

Modification of the Methods for the Extraction of Carboxypeptidase and Proteinase Activities from Sorghum Malts

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ABSTRACT

J. Inst. Brew. 109(1), 51–56, 2003

The effects of two extractants on the activities of carboxypeptidase and proteinase enzymes from three sorghum varieties germinated for 3 and 5 days were studied. In all three varieties, cysteine hydrochloride (Cyst. HCl) proved a better extractant than 2-mercaptoethanol (2-ME) as shown by the increase in carboxypeptidase and proteinase activities with 5-day malts. A three-way analysis of variance (ANOVA) on the effect of germination time, extractant and variety on the carboxypeptidase activities of the three malts showed that all the factors plus their possible four-way interactions highly significantly ($P < 0.001$) affected the extractability/activity of the enzyme. However, while the three factors had a highly significant ($P < 0.001$) effect on the proteinase activity, one of their four-way interactions, specifically, extractant/variety, had no significant effect. This suggests that, unlike the carboxypeptidase enzyme, the effectiveness of the extractants in enhancing the extractability/activity of the proteinase enzyme in all the sorghum malt samples was amongst other factors, not variety-dependent.

Key words: Carboxypeptidase, extractant, extraction, proteinase, sorghum malt.

INTRODUCTION

Protein extraction from plant materials presents some difficulty due to non-protein constituents such as phenolic compounds and their tannin derivatives^{22,24}. These phenolic compounds interfere with and even prevent the extraction of the proteins^{24,27}. Some antioxidants such as cysteine, 2-mercaptoethanol (2-ME), dithiothreitol (DTT), dithioerythreitol (DTE), glutathione, thioglycollate and a host of others^{4,12,24,41} are used as phenol-complexing agents or oxidase inhibitors. Several reports favour the use of varying concentrations of cysteine^{8,9,29} and 2-mercaptoethanol^{5,6,12,44} for the extraction of proteolytic enzymes from barley malts.

Unlike barley, some sorghum varieties have very high polyphenol/tannin contents^{14,36}. In some Nigerian varieties, this varies between 0.25% (catechin equivalent) and 2.92%³⁹. The presence of polyphenolic compounds in sor-

ghum can adversely affect the quantitative extraction of its proteins^{14,47}, reduce its food values²³ and make malt enzymes insoluble and inactive by complexing with them during extraction⁴². These compounds also inhibit the enzyme reactions and microbial activities needed in the brewing of sorghum beer^{14,36}. To prevent the inhibitory effects of tannins during brewing, sorghum grain especially the bird-resistant varieties are steeped in dilute formaldehyde¹⁵ or alkali^{11,16}. Initial work on the extraction of proteolytic activity from sorghum malt involved the use of 1.25 M NaCl which was only effective in extracting the albumin and globulin-types of proteins⁴⁵. A proportion of sorghum malt proteolytic enzymes, like those of barley malt, are not extractable with NaCl²⁷. Consequently, the EBC endopeptidase assay, for example, frequently measures less than 50% of the activity in the malt¹⁹. Mazhar *et al.*²⁹ reported an increase in the extractability of prolamin proteins (kafirins) from sorghum malt using varying concentrations of both t-butanol and 2-mercaptoethanol. Similarly, Evans and Taylor¹⁹, extracted both proteinase and carboxypeptidase enzymes from sorghum malts using a novel extractant, containing 0.6M lithium iodide plus 3.33 mM DTT, to enhance the extractability/activity of these enzymes. Recently, Agu and Palmer¹ reported a higher endopeptidase enzyme activity from sorghum malt using 0.2 M sodium acetate buffer containing 5 mM 2-ME as extractant. The proteolytic enzymes of malt are of considerable importance in brewing, particularly with the formation of free amino acids which are essential nutrients for yeast growth during fermentation¹³.

This paper describes the use of two extractants in enhancing the extractability/activity of the proteolytic enzymes from sorghum malt varieties. It also establishes by analysis of variance (ANOVA) the relationship between germination time, extractant and sorghum varieties. The fact that many workers have reported various increases in the activities of these enzymes using other extractants, emphasizes the need for more comprehensive studies on the use of other neutral salts and reducing agents in addition to those already investigated.

