PROLAMIN DEGRADING ACTIVITY IN SORGHUM MALT

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DECLARATION

I declare that the work forming the basis of this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other University.

D.J. Evans.

29th day of January 1978.

ABSTRACT.

A procedure for assaying proteinase and carboxypeptidase activity of sorghum malt has been developed. This assay was applied to determine how sorghum cultivars and malting conditions influence the development of sorghum malt proteinase and carboxypeptidase activity.

Through use of a novel extractant, (0,6M LiI/3,33aM dithiothreitol) the efficiency of extraction of sorghum malt was improved. Investigations into the purity of the sorghum prolamin used as substrate led to the discovery of a protein, not previously isolated from sorghum. This protein was called sorghum reduced-soluble protein as it was virtually identical in amino acid composition to the reduced-soluble protein of maize.

Malting time most strongly influenced the development of sorghum malt proteolytic activity. Furthermore, proteinase activity increased by less than two-fold during malting, whereas carboxypeptidase activity increased by approximately eight-fold.

This assay for sorghum malt proteinase and carboxypeptidase activity will provide a useful tool by which ultimately the sorghum beer industry can obtain a greater understanding of the physiological and biochemical processes which determine the brewing performance of its principal raw material, sorghum malt.

PREFACE

Some of this work has been accepted for publication in the *Journal of Cereal Science*. The paper is entitled "Isolation of Reduced-Soluble Protein from Sorghum Starchy Endosperm." and will appear in part 1 of the 1987 issue.

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CONTENTS.

LIST OF ABBREVIATIONS.

Dithiothreitol.....DTT
Free alpha-amino nitrogen.....FAN
High performance liquid chromatography....hplc
Gibberellic acid.....GA
Reduced-soluble protein.....RSP
Trichloroacetic acid.....TCA
Trinitrobenzenesulphonic acid.....TNBS

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1. INTRODUCTION.

In South Africa, the traditional alcoholic beverage of the black peoples is a beer made from sorghum malt. Originally, this beer was prepared in the home by the women. However, as the industrialisation process began, and the black peoples migrated towards the cities, this small scale preparation of sorghum beer proved insufficient to satisfy demand. To meet demand, the mining industry, other large employers and the municipalities began to produce beer and thus the sorghum beer industry came into being (Novellie, 1986).

Like the barley malting and brewing industry, the sorghum beer industry recognised the need for research in order to improve the quality of its raw materials and the efficiency of its process. One aspect of importance is the level of free amino acids and small peptides which are attained in wort or unfermented beer. Free amino acids and small peptides provide a source of nitrogen for the support of yeast growth during fermentation (MacWillian & Clapperton, 1969). It has recently been demonstrated that their level, as measured by the free alpha-amino nitrogen (FAN) content of wort, not only influenced the rate at which the alcoholic fermentation of wort by yeast proceeded, but also that a minimum level of amino acids and small peptides is required, if yeast is to convert the available wort sugar completely into alcohol (Pickerell, 1986). The origin of the free amino acids and small peptides in wort is mainly due to the hydrolysis of protein by the proteolytic enzymes of sorghum malt (Taylor & Boyd, 1986).

The quality of sorghum malt produced for brewing sorghum beer, has in the past been judged primarily upon its diastatic power: a combined measure of the alpha- and beta- amylase activity but predominantly a measure of alpha-amylase activity (SABS, 1970). However recently, because of the large contribution towards wort FAN made by proteases FAN in sorghum malt, an additional criterion of malt quality has been recommended: that of a minimum

FAN content for sorghum malt (Taylor, 1985). Because of the role that proteolytic enzymes play in generating malt FAN, this study of the prolamin degrading activity of sorghum malt was made. The term prolamin degrading activity has been chosen to describe collectively the proteolytic enzymes of sorghum malt which hydrolyse sorghum prolamin or kafirin, the enzymes principal, natural substrate.

The objectives of this study were:-
1) to develop a procedure to assay the prolamin degrading activity of sorghum malt,

2) to apply this assay to determine how the development of prolamin degrading activity during malting is influenced by:

a) the choice of sorghum cultivar

b) the conditions of malting temperature, moisture and time.

2 LITERATURE REVIEW.

2.1 Malting physiology and the mobilisation of storage reserves.

2.1.1 General.

Throughout the world, traditional foodstuffs and beverages have evolved which rely intrinsically upon malted cereal grain. Some of these preparations have remained esoteric, while others, in particular the brewing of beer from malted barley, have developed into vast international industries. However, because for the most part, the largest proportion of a cereal crop is processed as is, for example as flour or animal feed, new cereal varieties are bred for increased yield and disease resistance (Briggs et al, 1981) and malting quality is often neglected in national breeding programmes. The maltster therefore has to select from the available cultivars, the variety which best suits his requirements.

The process of malting is one of artificially initiating, manipulating and terminating cereal germination (Briggs et al, 1981; Novellie & de Schaepdrijver, 1984). Scientific studies of germinating seed physiology have however undoubtedly contributed to the advancement of malting technology. The first studies of germinating seed physiology were made on behalf of the barley malting and brewing industry (Brown & Morris, 1890), and although numerous other seeds subsequently have been investigated in this regard, most of the scientific literature relates to barley. The discussion which follows however will focus principally on sorghum but will refer to observations made with other germinating cereals where necessary.

2.1.2 Anatomy, ultrastructure and chemistry of the cereal grain.

2.1.2.1 Starchy endosperm.

Most of the reserve materials of cereal seeds are located in the starchy endosperm (Bewley & Black, 1978). Of these reserve materials, by far the most predominant is starch (Smith, 1967, Radley, 1976). Starch can in fact represent up to 70 per cent of the dry weight of sorghum grain (Watson, 1987).

Starch is packed or "laid down" in subcellular bodies called starch granules: the size and shape of the granules being characteristic of the cereal species (Manners, 1974). Sorghum starch granules tend to be spherical and of uniform shape (20 µm diam.)(Knight, 1969) in contrast, for example with barley starch granules which although spherical in shape, occur in two distinct groups: so-called large (25 µm diam.) and small (5 µm diam.) granules (Palmer, 1972). There is however little knowledge relating to the fine structure of starch granules although the observation that sections of the granules appear to comprise concentric rings have led to the postulation of some theories of how packing occurs (Buttrose, 1960). Also characteristic of the source of starch is the gelatinisation temperature, or the range of temperature over which the fine structure of the starch granule is irreversibly destroyed in the presence of water (Knight, 1969).

Studies by Schoch (1942) have shown that starch comprises a mixture of two polysaccharides, amylose and amylopectin. The proportions of amylose and amylopectin in starch can also be indicative of the starch's source (Bewley & Black, 1978; Knight, 1969) although it has been shown that most cereal starches contain between 15-35 per cent amylose (Greenwood, 1956). Some varieties of maize and sorghum, the so-called waxy mutants, can contain less than one per cent amylose (Nixon & Grishell, 1958).

Both amylose and amylopectin are polymers of D-glucose. Amylose,

being a *linear, long chain molecule* comprising glucose units joined by alpha-1,4 glucosidic bonds, whereas amylopectin is a branched chain polymer, *comprising linear chains of* glucose units linked by alpha-1,4 bonds and branching links of single alpha-1,6 bonds (Manners, 1974).

Also found in the starchy endosperm of cereal seeds to a *greater* or lesser extent is storage protein. Endosperm storage protein can account for six to twelve per cent of the dry weight of the cereal grain (Simmonds & Orth, 1973). Storage protein is located in discrete subcellular organelles called protein bodies and/or in an amorphous structure interspersed amongst the starch granules, often termed the protein matrix or interstitial protein (*Simmonds & Orth*, 1973). The so-called steely or horny regions of the starchy endosperm are characterised by relatively high proportions *of protein bodies and* interstitial protein compared with the floury endosperm (Seckinger & Wolf, 1973). The size of protein bodies range *from one to twenty µm diam.*, and like starch granules can be characteristic of a cereal species and to a certain extent upon the site of location *within a species* (Bewley & Black, 1978).

Osborne (1907) put forward a syste. of classification of seed proteins based upon protein solubility. Four groups of proteins were thus identified: water-soluble or albumin protein, salt-soluble or globulin protein, alcohol-soluble or prolamin *protein, and* glutelin protein, which was insoluble in water and aqueous solutions of salt and ethanol but could be extracted *under dilute acidic or alkaline conditions.* Ultrastructural studies of different sorghum varieties show that almost all protein bodies were two to *three µm diam. and comprised* mainly prolamin or kafirin (Johns & Brewster, 1916); while the interstitial protein consisted mainly *of glutelin (Seckinger &* Wolf, 1973; Taylor *et al*, 1984a). The bulk of cereal endosperm storage proteins are prolamins and glutelins (*Simmonds* & Orth, 1973, Beevers, 1976). In sorghum, kafirin and glutelin represent 4,5 and 3,8 per cent approx. respectively of the dry

grain weight (Taylor et al 1984c), whereas in barley, prolamin (hordein) and glutelin (hordenin) represent 4,0 and 4,5 per cent of dry grain weight respectively (Beavers, 1976). Because prolamin proteins represent such a large proportion of cereal proteins, their amino acid composition influence the nutritional value of the cereals. Since prolamin protein is generally deficient in the essential amino acid lysine, cereal protein is of poor nutritional quality (Bewley & Black, 1978).

A further characterisation of cereal proteins was made by Landry and Moureaux (1970), when they extracted additional prolamin type protein termed Gl-glutelin from residual maize glutelin by including the reducing agent, mercaptoethanol with aqueous alcohol. Jambunathan and Mertz (1973) using the Landry and Moureaux procedure identified the equivalent protein fraction in sorghum, which they initially termed alcohol soluble glutelin and latterly termed crosslinked kafirin (Guiragossian et al, 1978). Taylor et al (1984b) deaonstrated that crosslinked kafirin, like kafirin was also associated with sorghum protein bodies. Paulis and Wall (1977) identified a further cereal protein fraction by aqueous extraction of alcohol soluble glutelin in maize. This protein was termed water-soluble alcohol-soluble reduced glutelin and has also been termed reduced-soluble protein (Wilson et al, 1981). Evans et al (1987) have subsequently daonstrated the presence of reduced soluble protein in sorghum. Due largely to electrophoretic techniques and high performance liquid chromatography (hplc) proteins are now being isolated from cereals which are not defined by Osborne's classification. The need therfore has arisen for a new system of nomenclature such as the one devised by Wilson (1987).

Ultrastructural studies of sorghum grain (Hoseney et al, 1974; Bullins & Rooney, 1975) show that the starch granules and protein bodies/protein matrix of the endosperm are surrounded by cell walls. The chemical composition of sorghum endosperm cell

walls has been determined by Woolard *et al*(1976) and the major components appear to be pentosans which represent 2,5-5,0 per cent of the whole sorghum grain (D'Appolonia, 1973). The composition of sorghum endosperm cell walls bears sharp contrast to barley endosperm cell walls, the latter comprising 75 per cent beta-D-glucan (Fincher, 1975, Briggs & Morrall, 1984). The beta-D-glucan content of barley endosperm cell walls is unusually high for a cereal seed with its closest rival being rye which contains 48 per cent approx. beta-glucan in its cell walls (Burke *et al*, 1974).

2.1.2.2 *Aleurone.*

The non living, starchy endosperm tissue of cereal seeds is surrounded by a thin layer of living tissue called the aleurone (Bewley & Black, 1978; Toman & Laidman, 1979). The extent of the aleurone, like so many other properties of cereal seeds can be a feature of the species. In barley for example, the aleurone is normally three cells thick (Briggs, 1987), whereas in sorghum it is generally only one cell thick (Rooney & Clark, 1969). One feature of aleurone cells which distinguishes them from other cells is that they contain relatively large numbers of protein bodies which are often called aleurone grains (Palmer & Bathgate, 1976).

2.1.2.3 *Embryo.*

The embryo or germ is a living tissue which at maturation already comprises regions of specialised cells (Bewley & Black, 1978). These regions include the scutellum, the coleorhiza (embryonic root) and the coleoptile (embryonic shoot). Embryo morphology and the contribution of the embryo towards total seed weight varies greatly between different cereal species (Bewley & Black, 1978), and in sorghum contributes eight per cent approx. of total dry kernel weight (Hubbard *et al*, 1950). The

scutellum is an absorptive structure with highly specialised cells for transport. The epithelial cells of the scutellum have a high energy requirement to effect transport and to satisfy this large numbers of mitochondrion are *aligned alongside their* plasmalemma (Walker-Smith & Payne, 1984).

2.1.2.4 Pericarp and testa.

In cereal seeds, the pericarp (ovary wall) and testa (seed coat) are fused and completely envelop the aleurone layer and the embryo (Bewley & Black, 1978; Briggs, 1987). In sorghum, the testa is relatively rich in polyphenols and in some varieties, the polyphenol content (measured in *catechin equivalents) can be* as high as two per cent of the sorghum grain (Earp *et al*, 1981). These latter varieties are referred to as birdproof: the astringent bitterness imparted by the polyphenols safeguarding the seeds from degradation by birds (Hoseney *et al*, 1981; Mikola, J., 1983). Often associated *with the pericarp and testa* to a greater or lesser extent are microflora: yeasts, moulds and bacteria (Briggs, 1987). The pericarp of cereal seeds is rich in waxes. These waxes help to maintain the integrity of the seed and play a role in the control of water uptake (Briggs, 1987).

2.1.3 Germination of cereal grain.

2.1.3.1 Dormancy.

Practically all cereal grains undergo a period of dormancy after ripening during which time they are unable to germinate under normal conditions (Mayer & Shain, 1974). The phenomenon of dormancy has important implications for maltsters, as the production of a good quality malt *relies upon even or uniform* germination of grain (Briggs *et al*, 1981). To ensure even germination, the barley maltster routinely examines incoming grain for dormancy according to the procedure of Essery *et*

al (1955). This method has been modified for use with sorghum (Morrall *et al*, 1986). Because dormancy represents a delay between the time of harvesting and the time of malting, numerous researchers have investigated the phenomenon on behalf of the barley malting industry. *A number of chemical* and physical treatments have been shown to break dormancy, for example gibberellic acid (Pollock, 1959), heat-treatment (Essery & Pollock, 1957), cyanide (Major & Roberts, 1968) and hypochlorite (Blum & Gilbert, 1957). Various mechanisms have been proposed to *explain the action of these treatments and* in the light of these it would seem likely that the mechanism responsible for dormancy is complex (MacLeod, 1979).

2.1.3.2 Respiratory requirements *during germination.*

Once the cereal grain has been steeped in water and has begun to germinate, the oxygen requirement of the grain increases due to the onset of respiration (Briggs *et al*, 1981; MacLeod, 1979). But by the same hand, *steeping of the grain also poses* a physical barrier to the availability of oxygen to the seed. However to a large extent developments in steeping technology have helped to overcome this problem and enable the maltster to achieve necessary levels of moisture in the germinating seed without *impairing respiration and hence germination development.* Such developments in steeping technology, include incorporation of air rests and aeration of the steep water, and in the case of barley malting are reviewed by Brookes *et al* (1976). The use of additives in the steep water to suppress mould growth is common practice amongst *sorghum maltsters. The addition* of formaldehyde to steep water (Daiber, 1976) has a two-fold purpose, the suppression of mould growth, and the polymerisation of polyphenols in the testa. The latter function disabling the enzyme inhibitor potential of the polyphenols (Daiber, 1975).

2.1.3.3 Development of hydrolytic enzyme activity.

2.1.3.3.1 The role of plant hormones in the development of enzyme activity.

In the malting context, undoubtedly the most important of the plant hormones are the gibberellins. Gibberellic acid (GA) was first discovered in barley seeds in 1938 (Yomo, 1938). Paleg (1965) later postulated that GA was synthesised in the barley embryo shortly after the onset of germination and was then *secreted from the scutellum into the starchy endosperm.* The GA then migrated across the endosperm to the aleurone and induced the synthesis of hydrolytic enzymes by the aleurone cells. There is considerable evidence indicating that the aleurones of germinating barley and wheat synthesise alpha-amylase and protease in response to GA (Rowsell & Goad, 1964; Chrispeels & Varner, 1967; Jacobsen & Varner, 1967). In germinating maize, there is also evidence that the aleurone responds to GA by synthesising alpha-amylase and protease (Ingle & Hageman, 1965; Harvey & Oaks, 1974c). These results in maize however did not agree with the earlier findings of Dure (1960) who reported that alpha-amylase was synthesised exclusively in the scutellum. More recently, the biochemical mechanisms controlling the action of GA have become better understood (Akazawa & Miyata, 1972). In germinating sorghum however, GA does not appear to induce *hydrolytic enzyme synthesis in the aleurone.* Dalber and Novellie (1968) found that GA did not induce increases in alpha-amylase activity and postulated that *de novo* enzyme synthesis occurred exclusively in the scutellum; a postulation that has subsequently been supported by Palmer (1982). More recently Mundy et al (1983) were unable to detect alpha-amylase production by isolated sorghum aleurones in response to GA. The synthesis of proteolytic enzymes by sorghum aleurones has not been investigated however it seems unlikely that the aleurone of sorghum would produce proteolytic enzymes and not alpha-amylase considering the parallel nature of these two events in other germinating cereals. In support of this hypothesis, Taylor (unpublished data) applied the substrate film technique with gelatin (Okamoto et al, 1980) to samples of germinating

sorghum, and observed that dissolution of gelatin was localised around the scutellar interface.

The roles of other plant hormones during cereal germination are not so well understood. Cytokinins including ethylene have been shown to influence enzyme production in aleurones treated with GA (Clutterbuck & Briggs, *1973), and ethylene can counteract* the negative effects of abscisic acid upon germination (Jacobsen, 1973). Indole-acetic acid (IAA;auxin) generally appears to prevent or check germination of whole grains (Briggs ,1987), although MacLeod and F r (1966) have observed that auxin increases the response o Barley aleurones to GA.

2.1.3.3.2 Carbohydrases.

Alpha-amylase.

Studies of alpha-amylase in sorghum have shown that as with other cereals (Novellie, 1960a; Aimien, 1982), activity is practically *absent in resting grain but increases during germination* (Novellie,1960b; Aimien, 1980). The difference between sorghum and other cereals , for example wheat, barley and rice is the conclusive evidence that the sorghum *aleurone is not involved in de novo* synthesis of alpha-amylase (Mundy *et al*, 1983). It is generally believed that in sorghum alpha-amylase originates exclusively in the scutellum. The mechanisms controlling this process are not understood.

Beta-amylase.

In sorghum, beta-amylase like alpha-amylase appeared to be absent from the resting sorghum grain, but during germination its *activity increased* (Novellie, 1960b). In barley, there is no *de novo* synthesis of beta-amylase (Hardie, 1975), and in wheat, 88 per cent of the beta-amylase is present in an inactive

or latent form (Tronier & Ory, 1978). Meredith (1966) showed that latent forms of beta-amylase existed in other cereals. Adams *et al* (1975) suggested that the protein bodies of sorghum endosperm contained a linked beta-amylase/maltase system. The low diastatic powers of sorghum malts compared with barley malts have been attributed to the relatively low beta-amylase activity of sorghum malts (Novellie, 1968b). From the evidence available in other cereals, it would seem likely that sorghum beta-amylase is also present in the resting sorghum grain in an inactive form.

