CHAPTER III

RESULTS

3.1 DBE activities in cassava tubers from different ages and cultivars

Crude enzyme extracted from parenchyma of cassava tuber from Rayong1 and KU50 cultivars that harvested between 3-12 months were assayed for pullulanase and isoamylase activity as described in section 2.4.4. Both enzyme activities were expressed per wet weight and per mg protein. The results were shown in Table 3.1, Table 3.2, Figure 3.1 and Figure 3.2. Both pullulanase and isoamylase specific activities were higher in cassava cultivar KU50 and attended a maximum level at the 9 months old. Furthermore, previous studies on purifications of SBE and SS in our laboratory used 9 months old tubers. Therefore, the 9 months harvested of cassava tuber cultivar KU50 was used for purification and characterization of DBE.

Table 3.1 The activity of pullulanase in tubers of cassava cv. KU50 and Rayong1 at different ages

Cultivars	Age (months)	Activity*/kg tissue (units/kg tissue)	Specific activity (units/mg protein)
KU50	3	8.07	0.016
KU50	6	9.79	0.017
KU50	9	11.24	0.031
KU50	12	11.13	0.023
Rayong1	3	0	0
Rayong1	6	4.94	0.007
Rayong1	9	10.23	0.028
Rayong1	12	9.52	0.015

*Unit of activity is 1 μmol of reducing sugar/minute

Data are mean values of 5 duplicated experiments.

Table 3.2 The activity of isoamylase in tubers of cassava cv. KU50 and Rayong1 at different ages

Cultivars	Age (months)	Activity*/kg tissue (units/kg tissue)	Specific activity (units/mg protein)
KU50	3	103.08	0.200
KU50	6	85.90	0.148
KU50	9	72.04	0.198
KU50	12	84.87	0.174
Rayong1	3	38.64	0.070
Rayong1	6	52.49	0.076
Rayong1	9	58.23	0.115
Rayong1	12	80.82	0.129

*Unit of activity is 1 μmol of reducing sugar/minute

Data are mean values of 5 duplicated experiments.

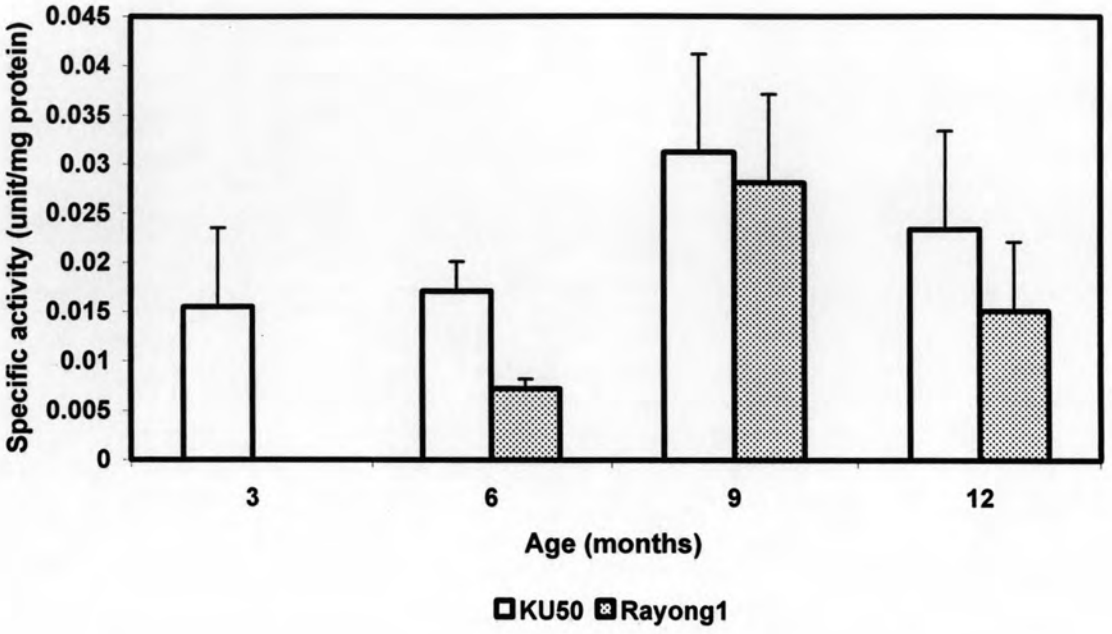


Figure 3.1 The specific activity of pullulanase in tubers of cassava cv.KU50 and Rayong1 at different ages

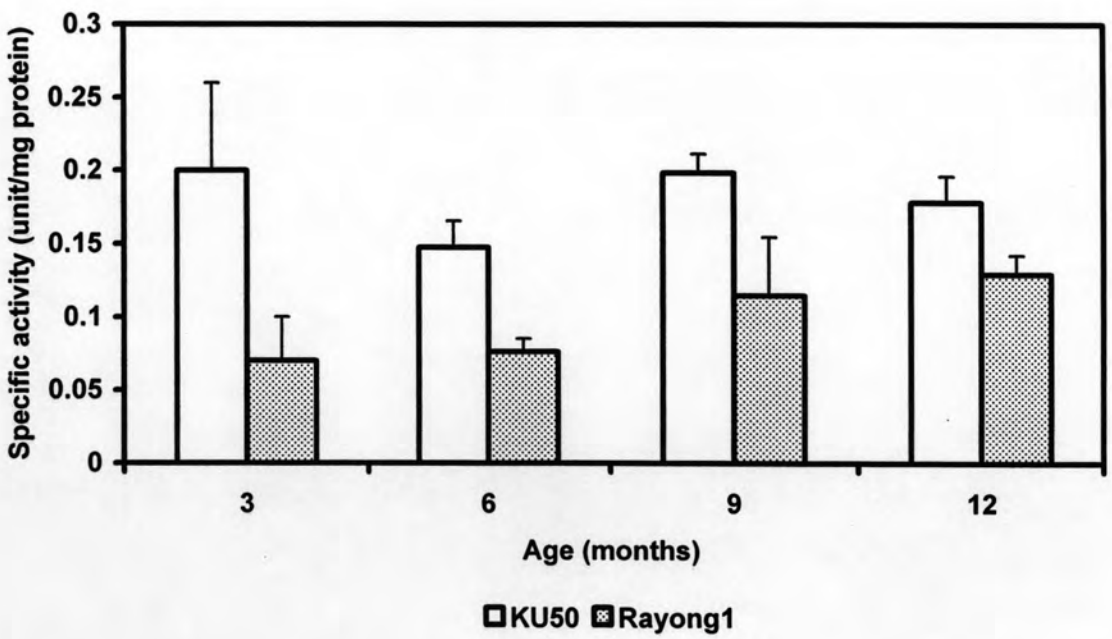


Figure 3.2 The specific activity of isoamylase in tubers of cassava cv.KU50 and Rayong1 at different ages

3.2 Purification of DBE from cassava tuber cv. KU50

To characterize DBE, 9 months old cassava tubers from cultivar KU50 were used for purification.

