Efficient production of active form of vitamin D_3 by microbial conversion

— Comprehensive approach from the molecular to the cellular level —

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Conversion processes of organic compounds using biocatalyst generally have high regio- and stereo-selectivity, and are becoming increasingly important for efficient production of chemicals. In addition, biocatalysis is less hazardous, less polluting and less energy-consuming than the conventional chemical method. We report the highly efficient bioconversion system using actinomycete *Rhodococcus erythropolis* to produce active form of vitamin D_3 currently used as a pharmaceutical. The improvement of performance of the enzyme used for the bioconversion has been achieved by the combination of evolutionary engineering and structure-based methods. Accordingly, the practical production efficiency of active form of vitamin D_3 has been substantially increased. In addition, we have succeeded in significant improvement of cellular permeability of vitamin D_3 by using nisin-treated cells, and have developed a new platform for vitamin D_3 hydroxylation process.

Keywords : Cytochrome P450, vitamin D_3, bioconversion, nisin, structural biology, protein engineering

1 Introduction

Vitamin D_3 (VD_3) is a fat-soluble secosteroid hormone involved in various important physiological functions in the human body[1]. Humans take in most of the VD_3 from food, and the ingested VD_3 is converted into active forms of VD_3 (25-hydroxyvitamin D_3 ($25(OH)VD_3$) and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2VD_3$)) in the liver and kidneys. These active forms of VD_3 are deeply involved in maintenance of calcium and phosphate homeostasis, cell reproduction and differentiation, immunity adjustment, and other functions in the human body. The deficiency of active forms of VD_3 due to genetic or environmental factors is known to cause diseases such as osteoporosis, rachitis, psoriasis, and hyperparathyroidism, and in fact, active forms of VD_3 are used as the treatment drug for such diseases[1].

Currently, the $1\alpha,25(OH)_2VD_3$ mainly used as drugs is manufactured by a chemical synthesis method, and it can be synthesized in approximately 20 reaction steps with cholesterol as the starting substance (Fig. 1A). However, the yield is only about 1 %[2]. It is commercially manufactured despite such low production efficiency, since the active form of VD_3 shows pharmacological effect at very low dose (0.5~several μg/day or less), but the price is extremely high. While the price of VD_3 sold as general-use reagent is about 7 yen per 1 mg, the price of $1\alpha,25(OH)_2VD_3$ is about 130,000 yen/mg, according to the catalog of Company S. To mass-produce such fine chemicals, the manufacture method becomes complex due to low reaction efficiency in the aforementioned chemical synthesis, and high cost must be paid to manufacture highly pure product that can be used as pharmaceuticals. Also, in the chemical synthesis method, it is difficult to conduct regio-selective hydroxylation for the steroid skeleton, and it is not a suitable method for manufacturing active form of VD_3 and its derivatives that may be pharmacologically effective.

As an alternative to this chemical synthesis method, the manufacture of $1\alpha,25(OH)_2VD_3$ using the microbial conversion capability has been realized[3][4]. The actinomycete *Pseudonocardia autotrophica* that conducts this microbial conversion has the capacity to convert the VD_3 added to the culture into $1\alpha,25(OH)_2VD_3$. Moreover, the reaction intermediate $25(OH)VD_3$ produced in this microbial conversion process is a valuable medical intermediate with the pharmacological effect equivalent to $1\alpha,25(OH)_2VD_3$, and the $25(OH)VD_3$ can be obtained in the same process. The price of $25(OH)VD_3$ sold as general-use reagent is about 40,000 yen per 1 mg, according to the catalog of Company S (Fig. 1B).

The biocatalytic conversion using the enzymes of organisms, in general, shows high regio-selectivity and stereoselectivity, and has major impact on the synthesis of chemical substances. Also, the biocatalytic conversion is a safe method compared to the conventional organic synthesis, in that it has very low emission of pollutants under mild reaction condition (ordinary temperature and pressure), and involves low energy consumption. The term "green chemistry" is used for the biocatalytic conversion technology with the aforementioned characteristics, as it is known as an environment-friendly synthesis. The production of active forms of VD_3 by *P. autotrophica* is an environment-friendly production method with all of

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these characteristics. However, the productivity has not been maximized since there are various issues that will be explained later. In this paper, the issues and the development goals of the microbial conversion that has been currently realized are presented. Then, the research methods used to solve the issues, how the methods were combined, and the ideas that turned out to be useful are explained to illustrate the construction of a highly efficient and high performing recombinant microbial conversion system.

Please note that, in this paper, the information pertaining to the VD_3 hydroxide production, which is currently being done commercially by pharmaceutical companies, cannot be disclosed, and we cannot present the actual figures for how much the developed technology contributed in increasing the efficiency of the production.

2 Issues that must be overcome and the development goal

The advantages of the activated VD_3 production technology by microbial conversion are as stated above. Currently, the companies employ the method of culturing the breeding strain of *P. autotrophica* that has high VD_3 hydroxylation capacity, adding the VD_3 and cyclodextrin (CD) that increases the solubility of VD_3, and accumulating the hydroxylated VD_3 while growing the microorganisms (Fig. 1B). In the early research, the wild strain *P. autotrophica* was used, and 200 μg/ml VD_3 was added two days after culturing, and accumulation of 45 μg/ml $25(OH)VD_3$ was observed after three days of culture[4]. Currently, the breeding strains are used for production, and the production efficiency is thought to have increased significantly.

