CHAPTER III

RESULTS

3.1 Partial purification of CGTase

Crude CGTase was partially purified from the culture broth of *Bacillus* sp. A11 by starch adsorption method as reported by Malai (1995). The yield of the enzyme was 78% with purification of 120 folds as shown in Table 10. The enzyme was then concentrated approximately 100 times by ultrafiltration and dialyzed against 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.5 (TBI). The preparation was used for separation by preparative gel electrophoresis Model 491 Prep Cell.

3.2 Isolation of CGTase isozymes

The concentrated partial purified enzyme was loaded onto a discontinuous preparative polyacrylamide gel electrophoresis, which performed on a Model 491 Prep cell (Figure 7). After the dye font reached the bottom of the gel, elution was followed by the use of Tris-glycine buffer, pH 8.3. Figure 8 shows elution profile of CGTase from preparative gel. CGTase was eluted between fractions 20-150. The highest dextrinizing activity was obtained at the same position as the protein peak. Samples from every 5 fractions eluted were subjected to discontinuous gel electrophoresis under non-denaturing condition and stained for dextrinizing activity, as presented in Figure 9. It is clearly illustrated that 5 distinct activity bands can be separated from each other by Prep cell. They were designated as bands 1,2,3,4 and 5 according to the mobility from

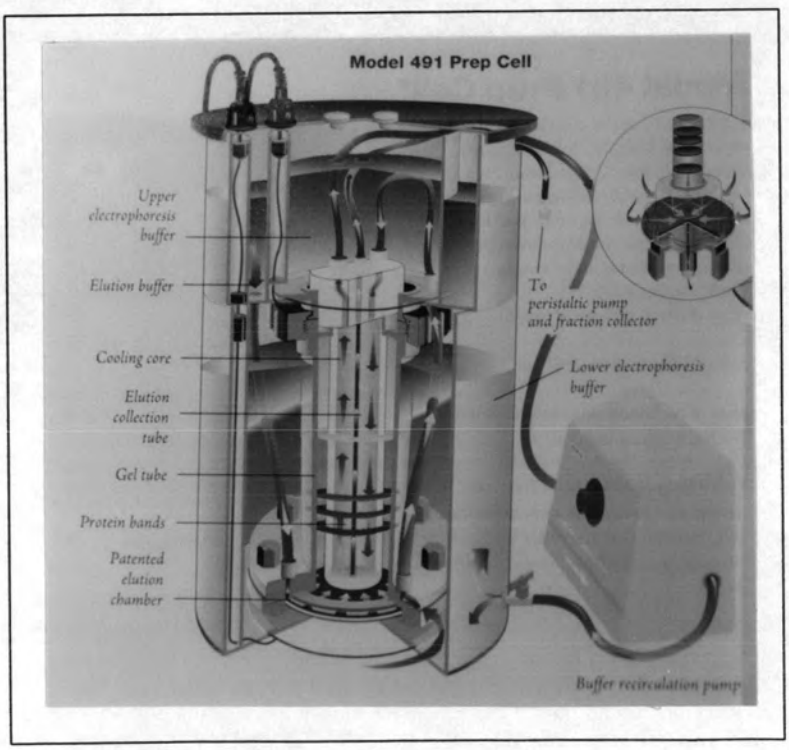


Figure 7. Preparative gel electrophoresis unit.

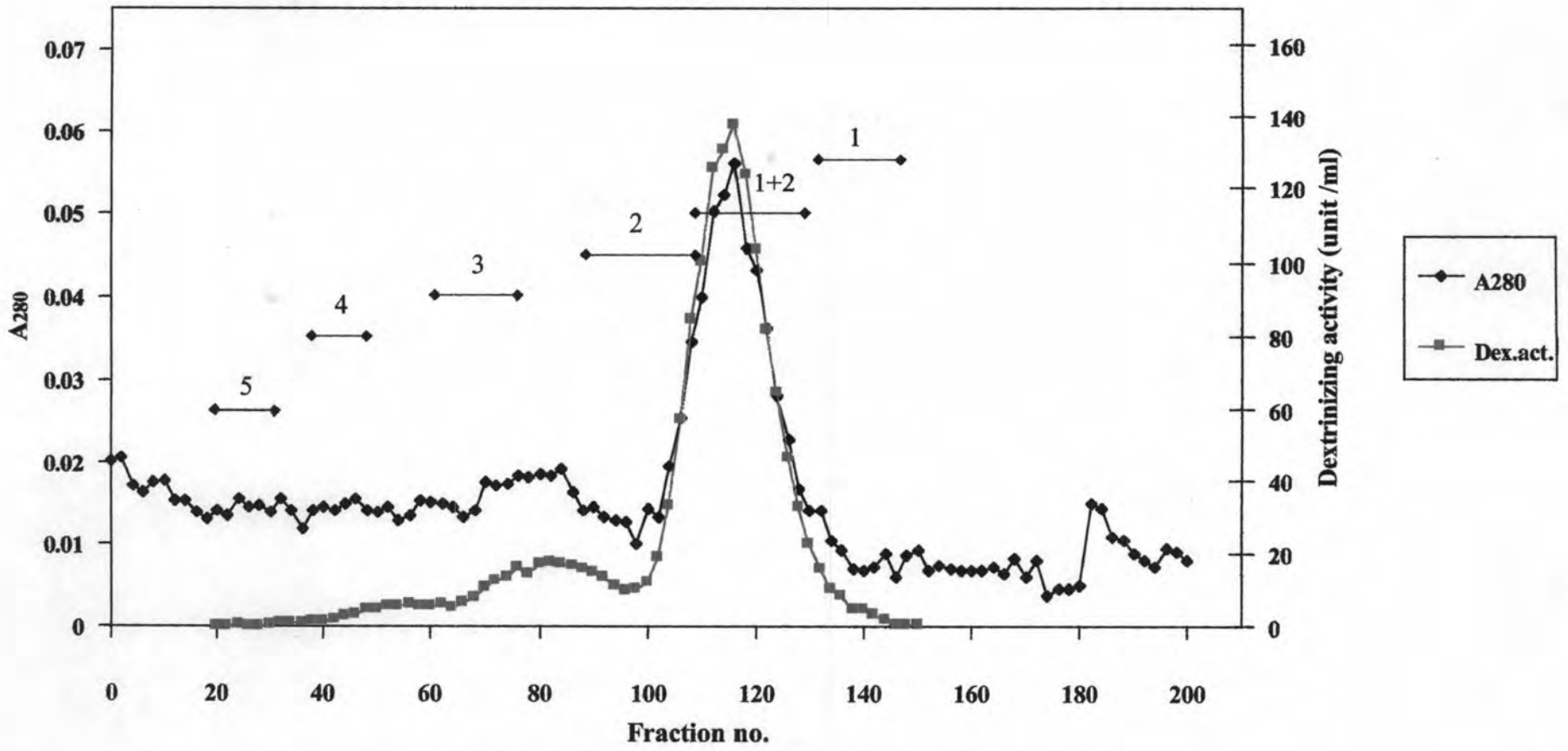


Figure 8. Elution profile of CGTase from preparative gel electrophoresis.

F 20-32 = band 5 , F 35-45 = band 4

F 57-76 = band 3 , F 83-107 = band 2

F108-130 = band 1+2 , F 135-150 = band 1

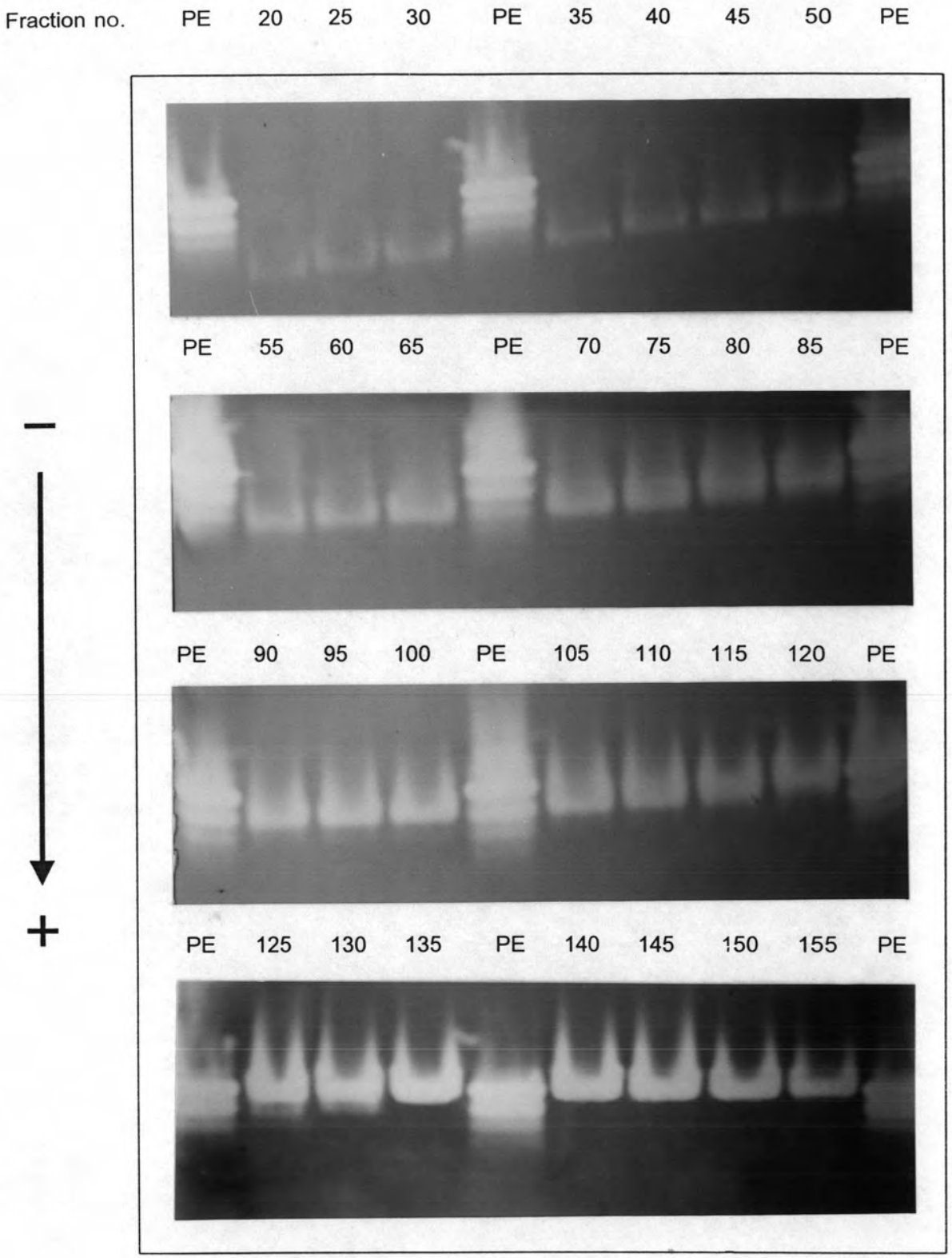


Figure 9. Non-denaturing PAGE pattern with amylolytic activity staining of every 5 fractions obtained from preparative gel electrophoresis.

