CHAPTER III

RESULTS

3.1 Purification of SBE from Cassava Tuber

Preliminary study of SBE in cassava tuber, separated into parenchyma and cortex, revealed that homogenate prepared from parenchymal tissue of the tuber contained most of the SBE activity while only trace amount of SBE was found in the cortex homogenate (data not shown). Therefore, purification of SBE was performed using parenchyma of cassava tubers.

3.1.1 Preparation of crude enzyme

Crude SBE was prepared from parenchyma tissue of cassava tuber as described in section 2.4.1. From the starting material of 750 g, 1,191 mg protein was obtained with 1,093 units of SBE activity (Table 3.1).

3.1.2 Precipitation with polyethyleneglycol

The crude SBE obtained in 3.1.1 was further purified by precipitation of contaminating proteins with 10 % polyethyleneglycol (PEG_{6000}) as described in section 2.4.2. Significant amount of proteins was removed, leaving 227 mg proteins in supernatant which contained the SBE activity. The enzyme recovered was 1,344 units with 6.4 folds of purification (Table 3.1).

3.1.3 DEAE-cellulose chromatography

The supernatant obtained from PEG precipitation was loaded on DEAE-cellulose prepared as described in section 2.4.3. Fractions of 4.5 ml were collected. The SBE activity was retained in the column when washed

with the starting buffer. It was eluted with NaCl gradient in fractions 125-138 at the salt concentration range 0.08-0.12 M NaCl. The chromatographic profile is shown in Figure 3.1. Fractions 125-138 were pooled for further purification.

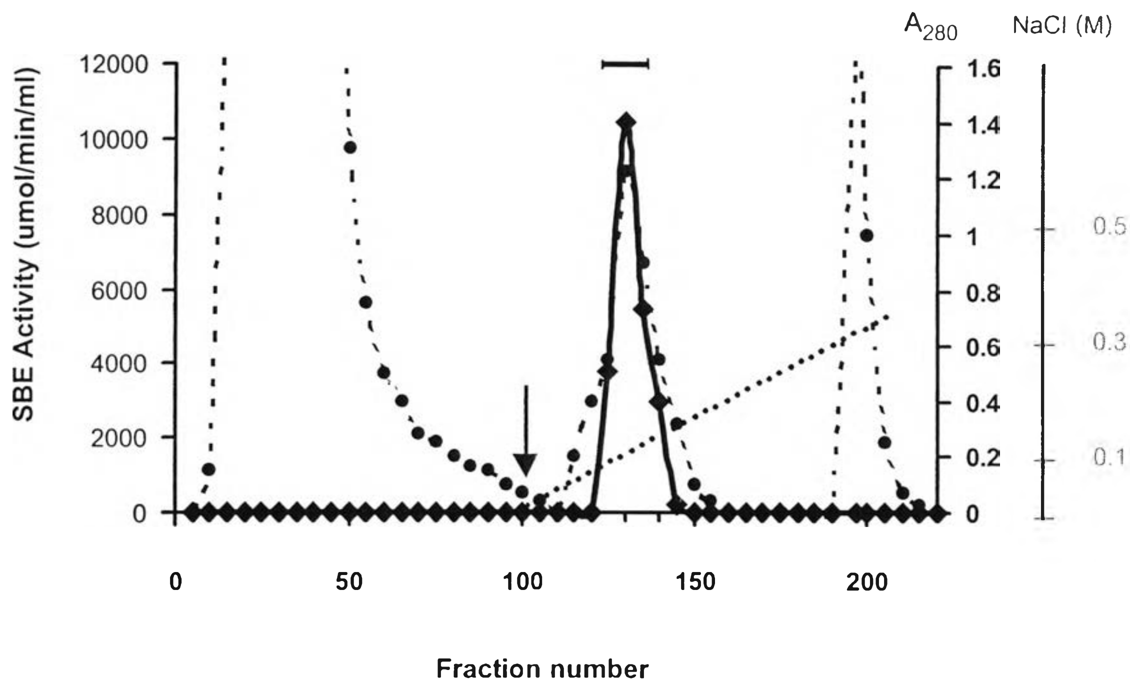

Figure 3.1 Elution profile of cassava SBE from DEAE-cellulose column.

The supernatant from PEG precipitation was loaded to DEAE-cellulose column chromatography. The column size 1.5 X 25 cm, fraction size 4.5 ml and flow rate 45 ml/hr.

●..........● = A_{280}

◆-----------◆ = SBE activity

.... = NaCl gradient

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3.1.4 Q-sepharose chromatography

The pooled fractions with SBE activity from DEAE-cellulose column in 3.1.3 was concentrated by 80% ammonium sulfate precipitation, resuspended in buffer, dialyzed and loaded to Q-sepharose column prepared as described in section 2.4.4. The SBE activity was still bounded to the column after washing with starter buffer. It was eluted at salt concentration of 0.14-0.26 M NaCl into fraction 95-115. Maximum SBE activity was found in fraction 107 with salt concentration 0.21 M. Fraction 95-115 were pooled for further study.

