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1,3-1,4-β-Glucanases and not 1,4-β-glucanases improve the nutritive value of barley-based diets for broilers

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ABSTRACT

Barley-based diets contain a significant proportion of highly soluble 1,3-1,4-β-glucans that display anti-nutritive properties for monogastric animals, in particular for poultry. Cleavage of mixed linked glucans by the addition of exogenous enzymes leads to a significant reduction in the degree of polymerization of the polysaccharide and a consequent reduction of digesta viscosity. Plant cell wall 1,3-1,4-β-glucans may be hydrolyzed by 1,3-1,4-β-glucanases (EC 3.2.1.73) or by 1,4-β-glucanases (EC 3.2.1.4), generally termed cellulases, although it remains unknown which enzymes are more effective in the degradation of feed mixed linked anti-nutritive glucans. Here we compared the capacity of two recombinant *Clostridium thermocellum* enzymes, 1,3-1,4-β-glucanase 16A (*Ct*Glc16A) and 1,4-β-glucanase 8A (*Ct*Cel8A), to improve the nutritive value of barley based diets for broilers. As already shown in a previous study (Ribeiro et al., 2012), *Ct*Glc16A is very effective to improve the performance of broilers fed with a highly viscous barley-based diet. In contrast, although remaining active and retaining its molecular integrity during passage through the GI tract, *Ct*Cel8A was unable to affect the nutritive value of the cereal based diet. *In vitro* studies revealed that *Ct*Glc16A and *Ct*Cel8A are equally effective in reducing the viscosity of a purified 1,3-1,4-β-glucan solution. However, the capacity of *Ct*Cel8A to cleave 1,3-1,4-β-glucans is significantly affected by the presence of the barley-based feed. Taken together the results suggest that *in vivo* 1,4-β-glucanases tend to act preferentially on cellulosic substrates and not on mixed linked glucans. Although further work is required to extend these observations to other cellulases, the data suggest that exogenous 1,3-1,4-β-glucanases but not 1,4-β-glucanases are obligatory enzymes to improve the nutritive value of barley based diets for broilers.

Abbreviations: GI, gastro intestinal; *Ct*Glc16A, 1,3-1,4-β-glucanase 16A; *Ct*Cel8A, 1,4-β-glucanase 8A; NSPs, non-starch polysaccharides; GH, glycoside hydrolase; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl 1-thio-β-D-galactopyranoside; SDS–PAGE, Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis; PC, phosphate citrate; DNSA, 3,5-DiNitroSalicylic Acid; MES, 2-N-morpholino-ethanesulfonic acid; DTT, dithiothreitol; BW, body weight; FCR, feed conversion ratio.

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1. Introduction

Cereal-based diets contain high levels of soluble non-starch polysaccharides (NSPs) that significantly affect the efficiency of the digestive process, impairing animal performance (Smits and Annison, 1996; Choct, 1997). Barley incorporation in poultry diets is limited by its high content in NSP, mainly soluble 1,3-1,4-β-glucans, which upon solubilization lead to an increase in digesta viscosity. An increase in digesta viscosity affects the interaction of the endogenous digestive enzymes with their target substrates, thus decreasing animal growth (Smits and Annison, 1996; Pettersson and Aman, 1989; Jozefiak et al., 2006). To reduce the negative effects associated with the presence of dietary 1,3-1,4-β-glucans, commercial enzyme mixtures expressing cellulase and hemicellulase activities are widely used to supplement broiler diets (Bedford and Morgan, 1996). Exogenous feed enzymes contribute to the hydrolysis of 1,3-1,4-β-glucans, leading to a reduction in digesta viscosity, an improvement of nutrients digestibility and an increase in feed intake (Bedford and Morgan, 1996; Choct, 2006).

Exogenous enzymes used to supplement poultry diets are usually obtained from the fermentation of cellulolytic fungi and consequently display a large range of polysaccharide specificities. However, enzymes required to improve the nutritive value of barley based diets for poultry need to cleave, specifically, 1,3-1,4-β-glucans. This highly soluble carbohydrate may be degraded by different types of glycoside hydrolases (GH; EC. 3.2.1), but in particular by 1,3-1,4-β-glucanases and 1,4-β-glucanases. Based on primary sequence similarities, GH are classified in families (Henrissat, 1991) and presently there are 133 families of GHs in the constantly updated Carbohydrate Active enZyme (CAZy) database (www.cazy.org). Enzymes expressing strict specificity for 1,3-1,4-β-glucans (EC 3.2.1.73), also generically termed by 1,3-1,4-β-glucanases, are currently grouped in GHs families 5, 7, 8, 9, 11, 12, 16, 17 and 26. In contrast, enzymes that participate in the cleavage of 1,4-β-glucans (EC 3.2.1.4), which are generally termed cellulases or 1,4-β-glucanases, belong to GHs families 5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 51, 74, 124 and 131.