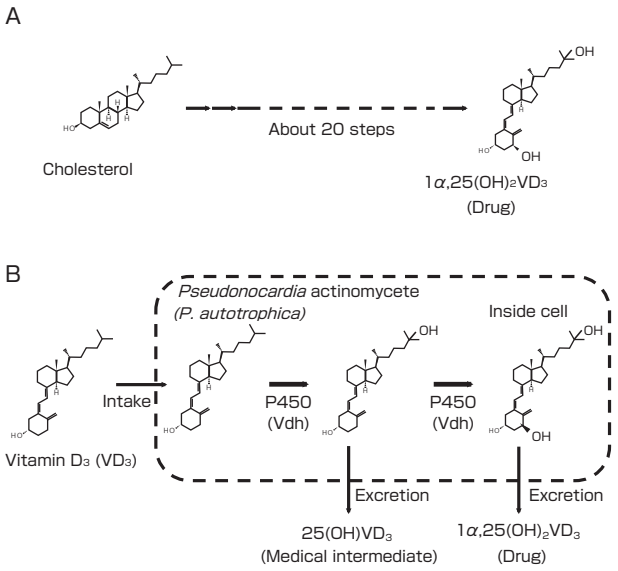

Fig. 1 Production method for active form of vitamin D3
Organic synthesis method (A) and microbial conversion method using *P. autotrophica* (B).

However, there are issues that must be improved in this method, and achievement of microbial conversion with dramatically increased performance can be expected by solving the issues. The issues and the expected causes are analyzed and summarized as a solution guideline as follows.

(1) Conversion efficiency: In the current microbial conversion, not all VD_3 added to the culture are converted, and there are remnant unreacted substrates. In the laboratory level analysis, over 30 % of unreacted substances are observed. Since the convertible amount may be dependent on the absolute amount of intracellular enzymes, the technology to stably express and accumulate a large amount of enzymes in the cell is necessary. By doing so, it may become possible to construct a conversion system where the majority of the added VD_3 can be converted into the active form.

(2) Conversion rate: In the currently conducted microbial conversion, over 100 hours is necessary for one conversion reaction. The main reasons are because the growth rate of the microorganisms is slow and because the enzyme reaction rate is slow since the VD_3 is a nonnative substrate for the enzyme. It is thought that the active forms of VD_3 of the same amount can be obtained in a shorter time by creating a variant enzyme with higher activity, and conducting the reaction in the microbial cell with a fast reproductive rate.

(3) Presence of the product of side reaction: The greatest issue in the microbial conversion is a production of side reaction products where the carbon-26 is hydroxylated. This 26-hydroxyvitamin D_3 ($26(OH)VD_3$) is eluted as a proximal or overlapping peak of the $25(OH)VD_3$ in high-performance liquid chromatography. Therefore, to produce pharmaceutical quality $25(OH)VD_3$, it is necessary to remove the $26(OH)VD_3$ completely. This is a factor that reduces the yield of $25(OH)VD_3$. This is caused by the low regio-selectivity of the enzyme reaction, and it is necessary to create a variant enzyme with improved regio-selectivity to control the 26-hydroxylation.

(4) Issue of cell membrane permeation: VD_3 is a fat-soluble vitamin with poor water solubility. Therefore, as mentioned above, cyclodextrin (CD) is added to the conversion culture along with VD_3, and VD_3 is dissolved by trapping it in the cyclic structure of the CD. Since the CD-VD_3 complex with relatively high molecular weight cannot permeate the cell membrane, it is necessary to devise a way where only the VD_3 separated from CD can permeate the membrane, or is made to permeate along with the CD. The rate of cell membrane permeation is the rate-limiting factor of this microbial conversion, and if the issue of membrane permeation is improved, both

the reaction efficiency (1) and the apparent reaction rate (2) can be expected to increase.

Of the above issues that must be solved, issues (1) to (3) pertain to the performance of the enzyme that is actually involved in the conversion reaction. Therefore, characterization of the enzyme and improvement by introduction of the mutation, as well as the mass accumulation technology of the enzyme in the cell are necessary. As it will be explained later, this enzyme is one of the enzyme group called cytochrome P450. Cytochrome P450 is the name of the enzyme group that holds the heme in the molecule and has the ability to insert the hydroxyl group into the hydrocarbon chain of various substances by receiving external electrons. It requires appropriate electron-supplying protein to be active. Therefore, it is necessary to look for a redox partner gene that allows the efficient transfer of electrons to this enzyme, and this gene must be coexpressed along with the enzyme. Issue (4) is related to the structure of the cell membrane itself, or the function of the transporter protein that allows a substance to permeate the membrane, and the cell that can obtain such information is desired.

To solve the above issues, we gathered data using the conversion host, which is a microorganism without the VD_3 hydroxylation capacity, is capable of mass recombinant expression, is easy to culture, reproduces quickly, and whose genome information can be utilized. This information was fed back to the *P. autotrophica* system. As an organic species that fulfilled this condition, we decided to use the *Rhodococcus* erythropolis host-vector system[5] that belonged to the same actinomycetes as *P. autotrophica* (Fig. 2).

