Production of Physiologically Active Chitosan Oligosaccharides at High Concentration by Immobilized Chitosanase

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Contents

Chapter 1 General Introduction

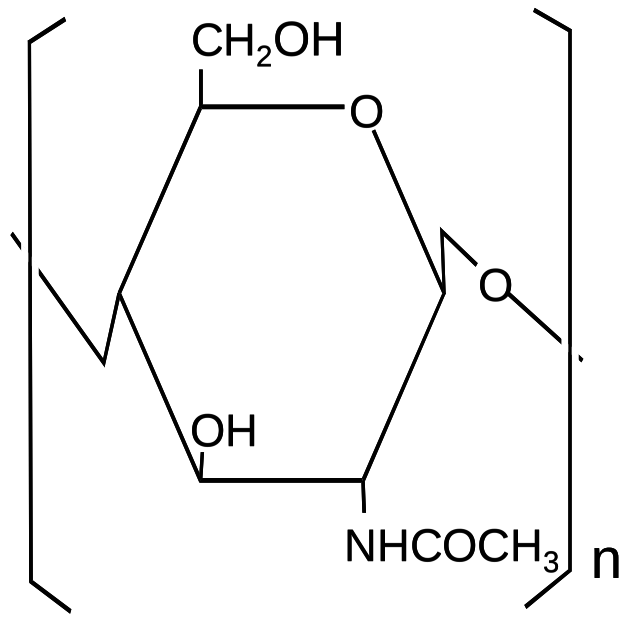
The researches on chitin and chitosan are in rapid progress in recent years. In particular, the oligomers of chitosan, namely chitosan oligosaccharides, arouse more and more attentions because of their functions. In this chapter, the previous researches relating to this study are reviewed to make the significance of this study clear, and the objectives are described.

1.1 Chitosan and chitosan oligosaccharides

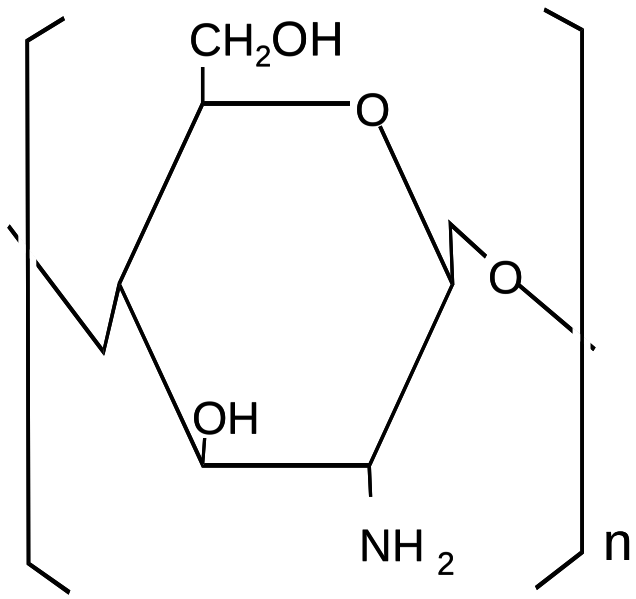
1.1.1 Chitin and chitosan

Chitin is the natural biopolymer that is abounding on the earth for the second next to the cellulose. It is a major component of arthropod and crustacean shells such as the lobsters, crabs and shrimps. Chitin is β(1→4)-linked glycan composed of 2-acetamido-2-deoxy-β-D-glucose, *i.e.* N-acetylglucosamine. Chitosan is a partially or fully acetylated chitin, which exists in the cell wall of some fungi such as the *Mucorales* strains (Juang *et al*., 2001). It is composed of 2-amino-2-deoxy-β-D-glucose, *i.e.* glucosamine (Matahira, 1997). The chemical structures of chitin and chitosan were shown in Fig. 1-1. Chitin is insoluble in water, aqueous alkaline and acid solutions. However, chitosan is soluble only in acidic aqueous solution (Hirano, 1987; Hirano *et al*., 2001).

Since the biodegradation of chitin is very slow in arthropod and crustacean shell waste, accumulation of large quantities of discards from processing of arthropod and crustaceans has become a major concern in the seafood processing industry. Disposal of discards has, thus, been a challenge in most of the shellfish-producing countries. Chitin can be isolated from these wastes. Therefore, production of value-added products such as chitin, chitosan and their derivatives and its applications in different fields are the utmost interest. Chitin and its deacetylated form, *i.e.* chitosan, aroused the interests of many researchers in the past few decades due to their potential broad range of industrial



Chitin



Chitosan

n= 5 Chitosan pentamer
n= 6 Chitosan hexamer

Fig. 1-1 Chemical structures of chitin and chitosan.

applications. However, only limited attention has been paid to food applications of these versatile biopolymers. Conversion into valuable by-products and alternative specialty materials has been identified as a timely challenge for researches and developments to expand numerous applications of chitinous polymers. In that sense, these biopolymers offer a wide range of unique applications including bioconversion for the production of value-added food products (Shahidi and Arachchi, 1999), preservation of foods from microbial deterioration, formation of biodegradable films, recovery of useful material from discards of food process, purification of water, clarification and deacidification of fruit juices, addition of antifungal properties to the apple juice (Roller *et al*., 1999), utilization as a support for immobilization in biotechnology (Juang *et al*., 2002; Carrara and Rubiolo, 1997), formation of membrane for separation (Huang *et al*., 2000), and utilization as promoters for plant growth and adsorbents for affinity chromatography (Kurita, 1998). Antimicrobial activity against bacteria and fungi (Shepherd *et al*., 1997; Begin and Calsteren, 1999), analgesic effect (Okamoto *et al*., 2002) and biological activating effect of chitin and chitosan (Minami *et al*., 1998) have also been reported. Recently, chitosan is used as a diet food because chitosan can adsorb fats and oil, and then promote them to get out from body. In addition, chitosan can prevent the constipation because chitosan is not digested. They would therefore be useful in various fields as advanced functional materials.

1.1.2 Chitosan oligosaccharides

In the FOSHU (‘**Fo**od for **S**pecified **H**ealth **U**se’ in Japan, ‘Functional Food’ in North America and Europe) published by Ministry of Health, Labour and Welfare of Japan in August 16th, 2002, oligosaccharides have more than 60 kinds, and they occupy more than 20% of all 300 kinds of particular health foods. So oligosaccharides will be focused more and more.

Oligosaccharides are 2~10 degree of polymer of monosaccharides, such as glucose, fructose, galactose, *etc*. They exist in the nature or are made from polysaccharide by hydrolysis, such as galacto-oligosaccharides from lactose, chitosan oligosaccharides

from chitosan and so on (Hayakawa, 1998).

1.1.2.1 Functions

Recent studies on chitin and chitosan have attracted interest for converting them to oligosaccharides, because the oligosaccharides are not only water-soluble but also show versatile functional properties such as antifungal activity, antimicrobial activity, antitumor activity, immuno-enhancing effects, enhancement of protective effects against infection. Properties of chitosan oligosaccharides, such as degree of polymerization (DP), degree of acetylation (DA), charge distribution and nature of chemical modification to the molecule strongly influence its observed biological activities. Therefore, molecular weight is considered as a principal characteristic of chitosan oligosaccharides that highly correlates to their biological activities.

(1) Antibacterial activity

Antibacterial activity of chitosan and its derivatives against several bacterial species has been recognized and it considered as one of the most important properties linked directly to their possible biological application. It was reported (Jeon and Kim, 2000 a, b; Jeon *et al*., 2001) that the growth of most bacteria tested was inhibited by chitosan oligosaccharides treatments and chitosan oligosaccharides with higher MW has higher antibacterial activity. Choi *et al*. (2001) showed that chitosan oligosaccharides mixture (MW 2000~30000Da; degree of deacetylation (DD) 91.5%) had the *in vitro* antibacterial activity against two representative oral pathogens, *Astinobacillus actinomycetemcomitans* and *Streptococcus mutans*. The antibacterial activity is influenced by a number of factors such as DP (Park *et al*., 2004; Park *et al*., 2002; Yun *et al*., 1999), level of DA (Chung *et al*., 2004; Tsai *et al*., 2002); type of microorganism (Gerasimenko *et al*., 2004; Uchida *et al*., 1989). Uchida *et al*. reported that chitosan oligosaccharides that mainly contained tetramer-hexamer more effectively inhibited *Escherichia coli* than chitosan oligosaccharides that mainly contained trimer-tetramer, which showed that chitosan oligosaccharides with DP being more than 5 have

antibacterial activity.

(2) Antiviral activity

Chitosan and chitosan oligosaccharides are reported to suppress viral infections in various biological systems. Researches for viral infections in plants have revealed that the treatment of chitosan on leaf surfaces can decrease the number of local necroses caused by different mosaic viruses (Pospieszny *et al*., 1991). Chitosan oligosaccharides are reported to stimulate immune and defense systems in animal cells. Bacon *et al*. (2000) showed that co-treatments of chitosan with antigen to mice could strongly increase the local and systemic immune and defense responses to influenza A and B viruses. Gama *et al*. (1991) observed that carboxymethyl and sulfated derivatives of chitosan could inhibit the replication of HIV-1 in cultured T-calls and human MT-4 lymphocytes. Chitosan oligosaccharides are also effective in preventing several phage infections (Kochkina and Chirkov, 2002).

(3) Antitumor activity

It was indicated that chitosan oligosaccharides could inhibit the growth of tumor cells by exerting immunoenhancing effects. Tokoro *et al*. (1989) have reported that a water-soluble N-acetyl-hexamer was able to enhance the protecting effect of BALB/c male mice against Listeria monocytogenes infection. It was also reported that N-acetyl-hexamer and hexamer had the growth-inhibitory effect against Meth-A solid tumor, and N-acetyl-hexamer was able to display chemotactic response of human neutrophils *in vitro* (Tokoro *et al*., 1988 a, b).

(4) Antioxidant and radical scavenging activities

Recently, the antioxidant activity of chitosan and its derivatives attracted a greater attention (Chiang *et al*., 2000a). Further, it has been observed that the radical scavenging properties of chitosan oligosaccharides are dependent on their degree of deacetylation and molecular weights. Based on the results obtained from studies being

carried out by using electron spin trapping techniques, chitosan oligosaccharides with molecular weight range of 1-3 kDa have been identified to have a higher potential to scavenge different radicals (Park *et al*., 2003a). In addition, highly deacetylated chitosan oligosaccharides are more preferable to scavenge DPPH (2, 2-diphenyl-1-picrylhydrazyl hydrate), hydroxyl, super-oxide and carbon-centered radicals (Je *et al*., 2004).

(5) Immunostimulant effects

Okamoto *et al*. (2003) reported that both oligomers of chitin and chitosan are effective in enhancing migratory activity of macrophages. When chitosan oligosaccharides with DP 2-8 were feed to rabbit, lysozyme activity in its blood serum increased two times (Hirano *et al*., 1991). And it was reported that physiological condition was not found any trouble as 4.5 mg/kg-body of chitosan oligosaccharides was feed during 11 days. It is known that chitosan oligosaccharides are safe for animals.

(6) Other functions

Application of chitosan oligosaccharides leads to control of blood cholesterol level, especially, they are capable of decreasing cholesterol level in the liver (Muzzarelli, 1997). Chitosan oligosaccharides prevent development of fatty liver caused by the action of hepatotrope poisons. Thanou *et al*. (2002) indicated that chitosan oligosaccharides had the functions of improving the liver function and decreasing the blood sugar level. Chitosan oligosaccharides have promotion effect on phytoalexin (especially with DP 6 and 7) (Kendra and Hadwiger, 1984) and promotion effect on chitinase (Hirano *et al*., 1989). Other elicitor activity, like increasing effect on enzyme activities in seedlings, was reported by Li *et al*. (1995). Chitosan oligosaccharides also have effect of lowering blood glucose level (Lee *et al*., 2003).

As stated above, chitosan oligosaccharides have various functions, so the effective utilization of them is expected. MW of chitosan oligosaccharides exerts their activities, and chitosan oligosaccharides with higher MW have higher activities. However, there is a problem about their viscosities of solutions after addition of chitosan oligosaccharides.

When MW is lower, the solution viscosity is lower and will be more benefiting for their applications. Therefore, thinking of both physiological activities and solubility, pentamer and hexamer of chitosan oligosaccharides have a widest utilities. So it is desired to produce the chitosan oligosaccharides containing pentamer and hexamer in higher content.

1.1.2.2 Preparation methods

Chemical and enzymatic methods are the widely used chitosan oligosaccharides production approaches.

(1) Chemical hydrolysis

Chemical hydrolysis is used more commonly in the industrial-scale production. However, chemical hydrolysis has some drawbacks to be commercialized, due to development of some toxic compounds, higher risk associated with the environmental pollution, and lower production yield.

Chitosan was early reported to be hydrolyzed under the strong hydrochloric acid condition for the production of chitosan oligosaccharides (Horowitz *et al*., 1957). Recently, nitric acid (Tømmeraas *et al*., 2001) and phosphoric acid (Jia and Shen, 2002) were also used to hydrolyze chitosan for obtaining chitosan oligosaccharides. In these methods, large amounts of acid would be needed. At the same time, some monosaccharide (D-glucosamine) could be produced, causing the low yield of oligosaccharides and the complex separation treatment. A large amount of waste acidic water would be generated as well. Therefore, lack of proper technology for the large-scale manufacturing of chitosan oligosaccharides production with desired molecular weight made it difficult for industrial use in the past years.

(2) Enzymatic hydrolysis

The enzymatic processes are preferable than chemical methods. This is due to minimized adverse chemical modifications of products during enzymatic hydrolysis and

promotion of their physiological activities. Enzymatic hydrolysis is carried out under mild conditions and few monosaccharide is produced. Enzymatic hydrolysis is recommended as one of the promising methods to produce bioactive chitosan oligosaccharides.

Differentially deacetylated chitosans have four different types of randomly distributed glycosidic bonds in their structures. These include linkages between two N-acetylated units (GlcNAc-GlcNAc), acetylated and deacetylated units (GlcNAc-GlcN), deacetylated and acetylated units (GlcN-GlcNAc) and two deacetylated units (GlcN-GlcN). Degradation of these glycosidic linkages of chitosan can be produced by many enzymes. The specificity of chitosanolytic enzymes (chitosanase) with respect to the cleavage of four different glycosidic linkages in partially N-deacetylated chitosan is determined by the identity of the reducing and non-reducing end and DD of chitosan. So far, a range of chitosanase has been reported from different of microorganisms including fungi (Kim *et al*., 1998; Muzarelli *et al*., 1994) and bacteria (Lee *et al*., 1996; Varum *et al*., 1996). Characteristic properties of chitosanase from different microorganisms were summerized by Kuroiwa (2005).

On the other hand, enzymes besides chitosanase have activities on degradation of glycosidic linkages of chitosan. Pantaleone *et al.* (1992) and Yalpani *et al.* (1994) reported that cellulase, pectinase, lipase and papain etc. can cleave chitosan for hydrolysis. Besides these, cellulose (Muraki *et al*., 1993), papain (Terbojevich *et al*., 1996; Lin *et al*., 2002), lipase (Muzzarelli *et al*., 1995), pectinase (Shin *et al*., 1998; Shin *et al*., 2001; Kittur *et al*., 2003a; Kittur *et al*., 2003b;), β-glycosidase (Zhang and Neau, 2001) and the mixture of cellulase, α-amylase and protease (Zhang *et al*., 1999) were reported for hydrolysis of chitosan. It is interesting to note that these enzymes are practical because of their low cost. However, activity of hydrolyzing chitosan of all of them is low. For producing large amount of chitosan oligosaccharides, large amount of enzyme has to be used and long time will be taken.

Therefore, it is expected to choose appropriate enzyme for effective production of chitosan oligosaccharides. Chitosanase is indicated to have excellent performances in

chitosan oligosaccharides production and its effective utilization is expected. However, the price of it is expensive. To decrease production cost, reuse of it is recommended.

1.2 Chitosanase and immobilization by the multipoint attachment method

1.2.1 Chitosanase

Chitosanase (EC3.2.1.99) is one of the enzymes that catalyze the hydrolytic degradation of chitosan. A series of oligosaccharides having a D-glucosamine as the reducing end-group are produced in reactions catalyzed by chitosanase. In the methods of chitosan hydrolysis, usage of enzyme is more effective for producing high degrees of polymerization oligosaccharides than chemical hydrolysis by acid. Chitosanase has potential of industrial applications in the utilization of the enormous chitosan and chitin substrates, which are waste from sea- food-processing.

Chitosanase from different organisms differ in their hydrolytic action patterns, which are dependent on the degree of polymerization and of acetylation of the substrate. Most of the chitosanase from various sources reported so far were endo-acting in nature, liberating predominantly mixture of dimers, trimers and oligomers of chitosan as the products of hydrolysis (Somashekar and Joseph, 1995). There are three types of chitosanase action: cleaves between GlcNAc or GlcN and GlcN; cleaves between GlcN and GlcN; cleaves between GlcN and GlcN or GlcNAc (Fukamizo *et al*., 1994). The mode of action of chitosanase on the substrates suggested that the enzyme required substrates with three or more glucosamine or N-acetylglucosamine residues for the expression of activity (Somashekar and Joseph, 1995).

The chitosanase from *Bacillus pumilus* BN-262 used in this study cleaves the linkages of GlcN-GlcN and GlcNAc-GlcN (Fukamizo *et al*., 1994). The effect of the degree of acetylation of chitosan on chitosanase activity had been studied (Kuroiwa, 2005). The chitosanase from *Bacillus pumilus* BN-262 used is most active for 100% deacetylated chitosan. Thus, in this study 100% deacetylated chitosan was used.

1.2.2 Enzyme immobilization by the multipoint attachment method

Enzyme immobilization has the advantages, such as repeating utilization of enzymes, being easy for separating enzymes from products, so it can decrease costs of products, especially for expensive enzyme. Chitosanase is an expensive enzyme, therefore, for industrial usage, the immobilization of enzyme is needed. Takano (1998) reported that chitosanase was successfully immobilized by multipoint attachment to agar gel and used more than for 5 times without activity loss. It suggested that the immobilized chitosanase was very stable. With the method of multipoint attachment, packed bed reactor (Takano, 1998; Kuroiwa, 2002) and membrane reactor (Izuta, 1999) had been used to produce chitosan oligosaccharides continuously for more than one month.

The immobilization of trypsin by multipoint attachment to aldehyde-agarose gels, which was improved from the immobilization of protein, was successful carried out by Guisan and Blanco (1987). In this method, hydroxyl groups in agarose support are activated to aldehyde groups, and then the aldehydes and free amino groups of enzyme react to form Schiff bases, which will realize the immobilization. There are a few combinations between enzyme and support that increases the stability of immobilized enzyme. On the other hand, the stability of immobilized enzyme can become better by increasing the aldehyde density on support, high pH and contacting time of enzyme and support (Guisan and Blanco, 1987; Guisan, 1988). In this case, the ε-amino group of lysine residue and the end amino group are used as the functional group. The amino group has the following properties: containing no active sites in all enzymes; existing mostly in the outside of enzymes for their polarity; react easily in the case of no protonation (Guisan, 1988).

Many enzymes were stably immobilized by multipoint attachment to agarose gel, such as penicillin G acylase (Guisan and Blanco, 1987), trypsin (Guisan, 1988), and so on. Being a cheaper support, agar gel that contains the constituents of the agarose had been well used as the substitute of the expensive agarose gel (Takano, 1998). Kuroiwa

(2002) revealed that the enzyme was immobilized only on the surface of the agar gel. Takano (1998) elucidated the procedures of chitosanase immobilization by multipoint attachment, which was shown in Fig. 1-2. Firstly, the hydroxyl groups of supports are etherified by adding glycidol to the NaOH solution and $NaBH_4$ (as oxidation inhibitor) and change to glycidyl, consequently change to glyoxyl by adding $NaIO_4$. Secondly, these activated gels with aldehyde groups and free amino groups of enzyme react to form Schiff bases. Finally, $NaBH_4$ are added for stabilizing Schiff bases and inactivating the aldehyde groups not reacted.

In those studies, the agarose or agar gel particles were used as the support material for chitosanase immobilization. However, the agarose or agar gel particles will be damaged under the high agitating speed and the gel particles size is difficult to be controlled for some specific sizes. So the utilization of agarose or agar gel particles as the supports for immobilization is still limited. New form of agar gel instead of particles is expected to be the support for immobilization. At the same time, some other new materials as the support for immobilization of chitosanase are also expected for producing chitosan oligosaccharides.

In practical applications, the activity recovery and repeated use of chitosanase are very important from the view of cost. Many immobilization techniques of enzymes have been employed and reviewed recently. There are many factors affecting the activity recovery and reusability of enzymes in immobilization process. Two of the most important factors are the choice of a support and the selection of an immobilization strategy. Thus, exploiting good supports and immobilization strategy have been attractive works for enzyme engineering.

1.3 Enzyme bioreactors for the production of chitosan oligosaccharides

Chitosan oligosaccharides are focused because of their physiological activities. Up to now, many enzyme bioreactors for producing chitosan oligosaccharides were reported.

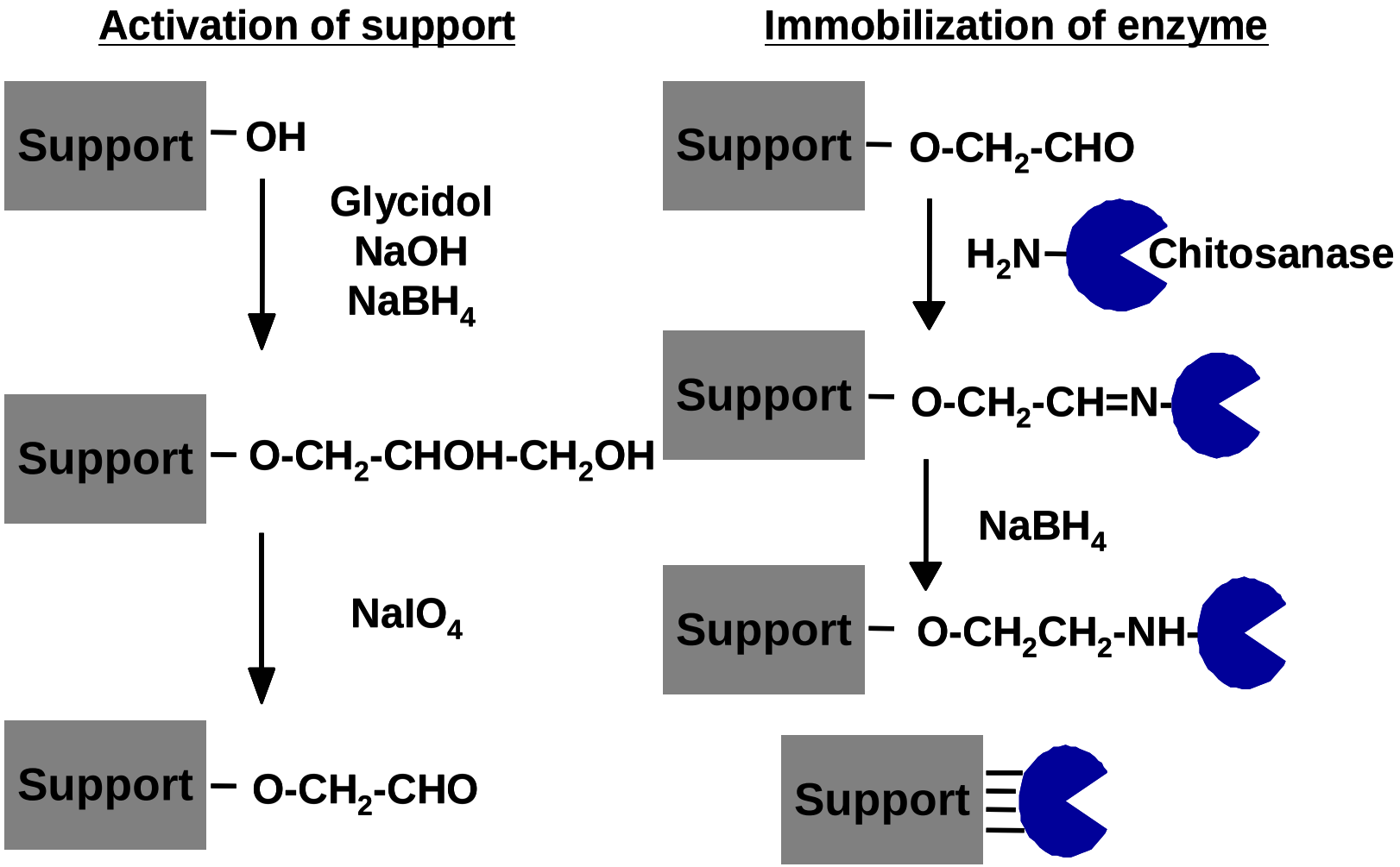


Fig. 1-2 Experimental procedures for the preparation of immobilized enzyme by multipoint attachment.

In the production of chitosan oligosaccharides, large amount of products with higher DP such as pentamer and hexamer that have more physiological activities as described above are expected to be produced. But pentamer and hexamer are the intermediate product of chitosan hydrolysis. So the control of the reaction progress is important. Immobilization of enzyme makes this control becoming easier. Following is different reactors being previously reported.

