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CARACTERIZACIÓN, DETECCIÓN Y MEJORA DE LOS TRIGOS ATACADOS POR HETERÓPTEROS

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Caracterización, detección y mejora de los trigos atacados por heterópteros

Tesis Doctoral

Silvia Aja Martínez

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Hace constar:

Que la memoria titulada **«CARACTERIZACIÓN, DETECCIÓN Y MEJORA DE LOS TRIGOS ATACADOS POR HETERÓPTEROS»** presentada por D.ª Silvia Aja Martínez para optar al grado de Doctor por la Universidad de Valencia, ha sido realizada en el Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC) bajo su dirección y que reúne las condiciones necesarias para ser defendida por su autor.

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INTRODUCCIÓN

Los cereales constituyen la principal fuente de energía y nutrientes para una amplia mayoría de la población mundial. En Europa, el cereal más consumido en alimentación humana es el trigo, seguido del arroz, el maíz y el centeno. Éstos han constituido la base de la alimentación humana. El trigo es el cereal de mayor consumo, se utiliza de distintas formas, como cereal para el desayuno, como harina panificable, en repostería, etc. Sin embargo, el principal destino del trigo es el proceso de panificación tanto artesanal como industrial, es decir, procesos que requieren una etapa de fermentación previa.

Proteínas del trigo

Las proteínas son el constituyente mayoritario de la harina de trigo después de los hidratos de carbono (representan el 12%), y tradicionalmente se han clasificado en albúminas, globulinas, gliadinas y gluteninas en función de su solubilidad en distintos solventes (Osborne, 1924). Las proteínas de almacenamiento, gliadinas y gluteninas, son los principales constituyentes del gluten.

Las proteínas de almacenamiento del trigo juegan un papel fundamental en el proceso de panificación. Tienen la habilidad de formar el gluten, una red viscoelástica necesaria para sostener al resto de los componentes del trigo, principalmente hidratos de carbono y retener el gas producido durante la fermentación. La funcionalidad del gluten es uno de los principales parámetros que gobierna la calidad de la harina y consecuentemente la calidad panadera del trigo (MacRitchie, 1987).

La calidad de la harina es un término difícil de definir, ya que dependiendo de este parámetro obtendremos un producto con unas características organolépticas distintas.

La calidad final del gluten depende de diversos factores, entre ellos: la variedad, condiciones medioambientales, nivel de infestación y condiciones post-cosecha. Cualquier variación en alguno de los anteriores factores, afecta a la capacidad de las proteínas de almacenamiento para formar el gluten (Zhu y Khan, 2001).

Infestación del trigo por insectos heterópteros

Desde 1930 se conoce que ciertos insectos heterópteros pueden atacar los granos de trigo en crecimiento. En Europa, Asia y Norte de África este ataque se ha asociado a ciertas especies de los géneros *Aelia* y *Eurygaster*, mientras que en Nueva Zelanda se atribuye a *Nysius huttoni*. En España, *Eurygaster austriaca seabrai* y *Aelia germari* se han identificado como las especies más frecuentes causantes del daño, y se conocen comúnmente como garrapatillo, también llamado paulilla o sampedrito (Figura 1).

Figura 1.–*Eurygaster* y *Aelia.*

El género *Aelia* pertenece a la familia de los pentatómidos, tienen entre 8 a 10 mm de longitud y su color es amarillento, en cambio, el género *Eurygaster* pertenece a la familia de los escutéridos y tiene 14 mm de longitud.

El garrapatillo se alimenta del jugo del grano de trigo en su fase lechosa, segregando unas enzimas proteolíticas, denominadas proteasas que debilitan las proteínas de la harina procedente de esos trigos, alterando así la red de gluten y por tanto, las propiedades viscoelásticas de la masa y su calidad panadera (Hariri y col, 2000), causando graves pérdidas económicas. La presencia de tan sólo 3-4 granos infestados entre 100 granos sanos producen una degradación significativa.

El ataque del garrapatillo se puede detectar a nivel del grano de forma

visual por la presencia de una mancha blanquecina que rodea una pequeña picadura negra (Meredith, 1970; Every, 1992) (Figura 2). Sin embargo, existen otros insectos que originan la misma apariencia pero no inoculan enzimas proteolíticas.

Figura 2.–Granos de trigo infestados por insectos heterópteros.
Fuente: Kent Jones y Amos, 1967.

Durante el proceso de amasado se forma el gluten, sobre el que actúan las proteasas produciéndose la degradación, siendo esta degradación mayor o menor en función del grado de ataque y del estadio de desarrollo del grano. El ataque se hace más visible durante la etapa de fermentación de la masa panaria, debido al mayor tiempo de actuación de las enzimas proteolíticas. Al prolongarse la fermentación aumenta la degradación del gluten, y se produce la liberación del agua que había sido retenida por este compuesto transformándose en una masa poco cohesiva, muy extensible e incapaz de mantener desarrollada la red de gluten y, por tanto, retener el gas producido durante los procesos fermentativos. El producto resultante se caracteriza porque posee un reducido volumen y una textura defectuosa (Hariri y col, 2000). Si los trigos infestados son molturados con otros sanos se obtienen harinas con actividad proteolítica excesiva.

Las enzimas procedentes de los insectos atacan a las cadenas peptídicas que forman el gluten de cualquier masa panaria, alterando los parámetros reológicos de la misma.

La composición del complejo enzimático inyectado por *Aelia y Eurygaster* no se conoce con exactitud, ya que no se ha aislado la enzima responsable de producir la degradación proteica, sólo se sabe que la proteasa procedente de los insectos responsables de la infestación en Rusia podría tener un pH óptimo de actuación de 7,2 (Kretovich, 1944).

Únicamente en el caso de *Nysius huttoni* se ha demostrado que la

enzima causante de la degradación de las proteínas son proteasas alcalinas cuya máxima actividad se presenta a pH 9, a una temperatura entre 35-40ºC y son inhibidas por el Co^{2+}, Mn^{2+}, Fe^{2+} y no lo son por el ácido etilendiamino tetraacético (EDTA) (Cressey y MacStay, 1987; Every, 1992).

Métodos de detección de trigos infestados

Actualmente los métodos utilizados para la detección de la infestación son determinaciones indirectas basadas en la modificación que sufren distintas propiedades de las masas, las cuales se correlacionan con la medida de los niveles de actividad proteolítica, de modo que valores anormales se atribuyen a un ataque por garrapatillo.

El método más ampliamente utilizado a nivel industrial es la determinación de la actividad proteolítica mediante el alveógrafo. Este equipo mide el efecto sobre la extensibilidad de la red de gluten. Otros métodos consisten en la determinación de la variación de la consistencia de la masa mediante el farinógrafo Brabender (Matsoukas y Morrison, 1990), o bien, la modificación de la extensibilidad de la masa por el extensógrafo (Kent-Jones y Amos, 1967).

Únicamente a nivel de laboratorio se han realizado determinaciones directas de la actividad proteolítica mediante métodos espectrofotométricos, basados en la hidrólisis de distintos sustratos. Los sustratos más usados han sido la hemoglobina (AACC, 1988) y la caseína o azocaseína (Kruger, 1973). Sin embargo, no se ha encontrado una buena correlación entre la actividad proteolítica determinada con estos sustratos y la calidad panadera de la harina, lo cual se ha atribuido a la imposibilidad de distinguir entre actividad exopeptidasa y endopeptidasa (esta última responsable del efecto ablandador del gluten) mediante estos métodos.

Otros métodos desarrollados para su detección son: una modificación del test de Zeleny (Greenaway y col, 1965), varios tests que determinan el ablandamiento del gluten (Kruger, 1971) y una modificación del test de sedimentación en SDS (Cressey y MacStay, 1987; Every, 1992) pero al igual que los métodos indirectos requiere una prolongada incubación y/o un equipamiento específico.

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OBJETIVOS

El objetivo general de la investigación es definir posibles métodos que permitan la detección y cuantificación de forma rápida y sencilla de la actividad proteolítica procedente de la infestación del trigo por insectos heterópteros.

Son objetivos particulares:

- Caracterización de los trigos infestados por insectos heterópteros mediante el análisis estructural de los granos infestados y la cuantificación de actividades enzimáticas.

- Caracterización de la naturaleza de las proteasas inoculadas por el garrapatillo, con el fin de identificar inhibidores específicos de su actividad, que sirvan para controlar e impedir su actuación sobre la red de gluten en todos aquellos procesos que requieran una etapa previa de fermentación.

- Evaluar la modificación ocasionada por el ataque de insectos heterópteros a distintos niveles de infestación e identificar la alteración de las proteínas de trigo infestadas, centrándose en el aislamiento de las fracciones proteicas y su caracterización.

- Analizar los productos solubles en agua procedentes de la hidrólisis del gluten producida por el ataque del insecto, y determinar la posible modificación de la calidad del gluten.

- Desarrollar alternativas para paliar el efecto negativo de la infestación, como la generación de nuevos enlaces covalentes entre las cadenas proteicas que confieran rigidez a la estructura reticular del gluten y contrarrestar el debilitamiento producido por las proteasas procedentes de los insectos.

PLAN DE TRABAJO

Para conseguir los objetivos propuestos, se diseñó el siguiente plan de trabajo:

- Análisis estructural de los granos infestados por insectos heterópteros y cuantificación de actividades enzimáticas. Se realizará en diversas variedades de trigo con distinto grado de infestación para determinar: i) Actividad proteolítica, mediante el análisis alveográfico. ii) Actividad diastática. iii) Actividad α y β amilasa. iv) Microscopía electrónica de barrido (SEM).

- Caracterización de las proteasas inoculadas por el garrapatillo, mediante la determinación de la actividad proteolítica frente a distintos sustratos. Los sustratos serán la hemoglobina y la azocaseína.

- Evaluación de la modificación ocasionada por el ataque de insectos heterópteros a distintos niveles de infestación. Se estudiarán las condiciones óptimas para la extracción de proteasas y se procederá a su purificación posterior.

- Identificación de la alteración de las diversas fracciones de proteínas de trigo infestado, mediante técnicas de electroforesis capilar.

- Determinación de la posible modificación de la calidad del gluten y análisis de los productos solubles en agua procedentes de la hidrólisis del gluten, mediante cromatografía líquida de alta resolución (HPLC) de exclusión molecular, y electroforesis en gel.

- Formación de enlaces cruzados entre las proteínas de trigo para paliar el efecto negativo de la infestación. Estos entrecruzamientos se realizarán mediante la adición de enzimas como la glucosa oxidasa, polifenol oxidasa y transglutaminasa, las cuales poseen distinto mecanismo de actuación.

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CAPÍTULO 1

Amylase activities in insect (*Aelia* and *Eurygaster)*-damaged wheat

C. M. Rosell [1], S. Aja [1], J. Sadowska[2]

ABSTRACT

The extent modification of wheat amylase activities caused by *Aelia* and *Eurygaster* attack on wheat grain was determined in different Spanish cultivars subjected to varying degrees of attack. High variation in diastatic activity, α amylase and ß amylase activities was found between cultivars, but no relationship could be established between these activities and bug damage within cultivars. Scanning electron micrographs of the cross-section of damaged kernels showed an empty cavity under the bite point. The surrounding cell walls and protein matrix were absent, but the starch granules were intact. Since wheat damaged by *Aelia* and *Eurygaster* does not have altered amylase activities, it appears that amylolytic enzymes were not involved in the alteration of bug-damaged wheat.

INTRODUCTION

It has been reported since the 1930s that certain heteropterous insects

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cause damage to wheat cultivars (Lorenz and Meredith, 1988). These insects (wheat bugs) feed on different stages of developing grain (Lorenz and Meredith, 1988; Swallow and Every, 1991). In Europe, The Middle East and North Africa the insects responsible for bug damage of wheat are species of the genera *Eurygaster* and *Aelia*, whilst in New Zealand the attack is attributed to *Nysius huttoni* (Lorenz and Meredith, 1988; Cressey *et al.*, 1987). In Spain, the insects that most frequently cause damage are *Aelia germari* and *Eurygaster austriaca* (Infiesta *et al.*, 1999).

Wheat bugs suck the milky nutrients from the immature grain by piercing it with their mouthparts and injecting their salivary juices, which contain very potent enzymes (Lorenz and Meredith, 1988; Swallow and Every, 1991). The most important and extensively studied of these enzymes are the proteases, since flour from bug-damaged wheat has poor quality. Dough prepared from this flour is very sticky and weak and produces loaves of poor volume and unsatisfactory texture (Hariri *et al.*, 2000).

Extensive research has been done on wheat damaged by *N. huttoni*. The protease of this insect is of alkaline nature and some inhibitors have been described (Cressey, 1987). Some authors (Cressey and McStay, 1987; Every *et al.*, 1990), studied its specificity for high-molecular-weight glutenin subunits (HMW-GS) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and determined that the hydrolysis products are acetic acid or SDS-soluble protein fragments. Less information is available about the effect caused by the insects *Aelia* and *Eurygaster*. In those genera the insect salivary proteases degrade gluten to water or alcohol soluble proteins (Kretovich, 1944). Recently, Sivri *et al.* (1998) reported a reduction in the gliadin and insoluble glutenin fractions using acid polyacrylamide gel electrophoresis (A-PAGE) and SDS-PAGE, respectively. In addition, a decrease in total glutenin, HMW-GS and low molecular weight glutenin subunits (LMW-GS) by reverse phase high performance liquid chromatography (RP-HPLC) was also reported (Sivri *et al.*, 1999).

A controversial point is reached when looking at the effect on starch, since reports vary as to the effect of insect damage on starch. Some authors have described an increase in the diastatic activity of bug-damaged wheat (Kretovich, 1944), whilst others (Kranz, 1935) found normal levels of this activity. Some photographs have even been published displaying starch damaged by wheat bugs (Hopf, 1938).

Evidence of starch damage was reported by Lorenz and Meredith (1988),

who showed severe starch attack due to different sucking insects (*Sidnia, Calocoris and Nysius*), although no differences in diastatic activity could be assessed. An increase in amylolytic activity was evident from scanning electron micrographs of starch from damaged wheat (Lorenz and Meredith, 1988), although later, other authors (Every *et al.*, 1990) found similar levels of α-amylase activity in both heavily infested wheat and control wheat.

No studies have been made on the starch characteristics of wheat damage by insects of the genera *Aelia* and *Eurygaste*r. Since the behaviour of European and New Zealand insects is different in some aspects of the proteolytic activity (Every *et al.*, 1990), it is important to demonstrate if they damage starch.

The purpose of this study was to determine the level of amylase activities in different bug-damaged wheat cultivars by using specific substrates for a and ² amylases and to compare that with the diastatic activity values. Scanning electron microscopy (SEM) was used for analysing the internal microstructure, especially starch granules, of infested kernels showing bite marks.

MATERIALS AND METHODS

Several Spanish bread wheat varieties (Marius, Soissons, Chamorro and Astral) with different degrees of bug damage were used in order to determine the intercultivar variability and their susceptibility to insect attack.

Wheat kernels from the 1999 crop were provided by the Asociacion Espanola de Tecnicos Cerealistas (AETC). Cereal α-amylase and β-amylase assay reagents (from Megazyme International Ireland Ltd, Wicklow, Ireland) were used to measure the α- and ß-amylase activities. Other chemical reagents were of analytical grade.

Wheat characteristics

All samples were tested for thousand-kernel weight (TKW), protein content and protein content per kernel (protein/K). Protein was determined using the Kjeldahl method (AACC, 1995). Specific wheat weight was determined with an Ohaus (NY, USA) chondrometer using a 1 L container. Four replicates were performed for each determination.

Proteolytic activity assessment

The proteolytic degradation was determined with a Chopin alveograph (Tripette et Renaud, Paris, France), following the AACC-approved method (1983). The deformation energy (W) of dough maintained for 3 hours at 25°C was determined in order to assess the proteolytic degradation as a percentage of the initial deformation energy (Berger *et al.*, 1974). Three replicates were performed for each determination.

Diastatic activity

Falling number was determined as a means of assessing diastatic activity. Falling numbers were measured on a falling number device (model 1800, Perten Instruments, Huddinge, Sweden) following the standarized AACC method (1995). Each value was the mean of four replicates.

Sample preparation

Extracts for measuring α-amylase activity were prepared by homogenising 10g of previously ground wheat in 50mL of 100mM sodium acetate buffer, pH 5.0, using a Virtis (NY, USA) homogeniser (3 x 10s strokes at 20,000 rpm). The homogenate was centrifuged (12,000 rpm, 15min, 15°C) and the supernatant was filtered through glass wool. The clear extracts were stored at 4°C for subsequent enzyme assays.

β-amylase extracts were prepared by mixing 5g of wholemeal in 50ml of 50mM Trizma, containing 150mM β-mercaptoethanol (ME), pH 7.0, using a Virtis homogeniser (3 x 10s strokes at 20,000rpm). Thereafter, the procedure described for preparing α-amylase extracts was followed.

Enzyme activities were analysed within 4 hours.

Assay of α-amylase activity

α-amylase activity was measured using a blocked p-nitrophenyl maltoheptaoside (BPNPG7) as substrate following the method reported by

McCleary and Sheenan (1987) and further adapted to a microplate reader by Rosell *et al.* (2001). The absorbance at 405 nm was measured using a microplate reader (Spectramax 190, Molecular Devices, CA, USA). One unit of α-amylase activity was defined as the amount of enzyme which releases 1 µmol of p-nitrophenol/hour under the defined assay conditions. In all cases, four replicates were assayed for each experimental point.

β-amylase activity measurements

β-amylase activity was assessed by using p-nitrophenyl-α-D-maltopentaose (PNPG5) as specific substrate, following the method described by McCleary and Codd (1989), and adapted to a microplate reader by Sirou *et al.* (1990) with minor modifications. Briefly, 30µL of substrate solution and 30µL of sample were pipetted into individual wells of a 96-well microplate. The enzyme reaction was allowed to proceed for 15 min at 30°C, then stopped by adding 150µL of 1% (w/v) Trizma base. The absorbance of the p-nitrophenol obtained was measured at 405nm in a microplate reader (Spectramax 190, Molecular Devices). One unit of β-amylase activity was defined as the amount of enzyme which releases 1 µmol of p-nitrophenol/min under the defined assay conditions. Each experimental point was the mean of four replicates.

Scanning electron microscopy

Kernels of bug-damaged wheat showing bite marks were carefully cut through the point of piercing. The specimens of cross-sectioned kernels (1.5 mm thick) were coated with gold and examined in a JSM 5200 microscope (JEOL, Tokyo, Japan) at accelerating voltage of 10 kV.

RESULTS AND DISCUSSION

Kernel weight and chemical composition

Before determining the enzyme activity in bug-damaged wheat, it was

necessary to characterise the wheat. Kernel and cultivar characteristics are summarized in Table 1.

The degree of damage was determined by the alveographic test. Samples with different degrees of proteolytic degradation (PD) within each cultivar were selected. No differences in any of the quality parameters tested (the standard deviation was less than 1% of the mean, therefore it is not presented with the data) were observed between the control and samples with different degree of damage.

Protein content was not affected by the bug attack (Table 1), which agrees with previous findings (Every *et al.*, 1990; Johnson and Miller, 1953; Greenaway *et al.*, 1965).

Decreases in specific wheat weight (Table 1), except for Chamorro cultivar, were observed with bug damage, although no relationship could be found between these values and the degree of damage.

Thousand-kernel weight and the amount of protein per kernel were not

Table 1.–**Characteristics of bread wheat cultivars used in this study.**

Cultivar	Samples	Specific weight (g/Hl)	TKW (g)	Protein (%)[a]	Protein/K (mg)	PD (%)
Marius	Control	80.1	41.1	11.9	4.9	0
	MD	77.0	39.5	11.9	4.7	36
	D	72.3	34.7	11.6	4.0	56
Soissons	Control	79.5	32.3	10.5	3.4	0
	MD	79.4	36.3	10.5	3.8	35
	D	69.4	32.7	10.3	3.4	53
Chamorro	Control	80.3	34.9	13.8	4.8	0
	MD	80.2	33.0	13.7	4.5	29
	D	80.8	34.8	14.4	5.1	42
Astral	Control	78.5	31.5	11.9	3.8	5
	MD	80.1	34.0	11.7	4.0	38
	D	73.5	33.8	11.6	3.9	61

[a] Percentage calculated on the basis weight for weight.

–Description of samples: Control, without proteolytic degradation; MD, medium damage; D, damaged samples.

–TKW: Thousand kernel weight.

–Protein/K: protein per kernel.

–PD : Proteolytic degradation determined by alveographic test.