3 Road to results

3.1 Isolation of the enzyme and identification of the gene

It was about 20 years ago when it was found that *P. autotrophica*, a rare actinomycete, possessed the ability to convert VD_3 to $1\alpha,25(OH)_2VD_3$. Due to the characteristic where it catalyzed the hydroxylation to the steroid skeleton, the enzyme that catalyzed this reaction was predicted to be a cytochrome P450, but the identification of the enzyme was not done successfully for a long time. Therefore, we started by searching the gene that encoded this enzyme. Since the genome sequence analysis had not been done for *P. autotrophica*, we attempted the purification of the enzyme directly from the cell extract using the VD_3 hydroxylation activity as the index. In general, the VD_3 hydroxylation activity could be detected by the coexistence of electron transferring protein that was needed for P450 to be active, and the target enzyme was confirmed as a P450 as predicted. However, purification was difficult due to the occurrence of the phenomenon where the VD_3 hydroxylation activity became undetectable during the purification process. After much trial and error, we found that salt (NaCl and others) had to be present in the reaction solution for this enzyme to be active, and it was possible to follow the activity to the final step of purification[6]. After identifying the N-terminal and internal amino acid sequences from the purified enzyme, we succeeded in cloning the gene that encoded this enzyme.

3.2 Reproduction of the VD_3 hydroxylation reaction in vitro

It was possible to produce the enzyme that showed VD_3 hydroxylation activity, by using the general overexpression system of the *E. coli*. However, it was necessary to add 5-aminolevulinic acid, a heme precursor, to the culture media to obtain the heme-containing holo enzyme. This is a method commonly used for the mass production of P450 enzyme using *E. coli* as a host cell. On the other hand, this enzyme could be obtained using *R. erythropolis* without adding 5-aminolevulinic acid to the culture. This was thought to be because the actinomycetes possess many P450 genes, the heme biosynthesis pathway functioned stably, and the level of intracellular heme could be maintained without depletion. Since 5-aminolevulinic acid is an expensive reagent, the microbial conversion using *R. erythropolis* as a host cell is advantageous when conducting the recombinant expression.

Next, the function of the enzyme was analyzed by *in vitro* reconstitution experiment. P450 required two electrons for one catalytic turnover of hydroxylation, and it was necessary to add the redox partner protein that supplied the electron to the assay system. Here, we used the commercially available redox partner proteins from spinach used widely for P450 assays. As a result, it was clarified that this enzyme continuously catalyzed the two-step hydroxylation from VD_3 to $25(OH)VD_3$ and from $25(OH)VD_3$ to $1\alpha,25(OH)_2VD_3$. Since $1\alpha(OH)VD_3$ was not detected, it was found that this

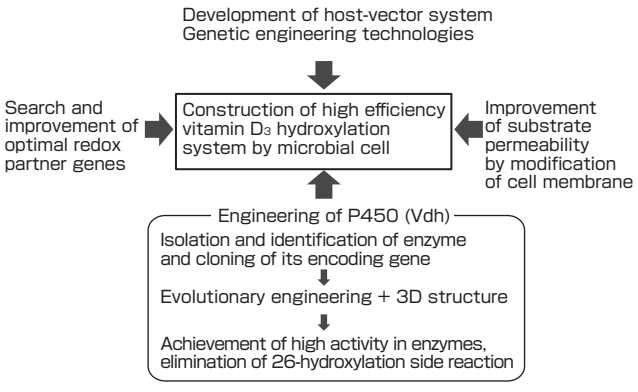

Fig. 2 Outline of R&D

enzyme first hydroxylated the carbon-25 of VD_3 and then hydroxylated the carbon-1 of 25(OH)VD_3. Also, a small amount of 26(OH)VD_3 was detected, and these substances matched the results detected in the cellular VD_3 conversion by *P. autotrophica*. We determined that this P450 was the enzyme actually responsible for microbial conversion, and named it vitamin D_3 hydroxylase (Vdh). The P450 will be called Vdh hereafter.

3.3 Intracellular conversion using the recombinant expression

Next we constructed the microbial conversion system that accomplished the VD_3 hydroxylation, using the recombinant cell of *R. erythropolis*. The VD_3 hydroxylation activity was very low with a single expression of Vdh, and it was necessary to coexpress some kind of redox partner protein. Therefore, we constructed a thiostrepton-inducible expression vector that contained genes encoding the Vdh and redox partner proteins (ferredoxin and ferredoxin reductase) derived from *R. erythropolis*. Then, co-expression was conducted in the *R. erythropolis* cell, and VD_3 was added to the culture. As a result, it was confirmed that the active forms of VD_3 were produced when the *R. erythropolis* cell was used. It has been reported that the redox partner that is capable of most efficiently supplying the electrons to P450 is not necessarily the protein with which P450 couples in the cell of the original organism[7]. This is thought to be because the electron transfer efficiency is affected drastically by the slight difference in the intracellular environment or the intracellular expression level of the gene. Therefore, we conducted the conversion tests by inserting various electron transfer protein genes to the aforementioned co-expression vectors, and looked for the redox partner that showed high VD_3 hydroxylation activity. As a result, it was found that the proteins called AciB and AciC from *Acinetobacter* were the most compatible partner to Vdh.