(1) Batch reactor

During the early period of enzymatic production of chitosan oligosaccharides, hydrolysis was carried out in stirred batch reactor. It was reported that the chitosan oligosaccharides were produced from chitosan by chitosanase from *Bacillus sp.* No. 7 (Izume and Ohtakara, 1987; Uchida *et al*., 1989). The oligosaccharides produced were mainly trimer and tetramer, and there were few monosaccharide produced. Jeon and Kim (2002a) reported that optimum conditions for hydrolyzing 1% chitosan (DD, 89%) using chitosanase from *Bacillus pumilus* BN-262 are 45 ^{o}C, pH 5.5 and reaction time was 1 h while Varum *et al*. (1996) hydrolyzed 65% deacetylated chitosan for 5 h under pH 5.5 and at 37 ^{o}C using chitosanase obtained from *Bacillus sp*. No. 7-M. The main disadvantage of the batch reactor system with free enzyme is the higher cost associated with large quantities of expensive enzymes that cannot be reused. And in these systems, lower yields are generally resulted from the limited ability to control DP.

There is the report about producing chitosan oligosaccharides using immobilized papain on chitin (Lin *et al*., 2002). The yield of chitosan oligomer with molecular weight under 1000 was 49.6% and oligomer with molecular between 500 and 2100 (degree of polymerization 3-7) was 11.0% by separating with nanofiltration and ultrafiltration membrane. But reuse of immobilized papain was not studied.

(2) Membrane reactor

The membrane reactor combined with immobilized enzyme and ultrafiltration membrane was used for the continuous production of chitosan pentamer and hexamer

(Izuta, 1999). The yield of pentamer and hexamer was 56% to the substrate in more than one month operation. Membrane reactor with ultrafiltration membrane could separate the target products (5, 6-oligosaccharide) simultaneously with the hydrolysis of chitosan, so high yield was obtained. But since the molecule weight of chitosan as substrate is high, so the productivity was not enough. And high concentration substrate is very difficult to be applied to the membrane reactor, so some other types of reactors are expected.

The enzyme reactor system along with an ultrafiltration (UF) membrane reactor was developed to produce chitosan oligosaccharides with relatively a higher DP (Jeon and Kim, 2002a). This system could hydrolyze substrate that equivalent to 11 batches used in the batch reactor with same amount of enzyme, and enabled effective production of relatively large amount of chitosan oligosaccharides at a low cost. The most important factor in the usage of an UF reactor system was the control of permeation rate that determines molecular size of chitosan oligosaccharides. However, UF membrane method did not allow continuous production of chitosan oligosaccharides due to the increased transmembrane pressure during the reaction. This was due to high viscosity of chitosan solution and fouling of membrane by accumulated substrate. Therefore, reduction of viscosity of chitosan prior to treatment in the UF membrane system was a requisite for a more effective continuous production.

Continuous production of chitosan oligosaccharides was feasible with combination of a column reactor packed with immobilized enzyme and the UF membrane reactor. The new system was named as dual reactor system (Jeon and Kim, 2000b). In the first step, chitosan is partially hydrolyzed by the immobilized enzyme prepacked in the column reactor and the product is supplied to the UF membrane system for the production of chitosan oligosaccharides. Partially hydrolyzed chitosan possesses low viscosity and does not create fouling problems under controlled conditions. This method ensures a greater productivity per unit enzyme, ability to control molecular weight distribution and more efficient continuous production process compared to those of conventional methods. But this system was complex, moreover,

chitosan solution was only 10 kg/m^3 (DD, 89%)

(3) Column reactors with immobilized enzyme

A method to produce chitosan oligosaccharides with higher DP was introduced by Jeon *et al*. (1998) using an immobilized chitosanase. In this system, the immobilization was tested with different carriers and the chitosanase immobilized on chitin exhibited the highest enzymatic activity.

In addition, Kuroiwa (2005) immobilized chitosanase from *Bacillus pumilus* BN-262 on agar gel with multipoint attachment method, and using packed bed reactor with immobilized chitosanase chitosan was hydrolyzed continuously for one month. The yield of pentamer and hexamer to the substrate was 35% even after one month operation. Kuroiwa *et al*. (2002) studied the reaction conditions for effective production of pentamer and hexamer and observed that it is greatly dependent on surface enzyme density, support particle size, temperature, agitation speed and initial substrate concentration. All these factors were found to be related to the reaction rate and mass transfer rate at the surface of the support materials. These all factors were correlated with Damköhler number (*Da*) defined as the ratio of the maximum reaction rate to the maximum mass transfer rate. The correlation indicated that the reaction conditions at a low *Da* value would cause a high yield of pentamer and hexamer oligosaccharides. Under the conditions of minimum *Da* for effective production of pentamer and hexamer, a packed –bed reactor was operated for one month continuously and resulted in a high yield of pentamer and hexamer. However, in the continuous reaction, 98% deacetylated chitosan was used instead of chitosan of 100% deacetylated chitosan because the former is easier to be dissolved and has lower viscosity. The yield of pentamer and hexamer was lower than that produced from 100% deacetylated chitosan and the production from 98% deacetylated chitosan resulted in different products, which causing the difficulties in products purification. Moreover, the concentration of chitosan solution was 2%. High concentration of chitosan as substrate will benefit to the productivity. But, high viscosity originated from high content of substrate will cause the difficulty of operation. So the

development of a bioreactor that enables to treat a high viscous chitosan solution is required.

1.4 Objectives of this study

As described above, many researches have attempted to improve production of physiological chitosan oligosaccharides by different enzymatic methods and reactors. However, all of them took low concentration of chitosan as substrate. The use of high concentration chitosan as a substrate will be benefit to the production of chitosan oligosaccharides in high concentration. However, chitosan solution in high concentration is highly viscous. The high viscosity of chitosan solution causes the difficulty of operation in a reactor. So the development of a new type of immobilized-enzyme bioreactor that enables the hydrolysis of the high viscous chitosan solution is required. And their operation method is also necessary for the efficient production of chitosan pentamer and hexamer. For these reasons, the objectives of this study are as follows:

(1) Development of a new bioreactor for the production of chitosan pentamer and hexamer in high concentrations by the enzymatic hydrolysis of the high concentration chitosan solution as substrate
(2) Development of an operation method of the bioreactor, which enables the production of chitosan pentamer and hexamer in higher concentrations by increasing the chitosan concentration solution as substrate

Based on the above mentioned background, firstly, a novel immobilized enzyme bioreactor was developed and characteristics of chitosan hydrolysis in the developed bioreactors were investigated in detail in Chapter 2. In Chapter 3, using the newly developed bioreactor, an operation method which enables the production of chitosan pentamer and hexamer in higher concentrations was studied and finally, a repeated

production of the objective substances, pentamer and hexamer, in higher concentration was carried out.

Chapter 2 Development of New Bioreactors for the Hydrolysis of High Viscous Chitosan Solution

2.1 Introduction

As stated in Chapter 1, in order to produce physiologically active chitosan oligosaccharides, the method using immobilized enzyme is more rational than that using chemical hydrolysis.

The advantage of the use of a highly concentrated substrate solution is that it allows the production of target products in high concentration, which will be increased the productivity and decrease the cost. In the hydrolysis reaction of chitosan, however, a high viscosity of substrate solution limits the operability of a bioreactor. Many enzyme-immobilized reactors were used for producing chitosan oligosaccharides. However, their uses were limited for producing chitosan oligosaccharides in high concentration because of viscosity of high concentration chitosan solution. An immobilized-enzyme bioreactor for such a highly viscous solution has not been developed. Therefore the development of a bioreactor that enables to hydrolyze a high viscous chitosan solution is required.

To overcome these problems, a novel immobilized enzyme bioreactor with a multidisk impeller was developed. Enzyme was immobilized on the disks, namely cotton cloth disk or agar-coated disk, by means of the multipoint attachment method. It is believed that using this type of reactor the slip velocity between the substrate solution and the enzyme immobilized on the support surface will be increased, which promotes mass transfer of the reaction mixture. The multiple disks provide a large surface area for the enzyme immobilization. Furthermore, stable immobilized enzyme will be obtained by means of the multipoint attachment method.

In this chapter, three kinds of impeller were developed, and the ability of the production of chitosan oligosaccharides, especially pentamer and hexamer, was examined. In the first part of this chapter, the hydrolysis of chitosan by free chitosanase was tested and its hydrolysis characteristics were examined. Secondly, using chitosanase

immobilized by multipoint attachment method, chitosan hydrolysis was carried out using the reactor with a cotton cloth multidisk impeller. Based on the results of reaction experiments and microscopic observation of the cotton cloth by scanning electronic microscope, the applicability of the cotton cloth disk for the support material of immobilization was discussed. Finally, agar gel-coated disk was used as the support for the immobilization of enzyme. The reactors with an agar gel-coated multidisk impeller were used for production of chitosan oligosaccharides. The production property of the target chitosan oligosaccharides, i.e. pentamer and hexamer, using these novel reactors were investigated.

2.2 Materials and methods

2.2.1 Enzyme

Chitosanase (EC.3.2.1.132) from *Bacillus pumilus* BN-262 was supplied by Meiji Seika Kaisha (Tokyo, Japan). Molecular weight of the chitosanase was 31,000 and its isoelectric point was 9.1. This is an endo hydrase, and the final products in chitosan hydrolysis by this enzyme are dimmers and trimmers of chitosan oligosaccharides. The enzyme powder contained 27.4% protein, as determined by the Bradford method (1976) using bovine serum albumin as the standard. The enzyme powder was used without further purification.

2.2.2 Substrate

Chitosan powder (100% deacetylation) was purchased from Funakoshi (Tokyo, Japan). The mean molecular weight was 370,000, as determined by the viscometric method (Wang *et al*., 1991). As the substrate solution, Chitosan powder (5 or 20 g) was added to 600 mL of deionized water and dissolved with 100 mL of 1 mol/L acetic acid by stirring. The final pH was adjusted to 5.6 with 5 mol/L NaOH solution, and the volume of the solution brought to 1 L to afford either a 5 or 20 kg/m^3 chitosan solution.

A 100-mL portion of the solution was used in each hydrolysis experiment to assay the activity of the immobilized enzyme and to produce oligosaccharides.

2.2.3 Development of impellers as support for immobilization of enzyme

2.2.3.1 Cotton cloth multidisk impeller

A cotton cloth multidisk impeller was combined with five sheets of cotton cloth in 45 mm diameter. Each sheet was tightened on circle stainless steel-hoop. Then they were perpendicularly fixed on the spindle by inserting the hole of cotton cloth and plastic cross and rubber ring in 1.9 mm diameter. There was the same center in the cotton cloth and the plastic cross, and the rubber ring was inserted between every 2 sheets to separate them. The picture of the cotton cloth multidisk impeller was shown in Fig. 2-1.

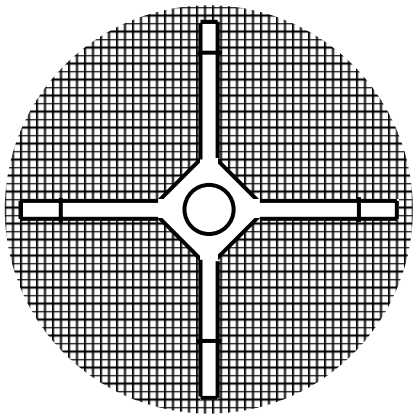
2.2.3.2 Agar gel-coated multidisk impeller

A multidisk impeller consisted of five disks of stainless steel wire mesh (18 mesh; diameter, 50 mm) supported by a plastic cross (thickness, 1 mm; Fig. 2-2a). The plates were fixed perpendicular to the stainless steel shaft, and a rubber ring (thickness, 1.9 mm) was inserted between each plate to maintain a constant spacing. The picture of the wire mesh multidisk impeller was shown in Fig. 2-2b. The impeller was dipped in a 6% agar solution that was kept at 100 °C, and then the impeller was removed from the solution and cooled to room temperature. The agar adhering to the mesh gelatinized into the flat mesh disks. The surface of the gel-coated disks became smooth because all the openings in the mesh were filled with the agar gel. The chitosanase was immobilized on the flat surface of agar gel-coated disks as described in 2.2.4.

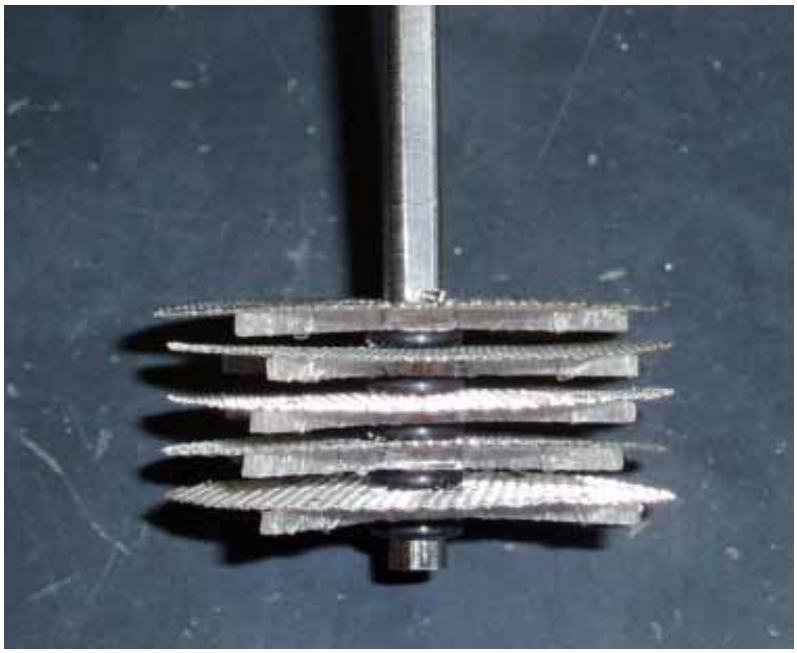
2.2.3.3 Agar gel-coated inclined multidisk impeller

The agar gel-coated inclined multidisk impeller was made similar to the agar gel-coated multidisk plate impeller. Two parts of each disk were cut and then each part

Fig. 2-1 Picture of cotton cloth multidisk impeller.



(a)



(b)

Fig. 2-2 Wire mesh multidisk impeller.
(a) Wire mesh disk plate.
(b) Picture of constructed impeller.

was inclined to up and down (Fig. 2-3a). So each disk joined together like in a shape of double helix. Assembly of the impeller was the same as described in 2.2.2. The picture of the agar gel-coated inclined multidisk impeller was shown in Fig. 2-3b. Method for the coating of inclined multidisk by agar gel was the same as described in 2.2.3.2. The chitosanase was immobilized on the agar gel-coated disks as described in 2.2.4.

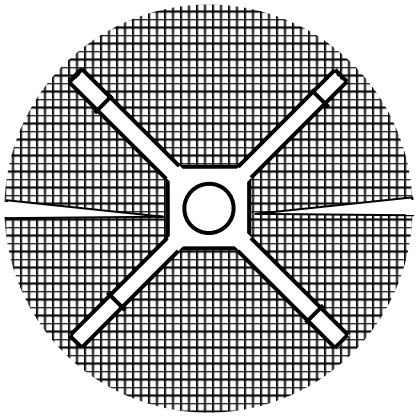
2.2.3.4 Experimental apparatus

A schematic drawing of reaction unit with impeller was shown in Fig. 2-4. The impeller was driven by a variable-speed motor, and the reactor made of glass (diameter, 56 mm; height, 115 mm) was placed in a thermostatted bath.

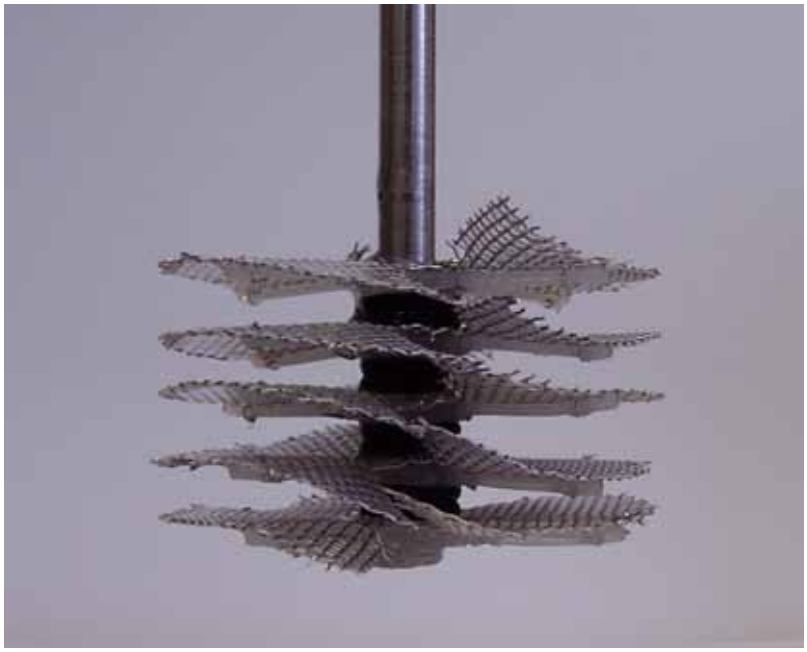
2.2.4 Immobilization of chitosanase

The immobilization of chitosanase was carried out using the multipoint attachment method reported by Ichikawa *et al.* (2002) was used. The impeller consisting of multidisk was using for the immobilization of chitosanase. The immobilization of chitosanase was in two steps. The activation of support, i.e. disks of impeller, was followed by the immobilization of chitosanase. The immobilization was done using the same unit as the hydrolysis reaction as shown in Fig. 2-4.

Firstly, the support (cotton cloth or agar-gel coated disks) was activated. Glyoxylated support as an activated support was prepared by etherification of support having hydroxyl group using glycidol and further oxidation by $NaIO_4$. The solution for the support activation was prepared by dissolving of $NaBH_4$ (0.63 g) as an antioxidant was dissolved in100 mL of 5 mol/L NaOH and 6 mL of glycidol suspension. The impeller having cotton cloth or agar-gel coated multidisk was stirred in the solution at 0.5 s^{-1} rotating speed for 18 h at 25 °C. Then, the impeller was washed with deionized water. The impeller with glycerylated support was stirred in 100 mL solution of 35 mmol/L $NaIO_4$ for 1 h at 25 °C. Finally, the resulting activated support was washed with deionized water and stored in deionized water at 4 °C until use.



(a)



(b)

Fig. 2-3 Wire mesh inclined multidisk impeller.
(a) Wire mesh disk plate with slit.
(b) Picture of constructed impeller.

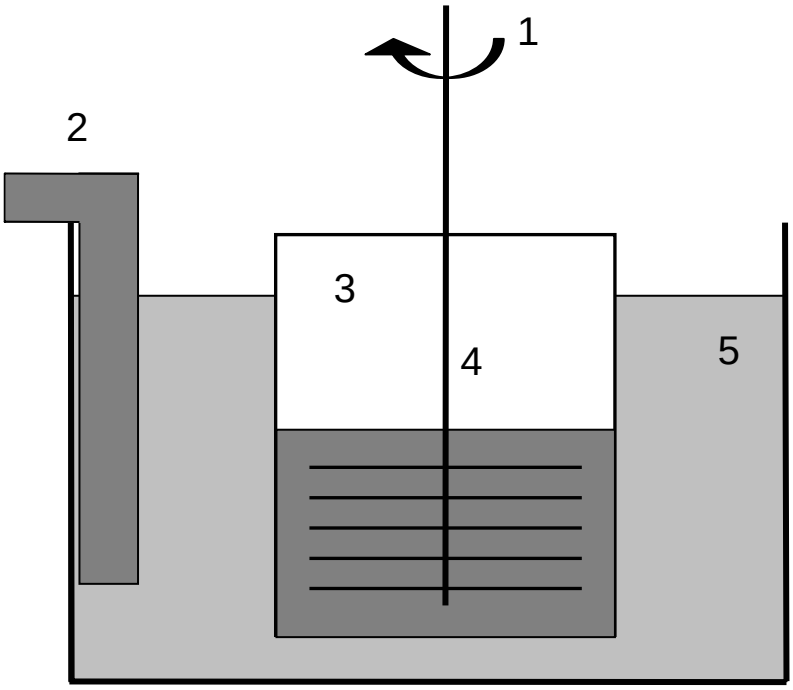


Fig. 2-4 Experimental apparatus.
1, motor; 2, heater; 3, reactor; 4, impeller; 5, water bath.

The next was the immobilization of chitosanase on the activated support. The impeller with activated support was stirred in the mixture of 114 mL of 0.2 mol/L borate buffer (pH 10.0) and 6 mL of chitosanase solution for 24 h at 25 °C. After that, 0.24 g of solid $NaBH_4$ was added to the solution and stirred for 30 min at 25 °C. This borohydride reduction constitutes a suitable end-point of the enzyme-support interactions because this reduction produces two complementary effects: stabilization of the enzyme-support attachments (Schiff's bases) already formed and reduction of unreacted aldehyde residues to inert hydroxyl ones (Guisan and Blanco, 1987). Then, the support immobilized chitosanase was washed with 0.1 mol/L phosphate buffer (pH 7.0) and deionized water, and stored in deionized water at 4 °C until use.

2.2.5 Chitosan hydrolysis by free chitosanase

Free chitosanase (0.5 mg/mL) 0.6 mL was added to chitosan solution (5 kg/m^3) 100 mL and the mixture was incubated at 35 °C with stirring by a magnetic stirrer. Samples were taken in certain interval and immediately heated in boiling water for 10 min to inactivate chitosanase.

2.2.6 Chitosan hydrolysis by chitosanase immobilized on the multidisk of impeller

Chitosan solution (5 or 20 kg/m^3) 100 mL was stirred with the chitosanase-immobilized impeller (described in 2.2.3.4) and kept at 35 °C (or 50 °C). The impeller speed was controlled by a speed-changeable motor. Samples were taken in a certain interval.

The repeated batch hydrolysis of chitosan using chitosanase-immobilized impeller was carried out for totally five times. After each reaction, the impeller was washed by deionized water and 0.1 mol/L phosphate buffer (pH 7.0). Samples of five times hydrolysis were all taken in the same time point.

2.2.7 Analysis

2.2.7.1 Quantitation of immobilized chitosanase

The amount of the immobilized enzyme was calculated by the difference of protein mass in the solutions between before and after immobilization.

The protein concentration was measured by Bio-Rad protein assay kit (Bradford, 1976). A 0.8 mL of sample solution was mixed with 0.2 mL of staining reagent, and kept at room temperature over 5 mins. Then the absorbance of the mixture was measured at 595 nm of wavelength by a spectrophotometer (UV-1200, Shimadzu. Kyoto, Japan) within 60 min after the mixing of sample and staining reagent. Using the same method, a calibration curve for chitosanase was obtained beforehand. The protein concentration in the sample solution was evaluated using the calibration curve. The chitosanase powder contained 27.4% protein, as determined by this Bradford method (1976) using bovine serum albumin as the standard.

2.2.7.2 Determination of reducing sugar concentration

The concentration of reducing sugar was measured by the method of modified Schales's (Imoto and Yagishita, 1971). Schales reagent was prepared by dissolving 0.25 g of $K_3[Fe(CN)_6]$ in 500 mL of 0.5 mol/L Na_2CO_3 solution. Sample (1.5 mL) that has been adequately diluted and 2.0 mL of Schales reagent were mixed. The mixture was heated for 20 min in a boiling water bath, and then it was quickly cooled in the bath filled with crushed ice and water. The mixture was centrifuged at 3000 rpm. The absorbance of the supernatant was measured at 420 nm of wavelength by (UV-1200, Shimadzu, Kyoto, Japan).

The activity of immobilized chitosanase was determined according the method of Uchida and Ohtakara (1988) which was measured by the quantity of reducing sugar produced. D-glucosamine (Sigma, St. Louis, MO, USA) was used as a reference compound. The relationship between its concentration and absorbance was measured and the linear range was determined. From this relationship, the concentration of reducing sugar was obtained. One unit of chitosan hydrolysis activity was defined as the amount of enzyme that liberated 1 μmol of D-glucosamine in 1 min. Based on this

definition, the specific activity of free chitosanase was 460 U/mg-protein.

2.2.7.3 Fractional determination of chitosan oligosaccharides

The concentrations of each chitosan oligosaccharide, from dimer to hexamer, were determined by high-performance liquid chromatography (HPLC). HPLC system constituted with pump (880-PU, JASCO, Tokyo, Japan), on-line degasser (model 152, Flom, Tokyo, Japan), column oven (model 556, Gasukuro Kogyo, Tokyo, Japan), RI detector (Shodex RI SE-51, Showa Denko, Tokyo, Japan) and integrator (C-R6A chromatopac, Shimadzu, Kyoto, Japan). The CAPCELL PAK NH_2 column (4.6 mm^{φ}×250mm, Shiseido, Tokyo, Japan) was used.

The HPLC operation was carried out with a 40:60:0.2:0.2 (volumetric ratio) mixtures of acetonitrile, deionized water, phosphoric acid and triethylamine as a mobile phase at a flow rate of 1.0 mL/min. The sample (1 mL) was added into 1 mL of acetonitrile. If a large amount of precipitate formed, it was centrifuged. Then it was filtrated by membrane filter with 0.45 μm pore size, and 20 μl of sample was injected into the column at 45 °C.

The concentrations of chitosan oligosaccharides in the samples were calculated by using the calibration curves between the areas of each chitosan oligosaccharides and their concentrations, which was obtained by using a standard sample (Seikagaku, Tokyo, Japan).

