Conferring new functions on lactic acid bacteria with cell surface adhesive proteins

細胞表層接着タンパク質による乳酸菌への新機能の付与

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CHAPTER 1

General Introduction

1.1 INTRODUCTION

Anchoring of proteins to the cell surface is a common theme in nature, and the processes governed by different surface proteins are bases of many biological phenomena, such as cell-cell recognition, signal transduction, adherence, colonization, and immunoreactions (1). The utilization of cellular surface anchoring systems for the display of heterologous proteins on cells have been developed into an active research area that holds great promise for a variety of biotechnological applications including production of whole-cell biocatalysts, microbial adsorbents, live vaccines, screening of novel proteins, and antibody production (2-4). Generally construction of these systems is accomplished by expressing a heterologous peptide or protein of interest as a fusion with various anchoring motifs, which are usually cell-surface proteins or their fragments. Depending on the characteristics of target and anchor proteins, N-terminal fusion, C-terminal fusion or sandwich fusion strategy can be considered (2).

1.2 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) have a long history of use in food production and preservation (5), and the importance of LAB as industrial microbes is just next to that of the yeast *Saccharomyces cerevisiae*. These bacteria are gram positive, non-spore forming, fastidious, acid tolerant, and strictly fermentative that secret lactic acid as the major end product of sugar fermentations (6). LAB are naturally present in media rich in organic nutrients such as

food products, and digestive tracts. They are a genetically diverse group of bacteria with GC contents varying from 34 to 53%, including rod shaped bacteria such as lactobacilli and cocci such as lactococci, enterococci, pediococci, and leuconostoc (7).

LAB are considered as safe and non pathogenic. They exhibit adjuvant properties, and are weakly immunogenic (8). Therefore, these bacteria are considered as attractive candidates for development of in vivo delivery vectors of biologically active molecules (e.g., antigens, enzymes or biological peptides) for vaccine and pharmaceutical purposes (9-13). It was shown that, extracellularly accessible antigens expressed on the surface of LAB are better recognized by immune system than those which are intracellular or secretory. Therefore, surface display is the technique of choice for making oral vaccine delivery vehicles with LAB and the research in cell surface display of LAB is aimed mainly at their use as oral vaccine vehicles (14).

1.3 SURFACE DISPLAY SYSTEMS FOR LAB

The anchoring domains that have been used for construction of protein display systems in LAB can be divided into three groups of cell wall bound proteins, cell membrane anchored proteins, and cell surface associated proteins.

1.3.1 Cell wall bound proteins

The most widely used surface anchoring system for LAB is based on the LPXTG motif anchor of cell wall bound proteins. This motif starts at the C-terminus with a short tail of positively charged residues that remain in the cytoplasm. Upstream of the cytoplasmic domain, a stretch of approximately 30 hydrophobic amino acids is preceded by the highly conserved

pentapeptide LPXTG. The charged tail and hydrophobic domain are thought to function as a temporary stop to position the LPXTG motif for proteolytic cleavage. Correct positioning results in cleavage between the threonine and glycine residues followed by amid-linkage of the threonine residue to the peptide crossbridge in the peptidoglycan of the cell wall, by the action of a sortase (15). This anchoring system is used for C-terminal fusion construct of target proteins. Prtp of *Lc. Lactis*, M6 protein of *Streptococcus pyogenes*, and Protein A of *Staphylococcus aureus* are among LPXTG type anchors which have been used widely for construction of surface display systems in *Lc. Lactis* and *Lactobacillus* (16-19).

1.3.2 Membrane bound proteins

The cell-membrane anchored proteins including transmembrane proteins and lipoproteins have also been used for protein display in LAB. In the case of transmembrane proteins, protein topology studies should be performed to specify suitable insertion sites. Insertion of target peptides in exterior loops between transmembrane domains may limit the insert size because it should be performed in a way that it does not disturb the topology of the membrane protein (20). However, display systems in which the target protein is simply linked at its N-terminus to a cytoplasmic membrane protein (LcnD, and PgsA) have also been described (21, 22).

Lipoprotein anchoring domains are characterized by a specific lipobox sequence including invariably a cystein residue located just after the cleavage site (23). These anchors can be used for fusion to the N-termini of target proteins. However, they detach from the cell-surface at specific growth phase (24).

1.3.3 Cell surface associated proteins

Nearly all bacterial cell wall hydrolases have a modular design, in which an active site degrades peptidoglycan and a cell wall binding domain immobilizes the enzyme on the peptidoglycan layer. The cell wall binding domain is often comprised of repeated amino acid sequences. The C-terminal region of lactococcal cell wall hydrolase AcmA contains three repeated sequences of 44 amino acids separated by streches of 21 to 31 amino acids rich in serine, threonine and asparagines residues. The association of this domain with the cell wall is of a non covalent nature and it can bind to the cells when it is added from the outside (25). BspA (26-28) and S-layer protein (SLP)(29) are also among the cell surface associated proteins which are anchored by charge interactions. SLPs form porous lattices of identical subunits completely covering the cell surface and may constitute up to 20% of the total cell protein content. These properties make them an attractive target for protein anchoring studies. However, expression of these proteins in heterologous hosts lacking these proteins such as *Lc. lactis* and *Lb. casei* resulted in secretion of the SLPs in the medium (30). This finding suggests that these cells lack a cell wall component required for proper attachment of SLPs and this may implicate that cell surface anchoring of these proteins is limited to the host from which the *slp* gene was isolated.

1.4 TWO MODES OF PROTEIN DISPLAY IN LAB

Depending on the type of anchoring domain, two modes of protein display can be considered for LAB including internal mode of display and external mode of display. In the case of internal mode, the fusion of target protein to the anchoring domain is expressed in the cell, and the protein is displayed on the surface of the expression host, whereas in the case of the external mode the expression host and display host are different from each other. If the association of the anchoring

domain with the cell surface is of a non covalent nature, it can bind to the cells when it is added from the outside. Therefore the fusion of the target protein to the anchoring domain can be produced in a suitable expression host capable of its correct folding and modifications. Then it can be purified and bound to the surface of desired protein display host cells which results in non genetically modified protein display system. A comparison between advantages and disadvantages of these two systems is given in Table 1.1.

TABLE 1.1 Comparison between two modes of protein display

	Internal	External
Control of display level	Limited	More flexible
Exposure of target protein outside of the cell wall	Incomplete	Full exposure
Anchoring stability	Often stable	Possibility of dissociation
Applicable host strains	_	Wider range (Production, Binding)

The systems based on the internal mode of protein display are often encountered with the problems in translocations and low levels of surface intensity of target proteins which have remarkable negative effects on the function of target proteins. In contrast, use of a display system based on the external mode of protein display can offer the advantage of full exposure of a target protein out of the cell wall, and the surface intensity of the protein can be adjusted by determining a suitable concentration for the protein and selection of a display host with a high binding capacity for the protein. However, regarding non covalent interaction of the target protein

with the cell surface, the possibility of dissociation of protein from the cell surface should be considered.

1.5 OVERVIEW OF THE PRESENT STUDY

Incomplete exposure of enzymes outside of the cell wall is often observed in the whole cell biocatalysts constructed based on the internal mode of protein display, and it limits access of large substrates such as starch which can not penetrate into the cell wall, to the enzymes. This limitation results in decreases in the apparent enzyme activities in these systems. This problem can be solved in a system based on the external mode of protein display because it results in full exposures of enzymes outside of the cell wall and it facilitates access of the substrates to the enzymes. However, the systems based on the external mode of protein display have been much less extensively studied compared with those based on the internal mode of protein display. Therefore, in chapter 2 ,we describe construction of α-amylase displaying LAB which are expected to be effective for direct fermentation of starch to lactate based on the external mode. In this study, *E. coli* was employed as the expression host and the C-terminal repeat region of peptidoglycan hydrolase (CPH) of *Lc. lactis* IL1403 was used as the anchoring domain. The efficiency of these systems is determined by cell surface binding and enzymatic activities of the hybrid biocatalysts and binding capacity and binding stability of display hosts. The fusion direction of an anchor protein to a target enzyme can induce conformational alterations in the fusion protein which affects its activities and the fusion of the anchor protein should be performed in a way that it does not make a steric hindrance for substrate binding to the enzyme. Therefore, the effect of the fusion direction on the activities of the cell-surface adhesive α-amylases was investigated, and several LAB

strains were examined for their binding capacities and stabilities for the cell-surface adhesive α-amylase. Finally, the effect of coexpression of molecular chaperones for increasing the production of the fusion protein in the soluble and active form in *E. coli* was investigated.

In chapter 3, we studied the possibility of production of cell-surface adhesive proteins in the methylotrophic yeast *Pichia pastoris*. This yeast is an attractive host for production of cell-surface adhesive proteins since compared with prokaryotic hosts such as *E. coli*, this organism has a better capability for the correct folding of recombinant proteins and disulfide bond formation. However, when a protein is expressed in this yeast, the influence of the post-translational modifications such as glycosylation on the protein properties should be considered. CPH contains several potential N-glycosylation sites, and attachment of glycoside chains at these sites may interfere with cell-surface binding activity of this domain. Therefore in this study, a CPH mutant devoid of the potential N-glycosylation sites (CPHM) was constructed which was expressed extracellularly in *P. pastoris*. The cell-surface binding activity of the constructed domain (CPHM) was studied and compared with that of the original domain (CPH) produced intracellularly in *E. coli*.

In chapter 4, we describe applicability of the external mode of protein display for increase in the survival of probiotics during passage through the upper gastrointestinal tract (GIT). The probiotic microorganisms can exert beneficial effects on human health if they are viable. Lactobacilli and bifidobacteria are most commonly used probiotics. However, these bacteria are not able to withstand the harsh acidity of the GIT. When these bacteria are mixed with starch or grown in the presence of starch, starch exerts a protective effect on the bacterial survival in acidic conditions. This fact is the basis for microencapsulation of bacteria within porous starch granules (bacterial core) which are then coated with amylose for the protection of probiotics. However, if the bacteria can not adhere to starch properly, it is not easy to

encapsulate them within starch granules because they may leak out of the pores. Therefore, the objective of this part of our research is to enhance delivery of viable microorganisms to the intestinal tract through conferring starch adhesion ability on them. We examined aggregation of bacteria with starch as an alternative technique for providing the bacterial core of microencapsulation. Compared with the previous method of preparing porous starch particles, this technique is easier, faster, and modification of starch granules is not required. In this way, the bacteria are entrapped between starch granules to take the advantage of protective effect of starch. Therefore, a cell-surface adhesive starch binding domain was constructed by fusion of CPH to a starch binding domain (SBD).The fusion protein was displayed on the surface of *Lb. casei* cells and suitable conditions for aggregation of bacteria with starch were determined. The aggregates were encapsulated with amylose and the survival of the cells under simulated conditions of upper GIT was examined.

Finally, the general conclusion was described in chapter 5.

CHAPTER 2

Bidirectional cell-surface anchoring function of the C-terminal repeat region of peptidoglycan hydrolase of *Lactococcus lactis* IL1403

2.1 INTRODUCTION

Microbial cell-surface display is potentially important in several areas of biotechnological applications, including construction of whole-cell biocatalysts (4, 31). The display of an enzyme on the cell surface by fusing it with one of cell-surface proteins of a host cell, (internal mode of protein anchoring) confers new functions on the host cell and the utilization of these systems for bioconversion processes is cost-effective because these enzymes can be readily recovered together with cells to reuse. However, the reduction in the apparent enzyme activity is an issue for these systems which is caused by incomplete exposures of enzymes on the outside of the cell and misfolded structures of biocatalysts. If an enzyme is not expressed on the outside of the cell wall, it is not accessible for a large substrate incapable of penetrating the cell wall. Therefore, decreases in the apparent enzyme activities are observed in these systems (32-36).

One possible solution for these problems is development of a whole-cell biocatalyst based on cell-surface adhesive enzymes which can bind to the cell-surface when added from the outside (external mode of protein anchoring). These enzymes can be constructed by a genetic fusion with a cell-surface adhesive domain. Use of this system can result in the full exposures of the enzymes outside of the cell wall and the enzymes can be folded correctly if produced in a suitable host strain.

The location of the fusion junction with the anchor protein is another important factor which

may influence the activity of the hybrid biocatalyst. For instance, activity of *Rhizopus oryzae* lipase whose active site is located at the C-terminal region, was strongly inhibited by fusion with a GPI anchor protein at the C-terminus, while fusing of the N-terminus of the lipase with the Flo1P flocculation functional domain enhanced the activity remarkably (36, 37).

It has been reported that the interaction of the C-terminal repeat region of peptidoglycan hydrolase (CPH) of *Lactococcus lactis* subsp. *cremoris* MG1363 with the cell wall of *Lc. lactis* cells is of a non-covalent nature and it can bind to these cells when added from the outside (38). For this reason, this domain is applicable for the production of the cell-surface adhesive enzymes. This domain has already been expressed in *Lc. lactis* for the display of peptides and proteins, and in all the cases it was fused at its N-terminus to a target protein (38-40).