MATERIALS AND METHODS

Sorghum varieties used

The sorghum grains used in this study were of the improved varieties ICSV400, SK5912 and KSV8, all obtained from the National Seeds Service, Zaria, Nigeria; Premier Seeds Nigeria, Ltd., Zaria, Nigeria and the Inter-

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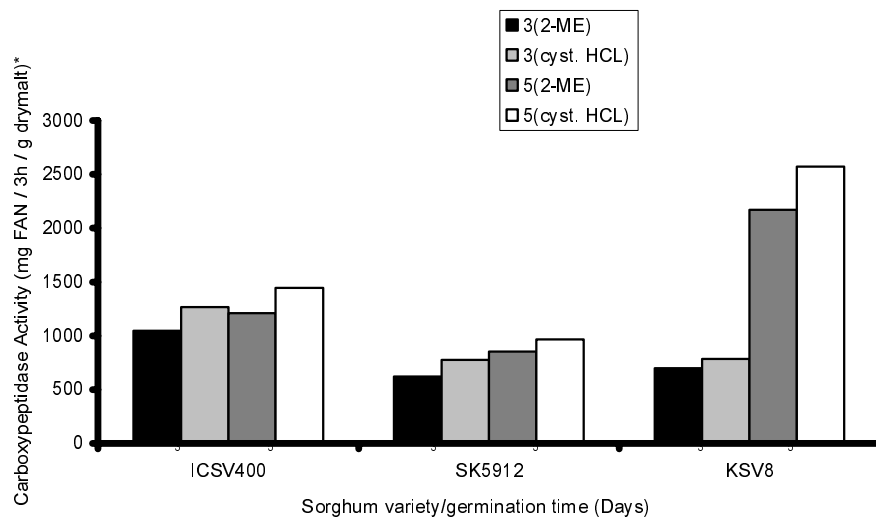


Fig. 1. Effect of extractants on the carboxypeptidase activity of sorghum malts. 2-ME = 2-mercaptoethanol; Cyst.HCl = cysteine hydrochloride. *Results are presented as means of three values.

Analysis of variance (ANOVA) for effect of extractants on carboxypeptidase activities of sorghum malts

| Sources of variation | Sums of squares | Degrees of freedom | Mean squares | F. ratio | P |
|----------------------|-----------------|--------------------|--------------|-----------|--------|
| Blocks | 100.72 | 2 | 50.36 | | |
| Germination time | 4065600.11 | 1 | 4065600.11 | 339082.58 | <0.001 |
| Extractant | 368044.44 | 1 | 368044.44 | 30695.94 | <0.001 |
| Variety | 3431846.72 | 2 | 1715923.36 | 143112.87 | <0.001 |
| Interaction 1 | 19228.45 | 1 | 19228.45 | 1603.70 | <0.001 |
| Interaction 2 | 4146909.72 | 2 | 2073454.86 | 172932.02 | <0.001 |
| Interaction 3 | 21885.39 | 2 | 10942.69 | 912.65 | <0.001 |
| Interaction 4 | 53652.39 | 2 | 26826.19 | 2237.38 | <0.001 |
| Error | 287.94 | 24 | 287.94 | | |
| Total | 12107555.88 | 35 | | | |

$F_{0.001} (1,24) = 14.03$

$F_{0.001} (2,24) = 9.34$

Interaction 1 = Germination time/extractant; Interaction 2 = Germination time/variety; Interaction 3 = Extractant/variety; Interaction 4 = Germination time/extractant/variety. Blocks = Replicates.

national Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Kano, Nigeria. While varieties ICSV400 and KSV8 were white in colour and virtually polyphenol/tannin-free, SK5912 was light-yellow and a low-tannin variety³⁹. All the grains had germinative energies of 96% and above and were not water-sensitive.

Sorghum malting

Sorting and cleaning. Grains for malting were sorted manually to remove broken kernels and foreign materials. Thereafter, sorted samples, in triplicate of 200 g batches, were surface-sterilized by immersion for 40 min in sodium hypochlorite solution having 1% (v/v) available chlorine. Subsequently, the grains were drained and washed four times in tap water as described by Morrall *et al.*³².

