
塩野義研究所年報

55

ANNUAL REPORT
OF
SHIONOGI
RESEARCH LABORATORIES

2005

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Production of Deacetyl 7-Aminocephalosporanic Acid with Recombinant Cephalosporin-C Deacetylase

AKIO TAKIMOTO¹ AND KENJI MITSUSHIMA²

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Bacillus subtilis SHS 0133 converts 7-aminocephalosporanic acid (7-ACA) to deacetyl 7-ACA with high efficiency. Cephalosporin-C deacetylase (CAH) was purified from *B. subtilis* and characterized. The K_m value for 7-ACA was 7.9 mM, and the inhibition of reaction products, deacetyl 7-ACA and acetic acid, was negligible. Therefore, it was considered that this enzyme would be useful for commercial production of deacetyl 7-ACA. The gene encoding CAH was cloned and expressed in *Escherichia coli*. The expressed CAH was an active form in the soluble fraction obtained after cell disruption. *E. coli* with the most powerful expression plasmid pCAH4312 produced *ca.* 5 g of CAH per liter on cultivation in a 30-l jar fermentor. The CAH could be purified with only a one non-column step, and immobilized on an anion exchange resin, KA-890 using glutaraldehyde. The immobilized enzyme obeyed Michaelis-Menten kinetics similar to that of the free enzyme. A 200-liter batch-type bioreactor was employed for deacetylation of 7-ACA. Ten kilograms of 7-ACA was completely converted to deacetyl 7-ACA at pH 8.0, 20 within 80 min. The reaction kinetics agreed well with a simulation model considered from mass-balance and Michaelis-Menten kinetics. The immobilized enzyme exhibited only a slight loss of the initial activity after repeated use (52 times).

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1. Introduction

The deacetyl cephalosporins serve as the starting material for many expanded-spectrum semisynthetic cephalosporins such as cefuroxime¹⁾ and cefcapene pivoxil hydrochloride (Flomox).^{2,3)} Flomox, which is highly active against a wide range of gram-positive and gram-negative bacteria, was synthesized from deacetyl 7-aminocephalosporanic acid (deacetyl 7-ACA). Deacetyl 7-ACA was obtained by enzymatic or chemical deacetylation of 7-aminocephalosporanic acid (7-ACA). Since the chemical route is slow and leads to lactone formation,⁴⁾ the enzymatic route has been the desired procedure.

Cephalosporin-C deacetylase (CAH, systematic name: cephalosporin-C acetylhydrolase) [EC. 3. 1. 1. 41] activities have been found in citrus peel,⁵⁾ actinomycetes,⁶⁾ fungi^{7, 8)} and *Bacillus subtilis*.⁹⁻¹¹⁾ Recently cephalosporin deacetylating enzymes from *Rhodospiridium toluloides*,¹²⁾ *Rhodotorula glutinis*,¹³⁾ *Agrobacterium radiobacter*,¹⁴⁾ and *Acremonium crysogenum*¹⁵⁾ have been well characterized. However, large-scale enzymatic deacetyl 7-ACA production has only used enzymes from *B. subtilis*.¹⁶⁾

To scale up production of 6-aminopenicillanic acid from penicillin G, Alvaro *et al.*¹⁷⁾ and Kobayashi *et al.*¹⁸⁾ used the immobilized penicillin acylase. However, in the case of industrial deacetyl 7-ACA production, a suitable bioreactor system could not be established due to the instability of 7-ACA and deacetyl 7-ACA at alkaline pH, low solubility at acidic pH and temperature instability. As a possibility toward resolving this issue, we recently found that a strain of *B. subtilis* produces a CAH which converts cephalosporins to deacetyl cephalosporins with high efficiency. The CAH displays wide substrate specificity and high k_{cat} values to various cephalosporins.¹⁹⁾

2. Characterization of cephalosporin-C deacetylase (CAH) from *Bacillus subtilis*¹⁹⁾

2-1. Purification of CAH

B. subtilis SHS 0133 was grown in a medium composed of 2.5% glucose, 0.75% corn steep liquor, 1.0% of a mixture of amino acids (Ajinomoto Co. Ltd., Kanagawa, Japan), 0.3% KH_2PO_4 and 0.8 mg/l $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (pH 7.0). Cultivation was carried out at 28 °C in a 30-l jar fermentor containing 20 l of medium. After 48 h of cultivation, 200 g of activated charcoal was added to the culture broth, which was then stirred for 2 h at 400 rpm. The crude enzyme solution was prepared by filtration and DEAE-Sephadex A-50 powder was added to 0.7% (w/v). The mixture was adjusted to pH 8.0 with 2 N NaOH and then stirred for 1 h. After filtration, the DEAE-Sephadex A-50 gel was washed with Tris-HCl buffer (50 mM, pH 8.0) containing 0.1 M NaCl, and the CAH activity was eluted with the same buffer containing 0.4 M NaCl. The enzyme solution was concentrated and desalted by ultrafiltration with an ultramembrane (MW cut off : 50,000) and fractionated by stepwise addition of solid ammonium sulfate to 40% and 95% saturation. The 40-95% precipitate was dissolved in 30 ml Tris-HCl buffer and dialyzed overnight against the same buffer. The dialyzate was subjected to high performance ion exchange column chromatography using a DEAE-Toyopearlpack 650 M column (2.2 x 20 cm) previously equilibrated with Tris-HCl buffer containing 0.15 M NaCl. CAH activity was eluted with a linear gradient (0.15-0.5 M NaCl) in 200 ml of the same buffer. After concentration, the enzyme solution was loaded onto preparative polyacrylamide gel. Polyacrylamide gel electrophoresis was conducted on a 2-mm-thick slab gel consisting of 7.5% (w/v) acrylamide separation gel. The CAH was stained by deacetylase activity according to the method of Higard and Spizizen.²⁰⁾ The visualized portion of the gel was sliced and crushed. The crushed gels were immersed in 200 ml Tris-HCl buffer overnight and removed by centrifugation. The extract from the gels was concentrated by ultrafiltration. Subsequently, this enzyme solution was applied to a Sephacryl S-300

column (2.5 x 100 cm), equilibrated with Tris-HCl buffer, in a conventional system (flow rate, 50 ml/h; fraction volume, 3 ml). The results of the above purification are summarized in Table I. Routinely, CAH activity was assayed in 0.1 M sodium acetate buffer (pH 6.8) and 11 mM *p*-nitrophenyl acetate (*p*-NPA) as a substrate. Enzyme activity was estimated spectrophotometrically at 400 nm. One unit of enzyme activity was defined as the amount producing 1 μ mol of *p*-nitrophenol at 30 .

Table I. Purification of CAH

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude enzyme	5,680	39,900	7.0	100	1.0
DEAE-Sephadex A-50	1,750	26,800	15.4	67	2.2
Ammonium sulfate	525	23,700	45.1	59	6.4
DEAE-Toyopearl 650M	168	19,000	113.0	48	16.1
Gel extraction (PAGE)	33	4,180	127.6	11	18.2
Sephacryl S-300	18	3,440	187.4	9	26.7

CAH activity was measured spectrophotometrically using *p*-NPA as a substrate.