Lactic acid bacteria (LAB) constitute an important group of industrial microorganisms that have been used widely for the fermentation and preservation of food products (41-43). We are interested in the construction of whole-cell biocatalysts based on LAB and the cell-surface adhesive enzymes for broadening or improvement of applications of these bacteria. For efficient construction of these whole-cell biocatalysts, three factors of cell-surface binding activity, enzymatic activity, and binding capacity of cells should be considered. CPH from *Lc. lactis* IL1403 is a homolog of CPH from *Lc. lactis* MG 1363. In this study, we investigated the capability of CPH from *Lc. lactis* IL1403 for the production of cell-surface adhesive enzymes in *Escherichia coli* using α-amylase (AMY) from *Streptococcus bovis* 148 as a target protein. The effects of the fusion direction on the binding activity of this domain for LAB cells and the enzymatic activities of the fusion proteins were studied and the binding capabilities of several LAB strains for these cell-surface adhesive α-amylases were investigated quantitatively. The whole-cell biocatalysts constructed in this study are expected to be effective for the direct fermentation of starch to lactate.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and growth conditions

E. coli XL1-Blue was used for construction of vectors and expression of heterologous proteins. It was grown in Luria-Bertani (LB) liquid medium or on LB agar plates at 37 °C. *Lc. lactis* subsp. *lactis* ATCC 19435 (American Type Culture Collection, Rockville, MD, USA), *Lactobacillus delbrueckii* subsp. *delbrueckii* ATCC 9649, *Lactobacillus casei* subsp. *casei* NRRL B-441 (Agriculture research service culture collection, Peoria, Illinois, USA), *Lb. casei* subsp. *rhamnosus* NRRL B-445, *Lactobacillus plantarum* NRRL B-531 were used for the binding assay. *Lc. lactis* subsp. *lactis* IL 1403 was used for the binding assay, and preparation of the genomic DNA. *Lactococcus* strains were grown at 30°C in M17 broth (Difco Laboratories, Detroit, MI, USA) containing 0.5% glucose (GM17). *Lactobacillus* strains were grown in MRS broth (Difco) at 37°C.

2.2.2 DNA manipulation

The C-terminal repeat coding region of peptidoglycan hydrolase gene (*acmA*) was PCR amplified from the chromosomal DNA of *Lc. lactis* IL1403 with cph-F and cph-R primers, and the PCR fragment was inserted into pQE21 derived from pQE31 (Qiagen GmbH, Hilden, Germany) with *Nco*I and *Bam*HI sites. The obtained plasmid was designated as pQCPH in which *cph* was fused with a hexa histidine tag gene at the N-terminus. The fragment including T5 promoter and α-amylase encoding gene (*amyA*) was prepared by PCR from pQE31amyA (44) with amyA-CF and amyA-CR primers. The PCR product was digested with *Xho*I and inserted into pQCPH which was digested with *Nco*I and made blunt using T4 DNA polymerase followed by cutting with *Xho*I. The resulting plasmid is pQAC in which *cph* was

fused to *amyA* at its N-terminus. To construct the C-terminus fusion of *cph* to *amyA*, the fragment including T5 promoter and CPH encoding gene was amplified from pQCPH with amyA-CF and cph-NR primers. The PCR product was digested with *Xho*I and *Bam*HI and introduced into the *Xho*I and *Bam*HI sites of the plasmid pQE31amyA. The obtained plasmid was designated as pQCA. The correctness of all of the constructs was confirmed by restriction digestion and sequencing.

TABLE 2.1 Oligonucleotide primers used

Primer	Sequence
cph-F	5′-tgcgcgccatgggtacttctaattccggtggttcaacagc-3′
cph-R	5′-gcggatccttatttaatacgaagatattgacc-3′
amyA-CF	5′-tctctcgagaaatcataaaaaatttatttgctttgtgagcg-3
amyA-CR	5′-ccttttagcccatctttattatagtttccag-3′
cph-NR	5′-aaggatccccttttaatacgaagatattgaccaattaaaatgg-3′

2.2.3 Expression studies

E. coli cells harboring the desired plasmids were grown overnight at 37°C in LB broth supplemented with 100 µg/ml ampicillin and 15 µg/ml tetracycline. The cells were then harvested by centrifugation and transferred to fresh LB broth containing the antibiotics as mentioned above, and incubated at 37°C until the OD_{600} reached 0.5. Isopropyl β-D -thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce the expression of target proteins. At the same time ampicillin was added to a final concentration of 400 µg/ml for plasmid maintenance. After further incubation for 4 h, the cells were collected and the expression was studied by resolving the whole cell extracts on 8% sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.4 Purification of fusion proteins

Proteins were purified under native conditions by metal affinity chromatography, utilizing the interaction between the histidine tag and a nickel chelate column (Ni-NTA superflow column (1.5 ml), Qiagen). The induced cells from a 100-ml culture were harvested by centrifugation, and were re-suspended in the binding buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole). Lysozyme was added to a final concentration of 1 mg/ml and the cell suspension was incubated for 1 h on ice. The cells were disrupted by sonication and the clear supernatant obtained by centrifugation was applied to the Ni-NTA column equilibrated with the binding buffer. The column was washed three times with the same buffer containing 20 mM imidazole and the bound proteins were eluted with the elution buffer, which was the same as the binding buffer except that it contained 250 mM imidazole. The buffer of the eluent was then exchanged to 50 mM sodium citrate buffer (pH 6.0) using PD-10 columns (GE Healthcare, Uppsala, Sweden). Purified proteins were subjected to 8% SDS-PAGE, and the bands were visualized by staining the gel using Coomasie Brilliant Blue R250. Gels were scanned using GT-F600 scanner (Epson, Suwa, Japan), and densitometrical analysis was performed with Scion image software (Scion, MD, USA) to quantify the proteins.

2.2.5 Binding assay and western blotting

LAB strains were grown as mentioned above until an OD_{660} of 1 was achieved. The cells from an 8-ml culture were dispersed in 0.5 ml GM17 medium containing the purified fusion protein at 2.4 mg/l, and incubated at 30°C for 2 h with gentle shaking. Free α-amylase was used in the control experiments at the same molar concentration with the fusion proteins. After

washing the cells with the citrate buffer, the cell pellets were resuspended in 2×SDS-PAGE loading buffer containing 20% (w/v) glycerol, 125 mM Tris-HCl (pH 6.8), 4% SDS, 5% (v/v) β-mercaptoethanol, 0.01% bromophenol blue and boiled for 5 min. The proteins were separated by 8% SDS-PAGE and electroblotted onto a PVDF membrane (Amersham Hybond-P, GE Healthcare Japan, Tokyo). Detection was performed with a horseradish peroxidase conjugated anti-pentahistidine antibody (Qiagen) using a chemiluminescence method (ECL plus western blotting detection system, GE Healthcare). The immunoblots were scanned and the amount of the fusion proteins bound to the cells was determined by densitometrical analysis of immunoblots as mentioned above using known amounts of the fusion proteins as standards.

2.2.6 Enzyme assays

α-Amylase activity for starch digestion was measured by the method of Giraud *et al.* (45) with some modifications. Briefly, 0.1 ml of appropriately diluted enzyme solutions were incubated with 0.8 ml of a solution containing 1% soluble starch (Nacalai Tesque, Kyoto) in the citrate buffer at 30°C with shaking at 100 rpm. The reaction was stopped by addition of 0.1 ml of 1 M H_2SO_4. Residual starch contents were determined colorimetrically at 620 nm by adding 0.1 ml of the reaction mixture to 2.4 ml of an iodine solution (1.2 g/l KI, 0.12 g/l I_2). One enzyme unit was defined as the disappearance of 1 mg iodine binding starch per min under the assay conditions. To determine the starch degradation activity of the fusion proteins in the cell-bound form, following the binding assay and washing the cells as described in the previous section, the cells were resuspended in 0.1 ml of the citrate buffer, and used for the starch digestion activity assay as mentioned above. For calculation of the specific activity of the enzyme in cell-bound form, the amount of the fusion proteins

determined by western blotting was used. The mean value of three replicates was reported. α-Amylase activity was also measured by an assay kit from Kikkoman Co. (Tokyo) using 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaoside (N3-G5-β-CNP) as substrate. The assay mixture contained 200 µl of reaction solution, 100 µl of the citrate buffer and 40 µl of cell suspension. The mixture was incubated at 37°C for 10 min, and the enzyme reaction was terminated by addition of 400 µl of the reaction stop solution supplied in the kit. The activity was assayed by measuring the absorbance of liberated 2-chloro-4-nitrophenol (CNP) at 400 nm. One unit of activity was defined as the amount of enzyme needed to release 1 µmol CNP per minute from N3-G5-β-CNP at 37°C.

2.2.7 Determination of dissociation rate constant

After performing the binding assay as described above, the cells were washed to remove unbound proteins, and incubated in the citrate buffer. At specified time intervals, the buffer was changed following centrifugation and a portion of the cells was withdrawn. The activity of the fusion proteins bound to the cells was determined with the α-amylase measurement kit as mentioned above. Since the dissociation of the proteins from cells can be regarded as a first-order reaction, the change in the activity of α-amylase bound to the cells (A) can be expressed as:

$$\frac{dA}{dt} = -kA \qquad (1)$$

where k is the dissociation rate constant. By integrating Eq. 1 with an initial condition of $A=A_0$ at $t=0$,

$$\ln\frac{A}{A_0} = -kt \qquad (2)$$

The dissociation rate constant was calculated based on the slope of ln A/A_0 vs t plot.

2.2.8 Coexpression of chaperones

For coexpression experiments, the chaperone plasmid set (Takara Bio Inc., Shiga) containing the plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16 was used. Initially, *E. coli* cells were transformed with one of the chaperone plasmids followed by transformation with pQCA. The coexpression was performed in LB medium containing 20 µg/ml chloramphenicol and 100 µg/ml ampicillin. The chaperone expression was initiated by addition of 0.5 mg/ml L-arabinose and/or 10 ng/ml tetracycline. When the OD_{600} reached 0.5, IPTG was added to a final concentration of 1 mM to induce expression of the fusion protein, and ampicillin was added to a final concentration of 400 µg/ml. After 4 h, the cells were harvested, resuspended in the binding buffer and sonicated. The amount of the protein in the soluble fraction was determined by SDS-PAGE as mentioned above, and the ratio of the amount of the protein in the soluble fraction to total amount of the expressed protein was considered as solubility percentage.

2.3 RESULTS

2.3.1 Expression and characterization of the fusion proteins

To investigate the effect of the fusion position on the cell-surface binding activity of CPH from *Lc. lactis* IL1403, two expression cassettes were constructed in which CPH was fused either at its N-terminus (pQAC) or at its C-terminus (pQCA) to α-amylase of *S. bovis* 148 (Fig. 2.1). The fusion proteins were expressed intracellularly in *E. coli* using the T5 promoter at 116 mg/l for AMY-CPH and 53 mg/l for CPH-AMY, and the observed molecular sizes for both the proteins were 100 kDa as expected.

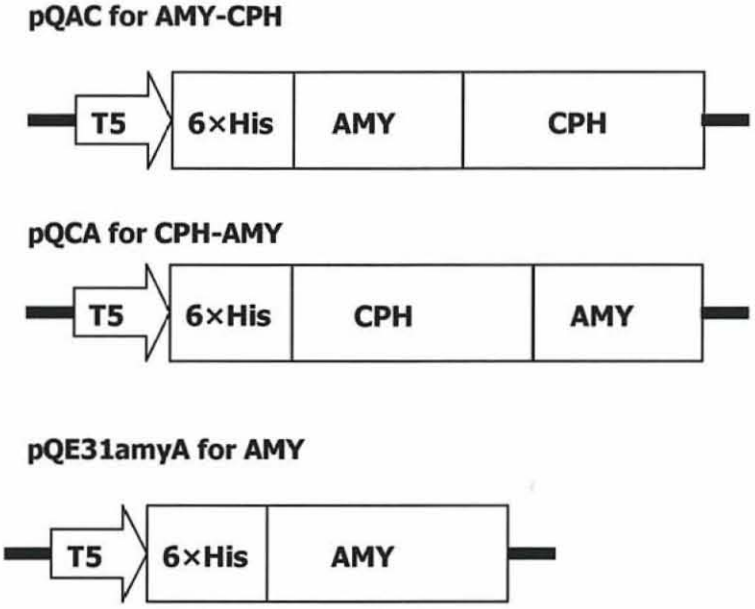


FIG 2.1 Structure of expression cassettes

The cells of *Lc. lactis* ATCC 19435 were mixed with the purified proteins and studied for the binding of the fusion proteins by western blotting. As shown in Fig. 2.2, both fusion proteins were associated with the cells. In addition, the antibody did not react with the cells not incubated with the purified fusion proteins, and also when the cells were incubated with free α-amylase expressed from pQE31amyA, no signal was detected (data not shown).

1 2

FIG. 2.2 Effect of fusion direction on the cell-surface binding activity of CPH. Lane 1, the cell bound AMY-CPH; lane 2, the cell bound CPH-AMY.

These results indicated that the binding of the fusion proteins to the cells was due to their CPH moiety and CPH was capable of binding to lactococcal cells either it was fused to α-amylase at its N-terminus or at its C-terminus. However, the number of bound molecules per cell was about three times more for CPH-AMY (1×10^3 molecules per cell) compared with AMY-CPH (3×10^2 molecules per cell).

When the specific activities of the fusion proteins in their free forms were determined for starch digestion (Table 2.2), it was about 11 times more for CPH-AMY (261 U/mg) compared with AMY-CPH (23 U/mg). Fusion of CPH to the N-terminus of AMY resulted in 40% decrease in the specific activity of AMY whereas that to the C-terminus of AMY resulted in 95% decrease in the specific activity. The binding of the fusion proteins to the cells resulted in 40% and 50% decrease in the specific activities of CPH-AMY (151 U/mg) and AMY-CPH (11 U/mg), respectively.

TABLE 2.2 Specific activity for starch digestion (U/mg)

Protein	Free form	Cell-bound form
AMY-CPH	23	11
CPH-AMY	261	151
AMY	426	–

2.3.2 Binding of CPH-AMY to LAB strains

In order to examine the binding capabilities of LAB strains for CPH-AMY, the purified protein was mixed with LAB cells. After incubation, the cells were examined for the binding of the

fusion protein by western blotting. CPH-AMY was observed to be associated with the cells of all the strains tested (Fig. 2.3). The antibody did not react with the cells not incubated with the fusion protein or incubated with free α-amylase.

