International Journal of Pure and Applied Science, Volume 3, Number 1, 2010

KINETIC STUDIES ON MALT AMYLASES AND PROTEASES OF A LOCAL VARIETY OF RICE

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ABSTRACT

Kinetic properties of the amylases and proteases of a local variety of dehusked unpolished rice grains ("Mottaikaruppan" variety) germinated for 4 days were studied. The optimum pH and temperature for the activities of malt amylase and protease were 5.0 & 7.2 and 60 & 50°C respectively. Under optimized conditions, the malt amylase and protease showed zero order kinetics for 25 and 165 minutes, respectively. The Km of the malt amylase and protease were 4.5 (Soluble starch) and 2.5gL^{-1} (Casein) respectively. The malted rice supernatant contained amylase and amyloglucosidase activities. Malt amylases of rice were stable over a wide range of pH (pH 4.0-8.0). Addition of 0.1gL^{-1} $CaCl_2$ to 0.01M acetate buffer (pH 5.0), enhanced the malt amylase activity. Malt amylase was stable at 4, 30 and 50°C for 3 days retaining 84.4, 84.4 and 51.6% of the initial activities respectively. Suitable pH for the storage of rice malt amylase was 5.5 at all the temperatures studied. Presence of calcium ions enhanced the thermostability of the rice malt amylase.

Keywords: Malting; Cereal Malts; Malt Amylases; Malt Proteases; Enzyme Kinetics; Enzyme Stability

INTRODUCTION

Cereal malts are rich in enzymes and soluble materials, which could be used in food industry (Finney, *et al.*, 1972). Malting of different cereals in food and brewing industries is in practice (Daussant, 1994). Malting of cereals like oat, millet, corn, wheat and rice (Sivaganeshan, *et al.*, 1993) has been studied. The kinetic properties of the enzymes of wheat (Lineback & Ponpipom, 1977), oat, barley (Briggs, 1963), corn (Balasubramaniam & Mahendran, 1990) and millet (Lineback & Ponpipom, 1977) have been studied extensively. A local variety of rice called "Mottaikaruppan", which has not been studied before was selected. Malting of rice grain could be carried out as required and quickly if the optimum conditions for the enzymes are studied. Further these enzymes also could be utilized in the food industries under optimized conditions. Malting would affect the properties of starch of the cereal flour (Kaminindski & Bushuk, 1969) and the hydrolysis of cereal proteins has not shown significant effect on cereal flour properties (Kaminindski & Bushuk, 1969). Thus during malting, while using the malt amylases, the interference of malt proteases need to be avoided to preserve the malt amylases from the proteolytic attack by malt proteases. Thus studying the kinetic properties of carbohydrases and proteases are essential. In this paper kinetic properties of endogenous □-amylase and proteases of the malted rice were studied in view of utilizing the malted rice and its enzymes in food industry.

MATERIALS AND METHODS

Materials

A variety of rice grain, "Mottaikaruppan" a local land variety to the Northern region of Sri Lanka was used in this study. The paddy was purchased from local market and dehusked for this study. Casein (sodium salt), p-Nitrophenyl-□-D-glucopyranoside (Catalogue No. N-1377) and Naringin (Catalogue No. N-1376) were from Sigma Chemical Company, USA. Soluble starch (Product No.30264, GPR) was from BDH Chemicals Ltd., UK. All the other chemicals used were of analytical grade. All the experiments were carried out in triplicate.

Malting of unpolished rice and Preparation of malted rice powder

Dehusked unpolished rice grains (10.0g) were steeped in distilled water (100.0mL) containing 0.1gL^{-1} $Na_2S_2O_5$ in a beaker for 12h and germinated in a moistened bag wetted with 0.15gL^{-1} $Na_2S_2O_5$ and kept in dark at 30°C. Germination of the dehusked unpolished rice grains was arrested on the 4th day, dried at 35°C to constant weight and powdered at room temperature in a domestic grinder. This powder was used for the extraction of malt enzymes.

Extraction and determination of amylases and proteases of malted rice powder

Rice malt amylase was extracted by mixing 1.0g of malted rice powder samples with $10gL^{-1}$ NaCl (10.0mL) for 15min at 30°C and centrifuging (MSE bench centrifuge) at 3000rpm for 5minutes. The supernatant was analysed for amylase activity (Bernfield, 1951). One unit of rice amylase (U) is the amount of enzyme, which releases 1.0μmole of glucose from $40gL^{-1}$ soluble starch in 1min at pH 5.0 and 60°C. The activity of malt amylase is presented as Ug^{-1} dry malt powder. Malt protease was extracted similar to malt amylase with 0.03M phosphate buffer (pH 7.5, 5.0mL) and the malt extract supernatant was analysed for protease activity (Anson, 1938). One unit of rice protease (U) is the amount of enzyme that liberates 1.0μmole of tyrosine from $10gL^{-1}$ casein in 1h at pH 7.2 and 50°C. The activity of malt protease is presented as Ug^{-1} dry malt powder.

