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Protease inhibition in cowpea pod-sucking bug species (Hemiptera: Heteroptera) using cereal and legume grain extracts

Abiola Elfrida Taiwo, Olalekan Joseph Soyelu and Raphael Emuebie Okonji
Selected grain extracts were assayed against digestive proteases of *Anoplocnemis curvipes*, *Clavigralla tomentosicollis* and *Mirperus jaculus* with a view to identifying suitable sources of protease inhibitors against the cowpea pests. Laboratory bioassays showed that grains of a local soybean variety *Kìší* and a cowpea landrace NG/SA/07/0098 contain potent inhibitors although the former produced a consistently higher level of inhibition in each of the three pests. Protease activity was highest in *C. tomentosicollis* (1.06 μmol/mg) followed by *M. jaculus* (0.94 μmol/mg) and *A. curvipes* (0.69 μmol/mg) but inhibition was highest in *M. jaculus*. The optimum temperature range for the inhibitors was 40-90°C while optimum inhibitory activity occurred in weak acidic to neutral media, pH 4-7. There was total protease inhibition in the pests when soybean and cowpea extracts were combined against gut extracts in soybean-biased volumetric ratios of 0.7:0.3 and 0.8:0.2. This synergistic efficacy was superior to those of two commercial products. The inhibitors were stable for 10-50 min at 80°C but got denatured within few minutes of exposure to higher temperatures. Obtained results showed that the soybean and cowpea are suitable sources of protease inhibitors in breeding exercises for development of transgenic cowpea varieties.

**Key words:** Grain extract, optimum pH, optimum temperature, protease inhibition, thermostability, transgenic cowpea.

**INTRODUCTION**

Cowpea, *Vigna unguiculata* (L.) Walp., is a staple food and source of cheap protein for poor and medium income earners in the tropics and subtropics (Tarawali et al., 2002). It is a good source of fodder for livestock and usually desired by farmers because of its role in maintaining soil fertility through nitrogen fixation (Eskandari and Ghanbari, 2009). The world cowpea production for 2014 was estimated at 5,588,947 tonnes,
of which 95.7% came from Africa (FAOSTAT, 2016). West Africa, with 80.9% of world total, is the key cowpea producing zone, mainly in the dry savannas and semiarid agro-ecological zones. Nigeria is the world’s leading producer of cowpea followed by Niger, Burkina Faso, Tanzania and Cameroon in descending order with 38.3, 28.4, 10.2, 3.4 and 3.1% of the world total, respectively. Despite this dominance by Nigeria, there has been a consistently low yield of cowpea per hectare of land cultivated (Ayodele and Oso, 2014).

Cowpea yield is known to be low because of heavy insect pest problems and virtually every part of the crop is attacked by insect pests (Nampala et al., 1999; Karungi et al., 2000). The crop is most vulnerable during the flowering and podding stages of its development when over 70% yield loss due to insect attack occurs (Akingbounge, 1982). The heteropteran pod-sucking bug species (PSBs) are the most important post-flowering pests of cowpea (Dreyer et al., 1994). The bug complex consists of various combinations of coreids [Clavigralla shadabi Dolling, C. tomentosicollis Stal, Anoplocnemis curvipes (Fabricius)], alydids [Riptortus dentipes Fabricius, R. acantharis (Dallas), Mirperus jaculus (Thunberg)] and pentatomids [Aspavia armigera Fabricius, Nezara viridula (Linnaeus)] (Mitchell, 2000). Nymph and adult bugs pierce and cut pod tissues with their styllets while injecting digestive enzymes through the salivary canal to liquefy the food into nutrient-rich slurry. The food slurry is ingested through the food canal and passed into the alimentary canal where it is further digested and absorbed (Cohen, 2000). The cellular and tissue damage caused by the injected salivary enzymes usually lead to shriveling of developing pods and seed malformation in mature pods (Soyelu and Akingbohungbe, 2006). Soyelu et al. (2007) also reported that proteases played a major role in tissue damage associated with pod penetration, extra-oral digestion and ingestion of food slurry. It was, therefore, concluded that feeding damage caused by PSBs on cowpea pods could be reduced significantly by using suitable protease inhibitors (PIs).