PE = Partial purified enzyme

the top of the non-denaturing gel. However, they were eluted from Prep cell in reverse order, *i.e.*, bands 5, 4, 3, 2, and 1, respectively.

Selected fractions which yield high purity of each band on non-denaturing gel were pooled : fractions 20 to 32 (band 5), 35 to 45 (band 4), 57 to 76 (band 3), 83 to 107 (band 2) and 135 to 150 (band 1). Although, fractions 108-130 showed highest protein and dextrinizing activity in the elution profile, they yielded contamination of bands 2 and 1 and were not used as purified fractions. The pooled fractions were dialyzed against TBI and concentrated for further study.

The pooled fractions (bands 1 to 5, respectively) were analyzed again by non-denaturing PAGE. Activity stains were performed, comparing between dextrinizing and CD-forming activity (Figure 10 a-b). The result shows that bands 1-4 exhibited both dextrinizing and CD-forming activity, while band 5 exhibited only dextrinizing activity, eventhough the amount of band 5 loaded was 10 times higher than band 1-4.

The recovery and yield of CGTase obtained at each purification step expressed in terms of dextrinizing activity are shown in Table 10. Specific activity of dextrinization increased through each step. The major band observed was band 1 which contained 1.8% of the total activity was purified up to 211 folds after preparative electrophoresis step.

3.3 Characterization of CGTase isozymes

Since, band 5 did not show CD-forming activity, only bands 1-4 were used for comparative study of CGTase activity.

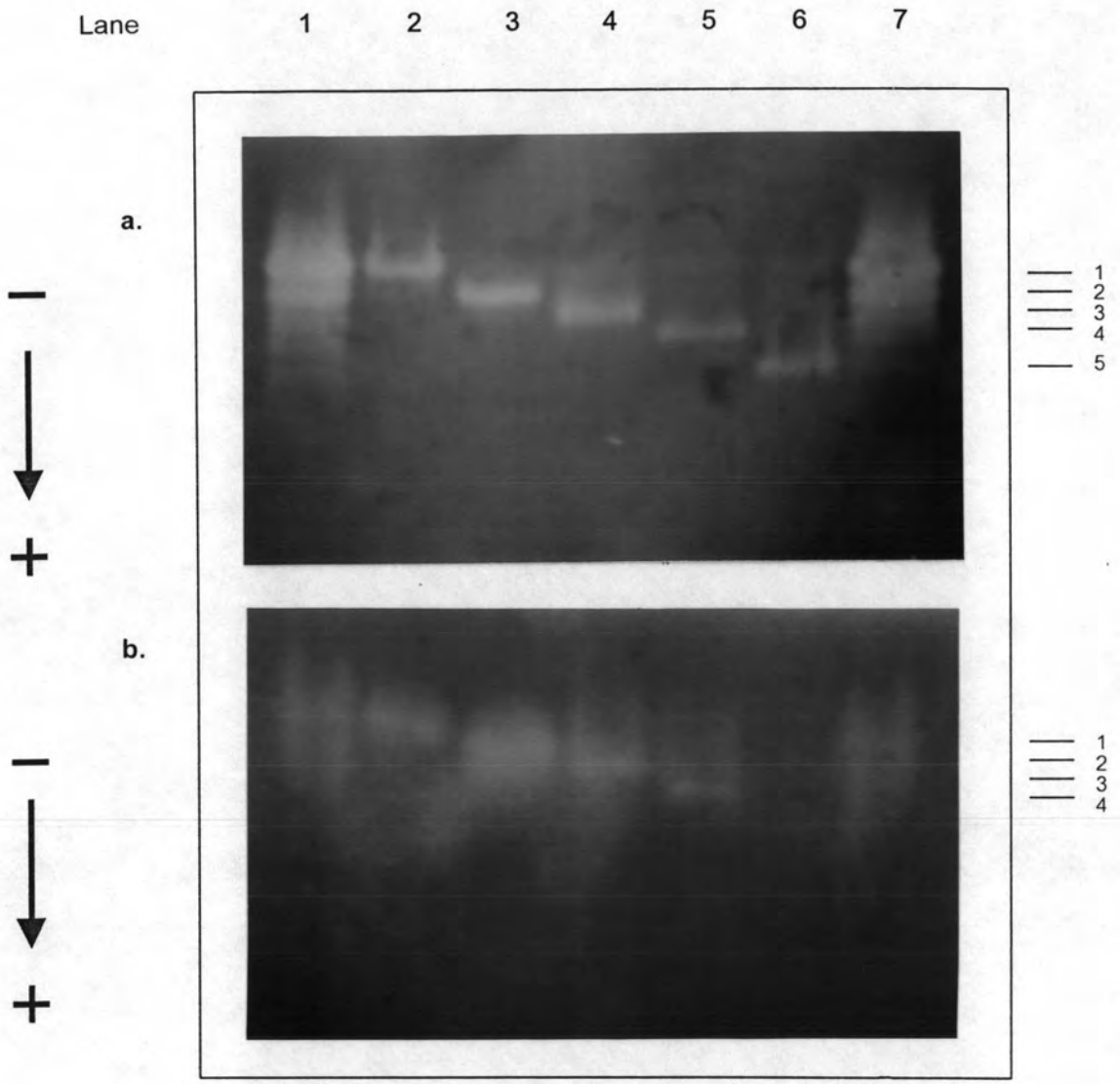


Figure 10. Non-denaturing PAGE of the enzyme bands 1 to 5 obtained from preparative gel electrophoresis.

a. Amylolytic activity staining by iodine solution
(0.2 units of dextrinizing activity)

b. CD-forming activity staining
(2.0 units of dextrinizing activity except for band 5, 20 units were applied)

Lane 1, 7. Partial purified enzyme
2. Band 1
3. Band 2
4. Band 3
5. Band 4
6. Band 5

Table 10. Purification of CGTase from *Bacillus* sp. A11

Purification step	Volume (ml)	Total activity (unit)*	Total protein (mg)	Specifc activity (unit/mg)	Purification fold	Yield (%)
Crude enzyme	20,000	480,000	23,508	20	1	100
Starch adsorption	5,000	375,980	153.7	2,446	120	78.3
Ultrafiltration	25	111,345	37.5	2,969	145	23.2
Preparative gel electrophoresis						
Band 1	28	8,550	1.98	4,318	211	1.8
Band 2	8	2,535	0.67	3,782	185	0.5
Band 3	7	604	0.50	1,207	59	0.12
Band 4	6	151	0.31	486	24	0.03
Band 5	5	2	0.06	35	2	0.0004

* Dextrinizing activity

The CGTase isozymes (bands 1 to 4) separated from preparative gel electrophoresis, were characterized in their properties such as carbohydrate content, molecular weight, isoelectric point, effects of pH and temperature on the enzyme activity, type of main product, amino acid composition and N-terminal amino acid sequence. The results were shown below.

3.3.1 Carbohydrate determination

3.3.1.1. Qualitative analysis by periodic acid-Schiff (PAS) staining

The native-PAGE of bands 1-5 was stained for glycoprotein, comparing with coomassie blue staining of protein as shown in Figure 11(a-b). The violet color of PAS stain developed at the positions of bands 1-5 and transferrin, a glycoprotein which was used as positive control, while no color developed at the position of hemoglobin which was used as negative control.

3.3.1.2. Quantitative analysis by phenol-sulfuric acid method

The total carbohydrate content of the enzyme bands 1-5 were determined by phenol-sulfuric acid method as described in Section 2.10.2.2., compared with standard curve of glucose (Appendix D). The result showed that each band contained different amount of carbohydrate and band 4 showed the highest content of carbohydrate of approximately 46.7% (w/w) (Table 11).

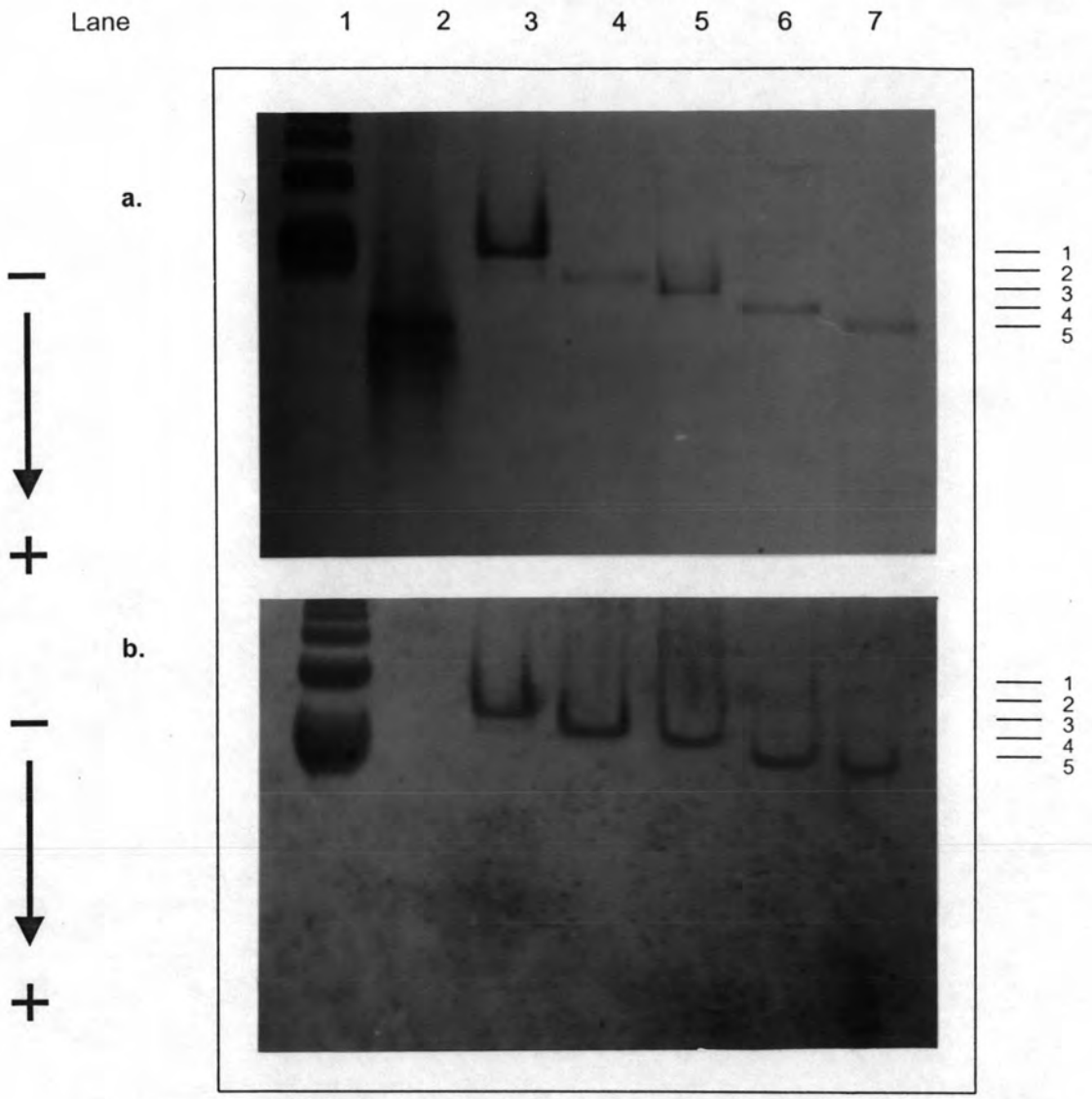


Figure 11. Non-denaturing PAGE of the enzyme bands 1 to 5 obtained from preparative gel electrophoresis.

