Immobilization of α-Chymotrypsin and Other Enzymes on Acylchitosan

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Summary

α-Chymotrypsin has been immobilized on acylchitosan, a chitosan derivative, using glutaraldehyde as an intermediate reagent ; the activity of the immobilized enzyme on acylchitosan was much higher than that on chitosan. The increase of the degree of substitution of acyl groups in acylchitosan as support was effective for the enzyme activity. The retained maximum activity of immobilized α-chymotrypsin was approximately 60% that of the native enzyme, obtained by the use of the butyrylchitosan with the degree of substitution of butyryl groups of 0.87.

Immobilized α-chymotrypsin showed a significant changes in optimal temperature and heat stability. The pH optimum of the fixed α-chymotrypsin was simillar to that of native enzyme.

Immobilized α-amylase and invertase on acylchitosan also showed a slight higher enzyme activity than that on chitosan.

Introduction

Reseach on chitin and its derivatives has developed rapidly, as witnessed by the publification of several books and monographs.

Chitin and chitosan have been used effectively as a support for various enzymes. Chitin has been resently used as a solid support to immobilize α-chymotrypsin,[1] α-galactosidase,[2] glucose isomerase,[3),8] glucoamylase,[4] α-amylase,[5] papain,[6] β-amylase,[7] and acid phosphatase[1] and chitosan to immobilize trypsin,[9,13] protease,[10] α-galactosidase[11] and urease. Immobilization of these enzymes on chitin and its derivative can be accomplished by means of such procedures as simple adsorption, crosslinking of enzyme and support with dialdehydes,[1)-11),13)-15] and enclosure in gel.[12]

But N-and O-acylated chitosan has not almost been reported to be available as support materials for enzymes. Acetyl, propionyl, and butyryl chitosans are prepared from chitosan by N-and O-acylation, and qualify them as good supports for enzyme immobilization, with anchoring bridge molecule with glutaraldehyde. Acyl groups in chitosan der-

ivative seem to establish hydrogen bonds with a protein.

This paper describes the immobilization of α-chymotrypsin on acylchitosan by glutaraldehyde and compares some of the properties of immobilized and free enzyme. Enzymes, invertase and α-amylase are also tried for immobilization.

Materials and Methods

Instrumentation

A SHIMAZU model UV-200 double beam spectrophotometer equipped with a printunit and an automatic strip-chart recorder was used.

Elemental analysis was performed at the Elemental Analysis Center Research Laboratory of Resourses Utilization of Tokyo Institute of Technology, Tokyo.

Reagents

Chitosan was supplied by Tokyō Kasei Kōgyō Co., Ltd. Tokyo Japan, and used in the form of powder. The degree of deacetylation of chitin was 83%, measured by the method of potentiometric titrations[16]. α-Chymotrypsin, invertase, and α-amylase were supplied by SIGMA Chemical Co, St. Louis. N-Acetyl-L-tyrosine ethyl ester (ATEE), Sucrose, and Soluble starch as substrates were purchased from Wakō Pure Chemicals Industries, LTD.

Preparation of Acylated Chitosans as Support Materials

Acylated Chitosans were prepared by the method of Hirano et al.[17] with a little modification as follows : chitosan (1 g) was dissolved in an aqueous solution of carboxylic acid (70 ml), and the corresponding prescribed amount of carboxlylic anhydride was added as shown in Table 1.

The mixture was kept at room temperature overnight to give the corresponding gel rigidly solidified in the whole solution of flask. The gel was milled in a mortor with a pestle and dialyzed or suspended in a large volume (~2 liters) of distilled water at room temparature for 3 days.