Control of cowpea pests has been mainly through application of synthetic insecticides but over-reliance on different chemicals and frequent misuse of the active ingredients have led to cases of poisoning in man, animals and the environment (Kopittke and Menzies, 2006; Ilavda, 2013, 2014; Dahiru et al., 2014). Efforts have, therefore, been directed towards finding non-chemical control strategies that are safer. Plant protease inhibitors are prime candidates for non-chemical control with highly proven inhibitory activity against insect pests (Lawrence and Koundal, 2002). The PIs act as defensive compounds against insects in direct assay or by expression in transgenic crop plants where they slow down digestion of plant materials thereby hindering insects from utilizing plant nutrients maximally. This ultimately renders the host plant unappealing to the herbivorous insects (Pueyo et al., 1995). Omitogun et al. (1999) demonstrated that crude lectin-enriched extract from African yam bean, Sphenostyles stenocarpa Harms, retarded the development of larval and adult C. tomentosicollis. Similarly, purified seed lectin from S. stenocarpa was toxic to the same bug species in an artificial cowpea seed system (Machuka et al., 1999; Okeola et al., 2000).

Proteases are proteolytic enzymes that catalyse the hydrolytic cleavage of specific peptide bonds in target proteins, thus being indispensable to the maintenance and survival of their host organism. Proteases are mostly classified according to the main catalytic amino acid residue in their active site in several groups, such as: serine proteases, cysteine proteases, aspartic proteases and metalloproteases, the latter having a metallic ion in their active site (Pouvreau, 2004; Habib and Fazili, 2007). Digestive physiology varies among bugs of the suborder Heteroptera because of the range of food sources. Heteropteran salivary proteases are most often of the serine protease class, as determined by inhibitor and substrate specificities and pH optima (Laurema et al., 1985; Cohen, 1993). The salivary gland protease activity is predominantly trypsin-like in some heteropterans (Cohen, 1993; Agusti and Cohen, 2000) while chymotrypsin-like protease activity predominates in some others (Cobleatch et al., 2001). On the contrary, midgut proteases are predominantly acidic proteases from both the cysteine and aspartic mechanistic classes (Houseman et al., 1985; Overney et al., 1998). Each bug species uses a specific complement of proteases belonging to a mechanistic class and this specificity makes the insects vulnerable because any suitable protein molecule that is able to bind to active sites on the protease group would have adverse effects on digestive activity of the enzymes. This interference with enzyme activity could serve as a basis for cowpea genome transformation to produce transgenic plants with improved level of cowpea resistance to the PSBs. High concentrations of PIs are often found in storage tissues of plants, such as tubers and seeds (Ryan, 1990), and there is, therefore, a need to assess plants that are available locally for their protease inhibition potentials against the cowpea pod-sucking bug species. In addition to this, the study also characterized PIs from selected plants with a view to establishing their suitability as control agents.