3.4 Two different approaches to enzyme improvement

In general, the enzymes produced by organisms are catalysts that specifically respond to certain substrates and are exclusive to some specific reaction. However, since VD_3 is not found in the soil where the *P. autotrophica* exists, it is thought that Vdh is not an enzyme that evolved for (is specific to) the hydroxylation and metabolism of VD_3. In fact, the VD_3 hydroxylation activity of the isolated and purified enzyme is fairly lower than the activity of the P450 with the specific function involved in the biosynthesis of some substance. Therefore, the VD_3 hydroxylation activity of Vdh is not at all optimized as an enzyme, and we believe it can be improved further.

There are two completely different approaches when introducing variations to improve the enzyme. One is the rational design where the 3D structure of the protein is analyzed and the mutation is introduced based on this structural information. While this is a powerful method in the case where the correlation of the structural functions is clear, since the 3D structure of the protein is a complex system composed of multiple parameters, there may be no simple correlation between the amino acid residue and the function. The other approach is the evolutionary engineering (directed evolution) where the genetic variation library to which the random mutation is introduced is created to screen the variants with improved performance. While much effort will be required to create the library and to verify the variations by assay, this may enable extracting the variations that may improve the enzyme function at any part of the sequence. In our research, we conducted improvement of enzymes using both methods, without choosing one of the two approaches (Fig. 2). As a result, the advantages of both the strategy of variation introduction based on structure and the strategy based on evolutionary engineering could be utilized, and we were successful in creating a useful variant.

3.4.1 High activation of the enzyme

The mutant that significantly improved the VD_3 hydroxylation activity was constructed by combining mutations generated by directed evolution. The quadruple mutant (Vdh-K1) that had the highest improvement showed about 12 times increase of 25-hydroxylase activity and about 25 times increase of 1α-hydroxylase activity compared to the wild type Vdh (Vdh-WT)[6][8]. Interestingly, the four mutations were located far from the active site, and it is difficult to find such mutations by rational design. The discovery of Vdh-K1 was the result of the maximization of the benefits of evolutionary engineering unfettered by structural information. On the other hand, we were able to infer why such variations brought about major activity improvement through the analysis of the 3D structure. Major structural change was observed between the Vdh-WT and Vdh-K1, and three of the four mutations might have induced such structural changes. This suggested that the activity increase of Vdh was not caused by an optimization of the substrate binding pocket, but by the orchestration of the conformational equilibrium between open and closed forms (Fig. 3)[8]. P450 is an enzyme involved in the detoxification and the biosynthesis of the secondary metabolites and various substances in nature, and there are many molecular species of the enzyme with wide substrate specificity. The conformational shift by introduction of a few mutations observed in our research may be a mechanism that allows in nature to adapt P450 to ever-emerging new conditions and substrates. Through the results obtained, the possibility of significantly improving the production efficiency of hydroxylated VD_3 was found. However, due to the issues in membrane permeability of the substrate that will be described later, major increase in the production efficiency of hydroxylated VD_3 had not been achieved simply by increasing the enzyme performance.

3.4.2 Completely eliminating the enzymatic side reaction

In the active form of VD_3 production by *P. autotrophica*, side reaction product, in which the carbon-26 is hydroxylated, was also produced at the rate of about 10 %. This was clearly an issue of the substrate recognition of the enzyme, and it was thought necessary to either fabricate the enzyme that tightly recognized the VD_3, or to fine-adjust the bonding orientation of the substrate in the substrate binding pocket. Therefore, we attempted to determine the crystal structure of Vdh in complex with VD_3, and based on the structural information, we introduced the mutations to the substrate-binding pocket. Vdh-WT had low substrate binding affinity, and substrate complex crystal could not be obtained. However, high activity mutant Vdh-K1 was successfully crystallized as the complex with VD_3, and we were able to clarify how the enzyme recognized VD_3 (Fig. 4)[8]. Of the amino acid residues that formed the substrate binding pocket, we focused on the amino acid residue positioned in proximity to the carbon-24 to -27 of VD_3, and the variant (I88V) with lowered percentage of side reaction was obtained by saturated mutagenesis for those residues[9]. The quintuple variant of Vdh-K1+I88V decreased the side reaction rate to about 1 % in the bioconversion test by *P. autotrophica*, and the 26(OH) VD_3 decreased to below detection limit in case of the single I88V variant. While this result was based on the structures, it was not entirely a rational design. It is extremely difficult to logically estimate which variation of what amino acid residue will reduce the side reaction. We succeeded in selecting the mutant that achieved the side reaction reduction by selecting the amino acid residues based on the 3D structure, and then by taking the strategy of saturated mutagenesis for the selected amino acid residues. Through this research result, we succeeded in increasing the production efficiency of the 25(OH)VD_3, and the development for practical application is currently in progress.

