VALUE ADDED WHEAT CRC PROJECT REPORT

Bug-Damage Protease and Wheat Qualtiy

Report on sabbatical visit by Prof. Dilek Sivri to Food Science Australia and Wheat CRC May – November 2002

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REPORT ON SABBATICAL VISIT BY PROF. DILEK SIVRI TO FOOD SCIENCE AUSTRALIA AND WHEAT CRC May – Nov., 2002

This report summarises the research that has been pursued during the sabbatical visit by Prof Dilek Sivri (of Hacettepe University, Ankara, Turkey) to Food Science Australia, and to other laboratories associated with the Value-Added Wheat CRC. The research has focused on the problem of bug damage to wheat grain in Turkey.

BACKGROUND TO THE PROBLEM OF BUG DAMAGE

Much of southern Europe and Russia is affected by the protease produced by the action of some insect species of Heteropterus on immature grain in the field, resulting in very weak dough and down-grading of grain to feed grade. This has long been a problem for the New Zealand wheat industry, but not in Australia.

Our recent enquiries of CSIRO Entomology indicate that there seems to be no reason for the problem not arising in Australia, except for good quarantine measures that have so far kept the relevant insects out. However, there is no assurance that the problem will not arise in Australia, due for example to a failure of quarantine.

For this reason, it is useful for Australia to develop awareness of the problem, and to develop diagnostic methods of detection. The development of a diagnostic test would also be in the interests of the Wheat CRC for possible royalty generation, given the apparently large size of the regions affected by bug damage, together with the severity of its effects on grain utilisation. There are several questions to address in considering the possibility of developing immuno-diagnostics for bug-damaged grain:

- **What is the extent of the problem?**
  - **Regions affected and sizes of grain harvests**

    The wheat bug (*Eurygaster* spp. and *Aelia* spp.) is widely distributed throughout Europe (except the northern parts), North Africa, the Middle East and the Near East/Asia. In New Zealand, an insect causing similar damage has been identified as *Nysius huttoni*. The economic importance of wheat-bug damage results from two fundamentally different effects on the wheat plant. Firstly, the bite of a single over-wintering adult can damage the stem, with the result that all the grains in the head do not develop. Secondly, damage can be caused to the grain in the ear by any of the five stages of new-generation nymphs or young adults; this type of attack can reduce baking quality. Losses due to cereal bugs are highly variable, depending on the population density of the insect, weather conditions, water availability, wheat cultivar and the duration of the crop growing period. A total of 10-15 million hectares under cereal cultivation are at risk (Paulian and Popov, 1980).

  - **Extent of damage by recent seasons; expected frequency in future**

    No statistical data is available. However, between 1967 and 1969, the presence of a single over-wintering adult per m^2 was estimated to cause a loss in yield of 30-340 kg/ha (3-12.6%) and higher losses were recorded in spring wheats. In some years, serious outbreaks of bug damage to wheat have been reported in several countries, including Turkey. On average, there is only one season of serious damage in every 10 years or so, although intervals between outbreak are highly variable. It seems likely that serious damage occurs in seasons when there has been a dry spring. Investigations carried out in a number of countries into the relationships between pest density and the number of kernels damaged, have shown that a single new-generation nymph (and later the adult) could feed on total of 40-55 wheat kernels during its development.

  - **Severity of damage; grain-value downgraded**

    Damage ranges from complete destruction, if the attack occurs during the milk-stage of maturity, to slight shrivelling of the grain, if the grain is attacked in late maturity. Whenever bug damage occurs, it causes varying degrees of yield loss, diminished seed grain value and reduced baking quality. Low levels of damage (even as low as 0.5%) could affect baking quality and damage levels of 8-10% of grains affected are considered unacceptable for breadmaking.