Values represent the mean of four replicates.

affected by the increasing percentage of proteolytic degradation (Table 1). Only in the cultivar Marius was there observed a steady decrease in TKW between the control and samples with different degree of damage. Lorenz and Meredith (1988) reported a decrease in kernel weight when different insects (*Sidnia*, *Calocoris* and *Nysius*) were introduced into the cage, although in that study the amount of damage was not described. In Europe and the Middle East, recent studies carried out on wheat damaged by pentatomid insects describe a reduction in kernel weight and protein per kernel as a result of attack (Hariri *et al.*, 2000; Karababa and Nazmi Ozan, 1998). However, the extent of the damage in different cultivars and the magnitude of the degree of attack were not considered in those reports. In New Zealand wheat samples from two different cultivars exhibiting a diverse range of *Nysius* attack, the kernel weight was reduced to 94-99% of that of undamaged grain in Rongotea cultivar, but no consistent relationship was detected in Kamaru cultivar (Every *et al.*, 1990).

The variability among cultivars observed in the present study might be attributed to differences in cultivar susceptibility to bug damage.

Diastatic activity

Falling number values are detailed in Table 2. In Marius cultivar a slight increase in falling number, representing a decrease in diastatic activity, was observed while in Astral cultivar the level of diastatic activity increased with the degree of damage (proteolytic degradation). No tendency could be detected with the extent of degradation in Soissons and Chamorro cultivars. Therefore it might be said that insect damage did not affect the falling number values, which are correlated with the diastatic activity. These data are supported by the results of Kranz (1935), but differ from those reported by other authors (Kretovich, 1944; Atasanova, and Popova, 1968), who found increased diastatic activity in grain damaged by *Aelia* and *Eurygaster*. It is likely that the difference could be attributable to cultivar variability. Results presented in this study suggest that diastatic activity may be more related to both cultivar and environmental conditions than to bug damage.

Most previous reports determined the diastatic activity rather than α and β-amylase activities, since there were no specific methods for separately measuring both amylase activities. In this study the amylase activities were

Table 2.–**Effect of wheat damage caused by *Aelia* and *Eurygaster* on amylase activities.**

Cultivar	Samples	Falling number (s)	α-amylase activity (µmol/h g)	β-amylase activity (µmol/min g)
Marius	Control	284 ± 8	1.758 ± 0.025	506.4 ± 22.2
	MD	284 ± 11	0.850 ± 0.069	356.3 ± 28.0
	D	326 ± 2	0.185 ± 0.011	259.0 ± 13.7
Soissons	Control	349 ± 28	0.254 ± 0.042	205.6 ± 30.1
	MD	363 ± 7	0.343 ± 0.006	148.4 ± 10.3
	D	284 ± 6	1.007 ± 0.019	211.7 ± 24.7
Chamorro	Control	375 ± 4	0.431 ± 0.010	328.8 ± 12.1
	MD	357 ± 6	0.822 ± 0.001	377.6 ± 10.4
	D	400 ± 15	0.250 ± 0.067	288.8 ± 5.9
Astral	Control	346 ± 11	0.170 ± 0.004	219.1 ± 27.3
	MD	327 ± 5	0.333 ± 0.019	323.0 ± 16.3
	D	303 ± 7	1.027 ± 0.001	231.0 ± 37.2

Description of samples: Control, without proteolytic degradation; MD, medium damage; D, damaged samples. Values are mean ± SD, n = 4.

assayed using specific substrates, since the contradictory results reported in the literature could be related to the measurement methods used to determine the amylase activity.

Amylase activities in bug damaged wheat

There was no consistent relationship between α-amylase activity and bug damage. In Marius cultivar the α-amylase activity of the undamaged wheat (control) was 10 times that of the most highly damaged wheat, which agrees with previous falling number values. Conversely, damaged samples from Soissons and Astral cultivars showed higher α-amylase values than the undamaged wheat. In Chamorro cultivar the α-amylase activity could hardly be related to bug attack.

This diversity observed in the α-amylase activity among cultivars might be due to the different growth stages at which the wheat was infested. Alternatively, the α-amylase activity may not have been related to bug attack,

but only to cultivar and environmental conditions.

High inter-cultivar variability was observed when analysing the β-amylase activity. No relationship was observed between β-amylase activity and the degree of bug damage within cultivars, with the exception of Marius cultivar. In Marius, β-amylase activity decreased with increasing damage level. The β-amylase activity in the most highly damaged Marius wheat was almost 50% of that in the undamaged wheat.

As previously observed for diastatic activity, β-amylase activity might be related to wheat variety and environmental conditions, and insect attack may not modify the levels of β-amylase.

Since the cultivar Marius showed a relationship between amylase activities and the degree of bug damage, a large number of samples from Marius cultivar with different degrees of damage were analyzed in order to confirm that tendency. However, no correlation was found when analyzing the amylase activities (α and β) of those wheat samples (unpublished results). This finding compels us to conclude that the initial results were casual and that wheat damage by *Aelia* and *Eurygaster* insects does not modify the amylase activities.

Therefore these results suggest that salivary amylases from *Aelia* and *Eurygaster* are not involved in the poor quality of bug-damaged wheat, which coincides with the behaviour of *N huttoni* described by Every *et al.*, (1990).

It has been stated that pests in Europe inject potent salivary enzymes which solubilise the wheat carbohydrate and proteins so that digested compounds can be sucked through the insect stylus (Kretovich, 1944). However, no studies on the purification and characterization of those enzymes has been reported. The data obtained in this study support the idea that insect salivary enzymes might not be involved in the feeding mechanism as was suggested by Doss (1980). In this case, the bug is feeding mechanism would consist of piercing the inmature grain with its stylus and sucking out the milky content of the grain.

SEM results

Some microscopic studies were carried out in order to confirm the previous results that amylase activity was not affected by *Aelia* and *Eurygaster* insects.

Wheat kernels attacked by insects have a black spot at the puncture point

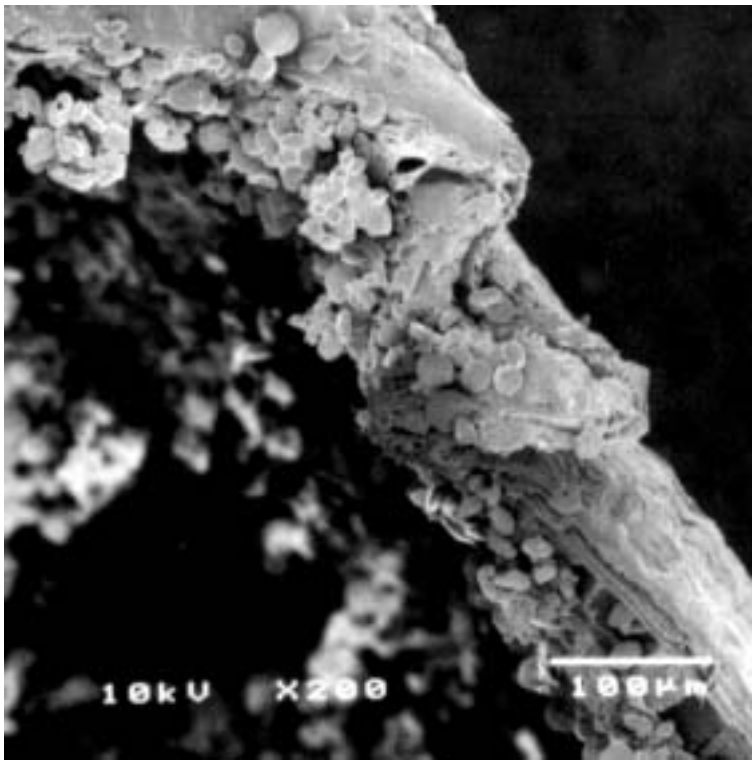


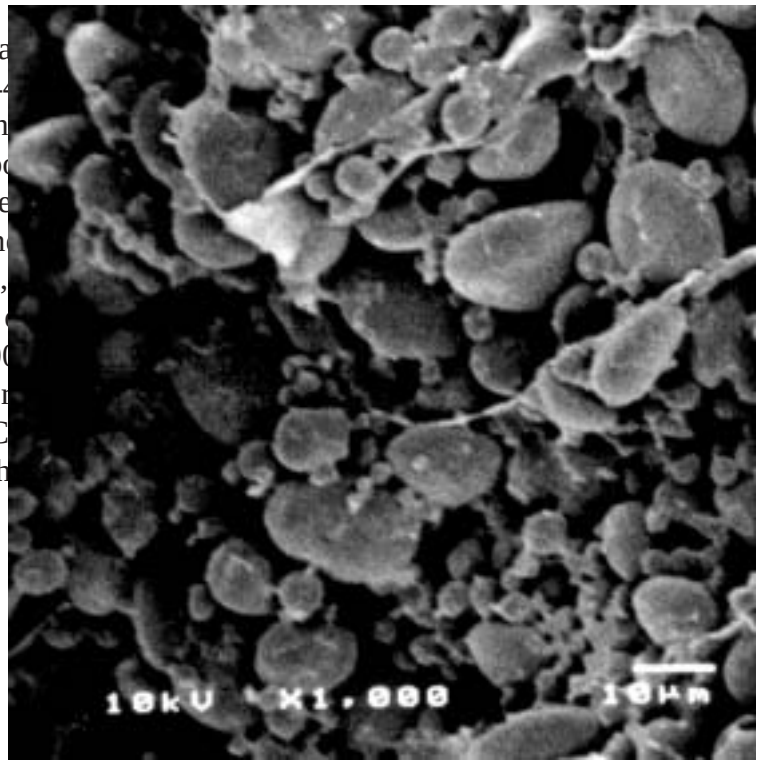
Figure 1.–Seed coat cross-section across piercing mark (200X). White arrow shows the empty endosperm underneath the bite point.

surrounded by a pale area. Wheat damaged by *Aelia* and *Eurygaster* showed the majority of bug puncture marks on the lateral surface of the kernel, and when multiple injuries were present, a clear line of feeding marks along the lateral surface could be observed. These examined kernels looked similar to those infested kernel shown by Every *et al.* (1990), who studied wheat grain damaged by *Nysius*.

Results of microscopic studies also suggested that injection of bug saliva did not affect amylase activity. The microstructure of endosperm in the damaged kernels was markedly changed, despite only a small black point being observed in the seed coat. Kernels were partly empty and the endosperm was completely destroyed in a large area underneath the bite point (Figure 1).

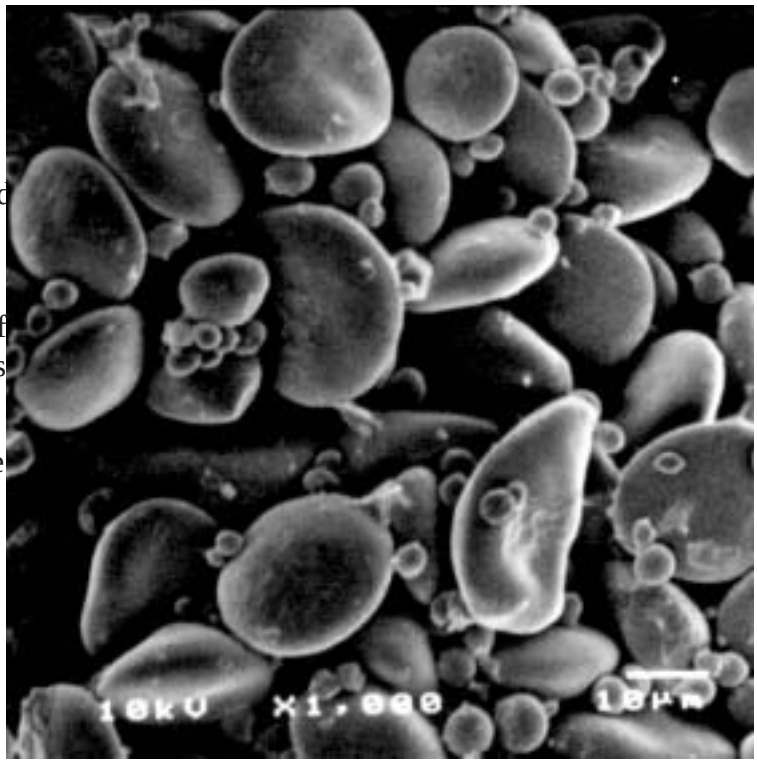
Typical changes in the remaining endosperm, ie the lack of an endosperm

Figure 2.–Micrograph of bug bite-damaged kernel endosperm (1000X).



protein matrix (Fig 2) confirmed high proteinase a
protein matrix might explain why Kretovich (194
traces of gluten from the infected parts of the in
cell walls in the endosperm near the puncture p
also the presence of cellulolytic enzymes in inse
B types of starch granules were intact. Some of th
appeared mechanically damaged on their surface,
to fingerprints of the smaller or B-type granules,
of the endosperm components (Rojas *et al.*, 20
tacked granules were not found (Figure 3). Ther
Eurygaster does not damage the starch granules. C
bug-attacked wheat showed severe damage to th
and Meredith, 1988).

Figure 3.–Micrograph of endosperm starch granules (1000X). White arrow shows a starch granule with mechanical damage on its surface.



CONCLUSION

High inter-cultivar and
ties (α and β) was found
of bug damage.

SEM examination of
showed unchanged shapes
damage.

Amylase activities are
and *Eurygaster*.

ACKNOWLEDGEMENTS

This study was financially supported by the European Union and Comisión Interministerial de Ciencia y Tecnologia (FEDER, 1FD97-0671-C02-01) and the Consejo Superior de Investigaciones Cientificas (CSIC, Spain). The authors would like to thank the Asociacion Espanola de Tecnicos Cerealistas (AETC) for providing samples. Part of this work belongs to the joint research programme between Polish Academy of Science and Consejo Superior de Investigaciones Cientificas (CSIC, Spain).

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CAPÍTULO 2

Caracterización de las proteasas procedentes de insectos heterópteros causantes de la degradación proteolítica

S. Aja[1], C. M. Rosell[1]

INTRODUCCIÓN

Ciertos insectos pentatómidos infestan las cosechas de trigo provocando regularmente la pérdida de dichas cosechas. La pérdida de calidad de los trigos como consecuencia del ataque de insectos ha sido descrita en Alemania, Rusia, España, Hungría, República Checa, Italia, Turquía, Irán, Iraq y Nueva Zelanda (Lorenz y Meredith, 1988). Las cuantiosas pérdidas económicas ocasionadas por estos ataques despertó el interés por determinar los agentes causantes de dicha infestación. En Europa, Asia y Norte de Africa este ataque se ha asociado a ciertas especies de los géneros *Aelia* y *Eurygaster* (Kent-Jones and Amos, 1967; Kent, 1987), mientras que en Nueva Zelanda se atribuye a *Nysius huttoni* (Cressey *et al.*, 1987). Un exhaustivo examen realizado en España entre muestras de trigo de la cosecha de 1997 reveló que *Eurygaster austriaca seabrai* y *Aelia germari* eran las especies más

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frecuentes en nuestro país (Infiesta *et al.*, 1999). Estos insectos perforan el grano de trigo y se alimentan del mismo en su fase lechosa. Al picar el grano introducen enzimas salivares, alterando la funcionalidad de los constituyentes del endospermo.

El ataque por estos insectos provoca un incremento excesivo de la actividad proteolítica, la cual origina la ruptura de las cadenas proteicas, concretamente las subunidades de mayor peso molecular de la glutenina (Swallow y Every, 1991). Esta acción se traduce en masas poco cohesivas, muy extensibles, con pegajosidades indeseables, incapaces de retener el gas que se produce durante la eatapa de fermentación. El producto resultante se caracteriza porque posee un reducido volumen y una textura defectuosa (Lorenz and Meredith, 1988; Haridi *et al.*, 2000).

En el caso del ataque por *Nysius huttoni*, se ha realizado la extracción, purificación y caracterización de la proteasa responsable de la degradación de las proteínas del gluten (Cressey, 1987; Every, 1993), demostrándose que se trata de una serin-proteasa soluble en agua, con una actividad óptima a pH 8,9 y 45°C. Además esta proteinasa no es inhibida por ácido etilendiamino tetraacético (EDTA) o N-etilmaleimida (NEMI) pero es inhibida por iones metálicos como Co^{2+}, Mn^{2+} y Fe^{2+}. Esta proteinasa no actúa sobre los substratos estándar de las proteasas como son hemoglobina, gelatina y N-benzoil-arginina etil ester; y cuando se incuba con harina no aumenta la cantidad de compuestos nitrogenados solubles en agua.

Son numerosos los estudios que dan por supuesto la existencia de proteasas como responsables de la degradación proteolítica de los trigos atacados por *Aelia* y *Eurygaster*, sin embargo en ningún estudio se ha comprobado la existencia de las mismas. Asimismo, a pesar del efecto proteolítico semejante que produce la infestación por *Nysius*, *Aelia* y *Eurygaster*, existen diferencias entre ellos. Por ello el objetivo de esta investigación fue la optimización de los métodos de extracción de las posibles proteasas exógenas responsables de la degradación, así como su purificación y posterior caracterización frente a distintos sustratos.

MATERIALES Y MÉTODOS

En este estudio se usó trigo comercial español con diferentes grados de infestación. La azocaseina, la hemoglobina (Sigma, España) y gluten vital

(Huici-Leidan, Navarra) se usaron como sustratos de proteasas. Los reactivos químicos utilizados fueron de grado analítico.

Obtención de las harinas de trigo

El trigo se limpió para eliminar las impurezas existentes. Posteriormente se acondicionó para ajustar el grado de humedad de la muestra a 15,5%. Por último se procedió a la molturación en un molino Perten de laboratorio.

Determinación de la degradación proteolítica

La degradación proteolítica fue determinada mediante el uso del Alveógrafo Chopin (Tripette y Renaud, Paris, France), según el método de la AACC (1983). Se determinó la energía de deformación (W) a las 3 horas a 25 ºC para determinar la degradación proteolítica, relacionándola con la energía de deformación inicial. Las determinaciones se realizaron por triplicado.

Extracción de las proteasas

Los extractos se prepararon suspendiendo 2 g de trigo molido en 20 ml de tampón Teorell y Stenhagen (citrato-fosfato-borato) usando un homogenizador Virtix (NY, USA) (3 x 10s) a 20,000 rpm. La suspensión se centrifugó (12,000rpm, 15 min, 15ºC), y el sobrenadante se filtró sobre lana de vidrio. Los extractos obtenidos se mantuvieron a 4 ºC hasta realizar la cuantificación de la actividad enzimática. La proteína extraída se cuantificó mediante el método de Bradford (1976) usando albúmina bovina como patrón.

Para la optimización de la extracción se analizó el efecto de la dilución, pH, fuerza iónica, presencia de ácido etilendiamino tetraacético (EDTA), presencia de agente reductor (mercaptoetanol, MCE), presencia de detergente (Tween), presencia de sales y tiempo de agitación necesario para la extracción.

Purificación de las proteasas

Se utilizaron distintas técnicas de separación para conseguir la purificación. Para la purificación por intercambio aniónico débil se utilizó una columna de DEAE-Sephacel (3,5 cm x 18 cm). Se inyectó 50 mL de extracto a 1,0 mL/min, usando como fase móvil Tris-HCl 50 mM a pH 6,0. La separación se realizó con un gradiente de cloruro sódico (0-500 mM), y se recogieron fracciones de 6,0 mL.

También se realizó una separación en columna de intercambio catiónico fuerte (SP-5PW, Phenomenex) en un cromatógrafo líquido de alta presión (HPLC) (Agilent), usando como fase móvil acetato sódico 5 mM a pH 5,0. El gradiente se realizó con cloruro sódico (0-500 mM). Se recogieron fracciones de 1,0 mL.

Determinación de la actividad azocaseína

La actividad endoproteolítica se determinó siguiendo el método de Preston y col (1978) con ligeras modificaciones. A 0,25 mL de azocaseína (1,5% (p/v) en 0,05 M tampón McIlvaine pH 5,4) se le añadieron 0,25 mL de extracto y se incubó 24 h a 40 ºC. La reacción se detuvo añadiendo 0,5 mL de ácido tricloroacético (TCA) 10% (p/v), se centrifugó durante 5 min a 20,000 rpm. A 0,150 mL de sobrenadante se le añadió 0,05 mL de hidróxido sódico 1,0 M, transcurridos 20 min a temperatura ambiente se midió la absorbancia en un lector de placas (Spectramax 190, Molecular Devices) a 440nm.