Kinetic properties of rice malt amylase

Amylolytic activity of rice malt amylase with time

Pre-incubated starch ($40gL^{-1}$)-0.02M acetate/acetic acid buffer solution (12.50mL, pH 5.0) and distilled water (11.25mL) were mixed with pre-incubated 1.25mL malted rice powder extract supernatant and incubated at 60°C. At definite time intervals samples were withdrawn and reducing sugar formed was determined (Miller, 1959).

Effect of pH on amylolytic activity of rice malt amylase

Starch ($40gL^{-1}$) – water solution (0.45ml) was mixed with 0.01M citrate – 0.02M phosphate buffer (0.50mL) of varying pH values (3.0-7.0) and malted rice extract supernatant (0.05mL). They were incubated for 10min at 60°C and the amylase activity was determined (Bernfield, 1951).

Effect of temperature on amylolytic activity of rice malt amylase

Soluble starch ($40gL^{-1}$) – 0.02M acetate/acetic acid buffer solution (pH 5.0, 0.50mL) and distilled water (0.45mL) were incubated with rice malt amylase (0.05mL) at different temperatures (4-80°C) for 10min and the amylase activity was measured (Bernfield, 1951).

Determination of Km and Vmax of rice malt amylase

Varying concentrations of soluble starch - 0.02M acetate/acetic acid buffer (pH 5.0, 0.95mL) solutions were incubated with rice malt amylase (0.05mL) at optimum temperature for 10minutes. The activity of amylase was measured and Km and Vmax values were calculated from Lineweaver Burk plot.

Effect of calcium ion on rice malt amylase activity

Soluble starch ($40gL^{-1}$) – 0.02M acetate/acetic acid buffer solution (pH 5.0) containing different concentrations of $CaCl_2$ ($0\text{-}0.5gL^{-1}$) was prepared and the amylase activity was determined under optimized conditions.

Identification of different carbohydrases of malted rice powder using specific substrates

Activities of different possible carbohydrases present in the malted rice flour extract were studied by using p-Nitrophenyl-alpha-D-glucopyranoside, soluble starch (Bernfield, 1951) and naringin (Ore, *et al.*, 1977) as substrates.

Storage stability of rice malt amylase extracted from rice malt powder

a) Effect of temperature on the stability of rice malt amylase

Supernatant of the malted rice powder extract (pH 6.17) containing 0.02% sodium azide was pre incubated at different temperatures (4, 30, 50, 60 and 70°C) and at definite time intervals samples were analysed for amylolytic activity (Bernfield, 1951).

b) Effect of pH on the stability of rice malt amylase

Supernatant of the malted rice powder extract containing 0.02% sodium azide at different pH values were pre incubated at different temperatures (4, 30, 50 and 60°C). Samples were withdrawn and analysed for amylolytic activity (Bernfield, 1951).

c) Effect of calcium ion on the stability of rice malt amylase

Malt extract in acetate/acetic acid buffer (pH 5.0) containing different concentrations of $CaCl_2$ and 0.02% sodium azide were pre incubated at different temperatures (4, 30 and 50°C). Controls were prepared without $CaCl_2$. On the fourth day, samples were withdrawn and amylase activity was determined (Bernfield, 1951).

Kinetic properties of rice malt protease

Rice malt protease activity with time

Supernatant of malted rice powder extract (20.0mL) was incubated with casein ($10gL^{-1}$) - 0.1M phosphate buffer (100mL, pH 7.2) at 50°C. Aliquots (3.0mL) were removed at different time intervals and proteolytic activity was determined (Anson, 1938).

Effect of pH on the proteolytic activity of rice malt protease

Casein ($20gL^{-1}$) – water solution (0.5mL) mixed with 0.1M phosphate buffer (2.0mL) of varying pH values (6.0-8.0) and incubated with supernatant of the malted rice powder extract (0.5mL) for 1h at 50°C. The proteolytic activity was determined as above.

Effect of temperature on the proteolytic activity of rice malt protease

Casein (sodium salt, $10gL^{-1}$) – 0.1M phosphate buffer (pH 7.2) solution (2.5mL) was incubated with malted rice supernatant (0.5mL) at different temperatures (30-100°C) for 1h and then the enzyme activity was measured.

Determination of Km and Vmax of rice malt protease

Varying concentrations of casein (sodium salt) - 0.1M phosphate buffer (pH 7.2) solutions (2.5mL) were incubated with rice malt protease (0.5mL) at optimum temperature for 1h and enzyme activity was assayed as described earlier.

