On the Presence and Activities of Proteolytic Enzymes in Vital Wheat Gluten

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ABSTRACT

Two vital wheat gluten samples were submitted to an Osborne type fractionation. The proteolytic activities of the resulting fractions were evaluated. Gluten contained endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase and Nα-benzoylarginine-p-nitroanilide hydrolase activities. After extraction with 0.5 M NaCl, and subsequently with 70% (v/v) ethanol, little activity remained in the extracted gluten. Upon autodigestion of gluten no (microbial) enzymes were released. High specificities of gluten-associated proteolytic enzymes were noted and their effects were clearly visible on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The sum of lysine, leucine, phenylalanine, tyrosine and arginine accounted for ca. 40–44% of the released amino acids, while they only make up ca. 18% of vital gluten proteins. Good correlations were found between the proportions of the amino acids released by the different Osborne fractions as a result of autodigestion, indicating that the gluten hydrolysing enzymes found in the different Osborne fractions are probably the same. Autodigestion of gluten proteins was reduced by ca. 73–76% upon addition of pepstatin A (0.2 mM), an inhibitor of aspartic proteases, and by c. 39–41% by phenylmethylsulphonyl fluoride (1.0 mM), a serine protease inhibitor.

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Keywords: proteolytic enzymes, vital wheat gluten.

INTRODUCTION

Literature data on the proteolytic enzymes associated with industrially produced vital wheat gluten are scarce. Haemoglobinase and azocaseinase activities have been demonstrated in vital wheat gluten1. Proteolytic activity was also detected2 in vital gluten by measuring the decrease in resistance to extension of a gluten-starch system when incubated for 12 h at pH 4.1. The sponge rheology changed both as a result of the low pH and gluten-associated proteolytic enzymes.

Dilute acetic acid dispersions of unheated hand-washed gluten showed a decrease in viscosity and an increase in non-protein nitrogen as a function of time, in contrast to gluten heat treated at 100°C3. A relationship was found between the degree of peptide bond scission during incubation of hand-washed gluten in water and the extent of gluten softening, mainly caused by native wheat proteases4. Increased gluten extensibility with long incubation times5 and totally different Farinograph characteristics for gluten incubated for 3 h in 0.2 M acetate buffer (pH 4.5) than for un-incubated gluten were also reported6. A starch-gel electrophoresis technique showed that the most active wheat proteases were associated with hand-washed gluten7. It also showed autodigestion of some pro-

ABBREVIATIONS USED: BAPA = Nα-benzoylarginine-p-nitroanilide; CPA = N-carbobenzoxy-L-phenylalanyl-L-alanine; DFP = diisopropylfluorophosphate; EDTA = tetra-sodium ethylenediamine tetraacetate; HMW = high molecular weight; LPA = L-leucine-p-nitroanilide; NEMI = N-ethylmaleimide; pHMB = p-hydroxymercuribenzoic acid; PMSF = phenylmethylsulphonyl fluoride; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA = trichloroacetic acid; TNBS = trinitrobenzene-sulphonic acid.
teins extracted from six different gluten samples after extraction with water \(\text{pH} 3-4\) of low ionic strength. Increased ionic strengths of the same buffer eliminated autodigestion \(^7\).

Results concerning the extractability of proteolytic enzymes from gluten proteins are contradictory. A fraction of the proteolytic enzymes remained associated with unextracted flour proteins after extraction of wheat flour in a single step with water \(^8,9\), Na acetate buffer \(^8,9\) or dilute acetic acid \(^9\) but repeated extraction of flour with water and 0.2 M acetic acid solubilised almost all proteolytic activity \(^9\). In contrast, gluten extracted with 0.2 M acetic acid retained 85\% of haemoglobin and 40\% of N-carbenzoyl-L-phenylalan-2-amine hydrolase activity \(^10\). Time-dependent softening of hand-washed gluten could be avoided by prior washing of gluten with 0.2 M NaCl \(^11\). Because 90\% of gluten hydrolysing activity was extractable with 0.1 M NaCl it was suggested that the proteases were albumins and globulins bound to the gluten during dough mixing \(^1\). Gluten extracted with 0.35\% \(\text{Na}_2\text{CO}_3\) retained 79\% of the serum albumin hydrolase activity in gluten \(^12\). In a glutenin extract, isolated from the same gluten, c. 24\% of the proteolytic activity was found \(^12\).

