Solvent Extraction Research and Development, Japan, **Vol.10 123 – 132 (2003)**
Received on December 14, 2002; Accepted on February 12, 2003

Characterization of the Surface Properties of Chitosanase under Heat Stress Condition Using Aqueous Two-Phase Systems and an Immobilized-Liposome Sensor System

Ho-Sup Jung, Hiroshi Umakoshi, Se-Young Son, Toshinori Shimanouchi and Ryoichi Kuboi*
Department of Chemical Science and Engineering, Graduate School of Engineering Science,
Osaka University, Toyonaka 560-8531, Japan

The surface properties of chitosanase from *Storeptomyces griseus* were characterized under heat stress conditions by using aqueous two-phase systems and by using an immobilized-liposome sensor. The surface net (*HFS*) and local hydrophobicities (*LH*), were first characterized by using the poly (ethylene glycol) (PEG) / dextran 100-200k (Dex) aqueous two-phase system at various temperatures (25~50°C). The *LH* values of dithiothreitol (DTT)-treated chitosanase showed a maximum at a specific temperature (45°C) while the *HFS* values gradually increased with increasing temperature from 40 to 50°C. At the same temperature conditions as above, the surface properties of chitosanase were also analyzed by using a sensor tip immobilized-liposome electrode. Similarly in the case of the variation of *LH* but not *HFS*, the detected signal (maxima in electric current) was maximized at 45 °C, and a linear correlation between the *LH* values and the signal values of the new immobilized-liposome sensor system was obtained. It was thus found that the new sensor system was effective for the characterization of the surface properties and *LH* values, of proteins, as well as the aqueous two-phase system method.

1. Introduction

Chitosan prepared from chitin through chemical *N*-deacetylation is one of the most abundant renewable polysaccharides. It is attracting more and more interest due to its potential application in medicine, industry and agriculture. Recently, chito-oligosaccharides have received much attention because they are not only water soluble, but possess distinctive biological activity such as antitumour activity [1], antifungal and antibacterial activity [2], immuno-enhancing effects [3], enhancement of protective effects against infection with some pathogens in mice [4]. Among the possible methodologies for preparation the chito-oligosaccharide, enzymic hydrolysis (i.e. chitinase and chitosanase) is a preferable method because of easy control, mild conditions of hydrolysis and low pollution of the environment [5]. The design and development of a simple process for the production and

separation of such hydrolytic enzymes is needed.

In a conventional bioprocess for the production of chitosanase, batch-fed fermentation has been utilized as a common industrial operation [6], where the concentration of glucose [7,8] or the specific growth rate [9] are normally monitored for the efficient production of chitosanase. The concept of a stress-mediated bioprocess has recently been proposed as an environment-friendly bioprocess with low energy-consumption and, sometimes, with high recovery yield [10-12]. The most important characteristic of this process is the utilization of the stress-response function of process-constituting elemental materials, such as bacterial cells, membrane and enzymes, by active control of the environmental stress. It has been reported that the specific stress can be optimized through the characterization of the surface properties of the elemental materials [13-15]. It is important therefore to evaluate the surface properties of the target protein under stress conditions by a simple evaluation method in order to control the stress-mediated bioprocess for the effective production and recovery of chitosanase.

In our series of studies, some methods for evaluating the surface properties of the biomaterials under stress conditions have been reported, such as an aqueous two-phase partitioning method [16], immobilized-liposome chromatography [17], and so on [18]. Among the above methods, the aqueous two-phase system (ATPS) is known to be suitable for the analysis of the surface properties of biomaterials because of the minimal impact on the structure and stability of the proteins [19]. It has also been reported that the surface properties of proteins during the denaturation/ refolding processes can be evaluated by using aqueous two-phase systems [20]. The observed local hydrophobicity (*LH*) of the proteins is found to play an important role in the protein denaturation or aggregate formation process [21], heat shock proteins [22], smart-polymer assemblies [23] and liposomes [24]. The analytical method using immobilized-liposome chromatography has recently been reported to be effective for the evaluation of the protein-liposome interaction under the stress conditions [17]. In addition, this interaction can be recalculated as a function of the surface properties of the protein and the liposome evaluated by the aqueous two-phase partitioning method [16]. It is expected that the liposome can also be immobilized on the surface of an inorganic electrode (sensor chip) based on the technique described previously and the immobilized-liposome sensor tip can be utilized as an effective evaluation method of the surface properties of proteins under stress.