MATERIALS AND METHODS

Field establishment

A susceptible cowpea variety (Ife Brown) was planted on a 0.04 ha plot at a spacing of 60 cm × 30 cm at two seeds per stand. The field was established for the purpose of obtaining pod-sucking bug species needed for bioassays in the laboratory. Metobromuron and metolachlor formulated as Galex 500 EC (pre-emergence herbicide)
Table 1. Sources of protease inhibitors assayed against enzyme extracts of three cowpea pod-sucking bug species.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Botanical name</th>
<th>Accession number, Common name or Local name</th>
<th>Family</th>
<th>Source</th>
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<tr>
<td>Cowpea</td>
<td>Vigna unguiculata</td>
<td>Ife Brown NG/SA/07/0098 NGB/06/047 NGB/VU/118</td>
<td>Fabaceae</td>
<td>IITA</td>
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<td></td>
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<td>NACGRAB</td>
</tr>
<tr>
<td>Soybean</td>
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<td>Kíshi</td>
<td>Poaceae</td>
<td>Local market</td>
</tr>
<tr>
<td>Maize</td>
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<td>TZM 158</td>
<td>Poaceae</td>
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<td>TZM 1457</td>
<td></td>
<td>NACGRAB</td>
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<tr>
<td>Sorghum</td>
<td>Sorghum bicolor</td>
<td>Oka baba</td>
<td>Gramineae</td>
<td>Local market</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>Pennisetum glaucum</td>
<td>Dawa</td>
<td></td>
<td>Local market</td>
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IITA: International Institute of Tropical Agriculture, Nigeria; NACGRAB: National Centre for Genetic Resources and Biotechnology, Nigeria.

were sprayed at the rate of 2.5 kg a.i./ha immediately after planting. Thereafter, weeding was done manually whenever the need arose.

**Insect rearing in the laboratory**

Nymph and adult A. curvipes, C. tomentosicollis and M. jaculus were collected from the cowpea field and reared in separate 90 cm \( \times \) 62 cm \( \times \) 62 cm laboratory cages at 26 ± 1°C and 73 ± 3% RH. The insects were provided with freshly cut young cowpea pods every 48 h. Freshly-emerged adults (≤ 2 days old) of each insect species were used for the bioassays.

**Preparation of enzyme extracts**

Enzyme samples were prepared as described by Oyebanji et al. (2014) with little modifications. Each insect was dissected in ice cold 0.09% NaCl under a Kyowa Optical Model SDZ-PL binocular microscope and a clean lateral cut was made through the length of the insect body to expose the digestive tract which was carefully extracted. The extracted digestive tracts were weighed and homogenized in 3 volumes of sodium phosphate buffer (pH 7.5) after which the solution was centrifuged at 4,000 rpm for 30 min at 4°C. The protease-rich supernatant gut extract was collected for bioassays.

**Preparation of plant extracts**

Grains of five cereal and five legume plants (Table 1) were used as sources of protease inhibitors. The grains were cleaned properly and blended separately into fine powder. Fifty grams (50 g) of each powder was dissolved in 3 volumes of 0.1 M potassium phosphate buffer (pH 7.0) and stirred thoroughly for 30 min. The mixture was stored in a refrigerator at 4°C for 24 h to ensure adequate release of plant juice. After 24 h, the mixture was brought out of the refrigerator, stirred evenly and the slurry was sieved with a cheese cloth. The obtained solution was centrifuged at 4000 rpm for 30 min at 4°C to obtain a pure extract. The supernatant was collected and frozen until needed for bioassays.

**Determination of protein concentration in enzyme and plant extracts**

The concentration of protein in enzyme extract of each insect species and plant extract was determined using Bradford (1976) method. The reaction mixture consisted of 0.01 ml distilled water in a test tube to which 0.01 ml extract was added and then shaken for some seconds. To this mixture was added 1.0 ml Bradford reagent. Blank was made of 0.02 ml distilled water and 1.0 ml Bradford reagent. The optical density was read at 540 nm using a Visible Camp Spectrophotometer.