Determinación de la actividad hemoglobina

La actividad exoproteolítica se determinó según el método de Bushuk y col (1971) con ligeras modificaciones. En primer lugar se desnaturalizó la hemoglobina en medio ácido, para ello se preparó una suspensión de hemoglobina al 1,0 % (p/v) y se ajustó el pH a 1,7 con ácido clorhídrico; transcurridos 30 min se ajustó el pH a 4,0 usando acetato sódico. A 0,25 mL de la suspensión de hemoglobina se añadieron 0,25 mL de extracto y se incubaron 24 h a 40ºC, la reacción se detuvo añadiendo 0,4 mL de TCA

(10% p/v), y se centrifugó 5 min a 20,000 rpm. Los péptidos solubles en TCA se cuantificaron a 280 nm en un lector de placas (Spectramax 190, Molecular Devices).

Una unidad de actividad corresponde a la liberación de un µmol de leucina por hora a pH 4 y 50ºC.

Determinación de la actividad hidrolítica frente a gluten vital

Se utilizó una suspensión de gluten vital (5%, p/v) en acetato sódico 50mM pH 4,5 como substrato de la proteasa. El extracto (100µL) procedente de trigo sano e infestado se incubó en una suspensión de gluten vital (500 µL) a 37°C durante 60 minutos. La suspensión se centrifugó 5 min a 20,000 rpm y la absorbancia del sobrenadante se determinó a 280nm en un lector de placas (Spectramax 190, Molecular Devices).

RESULTADOS

Optimización de la extracción de la proteasa presente en trigos infestados

Se utilizó trigo con un alto grado de infestación determinada visualmente por la presencia de numerosos granos atacados, cuya degradación proteolítica determinada por el método alveográfico fue de 60%. Se evaluó la influencia de diversos medios de extracción sobre la actividad proteolítica usando como substratos hemoglobina (substrato utilizado en el método de la AACC para detectar actividades proteolíticas) y azocaseína, además se realizó la cuantificación de la proteína extraída. En la tabla 1 se puede observar el efecto de las diversas condiciones utilizadas en la extracción. La dilución afectó negativamente a la actividad azocaseína y hemoglobina, seleccionándose diluciones de 1/5. La extracción de proteasas con actividad hemoglobina y azocaseína se favoreció a pH 4,5, utilizando alta fuerza iónica; la presencia de EDTA no afectó a la actividad azocaseína extraída, pero sí a la actividad hemoglobina, por ello se procedió a realizar extracciones en ausencia de EDTA. La presencia de agente reductor (ME), detergente y sales no produjeron ninguna mejora en la cantidad de proteasa

Tabla 1.–**Optimización de las condiciones de extracción de la proteasa presente en trigos infestados. Las condiciones de extracción se detallan en materiales y métodos.**

Condiciones		Proteína (mg/mL)	Actividad azocaseína (Ab_{330})	Actividad hemoglobina (U/mL)
Dilución	1/5	9,29	0,055	7,17
	1/10	9,86	0,018	6,11
pH	4,5	7,47	0,159	5,88
	6,0	8,64	0,081	5,16
	7,0	11,55	0,083	5,91
	8,0	13,11	0,084	5,75
	10,0	19,37	0,066	6,03
Fuerza iónica (mM)	0	7,80	0,021	3,78
	50	9,30	0,160	7,17
	100	8,80	0,425	9,79
EDTA (mM)	0	7,00	0,350	7,42
	1	6,62	0,375	5,11
	3	6,51	0,350	5,40
	5	7,31	0,360	4,60

extraída. Las condiciones óptimas seleccionadas para realizar la extracción de proteasa fueron en tampón 100mM a pH 4,5.

Purificación de la proteasa procedente de trigos infestados

Los ensayos de purificación se realizaron usando trigo sano y trigo infestado con un 60% de degradación proteolítica. Se aplicaron diversos métodos de purificación de proteínas, precipitación con sulfato amónico (resultados no mostrados), cromatografía de intercambio aniónico y catiónico.

Mediante precipitación de proteínas con sulfato amónico no se detectó ningún aumento de la actividad en las diversas fracciones obtenidas (resultados no mostrados). Cuando se realizó la separación por intercambio aniónico débil se observó un perfil de proteínas semejante en el extracto procedente de trigo sano comparado con el trigo infestado (Figura 1), aunque en el caso de trigo

sano se detectó mayor concentración de proteínas. En las fracciones recogidas se cuantificó la actividad hidrolítica frente hemoglobina (Figura 2) y azocaseína (Figura 3). Respecto a la actividad hemoglobina, fue mayor en las fracciones procedentes de trigo sano, además no se detectó un pico evidente de actividad frente a este substrato, se obtuvo un perfil muy dentado, indicando que no se realizó una separación efectiva de la posible proteasa.

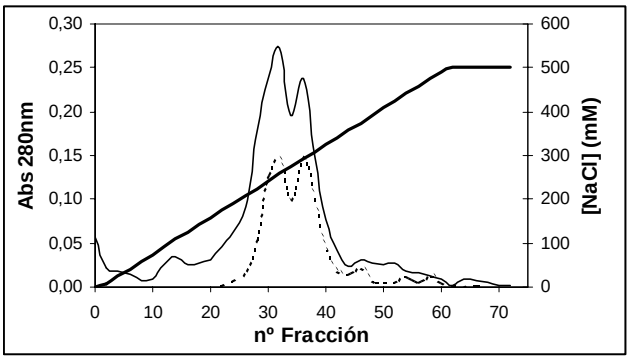
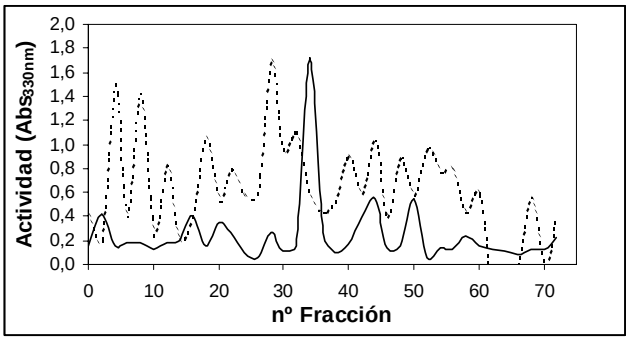


Figura 1.–Perfil proteico obtenido al separar por intercambio aniónico débil extractos procedentes de trigo sano (línea continua) y trigo infestado (línea discontinua). Además se representa el gradiente salino utilizado en la separación.

Figura 2.–Actividad hidrolítica frente a hemoglobina cuantificada en las fracciones obtenidas por intercambio aniónico débil procedentes de extractos de trigo sano (línea continua) y trigo infestado (línea discontinua).



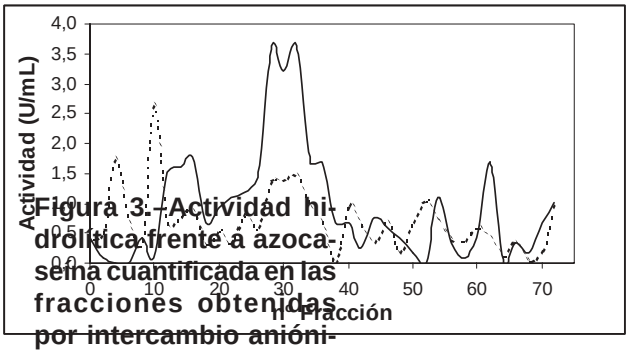


Figura 3.–Actividad hidrolítica frente a azocaseína cuantificada en las fracciones obtenidas por intercambio aniónico débil procedentes de extractos de trigo sano (línea continua) y trigo infestado (línea discontinua).

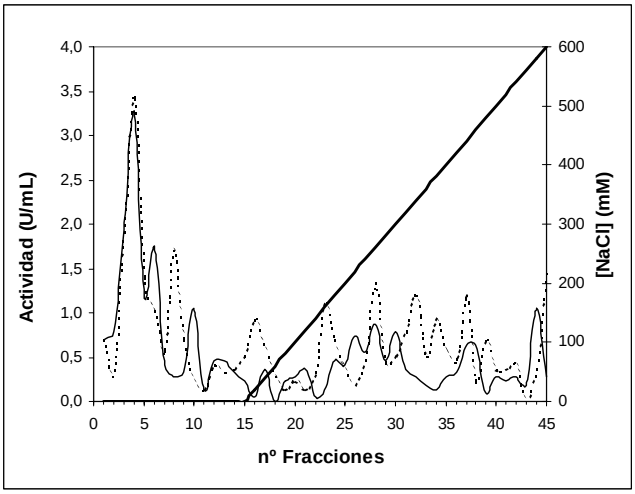


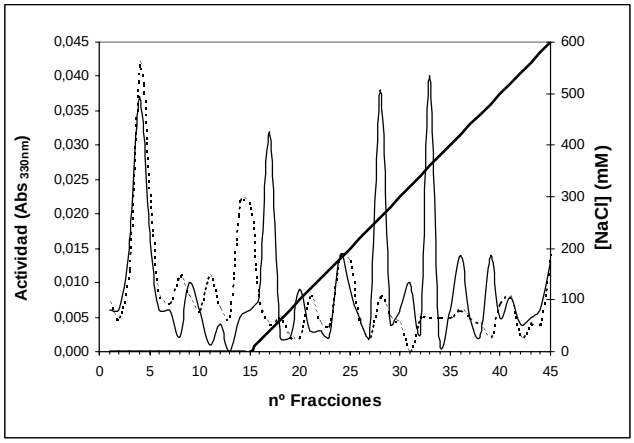
Figura 4.–Actividad hidrolítica frente a hemoglobina cuantificada en las fracciones obtenidas por intercambio catiónico fuerte procedentes de extractos de trigo sano (línea continua) y trigo infestado (línea discontinua). Además se representa el gradiente salino utilizado en la separación.

En el caso de la actividad frente azocaseína, en el extracto procedente de trigo sano se observó un pico de actividad frente a este substrato, mientras que en el caso del trigo infestado se obtuvo un perfil muy dentado sin evidencias de la presencia de alguna proteasa exógena.

Por tanto estos estudios comparativos del perfil de proteasas procedentes de trigo sano y trigo con degradación no permitieron detectar proteasas específicas procedentes de los insectos heterópteros.

Dada la ineficacia de la columna de intercambio aniónico débil para la purificación de la proteasa se procedió a realizarla utilizando el intercambio catiónico fuerte. En la figura 4 se puede observar la actividad hidrolítica

Figura 5.–Actividad hidrolítica frente a azocaseína cuantificada en las fracciones obtenidas por intercambio catiónico fuerte procedentes de extractos de trigo sano (línea continua) y trigo infestado (línea discontinua). Además se representa el gradiente salino utilizado en la separación.



frente a hemoglobina de las distintas fracciones obtenidas. Únicamente se detectó actividad frente a este substrato en el frente de proteínas, pero dicha actividad se cuantificó en los extractos procedentes de trigo sano e infestado, por lo cual no podría ser atribuida a proteasas procedentes de insectos.

Cuando se analizó la actividad frente a azocaseína (Figura 5), también se detectó actividad en el frente del cromatograma, y dicha actividad se determinó en ambos extractos. Además en el caso del trigo sano se observaron otros picos de actividad. Por tanto, la columna de intercambio catiónico fuerte tampoco permitió la separación de alguna proteasa procedente de los insectos causantes de la infestación.

Por lo tanto, los resultados obtenidos entre los cromatogramas de los trigos sanos y con degradación no permitieron detectar proteasas específicas procedentes de los insectos heterópteros. Ante los resultados obtenidos se puso en duda la idoneidad del substrato utilizado para la cuantificación de la actividad proteolítica.

Identificación de trigos con diversos grados de infestación

Se utilizaron mezclas de trigo sano e infestado y se evaluó su degradación proteolítica mediante el método alveográfico. Se seleccionaron mezclas con niveles de degradación de 15, 30, 45 y 60% y se determinó la actividad proteolítica utilizando como substratos la hemoglobina, azocaseína y el gluten (Figura 6), para determinar si con alguno de ellos existía buena correlación entre la degradación determinada con el método alveográfico y la actividad proteolítica.

En ninguno de los substratos utilizados se observó una correlación entre

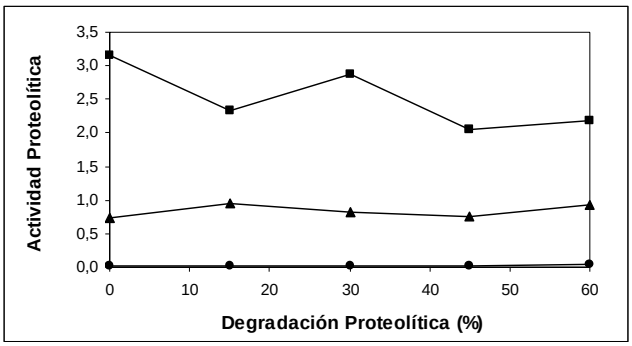


Figura 6.–Actividad hidrolítica de extractos, procedentes de mezclas de trigo sano e infestado con distinta degradación proteolítica, frente a distintos substratos. Azocaseína: círculos; hemoglobina: cuadrados; gluten: triángulos.

la actividad hidrolítica y la degradación proteolítica determinada mediante el método alveográfico.

Este valor se puede atribuir a que esa harina con un 60% de actividad proteolítica tenga proteasas de distintos orígenes que no hidrolizan la hemoglobina.

CONCLUSIONES

Los resultados obtenidos no permiten confirmar la existencia de determinados enzimas proteolíticos presentes en la saliva de los insectos *Aelia* y *Eurygaster* responsables de la degradación proteolítica de los trigos atacados por estos insectos, ya que no se pudo aislar la proteasa procedente del garrapatillo. Posiblemente los substratos usados no fueron los adecuados para medir la actividad proteolítica de estas enzimas.

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CAPÍTULO 3

Effect of *Aelia* spp. and *Eurygaster* spp. damage on wheat proteins

C.M. Rosell[1,2], S. Aja[1], S. Bean[3], G. Lookhart [3,4]

ABSTRACT

The effect of *Aelia* spp. and *Eurygaster* spp. wheat bugs on the protein fractions of different wheat cultivars has been studied by size-exclusion high-performance liquid chromatography (SE-HPLC) and free-zone capillary electrophoresis (FZCE). Those methods were used to quantify and characterize the extent of protein modification. A decrease in the amount of alcohol-insoluble polymeric proteins along with an increase in the alcohol-soluble polymeric proteins and gliadins were observed in damaged wheat. The high molecular weight (HMW) and low molecular weight (LMW) glutenin fractions were barely detected in the incubated damaged wheat from some cultivars, which indicated hydrolysis of those proteins by the bug proteinases. In damaged wheats, both incubated and unincubated, gliadin electrophoregrams revealed the presence of some new peaks with mobilities similar to the ω gliadins. The overall results suggest that the bug proteinases

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are potent enzymes that appear to be nonspecific because they hydrolyze all gluten proteins.

INTRODUCTION

Wheat is damaged by sucking insects, commonly called bugs, and that damage has produced important economic losses to millers and bakers (Lorenz and Meredith, 1988). Bug-damaged wheat has been characterized by a disrupted protein structure due to the action of some injected proteinases (Kretovich, 1944), although no alteration in the amylase activities and structure of the starch granules has been observed (Every *et al.*, 1990; Rosell *et al.*, 2002). Bug-damaged wheat causes reduced flour quality (Karababa and Ozan, 1998; and references cited herein), giving a softer dough and subsequently flat bread with low volume and unsatisfactory texture (Lorenz and Meredith, 1988; Hariri *et al.*, 2000).

In Europe, the Middle East, and North Africa, wheat damage has been attributed to two genera of heteropterous, *Aelia spp. and Eurygaster spp.*, and in New Zealand, *Nysius huttoni* is considered responsible for the wheat damage (Cressey *et al.*, 1987). Spain is one country where bug damage of wheat is considered an endemic problem. Infiesta *et al.* (1999) analyzed the bug damage in the 1997 wheat crop and found that *Eurygaster austriaca seabrai* and *Aelia germari* were the most frequent species present in bug damaged wheat. Nevertheless, scarce information has been reported about the protein modifications produced by these bugs.

Wheat attacked by *Nysius huttoni* showed high bug proteinase activity (Cressey, 1987; Every *et al.*, 1990; Every 1992). Different studies by PAGE and SDS-PAGE demonstrated that proteases from *N. huttoni* specifically hydrolyze high molecular weight glutenin subunits (HMW-GS), yielding degradation products with an electrophoretic mobility similar to that of ω-gliadins. However, *N. huttoni* protease does not affect the gliadin fraction (Cressey and McStay, 1987; Every *et al.*, 1990). In *Eurygaster* spp. damage, Yakovenko *et al.* (1973) reported a similar selective degradation of the glutenin fraction, although the hydrolysis products had an electrophoretic mobility similar to that of the α-gliadins. Kozmina and Tvorogova (1973) described a reduction in the relative intensity of some bands in the electrophoretic pattern of total unreduced glutenin and gliadin fractions, and the

appearance of two new bands with lower mobility in the gliadin pattern. Later, Sivri *et al.* (1998) confirmed by electrophoresis studies that bug damage caused by *E. maura* affected the gliadins and the reduced glutenin fraction, where the HMW-GS were the most susceptible fraction to bug attack. Less information is available about the damage caused by *Aelia* spp.

Nevertheless, most protein studies describe the changes in the protein pattern without quantifying the extent of the bug damage in specific protein fractions. Sivri *et al.* (1999) recently reported an attempt to quantified the effect of *E. maura* damage on the ratio of HMW-GS and LMW-GS among different wheat cultivars. These authors observed a high intercultivar variation when analyzing the ratio of HMW-GS and LMW-GS by RP-HPLC.

In Europe, bug damage is related to the disruption of polymeric glutenin proteins (HMW-GS, LMW-GS). Up to now, different approaches have been made to characterize those polymeric proteins although reduction of the proteins was always carried out. A better understanding of the effect of bug damage on wheat endosperm proteins might be reached by using size-exclusion high performance liquid chromatography (SE-HPLC), because this technique allows the characterization of wheat proteins without previous reduction and, hence, in their polymeric form (Gupta *et al.*, 1993; Larroque *et al.*, 1997).

High-performance capillary electrophoresis (CE) has emerged as a powerful technique in the characterization of cereal proteins (Bean *et al.*, 1998a; Bean and Lookhart, 2000a, b, 2001). CE allows high-resolution electrophoretic separation of the cereal proteins, based on different charge density (free-zone capillary electrophoresis, FZCE), size (SDS-CE) or isoelectric point (capillary isoelectric focusing, CIEF).

The aim of the present study was to analyze the extent of *Aelia* spp. and *Eurygaster* spp. damage on wheat proteins, in terms of both amount and size distribution, by using the high-resolving techniques of SE-HPLC and CE to help understand the real mechanism of bug action. Different cultivars of Spanish bread wheats with a wide range of damage were studied.

MATERIALS AND METHODS

Several Spanish bread wheat cultivars (Marius, Soissons, Chamorro, and Astral) with and without bug damage were used to study the damage to the

gluten proteins and the variability of damage on each cultivar due to insect attack. Wheat kernels (undamaged and damaged) from the 1999 crop of each cultivar were provided by the Asociacion Espanola de Tecnicos Cerealistas (AETC). Damaged samples were from the northern region of Spain, an endemic area of *Aelia* and *Eurygaster* infestation. In fact, a high number of insects was still present in the samples. Chemical reagents were purchased from Sigma (St Louis, MO) and were of the highest purity.

Proteolytic Activity Assessment

A black spot surrounded by a pale area was visually observed in the damaged wheat. However, the extent of the damage was quantified by using the Chopin Alveograph (Tripette et Renaud, Paris, France), following Approved Method 54-30 (AACC 2000). Berger *et al.*, (1974) found a good correlation between the deformation energy (W) change when the dough is allowed to stand at 25°C for 3 hr and the proteolytic degradation.

Sample Preparation

Alcohol-soluble proteins were obtained by vortexing 200 mg of whole meal with 1.0 mL of 50% 1-propanol for 5 min and centrifuging at 14,000 rpm for 2 min, as described Bean *et al.*, (1998b). These extracts contained albumins, globulins, gliadins and soluble polymeric glutenins. The residues were washed twice with the same solution and centrifuged at 14,000 rpm for 2 min. After extraction, pellets were freeze-dried and then the protein content was analyzed by combustion method using a nitrogen determinator (Leco Corp., St. Joseph, MI) according to Approved Method 46-30 (AACC 2000). A conversion factor of 5.7 was used to transform nitrogen values to protein content.

A sequential extraction was made to obtain each class of proteins. Gliadins were extracted with 50% propan-1-ol after previous removal of albumins and globulins as reported in Bean *et al.*, (1998a). Polymeric proteins were extracted by using both nonreducing and reducing conditions. Nonreduced polymeric proteins were obtained by mixing the pellet with 1.0 mL of 1% SDS centrifuged at 14,000 rpm for 2 min. The reduced polymeric

proteins were extracted with 1.0 mL 50% propan-1-ol containing 1 % DTT and then centrifuged at 14,000 rpm for 2 min. HMW-GS were prepared by acetone precipitation as described previously Bean and Lookhart (1998).