The gel free of carboxylic acid was collected by filtration or centrifugation and slowly dried at 60-65℃ in drying oven to give amorphous product. The product is soluble or swollen in dilute acids such as formic acid, acetic acid, and hydrochloric acid but almost insoluble in alkali solutions, methanol, ethanol, acetone and dimethylsulfoxide. The chemical structure of the product was confirm as described by Hirano et al., showing i. r. absorption for O-acyl gorups at 1750 cm^{-1} (C=O) and 1240 (C−O) and those for N-acyl groups at 1650 cm^{-1} (C=O) and 1540 cm^{-1} (N−H). The degree of substitution (d.s.) in

Table 1 Preparation of acylchitosans

Sample no.	Acyl Groups	Carboxylic Anhydride/Hexosaminide Residue	Yield (g)		Carbon/Nitrogen (W/W)	D.S.
			Gel	Drried		
1	Acetyl	47	36.1	0.85	7.04	1.11
2	〃	32	36.0	1.07	7.02	1.10
3	〃	16	46.3	0.74	6.68	0.90
4	〃	8	42.3	0.74	6.35	0.70
5	Propionyl	37	25.0	1.03	7.19	0.80
6	〃	24	30.0	1.03	7.48	0.91
7	〃	12	44.3	1.09	7.35	0.86
8	〃	6	40.0	1.12	7.27	0.83
9	Butyryl	30	45.0	1.01	8.13	0.87
10	〃	20	48.1	1.08	7.63	0.73
11	〃	10	37.8	0.92	6.62	0.43
12	〃	5	59.5	0.98	6.85	0.50

Chitosan used : 1 g

acylchitosan was calculated from the carbon/nitrogen ratio in the elemental analysis.

Immobilization Procedure of enzymes on acylchitosan and chitosan

The dried acylchitosan (0.5 g) was dissolved or swollen in 20 ml of 1% formic acid and the mixture was adjusted to pH 8 with 0.1 M borate buffer (pH 10.0). α-Chymotrypsin in 1 ml of borate buffer solution at pH 8 was added to the mixture in ice-water bath. Then, an aliquout of 25% glutaraldehyde was added so that final concentration of glutaraldehyde was approximately 2%. The reaction was allowed to proceed for one hour in ice-water bath with occational stirling, and a light-brown precipitate of immobilized enzyme formed stored overnight at 4℃. The granular product was collected by filtration, and washed on a glass funnel with 25 ml of distilled water, soaked with 25 ml of 3 M NaCl and 25 ml of 0.1 M borate buffer (pH 8) and finaly washed 50 ml of distilled water. The treatment was repeated 2 times. The immobilized enzyme on acylchitosan was lyophilized to give a powdered sample for enzyme assay.

Immobilized α-amylase was prepared by a little modifications of the case of α-chymotrypsin as follows ; the dried chitosan was dissolved or swollen in 0.5% acetic acid and the mixture was adjusted to pH 5.4 with 5 N-NaOH because α-amylase was apt to lose its activity in alkaline pH (over 7). The immobilized reaction was made in the same manner as α-chymotrypsin. The product was washed with distilled water, soaked 2 M NaCl, 0.1 M Acetate buffer (pH 7), 0.1 M potassium acetate buffer (pH 7) and 0.1 M potassum acetate at pH 3, and finally washed with distilled water. In the case of invertase

immobilization the procedure was the same as that of α-amylase.

Assay of Enzyme Activity

a) α-Chymotrypsin

Enzyme activity was determined by the method of Muzzarelli et al.[1] with a little modification as follows : enzyme solution (0.5 mg in 1 ml of phosphate buffer at pH 9.0) was added to the mixture of 1 ml of 2×10^{-3} M ATEE as substrate and 1 ml of disodium hydrogen orthophosphate and potassium dihydrogen orthophosphate, both 0.05 M as a buffer (pH 8.0), and allowed to react for 20 min at 30℃. The reaction was stopped in boiling water for 5 min. α-Chymotrypsin activity was determined by measuring the concentration decrease of ATEE at 237 nm by u.v. spectrum. Relative activity of immobilized α-chymotrypsin at a loading of 0.5 mg per 25 mg of dry support was measured in the following equation :

$$\text{Relative Activity}(\%)=100\times(A_i/A_n) \qquad (1)$$

A_i ; The amount of the ATEE consumed in a unit time by immobilized enzyme

A_n ; The amount of the ATEE consumed in a unit time by native enzyme

b) α-amylase

Determination of enzyme activity was carried out according to Somogyi-Nelson's methods[18] : enzyme solution (0.5 mg in 1 ml of acetate buffer at pH 6.0) was added to a mixture of 2 ml of 1% soluble starch as substrate and 10 ml of 0.2 M acetate buffer (pH 6.0), and the mixture was incubated for 20 min at 40℃ with the magnetic stirring. After incubation, the enzyme was inactivated by heating in boiling water for 5 min. Maltose formed as reducing sugar liberated in filtrate was determined by the method of Somogyi. Relative activity of immobilized α-amylase bound 0.5 mg of enzyme per 250 mg of dry support was measured in the same manner described above for equation (1). Here, A_i is the amount of maltose as reducing sugars produced in a unit time by immobilized enzyme, and A_n is that by native enzyme.