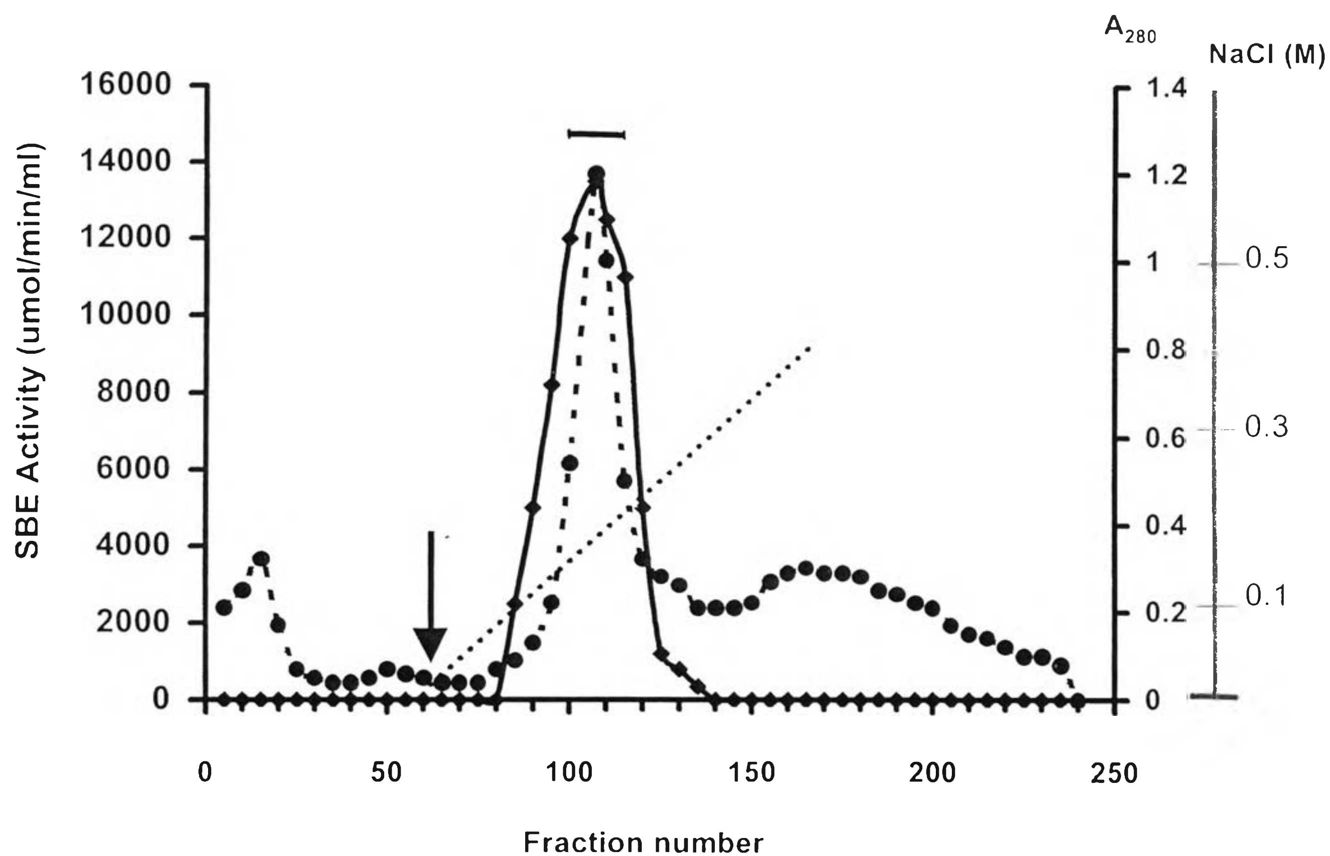

Figure 3.2 Elution profile of cassava SBE from Q-Sepharose column.

Active fractions from DEAE-cellulose column were applied to Q-Sepharose column chromatography. The column size was 1.0 X 15 cm, fraction size 2.0 ml, flow rate 20 ml/hr.

●.........● = A_{280}

◆------◆ = SBE activity

.... = NaCl gradient

3.1.5 Sephadex G-200 column chromatography

The pooled fractions from Q-Sepharose column containing SBE activity were concentrated with aquasorb and loaded to Sephadex G-200 column prepared as described in section 2.4.5. Proteins were separated into 2 peaks with SBE activity detected in the major peak. The column was also calculated with standard proteins: catalase (MW 232 kD), BSA (MW 68 kD), chymotrypsinogen (MW 27kD) and cytochrome c (MW 12 kD).

