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Title	A New Enzyme, Propioin Synthase (Acyloin Synthase) that Forms Propioin from Propionaldehyde プロピオンアルデヒドからプロピオインを形成する新酵素、プロピオイン合成酵素(アシロイン合成酵素)について
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A New Enzyme, Propioin Synthase (Acyloin Synthase) that Forms Propioin from Propionaldehyde

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Abstract

A new enzyme, propioin synthase (acyloin synthase) that was obtained from baker's yeast catalyzed the formation of acyloins, i.e., furoin, methyl furoin, acetoin, isobutyroin, valeroin, and propioin from the corresponding aldehyde.

The new enzyme was purified 270-fold from the crude preparation with a yield of 28% by protamine sulfate precipitation, ammonium sulfate fractionation and G-200 gel chromatography using citrate-phosphate buffer (0.1 M Na_2HPO_4-0.02 M citric acid, pH 6.8, containing 0.33 mM $MgSO_4$, 0.1 mM thiamine pyrophosphate, 2.5 mM $MnSO_4$ and 30 mM β-mercaptoethanol). The purified enzyme was homogeneous on disc gel electrophoresis. Enzyme activity was optimum at pH 6.8-7.0 and 37℃, and activity was stable at pH 7-8 and below 45℃. The activity was increased with addition of $FeSO_4 \cdot 7H_2O$, $MnSO_4$, thiamine pyrophosphate, β-mercaptoethanol, $MgSO_4$, $CaCO_3$, and NaCl, and decreased by inhibitor such as $CuSO_4$, $ZnSO_4$, $AgNO_3$, $HgCl_3$, iodoacetic acid, $FeCl_3 \cdot 6H_2O$ and $(NH_4)_2SO_4$. The molecular weight of the enzyme was 99,000 by sedimentation equilibrium analysis, and 100,000 by Sephadex G-200 column chromatography.

Propioin synthase was found in kernels of fresh corn and wheat grain; their specific activity was 0.00905 and 0.00750, respectively.

The study on a new enzyme, propioin synthase in baker's yeast[1)], and the several studies related to this new enzyme[2-9)] are summarized in this review. In 1988, the paper of propioin synthase was published[1)].

1. Acyloin synthase in baker's yeast

In 1966, flavor components of soy sauce such as furfuryl alchol, isoamyl alcohol, n-butyl acetate, ethyl maleate, ethyl levulinate, and ethyl caprate, were reported[2,3)], and major

product fermented from furfural by baker's yeast was found by gas-liquid chromatography (GLC) and infrared (IR) spectrophotometry to be furfuryl alcohol[4). In a study of fermentation products from aldehyde by yeast and other microorganisms it was found that furoin and

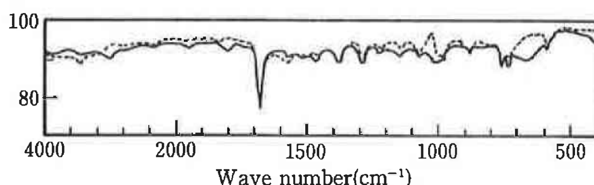
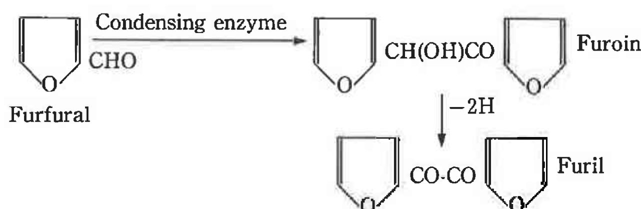


Fig. 1. IR spectrum of the precipitate in ether extract of the furfuryl alcohol ferment liquor.
——, Isolated ; ⋯⋯, authentic furoin.
Conditions: KRS-5, liquid cell, compensation method, 0.1−mm spacer.

(Fig. 1 was cited from Fig. 5 in J. Ferment. Technol., 47, 486 (1969).)

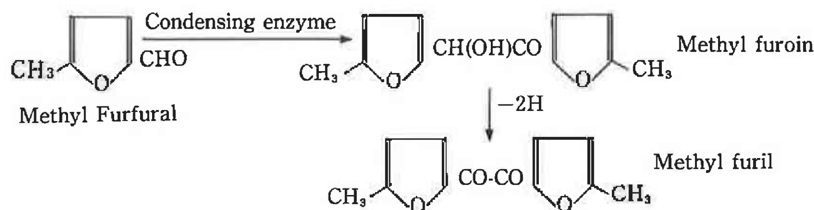
furil were produced from furfural, when 70% (by weight) baker's yeast was added to 6% furfural solution and this mixture was fermented for 3-7 days at 28-30℃[5].

