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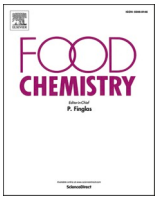

Influence of heat treatments on the functionality of soy protein hydrolysates in animal cell cultures

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ABSTRACT

Soy protein hydrolysates enhance integral viable cell density (IVCD) and recombinant protein production (Immunoglobulin, IgG) in cell cultures, but their functionality varies from batch-to-batch. This is undesirable since it affects both quantity and characteristics of the recombinant proteins. It is hypothesized that the variability of hydrolysates is due to variations in meal and hydrolysate processing treatments. To study this, hydrolysates were produced from meals heated at 121 °C/0–120 min. The heating decreased free amino acid and reducing monosaccharide contents in meals (0.72–0.27% and 3.3–2.6%) and hydrolysates (14.7–7.1% and 16.9–7.9%). Dry heating introduced large variation in the IVCD ((115–316%), but additional heating in suspension reduced it (131–159%). The decrease in IVCD variation corresponded with decreased variation in carboxymethyl-lysine (CML) and lysinoalanine (LAL) contents. Thus, meal and hydrolysate processing induced substantial variation in hydrolysate functionality. It is therefore critical to establish strict process controls for meal and hydrolysate production to ensure consistency.

1. Introduction

The functionality of soy protein hydrolysates used in cell culture applications may vary from batch-to-batch. In two separate studies, the IgG production in soy protein hydrolysate-supplemented cultures varied between 430 and 1350 mg/L (Li & Wen 2013) and 1900–4600 ng/L (Gupta et al., 2014). In the latter study, the gross composition was analyzed and was found to be quite similar for different batches of a soy protein hydrolysate (e.g., protein content varied between 56 and 58% w/w) (Gupta et al., 2014). This shows that variation in hydrolysate functionality cannot be explained using gross compositional parameters, i.e., total protein and total carbohydrate content. Since the soy protein hydrolysates are produced using the same starting raw material (soybean meal), it is hypothesized that the variability of hydrolysates is due to variations in meal and hydrolysate processing treatments. And to investigate the root-cause of the variation in hydrolysate functionality, it is necessary to focus on sub-compound classes, such as amino acids and carbohydrates.

Amino acids and carbohydrates are key sources of carbon and nitrogen that are essential for cell nutrition. They are however susceptible to modifications and degradation during soybean meal and hydrolysate processing. For instance, due to heat treatments, Maillard reaction may occur, in which free amino acids, peptides, and proteins react with reducing monosaccharides. As a result, free and/or essential amino acids and reducing monosaccharides (e.g., glucose) become unavailable for cellular nutrition and metabolism. This suggests that variations occurring in the contents of amino acids and monosaccharides due to processing may correlate with the variation in cell density and recombinant protein production.

The main heat treatments applied during soybean meal production are desolventization (removal of organic solvent) and toasting (inactivation of protease inhibitors). Typical time–temperature combinations for desolventization are 99–104 °C/ 18–20 min (Becker et al., 1953) and 177 °C/ 3–10 s (Netemeyer et al., 1982) and that for toasting are 99–116 °C/ 48–55 min (Becker et al., 1953), 79–115 °C/ 30 min (Netemeyer et al., 1982), and 165 °C/ 75–210 min (Demjanec et al., 1995). During hydrolysate production, process steps involving heat treatment are mixing (60 °C/ 30 min), enzymatic hydrolysis (50 °C/ 24 h), and enzyme inactivation (95 °C/ 10 min) (Gupta et al., 2015). An important difference between meal and hydrolysate processing is that heat treatment

Abbreviations: IVCD, integral viable cell density; IgG, Immunoglobulins; CD, chemically defined; CHO, Chinese hamster ovary; LAL, Lysinoalanine; MRP, Maillard reaction products; CML, Carboxymethyl lysine; HMF, 5-hydroxymethylfurfural; CLAA, cross-linked amino acids; DM, dry matter; CV, coefficient of variation.

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during meal processing is applied to dry substrate, whereas that during hydrolysate production is applied to substrate in suspension. This is relevant, since it is evident from literature that the effects of the Maillard reaction (typically expressed as degree of glycation) are much drastic in dry systems as compared to aqueous systems. For instance, a lactose-casein mixture with water activity = 0.33 reached 60% degree of glycation after heating for 20 h at 60 °C, whereas similar degree of glycation was achieved after ~ 70 h when the water activity was 0.98 (Malec et al., 2002). Regardless in dry or aqueous systems, in addition to the Maillard reaction, several chemical reactions may take place due to heating, such as decarboxylation, deamidation, and the formation of cross-linked peptides. Examples of reaction products formed in these heat-induced chemical reactions are furosine, CML, 5-hydroxymethylfurfural (HMF), lanthionine, and LAL (van Rooijen et al., 2014). These compounds have been reported to adversely affect viable cell density. With increase in HMF concentration from 2.5 to 100 mM in CHO-V79 cultures, the cell viability decreased from 95 to 71% (Durling et al., 2009). In HepG2 cells, addition of 38 mM of HMF decreased the cell viability by 50% (Severin et al., 2010). None of these studies reported effects on recombinant protein production.

Summarizing, amino acids, monosaccharides, Maillard reaction products (MRP), and cross-linked amino acids (CLAA), directly affect cell culture functionality. Additionally, through a second mechanism, these compounds may affect the enzymatic hydrolysis process and alter the peptide profiles consequently affecting the functionality. Despite the apparent reason to study the effects of variation in the contents of amino acids, monosaccharides, MRP, and CLAA in hydrolysates on cell culture functionality, no concrete data has been published so far.

In this study, changes in the content of amino acids, carbohydrates, MRP, and CLAA in soybean meal due to different heat treatments were studied. Then, to what extent these compositional effects from differently processed meals got transferred to hydrolysates and the impact of hydrolysate processing on hydrolysate composition was investigated. Finally, the functionality of hydrolysates was evaluated in CHO cell cultures.

