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Specificity of action of an insect proteinase purified from wheat grain infested by the New Zealand wheat bug, *Nysius huttoni*

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Abstract

A salivary proteinase from the New Zealand wheat bug (*Nysius huttoni*) was partially purified and analysed for substrate specificity by a variety of techniques on the following proteins: wheat, rye, barley and corn proteins, haemoglobin, bovine serum albumin, cytochrome c, cytochrome c oxidase, elastin, collagen, gelatine, keratin (hide powder), fibrin, azo-casein, α-casein, β-casein and κ-casein. The only proteins substantially hydrolysed (> 50%) by *Nysius*-proteinase were the high M_r glutenin subunits of wheat, the high M_r secalin and M_r 60,000 γ-secalin of rye, the D-hordeins of barley, the M_r 70,000, M_r 66,000 and M_r 58,000 C hordeins, and β-casein subunit of bovine milk. Sequence analysis of the peptide products of enzyme reaction on high M_r glutenin subunits, β-casein and κ-casein revealed that glutamine occupied the P1 position relative to the scissile bond at all cleavage sites. Proline in the P3 or P4 position, and particular residues in the P_1' position relative to the scissile bond may also be preferred structural features. A variety of fluorogenic substrates made from synthetic peptides with glutamine in the P1 position relative to the fluorogenic group were tested with the enzyme, but none reacted.

Keywords: Proteinase; Enzyme specificity; Wheat bug; *Nysius huttoni*; Cleavage site; Scissile bond; Cereal proteins; Casein

1. Introduction

The New Zealand wheat bug (*Nysius huttoni*) injects a salivary proteinase into immature wheat kernels while feeding. This proteinase remains in flour made from bug-damaged wheat and, in dough, digests gluten to produce slack, sticky dough and poor quality bread (Cressey and McStay, 1987; Every et al., 1989; Meredith and Best, 1985). The insect also injects proteases into immature seeds of a range of other plant species, inflicting various amounts of physical and chemical damage to the seeds (Every and Stufkens, 1999). Early attempts to develop an assay (Cressey, 1987; Cressey and McStay, 1987; Every et al., 1989; Every and Stufkens, 1999; Meredith and Best, 1985) showed that the enzyme did not act on several proteins and synthetic substrates commonly used for assay of proteases, such as haemoglobin, gelatine, bovine serum albumin, cytochrome C, Nα-benzoyl-L-arginine ethyl ester and benzoyl-L-tyrosine ethyl ester. The enzyme, however, was shown to specifically hydrolyse the high M_r glutenin subunits of wheat glutenin (Cressey, 1987; Cressey and McStay, 1987; Swallow and Every, 1991). Consequently a micro-assay for *Nysius*-proteinase was developed using acetic acid-insoluble glutenin (Every, 1990) or gluten (Every, 1991) as a substrate, and the enzyme was purified and characterised as a serine proteinase (Every, 1993).

This paper reports the activity of a proteinase from wheat damaged by *N. huttoni* on a range of proteins and synthetic fluorogenic peptide substrates. The site of *Nysius*-proteinase action on high M_r glutenin subunit 2, β-casein and κ-casein was also determined.

Abbreviations GS2, High M_r glutenin subunit 2; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed phase high pressure liquid chromatography; SDS, sodium dodecyl sulphate.

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2. Experimental

2.1. Enzymes, proteins and proteinase assays

Nysius proteinase was extracted from wholemeal flour of the New Zealand wheat cultivar, Otane, which was severely damaged by *N. huttoni*, and partially purified by ion exchange and hydrophobic interaction chromatography (Every, 1993). Proteinase activity was measured quantitatively by a SDS-protein gel assay (Every, 1991), and the specific activity of the enzyme preparation was determined as 56,000 Units/mg protein. Papain and α-chymotrypsin (Sigma Chemical Co.) were used as comparative controls in some assays. Dilute acetic acid-soluble glutenin (Sol-glutenin), dilute acetic acid-insoluble glutenin (Insol-glutenin) and gliadin were extracted from vital wheat gluten (Bunge, Australia) by the modified Osborne procedure described by Chen and Bushuk (1970). High M_r glutenin subunit 2 was isolated from wheat flour (cv. Kotare) by reversed phase (RP)-HPLC (Sutton, 1991). High M_r secalin of rye (cv. Gazelle) and D-hordein of barley (line Riso 1508) were isolated by ion exchange and gel permeation chromatography (Shewry et al., 1988). The glutenin, seculin and hordein proteins were reduced and pyridylethylated (Friedman et al., 1970) before chromatography. Proteins extracted from whole grain flour for SDS-PAGE were from wheat (cv. Kopara), barley (line CFL1831.1), rye (cv. Amelio) and sweet corn (cv. Early Chief). Gelatine was from Davis Gelatine (NZ) Ltd. All other proteins were from Sigma.