Steeping and germination. Two hundred gram (200 g) batches of each sorghum grain sample with initial moisture contents of between 9–11% were steeped to 32–35% moisture levels in 400 mL of deionized water at 30°C to give a grain/water ratio of 1:2. Steeping was done for 45 h in a cycle comprising: 6 h wet and 3 h dry. At the end of each 6 h wet steeping period, the grains were transferred

to a sieve, previously sterilized with the hypochlorite solution for the 3-hour air-rest. Properly rinsed grain batches were then spread in shallow wooden trays with fine mesh floors, kept in germination boxes in the dark and germinated for 3 and 5 days in an atmosphere of near water saturation at 30°C. At the end of each germination period, samples were removed and dried for 24 h in a forced-draught oven at 50°C^{34,35}. Roots and shoots were removed manually by rubbing against a sieve.

Enzyme extraction

The enzymes were extracted as outlined by Evans and Taylor¹⁹ with some modifications by Okolo and Ezeogu³⁸. Sorghum malt grists, 1.0 g, from each of the samples malted for 3 and 5 days was extracted separately with 15 mL of the 0.1 M citrate-phosphate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol (2-ME) or 0.2% (w/v) Cyst. HCl, for 2 h at 30°C using a Gallenkamp wrist-action shaker at 25 rev. min⁻¹. Thereafter, the extracts were centrifuged at 4000 g for 30 min and the supernatant fractions were assayed for carboxypeptidase and proteinase activities.

Assay of carboxypeptidase and proteinase activities

Substrate for the enzyme assays was bovine serum albumin (BSA). The BSA, 5.0 mg, was measured into each of the two test tubes (for control and test). An aliquot of 1.0 mL of each enzyme extract and 2.0 mL of the assay buffer, pH 4.6, were pipetted into each of the tubes while 1.0 mL of 15% (w/v) trichloroacetic acid (TCA) was added to the control. The test samples were incubated in a water bath at 50°C for 3 h with shaking every 15 min while the control samples were placed in a refrigerator for the same period (0 h). At the end of the 3 h incubation, 1.0 mL of 15% (w/v) TCA was added to the test samples to terminate the reaction.

Thereafter, the assay mixtures were centrifuged at 4000 g for 30 min using a Sigma 301 centrifuge. The clear supernatant which was obtained was then assayed for total nitrogen using the semi-micro Kjeldahl distillation method⁴⁰ and for free α -amino nitrogen (FAN) using the EBC ninhydrin method²⁸. Carboxypeptidase activity was calculated as the difference between FAN contents of the supernatants from 3 h and 0 h incubation reaction mixtures and expressed as: mg FAN/3h/g dry malt while proteinase activity was similarly calculated as the difference between nitrogen contents of the supernatants from 3 h and 0 h incubation reaction mixtures²⁰ and expressed as: mg N/3h/g dry malt.

Statistical analysis

The effects of germination time, extractant and variety, plus all their possible four-way interactions on the carboxypeptidase and proteinase activities from the three sorghum varieties were resolved by a three-way analysis of variance (ANOVA). Significance was accepted at $P \leq 0.05$ ²⁵.

RESULTS AND DISCUSSION

Consistent with earlier reports¹⁹, the terms proteinase and carboxypeptidase are used here to describe the proteolytic activities measured in terms of solubilization of total nitrogen and free α -amino nitrogen (FAN), respectively. In the absence of kafirin, the sorghum prolamins storage

protein^{19,46}, BSA was used as substrate contrary to earlier criticisms against the use of alien substrates^{9,17,18,21} since proteolytic activity is influenced by the amino acid sequence and tertiary structure of the substrate⁴³. To avoid this problem, Baxter⁵ assayed barley malt proteolytic activities using hordein, the barley prolamins storage protein as substrate.