2. Materials and methods

2.1. Materials

Hyland soybeans were received from L.I. Frank (Twello, The Netherlands). Technical hexane (24611.366; 95% v/v) was purchased from VWR International, Amsterdam, The Netherlands. L-lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride (608041) was purchased from Sigma Aldrich, Zwijndrecht, The Netherlands. Furosine.HCl (SC494), ε-*N*-carboxymethyl-L-lysine (SC1505), and lysinoalanine.HCl (SC1591) were purchased from PolyPeptide (Strasbourg, France). The materials required for size exclusion chromatography (Gupta et al., 2015) and for cell culture assay (Gupta et al., 2014) were described in detail previously.

2.2. Preparation of defatted soybean meal

Soybeans were milled using an ultra-centrifugal mill ZM 200 (Retsch, Haan, Germany) using a 0.5 mm sieve. The milled soybean meal was defatted using 1:10 (w/v) hexane in a Soxhlet extractor (60 °C). The hexane was refluxed for 5 cycles (~40 min/cycle). The defatted soybean meal obtained was denoted unheated meal, M_0, and separated into four equal parts. Three parts were heated using a Systec 5075 ELV autoclave (Hettich Benelux, Geldermalsen, The Netherlands) at 121 °C for 15 (M_{15}), 60 (M_{60}), or 120 min (M_{120}).

2.3. Preparation of soy protein hydrolysates

From the meals (M_0, M_{15}, M_{60}, and M_{120}), two sets of four hydrolysates each were prepared using the process described previously (Gupta et al., 2015). The first set was prepared as follows: M_0, M_{15}, M_{60}, and M_{120} meals were suspended (120 g/L) in Milli-Q water and hydrolyzed using Bromelain (5 g enzyme/100 g of meal) and Phyzyme (1 g enzyme/100 g of meal) at 60 °C/ 24 h. The enzymes were inactivated by heating the suspensions at 95 °C/ 10 min. The suspensions were cooled down, centrifuged (1350 g, 15 °C, 10 min), and filtered through a 10 kDa membrane (A/G Technology, Needham, Massachusetts, USA). The permeates were freeze dried and denoted as M_0H, $M_{15}H$, $M_{60}H$, and $M_{120}H$. For the second set of hydrolysates, an additional heating (95 °C/ 60 min) was applied to hydrolysate suspensions after enzyme inactivation. The permeates obtained after centrifugation and filtration were freeze dried and denoted as M_0H_{aq}, $M_{15}H_{aq}$, $M_{60}H_{aq}$, and $M_{120}H_{aq}$. The yield (%) of the process was calculated as g dry matter (DM) hydrolysate/g DM (meal) * 100.

2.4. Characterization of meals and hydrolysates

2.4.1. DM content

The DM content was analyzed using an Ohaus MB45 moisture analyzer (VWR International, Amsterdam, The Netherlands).

2.4.2. Ash content

The ash content was determined following the AOAC method (923.03) (AOAC, 1995).

2.4.3. Total protein content

The protein content (N * 5.7116) was determined following the AOAC method (993.13) using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). L-methionine was used for calibration and cellulose was used as the blank sample.

2.4.4. UV–visible spectra measurements

The UV–visible spectra of samples taken before hydrolysis, after enzyme inactivation, and ultrafiltration were measured after centrifugation (12,000 g, 15 °C, 5 min). The spectra were measured in quartz cuvettes in the range of 200–800 nm in a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4.5. Content of reducing end groups

The content of reducing end groups was determined using 4-hydroxybenzoic acid hydrazide assay as described elsewhere (Kühnel et al., 2010).

2.4.6. Free monosaccharide and oligosaccharide contents

The free mono-/oligosaccharides were analyzed using HPAEC-PAD (Dionex, Sunnyvale, California, USA) (Gupta et al., 2015).

2.4.7. Total neutral carbohydrate and uronic acid contents

The total neutral carbohydrate content was analyzed using GC-FID (Gupta et al., 2015) and the total uronic acid content was determined following the m-hydroxydiphenyl assay (Gupta et al., 2015).

2.4.8. Free and total amino acid contents

The free and total amino acid analyses were performed by Ansynth Service B.V. (Berkel en Rodenrijs, The Netherlands) following a procedure described elsewhere (Moore & Stein, 1963).

2.4.9. Size exclusion chromatography

Samples (20 μL of 2.0–2.5 mg protein/mL) were prepared in the eluent (30% v/v acetonitrile containing 0.1% v/v trifluoroacetic acid in Milli-Q water), filtered through 0.45 μm filters, and injected on a TSK gel G2000SWxl column (Tosoh Bioscience, Stuttgart, Germany) connected to an HPLC system (Thermo Scientific, Breda, The Netherlands). The elution profiles were recorded at 214 nm (Gupta et al., 2015).

2.4.10. CML, LAL, and furosine contents

Samples (10 mg) were hydrolyzed using 6 M HCl (1 mL) at 110 °C/24 h. The hydrolyzed samples were dried under a stream of nitrogen and redissolved in 1 mL of Milli-Q water, sonicated (10 min), and centrifuged (12,000 g, 15 °C, 5 min). The supernatants were diluted 5 times using 10 mM HCl containing 0.5 mg/L of L-lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride as internal standard. The MS signal intensity of L-lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride was used to verify the stability of MS signal throughout the UHPLC-MS run. The diluted samples (1 μL) were injected onto a BEH amide column (2.1*150 mm, 1.7 μm particle size, Waters, Milford, Massachusetts, USA) with an Acquity BEH amide vanguard pre-column (2.1*50 mm, 1.7 μm particle size, Waters, Milford, Massachusetts, USA) installed on an Accela UHPLC system (Thermo Scientific, Waltham, Massachusetts, USA). The UHPLC system was coupled to an LTQ VelosPro mass spectrometer (Thermo Scientific, San Jose, California, USA). The system was tuned with HMF in the positive mode with the ion transfer tube temperature of 300 °C and the source voltage of 3 kV. For selected reaction monitoring of CML (204 Da), LAL (233 Da), furosine (254 Da), and L-lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride (154 Da), the fragments were detected in m/z ranges of 130 ± 2.5, 198 ± 2.5, 130 ± 2.5, and 137 ± 2.5 respectively. The normalized collision energy was set at 35% for all compounds. The eluents A and B were 1% (v/v) formic acid in Milli-Q water and 1% (v/v) formic acid in acetonitrile, respectively. The elution profile used was: 0–2 min isocratic on 90% B, 2–10 min linear gradient from 90% to 40% B, 10–12 min isocratic 40% B, 12–13 min 40% B to 90% B, and 13–30 min isocratic on 90% B. The flow rate was 0.3 mL/minute and detections were performed at 214, 254, and 280 nm. CML, LAL, and furosine in the concentration range of 0.01 to 5 mg/L were used for calibration. To correct for any instability in MS signals, the peak areas of CML, LAL, and furosine in the samples and those of CML, LAL, and furosine standards were divided by the peak areas of L-lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride. Using the calibration data and the corrected peak areas, the contents of CML, LAL, and furosine were quantified.

