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PRODUCTION OF 5′-MONONUCLEOTIDES USING IMMOBILIZED 5′-PHOSPHODIESTERASE AND 5′-AMP DEAMINASE

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The preparations and properties of 5′-phosphodiesterase and 5′-AMP-deaminase immobilized on porous ceramics by covalent binding and the production of 5′-mononucleotides from RNA using these immobilized enzymes are described. Comparison tests for the properties of both immobilized and native enzymes were carried out. It was found that the pH optima of these immobilized enzymes were shifted toward the acidic side, and their practically operable pH regions were much more broadened. In such acidic pH regions, these immobilized enzymes also showed more excellent heat stabilities. These special characteristics of the immobilized enzymes were quite satisfactory for eliminating possible microbial contamination during the long-term operation of these immobilized enzyme systems. In continuous-column operations, more than 85% hydrolysis of RNA and complete conversion of 5′-AMP to 5′-IMP were maintained for more than 23 days when a 4% RNA solution was charged as the substrate.

INTRODUCTION

The synergetic effect of 5′-inosinic acid (5′-IMP) and 5′-guanylic acid (5′-GMP) for enhancing the meat flavor of monosodium glutamate (MSG) (1) has long been recognized in Japan. As shown in Fig. 1, if an equimolar

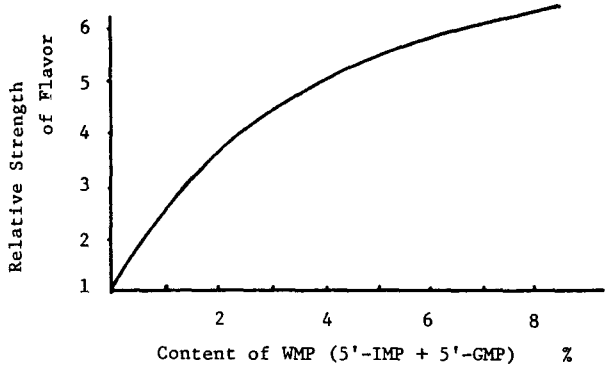

FIG. 1. Synergetic effect of 5′-IMP and 5′-GMP on the flavor of monosodium glutamate.

mixture of 5′-IMP and 5′-GMP is added to MSG, the meat flavor of MSG is greatly enhanced. For example, when 4% of such a nucleotide mixture is added to MSG, the flavor strength of the mixture increases to 5 times greater than that of MSG itself.

In 1961, Kuninaka and Sakaguchi (2) established an industrial process for producing 5′-IMP and 5′-GMP by enzymatic cleavage of RNA with microbial ribonuclease, i.e., 5′-phosphodiesterase, and 5′-AMP deaminase. Since then, several other enzymatic and microbial processes for the production of such ribonuleotides have been invented and operated commercially in Japan.

In 1973, the total production output of mixed 5′-IMP and 5′-GMP was estimated to be 2000–3000 tons/year. The total output of MSG fortified with such ribonucleotides reached approximately 40,000 tons/year, and the total sum of such fortified MSG was about 50 billion yens, i.e., about $180 million, per year.

The enzymatic cleavage of RNA established by Kuninaka and Sakaguchi is still one of the main commercial processes for the production of 5′-IMP and 5′-GMP in Japan. The schematic outline of the process is shown in Fig. 2. RNA isolated from yeast cells is first hydrolyzed by 5′-phosphodiesterase of *Penicillium* or other microorganisms to its constituent ribonucleotides such as 5′-AMP, -GMP, -CMP, and -UMP. Then, the nucleotide mixture is treated with 5′-AMP deaminase from *Aspergillus* or other microorganisms. From the resultant mixture, 5′-IMP and 5′-GMP are separated by an ion-exchange process and used as meat-flavor enhancer, and other pyrimidine nucleotides are used for pharmaceutical purposes.

