Correlating composition and functionality of soy protein hydrolysates used in animal cell cultures

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Thesis

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Abstract

Soy protein hydrolysates are often supplemented to chemically defined (CD) media in cell cultures, but there is little understanding of the effect of their composition on their functionality (viable cell density, total immunoglobulin (IgG), and specific IgG production). To identify the key parameters (e.g. compound classes) that determine their functionality, hydrolysates were prepared from different starting materials (meal, concentrates, and isolate) and from soybean meal that was heated for different time periods. The functionality of these hydrolysates were compared to those of industrial hydrolysates. From the comparison, it was shown that the variation in industrial and experimental processes of hydrolysate production induced larger variation in the functionality than the variation in starting materials. Moreover, it was observed that the correlations between the functionality and compositional parameters observed in one experiment were absent in the other experiments. During the study, it became apparent that the variations in other factors, like CD media and temperature during culturing also resulted in variation in functionality. The extent of variations in the functionality due to variation in CD media and temperature during culturing was equivalent to the variation caused by varying the hydrolysate composition. The functionality data of the different experiments were fitted with a model that described the relation between specific IgG production and viable cell density. Using the model, the maximum achievable total IgG production could be calculated for a culture condition. This information can provide directions for further optimization of hydrolysates to maximize total IgG production.

Contents

CHAPTER 1

General Introduction

Chinese hamster ovary (CHO) cells are often used in cell cultures to produce biopharmaceuticals, such as immunoglobulin (IgG) and interferon-γ. In these cultures, the chemically defined (CD) media are often supplemented, for instance by soy protein hydrolysates. Generally, the supplementation with hydrolysates has positive effects on the functionality, i.e. increased viable cell density and increased recombinant protein (e.g. monoclonal antibody) production [1–4]. However, the functionality in cell cultures is known to be variable. For example, the recombinant protein production in cultures supplemented with 29 batches of a soy protein hydrolysate varied from 0.43 to 1.06 g/L [5]. It is commonly believed that part of the observed variability in the functionality is due to variation in the hydrolysate composition.

The hydrolysates do not only contain proteinaceous material (peptides and amino acids), but also significant amounts of carbohydrates, minerals, isoflavones, and saponins. In addition to these compounds, many other compounds (e.g. lipids and organic acids) are present in lower amounts. The hydrolysate composition can vary due to changes in the raw material composition (soybean or meal composition) and/or processing treatments (meal or hydrolysate processing). The changes in the raw material may affect the contents of compounds (e.g. total protein content). The changes in the processing treatments result in modification of the existing compounds (e.g. protein glycation) and/or formation of new compounds (e.g. carboxymethyl lysine and lysinoalanine) in the hydrolysate. The influence of the composition of the hydrolysate on its functionality has been studied using different approaches. Examples are metabolic flux analysis, untargeted metabolomics, fractionation of hydrolysates, and supplementation of pure compounds. In metabolic flux analysis studies, functionalities in cultures (e.g. CD media supplemented with hydrolysates and non-supplemented media) were measured and linked to rates of consumption of substrates (e.g. glucose) and production of metabolites (e.g. lactate). However, these differences in the functionality were not linked to the composition of hydrolysates supplemented to cell cultures [6]. Attempts to make this link have been made in untargeted metabolomics studies, where the composition of hydrolysates was analyzed using methods, like nuclear magnetic resonance (NMR) [7] and near infrared spectroscopy

(NIR) [5]. The spectral signal intensity data obtained from these analyses were subsequently correlated to the functionality. Since in these studies the comparison of hydrolysates was based on their NMR and/or NIR spectra, no links to the individual compounds were made. In other studies, fractionation methods have been used to study the effects of different fractions of hydrolysates on the functionality. However, the composition of such fractions have not been described and linked to the functionality [3, 8]. In studies using supplementation with pure compounds, researchers have tried to provide this link between composition and functionality. However, it became apparent that the functionality of hydrolysates is not equivalent to the sum of functionality of that of pure compounds (e.g. amino acids or monosaccharides) [2, 9]. This is probably due to the fact the effect of each compound is influenced by the presence of other compounds in hydrolysates. Consequently, there is still no clear understanding of the influence of hydrolysate composition on its functionality in cell cultures. Hence, the aim of this thesis is to understand the effect of composition of hydrolysates using *in situ* approaches on its functionality in mammalian cell cultures.

1.1. Biopharmaceuticals

Biopharmaceuticals are amongst others protein-derived therapeutic molecules that are used for treatment, diagnosis and prevention of diseases, which are produced and extracted from genetically engineered expression systems [10]. Some examples of proteinaceous biopharmaceuticals are blood factors, interferons, cytokines, vaccines, hormones, recombinant proteins, and monoclonal antibodies [11]. Biopharmaceuticals do not include molecules that are extracted directly from their native sources, e.g. extraction of penicillin from yeast cultures [10].

The production of biopharmaceuticals depends on the viable cell density (cells/mL) and specific production ($g \cdot cell^{-1} \cdot day^{-1}$) in cell cultures. The specific production is the cellular efficiency of producing biopharmaceuticals [12]. These parameters, i.e. cell growth and specific production, depend on the expression system used for production of biopharmaceuticals (**Table 1.1**).

Table 1.1: Comparison of different expression systems [13, 14]

	Bacteria (*E. coli*)	**Yeast**	**Insect**	**Mammalian**
Growth rate	Very fast	Fast	Slow	Slow
Total production	Very high	High	High	Very low
e.g. Total Hirudin production (mg/L)	200-300	0.25-1500	0.4	0.05
Media cost	Very low	Low	High	Very high
Culture techniques	Very easy	Easy	Difficult	Very difficult
Complex glycosylation	No	Yes	Yes	Yes

1.2. Expression systems

Different expression systems, such as bacteria (mainly *E. coli*), yeast, insect, fungi, and mammalian cells, have been used to produce biopharmaceuticals. The *E. coli* expression system has been used mainly because of its rapid growth rate (integral viable cell density, cells·day·mL^{-1}), capacity for continuous culturing, relatively low costs of maintenance, and high production (g/mL) (**Table 1.1**).

A limitation of the *E. coli* expression system is that it cannot perform post-translational modifications, such as N- and O-linked glycosylation, disulfide-bond formation, and phosphorylation. These modifications are critical for the functionality of recombinant proteins [15]. The lack of proper folding adversely affects the structure, function, and stability and thereby the bioactivity of recombinant proteins [15]. Consequently, *E. coli* are used for expressing non-glycosylated recombinant proteins [16]. The yeast expression system has all the advantages as those of the *E. coli* expression system and in addition, it can perform glycosylation **(Table 1.1)**. However, the glycosylation patterns differ from those produced by human cells. The glycosylation patterns produced by insect cells are also different from those produced by human cells **(Figure 1.1)**.

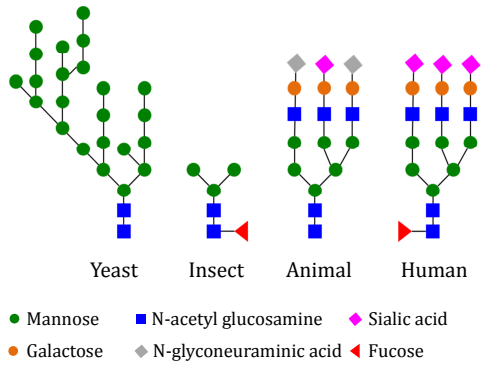

Figure 1.1: Glycosylation patterns produced by yeast, insect, animal, and human cells (adapted from [17])

Therefore, to produce proteinaceous biopharmaceuticals that have glycosylation patterns similar to that produced by human cells, expression systems based on animal cells are used [13, 18, 19]. Chinese hamster ovary (CHO) cells, baby hamster kidney-293 cells, mouse L-fibroblasts, and myeloma cells are commonly used for biopharmaceutical production [15, 18]. It has been indicated that 70% of all recombinant proteins that are produced are made using CHO cells [20]. To understand the current stature of animal cell cultures, it is important to see how it has evolved over time.

1.3. Development of animal cell cultures

The history of animal cell culture has been described in detail in 2 reviews [21, 22] and a selected list of important historical milestones is depicted in **Table 1.2**.

In 1878, Claude Bernard hypothesized that the cells isolated from an organism could be kept alive in their physiological state even after the death of the organism. Harrison proved this hypothesis by isolating nerve fibers from frog and cultivating them in *in vitro* cultures containing frog lymph. Later Burrows, a student of Harrison, together with Carrel used clotted blood plasma and embryonic extracts to grow avian and mammalian cells.

Table 1.2: Historical milestones in development of animal cell cultures [21, 22]

Timeline	Scientist	Achievements
1878	Bernard	Physiological state of cells can be maintained after the death of an organism
1885	Roux	Maintained chick embryonic cells in warm salt solutions
1907	Harrison	Isolated and cultured frog nerve cells
1910	Lewis and Lewis	Cultivated embryonic chick tissues in media containing bouillon
1911	Burrows	Used clotted blood plasma to grow avian and mammalian cells
1912	Lewis and Lewis	Cultivated chick tissues in salt solutions
1936	Baker	Cell culture media containing sera and blood protein digests
1948	Fischer*	Development of a CD medium for chick cells
1955	Eagle*	Development of a CD medium for mouse fibroblasts and HeLa cells
1964	Ham*	Development of a CD medium for CHO cells
1971	Takaoka and Katsuto	Development of a CD medium
1975	Kohler and Milstein	First hybridoma capable of secreting a monoclonal antibody
1977	Genentech	Production of Somatistatin
1978	Sato	Serum-free media containing hormones and growth factors
1982	Eli Lilly	Production of insulin, Humulin®
1985	n.m.#	Recombinant human growth hormone
1985	Collen	Recombinant tissue-type plasminogen activator
1985-1990	n.m.#	BSE and TSE crisis
1989	Amgen Inc.	Recombinant erythropoietin produced in CHO cells

* The medium was supplemented with chicken plasma [9], horse serum, human serum [23], or fetal calf serum [24];

n.m: not mentioned

Claude Bernard also pointed out that it was necessary to isolate and culture tissues in an artificial environment to understand their properties [25]. The first attempts (1912) to do this was by culturing cells in salt solutions (NaCl, $CaCl_2$, KCl, and $NaHCO_3$) of known compositions (CD media) [26]. In these solutions, the cells could be kept alive for up to 8-9 days. However, the cell growth was not as extensive as that observed in the media composed of nutrient agar, animal sera, or bouillon [26]. Attempts were made to understand the functionality of animal serum by separating it into fractions using alcohol-salt precipitation and exhaustive dialysis, but were unsuccessful [23]. Therefore, all the CD media developed subsequently [9, 23, 24] were supplemented with extracts from animal sources, such as ash of liver [27], horse serum [28], digests of blood [29], and digests of embryo tissues, egg white, fibrin, or rabbit brain [30].

In 1971, Takaoka and Katsuta successfully grew several cell lines in a CD medium without supplementing animal extracts [31]. However, periods of up to 6 months were required to adapt the cell lines in the CD medium without animal extracts [31]. Moreover, the cell lines that were successfully adapted lost some of their physiological properties (e.g. adherence and morphology) [31]. These issues were overcome with a major improvement in the formulation of CD medium in 1979. This was the addition of insulin, transferrin, putrescine, progesterone, and selenium to the CD medium [32]. The cell density in media supplemented with these compounds was $>$ 26 times higher than that in the non-supplemented medium [32]. The supplementation of individual compounds to the medium did not increase viable cell density, which illustrated the synergistic effects of these compounds [32]. While CD media were being developed further, the drawbacks of using animal extracts (e.g. serum) in cell culture were increasingly recognized. A substantial lot-to-lot variability was reported in the functionality and composition of the fetal calf sera [32–34]. The cell densities in human fetal lung cell cultures after 72 hours (initial incubation of $2 * 10^4$ cells/cm^2) supplemented with 117 different fetal calf sera varied between 6-195 $* 10^3$ cells/cm^2 [34]. The total protein, total lipid, and glucose contents in sera varied between 2-5 g/100 mL (164 samples), 140-440 mg/100 mL (60 samples), and 58-319 mg/100 mL (31 samples), respectively [33, 34]. Furthermore, the use of fetal calf sera and animal extracts became undesirable due to high risk of transmission of viruses, bacteria, and prions that might be present in sera or animal extracts [33]. Therefore, in recent years, substantial efforts have been made to find alternatives for serum.

1.4. Development and optimization of CD media and plant protein hydrolysates

To replace serum and other animal extracts from cell cultures, the approaches that have been developed are either to improve the composition of CD media or to use plant protein hydrolysates as supplements for CD media.

1.4.1. CD media

Since each cell line has unique nutritional requirements, one type of medium cannot be used for all cell lines. As a result, several CD media (e.g. ISCHO-CD™, PowerCHO™, and EX-CELL® CD CHO) are commercially available to culture cells of different origins. Although they are called chemically defined, their chemical composition has not been described in scientific literature, as discussed by van der Valk et al. [35]. Typically, CD media contain pure compounds of known structures, such as hormones, growth factors, protease inhibitors, synthetic polymers (e.g. copolymers of polyoxopropylene and polyoxyethylene) to protect against shear stress, vitamins, amino acids, trace elements, lipids, antibiotics, and attachment factors [35]. A detailed overview of studies evaluating different supplements in CD media is provided elsewhere [36].

1.4.2. Plant protein hydrolysates

Plant protein hydrolysates are produced from different raw materials, such as soy, cotton, rice, wheat, rapeseed, pea, and corn [4]. Soy protein hydrolysates are the ones that are most commonly supplemented to cell cultures. Typically, hydrolysates are supplemented at 2 g/L (w/v) to CD media [2, 3, 8, 37, 38]. The addition of hydrolysates to the CD medium substantially increases the protein and carbohydrate contents of the supplemented CD medium. To illustrate this, the composition of a CD medium and CD medium supplemented with 2 g/L of a soy protein hydrolysate is presented as an example below (**Table 1.3**). The addition of hydrolysates to the CD medium substantially increases the protein and carbohydrate contents of the supplemented CD medium (**Table 1.3**).

The functionality of hydrolysates has been evaluated in several studies [1, 3, 4, 8, 40]. To compare results from different experiments, the viable cell density and recombinant protein production data of hydrolysate-supplemented cultures were normalized by dividing them with the data of the non-supplemented CD medium. This results in relative viable cell density (%) and relative recombinant protein production (%). The relative viable cell density and relative recombinant protein production in the hydrolysate-supplemented CD medium in different studies [1, 3, 4, 8, 40], varied between 77-244% and 78-413%, respectively, relative to that in the CD medium (100%) (**Table 1.4**).

In a number of cases, the viable cell densities were lower in hydrolysate-supplemented CD media than that in the CD medium alone. However, in almost all cases the recombinant protein production was higher in hydrolysate-supplemented CD media than that in the CD medium. The observed effects of hydrolysate supplementation differ widely among the studies. This is because these studies have been performed with different cell lines, CD media, and hydrolysates produced from different raw materials and different processes.

Different cell lines

CHO-320 and ProCHO5 cells were cultivated in CD media supplemented with 2 g/L of a cotton meal hydrolysate. While, compared to the CD medium alone (100%), the relative viable cell density and relative recombinant protein production were 100 and 150% in CHO-320 culture, respectively, they were 244 and 413% in ProCHO5 culture, respectively [37].

Hydrolysates from same raw materials but different processes

The relative viable cell density and relative recombinant protein production were 179 and 153% in SP 2/0 culture supplemented with cotton meal hydrolysate (CNE50M) and 77 and 138% in SP 2/0 culture supplemented with another cotton meal hydrolysate (Hypep7504), respectively [4].

Hydrolysates supplemented at different concentrations

At 5 g/L the relative IgG production varied between 103 to 138%, whereas it varied between 83 to 138% when the same batches of a hydrolysate were supplemented at 15 g/L in CHO cell cultures, relative to the CD medium (100%) [43]. The latter study [43] also shows

Table 1.3: Composition of Iscove's modified Dulbecco's medium and Iscove's modified Dulbecco's medium supplemented with 2 g/L of a soy protein hydrolysate [39]

CD medium	Non-supplemented	Supplemented with HyPep™ 1510	% Increase
Other components	**mg/L**	**mg/L**	**%**
HEPES buffer	5958	5958	-
D-Glucose	4500	5094#	113
Sodium pyruvate	110	110	-
Phenol red sodium salt	15	15	-
Amino acids			
L-Lysine hydrochloride	146	222	52
L-Isoleucine	105	151	44
L-Leucine	105	185	176
L-Tyrosine disodium salt	104	146	40
L-Threonine	95	143	51
L-Valine	94	144	53
L-Cystine dihydrochloride	91	99	9
L-Arginine hydrochloride	84	166	98
L-glutamine + L-Glutamic acid	75 (0 + 75)	319**	325
L-Asparatic acid + L-Asparagine	55 (30 + 25)	201**	265
L-Phenylalanine	66	118	79
L-Histidine hydrochloride monohydrate	42	70	67
L-Serine	42	104	148
L-Proline	40	96	140
L-Glycine	30	80	167
L-Methionine	30	44	47
L-Alanine	25	77	208
L-Tryptophan	16	16*	0*
Vitamins			
Inositol	7.2	7.2*	-
Choline chloride	4	4*	-
D-Ca- Pantothenate	4	4*	-
Folic acid	4	4*	-
Nicotinamide	4	4*	-
Pyridoxal hydrochloride	4	4*	-
Thiamine hydrochloride	4	4*	-
Riboflavin	0.4	0.4*	-
D-Biotin	0.013	0.013*	-
Vitamin B 12	0.013	0.013*	-
Salts			
Sodium chloride	4505	4505*	-
Sodium bicarbonate	3024	3024*	-
Potassium chloride	330	330*	-
Calcium chloride dehydrate	219	219*	-
Sodium trihydrogen phosphate	109	109*	-
Magnesium sulfate	97.72	97.72*	-
Potassium nitrate	0.076	0.076*	-
Sodium selenite	0.017	0.017*	-

#Carbohydrate content(%) = 100 - *total amino acid*(%) - *ash*(%) - *moisture*(%)
*Data on hydrolysates is not available ** In hydrolysates, single values were given for glutamine + glutamic acid and asparagine + asparatic acid

that the effect of hydrolysates may vary from batch-to-batch. In another study, the recombinant protein production in cultures supplemented with 29 batches of a soy protein hydrolysate varied between 0.4-1.1 g/L [5]. While this variation in functionality can be due to cell culture parameters (e.g. pH and temperature [44]), CD media [45], and CD media supplements [46], it can also be due to the variation in the composition of hydrolysates supplemented to cell cultures.

Table 1.4: Effect of supplementing plant protein hydrolysates to CD media on relative viable cell density and relative recombinant protein production

Cell line	Source	Concentration % (w/v)	Viable cell density* (%)	Recombinant protein* (%)	Reference
CHO	Soy	0.1	145	160	[41, 42]
CHO-320	Pea	0.1	91	121	[1]
CHO-320	Wheat	0.1	111	121	[1]
CHO-320	Rice	0.1	95	107	[1]
CHO-320	Soy	0.2	150	272	[37]
CHO-320	Cotton	0.2	100	150	[37]
CHO-320	Soy	0.2	98	135	[8]
CHO-320	Soy	0.2	91	121	[40]
ME-750	Soy	0.2	107	112	[3]
ME-750	Wheat	0.2	148	188	[3]
ProCHO5	Soy	0.2	180	300	[37]
ProCHO5	Cotton	0.2	244	413	[37]
SP 2/0	Soy	0.1-0.5#	90	126	[4]
SP 2/0	Wheat	0.1-0.5#	131	155	[4]
SP 2/0	Wheat	0.1-0.5#	83	101	[4]
SP 2/0	Rice	0.1-0.5#	106	78	[4]
SP 2/0	Cotton	0.1-0.5#	77	138	[4]
SP 2/0	Soy	0.1-0.5#	154	115	[4]
SP 2/0	Wheat	0.1-0.5#	158	126	[4]
SP 2/0	Cotton	0.1-0.5#	179	153	[4]
SP 2/0	Pea	0.1-0.5#	167	154	[4]
CD medium	-	-	100	100	-

*Cell density and recombinant protein production are expressed as percentage relative to that in the CD medium
#The exact concentration of hydrolysate supplemented to the CD medium was not specified

1.5. Composition of plant protein hydrolysates

The total and free amino acid compositions, mineral composition, and molecular mass distribution of peptides of some industrial hydrolysates are described in the technical specifications of the hydrolysate manufacturers. Some examples of these hydrolysates are provided in **Table 1.5**. The protein (based on total amino acid content), ash, moisture, and total dry matter contents in hydrolysates varies between 53-84% (w/w), 7-14% (w/w), 3-4% (w/w), and 67-96% (w/w) in these hydrolysates, respectively. The carbohydrate contents were not reported. It was postulated that the carbohydrate content, calculated as 100 - *protein*% - *ash*% - *moisture*%, of the hydrolysates varied between 4-33% (w/w). Besides the technical specifications, only in one scientific study, the composition of an industrial hydrolysate was reported: 60% (w/w) peptides/amino acids, 20% (w/w) carbohydrates, 10% (w/w) minerals, and 0.0012% (w/w) phospholipids [8]. Although several cell culture studies have been performed with many experimental hydrolysates, in none of these studies this type of compositional information of experimental hydrolysates has been reported.

1.6. Factors affecting composition of hydrolysates

An overview of the factors that affect the composition and the functionality of soy protein hydrolysates is described in **chapter 2** of this thesis. From this, it is concluded that variation in the raw material (soybean or meal) composition and variation in the processing treatments (soybean, meal, or hydrolysate processing) affect the composition of soy protein hydrolysates (**Figure 1.2**).

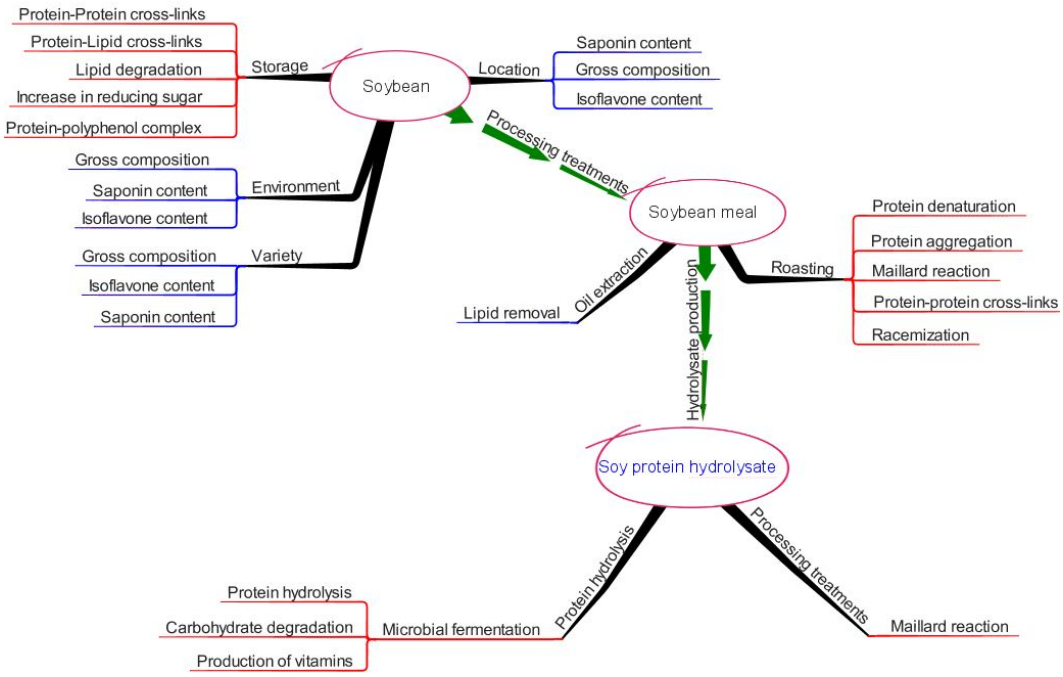

Figure 1.2: Quantitative (blue lines) and qualitative changes (red lines) in the composition of hydrolysate due to changes in raw material composition and processing treatments

These factors affect the compound classes (proteins, carbohydrates, isoflavones, and saponins) and sub compound classes (free amino acids, mono/oligosaccharides) that are present in the hydrolysates. In addition, it may result in formation of specific compounds (e.g. Maillard reaction products (MRP), cross-linked amino acids (CLAA)).

Table 1.5: Typical specifications of industrial soy protein hydrolysates [47]

Parameters	HyPep1510	HyPep1511	HyPep1512	Hy-Soy	Hy-Soy T	Hy-Soy 4D	N-Z-Soy BL4
Amino Nitrogen (%, AN)	2.0	2.7	2.2	1.9	1.8	1.9	2.7
Total Nitrogen (%, TN)	9.2	13.6	8.8	9.1	7.9	9.2	14.3
Ratio = AN/TN	21.7	20.0	25.0	21.0	22.8	20.4	18.9
Ash (%)	9.7	8.6	11.6	10.7	6.6	13.8	8.2
Moisture (%)	3.6	2.6	2.8	2.5	3.9	3.4	3.3
> 10 kDa (%)	0.0	0.1	0.0	0.0	0.0	1.7	0.5
5-10 kDa (%)	0.2	1.4	0.1	0.3	0.2	2.6	3.1
2-5 kDa (%)	4.6	10.1	3.2	5.3	5.0	9.1	13.0
1-2 kDa (%)	14.3	19.7	13.5	16.4	16.3	17.5	20.5
0.5-1 kDa (%)	22.8	25.0	21.1	23.5	23.8	20.4	25.2
< 0.5 kDa (%)	58.1	43.7	62.1	54.6	54.7	48.6	37.7
Calcium (%)	0.14	0.03	0.02	0.20	0.37	0.04	1.37
Iron (%)	0.0004	0.0	0.004	0.006	0.008	0.004	0.003
Magnesium (%)	0.03	0.0	0.14	0.25	0.29	0.22	0.04
Phosphorus (%)	0.03	0.25	0.41	0.27	0.75	0.44	0.14
Potassium (%)	3.4	0.7	2.5	3.0	2.4	2.7	0.2
Sodium (%)	3.30	4.04	3.44	2.7	1.78	3.25	0.93
Total amino acids (mg/g)	**568**	**767**	**525**	**542**	**609**	**536**	**843**
Alanine (mg/g)	26	30	25	23	25	25	30
Arginine (mg/g)	41	62	37	37	44	39	65
Asparagine/Aspartic acid (mg/g)	73	92	66	74	74	66	90
Cysteine (mg/g)	4	1	3	1	1	5	0
Glutamine/Glutamic acid (mg/g)	122	166	112	123	119	115	170
Glycine (mg/g)	25	29	24	22	20	25	35
Histidine (mg/g)	14	17	13	13	13	12	17
Isoleucine (mg/g)	23	28	22	20	21	25	25
Leucine (mg/g)	40	53	38	38	41	38	50
Lysine (mg/g)	38	52	32	39	42	34	106
Methionine (mg/g)	7	10	7	5	8	7	9
Phenylalanine (mg/g)	26	33	24	26	27	25	34
Proline (mg/g)	28	37	27	29	28	27	39
Serine (mg/g)	31	41	29	32	29	27	46
Threonine (mg/g)	24	29	22	22	22	22	27
Tyrosine (mg/g)	21	26	20	18	21	21	25
Valine (mg/g)	25	65	24	22	76	25	75
Total free amino acids (mg/g)	**45**	**28**	**68**	**63**	**35**	**56**	**20**

The changes in hydrolysate composition can be analyzed using conventional analytical methods (e.g. Dumas analysis for total nitrogen content), whereas for the analyses of changes in the specific compounds more specialized methods are needed. Different methods have been described and used in the literature to analyze the hydrolysate composition and to link it with the functionality.

1.7. Approaches to analyze hydrolysate composition and linking it to its functionality

It is clear from the previous section that the analyses of compound classes, sub compound classes, and specific compounds present in the hydrolysate and linking them to the functionality requires different approaches. Examples of these approaches are: 1) metabolic flux analysis (MFA), 2) untargeted metabolomics, 3) fractionation methods, and 4) supplementation with pure compounds.

1.7.1. Metabolic flux analysis

In this approach, the conversion of compounds from the medium by the cells is determined. The metabolic flux is the rate at which compounds are either consumed (substrates) or produced (metabolic end products) by cells [48]. In a cell culture assay, these fluxes are determined by measuring viable cell densities and concentrations of compounds (e.g. glucose, lactate, and amino acids) at different time points in the culture medium. From these values, specific rates of consumption or production are calculated for each compound. Using these rates, the fluxes of all reactions in the cellular metabolism are quantified. This is called metabolic flux analysis (MFA) [49]. The viable cell density in a meat hydrolysate-supplemented CD medium was 3.3 * 10^6 cells/mL as compared to 1.5 * 10^6 cells/mL in the CD medium [6]. As meat hydrolysates mainly contain peptides [50], this suggests that increase in protein content of CD media increased cell density. The IgG production in these cultures were not mentioned. Two major differences were observed between the metabolic fluxes in these cultures. Firstly, the flux towards the citric acid (TCA) cycle in the hydrolysate-supplemented medium was more than twice of that in the CD medium. In the TCA cycle, a large amount of energy (2 ATP, 2 $FADH_2$, and 6 NADH) is produced. This suggests that in hydrolysate-supplemented CD medium more energy was available than in the CD medium culture to support cell growth. Secondly, in the CD medium culture the flux of pyruvate towards lactate production was twice of that in the hydrolysate-supplemented CD medium. The lactate accumulation has been associated with decrease in cell growth and increase in cell death [51]. Although differences were identified between the metabolic fluxes of the two cultures, these differences were not linked to the composition of medium or medium containing hydrolysate.

1.7.2. Untargeted metabolomics

Using untargeted metabolomics studies, attempts have been made to link the composition of a hydrolysate to its functionality in cell cultures. The field of metabolomics has been developed

to characterize compounds that are present in biological samples [52]. Different separation and detection methods are used to identify and quantify metabolites, such as liquid and gas chromatography (LC/GC) coupled to mass spectrometry (MS), NMR, and NIR. For the LC-MS methods, MS signals obtained are compared with large databases to annotate compounds. The MS intensity can then be used to obtain the relative abundance of the compounds present in samples. For NIR and NMR methods, typically the spectra have been used without annotation of compounds. The approach of analysis, where these methods (NMR, NIR, and LC-MS) or others are used with or without annotation to analyze compounds without focusing on specific compounds is referred to as untargeted metabolomics. In all cases, the detector signals are directly correlated to viable cell density and recombinant protein production using statistical methods, such as principal component analysis (PCA) and partial least squares (PLS) regression [53, 54]. Recently, several studies have used this approach to analyze the functionality of protein hydrolysate in cell cultures (**Table 1.6**).

Table 1.6: Overview of untargeted metabolomics studies of hydrolysates

Analytical methods	Year	Statistical methods	Aim	
NMR	2007	PCA and PLS	Identify compounds that describe lot-to-lot variation	[7]
NIR	2010	PCA	Method to screen good and bad performing lots	[55]
NMR	2012	PCA and PLS	Identify compounds that describe lot-to-lot variation	[56]
NIR	2012	PCA and PLS	Predict functionality of hydrolysates	[57]
NIR, Raman, Fluorescence, and X-ray	2012	PCA and PLS	Predict functionality of hydrolysates	[57]
NIR	2013	PLS	Predict functionality of hydrolysates	[5]

PCA based on composition and functionality has been used to identify clusters of similar samples in a set of samples [55]. It is also used to identify the compounds due to which certain samples behave differently than others. Using PLS regression, lactate was identified to predict ($R^2 = 0.87$) the recombinant protein production in hydrolysate-supplemented cultures [5, 7, 56, 57].

1.7.3. Fractionation methods

Hydrolysates have been separated into fractions using different methods, like membrane filtration [2, 58], ethanol extraction [59], and chromatography [3, 8, 60]. In these studies, fractions with beneficial or detrimental effects have been obtained. The maximum viable cell density in cultures supplemented with 2 g/L of fractions were 7.5, 4.8, 3.4, 2.9, 2.8, and 3.9 * 10^6 cells/mL and that in the unfractionated hydrolysate was 5.6 * 10^6 cells/mL, respectively [59]. In these studies, the compounds present in different fractions were not identified [8, 58, 59, 61]. Consequently, there is no clear understanding of the relation of the composition of fractions on the cell culture functionality.

1.7.4. Supplementation with pure compounds

In few studies, the effects of supplementation with pure compounds like peptides, carbohy-

drates, isoflavones, saponins, lipids, and MRP have been studied in cell culture. It is important to note that the effects of compounds present in hydrolysates are not equivalent to the effects of pure compounds in cell culture. The average cell density in CD medium supplemented with rapeseed protein hydrolysate (2 g/L) was 138% as compared to 107% in CD medium added with a free amino acid mixture resembling the hydrolysate [62]. Nevertheless, a number of studies (*vide infra*) have been conducted using this approach. Some compounds or compound classes (e.g. peptides and carbohydrates) are functional at high concentrations, while others (e.g. lipids, isoflavones, and MRP) are toxic already at much lower concentrations.

Peptides

The supplementation with different types of peptides (e.g. amino acid composition, chain length, and concentration) has different effects on viable cell density and recombinant protein production. While peptide supplementation has been shown to only increase viable cell density [63], to only increase recombinant protein production, to increase both viable cell density and recombinant protein production [64], it has also been shown to decrease viable cell density and increase recombinant protein production [65]. The effects of peptides could not be reproduced by addition of equivalent amount of free amino acids [2, 9, 66]. Besides peptides, the effect of glutamine concentration has been studied. Glutamine is a major source of energy and nitrogen for mammalian cells. The maximum viable cell density and antibody production in high glucose-glutamine containing cultures were 3 * 10^6 cells/mL and 150 mg/L as compared to 11 * 10^6 cells/mL and 250 mg/L in low glucose-glutamine containing cultures [67]. This suggests that by optimizing glucose and glutamine concentrations, the productivity of cultures can be enhanced.