Figure 1 shows the IR spectrum of the precipitate in an ether extract of this ferment liquor. The precipitate was identified as furoin by comparison of its IR spectrum with that of authentic furoin. GLC also showed that this precipitate was furoin. A large amount of precipitate was produced when the ferment liquor was centrifuged to remove the cell of baker's yeast and the supernatant was left for 2-3 days at room temperature or in a refrigerator. This precipitaoe was also identified to be furoin by its IR spectrum and the results of GLC. The yield of furoin was about 3.4 mg from 1 l of ferment liquor. Furoin was oxidized to form furil when furoin was heated at 90℃. However, Neuberg et al.[10] reported that the product fermented from furil by baker's yeast with sugar was furoin. Accordingly, this reaction in living cells may involve the action of an enzyme. Reaction seemed to be as follows.



Methyl furoin and methyl furil seemed to be produced from methyl furfural to judge

from the IR spectra when methyl furfural was fermented by baker's yeast[6,7]. This reaction was as follows.



Other aldehydes were tested, such as acetaldehyde, isobutyladehyde, and valeraldehyde[8]. Acetoin was produced in large quantities when acetaldehyde was fermented

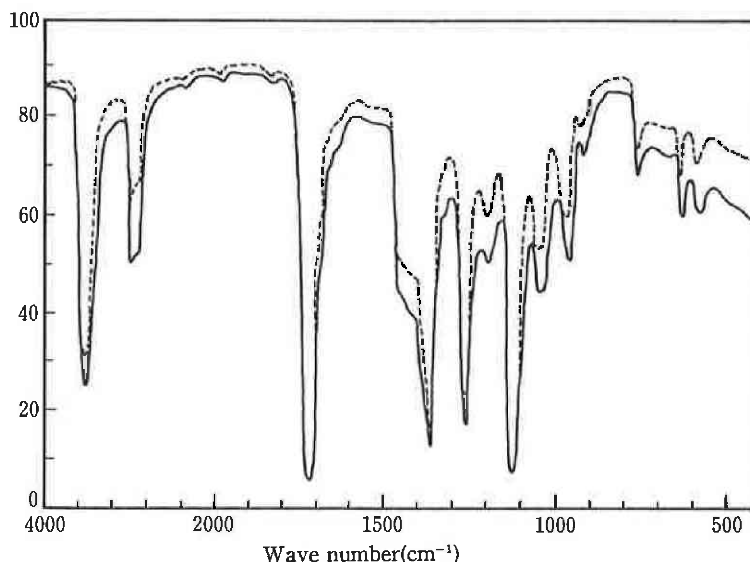


Fig. 2. IR spectum of acetoin produced from acetaldehyde.
——, Isolated ; ······, authentic acetoin.
Conditions: KRS-5, liquid cell, compensation method,
0.1−mm spacer.

(Fig. 2 was cited from Fig. 2 in Review of Living Science, 4, 29 (1971).)

by baler's yeast. Acetoin separated by GLC from the ferment liquor was identified by IR spectrophotometry (Fig. 2).

Figure 2 shows the IR spectra of the acetoin isolated and authentic acetoin. The yield of acetoin was about 6.0 ml from 1,l of ferment liquor. When isobutylaldehyde and valeraldehyde were fermented by baker's yeast, isobutyroin and valeroin were formed, to judge from

the results of GLC. The yield of isobutyroin was low (about 10 μl from 1 l of ferment liquor).

When 100 ml of a suspension of $CaCO_3$ containing 8% propionaldehyde was inoculated with 130g of baker's yeast and incubated at 30℃ for 1 day, propioin was formed[9]. A typical gas chromatogram of the ether extract of such an incubation mixture is shown in Fig. 3.

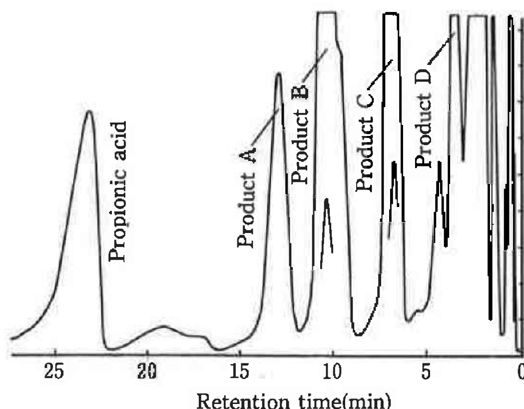


Fig. 3. Gas chromatogram of ether extract obtained from ferment liquor containing 5% $CaCO_3$, and 8% propionaldehyde.