The present work provides new data concerning the nature and the specificity of gluten hydrolysing proteolytic enzymes by studying their activity in different assays in the presence or absence of classic enzyme inhibitors. Also studied are the release of amino acids and the changes in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns and gel permeation profiles as a result of autodigestion.

**MATERIALS AND METHODS**

**Gluten and gluten fractions**

The moisture contents of gluten 1, 2 and 3 (Approved Methods of the AACC, 1983; 44-19) were 8-3\% \(\text{w} / \text{v}\) and 6-5\% \(\text{w} / \text{w}\), respectively.

Two gluten samples (gluten 2, North American and gluten 3, European) were fractionated by an Osborne approach \(^1\). To that end, gluten (10-0 g) was extracted sequentially with 0.5 M NaCl (1:10 \text{w/v}), 70\% \(\text{v} / \text{v}\) ethanol (1:10 \text{w/v}) and 0.05 M acetic acid (1:20 \text{w/v}) at 7°C. The residues from each extraction were freeze dried, weighed and protein contents \(\text{N} \times 5.7\) were determined by a micro-Kjeldahl procedure \(^13\). The gluten residue after extraction of gluten with 0.5 M NaCl is hereafter referred to as gluten A. Gluten B is the residue after extraction of gluten A with 70\% \(\text{v} / \text{v}\) ethanol and gluten C is the residue after extraction of gluten B with 0.05 M acetic acid. Fractionation and protein determination were performed at least in duplicate and the mean values are reported (Table I). The experimental error is defined as the difference \((\text{in} \%)\) between individual and mean value.

**Chemicals**

All analytical chemicals were reagent grade and supplied by Sigma-Aldrich (Belgium) unless specified otherwise.

**Enzyme assays**

In what follows, all centrifugation steps were at 8000 \(g\) (10 min, room temperature). In all the assay buffers used, 0.1\% \(\text{w} / \text{v}\) sodium azide was added. In the enzyme assay methods, all incubation steps were at 37°C with continuous magnetic stirring.

For autodigestion, gluten (0.05 g) was suspended in 0.05 M Na acetate buffer, \(\text{pH} 4.0\) (1.0 mL). After incubation (24 h), 10\% \(\text{w} / \text{v}\) sulphosalicylic acid \(0.4 \text{mL}\) was added and the precipitated proteinaceous material was removed by centrifugation. The supernatants were assayed for released amino acids and small peptides by the trinitrobenzenesulphonic acid (TNBS) reaction \(^14\). Supernatant (0.1 mL) and TNBS-reagent \([0.3\% \text{w} / \text{v}\] in 0.2 M Na phosphate buffer, \(\text{pH}\) 8.0) \(0.9 \text{mL}\) were incubated (20 min, 50°C) and the reaction was stopped with 0.2 M HCl (3.0 mL). Absorbance was measured at 340 nm. One unit of activity corresponded to liberation of one milligram leucine per h at pH 4.0 and 37°C.

The effect of five different inhibitors on gluten hydrolysing activity was also evaluated with this method. Inhibitors used were: tetradsodium ethylenediamine tetraacetate (EDTA, 2.0 mm; UCB, Belgium), N-ethylmaleimide (NEMI, 2.0 mm), p-hydroxy-mercuribenzoic acid (pHMB, 1.0 mm), phenylmethylsulphonyl fluoride (PMSF, 1.0 mm) and pepstatin A (0.2 mm).

Endoproteolytic activity was determined by modification of the method of Preston et al. \(^15\). Wheat gluten (0.02 g) was suspended in 0.2 M McIlvaine buffer, \(\text{pH} 5.5\) (1.0 mL) and azocasein [1.4\% w/v, dissolved in the same buffer] \(1.4 \text{mL}\) was added. After 12 h incubation, the reaction was
stopped by addition of cold (7°C) 10% (w/v) trichloroacetic acid (TCA) (2·0 mL) and precipitated proteins were removed by centrifugation. Sodium hydroxide (0·5 M) was added to an equal volume of supernatant. The mixture was allowed to stand for 20 min and absorbance was measured at 440 nm. The activity was reported as absorbance at 440 nm per h at pH 5·5 and 37°C.

