, and a 0.125 M bile salts equimolar mixture of sodium
160 glycodeoxycholate and sodium taurocholate. The pancreatic bovine trypsin and α-
161 chymotrypsin, and pancreatic porcine lipase were added to the mixture at the
162 enzyme:substrate ratios of 34.5, 0.4 and 24.8 units/mg of protein, respectively.
163 Pancreatic porcine colipase was added at an enzyme:substrate ratio of 1:895 (w:w).
164 Duodenal digestion was carried out for 60 min at 37ºC and stopped by rising
165 temperature to 95ºC for 10 min. Aliquots were taken before digestion and at the end of

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166 both gastric and duodenal phase. The simulated gastrointestinal digestion was carried
167 out in duplicate for each lunasin:IBB1 ratio.
168
169 *2.5. Analysis of digests by RP-HPLC-MS/MS*
170 Synthetic lunasin (at concentrations ranged from 0.125 to 1.50 mg/mL) and
171 samples collected during simulated gastrointestinal digestion were subjected to liquid
172 chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Samples were
173 analyzed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron,
174 Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik, Bremen,
175 Germany) and equipped with an electrospray ionization (ESI) source. The analyses were
176 carried out using a Mediterranea Sea_{18} column (150 × 2.1 mm, Teknokroma, Barcelona,
177 Spain). The injection volume was 50 µL and the flow rate was set at 0.2 mL/min.
178 Peptides were eluted with a linear gradient (0-70%) of solvent B [acetonitrile:TFA,
179 1000:0.27 (v:v)] in A [water:TFA, 1000:0.37 (v:v)] in 75 min. Spectra were recorded
180 over the mass/charge (*m/z*) range 200-3000. Each sample was analyzed in duplicate.
181 Data obtained were processed and transformed to spectra representing mass values
182 using the Data Analysis program (version 4.0, Bruker Daltonik). BioTools (version 3.1,
183 Bruker Daltonik) was used to process the MS/MS spectra and to perform peptide
184 sequencing.
185
186 *2.6. Cell viability assay*
187 Two human colorectal adenocarcinoma cell lines (Caco-2 and HT-29) were
188 obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).
189 HT-29 and Caco-2 cells were grown in McCoy´s medium (Lonza Group Ltd., Basel,
190 Switzerland) and Dulbecco´s Modified Eagle Medium (DMEM, Sigma Chemical),

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191 respectively, supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest, Nuaillé,
192 France), and 1% (v/v) penicillin/streptomycin/amphotericin B solution (Biowest). In the
193 case of Caco-2 cells, DMEM was also supplemented with 1% (v/v) non-essential amino
194 acids (Lonza Group Ltd.). Cell cultures were grown in a humidified incubator
195 containing 5% CO_2 and 95% air at 37ºC.

196 The effect of digests on cell viability was evaluated by using the MTT assay.
197 Caco-2 and HT-29 cells were seeded in 48-well plates (VWR International, Radnor, PA,
198 USA) at a density of 7×10^4 cells/cm^2, and incubated for 24 h. Cells were treated with
199 gastrointestinal digests (at total protein concentration of 0.3, 0.2 and 0.1 mg/mL) for 24
200 h, and washed with phosphate buffer saline (PBS, Lonza Group Ltd.). MTT solution
201 (0.5 mg/mL at final concentration) was added to each well, and cells were incubated for
202 2 h at 37ºC. The supernatant was aspirated and insoluble formazan crystals formed were
203 solubilized in dimetilsulfoxide:ethanol (1:1, v:v), measuring the absorbance at 570 nm
204 in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany).
205 Experiments were carried out in triplicate and the results were expressed as percentage
206 of viable cells compared to the control, and as IC_{50} value (protein concentration needed
207 to inhibit 50% of viable cells).

208

209 *2.7. Statistics*

210 Data were evaluated using one-way ANOVA followed by Bonferroni post hoc
211 test, and expressed as the mean ± SD of the different experiments carried out. GraphPad
212 Prism 5.0 software (San Diego, CA, USA) was used to perform statistical analyses.
213 Differences with a P value < 0.05 (*) were considered significant.

214

215 **3. Results**

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216 *3.1. Behavior of lunasin under gastric digestion*

217 Commercial BBI consisted in a mixture of two major BBI isoinhibitors, IBB1 218 and IBBD2, differing in potency and specificity against trypsin and chymotrypsin. 219 While IBB1 shows ability to inhibit both trypsin and chymotrypsin, IBBD2 only 220 inhibits trypsin.[22] By cation-exchange chromatography, both forms were isolated, being 221 IBB1 unbound to the Mono-S column whereas IBBD2 was eluted in the NaCl gradient. 222 From the functional point of view, obtained IBB1 demonstrated a high potency against 223 both trypsin and chymotrypsin showing K_i values at nanomolar level (30 ± 4 and 3 ± 1 224 nM, respectively). Its specific inhibitory activity against trypsin and chymotrypsin was 225 3828 ± 209 trypsin inhibitor unit/mg of protein and 2917 ± 292 chymotrypsin inhibitor 226 unit/mg of protein, respectively. Following reduction of disulphide bonds and further 227 alkylation of the cysteinyl sulphydryl groups, its inhibitory activities decreased in more 228 than 95%. In agreement with the inhibitory assays, the MS/MS analysis of inactive 229 IBB1 revealed the presence of a major peak corresponding to reduced/alkylated IBB1, 230 and a minor peak corresponding to the active isoinhibitor that represented 2% of total 231 IBB1. This indicated that residual IBB1 remained active after the inactivation process 232 carried out in this study.