3.2.1 Preparation of crude enzyme

Crude enzyme was prepared from 1.5 kilograms of parenchyma from cassava tuber as described in section 2.4.1. The amount of protein obtained was 358 mg with 15 units of pullulanase and 67.5 units of isoamylase.

3.2.2 Ammonium sulfate precipitation

Crude enzyme from cassava tuber was precipitated with 60% saturated ammonium sulfate to get rid of other contaminated proteins and starch granules. The proteins remained in the precipitate at this step was 152 mg. The activity of isoamylase and pullulanase dropped to 6.8 and 3.47 units as shown in purification table (Tables 3.3 and 3.4). The precipitate was dialyzed in starting buffer and stored at 4°C for further purification.

3.2.3 Ion exchange chromatography

The dialyzed ammonium sulfate precipitate from section 3.2.2 was loaded on DEAE-Sepharose column. The column was eluted with 3 column volumes of starting buffer followed with linear gradient of NaCl (0 - 0.3M) in starting buffer. Three major peaks of proteins were eluted (Figure 3.3). The first peak was the unbound protein which did not show both DBE activities. The second peak of bound protein was eluted at 0.10 M NaCl. This protein peak showed both pullulanase and isoamylase activities in different fractions. The third protein peak did not have DBE activity.

Two isoamylase peaks were eluted at 0.10 M NaCl (fractions no.131-142) and 0.15 M NaCl (fractions no.145-156). The latter coincided with pullulanase peak. The peaks were separately pooled, concentrated and dialyzed against starting buffer for use in the next step.

3.2.4 Gel filtration chromatography

The isoamylase and pullulanase samples from DEAE-Sepharose were separately loaded on Sephacryl-S200 column prepared as described in section 2.5.3. The results were shown in Figures 3.4 and 3.5. The specific activity of isoamylase obtained was 277.19 x 10^{-2} units/mg protein with 14.6 purification fold and the specific activity of pullulanase was 79.22 x 10^{-2} units/mg protein with 20 purification fold.

3.3 Native PAGE

Enzyme from each step of purification was electrophoresed in 7.5% native PAGE containing 0.4% w/v soluble starch or 0.2% amylopectin and stained for DBE activity as described in section 2.8. Crude enzyme from the enzyme preparation step showed colorless and blue band on the dark background. Enzyme from ammonium sulfate precipitation step showed the same pattern with crude enzyme. The isoamylase from DEAE-Sepharose and Sephacryl-S200 showed the colorless band of amylolytic activity. The enzyme with pullulanase activity from DEAE-Sepharose and Sephacryl-S200 showed the blue band (Figure 3.6). Amylolytic activity band from DEAE-Sepharose disappeared in Sephacryl S-200 column in pullulanase sample.

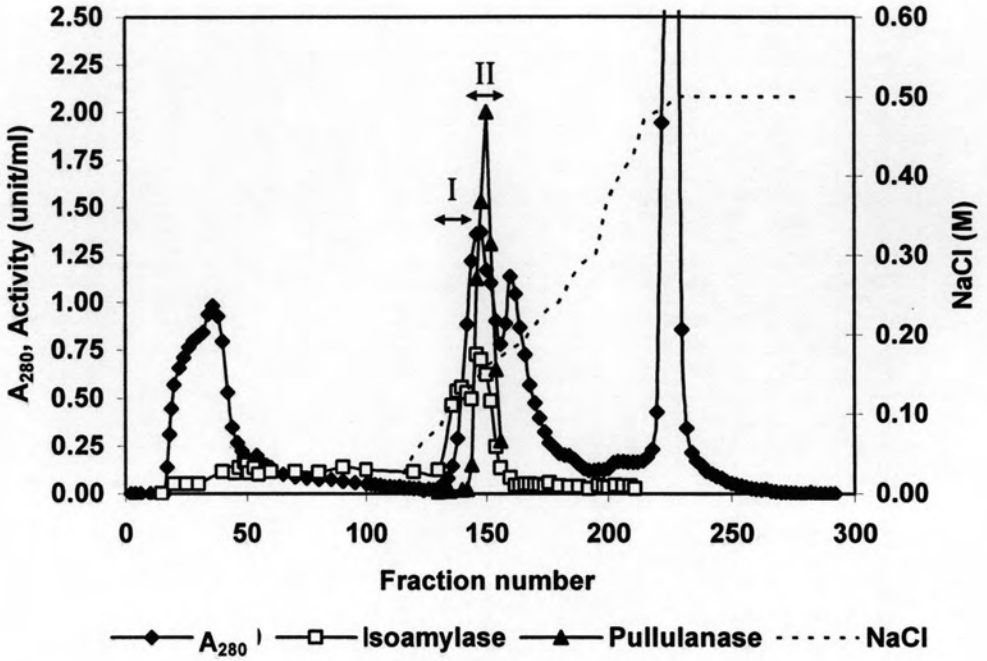


Figure 3.3 DEAE-Sepharose chromatographic profile of DBE

I = fractions pooled for isoamylase

II = fractions pooled for pullulanase

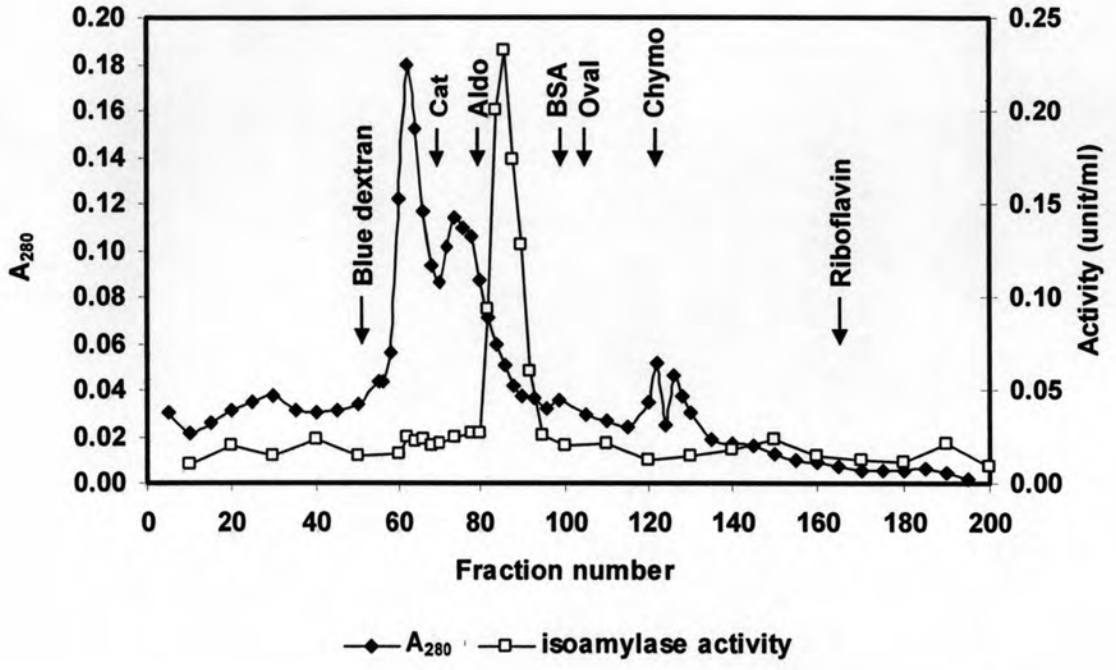


Figure 3.4 Chromatographic profile of isoamylase from Sephacryl S-200

Cat = Catalase	MW = 232,000 Da
Aldo = Aldolase	MW = 158,000 Da
BSA = Bovine serum albumin	MW = 66,000 Da
Oval = Ovalbumin	MW = 45,000 Da
Chymo = Chymotrypsinogen A	MW = 25,000 Da

