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BENEFICIAL FEATURES IN EP200

EP200 is a fermented soy protein concentrate with high digestible value and negligible anti-nutritional factors. An excellent choice for replacing costly protein meals in starter-diets. Appropriate for using in animal sectors.

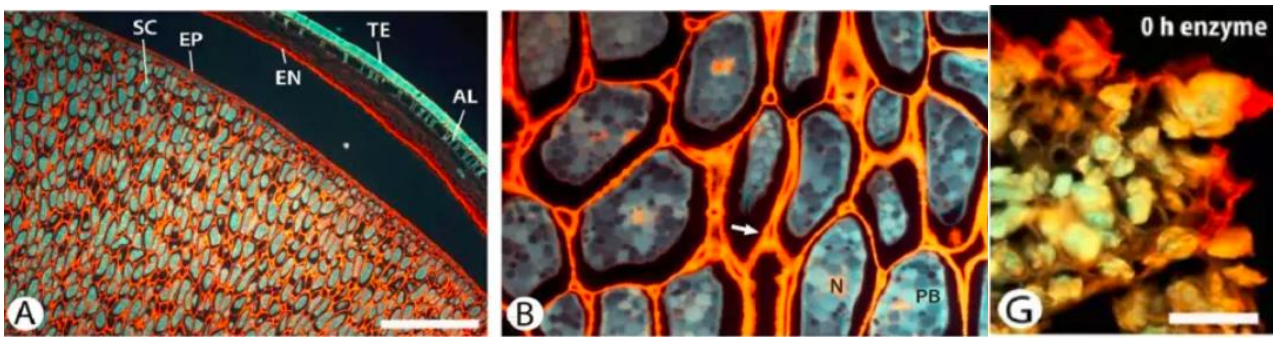
Contents

PROTEIN DIGESTIBILITY USING DIFFERENT METHODS

INTRODUCTION

The increase of digestible protein from EP200 and a competitor digestible soybean meal protein product was evaluated with several in vitro methods. In order to understand the results, first we need to describe how is protein stored in soybean? What happens after de-fatting and roasting to turn it into soybean meal (SBM)? What does it mean to evaluate the total free amino acid pool (TFAA)? And the total hydrolysable amino acid pool (THAA)? How can the visualization of proteins show protein turnover by either enzymatic or fermentation processes?

1. Protein (mainly glycinins and conglycinins) in soy is stored in compartments protected by thick cells walls of pectin, cellulose and hemicellulose. De-hulling, milling, de-fatting and roasting of soybeans, is not efficient at removing the inner cell walls that are encapsulating the protein in compartments (microscopy image A, B & G and Table 1).



Microscopy image 1. Thin sections of resin-embedded soybean showing the general seed microstructure of intact soybean tissue (A & B). Regions labelled with orange fluorescence are rich in pectin. The turquoise-blue color is autofluorescence from protein bodies within the storage cotyledons and from lignified cell walls. Storage cotyledons (SC), seed coat (TE-testa), epidermis (EP), endosperm (EN), aleurone layer (AL), protein bodies (PB), nucleus (N), arrows represent the pectin-rich regions. Bar = 500 µm in image A. Image B is a zoomed in of image A. Bar in image G = 100 µm (*Taken from Ravn et al., 2015*).

TABLE 1 COMPOSITION OF CARBOHYDRATES FORMING CELL WALLS STRUCTURES IN SOYBEAN AND RAPESEED.

Main structures in cell walls	Polysaccharide composition
	Soybean and rapeseed
cellulose	β-(1,4)-linked D-glucose
hemicellulose	Backbone of β-(1,4)-linked D-xylose, D-arabinose, D-mannose, D-glucose, and D-glucuronic acid branched ferulic acid
pectin	Backbone of α-(1,4)-linked D-galacturonic acid and α-(1,2)-linked L-rhamnose complexated with D-galactose, D-xylose and L-arabinose as substitutions

2. The free amino acid pool (TFAA) are readily digestible amino acids. There is a certain amount naturally present in soybean meals. It is expected that this amount will increase with fermentation or enzymatic treatment as the protein will be broken down into smaller pieces and single amino acids via a process known as hydrolysis (Image 2).

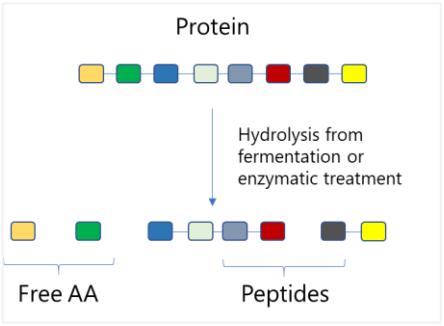


Image 2. Representation of protein hydrolysis that releases free amino acids and peptides.