**Protease activity**

Protease activity in the enzyme extract of each pod-sucking bug species was quantified spectrophotometrically as described by Morihara and Tsuzuki (1977) with slight modification. The reaction mixture consisted of 1 ml 1% (w/v) casein and 0.5 ml of the enzyme preparation. This was incubated in a water bath at 35°C for 30 min. The reaction was terminated by the addition of 3 ml cold 10% (w/v) Trichloroacetic acid (TCA). The mixture was allowed to stand at 4°C for 30 min, centrifuged at 3000 rpm for 10 min and the supernatant was collected for the determination of non-precipitated TCA protein (Lowry et al., 1951). One milliliter of the TCA protein was mixed with 5 ml Lowry’s reagent C, mixed thoroughly and incubated at room temperature for 10 min. Three fold diluted Folin-Ciocalteu’s phenol reagent (0.5 ml) was added to the mixture with shaking, and incubated at room temperature for 30 min. The optical density of the mixture was read at 760 nm and the amount of non-precipitated TCA protein was estimated as tyrosine from a standard curve of known concentrations of tyrosine. One unit of protease activity is defined as the quantity that is required to produce 100 μg of tyrosine in 1 ml of TCA filtrate under the above conditions.

**Protease inhibition and kinetics assays**

The assay was similar to the one described for protease activity except that 0.25 ml of each plant extract was incubated with 0.5 ml enzyme extract for at least 15 min before 1 ml 1% (w/v) casein was added. For comparison, separate assays were carried out in which the plant extracts were replaced with 0.25 ml commercial protease.
inhibitors, phenylmethylsulfonyl fluoride (PMSF) and α-ε-N-caproic acid, which inhibit serine and cysteine proteases. For kinetic analysis of inhibition, serial concentrations (5, 8, 10, 15, 17 and 20 μl) of each plant extract were added to the assay buffer prior to each bioassay. The concentration of the extract resulting in 50% protease inhibition (IC₅₀) was determined from a plot of remaining activity versus extract concentration.

**Temperature and pH activities**

The replicated inhibition assay mixtures were incubated at temperatures between 40 and 90°C to assess thermal effect on inhibitory activity and to determine the optimum temperature for each PI. Similarly, effect of pH on inhibitory activity and optimum pH were assessed by assaying the enzyme in buffers of different pH levels at 25°C. The buffer solutions used were citrate buffer pH 4.0 and 5.0, borate buffer pH 9.0 and, phosphate buffer pH 7.0 and 8.0. The absorbance was read at 366 nm. The thermostability of PIs in the best two plant sources (Kishi and NG/SA/07/0098) was also determined. Separate protease inhibition assays were repeated as earlier described using the two extracts but assay mixtures were incubated for 1 h at 40, 50, 60, 70 and 80°C. Inhibition activity was determined every 10 min and the length of time for which the PIs remained active was determined for each plant extract.

**Combined effect of Kishi and NG/SA/07/0098**

Grain extracts of the two plants with superior PIs were assayed together in nine separate volumetric ratios against the insect enzyme extracts. The constituted inhibition bioassay was as described earlier and combination ratios ranged from soybean-biased to equality and cowpea-biased.

**Statistical analysis**

Each bioassay was replicated three times and data obtained for total protein and protease activity in the pod-sucking bug species were subjected to analysis of variance (ANOVA) using SAS v. 9.0 (SAS, 2002). Activity of the plant-based protease inhibitors against enzyme extracts was also subjected to ANOVA. Mean values were separated using the Least Significant Difference (LSD) procedure at α = 0.05.

**RESULTS**

The total protein and corresponding protease activity in each pod-sucking bug species are presented in Table 2. *Anoplocnemis curvipes* had the highest concentration of protein (3.5 mg/ml) followed by *C. tomentosicollis* (2.5 mg/ml) and *M. jaculus* (2.1 mg/ml). However, the highest protease activity was recorded in *C. tomentosicollis* (1.1 μmol/mg) while *A. curvipes* (0.7 μmol/mg) had the least. Evidently, *C. tomentosicollis* and *M. jaculus* had higher protease to total protein ratios compared to *A. curvipes*. Extracts of legume grains had higher protein concentrations compared to the cereals (Table 3) and high concentrations were found in the local soybean variety *Kishi* and cowpea landrace NG/SA/07/0098.