2-2. Kinetic analysis of CAH

Kinetic analysis of the CAH was carried out at 37 and pH 7.0 using 0.1 M sodium phosphate buffer. K_m and V_{max} values were determined by double-reciprocal plots. For determination of the k_{cat} values, the concentrations of the CAH (as an octamer) were measured spectrophotometrically using the molar absorption coefficient of 406,000 $M^{-1}cm^{-1}$ at 280 nm, assuming a relative molecular weight of 280,000 for the octamer. Product inhibition studies for the CAH were carried out under the same assay conditions in the presence of the following products: 24 mM deacetyl 7-ACA or 400 mM acetate.

The CAH showed wide substrate specificity and followed Michaelis-Menten kinetics with various substrates. Table II shows the K_m and k_{cat} values for the substrates. From double-reciprocal plots, the K_m values for cephalosporin C, 7-ACA and *p*-NPA were estimated to be 24.0, 7.9 and 1.0 mM, and k_{cat} values to be 169, 98 and 201 s^{-1} , respectively. The inhibition kinetic parameters of the reaction products were also determined.

Table II. Kinetic parameters of CAH on various substrates

Substrate	K_m (mM)	V_{max} (μ mol/min/ml)	k_{cat} (s^{-1})	k_{cat} / K_m ($s^{-1}mM^{-1}$)
<i>p</i> -NPA	1.0	1.64	201	201.0
7-ACA	7.9	0.80	98	12.4
Cephalosporin C	24.0	1.38	169	7.0
Cephalothin	11.1	0.45	55	5.0
Cephapirin	13.9	0.65	80	5.8
Cephaloglycin	11.5	ND	ND	ND

ND, Not determined.

The activity for *p*-NPA was determined spectrophotometrically. Activities (except for *p*-NPA) were determined by following the substrate disappearance by reversed phase HPLC. When *p*-NPA was used as a substrate, the *p*-NPA concentration range was 0.11-11.0 mM; when 7-ACA was used, the 7-ACA concentration range was 1.0-20.0 mM; when other cephalosporins were used, each concentration range was 1.0-50.0 mM.

Acetate was a weak competitive inhibitor and the K_i value was 290 mM; on the other hand, deacetyl 7-ACA was a weak non-competitive inhibitor and the K_i value was 171 mM.

The reaction products, acetate and deacetyl 7-ACA, did not actually inhibit the deacetylation of 7-ACA. The purified CAH (15.7 $\mu\text{g/ml}$) hydrolyzed each substrate (8.3 mM; 7-ACA, cephalosporin C, cephalothin and cephalixin) with a yield of more than 99.5% within 40 min at 37 $^\circ\text{C}$. No by-products were observed by the HPLC analysis. Thus, the CAH has favorable features for industrial production of deacetyl cephalosporins because of its high k_{cat} values to various cephalosporins and negligible product inhibition. Therefore, we decided to breed a strain which could produce large amounts of the enzyme by means of genetic engineering with *Escherichia coli*.

3. Development of production methodology^{21, 22)}

3-1. Cloning of CAH gene

Reduced and *S*-carboxymethylated CAH prepared according to the procedure of Crestfield *et al.*²³⁾ was directly subjected to reversed-phase HPLC using a VydacC₁₈ column (a gradient of 0-70% acetonitrile in 0.1% trifluoroacetic acid for elution). HPLC-purified protein was dissolved in 0.1 M Tris-HCl buffer (pH 8.8) containing 2 M urea and incubated for 4 h at 37 $^\circ\text{C}$ with 1/100 volume (w/w) of *Achromobacter* proteinase I. The resulting peptide fragments were loaded on an HPLC column (Microbondapak C₁₈), equilibrated with 0.1% trifluoroacetic acid and eluted using a linear gradient from 0 to 60% acetonitrile over 60 min in the same solvent at a flow rate of 1.0 ml/min. To investigate the possibility of similarity to proteins with known sequences, the N-terminus part and five peptide fragments of the CAH were sequenced. Figure 1 shows the elution pattern of *Achromobacter* proteinase I-digested CAH by HPLC. The peaks indicated were collected and lyophilized. Gas-phase sequencing of five peptides (fragments 1 to 5) and the intact enzyme showed that fragment 4 corresponded to the N-terminus sequence. Fragment 5 was sequenced from residue 1 (Tyr) to residue 32 (Gly).

The following amino acid sequence was selected to design the oligonucleotide mixture to be used as hybridization probes: Glu-Met-Val-Asn-Trp-Ala. The 17-mer mixed-oligonucleotide probes [3' CT(C/T)TACCANTT(A/G)ACCCG 5' (N represents A/C/G/T)] representing all possible sequences of the

Fragment 1	L A F F K *
Fragment 2	D F S E F W K *
Fragment 3	V P V L M S I G L I D K
Fragment 4	M Q L F D L P L D Q L Q T Y K P E K
Fragment 5	Y H G Y N A S Y D G E I H E M V N W A L H G Y A A F G M L V R G
N-Terminus	M Q L F D L P L D Q L Q T Y K P E K T A P K D

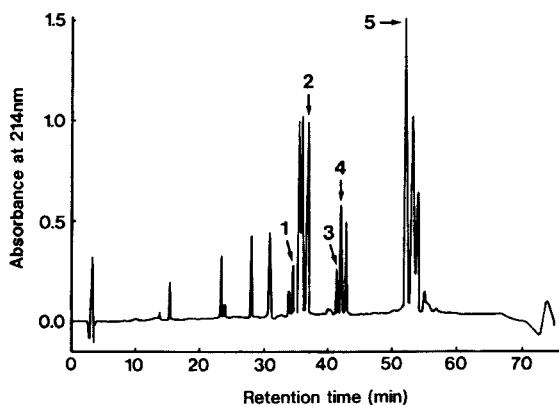


Fig. 1. Reversed-phase HPLC elution pattern of *Achromobacter* proteinase I-digested peptide fragments. The numbered peaks were used for N-terminal amino acid sequencing.

*Analysis stopped.

antisense strand were synthesized. The 5'-termini of the oligonucleotides were labeled radioactively and used in Southern hybridization with restriction enzyme-digested fragments *B. subtilis* SHS 0133 chromosomal DNA. The probes were found to hybridize to the 2 to 3 kb *DraI* fragment, ca. 4 kb *HindIII* fragments, and ca. 8 kb *PvuII* fragments. The chromosomal DNA was digested with *DraI*, and fragments ranging from 2 to 4 kb were ligated with *SmaI*-digested and dephosphorylated pUC13. The genomic library of *B. subtilis* SHS 0133 in *E. coli* HB101 was screened by colony hybridization as described by Grunstein and Hogness²⁴⁾ with the same probes, and two positive clones were selected. The recombinant plasmids obtained were designated pCAH01 and pCAH02. A preliminary analysis of the plasmids indicated that two or three *DraI* fragments were contained in pCAH01 and pCAH02, respectively, and one of these *DraI* fragments was found to hybridize to the mixed probes. Therefore, the desired *DraI* fragment was subcloned into the *SmaI* site of pUC13 and designated pCAH03. Restriction enzyme mapping of the plasmid pCAH03 carrying the 2.5-kb *DraI* fragment from *B. subtilis* chromosomal DNA was performed. Southern analysis showed that the mixed probes hybridized to the 0.24-kb *HindIII-EcoRI* fragment of the insert.