c) Invertase

Invertase activity was measured using sucrose as substrate by determining glucose formation with the glucose oxidase-chromogen procedure (glucostat method).

Soluble invertase activity was determined for 20 min at 40℃ by incubating the mixture containing 0.5 mg of enzyme in 10 ml solution which was 1 ml of 0.3 M sucrose, 5 ml of 0.04 M acetate buffer (pH 4-6), and 4 ml of distilled water. The reaction mixture was heated at 100℃ for 5 min to stop enzyme action, cooled, and analyzed for glucose content by glucostat method. Relative support-bound enzyme activity was measured in the same

manner described above equation, where A_i is the amount of glucose produced in a unit time by immobilized enzyme, and A_n is that by native one.

Results and Disscussion

Effect of d.s. of acyl groups in acylchitosan on the activity of α-chymotrypsin

The enzyme activity was expressed as percentage of the substrate transformation in terms of the percentage of the original soluble enzyme activity.

The enzyme activity was expected to be effected with d.s. of acyl groups in acylchitosan as support. As shown in table 2, the enzyme activity was slightly increased with an increase of d.s. of acyl groups in acetylchitosan, propionylchitosan, and butyrylchitosan, respectively. Maximum activity obtained by the use of the butyrylchitosan with d.s. of butyryl groups of 0.8was 60%. The activity of immobilized enzyme on chitosan reached at 22% of the substrate transformation. The value reported by Muzzarelli et al.[1] was 25% for chitosan-glutaraldehyde columns.

Table 2 Effect of d.s. of acyl groups in acylchitosan on the activity of α-chymotrypsin

Sample No.	Supports	D.S.	Conversion (%)	Relative Activity (%)
1	Acetyl	1.11	54	55
2	〃	1.10	49	50
3	〃	0.90	47	48
4	〃	0.70	41	41
5	Propionyl	0.80	53	53
6	〃	0.91	45	46
7	〃	0.86	48	48
8	〃	0.83	36	36
9	Butyryl	0.87	60	60
10	〃	0.73	54	54
11	〃	0.43	46	47
12	〃	0.50	49	49
13	Chitosan	0	23	22
14	Free enzyme		98	100

After incubation at 30℃ for 20 min in phosphate buffer (pH 7) with 2×10^{-3} M ATEE as substrate, enzyme activity was estimated from the decrease of ATEE in the reaction mixture.

Effect of pH

The influence of pH on the enzyme activity of both free and immobilized α-chymotrypsin was investigated, as shown in Fig. 1. The pH optimum for both the immobilized and free form of the enzyme was approximately pH 8. These pH value are the same as those reported by stanley at al[2] and Muzzarelli et al[1] for this enzyme immobilized on chitosan. However, free enzyme showed a slight lowering in the pH regions investigated. The immobilized enzyme in acylchitosan had a slight higher activity than that on chitosan, in pH range measured.

Effect of Temperature

For 20-min assay intervals at pH 8.0, the activity of immobilized enzyme increased with an increase of temperature, while showing a lillte decrease of that for free enzyme. Immobilized α-chymotrypsin was found to be more stable to heat, in comparsion with free form. The immobilized enzyme on acylchitosan had higher activity than that on other supports in temperarures form 20℃ to 50℃ invetigated (see Fig. 2).