RESULTS AND DISCUSSION

Kinetic properties of malt amylase of malted rice powder

Amylolytic activity of rice malt amylase with time

Malt amylase showed linear activity with $40gL^{-1}$ starch - 0.02M acetate buffer solution (pH 5.0) for 30min at 60°C (Figure 1). The drop in activity after 25min may be due to an inhibitory effect of the products formed or depletion of substrate, or a combination of both factors or denaturation of the enzyme. The digestive gland ∝-amylase activity of the tropical green mussel (*Perna viridis L.*) appears to remain linear for 1h (Sabapathy & Teo, 1992). The amylases from *Bacillus licheniformis* and *Aspergillus niger* showed linear activity for 10 and 50 minutes respectively (Venkadaramana, *et al.*, 2004 and Jansz, *et al.*, 1977). Therefore the malt amylase of Mottaikaruppan variety rice has shown the zero order kinetic for longer time than bacterial and lesser time than that of fungal and *Perna viridis L.* amylases (Sabapathy & Teo, 1992 and Jansz, *et al.*, 1977). It was decided to select the reaction time as 10min to maintain the zero-order kinetics through out the entire assay procedures.

Effect of pH on amylolytic activity of rice malt amylase

When the pH was increased from 3.0 to 4.0, sudden increase in the activity was observed ($101.0Ug^{-1}$ dry matter) and thereafter the activity of the enzyme increased gradually showing highest activity at pH 5.0 (Figure 2). At pH 7.0, 74.2% of the activity of pH 5.0 was expressed. Having a wide range of pH optimum is useful in using the malted rice with different carbohydrases and synergistic effects of the enzymes could be used efficiently in industrial food preparations (Fujii, *et al.*, 1988; Arasaratnam & Balasubramaniam, 1992 and Arasaratnam & Balasubramaniam, 1993). The pH optimum of corn malt amylase was 4.0 (Balasubramaniam & Mahendran, 1990) and that of sorghum was in the range of 3.7 to 4.0 (McCleary & Sheehas, 1987). The optimum pH for the activity of wheat flour α-amylase with BPNPG 7 was pH 5.2 (Michelenea and Castillo, 1984). Optimum pH of this enzyme falls within the optimal pH range of the activities of cereal amylases (pH 4.0-6.0). A wide range of pH optimum for the malt amylase activity shows the presence of more than one carbohydrase in the malted rice.

Effect of temperature on amylolyitc activity of rice malt amylase

Activity of rice malt amylase increased with the increase in temperature up to 60°C, followed by a rather sharp fall in activity (Figure 3). This indicated a quick denaturation of the enzyme by heat beyond 60°C. The optimum temperature for corn malt amylase activity was 50°C at pH 3.5 for 5min (Balasubramaniam &

Mahendran, 1990) and that of green mussel (Mollusc) digestive gland α-amylase was 43°C at pH 5.8 (Sabapathy & Teo, 1992). Optimum conditions for the activity of endogenous amylase obtained from malted paddy (where the variety was not specified) reported as 50°C at pH 4.5 (Sivaganeshan, *et al.*, 1993). The temperature optimum of malt amylase from Mottaikaruppan rice was higher than those of corn (Balasubramaniam & Mahendran, 1990) and paddy (Sivaganeshan, *et al.*, 1993). Thus this temperature optimum may be unique to this local variety of rice, which grows well in the dry zone of Sri Lanka.

Determination of Km and Vmax of rice malt amylase

When the concentration of the substrate (starch), was increased from 4-20gL^{-1}, there was a linear increase in amylase activity up to 5.0gL^{-1} of starch. The Km and Vmax values determined by Lineweaver-Burk plot of rice malt amylase for starch were 4.5gL^{-1} and 1278.77Ug^{-1} dry matter at pH 5.0 and 60°C. The Km values of ∝-amylase produced by *Aspergillus foetidus* were 1.14, 2.19 and 7.65$mgmL^{-1}$ for amylopectin, soluble starch and amylose respectively (Violet & Munier, 1989). The Vmax values for these substrates were 313, 606 and 1748mg/mg of protein/min respectively. As the amylase of malted Mottaikaruppan rice showed higher Km value, it can be used with higher substrate concentration in food industry than the amylases reported from *Bacillus licheniformis* 6346 (Venkadaramana, *et al.*, 2004) and *Aspergillus foetidus* (Violet & Munier, 1989).