a. Coomassie blue staining for protein (2 μg protein except for lane 1 and 2, 7 μg were applied)

b. PAS staining for glycoprotein (50 μg protein except for lane 1 and 2, 70 μg were applied)

Lane 1. Transferrin (positive control)

2. Haemoglobin (negative control)

3. Band 1

4. Band 2

5. Band 3

6. Band 4

7. Band 5

Table 11. Carbohydrate content of CGTase isozymes by phenol-sulfuric acid method

Band no.	Protein content (μg / μl)	Carbohydrate content		
		μg / μl	μg / μg protein	%w/w*
1	0.38	0.078	0.205	17.0
2	0.27	0.050	0.187	15.6
3	0.18	0.026	0.144	12.6
4	0.09	0.042	0.467	31.8
5	0.08	0.029	0.363	26.5

* %w/w = %carbohydrate content per total weight of isozyme

3.3.2 Molecular weight determination on SDS-PAGE

In the SDS-PAGE (Figure 12), purified bands 1-5 showed only one intense band at the same position on the gel. The molecular weight of this band was determined from a calibration curve of log of molecular weight of standard proteins and their Rf (Appendix E). The molecular weights of these five bands were equal and estimated to be approximately 72,000 daltons.

3.3.3 Determination of the isoelectric point

The enzyme bands 1-5 were also analyzed for their isoelectric points by separation on IEF gel electrophoresis, comparing to standard pI markers and their relative mobility against their pI (Appendix F). In the IEF gel, bands 1, 2, 3, 4 and 5 were focused at the pI of 4.73, 4.49, 4.40, 4.31 and 4.23, respectively (Figure 13). The faint thick bands were artifacts produced at the position where sample strips were placed.

3.3.4 Effects of pH on the isozyme activity

The effect of pH on the activity of enzyme bands 1-4 were determined for both the dextrinizing activity and the CD-forming activity as described in Section 2.6.1 and 2.6.2 at various pH's. Figure 14 (a-b) show that, bands 1 to 4 showed the same pattern of the pH-activity profile with the highest starch-dextrinizing activity showed in the pH range of 5.0-6.0. However, the CD-forming activity for bands 1 and 2 were highest in the pH range of 6.0-7.0, while activity of bands 3 and 4 were highest at pH 7.0 and 6.0, respectively.

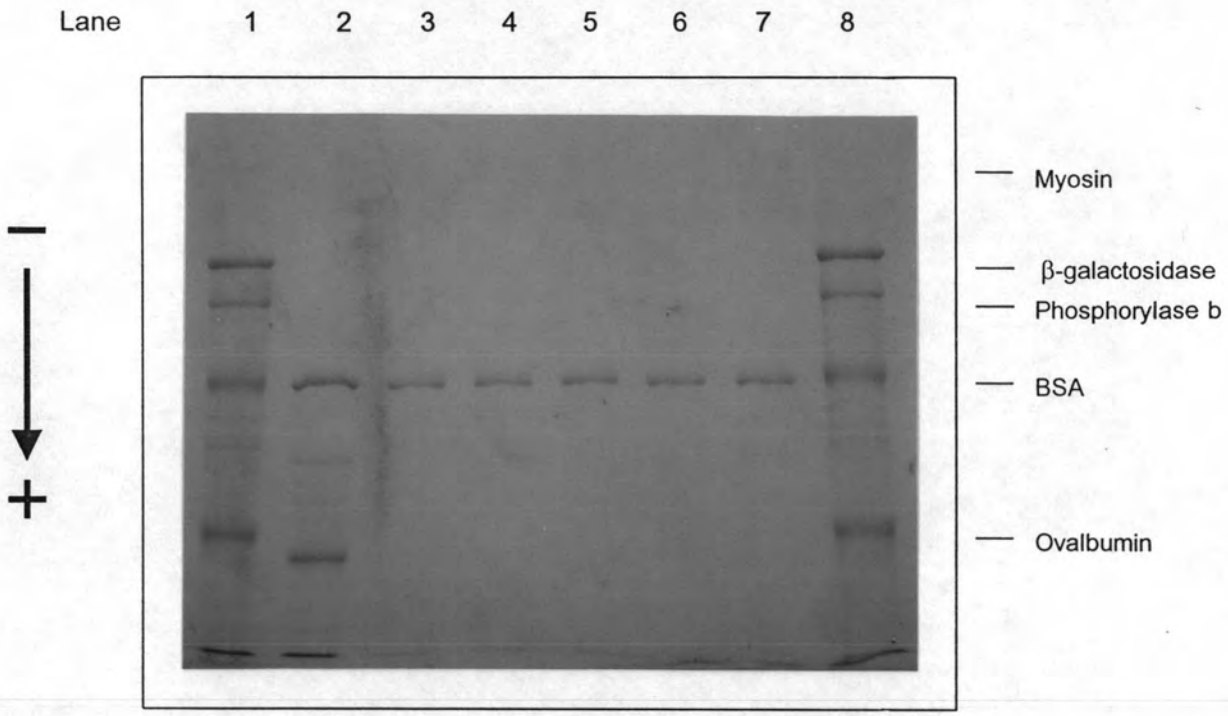


Figure 12. SDS-PAGE pattern of CGTase isozymes obtained from preparative gel electrophoresis.

Lane 1,8. Standard molecular weight protein ; myosin (200 kD) , β-galactosidase (116.25 kD) , phosphorylase b (97.4 kD) , BSA (66.2 kD) and ovalbumin (45 kD)

2. Partial purified enzyme (15 μg protein)
3. Band 1 (2 μg protein)
4. Band 2 (2 μg protein)
5. Band 3 (2 μg protein)
6. Band 4 (2 μg protein)
7. Band 5 (2 μg protein)

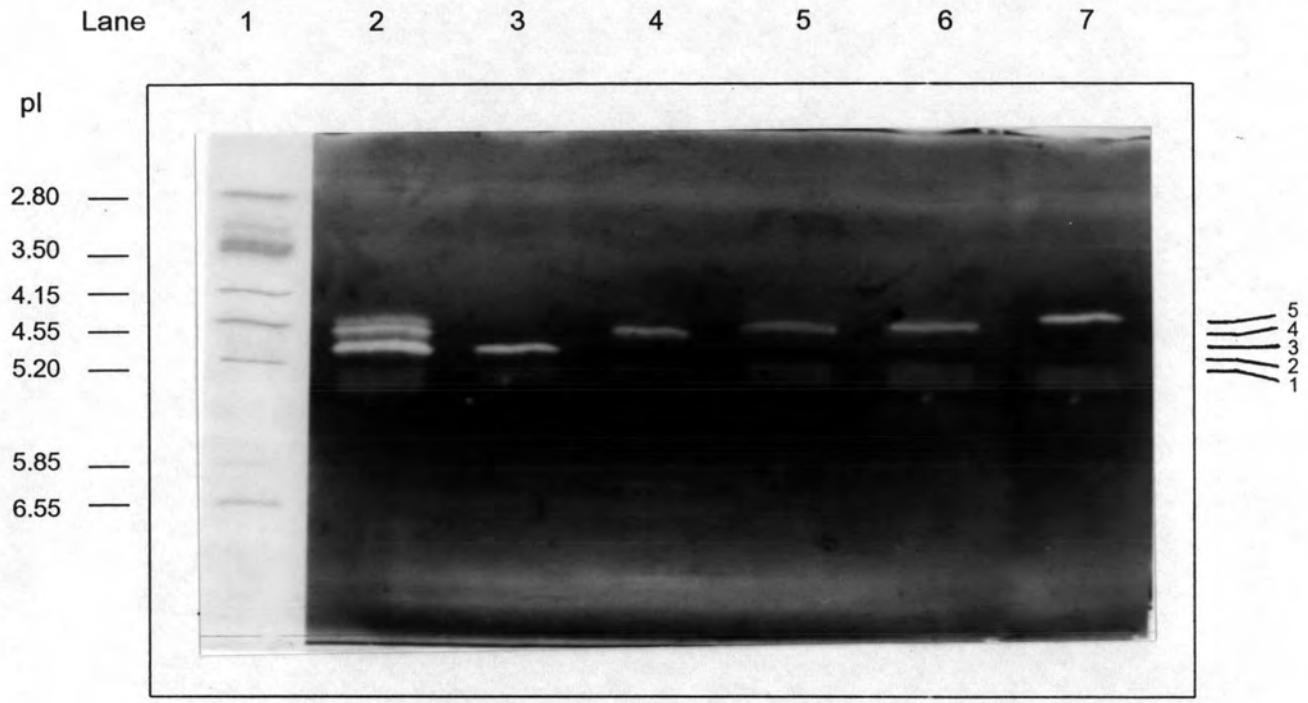


Figure 13. Isoelectrofocusing gel of CGTase isozymes using ampholyte pH 2.5-6.5.

