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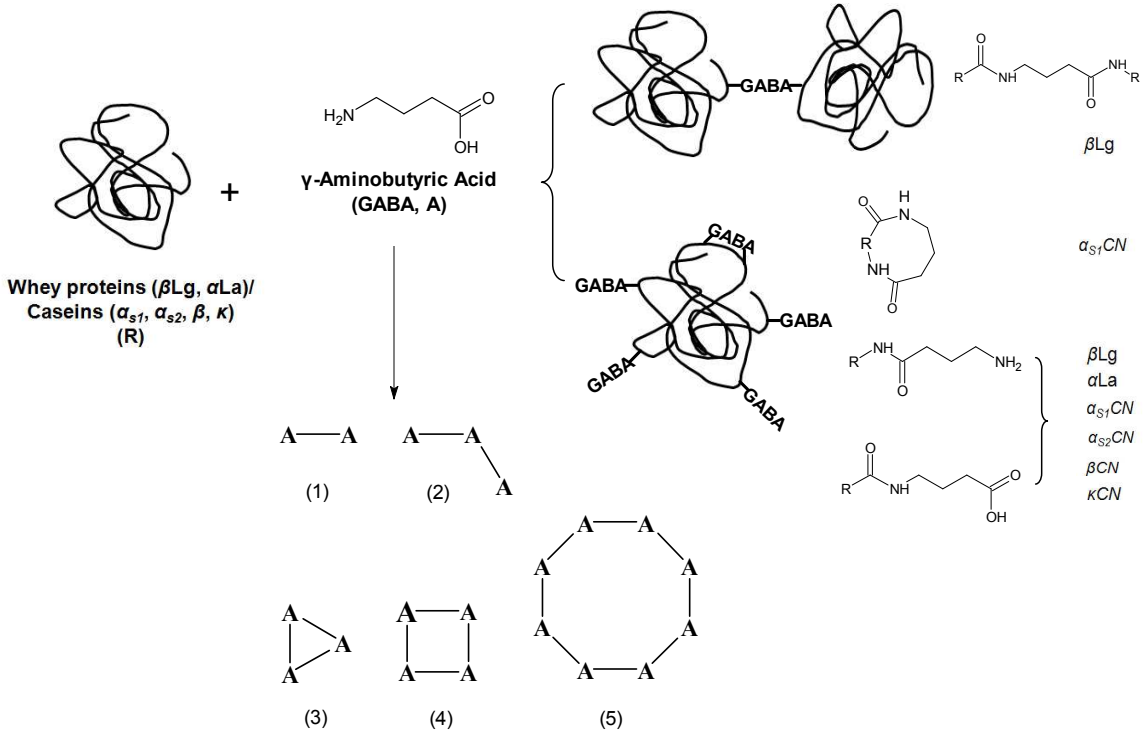
Graphical abstract



Fate of γ-aminobutyric acid is to react with whey proteins/caseins and itself polymerization during fortified milk production.

Highlighting the novelty of the work:

γ-Aminobutyric acid mainly cross-links β-Lg fraction and adducts with α-La or α_{s1}-casein fractions, and tends to form its linear or membered ring structure oligomers.

Interactions of γ-aminobutyric acid and whey proteins/caseins during fortified milk production

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ABSTRACT: The potential interactions between milk proteins (whey proteins and caseins) and γ-aminobutyric acid (GABA), alone and in combination, and the physiochemical properties of GABA-fortified milk were investigated in the present study. After mixing 0.05–1.0% (w/w, based on the solution) GABA with 0.6% (w/v) whey proteins and with 2.6% (w/v) caseins, sequential preheating (60 °C), homogenization (20 MPa), and pasteurization (72 °C for 15 s and 138 °C for 2 s) processes were used to simulate practical processing conditions for GABA-fortified milk production. GABA mainly cross-linked β-lactoglobulin (but had no remarkable cross-linking effect on caseins), adducted with α-lactalbumin and α_{s1}-casein fractions (not excluding other fractions in whey proteins/caseins) via the amide linkage under the sequential processing conditions, which was confirmed by electrophoresis, mass spectroscopy, and amino acid composition analysis. Characterization of the physiochemical properties (protein solubility, relative surface hydrophobicity, ζ-potential, particle size, and fluorescence intensity) of the GABA-fortified protein solutions substantiated the presence of the cross-links and adduction reactions. In addition, GABA tended to form its dimer, trimer (line-like structure) and 3–8 membered ring structure oligomers. The interaction between GABA and whey proteins/caseins provides insight into the evaluation of the food quality and chemical safety (due to protein/GABA derivatives) of GABA-fortified milk.

Keywords: γ-Aminobutyric acid, Milk proteins, Interaction, Sequential processes

1. Introduction

Growing interest in functional food development has been stimulated by the demand for partial or full alternatives to drugs and has helped reduce the side effects, promote health, and reduce disease risk.[1,2] Such foods are expected to render physiological benefits beyond their traditional nutritional value. In recent years, γ–aminobutyric acid (GABA) has typically served as an important functional factor playing an increasing role in the food industry.[3] It is a ubiquitous, four-carbon, non-protein amino acid, which exerts many physiological functions in humans and animals, mainly by acting as an inhibitory neurotransmitter in the central nervous system.[4,5] Because of the various physiological functions of GABA, such as its anti-hypertensive,[5,6] sedative,[7,8] anti-tumorigenic,[9] anti-diabetic,[10,11] anti-inflammatory,[12] and sleep-promoting activities,[13] extensive studies have focused on the enrichment of functional foods with GABA.[3]

Many foods or foodstuffs such as legumes,[14] meats,[15] cereal-based products,[16–18] beverages (Hou, Jeng, & Chen, 2010; Kim, Lee, Ji, Lee, & Hwang, 2009), vegetables,[19,20] and dairy products[22–24] have been successfully enriched with GABA by the method of biological conversion and have been endowed with physiological functions that have further increased the commercial value of these products. In general, these approaches to functional food production involve germination or fermentation by microorganisms, which involves *in vivo* enzymatic formation of GABA from glutamic acid in a reaction catalyzed by the enzyme glutamic acid decarboxylase.[25] However, for some other foods such as liquid pasteurized milk, this GABA fortifying strategy may not be suitable. Continuing attempts using some dairy products have been made, primarily in yoghurt[26] and cheese products.[22–24] Technically, the direct addition approach for GABA to fortify liquid milks is available according to

our previous work. [27] There appear to be increasing demand for simple and economical food processing procedures for the production of functional food.

Structurally, GABA contains one carboxylic group and one γ-amine group.[4] When GABA passes through the sequential treatments of preheating, homogenization, and pasteurization involved in the production of milk, the potential reactions between milk proteins and GABA and the effect of the processing conditions on the modification of GABA are unknown. However, according to our previous investigation,[27] GABA was mainly consumed by proteins and lactose during fortified milk production. Therefore, we hypothesized that potential reactions occur and these could induce interactive effects on the processing properties of liquid milks and consequently on the food quality of the resulting products.