Proteinase action on protein substrates was analysed by a SDS-protein gel assay (Every, 1991), a radial diffusion of enzyme in substrate-agar-gel method (Schumacher and Schill, 1972), SDS-PAGE (Every et al., 1998), acid-PAGE (Du Cros and Wrigley, 1979) and alkaline urea-PAGE (Creamer, 1991). Synthetic fluorogenic peptides were made by D. Harding (Massey University, NZ) and tested as protease substrates using a Shimadzu Fluorimeter. For all analyses 0.05 M Tris–HCl buffer (pH 8.9) was used for *Nysius* proteinase and 0.05 M Tris–HCl buffer containing 2 mM $CaCl_2$ (pH 7.8) was used for α-chymotrypsin. All tests were made in duplicate or triplicate.

2.2. Preparation of samples for gel electrophoresis

For SDS-PAGE, the samples (25 mg whole grain flours or 1 mg pure protein) were incubated at 37 °C for 2 h in 0.1 ml of 0.05 M Tris–HCl buffer containing 1000 U *Nysius* proteinase or 1 μg α-chymotrypsin where applicable. After incubation, the proteins and hydrolysis products were extracted by incubating the mixtures for 16 h at 40 °C with 0.1 ml of 4% SDS, 24% glycerol, 0.14 M Tris, 1% dithiothreitol and 0.001% bromophenol blue (pH 6.8), and separated by SDS-PAGE (12% polyacrylamide gel), and stained with Coomassie brilliant blue (Every et al., 1998).

Protein band intensities in the gel were measured on a Shimadzu scanning densitometer.

For acid-PAGE, gliadin samples (5 mg) were incubated at 37 °C for 2 h in 0.1 ml of 0.05 M Tris–HCl buffer containing 1000U *Nysius* proteinase or buffer without enzyme. After incubation, gliadin proteins were extracted by incubating the mixtures for 30 min with 1 M urea, and the clarified extracts were separated on 2.5–27% polyacrylamide gels (Gradient Laboratories Pty Ltd, Pyrmont, NSW, Australia) in sodium lactate buffer (pH 3.1) according to the methods of Du Cros and Wrigley (1979).

For alkaline urea-PAGE, samples of β- and κ-caseins (0.4 mg) were incubated at 37 °C for 2 h in 0.1 ml of 0.05 M Tris–HCl buffer (pH 8.9) containing 500 U *Nysius* proteinase. After incubation, the proteins and hydrolysis products were diluted 1:1 with electrophoresis sample buffer (i.e. 60 mM Tris–HCl, pH 7.6 containing 8 M urea, 0.2 M β-mercaptoethanol, 12.5% glycerol and 0.01% bromophenol blue), and separated by alkaline urea-PAGE (Creamer, 1991).

2.3. Peptide sequence analysis

Samples of purified, pyridylethylated high M_r glutenin subunit 2 (0.5 mg) were incubated at 37 °C for 4 h in 0.1 ml of 0.05 M Tris–HCl buffer (pH 8.9) containing 500 units of *Nysius* proteinase, and the reaction was stopped with 0.1 ml 8 M urea and 2% dithiothreitol in 0.1 M Tris–HCl buffer (pH 6.8). Samples of β- and κ-caseins (0.4 mg) were incubated at 37 °C for 6 h in 0.1 ml of 0.05 M Tris–HCl buffer (pH 8.9) containing 500 units of *Nysius* proteinase, and the reaction was stopped by the addition of 0.05 ml 1% trifluoroacetic acid.

Peptides from proteolysis of high M_r pyridylethylated glutenin subunit 2, β-casein and κ-casein were isolated by RP-HPLC, sequenced by an automated Edman procedure (Applied Biosystems 470A Protein Sequencer) and identified with peptide sequences in the original substrate proteins by the methods described by Sutton, 1991; Shewry et al., 1988 and Reid et al., 1991.

3. Results and discussion

3.1. Enzyme tests on protein substrates

Sol-glutenin was the most reactive substrate for *Nysius* proteinase in a radial diffusion assay (Table 1), while the reactions with gluten, Insol-glutenin and casein were weak. These protein substrates consist of many different protein subunits, and the weak enzyme reactions with gluten, Insol-glutenin and casein may be because only a fraction of the constituent protein subunits were hydrolysed and the unhydrolysed protein subunits obscured the detection of hydrolysed subunits. Other proteins did not react.