Because of the economic importance of this process, the authors have chosen the enzymes concerned as the targets for immobilization. Among several immobilization techniques examined for the preparation of these immobilized enzymes, the most promising one was the immobilization of the

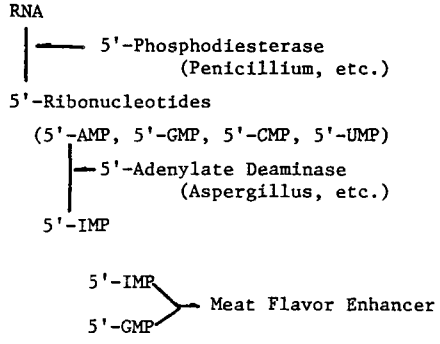

FIG. 2. Production of 5′-ribonucleotides by enzymatic cleavage of RNA.

enzymes on an inorganic support, i.e., porous ceramic developed by Corning Glass Works. The preparation methods, properties, and long-term operations of these immobilized enzymes are described in this paper, and some economic aspects concerning this process will be discussed.

MATERIALS AND METHODS

Enzyme Preparation

5'-Phosphodiesterase. This enzyme was obtained by culturing *Penicillium* CM932 in the medium comprising 5% glucose and 2% soluble vegetable protein for 3 days at 30°C with aeration. Proteins in the supernatant containing 5'-phosphodiesterase activity were precipitated by the addition of sodium sulfate. An aqueous extract of this precipitate was used without desalting. The 5'-phosphodiesterase activity of this extract was 120 U/ml, and the specific activity was 6.0 U/mg protein.

5'-AMP Deaminase. This enzyme, prepared from *Aspergillus*, was purchased from the Amano Pharmaceutical Co. Ltd., Japan. The crude enzyme powder was dissolved in water. The 5'-AMP deaminase activity of this solution was 97 U/ml, and the specific activity was 4.9 U/mg protein.

Enzyme Support

The enzyme support used was alkylamine porous ceramic Lot 1346 purchased from Corning Glass Works, Corning, New York, having an average pore diameter of 470 Å, a pore volume of 0.78 ml/g, and particle size from 20 to 30 mesh.

Immobilization of Enzymes on Porous Ceramic

The enzymes were bound to the alkylamine porous ceramic with glutaraldehyde according to the method of Weetall and Havewala (3). Details of the coupling procedures are described below.

Preparation of Porous Ceramic–CVB-5'-Phosphodiesterase. A quantity of 20 ml 0.01 M phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde was added to 5 g of the alkylamine porous ceramic. The reaction was carried out at room temperature for 2 h under vacuum. The beads were filtered off and washed with deionized water. The support material thus treated was mixed with 15 ml of enzyme solution, reacted for 2 h with stirring, then filtered and washed with deionized water.

Preparation of Porous Ceramic–CVB-5'-AMP Deaminase. Immobilized 5'-AMP deaminase was prepared in the same way as described above.

Methods for Analysis

Nucleotides. Mononucleotides (5'-AMP, -IMP, and -GMP) produced by the hydrolysis of RNA were determined chromatographically with a Liquid Chromatograph ALC 201 (Waters Associates). Paper chromatography was also applied for samples containing less impurities.

Activity of Immobilized Enzymes. For the assay of enzyme activity of porous ceramic–CVB-5'-phosphodiesterase, the substrate solution containing 4% RNA and 0.1 mM $ZnSO_4$ in 0.1 M acetate buffer (pH 4.5) was passed through a glass column packed with the immobilized 5'-phosphodiesterase at space velocities of 20–30 at 60°C. The units of enzyme activity were expressed as the amount (micromoles) of mononucleotides produced by 1 ml of the immobilized enzyme in 1 min.

The amounts of mononucleotides were determined spectrophotometrically using the mean molecular extinction coefficient value at 260 nm for mononucleotide, i.e., 11.5×10^3.

For the assay of enzyme activity of porous ceramic–CVB-deaminase, 0.1 M phosphate buffer (pH 6.5) containing 0.75% 5'-adenylic acid was passed through a column of the immobilized deaminase with S.V. 50 at 37°C. The units of enzyme activity were expressed as the amount (micromoles) of 5'-inosinic acid produced by 1 ml of the immobilized deaminase in 1 min.

Protein. The protein contents were determined by the colorimetric method of Lowry et al. (4) and calculated from a standard curve prepared with bovine serum albumin.