3. The acid hydrolysable amino acid pool (THAA) are the amino acids forming the proteins and peptides that will be broken down into single amino acid during acid treatment. In other words, those are the amino acids that need to undergo enzymatic hydrolysis during digestion because they packed together forming proteins. The term THAA refers to the harsh chemical treatment used in this analysis to detach them as single free amino acids from the proteins. For comparative purposes, in this report, THAA will be used as the entire protein pool that can be quantified. Not all the protein is 100% digestible. Specially, after heat-treatment (see above explanation). Therefore, the acid hydrolysis will not be 100% efficient.

4. Visualization of proteins in natural samples is commonly done via SDS-PAGE (stands for sodium-dodecyl-sulphate polyacrylamide gel electrophoresis). This is the most common visual method to investigate the profile of proteins present in a sample. The method uses a constant electrical current to mobilize the proteins through a porous gel. After the run, all proteins are separated and immobilized in the gel according to their size. Proteins travel from top to bottom. The bigger the size of the proteins, the upper the position of the band in the gel. A reference with known proteins is used (left lane). Low weight or peptides are immobilized at the bottom.

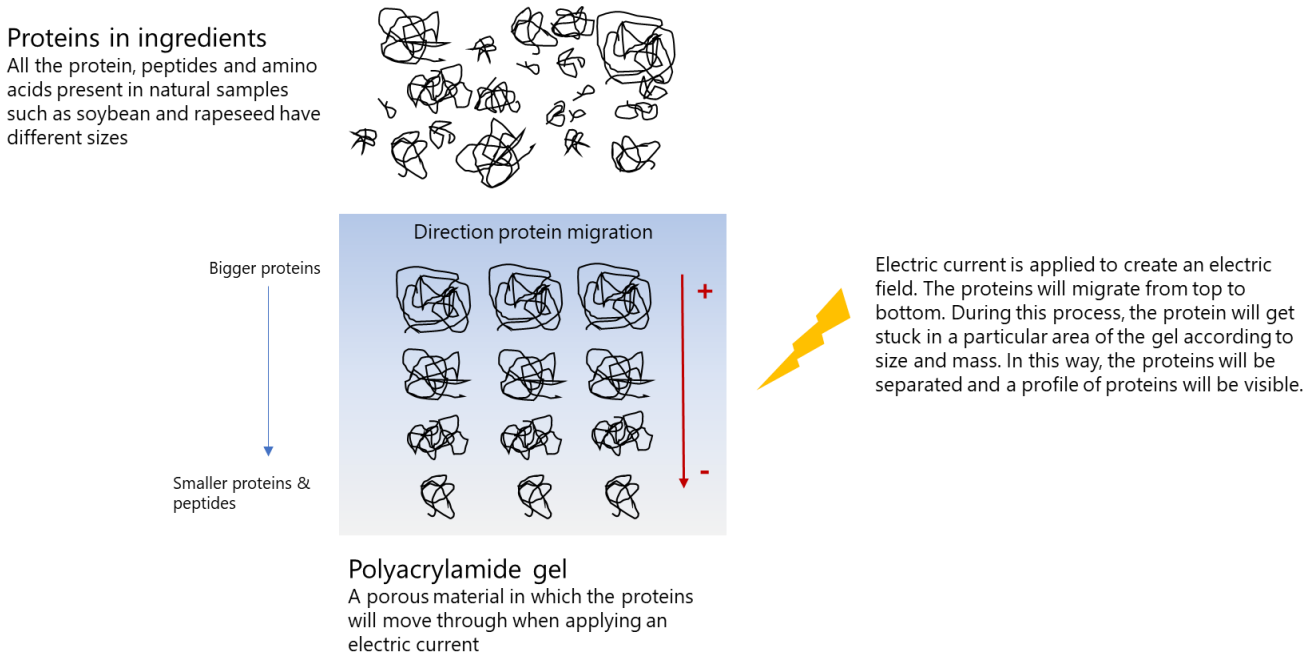
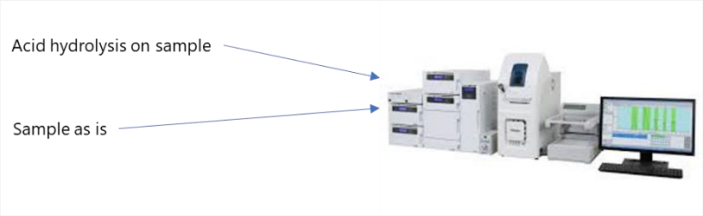


Image 3. Schematic representation of SDS-PAGE to investigate proteins in a sample.