Limit dextrinase.

Studies of limit dextrinase activity in germinating sorghum by Alsien *et al* (1983) show a rapid increase in activity in the endosperm and it was suggested that limit dextrinase may be present in the endosperm as a zymogen. This observation is in contrast to the situation in barley, where limit dextrinase has been shown to be synthesised *de novo* by the aleurone in response to GA (Hardie, 1975).

Beta-glucanase.

Studies of sorghum endo beta-glucanases have been made by Alsien *et al* (1983). It appeared that the enzymes did not exhibit activity when barley beta-glucan was used as substrate and that overall the levels in germinating sorghum were very low compared with germinating barley.

Pentosanase.

No studies of pentosanase activity have been made in germinating sorghum. However in barley, it has been shown that pentosanases are synthesised *de novo* by the aleurone (Taiz & Honigman, 1976).

2.1.3.3.3 Proteolytic enzymes.

Proteinase.

The term proteinase has been used here to describe the group of enzymes which are capable of hydrolysing the internal peptide bonds of proteins and polypeptides. Most of the work relating to the proteolytic enzymes of germinating cereals has been carried out on barley, however MacLeod (1979) has pointed out that much of this work has been difficult to interpret since natural substrates have seldom been used in enzyme assays. It is generally believed however that the proteolytic enzyme released from the aleurones of certain germinating cereals is a proteinase (Jacobsen & Varner, 1967; Rowsell & Goad, 1964) in view of the conditions of assay employed in their measurement.

Endoprotease activity was measured in germinating sorghum (Alyian et al. 1983) and found to act mainly in the endosperm. Garg and Virupaksha (1970) also demonstrated the increase in activity of an acid protease (proteinase) in germinating sorghum. Adams and Novellie (1974) showed that protease activity was associated with the protein bodies of resting sorghum.

Peptidase.

The term peptidase has been used here to classify all proteolytic enzymes capable of hydrolysing the external peptide bonds of

proteins and polypeptides, in other words, enzymes whose products of reaction are free amino acids. Mikola and coworkers have extensively studied the peptidases of germinating barley (Mikola, 1981) and have shown that carboxypeptidases are present mostly in the endosperm of germinating barley, while aminopeptidases are present mostly in the embryo. Both enzymes' activities increase rapidly after the onset of germination (Mikola & Kolehmainen, 1972). In germinating sorghum, Alsion *et al* (1983) have shown that protease (most likely carboxypeptidase, because of the acidic pH assay conditions) is absent from the endosperm but increases rapidly in the embryo. Little else is known about the peptidase activity of germinating sorghum. It is of interest to note here that Mikola and Kolehmainen (1972) have postulated that carboxypeptidase activity could arise from inactive precursors or zymogens in germinating barley, a mechanism which could explain their unusual pattern of development.

2.1.3.3.4 Other hydrolytic enzymes.

Other hydrolytic enzymes for example, phosphatase and ribonuclease have been shown to be associated with the protein bodies of resting sorghum (Adams & Novellie, 1974). In germinating barley, the equivalent enzymes have been shown to originate in the aleurone under the influence of GA treatment (Chrispeels & Varner, 1967; MacLeod *et al*, 1964).

2.1.3.3.5 Endosperm modification.

The pattern of modification in germinating sorghum endosperm appears to be markedly different to that of germinating barley. Modification in germinating barley appears to begin with cell wall degradation, specifically the action of a carboxypeptidase, termed solubilase (Forrest & Wainwright, 1977; Bamforth *et al*, 1979) in conjunction with the various beta-glucanases. The degradation of cell walls in germinating

barley is believed to be a rate limiting factor in the mobilisation of starch and protein (Palmer, 1975; Forrest & Wainwright, 1977). Although this phenomenon has not been investigated in germinating sorghum, cell wall degradation does not appear to be a limiting factor in endosperm mobilisation as the sorghum endosperm cell walls remained visibly unchanged even after the starch granules and protein bodies have been extensively modified (Glennie et al, 1983).

The major site of proteolysis in sorghum, is within and in the vicinity of the protein bodies of the endosperm, as it has been shown that kafirin and to a lesser extent glutelin were degraded most during malting (Taylor, 1983).

Ultrastructural studies of the degradation of starch granules by amylases have been made in barley (Briggs, 1987) and in sorghum (Glennie et al, 1983). These studies show extensive pitting of the starch granules during modification. Although beta-amylase is present in the resting endosperm of barley grain it does not appear to act upon undamaged starch granules (Sandstedt, 1958) or even upon partly degraded starch granules (Gunn, 1974). Therefore pitting of starch granules must be due to the action of alpha-amylase.

2.1.3.3.b Seedling metabolism.

Seedling metabolism is an aspect of malting physiology which represents a loss to the maltster (Briggs et al, 1981). In barley malting, this loss is greater than with sorghum since the rootlets are removed from the malted kernel prior to brewing. However seedling growth is greater in sorghum malting because of the high germinaton temperatures employed. One aspect of seedling metabolism which is relevant to malting physiology is the transport of the soluble degradation products of the endosperm to the growing seedling via the scutellum. The predominant soluble components of carbohydrate dissolution in the

endosperm are maltose and glucose. Both of these sugars are directly available for metabolism by the embryo (Bewley & Black, 1978). Similarly, the soluble products of endosperm proteolysis are transported across the scutellum to the growing seedling (Walker-Smith & Payne, 1984). These latter authors have also demonstrated the existence of a specific symport for the transport of dipeptides into the scutellum of germinating barley (Walker-Smith & Payne, 1984), that is separate to the mechanism for the uptake of amino acids by the scutellum (Higgins & Payne, 1977; Sopanen, 1976; Mikola, 1981). Thus free amino acids can also be generated in the scutellum and then utilised by the growing seedling.

2.2 Malt proteolytic enzymes and the sorghum beer brewing process.

2.2.1 General.

The preparation of sorghum wort has been described in detail by Novellie and de Schaepdrijver (1984). The proportions of malt and unmalted cereal or adjunct used in sorghum beer brewing are 30:70, whereas barley malt can represent from 50 to 100 per cent of the grist (adjunct and/or malt) in barley wort preparation (Canales, 1979). Also hops are not used in sorghum beer brewing. The context in which sorghum wort preparation is discussed here relates mainly to the opportunity it provides for increasing the FAN content of wort.

Very few direct comparisons can be made between sorghum and barley beer brewing. The event which is most similar to both processes however is probably mashing. Although even at this stage, the prime objective of barley beer brewing is to convert gelatinised starch into fermentable extract, whereas sorghum beer actually requires gelatinised starch in the final product. Despite these differences, a number of biochemical events are

common to both processes and therefore literature relating to barley beer brewing will be cited in this context where applicable. Briefly, sorghum wort preparation comprises four stages termed: souring, cooking, mashing and straining.

2.2.2 Souring.

Souring is a fermentation process whereby a malt slurry is inoculated with lactobacilli. It should be pointed out here that milling of sorghum malt is not the business of the brewer, as it is in the barley brewing industry. The sorghum beer brewer receives his malt pre-milled from the maltster. The malt used in souring represents 30 per cent approx. of the total malt used per brew, and ten per cent approx. of the total grist. This quantity of malt is blended with water into a ten per cent slurry. Fermentation proceeds for 20 to 48 hours at a temperature of 30°C approx. During souring, the lactic acid which gives sorghum beer its characteristic sour flavour is produced. Proteolysis during souring and the consequent generation of FAN has not been investigated, but the temperature and acidic pH conditions are favourable to proteolytic enzymes of sorghum malt (Barg & Virupaksha, 1970). It is known that the depletion of FAN from the malt slurry due to the growth of lactobacilli during souring is negligible (Watson, 1983). The sour is normally combined with the adjunct material, usually maize grits or sorghum grain and the mixture is then heated and cooked.

2.2.3 Cooking.

The main purpose of cooking is to gelatinise the starch so as to improve its susceptibility to amylosis during mashing (Briggs et al, 1981). However, a certain degree of liquifaction of starch must also take place in order to prevent the cooked adjunct from "setting". In barley beer brewing, this is achieved

by addition of a small amount of malt and/or industrial enzyme preparation to the cooker, coupled with a cooking programme with temperature stands which afford opportunity for limited liquifaction (Bradlee, 1977). In the sorghum beer brewing process the sour malt can contribute the alpha-amylase activity necessary for liquifaction during cooking in the sorghum beer brewing process. More recently, however the use of industrial preparations of alpha-amylase has become common practice (Novellie & de Schaepdrijver, 1986).

Because of the high temperatures at which cooking proceeds, there is little opportunity for generating FAN by proteolysis. Certainly no carry over of proteolytic activity to the mashing process can take place because the final temperature stand of the cooking process is usually at or just below boiling point. Unmalted cereal adjuncts have an inherently low FAN content (Jones & Pierce, 1964). However, because the proportion of adjunct in the grist is high in sorghum beer brewing, the adjunct can make a significant contribution towards the final FAN content of the wort (Taylor & Boyd, 1986). In barley beer brewing, adjuncts have been considered as diluents of the soluble protein and FAN content of barley wort. If adjuncts are to be used in high proportions in the grist formulation, they must contribute substantial quantities of soluble protein and FAN, in order to attain the proper amount of alpha-amino nitrogen required for a complete fermentation (Canales, 1979). Meilgaard (1976) has further shown that the spectrum of free amino acids in wort is also influenced by the type and proportion of adjuncts used in the grist, and has speculated that this situation must influence fermentation characteristics.

2.2.4 Mashing.

After cooking, the adjunct and sour is combined with a further quantity of sorghum malt, termed conversion malt, in the process referred to as mashing (Novellie & de Schaapdrijver, 1986).

Conditions of mashing are variable, depending on brewery equipment and raw materials, but generally the mash is maintained at 68°C for one to two hours at about pH 4. The main purpose of this process is to bring about the partial conversion of gelatinised starch, by the amylolytic enzymes of sorghum malt, to an acceptable level of fermentable sugars. It is largely because of the small proportion of conversion malt used during mashing (20 per cent of grist approx.), together with the low pH conditions, that the conversion of gelatinised starch does not proceed to completion (Glennie & Wight, 1986). This incomplete conversion ensures the presence of gelatinised starch in sorghum beer, thereby giving the beer its opaque character.

Apart from conversion of starch to dextrins and sugars, there is also a certain amount of FAN produced during mashing due to the action of proteolytic enzymes from the conversion malt. However the FAN produced is small when compared with that of the preformed FAN of the sorghum malt (Taylor & Boyd, 1986). In barley beer brewing, a specific temperature stand termed protein rest can be incorporated into the mashing regime to facilitate the solubilisation of protein and generation of FAN. The protein rest is generally held at 45 to 50°C for 10 to 30 minutes, the lower conditions of temperature being favourable to proteolytic activity (Briggs et al, 1981). Numerous papers recount the contribution of improved proteolytic activity during the protein rest towards increased FAN levels in wort (Prentice, 1976). Recently however it has been reported (Lewis & Mahnon, 1984; Muston et al,1986) that the amount of proteolysis which takes place during mashing is practically non-existent in barley wort production and that the protein rest and its influence upon FAN production are due to improved solubilisation of protein at lower temperatures.

2.2.5 Straining.

The final process in the procedure of sorghum wort preparation is straining. The purpose of straining is to remove insoluble, particulate material from the sweet wort. This is usually accomplished by centrifugation although in the past wort was strained through grass reed nets (Novellie, 1966). Also removed with the strainings are insoluble protein and gelatinised starch (Novellie & de Schaepdrijver, 1986) which improve the nutritional value of strainings, and render them readily saleable as animal feed.

2.2.6 Fermentation.

After straining, wort is pitched or inoculated with yeast and transferred to fermentation vessels. During fermentation, the growth of yeast is essential for the wort to be successfully fermented. The free amino acids and to a lesser extent small peptides in wort provide the essential nitrogen source for yeast growth. Because the proteolytic enzymes of sorghum malt, and the malting process, play such an active role in achieving wort FAN levels, it follows that a greater understanding of their mode of action and development during malting would lead to greater control over wort FAN levels.

2.3 The measurement of proteolytic activity in germinating cereals.

The measurement of proteolytic activity in germinating cereals has been accomplished by a wide variety of methods. This variation in methodology may be ascribed for the most part to differences in two broad but fundamental concepts of enzyme assay design. The first of these concepts concerns the choice of substrate, whilst the second relates to the means by which the enzyme reaction is followed.

One other aspect of proteolytic activity measurement which also leads to a significant source of variation is the method of isolation. Kringstad and Kilhovd (1955) demonstrated that proteolytic enzymes of barley malt could be categorised according to their solubility in different extractants. Thus three fractions of proteolytic activity emerged: water-soluble; salt-soluble and insoluble. Ineveart (unpublished results) demonstrated that similar fractions existed with sorghum malt and that approximately 40 per cent of the total proteolytic activity was apparently insoluble.

Traditionally, proteolytic activity in germinating cereals has been assayed using commercially available proteins or peptides as substrates. Thus bovine serum albumin (Kretovich *et al*, 1980), casein (Markovic *et al*, 1984), azocasein (Kruger, 1975), haemoglobin (Sandgren & Klang, 1950; Kringstad & Ohlsen, 1957; Burger, 1973) and gelatin (Enari *et al*, 1963) have all been used to assay proteinase activity. Similarly, dipeptides (Bopanen, 1976), carbobenzoxy derivatised dipeptides (Baxter, 1978; Breddam *et al*,1983), L-aminoacyl-beta-naphthylamides (Kolehmainen & Mikola, 1971), alpha-N-benzoyl-L-arginine ethyl ester and alpha-N-benzoyl-DL-arginine-p-nitroanilide (Burger *et al*, 1966) have been used to assay exopeptidase activity.

Recently however, the use of these substrates has been criticised since the proteolytic enzymes of germinating cereals do not encounter these proteins in nature. Baxter (1976) used hordein, the principal storage protein of barley, as substrate for barley malt proteolytic activity determinations. It was found that different activities were obtained using hordein as substrate compared with haemoglobin. As a consequence of this, Baxter recommended that natural substrates should be used to assay proteolytic activity in germinating cereals. The concept of employing natural protein substrates has also been emphasised by Sharpe (1982). Because of this, it was decided to assay sorghum malt proteolytic activity with its natural substrate.

The major storage protein of sorghum grain and the protein which is most extensively degraded during germination is sorghum prolamin or kafirin (Taylor, 1983). The amino acid composition of total kafirin comprises large amounts of glutamic acid (23 moles per cent), alanine (12 moles per cent), leucine (18 moles per cent) and proline (11 moles per cent) together with small quantities of lysine (<1 mole per cent)(Paulis & Wall, 1979). The high proportion of amino acids with hydrophobic side chains in total kafirin contribute to the molecule's hydrophobicity (Bigelow, 1967) and its consequent insolubility in aqueous solutions.

The measurement of the products of proteolytic activity in germinating cereals has been measured in terms of the release of trichloroacetic acid (TCA)-soluble nitrogen (Anson, 1938; Greenberg, 1955). Proteolytic enzyme incubation mixtures are treated with TCA to terminate further enzyme action and to precipitate unreacted protein substrate. Any nitrogenous material still in solution is identified as a product of proteolysis.

Both the concentration and the nature of the precipitant can influence the precipitation of protein (Davies et al, 1956). The distinction between precipitable nitrogen and soluble nitrogen can therefore only approximate the extent of proteolysis, as the choice of precipitant and its concentration is a compromise between ensuring the precipitation of unreacted protein substrate on the one hand, and preventing the precipitation of large molecular weight peptide products of proteolysis on the other. Despite this compromise, unlike most methods of identifying proteolysis products, the use of protein precipitants like TCA, does enable the degradation of substrate to be quantified. This is not possible for example with assays which utilise chromophore-linked substrates such as azocasein and the derivatised peptide substrates, or with the assay which utilises gelatin as substrate (Enari et al, 1963).

Quantification of TCA-soluble nitrogen can in addition be quantified by a variety of means. In the Anson method (1938) TCA-soluble nitrogen is estimated by UV absorption at 263nm, a measure of tyrosine and tryptophan residues. Use of the Kjeldahl digestion enables the TCA-soluble fraction to be quantified in terms of nitrogen (Bradstreet, 1965). Also acid hydrolysis of the TCA-soluble fraction enables it to be expressed in terms of FAN equivalents (Baxter, 1976). This latter procedure can lead to further variation since FAN can be measured by at least two methods: reaction with ninhydrin and reaction with trinitrobenzenesulphonic acid (TNBS)(Lie, 1973). Both of these methods do not detect proline as they measure alpha-amino nitrogen only. The two methods are however distinguished from each other in that ninhydrin reacts with peptide alpha-amino nitrogen poorly compared with TNBS (Lie, 1973). Another procedure used to follow the progress of proteolytic enzyme reactions in cereal seeds is to monitor the decrease in absorbance at 280nm, a measure of the decline in protein substrate (Harvey & Oaks, 1974a), however this procedure is not specific for proteolytic activity (Pomeranz & Meloan, 1971). In conclusion, certainly one of the reasons why it has been difficult to interpret levels of proteolytic activity in germinating cereals in an overall physiological context, has been due to constraints imposed by the methods used to assay proteolytic activity (MacLeod, 1979).

3. MATERIALS AND METHODS.

3.1. Preparation of sorghum malt used for development of the assay procedure.

Sorghum grain, cultivar: Barnard Red (2kg) was surface-sterilised as described by Morrall *et al* (1986), in nylon bags by immersion for 5 min in a 0,2% (m/v) solution of Wescodyne (a commercial disinfectant and detergent containing 1,6% available iodine). The grain was then washed and drained three times in running tap water for 3 min approx. This sterilisation and washing procedure was then repeated. The grain was then steeped in running tap water for 16h at 23 to 25°C with a cycle of 3h wet followed by a 1h air rest. The steeped grain was germinated according to a procedure of Daiber (unpublished results) in nylon bags at 28°C in a water-jacketed incubator (Forma Scientific, Marietta, Ohio, USA.) in which an atmosphere of 98% relative humidity was maintained throughout. During germination the grain was watered by periodically steeping it for short intervals in chlorinated water. Chlorinated water was used to inhibit mould growth. For each day of germination, the grain was immersed twice in a 0,2% (m/v) active Cl_2 solution of commercial bleach for alternate periods of 10 min and 5 min, after 8h and each full day of germination respectively. The germinating grain or green malt was harvested after 4 days of germination and then dried for 24h at 58°C in a forced draught oven. After drying, the roots and shoots were removed from the kernels. Sorghum malt kernels were milled in a "beater-type", water-cooled coffee mill (Janke & Kunkel, Staufen, W. Germany) for two 30 second periods, as required. The resultant flour is herein after referred to as malt kernels flour. The moisture content of the malt kernels flour was determined.

3.1.1. Moisture determinations.

Moisture determinations were performed according to the procedure of the Institute of Brewing (1983).