Although 50 mM sodium acetate buffer, pH 5.0, containing 0.2% (w/v) Cyst. HCl extracted endopeptidase enzymes from sorghum malt², greater proteinase and carboxypeptidase activities were extracted using citrate-phosphate buffer at a neutral rather than acidic pH²¹. Enari and Mikola¹⁸ employed the reducing agent, mercaptoethanol to extract an unstable proteinase that was previously not extractable²⁷ from barley malt. The 0.6 M lithium iodide (LiI) plus 3.33 mM dithiothreitol (DTT) was reported¹⁹ to extract far more proteinase and carboxypeptidase activities from some South African sorghum malts than all the extractants previously used. As a result of non-availability of both LiI and DTT, extraction of proteolytic activity from the sorghum malt varieties investigated was carried out using separately 2 mM 2-mercaptoethanol (2-ME) and 0.2% (w/v) Cyst. HCl, in 0.1 M citrate-phosphate buffer, pH 7.0.

Effect of extractants on carboxypeptidase activity

Effects of the two extractants (2-ME and Cyst. HCl) on the activity of carboxypeptidase enzyme in the three sorghum malts are illustrated in Fig. 1. The highest carboxypeptidase activities in ICSV400 (1446 mg FAN/3 h/g dry malt), SK5912 (964 mg FAN/3 h/g dry malt) and KSV8 (2571 mg FAN/3h/g dry malt) were recorded from samples germinated for 5 days and extracted with buffer containing Cyst. HCl as the extractant. On the other hand, the enzyme activity was generally lowest with 3-day malt samples extracted with buffer containing 2-ME as the extractant. The superiority of Cyst. HCl over 2-ME was further confirmed by various increases in the rates of carboxypeptidase activity between the two extractants in all the malt samples using either 3-day or 5-day malts (Table I). Preference for a 5-day germination period over a 3-day time was also demonstrated in various rates of the enzyme activity increase between the two germination periods using either of the two extractants (Table II). Our findings

TABLE I. Rates of increase in carboxypeptidase activity with extractants.

| Germination time (days)/extractant | Sorghum variety/rate of increase in carboxypeptidase activity (%) | | |
|------------------------------------|---|-------------|--------------|
| | ICSV400 | SK5912 | KSV8 |
| 3 (2-ME) | 1043 ¹ (21) ² | 620 (25) | 695 (13) |
| 3 (Cyst. HCl) | 1266 | 775 | 785 |
| 5 (2-ME) | 1210 (20) | 853 (13) | 2172 (18) |
| 5 (Cyst. HCl) | 1446 | 964 | 2571 |

3 & 5 = Days of germination; 2-ME = 2-mercaptoethanol; Cyst. HCl = Cysteine hydrochloride

¹ Carboxypeptidase activity (mg FAN/3 h/g dry malt)

² Figures in parentheses represent % rates of increase (mean of three values).

TABLE II. Rates of increase in carboxypeptidase activity with germination time.

| Germination time (days)/extractant | Sorghum variety/rate of increase in carboxypeptidase activity (%) | | |
|------------------------------------|---|-------------|--------------|
| | ICSV400 | SK5912 | KSV8 |
| 3 (2-ME) | 1043 ¹ (16) ² | 620 (38) | 695 (213) |
| 5 (2-ME) | 1210 | 853 | 2172 |
| 3 (Cyst. HCl) | 1266 (14) | 775 (24) | 785 (228) |
| 5 (Cyst. HCl) | 1446 | 964 | 2571 |

3 & 5 = Days of germination; 2-ME = 2-mercaptoethanol; Cyst. HCl = Cysteine hydrochloride

¹ Carboxypeptidase activity (mg FAN/3 h/g dry malt)

² Figures in parenthesis represent % rates of increase (mean of three values).