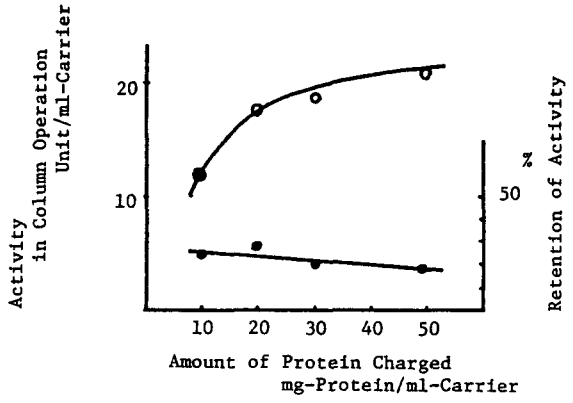

FIG. 3. Activity of porous ceramic–CVB-5'-phosphodiesterase in column operation. o—o: Activity in column operation; •—•: retention of activity.

RESULTS AND DISCUSSION

Preparation and Properties of Porous Ceramic-CVB-5'-Phosphodiesterase

Effects of the Amount of Enzyme Protein Charged on the Column Activity. The effects of the amount of enzyme protein charged to the unit volume of the support material in the preparation of PC-CVB-5'-phosphodiesterase are shown in Fig. 3. The activity of the immobilized 5'-phosphodiesterase in column operation increased in accordance with the increase of enzyme protein charged. On the other hand, the retention of activity in the immobilized enzyme decreased. In this case, when the amount of enzyme protein charged was in a range of 30–40 mg/ml carrier, nearly maximum activity of the immobilized enzyme, i.e., about 20 U/ml carrier, was obtained.

Optimum pH. The effect of the pH of the substrate solution on the activity of both native and immobilized enzymes was examined. As shown in Fig. 4, the optimum pH for the activity of the native enzyme is about 6.0, but that for the immobilized enzyme is shifted to 4.5. Also, the practically applicable pH range for the immobilized enzyme becomes much broader than that of the native enzyme.

Optimum Temperature. The temperature-activity relationships for both native and immobilized enzymes were also examined, and are shown in Fig. 5. The optimum temperature for the immobilized enzyme is about 80°C, and has shifted 20°C higher than that for the native enzyme.

pH Stability. The relationship between pH and enzyme stability was also examined. As shown in Fig. 6, the immobilized 5'-phosphodiesterase is

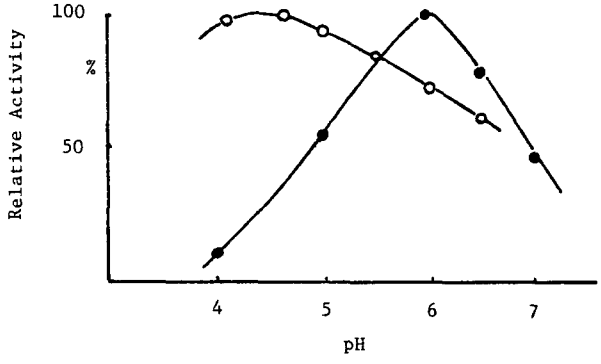

FIG. 4. Effect of pH on the activity of porous ceramic-CVB-5'-phosphodiesterase. o—o: Relative activity of immobilized enzyme (60°C, S.V. 20); •—•: relative activity of native enzyme (60°C, 30 min).

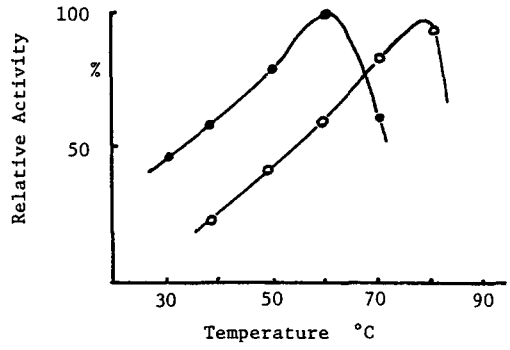

FIG. 5. Effect of temperature on the activity of porous ceramic-CVB-5'-phosphodiesterase. o—o: Relative activity of immobilized enzyme (pH 4.5, S.V. 30); •—•: relative activity of native enzyme (pH 5.5, 30 min).

quite stable over a wide range of acidic pH values compared with the native enzyme.

Heat Stability. The temperature–stability relationships for both native and immobilized enzymes were also examined, and are shown in Fig. 7. The heat-stability range of the immobilized enzyme shifted 10–15°C higher than that of the native enzyme.