The approximately 2.0-kb region between *DraI* and *EcoRI* was sequenced by the dideoxy chain termination method.²⁵⁾ Computer analysis of the sequence identified one major open reading frame (nucleotides 187 to 1140), with the capacity to encode a polypeptide of 318 amino acid residues (Fig. 2).

The deduced amino acid sequence contained the previously determined sequences from five *Achromobacter* proteinase I digested fragments of the CAH. The calculated molecular weight of the polypeptide was 35,571, which agreed well with the value of 35 kDa determined for the purified enzyme by SDS-PAGE. The deduced amino acid composition was essentially identical to that of the purified enzyme. These findings confirmed that the cloned sequence encoded the CAH from *B. subtilis* SHS 0133. No other significant homologies were found when either the nucleotide sequence or the predicted amino acid sequence was compared with the EMBL and GenBank nucleotide or SWISSPROT protein sequences. A purine-rich sequence, AAAGGGG, which probably serves as a ribosome-binding site^{26, 27)}, was found 12 nucleotides upstream from the initiation codon ATG (nucleotides 187 to 189). A pair of sequences, CAGGC (nucleotides 32 to 36) and GAAAGTCT (nucleotides 56 to 63), preceding the CAH structural gene were very similar to the consensus sequences of the -35 and -10 regions for the sigmaH form of *B. subtilis* RNA polymerase.^{28, 29)} As shown in Fig. 2, four tandem repeats of an A-rich box (AAACAAA) existed between the putative promoter and initiation codon. Overlapping the two A-rich boxes, two repeated sequences with a common sequence (AAAT--TTA-AAAACAAAG--GAA) were found immediately in front of the initiation codon. These sequences matched 19 of 24 bp of the common sequence. In the downstream region from termination codon TGA, an extended region of dyad symmetry was observed. This region is thought to be a rho-independent transcriptional termination site because a thymine-rich region exists just behind the stem-loop structure.

3-2. Construction of expression plasmids

Four ATG vectors have a synthetic unique *EcoRI* or *SstI* site between the SD sequence and the ATG codon. Digestion with *EcoRI* or *SstI* followed by blunt-ending with mung bean nuclease or Klenow fragment exposes the initiation codon. The CAH structural gene fragment, which starts at CAA, coding the second amino acid (Gln), and extends to the 3' noncoding region, was obtained. The strategy for constructing the expression plasmids is shown in Fig. 3.

The CAH structural gene fragment with a *SalI* site in the 3' noncoding region was inserted between *EcoRI*/mung bean nuclease and *SalI* sites of pTrp·pBR·Sn·ATG (n = 1, 2 or 3) for construction of the expression plasmids pCAH41, 42, and 43. In the same manner, the expression plasmid pCAH440 was constructed by ligating the CAH structural gene fragment digested with *XbaI* in the 3' noncoding region with the pTrp·pAT·Sst·ATG vector cleaved with the *SstI*/Klenow fragment and *XbaI*.

Plasmids pCAH41, 42, and 43 have a replication origin from pBR322 and an ampicillin-resistant marker (Ap^r). Therefore, these plasmids were improved to change the replication origin and resistant marker, and to

introduce the terminator region. The *PvuII-XbaI* fragment harboring the *trp* promoter and CAH structural gene from pCAH41, 42, or 43 was ligated to the pCAH211 cleaved with the *EcoRI/Klenow* fragment and *XbaI*, which contained a replication origin from pAT153, the Tc^r marker, and the 5S *rrmBT1T2* terminator, and the new expression plasmids pCAH411, 421, and 431 were constructed. To introduce the terminator region to pCAH440, the *XbaI-BamHI* region was changed to the *XbaI-BamHI* fragment of the pCAH211, and the new



Fig. 2. Nucleotide sequence of the 2.0 kb *DraI-EcoRI* fragment containing the *B. subtilis* SHS 0133 CAH structural gene. The deduced amino acid sequence is indicated above the nucleotide sequence by a single-letter code. Amino acids are numbered starting with the initiation methionine (M) as 1. The underlined amino acid sequences represent the previously determined sequences of purified CAH. The amino acid sequence from position 179 to 183, G-X-S-X-G, is boxed. The termination codon is designated by an asterisk. The probable Shine-Dalgarno (SD) sequence and the putative promoter (-35 and -10 regions) are shown by thick lines. The probable transcription terminator is indicated by arrows (and). The tandem repeats of the A-rich box and the repeated sequences constituting the A+T-rich block are indicated by thin and dashed line, respectively.

expression plasmid pCAH441 was constructed. The resulting plasmids were introduced into *E. coli* JM103, and tetracycline-resistant transformants were isolated.

3-3. Expression of CAH

The practical production of CAH with a 30-l jar fermentor was carried out as follows. The seed culture was performed in LB broth containing tetracycline (100 ml in a 500-ml flask) at 28 °C for 16 h on a rotary shaker at 200 rpm. The entire contents of the seed flask was added to 20 l of production medium, which contained (per liter) 80 g of glycerol, 20 g of casein hydrolysates, 3 g of yeast extract, M9 basal salts, and tetracycline, and was incubated at 37 °C for 24 h. The pH of the broth was maintained at 7.0 with 28% NH₄OH and 43% H₃PO₄. Agitation was maintained at 500 rpm, and air was sparged at a rate of 0.75 vol/vol/min at a pressure of 0 kg/cm² (gauge). Bacterial growth was monitored by measuring the A₆₅₀.

Table III shows the relationship between the SD-ATG sequences and the CAH activities expressed by the various *E. coli* transformants. *E. coli* JM103 harboring pCAH431 (the distance of the spacing was 13 nt) produced the highest level of CAH (1.04 kU/ml of culture). The CAH expression level with pCAH421 (11 nt) was slightly lower. However, pCAH411 (10 nt) and pCAH441 (11 nt) were far lower.