In incubated samples, whole meal (200 mg) was mixed with deionized water (0.5 mL) and kept under gentle magnetic stirring at 40°C for 3 hours, then the protein extraction was performed as described above.

Four different sets of samples from Marius, Soissons, Chamorro and Astral cultivars, with and without incubation, were run for each determination.

SE-HPLC Analysis

An Agilent 1100 Chromatograph was used for all the HPLC separations. Size-exclusion separation was performed by injecting 15 µL of sample at 1.0 mL/min of acetonitrile (ACN)/water (50:50) containing 0.1% (w/v) TFA into a Phenomenex BioSep SEC-4000 column (Phenomenex, Torrance, CA). Protein elution was monitored at 220nm.

Capillary Electrophoresis Analysis

Separations were made using a Beckman PACE 5510 instrument. Uncoated fused silica capillaries (Polymicro, Phoenix, AZ) of 50µm i.d. x 27 cm (20 cm L_D) were used for all separations.

Free-zone capillary electrophoresis (FZCE) was performed at the optimum separation conditions described by Bean and Lookhart, (2000b): 50mM iminodiacetic acid (IDA) containing 20% ACN and 0.05% hydroxypropyl methyl cellulose (HPMC), at 45°C and 30kV.

RESULTS AND DISCUSSION

Protein Size Modification by Bug-Damaged Attack

The proteolytic degradation of the wheat kernels used in this study was previously assessed by the alveographic method (Table 1).

Table 1.–**Proteolytic Activity of Bread Wheat Cultivars Determined by Alveographic Test.**

Cultivar		Proteolytic Activity (%)
Marius	Undamaged	0
	Damaged	56
Soissons	Undamaged	0
	Damaged	53
Chamorro	Undamaged	0
	Damaged	42
Astral	Undamaged	5
	Damaged	61

Values are means of three replicates.

The alcohol-soluble proteins comprise albumins, globulins, gliadins and some polymeric proteins. Typical SE-HPLC profiles are shown in Figure 1. The first peak corresponds to the alcohol-soluble polymeric proteins, the second peak corresponds to gliadins and the third peak corresponds to globulins and albumins with molecular mass < 30kDa (Larroque *et al.*, 1997). Instead of comparing the relative areas of the peaks for the different wheat cultivars, the quantitative method described by Bean *et al.* (1998b) was used for this purpose. The protein content of the remaining pellet, after the extraction of the alcohol-soluble proteins, was determined to quantify the amount of the alcohol-

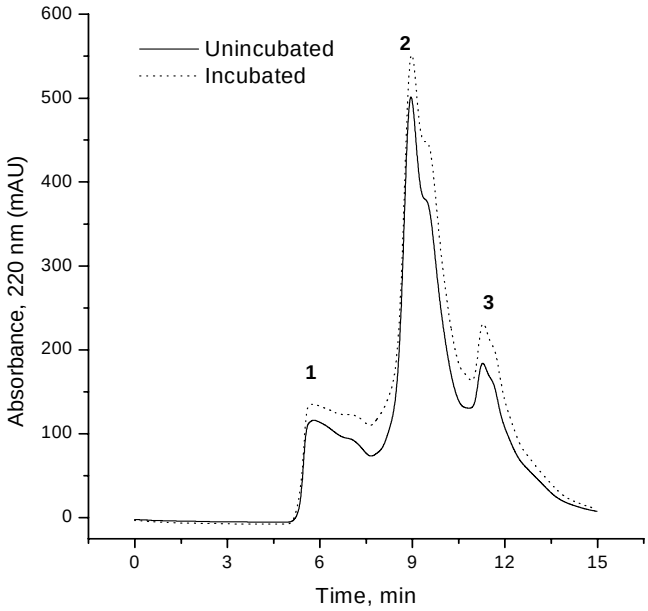

Figure 1.–Size-exclusion HPLC protein separation of alcohol-soluble extracts from wheat whole meal. Overlaid chromatograms of unincubated and incubated samples. Peaks: 1, alcohol-soluble polymeric proteins; 2, gliadins; 3, globulins and albumins with molecular mass <30 kDa.

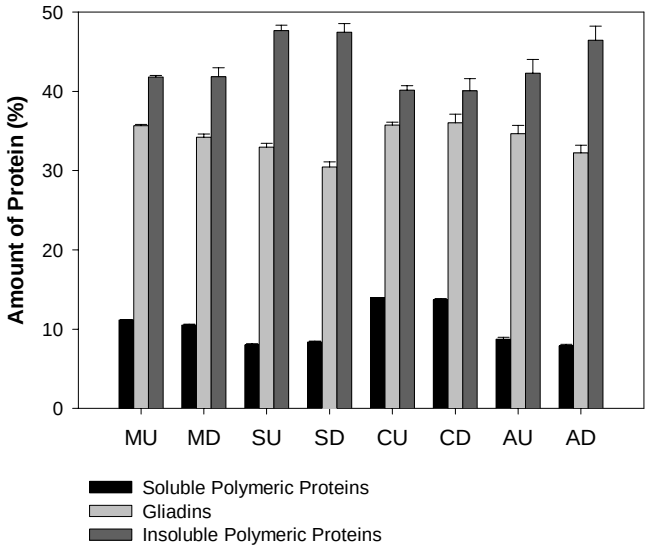

Figure 2. Effect of bug damage on the amount of proteins in various protein fractions. Cultivars: M, Marius; S, Soissons; C, Chamorro; A, Astral. Samples: U, undamaged; D, damaged. Error bars indicate standard deviations (n=4).

insoluble proteins. The alcohol-soluble proteins were calculated by subtracting them from the total protein amount. The protein content of each SE-HPLC protein fraction was obtained by relating the protein content (mg) with the area of the peaks obtained by SE-HPLC. For comparative purposes, the percentage of each class of proteins was calculated. Figure 2 shows that, along with a high intercultivar variability, no significant differences in the alcohol-soluble polymeric protein content were found between undamaged and bug-damaged wheat. However, with the exception of Chamorro, undamaged wheats had slightly higher amounts of gliadins than damaged wheat. Sivri *et al.* (1998) could not find a detectable effect of bug damage on the gliadins by using gel electrophoresis, but the quantitative SE-HPLC used in the present study showed differences. In Astral, the alcohol-insoluble polymeric proteins slightly increased with the bug attack. On the other hand, the total amount of alcohol-soluble proteins did not change with the bug attack, which means that the protein hydrolysis by bug proteinases led to changes in the protein size without modifying total alcohol-soluble protein content.

The SE-HPLC gliadin profile of incubated samples showed a large modification (Figure 1). These differences were observed in the undamaged and damaged samples, although the extent of the modification was varied, it was more pronounced in the damaged samples than the undamaged ones (Figure 3).

A decrease in the amount of alcohol-insoluble polymeric proteins was

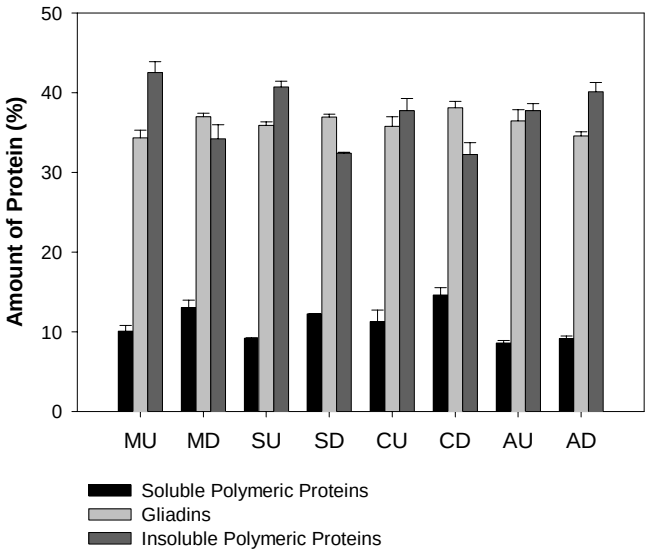

Figure 3. Effect of bug attack on the amount of proteins in various protein fractions after incubation at 40°C for 3 hours. Cultivars: M, Marius; S, Soissons; C, Chamorro; A, Astral. Samples: U, undamaged; D, damaged. Error bars indicate standard deviations (n=4).

observed in the incubated undamaged samples from Soissons and Astral, but no change was observed in undamaged Marius and Chamorro samples. The products derived from the hydrolysis of the alcohol-insoluble polymeric proteins eluted with the gliadins, yielding an increase of this fraction. The modification of the protein profile observed in the undamaged samples after incubation might be due to endogenous proteolytic enzymes, although no detectable activity was measured in Soissons by the alveographic method.

A severe decrease of the alcohol-insoluble polymeric proteins along with an increase of the alcohol-soluble polymeric proteins and gliadins were observed after incubation of the damaged samples. Nightingale *et al.* (1999) obtained analogous results when they studied the effect of *Fusarium spp* on wheat storage proteins by SE-HPLC. In that study, they also reported a large decrease of the HMW proteins concomitant with a redistribution of the proteins to LMW.

The intensity of the hydrolysis varied among cultivars, with Soissons being the most affected, although similar degradation activities were measured by the alveograph test. It may be that the protein structure of Soissons is more susceptible to bug proteinase hydrolysis. Similar findings were obtained by Every *et al.* (1998) and Sivri *et al.* (1999), who observed different cultivar susceptibilities to bug damage. They attributed this variability to intrinsic differences in glutenin structure. The changes observed

in the alcohol-soluble proteins supported the results of Kretovich (1944), where bug infection increases the protein solubility in alcohol and in water.

Effect of Bug Damage on the Gliadin Fraction

To determine the specific effect of the bug damage on the gliadins, these proteins were extracted following the removal of albumins and globulins and analyzed by FZCE. Damaged wheat from all the cultivars tested showed a decrease in a peak at the γ gliadin mobilities and new peaks appeared at the lowest mobilities in the ω gliadin range. Those results could be due to the hydrolysis of polymeric proteins, or insect feeding on immature kernels and causing interference with grain development and, in consequence, the laying down of specific proteins. The extent of the difference between undamaged and damaged wheat was greatly dependent on the cultivar. Soissons showed the greatest modification between undamaged and damaged samples, where some new peaks in the ω gliadin mobility zone were noted (Figure 4). In

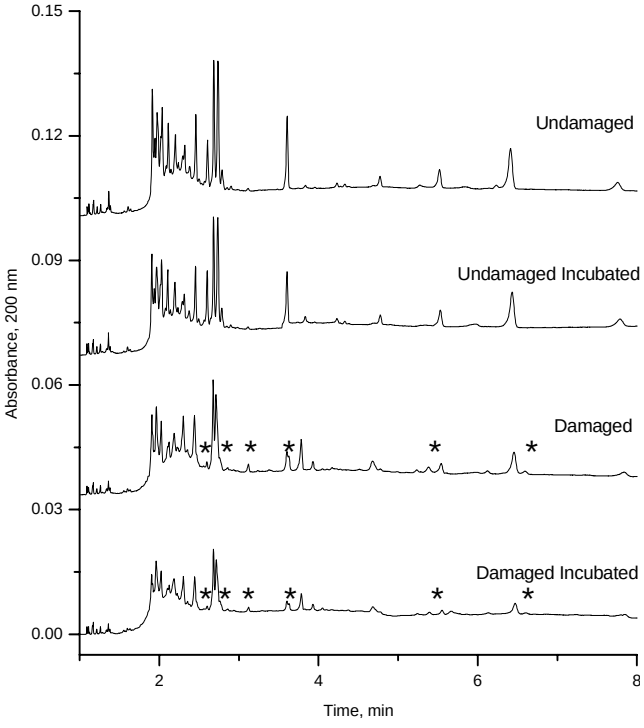

Figure 4.–Modification of the free-zone capillary electrophoresis (FZCE) pattern of gliadins from Soissons due to bug damage. Asterisks indicate differences among samples. Samples were pressure injected (0.5 psi) for 2 sec.

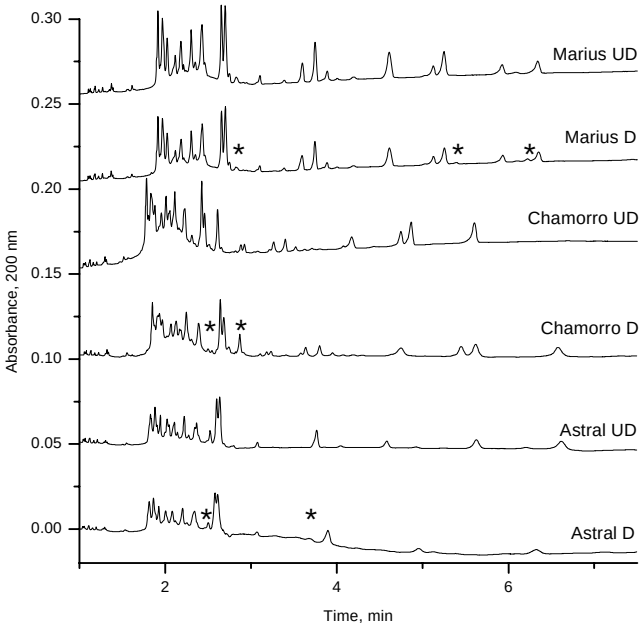

Figure 5.–Modification of the free-zone capillary electrophoresis (FZCE) pattern of gliadins from different cultivars due to bug damage. UD, undamaged wheat; D, damaged wheat. Asterisks indicate differences among samples.

the other cultivars, electrophoregrams of the gliadins also revealed differences between undamaged and damaged samples in the γ and ω gliadin mobility range (Figure 5).

Incubation of damaged samples caused a considerable decrease in the intensities of all the gliadin peaks without changing the mobilities. Only a slight decrease in intensity was observed in the undamaged samples. However, a very pronounced decrease was observed in the damaged samples, which contained smaller amounts of gliadin proteins (Figure 4). These results appeared to be contradictory because by SE-HPLC and protein quantification had shown that gliadin content increased with incubation. To explain those differences, the isolated gliadin fractions were analyzed by SE-HPLC. Once again, the relative amount of gliadins (peak 2) decreased after incubation (Figure 6). This difference can be explained by a size overlapping of the hydrolysis products produced by bug proteinase and the gliadins, which was not observed when gliadins were extracted after removal of the salt-soluble compounds. In fact, an increase of the salt-soluble compounds was observed after incubation when those fractions were analyzed by SE-HPLC (results not shown).

Changes in intensities and mobilities have been described in the bug-

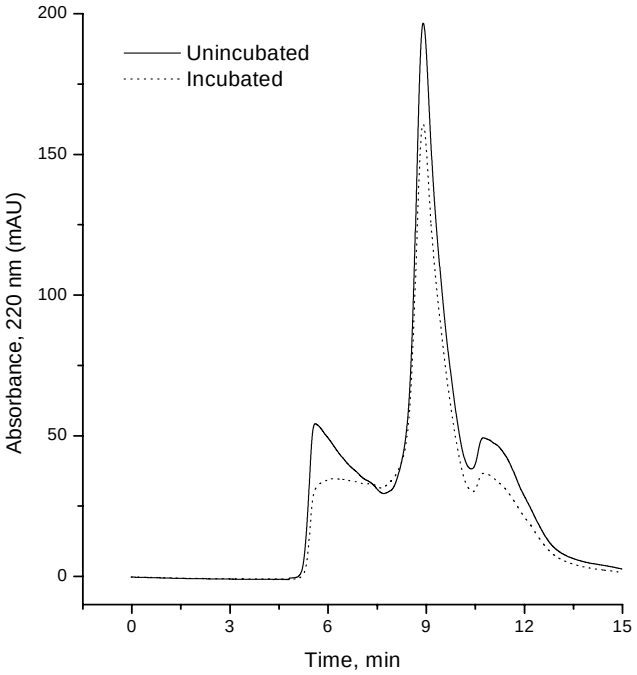

Figure 6.–Size-exclusion HPLC separation of gliadin fraction after removal of albumins and globulins.

damaged wheat caused by *E. maura,* but only after incubation (Sivri *et al.,* 1998). In this study, the effect of bug damage on gliadins was readily observed before incubation by using FZCE.

By using FZCE, some new peaks in the ω gliadin region were detected as well as a decrease of all gliadin peaks, the later being accentuated by the incubation. From these results, it is evident that gliadins are affected by bug damage, but the decrease in all gliadin proteins might be explained by a dilution effect due to the increase in the alcohol- and salt-soluble protein content or the presence of a nonspecific proteinase in bug insects.

In New Zealand, bug damage caused by *N. huttoni* does not modify the gliadin patterns but hydrolysis products from polymeric proteins with electrophoretic mobilities similar to ω gliadins have been detected (Cressey and McStay 1987; Swallow and Cressey 1987; Every *et al.,* 1990).

Effect of the Bug Damage on the Glutenin Fraction

Glutenin proteins extracted under nonreducing conditions were analyzed

by SE-HPLC. No differences could be attributed to bug attack because a high variability was observed between cultivars (results not shown). Comparison of the total area beneath the chromatograms showed a slight decrease of the amount of extracted glutenins in the incubated samples. This effect was observed in the undamaged and damaged wheat. Therefore, it should be more ascribed to endogenous protease activity rather than bug proteinase effect.

Glutenins extracted under reducing conditions were analyzed by FZCE. Comparison of the FZCE profiles of undamaged (unincubated and incubated) and damaged samples (unincubated) revealed no qualitative differences. However, a decrease of all the glutenin peaks was observed in the incubated damaged samples (results not shown).

It has been reported that bug proteinases hydrolyze glutenins, and within them they specifically degrade HMW-GS (Cressey and McStay, 1987; Sivri *et al.*, 1998, 1999). To identify the possible specificity of the bug proteinase, HMW-GS and LMW-GS were analyzed by FZCE. The electrophoregrams of the LMW-GS from undamaged samples did not show changes, even after incubation.

However, comparison of the electrophoregrams of LMW-GS from damaged samples showed a large decrease of the peaks after incubation, which was barely detected in Soissons and Chamorro cultivars (Figure 7).

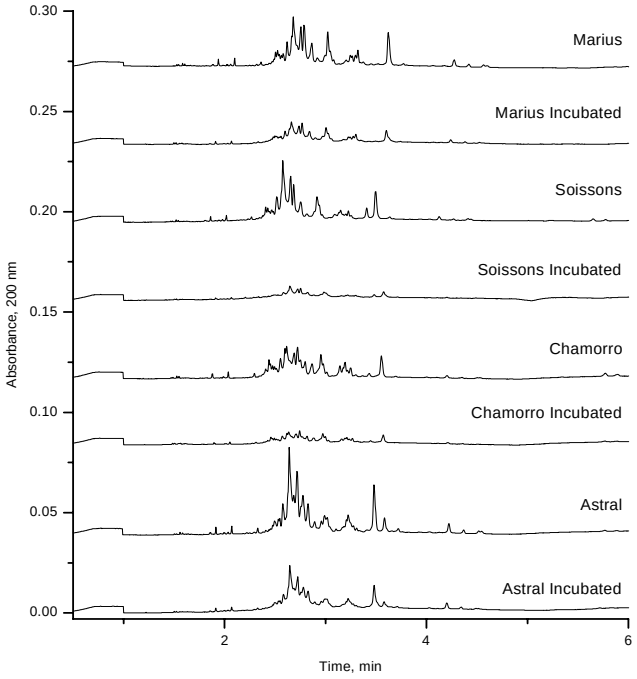

Figure 7.–Free-zone capillary electrophoresis (FZCE) of low molecular weight glutenin fractions from different bug-damaged cultivars. Samples were pressure-injected (0.5 psi) for 4 sec.