2.5. Error analysis

The analyses were performed as technical duplicates. The coefficient of variation (CV, standard deviation/average*100) in the contents (for each compound) of DM, proteins, ash, neutral carbohydrates, uronic acids, mono-/oligosaccharides, furosine, CML, and LAL were<10%. For total DM, proteins, carbohydrates, ash and composition of amino acids and carbohydrates, little variation was observed between meals and between hydrolysates. Therefore, the values for all meals and all hydrolysates were separately averaged and shown with corresponding standard deviations.

The free and total amino acid analyses were performed as single measurements. The viable cell density and IgG measurements were performed as triplicate measurements.

2.6. Cell culture

Preparation of hydrolysate stock solution: The hydrolysate stock solutions (4 % w/v) were prepared in the suspension medium (Gupta et al., 2014). The solutions were filtered (0.22 μm) to remove microorganisms that may be present in the hydrolysates.

2.6.1. Cell line adaptation, growth assay, and IgG production

The hydrolysate stock solution was diluted to final concentration of 0.4% (w/v) in the growth assay. The cell line adaptation, growth assay, and IgG production measurements were performed as described elsewhere (Gupta et al., 2014). The IVCD was calculated as the area under the growth curve from cell counts measured at different time points during the growth assay. The IgG production was measured using an enzyme-linked immunosorbent assay. The standard errors in IVCD and IgG production were lower than 10%.

Table 1A
Total amino acid (g/100 g DM) and mono-/oligosaccharide composition (g/100 g DM) in soybean meals heated at 121 °C for 0, 15, 60, and 120 min.

Amino acids (g/100 g DM)	M_0	M_{15}	M_{60}	M_{120}
Essential amino acids				
Valine	3.1	3.1	2.9	2.9
Phenylalanine	3.3	3.2	3.0	3.0
Lysine	3.9	3.5	3.1	2.6
Histidine	1.6	1.6	1.5	1.4
Leucine	4.8	4.8	4.5	4.4
Threonine	2.5	2.5	2.4	2.3
Isoleucine	2.9	2.9	2.7	2.7
Non-essential amino acids				
Arginine	4.8	4.5	4.2	3.9
Glutamic acid and Glutamine	11.7	11.7	10.9	10.8
Aspartic acid and Asparagine	7.1	7.0	6.5	6.4
Alanine	2.8	2.8	2.6	2.6
Tyrosine	2.4	2.3	2.2	2.1
Glycine	2.6	2.6	2.5	2.4
Proline	3.2	3.2	3.0	2.9
Serine	3.2	3.2	3.0	2.9
Total amino acids	**59.8**	**58.9**	**54.9**	**53.4**
Mono-/oligosaccharides* **(g/100 g DM)**				
Glucose	5.9	5.3	4.9	5.5
Galactose	7.3	6.1	6.1	6.8
Mannose	1.3	1.9	2.1	1.7
Rhamnose	0.2	0.4	0.5	0.6
Arabinose	2.6	2.5	2.6	2.7
Xylose	1.2	1.4	1.6	1.3
Uronic acids	0.9	1.1	1.0	1.0
Total mono-/oligosaccharides	**19.4**	**18.7**	**19.0**	**19.6**

* Standard error: <4%.

Table 1B
Free amino acid (mg/100 g DM) and mono-/oligosaccharide composition (g/100 g DM) in soybean meals heated at 121 °C for 0, 15, 60, and 120 min.

Amino acids (mg/100 g DM)	M_0	M_{15}	M_{60}	M_{120}
Essential amino acids#				
Valine	75	40	26	10
Phenylalanine	26	17	16	11
Lysine	24	13	11	6
Histidine	19	8	7	0
Leucine	11	6	5	3
Threonine	8	5	4	3
Isoleucine	7	5	3	0
Methionine	5	0	0	0
Non-essential amino acids				
Arginine	202	138	127	87
Glutamic acid	126	90	65	28
Aspartic acid	69	63	62	52
Asparagine	52	44	35	19
Alanine	29	26	24	22
Tyrosine	23	14	18	12
Glycine	14	9	8	5
Proline	13	10	9	6
Serine	13	8	7	5
Glutamine	4	0	0	0
Free amino acids	**720**	**496**	**427**	**269**
Mono-/oligosaccharides* **(g/100 g DM)**				
Glucose	2.8	2.5	2.2	2.4
Galactose	0.6	0.4	0.4	0.4
Mannose	<0.07	<0.07	<0.07	<0.07
Fructose	<0.07	<0.07	<0.07	<0.07
Reducing monosaccharide	**3.3**	**2.9**	**2.6**	**2.8**
Sucrose	5.0	4.5	4.1	4.6
Stachyose	3.4	2.8	2.2	2.7
Raffinose	0.1	0.1	0.3	0.2
Free mono-/oligosaccharides	**11.8**	**10.2**	**9.2**	**10.2**

* Standard error: <4%.
# Tryptophan was not analyzed.