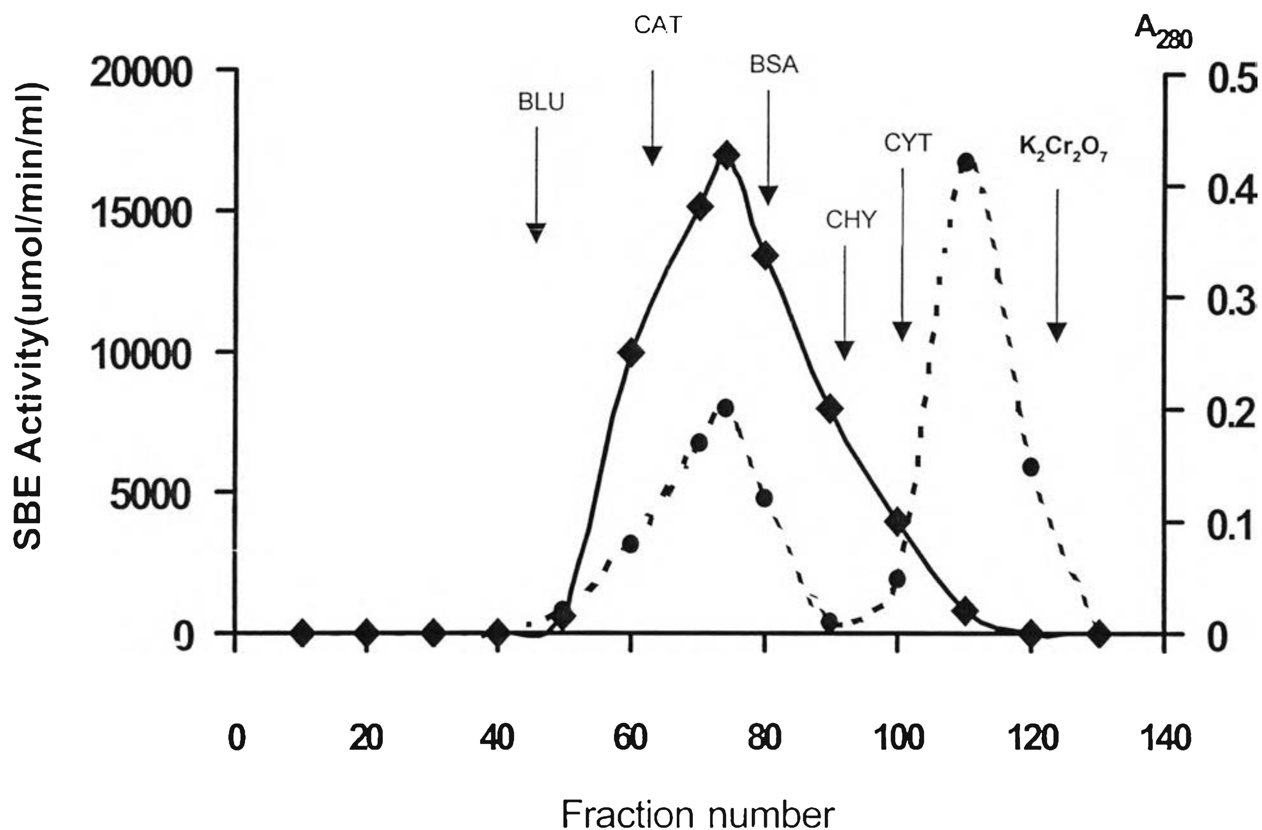

Figure 3.3 Elution profile of cassava SBE from Sephadex G-200 column.

Active fractions from Q-Sepharose column were loaded to Sephadex G-200 column chromatography. The column size was 2.4 X 55 cm, fraction size was 2.0 ml and the flow rate was 20 ml/hr.

●..........● = A_{280}

◆--------◆ = SBE activity

The molecular weight markers used were :-

BLU	=	Blue dextran	CHY	=	Chymotrypsinogen
CAT	=	Catalase	CYT	=	Cytochrome c
BSA	=	Bovine serum albumin	$K_2Cr_2O_7$	=	Potassiumdichromate

3.1.6 Monitoring of SBE purification

Table 3.1 summarized the overall purification achieved for SBE from all step described in 3.1.1 – 3.1.5. SBE was purified by 148.5 folds after Sephadex G-200 column chromatography but the yield of SBE was only 2 %.

Table 3.1 Purification of starch branching enzyme from cassava tubers.

Step	Protein (mg)	Total activity* (unit)	Specific activity (unit/mg)	Yield (%)	Purification (Fold)
Crude	1,191	1,093	0.92	100	1
$PEG_{Supernatant}$	227	1,344	5.92	123	6.4
DEAE-cellulose	36	662	18.4	60.6	19.9
Q-Sepharose	21.1	454	23.4	41.5	25.4
Sephadex G-200	0.15	20.5	137	2.0	148.5

*Starch branching enzyme activity was measured by the phosphorylase a stimulation method.

One unit is defined as mmol glucose incorporated into methanol-insoluble glucan per min.

See calculation method in Appendix D.

The purified SBE in each purification steps were further monitored by electrophoresis on non-denaturing starch-polyacrylamide gel and stained for both proteins and activity of SBE as shown in Figure 3.4. SBE appeared as reddish-brown band in the activity stain with iodine (section 2.7.1). SDS polyacrylamide gel electrophoresis of each purification steps were also performed as shown in Figure 3.5. Both gel patterns showed satisfactory purification of SBE from Sephadex G-200 column.

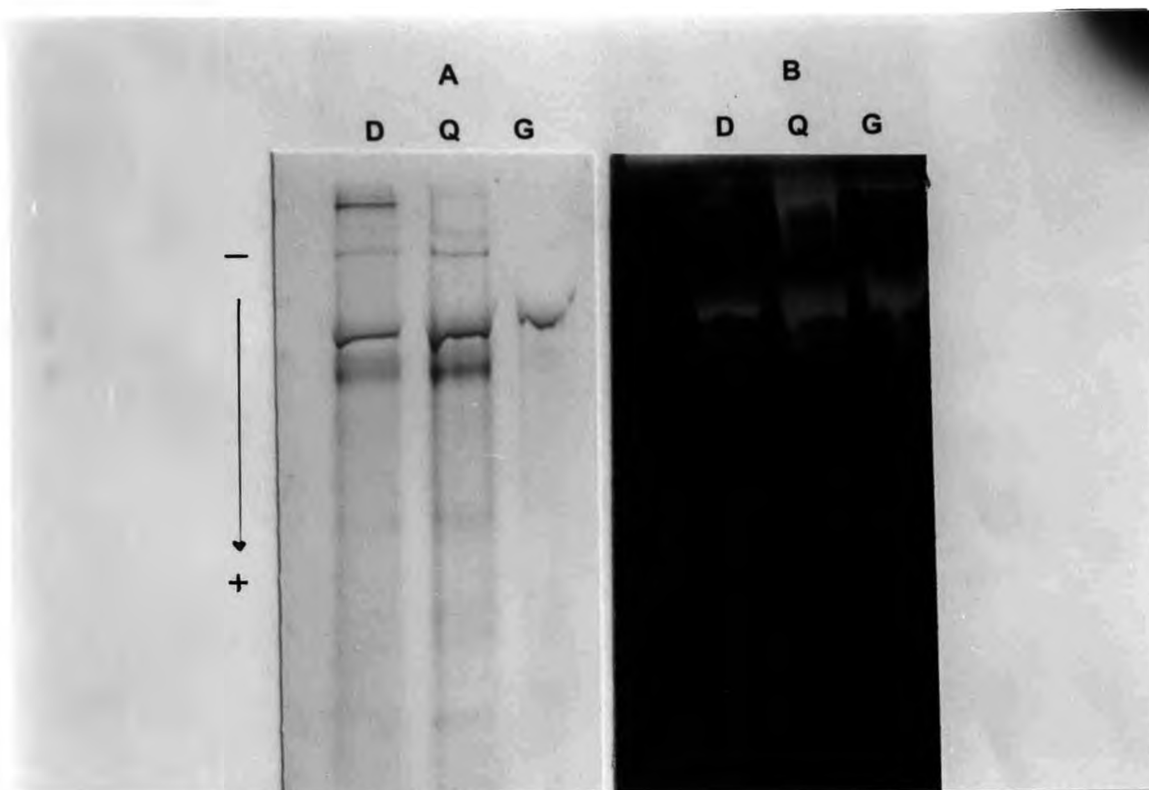

Figure 3.4 Staining of cassava SBE activity on Non-denaturing starch-polyacrylamide gel electrophoresis.