233 Both active and inactive IBB1 were used to prepare mixtures with lunasin at 234 different ratios in order to evaluate the resistance of this peptide to digestive enzymes in 235 the presence or absence of the BBI isoinhibitor. Mixtures were subjected to a two-stage 236 hydrolysis process simulating gastrointestinal digestion. Figure 1A-1D shows the UV- 237 chromatograms of the different digests obtained at the end of the gastric phase. 238 Chromatographic peaks (number or letters) corresponded to the lunasin- and IBB1- 239 derived peptides, respectively. Lunasin (peak 20) eluted after 43 minutes, with ions 240 confirming the identity of this peptide (5028.3 experimental mass, 5028.0, theoretical

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241 mass). Two peaks were visible for IBB1 (peak T). One of them eluted after 53 minutes
242 and corresponded to intact polypeptide (7858.6 experimental mass, 7858.8, theoretical
243 mass). The additional peak was detected at retention time of 55 min, which ions
244 suggested the presence of IBB1 lacking the C-terminal tetrapeptide ^{68}DKEN^{71} (7380.9,
245 theorical mass).

246 The percentage of intact lunasin after pepsin treatment was calculated by using a
247 lunasin's standard curve (0.125-1.5 mg/mL) analyzed in the same conditions (Table 1).
248 In the absence of IBB1, only 2.6% of lunasin was resistant to the action of pepsin,
249 indicating the high susceptibility of this peptide to the gastric enzyme. However, in the
250 presence of IBB1 at lunasin:IBB1 ratios of 1:1 and 1:2, more than 34% of lunasin
251 remained intact after pepsin digestion, without observing statistical differences between
252 both ratios. No significant differences were observed when lunasin was mixed with
253 inactive IBB1 in comparison with samples containing active IBB1, remaining more than
254 28% of this peptide intact after the gastric phase. The higher size of IBB1 in comparison
255 to that of lunasin could make this isoinhibitor to act as a "chaperone" encasing lunasin
256 and preserving its integrity during pepsin hydrolysis. In addition, it cannot be excluded
257 that the presence of a small percentage of active IBB1 during the inactivation process
258 was capable to protect lunasin from the effects of pepsin (Figure 1D).

259 The peptidic profiles of gastric digests in the presence of active IBB1 (Figures
260 1B-1C) were similar between them, but slightly different to that observed for lunasin's
261 digest in the absence of the isoinhibitor (Figure 1A). The HPLC-MS/MS analysis
262 allowed identifying peptides released from lunasin and inactive IBB1 during the
263 simulated gastrointestinal digestive process (Figure 2A-2D, Figure 3A, and
264 Supplementary Table S1). It is remarkable that no peptides derived from active IBB1
265 were identified in this study. In the gastric digests, up to 22 peptides derived from

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266 lunasin and 19 derived from inactive IBB1 were identified, although the presence or
267 absence of each one of these peptides depended on the mixture digested (Figures 2 and
268 3). As expected, the higher number of fragments released from lunasin was detected in
269 the digest of sample in the absence of IBB1, with 20 of 22 total identified peptides. Five
270 peptides, which sequences were ^{11}RKQLQGVNLTPCEKHIME^{28},
271 ^{17}VNLTPCEKHIME^{28}, ^{19}LTPCE^{28}, ^{20}TPCEKHIME^{28}, and ^{21}PCEKHIME^{28}, were
272 identified in the digest without IBB1 but not in those digests containing the isoinhibitor
273 in its active state. These fragments were released from the central region of lunasin sited
274 between amino acid 11 and 28, indicating that this area could be predominantly
275 enclosed and protected from the action of pepsin when the protease isoinhibitor is
276 present (Figure 2A-2D).
277 In the gastric digest of mixtures lunasin:inactive IBB1 (ratio 1:2), 19 peptides
278 released from inactive IBB1 were identified (Figure 3A and Supplementary Table S1),
279 confirming the vulnerability of inactive IBB1 to the action of this gastric enzyme, and
280 the consequent release of an important number of IBB1-derived fragments.
281
282 *3.2. Effects of pancreatic enzymes on lunasin*
283 Once completed the gastric phase, mixtures were digested with a combination of
284 pancreatic enzymes, and resulting digests were analyzed by HPLC-MS/MS (Figure 4)
285 to quantify intact lunasin (Table 1). In the absence of IBB1, the residual lunasin was
286 almost completely degraded after the action of pancreatic proteases (Table 1 and Figure
287 4A). However, active IBB1 exerted a protective role on lunasin against pancreatic
288 hydrolysis that could be explained by its ability to inhibit trypsin and chymotrypsin. The
289 enzymatic inhibitory properties of IBB1 together with its molecular structure supported
290 by a network of seven disulphide bridges are responsible of its resistance to duodenal

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291 digestion, thus the active isoinhibitor was clearly visible at the end of simulated
292 digestion at lunasin:IBB1 ratios 1:1 and 1:2 (Figure 4B and 4C, respectively). It is
293 remarkable that in contrast to the protection against the action of pepsin, inhibitory
294 effects on duodenal digestion were dose-dependent, being the resistance of lunasin more
295 evident when IBB1 was present at higher concentration (lunasin:IBB1 ratio 1:2). In this
296 case, the residual lunasin at the end of simulated gastrointestinal digestion was 5.3%
297 while the residual value for mixtures lunasin:IBB1 at ratio 1:1 was 1.8%. The latter
298 value was similar to that determined when mixtures lunasin:inactive IBB1 were
299 subjected to intestinal digestion (1.5% of residual lunasin, Table 1). As it has been
300 previously indicated, inactive IBB1 did not show trypsin and chymotrypsin inhibitory
301 activities. However, lunasin was slightly protected from the action of these pancreatic
302 enzymes when inactive IBB1 was present in the mixture. The small percentage of IBB1
303 remaining active during the inactivation process could be responsible for this slight
304 protection, although it cannot be excluded that the presence of the isoinhibitor (in both
305 active and inactive state) could be enough to encase lunasin and protect it from the
306 proteolytic action of trypsin and chymotrypsin. In addition, some of IBB1 derived
307 peptides might contribute on the protective action against lunasin's digestion.

