CHAPTER 3

RESULTS AND DISCUSSION

Figure 1 showed some samples of "Look Pang" of various uses - in making sweetened rice, vinegar and alcohol. The sources of "Look Pang", their intended uses, the isolates with code numbers, and amylolytic potent strain of each sample were given in Table 1.

α-amylase activity of the six samples of "Look Pang" were shown in Table 2. "Look Pang" No. 2 had the highest activity, followed by "Look Pang" No. 1, 5, 4 and 3, respectively. The dextrinization time of "Look Pang" No. 6 was more than 1 hours. The result suggested that "Look Pang" was not only the starter containing seed culture but also by itself was the source of enzyme.

An experiment was performed comparing the α-amylase producing capacity between one single potent strain from one sample and the mixture of all isolates from that same sample. As expected, the amylolytic producing capacity of mixed culture was higher than that of the single culture in all 5 samples, except sample No. 6 (Table 3). It was most likely that the mixed culture composed of more than one kind of amylase such as α-amylase, isoamylase and glucoamylase, which resulted in higher rate of starch hydrolysis. For sample No. 6, the activity was almost

the same both single and mixed culture, this could be explained that either they produced the same kind of amylase or low activity.

the cell morphological characteristics and sugar fermentation of the potent isolate. 1Y was shown in Figure 2. It showed the pseudomycelium with blastospores both in liquid and solid medium. This strain could ferment glucose, sucrose, maltose and soluble starch but not galactose, lactose and inulin. Trehalose, reffinose and α-methyl-D-glucoside were slightly formented. This strain was identified (with the co-operation of Dr. I. Banno, yeast taxonomy expert from [1]IFO) as Endomycopsis fibuligera. This strain differs from other strains of same species in some nutritional requirements. There was no growth on Wickerhams basal medium containing certain vitamins (33).

Table 4 was the modified medium for amylase production. The composition of the basal medium (Phaff et al) was modified only on carbon sources. Glucose, maltose and soluble starch were found to be good carbon sources for growth. Amylase was produced in the presence of maltose and soluble starch but not glucose. Soluble starch induced amylase production much better than maltose. By using half diluted YM medium, the amylase production was double.

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[1]IFO - the Institute for Fermentation of Osaka, Japan.

This medium of pH 6.0 which gave the highest activity was used for production of amylase.

The production of amylase in a 20 liters medium was graphically recorded in Figure 3. The growth and product formation was rather correlated. The pH of cultured medium dropped to 5.5 in the first few hours and then gradually increased, reaching pH 7.5 after 50 hours incubation time. The amylase activity of the harvested culture medium was 1.5-2.0 units per ml. This activity was almost the same to those produced by Endomyces sp. IFO 0111 (Hattori, 1961) and Endomycopsis fibuligera IFO 0108 (Fukumoto et al, 1960).

Figure 4 showed the elution patterns of the amylase from columns of DEAE cellulose (Figure 4a.) and Sephadex G-200 (Figure 4b.). The two peaks of major and minor were shown in Figure 4a. A minor peak was found to be an endolytic type of amylase hydrolyzing cyclodextrins (Figure 10 and Table 7). In Figure 4b, a single peak of amylase was obtained. The purity of enzyme was confirmed by disc-gel electrophoresis as one single band (Figure 5).

The steps of enzyme purification were given in Table 5. An amylase was purified 25-fold and 280 mg of purified enzyme protein were obtained from 20 liters of culture filtrate with 42% yield of total activity. The specific activity of the purified enzyme was 38 units per mg of protein. The molecular weight of the enzyme was 5.8×10^4.

The effect of pH and temperature on activity and stability of amylase were graphically shown in Figure 6. The maximum activity of the enzyme was at pH 5.5 (Figure 6a). The stability of the enzyme was between pH 4.0 and 9.0 (Figure 6c). The optimum temperature was at 55°C (Figure 6b), and enzyme activity was completely lost at 80°C (Figure 6d).

Figure 7 and Figure 8 showed the paper chromatograms of the hydrolytic products from potato starch, corn amylase, waxy corn amylopectin and oyster glycogen. Only glucose was released at all stages of hydrolysis from all substrates. From this result, one could concluded that this amylase was glucoamylase.

Figure 9 was the radioautogram of the reaction mechanism of the amylase. It showed the maltodextrins, labeled at the reducing end was hydrolyzed with accumulation of radioactive glucose and maltose. The former was the final product and the latter was an intermediate. Maltose was seen because of the relatively low maltase activity of the glucoamylase.