As a conclusion from such experimental results on the properties of immobilized 5'-phosphodiesterase, it can be said that the immobilized 5'-phosphodiesterase is more active and stable over a wide range of acidic pH values at higher temperature. And, these phenomena seem to be quite

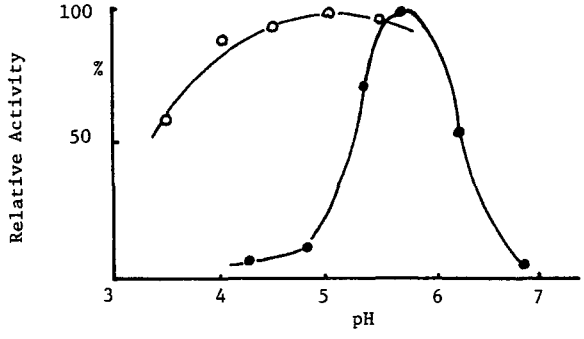

FIG. 6. Effect of pH on the stability of porous ceramic-CVB-5'-phosphodiesterase. o—o: Relative activity of immobilized enzyme (60°C, 13 h, with 2% RNA and 0.1 mM $ZnSO_4$); •—•: relative activity of native enzyme (60°C, 16 h).

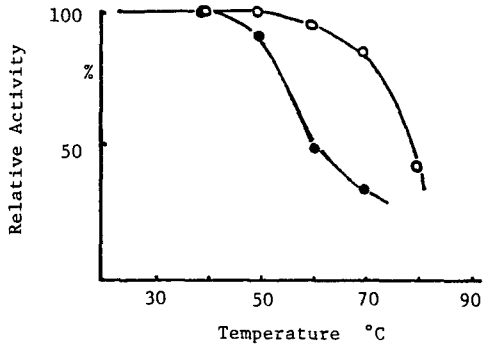

FIG 7. Effect of temperature on the stability of porous ceramic–CVB-5'-phosphodiesterase. o—o: Relative activity of immobilized enzyme (pH 4.5, 2 h, with 9% RNA and 0.1 mM $ZnSO_4$); •—•: relative activity of native enzyme (pH 5.5, 2 h).

satisfactory for eliminating microbial contamination in long-term column operations.

Effect of Zn^{2+} Ions During Continuous Operation of the Immobilized Enzyme. It was known that the native 5'-phosphodiesterase required Zn^{2+} ions as an enzyme activator. Therefore, the effect of Zn^{2+} ions during continuous operation of the immobilized 5'-phosphodiesterase was examined. The results are shown in Fig. 8. If there are no Zn^{2+} ions in the substrate solution, the enzyme activity is gradually lost. In contrast, when Zn^{2+} ions exist in the substrate solution at a concentration of 0.1 mM, the enzyme activity is quite stable even after 20 days' continuous operation. This result indicates the necessity for Zn^{2+} ions in this reaction.

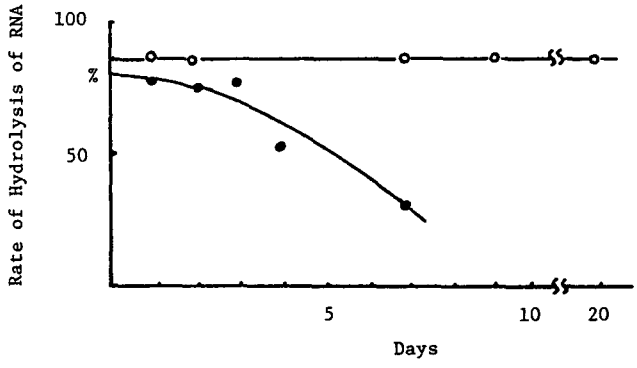

FIG. 8. Effect of Zn^{2+} ions during continuous operation of porous ceramic–CVB-5'-phosphodiesterase. o—o: 4% RNA, 0.1 mM $ZnSO_4$, pH 4.5, 55°C, S.V. 5; •—•: without $ZnSO_4$.

Effects of Temperature and Flow Rate (S.V.) of the Substrate Solution on the Rate of RNA Hydrolysis. The effects of temperature and flow rate (S.V.) of the substrate solution on the rate of RNA hydrolysis were examined using 4% RNA solution. As shown in Fig. 9, higher temperature and lower flow rate give higher rate of RNA hydrolysis. However, the differences caused by these effects are not very great. By choosing the best condition, nearly 90% hydrolysis of RNA can be obtained. This rate of hydrolysis is comparable with that obtained by batch hydrolysis of RNA using the native enzyme.