The present study was conducted to primarily explore the potential reactions between milk proteins and GABA or of GABA alone during the production of GABA-fortified milks. To achieve this objective, a simulated procedure for alternatives to practical processing conditions was used. The main fractions of milk proteins, namely whey proteins and casein, were separately mixed with GABA, and the resulting mixtures were then subjected to sequential processes. The structural and chemical changes in the processed proteins and GABA samples were also analyzed to determine the reactions between milk proteins and GABA. These changes are not only physical but chemical. On the one hand, the new substances that chemically generated during GABA fortification are uncertain; on the other hand, the effect of GABA addition on the physical stability of milk is still unclear.

2. Materials and methods

2.1. Materials and chemicals

Casein (90% protein, soluble sodium caseinate) was purchased from Murray Goulburn Cooperative Co. Ltd. (Murray Goulburn, VIC, Australia), and whey protein isolate (90% protein) was gifted from Hilmar Ingredients (Hilmar, CA, USA). GABA (99% purity) was ordered from Shanghai Bangcheng Chemical Co., Ltd. (Shanghai, China). 8-Anilino-1-naphthalene sulfonic acid (ANS) and chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein hydrolysis to yield amino acids were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade or higher and were purchased from Lingfeng Chemicals (Shanghai, China).

2.2. Fortification of whey proteins/caseins with GABA

In the milk, whey proteins and caseins contents were 0.6% or 2.6% (w/v), respectively. In this experiment, we used the same concentrations to simulate a practical milk process. Aqueous solutions of whey proteins/caseins were prepared by stirring their dry powders in distilled and deionized water until completely solubilized. GABA was added to the aqueous protein solutions with ranges of 0.05–1.0% (w/w, based on the milk solution) and thoroughly stirred to dissolve. It was observed that GABA concentration did not significantly affect the sample's pH, and the pH ranges were from 6.8 to 7.1. The samples were then subjected to the sequential treatments of 60 °C preheating, 20 MPa homogenization, and two types of pasteurization (72 °C for 15 s and 138 °C for 2 s). After cooling to room temperature, the samples were stored at 4 °C for further use. For measurement of the ζ-potential and particle size, the samples were freshly prepared.

101 **2.3. Protein solubility**

102 The prepared samples described above were used for the determination of protein solubility. For
103 caseins, the samples were diluted 4-fold in distilled and deionized water. These samples were then
104 centrifuged at 12,000 g for 20 min at 22 °C. The protein content of the supernatant was determined
105 using the biuret method.[28] The protein solubility was defined as the ratio of the measured content
106 versus the prepared content of protein.

107

108 **2.4. Surface hydrophobicity**

109 A hydrophobic fluorescence probe method using ANS was used to determine the surface
110 hydrophobicity of the GABA-fortified whey proteins/caseins solutions according to the method of
111 Hayakawa & Nakai[29] with a slight modification. The protein concentration was diluted to 0.02% (w/v)
112 with 100 mM phosphate buffer (pH 7.0), and 4.0 mL of this solution was then added to 20 μL of
113 0.04% (w/v) ANS (dissolved in above phosphate buffer). After keeping at 22 °C for 15 min, the
114 fluorescence intensity of the ANS-treated sample was recorded using a spectrofluorometer (Hitachi
115 650-60; Kyoto, Japan) with excitation (365 nm) and emission (470 nm) slits set at 5 nm. The surface
116 hydrophobicity of protein (whey/casein) without treatment of GABA was assigned a value of 1. The
117 data (relative surface hydrophobicity, RSH) were plotted against the hydrophobicity intensity of the
118 GABA-fortified whey proteins or caseins solutions.

119

120 **2.5. ζ-Potential and particle size**

121 The method of Cheng, Chen & Xiong[30] was used with a minor modification to characterize the

GABA-fortified whey proteins/caseins solutions. The abovementioned samples were freshly prepared and then diluted to 1.0% (m/v) in 0.01 mol L^{-1} phosphate buffer, pH 7.0 (for caseins; whey protein samples were not diluted). The particle size and ζ-potential of the diluted samples were measured using a Mastersizer Nano-ZS instrument (Malvern Instruments, Worcestershire, UK). The particle size and ζ-potential were expressed as a mean diameter Z-average and average of three freshly prepared samples, respectively.

2.6. Fluorescence intensity

A spectrofluorometer (Hitachi 650-60; Kyoto, Japan) was used to scan the fluorescence intensity of the prepared samples. The emission and excitation slits were set to 5 nm, as described by Agyare, Xiong, & Addo.[31] The test samples were diluted to 2.0 mg mL^{-1} with 50 mM Tris–HCl (pH 7.5), excited at 280 nm, and the emission intensities were recorded from 300–400 nm.

2.7. Electrophoresis

Protein patterns of the solutions were determined using SDS-PAGE, 5% stacking gel, and 12.5% running gel according to the method of Laemmli,[32] with a slight modification. Amount of sample in terms of 25 μg each one was loaded onto the gel and subjected to electrophoresis at a constant current. After separating, the gel was then subjected to sequential staining, destaining and densitometry determination.

2.8. Mass spectrometry

For the preparation of samples, GABA alone was subjected to the sequential treatments of 60 °C preheating, 20 MPa homogenization, and pasteurization at 138 °C for 2 s. The above samples were pre-frozen at -40 °C for 4 h and then subjected to vacuum drying at 0.02 MPa for 48 h. Molecular weight analysis was performed using high-performance liquid chromatography (HPLC) tandem electrospray positive-ionization mass spectrometry (ESI–MS) (Agilent 1100 LC/MSD SL; Agilent Technologies, Inc., Palo Alto, CA, USA). The mobile phase was a buffer containing acetonitrile and water (50:50, v/v; 0.1% formic acid, v/v) at a flow rate of 0.15 mL min^{-1}, and an AZorbax C8 column (200 × 4.6 mm i.d., Agilent Technologies). The LC column eluate was injected into the ion source (fragmentor voltage, 90 V), and the mass spectrometer m/z ratio range was 100–1000 in the full scan mode. For the mixture of whey proteins/caseins preparation, the molecular weights of the samples were quantified using an Applied Biosystems 4700 proteomics analyzer equipped with matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI–TOF) (Foster City, CA, USA).

2.9. Amino acid composition

The concentration of amino acids was determined as previously described.[33] The prepared samples of whey proteins/caseins (mixed with GABA) and control were hydrolyzed under vacuum using 6 N HCl at 110 °C for 22 h. Reverse-phase HPLC was performed on an Agilent 1100 (Agilent Technologies, CA, USA) assembly system to measure the amino acid content.

2.10. Statistical analysis

All determinations were performed in triplicate and expressed as means ± standard deviation, and the

164 data were analyzed using Statistix software, version 9.0 (Analytical Software, Tallahassee, FL). The
165 general linear model procedure was used to determine the statistical significance ($p < 0.05$) of the
166 differences between the means.
167

168 # 3 Results and discussion

169 **3.1. Influence of GABA addition on the physicochemical properties of whey proteins/caseins**

170 Solubility is often regarded as one of the most fundamental parameters for characterizing proteins and
171 is associated with the functional properties of the proteins.[34] Solubility also can be used to evaluate the
172 degree of protein modification. In the preparation of a GABA-fortified milk system, interactions can
173 potentially occur between milk proteins (whey proteins and caseins) and GABA, alone and in
174 combination, and the physiochemical properties of GABA-fortified milk. Fig. 1 shows a comparison
175 of the effects of pasteurization temperatures (72 °C and 138 °C) on protein solubility. For the whey
176 proteins, it can be observed that the solubilities of the 72 °C pasteurized protein samples were
177 dramatically higher than those of the 138 °C samples ($p < 0.05$), and the solubility significantly
178 increased as the amount of GABA increased ($p < 0.05$). The casein samples showed a similar behavior.
179 In general, protein solubility decreases when heated at a sufficiently high temperature because
180 denaturation due to exposure of hydrophobic groups occurs.[35] However, for the samples with added
181 GABA that were pasteurized at the same temperature, the increase in protein solubility suggested that
182 structural modification caused by GABA had occurred. Data obtained later also reflected changes in
183 protein solubility.