Carbohydrates

Typically, CD media contain glucose to support cell growth, although other carbohydrates also have been shown to significantly affect the cell culture functionality. In CHO-TF-70R cultures, the addition of glucose, fructose, galactose, and mannose at 20 mM concentration showed large differences in the viable cell densities (1.2, 0.6, 0, and 1.2 * 10^6 cells/mL) after 160 hours of culturing [68]. In these cultures, the tissue-plasminogen activator production were 3.2, 2.0, 2.5, and 3.6 mg/L, respectively [68]. Therefore, the functionality was comparable in cultures containing glucose or mannose, yet being higher than in cultures containing fructose and galactose.

Saponins and isoflavones

The saponin (0.20-0.26% w/w) and isoflavone (0.16-0.33% w/w) contents of soy protein hydrolysates are very low [69]. Thus, 0.4% (w/v) of hydrolysate supplementation to cell culture would result in saponin and isoflavone contents of 8-10 and 6-13 μg/mL, respectively. In CD media cultures, the effects of saponins and isoflavones have been tested at much higher concentrations than the concentrations expected in hydrolysate-supplemented cultures. For instance, the addition of saponins to a final concentration of 25-200 μg/mL, like soy

sapogenol B, reduced cell death by 25-87% caused by 2-acetoxy acetylaminofluorene in CHO cell cultures [70]. Conversely, supplementing isoflavones, like genistein-8-C-glucoside (0-100 μM), increased cell death by inducing apoptosis and genotoxicity in CHO cell cultures [71, 72].

Lipids

The supplementation with lipids, like sodium butyrate, has been reported to decrease cell growth in CHO cell cultures. CHO cells (3.5 * 10^4 cells) were cultured in tissue culture dishes. After 100 hours of culturing, the viable cell densities in non-supplemented medium and medium containing 0.5 mM sodium butyrate were 7 * 10^6 cells and 4 * 10^7 cells, respectively [73]. In another study, the viable cell densities in medium containing 0.5 mM of sodium butyrate were 83% lower than that in the non-supplemented medium cultures [74]. The effects on recombinant protein production were not discussed in these studies. As the total lipid content in soy protein hydrolysates is very low (0.0012% w/w) [8], it is expected that the lipids will not affect cell cultures.

Maillard reaction products and cross-linked amino acids

Certain compounds, like MRP (e.g. hydroxymethyl furfural, HMF) and CLAA (e.g. lysinoalanine, LAL), are not native to the raw materials used in hydrolysate production. Instead, they are produced during processing. The supplementation of HMF to a concentration of 2.5-100 mM in the CD medium increased cell death by 24-27% in CHO cells [75]. In HepG2 cells, supplementation to a concentration of 38 mM of HMF in the CD medium decreased cell viability by 50% [76]. In another study with CHO cells, the cell viability decreased from 68 to 22% with an increase in the HMF concentration from 80 to 140 mM in CD medium, relative to the CD medium (100%) [77]. The supplementation of other advanced MRP, like pyrazine, 2-ethylpyrazine, and 2,6-dimethylpyrazine to final concentrations of 232 mM, 313 mM, and 313 mM, respectively, to the CD medium caused toxicity and completely inhibited cell growth [78]. The effects of MRP and CLAA on the recombinant protein production have not been described in any of these studies.

1.8. Aim and outline of the thesis

From previous sections, it is clear that while the functionality of hydrolysates may vary from lot-to-lot, the factors that cause this variation have not been described. The variation in the functionality of hydrolysate can occur due to variation at the level of compound classes and/or the level of specific compounds that are present in the hydrolysate. Surprisingly, in most cases, either the composition of hydrolysates has not been analyzed systematically or it has not been analyzed at all and not been reported in the scientific literature. Furthermore, the composition of hydrolysates has not been linked to the functionality. Consequently, it is not clear how changes in the composition affect the functionality of hydrolysates.

The aim of this thesis is to identify the key parameters, at the level of compound classes, sub compound classes, and individual compounds, in the composition of soy protein hydrolysates that could determine the functionality in cell cultures. As proteins and carbohydrates consti-

tute 70-80% of the hydrolysate composition, it is hypothesized that these compound classes determine the hydrolysate functionality. The second aim is to identify whether variations in the hydrolysate composition are introduced by variations in the processing treatments or by variations in the starting material composition.

In **chapter 2**, a literature review is provided in which factors that affect the composition of the hydrolysates are described. In addition, the effect of different compound classes, sub compound classes, and specific compounds is discussed. To study the effect of variation in different compound classes, i.e. total protein, total carbohydrate, total saponin, and total isoflavone contents, hydrolysates are prepared from different starting materials (**chapter 3**). Next, to study the effect of different compound classes, sub compound classes, and specific compounds that are affected by processing, defatted soybean meal is prepared and heated for different times. From these meals, hydrolysates are prepared with and without extended heating in suspension. Subsequently, the gross composition and contents of free amino acids, mono-/oligosaccharides, and specific compounds, i.e. Maillard reaction products and cross-linked amino acids of hydrolysates are analyzed. The contents of all of these compounds are tested for correlations with the functionality (**chapter 4**). Finally, to compare the effects of experimental hydrolysates prepared from different starting materials and different processing treatments, industrial soy protein hydrolysates with similar gross composition are chosen. These hydrolysates are analyzed using a non-targeted metabolomics approach to analyze individual compounds. The compositional data of individual compounds is then correlated to the viable cell density and IgG production (**chapter 5**). In **chapter 6**, the data obtained in the previous chapters is combined and fitted with a model. The results were compared the variation induced by other factors, such as CD medium and temperature during culturing.

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CHAPTER 2

Factors causing compositional changes in soy protein hydrolysates and effects on cell culture functionality

Abstract

Soy protein hydrolysates significantly enhance cell growth and recombinant protein production in cell cultures. The extent of this enhancement in cell growth and IgG production is known to vary from batch-to-batch. This can be due to differences in the abundance of different classes of compounds (e.g. peptide content), the quality of these compounds (e.g. glycated peptides), or the presence of specific compounds (e.g. furosine). These quantitative and qualitative differences between batches of hydrolysates result from variation in the seed composition and seed/meal processing. Although a considerable amount of literature is available which describes these factors, this knowledge has not been combined in an overview yet. The aim of this review is to identify the most dominant factors that affect hydrolysate composition and functionality. While there is a limited influence of variation in the seed composition, the overview shows that the qualitative changes in hydrolysate composition result in the formation of minor compounds (e.g. Maillard reaction products). In pure systems, these compounds have a profound effect on the cell culture functionality. This suggests that presence of these compounds in soy protein hydrolysates may affect hydrolysate functionality as well. This influence on the functionality can be of direct or indirect nature. For instance, some minor compounds (e.g. Maillard reaction products) are cytotoxic; while other compounds (e.g. phytates) suppress protein hydrolysis during hydrolysate production resulting in altered peptide composition, and thus, affect the functionality.

Based on:

Abhishek J. Gupta, Harry Gruppen, Dominick Maes, Jan-Willem Boots, and Peter A. Wierenga, *Journal of Agricultural and Food Chemistry* (2013), **61**, (45), 10613-10625.

2.1. Introduction

The performance of a mammalian cell culture system is primarily determined by the product yield (production of recombinant proteins, such as immunoglobulin (IgG) and interferon-γ) and product quality (glycosylation of recombinant proteins) [1]. In many cell culture studies, viable cell density is used as a second parameter to describe the cell culture performance [1, 2]. This is because the recombinant protein production can be affected by cell growth. In some studies, recombinant protein production has been reported to increase with an increase in viable cell density [1, 2], while in other studies, recombinant protein production increased following a suppression in the viable cell density [3].

To improve the performance of mammalian cell culture processes, fetal calf serum has been used as a supplement for several decades. This supplement provides growth factors, proteins, lipids, attachment factors, minerals, hormones, and several trace elements that are important for promoting viable cell density and enhancing recombinant protein production [1, 4]. However, the use of fetal calf serum has become restricted due to the risk of transmissible diseases, like bovine spongiform encephalopathy. As a result, substantial research has been performed to identify alternatives for fetal calf serum. Currently, two alternatives have been described in literature: 1) to use chemically defined medium [5–8], or 2) supplement the basal chemically defined medium with plant protein hydrolysates [9–12]. The latter approach has become a common practice in the biopharma industry.

Industrially produced plant protein hydrolysates from several sources such as soy, cotton, rice, wheat gluten, pea, and rapeseed proteins are available for cell culture applications [13–15]. The viable cell density of the Chinese hamster ovary (CHO, CHO DG44/*dhfr*-/-) cells cultivated in HyQ® CDM4-CHO medium supplemented with 16 hydrolysates obtained from different sources varied from 106% to 144% relative to that in the chemically defined medium (100%) [2]. Although the enhancement in cell growth in culture supplemented with soy protein hydrolysate was the highest, the reason why soy protein hydrolysates performed better than other hydrolysates was not explained.

In **Table 2.1**, an overview of the influence of soy protein hydrolysate supplementation to chemically defined medium on viable cell density and recombinant protein production is provided. In this chapter, the viable cell density and recombinant protein production for chemically defined medium is set to 100%. The viable cell density and IgG production in hydrolysate-supplemented chemically defined medium ranged from 90-178% and 95-300%, respectively, as compared to that in the chemically defined medium (100%) (**Table 2.1**).

Several cell culture studies have been performed, in which various cell lines (e.g. CHO-320, ME-750, and WuT3) [10, 11, 17, 18], chemically defined media (e.g. RPMI-1640, 5:5:1 v/v of IMDM:Ham's F12:NCTC, and 2:1:1 v/v of DMEM:F12:RPMI) [11, 17, 18] supplemented with soy protein hydrolysates [9, 11, 12, 17] produced using different processes have been tested. The comparison of results obtained in these studies is limited, because these chemically defined media and hydrolysates have different chemical compositions and different cell lines have diverse nutritional requirements [1]. This comparison is further complicated by the

Table 2.1: Effect of supplementation of soy protein hydrolysates to chemically defined media on relative viable cell density and relative recombinant protein production

Cell line	Hydrolysate concentration % (w/v)	Relative viable cell density (%) (1)	Relative recombinant protein (%) (1,2)	Relative specific production (%) (3)	Reference
CHO-320	0.2	150	272	181	[16]
ProCHO5	0.2	178	300	169	[16]
CHO-320	0.2	91	121	133	[17]
CHO-320	0.1	100	116	115	[9]
CHO-320	0.1	91	108	119	[9]
ME-750	0.2	107	112	105	[11]
CHO-320	0.2	98	135	138	[17]
WuT3	0.1-1.0	94-152	95-155	87-102	[18]
CHO	0.1	145	160	110	[19, 20]
SP 2/0 hybridoma	0.1-0.5	90	126	140	[14]
SP 2/0 hybridoma	0.1-0.5	154	115	75	[14]
	CD medium	100	100	100	-

(1) Relative viable cell density and relative recombinant protein production are expressed as % relative to that in the chemically defined medium; (2) The values shown for relative recombinant protein production are for Interferon-γ, IgG, and unspecified recombinant proteins; (3) Relative specific production = Relative recombinant protein production/Relative viable cell density * 100%

fact that although the chemically defined media to which hydrolysates are supplemented are called chemically defined, an exact definition or composition of such media is not reported in scientific literature. This lack of comparability limits the understanding of the role of soy protein hydrolysates in enhancing cell culture functionality. Moreover, this results in wide variability in the functionality of soy protein hydrolysates in cell culture (**Table 2.1**). For several studies, while the viable cell density was low in hydrolysate supplemented cultures as compared to the chemically defined media, the recombinant protein production was always higher in the former than the latter. The beneficial effects of soy protein hydrolysate are attributed to complex composition, since they contain a large variety of different classes of compounds (e.g. peptides and carbohydrates). Typically, soy protein hydrolysates (60% peptides/amino acids and 20% carbohydrates) [17] are supplemented to chemically defined medium at 0.1-1.0% (w/v). A typical chemically defined medium like Iscove's modified Dulbecco's medium has an amino acid and glucose content of 1.2 g/L and 4.5 g/L, respectively. The hydrolysate supplementation to chemically defined medium significantly increases the protein content (50-500%) and carbohydrate content (4-44%) of the supplemented medium.

As a result, these compounds considerably enhance the cell culture functionality of chemically defined medium. Firstly, these compounds provide carbon and nitrogen to cells resulting in enhanced viable cell density and/or recombinant protein production [15, 21]. Secondly, there might be certain specific (key) compounds in hydrolysates that specifically enhance viable cell density or recombinant protein production [12, 17]. In previous work, phenyllactate, lactate, trigonelline, chiro-inositol, and X-190 (an unannotated peptide) were identified as key compounds in soy protein hydrolysates that correlated positively with CHO (CRL-11397)

integral viable cell density [12]. Lactate, ferulate, syringic acid, galactarate, adenine, and X-198 (an unannotated peptide) were shown to be the key compounds that correlated positively with IgG production [12, 22, 23].

In these studies [12, 22, 23], the variation in the concentration of the key compounds was linked to the batch-to-batch variability in the cell culture performance of soy protein hydrolysates. A comparison of supplementation of thirty batches of soy protein hydrolysate to chemically defined media to culture CHO (CRL-11397) cells showed that the relative integral viable cell density and relative total IgG production varied from 148-438% and 117-283%, respectively, relative to the chemically defined medium (100%) [12]. For 29 batches of another soy protein hydrolysate, the recombinant protein production ranged from 52-164% of the average recombinant protein production of all hydrolysates [24].

The extent of variability in the cell culture performance between different batches of a hydrolysate also depends on the cell lines used and the test concentration of hydrolysates. The relative IgG production in CHO cells ranged from 103-138% and that in avian cells ranged from 93-115% as measured for 16 batches of a soy protein hydrolysate (CD medium = 100%) [25]. When the same set of hydrolysates were supplemented at 0.5% (w/v) to CHO cells, the relative IgG production ranged from 103-138%, while it was 83-138% when hydrolysates were supplemented at 1.5% (w/v) concentration, respectively (CD medium = 100%) [25].

In addition to cell line and hydrolysate concentration, batch-to-batch variation in the hydrolysate functionality is influenced by variability in the gross composition and qualitative changes that occur in the chemical composition of hydrolysates. The gross composition includes the total content and composition of each class of compound present in the hydrolysate, for instance, total peptide content and content of individual peptides. The qualitative changes refer to the chemical modification reactions (e.g. cross-linking and glycation of proteins) that occur in hydrolysates. These reactions result in the formation of minor compounds, such as furosine, lysinoalanine (LAL), peptide-phytate complexes, and peptide-polyphenol complexes. Conventionally, these compounds are not included in the gross compositional analysis of hydrolysates. Therefore, qualitative changes could result in large differences in the hydrolysate functionality, while the gross composition of different batches of hydrolysate stays identical. Both gross and qualitative compositional changes occur in soy protein hydrolysates due to variations in processing and raw material (seed and/or meal) composition. While there is a lot of literature available on compositional (gross and qualitative) changes in soybean and its derived products, this knowledge, in relation to cell culture functionality, has not been combined in an overview yet.

In this review, we first discuss the effects of hydrolysate composition-both gross and qualitative on viable cell density and recombinant protein production in cell culture applications. Subsequently, it is discussed how variations in the hydrolysate composition may be induced by variations in the raw material and processing.

2.2. Chemical composition of soy protein hydrolysates

The major compounds present in soy protein hydrolysates are peptides, carbohydrates, and minerals. The typical gross composition of an industrial soy protein hydrolysate produced from defatted soybean meal is 60 ± 5% (w/w) peptides/amino acids, 20 ± 5% (w/w) carbohydrates and 10% (w/w) minerals[1] [17]. While the total carbohydrate and mineral contents of soy protein hydrolysate is known, the monosaccharide and mineral composition is not yet reported. For 30 batches of another industrial soy protein hydrolysate *Proyield Soy SE50MAF-UF* produced from defatted soybean meal, the protein content varied in a narrow range of 56-58% (w/w) [12]. This suggests that the batch-to-batch variability in the gross composition of a specific hydrolysate produced by a particular manufacturer is relatively small (< 5%).

In addition to peptides, carbohydrates, and minerals, several compounds like phytates (< 0.05%-0.2% in soy protein hydrolysates) [26], Maillard reaction products (MRP, e.g. pyrazines) [27] phenolic acids (227 mg/100 g protein in soy protein hydrolysates) [28], LAL[2], and racemized amino acids/peptides[2] may be present in low concentrations. These minor compounds are either present in the raw material (soybean or meal) or are produced as a result of chemical modification reactions that occur during processing. Although some of these compounds (e.g. racemized peptides) are still determined as 'proteins' in the total nitrogen analysis (e.g. Dumas method), their functionality is not the same as unmodified peptides. The concentrations of these minor compounds may be low, but they may have a large influence on the hydrolysate functionality.

2.3. Effect of compounds present in soy protein hydrolysates on cell culture functionality

Typically, soy protein hydrolysates are supplemented at 0.1-1.0% (w/v) concentration to the chemically defined medium in cell culture assays (**Table 2.1**). The optimal hydrolysate concentration varies depending on the cell line and the experimental set up. The optimal hydrolysate concentrations are determined from dose (hydrolysate concentration)-response (viable cell density/IgG production) curves. For CHO DG44/*dhfr* cells, the relative viable cell density at 0.2, 0.4, 0.6, 0.8, and 1.2% (w/v) hydrolysate concentration in chemically defined medium was 131, 155, 152, 133, and 105%, respectively [2]. The recombinant protein production was not reported. As the peak relative viable cell density is observed at 0.4% (w/v) hydrolysate concentration, this was considered as an optimum concentration. In WuT3 hybridoma cells, at 0.1, 0.2, 0.3, 0.5, and 1.0% (w/v) hydrolysate concentration, the relative viable cell density was 134, 150, 151, 152, and 94%, respectively [18]. At 0.1, 0.2, 0.3, 0.5, and 1.0% (w/v) hydrolysate concentration, the recombinant protein production was 117, 133, 145, 155, and 95%, respectively [18]. Thus, 0.5% (w/v) was the optimum concentration when both viable cell density and recombinant protein production were highest as compared to

[1] Variation in the mineral concentration was not specified
[2] Absolute concentrations are not available

other hydrolysate concentrations. In addition to cell line and experimental set up, the optimal hydrolysate concentration depends on the chemical composition of hydrolysates. Surprisingly, the composition of hydrolysates has not been described in any of the cell culture studies that evaluated the effect of hydrolysate supplementation. Consequently, there is no knowledge on the effect of compositional differences between different batches of a hydrolysate on the functionality. This limits the understanding of the role of compounds present in hydrolysate on the cell culture functionality. Nevertheless, recently some systematic studies, like chemometrics in combination with LC-MS or NMR, RP-HPLC fractionation methods, and supplementation or removal of pure and specific compounds has been performed to start understanding the role of individual compounds on the functionality. While the presence of peptides and carbohydrates contribute positively towards viable cell density and IgG production, minor compounds like racemized peptides, phytates, MRP, LAL, and polyphenols may reduce this positive effect. These are discussed below:

(a) ***Peptides***: The role of peptides in cell culture is two-fold. Firstly, the role of peptides is of nutritional character, where they act as a nitrogen source and support cell growth. Heidemann et al. (2000) demonstrated this nutritional effect by supplementing basal medium with 0.5% (w/v) wheat protein hydrolysate [15]. The hydrolysate supplementation increased the relative viable cell density and relative recombinant protein production by 105 and 114%, respectively, relative to the basal medium (100%) [15]. These effects on functionality due to hydrolysate supplementation could be reproduced by supplementing the basal medium with twice the amount of glutamine, asparagine, and four times the amount of serine present in the basal medium. The relative viable cell density and relative recombinant protein production in the fortified medium was 114% and 130%, respectively as compared to the non-fortified basal medium (100%) [15].

Secondly, certain peptides/peptide fractions exert a specific influence on viable cell density and/or recombinant protein production. Franek et al. (2000) used liquid chromatography to fractionate soy protein hydrolysate. From these fractions, a specific peptide fraction (0.2% w/v) exhibited 141% relative viable cell density and 213% relative IgG production as compared to the unfractionated hydrolysate (100%) in ME-750 hybridoma cells [11]. The other two fractions obtained exhibited lower relative viable cell density (88 and 84%) and relative IgG production (78 and 102%) as compared to the unfractionated hydrolysate (100%) [11]. In another study with synthetic peptides, supplementation at 0.2% (w/v) with Gly-Lys-Gly and Gly-His-Gly enhanced relative IgG production to 148% and 160%, while the relative viable cell density was suppressed to 88% and 80%, relative to the chemically defined medium (100%) [29]. Conversely, supplementation at 0.2% (w/v) with Gly-Gly-Gly enhanced relative viable cell density to 148% as compared to chemically defined medium (100%), while the relative IgG production was unaffected [29].

The specific effects are different from the nutritional effects because they cannot be reproduced by supplementing additional amino acids in the cell cultures [30]. In literature, the specific effects of peptides are referred to as survival factors, anti-apoptotic factors, or growth

factor like activity. These specific effects can probably be explained by an energetically efficient peptide transport into the cell as compared to that of free amino acids supplied by chemically defined medium [15, 31]. However, these mechanisms are not well understood.

The nutritional and specific effect of peptides depends on the molecular size. In general, hydrolysates with small peptides are functionally better than hydrolysates with large peptides. For example, extensively hydrolyzed (77% peptides < 1 kDa; 1% peptides > 10 kDa) and less-extensively hydrolyzed (18% peptides < 1 kDa; 26% peptides > 10 kDa) rapeseed protein hydrolysates supplemented to chemically defined media showed relative viable cell density of 133% and 85%, respectively (CD medium = 100%) [31]. Similar observations were reported when rapeseed protein hydrolysates made with Esperase 7.5L®, Neutrase 0.8L®, and Orientase 90N® were tested in cell culture. Esperase is a serine protease from *Bacillus lentus*, Neutrase is a neutral metallo-protease from *Bacillus amyloliquefaciens*, and Orientase is a serine protease from *Bacillus subtilis* [31]. All of the enzymes had low specificities. The activities of the enzymes were standardized using a hemoglobin standard allowing comparison of different enzymes. The hydrolysates had similar molecular size distribution of peptides, but the viable cell density observed was not similar. For instance, the hydrolysate made with Esperase enhanced the viable cell density 1.5 times higher than hydrolysates made with Neutrase [31]. This showed that hydrolysis with different enzymes resulted in formation of different peptides which affected the functionality of hydrolysates.

(b) ***Carbohydrates***: Carbohydrates are a source of carbon that supports cell growth. Different carbohydrates differ in the rates at which they are consumed by cells and hence, the maximum achievable viable cell density differs [32, 33]. Glucose, mannose, galactose, and fructose at 20 mM concentration were tested in a media formulation used for CHO-TF-70R cells.

While the average viable cell density after 140 hours of culturing in media containing glucose or mannose was 1.2 * 10^6 cells/mL, it was 0.7 * 10^6 cells/mL in media with galactose or fructose [32]. Furthermore, the consumption rate of glucose or mannose was much faster than galactose or fructose. After 150 hours of culturing, 12.5 mM of glucose/mannose and 2.5 mM of galactose/fructose was consumed by CHO-TF-70R cells [32].

In a similar study, Barngrover et al. (1985) studied the influence of fructose and galactose concentration on viable cell density of Vero African green monkey cells. After 6 days culture, the viable cell density was 27.5, 23.8, 11.3, and 16.3 * 10^5 cells/mL in 5, 10, 15, and 20 mM of galactose in L-15 (Leibovitz) medium [34]. On the other hand, the viable cell density was 23.8, 27.5, 23.8, and 16.8 * 10^5 cells/mL in 5, 10, 15, and 20 mM of fructose concentration in L-15 (Leibovitz) medium [34]. Therefore, a strategic selection of carbohydrates and concentration in cell culture can be used to enhance the recombinant protein production [35]. While the maximum viable cell density and tissue plasminogen activator production in 20 mM glucose medium was 1.6 * 10^6 cells/mL and 3.9 μg/mL, it was 1.9 * 10^6 cells/mL and 4.5 μg/mL in 5 mM glucose and 20 mM galactose medium [35].

(c) ***Racemized amino acids/Peptides***: The influence of racemized amino acids on viable cell density has been investigated in few studies. In HeLa [36] and chick embryo cell [37] cultures,

racemized D-amino acids did not inhibit or enhance viable cell density. Similarly, Naylor et al. (1976) tested racemized amino acids in several mammalian cell lines (e.g. LM (TK-), A9, HTC+, 6TG-11, CHO, and B16) and reported that racemized amino acids did not affect viable cell density [38].

(d) ***Chelating compounds (LAL, phytates, and MRP)***: The supplementation of phytic acid (0.27 mM to 2.7 mM) decreased viable cell density (93% to 45%) as compared to the non-supplemented control (100%) in BALB/c mouse 3T3 fibroblass [39]. This effect can be due to chelation of cations by phytic acid. In human and animal studies, LAL [40, 41], phytates [42], and MRP [43, 44] have been shown to chelate cations. Cations have an important role in mammalian cell culture. For example, magnesium controls the activities of many glycolytic enzymes in the Krebs cycle [45], and calcium and zinc are essential for cell growth and differentiation, apoptosis and recombinant protein production [46, 47].

(e) ***Maillard reaction compounds***: The Maillard reaction results in loss of essential amino acids (e.g. lysine), as the MRP cannot be metabolized in cell cultures. Moreover, Maillard reaction also results in formation of new compounds, such as pyrazines and hydroxymethyl furfural, which affect cell cultures. Pyrazine and its derivatives at 1% (w/v) concentration induced genotoxicity in the range of 0.4-29% in CHO cells [48]. More than 1% (w/v) pyrazine concentration was toxic and completely inhibited cell growth. Another MRP, hydroxymethyl furfural, at 0.1% (w/v) concentration exhibited similar genotoxic and growth inhibition effects in chick embryo fibroblasts [49].

(f) ***Peptide-polyphenol complexes***: The influence of peptide-polyphenol complexes was investigated by supplementing growth medium with sunflower meal hydrolysates containing 1% and 6% (w/w) of polyphenols. While the viable cell density and recombinant protein production for the former hydrolysate was 9 g biomass/L and 180 units of streptokinase $mL^{-1}{\cdot}hour^{-1}$, it was only 2 g biomass/L and 40 units of streptokinase $mL^{-1}{\cdot}hour^{-1}$ for the latter, respectively [50]. This clearly showed that high levels of polyphenol in hydrolysates inhibited cell growth and productivity. Although a large, but similar extent of variability is reported for polyphenol content in sunflower (1.4-6.1%) [50–53] and soybean meal (0.02-9%) [54–56], the polyphenol composition between the two meals differ greatly. While the sunflower meal primarily contains chlorogenic acid, caffeic, and quinic acids, soybean meals are rich in isoflavones, such as daidzein, glycitein, and genistein. In chemically defined cell cultures, influence of soy isoflavones on the cell culture functionality has also been investigated. The supplementation with genistein-8-C-glucoside at $> 10\ \mu$M concentration to the cell culture media resulted in drastic reduction of CHO cell viability, and apoptosis and genotoxicity was induced [57]. In addition to cytotoxicity, genotoxic effects on cell cultures due to polyphenols are reported. A 40-fold increase in chlorogenic acid concentration from 0.01 mg/mL to 0.4 mg/mL resulted in 40-fold increase in genotoxicity in CHO cells [58]. These effects increased further in the presence of metal ions, like manganese and copper [58].

In addition to a direct effect, the above-mentioned minor compounds influence the cell culture functionality by affecting hydrolysate composition. These compounds interfere with

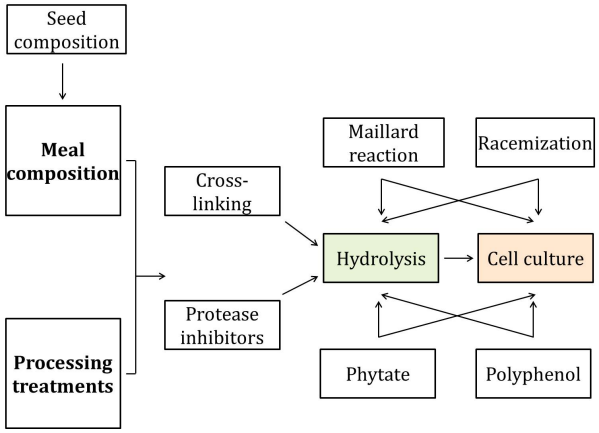

Figure 2.1: Schematic overview of chemical reactions and minor compounds that affect protein hydrolysis and functionality. The minor compounds are affected by meal composition and processing treatments

the production of peptides during enzymatic hydrolysis in hydrolysate production (**Figure 2.1**).

2.4. Production of soy protein hydrolysates

Soy protein hydrolysates for cell culture applications are produced by enzymatic hydrolysis of defatted soybean meal, soy protein concentrate, or protein isolate, followed by enzyme inactivation, coarse-filtration, ultrafiltration, and spray drying (**Figure 2.2**) [59]. The industrial enzyme preparations that are used for hydrolysis may contain carbohydrase side activities. Such side activities in Neutrase (0.025% on protein basis) resulted in release of 14% neutral sugars from the water unextractable solids fraction obtained from toasted soybean meal [60]. The hydrolysis reaction is stopped by inactivating the enzyme using a heat treatment. After enzyme inactivation, the solution is coarse-filtered to remove large and insoluble impurities, mainly polysaccharides, non-hydrolyzed proteins, and aggregated peptides. Coarse filtration is usually performed using filter aids, which are available in wide range of particle sizes (median particle size from 1.5-26 μm) [61]. The coarse filtration can be substantially hindered by polysaccharides and protein-lysophospholipids complexes [62]. After coarse filtration, the hydrolysate solution is ultrafiltered, typically using a 10 kDa membrane, to remove large molecular weight compounds, such as partially hydrolyzed peptides and endotoxins [59]. Endotoxins are complex lipopolysaccharides, which are a major component of the cell wall membrane of most gram negative bacteria. The presence of endotoxin in hydrolysate adversely affects its functionality. In B-9 cells, presence of 20 ng/mL endotoxins resulted in 30% reduced production of a recombinant human protein called Mullerian inhibiting substance [63]. After ultrafiltration, the hydrolysate is composed of only water-soluble compounds (including the minor compounds described in previous sections). Finally, the hydrolysate

solution is concentrated and spray dried.

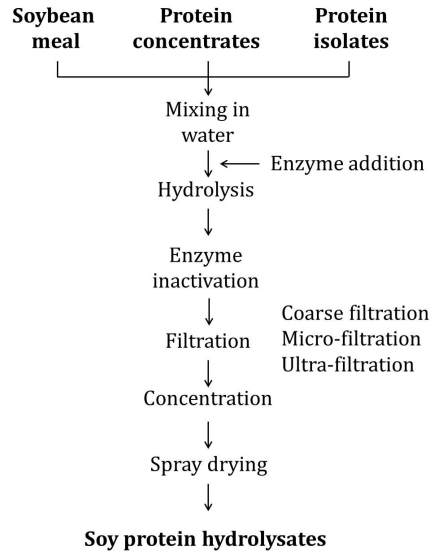

Figure 2.2: Schematic diagram for production of soy protein hydrolysates from soybean meal, protein concentrates, or isolates

Conventionally, industrial soy protein hydrolysates are characterized for molecular weight distribution of peptides. Other analyses may include determination of protein, ash, moisture, and amino acids [64].

2.4.1. Compounds affecting enzymatic hydrolysis of the soybean meal

The enzymatic hydrolysis of the soybean meal is affected by the minor compounds following three mechanisms: firstly, due to processing treatments, the substrate protein may get modified (e.g. MRP and cross-linked proteins). Secondly, compounds present in the soybean meal (e.g. protease inhibitors) may inhibit the enzyme used for hydrolysis. Thirdly, some compounds (e.g. polyphenols and phytates) can both modify the substrate and inhibit the enzyme (**Figure 2.1**). Since systematic data on soy protein hydrolysis in relation to these mechanisms are not available, references that describe effects on other types of proteins are included (**Table 2.2**). In addition, data on *in vitro* protein digestibility is not available and therefore, results from *in vivo* animal nutrition studies are used to illustrate the effects of these mechanisms on protein digestibility. These effects are then used as an indication to state that similar effects can be expected in *in vitro* protein hydrolysis during hydrolysate production.

Substrate modification

(a) ***Racemization***: In several studies, racemization, i.e. conversion of L-form to D-form, has been shown to occur in amino acids and peptides.