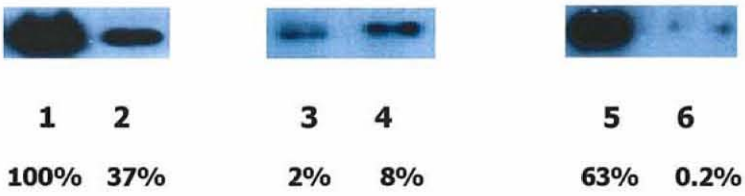


FIG. 2.3 Binding of CPH-AMY to LAB strains. Lane 1, *Lc. lactis* ATCC 19435; lane 2, *Lb. plantarum* NRRL B-531; lane 3, *Lc. lactis* IL 1403; lane 4, *Lb. casei* NRRL B-441; lane 5, *Lb. delbrueckii* ATCC 9649; lane 6, *Lb. casei* NRRL B-445. The estimated number of bound molecules of CPH-AMY to one cell of *Lc. Lactis* ATCC 19435 is 1×10^3 which was considered as 100% and the relative percentages for other cells were shown in the figure. No signal was detected for the cells incubated with citrate buffer or free α-amylase.

The highest binding capability was observed for *Lc. lactis* ATCC 19435 and the lowest one was observed for *Lb. casei* NRRL B-445. A high binding capability was observed for *Lb. delbrueckii* ATCC 9649 which was 63% of that of *Lc. lactis* ATCC 19435. Our calculations showed that 1×10^3 and 7×10^2 molecules of CPH-AMY bound to each cell of *Lc. lactis* ATCC 19435, and *Lb. delbrueckii* ATCC 9649, respectively. When the cells of *Lc. lactis* ATCC 19435 was incubated with a high concentration of CPH-AMY, the number of bound molecules increased remarkably. At the final concentration of 2.4 mg/ml for CPH-AMY in the binding assay mixture, the number of the bound molecules reached to 6×10^4 per cell.

2.3.3 Stable binding of CPH-AMY to the cell-surface

To study the stability of the binding of CPH-AMY to the cells of *Lc. Lactis* ATCC 19435, and *Lb. delbrueckii* ATCC 9649, the cells bound to CPH-AMY were incubated at a specified temperature and dissociation rate constants were determined based on the time courses of α-amylase activity on the cell-surfaces (Table 2.3).

TABLE 2.3 Dissociation rate constants of CPH-AMY for the cells at different temperatures (s^{-1})

Bacterial strain	4°C	30°C	37°C
Lb. delbrueckii ATCC 9649	$3.84 (\pm 1.07) \times 10^{-6}$ [a,b]	-	$6.96 (\pm 1.38) \times 10^{-6}$ [b]
Lc. lactis ATCC 19435	$1.08 (\pm 0.11) \times 10^{-4}$ [a,c]	$0.50 (\pm 0.25) \times 10^{-4}$ [c]	-

The statistical difference between each two values indicated with the same superscript (a or b or c) is significant at less than 5% probability.

The binding of CPH-AMY to the cells of *Lb. delbrueckii* ATCC 9649 was very stable, and the change in the activity of the bound protein during incubation was very low. The calculated dissociation rate constants at 4°C were 1.08×10^{-4} and 3.84×10^{-6} s^{-1} for *Lc. Lactis* ATCC 19435, and *Lb. delbrueckii* ATCC 9649 respectively. Increase in the temperature from 4 to 37°C resulted in 1.81 times increase in the dissociation rate constant for *Lb. delbrueckii* ATCC 9649. However, in the case of *Lc. Lactis* ATCC 19435, the dissociation rate constant at 30°C was 2.16 times lower than that at 4°C.

2.3.4 Effect of coexpression of chaperones on soluble expression of CPH-AMY

In the absence of chaperone coexpression, only 9% of CPH-AMY was present in the soluble form. To increase the solubility of CPH-AMY, coexpression of molecular chaperones was performed. When DnaK, DnaJ, and GrpE (pKJE7) were coexpressed with CPH-AMY, the solubility of the protein increased to 54%, and when other groups of chaperones were coexpressed the solubility of CPH-AMY increased to 34-35% (Table 2.4). The concentration of CPH-AMY in the soluble fraction increased three times by coexpression of trigger factor (pTf16) (16 mg/l) (46).

TABLE 2.4 Coexpression of chaperones

Chaperone plasmid	CPH-AMY in soluble fraction (%)	CPH-AMY in soluble fraction (mg/l)
–	9	5
pG-Tf2	36	7
pG-KJE8	34	7
pKJE7	54	12
pGro7	34	14
pTf16	35	16

GroES, GroEL and TF (trigger factor) were expressed from pG-Tf2; DnaK, DnaJ, GrpE, GroES and GroEL were expressed from pG-KJE8; DnaK, DnaJ and GrpE were expressed from pKJE7; GroES and GroEL were expressed from pGro7; TF was expressed from pTf16.

2.4 DISCUSSION

One of the objectives of this study is to develop an efficient system for the direct fermentation of starch to lactate by the construction of an α-amylase displaying lactic acid bacterium. Use of this system is advantageous over that of free α-amylase because the enzyme can be easily recovered together with cells to reuse. Starch is a large substrate which is not capable of penetrating the cell wall. For this reason, in order to achieve its efficient hydrolysis, the enzyme must be exposed on the outside of the cell wall in a way that it is accessible for starch. The display systems based on the internal mode of cell-surface anchoring are often associated with the limitation in the translocation of target proteins and mislocalization of a target protein can affect its activity negatively (32, 35, 47). In contrast, a display system based on the external mode of protein display can ensure the full exposure of a target protein outside of the cell. Therefore, a whole-cell biocatalyst based on the externally added cell-surface adhesive α-amylase was considered as a suitable selection for our purpose.

When CPH was fused at its C-terminus to α-amylase of *S. bovis,* as well as its N-terminal fusion, it was able to direct attachment of α-amylase molecules to the cells of *Lc. lactis* ATCC 19435 (Fig. 2.2). Therefore, it is a bidirectional anchor protein. However, interestingly for the C-terminal fusion construct, the number of the bound molecules increased three times. The change in the fusion direction may cause conformational alterations in the fusion protein leading to the better accessibility of CPH for cell-surface binding and increase in number of the bound molecules.

As shown in Table 2.2, the C-terminal fusion construct of CPH resulted in 11 times higher specific activity for the starch digestion compared with that of the N-terminal fusion

construct. The starch binding domains of this α-amylase are located at the C-terminus (48). Therefore, fusion of CPH to the N-terminus of α-amylase may help with more efficient adsorption of α-amylase onto starch and its degradation resulting in a higher specific activity for starch digestion.

It was observed that *Lc. lactis* ATCC 19435 was able to display 6×10^4 molecules per cell which is comparable with other surface display systems based on the internal mode of anchoring. The size of the α-amylase from *S. bovis* is very large (77 kDa), and usually an increase in the size of displayed proteins results in a decrease in the density of the proteins on the cell-surface. In the study by Maassen *et al.* (49), a surface density of 3.9×10^3 moleculse per cell was reported for the display of tetanus toxin fragment C (62 kDa) on the surface of *Lb. casei* using PrtP anchor protein. However, by the present system, a large protein (α-amylase, 77 kDa) was successfully displayed on the cell-surface at a density comparable to that of conventional systems.

The binding of CPH-AMY to the cells of *Lb. delbrueckii* ATCC 9649 was very stable and its dissociation rate constant at 37°C was 7×10^{-6} s^{-1} (the half life of the binding ($t_{1/2}$) was 28 h). The binding of this protein to the cells of *Lc. lactis* ATCC 19435 also was stable with dissociation rate constant of 5×10^{-5} s^{-1} at 30°C ($t_{1/2}$=4 h). It is known that lactate production by lactic acid bacteria is maximal during exponential growth phase. Therefore, for successful application of the constructed whole-cell biocatalysts in lactate production, suitable fermentation conditions should be specified for adjustment of duration of exponential growth phase with dissociation rate of the protein. These half lives are long enough for lactic acid fermentation if inoculm size is adequet and/or suitable growth conditions with high specific growth rates are used.

In the case of *Lc. lactis* ATCC 19435, the increase in the temperature resulted in the decrease

in the dissociation rate constant (Table 2.3). If the interaction between a protein and its ligand is mainly of a hydrophobic nature, the affinity for the ligand increases with an increase in temperature. However, it should not be the case for CPH. Because this domain is rich in Ser and Thr residues and it has a hydrophilic nature. Therefore, we can not exclude the involvement of temperature induced changes in the cell-surface structures in the decrease of the dissociation rate constant with the increase in the temperature. However, the exact reason for this observation remains to be clarified. The difference between the dissociation rate constants of *Lc. lactis* ATCC 19435 and *Lb. delbrueckii* ATCC 9649 at 4°C may be attributed to the difference in the target site of CPH. In addition, it has been reported that removal of peptidoglycan-associated polymers such as lipoteichoic acid by the acid treatment of lactococcal cells increased their binding capacity remarkably (38). Therefore, the difference between the binding capabilities of LAB for CPH-AMY can be attributed to the difference in cell-surface structures of these strains.

Coexpression of DnaK-DnaJ-GrpE with CPH-AMY showed the most pronounced effect on the prevention of the protein aggregation compared with that of other groups of chaperones (Table 2.4). However, regarding the expression level, the highest amount of CPH-AMY in the soluble form was observed for coexpression with trigger factor. This chaperone is assumed to play a role in protein folding because of its association with nascent polypeptides. Moreover, it can strengthen GroEL-substrate binding to facilitate protein folding or degradation (46). In future, other strategies such as fusion with solubility-enhancing proteins will be tried to improve production of CPH-AMY in the soluble form.

E. coli expression system with its ability of ease of genetic manipulation, low cost, and high expression level is the most attractive system for heterologous protein expression. The greatest disadvantage of this system is the formation of inclusion bodies and low refolding

yield. However, our results showed the effectiveness of coexpression of molecular chaperones as a suitable approach for increasing solubility of CPH-AMY in *E. coli*. In conclusion, the results of this study suggest that CPH is applicable for production of cell-surface adhesive enzymes in *E. coli*. However, a suitable fusion direction should be specified to ensure adequate cell-surface binding and enzymatic activities.

CHAPTER 3

Expression of C-terminal repeat region of peptidoglycan hydrolase of *Lactococcus lactis* IL1403 in methylotrophic yeast *Pichia pastoris*

3.1 INTRODUCTION

The display of proteins and peptides on the surface of cells by fusing them with the anchoring domains, has a broad range of potential biotechnological applications including the construction of diagnostic devices, vaccine delivery vehicles, and whole cell biocatalysts (2, 31, 50). Lactic acid bacteria (LAB) constitute a group of gram-positive bacteria which have extensively been used for the fermentation and preservation of food products (41-43). The nonpathogenicity of LAB has made them attractive for making oral vaccine delivery vehicles by the display of antigens (8, 51). The C-terminal repeat region of the peptidoglycan hydrolase (AcmA) of *Lactococcus lactis* subsp. *cremoris* MG1363 (CPH) is an anchoring domain which has been expressed in *Lc. lactis* for the display of proteins and peptides on the surface of this bacterium (27, 29, 38, 52). The interaction of this domain with the cell wall is of a non-covalent nature and since it can bind to cells when it is added from the outside, chimeric proteins need not to be expressed in the cells (38). For this reason, this mode of protein display can retain nongenetically modified status of the cells, and it is valuable for food and vaccine development (52).

In order to confer new properties on LAB by the display of desired proteins on them, we are interested to produce cell-surface adhesive proteins using CPH in the methylotrophic yeast *Pichia pastoris*. For many years, *Escherichia coli* has been used as a host microorganism for production of recombinant proteins due to its superior properties for

protein production including ease of genetic manipulation, low cost, and high expression level (53). However, as *E. coli* is a prokaryote, often it is not able to fold foreign proteins properly and perform other post-translational modifications such as disulfide bond formation (54). The formation of inclusion bodies is the main obstacle for protein production in this expression system, as the recombinant proteins must then be refolded into their native functional conformations using complex multistep processes that can lead to a significant decrease in the final yield of the protein (55). On the other hand, *P. pastoris* is a unicellular eukaryote that has many similarities to *E. coli* in terms of rapid growth, low cost, and ease of cloning foreign genes. Being a eukaryote, *P. pastoris* is capable of producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications that are essential for their functions including disulfide bond formation as the most important one (56). Several proteins generated as misfolded, insoluble inclusion bodies in *E. coli* obtained as soluble and correctly folded proteins when expressed in *P. pastoris*. For instance, when antigen 5 (Ag 5) was expressed in *P. pastoris*, it was shown to have the native structure of natural protein, but when it was expressed in *E. coli*, it did not show the native structure (57). In the study by Li *et al.* (58), successful secretory expression of herring antifreeze protein in *P. pastoris* was reported which exhibited full activity comparable with the native protein, whereas expression of this protein in *E. coli* resulted in the formation of inactive inclusion bodies which required further manipulation to get biological activity. In addition, *P. pastoris* has the capability of extracellular expression of recombinant proteins at high levels, and a simple purification of secreted proteins is possible due to the relatively low levels of native secreted proteins (56). Therefore, the use of *P. pastoris* is potentially more advantageous over that of *E. coli* or *Lc. lactis* with high nutritional requirement, and a costly cultivation (59), difficult genetic manipulation, and limited disulfide bond formation (60).