184 To verify the structural modification of proteins, RSH of these samples was further tested (Fig. 2).

185 Higher temperature pasteurization induced higher levels of RSH, and the effect, which was observed
186 not only for whey proteins but also for caseins, was remarkable ($p < 0.05$). Similarly, RSH of these
187 protein samples further increased with increasing amounts of GABA. We postulated that the proteins
188 were modified by GABA, which was thought to have induced thinning via an electrostatic shield or an
189 electric double layer that may have formed around the milk proteins.[36] Overall, the protein
190 conformations driven by heating and addition of GABA appeared to expose hydrophobic residues.

191 From the viewpoint of molecular aggregation and static electricity, the results of interactions
192 among proteins and GABA or of reactions with GABA alone could be attributed to protein clustering
193 and electric charge distribution. Fig. 3 illustrates the variations in the particle sizes and ζ-potentials
194 versus GABA concentration in the fortified milk preparation solutions. As shown in the figure, higher
195 temperature pasteurization (138 °C) induced remarkably higher particle sizes and lower ζ-potentials
196 relative than lower temperature pasteurization (72 °C). For both samples (whey proteins and caseins)
197 pasteurized at 72 °C or 138 °C, the particle size of the prepared solutions significantly decreased ($p <$
198 0.05) with increasing amounts of GABA, whereas the ζ-potentials remarkably increased ($p < 0.05$)
199 with increasing amounts of GABA. It was also observed that the casein samples had relatively larger
200 particle sizes (Fig. 3.A, B) and higher ζ-potentials (Fig. 3.A′, B′) than the whey protein samples. The
201 decrease in particle size may be attributed to the causes that once interacting with a partially denatured
202 protein, a charged molecule as GABA could help by increasing electrostatic repulsion among protein
203 chains. With regard to the ζ-potentials, the ionizable groups were confirmed to be distributed on the
204 surface of the protein cluster; therefore, the increases in ζ-potentials with decreases in particle size
205 may be attributed to the increase in the specific surface area of the particles.[37] Chemically, it could be

206 possible that the interaction between GABA with milk proteins was mostly through amino group
207 leaving the negatively charged carboxyl free.[38]

208 Among the selected physicochemical properties of the prepared GABA-fortified samples, the
209 obtained results matched well. In addition, these behaviors of the proteins and GABA strongly suggest
210 interactions among the milk proteins and GABA. Interactions such as these could alter the food
211 structure and generate new substances that potentially affect food safety adversely or, on the contrary,
212 improve the quality and/or safety of the milk. The physiochemical properties of the GABA-fortified
213 milk proteins suggest possible physical and chemical reactions and may consequently affect milk
214 quality.

215

216 **3.2. Structural modification of whey proteins/caseins**

217 To detect the influence of GABA-interacting proteins on the structure of resulting proteins, the
218 intrinsic fluorescence spectra between 300–400 nm were acquired to probe possible reactions. The
219 specific results of samples with 0.05–1.0% (w/w) of GABA added are shown in Fig. 4. We found that
220 GABA effectively increased the fluorescence intensity of the whey proteins. A similar tendency was
221 also observed for the casein samples. Aromatic amino acids, including phenylalanine, tryptophan, and
222 tyrosine, can produce fluorescence, and they exhibited different fluorescence spectra with maximum
223 absorption peaks at 282, 355, and 304 nm, respectively.[39] It is also known that tryptophan residues
224 have high quantum yield and mainly produce fluorescence.[40] It is very possible that the addition of
225 GABA causes tryptophan residues to expose. This result is consistent with the finding from RSH (Fig.
226 2) because tryptophan is also a very hydrophobic amino acid.[41] In addition, it was also observed that

these samples, especially for whey proteins, their absorption peaks marginally shifted from 333 nm to 336 nm as GABA concentration increased. Therefore, it is quite possible that interaction between milk proteins and GABA occurred.

3.3. Chemical modification of whey proteins/caseins and GABA

Electrophoresis under non-reducing and reducing conditions was performed to determine covalent cross-linkages between proteins and GABA as a result of sequential processing during fortified milk production. For the whey proteins, the band intensity of bovine serum albumin was not detected with increasing GABA concentrations, whereas bands for the β-lactoglobulin (β-lg) trimer appeared when the GABA concentration exceeded 0.5% (w/w). Increasing the concentration of GABA from 0.05% to 1.0% (w/w) led to increases in the β-lg dimer and polymers accumulated at the top of the stacking gels. However, β-lg and *α*-lactalbumin (α-la) monomers showed no substantial change (Fig. 5.A). After these samples were reduced by βME, the polymer at the top of the stacking gel and the β-Lg trimer completely disappeared, whereas the β-lg dimer decreased but remained visible (Fig. 5.A′). This result suggested that covalent bonds other than disulfides were formed during the sequential process (preheating, homogenization, and pasteurization) when GABA was added. The results of the casein samples to which GABA was added and which underwent sequential processing differed from those of the whey protein samples. Under non-reducing conditions, the polymer near the start of the separating gel substantially increased when GABA was increased from 0.05% to 1.0%, and the band intensities of these polymers were thicker than those of the control (no GABA addition but underwent sequential processing). The components of caseins exhibited no remarkable effects caused by GABA (Fig. 5.B).

248 After these polymers were treated with βME, the polymers at the top decreased but were still visible,
249 and the other main fraction (α_s-casein and β-casein) also recovered to some extent. Compared with the
250 control, the remaining band intensities of the samples showed no notable differences (Fig. 5.B′). To
251 quantify these factions (bonds) content for further accuracy, densitometry was also conducted (Fig. 6).
252 This result is strongly consistent with the mass spectra data.