Table 2.2: Influence of chemical reactions and minor compounds on the enzymatic hydrolysis of protein substrates

Reaction	Hydrolysis conditions	Substrate	Hydrolysis* (%)	Reference
Maillard reaction	Xylose added (1% and 3% soybean meal w/w); heat treated (120 °C, 160 °C) for 30 or 60 minutes; *in situ* hydrolysis; 48 hours	Soybean meal	8 to 46	[65]
Protein-polyphenol	BSA-quercetin (1:1 to 20:1 w/w); trypsin (E/S = 1:80); 35 °C; 4 hours	Bovine serum albumin	16 to 45	[66]
Protein-phytate	Pepsin (E/S = 1:250); pH 2.0-4.5; phytate 2 mg/mL; 37 °C; 30 minutes	Soybean 11S	43	[67]
Racemization	Immobilized crude porcine mucosa intestinal peptidases; 37 °C; 24 hours	Synthetic peptides	100	[68]
	Swine pepsin; pH 1.5; enzyme = 0.02-0.5 mg/mL; 20 hours; 40 °C	Synthetic peptides	93 to 99	[69]
Cross-linking	Wheat protein; 24 hours; pH 11.5; 65 °C; *in vivo* hydrolysis	Wheat protein	17	[70]
Saponins	1 mg/mL soy saponin; 1 mg/mL substrate; 0.1% α-chymotrypsin; 38 °C; pH 7.6; 3 hours	Glycinin	57	[71]
	1 mg/mL soy saponin; 1 mg/mL substrate; 0.1% α-chymotrypsin; 38 °C; pH 7.6; 3 hours	β-Conglycinin	27	[71]
Protease inhibitors	Crude soybean trypsin inhibitor type II S, 0-9.7 trypsin inhibitor activity (mg bovine trypsin inhibited per g feed); *in vivo* hydrolysis	Salmon	38 to 83	[72]
	Crude soybean trypsin inhibitor type II S, 0-1.48% trypsin inhibitor; *in vivo* hydrolysis	Trout	70 to 93	[73]

* Relative hydrolysis (%) = 100 - ((chemically modified substrate protein hydrolyzed (in the presence of minor compounds))/(unmodified substrate protein hydrolyzed (in the absence of minor compounds))) * 100

In these studies, combinations of extreme experimental conditions (pH 2-12; temperature = 25 °C-130 °C; heating time = 1-24 hours) have been used to induce racemization [74, 75]. However, these processing conditions never occur during hydrolysate production. The extent of racemization has not been investigated under representative conditions (neutral pH; temperature = 50 °C-100 °C; heating time = 0.5-1 hour). This is important because the occurrence of even very small extent of racemization in proteins and/or peptides greatly affects its hydrolysis [76]. *In vitro* hydrolysis of synthetic racemized and non-racemized tripeptides (Ala-L-Glu-Ala, Ala-D-Glu-Ala, Ala-L-Asp-Ala, Ala-D-Asp-Ala, Val-L-Asp-Val, Val-D-Asp-Val, Ala-L-Phe-Leu, Ala-D-Phe-Leu, Ala-Met-Ala.HCl, Ala-D-Met-Ala.HCl, Val-Met-Phe.HCl, and Val-D-Met-Phe.HCl) using intestinal peptidases was investigated. While tripeptides containing L-amino acid residues were completely hydrolyzed, the tripeptides containing D-amino acid residues were not hydrolyzed at all [68, 77]. In another study, peptic hydrolysis of benzyloxycarbonyl-L-Ala-L-Phe-L-Tyr, benzyloxycarbonyl-L-Ala-L-Phe-L-Leu-L-Ala, benzyloxycarbonyl-L-Ala-L-Gly-L-Phe-L-Tyr and their corresponding D-isomers was studied. While the rate of hydrolysis was 22, 95, and 584 nmoles/minute/mg pepsin for L-isomers, it was 1, 7, and 4 nmoles/minute/mg pepsin for D-isomers, respectively [69].

(b) ***Lysinoalanine (LAL) formation***: The processing treatments used during oil extraction from soybeans, production of the meal, and hydrolysates can lead to formation of compounds such as LAL, lanthionine, dehydroalanine, and β-aminoalanine. The cross-linking reaction and formation of these compounds is well described in literature [78, 79]. Soy proteins (defatted soy flour precipitated at pH 4.5, washed with water, and freeze dried) were heat treated at 100 °C-120 °C, pH 6.5 for 1-3 hours. The LAL content in these heat processed soy proteins was $<$ 40 to 130 $\mu g/g$ protein [80].

In industrial soy protein isolates, the LAL content has been reported to vary from 0-370 $\mu g/g$ protein [80]. Likewise, wheat protein processing (65 °C, 24 hours, pH 11.5) resulted in conversion of 15% lysine to LAL decreasing the protein digestibility from 93% (unprocessed) to 76% (processed) in miniature pigs [70].

Enzyme inhibition

(a) ***Protease inhibitors***: Protease inhibitors found in soybeans are Bowman-Birk and Kunitz inhibitors. The molecular size and structure of these inhibitors have been well described [81]. The specificity of these inhibitors is not only for trypsin and chymotrypsin, but also for elastase and several other serine proteases [40]. While the Kunitz inhibitor is heat-labile, Bowman-Birk is a heat-stable protease inhibitor [82]. In a starch matrix at 104 °C, the inactivation rate constants of the Kunitz and the Bowman-Birk inhibitor were 12 * 10^{-4} s^{-1} and 6.9 * 10^{-4} s^{-1}, respectively [83]. Thus, a strong heat treatment, such as autoclaving (121 °C/10 minutes, 15 p.s.i), is required to inactivate protease inhibitors [82]. This harsh processing initiates other reactions like Maillard reaction and racemization in the soybean meal. Generally, industrial defatted soybean meals retain some protease inhibitory activity (0-12.1 mg/g protein, **Table 2.3**). A wide variation in the protease inhibitory activity of soybean meals is reported in literature. For instance, in three samples of heated soy flours, trypsin inhibitory activity (TIA)

was 22, 11, and 4 mg trypsin inhibited per g sample [84].

Table 2.3: Prominent compositional differences between the under-processed, adequately-processed, and over-processed soybean meal

	Under-processed	Adequately-processed	Over-processed	Reference
Trypsin inhibitors (trypsin inhibited/protein (mg/g))	12.1	1.77	0	[85]
Protein solubility (%)	> 85	74-85	< 74	[86]
Lysine content (% dry matter)	3.5	3.0	2.7	[87]
Weight gain (g/chick)	605	643	596	[85]
Hunterlab (+a) color values	2.9	3.2	10.1	[88]
LAL (g/100 g of protein)	-	-	0.2	[89]
Protein content (%)	53.0	52.9	52.5	[88]
Moisture content (%)	11.2	10.9	7.5	[88]
Ash content (%)	6.2	6.3	6.1	[88]

The supplementation of Atlantic salmon (*Salmo salar*) diets with crude soybean trypsin inhibitor extract ranging from 0-9.7 TIA resulted in reduced protein digestibility from 83% to 38% [72]. The protein digestibility was calculated from the difference between total nitrogen of fish diet and faeces measured using micro-Kjeldahl method [90]. In a similar study with rainbow trout, the *in vivo* protein digestibility of fish meal containing 0-1.5% soybean protease inhibitors ranged from 93-70% [73]. As protease inhibitors affect *in vivo* protein digestibility, it is expected that *in vitro* protein hydrolysis during hydrolysate production also gets affected. This will affect the peptide composition of hydrolysate and consequently affect the cell culture functionality. A direct effect of protease inhibitors on the cell culture functionality is not expected because they are removed during the ultrafiltration step in hydrolysate production. (b) ***Free fatty acids***: Free fatty acids, like oleic, linoleic, and linolenic acid in soybean meal can act as protease inhibitors. In presence of oleic, linoleic, and linolenic acid (0.6 μmole/4 mL of assay solution), the chymotrypsin activity decreased by 30%, 40%, and 60%, respectively [91]. While oleic and linoleic acid totally inhibited trypsin, linolenic acid reduced trypsin activity by 50% [91].

Substrate modification and Enzyme inhibition

(a) ***Maillard reaction***: The background of Maillard reaction, factors affecting its rate of occurrence, and methods of analysis are well described in literature [92]. Maillard reaction in the soybean meal results in lysine modification, which is the site of action for enzymes like trypsin. This hinders the enzymatic hydrolysis of the soybean meal. The effect on hydrolysis was studied using heat-treated soybean meal (120 °C and 150 °C; 30 and 60 minutes) in presence of xylose (1% and 3%, soybean meal weight basis). The heat-treated samples and a non-heated control were enzymatically hydrolyzed in the rumen of a ram for 48 hours. The protein degradability for heat-treated soybean meal in presence of xylose was 47-85%, while it was 93% for untreated soybean meal [65]. Moreover, due to Maillard reaction, protein polymerization can occur. Kato et al. studied protein polymerization in ovalbumin stored on 1:1

(w/w) ratio with maltose, cellobiose, isomaltose, lactose, and melibiose stored for 0-20 days at 50 °C and 65% relative humidity. After 7 days, the polymers formed in ovalbumin-disaccharide mixtures ranged from 8-23% [93]. After 15 days of storage, the proportion of polymers formed increased to 13-44% [93]. These cross-linked protein polymers have reduced susceptibility towards enzymatic hydrolysis [94–96]. Since the composition (protein:carbohydrates) used in this study is similar to that of defatted soybean meal (**Table 2.4**), it is expected that similar reactions could occur, even though the data for cross-linking due to Maillard reaction in soybean meal has not been reported. In addition to substrate proteins, MRP affects enzyme activity.

Table 2.4: Composition (%) of soybeans, meals, protein concentrates, and isolates

	Soybeans	Meals	Concentrates	Isolates
Protein	31-48 [97, 98]	44-61 [97, 99]	62-69 [100]	85-96 [101]
Oil	12-24 [97, 102]	0.5-9.0 [97, 99]	0.5-1.0 [100]	0.5-1.0 [100]
Carbohydrates	33-44 [103]	32-38 [99]	17-25 [99]	0.3-0.6 [99]
Ash	4-5 [99]	6-7 [99]	3.8-6.2 [100]	2.5-6.3 [101]
Isoflavones	0.1-0.4 [104]	0.2-0.3 [54, 105]	0.02-0.3 [105]	0.1-0.3 [28, 105]
Phytates	1.4-2.3 [106]	1.3-4.1 [99]	1.3-2.2 [104]	1.0-1.7 [104]
Saponins	0.2-0.3 [107]	0.7 [108]	0.4 [109]	0.8 [104, 108]
Trypsin inhibitors	3.5-12.2 [104]	1.2-1.5 [85, 104]	0.8-1.1 [104]	0.1-2.9 [104]
Moisture	7.7-10.1 [110]	3.5-11.4 [97]	4-6 [100]	4-6 [100]

Carboxypeptidase A and aminopeptidase N were strongly inhibited by 0.5 mg/mL and 0.25 mg/mL of a low molecular weight fraction of MRP [94, 111]. However, the specific MRP present in the low molecular weight fraction were neither identified nor quantified. Therefore, a quantitative relation between the inhibition of hydrolysis and MRP could not be obtained.
(b) ***Protein-phytate interactions***: During processing of the soybean meal, complexes can be formed between proteins and phytate [112]. These complexes are insoluble and as a result, they are hydrolyzed at a slower rate than the non-complexed proteins [113]. In presence of 1 mg/mL phytate, 43% reduction in the peptic hydrolysis of soybean 11S proteins was observed [67]. Furthermore, phytate may directly inhibit enzyme. In soy flour, an increase in the phytate content by 2.1% resulted in decreased carboxypeptidase A activity by 14% [74]. In the presence of 10-90 mM phytate, 2.7-19.6% of trypsin activity was inhibited [114].
(c) ***Saponins***: Soy saponins suppress protein hydrolysis by forming enzyme-saponin and/or protein-saponin complexes. Addition of 1 mg/mL of soy saponins to 1 mg/mL of glycinin and 1 mg/mL of β-conglycinin reduced the protein hydrolysis by approximately 57% and 27%, respectively [71]. However, such high concentrations of saponins do not occur in soybean meal, soy protein concentrates, or soy protein isolates (**Table 2.4**).
(d) ***Protein-polyphenol complex***: Isoflavones are the major polyphenols present in defatted soybean meal (0.2-0.3 g/100 g, **Table 2.4**). These isoflavones form complexes with proteins during harvesting, storage, and seed/meal processing. This complex formation reduces the

susceptibility of proteins to enzymatic hydrolysis. The covalent binding of bovine serum albumin (BSA)-quercetin derivatives in 20:1, 10:1, 7:1, 5:1, and 2:1 (w/w) ratios lowered the hydrolysis by 16, 26, 30, 37, and 45%, respectively, as compared to the non-complexed BSA (100%) [66]. In addition to food proteins, complex formation between enzymes and polyphenols has been reported. More than 40% enzymatic activity was lost when chymotrypsin was complexed with chlorogenic acid [115]. The mechanism of protein-polyphenol complex formation and its influence on the protein hydrolysis is well described [116].

The concentration of above-mentioned minor compounds in hydrolysates is determined not only by the hydrolysate production itself, but also by the meal processing conditions. The variation in the seed composition influences the concentration of these minor compounds as well.

2.5. Soybean meal production

The harvested mature whole soybeans are cracked, dehulled, steam-heated, and pressed to obtain full-fat flakes. Subsequently, oil is extracted from the flakes to obtain defatted soybean meal (**Figure 2.3**) [117].

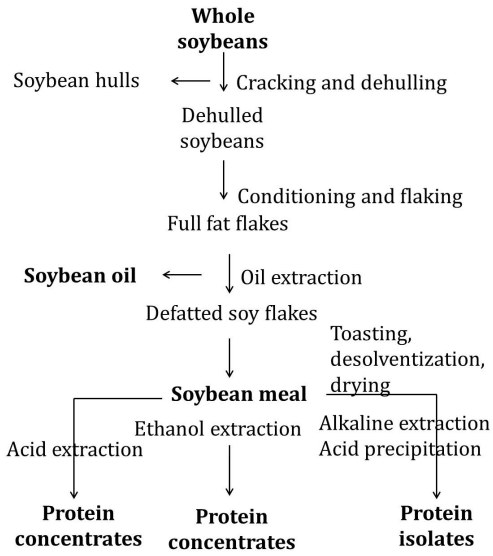

Figure 2.3: Schematic diagram for processing of soybeans to defatted soybean meals, soy protein concentrates, and isolates

Oil is extracted from the flakes using processes, such as extruder-expeller, continuous screw press, and solvent extraction. After oil extraction, solvent is removed from the residual meal by direct heating or steam. Subsequently, the defatted meal is toasted to inactivate protease inhibitors. A conventional and economic method of toasting is to use direct steam injection

at 120 °C for 30 minutes under atmospheric or pressurized conditions [118]. After toasting, the meal is ground to the desired particle size. The defatted soybean meal contains 44-61% protein, 32-38% carbohydrates, 6-7% ash, and 0.5-9% lipids (**Table 2.4**). The composition of the soybean meal is primarily affected by two factors, i.e. processing treatments and seed composition. The influence of these two factors on the meal composition is discussed further.

2.5.1. Influence of processing treatments

The processing treatments like desolventization and toasting cause qualitative changes (e.g. protein denaturation and aggregation) in the soybean meal. As a result of these processes, the nitrogen solubility index (% NSI) of the soybean meal decreases significantly.

$$\textbf{Nitrogen solubility index} = \frac{water\ soluble\ nitrogen}{total\ nitrogen} * 100$$

While in one study, a 70% decrease in NSI was reported [119], in another study an 18% decrease was reported [109]. This shows that NSI of defatted soybean meal strongly depends on the way they are processed. While a direct link between NSI and the hydrolysate composition has not been made, the variation in NSI is an indication of different (effects of) heat treatments in the production of the defatted soybean meal. In addition to differences in solubility, also the protease inhibition and occurrence of Maillard reaction will result from such heat treatments. For instance, during toasting under the warm and humid conditions, several amino acids, like lysine, glycine, arginine, cysteine, and methionine react with reducing sugars, each with their own reaction rate, and undergo Maillard reaction [120, 121]. In soybean meals after autoclaving for 4 hours at 15 p.s.i.g pressure, 41% lysine, 35% arginine, 17% histidine, and 16% tryptophan underwent Maillard reaction [122]. In many studies, 'redness' of samples has been used as an indicator to determine the extent of Maillard reaction. For low-Maillard-reacted to high-Maillard-reacted soybean meals, the Hunterlab values ranged from 2.9-10.1 +a (redness) [88]. Another approach to measure MRP is by binding free amino acid groups to binding agents like ortho-phthalaldehyde, 2,4,6-trinitrobenzenesulfonic acid, and fluorodinitrobenzene [92]. The MRP formed in later stages, such as furosine and hydroxymethyl furfural, can be determined using chromatographic methods.

Other reactions that simultaneously occur with Maillard reaction include racemization and cross-linking resulting in the formation of cross-linked and racemized peptides. As described previously, these compounds affect the functionality and influence the enzymatic hydrolysis of soybean meal. Depending on the processing conditions, soybean meal varies in quality, i.e. under-processed, adequately-processed, and over-processed (**Table 2.3**). Conventionally, different qualities of meals are characterized based on urease units, trypsin inhibitory activity, and protein solubility index. While under-processed meal scores high on these parameters, over-processed meal scores very low (**Table 2.3**). The over-processed meals have high concentrations of minor compounds (e.g. MRP and LAL) formed due to over-processing, while these compounds are either absent or present in trace amounts in under-processed and adequately-

processed meals. In spite of these differences, the gross composition of different meal qualities is similar. The protein contents of under-processed, adequately-processed, and over-processed meals were 53.0%, 52.9%, and 52.5%, respectively (**Table 2.3**). Despite their compositional equivalence, significant differences in their *in vivo* protein digestibility are reported, which is attributed to the presence/absence of minor compounds. In animal trials, the nutritional value of under-processed, adequately-processed, and over-processed meal, as evaluated by the weight gain of animals upon meal consumption for a test period, was 605, 643, and 596 g/chick, respectively [85].

2.5.2. Influence of seed composition

Soybeans are naturally variable from crop-to-crop because of genetic and environmental factors (e.g. temperature and rainfall). This natural variation of soybeans is carried over to the meals produced from them, thereby resulting in meals of different qualities from batch-to-batch. The influence of compositional variability of soybeans on the variability of meal composition has been demonstrated in several studies. For instance, soybeans were collected from Argentina, Brazil, U.S, India (high quality and low quality), and China and soybean meals were prepared from them using a common process. For six types of soybeans and soybean meals, the coefficient of variation (% CV) in protein, total essential amino acids, total non-essential amino acids, and fat contents were calculated.

$$\textbf{Coefficient of variation } (\%\ \text{CV}) = \frac{standard\ deviation}{mean} * 100$$

The CVs for protein, total essential amino acids, and total non-essential amino acids of the soybeans (10.4%, 9.9%, and 10.9%) were comparable to that of respective soybean meals (7.5%, 5.7%, and 7.6%) [123]. On the other hand, the CVs in fat contents of the soybeans and soybean meals were 6.5% and 20.1%, respectively [123]. Likewise, in another study the lysine-sucrose ratio ranged from 0.4-0.8 for 10 varieties of soybeans and from 0.5-0.9 for the soybean meals prepared from them [124]. This shows that the compositional differences in the soybeans are reflected in the composition of defatted soybean meals produced from them. Furthermore, when processing treatments are well-controlled, the seed processing does not induce changes in the gross composition (i.e. protein content). However, as described in the previous sections, processing treatments do induce changes that are not reflected in the conventional gross compositional analysis. For instance, variation in processing conditions, like temperature and pH, significantly influences the rate of Maillard reaction, while the gross composition may be unaffected [125].

In the next section, factors that influence the seed composition are described. Additionally, the resultant changes in the seed composition due to these factors are discussed.

2.6. Compositional variability of soybeans

The general composition of the soybeans is 31-48% protein, 33-44% carbohydrates, 12-24% lipids, and 4-5% ash (**Table 2.4**). In addition, other compounds, such as phytates, isoflavones,

and saponins, together contribute to less than 1% of the dry matter of the seed. These compounds are not typically included in standard analysis. However, they could be important, since for instance it has been shown that saponin fractions protected CHO cells (AS52) against DNA damage and cytotoxicity [126].

2.6.1. Genotype and Environment

The seed composition is affected not only by genotype but also by environmental factors like temperature, rainfall, and geography. In literature, there are a limited number of studies available that describe the influence of a specific factor on the seed composition. To show differences between studies % CV per study was calculated. Additionally, an overall effect was indicated by calculating overall % CV, where results from all the studies were combined together (**Table 2.5**). The compositional variation between different soybean varieties was higher due to genotype than due to environmental factors. The genotypic differences between soybean varieties resulted in CV of 4-21% for proteins and 4-11% for lipids. Due to environmental factors, the CV for these compounds varied between 3-13% and 3-11%, respectively. Smaller variation was observed for the carbohydrate content due to genotype (5-8%) and environment (2%), while large variations were observed for saponin (10-35%) and isoflavone (4-113%) contents due to all the factors. This is also apparent when variation in compound concentration (overall CV) is considered including all the factors. Saponins and isoflavones showed an overall CV of 33% and 84%, respectively, whereas proteins, lipids, and carbohydrates had a CV of 8%, 9%, and 10%, respectively. This suggests that due to genotype and environment, saponins and isoflavones are affected to a greater extent than proteins, lipids, and carbohydrates.

2.6.2. Post-harvest storage

In addition to genotypic and environmental factors that lead to gross compositional variation (e.g. protein content), post-harvest storage may lead to qualitative changes (e.g. lipolysis) in the seed composition.

Lipids: Nakayama et al. studied changes in the lipid composition of soybeans stored at 35 °C for 6 months. At the end of storage, 45% of total phospholipids were enzymatically hydrolyzed to phosphatidic acid and lysophosphatidylcholine [127]. Moreover, an increase in acid value of lipids from 0.13 to 0.90 mL (0.1 N KOH to titrate 1 g oil extracted from soybeans) was reported when soybeans were stored at 30 °C, 80% relative humidity for 10 months [128]. The lipolysis also resulted in an increase in the hydroperoxide content. These hydroperoxides can react with seed proteins and result in protein aggregation and formation of protein-lipid and protein-protein cross-links [129, 130]. The adverse effects of these cross-links on the peptide composition and hydrolysate functionality are described in the earlier sections.

Proteins: Increase in the acid value of seed lipids during storage affects protein solubility. In soybeans stored at 25 °C, 85% relative humidity (S1) and 35 °C, 85% relative humidity (S2) for 6 months, 20% (S1) and 45% (S2) reduction in NSI was observed [131]. This decrease in NSI is mainly due to precipitation of 11S proteins (isoelectric pH = 6.4) [132].

Table 2.5: Compositional variability[1,2] of soybeans due to genotype and environmental factors

Factor	Moisture	Protein	Lipids	Saponins	Isoflavones	Carbohydrates
Species	n = 12 [110] 7.7-10.1 (15.2) n = 24 [110] 36.2-41.4 (3.8)	n = 4 [98] 31.3-48.3 (20.7) n = 24 [110] 36.2-41.4 (3.8)	n = 24 [110] 20.9-25.1 (4.4) n = 6 [133] 19.8-26.7 (10.9)	n = 4 [107, 108, 134] 0.2-0.5 (35.3)	n = 15 [55] 0.3-0.9 (32.3) n = 6 [51] 0.1-0.2 (12.1)	n = 9 [56] 33.4-43.9 (8.4) n = 8 [103] 31.1-35.5 (4.7)
Temperature	not determined	n = 4 [135] 37.3-41.5 (4.1)	n = 4 [135] 23.5-25.4 (2.9)	not determined	n = 6 (Lee) [56] 0.32-0.34 (3.6) n = 7 (Fukuyataka) [56] 0.02-0.2 (112.5)	not determined
Rainfall	not determined	n = 4 (JS335) [136] 37.1-39.7 (3.0) n = 4 (KHSb2) [136] 32.2-41.6 (13.2)	n = 4 (JS335) [136] 18.5-20.1 (3.7) n = 4 (KHSb2) [136] 16.5-21.2 (10.6)	not determined	n = 7 (Kairyoshirome) [56] 0.06-0.2 (51.3) n = 7 (Lee) [56] 0.02-0.1 (106.4)	not determined
Location	n=3 (Clark) [137] 7.1-8.4 (8.2) n = 3 (Woodworth) [137] 7.4-8.5 (7.6)	n = 4 (Reyna52) [133] 38.8-42.3 (4.0) n = 4 (Conesita INTA) [133] 32.9-42.7 (11.4)	n = 4 (Rojas INTA) [133] 20.7-26.7 (11.3) n = 4 (Reyna52) [133] 20.7-22.2 (3.3)	n = 5 (PI437654) [107] 0.4-0.6 (18.1) n = 5 (Magellan) [107] 0.2-0.3 (10.1)	n = 4 (Hardin) [54] 0.05-0.2 (52.1) n = 4 (Corsoy79) [54] 0.08-0.2 (34.1)	n = 9 [99] 33.9-41.3 (1.9) n = 4 [99] 29.3-34.8 (2.1)
Overall CV	**9.5**	**7.6**	**9.4**	**32.6**	**83.9**	**9.7**

(1) All values are in percentages;
(2) Values in parenthesis are % coefficient of variation

Additionally, protein-lipid and protein-protein interactions occur due to lipolysis in soybeans stored at higher temperature and relative humidity [138].

Carbohydrates: During storage, 30-fold increase in reducing sugar content was observed when soybeans were stored at 35 °C, 82% relative humidity for 6 months [138]. This increase in reducing sugars was, however, species-dependent. While reducing sugar content increased from 0.2% to 8% in MD 27-51 cultivar, it remained constant at $< 0.2\%$ in SJ-4 and Palmetto cultivars over 9 months of storage [139].

While, due to genotype and environmental factors, there is variation in the gross composition, the functionality of soy protein hydrolysates is largely affected by variations in processing conditions. These conditions result in formation of minor compounds, such as Maillard reaction products and cross-linked peptides. In pure systems, these compounds have a profound effect on the cell culture functionality. This suggests that presence of these compounds in soy protein hydrolysates also affect cell growth and IgG production. Since these compounds are not included in the gross compositional analysis, a more detailed compositional analysis including minor compounds is needed to understand and control the variability in the functionality of soy protein hydrolysates.

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CHAPTER 3

Influence of protein and carbohydrate contents of soy protein hydrolysates on cell density and IgG production in animal cell cultures

Abstract

The variety of compounds present in chemically defined media as well as media supplements makes it difficult to use a mechanistic approach to study the effect of supplement composition on culture functionality. Typical supplements, such as soy protein hydrolysates contain peptides, amino acids, carbohydrates, isoflavones, and saponins. To study the relative contribution of these compound classes, a set of hydrolysates was produced containing 58-83% proteinaceous material and 5-21% carbohydrates. While the contents of the different compounds classes varied, the composition (e.g. elution profiles of peptides, carbohydrate composition) did not. The hydrolysates were supplemented to a chemically defined medium in cell culture, based on equal weight and on equal protein levels. The latter showed that an increase in the carbohydrate concentration significantly (p- value < 0.004) increased integral viable cell density (R = 0.7) and decreased total IgG (R = -0.7) and specific IgG production (R = -0.9). The extrapolation of effects of protein concentration showed that an increase in protein concentration increased total and specific IgG production and suppressed integral viable cell density. This showed that the generic effects of supplementing different concentrations of carbohydrates were apparent in cell cultures, despite the complexity of the composition of soy protein hydrolysates. Moreover, the balance between the total protein and total carbohydrates in the supplemented media determined the balance between integral viable cell density and total and specific IgG production.

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3.1. Introduction

The growth, viability, and productivity of host cells in cell cultures depend on the growth conditions (e.g. temperature) and the composition of the medium. Typically supplements, such as soy protein hydrolysates, are added to chemically defined (CD) medium to improve the cell culture functionality, i.e. increase viable cell density and recombinant protein production. Such supplementation resulted, for instance, in a 1.5 and 2.6 times increase in the viable cell density and interferon-γ production in Chinese hamster ovary (CHO)-320 cell cultures [1]. In another study, comparison of supplementation of 30 soy protein hydrolysates produced using the same raw materials and processes showed a 1.5-4.4 times increase in integral viable cell density (IVCD) (CHO-CRL11397) and 1.2-2.8 times increase in immunoglobulin (IgG) production for hydrolysate-supplemented cultures as compared to the CD medium [2]. The composition of these hydrolysates was analyzed using an untargeted metabolomics approach [2]. Phenyllactate, lactate, trigonelline, ferulic acid, chiro-inositol, and adenine were shown to explain part of the variability in IVCD (20-29%) and IgG production (22-30%) [2]. It is important to note that the effect of hydrolysate supplementation depends on the cells as well as on the CD medium used in the experiment. As a result, the exact contribution of the different compounds or compound classes in the CD medium or the supplemented hydrolysates is difficult to determine. In addition, the hydrolysates contain a large variety of different compounds (proteinaceous material, carbohydrates, minerals, lipids, isoflavones, saponins, and organic acids), which can affect the cell culture in different ways. The effect of varying the contents of different compound classes, i.e. proteins, carbohydrates, isoflavones, and saponins, present in the hydrolysates on the IVCD and immunoglobulin (IgG) production in hydrolysate-supplemented cultures has hardly received any attention. The proteinaceous material (further referred to as proteins) consists of partially hydrolyzed proteins, peptides, and amino acids. In this study, the relative importance of the different compound classes on cell culture functionality is studied.

The first effect of increased protein and carbohydrate concentration in the medium may be expected to be linked to the increased nutrient content of the medium [3]. Other effects may be due to the presence of specific compounds that have a more direct impact on the cells, such as β-alanine and 4-aminobutyric acid have been shown to be anti-apoptotic [4]. The specific effects have been shown by supplementing pure compounds to cell cultures. The supplementation with synthetic peptides has been shown to only increase viable cell density or to only increase recombinant protein production [5, 6]. At the same time, a decrease in viable cell density and increase recombinant protein production upon supplementation with peptides has also been reported [7]. In another study, supplementing isoflavones, like genistein-8-C-glucoside to a final concentration range of 5 to 110 μM in the CD media increased cell death up to $>$ 99% [8]. Conversely, addition of saponins, like soyasapogenol B (25-200 μg/mL), reduced cell death caused by 2-acetoxy acetylaminofluorene by 25 to 87% [9]. This shows that certain compounds may result in quite specific effects in the functionality. However, such studies with pure compounds have not yielded insights into the relative contribution of the

different compound classes on the functionality.

To study the role of the different compounds classes, hydrolysates were prepared from defatted soybean meal, soy protein concentrates (acid and ethanol extraction), and soy protein isolates, resulting in hydrolysates with different contents of protein, carbohydrate, isoflavone and saponin.

3.2. Materials and methods

3.2.1. Materials and chemicals

Defatted soybean meal (44% w/w proteins, 28% w/w carbohydrates, and 7% w/w ash on dry matter) was received from Archer Daniels Midland Foods (Decatur, IL, USA). Bromelain Br 2400 (2400 gelatin digestion units per gram of enzyme) was provided by Enzybel (Ath, Belgium). Phyzyme XP 10,000 (10,000 FTU per gram of enzyme; 1 FTU = amount of enzyme needed to release 1 μmol/minute of inorganic phosphate from 7.5 mM sodium phytate dissolved in 0.25 M sodium acetate buffer, pH 5.5 at 37 °C) was provided by Trouw Nutrition (Putten, The Netherlands). Iscove's modified Dulbeccos medium (31980-022), fetal calf serum (FCS) (10270-106), D-phosphate buffered saline (14190-094), 0.5% trypsin-EDTA (10x) (15400-054), penicillin-streptomycin (15140-122), HT supplement (11067-030), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (002024) were purchased from Invitrogen (Breda, The Netherlands). α-lactalbumin (L6010), aprotinin (A1153), bacitracin (11702), poloxamer 188 solution (P5556), L-glutamine (G7513), and Tween-20 (P2287) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Daidzein (049-28073) was purchased from Wako Pure chemical Industries (Osaka, Japan). Soluble soy polysaccharide (SSPS, Soyafibe-S-DA-100) was purchased from Fuji Oil Co. (Ibaraki, Japan). ISCHO-CD (91119) was purchased from Orange Medical (Tilburg, The Netherlands). Affinipure mouse anti-human IgG, F (ab') 2 fragment specific (209-005-097), Affinipure goat anti-human IgG, Fc (gamma) fragment specific (109-005-098), and human IgG (02-7102) were purchased from Sanbio (Uden, The Netherlands). The reagents for cell growth and IgG production assays were prepared as described previously [2].

3.2.2. Preparation of raw materials

Defatted soybean meal (M) was used to prepare acid (AC) and ethanol extracted (EC) soy protein concentrates and soy protein isolate (I).

Soy protein concentrate prepared using acid extraction (AC)

The concentrate (AC) was prepared from the meal (M). First, M was suspended in Milli-Q water (100 g/L). Next, the pH was adjusted to 4.6 using 2 M HCl, and the suspension was stirred at 40 °C for 30 minutes. The suspension was centrifuged (14,000 g, 15 °C, 30 minutes) and the pellet was resuspended (333 g wet weight of pellet/L) in Milli-Q water. The pH of the suspension was adjusted to 8.0 using 2 M NaOH and subsequently the suspension was freeze dried.

Soy protein concentrate prepared using ethanol extraction (EC)

The concentrate (EC) was prepared from the meal (M). M was suspended (100 g/L) in 60% (v/v) ethanol and the suspension was stirred at 40 °C for 30 minutes. The suspension was centrifuged (14,000 g, 15 °C, 30 minutes) and the pellet was resuspended (333 g wet weight of pellet/L) in Milli-Q water. The pH of the suspension was adjusted to 8.0 using 2 M NaOH and subsequently the suspension was freeze dried.

Soy protein isolate (I)

The isolate (I) was prepared from the meal (M) as described previously [10]. M was suspended (100 g/L) in 30 mM Tris-HCl buffer (pH 8.0) containing 10 mM β-mercaptoethanol and stirred for 1.5 hours at room temperature. The suspension was centrifuged (14,000 g, 15 °C, 30 minutes) and the supernatant was collected. The pH of the supernatant was adjusted to 4.6 using 2 M HCl. After 2 hours of stirring, the suspension was centrifuged (14,000 g, 15 °C, 30 minutes), the pellet obtained was suspended (333 g/L) in Milli-Q water, and the pH was adjusted to 8.0 using 2 M NaOH. Subsequently, the solution obtained was freeze dried.