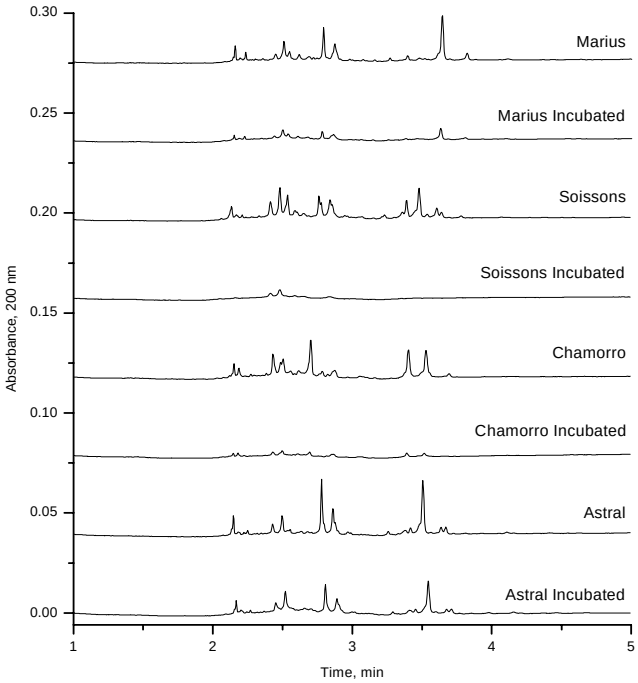

Figure 8.–Free-zone capillary electrophoresis (FZCE) of high molecular weight glutenin fractions from different bug-damaged cultivars. Samples were pressure-injected (0.5 psi) for 4 sec.

No differences were observed between unincubated undamaged, unincubated damaged HMW-GS, and incubated undamaged samples. HMW-GS electrophoregrams from incubated damaged samples showed a profound decrease of all the proteins and, again, they were hardly visible in Soissons and Chamorro cultivars (Fig. 8). Astral was the least affected by the bug attack; it showed the lowest modification of the HMW-GS and LMW-GS fractions. Those fractions (HMW-GS and LMW-GS) were also analyzed by using SDS-CE to determine a possible size change, but no differences were detected in any of them (results not shown). These findings indicate that the proteinases from Spanish bug pests hydrolyze glutenins specifically, but they do not have any specificity for HMW-GS or LMW-GS.

These results are similar to those obtained by Sivri *et al.* (1998), who reported a decreased of the LMW-GS and HMW-GS in damaged wheat caused by *Eurygaster,* but marked differences were observed among cultivars. Conversely, Every *et al.* (1990, 1998) only described a decrease of the HMW-GS in wheat damaged by *Nysius.*

CONCLUSIONS

Protein modification promoted by attack of bug pests can be quantified by using SE-HPLC combined with protein quantification. Some differences can be detected in the unincubated undamaged and damaged wheat, although they are largely dependent on the cultivar. When samples were incubated, bug proteinases produced profound decreases in the alcohol-insoluble polymeric proteins concomitant with an increase of the alcohol-soluble polymeric proteins, gliadins, and albumins and globulins. Free-zone capillary electrophoregrams of each protein fraction allow characterization of the protein disruption caused by bug proteinase and, in consequence, FZCE might be a useful tool for rapid determination of bug attack.

The high intercultivar variability suggests that further studies are needed to determine the intrinsic resistance of some cultivars to the bug proteinase attack.

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Novel raw materials, technologies and products, new challenge for the quality control[1]*, pp. 237-244 (2002)*

CAPÍTULO 4

Detection of cereal protein alterations by using free zone capillary electrophoresis

S. Aja[2], C.M. Rosell[2], C. Benedito[2]

The free zone capillary electrophoresis (FZCE) has emerged as a powerful tool to analyse the cereal protein, namely wheat proteins. A very fast, simple and easy method to separate gliadins and glutenins by FZCE has been previously reported.

However in this study, the utility of this technique to identify wheat protein alteration as insect infestation is presented. The storage proteins from wheat samples with different degree of insect attack were analysed by FZCE. The gliadin analysis revealed an increase in the peaks with the intensity of the infestation, mainly in the ω-gliadin region. From the qualitative point of view, the greatest differences were found in the region of the α and β gliadins with the increase of some peaks, which probably correspond to some hydrolysis products from the glutenins. Regarding the glutenin fraction, the insect attack produced a great modification in the electrophoretic profile of these proteins, mainly at the zone of the high electrophoretic mobility. In this

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case a decrease of the peaks was observed, confirming the main activity of the bug damage on the glutenin proteins. Incubation of the wheat samples made more evident the alteration of the proteins promoted by the insect attack. These results allow concluding that FZCE could be used as both rapid and sensitive technique for the detection of wheat protein modification or relations due to insect attack.

INTRODUCTION

Cereal proteins have been first classified on the basis of their solubility (Osborne, 1907) into four classes. This classification has been applied to all cereals. However, due to their widespread use and economic importance, the analysis and characterization of the cereal proteins have been a main research area for years. Several analytical techniques have been applied to separate the wheat proteins; among them could be cited size exclusion liquid chromatography (SE-HPLC) (Gupta *et al.*, 1993; Larroque *et al.*, 1997), and polyacrilamide gel electrophoresis (PAGE).

Lately, the free zone capillary electrophoresis (FZCE) has demonstrated to be a powerful tool to analyse the cereal proteins, namely wheat proteins (Bean *et al.*, 1998; Bean and Lookhart, 2000a; 2000b; 2001). This new technique allows obtaining rapid separations of the cereal proteins with a high resolution. Furthermore, the properties of the separations and the electrophoregram have better characteristics than the traditional polyacrylamide gel electrophoresis (PAGE) and the high performance liquid chromatography (HPLC).

Damage to wheat caused by heteropterous insects affects to many countries of the Europe, Middle East, North Africa and New Zealand (Swallow and Every, 1991). In Europe the damage is caused by certain species of *Aelia* and *Eurygaster,* and are commonly called “wheat bug” (Sivri *et al.*, 1998), while in New Zealand *Nysius huttoni* is the responsible of the wheat bug damage (Cressey *et al.*, 1987).

These insects attack the wheat kernel when developing and inject their salivary secretion in the grain. No increase in the amylase activities has been detected, neither alteration of the starch granules structure (Every *et al.*, 1990; Rosell *et al.*, 2002a). However, this secretion contains proteolytic enzymes that break down the gluten structure, resulting in wheat flour with

a reduced breadmaking quality (Lorenz and Meredith, 1988; Karababa and Nazmi Ozaan, 1998; Hariri *et al.*, 2000). Different studies by PAGE and SDS-PAGE demonstrated that bug proteases specifically hydrolyze high molecular weight glutenin subunits (HMW-GS), resulting in degradation products with an electrophoretic mobility similar to É-gliadins (Cressey and McStay, 1987; Rosell *et al.*, 2001b). *Nysius huttoni* protease does not affect the gliadin fraction (Cressey and McStay, 1987; Every, 1990), whilst *Eurygaster* spp. and *Aelia* protease does hydrolyze the gliadins (Sivri *et al.*, 1998; Rosell *et al.*, 2001b).

The aim of this study was to analyse the wheat protein alteration produced by insect infestation, and the extent of this modification with the intensity of the bug damage.

MATERIALS AND METHODS

A Spanish bread wheat variety, Soissons, generously provided by Harinera La Meta (Lerida, Spain) was used to study the modification of storage proteins by bug damage. All reagents were analytical grade.

Physical measurements and proteolytic degradation analysis

Wheat samples after an appropriate cleaning and tempering were milled in a laboratory Chopin Mill (Tripette and Renaud, Paris, France). The resulting flour was used for the gluten assessments and the alveograph analysis.

Wet gluten and gluten index were determined following the AACC standard method (1995). For analysing the gluten quality after resting, the wet gluten was kept for two hours at 25°C before the gluten index determination.

The alveograph analysis was carried out in a Chopin Alveograph (Tripette and Renaud, Paris, France), according to the AACC approved method (1983). The parameters determined were tenacity (P), extensibility (L), the deformation energy (W), and the curve configuration ratio (P/L) (see Rosell *et al.*, 2001a for a detailed description of these parameters). The proteolytic degradation was assessed by the alveograph test after a resting of 3 hours at 25°C (Berger, 1974; Rosell *et al.*, 2001b).

Sample preparation

Storage proteins were isolated by a sequential extraction, based on the Osborne method, but with some modifications reported by Bean *et al.* (1998). Briefly, 200 mg of wholemeal were suspended in 1.0 mL 50 mM Tris-HCl buffer pH 7.8, containing 100 mM KCl and 5 mM EDTA, after 5 min vortexing and centrifuging at 14,000 rpm for 2 min, the supernatant was discarded and the pellet was washed with water. Gliadins were obtained by mixing the washed pellet with 1.0 mL 50% 1-propanol, centrifuged at 14,000 rpm for 2 min. For the glutenin samples, the pellet obtained after gliadin extraction was mixed with 1.0 mL of 50% 1-propanol containing 1% dithiothreitol (DTT) for 10 min, and then centrifuged at 14,000 rpm for 2 min.

Capillary electrophoresis analysis

Beckman MDQ instrument (Beckman, Fullerton, CA) was used for all FZCE separations. Uncoated fused silica capillaries (Polymicro, Phoenix, AZ) of 50 μm id x 27 cm (20 cm L_D) were used for these analysis. The FZCE separations were made at the optimum separation conditions described by Bean and Lookhart (2000b) using 50 mM iminodiacetic acid (IDA) containing 20% ACN and 0.05% hydroxypropylmethylcellulose (HPMC) as a buffer, at 45°C and 30 kV. Gliadin samples were injected at 0.5 psi for 4 s, whilst glutenins were injected at 0.5 psi for 8 s. Proteins were detected by UV absorbance at 200 nm.

In incubated samples, wholemeal (200 mg) was mixed with deionized water (0.5 mL) and incubated at 35 °C for 3 hours, then the protein extraction was made as described above.

RESULTS AND DISCUSSION

Physical modification of wheat storage proteins by insect damage

Wheat damage by heteropterous insects is characterised by increased proteolytic activity, although they became evident after the incubation of the resulting dough. In order to quantify the extent of the dough alteration with

time, the alveograph parameters of a wheat flour of 41% proteolytic degradation were determined before and after incubation of the resulting dough (table 1). The incubation led to dough with decreased tenacity (P) and larger extensibility (L), resulting in a reduction of the curve configuration ratio (P/L), along with a decrease in the energy required for the deformation (W). Therefore, dough from bug damaged wheat will become very elastic and sticky as the proofing proceeds, as reported Lorenz and Meredith (1988).

Table 1.–**The influence of bug damage on the alveograph parameters.**

	Before incubation	**After 3 h incubation**
P (mm)	76	53
L (mm)	80	89
P/L	0.96	0.6
W (10^{-4} J)	212	125

In addition, the wet gluten and the gluten index were determined since the bug damage is associated to a degradation of storage proteins. In table 2 can be observed that a bug damaged wheat can have good values of gluten index (97.6%), therefore the identification of a bug damaged wheat can not be assessed by the gluten index, since normal values can be obtained. However, a low value of wet gluten was detected in the damaged wheat, which indicates that bug attack produced a reduction of the wheat storage proteins, but without affecting their quality. When the gluten from bug damaged wheat was incubated, a decrease of the gluten index was observed, and that was expected according to the results of the alveograph parameters, because the degradation diminishes the quality of the proteins.

Table 2.–**Physical gluten characteristics of a flour from bug damaged wheat.**

	Before incubation	**After 3 h incubation**
Wet gluten (%)	27.8 ± 0.9	28.2 ± 0.4
Gluten index (%)	92.5 ± 7.4	68.9 ± 1.8

Effect of insect infestation on wheat gliadins

Gliadins from wheat with different degrees of infestation were electrophoretically characterized (Figure 1). The bug damaged produced an increase in the peaks at the highest electrophoretic mobility, probably due to the presence of degradation products with an electrophoretic mobility

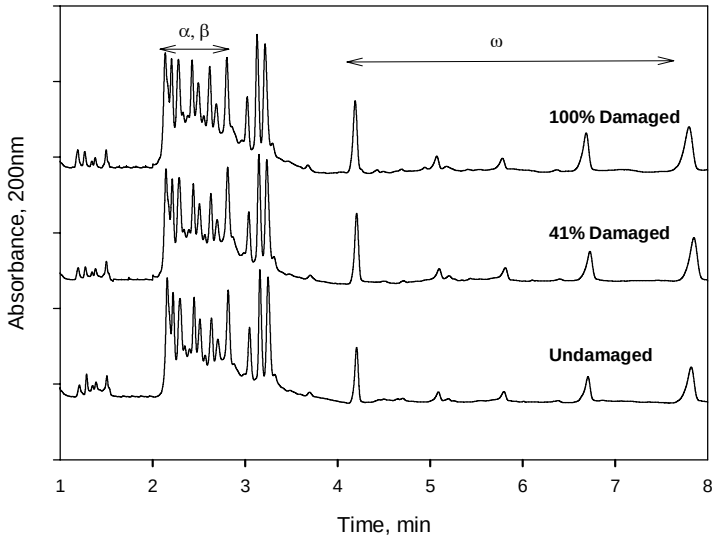

Figure 1.–Effect of different degree of bug damage on the electrophoregrams of the gliadin fraction.

similar to α and β gliadins, and that modification increase with the intensity of the damage. Yakovenko *et al.* (1973) described the degradation of the glutenin fraction by *Eurygaster spp* damage with a concomitant appearance of some products with similar mobilities as the α gliadins. In parallel, an increase in the peaks intensity was also observed at the lowest mobility in the ω gliadin range. These results agree with the previous ones reported by

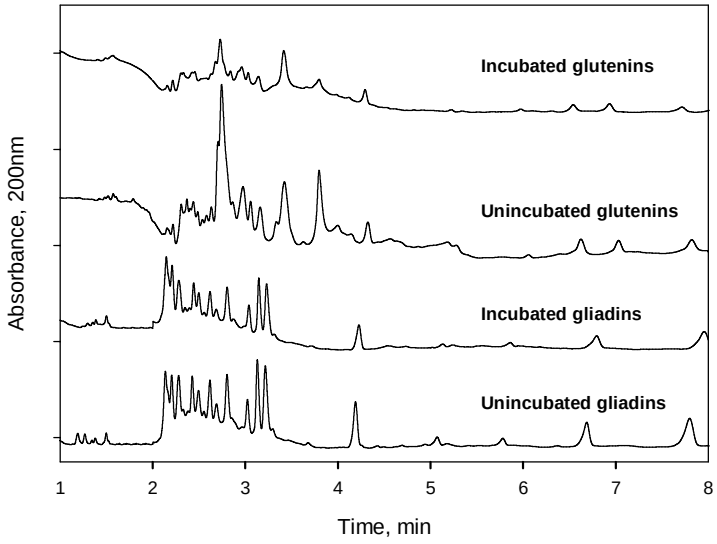

Figure 2.–Effect of the incubation on the FZCE pattern of the storage proteins due to bug damaged.

Rosell *et al.* (2001b), who described the major modification in the ω gliadin mobility zone, although the extent of the effect was greatly dependent on the wheat cultivar. Similar results were observed by Sivri *et al.* (1998) when analysed gliadins by A-PAGE, although only after incubation of the damaged samples.

The incubation of damaged samples produced a considerable decrease in the intensities of all the gliadin peaks, but without modifying the mobilities (Figure 2). The intensity decrease was readily dependent on the degree of damage (results not showed). Similar findings were described by Rosell *et al.* (2001b) and Sivri *et al.* (1998).

Wheat glutenin alteration by insect infestation

The FZCE of the glutenins from bug damage wheat revealed a reduction of some peaks and that decrease was dependent on the level of damage (Figure 3). Similar findings reported Sivri *et al.* (1998, 1999) and Cressey and McStay (1987), but both of them only found differences after the incubation. In this study by FZCE it was possible to identify the reduction in the intensity of some glutenins.

However, changes became more evident after incubation (Figure 2). The

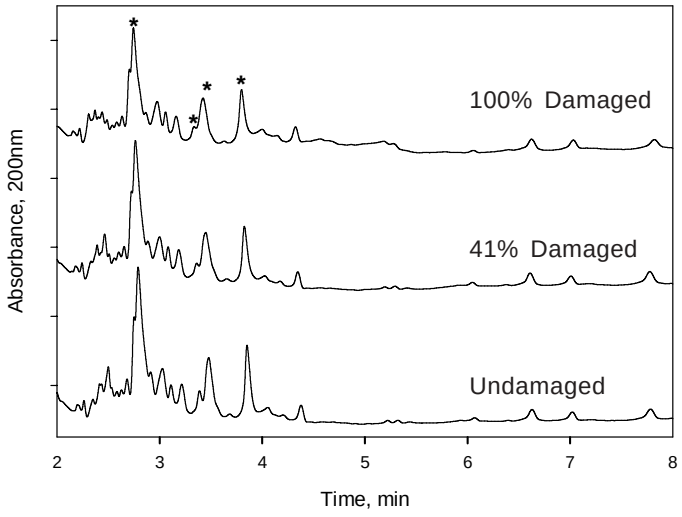

Figure 3.–Electrophoregrams of glutenin from wheat with different level of bug damage. Asterisk are drawn to indicate differences among samples.

electrophoregrams of the glutenins after incubation showed a great reduction in all the glutenin peaks, indicating the high specificity of the bug proteinases for these proteins.

CONCLUDING REMARKS

Bug damaged wheat has altered its breadmaking capacity due to the protein disruption, although up to now the protein alteration has been described by PAGE only after sample incubation. FZCE allows to detect the protein modification caused by bug proteinase, even without incubation it is possible to detect a decrease in some peaks of the glutenin fraction along with and increase in some peaks with similar mobility to the ω gliadins.

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CAPÍTULO 5

Wheat gluten hydrolysis by *Aelia* spp. and *Eurygaster* spp. damage and water soluble compounds released from gluten hydrolysis

S. Aja[1], G. Pérez[2], C.M. Rosell[1]

ABSTRACT

Wheat damage by heteropterous insects produces gluten hydrolysis giving different degradation products. In this study, the gluten content and the gluten quality assessed as gluten index were measured during incubation for different intervals (0, 1, 2, 3, 7, 24 hours). Simultaneously, the water soluble products released from gluten hydrolysis during incubation were analysed by SE-HPLC and SDS-PAGE. The results indicated that the amount of wet gluten remained constant even in the case of gluten isolated from damaged wheat, whereas the gluten index from damaged gluten showed a steady decrease with the incubation time suggesting an

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intense protein hydrolysis. A great amount of water soluble compounds were released from damaged gluten, increasing the relative proportion of compounds with molecular weight comprised between 15,000 and 30,000 during the first 3 hours of incubation. The SDS-PAGE studies under non reducing conditions revealed presence of six new bands from Mr 42,000 to 27,000 at 3 hours of incubation and they showed a progressive increase in their intensity with prolonged incubation time. The presence of some protein aggregates with Mr higher than 200,000 suggested the endoproteolytic activity of the insect proteases, and the analysis of those aggregates under reducing conditions indicated that disulphide bonds linked them. In addition, the use of gluten index is proposed as a parameter for objectively determining the insect attack.

INTRODUCTION

It is widely known that the preharvest attack of wheat by some *Heteropterous* insects yields wheat with reduced breadmaking quality (Hariri *et al.*, 2000; Lorenz and Meredith, 1988; Swallow and Every, 1991). This damage has been attributed to *Nysius huttoni* in New Zealand and to some species of *Aelia* and *Eurygaster* in Europe, Middle East and North Africa (Cressey *et al.*, 1987; Every *et al.*, 1992; Lorenz and Meredith, 1988). Spain is one of the countries that has undergone the wheat infestation by those insects, and the most frequent species responsible for that wheat damage are *Aelia germari* and *Eurygaster austriaca* (Infiesta *et al.*, 1999).

The feeding mechanism consists in piercing the insect mouthparts in the immature grain and then sucking the milky juices (Every *et al.*, 1990). The resulting mature wheat grains are partially empty in a large area underneath the puncture site, and surrounding that area the protein matrix is absent (Rosell *et al.*, 2002a). The wheat flour obtained from damaged wheat led to sticky and weak bread dough, which gives loaves of reduced volume and unacceptable texture (Hariri *et al.*, 2000; Karababa and Nazmi Ozan, 1998; Matsoukas and Morrison, 1990). Nevertheless, the damaged wheat does not show abnormal values of hectolitre weight, thousand- kernel weight and protein content (Every *et al.*, 1990; Lorenz and Meredith, 1991; Rosell *et al.*, 2002a); even usual values of diastatic and α-amylase activity have been

reported (Every *et al.*, 1990; Rosell *et al.*, 2002a). The unique anomalous characteristic of the bug-damaged wheat is a disrupted protein structure (Cressey, 1987; Kretovich, 1944).