In this study, the surface properties of chitosanase, such as surface net and local hydrophobicities, were first characterized by using the ATPS method under various conditions. Secondly, the immobilized liposome electrode was developed and was also applied to the characterization of the surface properties. The surface properties evaluated by both methods were compared. Based on these results, the effectiveness of the new sensor is discussed.

2. Experimental

2.1. Materials

Poly(ethylene glycol) (PEG) 1540, 4k, and 6k (average molecular weight (Mr): 1.5k, 3k and 7.5k, respectively) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Dextrane T-500 (Dex) was obtained from Amersham biosciences (Uppsala, Sweden). Chitosanase from *Streptomyces griseus*, and 16-mercaptohexadecanoic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The nonionic surfactant, Triton X-405, was purchased from Sigma. 1-Palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) was obtained from Avanti Polar lipid (Alabaster, AL, USA). Dithiothreitol (DTT), N-hydroxysuccinimide (NHS), dioxane and potassium hexacyano-ferrate (II) were purchased from Wako (Osaka, Japan). 1-Ethyl-3-(3-dimethlaminopropyl)-carbodiimide hydrochloride (WSC) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals of analytical grade were purchased from Wako Pure Chemicals (Osaka, Japan) and were used without further purification.

2.2. Characterization of the Surface Properties of Proteins under Stress Using the Aqueous Two-Phase Partitioning Method

Aqueous two-phase systems (ATPS) were prepared by weighing out the required amounts of stock solutions of PEG and Dex, together with buffer, protein, Triton and water. In this case, all the ATPS contained 10mM Tris-HCl buffer and the pH of the system was 8.0. The systems were equilibrated by mixing and then the systems were allowed to stand for in the water bath at the 25~60°C for 90min. Samples were withdrawn from the two phases and the protein concentration was analyzed to calculate the partition coefficient of the protein. The value of the hydrophobicity factor (*HF*) of the ATPS, which is defined as the hydrophobicity difference between the two phases, was determined from the partition coefficient of several hydroprobe amino acids according to a previous report [16]. The values of stress strength and those of surface properties were normalized by using the following equations [15].

$$\text{Normalized stress strength} = f(X_i) = |X_i - X_{iN}|/(X_{iM} - X_{iN})$$

where $f(X_i)$ is the functions of the characteristic variable X_i for each stress *i*. i.e. pH, concentration of GuHCl, C_{GuHCl} and temperature T. Subscript N and M denoted the native state and maximum value, respectively.

$$\text{Normalized surface property} = f(Y_j) = |Y_j - Y_{jN}|/(Y_{jM} - Y_{jN})$$

where $f(Y_j)$ is a function of surface property Y_j, i.e. *LH* and *HFS*. Subscript N and M denoted the native state and maximum value, respectively.

2.3. Preparation of the Immobilized-Liposome Electrode

POPC liposomes were prepared as previously described [25]. In brief, POPC (9.8mg/ml) dissolved in chloroform was dried in a 100ml round-bottom flask by rotary evaporation under reduced pressure. The lipid film was twice dissolved in diethylether, and the solvent was evaporated again. The obtained lipid film was kept under high vacuum for at

least 3hr and then hydrated with a 50mM potassium hexacyano-ferrate (II) solution in a 50mM potassium phosphate buffer (pH 7.5) at room temperature to form multilamellar vesicles. After the freeze-thawing treatment, the liposome size was adjusted by extrusion of the solution through polycarbonate membranes (diameter 100nm). The free WSC were removed from the sample by using size exclusion chromatography on Sepharose CL4B.

To fabricate the immobilized-liposome electrode, a self-assembled monolayer (SAM) using 16-mercaptohexadecanoic acid was formed on an Au electrode (2mm×2mm) as previously described [26]. To activate the SAM membrane, the above electrode was immersed in a solution of dioxane and distilled water (90:10) solution contained 17mM NHS plus 17mM WSC for 3~4hr. The liposome was then formed on the SAM layer by the amino conjugate method. After 1 hr, the liposome modified Au electrode was rinsed with phosphate buffer (pH7.5).