Soy extract/hydrolysate mixtures

Different amounts of M (24, 48, and 96 g) were each suspended in Milli-Q water (400 mL) and stirred at 60 °C for 30 minutes. The pH was adjusted to 4.6 using 2 M HCl and the suspensions were centrifuged (14,000 g, 15 °C, 30 minutes). Half of the volume of supernatants obtained was added to 6.5 g of soybean meal hydrolysate (MH) and the rest half to 6.2 g of soy protein isolate hydrolysate (IH). They were prepared from 24 g of M or I, respectively; the production of MH and IH is described in the next section. In this way, the dosage of extract to MH or IH corresponds to the amount of soluble material extracted from 12, 24, or 48 g of M. The soy extract/hydrolysate mixtures were freeze dried and denoted as MH-0.5E, MH-1.0E, MH-2.0E, IH-0.5E, IH-1.0E, and IH-2.0E.

3.2.3. Preparation of soy protein hydrolysates

Soy protein hydrolysates were prepared using the procedure described previously [11]. The raw materials (M, AC, EC, and I) were suspended (120 g/L) in Milli-Q water (2 L) and stirred at 60 °C for 30 minutes. Bromelain (5 g enzyme/100 g of raw material) and Phyzyme (1 g enzyme/100 g of raw material) were added to the suspensions. After 24 hours the suspensions were heated at 95 °C for 10 minutes and centrifuged (1350 g, 15 °C, 10 minutes). The supernatants were collected and filtered through a 10 kDa membrane (A/G Technology, Needham, MA, USA). After ultrafiltration, the permeates were freeze dried. The hydrolysates obtained were denoted as soybean meal hydrolysate (MH), acid extracted soy protein concentrate hydrolysate (ACH), ethanol extracted soy protein concentrate hydrolysate (ECH), and soy protein isolate hydrolysate (IH). The yield (%) of the process was calculated as the total amount of hydrolysate (g dry matter) divided by the total amount of starting material (g dry matter) * 100%.

3.2.4. Characterization of raw materials and hydrolysates

Dry matter content

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

Ash content

The ash content was determined following the AOAC method [12].

Total protein content

The protein content was analyzed following the AOAC method using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific, Waltham, MA, USA) [13]. L-methionine (1-20 mg) was used for calibration and cellulose was used as a blank. For soy materials, a nitrogen conversion factor of 5.71 was used [10]. For CD medium, a conversion factor of 6.66 was derived from the amino acid composition of Iscove's modified Dulbecco's medium [14].

Total neutral carbohydrate content and composition

Samples (7-10 mg) were hydrolyzed in 4 mL of 1 M sulphuric acid at 100 °C for 3 hours. The carbohydrates were derivatized to their alditol acetates and analyzed using gas chromatography-flame ionization detection with inositol as an internal standard [15].

Total uronic acid content

Samples (7-10 mg) were hydrolyzed in 4 mL of 1 M sulphuric acid at 100 °C for 3 hours. Subsequently, the total uronic acid content was determined following m-hydroxydiphenyl assay and quantified using galacturonic acid as standard [16].

Free monosaccharide content and composition

The free monosaccharides were analyzed using high performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA, USA) with post-column alkali addition. Fucose, mannose, galactose, arabinose, rhamnose monohydrate, xylose, glucose, glucuronic acid, and galacturonic acid in the concentration range of 5-50 μg/mL were used for quantification. Samples were standardized to $\sim$ 40 μg/mL of carbohydrate concentration and injected (10 μL) on a CarboPac PA1 column (Dionex). The eluents A, B, and C were 0.1 M NaOH, 1 M NaOAC in 0.1 M NaOH, and Milli-Q water, respectively. The flow rate was 0.3 mL/minute at 20 °C. The elution profile used was: 0.0-35.0 minutes 100% C, 35.0-35.1 minutes 100% A, 35.1-50.0 minutes 0-40% B, 50.1-55.0 minutes 100% B, 55.1-63.0 minutes 100% A, 63.1-78.0 minutes 100% C. For post-column alkali addition, 0.5 M NaOH with a flow rate of 0.1 mL/minute at 20 °C was used.

Oligosaccharide content and composition

The content and composition of oligosaccharides was analzyed using HPAEC-PAD. Sucrose, stachyose, and raffinose in the concentration range of 5-50 μg/mL were used for quantification.

Samples were standardized to $\sim$ 40 μg/mL of carbohydrate concentration and injected (10 μL) onto a CarboPac PA1 column. The eluents A and B were 0.1 M NaOH and 1 M NaOAC in 0.1 M NaOH, respectively, and the flow rate was 0.3 mL/minute at 20 °C. The elution profile used was: 0-10 minutes 0-5% B, 10-35 minutes 5-40% B, 35-40 minutes 40-100% B, 40-45 minutes 100% B, and 45-60 minutes 0-100% A.

Determination of carbohydrase activity

Bromelain and Phyzyme were analyzed for carbohydrase activities. SSPS (5 mg/mL in Milli-Q water) was incubated with Bromelain (5 g/100 g of SSPS) and Phyzyme (1 g/100 g of SSPS) at 60 °C for 24 hours. Samples were taken after 0, 1, 2, 3, 4, 8, and 24 hours, heated (100 °C, 10 minutes) to inactivate the enzymes, and analyzed using HPAEC-PAD for release of mono-/oligosaccharides.

Size exclusion chromatography

The molecular mass distribution of the peptides present in the hydrolysates was analyzed on an HPLC system (Thermo Scientific, Breda, The Netherlands). The eluent used was 30% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid in Milli-Q water. All samples and standards were prepared in the eluent and filtered through 0.45 μm filters. The samples (20 μL of 2.0-2.5 mg protein/mL) were injected onto a TSK gel G2000SWxl column (2SWXS3671) (Tosoh Bioscience, Stuttgart, Germany). The flow rate was 1 mL/minute and the detection was performed at 214 nm. The total peak area of each chromatogram was set to 100%. α-lactalbumin (14,200 Da), aprotinin (6,500 Da), bacitracin (1,423 Da), and phenylalanine (165 Da) (0.5 mg/mL) were used for calibration. The retention times were calculated for 0.5, 1, 2, 3, 5, and 10 kDa using a linear fit plotted between molecular masses and retention times of the standards. With the calculated retention times, the chromatograms were divided into molecular mass ranges of $<$ 0.5 kDa, 0.5-1 kDa, 1-2 kDa, 2-3 kDa, 3-5 kDa, and 5-10 kDa. As hydrolysates were ultrafiltered, no peptides larger than 10 kDa were detected. The peak areas (%) in each molecular mass range were calculated. In a given molecular mass range, the peak area indicated the amount of peptides (%) of that molecular mass range in the sample analyzed.

Reverse phase-high pressure liquid chromatography (RP-HPLC)

Samples (3-4 mg of protein/mL) were dissolved in 0.05 M Tris buffer (pH 7.5) containing 6 M urea and 5 g/L dithiothreitol. The samples and maltodextrin controls (DP-2, 6, 10, 14, and 20) were filtered through 0.45 μm filters and 20 μL of sample was injected on Thermo hypersil gold 100 * 4.6 mm (5 μm) column (Thermo Scientific) connected to an RP-HPLC system (Thermo Scientific). Eluent A was 1% (v/v) acetonitrile + 0.1% (v/v) trifluoroacetic acid in Milli-Q water and eluent B was 60% (v/v) acetonitrile + 0.1% (v/v) trifluoroacetic acid in Milli-Q water. The eluent profile used was: 0-10 minutes isocratic equilibration with 100% A, 10-75 minutes linear gradient 85% B, 76-81 minutes linear gradient 100% B, 82-92 minutes 0% B. The flow rate was 0.8 mL/minute and detection was performed at 214 nm. The

maltodextrin controls did not show any absorbance at 214 nm.

Isoflavone and saponin analysis

The extraction and analysis of isoflavones and saponins were performed as described elsewhere [17]. They were identified using UV and MS spectra. Purified soyasaponin Ab [18] and daidzein in the concentration range of 0.001 to 0.1 mg/mL were used for calibration and quantification.

The analyses were performed in duplicate and the errors in the measurements were calculated as standard deviation/mean * 100%. The errors in the contents and composition (for each compound) of dry matter, ash, protein, total neutral carbohydrate, total uronic acid, mono-/oligosaccharides, isoflavone and saponin were lower than 10%. Also, for the proportion of peptides present in each molecular mass range in size exclusion chromatography, the errors were lower than 10%.

3.2.5. Cell culture

Preparation of hydrolysate solutions

In a first set of cell culture experiments, hydrolysates were supplemented to the suspension CD medium on an equal weight basis (CCE_{wb}) to determine the combined influence of protein, carbohydrate, isoflavone, and saponin contents on IVCD and IgG production. In a second set, hydrolysates were supplemented on an equal protein basis (CCE_{pb}) to rule out the effects due to differences in protein concentration in the medium and to determine the combined effects of carbohydrate, isoflavone, and saponin contents on viable cell density and IgG production. For CCE_{wb}, the hydrolysates were dissolved to a final concentration of 4% (w/v) in the suspension CD medium just before use. In CCE_{wb}, the amount of hydrolysate added was standardized to the amount of protein equivalent to the suspension medium containing 4% (w/v) of MH. All hydrolysate solutions were filtered through 0.22 μm filters before use in cell culture.

Cell line adaptation, growth assay, and immunoglobulin production

CHO-2 cells were used to test the hydrolysates. The cell line adaptation, growth assay, and IgG production assay were carried out as described elsewhere [2]. Data were obtained from three separate experiments in which each sample was measured in triplicate. The suspension medium was used as negative control and suspension medium with 10% (v/v) FCS was used as positive control. The cell counting was based on the principle of trypan blue staining [19]. The integral viable cell density (* 10^5 cells·day/mL) was calculated from viable cell density. The total IgG production (μg/mL) was measured using an enzyme-linked immunosorbent assay [2]. The specific IgG production (fg·cell^{-1}·day^{-1}; fg = 10^{-15} g) was calculated as the ratio of total IgG production to IVCD.

Data correction for temperature gradient effects observed in the incubator

A temperature gradient was observed inside the incubator set at 37 °C. The temperature was highest at the center (position A; 38.0-38.5 °C) and lowest at positions away from the

center (position C; 37.0-37.5 °C) of the incubator. At positions between A and C, i.e. position B, the temperature was approximately an average of those at positions A and C (37.8-37.9 °C). For each sample, one flask was placed each at each position (A, B, and C). As a result, both IVCD and IgG production were low in flasks at position A, intermediate in flasks at position B, and high in flasks at position C (37.0-37.5 °C). The IVCD at positions A and B were 84 and 90%, relative to that at position C (100%). The IgG production at positions A and B were 64 and 88%, relative to that at position C (100%). Thus, a seemingly large standard deviation was observed between the triplicates. These observations coincide with previous findings, in which the relative viable cell densities were 15, 51, and 100% at 30, 33, and 37 °C, respectively [20]. To correct for this, the IVCD, total and specific IgG production data were rescaled to data at position C. After correcting the data for effects of temperature, the errors in the cell density and IgG production in all cultures were less than 10%.

Statistical data analysis

The corrected IVCD, total and specific IgG production data were analyzed using Tukey's multiple comparison tests for significant differences (p- value < 0.05) between samples. In addition, the cell culture data (IVCD, total IgG production, and specific IgG production) and protein or carbohydrate concentration in cell cultures were plotted and fitted with trend lines using linear regression. The correlation analysis (R and p- value < 0.05) was performed using the corrcoef function of Matlab®.

3.3. Results and discussion

3.3.1. Characterization of raw materials and hydrolysates

Raw materials

From the soybean meal (M), an aqueous extracted concentrate (AC), an ethanol extracted concentrate (EC), and an isolate (I) were produced. The protein content increased from 44 to 84% (w/w) and the carbohydrate content decreased from 28 to 4% (w/w) going from M, to AC, EC, and I (**Table 3.1**).

The isoflavone (0.25-0.29% w/w) and saponin (0.27-0.35% w/w) contents of M, AC, and I were higher than that of EC (0.07 and 0.12% w/w), respectively. The predominant isoflavone and saponin species present in all raw materials were genistin (52 ± 7% of the isoflavones) and soyasaponin Bb (72 ± 12% of the saponins), respectively (**Table 3.2**).

About 4-21% of dry matter could not be annotated to the compound classes analyzed in all samples. The unannotated dry matter was highest in carbohydrate-rich samples (e.g. 21% w/w in M) and lowest in high protein samples (e.g. 4% w/w in I). This suggests that part of the unannotated dry matter belongs to the carbohydrate fraction. In the compositional analysis of two untoasted soybean meals, similar gaps in the compositional analysis [21, 22] have been reported.

Table 3.1: Composition of raw materials, centrifugation pellets, ultrafiltration retentates, and soy protein hydrolysates (g per 100 g dry matter)

Sample	Yield (dry matter)	Protein*	Carbohydrate*	Ash*	Isoflavone*	Saponin*	Dry matter*
M	100	44.0	28.0	7.3	0.29	0.27	91
MH-P	55	42.5	34.3	6.1	0.20	0.40	95
MH-R	15	57.5	26.5	8.8	0.20	0.14	89
MH	23	58.0	20.8	10.3	0.21	0.01	86
AC	100	60.5	23.0	4.4	0.25	0.35	96
ACH-P	72	51.1	28.9	3.9	0.14	0.20	97
ACH-R	8	71.0	13.6	6.5	0.20	0.10	95
ACH	17	74.1	7.2	7.5	0.14	0.02	92
EC	100	58.6	20.0	7.0	0.07	0.12	95
ECH-P	61	50.2	30.5	5.8	0.02	0.17	97
ECH-R	13	67.0	15.0	8.8	0.03	0.04	95
ECH	22	71.4	5.9	10.0	0.03	0.01	92
I	100	83.8	3.7	5.0	0.25	0.27	94
IH-P	51	78.4	3.0	4.0	0.21	0.11	97
IH-R	20	82.1	3.0	5.9	0.14	0.12	96
IH	24	82.7	5.0	7.6	0.15	0.01	93

* Standard error: $< 5\%$

Characterization of pellets, retentates, and hydrolysates

In the production of hydrolysates, 17-24% of the total dry matter in the starting material was obtained as hydrolysates (**Table 3.1**). The remaining material (74 ± 5%) was present in the pellets and retentates. This effect was similar for the isolate and the meal, even though one would expect a higher yield of the hydrolysate prepared from the isolate. For all samples, 69 ± 6% of the protein and ash were present in the pellets and retentates, the remaining part (30 ± 5%) was present in the hydrolysates (**Figure 3.1**). This distribution was also valid for carbohydrates present in M and I, but not for AC and EC. In AC and EC, 99 ± 6% of the carbohydrate was present in pellets and retentates and only a small fraction (6 ± 1%) of carbohydrates was present in ACH and ECH (**Figure 3.1**).

The isoflavones and saponin contents in all raw materials were very low and could only be accurately determined in hydrolysates. In soy protein hydrolysates, the isoflavone contents of MH, ACH, and IH (0.14-0.21%) were higher than that of ECH (0.03%), whereas the saponin contents of all hydrolysates were quite similar (0.01-0.02%) (**Table 3.1**). The protein content increased from 58 to 83% going from MH, ACH, ECH, to IH (**Table 3.1**). The carbohydrate content of MH (20.8%) was higher than those of ACH, ECH, and IH. The carbohydrate contents in ACH, ECH, and IH ranged between 5.0-7.2%. Since most (87-89%) of the carbohydrates present in soy protein concentrates are insoluble [23], it is anticipated that they are completely removed during centrifugation and ultrafiltration.

Table 3.2: Isoflavone and saponin composition of raw materials, centrifugation pellets, ultrafiltration retentates, and soy protein hydrolysates (mg per 100 g dry matter)

Compounds	M*	MH-P*	MH-R*	MH*	AC*	ACH-P*	ACH-R*	ACH*	EC*	ECH-P*	ECH-R*	ECH*	I*	IH-P*	IH-R*	IH*
Daidzin	70	45	53	69	54	31	36	42	18	5	5	10	56	43	33	40
Glycitin	21	18	21	27	17	8	11	11	3	1	2	1	16	20	14	18
Genistin	127	95	91	90	123	87	71	66	38	15	15	18	119	118	72	67
Malonylgenistin	37	6	15	17	26	4	25	12	5	1	2	2	23	11	13	13
Genistein	37	8	11	11	32	8	8	8	6	1	1	2	33	17	11	10
Total isoflavones	292	172	191	214	252	138	151	139	70	23	25	33	247	209	143	148
Soyasaponin Ba	16	20	12	1	21	23	8	5	3	7	2	< 1	18	27	10	4
Soyasaponin Bd	< 13	< 6	< 3	5	< 18	< 14	< 1	1	3	< 1	< 1	< 1	18	< 8	< 10	8
Soyasaponin Bb	163	214	106	< 1	195	116	50	15	89	116	37	7	169	< 8	< 10	< 4
Soyasaponin Bc	27	6	17	< 1	29	< 14	6	< 1	14	21	4	< 1	26	< 8	13	< 4
Soyasaponin Bb'	35	60	< 3	< 1	55	< 14	3	< 1	9	22	1	< 1	39	76	84	< 4
Soyasaponin Ab	14	61	< 3	< 1	29	< 14	< 1	< 1	2	< 1	< 1	< 1	< 2	< 8	< 10	< 4
Soyasaponin Af	13	15	3	< 1	18	14	1	< 1	< 2	1	< 1	< 1	2	8	10	< 4
Total saponins	268	376	138	6	347	153	68	21	120	167	44	7	272	111	117	12

* Standard error: < 10%

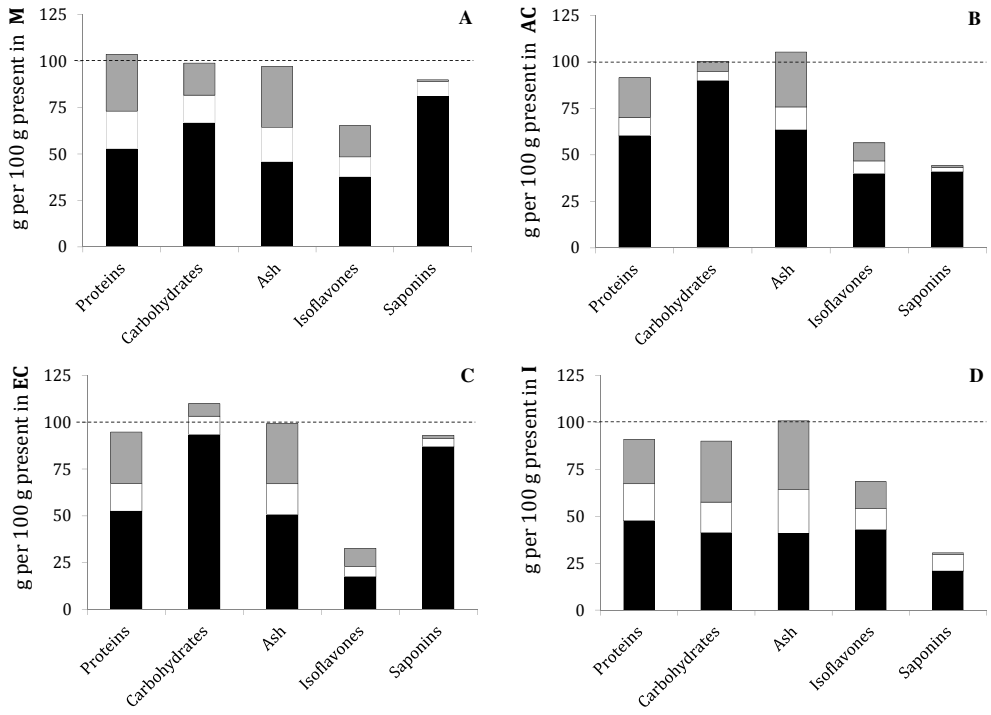

Figure 3.1: Distribution of protein, carbohydrate, ash, isoflavone, and saponin present in (A) M, (B) AC, (C) EC, and (D) I over centrifugation pellets (■), ultrafiltration retentates (□), and final hydrolysates (▣)

The presence of carbohydrates in ACH (7.2%) and ECH (5.9%) even after centrifugation and ultrafiltration could be due to three reasons. Firstly, part of the carbohydrate in hydrolysates could come from the glycosylated protein, β-conglycinin, present in the raw material. The carbohydrate content of β-conglycinin was reported to be 5.3% (w/w) [24]. Secondly, the enzymes used in the hydrolysis may contain carbohydrase activities. Thirdly, it has been proposed that microbial fermentation could occur during the enzymatic hydrolysis [25].

To test for the presence of carbohydrase activities, SSPS was incubated with Phyzyme and Bromelain. SSPS is a water-soluble polysaccharide extracted and refined from soybean and is composed of galactose, arabinose, rhamnose, xylose, fucose, glucose, and galacturonic acid [26]. Phyzyme did not exhibit any carbohydrase activity, whereas Bromelain released 2% of glucose and 11% of galacturonic acid present in SSPS (87% carbohydrate w/w, [27]). Thus, per 100 g of carbohydrate, 14.9 g of carbohydrate (2.3 g of glucose and 12.6 g of galacturonic acid) was released. Hence, 4.2, 3.4, 3.0, and 0.6% (w/w) carbohydrates is expected to be released during hydrolysis of M, AC, EC, and I, respectively, due to carbohydrase activity. Trace activities of amylase, cellulase, and hemicellulase have been previously reported in Bromelain [28]. However, this additional release of carbohydrate does not completely explain the carbohydrate contents of ACH (7.2% w/w) and ECH (5.9% w/w). This suggests that

microbial fermentation might also have occurred during enzymatic hydrolysis as proposed previously [29]. During this fermentation, high molecular mass carbohydrates are hydrolyzed into low molecular mass carbohydrates.

In addition to protein, carbohydrate, isoflavone, and saponin contents, their compositions were analyzed. The molecular mass distribution of peptides and RP-HPLC elution profiles (peptide composition) of the hydrolysates were similar (**Figure 3.2**).

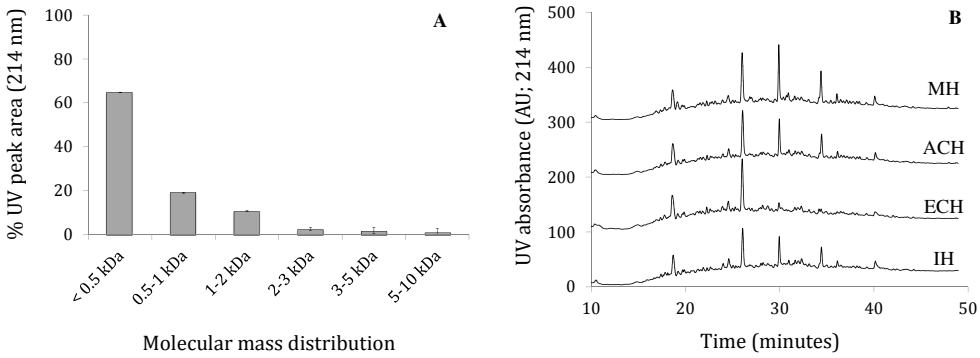

Figure 3.2: (A) Molecular mass distribution of peptides present in all soy protein hydrolysates. The error bars represent the standard deviation between molecular mass distributions of MH, ACH, ECH, and IH; (B) RP-HPLC elution profiles of soy protein hydrolysates

Most peptides in the hydrolysates (84 ± 0.6%) were smaller than 1 kDa. In addition to peptides, the constituent isoflavones, saponins (**Table 3.2**), and monosaccharides (**Table 3.3**) present in the hydrolysates were similar.

Summarizing, the hydrolysates varied in the contents, but not in the composition of protein, carbohydrates, isoflavones, and saponins. Consequently, the effect of different protein, carbohydrate, isoflavone, and saponin contents of hydrolysates could be assessed on IVCD and IgG production.

3.4. Effect of soy protein hydrolysates on IVCD, total IgG, and specific IgG production

The hydrolysate supplementation to the CD medium increased the cell viability as compared to the non-supplemented CD medium (**Figure 3.3**).

The hydrolysate supplementation also significantly (p- value < 0.05) increased IVCD, total and specific IgG production as compared to those in the CD medium. The IVCD, total IgG, and specific IgG production in CD medium were 51.9 * 10^5 cells·day/mL, 4.2 μg/mL, and 814 fg·cell^{-1}·day^{-1}, respectively. In the hydrolysate-supplemented cultures (CCE_{wb}), the IVCD, total IgG, and specific IgG production varied between 53-67 * 10^5 cells·day/mL, 5.4-7.4 μg/mL, and 987-1283 fg·cell^{-1}·day^{-1}, respectively. The cell viability was similar in all hydrolysate-supplemented cultures.

Table 3.3: Carbohydrate composition of raw materials, soy protein hydrolysates, and soy extract/hydrolysate mixtures (g per 100 g dry matter)

Sample	Arabinose*	Xylose*	Mannose*	Galactose*	Glucose*	Uronic acid*	Total
M	2.7	1.0	2.0	10.6	10.3	0.9	28
AC	4.1	1.5	1.2	10.3	4.9	1.2	23
EC	3.5	1.5	1.0	9.0	3.8	1.0	20
I	0.4	< 1.0	0.8	1.2	1.5	0.1	4
MH	< 0.4	< 1.0	2.3	6.4	9.8	2.3	21
ACH	< 0.4	< 1.0	1.1	1.1	2.7	2.3	7
ECH	< 0.4	< 1.0	1.1	1.1	1.6	2.1	6
IH	< 0.4	< 1.0	1.1	1.1	1.1	1.9	5
MH-0.5E	< 0.4	< 1.0	2.3	9.7	14.8	2.7	30
MH-1.0E	< 0.4	< 1.0	2.3	9.6	14.7	2.0	29
MH-2.0E	< 0.4	< 1.0	2.2	10.9	16.4	1.8	31
IH-0.5E	< 0.4	< 1.0	1.1	5.7	8.0	1.9	17
IH-1.0E	< 0.4	< 1.0	2.2	7.7	11.1	1.8	23
IH-2.0E	< 0.4	< 1.0	2.2	11.0	15.4	1.8	30

* Standard error: < 10%

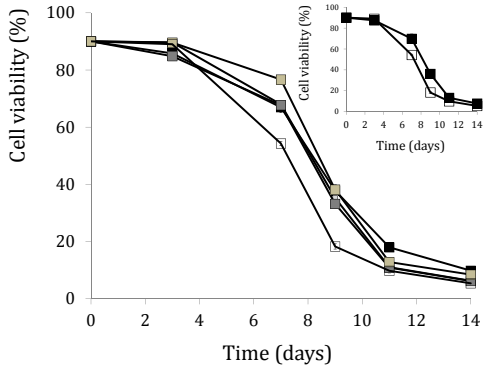

Figure 3.3: Cell viability in cultures supplemented with MH (■), ACH (□), ECH (■), IH (□), and CD medium (□) in CCE_{wb}; Inset: average cell viability in CD medium supplemented with hydrolysate (■) and CD medium (□)

3.4.1. Cell culture experiments on equal weight basis (CCE_{wb})

In CCE_{wb}, the hydrolysates were supplemented to the CD media on equal weight basis. The isoflavone and saponin concentrations in ACH (5.6 μg/mL isoflavones and 0.8 μg/mL saponins) were four-fold and three-fold higher than those in the ECH (1.2 μg/mL isoflavones and 0.3 μg/mL saponins) supplemented culture, respectively. Despite these differences, the IVCD, total and specific IgG production in ACH and ECH supplemented cultures were similar (p- value > 0.05). In a previous study, daidzein (1.7 μg/mL) and genistein (1.5 μg/mL) supplementation decreased cell density by approximately 5% and 25%, respectively [30]. In

the current study, daidzein was not detected and genistein was present in low concentrations at 0.32 and 0.08 μg/mL in ACH and ECH supplemented cultures, respectively. Conversely, their precursors, i.e. daidzin and genistin, were present in high concentrations. The daidzin concentrations were 1.7 and 0.4 μg/mL in ACH and ECH supplemented cultures, respectively. In these cultures, the genistin concentrations were 2.6 and 0.7 μg/mL, respectively. This suggests that in addition to isoflavones, their precursors did not have toxic effects in cell culture.

The protein concentration increased from 7.3 to 8.3 mg/mL and the corresponding carbohydrate concentration decreased from 6.4 to 5.7 mg/mL going from cultures supplemented with MH, ACH, ECH, to IH, respectively. The protein and carbohydrate concentrations in the non-supplemented CD medium were 5.0 and 5.5 mg/mL, respectively. In MH, ACH, ECH, and IH supplemented cultures, the IVCD were 67, 54, 53, and 58 * 10^5 cells·day/mL and the total IgG production were 6.6, 5.8, 5.4, and 7.4 μg/mL, respectively. The corresponding specific IgG production were 987, 1082, 1018, and 1283 fg·cell^{-1}·day^{-1} (**Figure 3.4**).

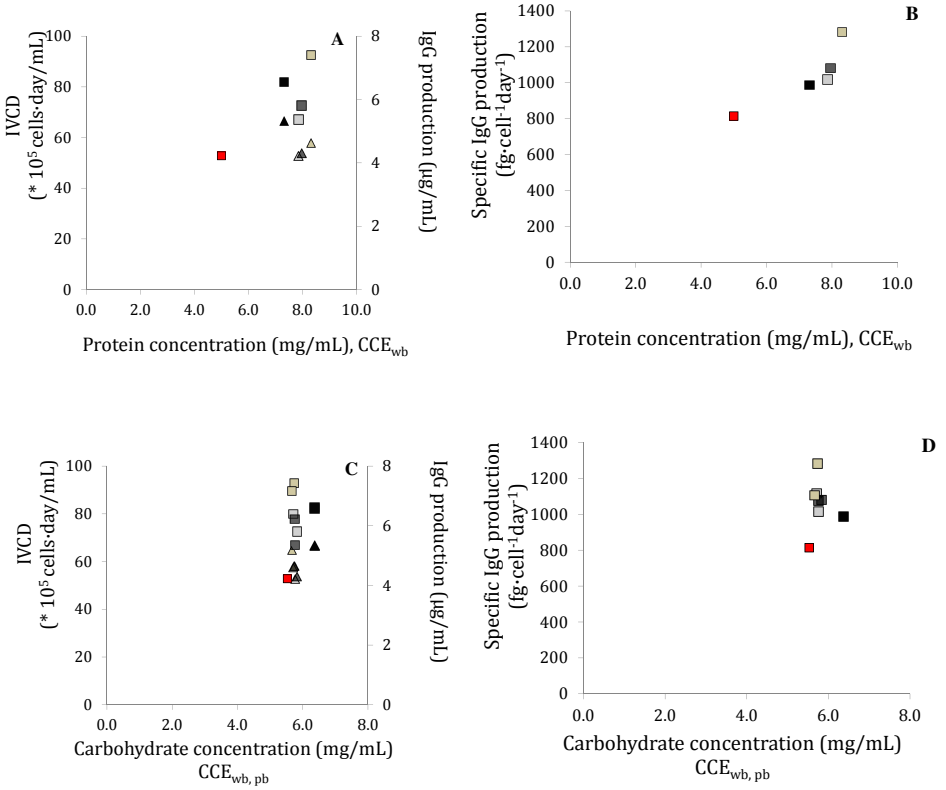

Figure 3.4: Influence of protein (A,B) and carbohydrate (C,D) concentration on cell density (△), total IgG (○), and specific IgG (□) production on equal protein (CCE_{pb}) and equal weight basis (CCE_{wb}); MH (▲ ● ■); ACH (△ ○ □); ECH (▲ ● ■); IH (△ ○ □); and CD medium (▲ ● ■)

This suggests that an increase in protein concentration, or decrease in carbohydrate concentration, decreased IVCD and increased total and specific IgG production. To separate the effects of proteins and carbohydrates, additional experiments were performed in which hydrolysates were supplemented on an equal protein basis to cell cultures. In the previous studies [31, 32], the notion of supplementing hydrolysates at equal protein basis to cell cultures has not been considered.

3.4.2. Cell culture experiments on equal protein basis (CCE_{pb})

In CCE_{pb}, the hydrolysates were supplemented to a final protein concentration of 7.2 ± 0.07 mg/mL in each culture was the same. In this way, any change in IVCD, total IgG, and specific IgG production can be related to the carbohydrate concentrations in cell culture. It was already shown in CCE_{wb} experiments that IVCD, total IgG, and specific IgG production were not influenced by the isoflavone and saponin contents of hydrolysates.

The carbohydrate concentrations in MH, ACH, ECH, and IH supplemented cultures were 6.4, 5.8, 5.7, and 5.7 mg/mL, respectively. In these cultures, the IVCD were 67, 58, 57, and 65 * 10^5 cells·day/mL. The total IgG production in these cultures were 6.6, 6.2, 6.4, and 7.2 μg/mL. As a result, the specific IgG production were 990, 1077, 1117, and 1107 fg·cell^{-1}·day^{-1} (**Figure 3.4**). This suggests that a decrease in carbohydrate concentration decreases IVCD, but increases the total IgG and specific IgG production. These observations are discussed in more detail in the next section.

3.5. Role of carbohydrates in cell cultures

To confirm the effect of carbohydrate on IVCD, total IgG, and specific IgG production, a water-soluble extract was prepared from M. This extract was added to MH and IH to create additional hydrolysate samples with a broader range of protein and carbohydrate contents (**Table 3.4**).

Table 3.4: Composition of soy extract/hydrolysate mixtures (g per 100 g dry matter)

Sample	Protein*	Carbohydrate*	Ash*	Isoflavone*	Saponin*	Dry matter
MH	58	21	10.3	0.21	0.01	86
MH-0.5E	35	30	13	0.48	0.08	88
MH-1.0E	36	29	14	0.27	0.04	88
MH-2.0E	26	31	17	0.29	0.04	92
IH	83	5	8	0.15	0.01	93
IH-0.5E	61	17	12	0.26	0.02	88
IH-1.0E	46	23	14	0.41	0.03	91
IH-2.0E	30	30	15	0.38	0.05	91
Extract	17	48	8	0.31	0.13	90

* Standard error: < 5%

Now, the protein and carbohydrate concentrations in CD media containing hydrolysates ranged from 6.0 to 8.3 mg/mL (CCE_{wb}) and 5.7 to 8.0 mg/mL (CCE_{wb} and CCE_{pb}), respectively.