Effect of calcium ion on rice malt amylase activity

Ca^{2+} is necessary for the structural integrity of α-amylase (Sirou, *et al.*, 1990). α-Amylases are known to contain at least one Ca^{2+} ion per molecule (intrinsic Ca^{2+}), which is involved in the stabilization of the molecular structure. This study was carried out to find whether addition of Ca^{2+} ions has any effect on the activity of the rice malt amylase. When the concentration of $CaCl_2$ was increased from 0 to 0.1gL^{-1} the enzyme activity increased from 118.14 to 120.05Ug^{-1} dry matter and remained the same with 0.2gL^{-1} $CaCl_2$ (Figure 4). Further increase of Ca^{2+} ions decreased the enzyme activity. Hence, maximum activity was obtained with 0.1 and 0.2gL^{-1} $CaCl_2$. The enzyme activity obtained in the absence of calcium ions was 2.39% less than the highest enzyme activity obtained with 0.1gL^{-1} $CaCl_2$. This result indicated that Ca^{2+} is not essential for the activity of malt amylases. However Ca^{2+} may be important for the stability of the enzyme. As the malted cereals have proteases in addition to amylases, addition of Ca^{2+} may be useful to preserve the amylase in malt enzymes extract supernatant, if they are not purified. Thus it is essential to study the malt amylase stability in presence of Ca^{2+}.

Using specific substrates to identify the type/s of amylase/s of malted rice powder

Malted cereals have shown □-amylase, □-amylase and amyloglucosidase activities (Forgarty, 1983). These three enzymes can act on starch but produce different products and their mode of actions are also different (Solomon & Levin, 1974). Among the several amylases, presence of α-amylase and β-amylase can be detected by the method reported by Bernfeld (1951). This method measures the reducing sugars produced by the amylolytic activity by allowing the reducing sugars to react with alkaline 3, 5-dinitro salicylic acid to form a coloured complex. While this test responds primarily to β-amylase, it also includes the products of α-amylase (Ramachandran, 1979). In the present study, 129.9Ug^{-1} dry matter malt amylase activity (both □- and □- amylases) was obtained when soluble starch was used as the substrate and enzyme activity was assayed at pH 5.0 and 60°C (Table 1). Here the activity was measured based on the method of Bernfeld (1951). Enzyme activity of 7.5Ug^{-1} dry matter was obtained with para-nitrophenyl-α-D-glucopyranoside (5gL^{-1}) at pH 5.0 and 60°C (Table 1). The para-nitrophenyl-α-D-glucopyranoside has terminal α, 1-4 link, which is not acted upon by α-amylase whereas amyloglucosidase being an exo-enzyme, hydrolyses it (Ramachandran, 1979). The presence of amyloglucosidase in the malted rice powder is confirmed by this experiment while it was 5.46% of the total amylase activity present in the malted rice powder. Naringinase is an enzyme mixture contains both α- rhamnosidase and β- glucosidase, and used for the hydrolysis of the substrate Naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside). The malt amylase enzyme of malted rice powder has not shown activity with the substrate Naringin (Table 1). This result confirmed the rice does not produce naringinase activity during malting.

Storage stability of malt amylase extracted from malted rice powder

a) Effect of temperature on the storage stability of rice malt amylase

Storage stability of rice malt amylase (pH 6.17) in the malted rice supernatant, extracted with 10gL^{-1} NaCl at 30°C was studied at 4, 30, 50, 60 and 70°C with time (0, ½, 1, 1½, 2 and 3h). The enzyme stored at 4, 30 and 50°C were more stable than those stored at higher temperatures (60 and 70°C). The enzyme retained 100%

activity at 4, 30 and 50°C at 3h (Figure 5). The enzyme lost 10 and 100% of the initial activity at 60 and 70°C respectively at 3h. Incubation of thermophilic amylolytic enzymes of *Aspergillus niger* (in the absence of the substrate) at 60°C resulted in 18 and 64% loss of its activity at 30 and 60min respectively, while at 70°C, 80% loss within 30 minutes (Gimbi & Kitabatake, 1968). Thermo stability studies on malt enzymes extracted from germinated African finger millet showed that malt extracts incubated at 40 and 50°C up to 4h retained 84 and 64% of α-amylase activities, respectively while more than 90% of the activity was lost 70 and 90°C when incubated for 40 and 10min, respectively (Balasubramaniam & Arasaratnam, 1989).
The enzyme stored at 4 and 30°C retained almost 85% of its initial activity on the third day (Figure 6) while that stored at 50°C lost 51.6% of the initial activity on the third day. The enzyme stored at 60 and 70°C, lost the total activity on the second and first days, respectively. These results indicated that at high temperatures (60 and 70°C) the rice malt amylase become labile and lost its activity completely. This enzyme is highly stable at 4 and 30°C without much loss in the enzyme activity and fairly stable and active at 50°C. Even though this enzyme is better than amyloglucosidase (which is unstable at 50°C (Balasubramainam and Arasaratnam, 1989), it is not stable as thermostable α-amylases. Studies on the malt amylase from corn flour also showed that it is more stable at 4°C than higher temperatures (Hagenimana, *et al.*, 1994). The corn malt amylase stored at 40 and 50°C lost its activity completely on the second and first day, respectively. The enzyme, which was kept at 30°C, lost 70% of its activity on the second day. Thus the malt amylase from Mottaikaruppan rice is more stable than the corn amylase (Hagenimana, *et al.*, 1994). Further it was decided to study the pH stability of malt amylase extracted from malted rice powder.