Preparation and Properties of Porous Ceramic–CVB-5'-AMP Deaminase

Effects of the Amount of Enzyme Protein Charged on the Column Activity. The effects of the amount of 5'-AMP deaminase charged to the unit volume of the support material on the various characteristics of the immobilized enzyme preparation were examined. As shown in Fig. 10, the maximum activity of immobilized 5'-AMP deaminase was obtained when the amount of enzyme protein charged was about 30 mg/ml carrier. In the range of higher charge of enzyme protein, the activity of the immobilized enzyme gradually decreased. This decrease might be caused by steric hindrance due to excess enzyme protein bound.

Optimum pH. The effect of the pH of the substrate (5'-AMP) solution on the activity of both native and immobilized enzymes was examined. As shown in Fig. 11, the optimum pH range for the immobilized enzyme was shifted to the acidic side and showed a wider range of pH optima in comparison with the native enzyme.

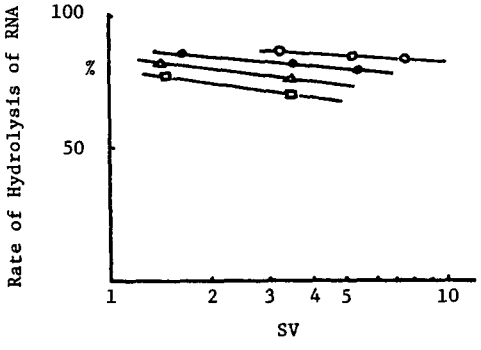

FIG. 9. Effects of temperature and flow rate (S.V.) on the rate of RNA hydrolysis. (RNA 4%, $ZnSO_4$ 0.1 mM, pH 4.5). ○—○: 60°C; ●—●: 55°C; △—△: 50°C; □—□: 45°C.

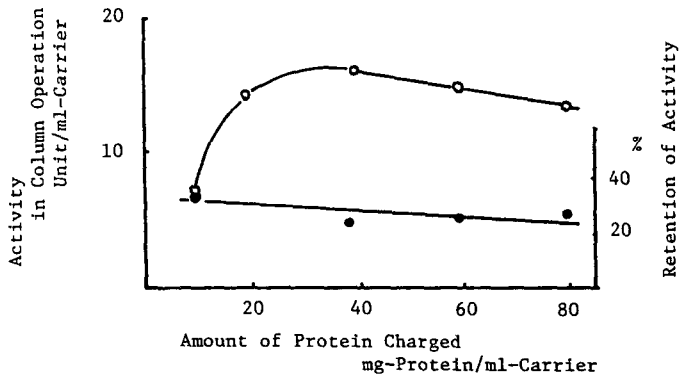

FIG. 10. Activity of porous ceramic–CVB-5'-AMP deaminase in column operation. o—o: Activity in column operation; •—•: retention of activity.

Optimum Temperature. The temperature–activity relationships for both native and immobilized enzymes were also examined. As shown in Fig. 12, the difference in temperature–activity relationships between native and immobilized enzymes was not so significant as that for 5'-phosphodiesterase. The immobilized enzyme showed relatively higher activity under a wider range of temperatures.

pH Stability. The relationship between pH and enzyme activity was examined for both native and immobilized enzymes. As shown in Fig. 13, the native enzyme is rather stable around the neutral pH region. In contrast, the immobilized enzyme is more stable in an acidic pH region.

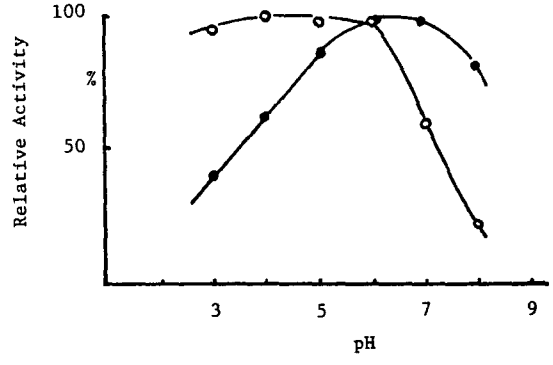

FIG 11. Effect of pH on the activity of porous ceramic–CVB-5'-AMP deaminase. o—o: Relative activity of immobilized enzyme (37°C, S.V. 50); •—•: relative activity of native enzyme (37°C, 30 min).