308 Of 20 lunasin-derived peptides identified in the gastric digest of IBB1 free-
309 sample, only three, corresponding to ^{24}KHIME^{28}, ^{28}EKIQGR^{33}, and
310 ^{29}KIQGRGDDDDDDDDD^{43}, appeared at the end of whole digestive process,
311 suggesting their resistance to the action of trypsin and chymotrypsin (Figure 2E). The
312 rest of peptides were not detected in the duodenal digest, indicating that without the
313 protective role of IBB1, pancreatic enzymes acted on those sequences released during
314 gastric phase resulting in the liberation of shorter peptides and/or free amino acids. Up
315 to 15 new peptides released from lunasin were identified in the hydrolyzate obtained at

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the end of simulated gastrointestinal digestion when IBB1 was not present (Figure 2E-2H and Supplementary Table S1). The release of eight of these peptides, corresponding to fragments ^{4}QHQQDSCR^{11}, ^{15}QGVNLTPCEK^{24}, ^{16}GVNLTPCEK^{24}, ^{17}VNLTPCEK^{24}, ^{19}LTPCEK^{24}, ^{25}HIME^{28}, ^{29}KIQGR^{33}, and ^{34}GDDDDDDDDD^{43}, could be explained by the action of trypsin on susceptible peptide bonds. The rest of them could be released by the combined action of trypsin and chymotrypsin used during simulated intestinal phase. None of peptides identified in the IBB1-free digest was identified in digests containing active IBB1, suggesting the ability of this isoinhibitor to protect both lunasin and derived peptides against the action of trypsin and chymotrypsin. It was also interesting the absence of those new peptides when the digestion was carried out on a mixture containing inactive IBB1. These results confirm that the presence of IBB1 in both active and inactive forms was enough to protect lunasin and derived fragments against digestion by duodenal enzymes.

Only four of nineteen peptides released from inactive IBB1 by the action of pepsin were degraded by trypsin and chymotrypsin, being not detected at the end of simulated gastrointestinal digestion. The sequences of these peptides were ^{24}CSDMRLNSCHSA^{35}, ^{43}LSYPAQC^{49}, ^{58}CYEPCKPSEDDKEN^{71}, and ^{61}PCKPSEDDKEN^{71}. The rest of peptides were identified at the end of digestion, indicating their resistance to the action of pancreatic enzymes (Figure 3). Among them, it was remarkable the presence of the trypsin inhibitory domain of IBB1 localized between amino acids ^{14}T and ^{22}K that could protect lunasin and its derived fragments for further digestion by trypsin.[22]

3.3. Anti-proliferative action of gastrointestinal digests against colon cancer cells

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340 Digests at the end of the simulated digestive process were assayed for their
341 effect against cell viability of two colon adenocarcinoma cell lines, HT-29 and Caco2,
342 using the MTT protocol. In order to evaluate the dose-response, the digests were proved
343 at the initial protein concentration (0.3 mg/mL) and once diluted in growth media 1.5
344 and 3-times. Digestion media in the absence of lunasin and IBB1 was assayed to
345 evaluate if any substances used to simulate digestion were capable to decrease cell
346 viability (Figure 5). An anti-proliferative effect was observed when the non-diluted
347 digest was added to HT-29 and Caco2 cells, with values of viable cells of 70.5 ± 8.0%
348 and 86.6 ± 4.0%, respectively. The anti-proliferative effect was significantly higher
349 when digests from lunasin:IBB1 mixtures were assayed. As shown in Figure 5A, all the
350 tested gastro-duodenal digests affected HT-29 cell viability in a dose-dependent
351 manner. Digest obtained in the absence of IBB1 provoked a decrease of viable cells of
352 52.4% at the highest protein concentration assayed. The IC_{50} value calculated for this
353 digest was 0.29 mg/mL. Since the amount of lunasin detected in this hydrolyzate was
354 very low (2.6%), the anti-proliferative activity seems to be mainly due to the peptides
355 released during lunasin digestion. However, these peptides did not exert any effect
356 against Caco2 cells (Figure 5B), indicating that their activity might be dependent on the
357 cell line studied. Higher anti-proliferative effects in both colon cancer cell lines were
358 observed when hydrolyzates obtained from mixtures containing lunasin and IBB1 were
359 assayed, indicating that both intact polypeptides lunasin and IBB1 and the peptides
360 released from them could cooperate to decrease viability of colon cancer cells. The
361 calculated IC_{50} values for these hydrolyzates were lower (0.16-0.23 mg/mL) when HT-
362 29 cells were treated with digests, suggesting the higher vulnerability of this cell line to
363 the action of lunasin, IBB1 and their derived-peptides.
364

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365 **4. Discussion**

366 Our findings indicate that, in the absence of IBB1, during the simulated gastric
367 phase, pepsin acts on lunasin hydrolyzing more than 97% of initial peptide and
368 releasing a great number of peptides. Previous studies have reported the ability of
369 pepsin to degrade lunasin, although the levels of residual peptide measured after
370 incubation with this gastric enzyme were different in each study. Two immunological
371 studies revealed the complete disappearance of both synthetic and soybean-isolated
372 lunasin after 2-minutes incubation with simulated gastric fluid containing pepsin in the
373 absence of BBI.[16,17] However, in BBI-free soymilk, a significant resistance (up to 60%)
374 of lunasin to pepsin treatment was reported.[18] The discrepancies among studies could be
375 due to multiple factors, such as the occurrence of other protease inhibitors, such as
376 Kunitz-trypsin and the isoinhibitor IBBD2, differences in lunasin:BBI ratio present in
377 products subjected to simulated pepsin digestion as well as methodology used to
378 evaluate the percentage of residual lunasin after pepsin proteolysis. To quantify lunasin,
379 the MS analysis used shows some advantages in comparison to Western-Blot and
380 ELISA methods. By one hand, this analysis allowed to quantifying lunasin that
381 remained intact after gastric and gastro-duodenal digestion.[26,27] In addition, the
382 application of MS to assess digests allowed, for first time, to identify peptides released
383 from lunasin by the action of digestive enzymes.