b) Effect of pH on the storage stability of rice malt amylase

Effect of pH on the storage stability of the α-amylase at 4, 30, 50 and 60°C was studied. Except at pH 4.5, the enzyme retained more than 90% of the initial activity compared with other pH values (5.0, 5.5 and 6.2) up to fourth day, when stored at 4°C (Table 2). Relative enzyme activity of about 96.9, 92.1 and 95.9% were observed at pH values 4.5, 5.0 and 5.5 respectively on the fourth day at 30°C, while the activities decreased at other pH values. The enzyme stored at pH 4.0 lost 28% of its initial activity, while that stored at pH 6.2 lost 10.8% of the initial activity on the fourth day (Table 2). At 50°C, at all pH values the enzyme lost fairly high amount of activity compared to that stored at 4 and 30°C (Table 2). The enzyme stored at pH 5.0 and 5.5 retained relatively high amount of activity 65.3 and 79.2% respectively. The malt amylase enzyme stored at pH 4.0 and 4.5 at 60°C lost the total activity on the first day. On the second day, the enzyme stored at pH 5.0, 5.5 and 6.2 at 60°C exhibited only 6.93, 8.16 and 7.84% relative activity, respectively (Table 2). These results revealed that the suitable pH for the storage of rice malt amylase is pH 5.5 at all the temperatures studied. This enzyme stored at pH 5.5 and at 60°C showed 8.16% relative activity on the second day. Similarly, when stored at pH 5.5 and at 4, 30 and 50°C showed relative activity of 96.9, 95.9 and 79.2% respectively on the fourth day. Earlier studies on temperature stability showed that the rice malt amylase enzyme was not found to be thermo stable at higher temperatures and it is more stable at low temperatures (4 and 30°C). In this experiment too, the enzyme stored at pH 5.5 and temperatures 4 and 30°C retained more activity on the fourth day when compared to other pH values and temperatures studied. Hence, the suitable condition for the storage of this enzyme is pH 5.5 at 4 or 30°C. Sweet potato α-amylase retained its activity in the pH range from pH 5.0 to 9.0, at 24h and at 4°C (Hagenimana, *et al.*, 1994). Studies on the pH stability of barley malt α-amylases at pH 3.6 and 30°C showed that the α-amylase I was very stable at this pH and at 5h 85% activity was retained, while α-amylase II was totally inactivated at 5 minutes (Fischer & Stein, 1960). However, addition of Ca^{2+} stabilized α-amylase II at low pH. Hence it was decided to study the effect of calcium ions on the storage stability of the rice malt amylase obtained from the malted rice powder of Mottaikaruppan rice.

c) Effect of calcium ions on the stability of rice malt amylase

Calcium ions improve the thermo stability of α-amylase by binding with the protein forming a tight intramolecular metal-chelate structure and stabilize the three-dimensional structure. Therefore folding of the enzyme is prevented (Sirou, *et al.*, 1990). Calcium performs a dual function: on the one hand, it maintains the protein in the proper configuration for biological activity; on the other hand, it stabilizes the secondary and tertiary structure, thus conferring to the amylase molecule its compact architecture (Bertoft, *et al.*, 1984). Addition of $CaCl_2$ of different concentrations has shown variation in the stability of malt amylase (Figure 7). The enzyme stored at 4°C was most stable without $CaCl_2$ followed by that with 0.01M $CaCl_2$ than with 0.001 and 0.1M $CaCl_2$. Similar observation was made at 30°C but the stability in presence of 0.1M $CaCl_2$ was almost same as that in the absence of $CaCl_2$. But at 50°C the enzyme stability was best in presence of 0.01M $CaCl_2$ followed with 0.001M and 0.1M when compared to the absence of $CaCl_2$. Therefore the

results indicted that at higher temperatures the enzyme was stabilized by Ca^{2+} (Figure 7). However at all the temperatures studied 0.01M $CaCl_2$ was the best followed by 0.001M $CaCl_2$. Further it can also be observed that at 4 and 30°C no calcium was needed to stabilize the enzyme. Sweet potato α-amylase was very heat labile in the absence of Ca^{2+} ions (Bertoft, *et al.*, 1984). Our observations can also be explained in the following manner. The experiments carried out with different substrates have shown that the rice malt extract supernatant contained more than one fraction of carbohydrases. Thus the fraction which was stable/ active at lower temperatures may not need calcium for its stabilization while the fraction which was stable/ active at higher temperature needed Ca^{2+} for its stabilization. Therefore studies have to be done in detail to find the difference in the effects of $CaCl_2$ at lower and higher temperatures on the rice malt amylases.