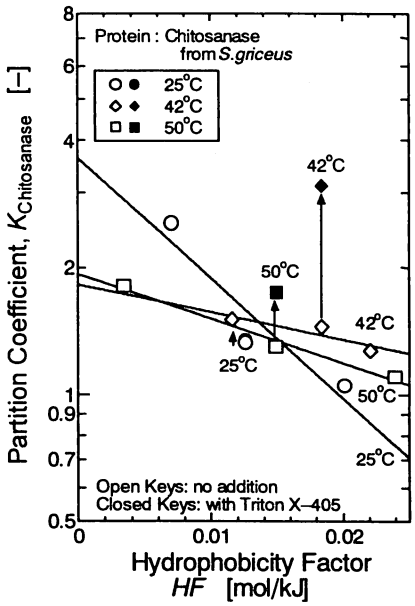

Fig. 1 Relationship between the Partition Coefficient of Chitosanase and the Hydrophobicity Factor (HF) of Aqueous Two-Phase Systems at Various Temperatures.

2.4. Measurement

A potentiostat /galvanostat (EG&G model 273) connected to a personal computer (EG&G Software Power Suit #270/250) was used for the electrochemical measurements. A conventional three-electrode cell, consisting of the Au electrode modified with liposome as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl electrode as a reference electrode was used for the electrochemical measurements. The concentration of chitosanase was determined by the Bradford method [27]. The other measurements were made as previously described[20].

3. Results and Discussion

3.1. Characterization of Surface Net and Local Hydrophobicities of Chitosanase from *S. griseus* Under Heat Stress Using Aqueous Two-Phase Systems.

The surface properties of chitosanase, such as surface net and local hydrophobicities, were characterized under heat stress by using a PEG/ Dex ATPS.

The surface net hydrophobicity (*HFS*) of chitosanase was first determined in the PEG/ Dex ATPS at various temperatures (25~60°C). **Figure 1** shows the relationship between the partition coefficient of chitosanase and the hydrophobicity of the ATPS (*HF*) at 25, 42, 50°C. The partition coefficient of chitosanase was, in general, moderately decreased

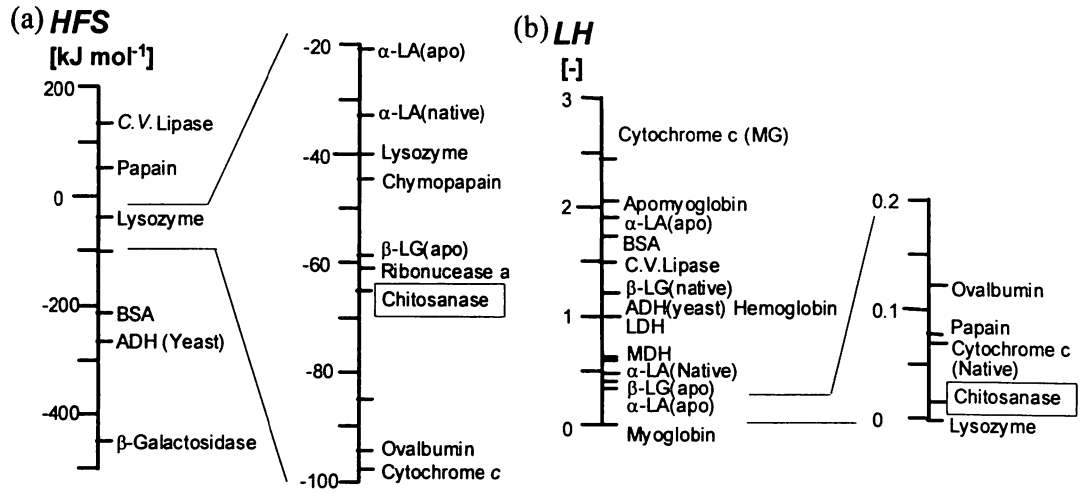

Fig. 2 Ladder of *HFS* and *LH* for various Proteins [16]

with increasing *HF* values, indicating that the chitosanase has a moderately hydrophobic surface. In addition, the *HFS* values increased with increasing temperature, showing that the chitosanase surface becomes hydrophobic under heat stress. The slope of the curve can, herewith, be defined as the surface net hydrophobicity (*HFS*) of chitosanase according to a previous paper [16]. The calculated *HFS* value of the chitosanase at 25°C was compared with that of other proteins [17] as shown in **Fig.2(a)**. The chitosanase was found to exhibit a medium hydrophobicity.