2.2.7.4 Microscopic observation of the structure of cotton cloth

The structure of dry cotton cloth was observed by a scanning electron microscope (JEOL JSM-500LV, Tokyo, Japan). A rectangular cotton cloth was cut and fixed on an aluminum SEM stub with a small droplet of graphite paste. Voltage used was 10 KV. Magnification was 35-1000×. All experiments were done under a low vacuum mode.

2.3 Results and discussion

2.3.1 Characteristics of chitosan hydrolysis by free chitosanase

Chitosanase from variable resources express different activities to different deacetylation degree of chitosan as described in 1.2.3. In this study, chitosanase from *Bacillus pumilus* was used. Kuroiwa (2002) reported that the chitosanase from *Bacillus pumilus* expressed the maximum activity to 100% deacetylated chitosan. So 100% deacetylated chitosan was used as the substrate in this study. Time course of chitosan hydrolysis by free chitosanase was shown in Fig. 2-5 and time courses of components of chitosan oligosaccharide concentrations were shown in Fig. 2-6.

It was shown in Fig. 2-5 that after the reaction started, the concentration of reducing sugar increased suddenly at the beginning, then the concentration of reducing sugar leveled off. The viscosity of the solution decreased with the increase of reducing sugar concentration. According to the report from chitosanase producing company (Matsunobu *et al*., 1996), the viscosity of chitosan solution decreased sharply at the beginning of hydrolysis, and after 30 min the viscosity decreased until 1/20 of the initial viscosity. At the same time, the hydrolysis yield was only 0.3%. It is known that the chitosanase was the endo-enzyme, which randomly cleaves chitosan molecule. It was reported that the chitosanase used in this study from *Bacillus pumilus* BN-262 reacted at positions like GlcN-GlcN and GlcNAc-GlcN (Fukamizo *et al*., 1994). It was indicated that there was enough GlcN-GlcN for cleavage by chitosanase at the beginning of reaction, therefore, the reaction speed was fast. However, with the proceeding of hydrolysis, it was considered that amounts of GlcN-GlcN decreased and thus reaction rate decreased.

Figure 2-6 showed the time courses of chitosan oligosaccharides concentration produced by chitosan hydrolysis using free chitosanase. At the initial period of reaction, the concentrations of the oligosaccharides produced increased. But chitosan pentamer and hexamer, being the target substances in this study, decreased with reaction time after they reached the maximum. The reason is that pentamer and hexamer are the intermediate products and are consequently hydrolyzed with proceeding of reaction.

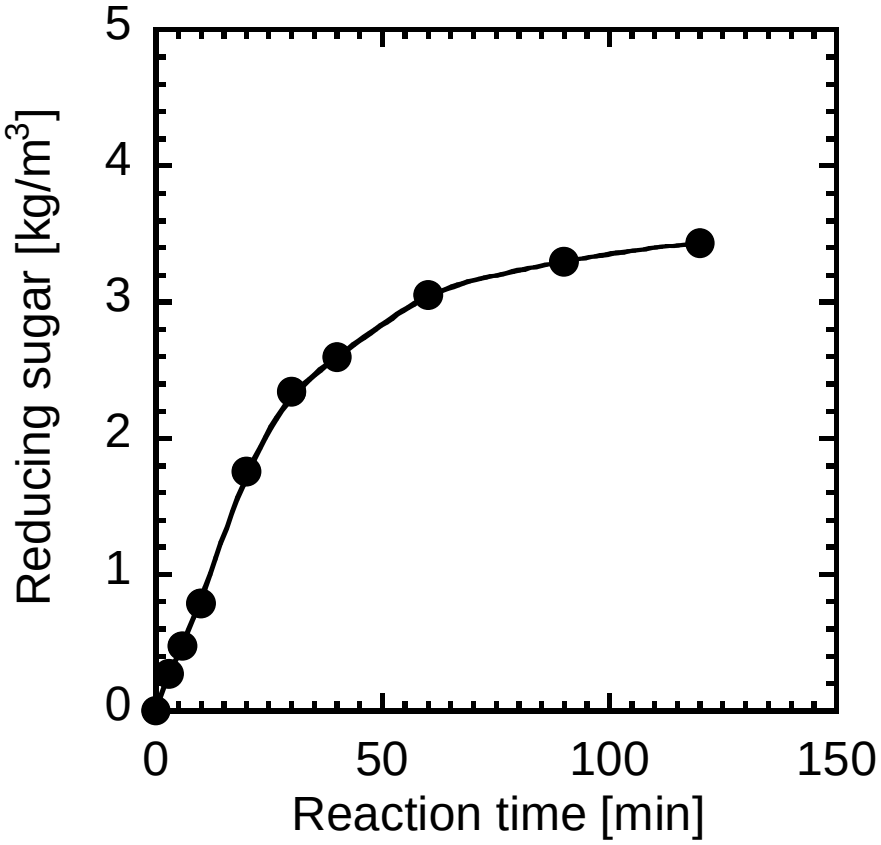


Fig. 2- 5 Typical time course of chitosan hydrolysis by free chitosanase.

Enzyme concentration: 38 U/100mL
Chitosan solution: 5 kg/m^3
Temperature: 35 ^{o}C

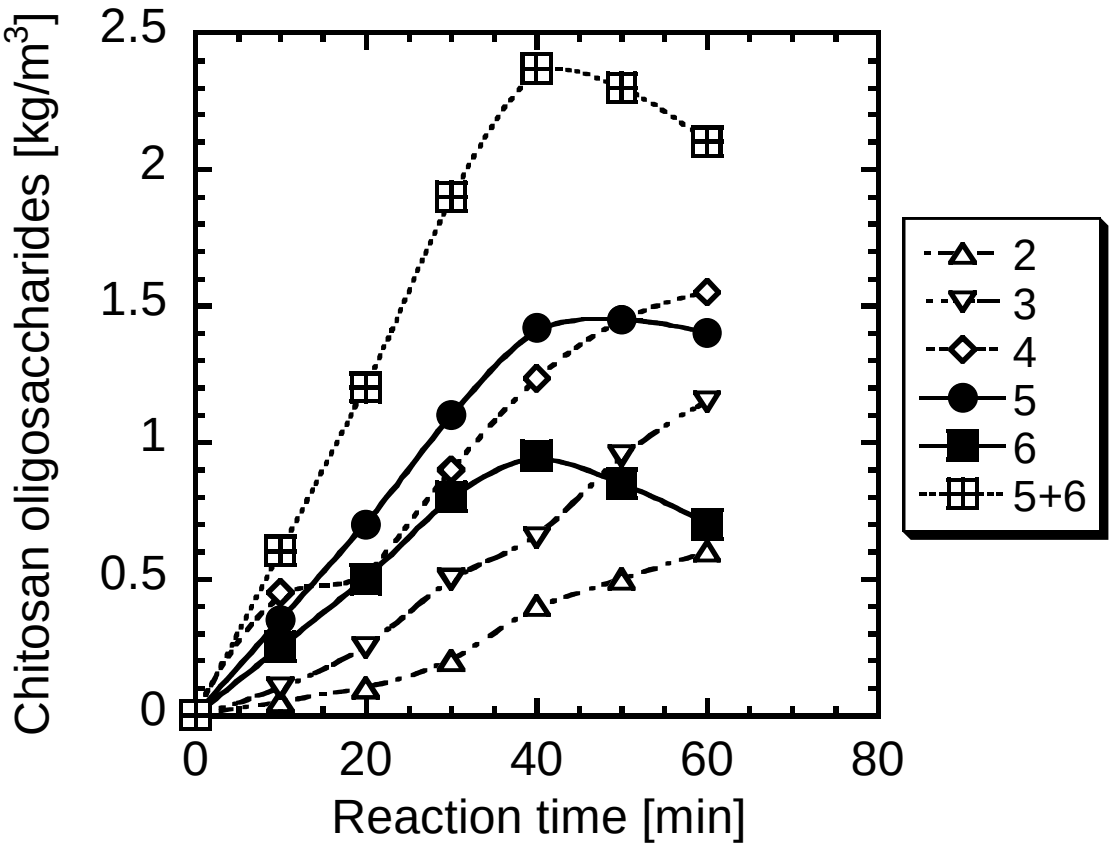


Fig. 2-6 Typical concentration time courses of chitosan oligosaccharides produced by free chitosanase.

Enzyme concentration: 38 U/100 mL
Chitosan solution: 5 kg/m^3
Temperature: 35 ^{o}C

The maximum value of the sum of pentamer and hexamer, $C_{(5+6)max}$, was 2.4 kg/m^3, which was 48 wt.% to the initial substrate (5 kg/m^3). On the other hand, the concentrations of dimer and trimer, as final products of the enzymatic hydrolysis, continuously increased with reaction time.

As shown in Fig. 2-6, in order to efficiently produce the physiologically active chitosan pentamer and hexamer, it is necessary that the hydrolysis of chitosan should be stopped at the time point when the concentration of pentamer and hexamer reaches the maximum. If free enzyme is mixed with substrate solution, it is difficult to separate enzyme from the mixture, and enzyme needs to be inactivated by heating for ceasing hydrolysis reaction. However, there are some problems with inactivating enzyme by heating, such as consumption of large amount of energy, impossibility of reuse of expensive enzyme, etc. Adding chemical reagents can also inactivate enzyme. But it will contaminate the products. On the other hand, if enzyme is immobilized, the enzyme is easy to be immediately separated from substrate and products. Thus, the enzyme can also be reused. For these reasons, the production of chitosan oligosaccharides by immobilized enzyme can have many advantages. In this part, cotton cloth and agar gel were tried for the immobilization of chitosanase as support material.

2.3.2 Cotton cloth multidisk impeller

Cotton cloth has many hydroxyls just as the agar gel, so the immobilization of chitosanase on it by multipoint attachment can be realized. Furthermore, cotton cloth is flexible and easy to cut, so can be easily designed. In this study, cotton cloth was formed to be a cotton cloth multidisk impeller as shown in Fig. 2-1. Chitosanase was immobilized on it and the impeller directly immobilizing chitosanase was used for producing chitosan oligosaccharides.

2.3.2.1 Immobilization yield of chitosanase

Immobilization yield is defined as the percentage of immobilized enzyme to the total enzyme used for immobilization. Immobilization yield of enzyme Y_I was

calculated by the following equation (2-1):

$$Y_I = \frac{E_0 - E_r}{E_0} \times 100 \qquad (2\text{-}1)$$

where, E_0 is original amount of the enzyme, and E_r is the remained enzyme concentration in the solution after immobilization.

The relationship between the enzyme concentration used for immobilization and the immobilization yield was shown in Fig. 2-7. The immobilization yield decreased with increasing the loading enzyme concentration. It was shown that for increasing the enzyme utilization efficiency, it was effective to decrease the loading enzyme concentration. However, decreasing the loading enzyme per area would decrease the specific activity of immobilized enzyme. So considering both the immobilization yield and the specific activity, the loading enzyme used for this study was lower than 2×10^4 mg-prot. /m^3.

2.3.2.2 Activity of immobilized chitosanase

The activity of immobilized enzymes varies with the immobilization method and the type of support material. In this study, firstly, chitosanase was immobilized on activated cotton cloth attached to impeller using the multipoint attachment method. The cotton cloth used as the support for immobilization is crossly weaved by a few strands of cotton threads, and each thread is twisted by many plies of fibers. However, the real surface area of the fibers is difficult to be measured. Accordingly, the apparent area was used for calculation of all following experiments, which was expressed as the two side surface areas of the cotton cloth. The activity of enzyme immobilized on cotton cloth was defined as the specific activity of immobilized enzyme activity per m^2 cotton cloth area [U/m^2]. The observed specific activity of immobilized chitosanase increased linearly with the loaded enzyme concentration (Fig. 2-8).

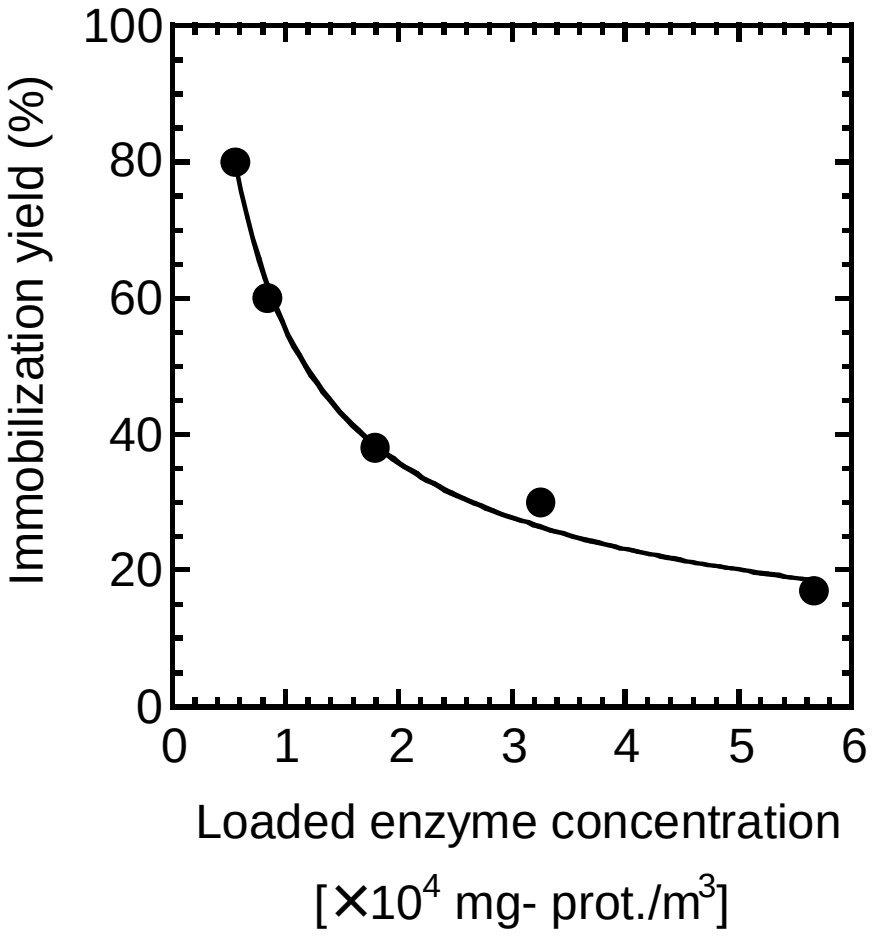


Fig. 2-7 Immobilization yield of chitosanase on cotton cloth by multipoint attachment.

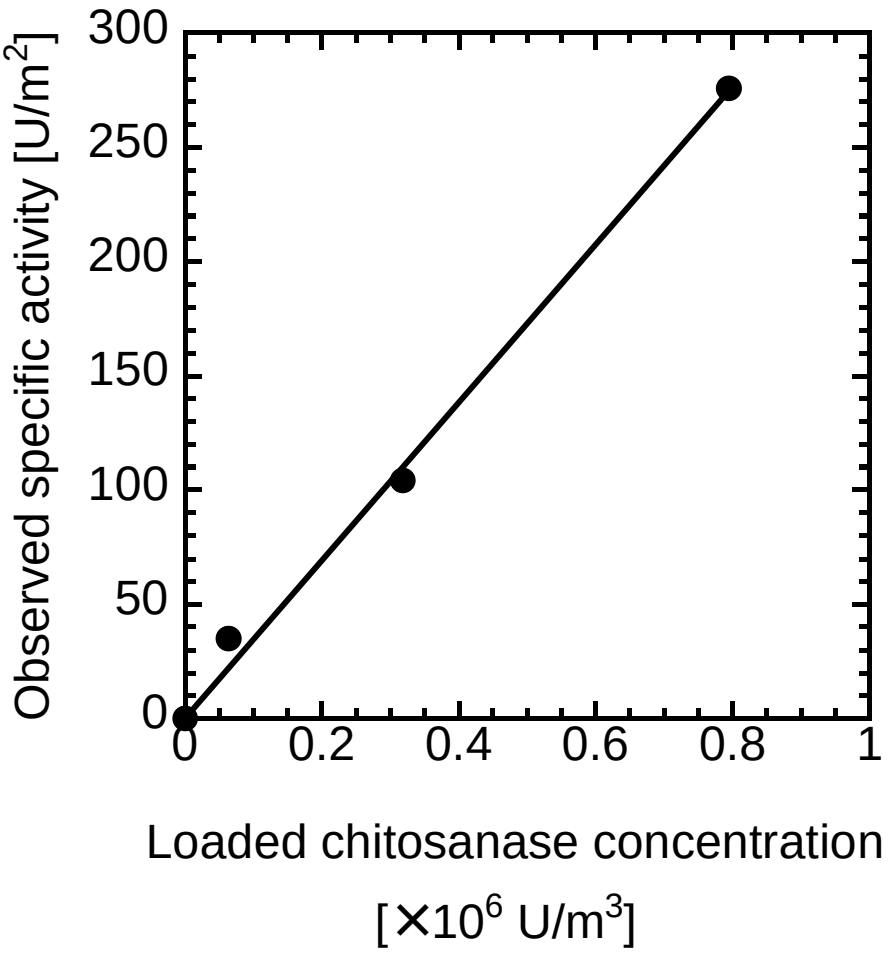


Fig. 2-8 Relationship between observed specific activity and loaded chitosanase concentration for immobilization.

Activity assay conditions:

Chitosan solution: 5 kg/m^3

Agitating speed: 1 s^{-1}

Temperature: 35 ^{o}C

2.3.2.3 Production of chitosan oligosaccharides using cotton cloth multidisk impeller

The new type reactor with cotton cloth multidisk impeller was developed and tried to produce chitosan oligosaccharides. From previous report (Kuroiwa, *et al*., 2002), it is clear that the oligosaccharide composition is affected by the activity of enzyme immobilized on the supports. Fig. 2-9 showed the time courses of each oligosaccharide concentration during hydrolysis of chitosan by enzymes immobilized on cotton cloth multidisk impeller having three different activities of 276, 104 and 53 U/m^2, respectively. The time courses of oligosaccharide compositions significantly differed from that using free enzyme. No hexamer and little pentamer were produced when chitosan was hydrolyzed using the immobilized enzymes having specific activity of 276 and 104 U/m^2, respectively (Fig. 2-9a, 9b). These oligosaccharides were produced when the immobilized enzymes having lower enzyme activity was used, and the maximum concentration of target products, pentamer and hexamer, increased to 1.1 kg/m^3 as specific activity decreased to 53 U/m^2 (Fig. 2-9c). These results showed that the chitosan substrate solution could be hydrolyzed by chitosanase immobilized on the cotton cloth multidisk impeller and chitosan oligosaccharides could be obtained.

2.3.2.4 Suitability of cotton cloth as the support material for immobilization

The maximum yield of target products, pentamer and hexamer, in each reaction is represented by the ratio of maximum concentration of pentamer and hexamer, $C_{(5+6)max}$, to initial chitosan concentration, C_0. Results of these experiments were summarized in Fig. 2-10. Decreasing the activity of immobilized enzyme on the support surface could not increase the yield of pentamer and hexamer at higher activities levels, however, decreasing more of the activity of immobilized enzyme on the support surface increased the yield of pentamer and hexamer at the lower activities level. Generally, at the higher enzyme activity on the surface of support the mass transfer rate would limit the production of intermediates as final product. So it could be concluded that at activity between 276 and 104 U/m^2, the mass transfer rate was the rate-limiting factor.

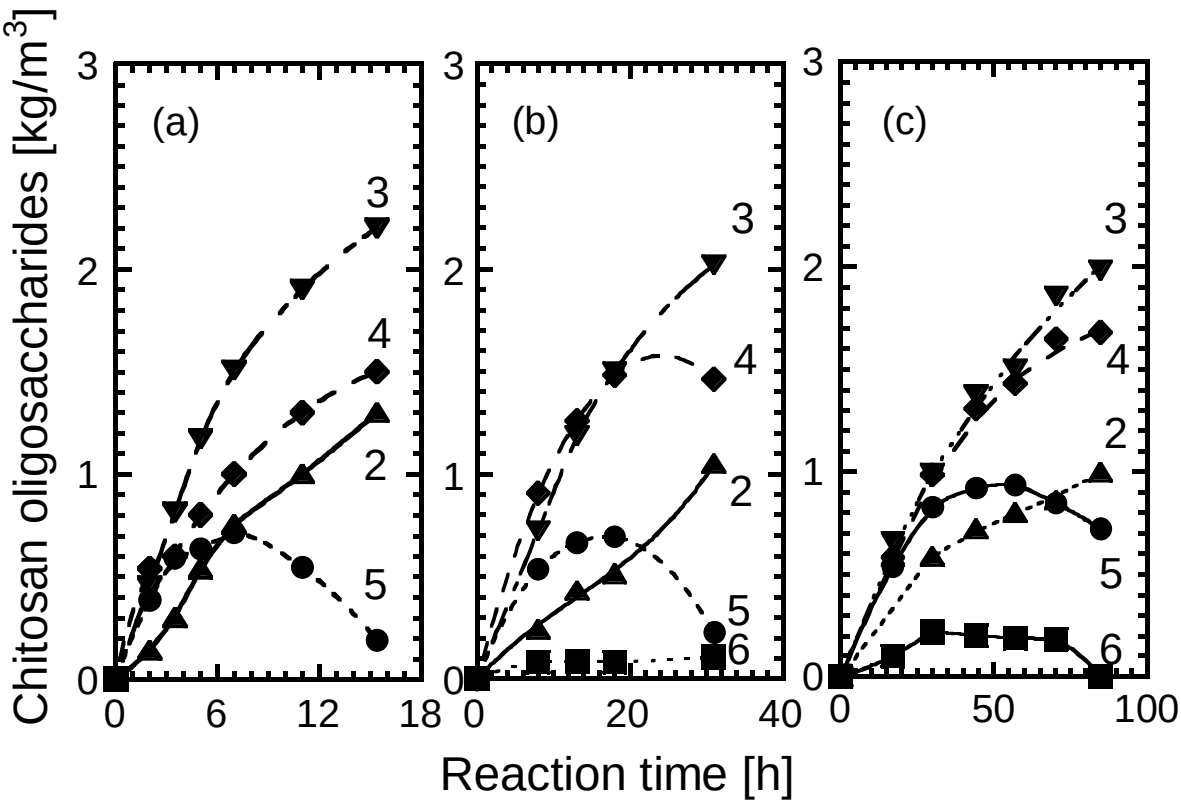


Fig. 2-9 Concentration time courses of chitosan oligosaccharides produced by chitosanase immobilized on cotton cloth multidisk impeller.

Enzyme activity: (a) 276, (b) 104 and (c) 35 U/m^2
Chitosan solution: 5 kg/m^3
Impeller speed: 1 s^{-1}
Temperature: 35 ^{o}C

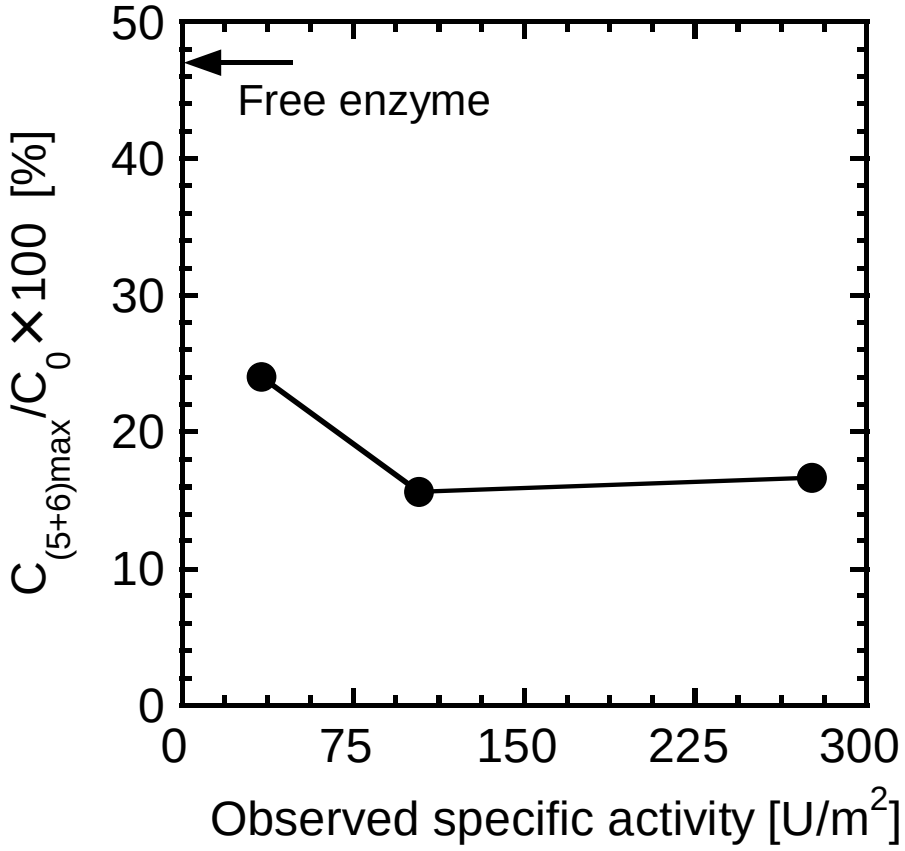


Fig. 2-10 Effect of observed specific activity on the maximum yield of pentamer and hexamer using cotton cloth multidisk impeller.

Chitosan solution: 5 kg/m^3
Impeller speed: 1 s^{-1}
Temperature: 35 ^{o}C

On the other hand, at the lower enzyme activity on the surface of support the reaction rate would limit the production rate and amount of intermediates such as pentamer and hexamer. That is, at activity between 104 and 53 U/m^2, the reaction rate was the rate-limiting factor.