Kinetic properties of malt protease of malted rice powder

Germination studies on dehusked unpolished rice grains of Mottaikaruppan rice revealed that even though ungerminated rice grains have not shown proteolytic activity during germination process the amount of malt protease started to increase gradually from the second day of germination (Miller, 1947). The proteolytic activity of flour from wheat was insignificant as far as the functional properties of the flour are concerned (Kaminindski & Bushuk, 1969) and hence, very little is known about the distribution or the molecular properties of these enzymes in malted cereal flour. In this studies we have given due consideration for the properties of the malted rice protease because the protease has not been characterized in the particular rice variety selected. Further the proteases should not affect the malt amylase if they are present together with the enzyme in the same extract obtained from malted rice. Thus if the pH and temperature optima for malt protease and amylase are different, it will be an additional advantage when applied in the food industry.

Proteolytic activity of rice malt protease with time

Malt protease showed linear activity with casein ($10gL^{-1}$) - 0.1M phosphate buffer (pH 7.2) for 165min at 50°C (Figure 8). Proteolytic activity of malted wheat flour was not linearly related to the time used for digestion (Senthuran, 1997). Proteolytic activity of acid protease obtained from mouldy bran showed linear relationship with reaction time up to 10min with casein (1%, w/v) at pH 6.5 and 30°C (Terp, *et al.*, 2000). As the malted rice protease showed zero order kinetics for 165min, it was decided to fix the incubation (hydrolysis) time for 1h.

Effect of pH on proteolytic activity of rice malt protease

The influence of pH on proteolytic activity of rice malt protease was studied using casein ($20gL^{-1}$) - 0.1M phosphate buffer of varying pH values as a substrate and at 50°C. When the pH was increased from 6.0 to 7.2, rice malt protease activity in the supernatant of malted rice powder extract increased and reached the highest value of $0.045Ug^{-1}$ dry matter at pH 7.2 and 50°C (Figure 9). 'Hordolisin' a serine protease purified from green malt is subtilisin like protease showed pH optima of 6.0 (MacDonald & Chen, 1964). Wheat flour contained a major protease showing optimum activity at pH 3.8 and a minor portease show ing optimum proteolytic activity at pH 4.4 (Mikola & Virtanen, 1980). Proteinases from 4-day germinated oat seeds were active at pH 3.8 (Briggs, 1963). Germinated rye grain contained proteases active at pH 3.8 (Wang & Grant, 1969). The protease activity obtained in rice malt extract supernatant showed optimum activity at pH 7.2. As the enzyme assay was carried out with casein as substrate, the proteolytic activity was not studied below pH 6.0. Therefore the presence of acid protease activity was not studied. This was not given due consideration as the aim was to use rice malt amylase and not malt protease. The optimum pH for malt amylase was 5.0 and the protease fraction at pH 6.0 showed 66.7% of the activity obtained at its optimum pH (7.2). Thus the rice malt amylase shall not be subjected to effective proteolysis at pH 5.0. The protelytic activity of the rice malt extract supernatant was very small at pH 5.0 and 60°C, it is feasible to use a non purified rice malt extract supernatant in industrial or food preparation.

Effect of temperature on proteolytic activity of rice malt protease

The proteolytic activity of the enzyme increased with increase in temperature from 30-50°C at pH 7.2 (Figure 10). Optimum activity of rice malt protease was 50°C at pH 7.2 with casein (sodium salt). Above this temperature, proteolytic activity decreased sharply due to thermal denaturation of the enzyme and lost the total activity at 100°C. Wheat flour contained proteases which were unstable at 50°C (Wang & Grant, 1969). Highest protease activity of malted wheat flour was 50°C (Senthuran, 1997). At 60°C the proteolytic activity obtained was 27.5% of that obtained at 50°C. Therefore if the enzymatic hydrolysis of starch is carried out with rice malt extract supernatant, the activity of protease shall not be pronounced and affect the amylolytic activity.

Determination of Km and Vmax of rice malt protease

The activity of protease in malt rice extract supernatant was linear up to $50gL^{-1}$ casein (sodium salt) in 0.1M phosphate buffer solution (pH 7.2) when incubated for 1h at 50°C. The Km value of rice malt protease for casein (sodium salt) was $2.5gL^{-1}$ and Vmax was $0.0882Ug^{-1}$ dry matter at pH 7.2 and 50°C. The Km and Vmax values for the proteolytic activity of protease of *Aspergillus niger* for casein were 9.09×10^{-2}M and $1.66 \times 10^{-1}\mu mol\ min^{-1}$ respectively at pH 6.5 and 30°C (Trep, *et al.*, 2000).

CONCLUSION

This present study was carried out with a locally available "Mottaikaruppan" variety of rice. The aim of this study was achieved by studying the kinetic properties of carbohydrases and proteases. The proteases showed low level of activity when compared to carbohydrases. Both groups of enzymes showed differences in their kinetic properties. The amylase showed variation in temperature stability from bacterial and fungal enzymes. The effects of $CaCl_2$ with the enzyme stability have shown that the carbohydrases in malted rice have different calcium requirements. Thus different fractions of the carbohydrases have to be separated and their kinetic properties need to be studied to obtain rice malt powder with different types of sugars. So that the products could be used in food industries to exert different desired properties.