The binding of the ribosome to the ribosomal binding site (RBS) on mRNA, the first step of translation, is known to be influenced by the length as well as the base composition of the spacing between SD sequence and

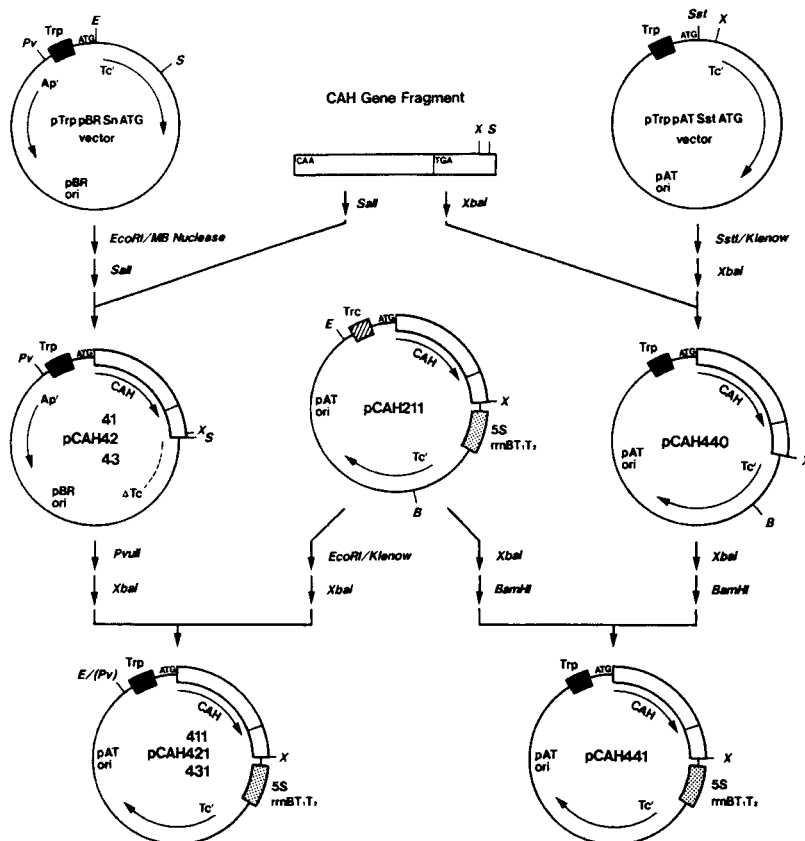


Fig. 3. Construction of various expression plasmids for CAH in *E. coli*. Symbols: Open bar, CAH structural gene and 3' noncoding region from *B. subtilis* SHS 0133; solid line, vector plasmid DNA; hatched bar, *trc* promoter; solid bar, *trp* promoter; stippled bar, 5S *rrmBT:T2* terminator; *EcoRI*, *PvuII*, *SalI*, *XbaI*, *SstI*, and *BamHI* cleavage sites are indicated by E, Pv, S, X, Sst, and B, respectively. Abbreviations: Klenow, Klenow fragment; MB nuclease, Mung bean nuclease.

an initiation codon.³⁰ Several groups have tried to improve the spacing for the expression of a desired gene. Marquis *et al.*³¹ reported that the optimal distance between the SD sequence and the initiation codon is dependent upon the promoter used. Sato *et al.*³² reported a suitable spacing of 13 nt for the expression of tuna growth hormone cDNA.

In view of these observations, we constructed expression plasmids with various lengths of spacing containing an AT rich region just before the initiation codon. Of these plasmids, pCAH431 (with a spacing of 13 nt) showed the highest activity of CAH (Table III). The results suggested that length of the spacing influenced the gene expression as described by Sato *et al.*³². However, the base composition of the spacing

Table III. CAH production in various expression plasmids.

Plasmid	SD-ATG	CAH production	
	Sequence	Activity (kU/ml)	
pCAH411	[S1] AAGG GTATCGAT AT	ATG	0.25
pCAH421	[S2] AAGG GTATCGAT ATT	ATG	0.93
pCAH431	[S3] AAGG GTATCGAT ACTAT	ATG	1.04
pCAH441	[S4] AAGG GTATCGAT AAT	ATG	0.32
pCAH400	AAGG GTATCGAT TCC	ATG	0.44

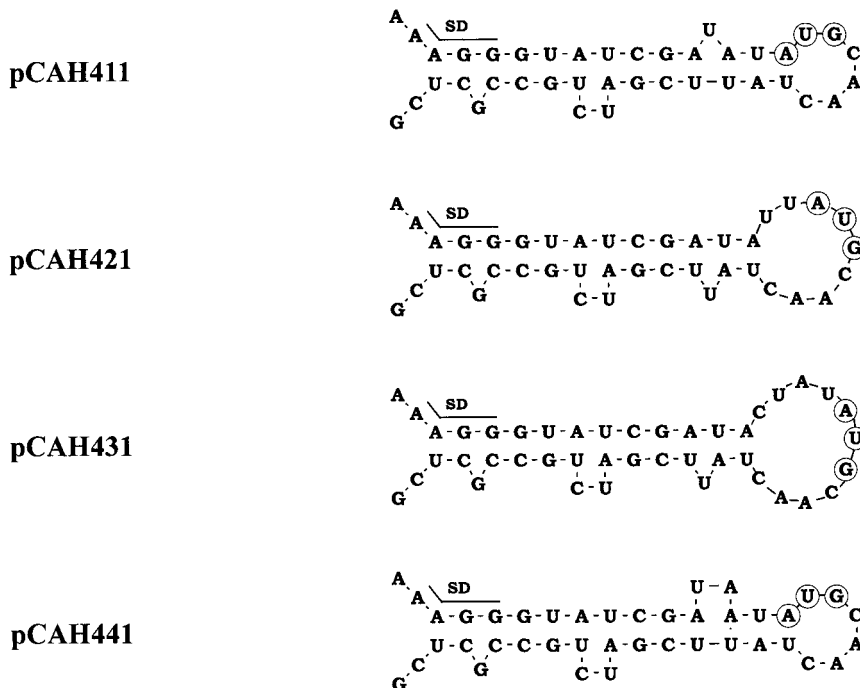


Fig. 4. Possible hairpin structure of mRNA derived from expression plasmids. The structures were predicted by computer analysis according to the method of Zuker and Stiegler.³³ The SD sequence is indicated by a solid line. The ATG initiation codon is circled.

would be another important factor because the expression levels of pCAH421 and pCAH441 differed in spite of their being the same length (11 nt). We therefore predicted the local secondary structure of mRNAs, including SD-ATG sequences, by computer analysis using the method of Zuker and Stiegler.³³⁾ Figure 4 shows the structure of each mRNA from the expression plasmid used. For all plasmids, a stem and loop structure was predicted between the SD sequence and the CAH coding region. In the higher level expression plasmids (pCAH421, pCAH431), the AUG initiation codon was found in the loop, suggesting that the RBS and ribosome would be easily accessible. On the other hand, in the lower level expression plasmids (pCAH411, pCAH441), the AUG codon was anchored in the stem structure. Such occlusion of the AUG codon by a stem-loop structure may cause the lower translational efficiency.

By alteration of the spacing, we succeeded in overexpression of a CAH gene in *E. coli* under the control of the *trp* promoter. Figure 5a shows the typical pattern of the CAH production by recombinant *E. coli* in a 30-l fermentor. The production of CAH protein was analyzed by SDS-PAGE of the soluble fraction (Fig. 5b). In agreement with the increase in CAH activity, the intensity of the protein corresponding to the CAH subunit (35 kDa) increased with the time and shows the main band at the end of cultivation. In batch culture of the recombinant *E. coli*, the amount of CAH reached 4.9 g per liter in a 20 h culture. The productivity of CAH is one of the highest expression cases of recombinant proteins in an *E. coli* host-vector system. Human growth hormone³⁴⁾ and human consensus interferon³⁵⁾ were reported to be produced at 1.75 g and 5.6 g per liter, respectively, with fed batch culture in which high cell density could be achieved. In the present case, a sufficient content of recombinant CAH could be obtained cost-effectively using a simple batch culture system.