3.2. Substrate preparation.

3.2.1. Preparation of total kafirin.

Sorghum grain, cultivar: Barnard Red (1,5kg) was passed through a rice-pearling machine until 20% by weight had been removed. Sorghum was pearled prior to extraction of total kafirin; this was done to remove the pericarp. The pericarp of sorghum grain has a testa which is rich in polyphenols (Glennie et al, 1982). It was believed that these polyphenols, if present as contaminants in the total kafirin could interfere with the enzyme assay. The inhibitory influence of polyphenols has been observed in the study of amylolytic enzymes in sorghum malt (Dalber, 1975). Baxter (1978) has also stated that polyphenolic contaminants of hordein preparations could inhibit the proteolytic enzymes of barley, malt when hordein was used as a substrate.

Pearled sorghum was ground in a DLFU, disc mill (Buhler-Miag, Braunschweig, W. Germany) at a disc gap of 0,2mm. The resultant flour (1kg) was extracted, by constant stirring, with 60% (v/v) tert-butanol plus 0,85% (m/v) dithiothreitol (DTT) (SI) for 1h at room temperature. The extraction mixture was then centrifuged at 7 500g for 10 min. The supernatant was collected and freeze-dried. The freeze-dried product was suspended in water and exhaustively dialysed against water at 4°C. The dialysed material was freeze-dried and then milled in the "beater-type", water-cooled coffee mill for two 30 second periods. This procedure extracts both kafirin and crosslinked kafirin (Taylor et al, 1984c). When these two fractions were isolated separately, they appeared to be virtually identical (Paulis & Wall, 1979; Taylor et al, 1984b). Because of this the term total kafirin has been used to describe this protein fraction. The protein content of the total kafirin was determined.

3.2.2. Nitrogen and protein determinations.

Aliquots (25mg approx.) of protein preparations were digested with conc. H_2SO_4 (0,5ml) containing 20% (m/v) K_2SO_4 and 0,1% (m/v) SeO_2 for 2,5h. Twelve drops (approx.) of H_2O_2 were added to each digestion flask after the first half hour of digestion to promote oxidation. After digestion, nitrogen was determined by an automated Berthelot phenol-hypochlorite method (Thomas et al, 1967). Protein content of the samples was determined from N x 6,25.

The ammonium sulphate produced during digestion was reacted under alkaline conditions with phenol and hypochlorite to give a blue colour: the Berthelot reaction (1859). This blue colour, indophenol, was quantified spectrophotometrically at 630nm. The chemistry of the Berthelot reaction is:

1) $2NH_3 + 2NaClO_4 \rightarrow 2NH_4Cl + 2NaOH$

2) 2 Phenol + $2NH_4Cl + 2NaOH \rightarrow$ 2 p-aminophenol + 2NaOH

3) 2 p-Aminophenol + 2 phenol + $O_2 \rightarrow$ 2 indophenol + $2H_2O$

3.3. Investigation into the extraction of a purified preparation of total kafirin.

3.3.1. Preparation of total kafirin for purification.

Sorghum grain, cultivar: Barnard Red, was passed through a rice-pearling machine until 25% by weight had been removed. Unpigmented, germ-free, starchy endosperm material was then hand-selected. The starchy endosperms were ground in the DLFU, disc mill at a disc gap of 0,2mm.

Total kafirin was prepared according to the method of Taylor et al, (1984c). Flour (80g) was extracted, by constant stirring, for 1h periods with three successive 400ml aliquots of distilled water at 4°C, to remove the albumins, globulins and low molecular weight nitrogen. After each extraction, a clear supernatant was obtained by centrifugation at 16 000g for 10 min. This supernatant was then decanted and discarded. After the water extraction, the residue was extracted, by constant stirring, at room temperature for two 1h periods and then overnight with three successive 400ml aliquots of 60% (v/v) tert-butanol plus 0,05% (w/v) DTT. Clear supernatants were obtained after each extraction by centrifugation at 16 000g for 10 min. The supernatants were combined and freeze-dried directly. The nitrogen content of the total kafirin preparation was determined (see 3.2.2.).

3.3.2. Purification procedure.

Total kafirin (1.5g) was suspended in distilled water (10ml) or 0,02M Na lactate/lactic acid buffer, pH 3,1 (10ml). Each suspension was then dialysed using 18/32" Visking tubing against *either* distilled water *or* 0,02M Na lactate/lactic acid buffer, pH 3,1 according to the following schedule. Initially suspensions were dialysed against a volume of extractant (20ml) for 16h at 4°C. The nitrogen content of the raffinate was determined. The suspensions were then further dialysed against 2 successive 250ml volumes of extractant for alternate periods of 8h and 16h at 4°C. Finally, both total kafirin suspensions were dialysed against 2 successive 250ml volumes of distilled water for alternate periods of 8h and 16h at 4°C. The suspensions were then rinsed from the dialysis tubing with water and centrifuged at 16 000g for 10 min. The supernatants and the residues were then separated and freeze-dried directly. The nitrogen contents of the lactate- and water-extracted residues and supernatants were determined.

The residues and supernatants, together with the tota. kafirin preparation were then analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid analysis .

3.3.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

SDS-PAGE was carried out in 12,4% acrylamide gels according to the procedure of Shewry *et al* (1977). Protein samples are denatured with SDS prior to electrophoresis. This confers upon the protein a constant charge which is independent of molecular composition. Proteins are then separated by electrophoresis according to their molecular size. An estimate of molecular weight is obtained, by reference to a series of internal protein standards. These protein standards were obtained from a Pharmacia electrophoresis calibration kit for low molecular weight proteins. The proteins together with their molecular weights (M_r) were: phosphorylase b, M_r 94 000; bovine serum albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; soybean trypsin inhibitor, M_r 20 100 and alpha-lactalbumin, M_r 14 400.

3.3.4. Amino acid analysis.

Amino acid analysis was performed using an LKB Alpha amino acid analyser. Duplicate samples were hydrolysed in 6M HCl for 22h, under vacuum, at 110°C (Satterlee, 1982). Separation of amino acids from the hydrolysates was made with a cation exchange resin. The amino acids were eluted from the resin with buffers of increasing pH. The amino acids were detected spectrophotometrically, after reaction with ninhydrin (Spackman *et al*, 1958).

3.4 Preparation of purified total kafirin for use as substrate.

Total kafirin was prep[illegible] from sorghum grain, which had been pearled until 20% by wei[illegible] had been removed, as described in 3.3.1. Total kafirin (80g protein) was suspended in 0,02M Na lactate/lactic acid buffer, pH 3,1 (400ml) and exhaustively dialysed at 4°C against 0,02M Na lactate/lactic acid buffer, pH 3,1. The dialysed suspe[illegible] of total kafirin was then recovered and centrifuged at [illegible]0g for 10 min. The supernatant was decanted and the residue was further extracted at 4°C for two 1h periods and then overnight with three successive 400ml volumes of 0,02M Na lactate/lactic acid buffer, pH 3,1. Clear supernatants were obtained after each extraction by centrifugation at 7 500g for 10 min. The residue was then resuspended in distilled water and exhaustively dialysed at 4°C against distilled water. The resultant dialysed suspension was freeze-dried directly and constituted the purified total kafirin preparation. The protein content of this preparation was determined as described in 3.2.2.

3.5. Development of procedure for extraction of proteolytic activity from sorghum malt.

3.5.1. General procedure for preparation of crude enzyme extracts of proteolytic activity during development of extraction conditions.

Malt kernels' flour (1g) was extracted, by constant stirring with magnetic stirrers and teflon-coated stirring bars in 50ml screw-top centrifuge tubes, for 1h at 38°C with extractant (15ml). The malt suspension was then centrifuged at 16 000g for 10 min. The liquid fraction was then decanted and centrifuged again at 16 000g for 10 min to obtain a clear supernatant.

The resultant supernatant was dialysed overnight at 4°C against water (3l). The dialysed solution was made up to volume (25ml) with water. The resultant extract was then assayed for proteinase and carboxypeptidase activity.

3.5.2. Proteinase and carboxypeptidase assay.

3.5.2.1. Incubations.

Enzyme extract (1ml), total kafirin (2.5% [illegible] in) and 0,3M citric acid/0,6M K_2HPO_4 buffer, pH 4,[illegible] were incubated in duplicate for 0 and 5h at 50°C in a shaking water bath. Teflon-coated magnetic stirring bars were placed into the incubation mixtures to ensure even suspension of [illegible] kafirin throughout incubation. Like all prolamin proteins, total kafirin by definition is only soluble in solutions of aqueous alcohol. Because enzymes are normally denatured in solutions of alcohol, the assay has been conducted in an environment where the total kafirin is not in solution. It was therefore necessary to ensure that total kafirin was kept in suspension during enzyme reactions. Incubation mixture tubes were sealed with parafilm to prevent evaporation losses during incubation. Reactions were stopped with 15% (w/v) TCA (1ml). Incubation mixtures were then immediately centrifuged at 10 000g for 10 min. The supernatants were then recovered and centrifuged again at 10 000g for 10 min. The supernatants were then collected and stored at 4°C until analysed for TCA-soluble nitrogen and FAN.

3.5.2.2. TCA-soluble nitrogen assay.

An aliquot (2ml) of clear supernatant was digested and assayed for nitrogen according to the procedure described in 3.2.2.

3.3.2.3. FAN assay.

An aliquot (0,4ml) of clear supernatant was assayed for FAN according to the EBC-ninhydrin procedure of Lie (1973). Ninhydrin causes oxidative decarboxylation of alpha-amino acids producing carbon dioxide, ammonia and an aldehyde. The reduced ninhydrin then reacts with the unreduced ninhydrin and the liberated ammonia to form a blue complex. The absorbance of this blue complex was assessed spectrophotometrically at 570nm.

3.3.2.4. Calculations of proteinase and carboxypeptidase activities.

The TCA-soluble nitrogen and FAN contents of duplicate supernatants were exmined. Proteinase activity was calculated from the difference between the nitrogen content of the 5 and 0h supernatants and expressed in terms of mgN/5h/g dry malt. Carboxypeptidase activity was calculated from the difference between the FAN content of 5 and 0h supernatants and expressed in terms of mgFAN/5h/g dry malt.

3.3.3. Extractability of sorghum malt proteolytic activity with 1,25M NaCl as extractant.

Malt kernels' flour (2g) was successively extracted , by constant stirring, for 1h periods at 4°C, with three aliquots (20ml) of 1,25M NaCl. At the end of each extraction period, clear supernatants were obtained by centrifugation as described in 3.3.1 The residual material was finally resuspended in 1,25M NaCl (20ml) and dialysed.

The three soluble extracts and the residual suspension were each dialysed overnight at 4°C against 0,125M NaCl (4l). The dialysed solutions were each made up to volume (25ml) with 0,125M

NaCl. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.4. Influence of extractant volume to malt mass ratio upon the extraction of sorghum malt proteolytic activity.

Malt kernels' flour was extracted as described in 3.5.1 with 5, 10, 15, 20 and 25ml volumes of water at 4°C. The resultant supernatants were each dialysed overnight at 4°C against a volume of water equal to 200 times the original volume of extractant. Enzyme extracts were prepared from the dialysed solutions as described in 3.5.1 and assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.5. Influence of temperature upon extraction of sorghum malt proteolytic activity.

Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.1 at temperatures of 4, 21, 30, 40 and 50°C using water as extractant. The resultant extracts were assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.6. Influence of extractant pH upon extraction of sorghum malt proteolytic activity.

Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.1 with 0.1M citric acid/0.2M Na_2HPO_4 buffers of pH 3.8, 4.6, 5.0, 6.0 and 7.0 (I=0.5M) as extractants. The ionic strength of the buffers were adjusted to 0.5M with NaCl. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.7. Extractability of sorghum malt proteolytic activity with a pH 7,0 buffer.

Malt kernels' flour (1g) was successively extracted for 1h periods at 30°C, by constant stirring, with 3 aliquots (15ml) of 0,1M citric acid/0,2M K_2HPO_4 buffer, pH 7,0 (I=0,5M). At the end of each extraction period, clear supernatants were obtained by centrifugation as described in 3.5.1. The residual material was finally resuspended in 15ml of the extractant.

Crude enzyme extracts were prepared from the resultant supernatants and residual suspension as described in 3.5.1. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.8. Influence of neutral sodium and lithium salts upon extraction of sorghum malt proteolytic activity.

Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.1 with 0,5M solutions of NaCl, NaBr, NaI, $NaClO_4$, NaSCN, LiCl, LiBr, LiI and $LiClO_4$ as extractants. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.9. Influence of different ionic strengths of lithium iodide upon extraction of sorghum malt proteolytic activity.

Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.1 with 0, 0,2, 0,4, 0,6, 0,8 and 1,0M solutions of LiI as extractants. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.10. Extractability of sorghum malt proteolytic activity with 0,6M LiI.

Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.7 with 0,6M LiI as extractant. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2)

3.5.11. Extractability of sorghum malt proteolytic activity with pH 7,0 buffer adjusted to an ionic strength of 0,6M with LiI.

Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.7. with 0,1% Citric acid/0,2M K_2HPO_4 buffer, pH 7,0/0.173M LiI (I=0,6M) as extractant. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.12. *Influence of dithiothreitol upon extraction of sorghum malt* proteolytic activity.

Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.1 with solutions of 0,6M LiI plus 0, 0,67, 1,67, 3,33, 5,00 and 6,67mM DTT as extractants. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2).

3.5.13. Comparison of the extractability of sorghum malt proteolytic activity with water,and 0,6M LiI plus 3,33mM DTT.

In this experiment two extractants were investigated: water and 0,6M LiI plus 3,33mM DTT. Extractions were performed in triplicate for both extractants. Crude enzyme extracts were prepared from malt kernels' flour as described in 3.5.7 with

either water or 0,6M LiI plus 3,33mM DTT as extractants. The resultant extracts were then assayed for proteinase and carboxypeptidase activity (3.5.2.).

3.5.14. Influence of extractant upon recovery of proteolytic activity from sorghum malts germinated for different times.

The malt samples used in this experiment were selected from malts prepared by Morrall et al (1986), from sorghum, cultivar: Barnard Red. Duplicate samples of malts germinated for 0,1,2,3,4,5 and 6 days at 28°C under medium conditions of moisture were used (see 3.10.1). The malt/resting grain samples were extracted with three different extractants: water, 0,6M LiI plus 3,33mM DTT, and 0,05M Na acetate/acetic acid buffer plus 0.2% (m/v) cysteine.HCl.

Crude enzyme extracts were prepared from malt/resting grain flour as described in 3.5.1 with each of the above extractants. In this experiment, the assay of proteinase and carboxypeptidase activity was conducted with total kafirin (5mg protein) as described in 3.5.2.1. In addition, total kafirin (5mg protein), 0.3M citric acid/0.6MK$_2$HPO$_4$ buffer, pH 4,58 (1ml) and water (1ml) was also incubated in duplicate for 0 and 5h at 50°C to determine the amount of nitrogenous material released from the substrate in the absence of enzyme extract.

3.6. Development of incubation conditions for assaying sorghum malt proteolytic activity.

3.6.1. Influence of pH upon sorghum malt proteolytic activity .

Crude enzyme extract was prepared from malt kernels' flour as described in 3.5.1 with 0,6M LiI plus 3,33mM DTT as extractant. Enzyme extract was incubated as described in 3.5.2.1 over a pH

range of 3,00 to 7,00. The buffers were all prepared from solutions of 0,3M citric acid.H_2O and 0,6M K_2HPO_4. Curves were statistically fitted to the data using polynomial regression analysis.

3.6.2. Influence of temperature and reaction time upon sorghum malt proteolytic activity.

Crude enzyme extract was prepared from malt kernels' flour as described in 3.6.1. Enzyme extract was incubated as described in 3.5.2.1 for 0,1,2,3,4,5,6 and 7 h at temperatures of 30, 40, 50, 55 and 60°C. In this experiment the TCA-soluble nitrogen and FAN contents of duplicate incubations were meaned and expressed in terms of mgN/g dry malt and mgFAN/g dry malt respectively. Progress curves of the release of TCA-soluble nitrogen at different temperatures were statistically fitted to the

3.6.3. Influence of temperature upon the stability of sorghum malt proteolytic activity.

Crude enzyme extract was prepared from malt kernels' flour as described in 3.6.1. The temperature stability of proteolytic activity was determined by pre-incubating enzyme extracts in duplicate for 1h at temperatures ranging from 25 to 60°C. The enzyme extracts were then assayed for proteolytic activity as described in 3.5.1.4.

3.6.4. Influence of substrate concentration upon sorghum malt proteolytic activity.

Crude enzyme extract was prepared from malt kernels' flour as described in 3.6.1. Enzyme extract was incubated as described in 3.5.2.1 with 0, 2,45, 4,9, 24,5, 49mg total kafirin (protein) for 0, 15, 30, 45, 60, 90 and 120 min. The nitrogen and FAN contents

of duplicate incubations were meaned and expressed in terms of μgN/g dry malt and μgFAN/g dry malt as described in 3.6.2. Progress curves of the release of TCA-soluble nitrogen and FAN at different substrate concentrations were statistically fitted to the data.

3.6.5. Influence of enzyme concentration upon sorghum malt proteolytic activity.

Crude enzyme extract was prepared from malt kernels' flour as described in 3.6.1. A range of enzyme concentrations was obtained by diluting the enzyme extract with water. Diluted enzyme extracts were assayed for proteolytic activity as described in 3.5.14. Proteinase and carboxypeptidase activity were plotted against enzyme concentration. Curves were fitted to the data by linear regression analysis.

3.7. Investigation into whether the release of TCA-soluble nitrogen from total kafirin was due to an enzymic or non-enzymic phenomenon.

3.7.1. Influence of heat-treatment upon release of TCA-soluble nitrogen from total kafirin.

Total kafirin was suspended in 0,3M citric acid/0,4M K_2HPO_4 buffer, pH 4,58, and pre-incubated in boiling water for 15 min. The rate of release of TCA-soluble nitrogenous material from heat-treated total kafirin was compared with that from total kafirin which had not been heat-treated. Ten test-tubes containing total kafirin (5,8mg protein), 0,3M citric acid/0,4M K_2HPO_4 buffer, pH 4,58 (1ml) was pre-incubated for 15 min in boiling water. Water (1ml) was then added to each of these tubes and they were incubated, in quintriplicate, for 0h and 5h at 50°C. Reactions were stopped with 15% (m/v) TCA (1ml). Incubation mixtures were then immediately centrifuged and the

supernatants stored as described in 3.5.2.1. This procedure was repeated without pre-incubation in boiling water for the control experiment.

The nitrogen and FAN contents of supernatants were expressed in terms of µgN/5,0mg total kafirin and µgFAN/5,0 total kafirin. Statistical analysis of the data was made using 2-way analysis of variance.

