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Application of the rat liver lysosome assay to determining the reduction of toxic gliadin content during breadmaking

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ABSTRACT

Enriched caricain was able to detoxify a major proportion of the gliadin in wholemeal wheat dough by allowing it to react for 5 h at 37 °C during the fermentation stage. A reduction of 82% in toxicity, as determined by the rat-liver lysosome assay, was achieved using 0.03% enzyme on weight of dough. Without enzyme, only 26% reduction occurred. The difference in reduction of toxicity achieved is statistically significant ($p < 0.01$). The results are very similar to those obtained in our previous work using an immuno assay and the same enzyme preparation. They confirm the value of caricain as a means of reducing the toxicity of gliadin and open the way for enzyme therapy as an adjunct to the gluten free diet. This approach should lead to better control over the elimination of dietary gluten intake in conditions such as coeliac disease and dermatitis herpetiformis.

1. Introduction

The prolamin group of proteins in wheat, rye, barley, triticale and oats are causative agents in coeliac disease (CD). These proteins cause damage to the small intestine of patients with CD which results in malabsorption of nutrients (McGough, 2005; Thompson, 1997). The incidence of CD is approximately 1% in both the United States and Europe (Molodecky et al., 2012; Rubio-Tapia, Ludvigsson, Brantner, Murray, & Everhart, 2012). The disease is of considerable interest in those countries in which the offending cereals are consumed. The symptoms normally observed are abdominal pain, bloating, diarrhoea, nausea and cramps. General malaise and tiredness are also common. These symptoms are often noticed in childhood and if left undiagnosed, can result in serious health problems including osteoporosis, anaemia, lymphomas and gastrointestinal cancers (Frick & Olsen, 1994). The primary treatment is the introduction of a gluten-free diet (Grodzinsky, Franzen, Hed, & Strom, 1992). Studies of the mechanism of the reaction to gluten have been important in research for alternative and supplementary treatments for CD. Comparison of remission coeliac and normal mucosal digestion of certain peptides have indicated defective digestion by remission coeliac mucosa (Cornell & Rivett, 1995). They found that synthetic peptides based on the A-gliadin sequence, were not completely digested by remission coeliac mucosa. Residues of toxic octapeptides remain from the latter digestion indicating that there is defective digestion in the small intestine of coeliacs, which is not the case in those who do not suffer from this condition.

Other workers have pointed out the difficulty of digesting certain proline-rich peptides that produce immunological reactions leading to intestinal damage (Hausch, Shan, Santiago, Gray, & Khosla, 2002). The 33-mer peptide of α-2 gliadin (Shan et al., 2002) is one such peptide that is difficult to digest, but the antigenicity of this peptide was reduced by the enzyme prolyl oligopeptidase (EC 3.4.21.26).

The gliadin digest that is prepared from the 67% ethanol extract in this work will be similar to the toxic mixture of oligopeptides obtained from gliadin in previous work (Townley, Cornell, Bhathal, & Mitchell, 1973) is a complex mixture of oligopeptides of average molecular weight 1500 Daltons. A fraction of this digest (Fraction 9), obtained by ion-exchange chromatography was shown to contain the most toxic oligopeptides when presented in the culture medium of biopsy specimens from patients with CD (Townley et al., 1973). Using the rat liver lysosome assay it was confirmed that the Fraction 9 was the most toxic of the ion-exchange fractions (Cornell & Townley, 1973). This gave confidence in the use of this assay as a screening test for the toxicity. Furthermore, it was shown later that the assay is well correlated with the foetal chick assay of (Mothes, Mühle, Müller, &

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Hekkens, 1985). In the caricain-treated 67% ethanol extract, we wish to determine if the toxicity of this digest has been reduced by the caricain.

A unified hypothesis (Cornell & Stelmasiak, 2007) recognised the involvement of immunological factors as well as an enzyme deficiency in CD. Within the context of this principle, the use of enzymes as a means of correcting an enzyme deficiency has become the subject of recent studies by our group. Our initial trials used an extract of porcine intestine and showed for the first time that enzyme therapy could become a complementary treatment to the gluten-free diet (Cornell et al., 2005). However, as a marketable preparation, we turned to the plant enzyme caricain (EC 3.4.22.30), which was identified as the active principle in papaya oleoresin for detoxification of gliadin. This was verified by lysosome assays on a highly purified caricain preparation (Cornell & Stelmasiak, 2011). Since then, the value of caricain extracts has been demonstrated in a clinical trial on patients with dermatitis herpetiformis (Zebrowska et al., 2014) a disease also involving a susceptibility to gluten, but which manifests itself in the form of a skin rash, blisters and itchiness (Fry, 1992; Kotze, 2013). The present work follows experiments (Buddrick, Cornell, & Small, 2015) in which it was shown that an enriched caricain preparation was able to considerably reduce the amount of immunoactive gliadin in wholemeal bread by allowing it to react in the dough during proofing. Accordingly, the purpose of the studies reported here has been to evaluate whether a corresponding reduction in toxic gliadin might be observed using the lysosome assay, which has been used in many of our previous studies for testing detoxification of gliadin by enzymes (Cornell, Doherty, & Stelmasiak, 2010; Cornell & Stelmasiak, 2011). It builds on other work using caricain as a mean of detoxifying gliadin, referred to as enzyme therapy.