384 The qualitative and quantitative data obtained confirm the protective role of
385 IBB1 against lunasin hydrolysis by both gastric and pancreatic enzymes. In the presence
386 of active IBB1 at both lunasin:IBB1 ratios, lunasin is partially resistant to the action of
387 pepsin, being more than 35% of original peptide observed in the gastric digest. Lunasin
388 protection is not likely associated to pepsin inhibition by IBB1 given its null or
389 extremely low inhibitory activity against this gastric enzyme.[28,29] The absence of

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390 peptides released from IBB1 during the gastric phase indicated the extraordinary
391 resistance of this isoinhibitor to pepsin. It is well known the role played by the
392 disulphide bridge network in maintaining its correct folding and functional structure,
393 being responsible its extraordinary resistance to digestive proteases and thermal
394 treatment.[20,30,31] Conformational changes resulting from reduction of disulphide bridges
395 and alkylation of the cysteinyl sulfhydryl groups almost completely abolish both trypsin
396 and chymotrypsin inhibitory activities of BBI, decreasing its resistance to enzyme and
397 to high temperature.[32] Our study confirms that after inactivation of IBB1 by
398 reduction/alkylation, no inactive IBB1 was visible after gastric digestion, although the
399 minimum quantity of IBB1 remaining active during the inactivation process was enough
400 to partly protect lunasin from pepsin action. IBB1-derived peptides released by the
401 action of pepsin might also contribute protecting lunasin against this gastric enzyme.

402 In the case of the simulated duodenal phase, the trypsin and chymotrypsin
403 inhibitory activity of IBB1 was responsible for the protective role on lunasin from
404 digestion by these enzymes. The absence of IBB1 in digested mixtures provoked the
405 almost complete disappearance of lunasin, as it had been previously reported by
406 Hernández-Ledesma et al.[18] when BBI-free soymilk samples were digested with
407 pancreatin during 60 min. However, the presence of BBI has been suggested to exert a
408 protective role from lunasin's digestion.[33] These authors, by using immunological
409 assays, reported that 3% of lunasin present in lunasin-enriched soybean was detected at
410 the end of a simulated pepsin-pancreatin digestion. In our study, when lunasin:IBB1
411 mixtures at 1:2 ratio were digested, more than 5% of lunasin remained intact. The
412 consumption of 25 g of soybean protein per day of a diet low in saturated fat and
413 cholesterol, recommended by the US Food and Drug Administration to reduce the risk
414 of heart disease, leads to a total intake of 0.94 g of lunasin.[33] Of this oral intake, 5% will

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415 survive gastrointestinal digestion resulting in 47 mg of biologically active lunasin, with
416 ability to act at both local and systemic levels.

417 Intact soybean lunasin and IBB1 have demonstrated to exert anti-proliferative
418 effects against colon cancer using different cell culture models. Dia and de Mejia[34,35]
419 reported the cytotoxic activity of soybean lunasin in colon cancer HT-29, KM12L4,
420 RKO, and HCT-116 cells. Treatment of human colorectal adenocarcinoma HT-29 cells
421 with IBB1 has been described to reduce the cell proliferation in a concentration and
422 time-dependent manner, with an IC_{50} value of 39.9 μM.[22] However, to our knowledge,
423 no information on the potential anti-proliferative effects of digests obtained from these
424 two bioactive peptides during their transit through the gastrointestinal tract was
425 available. In our study, a notable colon cancer HT-29 and Caco2 cells proliferation
426 inhibitory effect was provoked after cells incubation for 24 hours with hydrolyzates
427 obtained at the end of simulated gastrointestinal digestion. Remaining lunasin and IBB1
428 as well as shorter peptides released after the action of pepsin and duodenal enzymes
429 could be responsible for the observed effects. To date, only one fragment corresponding
430 to lunasin f(23-43), which sequence is ^{23}EKHIMEKIQGRGDDDDDDDDD^{43}, has
431 demonstrated higher anti-proliferative activity in human breast cancer MDA-MB-231
432 cells than complete lunasin.[36]

433 A petition has been filed with the Food and Drug Administration for a health
434 claim that soy protein consumption as part of a low fat diet may reduce the risk of
435 certain cancers, including colon cancer.[37] Among polypeptides contained in soy protein,
436 lunasin and naturally occurring protease inhibitors such as BBI and Kunitz-trypsin
437 inhibitor, have become the most extensively studied for their colon cancer preventive
438 properties.[38] Recently, a protein-enriched soybean meal hydrolyzate showing high
439 resistance to simulated gastrointestinal digestion was fractionated and evaluated for its

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440 effects on cell proliferation of colon cancer HCT-116 and Caco2 cells.[39] The highest
441 potency was demonstrated for both low molecular weight peptide fraction (<5 kDa) and
442 fraction containing polypeptides (10-50 kDa), although the sequences of responsible
443 peptides were not elucidated. In our study, at the end of simulated gastrointestinal
444 digestive process, 29 lunasin- and 24 IBB1-derived fragments have been identified,
445 although the presence of each one depended on the mixture digested. Since the final
446 digests have been found to exert potent anti-proliferative properties against colon cancer
447 HT-29 and Caco2 cells, peptides liberated from lunasin and IBB1 might be the main
448 responsible for the observed effects. So far, this is the first study reporting that peptides
449 directly released from lunasin and IBB1 during their transit through the gastrointestinal
450 tract possess anticancer activities. Further studies are currently ongoing to synthesize
451 these peptides and confirm their potential anti-proliferative effects.
452