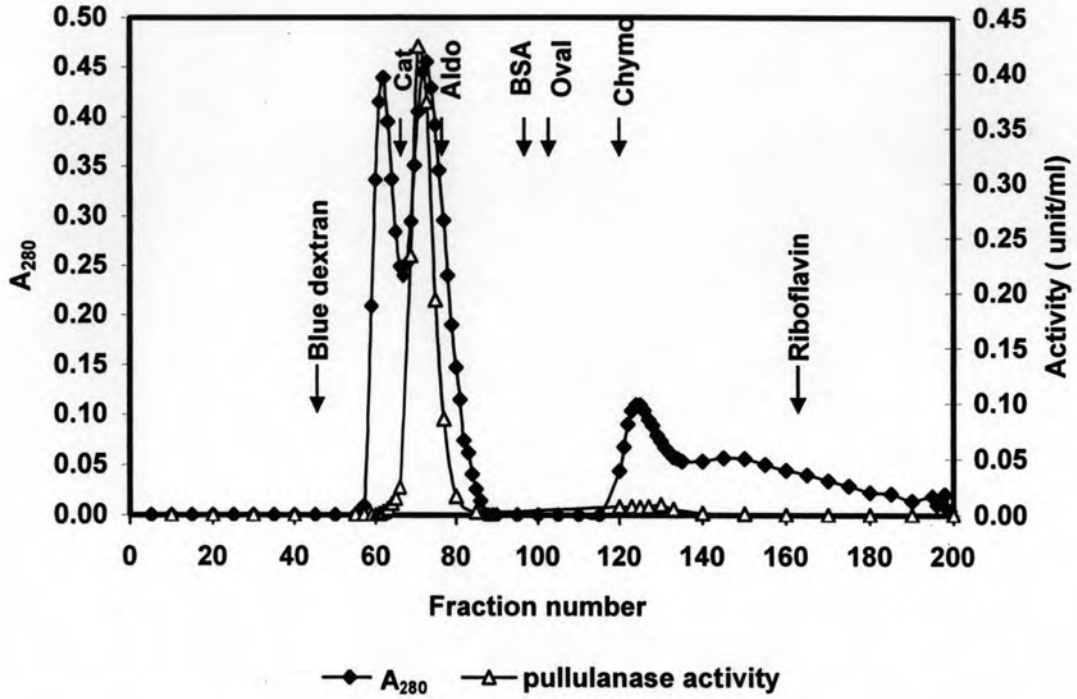


Figure 3.5 Chromatographic profile of pullulanase from Sephacryl S-200

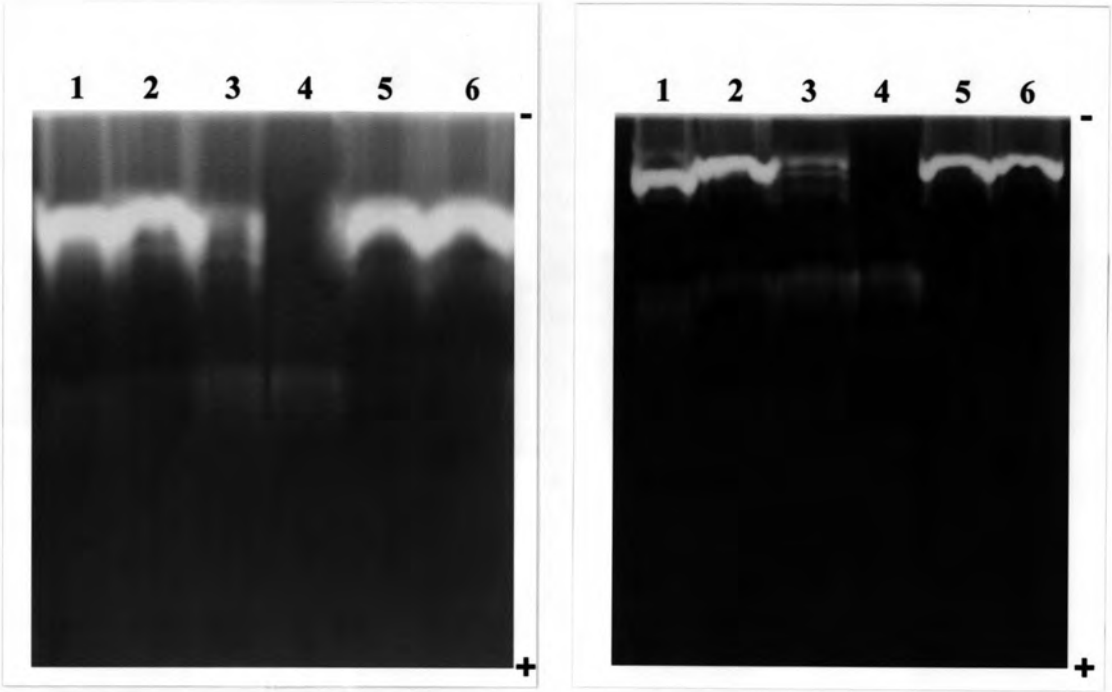
Cat = Catalase	MW = 232,000 Da
Aldo = Aldolase	MW = 158,000 Da
BSA = Bovine serum albumin	MW = 66,000 Da
Oval = Ovalbumin	MW = 45,000 Da
Chymo = Chymotrypsinogen A	MW = 25,000 Da

Table 3.3 Purification of isoamylase from cassava tuber cultivar KU50

Step	Total protein (mg)	Total activity (units)	Specific activity (10^{-2}units/mg protein)	Purification (fold)	Recovery (%)
Crude enzyme	358	67.5	18.9	1	100
0-60% ammonium sulfate	152	6.8	4.47	0.2	10
DEAE-Sepharose	4	2.43	60.8	3.2	3.6
Sephacryl S-200	0.57	1.58	277	14.6	2.3

Table 3.4 Purification of pullulanase from cassava tuber cultivar KU50

Step	Total protein (mg)	Total activity (units)	Specific activity (10^{-2}units/mg protein)	Purification (fold)	Recovery (%)
Crude enzyme	358	15.0	4.19	1	100
0-60% ammonium sulfate	152	3.47	2.28	0.5	23
DEAE-Sepharose	27	6.58	24.40	6	44
Sephacryl S-200	1.54	1.22	79.20	20	8



a) Soluble starch native PAGE b) Amylopectin native PAGE

Figure 3.6 Activity staining of cassava DBE on native PAGE

Lane 1 Crude enzyme ($4x10^{-3}$ units)

Lane 2 0-60% saturated ammonium sulfate ($4x10^{-3}$ units)

Lane 3 Pullulanase from DEAE-Sepharose ($7x10^{-3}$ units)

Lane 4 Pullulanase from Sephacryl S-200 ($7x10^{-3}$ units)

Lane 5 Isoamylase from DEAE-Sepharose ($6x10^{-3}$ units)

Lane 6 Isoamylase from Sephacryl S-200 ($6x10^{-3}$ units)

3.4 Characterization of DBE

3.4.1 Native molecular weight from gel filtration

K_{av} of isoamylase and pullulanase from Sephacryl-S200 was calculated. Standard proteins were loaded to Sephacryl S-200 and the calibration curve of standard proteins (Figure 3.7) was constructed and used to determine the molecular weight of DBE. The molecular weight of isoamylase and pullulanase were 98 and 175 kDa, respectively.