- **What control measures and testing is used at present?**

  Generally, bug damage in the kernel can be tested by visual examination. The presence of a small black mark in a discoloured area, framed by a halo, is a sign that the kernel was attacked at an advanced stage of maturity. Some methods of detection include gluten washing and Zeleny sedimentation tests with some modifications; these are applied to flour samples. The modification to these standard methods is that samples are incubated for an extended period (two hours) at room temperature or 37 $^{\circ}$C during testing. Test baking is also used for detection of bug damage in flours. Although, these tests are simple, they are time consuming and need laboratory facilities and expert operators.

- **What type of testing would be effective and needed by the industry?**

  For milling companies to test for bug damage during grain intake, the test must be simple, rapid, sensitive, quantitative, without the need for laboratory facilities or expert operators. Quantification of enzyme activity is important for flour samples, because the count of damaged kernels gives only an approximate estimate of the real effect on bread-making quality. There is the further factor that wheat varieties differ in their susceptibility to the bug protease, there being some tolerance to bug protease among strong wheats especially.

The following reference provides background to bug damage. Other references are provided in the attached publication (in press with ‘Cereals 2002’).

Sivri, D., and Koksel, H. Wheat bug protease: a protease with specific activity for gluten proteins. Pages 113-126 in, "Wheat Quality Elucidation: The Bushuk Legacy" (P.K.W.Ng and C.W.Wrigley, Eds) American Association of Cereal Chemists Inc., St Paul, MN.

ELUCIDATION OF THE MECHANISMS OF ACTION OF BUG PROTEASE
(Undertaken within VAW CRC Projects 1.1.1 and 3.1.2)

- **Proteome and SE-HPLC analysis of the action of bug enzyme on Katepwa flour**

Proteome analysis has been successful in identifying the degradation products of bug damage. Partly purified bug protease was added to the flour of a strong wheat (Katepwa) and protein composition was analysed at zero time and at 30 and 60 minutes. About 25 polypeptide spots appeared as a result of proteolytic activity from bug damage. Many of these were absent in the proteome map of the zero-time sample (control), but in other cases, they appeared to be due to the strengthening of components that were already present in the control sample. The changes caused by the protease indicate those aspects of flour-protein composition are critical to dough function, because the protease treatment used would have removed all rheological properties from the flour sample.

The main target of the proteolytic activity appears to be the large glutenin polymers. Their importance in providing dough strength has been demonstrated in other studies in the CRC. There is ongoing analysis of the results of bug damage by methods that would define size distribution, such as SE-HPLC. The proteome aspects of these studies were reported to the Cereal Chemistry Conference in Christchurch, NZ, and they were provided for publication in 'Cereals 2002'. See attached publication, entitled "Degradation of wheat-flour proteins by bug protease" by Sivri, D., Batey, I.L., Skylas, D.J., and Wrigley, C.W. 'Cereals 2002'. Proc. 52nd RACI Cereal Chemistry Conference' (in press). The paper is attached.

A more complete description of these studies has been prepared, extending the above account of these studies (publication details below). The further experiments have involved the use of SE-HPLC to determine the extent of change of molecular-weight distribution caused by bug protease action. This approach showed that bug protease reduced the proportion of very large glutenin polymers, as indicated by the changes in the SE-HPLC profile for the glutenin fraction that is extractable only after sonication in SDS solution.
The paper entitled "Variations in the gluten-protein composition and size distribution of polymeric protein due to degradation of wheat-flour proteins by bug protease" by D.Sivri, I.L.Batey, D.J.Skylas, L.Daqiq and C.W.Wrigley (It will be submitted for publication in Journal of Cereal Science) A draft version of the paper is attached.

- **Bug damage to grain prior to harvest**

Grain was tested for the Turkish varieties Gun (a hard red wheat with strong dough properties) and Gerek (a hard white wheat with weak dough properties). In both cases, sound and bug-damaged grain were obtained. The proteome maps were compared for the pair of Gun samples, but it did not prove possible to identify any polypeptides that might be of insect origin; nor were any quantitative differences observed at this polypeptide level. SE-HPLC, conducted on all the samples without incubation, showed that there was a considerable loss of the largest glutenin polymers for the bug-damaged samples. Presumably, this indicates that proteolysis had occurred in the immature grain following bug attack, although it is also possible that some of the loss of large glutenin occurred during the extraction with SDS solution. Nevertheless, this loss during extraction would be relatively small based on comparison with the experiment involving the addition of protease extract to Katepwa flour, described above. These experiments are being prepared for publication as a research paper.