The rates of the amylolytic reactions on various substrates were measured quantitatively from the initial velocities which was expressed as the specific activity (Table 6). Amylase, various maltodextrins, amylopectin and glycogen were hydrolyzed rapidly, while maltose and maltotriose were hydrolyzed less rapidly. Glucosides, such as p-nitrophenyl-α-glucoside and α-methyl-glucoside, were scarcely hydrolyzed. Thus the enzyme specificity was similar to those of other glucoamylases (Fukumoto,

1960 and Fukui, 1969). Raw starch and dextran were not hydrolyzed, a slight activity towards pullulan was detected. The ratio of the saccharifying activities on amylose and maltose was 5.0, which was close to the value of 4.0 (Hattori, 1961) and 3.5 (Fukui, 1969) reported for Endomyces glucoamylase (10, 7). No transglucosidase activity was detected. Maltotriitol was hydrolyzed quite rapidly but maltitol was not.

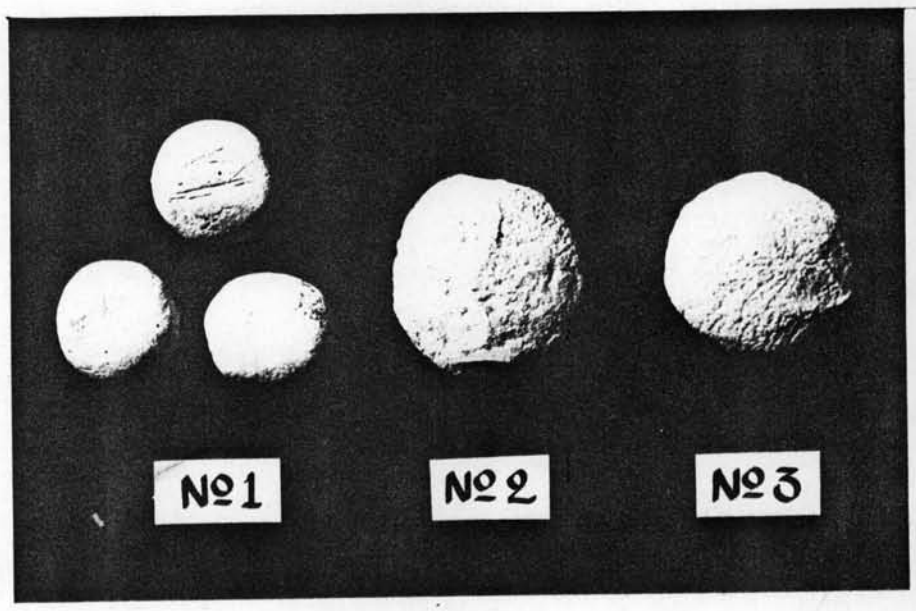


Fig. 1 Samples of"Look Pang"

No.1 "Look Pang Khao-mak"used in making sweetened rice

No.2 "Sar Nam-som"used in making vinegar

No.3 "Sar Lao"used in making alcohol

Table 1

Isolates from various sources of "Look Pang"

"Look Pang" Sample No.	Intended use	Sources	Isolates		Amylolytic Potent Strains
			Code No.	Classification	
1	sweetened rice	Ayuthya province	1 Y	Endomycopsis sp.	1 Y
			1 M	Rhizopus sp.	
			1 M M	Aspergillus niger group	
2	bakery	Monlohaeng Bakery Bangkok	2 Y	Endomycopsis sp.	2 Y
			2 M 1	Rhizopus sp.	
			2 M 2	Rhizopus sp.	
			2 M M	Aspergillus niger group	
3	sweetened rice	Vinegar Factory, Phrapradaeng	4 Y	Endomycopsis sp.	4 Y
			4 M	Rhizopus sp.	
4	alcohol	Chai Talae Chan Pen Vinegar Factory, Bangkok	5 Y	Endomycopsis sp.	5 Y
			5 M	Rhizopus sp.	
5	vinegar	Chai Talae Chantraphen Vinegar Factory, Bangkok	6 Y	Endomycopsis sp.	6 Y
			6 M	Rhizopus sp.	
6	alcohol	Mahakhun Distillery, Bangkok	7 Y	Endomycopsis sp.	7 M
			7 M	Rhizopus sp.	

Table 2

α-amylase activity of "Look Pang"

"Look Pang" Sample No.	Dextrinization time, min.	α-amylase unit
1	25	0.19
2	20	0.24
3	55	0.09
4	50	0.10
5	40	0.12
6	> 60	-

Table 3

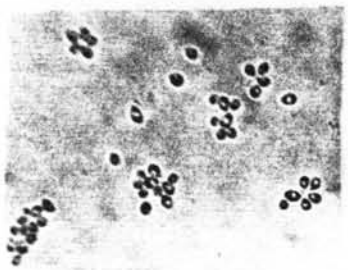
α-amylase producing capacity

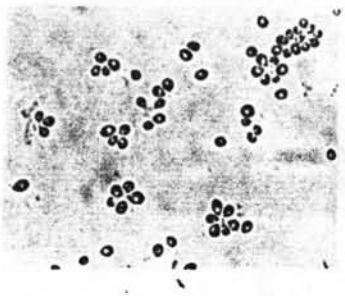
Sample No.	Culture code No.	Dextrinization time, min.	α-amylase Unit
1A	1Y	35	0.14
1B	1Y + 1M + 1MM	30	0.16
2A	2Y	40	0.12
2B	2Y + 2M1 + 2M2 + 2MM2	25	0.19
3A	4Y	35	0.14
3B	4Y + 4M	30	0.16
4A	5Y	40	0.12
4B	5Y + 5M	30	0.16
5A	6Y	45	0.11
5B	6Y + 6M	35	0.14
6A	7M	40	0.12
6B	7Y + 7M	45	0.11