A. Protein stain of the cassava SBE

B. Iodine stain of the cassava SBE

D ; DEAE-cellulose column (10 μg, 0.18 unit)

Q ; Q-Sepharose column (10 μg, 0.21 unit)

G ; Sephadex G-200 column (2 μg, 0.27 unit)

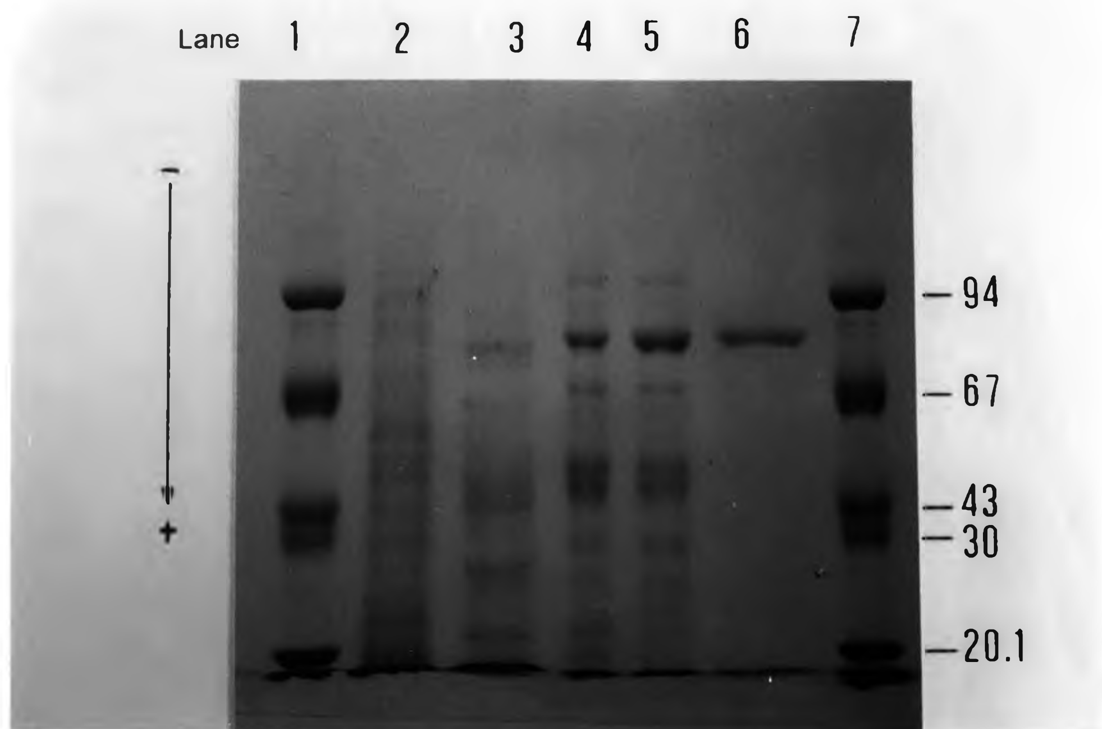

Figure 3.5 SDS-PAGE pattern of cassava SBE.

Lane 1,7	Standard molecular weight protein;-	
	Phosphorylase b (94 kD)	
	BSA (67 kD)	
	Ovalbumin (43 kD)	
	Carbonic anhydrase (30 kD)	
	Soybean trypsin inhibitor (20.1 kD)	
2	Crude enzyme	(20 ug)
3	PEG-supernatant	(20 ug)
4	DEAE-cellulose	(10 ug)
5	Q-sepharose	(10 ug)
6	Sephadex G-200	(10 ug)

3.2 Characterization of cassava SBE

3.2.1 Molecular weight determination

The native molecular weight of SBE was determined from molecular weight calibration curve (Figure 3.6) obtained from chromatography of standard proteins on Sephadex G-200 column as mentioned in 3.1.5. SBE was found to have the native molecular weight of 160 kD.

The molecular weight of SBE was also determined by SDS-PAGE (Figure 3.7) which included a series of standard proteins in the run. SBE was found to have the molecular weight of 80 kD on SDS-PAGE.