METHODS

The analysis of amino acids consisted of evaluating two pools of data. A) total hydrolysable amino acids (THAA; *in this report is referred as total quantifiable amino acids*). B) total free amino acids (TFAA). Both pools of data were evaluated using a chromatography method (post-column mcf-GC-MS). In which each individual amino acid from the samples is identified and quantified based on purified amino acid standards evaluated in the same run.

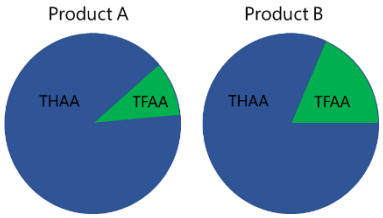


For THAA, the samples were subjected first to an acid hydrolysis under anoxygenic conditions (6 M HCl, 24 h, 106 ^{0}C), following by injection to the GC-MS, identification and quantification. The acid hydrolysis is a process that releases all amino acids bound to proteins. The hydrolysis generally destroys a few amino acids such as cysteine and arginine. Due to this, only 14 amino acids were able to be quantified.

The free amino acid pool is all amino acids that the GC-MS can detect without any pre-treatment. This means, TFAA measures amino acids that are not contained in proteins. Non-fermented SBM, EP200 and a competitor product (Hamlet protein) were compared in this analysis.

The comparison gives an indication of the fraction that the TFAA present in the THAA, and the THAA represents all protein there is in the sample.

This is represented in the following formula and the in the sketch on the right:



$$\%\ increase\ of\ free\ amino\ acids\ after\ processing\ of\ SBM = \left(\frac{TFAA_{product}}{THAA_{SBM}} \times 100\right) - TFAA_{SBM}$$

The visual analysis of proteins in EP200 and two competitors was carried out via gel electrophoresis. SDS-PAGE as described above. Furthermore, **protein solubility (PS)** and **nitrogen Solubility Index (NSI)** were also carried out on EP200. PS consists of determining the percentage of protein that is solubilized in a potassium hydroxide solution. NSI consists of measuring the amount of protein dispersed in water after blending a sample with water in a blender.

RESULTS

The results indicate that with lacto-fermentation of SBM, the amount of total quantifiable amino acids increases statistically significantly (T-test; $p < 0.05$) in comparison with the process used in making the competitor product (Table 1; Fig. 1). The total quantifiable amino acids were interpreted as all single amino acids released from proteins and peptides after acid hydrolysis. Because the method is not 100% efficient, not all amino acids were released and some were destroyed, therefore, the sum of all was not 1000 g (see table 2). The more accessible the protein is, the more will be broken down to single amino acids during acid hydrolysis. However, presence of fibrous cell walls encapsulating the protein in soy reduce the efficiency of the acid hydrolysis because the acid hydrolysis will not break down the fiber.

In regards of the free amino acid pool (formula explained in methods section), EP200 and the competitor product displayed minor increased. The sum of all free amino acids accounted for an increase of 3.1 % and 2.8 % respectively of the free

amino acid pool (Figure 2, right panel). This means that the method for making EP200 has the advantage of releasing more free amino acids in comparison with the method use by the competitor.

TABLE 2. TOTAL QUANTIFIABLE AMINO ACIDS AFTER ACID HYDROLYSIS (THAA). THE HIGHER THE DIGESTIBILITY OF PROTEIN IS, THE MORE EFFICIENT THE TREATMENT OF ACID HYDROLYSIS IS TO RELEASING SINGLE AMINO ACIDS. TOTAL FREE AMINO ACIDS (TFAA) PRESENT IN THE DIFFERENT SAMPLES.

THAA (corrected for CP)		Ala	Gly	Val	Leu	Ile	Thr	Pro	Asn	Ser	Met	Glu	Lys	Tyr	Asp	Total
Sample ID	Protein level (%)	g AA /kg protein														
Untreated SBM	48.5	40.6	35.9	44.4	71.4	41.5	25.5	44.8	5.5	38.1	11.6	94.8	34.6	13.3	101.2	603.1
EP200	50.5	55.4	45.8	59.2	95.7	55.7	26.0	60.6	5.3	44.4	10.0	109.3	42.1	16.8	133.7	760.1
Competitor product 1	55.8	41.3	37.0	44.5	71.0	41.2	24.8	45.0	5.0	35.7	9.6	86.5	31.0	10.8	97.8	581.3
14 amino acids were quantified. Cysteine, glutamine, tryptophan, phenylalanine, histidine and arginine are missing.																
TFAA corrected for CP		g AA/kg protein														
Untreated SBM		0.14	0.03	0.03	0.03	0.02	0.03	0.04	0.09	0.02	0.02	0.31	0.01	0.03	0.09	0.9
EP200		0.38	0.07	0.03	0.02	0.01	0.06	0.07	0.06	0.04	0.02	0.41	0.09	0.04	0.17	1.5
Competitor product 1		0.20	0.04	0.03	0.02	0.02	0.03	0.07	0.08	0.03	0.02	0.42	0.02	0.04	0.12	1.2