- **Identification of the proteolytic specificity of bug protease**

Studies conducted at EMAI, Camden, were directed towards identifying the amino-acid sequence likely to be attacked by the bug enzyme. This involved the use of several dye-labelled substrates, having specific amino-acid sequences. Release of the dye from any one of these indicates the amino-acid sequence that is susceptible to the protease. Two bug protease extracts were prepared from two different

freeze-dried insects sample (*Eurygaster* and *Aelia*) by using buffer (50 mM Tris-HCl pH 7.5, containing 0.9% NaCl). Enzyme extract were also prepared from bug-damaged wheat by using the same buffer. These experiments are being completed by Dr Ming Wu at EMAI, following the end of Prof Sivri's visit.

DEVELOPMENT OF BETTER DIAGNOSTICS FOR BUG-DAMAGED GRAIN
(Undertaken within VAW CRC Projects 1.1.1 and 1.1.2)

Given the dramatic damage that bug-damaged grain has if it is mixed with sound grain, it is critical that damaged grain should be identified as such at harvest, so as to avoid the admixture of damaged grain with sound grain. Therefore, a special accent of the studies during the sabbatical visit has been on the possible development of diagnostic procedures for the detection of 'bug-damaged' grain. A rough guide to the degree of sensitivity needed is provided by the requirement in Turkey, for example, that acceptable grain should have less than 5% of grains having signs of bug damage.

- **Use of photographic film to identify bug-damaged grain**

There have been published reports of the use of photographic film to determine protease activity, due to the action of protease on the gelatin layer of the film (references listed at the end of this section). This approach has been especially reported to suit the identification of protease isoenzymes following gel electrophoresis. This approach appeared to be promising as the basis of an on-the-spot test for detecting bug damage by placing a ground sample, mixed in water, onto the surface of the film, and afterwards determining the degree of damage to the gelatin layer by developing the film.

Initial experiments were conducted to evaluate photographic film test as a means of detecting the bug-damage protease. These were promising, in that a clear spot was seen on film following the incubation of whole-meal bug-damaged grain (50 mg plus 500µL water) on exposed B & W film (Agfa APX 25) for up to 2 hours at 37^{o}C. After washing off the whole-meal, the loss of gelatin layer was clearly seen on the film after 20 min. However, these experiments involved samples with very high proteolytic activity, namely, virtually 100% bug damage. The change in the appearance of the film was not evident when samples with lower levels of bug damage, such as would be encountered in practice. Attempts were made to increase the sensitivity of this test system, including the use of a buffer at the optimal pH of the enzyme (pH 9.5), but in this case the buffer itself caused a change in the appearance of the film. In addition, several forms of photographic film and paper were evaluated (colour as well as black-and-white, developed and undeveloped prior to testing), to determine which would be most suitable for this form of test, but none of them produced satisfactory results that would suit routine use.

Because it did not prove possible for this approach to provide definitive results at the degree of sensitivity required, and in the time necessary to be useful in practice, this approach was abandoned, mainly due to the slow response of this procedure. Nevertheless, these experiments provide the basis for possible further studies on return to Turkey.

Reports of the use of photographic film to determine protease activity:

Taufel, R. Friese and H. Ruttloff, 1974. Rapid assay of proteolytic enzyme on film material. J. Chromotography (93), 489-90.
Cheung, A.L., Ying, P., and Fischetti, V.A. 1991. A method to detect proteeinase activity using unprocessed X-ray films. Anal. Biochem. 193, 20-23.