The *LH* was determined by the PEG/ Dex aqueous two-phase system with and without 1mM Triton X-405. The partition coefficients with TritonX-405 are also shown as closed keys in **Fig.1**. The *LH* of the chitosanase can be defined as the incriminate change of the partition coefficient of the chitosanase in the PEG/ Dex ATPS with and without 1mM Triton X-405. The *LH* value of chitosanase at 25°C was compared with those for other proteins as shown in **Fig.2(b)**. Under normal conditions, the *LH* value was found to be small compared to other types of proteins. In addition, the value for chitosanase at 45°C was increased, indicating that the *LH* of chitosanase was increased especially at this specific temperature although the increment of the partition coefficient of chitosanase was not significantly increased at 25 and 50°C.

The evaluated surface properties were plotted against temperatures as shown in **Fig.3(a)**. An interesting bell-shaped curve was obtained for the temperature dependence of the *LH* values although the *HFS* values just increased at temperatures above 45°C. The above variation of the surface properties was caused by the conformational change of the chitosanase under heat stress as shown in previous work [28]. In order to compare the above results with the other proteins, the stress-response behaviors of the protein under stress were evaluated through the normalization of the surface properties (f (*HFS*) and f (*LH*)) and stress condition ($f(T)$) according to the previous report [15] as shown in **Fig. 4**.

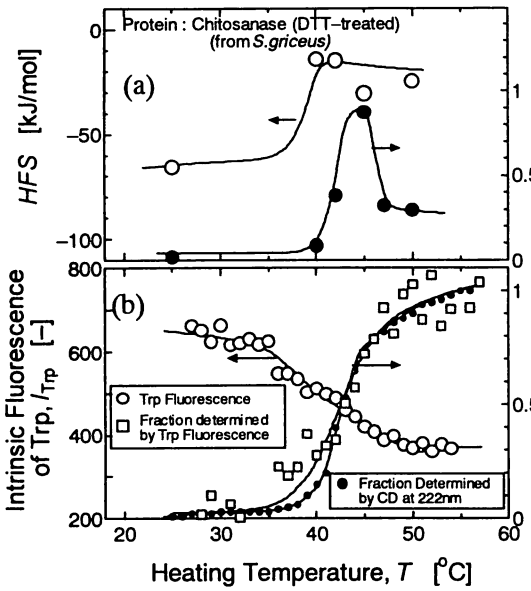

Fig. 3 Temperature Dependence of the Surface Properties of Chitosanase under Heat Stress Conditions. CD data: from [28]

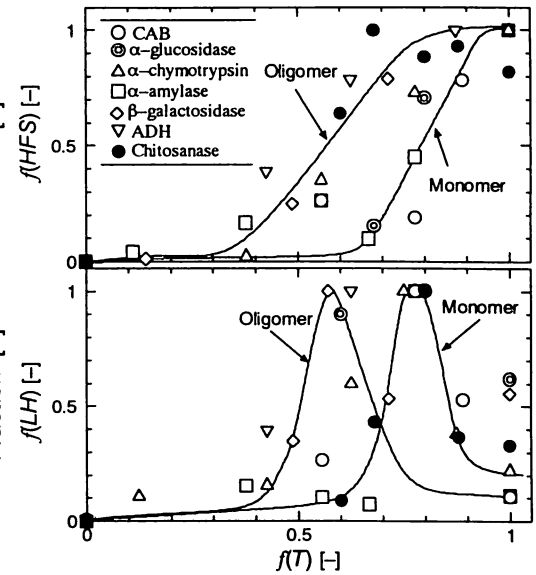

Fig. 4 Normalized Stress-Response of the Surface Properties of Various Proteins

The results obtained were found to be similar to the previous findings, except that the specific temperature to induce the conformational change of chitosanase was 45°C. The conformational change of chitosanase was also analyzed by using the intrinsic tryptophan fluorescence, and a decrease in tryptophan fluorescence is found at the temperature of 45°C (**Fig. 3(b)**). It has been reported that the chitosanase has a phase transition temperature at 45°C based on circular dichroism analysis (closed circles in **Fig. 3(b)**) [29]. The results obtained above correspond well with previous findings. It was thus found that the surface hydrophobicity of chitosanase increased at higher temperatures and the specific temperature for the induction of the conformational change of chitosanase from the native to the local hydrophobic intermediate state was 45°C.