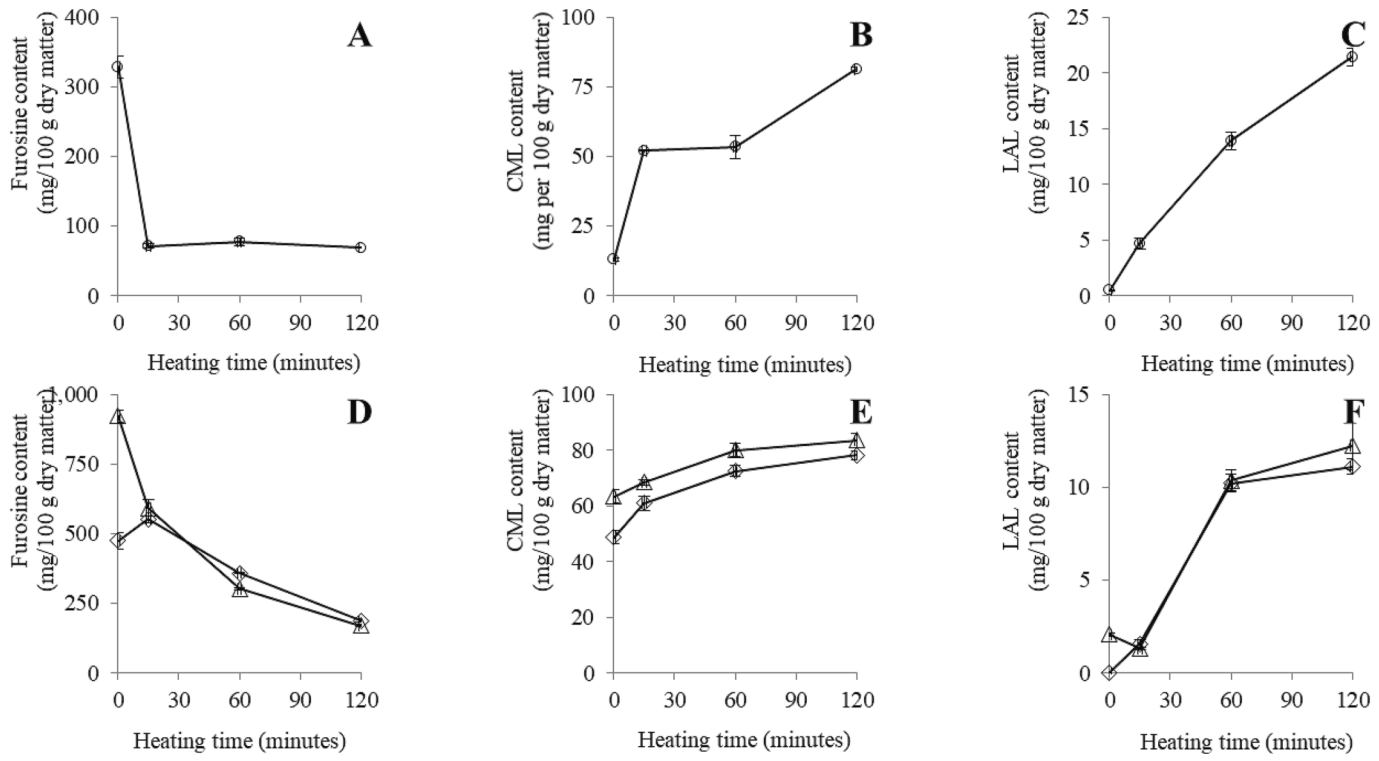

Fig. 1. Effect of heating time on furosine, CML, and LAL contents (mg/100 g DM) in (○) soybean meals (A-C) and soy protein hydrolysates (D-F) prepared with (△), and without (◇) additional heating in suspension.

3. Results and discussion

3.1. Characterization of defatted soybean meals

The total carbohydrate content and composition (19.3 ± 0.5% w/w DM) and ash (6.9 ± 0.2% w/w DM) content were not affected by different heat treatments (121 °C for 0, 15, 60, and 120 min) of the soybean meals (Table 1-A). The total protein content of the meals, however, decreased from 59.8% (M_0) to 53.4% (M_{120}) w/w DM with increase in duration (0–120 min) of the heat treatment (Table 1-A). This can be traced back to large decreases observed in the total contents of lysine (33%), arginine (19%), histidine (13%), and tyrosine (13%). Significant declines were noted in free amino acids as well; 100% of glutamine, methionine, isoleucine, and histidine and up to 73–87% of leucine, lysine, glutamic acid, and valine after 120 min of heat treatment (Table 1-B). Most of the free amino acids that had reacted (e.g., methionine, isoleucine, histidine, leucine, lysine, and valine) were essential amino acids that are required by CHO cells for optimal nutrition and metabolism (Carrillo-Cocom et al., 2015). Interestingly, the reactivity of free amino acids in the meals deviated from that reported in the literature (Ashoor & Zent, 1984), where lysine, glycine, tryptophan, and tyrosine were found to be most reactive. While the fraction of free amino acids present in the meals was small (0.5–1.2% of total amino acids), the decrease in the free amino acid content (0.72 (M_0) to 0.27% (M_{120}) w/w DM) (Table 1-B) was still significant.

Next to amino acids, the content of reducing monosaccharides (sum of glucose and galactose) decreased from 3.3% to 2.8% w/w DM after 120 min of heating (Table 1-B). Unlike, free and total amino acid contents, the reducing monosaccharides content did not decrease consistently.

Almost an equimolar loss of 3.2 and 3.0 mmol/100 g DM of free amino acids and reducing monosaccharides, respectively, had occurred after 120 min of heating. This indicates that they may have reacted to form MRP and cross-linked compounds.

3.1.1. MRP and CLAA in soybean meals

The consistent increase in the browning intensity of the meals confirmed the occurrence of the Maillard reaction (data not shown). When aqueous suspensions of meals were centrifuged, most brown colored products were retained in the pellets. This suggests that the brown colored products were either insoluble or that they were bound to the insoluble material in the meals.