The IVCD, total IgG, and specific IgG production data obtained from these cultures were plotted versus carbohydrate concentrations and fitted with linear regression. A positive correlation was observed between IVCD and carbohydrate concentration (R = 0.7). There was a negative correlation between carbohydrate concentration and total IgG (R = -0.7) and specific IgG production (R = -0.9) (**Figure 3.5**).

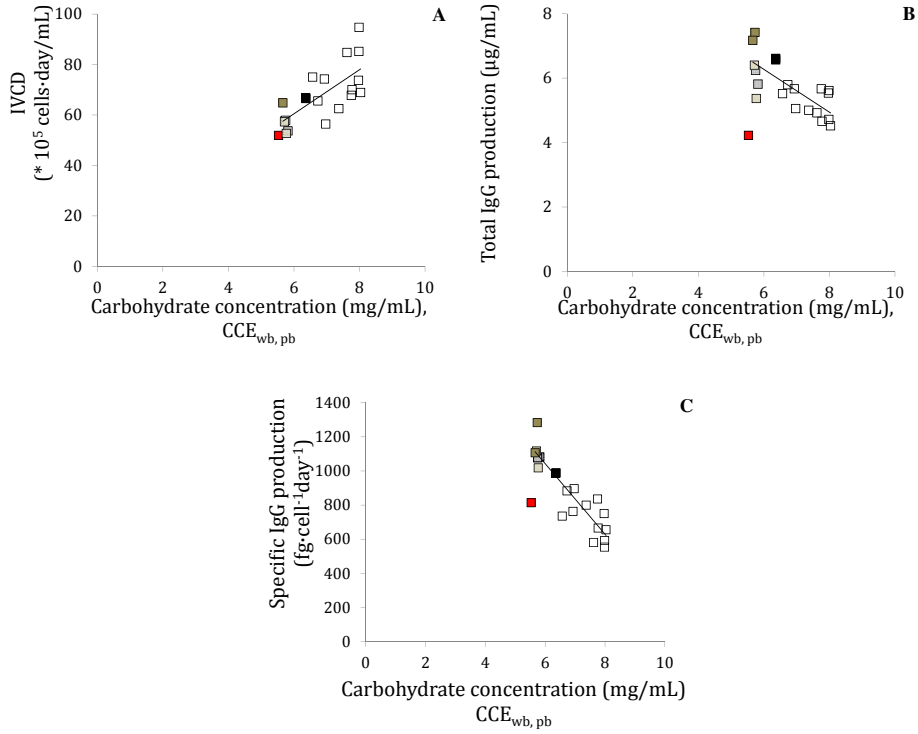

Figure 3.5: Influence of carbohydrate concentration on (A) cell density, (B) total IgG, and (C) specific IgG production, fitted with trend lines using linear regression on equal protein (CCE_{pb}) and equal weight basis (CCE_{wb}); MH (■); ACH (□); ECH (■); IH (□); and CD medium (■)

All correlations were significant at *p*- value < 0.004. In literature, little or no information is provided on the carbohydrate content of the CD media and/or hydrolysate-supplemented CD media. For instance, in one study, while the concentrations of pure monosaccharides supplemented to the CD medium have been indicated (e.g. 3.6 g/L or 20 mM galactose), the carbohydrate contents of the medium itself have not been determined [33]. In another study, the initial glucose concentration in the medium was indicated (2 g/L), whereas the carbohydrate content of the hydrolysate (Tryptose) supplemented at different concentrations (1-10 g/L) to the CD medium was not analyzed [34]. Consequently, the observation from our experiments cannot be compared to other data.

The positive correlations of carbohydrate content with cell growth and negative correlations

with total and specific IgG production suggest that in cultures containing high concentrations of carbohydrate, the cellular resources (e.g. ribosomes) are mainly used for cell division and the proteins synthesized are used for cell growth rather than for recombinant protein production [35]. Moreover, it is also suggested that high carbohydrate concentrations in cell cultures lead to high rates of aerobic glycolysis resulting in fast cell division and lactate accumulation. This has been referred to as the overflow metabolism [36, 37]. In a different study [35], similar negative associations between cell density and IgG production have been reported. In that study the cell density increased by 48% with a simultaneous decrease of 37% in the IgG production in untreated cultures as compared to the cultures treated with a growth inhibitor.

To determine the effects of protein concentration, the IVCD, total IgG, and specific IgG production data were extrapolated to zero supplemented carbohydrate concentrations. In hydrolysate-supplemented cultures, at zero carbohydrate concentration, the total IgG (10 μg/mL) and specific IgG (2283 fg·cell^{-1}·day^{-1}) were much higher, whereas the IVCD (8 * 10^5 cells·day/mL) was substantially lower than that in the non-supplemented CD medium (4.2 μg/mL, 814 fg·cell^{-1}·day^{-1}, and 51.9 * 10^5 cells·day/mL). Thus, an increase in protein concentration increased total IgG and specific IgG production and suppressed IVCD. While peptide supplementation has been shown to increase viable cell density [6], to increase recombinant protein production, to increase both viable cell density and recombinant protein production [5], it has also been shown to decrease viable cell density and increase recombinant protein production [7].

In the present study, the generic effects of increasing carbohydrate concentrations are apparent despite the fact that soy protein hydrolysates are complex mixtures of compounds. Although it has been shown previously that different types of peptides [38] and carbohydrates [33] are metabolized by cells at different rates, this was not observed in the current study. It is proposed that this is because hydrolysates used in this study contained similar constituent peptides and carbohydrates.

3.6. Conclusions

Although soy protein hydrolysates are complex mixtures of different compounds, the generic effects of supplementing different concentrations of carbohydrates were apparent. These effects were concentration dependent and large variations in the functionality could be correlated to the carbohydrate concentrations in cell culture.

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CHAPTER 4

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

Abstract

Soy protein hydrolysates are often supplemented to cell cultures to improve cell culture functionality (integral viable cell density (IVCD) and recombinant protein production). There is, however, little understanding of the effect of the hydrolysate composition on the functionality. Heating during defatting of the soybeans or during hydrolysate production, is expected to be an important factor, but the effects on composition and functionality of the hydrolysates are largely unknown. To study the effects of heating, hydrolysates were produced from meal heated for 0-120 minutes (121 °C). With increase in heating time, the free amino acid and reducing monosaccharide contents of the meals decreased from 0.72-0.27% and 3.3-2.6% respectively. Also, in the hydrolysates derived from these meals, large differences in the reducing monosaccharide (14.7-7.1 g/100 g dry matter) and free amino acid (16.9-7.9 g/100 g dry matter) contents were observed. The differences between the free amino acid contents of hydrolysates showed that the heat treatments applied to the meals hindered subsequent protein hydrolysis. In hydrolysate-supplemented cultures, the relative IVCD (115-316%) and relative specific IgG production (48-140%) varied substantially. However, when the same hydrolysates were subjected to extended heating in solution and then supplemented to cell cultures, the variation in functionality was significantly reduced while no significant changes in composition were observed. These results show that significant changes in functionality can be due to process-induced changes in hydrolysate composition, which are not quantified by typical analytical methods.

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4.1. Introduction

Soy protein hydrolysates produced from defatted soybean meals are commonly supplemented to chemically defined (CD) media in mammalian cell cultures. This is because they significantly enhance the functionality, expressed by integral viable cell density (IVCD) and recombinant protein production. For example, in Chinese hamster ovary (CHO)-320 cell cultures supplemented with hydrolysates the relative viable cell density and relative interferon-γ production were 150% and 260% higher, respectively, than that in the non-supplemented CD medium (100%) [1]. However, different batches of a hydrolysate may vary in their effect on the functionality. Two separate studies have shown that supplementation of different batches of industrial soy protein hydrolysates resulted in 19% and 25% variation (compared to the mean) in the recombinant protein production [2, 3]. In only one study the gross composition was analyzed and was found to be quite similar for all batches (e.g. protein content varied between 56-58% w/w) [2]. Therefore, the variation in the functionality of hydrolysates is expected to be due to differences in composition that are typically not analyzed in the gross composition. This may, for instance, be due to modification of compounds during heating of the starting material (defatted soybean meal) or that of the hydrolysates during and after production.

Soybean meal is heated after the defatting step to remove organic solvents (desolventization) and to inactivate protease inhibitors (toasting). Examples of the heat treatments applied to simulate desolventization are 99-104 °C for 18-20 minutes [4] and 177 °C using hexane vapor for 3-10 seconds [5]. Examples of the heat treatments applied to simulate toasting are 99-116 °C for 48-55 minutes [4], 79-115 °C for 30 minutes [5], and 165 °C for 75-210 minutes [6]. During hydrolysate production, heat treatment (95 °C, 10 minutes) is applied to inactivate the enzymes that are used to produce hydrolysates [7].

It is important to note that the heating is applied either to dry samples (soybean meal) or to samples in suspensions (hydrolysates). When considering literature, it is clear that in the dry state significant effects of the Maillard reaction are observed under relatively mild conditions. For instance, 47% of the native ovalbumin was glycated when 1:1 (w/w) mixture of native ovalbumin (0% glycation):saccharide (fructose or glucose) was heated at 45 °C (69% relative humidity) for 2 hours [8]. In another study, the degree of substitution/3-(2-furyl)-acryloyl-Ala-Lys-OH peptide molecule varied between 0.6-7.8 when 1:2 (w/w) mixture of 3-(2-furyl)-acryloyl-Ala-Lys-OH:saccharide was heated at 60 °C (65% relative humidity) for 1-4 hours [9]. In contrast to dry state heating, typically much harder heat treatments are needed to induce the Maillard reaction in liquid systems. This is because the Maillard reaction does not proceed as fast as in the dry state. For instance, in a lactose-casein mixture 60% of lysine residues were modified after 20 hours of heating at 60 °C at a water activity of 0.33, while a similar loss of lysine residues occurred only after $\sim$ 70 hours when the water activity was 0.98 [10]. In the Maillard reaction, free amino acids and lysine and arginine side chains react with reducing monosaccharides. Consequently, free amino acids and reducing monosaccharides (e.g. glucose) become unavailable for cellular nutrition and metabolism. Despite the apparent

reason to study dose-response curves with free amino acids and mono-/oligosaccharides, no concrete data has been published. Moreover, those studies that studied supplementation e.g. of glucose and glutamine [11], did not report on the initial concentrations of these compounds in the CD medium.

In addition to the Maillard reaction, other chemical reactions may take place during (prolonged) heating, such as decarboxylation, deamidation, and cross-linked peptide, e.g. lysinoalanine (LAL) formation. These reactions affect the contents of free amino acids and amino acid residues present in proteins, but when formed during the meal production, they can also affect the hydrolysis process. In addition to the loss of free amino acids and amino acid residues present in proteins and reducing monosaccharides, a wide variety of new compounds is formed during the Maillard and other heat-induced reactions. Due to the variety of the reactions, typically in studies on the Maillard reaction, a few Maillard reaction products (MRP) are used as indicators [12]. Examples are furosine, carboxymethyllysine (CML), and 5-hydroxymethylfurfural (HMF). Typical examples of cross-linked amino acids (CLAA) are LAL and lanthionine.

These MRP and CLAA can be used as markers of the heat treatment. In addition, some of these compounds may also have a direct influence on the cell culture functionality. When the concentration of HMF supplemented to CHO-V79 cultures was increased from 2.5 to 100 mM, the viability of cells decreased from 95 to 71% [13]. In another study, a 50% decrease in the viability of HepG2 cells was observed in cultures supplemented with 38 mM of HMF [14]. Unfortunately, these studies did not report the effects on the recombinant protein production.

In this study, defatted soybean meal was heated for different times in dry state. One part of the meals was hydrolyzed and the other part was both hydrolyzed and heated for prolonged period in suspension. The composition of meals and hydrolysates was analyzed and the effect of supplementation of the hydrolysates to CHO cells was tested.

4.2. Materials and methods

4.2.1. Materials

Hyland soybeans were received from L.I. Frank (Twello, The Netherlands). Technical hexane (24611.366; 95% v/v) was purchased from VWR International, 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 were purchased as described previously [7]. The materials required for cell culture assay were purchased and prepared as described previously [2].

4.2.2. 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 minutes/cycle). The defatted soybean meal obtained was denoted M_0 and divided 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 minutes (M_{120}).

4.2.3. Preparation of soy protein hydrolysates

From the meals (M_0, M_{15}, M_{60}, and M_{120}), two sets of four hydrolysates were prepared using the process described previously [7]. 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 raw material) and Phyzyme (1 g enzyme/100 g of meal) at 60 °C for 24 hours. Afterwards, the suspensions were shortly heated (95 °C, 10 minutes), cooled down, centrifuged (1350 g, 15 °C, 10 minutes) and filtered through a 10 kDa membrane (A/G Technology, Needham, MA, USA). The permeates obtained 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 extended heating (95 °C, 70 minutes) was applied to the hydrolysate suspensions during the enzyme inactivation step. Subsequently, the hydrolysate suspensions were centrifuged (1350 g, 15 °C, 10 minutes) and then filtered through 10 kDa. The permeates obtained 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 the total amount of hydrolysate (g dry matter) divided by the total amount of starting meal (g dry matter) * 100%.

4.2.4. Characterization of meals and hydrolysates

Dry matter content

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

Ash content

The ash content was determined following the AOAC method [15].

Total protein content

The protein content (N * 5.71, [16]) was determined following the AOAC method [17] using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific, Waltham, MA, USA). L-methionine (1-20 mg) was used for calibration and cellulose was used as a blank.

Ultraviolet (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 minutes). The spectra were measured in 1 cm quartz cuvettes in the range of 200-800 nm in a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Content of reducing end groups

The content of reducing end groups was determined using 4-hydroxybenzoic acid hydrazide (PAHBAH) assay as described elsewhere [18].

Free monosaccharide and oligosaccharide contents

The free mono-/oligosaccharides were analyzed using high performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA, USA) as described previously [7].

Total neutral carbohydrate and uronic acid contents

The total neutral carbohydrate content was analyzed using gas chromatography-flame ionization detection as described previously [7]. The total uronic acid content was determined following the m-hydroxydiphenyl assay as described previously [7].

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 [19].

Size exclusion chromatography (SEC)

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 method has been described previously [7].

CML, LAL, and furosine contents

Samples (10 mg) were hydrolyzed using 6 M HCl (1 mL) at 110 °C for 24 hours. Next, the hydrolyzed samples were dried under a stream of nitrogen and redissolved in 1 mL of Milli-Q water, sonicated (10 minutes), and centrifuged (12,000 g, 15 °C, 5 minutes). 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-13C6,15N2 hydrochloride was used to verify the stability of MS signal throughout the ultrahigh performance liquid chromatography (UHPLC)-mass spectrometry (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, MA, USA) with an Acquity BEH amide vanguard pre-column (2.1 * 50 mm, 1.7 μm particle size, Waters) installed on an Accela UHPLC system (Thermo Scientific, Waltham, MA, USA). The UHPLC system was coupled to an LTQ VelosPro mass spectrometer (Thermo Scientific, San Jose, CA, 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 minutes isocratic on 90% B, 2-10 minutes linear gradient from 90% to 40% B, 10-12 minutes isocratic 40% B, 12-13 minutes 40% B to 90% B, and 13-30 minutes isocratic on 90% B. The flow rate was 0.3 mL/minute and detection was 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.

Error analysis

The analyses were performed in duplicate and standard errors in the measurements were calculated as standard deviation/mean * 100%. The standard errors in the contents (for each compound) of dry matter, proteins, ash, neutral carbohydrates, uronic acids, mono-/oligosaccharides, furosine, CML, and LAL were less than 10%.

For the contents of total dry matter, proteins, carbohydrates, ash and composition of amino acids and carbohydrates, little variation was observed between different meals and between final hydrolysates. Therefore, the values for all meals and all hydrolysates were averaged and shown with the corresponding standard deviations. The free and total amino acid analyses were performed as single measurements.

4.2.5. Cell culture

Preparation of hydrolysate stock solution

The hydrolysate stock solution was prepared by dissolving hydrolysates to a concentration of 4% (w/v) in the suspension medium [2]. The hydrolysates solubilized completely in the suspension medium. The solutions were filtered (0.22 μm) to remove microorganisms that might be present in the hydrolysates.

Cell line adaptation, growth assay, and IgG production

The hydrolysate stock solution was diluted to a 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 previously [2]. The IVCD was determined by calculating the area under the growth curve from the cell counts measured at different time points during the growth assay. The total IgG production was measured using an enzyme-linked immunosorbent assay. The specific IgG production was calculated as the ratio of total IgG production to IVCD. The IVCD and total IgG production data were corrected for temperature gradient effects observed in the cell culture incubator as described previously [7]. After correction, the standard errors in IVCD and total IgG production were lower than 10%.

Correlation analysis (R and p- value) was performed using the corrcoef function of Matlab®.

4.3. Results and Discussion

4.3.1. Characterization of defatted soybean meals

There were no significant differences in total protein, total carbohydrate, and total ash contents between the 4 meals (M_0, M_{15}, M_{60}, and M_{120}). The average values were 56 ± 1.3%, 19 ± 0.5%, and 6.9 ± 0.2% on dry matter basis, respectively (**Table 4.1**).

Table 4.1: Gross composition (g/100 g dry matter) of soybean meals, centrifugation pellets, ultrafiltration retentates, and soy protein hydrolysates, values are averages ± standard deviations over all meals (M_0, M_{15}, M_{60}, and M_{120}) and hydrolysates (M_0H, $M_{15}H$, $M_{60}H$, $M_{120}H$, M_0H_{aq}, $M_{15}H_{aq}$, $M_{60}H_{aq}$, and $M_{120}H_{aq}$)

Sample	Yield (dry matter)	Protein content		Carbohydrate	Ash
		Dumas (N * 5.71)	Total amino acid analysis		
Meals	100	55.8 ± 1.3	56.7 ± 3.1	19.3 ± 0.5	6.9 ± 0.2
Centrifugation pellets	64 ± 4.3	53.5 ± 0.9	n.a.*	16.3 ± 1.4	6.5 ± 0.9
Ultrafiltration retentates	17 ± 4.0	66.8 ± 3.1	n.a.*	13.4 ± 1.7	9.1 ± 0.4
Hydrolysates	19 ± 0.3	65.3 ± 2.1	60.4 ± 1.6	12.5 ± 1.1	11.1 ± 0.6

n.a: not analyzed

Also, the total amino acid and total carbohydrate composition were similar. Hence, in **table 4.2** only the average values ± standard deviation are presented.

However, from M_0 to M_{120} a decrease of 63% in the free amino acid and of 16% in the reducing monosaccharide (sum of glucose and galactose) contents was observed (**Table 4.3**). This corresponds to a loss of 3.2 and 3.0 millimoles/100 g dry matter in free amino acids and reducing monosaccharides, respectively (**Table 4.3**).

The free amino acid content decreased from 0.72% (M_0) to 0.27% (M_{120}) (w/w) with increase in heating time (**Table 4.3**, **Figure 4.1 A**). This decrease was not observed in the total amino acid and total protein contents because of the low content of free amino acids, which was only 0.5-1.2% of the total amino acid content of the meals.

During heating, glutamine, methionine, isoleucine, and histidine had completely reacted. Moreover, up to 73-87% of leucine, lysine, glutamic acid, and valine reacted during heating. Most free amino acids that reacted substantially (e.g. methionine, isoleucine, histidine, leucine, lysine, and valine) are essential amino acids that are required by CHO cells for optimal nutrition and metabolism [20]. Interestingly, the reactivity of free amino acids in these complex hydrolysates deviates from that reported in the literature [21], where lysine, glycine, tryptophan and tyrosine were found to be most reactive.

The reducing monosaccharide content decreased by 21% in the first 60 minutes of heating (**Table 4.3**). Upon further heating to 120 minutes, an increase of 6% of reducing monosaccharides in the M_{120} sample was observed, relative to the M_{60} sample. This increase was confirmed with the PAHBAH assay (data not shown). This might be due to an increased degradation of large molecular mass carbohydrates upon prolonged heating.

Table 4.2: Total amino acid composition (g/100 g dry matter) and total carbohydrate composition (g/100 g dry matter) of soybean meals and soy protein hydrolysates, values are averages ± standard deviations over all meals and hydrolysates

Amino acid composition (g/100 g dry matter)	**Meals**	**Hydrolysates**
Essential amino acids		
Leucine	4.6 ± 0.2	4.4 ± 0.2
Lysine	3.3 ± 0.5	4.0 ± 0.3
Phenylalanine	3.1 ± 0.1	2.9 ± 0.2
Isoleucine	2.8 ± 0.1	2.7 ± 0.1
Valine	3.0 ± 0.1	3.0 ± 0.1
Threonine	2.4 ± 0.1	2.6 ± 0.1
Histidine	1.5 ± 0.1	1.5 ± 0.1
Non-essential amino acids		
Glutamic acid and Glutamine	11.3 ± 0.5	12.5 ± 0.2
Aspartic acid and Asparagine	6.8 ± 0.3	7.4 ± 0.2
Arginine	4.3 ± 0.4	4.9 ± 0.2
Proline	3.1 ± 0.1	3.3 ± 0.1
Serine	3.0 ± 0.2	3.3 ± 0.2
Alanine	2.7 ± 0.1	2.9 ± 0.1
Glycine	2.5 ± 0.1	2.8 ± 0.1
Tyrosine	2.2 ± 0.1	2.5 ± 0.1
Total	**56.6 ± 4.2**	**60.7 ± 3.3**
Carbohydrate composition (g/100 g dry matter)		
Mannose	1.8 ± 0.4	1.4 ± 0.2
Galactose	6.6 ± 0.6	4.3 ± 0.1
Glucose	5.4 ± 0.4	4.5 ± 1.0
Rhamnose	0.4 ± 0.2	< 0.2
Arabinose	2.6 ± 0.1	< 1.4
Xylose	1.4 ± 0.2	< 0.4
Uronic acids	1.0 ± 0.1	2.0 ± 0.1
Total	**19.3 ± 0.5**	**12.5 ± 1.1**

* Standard error: < 4%

Maillard reaction products and cross-linked amino acids in defatted soybean meals

The furosine, CML, and LAL contents of the unheated meal (M_0) were 329, 13, and 0.5 mg/100 g on dry matter basis, respectively (**Figure 4.1 B-D**). In literature, a much lower content of furosine (9.4 mg/100 g w/w) in soybean flour has been reported [22]. This suggests that the MRP and CLAA were formed either during milling of the soybeans or during the defatting of the meal, or that the soybeans already had high contents of MRP and CLAA. It has been reported that MRP accumulated in mung beans during storage at 33 °C for 600 days [23].

In M_{15}, M_{60}, and M_{120}, the furosine content did not change significantly and varied between 68.8-77.5 mg/100 g dry matter (**Figure 4.1 B**). In contrast to furosine, the CML and LAL contents increased from 52 to 81 and 5 to 22 mg/100 g dry matter, respectively, with increase

Table 4.3: Free amino acid composition (mg/100g dry matter) and mono-/oligosaccharide composition (g/100g dry matter) in soybean meals

Free amino acid (mg/100 g dry matter)	**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
Total	**720**	**496**	**427**	**269**
Mono-/oligosaccharides* (g/100 g dry matter)				
Glucose	2.75	2.48	2.23	2.4
Galactose	0.56	0.39	0.38	0.37
Mannose	< 0.07	< 0.07	< 0.07	< 0.07
Fructose	< 0.07	< 0.07	< 0.07	< 0.07
Reducing monosaccharide	**3.31**	**2.87**	**2.61**	**2.77**
Sucrose	4.96	4.47	4.08	4.6
Stachyose	3.44	2.83	2.23	2.66
Raffinose	0.13	0.07	0.28	0.15
Total	**11.84**	**10.24**	**9.2**	**10.18**

* Standard error: < 4%

in heating time (**Figure 4.1 C, D**). While the CML content has not been reported, a much higher LAL content of 150 mg/100 g protein (∼ 90 mg/100 g dry matter) has been reported in an industrial soybean meal [24]. In another study, the LAL content of soy protein isolates varied between 0-37 mg/100 g of protein (∼ 0-30 mg/100 g dry matter) [25]. While this shows that there is a large variation in the reported values of MRP and CLAA, it also shows that the contents of MRP and CLAA in meals prepared in the current study were lower than those reported in the industrial meal. Therefore, the effects of heating in industrial samples may even be more pronounced than the effects observed in this study.

As CML, LAL, and furosine are derived from lysine, the formation of these compounds was

compared to the loss of total lysine. The total lysine content M_0 and M_{120} were 3.85 and 2.59 g/100 g dry matter, respectively (data not specified in **Table 4.2**).

The difference between the two values corresponds to a loss of 9 millimoles of lysine/100 g dry matter. The corresponding increase in the CML and LAL contents were 0.33 and 0.09 millimoles/100 g dry matter, respectively. Hence, only 5% of the total lysine reacted to form CML and LAL. Furosine could not be included in this comparison as its content decreased with increase in heating time of the meals. Thus, the loss of total lysine is much higher than the formation of CML and LAL. Next to lysine, 5.1 g/100 g dry matter of other amino acids had reacted. The largest loss occurred in arginine (18%), while the average loss of other amino acids was quite similar (9 ± 2%). This suggests that in addition to CML and LAL, several other MRP and CLAA might have been formed. As a large variety of reaction products can be formed from different amino acids, not all the reaction products were individually studied.

The occurrence of the Maillard reaction was also obvious from the increase in the browning intensity of the meals (data not shown). When aqueous suspensions of meals were centrifuged, the brown colored products were mostly present in the pellets. This suggests that the brown colored products themselves were insoluble or that they were bound to the insoluble material in the meals. When meal suspensions were hydrolyzed with Bromelain and subsequently centrifuged, part of the color compounds stayed in solution as observed in the UV spectra of the supernatants (data not shown). When supernatants were ultrafiltered, most of the colored compounds were retained in the retentates, but the effect of heating was still visible in the permeates. It has been reported that brown compounds formed during heating of wheat gluten (150 °C for 1 hour) in the presence of glucose and water also became soluble after the tryptic hydrolysis [26].

4.3.2. Characterization of soy protein hydrolysates

During hydrolysate production, 81 ± 0.3% of the dry matter present in the meal was obtained as pellets and retentates and 19 ± 0.3% was obtained as hydrolysates (**Table 4.1**). The average protein (65 ± 2% on dry matter), carbohydrate (13 ± 1% on dry matter), and ash (11 ± 1% on dry matter) contents of all hydrolysates were quite similar (**Table 4.1**).

In hydrolysates prepared from meals without extended heating in suspension (M_0H, M_{15}H, M_{60}H, and M_{120}H), the contents of free amino acids and reducing monosaccharides decreased from 16.9-8.3 and 14.0-7.4 g/100 g dry matter, respectively (**Table 4.4**). This corresponds to a decrease from 124-63 and 78-41 mmol/100 g dry matter in free amino acids and reducing monosaccharides, respectively. Based on the mass balance, if all free amino acids (grams) and reducing monosaccharides (grams) present in the meals are transferred to the hydrolysates, one would expect free amino acid and reducing monosaccharide contents of 3.8-1.4 and 17.4-13.7 g/100 g dry matter, respectively, in the final hydrolysate. However, the measured contents of free amino acids and reducing monosaccharides in the hydrolysates varied between 16.9-8.3 and 14.0-7.4 g/100 g dry matter, respectively.

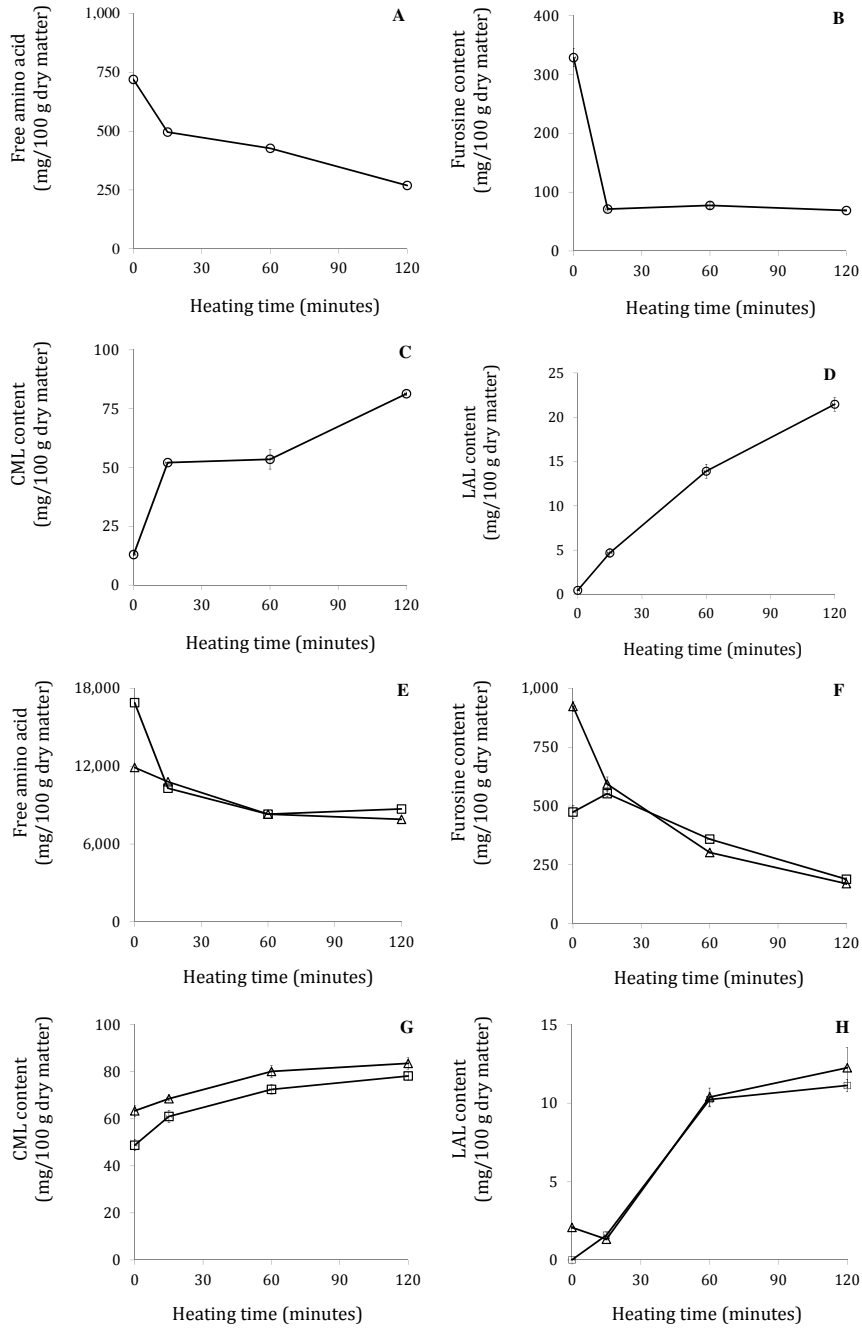

Figure 4.1: Effect of heating time on free amino acid, furosine, CML, and LAL content (mg/100 g dry matter) in (○) soybean meals (A-D, respectively) and soy protein hydrolysates (E-H, respectively) prepared from meals with (△), and without (□) extended heating in suspension

Table 4.4: Free amino acid composition (g/100g dry matter) and mono-/oligosaccharide composition (g/100g dry matter) in soy protein hydrolysates

Free amino acid (g/100 g dry matter)	M_0H	$M_{15}H$	$M_{60}H$	$M_{120}H$	M_0H_{aq}	$M_{15}H_{aq}$	$M_{60}H_{aq}$	$M_{120}H_{aq}$
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
Total	**16.9**	**10.3**	**8.3**	**8.7**	**11.9**	**10.8**	**8.3**	**7.9**
Mono-/oligosaccharides* (g/100 g dry matter)								
Glucose	1.50	2.56	0.94	2.62	3.19	1.38	2.53	0.56
Galactose	1.85	1.51	0.64	1.10	1.22	1.26	0.93	1.00
Mannose	0.93	0.93	0.75	1.04	0.90	0.99	1.00	0.82
Fructose	7.74	3.29	5.09	9.26	9.43	7.81	9.56	4.74
Reducing monosaccharide	**12.02**	**8.29**	**7.42**	**14.02**	**14.74**	**11.44**	**14.02**	**7.12**
Sucrose	0.13	0.37	0.74	0.11	0.33	0.55	0.08	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
Total	**12.15**	**8.66**	**8.16**	**14.13**	**15.07**	**11.99**	**14.1**	**7.16**

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

This shows that while the free amino acids were accumulated (369-615%) (**Figure 4.2 A**), reducing monosaccharides were lost (4-46%) (**Figure 4.2 B**) during hydrolysate production.