Exoproteolytic activity was determined by modification of the method of Bushuk et al.16. Wheat gluten (0·02 g) was suspended in 0·05 M Na acetate buffer, pH 4·0 (1·0 mL) and haemoglobin [1% w/v, dissolved in the same buffer] (1·0 mL) was added. After incubation (6 h), the reaction was stopped by addition of cold (7°C) 10% (w/v) TCA (2·0 mL) and precipitated proteins were removed by centrifugation. The supernatants were assayed for free \( \alpha \)-amino nitrogen with the TNBS reagent. One unit of activity corresponded to liberation of one milligram leucine per h at pH 4·0 and 37°C.

**N\( \alpha \)-benzoylarginine-\( p \)-nitroanilide (BAPA; Janssen Chimica, Belgium) and L-leucine-\( p \)-nitroanilide (LPA) were substrates for the detection of BAPA-ase and aminopeptidase activities respectively by modification of the method of Kruger19. Wheat gluten (0·02 g) was suspended in 0·2 M McIlvaine buffer (1·25 mL), pH 8·5 for BAPA-ase and pH 7·2 for LPA-ase activities, and the BAPA or LPA substrate solutions (0·25 mL) were added. The substrate solutions were prepared by dissolving BAPA or LPA (0·1 g) in dimethylsulphoxide (2·5 mL), and diluting the solution to 250·0 mL with 0·2 M McIlvaine buffer. After 4 h incubation, the reaction was stopped by addition of cold (7°C) 30% (v/v) acetic acid (0·5 mL). Precipitated proteins were removed by centrifugation. Absorbance was measured at 410 nm. One unit of activity corresponded to liberation of one \( \mu \)mol \( p \)-nitro-aniline per h at 37°C and pH 8·5 (BAPA) or 7·2 (LPA).

In all assays, activity was corrected for control values by incubating gluten suspensions and substrate solutions separately for the appropriate time, combining them and proceeding as described in the respective enzyme assays. Experimental error is defined as the difference (in %) between individual and mean value.

### Table I  Protein contents of total and fractionated gluten proteins determined by a micro-Kjeldahl method.

<table>
<thead>
<tr>
<th></th>
<th>Gluten 2</th>
<th></th>
<th>Gluten 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Protein</td>
<td>Total protein (g)</td>
<td>% Protein</td>
</tr>
<tr>
<td>Total gluten</td>
<td>76·9%</td>
<td>7·689 (100%)</td>
<td>70·6%</td>
</tr>
<tr>
<td>Gluten A</td>
<td>77·6%</td>
<td>7·119 (92·6%)</td>
<td>72·5%</td>
</tr>
<tr>
<td>Gluten B</td>
<td>78·1%</td>
<td>2·911 (37·9%)</td>
<td>71·4%</td>
</tr>
<tr>
<td>Gluten C</td>
<td>74·7%</td>
<td>2·068 (26·9%)</td>
<td>68·2%</td>
</tr>
</tbody>
</table>

The experimental error is <2%.

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**Gluten hydrolysing enzymes**

Gluten (0·05 g) was incubated for 72 h at 37°C in 0·05 M Na acetate buffer, pH 4·0 (1·0 mL), with or without 0·1% (w/v) Na azide. After incubation, the endo- and exoproteolytic activities of the gluten proteins were measured. In the exoproteolytic
enzyme assay, 1% (w/v) haemoglobin in 0·05 M Na acetate buffer, pH 4·0 (1·0 mL) was added. The further analysis was as described for the exoproteolytic assay. Endoproteolytic activities were evaluated by adding 1·4% (w/v) azocasein in 0·5 M McIlvaine buffer, pH 5·5 (1·4 mL), and proceeding as described for the endoproteolytic assay. The presence of bacterial and fungal organisms in gluten protein suspensions was checked at different time intervals using plate count agar (Oxoid). The plates were incubated for 72 h at 28°C.