3.4.2 Molecular weight of DBE from SDS-PAGE

Isoamylase and pullulanase were checked for purity and their molecular weight on 10% SDS-PAGE (Figure 3.8), the R_f value of each protein band was calculated and the molecular weight determined from the calibration curve of standard proteins (Figure 3.9). Isoamylase showed 2 major bands with molecular weight 41 and 34 kDa whereas pullulanase showed 3 major bands with molecular weight 54, 46 and 41 kDa. There was still some contamination of other proteins observed in the gel.

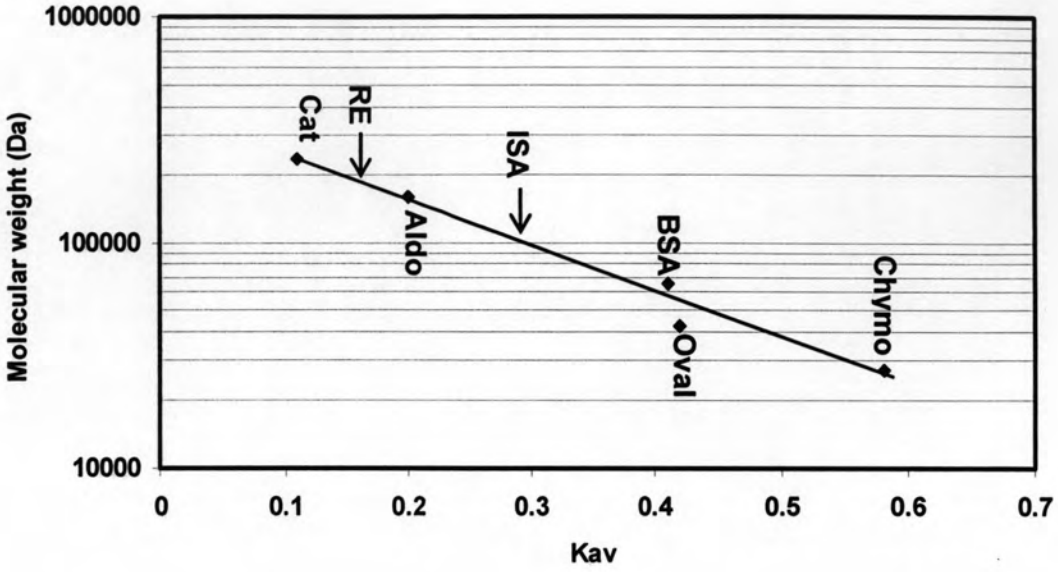


Figure 3.7 Calibration curve for native molecular weight determined by chromatography on Sephacryl S-200 column

Cat = Catalase	MW = 232,000 Da
Aldo = Aldolase	MW = 158,000 Da
BSA = Bovine serum albumin	MW = 66,000 Da
Oval = Ovalbumin	MW = 45,000 Da
Chymo = Chymotrypsinogen A	MW = 25,000 Da

RE = Pullulanase

ISA = Isoamylase

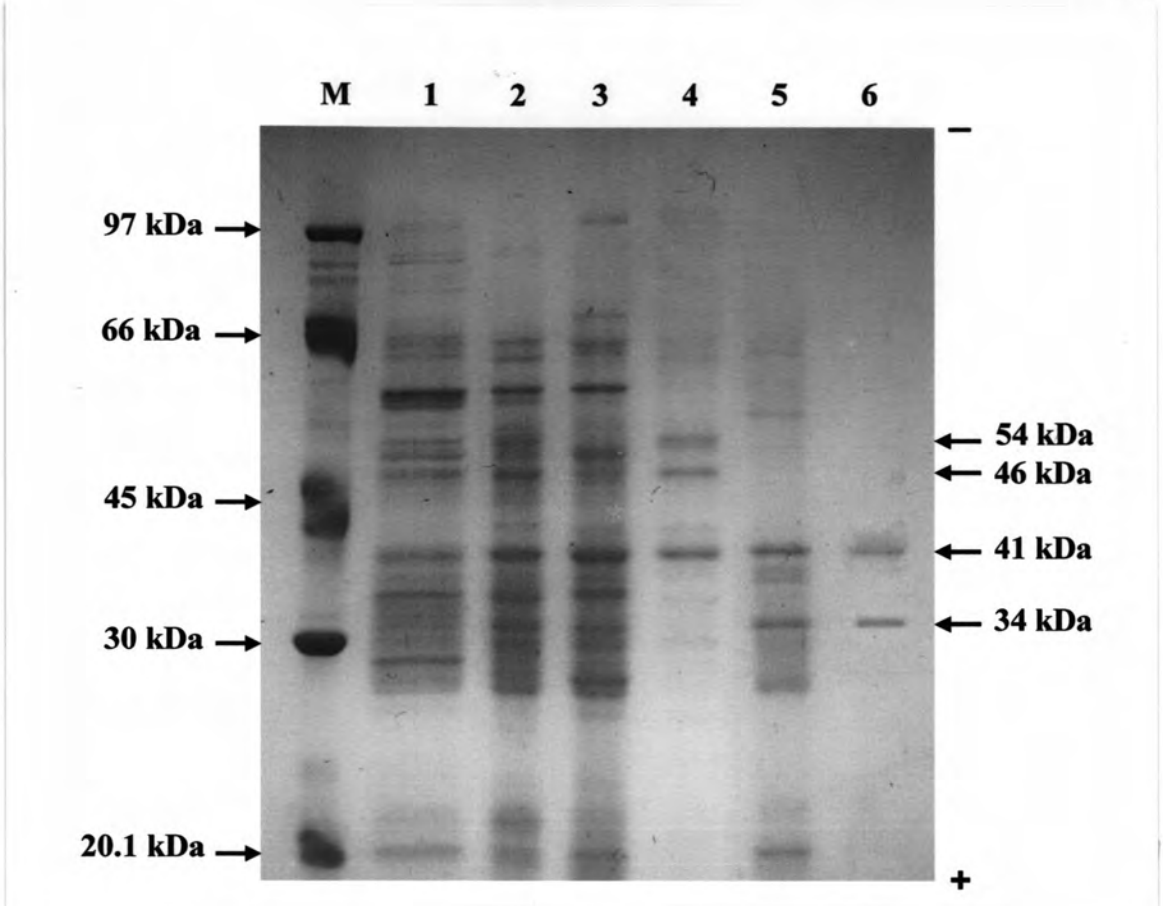


Figure 3.8 SDS-PAGE pattern of DBE

Lane M	Standard molecular weight proteins	
	Phosphorylase b (97 kDa)	
	BSA (66 kDa)	
	Ovalbumin (45 kDa)	
	Carbonic anhydrase (30 kDa)	
	Soybean trypsin inhibitor (20.1 kDa)	
Lane 1	Crude enzyme	20 μg
Lane 2	0-60% saturated $(NH_4)_2SO_4$	20 μg
Lane 3	Pullulanasae from DEAE Sepharose	20 μg
Lane 4	Pullulanase from Sephacryl S-200	5 μg
Lane 5	Isoamylase from DEAE Sepharose	7 μg
Lane 6	Isoamylase from Sephacryl S-200	5 μg

