Journal of Cereal Science 65 (2015) 290–297

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

Effects of natural protease inhibitors on high protease activity flours

Burcu Olanca [a, *], Dilek Sivri Ozay [b]

[a] National Food Reference Laboratory, Ministry of Food, Agriculture and Livestock, Fatih Sultan Mehmet St., N: 70, 06170, Yenimalle, Ankara, Turkey
[b] Hacettepe University, Food Engineering Department, 06800, Beytepe, Ankara, Turkey

ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form
20 August 2015
Accepted 24 August 2015

Keywords:
Wheat bug
Eurygaster spp.
Proteolytic activity
Plant inhibitors

ABSTRACT

Wheat grain damaged by wheat bug (*Eurygaster* spp.) contains the bug salivary secretion which hydrolyses the gluten needed for the dough quality of breadmaking due to its proteolytic activity. Since the protease inhibitors are widespread throughout the plant kingdom, the possibility of using some plants, especially food and feed legumes to decrease the proteolytic activity of flours milled from bug damaged wheats was investigated. The proteolytic activity was considerably inhibited by pefabloc-SC and EDTA-Na_2, suggesting that bug protease(s) included serine and metallo proteases. Extracts from cones of hops, seeds of grass pea, red kidney bean and sunflower caused reduction in the activity of bug protease(s). Effects of hop extract on electrophoretic, rheological properties of high protease activity flours milled from different bread wheat cultivars damaged by the bug were also studied. The dough development time and stability values of high protease activity flours increased considerably with hop extract at the lowest addition level (10:1, flour to hop extract ratio). The doughs supplemented with the hop extract had higher maximum resistance and lower extensibility values as compared to their controls. These results suggested that hop extract had improving effects on high protease activity flours due to the bug damage.

1. Introduction

A number of different species of wheat bugs in the *Eurygaster* (Scutelleridae) and *Aelia* (Pentatomidae) genera are responsible for economic losses due to low grain yield, a reduction of seed germination and poor gluten quality in many areas of the Near and Middle East, East and South Europe, North Africa and Russia (Paulian and Popov, 1980). Similar wheat damage associated with *Nysius huttoni* has been reported in New Zealand (Every, 1993). All parts of the wheat plant including leaves, stems and ears may be attacked, depending on the growth stage of the insects and of the wheat plants. A black central pinpoint on the grain surrounded by a pale or discoloured halo is the sign of bug damage. Damage to grain can range from complete destruction if the kernel is attacked in the milk-ripe stage, to slightly shriveled if attacked in later stage of kernel development. The grains damaged in milky stage are easily removed in the cleaning section of flour mills by sieving, aspiration and washing. Therefore, they do not cause major problems in terms of flour quality. However, grains damaged in late maturity retain their normal size and shape and are thus difficult to remove in the wheat cleaning section of the flour mill (Koksel et al., 2002). Flour milled from these grains exhibits weak dough properties and unsatisfactory baking quality due to its high content of protease(s) injected into grains by the insects during feeding (Matsoukas and Morrison, 1990; Paulian and Popov, 1980; Rosell et al., 2002; Sivri and Koksel, 2002). The bug protease(s) was more active on glutenin than on gliadin, especially on high molecular weight glutenin subunits (HMW-GS) (Sivri et al., 1999). There were intercultivar differences in the susceptibility of 50% 1-propanol insoluble (50PI) glutenin to the effects of *Eurygaster integriceps* protease(s) (Sivri et al., 2002). The conventional protease activity assays were not suitable for determination of bug protease(s) activity, since the wheat bug protease(s) had a great substrate specificity to gluten proteins and did not hydrolyse the substrates which are used in these assays. Wheat bug protease(s) activity was generally monitored by reducing in 50% 1-propanol insoluble glutenin with electrophoretic or spectrophotometric techniques. Its optimum reaction parameters were temperature of 35 °C and a neutral or slightly alkaline conditions (Sivri and

* Corresponding author.
E-mail address: olanca@gmail.com (B. Olanca).

http://dx.doi.org/10.1016/j.jcs.2015.08.007
0733-5210/

Koksel, 2002).

It is possible that breadmaking quality of bug damaged wheat could be improved by some modifications in milling and breadmaking process. Separation of damaged kernels by dry and/or wet cleaning methods and eliminating the high protease activity mill streams from straight flours (Koksel et al., 2002), as well as some physical treatments such as microwave, gamma irradiation and heat application under pressure (Dıraman, 2010; Sivri and Koksel, 1996) could increase the quality of bug damaged wheats. Oscillatory rheometer studies suggested that the addition of transglutaminase enzyme (1.5%) increased the dough strength of bug damaged flour (Koksel et al., 2001).

The proteinase inhibitors are widely distributed in the plant kingdom and certain storage organs, such as seeds from the Leguminosae family and tubers from the Solanaceae family, are excellent sources of proteinase inhibitors (Gatehouse, 1984). Other plants such as seeds of amaranth (Rodriguez et al., 1993), chickpea (Harsulkar et al., 1997), sunflower (Konarev et al., 2000), maize (Ellatif, 2014), and seeds or leaves of peppers (Montes et al., 2014) have been most extensively studied plants for their proteinase inhibitor contents. The most known proteinase inhibitors are active against digestive proteinases found in animals and microorganisms. The major role of them is plant protection by blocking the digestive proteolytic enzymes of invading pests (Gatehouse, 1984). It was reported that a phenylmethylsulphonyl floride (PMSF), a commercial serine protease inhibitor, was inhibited *Eurygaster* and *Nysius* proteases (Darkoh et al., 2010; Every, 1993). The protease from *Nysius huttoni* was insensitive to most proteinaceous inhibitors except for potato inhibitor I and some divalent metal ions such as Co^{2+}, Mn^{2+}, Fe^{2+} (Cressey, 1987; Every, 1993).