Percent protease inhibitory activity of ten grain extracts and two commercial protease inhibitors against enzyme extracts of the three PSBs are also presented in Table 3. The legume grain extracts had higher efficacy compared to the cereal plants. Extract of *Kishi* compared very well with PMSF in *A. curvipes* and with α-ε-N-caproic acid in *C. tomentosicollis* while it outperformed the two commercial inhibitors in *M. jaculus*. The two plant extracts with higher total protein had significantly higher inhibitory activity with PIs from the soybean being the most potent. The degree of protease inhibition in each PSB varied significantly depending on the plant extract tested. Also, proteases in *M. jaculus* were generally more susceptible to action of the PIs; yielding the highest inhibition of 76% when assayed against extract of *Kishi*.

Grain extracts inhibited protease activity in a dose-dependent manner with 50% inhibition concentration (IC₅₀) of 11.6, 13.1, 14.9 μg/ml (*Kishi*) and 10.9, 17.1, 8.9 μg/ml (NG/SA/07/0098) against *M. jaculus*, *C. tomentosicollis* and *A. curvipes*, respectively (Table 4). The optimum temperature and pH for protease inhibition by each plant extract are presented in Table 5. There was a noticeable variation in values recorded within and among the PSBs depending on plant extract. Generally, lower optimum temperatures (64.00 ± 6.36°C, range: 40-90°C) were recorded in assays involving *A. curvipes* compared to 77.00 ± 4.73°C (range: 60-90°C) for *C. tomentosicollis* and 80.00 ± 3.65°C (range: 50-90°C) for *M. jaculus*. There was also a noticeable difference in the optimum temperature of PIs from cereal and legume plants. Cereal-linked PIs acted best at higher temperatures (80.00 ± 4.78°C, range: 50-90°C) than those from legumes (67.33 ± 5.27°C, range: 40-90°C).

---

**Table 2.** Total protein concentration and protease activity in gut extracts of three cowpea pod-sucking bug species.

<table>
<thead>
<tr>
<th>Pod-sucking bug species</th>
<th>Protein concentration (mg/ml)</th>
<th>Protease activity (μmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anoplocnemis curvipes</em></td>
<td>3.545 ± 0.008</td>
<td>0.686 ± 0.005</td>
</tr>
<tr>
<td><em>Clavigralla tomentosicollis</em></td>
<td>2.525 ± 0.009</td>
<td>1.055 ± 0.007</td>
</tr>
<tr>
<td><em>Mirperus jaculus</em></td>
<td>2.115 ± 0.037</td>
<td>0.938 ± 0.032</td>
</tr>
<tr>
<td>LSD₀.₀₅</td>
<td>0.368</td>
<td>0.067</td>
</tr>
</tbody>
</table>

Values shown are average ± standard error.
Table 3. Total protein and protease inhibition effected by ten grain extracts and two commercial inhibitors against three cowpea pod-sucking bug species.