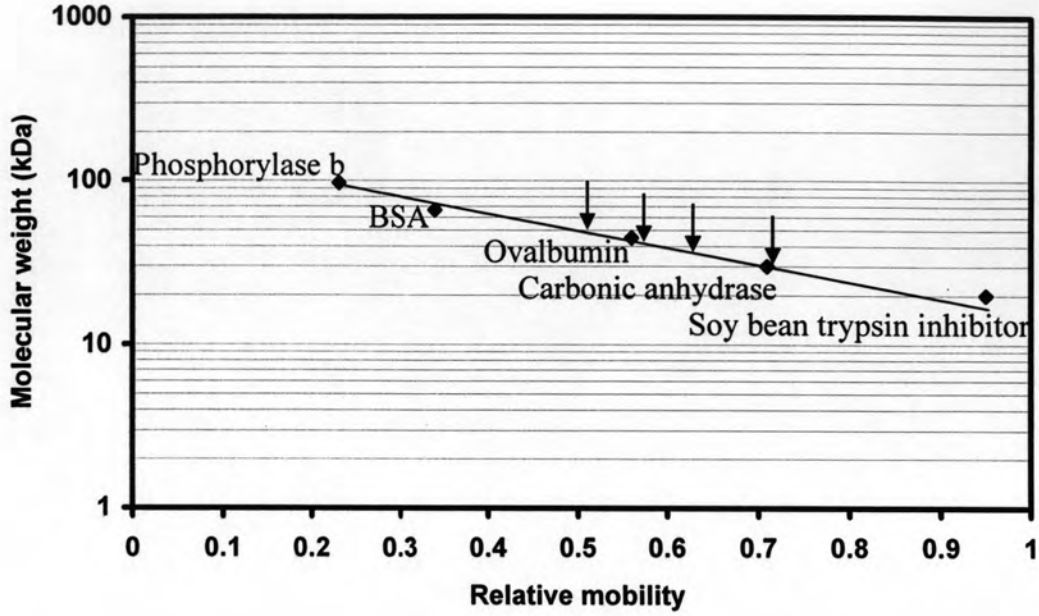


Figure 3.9 Calibration curve molecular weight markers on SDS-PAGE

The molecular weight of standard markers were plotted against theirs relative mobility on 10% SDS PAGE

Phosphorylase b	MW = 97 kDa
Bovine Serum Albumin	MW = 66 kDa
Ovalbumin	MW = 45 kDa
Carbonic anhydrase	MW = 30 kDa
Soybean trypsin inhibitor	MW = 20.1 kDa

3.4.3 Optimum pH for DBE activity

Isoamylase and pullulanase activities were assayed at various pH between 4.5-12. The result was shown in Figures 3.10 and 3.11. Both activities were presented as % relative activity with the highest activity referred as 100%. Isoamylase showed highest activity at pH 6.0 and the activity decreased to lower than 30% when the pH was more than 9.0. Pullulanase showed highest activity at pH 6.0. At pH below 5.0 and higher than 9.0, pullulanase activity was lower than 10%.

3.4.4 Optimum temperature for DBE activity

Isoamylase and pullulanase activities were assayed at various temperatures. The result was shown in Figure 3.12 and activity was expressed as % relative activity with the highest activity referred as 100%. Pullulanase has highest activity at 50°C. Isoamylase showed highest activity at 70°C. Pullulanase completely lost activity at 70°C but isoamylase lost 80% of its at 90°C.

3.4.5 Thermal stability of DBE

Isoamylase and pullulanase were tested for their stability by incubation at various temperatures up to 48 hr and then assayed activities as described in section 2.7. Isoamylase and pullulanase can remain their activity more than 80% after 48 hr of incubation at 4-37°C. Pullulanase lost its activity completely after 12 hr at 50°C. Isoamylase lost activity completely after 12 hr at 60°C. The results were shown in Figure 3.13 and Figure 3.14.

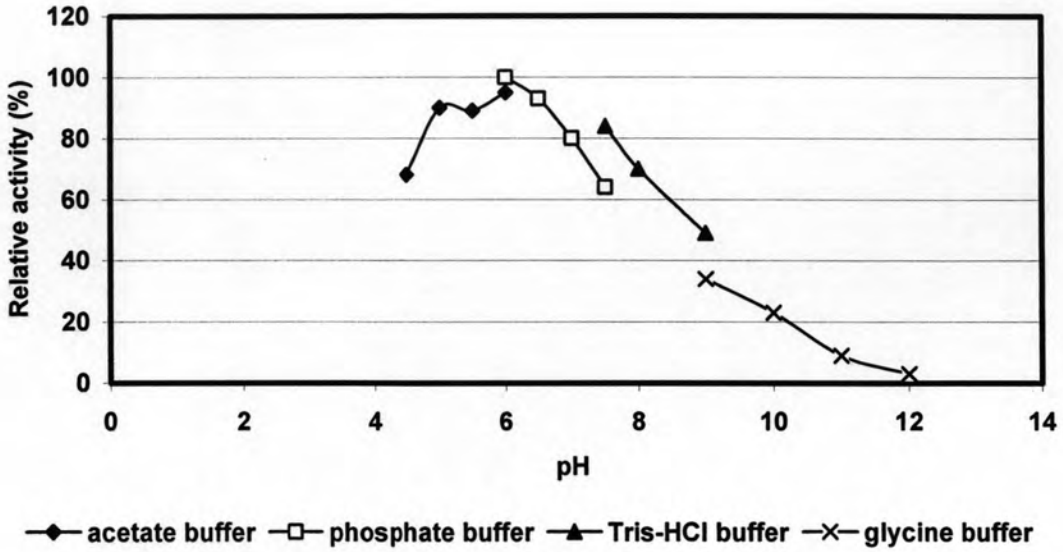


Figure 3.10 Optimum pH of isoamylase

Data was from 2 duplicated experiments.

