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Solubilisation of protein fractions induced by *Escherichia coli* phytase and its effects on *in vitro* fish digestion of plant proteins

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ABSTRACT

An *in vitro* assay simulating digestion in fish stomach was used to assess the effect of a bacterial phytase on different variables potentially affecting the digestibility of phosphorus and protein in eight plant ingredients: total protein solubility at different pH, differential solubility of protein fractions and potential bioavailability of amino acids after enzyme hydrolysis. Dephosphorylation of native phytate (IP6) significantly ($P<0.05$) increased protein solubility in all ingredients, with the exception of wheat flour. Such increase was measured both at acid and neutral pH in broad bean and peas, but only at acid pH in soybean and chickpea. The net effect was the result of increases in the solubility of specific protein fractions like convicilin, vicilin and legumin in peas and broad bean or glycinin and β-conglycinin in soybean. Minor increases in solubility as a result of IP6 hydrolysis by phytase were also identified in conglutin (lupin), gliadins and glutenins (wheat) and oleosin and napin (canola). *In vitro* assays evidenced that dephosphorylation of IP6 significantly affected ($P<0.001$) potential bioavailability of crude protein and phosphorus to a variable extent in the different ingredients tested. These results might help to get a better understanding of the mechanisms underlying IP6–protein interaction and the differences in potential bioavailability of proteins present in plant ingredients used in the formulation of aquafeeds.

1. Introduction

Aquafeed producers need to replace fishmeal by alternative protein sources due to its limited supply and high cost. Meals obtained from soybean, canola, broad bean, peas and lupin are some of the more interesting protein sources that can be potentially used in feeds for terrestrial and aquatic animals. However, one of the main constraints to their use is the presence of anti-nutritional factors like phytate (IP6), a salt of *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, which is present in variable proportions in plant seeds. Most of the phosphorus (P) bound to phytate (P–IP6) is excreted by fish into the water due to its low digestibility. In addition, this polyanionic molecule bounds to mineral cations, and also to cationic groups present in proteins and amino acids (AA). Since IP6 is a polyanionic molecule, its interactions with proteins are greatly affected by the pH (Cheryan, 1980). In an acidic environment, such as that existing in fish stomach, on

Abbreviations: AA, amino acids; BM, broad bean meal; CPI, chickpea protein isolate; FTU, phytase unit; IP6, *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; LUP, lupin meal; RSM, canola meal; OPA, *o*-phthaldialdehyde; P, phosphorus; PEAS, peas; SBM, soybean meal; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; WF, wheat flour; WM, wheat middlings.

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Table 1
Content of crude protein, total phosphorus (P) and P–phytate (P–IP6) as fed basis and enzymatic dephophorylation efficiency in the ingredients tested.

Plant ingredient	Dry Matter (g/kg)	Crude protein (g/kg)	Crude fat (g/kg)	Ash (g/kg)	Ca (g/kg)	Total P (g/kg)	P–IP6 (g/kg)	Ratio P–IP6/total P
Soybean meal[a]	880	470	19	62	2.9	6.4	4.2	0.66
Peas[b]	883	240	13	38	0.7	3.8	1.8	0.47
Broad bean meal[c]	881	250	19	23	0.7	5.3	3.0	0.57
Chickpea protein isolate[d]	925	900	17	35	1.8	8.7	4.5	0.52
Lupin meal[e]	908	430	89	36	2.9	3.2	1.8	0.56
Canola meal[f]	892	380	22	68	7.3	12.0	8.4	0.70
Wheat middlings[g]	880	150	35	48	1.2	9.5	7.4	0.78
Wheat flour[g]	892	110	20	6	0.5	3.5	1.0	0.29

[a] *Glicine max* supplied by Hamblet Protein, Horsens, Denmark.
[b] *Pisum sativum* supplied by Esteve Santiago SA, Valladolid, Spain.
[c] *Vicia faba* supplied by Esteve Santiago SA, Valladolid, Spain.
[d] *Cicer arietinum* supplied by Esteve Santiago SA, Valladolid, Spain.
[e] *Lupinus albus* supplied by Sel Chile S.A., Temuco, Chile.
[f] *Brassica napus* supplied by Esteve Santiago SA, Valladolid, Spain.
[g] *Triticum aestivum* (wheat middlings, 23% starch) supplied by Roquette, Laisa España, Barcelona, Spain.