β-Glucan is a major cell wall polysaccharide of barley endosperm cells and consists of a backbone of glucose residues connected by 1,4-β and 1,3-β-glycosidic bonds, generating a linear polysaccharide more soluble than cellulose (Xue et al., 2003). In barley β-glucan, 1,4-β linkages predominate with a ratio of 1,3-β to 1,4-β linkages of approximately 1:2.5 (Xue et al., 2003; Jamar et al., 2011). While 1,3-1,4-β-glucanases do not hydrolyze cellulose, 1,4-β-glucanases (cellulases) are able to degrade both cellulosic substrates and also the more soluble 1,3-1,4-β-glucans, by acting on the abundant 1,4-β-linkages of the mixed linked carbohydrate (Xue et al., 2003). The efficacy of enzyme mixtures currently used to supplement barley-based diets is presently evaluated by the levels of their 1,3-1,4-β-glucanase activity. However, it is usually unknown which GHs contribute to the total 1,3-1,4-β-glucanase activity of commercial enzyme mixtures; if the more specific 1,3-1,4-β-glucanases or the general 1,4-β-glucan cutters generally referred to as cellulases. In addition, it also remains to be established which enzymes are more effective in the hydrolysis of feed β-glucans. Although we have previously shown that a recombinant family 16 1,3-1,4-β-glucanase from *Clostridium thermocellum* is highly effective in improving the nutritive value of barley based-diets for broilers, it remains unknown if 1,4-β-glucanases are also competent enzymes to reduce the negative effects associated with the presence of 1,3-1,4-β-glucans.

Here, we have compared the capacity of a highly specific 1,3-1,4-β-glucanase and a typical 1,4-β-glucanase to improve the nutritive value of a barley-based diet for broilers. The enzymes were selected from the anaerobic thermophilic bacterium *C. thermocellum* and consisted in the previously described family 16 1,3-1,4-β-glucanase A (Ribeiro et al., 2012), *Ct*Glc16A, and the family 8 1,4-β-glucanase A, *Ct*Cel8A, also known as cellulase 8A (Cornet et al., 1983). The two enzymes were recombinantly expressed in *Escherichia coli*, purified to become free of other side activities, and used to supplement a barley based diet for broilers.

2. Materials and methods

2.1. Gene isolation and cloning into a prokaryotic expression vector

The thermostable 1,3-1,4-β-glucanase of *C. thermocellum*, termed *Ct*Glc16A (Ribeiro et al., 2012), is a modular enzyme containing an N-terminal glycoside hydrolase family 16 catalytic domain and a C-terminal dockerin. The gene was isolated and cloned into pET21a as described previously (Ribeiro et al., 2012) to generate pGH16. The thermostable 1,4-β-glucanase of *C. thermocellum*, termed *Ct*Cel8A, is also a modular enzyme containing an N-terminal glycoside hydrolase family 8 catalytic domain and a C-terminal dockerin (Cornet et al., 1983). The gene encoding *Ct*Cel8A (residues 396–1734) was amplified from *C. thermocellum* genomic DNA through PCR using the NZYProof DNA polymerase (NZYTech Ltd, Portugal) and the following primers: 5′-CTC**CATATG**GCAGGTGTGCCTTTTAAC-3′ and 5′-CAC**GGATCC**CTAATAAGGTAGGTGGGG-3′. Primers included *Nde*I and *Bam*HI engineered restriction sites (in bold) that allowed the subsequent cloning of the resulting nucleic acid into similarly restricted pET21a, generating the plasmid pGH8. Recombinant *Ct*Glc16A and *Ct*Cel8A contained an engineered C-terminal His6 tag thus allowing the direct purification of the two polypeptides by immobilized metal affinity chromatography (IMAC).

2.2. Expression and purification of CtGlc16A and CtCel8A

To express the proteins, *E. coli* BL21(DE3) cells harboring the appropriate recombinant plasmid, pGH16 or pGH8, were cultured in LB medium containing ampicillin (100 $\mu g\,ml^{-1}$) at 37 °C to mid-exponential phase (A_{600} 0.4). At this point,