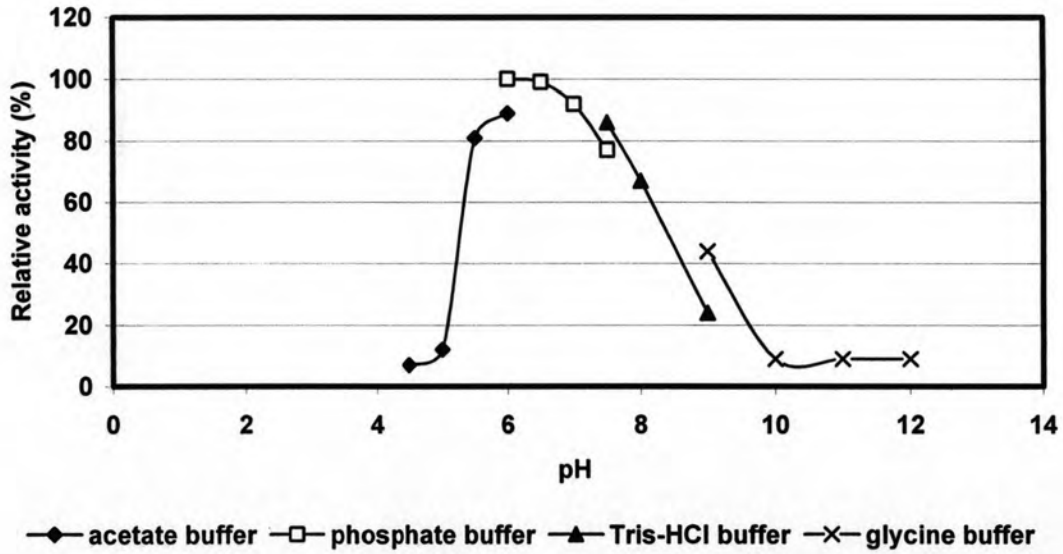


Figure 3.11 Optimum pH of pullylanase

Data was from 2 duplicated experiments.

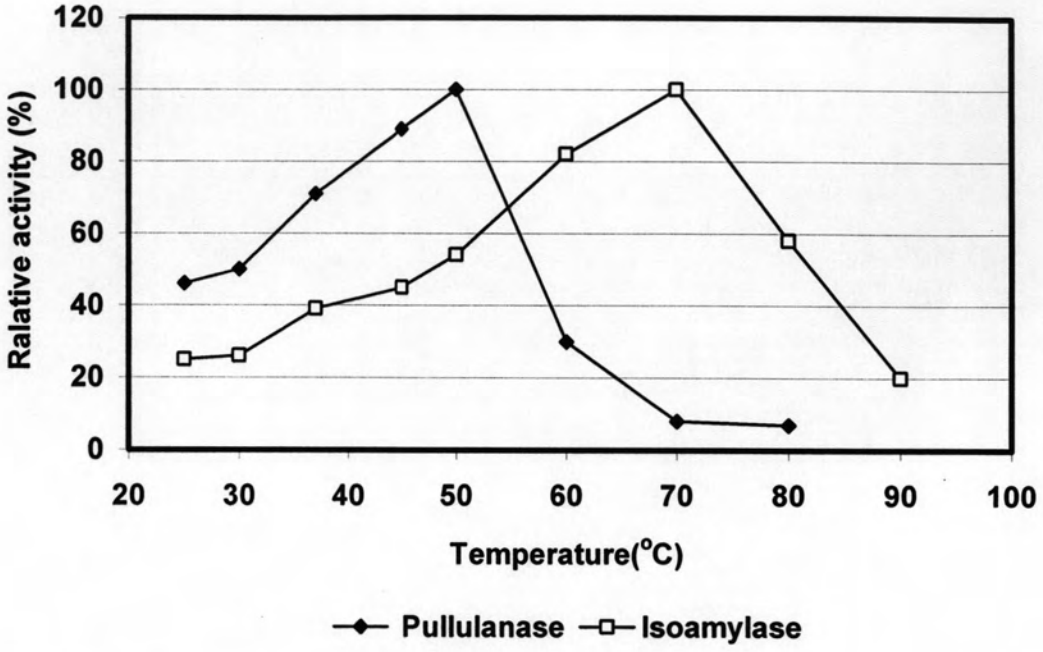


Figure 3.12 Optimum temperature of DBE

Data was from 2 duplicated experiments.

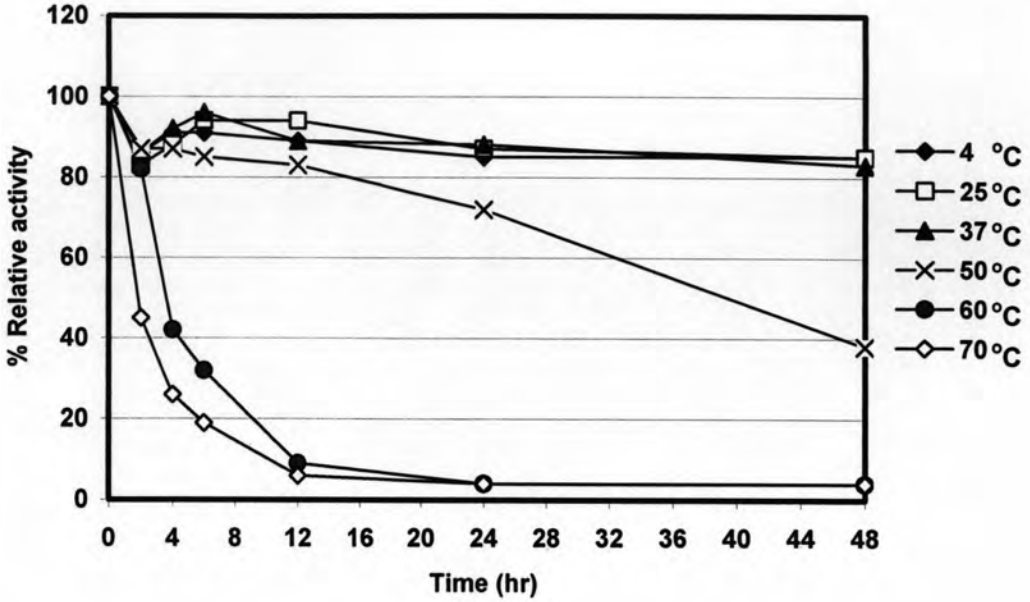


Figure 3.13 Thermal stability of isoamylase

Data was from 2 duplicated experiments.