A = single culture

B = mixed culture

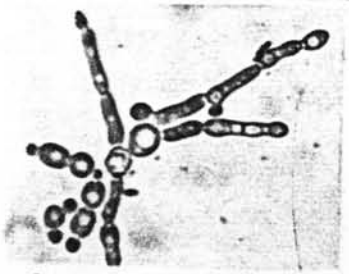
in solid medium

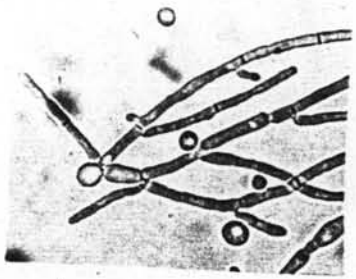


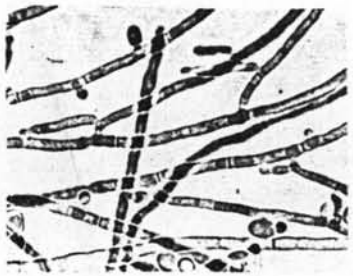


Glucose	+Y
Galactose	-
Sucrose	+Y
Maltose	+Y
Trehalose	-Y
Lactose	-
Raffinose	-Y
Inulin	-
Soluble starch	+Y
α-Methyl-D-glucoside	-Y

in liquid medium



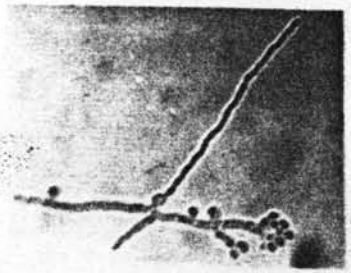


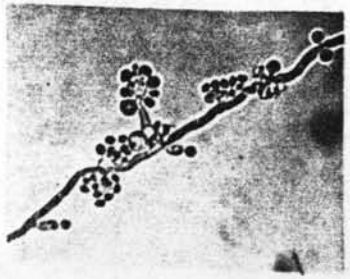


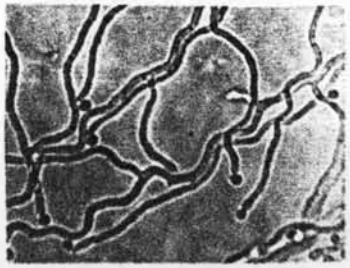
+Y = trace
-Y = slight
- = no

10μ

slide culture on corn meal agar







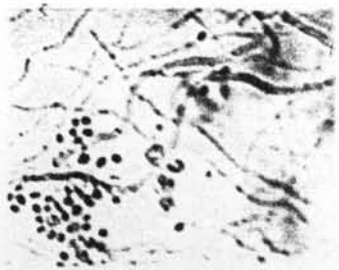


Fig. 2 Cell morphology and Fermentation of 1Y strain

Table 4

Medium Test

YM Basal medium
1.0% sugar
0.5% polypeptone
0.3% malt extract
0.3% yeast extract

Media	Initial pH	Final pH	Growth, Klett Unit	Activity (U/ml)
1. Glucose	7.0	3.8	33	0.00
2. Maltose	7.0	5.5	24	0.23
3. Soluble starch	7.0	6.3	30	0.68

Modified medium
0.5% soluble starch
0.2% polypeptone
0.1% malt extract
0.1% yeast extract

Media		Initial pH	Final pH	Growth, Klett unit	Activity (U/ml)
4.	Soluble starch	4.5	7.1	25	1.63
5.		5.0	7.0	24	1.66
6.		5.5	6.9	24	1.55
7.		6.0	7.3	22	1.80
8.		6.5	7.2	22	1.34
9.		7.0	7.3	20	1.30