However, when a protein is expressed in this yeast, the influence of the post-translational modifications such as glycosylation on the protein properties should be considered. The glycosylation of a foreign protein that is nonglycosylated in its native form may be regarded as a drawback for this system because it may affect the protein functions. CPH contains several potential N-glycosylation sites. In addition, it includes serine and threonine residues which may be subjected to O-glycosylation. The attachment of glycoside chains at these sites may affect the cell surface binding activity of CPH. In this study, therefore, we report the extracellular production of a mutant of CPH (CPHM) devoid of the potential N-glycosylation sites in the methylotrophic yeast *P. pastoris*. The cell-surface binding activity of the constructed domain was studied and compared with that of the original domain produced intracellularly in *E. coli*.

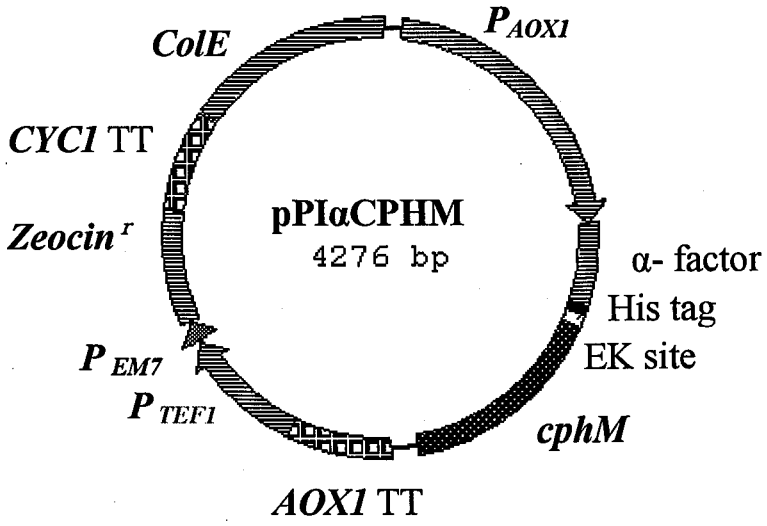
3.2 MATERIALS AND METHODES

3.2.1 Microorganisms and growth conditions

E. coli BL21 was used for the construction of vectors and the expression of heterologous proteins. It was grown in Luria-Bertani (LB) liquid medium or on LB agar plates at 37°C. *P. pastoris* GS115 (his4) (Invitrogen, Carlsbad, CA, USA) was used for protein expression. *Lactobacillus casei* subsp. *casei* NRRL B-441 (Agriculture research service culture collection, Peoria, IL, USA), *Saccharomyces cerevisiae* IFO0216 (Institute for Fermentation Osaka, Osaka), *E. coli* XL1-blue, and *Bacillus subtilis* 168 were used for the binding assay. *Lb. casei* NRRL B-441 was grown in MRS broth (Difco Laboratories, Detroit, MI, USA) at 37°C. *S. cerevisiae* IFO0216 was grown in YPD medium at 30°C. *E. coli* XL1-blue, and *B. subtilis* 168 were grown in LB medium at 37°C.

3.2.2 DNA manipulation

pQECPH (chapter 2) in which a hexa-histidine tag gene was attached at the N-terminus-encoding region of *cph*, was used as the template to amplify *cph* using 5′-tctctcgagaagagagaggctgaagcaatgagaggatcgcatcaccatcac-3′ and 5′-catgatgcggccgcttatttaatacgaagatattgacc-3′ as the forward and reverse primers, respectively. The PCR product was digested with *Xho*I and *Not*I (underlined) and subcloned into pPICZαC (Invitrogen) at the same restriction sites. The resultant plasmid is pPIαCPH. Site-directed mutagenesis was performed using a Quickchange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) to substitute five potential N-glycosylation sites using pPIαCPH as the template and three mutagenic primers 5′-acagctacaaataccaataCtaattcaCaAacaagctcaaccacttatac-3′ for N15T and N18Q, 5′-caagttcttcgtctaCtacaaCtagttcaacttcttcagg-3′ for N73T and N75T, and 5′-cgagttcaacttctaCctcttctgcagcttcaaGtacctctatccataaggttg-3′ for N168T and N174S (the converted nucleotides are shown in capital letters). After verification of the mutations by sequencing, the constructed plasmid (pPIαCPHM)(Fig. 1) and pPIαCPH were linearized using *Sac*I and introduced into *P. pastoris* by electroporation. The transformants were selected on YPDS medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol) containing 100 μg/ml zeocine (Invitrogen). The integration of the genes in the yeast chromosome was confirmed by genomic PCR using the 5′AOX1 primer (5′-gactggttccaattgacaagc-3′) and 3′AOX1 primer (5′-gcaaatggcattctgacatcc-3′), and the correctness of each gene sequence was confirmed by sequencing.