4. Production of deacetyl 7-ACA by immobilized enzyme³⁶⁻³⁸⁾

4-1. Purification of recombinant CAH

The cells were harvested by centrifugation from 1 liter of the culture broth of the *E. coli* JM103 harboring pCAH431. After the cells had been resuspended in 0.5 liter of sodium phosphate buffer (50 mM, pH 7.0), they were disrupted by three passages of the suspension through a Manton-Gaulin homogenizer (model 15M-8TA, APV Gaulin GmbH, Lubeck, Germany) at 8,000 psi. For the purification, 50 ml of the disrupted cell suspension was used. To remove cell debris, the suspension was incubated with 1.5 g of filter-aid (Micro-Cel T-38;

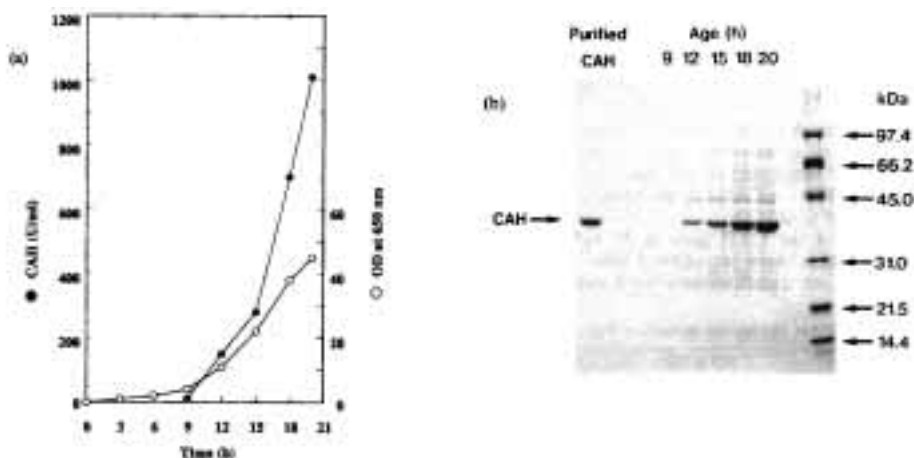


Fig. 5. a) Time course of CAH production of *E. coli* harboring pCAH431 in a 30-l jar fermentor. Symbols: \circ , cell growth (A650); \bullet , CAH activity in the soluble fraction from cell lysates (unit per ml of culture) b) SDS-PAGE analysis of the soluble fraction from cell lysates of *E. coli* JM103(pCAH431). The soluble fractions were prepared from cells harvested at various culture times and used as analysis samples. The numbers on the right indicate the molecular masses of the protein standards. The position of CAH is indicated by an arrow.

Manville Co., Ltd., CO, USA), at 30 °C for 1 h, and the cell lysate was clarified by filtration. Subsequently, the cell lysate was concentrated to 10 ml by ultrafiltration with a UK-50 ultramembrane (Advantech Co., Ltd., Tokyo, Japan). One-third of the concentrated cell extract was subjected to HPLC on DEAE-Toyopearl 650 M (Tosoh Co., Ltd., Tokyo, Japan). This step was repeated three times. The active fractions were pooled and concentrated. The homogeneity of the enzyme was examined by SDS-PAGE. Table IV summarizes the results of a typical purification process.

Table IV. Purification of recombinant CAH

Step	Total protein (mg)	Total activity (kU)	Specific activity (U/mg)	Yield (%)
Cell disruption	707	104	147	100
Micro-Cel T-38	384	75	196	72
DEAE-Toyopearl	314	66	210	63

4-2. Application to deacetyl 7-ACA production

We attempted to convert from 7-ACA to deacetyl 7-ACA using the purified enzyme, however, the reaction at optimal temperature is not applicable to industrial use because the product is unstable above room temperature. If the reaction is performed at 37 °C, deacetyl 7-ACA spontaneously changes to 3-hydroxymethyl-7-amino cephalosporanic lactone (deacetyl 7-ACA-LT). Therefore, the practical reaction conditions were optimized. Table V shows the K_m and V_{max} values for 7-ACA of the recombinant CAH at various temperatures. We decided to use recombinant CAH at 20 °C since the enzyme has sufficient activity for the conversion of 7-ACA at 20 °C. Figure 6a shows that 20 g of 7-ACA was converted to deacetyl 7-ACA in 99% yield at 20 °C within 100 min using 2.5 kU (corresponding to ca. 12 mg) of recombinant CAH. After the reaction was completed, there was a negligible amount of deacetyl 7-ACA-LT (not more than 0.2%), and impurities, such as 7-aminodeacetoxy cephalosporanic acid (7-ADCA) (about 0.1%), and N-acetyl-derivative (about 0.02%), derived from the material, as shown in Fig. 6b.

Table V. Michaelis constants of CAH for 7-ACA at various temperature

Temperature (°C)	V_{max} ($\mu\text{mol}/\text{min}/\text{ml}$)	K_m (mM)	V_{max} / K_m (min^{-1})
37	0.75	7.2	0.11
30	0.50	5.3	0.10
20	0.33	4.5	0.07

4-3. Construction of expression plasmid for large-scale production

In the expression plasmid pCAH431, the N-terminal region of hypothetical protein *ycgL* derived from *B. subtilis* chromosomal DNA and truncated β -lactamase gene are downstream of the CAH structural gene. To increase the plasmid stability in the cell, it was necessary to prevent unexpected expression of these ORFs by read-through from the CAH gene. As shown in Fig. 7, the expression plasmid pCAH4312 was constructed by eliminating the 0.2 kbp *VspI* - *XbaI* fragment in the truncated *ycgL* and the 0.23 kbp *SspI*-*DraI* fragment in the

truncated β -lactamase gene from pCAH431. This plasmid was introduced to *E. coli* strain JM109. Large-scale production of recombinant CAH in a fermentor was carried out as described previously.

4-4. Purification and immobilization of CAH

A concentrated cell suspension of 60 liters of culture broth of the *E. coli* JM109 harboring pCAH4312 was obtained by continuous centrifugation (at 10,000 rpm). After the cells were washed with 0.1 M sodium phosphate buffer, pH 7.5 containing 0.5% NaCl, the concentration of the cell suspension was adjusted to an optical density of 60 with the same buffer. Subsequently, the cells were disrupted by three passages of the