Lane 1. Standard pI marker proteins

2. Partial purified enzyme (02. unit of dextrinizing activity)

3. Band 1 (- -)

4. Band 2 (- -)

5. Band 3 (- -)

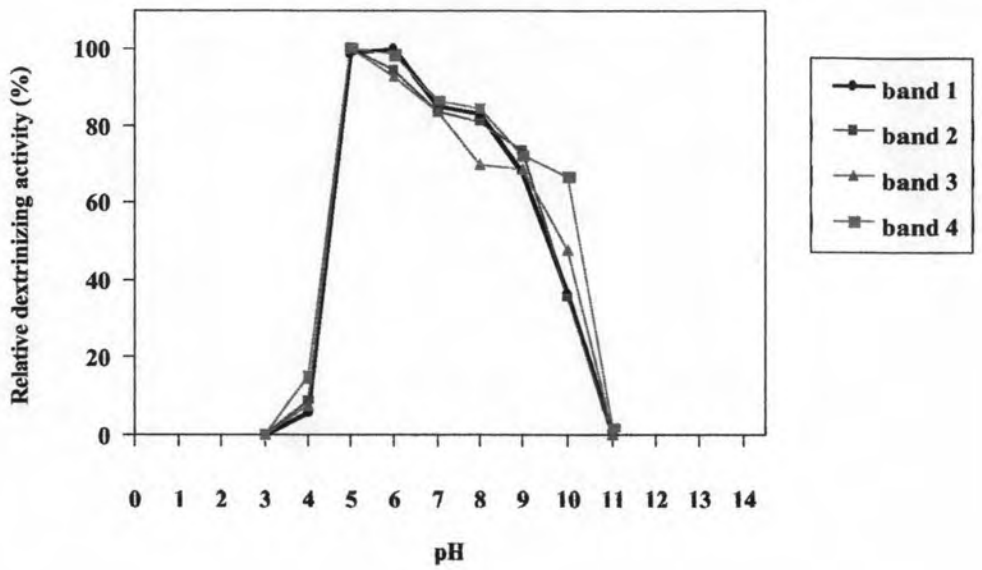
6. Band 4 (- -)

7. Band 5 (- -)

Table 12. Effect of pH on the CD-forming activities of CGTase isozymes

pH	Dilution limit (% Relative CD-forming activity)			
	Band 1	Band 2	Band 3	Band 4
3.0	- (0.00%)	- (0.00%)	- (0.00%)	- (0.00%)
4.0	2^2 (1.56%)	2^0 (0.78%)	2^2 (1.56%)	2^3 (12.50%)
5.0	2^6 (25.00%)	2^5 (25.00%)	2^6 (25.00%)	2^5 (50.00%)
6.0	2^8 (100.00%)	2^7 (100.00%)	2^7 (50.00%)	2^6 (100.00%)
7.0	2^8 (100.00%)	2^7 (100.00%)	2^8 (100.00%)	2^5 (50.00%)
8.0	2^6 (25.00%)	2^5 (25.00%)	2^5 (12.50%)	2^4 (25.00%)
9.0	2^6 (25.00%)	2^5 (25.00%)	2^5 (12.50%)	2^4 (25.00%)
10.0	2^4 (6.25%)	2^3 (6.25%)	2^4 (6.25%)	2^3 (12.50%)
11.0	2^1 (0.78%)	2^0 (0.78%)	2^1 (0.78%)	2^0 (1.56%)

a.



b.

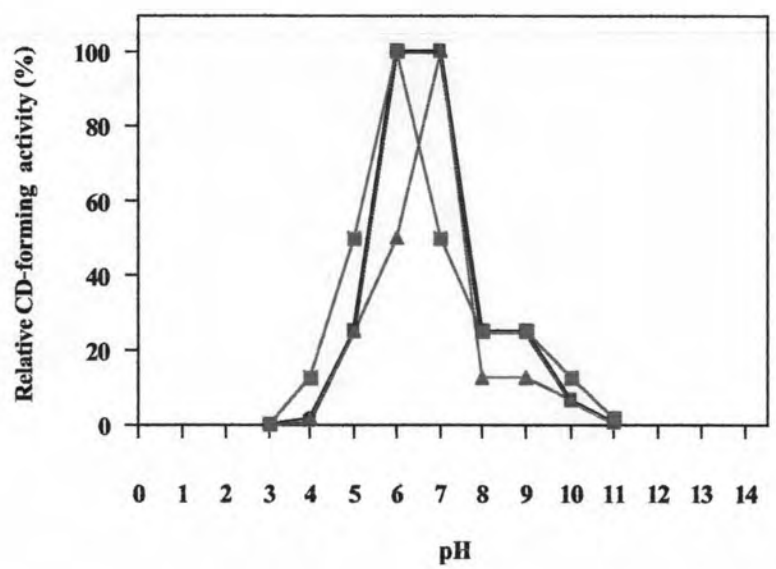


Figure 14. Effect of pH on the activities of CGTase isozymes.

a. Dextrinizing activity

b. CD-forming activity

3.3.5 Effects of temperature on the isozyme activity

The enzyme activities were also measured at various temperatures. The results showed that these 4 bands had the same pattern of temperature-activity profile for dextrinizing activity, with the optimum temperature of 60°C at pH 6.0. However, there were slightly different on the optimum temperatures of CD-forming activity. Band 1 and 2 had the highest activity at 40°C while band 3 had the optimum activity at 50°C. For band 4, the highest activity was shown at 50-60°C (Figure 15 (a-b)).

3.3.6 Analysis of cyclodextrin by HPLC

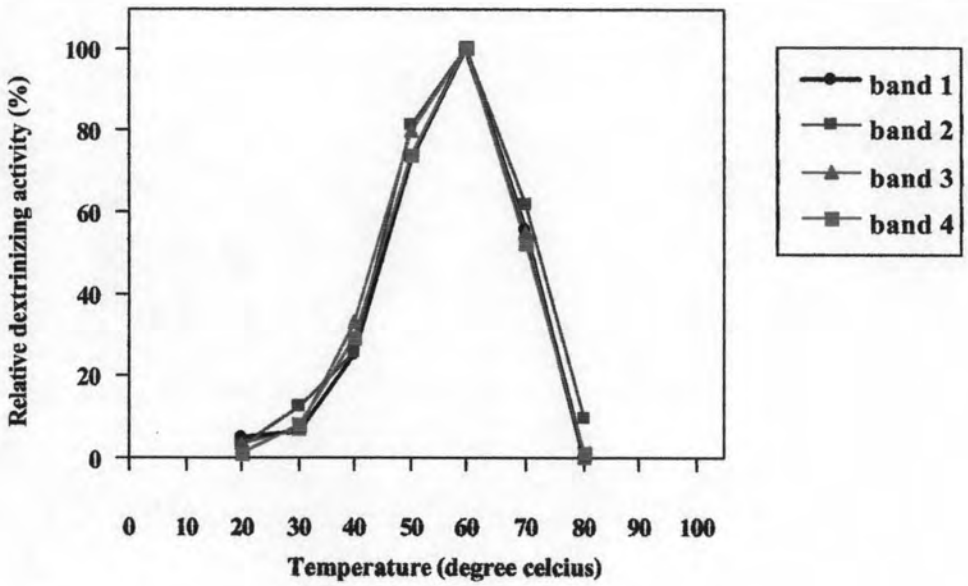
The CD products and linear oligosaccharides from the reactions of equal amount of bands 1-5 were analyzed by HPLC, comparing between non-treated and treated with β-amylase. To determine the real amount of cyclodextrins produced, the reaction products of these five bands were treated with 20 units of β-amylase at 25°C for an hour. This can differentiate the linear from the cyclic oligosaccharides by hydrolyzing the long chain oligosaccharides (*i.e.* G4-G7) to G2 and G3. However, the total amount of linear oligosaccharides occurred in the reaction products was also detected from the reaction without β-amylase treatment. The aim of this experiment was to determine whether the enzyme possesses some bands which can produce higher amount of linear oligosaccharides than CDs.

The retention times of the reaction products of bands 1-5 were compared with standard CDs and oligosaccharides (G1-G7). The retention times of standard α-,β-,

Table 13. Effect of temperature on the CD-forming activities of CGTase isozymes

Temp. (°C)	Dilution limit (% Relative CD-forming activity)			
	Band 1	Band 2	Band 3	Band 4
20	2^4 (6.25%)	2^3 (6.25%)	2^3 (3.13%)	2^1 (3.13%)
30	2^5 (12.50%)	2^4 (12.50%)	2^4 (6.25%)	2^3 (12.50%)
40	2^8 (100.00%)	2^7 (100.00%)	2^7 (50.00%)	2^5 (50.00%)
50	2^7 (50.00%)	2^6 (50.00%)	2^8 (100.00%)	2^6 (100.00%)
60	2^6 (25.00%)	2^3 (6.25%)	2^6 (25.00%)	2^6 (100.00%)
70	2^4 (6.25%)	2^2 (3.13%)	2^3 (3.13%)	2^4 (25.00%)
80	2^1 (0.78%)	- (0.00%)	2^0 (0.39%)	2^2 (6.25%)

a.



b.

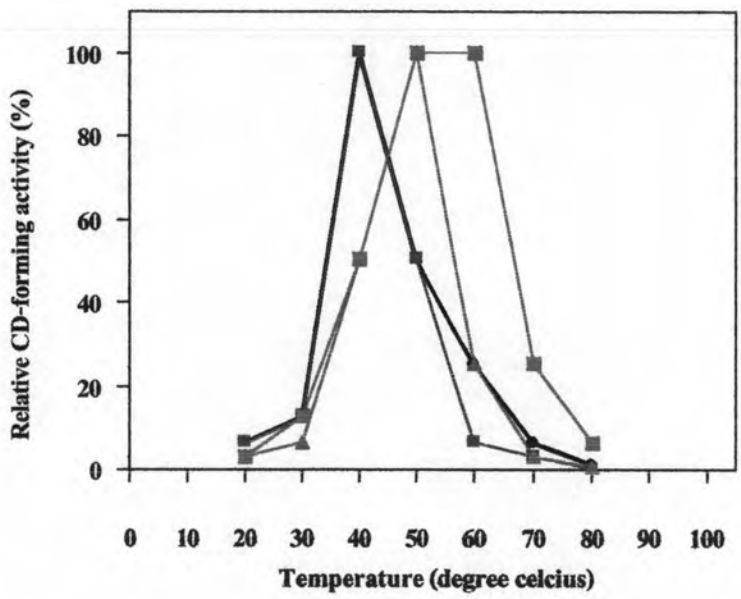


Figure 15. Effect of temperature on the activities of CGTase isozymes

a. Dextrinizing activity

b. CD-forming activity

and γ-CD were 5.54, 7.09, and 9.18 minutes, respectively (Figure 16). Those of oligosaccharides G1 to G7 were 2.38, 3.00, 3.86, 5.07, 6.75, 8.55, and 11.54 minutes, respectively (Table 12). The CD samples from every enzyme band, both treated and non-treated with β-amylase, showed a major peak with retention time corresponded to that of standard β-CD, but they gave some differences in other products, bands 1 and 2 showed a higher peak with retention time approximately 5.7 minute. which matched that of α-CD, while band 4 gave more of the product with retention time corresponded to γ-CD. Band 3 yielded almost equal amount of α- and γ-CD.

When comparing the peak of oligosaccharides in the non-treated products, band 1 and 4 showed the highest peak of G7 where band 2 and 3 showed highest amount of G1 and G2, respectively. For band 5, only linear oligosaccharides were detected with no CD products. The results obtained were different from those with β-amylase treatment in that the amount of G7, which highest in bands 1 and 4 was decreased and yielded the increasing of G2 and G3 instead. The same result occurred in bands 2 and 3. However, G1 was increased in only band 2 (Figure 16). The ratios of the reaction products from these 5 bands were summarized in Table 13.