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CPH:  KREAEAMRGSHHHHHHGTSNSGGSTATNTNNNSNTSSTTYTVKS 28
CPHM: ----------------------------T--Q---------- 
GDTLWGISQKYGISVAQIQSANNLKSTVIYIGQKLVLTTSSSSSNTNSST 78
-----------------------------------------T-T---
SSGNSAGTTTPTTSVTPAKPASQTTIKVKSGDTLWGLSVKYKTTIAQLKS 128
-------------------------------------------------
WNHLNSDTIFIGQNLIVSQSAGSSSSSTGSSSASTSSTSNSSAASNTSIH 178
-----------------------------------T-----S----
KVVKGDTLWGLSQKSGSPIASIKAWNHLSSDTILIGQYLRIK*
----------------------------------------
```

FIG 3.1 Structure of pPIαCPHM and amino acid sequences of CPHM and CPH.

The plasmid pPIαCPH has the same structure as that of pPIαCPHM except that *cphM* was replaced with *cph*. The arrow represents the Kex2 cleavage site.

3.2.3 Expression of CPH in *E. coli*

E. coli BL21(DE3) cells harboring pQECPH were grown overnight at 37°C in LB broth supplemented with 100 µg/ml ampicillin. The cells were then harvested by centrifugation and transferred to fresh LB broth containing the antibiotic as mentioned above, and incubated at 37°C until the OD_{600} reached 0.5. Isopropylthiogalactoside was added to a final concentration of 1 mM to induce the expression of the target protein. At the same time, ampicillin was added to a final concentration of 400 µg/ml for the plasmid maintenance. After further incubation for 4 h, the cells were collected and the expression was studied by resolving the whole cell extract on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.4 Expression of CPHM in *P. pastoris*

To select a clone with a high expression level of CPH or CPHM, transformants were inoculated into 2 ml of buffered glycerol complex medium (BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6), 1.34% yeast nitrogen base (Difco Laboratories, Detroit, MI, USA), 4×10^{-5} % biotin, 1% (w/v) glycerol) and cultivated at 30°C with shaking at 240 spm until an OD_{600} of 6 was achieved. Then, the expression was induced by suspending 10 OD units of cells in 10 ml of buffered methanol complex medium (BMMY: the same composition as that of BMGY medium except for glycerol, which was replaced with methanol at 0.5%(v/v)) in 100 ml test tubes. The cells were cultivated for 4 days with addition of 0.05 ml of methanol every 24 h. For flask-scale expression studies, induction was initiated at an OD_{600} of 2 and methanol was added to 1%(v/v) every 24 h.

3.2.5 Protein purification

Proteins were purified by metal affinity chromatography, utilizing the interaction between the histidine tag and a nickel chelate column (Ni-NTA superflow column (Qiagen)). For purification of CPHM, ammonium sulfate was added to the culture supernatant at 80% saturation and pH 6. The precipitate collected by centrifugation was dissolved in binding buffer (50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, 10 mM imidazole), and insoluble materials were removed by centrifugation. The supernatant was desalted using a PD-10 column (GE Healthcare, Uppsala, Sweden) and it was applied to the Ni-NTA column, which had already been equilibrated with the binding buffer. After washing the column with the same buffer containing 20 mM imidazole, bound proteins were eluted with the elution buffer which was the same as the binding buffer except that it contained 250 mM imidazole. For the purification of CPH from *E. coli*, IPTG-induced cells were suspended in the binding buffer and disrupted by sonication. After centrifugation, the native CPH in the supernatant was purified as described above.

The purified proteins were subjected to 12.5% SDS-PAGE, and the protein bands were visualized by staining the gel using Coomasie Brilliant Blue R250. Gels were scanned and densitometrical analysis was performed to quantify the protein.

3.2.6 Binding of proteins to cells

The microorganisms were grown in appropriate conditions as mentioned above until an OD_{660} of 1 was achieved. Cells from 1 ml culture for the bacteria and 0.8 ml culture for the yeast were dispersed in GM17 medium (M17 (Difco) including 0.5% glucose) containing the purified proteins at 0.24 μM, and incubated at 30°C for 2 h with gentle shaking. After washing the cells three times with phosphate buffered saline (PBS) (50 mM potassium

phosphate (pH 7.2), 150 mM NaCl) containing 0.1% (w/v) Tween 20, the obtained pellet was resuspended in 2×SDS-PAGE loading buffer containing 20% (w/v) glycerol, 125 mM Tris-HCl (pH 6.8), 4% SDS, 5% (v/v) β-mercaptoethanol, and 0.01% bromophenol blue, and boiled for 5 min. The proteins were separated by 12.5% SDS-PAGE and electroblotted onto a PVDF membrane (Amersham-Hybond P, GE Healthcare, Buckinghamshire, UK). CPH and CPHM were detected with a horseradish peroxidase-conjugated anti-pentahistidine antibody (Qiagen) using a chemiluminescence method (ECL plus western blotting detection system (GE Healthcare)), and densitometrical analysis was performed to quantify the bound proteins.

3.2.7 N-terminal sequencing

The purified proteins were subjected to SDS-PAGE and electroblotted onto a PVDF membrane (Immobilon P^{SQ}, Millipore, Bedford, MA, USA). The membrane was stained with Coomassie brilliant blue R250 and the bands were excised to determine the N-terminal sequence. N-terminal sequencing was outsourced to Aproscience Co., Ltd. (Tokushima, Japan) and determined by the Edman method.

3.2.8 Determination of dissociation rate constant

The binding assay was performed as described above except that 8 ml of the cells of *Lb. casei* B-441 were dispersed in GM17 medium containing the purified proteins at 0.5 mg/l, and after washing cells to remove unbound proteins, the cells were incubated in PBS at 4°C. At specified time intervals, the buffer was changed following centrifugation and a portion of the cells was withdrawn. The time courses of the amounts of CPH and CPHM bound to the cells were analyzed by western blotting followed by densitometrical quantification of protein

bands. Because the dissociation of proteins from cells can be regarded as a first-order reaction, a change in the concentration of a complex is given as

$$\frac{dX}{dt} = -k_{off}X \qquad (1)$$

where X is the concentration of CPH (or CPHM) and k_{off} is the dissociation rate constant. By integrating Eq. (1), (X=X_0 at t=0),

$$\ln X - \ln X_0 = -k_{off}t\,. \qquad (2)$$

ln$X$ was plotted against time and k_{off} was calculated from the slope of the curve.

3.3 RESULTS

3.3.1 Binding of CPH produced intracellularly in *E. coli* to *Lactobacillus* cells

CPH from *Lc. lactis* IL1403 is a homolog of CPH from *Lc. lactis* MG1363. To investigate the cell-surface binding activity of CPH from *Lc. lactis* IL1403, this domain was expressed intracellularly in *E. coli* using the T5 promoter. To facilitate the purification of CPH, a hexa-histidine tag was introduced at its N-terminus. After IPTG induction, there was an obvious extra band at the molecular size of 32 kDa. Analysis of the soluble and insoluble fractions of the induced *E. coli* cell extracts indicated that 20% of the protein was present in the soluble fraction. The soluble protein was purified under native conditions by metal affinity chromatography (data not shown) and it was mixed with cells of *Lb. casei* B-441. After incubation, cells were harvested and examined for the presence of bound CPH by western blotting using the anti-histidine tag antibody. As shown in Fig. 3.2, CPH was observed to be associated with the cells. In addition, the antibody did not react with the cells which were not incubated with the purified CPH and the cells which were incubated with the proteins purified from the extract of *E. coli* cells containing the vector without the insert (data

not shown). Therefore, CPH is capable of binding to the cell surface when produced intracellularly in *E. coli*.

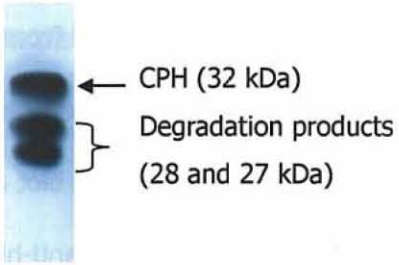

FIG. 3.2 Binding of CPH produced in *E. coli* to *Lb. casei* B-441 cells.

The CPH bound to cells was detected using the anti-histidine tag antibody. No signal was detected in the control experiment, in which the cells were mixed with elution buffer instead of CPH.

3.3.2 Mutation of N-glycosylation sites of CPH and selection of positive transformants

To delete the potential N-linked glycosylation sites (Asn^{15}-Asn-Ser, Asn^{18}-Thr-Ser, Asn^{75}-Ser-Ser, Asn^{168}-Ser-Ser, and Asn^{174}-Thr-Ser) of CPH, *Pichia* vector containing *cph*, pPIαCPH, was mutated from these asparagines residues to Thr, Glu, Thr, Thr, and Ser, respectively, resulting in the plasmid construct pPIαCPHM (Fig. 3.1). Since a new N-glycosylation site appeared at Asn^{73} by substitution of Asn^{75} to Thr, Asn^{73} was also mutated to Thr. After introduction of these two plasmids into *P. pastoris* GS115 by electroporation, the selected transformants , were cultured in BMGY broth, followed by methanol induction in BMMY and assayed for the secretion of the proteins by dot-blotting of the culture supernatant onto a PVDF membrane followed by detection with the anti-histidine tag antibody. All the selected clones exhibited positive indication for the presence of CPHM in the culture supernatant after induction with methanol. However, no positive clone for CPH was

observed. A clone with the highest level of secreted CPHM production was selected for further studies.

3.3.3 Characterization of CPHM secreted from *P. pastoris*

When the culture supernatant of the induced cells of GS115 (pPIαCPHM) was resolved on 12.5% SDS-PAGE and subjected to western blot analysis, two protein bands, corresponding to 50 and 35 kDa, were reacted with the anti-histidine antibody (Fig. 3.3A). After His-tag affinity chromatography, the purified proteins (Fig. 3.3B and 3.3C) were subjected to N-terminal sequence analysis. It showed that both protein bands contained Met-Arg-Gly-Ser-His-His-His at their N-termini. Therefore, the histidine tag was retained and the α-mating factor signal peptide was completely processed.

The cell surface binding activity of the produced proteins was examined by mixing cells of *Lb. casei* B-441 with the purified proteins. The protein binding was studied by western blotting. The 50-kDa band was capable of binding to the cells, whereas the 35-kDa band did not bind to the cells of this bacterium (Fig. 3.3D). The anti-histidine tag antibody did not react with the cells which were incubated with the proteins purified from the supernatant of the host strain with the chromosomal integration of the vector without the insert (data not shown).

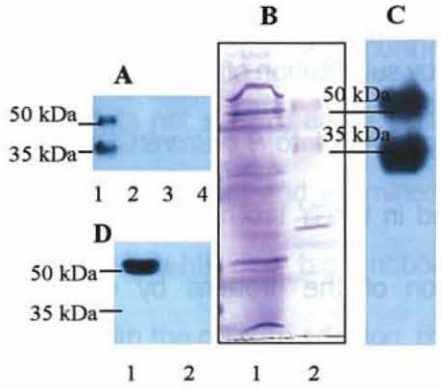

FIG. 3.3 Expression, purification, and binding of CPHM produced in *P. pastoris*. (A) Western blot of CPHM expressed in *P. pastoris*. Lane 1, supernatant of GS115/pPIαCPHM; lane 2, supernatant

of host strain (GS115); lane 3, cell pellet of GS115/pPIαCPHM; lane 4, cell pellet of GS115. (B) SDS-PAGE of culture supernatant of GS115/pPIαCPHM (lane1) and purified CPHM (lane2). (C) Western blot of pure CPHM (the same sample as that for lane 2 of panel B). (D) Binding of CPHM to the cells of *Lb. casei* B-441. CPHM bound to the cells was detected using the anti-histidine tag antibody (lane 1) and no signal was detected for the cells incubated with elution buffer instead of CPHM (lane 2).

3.3.4 Binding of CPHM and CPH to other cells

When the binding of purified CPHM to the cells of *E. coli* XL1-Blue, *B. subtilis* 168, and *S.cerevsiae* IFO0216 was examined by western blotting, it was observed that in addition to that of gram-positive bacteria including *B. subtilis* 168 and *Lb. casei* B-441, this domain was also able to bind to the surface of a gram-negative bacterium (*E. coli* XL1-Blue) and a yeast (*S.cerevsiae* IFO0216) (Fig. 3. 4).

1 2 3 4

FIG. 3.4 Binding of CPHM produced in *P. pastoris* to cells. CPHM bound to cells was detected using the anti-histidine tag antibody and no signal was detected for the cells incubated with elution buffer instead of CPHM. Lane 1, *B. subtilis* 168; lane 2, *E. coli* XL1-Blue; lane 3, *Lb. casei* B-441; lane 4, *S.cerevisiae* IFO0216.

The number of bound molecules of CPHM (produced in *P. pastoris*) per OD unit of cells and

the number of bound molecules of CPH (produced in *E. coli*) per OD unit of cells were calculated based on the densitometrical analysis of the western blot (Table 3.1)

TABLE 3.1 Binding of CPH and CPHM to cells

Microorganism	Number of bound molecules per OD unit		Ratio
	CPH	CPHM	
B. subtilis 168	6×10^{12}	4×10^{12}	0.67
E. coli XL1-Blue	3×10^{12}	3×10^{12}	1
Lb. casei NRRL B-441	6×10^{11}	3×10^{12}	5
S. cerevisiae IFO0216	2×10^{12}	6×10^{12}	3

These results indicated that the number of bound molecules per OD unit of *Lb. casei* B-441 cells for CPHM was 5 times more than that of CPH. In the case of *S.cerevisiae* IFO0216, 3 times increase in the number of bound molecules per OD unit of cells was observed for CPHM compared with CPH. However for *B. subtilis* 168, the number of bound molecules of CPHM per OD unit was 1.5 times lower than that of CPH, and in the case of *E. coli* XL1-Blue no difference between these values was observed.

3.3.5 Stable binding of CPHM and CPH on the cell surface of *Lb. casei*

The stability of the binding of CPHM secreted in *P. pastoris*, and CPH produced intracellularly in *E. coli* to cells of *Lb. casei* B-441 was studied by incubating the cells bound to the proteins in PBS at 4°C. As shown in Fig. 3.5, about 80% of CPHM remained bound to the cells after 3

h of incubation. The rate constants for the dissociation of the proteins from the cells (k_{off}) were estimated based on the time courses of the amount of the bound proteins. The k_{off} of CPHM produced in *P. pastoris* ($2\times10^{-5}\ s^{-1}$) was 3.5 times lower than that of CPH produced intracellularly in *E. coli* ($7\times10^{-5}\ s^{-1}$), and the difference was statistically significant at a 95% confidence level. These low dissociation rate constants are comparable with those of antibodies with very high affinities (61), and it shows the specificity of the binding.

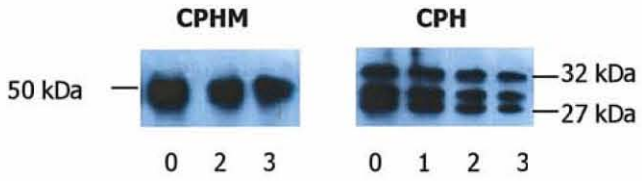

FIG. 3.5 Stable binding of CPHM and CPH to cells of *Lb. casei*. Cells bound to CPH or CPHM were incubated in PBS buffer at 4°C. The samples were taken at the time (h) indicated in the figure and were analyzed for the amount of the bound protein by western blotting using the anti-histidine tag antibody. The dissociation rate constant (k_{off}), estimated as described in materials and methods, was $2\times10^{-5}\ s^{-1}$ for CPHM, and $7\times10^{-5}\ s^{-1}$ for CPH.

3.4 DISCUSSION

The methylotrophic yeast *P. pastoris* is a popular expression host which has widely been used to produce various prokaryotic and eukaryotic proteins. In contrast to the prokaryotic recombinant expression systems such as those based on *E. coli*, the *P. pastoris* system has the ability to perform many of the post-translational modifications such as correct folding, disulfide bond formation, and proteolytic processing (56). The *P. pastoris* expression system has successfully been used to produce proteins that are highly disulfide-bonded whereas prokaryotic systems have been generally unsuccessful in achieving this (62). Furthermore,

extracellular expression in *P. pastoris* is an attractive option because this yeast secretes low-levels of endogenous proteins; therefore secretion of the expressed proteins may be considered as an effective purification step (56). Considering these advantages, we investigated the possibility of production of cell-surface adhesive proteins in this system in order to confer new properties on cells using these proteins.