3.7.2. Influence of incubation time upon release of TCA-soluble nitrogen from total kafirin.

A progress curve of the release of TCA-soluble nitrogen from total kafirin incubated in the absence of proteolytic enzymes from sorghum malt was determined. Total kafirin (4,9mg protein), 0,3M citric acid/0,4M K_2HPO_4 buffer, pH 4,38 (1ml) and water (1ml) were incubated in duplicate for 0, 1, 2, 3, 4, 5, 6 and 7h at 58°C. Reactions were stopped with 15% (m/v) TCA (1ml). Incubations mixtures were then immediately centrifuged and the supernatants stored as described in 3.5.2.1

The nitrogen contents of duplicate supernatants were meaned and expressed in terms of µgN/4,9mg total kafirin. Nitrogen levels were plotted against reaction time. A progress curve was statistically fitted to the data by polynomial regression analysis.

3.7.3. Influence of pH upon release of TCA-soluble nitrogen from total kafirin.

The rate of release of TCA-soluble nitrogen from total kafirin incubated in the absence of sorghum malt proteolytic enzymes was determined over a pH range of 3 to 10. Total kafirin (5,0mg protein), buffer (1ml) and water (1ml) were incubated in duplicate for 0 and 5h at 58°C. Reactions were stopped with

15% (m/v) TCA (1ml). Incubation mixtures were then immediately centrifuged and the supernatants stored as described in 3.5.2.1. The citric acid/phosphate buffers were prepared by mixing solutions of 0,1M citric acid.H_2O and 0,4M K_2HPO_4 to give the required pH. The lactic acid/NaOH buffers were prepared by mixing solutions of 0,1M lactic acid and 0,1M NaOH to give the required pH. The other buffers were prepared according to the procedures described by McKenzie (1969).

The nitrogen contents of the supernatants were expressed in terms of mgN/5,0mg total kafirin. Statistical analysis of the data was made using 2-way analysis of variance.

3.8. Influence of reaction time upon sorghum malt proteolytic activity assayed at 30 and 50°C with purified total kafirin as substrate.

Crude enzyme extract was prepared, in duplicate, from malt kernels' flour as described in 3.6.1. Enzyme extract was incubated as described in 3.5.2.1 for 0, 1, 2, 3, 4, 5 and 6h at 30 and 50°C with purified total kafirin (5mg protein) as substrate. Progress curves of the release of TCA-soluble nitrogen and FAN at different were determined as described in 3.6.2.

3.9. Extractability of proteolytic activity from sorghum malts prepared from different cultivars.

3.9.1. Preparation of malts.

The sorghum cultivars used in this experiment were produced in the annual cultivar trials of the Department of Agriculture, at the farm Nooitgedacht/Ermelo, North-eastern Transvaal. Aliquots of sorghum cultivars (50g) were malted according to the procedure

described in 3.1, except that germination proceeded for six days. Sorghum malt (*kernels plus roots and shoots*) was ground in the beater-type mill. The moisture contents of the samples were determined (3.1.1.).

3.9.2. Proteolytic activity determinations.

Crude enzyme extract was prepared, in duplicate, from malt flour (1g) as described in 3.5.7 with 0,6M LiCl plus 3,33mM DTT as extractant. The enzyme extracts were then assayed for proteinase and carboxypeptidase activity as described in 3.5.14. Statistical analysis of the resultant data was made with 2-way analysis of variance.

3.10. *Influence of malting conditions upon* the development of sorghum malt proteolytic activity.

3.10.1. Preparation of malts.

The malts used in this experiment were prepared by Morrall *et al* (1986). Sorghum grain, cultivar: Barnard Red was surface-sterilised and steeped according to the procedure described in 3.1. After steeping, the imbibed grain was germinated in the water-jacketed incubator in an atmosphere of near water saturation with a continuous flow of moist air. Samples were germinated for 6 days at three temperatures: 24°, 28° and 32°C. At 8h intervals, a weighed quantity of water was sprayed onto the germinating grain using an atomiser spray whilst at the same time the malt was turned.

Three watering treatments were used:

Low- just enough water was applied to maintain the germinating grain at a constant fresh weight.

Medium- at the end of each 6h period, the malt felt damp but not wet, ie. there was no surface film of water on the malt.

High- sufficient water was applied so that none drained off the malt but at the end of each 6h period, the grain just felt wet.

At 12h intervals, before spraying, three to five samples (50-100g) were taken from the germinating grain bed, and dried in a forced-draught oven for 24h at 50°C. Of these samples two were selected from those germinated for whole day intervals for analysis. The malts were milled in the beater-type mill. The moisture contents of the resultant flour was determined (3.1.1.).

3.10.2. Proteolytic activity determinations.

Crude enzyme extracts were prepared from malt flour (1g) as described in 3.6.1. Enzyme extracts were assayed for proteinase and carboxypeptidase activity as described in 3.6.14. The proteinase and carboxypeptidase activities of duplicate malts were meaned. The resultant data was statistically analysed using 3-way analysis of variance.

3.11. Statistical analysis.

3.11.1. Curve fitting.

Curves were fitted to the data by regression analysis as described, according to the procedures and models devised by van der Walt (unpublished data). Error bars were calculated for each point on the curve from the residual variance, ie the variance of the data which was not due to regression (Dise & Lentner, 1978).

$$E = W_{N,n,\alpha}(s_N^2/n)^{1/2}$$

where E = length of error bar or extreme range

s_N^2 = residual variance with N degree of freedom.

n = number of repeat determinations for each point on the curve

$W_{N,n,\alpha}$ = studentised extreme range value for residual variance with N degrees of freedom and n repeats per point on the curve for a degree of confidence α.

3.11.2. Analysis of variance.

Analysis of variance using a fixed model was performed on relevant data as described by Winer (1971).

4. RESULTS.

4.1. Analysis by SDS-PAGE, of total kafirin preparation used as substrate .

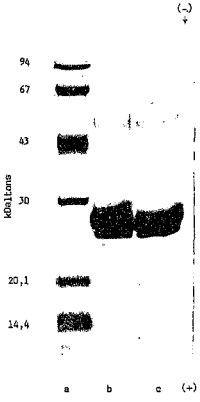
Figure 1 shows the separation by SDS-PAGE of two total kafirin preparations. Lane b represents a total kafirin preparation used as substrate for sorghum malt proteolytic activity determinations. This preparation was extracted directly from pearled sorghum grain flour with 60%(v/v) tert-butanol plus 0,05% (m/v) dithiothreitol (DTT). Lane c also represents a preparation of total kafirin. This latter preparation of total kafirin was extracted from pearled sorghum grain flour with 60% (v/v) tert-butanol plus 0,05% (m/v) DTT after exhaustive extraction of albumin and globulin proteins. The material prepared for use as substrate had a virtually identical electrophoretic mobility to total kafirin prepared by a standard procedure (Taylor et al,1984c).

4.2. Development of procedure for extraction of sorghum malt proteolytic activity.

4.2.1. Extractability of sorghum malt proteolytic activity with 1,25M NaCl as extractant.

Figure 2 shows the extractability of proteinase activity from sorghum malt with 1,25M NaCl. It can be seen that 30% of the total proteinase activity was recovered after a single extraction. However, 44% of the activity was apparently inextractable and remained in the residue fraction. Figure 3 shows the extractability of carboxypeptidase activity from sorghum malt. After a single extraction of sorghum malt, 36% of the total activity was recovered, whilst 44% was inextractable and remained in the residue fraction.

FIGURE 1. Analysis by SDS-PAGE of total kafirin used as substrate.



a. Molecular Weight Standards.
b. Total Kafirin (Substrate preparation).
c. Total Kafirin.

FIGURE 2. The extractability of proteinase activity from sorghum malt with 1.25M NaCl.

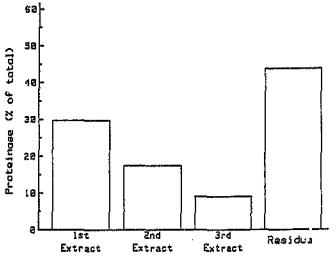
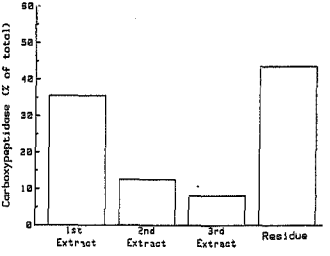


FIGURE 3. The extractability of carboxypeptidase activity from sorghum malt with 1.25M NaCl.



4.2.2. Influence of extractant volume to malt mass ratio upon the recovery of proteolytic activity and water-soluble protein from sorghum malt.

The recovery of proteolytic activity from sorghum malt after a single extraction with water was investigated. The recovery of both proteinase and carboxypeptidase activity was influenced by the extractant volume to malt mass ratio (Table I), up until a ratio of 15:1. Further increases in the ratio did not greatly improve the recovery of proteolytic activity from sorghum malt.

Table I also shows the protein contents of the enzyme extracts. The recovery of water-soluble proteins or albumins from sorghum malt was influenced by the extractant volume to malt mass ratio up until a ratio of 20:1. It appeared therefore that the recovery of proteolytic activity from sorghum malt was not directly related to the recovery of protein. An extractant volume to malt mass ratio of 15:1 was chosen for further investigations. It was felt that use of a higher ratio would dilute the enzyme activity without appreciably increasing its recovery.

4.2.3. Influence of extraction temperature upon the recovery of proteolytic activity from sorghum malt.

In this investigation, water was used as an extractant, and a single extraction of sorghum malt was made. Previously, proteolytic activity was extracted from sorghum malt at 4°C. Figure 4 shows that marginal improvements in recovery of proteinase activity were made at higher temperatures (21 to 48°C). The recovery of carboxypeptidase activity from sorghum malt appeared to be optimal at an extraction temperature in the range of 21 to 30°C (Fig. 5). For future investigations proteolytic activity was extracted from sorghum malt at 30°C.

Table I. The influence of extractant volume to malt mass ratio upon the recovery of proteolytic activity and water-soluble protein from sorghum malt.

Extractant volume: malt mass (ml/g)	Proteinase (μgN/5h/g dry malt)	Carboxypeptidase (μgN/5h/g dry malt)	Recovery of protein (mg/g dry malt)
5:1	418,4	63,9	3,52
10:1	712,5	117,3	4,38
15:1	1129,6	158,1	4,69
20:1	1186,0	159,6	3,47
25:1	1149,8	157,9	3,47

FIGURE 4. The influence of extraction temperature upon the recovery of sorghum malt proteinase activity.

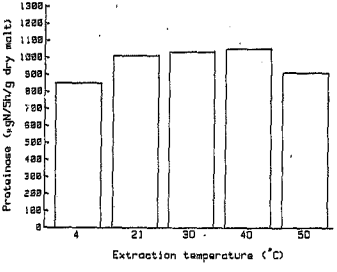
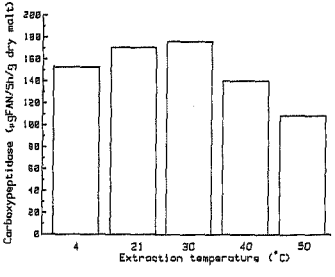


FIGURE 5. The influence of extraction temperature upon the recovery of sorghum malt carboxypeptidase activity



4.2.4. *Influence of extractant pH upon the recovery of proteolytic activity from sorghum salt.*

In this investigation, a single extraction of sorghum salt was made. The extractants were citric acid/K_2HPO_4 buffers. The ionic strength of each buffer was adjusted to 0,5M by addition of NaCl. The recovery of both proteinase and carboxypeptidase activity was dependant upon the pH of the extractant (Fig. 6 and Fig. 7). A pH 7,8 buffer extracted almost twice as much proteolytic activity from sorghum salt than did a pH 3,8 buffer.

4.2.5. Extractability of sorghum salt proteolytic activity with a pH 7,8 buffer as extractant.

The ionic strength of the extractant buffer used in this experiment was 0,5M. Figure 8 shows that 50% of the total proteinase activity was recovered from sorghum salt after a single extraction was made. The proportion of total salt proteinase activity which was apparently inextractable was 35%. The extractability of carboxypeptidase activity from sorghum salt was virtually identical to that of proteinase activity with this extractant (Fig. 9)

4.2.6. Influence of neutral salt solutions upon the recovery of proteolytic activity from sorghum salt.

In this investigation a single extraction of sorghum salt was made. A 0,5M solution of LiI recovered more proteinase and carboxypeptidase activity from sorghum salt than did 0,5M solutions of other neutral salts (Fig. 10 and Fig. 11). Compared with NaCl, a traditional protein extractant, LiI gave a marginal improvement in recovery of proteolytic activity from sorghum salt.

FIGURE 6. The influence of extractant pH upon the recovery of sorghum malt proteinase activity.

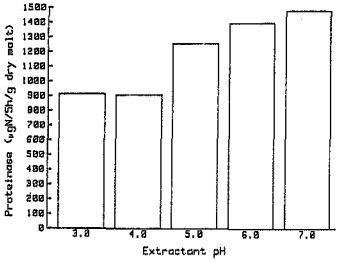


FIGURE 7. The influence of extractant pH upon the recovery of sorghum malt carboxypeptidase activity.

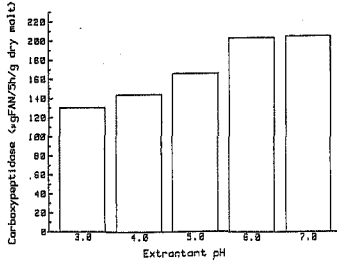


FIGURE 8. The extractability of proteinase activity from sorghum malt with 0.1M citric acid/0.2M K_2HPO_4 buffer, pH 7.0 plus 0.073M NaCl.

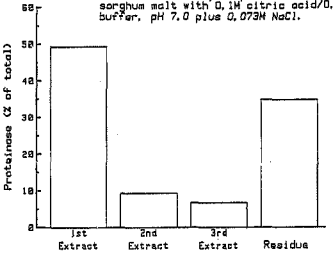


FIGURE 9. The extractability of carboxypeptidase activity from sorghum malt with 0.1M citric acid/0.2M K_2HPO_4 buffer, pH 7.0 plus 0.073M NaCl.

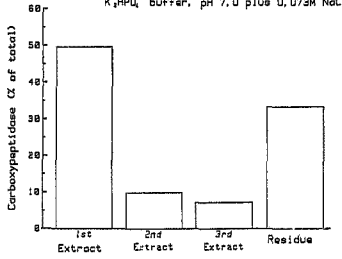


FIGURE 10. The influence of 0.5M solutions of neutral salt upon the recovery of sorghum malt proteinase activity.

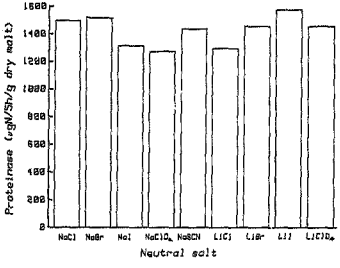
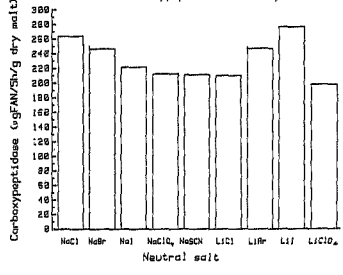


FIGURE 11. The influence of 0.5M solutions of neutral salt upon the recovery of sorghum malt carboxypeptidase activity.



4.2.7. *Influence of ionic strength of LiI upon the recovery of proteolytic activity from sorghum malt.*

In this investigation, a single extraction of sorghum malt was made. The recovery of both proteinase and carboxypeptidase activity appeared to be dependent upon the ionic strength of LiI. A solution of 0,6M LiI extracted more proteolytic activity from sorghum malt than did LiI at other ionic strengths (Fig. 12 and Fig. 13).

4.2.8. *Extractability of sorghum malt proteolytic activity with 0,6M LiI as extractant.*

Figure 14 shows that 40% of the total proteinase activity of sorghum malt was recovered after a single extraction, whilst 25% was apparently inextractable and remained in the residue. It can be seen from Figure 15 that 65% of the total carboxypeptidase activity was recovered after a single extraction with 0,6M LiI, whilst 30% of total carboxypeptidase activity remained in the residue.

4.2.9. *Extractability of sorghum malt proteolytic activity with a pH 7,8 buffer adjusted to an ionic strength of 0,6M with LiI as extractant.*

In this investigation, an attempt was made to combine extractant properties which improved the extractability of proteolytic activity from sorghum malt. Figure 16 shows that 45% of the total proteinase activity of sorghum malt was recovered after a single extraction, whilst 35% of the total activity was apparently inextractable and remained in the residue. It can be seen from Figure 17 that 57% of the total carboxypeptidase activity was recovered after a single extraction of sorghum malt. The residue contained about 35% of the total carboxypeptidase activity.

FIGURE 12. The influence of ionic strength of LiI upon the recovery of sorghum malt proteinase activity.

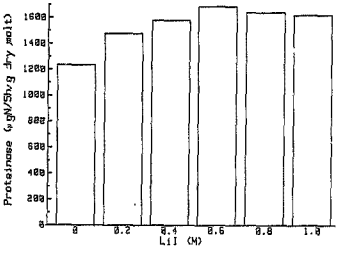


FIGURE 13. The influence of ionic strength of LiI upon the recovery of sorghum malt carboxypeptidase activity.

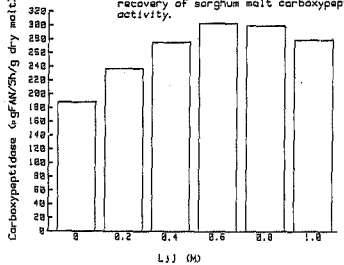


FIGURE 14. The extractability of proteinase activity from sorghum malt with 0.6M LiI.

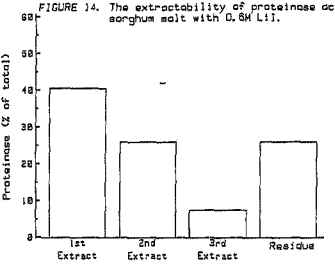


FIGURE 15. The extractability of carboxypeptidase activity from sorghum malt with 0.6M LiI.

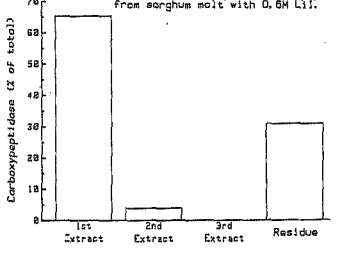


FIGURE 16. The extractability of proteinase activity from sorghum malt with 0.1M citric acid/0.2M K_2HPO_4 buffer, pH 7.0 plus 0.173M LiI.

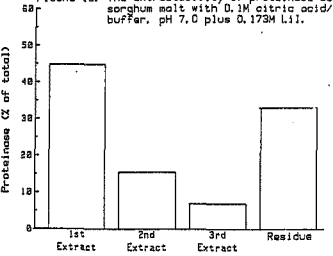
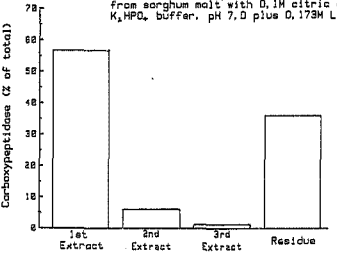


FIGURE 17. The extractability of carboxypeptidase activity from sorghum malt with 0.1M citric acid/0.2M K_2HPO_4 buffer, pH 7.0 plus 0.173M LiI.