The furosine content of the unheated meal (M_0) was rather high at 329 mg/100 g DM. After heating for 120 min, the furosine content decreased to 78 mg/100 g DM (Fig. 1-A). Much lower furosine content (9.4 mg/100 g w/w) in soybean flour has been reported in literature (Rufián-Henares et al., 2009). It has also been shown that the furosine content starts to decrease in severely heated samples since it is a marker of early stages of the Maillard reaction (Erbersdobler & Somoza, 2007; Taş & Gökmen, 2019). This implies that the starting meal M_0 had reached advanced stages of the Maillard reaction. It may be that these reactions had initiated and advanced during storage of soybean seeds, milling process, or defatting process of the meals. It has been reported that MRP accumulated in mung beans during storage at 33 °C for 600 days (Murthy et al., 2003).

In contrast to furosine, CML (Fig. 1-B) and LAL (Fig. 1-C) contents increased from 13 to 81 mg/100 g DM and from 0.5 to 22 mg/100 g DM, respectively, after 120 min of heating. Much higher LAL content of ~ 90 mg/100 g DM (150 mg/100 g protein) in soybean meal has been reported in literature (Aymard et al., 1978). This suggests that the effects of heating in commercial meals can be even more pronounced than the effects observed in this study. CML data for soybean meals, protein concentrates, or isolates could not be found in literature.

As CML and LAL are derived from lysine, the formation of these compounds was compared to the loss of total lysine. After 120 min of heating, the total lysine content decreased by 8.72 mmol/100 g DM and CML and LAL contents increased simultaneously by 0.33 and 0.09 mmol/100 g DM, respectively. Thus, the amount of lysine that had reacted was 21 times higher than the total CML and LAL content measured in the meals. In addition to lysine, several other amino acids

Table 2A
Total amino acid (g/100 g DM) and mono-/oligosaccharide composition (g/100 g DM) in soy protein hydrolysates.

Amino acids(g/100 g DM)	M_0H	$M_{15}H$	$M_{60}H$	$M_{120}H$	M_0H_{aq}	$M_{15}H_{aq}$	$M_{60}H_{aq}$	$M_{120}H_{aq}$
	Dry heating				Dry heating + heating in suspension			
Essential amino acids								
Valine	3.07	3.07	2.79	2.82	3.00	2.96	2.84	2.88
Phenylalanine	3.13	3.05	2.73	2.71	2.94	2.83	2.73	2.89
Lysine	4.37	4.22	3.91	3.58	3.46	4.14	3.99	3.79
Histidine	1.62	1.61	1.49	1.44	1.53	1.55	1.53	1.51
Leucine	4.54	4.55	4.28	4.20	4.42	4.45	4.35	4.38
Threonine	2.71	2.65	2.58	2.49	2.55	2.57	2.61	2.62
Isoleucine	2.82	2.81	2.57	2.58	2.73	2.71	2.61	2.67
Non-essential amino acids								
Arginine	5.12	4.98	4.75	4.65	4.83	4.96	4.90	4.77
Glutamic acid + Glutamine	12.70	12.63	12.38	12.13	12.06	12.18	12.41	12.70
Aspartic acid + Asparagine	7.15	7.71	7.43	7.15	7.57	7.59	7.58	7.34
Alanine	3.01	2.93	2.82	2.77	2.87	2.87	2.85	2.87
Tyrosine	2.65	2.59	2.43	2.34	2.45	2.52	2.43	2.46
Glycine	2.89	2.86	2.74	2.69	2.78	2.79	2.77	2.80
Proline	3.40	3.38	3.32	3.21	3.27	3.27	3.34	3.37
Serine	3.42	3.41	3.38	3.17	2.89	3.37	3.43	3.39
Total amino acids	**62.6**	**62.5**	**59.6**	**57.9**	**59.4**	**60.8**	**60.4**	**60.4**
Mono-/oligosaccharides*(g/100 g DM)								
Glucose	3.82	4.73	5.99	3.90	3.78	5.50	5.45	3.18
Galactose	4.35	4.17	4.18	4.17	4.31	4.49	4.31	4.29
Mannose	1.40	1.11	1.55	1.58	1.39	1.55	1.59	1.29
Uronic acids	1.92	1.93	2.17	2.14	1.98	1.99	1.96	1.94
Total mono-/oligosaccharides	**11.49**	**11.94**	**13.89**	**11.80**	**11.45**	**13.53**	**13.32**	**10.66**

* Standard error: <4%.

Table 2B
Free amino acid (g/100 g DM) and mono-/oligosaccharide composition (g/100 g DM) in soy protein hydrolysates.