Our result indicated that when CPH was produced intracellularly in *E. coli*, about 20% of the protein was present in the soluble fraction, which was in the active form and it could bind to the cells tested (Fig. 3.2). However, when CPH is fused with a desired protein to produce a cell-surface adhesive protein in *E. coli*, the fusion protein may not be correctly folded, and is mostly deposited as inclusion bodies with a usually low refolding yield. When CPH was fused with the α-amylase of *Streptococcus bovis*, only 9% of the protein was found in the soluble fraction (data not shown). Therefore, *P. pastoris* was considered as a more appropriate host for the expression of these proteins regarding its better capability for the correct folding of the proteins.

CPH is a prokaryotic protein and it contains five potential N-glycosylation sites which may be subjected to N-glycosylation in the yeast (Fig. 3.1). To avoid the possible interference of N-glycan chains with the cell-surface binding activity of this domain, these sites were substituted by site-directed mutagenesis. Our attempts for expression of native CPH in *P. pastoris* were not successful. However, we succeeded to express the mutated domain (CPHM) in *P. pastoris*. The reason for this observation remains to be clarified. When the mutated domain (CPHM) was expressed in *P. pastoris*, the apparent molecular sizes of the observed bands (50 and 35 kDa) were larger than that of the original domain (CPH) produced in *E. coli* (32 kDa) (Fig. 3.3A). Because the complete removal of the signal peptide was confirmed by N-terminal sequencing, and CPHM does not contain any N-glycosylation

sites, the increase in the apparent molecular size is attributed to O-glycosylation. The results of the cell binding assays showed that only the 50-kDa band was able to bind to the cell surfaces. Because both bands had the same N-terminal sequences, the 35-kDa band may have been derived from the 50 kDa band by proteolytic degradation at the C-terminal region.

It has been reported that the homolog of CPH from *Lc. lactis* MG 1363 binds noncovalently to a broad range of gram-positive bacteria (11). However, our result indicated that in addition to gram-positive bacteria, CPH and CPHM were able to bind to the surfaces of gram-negative bacteria and yeast, albeit to different extents (Table 3.1 and Fig. 3.4). CPH contains three homologous repeats called the LysM repeats, and it has been suggested that the LysM domain is a general peptidoglycan-binding module, although the precise component to which it can bind has not yet been established (63).

In the present study, it was observed that the dissociation rate constant of CPHM for the cells of *Lb. casei* B-441 decreased 3.5 times compared with that of CPH as a result of O-glycosylation in *P. pastoris* (Fig. 3.5), which indicated that the binding of O-glycosylated CPHM to the cells was stronger than that of native CPH. However, Boraston *et al.* (64) showed that O-glycosylation did not affect the adsorption of a carbohydrate-binding module produced in *P. pastoris* to cellulose because the O-linked glycans were far from the binding site and glycans at those positions were unlikely to affect the binding. Therefore, the effect of O-glycan chains on the binding to a ligand depends on the spatial conformation of the protein. In another study by Munro *et al.* (65), a role for O-glycosylation in the adhesion of *Candida albicans* to buccal epithelial cells was demonstrated by the significant reduction in adherence to the epithelial cells in the mutants with a reduced level of mannosyltransferase activity and truncated O-mannan. Our comparison of the numbers of bound protein molecules per OD unit cells of O-glycosylated CPHM and nonglycosylated CPH for cells of *Lb. casei* B-441 and

S.cerevisiae IFO0216 (Table 3.1) suggested a positive role for the involvement of O-glycan chains in the binding of CPHM to the surfaces of these cells. However, in the case of *E. coli* XL1-Blue, no effect was observed and a negative effect was observed for *B. subtilis* 168. Considering the differences in cell-surface structures of these microorganisms, these results indicated a cell-dependent role for the involvement of O-glycoside chains through interaction with the cell-surface structures in the adherence to cell-surfaces.

In conclusion, our results suggested the capability of CPHM for the production of cell-surface adhesive proteins in *P. pastoris*. Several applications may be considered for these proteins including an enhancement of the delivery of viable probiotics to the human gastrointestinal tract. Several genera of LAB including *Lb. casei* are well-known probiotics that can contribute to human health by maintaining or improving intestinal microbial balance. Many beneficial effects are exerted by probiotics (66, 67). However, their viabilities must be maintained during the product storage, and they must be able to survive the adverse conditions of the gastrointestinal tract (GIT) before reaching their site of action. It was shown that the microencapsulation of probiotics with starch or other food grade polymers can help enhance the survival of probiotics during storage, during passage through the human GIT, and when being incorporated in dairy products, because microencapsulation segregates the cells from adverse environments; thus, potentially reducing cell injuries (67, 68, Valton, T. *et al.*, European patent 1999-WO9952511).

The fusion of CPHM with a starch binding domain (SBD) will confer nongenetically modified probiotic strains with the capability of binding to starch, which can facilitate their microencapsulation with starch. Therefore, the results of the present study may contribute to the development of probiotic production technologies.

CHAPTER 4

A new strategy for enhancement of microbial viability in simulated gastric conditions based on the display of starch binding domain on the cell-surface

4.1 INTRODUCTION

Probiotics are live microbial food supplements, which benefits the health of consumers by improving their intestinal microbial balance (69). These bacteria have been increasingly included as functional ingredients in dairy products such as yoghurts and fermented milks (70). Since the viability and activity of a probiotic is essential at the site of action, it must survive passage through the upper gastrointestinal tract (GIT), and it must be able to function in the gut environment (71). Most commonly used probiotics are lactobacilli and bifidobacteria (72). However, several studies indicate that most of these bacteria may not be able to withstand the harsh acidity of the GIT (73, 74). Different approaches have been proposed to protect probiotics from environmental stresses including appropriate selection of acid resistant strains, stress adaptation, and microencapsulation (75). Microencapsulation segregates cells from the adverse environment; thus it reduces the cell injuries. Food grade polymers such as alginate, chitosan, carboxymethyl cellulose, carrageenan, gelatin, pectin and starch are mainly applied for microencapsulation using different technologies (76). Valton *et al.* have recently developed a microencapsulation technology that involves entrapping bacteria in the hollow core of partially hydrolyzed starch granules (bacterial core), which are then encapsulated in an outer coating of amylose (Valton, T. *et al.* European patent

1999-WO9952511). The aim of this technology is to protect the probiotic bacteria from adverse environmental conditions during processing, during storage in products, and during passage through the GIT, and it is based on the fact that starch granules can be used to protect living microbes from environmental stresses (Valton, T. *et al.* European patent 1999-WO9952511). It is expected that bacterial adhesion to starch can facilitate encapsulation of the bacteria when this technology is used (67). Because adherence of the bacteria to starch will increase the density of the cells entrapped in the starch granules, and lower number of the cells may leak out of the pores of the porous starch granules when preparing the bacterial core. In addition, it is known that an increase in the initial cell load of probiotic products results in an increase in the bacterial survival on exposure to the GIT conditions. Therefore, it is expected that adhesion to starch can enhance the delivery of viable probiotics to the intestinal tract. However, not all of the desired probiotics possess proper starch adhesion abilities. In the study by Crittenden *et al.* (77), several strains of bifidobacteria were examined for adhesion to starch, and it was shown that starch adhesion was not characteristic of all of the bifidobacteria tested. Moreover, using genetic engineering techniques to confer starch binding ability on probiotics is not favorable because of consumers' concerns about genetically modified foods.

In chapter 2 and chapter 3, we showed that the C-terminal region of peptidoglycan hydrolase (CPH) of *Lactococcus lactis* IL1403 is an efficient anchoring domain for the display of heterologous proteins on cells which can bind to the cell surface when it is added from the outside. When fusion of CPH to α-amylase was produced in *E. coli,* CPH was able to direct binding of the enzyme to the cell surfaces of various lactic acid bacteria. For this reason, this domain can be used to confer new properties on the cells without making any genetic modifications in them. In this chapter, therefore, we investigated the capability of CPH for the

display of a starch binding domain on the surface of *Lactobacillus casei* cells, and aggregation of the cells with starch was examined as an alternative technique of providing the bacterial core of microencapsulation. This is the first report to demonstrate potential applicability of the cell-surface display technology for enhancement of delivery of viable microorganisms to the intestinal tract.

4.2 MTERIALS AND METHODS

4.2.1 Bacterial strains and growth conditions

E. coli XL1-Blue was used for the construction of vectors and the expression of heterologous proteins. It was grown in Luria-Bertani (LB) liquid medium or on LB agar plates at 37°C. *Lactobacillus casei* NRRL B-441 was used for the binding assay, and it was grown at 37°C in MRS broth (Difco Laboratories, Detroit, MI, USA).

4.2.2 DNA manipulation

The gene encoding the linker and the first nine amino acid residues of the starch binding domains of the α-amylase of *Streptococcus bovis* was prepared by PCR from pQE31amyA (44) with 5′-aaggatccgggccaagctagccaagcagctc-3′ and 5′-gcgccaattatctgggttttgg-3′ as forward and reverse primers respectively. The amplified fragment was digested with *Bam*HI and *Bst*XI and inserted at the same restriction sites into pQCA (chapter 2). The obtained plasmid was designated as pQCLS (Fig. 4.1). The correctness of the construct was confirmed by restriction digestion and sequencing.

FIG. 4.1 Structure of expression cassette

4.2.3 Expression studies

E. coli cells harboring the desired plasmids were grown overnight at 37°C in LB broth supplemented with 100 µg/ml ampicillin and 15 µg/ml tetracycline. The cells were then harvested by centrifugation and transferred to fresh LB broth containing the antibiotics mentioned above, and incubated at 37°C until the OD_{600} reached 0.5. Isopropyl β-D -thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce the expression of the target protein. At the same time ampicillin was added to a final concentration of 400 µg/ml for plasmid maintenance. After further incubation for 4 h, the cells were collected and the expression was studied by resolving the whole cell extracts on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.4 Purification of the fusion protein

Proteins were purified under native conditions by metal affinity chromatography, utilizing the interaction between the histidine tag and a nickel chelate column (Ni-NTA superflow column (1.5 ml), Qiagen GmbH, Hilden, Germany). The induced cells from a 100-ml culture were harvested by centrifugation, and were re-suspended in the binding buffer (50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, 10 mM imidazole). Lysozyme was added to a final concentration of 1 mg/ml and the cell suspension was incubated for 1 h on ice. The cells were disrupted by sonication and the clear supernatant obtained by centrifugation was applied to the Ni-NTA column equilibrated with the binding buffer. The column was washed three times with the same buffer containing 20 mM imidazole and the bound proteins were eluted with the elution

buffer which was the same as the binding buffer except that it contained 250 mM imidazole. The buffer of the eluent was then exchanged to 20 mM Tris-Cl buffer (pH 8.0) by ultrafiltration. The protein preparation was applied to an anion exchange column (SuperQ-5PW, Tosoh) equilibrated with 20 mM Tris-Cl buffer (pH 8.0). The absorbed proteins were eluted by a linear NaCl gradient (0-1M). Protein elution was monitored using a UV detector, and the desired fraction was collected and desalted by ultrafiltration. Purified proteins were subjected to 12.5% SDS-PAGE, and the bands were visualized by staining the gel using Coomasie Brilliant Blue R250. Gels were scanned using GT-F600 scanner (Epson, Suwa, Japan), and densitometrical analysis was performed with Scion image software (Scion, Maryland, USA) to quantify the proteins.

4.2.5 Cell-surface binding assay

Lb. casei cells were grown as mentioned above until an OD_{660} of 1 was achieved. The cells from a 1.5-ml culture were dispersed in 0.15 ml MRS medium containing the purified fusion protein at 0.12 mg/ml, and incubated at 30°C for 2 h with gentle shaking. After washing the cells twice with 0.1 M phosphate buffer (pH 7.0) (PB), the cell pellets were resuspended in 2×SDS-PAGE loading buffer containing 20% (w/v) glycerol, 125 mM Tris-HCl (pH 6.8), 4% SDS, 5% (v/v) β-mercaptoethanol, 0.01% bromophenol blue and boiled for 5 min. Binding of the protein to the cells was studied by 12.5% SDS-PAGE followed by CBB staining, and the amount of the fusion protein bound to the cells were determined by densitometrical analysis of CBB stained gels as mentioned above.

4.2.6 Starch binding assay

200 µl of the protein purified by the his-tag column (0.06 mg/ml in PB) was mixed with an

equal volume of a suspension of starch granules (Corn starch, Sigma-Japan, Tokyo, Japan,) (10 mg/ml) in the same buffer and incubated at 37°C for 3 h with gentle shaking. After centrifugation, the supernatant was examined for the presence of unbound proteins by SDS-PAGE.

4.2.7 Aggregation of bacteria with starch and microencapsulation

After the performing binding assay as mentioned above, the cells were washed and resuspended in PB to a final density of 1×10^9 cells ml^{-1}. Equal volumes of the cell suspension and starch granules suspended in PB, were mixed for 30 min and allowed to stand at room temperature for 1h. Formation of aggregates was studied both visually and with phase contrast microscopy. To determine the percentage of the cells adhering to starch under these conditions, after sedimentation, 0.5 ml sample was taken from below the surface of the liquid. Optical density at 540 nm was measured and compared with those of the controls (bacteria without starch, and starch without bacteria) to calculate the starch adhesion percentage as described by Crittenden et al (77). For coating of the aggregates with amylose, an 1% solution of amylose in water (amylose from potato, Sigma) was prepared by heating it to a temperature of 170°C in a pressure heater (Taiatsu Techno, Tokyo, Japan) which was then cooled down to 37°C. The aggregates were mixed gently with 0.5 ml of the amylose solution and the coating was allowed to form overnight at 4°C.

4.2.8 Survival of cells in simulated gastric juice

The simulated gastric juice was prepared as described by Lian *et al.* (78), which was a pepsin solution (3 g l^{-1}) in saline (0.5%). The juice was prepared freshly, and its pH was adjusted to 2.0 or 3.0 with 5 M HCl. The amylose coated cells were mixed with 1 ml of the filter sterilized