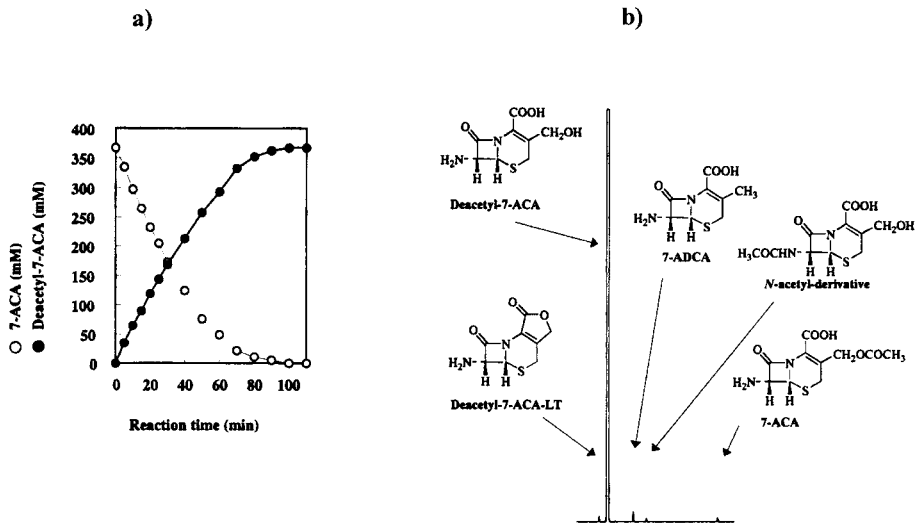


Fig. 6. a) Time course of the deacetylation reaction of 7-ACA at 20 °C. Symbols: ○, 7-ACA concentration (mM); ●, deacetyl-7-ACA concentration (mM). b) HPLC profile of reaction products of 7-ACA by CAH. Abbreviations: Deacetyl-7-ACA-LT, 3-hydroxymethyl-7-aminocephalosporanic lactone; 7-ADCA: 7-aminodeacetoxy cephalosporanic acid.

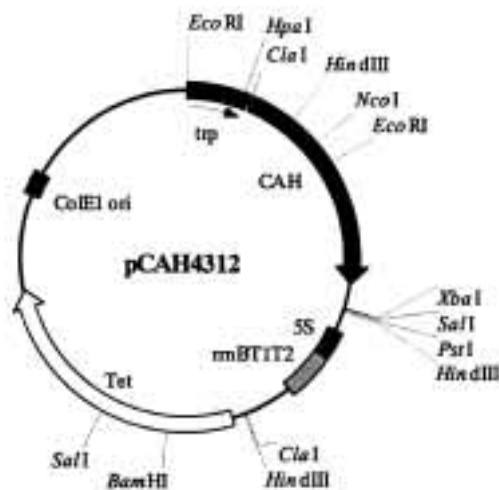


Fig. 7. The recombinant plasmid pCAH 4312 for overexpression of CAH protein. The bold arrows indicate the CAH gene and tetracycline resistance gene (Tet). The locations of the *trp* promoter (*trp*), 5S *rrmBT1T2* terminator (5S, *rrmBT1T2*), ColE1 replication origin (ColE1 ori) are shown.

suspension through an APV-Gaulin high-pressure homogenizer at 8,000 psi. Micro-Cel T-38 was added to the APV-Gaulin processed homogenate at a final concentration of 3%, and the mixture was maintained at 35 °C for 1 h. Because the cell debris and majority of *E. coli* proteins became adsorbed to the Micro-Cel T-38, they were removed by filtration. After the clarified cell extract was concentrated by ultrafiltration and sterilized by microfiltration, it was stored at 4 °C until use.

The enzyme activity of free recombinant CAH was measured using 7-ACA as a substrate. Twenty grams of 7-ACA was dissolved in 80.7 mM borate-ammonium buffer, pH 8.0 (buffer A). The pH was adjusted to 8.0 with 4 M aqueous ammonia. After the volume had been adjusted to 230 ml with the same buffer, the reaction was initiated by addition of the enzyme solution. The mixture was incubated at 20 °C with continuous agitation. The pH was controlled at 8.0 with 4 M aqueous ammonia. The formation of deacetyl 7-ACA was measured by HPLC. One unit of enzyme activity was defined as the amount that converts 1 μmol of 7-ACA to deacetyl 7-ACA in one min. To measure the activity of the immobilized enzyme, the method described above was used. To initiate the reaction, 24 g of immobilized enzyme was added instead of enzyme solution. The mixture was incubated with agitation (280 rpm) at 20 °C.

The partially purified CAH was immobilized on the basic anion resin, KA890 (Sumitomo Kagaku Kogyo Co., Ltd, Japan) with the following procedure. After 10 kg of KA890 was washed with 300 liters of water and 100 liters of 0.04 M H₂SO₄, it was suspended in 17.7 liters of 22.3 mM H₃BO₃ - Na₂B₄O₇ buffer, pH 8.5. In order to activate the resin, glutaraldehyde was added to the suspension with continuous stirring to a final concentration of 11% and incubated for 30 min at 10 °C. Subsequently, the activated resin was washed with 300 liters of 1 M sodium phosphate buffer, pH 7.5 and suspended in 32 liters of the same buffer. The CAH solution (ca. 1.3 Mega-units) was added to the suspension and incubated at 30 °C for 4 h. The resulting immobilized enzyme was washed with 15 liters of 0.1 M sodium phosphate buffer, pH 7.5 containing 50 mM glycine and 150 liters of buffer A. The immobilized enzyme was stored in buffer A at 5 °C until use.

The enzymatic properties of the immobilized CAH were compared with those of the free enzyme. Thermal stability studies were carried out using a water bath at different temperatures from 20 to 60 °C. After incubation for 1 h, the samples were stored on ice before being assayed in the usual manner. The immobilized enzyme was stable below 50 °C, which was almost the same as that of the free enzyme. The immobilized enzyme exhibited slight loss (about 4%) of activity at 5 °C, pH 8.0 for two years. The effects of pH on the stability were determined after preincubation at 20 °C for 60 min in buffers and found to be stable between pH 5 and 10 (Fig. 8). The pH tolerance of the enzyme was thus increased by immobilization.

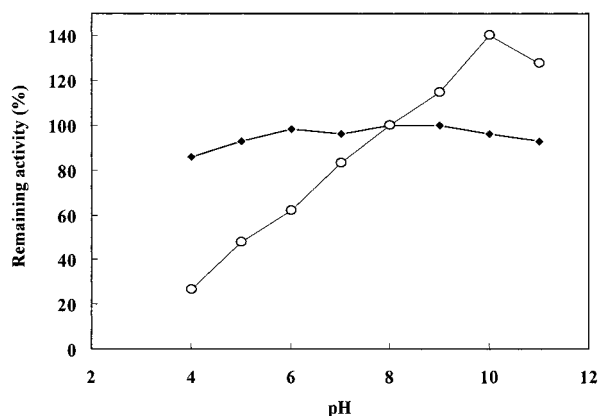


Fig. 8. Effect of pH on the stability of free and immobilized CAH. ○ Free enzyme, ● immobilized enzyme. The activities were determined after preincubation at 20 °C for 60 min in various buffers. The buffers used were 0.1 M sodium acetate (pH 4 - 5), 0.1 M sodium phosphate (pH 6 - 7), 0.1 M Tris-HCl (pH 8 - 10).