The ether extract was concentrated by evaporation of the ether at about 40℃ and studied by gas-liquid chromatography.

Conditions: Column, Hyprose ; column temperature, 115℃; carrier gas, He ; flow rate, 60 ml/min; sensitivity, 1mV; bridge current, 110mA.

(Fig. 3 was cited from Fig. 1 in J. Ferment. Technol ., 50, 850 (1972).)

Product A was identified to be propioin by GLC and by comparison of its spectrum with

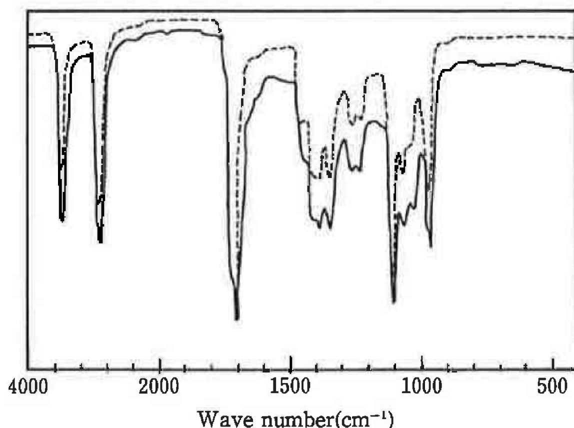


Fig. 4. IR spectrum of product A fraction ——, Product A ; ······, anthentic propioin.

Conditions: KRS-5, liquid cell, compensation method, 0.1−mm spacer.

(Fig. 4 was cited from Fig. 2 in J. Ferment . Technol., 50, 850 (1972).)

that of authentic propioin (Fig. 4).

Product B, which was abundant, was not identified.

These results suggested that baker's yeast contained an enzyme that catalyzed the

formation of acyloins from the corresponding aldehyde. The reaction was as follows.

$$2\ RCHO \longrightarrow RCHOHCOR$$

According to Singer[11], as written in Methods in Enzymology, the enzyme, plant carboxylase catalyzes reactions 1 and 2 below, but not reaction 3. Reaction 4, which may also occur with yeast enzyme as well as with plant carboxylase, is the sum of reactions 1 and 2.

$$RCOCOOH \rightarrow RCHO + CO_2 \quad (1)$$

$$RCOCOOH + R'CHO \rightarrow RCHOHCOR' + CO_2 \quad (2)$$

$$RCHO + R'CHO \rightarrow RCHOHCOR' \quad (3)$$

$$2RCOCOOH \rightarrow RCHOHCOR + 2CO_2 \quad (4)$$

In addition, as written in Enzyme Nomenclature, pyruvate decarboxylase, (i.e., carboxylase or ketoacid carboxylase) catalyzes reaction 5[12]. This enzyme also catalyzed acyloin formation. If pyruvic acid is the 2-oxoacid in reaction 5, the reactions are 6 and 7. The sum of these reactions is 8.

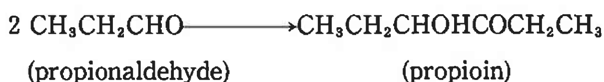
$$\text{A 2-oxoacid} = \text{an aldehyde} + CO_2 \quad (5)$$

$$CH_3COCOOH \rightarrow CH_3CHO + CO_2 \quad (6)$$

$$CH_3COCOOH + CH_3CHO \rightarrow CH_3CHOHCOCH_3 + CO_2 \quad (7)$$

$$2CH_3COCOOH \rightarrow CH_3CHOHCOCH_3 + 2CO_2 \quad (8)$$

Plant carboxylase does not catalyze reaction 3, but the new enzyme that formed propioin from propionaldehyde catalyzed reaction 3 which is written as follows.



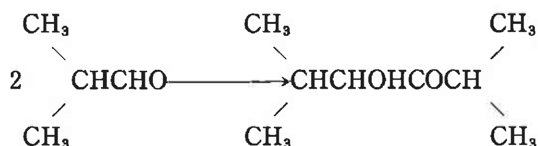
$$2\ CH_3CH_2CHO \longrightarrow CH_3CH_2CHOHCOCH_2CH_3$$

(propionaldehyde) (propioin)

This enzyme produced propioin from propionaldehyde itself without ketoacid. Therefore, the enzyme is considered a new enzyme to catalyze the formation of acyloins such as furoin, methyl furoin, acetoin, isobutyroin, valeroin, and propioin from the corresponding aldehyde. The reactions with acetaldehyde, isobutylaldehyde, and valeraldehyde were as follows.