It has been reported that insect infestation affects the glutenin and gliadin fraction of wheat proteins, showing increased specificity towards the high molecular weight glutenin subunits (HMW-GS) (Cressey and McStay, 1987; Rosell *et al.*, 2002b; Sivri *et al.*, 1999). Several methods like reverse-phase high performance liquid chromatography (RP-HPLC) (Rosell *et al.*, 2002b; Sivri *et al.*, 1999; 2002), free zone capillary electrophoresis (FZCE) (Aja *et al.*, 2002; Rosell *et al.*, 2002b), size-exclusion high performance liquid chromatography (SE-HPLC) (Rosell *et al.*, 2002b), and gel electrophoresis (PAGE and SDS-PAGE) (Sivri *et al.*, 1998) have been applied for assessing the protein degradation due to insect attack. Little attention has been paid to the released products during protein hydrolysis. The degradation products from gluten hydrolysis are water or alcohol soluble proteins (Kretovich, 1944) in the case of *Eurygaster* and *Aelia* infestation, while *Nysius* attack does not increase the amount of water soluble nitrogen after incubation (Swallow and Every, 1991). In fact, a quantitative test has been reported for *Nysius* proteinase in bug damaged wheat, which essentially measures the amount of aqueous SDS-soluble gluten protein after incubation of gluten with the enzyme extract from bug-damaged wheat (Every 1991; 1992; 1993). However, concerning *Aelia* and *Eurygaster* no further characterisation of the water soluble products has been reported, despite it could be an important way to determine specific products for developing rapid detection assays.

The aim of the present study was to analyse the water soluble products released from gluten hydrolysis produced by insect attack. In addition the simultaneous gluten hydrolysis was followed in order to determine the possible modification of the gluten quality.

MATERIALS AND METHODS

Sound and damaged wheat grains from Bolero cultivar were provided by La Meta (Lérida, Spain). Wheat characteristics were protein 12.0% and 11.0% (based on 14% moisture content) for the sound wheat and visually damaged wheat, respectively. Chemical reagents of the highest purity were purchased from Sigma (St Louis, MO).

Gluten determination and proteolytic degradation assessment in wheat samples

Wholemeal flour was prepared on a falling number mill type 3100 for the various analytical tests. Wet gluten and gluten index were determined according to the AACC standard method (AACC, 1995). Washed gluten was kept in a shaking water bath at 37°C for different time intervals, and subsequently wet gluten and gluten index were determined by the standard method previously described.

The proteolytic degradation was quantified by using the Chopin Alveograph (Tripette et Renaud, Paris, France) as previously described by Berger *et al.* (1974) and Rosell *et al.* (2002a, b), because a good correlation has been described between the deformation energy (W) change when the dough is allowed to stand at 25°C for 3 hours and the proteolytic activity. One unit of enzyme activity was arbitrarily defined as the reduction of the deformation energy after 3 hours of incubation at 25°C. No proteolytic activity was detected in the sound wheat, whereas the damaged wheat showed a proteolytic activity of 1.64 mU/g of wheat.

Extraction of the water-soluble fraction from incubated gluten

Wet gluten from damaged wheat and sound wheat were obtained by using the Glutomatic. Wet gluten (200 mg) was incubated at 37°C in a shaking water bath for different time intervals; afterward it was suspended in 1.0 mL of distilled water, vortexed for 5 min and centrifuged at 15,700 x g for 2 min. In order to ensure the absence of any remaining soluble starch the supernatant containing all the water-soluble compounds was mixed with three volumes of ethanol, kept overnight at 4°C and then centrifuged at 15,700 x g for 5 min. The supernatant was freeze-dried and stored for further analysis.

SE-HPLC analysis

An Agilent 1100 Liquid Chromatograph was used for all the HPLC separations. Samples previously freeze dried were dissolved in 100 µL of

distilled water. Size exclusion separation was performed by injecting 20 µL of sample at 0.4 mL/min of acetonitrile (ACN):water (20:80) containing 0.05% (w/v) trifluoroacetic acid (TFA) into a TosoHaas TSK-gel™ G3000 PW_{XL} column (TosoHaas GmbH, Stuttgart, Germany). Protein elution was monitored at 210nm. Different molecular weight proteins were used for assessing the molecular weight of the different eluted fractions. The standard proteins used for calibrating the column were: apoferritin (443,000), ß-amylase (200,000), alcohol dehydrogenase (150,000), albumin (66,000), carbonic anhydrase (29,000), myoglobin (17,600), and cytochrome C (12,400). Data quantification was performed using the chromatograph data analysis software (Hewlett Packard HPLC Chemstation ver.A.05). The chromatogram integration parameters were uniformly applied to all the chromatograms to quantify the distribution of the molecular weight compounds in the water-soluble fraction released during gluten incubation.

Gel electrophoresis

Protein composition of water extracts was analysed by SDS-PAGE (stacking gel of 4% (w/v) acrylamide and resolving gel of 12% (w/v) acrylamide) according to Laemmli (1970). A Mini Protean II Dual Slab Cell (Bio-Rad Laboratories, Hercules, USA) was employed to perform electrophoretic runs working at constant voltage (150 V) until the front reached the end of the gel. Water extracts were dissolved in 0.125M Tris/HCl (pH 6.8) containing 2% SDS, 10% glycerol, 0.05% bromophenol blue and 2% ″-mercaptoethanol (reducing conditions) or without ″-mercaptoethanol (unreducing conditions). The protein bands were stained using silver stain.

Densitometry and quantification of protein bands

Gels were analyzed by densitometry in an image master VDS (Pharmacia Biotech Inc., Uppsala, Sweden) using the software image master VDS. A blank lane was used to obtain the background signal. The volume of protein band (integrated optical density, IOD) was represented by the expression

$$IOD = (\text{mean intensity} - \text{background}) \times \text{band area}$$

The standard curve for silver staining IOD vs quantity of protein was performed using carbonic anhydrase (C 7025 Sigma Chemical Co., St. Louis, MO, USA) as standard. The silver stained IOD showed a linear response between 0.1 and 10 μg of protein with a regression coefficient r^2= 0.915, and a lineal equation y= 639.5x + 205.49. At least three determinations per point were made and average values were determined.

Statistical Analysis

All reported results are the means of at least four replicates. Results were analyzed by the one-way analysis of variance procedure using Infostat, Statistical software (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentine). The comparison among means was done by the Tukey test, and the significant differences were calculated at $P<0.05$.

RESULTS AND DISCUSSION

Effect of insect damage on the gluten characteristics

The gluten softening effect promoted by the insect infestation of wheat and a subjective method to quantify that effect has already been reported (Cressey and McStay, 1987; Handford, 1967). In this study, although the objective was to analyse the water soluble products from protein hydrolysis, it seemed necessary to previously determine the characteristics of the gluten proteins from sound and damaged wheat. That analysis was performed by assessing the wet gluten content and gluten quality during incubation at different time intervals.

Figure 1 shows the effect of insect damage on the amount of wet gluten compared to that of sound wheat. No significant differences ($P<0.05$) were found in the amount of wet gluten as a consequence of the insect damage, even when wheat from different cultivars was tested, but these results could be attributed to cultivar variability, because genetic factors and environmental conditions determine the wheat grain composition (Rosell *et al.*, 2002a; Sivri *et al.*, 2002). However, when a careful selection of the damaged kernels is done, abnormally low values of wet gluten are usually found in insect

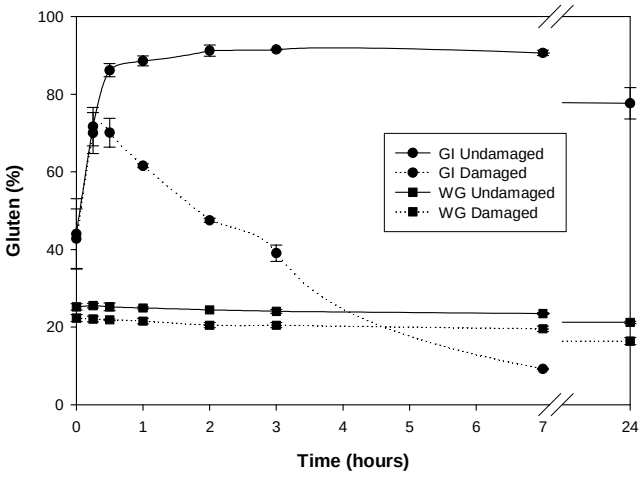

Figure 1.–Effect of incubation at 37°C on the amount of wet gluten (WG) and the quality of gluten (gluten index, GI) from damaged and sound wheat.

infested wheat (Karababa and Nazmi Ozan, 1998; Kretovich, 1944; Lorenz and Meredith, 1988). The amount of wet gluten remained constant during the incubation time, even in the case of the gluten isolated from damaged wheat. Only a slight decrease was observed after 24 hours incubation in the sound and damaged samples. These results indicate that the hydrolysis effect promoted by the insect attack is mainly of endoproteolytic type as previously stated by Cressey and McStay (1987), since the amount of wet gluten remains constant.

A different pattern was observed for the gluten index. No significant differences were observed in the gluten index when fresh gluten from sound and damaged wheat was analysed. In the sound wheat the gluten index underwent a great increase within the first 30 min of incubation, then it remained constant with time and a drop was only observed after 24 hours incubation. The initial increase indicates that the polymerisation of the gluten proteins takes place during that period, becoming stronger and in consequence increasing the retained portion of the gluten in the centrifuge sieve. Different wheat varieties have been tested and the same trend was always observed (results not shown). Therefore, consistent gluten index values only can be attained from wet gluten rested at least 30 minutes after washing, otherwise a great variation within a sample can be obtained.

Regarding the gluten isolated from damaged wheat, a small increase of the gluten index was exhibited during the first 15 min incubation, but beyond that time a steady decrease was obtained, suggesting a very rapid hydrolysis

process involving a size redistribution of the gluten proteins (Rosell *et al.*, 2002b). Microbial counts on plate count agar after incubation at 30°C for 24 hours did not reveal any contamination; therefore no microbial hydrolysis was produced in the samples during incubation.

This method could also serve for measuring the protein degradation by comparing the gluten index values between 30 min and 1.5 hours after gluten washing, since sound wheat gives a constant gluten index during that period in contrast to damaged wheat, which shows a reduced gluten index after resting.

Characterisation of the water soluble compounds by SE-HPLC

Although no evident change was observed in the amount of wet gluten as a consequence of the insect damage, the water soluble compounds were extracted and analysed by size exclusion chromatography. Similar SE-HPLC chromatogram pattern was observed between the water soluble compounds isolated from damaged and undamaged gluten (Figure 2). No statistically significant differences ($P<0.05$) were observed in the total area beneath the chromatograms, as was expected, since damaged wheat does not have abnormal values of protein content, thousand kernel weight, specific weight (Karababa and Nazmi Ozan, 1998; Lorenz and Meredith, 1988; Rosell *et al.*, 2002a), even no significant differences have been found in the alcohol

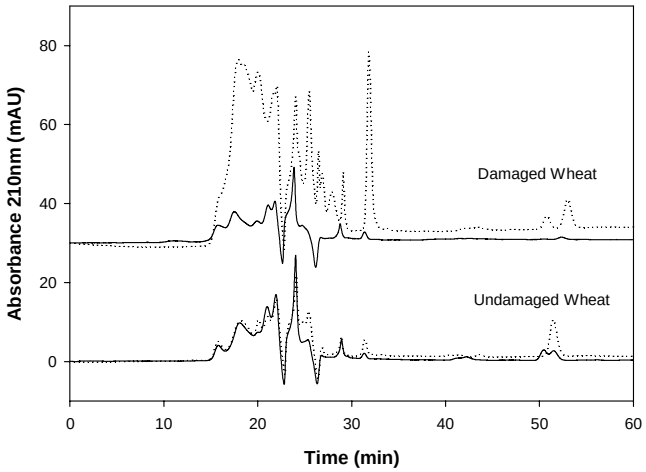

Figure 2.–Size exclusion HPLC separation of the water-soluble fraction isolated from damaged and sound wheat gluten. Overlaid chromatograms of unincubated (—) and 3 hours incubated (….) samples.

soluble polymeric proteins, only a slightly lower gliadin content has been detected (Rosell *et al.*, 2002b). However, a really different chromatogram was observed after a 3 hour incubation, sound wheat only showed a slight increase in some peaks, which could be attributed to endogenous protease. Conversely, a great increase in the amount of water soluble compounds was observed in the damaged sample compared to the sound gluten. Despite no variation being detected in the wet gluten, some of the products released during hydrolysis were soluble in water, and had a wide range of molecular weight. This result agrees with previous studies focused on the modification of gliadins and glutenins from damaged wheat during incubation, showing a considerable decrease after incubation, the highest degradation being produced on the high molecular weight glutenin subunits (Rosell *et al.*, 2002b; Sivri *et al.*, 1998; 2002).

The sum of the total area beneath the chromatogram was used to quantify the total amount of water soluble compounds released as a consequence of the gluten hydrolysis (Figure 3). The incubation of sound gluten barely produced an increase in the amount of water soluble protein compounds, only evident after 7 hours of incubation. In opposition, the damaged gluten liberated a high amount of water soluble compounds even after a short incubation.

When the size distribution of the water soluble compounds was analysed (Table 1), a modification of the distribution during incubation was observed, and the extent of that was different in the sound and damaged wheat. The majority of the water soluble products released from gluten hydrolysis had

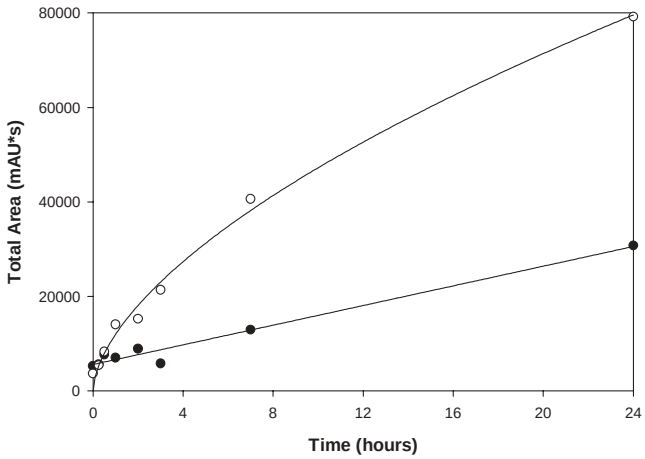

Figure 3. Effect of insect damage on the amount (in mAU*sec) of water-soluble compounds analysed by SE-HPLC. The water-soluble fractions were isolated from damaged (filled circles) and sound (empty circles) wheat gluten during incubation at 37°C.

Table 1.–**Molecular weight distribution of the water-soluble fraction from damaged and sound wheat gluten during incubation at 37°C determined by SE-HPLC. Values are expressed as percentage of the total areas.**

	Sound wheat incubation time (min)					Damaged wheat incubation time (min)				
Molecular weight	**0**	**30**	**60**	**120**	**180**	**0**	**30**	**60**	**120**	**180**
> 70,000	61.0^{b}	61.1^{b}	59.7^{b}	61.1^{b}	59.6^{b}	63.3^{a}	65.5^{a}	65.1^{a}	63.7^{a}	63.8^{a}
70,000-30,000	26.2^{b}	28.1^{a}	29.1^{a}	27.6^{a}	29.8^{a}	29.0^{a}	24.9^{c}	23.2^{d}	21.1^{e}	17.8^{f}
30,000-20,000	1.2^{c}	1.2^{c}	1.4^{c}	1.7^{c,d}	0.8^{c,e}	0.0^{f}	1.0^{c}	2.2^{b}	2.4^{b}	3.9^{a}
20,000-15,000	0.0^{e}	0.8^{c}	0.8^{c}	0.9^{c}	1.0^{c}	0.0^{e}	0.5^{d}	1.3^{b}	2.4^{a}	2.5^{a}
15,000-10,000	3.8^{b}	3.2^{c}	2.9^{c,d}	2.2^{e}	2.6^{d}	4.7^{a}	1.6^{f}	1.8^{f}	1.8^{f}	1.4^{f,g}
< 10,000	7.8^{c}	5.6^{f}	6.1^{e}	6.4^{e}	7.3^{d}	3.0^{g}	6.5^{e}	6.5^{e}	8.5^{b}	10.6^{a}

Different letters within a row mean significant differences (Pd»0.05).

a molecular size higher than 30,000 and a high proportion of molecular size smaller than 10,000 was also found. Small but statistically significant variations were observed in the size distribution of the water soluble compounds from sound gluten during incubation. No variation in the percentage of the fraction with molecular weight higher than 70,000 was detected during the incubation of sound and damaged gluten. Regarding damaged gluten, a progressive decrease in the relative proportion of the fractions with molecular weight between 70,000-30,000 and 15,000-10,000 was observed with the incubation. In contrast, the percentage of the fractions with molecular weight between 30,000-20,000 and lower than 10,000 showed an increase during the same period. In the range 20,000-15,000 the fraction from sound gluten showed a small increase at 30 min of incubation and no significant change was observed after 30 min. In the same range the damaged gluten showed a steady increase of the compounds during the incubation period. Therefore water soluble compounds released from protein hydrolysis had a completely different behaviour when they were from damaged samples.

SDS-PAGE of the water soluble compounds

The water soluble compounds were also analysed by SDS-PAGE in order

to obtain some additional information about those compounds. When the water soluble extracts were analysed by SDS-PAGE in unreducing conditions (Figure 4) only a band of Mr 35,000 appeared when the sound gluten was incubated during 0, 3, 7 h and their IOD did not change significantly (144±54; 113±68 and 139±47 respectively). However, when the sound gluten was incubated during 24 h, numerous and very intense protein bands were present,

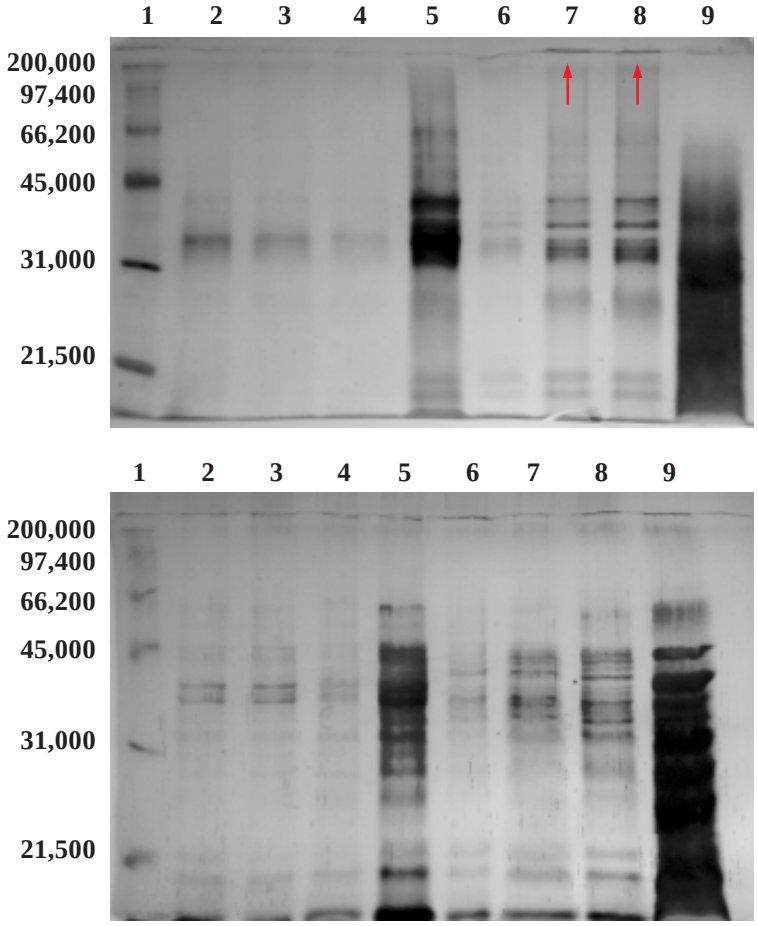

Figure 4.–Pattern of SDS-PAGE gels (12 % acrylamide) under unreducing conditions (upper gel) and reducing conditions (lower gel) of water- soluble fractions from damaged and sound wheat gluten.

Lane 1: molecular mass marker Myosin 200,000; Phosphorylase b 97,400; Serum albumin 66,200; Ovalbumin 45,000; Carbonic anhydrase 31,000; Soybean trypsin inhibitor 21,500. Lane 2 – 5: water soluble fractions from sound wheat incubated 0, 3, 7 and 24 h. Lane 6 – 9: water soluble fractions from damaged wheat incubated 0, 3, 7 and 24 h.

which correspond to the hydrolysis products resulting from the intrinsic proteolytic activity of the sound gluten. Those results are in agreement with the findings of Bleukx *et al.* (1997), describing the proteolytic activity associated with vital gluten.