253 To further verify the effect of GABA on cross-linking of the whey proteins and caseins, the
254 molecular weights of the prepared samples, which were mixtures of whey proteins/caseins and 1.0%
255 (w/w) GABA, were also determined by MALDI–TOF–MS. Fig. 7.A shows the presence of the
256 fractions, which had molecular weights of 14,678 Da, 18,264 Da, and 18,325 Da. In contrast to the
257 previously reported values for α-la (molecular weight of 14,178 Da) and β-lg (molecular weights of
258 18,363 Da and 18,277 Da, variants A and B, respectively),[42,43] the former (labeled "a") is notably
259 different from the literature value. The found molecular mass corresponds to a GABA-α-la adduct
260 with a 6:1 stoichiometry, the resulting molecular mass was very close (1α-la + 6GABA – $6H_2O$,
261 14,688 Da) to the observed value for the α-la molecular mass. For β-lg (labeled "b_1" and "b_2"), the
262 observed and theoretical values were similar. In other words, the peaks "a", "b_1" and "b_2" at 14,678 Da,
263 18,264 Da, and 18,325 Da were α-la+6GABA derivatives and β-lg genetic variants B and A,
264 respectively. Based on the theoretical value of the variant A of β-lg, its dimer mass should be 36,708
265 Da (2β-lg – $1H_2O$), which is very close to the observed molecular weight of 36,718 Da (labeled "c").
266 In the SDS-PAGE experiment, we observed that the non-reduced fraction markedly increased with
267 increasing GABA amount (Fig. 5.A′). It was logically believed that β-lg dimer formation (labeled "c",
268 which via non-SS covalent bonds). GABA also adducted with β-lg monomer. The theoretical

β-lg–GABA–β-lg molecular weight is 36,793 Da (2β-lg + 1GABA − $2H_2O$), which is in close agreement with the observed value (molecular weight of 36,753 Da, as labeled "d"). For casein samples, four main fractions, which had molecular weights of 18,711 Da, 23,201 Da, 23,949 Da, and 24,331 Da, were observed (Fig. 7.B); however, these molecular masses were approximate to the theoretical values of α_{s1}, β except κ and α_{s2} fractions (molecular weights of 23,164 Da, 23,983 Da, 19,038 Da, and 25,388 Da, respectively) in caseins, as reported in the literature.[44] For the peaks "e" and "h", the corresponding molecular weights obviously deviated from the values for the κ and α_{s2} fractions in the caseins. It was noticed that no κ-casein fraction was present in the gels on the casein samples (Fig. 5.B, B′), the peak "e" did not denote the κ-casein fraction. Moreover, the m/z at 24,331 (peak "h" in Fig. 7) could not be the α_{s2} fraction. For the other casein fractions, the β-casein fraction (peak "g") was particularly consistent with its monomer, whereas for the α_{s1} fraction (peak "f"), the observed value was moderately higher than its theoretical value; it could be adducted with one molecule of GABA (namely $1\alpha_{s1}$ + 1GABA − $2H_2O$). The peaks "e" and "h" have not been identified to κ-casein and α_{s2} fractions, respectively. This could be these two fractions degradation caused by hydrolysis during the casein harvest or their variants. As previously determined,[27] the decreasing of of free amine and carboxyl content echo above MS data herein further confirmed this hyphothesis.

From the above mentioned data, we hypothesized that particular potential chemical reactions between the milk proteins and GABA occurred, as shown in Fig. 8. For the whey proteins, the non-reduced band in the gels indicated β-lg cross-linking. When MALDI–TOF–MS was used to test the molecular weight, it was confirmed that GABA played a role in producing cross-linked β-lg and adducts with α-la. The decreased free amine and carboxyl content suggested that reaction (**A**) was

290 mainly responsible for β-lg and GABA cross-linkage because reactions (**C**) and (**D**) did not deplete
291 free amines and carboxyl. However, we could not exclude the reactions (**B**), (**C**), and (**D**) from
292 involvement in the β-lg and GABA adduction, and not specify reactions (**B**), (**C**), and (**D**) for α-la and
293 GABA adduction. Other techniques (e.g., high-resolution MS and restricted enzymatic hydrolysis)
294 were introduced into the current study that of necessary for identification of the specific fractions
295 which participate in these reactions. Similarly, for caseins, there was no band of a notable
296 concentration or new bands. However, the decreases in free amine and carboxyl content of the caseins
297 illustrated the reactions due to GABA addition. MALDI–TOF–MS analysis suggested that reaction (**B**)
298 could have accounted for the α_{s1} fraction in the caseins and GABA adduction. Moreover, we could not
299 exclude reactions (**C**) and (**D**) for α_{s1} or the other fractions in the caseins.

300 Chemical bonds were formed via the reaction of the carboxyl groups (or amine groups) of the
301 non-protein amino acids such as GABA with the amine groups (or carboxyl groups) of the protein
302 amino acids. To identify the acid hydrolysis products of these covalent bonds, the amino acid
303 composition of the resulting milk proteins was measured using hydrochloric acid (Table 1). For most
304 amino acids, there was no significant difference in the amount lost between the samples and control.
305 However, we observed that proline showed appreciable losses, particularly for the caseins with GABA
306 samples. As reported, proline is an amino acid that has been shown to be very susceptible to
307 oxidation,[45] which could be attributed to oxidative potential in the sequential processes altered by
308 GABA. This result suggested that amidation and acetylation reactions dominate main chemical
309 interaction between whey proteins/casein and GABA.

310 The GABA behavior during the sequential processes was also determined by HPLC–ESI–MS,

311 and the results are shown in Fig. 9. It can be seen that the GABA monomer tended to form its dimer-
312 and trimer line-like structure oligomers (Fig. 9.A, E) and 3–8 ring-like structure oligomers through
313 terminal amines and carboxyl-forming peptide bonds (Fig. 9.B, C, D, F). A previous experiment
314 indicated that GABA was reduced under sequential processing conditions.[27] In the present study,
315 GABA that had converted to its ring or line-like oligomers accounted for the GABA loss.
316

317 ## 4. Conclusions

318 In conclusion, this study explored the potential interactions of GABA and whey proteins/caseins
319 during fortified milk production. The result indicated that GABA mainly cross-links β-lg fraction and
320 adducts with α-la or α_{s1}-CN fractions both through amidation and acetylation reactions, whereas these
321 interactions consequently induced GABA loss and improved solubility of milk proteins. Moreover,
322 GABA also tended to form its linear or membered ring structure oligomers, which partially
323 contributed to GABA loss. Overall, the findings of this work could provide a rational consideration for
324 the GABA-fortified bovine milk production.
325

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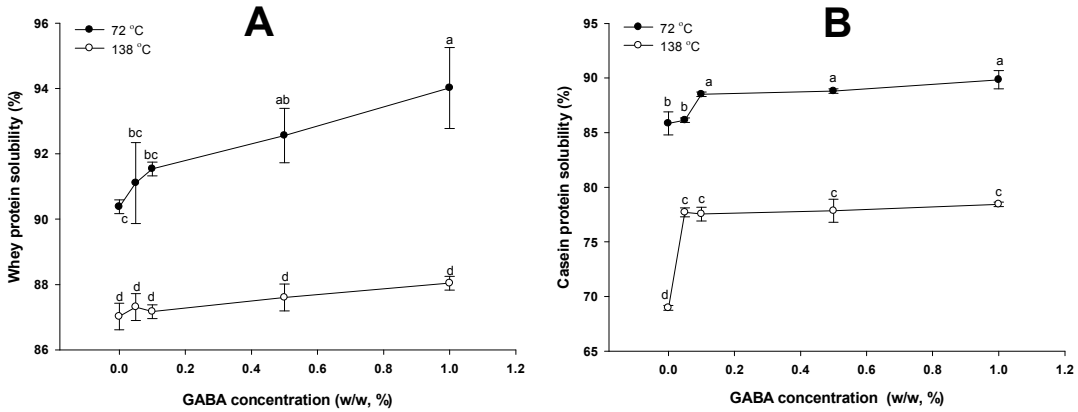


Fig. 1 Solubility of whey proteins (A) and caseins (B) mixed with different GABA concentrations (0.05–1.0%, w/w) and then treated by sequential 60 °C heating, 20 MPa homogenization, and 72 °C or 138 °C pasteurization.