3.5 Processing the cell

In producing the active form of VD_3 by R. erythropolis with recombinant expression of Vdh, the final major issue was the cell membrane permeability. This is not a unique problem of *R. erythropolis* but a similar issue was observed also for *P. autotrophica*. In *R. erythropolis*, no correlation was recognized in the conversion rate of hydroxylated VD_3 and the amount of intracellular enzyme. Even if the expression level of Vdh was changed, the conversion rate was fixed at a certain rate[10]. Even the Vdh-K1 (see section 3.4.1) in which dramatic high activity was confirmed using the *in vitro* reconstitution system did not show much significant difference from the wild type when the conversion was done in the *P. autotrophica* cell. This meant that the performance of the enzyme inside the cell was not utilized, and it was estimated that the cell membrane permeation of substrate VD_3 acted as the rate limiting factor. The VD_3 is a fat-soluble steroid and solubility in the water is extremely low. Therefore, in the current microbial conversion, the solubility is increased by adding CD to the culture to trap the VD_3 in the cyclic structure of the CD. In fact, the VD_3 hydroxylation of Vdh increases dramatically by the addition of CD to the solution both *in vivo* and in vitro experiments. However, the permeation of the high molecular weight CD-VD_3 complex through the cell membrane is difficult,

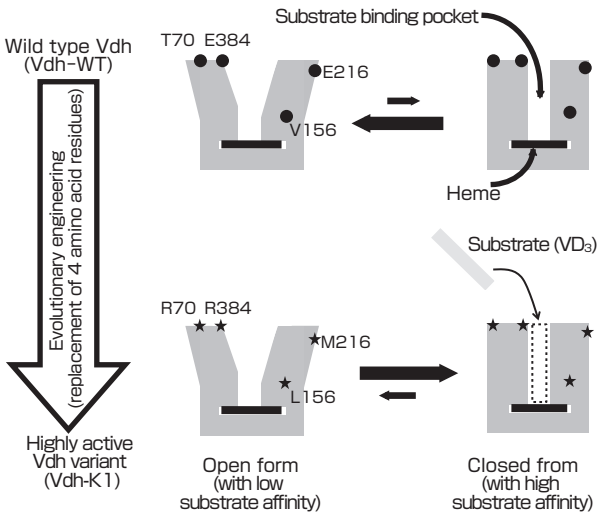

Fig. 3 Structural mechanism of activity enhancement of P450 Vdh

In general, P450 is in equilibrium of open and closed structures, and the substrate is likely to bind with the closed structure. The equilibrium between these structures shift greatly according to the variation selected by evolutionary engineering. The population in closed form increased and the activity was enhanced.

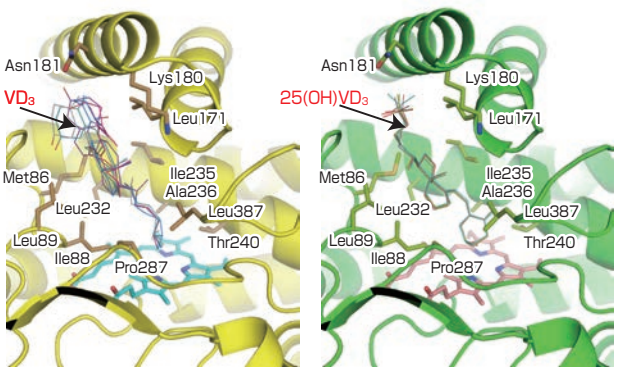

Fig. 4 Recognition mechanism of substrate in an anti-parallel orientation by P450 Vdh

The VD_3 (left) and 25(OH)VD_3 (right) can bond to the enzyme in an anti-parallel orientation, and that gives them the capacity for two-step hydroxylation to 1α,25(OH)$_2VD_3$. The amino acids that were candidates for variations to eliminate the side reaction were selected from the detailed structural information of the substrate binding site.

and the VD_3 is thought to enter the cell diffusely after it breaks off from CD. However, the actual mechanism of the intake of VD_3 into the cell is completely unknown (Fig. 5). Therefore, considering the possibility that the VD_3 may be transported by some kind of membrane protein (transporter), we attempted the identification of a gene that might increase the VD_3 conversion activity, by conducting the random gene destruction experiment using transposon and by using the *R. erythropolis* genome information. However, we have not been able to find such genes.

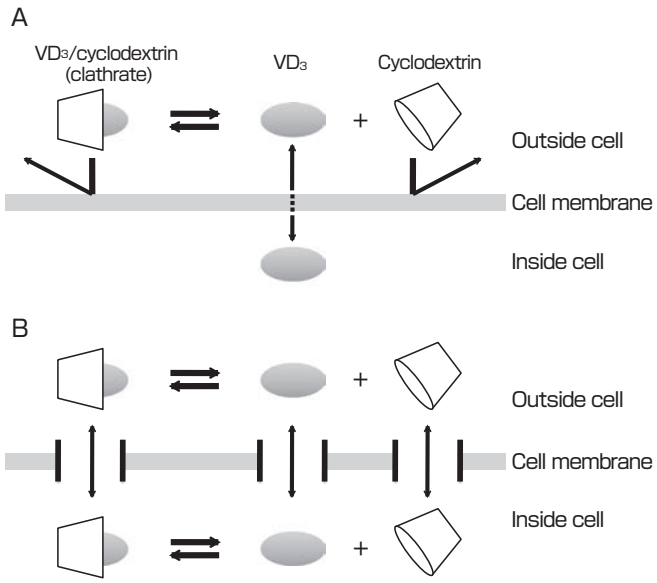

Fig. 5 Conceptual diagram of the permeability of cell membrane

In the cell membrane in an ordinary state (A), only VD3 may permeate the membrane by natural diffusion. In the cell membrane with pores created by nisin-treatment (B), the low molecular weight substance including cyclodextrin can move through freely.