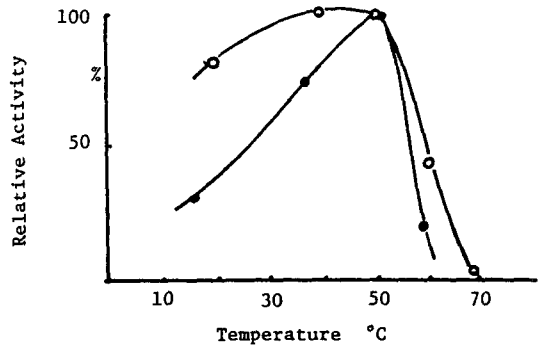

FIG. 12. Effect of temperature on the activity of porous ceramic–CVB-5′-AMP deaminase. o—o: Relative activity of immobilized enzyme (pH 5.0, S.V. 50); •—•: relative activity of native enzyme (pH 6.5, 30 min).

Heat Stability. The temperature–stability relationships for both native and immobilized 5′-AMP deaminases were also examined. As shown in Fig. 14, the immobilized enzyme is much improved in stability at higher temperature than the native enzyme.

From these results, it was shown that both immobilized 5′-phosphodiesterase and 5′-AMP deaminase have pH optima and stability in the acidic pH region. The derivatives are also more stable at higher temperature than each native enzyme. These properties seem to be very favorable for continuous operation without microbial contamination.

Effects of Temperature and Flow Rate (S. V.) of the Substrate Solution on the Conversion Rate of 5′-AMP to 5′-IMP. The effects of temperature and

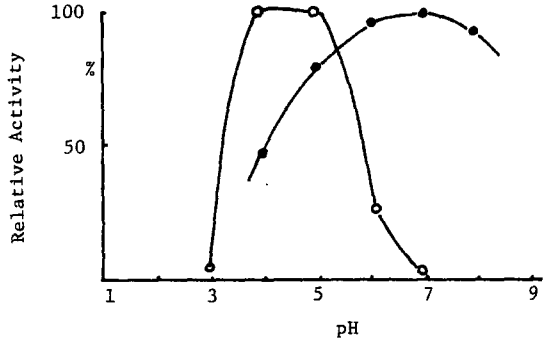

FIG. 13. Effect of pH on the stability of porous ceramic–CVB-5′-AMP-deaminase. o—o: Relative activity of immobilized enzyme (37°C, 20 h, with AMP solution); •—•: relative activity of native enzyme (37°C, 20 h).

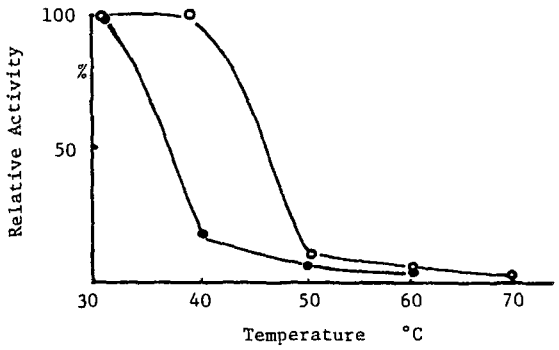

FIG. 14. Effect of temperature on the stability of porous ceramic-CVB-5'-AMP deaminase. o—o: Relative activity of immobilized enzyme (pH 5.0, 18 h, with AMP solution); •—•: relative activity of native enzyme (pH 6.5, 18 h).

flow rate (S.V.) of the substrate solution on the rate of conversion from 5'-AMP to 5'-IMP was examined using the enzymatic hydrolyzate of 4% RNA solution by 5'-phosphodiesterase. As shown in Fig. 15, the conversion rate of 5'-AMP to 5'-IMP was independent of temperature in the region of 40–50°C. Further, 100% conversion was achieved even at a very high flow rate such as S.V. 30.