3.3.7 Amino acid composition of CGTase isozymes

The enzyme bands 1 to 5 were hydrolyzed with 6 M HCl at 110°C for 22 hours in the Waters Pico-Tag Workstation. In this pretreated method. tryptophan and cysteine couldn't be detected. The amino acid mixtures obtained were analyzed on a Pico-Tag column and calculated for their amino acid composition. Chromatograms of various amino acid standards and enzyme samples were shown in Figure 17 and

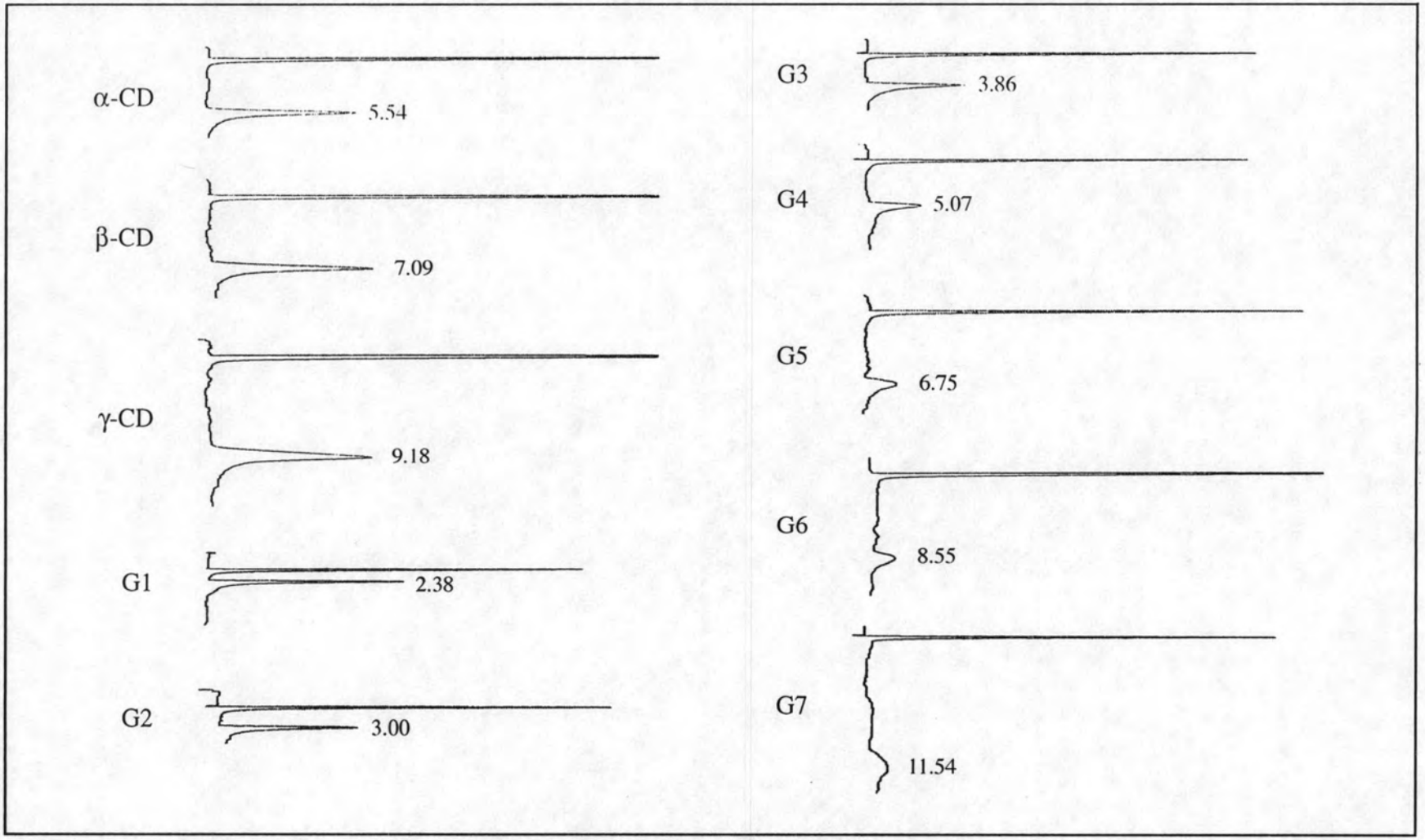


Figure 16. HPLC chromatograms of standard cyclodextrins , linear oligosaccharides (G1-G7) , and reaction products from CGTase isozymes both treated and non-treated with β-amylase. Supelco-NH_2 column was used. Acetonitrile : water (75 : 25) (v/v) was used as eluent at 2 ml/min flow rate.

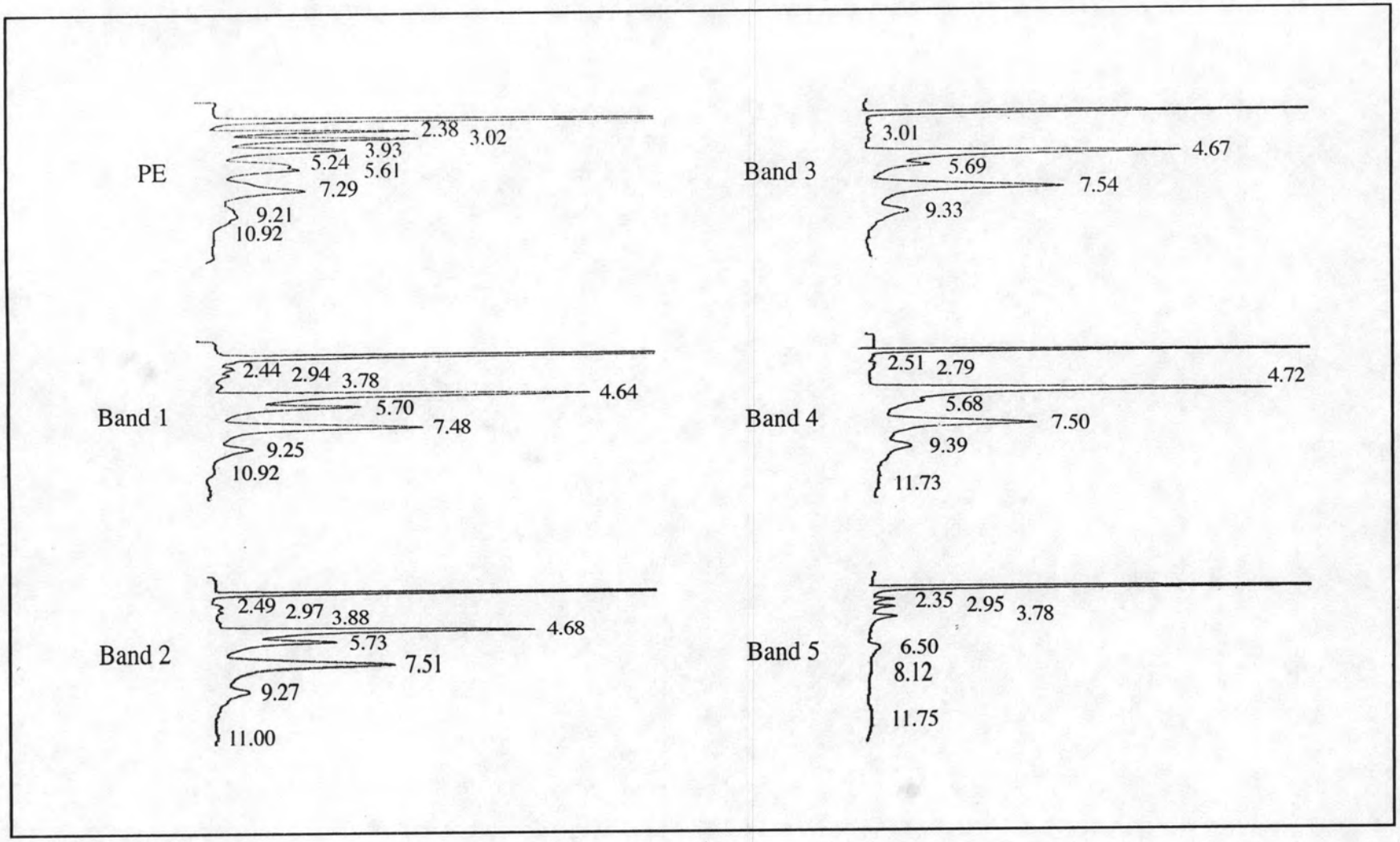


Figure 16. (continued)

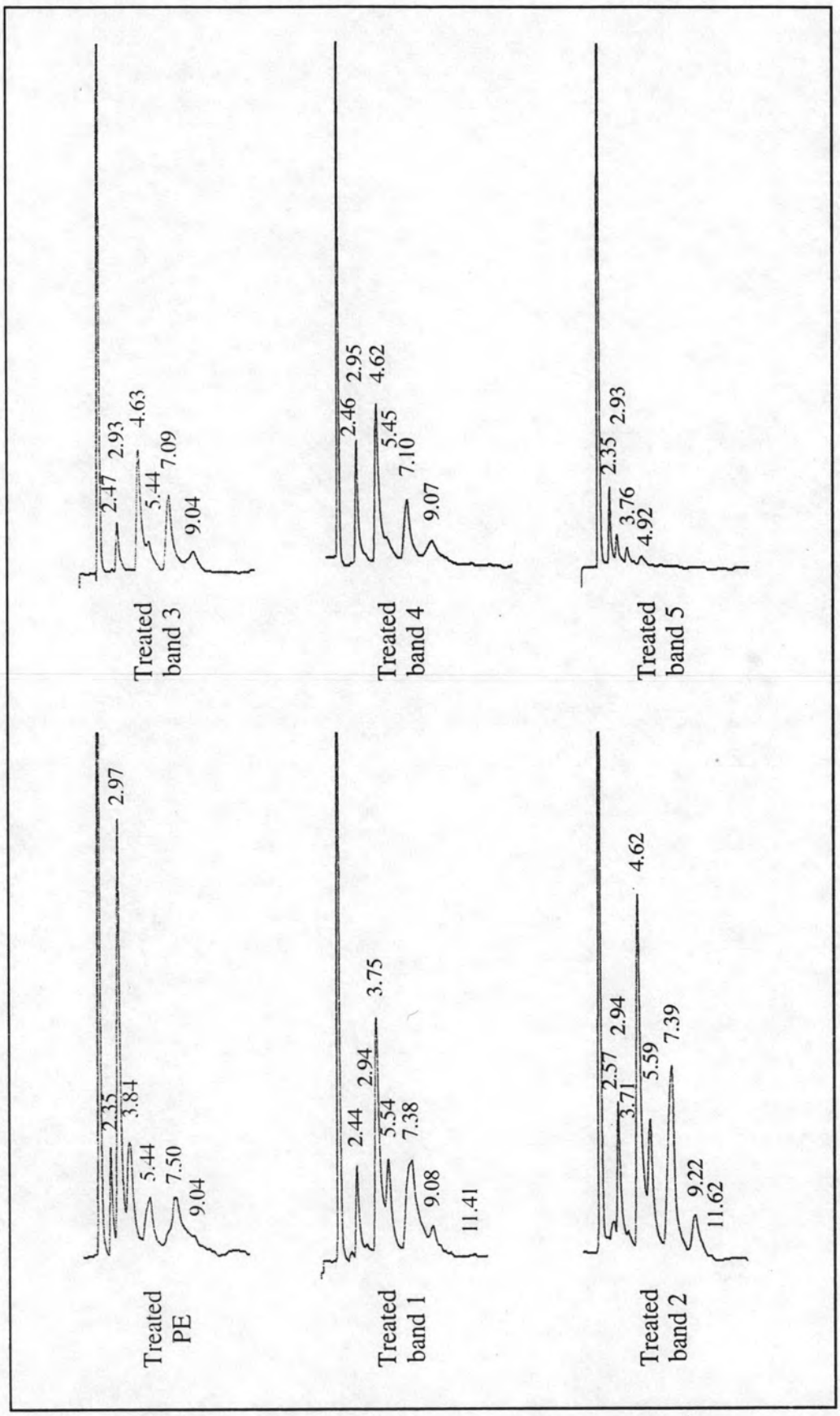


Figure 16. (continued)