2. Materials and methods

2.1. Preparation of enriched enzyme

The procedure was based on that previously described (Buddrick et al., 2015). A column (3.2 × 20 cm) of CM Sephadex C-50 (Pharmacia, Sweden) was equilibrated with 0.02 mol/L phosphate buffer adjusted to pH 4.6 in a cold room at 5 °C. A sample (2.5 g) of dry papaya latex, MG 50.000 (Enzyme Solutions, Pty Ltd, Melbourne) dissolved in starting buffer (30 mL) was applied to the column and the eluate monitored for protein at 280 nm. After the unabsorbed material eluted, the pH was increased with a pH 6.5 phosphate buffer (0.02 mol/L). Further elution using the same buffer involved application of a gradient from 0.1 to 0.3 mol/L sodium chloride in the phosphate buffer, followed by 0.8 mol/L NaCl in the same buffer. Fractions corresponding to the peaks at 280 nm were monitored, but only those obtained with the latter eluant were dialysed against distilled water (×3 changes) and freeze dried to obtain enriched caricain. The yield obtained was 16% of the applied crude papaya latex. Assays of the enriched enzyme were carried out using an established method (Gravett, Viljoen, & Oosthuizen, 1991), based on the use of benzoylarginine *p*-nitroanilide (BAPNA).

2.2. Preparation of bread doughs

Organically grown wheat grain of mixed variety (11.7% protein, dry basis) from the Laucke Flour Mill (Bridgewater, Victoria, Australia) was freshly milled to provide wholemeal flour for breadmaking. The mill used was a bench top unit (Grain Master Whisper Mill, Korea) which uses upright blades spinning at high speed (10,000 rpm) producing a relatively fine meal with increased surface area. For dough preparation, a bench mixer with 10 different speeds (Kitchen Aid Heavy Duty, Model 5KPM50, Benton Harbour, USA) was utilised. The meal (100%), water (70%), red palm oil (5%), salt (2%) and instant dry yeast (0.2%) were firstly mixed at slow speed (setting 2, 4 min), followed by fast speed (setting 7, 6 min) until a dough was achieved. For samples treated with enriched caricain was incorporated (0.03% on a dough weight basis) after complete dough development had been achieved, using a slow mixing speed. The wheat dough was bulk fermented (5 h) at a temperature of 37 °C. The two doughs of interest (with enzyme added and control without) were weighed (180 g) and placed into bread tins prior to the final proof at 37 °C for 45 min. Baking was achieved at 230 °C for 10 min followed by a further 15 min at 200 °C in order to bake the bread evenly without causing an increase in crust colour.

2.3. Extraction of gliadin from bread samples

Samples of bread (6.00 g) made without enriched caricain and with 0.03% enriched caricain (on dough weight) were mixed with 18 mL of aqueous ethanol (80% v/v). This gave an ethanol concentration of approximately 67% v/v, and samples were extracted for 48 h at room temperature with constant agitation in order to extract prolamins (Mothes, Osman, Seilmeier, & Wieser, 1999). The mixture was then filtered (Whatman No. 1) and the filtrate rotary evaporated at 45 °C. The dry material from each extraction was then treated with pepsin, trypsin and pancreatin (Cotazym) as previously described (Cornell et al., 2010) and each digestion product diluted to 3.00 mL with phosphate-buffered saline (PBS). In each case, the concentration of gliadin present was approximately 50 mg/mL, estimated from the protein content of the flour used for aqueous ethanol extraction.

2.4. Preparation of rat liver lysosomes

Lysosomes were prepared from livers from two Wistar rats as described previously (Sawant, Desai, & Tappel, 1964). The lysosome fraction was dispersed in 2.0 mL of PBS and the concentration adjusted so that a 0.10 mL aliquot in 3.0 mL PBS gave an absorbance reading of 0.8 at 405 nm in a UV/visible spectrophotometer. This suspension of lysosomes was used in all the assays described.

2.5. Lysosome assay

Each of the bread extracts (0.10 mL) was incubated with the suspension of lysosomes for 1.5 h at 37 °C and then mixed with 3 mL of PBS before reading the absorbance at 405 nm. Controls without incubation were also run and calculations made of the percentage reduction in absorbance based on the means of triplicate incubations and controls as described previously (Cornell et al., 2010). A large reduction in absorbance indicates a high degree of toxicity. For the purpose of comparison, incubations of the suspension of lysosomes (0.10 mL) were also carried out in a series of tubes containing a peptic-tryptic-pancreatic digest of gliadin (0.10 mL), each tube containing 5, 3 or 1 mg of gliadin. These latter incubations were important as they could be used to estimate the amount of gliadin in each bread extract, using a calibration curve. The detoxification achieved (*D*%) was calculated from:

$$D = [\text{initial gliadin (mg)} - \text{gliadin from calibration curve (mg)}] \times 100/\text{initial gliadin (mg)},$$

where the initial gliadin was 5 mg (0.1 mL of 50 mg/mL solution). The calibration curve was plotted from known amounts of gliadin using an Excel program.