Amino acids (g/100 g DM)	M_0H	$M_{15}H$	$M_{60}H$	$M_{120}H$	M_0H_{aq}	$M_{15}H_{aq}$	$M_{60}H_{aq}$	$M_{120}H_{aq}$
	Dry heating				Dry heating + heating in suspension			
Essential amino acids#								
Valine	0.9	0.5	0.4	0.4	0.7	0.5	0.4	0.4
Phenylalanine	1.4	0.7	0.5	0.6	1.1	0.7	0.5	0.6
Lysine	1.6	1	0.8	0.7	0.5	1.1	0.8	0.7
Histidine	0.3	0.2	0.2	0.1	0.2	0.2	0.2	0.2
Leucine	2.2	1.4	1.1	1.2	1.8	1.5	1.1	1.1
Threonine	0.6	0.4	0.3	0.3	0.4	0.4	0.3	0.3
Isoleucine	0.6	0.2	0.2	0.2	0.4	0.2	0.2	0.2
Methionine	0.4	0.3	0.2	0.2	0.3	0.3	0.2	0.2
Non-essential amino acids								
Arginine	2.5	1.6	1.3	1.4	2	1.8	1.5	1.3
Glutamic acid	1.4	0.7	0.5	0.6	1.1	0.7	0.5	0.6
Aspartic acid	0.3	0.4	0.3	0.3	0.5	0.3	0.3	0.3
Asparagine	0.5	0.3	0.3	0.3	0.4	0.4	0.3	0.3
Alanine	1.1	0.7	0.6	0.7	0.9	0.8	0.7	0.6
Tyrosine	1.1	0.6	0.5	0.6	0.9	0.7	0.5	0.5
Glycine	0.4	0.3	0.2	0.2	0.3	0.3	0.3	0.2
Proline	0.3	0.2	0.1	0.1	0.2	0.2	0.1	0.1
Serine	0.7	0.5	0.5	0.5	0.3	0.6	0.5	0.4
Glutamine	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.3
Free amino acids	**16.9**	**10.3**	**8.3**	**8.7**	**11.9**	**10.8**	**8.3**	**7.9**
Mono-/oligosaccharides*(g/100 g DM)								
Glucose	1.5	2.6	0.9	2.6	3.2	1.4	2.5	0.6
Galactose	1.9	1.5	0.6	1.1	1.2	1.3	0.9	1.0
Mannose	0.9	0.9	0.8	1.0	0.9	1.0	1.0	0.8
Fructose	7.7	3.3	5.1	9.3	9.4	7.8	9.6	4.7
Reducing monosaccharide	**12.0**	**8.3**	**7.4**	**14.0**	**14.7**	**11.4**	**14.0**	**7.1**
Sucrose	0.1	0.4	0.7	0.1	0.3	0.6	0.1	0.04
Stachyose	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
Raffinose	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
Free mono-/oligosaccharides	**12.2**	**8.7**	**8.2**	**14.1**	**15.1**	**12.0**	**14.1**	**7.2**

* Standard error: <4%.
# Tryptophan was not analyzed.

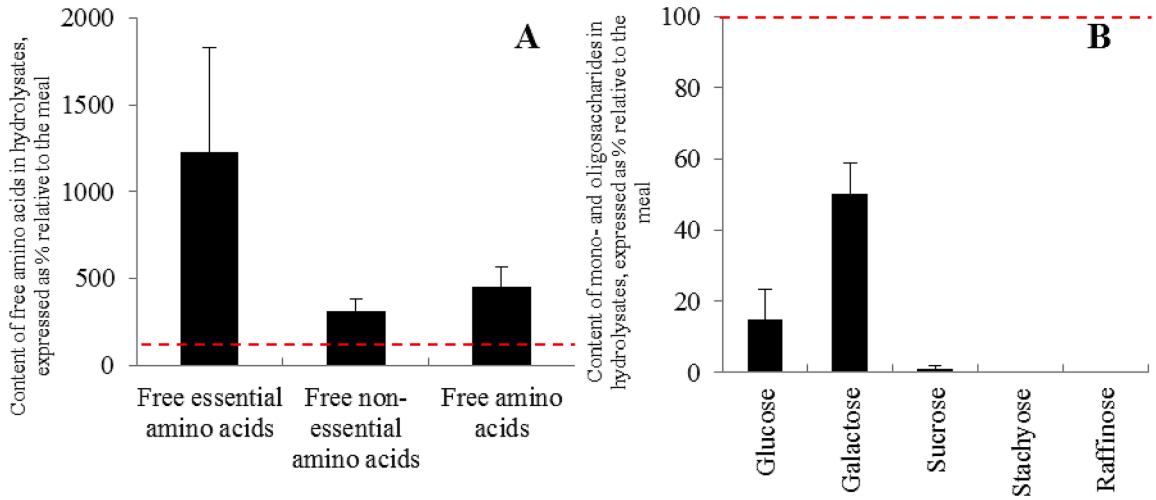

Fig. 2. Amount of (A) free amino acids and (B) mono-/oligosaccharides in soy protein hydrolysates, relative to the meal (100%, indicated by red line) calculated using a mass balance, average and standard deviation over all hydrolysates.

had reacted indicating that a large variety of MRP and CLAA might have been formed. But because of the complexity, not all the reaction products could be individually studied.

3.2. Characterization of soy protein hydrolysates

During hydrolysate production, 81 ± 0.3% w/w DM of the meals was separated as pellets/retentates and 19 ± 0.3% w/w DM was obtained as hydrolysates. Similar yield and mass balance has been reported by Gupta et al., 2015. All hydrolysates had similar average protein (61 ± 2% w/w DM), carbohydrate (13 ± 1% w/w DM), and ash (11 ± 1% w/w DM) contents (Table 2-A).

Similar to the meals, the trend of increase in browning intensity with increase in the duration (0 to 120 min) of heat treatment was apparent in the derived hydrolysates (data not shown). This may be because the insoluble brown products present in the meals got partially solubilized during hydrolysis. Similar solubilization of Maillard related brown compounds has been reported after tryptic hydrolysis of wheat gluten heated at 150 °C/ 1 h in presence of glucose and water (Fogliano et al., 1999).

The elution profiles and molecular weight distributions of hydrolysates prepared from meals heated for 15, 60, and 120 min were similar (Refer Supplementary material) suggesting that the longer dry heating (15–120 min) and heating in suspension (95 °C/60 min) did not notably impact the peptide profiles. The hydrolysates prepared from the unheated meal, M_0H (71 ± 0.1 %) and M_0H_{aq} (65 ± 0.4%), had slightly higher proportion of small peptides < 500 Da as compared to hydrolysates prepared from heated meals M_{15}, M_{60}, and M_{120} (60 ± 2.5%) (Refer Supplementary material). This implied that the hydrolysis was hindered in meals heated for 15, 60, and 120 min. This inference was confirmed with lower free amino acid contents (9.1 ± 2.5% w/w DM) in hydrolysates prepared from heated meals M_{15}, M_{60}, and M_{120} as compared to those of the hydrolysates prepared from the unheated meal (14.4 ± 3.5% w/w DM) (Table 2-B). These differences in hydrolysis can be attributed to the occurrence of advanced Maillard and cross-linking reactions.