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535 **Figure legends**

536 Figure 1. UV-chromatograms obtained after the gastric phase of the digestive process
537 simulating the gastrointestinal digestion of lunasin:IBB1 mixtures at ratios of (A) 1:0
538 (w:w), (B) 1:1 (w:w), (C) 1:2 (w:w), and (D) 1:2 (w:w) with IBB1 inactive.
539 Chromatographic peaks (number or letters) corresponding to the lunasin- and IBB1-
540 derived peptides, respectively, were identified by mass spectrometry in tandem, and
541 shown in Supplementary Table S1.
542

543 Figure 2. Lunasin-derived peptides identified in the digests obtained after the (A-D)
544 gastric and (E-H) intestinal phase of the simulated gastrointestinal digestion from
545 lunasin:IBB1 mixtures at ratios of (A and E) 1:0 (w:w), (B and F) 1:1 (w:w), (C and G)
546 1:2 (w:w), and (D and H) 1:2 (w:w) with IBB1 inactive. Susceptible peptide bonds in
547 absence of IBB1 but resistant in the presence of this isoinhibitor are indicated with an
548 arrow.
549

550 Figure 3. IBB1-derived peptides identified in the digests obtained after the (A) gastric
551 and (B) intestinal phase of the simulated gastrointestinal digestion from lunasin:IBB1
552 inactive mixture (1:2, w:w).
553

554 Figure 4. UV-chromatograms obtained at the end of simulated gastrointestinal digestion
555 of lunasin:IBB1 mixtures at ratios of (A) 1:0 (w:w), (B) 1:1 (w:w), (C) 1:2 (w:w), and
556 (D) 1:2 (w:w) with IBB1 inactive. Chromatographic peaks (number or letters)
557 corresponding to the lunasin- and IBB1-derived peptides, respectively, were identified
558 by mass spectrometry in tandem, and shown in Supplementary Table S1.
559

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560 Figure 5. Effect on proliferation of human colon adenocarcinoma (A) HT-29 and (B)
561 Caco-2 cells shown by the digests obtained at the end of simulated gastrointestinal
562 digestion from digestion medium without lunasin and IBB1, and lunasin:IBB1 mixtures
563 at different ratios and protein concentration (■) 0.1 mg/mL, (■) 0.2 mg/mL, and (□) 0.3
564 mg/mL. Cell viability was evaluated by the MTT assay. Experiments were carried out in
565 triplicate, and the results were expressed as percentage of viable cells compared to
566 control non-treated cells, considered as 100%. Different letters denote statistically
567 significant differences ($p < 0.05$) between digests at the same protein concentration and
568 the corresponding dilution of digestion medium without peptides.
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570 **Table 1.** Intact lunasin (expressed as % of initial intact lunasin) measured in the digests
571 obtained after the gastric and the intestinal phases of the digestion process simulating
572 gastrointestinal conditions from the lunasin:IBB1 mixtures at different ratios. Digestion
573 was carried out in duplicate and two HPLC-MS/MS analysis were run for each digest.
574

Lunasin:IBB1 ratio (w:w)	**Intact lunasin (%)**	
	Gastric digest	**Gastric + Intestinal digest**
1:0	2.6 ± 0.4^{a}	0.1 ± 0.1^{a}
1:1	34.3 ± 3.7^{b}	1.8 ± 0.4^{b}
1:2	35.1 ± 2.8^{b}	5.3 ± 0.4^{c}
1:2 (inactive IBB1)	28.4 ± 3.6^{b}	1.5 ± 0.2^{b}

575
576 $^{a\text{-}c}$ Different superscript letters within the same column denote statistically significant
577 differences ($p < 0.05$)