4.2.10. Influence of dithiothreitol plus 0,6M LiI upon the recovery of proteolytic activity from sorghum malt.

In this investigation, a single extraction of sorghum malt was made. The incorporation of DTT with 0,6M LiI appeared to marginally enhance the recovery of proteinase activity from sorghum malt up until a concentration of 3,33mM DTT (Fig. 18). The influence of DTT upon the recovery of carboxypeptidase activity from sorghum malt is shown in Figure 19. It appeared that higher concentrations of DTT possibly reduced the recovery of carboxypeptidase activity from sorghum malt. A solution of 0,6M LiI plus 3,33mM DTT was chosen for further investigation as an extractant.

4.2.11. Comparison of the extractability of sorghum malt proteolytic activity using either water or 0,6M LiI plus 3,33mM dithiothreitol as extractants.

Table II shows the proteinase activities of 3 consecutive soluble extracts made from sorghum malt using water and 0,6M LiI plus 3.33mM DTT as extractants. The proteinase activities of the residues from each extraction are also shown. The analysis of variance table shows that proteinase activity was strongly influenced by extraction stage but not by extractant. In other words although the amount of proteinase activity recovered at each stage of extraction was different, the total amount of activity recovered from sorghum malt with each extractant was the same. There was however a significant interaction between the extraction stage and the extractant. This means that the extractant influenced the amount of proteinase activity recovered at a particular stage of extraction. Water recovered less proteinase activity from sorghum than 0,6M LiI plus 3,33mM DTT after a single extraction ($p<0,05$). The amount of proteinase

FIGURE 18. The influence of dithiothreitol plus 0.6M LiI upon the recovery of sorghum malt proteinase activity.

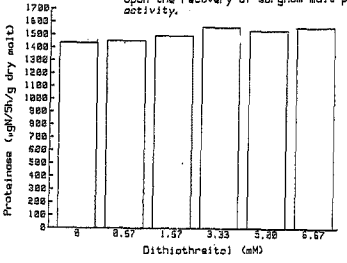


FIGURE 19. The influence of dithiothreitol plus 0.6M LiI upon the recovery of sorghum malt carboxypeptidase activity.

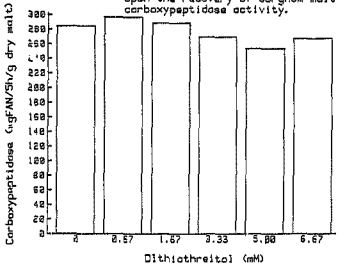


Table II. Comparison of the extractability of sorghum malt proteinase activity using water and 0,6M LII plus 3,33mM dithiothreitol as extractants.

Proteinase activity (µgN/5h/g dry malt)

Extractant / Extraction stage	Water	0,6M LII/3,33mM DTT	mean
1st extract	1873,7	1538,8	1302,3
2nd extract	512,2	485,6	498,9
3rd extract	376,4	283,3	329,9
Residue	1363,8	747,1	1055,5
means	831,5	761,7	796,6

Analysis of variance table: fixed model.
Mean square of extractant and extraction stage are compared with error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Extraction stage	3	1258440	112,2	<0,001
Extractant	1	29252	2,6	0,126
Interaction	3	289531	25,8	<0,001
Error	16	11216		
Total	23	218983		

activity in the water-extracted residue was greater than that in the residue extracted with 0,6M LiI plus DTT. Water recovered 33,7% of the total activity after a single extraction compared with the 48% recovery obtained with 0,6M LiI plus DTT. Also 43% of the total proteinase activity remained in the water-extracted residue compared with 23% in the 0,6M LiI plus DTT- extracted residue.

Table III shows a comparison of the extractability of carboxypeptidase activity from sorghum malt with water and 0,6M LiI plus DTT as extractants. Like the proteinase activities, the carboxypeptidase activities recovered at each stage of extraction were different. The total carboxypeptidase activity of sorghum malt was however the same irrespective of whether water or 0,6M LiI plus DTT was used as an extractant.

With 0,6M LiI plus DTT 55% of the total carboxypeptidase activity was recovered after a single extraction whilst 28% remained in the residue. With water, 35% of the total activity was recovered after a single extraction whilst 62% remained in the residue.

4.2.12. Influence of extractant upon the recovery of proteolytic activity from sorghum malt germinated for different times.

Table IV shows the influence of different extractants upon the recovery of proteinase activity from sorghum malt harvested at different germination times. The malt used in this study was germinated at 28°C. Throughout germination, a fixed level of watering was applied to the germinating grain. The acetate buffer plus cysteine hydrochloride was investigated as an extractant of proteolytic activity from sorghum malt as it had already been used in this regard by Aisien (1982).

Analysis of variance shows that malt proteinase activity was strongly influenced both by the choice of extractant and by germination time. There was also significant interaction of

Table III. Comparison of the extractability of sorghum malt carboxypeptidase activity using water and 0,6M LiI plus 3,33mM dithiothreitol as extractants.

Carboxypeptidase activity
(pgFAN/5h/g dry malt)

Extractant / Extraction stage	Water	0,6M LiI/3,33mM DTT	mean
1st extract	125,1	196,6	161,8
2nd extract	38,1	19,7	24,9
3rd extract	12,6	10,5	11,6
Residue	224,4	180,3	162,4
means	98,8	82,3	90,2

Analysis of variance table: fixed model.
Mean square of extractant and extraction stage are compared with error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Extraction stage	3	41583	52,8	<0,001
Extractant	1	1488	1,9	0,188
Interaction	3	9961	12,7	<0,001
Error	16	787		
Total	23	7335		

Table IV. Influence of extractant upon the recovery of proteinase activity from sorghum malt germinated at different times.

Proteinase activity (µgN/Sh/g dry malt)

Extractant	0,6M LiI/ 3,33mM DTT	Water	Na acetate buffer, pH 5,0/ 0,2% *cysteine.HCl*	mean
Germination time (days)				
0	762	580	243	516
1	1180	640	616	812
2	1329	1028	701	1086
3	1380	874	694	1049
4	1401	831	944	1065
5	1234	762	858	951
6	1356	679	1099	1045
mean	1241	770	793	935

Analysis of variance table: fixed model.
Mean square of extractant and germination time are compared with *error mean square*.

Source	Degrees of freedom	Mean square	F-value	Probability
Germination time	6	261285	20,9	<0,001
Extractant	2	984295	78,7	<0,001
Interaction	12	33782	2,7	0,023
Error	21	12514		
Total	41	102529		

these two effects ($p<,05$). In other words the apparent rate of development of sorghum malt proteinase activity was influenced by the nature of the extractant used to recover the proteinase from sorghum malt.

For all germination times, 0,6M LiI plus DTT recovered more proteinase activity from sorghum malt than either water or acetate buffer plus cysteine.HCl. Table V shows that the recovery of carboxypeptidase activity from malts germinated at different times was also influenced by the extractant chosen to isolate the enzyme from the malt. As was the case with the proteinase activity, there was a highly significant interaction of the effects of germination time and extractant. But at all germination times, 0,6M LiI plus DTT extracted more carboxypeptidase activity than the other extractants.

The extractant 0,6M LiI plus 3,33mM DTT consistently recovered more proteolytic activity from sorghum malt after a single extraction. It was therefore chosen as the extractant for sorghum malt proteolytic activity in future investigations.

Table V. Influence of extractant upon the recove carboxypeptidase activity from sorghum malt germinated at different times.

Carboxypeptidase activity (pgFAN/5h/g dry malt)

Extractant	8,6M LiI/ 3,33mM DTT	Water	Na acetate buffer, pH 5,0/ 0,2% cysteine.HCl	mean
Germination time (days)				
0	32	0	0	11
1	103	58	27	63
2	161	91	71	114
3	186	116	74	125
4	221	95	185	140
5	257	109	132	166
6	240	88	117	155
mean	177	79	75	110

Analysis of variance table: fixed model.
Mean square of extractant and germination time are compared with error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Germination time	6	18367	98,6	<0,001
Extractant	2	46465	229,2	<0,001
Interaction	12	1648	8,1	<0,001
Error	21	203		
Total	41	5538		

4.3. Development of incubation conditions for assaying sorghum malt proteolytic activity.

4.3.1. Influence of pH upon sorghum malt proteolytic activity.

Figure 20 shows that the optimum pH for proteinase activity was in the range of pH 4,25 to 4,50. Carboxypeptidase activity had a broader pH optimum in the range of pH 4,00 to 5,00 (Figure 21). A pH of 4,50 was chosen to assay the proteolytic activity of sorghum malt.

4.3.2. Influence of temperature and reaction time upon sorghum malt proteolytic activity.

Figure 22 shows the influence of temperature upon the progress curves of TCA-soluble nitrogen formation due to sorghum malt proteinase activity degrading total kafirin. Curves of the form

$$P = A + BC^t + Dt$$

where P= TCA-sol N
t= time
and A,B,C, and D are constants,

provided the best lines of fit for all the progress curves obtained at different temperatures. This was because the release of product with time occurred in two distinct phases. The initial phase of reaction was best explained by an exponential release of product with time. The second phase of product formation was linear with respect to time. Determination of initial rates of reaction were not meaningful due to the absence of points at the initial stage of the reaction.

FIGURE 20. The influence of pH upon sorghum malt proteinase activity.

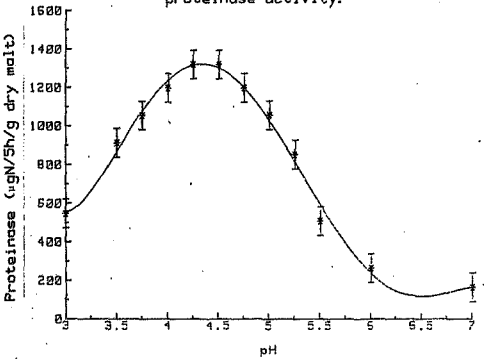


FIGURE 21. The influence of pH upon sorghum malt carboxypeptidase activity.

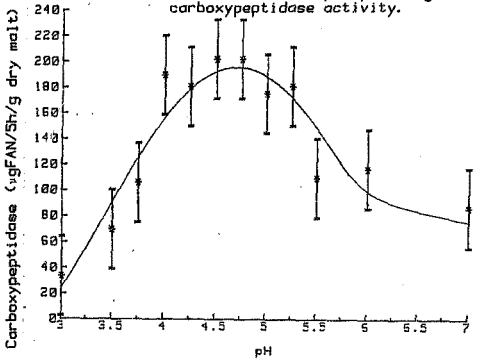
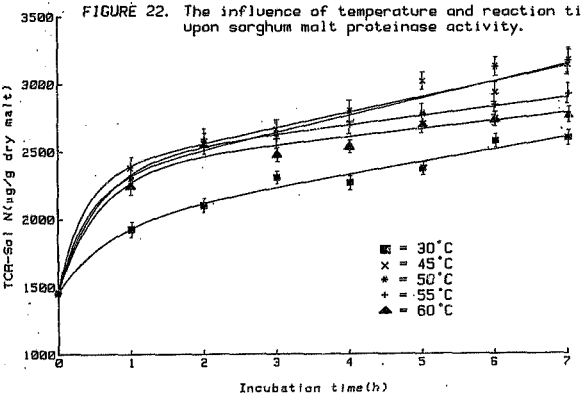


FIGURE 22. The influence of temperature and reaction time upon sorghum malt proteinase activity.



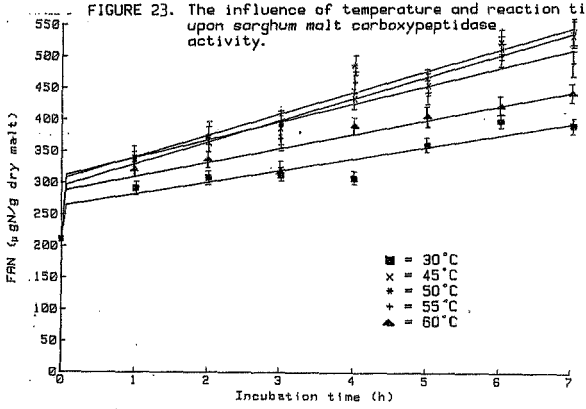
It can be seen that an incubation temperature of 30°C gave an exponential release of TCA-soluble nitrogen until an incubation time of between two and three hours. Thereafter the release of product was linear with time. For the higher incubation temperatures (45° to 60°C), the time taken to enter the linear phase was between one to two hours.

The maximum amount of nitrogen released during these incubations never represented more than a seven per cent degradation of the total kafirin. At the higher incubation temperatures (45 to 60°C), approximately five per cent of the total kafirin was degraded into TCA-soluble nitrogen during the exponential phase of the reaction. At 30°C, only approximately three per cent of the total kafirin was degraded into TCA-soluble nitrogen during the first phase of the reaction.

The influence of temperature upon the progress curves of FAN formation due to sorghum malt carboxypeptidase activity are shown in Figure 23. Again, the curves were characterised by an initial rapid release of product. For all the incubation temperatures, this phase was finished after one hour. Because of the absence of information during this initial phase of reaction, an exponential relationship could not be demonstrated. For this reason straight lines were drawn to represent the product released during the first hour of incubation. After the first phase, the reaction entered a linear phase at all the incubation temperatures.

The methods used to assay the products, TCA-soluble nitrogen and FAN were not sensitive enough to measure the small amount of product released at the start of the initial phase of the proteinase and carboxypeptidase reactions. Because of this it was not possible to determine the initial rates of reaction. An incubation time of five hours at 30°C was chosen to assay proteolytic activity in sorghum malt. Although this gave a net rate of reaction, the amount of product released could be measured accurately and reproducibly.

FIGURE 23. The influence of temperature and reaction time upon sorghum malt carboxypeptidase activity.



4.3.3. Influence of temperature upon the stability of sorghum malt proteolytic activity.

Table VI shows that both proteinase activity and carboxypeptidase activity from sorghum malt declined rapidly after one hour pre-incubation of the enzyme extracts at temperatures exceeding 30°C.

4.3.4. Influence of substrate concentration upon sorghum malt proteolytic activity determinations.

In this investigation, attempts were made to measure the amount of product released during the initial phase of the reaction, in order to obtain a measure of the initial rate of reaction. Figure 24 shows the progress curves of TCA-soluble nitrogen formation due to proteinase activity in the presence of differing amounts of total kafirin. The general shapes of these progress curves were characterised by an initial rapid release of product followed by a linear phase. The initial rates of reaction appeared similar for the three highest levels of total kafirin investigated. The overall amount of TCA-soluble nitrogen released, increased however with increasing total kafirin concentration.

The rates of proteinase activity during the linear phase of reaction appeared to be hardly influenced by increases in total kafirin concentration. The amount of TCA-soluble nitrogen released during the initial exponential phase of the reaction represented approximately five per cent of the total kafirin irrespective of the total kafirin concentration.

Figure 25 shows the progress of PAN formation due to carboxypeptidase in the presence of different amounts of total kafirin. The general shapes of these progress curves were also

Table VI. The stability of sorghum salt proteinase and carboxypeptidase activity after pre-incubation of enzyme extract for 1 hour at different temperatures.

Pre-incubation temperature(°C)	Proteinase		Carboxypeptidase	
	activity (pgN/Sh/ g dry salt)	survival (% of control)	activity (pgFAM/Sh/ g dry salt)	survival (% of control)
Control ie. no pre-incubation	1411	100	317	100
30	1393	99	306	96
50	1025	73	241	76
60	572	41	131	41
70	184	13	37	12
80	169	12	28	8

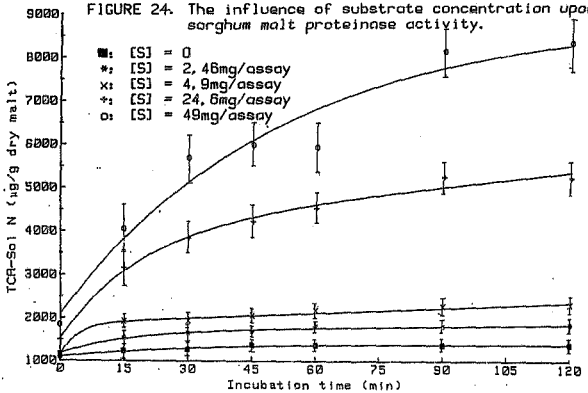
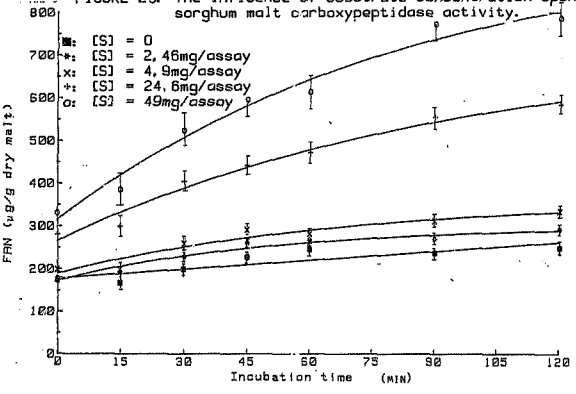


FIGURE 24. The influence of substrate concentration upon sorghum malt proteinase activity.

FIGURE 25. The influence of substrate concentration upon sorghum malt carboxypeptidase activity.



characterised by an exponential release of product followed by a linear phase except for when total kafirin was absent from the incubation ie. zero substrate concentration. Again, although the initial rates of carboxypeptidase activity appeared similar, the overall amount of FAN released increased with increasing total kafirin concentration.

Because of these unusual results it was decided to investigate conditions of substrate saturation indirectly by observing the influence of enzyme concentration upon proteolytic activity.

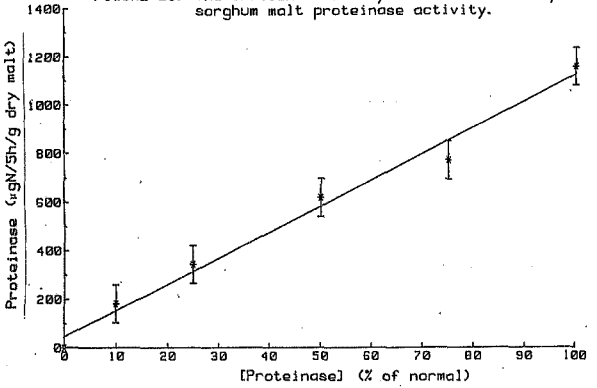
4.3.5. Influence of enzyme concentration upon sorghum malt proteolytic activity determinations.

Figure 26 shows the influence of enzyme concentration upon the proteinase activity of sorghum malt. At a substrate concentration of 4,9mg total kafirin, the proteinase activity was directly proportional to the enzyme concentration. Figure 27 shows that carboxypeptidase activity was also directly proportional to enzyme concentration at a substrate concentration of 4.9mg total kafirin. A substrate concentration of 5mg total kafirin per enzyme assay was therfore chosen for further investigation.