**Amino acid analysis**

The supernatants obtained after autodigestion of gluten proteins and precipitation of the solubilised proteins and peptides with 10% (w/v) sulphosalicylic acid (as above) were filtered (Millipore filter Type GVWP 0·22 μm). Filtrate (50·0 μL) was injected onto a Pharmacia LKB Biochrom 4151 Alpha Plus Amino Acid Analyser 4·6 × 200 mm (Pharmacia Biotech, Sweden). Lithium-citrate was used as a buffer system (buffer 1: 0·2 M, pH 2·8; buffer 2: 0·3 M, pH 3·0; buffer 3: 0·5 M, pH 3·15; buffer 4: 0·9 M, pH 3·5; buffer 5: 1·65 M, pH 3·55; flow rate 20 mL/h) and the amino acids were detected with ninhydrin. Peak areas and retention times were registered with a Shimadzu C-R6A Chromatopac integrator. The experimental error is c. 4%.

**Characterisation of digested proteins by gel permeation chromatography**

Changes in molecular size distribution of solubilised vital gluten proteins as a result of autodigestion were studied by gel permeation chromatography (Superose 12 10/30 column, Pharmacia Biotech, Sweden). Gluten proteins (0·05 g) were incubated (0, 24 and 72 h) in 0·05 M Na acetate buffer, pH 4·0, 0·1% (w/v) Na azide (1·0 mL). After centrifugation, the supernatant (0·2 mL) was applied onto the gel permeation column and eluted at 0·4 mL/min with 0·05 M Na acetate buffer, pH 4·0. The column was maintained at 7°C. Its void volume was determined with Blue Dextran 2000, the total volume with Blue 2·0 (Pharmacia Biotech, Sweden) were ribonuclease A (13·7 k), bovine serum albumin (67 k), ovalbumin (43 k), carbonic anhydrase (30 k), soybean trypsin inhibitor (20·1 k) and α-lactalbumin (14·4 k).

Gels were stained overnight with 0·025% (w/v) Coomassic Brilliant Blue R250 in 40% (v/v) methanol containing 7% (v/v) acetic acid. Destaining was with a 5% (v/v) methanol solution containing 7% (v/v) acetic acid until background was clear.

**RESULTS AND DISCUSSION**

**Formation of (microbial) gluten hydrolysing enzymes**

When native wheat gluten (gluten 1, European) was incubated in 0·05 M Na acetate buffer, pH 4·0, 0·1% (w/v) Na azide at 37°C, an increase in free α-amino nitrogen was observed as a function of time (Fig. 1). When gluten suspensions, heated for 15 min at 100°C and incubated for 72 h at 37°C, were subjected to the autodigestion assay,
no amino acids were released, and SDS-PAGE patterns identical to those of native gluten were obtained. It seemed obvious that gluten proteins were hydrolysed by proteolytic enzymes although it was not clear whether this was due to enzymes formed during autodigestion.

Endo- and exoproteolytic activities of gluten (gluten 1), incubated for 72 h in the presence or absence of 0.1% (w/v) Na azide, were evaluated and compared with the activities of un-incubated gluten. Growth of bacterial and fungal organisms was evaluated on plate count agar. Results showed that, although growth of bacterial organisms was slowed down considerably by the addition of 0.1% (w/v) Na azide, after 72 h incubation growth of both bacterial and fungal organisms was noticed, even in the presence of sodium azide. Surprisingly, more endo- and exoproteolytic activities were found in fresh gluten than in gluten incubated for 72 h. The presence of 0.1% (w/v) Na azide had no effect on the activity of the proteolytic enzymes associated with gluten proteins.

**Proteolytic enzymes associated with gluten proteins**

To gain better insight in the nature and levels of the gluten hydrolysing enzymes, two gluten samples (gluten 2 and 3) with different breadmaking properties were fractionated by an Osborne approach. The proteolytic activities of these gluten fractions were studied with five different enzyme assays. Table II shows that vital wheat gluten contain endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase and BAPA-ase activities. Gluten 3 in general had higher activities in all tested enzyme assays than gluten 2.

Gluten B had more than 15% of the original proteolytic activity only when haemoglobin or CPA were used as substrate. With other substrates, its activity was substantially lower. The extraction of gluten proteins with dilute acetic acid after the extraction with 0.5 M Na chloride and 70% (v/v) ethanol barely changed the activity of the gluten residue. It seems that the proteolytic enzymes are not sig-
Table II  Endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase, N\textsubscript{2}-benzoylarginine-\textit{p}-nitroanilide hydrolase (BAPA-ase) and gluten hydrolysing activities of total and fractionated gluten proteins. The gluten residue after extraction of total gluten with 0·5\% sodium chloride is hereafter referred to as gluten A. Gluten B is the residue after extraction of gluten A with 70\% (v/v) ethanol and gluten C is the residue after extraction of gluten B with 0·05\% acetic acid.