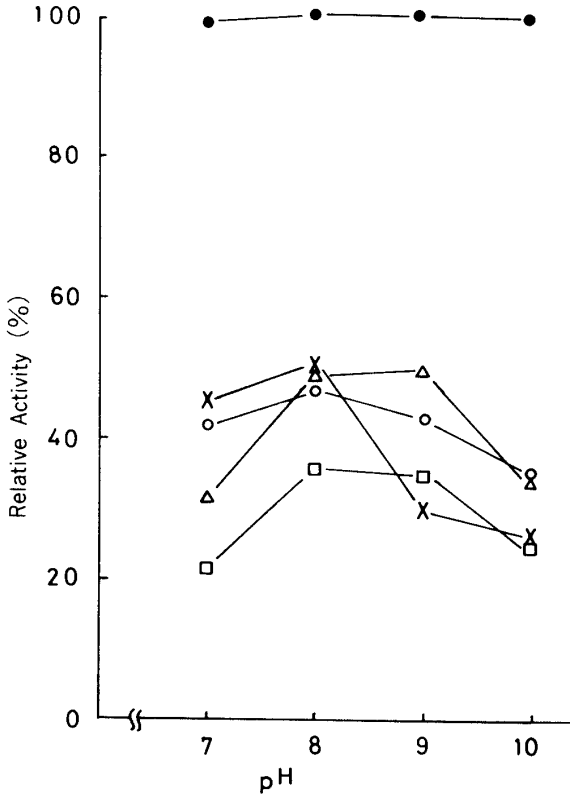

Fig. 1 Effect of pH on α-chymotrypsin activity
Activity based on 20-min assay at 30 ℃ using phosphate buffer. Symbols : (●), Free enzyme ; (○), Acetylchitosan (d.s.=1.11) ; (△), Propionylchitosan (d.s.=0.80) ; (×), Butyrylchitosan (d.s.=0.83) ; and (□), Chitosan

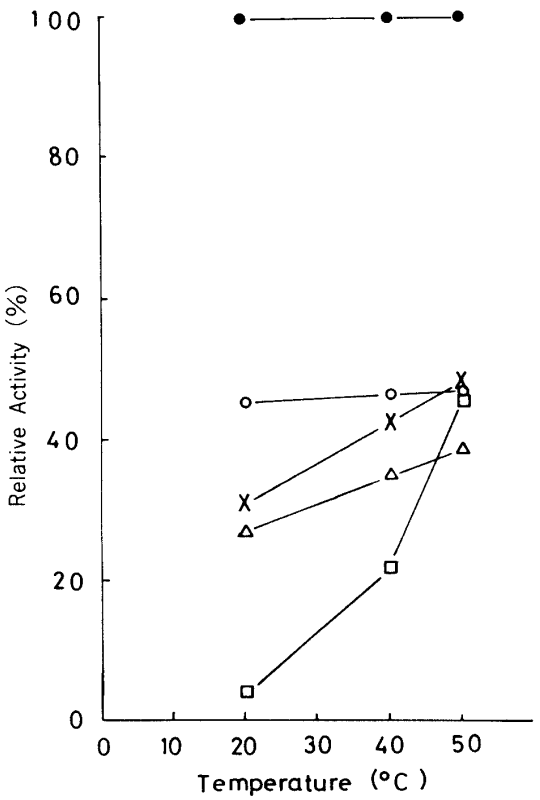

Fig. 2 Effect of temperature on α-chymotrypsin activity
Activity based 20-min assays at pH 8.0 in phosphate buffer. Symbols : (●), Free enzyme ; (○), Acetylchitosan (d.s.=1.11) ; (△), Propionylchitosan (d.s.=0.08) ; (×), Butyrylchitosan (d.s.=0.83) ; and (□), Chitosan

Continuous reaction

Continuous reaction was carried out using 1.3 cm dia. × 30 cm column packed with the chitosan-α-chymotrypsin and with the acetylchitosan-α-chymotrypsin. The result is shown in Fig.3. During 20 days operation, the conversion power of the column was approximately 45-70% for acetylchitosan-column and 40-65% for chitosan-column. The continous enzyme activity for acetylchitosan column showed slightly higher value than that for chitosan-column. The enzyme activity on these columns continued without significant drop in activity over 20 days.