As expected, due to hydrolysis, the free amino acid contents of hydrolysates (7.9–16.9% DM) were significantly higher than those in the meals (Fig. 2-A). This was however not the case with reducing monosaccharide contents. Based on the mass balance, if all reducing monosaccharides present in meals were transferred to hydrolysates, one would expect reducing monosaccharide contents of 13.7–17.4% DM in hydrolysates. But the reducing monosaccharide content in hydrolysates varied between 7.12 and 14.74% DM, suggesting a loss of 4.8–51.7%

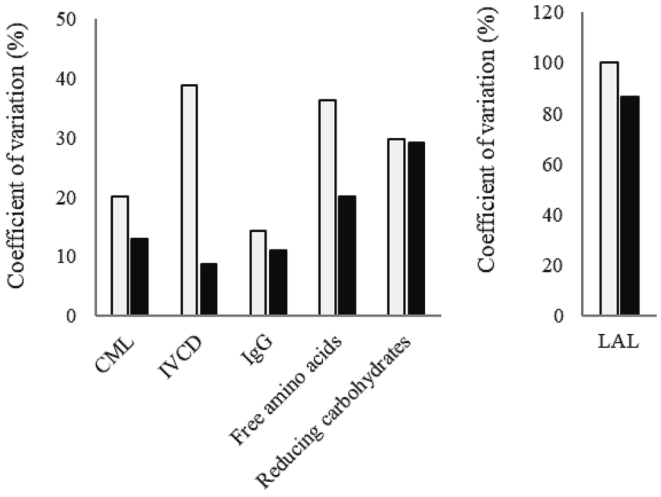

Fig. 3. Variation (CV%) in IVCD and IgG production and CML, free amino acids, reducing carbohydrates, and LAL contents in hydrolysates prepared with (■) and without (■) additional heating in suspension.

during hydrolysate production (except $M_{60}H_{aq}$; 2.5% gain) (Fig. 2-B). In comparison to the meals, the amount of glucose, galactose, and sucrose were lower and stachyose and raffinose were not detectable in the hydrolysates. Considerable amounts of mannose (0.75–1.04% w/w DM) and fructose (3.29–9.56 % w/w DM) were found in hydrolysates, but not in the meals (Table 2-B). These differences in the carbohydrate content and composition between meals and hydrolysates indicate the occurrence of microbial fermentation. While this has been proposed (Richardson et al., 2015), the occurrence of fermentation during hydrolysate production has not actually been studied or reported.

3.2.1. MRP and CLAA in soy protein hydrolysates

Basis the mass balance, one would expect CML and LAL contents of 68.4–428.4 and 2.6–113.2 mg/100 g DM in hydrolysates, respectively. However, the CML and LAL contents of all hydrolysates varied between 49 and 84 and 0–12 mg/100 g dry matter, respectively (Fig. 1 D-F). It can be calculated that from the unheated meal M_0 that 82 ± 15% CML and 40 ± 56% LAL was transferred to hydrolysates (M_0H and M_0H_{aq}). And, from the heated meals (M_{15}, M_{60}, and M_{120}), only 23 ± 4% CML and 10 ± 4% LAL was transferred to the hydrolysates. This shows that for hydrolysates prepared from heated meals, large amounts of CML and