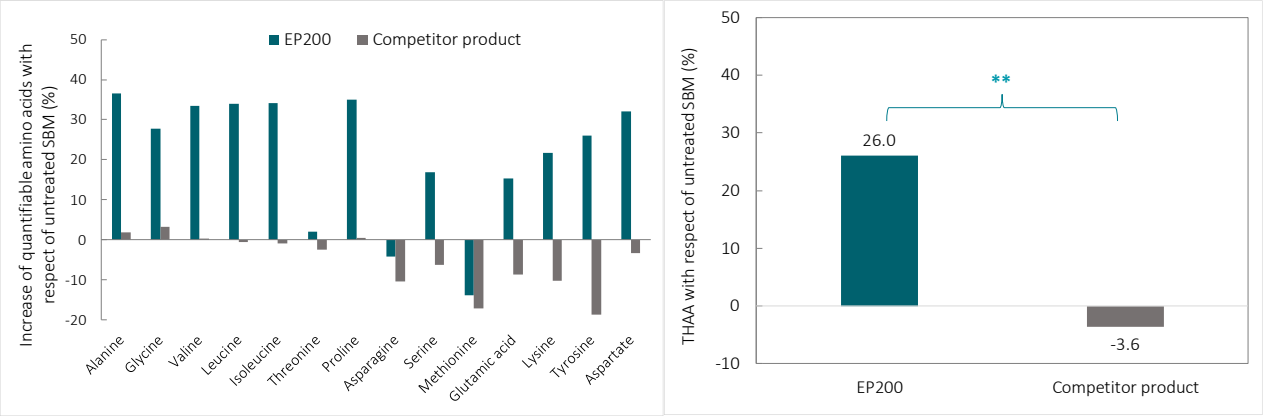


FIGURE 1 THE TOTAL QUANTIFIABLE AMINO ACIDS (THAA) INCREASE IS BASED ON THE COMPARISON OF EP200 AND COMPETITOR PRODUCT 1 AGAINST NON-FERMENTED SBM. A *T-TEST* INDICATED THE DIFFERENCE OF QUANTIFIABLE AMINO ACIDS IN EP200 AND THE COMPETITOR PRODUCT WAS SIGNIFICANT (*P = 0.00008; BLUE STARS*). THE QUANTIFIABLE AMINO ACIDS ARE EACH SINGLE AMINO ACID RELEASED FROM PROTEIN AND PEPTIDES. THE MORE ACCESIBLE THE PROTEIN IS, THE MORE EFFICIENT THE ACID HYDROLYSIS WILL BE AT BREAKING DOWN THE PROTEIN DOWN TO SINGLE AMINO ACIDS.

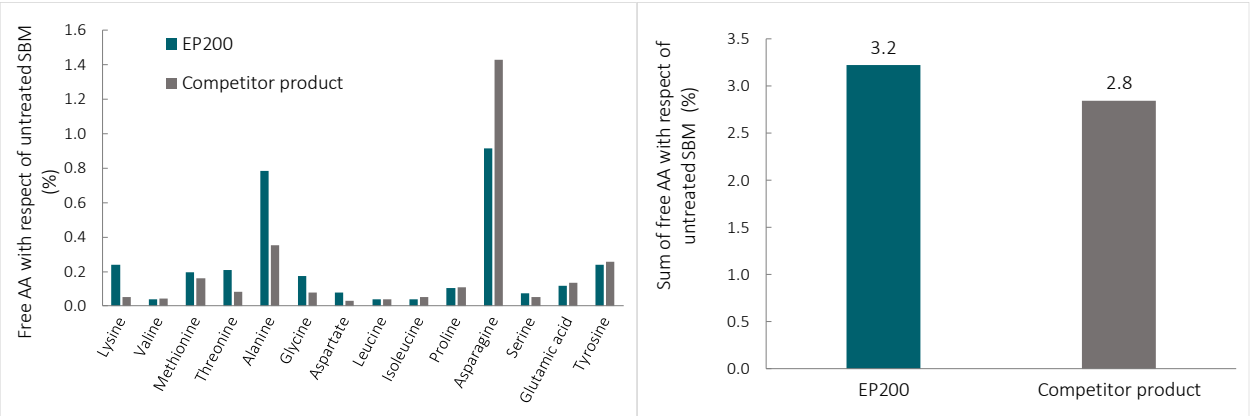


FIGURE 2. THE EXTENT OF FREE AMINO ACIDS IN EP200 AND THE COMPETITOR PRODUCT.