4.4. Investigation of the purity of total kafirin substrate preparations.

During the investigation into the influence of enzyme concentration upon sorghum malt proteolytic activity, it was observed that a small but detectable amount of TCA-soluble nitrogen was released from total kafirin when incubated in the absence of sorghum malt proteolytic enzymes ie. zero enzyme concentration. Three investigations were made in an attempt to determine the nature of this release of nitrogen.

FIGURE 26. The influence of enzyme concentration upon sorghum malt proteinase activity.



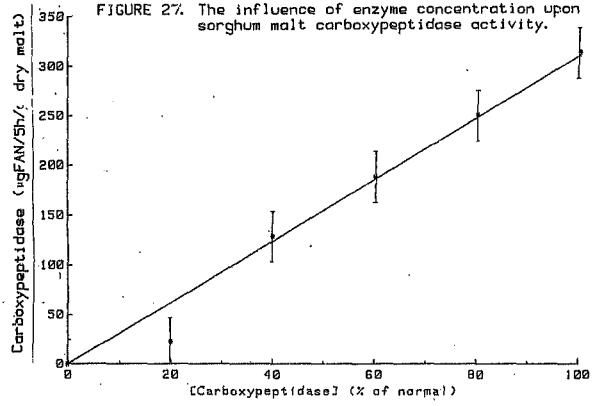


FIGURE 27. The influence of enzyme concentration upon sorghum malt carboxypeptidase activity.

4.4.1. Influence of immersing total kafirin in boiling water upon its subsequent release of TCA-soluble nitrogen.

Immersion of a total kafirin suspension in boiling water for 15 min. was carried out to eliminate any proteolytic activity that may have been associated with it. Table VII shows that there was a significant release of TCA-soluble nitrogen from total kafirin during a five hour incubation period. Pre-heating the total kafirin for 15 min appeared to increase the amount of TCA-soluble nitrogen released at any one time. However, the net amount of TCA-soluble nitrogen released between zero and five hours was not influenced by the pre-heating treatment, since there was no significant interaction of incubation time with pre-heat treatment of total kafirin.

Table VIII shows that there was no influence of incubation time upon the FAN released from total kafirin.

4.4.2. Influence of incubation time upon release of TCA-soluble nitrogen from total kafirin.

Figure 28 shows the influence of incubation time upon the release of TCA-soluble nitrogen from total kafirin. It can be seen that there was a linear relationship between the release of TCA-soluble nitrogen from total kafirin and incubation time.

4.4.3. Influence of incubation pH upon release of TCA-soluble nitrogen from total kafirin.

Tables IX and X show the results of two experiments whereby the influence of incubation pH upon TCA-soluble nitrogen release from total kafirin was studied. Both Table IX and Table X show that the effects of both incubation time and incubation pH were highly significant. However, the interaction between the two effects

Table VII. Influence of immersing total kafirin in boiling water upon the release of TCA-soluble nitrogen contaminants.

TCA-sol N (µg/5mg total kafirin)

Heat-treatment	Total kafirin		
	Control	Boiled	mean
Incubation time (h)			
0	17,8	19,5	18,3
5	23,8	24,8	23,5
means	20,8	21,8	20,9

Analysis of variance table: fixed model.
Mean square of heat-treatment and incubation time are compared with
error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Incubation time	1	136,2	43,7	<0,001
Heat-tmnt	1	16,2	5,2	0,037
Interaction	1	2,9	0,9	0,358
Error	16	3,1		
Total	19	18,8		

Table VIII. Influence of immersing total kafirin in boiling water upon the release of free α-amino nitrogen contaminants.

FAN (µg/5mg total kafirin)

Heat-treatment	Total kafirin		
	Control	Boiled	mean
Incubation time (h)			
0	1,84	2,32	2,08
5	2,30	2,68	2,49
means	2,07	2,50	2,29

Analysis of variance table: fixed model.
Mean square of heat-treatment and incubation time are compared with
error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Incubation time	1	0,84	2,38	0,133
Heat-tmnt	1	0,92	2,76	0,116
Interaction	1	0,01	0,04	0,849
Error	16	0,34		
Total	19	0,38		

FIGURE 28. The influence of incubation time upon the release of TCA-soluble nitrogen contaminants from total kafirin.

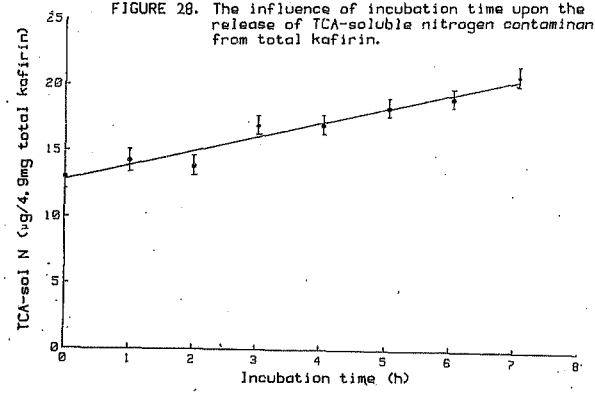


Table IX. Influence of pH upon the release of TCA-soluble nitrogen contaminants from total kafirin. Trial 1.

TCA-solN (µg/5mg total kafirin)

Incubation time (h)		0	5	mean
pH at 50°C	Buffer type			
4,15	0,3M cit/0,6M Phos	11,3	18,0	14,6
4,58	" "	12,1	21,4	16,7
5,28	" "	12,8	19,6	16,2
6,16	" "	12,8	15,0	10,5
7,58	0,1M KCl/0,1N aOH	9,4	11,7	10,5
8,71	" "	9,4	12,4	10,9
9,52	" "	9,8	12,1	10,5
mean		11,0	15,7	13,4

Analysis of variance table: fixed model.
Mean square of pH and incubation time are compared with error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Incubation time	1	158,9	176,2	<0,001
pH	6	28,9	32,1	<0,001
Interaction	6	8,8	8,9	<0,001
Error	14	0,9		
Total	27	14,6		

Table X. Influence of pH upon the release of TCA-soluble nitrogen contaminants from total kafirin. Trial 2.

TCA-solN (µg/5mg total kafirin)

Incubation time (h)		0	5	mean
pH at 58°C	Buffer type			
2,58	0,2M KCl/0,2M HCl	8,3	10,2	9,2
3,03	0,1M lactic/0,1M NaOH	24,1	24,4	24,2
4,00	" "	19,9	21,8	20,9
4,55	" "	19,9	21,1	20,5
5,05	" "	18,4	20,3	19,4
6,04	" "	19,9	19,2	19,5
6,15	0,3M cit/0,1M Phos	11,3	11,7	11,5
7,10	" "	9,4	10,2	9,8
6,33	0,1M lactic/0,1M NaOH	19,2	21,8	20,5
7,52	0,1M KCl/0,1M NaOH	8,7	10,2	9,4
8,82	" "	10,2	10,2	10,2
9,70	" "	9,8	10,5	10,2
mean		14,9	15,9	15,4

Analysis of variance table: fixed model.
Mean square of pH and incubation time are compared with error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Incubation time	1	12,8	25,4	<0,001
pH	11	133,9	267,8	<0,001
Interaction	11	0,9	1,9	0,076
Error	24	0,5		
Total	47	32,1		

was significant for the data in Table IX but not for the data in Table X. A significant interaction between incubation pH and incubation time means that the net rate of release of TCA-soluble nitrogen from kafirin over five hours was also influenced by incubation pH. Table IX shows that the net release of nitrogen over the pH range: 4,15 to 5,20 was greater than that released over the pH range: 7,58 to 9,52 ($p<0,05$). The data in Table X however shows that there was no influence of pH upon the rate of release of peptides from total kafirin. It appears therefore that the composition of the buffer ie. lactic acid/NaOH versus citric acid/Na_2HPO_4 influenced the rate of release of peptides from total kafirin more than the buffer pH.

4.4.4. Purification of total kafirin.

Because of the presence of TCA-soluble nitrogen contaminants, the total kafirin was further purified. This was achieved by suspending the total kafirin in either water or a sodium lactate/lactic acid buffer, pH 3,1. The suspensions were then subjected to exhaustive dialysis. The amount of nitrogen which was dialysed from the total kafirin - represented 8,2 per cent approx. of the total nitrogen. The suspensions were then centrifuged and the residues and supernatants were freeze-dried. The freeze-dried products were all white powders and comprised 98 per cent approx. protein. The freeze-dried supernatant from the lactate-extracted total kafirin represented about 3,5 per cent of the total nitrogen, whereas that from the water-extracted total kafirin represented 1,5 per cent of total nitrogen.

The amino acid compositions of the various protein fractions are shown in Table XI. The residual proteins derived from both water and lactate extraction of total kafirin appear identical and have a similar amino acid profile to total kafirin. Both supernatant fractions also have virtually identical amino acid compositions. The lactate buffer therefore does not differ from water with respect to the nature of the proteins it extracts from total

TABLE XI: *Amino acid composition (moles %) of total kefirin before and after extraction with water and 0,02M lactate buffer, pH 3,1.*

Amino Acid	Total kefirin	Total kefirin extracted with water		Total kefirin extracted with 0,02M lactate, pH 3,1	
		residue	supernatant	residue	supernatant
Asx	4,8	4,9	0,5	4,9	0,4
Thr	2,6	2,5	4,6	7,6	4,2
Ser	4,7	4,7	4,2	5,5	4,2
Glx	20,0	20,7	12,7	20,2	13,3
Pro	11,2	11,5	24,9	10,2	23,8
Gly	2,7	2,5	8,7	2,3	8,9
Ala	15,6	15,6	6,1	15,8	6,4
Cys/2	0,7	1,5	6,2	1,0	6,3
Val	5,6	5,2	6,1	5,3	6,2
Met	1,7	1,2	1,0	1,1	1,2
Ile	4,1	4,0	2,6	4,2	2,8
Leu	15,4	15,4	8.5	15,7	8,7
Tyr	3,0	3,3	1,9	3,3	1,8
Phe	4,7	4,7	2,0	5,1	2,0
His	1,6	1,3	7,5	1,3	7,2
Lys	0,2	0,1	0,4	0,2	0,2
Arg	1,2	0,9	2,1	1,3	2,4

kafirin. However, the amino acid profile of the supernatant protein was different to that of total kafirin. It was characterised by a very high proline content (24-25 mole per cent), together with high amounts of histidine (7 mole per cent) and cysteine (6 mole per cent) and low amounts of aspartic acid and lysine (<1 mole per cent).

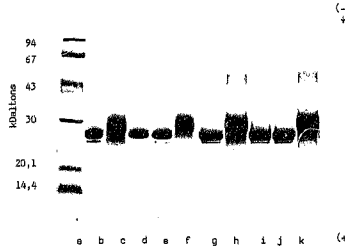
Separation of the various protein fractions by SDS-PAGE is shown in Figure 29. The results obtained are in agreement with the observations made from analysis of the amino acid compositions. There appeared to be no difference between the band pattern of the lactate-extracted residue (lanes e and j) and the water-extracted residue (lanes b and g). The same applied to the lactate- and water-extracted supernatants (lanes f and k, and c and h respectively). There also appeared to be no difference in electrophoretic pattern between the residual fractions and the total kafirin (lanes d and i), whereas the supernatant fractions gave a completely different pattern. Even at high sample loading of total kafirin (lane j), the supernatant fraction was not visible. This was in agreement with the nitrogen determination which showed that the supernatant fraction represented a small proportion of total kafirin.

The supernatant protein appears to comprise a major band of apparent M_r 27 000 to 31 000 with a mid-value of 28 000 and a minor band with an apparent M_r of 49 000. Because of the isolation of this water-soluble protein from total kafirin, the residual protein must have therefore represented a more pure form of prolamin protein.

4.5. Influence of reaction time upon sorghum malt proteolytic activity assayed at 30 and 50°C, with purified total kafirin as substrate.

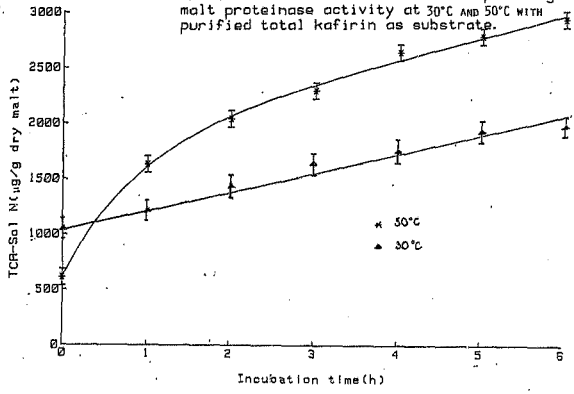
Figure 30 shows the rate of formation of TCA-soluble nitrogen due

FIGURE 29. Separation by SDS-PAGE of total kafirin, before and after extraction with water and 0,02M lactate buffer, pH3,1.



a. Molecular weight standards.
b. Residue:water extraction (50 µg).
c. Supernatant:water extraction (50 µg).
d. Total kafirin (50 µg).
e. Residue: lactate extraction (50 µg).
f. Supernatant: lactate extraction (50 µg).
g. Residue: water extraction (100 µg).
h. Supernatant: water extraction (150 µg).
i. Total kafirin (100 µg).
j. Residue: lactate extraction (100 µg).
k. Supernatant: lactate extraction (150 µg).

FIGURE 30. The influence of incubation time upon sorghum malt proteinase activity at 30°C AND 50°C WITH purified total kafirin as substrate.



to proteinase activity from sorghum malt at 30 and 50°C. The progress curve at 50°C was characterised by an initial exponential phase of product formation followed by a linear phase. There was a 4,63 degradation of purified total kafirin during the exponential phase. However at 30°C the formation of TCA-soluble nitrogen due to proteinase activity from sorghum malt showed a linear release of product with time for the *duration of the reaction. Figure 3*1 shows the formation of FAN due to carboxypeptidase activity from sorghum malt at 30°C. Here there was evidence of a slight curvilinear relationship for product formation during the initial stages of reaction. Thereafter the reaction proceeded at a constant rate.

4.6. Extractability of proteolytic activity from sorghum malts prepared from different cultivars.

The sorghum cultivars used in this study were all grown under identical environmental conditions. The grains were all malted according to the same schedule for six days. Table XII shows the extractability of proteinase activity from these sorghum malts. Both the cultivar and the extraction stage had a highly *significant effect* upon the amount of proteinase activity recovered ($p<0,001$). Sorghum cultivar SNK 3377 produced the malt with the highest total proteinase activity, whilst after a single extraction the greatest amount of proteinase activity was recovered from sorghum malt prepared from *cultivars DC 75. The* cultivar PNR 8433 produced the malt with the lowest amount of total proteinase activity. This malt also yielded the least amount of proteinase activity after a single extraction.

It can also be seen from Table XII that there was a significant interaction between cultivar and extraction stage ($p<0,05$). In other words, the amount of proteinase activity extracted from sorghum malts at a given stage of extraction was dependent upon the sorghum cultivar. Thus the first soluble extracts of different sorghum malts may provide different proportions of the

FIGURE 31. The influence of incubation time upon sorghum malt carboxypeptidase activity at 30 C with purified total kafirin as substrate.

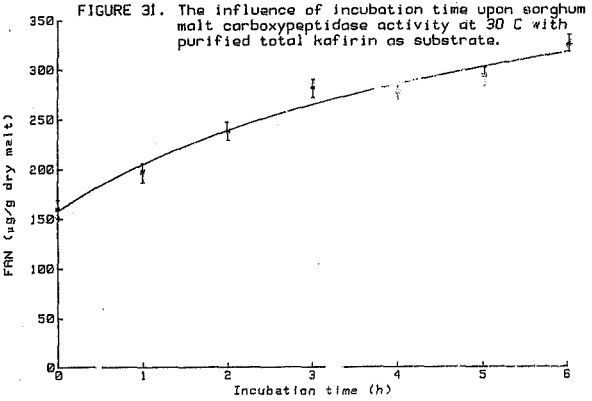


Table XII. Extractability of proteinase activity from sorghum malts prepared from different cultivars.

Proteinase activity (pgN/Sh/g dry malt)

Extraction stage / Cultivar	1st extract	2nd extract	3rd extract	Residue	mean
Barnard Red	1002	535	230	623	617
DC 34	1118	394	178	630	578
Breyt. Red	1334	418	216	656	654
NK 190	989	481	205	584	525
PNR 8433	796	438	326	468	505
SNK 3377	1285	775	228	948	809
PNR 8469	1291	682	284	678	712
PNR 8311	1149	569	372	661	687
DC 75	1519	612	147	513	698
NK 283	1035	472	219	675	600
mean	1152	529	231	642	638

Analysis of variance table: fixed model.
Mean square of extraction stage and cultivar are compared with error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Cultivar	9	68343	4,1	<0,001
Extraction stage	3	2940490	176.0	<0,001
Interaction	27	33053	2,0	0,024
Error	40	16706		
Total	79	139285		

total proteinase activities. However, determination of the correlation coefficients between proteinase activity at each extraction stage and the total activity for the different malts showed significant correlation ($p<0.05$) for the first and second extracts and the residue (Table XIII). In other words the proteinase activities of the first and second extracts and the residue did represent a fixed proportion of the total activity. The significant interaction between extraction stage and cultivar, as estimated by 2-way analysis of variance was probably largely due to the lack of correlation between the proteinase activity of the third extract and the total activity.

Table XIV shows the extractability of carboxypeptidase activity from sorghum malts prepared from different sorghum cultivars. Again both the cultivar and extraction stage influenced the carboxypeptidase activity recovered from sorghum malt. Cultivar: PNR 8433, which resulted in a malt with low proteinase activity, yielded the highest level of total carboxypeptidase activity. Cultivar: SNK 3327 yielded marginally more carboxypeptidase activity after a single extraction than did other cultivars. Cultivar: DC 78 produced the malt with the lowest total carboxypeptidase activity after a single extraction, whilst PNR 8311 yielded the least carboxypeptidase activity after a single extraction. These results suggested that carboxypeptidase and proteinase activity were independent cultivar related phenomena.

There was no interaction between cultivar and extraction stage for carboxypeptidase activity ($p>0.05$). This indicated that at each stage of extraction, a fixed proportion of the total carboxypeptidase activity was recovered.

4.7. Influence of malting conditions upon the development of sorghum malt proteolytic activity.

Table XV shows the influence of germination conditions upon the

Table XIII. Correlation coefficients for relationship between total sorghum malt proteinase activity and proteinase activity recovered at different stages of extraction.

Relationship	Degrees of freedom	Correlation coefficient	Probability
1st extract with total	8	0,79	<0,05
2nd extract with total	8	0,88	<0,05
3rd extract with total	8	-0,18	>0,50
Residue with total	8	0,73	<0,05

Table XIV. Extractability of carboxypeptidase activity from sorghum malts prepared from different cultivars.