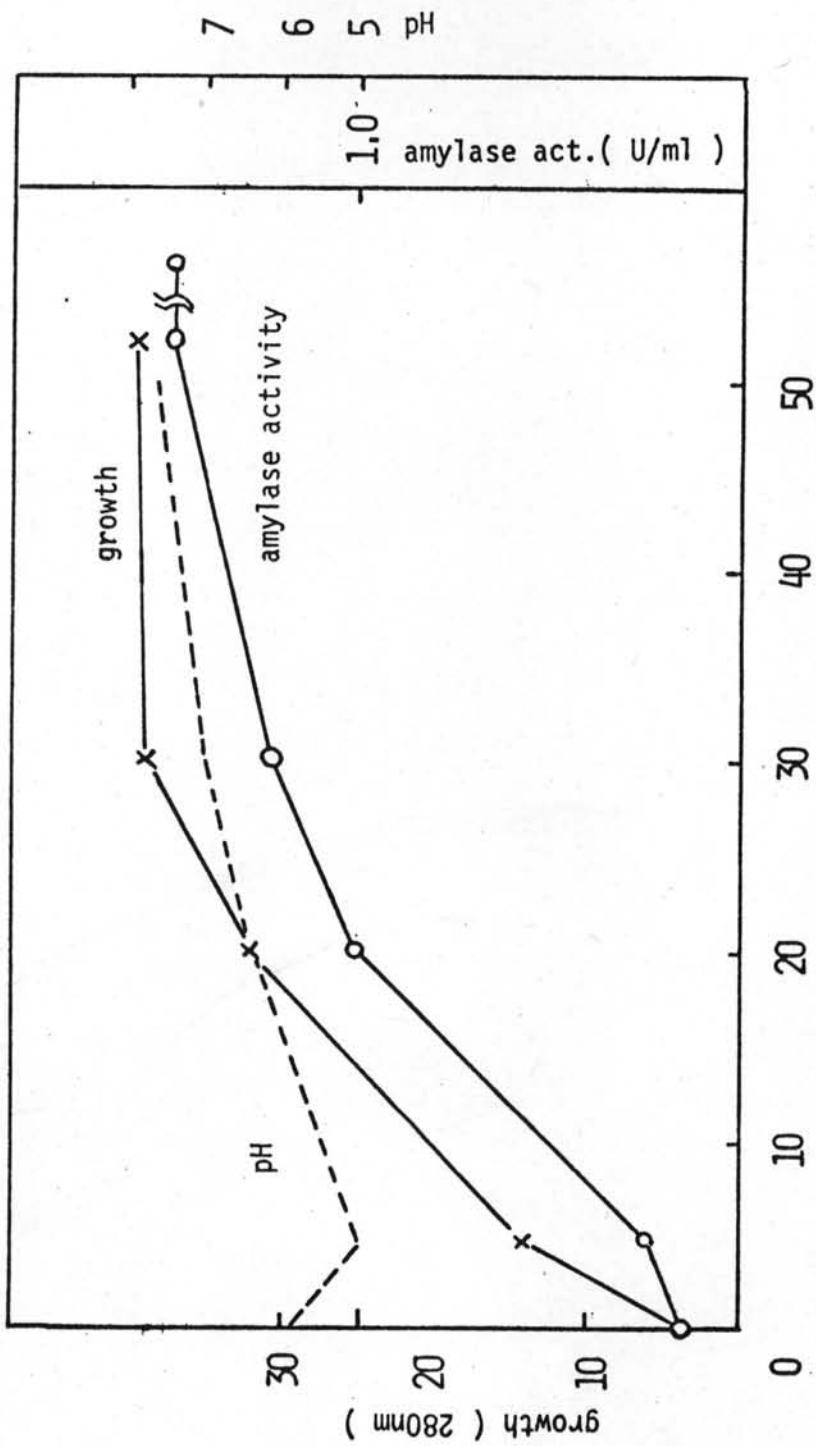


Fig. 3 Formation of the amylase from Endomycopsis sp.