Table 14. Retention time of standard CDs and linear oligosaccharides on HPLC Supelco-NH_2 column

Standard	Retention time (min)
Glucose (G1)	2.38
Maltose (G2)	3.00
Maltotriose (G3)	3.86
Maltotetraose (G4)	5.07
Maltopentaose (G5)	6.75
Maltohexaose (G6)	8.55
Maltoheptaose (G7)	11.54
α-CD	5.54
β-CD	7.09
γ-CD	9.18

Table 15. Comparison of cyclodextrin products, linear oligosaccharides and product ratios of CGTase isozymes by HPLC method

Band no.	Yield of CD (%conversion)				Product ratio	
	α-CD	β-CD	γ-CD	total	α:β:γ-CD	G_1:G_2:G_3:G_4:G_5:G_6:G_7
Without β-amylase treatment						
PE	1.00	2.19	1.01	4.20	8 : 18 : 7	7 : 10 : 11 : 6 : 0 : 0 : 0.4
1	2.15	3.93	1.13	7.21	10 : 18 : 5	13 : 10 : 7 : 0 : 0 : 0 : 26
2	2.18	4.17	1.13	7.48	9 : 18 : 5	11 : 10 : 7 : 0 : 0 : 0 : 3
3	1.18	4.41	1.24	6.83	5 : 18 : 5	0 : 10 : 0 : 0 : 0 : 0 : 0
4	1.12	4.07	1.45	6.64	5 : 18 : 7	8 : 10 : 0 : 0 : 0 : 0 : 26
5	0	0	0	0.00	0 : 0 : 0	7 : 10 : 17 : 0 : 0 : 0 : 4
With β-amylase treatment						
PE	1.14	2.46	1.02	4.62	8 : 18 : 7	2 : 10 : 5 : 0 : 0 : 0 : 0
1	1.50	3.08	0.79	5.37	9 : 18 : 5	2 : 10 : 3 : 0 : 0 : 0 : 0.4
2	1.68	3.38	0.72	5.78	9 : 18 : 4	3 : 10 : 2 : 0 : 0 : 0 : 0.3
3	0.98	3.26	1.26	5.50	5 : 18 : 7	2 : 10 : 0 : 0 : 0 : 0 : 0
4	0.79	3.54	1.58	5.91	4 : 18 : 8	1 : 10 : 0 : 0 : 0 : 0 : 0
5	0	0	0	0.00	0 : 0 : 0	16 : 10 : 8 : 4 : 0 : 0 : 0

PE = Partial purified enzyme

Table 16. Summarization of properties of CGTase isozymes

Band no.	MW (kD)	pI	pH optimum	Temp. optimum	Carbohydrate content (%w/w)	Product ratio (α:β:γ-CD)
1	72	4.73	6.0-7.0	40°C	17.0	10:18:5
2	72	4.49	6.0-7.0	40°C	15.6	9:18:5
3	72	4.40	7.0	50°C	12.6	5:18:5
4	72	4.31	6.0	50-60°C	31.8	5:18:7

18(a-f). The chromatograms of bands 1-5 showed very high amount of glycine. This may be caused by the glycine in elution buffer of preparative electrophoresis which were not completely removed. Therefore, composition of glycine was not calculated in views of inaccuracy. Cystine, which should not have been detected under the condition used, was also present in the chromatogram. It may be caused by some artifact from the buffer. The amino acid compositions of the enzyme bands 1 to 5 obtained from preparative gel electrophoresis were presented in Table 15. The result showed that their amino acid compositions were different, especially in Asp, Glu, His, Arg, Ala and Pro. However, the amounts of Met were similar and less than other components. Furthermore, band 1 was rich in Ala, while contained little His. Bands 2, 3, 4, and 5 were rich in Asp but the other compositions were different. It should be noted that no Thr was found in band 4 while rather higher content was found in bands 1, 2, 3 and 5.

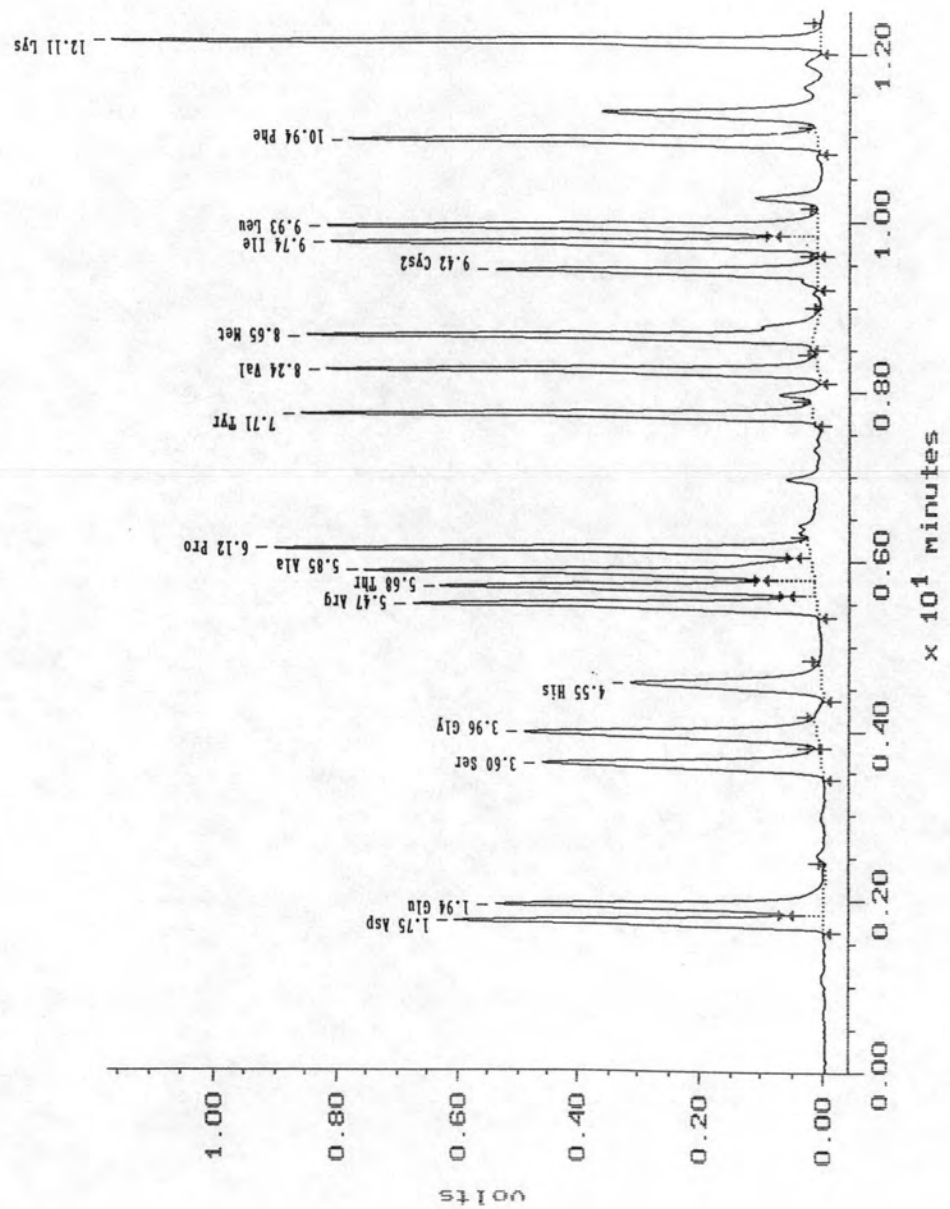


Figure 17. Chromatogram of various amino acid standards from Amino acid analyzer

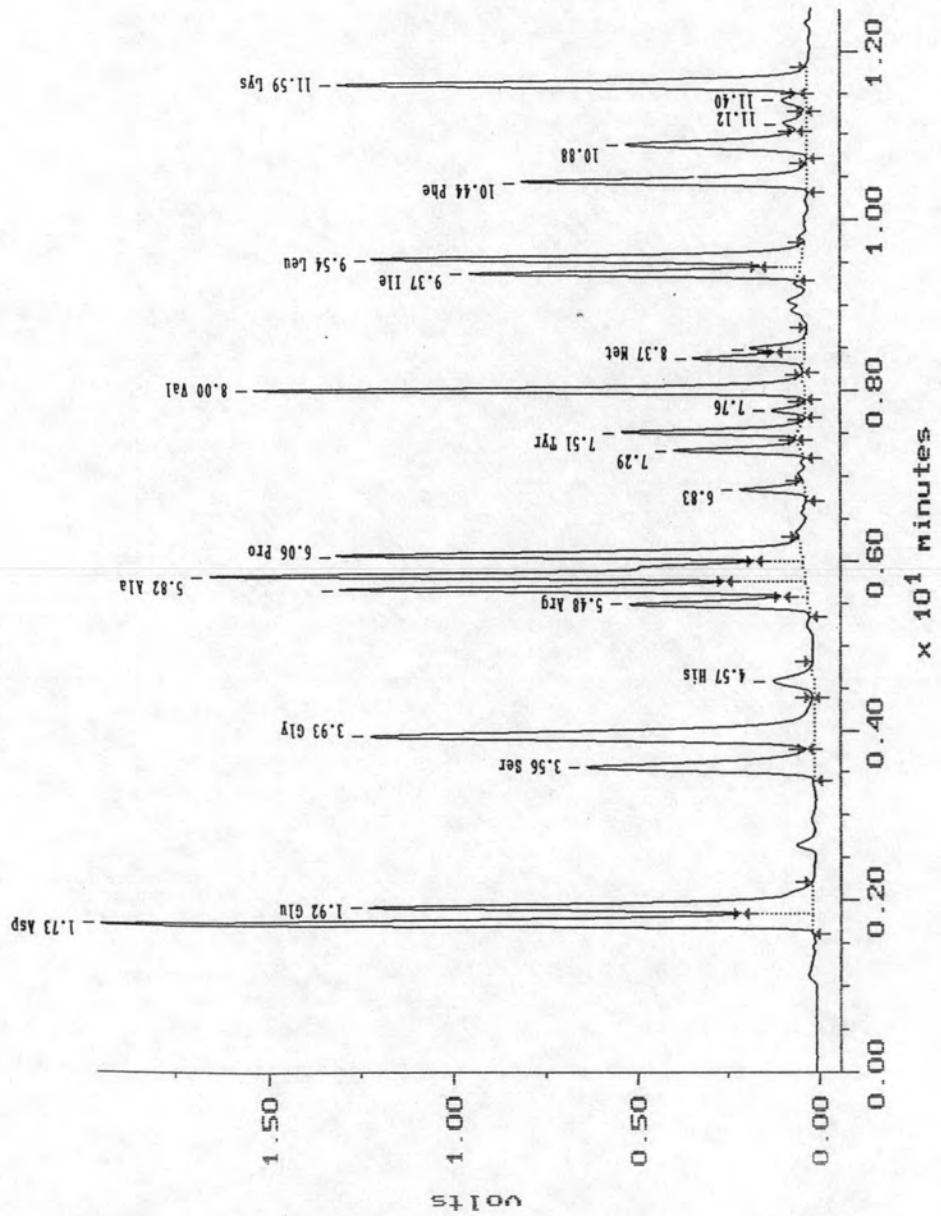


Figure 18. Chromatogram of amino acid composition of CGTase isozymes from Amino acid analyzer