TABLE 3. LEVEL OF PS AND NSI IN EP200 IN COMPARISON WITH AN UNTREATED SBM.

	Crude protein	Protein dispersibility - PDI (KOH dispersible)	Nitrogen solubility (KOH)	TIA
	%	%	%	mg/g
Soybean meal (SBM)	48.7	37,3	77	1.5
EP200	50.9	37.4	73	<0.5
Competitor 1	55.8	24.5	44	<0.5
Competitor 2	52.8	21.6	41	1.2

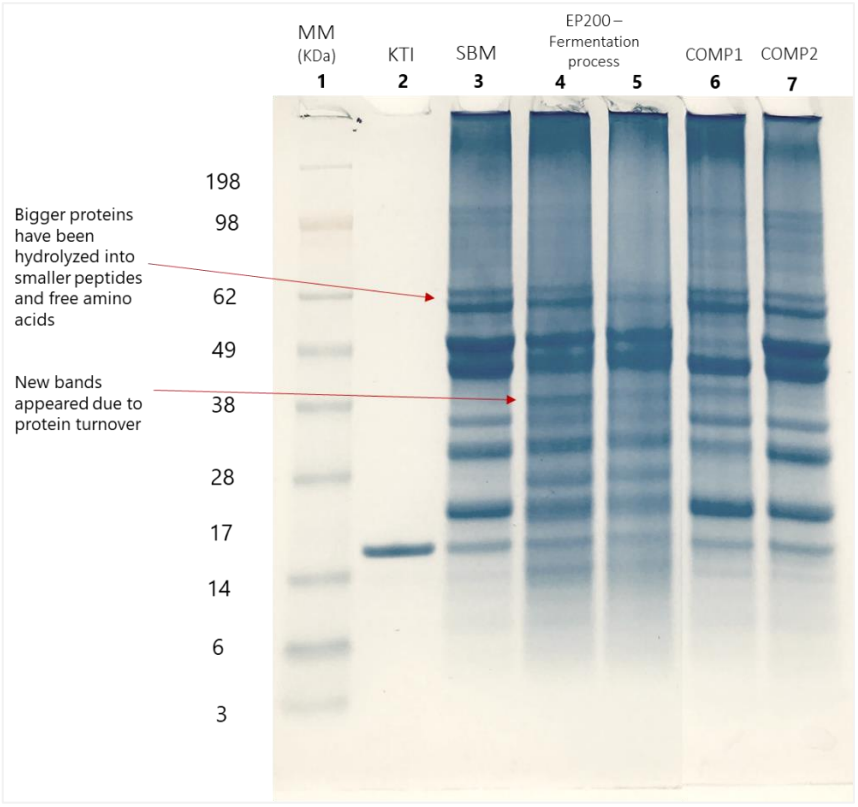


FIGURE 3. PROTEIN PROFILE OF EP200 AND OTHER SOY PRODUCTS.

SDS-PAGE on EP200 vs two competitor soy products	
Lanes	Description
1	Molecular Marker (MM)
2	Kunitz trypsin inhibitor (KTI)
3	Soybean meal (SBM)
4	EP200 – fermentation process
5	EP200 – fermentation process
6	Competitor 1
7	Competitor 2

Conclusions

Based on the different analyses, it seems that the lacto-fermentation of SBM in EP200, makes protein more digestible in comparison with the process used by other soy products. For instance, the quantification of amino acids (THAA & TFAA) showed that there is more digestible protein present in EP200. Lysine and methionine are higher in EP200 in comparison with the competitor product. The protein profile of EP200 and two other competitor soy products via SDS-PAGE showed the disappearance of bigger proteins (the top of the gel) while the appearance of medium and smaller protein/peptides in the gel due to protein turnover carried out by bacterial processes during fermentation. Finally, %PDI and %KOH values indicated that EP200 contains more available protein for digestion whilst TIA values are negligible in comparison with the two competitors.

The microbial enzymes of the lactic acid bacteria used in the processing of EP200 that can release protein within cell wall hemicellulose and cellulose structures (Cai et al., 1999), and thereby turning over the protein in soy into a more digestible pool of microbial proteins, peptides and to some extend free amino acids.

Hydrolysis of Trypsin inhibitors

Introduction

There are a few compounds in soybean that impairs protein digestion. These compounds are naturally present in the plant as a result of an evolutionary trait. The two most important are lectins and trypsin inhibitors. Lectins are proteins that have a high affinity for binding to carbohydrates, creating complex structures that are difficult to digest. Lectins also bind to the gut mucosa prompting an inflammation reaction. The second ones are trypsin inhibitors. These are small peptides characterized by their ability to bind to trypsin and chymotrypsin proteases. Trypsin is the most important pancreatic enzyme which role is to mature all other pancreatic enzymes but also to break down protein at the small intestine. In soy, there are two main trypsin inhibitors, the Kunitz and the Bowman-Birk inhibitors. Both small peptides name after the scientist who discovered them. The Kunitz inhibitor is the most abundant of the two.