4-5. Conversion of deacetyl 7-ACA from 7-ACA using immobilized enzyme

The conversion was performed by agitating (330 rpm) a reaction mixture comprising immobilized enzyme (32 g), 40 g of 7-ACA and 280 ml of buffer A in a beaker at 20 °C. The pH was continuously controlled to 8.0 with 4 M aqueous ammonia. The immobilized enzyme obeyed Michaelis-Menten kinetics similar to the free enzyme indicating that there was no effect on the active site of CAH by glutaraldehyde immobilization. The K_m^{app} value of the immobilized enzyme for 7-ACA was 38.1 mM in buffer A. There was no significant change of the K_m^{app} by immobilization. Product inhibition studies for the immobilized enzyme were carried out under the same conditions in the presence of 1 - 385 mM deacetyl 7-ACA or 1 - 380 mM acetic acid. These reaction products did not inhibit the deacetylation of 7-ACA. Because K_i^{app} values for these reaction products are considered to be much larger than the K_m^{app} for 7-ACA, the theoretical predictions were calculated based on the simple Michaelis-Menten model.

$$- \frac{dSV}{dt} = \frac{V_{max} \cdot Vp \cdot S}{K_m^{app} + S} \quad (1)$$

$$\frac{dV}{dt} = \frac{1}{N} \cdot \frac{V_{max} \cdot Vp \cdot S}{K_m^{app} + S} \quad (2)$$

where S is the concentration of 7-ACA, V_{max} is the maximum reaction rate per liter, Vp is the volume of immobilized enzyme, V is the volume of reaction mixture, and N is the concentration of aqueous ammonia for the pH control. Equations (1) and (2) can be easily solved to give

$$t = - \frac{V_0 \cdot (S_0 + N) \cdot K_m^{app}}{V_{max} \cdot Vp \cdot N} \left\{ \ln \frac{S \cdot (S_0 + N)}{S_0 \cdot (S + N)} + \left[N - \frac{N^2}{K_m^{app}} \right] \left[\frac{1}{S + N} - \frac{1}{S_0 + N} \right] \right\} \quad (3)$$

where t is the reaction time, V_0 is the initial volume of reaction mixture, S_0 is the initial concentration of 7-ACA. Experimental time course studies with various substrate concentrations agreed well with the simulation model. Figure 9 shows a typical experimental result of conversion from 7-ACA to deacetyl 7-ACA.

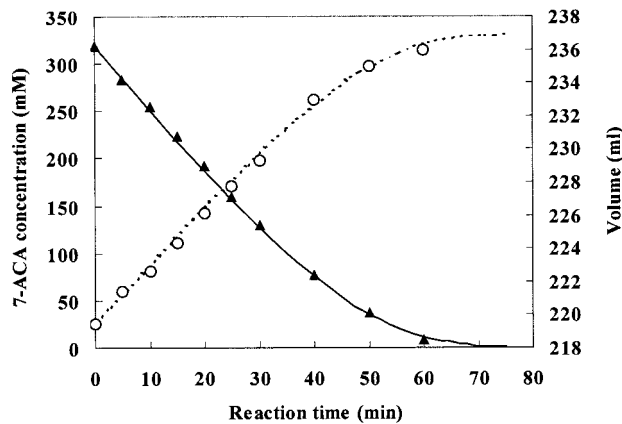


Fig. 9. Experimental and simulated data for the time course of deacetylation reaction of 7-ACA at pH 8.0, 20 °C by immobilized CAH. Experimental data for 7-ACA (▲) and volume of reaction mixture (○), and simulated results for 7-ACA (black line), volume of reaction mixture (broken line).

4-6. Crystallization of deacetyl 7-ACA

After the conversion was completed, the immobilized enzyme was removed by filtration and washed with buffer A. The reaction mixture and washing solution were mixed. The mixture was adjusted to pH 4.0 with 20% H₂SO₄, and stirred for 1 h at 7 °C to mature the crystals. The crystals were collected by centrifugation and washed with 60% methanol. After drying, the deacetyl 7-ACA crystals were obtained.

4-7. Pilot-scale production of deacetyl 7-ACA

For production of deacetyl 7-ACA using immobilized CAH, there are two types of reactors. One is a column reactor, and the other is a batch reactor. Konecny *et al.*³⁹⁾ used a column reactor for 7-ACA production. However, when the reaction was carried out at a high concentration of substrate, the pH at the bottom of the column decreased and deacetyl 7-ACA was deposited. Thus, a column reactor was not suitable for industrial levels of production, and we selected the batch reactor instead. In order to minimize the size of the reactor, deacetyl 7-ACA should be produced at high concentration. Therefore, we carried out the reaction at pH 8.0 based on consideration of the solubility of deacetyl 7-ACA. In order to prevent nonenzymatic hydrolysis and breakdown of 7-ACA at alkaline pH, 7-ACA was fed as slurry.

The pH stability of the enzyme was increased by immobilization. Similar broad pH tolerance of an immobilized enzyme was reported by Schaaf *et al.*⁴⁰⁾ for immobilized isochorismate hydroxymutase and Byuiyan *et al.*⁴¹⁾ for L-rhamnose isomerase. Such pH stability over a wide range is very advantageous for re-use of the immobilized enzyme, especially, in case of deacetyl 7-ACA production. Since the pH decreased because of the formation of acetic acid due to hydrolysis of 7-ACA, continuous alkaline addition to the reaction mixture was required. On an industrial scale, concentrated alkali should be used to avoid an increase of reaction volume. The reactor became very stable and reusable, once immobilized CAH tolerated the local pH increase. Further, aqueous ammonium was preferred over NaOH for pH control.

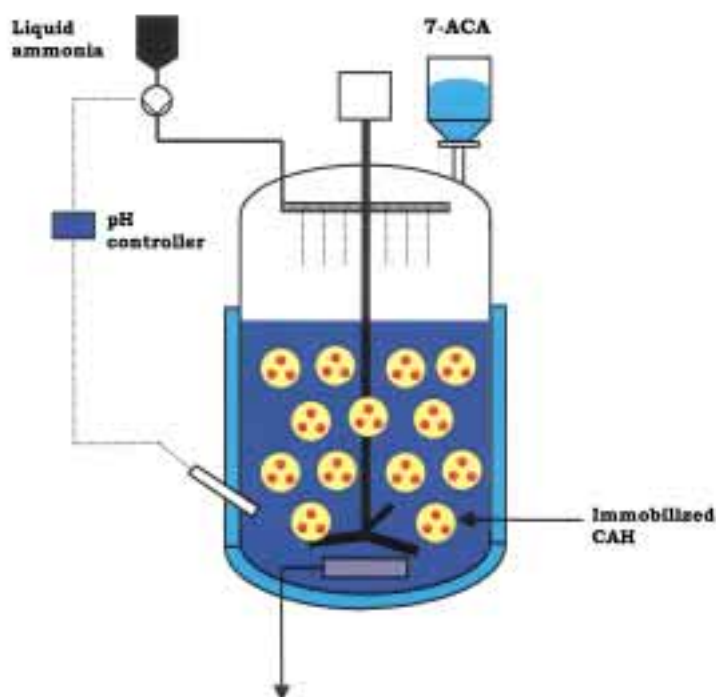


Fig. 10. A batch-type reactor with immobilized CAH.