<table>
<thead>
<tr>
<th></th>
<th>Glutens 2</th>
<th>Glutens 3</th>
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<tbody>
<tr>
<td></td>
<td>U/g protein</td>
<td>Total U</td>
</tr>
<tr>
<td>Endoproteolytic activity(^{a,f})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total gluten</td>
<td>0·087</td>
<td>0·667</td>
</tr>
<tr>
<td>Gluten A</td>
<td>0·027</td>
<td>0·200</td>
</tr>
<tr>
<td>Gluten B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gluten C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Exoproteolytic activity(^{b,f})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total gluten</td>
<td>7·290</td>
<td>56·049</td>
</tr>
<tr>
<td>Gluten A</td>
<td>6·955</td>
<td>51·237</td>
</tr>
<tr>
<td>Gluten B</td>
<td>3·232</td>
<td>9·467</td>
</tr>
<tr>
<td>Gluten C</td>
<td>3·824</td>
<td>7·995</td>
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<tr>
<td>Carboxypeptidase activity(^{b,g})</td>
<td></td>
<td></td>
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<tr>
<td>Total gluten</td>
<td>12·062</td>
<td>92·742</td>
</tr>
<tr>
<td>Gluten A</td>
<td>11·699</td>
<td>86·190</td>
</tr>
<tr>
<td>Gluten B</td>
<td>9·170</td>
<td>26·697</td>
</tr>
<tr>
<td>Gluten C</td>
<td>5·265</td>
<td>11·008</td>
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<tr>
<td>Aminopeptidase activity(^{c,e})</td>
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<tr>
<td>Total gluten</td>
<td>0·986</td>
<td>7·581</td>
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<tr>
<td>Gluten A</td>
<td>0·573</td>
<td>4·221</td>
</tr>
<tr>
<td>Gluten B</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Gluten C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BAPA-ase activity(^{d,f})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total gluten</td>
<td>0·095</td>
<td>0·728</td>
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<tr>
<td>Gluten A</td>
<td>0·062</td>
<td>0·453</td>
</tr>
<tr>
<td>Gluten B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gluten C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Autodigestion(^{b,g})</td>
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<td></td>
</tr>
<tr>
<td>Total gluten</td>
<td>0·734</td>
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<tr>
<td>Gluten A</td>
<td>0·591</td>
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</tr>
<tr>
<td>Gluten B</td>
<td>0·257</td>
<td>0·749</td>
</tr>
<tr>
<td>Gluten C</td>
<td>0·235</td>
<td>0·333</td>
</tr>
</tbody>
</table>

\(^{a}\) The activity is reported as the absorbance at 440 nm per h at pH 5·5 and at 37\°C.
\(^{b}\) One unit of activity corresponded to liberation of one milligram leucine per h at pH 4·0 and at 37\°C.
\(^{c,d}\) One unit of activity corresponded to liberation of one \textit{lmol} \textit{p}-nitro-aniline per h at pH 7·2 or 8·5\(^{c}\) and at 37\°C.
\(^{f}\) The experimental error is <3\%; <6\%; <10\%.

Significantly associated with glutenins or that they are inactivated by extraction with 70\% (v/v) ethanol. Ikhsanova \textit{et al.}\(^{12}\) reported that, after the isolation of the gliadin fraction from gluten proteins with 70\% (v/v) ethanol, 32\% of the proteolytic activity of gluten remained in the residue. No activity was found in the isolated gliadin fraction. Kaminski and Bushuk\(^7\), however, showed that, in a sequential Osborne type fractionation, the proteolytically most active part of flour was mainly present in the 70\% (v/v) ethanol extractable fraction. In our hands, a 70\% (v/v) ethanol extract showed no exoproteolytic activity, implying denaturation of the enzymes in this solvent.

**Autodigestion of fractionated vital gluten proteins**

Table II also lists the release of amino acids and small peptides as a result of autodigestion. The
recoveries of the gluten hydrolysing activities of the different gluten fractions are comparable to the recoveries of the exoproteolytic activities, although c. 10 times more amino acids were released in the exoproteolytic assay than in the autodigestion assay. Different authors have noticed a higher release of amino acids from haemoglobin than from gluten proteins. The insolubility of the gluten proteins and the high sensitivity of the gluten to salts, resulting in low solubility, are possible explanations.