Typical values for anti-nutritional factors in whole soybeans are: Kunitz trypsin inhibitor 30.3 mg/g CP and Bowman-Birk inhibitor 10.7 mg/g CP, Lectins 50-200 mg/g CP, Saponins 0.5-0.6 %, Phytic acid 0.6 % (Van Eys et al., 2004). Depending on the quality of the SBM these can be lowered, but the protein digestibility generally is also lower due to over roasting forming Maillard reactions (strong binding of carbohydrates and protein due to overheating).

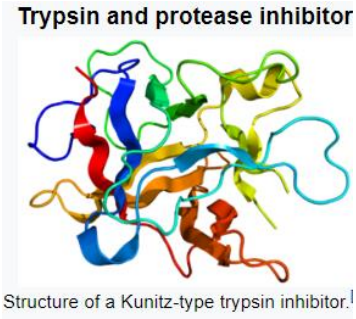


Image 4. Structure of the Kunitz trypsin inhibitor. A small peptide capable to impair the activity of trypsin a pancreatic protease (image source, Wikipedia).

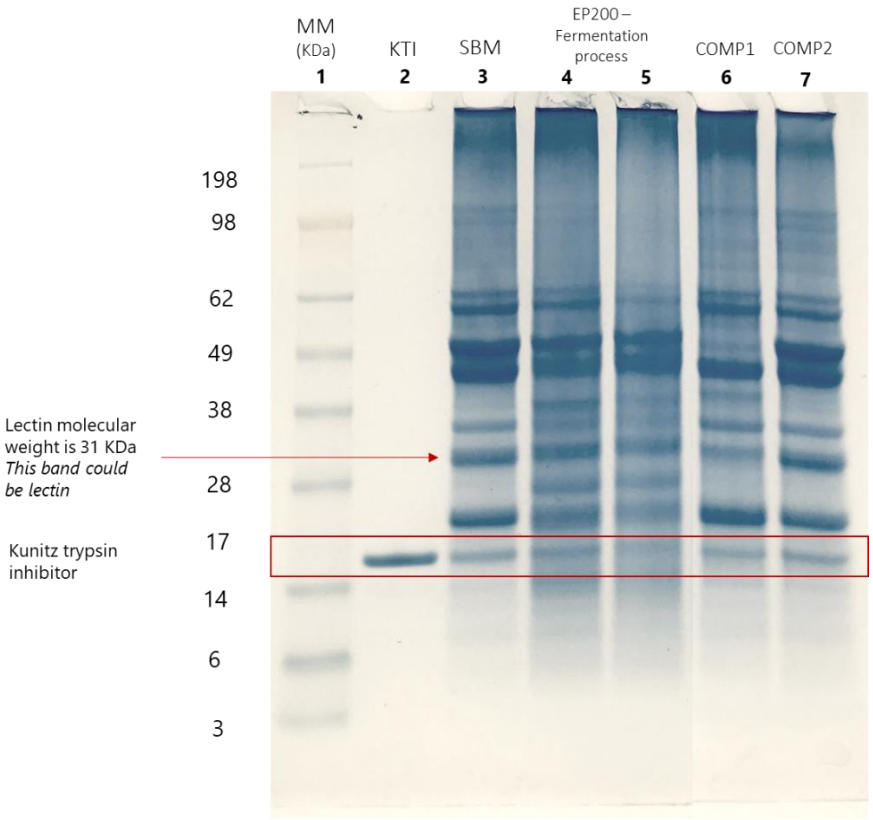
Methods

We quantified the amount of trypsin inhibitors in EP200 using the visual method SDS-PAGE to observe the breakdown of the trypsin inhibitors with the fermentation process used for making EP200. The technique is described in page 3 (image 3).

RESULTS

SDS-PAGE showing the protein profile of soybean meal, EP200 and two digestible soy competitor products. Lane 2 is the purified Kunitz trypsin inhibitor that is typically present in soy at a level of 30.3 mg/g.

In parallel, a commercial lab investigated the trypsin inhibition activity (ISO-14902). The results were negligible (< 0.5 mg/g).



