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The effect of legume protease inhibitors on native milk and bacterial proteases

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ABSTRACT

Protease inhibitors from legume seed extracts (soybean, cowpea and marama beans) and purified soybean protease inhibitor were evaluated with regards to their abilities to inhibit proteases produced by important milk contaminating bacteria, i.e. *Bacillus* spp. and *Pseudomonas* spp., and native milk protease, plasmin. Although heat treatment is the most common mean of inactivating enzymes, some heat-stable enzymes can survive the ultra-high temperature (UHT) processing of milk and cause sensory and consistency defects during storage at room temperature. The legume protease inhibitors reduced the activity of plasmin and proteases produced by *Bacillus* spp. by up to 94% and 97%, respectively, while it showed low inhibitory activity towards *Pseudomonas fluorescens* proteases (19%) in a buffer system. The protease inhibitors reduced the activity of plasmin (41%) and *Bacillus* proteases (50%) in UHT milk, however to a lesser extent as compared to inhibition in the buffer system; while it had little or no effect on proteases form *Pseudomonas* spp. Legume protease inhibitors show great potential in preventing or reducing proteolytic activity of *Bacillus* proteases and plasmin and may be exploited in various applications where these proteases cause sensory or consistency defects in the product.

1. Introduction

Proteolysis of UHT milk during storage at ambient temperature limits both the shelf life and market potential of the milk (Datta & Deeth, 2001) and has been attributed to extracellular heat-stable proteases produced by psychrotrophic bacterial contaminants of raw milk and natural milk alkaline serine protease, plasmin (Datta & Deeth, 2003; Grufferty & Fox, 1988; Visser, 1981). Protease activity in UHT milk is associated with changes in the flavour and viscosity of the milk, with the eventual formation of a gel (Chen, Daniel, & Coolbear, 2003; Datta & Deeth, 2003; McMahon, 1995). Off-flavours, such as bitterness, in UHT milk are associated with the release of tyrosine in the milk (Datta & Deeth, 2003; Gebre-Egziabher, Humbert, & Blankenagel, 1980), while viscosity changes are associated with the hydrolysis of caseins (Chen et al., 2003; Datta & Deeth, 2003). During the course of the latter, the enzymes release the β-lactoglobulin–κ-casein complex (βκ-complex), formed during heat treatment, from the casein micelle. Subsequent aggregation of the released βκ-complexes forms a three-dimensional cross-linked protein network, which causes gelation of the milk (McMahon, 1995).

Bacterial contaminants are mainly introduced into milk within the interior of the udder, the cow's teats and milking and storage equipment (Law & Mabbitt, 1983). Contaminating milk bacteria often isolated from refrigerated milk and associated with proteolysis in milk are mainly species from *Pseudomonas*, particularly *Pseudomonas fluorescens* (Fairbairn & Law, 1986; Kohlmann, Nielsen, & Ladisch, 1991; Matselis & Roussis, 1998). In addition, *Bacillus* species are abundant in the environment and can contaminate milk during production, handling and processing (Matta & Punj, 1999; Phillips & Griffiths, 1990). Although the contaminating milk bacteria are mainly psychrotrophs that are eliminated by the UHT process, many of their enzymes survive and remain active in the derived dairy products where they can cause problems during storage (Chen et al., 2003; Sørhaug & Stepaniak, 1997). The plasmin system is complex and not only comprise plasmin, but also plasminogen (plasmin precursor), plasminogen activators, plasmin inhibitors and inhibitors of plasminogen activators (Upadhyay, McSweeney, Magboul, & Fox, 2004). Plasminogen is more heat stable than its active form and can be activated by cleavage of a single peptide bond by even more heat-stable activators (Aroonkamonsri, Aroonkamonsri, & Kakuda, 1996; Lu & Nielsen, 1993).

Various studies show the potential of protease inhibitors in preventing or reducing adverse effects of proteases in food

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products. Examples of this include the use of α-2-macroglobulin to inhibit protease activity in various fish species that previously prevented the use of these species in processed fish products (Lorier & Aitken, 1990), and the use of protease inhibitors to improve the gel properties of frozen squid muscle (Peréz-Mateos, Montero, & Gómez-Guillén, 2002). Legume seeds contain protease inhibitors with the ability to inactivate proteases by various mechanisms. The objective of this study was to examine the effect of protease inhibitors from legume seeds, i.e. soybeans (*Glycine max* (L.) Merr), marama beans (*Tylosema esculentum* (Burch) A. Schreib) and cowpeas (*Vigna unguiculata* (L.) Walp), on the proteolytic activity of native milk protease plasmin and proteases from bacterial contaminants previously isolated from milk, all known to cause adverse effects during the storage of UHT milk.

2. Materials and methods

2.1. Materials and chemicals

Savinase and Alcalase were supplied by Novozymes, Johannesburg, South Africa. Plasmin, bovine trypsin, azocasein, bovine serum albumin (BSA), purified soybean trypsin inhibitor and Tyr-Leu were obtained from Sigma–Aldrich, Kempton Park, South Africa. The rest of the chemicals and standards were of analytical grades and obtained from Sigma–Aldrich or Merck (Johannesburg, South Africa) unless otherwise stated.