simulated gastric juice, and incubated at 37°C. At specified time intervals, the gastric juice was removed after centrifugation and the cells were washed once with PB following with two washes with saline. The cells were then resuspended in PB containing 30 U ml^{-1} α-amylase (Megazyme, Bray, Ireland) and incubated at 40°C for 20 min to aid release of the cells from the encapsulating materials. Viable bacteria were enumerated on MRS-agar after incubation for 24 h at 37°C and the survival percentage was determined by dividing the final viable population (cfu ml^{-1}) with the initial viable population (cfu ml^{-1}) of the *Lb. casei* cells inoculated to the simulated gastric juice.

4.3 RESULTS

4.3.1 Expression and purification of the fusion protein

In chapter 2, we showed that fusion of CPH to the C-terminus of α-amylase of *S. bovis* resulted in the decrease in the starch degradation activity of this enzyme compared with fusion to it N-terminus. The starch binding domain of this α-amylase is located at its C-terminus. Fusion of CPH at the C-terminus of this enzyme can make steric hindrance for binding of starch to the starch binding domain and it causes the decrease in the enzyme activity. Thus the C-terminus of this starch binding domain is necessary for its function and it should be free. For this reason in this study, the capability of CPH for the construction of a cell-surface adhesive starch binding domain was investigated by fusion of CPH to the N-terminus of the starch binding domain (Fig. 4.1). The fusion protein was expressed intracellularly in *E. coli* using the T5 promoter at 0.35 g l^{-1}. The molecular size was 56 kDa as expected and 75% of the protein was present in the soluble fraction. When the protein was purified under native conditions by the histidine-tag affinity chromatography, two additional

bands at the molecular weight of 73 and 71 kDa were also present in the protein preparation (Fig. 4.2, lane 1). After incubation of the purified proteins with starch, no bands for target protein, and the protein at 71 kDa were detected in the supernatant, and the concentration of the protein at 73 kDa in the supernatant was lower than that of the initial one (Fig. 4.2, lane 2). These results showed that in addition to the target protein, these two proteins also were able to adhere to starch. Therefore, in order to confirm the activity of the target protein for adhesion to starch, these bands were successfully separated from CPH-SBD by the anion exchange chromatography (Fig. 4.2, lane 4). The result of the starch binding assay showed that the purified protein is in the active form and it is able to adhere to starch (data not shown).

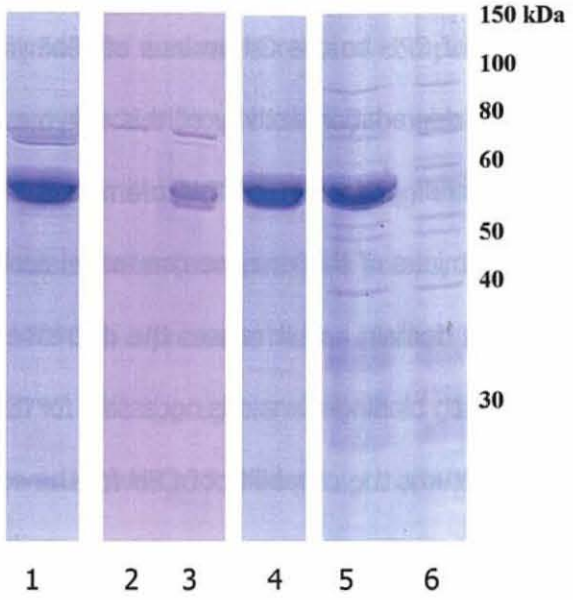

FIG. 4.2 Purification of CPH-SBD, its binding to starch, and to *Lb. casei* cells. Lane 1, nickel chelate column purified protein preparation; lane 2, supernatant after starch binding assay; lane 3, control without starch; lane 4, ion exchange chromatography purified CPH-SBD; lane 5, cells

bound to CPH-SBD; lane 6, cells only .

4.3.2 Binding of CPH-SBD to the surface of *Lactobacillus* cells

The cells of *Lb. casei* were incubated with the purified CPH-SBD and studied for binding of the protein by SDS-PAGE. As shown in Fig. 4.2 (lane 5), the protein was observed to be associated with the cells, and the result of densitometrical analysis showed that 6×10^4 molecules of CPH-SBD bound to each cell of *Lb. casei*.

4.3.3 Aggregation of the bacteria with starch

For aggregation of bacteria with starch, it is necessary to specify an optimal ratio between bacteria and starch. Therefore, dependence of aggregate formation on starch concentration at a constant cellular density was investigated. Free cells mixed with starch, and starch without cells were used as controls. For each case, we compared the volume of the sediment formed in the samples containing the bacteria bound to CPH-SBD and starch with those of the controls visually. The result is shown in Table 4.1.

TABLE 4.1 Comparison of sediment formation at different starch concentrations

Starch concentration (mg ml $^{-1}$)	BPS[1]	BS[2]	S[3]
1	+	+	+
2	++	+	+
5	+++	+	+
10	+	+	+

[1]: Mixture of bacteria displaying CPH-SBD and starch

[2]: Mixture of bacteria and starch

[3]: Starch only

The volumes of the sediments formed after 1 h standing of the samples at room temperature, were almost the same for the controls in all the cases tested. When the starch concentration was 5 mg ml^{-1}, the volume of the formed sediment in the sample containing the cells bound to the fusion protein and starch was remarkably larger than those of the controls. When the adhesion percentage of the cells to starch granules was measured under these conditions, it was 32% for the bacteria bound to CPH-SBD and 4% for the free cells.

Formation of aggregates was confirmed by microscopic observation for the bacteria bound to CPH-SBD (Fig. 4.3), whereas the starch granules were separated from each other in the case of the free cells (data not shown).

FIG. 4.3 Aggregation of *Lb. casei* cells displaying CPH-SBD with starch granules

4.3.4 Survival of cells in the simulated gastric juice

When free cells of *Lb. casei* were subjected to the simulated gastric juice at pH 3.0 or 2.0 for 1 h, the survival of the cells was 0.074% and 0.002% respectively. However, when the amylose coated bacterial aggregates were subjected to the gastric juice for 1 h, the survival

was increased to 64% and 6% at pH 3.0 and 2.0 respectively (Fig. 4.4).

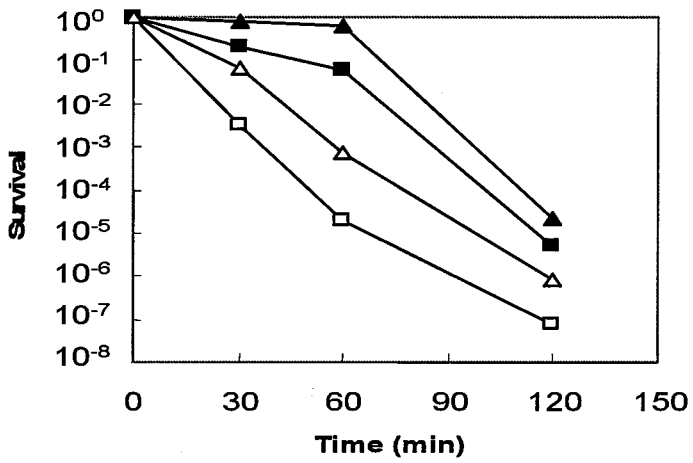

FIG. 4.4 Timecourse of survival of *Lb. casei* cells in the simulated gastrointestinal conditions. Open triangle and filled triangle: free cells and amylose coated aggregates respectively (pH 3.0); open circle and filled circle: free cells and amylose coated aggregates respectively (pH 2.0).

TABLE 4.2 Effect of system components on survival

Bacteria	CPH-SBD	Starch	Amylose	Survival (%)
+	+	+	+	64
+	-	+	+	37
+	+	-	+	7
+	+	+	-	11
+	-	-	-	0.074

We compared the effects of different components of the system on the survival at pH 3.0 (Table 4.2). It was observed that the survival of the bacterial aggregates in the absence of amylose coating was 11%, and when the bacteria bound to the fusion protein was coated with amylose without aggregation with starch, the survival was 7%. Incorporation of CPH-AMY to the system resulted in 27% increase in the survival.

4.4 DISSCUSSION

In the study by Wang *et al.* (79), it has been reported that survival of bifidobacteria in acidic conditions enhanced as a result of growth in the presence of starch and also mixing with the starch granules. However, the exact mechanism for the protective effect of starch is not known, and the adhesion of these bacteria to the starch granules and the bulking capacity of the starch which may markedly modify the pH of stomach were considered as possible explanations for these observations. The result of their study suggested that, the starch granules can be used to protect living microbes from environmental stress factors, and this fact is the basis for development of the technique of encapsulating probiotics within starch granules (Valton, T. *et al.* European patent 1999-WO9952511). The objective of the present study is, therefore, to enhance delivery of viable microorganisms to the intestinal tract through conferring starch binding ability on them. In this way, the bacteria are entrapped

between starch granules in order to take advantages of the protective effect of starch. Our result showed that CPH was able to direct binding of SBD of α-amylase of *S. bovis* to the cell-surface of *Lb. casei*, and the results of the starch binding assay showed that the SBD fused with CPH was in the active form and it could bind to the starch granules. Therefore, when the fusion protein was produced in *E. coli*, it was able to direct adhesion of the cells to the starch granules. We examined aggregation of the cells with starch as an alternative protective strategy for entrapping bacteria between the starch granules. Compared with the previous method of entrapping bacteria within the porous starch granules which were prepared by an enzymatic digestion, this technique is much simpler and faster. Moreover, the starch granules can be used in their intact forms without any modifications. When the amylose coated bacterial aggregates were subjected to low pH, there was a significant increase in the survival (64% at pH 3.0, and 6% at pH 2.0), compared with the free cells (0.074% at pH 3.0, and 0.002% at pH 2.0) under the same conditions. These results indicate the effectiveness of the developed technique for protection of the bacteria. As shown in Table 4.2, the protective effect of the entrapment of the bacteria between the starch granules with the aid of CPH-SBD on the survival was comparable with that of using only the amylose coating (11% and 7% respectively). In addition, 1.73 times increase in the bacterial survival was observed as a result of the aggregation of the bacteria with starch using CPH-SBD

compared with the mixing of the free cells with starch (64% and 37% respectively). The most pronounced positive effect on the survival was observed when all of the components (fusion protein, starch, and amylose) acted together. In conclusion, the results of this study demonstrated the effectiveness of the cell-surface display technique for protection of the cells from adverse gastric conditions, and also it is expected that the constructed fusion protein can result in an improvement of the known technology of encapsulating probiotics in the porous starch granules.

CHAPTER 5

General conclusion

Utilization of enzymes displayed on the cell surface in bioconversion processes is cost effective since the enzymes can be easily recovered together with cells to reuse. However, the reduction in the apparent enzyme activity is an issue when internal mode of protein display is used, which is caused by incomplete exposures of enzymes outside of the cell and misfolded structures of biocatalysts. These problems can be circumvented in a display system based on the external mode of protein display. However, optimal performance of these systems is dependent on the efficiency of the expression host and the display host. Therefore, in the present study construction of a whole cell biocatalyst based on the cell surface adhesive enzymes was studied using *E. coli* as the expression host and the α-amylase as the target enzyme. However, as *E. coli* is a prokaryote, its ability for correct folding of proteins is limited and formation of inclusion bodies is the main obstacle of this expression system. In contrast, *P. pastoris* is a eukaryotic host which is capable of producing soluble, correctly folded recombinant proteins that have gone under all the post-translational modifications necessary for their functions. For this reason, we investigated the possibility of production of cell surface adhesive proteins in this expression host, and finally we showed the potential applicability of the external mode of protein display for enhancement of delivery of viable microorganisms to the intestinal tract. The result of each step of this research is summarized as followings:

In chapter 2, we showed that when the C-terminal region of peptidoglycan hydrolase of *Lc. lactis* IL1403 was produced in *E.coli*, it was in the active form and when it was fused to the

α-amylase of *Streptococcus bovis*, it could direct binding of the enzyme to the cell surface of *Lc. lactis* ATCC 19435. The positive effect of change in the fusion direction on the cell-surface binding activity and enzymatic activity of the cell surface adhesive enzyme was demonstrated, and the capabilities of several strains of lactic acid bacteria for binding to this enzyme were studied. Among the LAB tested, *Lc. lactis* ATCC 19435 showed the highest binding capability and *Lb. delbrueckii* showed the highest binding stability. Furthermore, the effect of coexpression of molecular chaperones on improvement of the production of the adhesive enzyme in the soluble form was studied.

In chapter 3, we constructed a mutant of CPH devoid of the potential N-glycosylation sites to avoid the possible interference of attached N-glycoside chain with the cell surface binding activity of this domain. This domain was successfully produced extracellularly in *P. pastoris* in the active form, and we observed a remarkable increase in the binding stability of this domain compared with that of the original domain produced in *E. coli* as a result of O-glycosylation in *P. pastoris*. We showed that in addition to gram-positive bacteria, this domain is able to bind to the cell surfaces of gram-negative bacteria and yeast. Therefore, it is a suitable fusion partner for the construction of a variety of protein display systems.

In chapter 4, we showed the effectiveness of the external mode of protein display for protection of microorganisms under simulated intestinal conditions. When CPH was fused to the starch binding domain (SBD) of α-amylase of *Streptococcus bovis* , it was able to direct binding of SBD to the cell surface of *Lb. casei* cells. Therefore, binding of the cells to starch was mediated by the fusion protein. Then the suitable conditions for aggregation of these bacteria with starch were determined. The formed aggregates were coated with amylose and subjected to simulated gastric juice. As a result, 64% increase in the survival of protein coated bacteria compared with free cells was observed.

In conclusion, external mode of protein display is useful in the applications for which the use of genetically modified organisms is not favorable such as foods and vaccines, and also it should be used when the full exposures of target proteins outside of the cell are required such as a biocatalyst with a huge substrate incapable of penetrating the cell wall. The result of the present study demonstrated for the first time the applicability of *P. pastoris* as a protein expression system for the development of protein display systems based on the external mode. In addition, for the first time, the efficiency of *E. coli* for production of cell-surface adhesive enzymes and cell-surface adhesive starch binding domain was demonstrated, and we showed the importance of the fusion direction on the activities of cell surface adhesive proteins. The cell-surface associated domains have also been studied by other research groups. However, most of these binding domains can bind to specific species or they are not able to bind to the microbes of industrial value (80, 81). For example the cell wall binding domain of *Staphylococcus aureus* autolysin was able to bind to the surface of some of gram-positive bacteria but it was not able to bind to the surface of *Lactobacillus sp.* and *E. coli*, and the treatment of these bacteria with trichloroacetic acid was required for binding of this domain which gives non living status to the cells. In the case of *Saccharomyces cerevisiae* even after acid treatment, binding of the domain was not observed (80). However, the result of the present study demonstrated the capability of CPH for binding to the surface of gram-positive and gram-negative bacteria and yeast cells. This finding opens new lines of research for construction of protein display systems on other microorganisms especially on yeast.

REFERENCES

1. Westerlund B., and Korhonen T.K. 1993. Bacterial proteins binding to the mammalian extracellular matrix. Mol. Microbiol. 9: 687-694.
2. Lee S. Y., Choi J. H., and Xu Z. 2003. Microbial cell-surface display. Trends Biotechnol. 21: 45-52.
3. Hansson M., Samuelson P., Gunneriusson E., and Stahl S. 2001. Surface display on gram positive bacteria. Comb. Chem. High throuput Screen. 4: 171-184.
4. Kondo A., and Ueda M. 2004. Yeast cell-surface display- applications of molecular display. Appl. Microbiol. Biotechnol. 64: 28-40.
5. Mckay L. L., and Baldwin K. A. 1990. Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol. Lett. 87: 3-14.
6. Axelsson L. 1998. Lactic acid bacteria: classification and physiology. In Lactic acid bacteria: Microbiology and functional aspects, Salminen S., and Wright A. W. (eds), Marcel Dekker Inc., New York, pp. 1–72.