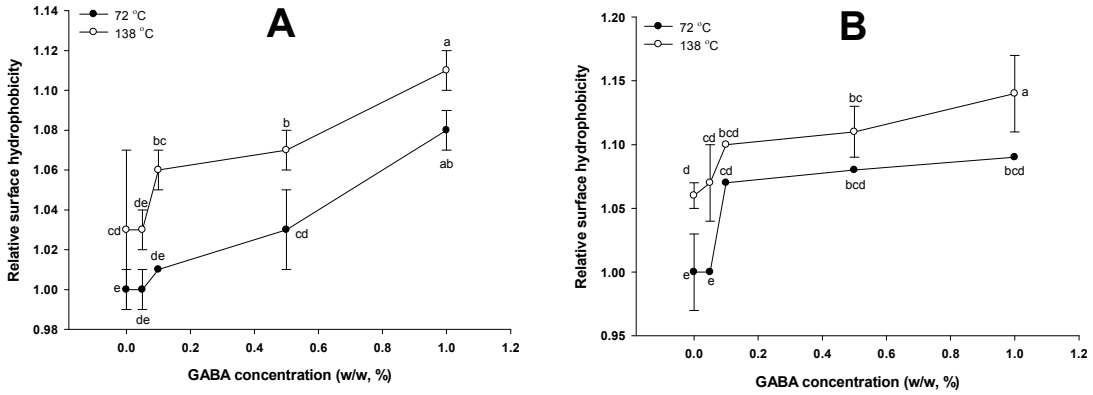


Fig. 2 Relative surface hydrophobicity of whey proteins (A) and caseins (B) mixed with 0.05–1.0% (w/w) GABA and then subjected to sequential 60 °C heating, 20 MPa homogenization, and 72 °C or 138 °C pasteurization.

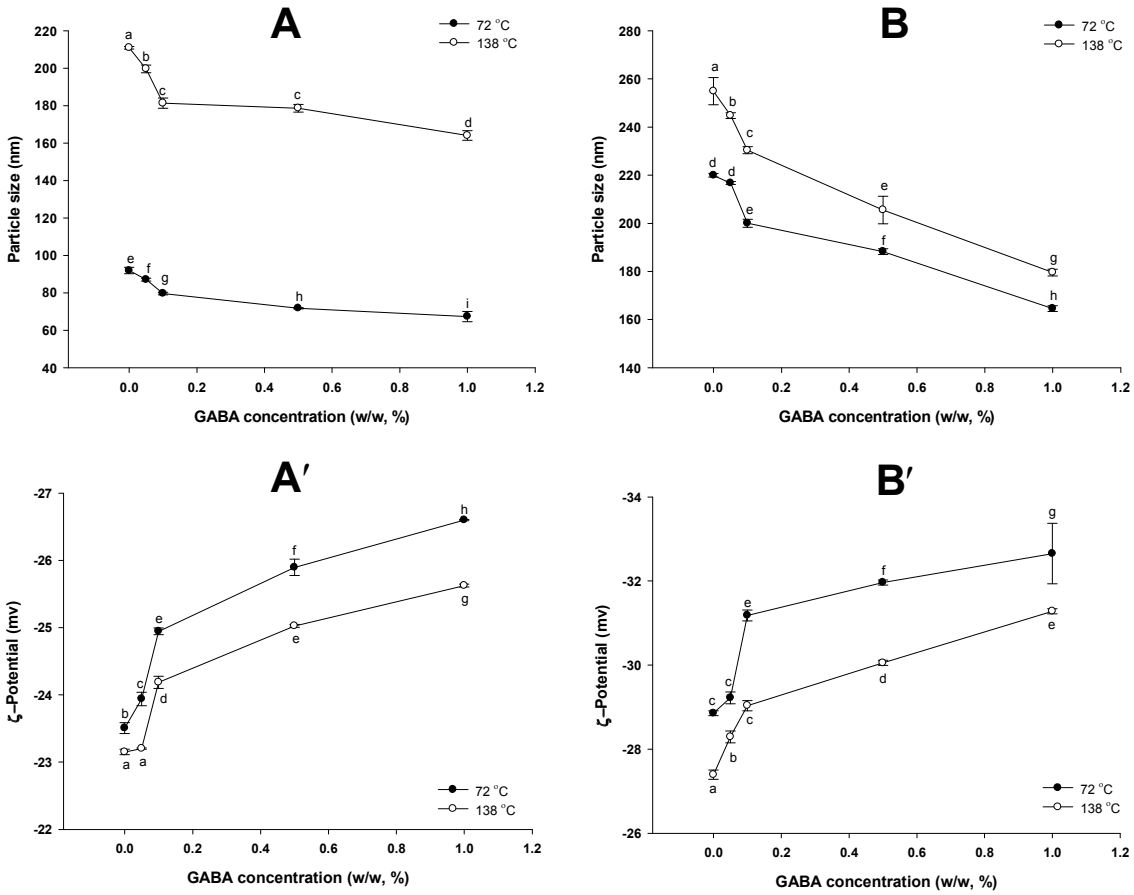


Fig. 3 Effects of the main fractions (A and A′: 0.6% whey proteins; B and B′: 2.6% caseins) with 0.05–1.0% (w/w) added GABA subjected to 72 °C for 15 s and 138 °C for 2 s after 60 °C preheating and 20 MPa homogenization on particle size and ζ-potential of the mixture.

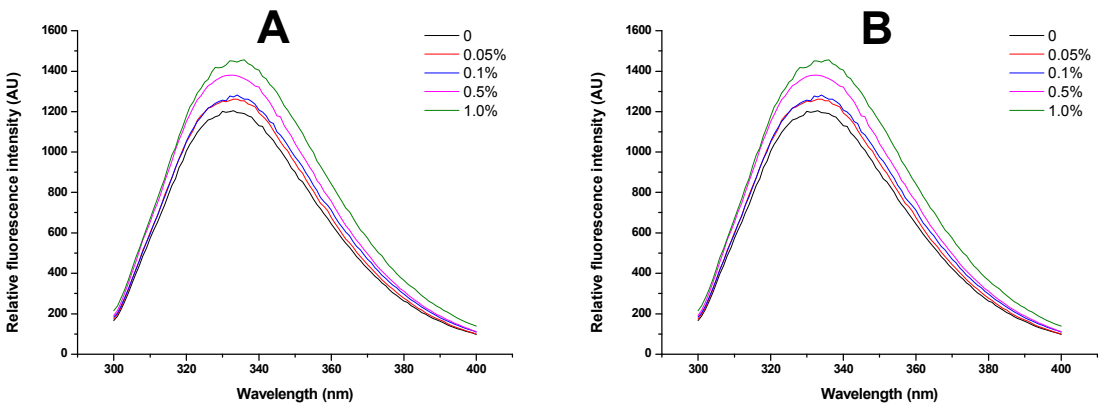


Fig. 4 Fluorescence spectra of whey proteins (A) and caseins (B) mixed with or without 0.05–1.0% (w/w) GABA and then subjected to 138 °C for 2 s pasteurization after 60 °C preheating and 20 MPa homogenization.