Long-Term Operation of Immobilized 5'-Phosphodiesterase and 5'-AMP Deaminase

Long-term operation of the immobilized 5'-phosphodiesterase was carried out using a substrate solution that contained 4% yeast RNA and

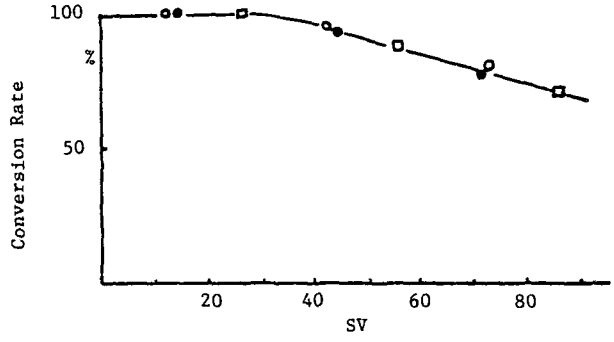

FIG. 15. Effects of temperature and flow-rate(SV) on the conversion rate of 5'-AMP to 5'-IMP. o—o: 40°C, •—•: 45°C, □—□: 50°C.

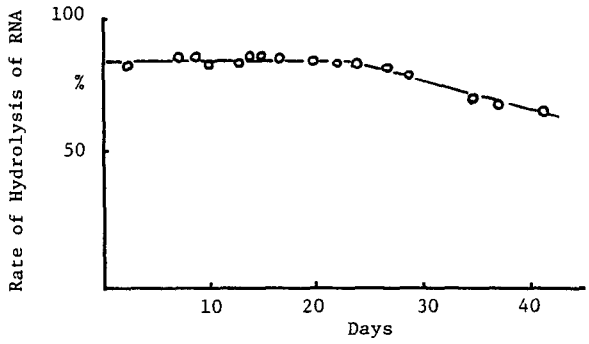

FIG. 16. Continuous operation of porous ceramic-CVB-5'-phosphodiesterase (substrate: RNA 4%, $ZnSO_4$ 0.1 mM, pH 4.5; S.V. 2.5, 55°C).

0.1 mM $ZnSO_4$ adjusted to pH 4.5. The substrate solution was kept at 55°C and passed through the column with a downward flow at S.V. 2.5. As shown in Fig. 16, about 85% hydrolysis was observed continuously for 23 days. The half-life of the immobilized enzyme was estimated to be about 49 days.

In the case of immobilized 5'-AMP deaminase, the enzymatic hydrolyzate of 4% RNA solution was adjusted to pH 4.75 and then used as a substrate solution. This substrate solution was kept at 50°C and passed through the enzyme column with a downward flow at S.V. 5. As shown in Fig. 17, 100% conversion was maintained for 33 days. The half-life could not be estimated at this stage.

Continuous operation of a mixed-bed enzyme column containing 2 vol immobilized 5'-phosphodiesterase and 1 vol of immobilized 5'-AMP deaminase was also carried out using different flow rates. As shown in Fig.

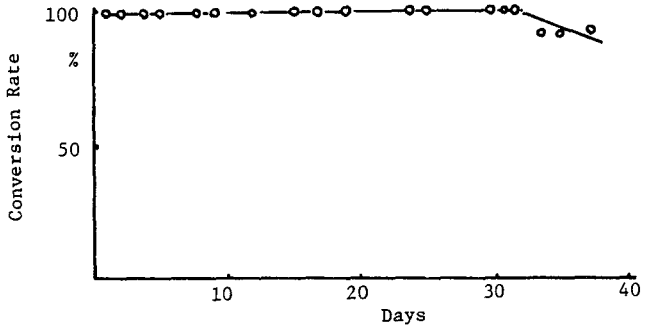

FIG. 17. Continuous operation of porous ceramic-CVB-5'-AMP deaminase (substrate: enzymatically hydrolyzed solution of 4% RNA, pH 4.7; S.V. 5.0, 50°C).

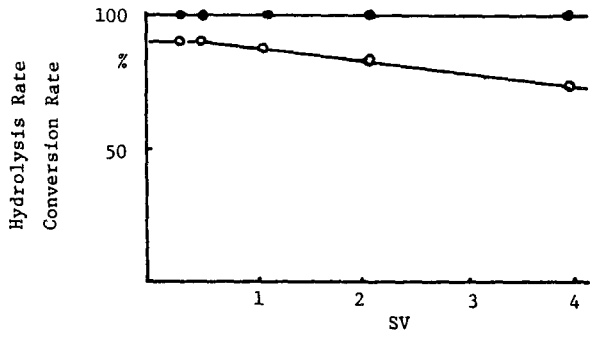

FIG. 18. Hydrolysis and conversion in one column. Mixed-bed enzyme column: PC-CVB-5'-phosphodiesterase : PC-CVB-5'-AMP deaminase = 2 : 1 (vol/vol); substrate: RNA 4%, $ZnSO_4$ 0.1 mM, pH 4.7, 50°C. o—o: Hydrolysis rate of RNA; •—•: conversion rate of 5'-AMP to 5'-IMP.