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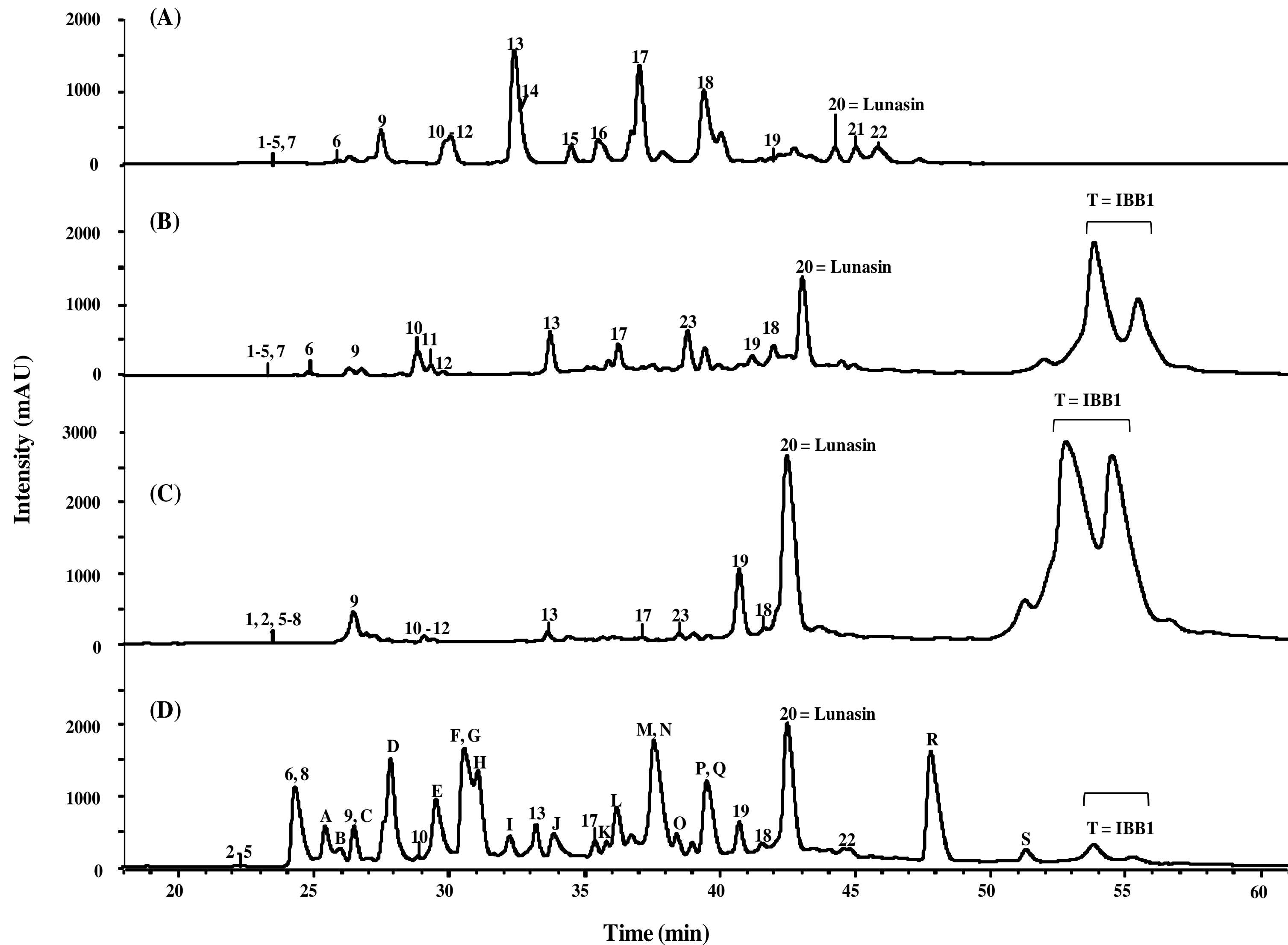

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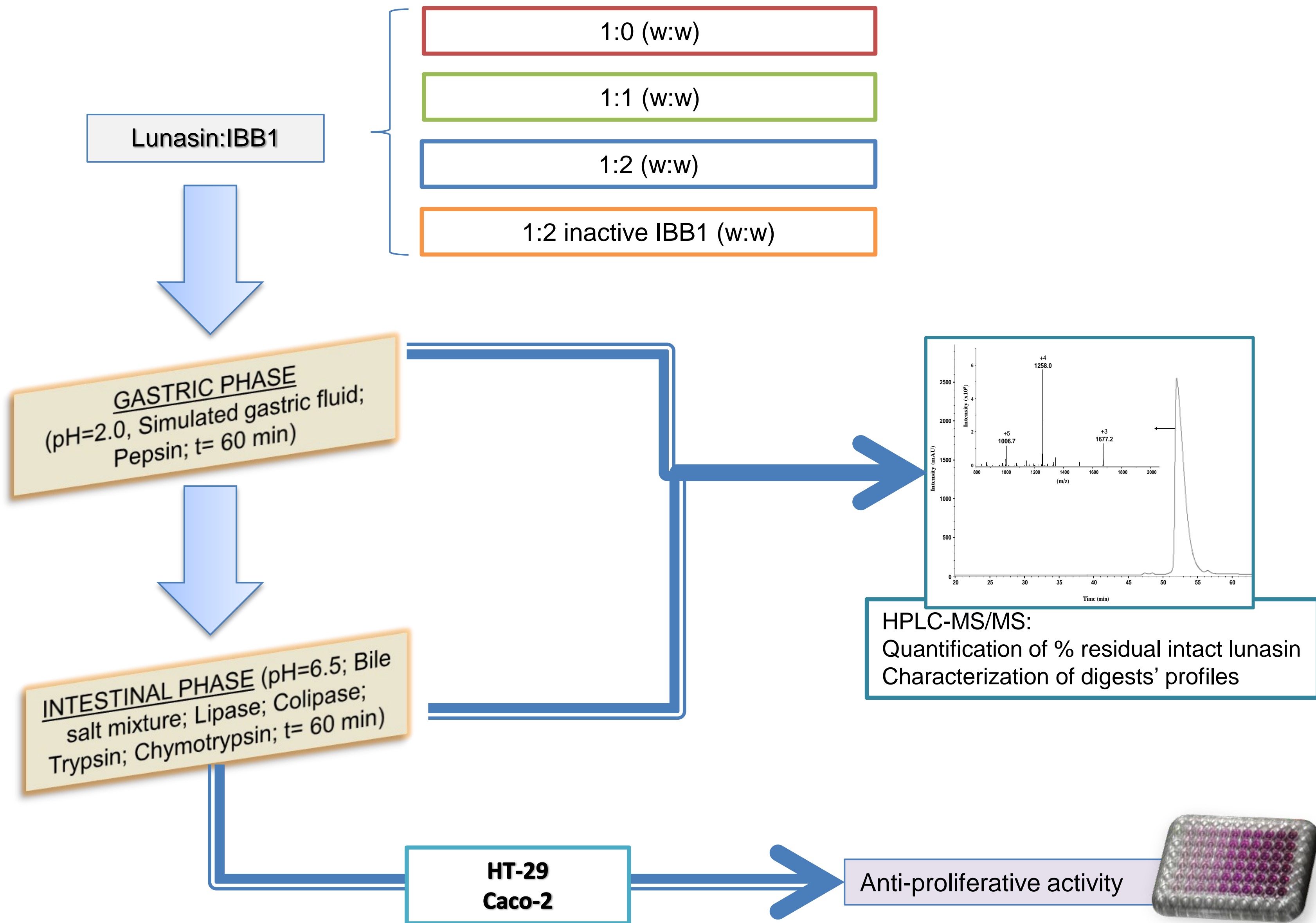