When the substrate was agitated by the impeller with immobilized chitosanase, impeller speed would affect the mass transfer, which would affect the composition of chitosan oligosaccharides. Effect of impeller speed on the maximum yields of target products was shown in Fig. 2-11. Based on these results, no regular tendency was found for the effects of impeller speeds. It is known that the impeller speed affects the rate of mass transfer between the bulk fluid and the surface of the immobilizing support, and a higher impeller speed usually gives a larger mass transfer rate. The higher mass transfer rate near the support surface is important to improve the yield of pentamer and hexamer (Kuroiwa *et al*., 2002). But the results in Fig. 2-11 did not show this tendency, where the impeller speed irregularly affected the maximum yield of chitosan oligosaccharides.

The surface activity of immobilized enzyme on supports affects the reaction rate. When the surface enzyme activity was very high, no or only a little pentamer and hexamer were produced. On the other hand, when the enzyme activity was reduced, pentamer and hexamer were produced and the maximum yield increased with decreasing surface enzyme density. But, even when the reaction rate was affected (data not shown), the specific activity of immobilized enzyme hardly affected the yield of pentamer and hexamer produced. Above results also showed the impeller speed could not affect the maximum yield of target products (Fig. 2-11). There should be other factors affecting the mass transfer rather than impeller speed and enzyme activity. The contact between the enzymes immobilized on support surface and substrate was suggested as an important factor. To elucidate it, the microstructure of cotton cloth was observed by scanning electronic microscope (SEM). Figure 2-12 showed SEM pictures of cotton cloth. As shown in the pictures, the cotton cloth had a structure that knit threads grating-like. Many fibers were bound into one thread. Chitosanase would be immobilized on these fibers. There were many narrow spaces between fibers.

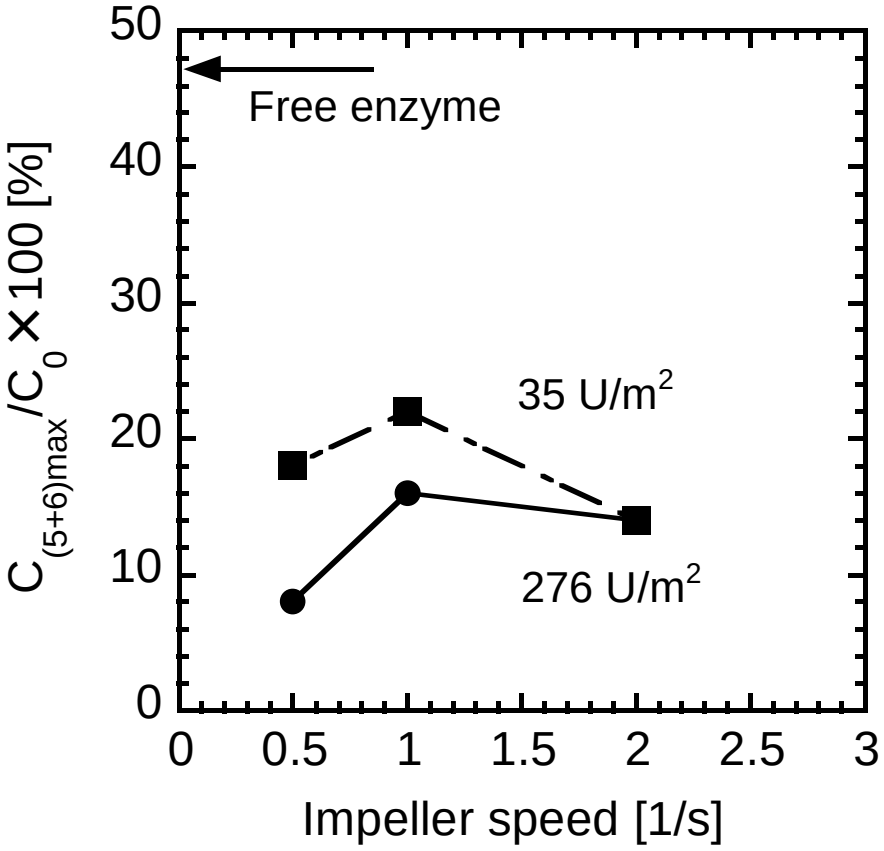
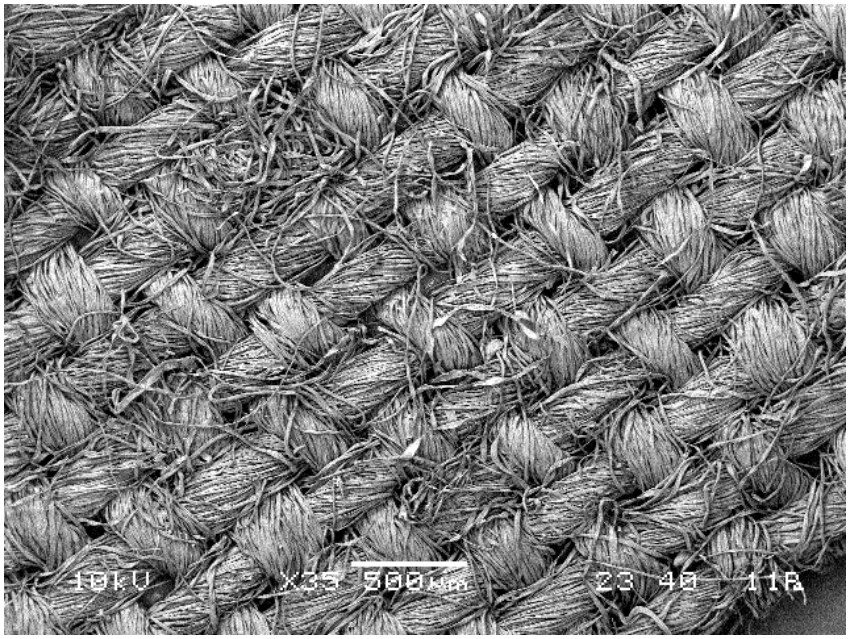
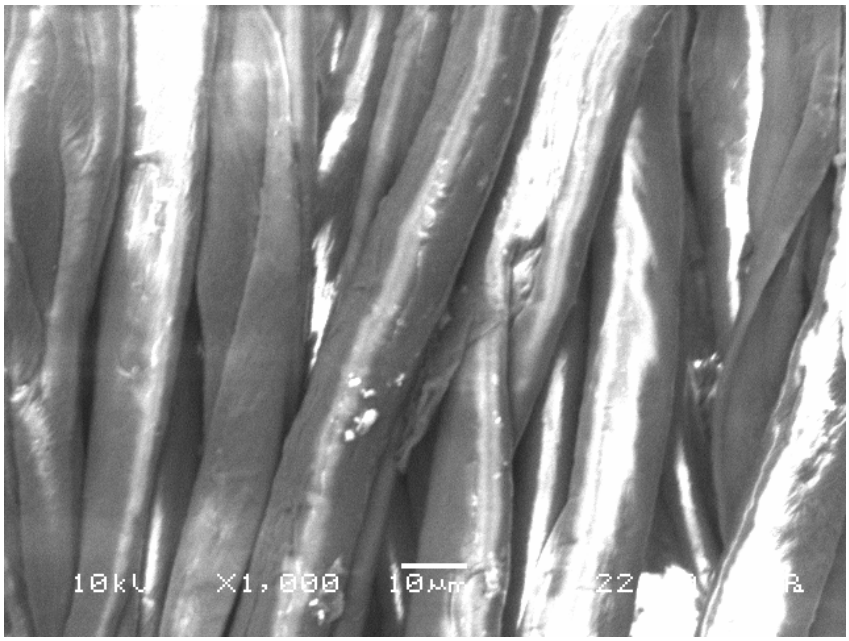


Fig. 2-11 Effect of agitating speed on maximum yield of pentamer and hexamer using cotton cloth multidisk impeller.

Chitosan solution: 5 kg/m^3



500 μm



10 μm

Fig. 2-12 SEM pictures of cotton cloth.

Chitosan solution is relatively viscous. Once some substrate solution is trapped in this narrow space between fibers, it will be difficult to replace with a bulk solution even in high agitation condition. If the substrate supply from bulk cannot be achieved continuously, pentamer and hexamer generated as intermediate products will be further hydrolyzed and their yields will be low. This could be illustrated by the schematic figure shown as Fig. 2-13. Even changing the impeller speed, the mass transfer inside the cotton cloth cannot be promoted. For this reason, the effects of impeller speeds and enzyme densities on yields in this study were not remarkable.

In previous reports (Albayrak *et al*., 2001; 2002), the knitted cotton cloth was used as the support for immobilization of β-galactosidase, and about 50 mg enzyme was immobilized onto one gram of tosylated cotton cloth with a protein coupling efficiency of more than 85%. The enzyme immobilized on cotton cloth had been used in a plug-flow reactor for continuous production of galacto-oligosaccharides from lactose. Their results showed that the reactor performance was stable, and there was no loss of enzyme activity for the 15-day period studied, and immobilized enzyme on cotton cloth also showed same patterns and level of galacto-oligosaccharides formation from lactose as those from free enzyme reaction, indicating that the reaction kinetics was not affected by immobilization and there was no significant diffusion limitation in the immobilized enzyme. But in this study, it seemed having serious mass transfer limitation by this support. The activation method in this study was different from that report, and the reactor type was also different. Results suggested that it was important that there was no or little diffusion limitation effect in immobilized enzyme reaction for the production of intermediates and the immobilized enzyme should be stable enough. The stirring type reactor was suggested to produce chitosan oligosaccharides at high concentrations for their high viscosity, and the cotton cloth was tried to be used as the support material for immobilization of chitosanase. However, for producing high yield of pentamer and hexamer, results showed that the cotton cloth was not suitable support for immobilization of chitosanase because of it microstructure. Thus, a kind of support with following character for replacement is needed: mass transfer near the immobilized

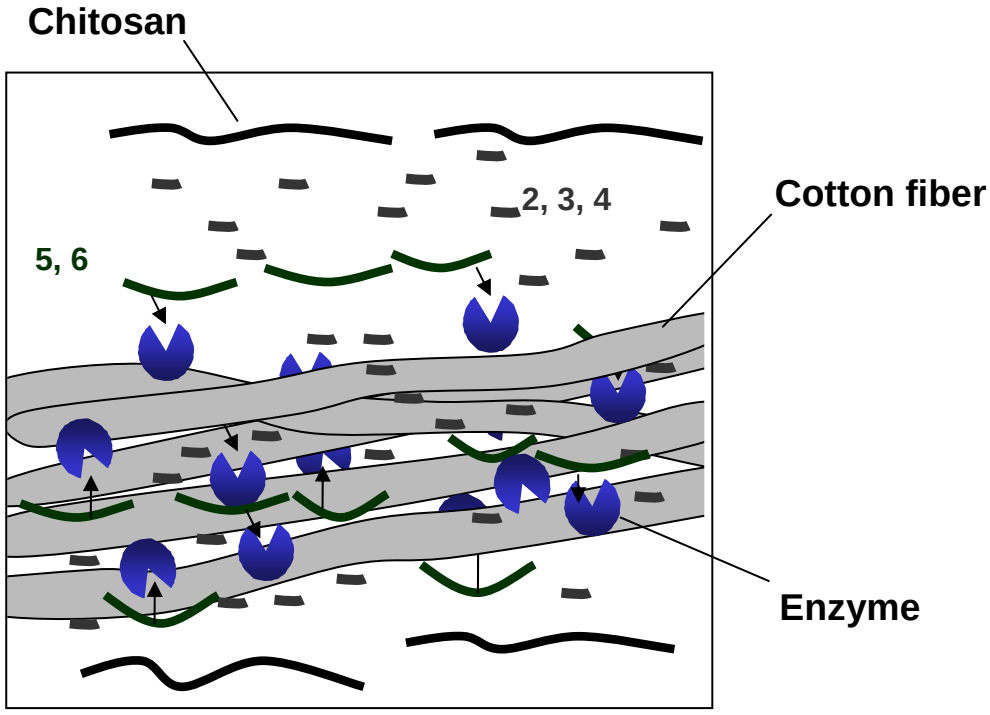


Fig. 2-13 Schematic illustrations of reaction mechanism of chitosan hydrolysis near cotton cloth fiber.

enzyme should be promoted by easy operations such as agitation.

2.3.3 Agar gel-coated multidisk impellers

In this study, the cotton cloth was used for immobilization of enzyme firstly. But as stated above, high yield of targets products could not be obtained, because their microstructure limited the mass transfer between substrate and enzyme surface. It was previously reported (Kuroiwa *et al*., 2003) that agar gel was used as support of immobilized chitosanase and high yield of pentamer and hexamer was obtained. Similar with that of cotton cloth multidisk impeller, agar gel-coated multidisk impellers were prepared and used for producing high yield of oligosaccharides. The productivity of pentamers and hexamers was evaluated.

2.3.3.1 Activity of immobilized chitosanase

The activity of immobilized chitosanase on the support surface was presented by specific activity per surface area. Chitosanase was immobilized on activated agar gel-coated onto mesh disks using the multipoint attachment method. Because it was previously confirmed that chitosanase is immobilized only on the surface of agar gel particles (kuroiwa, 2005), it is believed that chitosanase must be immobilized only on the surface of the agar gel coating the mesh disks in this study. The specific activity of the immobilized enzyme can therefore be represented as the activity per unit surface area of the support (U/m^2). The surface area of the supports was determined from their geometry, because they had smooth surfaces. The observed specific activity of immobilized chitosanase increased linearly with the loaded enzyme concentration (Fig. 2-14). No protein was detected in the residual solution after immobilization at any loaded enzyme concentration. This result suggests that almost all loaded enzyme was immobilized on the support. Consequently, the observed specific activity was proportional to the loaded enzyme concentration.

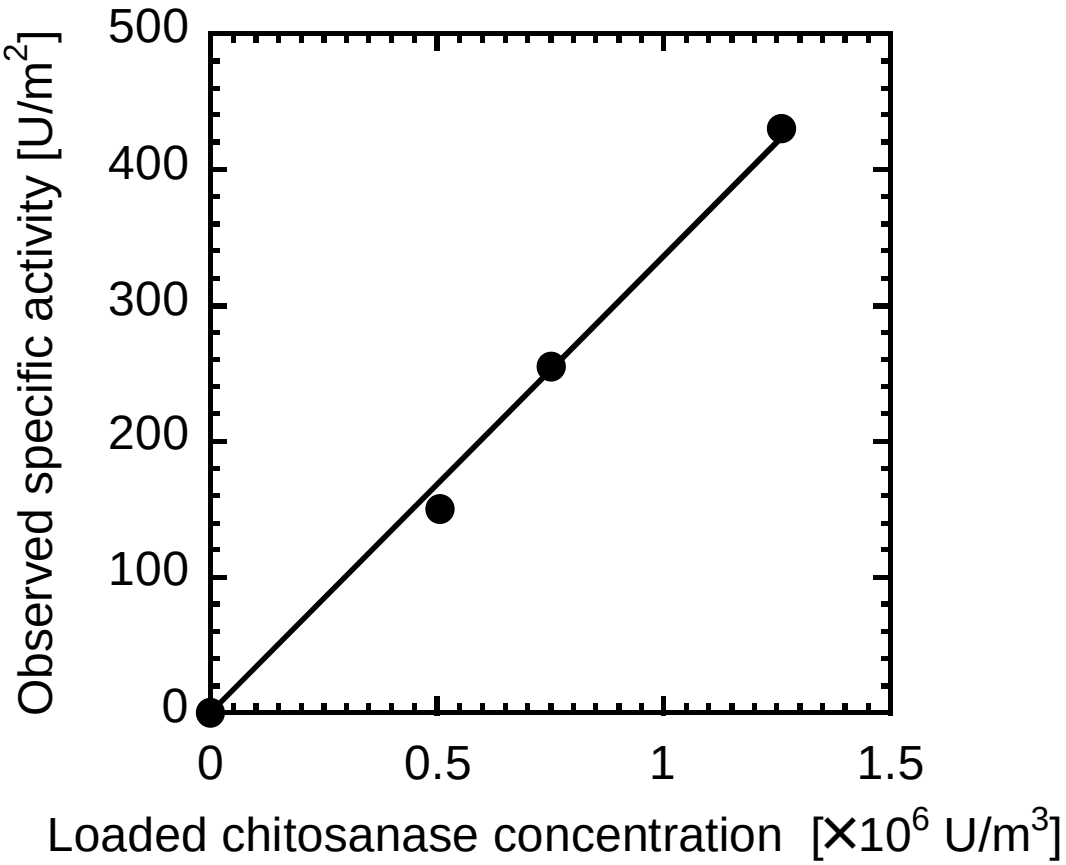


Fig. 2-14 Relationship between observed specific activity and loaded chitosanase concentration for immobilization.

Activity assay condition:

Chitosan solution: 5 kg/m^3

Impeller speed: 2 s^{-1}

Temperaure: 35 ^{o}C

2.3.3.2 Stability of immobilized chitosanase

To determine the stability of chitosanase immobilized on the agar gel-coated impeller disks, batch hydrolysis of chitosan was carried out five times at 35 °C using the same immobilized chitosanase. After each reaction, the impeller bearing the immobilized chitosanase was washed with distilled water and 0.1 mol/L phosphate buffer (pH 7.0) and then stored in water at 4 °C until the next use. Reactions were carried out once a day. The time courses of the first, third, and fifth batches were almost identical (Fig. 2-15), which clearly indicates that the activity of the immobilized chitosanase did not change over the course of the five batch reactions and that multipoint attachment to the agar gel resulted in highly stable immobilized enzyme.

2.3.3.3 Effect of impeller speed on production of chitosan oligosaccharides

Agitation is important in heterogeneous reaction systems. In solid-liquid systems, agitation accelerates the reaction by promoting mass transfer at the solid-liquid interface when the mass transfer is the rate-limiting step. The typical time courses of oligosaccharide concentrations (dimer to hexamer) produced by hydrolysis at impeller speeds of 1 and 2 s^{-1} indicate that the concentrations of all the oligosaccharides increased as the reaction progressed at reactions times up to 4-5 h (Fig. 2-16). After the concentrations of pentamer and hexamer reached their maximum values, the concentrations decreased as the reaction proceeded, owing to enzymatic hydrolysis of the intermediates. The time courses of oligosaccharide concentrations at the two impeller speeds differed: the total maximum yield of the two target products at 2 s^{-1} was higher than that at 1 s^{-1}.

Because the impeller speed affected the product composition, the effect of impeller speed on the yield of target products was investigated at various observed specific enzyme activities under 1, 2 and 4 s^{-1} of impeller speed. The experiments were carried out with 5 and 20 kg/m^3 chitosan solutions. Except at the lowest observed specific activity (79 U/m^2), the yields of target products at 2 and 4 s^{-1} were higher than the yields at 1 s^{-1} (Fig. 2-17). However, no significant increase in yield was obtained by

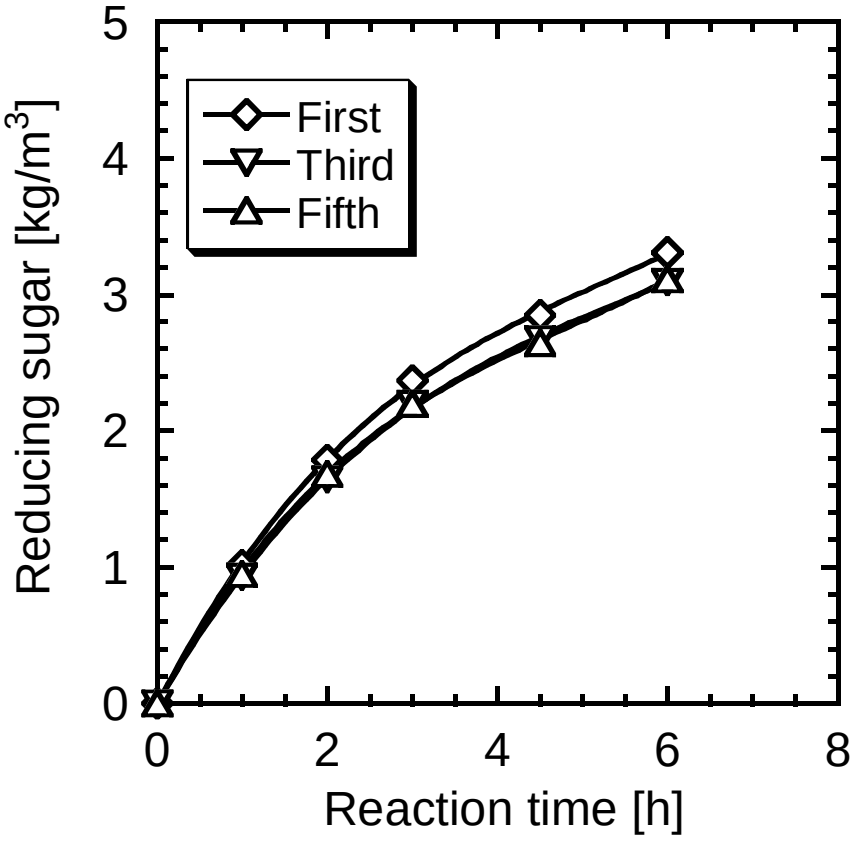


Fig. 2-15 Repeated chitosan hydrolysis by chitosanase immobilized on agar gel-coated multidisk impeller.

Chitosan solution: 5 kg/m^3
Impeller speed: 2 s^{-1}
Temperature: 35 ^{o}C

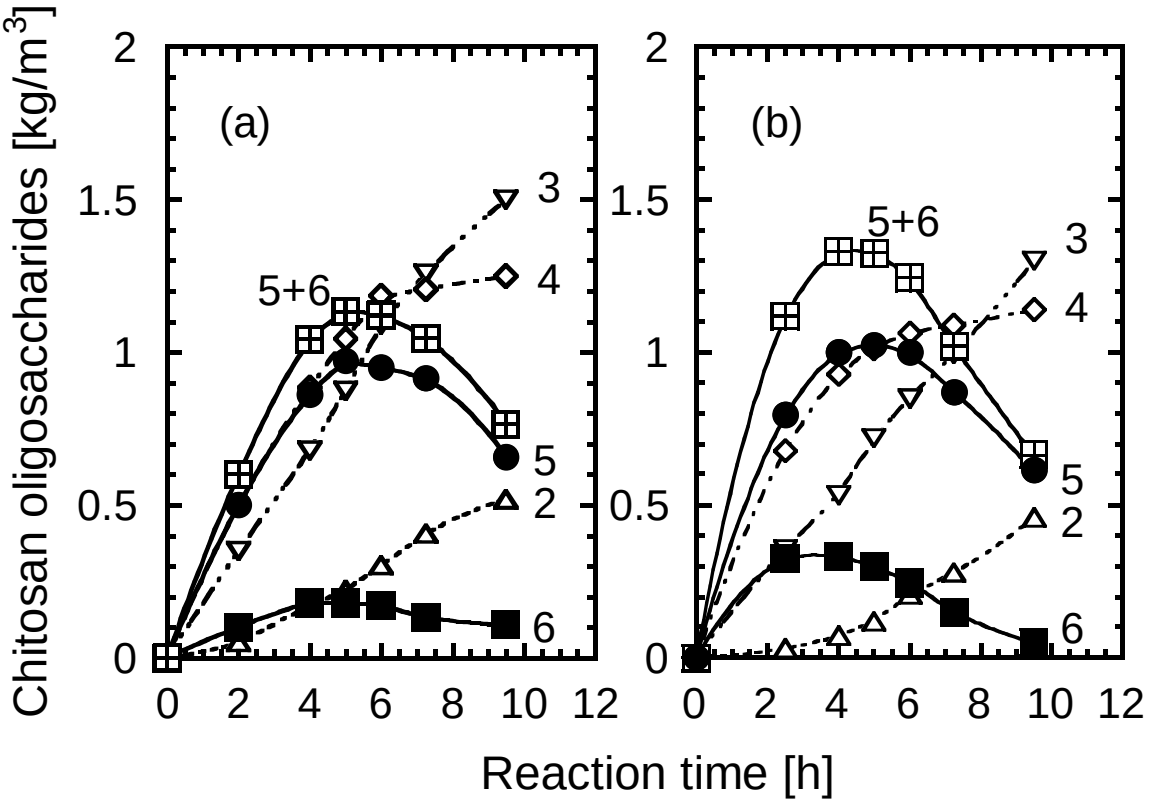


Fig. 2-16 Concentration time courses of chitosan oligosaccharides produced by chitosanase immobilized on the agar gel-coated multidisk impeller.