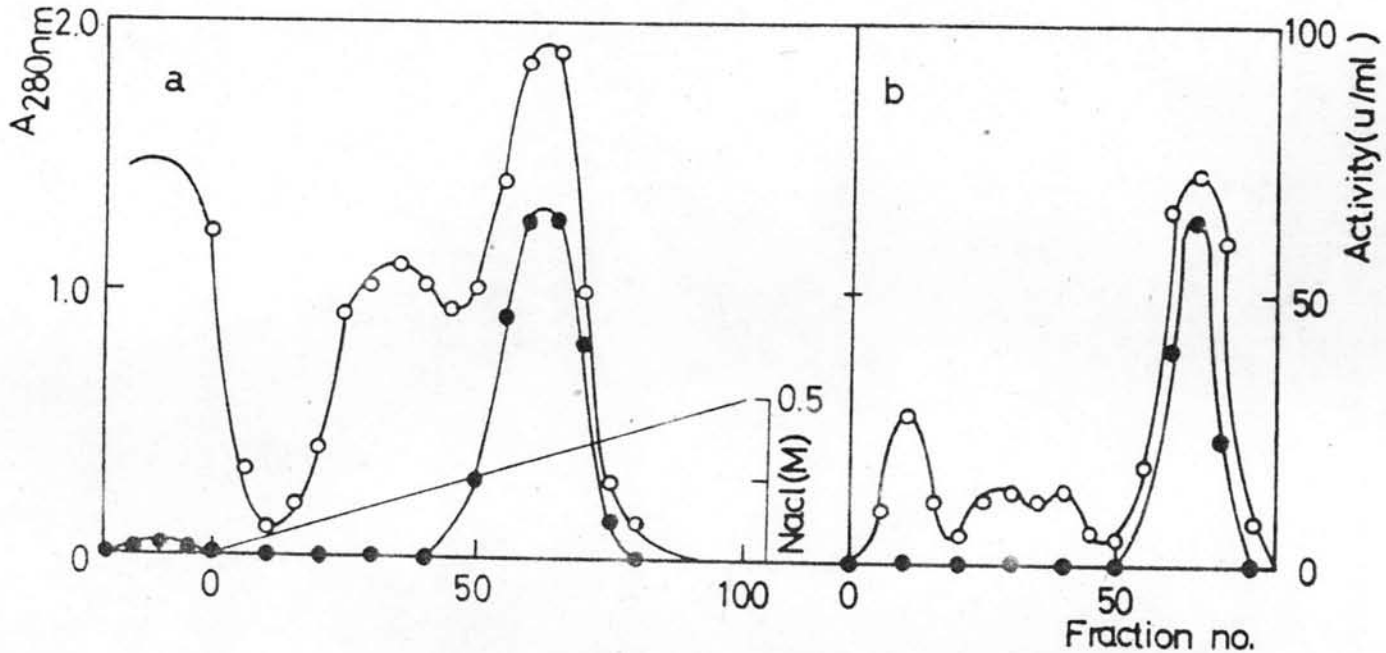


Fig. 4. Elution patterns of amylase from columns of DEAE-cellulose (Fig. 1a) and Sephadex G-200 (Fig. 1b). Fractions of 10 ml of effluent were collected. ○—○, absorbance at 280 nm; ●—●, amylase activity; ——, concentration of NaCl.

Table 5

Enzyme purification

Step of purification	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Crude enzyme	20,000	16,000	24,000	1.5
2. 50-70% $(NH_4)_2SO_4$	180	2,000	16,000	8.2
3. DEAE-cellulose	200	500	11,000	23
4. Sephadex G-200	13	280	10,000	38