SDS-PAGE. Protein profile of EP200 and two more protein products with the focus on KTI hydrolysis	
Lanes	Description
1	Molecular Marker (MM)
2	Kunitz trypsin inhibitor (KTI)
3	Soybean meal (SBM)
4	EP200 – fermentation process
5	EP200 – fermentation process
6	Competitor 1
7	Competitor 2

Conclusions

The fermentative process used in the making of EP200 reduces the level of the anti-nutritional factors. In this case, we focused on investigating the Kunitz trypsin inhibitor (KTI) as it is the anti-nutritional factor present in soy at a higher level. The trypsin inhibition activity assay also showed negligible activity. This means that the low level of trypsin inhibitors after the fermentative process of EP200 have no activity against trypsin and chymotrypsin, the pancreatic proteases. The lactic acid bacteria used for the fermentation of EP200 is well recognized for processing a full enzymatic machinery of extracellular proteases (Mora et al., 2003).

Hydrolysis of phytate

Introduction

Phytate or phytic acid (known as inositol hexakisphosphate (IP6), inositol polyphosphate, or phytate when in salt form) is the phosphate ester of inositol. It contains six phosphate groups. It plays a significant nutritional role as the principal storage form of phosphorus in many plant tissues, especially bran and seeds. It is also present in many legumes, cereals, and grains. Phytate has a strong binding affinity to the dietary minerals, calcium, iron, and zinc, inhibiting their absorption. Therefore, it is considered an anti-nutritional factor.

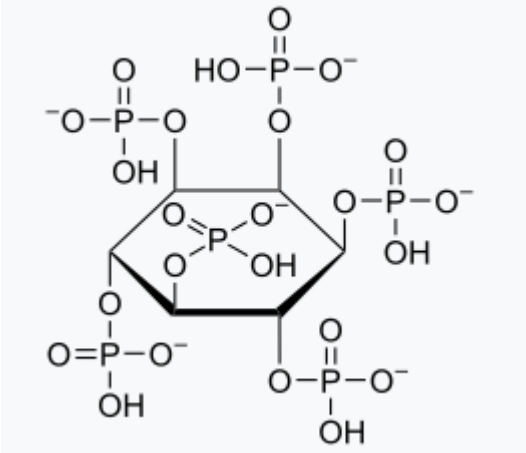


Image 5. Phytate molecule. Metabolites of phytic acid are called lower inositol polyphosphates. Examples are inositol penta- (IP5), tetra- (IP4), and triphosphate (IP3).

Methods

The hydrolysis of phytate occurring during the fermentative process of the making of EP200, was analyzed via chromatography. In this case, we used a post-column Hilic-liquid chromatography coupled with mass spectroscopy method to investigate a few of the metabolites produced from the hydrolysis of phytate from the microbial fermentation.

We followed the signal of myo-inositol and inositol-x-phosphate, likewise, glucose. Following the peak intensity signal of these molecules gives an indication of the extent of phytate hydrolysis during fermentation. The results are qualitative.

Results

The metabolite screening with chromatography showed an increase in glucose, myo-inositol and inositol-x-phosphate. Myo-inositol is backbone molecule of phytate (the molecule without any phosphate group), while inositol-x-phosphate is myo-inositol with only one phosphate substitution in an undetermined position). Both are the final products of the hydrolysis of phytate.