Chitosan solution: 5 kg/m^3
Impeller speed: (a) 1 and (b) 2 s^{-1}
Temperature: 35 ^{o}C

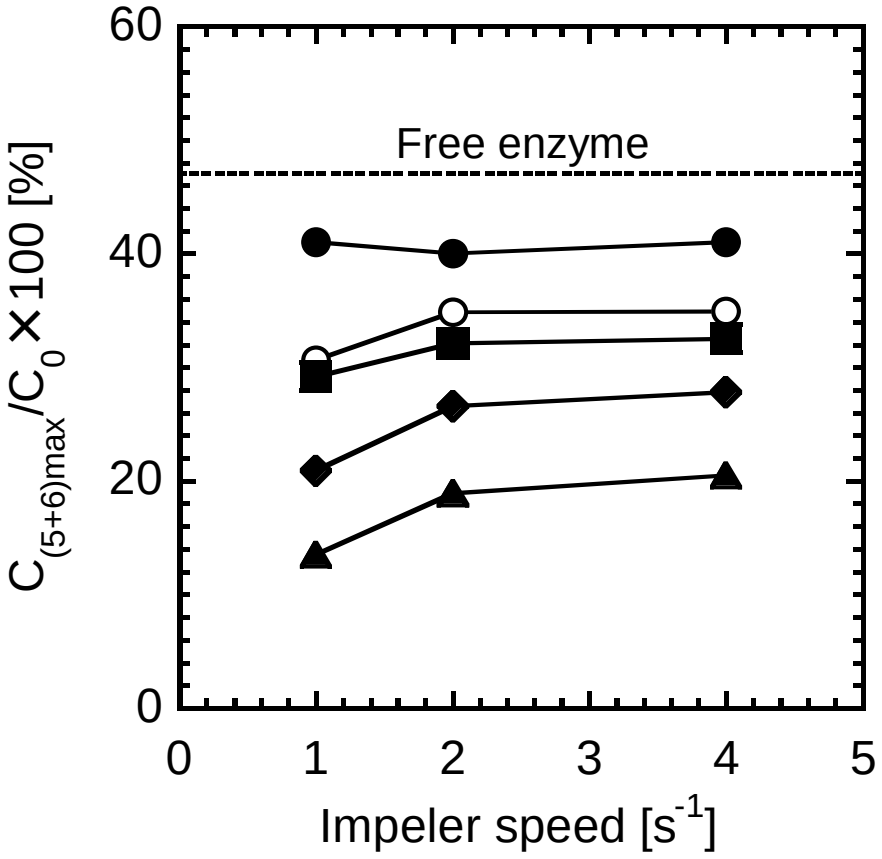


Fig. 2-17 Effect of agitating speed on the maximum yield of pentamer and hexamer by chitosanase immobilized on the agar gel-coated multidisk impeller.

Chitosan solution: 5 kg/m^3
Temperature: 35 ^{o}C

increasing the impeller speed from 2 to 4 s^{-1}, even at the higher observed specific activity. The same trend was observed at both chitosan concentrations (5 and 20 kg/m^3).In the range of a lower yield of target products, the mass-transfer rate should limit the overall production of intermediates such as pentamer and hexamer. Nevertheless, the increase in impeller speed did not improve the yield of target products. It is believed that the reason for the lack of improvement was that, owing to the high viscosity of the chitosan solution, the intensity of liquid turbulence near the enzyme support surface was not improved by the increased impeller speed.

At the lower observed specific activity (79 U/m^2), the impeller speed did not affect the maximum yield of target products. At this activity level, the reaction rate at the support surface would limit the overall production rate and the amount of intermediates produced. Then a higher yield of target products was obtained over a wide range of impeller speeds, and the yields approached the yield in the homogeneous reaction (free-enzyme system).

In this study, an immobilized enzyme bioreactor was newly developed. In order to promote mass transfer well cotton cloth was instead by agar gel using as the support for immobilization. An agar gel-coated multidisk impeller was used for immobilization of chitosanase and used for hydrolysis of chitosan. To obtain a high yield of intermediate, pentamer and hexamer, a larger mass transfer rate would be expected. Generally, for mixing a viscous liquid, a propeller type of impeller is often used. Chitosan solution is viscous and a high yield of target products, pentamer and hexamer could be obtained only at the larger mass transfer rate. So basing on the multidisk impeller, an agar gel-coated inclined multidisk impeller was prepared, which moved in a spiral and shown in Fig. 2-3. It had the same surface area with the multidisk impeller. It was expected that mass transfer of the substrate solution would be promoted better than the agar gel-coated multidisk impeller.

Using the agar gel-coated inclined multidisk impeller immobilizing chitosanase having various specific activities, hydrolysis reaction of chitosan was performed under 1, 2 and 4 s^{-1}. The results were shown in Fig. 2-18. As the similar to flat agar gel-coated

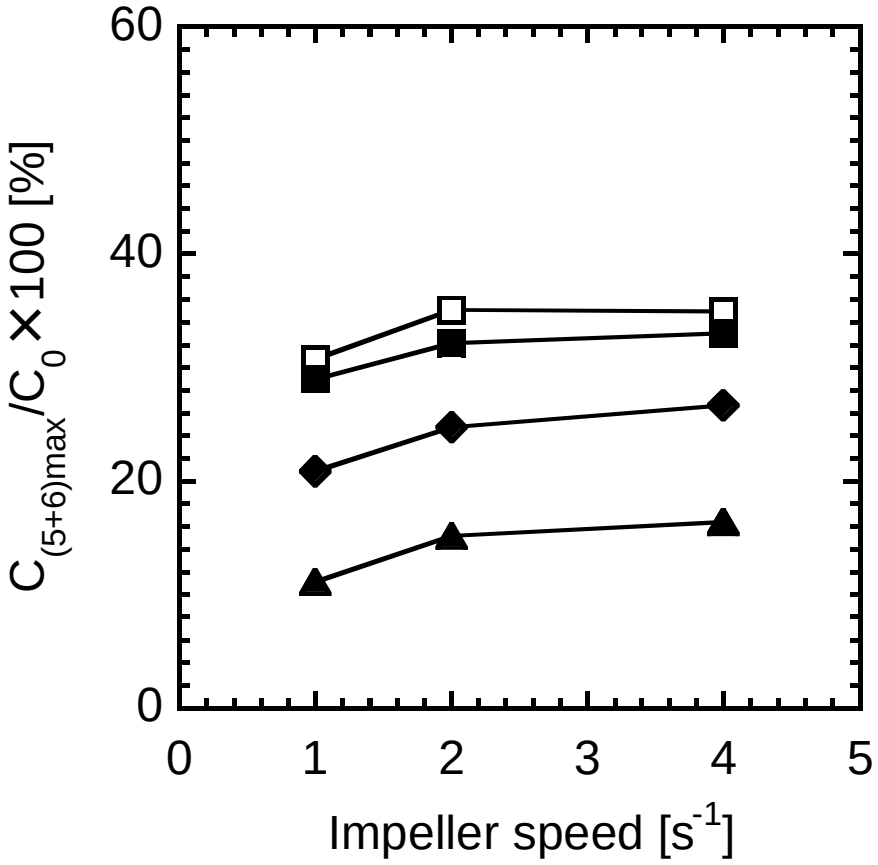


Fig. 2-18 Effect of agitating speed on the maximum yield of pentamer and hexamer by chitosanase immobilized on the agar gel-coated inclined multidisk impeller.

Chitosan solution: 5 kg/m^3
Temperature: 35 ^{o}C

multidisk impeller, the yields of target products at 2 and 4 s^{-1} were higher than the yields at 1 s^{-1}. And also, no significant increase in yield was obtained by increasing the impeller speed from 2 to 4 s^{-1}, even at a higher observed specific activity. The same trend was observed at both chitosan concentrations of 5 and 20 kg/m^3.

Although, the inclined multidisk impeller that was expected to realize better mass transfer than flat multidisk impeller, no significant difference in production of target substance was observed between two types of impeller.

2.3.3.4 Effect of activity at the support surface on the maximum yield of pentamer and hexamer

In above investigation of the effect of agitation on chitosan hydrolysis, it was indicated that the composition of oligosaccharides produced and the maximum yield of the target hexamer and pentamer were also affected by the immobilized enzyme activity at the support surface. Figure 2-19 shows the relationship between the maximum yield of target products and specific activity of immobilized enzyme at an impeller speed of 2 s^{-1}. The data obtained by using two types of impeller are plotted in the same figure. The maximum yield of the target products increased with decreasing surface enzyme activity. As the surface enzyme activity decreased, the reaction rate became lower. On the one hand, if the reaction rate was greater than the mass-transfer rate, intermediates of the hydrolysis reaction would exist mainly in a liquid film near the immobilized enzyme molecules and would be degraded successively to smaller oligosaccharides because the rate of supply of substrate was low. On the other hand, if the reaction rate became low and the mass-transfer rate was greater than the reaction rate, higher-molecular-weight substrates would exist along with intermediates near the enzyme because the supply of unreacted substrate would exceed the consumption of substrates at the support surface. Consequently, the yield of intermediates would approach that in homogeneous reaction (free-enzyme system).

The 20 kg/m^3 chitosan solution was more viscous than the 5 kg/m^3 solution, and no additional chitosan would dissolve in 0.1 mol/L acetic acid solution at 35 °C. It was

attempted to hydrolyze the 20 kg/m^3 chitosan solution at two temperatures, 35 and 50 °C. The data plotted at observed specific activities of 115 U/m^2 (open circle) and 320 U/m^2 (open square) in Fig. 2-19 were obtained using the same immobilized enzyme, the former at 35 °C and the latter at 50 °C, respectively. The activity was determined from the initial progress curve of hydrolysis measured by the modified Schales's method at each temperature. The maximum yield obtained at 50 °C decreased for the higher reaction rate, compared with that at 35 °C. These results indicated that the maximum yield of target products was determined by the surface enzyme activity, regardless of the initial substrate concentration and reaction temperature under high-agitation conditions (2 s^{-1}), whether it is the multidisk impeller or the inclined multidisk impeller. There were no significant difference between multidisk impeller and the inclined multidisk impeller.

It was reported that when enzyme was immobilized on agar gel, it was only immobilized on the surface of agar gel (Kuroiwa, 2005). It can be illustrated by the schematic figure shown in Fig. 2-20. It is different from that of cotton cloth (Fig. 2-13), where chitosanase was also immobilized in the internal part of support. For the reaction using agar gel, mass transfer would not be affected by the inner structure of agar gel. Thus, using agar gel as the support material, high yield of chitosan pentamer and hexamer can be obtained, while usage of cotton cloth resulted in little production of chitosan pentamer and hexamer.

2.3.3.5 Productivity of pentamer and hexamer

As discussed above, to obtain high yields of target products (pentamer and hexamer), the surface activity of immobilized chitosanase must be low. However, a low activity prolongs the reaction time. A plot of the relationship between the observed specific activity of immobilized enzyme and the time required to achieve the maximum yield of target products, $T_{(5+6)max}$, indicates that $T_{(5+6)max}$ was inversely proportional to

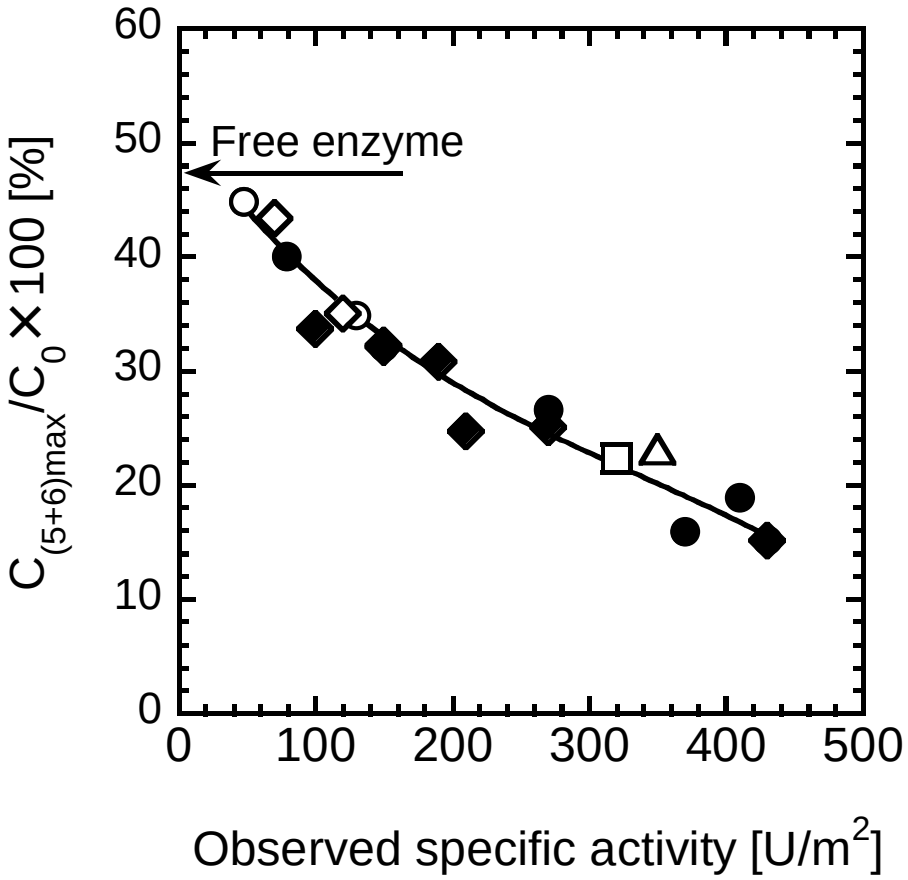


Fig. 2-19 Effect of observed specific activity on the maximum yield of pentamer and hexamer.

symbol	Substrate concentration kg/m^3	Reaction temperature °C	Impeller
●	5	35	Multidisk
○	5	50	Multidisk
□	20	50	Multidisk
◆	5	35	Inclined Multidisk
◇	20	50	Inclined Multidisk
△	20	50	Inclined Multidisk

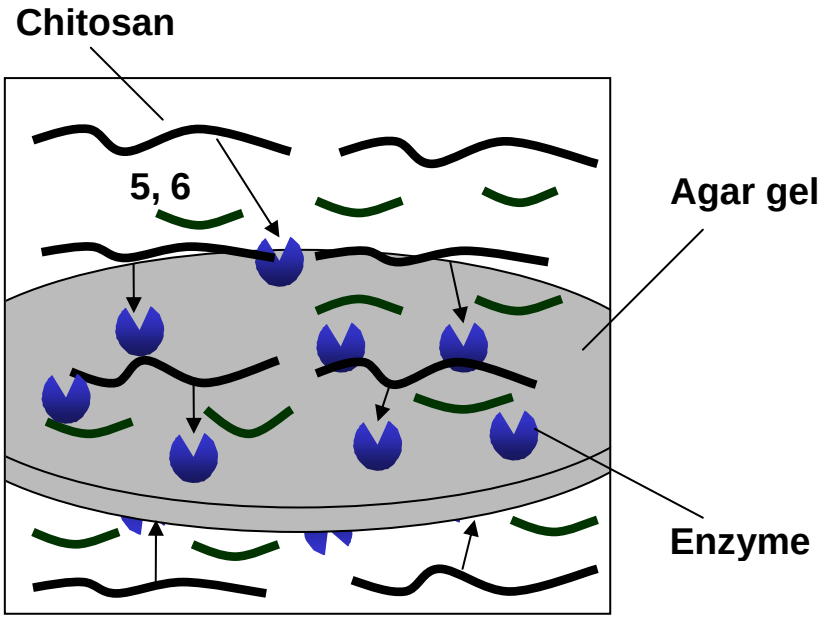


Fig. 2-20 Schematic illustrations of reaction mechanism of chitosan hydrolysis near surface of agar gel plate.

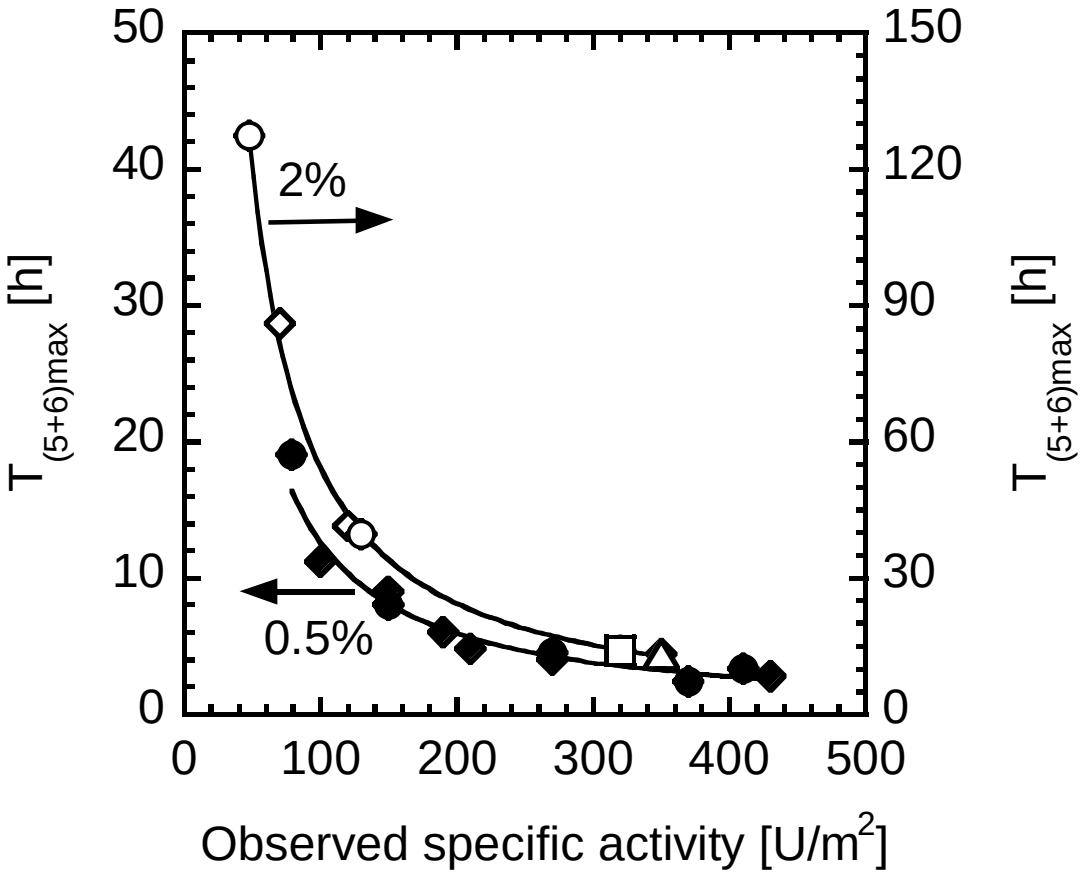


Fig. 2-21 Relationship between observed specific activity and time required for obtaining maximum yield of pentamer and hexamer.
Symbols are the same as in Fig. 2-19.

the observed specific activity (Fig. 2-21). Comparison of the two chitosan concentrations (20 and 5 kg/m^3) reveals that hydrolysis of the 20 kg/m^3 solution required a longer reaction time in proportion to the initial substrate concentration. That is, the concentration was 4 times as high; the reaction time was also 4 times as long. To maximize the production of target products, it is desirable that a high yield of highly concentrated product be obtained in a short time. However, shortening $T_{(5+6)max}$ by increasing the surface activity of immobilized chitosanase is not compatible with the production of target substances in high yield and at a high concentration, as discussed above. To evaluate the productivity of batch operations, the time required for the preparation of raw material and for recovery of products must generally be considered, but this time might be independent of the surface enzyme activity. To determine the optimal surface enzyme activity for the production of the target substances, the productivity for the reaction period alone was evaluated using $C_{(5+6)max}/T_{(5+6)max}$ as an index (Fig. 2-22). The results shown in the figure clearly indicated that an observed specific activity of about 340 U/m^2 gave the maximum productivity, regardless of the initial substrate concentration. When the concentration of substrate increased, $T_{(5+6)max}$ became longer but the concentration of target products became higher. This might be the reason for the similar productivities observed at chitosan concentrations of 20 and 5 kg/m^3. At a surface enzyme activity of 340 U/m^2, the maximum yield of the target pentamers and hexamers was estimated to be about 22% from Fig. 2-19. This value is higher than that obtained by means of chemical hydrolysis (the reported total yield of pentamers and hexamers is about 15% in the hydrolysis of 50 kg/m^3 chitosan in hydrochloric acid solution (Horowitx *et al*., 1957)). To obtain a higher yield of the target products, the surface activity of immobilized enzyme must be lower, with the concomitant sacrifice in productivity caused by the increase in $T_{(5+6)max}$. A surface enzyme activity lower than 160 U/m^2 afforded a high yield (>30%, Fig. 2-19). In practice, to optimize the reactor performance a compromise between the yield of products and the productivity of the process must be found. The results shown in Figs.

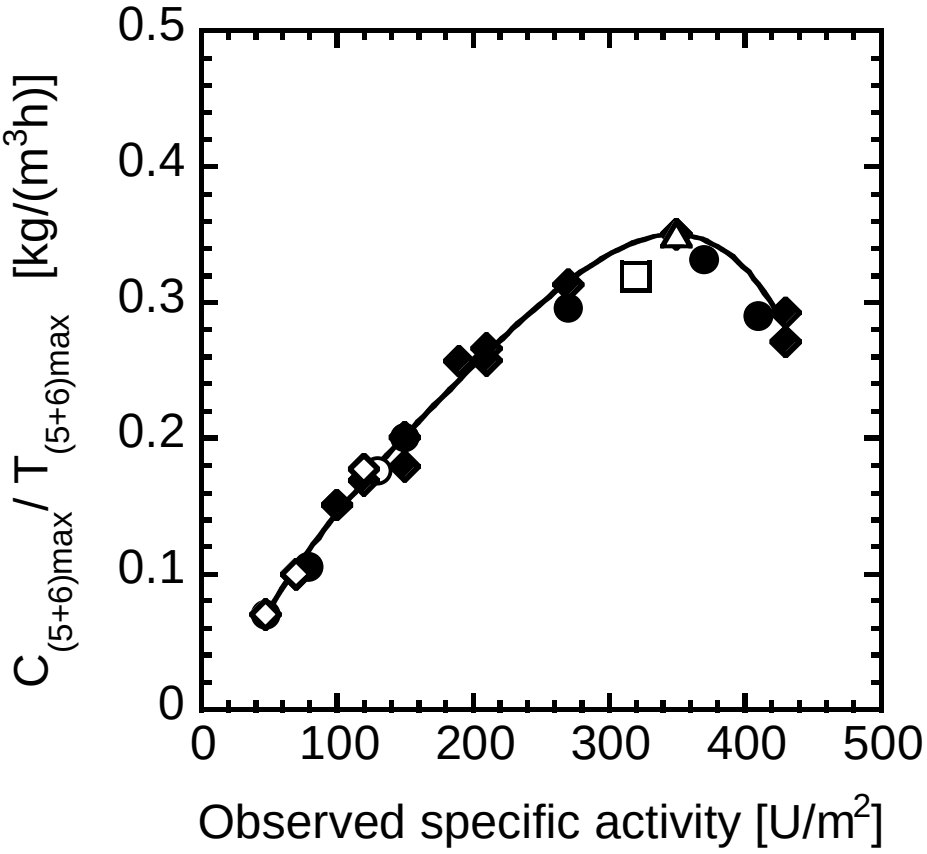


Fig. 2-22 Productivity of pentamer and hexamer under various observed specific activities of immobilized chitosanase. Symbols are the same as in Fig. 2-19.

2-19 and 2-22 would be useful for the determination of the optimum operation conditions. In addition, increasing the surface area for immobilization of the enzyme (for instance, by increasing the number of impeller disks) could improve the productivity of the reactor without decreasing the yield of the target products.

2.4 Conclusions

The new bioreactors for hydrolyzing highly viscous chitosan solutions for the production of physiologically active oligosaccharides (pentamer and hexamer) were developed. Cotton cloth and agar gel were used as supports of immobilization, respectively. Chitosan was hydrolyzed by free enzyme and immobilized enzyme for producing chitosan oligosaccharides. The production efficiency of chitosan oligosaccharides, especially pentamer and hexamer was evaluated. Following conclusions were obtained.

(1) The reactor with a cotton cloth multidisk impeller directly immobilized chitosanase was examined for production of chitosan oligosaccharides. The yield of pentamer and hexamer was lower than that by free enzyme. SEM observation showed that there were many narrow spaces between cotton fibers. This microstructure of cotton cloth might cause the mass transfer limitation inside the cotton cloth. It was concluded that the cotton cloth is not suitable as the support material of immobilized chitosanase for producing high yield of chitosan pentamer and hexamer.

(2) The reactor equipped with agar gel-coated multidisk impellers, where chitosanase was directly immobilized onto the agar gel-coated disks by the multipoint attachment method, was developed. The stability of immobilized enzyme was confirmed by five repetitions of the batch reaction. Using this new reactor, 9.0 kg/m^3 of pentamer and hexamer was produced by hydrolyzing of 2% chitosan solution as substrate solution. The maximum yield of pentamer and hexamer

reached to 45%. This value of the yield is the same as the maximum yield performed in the batch hydrolysis reaction using free-enzyme.

(3) The productivity of pentamer and hexamer in the new reactor was evaluated using two indexes, *i.e.*, the maximum yield of target products and the reaction time required for the maximum yield. The optimal value of the observed specific activity of immobilized enzyme was found for maximizing the productivity of pentamer and hexamer.

Chapter 3 Production of Chitosan Oligosaccharides at High Concentration by Chitosanase Directly Immobilized on the Agar Gel-Coated Multidisk Impeller

3.1 Introduction

Production of target substances at high concentrations saves time, reduces the cost of condensation of the substances, and increases productivity. A high concentration of product requires a high concentration of the substrate raw material. However, because chitosan solutions are highly viscous even at low concentrations, the use of a high-concentration chitosan solution during hydrolysis would limit the operability of a bioreactor. The saturation concentration of chitosan in 0.1 mol/L acetic acid solution at 35 °C and at pH 5–6 is 20 kg/m^3, so chitosan solutions with concentrations higher than 20 kg/m^3 cannot be prepared directly. A method for the production of chitosan oligosaccharides, especially pentamer and hexamer, with immobilized enzyme and with chitosan solutions more concentrated than 20 kg/m^3 has not yet been developed.