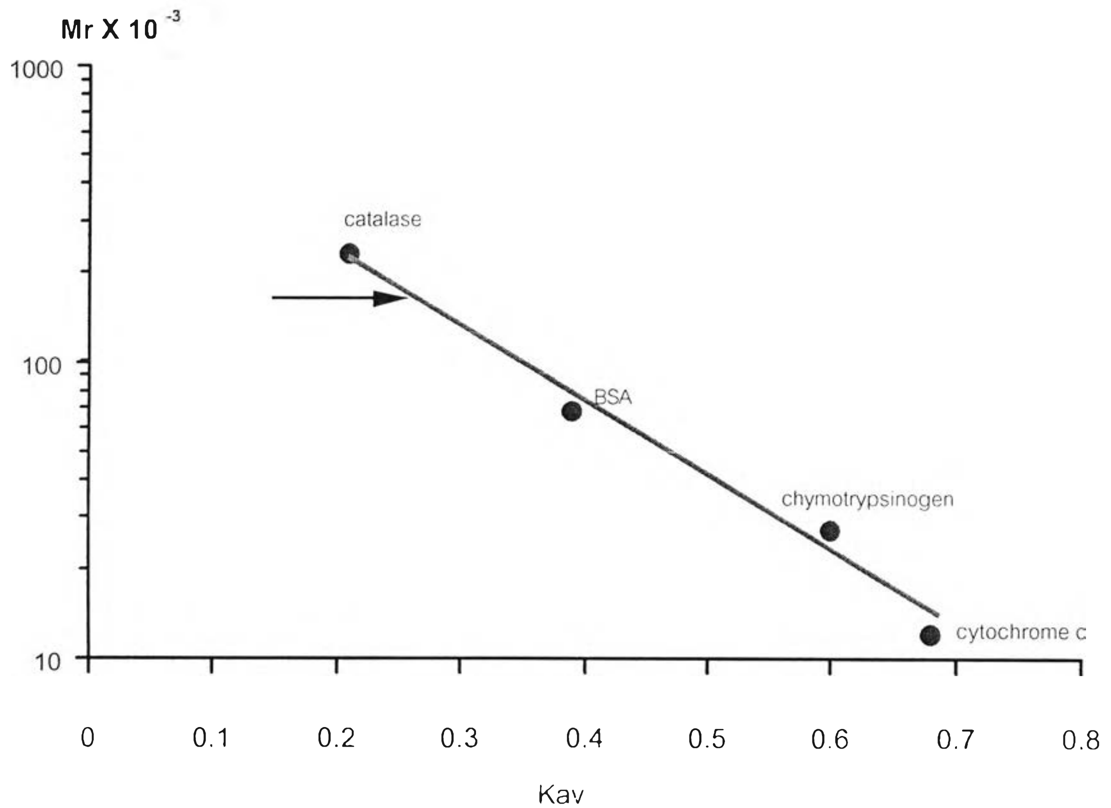

Figure 3.6 Calibration curve of molecular weight markers from Sephadex G-200 column.

The calibration curve was plotted from relative molecular weight of the standard marker proteins against their Kav values. The standard markers used were catalase (232 kD), BSA (68 kD), chymotrypsinogen (27 kD) and cytochrome c (12 kD). The arrow indicates Kav of the cassava SBE.

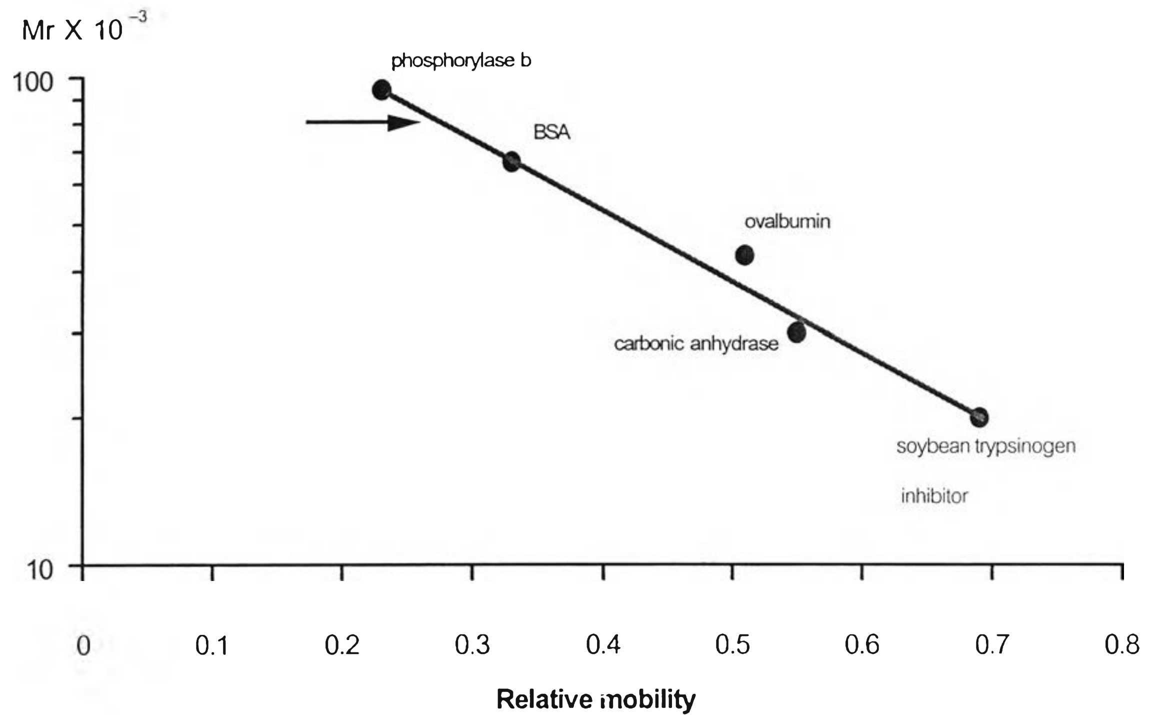

Figure 3.7 Calibration curve of relative molecular weight markers on SDS-PAGE.

The relative molecular weights of the standard markers were plotted against their relative mobility on the SDS-PAGE. The standards were phosphorylase b (94 kD), BSA (67 kD), ovalbumin (45 kD), carbonic anhydrase (30 kD) and soybean trypsin inhibitor (20.1 kD). The arrow indicates the relative mobility of cassava SBE.

3.2.2 Effect of pH on SBE activity

To determine the effect of pH on the activity of SBE, the enzyme was assayed at various pH's at 30 ^{o}C as describe in section 2.8. The cassava SBE was shown to have highest activity at pH 7.0 (Figure 3.8).

3.2.3 Effect of temperature on SBE activity

The effect of temperatures on the cassava SBE activity was also investigated by incubation of the reaction mixture at various temperatures as described in section 2.9. The result is shown in Figure 3.9. The highest cassava SBE activity was obtained at 37 ^{o}C.

3.2.4 Temperature stability

The stability of the cassava SBE at different temperatures was studied by incubating aliquots of the enzyme in a shaking waterbath at various temperatures for 30 min before the activity was assayed at 37 ^{o}C as described in section 2.10 compare to enzyme incubated at 25 ^{o}C, 0 time as 100 % activity. It was found that the cassava SBE activity was lost when the temperature was over 45 ^{o}C (Figure 3.10).