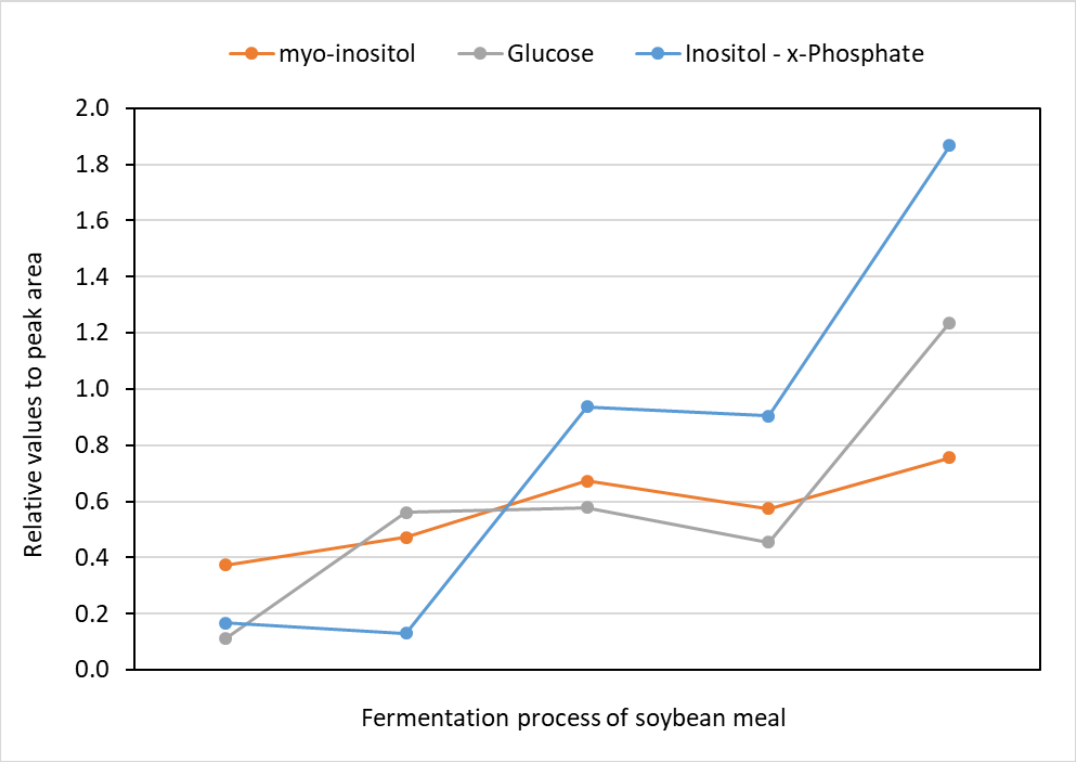


FIGURE 4. MYO-INOSITOL, INOSITOL-X-PHOSPHATE AND GLUCOSE INCREASE DURING THE FERMENTATIVE PROCESS.

CONCLUSIONS

The results indicate, that the microbial process during the fermentation reduces phytate to a high extent. This is based from following the increase of the two metabolites formed from the total hydrolysis of phytate.

REDUCTION OF SAPONINS

INTRODUCTION

Saponins are complex molecules found in many plants including soybeans. They are formed by a carbohydrate chain and steroids, steroid alkaloids or triterpenes. When dissolved in water they form a stable soapy froth. Because of the presence of both a hydrophilic (sugar) and hydrophobic (triterpenes and steroids) they act as emulsifiers and foaming agents. Soy saponins are divided in 2 groups: group A saponins have and undesirable astringent taste, typical for some soy products, and group B saponins have the health promoting properties.

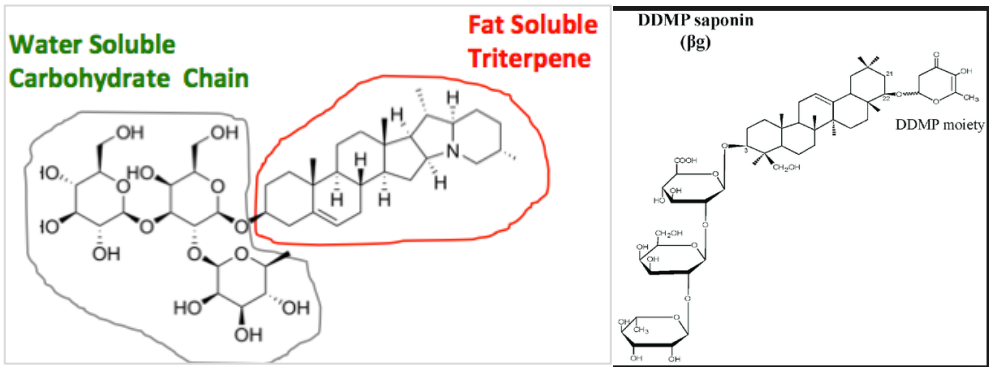


Image 6. Schematic representation of the backbone structure of saponins.

Methods

The analysis of saponins in EP200 was carried out by a commercial laboratory. The most common method for quantification of saponins is chromatography via high performance liquid chromatography using purified standards of saponin compounds.

Results

Saponin analysis showed a reduction of the different saponins analyzed at a range of 5-43 % depending of the saponin molecule. Ab saponins which are generally accepted to be toxic to fishes and to create the astringent flavor, reduced 33 % in EP200 in comparison with untreated soybean meal. Likewise, in the rest of the saponins, the molecules with a DDMP group (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) were also about 39 %.

TABLE 4. QUANTIFICATION OF DIFFERENT SAPONIN-COMPOUNDS IN SOY.

Soy saponins	*Ab*	*Ba-DDMP*	*Bb*	*Bb-DDMP*	*Bc*	*Bc-DDMP*	*Sum*
	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
Soybean meal	0.56	0.11	2.15	1.47	0.94	0.75	5.98
EP200 (Batch 1)	0.38	0.08	2.03	0.88	0.88	0.43	4.68
EP200 (Batch 2)	0.37	0.07	2.06	0.87	0.89	0.42	4.68
Reduction (%)	33.0	31.8	4.9	40.5	5.9	43.3	21.7

Conclusions

The fermentation process used in the making of EP200 is capable to reduce saponins up to 43 % in comparison with soybean meal which is already lower in saponins. Particularly the saponins that are causative of toxic effects in fishes are reduced to a major extent.

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