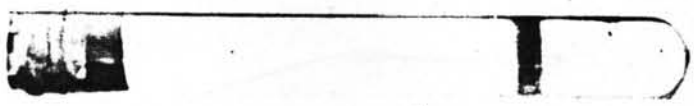


Fig. 5 Polyacrylamide gel electrophoresis of the purified amylase

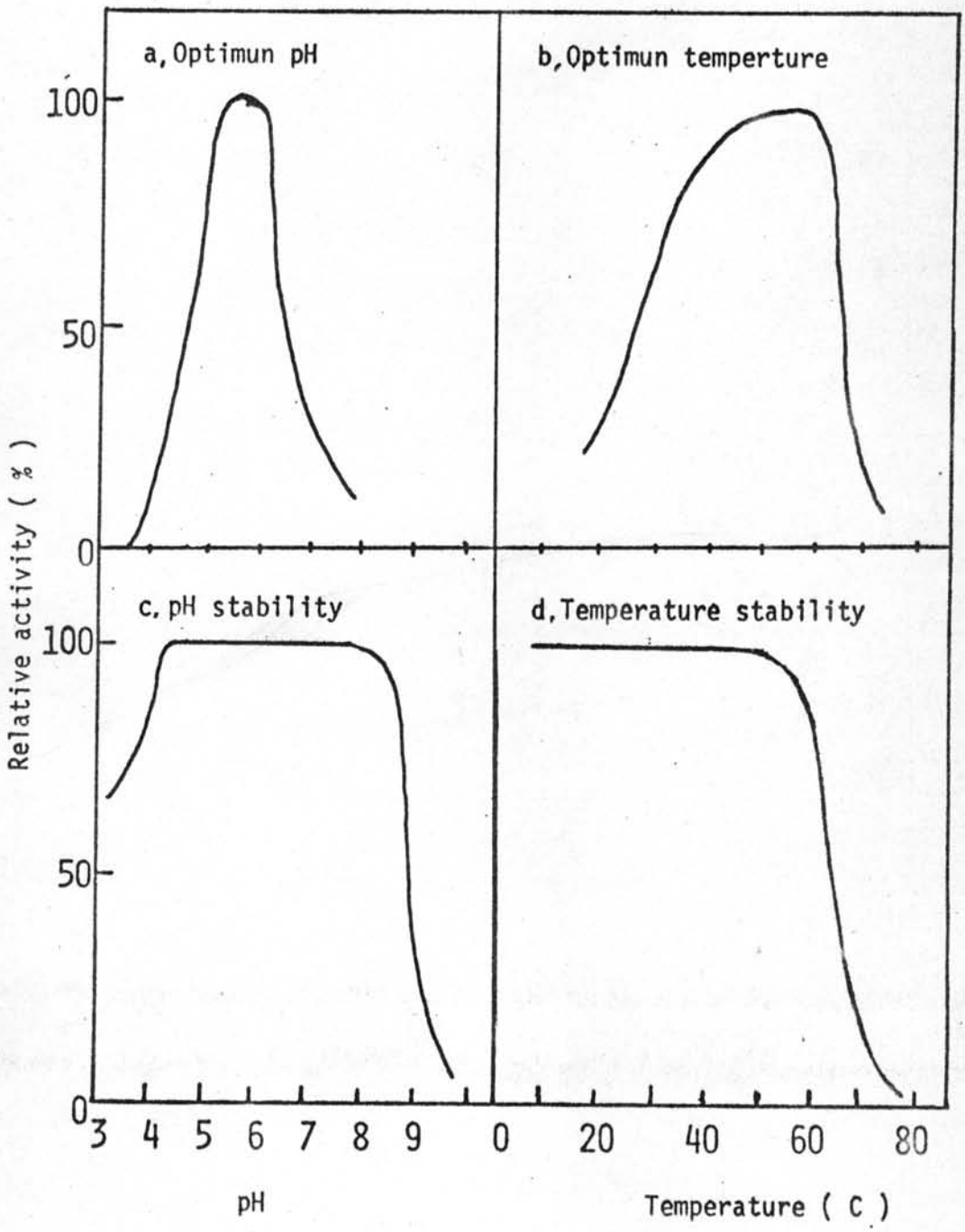


Fig. 6. Effect of pH and temperature on activity and stability of amylase.

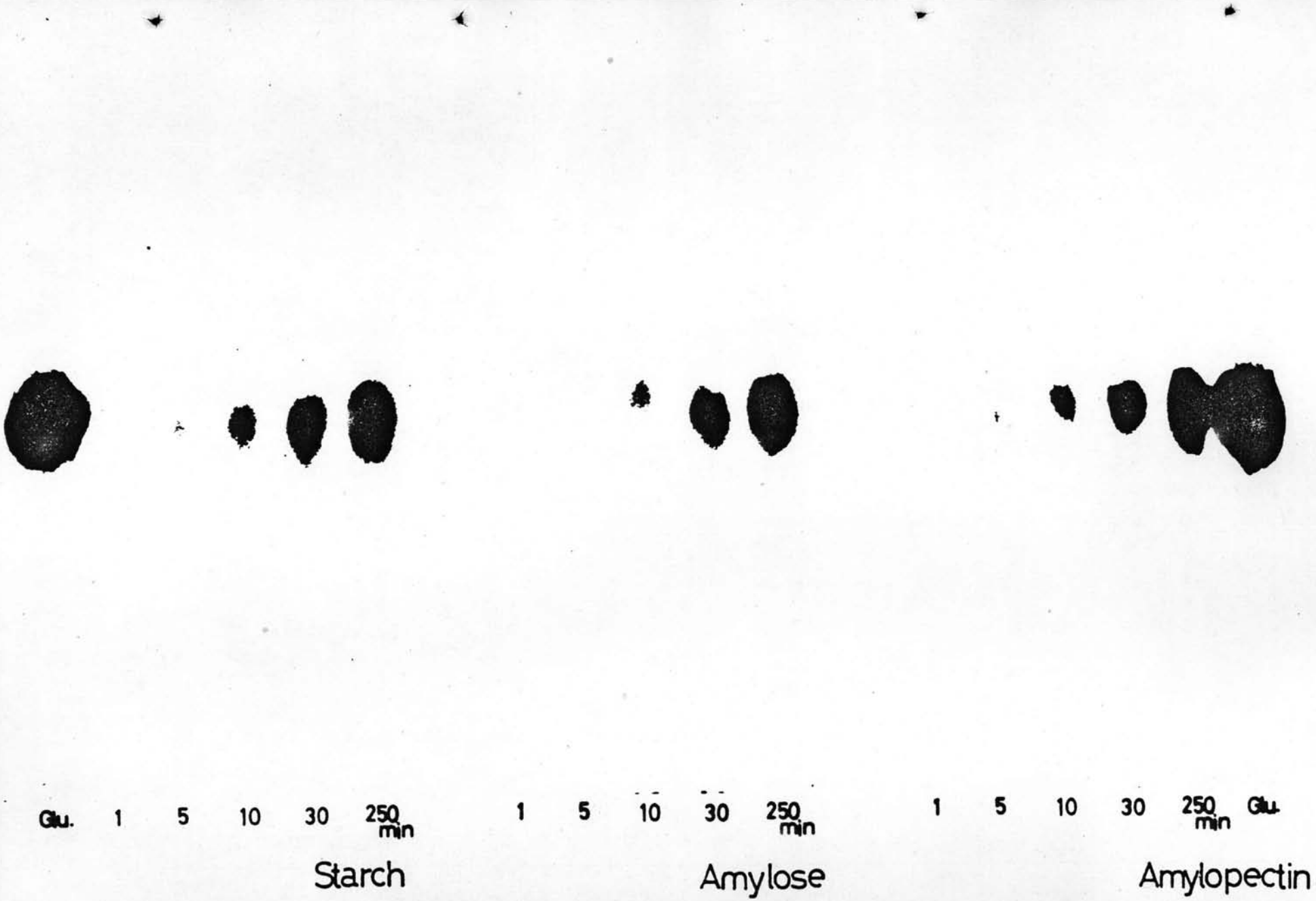


Fig. 7 Paper chromatogram of amylase hydrolysis on starch, amylose, and amylopectin