In Chapter 2, the bioreactor of agar gel-coated multidisk impellers with directly immobilized chitosanase was developed for the production of chitosan oligosaccharides. The chitosanase immobilized on the impeller was highly stable and produced pentamer and hexamer at a concentration of 9 kg/m^3 from a 20 kg/m^3 chitosan solution.

In this chapter, the use of the bioreactor described above to produce chitosan pentamer and hexamer at a higher concentration was investigated. The chitosan concentration was increased by stepwise addition of chitosan powder as the hydrolysis reaction proceeded. Then lactic acid was used to solubilize the chitosan powder in stead of acetic acid because lactic acid has higher solubility of chitosan and has no odor. The solubility of chitosan also increases with decreasing pH of the solution. But as the pH affects the enzyme activity , chitosan powder was added while controlling the pH. The pH range which could be reduced was determined from the relation of the enzyme activity and pH. The effective timing of powder addition and the productivity of target products in the hydrolysis of high-concentration chitosan were also investigated. Finally,

the stability of the immobilized enzyme in highly viscous solution was determined from the repeated use of it in the production of chitosan pentamer and hexamer at high concentration.

3.2 Materials and methods

3.2.1 Enzyme

Chitosanase used was the same as described in 2.2.1.

3.2.2 Substrate

Chitosan solutions with concentrations of 5 and 20 kg/m^3 were prepared as follows. Chitosan powder (1.5 or 6 g) was added to 100 mL of deionized water and dissolved with 70 mL of 1 mol/L lactic acid by stirring. The final pH was adjusted to 5.6 with 5 mol/L NaOH solution, and the volume of the solution was brought to 300 mL to afford either a 5 or 20 kg/m^3 chitosan solution. A 100-mL portion of the solution was used in each hydrolysis experiment to assay the activity of the immobilized enzyme and to produce oligosaccharides.

A 50 kg/m^3 chitosan solution was prepared by adding the chitosan powder directly to the reactor stepwise after the start of the hydrolysis of the 20 kg/m^3 chitosan solution. One gram of chitosan powder was added each time. The timing of addition was determined by monitoring the torque required to agitate the reaction solution. The pH of the starting 20 kg/m^3 chitosan solution was about 3.5 (that is, not adjusted to 5.6). Because the pH of the reaction solution increased with each addition of chitosan powder, 0.2 mL of lactic acid was also added with the powder when the pH of the reaction solution rose above ca. 4. Chitosan powder was added a total of three times, at which point the final chitosan concentration reached 50 kg/m^3.

3.2.3 Reactor equipped with the agar gel-coated multidisk impeller

The agar-coated multidisk impeller was shown in 2.2.3.2.

A schematic illustration of the reaction unit with impeller was shown in Fig. 3-1. The impeller was driven by a variable-speed motor and a torque meter was set on it. And a pH probe was inserted into the reactor. The reactor was made of plastic (diameter, 56 mm; height, 115 mm). The working volume of the reactor was 100 mL. The reactor was placed in a thermostatted bath.

3.2.4 Immobilization of chitosanase on agar gel-coated multidisk impeller

The method was the same as described in 2.2.4.

3.2.5 Chitosan hydrolysis by free chitosanase

Hydrolysis reaction of 5 and 20 kg/m^3 chitosan solution by free enzyme was the same as described in 2.2.5. For the hydrolysis of 20 kg/m^3 chitosan, 126 U of chitosanase was used for 100mL of reaction solution.

3.2.6 Production of chitosan oligosaccharides at high concentration by free chitosanase

For the hydrolysis reaction of 50 kg/m^3 chitosan by free chitosanase, the substrate concentration was increased by the method of stepwise addition of chitosan powder. 126 U of chitosanase was used for 100 mL of reaction solution.

3.2.7 Chitosan hydrolysis at high concentration by chitosanase immobilized on the agar gel-coated multidisk impeller

Production of chitosan oligosaccharides at high concentration using the reactor equipped with the agar gel-coated multidisk impeller immobilizing chitosanase was carried out as described in 2.2.6. The substrate solution of 50 kg/m^3 was prepared by the stepwise addition of chitosan powder.

3.2.8 Analysis

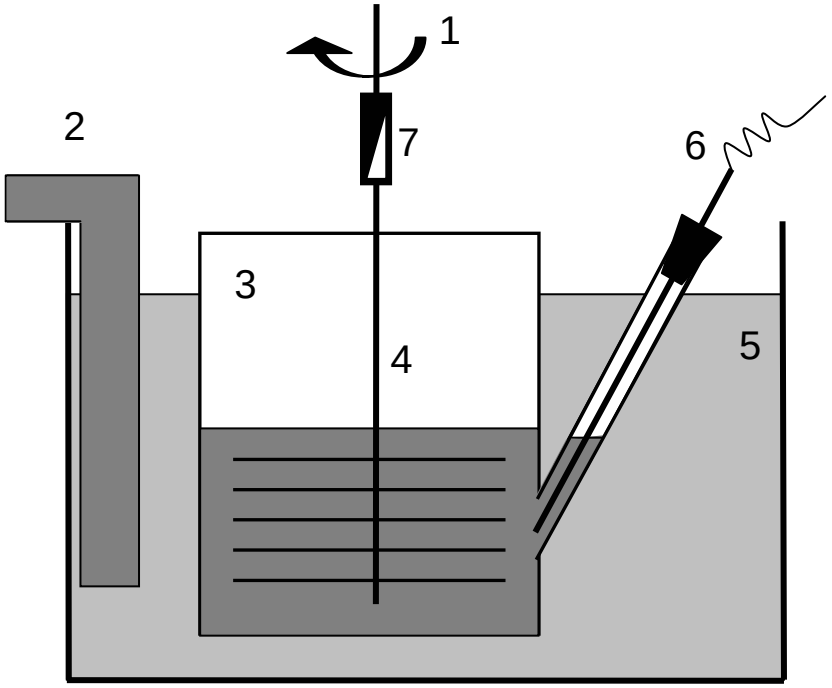


Fig. 3-1 Experimental apparatus.
1, motor; 2, heater; 3, reactor; 4, impeller;
5, water bath; 6, pH probe; 7, torque meter.

3.2.8.1 Determination of reducing sugar concentration

Concentration of reducing sugar was measured by the modified Schales's method, as described in 2.2.7.2.

3.2.8.2 Fractional determination of chitosan oligosaccharides

Concentrations of chitosan oligosaccharides were measured by HPLC as described in 2.2.7.3.

3.3 Results and discussion

3.3.1 Effect of acid type on the activity of chitosanase

The average molecule weight of chitosan used in this study was 370,000, and it was difficult to be dissolved in acetic acid exceeding 20 kg/m^3. So far, acetic acid was used to dissolve chitosan. In this part, in order to produce chitosan oligosaccharides at higher concentration, lactic acid was used to dissolve chitosan instead of acetic acid. As a result, chitosan could be dissolved in a lactic acid solution more easily than in an acetic acid solution, and the time necessary for preparing 20 kg/m^3 chitosan solution was shortened. Furthermore, lactic acid has no odor, whereas acetic acid smells strong. Usage of mild chemicals is preferable especially in the food processing. In order to examine whether lactic acid is suitable for producing chitosan oligosaccharides, effects of it on the activity of chitosanase and the production time course of chitosan oligosaccharides in batch reaction were investigated using free chitosanase.

Chitosan dissolved in lactic acid was hydrolyzed by free enzyme, and the time course of the reducing sugar produced was shown in Fig. 3-2. For comparison, the result obtained in acetic acid was also shown in this figure. From Fig. 3-2, it was shown that the reaction rate in lactic acid solution was almost the same as that in acetic acid solution. The concentration time course of each chitosan oligosaccharides in lactic acid were shown in Fig. 3-3. The maximum concentration of chitosan pentamer and hexamer was 2.5 kg/m^3 and the yield to the initial substrate (5 kg/m^3) based on the weight was

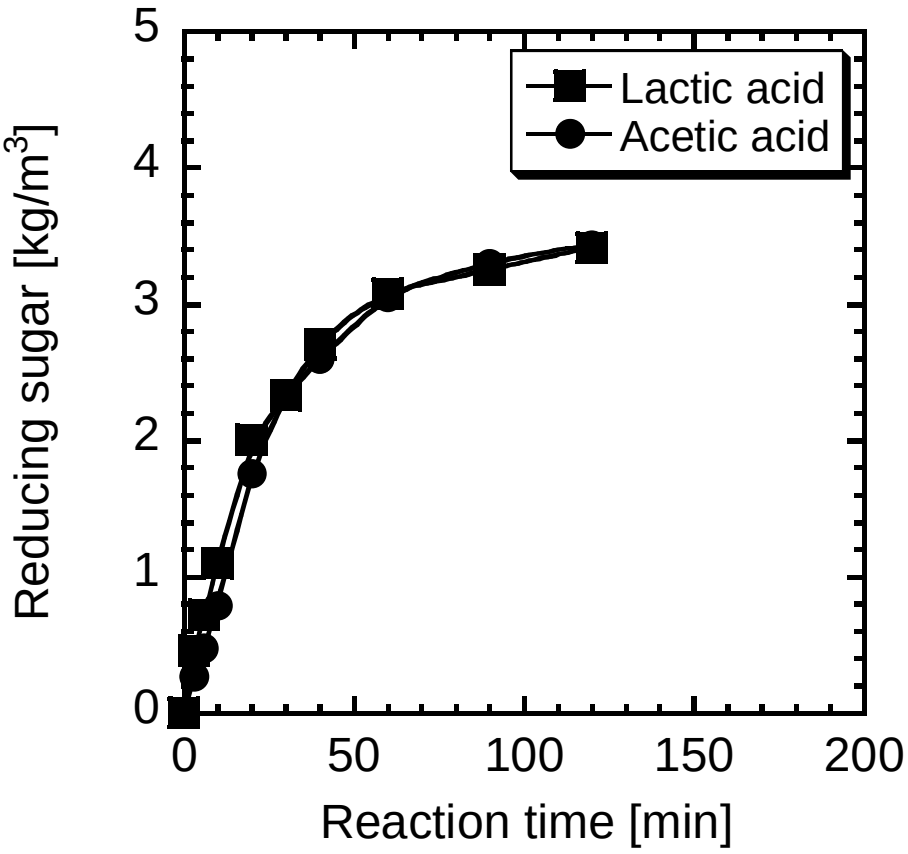


Fig. 3-2 Comparison of chitosan hydrolysis rate by free chitosanase in acetic acid solution and in lactic acid solution.

Enzyme concentration: 38 U/100mL
Chitosan solution: 5 kg/m^3
Temperature: 35 ^{o}C

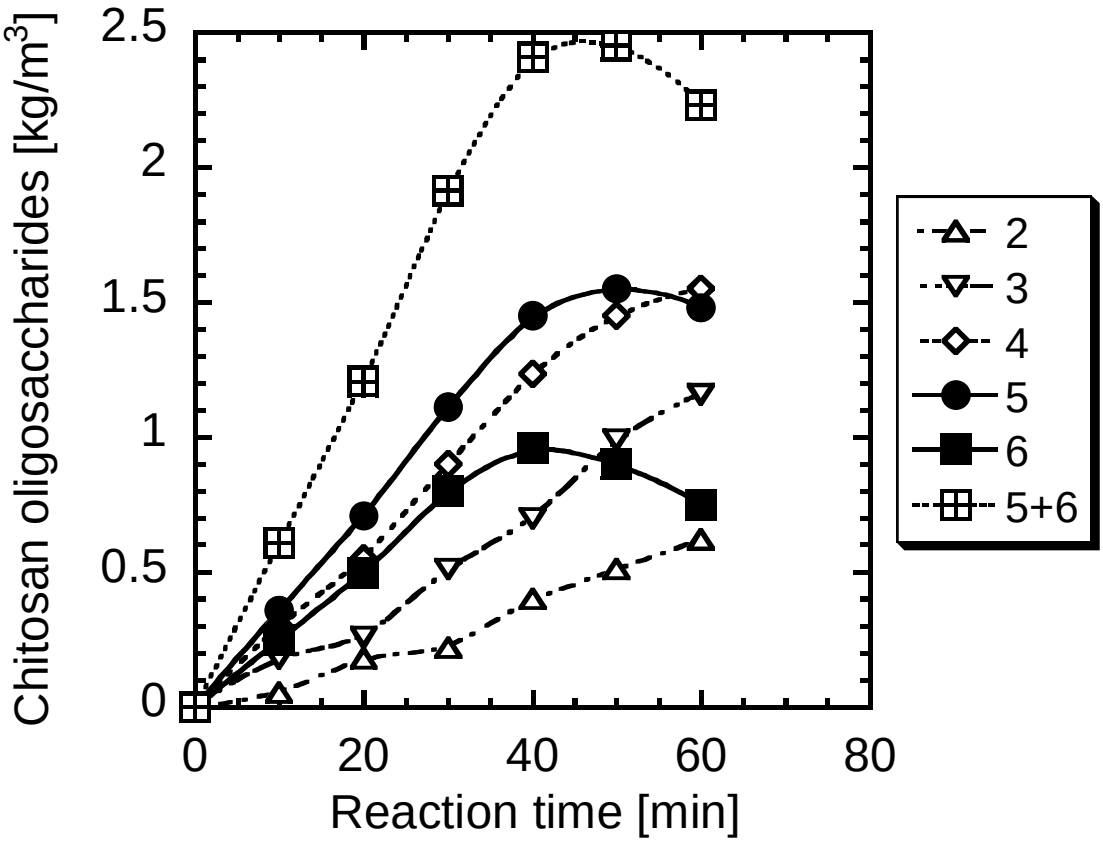


Fig. 3-3 Typical concentration time courses of chitosan oligosaccharides produced by free chitosanase.

Enzyme concentration: 38 U/100 mL
Chitosan solution: 5 kg/m^3
(Prepared using lactic acid)
Temperature: 35 ^{o}C

50%. It was the same as that in acetic acid as shown in Fig. 2-6, where the maximum concentration of pentamer and hexamer was 2.4 kg/m^3 and the yield was 48%. In addition, a similar time course of each chitosan oligosaccharides was also obtained in the two different acid solutions.

Considering no difference in the hydrolysis of chitosan by chitosanase between lactic acid and acetic acid, as described above and from higher solubility and odorless, lactic acid was used for dissolving chitosan to produce chitosan oligosaccharides at higher concentration instead of acetic acid.

3.3.2 Effect of pH on the activity of immobilized chitosanase

In this study, the concentration of chitosan was increased by stepwise addition of chitosan powder as the hydrolysis reaction proceeded. When the chitosan powder dissolved in the reaction solution, the pH of the solution increased. Because the solubility of chitosan decreases with increasing pH, keeping the pH of the reaction solution low is preferable for solubilizing the chitosan powder added. However, the activity of the enzyme also depends on pH. Because both the chitosan solubility and the enzyme activity depend on pH, we set about determining how the pH range varied during the hydrolysis reaction.

Ichikawa *et al.* (2002) reported that the optimum pH for hydrolysis of chitosan by immobilized chitosanase on agar gel in acetic acid is pH 4–6, whereas the optimum pH for free chitosanase is pH 5.6. In this study, lactic acid was used to dissolve the chitosan, because it has higher chitosan solubility and is odorless. To determine the effect of pH on the activity of immobilized chitosanase for the hydrolysis of chitosan dissolved in lactic acid, we investigated the activity of immobilized chitosanase at various pH values (Fig. 3-4). The results indicated that the activity of the immobilized chitosanase was higher at pH 4–6, as was the case for acetic acid.

To facilitate the dissolution of the chitosan powder, the pH of the reaction solution must be as low as possible in the range over which the hydrolysis reaction can proceed. On the basis of the data shown in Fig. 3-4, we decided that the pH of the solution could

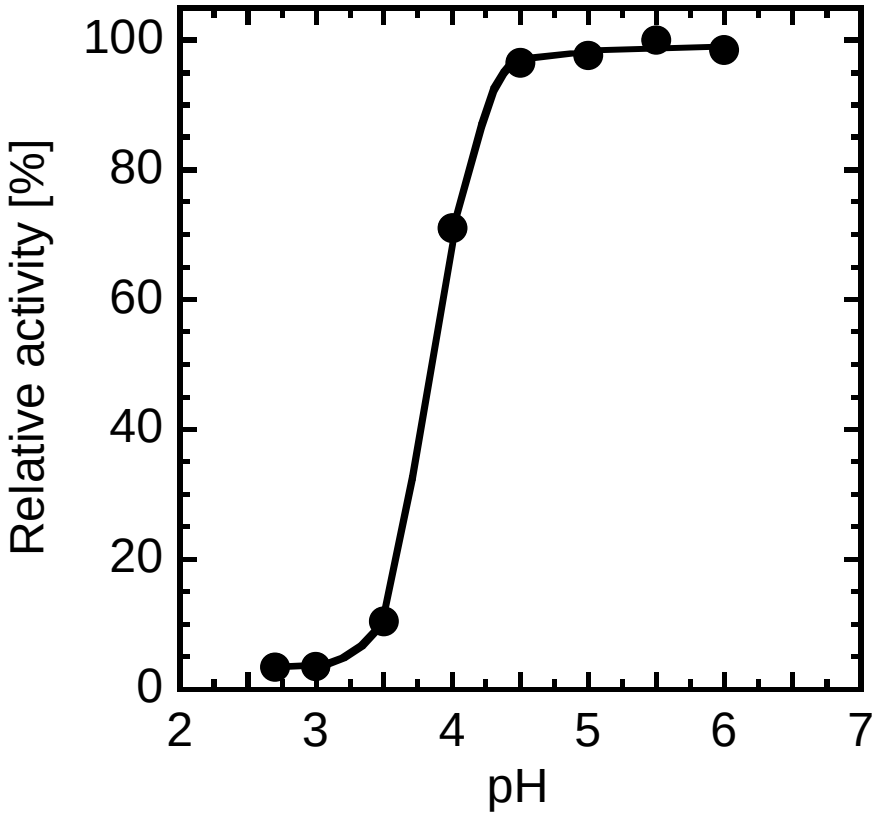


Fig. 3-4 pH dependence of activity of immobilized chitosanase.

Chitosan solution: 5 kg/m^3
Impeller speed: 2 s^{-1}
Temperature: 35 ^{o}C

be reduced to 3.5, at which value the activity of the immobilized chitosanase was about one-tenth of the highest activity, at pH 4.5–6. By means of the following experiment, we confirmed that when the pH was raised again, the activity of the immobilized chitosanase could be recovered. The hydrolysis reaction was started at pH 5.6. After 40 min, the pH of the reaction solution was adjusted to 3.3 with lactic acid and kept there for 40 min. Then the pH was increased to 4.5 with 5 mol/L NaOH solution and allowed to remain there for 40 min. The time courses of reducing sugar concentration and pH in the reaction solution are shown in Fig. 3-5. During the initial 40-min period, reducing sugar was produced rapidly because of the high activity of the immobilized enzyme at pH 5.6. After the pH was lowered to 3.3, the reaction rate became slower because the pH was not optimum for the activity of immobilized chitosanase. After the pH was increased from 3.3 to 4.5, however, the reaction rate recovered to the same level observed in the initial stage at pH 5.6.

3.3.3 Hydrolysis of high-concentration chitosan by free chitosanase

In order to know the reaction profile and maximum yield of target product (pentamer and hexamer) obtained in the hydrolysis of high concentration chitosan prepared with lactic acid solution, 20 and 50 kg/m^3 of chitosan solution was hydrolyzed by free chitosanase. The concentration time course of chitosan oligosaccharides in the hydrolysis of 20 kg/m^3 chitosan was shown in Fig. 3-6. The maximum concentration of pentamer and hexamer was obtained at 80 minutes. The maximum concentration of pentamer and hexamer was 10.3 kg/m^3. The yield to the initial substrate was 52%, which was almost the same as that in the hydrolysis of 5 kg/m^3 chitosan as shown in Fig. 2-6.

The chitosan solution of 50 kg/m^3 was prepared by the stepwise addition of chitosan powder while proceeding the hydrolysis reaction. The starting concentration of chitosan was 20 kg/m^3. In order to increase the chitosan concentration stepwisely, one gram of chitosan powder was added into 100 ml of the reaction solution in each addition when the no more increase of the viscosity of the reaction solution was confirmed.

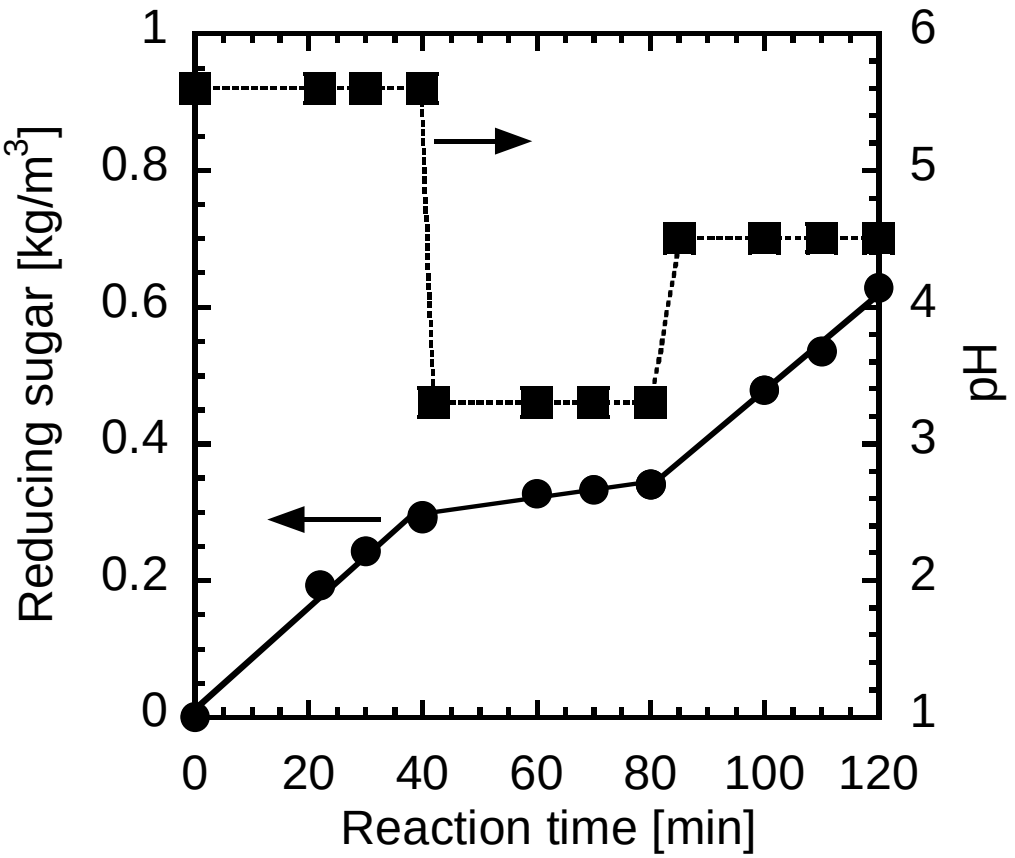


Fig. 3-5 Activity recovery of immobilized chitosanase by pH change.

Specific activity: 175 U/m^2
Chitosan solution: 5 kg/m^3
Impeller speed: 2 s^{-1}
Temperature: 35 ^{o}C

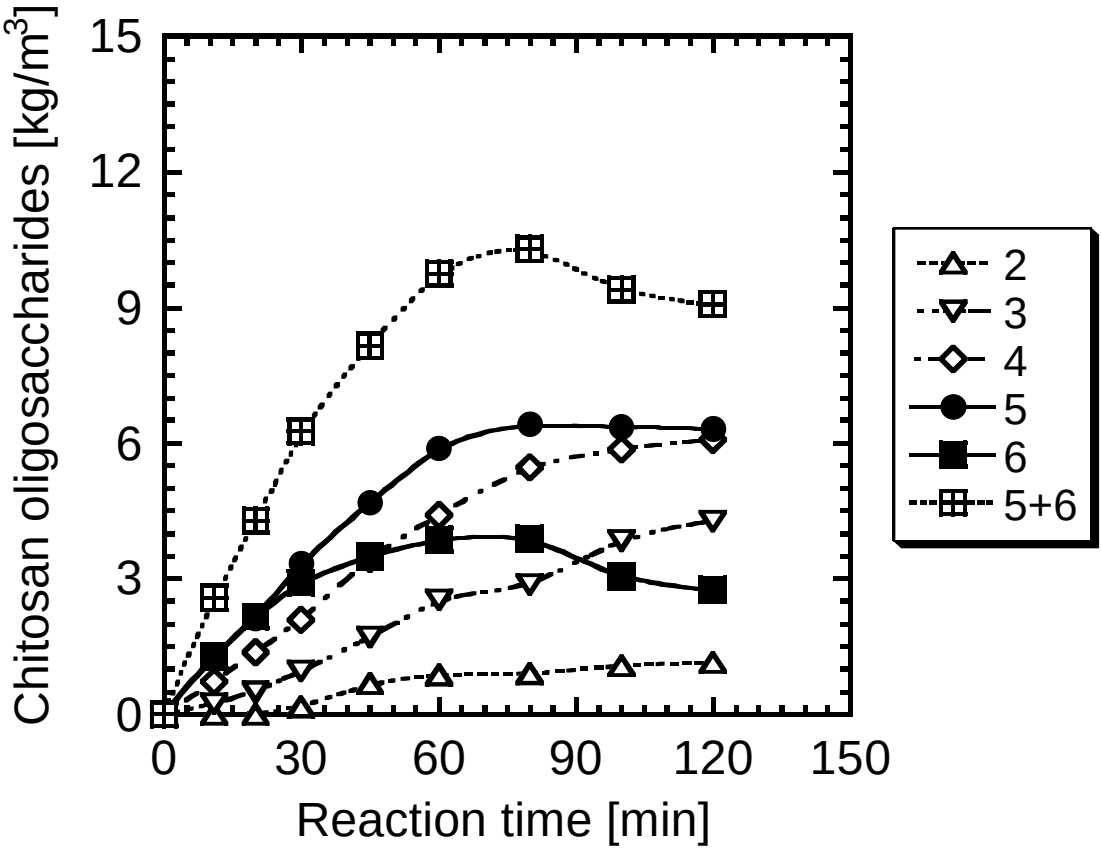


Fig. 3-6 Time courses of chitosan oligosaccharide concentrations in 20 kg/m^3 chitosan hydrolysis by free enzyme.