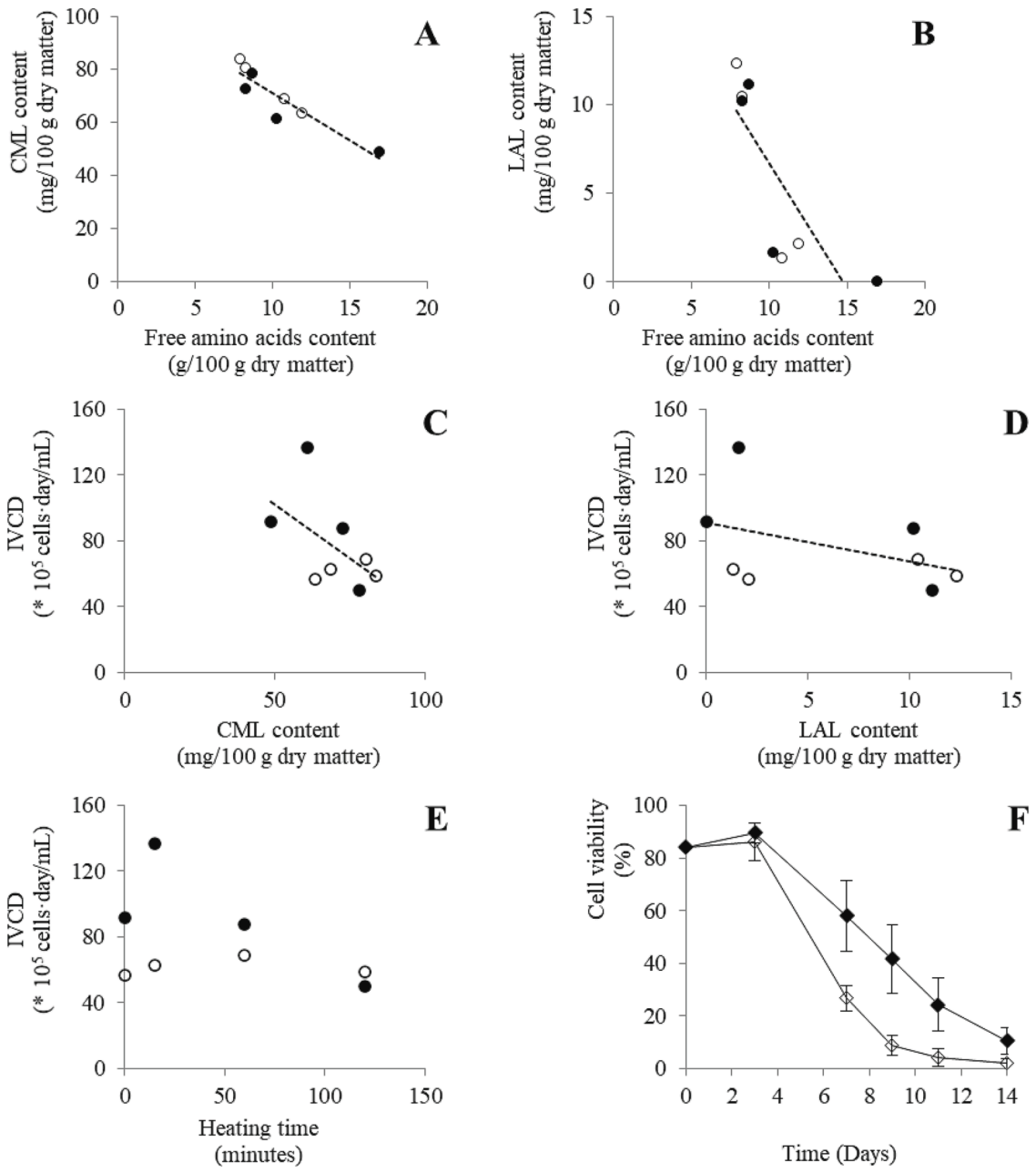

Fig. 4. Correlations between (A) CML-free amnio acid, (B) LAL-free amino acid, (C) IVCD-CML, (D) IVCD-LAL contents, (E) IVCD-heating time in cultures containing hydrolysates prepared with (○) and without (●) additional heating in suspension and (E) average cell viability in hydrolysate-supplemented CD medium (◆) and unsupplemented CD medium (◇) cultures.

LAL were removed during centrifugation and ultrafiltration steps of hydrolysate production.

The dry heating of meals introduced large variation in the CML (65.1 ± 13.0 mg/100 g DM; CV = 20%) and LAL contents (5.7 ± 5.8 mg/100 g DM; CV = 100%) in the resultant hydrolysates. This variation in the CML (73.9 ± 9.5 mg/100 g DM; CV = 13%) and LAL (6.5 ± 5.6 mg/100 g DM; CV = 86%) contents in the hydrolysates reduced substantially with additional heating in suspension (Fig. 3). Similar trend of decrease in variation with additional heating in suspension was observed for free amino acids and reducing carbohydrates as well (Fig. 3).

The CML (R = -0.9, p-value = 0.001) and LAL contents (R = -0.8, p-value = 0.01) correlated with the free amino acid contents in the hydrolysates (Fig. 4-A, B). No correlations were observed between CML, LAL, furosine and the reducing monosaccharide contents. This could be because mono-/oligosaccharides were more affected by fermentation than by the Maillard and cross-linking reactions during hydrolysate production.

These compositional effects on amino acids, carbohydrates, MRP, and CLAA may be quite significant for the nutritional and functional quality of hydrolysates used in cell cultures.

3.3. *Effects of hydrolysates on the cell culture functionality*

In all cases, the hydrolysate supplementation to CD medium enhanced cell viability, IVCD, and IgG production and decreased cell death as compared to the unsupplemented medium (set at 100%). The IVCD, IgG production, and cell death in hydrolysate-supplemented media were 115–316%, 148–200%, and 53–91%, respectively.

For all hydrolysates, the extent of enhancement in IVCD was much variable (CV_{IVCD} = 37%) than the IgG production (CV_{IgG} = 12.5%) and

depended on the heat treatments carried out on meals and hydrolysates. A large variation in the IVCD (115–316%; CV = 38.7%) was observed in the hydrolysates prepared from dry heated meals (121 °C/0–120 min) as compared to hydrolysates (131–159%; CV = 8.6%) prepared with additional heating in suspension (95 °C/ 60 min) (Fig. 3; Fig. 4-E). The variation in IgG production in cultures supplemented with hydrolysates prepared with (CV = 11%) and without (CV = 14.3%) additional heating in suspension was limited. Similar to IVCD, trends of decrease in variability were also noted in the CML, LAL, free amino acids, and the reducing carbohydrate contents (Fig. 3).

Weak negative correlations were observed between IVCD-CML (R = -0.534) and IVCD-LAL (R = -0.430), but not with IgG production (Fig. 4-C, D). This suggests that MRP and CLAA directly influenced the cell growth. Similar findings have been reported by Durling et al. (2009), where cell viability decreased from 95 to 71% with increase in HMF concentration from 2.5 to 100 mM in CHO-V79 cultures. In another study, as high as 50% decrease in cell viability was reported in HepG2 cells with addition of 38 mM of HMF (Severin et al., 2010).

In the current study, only the indicator compounds (CML, LAL, and furosine) of the Maillard and cross-linking reactions were analyzed. From the data, it is obvious that several diverse MRP and CLAA may have formed and that these compounds may have affected cell culture functionality. Furthermore, note that the LAL content (22 mg/100 g DM) reported for our most extreme sample (M_{120}; meal heated at 121 °C/ 120 min) was 4 times lower than the LAL content (~90 mg/100 g DM; 150 mg/100 g protein) reported for an industrial meal (Aymard et al., 1978). This suggests that the effects of industrial processing on MRP and CLAA can be even more drastic. Analyzing and studying these changes in hydrolysates and hydrolysate-supplemented cell cultures is challenging due to complex composition and interplay of compounds, like amino acids, carbohydrates, MRP and CLAA, on the functionality.

The results obtained in this study indicate that MRP and CLAA compounds may inhibit cell growth, but not affect recombinant protein production. These effects could however be cell line specific. The study shows significant effects of heat treatments on the composition and functionality of protein hydrolysates. Understanding these effects is very important since protein hydrolysates are commonly used in infant, human, and animal nutrition applications.

CRediT authorship contribution statement

Abhishek J. Gupta: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Jan-Willem Boots:** Conceptualization, Methodology, Writing – review & editing. **Harry Gruppen:** Conceptualization, Methodology, Writing – review & editing. **Peter A. Wierenga:** Conceptualization, Methodology, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136914.

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