Extraction stage	Carboxypeptidase activity (µgFAN/5h/g dry malt) 1st extract	2nd extract	3rd extract	Residue	mean
Cultivar					
Barnard Red	156	8	0	76	60
DC 34	138	8	0	91	64
Greyt. Red	131	18	22	84	64
NK 190	126	46	0	77	63
PNR 8433	159	83	40	155	109
SNK 3377	169	36	3	122	82
PNR 8469	168	42	13	123	86
PNR 8311	126	38	0	97	63
DC 75	127	0	0	52	45
NK 283	142	22	0	94	64
mean	146	29	8	97	70

Analysis of variance table: fixed model.
Mean square of extraction stage and cultivar are compared with error mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Cultivar	9	2579	7,3	<0,001
Extraction stage	3	80684	229,3	<0,001
Interaction	27	435	1,2	0,267
Error	40	352		
Total	79	3685		

Table IV. Influence of germination conditions upon the development of proteinase activity in sorghum malt.

Proteinase activity (μgN/5h/g dry malt)

Temperature / Moisture	24°C			28°C			32°C		
	Low	Med	High	Low	Med	High	Low	Med	High
Germination time (days)									
1	1287	916	1091	951	1188	1121	986	1098	1043
2	1162	1359	1588	1266	1329	1257	942	1364	1435
3	1338	1387	1198	1189	1380	1195	1298	1474	1488
4	1366	1684	1318	1254	1481	1298	1333	1292	1079
5	1296	1267	1086	1195	1234	1287	1078	1813	1064
6	1098	943	1042	1061	1356	1092	1086	1048	1002

At 0 days germination the proteinase activity was 762μgN/5h/g dry malt.

development of proteinase activity in sorghum malt. The zero day malt used in this experiment was the same for all the malting conditions investigated, since at this stage germination temperature, moisture and time can not influence the development of proteolytic activity. The proteinase activities of all the malts represented the mean value of two independent determinations. It can be seen from Table XV that at all conditions of temperature and moisture, the proteinase activity of sorghum malt increased by about two-fold up until ..y four. Thereafter the proteinase activity of the malt declined.

Table XVI shows the statistical evaluation of the data in Table XV by 3-way analysis of variance. Only germination time had a significant effect upon the proteinase activity of sorghum malt ($p<0.001$). Maximum levels of proteinase activity in sorghu. were achieved after four days germination.

Table XVII shows the influence of germination conditions upon the development of carboxypeptidase activity in sorghum malt. As for the proteinase activities, the carboxypeptidase activities of all the malts represented the mean values of two independent determinations. It can be seen from Table XVII that for all moisture and temperature conditions the carboxypeptidase activity increases with germination time by up to about eight-fold. The rate of this increase was most rapid during the first day of germination.

Table XVIII shows the statistical evaluation of the data in Table XVII by 3-way analysis of variance. The conditions of germination temperature, moisture and time all strongly influenced the carboxypeptidase activity of sorghum malt. A germination temperature of 24°C produced malts with higher carboxypeptidase activity than did either 28° or 32°C. A medium moisture treatment during germination produced malts with higher carboxypeptidase activity than low or high conditions of moisture. Maximum levels of carboxypeptidase activity were obtained in sorghum malts germinated for between four and six

Table XVI. Statistical evaluation of the data in Table XV by 3-way analysis of variance.

Analysis of variance: fixed model.
Mean square of germination temperature, moisture and time, plus their 2-way interactions are compared with residual mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Temperature	2	19549	1,5	0,249
Moisture	2	35343	2,7	0,092
Time	5	125465	9,6	<0,001
Temp X Moist	4	17425	1,3	0,293
Temp X Time	10	16743	1,3	0,265
Moist X Time	10	18838	1,4	0,234
Residual	28	13077		

Cell means for Temp X Time interaction

Temp.(°C) / Time(days)	24	28	32	mean
1	1096	1084	1042	1074
2	1343	1284	1247	1291
3	1271	1285	1404	1318
4	1429	1338	1235	1334
5	1216	1212	1049	1159
6	1028	1170	1045	1081
mean	1231	1224	1170	1209

Cell means for Moist X Time interaction

Moist. / Time(days)	Low	Med	High	mean
1	1075	1063	1085	1074
2	1123	1351	1400	1291
3	1249	1367	1294	1318
4	1312	1459	1226	1334
5	1187	1171	1119	1159
6	1082	1116	1045	1081
mean	1172	1258	1195	1208

Cell means for Temp X Moist interaction

Moist. / Temp(°C)	Low	Med	High	mean
24	1253	1232	1207	1231
28	1153	1327	1192	1224
32	1111	1215	1185	1170
mean	1172	1258	1195	1208

Table XVII. Influence of germination conditions upon the development of carboxypeptidase activity in sorghum malt.

Carboxypeptidase activity (µgFAN/5h/g dry malt)

Temperature	24°C			28°C			32°C		
Moisture	Low	Med	High	Low	Med	High	Low	Med	High
Germination time (days)									
1	148	127	134	117	103	127	129	145	154
2	166	244	224	63	181	172	134	202	171
3	227	230	235	179	186	184	165	176	214
4	240	276	239	173	221	198	202	237	189
5	229	238	198	189	257	192	212	202	159
6	226	210	201	201	268	177	209	202	154

At 0 days germination, the carboxypeptidase activity was 32µgFAN/5h/g dry malt.

Table XVIII. Statistical evaluation of the data in Table XVII by 3-way analysis of variance.

Analysis of variance: fixed model.
Mean square of germination temperature, moisture and time, plus their 2-way interactions are compared with residual mean square.

Source	Degrees of freedom	Mean square	F-value	Probability
Temperature	2	4957	15,8	<0,001
Moisture	2	2729	8,7	0,002
Time	5	9098	28,9	<0,001
Temp X Moist	4	186	0,3	0,849
Temp X Time	10	644	2,1	0,063
Moist X Time	10	1012	3,2	0,013
Residual	28	315		

Cell means for Temp X Time interaction

Temp.(°C) Time(days)	24	28	32	mean
1	134	116	143	131
2	211	172	169	184
3	231	183	185	200
4	251	197	209	219
5	222	213	191	208
6	213	213	188	205
mean	210	182	181	191

Cell means for Moist X Time interaction

Moist. Time(days)	Low	Med	High	mean
1	129	125	138	131
2	154	209	189	184
3	198	197	211	200
4	205	244	209	219
5	218	232	183	208
6	213	224	177	205
mean	184	205	185	191

Cell means for Temp X Moist interaction

Moist. Temp(°C)	Low	Med	High	mean
24	205	221	205	210
28	170	201	175	182
32	175	194	174	181
mean	184	205	185	191

days, depending on the moisture conditions. Thus there was significant interaction between moisture and time. The interaction between temperature and time was not significant at a five per cent level of confidence. It appears that the maximum level of carboxypeptidase activity was produced with a four day malt germinated at 24°C under medium conditions of moisture.

5. DISCUSSION.

Throughout the course of this study, the release of TCA-soluble nitrogen by proteolysis was always far greater than the release of FAN. Since FAN is largely a measure of free amino acids, it was concluded that TCA-soluble nitrogen was predominantly a measure of peptides. Thus proteolytic activity quantified in terms of TCA-soluble nitrogen and FAN was believed to be *indicative of proteinase and exopeptidase activity* respectively. The simultaneous measurement of proteinase and exopeptidase activity by distinguishing between the rate of release of peptides and free amino acids has also been employed by Baxter (1976).

The incubation conditions of temperature and pH under which sorghum malt proteolytic activity was initially assayed, were chosen from the results of a preliminary study by Taylor (unpublished results). Acidic pH conditions (pH 4,3) were employed throught this study. Of the exopeptidases found in germinating seeds, only the carboxypeptidases have acidic pH optima (Ashton, 1976; Mikola, J., 1983), thus proteolytic activity measured in terms of FAN release was more specifically referred to as carboxypeptidase activity.

The first stage in the development of a procedure for assaying sorghum malt proteolytic activity was to investigate the extraction of malt proteolytic enzymes. The development of a satisfactory extraction procedure for recovering proteolytic enzymes from sorghum malt was complicated by the apparently insoluble nature of the proteolytic enzymes. In an attempt to increase the recovery of proteolytic activity from sorghum malt, a variety of extraction procedures (Baxter, 1976; Moureaux, 1979; Preston and Kruger, 1976) employed in studies of proteolytic activity with other germinating cereals were also investigated. All of these extractants recovered less proteinase activity from sorghum malt than 1,25M NaCl after a single extraction (Taylor,

unpublished results). Sodium chloride (1,25M NaCl) was chosen as an extractant of proteolytic activity from sorghum malt because it had proven to be an efficient extractant of albumins and globulins from sorghum grain (Taylor et al, 1984c). The albusins and globulins of cereals are predominantly metabolic proteins (Wilson, 1981) and thus probably include the proteolytic enzymes.

After a single extraction of sorghum malt with 1,25M NaCl, 34% of the total proteinase activity was recovered. It can therefore be inferred that the extractants of Baxter, Moureaux, and Preston and Kruger recovered a smaller proportion of total proteinase activity than this.

Because of the limited success experienced in solubilising sorghum malt proteolytic activity, a more systematic approach was adopted to develop a more efficient extraction procedure. The objectives of this approach were two-fold. Firstly, and most importantly, for the purpose of the assay measure, the amount of proteolytic activity recovered after a single extraction of sorghum malt should be increased. Secondly, the proportion of inextractable proteolytic activity should be reduced.

In order to achieve these objectives, a number of parameters that have been shown to enhance the solvation of proteins were incorporated stepwise into extraction procedures. These procedures were then assessed with respect to their influence upon the recovery of both proteinase and carboxypeptidase activity from sorghum malt. Firstly, it was found that the amount of proteinase and carboxypeptidase activity and protein extracted from sorghum malt were all influenced by the extractant volume to malt mass ratio. At higher ratios the amount of proteolytic activity and protein extracted became constant. Thus at lower ratios it appeared that water-soluble components of sorghum malt saturated the solvent. Similarly, Taylor et al (1984c) found that the extractant volume influenced the amount of protein extracted from a given mass of sorghum grain.

Secondly, it is known that a number of parameters can act as structural perturbants of macromolecules (Von Hippel & Schleich, 1969). Structural perturbants disrupt the native conformation of macromolecules, and this situation can in turn lend itself to increased macromolecular solubility. Some of these parameters: temperature, pH and neutral salts were investigated with respect to their influence upon the extraction of proteolytic activity from sorghum malt.

Previous extractions of sorghum malt proteolytic activity were conducted at 4°C. This was because enzymes can be inactivated at higher temperatures (Lehninger, 1975). Plant proteinases however are unusually temperature-stable (Greenberg, 1955). Sorghum malt proteolytic enzymes can also withstand moderately high temperatures since their extraction was improved at 38°C.

Buffers of various composition and pH have been used to extract proteolytic activity from germinating cereals. With maize for example, acetate buffer, pH 3,9 (Moureaux, 1979) and phosphate buffer, pH 7.2 (Dencheva & Nikolova, 1984) have both been used as extractants of proteolytic activity. The extraction of proteolytic activity from sorghum malt was strongly influenced by pH. The use of a pH 7,0 buffer with an ionic strength of 0,5M resulted in a greater recovery of proteolytic activity after a single extraction compared with 1,25M NaCl. Recently Dencheva and Nikolova (1984) found that the extraction of proteolytic activity from germinating maize was best accomplished using 0,05M phosphate buffer, pH 7,2 plus 2mM 2-mercaptoethanol.

Protein solubility can change in solutions of different neutral salts. This phenomenon has led to the formulation of the Hofmeister or lyotropic series of ions and has been reviewed by Von Hippel and Schleich (1969). Both the anions and cations of a neutral salt can influence a protein's solubility. The lyotropic series predicts that $Li^+ > Na^+ > K^+$ and $SCN^- > ClO4^- > I^- > Br^- > Cl^- > F^-$ for the salting-in

or solubilisation of proteins. The relationship between the amount of proteolytic activity extracted from sorghum malt with solutions of different neutral salt however was inexplicable in terms of the lyotropic series. Staker and Gortner (1931) investigated solutions of neutral salts for their ability to recover albumins and globulins from cereals. The recovery of albumins and globulins from most of the cereals studied, with the notable exception of sorghum, were in agreement with predictions made from the lyotropic series. Lithium iodide, a neutral salt with good salting-in properties, did however recover more of both proteinase and carboxypeptidase activity from sorghum malt compared with the other neutral salts investigated. Further investigation of LiI in solutions of varying ionic strength showed that the amount of proteolytic activity recovered from sorghum malt after a single extraction was optimal with 0,6M LiI.

Comparison of the two extractants: 0,6M LiI and pH 7,0 buffer with respect to their influence upon the extractability of proteolytic activity revealed that both extractants had their advantages. The pH 7,0 buffer recovered a greater proportion of proteolytic activity after a single extraction, whereas 0,6M LiI succeeded in substantially reducing the proportion of inextractable proteolytic activity in the malt. In an attempt to combine the merits of these two extractants into one, a pH 7,0 buffer was adjusted to an ionic strength of 0,6M with LiI. This extractant was however not as successful as 0,6M LiI in reducing the proportion of inextractable proteolytic activity in sorghum malt.

Thirdly, the incorporation of reducing agent (DTT) with 0,6M LiI resulted in slight improvements in the recovery of proteinase activity from sorghum malt. Enari and Nikola (1967) extracted a highly unstable fraction of proteolytic activity from barley malt due to the incorporation of mercaptoethanol into their extraction procedure. They proposed that this activity could be the so-called insoluble proteolytic activity of barley malt described by Krinstad and Kilhovd (1955). Certain cereal proteins are

known to be extensively *crosslinked by intra*-molecular disulphide *bridges* (Wall, 1971). These proteins may only be solubilised after cleavage of disulphide bonds with reducing agents such as mercaptoethanol and dithiothreitol. Dithiothreitol plus 0.6M LiI did not further reduce the proportion of *either* proteinase or carboxypeptidase *activity in the residue*, compared with 0.6M LiI *alone*. It appeared therefore that cleavage of intermolecular disulphide bonds had influenced the solubility of the proteolytic enzymes without permanently affecting their activity, since *there* was no significant difference *between total proteinase* and *carboxypeptidase* activities of salt extracted in the presence and absence of DTT. Presumably any intermolecular disulphide bridges, which were cleaved and which must remain intact in order to retain proteolytic activity, were reoxidised during *dialysis* of the enzyme extracts.

The choice of extractant used for isolating proteolytic activity from sorghum malt not only influenced the amount of activity recovered after a single extraction. Progress curves of the development of enzyme activity in *germinating seeds* can be obtained by *monitoring enzyme* activity in seeds which have been *germinated* for different periods of time (Harvey & Oaks, 1974b; Aisien, 1982; Enari & Mikola, 1977). When different extractants were used to recover proteolytic activity from sorghum *malts* germinated for different times, *the resultant progress* curves were strongly *dependent* upon the choice of extractant. Use of 0.6M LiI plus DTT always extracted more proteolytic activity than the other two extractants investigated. The 0.05M acetate buffer, pH 5.0 plus cysteine.HCl was employed by Aisien *(1982)* as an extractant, in the study of proteolytic activity of *germinating sorghum*. *This* extractant indicated a 4-fold increase *in* proteinase activity during germination compared with the 2-fold increase observed with 0.6M LiI plus DTT. The extractants employed in this study did not solubilise the total *sorghum malt* proteolytic activity. However, *the fact* that 0.6M LiI plus DTT *succeeded in recovering more* proteolytic activity from sorghum malt than either acetate buffer or water at all germination times

indicated that it was a prudent choice of extractant.

The procedure finally chosen to extract proteolytic activity from sorghum malt, namely that which utilised 0,6M LiI plus DTT as an extractant, was not ideal, since not all the proteolytic activity was extracted in the first extract and a considerable proportion still remained in the residue after three extractions. However, compared with water and the extractant employed by Aisien (1982) *to isolate proteolytic activity from germinating* sorghum, considerable progress had been made both in terms of reducing the inextractable proportion of proteolytic activity and with respect to increasing the amount of activity *recovered after a single* extraction of sorghum malt.

Having developed an extraction procedure which improved the recovery of proteolytic activity from sorghum malt, it was necessary to verify that proteolytic activity was being assayed under optimum conditions. The proteinase activity of sorghum malt had a pH optimum in the range of pH 4,25 to 4,50. Other workers have found acidic pH optima for proteinase activity in germinating cereals. The pH optima of proteinase activity in germinating barley, sorghum, maize and wheat has been found to be *pH 3,8.* (Baxter, 1976), *pH* 3,9 (Garg & Virupaksha, 1970), 3,8 (Moureaux, 1979) and 4,0 (Preston & Kruger, 1976) respectively. The carboxypeptidase activity compared with proteinase activity, had a *relatively broad pH optimum in the range of pH* 4,0 to pH 5,25. This apparently broad pH optimum may have been due to the variability of the data. However it is also possible that this result was indicative of a number of different carboxypeptidase enzymes being present in sorghum malt. With barley malt, five different carboxypeptidase enzymes have been isolated with various acidic pH optima (Mikola, L., 1983). For the purpose of the assay procedure an incubation pH of 4,50 was chosen as being optimal for both proteinase and carboxypeptidase activity.

The selection of a suitable incubation temperature and reaction time for the assay of sorghum malt proteolytic activity was

complicated by the unusual nature of the progress curve, for both proteinase and carboxypeptidase activity. These progress curves were essentially biphasic in character for both proteinase and carboxypeptidase activity over the range of incubation temperatures studied.

In an attempt to explain these kinetics, the possibility that the proteolytic enzymes were not operating under conditions of substrate saturation was considered. The drop in reaction rate experienced after the initial, rapid phase might then be due to a depletion of substrate. However, from theoretical considerations (Dixon & Webb, 1979) it seemed unlikely that substrate limitation was the cause of the rapid release of product during the initial phase of reaction, since the degradation of substrate did not exceed 20% during an enzyme assay. The greatest extent of reaction during incubations of malt proteolytic activity, ie. the most product solubilised, never represented more than a seven per cent degradation of total kafirin. However TCA-soluble product is only an estimation of the extent of peptide bond cleavage, especially when the cleavage is due to proteinase action.

An alternative explanation for the drop in reaction rate during the first phase of the reaction was that temperature-labile proteolytic enzymes were being denatured. Such a hypothesis presupposes the existence of a multi-proteolytic enzyme system for sorghum malt, however such a system is not unreasonable considering that two proteinases and five carboxypeptidases have been identified in barley malt (Mikola, J., 1983). The existence of isozymes of alpha-amylase in barley malt characterised by differences in both pH and temperature optima has been demonstrated by Bertoft et al (1984). The rapid release of product during the initial phase of reaction could then be due to the combined action of both temperature-labile and temperature-stable proteolytic enzymes. And the second linear phase of reaction could then be due to the continued activity of temperature-stable proteolytic enzymes. Two criticisms of this explanation are that firstly the enzymes responsible for the

second phase of the reaction appear temperature-stable to the extent that they were not noticeably denatured after seven hours incubation at 60°C. Secondly, the enzymes denatured over the first phase of the reaction would have to be temperature-labile at 30°C.