7. Stiles M. E., and Holzapfel W. H. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36: 1-29.
8. Pouwels P. H., Leer R. J., Shaw M., den Bak-Glashouwer M-J. H., Tielen F. D., Smit E., Martinez B., Jore J., and Conway P. L. 1998. Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. Int. J. Food Microbiol. 41: 155-167.
9. Mannam P., Jones K. F., and Geller B. L. 2004. Mucosal vaccine made from live, recombinant *Lactococcus lactis* protects mice against pharyngeal infection with *Streptococcus pyogenes*. Infect. Immun. 72:3444-3450.
10. Robinson, K., Chamberlain L. M., Schofield K. M., Wells J. M., and Le Page R. W. F. 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nat.

Biotechnol. 15:653-657.

11. Seegers, J. F. M. L. 2002. *Lactobacilli* as live vaccine delivery vectors: progress and prospects. Trends Biotechnol. 20:508-515.

12. Shaw, D. M., Gaerthe B., Leer R. J, Van der Stap J. G. M. M., Smittenaar C., Heijne den Bak-Glashouwer M. J. , Thole J. E. R., Tielen F. J., Pouwels P. H., and Havenith C. E. G.. 2000. Engineering the microflora to vaccinate the mucosa: serum immunoglobulin G responses and activated draining cervical lymph nodes following mucosal application of tetanus toxin fragment C-expressing lactobacilli. Immunology 100:510-518.

13. Xin, K. Q., Hoshino Y. , Toda Y. , Igimi S., Kojima Y., Jounai N., Ohba K., Kushiro A., Kiwaki M. , Hamajima K. , Klinman D., and Okuda K. 2003. Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. Blood 102:223-228.

14. Bermudez-Humaran L. G., Cortes-Perez N. G., le Loir Y., Alcocer-Gonzalez J. M., Tamez-Guerra R. S., Montes de Oca-Luna R., and Langella P. 2004. An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. J. Med. Microbiol. 53: 427-433.

15. Navarre W. W. and Schneewind O. 1994. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram positive bacteria. Mol. Microbiol. 14: 115-121.

16. Norton P. M. Brown H. W. G., Wells J. M., Macpherson A. M., Wilson, P. W., and Le Page R. W. F. 1996. Factors affecting the immunogenicity of tetanus toxin fragment C expressed in *Lactococcus lactis*. FEMS Immunol. Med. Microbiol. 14: 167-177.

17. Pouwels P. H., Leer, R. J., and Boersma W. J. A. 1996. The potential of Lactobacillus as a carrier for oral immunization: Development and preliminary characterization of vector systems for targeted delivery of antigens. J. Biotechnol. 44: 183-192.

18. Piard J. C., Hautefort I., Fischetti V. A., Ehrlich S. D., Fons M., and Gruss A. 1997a. Cell wall anchoring of *Streptococcus pyogenes* M6 protein in various lactic acid bacteria. J. Bacteriol. 179: 3068-3072.

19. Steidler L., Viaene J., Fiers W., and Remaut E. 1998. Functional display of a heterologous protein on the surface of *Lactococcus lactis* by mean of cell wall anchor of *Staphylococcus aureus* protein A. Appl. Environ. Microbiol. 64: 342-345.

20. Fischetti V. A., Pancholi V., and Schneewind O. 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. Mol. Microbiol. 4: 1603-1605.

21. Franke C. M., Leenhouts K. J., Haandrikman A. J., Kok J., Venema G., and Venema K. 1996. Topology of LcnD, a protein implicated in the transport of bacteriocins from *Lactococcus lactis*. J. Bacteriol. 178: 1766-1769.

22. Narita J., Okano K., Kitao T., Ishida S., Sewaki T., Sung M-H., Fukuda H., and Kondo A. 2006. Display of α-amylase on the surface of *Lactobacillus casei* cells by use of the PgsA anchor protein, and production of lactic acid from starch. Appl. Environ. Microbiol. 72: 269-275.

23. Sutcliffe I. C., and Russell R. B. 1995. Lipoproteins of gram positive bacteria. J. Bacteriol. 177: 1123-1128.

24. Kim J-H., Park I-S., and Kim B-G. 2005. Development and characterization of membrane surface display system using molecular chaperone, prsA, of Bacillus subtillis. Biochem. Biophys. Res. Commun. 334: 1248-1253.

25. Buist G., Kok J., Leenhouts K. J., Babrowska M., Venema G., and Haandrikman A. J. 1995. Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J. Bacteriol. 177: 1554-1563.

26. Turner M. S., Timms P., Hafner L. M., and Giffard P. M. 1997. Identification and characterization of a basic cell-surface-located protein from *Lactobacillus fermentum* BR11. J. Bacteriol. 179: 3310-3316.

27. Turner M. S., and Giffard P. M. 1999. Expression of *Chlamydia psittaci* and human immunodeficiency virus-derived antigens on the cell-surface of *Lactobacillus fermentum* BR11 as fusions to BspA. Infect. Immun. 67: 5486-5489.

28. Hung J., Rathsam C., Jacques N. A., and Giffard P. M. 2002. Expression of a streptococcal glucosyltransferase as a fusion to a solute-binding protein in Lactobacillus fermentum BR11. FEMS Microbiol. Lett. 211: 71-75.

29. Avall-Jaaskelainen S., Kyla-Nikkila K., Kahala M., Miikkulainen-Lahti T., and Palva A. 2002. Surface display of foreign epitopes on the *Lactobacillus brevis* S-layer. Appl. Environ. Microbiol. 68: 5943-5951.

30. Callegari M. L., Riboli B., Sanders J. W., Cocconcelli P. S., Kok J., Venema G., and Morelli L. 1998. The S-layer gene of *Lactobacillus helveticus* CNRZ 892: cloning, sequence and heterologous expression. Microbiol. 144: 719-726.

31. Chen W., and Georgiou G. 2002. Cell surface display of heterologous proteins: from high throughput screening to environmental applications. Biotechnol. Bioeng. 79: 496-503.

32. Van Der Vaart J. M., Biesebeke R., Chapman J. W., Toschka H. Y., Klis F. M., and Verrips T. 1997. Comparison of cell wall proteins of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. Appl. Environ. Microbiol. 63: 615-620.

33. Strauss A., and Gotz F. 1996. In vivo immobilization of enzymatically active polypeptides on the cell surface of *Staphylococcus carnosus*. Mol. Microbiol. 21: 491-500.

34. Schreuder M. P., Mooren A. T. A., Toschka H. Y., Verrips T., and Klis F. M.1996. Immobilizing proteins on the surface of yeast cells. Trends Biotechnol. 14: 115-120.

35. Wan H-M., Chang B-Y., and Lin S-C. 2002. Anchorage of cyclodextrin glucanotransferase on the outer membrane of *Escherichia coli*. Biotechnol. Bioeng. 79: 457-464.

36. Washida M., Takahashi S., Ueda M., and Tanaka A. 2001. Spacer-mediated display of active lipase on the yeast cell surface. Appl. Microbiol. Biotechnol. 56: 681-686.

37. Matsumoto T., Fukuda H., Ueda M., Tanaka A., and Kondo A. 2002. Construction of yeast strains with high cell surface lipase activity by using novel display systems based on the Flo1p flocculation functional domain. Appl. Environ. Microbiol. 68: 4517-4522.

38. Steen A., Buist G., Leenhouts K.J., El Khattabi M., Grijpstra F., Zomer A. L., Venema G., Kuipers O. P., and Kok J. 2003. Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. J. Biol. Chem. 278: 23874-23881.

39. Åvall-Jääskeläinen S., Lindholm A., and Palva A. 2003. Surface display of the receptor-binding Region of the *Lactobacillus brevis* S-Layer protein in *Lactococcus lactis* provides nonadhesive Lactococci with the ability to adhere to intestinal epithelial cells. Appl. Environ. Microbiol. 69: 2230-2236.

40. Lindholm A., Smeds A., and Palva A. 2004. Receptor binding domain of *Escherichia coli* F18 fimbrial adhesin FedF can be both efficiently secreted and surface displayed in a functional form in *Lactococcus lactis*. Appl. Environ. Microbiol. 70: 2061-2071.

41. Konings W., Kok J., Kuipers O. S., and Poolman B. 2000. Lactic acid bacteria: the bugs of new millennium. Curr. Opinion Microbiol. 3: 276-282.

42. Ross R. P., Morgan S., and Hill C. 2002. Preservation and fermentation: past, present and

future. Int. J. Food Microbiol. 79: 3-16.

43. Schnürer J., and Magnusson J. 2005. Antifungal lactic acid bacteria as biopreservatives. Trends Food Sci. Technol. 16: 70-78.

44. Shigechi H., Koh J., Fujita Y., Matsumoto T., Bito Y., Ueda M., Satoh E., Fukuda H., and Kondo A. 2004. Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and α-amylase. Appl. Environ. Microbiol. 70: 5037-5040.

45. Giraud E., Gosselin L., Marin B., Parada J. L., and Raimbault M. Purification and characterization of an extracellular amylase from *Lactobacillus plantarum* strain A6. 1993. J. Appl. Bacteriol. 75: 276-282.

46. Nishihara K., Kanemori M., Yanagi H., and Takashi Y. 2000. Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli.* Appl. Environ. Microbiol. 66: 884-889.

47. Dieye Y., Hoekman A. J. W., Clier F., Juillard V., Boot H. J., and Piard J-C. 2003. Ability of *Lactococcus lactis* to export viral capsid antiges: a crucial step for development of live vaccines. Appl. Environ. Microbiol. 69: 7281-7288.

48. Matsui Y., Okada S., Uchimura T., Kondo A., and Satoh E. 2007. Determination and analysis of the starch binding domain of *Streptococcus bovis* 148 raw-starch hydrolyzing alpha-amylase. J. Appl. Glycosci. 54: 217-222.

49. Maassen C. B. M., Laman J. D., Heijne den bak - Glashouwer M. J., Tielen F. J., van Holten - Neelen J. C. P. A., Hoogteijling L., Antonissen C., Leer R. J., Pouwels P. H., Boersma W. J. A., and Shaw D. M. 1999. Instruments for oral disease intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. Vaccine,

17: 2117-2128.

50. Wernèrus H. and Stefan S. 2004. Biotechnological applications for surface engineered bacteria. Biotechnol. Appl. Biochem. 40: 209-228.
51. Lee J-S., Poo H., Han D.P., Hong S-P., Kim K., Cho M.W., Kim E., Sung M-H., and Kim C-J. 2006. Mucosal immunization with surface displayed severe acute respiratory syndrome corovirus spike protein on *Lactobacillus casei* induces neutralizing antibodies in mice. J. Virol. 80: 4079-4087.
52. Bosma T., Kanninga R., Neef J., Audouy S. A. L., van Roosmalen M. L., Steen A., Buist G., Kok J., Kuipers O. P., Robillard G., and Leenhouts K. 2006. Novel surface display system for proteins on non genetically modified gram positive bacteria. Appl. Environ. Microbiol. 72: 880-889.
53. Baneyx F. 1999. Recombinant protein expression in *Escherichia coli*. Curr. Opinion Biotechnol. 10: 411-421.
54. Yin J., Li G., Ren X., and Herrler G. 2007. Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. J. Biotechnol. 127: 335-347.
55. Singh S. M., and Panda A. K. 2005. Solubilization and refolding of bacterial inclusion body proteins. J. Biosci. Bioeng. 99: 303-310.
56. Patrick S. M., Fazenda M. L., McNeil B., and Harvey L. M. 2005. Heterologous protein production using *Pichia pastoris* expression system. Yeast 22: 249-270.
57. Monsalve R. I., Lu G., and King T. P. 1999. Expression of recombinant venom allergen, antigen5 of yellowjacket (*Vepsula vulgaris*) and paper wasp (*Polistes annularis*) in bacteria or yeast. Protein Express. Purif. 16: 410-416.
58. Li Z., Xiong F., Lin Q., d'Anjou M., Daugulis A. J., Yang D. S.C., and Hew C.L. 2001. Low

temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Express. Purif. 21: 438-445.

59. de Boer J. P., Teixeira de Mattos M. J., and Neijssel O.M. 1990. D (-) lactic acid production by suspended and aggregated continuous cultures of *Bacillus laevolacticus*. Appl. Microbiol. Biotechnol. 34: 149-153.
60. Guimaraes V. D., Gabriel J. E., Lefevre F., Cabanes D., Gruss A., Cossart P., Azevedo V., and Langella P. 2005. Internalin-expressing *Lactococcus lactis* is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. Microb. Infect. 7: 836-844.
61. Thompson J., Pope T., Tung J-S, Chan C., Hollis G., Mark G., and Johnson K. S. 1996. Affinity maturation of a human high affinity monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity. J. Mol. Biol. 256: 77-88.
62. White C. E., Kempi N. M., and Komives E. A. 1994. Expression of highly disulfide-bonded proteins in *Pichia pastoris*. Structure 2: 1003-1005.
63. Bateman A., and Bycroft M. 2000. The structure of a LysM domain from E. coli membrane-bound lytic murein transglycosylase D (MltD). J. Mol. Biol. 299: 1113-1119.
64. Boraston A. B., Sandercock L. E., Antony R., Warren J., and Kilburn D.G. 2003. O-glycosylation of a recombinant carbohydrate-binding module mutant secreted by *P. pastoris*. J. Mol. Microbiol. Biotechnol. 5: 29-36.
65. Munro C. A., Bates S., Buurman Ed. T., Hughes H. B., MacCallum D. M., Bertram G., Atrih A., Ferguson M. A. J., Bain J. M., Brand A., Hamilton S., Westwater C., and other 4 authors. 2005. Mnt1p and mnt2p of *Candida albicans* are partially redundant α-1, 2-mannosyltransferases that participate in *O*-Linked mannosylation and are required for

adhesion and virulence. J. Biol. Chem. 280: 1051-1060.

66. Kaur I. P., Chopra K., and Saini A. 2002. Probiotics: potential pharmaceutical applications. Eur. J. Pharm. Sci. 15: 1-9.

67. Sandholm T. M., Myllärinen P., Critenden R., Mogensen G., Fondén R., and Saarela M. 2002. Technological challenges for future probiotic foods. Int. Dairy J., 12, 173-182.

68. Kailasapathy K. 2006. Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. LWT 39: 1221-1227.

69. Fuller R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66: 365-378.

70. Desmond C. B., Corcoran M., Coakley M., Fitzgerald G. F., Ross R.P., and Stanton C. 2005. Development of dairy-based functional foods containing probiotics and prebiotics. Australian J. Dairy Technol. 60: 121-126.

71. Collins J.K., Thornton and G., Sullivan G. O. 1998. Selection of probiotic strains for human applications. Int. Dairy J. 8: 487-490.

72. Daly C., and Davis R. 1998. The biotechnology of lactic acid bacteria with emphasis on applications in food safety and human health. Agric. Food Sci. 7: 219-250.

73. Conway P. L., Gorbasch S. L., and Goldin B. R. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. dairy Sci. 70: 1-12.

74. Lankaputhra W. E. V., and Shah N. P. 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. In the presence of acid and bile salts. Cultured Dairy Products J. 30: 2-7.

75. Gismondo M. R., drago L., and Lombardi A. 1999. Review of probiotics available to modify gastrointestinal flora. Int. J. Antimocrobial Agents 12: 287-292.

76. Anal A. K., and Singh H. 2007. Recent advances in microencapsulation of probiotics for industrial applications and targetd delivery. Trends Food Sci. Technol. 18: 240-251.

77. Crittenden R., Laitila A., Forssell P., Matto J., Saarela M., Mattilla-Sandholm T., and Myllarinen P. 2001. Adhesion of bifidobacteria to granular starch and its implications in probiotic technologies. Appl. Environ. Microbiol. 67: 3469-3475.

78. Lian W-H., Hsiao H-C., and Chou C-C. 2003. Viability of microencapsulated bifidobacteria in simulated gastric juice and bile solution. Int. J. Food Microbiol. 86: 293-301.

79. Wang X., Brown I. L., Evans A. J., and Conway P. L. 1999. The protective effect of high amylose maize (amylomaize) starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract. J. Appl. Microbiol. 87: 631-639.

80. Loessner M. J., Kramer K., Ebel F., and Scherer S. 2002. C-terminal domains of *Listeria monocytogens* bacteriophage murein hydrolases determine specific recognition and high-affinity binding to bacterial cell wall carbohydrates. Mol. Microbiol. 44: 335-349.

81. Ahmed A. B. F., Noguchi K., Asami Y., Nomura K., Fuji H., Sakata M., Tokita A., Noda K., and Kuroda A. 2007. Evaluation of cell wall binding domain of *Staphylococcus aureus* autolysin as affinity reagent for bacteria and its application to bacterial detection. J. Biosci. Bioeng. 104: 55-61.

RELATED PUBLICATIONS

1. Tarahomjoo S., Katakura Y., and Shioya S. Bidirectional cell-surface anchoring function of the C-terminal repeat region of peptidoglycan hydrolase of *Lactococcus lactis* IL1403. Accepted (J. Biosci. Bioeng.). 2007.
2. Tarahomjoo S., Katakura Y., and Shioya S. Expression of C-terminal repeat region of peptidoglycan hydrolase of *Lactococcus lactis* IL1403 in methylotrophic yeast *Pichia pastoris.* Accepted (J. Biosci. Bioeng.). 2007.
3. Tarahomjoo S., Katakura Y., and Shioya S. A new strategy for enhancement of microbial viability in simulated gastric conditions based on the display of starch binding domain on the cell-surface. Submitted (J. Biosci. Bioeng.). 2008.

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