We constructed a water jacketed reactor shown in Fig. 10 for the pilot scale production of deacetyl 7-ACA from 7-ACA. The reactor was run under the optimum conditions. The slurry of 7-ACA was fed to the reactor continuously at a flow rate of 2.75 liters/min.

The following rate equations of the reaction catalyzed by immobilized CAH are expressed by Equations (4), (5) and (6).

$$\frac{dV}{dt} = F + F_w + \frac{1}{N} \cdot F \cdot (S_s + C_s) + \frac{1}{N} \cdot \frac{V \max \cdot V_p \cdot S}{K_m^{app} + S} + \frac{1}{N} \cdot V \cdot kd \cdot S \quad (4)$$

$$\frac{dVS}{dt} = F \cdot S_s - \frac{V \max \cdot V_p \cdot S}{K_m^{app} + S} - V \cdot kd \cdot S \quad (5)$$

$$\frac{dVC}{dt} = F \cdot C_s + \frac{V \max \cdot V_p \cdot S}{K_m^{app} + S} - V \cdot kd' \cdot S \quad (6)$$

where F is the flow rate for feeding, F_w is the flow rate for line-washing, S_s is the amount of 7-ACA in the slurry (mmol/liter), C_s is the amount of deacetyl 7-ACA in the slurry (mmol/liter), C is the concentration of deacetyl 7-ACA in the reaction mixture, kd and kd' are spontaneous degradation rates for 7-ACA and deacetyl 7-ACA, respectively.

The simulation data agreed fairly well with the experimental results as presented in Fig. 11. After the completion of conversion, the solutions containing the product were pooled. The product deacetyl 7-ACA was then crystallized. Reverse phase HPLC showed that the purity of the deacetyl 7-ACA was not less than 97.5% with only a negligible amount of deacetyl 7-ACA lactone.

The stability of the immobilized enzyme after repeated use was determined by measuring the reaction time needed for complete conversion from 7-ACA to deacetyl 7-ACA. In this experiment, pH was controlled by addition of 4 M NaOH or 4 M aqueous ammonia. The immobilized enzyme was used 22 times and 52 times in these reaction mixtures, respectively. As shown in Fig. 12, the immobilized enzyme was very stable when 4 M aqueous ammonia was used to adjust the pH. In the case of 4 M NaOH, the reaction time was prolonged 30% after such repeated use. The immobilized enzyme was very stable and exhibited only a slight decrease in activity upon repeated use. Moreover, the pilot-scale conversion from 7-ACA to deacetyl 7-ACA was

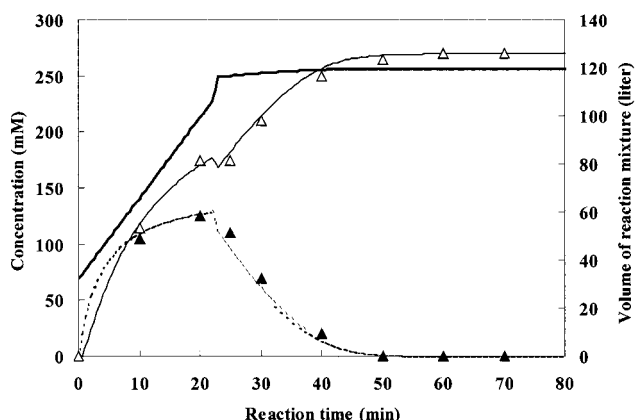


Fig. 11. Experimental and simulated data for time course of deacetylation reaction of 7-ACA at pH 8.0, 20 °C using batch-type reactor on a pilot-scale. Experimental data for deacetyl 7-ACA (△), 7-ACA (○) and simulated results for deacetyl 7-ACA (black line), 7-ACA (broken line) and volume of reaction mixture (bold line).

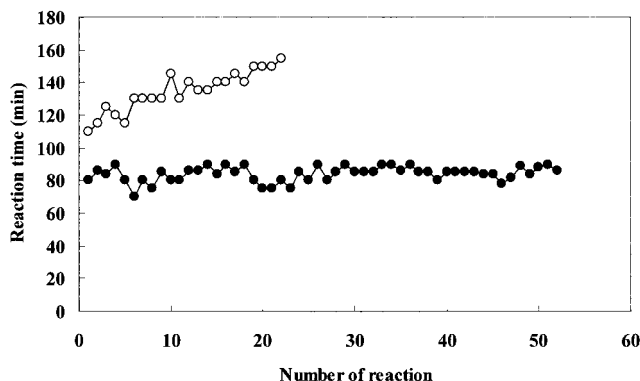


Fig. 12. Reusability of immobilized CAH on the pilot scale batch-type reactor. Symbols: NaOH (○) and aqueous ammonia (●) for neutralization.

successfully conducted 52 times over a period of 70 days without significant loss of immobilized enzyme activity.

Thus, this reaction system using immobilized enzyme has made possible the industrial production of CAH. Chemical deacetylation is the primary method used to produce deacetyl 7-ACA industrially, however, there are problems with environmental and safety issues due to the large quantities of hazardous chemical used. Immobilized CAH will have great success in the pharmaceutical industry in reducing process costs while having a positive environmental impact. In addition, the methods and simulations described here have been applied to the commercial production of deacetyl 7-ACA which provided the starting material for cefcapene pivoxil hydrochloride synthesis.

5. Concluding remarks

We were able to establish an expression and purification system of CAH. The CAH overexpressed in *E. coli* JM109 was purified by a one non-column step, and immobilized on the anion exchange resin, KA-890 using glutaraldehyde. The activity yield of immobilized enzyme was approximately 55% that of the free enzyme. This immobilized enzyme obeyed Michaelis-Menten kinetics similar to that of free enzyme. A 200-l batch-type bioreactor with a water jacket was employed for deacetylation of 7-ACA using CAH immobilized on KA-890 resin. Comparing this batch-type bioreactor with a conventional column-type one shows that the advantages of the batch-type bioreactor are that it allows facile and rapid pH adjustment of reaction mixture and 7-ACA, which is very unstable in buffer, can be introduced as a slurry. These advantages greatly facilitated large-scale production. Ten kilograms of 7-ACA could be completely converted to deacetyl 7-ACA at pH 8.0 and 20 min within 80 min. The reaction kinetics agreed well with a simulation model based on mass-balance and Michaelis-Menten kinetics. The immobilized enzyme exhibited only a slight loss of the initial activity after repeated use (52 times) over a period of 70 days.

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Dr. Kenji Mitsushima was born in Osaka in 1949. He completed the doctoral program of the Department of Fermentation Technology, Graduate School of Engineering, Osaka University and received his Ph.D. degree in 1978. In the same year he joined Shionogi & Co., Ltd. and worked at the Manufacturing Division and the Research Laboratories for more than 20 years. He was in charge of the development of manufacturing process of the recombinant proteins by biotechnology and the development of bio-processes using enzymatic or microbial reactions for the production of chemical products. Since 2005, he has been with the Pharmaceutical Affairs Department and supports Shionogi's research and development through various activities in industrial associations. His hobby is the appreciation of Japanese classical performing arts (including *Rakugo*, *Bunraku*, *Kyogen*).

List of Staff Publications

Physical and Analytical Chemistry

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