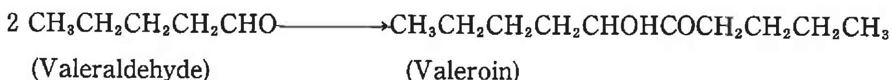
$$2\ CH_3CHO \longrightarrow CH_3CHOHCOCH_3$$

(Acetaldehyde) (Acetoin)



$$2\ (CH_3)_2CHCHO \longrightarrow (CH_3)_2CHCHOHCOCH(CH_3)_2$$

(Isobutylaldehyde) (Isobutyroin)



$$2\ CH_3CH_2CH_2CH_2CHO \longrightarrow CH_3CH_2CH_2CH_2CHOHCOCH_2CH_2CH_2CH_3$$

(Valeraldehyde) (Valeroin)

Propioin synthase was then purified from baker's yeast[1].

2. Propioin synthase purified form baker's yeast

2-1 Preparation of the crude enzyme solution First 160g of commercial baker's yeast (Oriental Yeast Co., Ltd.) and 40g of alumina were added to 160ml of citrate-phosphate buffer A (0.05M Na_2HPO_4-0.01M citrate pH 6.8) and the yeast cells were disrupted with sonic oscillator (60kHz, 200w) in an ice bath containing NaCl for 60min. The cellular debris was removed by centrifugation (Kubota KR-20000) at 28,700×g for 10min. The resulting supernatant was stored at −15℃, and used as the crude enzyme solution.

2-2 Buffer for Sephadex G-200 gel chromatography

Citrate-phosphate buffer solution B (0.1 M Na_2HPO_4-0.02 M citrate, pH 6.8, containing 0.33 mM $MgSO_4$, 0.1 mM thiamine pyrophosphate, 2.5 mM $MnSO_4$ and 30 mM β-mercaptoethanol) was used.

2-3 Assay The enzyme activity was assayed as the amount of propioin formed from propionaldehyde. In a 10-ml test tube, 0.1-0.4 ml of a different citrate-phosphate buffer (0.1 M Na_2HPO_4-0.02 M citrate, pH 6.8, containing 1.0 mM $CaCO_3$, 0.33 mM $MgSO_4$, 0.1 mM thiamine pyrophosphate, and 1.7 mM $MnSO_4$), and 0.05-0.2 ml of the enzyme solution were mixed and 5-20 μl of propionaldehyde was added to this mixture. The mixture was incubated at 30℃.

After 30 min of incubation, the mixture was heated for 30_s in boiling water, and 30-120 μl of an internal standard (0.5 mg ml^{-1} ethyl malonate) was added. Then the mixture was used in GLC. One unit of enzyme activity was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of propioin per minute under these conditions.

Figure 5 shows a gas chromatogram taken to measure the activity of the enzyme obtained from step 5 in the next section 2-4.

2-4 Purification of the enzyme

Step 1 First, 2 .6 ml of 2% protamine sulfate solution was added to 26 ml of the crude enzyme solution and the mixture was stirred in an ice bath for 15-30 min and centrifuged at 28,700×g for 10 min. The precipitate was removed.

Step 2 Next, 5.88 g of solid ammonium sulfate (40% saturation) was added slowly to 24.4 ml of the supernatant obtained from step 1 with stirring in an ice bath for 20-30 min and centrifuged at 28,700×g for 10 min. The precipitate was removed.

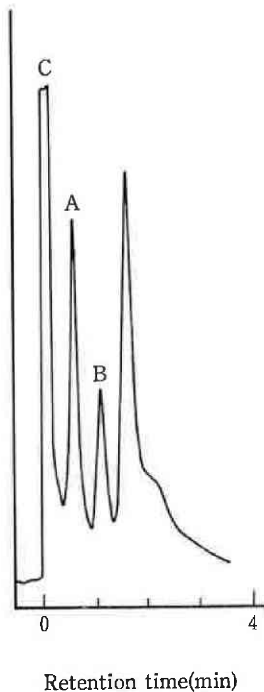


Fig. 5. Gas chromatogram for measurement of the enzyme activity. A, Propioin formed ; B, internal standard (ethyl malonate) ; C, propionaldehyde.
Conditions: Column, Hyprose; Column temperature, 85℃; carrier gas, N_2; flow rate, 90ml/min ; range, 10; attenuation, 4; injection volume, 2μl; chart speed, 2 mm/min.