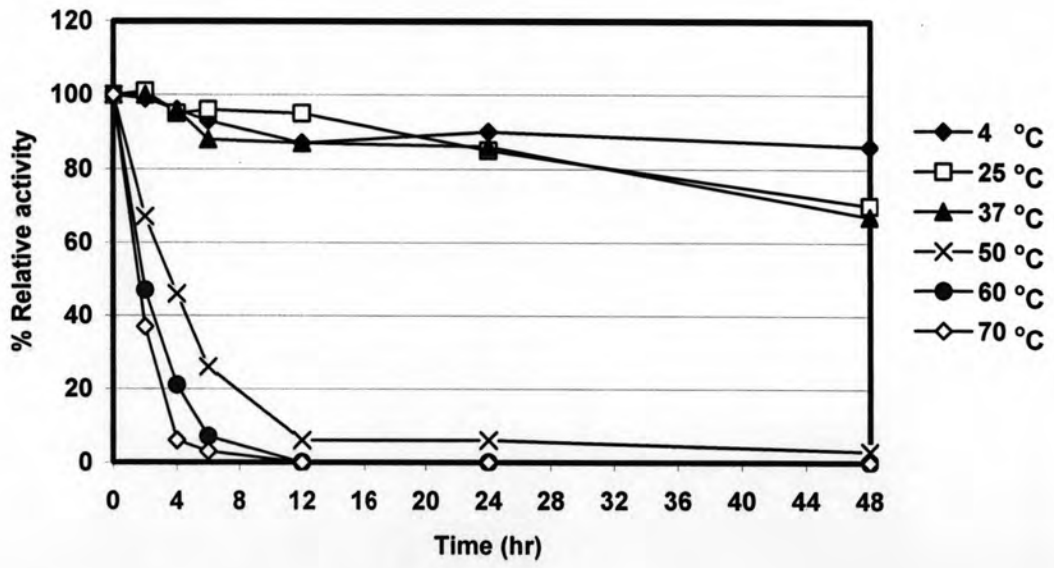


Figure 3.14 Thermal stability of pullulnase

Data was from 2 duplicated experiments.

3.4.6 Effect of sulfhydryl reagents on DBE activity

Isoamylase and pullulanase (5 mUnit) were assayed with sulfhydryl reagents to monitor the effect of SH-group on their activities as described in section 2.4.7.5. The result was shown in Figures 3.15 and 3.16. The control was the reaction mixture without sulfhydryl reagent. Increasing DTT, GSH and β-mercaptoethanol concentration caused increase activity of isoamylase and pullulanase. On the other hand, NEM and IAA caused decrease activity of isoamylase and pullulanase.

3.4.7 Effect of divalent metal ions on DBE activity

Isoamylase and pullulanase (5 mUnit) were assayed on the effect of divalent metal ions on its activity as described in section 2.4.7.6. The control was the reaction mixture without adding divalent metal ion and presented as 100% relative activity. The result was shown in Figures 3.17 and 3.18. Cu^{2+} was strong inhibitor of isoamylase and pullulanase. Ni^{2+} was strong inhibitor of pullulanase and has small effect on isoamylase. Mg^{2+} has no effect on isoamylase and pullulanase. Low concentration of Co^{2+} increased activity of both DBE. Mn^{2+} increased activity of pullulanase but inhibited activity of isoamylase.

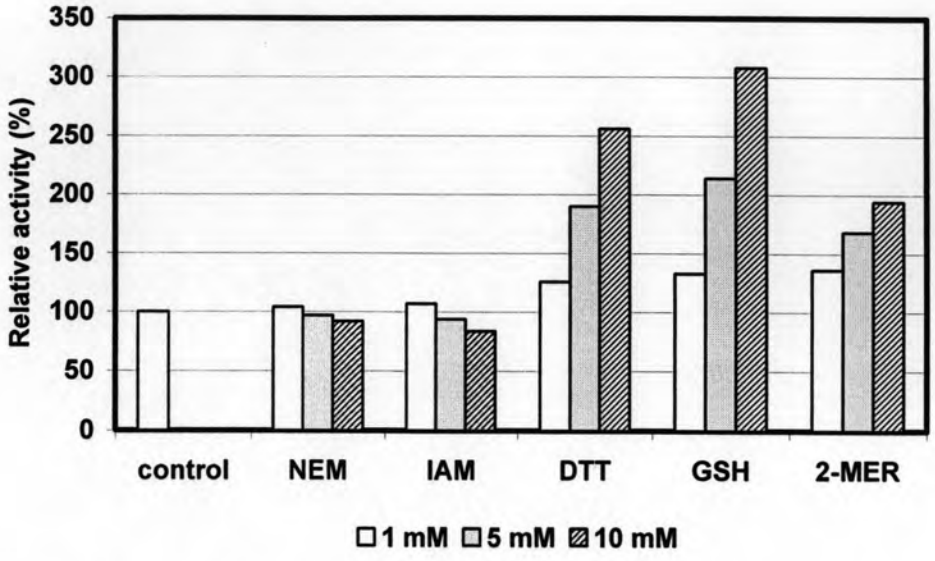


Figure 3.15 Effect of sulfhydryl reagents on isoamylase activity
Data was average of 2 duplicated experiments.

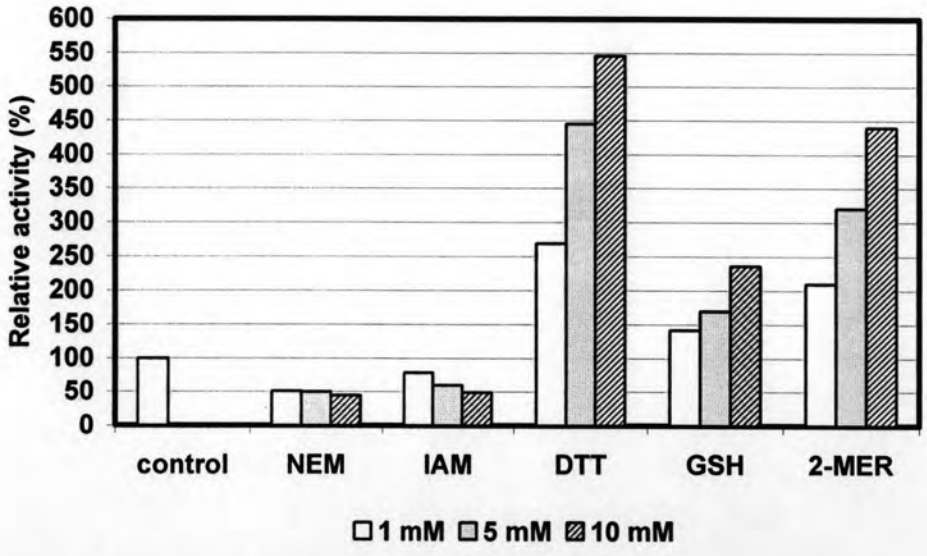


Figure 3.16 Effect of sulfhydryl reagents on pullulanase activity
Data was average of 2 duplicated experiments.

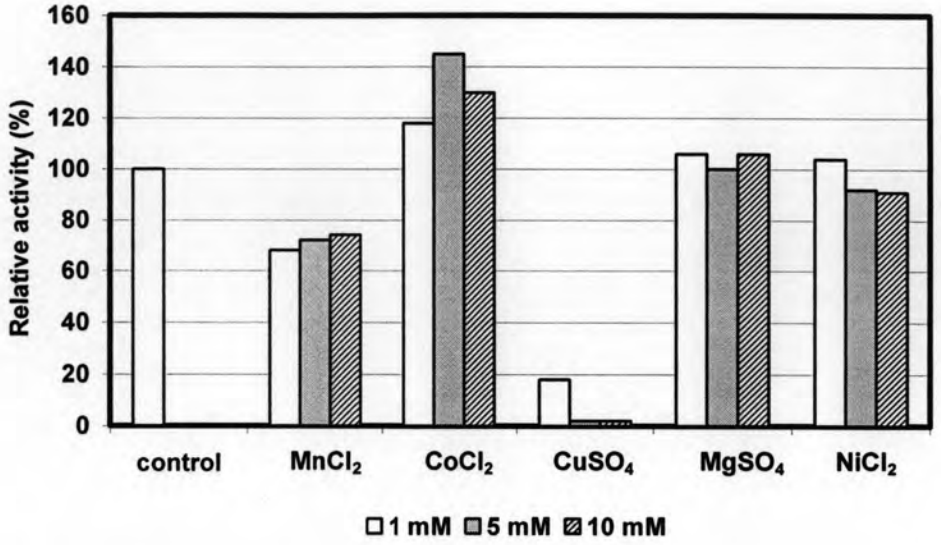


Figure 3.17 Effect of divalent metal ions on isoamylase activity
Data was average of 2 duplicated experiments.