To determine the effect of storage temperature of the enzyme activity at temperature –20 ^{o}C, 4 ^{o}C and 30 ^{o}C for a period of time from 1 to 4 weeks before assaying the remaining activity as described in section 2.10. The result is shown in Figure 3.11. The cassava SBE activity decreased slowly if the enzyme was stored at –20 ^{o}C (Figure 3.11).

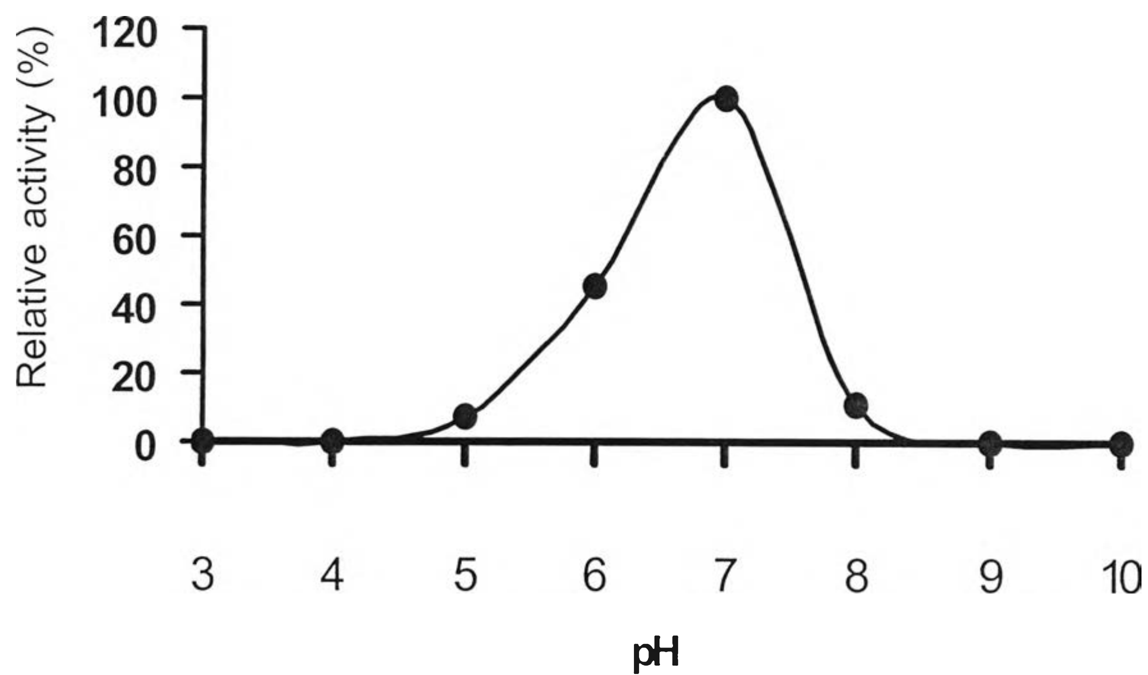

Figure 3.8 Effect of pH on cassava SBE activity.

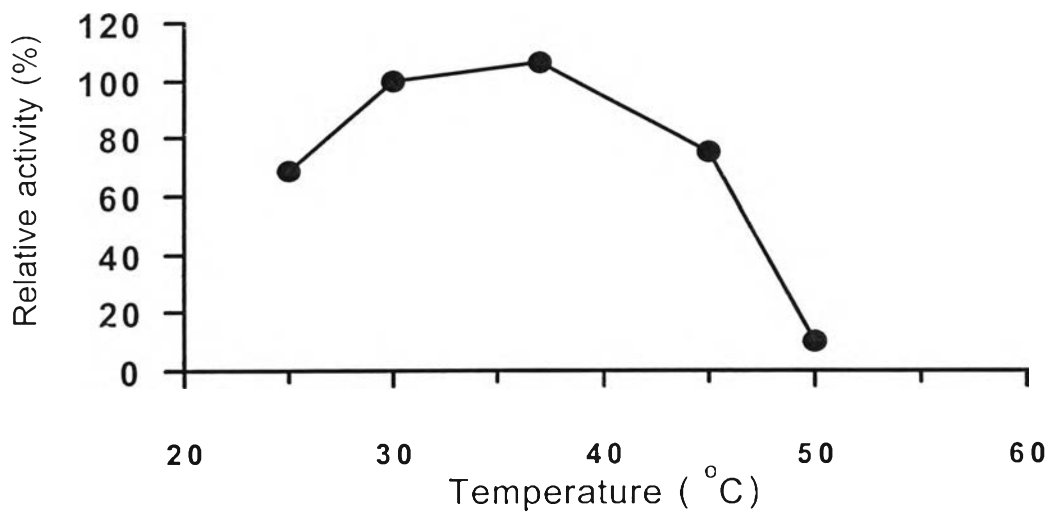

Figure 3.9 Effect of temperature on cassava SBE activity.

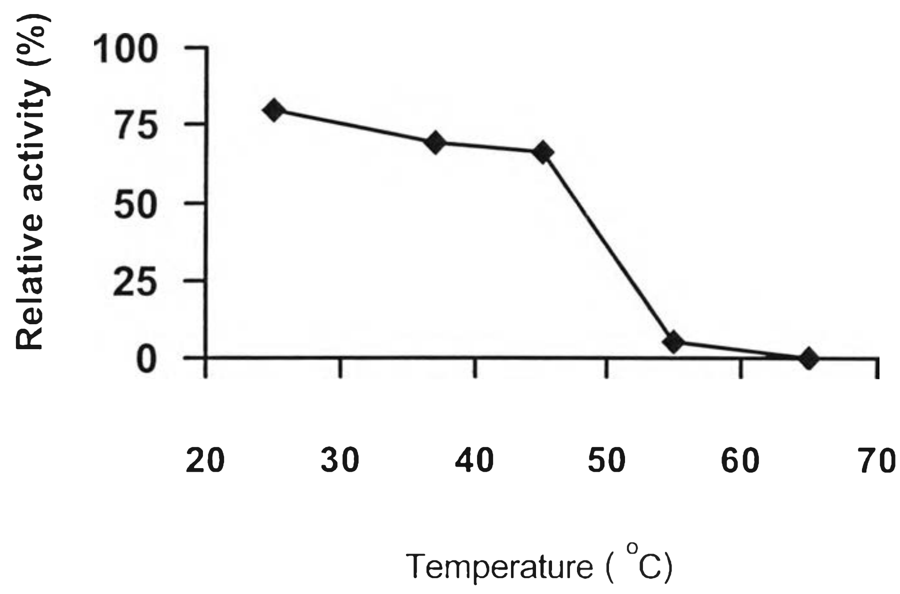

Figure 3.10 Temperature stability of cassava SBE.