Then 3.46 g more of solid ammonium sulfate (60% saturation) was added to 26.2 ml of the supernatant with stirring in an ice bath for 20-30 min and the mixture was centrifuged at 28,700×g for 10 min. The precipitate was dissolved in 6.6 ml of citrate-phosphate buffer A.

Step 3 Water was added to 6.6 ml of the enzyme solution from step 2 to obtain 66 ml of the enzyme solution, and 33 ml of the suspension containing 33 g of $Ca_3(PO_4)_3$ was added with stirring in an ice bath for 20-30 min. After removal of $Ca_3(PO_4)_2$ by centrifugation at 28,700×g for 10min, 34 g of ammonium sulfate (60% saturation) was added to 87.1 ml of supernatant with stirring under the same conditions, and the precipitate obtained by centrifugation at 28,700×g for 30 min was dissolved in 5.1 ml of citrate-phosphate buffer A.

Step 4 The enzyme solution obtained from step 3 was then chromatographed at 0℃

with a Sephadex G-200 column (18 mm×400 mm). The column of the ascending type was equilibrated with the above citrate-phosphate buffer (B). The effluent fractions (25 ml) containing the activity were collected and 9.8 g (60% saturation) of solid ammonium sulfate was added with stirring in an ice bath for 20-30 min. The precipitate was collected by centrifugation (Hitachi SCP 85H) at 37,300×g for 10 min and dissolved in 5.3 ml of the citrate-phosphate buffer A.

Step 5 The enzyme solution from step 4 was chromatographed again and the effluent fractions (25 ml) containing the activity were collected. To them, 25 ml of acetone (−15℃) was added slowly in an ice bath (−15℃) containing NaCl, with stirring for 1-2 min. The precipitate was collected by centrifugation at 28,700×g for 10 min, and dissolved in the citrate-phosphate buffer A. The insoluble residue was removed by centrifugation (0℃) at 28,700×g for 5 min, and 8.1 ml of the purified enzyme solution was obtained and stored at −6℃.

2-5 Enzyme purification. The enzyme activity in the effluent fraction from the Sephadex G-200 column was lost if a routine buffer such as phosphate buffer was used, but propioin synthase was purified by Sephadex G-200 column chromatography with citrate-phosphate buffer B which contained thiamine pyrophosphate, $MnSO_4$ $MgSO_4$, and β-mercaptoethanol. The purification of propioin synthase is summarized in Table 1.

Table 1. Purification of propioin synthase.

Purification step	Volume (ml)	Total activity (u)	Total protein (mg)	Specific activity (u mg^{-1})	Purification (fold)	Yield(%)
Crude enzyme	26.0	4.30	1270	0.0034	1	100
Protamine sulfate ppt	24.4	3.75	708	0.0053	1.6	87
Ammonium sulfate ppt	6.6	4.12	342	0.012	3.6	96
$Ca_3(PO_4)_2$ treatment	5.1	2.55	157	0.016	4.8	59
First gel filtration	5.3	1.32	13.0	0.102	30	31
Second gel filtration	8.1	1.19	1.3	0.91	270	28

Polyacrylamide disc gel electrophoresis of the enzyme obtained from step 5 is shown in Fig 6. A single protein band was found, indicating the electrophoretic homogeneity of the preparation.

2-6 Properties of the enzyme

Molecular weight of the enzyme The enzyme solution was put on a Sephadex G-200

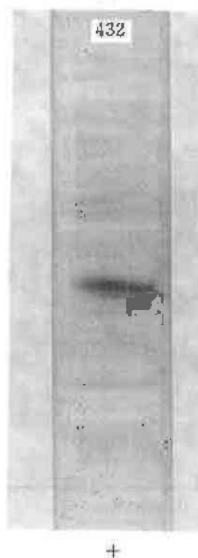


Fig. 6. Polyacrylamide disc gel electrophoresis of the purified new enzyme.
The gel was stained with amido black. The amount of protein applied was 28.7 μg.

(Fig. 6 was cited from Fig. 2 in J. Ferment. Technol., 66, 7 (1988).)

column (26.4 mm × 700 mm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, and the molecular weight of this enzyme was estimated to be 100,000 from the results.

The enzyme solution (0.37 mg ml^{-1}) was centrifuged to equilibrium for 12 h at 7000 rpm and 20℃ in a double-sector cell. From the linear relationship between ln (A_{280}) and $(radius)^2$, the molecular weight was estimated to be 99,000, as shown in Fig 7.