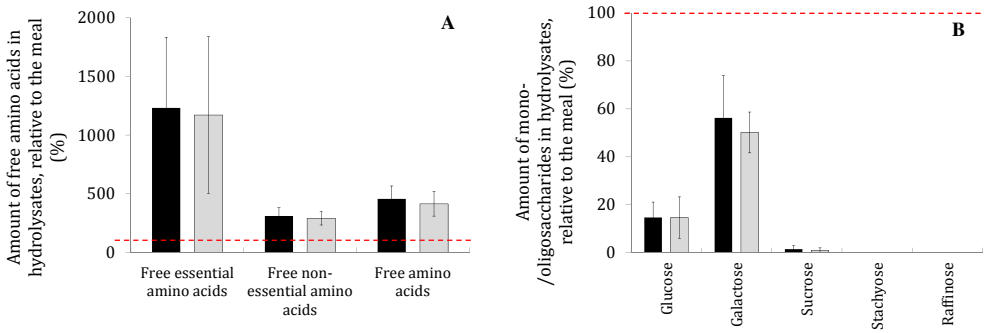

Figure 4.2: Amount of (A) free amino acids and (B) mono-/oligosaccharides in soy protein hydrolysates prepared with (□) and without (■) extended heating in suspension, relative to the meal (100%, indicated by dashed red line) calculated using a mass balance, averages and standard deviations were calculated over all hydrolysates

Despite a large increase in free amino acid contents due to the hydrolysis, there was a clear trend of decrease in total free amino acid content with heating of the meals. This suggests that the Maillard and cross-linking reactions induced in the meals hindered subsequent protein hydrolysis. The differences in hydrolysis were apparent in the SEC elution profiles of the hydrolysates (**Figure 4.3**). The M_0H hydrolysate prepared from the unheated meal contained more low molecular mass peptides (< 500 Da) and free amino acids than the $M_{120}H$ hydrolysate prepared from the meal heated for 120 minutes.

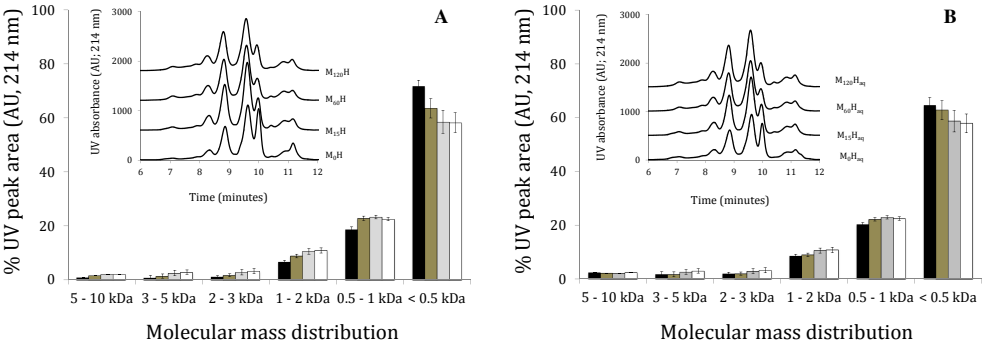

Figure 4.3: Molecular mass distribution of peptides present in hydrolysates prepared from meals (A) without (■) M_0H (■) $M_{15}H$ (□) $M_{60}H$ (■) $M_{120}H$ and (B) with extended heating in suspension (■) M_0H_{aq} (■) $M_{15}H_{aq}$ (□) $M_{60}H_{aq}$ (■) $M_{120}H_{aq}$, insets show the raw data of the SEC elution profiles

The amount of glucose, galactose, and sucrose were lower in hydrolysates as compared to those in the meals (**Figure 4.2 B**). In addition, the oligosaccharides stachyose and raffinose were not detectable in the hydrolysates. At the same time, mannose and fructose were present in negligible amounts in the meals, but they were present in considerable amounts in hydrolysates. The differences in the carbohydrate content and composition in hydrolysates as compared to the meals suggest the occurrence of microbial fermentation. While this has been proposed [27], the occurrence of fermentation during hydrolysate production has not actually been studied.

The free amino acid content of the hydrolysate prepared from the unheated meal with extended heating in suspension (M_0H_{aq}) was 30% lower than that in M_0H (**Figure 4.1 E**). For remaining hydrolysates prepared from heated meals (15-120 minutes) with and without extended heating in suspension, the free amino acid contents were similar (**Table 4.4**). In addition, the range of variation in free reducing monosaccharides (7.1-14.7 g/100 g dry matter) was similar in all hydrolysates (**Table 4.4**). Therefore, the extended heating in suspension did not influence the free amino acid and reducing monosaccharide contents.

Maillard reaction products and cross-linked amino acids in soy protein hydrolysates

The furosine, CML, and LAL contents of hydrolysates varied between 170-923, 49-84, and 0-12 mg/100 g dry matter, respectively (**Figure 4.1 F-H**). It can be calculated that only 23 ± 4% of CML and 10 ± 4% of LAL present in heated meals was transferred to the hydrolysates. This suggests that CML and LAL were bound to peptides and were removed during centrifugation and ultrafiltration.

The furosine, CML, and LAL contents of all hydrolysates prepared from heated meals (M_{15}, M_{60}, and M_{120}) were quite similar, but different from hydrolysates prepared from the unheated meal (M_0). The furosine, CML, and LAL contents (923, 63, and 2.1 mg/100 g dry matter, respectively) of M_0H_{aq} were higher than those of the M_0H (474, 49, and 0 mg/100 g dry matter). The high contents of furosine, CML, and LAL corresponded with low free amino acid content (11.9 g/100 g dry matter) in M_0H_{aq}.

Also after centrifugation and ultrafiltration, CML ($R = -0.9$, p- value $= 0.001$) and LAL ($R = -0.8$, p- value $= 0.01$) correlated significantly with free amino acids. Hence, LAL and CML can be used as markers for the effects of the Maillard and cross-linking reactions in hydrolysates. The correlation between furosine and free amino acid contents were not significant. Moreover, CML, LAL, and furosine contents did not correlate with that of reducing monosaccharides. This was probably because mono-/oligosaccharides were more significantly affected by fermentation than by the Maillard reaction during hydrolysate production.

4.4. Effects of hydrolysates on the cell culture functionality

For all hydrolysates, despite intensive heat treatments, the cell death was lower in hydrolysate-supplemented CD media than that in the CD medium alone (**Figure 4.4 A**). Also, the IVCD and total IgG production were higher in hydrolysate-supplemented CD media than in the CD

medium alone. The IVCD and specific IgG production varied substantially between 5-14 * 10^6 cells·day/mL and 419-1218 fg/cell·day, respectively. This corresponds to a variation in 115-316% relative IVCD and 48-140% relative specific IgG production in hydrolysate-supplemented cultures (CD medium = 100%) (**Figure 4.4 B**).

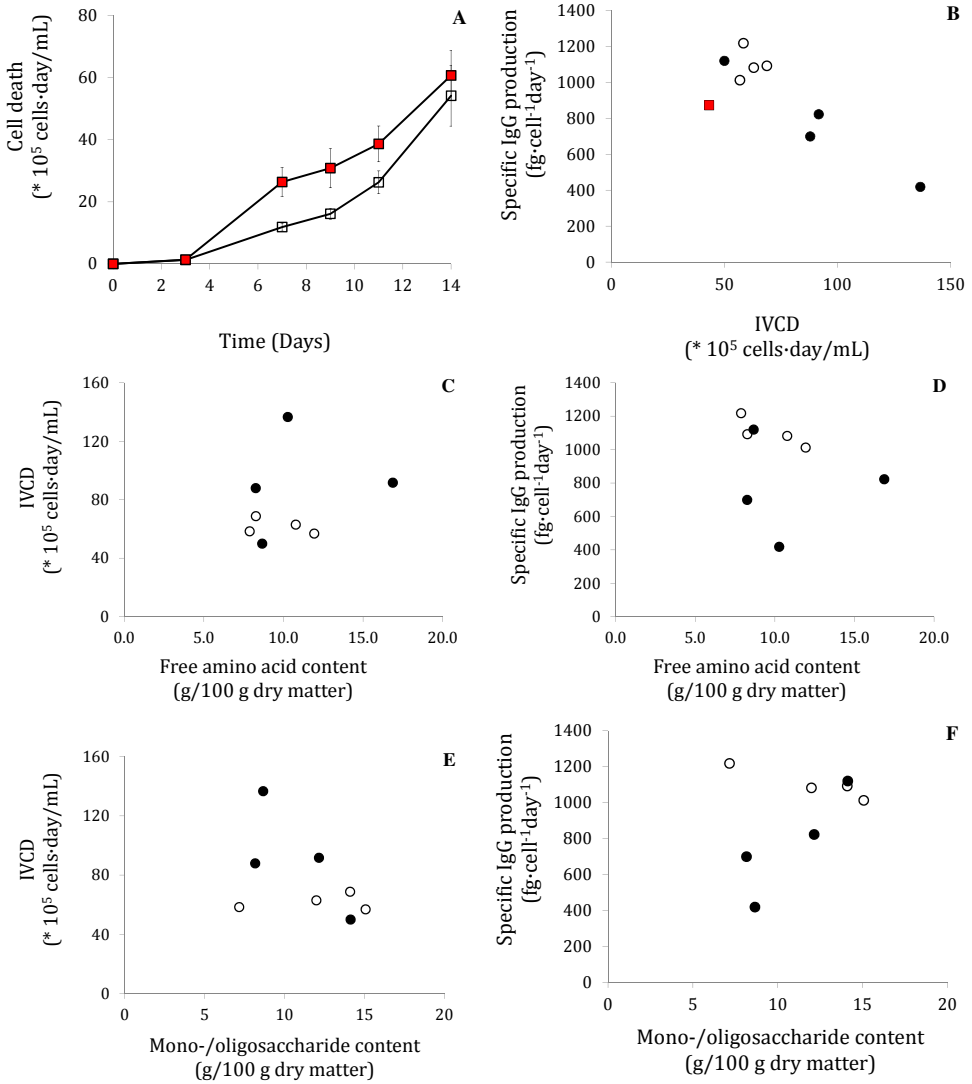

Figure 4.4: (A) Average cell death in hydrolysate-supplemented (□) and CD medium (■) culture, (B) IVCD and specific IgG production in cultures supplemented with hydrolysates prepared with (○) and without (●) extended heating in suspension, (■) CD medium, and IVCD and specific IgG production correlated with (C, D) free amino acid and (E, F) mono-/oligosaccharide content in hydrolysates

In cultures supplemented with hydrolysates prepared from heated meals without extended heating in suspension, the relative IVCD correlated negatively (R = -0.97, *p*- value = 0.02) with relative specific IgG production (**Figure 4.4 B**). This coincides with literature, in which it was shown that the viable cell density increased from 0.49 to 2.95 * 10^6 cells/mL and specific erythropoietin production decreased from 0.49 to 0.09 $\mu g \cdot /10^6$ cells·h in CHO cells cultivated between 30-37 °C [28].

The relative IVCD and relative specific IgG production did not correlate with the concentrations of free amino acid and mono-/oligosaccharide in hydrolysate-supplemented cultures (**Figure 4.4 C-F**). Since CML, LAL, and furosine were not present in the CD medium, the final concentrations in the medium after hydrolysate supplementation could be calculated. The concentrations of CML, LAL and furosine in CD media varied between 10-15 μM, 0-2 μM, and 37-108 μM, respectively. The concentrations of CML, LAL, and furosine did not correlate with functionality. In literature, the effect of the supplementation of Maillard reaction products (e.g. HMF) has been tested at 400-1000 times concentrations higher [13, 14] than that reported in the current study.

When hydrolysates prepared from heated meals were subjected to extended heating in suspension, the variation in IVCD (6-7 * 10^6 cells·day/mL) and specific IgG production (1012-1218 fg/cell·day) was substantially reduced (**Figure 4.4 B**). This variation corresponds to a range of 131-159% and 116-140% in relative IVCD and relative specific IgG production, respectively. In these cultures, CML (12-16 μM), LAL (0-2 μM), and furosine (33-181 μM) varied in the same range as cultures supplemented with hydrolysates prepared without extended heating in suspension. None of these compounds present in the hydrolysates, which were heated in the suspension, correlated significantly with the functionality. This suggests that the variation in the functionality could be due to compounds that were neither covered in the gross compositional analyses nor in the analyses of free amino acids and mono-/oligosaccharides.

Different heat treatments induced changes in the meal composition, which consequently resulted in large effects on the functionality of hydrolysates (**Figure 4.4 B**). In our experimental meal with the longest heating time (120 minutes), the LAL content was 4 times lower than the reported value for an industrial meal (150 mg/100 g protein or $\sim$ 90 mg/100 g dry matter) [24]. Based on this, it may be expected that these heat induced effects are also relevant for hydrolysates produced from industrial meals.

4.5. Conclusions

The differences in the free amino acid, CML, and LAL contents introduced in the meals due to heat treatments were partly transferred to the hydrolysates. While the extended heating in suspension did not affect the composition, the variation in the functionality of hydrolysates was substantially reduced.

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CHAPTER 5

Chemometric analysis of soy protein hydrolysates used in animal cell culture for IgG production - an untargeted metabolomics approach

Abstract

Soy protein hydrolysates are used as the most cost effective medium supplement to enhance cell growth and recombinant protein productivity in cell cultures. Such hydrolysates contain diverse classes of compounds, such as peptides, carbohydrates and phenolic compounds. To identify if specific compounds dominate the functionality of hydrolysates in cell cultures, thirty samples of hydrolysates with different cell culture performances were analyzed for chemical composition using an untargeted metabolomics approach. Out of 410 detected compounds, 157 were annotated. Most of the remaining 253 compounds were identified as peptides, but could not be annotated. All compounds were quantified relatively, based on their average signal intensities. The relative integral viable cell density (IVCD) and relative total immunoglobulin (IgG) production, relative to the CD medium (100%), ranged from 148 to 438% and 117 to 283%, respectively. Using bootstrapped stepwise regression (BSR), the compounds with the highest inclusion frequency were identified. The most important compound, i.e. phenyllactate and ferulate explained 29% and 30% of the variance for relative IVCD and total IgG production, respectively. Surprisingly, all compounds identified in the BSR showed a positive correlation with relative IVCD and relative total IgG production. This knowledge can be applied to monitor the production and accumulation of these compounds during the production process of hydrolysates. Consequently, the processing conditions can be modulated to produce soy protein hydrolysates with enhanced and consistent cell culture performance.

Based on:

Abhishek J. Gupta, Jos A. Hageman, Peter A. Wierenga, Jan-Willem Boots, and Harry Gruppen, *Process Biochemistry* (2014), **49**, 309-317.

5.1. Introduction

In mammalian cell culture processes, fetal calf serum (FCS) has been used as an essential nutrient supplement for several decades. It is composed of growth factors, transferrin, bovine serum albumin, anti-proteases, attachment factors, minerals, hormones, and several trace element that are important for promoting cell growth and enhancing recombinant protein production [1, 2]. However, in recent years, the use of FCS has become undesirable due to the risk of transmissible diseases like mad cow disease, bovine spongiform encephalopathy, and transmissible spongiform encephalopathy. As a result, substantial research is now performed to identify alternatives for FCS. In recent years, two approaches have been developed which are to culture cells either in chemically defined (CD) basal medium supplemented with low molecular weight CD supplements (e.g. CD CHO, EXCELL, and CDM4CHO) [3–6], or CD medium supplemented with plant protein hydrolysates obtained from a variety of sources like soy, rice, wheat, rapeseed, cotton, and pea [7, 8]. Out of the two, the supplementation with plant protein hydrolysates to CD medium is commonly practiced in the biopharma industry. The plant protein hydrolysates significantly enhance the viable cell density and productivity much higher than the CD medium alone [9–11], but they may suffer from variability. When mouse embryo (ME-750) cells were cultured in presence of soy and wheat protein hydrolysates, the relative viable cell density (7% and 48%) and relative immunoglobulin (IgG) production (12% and 88%) was significantly higher than the CD medium [12]. This enhanced performance is due to the complex composition of hydrolysates. They contain a wide variety of different classes of molecules (peptides, but also non-protein compounds, such as carbohydrates) [7, 13], that are beneficial to cell culture performance. In addition, the performance of hydrolysates can be affected by several compounds that are present in minor concentrations, such as phenolics, lipids, organic acids, and DNA-RNA bases. However, while beneficial, the complexity hinders the understanding and control over the functionality of hydrolysates in cell culture medium. To improve the understanding and control of the hydrolysate functionality, it is hypothesized that several key compounds in hydrolysates significantly influence the viable cell density and IgG production. To identify these compounds, two different approaches, i.e. chemometrics and fractionation, have been used. In the chemometric approach, samples of commercial hydrolysates were analyzed for chemical composition using 1D proton nuclear magnetic resonance [14, 15]. Subsequently, the chemical composition and recombinant protein production data were analyzed using principal component analysis and partial least squares regression. A positive correlation between lactate and recombinant protein production was reported. However, no other compounds present in the hydrolysates were included in the analysis. Other methods that have been used for analyzing composition of soy protein hydrolysates include near infrared spectroscopy, Raman spectroscopy, 2D fluorescence, and X-ray fluorescence [16–18]. In the fractionation approach, hydrolysates were fractionated using liquid chromatography to obtain fractions that had high cell culture performance. In a study, the peptide part of the soy protein hydrolysates was fractionated and a fraction was isolated that showed 41% higher relative viable cell density and 113% higher relative IgG

production as compared to the parental soy protein hydrolysate [12]. This indicates that in a complex hydrolysate, certain compounds are present that positively influence the cell culture performance. However, these compounds were not identified in that study. The identification of such compounds requires a comprehensive analysis of hydrolysates, which can be achieved using an untargeted metabolomics approach. An untargeted metabolomics approach is useful for comparative screening of complex samples in which analysis is aimed at measuring as many compounds as possible in a sample. In untargeted metabolomics, samples are analyzed using liquid chromatography coupled to mass spectrometry. All the compounds detected in a sample are semi-quantified using mass spectrometry (MS). Some of the compounds can then be annotated based on database searches using MS/MS fragmentation patterns. In this way, information becomes available on as many compounds as possible in a given sample [19]. In the present study, soy protein hydrolysates were chosen that showed differences in the cell culture performance. These hydrolysates were analyzed for chemical composition using an untargeted metabolomic approach. In addition, the hydrolysates were tested in cell culture assays to determine viable cell density and IgG production. Subsequently, the compositional and functionality data were combined and chemometric methods were used to identify the key compounds that significantly explained the viable cell density and IgG production. Pure preparations of key compounds were tested in cell culture experiments to validate the statistical findings.

5.2. Materials and methods

5.2.1. Materials and chemicals

Thirty samples of an ultrafiltered soy protein hydrolysate that had different cell culture performances were provided by FrieslandCampina Domo (Amersfoort, The Netherlands). The samples belonged to different batches of one product. Thus, samples were prepared using a common process, raw materials, and enzymes. However, different batches of raw materials and enzymes were used to produce them. Iscove's modified Dulbecco's medium (IMDM) (31980-022), fetal calf serum (10270-106), D-phosphate buffered saline (D-PBS) (14190-094), trypsin 0.5% (10X) with EDTA-4Na solution (15400-054), penicillin-streptomycin solution (15140-122), HT supplement (11067-030) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (002024) were obtained from Invitrogen (Breda, The Netherlands). Tween-20 solution was purchased from VWR international (Amsterdam, The Netherlands). Pluronic F-68 (P5556), syringic acid (S6881), sodium L-lactate (L7022), methyl L-3 phenyllactate (68193), trigonelline hydrochloride (T5509), N-acetyl methionine (01310), D (±) chiro-inositol (468045), mucic acid (galactaric acid) (M89617), adenine (A2786), and L-glutamine (G7513) were obtained from Sigma-Aldrich (Zwijndrecht,The Netherlands). Methyl ferulate (02828M) was purchased from Apin chemicals (Oxfordshire, UK). ISCHO-CD (91119) was purchased from Orange Medical B.V (Tilburg, The Netherlands). Affinipure mouse anti-human IgG F (ab') 2 fragment specific (209-005-097), Affinipure goat anti-human IgG, Fc

(gamma) fragment specific (109-005-098), and human IgG (02-7102) were purchased from Sanbio B.V. (Uden, The Netherlands). For growth assays, the adherent medium was prepared by supplementing IMDM with 10% (v/v) FCS and 1% (v/v) penicillin-streptomycin. The suspension medium was prepared by supplementing ISCHO-CD with 1% (v/v) pluronic F-68, 1% (v/v) HT supplement, 4% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin. For ELISA experiments, the coating buffer was prepared by dissolving 5 mg/mL of capture antibody in D-PBS. Wash buffer was prepared with 0.05% (v/v) Tween-20 solution in a mixture of D-PBS and Millipore water (1:1). Blocking and dilution buffer were prepared using 2% (w/v) and 0.33% (w/v) EM-7 sodium caseinate (FrieslandCampina DMV, Veghel, The Netherlands) in wash buffer. Detection buffer was prepared with 1:6500 (v/v) detection antibody in dilution buffer.

5.2.2. Cell culture

Cell line adaptation

Chinese hamster ovary (CHO) cells are the most commonly used cell lines within biopharmaceutical industry. In this industry, it has been primarily cultivated under suspension culture conditions. Therefore, to mimic this industrial setup, it was considered to use CHO cells in suspension. The cell line adaptation and growth assay was performed using methods adapted from Gupta et al. (2011) [20]. CHO cells (CRL-11397) were obtained from an in-house cell bank stored under liquid nitrogen. CHO cells were cultured in 75 cm^2 T-flasks containing adherent medium and incubated at 37 °C and 5% (v/v) CO_2. When the cells reached approximately 80% confluence, they were washed with D-PBS and detached from the flasks using 1 mL trypsin-EDTA. The cell suspension was centrifuged at 1200 rpm for 3 minutes at 20 °C (centrifuge 5810R V2.1, VWR International, Amsterdam, The Netherlands) to remove the adherent medium, trypsin-EDTA and dead cells. During this step, all the FCS was removed from the culturing medium and the pellet was resuspended in FCS-free suspension medium and cells were counted using Cedex HiRes (Innovatis, Bielefeld, Germany). 20 * 10^6 viable cells were transferred to 125 mL baffled flasks and the final volume was adjusted to 40 mL using suspension medium. The flasks were placed on an orbital shaker with an agitation rate of 125 rpm maintained in an incubator at 37 °C and 5% (v/v) CO_2 for 4 days before starting the growth assay. Thus, the cells were adapted for one passage in suspension before starting the growth assay. The direct passage from adherent culture to suspension culture can make the cells more sensitive to test conditions. Therefore, several experimental controls were used after adaptation and the growth assay to check the sensitivity and physical state of the cells. Based on these controls, the experiment was either continued or discarded.

Growth assay

After the adaptation period, the physical condition of cells was evaluated using experimental controls like cell viability and number of viable cells. If the viability of cells was > 85-95% and cells had doubled approximately 3.5-4 times during the adaptation, the growth assay could

be started otherwise, the cells were considered unhealthy and discarded. After adaptation, the suspension culture was centrifuged at 1200 rpm at 20 °C for 3 minutes. The pellet was resuspended in the suspension medium. 5 * 10^6 viable cells were transferred to 125 mL baffled flask containing suspension medium. The seeding density in each baffled flask was 2 * 10^5 cells/mL. The soy hydrolysates were suspended in suspension medium, filtered through 0.22 μm filters and added to a final concentration of 0.4% (w/v) to the baffled flasks. The total volume in each baffled flask was 25 mL. Suspension medium was the negative control and suspension medium with 10% (v/v) FCS was the positive control. All samples and controls were tested in triplicates. The flasks were placed on an orbital shaker with an agitation rate of 125 rpm maintained in an incubator at 37 °C and 5% (v/v) CO_2. The growth assay was run for 14 days. At each time point, i.e. on days 3, 7, 9, 11, and 14, 150 μL (in duplicate) of cell suspension was drawn per flask to count the cells. Therefore, six measurements of cell count were available per sample and per time point. This was done to verify the reproducibility of the experiment. For each sample, the cell counts of the above-mentioned time points were converted to integral viable cell density (IVCD, * 10^5 cells·day/mL) using linear interpolation. On day 11, 150 μL of cell suspension per flask was transferred to a 96-well V-bottom plate. The plate was centrifuged at 1200 rpm for 3 min. The supernatant was harvested in a 96-well flat bottom plate and frozen for IgG production measurement.

5.2.3. Immunoglobulin production measurement

IgG production was measured using an enzyme-linked immunosorbent assay (ELISA). 96 well plates were coated with 100 μL of coating buffer and incubated overnight at 4 °C. The supernatants drawn during the growth assay were thawn and diluted using dilution buffer. The plates were washed 3 times with 200 μL wash buffer and 200 μL well blocking buffer was added to the plate. The plates were incubated for 50 minutes at 25 °C. The plates were washed 3 times with 200 μL wash buffer. The calibration curve (in duplicate) in the concentration range from 0.98 to 500 ng/mL was prepared by diluting human IgG in dilution buffer. For this, two rows (12-wells each) in the ELISA plate were used. In each row, 10 wells were filled with 100 μL of IgG standards (0.98-500 ng/mL) and 2 wells were used for sample blanks. The remaining 78 wells in the plate were filled with 100 μL diluted supernatants. The supernatants were tested in triplicates at each of the four dilutions (250X, 500X, 1000X, and 2000X). Thus, one sample was analyzed twelve times in order to check the reproducibility of the method. The plates were covered with aluminium foil and incubated for 50 minutes at 37 °C at 350 rpm. The plates were washed with 200 μL wash buffer and 100 μL well detection buffer was added. The plates were covered with aluminium foil and incubated again for 50 minutes at 37 °C on the shaker. The plates were washed 3 times with 200 μL wash buffer. 100 μL/well of ABTS was added and the color development was observed visually. After 10 minutes, 100 μL of 0.1 M citric acid was added to stop the reaction and extinction was read at 410 nm. The IgG production is reported as total IgG production (ng/mL) and specific IgG production(total IgG production/IVCD, fg·cells^{-1}·day^{-1}).

5.2.4. Compositional analysis of hydrolysates

The compositional analysis was performed by Metabolon Inc. (North Carolina, U.S) using an untargeted metabolomics approach [21, 22]. The analysis was carried out using ultra high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS^2) and gas chromatographic/mass spectrometric (GC/MS)methods. The details of sample preparation, analytical methods used, and data analysis is described in detail by Evans et al. [21] and Sha et al. [22]. All hydrolysates were analyzed in triplicates. The compounds detected in samples during the analysis run were identified by matching retention time, molecular mass (m/z), and MS/MS spectra against in-house reference library entries created from authentic standard compounds. In the MS chromatogram of a hydrolysate, each peak represented a compound and the peak area corresponded to its relative concentration in the hydrolysate. The validation of data obtained from the analysis is explained in the literature [21–23]. The variation in the analytical process was monitored using quality control standards. The quality control standards were prepared by mixing an aliquot of 30 soy hydrolysate samples. These standards were injected after every five samples during the whole run. The relative standard deviation in the quantification of these standards was 9%.

5.2.5. Statistical analysis

Data pretreatment

Out of all compounds in the metabolomics data, 97 compounds were detected in some, but not all, of the hydrolysates and were excluded from the dataset. The remaining 313 compounds were included in the final data analysis. The semi-quantitative data of the concentrations of compounds was based on the MS-intensities. For these compounds, average values were calculated from triplicate measurements. This semi-quantitative data was natural log transformed to correct for the fact that the data was not normally distributed. The normality of the dataset was assessed by the linearity of the normal probability plots. The cell culture performance of hydrolysates was analyzed in five separate cell culture batch experiments. Therefore, the response variables were corrected for the variations that may result from these batch-wise experiments. For every batch of experiments, the means of IgG or IVCD values were calculated and subtracted from the individual IgG or IVCD values. Finally, the natural log transformed biochemical dataset and the corrected response variables were integrated in a database, which was analyzed further using in-house developed Matlab procedures (Matlab® R2009b version 7.9.0.529).

Two-mode cluster analysis

Two-mode cluster analysis is a descriptive statistical technique. The Matlab procedure for two-mode cluster analysis was based on the principle of two-mode k-means clustering. This procedure is described in detail elsewhere [24, 25]. It clustered simultaneously compounds, hydrolysates, and response variables. It showed which group of compounds behaved identically for which hydrolysates and determined the compounds that were related to the response

variables [24, 25]. In two-mode clustering, the number of compound and sample clusters is important. This optimal cluster combination is determined using a 2D scree chart. In this chart, sum of squared distances between data points for every cluster combination is plotted against the number of sample and compound clusters. The combination of clusters for which the decrease in sum of squared distances ('within-cluster distance') was not steep anymore was considered optimal.

Bootstrapped stepwise regression

The Matlab script for bootstrapped stepwise regression was based on the principle of bootstrapping and stepwise regression. Stepwise regression yields a regression model with a short list of compounds that explain IVCD or total IgG production in the best possible way. In the first step of stepwise regression, the compound that explained as high a variance as possible is added to the regression model. In each succeeding step, a compound that had the highest added variance in the orthogonal direction is added to the model. This was carried out for four steps so that only the most important compounds were added in the model. However, due to large number of parameters in the dataset, stepwise regression can suffer from over-parameterization. This was overcome using a bootstrap method in which only a small subset of parameters was included in each step of an iterative process to reduce the risk of over-parameterization. In a metabolomics dataset, it was expected that a set of compounds can have almost identical explanatory properties for either IVCD or total IgG production. Bootstrapped stepwise regression (BSR) allowed identification of compounds that had nearly identical explanatory properties for a response variable. Bootstrapping is a resampling technique that randomly draws with replacement from the original data. During resampling, some samples and compounds were chosen while other samples and compounds were left out. Afterwards, ordinary stepwise linear regression was applied to this resampled subset and this allowed other compounds to be chosen in the stepwise model. By repeating this many times ($\sim$ 10,000X), a list of compounds that had similar explanatory properties for a response variable was generated. For all compounds, the frequencies that a compound had been selected in a stepwise model were recorded. The higher the frequency, the more often a compound had been chosen and the more important a compound was. This was ideal for selecting a 'marker' compound. If a compound from the overall stepwise model could not be measured, one of the alternatives from the BSR could be measured (the term 'overall' indicates that no bootstrapping/resampling have been applied and the complete dataset is used). In BSR, over-fitting was avoided in two ways. Firstly using a stepwise linear model and secondly, by selecting only a few most important compounds.

2D correlation maps

The Matlab script was composed to calculate correlation coefficients between response variables and compounds identified in the BSR model. Only significant correlations (p- value < 0.05) were selected to make the correlation plots. The plotting was done using classical multidimensional scaling (CMDS). In CMDS, the higher dimensional distances between the

compounds were retained as best as possible and plotted in a 2D space. In this space, the compounds that had correlation coefficients ≥ threshold correlation coefficient (-1 to +1) were connected in a network.

5.2.6. Validation assay

The key compounds for IVCD and IgG production that were identified in the statistical analysis were tested in a separate cell culture experiment to determine their effects in cell culture. The key compounds analyzed were phenyllactate, lactate, trigonelline, chiro-inositol, N-acetyl methionine, methyl ferulate, syringic acid, mucic acid (galactaric acid), and adenine. The quantification of these compounds in hydrolysates was limited due to interference caused by the complex matrix of hydrolysates. However, several patents on cell culture medium formulations for mammalian cells already describe the use of some key compounds like inositol, adenine (nucleotides), and nicotinamide (trigonelline-related compounds) in the range 0.0005-0.002% [26], 0.000001-0.0075% [27], and 0.0001-0.06% [28], respectively. Additionally, use of lactate (0.001%) in Dulbeccos modified Eagle medium for cell culture is described by Lampe et al. [29]. Based on these patents, it was hypothesized that chemically defined medium used in this study might already contain some key compounds identified in this study. Additionally, based on the positive correlations found between key compounds and the functionality, a concentration of 0.01%, which was at the higher end of the ranges suggested in the patents, was chosen for the validation. Nevertheless, it is important to note that too high concentration of key compounds in cell culture assay can potentially cause cytotoxicity and result in reduced IVCD and IgG production. A modified medium was prepared by adding a test compound to the suspension medium at 0.01% (w/v) concentration. The suspension medium as such was used as a control to evaluate the performance of the modified medium. Adapted and viable CHO cells were transferred to all the flasks containing modified medium and control suspension medium and growth assay was performed. Viable cell counts were measured and the IVCD were calculated. Total IgG production was measured and specific IgG production was calculated as the ratio of Total IgG production and IVCD.

5.3. Results and discussion

5.3.1. Cell culture performance of soy protein hydrolysates

As expected, the selected hydrolysates showed marked differences in the IVCD, specific IgG production, and total IgG production (**Figure 5.1**).

The corrected IVCD and total IgG production ranged from 40 to 118 * 10^5 cells·day/mL and 1900 to 4600 ng/mL, respectively. This corresponds to a range of 148-438% for relative IVCD and 117-283% for relative total IgG production as compared to the CD medium (100%). The values obtained for some hydrolysates were even higher than those obtained for the positive control (ISCHO-CD medium + 10% (v/v) FCS; 158.3 * 10^5 cells·day/mL and 3318 ng/mL). This shows that if the quality can be controlled, soy protein hydrolysates can be used

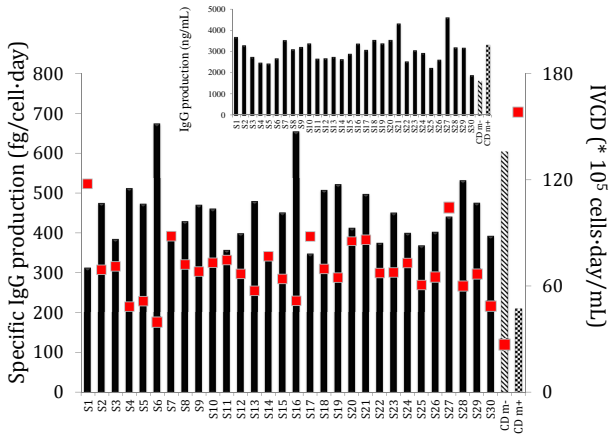

Figure 5.1: Effect of 30 extremely variable soy hydrolysates on IVCD (■), specific IgG production (■), and total IgG production in CHO cell culture, CDm- is non-supplemented ISCHO-CD medium, and CDm+ is ISCHO-CD medium supplemented with fetal calf serum

to successfully replace FCS from CD medium, while maintaining the total IgG production. The specific IgG production for the soy protein hydrolysates ranged from 313 to 673 fg·cells^{-1}·day^{-1}. Although variation in the functionality of hydrolysates has been briefly described [16, 30], to our knowledge, this is the first time the data are reported.