which the pH is below the pKa of proteins, the anionic phosphate groups of IP6 are strongly bound to the basic amino acid residues of arginine, lysine and histidine (Cosgrove, 1966). It has been demonstrated that IP6 can inhibit pepsin activity *in vitro* by the formation of binary protein–IP6 complexes (Vaintraub and Bulmaga, 1991; Morales et al., 2011). In addition, the formation of ternary complexes IP6-divalent cation–protein is favoured in an alkaline environment such as this existing in the intestine (Vaintraub and Bulmaga, 1991). The susceptibility of a given plant protein to form such complexes with IP6, which are insoluble and quite resistant to the hydrolysis by digestive proteases (Richie and Garling, 2004) will depend on its molecular structure and more precisely on the relative presence of different amino-groups.

Phytases are a special class of phosphatases that catalyse the sequential hydrolysis of IP6 to less phosphorylated *myo*-inositol derivatives and inorganic phosphate. Most commercial microbial phytases react efficiently under the conditions present in the stomach. One of the main commercial phytases used in animal nutrition is produced from *Escherichia coli* and has two pH optima at 2.5 and 4.5 (Elkhalil et al., 2007). It has been demonstrated that the addition of phytase to plant ingredients used in fish nutrition improves P availability and also prevents binding of IP6 to protein, this resulting in an increased nutritive utilization (Storebakken et al., 1998). It has been also suggested that a number of factors such as pH, nature of the protein source and the presence of digestive proteases may determine the net effect of phytase on protein bioavailability within fish stomach (Morales et al., 2011). Details of the specific effects of such factors on the action of phytase efficacy have not been elucidated yet and, as indicated in the authoritative review performed by Kumar et al. (2011), more research is needed to obtain a better insight into the mechanisms underlying phytase–protein interactions and subsequent availability of proteins and AA after digestion in fish.

The present study was designed to assess the effects of native IP6 in the digestive bioavailability of proteins present in different plant ingredients, currently or potentially used in commercial aquafeeds, by evaluating changes in: (i) the solubility profile of total protein and native IP6 under different pH, (ii) the solubility of specific protein fractions and (iii) the release of AA and soluble P after digestion by acid proteases using an *in vitro* model simulating fish stomach.

2. Materials and methods

2.1. Ingredients and enzymes

Enzymatic dephosphorylation of IP6 was carried out using a bacterial 6–phytase, EC 3.1.3.26, from *E. coli* expressed in *Pichia pastoris* (Quantum Phytase 2500 XT; AB Enzymes, Darmstadt, Germany). A preliminary semi–purification using ammonium sulphate was carried out to eliminate additives and excipients of the commercial product (Wingfield, 1998). Phytase was extracted by diluting 1 g of product in 5 mL of 200 mM sodium acetate buffer, pH 5.5, at 4 °C and mixing overnight the extract with $(NH_4)_2SO_4$ at 75% saturation. The precipitate was then collected by centrifugation at 14,000 × *g* for 30 min, suspended in 200 mM sodium acetate buffer, pH 5.5 and dialyzed 12 h at 4 °C against the same buffer solution. Residual phytase activity and soluble protein were determined in dialysate, being this frozen at −20 °C until use.

The *in vitro* assay simulating conditions in fish stomach was performed using gastric enzyme extract obtained from rainbow trout *Oncorhynchus mykiss*. Fish sampling and preparation of enzyme extracts were carried out as described by Morales and Moyano. (2010). Assays were conducted on eight selected plant sources commonly used as ingredients in fish feeds (Table 1): soybean meal, SBM (*Glycine max*); peas, PEAS (*Pisum sativum*); broad bean meal, BM (*Vicia faba*); chickpea protein isolate, CPI (*Cicer arietinum*); lupin meal, LUP (*Lupinus albus*); canola meal, RSM (*Brassica napus*); wheat middlings, WM; wheat flour, WF (*Triticum aestivum*).