Investigations into the thermal stability of sorghum malt proteolytic activity indicated that both proteinase and carboxypeptidase activity had virtually identical thermal stabilities. A possible explanation for this observation is that carboxypeptidase may be strongly dependent upon proteinase activity for the generation of its substate. Proteinase activity has been shown to be the rate-limiting step in the degradation of proteins to amino acids during the mashing of barley malt (Gopanen et al, 1968). In other words, barley malt carboxypeptidase activity was present in excess. If this was the case with sorghum malt, it could explain why the reaction kinetics of proteinase and carboxypeptidase activity were also similar.

Sorghum malt proteolytic enzymes appear to be temperature-stable at 30°C. Pre-incubation of the enzyme extracts for one hour at this temperature did not result in any appreciable loss of activity. The amount of product released at 30°C during the initial phase of both proteinase and carboxypeptidase reactions was however noticeably less than at higher temperatures. At this stage it was not possible to provide an explanation for the rapid release of product at 30°C, other than it did not appear to be due to either substrate limitation or to a thermal inactivation of isoenzymes.

The investigation into the temperature stability of sorghum malt proteolytic activity demonstrated that there was a rapid loss of activity with increasing temperature. This investigation was however conducted with the crude enzyme extract in the absence of excess substrate. It is known that in the presence of excess substrate, the temperature stability of enzymes increases (Dixon

& Webb, 1979; Godfrey, 1983). It appeared that this was the case for sorghum malt proteolytic enzymes. The net amount of product released during the initial, rapid phase of reaction was approximately equal over the temperature range of 45 to 60°C. *If in the presence of excess* substrate, the proteolytic enzymes were progressively denatured with increasing temperature, a concomitant reduction in the net amount of product released during the initial phase of reaction would be expected. Barley malt proteolytic enzymes, in the presence of excess protein have also been shown to be highly resistant to thermal denaturation. When germinating barley is kilned, there is no appreciable loss of proteolytic activity in 24 hours at 65°C (Baxter *et al*, 1978). However during mashing, in which the protein *concentration is such* less than in the germinating grain, all proteinase activity is destroyed in 15 min at 70°C (Kringstad & Ohlsen, 1957).

To summarise, it appears that thermal fractionation of proteolytic activity during incubation could explain in part the rapid release of product observed during the initial phase of proteinase and carboxypeptidase reactions. There is also good reason to suppose that some sorghum malt proteolytic enzymes may *withstand temperatures of 60°C for seven hours* without losing their activity. However the rapid release of product during the initial phase of reaction at 30°C was inexplicable in terms of thermal fractionation.

Considering the practicalities of the assay procedure, the problem of temperature-labile enzymes would be of academic interest, if the initial rate of reaction was used to measure both proteinase and carboxypeptidase activity. However, the *accurate measurement of initial reaction* rate was not possible. This was because the assay procedures for both FAN and TCA-soluble nitrogen were not sensitive enough to detect reliably the small amount of product released during the initial stage of the reaction. Thus it was decided to incubate sorghum malt proteolytic activity for a period of five hours at 50°C for

routine assays. These conditions were chosen since they resulted in a sufficient release of product to give reproducible determinations of both proteinase and carboxypeptidase activity, but it must be stated that they provided a measure of the net rate of reaction. With certain assay procedures, especially those which have industrial applications, it is however often impractical or meaningless to use the initial rate of reaction as a measure of enzyme activity (Fullbrook, 1983). The choice of 50°C as incubation temperature and five hours as incubation time was made for two reasons. Firstly, because it appeared that 50°C resulted in a faster, initial rate of reaction than did other temperatures. And secondly, because after an incubation time of five hours, an incubation temperature of 50°C gave rise to an amount of product release which could be reproducibly measured. Incubation temperatures of 37°C (Baxter, 1976), 40°C (Harvey & Oaks, 1974a) have been employed to assay proteolytic activity in germinating barley and maize respectively. These procedures have employed reaction times considerably less than five hours, the progress curves of product release have in addition been shown to be linear with respect to time.

An investigation into the influence of substrate concentration upon proteolytic activity determinations confirmed that progress curves of product release were biphasic in character. With increased levels of substate concentration, however there was an accompanying increase in the amount of product released during the reaction. Despite the observed increase in product release with increasing substrate concentration, the degradation of substrate after a 2h period at 50°C never represented more than approximately 6%. Since problems with substrate limitation are not generally encountered until 20% substrate degradation has been achieved (Dixon & Webb, 1979), these results were rather difficult to explain. In view of this it was decided to investigate conditions of substrate saturation indirectly by investigating the influence of enzyme concentration upon proteolytic activity.

Enzyme assays are only valid if the amount of enzyme used is directly proportional to its rate of reaction. This condition is only satisfied when the enzyme is operating under conditions of substrate saturation and the pH and temperature of the reaction remain constant (Wynn, 1973). At a substrate concentration of 5mg total kafirin per assay, the net rates of both proteinase and carboxypeptidase activity over five hours at 50°C were proportional to their respective concentrations. Since the assay procedure was operating under conditions of substrate saturation, why then did higher substrate concentrations have such a profound effect upon the amount of product released over the initial phase of reaction? In order to answer this question it was proposed that the purity of the total kafirin preparation used as a substrate should be investigated.

When total kafirin was incubated in the absence of proteolytic enzymes from sorghum malt, it gave rise to a release of TCA-soluble nitrogenous material. Because this release of nitrogen could not be detected in terms of FAN release, it was believed that the nitrogen was in the form of TCA-soluble, hence small, peptides rather than free amino acids. Under the pH and temperature conditions of the assay procedure for proteolytic activity, this release of peptides was linear with respect to time. It was therefore due to a different mechanism to that which controlled nitrogen release in the presence of proteolytic enzymes from sorghum malt. Because of the linear nature of this release of peptides from total kafirin with time, it could not therefore account for the rapid release of product previously observed during the initial phase of the proteinase reaction.

Attempts to determine whether the release of peptide material from total kafirin was due to an enzymic phenomenon were inconclusive. However, these investigations did indicate that a lactic acid/NaOH buffer, pH 3.0 resulted in an almost instantaneous release of peptide contaminants from total kafirin. It was believed that both the pH and the lactic acid component of

this buffer were responsible for its success in rapidly liberating peptides from total kafirin. Lactic acid has been used during industrial starch preparations since it dispersed the protein matrix of maize (Cox *et al*, 1944). Also sodium lactate/lactic acid buffers have been used extensively in the electrophoresis of prolamin proteins in cereals (Shewry *et al*, 1977). A sodium lactate/lactic acid buffer, pH 3,1, as used by Lookhart et al (1985) in the electrophoresis of prolamins, was used to extract peptide contaminants from total kafirin. This purification procedure resulted in a preparation of total kafirin which was apparently free from peptide contaminants. Apart from the extraction of peptide material however a water-soluble protein was also extracted from the total kafirin. This protein accounted for 3,5% of the nitrogen in the total kafirin preparation.

A protein has been isolated from preparations of maize by a similar procedure to that which was used for purifying total kafirin. This protein has been variously called water-soluble alcohol-soluble reduced glutelin (Paulis & Wall, 1977), reduced-soluble protein (Wilson *et al*, 1981), glutelin-2 (Ludvid *et al*, 1983) and proline-rich alcohol-soluble reduced glutelin (Esen *et al*, 1985). In keeping with a recent nomenclature for maize proteins (Wilson, 1987), this maize protein has been referred to as reduced-soluble protein (RSP).

Comparisons of the amino acid composition of the water-soluble protein extracted from total kafirin and maize RSP showed them to be virtually identical, except for the levels of glutamic acid. Because of this, the water-soluble protein extracted from total kafirin has also been called reduced-soluble protein. Furthermore the apparent M_r of the major component of maize RSP by SDS-PAGE has been reported in the range of 27 500 (Esen *et al*, 1981) to 28 000 (Vitale *et al*, 1982; Ludvid *et al*, 1984). Thus the apparent M_r of the predominant component of sorghum RSP was virtually identical to its maize counterpart. The fact that RSP, a protein with a

rather unusual amino acid composition, occurs in sorghum as well as maize, adds further credence to the suggestion that it has an important physiological function that has constrained changes in composition during evolution (Esen et al, 1982).

With respect to the assay procedure, the discovery of RBP in preparations of total kafirin meant that unwittingly an impure protein preparation had been employed as a substrate for sorghum malt proteolytic activity. Using total kafirin, from which RBP had been exhaustively extracted, as a substrate showed that at 50°C the formation of TCA-soluble nitrogen by sorghum malt proteinase activity still appeared to be biphasic. However at 30°C the release of TCA-soluble nitrogen was linear with respect to time. This implied that the rapid release of TCA-soluble nitrogen previously encountered with sorghum malt proteinase activity at 30°C was due to the presence of RBP in the total kafirin.

The reaction kinetics of the sorghum malt proteolytic enzymes in the absence and presence of RBP suggested that RBP was degraded in preference to total kafirin. The exponential nature of the release of nitrogen in the presence of RBP could then be due to RBP becoming increasingly substrate limiting. In support of this explanation the amount of nitrogen released during the exponential phase was approximately equal to the amount of nitrogen in the total kafirin preparation that was accounted for by RBP.

The exponential phase of product release was evident at an incubation temperature of 50°C both in the presence and absence of RBP in total kafirin. It therefore appeared that in crude enzyme extracts of sorghum malt there were proteolytic enzymes that were thermolabile even under conditions of substrate saturation. It would therefore seem probable that the exponential phase of product release originally observed with higher temperatures was due to the preferential degradation of RBP, together with an inactivation of some of the proteolytic

enzymes.

The progress curve of FAN release at 30°C with purified total kafirin showed a marginal deviation from linearity. Why carboxypeptidase activity should exhibit reaction kinetics which apparently differ from those of proteinase activity after a previous period of parity is not understood. However it is possible that the breakdown products of purified total kafirin by proteinase enzymes provided a complex substrate which complicated the reaction kinetics of carboxypeptidase activity.

The extractability of sorghum malt proteolytic activity from different sorghum malts showed that although the total activity was not extracted after a single extraction, the amount recovered did represent a fixed proportion of the total activity. Approximately 45 per cent of the total proteinase and carboxypeptidase activity was recovered from sorghum malt after a single extraction.

Significant differences in both proteinase and carboxypeptidase activity could be attributed to cultivar effects. Furthermore, cultivars which resulted in high levels of proteinase activity did not necessarily result in high levels of carboxypeptidase activity and vice-versa. This suggested that the levels of proteinase and carboxypeptidase activity achieved during malting were under independent control. It was not possible however to conclude that differences in the levels of proteolytic activity were due to a cultivar-related phenomenon of sorghum. This was because the experiment did not investigate either the influence of the environment or the season upon the proteolytic activity of sorghum malt prepared from different cultivars. Dalbar (1980) however has demonstrated that the diastatic power of sorghum malt is a cultivar-related phenomenon. Morgan *et al* (1983) have also observed significant varietal differences for endoprotease activity in barley malt, but were also unable to attribute this conclusively to a cultivar-related phenomenon.

Concerning the influence of malting conditions, the methods of germination employed were different from those reported in the literature so that it is practically meaningless to make direct comparisons between the results of this study and those of other workers. Any comparisons that are drawn are based solely upon generalised trends.

In contrast with barley, there was no increase in either proteinase or carboxypeptidase activity during steeping. During steeping of barley, proteinase and carboxypeptidase activity increased by several fold (Narziss & Friedrich, 1970). However the raison d'etre for preparing the malts used in this study was to investigate the influence of germination conditions upon the development of sorghum malt properties which are of interest to brewers. The steeping conditions could therefore be suboptimal (Morrall, personal communication).

During germination, a less than two-fold increase in proteinase activity was observed. This increase was somewhat less than the 2,5-fold increase in proteinase activity of germinating sorghum reported by Garg and Virupaksha (1970). The proteinase activity of barley malt increased by about 20-fold during germination (Enari & Mikola, 1977).

The mechanism by which proteolytic activity increases in germinating sorghum is not known. In contrast to germinating barley however it appeared that more than half of the maximum observed solubilised proteinase activity in sorghum malt was already present in the resting grain. The proportionately small increase in proteinase activity observed during sorghum germination could explain why the conditions of temperature and moisture did not influence the proteinase activity development.

In sharp contrast to proteinase activity, the carboxypeptidase activity of sorghum malt only appeared in appreciable quantity after the onset of germination. The carboxypeptidase activity of resting sorghum grain was almost too low to detect. During

germination, however up to an 8-fold increase in activity was observed. The rate and extent of this increase was dependent upon the germination temperature, moisture and time. A rapid, large increase in exopeptidase activity has also been observed in germinating sorghum by Aisien (1980). In germinating barley, Baxter *et al* (1978) have observed that peptidase activities were generally lower at lower levels of moisture. With respect to the influence of temperature upon the development of proteolytic activity in germinating barley, it has been observed that in contrast to sorghum the development of endopeptidase activity rather than carboxypeptidase activity was influenced more by temperature (Baxter & O'Farrell, 1980). Baxter and O'Farrell explained this observation in terms of the requirement for protein synthesis of endopeptidase , whereas carboxypeptidase was already present in the resting barley grain in an inactive form.

This investigation showed that sorghum germinated for four days at 24°C under medium moisture conditions produced malts with optimal levels of proteinase and carboxypeptidase activity. In an earlier investigation, the same malts that were used in this study were analysed for diastatic power and FAN (Morrall *et al*, 1986). Both diastatic power and FAN are malt properties which are of importance to brewing performance. At present proteolytic activity is not a property by which malt quality is assessed. This is because up until now it has not been possible to investigate the relationship between malt proteolytic activity and brewing performance. However the germination conditions which produced malts with optimal proteolytic activities also had levels of diastatic power and FAN which would be acceptable to brewers. These germination conditions will be of direct benefit to sorghum maltsters if future investigations demonstrate that high proteolytic activity leads to better brewing performance.

6. CONCLUSIONS.

A procedure has been developed by which the proteolytic activity of sorghum malt can be assayed. This assay is an improvement on previous methods of determining proteolytic activity in sorghum malt for a number of reasons. Firstly, the inextractable nature of sorghum malt proteolytic enzymes has been recognised. In view of this an extraction procedure has been developed which considerably improved the recovery of proteolytic activity from sorghum malt compared with previous choices of extractant.

Secondly, the natural substrate of the proteolytic activity, total kafirin, has been used for the assay. Initially, the use of total kafirin as substrate presented problems, since the procedure used for its preparation also extracted a water-soluble protein from sorghum. This protein had not previously been isolated from sorghum. However, it has now been identified as sorghum RSP since its amino acid composition and apparent M_r were almost identical to the RSP found in maize.

Sorghum malt proteolytic activity appeared to have a greater affinity for sorghum RSP than for total kafirin. This resulted in unusual reaction kinetics due to the apparent preferential degradation of RSP over total kafirin by the proteolytic enzymes. Through employing total kafirin from which RSP had been extracted, the problems with the reaction kinetics have largely been resolved.

Thirdly, the products of the reaction have been measured in terms of the amount of nitrogen released. Thus it is possible to quantify the actual extent of substrate degradation by the enzymes. This is not possible with a number of assays commonly in use for measuring proteolytic activity in malted cereals. Full experimental details for the recommended procedure for assaying proteolytic activity in sorghum malt are given in the appendix.

This procedure for assaying proteolytic activity has been applied to determine how the conditions of malting influence the development of proteolytic activity in sorghum malt. Of great practical importance, the malting conditions which favoured the development of maximal levels of proteinase and carboxypeptidase activity also resulted in malts of good quality for brewing. Furthermore, these malts were produced under conditions which did not require either excessive germination times or moisture treatments. Thus it is probable that malts can be produced on an industrial scale which possess both high levels of proteolytic activity together with specified levels of other malt quality parameters, eg. diastatic power and free alpha-amino nitrogen, which are of importance to brewing performance.

This assay for sorghum malt proteolytic activity will provide a useful tool for the Sorghum Beer Industry. However in order for the industry to derive maximum benefit from its application, ultimately the assay must be applied to determine precisely how malt proteolytic activity affects brewing performance.

APPENDIX. Recommended procedure for determining proteolytic activity in sorghum malt.

Preparation of total kafirin.

Sorghum grain was passed through a rice-pearling machine until 20% by weight had been removed. Pearled sorghum was ground in a DLFU disc mill (Buhler-Miag, Braunschweig, W. Germany) at a disc gap of 0,2mm. Flour (1kg) was extracted, by constant stirring, for 1h periods with three successive 5l aliquots of distilled water at 4°C, to remove the albumins, globulins and low molecular weight nitrogen. After each extraction, a clear supernatant was obtained by centrifugation at 16 000g for 10 min. This supernatant was then decanted and discarded. After the water extraction, the residue was extracted, by constant stirring, at room temperature for two 1h periods and then overnight with three successive 5l aliquots of 60% (v/v) tert-butanol plus 0,05% (m/v) dithiothreitol (DTT). Clear supernatants were obtained after each extraction by centrifugation at 16 000g for 10 min. The supernatants were combined and freeze-dried directly. The freeze-dried material (Mg) was suspended in a volume (5xM ml) of 0,02M Na lactate/lactic acid buffer, pH 3,1. The suspension was then exhaustively dialysed against 0,02M Na lactate/lactic acid buffer, pH 3,1. The suspension was then recovered and centrifuged at 10 000g for 10 min. The resultant supernatant was then collected and discarded. This extraction/dialysis procedure of the freeze-dried material was repeated at least three times. Finally the residue was suspended in distilled water and exhaustively dialysed against distilled water at 4°C. The suspension was then freeze-dried directly. The resultant powder constituted the total kafirin preparation. The protein content of the total kafirin was determined.

Extraction of proteolytic activity from sorghum malt.

Sorghum malt was milled in a "beater-type", water-cooled coffee mill (Janke & Kunkel, Staufen, W. Germany) for two 30s periods. The moisture content of the resultant flour was determined. Flour (1g) was extracted in duplicate, by constant stirring, for 1h at 30°C with 0,6M Li1 plus 3,33mM dithiothreitol (15ml). The malt suspension was then centrifuged at 16 000g for 10 min. The liquid fraction was recovered and centrifuged again at 16 000g for 10 min to obtain a clear supernatant.

The resultant supernatant was dialysed against water (3l) for 16h at 4°C. The dialysed solution was made up to volume (25ml) with water. The resultant extract was assayed for proteinase and carboxypeptidase activity.

Proteinase and carboxypeptidase assays.

Enzyme extract (1ml), total kafirin (5,0mg protein) and 0,1M citric acid/0,2M K_2HPO_4 buffer, pH 4,50 (1ml), was incubated in duplicate for 0h and 5h at 30°C. Reactions were stopped with 15% (m/v) TCA (1ml). Incubation mixtures were then immediately centrifuged at 18 000g for 10 min. The supernatants were then collected and stored at 4°C until analysed for TCA-soluble nitrogen and FAN.

The nitrogen and FAN contents of duplicate supernatants were assayed. Proteinase activity was calculated from the difference between the TCA-soluble nitrogen content of the 5h and 0h supernatants and expressed in terms of µgN/5h/g dry malt. Carboxypeptidase activity was calculated from the difference between the FAN content of 5h and 0h supernatants and expressed in terms of µgFAN/5h/g dry malt.

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