The water soluble compounds extracted from incubated damaged gluten showed six bands between Mr 42,000 and 27,000 and a progressive increase in their intensity was observed during the incubation time (1.80±0.13 fold between 3 and 7 h). In addition, two bands of Mr lower than 20,000 appeared with the incubation but their intensity did not change up to 7 hours of incubation, and also a band retained between the stacking gel and the resolution gel was observed at 3 and 7 h of incubation, likely due to large protein aggregates. The intensity of this band slightly decreased under reducing conditions. In this condition a great number of bands appeared in the water soluble sample from damaged wheat, namely nine new bands of molecular weight between 45,000 and 20,000 appeared indicating that large polymers composed of polypeptides linked by disulfide bonds were released during the incubation of damaged wheat gluten. These results are in agreement with those observed when analysing wet gluten and indicate that the insect enzyme is an endoprotease like the *Nysius* proteinase reported by Cressey and McStay (1987) and Every (1993).

The unique information related to water soluble compounds from damaged wheat came from the increase in water soluble nitrogen values reported by Kretovich (1944), who also indicated that those compounds could be peptones, peptides and amino acids but our results show that proteins and peptide aggregates of high molecular weight (higher than 200,000) were also released during incubation and they were water soluble.

CONCLUSION

The measurement of the gluten index before and after resting could be a very useful way to detect damaged wheat produced by heteropterous insects. The hydrolysis of wheat proteins lead to the release of water soluble compounds and their amount increased with the incubation time. The proteins and peptides released have a wide range of molecular weight, from peptide aggregates linked by disulfide bonds higher than 200,000 to small peptides lower than 10,000.

ACKNOWLEDGEMENTS

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CAPÍTULO 6

Wheat flour proteins as affected by transglutaminase and glucose oxidase

C.M. Rosell[1,2] , Jinshui Wang[1], S. Aja[1], S. Bean[3], G. Lookhart[3,4]

ABSTRACT

Enzymes are good tool to modify wheat proteins by creating new bonds between the protein chains. In this study, the effect of the addition of glucose oxidase (GO) and transglutaminase (TG) on the wheat flour proteins is presented. The modification of wheat proteins was determined by analyzing the changes in gluten quality, alveograph parameters, and protein modifications. The amount of wet gluten increased with the addition of GO and TG, but the gluten quality was not improved in any case. Regarding the alveograph parameters, the effect of GO was readily evident obtaining wheat dough with higher tenacity and lower extensibility than the control, while TG led to doughs with lower tenacity and that were also less extensible. The protein modifications were characterized by free-zone capillary electrophoresis

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(FZCE). FZCE data indicated that TG polymerizes mainly glutenins and, of those, the high molecular weight glutenin subunits were the most affected.

INTRODUCTION

Cereal storage proteins of wheat play a fundamental role in the breadmaking process. They have the ability to form gluten, a necessary network to amalgamate the other wheat components, mainly carbohydrates, and the gas produced during proofing. The gluten characteristics are one of the main parameters that govern flour quality and subsequent breadmaking quality of the wheat (MacRitchie, 1987). Several factors affect the final gluten quality such as cultivar, environmental conditions, insect infestation level, and postharvest conditions. Variability in any of them could result in a reduced capacity of the storage proteins to form gluten (Zhu and Khan, 2001). Numerous breeding programs have attempted to improve breadmaking quality of wheat, however some new cultivars developed are not appropriate for breadmaking and require some protein modifications.

Dough conditioners have been developed to overcome some deficiencies in the breadmaking quality of the wheat gluten. The oxidizing agents, ascorbic acid, azodicarbonamide, and potassium bromate are the most commonly used (Tsen, 1969). However, recent indications that some of them may cause cancer are decreasing their use (Wolf *et al.*, 1998).

The use of enzymes is the best alternative to the chemical compounds because they are generally recognized as safe (GRAS) and do not remain active after baking. Among the enzymes that can confer strength to the dough are transglutaminases (TG) and glucose oxidases (GO). These enzymes act through different catalytic mechanisms and may induce changes in the polymerized form of the glutenin subunits and maybe transform soluble proteins into insoluble ones. Transglutaminase (EC 2.3.2.13) is an acyl transferase that catalyzes inter- or intramolecular crosslinking through the formation of peptide bonds between glutamine and lysine residues. A number of food applications of TG focus on increasing the functional value of milk, meat and fish proteins (Zhu *et al.*, 1995; Motoki and Seguro, 1998).

Gerrard *et al.* (1998) reported an improvement of the breadcrumb strength similar to that produced by oxidizing agents. Glucose oxidase (EC 1.1.3.4) (GO) catalyzes the oxidation of glucose, producing a molecule of hydrogen peroxide

that can cause either from disulfide bonds between proteins (Haaralsita and Pullinen, 1992), or form tyrosine cross-links, whose role in the gluten structure was recently reported (Tilley *et al.*, 2001). The addition of GO improves the loaf volume of bread and the crumb grain (Vemulapalli *et al.*, 1998).

The purpose of this study was to modify the wheat proteins to improve dough rheological properties and gluten strength through the addition of TG or GO.

MATERIALS AND METHODS

Wheat grain from Bolero cultivar wheats was provided by Porta S.A., Huesca, Spain. Wheat characteristics were 81.3kg/hL test weight, 32.2g thousand kernel weight, and 12.4% protein (based on 12% moisture content). Chemical reagents were purchased from Sigma (St Louis, MO) and were of the highest purity. Transglutaminase (100 TG U/g) was a gift from Ajinomoto Co., Japan. Glucose oxidase (500 GO U/g) was kindly provided by Novo Nordisk, Madrid, Spain.

Milling process

To ensure a uniform distribution, enzymes were added to the tempering solution during the milling process because Haros *et al.* (2002) reported this method was a good alternative for the enzyme addition. After appropriate cleaning, a 600 g sample lot of wheat kernels was tempered to 15.5% moisture in a Chopin conditioner by adding the necessary amount of water (unless otherwise specified). For the tempering, wheat kernels were kept at 20-25°C for 16 hours and then milled in a laboratory Chopin mill. Different enzyme concentrations were added to the tempering water for the enzyme-pretreated wheat. The concentrations of GO were 0.4-4.0 enzyme activity units per gram of kernel, and up to 2.0 enzyme activity units per gram of kernel in the case of TG. Four sets of samples were milled for each treatment.

Enzyme Activity Determination

The enzyme activity was measured in the resulting flour from each

tempering conditions. Transglutaminase activity was spectrophotometrically measured as described Folk and Cole (1966) by using the hydroxamate method. One unit of TG is defined as the amount of enzyme that release 1 µmol of hydroxamic acid in 1 min at 37°C.

The GO activity was measured by using glucose as substrate, and coupling that reaction to the o-dianisidine oxidation in the presence of peroxidase. One unit of GO is defined as the amount of enzyme that oxidizes 1 µmol of o-dianisidine/min at 25°C.

Two extractions were made from each sample and four replicates per extract.

Physical Measurements

Wet gluten and gluten index were determined according to the Approved Method (AACC 2000).

The alveograph test (Chopin, Tripette et Renaud, Paris, France), followed the Approved Method. The parameters registered were tenacity (P), extensibility (L), the ratio of work input to deformation or energy and the deformation curve (P/L), (Rosell *et al.*, 2001).

Sample Preparation for Protein Characterization

A sequential extraction was obtained for each class of proteins. Albumins and globulins were preextracted from the flours as reported by Bean *et al.* (1998). Gliadins were then extracted from that precipitate by vortexing 200 mg of the preextracted wheat flour with 1.0 mL 1-propanol and water (50:50, v/v) for 5 min and then centrifuging at 15,700 x g for 2 min as reported in Bean and Lookhart (1998). The precipitates were extracted twice more with the same solution and centrifuged at 15,700 x g for 2 min; the last two supernatants were discarded. Glutenins were obtained by mixing the gliadin-free pellet with 1.0 mL 1-propanol and water (50:50, v/v), containing 65 mM dithiothreitol and then centrifuged at 15,700 x g for 2 min. High molecular weight (HMW) and low molecular weight (LMW) glutenin subunits (GS) were prepared by acetone precipitation as described by Bean and Lookhart (2000).

Four repetitions of each sample were made for each determination.

Capillary Electrophoresis Analysis

Separations were made using a Beckman PACE 5510 instrument. Uncoated fused silica capillaries (Polymicro, Phoenix, AZ) of 50μm id x 27 cm (20 cm L_D) were used for all separations.

Free-zone capillary electrophoresis (FZCE) was performed with 50mM iminodiacetic acid (IDA) in acetonitrile and hydroxypropyl methyl cellulose (HPMC) and water (20:0.05:79.95, v/v) at 45°C and 30kV, the optimum separation conditions described by Bean and Lookhart (2000).

RESULTS AND DISCUSSION

Physical Properties of Enzyme-Treated Flours

The effect of the enzyme treatment on the gluten properties was assessed by determining both the wet gluten and the gluten index values. Enzyme treatment led to flours with modified gluten characteristics as in Table 1. The amount of wet gluten slightly increased with a high dosage of GO and TG. In GO, the wheat treatment with 2.0 and 4.0 U/g led to high wet gluten content; the same result was obtained with the TG treatment at dosage > 1.0 U/g. These results could be attributed to the presence of some compounds bonded to gluten due to the enzyme activity. The gluten quality determined by the gluten index values was not modified by the addition of increasing concentrations of GO. Conversely, the addition of TG decreased the gluten index. To determine whether these enzymes have a synergistic effect, a sample was tempered using the lowest concentration of GO and TG. The final effect was similar to that observed by adding GO. It should be expected that the gluten index would increase with the enzyme treatment because the enzymes used have the ability to form new covalent bonds. No modification was observed with GO, lower gluten index values were obtained when kernels were treated with high TG concentrations, although the values (with the exception of the treatment with 1.0 and 1.5 TG U/g of kernel) were still in the optimum gluten index range for breadmaking (60-90) as reported by Perten (1990). These results differ from Larre *et al.* (2000), who found a decrease of gluten solubility after TG treatment of gluten due to the polymerization

Table 1.–**Effect of the Enzyme Treatment During Milling on Gluten Properties Determined by Wet Gluten Content and Gluten Index.**

Enzyme	Dosage (U/g of kernel)	Enzyme activity[a] (U/g of flour)	Wet gluten (%)[b]	Gluten Index (%)[b]
Control	0.0	–	33.0 ± 0.3	81.2 ± 1.1
Glucose Oxidase(GO):				
	0.4	0.10 ± 0.01	32.9 ± 0.9	80.3 ± 7.9
	2.0	0.59 ± 0.03	35.9 ± 0.5	82.1 ± 2.3
	4.0	0.94 ± 0.04	35.5 ± 1.5	83.1 ± 4.3
Transglutaminase(TG):				
	0.01	n.d.	32.8 ± 0.7	79.4 ± 5.3
	0.05	0.01 ± 0.00	33.7 ± 0.1	80.8 ± 0.4
	0.1	0.02 ± 0.00	31.4 ± 0.5	88.6 ± 0.0
	1.0	0.22 ± 0.03	36.2 ± 1.3	65.9 ± 0.7
	1.5	0.32 ± 0.02	34.3 ± 0.0	66.5 ± 0.2
	2.0	0.52 ± 0.03	35.0 ± 0.2	67.1 ± 0.1
TG + GO	0.01 + 0.4	n.d.	33.9 ± 0.4	80.0 ± 4.5

[a] Remaining enzyme activities in the resulting flour
[b] Mean ± standard deviation obtained from four assays per wheat sample

reaction. The difference could be due to the lower amount of enzyme used in this study (at least 150 times lower). Consequently, the polymerization promoted by the enzyme treatment might not be sufficient to produce a size change detectable by a gross mechanical method like the gluten index.

Alveographic parameters were also determined because it is an extended method used to assess breadmaking quality of the flours. Table 2 shows that GO addition increased the tenacity, and the effect increased with the enzyme concentration. Conversely, the extensibility (L) was largely reduced with treatment at 2.0 U/ g of kernel, showing no further decrease at the highest GO concentration tested (4.0 U/g of kernel). As a consequence, the deformation curve ratio (P/L) when adding 2.0 U/g of kernel was twice that of the control flour (without enzyme treatment). Therefore, some types of deficiencies in breadmaking quality of wheat

Table 2.–**Effect of Enzyme Treatments on Alveographic Parameters of flour[a].**

Enzyme	Dosage (U/g of kernel)	P (mm)	L (mm)	P/L	W (10^{-4} J)
Control	0.0	41.0 ± 1.7	164.0 ± 6.7	0.25 ± 0.00	143 ± 0
Glucose Oxidase (GO):					
	0.4	38.0 ± 0.0	168.0 ± 14.0	0.23 ± 0.01	145 ± 8
	2.0	50.0 ± 1.3	99.0 ± 3.3	0.51 ± 0.01	138 ± 11
	4.0	57.0 ± 0.3	97.0 ± 2.7	0.59 ± 0.01	130 ± 7
Transglutaminase (TG):					
	0.01	41.0 ± 0.0	162.0 ± 0.0	0.25 ± 0.00	148 ± 0
	0.05	37.0 ± 0.0	159.0 ± 0.0	0.24 ± 0.00	144 ± 1
	0.1	38.0 ± 0.7	160.0 ± 0.0	0.24 ± 0.00	144 ± 2
	1.0	34.0 ± 0.0	156.0 ± 3.8	0.22 ± 0.03	125 ± 11
	1.5	34.0 ± 0.7	158.0 ± 1.3	0.22 ± 0.00	116 ± 1
	2.0	31.0 ± 1.3	137.0 ± 2.0	0.23 ± 0.01	109 ± 5
TG + GO	0.01 + 0.4	41.0 ± 2.0	142.0 ± 11.0	0.29 ± 0.01	144 ± 5

[a] Mean ± standard deviation obtained from four assays per wheat sample

flour could be overcome by GO treatment. Studies conducted with an extensigraph showed that the addition of GO resulted in dough that was less extensible and more resistant than the control (Poulsen and Bak Hostrup, 1998).

The addition of TG also modified the alveographic parameters. However, in this case, the TG treatment yielded a steady decrease of tenacity (P) with the increase of enzyme concentration. The effect on the extensibility was not so clear, only a slight decrease could be observed. In addition, a decrease of deformation energy (W) was observed.

A synergistic effect of the TG and GO, in terms of decrease in extensibility, was also obtained.

The results obtained with both techniques revealed that the enzyme treatment modified the properties of the proteins of wheat flour. Comparison of the effects of both enzymes (GO and TG) on the gluten quality and alveographic parameters indicates that higher effect is promoted by GO treatment than by TG.

Protein Modification by FZCE

Separation of the wheat proteins extracted from flours treated with and without enzyme solutions were performed by SE-HPLC. No differences in the total areas beneath the chromatogram curves were detected either in the alcohol-soluble proteins or the glutenins (results not shown). Vemulapalli and Hoseney (1998) also found that GO did not act directly on gluten proteins. Only the treatment with the highest concentration of TG (2.0 U/g of kernel) produced a slight decrease in the glutenin fraction (results not shown). Therefore, no noticeable change in the size of the proteins was produced by the enzyme treatment, with the exception of above-mentioned TG treatment. Larre *et al.* (2000) described a decrease of the gluten solubility with the polymerization reaction promoted by TG. In addition, they reported that the extent of polymerization and, in consequence, the amount of polymerized products was largely related to the quantity of enzyme. This would explain why this study found a decrease in the glutenins only at the highest TG concentration, which still was very low compared with Larre *et al.* (2000).

The flour proteins were fractionated and analyzed by FZCE to better

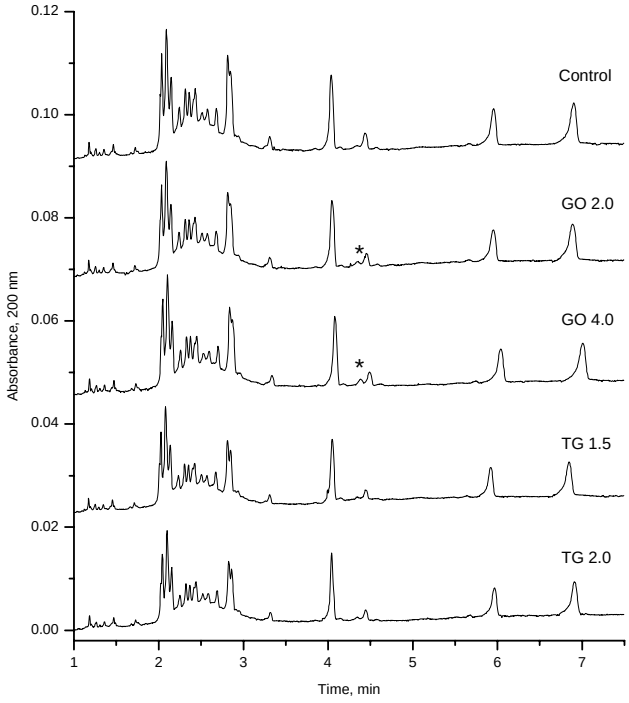

Figure 1.–Effect of enzyme treatments with glucose oxidase (GO) and transglutaminase (TG) on electropherograms of gliadin fraction. Numbers refer to enzyme concentration used in the treatment (U/g of kernel). Asterisks indicate differences among samples. Separations were in an uncoated capillary 50 µm i.d. x 27 cm long (20 cm L_D) at 45°C and 30 kV. Samples were pressure-injected (0.5 psi) for 4 sec.

understand the effect of various enzyme treatments. Comparison of the gliadin electrophoregram from GO-treated flours revealed the increase of a peak at the low mobility region, which increased with the enzyme concentration (Figure 1). The profile of the gliadins from TG-treated samples showed a decrease in the amount of all gliadin types. That effect was more pronounced at the highest enzyme concentration (2.0 U/g of kernel). There was a decrease in the intensity of the SDS-PAGE bands of gluten proteins that had been treated with TG; the LMW-GS and gliadins, and within the latter, γ gliadins were the most affected (Larre *et al.* 2000). However, in the present study, a reduction of all the gliadin peaks was observed.

No differences were detected in the glutenin electrophoregrams from GO-treated samples. These findings agree with previous results of Vemulapalli and Hoseney (1998), that GO did not affect gluten solubility and viscosity properties, and the effect on dough might be due to its oxidizing action on the water-soluble fraction.

Conversely, TG treatment induced several changes in the glutenin FZCE. In Figure 2, there is a progressive decrease of some peaks at the

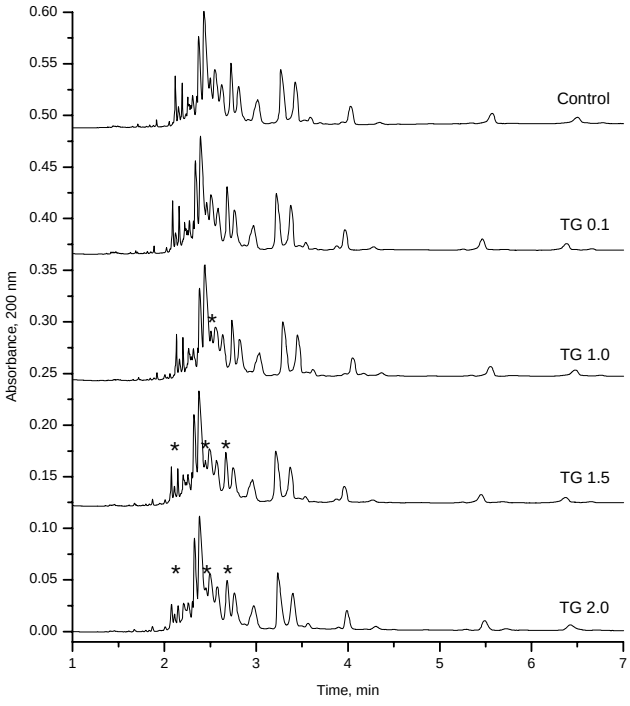

Figure 2.–FZCE profile of glutenins from flours treated at different transglutaminase (TG) concentrations. Numbers refer to the enzyme concentration (U/g of kernel) during milling. Asterisks indicate differences among samples. FZCE conditions as described in Figure 1.