During autodigestion, (poly)peptides were released by the four different fractions of gluten 2 and 3. A part of this proteineous material precipitated when adding 0.2 M Na phosphate buffer (pH 8.0) in the free \( \alpha \)-amino nitrogen assay (TNBS reaction).

The amino acids released by gluten hydrolysing enzymes are listed in Table III. Noteworthy are the very low proportions of glycine, proline, glutamine and glutamic acid released, and the high proportions of lysine, leucine, phenylalanine, tyrosine and arginine, especially when the levels of the released amino acids are compared with normal levels of these amino acids in vital wheat gluten. Lysine, leucine, phenylalanine, tyrosine and arginine together account for c. 40–44% of the released amino acids, while in vital gluten they account for c. 18%.

A high quantity of tryptophan is released by both total gluten and gluten A. Wookey reported that the tryptophan content of gluten proteins is quite low. Released glycine, proline, glutamine and glutamic acid make up c. 9–10%, while gluten proteins contain c. 52% of these amino acids. This indicates that the gluten hydrolysing enzymes have a high specificity for peptide bonds adjacent to lysine, leucine, phenylalanine, tyrosine, arginine and probably tryptophan but a rather low specificity for peptide bonds adjacent to glycine, proline, glutamine and glutamic acid.

The results are in agreement with those of Grant and Wang, who found a release of high levels of leucine and phenylalanine by the action of endogenous proteolytic enzymes in a 0.2 M acetic acid extract of wheat flour. When haemoglobin was added to wheat flour extract, high amounts of histidine, lysine and arginine were also found. The amino acid fraction was low in glutamine, glutamic acid and proline, in agreement with our results.
For both gluten samples 2 and 3, the quantities of the different amino acids, released by total gluten and gluten A, B and C, correlated well with one another ($r^2$ between 0.783 and 0.974, $P<0.001$ for gluten 2, $r^2$ between 0.796 and 0.952, $P<0.001$ for gluten 3). It is reasonable to assume, therefore, that the same proteolytic enzymes are responsible for the hydrolysis of gluten proteins in the different gluten fractions. Their varying extractability in different solvents is probably related to their binding to different proteins.

In the autodigestion of total gluten 2 and 3, good correlations existed between the quantities of amino acids released by the total gluten materials ($r^2=0.923$, $P<0.001$). The same was observed when the profiles of the subfractions were compared with each other (gluten A: $r^2=0.969$, gluten B: $r^2=0.961$ and gluten C: $r^2=0.965$; $P<0.001$). This indicates that the same gluten hydrolysing enzymes are present in both gluten 2 and 3.

**Autodigestion of vital gluten proteins as a function of time**

Increased amounts of proteins solubilised during the incubation of gluten in 0.05 M Na acetate buffer, pH 4.0, were observed using gel permeation chromatography. The solubilisation, measured at 280 nm with bovine serum albumin as standard, increased from 7.6 to 17.3% and from 9.2 to 21% after 3 days incubation for gluten 2 [Fig. 2(a)] and 3 [Fig. 2(b)], respectively. Figures 2(a) and 2(b) suggest that the endoproteolytic enzymes have a high specificity in their action on gluten proteins and lead to the formation of reasonably well resolved protein peaks that, after the different incubation times, had constant elution volumes. Peak 5 is from Na azide. Protein peaks 6 and 7 eluted later than expected. This is probably due to hydrophobic interaction with the Superose column.

The residues after autodigestion of vital gluten proteins were reduced. SDS-PAGE patterns (Fig. 3) obtained after 0 h and 72 h incubation differed for both gluten 2 and gluten 3. The high and low molecular weight glutenin subunits, the gliadins and the HMW albumins and globulins (terminology of MacRitchie et al.22) disappeared as a function of time. The fact that the proteins disappeared with longer incubation times was not due to increased solubilisation since new bands arose in the $M_r$ 30–33 k region. These results are in agreement with those of Kawamura and Yonezawa10. These authors noticed with SDS-PAGE that an HMW subunit of $M_r$ 90 k disappeared as a function of time and that new protein bands in the region of $M_r$ 26–28 k and a new protein band of $M_r$ 68 k appeared.