Fig.8 Paper chromatogram of amylase hydrolysis on glycogen

ST = standard glucose

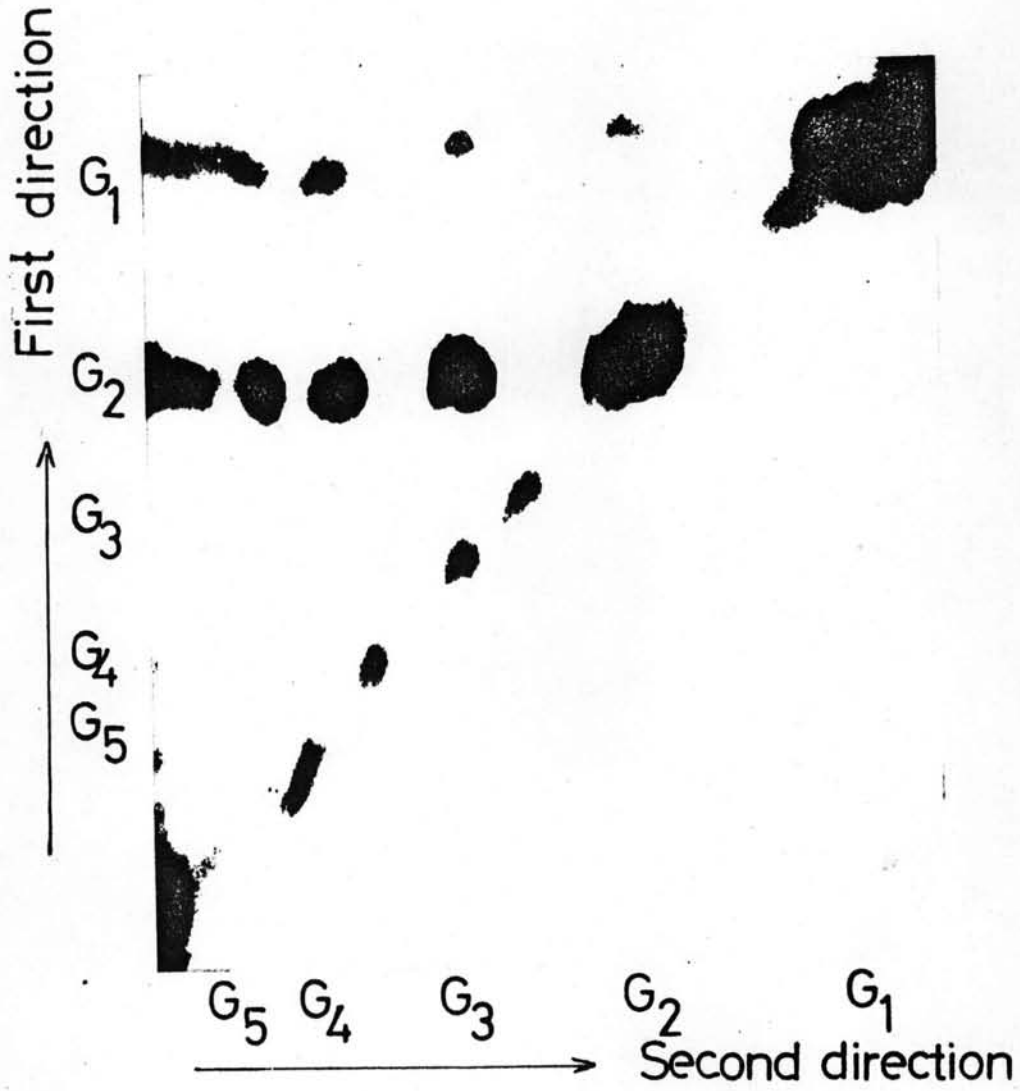


Fig.9 Radioautogram to show the action of the amylase on maltodextrin labeled at the reducing end