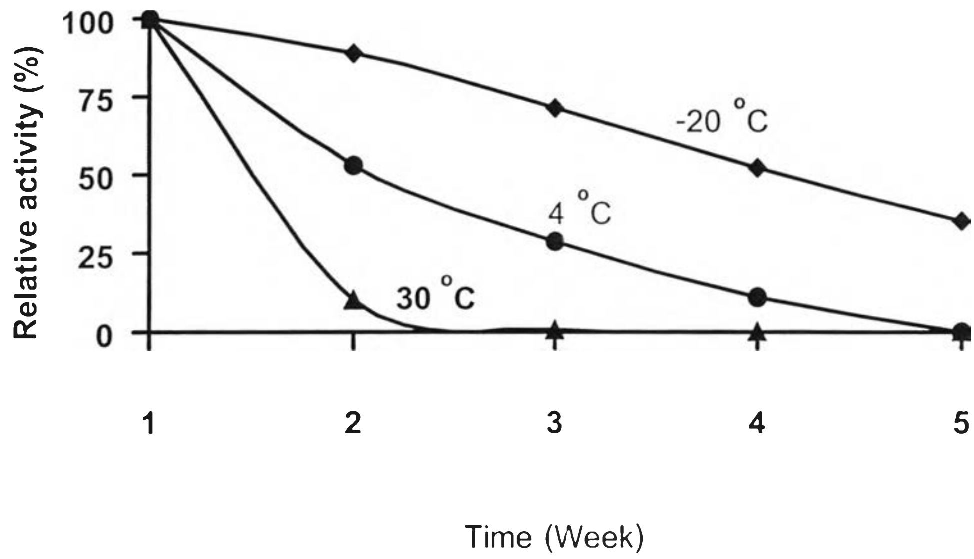

Figure 3.11 Storage temperature of cassava SBE.

3.2.5 Isoelectric point (pI)

The desalted cassava SBE from the Sephadex G-200 column was analyzed for its pI value by separation on IEF gel electrophoresis, comparing to standard pI markers and their relative mobility against their pI's (Figure 3.12) as described in section 2.11. The protein staining pattern of purified SBE on the IEF gel (lane 1) revealed one sharp protein band with a smear area at the acidic end (Figure 3.13). The pI of the sharp band was 5.4.

3.2.6 Effect of glycans on the cassava SBE activity

To study the effect of glycans of different sizes and structures on SBE activity, maltose, pentose, amylose, dextrin, amylopectin, glycogen and starch, at the concentration 1.0 mg/ml were added with other components to the reaction mixture. It was found that the activity increased 5.7, 2.4, 2.0 and 1.9 folds with the addition of starch, glycogen, amylopectin and dextrin, respectively (Table 3.2).

3.2.7 Effect of glycans on precipitation of SBE reaction products

In the SBE assay process, after the reaction was stopped, the radioactive product was precipitated by methanol for radioactive determination and glycogen was added as a carrier to help the precipitation process. To study on the effect of other glycans as alternative carrier in the precipitation process, glycogen, starch, amylopectin and dextrin was added at the concentration 1.0 mg/ml in place of glycogen and the amount of radioactive product monitored. As shown in Table 3.3, all the compounds tested has no different effect compared to glycogen.