Enzyme concentration: 126 U/100mL
Temperature: 35 ^{o}C

Chitosan powder was added totally three times at 4, 13 and 30 minutes after the beginning of the reaction, and then the final chitosan concentration reached to 50 kg/m^3.

Chitosan dissolves more easily in lower pH, but the pH of solution increases with dissolving the chitosan powder. So the pH of the reaction solution was kept at about 4.5 by using lactic acid until the next addition. Time courses of the concentrations of each chitosan oligosaccharides produced were shown in Fig. 3-7. The maximum concentration of pentamer and hexamer produced was 25.8 kg/m^3 and the yield to the initial substrate (50 kg/m^3) was about 52%. This maximum yield of target products is slightly higher than those obtained in the hydrolysis reaction using 5 and 20 kg/m^3 chitosan as shown in Fig. 2-6 and Fig. 3-6, respectively. From this, it is indicated that the stepwise addition of chitosan powder can realize the high concentration of chitosan and is very effective for the production of the chitosan pentamer and hexamer.

3.3.4 Effect of addition timing in stepwise addition of chitosan powder on the production of pentamer and hexamer

In a heterogeneous reaction system such as an immobilized enzyme reaction, the intensity of agitation is an important determinant of the reaction rate and mass transfer rate, especially in viscous solutions. The rates of both reaction and mass transfer remarkably affect the yield of chitosan oligosaccharides (Kuroiwa *et al.*, 2002). In Chapter 2, we investigated on the effect of impeller speed on the yield of chitosan oligosaccharides in the hydrolysis of 5 and 20 kg/m^3 chitosan solutions using the same reactor as used in this study. No significant increase in yield was obtained when the impeller speed was increased from 2 to 4 s^{-1}, although at slower impeller speeds the yield of target products did increase with increasing impeller speed. Because the chitosan solution we used in this study was more concentrated and more viscous than the 20 kg/m^3 solution, the impeller speed was set at 4 s^{-1}. Impeller speeds higher than 4 s^{-1} caused the solution to rise along the impeller shaft, and then the solution could not be agitated well.

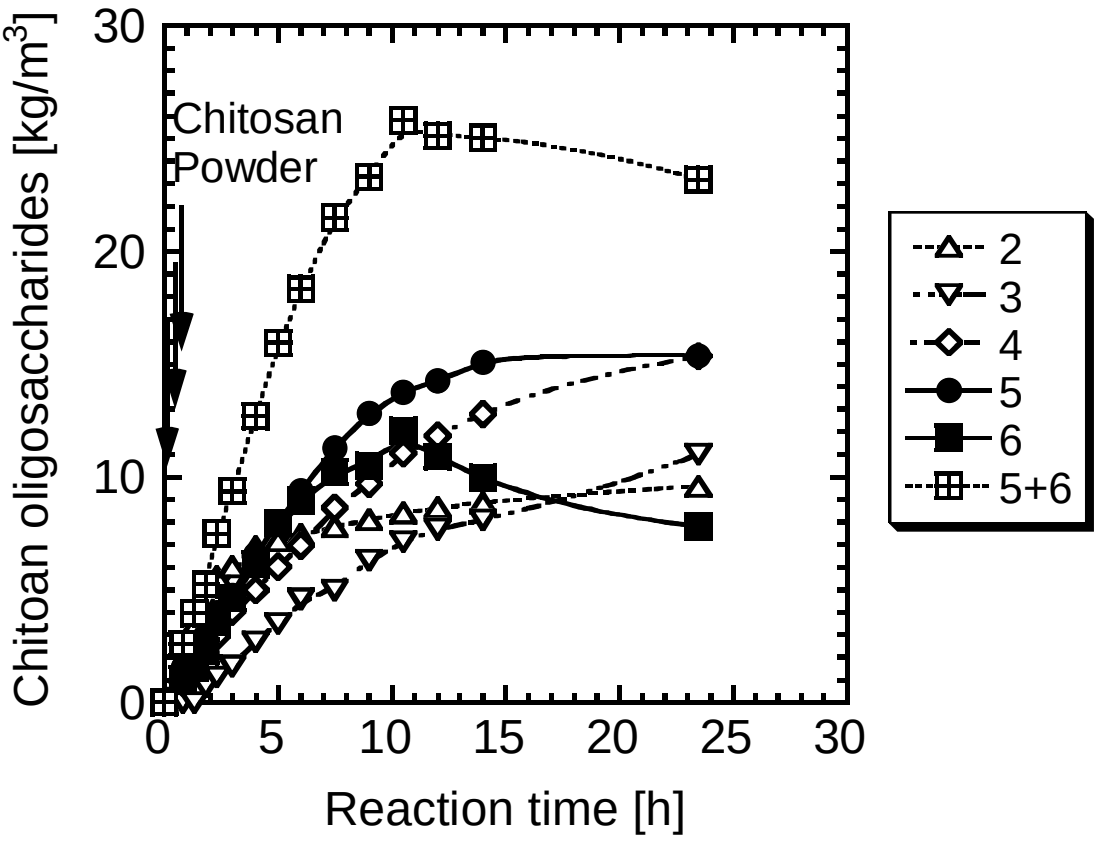


Fig. 3-7 Time courses of chitosan oligosaccharide concentrations in hydrolysis of high concentration chitosan by free chitosanase. Chitosan concentration was increased from 20 to 50 kg/m^3 by stepwise addition of chitosan powder.

Enzyme concentration: 126 U/100mL
Addition time: 4 min, 13 min, 30 min
Temperature: 35 ^{o}C

Chitosan solution is highly viscous even at low concentration, and the saturation concentration is about 20 kg/m^3 at pH 5–6 and 35 °C in lactic acid solution. Therefore, preparing a solution with a concentration exceeding 20 kg/m^3 in one step is difficult. In this study, stepwise addition of chitosan powder enabled a higher concentration chitosan solution, and the pH of the reaction solution was temporarily lowered to facilitate the dissolution of the added chitosan powder. The timing of powder addition was determined from the change in the torque necessary for agitating the reaction solution, because the torque reflects the viscosity change in the reaction solution: the viscosity increases as the chitosan powder dissolves, and then decreases as the chitosan is hydrolyzed.

To determine the appropriate timing for powder addition, we added chitosan powder consecutively by one of two methods: a long-interval method and a short-interval method. In the long-interval method, the powder was added when the torque decreased to half of the maximum value observed after the previous addition (Fig. 3-8a). In the short-interval method, the new powder was added when the torque reached its maximum after the previous addition (Fig. 3-8b). At the addition stage, the pH was adjusted to ca. 4 with lactic acid. When the final chitosan concentration reached 50 kg/m^3, the pH was adjusted to 4.5–5.0.

The temporal variation in the concentration of chitosan oligosaccharides produced by the two methods of stepwise addition is shown in Fig. 3-9a and Fig. 3-9b. The maximum concentrations of the target pentamer and hexamer were 10.6 kg/m^3 for the long-interval method and 14.9 kg/m^3 for the short-interval method. In the long-interval method (a), a broad peak with lower pentamer and hexamer concentrations was obtained. In contrast, in the short-interval method (b), a narrower peak with higher total concentration of the target products was obtained. These results suggest that the homogeneity of the substrate is important for production of the target products at a higher concentration. That is, because pentameric and hexameric chitosan oligosaccharides are intermediates of chitosan hydrolysis, the presence of substrates and products with widely different hydrolysis histories lowers the yield of the target

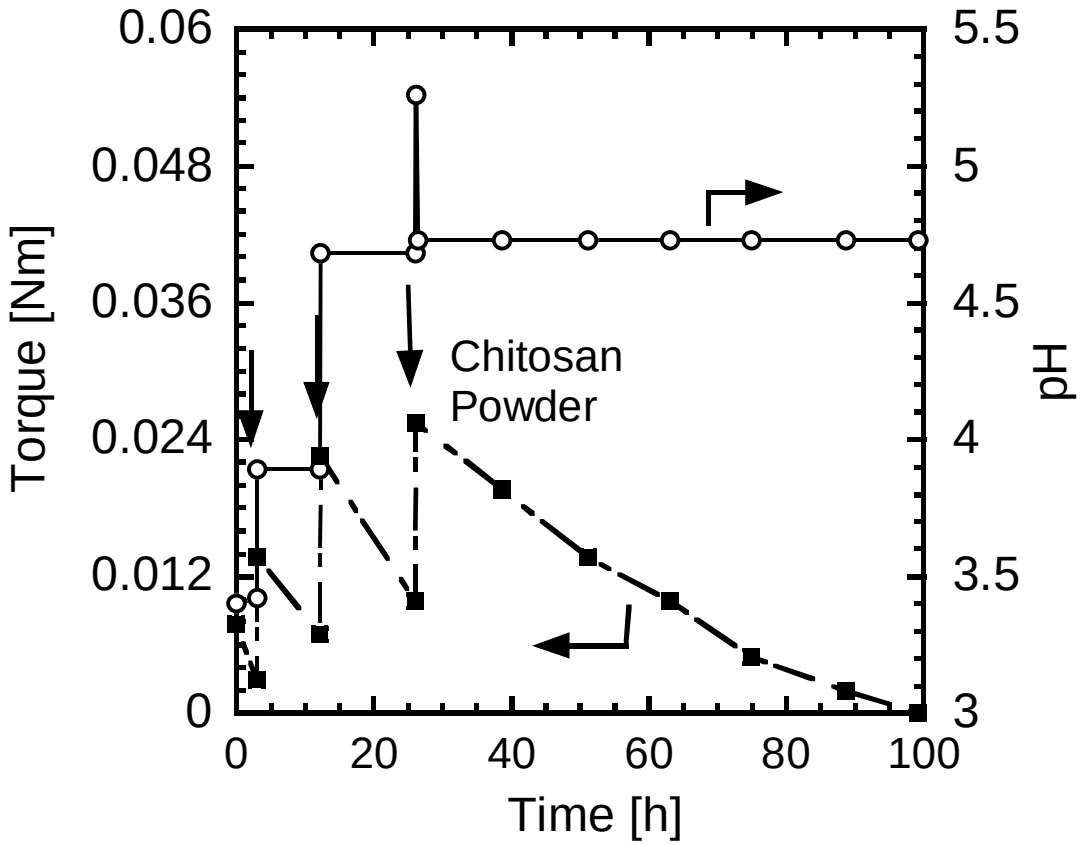


Fig. 3-8 (a) Changes in pH and torque values in 50 kg/m^3 chitosan hydrolysis by immobilized chitosanase. Chitosan concentration was increased from 20 to 50 kg/m^3 by adding the chitosan powder three times with long-interval method. Arrows show the addition time of chitosan powder.

Specific activity: 307 U/m^2
Impeller speed: 4 s^{-1}
Temperature: 35 ^{o}C

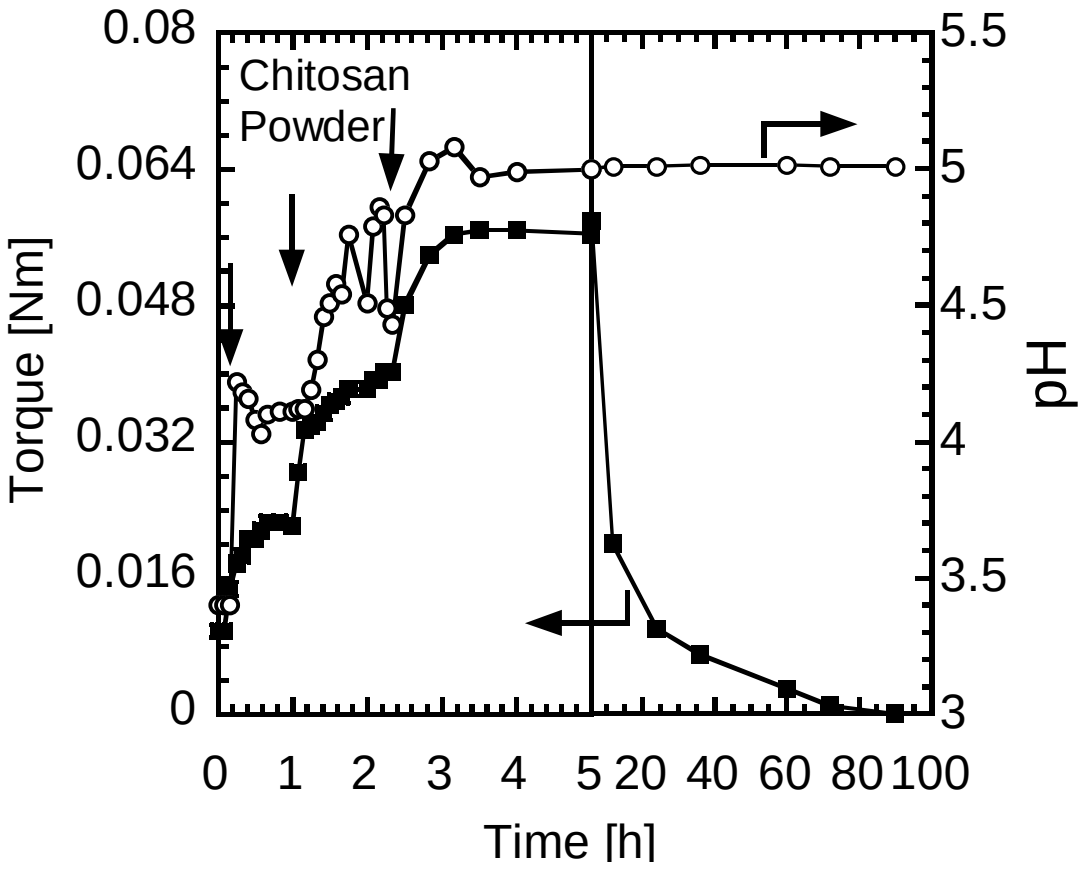


Fig. 3-8 (b) Changes in pH and torque values in 50 kg/m^3 chitosan hydrolysis by immobilized chitosanase. Chitosan concentration was increased from 20 to 50 kg/m^3 by adding the chitosan powder three times with short-interval method. Arrows show the addition time of chitosan powder.

Specific activity: 307 U/m^2
Impeller speed: 4 s^{-1}
Temperature: 35 ^{o}C

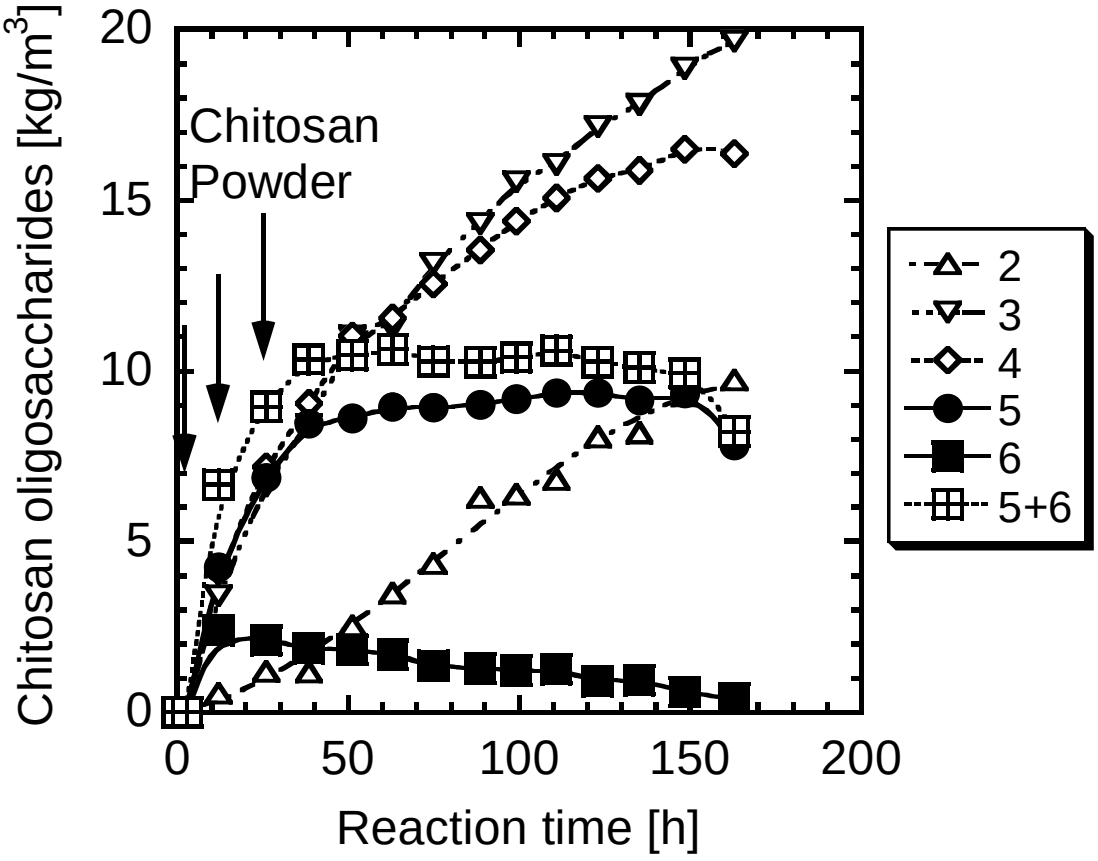


Fig. 3-9 (a) Time courses of chitosan oligosaccharide concentrations in 50 kg/m^3 chitosan hydrolysis by chitosanase immobilized on agar gel-coated multidisk impeller. Chitosan powders were added three times by long-interval method.

Specific activity: 307 U/m^2
Impeller speed: 4 s^{-1}
Temperature: 35 ^{o}C

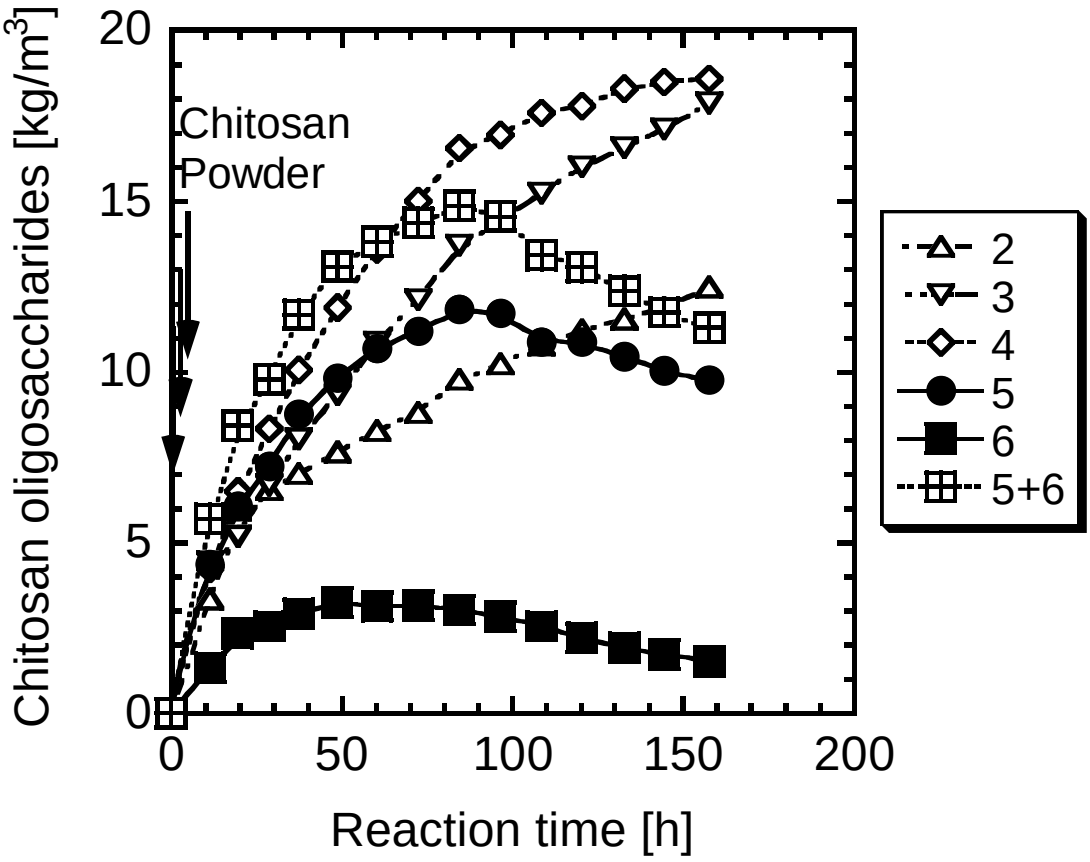


Fig. 3-9 (b) Time courses of chitosan oligosaccharide concentrations in 50 kg/m^3 chitosan hydrolysis by chitosanase immobilized on agar gel-coated multidisk impeller. Chitosan powders were added three times by short-interval method.

Specific activity: 307 U/m^2
Impeller speed: 4 s^{-1}
Temperature: 35 ^{o}C

products. In the long-interval method, the distribution of molecular weights of chitosan in the reaction solution would be wide owing to delayed addition of native chitosan powder during the hydrolysis reaction. In contrast, in the short-interval method, the molecular weight distribution in the reaction mixture would be relatively narrow because the addition of chitosan powder was finished at a fairly early stage of the hydrolysis reaction. Therefore, the peak concentration of the intermediate products became sharp, and a higher maximum concentration was obtained with the short-interval method.

The effects of the addition method on the maximum concentration of pentamer and hexamer, $C_{(5+6)max}$, obtained with immobilized chitosanases having different activities are summarized in Table 1. The value of $C_{(5+6)max}$ for the short-interval method was higher than that for the long-interval method for each of the two different activity of immobilized chitosanases. From the viewpoint of the yield of the target products, the short-interval method for the addition of chitosan powder was more favorable than the long-interval method for the production of chitosan pentamer and hexamer.

3.3.5 Effect of activity at the support surface on the maximum yield of pentamer and hexamer at high concentration

It was stated in chapter 2 that the composition of oligosaccharides produced and the maximum yield of the target hexamer and pentamer were affected by the immobilized enzyme activity at the support surface. The maximum yield of pentamer and hexamer increased with decreasing the activity per support surface. These results were obtained for the hydrolysis of 5 and 20 kg/m^3 chitosan solution. In this part, effect of surface activity of immobilized chitosanase on maximum yield of chitosan oligosaccharides produced from higher concentration chitosan prepared by the stepwise addition (short-interval) of chitosan powders was also investigated. Generally, as temperature increases, the viscosity of a solution decreases while the solubility of solid substances increases. Taking account of both the viscosity and the solubility, a higher

Table 1 Comparison of maximum concentrations of pentamer and hexamer produced by long-interval and short-interval stepwise addition of chitosan powder.

Observed specific activity* [U/m^2]	$C_{(5+6)max}$ [kg/m^3]	
	Long-interval	Short-interval
820	4.4	8.3
307	10.6	14.9

temperature is preferable to increase the concentration of chitosan and for its hydrolysis. On the other hand, the activity and the stability of enzymes also depend on temperature. It was reported that the activity of chitosanase immobilized on agar gel by multipoint attachment method was remained almost all even after being incubated for 250 h at 50 °C and decreased to about 75% of initial activity after 200 h at 55 °C (Takano, 1998). Based on these data, the production of chitosan oligosaccharides at higher concentration was also tried at 50 °C.

Hydrolysis of chitosan was carried out at two temperatures (35 and 50 °C) and at various surface activities of enzyme. The yields of pentamer and hexamer were shown in Fig. 3-10. The activity was determined from the initial reaction curve of hydrolysis measured by the modified Schales's method in each reaction. A higher maximum yield was obtained at lower surface activity. These results indicated that the maximum yield of target products was determined by the surface enzyme activity, regardless of the reaction temperature.

As is obvious from the figure, the highest yield of chitosan pentamer and hexamer, which was 52% (26.0 kg/m^3) and was the same as that produced by free chitosanase, was obtained at the enzyme activity of 48 U/m^2. A high yield (>30%) was obtained at surface enzyme activities under *ca.* 400 U/m^2. The results suggest that the stepwise addition of chitosan powder was also effective for the production at high concentration using immobilized enzyme.