Therefore, we changed our way of thinking and investigated whether the CD-VD_3 complex could be delivered directly to the intracellular enzyme by physically making a hole in the cell. We focused on the antibacterial substance called nisin[11]. Nisin is an antibacterial peptide composed of 34 amino acids derived from *Lactococcus lactis*, and is approved as food additive. The action mechanism of nisin has been studied thoroughly, and it shows antibacterial activity by creating pores with diameters of about 2-2.5 nm in the membrane of the gram-positive bacteria, and the intracellular low molecular substances leak outside of the cell through the pores[12]. Although excessive addition of nisin will cause bacteriolysis, the *R. erythropolis* cell is characterized by bacteriolysis resistance compared to other bacteria. Therefore, by adjusting the amount of nisin added, it is possible to create a unique situation where pores will be formed while the cell structure is maintained without bacteriolysis. In theory, proteins such as ferredoxin and P450 cannot leave the cell, so such a cell can be used as a reaction vessel packed with high concentration of enzymes. To experimentally investigate whether CD and VD_3 could freely enter and leave the cell through the pores, green chemiluminescence CD was added to the nisin-treated cell, to observe the intracellular intake of the substances. As a result, it was confirmed that the luminescence level from the cell increased depending on the nisin concentration and treatment time, and the pores could be used as the passage of CD.

Next, the nisin-treated cell was used to conduct the VD_3 hydroxylation experiment under various conditions, and we found that the hydroxylation capacity increased depending on the amount of enzyme present in the nisin-treated cell, unlike the untreated cells. Moreover, it was found that it was important to have a NADH regeneration system in the

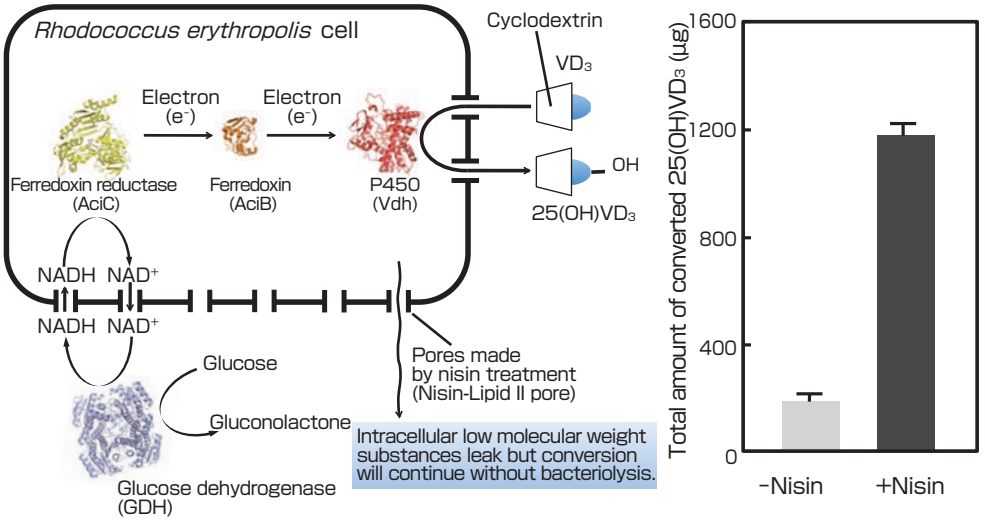

Fig. 6 Conceptual diagram of the hydroxylated VD_3 production using the nisin-treated *R. erythropolis* cell

reaction system and to have a stable redox partner in the cell[10]. Glucose dehydroxylase (GDH) was used as the NADH regenerator, and the reaction system using the wild-type Vdh, in which the AciB and AciC derived from *Acinetobacter* were co-expressed, was constructed as a highly stable redox partner (Fig. 6). The hydroxylated VD_3 productivity of the nisin-treated cell was observed using this system. As a result, it was confirmed that hydroxylation efficiency was several times higher in the nisin-treated cells, compared to that of the untreated cells. It was also found that when the reaction, where one cycle consisted of a 16-hour reaction, was repeated in the nisin-treated cell, the VD_3 hydroxylation rate per cycle increased to maximum 90 % (less than 50 % in untreated cells), and the total yield of 25(OH)VD_3 after four reaction cycles was about six times higher compared to the untreated cell[10]. The conversion reaction system using nisin could convert 90 % of the VD_3 in a short time, and significantly increased the production efficiency of the 25(OH)VD_3. Moreover, since the nisin-treated cell used the buffer system as the reaction solution instead of culture media, the amount of foreign substances could be reduced. Also, the cells could be recovered and reused, and this is an effective method in the case where the productivity must be raised by increasing the number of reactions using the substrate with low solubility. If this technology could be used in *P. autotrophica*, we believe we can create a production system where highly active enzyme Vdh-K1 can be maximized.