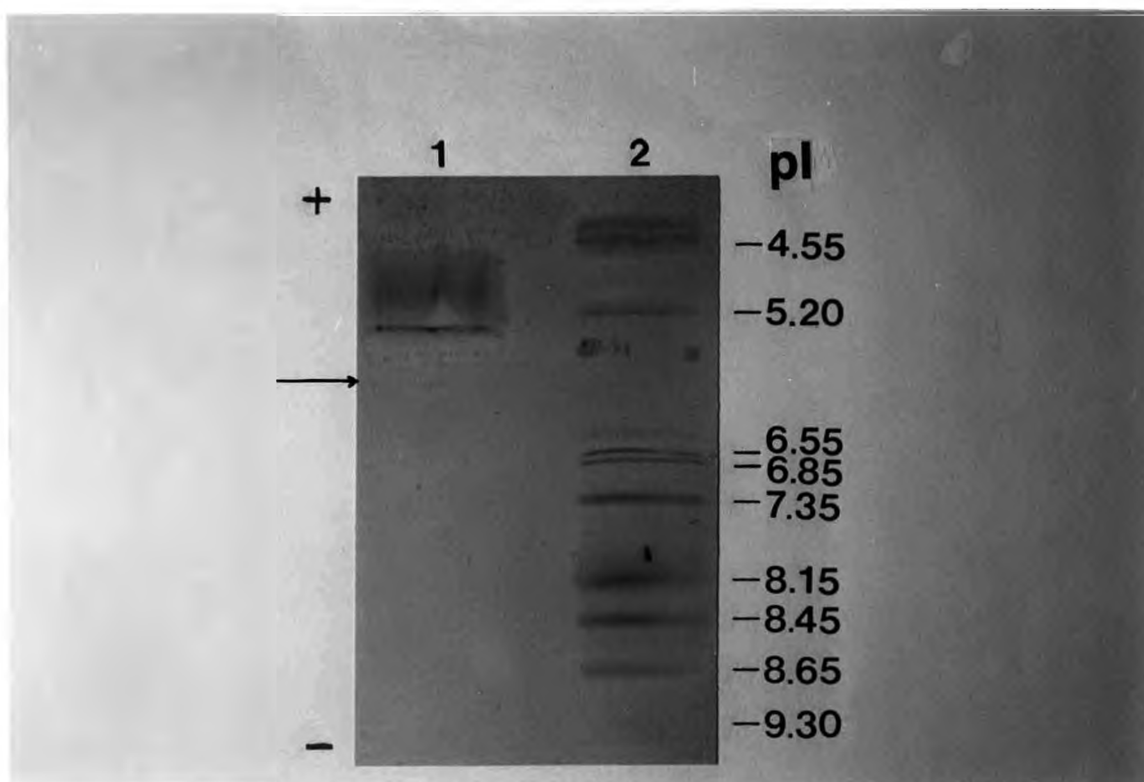

Figure 3.12 Polyacrylamide isoelectrofocusing of cassava SBE.

Lane 1 Purified cassava SBE from Sephadex G-200 column

Lane 2 Standard pI markers :

Soybean trypsin inhibitor	(pI 4.55)
β-lactoglobulin	(pI 5.20)
Human carbonic anhydrase B	(pI 6.55)
Myogloblin-acidic band	(pI 6.85)
Myogloblin-basic band	(pI 7.35)
Lentil lectin-acidic band	(pI 8.15)
Lentil lectin-middle band	(pI 8.45)
Lentil lectin-basic band	(pI 8.65)
Trypsinogen	(pI 9.30)

The arrow indicates point of sample applications.

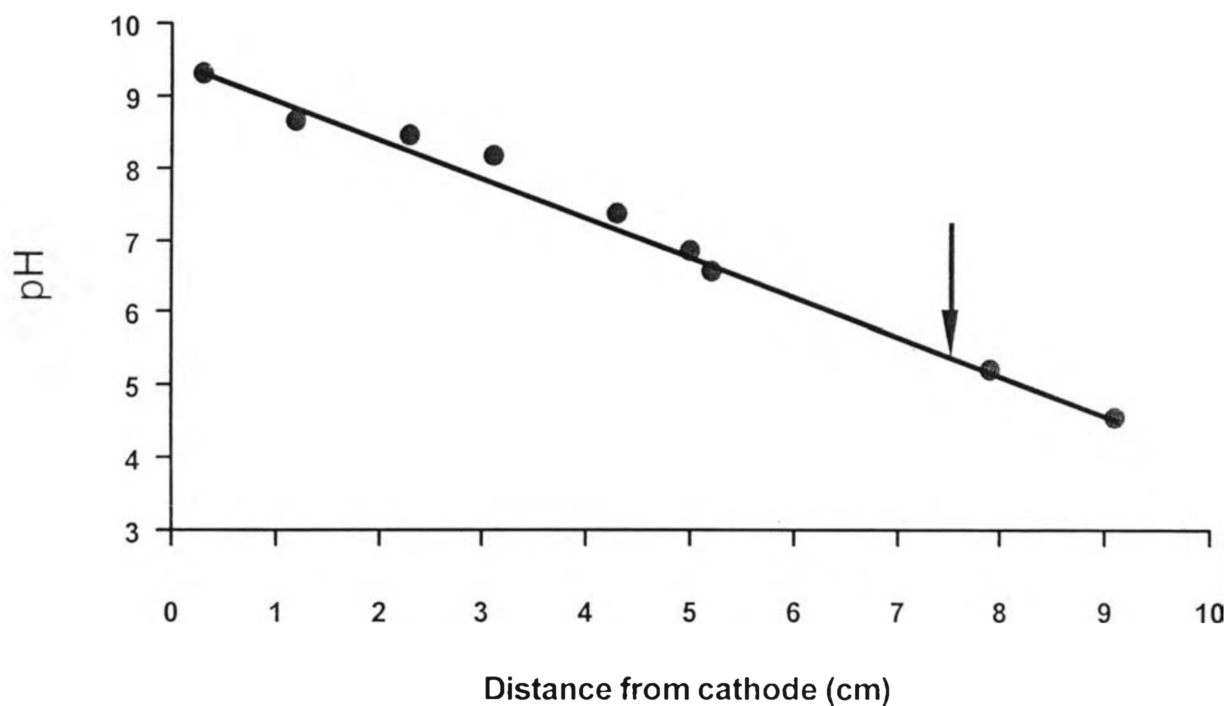

Figure 3.13 Calibration curve of standard pI markers.

The migration distance from cathode of the pI standard markers (as describe in Figure 3.12) were measured and plotted against pI'S. The arrow indicates migration position of the cassava SBE.

Table 3.2 Effect of some Glycans on cassava SBE activity.

Glycans	**Activity** (unit/ml)	**Relative activity** (Fold)
G1P (control)	755	1
G1P + Maltose (5 mg/ml)	524	0.7
G1P + Pentose (5 mg/ml)	609	0.8
G1P + α-Cyclodextrin (1 mg/ml)	680	0.9
G1P + β-Cyclodextrin (1 mg/ml)	795	1.1
G1P + Amylose (1 mg/ml)	794	1.1
G1P + Dextrin (1 mg/ml)	1,502	1.9
G1P + Amylopectin (1 mg/ml)	1,536	2.0
G1P + Glycogen (1 mg/ml)	1,823	2.4
G1P + Starch (1 mg/ml)	4,338	5.7

Table 3.3 Assay of cassava SBE activity using different carbohydrate as carrier for product precipitation.

Carrier		**CPM**	Fold
Glycogen (control)	(10 mg/ml)	9,788	1
Glycogen	(1 mg/ml)	8,516	0.87
Starch	(1mg/ml)	9,140	0.93
Amylopectin	(1 mg/ml)	9,486	0.97
Dextrin	(1 mg/ml)	9,846	1.01