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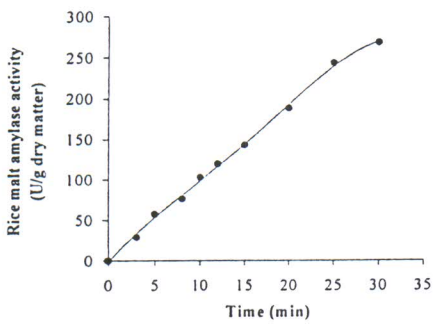

Figure 1: Effect of time on the amylolytic activity of rice malt amylase on starch ($40gL^{-1}$) at pH 5.0 and 60°C.

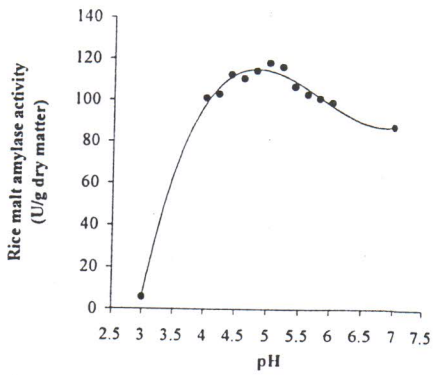

Figure 2: Effect of pH on amylolytic activity of rice malt amylase on starch ($40gL^{-1}$) at 60°C.

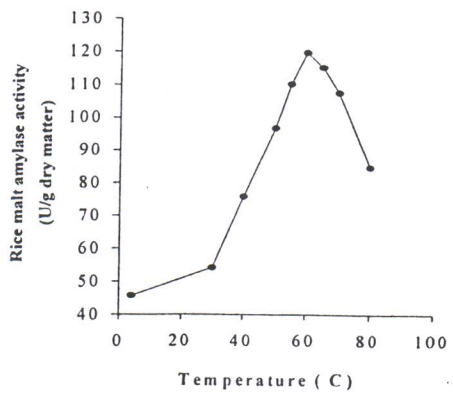

Figure 3: Effect of temperature on amylolytic activity of rice malt amylase on starch ($40gL^{-1}$) at pH 5.0.

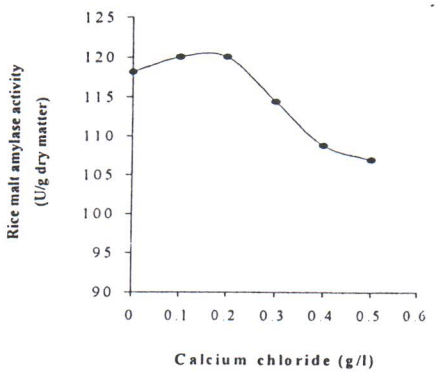

Figure 4: Influence of calcium ions on amylolytic activity of rice malt amylase.
Assay mixtures contained malted rice supernatant, starch ($40gL^{-1}$) – water solution and 0.02M acetate/acetic acid buffer (pH 5.0) with varying $CaCl_2$ concentrations were incubated at 60°C for 10 minutes.

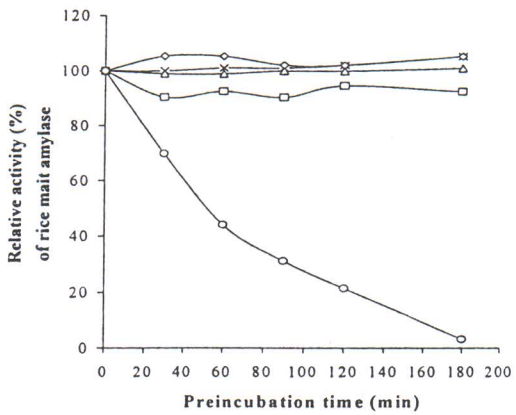

Figure 5: Effect of temperature on the stability of malt amylase extracted from malted rice powder with $10gL^{-1}$ NaCl at pH 6.17 and 30°C. (◊), 4°C; (x), 30°C; (Δ), 50°C; (□), 60°C and (○), 70°C.
Note: The activity of the enzyme at zero time in each temperature studied was calculated and considered as initial activity (100%). Then, the enzyme activity calculated at different time intervals in each temperature studied were converted into percentage of the initial activity and considered as relative activity.