<table>
<thead>
<tr>
<th>Source of protease inhibitor</th>
<th>Protein concentration (mg/ml)</th>
<th>Percentage inhibition</th>
<th>(^\d)LSD(_{0.05})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Anoplocnemis curvipes</strong></td>
<td><strong>Clavigralla tomentosicollis</strong></td>
</tr>
<tr>
<td><strong>Kishi</strong></td>
<td>1.457 ± 0.012</td>
<td>61.07 ± 0.08</td>
<td>37.80 ± 0.19</td>
</tr>
<tr>
<td>NG/SA/07/0098</td>
<td>0.776 ± 0.006</td>
<td>16.32 ± 0.17</td>
<td>22.65 ± 0.07</td>
</tr>
<tr>
<td>NGB/06/047</td>
<td>0.740 ± 0.007</td>
<td>20.69 ± 0.09</td>
<td>20.37 ± 0.04</td>
</tr>
<tr>
<td>NGB/Vu/118</td>
<td>0.714 ± 0.011</td>
<td>11.66 ± 0.06</td>
<td>18.10 ± 0.05</td>
</tr>
<tr>
<td>Ife Brown</td>
<td>0.721 ± 0.005</td>
<td>18.00 ± 0.08</td>
<td>5.97 ± 0.02</td>
</tr>
<tr>
<td>TZM 212</td>
<td>0.716 ± 0.007</td>
<td>19.24 ± 0.08</td>
<td>6.35 ± 0.01</td>
</tr>
<tr>
<td>TZM 1457</td>
<td>0.701 ± 0.013</td>
<td>8.75 ± 0.03</td>
<td>7.77 ± 0.02</td>
</tr>
<tr>
<td>TZM 158</td>
<td>0.644 ± 0.011</td>
<td>6.71 ± 0.02</td>
<td>0.57 ± 0.01</td>
</tr>
<tr>
<td>Sorghum</td>
<td>0.679 ± 0.002</td>
<td>0.73 ± 0.01</td>
<td>5.02 ± 0.03</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>0.612 ± 0.004</td>
<td>10.50 ± 0.03</td>
<td>18.48 ± 0.07</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td></td>
<td>67.80</td>
<td>100.00</td>
</tr>
<tr>
<td>α-ε-N-caproic acid</td>
<td>100.00</td>
<td>41.00</td>
<td>39.40</td>
</tr>
</tbody>
</table>

\(^{1,2}\)Separate mean values (± s.e.) within and among the pod-sucking bug species, respectively.

Table 4. Inhibition concentration (IC\(_{50}\)) of ten grain extracts acting on proteases of three cowpea pod-sucking bug species.

<table>
<thead>
<tr>
<th>Grain extract</th>
<th>Inhibition concentration (μg/ml)</th>
<th><strong>Anoplocnemis curvipes</strong></th>
<th><strong>Clavigralla tomentosicollis</strong></th>
<th><strong>Mirperus jaculus</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kishi</strong></td>
<td>14.899</td>
<td>13.074</td>
<td>11.560</td>
<td></td>
</tr>
<tr>
<td>NG/SA/07/0098</td>
<td>8.859</td>
<td>17.084</td>
<td>10.934</td>
<td></td>
</tr>
<tr>
<td>NGB/06/047</td>
<td>11.163</td>
<td>16.189</td>
<td>11.582</td>
<td></td>
</tr>
<tr>
<td>NGB/Vu/118</td>
<td>12.721</td>
<td>11.057</td>
<td>16.526</td>
<td></td>
</tr>
<tr>
<td>Ife Brown</td>
<td>12.036</td>
<td>8.696</td>
<td>9.229</td>
<td></td>
</tr>
<tr>
<td>TZM 212</td>
<td>10.412</td>
<td>11.317</td>
<td>14.185</td>
<td></td>
</tr>
<tr>
<td>TZM 1457</td>
<td>10.059</td>
<td>9.164</td>
<td>14.839</td>
<td></td>
</tr>
<tr>
<td>TZM 158</td>
<td>13.154</td>
<td>11.900</td>
<td>10.575</td>
<td></td>
</tr>
<tr>
<td>Sorghum</td>
<td>14.733</td>
<td>9.784</td>
<td>15.655</td>
<td></td>
</tr>
<tr>
<td>Pearl millet</td>
<td>13.598</td>
<td>7.947</td>
<td>9.069</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Optimum conditions for protease inhibitory activity against the three cowpea pod-sucking bug species.

<table>
<thead>
<tr>
<th>Grain extract</th>
<th>Optimum temperature (°C)</th>
<th>Optimum pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anoplocnemis curvipes</td>
<td>Clavigralla tomentosicollis</td>
</tr>
<tr>
<td>Kìshi</td>
<td>40.0</td>
<td>60.0</td>
</tr>
<tr>
<td>NG/SA/07/0098</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>NGB/06/047</td>
<td>90.0</td>
<td>80.0</td>
</tr>
<tr>
<td>NGB/Vu/118</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Ife Brown</td>
<td>40.0</td>
<td>90.0</td>
</tr>
<tr>
<td>TZM 212</td>
<td>50.0</td>
<td>90.0</td>
</tr>
<tr>
<td>TZM 1457</td>
<td>70.0</td>
<td>90.0</td>
</tr>
<tr>
<td>TZM 158</td>
<td>50.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Sorghum</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>90.0</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Table 6. Percent protease inhibition due to different volumetric combinations of Kìshi and NG/SA/07/0098 extracts.