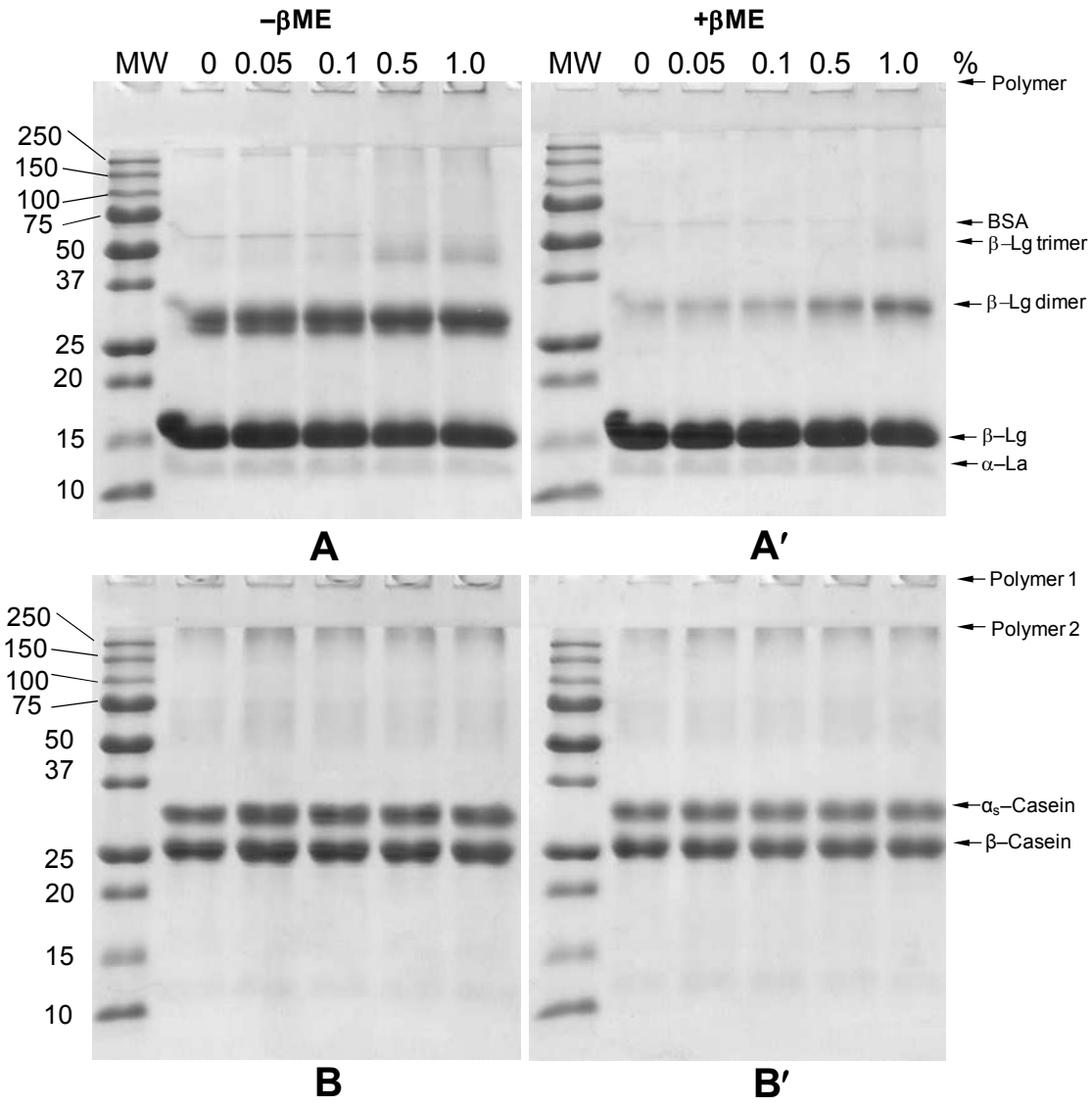


Fig. 5 SDS-PAGE of whey proteins (upper panel: A and A′) and caseins (lower panel: B and B′) under non-reducing (–βME) and reducing (+βME) conditions; These proteins were mixed with 0.05–1.0% (w/w) GABA and then subjected to 138 °C for 2 s after 60 °C preheating and 20 MPa homogenization. α-la: α-lactalbumin; β-lg: β-lactoglobulin; BSA: bovine serum albumin; MW: molecular weight marker (kDa).

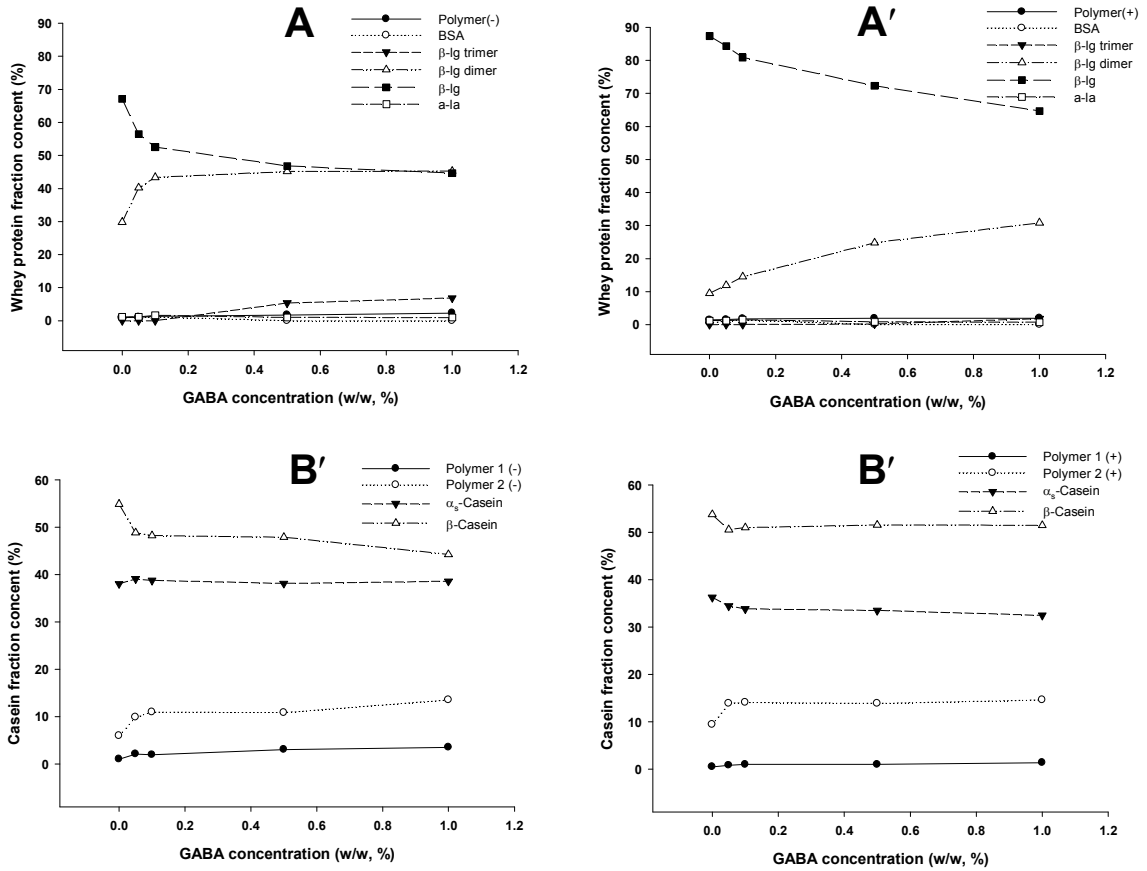


Fig. 6 Densitometry determination of protein fractions (A and A′: whey proteins; B and B′: caseins) based on the SDS-PAGE result (in Fig. 5). The "-" and "+" denote the sample treated without and with βME, respectively.