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VNLTPCEKHIME
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LTPCEKHIME
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LTPCE
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(B)
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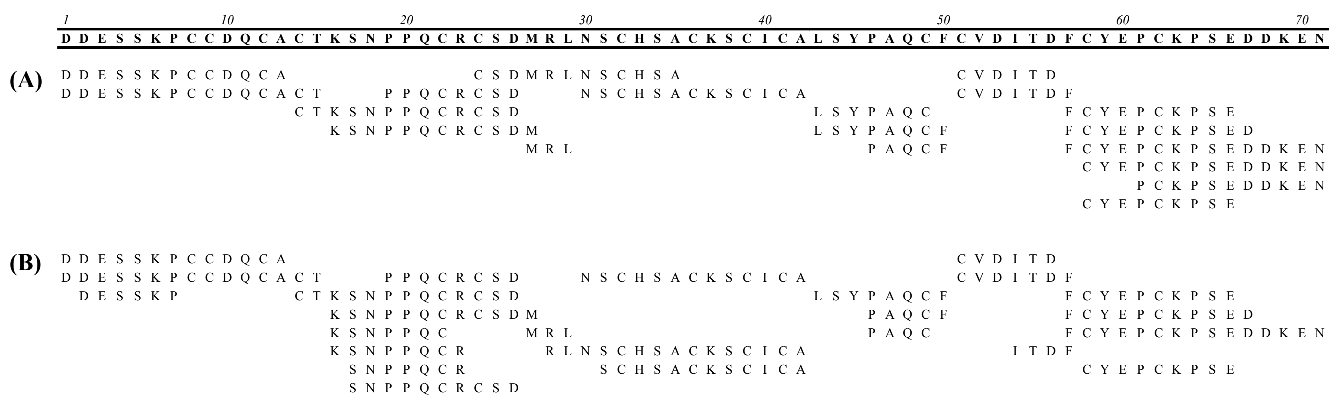

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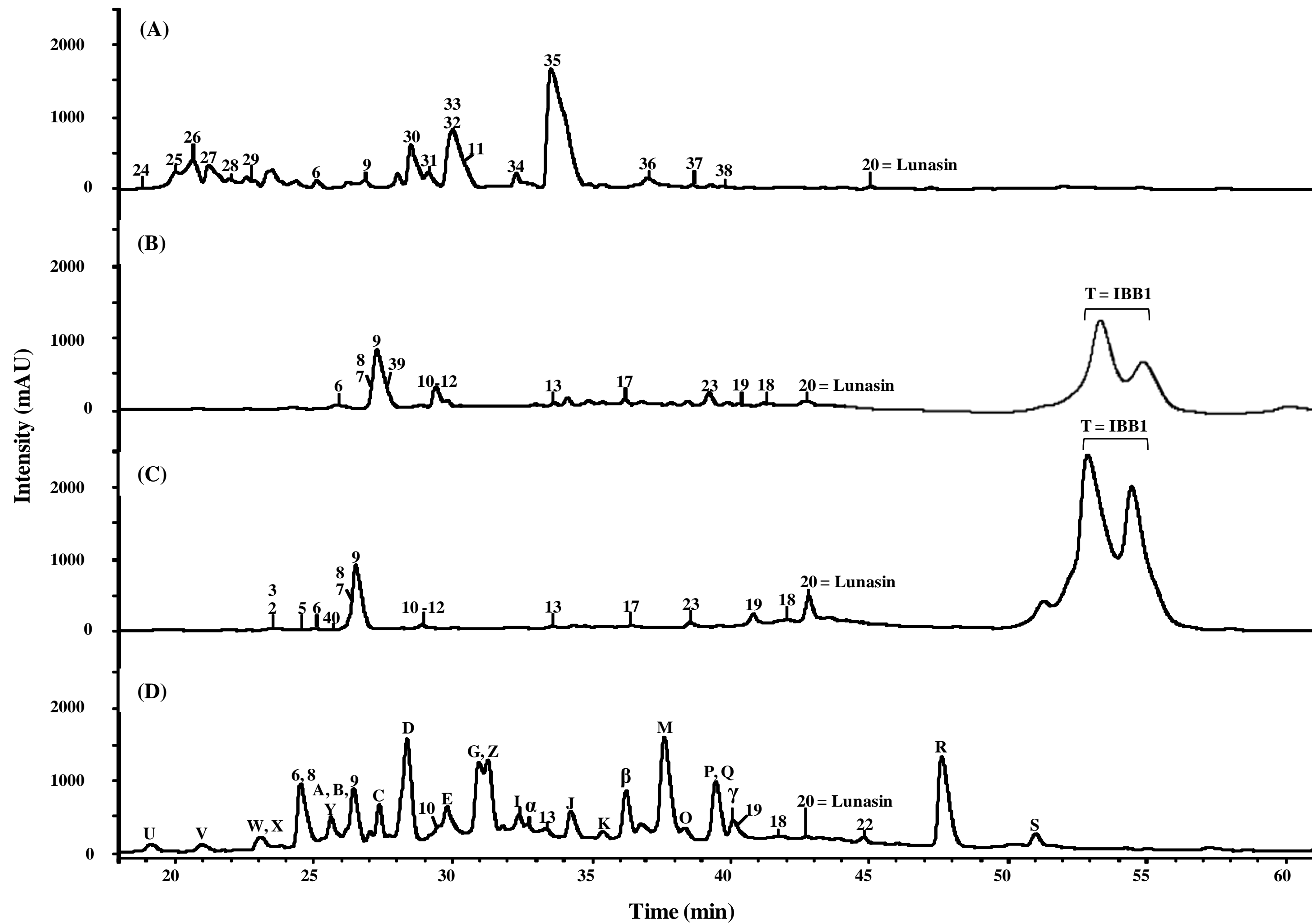