3.2. Detection of Heat-Exposing Chitosanase Using Immobilized-Liposome Electrode Sensor System

The surface properties of chitosanase were also characterized by the immobilized-liposome electrode sensor system [30]. As shown in **Fig. 5**, the electrolyte-entrapped liposomes were fabricated on the electrode. After the sample of chitosanase

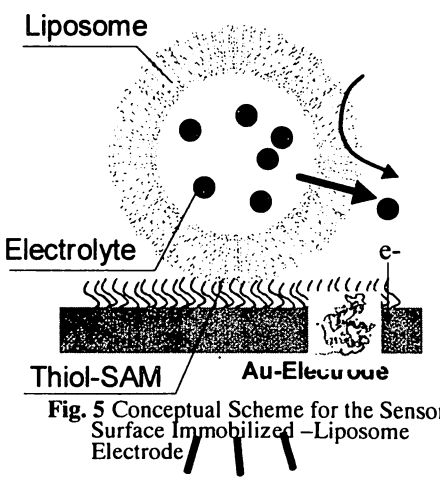

Fig. 5 Conceptual Scheme for the Sensor Surface Immobilized -Liposome Electrode

was

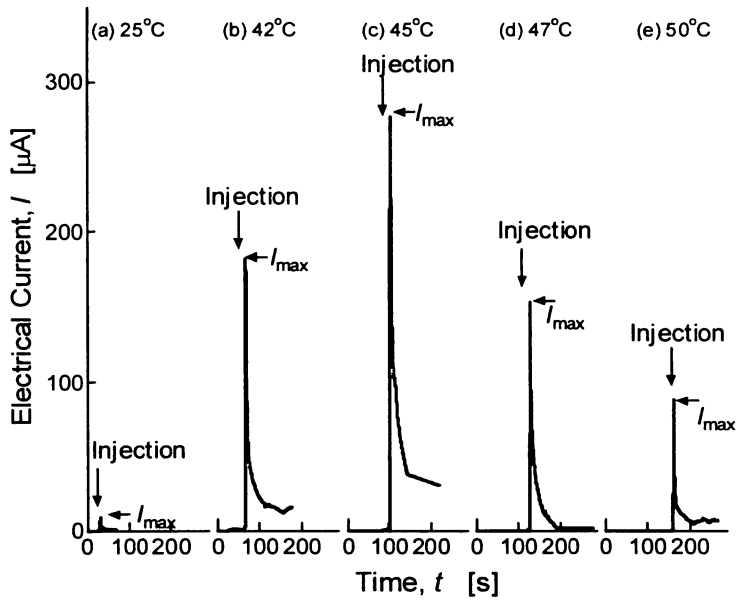

Fig. 6 Variation of Electrochemical Signal as a Response of Protein-Liposome Interaction at Various Temperatures

applied for the sensing system at various temperatures, the output current of the sensor chip, which can be detected as a result of leakage of electrolyte from the liposome because of the perturbation of the membrane, was measured kinetically. **Figure 6** shows the rate of change of the output current in the presence of chitosanase for various temperature conditions. At all conditions, the electrical current values were increased and maximized just after the samples were injected. At a temperature of 45°C, the highest value of the maximum current could be obtained although the values were not significantly increased at other temperatures tested here.

It has been reported that the protein in the intermediate (molten-globule, MG) state has the ability to interact with phospholipid membranes (liposome and biological membrane) because of the increase of the *LH* value under the stimuli conditions [31,32]. It has also been reported that such a protein-membrane interaction can be analyzed by using immobilized-liposome chromatography [17]. Recently, a gel support immobilizing the calsein-entrapping liposome has been utilized for the detection of this interaction, as the addition of the model protein (bovine carbonic anhydrase) at the intermediate state in the presence of a denaturant (1M GuHCl) increased the calsein leakage from the liposome [33]. It was thus found that the immobilized-liposome on the sensor tip was effective for the detection of the liposome-protein interaction under stress conditions.

3.3. Comparison of Surface Properties Evaluated by Aqueous Two-Phase Systems and the Immobilized-Liposome Electrode Sensor System.

The surface properties of chitosanase evaluated by using the ATPS and by using the

liposome-immobilized stress sensor system were compared. **Figure 7** shows the dependence of the surface properties, determined by using the ATPS, on the maximum electric current, determined by the stress sensor system, under heat stress. In the case of *HFS*, a clear relationship cannot be observed between these values (**Fig. 7(a)**). On the other hand, a linear correlation between the values can be obtained in the case of *LH* (**Fig. 7(b)**).