5.3.2. Chemical composition of hydrolysates

The protein content of all hydrolysates varied from 55.6 to 58.1% (56.8 ± 0.8%). 410 compounds were detected and semi-quantified in soy hydrolysates. Out of these, 157 compounds were annotated and the remaining 253 were labeled as X-compounds. An important reason for high number of X-compounds was because, the library used to identify the compounds was not specifically designed for the analysis of protein hydrolysates. Thus, these X-compounds were analyzed further using their MS-fragmentation patterns. Subsequently, they were identified to be peptides; however, an exact annotation was not fully achieved. All the compounds measured could be categorized in five major classes, i.e. X-compounds, amino acids and peptides, carbohydrates, lipids, and DNA-RNA bases (in decreasing order of number of compounds present in a class).

5.3.3. Two-mode cluster analysis

Two-mode cluster analysis was used to cluster compounds, hydrolysates, and response variables. The optimal cluster combination needed to perform the analysis was determined using a 2D scree plot. The optimal point was identified at 6 hydrolysates and 8 compounds clusters. The two-mode cluster plot gave a descriptive overview of the compositional differences

between the hydrolysates grouped in different clusters (**Figure 5.2**).

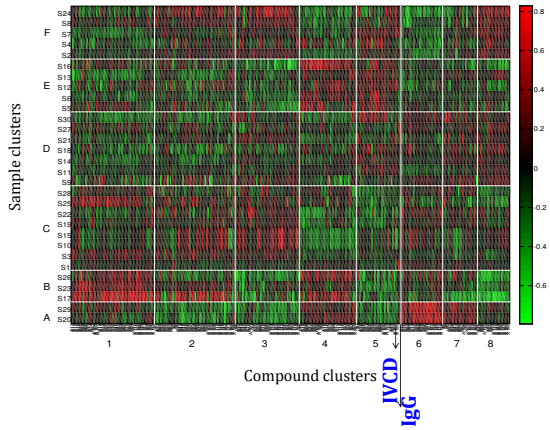

Figure 5.2: Two-mode cluster analysis of 30 soy protein hydrolysates. Each line on the x-axis represents a compound. Red color indicates compound concentration above average, black color indicates an average concentration, and the green color indicates compound concentration below average. Response variables are labeled in blue and indicated with a black arrow

Within a compound cluster, the compounds were positively correlated to each other. The response variables (IVCD and total IgG production) were clustered in compound cluster 5. This suggested that cluster 5 compounds (adenine, syringic acid, phenyllactate, lactate, homoserine, chiro-inositol, and X-191) were positively correlated to the response variables. These compounds are identified in BSR and correlation analysis as well. For instance, cluster D was rich in cluster 5 compounds and the response variables and conversely, cluster B was poor in both cluster 5 compounds and the response variables. In order to confirm these findings and to calculate the explained variance of the compounds, the dataset was analyzed using BSR analysis.

5.3.4. Bootstrapped stepwise regression

The key compounds that significantly (p- value < 0.05) explained the variability in the response variables are shown in **Figures 5.3** and **5.4**. The key compounds identified in BSR were largely present in the compound cluster 5 identified in two-mode cluster analysis. Although few of these compounds have been previously identified to influence IVCD or IgG production, most of them have not been mentioned before. This indicates that the key compounds identified are relevant for CHO cells (CRL-11397) and hydrolysates tested in this study. However, with different cell cultures and type of hydrolysates, different key compounds might be identified.

Cell growth

Phenyllactate, lactate, trigonelline, chiro-inositol, and X-190 had the highest explained

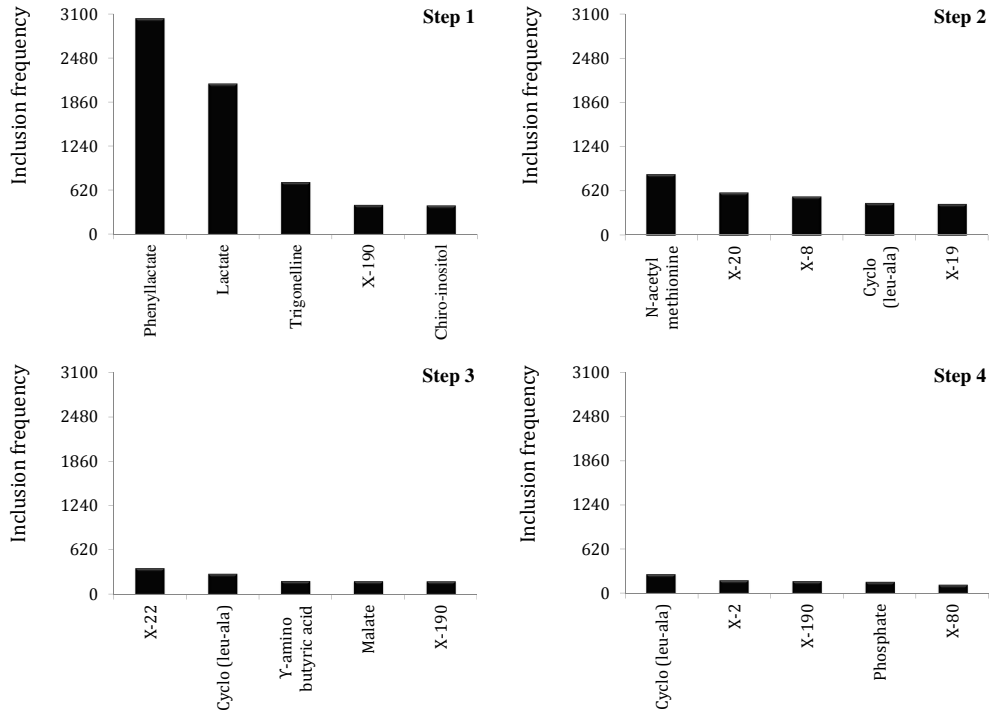

Figure 5.3: Inclusion frequency of five compounds appearing at the first, second, third, or fourth step in the bootstrapped stepwise regression for IVCD

variance for IVCD (**Table 5.1**).

Table 5.1: Explanatory power (variance) of the key compounds at the first step in the bootstrapped stepwise regression for IVCD

Compound	% Explained variance* (R^2)	Two-mode compound cluster
Phenyllactate	29	5
Lactate	28	5
Trigonelline	20	7
Chiro-inositol	20	4
X-190	10	2

* % explained variance is the average calculated from the repeated stepwise linear regressions in BSR

These compounds explained 10-29% of the variance in the IVCD. In steps 2, 3, and 4, N-acetyl methionine, cyclo (Leu-Ala), γ-amino butyric acid, malate, phosphate, and several unknowns were identified (**Figure 5.3**).

These compounds had much lower inclusion frequencies than the compounds identified at step 1. Moreover, in step 1, phenyllactate and lactate had much higher inclusion frequencies as

compared to trigonelline, chiro-inositol, and X-190. This suggested that these two compounds had a profound effect on the IVCD as compared to all the other compounds. Many compounds like phenyllactate, γ-amino butyric acid, malate, and phosphate have not been reported before to enhance IVCD in CHO-2 cells. On the other hand, compounds like lactate and peptides compounds have been extensively studied in CHO cell culture [12, 15, 31, 32]. With regard to lactate, contradictory results have been reported in literature. Lactate has been regarded as an inhibitor of cell growth and productivity [33, 34]. Conversely, a positive effect of lactate on viable cell density and productivity in cell culture is also reported ([14, 15, 31], this study). These differences can be due to the use of different cell lines in these studies. Besides lactate, several peptides were identified to affect IVCD. This included several X-compounds, N-acetyl methionine, and cyclo (Leu-Ala). N-acetyl methionine (methionine derivative) is an essential amino acid that supports cell growth [35, 36]. Short chain peptides have been identified to exert both an aspecific nutritional and a specific anti-apoptotic growth promoting effect in cell culture [12, 32]. Nutritionally, short chain peptides can be hydrolyzed easily by cell derived peptidases and the resulting amino acids are transported into the cells where they are consumed for energy generation and protein synthesis. Few studies have been also performed to evaluate the effects of trigonelline and inositol [37–39]. Trigonelline is closely associated to niacin, which is a water soluble vitamin. Surprisingly, several other vitamins and lipids like nicotinate, caproate, isovalerate, glycerophosphorylcholine, and choline that were analyzed showed only a very weak or no significant correlation with IVCD and IgG production. In addition, trace mineral analysis was carried out for 10 lots. This data was combined with the metabolomics data and analyzed using BSR. Surprisingly, none of the trace minerals (e.g. cadmium, copper, and chromium) were identified as key compounds in BSR analysis.

Total IgG production

Ferulate, syringic acid, galactarate, adenine, and X-198 had the highest explained variance for total IgG production (**Table 5.2**). These compounds explained 21-30% of the variance in the total IgG production. In steps 2, 3, and 4, trigonelline and unknowns were identified to significantly influence the total IgG production (**Figure 5.4**).

Table 5.2: Explanatory power (variance) of the key compounds at the first step in the bootstrapped stepwise regression for total IgG production

Compound	% Explained variance* (R^2)	Two-mode compound cluster
Ferulate	30	3
Syringic acid	26	5
Galactarate	22	3
Adenine	22	7
X-198	21	2

* % explained variance is the average calculated from the repeated stepwise linear regressions in BSR

Unlike cell growth, the total IgG production was influenced by phenolics (ferulate and syringic acid), nucleotide (adenine), oxidized sugar (galactarate), and several peptides. Amongst

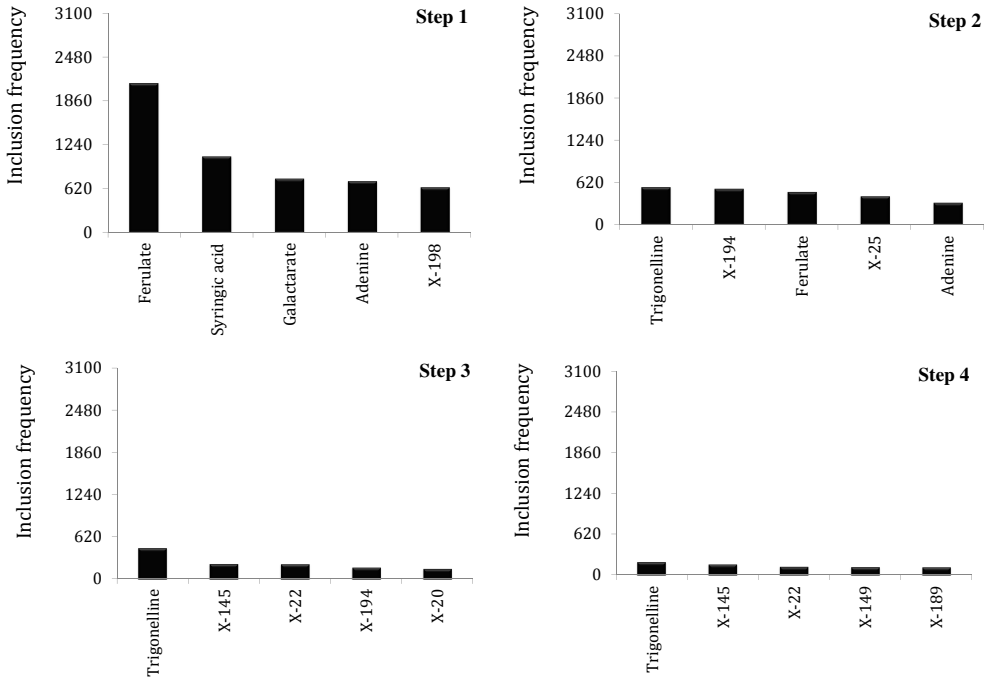

Figure 5.4: Inclusion frequency of five compounds appearing at the first, second, third, or fourth step in the bootstrapped stepwise regression for IgG production

these key compounds, the effects of ferulate, syringic acid, galactarate, and trigonelline on total IgG production enhancement have not been reported before. Adenine has been reported to arrest cell growth and enhance the productivity of cells [40, 41]. Regarding peptides, it has been observed that some peptides enhanced the recombinant protein yield and suppressed IVCD, while others promoted both recombinant protein yield and cell growth [32]. This was also observed in the present study, where X-194, X-198, X-25, X-145, X-149, and X-189 enhanced only total IgG production while X-22 and X-20 promoted both IVCD and IgG production. In BSR, most of these peptides had similar inclusion frequencies, which suggests that the role of one peptide could be fulfilled by another peptide. This was also observed in the principal component analysis on proteinaceous compounds (mostly not annotated) which showed that most peptides had similar effects on the functionality (data not shown). Furthermore, the protein content of all hydrolysates varied in a narrow range, i.e. 55.6-58.1% and 1.9-2.3%, respectively. This suggested that the variation in the peptide composition was not significant enough for them to be identified as sources of variation in functionality between hydrolysates. As a result, very few peptides were identified to be key compounds in BSR.

5.3.5. Correlation analysis of the key compounds and response variables

Although the key compounds that significantly influenced the response variables were identified using BSR, the nature of influence (positive or negative) was not revealed between the compounds and the response variables. This was investigated using 2D correlation maps. An overview of the relationship between the key compounds and the response variables was generated using 2D correlation maps. The correlation maps for IVCD and total IgG production are shown in **Figure 5.5**. In correlation maps, not only compound-response variable correlations, but also compound-compound correlations are observed. The latter information gives an insight into the complex interactions of the compounds present in hydrolysates. Thickness of the lines in the map indicates the strength of correlations. In **Figure 5.5**, all the correlations are positive as both maps are drawn with blue lines, else red lines would have been observed.

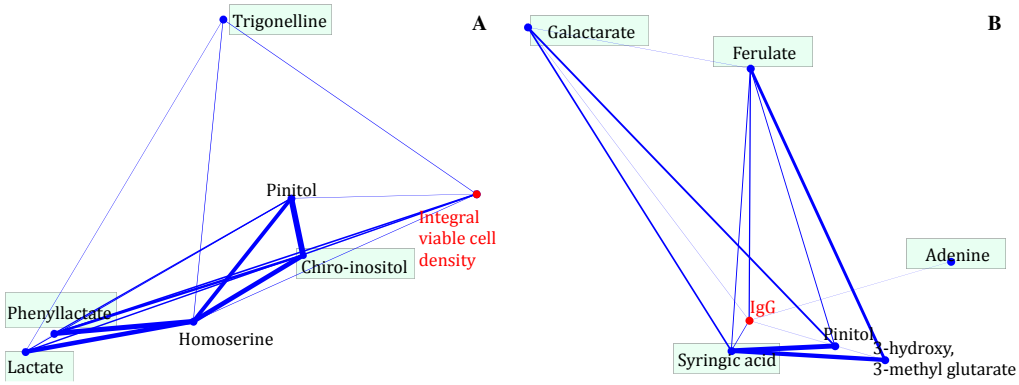

Figure 5.5: 2D correlation map of compounds and (A) IVCD and (B) IgG production. Compounds in the green boxes are identified in bootstrapped stepwise regression and two-mode cluster analysis. Thickness of the lines indicates the strength of the correlation between the threshold of 0.4 ± 1.0. Positive and negative correlations are indicated by blue and red lines, respectively

For instance, pinitol is weakly correlated to IVCD, but very strongly correlated to chiro-inositol (**Figure 5.5 A**). Likewise, syringic acid is weakly correlated to total IgG but strongly correlated to pinitol (**Figure 5.5 B**). In the correlation maps of IVCD the compounds plotted were phenyllactate, lactate, chiro-inositol and trigonelline. These compounds also appeared in the BSR analysis and in the cluster 5, which was identified in two-mode clustering. Besides these compounds, homoserine and pinitol had high positive correlations with IVCD. Although, these compounds appeared in step 1 in BSR analysis, their inclusion frequencies were lower than that of chiro-inositol (data not shown). In the correlation maps for total IgG production the compounds plotted were ferulate, syringic acid, adenine, and galactarate. These compounds were also identified in BSR and two-mode cluster analysis, showing that these compounds were indeed important.

5.3.6. Experimental validation

Pure preparations of key compounds of IVCD (KC) (phenyllactate, lactate, trigonelline, chiro-inositol, and N-acetyl methionine) and total IgG production (KI) (ferulate, syringic acid, galactarate, adenine, and trigonelline) were tested in validation experiments (**Figure 5.6**).

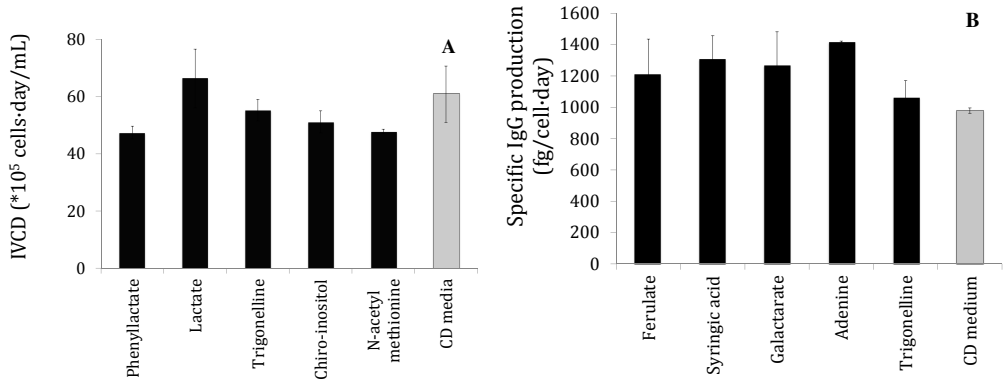

Figure 5.6: Experimental validation of key compounds at 0.01% (w/v) concentration in the suspension medium in CHO cell culture. (A) IVCD and (B) Specific IgG production ($fg{\cdot}cells^{-1}{\cdot}day^{-1}$). All values are significantly different (p- value < 0.05) than the CD medium

The relative IVCD with KC compounds ranged between 78 and 109% as compared to the control medium (100%). Except for lactate, the addition of KC compounds to the control medium did not enhance IVCD. This might be due to high test concentrations (0.01%) of the key compounds in cell culture assay. In addition, this also suggests the possible presence of key compounds in the chemically defined medium used for cell culture assay. It must be noted that the concentrations of the key compounds tested in the assay were not optimized. At the same time, it was confirmed that all the KI compounds enhanced total and specific IgG production as compared to the control medium. The total and specific IgG production by KI compounds ranged from 108 to 149% and 108 to 144%, respectively, as compared to the control (100%), showing the effect of the presence of these compounds on the IgG production. This indicates that metabolomics in combination with a chemometric approach can indeed help to identify the key compounds, which will help to control and enhance the functionality of hydrolysates.

5.4. Conclusions

Soy protein hydrolysates contain not only proteinaceous, but also non-proteinaceous compounds. In the current setup of CHO cells (CRL-11397) and soy protein hydrolysates, two groups of five unique non-proteinaceous compounds were found to explain the most variation in IVCD and total IgG production. While the approach can be applied to different cell lines and hydrolysates, the key compounds identified in such experiments are expected to be different from the ones identified in this study. Firstly, this knowledge can be used to

control the concentrations of key compounds in raw materials. Secondly, during the hydrolysate production, the compounds can be monitored and controlled by adjusting processing conditions (e.g. heat treatment) to produce soy protein hydrolysates with enhanced and more consistent cell culture performance.

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CHAPTER 6

General Discussion

The first aim of this thesis was to identify the key parameters (compound classes, sub compound classes, and individual compounds) in the composition of soy protein hydrolysates that determine their functionality (viable cell density, total immunoglobulin (IgG), and specific IgG production) as supplements in cell culture experiments (**Figure 6.1**). The key parameters are those compounds that correlate significantly (p- value < 0.05) with the functionality. The second aim was to identify whether these variations in the hydrolysate composition are introduced by variations in the processing treatments or by variations in the composition of starting material.

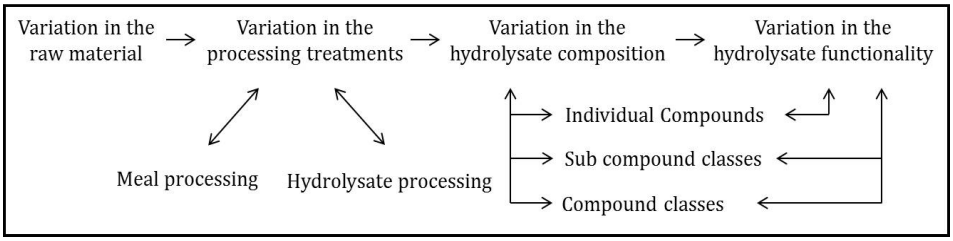

Figure 6.1: Schematic overview of factors affecting the composition of hydrolysates and the relation between the composition and the functionality of hydrolysates used in cell cultures

Rather than studying the effect of supplementation of single compounds in cell culture, in this study, three different sets of soy protein hydrolysates were analyzed with respect to their composition as well as their functionality. These were (1) hydrolysates prepared from different starting materials (meal, 2 concentrates, and one isolate) derived from an industrial soybean meal, (2) 30 batches of an industrial hydrolysate produced from the same soybean meal mentioned in (1), and (3) hydrolysates prepared from an experimental soybean meal that was heated for different time periods. One part of each hydrolysate from (3) was subjected to an extended heating in suspension during hydrolysate production.

To describe the functionality of supplements in cell culture experiments, typically cell growth (also expressed as integral viable cell density, IVCD), total antibody (in this thesis IgG) production, and specific antibody production (total antibody production per cell) are used as result parameters. Total antibody production is expected to be maximal when both IVCD (cells·day/mL) and specific IgG production (SIP, $g \cdot cell^{-1} \cdot day^{-1}$) are maximal. To compare results from different experiments, the IVCD and specific IgG production data of hydrolysate-supplemented cultures were normalized by dividing them with the data of the non-supplemented CD medium of each set of experiments. This results in relative (r)IVCD (%) and relative (r)SIP (%) values that were plotted for the different experiments (**Figure 6.2**).

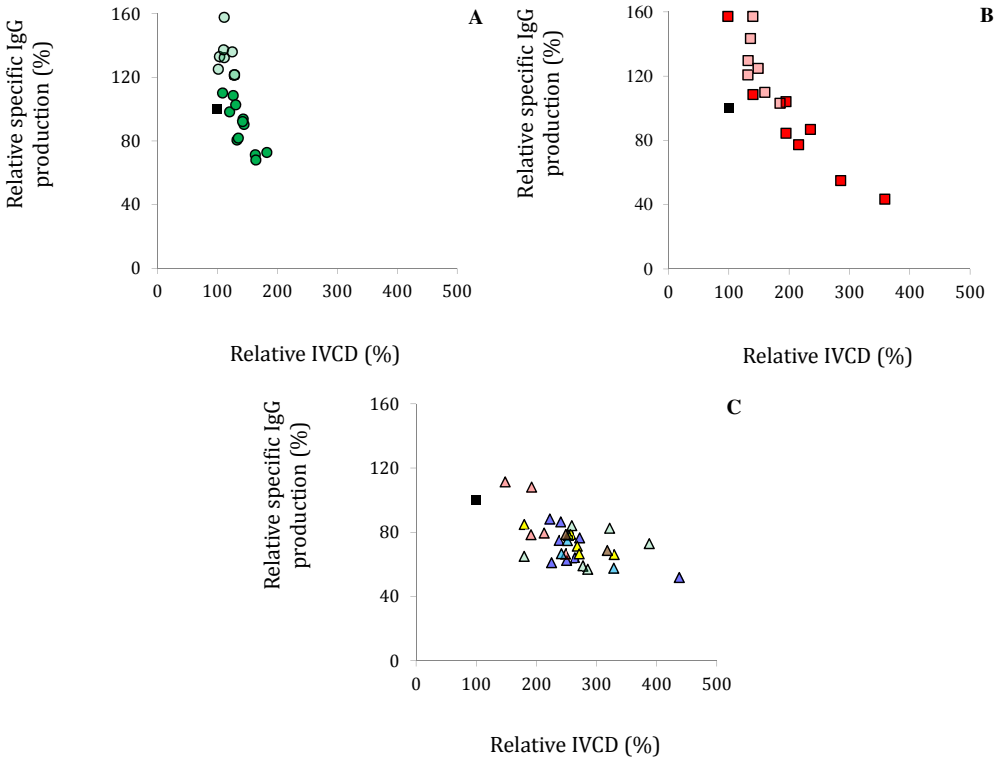

Figure 6.2: Correlation between rIVCD and rSIP in cultures supplemented with (A) hydrolysates (●) and extract/hydrolysate mixtures (●) produced from different starting materials **(chapter 3)**, (B) hydrolysates produced following different processing treatments (with (●) and without (●) extended heating in suspension) **(chapter 4)**, (C) different batches of a hydrolysate produced in industrial processes, different color of markers (△, △, △, △, △, ▲) indicate different hydrolysate clusters **(chapter 5)**, and (■) in non-supplemented CD medium culture (100%)

For each set of hydrolysates, there is an inverse relation between rIVCD and rSIP. That an increase in cell growth is accompanied by a consequent decrease in specific antibody production

has been shown before in literature [1–5]. To describe this relation between cell growth rate and specific antibody production, a model has been proposed [6] (**Equation 1**).

$$\mathbf{q} = \frac{C}{\mu + k} \quad \textbf{(Equation 1)}$$

In equation 1, q is the antibody production in molecules per cell per day, and C and k are the fitting parameters. C = α * β * E * D, in which α (day^{-1}) is the coefficient of mRNA synthesis as the first order of the number of the corresponding gene, β (day^{-1}) is the coefficient of mRNA translation rate assumed as the first order of the number of the mRNA molecule, E (dimensionless) is the efficiency for splicing, and D ($cell^{-1}$) is the copy number of the gene in a cell. The μ (day^{-1}) is the specific growth rate of mammalian cells and k (day^{-1}) is the coefficient of the mRNA decomposition rate assumed as first order of the number of mRNA molecules [6]. In short, in this model, a high value of C indicates faster rates of mRNA synthesis and translation for the production of antibody. The value of C and k represents the maximum achievable total IgG production for that system. Although in several studies the enhancement of the specific antibody production has been achieved by reducing the cell growth rate, e.g. by decreasing the temperature [5] or supplementation with sodium butyrate [2], it seems that the model has been used in only three studies [6–8].

In this thesis, the specific growth rate was not determined, but the rIVCD (%) can be taken as an approximation for specific growth rate (μ). In this way, the interpretation of parameters changes, but still parameters C and k can be determined by fitting equation 1 to the experimental data from different experiments (**Figure 6.2**).

For the hydrolysates that were produced on laboratory scale, a quite good fit ($R^2 = 0.67$ and 0.85) is obtained (**Figure 6.3**). For the industrial hydrolysates the data is more scattered ($R^2 = 0.37$), which suggests a higher extent of variation in the industrial processes, but still the trend seems to agree with the model (**Figure 6.3**, **Table 6.1**).

Table 6.1: Model parameters and efficiency of IgG production for the functionality data in hydrolysates produced with different starting materials (**chapter 3**), hydrolysates produced following different processing treatments (**chapter 4**), and different batches of a hydrolysate produced in industrial processes (**chapter 5**)

	Overall	Starting materials	Processing treatments	Industrial processes	Average	Standard error (%)
R^2	0.73	0.67	0.85	0.37	0.63	38
C	231	137	203	355	231	48
k	68	0	20	226	79	153
Efficiency of IgG production*	74	99	88	53	80	30
St.dev. of efficiency**	16	14	11	10	24	-

* % of maximum IgG production over all samples;
** Calculated over all samples

The derived model parameters: basic rate of IgG production (C) and k (mRNA decomposition rate) are quite different between the experiments. The values are highest for the cultures

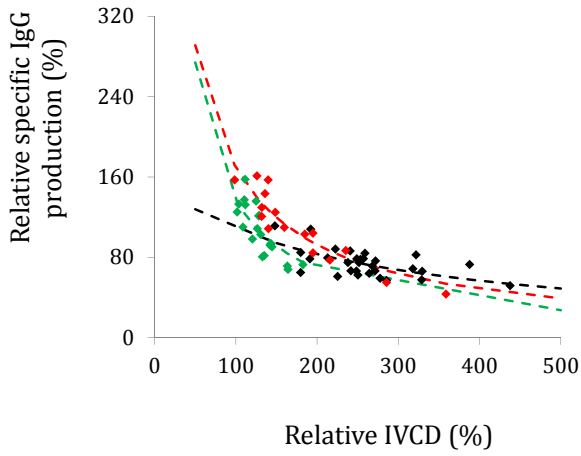

Figure 6.3: Fitted models of rIVCD and rSIP production in cultures supplemented with hydrolysates produced from different starting materials in **chapter 3** (green), hydrolysates produced following different processing treatments in **chapter 4** (red), and different batches of a hydrolysate produced in industrial processes in **chapter 5** (black), Model data were extrapolated from 50-500% rIVCD. Markers indicate experimental data for each experiment, R^2 is the coefficient of determination and C and k are the model fitting parameters (equation 1)

supplemented with industrial hydrolysates. Unfortunately, the values of C and k obtained in different chapters cannot be compared to literature. This is because, in this chapter, rIVCD was used as a measure of specific growth rate, while in the original work of Takahashi [6] or in the other sources that used this approach [7, 8], specific growth rate has been used. Nevertheless, the model (equation 1) seems to be a promising tool to compare the functionality of hydrolysates in different cultures. Using the fitted value of C, the efficiency of a supplement can be calculated to describe how close the total production is to the maximum possible production (**Equation 2**).

$$\textbf{Efficiency of IgG production (\%)} = \frac{Total\ IgG\ production}{C} * 100 \quad \textbf{(Equation 2)}$$

Interestingly, for the lab-scale hydrolysates a higher average efficiency (94 ± 14%) is observed than for the industrial hydrolysates (53 ± 10%). This shows that the used model can be helpful in giving directions for further improvements and optimization.

A surprising observation is that the data from all experiments fall closely together onto one curve (**Figure 6.3**), despite the fact that hydrolysates were prepared by using different starting materials and processing treatments. This indicates that there were no major systematic differences in the cell metabolism under different conditions (e.g. presence of highly toxic compounds). To relate the variation in the functionality to the composition, the composition

of hydrolysates was analyzed at different levels. (1) Compound classes (total protein, total carbohydrate, total ash, total isoflavones, and total saponins) (**Figure 6.4**). (2) Sub compound classes (free amino acids, elution profile of peptides, size distribution of peptides, and mono-/oligosaccharides, individual isoflavones, individual saponins) and specific compounds (CML, LAL, and furosine) (**Figure 6.4**). (3) Individual compounds following a non-targeted metabolomics approach (**Figure 6.4**).

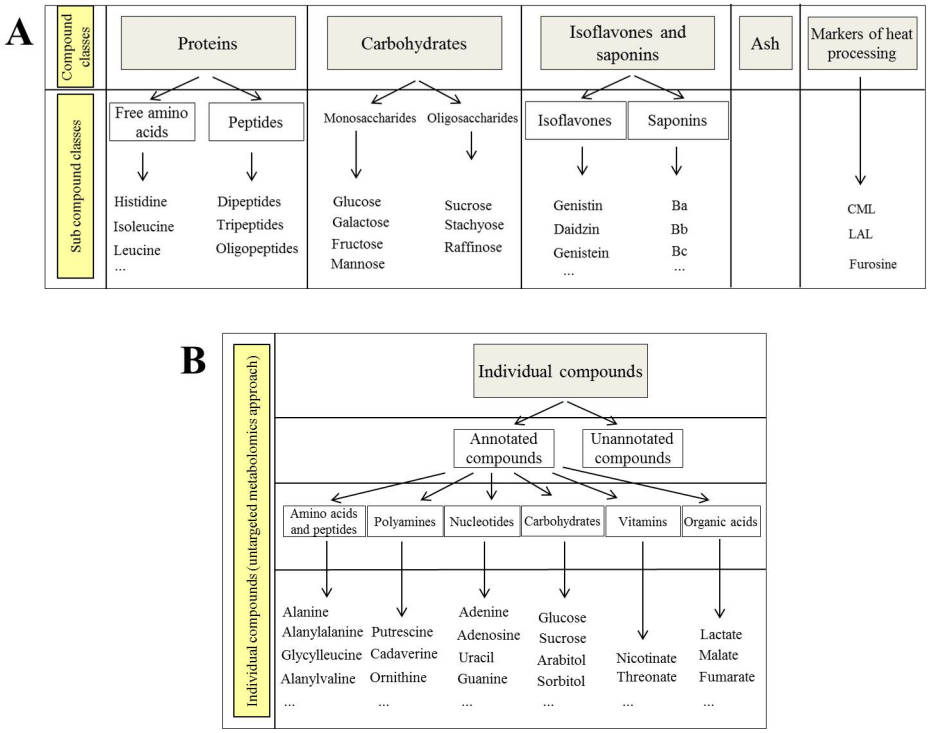

Figure 6.4: Overview of analyses of (A) compound classes, sub compound classes, and specific compounds, and (B) individual compounds analyzed in different chapters

In the hydrolysates prepared from different starting materials, the variation in total carbohydrate content correlated with a decrease in rSIP (**chapter 3**). From this, a hypothesis might be formed on the effect of gross composition on the functionality (**Figure 6.2 A**). However, for the hydrolysates prepared from heat-treated meals (**chapter 4**) the total protein and total carbohydrate content were rather constant, while still a large variation in rIVCD and rSIP was observed (**Figure 6.2 B**). The rIVCD correlated negatively with the rSIP. Surprisingly, both rIVCD and rSIP did not correlate with any of the compound classes or sub compound classes (total content of free amino acids, mono-/oligosaccharides) or specific compounds (CML, LAL, and furosine) analyzed.