18, if the flow rate (S.V.) is kept less than 1.0, the rate of RNA hydrolysis is nearly 90%. If the S.V. is kept less than 2.5, more than 80% RNA hydrolysis can be obtained On the other hand, the conversion rate of 5'-AMP to 5'-IMP is always 100% at any flow rate below S.V. 4. These data show the practical applicability of such mixed-bed enzyme columns in commercial operation.

Some Economic Remarks on the Immobilized Enzyme Process for Production of 5'-Mononucleotides from RNA

In Fig. 19, process flow diagrams for continuous reaction using both immobilized enzymes and batch technique with native enzymes are described. In this diagram, the following assumptions were made:

1. 1000 kg yeast RNA are cleaved to the mononucleotide mixture containing 5'-IMP, -GMP, -CMP, and -UMP within 24 h.
2. The concentration of RNA feed-solution is 4% in the case of the immobilized enzyme process, and 10% in the batch process using native enzymes.
3. The rate of RNA hydrolysis is maintained at 80%, and the rate of deamination of 5'-AMP is kept at 100%.
4. Both immobilized enzymes, i.e., 5'-phosphodiesterase and 5'-AMP deaminase, are renewed every 30 days.

From these assumptions, the volume of the reactors in both systems was calculated as shown in Fig. 19. The volume of the enzyme reactors in the immobilized enzyme system is much smaller than that in the batch system using native enzymes as far as the enzymatic cleavage steps are concerned.

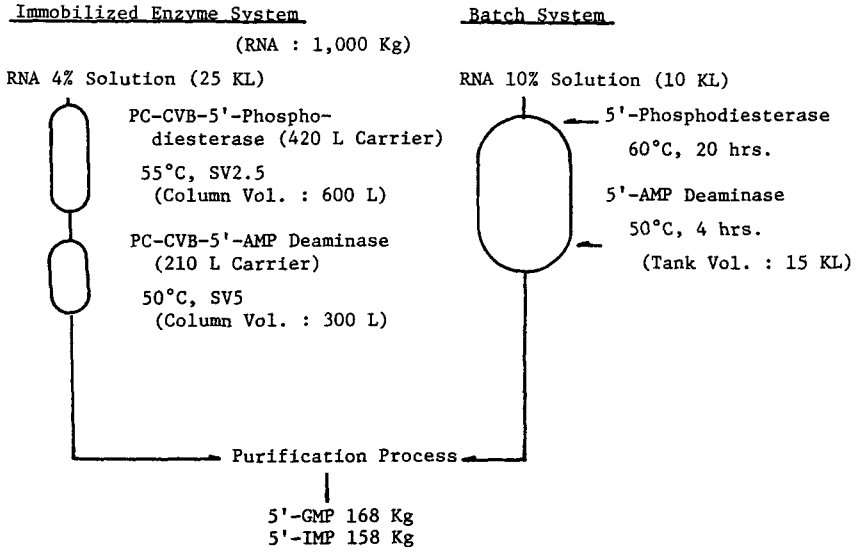

FIG. 19. Flow diagrams of enzymatic processes for the production of 5'-mononucleotides from RNA.

Enzyme costs for obtaining the same amount of 5'-IMP and -GMP mixture in the batch system and immobilized enzymes system were calculated from the tentative prices of native enzymes and support materials available in Japan. In the immobilized enzyme process, the cost of native enzymes becomes very low, i.e., less than 10% of that for the batch process. However, the cost of the immobilization support must also be considered. The total cost of native enzyme and support material is assumed to be about 1/4 the enzyme cost for the batch process.

In the enzymatic cleavage process, the cost of RNA itself constitutes a large part of the variable cost, and the enzyme cost a rather small part. Therefore, the merits of the immobilized enzyme process must come mostly from decreasing fixed costs, such as labor and construction costs.

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