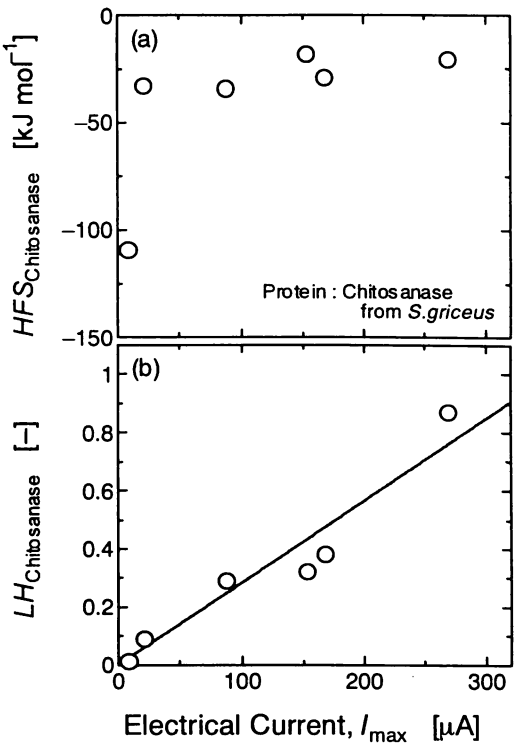

Fig. 7 Relationship between the Current Values of the Immobilized-Liposome Electrode Sensor and the Surface Properties of Chitosanase

In our previous studies, the surface properties (*LH* and *HFS*) determined by using the ATPS have been compared with other parameters, which have been determined by using other methods [33]. The values of *HFS* and *LH* corresponds well with the accessible surface area of solutes and the intensity of a hydrophobic fluorescence probe (8-Anilino-1-naphtalene-sulfonate, ANS), respectively [33]. In addition, the value of *LH* has been shown to be correlated with the intensity of the interaction between the protein and membrane under the various stresses and, therefore, to be an important factor in the determination of the protein destiny in the cellular environment, such as refolding [17], translocation [30], aggregation [21], and so on [31]. It has been reported that the intensity of the protein-liposome interaction determined by immobilized-liposome chromatography could be correlated with the *LH* value because of the increase of membrane fluctuation induced by the hydrophobic interaction of protein under stress conditions. The above correlation shows that the micro sensor tip immobilized-liposome electrode can also be utilized as a tool for the characterization of the surface properties, the *LH* value, of protein under stimuli as well as the aqueous two-phase partitioning method and, also, immobilized- liposome chromatography. In the case of the sensor system, there are some benefits for this analytical method such as (i) low sample quantity, (ii) short analytical time, and (iii) multi-array. The possibility of utilizing the immobilized-liposome electrode sensor system as an analytical system for the surface properties of proteins was thus confirmed by comparing their results with those determined by using the ATPS.

4. Conclusion

The surface properties of chitosanase were first evaluated by using an ATPS. The specific heating temperature for the induction of the specific conformational state with the maximum *LH* value of the chitosanase was found to be 45°C. The chitosanase under the above heat condition was secondly analyzed by using the immobilized-liposome electrode sensor system. A simple linear correlation was found between the *LH* values of chitosanase measured by the ATPS and the observed electrical current, I_{max}, implying that the former ATPS method for the measurement of *LH* values can be substituted by the latter new sensing system. In the latter system, the surface properties of liposomes can easily be varied by varying their membrane composition and they can be located on a sensor chip.

The authors thank Prof. Dr. Kwan Kim (Don-A University, Korea) for fruitful discussions. This work was partly supported by a Grand-in-Aid for Scientific Research (Nos. 12450333, 13750637, and 14655310) of The Ministry of Education, Science, Sports and Culture of Japan and the Sumitomo Foundation (No. 010400). One of authors (H.-S. J.) acknowledges the financial support from the Foundation for C&C Promotion and the Sasakawa Scientific Research Grant from The Japan Science Society.