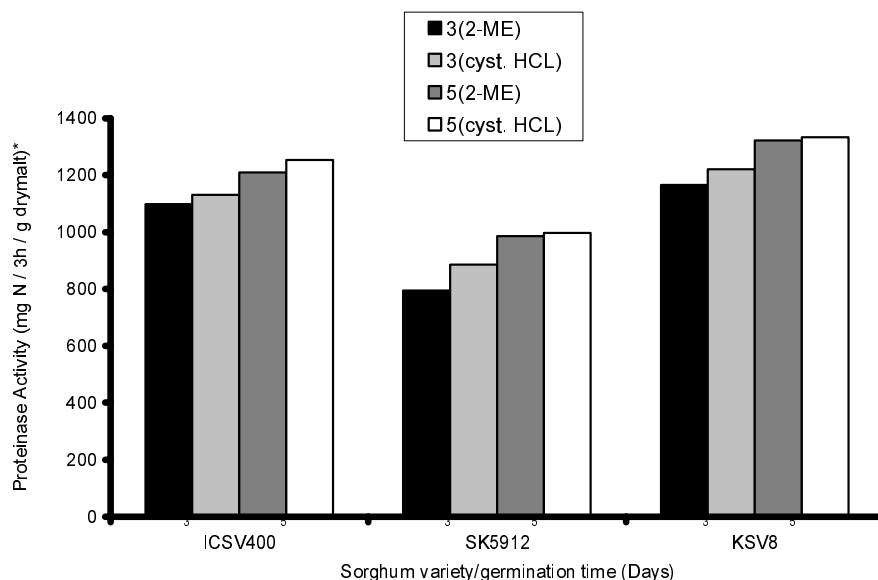


Fig. 2. Effect of extractants on the proteinase activities of sorghum malts. 2-ME = 2-mercaptoethanol; Cyst.HCl = cysteine hydrochloride. *Results are presented as means of three values.

Analysis of variance (ANOVA) for effects of extractants on proteinase activities of sorghum malts

| Sources of variation | Sums of squares | Degrees of freedom | Mean squares | F. ratio | P |
|----------------------|-----------------|--------------------|--------------|----------|--------|
| Blocks | 857.16 | 2 | 428.58 | | |
| Germination time | 162812.25 | 1 | 162812.25 | 1155.84 | <0.001 |
| Extractant | 15006.25 | 1 | 15006.25 | 106.53 | <0.001 |
| Variety | 770222.0 | 2 | 385111.0 | 2733.99 | <0.001 |
| Interaction 1 | 3192.25 | 1 | 3192.25 | 22.66 | <0.001 |
| Interaction 2 | 1734.0 | 2 | 867.0 | 6.16 | <0.05 |
| Interaction 3 | 458.0 | 2 | 229.0 | 1.62 | NS |
| Interaction 4 | 3098.0 | 2 | 1549.0 | 10.99 | <0.001 |
| Error | 3380.84 | 24 | 140.86 | | |
| Total | 960760.75 | 35 | | | |

$F_{0.001}(1,24) = 14.03$. $F_{0.05}(1,24) = 4.26$. NS = Not significant.

$F_{0.001}(2,24) = 9.34$. $F_{0.05}(2,24) = 3.40$.

Interaction 1 = Germination time/extractant; Interaction 2 = Germination time/variety; Interaction 3 = Extractant/variety; Interaction 4 = Germination time/extractant/variety. Blocks = Replicates.

with both extractants and times of germination are analogous to those of Evans and Taylor¹⁹ who reported superior increases in carboxypeptidase enzyme activity with lithium iodide plus dithiothreitol over a sodium acetate buffer at all germination times up to 6 days. Variety KSV8 did not only record the highest overall carboxypeptidase activity with its 5-day malts using Cyst.HCl as extractant, but had by several times the highest rates of increase in the enzyme activity between the two germination times and the two extractants studied.

A three-way analysis of variance (ANOVA) results (Fig. 1) showed that germination time, extractant and variety plus all their possible four-way interactions highly significantly ($P < 0.001$) affected the extractability/activity of the carboxypeptidase enzyme in all the sorghum malts investigated. This suggests that the effectiveness of the extractants in enhancing the extractability/activity of the carboxypeptidase enzyme in all the sorghum malt samples was dependent on the three factors and their possible interactions acting concertedly. These conclusions are in total agreement with earlier reports³⁸. Evans and Taylor²⁰ re-

ported a highly significant effect ($P < 0.001$) of cultivar and other factors on carboxypeptidase activity in some South African sorghum varieties.