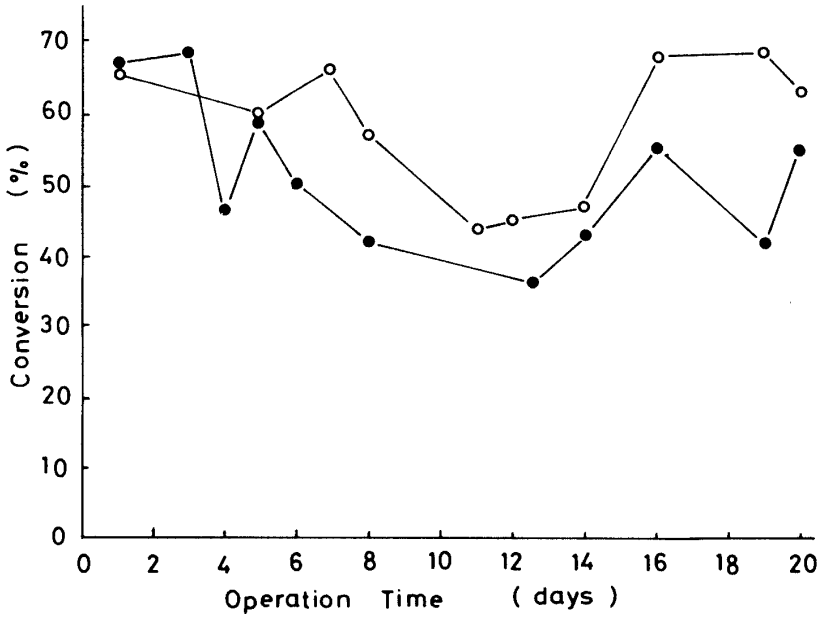

Fig. 3 Continuous reaction of α-chymotrypsin immobilized on acylchitosan and chitosan.
Symbols : (○), Acetylchitosan (d.s.=1.10) ; (●), Chitosan

Other enzyme activity

The relative activities of both free and immobilized enzymes are shown in Table 3. α-Amylase activity was estimated by the determination of maltose formed as reducing sugars in reaction mixture after incubation with soluble starch as substrate in phosphate buffer (pH 6.0) for 20 min at 40℃. Invertase activity was analyzed for glucostat procedure after enzymic reaction with saccharose as substrate in 0.04 M acetate buffer (pH 4.6) for 20 min at 40℃. The activity of immobilized α-amylase on acylchitosan was slightly higher than that on chitosan and free enzyme. Immobilized α-amylase on propionylchitosan had maximum activity of 125% as relative activity. In the case of invertase, enzyme activity was dropped by immobilization. However the relative activity of immobilized enzyme on acetylchitosan and propionylchitosan was 28% in comparison with those on butyrylchitosan (18%) and chitosan (23%) as supports.

Table 3 Activity of immobilized enzymes on acylchitosan

Supports	Coversion (%)		Relative Activity (%)	
	α-Amylase[1]	Invertase[2]	α-Amylase	Invertase
Acetylchitosan (No. 1)	17	28	106	28
Propionylchitosan (No. 5)	25	28	125	28
Butyrylchitosan (No. 9)	22	18	118	18
Chitosan	10	23	88	23
Free enzyme	15	100	100	100

1) After incubation at 40 ℃ for 20 min in 0.2 M phosphate buffer (pH 6) with 1 % soluble starch as substrate, enzyme activity was estimated by the determination of glucose produced in reaction mixtures.

2) After incubation at 40 ℃ for 20 min in 0.04 M acetate buffer (pH 4.6) with 0.3 M saccharose as substrate, enzyme activity was estimated by the same manner as that of α-amylase.

Conclusion

Chitin and its derivatives, supports for immobillization of enzyme should be excellent in presentation, solidity and durability, and also less expensive, non toxic especially for food processing. From these points of view, the author tried the immobilization of enzymes on acylchitosan-glutaraldehyde, expected to have a higher activity than that on chitosan.

Immobilized enzymes on acylchitosan had higher activity than that on chitosan. Activity of immobilized enzymes on acylchitosan increased with an increase of d.s. in it. The advantages with powdered acylchitosan as support are expected to be simple column-packing for continous enzyme reaction and possible use in large scale. Acylchitosan, an industrially available product, would find application as an enzyme support or carrier in the food and biomedical sciences and chemical industries.

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