3.3.6 Productivity of pentamer and hexamer using the agar gel-coated multidisk impeller at high concentration

As previously discussed in 2.3.5.5 and 3.3.6, the surface activity of immobilized chitosanase must be low for obtaining a high yield of target products (pentamer and hexamer). However, a low activity prolongs the reaction time. Also in the hydrolysis of 50 kg/m^3 chitosan solution, the time required to achieve the maximum yield of target products, $T_{(5+6)max,}$ was inversely proportional to the observed specific activity (Fig. 3-11), regardless of reaction temperature.

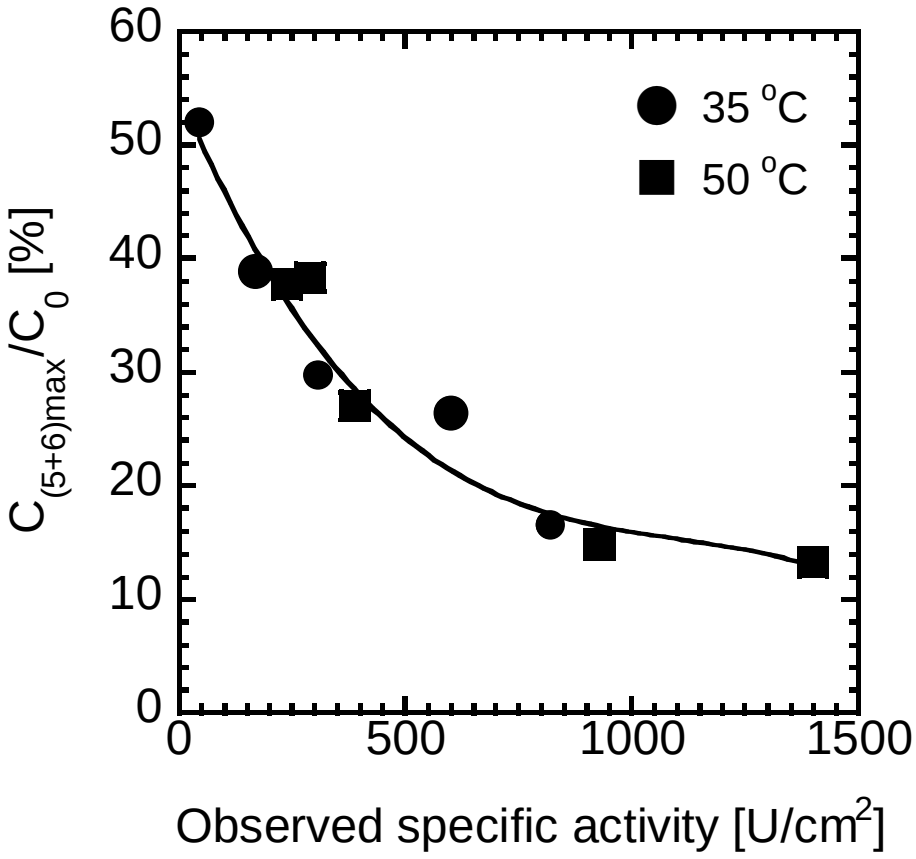


Fig. 3-10 Effect of observed specific activity of immobilized chitosanase on maximum yield of pentamer and hexamer in 50 kg/m^3 chitosan hydrolysis with stepwise addition of chitosan powder.

Impeller speed: 4 s^{-1}

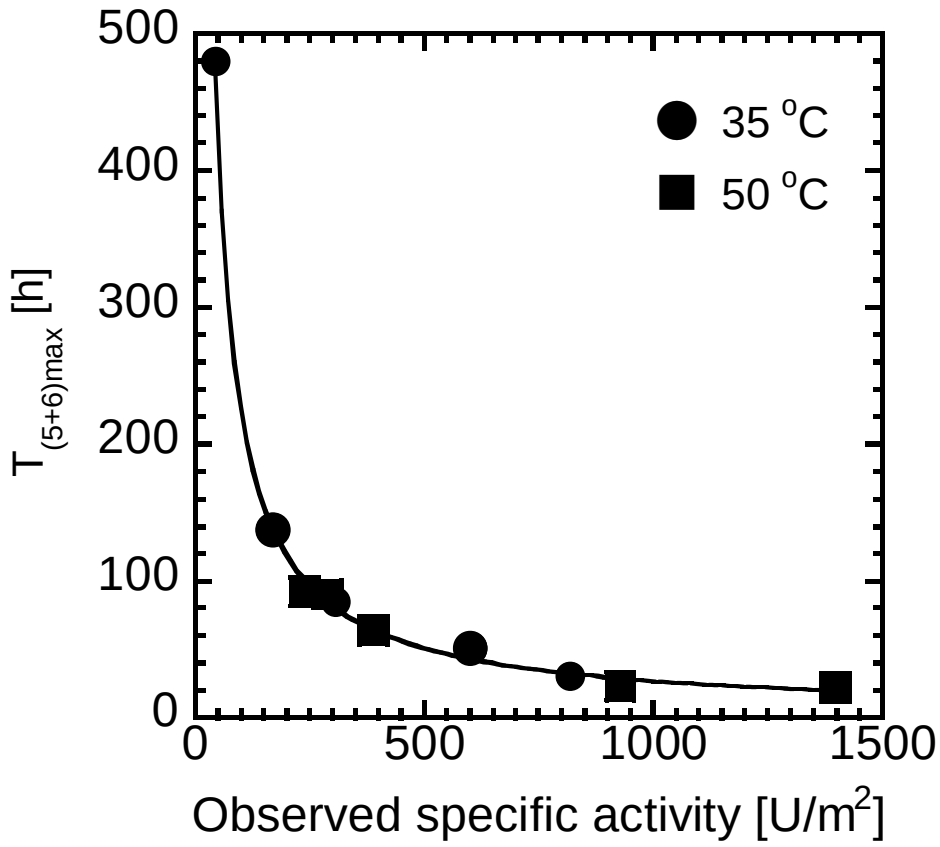


Fig. 3-11 Relationship between observed specific activity of immobilized chitosanase and reaction time required for obtaining the maximum yield of pentamer and hexamer in 50 kg/m^3 chitosan hydrolysis.

Impeller speed: 4 s^{-1}

Although a high yield of target products of 52% was obtained at observed specific activity of 48 U/m^2 obtained was as high as that produced by free enzyme, it took a very long time of about three weeks. Shortening $T_{(5+6)max}$ by increasing the surface activity of immobilized chitosanase is not compatible with the production of target substances in high yield and at a high concentration, as discussed previously. To evaluate the efficiency of reaction under different conditions, the productivity was plotted in Fig. 3-12. The results showed that an observed specific activity of about 930 U/m^2 gave the maximum productivity of 0.32 kg/(m^3h), regardless of the reaction temperature. Then the maximum yield of the target pentamer and hexamer was estimated to be about 17% from Fig. 3-10.

In comparison with the productivity were obtained from the hydrolysis of 5 and 20 kg/m^3 chitosan solution (Fig. 2-22), the surface enzyme activity for obtaining the maximum productivity shifted to higher value. Although the maximum productivity of target substances was not improved by increasing the substrate concentration, the target substances were produced at higher concentration.

From these results, it is clear that the production of pentamer and hexamer from high-concentration chitosan carried out using the newly developed bioreactor could be successfully. It can overcome the handling difficulty of highly viscous chitosan, and then realize the production of chitosan pentamer and hexamer at high concentration and in a high yield. In practice, to optimize the reactor performance, a compromise between the yield of products and the productivity of the process must be considered. To get a relatively high yield of pentamer and hexamer, it is necessary to reform the hydrolysis at a low surface enzyme activity even sacrificing the productivity in some extent. The results shown in Figs. 3-10 and 3-12 would be useful for the determination of the optimum operation conditions of producing chitosan oligosaccharides at high concentration.

For improvement of the productivity, it is necessary to increase the surface area per volume of reactor for immobilization of the enzyme.

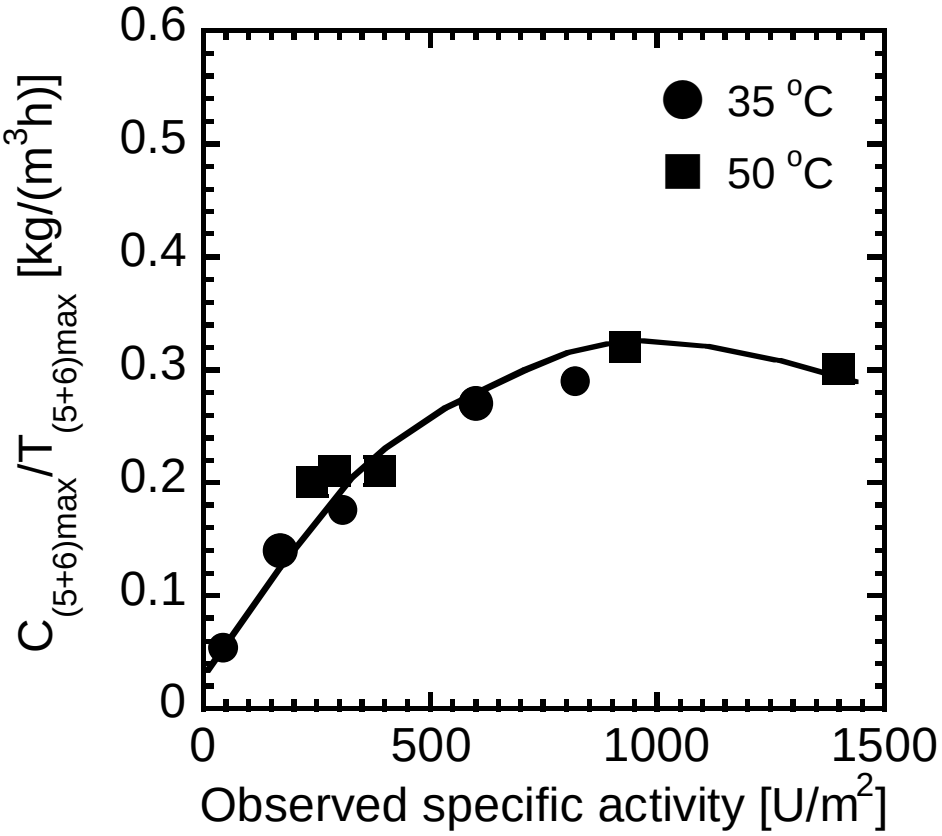


Fig. 3-12 Productivity of pentamer and hexamer under various specific activities of immobilized chitosanase.

Chitosan solution: 50 kg/m^3
Impeller speed: 4 s^{-1}

3.3.7 Repeated production of pentamer and hexamer at high concentration

To verify the stability and the re-usability of the bioreactor used in this study, we investigated repeated batch production of chitosan pentamer and hexamer using the same immobilized enzyme three times. Generally, as temperature increases, the viscosity of a solution decreases and the solubility of a solid substance increases. From the viewpoint of chitosan viscosity, solubility, and hydrolysis, a higher temperature is preferable to a higher concentration of chitosan. However, the activity and stability of enzymes also depend on temperature. The substrate concentration was increased to 50 kg/m^3 by the short-interval stepwise addition method (Fig. 3-8b). When the concentration of pentamer and hexamer reached a plateau, the impeller and the reactor were washed, and then the next reaction was started. As shown in Fig. 3-13, the time courses of the concentrations of reducing sugars and the target oligosaccharides were almost identical in the three batch reactions. This result shows that the chitosanase immobilized on the agar gel-coated multidisk impeller was highly stable during the reaction at 50 °C in a highly viscous solution. The maximum concentration of pentamer and hexamer was about 20.0 kg/m^3 (a 40% yield with respect to the amount of substrate used). To our knowledge, there have been no previous reports of the production of pentamer and hexamer at concentrations higher than 10 kg/m^3. Thus, the bioreactor presented here has great advantages in terms of both the stability and the yield of the target products at high concentration. The reactor should be useful for the practical production of physiologically active chitosan oligosaccharides. We believe that the production method developed in this study will find industrial application.

3.4 Conclusions

In this chapter, the production of chitosan pentamer and hexamer at high concentration was investigated using the chitosanase immobilized on the agar gel-coated multidisk impeller. The following conclusions were obtained.

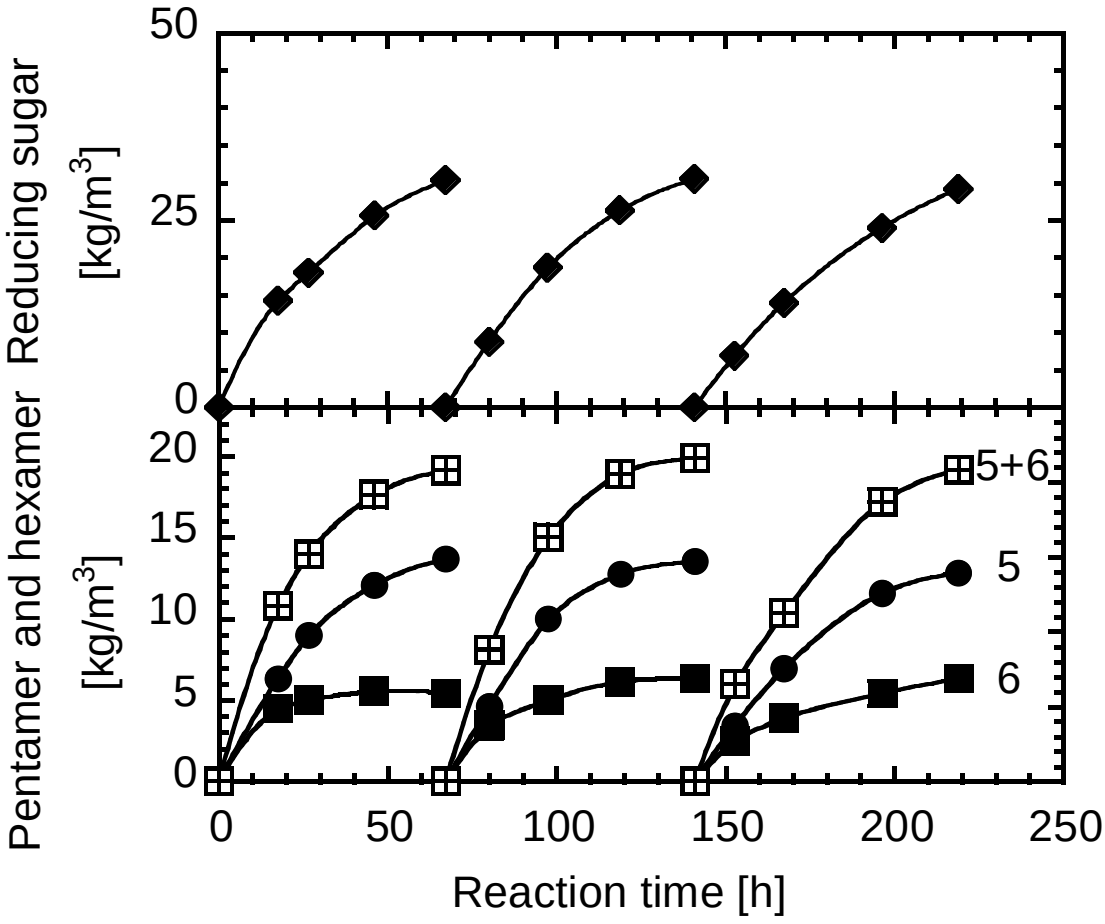


Fig. 3-13 Repeated production of pentamer and hexamer at high concentration using a multi agar gel-coated disk impeller immobilizing chitosanase.

Specific activity: 209 U/m^2
Chitosan solution: 50 kg/m^3
Impeller speed: 4 s^{-1}
Temperature: 50 ^{o}C

(1) For the production of chitosan oligosaccharides at high concentration, lactic acid was more suitable than acetic acid as the acid, because it did not affect the activity of chitosanase and the yield of pentamer and hexamer. Moreover, the solubility of chitosan in lactic acid solution was much higher than that in acetic acid solution, and lactic acid is odorless.

(2) The concentration of chitosan could be increased up to 50 kg/m^3 by the stepwise addition of chitosan powder into the reaction solution and by pH adjustment.

(3) The addition timing of chitosan powder was examined with monitoring the change of the torque for agitating the reaction solution. A short-interval addition of chitosan powder was more efficient than the long interval addition for the production of pentamer and hexamer as target products.

(4) Using a highly concentrated 50 kg/m^3 chitosan solution prepared by the short-interval addition of chitosan powder, 25.9 kg/m^3 of pentamer and hexamer was obtained and the yield to initial substrate reached to 52%. These values were the same as those obtained by free enzyme.

(5) The productivity of pentamer and hexamer was also evaluated for the hydrolysis of 50 kg/m^3 of high chitosan solution. The optimal surface specific activity of immobilized enzyme was found for maximizing the productivity of pentamer and hexamer.

(6) Three times repeated batch hydrolysis of 50 kg/m^3 chitosan was performed at 50 °C using the same immobilized enzyme. The activity of immobilized enzyme did not change during the three batch reactions. In these repeated batch hydrolysis reactions, 20.0 kg/m^3 of pentamer and hexamer (40%) was produced in each reaction.

Chapter 4 General Conclusion

In this chapter, the results obtained in previous chapters are summarized as conclusion, and the prospects for further studies are discussed.

4.1 Conclusions

Chitosan oligosaccharides, especially pentamer and hexamer, have many functions, so they have been applied in many fields, such as functional foods, pharmaceuticals, *etc*. For the efficient production of chitosan oligosaccharides, especially pentamer and hexamer, the usage of immobilized chitosanase is useful and effective as revealed in the previous studies in our laboratory (Hanamura, 1996; Takano, 1999; Kuroiwa, 2002). In previous studies, the concentration of chitosan pentamer and hexamer produced was only 7 kg/m^3. Higher concentration of chitosan will benefit to the production of higher concentration of chitosan oligosaccharides. However, chitosan is highly viscous and the high viscosity of substrate solution will cause the difficulty of operation in a reactor. So the development of a new type of immobilized-enzyme bioreactor that enables to hydrolyze a highly viscous chitosan solution was expected. Because the target products, *i.e.*, pentamer and hexamer, are the intermediates of the hydrolysis reaction, one important aspect in this study is how to improve the yield of target products with proper conditions, which can be realized by promoting a good mass transfer rate. So the development of a new type of immobilized-enzyme bioreactor that enables the hydrolysis of the high viscous chitosan solution is required. And their operation method is also necessary for the efficient production of chitosan pentamer and hexamer. For these reasons, the objectives of this study were as follows:

(1) Development of a new bioreactor for the production of chitosan pentamer and hexamer at high concentrations by the enzymatic hydrolysis of the high-concentration chitosan solution.
(2) Development of an operation method of the bioreactor, which enables the production of chitosan pentamer and hexamer at higher concentrations by increasing

the chitosan concentration as a substrate solution.

In Chapter 2, based on the above mentioned background, a novel immobilized enzyme bioreactor was developed. (1) It is applicable to highly viscous solutions; (2) it has good mass-transfer properties; (3) it has a large surface area available for enzyme immobilization; (4) it exhibits high enzyme stability. A reactor equipped with multidisk impeller directly immobilized chitosanase was newly proposed and studied in detail. This type of reactor will increase the slip velocity between the substrate solution and the enzyme immobilized on the support surface, which promotes mass transfer. Because no solid-solid collisions occur, support for immobilization was used as impeller being an agitator. The multiple disks provide a large surface area for enzyme immobilization. Furthermore, stable immobilized enzyme can be obtained by means of the multipoint attachment method.

Two kinds of support materials were used for immobilization of chitosanase. Firstly, cotton cloth was used as the support material for immobilization of chitosanase. Cotton cloth has many hydroxyl groups, so the immobilization of chitosanase on it by multipoint attachment can be realized. Cotton cloth is also flexible and easy to cut, so it can be easily designed. The reactor with a cotton cloth multidisk impeller directly immobilized chitosanase was examined for production of chitosan oligosaccharides. The yield of pentamer and hexamer was considerably lower than that obtained by free enzyme. The effect of impeller speeds on the yield did not show a regular tendency. The effect of densities of immobilized enzyme was not remarkable. SEM observation showed that there were many narrow spaces between cotton fibers. This microstructure of cotton cloth might cause the mass transfer limitation inside the cotton cloth. Based on these results, it was concluded that the cotton cloth was not suitable to be used as the support material of immobilized chitosanase for producing high yield of chitosan pentamer and hexamer.

Secondly, agar gel-coated multidisk impellers were developed to replace the cotton cloth impeller. Immobilization of chitosanase on agar gel particles by multipoint

attachment was examined previously in our laboratory (Takano, 1998; Kuroiwa, 2005). It was shown that there was not the mass transfer limitation inside the agar gel support because chitosanase was immobilized on the surface of agar gel. Therefore, using agar gel as a support material, a multi agar gel-coated disk plate type reactor that enables to hydrolyze a high viscous chitosan was newly developed. The reactor equipped with agar gel-coated multidisk impellers, where chitosanase was directly immobilized onto the agar gel-coated disks by the multipoint attachment method.

The stability of immobilized enzyme was confirmed by five repetitions of the batch reaction. Using this new reactor, 9 kg/m^3 of pentamer and hexamer was produced by hydrolyzing of 20 kg/m^3 chitosan solution. The maximum yield of pentamer and hexamer reached to 45%. This value of the yield is the same as the maximum yield obtained in the batch hydrolysis reaction using free-enzyme. The productivity of pentamer and hexamer in the new reactor was evaluated using two indexes, *i.e.*, the maximum yield of target products and the reaction time required for the maximum yield. The optimal value of the observed specific activity of immobilized enzyme was found for maximizing the productivity of pentamer and hexamer.

In Chapter 3, using the agar gel-coated multidisk impeller developed in Chapter 2, the production of higher concentration of chitosan oligosaccharides was investigated. For the production of chitosan oligosaccharides at high concentration, lactic acid was more suitable than acetic acid as an acidic solvent, because it did not affect the activity of chitosanase and the yield of pentamer and hexamer. Moreover, the solubility of chitosan in lactic acid solution was much higher than that in acetic acid solution. The optimum pH range was between 4.5 and 6. The pH change between 3.3 and 5.6 did not affect the activity recovery of immobilized chitosanase recovered.

As a new method for the practical production of chitosan pentamer and hexamer at higher concentration, a stepwise addition method was developed for increasing the chitosan concentration as a substrate solution. In this method, chitosan powder was added stepwise while proceeding the hydrolysis reaction. Starting chitosan solution was 20 kg/m^{3}and the chitosan concentration was increased up to 50 kg/m^3 by the addition of

new chitosan powder three times. The timing of chitosan powder addition was examined with monitoring the change of the torque of the reaction solution. A short-interval addition of chitosan powder was more efficient than the long-interval addition for the efficient production of pentamer and hexamer as target products.

The productivity of pentamer and hexamer was also evaluated in the hydrolysis of using high-concentration chitosan solution of 50 kg/m^3. The optimal surface observed specific activity of immobilized enzyme was found for maximizing the productivity of pentamer and hexamer.

Three times repeated batch hydrolysis of 50 kg/m^3 chitosan was performed at 50 °C using the same immobilized enzyme. The activity of immobilized enzyme did not change during the three batch reactions. In this repeated batch hydrolysis reaction, 20.0 kg/m^3 of pentamer and hexamer was produced in each reaction.

Using a highly concentrated substrate of 50 kg/m^3 chitosan solution prepared by the short-interval addition of chitosan powder, 25.9 kg/m^3 of pentamer and hexamer was obtained and the yield to initial substrate reached to 52%. The production of high-concentration chitosan oligosaccharides was successfully carried out for the first time by using a newly developed reactor in this study.

4.2 Prospects for further studies

In this study, the concentration of chitosan was increased by using the stepwise addition method of chitosan powder, and 50 kg/m^3 solution was successfully prepared. This stepwise addition method of substrate might enable a higher chitosan concentration exceeding 50 kg/m^3 and then the chitosan pentamer and hexamer could be produced at a higher concentration.

In the case that the target products are intermediates of the hydrolysis of substrate, a high reaction rate at the support surface of immobilized enzyme causes a low yield of the target products, as described in this thesis. Therefore, it is necessary to maintain a low specific enzyme activity at the support surface for obtaining the target products with a high yield. However, the reaction time required for it becomes longer and the

productivity decreased inevitably. The maximum productivity of chitosan pentamer and hexamer was obtained at the 15% yield of the target products to substrate in this study. In order to satisfy both the productivity and the yield of target products, the surface area of the support for enzyme immobilization per reactor volume must be increase. It may be achieved by increasing the number of disk of impeller in the newly developed reactor.

A paced-bed reactor is usually used for industrial production of valuable substances, because a large amount of immobilized enzyme can be packed per unit volume of the reactor, compared with a stirred tank reactor. However, it is very difficult to apply the packed-bed reactor to highly viscous solution such as chitosan solution. As a strategy for the application of pack-ed reactor to chitosan solution at high concentration, two-steps reaction system using the newly developed stirred reactor and the packed-bed reactor is considered. That is, as the first step, the concentration of chitosan is increased in the newly developed stirred reactor by the stepwise addition method and the hydrolysis reaction is continued in it for some time. The second step is the hydrolysis in the packed-bed reactor. After decreasing the viscosity of the reaction solution in some degree in the stirred reactor, the solution is removed from the stirred reactor and then fed to the packed-bed reactor. In this system, the first step is batchwise and the second step is operated continuously. Since the packed-bed reactor has a large surface area of support for enzyme immobilization, the productivity of chitosan oligosaccharides must be improved by using this two-steps system, while keeping a high yield of them. This will be also an important future work.

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