When the hydrolysates prepared from heated meals were subjected to extended heating

in suspension, the variation in rIVCD and rSIP was substantially reduced. Moreover, the rIVCD and rSIP did not correlate with each other. The rIVCD and rSIP did not correlate with other sub compound classes. At the same time, the total content of free amino acids and mono-/oligosaccharides was not significantly affected. Consequently, the variation in the functionality could be due to compounds that were covered neither in the gross compositional analyses nor in the analyses of free amino acids, mono-/oligosaccharides. Therefore, another analytical tool was needed to identify and quantify a larger set of compounds. This can be done with non-targeted metabolomics. This technique was used for industrial hydrolysates, which had similar gross composition, but showed a significant variation in the functionality (**Figure 6.2 C**). In these experiments, 10 unique key compounds correlated significantly with the functionality (**chapter 5**).

From the combined results, it is concluded that similar effects on functionality can be induced either by changing the starting material or by changing the heat treatments used for hydrolysate production. Moreover, in different experiments, certain correlations between functionality and a compositional parameter may be observed. However, from combining and comparing different methods to induce variation it becomes apparent that the correlations observed in one experiment can be absent in other experiments.

6.1. Effect of hydrolysate composition on the functionality

When hydrolysates were prepared from meals heated for different time periods, the extent of variation in the functionality (particularly in rIVCD) was larger than that observed in the hydrolysates prepared from meal, isolate, and concentrates (**Figure 6.2 A, B**). This means that factors that affect the gross composition of soybeans, such as genotype [9] and environment [10], are expected to have a small effect on the functionality of hydrolysates. Rather, the variation in functionality seems to be mostly affected by variations in the composition due to variation in the processing of the meal and the hydrolysate. This is in line with expectations based on existing literature, where the link to hydrolysate functionality was not made, but large effects of processing treatments on the composition of hydrolysate was concluded (**chapter 2**).

Heat-induced changes in the meal composition resulted in large effects on functionality of the derived hydrolysates. The extent of effects of heat treatment can be indicated by the LAL content. In the experimental meal with the highest heat treatment, the LAL content was 4 times lower (**chapter 4**) than the reported value for an industrial meal (150 mg/100 g protein or $\sim$ 90 mg/100 g dry matter) [11]. Based on this, it is expected that indeed heat treatment of the meals is one of the important factors that contribute to the functionality of the derived hydrolysate.

Still, the question was if the factors identified in the previous chapters were also relevant for commercial hydrolysates. Therefore, 30 batches of an industrial hydrolysate were analyzed using non-targeted metabolomics analysis (**chapter 5**). An important point of attention is that this approach does not provide an indication of the completeness of the analysis. In addition, out of the 410 reported compounds, only 157 compounds could be annotated. This is most likely

due to the fact that the database used to annotate compounds is not completely untargeted, but was developed specifically to identify metabolites in cells, tissues, and biological fluids, like blood, urine, and saliva. Therefore, some compounds, such as LAL were not annotated in the metabolomics analysis, even though they were identified and quantified with other methods (**chapter 4**). This means that for future applications of this technique for this type of studies, the compound databases used for annotation should be adapted to be more fitting for hydrolysates.

From the three studies (**chapters 3, 4, and 5**), no clear conclusions can be drawn about the key parameters that explain the functionality. Still, the functionality curves (**Figure 6.3**) in all studies fall together, which may mean that there is a common factor in the composition of hydrolysates that relates to functionality. By comparing results from **chapters 3 and 4**, it seems that the total protein and total carbohydrate content do not determine the functionality. At the same time, the functionality in **chapter 3** correlates with the protein and carbohydrate content. This may be due to the free amino acids and mono-/disaccharides. Also, in **chapter 4**, a large variation was observed in the content of free amino acids and mono-/disaccharides in the hydrolysates. Although the correlations between these sub compound classes and functionality could not be quantitatively confirmed, it seems important to investigate this relation, as these compounds are easily consumed by the cells. Unfortunately, the free amino acid concentrations in the CD medium used in **chapters 3, 4, and 5** were not analyzed. However, using the typical composition of Iscove's Dulbecco modified medium (**chapter 1**, **Table 1.3**), it was calculated that the hydrolysate supplementation results in an increase in the total free amino acid and monosaccharide concentrations of 88% and 113%, respectively (**chapter 1**).

6.2. Sources of variation in hydrolysate composition-raw material and processing

To understand the variation in hydrolysate composition, it is important to know to what extent different compounds are transferred from the starting material to the final hydrolysate. The transfer of content for a compound from the starting material to the final hydrolysate was calculated using the **equation 3**.

$$\textbf{Transfer of content (\%)} = \frac{M_x\ hydrolysate}{M_x\ starting\ material} * 100 \quad \textbf{(Equation 3)}$$

In equation 3, M_x hydrolysate is the amount (g) of a compound in hydrolysate produced from 100 g of starting material and M_x starting material is the amount (g) of the same compound present in 100 g of starting material.

In the laboratory scale procedure that mimicked the industrial process of hydrolysate production, it was shown that 19-21% of the dry matter present in the starting material was obtained as hydrolysates (**Table 6.2**). For some compounds the transfer to hydrolysate is similar to that of the dry matter, while for others it is not.

Table 6.2: Transfer of different compound classes and compounds from starting material to the final hydrolysate

Chapter	Compounds/compound class	Content (%) in starting material	Content (%) in hydrolysates	Transfer of content (%)[b]	Relative transfer of content (%)[e]	Variation in starting material (%)[a]	Variation in hydrolysate (%)[a]	Transfer of variation (%)[c]
3	Dry matter	91-96	86-93	21	100	2	4	154
4	Dry matter	84-93	84-89	19	100	5	2	38
3	Total protein[d]	44-84	58-83	25	125	27	14	54
4	Total protein[d]	54-57	62-67	22	110	2	3	135
3	Total carbohydrate[d]	4-28	5-21	15	75	56	77	136
4	Total carbohydrate[d]	19-20	11-14	12	60	2	9	358
3	Isoflavone[d]	0.07-0.29	0.03-0.21	13	65	46	57	124
3	Saponin[d]	0.12-0.35	0.01-0.02	1	5	38	40	105
3	Ash[d]	4-7	8-10	32	160	24	17	70
4	Ash[d]	6.8-7.1	10-12	31	155	2	6	252
4	Free amino acids[d]	0.27-0.72	7.9-16.9	430	2150	36	29	79
4	Mono-/oligosaccharides[d]	9.2-11.8	7.2-15.1	20	100	10	27	273
4	CML[d]	13-81	49-84	38	190	52	17	32
4	LAL[d]	0.5-21.5	0-12	18	90	86	86	100
4	Furosine[d]	69-329	170-923	81	405	87	56	64

[a] Variation in starting material or variation in hydrolysate (%) = standard deviation/mean * 100%;
[b] % transferred from starting material to hydrolysate; Starting material = 100%;
[c] Transfer of variation (%) = variation (%) in hydrolysate/variation (%) in starting material * 100%;
[d] Values are in g or mg/100 g dry matter;
[e] Relative transfer of content (%) = transfer of content (%) for a compound/transfer of content (dry matter) * 100%;

These compounds were accumulated (or produced) or were lost during hydrolysate production. This is clearly seen when the transfer of content is normalized with the transfer (20 ± 3%) of dry matter (**Equation 4**, **Table 6.2**, Relative transfer of content).

$$\textbf{Relative transfer of content (\%)} = \frac{Transfer\ of\ content_{\ \text{compound}}}{Transfer\ of\ content_{\ \text{dry matter}}} * 100 \quad (\textbf{Equation 4})$$

The substantially high accumulation (relative transfer of content (%) - 100%) of free amino acids shows that they were produced during hydrolysate production. The accumulation of CML (90%) and furosine (305%) is attributed to the liberation of protein bound CML and fructosyl lysine during the hydrolysis process. The significant loss of saponins may indicate instability of the compounds during processing. A 50% reduction in the total saponin concentration has been reported when defatted soy flour was heated at 80 °C for 60 minutes [12].

Despite the large loss in the amount of saponins, the variation in the saponin content of the hydrolysate was similar as that in the meals (40 and 38%, respectively). The variation of compounds in starting material and in hydrolysates was calculated as coefficient of variation (CV, standard deviation/mean * 100%) (**Table 6.2**). The change (increase or decrease or no change) in variation from starting material to hydrolysate was expressed as the transfer of variation (**Equation 5**).

$$\textbf{Transfer of variation (\%)} = \frac{V_{\text{x}}\ hydrolysate}{V_{\text{x}}\ starting\ material} * 100 \quad (\textbf{Equation 5})$$

In equation 5, V_x hydrolysate is the variation in compound content in hydrolysate and V_x starting material is the variation in compound content in starting material.

While the transfer of variation (54%) of the total protein content was low in **chapter 3**, in **chapter 4**, the transfer of variation (135%) was high. In addition to total protein, there were large differences in the transfer of variations in the total carbohydrate and total ash content between different the two studies. This shows that there is no generic trend for the transfer of variation for different compounds from the starting material to the hydrolysate.

6.3. Other factors that cause variation in the functionality

During the research it was found that the functionality in cell cultures was also strongly affected by other factors than the variation in the analyzed composition of hydrolysates. These were, amongst others, the variation in CD media and the temperature during culturing. In this section, the effect of variations in these factors is placed in perspective of the variation induced by hydrolysate composition.

6.3.1. CD media and chemically defined supplements

While CD media are labeled 'chemically defined', the exact composition is typically not reported in articles that describe experiments using these media. It has also been shown in a few cases

that for one type of CD media, the functionality varied between batches [13] and with storage time [14]. With respect to the latter, it was shown that with increased storage time (22 ± 4 °C, 12 weeks) of the CD medium (powder), the rIVCD decreased from 117 to 66% (CV = 15%, R = -0.74, *p*- value < 0.001) and the rSIP increased from 82 to 144% (CV = 17%, R = -0.60, *p*-value < 0.0001). This is presented in **Figure 6.5**.

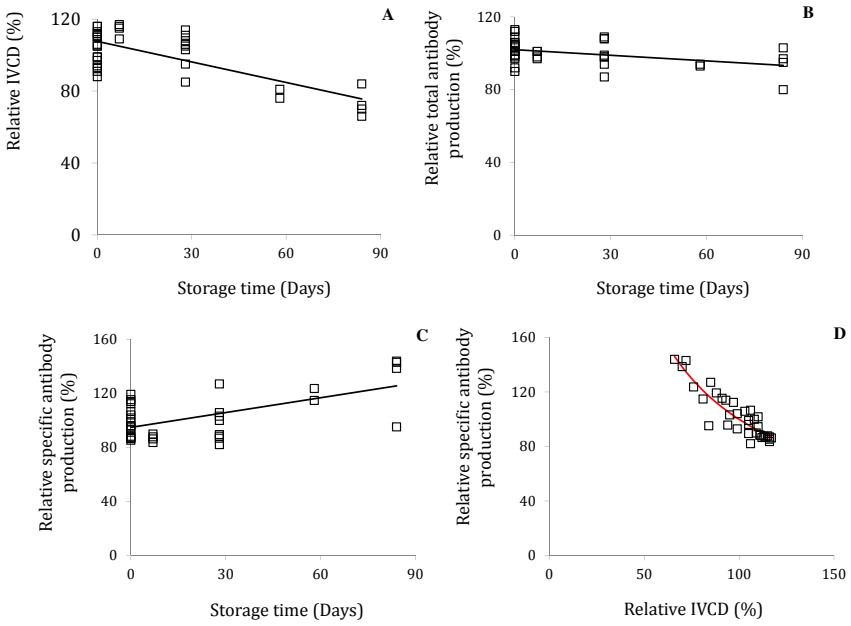

Figure 6.5: Effect of storage time of CD media powder on (A) rIVCD, (B) relative total antibody, and (C) relative specific antibody production in CHO cells. (D) relative IVCD versus relative specific antibody production (fitted with equation 1) (data taken from Hakemeyer et al. [14])

Unfortunately, the study on the effect of storage time of CD medium functionality [14] did not report on the compositional changes in the CD medium. In the other study mentioned [13], the differences in the functionality of 4 batches of CD media were compared. The viable cell density in 4 batches of CD media measured at day 4 varied between 10-16 * 10^5 cells/mL (CV = 21%) [13]. In this study, the composition of these batches were analyzed using metabolomic profiling. Most of the compounds (including amino acids) showed little variation in content, but indications were obtained for light-induced degradation of riboflavin. These effects were confirmed in a second experiment, in which fresh CD media was subjected to light at room temperature for 2 days.

In addition to CD media, different batches of chemically defined supplements (e.g. poloxamer 188) added to CD media have been shown to result in significant lot-to-lot variation in the functionality [15]. Poloxamer 188 is a surfactant, which is supplemented to CD medium to protect the cells against damage caused by agitation and sparging [16]. A large variation in cell

viability (65-98%) and viable cell density (5-43 * 10^6 cells/mL, CV = 69%) was observed in CHO cell cultures supplemented with 7 different batches of poloxamer 188 [15]. Surprisingly, this variation in the IVCD is substantially higher than all the other factors discussed in the previous chapters of this thesis. Unfortunately, the variation in the antibody production was not reported.

Inevitably, during the course of the thesis research, different batches of CD media were used. These also showed variation in the functionality, as expressed by the CV (**Table 6.3**).

Table 6.3: IVCD, total IgG, and specific IgG production in 3 different batches (A, B, and C) of a CD medium and CD medium supplemented with two different batches of an industrial hydrolysate (MH1 and MH2) (additional experiments not included in **chapters 3, 4, and 5**)

I	**IVCD (* 10^6 cells·day/mL)**	**Total IgG production (μg/mL)**	**Specific IgG production** (fg/cells·day)
Lot A	8.1	6.3	778
Lot B	7.4	5.5	743
Lot C	8.4	4.6	548
CV (%) *	**6**	**15**	**18**
II			
A + MH1	19.1	12.4	649
A + MH2	20.1	15.0	746
CV (%) *	**4**	**13**	**10**
B + MH1	15.8	12.0	759
B + MH2	17.5	9.9	566
CV (%) *	**7**	**14**	**20**
C + MH1	17.9	8.5	475
C + MH2	19.5	11.5	590
CV (%) *	**6**	**21**	**16**
III			
A + MH1	19.1	12.4	649
B + MH1	15.8	12.0	759
C + MH1	17.9	8.5	475
CV (%) *	**10**	**20**	**23**
A + MH2	20.1	15.0	746
B + MH2	17.5	9.9	566
C + MH2	19.5	11.5	590
CV (%) *	**7**	**21**	**15**

* CV = standard deviation/mean * 100%

The IVCD showed a small variation between different batches of CD media (CV = 6%), but a larger difference was observed in the total IgG production (CV = 15%). Surprisingly, a similar variation (CV = 19%) of IgG production was observed for cultures where one CD media was supplemented with different industrial hydrolysates, although in that case the variation in IVCD was larger (CV = 23%) (**chapter 5**). Similarly, the variation in IVCD (CV = 21%) reported in reference [13] due to variation between different batches of CD media was also higher than that observed in this thesis (CV = 6%). These results show that, in contrast to common belief, the functionality and the composition of CD media are not necessarily constant. Moreover, the extent of variation in functionality in CD media is shown to be in the same range as that observed for the hydrolysates.

Supplementation of lots A and B of CD medium with two different hydrolysates showed similar variation in IVCD (CV = 4-7%), total IgG production (CV = 13-14%) as the CD media (**Table 6.3 II**). For lot C, an increase in the variation of IgG production (CV = 21%) was observed. This could be due to specific interaction effects between the CD medium and supplemented hydrolysate, as indicated by lower total IgG production in lots B and C supplemented with MH2 and MH1, respectively.

As already indicated before, another important aspect regarding CD medium is that its composition has not been described in scientific literature [17]. Even in the studies that investigated the effects of supplementation of compounds, like glucose and glutamine [18], that are already present in CD medium, the initial content of these compounds in the CD medium have not been taken into account. This further strengthens the conclusion that variation in the CD media and the chemically defined supplements is equally important as variation in hydrolysate composition with respect to the variation in the cell culture functionality.

6.3.2. Temperature

Even if experiments are performed with a stable cell line, a single batch of CD medium and supplement, the functionality may still vary due to small but significant differences in temperature during culturing.

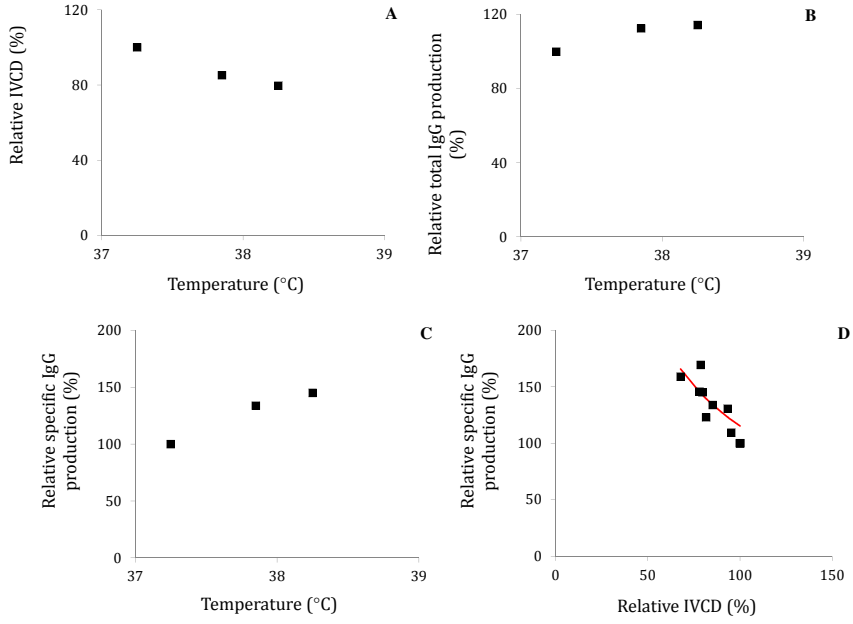

Figure 6.6: Effect of temperature on (A) rIVCD, (B) total IgG, (C) rSIP, and (D) rIVCD and rSIP (fitted with equation 1) in CD medium culture

In **chapters 3** and **4**, it was discussed that a correction was made for the temperature differences observed inside the incubator used for culturing cells. The temperature varied between 37.0-38.5 °C at different positions inside the incubator. The rIVCD, total IgG, and rSIP were substantially affected by a mere 1.5 °C change in temperature during cell culture (**Figure 6.6**).

The CV's in rIVCD, total IgG, and rSIP in CD medium culture were 13, 13, and 20%, respectively. The CV's in rIVCD and rSIP in CD medium supplemented with hydrolysates produced from different starting materials were 16 and 24%, respectively. This means that the effect of variation in temperature (1.5 °C difference during culturing) on the functionality has the same effect as variation in protein content (28-83%) of hydrolysates (**chapter 3**). However, such large variations in the protein and carbohydrate content or processing treatments are typically not observed in different batches of an industrial soy protein hydrolysate. Therefore, it is concluded that the variation in temperature during cell culturing has an equal impact on the variation in functionality than that caused by the lot-to-lot variation in the hydrolysates.

6.4. Concluding remarks

To compare and summarize the effects on the functionality observed in the different **chapters 3**, **4**, and **5**, and those of CD media, CD supplements, and temperature during culturing, these data are combined in **Table 6.4**.

Table 6.4: Combined overview of variation in rIVCD and rSIP in different chapters and from literature

Varying factors	Cell line	rIVCD (%)	CV (%)	rSIP (%)	CV (%)	Reference
Starting materials	CHO-2	102-183	16	68-158	24	**chapter 3**
Processing treatments	CHO-2	98-359	38	43-161	32	**chapter 4**
Industrial processes	CHO-2	148-438	23	52-111	19	**chapter 5**
Temperature during culturing	CHO-2	68-100	13	100-169	20	**chapters 3, 4, and 5**
Storage of CD media	CHO	66-117	15	82-144	17	[14]
Composition of CD media	n.d.	60-100	21	n.a.	n.a.	[13]
Composition of CD media	CHO-C4	100-88	6	100-140	15	**this chapter***
Supplements	CHO-DUXB11	12-100	69	n.a.	n.a.	

* Collected during this thesis research
n.d.: not described
n.d.: not analyzed

From the **table 6.4**, it is clear that each of the reported factors can result in significant variation of the final functionality. The variation in the composition of starting materials in this study, however, is much larger than the natural variation in the soybean composition. Therefore, the actual range of variation in the functionality due to variation in soybean composition is expected to be much smaller. Hence, it is concluded that the processing treatments and industrial processes are the most critical factors that determine hydrolysate functionality in cell cultures (**Figure 6.7**).

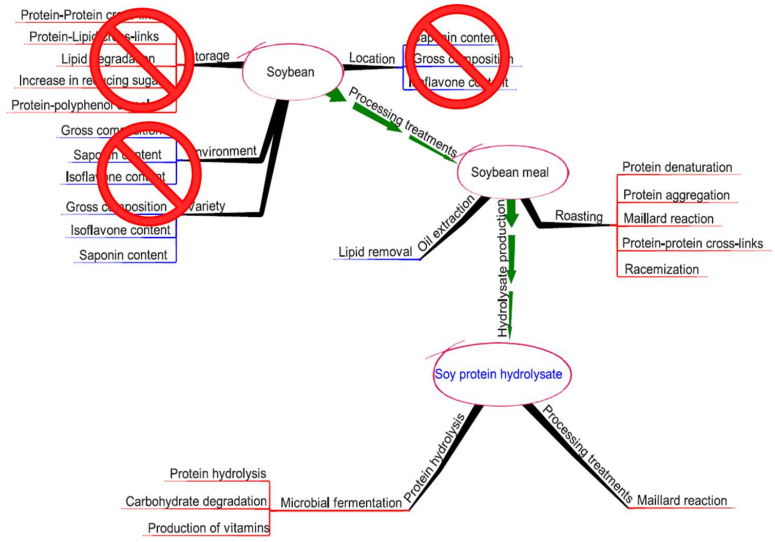

Figure 6.7: Stages at which changes in the compound classes (blue lines) and specific compounds (red lines) can occur in the composition of a soy protein hydrolysate (taken from **chapter 1**). Based on conclusions from this work, stages with the least influence on the final functionality of the hydrolysates are ruled out

Still, the culturing conditions have a significant influence on the culture functionality. To compare the effects of hydrolysates and culturing conditions on cell culture functionality, the data were fitted with $q = C/(\mu + k)$ model (**Figure 6.8**) proposed in literature [6].

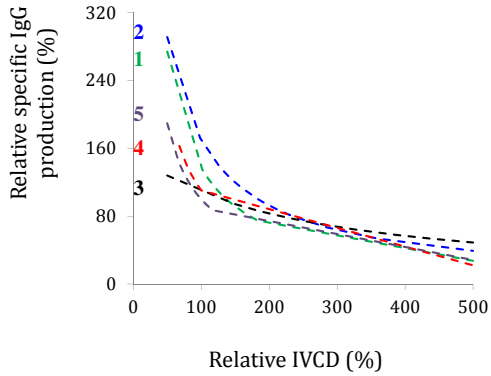

Figure 6.8: Fitted models of rIVCD and rSIP in hydrolysate-supplemented cultures in hydrolysates prepared using different starting materials (1), processing treatments (2), and industrial processes (3), and fitted models of rIVCD and rSIP as affected by temperature during culturing (4), and storage of CD media (5)

The data obtained from literature are in the same range as those obtained in this thesis. This is slightly surprising since it was obtained using a different cell line (unspecified CHO clone) as well as under different conditions (e.g. media) [14]. The benefit of the model is that it allows comparison of results obtained in different experiments based on the fitting parameters, C, k, and μ (**Table 6.5**).

Table 6.5: Model parameters for the functionality data in hydrolysates produced with different starting materials (**chapter 3**), hydrolysates produced following different processing treatments (**chapter 4**), different batches of a hydrolysate produced in industrial processes (**chapter 5**), variation in CD media, and temperature during culturing

	Starting materials#	**Processing treatments#**	**Industrial processes#**	**Temperature during culturing#**	**Storage of CD media [14]***
R^2	0.67	0.85	0.37	0.79	0.82
C	137	203	355	110	110
k	0	20	226	0	10

CHO-2 cells (data collected in this thesis)
* Unspecified CHO clone

Also for the data for effects of storage media, a good fit ($R^2 = 0.82$) was obtained. The values for C and k are similar for the starting materials, temperature during culturing, and storage of the CD media experiments. Similar values of C and k suggest that the rate of mRNA synthesis and translation for production of antibody and decomposition of mRNA were similar in these experiments. A large difference is observed in the processing, specifically in the industrial processes experiments. This suggests a substantial difference in the production and stability of mRNA and translation for production of antibodies. In other words, this suggests that there was a difference in the cellular mechanism in cultures supplemented with industrial hydrolysates as compared to those produced in the laboratory. Using this, one can distinguish the change in specific IgG production due to variation in the cell growth from that due to the variation in cellular mechanism. In addition, the information on maximum possible antibody production for a certain hydrolysate or a culture condition could be derived using the model. Such information is useful in giving directions for further improvement and optimization of hydrolysates or culture conditions to maximize total antibody production.

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Summary

Soy protein hydrolysates are commonly supplemented to chemically defined (CD) media in cell culture applications to enhance integral viable cell density (IVCD) and recombinant protein production. However, the functionality may vary when different batches of a hydrolysate are supplemented in cell cultures. Although it seems likely that this variation in the functionality of hydrolysates is due to variation in its composition, this relation has not yet been systematically studied. The first aim of this thesis was to identify the extent to which the functionality varied in hydrolysate-supplemented cultures due to different starting materials and processing treatments. The second aim was to identify the key parameters (compound classes, sub compound classes, and compounds) that significantly affected the functionality of soy protein hydrolysates.

To study the relative contribution of total proteins, total carbohydrates, total isoflavones, and total saponins, hydrolysates were produced from different starting materials (meal, acid and ethanol extracted concentrates, and isolate) (**chapter 3**). While the contents of total protein, total carbohydrate, total isoflavones, and total saponins varied, the type of peptides (elution profile of peptides), mono-/oligosaccharides, individual isoflavones, and individual saponins present in the hydrolysates were similar. The hydrolysates were supplemented to a CD medium in cell culture based on equal weight and on equal protein levels. The functionality was not affected by the isoflavone and saponin contents of the hydrolysates. However, an increase in the total carbohydrate content significantly (p- value < 0.004) increased IVCD and decreased total immunoglobulin (IgG) and specific IgG production. An opposite effect of the total protein content, i.e. an increase in the total and specific IgG production and suppression of IVCD, was observed. This suggested that the total protein and total carbohydrate contents of hydrolysates determined its functionality in cell cultures.

To study the effect of heating on hydrolysate composition and functionality, an experimental soybean meal was produced and heated for different times in dry state (**chapter 4**). One part of the meals was hydrolyzed and the other part was both hydrolyzed and heated for prolonged time in suspension. With increasing heating time, the free amino acid and mono-/oligosaccharide contents decreased in the meals. The contents of typical indicators of the Maillard and cross-linking reactions, i.e. carboxymethyl lysine (CML) and lysinoalanine (LAL) increased with increase in heating time of the meals. The initial differences in the contents of CML, LAL, furosine, free amino acid, mono-/oligosaccharides in the meals were also reflected

in the final hydrolysates. When these hydrolysates were supplemented to cell cultures, a large variation was observed in the functionality. However, the variation in the functionality reduced substantially when cultures were supplemented with hydrolysates that were heated in suspension. Surprisingly, no significant differences could be identified in the composition of the hydrolysates that were produced from meals heated in dry state with and without extended heating in suspension.

Still, the question was if the factors identified in the previous chapters were also relevant for commercial hydrolysates. Therefore, 30 batches of an industrial hydrolysate were analyzed using non-targeted metabolomics analysis (**chapter 5**). In these hydrolysates, 410 compounds were detected from which 157 were annotated. The remaining 253 compounds were identified as peptides, but could not be annotated. All the compounds were semi-quantified relatively based on their signal intensities. As the analysis was performed in triplicate, the average signal intensities were calculated for each compound in each hydrolysate. This data was combined with the functionality data and normalized before performing bootstrapped stepwise regression. From the bootstrapped stepwise regression, the key compounds were identified that significantly correlated with the functionality. The most important key compounds, i.e. phenyllactate and ferulate, explained 29% and 30% of the variance in IVCD and total IgG production, respectively. All the key compounds correlated positively with IVCD and total IgG production.

In addition to hydrolysates, it was found that the functionality in cell culture was affected by variation in the CD media and temperature during culturing (**chapter 6**). To place the effect of variations in these factors in the perspective of the variation induced by hydrolysates, the data from literature and additional experiments were combined with the data from different chapters. The data could be fitted nicely with an existing model proposed in the literature. In this way, it became clear that the effects of different culture conditions and even different cell lines could be studied together. From the model, it was found that the processing treatments were the most critical factor in determining the functionality of hydrolysates. The effect of starting materials on the hydrolysate functionality was similar to the effects induced by variation in the CD media and temperature during culturing. This suggested that the effects of variation in CD media and temperature during culturing were equally important in relation to the variation in the functionality. Furthermore, the information on maximum achievable total IgG production in a culture condition could be derived from the model. This information is useful in giving directions for further improvement and optimization of hydrolysates or culture conditions to maximize total antibody production.

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About the author

Abhishek Jawaharlal Gupta was born on 29th September, 1981 in Anand, India. After finishing his schooling (Kendriya Vidyalaya, V.V.Nagar) in 1999, he started his Bachelor studies (B.Tech) in Dairy Technology at S.M.C College of Dairy science, Anand Agricultural university (Anand). After graduating in 2003, he started to work at Mehsana District Co-operative Milk Producer's Union Ltd (Mehsana). In 2005, he moved to Netherlands to continue with Master studies in Food technology. During master studies, he did a thesis on optimizing the fermentation of quarg-like products at FrieslandCampina (previously, Campina Innovation, Wageningen). This was followed with an internship on studying the immunomodulatory effects of bioactive food ingredients in *in vitro* gut models at FrieslandCampina (previously, DMV-International, Wageningen). Since 2007 he works as a researcher at FrieslandCampina (Wageningen), where he is studying the relation between the composition and functionality of plant protein hydrolysates used in biopharma industry.
From January'2010 till July'2015, together with his job at FrieslandCampina he carried out the research described in this PhD thesis. Currently, he is continuing to work at FrieslandCampina to apply the learnings of the PhD thesis into practice.

List of publications

Abhishek J. Gupta, Harry Gruppen, Dominick Maes, Jan-Willem Boots, and Peter A. Wierenga, Factors causing compositional changes in soy protein hydrolysates and the effects on cell culture functionality, *Journal of Agricultural and Food Chemistry* **2013**, 61, (45), 10613-10625.

Abhishek J. Gupta, Jos A. Hageman, Peter A. Wierenga, Jan-Willem Boots, and Harry Gruppen, Chemometric analysis of soy protein hydrolysates used in animal cell culture for IgG production-an untargeted metabolomics approach, *Process Biochemistry* **2014**, 49, (2), 309-317.

Abhishek J. Gupta, Peter A. Wierenga, Jan-Willem Boots, and Harry Gruppen, Influence of protein and carbohydrate contents of soy protein hydrolysates on cell density and IgG production in animal cell cultures. *Submitted for publication*

Abhishek J. Gupta, Jan-Willem Boots, Harry Gruppen, and Peter A. Wierenga, Influence of heat treatments on the functionality of soy protein hydrolysates in animal cell cultures. *Submitted for publication*

Abhishek J. Gupta, Mireille Maria Gadellaa, and Dominick Yves Willy Maes, Culture medium for eukaryotic cells. *International patent application: WO2012030217*

Abhishek J. Gupta, Mireille Maria Gadellaa, and Dominick Yves Willy Maes, Culture medium for eukaryotic cells. *International patent application: WO2013133714*

Abhishek J. Gupta, Mireille Maria Gadellaa, and Dominick Yves Willy Maes, Culture medium for eukaryotic cells. *International patent application: WO2013133715*

Overview of completed training activities

Discipline specific courses

Summer school glycosciences, Wageningen, The Netherlands, 2010
Advanced food analysis, Wageningen, The Netherlands, 2010
Basic statistics, Wageningen, 2009
Magnetic resonance in food, Wageningen, The Netherlands, 2012
Reaction kinetics in food science, Wageningen, The Netherlands, 2012
Biorefinery for food and fuel and materials, Wageningen, The Netherlands, 2013
Introduction to R for statistical analysis, Wageningen, The Netherlands, 2013
Industrial food proteins, Wageningen, The Netherlands, 2013
The challenges and relevance of bioprocessing in today's life sciences and biotechnology, Wageningen, The Netherlands, 2014
Netherlands biotechnology congress-15, Biotechnology by Dutch Design, Ede, The Netherlands, 2014
Multivariate analysis for food/data sciences, Wageningen, The Netherlands, 2014

General courses

Ph.D. introduction week, Baarlo, The Netherlands, 2010
Ph.D. competence assessment, Wageningen, The Netherlands, 2010
Scientific publising, Wageningen, The Netherlands, 2012
Scientific writing, Wageningen, The Netherlands, 2012
Career perspectives, Wageningen, The Netherlands, 2012
Mobilising scientific network, Wageningen, The Netherlands, 2013
Data management, Wageningen, The Netherlands, 2013
Reviewing a scientific paper, Wageningen, The Netherlands, 2013

Other activities

Preparation of PhD project proposal, 2010
Ph.D. study trip, Singapore and Malaysia, 2012
B.Sc. and M.Sc. thesis presentations, 2010-2015
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