(a) Purified CGTase

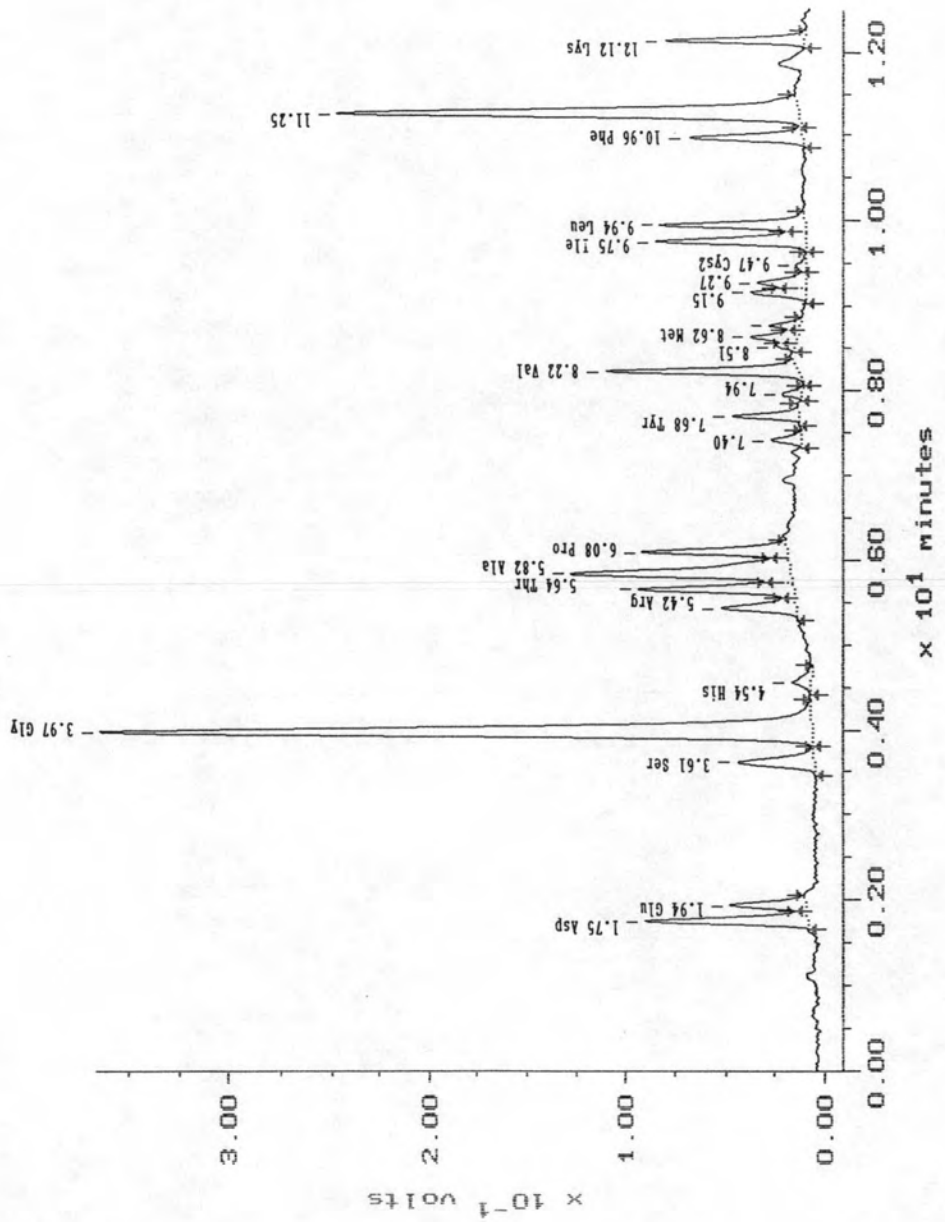


Figure 18. (continued)

(b) Band 1

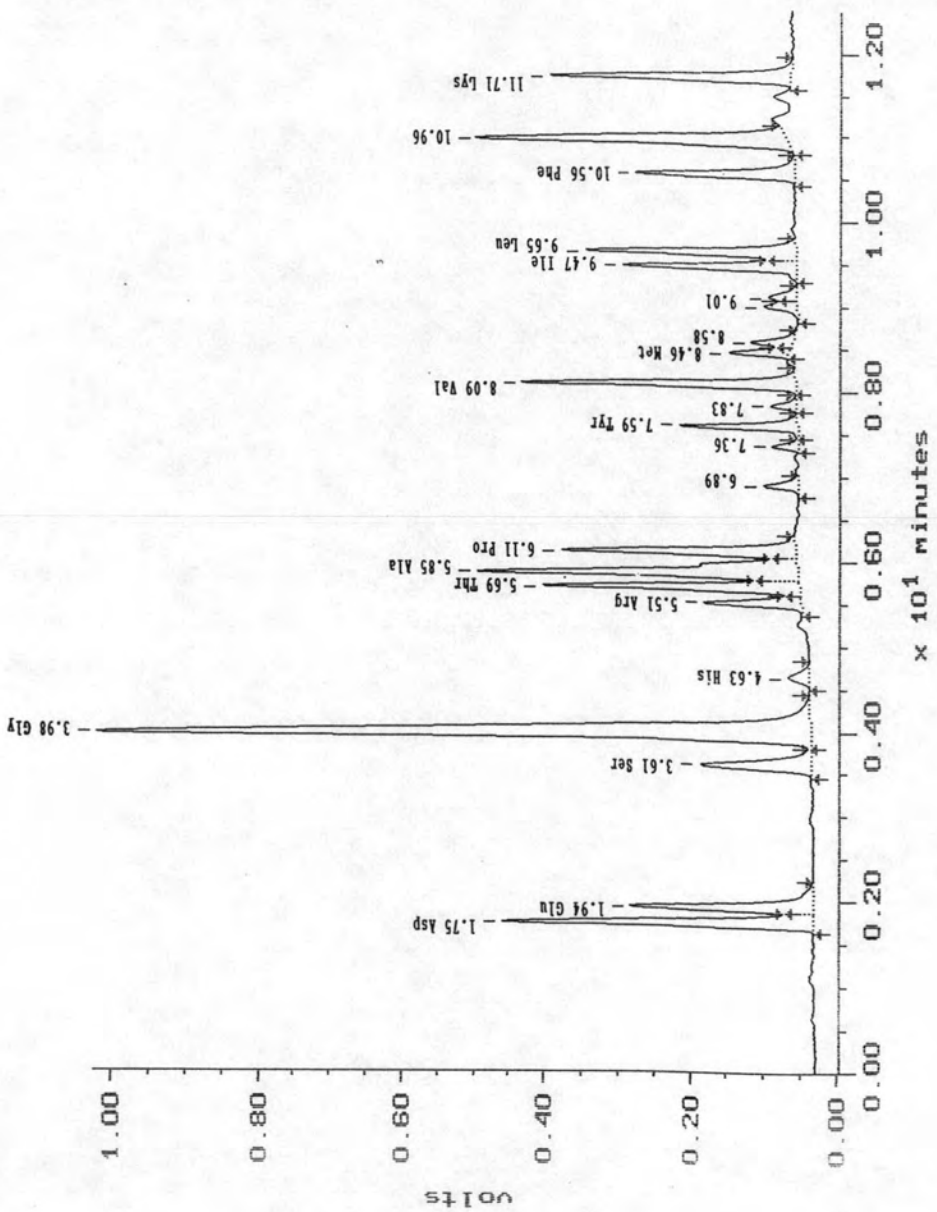


Figure 18. (continued)