Effect of extractants on proteinase activity

The effects of the two extractants (2-ME and Cyst. HCl) on the activities of the proteinase enzyme from the three sorghum malts at each germination time are shown in Fig. 2. The highest proteinase activities were observed using the 5-day malts in all the sorghum varieties with Cyst. HCl as the extractant. As with the carboxypeptidase activity, proteinase enzyme activity was lowest with the 3-day samples when 2-ME was used as extractant. Generally, KSV8 recorded the highest overall proteinase activity (1333 mg N/3 h/g dry malt) with its 5-day malts using Cyst. HCl as extractant followed by ICSV400 (1254 mg N/3 h/g dry malt) while SK5912 had the least activity (997 mg N/3 h/g dry malt).

A three-way analysis of variance (ANOVA) results (Fig. 2) revealed that while the three factors of germination time, extractant and variety highly significantly ($P <$

TABLE III. Rates of increase in proteinase activity with extractants.

| Germination time (days)/extractant | Sorghum variety/rate of increase in proteinase activity (%) | | |
|---------------------------------------|--|--------------|---------------|
| | ICSV400 | SK5912 | KSV8 |
| 3 (2-ME) | 1098 ¹ (3) ² | 795 (11) | 1165 (5) |
| 3 (Cyst. HCl) | 1131 | 885 | 1221 |
| 5 (2-ME) | 1210 (4) | 986 (1.0) | 1322 (0.8) |
| 5 (Cyst. HCl) | 1254 | 997 | 1333 |

3 & 5 = Days of germination; 2-ME = 2-mercaptoethanol; Cyst. HCl = Cysteine hydrochloride

¹Proteinase activity (mg N/3 h/g dry malt)

²Figures in parentheses represent % rates of increase (mean of three values).

TABLE IV. Rates of increase in proteinase activity with germination time.

| Germination time (days)/extractant | Sorghum variety/rate of increase in proteinase activity (%) | | |
|---------------------------------------|--|-------------|--------------|
| | ICSV400 | SK5912 | KSV8 |
| 3 (2-ME) | 1098 ¹ (10) ² | 795 (24) | 1165 (13) |
| 5 (2-ME) | 1210 | 986 | 1322 |
| 3 (Cyst. HCl) | 1131 (11) | 885 (13) | 1221 (9) |
| 5 (Cyst. HCl) | 1254 | 997 | 1333 |

3 & 5 = Days of germination; 2-ME = 2-mercaptoethanol; Cyst. HCl = Cysteine hydrochloride

¹Proteinase activity (mg N/3 h/g dry malt)

²Figures in parentheses represent % rates of increase (mean of three values).

0.001) affected the proteinase activities in the three sorghum malts, one of their four-way possible interactions, specifically, extractant/variety, had no significant effect. This was equally evident in the marginal increases in proteinase activity observed with Cyst. HCl over 2-ME in all the sorghum malt samples (Table III). The implication is that unlike the carboxypeptidase enzyme, the effectiveness of the extractants in enhancing the extractability/activity of the proteinase enzyme in the sorghum malt samples was amongst other factors, not variety-dependent.

It is noteworthy that as in carboxypeptidase, preference for a 5-day germination time over a 3-day period was demonstrated in various increases in proteinase activity in all the sorghum malts (Table IV) using either of the extractants. It is equally remarkable that the proteinase enzyme activity increases in all the malt varieties were moderate or not as pronounced as in the carboxypeptidase enzyme. Although several-fold increases in proteinase activity were reported during sorghum germination^{3,21,26,37} and barley germination^{6,7,10,30,31,33}, our results are in consonance with the moderate increase reported earlier by Evans and Taylor^{19,20}.

CONCLUSION

We have reported in this work the effects of two extractants on the extractability/activity of the proteolytic enzymes from sorghum malts. Cyst. HCl proved a better

extractant with 5-day malts than 2-mercaptoethanol (2-ME). Further studies should be undertaken using other neutral salts and reducing agents in addition to those already investigated. Such studies should take into cognizance the standardization of the concentrations of various extractants as well as a uniformity of the experimental conditions for a possible optimisation of the extractability/activity of sorghum malt proteolytic enzymes.

ACKNOWLEDGEMENTS

This research was supported by the International Foundation for Science, Stockholm, Sweden, and the Organisation for the Prohibition of Chemical Weapons, The Hague, The Netherlands through a grant No. E/3326-1 to A. C. Ogbonna.

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(Manuscript accepted for publication December 2002)