**Effect of inhibitors on proteolytic enzymes in vital wheat gluten**

Enzymes responsible for the hydrolysis of the gluten proteins are mainly aspartic and serine proteases. The addition of pepstatin decreased the hydrolysis of gluten proteins by c. 73–75%. PMSF, an inhibitor of serine proteases, decreased the hydrolysis by c. 39–41%. In a previous study10, the disappearance of an $M_r$ 90 k HMW subunit (SDS-PAGE) was prevented by pepstatin (c. 75%) and by diisopropylfluorophosphate (DFP) (c. 70%), a serine protease inhibitor. Upon simultaneous addition of pepstatin and DFP, the disappearance of the $M_r$ 90 k HMW subunit was totally prevented. A drop in viscosity of a 0.2 M acetic acid solution was completely overcome by pepstatin and to a lesser extent by DFP10. EDTA had no effect on the activity of the gluten hydrolysing enzymes.

One sulphydryl protease inhibitor, $\beta$HMB, decreased the hydrolysis of gluten by c. 14–27% while a second (NEMI) had no effect. The fact that $\beta$HMB inhibits the proteolytic activity of gluten associated enzymes, whereas NEMI does not, does not necessarily imply that one of these enzymes is a sulphydryl protease. A wheat bran carboxypeptidase (serine protease) was inhibited c. 35% by 1.0 mM $\beta$HMB, while other sulphydryl inhibitors had no effect17. The fact that NEMI had no effect on the activity of the gluten associated enzymes is in contradiction with earlier results8, where the unextractable proteolytic enzymes were inhibited by sulphhydryl protease inhibitors.

Serine proteases have specificities for peptide bonds adjacent to lysine, arginine, tyrosine, phenylalanine, tryptophan and alanine while aspartic proteases are specific for peptide bonds adjacent to phenylalanine, tryptophan and tyrosine30. The high levels of the amino acids lysine, phenylalanine, tyrosine and arginine, released as a result of autodigestion of vital wheat gluten, may well be the result of the action of aspartic and serine proteases.
Figure 2  Gel permeation chromatography of solubilised proteins as a result of autodigestion of vital gluten proteins as a function of time: (a) gluten 2; (b) gluten 3. Elution profiles shown are for control gluten and gluten incubated for 24 and 72 h.
bands with molecular weights of c. $M_r$ 30–33 k are formed.

Gluten hydrolysing enzymes, found in the different Osborne gluten fractions are probably the same enzymes because relative quantities of the different amino acids, released by total gluten, gluten A, B and C, were quite comparable. The enzymes have different extractabilities presumably due to their association with different proteins.

Inhibitor studies showed that aspartic and serine proteases are responsible for the hydrolysis of gluten proteins. Taking our results with those of previous studies\textsuperscript{10,31}, the data lead to the conclusion that one or more aspartic and serine endoproteases are responsible for gluten hydrolysis. Carboxypeptidases with acid pH optima probably also contribute to the hydrolysis of gluten proteins, because plant carboxypeptidases in general are enzymes with a serine mechanism\textsuperscript{32}.

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CONCLUSIONS

This study confirms previous results\textsuperscript{1,2} that proteolytic enzymes are associated with vital wheat gluten.

The gluten associated enzymes hydrolyse azocasein, haemoglobin, CPA, LPA, BAPA and gluten. Analysis of amino acids released as a result of autodigestion shows that the gluten hydrolysing enzymes have a high specificity for peptide bonds adjacent to lysine, leucine, phenylalanine, tyrosine, arginine and probably tryptophan but a very low specificity for peptide bonds adjacent to glycine, proline, glutamine and glutamic acid.

The effects of the gluten hydrolysing enzymes are clearly visible on SDS-PAGE. New protein

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{SDS-PAGE patterns of residue proteins after autodigestion of vital gluten proteins as a function of time (lanes 1–3: gluten 2; lanes 3–7: gluten 3; lanes 4 and 8: molecular weight markers). SDS-PAGE patterns shown are for control gluten (lanes 1 and 5) and gluten incubated for 24 h (lanes 2 and 6) and 72 h (lanes 3 and 7).}
\end{figure}

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