(c) Band 2

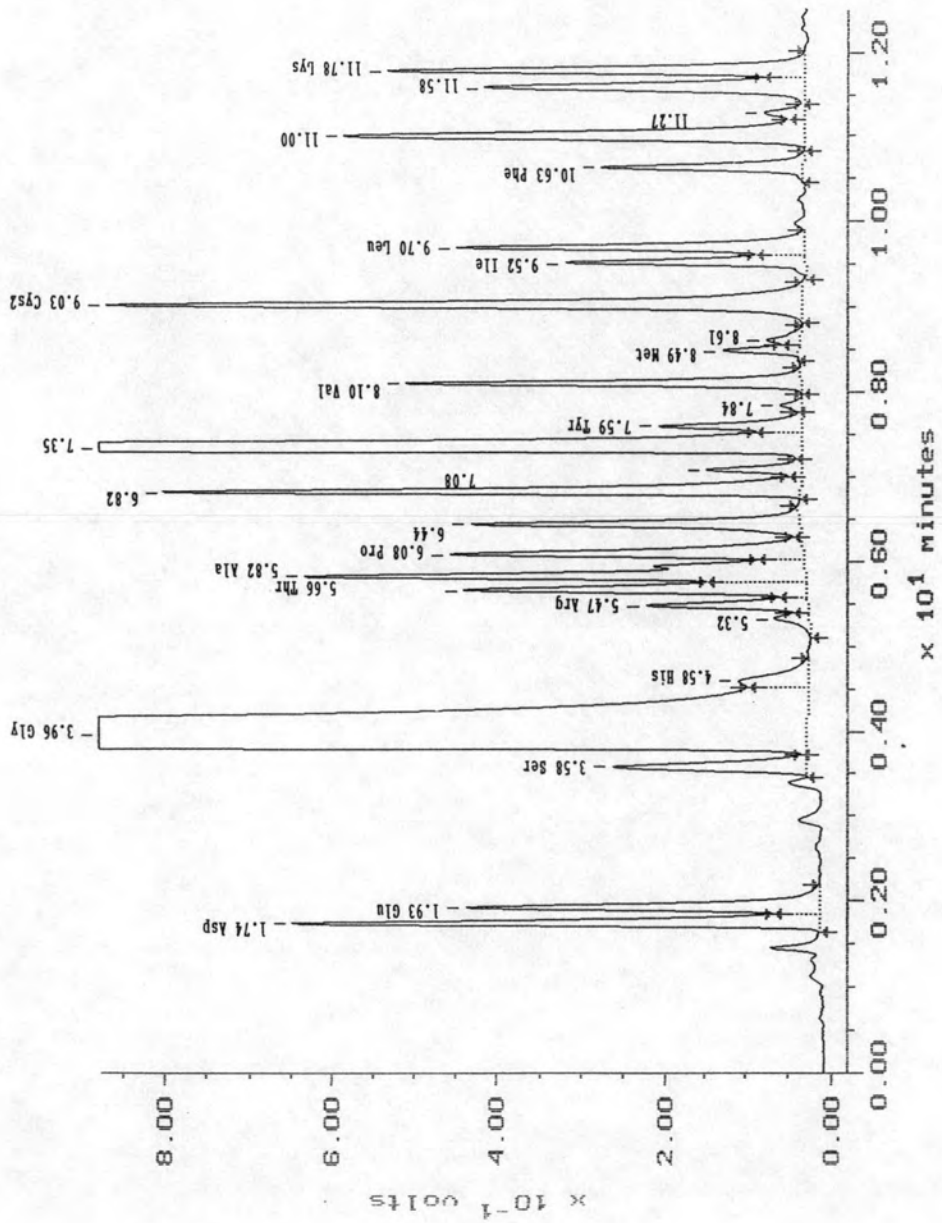


Figure 18. (continued)

(d) Band 3

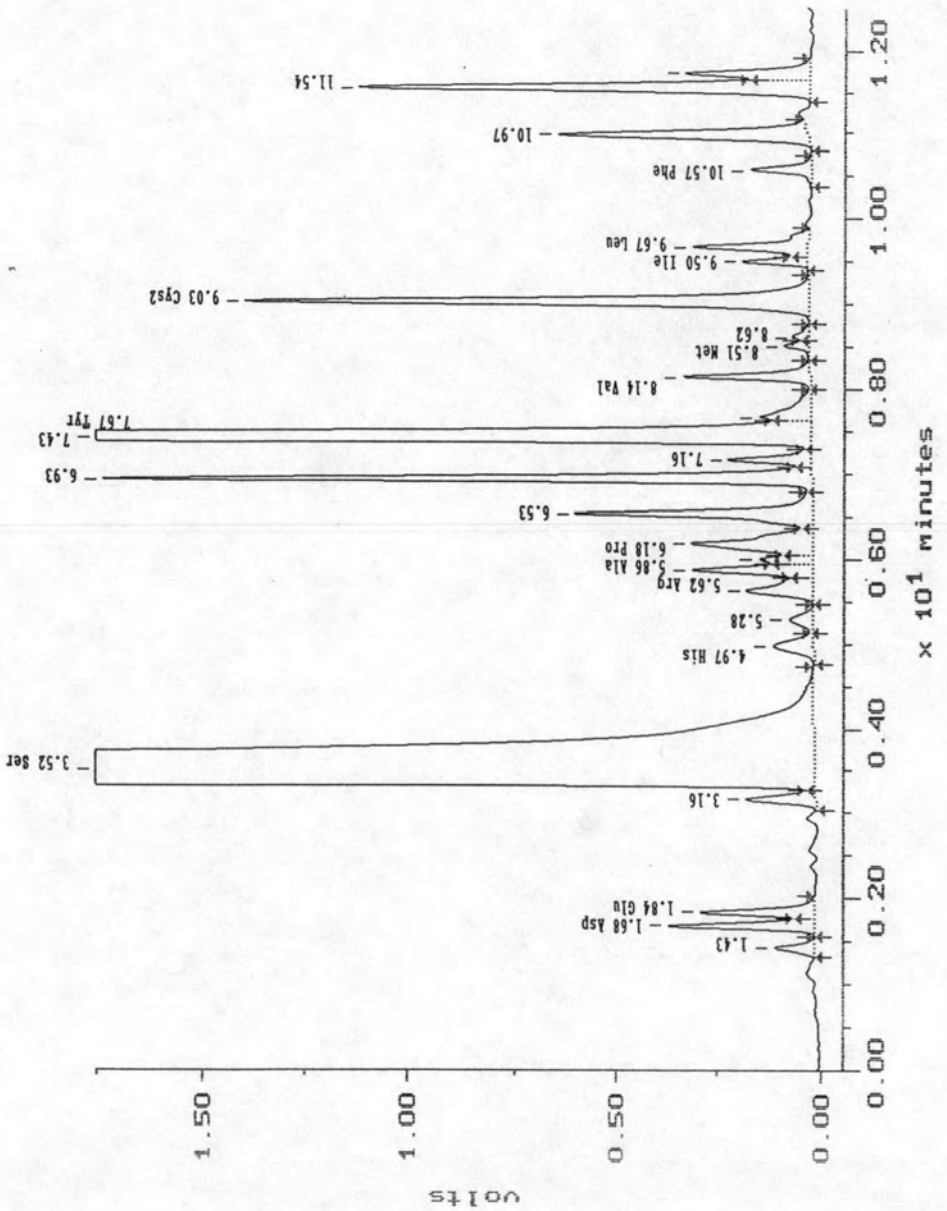


Figure 18. (continued)

(e) Band 4

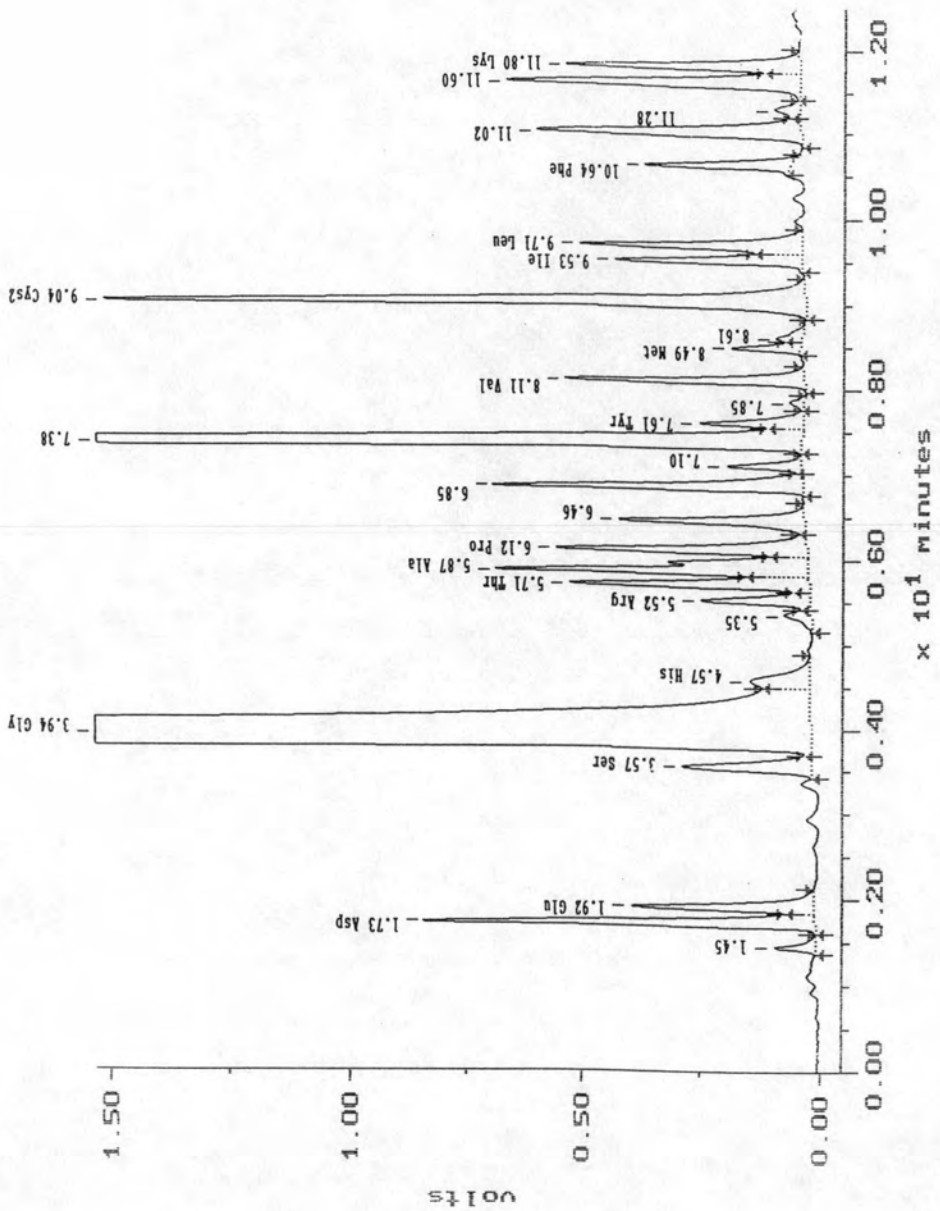


Figure 18. (continued)

(f) Band 5

Table 17. Amino acid composition of CGTase isozymes

Amino acid	Content (mol%)					
	P*	Band 1	Band 2	Band 3	Band 4	Band 5
Asp	14.35	10.04	13.25	13.15	12.24	14.63
Glu	10.04	4.79	8.90	9.96	10.33	7.59
Ser	5.43	6.45	5.95	5.85	**	5.90
Gly	8.90	**	**	**	**	**
His	1.29	2.11	1.64	3.96	7.79	5.42
Arg	2.87	4.80	3.53	3.78	8.62	3.89
Thr	8.17	10.09	10.43	9.98	-	9.89
Ala	9.63	15.41	11.74	11.39	8.42	11.15
Pro	5.49	7.41	6.17	6.55	11.37	6.51
Tyr	2.94	2.93	3.76	3.41	6.66	3.51
Val	8.64	9.43	10.02	9.04	10.71	8.41
Met	1.11	2.16	1.40	1.10	1.34	1.37
Cys2	Not determined					
Ile	4.91	7.85	6.63	5.67	4.54	6.56
Leu	6.32	6.53	6.18	6.49	8.31	6.56
Trp	Not determined					
Phe	4.31	5.53	5.62	4.42	4.30	4.38
Lys	5.59	4.46	4.77	5.26	5.38	4.30

P* = Purified enzyme from DEAE column by Laloknam (1997) analyzed in parallel

** = Not calculated because of glycine contamination in the enzyme sample