In this study, specific commercial inhibitors for all protease groups (acidic-, sulfhydryl-, serine- or metallo-proteases) were used to determine the type(s) of wheat bug (*Eurygaster* spp.) protease(s). Various plants, especially seeds from the Leguminosae family due to their high protease inhibitor contents were assayed for inhibiting/reducing activity of wheat bug (*Eurygaster* spp.) protease(s). In addition, hop (*Humulus lupulus* L.), used in preparation of sourdough to improve some dough and bread properties and delay bread staling in some European countries (Gocmen et al., 1997; Umezama, 1977), was also used to decrease the protease activity of bug damaged flours. Hop cones are rich in terpenes, bitter acids, chalcone, flavonol glycosides and catechins (Zanoli and Zavatti, 2008). Because of these biologically active compunds, it has anti-inflammatory, antioxidant, antilipoperoxidative, anti-angiogenic, antiproliferative, apoptotic, anti-infective effects. Effects of hop (bitter) extract on electrophoretic, rheological properties of high protease activity flours milled from different bread wheat cultivars damaged by wheat bug (*Eurygaster* spp.) were also studied.

2. Materials and methods

2.1. Materials

A heavily bug (*Eurygaster* spp.) damaged wheat (cv. Bezostaya) sample was used to obtain crude enzyme extract (CEE). The flour of a Canadian hard red spring wheat cultivar (cv. Katepwa) was used as the substrate of the wheat bug protease(s). Two hard red wheat samples (cv. Gun-91 and breeding line Ankara-S6) and two semi-hard white wheat samples (cv. Kirac-66 and breeding line Ankara-S5) damaged by the bug (*Eurygaster* spp.) were selected due to their similar protein contents and bug damage levels. All these samples were ground in a Buhler experimental mill into straight grade flour.

Commercial protease inhibitors set (Roche) including antipain-dihydrochlorid (inhibitor of papain, trypsin, cathepsin A and B), bestatin (inhibitor of amino peptidases), chymostatin (inhibitor of α-, β-, γ-, δ-chymotrypsin), E-64 (inhibitor of cysteine proteases), leupeptin (inhibitor of serine and cysteine proteases, such as plasmin, trypsin, papain, and cathepsin B), pepstatin (inhibitor of aspartate proteases), phosphoramidon (inhibitor of metallo-endopeptidases, specifically thermolysine), pefabloc-SC (inhibitor of serine proteases), ethylene diamine tetra acetic acid disodium salt (EDTA-Na_2, inhibitor of metalloproteases), aprotinin (inhibitor of serine proteases), and other inhibitors such as 3,4-dichloroisocoumarin (inhibitor of serine proteases), N_α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK-HCl, inhibitor of serine proteases), α_2-macroglobulin from human blood plasma (inhibitor of endoproteases), trypsin-chymotrypsin inhibitor from soybean, trypsin inhibitors from soybean and henn egg white were purchased from Sigma company.

Dry seeds of amaranth (*Amaranthus hypochondriacus*), red and green lentils (*Lens culinaris*), chickpea (*Cicer arietinum*), maize (*Zea mays*), red kidney bean (*Phaseolus vulgaris* var. Linden), soybean (*Glycine max*), common bean (*P. vulgaris*), seeds and pods of cowpea (*Vigna unguiculata*), seeds of sunflower (*Helianthus annuus*), tuber and shell of potato (*Solanum tuberasum*) and fruit of pepper (*Capsicum annuum*) were purchased from local markets. Feed legumes such as dry seeds of common vetch (*Vicia sativa* L.), woolypod vetch (*Vicia villosa* spp. glabrescens syn: dasycarpa), hairy vetch (*Vicia villosa Roth*), grass pea (*Lathyrus sativus* L.), narbon vetch (*Vicia narbonensis* L.), Hungarian white and pink vetch (*Vicia pannonica* Crantz.) were obtained from experimental plots of Field Crops Improvement Center (Ankara, Turkey). Dry pellets of hops (*Humulus lupulus* L., aroma and bitter types) prepared from hop cones were obtained from Anadolu Efes Brewery Company (Turkey). High molecular weight protein marker (wide range) for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and other chemicals were obtained from Sigma company.

2.2. Methods

2.2.1. Chemical and physicochemical analysis

Moisture contents, Zeleny sedimentation values, protein contents of bug damaged wheat flours were determined according to the methods of AACC Metot No: 44.19.01; 56.60.01; 46.12.01, respectively (AACC, 2010). The levels of bug damage in wheat flours were determined by modified sedimentation test. This test involved incubation of samples with a solution of bromphenol blue for 120 min at 37 °C prior to adding the lactic acid solution. Subsequent steps of the procedure were carried as in the Zeleny sedimentation test (Greenaway et al., 1965). Changes (%) between Zeleny and modified sedimentation test values were indicated the level of bug damage in the flour.

2.2.2. Extraction of wheat bug (Eurygaster spp.) protease(s)

Crude enzyme extract (CEE) was obtained from a heavily bug (*Eurygaster* spp.) damaged wheat (cv. Bezostaya) sample according to the method of Sivri et al. (2002). The bug damaged wheat sample was ground in a laboratory hammer mill (Falling Number Mill AB, type 120, Sweden). The whole meal (300 g) was extracted with distilled water (1500 mL) by magnetic stirring for 24 h at 4 °C and centrifuged for 10 min at 15 000× *g* at 4 °C. The residue was re-extracted with distilled water at the same conditions. The supertanants were pooled and freeze-dried. The resulting dry material was used as a crude enzyme extract (CEE).

2.2.3. Preparation of commercial protease inhibitors

Protease inhibitors were generally prepared at maximum