Effects of pH activity and stability of the enzyme The enzyme was most active at pH 6.8-7.0 and was stable at pH 7-8 on incubation for 30 min at 30℃. It lost almost all of its activity at pH 3.

Effect of temperature on activity and stability of the enzyme The optimum temperature for the enzyme dissolved in citrate-phosphate buffer A was 37℃. The enzyme dissolved in citrate-phosphate buffer (0.1 M Na_2HPO_4-0.02 M citrate pH 6.7) was stable at up to 45℃. Its activity was completely lost on incubation for 5 min at 60℃.

The effects of various enhancers and inhibitors of the enzyme The effects of various compounds as enhancers were examined. $FeSO_4 \cdot 7H_2O$, $MnSO_4$, thiamine pyrophosphate, β-mercaptoethanol, and $MgSO_4$ increased the activity strongly, and $CaCO_3$ and NaCl caused slight increases. The activity was inhibited by $CuSO_4$, $ZnSO_4$, $SnCl_2$, $(CH_3COO)_2$ Pb, $FeCl_3 \cdot 6H_2O$, $(NH_4)_2SO_4$, NH_4Cl, Na_2SO_4 and EDTA. At the concentration of 806 mM $(NH_4)_2SO_4$

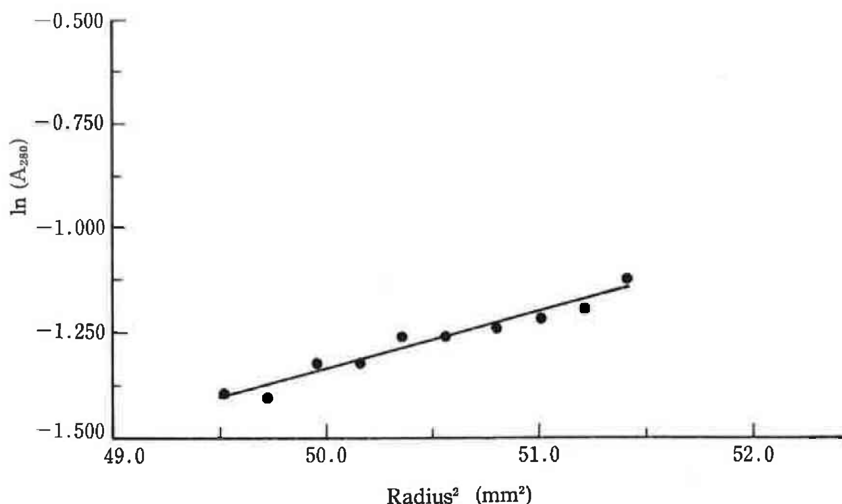


Fig. 7. Sedimentation equilibrium analysis of the purified enzyme.

completely inhibited the activity, so excess $(NH_4)_2SO_4$ must be removed by washing with water when it is used in steps 2-4. The enzyme was also inhibited by $AgNO_3$, $HgCl_3$, and iodoacetic acid, suggesting that it is an SH enzyme.

3. Propioin synthase obtained from grain

3-1 Corn To 100 ml of citrate-phosthate buffer A, 160g of fresh corn and 30g of alumina were added and the mixture was homogenized at 10,000 rpm for 10 min in an ice bath containing NaCl. The cell debris was removed by centrifugation at $28{,}700 \times g$ for 10 min. The supernatant was collected, stored at −50℃, and used as the crude enzyme solution. Solid ammonium was added slowly to the supernatant to 50% saturation with stirring in an ice bath for 20-30 min and the mixture was centrifuged at $28{,}700 \times g$ for 10 min. The precipitate and the yellow substance floating on the supernatant were dissolved separately in citrate-phosphate buffer A and these enzyme solutions were stored at −50℃. The specific activities of the enzyme solution obtained from the precipitate and the yellow substance were 0.00469 and 0.00905, respectively.

3-2 Wheat Fifty grams of wheat and 30 g of alumina were added to 100 ml of citrate-phosphate buffer and the mixture was homogenized at 14,000 rpm for 10 min. The other steps were the same as those described above. The precipitate obtained from the crude enzyme solution with 50% saturation of ammonium was dissolved in citrate-phosphate buffer

A and this enzyme solution was stored at −50℃. The specific activity of the enzyme was 0.0075.

Propioin synthase was not found in crude enzyme solutions obtained from millet, brown rice, and buckwheat.

Acknowledgments

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