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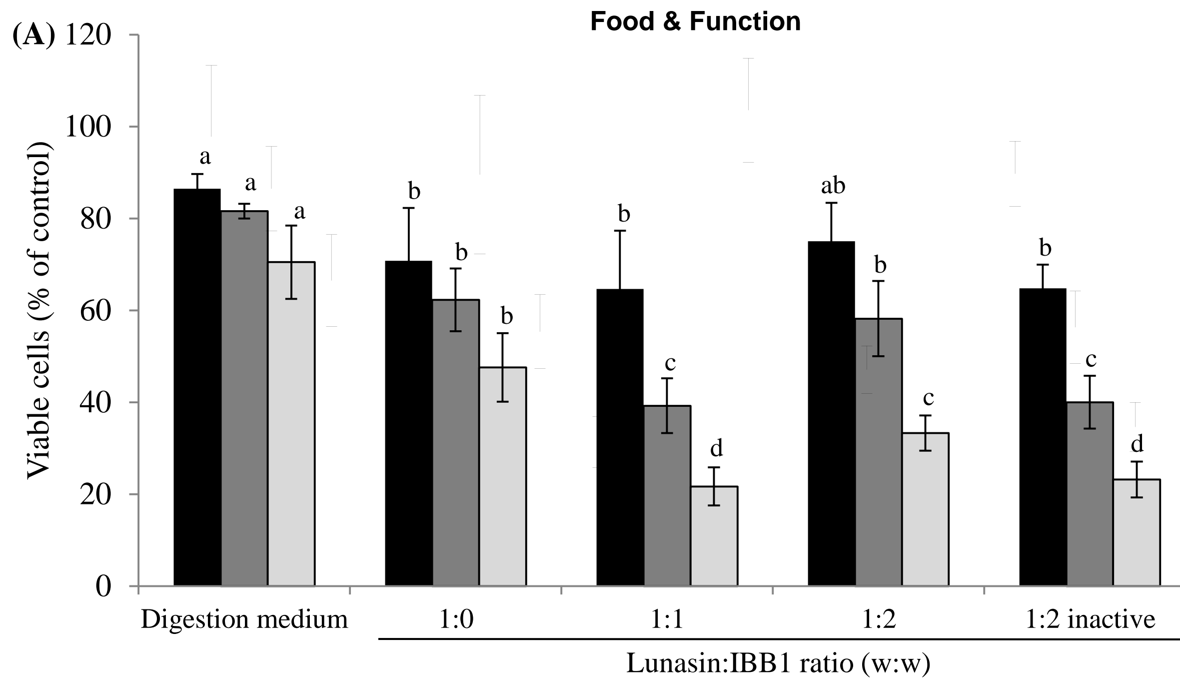

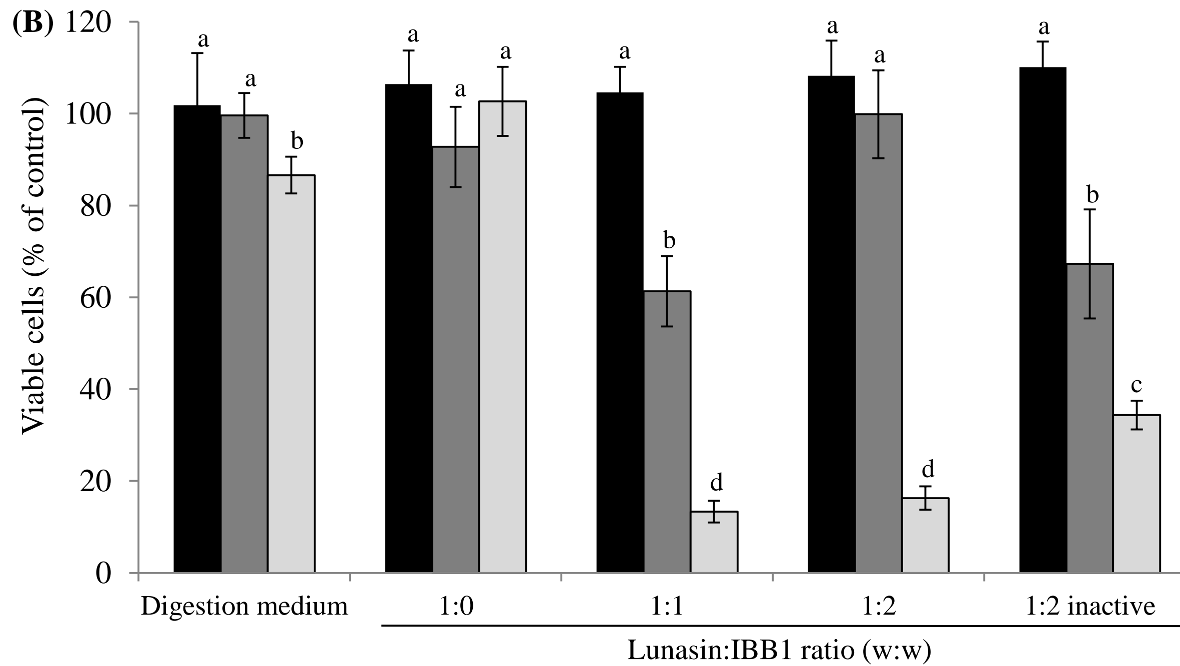

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