Table 6

Substrate specific activity of amylase

Substrates		Specific activity (units/mg)	
		Reducing power	Glucose oxidase
0.002 M	Maltose		4.97
	Maltotriose		13.2
	Maltotetraose		25.6
	Maltohexaose		25.7
	Maltoheptaose		25.9
	Maltooctaose		25.9
	Maltodextrin (Hayashibara Co. DP=100)		24.9
	p-Nitrophenyl-α-glucoside		1.1
	α-methyl glucoside		2.0
	Maltitol		0.0
	Maltotriitol		9.16
	Maltotetraitol		14.5
1.0%	Corn amylose (Sigma Co.)	20	
	Waxy corn amylopectin	27	
	Oyster glycogen (Wako. Chem.)	30	
	Pullulan	2.6	
	Dextran	0.0	
	Raw starch (Potato)	0.0	

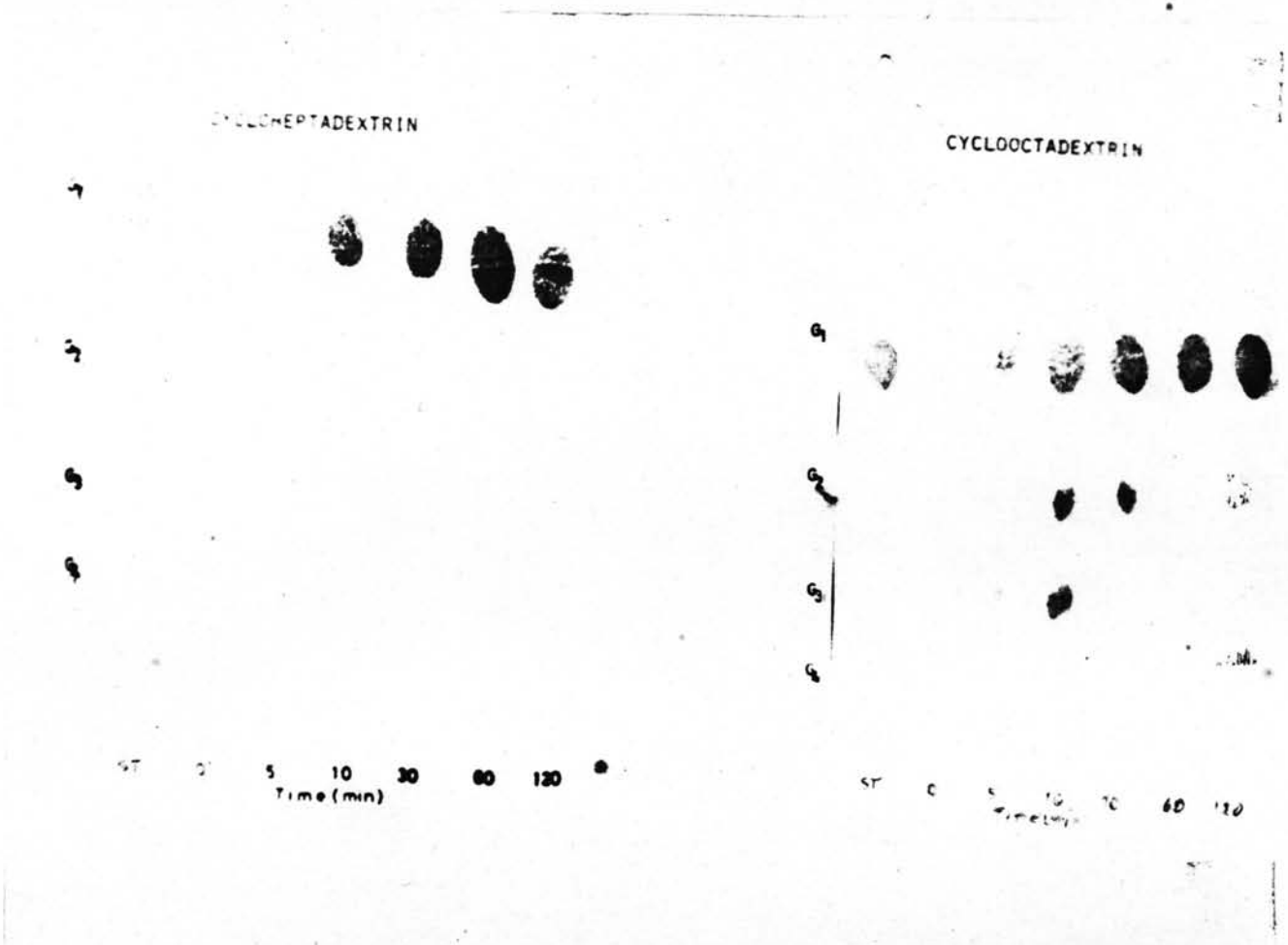


Fig. 10 Paper chromatogram of endolytic amylase hydrolysis on cycloheptadextrin, and cyclooctadextrin.

Table 7

Substrate specific activity of an endolytic type of amylase hydrolyzing cyclodextrin

Substrates	Specific activity units/mg (Reducing power)
Cyclohexadextrin	3.2
Cycloheptadextrin	37
Cyclooctadextrin	42