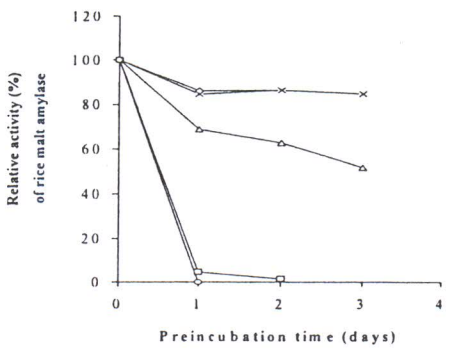

Figure 6: Effect of temperature on the stability of rice malt amylase at pH 6.17. Malt amylase was extracted from malted rice powder using $10gL^{-1}$ NaCl at pH 6.17 and 30°C. (◊), 4°C; (x), 30°C; (Δ), 50°C; (□), 60°C and (○), 70°C.
Note: The activity of the enzyme at zero time in each temperature studied was calculated and considered as initial activity (100%). Then, the enzyme activity calculated at different time intervals in each temperature studied were converted into percentage of the initial activity and considered as relative activity.

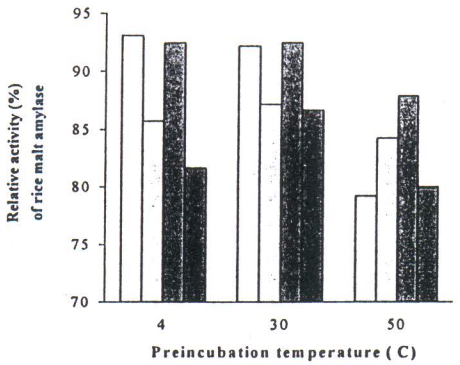

Figure 7: Effect of Ca^{2+} ions on the stability of rice malt amylase at different temperatures. ( ), without $CaCl_2$; ( ), 0.001M $CaCl_2$; ( ), 0.01M $CaCl_2$; ( ), 0.1M $CaCl_2$.

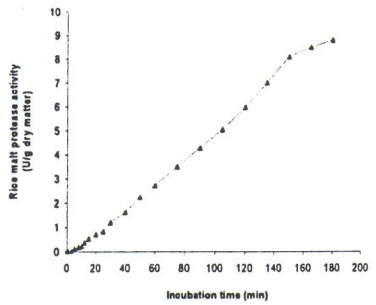

Figure 8: Effect of time on the proteolytic activity of rice malt protease on casein ($10gL^{-1}$) – 0.1M phosphate buffer at pH 7.2 and 50°C.

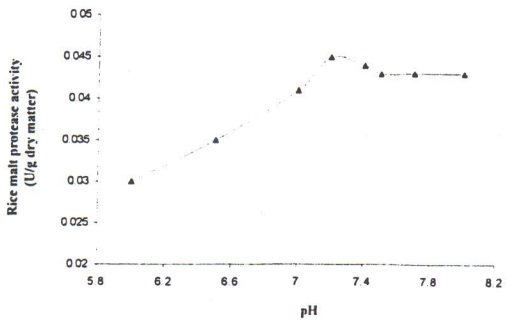

Figure 9: Effect of pH on the activity of rice malt protease with casein ($20gL^{-1}$) 50°C.

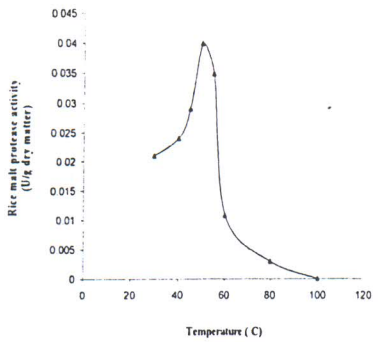

Figure 10: Effect of temperature on proteolytic activity of rice malt protease with casein ($10gL^{-1}$) – 0.1M phosphate buffer (pH 7.2) solution.

Table 1: Detection of different amylases present in the malted rice powder of "Mottaikaruppan" rice, using different enzyme specific substrates.

Type of Substrate	Amylolytic activity (Ug^{-1} dry matter)
Naringin ($5gL^{-1}$)	0.00
Starch soluble ($20gL^{-1}$)	129.87
p-Nitrophenyl-α-D-glucopyranoside ($5gL^{-1}$)	7.51

Table 2: Effect of pH on the storage stability of rice malt amylase at 4, 30, 50 and 60°C.

Day	4°C				30°C					50°C					60°C				
	4.5	5.0	5.5	6.2	4.0	4.5	5.0	5.5	6.2	4.0	4.5	5.0	5.5	6.2	4.0	4.5	5.0	5.5	6.2
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1	93.5	94.3	97.9	97.1	74.0	96.9	95.1	95.9	94.1	0	58.8	84.2	88.8	75.5	0	0	20.8	26.5	24.5
2	90.9	93.1	96.9	96.1	73.0	98.8	95.1	97.9	92.2	-	45.7	75.6	85.7	65.7	-	0	6.9	8.2	7.8
4	88.7	93.1	96.9	94.1	72.0	96.9	92.1	95.9	89.2	-	33.3	65.3	79.2	45.4	-	-	-	-	-