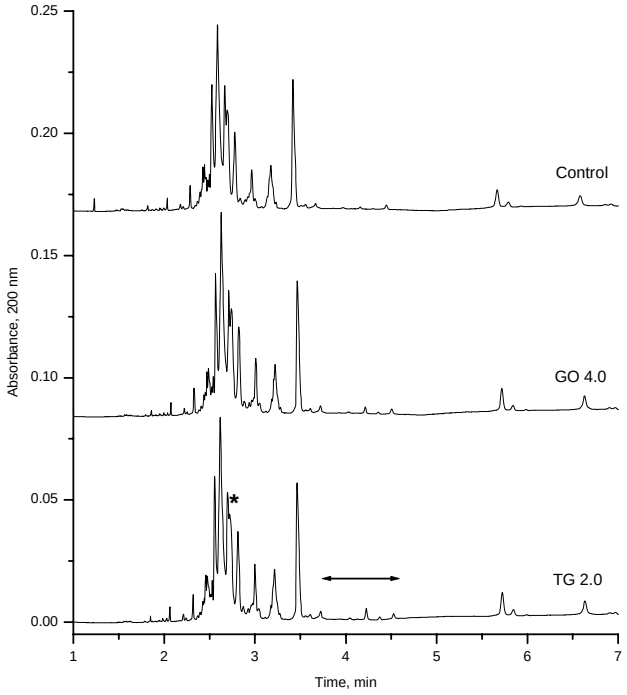

Figure 3.–Electropherogram profiles of LMW-GS from flours subjected to enzyme treatments with glucose oxidase (GO) and transglutaminase (TG). Numbers refer to enzyme concentration used in the treatment (U/g of kernel). Asterisk indicate differences among samples. Arrow indicates range where differences were detected. FZCE conditions are the same as Figure 1.

higher mobility region. To better separate the glutenins, the LMW and HMW glutenin fractions were analyzed by FZCE. Only at the highest TG treatment were differences found in the LMW-GS pattern obtained from the protein extracts from flours treated with GO and TG (Figure 3). Those differences were small, a decrease in one peak and also an increase in the peaks located at low electrophoretic mobilities. As previously indicated, Larre *et al.* (2000) reported a decrease in the SDS-PAGE bands corresponding to LMW-GS when gluten was modified by TG. In this study, we were able to detect the specific peaks modified by the TG reaction by using FZCE.

Electrophoregrams of the HMW-GS from GO-treated samples, again did not reveal changes in the protein profile (results not shown). On the contrary, increasing the TG concentration produced a progressive decrease in the height of some HMW-GS peaks (Figure 4), at 2.1, 2.2, 2.7, and 2.8 minutes of retention time. These results were similar to the findings of Larre *et al.* (2000), where a reduction of the SDS-PAGE band intensity of the HMW-GS, along with the pre-

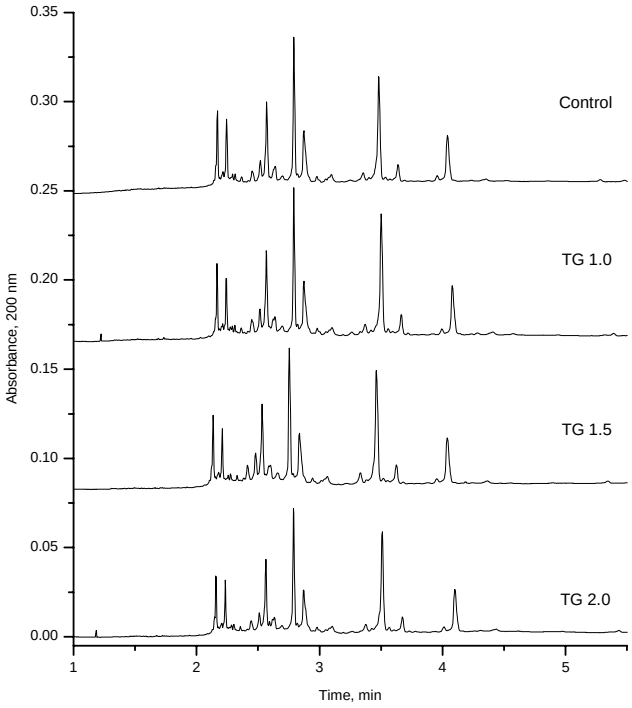

Figure 4.–Effect of transglutaminase (TG) treatment on the HMW-GS electrophoregram patterns. Numbers refer to enzyme concentration used (U/g of kernel). FZCE conditions are the same as Figure 1.

sence of new bands corresponding to higher molecular weight molecules, were described.

CONCLUSIONS

The addition of TG or GO modified wheat storage proteins. The changes promoted by GO treatment exhibited very pronounced effects on the physical properties of wheat flour but did not introduce changes in the protein profiles. This might be attributed to the presence of oxygen (involved in the enzyme reaction) in both the gluten index and the alveographic analysis, which was not as available during protein extraction and, in consequence, no oxidative effects were observed in the protein profile. On the other hand, the effects promoted by TG treatments were readily apparent on the protein electrophoretic properties. By using FZCE, the specific protein fraction affected by the enzyme can be detected.

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Recent advances in enzymes in grain processing[1], *pp. 101-106 (2003)*

CAPÍTULO 7

Improvement of cereal protein network through enzyme treatment

S. Aja[2], Jinshui Wang[2] and C.M. Rosell[2]

INTRODUCTION

Cereal grains are worldwide used as food for humans or as feed for animals (Bietz *et al.*, 1994). Cereal proteins have important functional properties in many foods (Bean *et al.*, 2000) and due to their widespread use and economic importance, they have been studied, analysed and characterised for years.

Cereal storage proteins of wheat play a fundamental role in the breadmaking process, because they have the ability to form gluten, a network necessary to hold other wheat components, mainly carbohydrates, and gas produced during fermentation. The formation of gluten during dough-mixing and breadmaking is very complex and depends on the variety, insect infestation, and environmental and post-harvest conditions. Those factors determine the resulting breadmaking quality of wheat, and could yield wheat with a reduced gluten forming capacity.

The formation of different linkages between wheat proteins could be a method for increasing the strength of the gluten network and in con-

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sequence for improving the breadmaking quality of soft wheat or wheat with a reduced quality. The used of enzymes labeled as GRAS (Generally Recognized As Safe) could be the best alternative to cross-link chemical compounds. Enzymes like glucose oxidase (GO), polyphenol oxidase (PPO) and transglutaminase (TG) can catalyse the formation of some new bonds among proteins, modifying the resulting gluten network. In fact, an improvement of the breadcrumb by the addition of GO and TG to the breadmaking process has already been reported (Gerrard *et al.*, 1998; Vemulapalli *et al.*, 1998).

In this study, the potential use of various enzymes that act through different catalytic mechanism (GO, PPO, TG) as gluten reinforces is presented. The modification of dough rheological properties was evaluated by using the farinograph and alveograph. In addition, the gluten modification by enzyme treatment was assessed by the gluten index and the use of free zone capillary electrophoresis (FZCE) allowed to determe the changes undergone by the gliadins and glutenins.

MATERIALS AND METHODS

Materials kernels from wheat cultivar Bolero were provided by Porta S.A.(Huesca, Spain). Glucose oxidase (500 glucose oxidase units, GODU/g) and polyphenol oxidase (1552 polyphenol oxidase units, LAMU/ml) were a gift from Novo Nordisk (Madrid, Spain). Transglutaminase (100 transglutaminase units per gram) was kindly supplied by Ajinomoto Co (Inc. Japan). Chemical reagents of the highest purity were from Sigma (St Louis, MO).

Milling process

In order to ensure an uniform distribution, enzymes were added during tempering as previously described (Haros *et al.*, 2002). The enzyme concentration added in the tempering solution was: GO 4.0 U/g of kernel, PPO 2.0 U/g of kernel, and 1.0 U/g of kernel in the case of TG. Wheat flour was obtained by milling in a laboratory Chopin Mill (Tripette and Renaud, Paris, France).

Physical measurements

Gluten Index was determined following the AACC standard method (1995). For analyzing the gluten quality after resting, the wet gluten was kept for 2 h at room temperature before the gluten index determination. The alveograph analysis was carried out in a Chopin Alveograph (Tripette and Renaud, Paris, France), according to the AACC approved method (1983). The parameters determined were tenacity (P), extensibility (L), the deformation energy (W), and the curve configuration ratio (P/L) (see Rosell *et al.*, 2001; for a detailed description of these parameters). Farinograph analysis was performed in a Brabender Farinograph (Duisburg, Germany) according to AACC standard method (1983) with the following modification, the mixing was stopped after 4 minutes of reaching the maximum consistency (500 Brabender Units, BU), and kept for 2 h at room temperature, then dough consistency was again determined. Dough samples withdrawn at the maximum consistency were lyophilized and further used for the FZCE analysis.

Sample preparation for protein analysis

Proteins were isolated following the method reported by Bean *et al.* (1998). Briefly, 200 mg of lyophilized dough were suspended in 1.0 mL 50 mM Tris-HCl buffer (pH 7.8), containing 100 mM KCl and 5 mM EDTA. After 5 min vortexing and centrifuging at 14,000 rpm for 5 min, the supernatant was discarded and the pellet was washed twice with water. Gliadins were obtained by mixing the albumins and globulin pellet free with 1.0 mL 50% 1-propanol, vortexing for 5 min and centrifuging at 14,000 rpm for 5 min. For the glutenin samples, the pellet obtained after gliadin extraction was mixed with 1.0 mL of 50% 1-propanol containing 1% dithiothreitol (DTT), vortexing for 30 min, and then centrifuged at 14,000 rpm for 5 min.

FZCE analysis

A Beckman MDQ instrument (Beckman, Fullerton, CA) was used for all FZCE separations. Uncoated fused silica capillaries (Composite Metal Services Ltd, Worcester, UK) of 50μm id x 27 cm (20 cm L_D) were used

for all separations. The FZCE separations were performed at the optimum separation conditions described by Bean and Lookhart (2000) using 50 mM iminodiacetic acid (IDA) containing 20% ACN and 0.05% hydroxypropylmethylcellulose (HPMC) as a buffer, at 45°C and 30 kV. Gliadin and glutenins samples were injected at 0.5 psi for 4 s. Proteins were detected by UV absorbance at 200 nm.

RESULTS AND DISCUSSION

Effect of enzyme treatment on the gluten quality

In order to test the effect of the enzyme treatment on the reinforcement of the gluten, the quality of the wheat gluten was assessed by the gluten index (Figure 1). All the enzyme treatments tested promoted an increase in the gluten index, the greatest effect being produced by GO addition. This result might indicate the formation of new bonds among gluten proteins, which would increase the polymerization degree of the proteins and in consequence would decrease the protein solubility. This result supported the finding of Larré *et al.* (2000), who reported reduced gluten solubility after TG treatment. Conversely, Rosell *et al.* (2002) described that no modification of gluten index was found by GO treatment and that a decrease was observed with TG treatment, the observed difference could be due to the different wheat quality, since Basman *et al.* (2002) described a great effect of TG addition on soft wheat and also a detrimental effect of the TG addition when increasing concentrations of TG were used.

When analyzing the evolution of gluten index during resting, a progressive increase in the gluten index was observed after short incubation period (30 min). Beyond that time no modification was found (unpublished results),

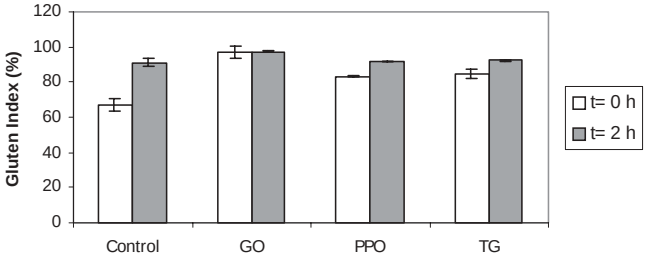

Figure 1.–The effect of enzyme treatment on the wheat gluten index, before and after resting. GO: glucose oxidase, PPO: polyphenol oxidase, TG: transglutaminase.

probably because the formation of the linkages among wheat storage proteins requires some time untill the gluten network is completely developed. No additional improvement of the gluten index was observed by GO treatment after 2 h resting, and only a slight increase was observed in the PPO and TG samples after 2 h resting. However, when comparing the gluten index values after 2 h resting, no significant differences were found among control and the different enzyme treatments. In all cases similar values of the gluten index were obtained, which indicates that there is probably a limit for the gluten index and that additional linkages do not improve the gluten quality. It should be stressed that the addition of oxidases and TG seems to accelerate the polymerization of the gluten proteins, since higher values were obtained from the beginning and no further improvement was observed after resting.

Influence of enzyme treatment on dough rheological properties

In figure 2 the alveographic behavior of different enzyme treated dough is shown. A substantial difference was produced by enzyme addition, all the enzymes tested led to an increase in the tenacity (P), a considerable reduction in the extensibility (L). The greatest effect was promoted by GO followed by PPO and TG. The action of the oxidases on the viscoelastic properties of dough is more evident than the effect of TG. Similar results were obtained by Rosell *et al.* (2002) when adding increasing concentrations of GO and

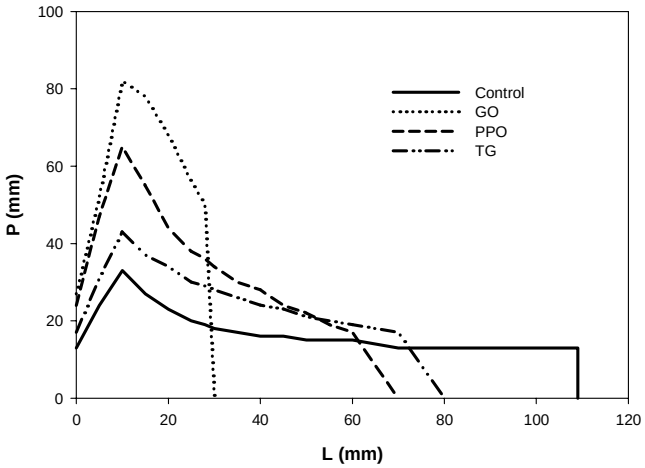

Figure 2.–The effect of enzyme treatment on the wheat gluten index, before and after resting. GO: glucose oxidase, PPO: polyphenol oxidase, TG: transglutaminase.

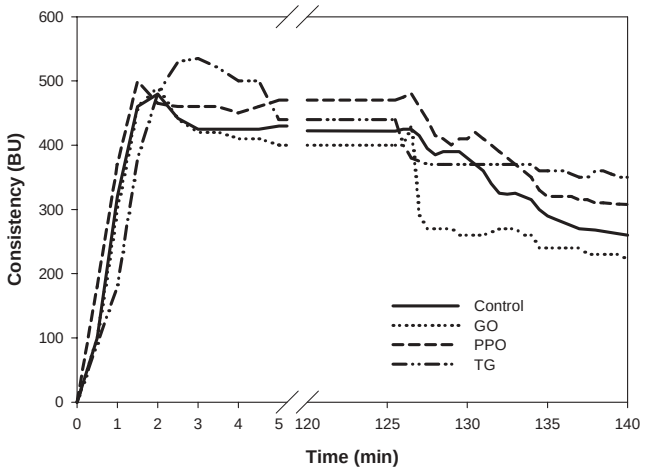

Figure 3.–The effect of enzyme treatment on the dough mixing properties, before and after resting, measured by the farinograph. GO: glucose oxidase, PPO: polyphenol oxidase, TG: transglutaminase.

TG, although in that study the addition of TG did not produce a clear effect on the tenacity.

In order to determine the effect of the enzyme treatment on the dough mixing properties the farinograph analysis was carried out (Figure 3). The addition of TG decreased the water absorption, and increased the development time, which is in agreement with the results reported by Basman *et al.* (2002).

To study the effect of the different enzymes on dough stability after a long incubation period, dough consistency was measured after 2 h resting. Good stability of dough was observed during the first 5 min after resting, but beyond that time a great reduction of the consistency was obtained. The highest drop in dough consistency was observed with the addition of GO, whereas the presence of TG conferred good stability to dough. Intermediate stability was obtained with PPO. Likely, the linkages promoted by the TG reaction prove essential for keeping the gluten structure during time.

Modification of the electrophoretic properties of storage proteins by the addition of enzymes

The electrophoretic pattern of the storage proteins from the different enzyme treatments can be observed in Figure 4. The most evident change in the gliadins (A) was the quantitative decrease in the control gliadins,

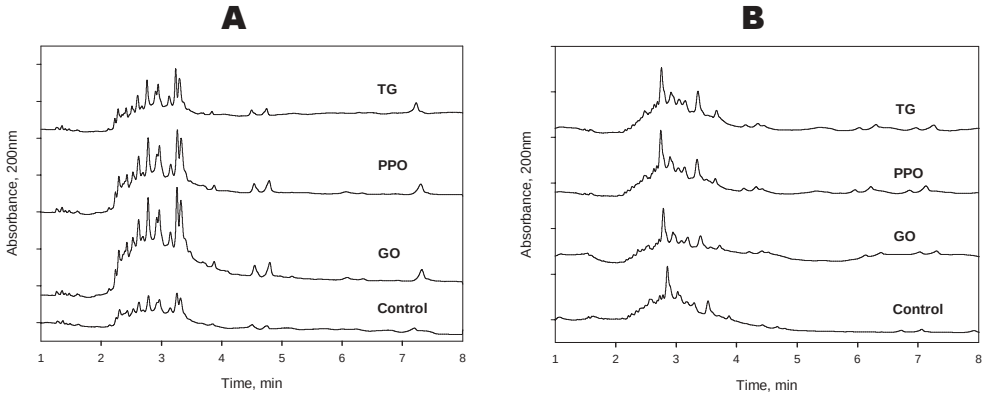

Figure 4.–The effect of the different enzyme treatments on the FZCE patterns of the wheat storage proteins. A, gliadins; B, glutenins. GO: glucose oxidase, PPO: polyphenol oxidase, TG: transglutaminase.

suggesting that part of gliadins could become insoluble due to polymerization during gluten formation. The level of gliadins in the enzyme treated dough did not undergo such a great drop; it is likely that the new links generated by the enzyme reaction could interfere in the gliadin polymerization. It seems that the GO treatment promoted the greatest interference, since high amount of gliadins were extracted from that sample.

Minor changes were observed in the FZCE pattern of the glutenins (Figure 4B), with the exception of the GO samples, which showed a decrease in all the peaks. These results sugest that the GO treatment modifies the glutenins to large extent, polymerising them and in consequence decreasing their alcohol solubility. However, further experiments are needed to demonstrate the specificity of these enzymes for the wheat storage proteins.

CONCLUSIONS

From the above results it could be concluded that reinforcement of wheat gluten proteins can be accomplished by using different enzymes such as a transferase (TG) or some oxidases (PPO and GO). This way of creating new bonds between proteins could be an excellent method for obtaining a protein network, in cereals without gluten-forming capacity such as rice or maize. This could provide a tool for obtaining gluten-free products for celiac persons.

ACKNOWLEDGEMENTS

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CONCLUSIONES

El estudio de la caracterización, detección y mejora de los trigos atacados por insectos heterópteros ha permitido establecer las siguientes conclusiones:

- Los trigos infestados muestran una alta variabilidad en las actividades amilásicas (α y β), pero estos cambios no están relacionados con el grado de ataque. La observación por microscopía electrónica de barrido de los granulos de almidón confirma que las actividades amilásicas no están involucradas en la alteración del trigo atacado por *Aelia* y *Eurygaster.*

- No se han podido aislar las enzimas proteolíticas procedentes de la saliva de los insectos *Aelia* y *Eurygaster* responsables de la degradación proteolítica de los trigos, posiblemente los sustratos usados no fueron los adecuados para medir la actividad proteolítica de estas enzimas.

- Las modificaciones proteicas causadas por el ataque de insectos heterópteros pueden ser cuantificadas empleando la combinación de la técnica SE-HPLC y cuantificación de proteínas. La extensión de la alteración de las proteínas depende de la variedad de trigo. La infestación produce una disminución de las proteínas poliméricas insolubles en alcohol y gliadinas, y un aumento de las proteínas solubles en sal.

- La electroforesis capilar zonal permite detectar la modificación proteica causada por la infestación por garrapatillo, observándose incluso sin incubación una disminución de las gluteninas y la aparición de picos con una movilidad electroforética similar a las ω-gliadinas.

- La determinación del índice de gluten inicial y después de una hora de incubación puede utilizarse como una herramienta capaz de detectar el grado de ataque producido por los insectos.

- Los productos de hidrólisis procedentes de las proteínas del trigo son solubles en agua y poseen pesos moleculares comprendidos entre más de 200,000 y menos de 10,000.

- La red proteica de las proteínas del trigo puede mejorarse mediante la utilización de distintos enzimas, como una transferasa (transglutaminasa) o algunas oxidasas (polifenol oxidasas y glucosa oxidasa). La adición de transglutaminasa y glucosa oxidasa modifica las propiedades funcionales de las proteínas de almacenamiento. Los cambios producidos por la glucosa oxidasa afectan a las propiedades físicas de la harina de trigo pero no introducen cambios en los perfiles de proteínas. Esto puede atribuirse a la ausencia de oxigeno durante la extracción de proteínas. Los efectos producidos por el tratamiento con transglutaminasa son evidentes en los perfiles electroforéticos de las proteínas. Esta forma de crear nuevos enlaces entre las proteínas podría ser un buen método para obtener redes proteicas en cereales sin gluten, como el arroz y el maíz.