5. References

[1] A. Tokoro, N. Tatewaki, K. Suzuki, T. Mikami, S. Suzuki, M. Suzuki, Chem. Pharm. Bull., 36(2),784 (1998)

[2] D.F. Kendra, L.A. Hadwiger, Exp. Mycol., 8, 276 (1984)

[3] Y.J. Jeon, S.K. Kim, Carbohydr. Polym., 41, 133 (2000)

[4] Y.J. Jeon, P.J. Park, S.K. Kim, Carbohydr. Polym., 44, 71 (2001)

[5] M. Izume, M. Ohtakara, Agric. Biol. Chem., 51, 1189 (1987)

[6] K. Kim, A.L. Creagh, C.A. Haynes, Korean Soc. Biotech. Bioeng., 3, 71 (1998)

[7] G.L. Kleman, J. J. Chalmers, G.W. Lulli, W.R. Strohl, Appl. Environ. Microb., 57, 918 (1991)

[8] Y.L. Huang, S.Y. Li, B.A.A. Dremel, U. Bilitewski, R.D. Schimid, J. Biotechnol., 18, 161 (1991)

[9] A.S. Saint, P. Perlot, G. Goma, P. Soucaille, Biotechnol. Lett., 16, 831 (1994)

[10] R. Kuboi, H. Umakoshi, I. Komasawa, Biotechnol. Prog., 11(2), 202 (1995)

[11] H. Umakoshi, K.Yano, R. Kuboi, I. Komasawa, Biotechnol. Prog., 12(1), 51 (1996)

[12] H.Ota, K. Yamahara, H. Umakoshi, R. Kuboi, Solv. Extr. Res. Dev. Japan, 6, 151 (1999)

[13] H. Umakoshi, R. Kuboi, I. Komasawa, T. Tsuchido, Y. Matsumura, Biotechnol. Prog., 14(2), 210 (1998)

[14] H. Umakoshi, Y. Fukuta, R. Kuboi, Biotechnol. Prog., 14(6), 909 (1998)

[15] K. Yamanhara, H. Ota, R. Kuboi, J. Chem. Eng. Japan, 31(5), 795 (1998)
[16] R. Kuboi, K. Yano, I. Komasawa, Solv. Extr. Res. Dev. Japan, 1, 42 (1994)
[17] M. Yoshimoto, T. Shimanouchi, H. Umakoshi, R. Kuboi, J. Chromatogr. B, 743(1-2), 93 (2001)
[18] S. Morita, H. Umakoshi, R. Kuboi, J. Biosci. Bioeng., 90(2), 157 (2000)
[19] P. A. Albertsson, Partitioning of cell particles and macromolecules, 3rd ed., Wiley-Interscience, New York (1986)
[20] R. Kuboi, K. Yano, H. Tanaka, I. Komasawa, J. Chem. Eng. Japan, 26(3), 286 (1993)
[21] R. Kuboi, K.Yamahara, H. Ota, J. Chem. Eng. Japan, 30(6), 1119 (1997)
[22] K. Yano, T. Hasegawa, R. Kuboi, I. Komasawa, T. Tsuchido, J. Chem. Eng. Japan, 27(6), 808 (1994)
[23] N. Yoshimoto, T. Hashimoto, M. M. Felix., H. Umakoshi, R. Kuboi, Biomacromol., in press (2003)
[24] R. Kuboi, M. Yoshimoto, P. Walde, P. L. Luisi, Biotechnol. Prog., 13, 828 (1997)
[25] D.V. Lynch, P. L. Steponkus, Biochim. Biophys. Acta, 984, 267 (1989)
[26] R. Michael, M. M. Vladimir, N. Ivan, T. Vladimir, R. Vlastimil, S. W. Otto, Analytica Chimica Acta, 392, 77 (1999)
[27] M. Bradford, Anal. Biochem., 72, 248 (1976)
[28] K. Yano, A. Wakayama, R. Kuboi, I. Komasawa, Bunseki Kagaku, 42(11), 673 (1993)
[29] Y. Honda, T. Fukamizo, T. Okajima, S. Goto, I. Boucher, R. Brzezinski, Biochim. Biophys. Acta, 1429(2), 365 (1999)
[30] A. J. Bäumner, R. D. Schmid, Biosensor & Bioelectronics, 13, 519 (1998)
[31] H. Umakoshi, M. Yoshimoto, T. Shimanouchi, R. Kuboi, I. Komasawa, Biotechnol. Prog., 14(2), 218 (1998)
[32] M. Yoshimoto, P. Walde, H. Umakoshi, R. Kuboi, Biotechnol. Progr., 15(4), 689 (1999)
[33] X.-Y. Liu, Q. Yang, C. Nakamura, J. Miyake, J. Chromatogr. B, 750(1), 51 (2001)