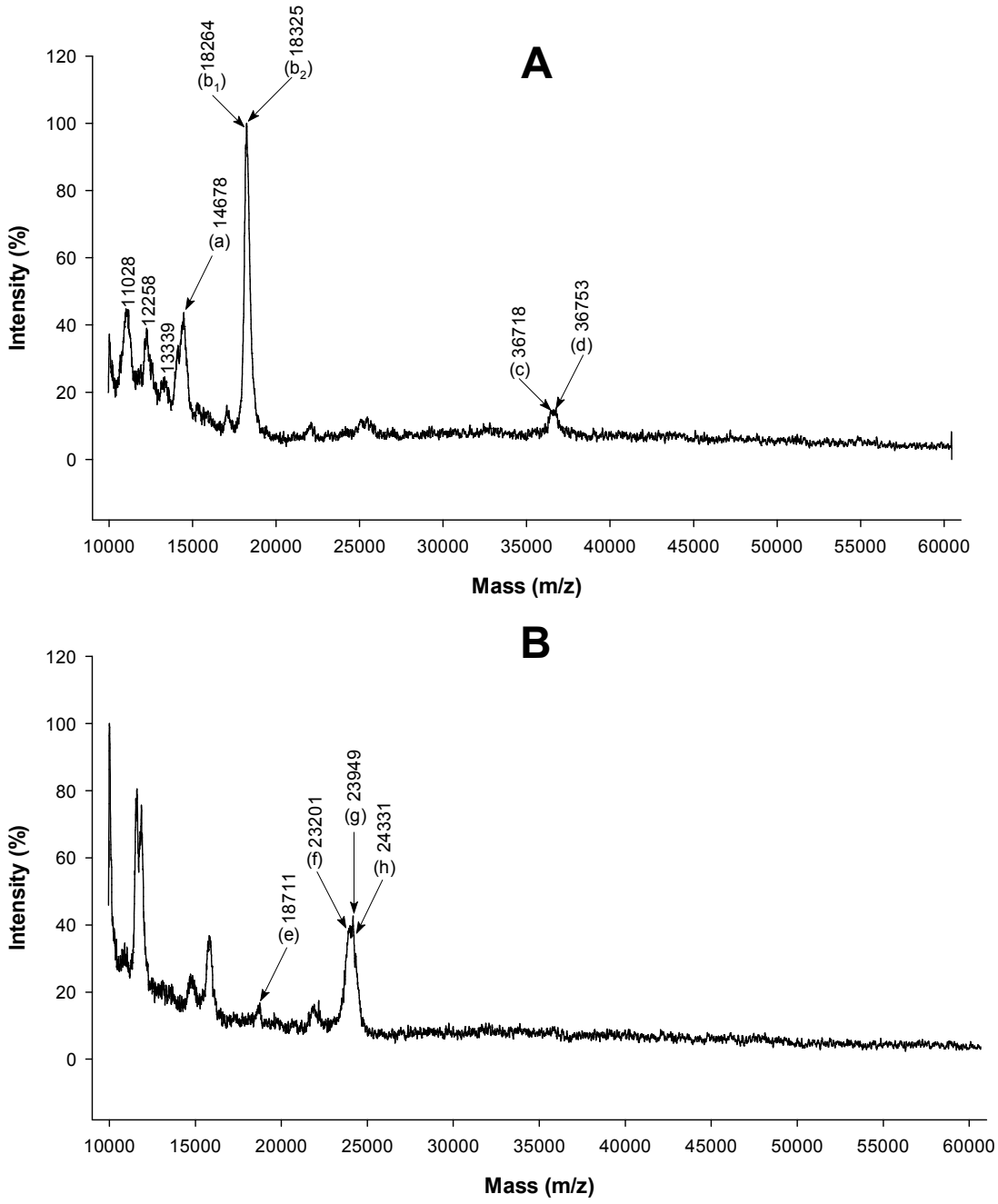


Fig. 7 MALDI–TOF mass spectra of the processed whey proteins (A) and caseins (B) The proteins were mixed with 1.0% (w/w) GABA and then subjected to sequential treatment of 60 °C preheating, 20 MPa homogenization, and 138 °C for 2 s.