<table>
<thead>
<tr>
<th>Combined ratio (Soybean: Cowpea)</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anoplocnemis curvipes</td>
</tr>
<tr>
<td>0.5:0.5</td>
<td>100</td>
</tr>
<tr>
<td>0.4:0.6</td>
<td>93</td>
</tr>
<tr>
<td>0.3:0.7</td>
<td>18</td>
</tr>
<tr>
<td>0.2:0.8</td>
<td>36</td>
</tr>
<tr>
<td>0.1:0.9</td>
<td>58</td>
</tr>
<tr>
<td>0.8:0.2</td>
<td>100</td>
</tr>
<tr>
<td>0.7:0.3</td>
<td>100</td>
</tr>
<tr>
<td>0.6:0.4</td>
<td>99</td>
</tr>
<tr>
<td>0.9:0.1</td>
<td>100</td>
</tr>
</tbody>
</table>

While Kìshi, TZM 212 and sorghum extracts were more active in weakly-acidic medium (pH 6.0), the cowpeas (Ife Brown, NG/SA/07/0098, NGB/06/047 and NGB/Vu/118) inhibited proteases best at pH 7.0. Generally, optimum pH for the protease inhibitors ranged from weak acidity (pH 4.0) to neutral (pH 7.0).

Percent protease inhibition caused by nine volumetric ratios of the two superior plant extracts is presented in Table 6. There was a significant (**P < 0.01) intra- and interspecific variation in inhibitory activity depending on the extract ratio used. The combined protease inhibitory effect was synergistic for most of the extract ratios considered and inhibition efficacy was superior to those of two commercial inhibitors. Soybean-biased ratios of 0.7:0.3 and 0.8:0.2 caused total protease inhibition (100%) in all the three pod-
Table 7. Thermostability of protease inhibitors in Kìshí and NG/SA/07/0098 extracts.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Grain extract</th>
<th>Thermostability (min)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anoplocnemis curvipes</td>
<td>Clavigralla tomentosicollis</td>
</tr>
<tr>
<td>40</td>
<td>Kìshí</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>NG/SA/07/0098</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>50</td>
<td>Kìshí</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>NG/SA/07/0098</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>60</td>
<td>Kìshí</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>NG/SA/07/0098</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>70</td>
<td>Kìshí</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>NG/SA/07/0098</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>80</td>
<td>Kìshí</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>NG/SA/07/0098</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>90</td>
<td>Kìshí</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NG/SA/07/0098</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Thermostability could not be determined at 90°C because protease inhibitors were denatured very quickly when incubated.

sucking bug species. Generally, cowpea-biased ratios effected lower protease inhibition, the lowest at 18% when 0.3:0.7 extract ratio was assayed against the gut extract of A. curvipes. The thermostability of PIs from the two plants is shown in Table 7. The protease inhibitors were more stable at lower temperatures, for as long as 1 h at 40 and 50°C. However, thermostability could not be determined at temperatures higher than 80°C because the inhibitors were denatured.