4 Future developments and issues

This R&D was conducted to find an efficient production method of active form of VD_3 by microbial conversion and to construct an excellent conversion system in terms of efficiency and cost by overcoming the issues of conversion by wild-type strains that are currently done by the companies. The conversion system of *R. erythropolis* cell treated with nisin that was constructed in this research may realize a production efficiency that surpasses the system using *P. autotrophica*. Currently, we are engaging in the investigation of whether further efficiency can be achieved in the electron transfer between the redox partner and P450. Since the structural stability of the highly active mutant (Vdh-K1) is reduced, we think it is necessary to construct a system that promotes activity while maintaining the thermostability. There are already reports that the electron transfer efficiency has been increased in P450 and the activity is increased[13], and the introduction of the mutation on the ferredoxin binding region is expected to increase the conversion performance. On the other hand, the substance conversion technology that combines CD and the formation of pores by nisin treatment can be applied widely to the conversion system of substances where the highly hydrophobic, poorly soluble substances and CD are used as carriers. In general, the achievement of high efficiency in the microbial conversion of fat-soluble substance is very difficult, and we think there is high value in such usage. In the future, we would like to evaluate the usage value of nisin and CD in other microbial conversion systems.

Acknowledgements

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References

[1] G. Jones, S. A. Strugnell and H. F. DeLuka: Current understanding of the molecular actions of vitamin D, *Physiol. Rev.*, 78 (4), 1193-1231 (1998).
[2] G. D. Zhu and W. H. Okamura: Synthesis of vitamin D (calciferol), *Chem. Rev.*, 95 (6), 1877-1952 (1995).
[3] J. Sasaki, A. Miyazaki, M. Saito, T. Adachi, K. Mizoue, K. Hanada and S. Omura: Conversion of vitamin D_3 to 1α, 25-dihydroxyvitamin D_3 via 25-hydroxyvitamin D_3 using Amycolata sp. strains, *Appl. Microbiol. Biotechnol.*, 38 (2), 152-157 (1992).
[4] K. Takeda, T. Asou, A. Matsuda, K. Kimura, K. Okamura, R. Okamoto, J. Sasaki, T. Adachi and S. Omura: Application of cyclodextrin to microbial conversion of vitamin D_3 to 25-hydroxyvitamin D_3 and 1α, 25-dihydroxyvitamin D_3, *J. Ferment. Bioeng.*, 78 (5), 380-382 (1994).
[5] T. Nakashima and T. Tamura: A novel system for expressing recombinant proteins over a wide temperature range from 4 to 35ºC, *Biotechnol. Bioeng.*, 86 (2), 136-148 (2004).
[6] Y. Fujii, H. Kabumoto, K. Nishimura, T. Fujii, S. Yanai, K. Takeda, N. Tamura, A. Arisawa and T. Tamura: Purification, characterization, and directed evolution study of vitamin D_3 hydroxylase from *Pseudonocardia autotrophica, Biochem. Biophys. Res. Commun.*, 385 (2), 170-175 (2009).
[7] Y. Khatri, M. Girhard, A. Romankiewcz, M. Ringle, F. Hannemann, V. B. Urlacher, M. C. Hutter and R. Bernhardt: Regioselective hydroxylation of norisoprenoids by CYP109D1 from *Sorangium cellulosum* So ce56, *Appl. Microbiol. Biotechnol.*, 88 (2), 485-495 (2010).
[8] Y. Yasutake, Y. Fujii, W.-K. Cheon, A. Arisawa and T. Tamura: Structural evidence for enhancement of sequential vitamin D_3 hydroxylation activities by directed evolution of cytochrome P450 vitamin D_3 hydroxylase, *J. Biol. Chem.*, 285 (41), 31193-31201 (2010).
[9] K. Nishimura, Y. Fujii, A. Arisawa, T. Tamura and Y. Yasutake: Improvement of vitamin D hydroxylase, Jpn. Kokai Tokkyo Koho JP 2011-115078 (June 16, 2011).
[10] N. Imoto, T. Nishioka and T. Tamura: Permeabilization induced by lipid II-targeting lantibiotic nisin and its effect on the bioconversion of vitamin D_3 to 25-hydroxyvitamin D_3 by *Rhodococcus erythropolis*, *Biochem. Biophys. Res. Commun.*, 405 (3), 393-398 (2010).
[11] W. Liu and J. N. Hansen: Some chemical and physical properties of nisin, a small protein antibiotic produced by *Lactococcus lactis, Appl. Environ. Microbiol.*, 56 (8), 2551-2558 (1990).

[12] H. E. Hasper, B. Kruijff and E. Breukink: Assembly and stability of nisin-lipid II pores, *Biochemistry*, 43 (36), 11567-11575 (2004).
[13] L. S. Koo, C. E. Immoos, M. S. Cohen, P. J. Farmer and P. R. Ortiz de Montellano: Enhanced electron transfer and lauric acid hydroxylation by site-directed mutagenesis of CYP119, *J. Am. Chem. Soc.*, 124 (20), 5684-5691 (2002).

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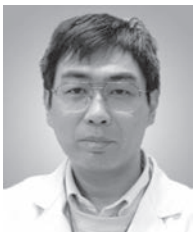
Completed the doctorate course at the Graduate School of Science, Hokkaido University in 2004. Industry-academia-government collaboration researcher, Graduate School of Science, Hokkaido University. Doctor (Science). Researcher, Research Institute of Genome-based Biofactory, AIST in April 2005. Researcher, Bioproduction Research Institute, AIST in April 2010. Currently engages in the researches for the VD_3 hydroxylation reaction described in this paper, as well as the biosynthesis enzyme of new antibiotic material and the structural function analysis and achievement of high function for useful function proteins such as the medical diagnostic enzymes. In this paper, was in charge of the structural research, functional analysis, and functional alteration of the proteins involved in microbial conversion.