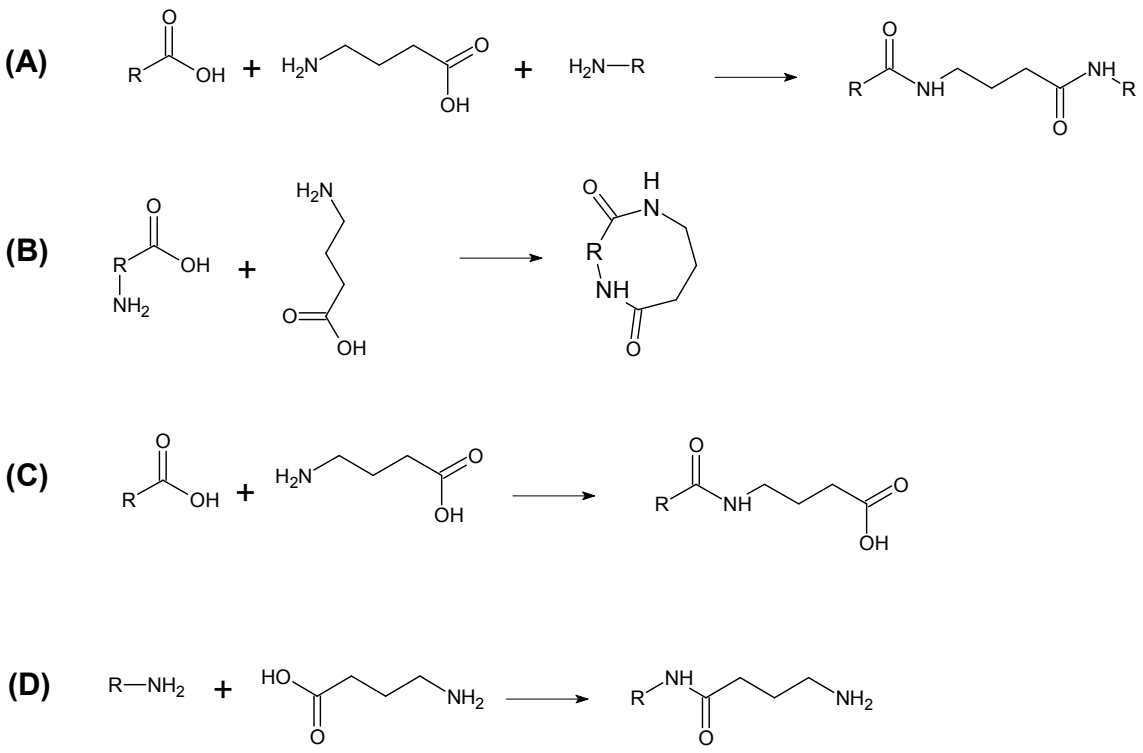
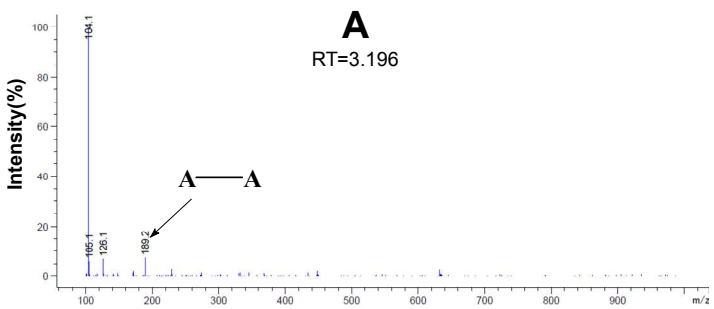
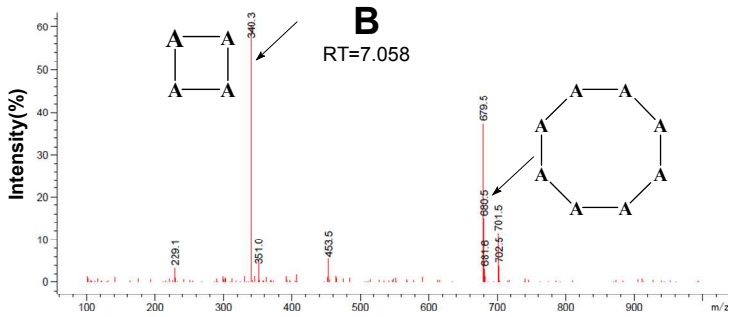
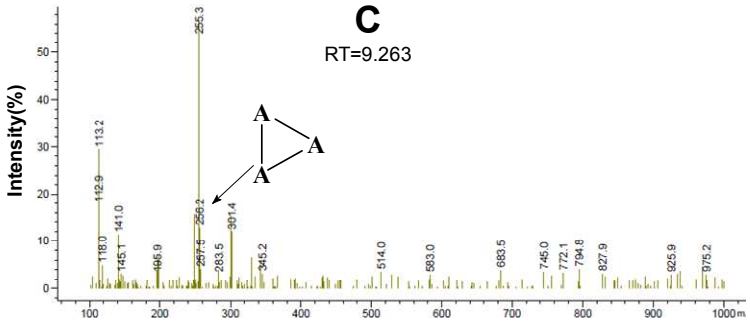
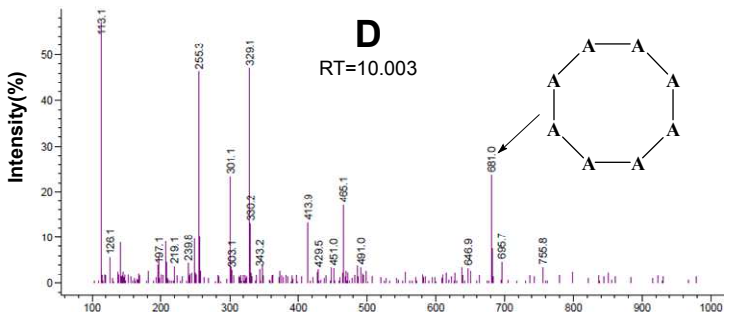


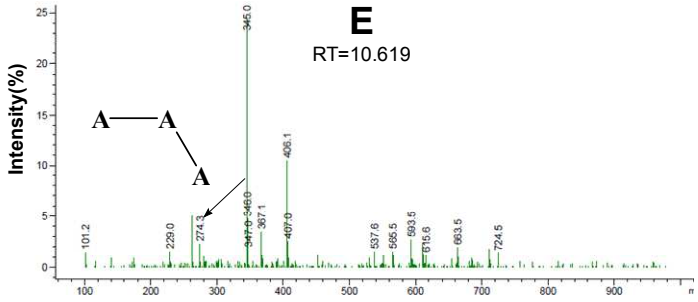
Fig. 8 Hypothesized chemical reactions between the whey proteins or caseins and GABA; The symbol "R" denotes protein.


A
RT=3.196
Intensity(%)
m/z


B
RT=7.058
Intensity(%)
m/z


C
RT=9.263
Intensity(%)


D
RT=10.003
Intensity(%)


E
RT=10.619
Intensity(%)

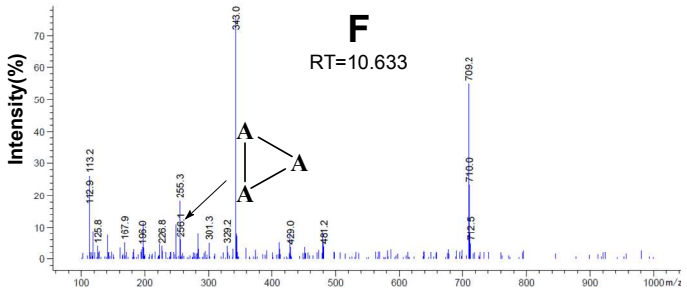

F
RT=10.633
Intensity(%)
m/z

Fig. 9 High-performance liquid chromatography–electrospray ionization mass spectrometry of GABA after undergoing sequential treatment of 60 °C heating, 20 MPa homogenization, and 138 °C pasteurization for 2 s; The symbol "A" represents GABA, and the amide linkage represents cross-linked GABA.

Table 1 Amino acid composition of whey proteins and caseins mixed with or without 0.1–1.0% (w/w) GABA and then subjected to 138 °C for 2 s after 60 °C preheating and 20 MPa homogenization[1]

Amino acids (mg/g)[2]	Whey			Casein		
	0	0.1%	1%	0	0.1%	1%
Asx	86.06	85.41	86.40	52.41	52.47	52.01
Glx	167.21	168.00	166.95	178.53	178.27	176.99
Ser	37.06	36.61	36.81	42.22	42.84	41.87
His	12.46	12.56	12.72	21.19	21.03	20.38
Gly	12.31	12.60	13.12	14.29	13.84	14.02
Thr	55.36	54.86	55.06	31.71	31.80	31.85
Arg	22.18	22.08	21.68	28.14	28.08	27.93
Ala	51.21	51.49	50.57	22.43	22.40	22.19
Tyr	24.16	24.40	24.80	40.64	41.17	40.19
Val	48.59	48.68	49.48	48.93	48.13	48.15
Met	22.31	22.01	21.58	21.25	22.19	21.65
Phe	26.56	26.10	25.45	40.72	40.72	39.81
Ile	53.24	52.90	53.12	38.33	37.52	37.24
Leu	99.94	98.90	99.97	70.10	70.03	69.35
Lys	80.74	80.82	82.45	57.50	57.34	58.18
Pro	55.91	53.60	53.87	109.46	99.68	96.17
Total	868.59	864.76	869.02	819.51	809.08	799.68

[1]Whey proteins and caseins were fortified by 0.1% and 1.0% (w/w) GABA, respectively.

[2]Asx and Glx represent combined Asn and Asp and combined Gln and Glu, respectively.