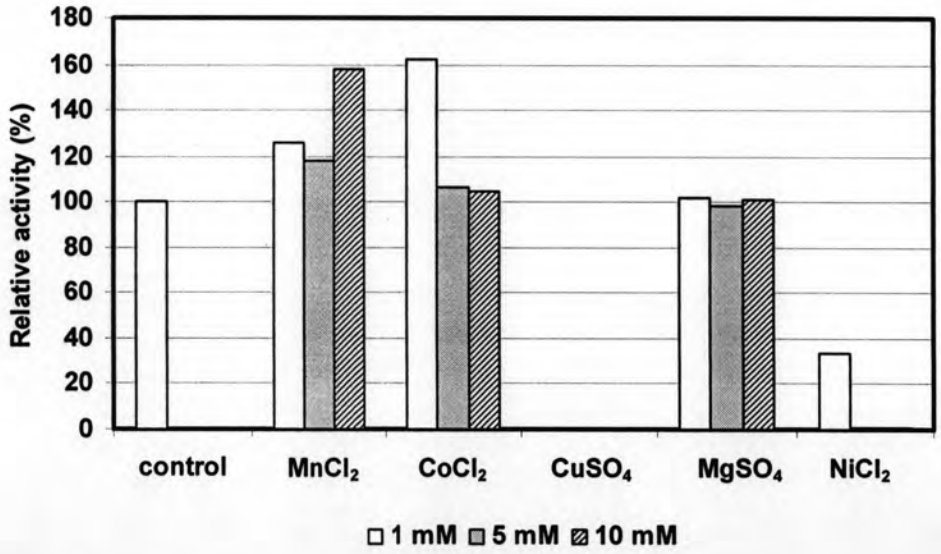


Figure 3.18 Effect of divalent metal ions on pullulanase activity
Data was average of 2 duplicated experiments.

3.4.8 Comparison of DBE activity with various substrates

To compare the substrate specificity, 1% w/v of amylose, amylopectin, pullulan, glycogen and soluble starch were used as substrates. The activity was assayed as described in section 2.7. The highest activities of pullulanase and isoamylase detected were taken as 100% activity. Isoamylase preferred amylopectin, amylose, soluble starch and glycogen in decreasing order respectively but did not hydrolyze pullulan. Pullulanase hydrolyzed pullulan, amylopectin, soluble starch and amylose in decreasing preference but did not debranch glycogen. The result was shown in Table 3.5.

Table 3.5 Comparison of DBE activity with various substrates

Substrate	Relative activity (%)	
	Pullulanase	Isoamylase
Pullulan	100	0
Amylopectin	54	100
Amylose	21	77
Glycogen	0	42
Soluble starch (from potato)	41	94

3.4.9 Kinetic parameters, K_m and V_{max} of DBE

Isoamylase (5 mUnit) was assayed with varying concentrations of amylopectin at 70°C for 10 minutes and measured the activity as described in section 2.11. From Lineweaver-Burk plot of isoamylase (Figure 3.19), K_m for amylopectin was calculated at 21.14 mg/ml and V_{max} was 52.10 nmol maltose/min. Pullulanase (8 mUnit) was assayed with varying concentrations of pullulan at 50°C for 10 minutes and measured the activity as described in section 2.11. From Lineweaver-Burk plot of pullulanase

(Figure 3.20), K_m and V_{max} of pullulan were 39.49 mg/ml and 35.31 nmol maltose/min.

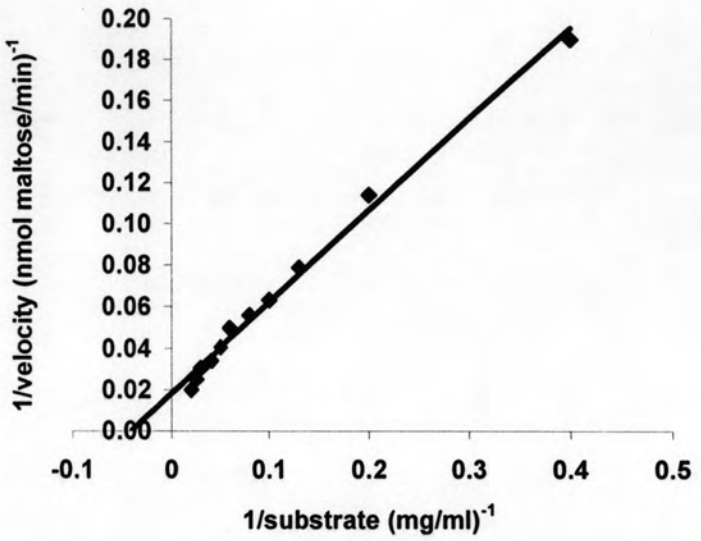


Figure 3.19 Lineweaver-Burk plot of isoamylase activity with amylopectin as substrate

Data from 3 duplicated experiments.

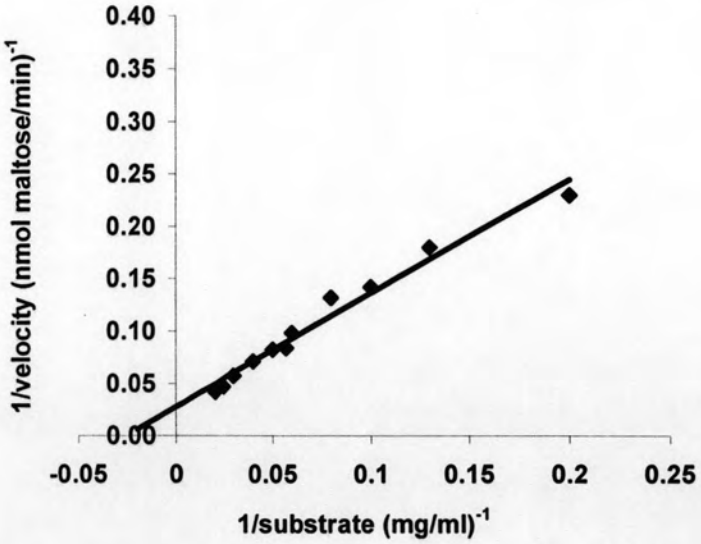


Figure 3.20 Lineweaver-Burk plot of pullulanase activity with pullulan as substrate

Data from 3 duplicated experiments.