Tomohiro TAMURA



Completed the courses at the Faculty of Medicine, The University of Tokushima in 1993. Research Fellow of the Japan Society for the Promotion of Science. Doctor (Medicine). Post-doctorate at the Research Unit for Molecular Structural Biology, Max Planck Institute of Biochemistry in 1994. Joined the Hokkaido National Industrial Research Institute, Agency of Industrial Science and Technology (currently, AIST) in 2000. Professor of the Graduate School of Agriculture, Hokkaido University in 2002. Leader of Proteolysis and Protein Turnover Research Group, Bioproduction Research Institute, AIST in 2011. Visiting professor of the Graduate School of Agriculture, Hokkaido University. Currently engages in the development of expression platform that allows the use of the *Rhodococcus* actinomycetes for diverse purposes. In this paper, planned and summarized the research for overall VD_3 hydroxylation reaction.

Discussion with Reviewers

1 Overall comment

Comment (Kazunori Nakamura, Biomedical Research Institute, AIST)

Overall, the specific reaction system is not clearly stated in the paper. Also, since you do not state the specific figures for the cost and efficiency of the conventional method, it is difficult to determine quantitatively how much increased productivity and cost reduction were achieved in this research.

Answer (Yoshiaki Yasutake, Tomohiro Tamura)

I added the explanation pertaining to the reaction system as you indicated. However, this reaction system is a technology that is currently being used in production, and is a corporate secret. Therefore, I cannot disclose any specific information including the production efficiency. I added and modified the descriptions to the extent that I can disclose at this point.

2 Description of specific production method

Comment (Kazunori Nakamura)

In the description relating to bioconversion rate, you say that the issues are the slow rate of growth of the microorganisms and the reaction speed of the enzymes, but it is difficult to know which phase is a more serious problem since you do not describe the specific production method. For example, do you convert using non-growing cells after culturing the microorganisms, or using growing microorganisms? Please describe the specific production method in detail.

Answer (Yoshiaki Yasutake, Tomohiro Tamura)

As mentioned in Discussion 1, the various information relating to the current hydroxylated VD3 production system cannot be disclosed. Therefore, please understand that I cannot provide comparisons using actual figures. I added and modified the descriptions to the extent that I can disclose at this point.

3 Future issues in realization

Question (Motoyuki Akamatsu, Human Technology Research Institute, AIST)

You write that you developed a production method with extremely high production efficiency in this research, and you aim for further efficiency of electron transfer in "4. Future developments and issues". Why do you need further efficiency? Pertaining to this, you mention that this is a joint research with Micro Biopharm Japan Co., Ltd. Do you need further R&D for actual production? Is the electron transfer efficiency the barrier for the production? Please describe any other barriers, if any, to clarify the positioning of the results.

Answer (Yoshiaki Yasutake, Tomohiro Tamura)

This R&D is an effort to enhance the efficiency and refinement of the production method using *P. autotrophica* that has already been realized. The productivity has greatly improved through the modification of the enzymes based on evolutionary engineering and 3D structure, as well as changes in the organic species. However, the thermostability of the enzyme with increased activity by the introduction of variation is low, and the issue that the enzyme cannot be accumulated in large amounts in the cell became apparent. Therefore, as a method for increasing activity while maintaining enzyme stability, we think the increased efficiency of electron transfer for P450 is necessary, and we infer that there are rooms for improvement in the Vdh, AciB, and C systems. By advancing the electron transfer efficiency, I think it is possible to go beyond the current production efficiency. On the other hand, the barrier in using the reaction system by *R. erythropolis* in actual production is not a matter of production efficiency, but is the problem of using recombinant bacteria. Currently, the company uses the breeding strain and does not engage in production by recombinant bacteria. To change the bacteria type and to start up the production system using the recombinant bacteria, various procedures including safety tests will become necessary, as well as additional facility investment to build such a plant. The market for active form of VD_3 is expected to grow further, and I expect the production using the technology we developed will be in demand in the future. I modified the paper according to your comment.

4 Significance of high activation of enzyme in the production process

Comment (Kazunori Nakamura)

You describe the increase of the activity of the enzyme itself in achieving high activation. I think you need to provide some description on how effective this activity increase is in the actual production process. I think it will be better if the reader can image how much cost reduction can be ultimately achieved by such a process.

Answer (Yoshiaki Yasutake, Tomohiro Tamura)

In the laboratory level analysis, high enzyme activation or the increased intracellular accumulation of enzymes were achieved, yet these have not led to the dramatic improvement of the production volume of 25(OH)VD_3. This may be because VD_3 is a fat-soluble substance, and transfer into and out of the cell is limited greatly compared to water-soluble molecules. The conversion rate was improved significantly by increasing the solubility by trapping the VD_3 in cyclodextrin, but the improvement of activity dependent on the accumulation volume of the intracellular enzyme was not confirmed. As described in the paper, we obtained the result that the conversion activity could be increased by removing the barrier of VD_3 cell membrane permeability by using the antibacterial substance nisin. However, when the nisin-treated cell was used, the production volume of the hydroxylated VD_3 increased according to the accumulation volume of the intracellular enzyme. I predict that similar effect will be obtained if highly active enzymes are used. In the near future, if we could use the system, in which the membrane permeability is improved using nisin, in actual production, I think the highly active enzyme can exert its potential sufficiently.