2.2. Protease inhibitor extraction

2.2.1. Preparation of flours

Seeds from marama bean were dehulled using a cracker (WMC Sheet Metal Works, Tzaneen, South Africa) and soybeans were dehulled using a Tangential Abrasive Dehulling Device (TADD). After the dehulled seeds were milled in a food blender, the flour was defatted twice with n-hexane (1:5 m/v) for an hour each time, air-dried and milled again. This process was repeated and defatted flour was placed in a fume hood overnight to remove the remaining hexane. The defatted flour was milled again to pass through a 1000 μm mesh. Due to the low fat content of cowpeas, it was milled to pass through a 1000 μm mesh and used for protein extraction without any defatting.

2.2.2. Protein extraction

The protein was extracted following the protocol of Maggo, Malhotra, Dhawan, and Singh (1999), with a few modifications. Flours were extracted with 0.1 M phosphate buffer, pH 7.5, at a ratio (flour:buffer) of 1:20 (m/v) for 4 h at room temperature in a shaking water bath. The suspension obtained was centrifuged at 10 000 × *g* for 30 min and the supernatant was collected and used for determining the protein and total phenolic contents and the trypsin inhibitor activity.

2.3. Characterisation of protein extract

2.3.1. SDS-PAGE

The method described by Taylor, Bean, Ioerger, and Taylor (2007) was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots of protein extracts (15 μL) containing 15 μg protein was loaded onto 4–12% polyacrylamide gradient gels. Gels were stained with Coomassie Brilliant Blue R-250.

2.3.2. Native-PAGE

Native-PAGE was performed using the NativePAGE™ Novex® Bis-Tris Gel system (Invitrogen™, Johannesburg, South Africa). The Native-PAGE gels were stained for trypsin inhibitor activity as described by Ee, Zhao, Rehman, and Agboola (2008).

2.3.3. Quantification of protein concentration in crude protein extracts

The method of Bradford (1976), with the following modifications, was used to determine the protein concentration of the crude protein extracts. Aliquots of these extracts (20 μL) were pipetted into the wells of a 96-well polystyrene microtiter plate, followed by addition of 40 μL of Bradford's Reagent. The final volume in the wells was made up to 200 μL with the addition of dH_2O and the absorbance was measured at 595 nm against a reagent blank. A BSA standard curve was prepared and the protein concentration in the crude protein extracts was determined using the standard curve. Results were expressed as mg protein per mL crude extract.

2.3.4. Quantification of total phenolic content in crude protein extracts

The total phenolic content in the crude protein extracts was determined using the Folin-Ciocalteu procedure described by Hagerman, Harvey-Mueller, and Makkar (2000). A catechin standard curve was prepared and used to determine the total phenolic content in the protein extracts. Results were expressed as catechin equivalents (CE, mg catechin equivalents/100 mg sample) on dry basis. The dilution factor of flour to buffer (1 g: 20 mL) were taken in account in calculating the dry basis of the protein extracts.

2.3.5. Quantification of trypsin inhibitor activity in crude protein extracts

Equal volumes of protein extracts (60 μL) and trypsin (375 protease units (PU)/mL) were mixed and incubated for 1 h at 25 °C. The activity of trypsin inhibitor was assayed by determining the residual trypsin activity following the method of Secades and Guijarro (1999) using azocasein as substrate and bovine trypsin as standard enzyme. A soybean trypsin inhibitor standard curve was prepared and used to determine the trypsin inhibitor activity (TIA) in the protein extracts. Results were expressed as trypsin inhibitor units (TIU) per mL by determining the trypsin units inhibited per ml extract relative to that of controls that contained only enzyme and no crude extract.

2.4. Inhibition of bacterial protease and plasmin activity in buffer

2.4.1. Bacterial protease inhibition assay

Alcalase and Savinase are alkaline proteases (E.C. 3.4.21.62) produced by *Bacillus licheniformis* and *Bacillus lentus*, respectively. *P. fluorescens* (ATCC 13525) (Quantum Biotechnologies, Randburg, South Africa) was cultured in tryptone soy broth (TSB) for 3 days at 25 °C. Bacterial cells were removed by centrifugation at 24 000 × *g* for 10 min at 5 °C. The resulting supernatants were stored at −20 °C until used as crude protease source.

One volume (60 μL) of either Savinase, Alcalase or *P. fluorescens* crude protease (at activities of 375 PU/mL as determined by the azocasein assay (Section 2.3.5) was mixed with one volume of crude extract from either soybeans or marama beans or cowpeas or purified soybean trypsin inhibitor at concentrations of 200, 287.5 and 375 TIU/mL. Inhibition of the bacterial proteases by the protease inhibitors from the various legume seeds was evaluated using the same method described in Section 2.3.5 except that bovine trypsin was replaced by the bacterial proteases. One unit of enzyme activity was defined as the amount that yielded an increase of 0.01 in the absorbance at 420 nm in 30 min at 30 °C. The protease inhibitor activity (PIA) was defined as the percentage of protease units inhibited (PUI) relative to that of controls that contained only