**DISCUSSION**

The protease inhibitors are basic agents of plant defense and an attempt was made in this study to exploit the mechanism of defense with a view to identifying suitable plant sources of PIs against cowpea pod-sucking bug species. Two legume plants were found to be good sources of PIs against the PSBs and when assayed together against the enzyme extracts, they deactivated proteases synergistically. Many of the biosynthesised defensive chemicals in plants act either alone or in concert to contribute to the resistance of plants against insect pests (War et al., 2012). A combination of protease inhibitors holds promise of higher success rate than when each of them is applied singly (Koiwa et al., 1997; Vain et al., 1998). Insect herbivores have successfully developed multiple mechanisms of adaptation to overcome defensive effects of plant protease inhibitors (Gatehouse, 2011) especially when acting alone but combined effect of a mixture of PIs may not be broken easily.

Generally, the legume plants had higher total protein concentration and more efficient PIs compared to the cereals. The PIs, especially trypsin and chymotrypsin inhibitors, are widespread among legume crops (Clemente et al., 2015) and this may explain the reason for higher inhibitory efficacy achieved by the legumes because trypsin and chymotrypsin play important roles in extra-oral digestion. The first PI to be isolated and characterized was Kunitz’s soybean trypsin inhibitor (KTI) (Kunitz, 1947). The KTI is primarily an inhibitor of trypsin, but also weakly inhibits chymotrypsin. McGrain et al. (1992) reported a slow phase of KTI accumulation in soybean during seed maturation followed by a rapid phase when the seeds reached half their maximum fresh weight and over 80% of maximum length. Thus, a good level of KTI accumulation is attained in matured soybean seeds.

The PIs from cereal crops were more structurally stable than those from the legumes. This was evident in their ability to function at higher temperatures and more acidic environments compared to lower temperatures and mostly neutral media required by legume-linked PIs. The stability is likely due to stronger disulfide bonds linking amino acids in the storage protein of cereal crops. Disulfide bonds play an important role in determining the structure and properties of storage proteins and they are formed between sulphhydryl groups of cysteine residues, either within a single protein (intrachain) or between proteins (interchain) (Koehler and Wieser, 2013). Cereals have higher content of cysteine residues than legumes (Mlyneková et al., 2014) and this gives room for formation of more disulfide bridges. For instance, the protein fraction in wheat (glutenins) has fifty disulfide bridges compared to twenty two possessed by glycinin from soybean (Carbonaro et al., 2015). The higher number of disulfide bonds in cereals play an important role in enabling stability of proteins to a variety of chemical and
physical denaturants, including low pH, chaotropes, high temperature and pressure (Mills et al., 2009). The protein fraction of plants with high cysteine content is typically quite resistant to heat denaturation and proteolytic digestion. Therefore, for the health of man and animals, these traits are usually avoided when developing transgenic crops.

Because of the key roles played by proteases in digestion, considerable interest has been generated over the years about the effects of PIs that could be present in the food chain. If present, they can impair nutritional quality and food safety by altering the digestive processes and interfering with growth and development (Ryan, 1990; Clemente et al., 2015; vaz Patto et al., 2015). A few of the PIs tested in this study acted at 90°C but none of them was able to sustain its activity beyond 50 min at 80°C, losing their potency and getting denatured. Instability of a PI below 100°C is one of the desirable traits that guarantee safety of man and animals when such PI is incorporated into a food source (Ryan, 1990).

The tropical region has a great floral diversity from which more suitable sources of PIs against the PSBs and other major insect pests could be identified. However, it is advisable to use less susceptible proteases, such as the ones in C. tomentosicollis, when screening plant extracts for good PIs. Cowpea is severely attacked by insect pests at every stage of its growth (Asiwe et al., 2005) and its production cannot be successful without insecticide application (Nabiyre et al., 2003; Dugje et al., 2009; Kamara et al., 2010). Genetic modification of cowpea cultivars using genes that mediate protease inhibition in Kíšhi and NG/SA/07/0098 may serve as a basis for producing transgenic cowpea lines. This may eventually reduce the heavy dependence on synthetic chemical insecticides for cowpea production.

Conflict of Interests

The authors have not declared any conflict of interests.

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- African Journal of Biotechnology
- Journal of Developmental Biology and Tissue Engineering