- **Dye-coupled glutenin as a substrate for the protease of bug-damaged grain**

Azo-glutenin was prepared according to the method of Tomerelli *et al*. (1949) J. Lab. Clin. Med. 34: 428. Soluble glutenin (deaminated) was obtained from Dr John Pearce, of Manildra Group.

Milled grain (100 mg of sound or bug-damaged) was mixed with azo-glutenin (25 mg) in 1 mL 0.05M Tris-HCl buffer (pH 9.5). After incubation for one hour at 37^{o} C, 8 mL 10% trichloro acetic acid was added, and the mixture was filtered through Whatman 1 filter paper. To the filtrate (0.5 mL), 0.5 mL 10% NaOH was added, the absorbance was determined at 440 nm. Bug-damaged grain gave an absorbance three times that for the sound grain.

This dyed substrate is appropriate for the bug enzyme, whereas conventional protease substrates, such as azo-casein and denatured haemoglobin which are not attacked by the bug protease. The azo-glutenin substrate thus has the promise of providing a means of simple detection of bug protease, especially if the substrate could be coupled to/immobilised onto a solid support so that proteolytic activity could be seen as the removal of the azo dye. This approach provides the basis for further studies on return to Turkey.

- **Proteome analysis to identify marker proteins for bug-damaged grain**

Proteome analysis was applied to control and bug-damaged samples (same genotype and growth location) with the aim of finding polypeptides that would be indicative of the attack by the insect on the damaged grain. Some minor differences in the maps were identified with one of two methods of protein staining, but it did not prove possible to obtain sequence information that would provide a distinguishing basis for a diagnostic test. On her return to Turkey, Prof Sivri intends to prepare new samples for this type of analysis by dissecting the grain portion immediately surrounding the point of bug attack.

- **Development of a test kit for bug damage, using immuno-reaction to the bug protease**

Interaction with staff at EMAI (James Chin's group) indicates the possibility of developing a test system, based on the specificity of antibodies. This would involve the production of a polyclonal set of antibodies using an extract of the causal insects as the antigen. This polyclonal antibody preparation would then be used to attempt distinction between sound and damaged samples of the same wheat variety, thereby permitting the identification of a suitable antibody preparation for developing an immuno-assay. Time did not permit this approach to be pursued, and it was not considered to be sufficiently urgent to the CRC's objectives for it to be continued immediately after the departure of Prof Sivri. Nevertheless, it may be possible to pursue this approach at a later date, possibly involving a visit to EMAI by a post-graduate student from Prof Sivri's university.

BLENDING STUDIES

Suitability of very strong Australian wheats for the Turkey (*et alia*) market (Relevant to VAW CRC Project 2.1.1)

Australia has close wheat-trade ties with Turkey, providing strong wheat for blending with Turkish grain that has a degree of bug damage. The possibility of on-going research collaboration is being explored whereby assistance would be provided to Australian breeding work to suit the dough properties of new Australian varieties for this trade opportunity. If this proves possible, it would involve Geoff Cornish (SARDI, Adelaide) in sending flour samples of promising Australian strong wheats for Prof Sivri to evaluate in Turkey with bug-damaged flour added, involving protease and baking tests.

SOLUBILISATION OF GLUTEN FOR INGREDIENT USE

Use of the bug protease to modify gluten properties for food uses (Relevant to VAW CRC Project 2.1.9)

Bug enzyme was evaluated as a possible gluten-solubilising agent. Initial experiments suggest that the action of the enzyme may not be so extreme as was initially assumed, but that its effects in rendering gluten ineffective in baking may involve the rupture of relatively few peptide bonds in the glutenin molecules (based mainly on the results of SE-HPLC analyses). However, further experiments are being pursued on the possible use of the bug protease as a solubilizing agent for modifying gluten for ingredient uses, particularly in association with other agents. Prof Sivri visited the Food Science labs at Werribee for discussions on these experiments with Dr Li Day, who will continue these studies in collaboration with Prof Sivri.